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Peripheral Haemodynamic Studies In Patients With Cirrhosis And Portal Hypertension

By
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A thesis submitted for the degree of
Doctor of Philosophy (Ph.D.)

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DEDICATION

This thesis is dedicated to my parents, my wife and my daughters Eman, Salma, Amal, and Sarah.

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ABSTRACT OF THESIS

Peripheral Haemodynamic Studies In Patients With Cirrhosis And Portal Hypertension.

Background & Objectives: Patients with liver cirrhosis and portal hypertension have systemic vasodilatation and activated neurohumoral systems. These abnormalities worsen with disease severity and are responsible for the development of its associated complications. The aims of the present series of studies were to evaluate the contribution of endogenous angiotensin II (ANG II) to the maintenance of basal and sympathetically-stimulated peripheral vascular tone, to assess the contribution of endothelin-1 and nitric oxide (NO) to the maintenance of basal peripheral vascular tone; to determine the forearm vascular responses to exogenous ANG II, endothelin-1, and noradrenaline; and to study the role of endogenous NO release in mediating the peripheral vascular response to ANG II and to endothelin receptor antagonism.

Methods: In patients with cirrhosis and matched healthy controls, bilateral forearm blood flow (FBF) was measured using venous occlusion plethysmography at baseline, and after unilateral sub-systemic, intra-brachial infusions of incremental doses of agonists or selective receptor antagonists, with or without the application of lower body negative pressure (LBNP) or 'NO-clamp': a balanced co-infusion of L-NMMA (a NO synthase inhibitor) and sodium nitroprusside (an exogenous NO donor) to block endogenous NO release and restore normal basal blood flow respectively. Baseline systemic haemodynamic parameters, and plasma hormonal concentrations, were also measured using electrical bioimpedance and radioimmuno-assay respectively.

Results: Noradrenaline, angiotensin II, endothelin-1, BQ-788 (a selective endothelin type B receptor antagonist), and L-NMMA reduced FBF and, whilst responses to noradrenaline, BQ-788, and L-NMMA were similar across groups, those to ANG II and endothelin-1 were greater in the healthy controls than in patients with cirrhosis. Both losartan (a selective ANG II type 1 receptor antagonist) and BQ-123 (a selective endothelin type A receptor antagonist) increased FBF, but responses to losartan were enhanced only in patients with advanced cirrhosis, and those to BQ-123 were enhanced in patients with pre-ascitic cirrhosis. LBNP caused similar reductions in FBF during both saline and losartan infusions. However, responses to LBNP were less in patients with advanced cirrhosis only. In the presence of the 'NO-clamp', the ANG II mediated vasoconstriction was enhanced in patients with cirrhosis, unchanged in controls, and became similar in both groups. In contrast, the BQ-123-induced vasodilatation was abolished in controls and attenuated in patients during the 'NO-clamp', with FBF remaining significantly greater in patients with cirrhosis.

Conclusions: These studies indicate that ANG II contributes to the maintenance of basal peripheral vascular tone only in advanced cirrhosis, but not to sympathetically-stimulated vascular tone. The impaired responses to ANG II and endothelin-1 are apparent in early cirrhosis, whilst those to LBNP are impaired only in advanced cirrhosis despite normal responses to noradrenaline infusion. In addition, the enhanced BQ-123 vasodilatation suggests an activated endothelin system and a greater contribution of endogenous ET-1 to the maintenance of basal forearm vascular tone in patients with pre-ascitic cirrhosis. These findings suggest a compensated vasodilated state and a role of ANG II, endothelin-1 and the cardiopulmonary baroreceptors in the pathogenesis and perpetuation of the haemodynamic derangements of early and advanced cirrhosis respectively. Moreover, there is an important interaction between the endogenous tonic vasopressor and vasodepressor systems since NO generation largely mediates the vasodilatation response to ET_A receptor antagonism, and, in patients with cirrhosis, the impairment of ANG II vasoconstriction.

DECLARATION

I declare that the composition of this thesis and the work presented herein is my own. I also declare that this thesis was written by me. I performed the experiments included in this thesis while holding a honorary clinical assistant and research fellow post at the Department of Medicine in the Royal Infirmary of Edinburgh, together with my full-time studentship in the Faculty of Medicine, Edinburgh University, in collaboration with the Clinical Research Centre in the Western General Hospital in Edinburgh, and under supervision of Professor Peter C. Hayes, Professor of Hepatology, the Royal Infirmary of Edinburgh.

Blood assays were performed with the technical assistance of Neil R. Johnson (Clinical Pharmacology Unit, Western General Hospital, Edinburgh) and Rhona Stevens (Department of Clinical Biochemstery, Royal Hospital for Sick Children, Edinburgh). All the work described in this thesis has been presented in scientific meetings and has been published, accepted, or submitted for publication. Papers and communications arising from this work are listed in the Appendix. I have not presented the studies included in this thesis in candidature for any other degree, diploma or qualification.

