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**CARDIOVASCULAR EFFECTS OF THE SIRTUIN
AND
UROCORTIN SYSTEMS IN HUMANS**

BY

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**A Thesis presented for the degree of Doctor of Medicine at the
University of Edinburgh**

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ABSTRACT

Background Cardiovascular disease continues to remain a leading cause of morbidity and mortality in both developing and developed worlds. The sirtuin and urocortin systems are novel hormone systems in humans with an emerging role in cardiovascular physiology and pathophysiology. Through a series of studies, this thesis examines the cardiovascular effects of SRT2104 (a novel small molecule SIRT1 activator) in otherwise healthy cigarette smokers and in patients with type 2 diabetes mellitus, and of urocortins 2 and 3 in healthy volunteers and in patients with heart failure.

Methods Twenty-four otherwise healthy cigarette smokers and 15 subjects with stable type 2 diabetes participated in a randomised, double blind, placebo controlled, crossover trial and received 28 days of oral SRT2104 (2.0 g/day) or matched placebo. Plasma SRT2104 concentrations, serum lipid profile, plasma fibrinolytic factors, markers of platelet and monocyte activation and pulse wave analysis and velocity were measured at baseline and the end of each treatment period together with an assessment of forearm blood flow during intra-arterial bradykinin, acetylcholine and sodium nitroprusside infusions. The pharmacodynamic profile of urocortins 2 and 3 were assessed in 18 healthy male volunteers recruited into a series of randomised, double blind, placebo controlled, crossover studies. Bilateral forearm venous occlusion plethysmography was performed during incremental intra-arterial infusions of urocortin 2 (3.6-120 pmol/min), urocortin 3 (1.2-36 nmol/min) and substance P (2-8 pmol/min) in the presence or absence of inhibitors of cyclooxygenase (aspirin), cytochrome P450 metabolites of arachidonic acid (fluconazole) and nitric oxide synthase (L-N^G-monomethyl-arginine (L-NMMA)). Finally, 12 patients with stable heart failure (New York Heart Association (NYHA) II-IV) and 10 age- and sex-matched healthy volunteers were recruited to attend once each. Bilateral forearm arterial blood flow was measured using forearm venous occlusion plethysmography during incremental intra-arterial infusions of urocortin 2 (3.6-36 pmol/min), urocortin 3 (360-3600 pmol/min) and substance P (2-8 pmol/min).

Results SRT2104 was safe and well tolerated in otherwise healthy cigarette smokers and subjects with type 2 diabetes mellitus. There were no significant differences in fibrinolytic or blood flow parameters between placebo and SRT2104. Treatment with SRT2104 was associated with a significant reduction in augmentation pressure ($P=0.0273$) and a trend towards improvement in the augmentation index (AIx) and corrected augmentation index ($0.10 > P > 0.05$ for both) without significant changes in pulse wave velocity (PWV) and time to wave reflection (Tr) ($P > 0.05$). Administration of SRT2104 had a favourable effect on lipid profile in otherwise healthy cigarette smokers in comparison to placebo. Urocortins 2 and 3 evoked arterial vasodilatation ($P < 0.0001$) without tachyphylaxis but with a slow onset and offset of action. Inhibition of nitric oxide synthase with L-NMMA reduced vasodilatation to substance P and urocortin 2 ($P \leq 0.001$ for both) but had little effect on urocortin 3 ($P > 0.05$). Neither aspirin nor fluconazole affected vasodilatation induced by any of the infusions ($P > 0.05$ for all).

In the presence of all three inhibitors, urocortin 2- and urocortin 3-induced vasodilatation were attenuated ($P < 0.001$ for all) to a greater extent than with L-NMMA alone ($P \leq 0.005$). The vasodilatory effects of urocortins 2 and 3 were preserved in patients with heart failure.

Conclusion Activation of SIRT1 through SRT2104 improved lipid profile but did not produce demonstrable differences in vascular or platelet function with some effect on measures of arterial stiffness. Urocortins 2 and 3 appear to be potent arterial vasodilators whose vasomotor responses remained preserved in patients with heart failure and were at least partly mediated via the endothelium. Both hormone systems hold potential in their role in cardiovascular disease in man but require further studies to help translate findings of this thesis to clinical practice.



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Background: The sirtuin and urocortin systems are novel hormone systems in humans with an emerging role in the functioning of the heart in health and in disease states. Through a series of studies, this thesis examines the effects of SRT2104 (a novel small molecule SIRT1 activator) in otherwise healthy cigarette smokers and in patients with type 2 diabetes mellitus, and of urocortins 2 and 3 in healthy volunteers and in patients with heart failure.

Methods: Twenty-four otherwise healthy cigarette smokers and 15 subjects with stable type 2 diabetes participated in a series of studies and received 28 days of oral SRT2104 (2.0 g/day) or matched placebo. Through a range of tests, we measured blood levels of SRT2104 and assessed its effects on blood cholesterol, stiffness of blood vessels, blood clotting parameters and on blood flow in the forearm at baseline and at the end of each treatment period. The profile of urocortins 2 and 3 were assessed in eighteen healthy male volunteers recruited in a series studies during the infusion of urocortin 2, urocortin 3 and substance P in the presence or absence of specific chemicals (using aspirin, L-NMMA and fluconazole) which block receptors found in the lining of blood vessels. Finally, twelve patients with stable heart failure and ten age and sex-matched healthy volunteers had forearm blood flow measured during incremental infusions of Ucn 2, Ucn 3 and Substance P.

Results: SRT2104 was safe and well tolerated in otherwise healthy cigarette smokers and subjects with type 2 diabetes mellitus. There were no significant differences in blood clotting or blood flow variables between placebo and SRT2104. Treatment with SRT2104 was associated with an improvement in some measures of blood vessel elasticity. Administration of SRT2104 had a favourable effect on lipid profile in otherwise healthy cigarette smokers in comparison to placebo. Urocortins 2 and 3 increased blood flow in the

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forearm without a decrease in effect on repeated administration but with a slow onset and offset of action. There was no effect on the increase in blood flow to urocortins 2 and 3 in the presence or absence of aspirin or fluconazole. In the presence of all three inhibitors, urocortin 2 and urocortin 3 induced increases in forearm blood flow were attenuated to a greater extent than with L-NMMA alone. The blood flow increasing effects of urocortins 2 and 3 were preserved in patients with heart failure.

Conclusion: Activation of SIRT1 through SRT2104 improved lipid profile but did not produce demonstrable differences in blood vessel or platelet function with some effect on measures of blood vessel stiffness. Urocortins 2 and 3 appear to be potent hormones that increase blood flow whose responses remained preserved in patients with heart failure and whose effects were at least partly mediated via the lining of the blood vessels. Both hormone systems hold potential in their role in cardiovascular disease in man but require further studies to help translate the findings of this thesis into clinical practice.

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I dedicate this thesis

to my family

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ABBREVIATIONS

β-blockers	beta-blockers
β-cells	beta-cells
ACE	angiotensin-converting enzyme
ADP	adenosine diphosphate-ribosylation
AIx	augmentation index
Akt	protein kinase B pathways
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	analysis of variance
AP	augmentation pressure
apoE^{-/-}	apolipoprotein E
ATPase	adenosine triphosphatase
ATR1	angiotensin II receptor type 1
AUC_{0-t}	area under the curve from zero to time
AVP	arginine vasopressin
BaCl₂	barium chloride
Ca²⁺	calcium
CaMKKβ	calcium/calmodulin-dependent protein kinase beta
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
cGMP	cyclic guanosine monophosphate
CK	creatinine kinase
CRH	corticotrophin-releasing hormone
CRH-BP	Corticotrophin-releasing hormone-binding protein
CRH-R	CRH receptors
C-terminal	carboxyl terminal
DCCT	Diabetes Control and Complications Trial
DNA	deoxyribose nucleic acid
EDHF	endothelium-derived hyperpolarising factor
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay

eNOS	endothelial nitric oxide synthase
ERK	extracellular receptor kinase
FBF	forearm blood flow
FITC	fluorescein isothiocyanate
FoxO1	forkhead box O1 transcription factor acetylation
GMP	Good Medical Practice
GPCR	G protein-coupled receptor
HCG	serum human chorionic gonadotrophin
HDACs	histone deacetylases
HDL	high-density lipoprotein
HUVEC	human umbilical vein endothelial cells
<i>I_{Ca}</i>	L-type calcium channel
IGF-1	insulin-like growth factor-1
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
LKB1	serine threonine liver kinase B1
L-NAME	N ^G -nitro-L-arginine methyl ester
L-NMMA	L-N ^G -monomethyl-arginine
LXR	liver X receptor
LXR-ATP	liver X receptor-adenosine triphosphate
Mac-1	membrane activated complex-1
MAPK	mitogen-activated protein kinases
MEK	MAPK/ERK kinase
MHRA, UK	Medicines and Healthcare products Regulatory Authority
MLP	muscle specific LIM protein
mRNA	messenger ribonucleic acid
MSNA	muscle sympathetic nerve activity
NAD⁺	nicotinamide adenine dinucleotide-dependent deacetylases
NCBI	National Centre for Biotechnology Information.
NF-κB	nuclear factor kappa B
NO	nitric oxide
NOS	nitric oxide synthase

N-terminal	amino terminal
NYHA	New York Heart Association
ODQ	1H-[1,2,4] oxadiazolo [4,2-a] quinoxalin-1-one
PAI-1	plasminogen activator inhibitor type 1
PCSK9	proprotein convertase subtilisin/kexin 9
PE	phycoerythrin
PGC-1α	coactivator-1 alpha
PKA	protein kinase A
PMA	platelet-monocyte aggregation
PP	pulse pressure
PPACK	D-Phenylalanine-L-propyl-L-arginine chloromethyl ketone
PPAR-γ	peroxisome proliferators-activated receptor gamma
PTP	protein tyrosine phosphatase
PWA	pulse wave analysis
PWV	pulse wave velocity
rRNA	ribosomal ribonucleic acid
sCD40L	soluble CD40 ligand
SD	standard deviation
SEM	standard error of the mean
SERCA	sarcoplasmic and endoplasmic reticulum Ca ²⁺ ATPase
SIRT1	Silent Information Regulator Two 1
SNP	sodium nitroprusside
Tg/apoE^{-/-}	transgenic/apolipoprotein E
TIMP3	tissue inhibitory metalloproteinase 3
Tmax	time taken to reach the maximum
t-PA	tissue-plasminogen activator
Tr	time to reflected wave
Ucn	urocortin
VCAM-1	vascular cell adhesion molecule-1
VSMC	vascular smooth muscle cells

DECLARATION

This thesis represents research undertaken in the British Heart Foundation Centre for Cardiovascular Science, University of Edinburgh during the period August 2009 to August 2012.

The work in this thesis is my own. I was personally involved in carrying out all the studies involved in this thesis with the following exceptions. Trained nurses at the Clinical Research Facility, Royal Infirmary of Edinburgh and Western General Hospital performed the pulse wave analysis studies. Dr Radzi Noh was involved in the supervision of the pulse wave studies carried out at the Western General Hospital as part of the Phase 1 trial with SRT2104. Eric Thomson and Neil Johnston carried out the fibrinolytic assays. Sirtris, a GSK company funded the Phase 1 trial with SRT2104 and were involved in the design, monitoring and analysis of the data. They have provided permission to include their data as part of my thesis.

Chapters 1 (part of), 3 and 5 have been published in peer-reviewed journals. Chapter 4 has been accepted for publication and the contents of Chapter 6 form a part of a manuscript that is under review. No material has previously been submitted for any other professional degree or qualification.

Sowmya Venkatasubramanian
May 2016

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CHAPTER 1

CARDIOVASCULAR EFFECTS OF THE SIRTUIN AND UROCORTIN SYSTEMS IN HUMANS

Venkatasubramanian S, Newby DE, Lang NN.
Urocortins in heart failure.
Biochem Pharmacol 2010;**80**:289-296.

1.1 INTRODUCTION

Cardiovascular disease continues to remain a leading cause of mortality and morbidity in both the developed and developing worlds. This presents an ever increasing need to understand the underlying mechanism of the pathological processes involved in order to aid the development of novel treatment targets. The sirtuin and urocortin systems are novel hormone systems in humans with an emerging role in cardiovascular physiology and pathophysiology. This thesis will focus on the cardiovascular effects of activation of the sirtuin and urocortin systems in health and in disease.

Endothelial dysfunction is a recognised pathological process associated with aging and in disease processes such as diabetes mellitus and heart failure. Both hormone systems appear to play a crucial role in maintaining endothelial homeostasis and have therefore generated immense interest in their contribution to cardiovascular health. Through a series of studies in healthy volunteers, cigarette smokers, patients with type 2 diabetes and patients with heart failure, this thesis will focus on the cardiovascular effects of activation of the sirtuin and urocortin systems.

This chapter provides a brief overview of the sirtuin and urocortin systems.

1.2 SIRTUINS

Sirtuins represent a class of highly conserved nicotinamide adenine dinucleotide (NAD⁺)-dependent deacetylases with seven identified members in mammals [Frye 1999; Frye 2000]. The members are designated silent information regulator two (SIRT) 1 to 7 and have distinct tissue and subcellular distributions [Borradaile and Pickering 2009]. The role of sirtuins in metabolism, cardiovascular health and particularly in diseases of ageing has been of increasing interest in recent years.

1.2.1 BIOLOGY OF SIRTUINS

1.2.1.1 *Molecular structure and tissue distribution*

Sirtuins are characterised by a conserved 275 amino acid catalytic core and unique additional amino (N)-terminal and carboxyl (C)-terminal sequences of variable length [Maiese *et al* 2011] (Figure 1.1). They differ in their cellular localisation, tissue distribution and target substrates in mammals. SIRT1, SIRT6 and SIRT7 localise primarily to the nucleus; SIRT3, SIRT4 and SIRT5 localise to mitochondria; and SIRT2 localises to the cytosol [Hubbard and Sinclair 2014]. SIRT1 is currently the best-known member of this class and has been shown to play a critical role in all major metabolic organs and tissues [Dong 2012].

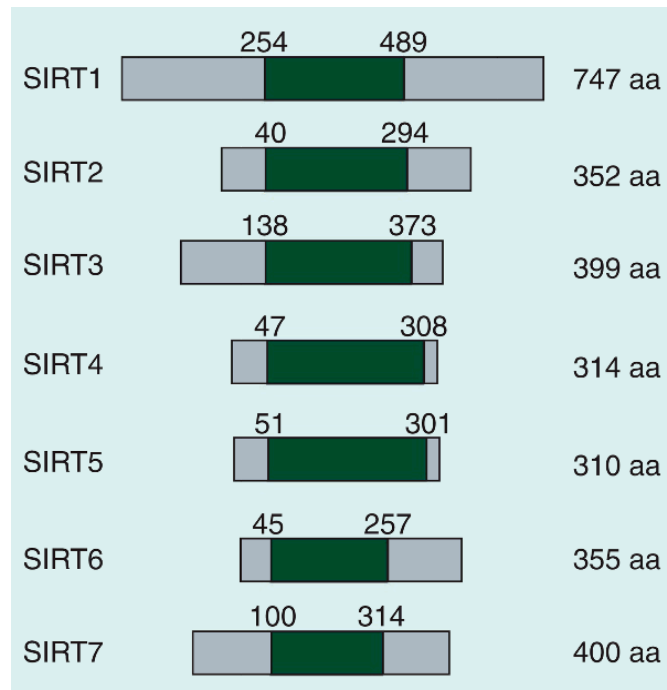


Figure 1.1. Human sirtuin proteins and their domain structure. The protein sequences and annotations are based on the NCBI Gene data set [Dong *et al* 2012]. SIRT - silent information regulator two; NCBI - National Centre for Biotechnology Information.

SIRT1 is highly expressed in the vascular endothelium [Edirisinghe and Rahman 2010] as well as in the brain, heart, liver, pancreas, skeletal muscle, spleen and adipose tissues [Maise *et al* 2011; Stein and Matter 2011; Sundaresan *et al* 2011; Chong *et al* 2012]. SIRT3 and SIRT4 are predominantly mitochondrial proteins and are thus highly expressed in tissues rich in mitochondria, including brown adipose tissue, liver, heart and brain. SIRT4 is also highly expressed in pancreatic beta (β)-cells and has been demonstrated to play key roles in the regulation of insulin secretion [Haigis *et al* 2006; Ahuja *et al* 2007]. Table 1.1 illustrates the differential cellular localisation and tissue distribution of the members of the sirtuin family.

TABLE 1.1 Cellular and tissue distribution of the members of the sirtuin family

Gene	Expression levels		Cellular compartmentation	Putative target genes	Potential link with diseases
	High	Low			
SIRT1	Br, Te, Sk, Ki (+++), Th, Ut (++)	Li, Sp, He Lu, Ov, BM	Nuclear	p53, Ku70, NFκB, PGC-1α, MEF2D, MyoD, PPARγ, FOXO, p300, AceCS1, tat	ageing, obesity, Insulin resistance, inflammation, diabetes, heart failure, axonal degeneration, AIDS
SIRT2	Br, Sk (+++), Li, Te, Ki, He (++)	Th, Lu, BM, Ut, Ov, Sp	Cytoplasmic	α-tubulin	downregulated in human gliomas
SIRT3	Ov (+++), most other organs (++)		Mitochondrial	PGC-1α, AceCS2	adaptive thermogenesis, overexpressed in node-positive breast cancer
SIRT4	Br, Te, He, Lu (+++), Li, Sk, Ki, Th, Ut, Ov (++)	BM, Sp	Mitochondrial	Glutamate dehydrogenase	inhibits amino acid-stimulated insulin secretion
SIRT5	Br, Te, Sk, Ki, He (+++) Li, Ov, Lu, Th, Ut, BM (++)	Sp	Mitochondrial	unknown	unknown
SIRT6	Fetal Br (+++), Br, Li, Te, Sk, Ki, He, Ov (++)	Sp, Th, Ut, BM, Lu	Nuclear	DNA polβ	age-related diseases
SIRT7	Br, Te, Ki Sp, Te, Li, Ki, Pa (proteins)	Lower in other organs Br, Sk, He (proteins)	Nuclear	RNA polymerase Pol I	highly expressed in thyroid cancers, overexpressed in node-positive breast cancer

Br - brain; Te - testis; He - heart; Ki - kidney; Sk - skeletal muscle; Li - liver; Lu - lung; Sp - spleen; Ov - ovary; Ut - uterus; BM - bone marrow; Th - thyroid; NFκB - nuclear factor kappa B; PGC-1α - coactivator-1 alpha; PPARγ - peroxisome proliferators-activated receptor gamma; AceCS1 - acetyl coenzyme A synthetase; Pol I - RNA polymerase I; RNA - Ribonucleic acid.

[Dali-Youcef *et al* 2007]

1.2.1.2 Enzymatic activity of sirtuins

Sirtuins are class III histone deacetylases (HDACs) that are largely regulated by cellular NAD⁺ availability and preferentially target non-histone proteins [Carafa *et al* 2012]. Deacetylation and adenosine diphosphate (ADP)-ribosylation are the two main chemical reactions catalysed by sirtuins. SIRT1, which is the closest homologue of the yeast Sir2, deacetylates both histone and non-histone proteins resulting in a wide range of physiological and metabolic effects [Carafa *et al* 2012]. Target proteins for SIRT1 include peroxisome proliferators-activated receptor gamma (PPAR-γ) and its coactivator-1 alpha (PGC-1α), forkhead transcriptional

factors, serine threonine liver kinase B1 (LKB1), nuclear factor-kappa beta (NF- κ B) and protein tyrosine phosphatase (PTP) [Chong *et al* 2012]. SIRT2 is a ubiquitous, nuclear and cytoplasmic protein deacetylase that inhibits adipocyte differentiation by regulating forkhead box transcription factor (FoxO1) acetylation [Borradaile and Pickering 2009].

1.2.2 PHYSIOLOGY OF SIRTUINS

There is an abundance of data demonstrating the role of mammalian sirtuins in metabolic processes such as gluconeogenesis [Sun *et al* 2007; Chen *et al* 2010; Li *et al* 2011], lipid metabolism, oxidative stress as well as on the benefits of calorie restriction. SIRT1 is found to be upregulated in tissues of calorie-restricted animals and through its interaction with PGC-1 α , plays a central role in control of energy metabolism and serves as a key mediator of the effects of calorie restriction [Cohen *et al* 2004]. This has led to immense interest in the role of sirtuins in the ageing process and diseases associated with ageing.

1.2.2.1 Cardiovascular effects

The cardiovascular benefits of SIRT1 activation have been well elucidated in several studies and appear to arise from its effects on maintaining endothelial homeostasis, effects on cardiac cell hypertrophy, direct effects as a vasodilator, antioxidative properties and its role in promoting myocardial recovery in the setting of ischaemia reperfusion injury. Below is a short summary of the cardiovascular effects of SIRT1 activation.

Cardiac effects

Data from animal models and *in vitro* studies suggest that sirtuins, in particular SIRT1, play an important role in the development and function of cardiomyocytes. A direct role for SIRT1 and SIRT7 in the development of the heart has been demonstrated by Cheng *et al* [2003] and Vakhrusheva *et al* [2008] in mouse models. Cheng *et al* demonstrated that SIRT1 mutant or deficient mice showed defects in cardiac septation [Cheng *et al* 2003]. Similarly, Vakhrusheva *et al* [2008] demonstrated SIRT7 deficiency associated cardiac hypertrophy and inflammatory cardiomyopathy. Shan *et al* [2012] report participation of SIRT1 in the development of congenital heart disease.

SIRT1 appears to play a role in the development of cardiomyocyte hypertrophy in mouse models. The anti-hypertrophy effects of SIRT1 may be mediated through adenosine monophosphate-activated protein kinase (AMPK) and Akt (also known as protein kinase B) pathways [Chan *et al* 2008]. Through PPAR- γ , SIRT1 prevents phenylephrine-induced neonatal cardiomyocyte hypertrophy and phenylephrine-induced downregulation of fatty acid oxidation genes [Maiese *et al* 2011]. SIRT3 and SIRT6 negatively regulate cardiac hypertrophy by inhibiting insulin-like growth factor-1 (IGF-1)-Akt signalling cascade [Giblin *et al* 2014]. Deletion of SIRT6 in mouse heart results in the development of cardiac hypertrophy [Giblin *et al* 2014].

Although increased expression of SIRT1 is associated with cardioprotective effects, Alcendor *et al* have shown that SIRT1 activation can serve as a double-edged sword depending on the degree of activity increase [Alcendor *et al* 2007]. In transgenic mice, mild to moderately increased expression of SIRT1 reduced myocardial hypertrophy, interstitial fibrosis and senescence markers. On the other hand, high levels of SIRT1 expression resulted in development of cardiomyopathy.

There appears to be an emerging role for sirtuins, especially SIRT1 and SIRT3 in the pathophysiology of heart failure. SIRT1 is normally localised to the cytoplasm in normal human cardiomyocytes. Tanno *et al* [2010] demonstrated, that in chronic heart failure, there occurs a nuclear translocation of SIRT1. It is felt that this nuclear accumulation might be an adaptive mechanism of cardiomyocytes in heart failure, given the cell protective effects of nuclear SIRT1. Resveratrol, a naturally occurring polyphenolic compound and a potent Sir2 homolog activator, prevents the development of concentric cardiac hypertrophy and cardiac dysfunction in spontaneously hypertensive rats [Thandapilly *et al* 2010]. A reduced expression of SIRT6 has been demonstrated in failing human and hypertrophic mouse hearts [Giblin *et al* 2014].

Effects on vascular and endothelial function

Endothelial dysfunction is considered a strong precursor of cardiovascular disease. Recent clinical studies suggest that endothelial dysfunction during ageing is associated with oxidative stress in endothelial cells [Donato *et al* 2007]. SIRT1 plays an important role in the oxidative stress response in different cell types. Given that

SIRT1 is highly expressed in vascular endothelial cells, it is therefore likely to play a key role in controlling endothelial cell function. SIRT1 directly controls endothelium-dependent vasodilatation and also regulates endothelial nitric oxide synthase (eNOS) expression and production of nitric oxide (NO) [Mattagajasingh *et al* 2007]. Inhibition of SIRT1 expression and activity in human umbilical vein endothelial cells (HUVEC) induces premature senescence through decreased eNOS expression and increased plasminogen activator inhibitor-1 (PAI-1) expression [Ota *et al* 2007; Csizar *et al* 2008]. In arteries isolated from humans with coronary heart disease, NO-dependent vasorelaxation in response to resveratrol is lost [Cruz *et al* 2006], although some dilatation was still observed from NO-independent pathways.

There is direct evidence for the role of SIRT1 expression and activity in age-associated endothelial dysfunction. Donato *et al* demonstrated reduced expression of SIRT1 in the endothelial cells obtained from older humans in comparison to younger humans with corresponding differences in age-related vascular endothelial function [Donato *et al* 2001]. *In vitro*, resveratrol blocks adhesion of monocytes and granulocytes to endothelial cells by inhibiting expression of vascular cell adhesion molecule-1 (VCAM-1) [Ferrero *et al* 1998].

Apart from direct effects on endothelial cell function, SIRT1 may also have a role to play in regulating blood pressure through its effects on vascular smooth muscle cells (VSMC). Miyazaki identified a direct role of SIRT1 in the control of blood pressure via regulation of angiotensin II receptor type 1 (ATR1) expression in VSMC

[Miyazaki *et al*]. Resveratrol has been shown to inhibit VSMC proliferation through p53-independent mechanisms [Baur and Sinclair 2006] and through inhibition of extracellular receptor kinase (ERK) activation [El-Mowafy *et al* 2008] in rat pulmonary arterial cell lines and human coronary smooth muscle cell lines respectively. SIRT1 activation enhances liver X receptor (LXR)-mediated inhibition of smooth muscle cell proliferation and foam cell formation in mice models [Li *et al* 2007].

SIRT1, a key regulator of the inflammatory process, also has a role in preventing atherosclerosis through different mechanisms. Endothelial cell-specific SIRT1 transgenic mice (SIRT1-transgenic/apolipoprotein E (Tg/apoE^{-/-})mice) showed improvement in high fat-induced impairment in endothelium-dependent vasodilatation and had fewer atherosclerotic lesions [Zang *et al* 2008]. This was mediated by endothelial specific expression of SIRT1, leading to inhibition of endothelial apoptosis and improved endothelial function. In these mice models, there was no change in blood levels of serum lipids or glucose, suggesting that these changes are very much at the level of endothelial cells. Other mechanisms involved in the anti-atherosclerotic effects of SIRT1 include regulation of tissue inhibitory metalloproteinase 3 (TIMP3) expression [Stein and Matter 2011], calcium/calmodulin-dependent protein kinase beta (CaMKK β) [Wen *et al* 2013], LXR-adenosine triphosphate (ATP) and nuclear factor kappa beta (NF- κ β) pathways [Zeng *et al* 2013].

Role in myocardial stress and ischaemia

Research data suggest a significant role of SIRT1 in ischaemia-related myocardial injury. Monocytic SIRT1 expression is reduced in patients with stable coronary artery disease and acute coronary syndromes [Breitenstein *et al* 2013]. Treatment with resveratrol, a well-known SIRT1 activator, during myocardial ischaemia reperfusion in rats, reduces rhythm disturbances, cardiac infarct size [Dernek *et al* 2004] and plasma levels of lactate dehydrogenase (LDH) and creatinine kinase (CK) [Hung *et al* 2004]. In murine models, SIRT1 protects the heart from ischaemia reperfusion injury through activation of FoxO1 and decreases in oxidative stress [Hsu *et al* 2010]. The anti-atherosclerotic effects of SIRT1 activation combined with its effects on angiogenesis [Fukida *et al* 2006; Guarani and Potente 2010] makes it a potential tool in the management of ischaemic heart disease.

1.2.2.2 *Effects on lipid metabolism*

The exact mechanism by which SIRT1 activation leads to an improvement in lipid metabolism in man has not yet been fully elucidated. However, several potential mechanisms have been implicated in animal models and appear promising. SIRT1 modulates hepatic lipid homeostasis in SIRT1 knockout and transgenic mouse models [Purushotham *et al* 2009; Wang *et al* 2010]. PPAR- γ plays an important role in adipogenesis by increasing free fatty acid uptake and decreased lipolysis. In white adipose tissue, SIRT1 mobilises fatty acids and prevents preadipocyte differentiation via PPAR- γ [Borradaile and Pickering 2009].

Liver X receptors are nuclear receptors that are involved in cholesterol and lipid homeostasis. Li and colleagues have shown that SIRT1 deacetylates and positively regulates nuclear LXRs, which could alter cholesterol transport and metabolism [Li *et al* 2007]. SIRT3, SIRT4 and SIRT6 also play a role in regulating fatty acid oxidation [Hirshey *et al* 2010; Kim *et al* 2010; Nasrin *et al* 2010; Kendrick *et al* 2011].

1.2.3 SMALL MOLECULE ACTIVATORS OF SIRT1 ACTIVITY

Several small molecule SIRT1 activators have been developed given the varied effects of SIRT1 activation on different metabolic processes. Initially identified SIRT1 activators were all plant polyphenols. Plant derived foods contain reactive polyphenols with a role in oxidative stress. Resveratrol is a well-known polyphenolic compound found in red wine that is a potent activator of SIRT1 activity [Howitz *et al* 2003]. Isoflavanes and silibinin induce SIRT1 expression in cardiomyocytes [Zhou *et al* 2006] and renal proximal tubular cells [Rasbach and Schnellmann 2008]. Since the discovery of resveratrol, several other synthetic small molecule activators (SRT1460, SRT2183, SRT1720) have been identified which are several-fold more potent than resveratrol [Milne *et al* 2007]. Data focussing on the beneficial effects of these and several other small molecule SIRT1 activators in man are limited with varying results and require more work to show consistent and safe beneficial effects of SIRT1 activation. With advancing age of the general population, there is an increase in the incidence of age-related diseases such as metabolic syndromes, diabetes, cardiovascular disease and cancers. Therefore, development of

a drug with a potential to address these disease processes remains a focus of great interest amongst researchers.

1.3 THE UROCORTIN - CORTICOTROPHIN-RELEASING HORMONE (CRH) SYSTEM

Urocortins belong to the corticotrophin-releasing hormone (CRH) family which includes CRH, fish urotensin I, frog sauvagine, urocortin 1, urocortin 2 and urocortin 3 [Boonprasert *et al* 2008] (Figure 1.2). Corticotrophin-releasing hormone is produced in the brain in response to stress, has central effects upon behaviour, and exerts a variety of peripheral responses. However, CRH is unlikely to have major effects upon cardiac function as it is not expressed locally and its plasma concentrations are very low.

In 1995, Vaughan and colleagues observed urotensin-like immunoreactivity in the Edinger Westphal nucleus and lateral superior olive regions of the adult rat brain. It was named urocortin (now known as urocortin 1) to reflect its similarities of structure and biological properties to urotensin (suckerfish urotensin) and rat CRH [Vaughan *et al* 1995]. It is believed to be the second endogenous mammalian ligand for CRH receptors (CRH-R) [Skelton *et al* 2000]. Subsequently, two further paralogues of CRH were identified; urocortin 2 and urocortin 3. Human CRH and urocortin 1 genes have been localised to chromosomes 8 (8q13) and 2 (2p23-p21) respectively. Urocortin 2 and urocortin 3 have prominent cardiovascular roles and are expressed in the heart. In contrast to CRH, the urocortins do not increase corticosterone secretion and do not appear to have any physiologic role in the regulation of the hypothalamic-pituitary-adrenal axis [Davidson *et al* 2009; Davidson and Yellon 2009].

	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
CRH	A	R	A	E	Q	L	A	Q	Q	A	H	S	N	R	K	L	M	E	I	I
Urocortin	A	R	T	Q	S	Q	R	E	R	A	E	Q	N	R	I	I	F	D	S	V
Urocortin	A	R	Y	K	A	A	R	N	Q	A	A	T	N	A	Q	I	L	A	H	V
Urocortin	D	K	A	K	N	L	R	A	K	A	A	A	N	A	Q	L	M	A	Q	I
Sauvagine	E	K	Q	E	K	E	K	Q	Q	A	A	N	N	R	L	L	L	D	T	I
Urotensin	A	R	I	E	N	E	R	E	Q	A	G	L	N	R	K	Y	L	D	E	V

Figure 1.2. Amino acid sequences of CRH and its analogue peptides. Sequences shown are that of mammalian CRH and urocortins, amphibian sauvagine and teleost urotensin 1. Highlighted sequences represent similarity to CRH. CRH - corticotrophin-releasing hormone.

1.3.1 CRH RECEPTORS

The effect of CRH and urocortins is mediated via CRH receptors. These seven transmembrane G protein-coupled receptors are members of the secretin family [Hillhouse and Grammatopoulos 2006] and the human CRH receptor gene has been localised to chromosomes 17 (17q12-qter) and 7 (7p21-p15) [Vamvakopoulos and Sioutopoulou 1994; Meyer *et al* 1997]. Two subtypes of CRH receptors have been identified in mammals and rodents; corticotrophin-releasing hormone receptor 1 (CRH-R1) and -R2. Structurally, the two subtypes exhibit considerable divergence at the N-terminus, consistent with their distinct pharmacological properties. Furthermore, three splice variants of CRH-R2 have been identified. These variants differ in the structure of their N-terminal extracellular domain. R2a and R2b have been observed in rodents and in man, whilst R2g is specific to humans (isolated in the limbic regions of the human brain) [Hillhouse and Grammatopoulos 2006]. It is, however, unclear whether the g splice variant has any specific physiological role. Low homology of the extracellular domains of CRH-R1 and -R2 account for differences in their ligand specificity [Hillhouse and Grammatopoulos 2006]. Urocortin 1 and CRH both act at CRH-R1 but the affinity of urocortin 1 for CRH-R2 is more than 10-fold higher than that of CRH [Skelton *et al* 2000]. While urocortin 1 can activate both receptors, urocortins 2 and 3 are potent and specific agonists at CRH-R2 [Vaughan *et al* 1995; Hsu and Hsueh 2001] with little effect at CRH-R1.

1.3.2 BIOLOGY OF UROCORTINS

1.3.2.1 *Anatomy (tissue distribution of urocortins and CRH receptors)*

Immunoreactivity to the urocortins and their receptors has been demonstrated in the central nervous, digestive, reproductive, cardiovascular, immune and endocrine systems, suggesting important roles throughout the body [Oki and Sasano 2004]. In the brain, urocortin 1 is most prominent in the Edinger Westphal nucleus and lateral superior olive. Urocortin 1 messenger ribonucleic acid (mRNA) or immunoreactivity has also been reported in other regions of the brain, such as the cerebellum and hypothalamus [Hillhouse and Grammatopoulos 2006], and it appears to be co-localised with dopamine in the basal ganglia and hypothalamus. Urocortin 1 mRNA is also expressed in VSMC and in cardiac myocytes. Urocortin 2 has a similar distribution in the central nervous system in mice and rats, but is also seen in high concentrations in the peripheral tissues including the heart, adrenals, placenta, stomach, ovary, skin, gastrointestinal tract, uterine smooth muscle, skeletal muscle and peripheral blood vessels [Hillhouse and Grammatopoulos 2006].

The distribution of urocortin is distinct. In the central nervous system, it is demonstrable in regions of high CRH-R2 expression, supporting the notion that it is an endogenous ligand [Hillhouse and Grammatopoulos 2006]. In humans, urocortin 3 is also seen in peripheral tissues such as adrenals, heart and kidney – particularly in the distal tubules [Takahashi *et al* 2004].

Corticotrophin-releasing hormone receptor 1 is predominantly found in the central nervous system. In addition to its central nervous system expression,

CRH-R2 is found in peripheral tissues such as the gut, heart, lymphocytes and adrenals. In humans, urocortin 1 and CRH-R2a have been identified in all four chambers of the heart, suggesting that urocortin acts in an autocrine or paracrine fashion through CRH receptors [Kimura *et al* 2002]. In contrast to rats where CRH-R2b is the predominant splice variant in the heart and VSMC, humans appear to predominantly express CRH-R2a in these tissues. CRH-R2 has also been characterised in the human left ventricle and intramyocardial blood vessels [Wiley and Davenport 2004]. In humans, both CRH-R1 and -R2 are found in the periphery, although their specific role remains to be fully characterised in human physiology and pathophysiology.

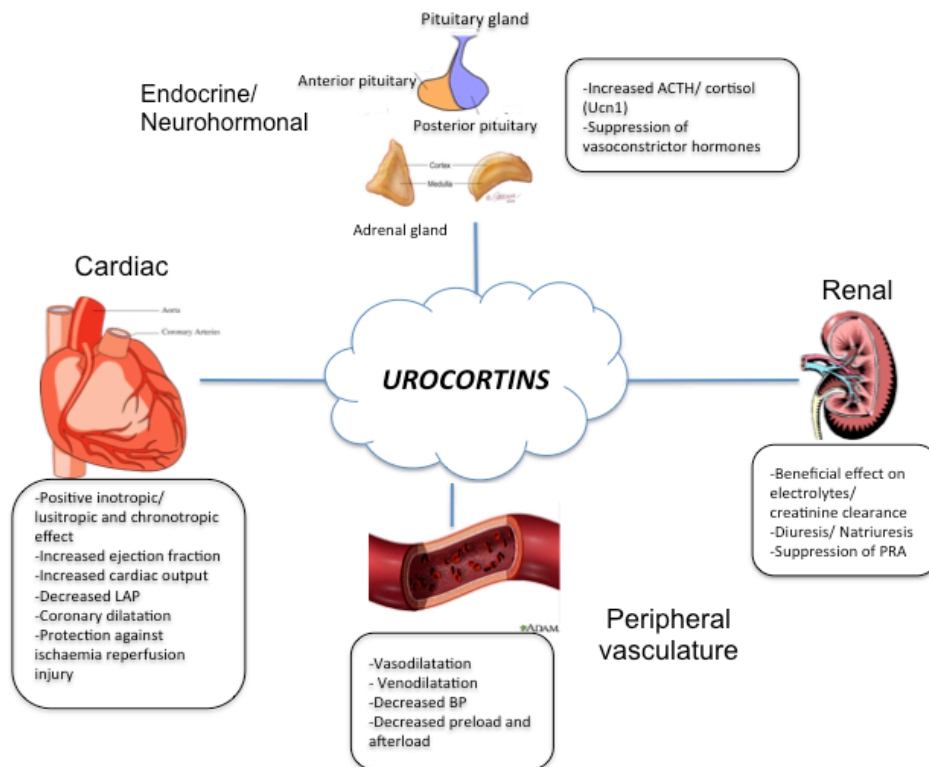


Figure 1.3. The effects of urocortins on multiple organ systems in heart failure.

Urocortins, through different mechanisms exerts a multitude of beneficial effects on the various organ systems as demonstrated by animal and human models.

ACTH - adrenocorticotrophic hormone; Ucn 1 - urocortin 1; LAP - left atrial pressure; BP - blood pressure; PRA - plasma renin activity.

1.3.2.2 *Biochemistry*

Molecular structure and Pharmacokinetics

Urocortin is a 40 amino acid-containing neuropeptide, related to urotensin (63% sequence identity) and corticotrophin-releasing hormone (45% sequence identity) [Vaughan *et al* 1995]. Rat and human urocortin bear 95% homology to each other. The precursor protein contains 122 amino acid residues with an N-terminal methionine and consensus signal peptide sequence, whilst the carboxy terminus of the precursor contains the C-terminal amidated peptide of urocortin. The CRH analogue peptides possess a helical conformation with varying degrees of amphipathicity. The amphipathic N-terminal helices could play a crucial role in selectivity of the analogues to CRH-R1, whereas it may not be as important for CRH-R2 binding [Grace *et al* 2007]. The parent protein is half the length of urotensin and CRH precursors with little sequence similarity to either [Hillhouse and Grammatopoulos 2006]. Urocortin 2 shows moderate homology with human and rat CRH (34%), urocortin 1 (43%) and urocortin 3 (37-40%). The half-life of urocortin 1 in healthy humans and those with stable heart failure is approximately 50 minutes [Davis *et al* 2004; Davis *et al* 2005]. Urocortin 2 has a shorter half-life of 10 minutes in healthy humans [Davis *et al* 2007a]. The exact half-life of urocortin 3 is not yet known, but it appears to have a more rapid onset and shorter duration of action in animal studies [Rademaker *et al* 2006].

CRH Binding Protein

Corticotrophin-releasing hormone-binding protein (CRH-BP) is a 37-kDa protein that was first isolated in human plasma in 1989 [Behan *et al* 1989] and binds to both

CRH and urocortin 1. Given that the expression of CRH-BP overlaps that of both CRH and urocortin in the central nervous system, it has been proposed that the CRH-BP plays a role in the modulation of the action of urocortin and CRH at these sites. In humans, CRH-BP has been detected in the brain, pituitary, liver and placenta [Potter *et al* 1992; Petraglia *et al* 1993; Lovejoy *et al* 1998]. In ovine models of heart failure [Rademaker *et al* 2002] the half-life of urocortin 1 was markedly prolonged compared to human models. This has been attributed to the possible role of CRH-BP in clearance of urocortin 1 in humans. However, the exact role of these binding proteins remains to be fully elucidated in health and disease states.

Intracellular signalling pathways

The urocortins bind to G protein-coupled CRH (R1 and R2) receptors to induce conformational changes in the receptor that activate intracellular signalling pathways (Figure 1.4). In most cells, this involves adenylyl cyclase and cyclic adenosine monophosphate (cAMP). Indeed, Kageyama and colleagues demonstrated that urocortin 2 induces vasorelaxation in VSMC via CRH-R2 in association with increased cAMP accumulation via activation of adenylate cyclase [Kageyama *et al* 2003a; Kageyama *et al* 2005]. Mitogen-activated protein kinases (MAPK) are also implicated in urocortin-mediated vasodilatation [Kaageyama *et al* 2003a] as well as in the cardioprotective role of urocortin in response to ischaemic or hypoxic injury [Brar *et al* 2000]. However, in some studies, inhibition of the cAMP or protein kinase A (PKA) pathway has failed to inhibit the effects of CRH and its related peptides, suggesting the involvement of other signalling mechanisms.

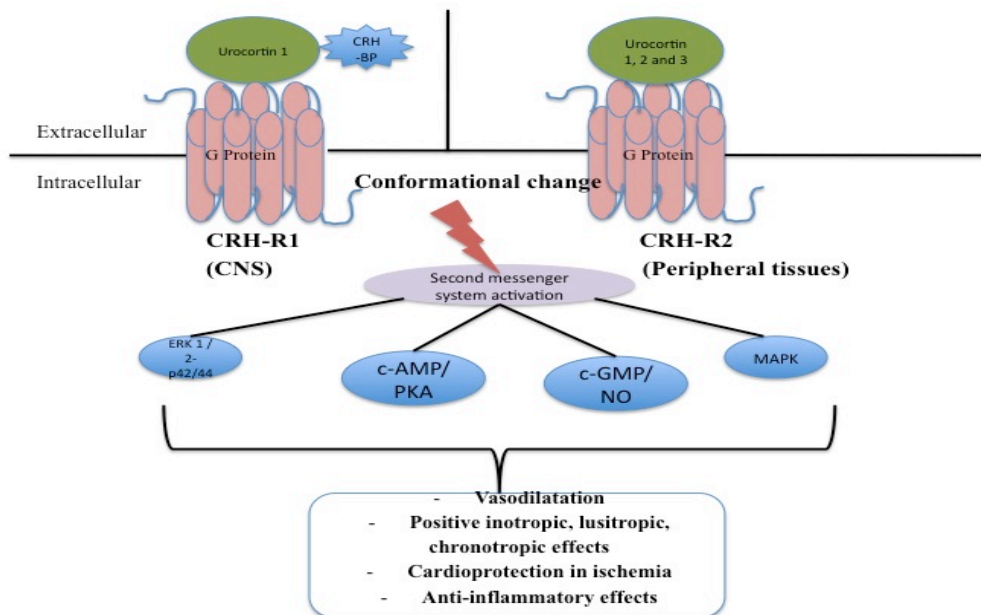


Figure 1.4. Schematic of urocortin intracellular signalling pathway.

Urocortins bind to CRH receptors to induce conformational changes in the G protein-coupled receptors and activate the second messenger systems. CRH-BP has a greater affinity to urocortin 1 and may play a role in its metabolism. Urocortin 1 binds to both CRH-R1 and -R2, whereas urocortins 2 and 3 are potent, specific CRH-R2 agonists.

CRH-BP - corticotrophin-releasing hormone binding protein; CRH-R - corticotrophin-releasing hormone receptor; CNS - central nervous system; ERK 1/2-p42/44 - extracellular signal-related kinases 1/2-p42/44; c-AMP - cyclic adenosine monophosphate; PKA - protein kinase A; c-GMP - cyclic guanosine monophosphate; NO - nitric oxide; MAPK - mitogen-activated protein kinase.

Corticotrophin-releasing hormone and related agonists can evoke the endothelial release of nitric oxide via nitric oxide synthase with subsequent accumulation of cyclic guanosine monophosphate (cGMP). This endothelium-dependent mechanism is important in CRH or urocortin-induced relaxation in placental vasculature [Clifton *et al* 1995] and peripheral arteries, and has been studied in isolated arterial segments such as the human internal mammary artery graft [Chen *et al* 2005] and in rat coronary artery [Huang *et al* 2002]. The nitric oxide and cGMP-dependent component of this vasodilator effect is mediated via activation of calcium-activated potassium channels in underlying vascular smooth muscle. Indeed, the vasorelaxant effect of urocortin is blunted in the presence of N^G-nitro-L-arginine methyl ester (L-NAME) a nitric oxide synthase inhibitor and ODQ (1H-[1,2,4] oxadiazolo [4,2-a] quinoxalin-1-one; inhibitor of guanylyl cyclase) [Chen *et al* 2005]. There is increasing evidence that urocortin and CRH-related peptides play an important role in cell survival mechanisms in a number of systems. Similar to CRH, urocortin 1 activates the MAPK extracellular signal-related kinases (ERK) 1/2-p42/44 signalling cascade in *in vitro* cultures of isolated rat cardiac myocytes, which is inhibited by blockade of MEK 1/2. This signalling cascade mediates the cardioprotective function of urocortin in stimulated hypoxia or ischaemia [Brar *et al* 2000]. Urocortins also possess anti-inflammatory properties that appear to be mediated via pro-apoptotic effects on macrophages via a direct effect on pro-apoptotic Bcl-2 related proteins [Tsatsanis *et al* 2005].

1.3.2.3 *Physiology of urocortins*

Cardiovascular actions

The important roles of urocortins in the regulation of normal cardiovascular physiology are being increasingly recognised. Genetically engineered mice lacking CRH-R2 are resistant to otherwise marked urocortin-evoked changes in cardiac performance and blood pressure [Coste *et al* 2000]. Studies, which are largely preclinical, have so far demonstrated a favourable effect of urocortins on haemodynamic and neurohumoral regulation. Urocortins 1, 2 and 3 produce positive inotropic and lusitropic effects, reduction in the mean arterial pressure due to decreased peripheral vascular resistance, and increased coronary perfusion in rodent and ovine studies.

Vascular effects

Central administration of CRH in rats produces a pressor response, which appears to be mediated through CRH-R1 receptors [Briscoe *et al* 2000]. Peripheral administration of CRH produces a depressor response, which is blocked by a helical CRH (a non-selective CRH antagonist), and not by antalarmin (selective CRH-R1 antagonist). As a peptide, CRH has limited access to the central nervous system. This suggests that the hypotensive effect is mediated via peripheral CRH-R2 receptors. CRH-R2 may have a more prominent role in changes in arterial pressure in comparison to CRH-R1. CRH-R2 may contribute to the maintenance of basal vascular tone in mice. Indeed, mice deficient in CRH-R2 receptors are hypertensive with no fall in the mean arterial pressure in response to exogenously administered

urocortin [Coste *et al* 2002]. It remains unknown whether urocortins and CRH receptors have a similar role in the maintenance of basal vascular tone in humans.

Intravenous administration of CRH produces vasodilatation in rats and a consequent fall in blood pressure with a reflex increase in heart rate, although this is not observed in sheep and is seen only with relatively high doses in higher primates like monkeys and humans. In anaesthetised rats, intravenous injection of human urocortin 2 reduced basal systemic blood pressure in a dose-dependent fashion [Chen *et al* 2003]. As demonstrated by Vaughan and colleagues [1995], urocortin 1 also possesses a potent and long lasting hypotensive action. Decrease in mean arterial pressure of 18 ± 1 mmHg was observed with urocortin 1 in rats and lasted for almost 2 hours. Thomas Dieterle's group [Dieterle *et al* 2009] studied the effects of urocortin 2 injection in control and hypertensive rats. They showed an immediate and sustained lowering of blood pressure in hypertensive rats with no rise in heart rate. This effect on the blood pressure was seen for up to 12 hours after intraperitoneal injection of urocortin 2.

Several mechanisms have been postulated for the blood pressure lowering effects of urocortins. These may include a direct smooth muscle relaxant effect in combination with an associated reduction in plasma concentrations of vasoconstrictor hormones, such as endothelin 1, angiotensin II and arginine vasopressin (AVP), as seen in animal models of heart failure (see section 1.3.3.3 on Neurohormonal effects). *Ex vivo* studies in the human internal mammary artery [Chen *et al* 2005] and in the

rat coronary artery [Huang *et al* 2002] suggest both endothelium-dependent and -independent components for vasorelaxation. In isolated rat coronary artery [Huang *et al* 20002], potent vasorelaxant effect of urocortin 1 was observed with an IC_{50} of 2.24 nM, in the presence of an intact endothelium. The endothelium-dependent component appears to be, at least in part, mediated by nitric oxide via cGMP, as outlined above. Huang and colleagues [2002] have also shown the role of activation of barium chloride (BaCl₂) potassium channels in arterial smooth muscle cells, mediating the endothelial component of urocortin-induced coronary relaxation. Indeed, these studies also demonstrate a blunted, but not abolished, vasorelaxant response to urocortin in endothelium-denuded arterial segments, further suggesting the role of additional endothelium-independent mediators. Endothelium-independent regulation of vascular tone appears to involve calcium (Ca²⁺)-independent phospholipase A₁ and store operated Ca²⁺ entry modulation [Smani *et al* 2007]. Other mechanisms demonstrated in mediating urocortin-induced vasorelaxation include the MAPK and protein kinase A pathway [Kageyama *et al* 2003a].

Cardiac effects

It is not entirely clear whether urocortins mediate their protective effects upon cardiac contractility via a direct effect on cardiac myocytes or via sympathetic stimulation in response to reduced peripheral resistance. Brar and colleagues [2000] have demonstrated the presence of a 22 kDa urocortin 1 precursor protein in neonatal rat cardiac myocytes and release of urocortin into the supernatant of cardiac myocytes exposed to stimulated ischaemia or hypoxia, suggesting endogenous

release of urocortin from ischaemic cardiac myocytes. This may suggest potential direct and local action of urocortins on cardiac myocytes, mediated via CRH receptors.

To determine whether urocortin induced direct effects on contractility of cardiac myocytes, Yang and colleagues [2006] observed the effects of application of 100 nmol/L of urocortin 2 to isolated adult rabbit ventricular cardiomyocytes. They showed a progressive enhancement in myocyte contractility with reduction in diastolic length and concluded that urocortins exert direct positive inotropic as well as lusitropic effects. These effects were mediated via activation of CRH-R2 and subsequent stimulation of protein kinase A activity, leading to augmentation of L-type calcium channel (I_{Ca}), and sarcoplasmic and endoplasmic reticulum Ca^{2+} ATPase (SERCA)-mediated Ca^{2+} uptake into the sarcoplasmic reticulum.

Immediate improvement of left ventricular fractional shortening and circumferential fibre shortening velocity were noted following acute injection of urocortin 2 in rats [Dieterle *et al* 2009]. These beneficial effects were preserved even at 5 weeks after treatment. In ovine models, intravenous injection of urocortin 1 causes a marked dose-dependent increase in cardiac output and contractility, which is sustained at 24 hours post injection [Parkes *et al* 2001].

Effects in humans

A small number of clinical studies have carried forward data obtained from animal studies, to look at the effects of exogenous administration of urocortins on cardiovascular and neurohormonal responses.

In contrast to the favourable haemodynamic responses seen in normal sheep, infusion of urocortin 1 in healthy humans caused no change in haemodynamic variables, nor did it affect the plasma concentrations of humoral factors, such as aldosterone or arginine vasopressin. The pharmacokinetics of urocortin 1 were similar in normal humans and in subjects with heart failure: plasma urocortin 1 half-life of 52 ± 3 minutes in healthy volunteers and 54 ± 3 minutes in those with heart failure [Davis *et al* 2005]. Pemberton and colleagues [Davis *et al* 2005] did not observe any increase in urinary urocortin 1 following its infusion suggesting that urocortin 1 is not excreted in the kidneys. Consistent with its predicted cardiovascular profile, urocortin 2 infusion caused a dose-dependent increase in cardiac output with a decrease in mean arterial and diastolic blood pressure, and systemic vascular resistance [Davis, Pemberton *et al* 2007a]. The effects of systemic urocortin 3 in humans are yet to be explored, although recently, Gheorghide *et al* [2013] have examined the effects of intravenous human stresscopin (a 40 amino acid peptide, derived from the same gene as urocortin 3 in patients with heart failure. Wiley and Davenport [2004] have demonstrated that urocortin 3, like urocortin 2, causes vasodilatation in isolated human internal mammary artery segments and that this effect is mediated via the direct effect of urocortin 3 on the VSMC. Although not yet

assessed in man, urocortin 3 is likely to exert cardiovascular effects similar to those evoked by urocortin 2.

Cardioprotective effects - role in ischaemia reperfusion injury

Urocortins appear to have a cardioprotective role and indeed, urocortin expression and peptide release is increased by ischaemia [Brar *et al* 1999].

Urocortin 1 reduces myocyte cell death caused by ischaemia reperfusion injury. This appears to be mediated via several mechanisms including upregulation of cardiotrophin 1 expression [Talwar *et al* 2000], stimulation of heat shock protein [Brar *et al* 2002] and natriuretic peptides, and attenuation of calcium insensitive phospholipase A2 gene expression [Lawrence *et al* 2003]. *In vitro* secretion of urocortin is also enhanced by inflammatory cytokines such as interleukin-6, interleukin-1 and tumour necrosis factor alpha: factors that are elevated in patients with heart failure and acute coronary syndromes.

Urocortins 2 and 3 protect neonatal rat cardiac myocytes *in vitro* when administered before hypoxia or at the point of reoxygenation. Urocortins 2 and 3 also protect the adult rat heart *ex vivo* and acts via the MAPK pathway to reduce the infarct size of a perfused intact rat heart exposed to regional ischaemia [Brar *et al* 2004]. Brar and colleagues have demonstrated that urocortin induces ERK 1/2-p42/44 phosphorylation in neonatal rat cardiac myocytes and that inhibition of MEK 1/2 inhibits its cardioprotective effects [Brar *et al* 2004].

Nitric oxide is recognised as a key determinant of vascular health. It acts as a potent vasodilator, inhibits expression of several proinflammatory cytokines and chemokines, and plays a key role in vascular smooth muscle proliferation, platelet aggregation and endogenous fibrinolysis [Behrendt and Ganz 2002; Ganz and Vita 2003; Yang *et al* 2008]. Whilst urocortin may act via nitric oxide to mediate cardiovascular protective effects [Yang *et al* 2008], studies to date have not yet completely elucidated the effect of urocortins on the endothelium or nitric oxide.

1.3.3 UROCORTINS AND HEART FAILURE

Given the potent vasorelaxant and inotropic effects of the urocortins, interest has grown in their role in the pathophysiology and potential therapeutic utility in the treatment of heart failure. A number of studies have demonstrated increased concentrations of urocortin 1 in cardiac tissue [Ikeda *et al* 1998; Nishikimi *et al* 2000] and plasma [Rademaker *et al* 2002; Ng *et al* 2004; Charles *et al* 2006] in experimental and clinical heart failure. Nishikimi and colleagues [2000] demonstrated upregulation of expression of urocortin 1 mRNA in left ventricular hypertrophy. They also demonstrated increased urocortin 1 immunoreactivity in the failing heart. This has been cited as possible evidence that urocortins play a role in the pathophysiology of cardiac hypertrophy and heart failure.

1.3.3.1 *Animal models*

Preclinical studies of heart failure have previously examined the roles of urocortin 1 and 2 whilst urocortin 3 remains less well characterised. The beneficial

cardiovascular and neurohumoral responses of urocortins are preserved, and may be augmented in the presence of heart failure.