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LIST OF ABBREVIATIONS

4,5-PIns,	phosphatidylinositol 4,5-biphosphate.
ACE,	angiotensin converting enzyme.
ANG II,	angiotensin II.
ANOVA,	analysis of variance.
ANP,	atrial natriuretic peptide.
AT-1 receptor,	angiotensin II type 1 receptor.
AT-2 receptor,	angiotensin II type 2 receptor.
Big ET-1,	big endothelin-1.
BMI,	body mass index.
BNP,	brain natriuretic peptide.
BP,	blood pressure.
BSA,	body surface area.
cAMP,	cyclic adenosine monophosphate.
cGMP,	cyclic guanosine monophosphate.
CGRP,	calcitonin-gene-related peptide.
CI,	cardiac index.
CNP,	c-type natriuretic peptide.
CO,	cardiac output.
COX,	cyclo-oxygenase.
CV,	coefficient of variation
CVP,	central venous pressure.
DAG,	diacylglycerol.
DHV,	dorsal hand vein.
ECE,	endothelin converting enzyme.
EDHF,	endothelium-derived hyperpolarizing factor.
EDRF,	endothelium-derived relaxing factor.
eNOS,	endothelial nitric oxide synthase.
ET-1,	endothelin-1.
ET_A,	endothelin type A receptor.
ET_B,	endothelin type B receptor.
FBF,	forearm blood flow.
GFR,	glomerular filteration rate.
HR,	heart rate.
iNOS,	inducible nitric oxide synthase.
IP3,	Inositol triphosphate.
LBNP,	lower body negative pressure.
L-NMMA,	L-N^G-monomethyl-arginine.
MAP,	mean arterial pressure.
MW,	molecular weight.
Nad,	noradrenaline.
NO,	nitric oxide.
NOS,	nitric oxide synthase.
nNOS,	neuronal nitric oxide synthase.
PAF,	platelet activating factor.
PGI_2,	prostacyclin.
PKC,	protein kinase subtype C.

PLC,	phospholipase C.
PRA,	plasma renin activity.
RAS,	renin angiotensin system.
RBF,	renal blood flow.
RIA,	radioimmunoassay.
SD,	standard deviation.
SEM,	standard error of the mean.
SI,	stroke index.
SNP,	sodium nitroprusside.
SNS,	sympathetic nervous system.
SP,	substance P.
SR,	sarcoplasmic reticulum.
SV,	stroke volume
SVR,	systemic vascular resistance.
TIPSS,	transjugular intrahepatic portosystemic stent shunt.
TNF-α,	tumour necrosis factor-alpha.
TPVRI,	total peripheral vascular resistive index.
VIP,	vasoactive intestinal peptide.
VSMC,	vascular smooth muscle cell.

ACKNOWLEDGEMENTS

Professor Peter C. Hayes who provided valuable advice and critical discussions during the whole research period supervised this work. He also encouraged me in during all preparation stages of this thesis. I am most grateful to him for his help understanding, support and assistance. Professor Hayes was of great help to me even before my arrival in Edinburgh. His supervision will never be forgotten. Indeed, I am indebted to him.

I very much thank Professor David J Webb for his excellent collaboration and generous support efforts especially during the production of publications arising from this work. Professor Webb gave me full access to all the facilities of the Clinical Research Centre at the Western General Hospital. I have learned a lot about writing and organizational skills from Professor Webb for which I am most grateful.

All my sincere thanks and deep gratitude is due to Dr. David E. Newby for his collaborative efforts, for providing the best example, for teaching me the forearm blood flow technique, and for his understanding, advice and broad support during all stages of development of this work I have also learned a lot of research skills from Dr. Newby for which I am most grateful.

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I wish to express my thanks to all members of the Liver Unit, Department of Medicine, Royal Infirmary of Edinburgh, and Clinical Pharmacology Unit and Research Centre, Western General Hospital, for the friendly and supportive environment during conduct of this work.

I wish to thank my parents, my wife, and my daughters for their patience, understanding, and extreme support throughout period of conducting this research and preparation of this thesis.

Before all and after all, my sincere thanks, praise, and gratitude are due to GOD for his uncountable blessings.

Ahmed Helmy Salem

CHAPTER 1

INTRODUCTION AND AIMS

1. Haemodynamics of cirrhosis and portal hypertension

1.1. Historical background:

1.1.1. Liver cirrhosis:

Liver cirrhosis is a serious chronic disease that has been recognised for many centuries. Hippocrates wrote "in cases of jaundice, it is a bad sign when the liver becomes hard" (Chadwick & Mann 1950). Matthew Baillie, a Lanarkshire-born physician, has provided the earliest illustration of a cirrhotic liver in 1793. However, Laennec was the first physician to use the term 'cirrhosis' early in the nineteenth century. Later in the same century, William Osler wrote that in cirrhosis "the prognosis is, as a rule, bad" (Osler 1892). At present, cirrhosis of the liver is known to have a variety of causes, and is one of the greatest health problems worldwide.

1.1.2. Portal Hypertension:

Portal hypertension has also been recognised for a number of centuries, and was identified as a potential cause of gastrointestinal bleeding by Vesaluis and Morgagni (Sandblom 1993). Oesophageal varices were shown to be a site of communication between the systemic and portal venous systems by Sappey in the nineteenth century (Sandblom 1993). The term 'portal hypertension' was first used in 1906 (Gilbert & Villaret 1906), and, until 1937, the increase in portal pressure could only be measured directly at laparotomy (Sandblom 1993). Although under investigation for 50 years, the pathogenesis of portal hypertension remains poorly characterised and incompletely understood.

1.1.3. Haemodynamics of portal hypertension:

The first evidence of increased total plasma volume in the patients with cirrhosis was obtained in 1946 (Perera 1946). The association between portal hypertension and a hyperdynamic circulatory state was first described by Kowalski & Abelmann in 1953, subsequently confirmed by others (Murray et al 1958; Kontos et al 1964), and reviewed later by Martini et al in 1972. The presence of marked splanchnic vasodilatation in patients with cirrhosis was reported by Kotelanski et al 1972. However, in the early 1960s, many studies showed that the incidence of arterial hypertension is 10 times less in patients with cirrhosis than the general population,

and that arterial hypertension in these patients disappears with the development of portal hypertension (Lowke 1962; Mashford et al 1962). Plevris and colleagues have shown inverse correlation between the arterial blood pressure and the severity of liver disease (Plevris et al 1990).

1.2. Description:

The haemodynamic changes of portal hypertension refer mainly to increased cardiac output (CO), increased heart rate (HR), reduced systemic vascular resistance (SVR), and splanchnic vasodilatation. The severity of these changes correlates with the clinical indices of hepatic dysfunction (Braillon et al 1986; Schrier et al 1988; Bendtsen et al 1990; Meng et al 1994). A list of the components of the hyperdynamic syndrome in cirrhosis is shown in Table 1-1.

1.3. Importance and sequels

The hyperdynamic disturbances associated with portal hypertension are no longer an academic curiosity. Indeed, it is influenced by the aetiology of liver disease, which is most frequently alcohol-related in western countries. Moreover, many studies have shown that certain haemodynamic characteristics, including the low arterial blood pressure, have prognostic significance (Llach et al 1988, Gluud et al 1988). In addition, the hyperdynamic disturbances may be responsible for the development of the life-threatening complications of cirrhosis including ascites, hepatic encephalopathy, variceal haemorrhage (Schrier et al 1988), and hepatorenal failure (Epstein et al 1970; Badalamenti et al 1993). These complications, especially variceal haemorrhage, are serious and may lead directly to death (D'Amico et al 1986). Therefore, a better understanding of the mechanism(s) underlying this hyperdynamic circulation is essential for the development of therapeutic, and possibly preventive, interventions.

Table 1-1: The hyperdynamic circulatory syndrome of cirrhosis.

Organ	Changes
Cardiovascular system	↑ Cardiac output & ↑ heart rate
	↓ Mean arterial pressure
	↓ Systemic vascular resistance
	↓ Peripheral oxygen utilization
	↑ Plasma volume
	Cardiac dysfunction and ? heart failure
Lungs	↓ Diffusion capacity
	↓ Partial pressure of oxygen
	Hepatopulmonary syndrome
Kidney	↓ → Renal blood flow
	↓ Glomerular filteration rate
	Na^+ and water retention
	Hepatorenal syndrome
Brain	↓ → Cerebral blood flow
	? Encephalopathy
	? Brain oedema
Liver and splanchnic circulation	Portal hypertension
	↑ Portal blood flow
	↓ → ↑ Hepatic blood flow
	↑ Sinusoidal vascular resistance
	opened portosystemic collaterals
Skin and muscle	↑→ Skin blood flow
	↑→ Muscle blood flow

↓ = decrease. → = normal. ↑ = increased.

The haemodynamic changes are related to liver pathology as evidenced by the significant rise in the mean arterial pressure (MAP) and SVR, and the reduction in the cardiac index (CI) following orthotopic liver transplantation (Navasa et al 1993; Gadano et al 1995). The nature of the relationship between liver pathology, portal hypertension, SVR, and renal blood flow is unclear, although there is evidence of a

hepatorenal reflex (Jalan et al 1997). However, the haemodynamic changes also exist in association with pre-hepatic portal hypertension (e.g. due to portal vein thrombosis or hepatic schistosomiasis) in the absence of cirrhosis (Bosch et al 1988), suggesting an independent role of high portal pressure in the pathogenesis of circulatory changes in cirrhosis.

1.4. Pathogenesis:

Portal hypertension is initiated by the increased hepatic vascular resistance within the cirrhotic liver [the backward theory], and is exacerbated by the increase in portal blood flow [the forward theory] (Vorobioff et al 1983; Benoit et al 1985; Bosch et al 1988; Grose & Hayes 1992; Figure 1-1).

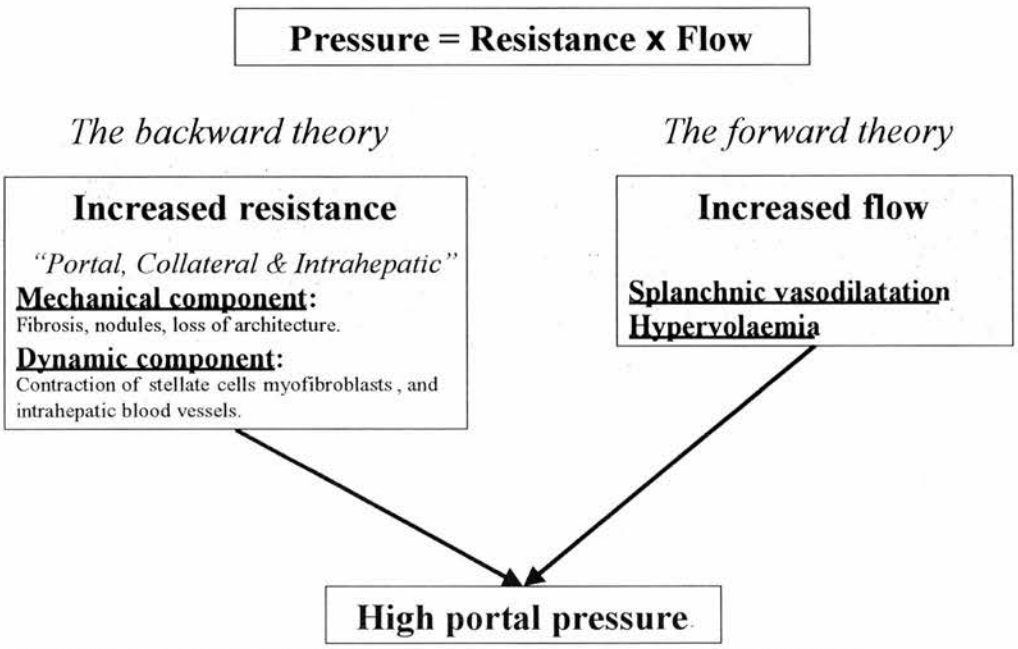


FIGURE 1-1: PATHOGENESIS OF PORTAL HYPERTENSION.