Intravenous infusion of urocortin 1 in an ovine model of heart failure attenuates the haemodynamic deterioration and harmful neurohormonal activation associated with heart failure [Rademaker *et al* 2007]. These hormones include renin, angiotensin II, aldosterone, endothelin-1, vasopressin and catecholamines that, along with sympathetic nervous system activation, combine to exert the haemodynamic and endocrine hallmarks of heart failure. Furthermore, in this model, urocortin 1 also protected renal function. The preservation of cardiac output with urocortin 1 infusion may be partly due to its inotropic actions, in addition to its coronary arterial vasodilator effects and improved cardiac bioenergetics. When infused at the onset of left ventricular pacing, urocortin 1 infusion restricts the increase in left atrial pressure and attenuates the reduction in cardiac output. It also has lusitropic and venodilating effects [Sanz *et al* 2002]. These results highlight that treatment with urocortin 1 may be beneficial in the treatment of heart failure initiated early in the disease. Of note, these favourable haemodynamic effects persisted during prolonged (4-day) infusion of urocortin 1.

In keeping with a favourable haemodynamic profile of urocortin 1 in ovine heart failure, urocortin 2 infusion in muscle specific LIM protein (MLP) deficient mice, a model of dilated cardiomyopathy, caused a dramatic improvement in cardiac output and left ventricular function, enhanced cardiac contractility and reduced systolic load [Bale *et al* 2004]. The enhancement in ejection fraction by urocortin 2 is partly

attributed to reduction in arterial load: this may prove more notable in the failing heart where there is an afterload mismatch.

Patients with heart failure are likely to receive any new treatment in conjunction with conventional evidence based therapy. Hence it is important to ensure that any potential new treatment does not interact with and has benefits in addition to drugs such as angiotensin-converting enzyme (ACE) inhibitors and beta (β)-blockers. Rademaker and colleagues [Rademaker *et al* 2008] assessed the combined effects of captopril with urocortin 2 in sheep with pacing-induced heart failure. Combined treatment of urocortin 2 with captopril augmented the decrease in total peripheral resistance by an additional 20% compared with either agent alone. One of the potential drawbacks of treatment with ACE inhibitors is its profound hypotensive effect, which in the presence of heart failure can compromise blood flow to vital organs, such as the kidneys. When used in combination with an ACE inhibitor, urocortin 2 evoked an additional decrease in peripheral resistance without further reduction in the systolic blood pressure. This makes the combination of these agents an attractive tool in the management of heart failure. In addition, combination of urocortin 2 with captopril improved cardiac performance, and decreased peripheral resistance and ventricular filling pressures in association with reductions in plasma aldosterone and endothelin-1 concentrations. In a murine acute heart failure model, pretreatment with a beta-adrenergic receptor antagonist, did not affect the inotropic or lusitropic actions of urocortin 2 *in vivo* indicating that its actions are independent of beta-adrenergic receptors [Bale *et al* 2004].

The effects of combined treatment with urocortin 2 and furosemide has been studied recently [Rademaker *et al* 2009]. When this combination was administered to sheep with pacing-induced heart failure, it caused increased diuresis, natriuresis and sustained increase in creatinine excretion and clearance without additional potassium elimination. Urocortin 2 alone or in combination increased cardiac output and contractility whilst furosemide had no effect. Importantly, the combination of the two drugs, produced reversal of furosemide-induced increase in plasma renin activity and a greater decrease in plasma aldosterone and vasopressin concentrations. These beneficial effects of urocortin 2 may be mediated by an increase in cardiac output and consequently improved renal perfusion. Indeed, improvements in glomerular filtration rate, urine volume and sodium excretion have been demonstrated in the ovine model of heart failure [Rademaker *et al* 2005b]. In addition to increased cardiac output and renal vasodilatation, other postulated mechanisms include a direct tubular action of urocortin 2 [Hsu and Hsueh 2001] as well as attenuation of anti-natriuretic and anti-diuretic factors resulting in increased diuresis and natriuresis [Rademaker *et al* 2009].

As already noted, the haemodynamic and humoral responses of urocortin 3 in experimental heart failure are less well studied than urocortin 1 or 2. In a study of sheep with pacing-induced heart failure [Rademaker *et al* 2006], the haemodynamic responses produced by urocortin 3 were similar to that produced by equivalent doses of urocortin 1 or 2. Urocortin 3 caused a marked dose-dependent improvement in cardiac output and reduction in peripheral resistance and left atrial pressure. This was associated with a reduction in mean arterial pressure, beneficial effect on hormonal

responses (attenuation of vasoconstrictor peptide systems) and improved renal function (dose-dependent increases in urine volume, sodium and creatinine excretion). The onset and duration of action was much shorter than that of urocortin 1 or 2.

1.3.3.2 *Patients with heart failure*

Plasma concentrations of urocortins are elevated in patients with heart failure. Ng and colleagues [2004] found higher plasma urocortin 1 concentrations in men with heart failure as well as in elderly patients. There appeared to be an inverse relationship between plasma urocortin concentrations and New York Heart Association (NYHA) class. In more severe heart failure, as reflected by NYHA classes III or IV and low left ventricular ejection fraction, plasma urocortin concentrations appeared to be suppressed, suggesting that upregulation of the urocortin system in early heart failure may be cardioprotective. In keeping with this report, more recent work by Wright and colleagues [2009] has demonstrated elevated concentrations of plasma urocortin 1 in patients with heart failure with positive relationships to other circulating neurohormones such as brain natriuretic peptide, adrenomedullin and endothelin-1. However, in contradiction to findings from Ng *et al* [2004], Wright's group [Wright *et al* 2009] noted an inverse relationship of the level of plasma urocortin 1 to left ventricular ejection fraction, with a linear increase in plasma concentration of urocortin 1 with increasing NYHA class. It is possible that this difference in results is attributable to differences in the immunoassay used or potential effect of CRH-BP on the assay performance. Further

research is, however, required to establish the relationship between urocortin concentrations and NYHA class.

Systemic intravenous infusions of urocortin 1 and 2 have been administered to a small number of patients with heart failure. Urocortin 1 infusion increased corticotrophin and cortisol, but produced no changes in haemodynamic, renal or neurohormonal parameters [Davis *et al* 2005]. Infusion of urocortin 2 [Davis *et al* 2007a] evoked an increase in cardiac output with peripheral vasodilatation and a small increase in heart rate. Consistent with findings from the ovine model, systolic blood pressure fell in patients with congestive cardiac failure but not in control subjects [Davis *et al* 2007b]. It has been hypothesised that, in the presence of heart failure, the urocortin-induced rise in cardiac output is insufficient to compensate for the pronounced decrease in systemic vascular resistance [Davis *et al* 2007b]. However, this phenomenon may otherwise be explained by heightened peripheral sensitivity to the vasodilator effects of urocortin in the presence of heart failure. In agreement with this suggestion, administration of a CRH-R2 antagonist increases mean arterial blood pressure in sheep with heart failure but not in those without [Rademaker *et al* 2005c]. When administered in acute decompensated heart failure [Wandy Chan *et al* 2013], urocortin 2 markedly augmented cardiac output without significant reflex tachycardia. More recently, intravenous administration of human stresscopin (JNJ-39588146) has shown to increase cardiac index and reduction in systemic vascular resistance in chronic heart failure and reduced ejection fraction [Gheorghide *et al* 2013].

Apart from the positive influences on cardiovascular parameters when used in treatment in heart failure, urocortins may serve as potential biomarkers in identification of early heart failure, in combination with other biomarkers such as brain natriuretic peptide [Wright *et al* 2009].

1.3.3.3 Neurohormonal effects of urocortins in heart failure

As noted above, urocortins cause a pronounced suppression of vasoconstrictor hormones in animal models of heart failure, which further supports its potential therapeutic role. However, studies of urocortins 1 and 2 in humans have only shown modest changes in neurohormonal activity. It is important to note that in man, infusion of urocortin 2 in healthy volunteers and in patients with heart failure does not alter plasma adrenocorticotrophic hormone or cortisol concentrations. Table 1.2 summarises the effects of urocortins on neurohormonal activity in ovine and human experiments.

1.3.4 FUTURE OF UROCORTINS

There is increasing evidence that urocortins have several potential uses in management of cardiovascular conditions such as hypertension, ischaemic heart disease and heart failure. The immediate and sustained blood pressure lowering effects by urocortin 2 [Dieterle *et al* 2009] appears to pose a novel and attractive approach for antihypertensive treatment. The favourable effects on haemodynamics, renal and neurohumoral mechanisms have generated much interest in the use of urocortins in heart failure. In particular, the positive inotropic effect, combined with its ability to reduce peripheral arterial resistance, favours use in this group of

patients. In humans, studies to date have largely looked at combined systemic effects of urocortins. Direct arterial and venous effects of urocortins have not yet been described in man. It has not been possible to tease out the relative contribution of urocortin-induced changes in haemodynamic variables on the augmentation of cardiac output. Further studies are required to look at potential effects of long term administration of these peptides.

In the search for novel treatments for heart failure, the focus is on urocortins 2 and 3. Indeed, urocortin 1 has no haemodynamic effects in man and also bears the potential to induce unwanted side effects by activating CRH-R1 and stimulation of the hypothalamus-pituitary axis. However, it may have a role as an early biomarker of heart failure.

In conclusion, urocortins are emerging as an important group of peptidic mediators with important roles in human physiology and pathophysiology. There are several other potential applications of this group of peptides, including roles in appetite suppression, in muscle wasting and central nervous system disorders to name a few. However, their major effects in the cardiovascular system implicate them as potential therapeutic targets in a range of processes, particularly heart failure.

TABLE 1.2 Neurohormonal effects of urocortins in human and ovine models

	Humans					Sheep				
	Healthy		Heart Failure		Ucn2	Healthy		Heart Failure		
	Ucn 1	Ucn 2	Ucn 1	Ucn 2		Ucn 3	Ucn1	Ucn 1	Ucn 2	Ucn 3
cAMP	=	↑	=	=	=	=		↓ ¹ ↑ ²	↓	↓↓
cGMP	↑	↑		=						
ACTH	↑↑	=	↑	↑	↑	↑↑		↑	↑	↑↑
Cortisol	↑↑	=	↑	=	↑	↑↑		↑	↑	↑↑
GH	=									
Ghrelin	↓	=	=	=						
LH/FSH/Prolactin	=									
TSH	=									
AVP	↑	=	=		↑	↑↑		↓↓	↑	↓↓
ANP	=		=		↓	↓		↓	↓↓	↓↓
BNP	=		=		↑	↑		↓↓	↓↓	↓↓
NT – BNP	=	=	=	↑				↓		
PRA	=	↑↑	=	=	=	=		↓	↓↓	↓↓
Aldosterone	=	↑	=		↓	=		↓	↓↓	↓↓
Adrenaline	=	↓	=	=	↓	↓		↓	↓↓	↓↓
Nor adrenaline	=	↑↑	=	=	=	=		↓	↓	↓
Endothelin	=	=	=	=	=	=		↓↓	↓↓	↓↓
Adrenomedullin	=	=	=	↑						
Insulin		=	=	=						
Angiotensin II		↑↑								
Glucose			=					=		

¹ Suppression of cAMP by Ucn 1 in LV pacing induced heart failure in sheep – as an acute effect.

² On prolonged exposure to Ucn 1 cAMP levels were substantially increased with a lag between the onset of haemodynamic and hormonal effects. Fields left blank - no available data.

‘↑’ - Increase, ‘↓’ - Decrease, ‘=’ No change.

cAMP - cyclic adenosine monophosphate; cGMP - cyclic guanosine monophosphate; ACTH - adrenocorticotrophic hormone; GH - growth hormone; LH - leutinising hormone; FSH - follicle stimulating hormone; TSH - thyroid stimulating hormone; AVP - arginine vasopressin; ANP - atrial natriuretic peptide; BNP - brain natriuretic peptide; NT - N-terminal; PRA - plasma renin activity; Ucn - urocortin; LV - left ventricular.

1.4 AIMS

Through a series of studies, the aims of this thesis were to examine the cardiovascular effects of

1. activation of SIRT1 through the use of SRT2104, a novel small molecule SIRT1 activator in otherwise healthy cigarette smokers and patients with type 2 diabetes mellitus, and of
2. urocortins 2 and 3 in healthy volunteers and in patients with heart failure.

1.5 HYPOTHESES

In otherwise healthy cigarette smokers we hypothesised that activation of SIRT1 would result in (Chapter 3):

- Reversing of vasomotor and fibrinolytic dysfunction.
- A reduction in markers of platelet and monocyte activation.

In otherwise healthy cigarette smokers and patients with type 2 diabetes mellitus, activation of SIRT1 would result in (Chapter 4):

- Improvement in measures of arterial compliance.

In healthy male volunteers (Chapter 5) and patients with heart failure (Chapter 6), we hypothesised that, acting via CRH-R2, urocortins 2 and 3:

- Cause forearm arterial vasodilatation in a dose-dependent manner that is at least in part, determined by the endothelium and nitric oxide release.
- Have greater vascular effects in patients with heart failure.

CHAPTER 2

METHODS

2.1 GENERAL

2.1.1 ETHICAL CONSIDERATION

All studies were approved by the local Research Ethics Committee (Lothian Research Ethics Committee and Berkshire Research Ethics Committee) and carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants prior to the study. Studies pertaining to the sirtuin system were part of a Phase 1 clinical trial and were given Clinical Trial Authorisation by the Medicines and Healthcare products Regulatory Authority (MHRA, UK). All studies were carried out at the MHRA Phase 1 accredited Wellcome Trust Clinical Research Facility at the Royal Infirmary of Edinburgh.

2.2 STUDY PARTICIPANTS

This thesis was based upon four study groups: the first part of the thesis evaluates the cardiovascular effects of sirtuins in otherwise healthy cigarette smokers and patients with type 2 diabetes mellitus. These two seemingly disparate groups would be deemed as having endothelial dysfunction, albeit through different mechanisms.

The second part of the thesis serves to evaluate the cardiovascular effects of urocortins in healthy volunteers and in patients with heart failure.

2.2.1 HEALTHY CIGARETTE SMOKERS AND PARTICIPANTS WITH TYPE 2 DIABETES MELLITUS

Twenty-four otherwise healthy cigarette smokers and 15 participants with stable type 2 diabetes, aged between 18 and 70 years, were eligible for the study. Healthy cigarette smokers were required to have smoked ≥ 10 cigarettes daily for at least 1 year. Participants with type 2 diabetes were non-smokers and were selected on the basis of having a diagnosis of type 2 diabetes mellitus for at least 6 months prior to inclusion in the study, with no change in medications having been made for at least the preceding 3 months, a fasting blood glucose ≤ 13.9 mmol/L (250 mg/dL) and Diabetes Control and Complications Trial (DCCT)-aligned HbA1c $< 9\%$ (75 mmol/mol) on screening. Exclusion criteria included the presence of significant comorbidities, chronic illness, renal or liver impairment, history of gastrointestinal diseases or previous surgical procedures that would influence drug absorption, history of alcoholism, history of neoplastic disease within the last 5 years, a positive urinary test for recreational drugs, pregnancy and participation in other clinical trials

or blood donation within the last 3 months. Patients with type 2 diabetes mellitus on ACE inhibitors, antiplatelet or anticoagulant therapies were excluded from the study. Tests for pregnancy (serum human chorionic gonadotrophin (HCG) concentrations at screening and urinary HCG concentrations at study visits) were conducted on all female participants of child-bearing potential.

2.2.2 HEALTHY VOLUNTEERS AND PATIENTS WITH HEART FAILURE

Eighteen healthy non-smoking male volunteers were recruited into a series of vascular studies. Participants had no documented previous medical history, were taking no regular medication and tested negative in a urinary toxicology screen (Nova Test, One step Diagnostic Rapid Test, CA, USA) for recreational drugs.

A further 12 patients with stable heart failure (NYHA class II-IV) and 10 age- and sex-matched healthy volunteers were recruited to attend once each. Healthy volunteers had no previous documented medical history and were on no regular medications. Patients with heart failure were eligible if echocardiography had confirmed left ventricular ejection fraction $<35\%$, left ventricular end diastolic diameter >5.5 cm and fractional shortening $<20\%$ within the preceding 12 months.

Patients were required to have been on maximally tolerated doses of ACE inhibitor and beta-blocker for at least 3 months. Participants were excluded if they had a systolic blood pressure >190 mmHg or <90 mmHg, or if they were known to have untreated ventricular arrhythmias or haemodynamically significant valvular heart disease. Patients with other severe or significant comorbidities including bleeding diathesis, renal or hepatic failure, anaemia or a recent infective or inflammatory condition were also excluded. In addition, women of child-bearing potential were excluded from the study.

2.3 STUDY METHODOLOGY

2.3.1 FOREARM VENOUS OCCLUSION PLETHYSMOGRAPHY

Forearm venous occlusion plethysmography as a technique to assess human vascular physiology *in vivo* has been around for more than half a century. It has been well described in literature and is considered a ‘gold standard’ in the assessment of vascular function in health and disease [Wilkinson and Webb 2001]. It provides an accurate, reproducible, minimally invasive method of assessing novel vasoactive drugs and hormones in man, allowing for the use of sub-systemic doses. Bilateral

venous occlusion plethysmography is now considered the preferred method of assessing response to vasoactive drugs/hormones instead of unilateral plethysmography, with the non-infused forearm serving as a contemporaneous control.

Basic principles of forearm venous occlusion plethysmography

Venous occlusion plethysmography works on the principle that if venous return from the arm is obstructed and arterial inflow continues uninterrupted, the rate of forearm swelling is proportional to the rate of arterial inflow [Benjamin *et al* 1995]. It measures the total forearm blood flow, excluding flow through the hand, where blood flow is predominantly through the skin and highly influenced by environmental factors. A blood pressure cuff around the wrist inflated to well above systolic pressures (usually 200 mmHg), serves to exclude the hand circulation. An upper cuff (around the upper part of arm) is intermittently inflated and deflated (to 40 mmHg, inflating for 10 seconds followed by a deflation period of 5 seconds) allowing intermittent occlusion of venous return, whilst maintaining arterial flow [Wilkinson and Webb 2001]. Changes in forearm volume measured by the

mercury-in-silastic strain gauges applied across the widest part of the forearm, is expressed as mL/100 mL forearm tissue/minute.

This technique has been extensively used in assessing vascular and endothelial responses to intra-arterial infusion of vasodilators such as acetylcholine [Kubo *et al* 1991; Hirooka *et al* 1992; Walker *et al* 2001; Lang *et al* 2008a, Lang *et al* 2008b; Maclay *et al* 2009; Japp *et al* 2010], bradykinin [Mason and Melon 1965; Labinjoh *et al* 2001; Pretorius *et al* 2002; Witherow *et al* 2003; Pretorius and Brown 2010; Ozkor *et al* 2011] and substance P [Tagawa *et al* 1997; Newby *et al* 1997a; Newby *et al* 1997b; Newby *et al* 1999a; Newby *et al* 1999b] and vasoconstrictors such as endothelin [Strachan and Webb 2002] in addition to assessing factors influencing the maintenance of peripheral vascular tone [Helmy *et al* 2001; Helmy *et al* 2003]. Several groups have demonstrated within-subject reproducibility and reliability of bilateral occlusion plethysmography [Petrie *et al* 1998; Walker *et al* 2001]. Walker *et al* [2001] and Petrie *et al* [1998] have demonstrated that changes in absolute forearm blood flow is a more reliable way of expressing responses to vasodilators whereas percentage changes may be more appropriate to represent response to vasoconstrictor agents. Forearm plethysmography is able to detect as

much as a 4 to 8-fold shift in dose-response relationships when comparing within-day and between-day responses for vasodilator agents [Newby *et al* 1997a], thus making it a sensitive tool for detecting changes in forearm blood flow. Previous work has assessed intra-subject variability and determined the coefficient of variation for forearm blood flow across resting conditions and a range of agonists to be between 24 to 27% [Walker *et al* 2001].

Study technique

Forearm plethysmography was performed with the participant lying supine, in a quiet, temperature controlled room (22-25°C). Volunteers fasted for 4 hours prior to the study and refrained from alcohol and caffeine for 24 hours prior to the study. Venous cannulae (17-G) were inserted into large subcutaneous veins in the antecubital fossae of both arms at the start of the study to facilitate periodic venous sampling as per study protocol. Heart rate and blood pressure were monitored at regular intervals throughout the study with a semi-automated oscillometric sphygmomanometer (Omron 705IT, Omron Healthcare, UK) (Figure 2.1).

Participants underwent brachial artery cannulation of the non-dominant forearm with a 27-standard wire gauge steel needle (Coopers Needle Works Ltd, Birmingham, UK) and received intra-brachial infusions of vasoactive agents as per protocol. These include acetylcholine (Chem. Pharm Fabrik GmbH, Germany), bradykinin (American Peptide Co, CA, USA), sodium nitroprusside (Hospira Inc, Lake Forest, IL, USA), urocortin 2 (Neurocrine Biosciences Inc, San Diego, CA, USA), urocortin 3 (GenScript, NJ, USA) and substance P (Clinalfa Basic, Bachem Distribution Services GmbH, Germany). Each drug dose was infused for a total of 6 minutes through the brachial artery cannula. Forearm arterial blood flow was measured using mercury-in-silastic strain gauges around the widest part of the forearm in both infused and non-infused forearms. Measurements were taken after the first 3 minutes of infusion, allowing for equilibration of blood flow. A 20 to 30-minute saline washout followed each period of drug administration, allowing forearm blood flow to return to baseline. Plethysmography data was analysed using LabChart (ADInstruments, Australia, version 7.1).

Forearm venous occlusion plethysmography was used in assessment of vascular responses to SRT2104 (*Chapter 3*) and urocortins (*Chapters 5 and 6*).

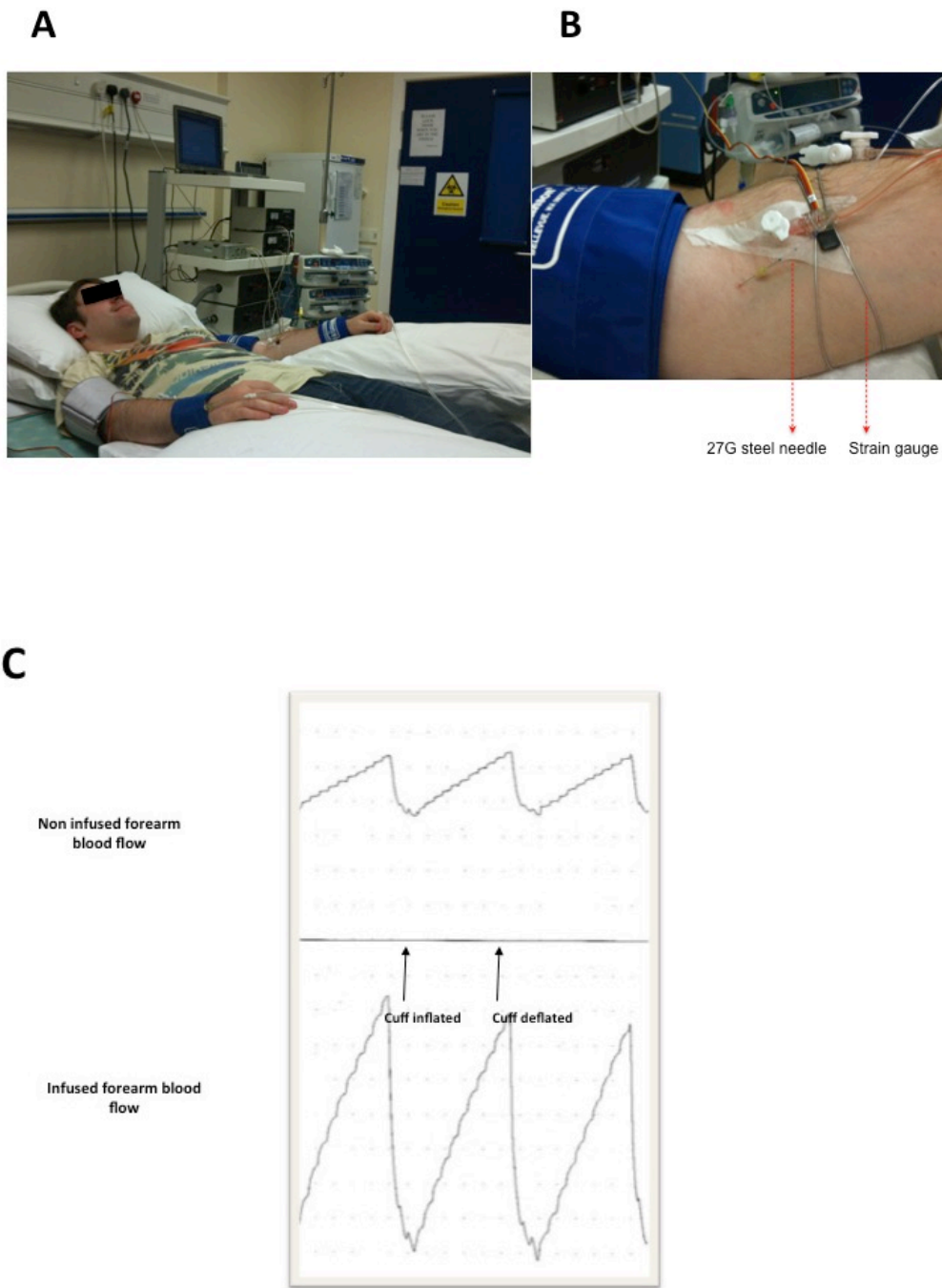


Figure 2.1. (A) Participant set-up for forearm venous occlusion plethysmography. (B) 27-G steel needle cannulated in left brachial artery with mercury-in-silastic strain gauge wrapped around widest part of forearm. (C) Typical LabChart data obtained during plethysmography showing non-infused and infused forearm blood flow.

2.3.2 FLOW CYTOMETRY

Flow cytometric measurements of platelet-monocyte aggregation (PMA), platelet surface expression of P-selectin and monocyte Mac-1/CD11b expression were performed to assess effect of SRT2104 on markers of platelet and monocyte activation in otherwise healthy cigarette smokers (*Chapter 3*). These assessments were carried out at baseline and at the end of each treatment period as described previously [Sarma *et al* 2002; Harding *et al* 2004a; Harding *et al* 2004b].

Basic principles of flow cytometry

Flow cytometry uses the principle of light scatter and fluorescence emitted by fluorochrome tagged cells to measure specific properties such as size (represented by forward angle light scatter) and internal complexity (represented by right-angled scatter) of a large number of individual cells.

Before flow cytometric analysis, cells in suspension are fluorescently labelled, typically with fluorescently conjugated monoclonal antibodies. Antibodies conjugated to fluorescent dyes can bind specific proteins on cell membranes or inside cells. When labelled cells are passed by a light source, the fluorescent molecules are

excited to a higher energy state. Upon return to their resting states, fluorochromes emit light energy at higher wavelengths. The use of multiple fluorochromes, each with similar excitation wavelengths and different emission wavelengths, allows several cell properties to be measured simultaneously. In the flow cytometer, the suspended cells pass through a flow chamber, and at a rate of 1,000 to 10,000 cells per minute, through a focussed beam of laser. After fluorescent activation of the fluorophore at the excitation wavelength, a detector processes the emitted fluorescence and light scatter properties of each cell. The resulting information is displayed in a histogram or two-dimensional dot-plot format (Figure 2.2).

In samples anticoagulated with D-Phenylalanine-L-propyl-L-arginine chloromethyl ketone (PPACK, a direct thrombin inhibitor), Harding *et al* [2007] were able to demonstrate a mean coefficient of variation of 7.8% for platelet-monocyte aggregates. Similarly, Burdess *et al* [2012] have also demonstrated good reproducibility (both within-day and between-day) of platelet-monocyte aggregates, P-selectin and CD40 ligand. Harding *et al* [2007] also demonstrated that in immune-fixed samples stored at 4°C, platelet-monocyte aggregates remained stable over a 24 hour period.

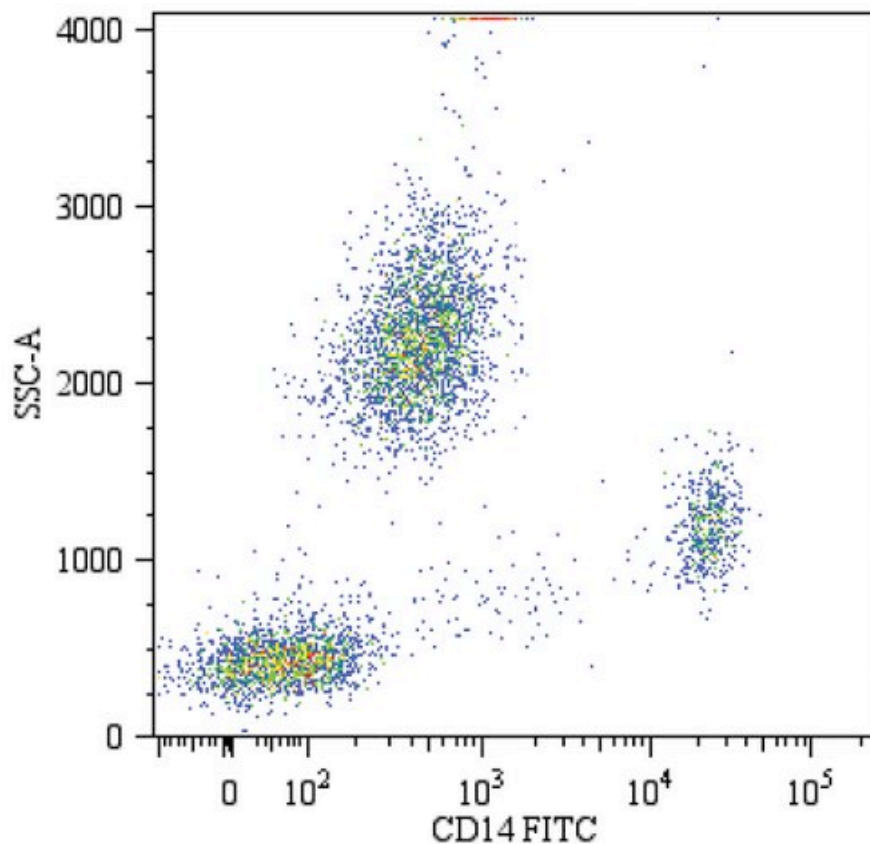


Figure 2.2. Typical flow cytometric dot-plot representation of CD14-FITC tagged human peripheral blood leucocytes. CD - cluster of differentiation; SSC-A - side scatter-angle; FITC - fluorescein isothiocyanate.

Study technique

Peripheral venous blood was drawn from a large antecubital vein and anticoagulated with PPACK (75 μ mol, Cambridge Biosciences, UK) and immunolabelled within 5 minutes of phlebotomy for subsequent flow cytometric analysis. Directly conjugated monoclonal antibodies were obtained from DakoCytomation

(Cambridge, UK) and Serotec (Oxford, UK). Samples were stained with the following conjugated monoclonal antibodies: phycoerythrin (PE)-conjugated CD14, PE-conjugated CD62p, PE-conjugated CD11b, fluorescein isothiocyanate (FITC)-conjugated 42a, and FITC-conjugated CD14 and appropriate control isotypes. Once stained, samples were incubated for 20 minutes at room temperature before being fixed with FACS-Lyse (Becton-Dickinson, Oxford, UK). All samples were analysed using a FACS Calibur flow cytometer using CellQuestPro software (Becton-Dickinson, Oxford, UK). Platelet-monocyte aggregation, P-selectin and Mac-1/CD11b values expressed as a percentage, were directly obtained from the FACS Calibur flow cytometer.

2.3.3 PULSE WAVE ANALYSIS AND VELOCITY

Measurement of arterial stiffness is widely used in both clinical practice and research. Arterial stiffness, as a marker of cardiovascular risk, is not a new concept. Pulse wave velocity (PWV), measured through peripheral arterial applanation tonometry is the current gold standard for determining arterial stiffness. Pulse wave analysis (PWA) and velocity provide a non-invasive, reproducible and relatively inexpensive method of measuring arterial stiffness. A number of studies have

examined the ability of arterial stiffness to predict the risk of future fatal and non-fatal cardiovascular events and total mortality [Vlachopoulos *et al* 2010].

Pulse wave analysis and velocity were measured in otherwise healthy cigarette smokers and patients with type 2 diabetes mellitus before and after administration of SRT2104 (*Chapter 4*).

Basic principles of pulse wave analysis and velocity

Arterial stiffness partly depends on smooth muscle tone. The shape of the arterial waveform provides a measure of systemic arterial stiffness and can be assessed non-invasively using pulse wave analysis [Wilkinson *et al* 2002]. O'Rourke and colleagues [O'Rourke and Gallagher 1996] developed the technique of pulse wave analysis to allow non-invasive measurement of central pressure waveforms using applanation tonometry. A meta-analysis of 17 original articles measuring aortic pulse wave velocity and cardiovascular outcomes by Vlachopoulos *et al* [2010] has shown that aortic pulse wave velocity is a strong predictor of future cardiovascular events. Pulse wave analysis has been shown to be a simple way of assessing endothelial vasomotor function [Wilkinson *et al* 2002]. It has also been shown to be

a simple and reproducible technique [Wilkinson *et al* 1998], allowing easy use in large scale studies. Wilkinson *et al* [1998] have reported extremely low standard deviation (SD) for measurement differences (both within-observer and between-observer reproducibility; 5.37% and 3.8% respectively) for pulse wave analysis.

Pulse wave analysis derives an aortic pulse pressure waveform (Figure 2.3) from the radial artery wave via a mathematical transfer function. The arterial pressure waveform is a composite of the forward pressure wave created by ventricular contraction and a reflected wave generated by peripheral vascular resistance [Lundback *et al* 2009]. The augmentation pressure is the pressure difference between the second and first systolic peaks. The augmentation index (AIx), augmentation pressure as a percentage of the pulse pressure, is a measure of systemic arterial stiffness and wave reflection. Corrected augmentation index represents the AIx corrected for heart rate [Wilkinson *et al* 2000]. The time to wave reflection (Tr) declines with increasing arterial stiffness, and provides a surrogate measure of aortic pulse wave velocity [Mills *et al* 2008]. Pulse wave velocity was calculated by measuring the time for the pulse wave to travel between the carotid and femoral arteries.

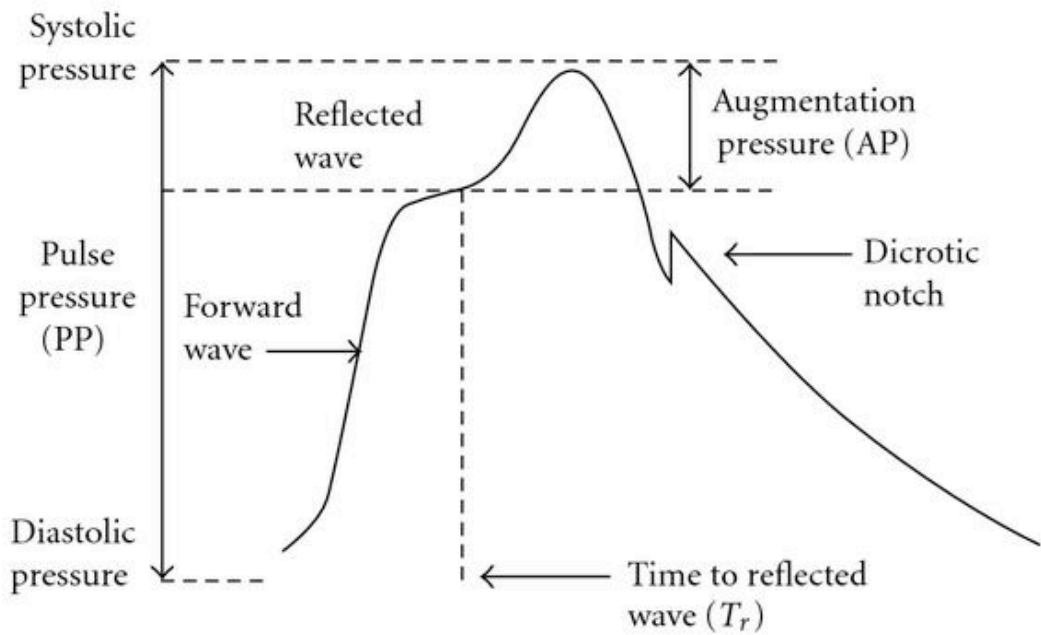


Figure 2.3. Aortic pulse pressure waveform. Systolic and diastolic pressures are the peak and trough of the waveform. Augmentation pressure is the difference between the second and first systolic peaks. Augmentation index is the augmentation pressure as a percentage of pulse pressure. Time to reflection is calculated as the time at the onset of the ejected pulse waveform to the onset of the reflected wave.

Study technique

All studies were performed in a quiet, temperature controlled (22-25°C) room. Participants were fasted and asked to refrain from smoking for 10 hours, and abstain from caffeine and alcohol for 24 hours prior to assessment. Subjects remained supine for at least 30 minutes before any recordings were commenced. Systolic and diastolic blood pressures were recorded using a non-invasive oscillatory sphygmomanometer (Omron705IT).

Pulse wave analysis of the radial artery was performed at the wrist using micromanometer applanation tonometry (Millar Instruments, Texas) and the SphygmoCor™ system (AtCor Medical, Sydney) in accordance with the manufacturer's recommendations. The SphygmoCor pulse wave analysis system uses applanation tonometry to record non-invasively a high fidelity peripheral arterial blood pressure waveform. From peripheral measurements, the PWA software derives central aortic blood pressure waveform and a range of central arterial indices. At least three independent waveform analyses were obtained from each subject, with measurements only accepted upon meeting SphygmoCor™'s internal quality control criteria (Figure 2.4).

As part of the study protocol, the process of data collection of pulse wave analysis and velocity was carried out by a trained research nurse at the Wellcome Trust Clinical Research Facility at the Royal Infirmary of Edinburgh (otherwise healthy cigarette smokers) and Western General Hospital (participants with type 2 diabetes). The operator performing the analysis was kept constant for each participant throughout the study.

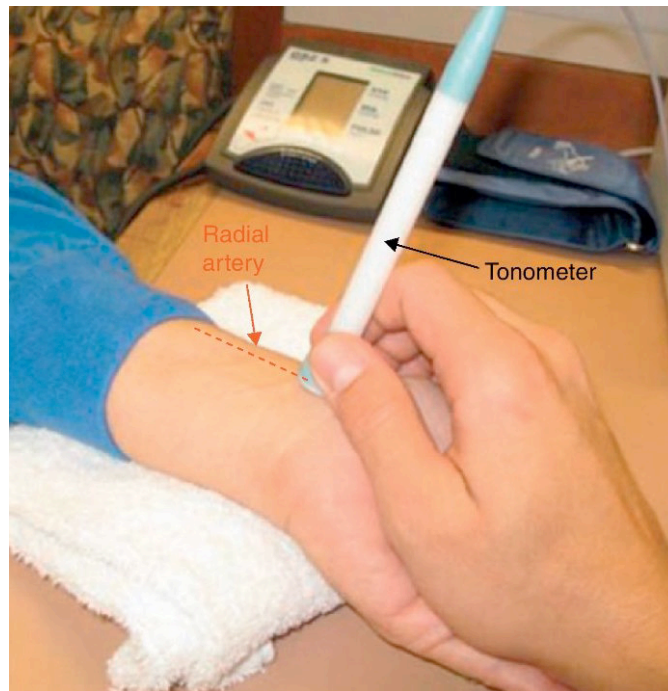


Figure 2.4. Applanation tonometry apparatus. **Top panel:** Application of the Millar tonometer against the radial artery. **Bottom panel:** The SphygmoCor applanation tonometry apparatus.

2.4 BLOOD ANALYTES

2.4.1 HAEMATOLOGICAL AND BIOCHEMICAL ANALYTES

Venous cannulae (17-G) were placed in the antecubital fossa in both forearms to facilitate periodic venous blood sampling throughout the study as per protocol. Blood sampling was carried out at baseline and at periodic intervals (as per protocol) for the assessment of full blood count, liver and renal function tests, cholesterol and blood glucose levels. Analysis was performed by the local clinical biochemistry and haematology reference laboratories using an automated haematology analyser (XE-2100, Sysmex Corporation (Japan)) and ACL TOP, Instrumentation Laboratory, (Bedford, MA, USA), an automated chemistry analyser using colourimetric, kinetic and enzymatic ultraviolet and colour assays (AU2700/ AU640 analysers, Beckman and Coulter, UK Ltd), ion selective electrodes (sodium, potassium and chloride assays) and two-point and multiple point rate assays (Ortho Clinical Vitros 250 analyser, Minnesota, USA).

2.4.2 MEASUREMENT OF ENDOGENOUS FIBRINOLYSIS AND MARKERS OF PLATELET AND MONOCYTE ACTIVATION

Bradykinin is an endothelium-dependent vasodilator that also stimulates the release of stored tissue-plasminogen activator (t-PA) from the vascular endothelium.

[Brown *et al* 1997; Brown *et al* 2000; Witherow *et al* 2001; Witherow *et al* 2003].

Paired venous blood samples were obtained from each forearm before and during the infusion of intra-arterial bradykinin (*Chapter 3*). Samples were collected into acidified buffered citrate (Stabilyte™, Trinity Biotech plc, Bray, Ireland) and citrate (BD Vacutainer, Becton-Dickinson, Oxford, UK) for determination of t-PA and PAI-1 concentrations respectively. Samples were placed on ice before centrifuging at 2000 g for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at -80°C before further analysis. Venous blood samples were collected into ethylene diamine tetraacetic acid (EDTA) at the beginning and end of the vascular study to determine haematocrit.

Plasma t-PA antigen and activity (t-PA Combi Actibind t-PA ELISA kit, Technoclone, Vienna, Austria) and PAI-1 antigen and activity (Elitest PAI-1 Antigen

and Zymutest PAI-1 Activity, Hyphen Biomed, France) concentrations were determined by enzyme-linked immunosorbent assays (ELISAs). Intra-assay coefficients of variation were 7.0% and 5.5% for t-PA and PAI-1 antigen, 4.0% and 2.4% for t-PA and PAI-1 activity. Inter-assay coefficients of variability were 4.0%, 7.3%, 4.0% and 7.6% respectively. The sensitivities of the assays were 0.5 ng/mL, 0.10 IU/mL, 2.5 ng/mL and 5 IU /mL respectively.

Venous blood was collected into citrate at baseline and after each dosing period of SRT2104 to assess plasma soluble CD40 ligand (sCD40 ligand) concentrations. Blood was centrifuged at 1500 g for 15 minutes at 4°C, and plasma was decanted and stored at -80°C for further analysis by ELISA (Bender Medsystems, UK).

2.4.3 PHARMACOKINETIC ASSAY OF SRT2104

Venous blood samples were taken into pre-labelled heparinised sodium tubes for pharmacokinetic assessment of plasma SRT2104 concentrations (Simbec Laboratories Limited, UK). Serial blood samples were collected on days 1, 28 and 56 immediately prior to (0 min) and 15, 30, 60, 120, 180, 240, 480, 720 and 1440 min following study medication. Plasma was separated by centrifugation of

whole blood at 1500 g at 4°C for 15 minutes, and decanted and stored at -80°C until analysed.

Plasma concentrations of SRT2104 were measured using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) in positive ion mode. High-pressure liquid chromatography was performed using Betasil silica–100 columns utilising a Phenomenex C18 guard column [Hoffmann *et al* 2012].

2.5 DATA ANALYSES AND STATISTICS

Forearm blood flow data were analysed as described previously [Newby *et al* 1997b]. In *Chapter 3*, fibrinolysis and forearm blood flow data were analysed using linear mixed model repeated measures analysis of covariance. Treatment differences were investigated in a model adjusting for period, treatment by period, vasodilator dose, treatment by vasodilator dose, and vasodilator dose by period using SAS[®] for UNIX (Version 9.1.3 or higher) (SAS Institute, Cary, North Carolina). Values for these parameters are expressed as model adjusted (least square means) and 95% confidence intervals. Between-day reproducibility of

forearm venous occlusion plethysmography data was assessed using the Bland-Altman method and coefficient of reproducibility was determined for 95% confidence intervals using the Student's *t*-test distribution. All other values are expressed as mean±SD.

Estimated net release of t-PA and PAI-1 antigen and activity was measured with intra-brachial infusion of bradykinin. It was defined as the product of forearm plasma flow (based on blood flow and haematocrit) and the difference in plasma antigen (or activity) concentrations between the two forearms.

$$\text{Estimated net t-PA release} = \text{FBF} \times (1-\text{Hct}) \times ([\text{t-PA}]_{\text{inf}} - [\text{t-PA}]_{\text{non-inf}})$$

Based on previous power calculations [Labinjoh *et al* 2001], a sample size of 20 gives 80% power to detect a change in net t-PA antigen release of 27.0 ng/100 mL of tissue/minute, assuming a standard deviation of 40.0 and a two-sided $P < 0.05$ (paired *t*-test).

For pulse wave analysis and velocity, data was analysed, where appropriate, using repeated measures analysis of covariance on the change from baseline for all parameters. Initially, analyses were conducted separately on cohorts (otherwise healthy cigarette smokers and patients with type 2 diabetes). Because of the small sample size and similar trends for the two cohorts, these data were pooled post-hoc. Treatment differences were investigated in a model adjusting for baseline, period, treatment by period and treatment by cohort using SAS® for UNIX (Version 9.1.3 or higher). Unless stated otherwise, values are expressed as mean±SD. Tests for treatment effect were two-sided with a significance level of 0.05.

For the studies assessing effect of urocortins in healthy volunteers and patients with heart failure (*Chapters 5 and 6*), data was analysed, where appropriate, by analysis of variance (ANOVA) with repeated measures, and paired and unpaired Student's *t*-test as appropriate (Graph-Pad Prism, GraphPad Software, San Diego, CA, USA). Significance was taken at 5%. At a level of significance of 5%, a sample size of 12, gives a 80% power of detecting a change in forearm blood flow of 0.7 mL/100 mL/minute. These power calculations are based on a

standard deviation of 0.53 mL/100 mL/minute [Newby *et al* 1997a; Lang *et al* 2008b; Lang *et al* 2008c]. All results are expressed as mean±standard error of the mean (SEM), unless otherwise stated.

CHAPTER 3

CARDIOVASCULAR EFFECTS OF A NOVEL SIRT1 ACTIVATOR, SRT2104, IN OTHERWISE HEALTHY CIGARETTE SMOKERS

Venkatasubramanian S, Noh RM, Daga S *et al.*
Cardiovascular effects of a novel SIRT1 activator, SRT2104,
in otherwise healthy cigarette smokers.
J Am Heart Assoc 2013;**2**:e000042.

3.1 SUMMARY

Background We examined the effect of the oral SIRT1 activator, SRT2104, on cardiovascular function in otherwise healthy cigarette smokers.

Methods Twenty-four otherwise healthy cigarette smokers participated in a randomised, double blind, placebo controlled, crossover trial and received 28 days of oral SRT2104 (2.0 g/day) or matched placebo. Plasma SRT2104 concentrations, serum lipid profile, plasma fibrinolytic factors and markers of platelet and monocyte activation were measured at baseline and the end of each treatment period together with an assessment of forearm blood flow during intra-arterial bradykinin, acetylcholine and sodium nitroprusside infusions.

Results Three hours post-dose, mean plasma SRT2104 concentration was 1328 ± 748 ng/mL after 28 days of active treatment. In comparison to placebo, serum lipid profile improved during SRT2104 administration with reductions in serum total cholesterol (-11.6 ± 20 versus $+6 \pm 21$ mg/dL), low-density lipoprotein (LDL) (-10 ± 17 versus $+3 \pm 21$ mg/dL) cholesterol, and triglyceride (-39.8 ± 77 versus $+13.3 \pm 57$ mg/dL) concentrations ($P < 0.05$ for all). All vasodilators produced a dose-dependent increase in blood flow ($P < 0.0001$) that was similar during each treatment period ($P > 0.05$ for all). No significant differences in fibrinolytic or blood flow parameters were observed between placebo and SRT2104.

Conclusion SRT2104 appears to be safe and well tolerated, and associated with an improved lipid profile without demonstrable differences in vascular or platelet function in otherwise healthy cigarette smokers.

3.2 INTRODUCTION

Originally identified in yeast, sirtuins represent a class of highly conserved NAD-dependent histone deacetylases that have seven identified members in mammalian species [Frye 1999; Frye 2000]. They have been implicated in the beneficial effects of calorie restriction, on longevity in several species, and are promising drug targets for a variety of diseases of ageing [Tang 2011]. Sirtuin 1 is the best-known member of this class of proteins and is expressed broadly in multiple tissues and highly expressed in the vascular endothelium [Edirisinghe and Rahman 2010]. SIRT1 inhibition is associated with vascular dysfunction and arterial thrombosis [Breitenstein *et al* 2011] as well as alterations in fibrinolysis [Ota *et al* 2007]. Conversely, SIRT1 activation is associated with improved endothelial function [Mattagajasingh *et al* 2007], enhanced lipid metabolism [Lomb *et al* 2010] and inhibition of atherogenesis [Stein *et al* 2010].

Smoking tobacco remains one of the most important and consistent modifiable risk factors for coronary heart disease, and is associated with an up to 7-fold increased risk of non-fatal myocardial infarction [Teo *et al* 2006]. It is associated with both accelerated atherosclerosis [Ambrose and Barua 2004] and a propensity to acute coronary thrombosis [Burke *et al* 1997; Zieske *et al* 2005]. This is mediated through a variety of mechanisms including alterations in vascular, endothelial, fibrinolytic and platelet function [Newby *et al* 1999b; Newby *et al* 2001; Harding *et al* 2004; Lang *et al* 2008b]. The precise cellular mechanism for these effects is as yet

unknown but cigarette smoke is associated with oxidative stress, eNOS acetylation, and increased endothelial cell senescence that has been attributed to reduced SIRT1 levels [Edirisinghe and Rahman 2010].

To date, there have been few clinical studies to assess the effect of SIRT1 activation *in vivo* in humans. Therefore, the aim of the present study was to examine the *in vivo* effects of a novel oral SIRT1 activator, SRT2104, on the lipid profile, and vascular, endothelial and platelet function in otherwise healthy cigarette smokers. We hypothesised that SIRT1 activation could improve the cardiovascular risk profile, and reverse or improve the vascular and endothelial dysfunction associated with cigarette smoking.

3.3 METHODS

The study was approved by the Research Ethics Committee, was given Clinical Trial Authorisation by the MHRA, UK, and carried out at the MHRA Phase 1 accredited Wellcome Trust Clinical Research Facility at the Royal Infirmary of Edinburgh, UK between June 2010 and September 2011. Written informed consent was obtained from each volunteer and the study was carried out in accordance with the Declaration of Helsinki.

3.3.1 STUDY PARTICIPANTS

Twenty-four otherwise healthy male and female volunteers aged between 18 and 70 years who smoked ≥ 10 cigarettes daily for at least 1 year were eligible for the study. Exclusion criteria are as described in *Chapter 2*. Eligibility of participants including absence of relevant past medical history was confirmed through a standardised proforma completed by the registered General Practitioners after informed consent. Tests for pregnancy (serum HCG concentrations at screening and urinary HCG concentrations at study visits) were conducted on all female participants of child-bearing potential.

3.3.2 STUDY DESIGN

This was a prospective randomised, double blind, placebo controlled, crossover study (1:1, SRT2104:placebo). Subjects were randomised to receive 2.0 g daily of oral SRT2104 or matched placebo (Sirtris a GSK Company, Cambridge, MA, USA) for a 28-day period, followed by crossover to the alternate study arm for another 28 days, giving a total dosing duration of 56 days. An end of study visit was conducted at Day 70 with a phone call follow up on Day 86. Assessment of drug safety, tolerability and efficacy on vascular function was carried out at baseline, during and at the end of each treatment period (Figure 3.1).

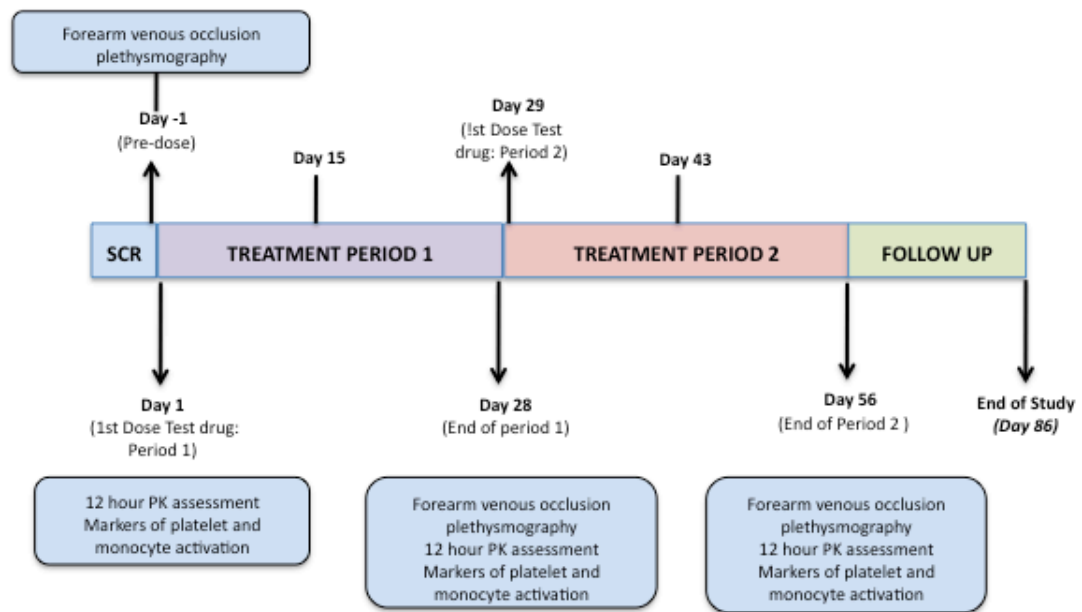


Figure 3.1. Schematic representation of study design. SCR - screening; PK - pharmacokinetic.

3.3.3 VASCULAR STUDIES

Vascular studies were undertaken before and at the end of each 28-day trial period. All studies were performed with the patient lying supine in a quiet, temperature controlled (22-25°C) room. Participants were fasted and asked to refrain from smoking for 10 hours prior to the study and avoid caffeine and alcohol for 24 hours prior to the study. Venous cannulae (17-G) were inserted into large subcutaneous veins in the antecubital fossae of both arms at the start of the study to facilitate periodic venous sampling. Supine heart rate and blood pressure were monitored at intervals throughout the study using a semi-automated non-invasive oscillometric sphygmomanometer (Omron 705IT, Omron Healthcare, UK).

Forearm venous occlusion plethysmography

Forearm blood flow was measured in the infused and non-infused forearms using forearm venous occlusion plethysmography as described in *Chapter 2*. After a 20-minute baseline infusion with 0.9% saline, incremental intra-arterial doses of bradykinin at 100, 300 and 1000 pmol/minute (an endothelium-dependent vasodilator that evokes t-PA release), acetylcholine at 5, 10 and 20 $\mu\text{g}/\text{minute}$ (an endothelium-dependent vasodilator that does not evoke t-PA release) and sodium nitroprusside at 2, 4 and 8 $\mu\text{g}/\text{minute}$ (an endothelium-independent vasodilator that does not evoke t-PA release) were infused for 6 minutes at each dose, with a 30-minute 0.9% saline washout infusion between drugs (Figure 3.2). The order of drugs was randomised between subjects but kept constant for each subject across the three visits.

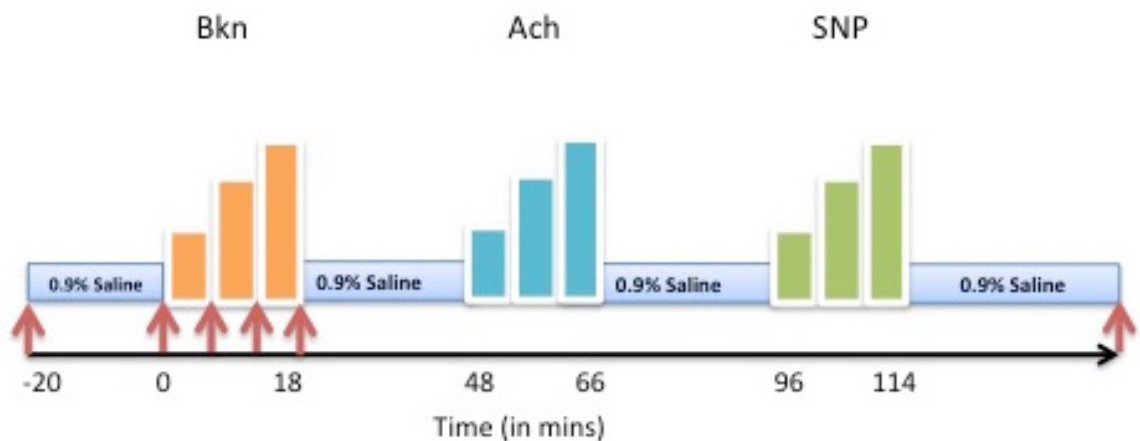


Figure 3.2. Schematic representation of forearm intra-arterial infusion protocol: Incremental intra-arterial infusion of bradykinin (Bkn) (100, 300, 1000 pmol/min); acetylcholine (Ach) (5, 10, 20 $\mu\text{g}/\text{min}$) and sodium nitroprusside (SNP) (2, 4, 8 $\mu\text{g}/\text{min}$) in the presence of saline washout between the peptides. **Red arrow:** Time points for blood sampling during plethysmography.