Over the last 4 decades, many theories have been proposed to explain the pathogenesis of the hyperdynamic circulatory changes and its complications especially ascites formation, in patients with cirrhosis. In general, these haemodynamic changes have been linked to the development of portal hypertension and liver dysfunction, but the exact mechanism remains controversial (Badalamenti et al 1993, Wong et al 1997). The following theories have been proposed:

1.4.1. The "Underfill Theory":

This theory suggests that the imbalance between hepatic sinusoidal hydrostatic and oncotic pressures leads to the accumulation of hepatic lymph in the peritoneal cavity, with a subsequent reduction in the effective arterial blood volume that stimulates the vasoconstrictor systems, and triggers renal sodium and water retention (Figure 1-2). However, this theory supposes that the plasma volume and CO would be low, which is not the case even in the pre-ascitic stage of the disease (Witte et al 1971; Epstein 1979).

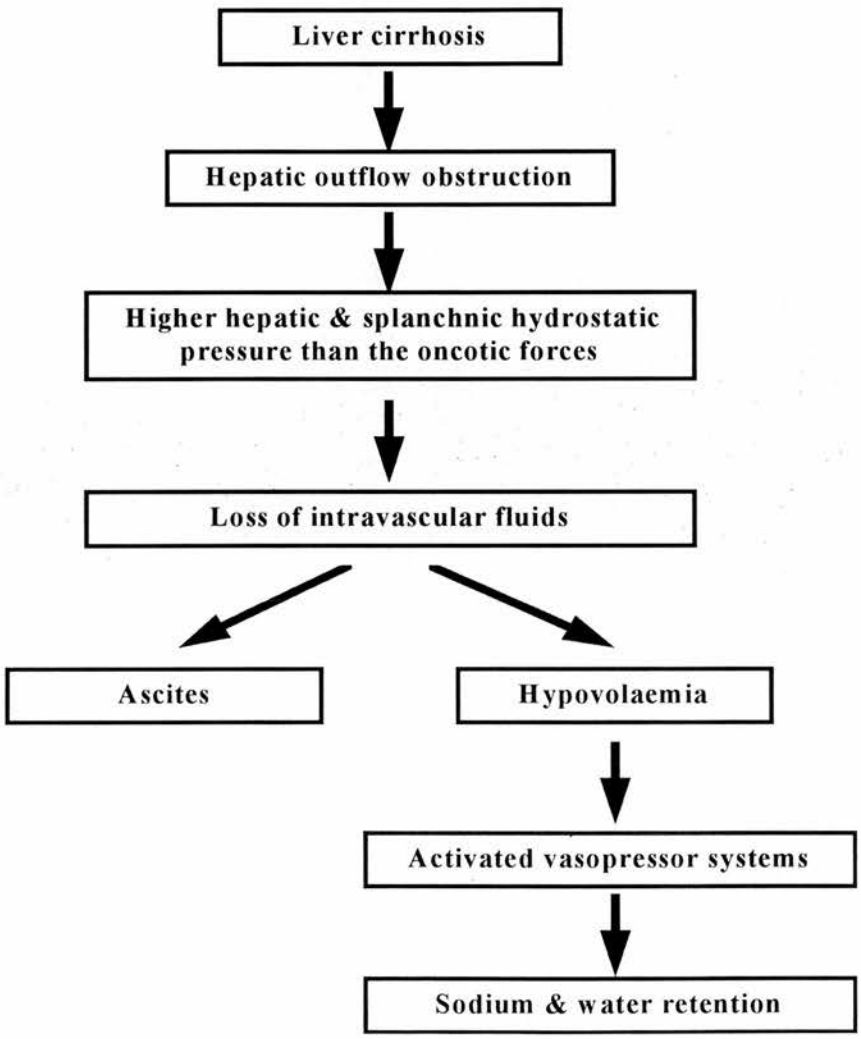


FIGURE 1-2: DIAGRAM OF THE UNDERFILL THEORY.

1.4.2. The "Overflow Theory":

This theory" proposes that renal sodium retention stimulated by liver dysfunction and/or portal hypertension, perhaps via a humoral factor or the autonomic nervous system, produces expansion of plasma volume and CO (Figure 1-3). This does not explain the disproportionate increase in the vascular compartment, reduction in SVR, and the activated vasoconstrictor systems found in these patients (Lieberman et al 1970).

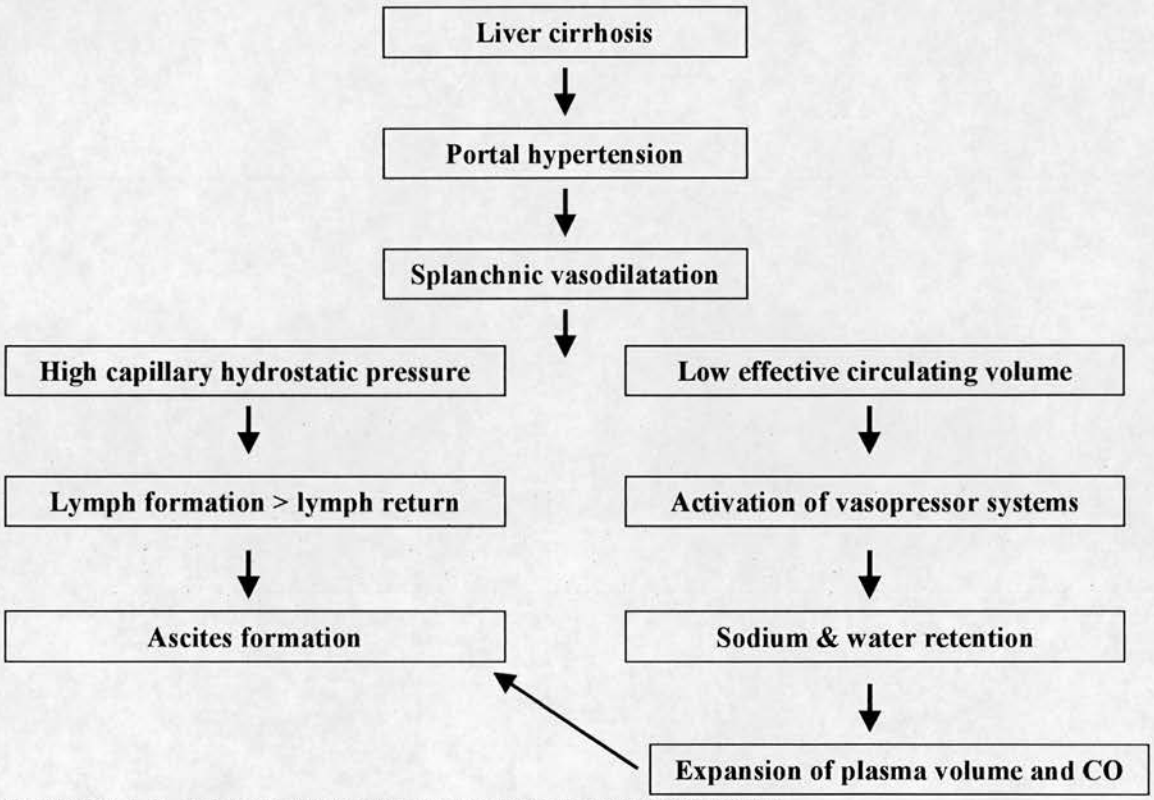


FIGURE 1-3: DIAGRAM OF THE OVERFLOW THEORY.
CO, cardiac output.

1.4.3. The "Peripheral Arterial Vasodilatation Theory":

This theory (Figure 1-4) superseded and unified the strongest aspects of its predecessors, and proposes that portal hypertension and other factors stimulate peripheral vasodilatation leading to a reduction in the SVR and "effective" hypovolemia. This results in compensatory activation of the vasoconstrictor system including the renin-angiotensin system (RAS), the sympathetic nervous system (SNS), and the anti-diuretic hormone (ADH), in an attempt to restore normal SVR.

This usually succeeds in increasing the blood volume and restoring blood pressure in patients with pre-ascitic cirrhosis and mild to moderate portal hypertension, and as a result, inactivates the vasoconstrictor systems either partial or complete (Schrier et al 1988).