Blood sampling

Paired venous blood samples were obtained from each forearm before and during the infusion of intra-arterial bradykinin for estimation of plasma t-PA and PAI-1 antigen and antibody as described in *Chapter 2*.

Platelet and monocyte activation

Flow cytometric measurements of platelet-monocyte aggregation, platelet surface expression of P-selectin and Mac-1/CD11b were performed at baseline and at the end of each treatment period as described in *Chapter 2*. Venous blood was also collected at baseline and after each dosing period to assess plasma sCD40 ligand concentrations as described in *Chapter 2*.

3.3.4 SAFETY AND PHARMACOKINETIC ANALYSES

Venous blood samples were collected at fortnightly intervals to measure haematological and biochemical analytes including full blood count, coagulation profile, liver and renal function, creatine phosphokinase, lactate dehydrogenase, lipid profile and free fatty acids. Analyses were conducted by the regional clinical haematology and biochemistry reference laboratories as described in *Chapter 2*.

Serial venous blood samples were collected following study medication for pharmacokinetic assessment of plasma SRT2104 concentrations as described in *Chapter 2*.

3.3.5 DATA ANALYSIS AND STATISTICS

Plethysmographic data were analysed as described previously [Newby *et al* 1997b], Estimated net release of t-PA and PAI-1 antigen and activity was defined as the product of forearm plasma flow (based on blood flow and haematocrit) and the difference in plasma antigen (or activity) concentrations between the two forearms. Based on previous power calculations [Labinjoh *et al* 2001], a sample size of 20 gives 80% power to detect a change in net t-PA antigen release of 27.0 ng/100 mL of tissue/minute, assuming a standard deviation of 40.0 and a two-sided $P < 0.05$ (Student's paired *t*-test). To account for a 20% dropout rate, we recruited 24 subjects.

Fibrinolysis and forearm blood flow data were analysed as described in *Chapter 2*.

3.4 RESULTS

3.4.1 STUDY PARTICIPANTS

Volunteers had a mean age of 38 ± 13 years (median 37 years), relatively equal sex distribution (58% male) and were normotensive without any significant coexisting medical conditions. Volunteers had a body mass index of 25 ± 4 kg/m² and a mean cigarette consumption of 17 ± 6 cigarettes per day over 21 ± 14 years. The mean urinary cotinine concentration at screening was 1352 ± 950 ng/mL. All 24 volunteers completed all study visits. Prior to drug administration, one subject was withdrawn from the study due to problems with venous access and was replaced.

3.4.2 PHARMACOKINETICS, TOLERABILITY AND SAFETY

Three hours post-dose, mean plasma SRT2104 concentration was 1328 ± 748 ng/mL after 28 days of active treatment (Figure 3.3). The median plasma SRT2104 concentration after 28 days of treatment was 366 ng/mL (IQR 940 ng/mL). The median time at which the maximum plasma concentration was observed (T_{max}) on Day 28 of dosing was 3.05 hours, which coincided well with study measurements performed on those days (2 to 4 hours post-dose). The geometric mean area under the curve ($AUC_{0-\tau}$) was 6412 hr ng/mL. Consistent with previous observations [Hoffmann *et al* 2012] there was substantial inter-subject variability in exposure during this study. Plasma SRT2104 concentration was below level of quantification (<0.521 ng/ml) after treatment with placebo.

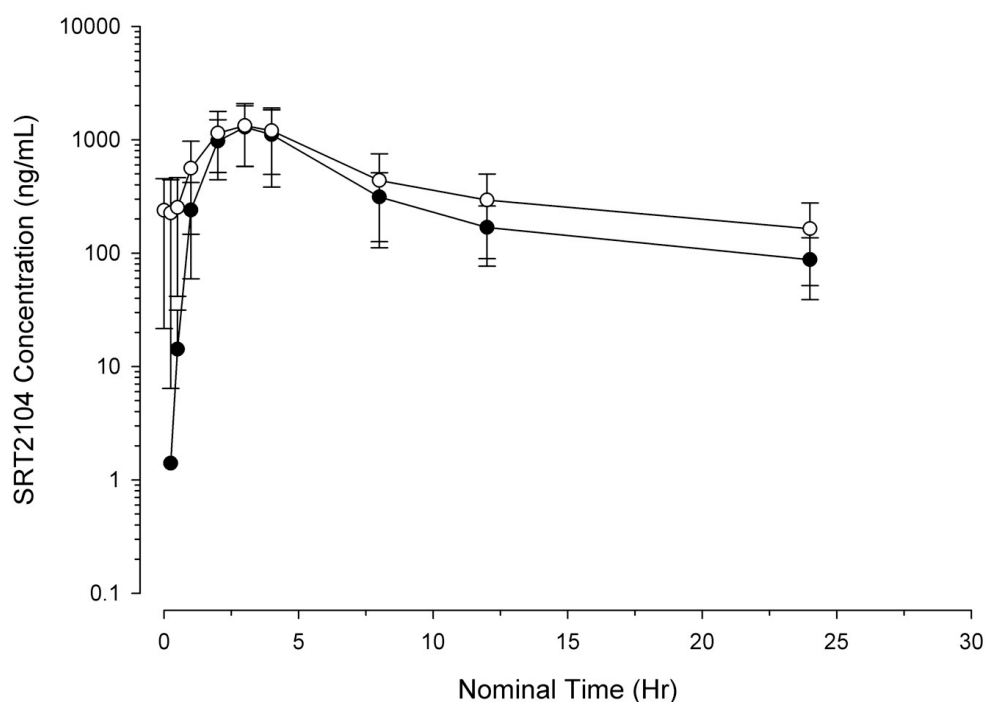


Figure 3.3. Pharmacokinetics: Mean plasma concentration - time curve following oral dosing of SRT2104 on **Day 1** (closed circles) and **Day 28** (open circles). Data presented as Mean \pm SD. Hr - hour; SD - standard deviation.

All subjects tolerated study medication well. Commonly reported side effects included headache (25%), rhinitis, nasopharyngitis and respiratory tract symptoms (17%) (Table 3.1). The reported adverse events were mild in intensity and resolved without any intervention or sequelae. There were no meaningful differences in the number of events between active treatment and placebo. There was only one reported serious adverse event in the study (SRT2104 arm) of traumatic facial bone fracture that was considered unrelated to SRT2104.

Blood pressure and heart rate remained unchanged throughout the study ($P>0.05$). There were no effects on cardiac rhythm or the 12-lead electrocardiogram, and specifically there were no effects on the corrected or uncorrected QT intervals. There were no clinically significant adverse effects involving any of the clinical haematological or biochemical analytes.

3.4.3 LIPID PROFILE

Treatment with SRT2104 had a favourable effect on the lipid profile. A statistically significant period effect was observed in the analysis of total and LDL cholesterol concentrations. Baseline values were higher in subjects receiving placebo in the first period. Regardless of treatment arm, the level of change from baseline was greater in treatment period 2 for total and LDL cholesterol, and less in treatment period 2 for triglycerides. Adjusted summaries combined over treatment periods are presented in Table 3.2. There was a reduction in total and LDL cholesterol as well as triglyceride concentrations. There was no effect on high-density lipoprotein (HDL)

concentrations and the 7% fall in total cholesterol was attributable to the 11% fall in LDL cholesterol concentrations.

TABLE 3.1 List of adverse and serious adverse events

System	Symptom (AE/ SAE)	Number of events	
		Placebo (N=24)	SRT2104 (N=24)
Any Event		18	18
Nervous System Disorders	Headache	4	6
	Paraesthesia	0	2
	Hypoaesthesia	1	2
	Carpal Tunnel Syndrome	0	1
	Presyncope	1	1
	Burning Sensation	0	1
	Sciatica	0	1
Respiratory, Thoracic and Mediastinal Disorders	Oropharyngeal pain	1	2
	Rhinorrhoea	0	1
Gastrointestinal Disorders	Flatulence	1	0
	Mouth ulceration	0	1
	Abdominal pain upper	1	0
	Haemorrhoids	1	0
Reproductive System and Breast Disorders	Dysmenorrhea	3	1
Musculoskeletal and Connective Tissue Disorders	Back pain	2	0
	Musculoskeletal chest pain	1	0
	Musculoskeletal pain	1	0
	Myalgia	0	1
Investigational	Blood Bilirubin Increased	1	1
	CreatininePhosphokinase Increased	1	0
	LDH Increased	1	0
	AST Increased	1	0

Continued...

General Disorders and Administration Site Conditions	Influenza like illness	1	0
	Fatigue	1	0
	Catheter site pain	0	1
	Catheter site rash	1	0
	Catheter site related reaction	1	0
	Catheter site swelling	1	0
	Oedema peripheral	0	1
	Pyrexia	0	1
	Swelling	0	1
Infections and Infestations	Nasopharyngitis	0	1
	Rhinitis	2	2
	Upper respiratory tract infection	1	0
	Oral Herpes	0	1
	Respiratory tract infection	0	1
Injury, Poisoning and Procedural Complications	Contusion	1	0
	Excoriation	1	1
	Arthropod bite	1	0
	Facial bones fracture	0	1
	Laceration	1	0
Skin and Subcutaneous Tissue	Rash	1	0
Metabolism and Nutrition Disorders	Decreased appetite	0	1
Vascular Disorders	Phlebitis	0	1
Eye Disorders	Eye pain	1	0
	Vision blurred	0	1
Ear and Labyrinthine Disorders	Motion sickness	0	1
Surgical and Medical Procedures	Vasectomy	1	0

TABLE 3.2 Effect of SRT2104 on serum lipid concentrations

	Placebo	SRT2104	P value
	N=22	N=20	
Total Cholesterol			
(mg/dL)			
Baseline	174±54	176±50	
Day 28/56	180±51	164±47	
Change from Baseline	6±21	-12±20*	0.001
HDL Cholesterol			
(mg/dL)			
Baseline	46±11	47±10	
Day 28/56	50±13	51±16	
Change from Baseline	3±7	4±7	0.81
LDL Cholesterol			
(mg/dL)			
Baseline	99±41	99±37	
Day 28/56	102±37	88±34	
Change from Baseline	3±21	-10±17*	0.006
Triglycerides			
(mg/dL)			
Baseline	133±110	140±114	
Day 28/56	146±149	100±67	
Change from Baseline	13±57	-40±77*	0.02

* = P value <0.05.

SD - standard deviation; HDL - high-density lipoprotein; LDL - low-density lipoprotein. Values expressed as mean±SD.

3.4.4 VASOMOTOR FUNCTION

Non-infused forearm blood flow remained unchanged throughout all assessment periods, as were the pre-dose measurements of blood flow in the infused arm between visits ($P>0.05$). Baseline blood flow remained unchanged throughout the study ($P>0.05$). There was a dose-dependent increase in the infused forearm blood flow with all three agonists (acetylcholine, bradykinin and sodium nitroprusside) in the presence of either SRT2104 or placebo ($P<0.0001$ for all three agonists; Figure 3.4). There were no significant differences in response to either endothelium-dependent or -independent vasodilators in the presence of SRT2104 compared to placebo (bradykinin, $P=0.17$; acetylcholine, $P=0.17$; sodium nitroprusside, $P=0.90$: placebo *versus* SRT2104). There were no differences in forearm vasodilatation between the baseline and placebo visits of the study for all three agonists ($P=0.56$, $P=0.40$, $P=0.29$ for bradykinin, acetylcholine and sodium nitroprusside, respectively) confirming good reproducibility of the measurements (Table 3.3).

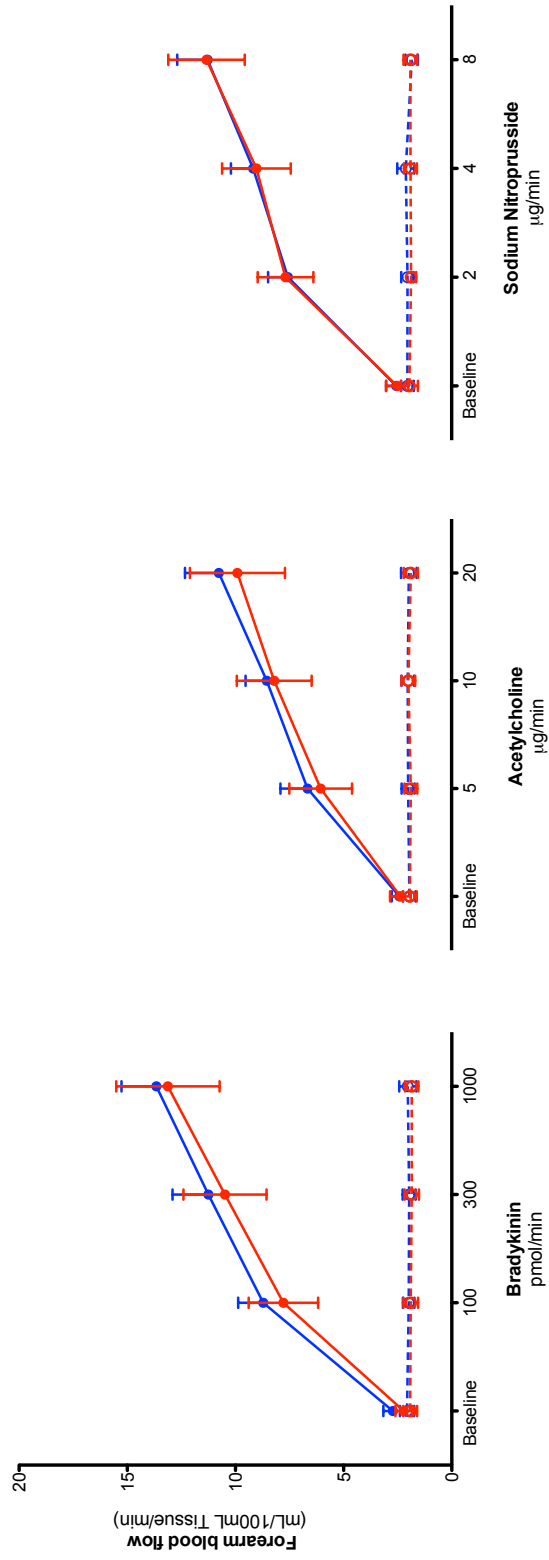


Figure 3.4 Effect of Bradykinin (100, 300, 1000 pmol/min), Acetylcholine (5, 10, 20 µg/min) and Sodium Nitroprusside (2, 4, 8 µg/min) on absolute forearm blood flow. **Blue:** Placebo; **Red:** SRT2104; **Closed circle:** Infused forearm blood flow; **Open circle:** Non-infused forearm blood flow. Data presented as Mean±95% Confidence Intervals.

TABLE 3.3 Between-day repeatability of forearm blood flow

Drug	Dose	Mean of differences in forearm blood flow (mL/100 mL/min)	Coefficient of Repeatability (mL/100 mL/min)
Bradykinin	100	-0.3	5
(pmol/min)	300	0.5	6
	1000	-0.2	7
Acetylcholine	5	-0.1	8
(µg/min)	10	-0.5	8
	20	0.3	7
Sodium Nitroprusside	2	0.2	5
(µg/min)	4	0.3	6
	8	0.7	6

Between-day reproducibility (Baseline *versus* Placebo visit) in absolute forearm blood flow for Bradykinin (100, 300, 1000 pmol/min), Acetylcholine (5, 10, 20 µg/min) and Sodium Nitroprusside (2, 4, 8 µg/min).

3.4.5 ENDOGENOUS FIBRINOLYSIS, AND MONOCYTE AND PLATELET ACTIVATION

There was a dose-dependent increase in bradykinin-evoked net t-PA antigen and activity release ($P < 0.0001$ for both) in the infused arm that was unaffected by SRT2104 ($P = 0.37$, $P = 0.13$, placebo *versus* SRT2104, for net t-PA antigen and activity, respectively; Table 3.4). Plasma PAI-1 activity decreased with time during all study visits ($P < 0.05$) consistent with its circadian variation and t-PA release. Plasma PAI-1 antigen and activity concentrations were similar in both treatment arms ($P = 0.89$, $P = 0.66$, placebo *versus* SRT2104, for plasma PAI-antigen and activity respectively). SRT2104 had no effect on markers of *in vivo* platelet or monocyte activation (Figure 3.5).

TABLE 3.4 Effect of SRT2104 on Endogenous Fibrinolysis

(LS Means + 95% CI)	Placebo				SRT2104			
	<i>Bradykinin Dose (pmol/min)</i>				<i>Bradykinin Dose (pmol/min)</i>			
	Saline	100	300	1000	Saline	100	300	1000
Baseline								
Net release tPA Antigen (ng/100mL tissue/min)	0.5 (-8.6, 9.7)	4.7 (-4.4, 13.9)	8.8 (-0.3, 18.0)	28.8 [§] (19.6, 37.9)	-1.0 (-10.9, 9.0)	5.4 (-4.5, 15.4)	5.6 (-4.7, 16.0)	38.3* [§] (28.4, 48.3)
Net release tPA Activity (ng/100mL tissue/ min)	-0.0 (-4.7, 4.6)	3.7 (-1.0, 8.3)	8.6 (3.9, 13.2)	17.6 [§] (12.9, 22.2)	0.0 (-5.0, 5.1)	3.4 (-1.7, 8.4)	6.5 (1.3, 11.8)	18.6* [§] (13.5, 23.6)
Net PAI-I Antigen (ng/100mL tissue/min)	-5.7 (-68.2, 56.8)	---	---	2.9 (-59.6, 65.5)	-1.7 (-69.7, 66.3)	---	---	-86.0* (-154.0, -18.0)
Net PAI-I Activity (ng/100mL tissue/min)	-0.0 (-1.2, 1.2)	---	---	-2.3 (-3.5, -1.1)	0.0 (-1.3, 1.4)	---	---	3.9* (-5.2, -2.6)
Day 28/56								
Net release tPA Antigen (ng/100mL tissue/min)	-1.05 (-8.0, 5.9)	3.9 (-3.1, 10.8)	8.8 (1.7, 15.9)	22.4 (15.1, 29.7) [§]	-0.1 (-6.7, 6.6)	2.6 (-4.3, 9.6)	9.3 (2.3, 16.2)	30.7* [§] (23.6, 37.8)
Net release tPA Activity (ng/100mL tissue/ min)	-0.1 (-3.2, 3.0)	2.4 (-0.8, 5.5)	5.8 (2.6, 9.0)	13.3 (10.1, 16.6) [§]	0.0 (-3.0, 3.0)	2.2 (-0.9, 5.3)	7.0 (3.8, 10.1)	18.1* [§] (14.9, 21.3)
Net PAI-I Antigen (ng/100mL tissue/min)	-4.8 (-52.2, 42.6)	---	---	-20.6 (-70.4, 29.3)	-0.1 (-45.4, 45.2)	---	---	-43.7* (-92.4, 5.1)
Net PAI-I Activity (ng/100mL tissue/min)	-0.0 (-1.2, 1.2)	---	---	-3.6 (-4.9, -2.4)	0.2 (-1.0, 1.3)	---	---	-3.3* (-4.5, -2.1)

Data presented as LS Mean±95% Confidence Intervals *P>0.05, SRT2104 versus Placebo; [§]P<0.0001, for dose response to agonist.

LS - Least square; t-PA - tissue plasminogen activator; PAI-I - plasminogen activator inhibitor-1.

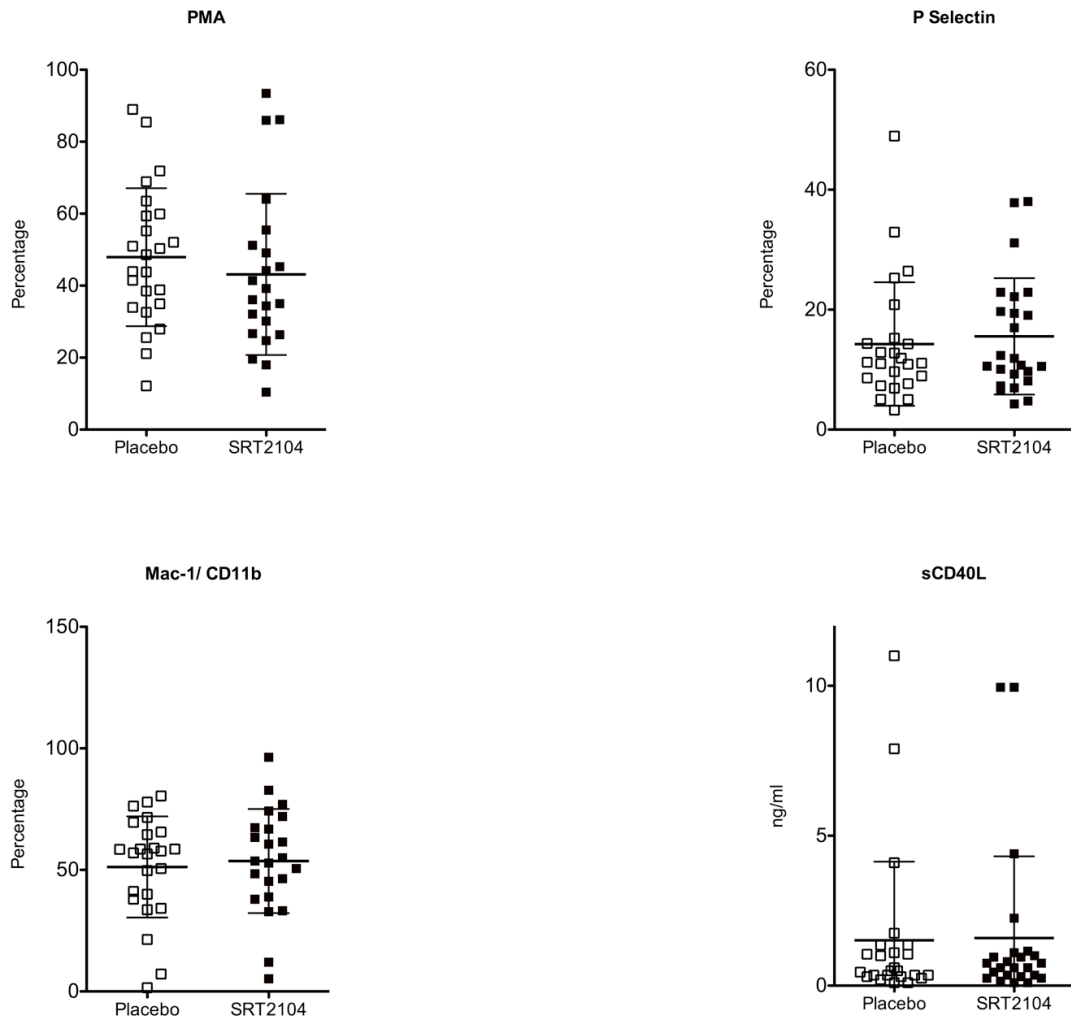


Figure 3.5. Effect of SRT2104 on markers of platelet and monocyte activation. PMA - platelet-monocyte aggregate; sCD40L - soluble CD40 ligand; Mac-1 - membrane activated complex-1; CD - cluster of differentiation; SD - standard deviation. Data presented as Mean \pm SD.

3.5 DISCUSSION

In this randomised, double blind, placebo controlled, crossover trial of otherwise healthy cigarette smokers, we have demonstrated that oral SRT2104 is safe and well tolerated at a dose of 2.0 g daily. Importantly, we have shown that treatment with SRT2104 was associated with an 11% mean reduction in serum LDL cholesterol concentrations, but without demonstrable differences in vasomotor function, endothelial function, or platelet activation assessments compared to placebo. The favourable effects on lipid profile suggest that SIRT1 activation may have a beneficial role in patients at risk of developing, or with established, cardiovascular disease.

Elevated serum cholesterol is an established risk factor for atherosclerosis and coronary heart disease. In general, coronary heart disease risk is reduced by 2 to 3% for each 1% decrease in total cholesterol concentrations [Gotto 1999]. We observed a 7% mean reduction in serum total cholesterol and an 11% mean reduction in LDL cholesterol concentrations without affecting serum HDL cholesterol concentrations. The mechanism of this lipid-lowering effect is not entirely clear, but is consistent with observations associated with SIRT1 activation in animals. Resveratrol (3,5,4'-trihydroxy-trans-stilbene), is a naturally occurring polyphenolic compound that is believed to confer health benefits through SIRT1 activation [Yu *et al* 2012]. Resveratrol has been found to lower plasma triglycerides and cholesterol accumulation in guinea pigs [Zern *et al* 2003] and to suppress atherogenic lesion formation in apoE^{-/-} deficient mice [Do *et al* 2008]. Indeed, SRT2104 also lowers

triglyceride levels in preclinical murine models of dyslipidaemia, diabetes, and obesity as well as improving insulin sensitivity and metabolic function in these animals [Qi *et al* 2010]. One mechanism whereby SIRT1 activators, such as SRT2104, could improve lipid profiles may involve a positive regulatory effect on LXRs, nuclear receptors involved in cholesterol and lipid homeostasis. Nuclear receptor LXR is a substrate for SIRT1. Li and colleagues have shown that SIRT1 deacetylates and positively regulates this receptor, potentially altering cholesterol transport and metabolism [Li *et al* 2007]. Although the exact mechanism of the improved lipid profiles seen with SIRT1 activation remains to be determined, our findings would suggest that SIRT1 activation could provide a therapeutic adjunct to current lipid lowering strategies, leading to improvements in cardiovascular disease pathophysiology and thus clinical outcomes.

There are currently no published data directly examining the effects of SIRT1 activation on vasomotor function or endogenous fibrinolysis *in vivo* in man. Despite the several beneficial effects of SIRT1 activation on endothelial function observed in preclinical *in vitro* studies [Mattagajasingh *et al* 2007; Ota *et al* 2007; Csiszar *et al* 2008; Orimo *et al* 2009; Donato *et al* 2011], we were unable to demonstrate improvements in vascular, endothelial or platelet function in these otherwise healthy smokers. Why was this?

Did we use appropriate and sufficiently sensitive techniques? Forearm venous occlusion plethysmography is a well established technique that has been used extensively over the years to study human vascular physiology and has been

considered one of the gold standards in the assessment of vascular function in health and disease [Wilkinson and Webb 2001]. Using endothelium-dependent (bradykinin and acetylcholine) and -independent (sodium nitroprusside) vasodilators, we observed a dose-dependent increase in forearm arterial vasodilatation with all three agonists. Our results are comparable with those reported in previously published studies [Heitzer *et al* 1996; Butler *et al* 2001; Noma *et al* 2005; Lang *et al* 2008b], in otherwise healthy cigarette smokers including impaired t-PA release [Newby *et al* 2001; Pretorius *et al* 2002; Takashima *et al* 2007]. Moreover, our data had low variance and were highly reproducible when we compared the baseline responses with those obtained during placebo administration. Similarly, flow cytometric analysis is considered a sensitive gold standard for measurement of *in vivo* platelet activation. We have previously shown that in patients with peripheral arterial disease, measurements of platelet-monocyte aggregates are reproducible and consistently reflect other markers of platelet and monocyte activation [Burdess *et al* 2012]. In the present study, we again report comparable levels of platelet-monocyte aggregation [Harding *et al* 2004; Lupia *et al* 2010] that were reproducible between visits.

There is a body of published data that confirms a strong association between cigarette smoking, endothelial dysfunction and impaired endogenous fibrinolysis [Newby *et al* 1997b; Newby *et al* 1999b; Lang *et al* 2008b; Edirisinghe and Rahman 2010; Xue *et al* 2011]. We were interested to see if this vascular and endothelial dysfunction could be improved or reversed by SIRT1 activation. There could be numerous explanations as to why we failed to achieve improvement with SRT2104

on these parameters in the current study. One possibility is that the SRT2104 exposure achieved in this study did not lead to adequate or consistent SIRT1 activation, which would be required to reverse the vascular and endothelial dysfunction in these smokers. Unfortunately, there is no current biomarker for SIRT1 activation or the ability to measure SIRT1 activation directly in humans. Therefore, we do not have a good understanding of the pharmacokinetic-pharmacodynamic relationship between SRT2104 drug exposure and SIRT1 activation. Although we were able to demonstrate improved lipid profiles, it is unclear whether the same exposure levels would also lead to improved vascular and endothelial function. There are at least 70 known substrates for SIRT1. SRT2104 may differentially deacetylate certain substrates in preference to others, depending on the precise interaction between SRT2104 and the substrates as well as the level and activity of the substrates in a particular disease state. It is also possible that certain abnormalities may be reversed more readily than others through SIRT1 activation. While a 28-day exposure may be adequate for observing improvement in lipid profiles, longer treatment may be required to reverse some of the vascular and endothelial abnormalities. The small sample size of our study may also be a potential limitation. SRT2104 is the first selective SIRT1 activator to be studied in human clinical trials. As the biology of SIRT1 becomes more established and additional data are gathered from small exploratory trials such as this one, the optimal approach for developing SIRT1 activators and identifying disease states with the greatest therapeutic potential will become better defined.

In conclusion, we have demonstrated that the oral SIRT1 activator, SRT2104, is safe and well tolerated in otherwise healthy cigarette smokers and provides positive effects on lipid profiles, but were unable to demonstrate beneficial effects on vascular, endothelial or platelet function compared to placebo.

CHAPTER 4

EFFECTS OF THE SMALL MOLECULE SIRT1 ACTIVATOR, SRT2104 ON ARTERIAL STIFFNESS IN OTHERWISE HEALTHY CIGARETTE SMOKERS AND SUBJECTS WITH TYPE 2 DIABETES MELLITUS

Venkatasubramanian S, Noh RM, Daga S *et al.*

Effects of the small molecule SIRT1 activator, SRT2104 on arterial stiffness in otherwise healthy cigarette smokers and subjects with type 2 diabetes mellitus.

Open Heart 2016;3e000402.

4.1 SUMMARY

Background Arterial stiffness increases with age, and is associated with adverse cardiovascular outcome including increased mortality. The effect of the oral small molecule SIRT1 activator, SRT2104, on arterial stiffness was examined in otherwise healthy cigarette smokers and subjects with type 2 diabetes mellitus.

Methods Twenty-four otherwise healthy cigarette smokers and 15 people with stable type 2 diabetes were randomised in a double blind, placebo controlled, crossover trial and received 28 days of oral SRT2104 (2.0 g/day) or matched placebo. Blood pressure was measured using non-invasive oscillatory sphygmomanometry. Pulse wave analysis and velocity were measured using applanation tonometry at baseline and the end of each treatment period. Due to the small sample size and similar trends for both groups, data for the two groups were pooled (post-hoc analysis).

Results Compared to placebo, treatment with SRT2104 was associated with a significant reduction in augmentation pressure ($P=0.02$) and a trend towards improvement in the augmentation index and corrected AIx ($P>0.05$ for both). However, no changes were observed in pulse wave velocity and time to wave reflection ($P>0.05$). Systolic and diastolic blood pressures remained unchanged throughout the study. Treatment by cohort interaction was not significant for any of the pulse wave parameters, suggesting that the response to SRT2104 in otherwise healthy smokers and people with diabetes was consistent.

Conclusion SRT2104 may improve measures of arterial stiffness in otherwise healthy cigarette smokers and in subjects with type 2 diabetes. Definitive conclusions are not possible given the small sample size and exploratory nature of this analysis.

4.2 INTRODUCTION

The enzyme sirtuin belongs to the sirtuin family of nicotinamide adenine dinucleotide-dependent histone deacetylases and is highly expressed in the vascular endothelium [Edirisinghe and Rahman 2010]. In addition to other characteristics, its activation is associated with improved endothelial function [Mattagajasingh *et al* 2007] and inhibition of atherogenesis [Stein *et al* 2010]. Particular interest has focused on the potential of therapeutic SIRT1 activators to act as anti-ageing agents.

Arterial stiffness rises with age and is recognised to be an independent predictor of cardiovascular risk [Cockcroft and Wilkinson 2002]. In particular, elevations in pulse pressure and aortic stiffness are associated with increased risk of coronary events and overall mortality [Vlachopoulos *et al* 2010]. Indeed, central aortic stiffness is associated with the presence of coronary atherosclerosis and ischaemic heart disease [McLeod *et al* 2004].

Cigarette smoking and diabetes mellitus are significant risk factors for the development of cardiovascular disease. A wealth of data has established a strong correlation between diabetes and cigarette smoke exposure with increased aortic

stiffness, endothelial dysfunction and cardiovascular risk [Rehill *et al* 2006; Binder *et al* 2008; Stehouwer *et al* 2008; Roos *et al* 2011]. New pharmacological strategies that improve arterial compliance would therefore be highly relevant to these groups at increased cardiovascular risk.

The aims of the present study were to assess the effect of the oral SIRT1 activator, SRT2104, on measures of arterial compliance in otherwise healthy cigarette smokers and patients with type 2 diabetes. It was hypothesised that SIRT1 activation in these ‘at risk’ groups could lead to an improvement in arterial compliance and therefore reduce their cardiovascular risk.

4.3 METHODS

The study was approved by the Berkshire Research Ethics Committee, received Clinical Trial Authorisation from the MHRA, UK, and was conducted at the MHRA Phase I accredited Wellcome Trust Clinical Research Facility at the Royal Infirmary of Edinburgh and Western General Hospital, UK between June 2010 and September 2011 (EudraCT #: 2009-016765-28; Clinical trials identifier: *NCT01031108*). Written informed consent was obtained from each volunteer and the study was carried out in accordance with the Declaration of Helsinki.

4.3.1 STUDY PARTICIPANTS

Twenty-four otherwise healthy cigarette smokers and 15 participants with stable type 2 diabetes, aged between 18 and 70 years, were eligible for the study. Inclusion and exclusion criteria for the study are as outlined in *Chapter 2*.

4.3.2 STUDY DESIGN

This was a prospective randomised, double blind, placebo controlled, crossover study as described in *Chapter 2*. Subjects were randomised to receive 2.0 g daily of oral SRT2104 or matched placebo for a 28-day period, followed by crossover to the alternate study arm for a further 28 days, giving a total dosing duration of 56 days. An end of study visit was conducted at Day 70 with a telephone call follow up on Day 86. Measures of arterial stiffness were undertaken prior to and at the end of each 28-day trial period.

All studies were performed in a quiet, temperature controlled (22-25°C) room. Participants were fasted and asked to refrain from smoking for 10 hours, and abstain from caffeine and alcohol for 24 hours prior to assessment. Subjects remained supine for at least 30 minutes before any recordings were commenced. Systolic and diastolic blood pressures were recorded using a non-invasive oscillatory sphygmomanometer.

Pulse wave analysis of the radial artery was performed at the wrist using micromanometer applanation tonometry and the SphygmoCor™ system in accordance with the manufacturer's recommendations as described in *Chapter 2*. At least three independent waveform analyses were obtained from each subject, with

measurements only accepted upon meeting SphygmoCor™ quality control criteria. The operator performing the analysis was kept constant for each participant throughout the study.

4.3.3 BLOOD SAMPLING

Venous blood samples were collected and analysed at fortnightly intervals to measure haematological and biochemical analytes including full blood count, coagulation profile, liver and renal function, creatinine kinase, lactate dehydrogenase and lipid profile as described in *Chapter 2*.

4.3.4 DATA ANALYSIS AND STATISTICS

As described in *Chapter 2*, data were analysed, where appropriate, using repeated measures analysis of covariance on the change from baseline for all parameters. Due to the small sample size and similar trends for the two cohorts (otherwise healthy cigarette smokers and subjects with type 2 diabetes mellitus) data was pooled post-hoc. Unless stated otherwise, values are expressed as mean±SD. Tests for treatment effect were two-sided with a significance level of 0.05.

4.4 RESULTS

4.4.1 BASELINE CHARACTERISTICS

Participants in the study had a mean age of 45±15 years and were predominantly male (68%). Subjects in the type 2 diabetes cohort were older (mean age 58±8 years) when compared to the participants in the otherwise healthy smokers group

(mean age 38 ± 13 years). All subjects were normotensive with comparable systolic and diastolic blood pressures at baseline (Table 4.1). No clinically significant changes in haematological or biochemical analytes occurred throughout the study. Biochemical measures of renal function (serum urea, creatinine and electrolytes) were within normal limits at baseline and remained unchanged with placebo and treatment with SRT2104 in both sub-groups (Table 4.2).

4.4.2 BLOOD PRESSURE

Resting systolic and diastolic blood pressures remained unchanged throughout the study with no significant differences between treatment and placebo treatment periods.

TABLE 4.1 Baseline characteristics of participants who were otherwise healthy cigarette smokers or who had type 2 diabetes mellitus

	Otherwise Healthy Cigarette Smokers (n=24)	People with Type 2 Diabetes (n=15)
Mean age (yrs)	38±13	58±8
Sex		
Male (%)	14 (58%)	13 (87%)
Female (%)	10 (42%)	2 (13%)
Baseline Blood pressure (mmHg)		
Systolic	129±6	133±7
Diastolic	77±2	80±3
Heart Rate (bpm)	68±1	77±5
Body Mass Index (kg/m²)	25±4	30±4
Smoking History		
No. of cigarettes/ day	17±6	---
No. of pack years	16	---
Urinary cotinine concentration (ng/mL)	1352±950	---
Glycaemic Profile		
Baseline blood glucose (mg/dL)	85±0	144±2
HbA1c (%)	---	7.4±0.8
Concomitant Medications		
1. Antiplatelet agents (%)	---	5 (33%)
2. Antihypertensive agents		
ARB (%)	---	3 (20%)
Diuretics (%)	---	2 (13%)
3. Lipid lowering agents (%)	---	10 (67%)

Values expressed as mean±standard deviation. ARB - angiotensin receptor blocker.

TABLE 4.2 Changes in biochemical measures of renal function in otherwise healthy cigarette smokers and participants with type 2 diabetes mellitus administered placebo and SRT2104

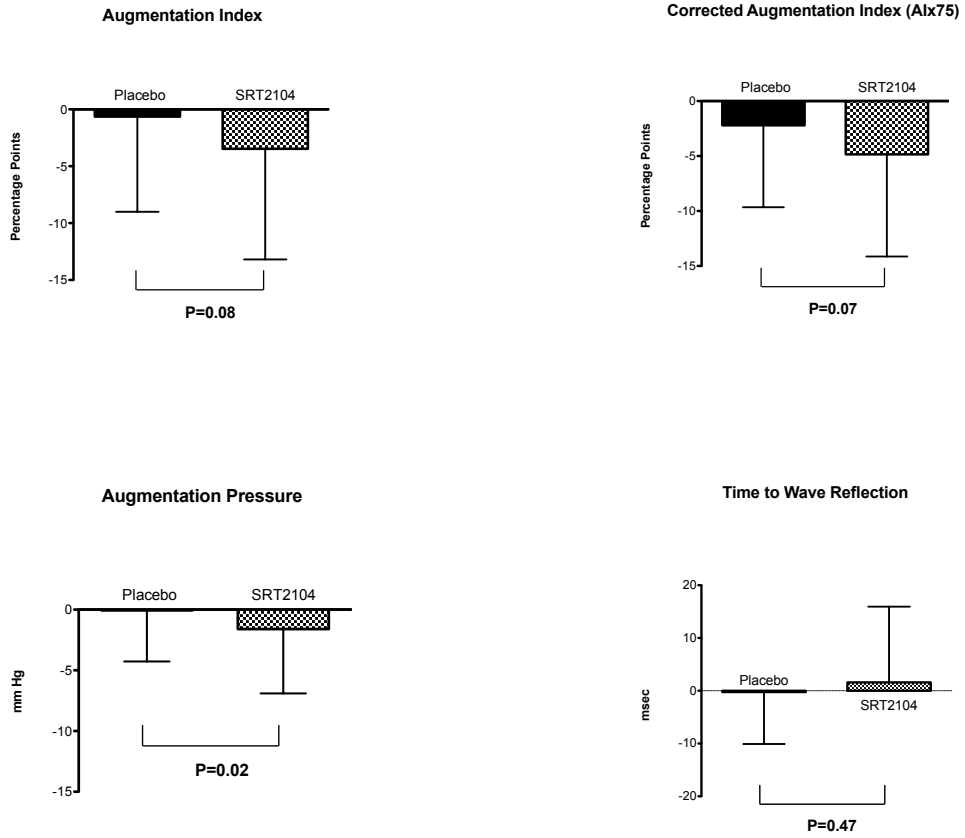
	Otherwise Healthy Cigarette Smokers (n=24)				Participants with Type 2 Diabetes (n=15)			
	Treatment period 1: Placebo (n=13)		Treatment period 1: SRT2104 (n=11)		Treatment period 1: Placebo (n=8)		Treatment period 1: SRT2104 (n=7)	
	Day 1	Day 28	Day 1	Day 28	Day 1	Day 28	Day 1	Day 28
Serum urea (mg/dL)	17±3	14±3	11±3	14±3	17±3	17±3	17±6	14±3
Serum creatinine (mg/dL)	0.8±0.1	0.8±0.1	0.7±0.1	0.8±0.1	0.8±0.1	0.8±0.1	0.9±0.1	0.9±0.1
Electrolytes:								
Sodium (mmol/L)	140±2	140±2	140±2	140±1	139±1	139±3	138±2	139±3
Potassium (mmol/L)	4.0±0.4	4.0±0.4	4.0±0.4	4.0±0.2	4.0±0.3	4.0±0.3	4.0±0.2	4.0±0.4
Chloride (mmol/L)	109±3	107±3	108±3	107±2	104±4	104±3	103±2	104±2

4.4.3 PULSE WAVE ANALYSIS AND VELOCITY

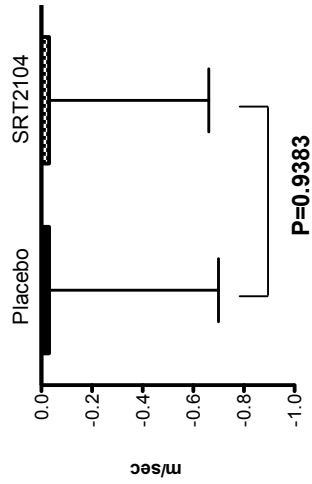
In a combined analysis of otherwise healthy cigarette smokers and participants with type 2 diabetes, a reduction in the augmentation pressure was observed in subjects receiving SRT2104 compared with placebo (mean change from baseline: SRT2104 -1.60 (5.304) *versus* placebo -0.06 (4.205); P=0.02) and a trend towards improvement in the augmentation index (mean change from baseline in AIx: placebo -0.64 (8.361) *versus* SRT2104 -3.47 (9.728); P=0.08) and the corrected AIx (mean change from baseline AIx 75: placebo -2.2- (7.453) *versus* SRT2104 -4.84 (9.299); P=0.07) (Figure 4.1A). Pulse wave velocity and time to wave reflection remained unchanged between placebo and treatment arms (P>0.05 for both parameters; Figure 4.1B). The effects of SRT2104 administration on measures of arterial compliance were consistent across the two cohorts. For example, in the SRT2104 arm, mean augmentation index at 75 beats/minute was reduced for both healthy smokers and participants with type 2 diabetes (-4.97 *versus* -4.63, respectively). A statistical interaction between cohort and treatment was not observed (P>0.05 for all variables tested).

Figure 4.1

A:



B:



C:

Mean (SD)	Augmentation Index Corrected		Augmentation Pressure (mmHg)		Time to Wave Reflection (ms)		Pulse Wave Velocity (m/sec)	
	Baseline (n=39)	Day 28/56 (n=39)	Baseline (n=39)	Day 28/56 (n=39)	Baseline (n=39)	Day 28/56 (n=39)	Baseline (n=39)	Day 28/56 (n=39)
Placebo (n=37)	14.61 (16.25)	12.13 (16.05)	6.41 (6.63)	6.24 (7.26)	147.65 (12.73)	147.28 (10.89)	7.26 (1.82)	7.29 (1.90)
SRT2104 (n=39)	12.00 (18.44)	7.29 (17.41)	4.81 (8.09)	4.81 (8.09)	149.24 (17.27)	149.24 (17.27)	7.23 (1.95)	7.23 (1.95)

Figure 4.1. Effect of treatment with SRT2104 on measures of arterial compliance in otherwise healthy cigarette smokers and participants with type 2 diabetes mellitus - Change from baseline. **Panel A:** Pulse wave analysis - augmentation index (AIx), corrected augmentation index (AIx75), augmentation pressure (AP) and time to wave reflection (Tr); **Panel B:** Pulse wave velocity (PWV). Solid column: Placebo; Checked column: SRT2104; **Panel C:** Baseline parameters of measures of arterial compliance - combined data. SD - standard deviation.

4.4.4 TOLERABILITY AND SAFETY

Subjects in both study groups (healthy cigarette smokers and patients with type 2 diabetes) tolerated the study medication well. There were no meaningful differences in the number of adverse events between active treatment and placebo. All reported adverse events were mild in intensity and resolved without any intervention or sequelae (Table 4.3). Headaches occurred with nearly equal frequency in the treatment (SRT2104) group in both cohorts. Subjects with type 2 diabetes appeared to have more frequent gastrointestinal disturbances, such as diarrhoea and nausea in comparison to healthy smokers. Elevated liver enzymes (alanine transaminase) resulted in withdrawal of one subject in the placebo period (Day 36) of the diabetes group. There was only one reported serious adverse event in the study (SRT2104 arm of healthy cigarette smokers) of traumatic facial bone fracture that was considered unrelated to SRT2104.

TABLE 4.3 Summary of treatment emergent adverse events occurring in two or more subjects in otherwise healthy cigarette smokers (OHS) and participants with type 2 diabetes mellitus (T2DM)

System Class	Organ	Adverse event	Number of Events			
			OHS		T2DM	
			Placebo (N=24)	SRT2104 (N=24)	Placebo (N=14)	SRT2104 (N=15)
Any Event			18	18	11	14
Nervous System Disorders		Any event	6 (25%)	11 (46%)	1 (7%)	7 (47%)
		Headache	4	6	1	5
		Paraesthesia	0	2	0	1
		Hypoaesthesia	1	2	0	0
		Presyncope	1	1	0	0
Respiratory, Thoracic and Mediastinal Disorders		Any event	1 (4%)	3 (13%)	0	3 (20%)
		Oropharyngeal pain	1	2	0	0
		Rhinorrhoea	0	1	0	1
Gastrointestinal Disorders		Any event	3 (13%)	1 (4%)	4 (29%)	8 (53%)
		Diarrhoea	0	0	2	4
		Nausea	0	0	1	2
		Abdominal pain upper	1	0	0	1
		Dyspepsia	0	0	0	2
Reproductive System and Breast Disorders		Any event	3 (13%)	1 (4%)	0	0
		Dysmenorrhea	3	1	0	0
Musculoskeletal and Connective Tissue Disorders		Any event	4 (17%)	1 (4%)	0	2 (13%)
		Back pain	2	0	0	0
Investigational		Any event	2 (8%)	1 (4%)	1 (7%)	1 (7)
		Blood Bilirubin Increased	1	1	0	0
		Alanine Amino Transferase Increased	0	0	1	0
		Abnormal liver function test	0	0	0	1
General Disorders and Administration Site Conditions		Any event	4 (17%)	4 (17%)	2 (14%)	3 (20%)
		Influenza like illness	1	0	0	1
		Fatigue	1	0	1	1

Continued...

Infections and Infestations	Any event	3 (13%)	5 (21%)	3 (21%)	1 (7%)
	Nasopharyngitis	0	1	3	0
	Rhinitis	2	2	0	0
	Upper respiratory tract infection	1	0	0	1
Injury, Poisoning and Procedural Complications	Any event	4 (17%)	2 (8%)	3 (21%)	1 (7%)
	Contusion	1	0	1	0
	Excoriation	1	1	0	0
Skin and Subcutaneous Tissue	Any event	1 (4%)	0	1 (7%)	3 (20%)
	Pruritis	0	0	0	2
Metabolism and Nutrition Disorders	Any event	0	1 (4%)	2 (14%)	2 (13%)
	Hypoglycaemia	0	0	1	2
Vascular Disorders	Any event	0	1 (4%)	0	2 (13%)
	Flushing	0	0	0	2

4.5 DISCUSSION

This randomised, double blind, placebo controlled, crossover study demonstrated for the first time that the oral SIRT1 activator, SRT2104, may improve arterial compliance in otherwise healthy cigarette smokers and in people with type 2 diabetes, without affecting resting measures of blood pressure.

The assessment of arterial stiffness is increasingly being used in clinical practice as an independent measure of cardiovascular risk, including those in high-risk groups [Laurent *et al* 2006]. Ageing is associated with an increase in the stiffness of large elastic arteries induced by structural alterations in the vascular media such as an increase in collagen and a decrease in elastin content [Weiss and Fontana 2011]. This process of biological ageing is accelerated in the presence of conditions such as diabetes mellitus and hypertension. Semba *et al* [2009] and Hoffman *et al* [2013] have demonstrated an association between the presence of advanced glycation end products and increased arterial stiffness. Indeed, vascular change induced by cigarette smoke is considered to be a model of accelerated vascular ageing. The relationship between tobacco exposure [Rehill *et al* 2006; Binder *et al* 2008; Kubozono *et al* 2011], diabetes [Stehouwer *et al* 2008; Roos *et al* 2011; Bruno *et al* 2012] and increased arterial stiffness is well established.

Calorie restriction can attenuate age-related arterial stiffness in animal models through reduced oxidative stress and altered endothelial nitric oxide bioavailability [Weiss and Fontana 2011]. Indeed, calorie restriction can extend lifespan in lower

organisms and mammals, and improves several metabolic and inflammatory parameters [Lane *et al* 2001; Roth *et al* 2001; Lane *et al* 2002; Heilbronn and Ravussin 2003; Heilbronn and Ravussin 2005; Heilbronn *et al* 2006]. SIRT1 has been implicated as an important mediator of life span extension mediated by calorie restriction [Barzilai *et al* 1998; Labinsky *et al* 2006; Timmers *et al* 2011]. The current hypothesis, therefore, was that activation of SIRT1 may inhibit this process of vascular ageing and be associated with improvements in arterial stiffness.

No studies have examined the direct effect of SIRT1 activation on measures of arterial compliance. Botden *et al* [2012] were unable to demonstrate an improvement in augmentation index, or central or peripheral blood pressure following treatment with red wine polyphenols. In the present study, a 28-day period of treatment with the oral SIRT1 activator SRT2104 was associated with a reduction in augmentation pressure and trends towards improvement in AIx and corrected AIx. Augmentation pressure and index are measures of arterial compliance and wave reflection from small to medium sized arteries. As such, they can be influenced by endothelial function and a number of other dynamic and functional factors, such as heart rate and peripheral circulatory tone [Laurent *et al* 2006; Stehouwer *et al* 2008]. Preclinical studies have demonstrated improved vascular function with SIRT1 activation [Mattagajasingh *et al* 2007; Ota *et al* 2007; Csiszar *et al* 2008; Donato *et al* 2011], and this may explain our observations of improvement in dynamic measures of arterial stiffness following short term administration of SRT2104.

Pulse wave velocity is a more direct measure of arterial stiffness that is determined by the structural and physical composition of the arterial wall [Roos *et al* 2011]. Changes in pulse wave velocity are therefore more gradual and less dependent on the function of small to medium sized arteries. In the present study, a change in PWV was not observed with SRT2104 administration. This is perhaps not surprising given the short time period of exposure to SRT2104 (28 days) and the brief period of observation. An improvement in pulse wave velocity might be anticipated with a longer period of treatment with SRT2104, to allow more favourable structural changes in the larger arterial tree.

4.5 STUDY LIMITATIONS

Some limitations of this trial should be considered. Although favourable trends in parameters of arterial compliance were observed, some did not achieve statistical significance. This may partly be attributed to the trial being designed specifically to examine the acute effects of treatment with SRT2104. A longer period of treatment may be required for benefits to emerge on variables such as pulse wave velocity, that involve structural changes in the arterial wall. Moreover, the sample sizes of the two groups examined were small. Two disparate populations were studied in this trial, in whom the mechanisms of vascular dysfunction may be very different. Moreover, there was a significant inter-subject variability in the mean plasma concentration of SRT2104. A higher peak plasma concentration was achieved in otherwise healthy cigarette smokers compared to subjects with type 2 diabetes ($P < 0.05$ for mean plasma concentration of SRT2104 at 3 hours post dose - otherwise healthy cigarette

smokers *vs* subjects with type 2 diabetes) which may account for some of the differences in response seen between the two groups. However, the direction of beneficial effects on treatment with SRT2104 was similar between the two groups, providing reassurance of a consistency of effect and allowing the post-hoc presentation of the results pooled across the two groups. We do acknowledge that given the disparate nature of the two groups, the results from this study can only be considered to be hypothesis generating.

4.6 CONCLUSION

The present study has provided evidence that suggests treatment with the oral SIRT1 activator, SRT2104, may lead to an improvement in measures of arterial compliance in otherwise healthy cigarette smokers and people with type 2 diabetes. The exact mechanism of this improved arterial compliance and the effects of prolonged treatment with SRT2104 on vascular health remain to be elucidated. Given that aortic stiffness and endothelial function are key factors in predicting cardiovascular outcomes, identification of novel pharmacological means of improving these predictive parameters is important and highly relevant in populations with known cardiovascular risk factors.

CHAPTER 5

VASCULAR EFFECTS OF UROCORTINS 2 AND 3 IN HEALTHY VOLUNTEERS

Venkatasubramanian S, Griffiths ME, McLean SG *et al.*
Vascular effects of urocortins 2 and 3 in healthy volunteers.
J Am Heart Assoc 2013;**2**(1):e004267.

5.1 SUMMARY

Background Urocortin 2 and urocortin 3 are endogenous peptides with an emerging role in cardiovascular pathophysiology. We assessed their pharmacodynamic profile and examined the role of the endothelium in mediating their vasomotor effects *in vivo* in man.

Methods Eighteen healthy male volunteers (23±4 years) were recruited into a series of randomised, double blind, placebo controlled, crossover studies using bilateral forearm venous occlusion plethysmography during intra-arterial urocortin 2 (3.6-120 pmol/min), urocortin 3 (1.2-36 nmol/min) and substance P (2-8 pmol/min) in the presence or absence of inhibitors of cyclooxygenase (aspirin), cytochrome P450 metabolites of arachidonic acid (fluconazole) and nitric oxide synthase (L-N^G-monomethyl-arginine citrate (L-NMMA)).

Results Urocortins 2 and 3 evoked arterial vasodilatation ($p < 0.0001$) without tachyphylaxis but with a slow onset and offset of action. Inhibition of nitric oxide synthase with L-NMMA reduced vasodilatation to substance P and urocortin 2 ($P \leq 0.001$ for both) but had little effect on urocortin 3 ($P > 0.05$). Neither aspirin nor fluconazole affected vasodilatation induced by any of the infusions ($P > 0.05$ for all). In the presence of all three inhibitors, urocortin 2- and urocortin 3-induced vasodilatation was attenuated ($P < 0.001$ for all) to a greater extent than with L-NMMA alone ($P \leq 0.005$).

Conclusion Urocortins 2 and 3 cause potent and prolonged arterial vasodilatation without tachyphylaxis. These vasomotor responses are at least partly mediated by endothelial nitric oxide and cytochrome P450 metabolites of arachidonic acid. The

role of urocortins 2 and 3 remains to be explored in the setting of human heart failure but they have the potential to have major therapeutic benefits.

5.2 INTRODUCTION

Urocortin peptides, especially urocortins 2 and 3, have prominent cardiovascular roles and are expressed in the heart. Although related to corticotrophin-releasing hormone, they do not appear to have any role in the regulation of the hypothalamic-pituitary-adrenal axis [Davidson *et al* 2009a; Davidson and Yellon 2009b]. The effects of CRH and the urocortins are mediated via two G protein-coupled receptors: CRH-R1 and CRH-R2. Whilst CRH-R1 is predominantly expressed in the brain and not in the heart, CRH-R2 is expressed in the myocardium and vascular smooth muscle [Wiley and Davenport 2004; Davidson and Yellon 2009b]. It is found in human coronary artery microvascular endothelial cells and has been detected in the endothelium of a variety of peripheral vascular beds. Urocortin 1 activates both receptors whilst urocortins 2 and 3 are potent selective agonists at CRH-R2 but have no effect on CRH-R1 [Venkatasubramanian *et al* 2010].