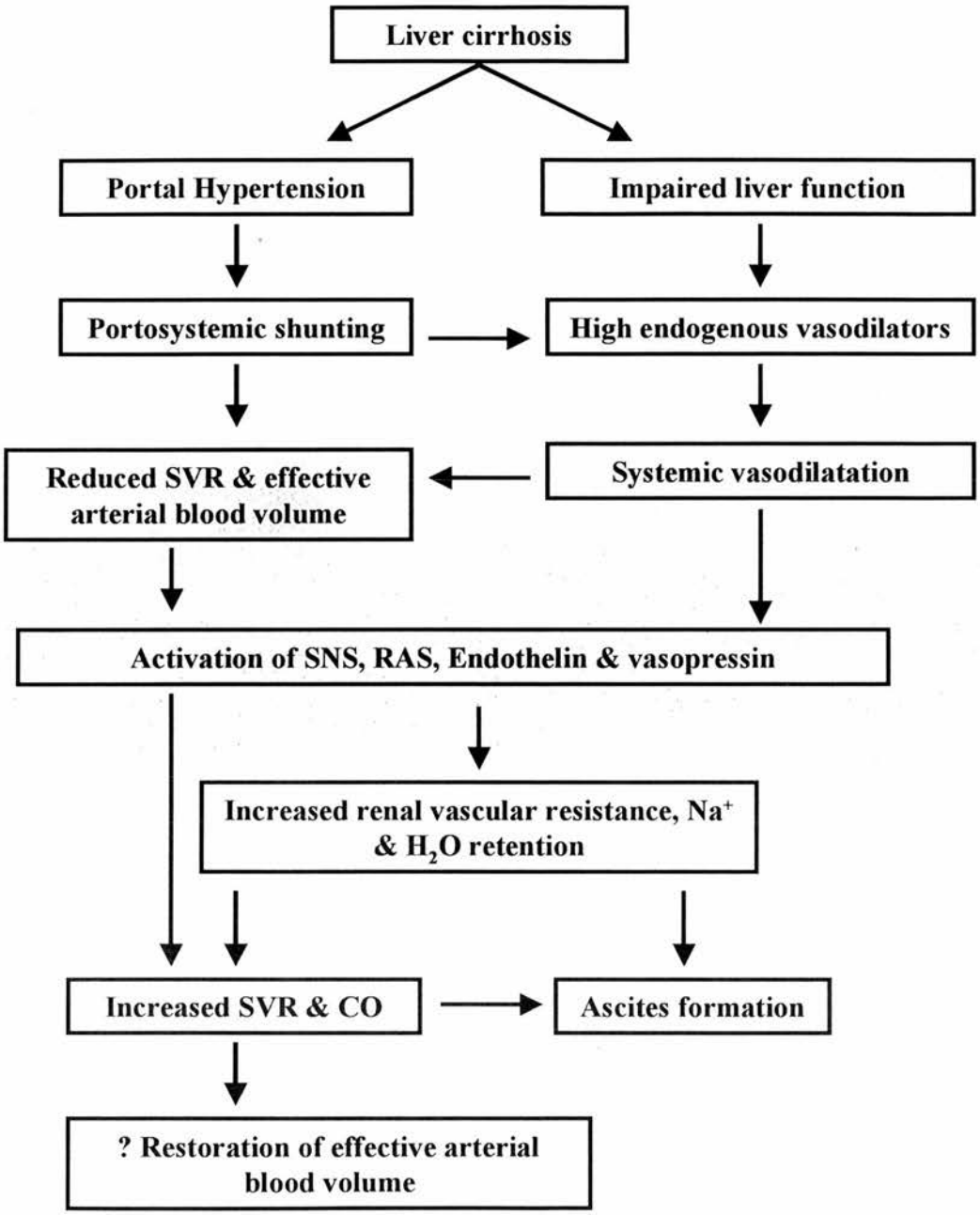


FIGURE 1-4: DIAGRAM OF THE PERIPHERAL ARTERIAL VASODILATATION THEORY.
CO, cardiac output. RAS, renin angiotensin system. SNS, sympathetic nervous system SVR, systemic vascular resistance.

In patients with severe portal hypertension, however, the increase in blood volume is not sufficient to normalise the SVR, the vasoconstrictor systems become more activated with a continued renal fluid retention. Ascites then develop as a result of fluid sequestration from the portal venous system into the peritoneal cavity (Rahman et al 1992). Recently, the peripheral arterial vasodilatation theory has been challenged (Wong et al 1997) following demonstrations in some patients that the hyperdynamic syndrome is only evident when they are seated (Iwao et al 1997; Bernardi et al 1992) and that the increase in CO precedes peripheral vasodilatation (Lewis et al 1992). This suggests that primary arteriolar dilatation, followed by a compensatory increase in CO, is not the main mechanism underlying the development of the hyperdynamic circulation. Rather, the increase in CO, with a compensatory reduction in SVR has been proposed to result from an increase in the cardiac preload (Bernardi & Trevisani 1997).

1.5. Site of initial vasodilatation:

Traditionally, the cutaneous and muscular vascular territories have been proposed as the main site of dilatation (Kontos et al 1964). However, this has recently been challenged by *in vivo* studies, which showed that the brachial and femoral components of the CO are actually reduced in patients with cirrhosis and ascites (Fernandez-Seara et al 1989; Maroto et al 1993). Many studies in animals and humans with cirrhosis have indicated that vasodilatation occurs mainly in the splanchnic bed (Vorobioff et al 1984; Sato et al 1987; Maroto et al 1993; Iwao et al 1997), but cutaneous and muscular vasodilatation may also contribute to the reduction in SVR (Bernardi & Trevisani 1997; Fernandez-Seara et al 1989; Steele et al 1994). Teleologically, this makes sense, as one would believe that amongst the first changes seen might be a direct attempt to increase blood flow to the liver, which has been reduced due to the increased sinusoidal resistance.

1.6. Aetiology of the initial vasodilatation:

The exact cause of the initial vasodilatation remains unclear. Whatever the cause of the hyperdynamic circulation, it is evident that SVR falls in patients with cirrhosis and persists despite activation of the vasoconstrictor systems (Laragh 1962). This fall

in SVR may result from impaired responses to the activated vasoconstrictor systems (Lunzer et al 1975; MacGilchrist et al 1991) due to receptor or post receptor defects and/or increased activity of vasodilators such as atrial natriuretic peptide (ANP; Iwao et al 1997). Additionally, increased nitric oxide (NO) release (Battista et al 1997; Martin et al 1998) secondary to either increased shear stress (Bomzon & Blendis 1994) or activation of inducible NO synthase (iNOS; Vallance & Moncada 1991) may be involved.

1.7. Then vasoconstricted territories in cirrhosis:

In the presence of low SVR, activation of the vasoconstrictor systems would be expected in patients with cirrhosis, and is believed to contribute to renal arteriolar constriction (Arroyo et al 1988), and ultimately to development of the hepatorenal syndrome (Badalamenti et al 1993; Forrest et al 1996). In addition, cerebral vasoconstriction has been reported in these patients (Aggarwal et al 1994; Dillon et al 1995). Doppler ultrasound studies have also shown brachial and femoral vasoconstriction (Fernandez-Seara et al 1989; Maroto et al 1993), and thermography studies showed reduced peripheral blood flow (Steele et al 1994; Forrest et al 1995). However, the presence of pulmonary vasodilatation that develops in the context of a hepato-pulmonary syndrome (Matsubara et al 1984) indicates that the vasoconstriction is not universal.

1.8. Circulating blood volume in cirrhosis:

Central blood volume is the volume of blood present in the central vascular compartments, and is responsible for stimulation of the low-pressure cardiopulmonary and arterial receptors. Studies measuring mean transit time of an indicator calculated central blood volume and found that it is reduced in patients with cirrhosis and ascites, but peripheral blood volume is significantly increased (Wong et al 1994; Henriksen & Moller 1996). Moreover, central blood volume correlates directly with total SVR and inversely with portal pressure and the activity of the SNS (Henriksen & Moller 1996). Also, central blood volume is reduced in animals with portal hypertension (Colombato et al 1996). In addition, central blood volume can be assessed indirectly by measuring the activity of neurohumoral vasoconstrictor

systems (Henriksen et al 1998). Indeed, most patients with cirrhosis and ascites have activated vasoconstrictor systems, which can be suppressed by the administration of vasopressor agents or by the expansion of the plasma volume (Wong et al 1979; Shapiro et al 1985; Gines et al 1997; Jalan & Hayes 2000). These findings indicate that arterial vasodilatation causes an abnormal distribution of the total blood volume and a reduction in the effective arterial blood volume.

1.9. Haemodynamics and the stage of cirrhosis:

1.9.1. Stage of pre-ascitic cirrhosis:

Patients in the pre-ascitic stage of cirrhosis have moderate portal hypertension, increased total blood volume, increased CO, decreased SVR, and normal arterial pressure (Bosch et al 1988; Bernardi & Trevisani 1997). The changes in CO and SVR are more apparent in the supine position, and are probably caused by misdistribution of total blood volume, with increased blood volume in the splanchnic circulation that moves to the central circulation on lying down (Bernardi et al 1992; Bernardi & Trevisani 1997). In addition, when patients with pre-ascitic cirrhosis are given a high sodium diet, their central blood volume failed to increase, and the activity of vasoconstrictor systems are not suppressed, confirming misdistribution of total blood volume (La Villa et al 1992). Compared with healthy volunteers, patients with pre-ascitic cirrhosis show increased mesenteric blood flow and normal femoral blood flow, as measured by Doppler ultrasonography. This indicates the early onset of splanchnic arterial vasodilatation and confirms total blood volume misdistribution (Iwao et al 1997).

Despite their apparently normal renal function, these patients fail to escape the sodium-retaining effect of mineralocorticoids, and are unable to excrete a sodium load (Wood et al 1988; La Villa et al 1992; Gines & Schrier 1997). Moreover, natriuresis in these patients is significantly less when they remain upright compared with the supine position (Bernardi et al 1993) Also, in the upright position, patients with pre-ascitic cirrhosis have higher plasma aldosterone concentrations than healthy volunteers, and their plasma ANP concentrations are normal. On assuming the supine position, however, the plasma concentrations of aldosterone become normal, and

plasma ANP concentrations increase markedly (La Villa et al 1992; Bernardi et al 1993). Misdistribution of blood volume may be the cause of these hormonal changes, and may explain the subtle sodium retention observed when these patients are supine.

1.9.2. Stage of cirrhosis and ascites:

Patients with cirrhosis and ascites have severe portal hypertension, markedly increased intrahepatic vascular resistance, increased total blood volume, high CO, low SVR, and low arterial pressure (Groszmann 1994; Moller et al 1997). These haemodynamic changes are associated with persistent activation of the vasoconstrictor systems including the RAS, the SNS, the endothelin system, and vasopressin (Bichet et al 1982; Martin et al 1998). This activation is not affected by postural changes (Bernardi et al 1995). Moreover, the use of pharmacological agents to antagonise the effects of these vasoconstrictor systems produced marked reduction in total SVR and systemic hypotension. This indicates that activation of the vasoconstrictor systems in patients with decompensated cirrhosis constitutes a compensatory mechanism to maintain integrity of the systemic circulation (Schroeder et al 1976; Claria et al 1991; Esler et al 1992).

Patients with cirrhosis and ascites show elevated plasma ANP concentrations and reduced renal responses to this peptide (Gines et al 1988). The combination of low SVR, systemic hypotension, and marked activation of the vasoconstrictor systems is consistent with the peripheral vasodilatation theory (Schrier et al 1988). Furthermore, patients in the ascitic stage of cirrhosis have functional renal abnormalities. These include: 1) sodium retention, secondary to increased tubular reabsorption of sodium, with subsequent ascites and oedema formation; 2) progressive impairment in the ability to excrete solute-free water; 3) renal arteriolar vasoconstriction. These changes may lead to the development of dilutional hyponatremia, refractory ascites, and hepatorenal syndrome (Gines et al 1997). Substantial improvement in renal abnormalities is associated with increasing central blood volume and suppressing the activity of the neurohumoral systems (Shapero et al 1985; Guevara et al 1998; Soper et al 1996).

2. The vasoconstrictor systems in cirrhosis

2.1. Mechanism of action of vasoconstrictors:

Vasoconstriction may result from sympathetic stimulation (noradrenaline; Nad), endocrine hormones (ADH and angiotensin II; ANG II), locally produced hormones such as ET-1 (Gurney 1994), or by the myogenic response to pressure (Meninger & Davis 1992). In fact, plasma hormones (e.g., ANG II) may be produced locally, and the locally produced ones (e.g., ET-1) may have a humoral role.

Despite the diversity of vasoconstrictor agonists and their receptors, their mechanism of action is very similar. Binding of a vasoconstrictor agonist to its specific receptor, which is located on the vascular smooth muscle cells (VSMC), induces a cascade of second messengers leading ultimately to an increase in intracellular calcium, which is the signal for smooth muscle contraction (Figure 1-5). Briefly, the binding of vasoconstrictors to their G-protein coupled receptors causes hydrolysis of 4,5 phosphatidylinositol (4,5-PIns), resulting in the generation of two second messengers, inositol triphosphate ($InsP_3$) and diacylglycerol (DAG). The former mobilizes intracellular Ca^{2+} to activate protein kinase subtype C (PKC), which is one of the main transducers of the vasoconstrictor effects (Lee & Severson 1994), and Ca^{2+} calmodulin dependent kinases with subsequent phosphorylation of the myosin light chain and initiation of vasoconstriction (Rasmussen et al 1987). DAG is an endogenous PKC activator, which plays an important role in signal transduction of the contractile responses of the VSMC. It is generally accepted that activation of PKC, measured by its translocation from the cytosol to cell membrane, plays an important role in agonist-induced contraction of VSMC not only through phosphorylation of myosin light chains but also through modulating Ca^{2+} channel activity, Ca^{2+} pump activity, and the sensitivity of the proteins involved in controlling the contractile process, such as calponen and desmin, to Ca^{2+} (Andrea & Walsh 1992).