The role of urocortins in cardiovascular physiology and pathophysiology, particularly heart failure, has become increasingly apparent. Intravenous urocortin 1 causes marked vasodilatation in mice via CRH-R2 [Bale *et al* 2000; Coste *et al* 2000]. Furthermore, mice lacking CRH-R2 receptors are hypertensive, suggesting a role for urocortin in the maintenance of basal vascular tone [Coste *et al* 2000]. Systemic administration of urocortin 2 in humans increases

cardiac output, heart rate and left ventricular function while decreasing systemic vascular resistance and these effects may be amplified in the setting of heart failure [Davis *et al* 2007a; Davis *et al* 2007b]. Urocortin 3 has not previously been administered to humans but, in an ovine model, both urocortins 2 and 3 appeared to produce similar cardiovascular effects. [Rademaker *et al* 2005b; Rademaker *et al* 2006]. Although urocortins 2 and 3 each activate the same receptor, potential differences in their cardiovascular therapeutic utility may arise from their differing pharmacokinetic and pharmacodynamic profile [Davidson *et al* 2009a].

The cardiovascular responses of urocortins represent an amalgamation of systemic actions. However, their direct *in vivo* arterial vasomotor effects have never been examined in man. Moreover, the role of the endothelium in the mediation of these responses is unknown. Therefore, our study aims were to conduct the first comparative clinical assessment of local arterial vasomotor effects of urocortins 2 and 3, and determine the role of the endothelium in the mediation of these effects.

5.3 METHODS

All studies were approved by the local Research Ethics Committee and carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants prior to the study.

5.3.1 STUDY PARTICIPANTS

Eighteen healthy non-smoking male volunteers were recruited into a series of vascular studies. Participants had no documented previous medical history, were taking no regular medication and tested negative in a urinary toxicology screen for recreational drugs.

5.3.2 VASCULAR STUDIES

All studies were conducted using a randomised, double blind, placebo controlled, crossover design. They were performed with the patient lying supine, in a quiet, temperature controlled room (22-25°C). Volunteers fasted for 4 hours prior to the study and refrained from alcohol and caffeine for 24 hours prior to the study. Venous cannulae (17-G) were inserted into large subcutaneous veins in the antecubital fossae of both arms at the start of the study to facilitate periodic venous sampling. In view of the theoretical risk of alterations in body temperature [Telegdy and Adamik 2008] and blood glucose concentrations [Li *et al* 2007] with the first-in-human administration of urocortin 3, tympanic temperature (Genius 2 tympanic Thermometer, Coviden, Boston, USA) and capillary blood glucose measurements (Advantage Accucheck blood glucometer, USA) were performed at baseline and after each dose of urocortin 3. Heart rate and blood pressure were monitored at regular intervals throughout the study with a semi-automated oscillometric sphygmomanometer.

Subjects underwent brachial artery cannulation in the non-dominant forearm with a 27-standard wire gauge steel needle. Forearm blood flow was measured in the

infused and non-infused forearms using bilateral venous occlusion plethysmography as described previously [Newby *et al* 1997b; Newby *et al* 1999 b].

5.3.3 PHARMACODYNAMIC STUDY

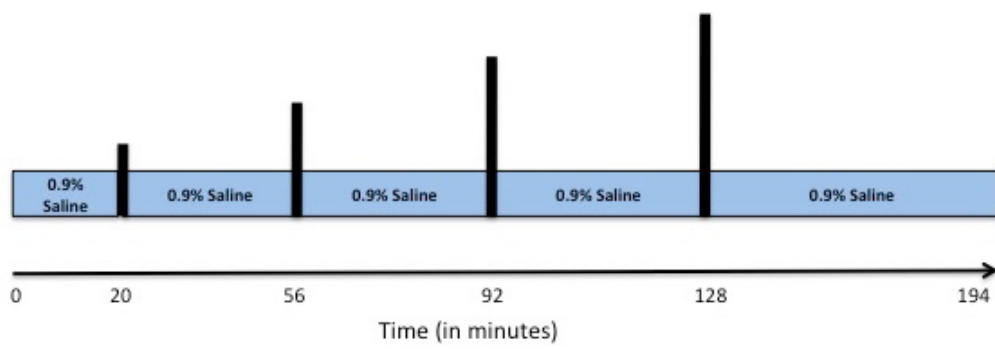
Eight healthy volunteers attended on four occasions (Protocol 1) separated by at least one week (Figure 5.1A). After an initial infusion of normal saline (0.9%) for 20 minutes, volunteers received discontinuous (Protocol 1a) or continuous (Protocol 1b) incremental intra-arterial doses of urocortin 2 (3.6-120 pmol/min) or urocortin 3 (1.2-36 nmol/min) interspersed with saline infusions between doses as appropriate.

5.3.4 MECHANISTIC STUDY

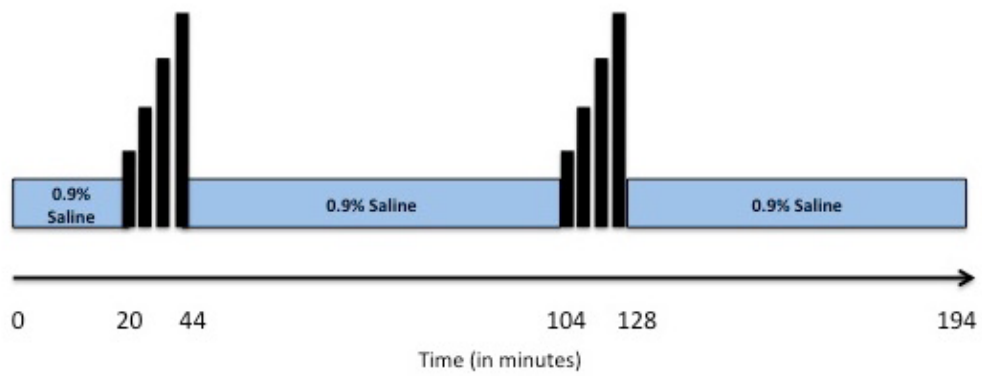
A further 10 healthy volunteers (Protocol 2) attended on each of five occasions to receive incremental intra-arterial doses of urocortin 2 (3.6-36 pmol/min), urocortin 3 (1.2-12 nmol/min) and substance P (2-8 pmol/min; a control endothelium-dependent vasodilator that evokes endothelial t-PA release (Figure 5.1B)). These infusions were administered in the presence of 1) placebo, 2) oral aspirin 600 mg (cyclooxygenase inhibition), 3) nitric oxide clamp (nitric oxide synthase inhibition; see below), 4) intra-arterial fluconazole (1.2 µmol/min; inhibition of cytochrome P450 metabolites of arachidonic acid), and 5) a combination of oral aspirin, intra-arterial fluconazole and the nitric oxide clamp.

Protocol 1

Protocol 1a:

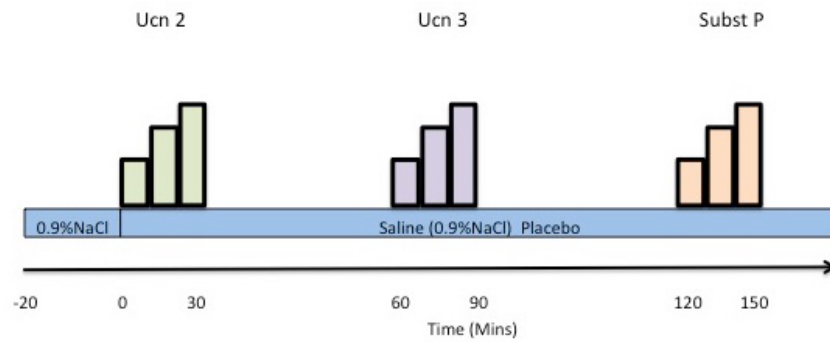


Protocol 1b:

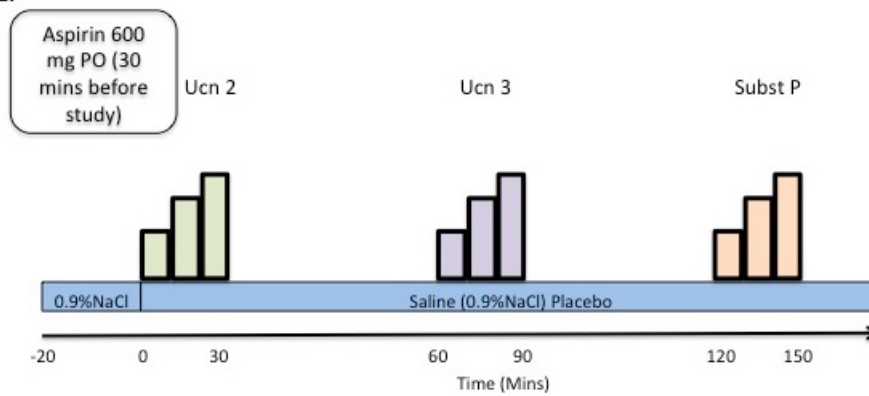


Protocol 2

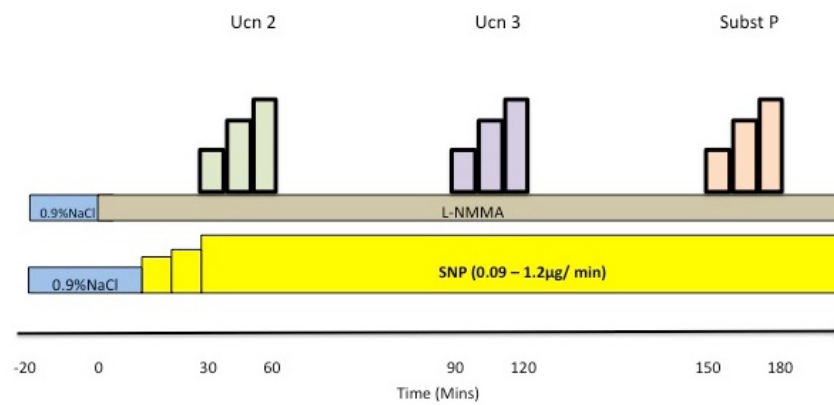
1.



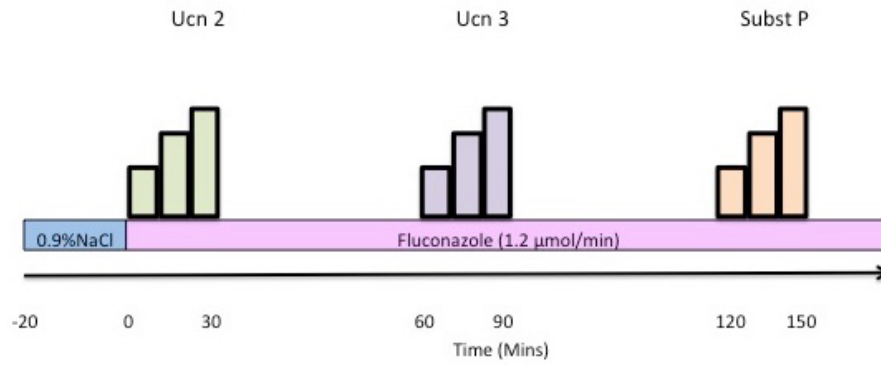
2.



3.



4.



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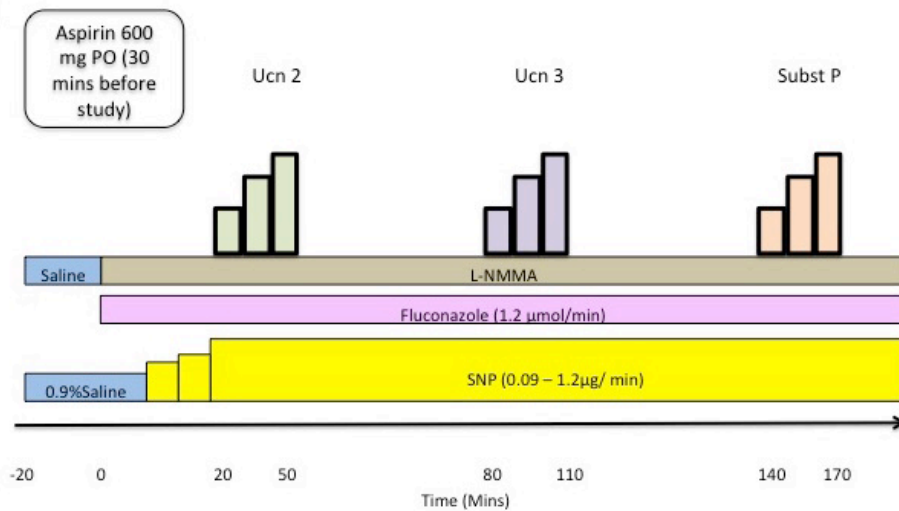


Figure 5.1. Schematic representation of study protocols. **A) Protocol 1:** Incremental intra-arterial doses of urocortin 2 (Ucn 2; 3.6-120 pmol/min) and urocortin 3 (Ucn 3; 1.2-36 nmol/min) in the presence (**Protocol 1a**) and absence (**Protocol 1b**) of saline washout. **B) Protocol 2:** Incremental intra-arterial infusions of Ucn 2 (3.6-36 pmol/min), Ucn 3 (1.2-12 nmol/min) and substance P (subs P; 2-8 pmol/min) in the presence of 1) saline placebo, 2) oral aspirin, 3) nitric oxide clamp, 4) intra-arterial fluconazole and 5) combination of oral aspirin, fluconazole and nitric oxide clamp. L-NMMA - L-N^G-monomethyl-arginine; SNP - sodium nitroprusside.

The nitric oxide clamp was used to determine the contribution of nitric oxide to urocortin-induced vasodilatation. Following baseline saline infusion, the nitric oxide synthase inhibitor, (L-NMMA; 8 μ mol/min), was infused intra-arterially. To compensate for L-NMMA-induced basal vasoconstriction, forearm blood flow was returned to baseline using a titrated dose of the exogenous nitric oxide donor sodium nitroprusside (SNP; 90-1200 ng/min). Once baseline blood flow had been restored, this dose of SNP was co-infused with L-NMMA and continued throughout the study. This arrangement allows a constant 'clamped' delivery of exogenous nitric oxide whilst endogenous nitric oxide synthase activity is abolished.

The order of urocortin 2, urocortin 3 and substance P infusions was randomised between subjects, but kept constant for all visits of each individual subject. The order of infusion of inhibitors was also randomised in a double blind manner.

5.3.4 VENOUS SAMPLING

Blood sampling was carried out at baseline for the assessment of full blood count, liver and renal function tests, cholesterol and blood glucose levels. Analysis was performed by the local clinical biochemistry and haematology reference laboratories.

5.3.5 DATA ANALYSIS AND STATISTICS

Forearm blood flow data were analysed as described previously [Newby *et al* 1997b]. A normal distribution of the data was demonstrated using D'Agostino and Pearson omnibus normality test. All variables are reported as mean \pm SEM using

repeated-measure ANOVA with post-hoc Bonferroni corrections and two-tailed Student's *t*-test as appropriate. Significance was taken as two-sided $P < 0.05$.

5.4 RESULTS

5.4.1 STUDY PARTICIPANTS

All volunteers were young healthy men (23 ± 4 years). Both urocortin 2 and urocortin 3 produced marked localised flushing in the infused arm along with facial flushing at the highest doses. Volunteers also experienced heightened awareness of their heart beat during, and immediately after, the highest dose of urocortin 3 (36 nmol/min). All symptoms were self-limiting, well tolerated and short-lived. Substance P also induced localised flushing of the infused forearm, which was self-limiting. There were no clinically significant changes in the standard haematological and biochemical analytes including full blood count, blood glucose, cholesterol, and renal and hepatic function throughout the study (data on file). Capillary blood glucose and body temperature (tympanic) remained unchanged during all doses of urocortin 3 (data on file).

Systolic blood pressure and non-infused forearm blood flow remained unchanged at all doses with all three peptides across both protocols. However, at the highest infused dose of urocortin 3 (36 nmol/min), there was a sinus tachycardia ($+22 \pm 2 \text{ beats/min}$; ANOVA, $P < 0.0001$) with an associated drop in diastolic blood pressure ($-8.5 \pm 0.8 \text{ mmHg}$; ANOVA, $P = 0.004$; Figure 5.2) that was not seen with either substance P or urocortin 2 infusions.

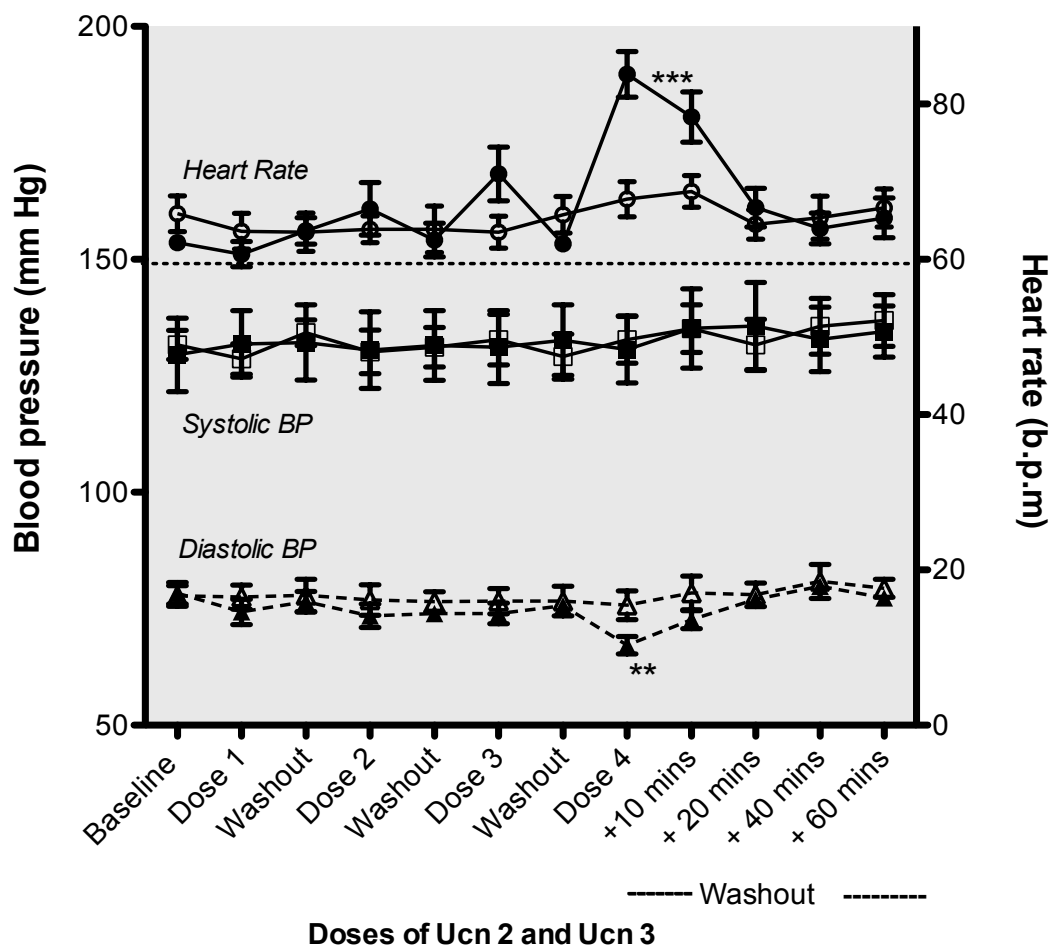


Figure 5.2. Haemodynamic responses to intra-arterial infusion of urocortin 2 (Ucn 2; 3.6-120 pmol/min) and urocortin 3 (Ucn 3; 1.2-36 nmol/min): At a dose of 36 nmol/min, Ucn 3 evoked a transient tachycardia associated with a drop in diastolic blood pressure. **Open symbols:** Ucn 2; **Closed symbols:** Ucn 3; **Circle:** Heart rate; **Square:** Systolic blood pressure; **Triangle:** Diastolic BP; *****:** $P < 0.0001$; ****:** $P = 0.004$; **Dose 1** = 3.6 pmol/min Ucn 2 or 1.2 nmol/min Ucn 3; **Dose 2** = 12 pmol/min Ucn 2 or 3.6 nmol/min Ucn 3; **Dose 3** = 36 pmol/min Ucn 2 or 12 nmol/min Ucn 3; **Dose 4** = 120 pmol/min Ucn 2 or 36 nmol/min Ucn 3.

5.4.2 PHARMACODYNAMIC EFFECTS OF UROCORTIN 2 AND 3

Both urocortin 2 and urocortin 3 evoked dose-dependent arterial vasodilatation in the infused arm (two-way ANOVA, $P < 0.0001$; Figure 5.3).

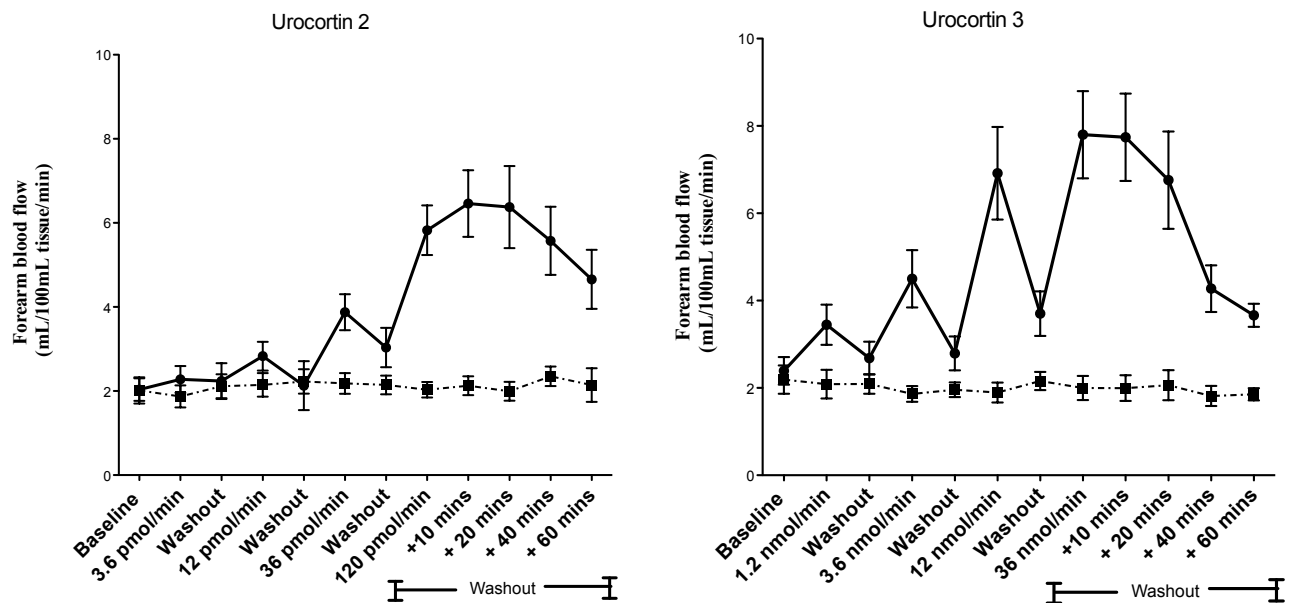


Figure 5.3. Forearm arterial blood flow responses to increasing doses of urocortin 2 (Ucn 2) and urocortin 3 (Ucn 3). **Circle:** infused forearm blood flow; **Square:** non-infused forearm blood flow. $P < 0.0001$ at all doses.

Maximal vasodilatation with urocortin 2 was apparent approximately 10 minutes after cessation of the 120 pmol/minute infusion (paired Student's *t*-test, Ucn 2 120 pmol/min *versus* +10 minutes washout; $P = 0.04$) and thereafter the blood flow gradually returned towards baseline. This was in contrast to the effect seen with

urocortin 3, where the maximum vasodilatory response was immediate (Figure 5.4A). The offset of vasodilatation was prolonged with both peptides although urocortin 2 took longer than urocortin 3 to return to baseline (Figure 5.4A).

The vasodilator effects of both peptides appeared to be reproducible within-day, with no evidence of tachyphylaxis (two-way ANOVA, $P > 0.05$ for all; Figure 5.4.B).

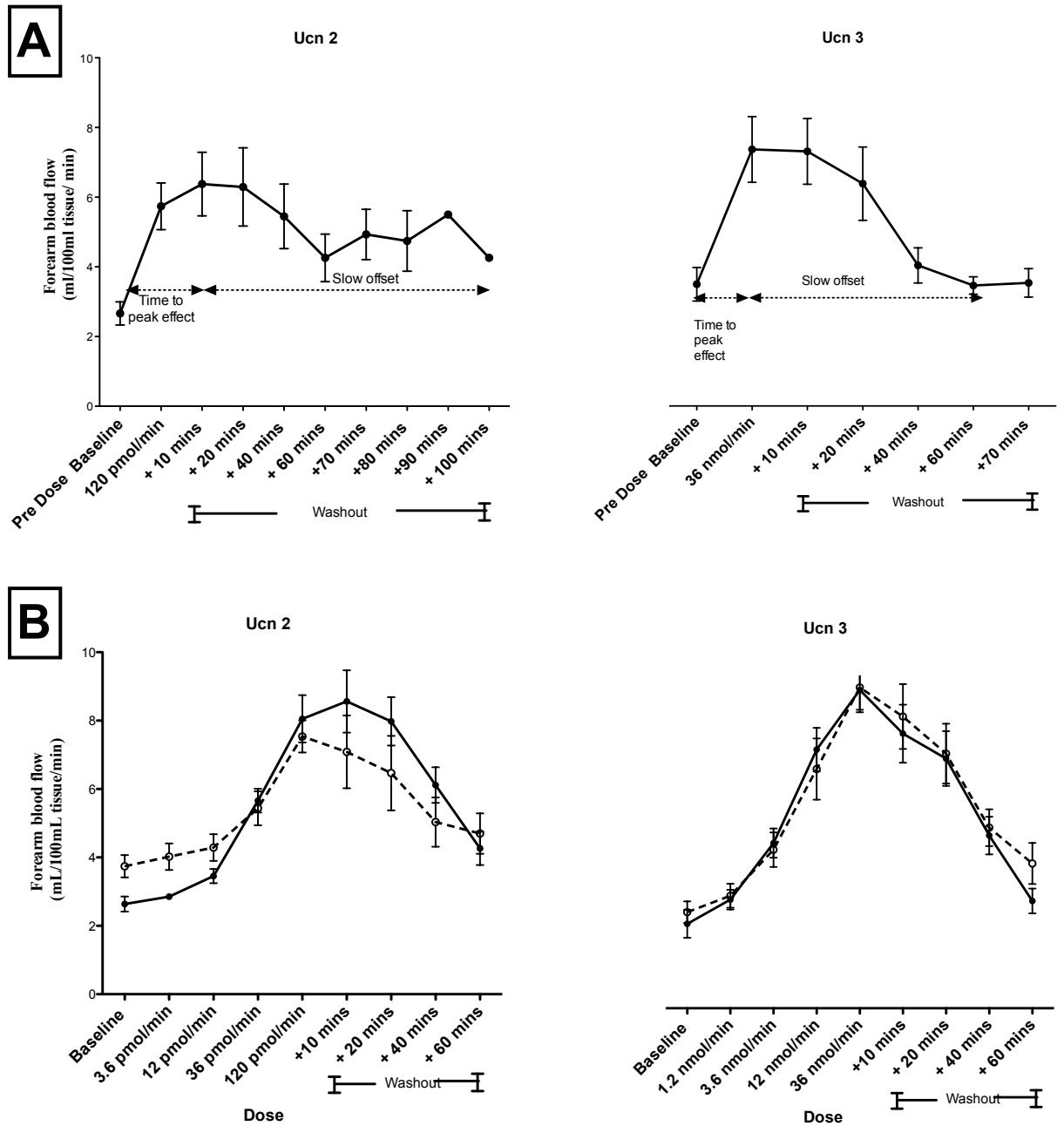


Figure 5.4. Pharmacodynamics of urocortin 2 (Ucn 2) and urocortin 3 (Ucn 3). **A**) Onset and offset of vasodilatory effect of Ucn 2 (left) and Ucn 3 (right) after infusion of highest dose; **B**) Within-day reproducibility of Ucn 2 (left) and Ucn 3 (right) (P =non-significant, 1st Dose response *versus* 2nd Dose response; Ucn 2 and Ucn 3). **Closed circle:** 1st Dose Response; **Open circle:** 2nd Dose Response.

5.4.3 ENDOGENOUS FIBRINOLYTIC FACTORS

Preliminary data showed no effect of urocortin 2 or 3 on endothelial release of t-PA and PAI-1.

5.4.4 MECHANISM OF VASODILATATION

Baseline forearm arterial blood flow was unaffected by oral aspirin or intra-arterial fluconazole, and the co-infusion of sodium nitroprusside restored baseline blood flow during L-NMMA administration (two-way ANOVA, $P > 0.05$ for all). Inhibition of nitric oxide synthase reduced arterial vasodilatation to substance P and urocortin 2 (two-way ANOVA, $P \leq 0.001$ for both) but had no apparent effect on urocortin 3-induced vasodilatation (two-way ANOVA, $P = 0.36$). Neither inhibition of cyclooxygenase with aspirin nor cytochrome P450 metabolites of arachidonic acid with fluconazole affected the vasodilatation induced by the urocortins or substance P (two-way ANOVA, $P > 0.05$ for all; data on file). In the presence of all three inhibitors, substance P, urocortin 2 and urocortin 3-induced vasodilatation was further attenuated (two-way ANOVA, $P < 0.001$ for all), but not completely abolished. Combined inhibition of cyclooxygenase, nitric oxide synthase and cytochrome P450 metabolites of arachidonic acid produced a greater reduction in vasodilatation than the nitric oxide clamp alone (two-way ANOVA, $P \leq 0.005$ for urocortins 2 and 3; Figure 5.5).

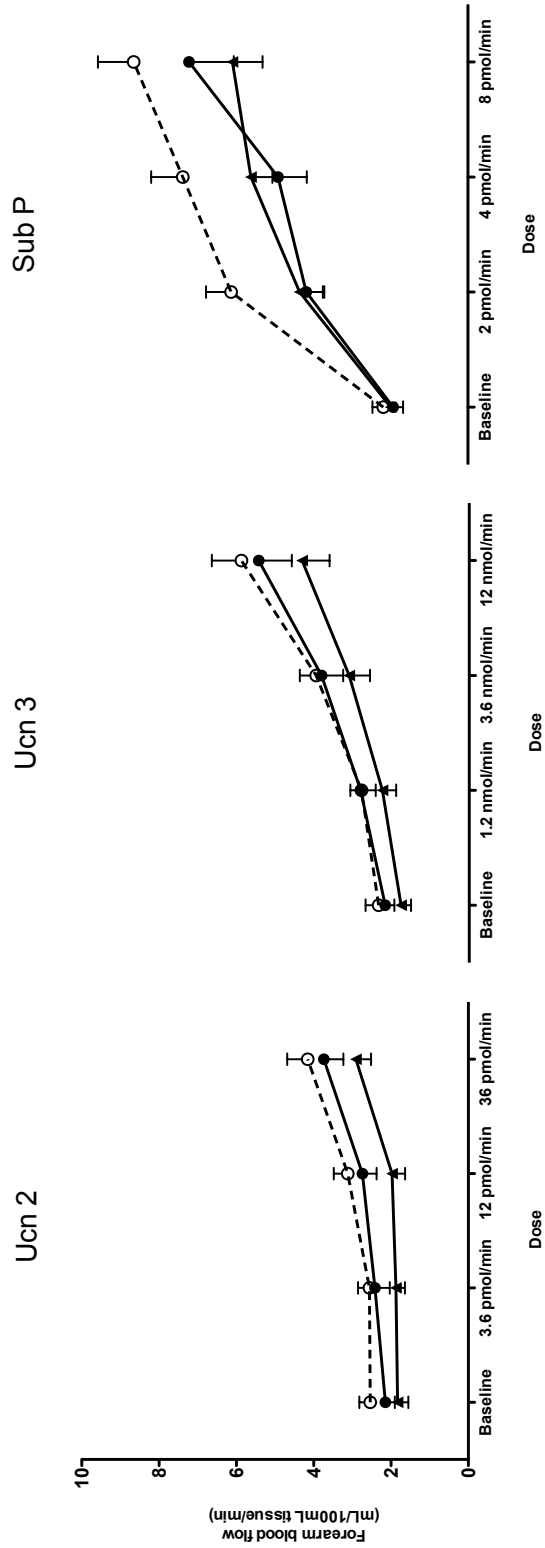


Figure 5.5. Vasomotor effects of inhibition of endothelial nitric oxide synthase, cyclooxygenase and cytochrome P450 metabolites of arachidonic acid on urocortin 2, urocortin 3 and substance P-mediated vasodilatation. **Open circle:** nitric oxide clamp; **Closed triangle:** combined aspirin (600 mg), nitric oxide clamp and fluconazole (1.2 $\mu\text{mol}/\text{min}$). **Ucn** - urocortin; **Sub P** - substance P.

5.5 DISCUSSION

This study represents the first-in-human administration of urocortin 3 and demonstrates that both urocortins 2 and 3 directly evoke potent and prolonged arterial vasodilatation that is, at least in part, mediated by the endothelium. These findings are of direct relevance not only to our understanding of human cardiovascular physiology but also inform the development of therapies targeting the urocortin system for the treatment of conditions such as heart failure.

The forearm arterial vasodilator effects of urocortin 2 and urocortin 3 are consistent with data from *in vitro* [Huang *et al* 2002; Sanz *et al* 2002; Wiley and Davenport 2004; Chen *et al* 2005; Smani *et al* 2010] and preclinical animal studies [Chen *et al* 2003; Dieterle *et al* 2009]. However, in contrast with existing preclinical data, we observed a more marked difference in potency between the two peptides. Although preclinical studies have suggested urocortin 2 is 10-fold more potent [Hsu and Hsueh 2001; Fekete and Zorrilla 2007]. Wiley and Davenport [2004] showed equipotency of urocortins 2 and 3 in isolated human internal mammary arterial segments. In contrast, here we observed that a 300-fold higher dose of urocortin 3 was required to evoke comparable vasomotor effects in the human forearm arterial circulation. This discrepancy underlines the importance of a direct head-to-head assessment *in vivo* in man, without which the extrapolation of preclinical data may be deceptive.

Urocortins 2 and 3 are specific agonists at the G protein-coupled CRH-R2 receptors, mediating their effects through a cascade of intracellular signalling pathways

including adenylyl cyclase, cAMP [Kageyama *et al* 2003a; Kageyama *et al* 2003b], and mitogen-activated protein kinases [Brar *et al* 2000]. Other well characterised G protein-coupled receptor (GPCR) agonists such as bradykinin [Wetherow *et al* 2001; Wetherow *et al* 2003], substance P [Newby *et al* 1997b] and protease activated receptor type 1 activating peptide (SFLLRN) [Gudmundsdottir *et al* 2006; Lang *et al* 2008b] evoke vasodilatation with rapid onset and offset in the human forearm arterial circulation. Unlike these agonists, the maximal vasodilator effect evoked by urocortin 2 in this study was apparent approximately 10 minutes following completion of the highest dose (Protocol 1a). In addition to a late maximal response with urocortin 2, we also observed a prolonged offset. Even at 100 minutes post urocortin 2 administration, infused forearm blood flow remained elevated. Although not as lengthy as the effect evoked by urocortin 2, urocortin 3-evoked vasodilatation was also prolonged and took one hour for blood flow to return to baseline after discontinuation of the infusion. This prolonged offset of effect is unusual for G protein-coupled receptor agonists, although a similar time course has been observed in response to apelin [Japp *et al* 2008] and vasopressin [Affolter *et al* 2003], and is thought to be the result of prolonged receptor occupancy. *In vitro* studies by Hoare *et al* [2005] have demonstrated differing affinities of urocortin 2 and 3 to the CRH-R2 receptor determined by the affinity of the extracellular domains of the CRH receptors to these agonists. It remains to be established whether urocortin 2 induces receptor transformation, thereby promoting prolonged binding to CRH-R2 and a delayed maximal response. However, when assessed in isolation, it is clear that the direct vasomotor effects of urocortin 2 and urocortin 3 are more prolonged than previously reported.

The vasodilator effects of both peptides showed good within-day reproducibility without evidence of tachyphylaxis. These are important properties especially with potential applications in extended or chronic therapies where predictable and reproducible pharmacologic and haemodynamic effects are needed.

Several mechanisms have been proposed to explain the mechanistic pathways of urocortin-mediated vasorelaxation. Studies to date suggest that the mechanism involved may depend on the species or vascular bed in question. In rats, both endothelium-dependent [Jain *et al* 1999; Huang *et al* 2002] and -independent components are implicated [Schilling *et al* 1998]. Grossini *et al* demonstrated that urocortin 2-mediated vasorelaxation in the coronary arteries of anaesthetised pigs was mediated by nitric oxide [Grossini *et al* 2008]. However, urocortin-mediated vasodilatation appeared to be independent of endothelial integrity in isolated human coronary and internal mammary artery segments [Wiley and Davenport 2004; Smani *et al* 2010]. In the current study, the nitric oxide clamp appeared to cause modest inhibition of urocortin 2-mediated vasodilatation and appeared to be marginally more pronounced with urocortin 2 compared to urocortin 3. Inhibition of the cytochrome P450 metabolites of arachidonic acid with fluconazole alone did not have an appreciable effect on urocortin-mediated vasodilatation, but its addition enhanced the inhibitory effect of the nitric oxide clamp. This suggests that endogenous nitric oxide and the cytochrome P450 metabolites of arachidonic acid may have a close interrelationship and can compensate for one another to maintain vascular tone. A similar effect has previously been described for endothelium-derived hyperpolarising

factor (EDHF), whereby its relative importance increases in the face of impaired nitric oxide bioavailability under conditions of oxidative stress. Whilst urocortin 2 and urocortin 3-mediated vasodilatation was inhibited by the combination of all three inhibitors, it was not abolished and a substantial degree of vasomotor activity remained. We cannot exclude a contribution from other endothelial pathways such as residual 'fluconazole insensitive' EDHF although the results do suggest a substantial contribution from direct smooth muscle activation. Whilst the role of the endothelium is substantial, a large contribution from endothelium-independent vasodilator mechanisms may be seen as an advantage in the potential therapeutic applications of CRH-R2 agonism. Arguably, this would allow a more predictable response from the manipulation of urocortin (2 or 3) as a therapy for cardiovascular conditions, the vast majority of which would be expected to be associated with impaired endothelial homeostatic mechanisms but preserved smooth muscle activity.

This study was designed to assess locally active, sub-systemic doses of urocortin 2 and 3 in the forearm arterial vasculature. Indeed, the forearm venous occlusion plethysmography model allows these local effects to be studied at doses 10 to 100-fold lower than that usually expected or required to elicit a systemic response. It would appear, however, that there was a degree of systemic overspill associated with the top dose of urocortin 3 tested. The observed decrease in diastolic blood pressure and increase in heart rate observed during and after the infusion of the top dose of urocortin 3 is notable particularly as it occurred in the absence of any change in blood flow in the contralateral non-infused forearm. Usually, in the case of systemic overspill, a corresponding change in the 'control' non-infused arm would

be observed [Newby *et al* 1997a], but this was not the case with the infusion of the dose of urocortin 3. We therefore hypothesise that the hypotensive effect observed reflects vasodilatation in another more sensitive vascular bed, such as the splanchnic circulation, with consequent reflex tachycardia. Absence of a similar haemodynamic response to the highest dose of urocortin 2 may be the result of lower, sub-systemic doses of the less potent peptide urocortin subtype or, conceivably, a differential in the relative sensitivity of the splanchnic and forearm arterial circulation to urocortin 2. Systemic doses of up to 100 µg of urocortin 2 have, however, been shown to increase heart rate and decrease diastolic blood pressures in healthy adults [Davis *et al* 2007a].

5.6 STUDY LIMITATIONS

The size of our study population was relatively small. However, we have previously described the influence of a range of factors on blood flow in the forearm vasculature using sample sizes of ≤ 12 subjects [Newby *et al* 1997a; Wilkinson and Webb 2001; Gudmundsdottir *et al* 2006; Gudmundsdottir *et al* 2008; Japp *et al* 2008; Lang *et al* 2008a; Lang *et al* 2008b; Lang *et al* 2008c]. As with most other physiological studies, we have not performed multiple testing correction for the data obtained.

We studied the changes in forearm blood flow during brief, local intra-brachial infusions of urocortins 2 and 3. The apparent differences between urocortin 2 and 3

observed in our study may reflect, in part, differences in the tissue exposure achieved between the two peptides.

We do not as yet know the effects of prolonged infusions of these peptides. Moreover, the effects of urocortins 2 and 3 in patients with heart failure remain to be explored. Although there is good concordance between the vasomotor responses observed in the forearm resistance vessels and other vascular beds [Wilkinson and Webb 2001], further studies will be required to explore their systemic effects in health and disease in humans.

5.7 CONCLUSION

We have demonstrated that urocortin 2 and urocortin 3 evoke potent prolonged arterial vasodilatation and their effects are at least partly dependent upon endothelial nitric oxide and cytochrome P450 metabolites of arachidonic acid. These data provide important insights into human cardiovascular physiology and they will inform the development of further therapies directed towards the urocortin pathway. The *in vivo* role of this endogenous peptide system in patients with heart failure and the role of CRH-R2 receptor in human health and disease remain to be explored.

CHAPTER 6

EFFECT OF UROCORTIN 2 AND 3 ON FOREARM ARTERIAL BLOOD FLOW IN PATIENTS WITH HEART FAILURE

6.1 SUMMARY

Background Urocortin 2 and urocortin 3 may play a role in the pathophysiology of heart failure and are emerging as potential therapeutic targets. We assessed the vasomotor effects of urocortin 2 and urocortin 3 in patients with heart failure and in age- and sex-matched healthy volunteers using an *in vivo* human forearm arterial model.

Methods Twelve patients with stable heart failure (NYHA II-IV; 61±2 years) and 10 age- and sex-matched healthy volunteers (58±2 years) attended once each. Bilateral forearm arterial blood flow was measured using forearm venous occlusion plethysmography during incremental intra-arterial infusions of urocortin 2 (3.6-36 pmol/min), urocortin 3 (360-3600 pmol/min) and substance P (2-8 pmol/min).

Results Urocortin 2, urocortin 3 and substance P induced dose-dependent forearm arterial vasodilatation. There was no difference in the magnitude of forearm arterial vasodilatation between the two groups ($P>0.05$) for all three peptides. At the highest dose infused, urocortin 3 induced a transient tachycardia ($P<0.05$) in both groups associated with a drop in diastolic blood pressure in healthy volunteers ($P=0.02$) suggesting systemic spillover from the infused forearm. However, non-infused forearm blood flow remained unchanged in both groups ($P>0.05$).

Conclusion The acute forearm arterial vasodilator effects of urocortin 2 and urocortin 3 are preserved in patients with heart failure. These findings provide

further evidence of the relevance of the urocortin 2 and urocortin 3 in patients with heart failure and reinforce the need to further explore this pathway in clinical trials.

6.2 INTRODUCTION

Despite considerable advances in treatment, heart failure continues to carry a poor prognosis with high morbidity and mortality. Hence, there remains a major interest in the development of novel therapeutic agents for this debilitating condition. The recent decades have seen significant advances in therapeutic interventions for patients with chronic heart failure. However, the mainstay of treatment in acute heart failure involves the use of diuretics, inotropes and vasodilators. There have been relatively few recent trials investigating the use of novel agents in acute heart failure [Konstam *et al* 2007; Voors *et al* 2011; Teerlink *et al* 2013]. Urocortins are endogenous vasoactive peptides that are increasingly recognised to play an important cardiovascular homeostatic role and the potential for their manipulation in the treatment of heart failure has become the focus of a growing body of research.

We have confirmed that urocortin 2 and 3 are potent arterial vasodilators, the effects of which are reproducible and well tolerated in healthy male volunteers [Venkatasubramanian *et al* 2013]. Previous studies in animal models of heart failure [Ng *et al* 2004; Davis *et al* 2005; Rademaker *et al* 2005b; Rademaker *et al* 2006; Boonprasert *et al* 2008; Rademaker *et al* 2008; Rademaker *et al* 2009], as well as studies in heart failure patients [Davis *et al* 2007b (urocortin 2)], suggest that there is great scope for urocortins as novel biomarkers and as potential therapeutic agents in

heart failure. More recently, Wandy Chan *et al* [2013] have demonstrated beneficial haemodynamics of urocortin 2 infusion in acute decompensated heart failure in man. Recent studies by Gheorghiade *et al* [2013] demonstrated increased cardiac output and reduced systemic vascular resistance with intravenous administration of JNJ-39588146 (a synthetic analogue of human stresscopin) in patients with stable heart failure. Human stresscopin is a 40 amino acid peptide, derived from the same gene as urocortin 3. The systemic effects of urocortin 3 remain to be elucidated in man. Moreover, no clinical study has examined the regional effects of both urocortin 2 and 3 (as distinct from the amalgam of systemic effects) in heart failure in man, nor has a direct head to head comparison of their effects been made in these patients.

With this in mind, we set out to explore the local vasomotor effects of these peptides in patients with heart failure.

6.3 METHODS

All studies were approved by the local Research Ethics Committee and carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants prior to the study.

6.3.1 STUDY PARTICIPANTS

Twelve patients with stable heart failure (NYHA II-IV) and 10 age- and sex-matched healthy volunteers were recruited to attend once each as described in *Chapter 2*.

6.3.2 VASCULAR STUDIES

Studies were performed as described in *Chapter 2*. Forearm blood flow was measured in the infused and non-infused forearms using bilateral venous occlusion plethysmography as described previously [Newby *et al* 1997b; Newby *et al* 1999b].

Subjects attended once each to receive incremental intra-arterial infusions of urocortin 2 (3.6-36 pmol/min), urocortin 3 and substance P (substance P; 2-8 pmol/min) (Figure 6.1). The order in which urocortin 2, urocortin 3 and substance P were infused was randomised. Urocortin 3 was initially infused at a dose of 1200-12000 pmol/minute (n=4 patients with heart failure, n=2-matched healthy controls). Data analysis from the first 4 patients with heart failure demonstrated a significant drop in systolic blood pressure at the highest dose infused in patients with heart failure. The protocol was therefore revised leading to reduction in the dose of

urocortin 3 to 360-3600 pmol/minute following appropriate approvals. All subsequent participants (n=8) thereafter, received the reduced dose of urocortin 3. Haemodynamic data from the first 4 patients with heart failure and first 2-matched healthy volunteers is therefore presented separately in the results section and not included in the main analysis.

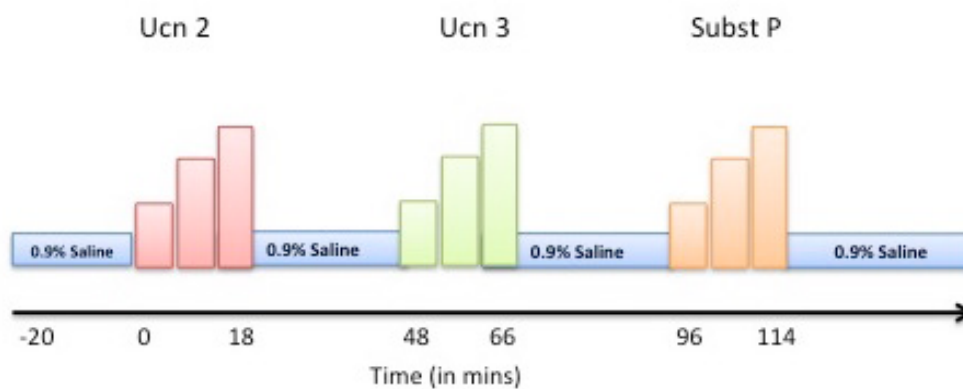


Figure 6.1. Schematic representation of study protocol - incremental intra-arterial infusion of urocortin 2 (3.6-36 pmol/min), urocortin 3 (360-3600 pmol/min) and substance P (2-8 pmol/min) in the presence of a saline washout between peptides. Ucn - urocortin; Subst P - substance P.

6.3.3 VENOUS SAMPLING

Baseline blood samples were drawn for assessment of full blood count, renal function, blood glucose and cholesterol levels at the start of each study. Analysis was performed by the local clinical biochemistry and haematology reference laboratories.

6.3.4 DATA ANALYSIS AND STATISTICS

Data was analysed, where appropriate, by ANOVA with repeated measures, and paired and unpaired Student's *t*-test as appropriate. Statistical significance was taken at the 5% level. All results are expressed as mean±SEM, unless otherwise stated.

6.4 RESULTS

Patients with heart failure were predominantly male. Baseline characteristics of patients with heart failure and healthy volunteers are shown in Table 6.1. Patients with heart failure were well established on standard heart failure therapy. All patients were receiving treatment with an ACE inhibitor or angiotensin receptor blocker. The majority of patients were receiving treatment with a beta-blocker. One patient was intolerant of beta-blockers but was receiving treatment with ivabradine.

TABLE 6.1 Baseline characteristics of subject

	Patients with heart failure	Healthy volunteers
Age, years	61±2	58±2
Sex		
Male	9 (75%)	7 (70%)
Female	3 (25%)	3 (30%)
NYHA class		n/a
I	0	
II	11	
III	1	
IV	0	
Concomitant medications		
ACEi/ ARB	12 (100%)	n/a
Beta-blockers	10 (83%)	n/a
MRA	7 (58%)	n/a
Loop diuretic	8 (67%)	n/a
Digoxin	3 (25%)	n/a
Ivabradine	1 (8%)	n/a

Mean±SEM or n (%).

SEM - standard error of the mean; NYHA - New York Heart Association; ACEi - angiotensin-converting enzyme inhibitor; ARB - angiotensin receptor blocker; MRA - mineralocorticoid receptor antagonist.

The intra-brachial infusion of all three drugs was well tolerated with no adverse events. Intra-brachial infusion of urocortin 2 and 3 produced localised, self-limiting forearm flushing and some facial flushing in both groups of participants as noted in previous studies [Venkatasubramanian *et al* 2013].

6.4.1 RESPONSE TO UROCORTIN 3 AT HIGHER INFUSED DOSES (PILOT DATA)

The first 4 patients with heart failure and the first 2-matched healthy volunteers received higher incremental intra-arterial infusion of urocortin 3 at 1200, 3600 and 12000 pmol/minute, in addition to urocortin 2 and substance P at the stated doses.

All three peptides (urocortin 2, urocortin 3 and substance P) evoked dose-dependent forearm arterial vasodilatation ($P < 0.05$ for all, ANOVA; Figure 6.2A). Urocortin 3 also produced a drop in systolic blood pressure at the higher doses in patients with heart failure ($p < 0.05$). Patients with heart failure were symptomatic with dizziness and angina as a result of this fall in systolic blood pressure. As a consequence, the dose of urocortin 3 was reduced to 360-3600 pmol/minute for the remaining participants in the study. The data from these participants has not been included in the main data analysis. Figure 6.2B shows the haemodynamic response to urocortin 2, urocortin 3 and substance P in the pilot data group of patients with heart failure and healthy volunteers.

Forearm arterial blood flow

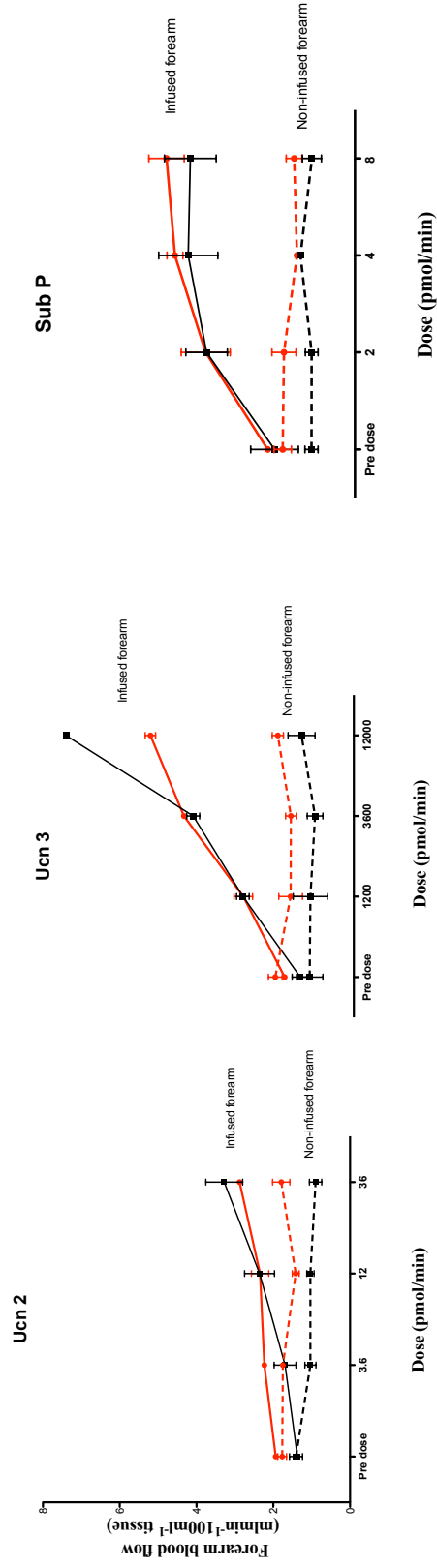
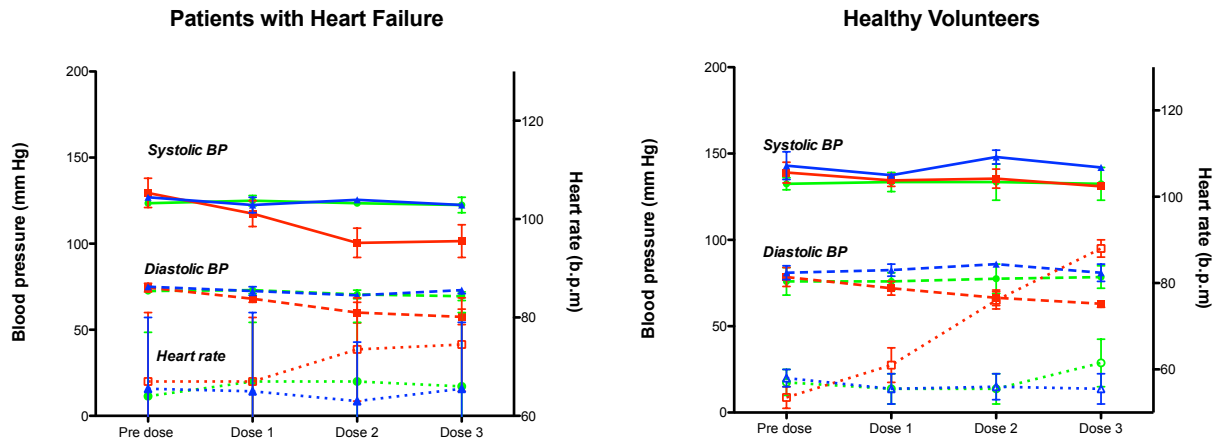


Figure 6.2. A: Change in absolute forearm arterial blood flow (n=4) to Ucn 2 (3.6-36 pmol/min), Ucn 3 (1200-12000 pmol/min) and Sub P (2-8 pmol/min). **Solid red line:** Infused forearm blood flow - patients with heart failure; **Broken red line:** Non-infused forearm blood flow - patients with heart failure; **Solid black line:** Infused forearm blood flow - healthy volunteers; **Broken black line:** Non-infused forearm blood flow - healthy volunteers. Ucn - urocortin; Sub P - substance P.

Haemodynamic parameters

B:



C:

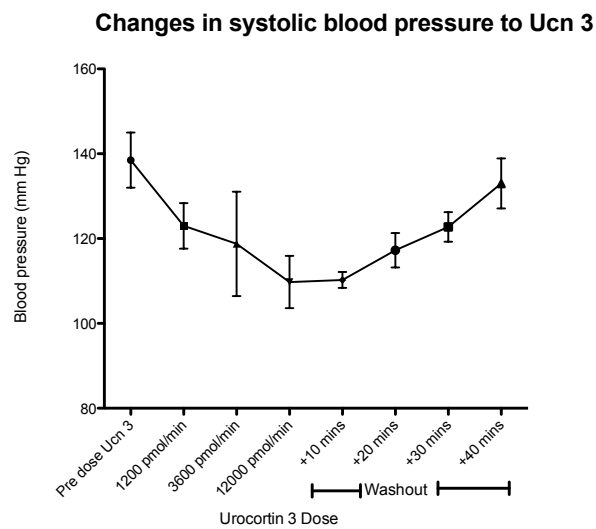
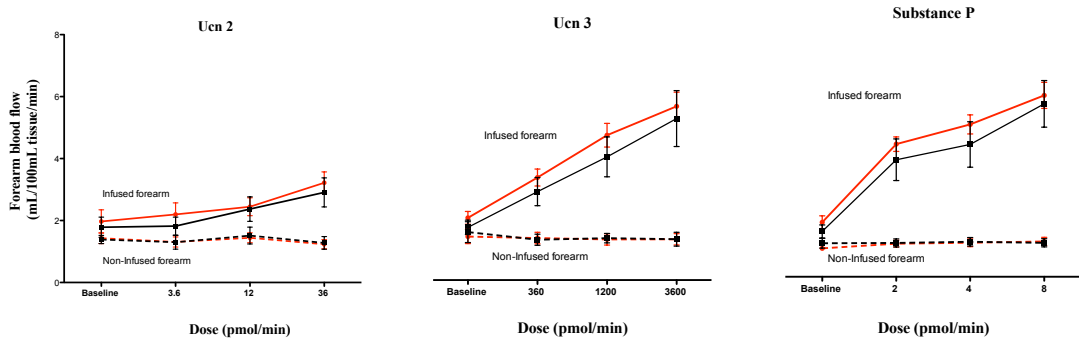


Figure 6.2. B: Changes in systolic, diastolic blood pressure and heart rate to intra-arterial Ucn 2 (green line) (3.6-36 pmol/min), Ucn 3 (red line) (1200-12000pmol/min) and Sub P (blue line) (2-8 pmol/min) in patients with heart failure (n=4) (left top panel) and in age- and sex-matched healthy volunteers (n=2) (Right top panel). **C:** Changes in systolic pressure during and after intra-arterial Ucn 3 at the higher doses of 1200-12000 pmol/minute in patients with heart failure. Ucn - urocortin; Sub P - substance P.

6.4.2 MAIN DATA ANALYSIS

Urocortin 2, urocortin 3 and substance P all evoked dose-dependent forearm arterial vasodilatation ($P < 0.0001$ for all, ANOVA). There were no differences in forearm vasodilatation between the two groups for urocortin 2, urocortin 3 and substance P ($p = 0.63$, $p = 0.44$ and $p = 0.49$ respectively). Systolic and diastolic blood pressure and heart rate remained unchanged in both groups in response to urocortin 2 and substance P. At the highest dose, urocortin 3 induced a transient tachycardia in both groups ($p = 0.0009$ and $p = 0.03$; healthy volunteers and patients with heart failure respectively) accompanied by a drop in diastolic blood pressure ($p = 0.03$) in healthy volunteers. Mean systolic and diastolic blood pressures were numerically lower at the maximal dose of urocortin 3 in patients with heart failure, but this did not achieve statistical significance ($p = 0.20$ and $p = 0.39$ for systolic and diastolic blood pressures respectively). Non-infused forearm blood flow remained unchanged throughout the study (Figure 6.3).

A: Forearm Blood Flow



B: Haemodynamic Parameters

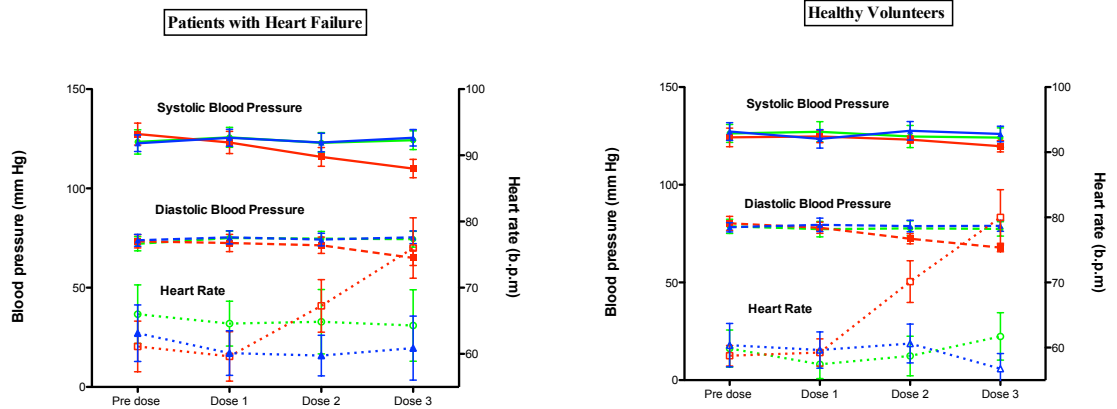


Figure 6.3. **A:** Change in absolute forearm arterial blood flow to Urocortin 2 (3.6-36 pmol/min), urocortin 3 (360-3600 pmol/min) and substance P (2-8 pmol/min) in patients with heart failure and in healthy volunteers. Solid red line: Infused forearm blood flow - patients with heart failure; Broken red line: Non-infused forearm blood flow - patients with heart failure; Solid black line: Infused forearm blood flow - healthy volunteers; Broken black line: Non-infused forearm blood flow - healthy volunteers. **B:** Non-invasive haemodynamic responses to intra-arterial urocortin 2 (green line), urocortin 3 (red line) and substance P (blue line) in patients with heart failure (lower left panel) and healthy controls (lower right panel).

6.5 DISCUSSION

This study demonstrates that the arterial vasodilator effects of urocortin 2 and 3 are preserved in patients with heart failure. We observed a dose-dependent increase in forearm arterial blood flow with all three peptides.

Subjects in both groups were appropriately age- and sex-matched. Patients with heart failure were well established on standard heart failure therapy. Healthy volunteers included participants with no other significant past medical history on no regular medications.

Substance P is an endothelium-dependent agonist [Hirooka *et al* 1992; Newby *et al* 1997b; Tagawa *et al* 1997; Newby *et al* 1999a; Newby *et al* 1999b]. Hirooka *et al* [1992] and Takeshita *et al* [1996] have shown that endothelium-dependent forearm arterial vasodilatation mediated by muscarinic receptor (in response to acetylcholine) was attenuated in patients with heart failure, but endothelium dependent vasodilatation in response to substance P remained preserved. Although the exact mechanism behind this difference in response is not clear, it is postulated that part of this may be due to reduced endothelium-derived relaxing factor release secondary to muscarinic receptor stimulation in patients with heart failure. In keeping with these findings, we did not observe a significant difference in the vasomotor responses to substance P between groups. This partly may also be explained by the assessment of patients who had stable heart failure symptoms who were receiving robust heart failure treatment.

Impaired endothelial function is a recognised feature and an independent predictor of adverse outcome in patients with heart failure [Kubo *et al* 1991; Nikolic *et al* 1992; Linke *et al* 2001; de Berrazueta *et al* 2010; Takishima *et al* 2012; Yang *et al* 2015]. There is therefore a theoretical concern that urocortin would be less effective in patients with heart failure because of this concomitant endothelial dysfunction. However, we have been able to demonstrate that urocortin 2 and urocortin 3 evoked normal forearm arterial vasodilatory responses in our patients with heart failure. This suggests that either endothelial dysfunction does not have a meaningful impact on the actions of urocortin or our subjects did not have significant endothelial dysfunction. As discussed above, our patients did not demonstrate impaired substance P-induced vasodilatation suggesting preserved endothelial function. This may reflect that our patients were stable, well treated and receiving optimal medical therapy. Whether similar findings would be observed in patients with decompensated heart failure remains to be established.

The advantage of the forearm plethysmography technique is the ability to study localised, sub-systemic effects of investigational agents at locally active but sub-systemic doses. We therefore, expected to observe no major changes in haemodynamic parameters in both groups. We did however, notice a dose-related tachycardia and drop in diastolic blood pressure in our study with healthy volunteers with urocortin 3 [Venkatasubramanian *et al* 2013]. This change in heart rate and diastolic blood pressure at the highest dose of urocortin 3 was attributed to a potential systemic spillover, potentially stimulating other vascular beds

(eg. splanchnic), but without appreciable effect on contralateral non-infused forearm blood flow. We observed a similar trend in patients with heart failure, but in addition, we observed numerically lower systolic blood pressures in patients with heart failure during the highest dose of urocortin 3. This was more pronounced at the higher infused dose of urocortin 3 in the pilot group. This may be explained by the potential lack of compensatory responses to vasodilatation in heart failure leading to lower systolic and diastolic blood pressures. Moreover, all patients were receiving maximal tolerated doses of beta-blockers, which might explain the relative lack of tachycardia in this group. In previous studies, systemic infusion of urocortin 2 in patients with heart failure [Davis *et al* 2007a] produced a drop in both systolic and diastolic blood pressures associated with a minimal tachycardia. This was attributed to blunted baroreceptor responses in response to the vasodilator effects of urocortin 2 in patients with heart failure. The lack of a similar trend to fall in blood pressure in response to urocortin 2 in heart failure patients in our study may be as a result of lower plasma concentrations of urocortin 2 achieved at the doses infused in comparison to urocortin 3.

There have been very few recent novel agents to be used in the management of acute heart failure. Serelaxin appeared to show some promise in this group of patients. In the RELAX-AHF study, intravenous serelaxin infusion was associated with dyspnoea relief and improvement in other clinical outcomes in acute heart failure. In addition, a significant late (180-day) mortality benefit (both cardiovascular and all cause mortality) was observed in the serelaxin group [Teerlink *et al* 2013]. Serelaxin and urocortin both act via G protein-coupled receptors with some overlap in their

cardiovascular molecular biology and physiologic effects [Felker *et al* 2009; Venkatasubramanian *et al* 2010]. Urocortins may therefore hold potential in the treatment of acute heart failure.

6.6 STUDY LIMITATIONS

The size of our study population was relatively small. However, the study was adequately powered to demonstrate marked changes in forearm arterial blood flow.

The degree of forearm vasodilatation evoked by urocortin 3 appeared to be greater than that produced by urocortin 2 in both healthy volunteers and in patients with heart failure. This difference may be a result of varying plasma concentrations achieved at the doses infused. Plasma assays of urocortin 2 and 3 would help better understand the difference in dose-response relationship for the two peptides but are currently not available for clinical use.

6.7 CONCLUSION

Forearm arterial vasodilator effects of urocortin 2 and urocortin 3 are preserved in patients with heart failure when compared with age- and sex-matched healthy volunteers. Studies to make head-to-head comparisons of the systemic haemodynamic effects of both urocortin 2 and urocortin 3 in patients with heart failure will be important to inform the clinical development of what appears to be a ripe target for heart failure pharmacotherapy.

CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

7.1 INTRODUCTION

Cardiovascular disease continues to be a leading cause of mortality worldwide. In the United Kingdom, death from cardiovascular disease accounts for more than a quarter of all deaths with an estimated cost of £19 billion per year. Heart failure continues to remain a major healthcare burden with an estimated 550,000 people living with heart failure in the UK. Unsurprisingly, research into novel targets for cardiovascular disease and heart failure remains a major focus. This thesis has concentrated upon the effects of two such novel hormone systems and their effects on the cardiovascular system in health and in disease. The results from this thesis have helped inform the cardiovascular effects of these systems and serve as a platform upon which future studies can be based upon, in order to successfully translate the findings from bench to bedside.

The first part of the thesis focussed upon the effects of activation of the sirtuin system in man. With advancing years, the pathophysiology of ‘diseases of ageing’ and hence, novel targets to tackle them, has generated a lot of interest. SRT2104 is a selective small molecule activator of the silent information regulator two 1, which has been implicated in the beneficial effects of calorie restriction and on longevity in several species. The effects of SIRT1 activation in animal models showed promising results on fasting blood glucose levels, body weight and triglyceride and plasma lipid concentrations. Previous studies with SRT2104 in man had demonstrated that it was safe for use in man. This study was therefore undertaken as a Phase 1 clinical trial to examine the *in vivo* effects of SRT2104 on lipid profile, and vascular, endothelial and platelet function in otherwise healthy cigarette smokers and in patients with type 2 diabetes mellitus.

The second part of the thesis focused upon the *in vivo* vascular effects of the urocortin system using urocortins 2 and 3. For the first time, we demonstrated the direct *in vivo* arterial vasomotor effects of urocortin 2 and 3 in healthy volunteers and in patients with stable heart failure. The studies forming part of this thesis represent the ‘first-in-human’ administration of urocortin 3.

7.2 SUMMARY OF FINDINGS

7.2.1 CARDIOVASCULAR EFFECTS OF NOVEL SIRT1 ACTIVATOR, SRT2104, IN OTHERWISE HEALTHY CIGARETTE SMOKERS

As a novel potent small molecule activator of silent information regulator two 1, SRT2104, has demonstrated significant activity in *in vivo* mice models of diabetes, effectively improving glucose and insulin homeostasis in diet-induced obese mice and *ob/ob* mice. Little is known about its cardiovascular effects in man. Safety and toxicity studies with SRT2104 [Hoffman *et al* 2010] have shown that doses up to 3.0 g/day are well tolerated in humans without any dose-related side effects. In keeping with this, we observed that once daily administration of SRT2104 for 28 days at a dose of 2.0 g/day was safe and well tolerated in otherwise healthy cigarette smokers and in patients with type 2 diabetes mellitus. Any reported side effects were mild and resolved without any sequelae. As with previous studies with oral SIRT1 activators, we only examined the effects over a 28-day dosing period. The effects of prolonged SIRT1 stimulation remain to be explored.

As part of series of early studies investigating novel SIRT1 activators, we examined the pharmacokinetics of SRT2104. Previous Phase I trials with SRT2104 have demonstrated a substantial food effect in the levels of exposure achieved [Hoffman *et al* 2012; Libri *et al* 2012]. Therefore, in our study, all participants were given a prescribed standard meal (breakfast) to ensure improved drug absorption. Despite substantial inter-subject variability in exposure, we were able to demonstrate a steady plasma concentration of the drug over a 24-hour period with a once a day dosing regime. The median time at which maximum plasma concentration was observed (T_{max}) on Day 28 of dosing was 3 hours, which coincided well with vascular study measurements performed on those days. The T_{max} observed was in keeping with previous pharmacokinetic studies with SRT2104 [Hoffman *et al* 2012; Libri *et al* 2012].

SRT2104 was identified as a SIRT1 activator in a high throughput screen of a diverse library of 290,000 compounds [Milne *et al* 2007]. SIRT1 activity is increased by SRT2104 due to lowering of the K_m (Michaelis constant) of its acetylated protein substrate, resulting in an approximately 2-fold increase in activity. In our study, we have been able to demonstrate that oral dosing with SRT2104 was safe and, at the given dose, was able to achieve a steady plasma concentration. However, given that we did not directly measure the level of SIRT1 expression, we have been unable to demonstrate target engagement of SRT2104. The variability in target engagement at the plasma concentrations of SRT2104 achieved may well be a reason for the lack of vascular effects seen with the 28-day dosing schedule.

In recent years, the focus of basic and epidemiological investigations has shifted to factors or mechanisms involved in the early subclinical phases of atherosclerosis. Several inflammatory cells and their activation markers play an important role in the formation and progression of atherosclerosis [Furman *et al* 1998; Furman *et al* 2001; Libby 2002]. Inflammation plays a key role in the pathogenesis of atherosclerosis and its complications. SIRT1, a key regulator of the inflammatory process, also has a role in preventing atherosclerosis through different mechanisms. There is also *ex vivo* evidence to support that SIRT1 plays a role in directly controlling endothelium-dependent vasodilatation and eNOS expression [Mattagajasingh *et al* 2007]. There are no studies directly examining the effect of SIRT1 activation on endothelial function *in vivo*. Based on available preclinical data, we hypothesised that activation of SIRT1 through SRT2104 would improve endothelial and platelet function in man, and serve to improve the cardiovascular risk profile in otherwise healthy cigarette smokers. However we did not demonstrate any differences in vascular, endothelial or platelet function with SRT2104.

Elevated serum cholesterol is an established risk factor for atherosclerosis and coronary heart disease. Despite the lack of effect on direct measures of vascular function, we did observe positive and significant improvements in the lipid profile in the otherwise healthy cigarette smokers. We observed a 7% mean reduction in serum total cholesterol and an 11% mean reduction in LDL cholesterol concentrations. Similar reductions in serum total and LDL cholesterol have been demonstrated with previous studies in elderly volunteers [Libri *et al* 2012] and patients with type 2 diabetes mellitus [Baksi *et al* 2014]. The exact mechanism for this reduction is not entirely clear, but a

positive regulatory effect on LXR proteins has been postulated. Our findings suggest that SIRT1 activation could provide a useful adjunct to lipid-lowering therapies, thereby improving cardiovascular risk profiles.

As part of the assessment of vascular function, we then examined the effects of SRT2104 on measures of arterial compliance.

7.2.2 EFFECTS OF SRT2104 ON ARTERIAL STIFFNESS IN OTHERWISE HEALTHY CIGARETTE SMOKERS AND PATIENTS WITH TYPE 2 DIABETES MELLITUS

Both endothelial dysfunction and arterial stiffness commonly coexist in patients at increased risk of cardiovascular disease. Arterial stiffness is recognised as an independent predictor of cardiovascular risk. Calorie restriction can attenuate age-related arterial stiffness in animal models. No studies have examined the direct effect of SIRT1 activation on measures of arterial compliance. Having established the effect on measures of endothelial and platelet function in *Chapter 3*, we set out to examine the effect of SIRT1 activation on measures of arterial compliance.

Chapter 4 presents a combined analysis of treatment with SRT2104 of otherwise healthy cigarette smokers and subjects with type 2 diabetes mellitus on measures of arterial compliance. Treatment with SRT2104 was associated with a significant reduction in augmentation pressure and a trend towards improvement in augmentation index and corrected AIx. There were no changes observed in pulse wave velocity or time to wave reflection. The lack of change in PWV may result from the short duration of exposure to treatment, which may be inadequate to induce structural

changes in the arterial wall. The duration of treatment may, however, be adequate to improve dynamic measures such as augmentation pressure and index. It would be interesting to see whether prolonged administration of SRT2104 renders positive changes in all measures of arterial compliance.

In summary, activation of SIRT1 through SRT2104 in healthy cigarette smokers and subjects with type 2 diabetes mellitus was safe and well tolerated and was associated with an improvement in lipid profile. It did not produce any demonstrable differences in vascular, endothelial or platelet functions and had some effect on measures of arterial stiffness.

7.2.3 VASCULAR EFFECTS OF UROCORTINS 2 AND 3 IN HEALTHY VOLUNTEERS

Urocortin 2 and 3 are endogenous vasoactive peptides that belong to the corticotrophin-releasing hormone family with prominent cardiovascular roles. Urocortin 2 and 3 are potent selective agonists at CRH-R2 - a G protein-coupled receptor, which is expressed in the myocardium and vascular smooth muscle [Wiley and Davenport 2004; Davidson and Yellon 2009]. Recent years have seen an increasing interest, especially for urocortin 2, for its role in heart failure.

Ex vivo and animal studies have shown that urocortins are potent vasodilators of different arteries from various animal species [Huang *et al* 2002; Chen *et al* 2005; Smani *et al* 2007; Smani *et al* 2010]. The beneficial haemodynamics of urocortin 2 and 3 have been demonstrated in sheep with heart failure [Rademaker *et al* 2005b; Rademaker *et al* 2006]. Similar beneficial effects of urocortins have been seen in small

human clinical studies of healthy volunteers and patients with heart failure [Davis *et al* 2004a; Davis *et al* 2007; Wandy Chan *et al* 2013]. However no studies have examined the direct *in vivo* effects of urocortin 2 and 3 on human vasculature or indeed their mechanism of action. We therefore set out to examine the effect of *in vivo* infusions of urocortin 2 and 3 on human forearm circulation.

Through a series of studies, using the well-validated technique of forearm venous occlusion plethysmography, we were able to demonstrate that both urocortin 2 and 3 were potent forearm arterial vasodilators. As expected with any vasodilatory peptide, we observed localised flushing in the infused forearm at the highest doses of urocortin 2 and 3. These were, however, self-limiting and well tolerated by all volunteers.

We observed a significant discrepancy in the potency of the two peptides. Preclinical data suggest that urocortin 2 is 10-fold more potent than urocortin 3 [Hsu and Hsueh 2001; Fekete and Zorrilla 2007]. In contrast, we observed that a nearly 300-fold higher dose of urocortin 3 was required to evoke comparable arterial effects in the human forearm circulation. This discrepancy in potency was observed both in healthy volunteers and in later studies with patients with heart failure. Differences in the commercial manufacturing process of the two peptides may have contributed to the observed discrepancy. The commercially synthesised peptide was further processed and filtered, at a local Good Medical Practice recognised unit, into smaller strength vials to allow ease of administration. It is therefore possible that this process resulted in loss of some peptide activity. Plasma assay of urocortin 2 and 3 at the infused doses would

have added useful information of plasma concentrations achieved to explain the discrepancy.

Ex vivo and animal data suggests that urocortin 3 has a shorter half-life than urocortin 2 and has a more rapid onset of maximal activity [Kageyama *et al* 2003a; Rademaker *et al* 2006]. The half-life of urocortin 2 in healthy humans was found to be 10 minutes [Davis *et al* 2007a]. In keeping with preclinical data, the maximal vasodilatory effect of urocortin 3 in our study was immediate whereas with urocortin 2, the maximal vasodilatory effect was apparent 10 minutes after completion of the highest infused dose. In addition to the late maximal response with urocortin 2, we also observed a prolonged offset of the vasodilatory effect with urocortin 2 with elevated forearm blood flow even at 100 minutes after urocortin 2 administration. Urocortin 3-evoked vasodilatation was also prolonged, but shorter than that observed for urocortin 2 with a return to baseline blood flow at 60 minutes after urocortin 3 administration. This prolonged offset is unusual for G protein-coupled receptor agonists and thought to be the result of prolonged receptor occupancy. The vasodilatory effects of both peptides were reproducible within-day without any evidence of tachyphylaxis.

Having established that urocortin 2 and 3 are potent vasodilators, we then sought to determine the mechanistic pathways of urocortin-mediated vasorelaxation. Use of a nitric oxide clamp caused modest inhibition of urocortin 2-mediated vasodilatation. This effect appeared to be more pronounced with urocortin 2 compared with urocortin 3. There was no appreciable difference in urocortin-mediated vasodilatation with either individual inhibition of cyclooxygenase pathway (with aspirin) or cytochrome P450

metabolites of arachidonic acid (using fluconazole). However, in combination with the nitric oxide clamp, an enhanced inhibitory effect was observed. We also observed that inhibition of nitric oxide and cytochrome P450 metabolites of arachidonic acid in combination did not completely abolish urocortin-mediated vasodilatation, which suggests that there remains a large contribution from endothelium-independent mechanisms. This is in keeping with observed preclinical data in animal and human arterial segments [Jain *et al* 1999; Huang *et al* 2002; Chen *et al* 2005; Grossini *et al* 2008; Smani *et al* 2010].

7.2.4 EFFECT OF UROCORTIN 2 AND 3 ON FOREARM ARTERIAL BLOOD FLOW IN PATIENTS WITH HEART FAILURE

After establishing the local vasomotor effects of urocortin 2 and 3 in healthy volunteers, we proceeded to compare the effects of intra-arterial infusion of the two peptides in the human forearm circulation in healthy volunteers and patients with heart failure. In keeping with the findings in healthy volunteers, we were able to demonstrate that urocortin 2 and 3 induced dose-dependent forearm arterial vasodilatation in patients with heart failure. However, there was no difference in the magnitude of forearm vasodilatation between patients with heart failure and their age- and sex-matched healthy controls. This may be explained by the fact that the patients in our study were well established on appropriate heart failure therapies or the fact that, as observed in our mechanistic study, there is a large endothelium-independent component of urocortin-mediated vasodilatation. However, this preservation of vasodilatation suggests that urocortin remains a valuable potential agent for the treatment of heart failure.

7.3 FUTURE DIRECTIONS

7.3.1 SIRTUINS IN CARDIOVASCULAR AND METABOLIC HEALTH

Scientific interest in the benefits of sirtuins and SIRT modulators in man is still at its relative infancy. Literature to date has identified SIRT1 as the most critical modulator of vascular function, with several reported laboratory and animal studies demonstrating its prominent role in the regulation of vascular homeostasis and diseases through its action at multiple cellular levels. Translating these observed benefits into successful clinical trials remains a challenge. In recent years, pharmacological modulation of sirtuins to allow development of potent SIRT1 activators has been widely studied. Indeed, there have been several *in vivo* and *ex vivo* studies and clinical trials designed to explore the effects of SIRT1 activation and inhibition through resveratrol and other sirtuin modulators and inhibitors [Grozinger *et al* 2001; Mai *et al* 2005; Camins *et al* 2010; Orecchia *et al* 2011; Patel *et al* 2011; Sanchez-Fidalgo *et al* 2012; Gano *et al* 2014]. Summarised below is an overview of sirtuin activators and inhibitors that modulate different cellular targets involved in vascular homeostasis and diseases (Figure 7.1).

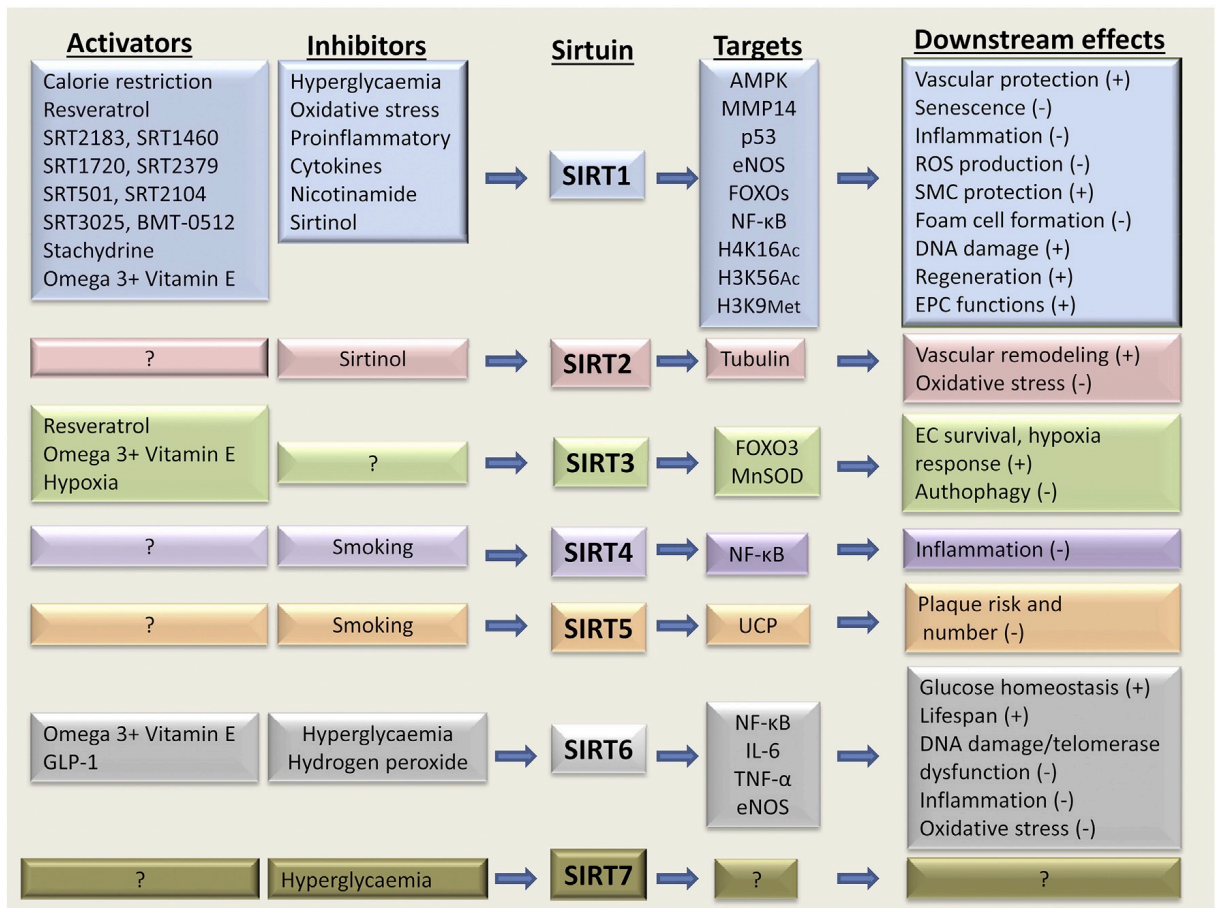


Figure 7.1. An overview of sirtuin activators/inhibitors that modulate different cellular targets involved in vascular homeostasis and diseases.

AMPK - AMP-activated protein kinase; eNOS - endothelial nitric oxide synthase; FOXO - Forkhead box O; IL-6 - Interleukin 6; MMP-14 - metalloproteinase14; MnSOD - manganese superoxide dismutase; NF-kB - nuclear factor-kappa B; TNF- α - tumor necrosis factor alpha; p53 - tumor protein; UCP - uncoupling proteins; SMC - smooth muscle cell; ROS - reactive oxygen species; EPC - endothelial progenitor cells; (+) - positively regulated; (-) - negatively regulated; (?) - unknown.

[D'Onofrio *et al* 2015].

Very few clinical trials have examined the effect of SIRT1 activation in the prevention or treatment of endothelial dysfunction in man. We have been the first to evaluate this. Interestingly, although preclinical and animal data suggest that SIRT1 stimulation has beneficial effects on eNOS activation, endothelial function and in the maintenance of vascular homeostasis, we were unable to demonstrate any significant differences in vasomotor or endothelial function.

Although SRT2104 is a potent small molecule activator of SIRT1, we did not directly measure the change in the level of SIRT1 expression with SRT2104. Correlation of plasma concentrations of SRT2104 achieved with measured changes in SIRT1 mRNA expression may help understand the relationship between target engagement and observed physiological changes.

An important finding in our study was the significant improvement in lipid profile in otherwise healthy cigarette smokers. Pharmacological SIRT1 activation lowers plasma LDL cholesterol concentrations by inhibiting proprotein convertase subtilisin/kexin 9 (PCSK9) secretion, thereby increasing hepatic LDL receptor availability and subsequently LDL cholesterol clearing [Miranda *et al* 2015]. It is also postulated that SIRT1 activators like SRT2104 could involve a positive regulatory effect on liver X receptor proteins. We noticed a 7% reduction in serum total cholesterol with an 11% reduction in LDL cholesterol in our study. Although the exact mechanism of this reduction is not entirely clear, this lipid-lowering effect makes SIRT1 activation an attractive adjunct in the treatment of lipid disorders. It would however be important to first establish the efficacy and safety of its long term administration.

The role of SIRT1 activation in metabolic disorders and type 2 diabetes mellitus remains a major focus of interest. Animal data have abundantly demonstrated that SIRT1 activation leads to improved glucose and insulin homeostasis in *ex vivo* and animal models. Clinical trials to date have however been unable to reproduce this improvement in glucose concentrations in human studies. Baksi *et al* [2014] were unable to demonstrate an improvement in glucose or insulin control in patients with

type 2 diabetes mellitus following 28 days of treatment with SRT2104, which they attributed to a large between-subject variability in the pharmacokinetics of the drug. Similar to my own findings with SRT2104, they observed an improvement in the lipid profiles. In a small Phase 1 trial in elderly individuals, Libri *et al* [2012] observed no changes in oral glucose tolerance tests, but once again, did see an improvement in lipid profile. Results from the diabetic arm of our Phase 1 trial showed no discernible effects on vascular, fibrinolytic, lipid or platelet function in patients with type 2 diabetes mellitus. An important and significant weight loss in diabetic patients was also observed in this trial, the mechanism for which is unclear.

These lead to an exciting and important question – could SIRT1 activation be useful as a tool to aid weight loss, lower total and LDL cholesterol and therefore, indirectly, have an impact on cardiovascular risk and health? Are these benefits likely to be sustained with prolonged administration of SIRT1 activators and does chronic SIRT1 stimulation have any deleterious effects? These questions remain unanswered. Table 7.1 lists current ongoing clinical trials with different oral SIRT1 activators.

TABLE 7.1 SIRT1 modulators in clinical trials

Compound	Sponsor	Status	Phase	Condition	NIH code
Trans-resveretrol from <i>Polygonum cuspidatum</i>	Kho Teck Puat Hospital	Completed	1	Type 2 diabetes	NCT01677611
Resveretrol	InCor Heart Institute	Recruiting	-	Vascular system injuries, endothelial dysfunction	NCT01668836
Resveretrol	University of Aarhus	Completed	-	Metabolic syndrome, obesity	NCT01150955
Omega 3 and vitamin E supplementation	Tehran University of Medical Sciences	Enrolling by invitation	4	Coronary artery disease	NCT02011906
Polyphenols contained in red grape cells	Tel Aviv University	Recruiting	0	Type 2 diabetes	NCT01938521
SRT2104	Sirtris,GSK Company	Completed	1	Type 2 diabetes	NCT01031108
SRT2104	GlaxoSmithKline	Completed	2	Type 2 diabetes	NCT01018017
SRT2104	Sirtris,GSK Company	Completed	1	Type 2 diabetes	NCT00937872
SRT2104	Sirtris,GSK Company	Completed	2	Type 2 diabetes	NCT00937326
SRT2104	Sirtris,GSK Company	Completed	1	Type 2 diabetes	NCT00938275
SRT2104	GlaxoSmithKline	Completed	1	Type 2 diabetes	NCT00933062
SRT2104	Sirtris,GSK Company	Completed	1	Type 2 diabetes	NCT00933530
SRT2379	GlaxoSmithKline	Completed	1	Type 2 diabetes	NCT01018628
SRT3025	Sirtris,GSK Company	Completed	1	Type 2 diabetes	NCT01340911

[D'Onofrio et al 2015]

Although SIRT1 is considered the most critical sirtuin for the maintenance of vascular homeostasis in preclinical models, the fact that we did not observe any change in markers of endothelial function, leads us to question the potential role of other sirtuins in cardiovascular health in man. Together with SIRT4 and 5, SIRT3 is localised to the mitochondria. Mitochondrial dysfunction plays a central role in several cardiovascular diseases such as cardiomyopathy, heart failure, pulmonary hypertension and endothelial dysfunction [Wallace 2000; Madamanchi *et al* 2005]. SIRT3, through its effects on mitochondrial function, may play a role in cardiovascular disease states. Similarly, SIRT6 has been shown in *ex vivo* studies to play a role in cardiovascular disease including cardiac hypertrophy, heart failure, myocardial hypoxic damage and metabolism [Cai *et al* 2012; Sundaresan *et al* 2012; Maksin-Matveev *et al* 2015]. The role of these sirtuins remains unexplored in man.

The role of sirtuin activators may hold the key to several important and relevant metabolic disease processes in today's world. However, findings from this thesis, in addition to other available literature suggests that at least in the short term, SIRT1 activation may not necessarily have a direct impact on vascular function, but may become an important tool in altering metabolic risk factors for cardiovascular health. More information is required through well-designed clinical trials and perhaps more potent small molecule activators of SIRT1 to understand the consequences of chronic sirtuin stimulation in man.

7.3.2 UROCORTIN RECEPTOR ANTAGONISTS

Urocortins 2 and 3 exert their effects via G protein-coupled CRH-R2 receptors for which they are highly specific and potent. CRH-R2 receptors are expressed in the heart, vascular smooth muscle cells and intramyocardial blood vessels. In mice, CRH-R2 receptors may contribute to the maintenance of basal vascular tone. It remains unknown whether urocortins and their receptors have a similar role in the maintenance of basal vascular tone in humans.

Astressin 2b is a synthetic, highly potent and long lasting antagonist with a greater than a 100-fold selectivity for CRH-R2 receptors. Like most antagonist molecules, stressin 2b has been used (in animal studies) to characterise the role of CRH-R2 receptors and to demonstrate that urocortins 2 and 3 act through these receptors. Urocortin receptor antagonism is as yet unexplored in humans and as such its role in the maintenance of basal arterial vascular tone or indeed its clinical implications, is unknown. There is no doubt that understanding the basic mechanism of interaction of urocortins with their receptors, through the use of an antagonist, will aid in understanding the pharmacology of these peptides.

Given that forearm venous occlusion plethysmography allows examination of local vascular effects without the potential of systemic effects, this technique would be best suited to study novel peptides like stressin 2b. In fact, as part of my initial work on urocortins, we did procure commercially available stressin 2b, but had significant problems with the bioactivity and solubility of the peptide to allow safe use in humans.

This was therefore abandoned. Perhaps, future preparations of commercially available astressin 2b will allow its safe and easy use in human studies.

7.3.3 UROCORTINS IN HEART FAILURE

Chronic heart failure and acute decompensated heart failure continue to remain a major cause of hospitalisation, morbidity and mortality. Despite this, there have been only a couple of heart failure drugs with novel mechanisms of action approved for use in the United States [Hanigan *et al* 2016] since 2001 (Figure 7.2). Hence, there remains an increasing need for newer therapies for this rather debilitating condition.

There have been more promising new therapies for chronic heart failure than acute decompensated heart failure in recent years. Findings from recent trials using LCZ696 (PARADIGM-HF) [McMurray *et al* 2014] and ivabradine (SHIFT) [Swedberg *et al* 2010] in chronic heart failure have been major landmarks. The result for acute decompensated heart failure therapies has been less promising.

Nesiritide, a recombinant brain natriuretic peptide analogue, was the last medication to receive Food and Drug Administration approval for the treatment of acute heart failure in 2001. Through vasodilatation and reduction in pulmonary capillary wedge pressure, nesiritide was thought to improve dyspnoea scores in patients. This drug was later withdrawn, as results from the ASCEND-HF trial [Gottlieb *et al* 2013] showed no beneficial clinical outcomes. More recently, early clinical trials with serelaxin (RELAX-AHF trial) [Teerlink *et al* 2013] and omecamtiv mercabil

(ATOMIC-AHF trial) [Teerlink *et al* 2016] have been more promising, but remain under evaluation in ongoing Phase 2 and 3 trials.

The results from my thesis leave no doubt that urocortins 2 and 3 are potent vasodilators whose effects remain preserved in patients with heart failure. Also, results from my thesis help better understand the mechanism of action and local arterial effects of these potent vasodilators and also allows a head to head comparison between urocortin 2 and 3. In order to translate these results to clinical use, it is vital that the effects observed are reproduced in studies with systemic infusions of these peptides. There is already published work with systemic infusions of urocortins 1 and 2 in healthy volunteers and patients with stable heart failure [Davis *et al* 2004; Davis *et al* 2005; Davis *et al* 2007a; Davis *et al* 2007b]. In acute heart failure, urocortin 2 has been shown to augment cardiac output without significant reflex tachycardia [Wandy Chan *et al* 2013]. This was however associated with a transient fall in renal indices concurrent with urocortin 2 induced reductions in blood pressure.

The effects of urocortin 3 in man are relatively less well known. Gheorghide *et al* have recently examined the effects of systemic human stresscopin (JNJ-39588146) in patients with stable heart failure with favourable effects on cardiac indices and haemodynamic variables [Gheorghide *et al* 2013]. Colleagues in my own group have conducted a study examining the effects of systemic urocortin 2 and 3 on cardiac index, haemodynamic measures and peripheral vascular resistance in healthy volunteers and patients with stable heart failure. This study demonstrated that both peptides cause vasodilatation, reduce peripheral resistance and increase cardiac output in health and

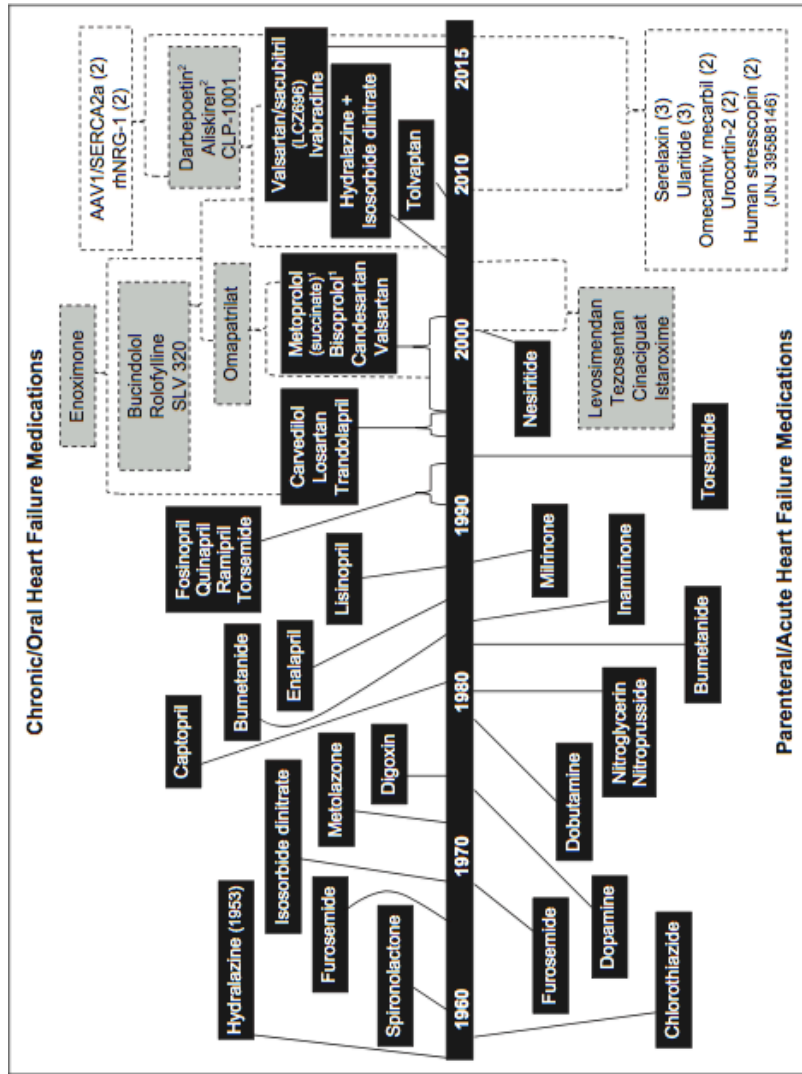


Figure 7.2. Timeline of FDA approval and development of heart failure medications. **Black box** - medications that have been approved and in use for treatment of heart failure in the USA; **Grey box** with dashed borders - development suspended due to failed phase 3 studies; **white box** with dashed borders - investigational medications with promising phase 2 and/or 3 data. (1): Approved by FDA for non-heart failure indications(s) prior to this period; (2): Approved by the FDA for heart failure indications. AAV1/SERCA1a: Adeno-associated virus type I/sarcoplasmic reticulum calcium ATPase gene transfer; FDA: Food and Drug Administration; rhNRG-1: recombinant human neuregulin 1. [Hanigan *et al* 2016]

disease. These results are in keeping with that seen in previous studies with systemic urocortin 2 infusion in stable heart failure.

Heart failure is associated with an increased neurohormonal activity as evidenced by increased central sympathetic activity and increased plasma norepinephrine from activated sympathetic fibres [Pepper and Lee 1999]. Studies have shown that muscle sympathetic nerve activity (MSNA) is also exaggerated in patients with heart failure [Leimbach *et al* 1986]. Thus, drugs that reduce MSNA may have major benefits in the treatment of heart failure. In preclinical models, infusions of urocortin 2 and 3 reduce cardiac sympathetic nerve activity in conscious sheep [Charles *et al* 2010; Charles *et al* 2011]. These results were however not reproduced in human MSNA responses to urocortin 2 in health or heart failure [Wandy Chan *et al* 2015. Doi:10.1111/1440-1681.12449].

The role of urocortins in heart failure continues to hold promise. Current data available have only been through small clinical trials with relatively small numbers of patients enrolled. In order to be able to translate these findings into meaningful therapeutic options, further work is required. As potent vasodilators, we have seen significant reductions in mean arterial pressures and peripheral vascular resistance with these peptides. It is possible that this profound vasodilatory effect may in fact serve to be a useful antihypertensive treatment in man. However, in the setting of acute heart failure, this may prove to be detrimental with reduced renal perfusion. Hence, it is important that we continue to carefully examine the effects of prolonged infusions of urocortin 2 and 3 in patients with heart failure.

7.3.4 UROCORTINS AS BIOMARKERS

In addition to serving as a potential therapeutic tool in the management of heart failure, it is possible that urocortins may serve as diagnostic biomarkers in heart failure and myocardial ischaemia. To date, there have been a small number of studies that have looked at urocortin concentrations in patients with heart failure and their proposed utility as biomarkers. Ng *et al* [2004] showed that plasma urocortin concentrations were elevated in patients with heart failure, especially in early stages and that the concentrations declined with increasing severity of disease. Contrary to this, several other authors [Wright *et al* 2009; Gruson *et al* 2010; Tang *et al* 2010; Yildirim *et al* 2014. Doi:10.5152/akd.2014.5793] have demonstrated that urocortin concentrations increase as the severity of heart failure increases. These studies suggest that the concentrations of urocortin in heart failure correlate well with other heart failure related neurohormones but, on their own, urocortins may not serve as a good marker of heart failure. However, when used in conjunction with other markers such as the natriuretic peptides, urocortins provide additional and independent information. In my thesis, we were unable to perform urocortin 2 and 3 assays due to lack of robust commercially available assays, but this information will no doubt add to our understanding of the relation between plasma concentrations of urocortins and the observed physiological changes.

7.4 CONCLUDING REMARKS

Findings from my thesis suggest that the sirtuin and urocortin hormone systems may hold great promise as potential targets in cardiovascular health. From cardiovascular risk factor prevention to heart failure, the two hormone systems do seem to hold their place in today's world of research. Since the initial description of the human sirtuin genes in 1999, a great deal of work has been done in the field of sirtuin biology. The translation of these findings into effective treatment modalities in man remains an area of interest and challenge. With a global increasing burden of heart failure, further work is required to examine the potential role of urocortins as a viable, safe treatment option in acute decompensated heart failure through larger scale studies. Further work is required to uncover the full potential of these hormone systems in man.

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Commentary

Urocortins in heart failure

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ABSTRACT

Despite modern advances in the treatment of the causes and consequences of cardiovascular illness, heart disease and heart failure remain a leading cause of death in the western world. Many novel peptides are emerging as biomarkers and potential therapeutic tools for this debilitating condition. Urocortins represent one such group of peptides whose role in normal cardiovascular physiology and disease states is now increasingly being recognized. The cardiovascular effects of the urocortins are mediated via corticotrophin-releasing hormone (CRH) receptors through a variety of intra-cellular signaling pathways. Studies to date have demonstrated a favourable effect of urocortins on hemodynamic and neurohumoral regulation. They cause relaxation of the vasculature as well as having positive inotropic, chronotropic and lusitropic effects on the heart. This makes the urocortins a potentially attractive target in the treatment of heart failure. Indeed, a number of studies have demonstrated increased urocortin activity in experimental and clinical heart failure, with apparent augmented responses in these states. This article provides a review of the role of urocortins in normal cardiovascular physiology and in the pathophysiology of heart failure.

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1. Introduction

Despite modern advances in the treatment of the causes and consequences of cardiovascular illness, heart disease remains a leading cause of death in the western world. In particular, heart failure continues to carry a poor prognosis with a considerable burden on the health care system throughout the world: the estimated direct cost for heart failure in the United States was \$30 billion in 2006 [1]. Many novel peptides are emerging as biomarkers and potential therapeutic tools for this debilitating condition. Urocortins represent one such group of peptides whose role in normal cardiovascular physiology and disease states is now being increasingly recognized. After their initial discovery in 1995 [2], subsequent research has furthered understanding of their mechanisms, predominantly in pre-clinical models, with expansion of this knowledge into potential therapeutic applications in humans. This article provides a review of the role of urocortins in normal cardiovascular physiology and in the pathophysiology of heart failure.

2. The urocortin–CRH system

Urocortins belong to the corticotrophin-releasing hormone (CRH) family which includes CRH, fish urotensin I, frog sauvagine,

urocortin 1, urocortin 2 and urocortin 3 [3] (Fig. 1). CRH is produced in the brain in response to stress, has central effects upon behaviour, and exerts a variety of peripheral responses. However, CRH is unlikely to have major effects upon cardiac function as it is not expressed locally and its plasma concentrations are very low.

In 1995, Vaughan et al. [4] observed urotensin-like immunoreactivity in the Edinger Westphal nucleus and lateral superior olive regions of the adult rat brain. It was named urocortin (now known as urocortin 1) to reflect its similarities of structure and biological properties to urotensin (suckerfish urotensin) and rat CRH. It is believed to be the second endogenous mammalian ligand for CRH receptors [5]. Subsequently, two further paralogues of CRH were identified—urocortin 2 and urocortin 3. Human CRH and urocortin 1 genes have been localized to chromosomes 8 (8q13) and 2 (2p23–p21), respectively. Urocortin 2 and urocortin 3 have prominent cardiovascular roles and are expressed in the heart. In contrast to CRH, the urocortins do not increase corticosterone secretion and do not appear to have any physiologic role in the regulation of the hypothalamic–pituitary–adrenal axis [6,7].

2.1. CRH receptors

The effect of CRH and urocortins is mediated via CRH receptors (CRH-R). These seven transmembrane G-protein coupled receptors are members of the secretin family [8] and the human CRH-R gene has been localized to chromosomes 17 (17q12–qter) and 7 (7p21–p15) [9,10]. Two subtypes of CRH receptors have been identified in mammals and rodents—CRF-R1 and -R2. Structurally, the two

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	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
CRH	S	E	E	P	P	I	S	L	D	L	T	F	H	L	L	R	E	V	L	E	M
Urocortin		D	D	P	P	L	S	I	D	L	T	F	H	L	L	R	T	L	L	E	L
Urocortin 2				V	I	L	S	L	D	V	P	I	G	L	L	R	I	L	L	E	Q
Urocortin 3				F	T	L	S	L	D	V	P	T	N	I	M	N	I	L	F	N	I
Sauvagine		E	G	P	P	I	S	L	D	L	S	L	E	L	L	R	K	M	I	E	I
Urotensin 1	N	D	D	P	P	I	S	L	D	L	T	F	H	L	L	R	N	M	I	E	M
	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	
CRH	A	R	A	E	Q	L	A	Q	Q	A	H	S	N	R	K	L	M	E	I	I	
Urocortin	A	R	T	Q	S	Q	R	E	R	A	E	Q	N	R	I	I	F	D	S	V	
Urocortin 2	A	R	Y	K	A	A	R	N	Q	A	A	T	N	A	Q	I	L	A	H	V	
Urocortin 3	D	K	A	K	N	L	R	A	K	A	A	A	N	A	Q	L	M	A	Q	I	
Sauvagine	E	K	Q	E	K	E	K	Q	Q	A	A	N	N	R	L	L	L	D	T	I	
Urotensin 1	A	R	I	E	N	E	R	E	Q	A	G	L	N	R	K	Y	L	D	E	V	

Fig. 1. Amino acid sequences of CRH and its analogue peptides. Sequences shown are that of mammalian CRH and urocortins, amphibian sauvagine and teleost urotensin 1. Highlighted sequences represent similarity to CRH.

subtypes exhibit considerable divergence at the N terminus, consistent with their distinct pharmacological properties. Furthermore, three splice variants of CRH-R2 have been identified. These variants differ in the structure of their N-terminal extra-cellular domain. R2 α and R2 β have been observed in rodents and in man, whilst R2 γ is specific to humans (isolated in the limbic regions of the human brain) [8]. It is, however, unclear whether the γ splice variant has any specific physiological role. Low homology of the extra-cellular domains of CRH-R1 and -R2 accounts for differences in their ligand specificity [8]. Urocortin 1 and CRH both act at CRH-R1 but the affinity of urocortin 1 for CRH-R2 is more than 10-fold higher than that of CRH [5]. Whilst urocortin 1 can activate both receptors, urocortins 2 and 3 are potent and specific agonists at CRH-R2 [4,11] with little effect at CRH-R1.

3. Biology of urocortins

3.1. Anatomy (tissue distribution of urocortins and CRH receptors)

Immunoreactivity to the urocortins and their receptors has been demonstrated in the central nervous, digestive, reproductive, cardiovascular, immune and endocrine systems, suggesting important roles throughout the body [12]. In the brain, urocortin 1 is most prominent in the Edinger Westphal nucleus and lateral superior olive. Urocortin 1 mRNA or immunoreactivity has also been reported in other regions of the brain, such as the cerebellum and hypothalamus [8], and it appears to be co-localized with dopamine in the basal ganglia and hypothalamus. Urocortin 1 mRNA is also expressed in vascular smooth muscle cells and in cardiac myocytes. Urocortin 2 has a similar distribution in the central nervous system in mouse and rats, but is also seen in high concentrations in the peripheral tissues including the heart, adrenals, placenta, stomach, ovary, skin, gastrointestinal tract, uterine smooth muscle, skeletal muscle and peripheral blood vessels [8].

The distribution of urocortin 3 is distinct. In the central nervous system, it is demonstrable in regions of high CRH-R2 expression,

supporting the notion that it is an endogenous ligand [8]. In humans, urocortin 3 is also seen in peripheral tissues such as adrenals, heart and kidney—particularly in the distal tubules [13].

CRH-R1 is predominantly found in the central nervous system. In addition to its central nervous system expression, CRH-R2 is found in peripheral tissues such as the gut, heart, lymphocytes and adrenals. In humans, urocortin 1 and CRH-R2 α has been identified in all four chambers of the heart, suggesting that urocortin acts in an autocrine or paracrine fashion through CRH-Rs [14]. In contrast to rats where CRH-R2 β is the predominant splice variant in the heart and vascular smooth muscle cells, humans appear to predominantly express CRH-R2 α in these tissues. CRH-R2 has also been characterized in the human left ventricle and intra-myocardial blood vessels [15]. In humans, both CRH-R1 and -R2 are found in the periphery, although their specific role remains to be fully characterized in human physiology and pathophysiology.

3.2. Biochemistry

3.2.1. Molecular structure

Urocortin is a 40 amino acid-containing neuropeptide, related to urotensin (63% sequence identity) and CRH (45% sequence identity) [4]. Rat and human urocortin bear 95% homology to each other. The precursor protein contains 122 amino acid residues with an N-terminal methionine and consensus signal peptide sequence, whilst the carboxy terminus of the precursor contains the C terminally amidated peptide of urocortin. The CRH analogue peptides possess an α helical conformation with varying degrees of amphipathicity. The amphipathic N-terminal helices could play a crucial role in selectivity of the analogues to CRH-R1, whereas it may not be as important for CRH-R2 binding [16]. The parent protein is half the length of urotensin and CRH precursors with little sequence similarity to either [8]. Urocortin 2 shows moderate homology with human and rat CRH (34%), urocortin 1 (43%) and urocortin 3 (37–40%). The half-life of urocortin 1 in healthy humans and those with stable heart failure is approximately 50 min [17,18]. Urocortin 2 has a shorter half-life of 10 min in

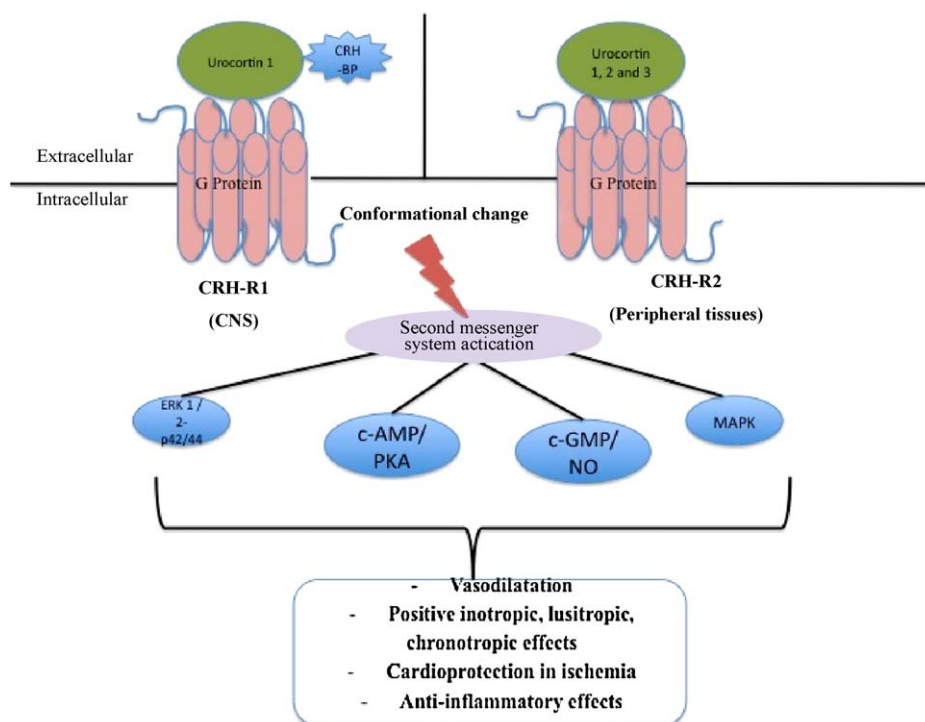


Fig. 2. Schematic of Urocortin intra-cellular signaling pathway. Urocortins bind to CRH receptors to induce conformational changes in the G protein receptors and activate the second messenger systems. CRH-BP has a greater affinity to Urocortin 1 and may play a role in its metabolism. Urocortin 1 binds to both CRH-R1 and -R2, whereas Urocortins 2 and 3 are potent, specific CRH-R2 agonists. CRH-BP: corticotrophin releasing hormone-binding protein; CRH-R: corticotrophin releasing hormone receptor; CNS: central nervous system; ERK 1/2-p42/44: extra-cellular signal-related kinases 1/2-p42/44; c-AMP: cyclic adenosine monophosphate; PKA: protein kinase A; cGMP: cyclic guanosine monophosphate; NO: nitric oxide; MAPK: mitogen-activated protein kinase.

healthy humans [19]. The exact half-life of urocortin 3 is not yet known, but it appears to have a more rapid onset and shorter duration of action [20].

3.2.2. CRH binding protein

Corticotrophin releasing hormone-binding protein (CRH-BP) is a 37 kDa protein that was first isolated in human plasma in 1989 [21] and binds to both CRH and urocortin 1. Given that the expression of CRF-BP overlaps that of both CRH and urocortin 1 in the central nervous system, it has been proposed that the CRH-BP plays a role in the modulation of the action of urocortin and CRH at these sites. In humans, CRH-BP has been detected in the brain, pituitary, liver and placenta [22–24]. In ovine models of heart failure [25], the half-life of urocortin 1 was markedly prolonged compared to human models. This has been attributed to the possible role of CRF-BPs in clearance of urocortin 1 in humans. However, the exact role of these binding proteins remains to be fully elucidated in health and disease states.

3.2.3. Intra-cellular signaling pathways

The urocortins bind to G-protein coupled CRH (R1 and R2) receptors to induce conformational changes in the receptor that activate intra-cellular signaling pathways (Fig. 2). In most cells, this involves adenyl cyclase and cyclic adenosine monophosphate (cAMP). Indeed, Kageyama et al. demonstrated that urocortin 2 induces vasodilatation in vascular smooth muscle cells via CRH-R2 in association with increased cAMP accumulation via activation of adenylate cyclase [26,27]. Mitogen-activated protein kinases (MAPK) are also implicated in urocortin-mediated vasodilatation [26] as well as in the cardioprotective role of urocortin in response to ischemic or hypoxic injury [28]. However, in some studies, inhibition of the c-AMP or protein kinase A (PKA) pathway has failed to inhibit the effects of CRH and its related

peptides, suggesting the involvement of other signaling mechanisms.

CRH and related agonists can evoke the endothelial release of nitric oxide via nitric oxide synthase with subsequent accumulation of cyclic guanosine monophosphate (cGMP). This endothelium-dependent mechanism is important in CRH or urocortin-induced relaxation in placental vasculature [29] and peripheral arteries, and has been studied in isolated arterial segments such as the human internal mammary artery graft [30] and in rat coronary artery [31]. The nitric oxide and cGMP-dependent component of this vasodilator effect is mediated via activation of calcium-activated potassium channels in underlying vascular smooth muscle. Indeed, the vasorelaxant effect of urocortin is blunted in the presence of L-NAME (N^G -nitro-L-arginine methyl ester; a NOS inhibitor) and ODQ (1H-[1,2,4] oxadiazolo [4,2-a] quinoxalin-1-one; inhibitor of guanylyl cyclase) [30].

There is increasing evidence that urocortin and CRH-related peptides play an important role in cell survival mechanisms in a number of systems. Similar to CRH, urocortin 1 activates the MAPK extra-cellular signal-related kinases (ERK) 1/2-p42/44 signaling cascade in *in vitro* cultures of isolated rat cardiac myocytes, which is inhibited by blockade of MEK 1/2. This signaling cascade mediates the cardioprotective function of urocortin in stimulated hypoxia or ischemia [28]. Urocortins also possess anti-inflammatory properties that appear to be mediated via pro-apoptotic effects on macrophages via a direct effect on pro-apoptotic Bcl-2 related proteins [32].

3.3. Physiology of urocortins

3.3.1. Cardiovascular actions

The important roles of urocortins in the regulation of normal cardiovascular physiology are being increasingly recognized.

Genetically engineered mice lacking CRH-R2 are resistant to otherwise marked urocortin-evoked changes in cardiac performance and blood pressure [33]. Studies, which are largely pre-clinical, have so far demonstrated a favourable effect of urocortins on hemodynamic and neurohumoral regulation. Urocortins 1, 2 and 3 produce positive inotropic and lusitropic effects, reduction in the mean arterial pressure due to decreased peripheral vascular resistance, and increased coronary perfusion in rodent and ovine studies.

3.3.1.1. Vascular effects. Central administration of CRH in rats produces a pressor response, which appears to be mediated through CRH-R1 receptors [34]. Peripheral administration of CRH produces a depressor response, which is blocked by α helical CRH (a non-selective CRH antagonist), and not by antalarmin (selective CRH-R1 antagonist). As a peptide, CRH has limited access to the central nervous system. This suggests that the hypotensive effect is mediated via peripheral CRH-R2 receptors. CRH-R2 may have a more prominent role in changes in arterial pressure in comparison to CRH-R1. CRH-R2 may contribute to the maintenance of basal vascular tone in mice. Indeed, mice deficient in CRH-R2 receptors are hypertensive with no fall in the mean arterial pressure in response to exogenously administered urocortin [35]. It remains unknown whether urocortins and CRH-R have a similar role in the maintenance of basal vascular tone in humans.

Intravenous administration of CRH produces vasodilatation in rats and a consequent fall in blood pressure with a reflex increase in heart rate, although this is not observed in sheep and is seen only with relatively high doses in higher primates like monkeys and humans. In anesthetized rats, intravenous injection of human urocortin 2 reduced basal systemic blood pressure in a dose-dependent fashion [36]. As demonstrated by Vaughan et al. [4],

urocortin 1 also possesses a potent and long lasting hypotensive action. Decrease in mean arterial pressure of 18.3 ± 0.7 mm Hg was observed with urocortin 1 in rats and lasted for almost 2 h. Dieterle et al. [37] studied the effects of urocortin 2 injection in control and hypertensive rats. They showed an immediate and sustained lowering of blood pressure in hypertensive rats with no rise in heart rate. This effect on the blood pressure was seen for up to 12 h after intraperitoneal injection of urocortin 2.

Several mechanisms have been postulated for the blood pressure lowering effects of urocortins. These may include a direct smooth muscle relaxant effect in combination with an associated reduction in plasma concentrations of vasoconstrictor hormones, such as endothelin 1, angiotensin II and arginine vasopressin (AVP), as seen in animal models of heart failure (see Section 4.3 on Neurohormonal effects).

Ex vivo studies in the human internal mammary artery [30] and in the rat coronary artery [31] suggest both endothelium-dependent and independent components for vasorelaxation. In isolated rat coronary artery [31], potent vasorelaxant effect of urocortin 1 was observed with an IC_{50} of 2.24 nM, in the presence of an intact endothelium. The endothelium-dependent component appears to be, at least in part, mediated by nitric oxide via cGMP, as outlined above. Huang et al. [31] have also shown the role of activation of barium chloride ($BaCl_2$) sensitive potassium channels in arterial smooth muscle cells, mediating the endothelial component of urocortin-induced coronary relaxation. Indeed, these studies also demonstrate a blunted, but not abolished, vasorelaxant response to urocortin in endothelium-denuded arterial segments, further suggesting the role of additional endothelium-independent mediators. Endothelium-independent regulation of vascular tone appears to involve calcium (Ca^{2+}) independent phospholipase A_1 and store-operated Ca^{2+} entry

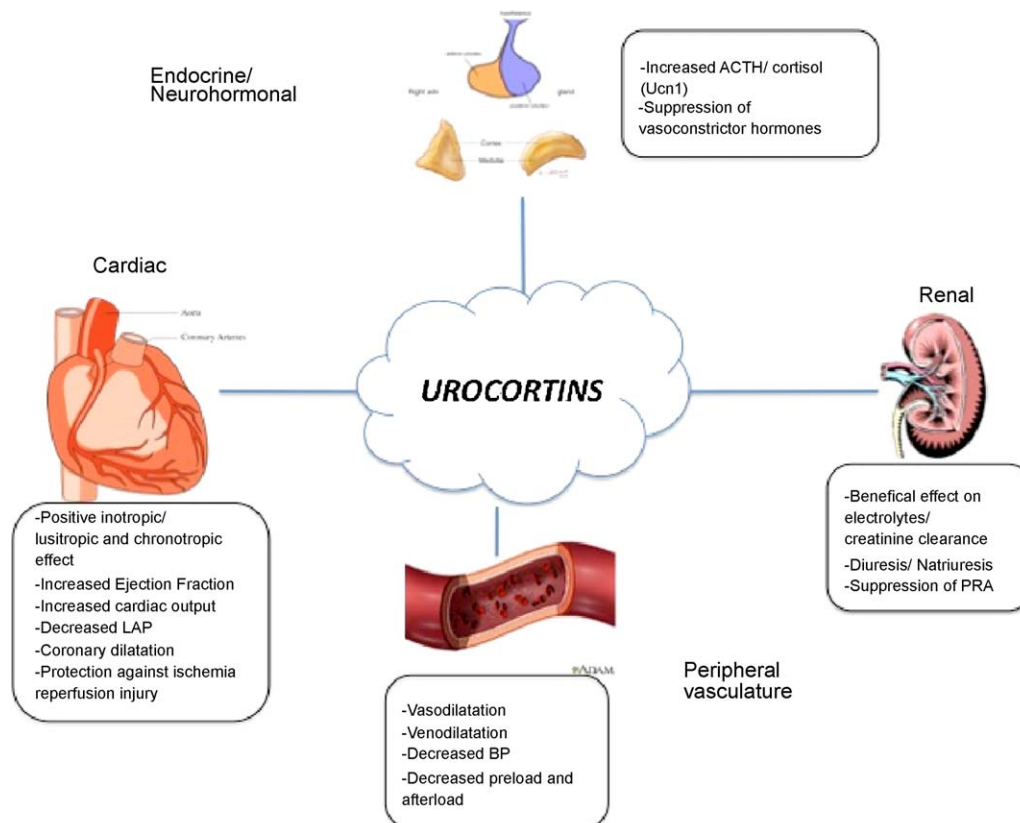


Fig. 3. Beneficial effects of urocortins in heart failure. The beneficial effects of urocortins on various systems as seen in animal and human models of heart failure. ACTH: adrenocorticotrophic hormone; Ucn 1: urocortin 1; LAP: left atrial pressure; BP: blood pressure; PRA: plasma renin activity.

modulation [38]. Other mechanisms demonstrated in mediating urocortin-induced vasorelaxation include the MAPK and PKA pathway [26].

3.3.1.2. Cardiac effects. It is not entirely clear whether urocortins mediate their protective effects upon cardiac contractility via a direct effect on cardiac myocytes or via sympathetic stimulation in response to reduced peripheral resistance. Brar et al. [28] have demonstrated the presence of a 22 kDa urocortin 1 precursor protein in neonatal rat cardiac myocytes and release of urocortin into the supernatant of cardiac myocytes exposed to stimulated ischemia or hypoxia, suggesting endogenous release of urocortin from ischemic cardiac myocytes. This may suggest potential direct and local action of urocortins on cardiac myocytes, mediated via CRH-R.

To determine whether urocortin induced direct effects on contractility of cardiac myocytes, Yang et al. [39] observed the effects of application of 100 nmol/L of urocortin 2 to isolated adult rabbit ventricular cardiomyocytes. They showed a progressive enhancement in myocyte contractility with reduction in diastolic length and concluded that urocortins exert direct positive inotropic as well as lusitropic effects. These effects were mediated via activation of CRH-R2 and subsequent stimulation of PKA activity, leading to augmentation of L-type calcium channel (I_{Ca}), and sarcoplasmic and endoplasmic reticulum Ca^{2+} ATPase (SERCA)-mediated Ca^{2+} uptake into the sarcoplasmic reticulum.

Immediate improvement of left ventricular fractional shortening and circumferential fibre shortening velocity were noted following acute injection of urocortin 2 in rats [37]. These beneficial effects were preserved even at 5 weeks after treatment. In ovine models, intravenous injection of urocortin 1 causes a marked dose-dependent increase in cardiac output and contractility, which is sustained at 24 h post injection [40].

3.3.1.3. Effects in humans. A small number of clinical studies have carried forward data obtained from animal studies, to look at the effects of urocortins on cardiovascular and neurohormonal responses.

In contrast to the favourable hemodynamic responses seen in normal sheep, infusion of urocortin 1 in healthy humans caused no change in hemodynamic variables, nor did it affect the plasma concentrations of humoral factors, such as aldosterone or arginine vasopressin. The pharmacokinetics of urocortin 1 were similar in normal humans and in subjects with heart failure: plasma urocortin 1 half-life of 52 ± 3 min in healthy volunteers and 54 ± 3 min in those with heart failure [18]. Pemberton and colleagues did not observe any increase in urinary urocortin 1 following its infusion suggesting that urocortin 1 is not excreted in the kidneys. Consistent with its predicted cardiovascular profile, urocortin 2 infusion caused a dose-dependent increase in cardiac output with a decrease in mean arterial and diastolic blood pressure, and systemic vascular resistance [19]. The effects of systemic urocortin 3 in humans are yet to be explored. Wiley and Davenport [15] have demonstrated that urocortin 3, like urocortin 2, causes vasodilatation in isolated human internal mammary artery segments and that this effect is mediated via the direct effect of urocortin 3 on the vascular smooth muscle cells. Although not yet assessed in man, urocortin 3 is likely to exert cardiovascular effects similar to those evoked by urocortin 2.

3.3.2. Cardioprotective effects—role in ischemia reperfusion injury

Urocortins appear to have a cardioprotective role and indeed, urocortin expression and peptide release is increased by ischemia [41].

Urocortin 1 reduces myocyte cell death caused by ischemia reperfusion injury. This appears to be mediated via several mechanisms including up-regulation of cardioprotectin 1 expression

[42], stimulation of heat shock protein [43] and natriuretic peptides, and attenuation of calcium-insensitive phospholipase A2 gene expression [44]. *In vitro* secretion of urocortin is also enhanced by inflammatory cytokines such as interleukin-6, interleukin-1 and tumour necrosis factor α : factors that are elevated in patients with heart failure and acute coronary syndromes [64].

Urocortins 2 and 3 protect neonatal rat cardiac myocytes *in vitro* when administered before hypoxia or at the point of reoxygenation. Urocortins 2 and 3 also protect the adult rat heart *ex vivo* and acts via the MAPK pathway to reduce the infarct size of a perfused intact rat heart exposed to regional ischemia [45]. Brar et al. [45] have demonstrated that urocortin induces ERK 1/2-p42/44 phosphorylation in neonatal rat cardiac myocytes and that inhibition of MEK 1/2 inhibits its cardioprotective effects.

Nitric oxide is recognized as a key determinant of vascular health. It acts as a potent vasodilator, inhibits expression of several pro-inflammatory cytokines and chemokines, and plays a key role in vascular smooth muscle proliferation, platelet aggregation and endogenous fibrinolysis [46–48]. Whilst urocortin may act via nitric oxide to mediate cardiovascular protective effects [46], studies to date have not yet completely elucidated the effect of urocortins on the endothelium or nitric oxide.

4. Urocortins and heart failure

Given the potent vasorelaxant and inotropic effects of the urocortins, interest has grown in their role in the pathophysiology and potential therapeutic utility in the treatment of heart failure. A number of studies have demonstrated increased concentrations of urocortin 1 in cardiac tissue [49,50] and plasma [25,51,52] in experimental and clinical heart failure. Nishikimi et al. [49] demonstrated up-regulation of expression of urocortin 1 mRNA in left ventricular hypertrophy. They also demonstrated increased urocortin 1 immunoreactivity in the failing heart. This has been cited as possible evidence that urocortins play a role in the pathophysiology of cardiac hypertrophy and heart failure (Fig. 3).

4.1. Animal models

Pre-clinical studies of heart failure have previously examined the roles of urocortins 1 and 2 whilst urocortin 3 remains less well characterized. The beneficial cardiovascular and neurohumoral responses of urocortins are preserved, and may be augmented in the presence of heart failure.

Intravenous infusion of urocortin 1 in an ovine model of heart failure attenuates the hemodynamic deterioration and harmful neurohormonal activation associated with heart failure [53]. These hormones include renin, angiotensin II, aldosterone, endothelin-1, vasopressin and catecholamines that, along with sympathetic nervous system activation, combine to exert the hemodynamic and endocrine hallmarks of heart failure. Furthermore, in this model, urocortin 1 also protected renal function. The preservation of cardiac output with urocortin 1 infusion may be partly due to its inotropic actions, in addition to its coronary arterial vasodilator effects and improved cardiac bioenergetics. When infused at the onset of left ventricular pacing, urocortin 1 infusion restricts the increase in left atrial pressure and attenuates the reduction in cardiac output. It also has lusitropic and venodilating effects [54]. These results highlight that treatment with urocortin 1 may be beneficial in the treatment of heart failure initiated early in the disease. Of note, these favourable hemodynamics persisted during prolonged (4 day) infusion of urocortin 1.

In keeping with a favourable hemodynamic profile of urocortin 1 in ovine heart failure, urocortin 2 infusion in MLP (muscle specific LIM protein) deficient mice, a model of dilated cardiomy-

opathy, caused a dramatic improvement in cardiac output and left ventricular function, enhanced cardiac contractility and reduced systolic load [55]. The enhancement in ejection fraction by urocortin 2 is partly attributed to reduction in arterial load: this may prove more notable in the failing heart where there is an afterload mismatch.

Patients with heart failure are likely to receive any new treatment in conjunction with conventional treatment. Hence it is important to ensure that any potential new treatment does not interact with drugs such as ACE inhibitors and β blockers. Rademaker et al. [56] assessed the combined effects of captopril with urocortin 2 in sheep with pacing-induced heart failure. Combined treatment of urocortin 2 with captopril augmented the decrease in total peripheral resistance by an additional 20% compared with either agent alone. One of the potential drawbacks of treatment with ACE inhibitors is its profound hypotensive effect, which in the presence of heart failure can compromise blood flow to vital organs, such as the kidneys. When used in combination with an ACE inhibitor, urocortin 2 evoked an additional decrease in peripheral resistance without further reduction in the systolic blood pressure. This makes the combination of these agents an attractive tool in the management of heart failure. In addition, combination of urocortin 2 with captopril improved cardiac performance, decreased peripheral resistance and ventricular filling pressures in association with reduction in plasma aldosterone and endothelin-1 concentrations. In a murine acute heart failure model, pre-treatment with a β -adrenergic receptor (AR) antagonist, did not affect the inotropic or lusitropic actions of urocortin 2 *in vivo* indicating that its actions are independent of β -adrenergic receptors [55].

The effects of combined treatment with urocortin 2 and furosemide has been studied recently [57]. When this combination was administered to sheep with pacing-induced heart failure, it caused increased diuresis, natriuresis and sustained increase in creatinine excretion and clearance without additional potassium elimination. Urocortin 2 alone or in combination increased cardiac output and contractility whilst furosemide had no effect. Importantly, the combination of the two drugs, produced reversal of furosemide-induced increase in plasma renin activity (PRA) and

a greater decrease in plasma aldosterone and vasopressin concentrations. These beneficial effects of Ucn 2 may be mediated by an increase in cardiac output and consequently improved renal perfusion. Indeed, improvements in glomerular filtration rate, urine volume and sodium excretion have been demonstrated in the ovine model of heart failure [58]. In addition to increased cardiac output and renal vasodilatation, other postulated mechanisms include a direct tubular action of Ucn 2 [11] as well as attenuation of anti-natriuretic and anti-diuretic factors resulting in increased diuresis and natriuresis [57].

As already noted, the hemodynamic and humoral responses of urocortin 3 in experimental heart failure are less well studied than urocortin 1 or 2. In a study of sheep with pacing-induced heart failure [20], the hemodynamic responses produced by urocortin 3 were similar to that produced by equivalent doses of urocortin 1 or 2. Urocortin 3 caused a marked dose-dependent improvement in cardiac output and reduction in peripheral resistance and left atrial pressure. This was associated with a reduction in mean arterial pressure, beneficial effect on hormonal responses (attenuation of vasoconstrictor peptide systems) and improved renal function (dose-dependent increases in urine volume, sodium and creatinine excretion). The onset and duration of action was much shorter than that of urocortin 1 or 2.

4.2. Patients with heart failure

Plasma concentrations of urocortins are elevated in patients with heart failure. Ng et al. [51] found higher plasma urocortin 1 concentrations in men with heart failure as well as in elderly patients. There appeared to be an inverse relationship between plasma urocortin concentrations and New York Heart Association (NYHA) class. In more severe heart failure, as reflected by NYHA class III or IV and low left ventricular ejection fraction, plasma urocortin concentrations appeared to be suppressed, suggesting that up-regulation of the urocortin system in early heart failure may be cardioprotective. In keeping with this report, more recent work by Wright et al. [59] has demonstrated elevated concentrations of plasma urocortin 1 in patients with heart failure with positive relationships to other circulating neurohormones such as

Table 1
Neurohormonal effects of Urocortins in human and ovine models.

	Humans				Sheep					
	Healthy		Heart failure		Healthy			Heart failure		
	Ucn 1 [17]	Ucn 2 [19]	Ucn 1 [18]	Ucn 2 [62]	Ucn2 [58]	Ucn 3 [20]	Ucn1	Ucn 1 [53,63]	Ucn 2 [58]	Ucn 3 [20]
cAMP	=	↑	=	=	=	=		↓ ^{a†b}	↓	↓↓
cGMP	↑	↑		=						
ACTH	↑↑	=	↑	↑	↑	↑↑		↑	↑	↑↑
Cortisol	↑↑	=	↑	=	↑	↑↑		↑	↑	↑↑
GH	=									
Ghrelin	↓	=	=	=						
LH/FSH/Prolactin	=									
TSH	=									
AVP	↑	=	=		↑	↑↑		↓↓	↑	↓↓
ANP	=		=		↓	↓		↓	↓↓	↓↓
BNP	=		=		↑	↑		↓↓	↓↓	↓↓
NT-BNP	=	=	=	↑				↓		
PRA	=	↑↑	=	=	=	=		↓	↓↓	↓↓
Aldosterone	=	↑	=	=	↓	=		↓	↓↓	↓↓
Adrenaline	=	↓	=	=	↓	↓		↓	↓↓	↓↓
Nor adrenaline	=	↑↑	=	=	=	=		↓	↓	↓
Endothelin	=	=	=	=	=	=		↓↓	↓↓	↓↓
Adrenomedullin	=	=	=	↑						
Insulin	=	=	=	=						
Angiotensin II		↑↑								
Glucose			=					=		

Fields left blank, no available data; (↑) increase, (↓) decrease, (=) no change.

^a Suppression of cAMP by Ucn 1 in LV pacing-induced heart failure in sheep—as an acute effect.

^b On prolonged exposure to Ucn 1 cAMP levels were substantially increased with a lag between the onset of hemodynamic and hormonal effects.

brain natriuretic peptide, adrenomedullin and endothelin-1. However, in contradiction to findings from Ng et al., Wright's group noted an inverse relationship of the level of plasma urocortin 1 to left ventricular ejection fraction, with a linear increase in plasma concentration of urocortin 1 with increasing NYHA class. It is possible that this difference in results is attributable to differences in the immunoassay used or potential effect of CRH-BP on the assay performance. Further research is, however, required to establish the relationship between urocortin concentrations and NYHA class.

Systemic intravenous infusions of urocortins 1 and 2 have been administered to a small number of patients with heart failure. Urocortin 1 infusion increased corticotrophin and cortisol, but produced no changes in hemodynamic, renal or neurohormonal parameters [18]. Infusion of urocortin 2 [19] evoked an increase in cardiac output with peripheral vasodilatation and a small increase in heart rate. Consistent with findings from the ovine model, systolic blood pressure fell in patients with congestive cardiac failure but not in control subjects [60]. It has been hypothesised that, in the presence of heart failure, the urocortin-induced rise in cardiac output is insufficient to compensate for the pronounced decrease in systemic vascular resistance [60]. However, this phenomenon may otherwise be explained by heightened peripheral sensitivity to the vasodilator effects of urocortin in the presence of heart failure. In agreement with this suggestion, administration of a CRH-R2 antagonist increases mean arterial blood pressure in sheep with heart failure but not in those without [61].

Apart from the positive influences on cardiovascular parameters when used in treatment in heart failure, urocortins may serve as potential biomarkers in identification of early heart failure, in combination with other biomarkers such as brain natriuretic peptide [59].

4.3. Neurohormonal effects of urocortins in heart failure

As noted above, urocortins cause a pronounced suppression of vasoconstrictor hormones in animal models of heart failure, which further supports its potential therapeutic role. However, studies of urocortins 1 and 2 in humans have only shown modest changes in neurohormonal activity. It is important to note that in man, infusion of urocortin 2 in healthy volunteers and in patients with heart failure does not alter plasma adrenocorticotrophic hormone or cortisol concentrations. Table 1 summarizes the effects of urocortins on neurohormonal activity in ovine and human experiments.

5. Future of urocortins

There is increasing evidence that urocortins have several potential uses in management of cardiovascular conditions such as hypertension, ischemic heart disease and heart failure. The immediate and sustained blood pressure lowering effects by urocortin 2 [37] appears to pose a novel and attractive approach for antihypertensive treatment. The favourable effects on hemodynamics, renal and neurohumoral mechanisms have generated much interest in the use of urocortins in heart failure. In particular, the positive inotropic effect, combined with its ability to reduce peripheral arterial resistance, favours use in this group of patients. In humans, studies to date have largely looked at combined systemic effects of urocortins. Direct arterial and venous effects of urocortins have not yet been described in man. It has not been possible to tease out the relative contribution of urocortin-induced changes in hemodynamic variables on the augmentation of cardiac output. Further studies are required to look at potential effects of long-term administration of these peptides.

In the search for novel treatments for heart failure, the focus is on urocortins 2 and 3. Indeed, urocortin 1 has no hemodynamic

effects in man and also bears the potential to induce unwanted side effects by activating CRH-R1 and stimulation of the hypothalamus–pituitary axis. However, it may have a role as an early biomarker of heart failure.

The potential application of urocortins in protection from ischemia reperfusion injury remains of major interest. Evidence available from pre-clinical models suggests that urocortins may have a role in protecting against ischemic reperfusion injury and in limiting infarct size. This has yet to be evaluated in man.

In conclusion, urocortins are emerging as an important group of peptidic mediators with important roles in human physiology and pathophysiology. This review provides an overview of their effects on the cardiovascular system alone. There are several other potential applications of this group of peptides, including roles in appetite suppression, in muscle wasting and central nervous system disorders to name a few. However, their major effects in the cardiovascular system implicate them as potential therapeutic targets in a range of processes, particularly heart failure.

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Vascular Effects of Urocortins 2 and 3 in Healthy Volunteers

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Background—Urocortin 2 and urocortin 3 are endogenous peptides with an emerging role in cardiovascular pathophysiology. We assessed their pharmacodynamic profile and examined the role of the endothelium in mediating their vasomotor effects in vivo in man.

Methods and Results—Eighteen healthy male volunteers (23 ± 4 years) were recruited into a series of double-blind, randomized crossover studies using bilateral forearm venous occlusion plethysmography during intra-arterial urocortin 2 (3.6 to 120 pmol/min), urocortin 3 (1.2 to 36 nmol/min), and substance P (2 to 8 pmol/min) in the presence or absence of inhibitors of cyclooxygenase (aspirin), cytochrome P450 metabolites of arachidonic acid (fluconazole), and nitric oxide synthase (L-NMMA). Urocortins 2 and 3 evoked arterial vasodilatation ($P<0.0001$) without tachyphylaxis but with a slow onset and offset of action. Inhibition of nitric oxide synthase with L-NMMA reduced vasodilatation to substance P and urocortin 2 ($P\leq 0.001$ for both) but had little effect on urocortin 3 ($P>0.05$). Neither aspirin nor fluconazole affected vasodilatation induced by any of the infusions ($P>0.05$ for all). In the presence of all 3 inhibitors, urocortin 2- and urocortin 3-induced vasodilatation was attenuated ($P<0.001$ for all) to a greater extent than with L-NMMA alone ($P\leq 0.005$).

Conclusions—Urocortins 2 and 3 cause potent and prolonged arterial vasodilatation without tachyphylaxis. These vasomotor responses are at least partly mediated by endothelial nitric oxide and cytochrome P450 metabolites of arachidonic acid. The role of urocortins 2 and 3 remains to be explored in the setting of human heart failure, but they have the potential to have major therapeutic benefits.

Clinical Trial Registration—<http://www.clinicaltrials.gov/>. Unique identifier: NCT01096706 and NCT01296607. (*J Am Heart Assoc.* 2013;2:e004267 doi: 10.1161/JAHA.112.004267)

Key Words: forearm plethysmography • nitric oxide • urocortin 2 • urocortin 3 • vasodilatation

Urocortin peptides, especially urocortins 2 and 3, have prominent cardiovascular roles and are expressed in the heart. Although related to corticotrophin-releasing hormone (CRH), they do not appear to have any role in the regulation of the hypothalamic-pituitary-adrenal axis.^{1,2} The effects of CRH and the urocortins are mediated via 2 G-protein-coupled receptors: CRH-R1 and CRH-R2. Although CRH-R1 is predominantly expressed in the brain and not in the heart, CRH-R2 is expressed in the myocardium and vascular smooth muscle.^{2,3}

It is found in human coronary artery microvascular endothelial cells and has been detected in the endothelium of a variety of peripheral vascular beds. Urocortin 1 activates both receptors, whereas urocortins 2 and 3 are potent selective agonists at CRH-R2 but have no effect on CRH-R1.⁴

The role of urocortins in cardiovascular physiology and pathophysiology, particularly heart failure, has become increasingly apparent. Intravenous urocortin 1 causes marked vasodilatation in mice via CRH-R2.^{5,6} Furthermore, mice lacking CRH-R2 receptors are hypertensive, suggesting a role for urocortin in the maintenance of basal vascular tone.⁶ Systemic administration of urocortin 2 in humans increases cardiac output, heart rate, and left ventricular function while decreasing systemic vascular resistance, and these effects may be amplified in the setting of heart failure.^{7,8} Urocortin 3 has not previously been administered to humans, but in an ovine model, both urocortin 2 and urocortin 3 appeared to produce similar cardiovascular effects.^{9,10} Although urocortin 2 and urocortin 3 each activate the same receptor, potential differences in their cardiovascular therapeutic utility may arise from their differing pharmacokinetic and pharmacodynamic profiles.¹

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The cardiovascular responses of urocortins represent an amalgamation of systemic actions. However, their direct in vivo arterial vasomotor effects have never been examined in humans. Moreover, the role of the endothelium in the mediation of these responses is unknown. Therefore, our study's aims were to conduct the first comparative clinical assessment of local arterial vasomotor effects of urocortins 2 and 3 and to determine the role of the endothelium in the mediation of these effects.

Methods

All studies were approved by the local research ethics committee and carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants prior to the study.

Study Participants

Eighteen healthy nonsmoking male volunteers were recruited into the series of vascular studies. Participants had no documented medical history, were taking no regular medications, and tested negative in a urinary toxicology screen (Nova Test, One Step Diagnostic Rapid Test, CA) for recreational drugs.

Vascular Studies

All studies were conducted using a double-blind, randomized, controlled crossover design. They were performed with the patient lying supine in a quiet, temperature-controlled room (22°C to 25°C). Volunteers fasted for 4 hours prior to the study and refrained from alcohol and caffeine for 24 hours prior to the study. Venous cannulae (17G) were inserted into large subcutaneous veins in the antecubital fossae of both arms at the start of the study to facilitate periodic venous sampling. In view of the theoretical risk of alterations in body temperature¹¹ and blood glucose concentrations¹² with the first-in-human administration of urocortin 3, tympanic temperature (Genius 2 Tympanic Thermometer, Coviden, Boston, MA) and capillary blood glucose (Advantage Accucheck blood glucometer, USA) measurements were performed at baseline and after each dose of urocortin 3. Heart rate and blood pressure were monitored at regular intervals throughout the study with a semiautomated oscillometric sphygmomanometer (Omron 705IT).

Subjects underwent brachial artery cannulation in the nondominant forearm with a 27 standard wire-gauge steel needle. Forearm blood flow was measured in the infused and noninfused forearms using bilateral venous occlusion plethysmography as described previously.^{13,14}

Pharmacodynamic Study

Eight healthy volunteers attended on 4 occasions (protocol 1) separated by at least 1 week (Figure 1A). After an initial infusion of normal saline (0.9%) for 20 minutes, volunteers received discontinuous (protocol 1a) or continuous (protocol 1b) incremental intra-arterial doses of urocortin 2 (3.6 to 120 pmol/min; Neurocrine Biosciences, Inc, San Diego, CA) or urocortin 3 (1.2 to 36 nmol/min; GenScript, NJ) interspersed with saline infusions between doses as appropriate.

Mechanistic Study

An additional 10 healthy volunteers (protocol 2) attended on each of 5 occasions to receive incremental intra-arterial doses of urocortin 2 (3.6 to 36 pmol/min), urocortin 3 (1.2 to 12 nmol/min), and substance P (2 to 8 pmol/min; a control endothelium-dependent vasodilator that evokes endothelial tissue plasminogen activator release; Clinalfa Basic, Bachem Distribution Services GmbH, Germany) (Figure 1B). These infusions were administered in the presence of (1) a placebo, (2) oral aspirin 600 mg (cyclo-oxygenase inhibition), (3) a "nitric oxide clamp" (nitric oxide synthase inhibition; see below), (4) intra-arterial fluconazole (1.2 μ mol/min; inhibition of cytochrome P450 metabolites of arachidonic acid), and (5) a combination of oral aspirin, intra-arterial fluconazole, and the nitric oxide (NO) clamp.

The nitric oxide clamp was used to determine the contribution of nitric oxide to urocortin-induced vasodilatation. Following baseline saline infusion, the nitric oxide synthase inhibitor L-N(G)-monomethyl arginine citrate (L-NMMA; 8 μ mol/min; Clinalfa Basic, Bachem Distribution Services GmbH, Germany) was infused intra-arterially. To compensate for L-NMMA-induced basal vasoconstriction, forearm blood flow was returned to baseline using a titrated dose of the exogenous nitric oxide donor sodium nitropruside (SNP; 90 to 1200 ng/min; Hospira Inc, Lake Forest, IL). Once baseline blood flow had been restored, this dose of SNP was coinfused with L-NMMA and continued throughout the study. This arrangement allows a constant "clamped" delivery of exogenous nitric oxide while endogenous nitric oxide synthase activity is abolished.

The order of urocortin 2, urocortin 3, and substance P infusions was randomized between subjects but kept constant for all visits of each individual subject. The order of infusion of inhibitors was also randomized in a double-blind manner.

Venous Sampling

Blood sampling was carried out at baseline for the assessment of full blood count, liver and renal function tests,

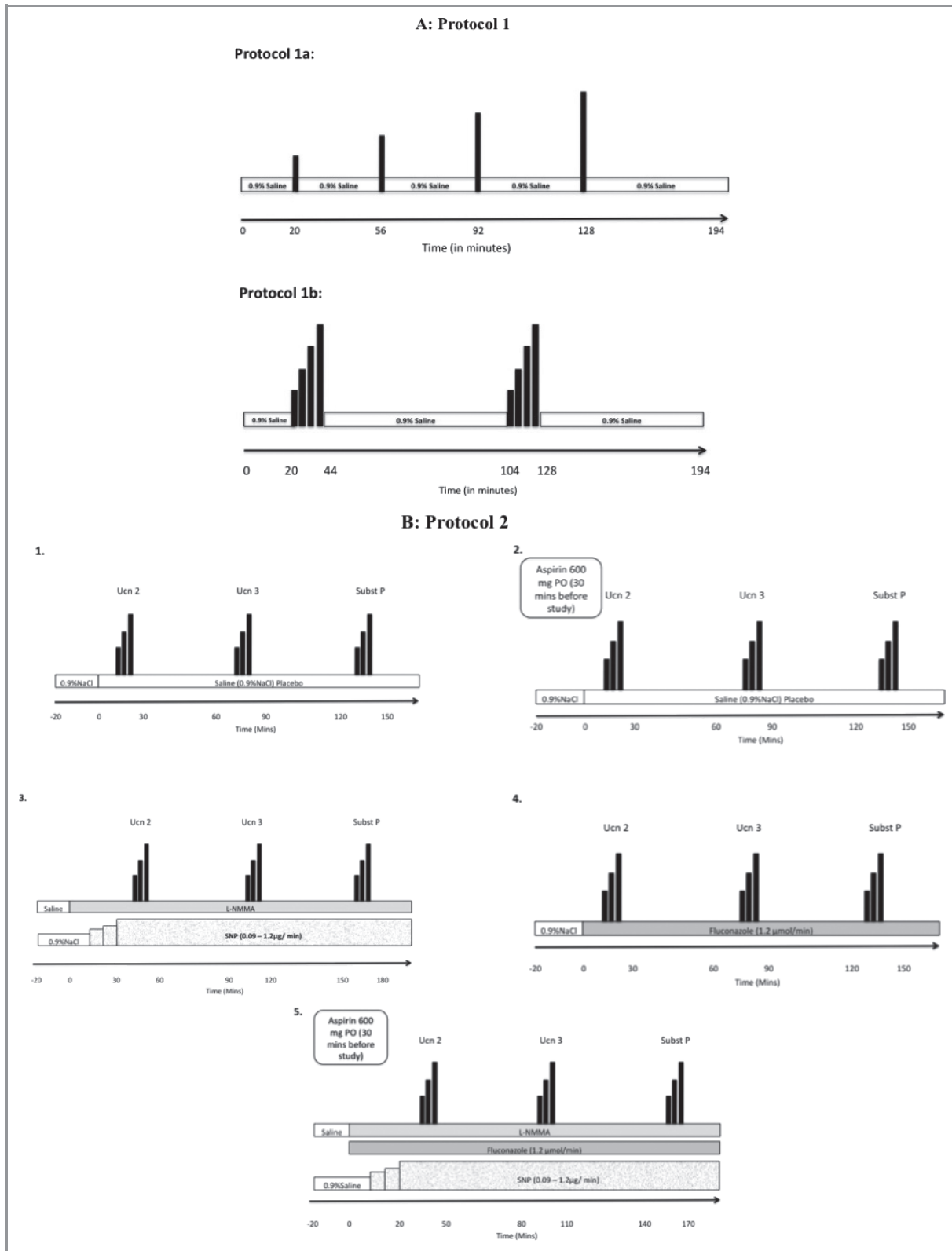


Figure 1. Schematic representation of study protocols. A, Protocol 1—incremental intra-arterial doses of urocortin 2 (Ucn 2; 3.6 to 120 pmol/min) and urocortin 3 (Ucn 3; 1.2 to 36 nmol/min) in the presence (protocol 1a) and absence (protocol 1b) of saline washout. B, Protocol 2—incremental intra-arterial infusions of Ucn 2 (3.6 to 36 pmol/min), Ucn 3 (1.2 to 12 nmol/min), and substance P (subst P; 2 to 8 pmol/min) in the presence of (1) saline placebo, (2) oral aspirin, (3) “nitric oxide” clamp, (4) intra-arterial fluconazole, and (5) a combination of oral aspirin, fluconazole, and nitric oxide clamp. L-NMMA indicates L-N(G)-monomethyl arginine citrate.

cholesterol, and blood glucose levels. Analysis was performed by the local clinical biochemistry and hematology reference laboratories.

Data Analysis and Statistics

Forearm blood flow data were analyzed as described previously.¹³ A normal distribution of the data was demonstrated using the D'Agostino & Pearson omnibus normality test. All variables are reported as mean±SEM using repeated-measures analysis of variance (ANOVA) with post hoc Bonferroni corrections and a 2-tailed Student *t* test as appropriate (Graph-Pad Prism, GraphPad Software, San Diego, CA). Significance was taken as 2-sided $P < 0.05$.

Results

Study Participants

All volunteers were young healthy men (23 ± 4 years). Both urocortin 2 and urocortin 3 produced marked localized flushing in the infused arm along with facial flushing at the highest doses. Volunteers also experienced heightened awareness of their heartbeat during and immediately after the highest dose of urocortin 3 (36 nmol/min). All symptoms were self-limiting, well tolerated, and short-lived. Substance P also induced localized flushing of the infused forearm, which was self-limiting. There were no clinically significant changes in the standard hematological and biochemical analytes including full blood count, blood glucose, cholesterol, and renal and hepatic function throughout the study (data on file). Capillary blood glucose and body temperature (tympenic) remained unchanged during all doses of urocortin 3 (data on file).

Systolic blood pressure and noninfused forearm blood flow remained unchanged at all doses with all 3 peptides across both protocols. However, at the highest infused dose of urocortin 3 (36 nmol/min), there was a sinus tachycardia ($+22 \pm 2$ beats/min; ANOVA, $P < 0.0001$) with an associated drop in diastolic blood pressure (-8.5 ± 0.8 mm Hg; ANOVA $P = 0.004$; Figure 2) that was not seen with either substance P or urocortin 2 infusions.

Pharmacodynamic Effects of Urocortins 2 and 3

Both urocortin 2 and urocortin 3 evoked dose-dependent arterial vasodilatation in the infused arm (2-way ANOVA, $P < 0.0001$) (Figure 3).

Maximal vasodilatation with urocortin 2 was apparent ≈ 10 minutes after cessation of the 120 pmol/min infusion (paired Student *t* test, Ucn 2 120 pmol/min versus +10-minute washout; $P = 0.04$), and thereafter the blood flow

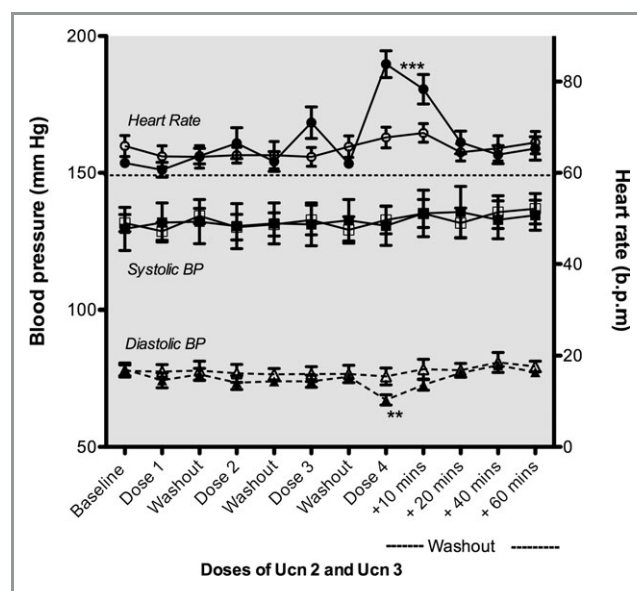


Figure 2. Hemodynamic responses to intra-arterial infusion of urocortin 2 (Ucn 2; 3.6 to 120 pmol/min) and urocortin 3 (Ucn 3; 1.2 to 36 nmol/min). At a dose of 36 nmol/min, Ucn 3 evoked transient tachycardia associated with a drop in diastolic blood pressure. Open symbols, Ucn 2; closed symbols, Ucn 3; circle, heart rate; square, systolic blood pressure (BP); triangle, diastolic BP; *** $P < 0.0001$; ** $P = 0.004$; dose 1=3.6 pmol/min Ucn 2 or 1.2 nmol/min Ucn 3; dose 2=12 pmol/min Ucn 2 or 3.6 nmol/min Ucn 3; dose 3=36 pmol/min Ucn 2 or 12 nmol/min Ucn 3; dose 4=120 pmol/min Ucn 2 or 36 nmol/min Ucn 3; bpm indicates beats per minute.

gradually returned toward baseline. This was in contrast to the effect seen with urocortin 3, for which the maximum vasodilatory response was immediate (Figure 4A). The offset of vasodilatation was prolonged with both peptides, although urocortin 2 took longer than urocortin 3 to return to baseline (Figure 4A).

The vasodilator effects of both peptides appeared to be reproducible within a day, with no evidence of tachyphylaxis (2-way ANOVA, $P > 0.05$ for all; Figure 4B).

Endogenous Fibrinolytic Factors

Preliminary data showed no effect of urocortin 2 or urocortin 3 on endothelial release of tissue plasminogen activator and plasminogen activator inhibitor-1 (data on file).

Mechanism of Vasodilatation

Baseline forearm arterial blood flow was unaffected by oral aspirin or intra-arterial fluconazole, and the coinfusion of SNP restored baseline blood flow during L-NMMA administration (2-way ANOVA, $P > 0.05$ for all). Inhibition of nitric oxide synthase reduced arterial vasodilatation to substance P and urocortin 2 (2-way ANOVA, $P \leq 0.001$ for both) but had no

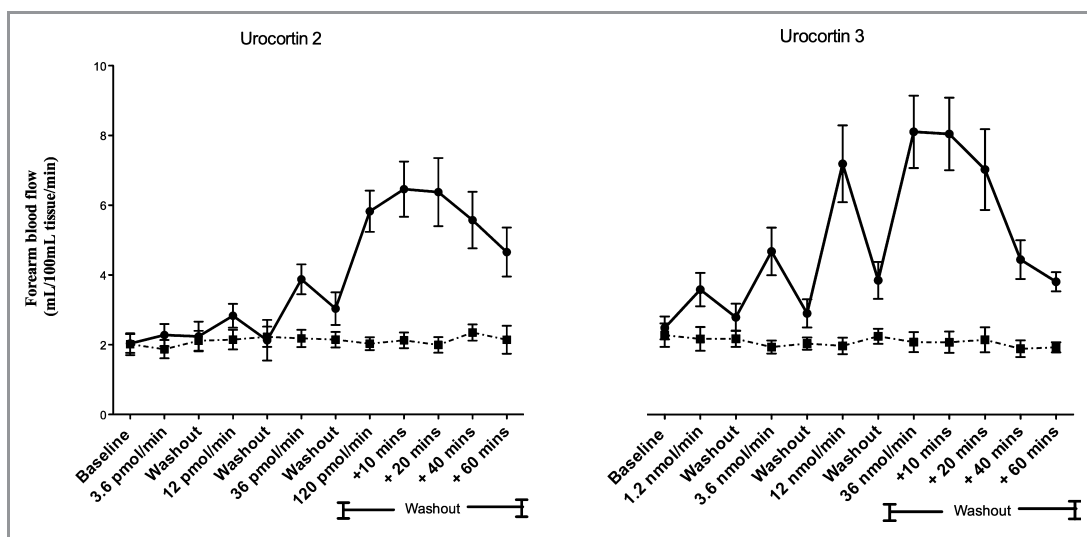


Figure 3. Forearm arterial blood flow responses to increasing doses of urocortin 2 (Ucn 2) and urocortin 3 (Ucn 3). Circle, infused forearm blood flow; square, noninfused forearm blood flow. $P < 0.0001$ at all doses.

apparent effect on urocortin 3–induced vasodilatation (2-way ANOVA, $P = 0.36$). Neither inhibition of cyclo-oxygenase with aspirin nor cytochrome P450 metabolites of arachidonic acid with fluconazole affected the vasodilatation induced by the urocortins or substance P (2-way ANOVA, $P > 0.05$ for all; data on file). In the presence of all 3 inhibitors, substance P–, urocortin 2–, and urocortin 3–induced vasodilatation was further attenuated (2-way ANOVA, $P < 0.001$ for all) but not completely abolished. Combined inhibition of cyclo-oxygenase, nitric oxide synthase, and cytochrome P450 metabolites of arachidonic acid produced a greater reduction in vasodilatation than the nitric oxide clamp alone (2-way ANOVA, $P \leq 0.005$ for urocortins 2 and 3; Figure 5).

Discussion

This study represents the first administration in humans of urocortin 3 and demonstrates that both urocortin 2 and urocortin 3 directly evoke potent and prolonged arterial vasodilatation that is, at least in part, mediated by the endothelium. These findings are of direct relevance not only to our understanding of human cardiovascular physiology but also inform the development of therapies targeting the urocortin system for the treatment of conditions such as heart failure.

The forearm arterial vasodilator effects of urocortin 2 and urocortin 3 are consistent with data from *in vitro*^{3,15–18} and preclinical^{19,20} animal studies. However, in contrast with existing preclinical data, we observed a more marked difference in potency between the 2 peptides. Although preclinical studies have suggested urocortin 2 is 10-fold more potent,^{21,22} Wiley et al³ showed equipotency of urocortins 2

and 3 in isolated human internal mammary arterial segments. In contrast, here we observed that a 300-fold-higher dose of urocortin 3 was required to evoke comparable vasomotor effects in human forearm arterial circulation. This discrepancy underlines the importance of a direct head-to-head assessment *in vivo* in humans, without which the extrapolation of preclinical data may be deceptive.

Urocortins 2 and 3 are specific agonists at the G-protein-coupled CRH-R2 receptors, mediating their effects through a cascade of intracellular signaling pathways including adenylyl cyclase, cyclic adenosine monophosphate,^{23,24} and mitogen-activated protein kinases.²⁵ Other well-characterized G-protein-coupled receptor agonists such as bradykinin,^{26,27} substance P,²⁸ and protease activated receptor type 1 activating peptide^{29,30} evoke vasodilatation with rapid onset and offset in human forearm arterial circulation. Unlike these agonists, the maximal vasodilator effect evoked by urocortin 2 in this study was apparent ≈ 10 minutes following completion of the highest dose (protocol 1a). In addition to a late maximal response with urocortin 2, we also observed a prolonged offset. Even 100 minutes post–urocortin 2 administration, infused forearm blood flow remained elevated. Although not as lengthy as the effect evoked by urocortin 2, urocortin 3–evoked vasodilatation was also prolonged and took 1 hour for blood flow to return to baseline after discontinuation of the infusion. This prolonged offset of effect is unusual for G-protein-coupled receptor agonists, although a similar time course has been observed in response to apelin³¹ and vasopressin,³² and is thought to be the result of prolonged receptor occupancy. *In vitro* studies by Hoare et al³³ have demonstrated differing affinities of urocortin 2 and urocortin 3 to the CRF-R2 receptor determined by the affinity of the

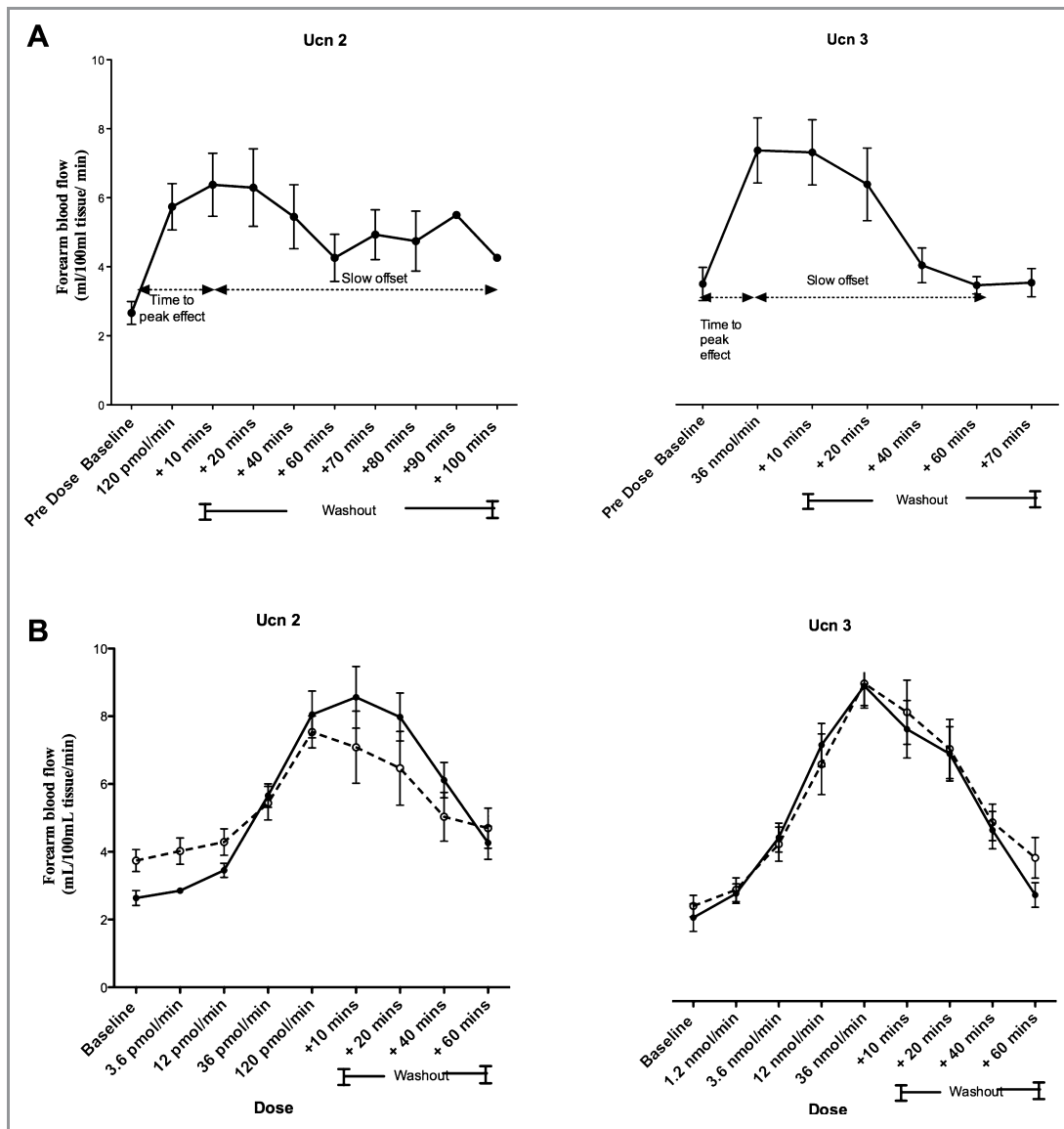


Figure 4. Pharmacodynamics of urocortin 2 (Ucn 2) and urocortin 3 (Ucn 3). A, Onset and offset of vasodilatory effect of Ucn 2 (left) and Ucn 3 (right) after infusion of highest dose. B, Within-day reproducibility of Ucn 2 (left) and Ucn 3 (right); P =nonsignificant, first dose response vs second dose response; Ucn 2 and Ucn 3. Closed circle: first dose response; open circle, second dose response.

extracellular domains of the CRF receptors to these agonists. It remains to be established whether urocortin 2 induces receptor transformation, thereby promoting prolonged binding to CRH-R2 and a delayed maximal response. However, when assessed in isolation, it is clear that the direct vasomotor effects of urocortin 2 and urocortin 3 are more prolonged than previously reported.

The vasodilator effects of both peptides showed good within-day reproducibility without evidence of tachyphylaxis. These are important properties, especially for potential applications in extended or chronic therapies in which predictable and reproducible pharmacologic and hemodynamic effects are needed.

Several mechanisms have been proposed to explain the mechanistic pathways of urocortin-mediated vasorelaxation. Studies to date have suggested that the mechanism involved may depend on the species or vascular bed in question. In rats, both endothelium-dependent^{17,34} and -independent components were implicated.³⁵ Grossini et al³⁶ demonstrated that urocortin 2-mediated vasorelaxation in the coronary arteries of anaesthetized pigs was mediated by nitric oxide. However, urocortin-mediated vasodilatation appeared to be independent of endothelial integrity in isolated human coronary and internal mammary artery segments.^{3,16} In the current study, the NO clamp appeared to cause modest inhibition of urocortin 2-mediated vasodilatation and

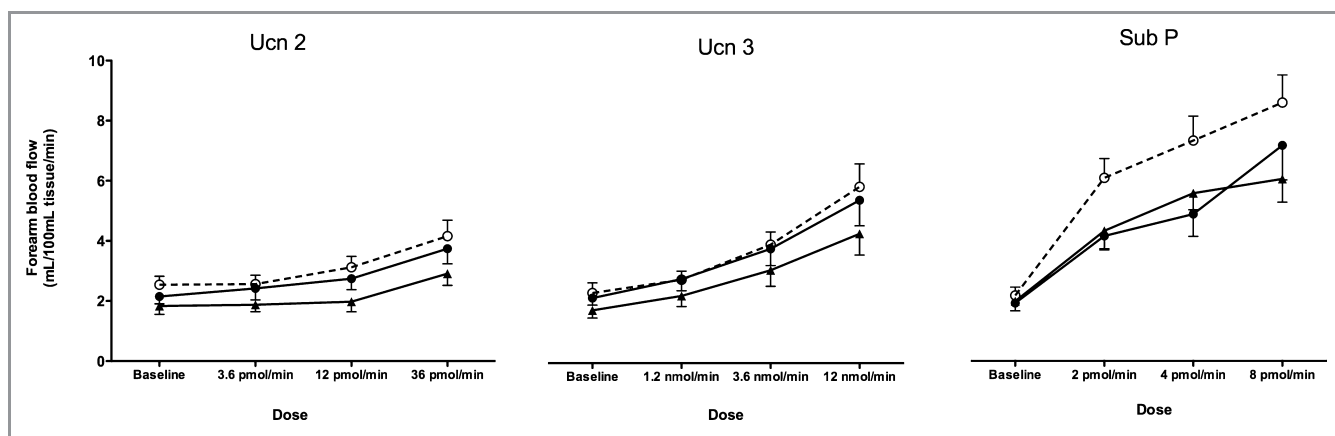


Figure 5. Vasomotor effects of inhibition of endothelial nitric oxide synthase, cyclooxygenase, and cytochrome P450 metabolites of arachidonic acid on urocortin 2-, urocortin 3-, and substance P-mediated vasodilatation. Open circle, placebo; closed circle, nitric oxide clamp; closed triangle, combined aspirin (600 mg), nitric oxide clamp, and fluconazole (1.2 μ mol/min).

appeared to be marginally more pronounced with urocortin 2 compared with urocortin 3. Inhibition of cytochrome P450 metabolites of arachidonic acid with fluconazole alone did not have an appreciable effect on urocortin-mediated vasodilatation, but its addition enhanced the inhibitory effect of the NO clamp. This suggests that endogenous nitric oxide and cytochrome P450 metabolites of arachidonic acid may have a close interrelationship and can compensate for one another to maintain vascular tone. A similar effect has previously been described for endothelium-derived hyperpolarizing factor (EDHF), whereby its relative importance increases in the face of impaired NO bioavailability under conditions of oxidative stress. Although urocortin 2- and urocortin 3-mediated vasodilatation was inhibited by the combination of all 3 inhibitors, it was not abolished, and a substantial degree of vasomotor activity remained. We cannot exclude a contribution from other endothelial pathways such as residual “fluconazole-insensitive” EDHF, although the results do suggest a substantial contribution from direct smooth muscle activation. Although the role of the endothelium is substantial, a large contribution from endothelium-independent vasodilator mechanisms may be seen as an advantage in the potential therapeutic applications of CRH-R2 agonism. Arguably, this would allow a more predictable response from the manipulation of urocortin (2 or 3) as a therapy for cardiovascular conditions, the vast majority of which would be expected to be associated with impaired endothelial homeostatic mechanisms but preserved smooth muscle activity.

This study was designed to assess locally active, sub-systemic doses of urocortins 2 and 3 in forearm arterial vasculature. Indeed, the forearm venous occlusion plethysmography model allows these local effects to be studied at doses 10- to 100-fold lower than that usually expected or required to elicit a systemic response. It would appear,

however, that there was a degree of systemic overspill associated with the top dose of urocortin 3 tested. The observed decrease in diastolic blood pressure and increase in heart rate observed during and after the infusion of the top dose of urocortin 3 is notable, particularly as it occurred in the absence of any change in blood flow in the contralateral noninfused forearm. Usually, in the case of systemic overspill, a corresponding change in the “control” noninfused arm would be observed,²⁸ but this was not the case with the infusion of the dose of urocortin 3. We therefore hypothesize that the hypotensive effect observed reflects vasodilatation in another, more sensitive vascular bed, such as splanchnic circulation, with consequent reflex tachycardia. Absence of a similar hemodynamic response to the highest dose of urocortin 2 may be the result of lower sub-systemic doses of the less potent peptide urocortin subtype or, conceivably, a differential in the relative sensitivity of splanchnic and forearm arterial circulation to urocortin 2. However, systemic doses up to 100 μ g of urocortin 2 have been shown to increase heart rate and decrease diastolic blood pressures in healthy adults.⁷

Study Limitations

The size of our study population was relatively small. However, we have previously described the influence of a range of factors on blood flow in forearm vasculature using sample sizes of ≤ 12 subjects^{28–31,37–39} As with most other physiological studies, we have not performed multiple testing correction for the data obtained.

We studied the changes in forearm blood flow during brief local intrabrachial infusions of urocortins 2 and 3. The apparent differences between urocortin 2 and urocortin 3 observed in our study may reflect, in part, differences between the 2 peptides in the tissue exposure achieved.

We do not as yet know the effects of prolonged infusions of these peptides. Moreover, the effects of urocortins 2 and 3 in patients with heart failure remain to be explored. Although there is good concordance between the vasomotor responses observed in the forearm resistance vessels and other vascular beds,⁴⁰ further studies will be required to explore their systemic effects in health and disease in humans.

Conclusions

We have demonstrated that urocortin 2 and urocortin 3 evoke potent prolonged arterial vasodilatation and that their effects are at least partly dependent on endothelial nitric oxide and cytochrome P450 metabolites of arachidonic acid. These data provide important insights into human cardiovascular physiology, and they will inform the development of further therapies directed toward the urocortin pathway. The in vivo role of this endogenous peptide system in patients with heart failure and the role of CRH-R2 in human health and disease remain to be explored.

Acknowledgments

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Disclosures

None.

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Cardiovascular Effects of a Novel SIRT1 Activator, SRT2104, in Otherwise Healthy Cigarette Smokers

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Background—We examined the effect of the oral SIRT1 activator SRT2104 on cardiovascular function in otherwise healthy cigarette smokers.

Methods and Results—Twenty-four otherwise healthy cigarette smokers participated in a randomized double-blind, placebo-controlled crossover trial and received 28 days of oral SRT2104 (2.0 g/day) or matched placebo. Plasma SRT2104 concentrations, serum lipid profile, plasma fibrinolytic factors, and markers of platelet and monocyte activation were measured at baseline and at the end of each treatment period together with an assessment of forearm blood flow during intra-arterial bradykinin, acetylcholine, and sodium nitroprusside infusions. Three hours postdose, mean plasma SRT2104 concentration was 1328 ± 748 ng/mL after 28 days of active treatment. Compared with placebo, serum lipid profile improved during SRT2104 administration, with reductions in serum total cholesterol (-11.6 ± 20 versus 6 ± 21 mg/dL), low-density lipoprotein cholesterol (-10 ± 17 versus 3 ± 21 mg/dL), and triglyceride (-39.8 ± 77 versus 13.3 ± 57 mg/dL) concentrations ($P < 0.05$ for all). All vasodilators produced a dose-dependent increase in blood flow ($P < 0.0001$) that was similar during each treatment period ($P > 0.05$ for all). No significant differences in fibrinolytic or blood flow parameters were observed between placebo and SRT2104.

Conclusions—SRT2104 appears to be safe and well tolerated and associated with an improved lipid profile without demonstrable differences in vascular or platelet function in otherwise healthy cigarette smokers.

Clinical Trial Registration—<http://www.clinicaltrials.gov>. Unique identifier: NCT01031108. (*J Am Heart Assoc.* 2013;2:e000042 doi: 10.1161/JAHA.113.000042)

Key Words: cigarette smokers • endothelium • forearm plethysmography • platelet activation • SIRT1 • sirtuins • vascular

Originally identified in yeast, sirtuins represent a class of highly conserved nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylases that have 7 identified members in mammalian species.^{1,2} They have been implicated in the beneficial effects of calorie restriction on longevity in several species and are promising drug targets for a variety of diseases of aging.³ Sirtuin (silent mating type information regulation 2 homolog) 1 (SIRT1) is the best-known

member of this class of proteins and is expressed broadly in multiple tissues and highly expressed in the vascular endothelium.⁴ SIRT1 inhibition is associated with vascular dysfunction and arterial thrombosis⁵ as well as alterations in fibrinolysis.⁶ Conversely, SIRT1 activation is associated with improved endothelial function,⁷ enhanced lipid metabolism,⁸ and inhibition of atherogenesis.⁹

Smoking tobacco remains one of the most important and consistent modifiable risk factors for coronary heart disease and is associated with an up to 7-fold increased risk of nonfatal myocardial infarction.¹⁰ It is associated with both accelerated atherosclerosis¹¹ and a propensity for acute coronary thrombosis.^{12,13} This is mediated through a variety of mechanisms including alterations in vascular, endothelial, fibrinolytic, and platelet function.^{14–17} The precise cellular mechanism for these effects is as yet unknown, but cigarette smoke is associated with oxidative stress, endothelial nitric oxide synthase acetylation, and increased endothelial cell senescence that has been attributed to reduced SIRT1 levels.⁴

To date, there have been few clinical studies to assess the effect of SIRT1 activation in vivo in humans. Therefore, the

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aim of the present study was to examine the in vivo effects of a novel oral SIRT1 activator, SRT2104, on the lipid profile and vascular, endothelial, and platelet function in otherwise healthy cigarette smokers. We hypothesized that SIRT1 activation could improve the cardiovascular risk profile and reverse or improve the vascular and endothelial dysfunction associated with cigarette smoking.

Methods

The study was approved by the Research Ethics Committee, was given Clinical Trial Authorization by the Medicines and Healthcare products Regulatory Authority (MHRA), and carried out at the MHRA Phase 1 accredited Wellcome Trust Clinical Research Facility at the Royal Infirmary of Edinburgh, United Kingdom, between June 2010 and September 2011. Written informed consent was obtained from each volunteer, and the study was carried out in accordance with the Declaration of Helsinki.

Study Participants

Twenty-four otherwise healthy male and female volunteers aged between 18 and 70 years who smoked ≥ 10 cigarettes daily for at least 1 year were eligible for the study. Exclusion criteria included the presence of significant comorbidities, chronic illness, renal or liver impairment, history of gastrointestinal diseases or surgeries influencing drug absorption, history of alcoholism, history of neoplastic disease within the last 5 years, a positive urinary test for recreational drugs, pregnancy, and participation in other clinical trials or blood

donation within the last 3 months. Eligibility of participants including absence of relevant medical history was confirmed through a standardized form completed by the registered general practitioners after informed consent. Tests for pregnancy (serum human chorionic gonadotrophin [HCG] concentrations at screening and urinary HCG concentrations at study visits) were conducted on all female participants of child-bearing potential.

Study Design

This was a prospective double-blind, randomized, placebo-controlled crossover study (1:1 SRT2104:placebo). Subjects were randomized to receive 2.0 g daily of oral SRT2104 or matched placebo (Sirtris Pharmaceuticals Inc) for a 28-day period, followed by crossover to the alternate study arm for another 28 days, giving a total dosing duration of 56 days. An end-of-study visit was conducted on day 70, with a phone call follow-up on day 86. Assessment of drug safety, tolerability, and efficacy on vascular function was carried out at baseline and during and at the end of each treatment period (Figure 1).

Vascular Studies

Vascular studies were undertaken before and at the end of each 28-day trial period. All studies were performed with the patient lying supine in a quiet temperature-controlled (22°C to 25°C) room. Participants were fasted and asked to refrain from smoking for 10 hours before the study and to avoid caffeine and alcohol for 24 hours before the study. Venous

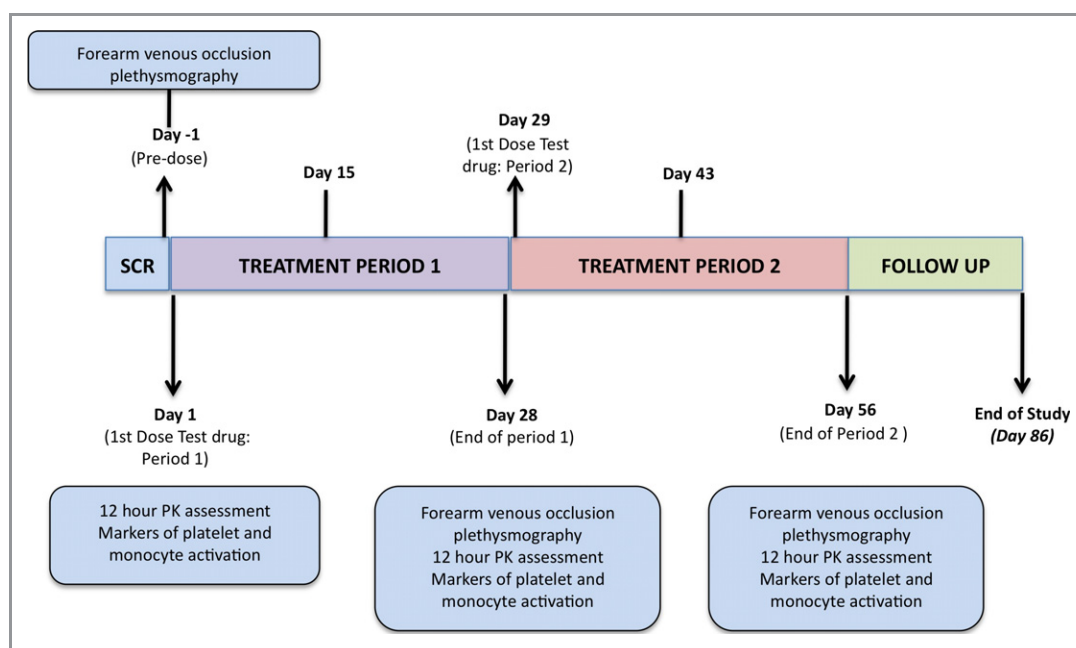


Figure 1. Study design: schematic representation of study design. PK indicates pharmacokinetic; SCR, screening.

cannulas (17G) were inserted into large subcutaneous veins in the antecubital fossae of both arms at the start of the study to facilitate periodic venous sampling. Supine heart rate and blood pressure were monitored at intervals throughout the study using a semiautomated noninvasive oscillometric sphygmomanometer (Omron 705 IT).

Forearm Venous Occlusion Plethysmography

Forearm blood flow was measured in the infused and noninfused forearms using forearm venous occlusion plethysmography as described previously.¹⁷ Subjects underwent brachial artery cannulation in the nondominant forearm with a 27 standard-wire-gauge steel needle. After a 20-minute baseline infusion with 0.9% saline, incremental intra-arterial doses of bradykinin (American Peptide Co) at 100, 300, and 1000 pmol/min (an endothelium-dependent vasodilator that evokes tissue plasminogen activator [t-PA] release); acetylcholine (Chem. Pharm Fabrik GmbH) at 5, 10, and 20 $\mu\text{g}/\text{min}$ (an endothelium-dependent vasodilator that does not evoke t-PA release); and sodium nitroprusside (Hospira Inc) at 2, 4, and 8 $\mu\text{g}/\text{min}$ (an endothelium-independent vasodilator that does not evoke t-PA release) were infused for 6 minutes at each dose, with a 30-minute 0.9% saline washout infusion between drugs. The order of drugs was randomized between subjects but kept constant for each subject across the 3 visits.

Blood Sampling

Paired venous blood samples were obtained from each forearm before and during the infusion of intra-arterial bradykinin. Samples were collected into acidified buffered citrate (Stabilyte; Trinity Biotech Plc) and citrate (BD Vacutainer; BD UK Ltd) for determination of t-PA and plasminogen-activator inhibitor type 1 (PAI-1) concentrations, respectively. Samples were placed on ice before centrifuging at 2000g for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at -80°C before further analysis. Venous blood samples were collected into EDTA at the beginning and end of the vascular study to determine hematocrit.

Plasma t-PA antigen and activity (t-PA Combi Actibind t-PA ELISA kit; Technoclone, Vienna, Austria) and PAI-1 antigen and activity (Elitest PAI-1 Antigen and Zymutest PAI-1 Activity; Hyphen Biomed) concentrations were determined by enzyme-linked immunosorbent assays (ELISAs).

Platelet and Monocyte Activation

Flow-cytometric measurements of platelet-monocyte aggregation (PMA) and platelet surface expression of P-selectin and monocyte CD11b expression (Mac-1/CD11b) were performed at baseline and at the end of each treatment

period as described previously.^{16,18,19} Briefly, peripheral venous blood was drawn from a large antecubital vein and anticoagulated with the direct thrombin inhibitor D-phenylalanyl-L-arginine chloromethyl ketone (75 $\mu\text{mol}/\text{L}$ PPACK; Cambridge Biosciences) and immunolabeled within 5 minutes of phlebotomy for subsequent flow cytometric analysis. Directly conjugated monoclonal antibodies were obtained from DakoCytomation and Serotec. Samples were stained with the following conjugated monoclonal antibodies: phycoerythrin (PE)-conjugated CD14, PE-conjugated CD62p, PE-conjugated CD11b, fluorescein isothiocyanate (FITC)-conjugated 42a, and FITC-conjugated CD14 and appropriate control isotypes. Once stained, samples were incubated for 20 minutes at room temperature before being fixed with FACS-Lyse (Becton-Dickinson). All samples were analyzed using a FACS Calibur flow cytometer using CellQuestPro software (Becton-Dickinson).

Venous blood was collected in citrate at baseline and after each dosing period to assess plasma-soluble CD40 ligand (sCD40L) concentrations. Blood was centrifuged at 1500g for 15 minutes at 4°C, and plasma was decanted and stored at -80°C for further analysis by ELISA (Bender Medsystems).

Safety and Pharmacokinetic Analyses

Venous blood samples were collected biweekly to measure hematological and biochemical analytes including full blood count, coagulation profile, liver and renal function, creatine phosphokinase, lactate dehydrogenase, lipid profile°C and free fatty acids. Analyses were conducted by the regional clinical hematology and biochemistry reference laboratories using an automated hematology analyzer (XE2100, Sysmex Corporation and ACL TOP, Instrumentation Laboratory), an automated chemistry analyzer using colorimetric, kinetic and enzymatic ultraviolet and color assays (AU2700/AU640 analyzers, Beckman & Coulter), ion-selective electrodes (sodium, potassium, and chloride assays) and 2-point and multiple-point rate assays (Ortho Clinical Vitros 250 analyzer).

Venous blood samples were taken into prelabeled heparinized sodium tubes for pharmacokinetic assessment of plasma SRT2104 concentrations (Simbec Laboratories Limited). Serial blood samples were collected on days 1, 28, and 56 immediately before (0 minutes) and 15, 30, 60, 120, 180, 240, 480, 720 and 1440 minutes following study medication. Plasma was separated by centrifugation of whole blood at 1500g at 4°C for 15 minutes, and decanted and stored at -80°C until analyzed.

Methodology of SRT2104 Analysis

Plasma concentrations of SRT2104 were measured using liquid chromatography with tandem mass spectrometry

detection in positive ion mode. High-pressure liquid chromatography was performed using Betasil silica–100 columns using a Phenomenex C18 guard column.²²

Data Analysis and Statistics

Plethysmographic data were analyzed as described previously.²¹ Estimated net release of t-PA and PAI-1 antigen and activity was defined as the product of forearm plasma flow (based on blood flow and hematocrit) and the difference in plasma antigen (or activity) concentrations between the 2 forearms. On the basis of previous power calculations,²⁰ a sample size of 20 gives 80% power to detect a change in net t-PA antigen release of 27.0 ng/100 mL of tissue per minute, assuming a standard deviation of 40.0 and a 2-sided $P < 0.05$ (paired t test). To account for a 20% dropout rate, we recruited 24 subjects.

Fibrinolysis and forearm blood flow data were analyzed using a linear mixed-model repeated-measures analysis of covariance. Treatment differences were investigated in a model adjusting for period, treatment by period, vasodilator dose, treatment by vasodilator dose, and vasodilator dose by period using SAS for UNIX (version 9.1.3 or higher; SAS Institute). Values for these parameters are expressed as model adjusted (least square means) and 95% confidence intervals. Between-day reproducibility of forearm venous occlusion plethysmography data was assessed using the Bland–Altman method, and coefficient of reproducibility was determined for 95% confidence intervals using the Student t distribution. All other values are expressed as mean \pm SD.

Results

Study Participants

Volunteers had a mean age of 38 ± 13 years (median, 37 years) and relatively equal sex distribution (58% male) and were normotensive without any significant coexisting medical conditions. Volunteers had a body mass index of 25 ± 4 kg/m² and a mean cigarette consumption of 17 ± 6 cigarettes per day over 21 ± 14 years. The mean urinary cotinine concentration at screening was 1352 ± 950 ng/mL. All 24 volunteers completed all study visits. Before drug administration, 1 subject was withdrawn from the study because of problems with venous access and was replaced.

Pharmacokinetics, Tolerability, and Safety

Three hours postdose, mean plasma SRT2104 concentration was 1328 ± 748 ng/mL after 28 days of active treatment (Figure 2). The median plasma SRT2104 concentration after 28 days of treatment was 366 ng/mL (IQR, 940 ng/mL). The median time at which the maximum plasma concentration

was observed (T_{max}) on day 28 of dosing was 3.05 hours, which coincided well with study measurements performed on those days (2 to 4 hours postdose). The geometric mean area under the curve ($AUC_{0-\tau}$) was 6412 h \cdot ng/mL. Consistent with previous observations,²² there was substantial intersubject variability in exposure during this study.

All subjects tolerated study medication well. Commonly reported side effects included headache (25%) and rhinitis, nasopharyngitis, and respiratory tract symptoms (17%) (Table 1). The reported adverse events were mild in intensity and resolved without any intervention or sequelae. There were no meaningful differences in the number of events between active treatment and placebo. There was only 1 reported serious adverse event in the study (SRT2104 arm): a traumatic facial bone fracture that was considered unrelated to SRT2104.

Blood pressure and heart rate remained unchanged throughout the study. There were no effects on cardiac rhythm or the 12-lead electrocardiogram, and specifically there were no effects on the corrected or uncorrected QT intervals. There were no clinically significant adverse effects involving any of the clinical hematological or biochemical analytes.

Lipid Profile

Treatment with SRT2104 had a favorable effect on the lipid profile. A statistically significant period effect was observed in the analysis of total and low-density lipoprotein (LDL) cholesterol concentrations. Baseline values were higher in subjects receiving placebo in the first period. Regardless of treatment arm, the level of change from baseline was greater in period 2 for total and LDL cholesterol and less in period 2 for triglycerides. Adjusted summaries combined over period are presented in Table 2. There was a reduction in total and LDL cholesterol as well as triglyceride concentrations. There

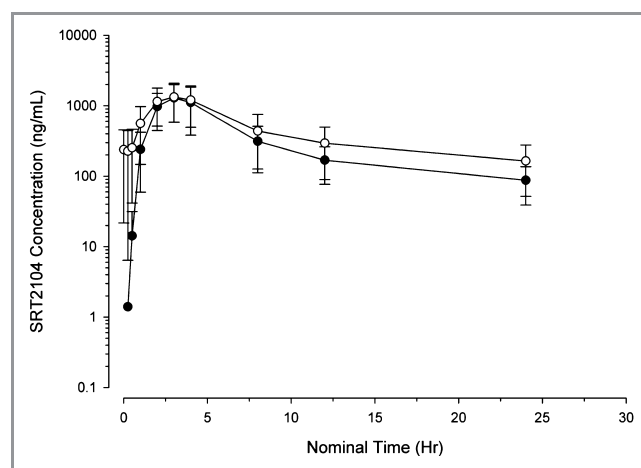


Figure 2. Pharmacokinetics: mean plasma concentration–time curve following oral dosing of SRT2104 on day 1 (closed circles) and day 28 (open circles). Data presented as mean \pm SD.

Table 1. List of Adverse and Serious Adverse Events

System	Symptom (AE/SAE)	Number of Events	
		Placebo (n=24)	SRT2104 (n=24)
Any event	—	18	18
Nervous system disorders	Headache	4	6
	Paresthesia	0	2
	Hypoesthesia	1	2
	Carpal tunnel syndrome	0	1
	Presyncope	1	1
	Burning sensation	0	1
	Sciatica	0	1
Respiratory, thoracic, and mediastinal disorders	Oropharyngeal pain	1	2
	Rhinorrhea	0	1
Gastrointestinal disorders	Flatulence	1	0
	Mouth ulceration	0	1
	Abdominal pain upper	1	0
	Hemorrhoids	1	0
Reproductive system and breast disorders	Dysmenorrhea	3	1
Musculoskeletal and connective tissue disorders	Back pain	2	0
	Musculoskeletal chest pain	1	0
	Musculoskeletal pain	1	0
	Myalgia	0	1
Investigational	Blood bilirubin increased	1	1
	Creatinine phosphokinase increased	1	0
	LDH increased	1	0
	AST increased	1	0
General disorders and administration site conditions	Influenza-like illness	1	0
	Fatigue	1	0
	Catheter site pain	0	1
	Catheter site rash	1	0
	Catheter site-related reaction	1	0
	Catheter site swelling	1	0
	Edema peripheral	0	1
	Pyrexia	0	1
Swelling	0	1	
Infections and infestations	Nasopharyngitis	0	1
	Rhinitis	2	2
	Upper respiratory tract infection	1	0
	Oral herpes	0	1
	Respiratory tract infection	0	1
Injury, poisoning, and procedural complications	Contusion	1	0
	Excoriation	1	1
	Arthropod bite	1	0
	Facial bones fracture*	0	1
	Laceration	1	0

Continued

Table 1. Continued

System	Symptom (AE/SAE)	Number of Events	
		Placebo (n=24)	SRT2104 (n=24)
Skin and subcutaneous tissue	Rash	1	0
Metabolism and nutrition disorders	Decreased appetite	0	1
Vascular disorders	Phlebitis	0	1
Eye disorders	Eye pain	1	0
	Vision blurred	0	1
Ear and labyrinthine disorders	Motion sickness	0	1
Surgical and medical procedures	Vasectomy	1	0

*Serious Adverse Event.

AE indicates adverse event; SAE, serious adverse event; LDH, lactate dehydrogenase; AST, aspartate aminotransferase.

Table 2. Effect of SRT2104 on Serum Lipid Concentrations

	Placebo (n=22)	SRT2104 (n=20)
Total cholesterol (mean±SD), mg/dL		
Baseline	174±54	176±50
Day 28/56	180±51	164±47
Change from baseline	6±21	-12±20*
HDL cholesterol, mg/dL		
Baseline	46±11	47±10
Day 28/56	50±13	51±16
Change from baseline	3±7	4±7
LDL cholesterol, mg/dL		
Baseline	99±41	99±37
Day 28/56	102±37	88±34
Change from baseline	3±21	-10±17*
Triglycerides, mg/dL		
Baseline	133±110	140±114
Day 28/56	146±149	100±67
Change from baseline	13±57	-40±77*

* $P<0.05$.

HDL indicates high-density lipoprotein; LDL, low-density lipoprotein.

was no effect on high-density lipoprotein concentrations, and the 7% fall in total cholesterol was attributable to the 11% fall in LDL cholesterol concentrations.

Vasomotor Function

Noninfused forearm blood flow remained unchanged throughout all assessment periods, as were the predose measurements of blood flow in the infused arm between visits ($P>0.05$). There was a dose-dependent increase in the infused forearm blood flow with all 3 agonists (acetylcholine,

bradykinin, and sodium nitroprusside) in the presence of either SRT2104 or placebo ($P<0.0001$ for all 3 agonists; Figure 3). There were no significant differences in response to either endothelium-dependent or -independent vasodilators in the presence of SRT2104 compared with placebo (bradykinin, $P=0.1169$; acetylcholine, $P=0.1683$; sodium nitroprusside, $P=0.9039$: placebo versus SRT2104). There were no differences in forearm vasodilatation between the baseline and placebo visits of the study for all 3 agonists ($P=0.5649$, $P=0.4009$, and $P=0.2908$ for bradykinin, acetylcholine, and sodium nitroprusside, respectively), confirming the good reproducibility of the measurements (Table 3).

Endogenous Fibrinolysis and Monocyte and Platelet Activation

There was a dose-dependent increase in bradykinin-evoked net t-PA antigen and activity release ($P<0.0001$ for both) in the infused arm that was unaffected by SRT2104 ($P=0.3691$ and $P=0.1377$, placebo versus SRT2104, for net t-PA antigen and activity, respectively; Table 4). Plasma plasminogen activator inhibitor-1 (PAI-1) activity decreased with time during all study visits ($P<0.05$), consistent with its circadian variation and t-PA release. Plasma PAI-1 antigen and activity concentrations were similar in both treatment arms ($P=0.8877$ and $P=0.6635$, placebo versus SRT2104, for plasma PAI antigen and activity, respectively).

SRT2104 had no effect on markers of in vivo platelet or monocyte activation (Figure 4).

Discussion

In this randomized, double-blind, placebo-controlled crossover trial of otherwise healthy cigarette smokers, we have demonstrated that oral SRT2104 is safe and well tolerated at a dose of 2.0 g daily. Importantly, we have shown that

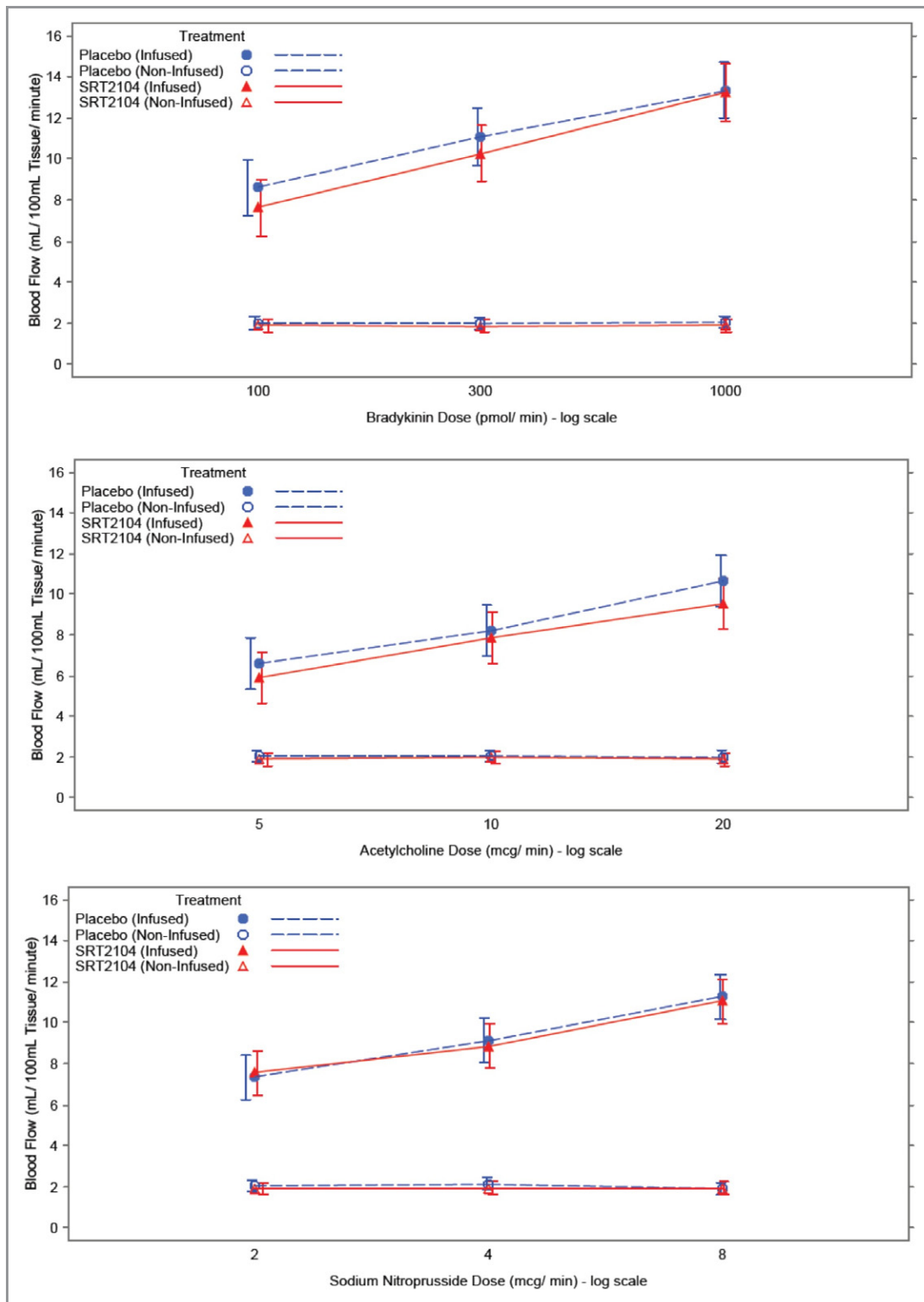


Figure 3. Effect of bradykinin (100, 300, 1000 pmol/min), acetylcholine (5, 10, 20 $\mu\text{g}/\text{min}$), and sodium nitroprusside (2, 4, 8 $\mu\text{g}/\text{min}$) on absolute forearm blood flow. Blue, placebo; red, SRT2104; closed circle, infused forearm blood flow; open circle, noninfused forearm blood flow. Data presented as mean \pm 95% confidence interval.

treatment with SRT2104 was associated with an 11% mean reduction in serum LDL cholesterol concentrations, but without demonstrable differences in vasomotor function,

endothelial function, or platelet activation assessments compared with placebo. The favorable effects on lipid profile suggest that SIRT1 activation may have a beneficial role in

Table 3. Between-Day Repeatability of Forearm Blood Flow

Drug	Dose	Mean of Differences in Forearm Blood Flow (mL/100 mL per minute)	Coefficient of Repeatability (mL/100 mL per minute)
Bradykinin, pmol/min	100	−0.3	5
	300	0.5	6
	1000	−0.2	7
Acetylcholine, μg/min	5	−0.1	8
	10	−0.5	8
	20	0.3	7
Sodium nitroprusside, μg/min	2	0.2	5
	4	0.3	6
	8	0.7	6

Between-day reproducibility (baseline vs placebo visit) in absolute forearm blood flow for bradykinin (100, 300, 1000 pmol/min), acetylcholine (5, 10, 20 μg/min), and sodium nitroprusside (2, 4, 8 μg/min).

patients at risk of developing or with established cardiovascular disease.

Elevated serum cholesterol is an established risk factor for atherosclerosis and coronary heart disease. In general, coronary heart disease risk is reduced by 2% to 3% for each 1% decrease in total cholesterol concentrations.²³ We observed a 7% mean reduction in serum total cholesterol and an 11% mean reduction in LDL cholesterol concentrations without affecting serum high-density lipoprotein cholesterol concentrations. The mechanism of this lipid-lowering effect is not entirely clear but is consistent with observations associated with SIRT1 activation in animals. Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is a naturally occurring polyphenolic compound that is believed to confer health benefits through SIRT1 activation.²⁴ Resveratrol has been found to lower plasma triglycerides and cholesterol accumulation in guinea pigs²⁵ and to suppress atherogenic lesion formation in apolipoprotein E-deficient mice.²⁶ Indeed, SRT2104 also lowers triglyceride levels in preclinical murine models of dyslipidemia, diabetes, and obesity as well as improving insulin sensitivity and metabolic function in these animals.²⁷ One mechanism whereby SIRT1 activators such as SRT2104 could improve lipid profiles may involve a positive regulatory effect on liver X receptor proteins (LXRs), nuclear receptors involved in cholesterol and lipid homeostasis. Nuclear receptor LXR is a substrate for SIRT1. Li and colleagues have shown that SIRT1 deacetylates and positively regulates this receptor, potentially altering cholesterol transport and metabolism.²⁸ Although the exact mechanism of the improved lipid profiles seen with SIRT1 activation remains to be determined, our findings would suggest that SIRT1 activation could provide a therapeutic

adjunct to current lipid-lowering strategies, leading to improvements in cardiovascular disease pathophysiology and thus clinical outcomes.

There are currently no published data directly examining the effects of SIRT1 activation on vasomotor function or endogenous fibrinolysis in vivo in humans. Despite the several beneficial effects of SIRT1 activation on endothelial function observed in preclinical in vitro studies,^{6,7,29–31} we were unable to demonstrate improvements in vascular, endothelial, or platelet function in these otherwise healthy smokers. Why was this?

Did we use appropriate and sufficiently sensitive techniques? Forearm venous occlusion plethysmography is a well-established technique that has been used extensively over the years to study human vascular physiology and has been considered a gold standard in the assessment of vascular function in health and disease.³² Using endothelium-dependent (bradykinin and acetylcholine) and -independent (sodium nitroprusside) vasodilators, we observed a dose-dependent increase in forearm arterial vasodilatation with all 3 agonists. Our results are comparable with those reported in previously published studies^{17,33–35} in otherwise healthy cigarette smokers including impaired t-PA release.^{15,36,37} Moreover, our data had low variance and were highly reproducible when we compared the baseline responses with those obtained during placebo administration. Similarly, flow cytometric analysis is considered a sensitive gold standard for measurement of in vivo platelet activation. We have previously shown that in patients with peripheral arterial disease, measurements of platelet–monocyte aggregates are reproducible and consistently reflect other markers of platelet and monocyte activation.³⁸ In the present study, we again report comparable levels of platelet–monocyte aggregation^{19,39} that were reproducible between visits.

There is a body of published data that confirms a strong association between cigarette smoking, endothelial dysfunction, and impaired endogenous fibrinolysis.^{4,14,17,21,40} We were interested to see if this vascular and endothelial dysfunction could be improved or reversed by SIRT1 activation. There could be numerous explanations why we failed to achieve improvement with SRT2104 in these parameters in the current study. One possibility is that the SRT2104 exposure achieved in this study did not lead to adequate or consistent SIRT1 activation, which would be required to reverse the vascular and endothelial dysfunction in these smokers. Unfortunately, there is no current biomarker for SIRT1 activation or the ability to measure SIRT1 activation directly in humans. Therefore, we do not have a good understanding of the pharmacokinetic–pharmacodynamic relationship between SRT2104 drug exposure and SIRT1 activation. Although we were able to demonstrate improved lipid profiles, it is unclear whether the same exposure levels

Table 4. Effect of SRT2104 on Endogenous Fibrinolysis

LS Means±95% CI	Placebo						SRT2104					
	Bradykinin Dose, pmol/min			Bradykinin Dose, pmol/min			Bradykinin Dose, pmol/min			Bradykinin Dose, pmol/min		
	Saline	100	300	1000	300	1000	Saline	100	300	1000	300	1000
Net release t-PA antigen, ng/100 mL tissue per minute	0.5 (-8.6 to 9.7)	4.7 (-4.4 to 13.9)	8.8 (-0.3 to 18.0)	28.8* (19.6, 37.9)	8.8 (-0.3 to 18.0)	28.8* (19.6, 37.9)	-1.0 (-10.9 to 9.0)	5.4 (-4.5 to 15.4)	5.6 (-4.7 to 16.0)	38.3* [†] (28.4 to 48.3)	5.6 (-4.7 to 16.0)	38.3* [†] (28.4 to 48.3)
Net release t-PA activity, ng/100 mL tissue per minute	-0.0 (-4.7 to 4.6)	3.7 (-1.0 to 8.3)	8.6 (3.9 to 13.2)	17.6* (12.9 to 22.2)	8.6 (3.9 to 13.2)	17.6* (12.9 to 22.2)	0.0 (-5.0 to 5.1)	3.4 (-1.7 to 8.4)	6.5 (1.3 to 11.8)	18.6* [†] (13.5 to 23.6)	6.5 (1.3 to 11.8)	18.6* [†] (13.5 to 23.6)
Net PAI-1 antigen, ng/100 mL tissue per minute	-5.7 (-68.2 to 56.8)	—	—	2.9 (-59.6 to 65.5)	—	2.9 (-59.6 to 65.5)	-1.7 (-69.7 to 66.3)	—	—	-86.0 [†] (-154.0 to -18.0)	—	-86.0 [†] (-154.0 to -18.0)
Net PAI-1 activity, LS mean±95% c	-0.0 (-1.2 to 1.2)	—	—	-2.3 (-3.5 to -1.1)	—	-2.3 (-3.5 to -1.1)	0.0 (-1.3 to 1.4)	—	—	3.9 [†] (-5.2 to -2.6)	—	3.9 [†] (-5.2 to -2.6)
Day 28/56												
Net release t-PA antigen, ng/100 mL tissue per minute	-1.05 (-8.0 to 5.9)	3.9 (-3.1 to 10.8)	8.8 (1.7 to 15.9)	22.4 (15.1 to 29.7)*	8.8 (1.7 to 15.9)	22.4 (15.1 to 29.7)*	-0.1 (-6.7 to 6.6)	2.6 (-4.3 to 9.6)	9.3 (2.3 to 16.2)	30.7* [†] (23.6 to 37.8)	9.3 (2.3 to 16.2)	30.7* [†] (23.6 to 37.8)
Net release t-PA activity, ng/100 mL tissue per minute	-0.1 (-3.2 to 3.0)	2.4 (-0.8 to 5.5)	5.8 (2.6 to 9.0)	13.3 (10.1 to 16.6)*	5.8 (2.6 to 9.0)	13.3 (10.1 to 16.6)*	0.0 (-3.0 to 3.0)	2.2 (-0.9 to 5.3)	7.0 (3.8 to 10.1)	18.1* [†] (14.9 to 21.3)	7.0 (3.8 to 10.1)	18.1* [†] (14.9 to 21.3)
Net PAI-1 antigen, ng/100 mL tissue per minute	-4.8 (-52.2 to 42.6)	—	—	-20.6 (-70.4 to 29.3)	—	-20.6 (-70.4 to 29.3)	-0.1 (-45.4 to 45.2)	—	—	-43.7 [†] (-92.4 to 5.1)	—	-43.7 [†] (-92.4 to 5.1)
Net PAI-1 activity, LS mean±95% c	-0.0 (-1.2 to 1.2)	—	—	-3.6 (-4.9 to -2.4)	—	-3.6 (-4.9 to -2.4)	0.2 (-1.0 to 1.3)	—	—	-3.3 [†] (-4.5 to -2.1)	—	-3.3 [†] (-4.5 to -2.1)

Data presented as LS mean±95% confidence interval. t-PA, tissue plasminogen activator; PAI-1, plasminogen-activator inhibitor type 1.

*P<0.0001, for dose response to agonist.

[†]P>0.05, SRT2104 vs placebo.

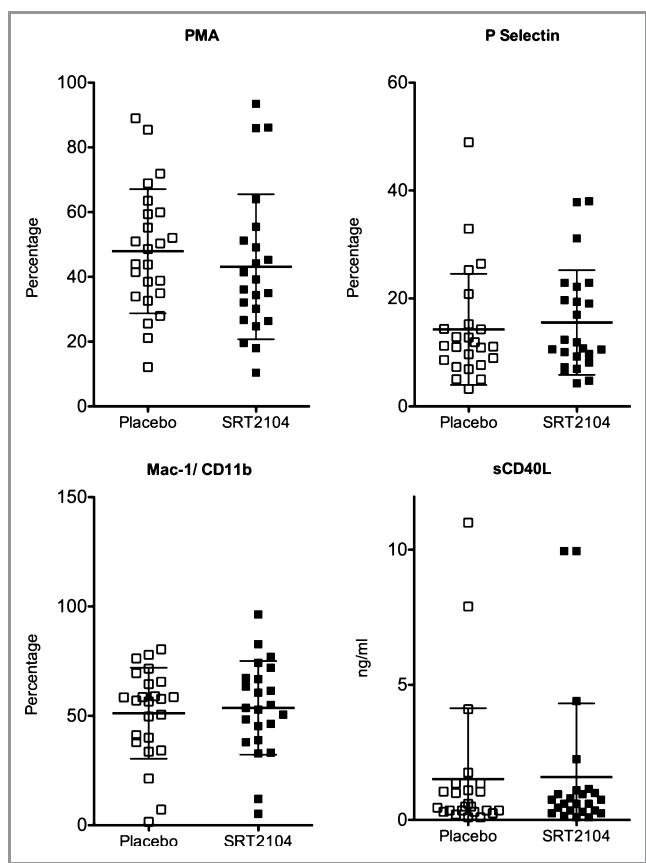


Figure 4. Effect of SRT2104 on markers of platelet and monocyte activation. Data presented as mean \pm SD. PMA indicates platelet–monocyte aggregate; Mac-1, macrophage-1 antigen; sCD40L, soluble CD40 ligand.

would also lead to improved vascular and endothelial function. There are at least 70 known substrates for SIRT1. SRT2104 may differentially deacetylate certain substrates in preference to others, depending on the precise interaction between SRT2104 and the substrates as well as the level and activity of the substrates in a particular disease state. It is also possible that certain abnormalities may be reversed more readily than others through SIRT1 activation. Although a 28-day exposure may be adequate for observing improvement in lipid profiles, longer treatment may be required to reverse some of the vascular and endothelial abnormalities. The small sample size of our study may also be a potential limitation. SRT2104 is the first selective SIRT1 activator to be studied in human clinical trials. As the biology of SIRT1 becomes more established and additional data are gathered from small exploratory trials such as this one, the optimal approach for developing SIRT1 activators and identifying disease states with the greatest therapeutic potential will become better defined.

In conclusion, we have demonstrated that the oral SIRT1 activator SRT2104 is safe and well tolerated in otherwise healthy cigarette smokers and provides positive effects on

lipid profiles, but were unable to demonstrate beneficial effects on vascular, endothelial, or platelet function compared with placebo.

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Disclosures

Drs Venkatasubramanian, Noh, Langrish, Joshi, Mills, Lang, and Newby report no disclosures; Dr Daga is currently an employee of GlaxoSmithKline, United Kingdom; Drs Hoffmann, Jacobson, and Vlasuk are employees of Sirtris Pharmaceuticals, Massachusetts, and own stock; Dr Waterhouse is an employee of GlaxoSmithKline, Pennsylvania, and owns stock.

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openheart Effects of the small molecule SIRT1 activator, SRT2104 on arterial stiffness in otherwise healthy cigarette smokers and subjects with type 2 diabetes mellitus

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ABSTRACT

Objective: Arterial stiffness increases with age, and is associated with adverse cardiovascular outcome including increased mortality. The effect of the oral small molecule SIRT1 activator, SRT2104, on arterial stiffness was examined in otherwise healthy cigarette smokers and participants with type 2 diabetes mellitus.

Methods: 24 otherwise healthy cigarette smokers and 15 people with stable type 2 diabetes were randomised in a double-blind placebo-controlled crossover trial and received 28 days of oral SRT2104 (2.0 g/day) or matched placebo. Blood pressure was measured using non-invasive oscillatory sphygmomanometry. Pulse wave analysis and velocity were measured using applanation tonometry at baseline and the end of each treatment period. Owing to the small sample size and similar trends for both groups, data for the two groups were pooled (post hoc analysis).

Results: Compared to placebo, treatment with SRT2104 was associated with a significant reduction in augmentation pressure ($p=0.0273$) and a trend towards improvement in the augmentation index and corrected augmentation index ($p>0.05$ for both). However, no changes were observed in pulse wave velocity and time to wave reflection ($p>0.05$). Systolic and diastolic blood pressures remained unchanged throughout the study. Treatment by cohort interaction was not significant for any of the pulse wave parameters, suggesting that the response to SRT2104 in otherwise healthy smokers and people with diabetes was consistent.

Conclusions: SRT2104 may improve measures of arterial stiffness in otherwise healthy cigarette smokers and in participants with type 2 diabetes. Definitive conclusions are not possible given the small sample size and exploratory nature of this analysis.

Trial registration number: NCT01031108.

INTRODUCTION

The enzyme sirtuin (silent mating-type information regulation 2 homologue) 1 (SIRT1)

KEY QUESTIONS

What is already known about this subject?

► Among the seven known sirtuins, SIRT1 has been identified as the most critical modulator of vascular function. Animal and laboratory studies have amply demonstrated its prominent role in the regulation of vascular homeostasis and diseases. However, little is known about their direct vascular effects in man.

What does this study add?

► The present study has provided evidence that suggests treatment with the oral SIRT1 activator, SRT2104, may lead to an improvement in measures of arterial compliance in otherwise healthy cigarette smokers and people with type 2 diabetes. The exact mechanism of this improved arterial compliance and the effects of prolonged treatment with SRT2104 on vascular health remain to be elucidated.

How might this impact on clinical practice?

► Given that aortic stiffness and endothelial function are key factors in predicting cardiovascular outcomes, identification of novel pharmacological means of improving these predictive parameters is important and highly relevant in populations with known cardiovascular risk factors.

belongs to the sirtuin family of nicotinamide adenine dinucleotide-dependent histone deacetylases and is highly expressed in the vascular endothelium.¹ In addition to other characteristics, its activation is associated with improved endothelial function² and inhibition of atherogenesis.³ Particular interest has been focused on the potential of therapeutic SIRT1 activators to act as anti-ageing agents.

Arterial stiffness rises with age and is recognised to be an independent predictor of

cardiovascular risk.⁴ In particular, elevations in pulse pressure and aortic stiffness are associated with increased risk of coronary events and overall mortality.⁵ Indeed, central aortic stiffness is associated with the presence of coronary atherosclerosis and ischaemic heart disease.⁶

Cigarette smoking and diabetes mellitus are significant risk factors for the development of cardiovascular disease. A wealth of data has established a strong correlation between diabetes and cigarette smoke exposure with increased aortic stiffness, endothelial dysfunction and cardiovascular risk.^{7–10} New pharmacological strategies that improve arterial compliance would therefore be highly relevant to these groups at increased cardiovascular risk.

The aims of the present study were to assess the effect of the oral SIRT1 activator, SRT2104, on measures of arterial compliance in otherwise healthy cigarette smokers and patients with type 2 diabetes. It was hypothesised that SIRT1 activation in these 'at risk' groups could lead to an improvement in arterial compliance and therefore reduce their cardiovascular risk.

METHODS

The study was approved by the Berkshire Research Ethics Committee, received Clinical Trial Authorisation from the Medicines and Healthcare products Regulatory Agency (MHRA, UK), and was conducted at the MHRA phase I accredited Wellcome Trust Clinical Research Facility at the Royal Infirmary of Edinburgh, UK between June 2010 and September 2011 (EudraCT #: 2009-016765-28; Clinical trials identifier: NCT01031108). Written informed consent was obtained from each volunteer and the study was carried out in accordance with the declaration of Helsinki.

Study participants

Twenty-four otherwise healthy cigarette smokers and 15 participants with stable type 2 diabetes, aged between 18 and 70 years, were eligible for the study. Healthy cigarette smokers were required to have smoked ≥ 10 cigarettes daily for at least 1 year. Participants with type 2 diabetes were non-smokers and were selected on the basis of having a diagnosis of type 2 diabetes mellitus for at least 6 months prior to inclusion in the study, with no change in medications having been made for at least the preceding 3 months, a fasting blood glucose ≤ 13.9 mmol/L (250 mg/dL) and diabetes control and complications trial-aligned HbA1c $< 9\%$ (75 mmol/mol) on screening. Exclusion criteria included the presence of significant comorbidities, chronic illness, renal or liver impairment, history of gastrointestinal diseases or previous surgical procedures that would influence drug absorption, history of alcoholism, history of neoplastic disease within the last 5 years, a positive urinary test for recreational drugs, pregnancy and participation in other clinical trials or blood donation within the last

3 months. Patients with type 2 diabetes mellitus on ACE inhibitors, antiplatelet or anticoagulant therapies were excluded from the study. Tests for pregnancy (serum human chorionic gonadotropin (HCG) concentrations at screening and urinary HCG concentrations at study visits) were conducted on all female participants of childbearing potential.

Study design

This was a prospective double-blind randomised placebo-controlled cross-over study. Participants were randomised to receive 2.0 g daily of oral SRT2104 or matched placebo (Sirtris, a GSK company, Massachusetts, USA) for a 28-day period, followed by cross-over to the alternate study arm for a further 28 days, giving a total dosing duration of 56 days. An end of study visit was conducted at day 70 with a telephone call follow-up on day 86. Measures of arterial stiffness were undertaken prior to and at the end of each 28-day trial period. [Figure 1](#) outlines participant enrolment, intervention allocation, follow-up and data analysis for both groups.

All studies were performed in a quiet temperature controlled (22–25°C) room. Participants were fasted and asked to refrain from smoking for 10 h, and abstain from caffeine and alcohol for 24 h prior to assessment. Participants remained supine for at least 30 min before any recordings were started. Systolic and diastolic blood pressures were recorded using a non-invasive oscillatory sphygmomanometer (Omron705 IT, Omron Healthcare Europe, the Netherlands).

Pulse wave analysis of the radial artery was performed at the wrist using micromanometer applanation tonometry (Millar Instruments, Texas, USA) and the SphygmoCor system (AtCor Medical, Sydney, Australia) in accordance with the manufacturer's recommendations. Briefly, pulse wave analysis derives an aortic pulse pressure waveform from the radial artery wave via a mathematical transfer function. The arterial pressure waveform is a composite of the forward pressure wave created by ventricular contraction and a reflected wave generated by peripheral vascular resistance.¹¹ The augmentation pressure is the pressure difference between the second and first systolic peaks. The augmentation index, augmentation pressure as a percentage of the pulse pressure, is a measure of systemic arterial stiffness and wave reflection. Corrected augmentation index represents the augmentation index corrected for heart rate.¹² The time to wave reflection declines with increasing arterial stiffness, and provides a surrogate measure of aortic pulse wave velocity.¹³ At least three independent waveform analyses were obtained from each participant, with measurements only accepted on meeting SphygmoCor quality control criteria. Pulse wave velocity was calculated by measuring the time for the pulse wave to travel between the carotid and femoral arteries. The operator performing the analysis was kept constant for each participant throughout the study.

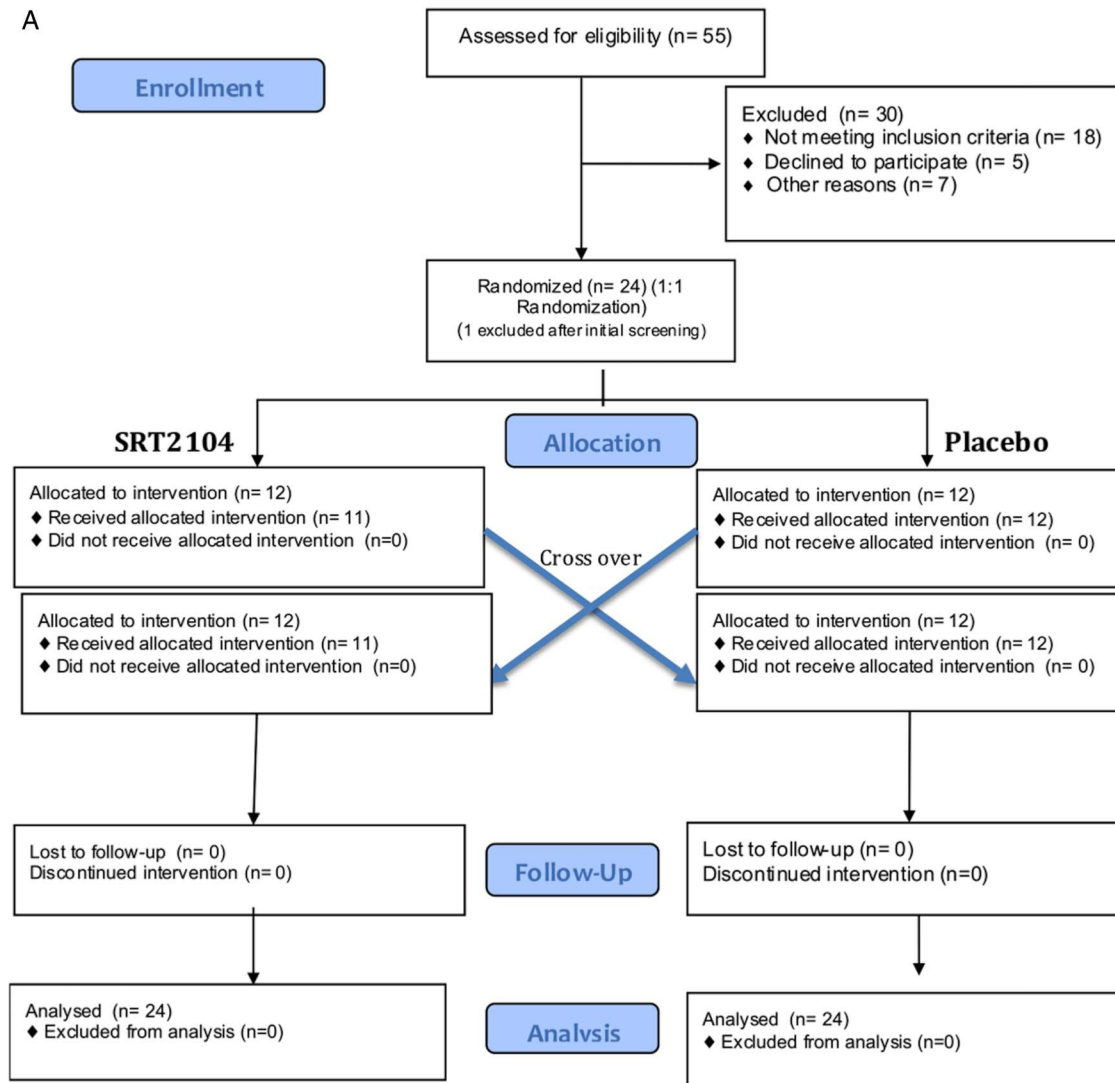


Figure 1 Consolidated Standards of Reporting Trials flow diagram representing participant enrolment, intervention allocation, follow-up and data analysis. (A) Recruitment of otherwise healthy cigarette smokers; (B) participants with type 2 diabetes mellitus.

Blood sampling

Venous blood samples were collected at fortnightly intervals to measure haematological and biochemical analytes including full blood count, coagulation profile, liver and renal function, creatine kinase, lactate dehydrogenase and lipid profile. Analyses were conducted by the regional clinical haematology and biochemistry reference laboratories using an automated haematology analyser (XE2100, Sysmex Corporation (Japan) and ACL TOP, Instrumentation Laboratory), an automated chemistry analyser using colorimetric, kinetic and enzymatic ultraviolet and colour assays (AU2700/AU640 analysers, Beckman and Coulter), ion selective electrodes (sodium, potassium and chloride assays) and two point and multiple point rate assays (Ortho Clinical Vitros 250 analyser, USA).

Data analysis and statistics

Data were analysed, where appropriate, using repeated measure analysis of covariance on the change from

baseline for all parameters. Initially, analyses were conducted separately on cohorts. As a result of the small sample size and similar trends for the two cohorts, these data were pooled post hoc. Treatment differences were investigated in a model adjusting for baseline, period, treatment by period and treatment by cohort using SAS for UNIX (V.9.1.3 or higher) (SAS Institute, Cary, North Carolina, USA). Unless stated otherwise, values are expressed as mean±SD. Tests for treatment effect were two-sided with a significance level of 0.05.

RESULTS

Baseline characteristics

Participants in the study had a mean age of 45±15 years and were predominantly male (68%). Participants in the type 2 diabetes cohort were older (mean age 58±8 years) when compared with the participants in the otherwise healthy smokers group (mean age 38±13 years). All participants were normotensive with comparable systolic

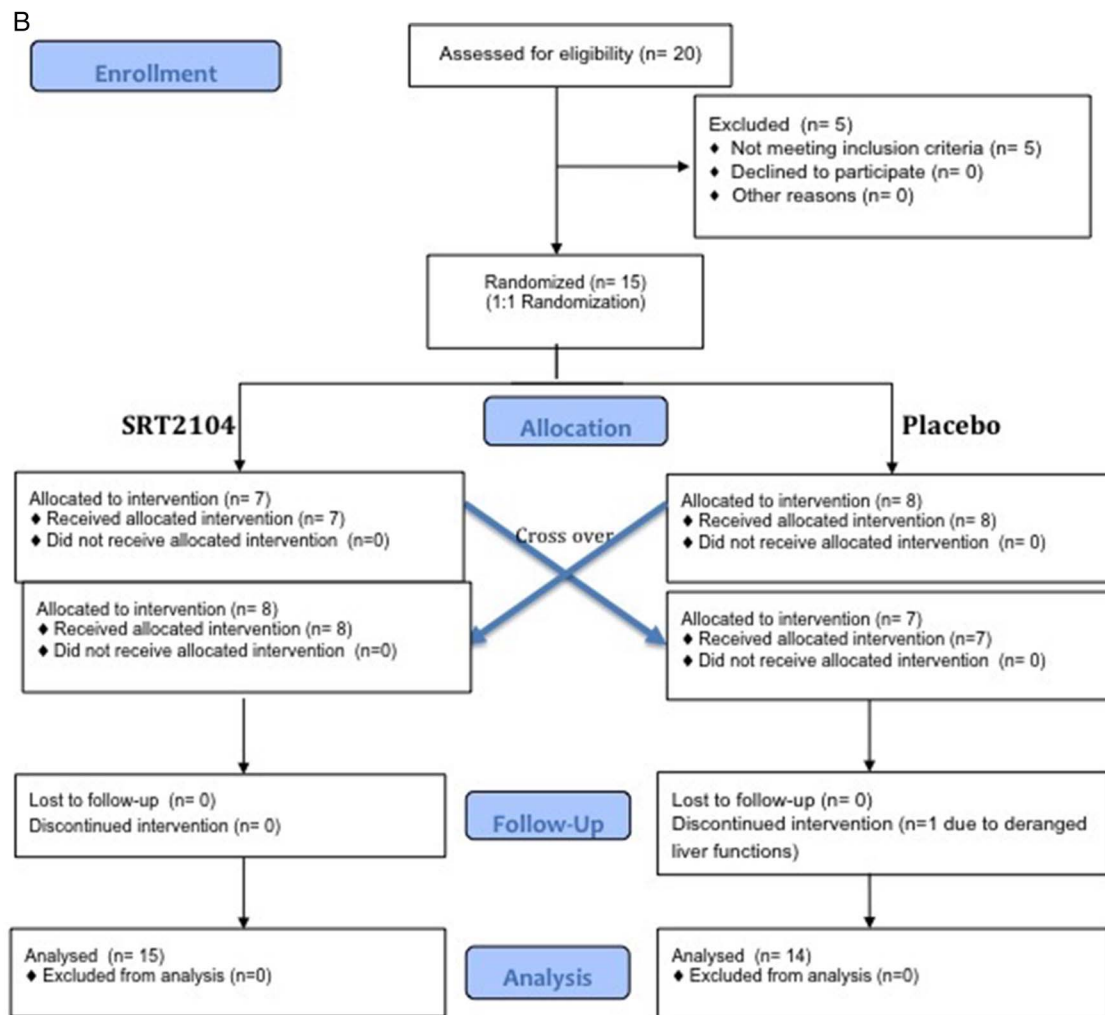


Figure 1 Continued

and diastolic blood pressures at baseline (table 1). No clinically significant changes in haematological or biochemical analytes occurred throughout the study. Biochemical measures of renal function (serum urea, creatinine and electrolytes) were within normal limits at baseline and remained unchanged with placebo and treatment with SRT2104 in both subgroups (table 2).

Blood pressure

Resting systolic and diastolic blood pressures remained unchanged throughout the study with no significant differences between treatment and placebo treatment periods.

Pulse wave analysis and velocity

In a combined analysis of otherwise healthy cigarette smokers and participants with type 2 diabetes, a reduction in the augmentation pressure was observed in participants receiving SRT2104 compared with placebo (mean change from baseline: SRT2104 -1.60 (5.304) vs placebo -0.06 (4.205); $p=0.0273$) and a trend towards improvement in the augmentation index (mean change

from baseline in AIx: placebo -0.64 (8.361) vs SRT2104 -3.47 (9.728); $p=0.0813$) and the corrected augmentation index (mean change from baseline AIx75: placebo -2.2 (7.453) vs SRT2104 -4.84 (9.299); $p=0.0747$) (figure 2A). Pulse wave velocity and time to wave reflection remained unchanged between placebo and treatment arms ($p>0.05$ for both parameters; figure 2B). The effects of SRT2104 administration on measures of arterial compliance were consistent across the two cohorts. For example, in the SRT2104 arm, mean augmentation index at 75 bpm was reduced for healthy smokers and participants with type 2 diabetes (-4.97 vs -4.63 , respectively). Measures of arterial compliance and stiffness for the individual cohorts have been presented in the online supplementary table S1. A statistical interaction between cohort and treatment was not observed ($p>0.05$ for all variables tested).

Tolerability and safety

Participants in both study groups (healthy cigarette smokers and patients with type 2 diabetes) tolerated the study medication well. There were no meaningful

Table 1 Baseline characteristics of participants who were otherwise healthy cigarette smokers or who had type 2 diabetes mellitus

	Otherwise healthy cigarette smokers (n=24)	People with type 2 diabetes (n=15)
Mean age (years)	38±13	58±8
Sex		
Male	14 (58)	13 (87)
Female	10 (42)	2 (13)
Baseline blood pressure (mm Hg)		
Systolic	129±6	133±7
Diastolic	77±2	80±3
Heart rate (bpm)	68±1	77±5
Body mass index (kg/m ²)	25±4	30±4
Smoking history		
Number of cigarettes/day	17±6	–
Number of pack years	16	–
Urinary cotinine concentration (ng/mL)	1352±950	–
Glycaemic profile		
Baseline blood glucose (mg/dL)	85±0	144±2
HbA1c (%)	–	7.4±0.8
Concomitant medications		
Antiplatelet agents	–	5 (33%)
Antihypertensive agents	–	3 (20%)
ARB	–	2 (13%)
Diuretics	–	10 (67%)
Lipid lowering agents	–	13 (86%)
Hypolycaemic agents	–	4 (27%)
Biguanide	–	2 (13%)
Sulfonamide	–	1 (6%)
Thiazolidine	–	2 (13%)
Insulin	–	–
Others	–	–

Values expressed as mean±SD.

ARB, angiotensin receptor blocker; HbA1c, haemoglobin A1c.

differences in the number of adverse events between active treatment and placebo. All reported adverse events were mild in intensity and resolved without any intervention or sequelae (table 3). Headaches occurred with nearly equal frequency in the treatment (SRT2104) group in both cohorts. Participants with type 2 diabetes appeared to have more frequent gastrointestinal disturbances, such as diarrhoea and nausea in comparison with healthy smokers. Elevated liver enzymes (alanine transaminase) resulted in withdrawal of one participant in the placebo period (day 36) of the diabetes group. There was only one reported serious adverse event in the study (SRT2104 arm of healthy cigarette smokers) of traumatic facial bone fracture that was considered unrelated to SRT2104.

DISCUSSION

This randomised double-blinded cross-over study demonstrated for the first time that the oral SIRT1 activator, SRT2104, may improve arterial compliance in otherwise healthy cigarette smokers and in people with type 2 diabetes, without affecting resting measures of blood pressure.

The assessment of arterial stiffness is increasingly being used in clinical practice as an independent measure of cardiovascular risk, including those in high-risk groups.¹⁴ Ageing is associated with an increase in the stiffness of large elastic arteries induced by structural alterations in the vascular media such as an increase in collagen and a decrease in elastin content.¹⁵ This process of biological ageing is accelerated in the presence of conditions such as diabetes mellitus and hypertension. Semba *et al*¹⁶ and Hofmann *et al*¹⁷ have demonstrated an association between the presence of advanced glycation end products and increased arterial stiffness. Indeed, vascular change induced by cigarette smoke is considered to be a model of accelerated vascular ageing. The relationship between tobacco exposure,^{9 10 18} diabetes^{7 8 19 20} and increased arterial stiffness is well established.

Calorie restriction can attenuate age-related arterial stiffness in animal models through reduced oxidative stress and altered endothelial nitric oxide bioavailability.¹⁵ Indeed, calorie restriction can extend lifespan in lower organisms and mammals, and improves several metabolic and inflammatory parameters.^{21–26} SIRT1 has been implicated as an important mediator of lifespan

Table 2 Changes in biochemical measures of renal function in otherwise healthy cigarette smokers and participants with type 2 diabetes mellitus administered placebo and SRT2104

	Otherwise healthy cigarette smokers (n=24)				Participants with type 2 diabetes (n=15)			
	Treatment period 1: placebo (n=13)		Treatment period 1: SRT2104 (n=11)		Treatment period 1: placebo (n=8)		Treatment period 1: SRT2104 (n=7)	
	Day 1	Day 28	Day 1	Day 28	Day 1	Day 28	Day 1	Day 28
Serum urea (mg/dL)	17±3	14±3	11±3	14±3	17±3	17±3	17±6	14±3
Serum creatinine (mg/dL)	0.8±0.1	0.8±0.1	0.7±0.1	0.8±0.1	0.8±0.1	0.8±0.1	0.9±0.1	0.9±0.1
Electrolytes								
Sodium (mmol/L)	140±2	140±2	140±2	140±1	139±1	139±3	138±2	139±3
Potassium (mmol/L)	4.0±0.4	4.0±0.4	4.0±0.4	4.0±0.2	4.0±0.3	4.0±0.3	4.0±0.2	4.0±0.4
Chloride (mmol/L)	109±3	107±3	108±3	107±2	104±4	104±3	103±2	104±2

Values expressed as mean±SD.

extension mediated by calorie restriction.^{27–29} The current hypothesis, therefore, was that activation of SIRT1 may inhibit this process of vascular ageing and be associated with improvements in arterial stiffness.

No studies have examined the direct effect of SIRT1 activation on measures of arterial compliance. Botden *et al*³⁰ were unable to demonstrate an improvement in augmentation index or central or peripheral blood

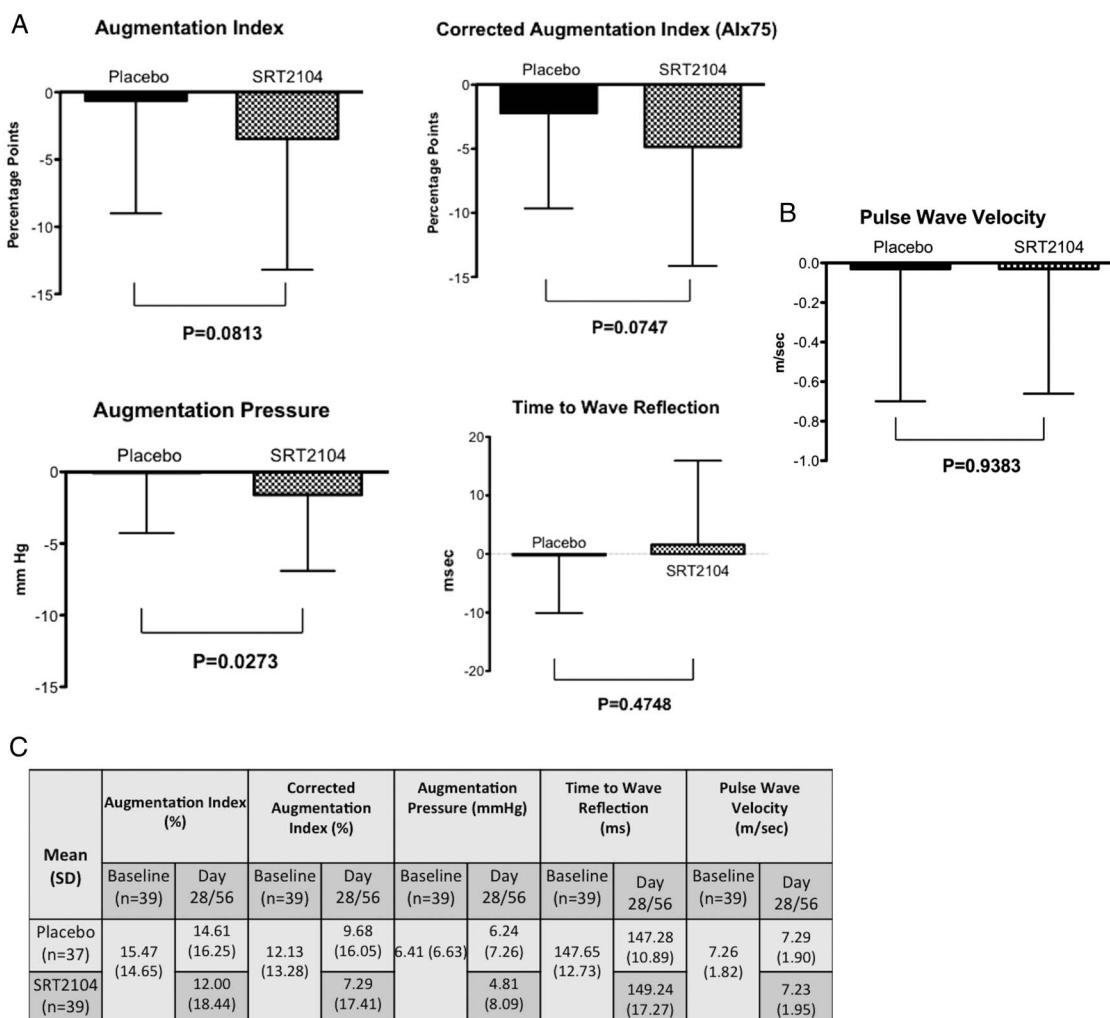


Figure 2 Effect of treatment with SRT2104 on measures of arterial compliance in otherwise healthy cigarette smokers and participants with type 2 diabetes mellitus—change from baseline. (A) Pulse wave analysis—augmentation index, corrected augmentation index, augmentation pressure and time to wave reflection. (B) pulse wave velocity. Solid column: placebo; checked column: SRT2104. (C) baseline parameters of measures of arterial compliance-combined data.

Table 3 Summary of treatment emergent adverse events occurring in two or more participants in OHS and participants with T2DM

System organ class	Adverse event	Number of events			
		OHS		T2DM	
		Placebo (n=24)	SRT2104 (n=24)	Placebo (n=14)	SRT2104 (n=15)
Any event		18	18	11	14
Nervous system disorders	Any event	6 (25%)	11 (46%)	1 (7%)	7 (47%)
	Headache	4	6	1	5
	Paraesthesia	0	2	0	1
	Hypoaesthesia	1	2	0	0
	Presyncope	1	1	0	0
Respiratory, thoracic and mediastinal disorders	Any event	1 (4%)	3 (13%)	0	3 (20%)
	Oropharyngeal pain	1	2	0	0
	Rhinorrhoea	0	1	0	1
Gastrointestinal disorders	Any event	3 (13%)	1 (4%)	4 (29%)	8 (53%)
	Diarrhoea	0	0	2	4
	Nausea	0	0	1	2
	Abdominal pain upper	1	0	0	1
	Dyspepsia	0	0	0	2
Reproductive system and breast disorders	Any event	3 (13%)	1 (4%)	0	0
	Dysmenorrhoea	3	1	0	0
Musculoskeletal and connective tissue disorders	Any event	4 (17%)	1 (4%)	0	2 (13%)
	Back pain	2	0	0	0
Investigational	Any event	2 (8%)	1 (4%)	1 (7%)	1 (7)
	Blood bilirubin increased	1	1	0	0
	Alanine amino transferase increased	0	0	1	0
	Abnormal liver function test	0	0	0	1
General disorders and administration site conditions	Any event	4 (17%)	4 (17%)	2 (14%)	3 (20%)
	Influenza like illness	1	0	0	1
	Fatigue	1	0	1	1
Infections and infestations	Any event	3 (13%)	5 (21%)	3 (21%)	1 (7%)
	Nasopharyngitis	0	1	3	0
	Rhinitis	2	2	0	0
	Upper respiratory tract infection	1	0	0	1
Injury, poisoning and procedural complications	Any event	4 (17%)	2 (8%)	3 (21%)	1 (7%)
	Contusion	1	0	1	0
	Excoriation	1	1	0	0
Skin and subcutaneous tissue	Any event	1 (4%)	0	1 (7%)	3 (20%)
	Pruritus	0	0	0	2
Metabolism and nutrition disorders	Any event	0	1 (4%)	2 (14%)	2 (13%)
	Hypoglycaemia	0	0	1	2
Vascular disorders	Any event	0	1 (4%)	0	2 (13%)
	Flushing	0	0	0	2

OHS, otherwise healthy cigarette smokers; T2DM, type 2 diabetes mellitus.

pressure following treatment with red wine polyphenols. In the present study, a 28-day period of treatment with the oral SIRT1 activator SRT2104 was associated with a reduction in augmentation pressure and trends towards improvement in augmentation index and corrected augmentation index. Augmentation pressure and index are measures of arterial compliance and wave reflection from small to medium sized arteries. As such, they can be influenced by endothelial function and a number of other dynamic and functional factors, such as heart rate

and peripheral circulatory tone.^{7 14} Preclinical studies have demonstrated improved vascular function with SIRT1 activation,^{2 31-33} and this may explain our observations of improvement in dynamic measures of arterial stiffness following short-term administration of SRT2104.

Pulse wave velocity is a more direct measure of arterial stiffness that is determined by the structural and physical composition of the arterial wall.⁸ Changes in pulse wave velocity are therefore more gradual and less dependent on the function of small to medium sized arteries. In

the present study, a change in pulse wave velocity was not observed with SRT2104 administration. This is perhaps not surprising given the short-time period of exposure to SRT2104 (28 days) and the brief period of observation. An improvement in pulse wave velocity might be anticipated with a longer period of treatment with SRT2104, to allow more favourable structural changes in the larger arterial tree.

STUDY LIMITATIONS

Some limitations of this trial should be considered. Although favourable trends in parameters of arterial compliance were observed, some did not achieve statistical significance. This may partly be attributed to the trial being designed specifically to examine the acute effects of treatment with SRT2104. A longer period of treatment may be required for benefits to emerge on variables such as pulse wave velocity that involve structural changes in the arterial wall. Moreover, the sample sizes of the two groups examined were small. Two disparate populations were studied in this trial, in whom the mechanisms of vascular dysfunction may be very different. However, the direction of beneficial effects on treatment with SRT2104 was similar between the two groups, providing reassurance of a consistency of effect and allowing the post hoc presentation of the results pooled across the two groups.

Conclusion

The present study has provided evidence that suggests treatment with the oral SIRT1 activator, SRT2104, may lead to an improvement in measures of arterial compliance in otherwise healthy cigarette smokers and people with type 2 diabetes. The exact mechanism of this improved arterial compliance and the effects of prolonged treatment with SRT2104 on vascular health remain to be elucidated. Given that aortic stiffness and endothelial function are key factors in predicting cardiovascular outcomes, identification of novel pharmacological means of improving these predictive parameters is important and highly relevant in populations with known cardiovascular risk factors.

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preparation and review of the manuscript. BW, EH, EWJ and DEN were involved in planning of the trial, data analysis and statistics and review of the manuscript.

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Competing interests SV, RMN, JPL, NLM, NNL, BMF and DEN—no disclosures; SD is currently an employee of GlaxoSmithKline and owns GSK stock, UK; EH and EWJ are employees of Sirtris Pharmaceuticals, Massachusetts, USA and own stock; BRW is an employee of GlaxoSmithKline, Pennsylvania, USA and owns stock.

Patient consent Obtained.

Ethics approval Berkshire Research Ethics Committee.

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Data sharing statement No additional data are available.

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CLINICAL TRIALS

Cardiovascular effects of urocortin 2 and urocortin 3 in patients with chronic heart failure

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Keywords cardiac, heart failure, inotrope, urocortin, vasodilator

AIMS

Urocortin 2 and urocortin 3 may play a role in the pathophysiology of heart failure and are emerging therapeutic targets. We aimed to examine the local and systemic cardiovascular effects of urocortin 2 and urocortin 3 in healthy subjects and patients with heart failure.

METHODS

Patients with heart failure ($n = 8$) and age and gender-matched healthy subjects ($n = 8$) underwent bilateral forearm arterial blood flow measurement using forearm venous occlusion plethysmography during intra-arterial infusions of urocortin 2 (3.6 – 36 pmol min⁻¹), urocortin 3 (360 – 3600 pmol min⁻¹) and substance P (2 – 8 pmol min⁻¹). Heart failure patients ($n = 9$) and healthy subjects ($n = 7$) underwent non-invasive impedance cardiography during incremental intravenous infusions of sodium nitroprusside (573 – 5730 pmol kg⁻¹ min⁻¹), urocortin 2 (36 – 360 pmol min⁻¹), urocortin 3 (1.2 – 12 nmol min⁻¹) and saline placebo.

RESULTS

Urocortin 2, urocortin 3 and substance P induced dose-dependent forearm arterial vasodilatation in both groups ($P < 0.05$ for both) with no difference in magnitude of vasodilatation between patients and healthy subjects. During systemic intravenous infusions, urocortin 3 increased heart rate and cardiac index and reduced mean arterial pressure and peripheral vascular resistance index in both groups ($P < 0.01$ for all). Urocortin 2 produced similar responses to urocortin 3, although increases in cardiac index and heart rate were only significant in heart failure ($P < 0.05$) and healthy subjects ($P < 0.001$), respectively.

CONCLUSION

Urocortins 2 and 3 cause vasodilatation, reduce peripheral vascular resistance and increase cardiac output in both health and disease. These data provide further evidence to suggest that urocortins 2 and 3 continue to hold promise for the treatment of heart failure.

WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Urocortins 2 and 3 are emerging therapies for treating heart failure.
- Urocortins 2 and 3 reduce peripheral vascular resistance and increase cardiac output in both health and disease.

WHAT THIS STUDY ADDS

- This is the first direct head-to-head comparison of urocortin 2 and urocortin 3 in man.
- These data provide further evidence that urocortin 2 and urocortin 3 hold major potential for the treatment of heart failure.

Introduction

There are almost six million people living with heart failure in the USA. This comes at an annual cost to the US economy of over \$30 billion [1]. Despite many evidence-based therapies for patients with chronic heart failure, treatments for acute heart failure are limited, less well developed and have not been shown to improve clinical outcomes. Indeed the use of inotropic agents has been associated with harm [2–4]. However, the tentative but promising improvements in clinical outcome seen with serelaxin in the recent RELAX-AHF study [5] has renewed enthusiasm for the assessment of novel vasoactive mediators in this important patient group.

The urocortins are an endogenous peptidic hormone group comprising urocortin 1, urocortin 2 and urocortin 3 with vasodilator, inotropic and lusitropic effects [6]. Urocortins 2 and 3 counteract many of the effects of corticotrophin-releasing hormone (CRH) [7–10] and act on the CRH-receptor 2 (CRH-R2), a G-protein coupled receptor that is expressed abundantly in the heart and peripheral vasculature [11, 12]. Urocortin 2 causes vasodilatation in healthy volunteers, augments cardiac output and reduces vascular resistance in healthy humans, patients with acute decompensated, and also chronic stable, heart failure [13–16].

A recombinant acetate salt of urocortin 3 (JNJ-39 588 146 [recombinant stresscopin]), improved cardiac output whilst reducing vascular resistance in a multicentre study of patients with chronic stable heart failure [17]. We recently demonstrated that urocortins 2 and 3 increase forearm blood flow in young healthy volunteers [15]. To date, no clinical study has separated the regional from systemic effects of urocortins 2 and 3 in heart failure or conducted a direct head to head comparison of their effects.

We aimed to evaluate and compare the local and systemic cardiovascular effects of urocortins 2 and 3 in patients with heart failure and healthy subjects by assessing (i) local forearm arterial blood flow using venous occlusion plethysmography and (ii) cardiac output and vascular resistance using thoracic bioimpedance cardiography. We hypothesized that urocortins 2 and 3 would cause vasodilatation, reduced peripheral vascular resistance and increased cardiac output in both healthy subjects and patients with heart failure.

Methods

Both studies were approved by the local research ethics committee (South East Scotland REC 01) and carried out in accordance with the Declaration of Helsinki. The registered clinical trials on UKCRN were ID 10749 and 13002.

Written informed consent was obtained from all participants prior to the study.

Study participants

Patients with heart failure were eligible if they were aged 18–80 years, New York Heart Association (NYHA) symptom class II–III and had echocardiographically confirmed left ventricular ejection fraction <35% with left ventricular end diastolic diameter > 5.5 cm. Patients were required to be receiving maximally tolerated doses of angiotensin-converting enzyme inhibitor and β -adrenoceptor blocker therapies for at least 3 months. Healthy subjects had no significant previous medical history and were on no regular medications. Exclusion criteria for both groups included systolic blood pressure > 190 mmHg or <90 mmHg, haemodynamically significant valvular heart disease and other severe or significant co-morbidities including bleeding diathesis, renal or hepatic failure, anaemia or recent infective/inflammatory conditions. Women of child bearing potential were also excluded.

Protocol A: local vascular study

This was a randomized study (Figure 1A) of eight patients with heart failure and eight age and gender-matched healthy subjects. Subjects attended once each to receive incremental intra-arterial infusions of urocortin 2 (3.6, 12, 36 pmol min⁻¹, molecular weight 4450.3 g mol⁻¹), urocortin 3 (360, 1200, 3600 pmol min⁻¹, molecular weight 4137.9 g mol⁻¹) and substance P (2, 4, 8 pmol min⁻¹ [control endothelium-dependent vasodilator]) for 6 min at each dose with a 30 min washout period between agents.

Forearm venous occlusion plethysmography studies were performed with the subject lying supine, in a quiet, temperature-controlled room (22–25°C). Participants fasted for 4 h prior to the study and refrained from alcohol and caffeine for 24 h prior to the study. Venous cannulae (17G) were inserted into large subcutaneous veins in the antecubital fossae of both arms at the start of the study to facilitate periodic venous sampling. Heart rate and blood pressure were monitored at regular intervals throughout the study with a semi-automated oscillometric sphygmomanometer (Omron 705IT). Participants underwent brachial artery cannulation of the non-dominant forearm with a 27 standard wire-gauge steel needle for agent infusion. Forearm blood flow was measured in the infused and non-infused forearms using bilateral venous occlusion plethysmography as described previously [18, 19].

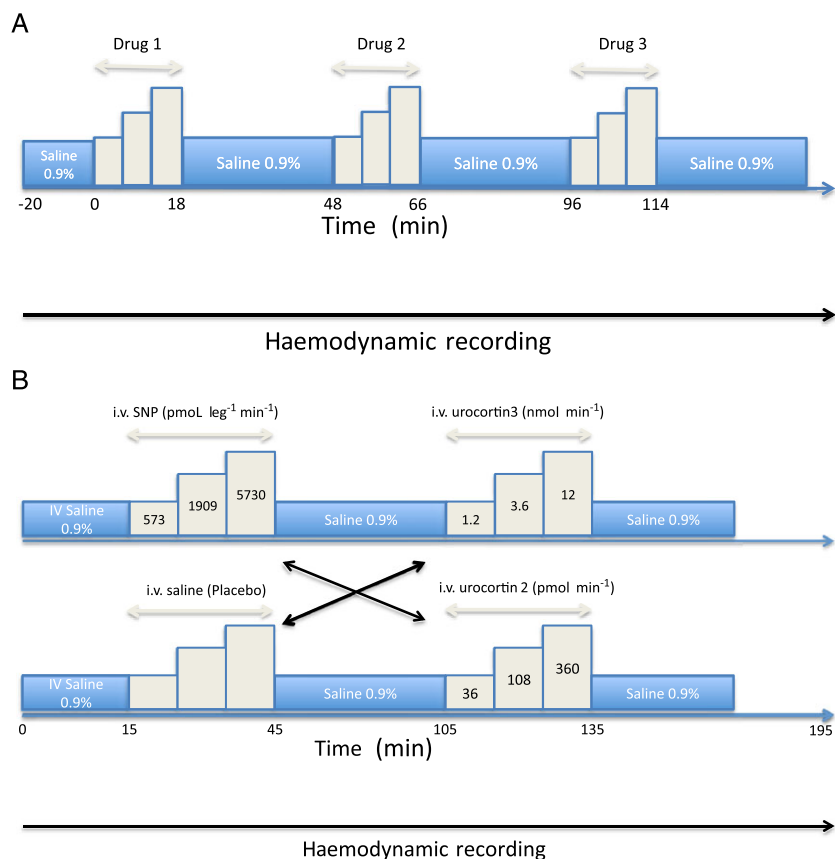


Figure 1

A) Schematic representation of study protocol A – vascular study and B) schematic representation of study protocol B – systemic study. A) forearm blood flow. B) systemic haemodynamic responses

Protocol B: systemic study

This was a randomized, double-blind, placebo controlled crossover study (Figure 1B). Three patients with heart failure and three volunteers participants were involved in both studies. Patients with heart failure ($n = 9$) and healthy subjects ($n = 7$) were recruited. Participants had venous cannulae inserted into both antecubital fossae and attended on two occasions, receiving intravenous infusions of saline (placebo) or SNP ($573, 1909, 5730 \text{ pmol kg}^{-1} \text{min}^{-1}$) followed by either urocortin 2 ($36, 108, 360 \text{ pmol min}^{-1}$) or urocortin 3 ($1.2, 3.6, 12 \text{ nmol min}^{-1}$). On the second visit, participants received the two remaining agents not administered on visit 1. Each agent was given in three ascending doses for 10 min at each dose. A 1 h saline washout was given between agents and a further 30 min of saline washout was administered after the cessation of the second agent.

Haemodynamic monitoring. Haemodynamic measurements were recorded throughout the study. Cardiac output, blood pressure and stroke volume were recorded using non-invasive thoracic impedance cardiography (NCCOM3-R7, BioMed, CA, USA or Cardioscreen 1000, Medis, Germany) and oscillometric sphygmomanometry (Omron HEM-705CP, Omron, Matsusaka, Japan). Values were indexed to body surface area where appropriate and peripheral

vascular resistance index was calculated using recorded measurements ($\text{PVRI} = \text{MAP}/\text{CI}$).

Safety. Study stopping criteria were in place to ensure participant safety. Criteria included a drop in diastolic blood pressure of $>25 \text{ mmHg}$, fall in heart rate below $50 \text{ beats min}^{-1}$ or rise above $120 \text{ beats min}^{-1}$, at the request of the participant, attending nurse or the attending physician.

Venous sampling. Baseline blood samples were drawn for assessment of full blood count, renal function, glucose and cholesterol concentrations at the start of each study. The local clinical biochemistry and haematology reference laboratories performed analysis.

Data analysis and statistics. Data were collected in a double-blind fashion for both studies. Data were analyzed, where appropriate, by analysis of variance (ANOVA, one way and two way with repeated measures where appropriate). All results in figures are expressed as mean \pm SEM. All statistical analysis was performed with GraphPad Prism, version 6 (GraphPad Software, San Diego, CA, USA). Statistical significance was taken as two-sided $P < 0.05$.

Results

Patients with heart failure and healthy subjects recruited were predominantly male and middle-aged. Heart failure patients had greater BMI compared with healthy subjects. Participant characteristics are shown in Table 1. Patients with heart failure were receiving maintenance heart failure therapy. Patients and volunteers were age and gender-matched in protocol A and age matched alone in protocol B. Three patients with heart failure and three volunteer participants were recruited to both studies.

Protocol A – vascular study

The intra-brachial infusion of all three drugs was well tolerated with no adverse effects. Intra-brachial infusion of urocortin 2 and 3 produced localized, self-limiting forearm flushing and some facial flushing in both groups of participants as noted in previous studies [15].

Urocortin 2, urocortin 3 and substance P all evoked dose-dependent forearm arterial vasodilatation in both participant groups (mean changes across the three doses [95% CI] from baseline as follows: urocortin 2 +60% [9–111] $P < 0.05$, +72% [21–123] $P < 0.01$; urocortin 3 +167% [100–237] $P < 0.0001$, +151% [82–219] $P < 0.0001$; substance P +227% [130–326] $P < 0.0001$, +155% [57–253] $P < 0.001$ for healthy controls and heart failure patients, respectively; Figure 2A). There were no significant differences in changes in forearm blood flow between heart failure patients and healthy subjects (urocortin 2 +12% [–40 to 63%] $P = 0.84$, urocortin 3 –18% [–86 to +50%], $P = 0.80$; substance P –72% [–170 to +26%] $P = 0.19$).

Blood pressure and heart rate remained unchanged in both groups in response to urocortin 2 and substance P. At the highest dose, urocortin 3 induced a transient tachycardia compared with baseline in both groups (heart failure +14.9 beats min^{-1} [20.9 to 8.9], healthy volunteers +21.3 mmHg [27.3 to 15.3] both $P < 0.0001$) that was accompanied by a drop in systolic (–17.4 mmHg [–7.9 to –26.9], $P < 0.0001$) and diastolic blood pressure in patients with heart failure (–8.4 mmHg [–3.5 to –13.3], $P < 0.001$) and a drop in diastolic blood pressure alone in healthy subjects (–12.4 mmHg [–7.5 to –17.3], $P < 0.0001$, Figure 2B). Non-infused forearm blood flow remained unchanged throughout the study (data not shown).

Protocol B – systemic study

Participants displayed hypotension at the higher doses of SNP initially administered in the study (17.2 nmol $\text{kg}^{-1} \text{min}^{-1}$), frequently reaching study stopping criteria. A dose reduction to SNP (maximum dose of 5730 pmol- $\text{kg}^{-1} \text{min}^{-1}$) was made after two healthy subjects completed the study. For the urocortins, hypotension reaching study stopping criteria was met in one heart failure patient for urocortin 2 and two heart failure patients and one healthy subject for urocortin 3. Participants described dose-dependent symptoms of tachycardia and a warm sensation with both urocortins, but there were no significant adverse events attributable to either urocortin 2 or urocortin 3. Full blood count and serum biochemistry was unchanged between the two study visits (Table 1).

Urocortin 2 had no significant effect on cardiac index in healthy subjects and heart rate in heart failure patients when compared with saline placebo ($P > 0.05$ for both, see Figure 3

Table 1

Participant characteristics for protocol A and B

	Protocol A		$P = 0.84$	Protocol B		$P = 0.56$
	Heart failure patients ($n = 8$)	Healthy subjects ($n = 8$)		Heart failure patients ($n = 9$)	Healthy subjects ($n = 7$)	
Age (years)	55.5 [52.25–67]	57 [53–65.75]		58 [50–66.5]	58 [45–66]	
BMI	30 ± 5.4	25.1 ± 2.3	$P = 0.03$	32.2 ± 4.3	25.9 ± 1.9	$P = 0.003$
Gender	5 M, 3F	5 M, 3F		7 M, 2F	4 M, 3F	
ACEi/ARB	8 (100)	n/a		9 (100)	n/a	
BB	6 (75)	n/a		7 (78)	n/a	
MRA	5 (63)	n/a		8 (89)	n/a	
Digoxin	1 (13)	n/a		4 (44)	n/a	
Loop diuretic	6 (75)	n/a		6 (67)	n/a	
Haemoglobin (visit 1 vs visit 2, g l^{-1})	n/a	n/a		136.0 ± 14.46 vs 133.0 ± 14.96 ($P > 0.99$)	143.7 ± 7.111 vs 135.3 ± 8.524 ($P > 0.99$)	
Creatinine (visit 1 vs visit 2, $\mu\text{mol l}^{-1}$)	n/a	n/a		109.8 ± 39.66 vs 116.3 ± 74.95 ($P > 0.99$)	75.29 ± 8.635 vs 72.67 ± 7.840 ($P > 0.99$)	

n (%), mean ± SD, median [interquartile range]

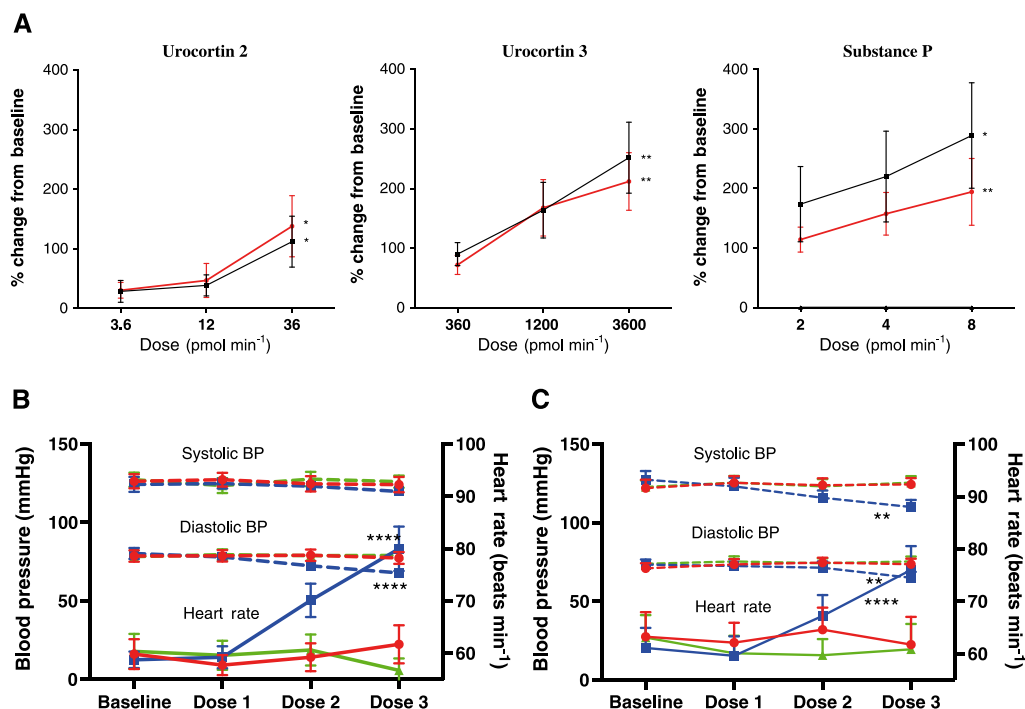


Figure 2

Haemodynamic responses during local administration of urocortin 2, urocortin 3 and substance P (protocol A). A) Percentage change in forearm arterial blood flow to urocortin 2 (3.6–36 pmol min⁻¹), urocortin 3 (360–3600 pmol min⁻¹) and substance P (2–8 pmol min⁻¹) in patients with heart failure (red) and in healthy subjects (black). B) Non-invasive systemic hemodynamic responses to intra-arterial urocortin 2 (red), urocortin 3 (blue) and substance P (green) in healthy subjects (A) and patients with heart failure (B). (**** = $P < 0.0001$, ** = $P < 0.01$, * = $P < 0.05$)

and Table 2 for results). Otherwise urocortin 2 and urocortin 3 increased cardiac index and heart rate and reduced mean arterial pressure and peripheral vascular resistance index in both patients with heart failure and healthy subjects ($P < 0.05$ for all). There was no effect of either urocortin 2 or urocortin 3 on stroke volume ($P > 0.05$ for both).

At the doses used, urocortin 3 caused greater mean increases than urocortin 2 in cardiac index (+13.4 [+4.1 to +22.6], $P < 0.01$) and heart rate (+11.0 [+2.9 to +19.2], $P < 0.01$) and greater mean reductions in mean arterial pressure (+4.4 [0.0 to +8.7], $P < 0.05$) and peripheral vascular resistance index (+11.2 [2.2 to +20.1], $P < 0.01$) in patients with heart failure. No such haemodynamic differences existed between urocortin 2 and urocortin 3 in healthy subjects. No haemodynamic differences existed between participant groups for both urocortin 2 and urocortin 3 ($P > 0.05$ for all). Following cessation of the intravenous infusions, haemodynamic variables returned to baseline after 40–60 min (for example, cardiac index, Figure 4). Over the infusion and washout period, urocortin 3 again caused a greater increase in cardiac output ($P < 0.0001$) than urocortin 2 in patients with heart failure but not in healthy subjects ($P = 0.48$).

Discussion

For the first time, we report the local vascular and systemic effects of urocortins in both patients with heart failure and

healthy subjects. We demonstrate that both urocortin 2 and urocortin 3 increase cardiac index and reduce peripheral vascular resistance and their parenteral administration is feasible, safe and well tolerated. We conclude this haemodynamic profile suggests that urocortins 2 and 3 hold major potential for the treatment of acute heart failure.

Forearm venous occlusion plethysmography combined with intra-arterial cannulation allows the assessment of local, sub-systemic vasomotor effects of peptides without exerting a systemic response. This is particularly useful in the study of novel compounds, such as urocortin 2 and urocortin 3. We have previously demonstrated that urocortin 2 and 3 cause vasodilatation in healthy subjects and that is in part mediated by endothelium-dependent factors such as nitric oxide and endothelium derived hyperpolarizing factor [15]. Impaired endothelial function is a recognized feature and an independent predictor of adverse outcome in patients with heart failure [20–24]. There is therefore a theoretical concern that urocortin would be less effective in patients with heart failure because of this concomitant endothelial dysfunction. However, we demonstrate that urocortin 2 and urocortin 3 evoked normal forearm arterial vasodilatory responses in our patients with heart failure. This suggests that either endothelial dysfunction does not have a meaningful impact on the actions of urocortin or our subjects did not have significant endothelial dysfunction. Interestingly, our patients did not demonstrate impaired substance P induced vasodilatation suggesting preserved endothelial function. This may reflect that the patients studied had stable heart

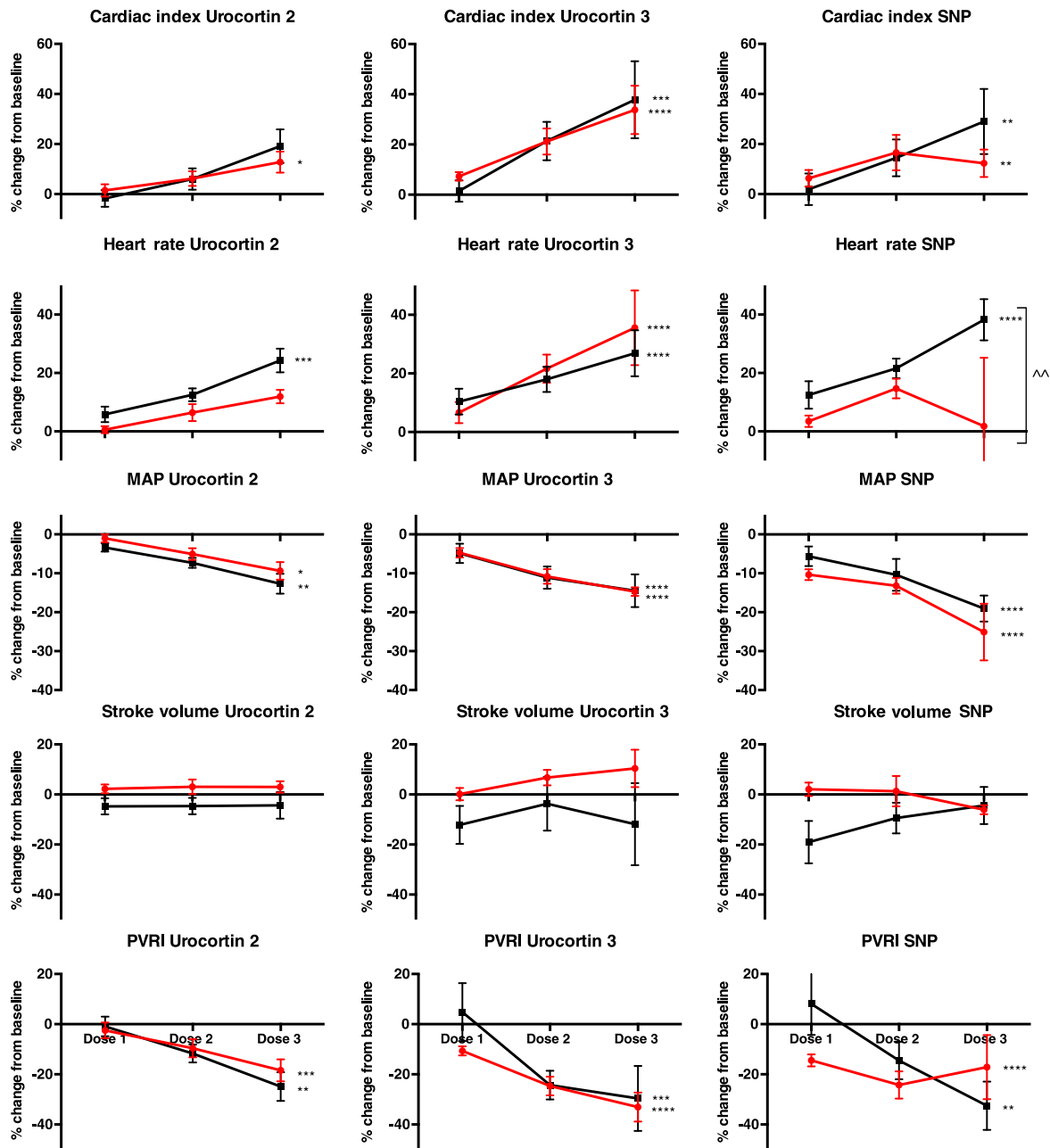


Figure 3

Changes in haemodynamic responses from baseline following systemic infusion (protocol B). Haemodynamic responses to infusions of urocortin 2, urocortin 3 and SNP in healthy subjects (black) and patients with heart failure (red) at doses 1–3 (D1–D3). * represents significant differences from saline placebo (not shown) across the three doses (see Table 2). ^ represents significant differences between participant groups. (**** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; ^^ $P < 0.01$)

failure symptoms and were well-treated and receiving optimal medical therapy. Whether similar findings would be observed in patients with decompensated heart failure remains to be established.

During local intra-arterial infusions of the urocortins, we were not anticipating observing any major changes in haemodynamic variables in either group. However, we found dose-related increases in heart rate and falls in diastolic blood pressure, especially with urocortin 3 in healthy subjects. This

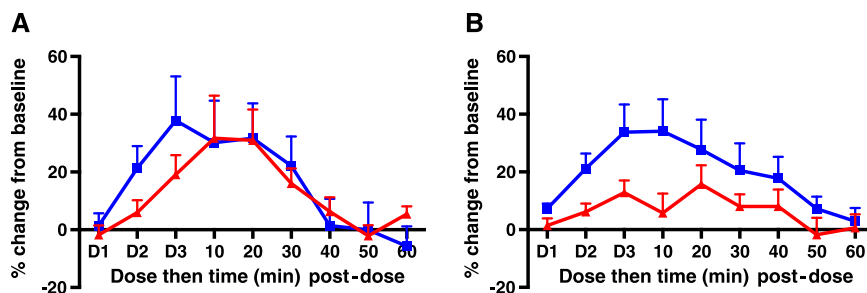
suggests that at the doses used here, we had systemic spill over of urocortin such that we achieved vasoactive blood concentrations outwith the forearm. The doses where systemic spill over was seen helped guide the dosing regime used in protocol B. We have seen systemic spill over previously with other compounds such as substance P [25] and this is heralded by systemic flushing and rises in the contralateral non-infused forearm blood flow. However, here we observed no change in contralateral non-infused forearm

Table 2

Results of systemic infusion of urocortin 2 and 3

	Urocortin 2	Urocortin 3	SNP
Healthy volunteers			
Cardiac index	+11.9 [−3.1, +26.9] <i>P</i> = 0.17	+25.4 [+9.8, +41.0]***	+21.7 [+5.5, +37.8]**
Heart rate	+17.2 [+7.2, +27.1]***	+25.0 [+14.7, +35.4]****	+29.0 [+18.4, +39.8]****
MAP	−8.0 [−2.6, −13.5]**	−10.8 [−5.1, −16.4]****	−13.0 [−7.2, −18.8]****
SV	−3.7 [−13.8, +6.4] <i>P</i> = 0.77	−6.3 [−16.9, +4.2] <i>P</i> = 0.40	−6.8 [−17.4, +3.9] <i>P</i> = 0.35
PVRI	−17.6 [−3.5, −31.7]**	−23.1 [−8.4, −37.8]***	−22.1 [−6.9, −37.3]**
Heart failure			
Cardiac index	+10.1 [+1.0, +19.3]*	+23.5 [+14.3, +32.7]****	+14.2 [+3.8, +24.7]**
Heart rate	+7.1 [−0.8, +15.0] <i>P</i> = 0.1	+18.1 [+10.0, +26.3]****	+7.4 [−1.8, +16.6] <i>P</i> = 0.16
MAP	−4.8 [−0.5, −9.0]*	−9.1 [−4.8, −13.5]****	−13.6 [−8.7, −18.5]****
SV	+2.7 [−2.4, +7.8] <i>P</i> = 0.51	+4.3 [−0.9, +9.5] <i>P</i> = 0.14	+0.7 [−4.8, +6.3] <i>P</i> = 0.99
PVRI	−14.0 [−5.3, −22.7]***	−25.2 [−16.2, −34.2]****	−22.1 [−12.2, −32.0]****

Mean difference [95% CI] of each agent with saline placebo across the three administered doses. * represents significant differences from placebo. (*****P* < 0.0001, ****P* < 0.001, ***P* < 0.01, **P* < 0.05).

**Figure 4**

Duration of haemodynamic response (min) to intravenous urocortin 2 and urocortin 3 (protocol B). Effects of urocortin 2 (red) and urocortin 3 (blue) last 40–60 min after cessation of dose 3 (D3) before returning to baseline. At the doses used, urocortin 3 caused a greater increase in cardiac output compared with urocortin 2 in patients with heart failure (*P* < 0.0001) but not healthy subjects (*P* = 0.48)

blood flow although subjects did develop some skin flushing. This suggests that other vascular beds, such as the dermal and splanchnic circulation, are more sensitive to the actions of the urocortins than the forearm circulation. The increase in heart rate at the highest dose of urocortin 3 was less pronounced in patients with heart failure and this was likely due to concomitant β -adrenoceptor blocker therapy. Furthermore the associated lower systolic and diastolic blood pressures in this group may be explained by a relative lack of compensatory tachycardia response to vasodilatation in these patients.

During systemic intravenous infusions, we observed increases in cardiac output with both urocortins. This increase in cardiac output is likely to occur in response to the systemic vasodilatation induced by the urocortins. Although there was no change in stroke volume for either urocortin, this is not a direct measurement of inotropy and we cannot exclude a

direct inotropic effect of urocortin on the heart, as previously seen with urocortin 2 in rodents [26]. At the doses we administered, urocortin 3 exerted more marked haemodynamic effects than urocortin 2 in patients but not healthy subjects. This may reflect the differences in doses we employed, but may also reflect important differences between these two agents. This needs further exploration to determine which urocortin subtype is the most promising for clinical therapeutic development.

Differences in biological effects between the urocortins can be explained by the structural differences that exist between the two agents, generating conformational changes in the G protein coupled receptor, in turn altering secondary messenger systems that are responsible for creating the biological effects seen. However it may not be this simple and there may be other systems involved. Promising preclinical studies suggest a role for the urocortins in the inhibition

of cardiac sympathetic nerve activity [SNA] [27, 28], often overactive in patients with heart failure. Although Ucn2 has been reported to increase skeletal muscle SNA in humans [29], this should not necessarily be seen as a discrepant finding as SNA responses typically are regionally differentiated.

Increases in heart rate have not been reported in recent studies with urocortin 2/stresscopin [16, 17]. However higher doses of urocortin 2 [14], comparable with the doses we have used here, saw similar increases in heart rate.

There may be concern regarding the clinical use of a heart failure therapy that is both positively chronotropic and inotropic. This combination of effects might predispose to increased myocardial oxygen consumption and potential arrhythmia, particularly in patients with coronary artery disease. We observed no episodes of arrhythmia or adverse effects in our study and, indeed, urocortin 2 has been shown to have anti-arrhythmic effects that would be hugely beneficial in treating this group of patients [30, 31]. However, our infusions were brief and adverse effects may be seen with longer infusions or in those patients with decompensated heart failure. In addition, avoidance of significant hypotension remains an important consideration, especially in patients with already low perfusion pressure or renal impairment. Results from our study suggest that dose titration during administration may be needed to optimize cardiac output and vasodilatory responses, whilst avoiding the unwanted effects of significant tachycardia or hypotension.

Study limitations

This study included only patients with stable heart failure who were prescribed evidence-based heart failure therapy that may have affected the response of these agents. However, there was no evidence of diminished effect with concomitant medical therapy in patients with heart failure. We did not include patients with acute decompensated heart failure but we did compare with healthy control subjects, not used in a similar sized study with urocortin 2 [14]. Although our results cannot be extrapolated to the setting of acute heart failure, there appears to be no reason why the beneficial effects seen in this study cannot be replicated in the acute heart failure setting provided an appropriate, controlled dosing regime is used. Furthermore studies using urocortin 2, and derivatives thereof, in the acute setting have already been carried out successfully. There does however remain a clear need for urocortin 3 to be trialled in this group of patients.

We observed increases in cardiac index in protocol B but it should be reinforced that this study was not designed to compare the relative contributions of chronotropy, inotropy and vasodilatation to the changes in cardiac index we recorded. Invasive studies would be required for this. Furthermore, the molar concentrations of urocortin 2 and urocortin 3 at each dose were different and differences in efficacy may reflect the differences in dose used and not true differences in potency.

Finally although there was no change in stroke volume for either agent, we did not conduct invasive haemodynamic monitoring which would be required for assessment of true inotropy and also useful for assessment of pulmonary capillary wedge pressure (PCWP). Surprisingly two recent studies did not detect a statistically significant reduction in

PCWP in patients with heart failure with either urocortin 2/stresscopin [16, 17]. However a clear trend toward a reduction in PCWP was seen in both studies.

In conclusion, we demonstrate that both urocortin 2 and urocortin 3 increase cardiac index and reduce peripheral vascular resistance. Their parenteral administration was feasible, safe and well tolerated. We conclude that these data provide further evidence suggesting urocortin 2 and urocortin 3 continue to hold promise for the treatment of heart failure.

Competing Interests

All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare no support from any organization for the submitted work, no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years and no other relationships or activities that could appear to have influenced the submitted work.

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