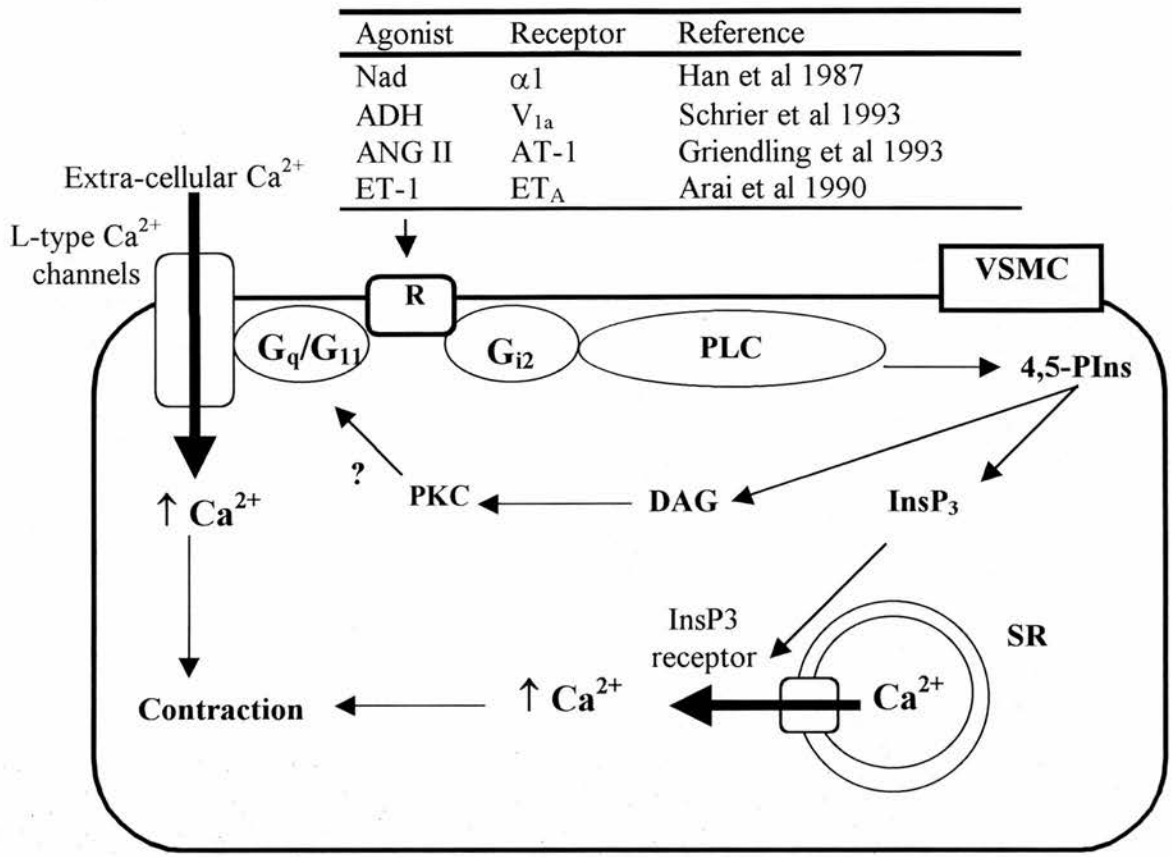


FIGURE 1-5: MECHANISM OF ACTION OF ASOCONSTRICTORS.
ADH, anti-diuretic hormone. ANG II, angiotensin II. DAG, diacylglycerol. ET-1, endothelin 1. G_{i2}, G protein of the G_i family. G_q/G_{11}, G protein of the G_q family. InsP3, inositol triphosphate. Nad, noradrenaline. 4,5 PIns, phosphatidylinositol 4,5-biphosphate. PLC, phospholipase C. PKC, protein kinase subtype C. R, receptor. SR, sarcoplasmic reticulum. VSMC, vascular smooth muscle cell.

2.2. The sympathetic nervous system (SNS):

2.2.1. The SNS in health:

The SNS has a central role in maintaining cardiovascular homeostasis (Hjemdahl 1990). This control system consists of peripheral receptors, which are responsive to changes in cardiovascular stability, afferent, central, and efferent integrated pathways, and neurohumoral effectors that ultimately modulate vascular tone (Abraham & Schrier 1994).

Baroreceptors represent an integral part of the regulatory system involved in the maintenance of circulatory stability (Schrier 1990). High-pressure arterial baroreceptors such as those found in the carotid sinus and aortic arch are present within the arterial vascular tree and will respond to changes in arterial pressure, which, in turn, is dependent on both CO and SVR (Abraham & Schrier 1994). Low-pressure cardiopulmonary baroreceptors respond to changes in cardiac preload, which is dependent on venous return to the heart and central blood volume (Mohanty et al 1985).

The reduction in cardiac preload (Stevens et al 1965) or arterial pressure (Schrier 1988), which is sensed by the low-pressure cardiopulmonary or the high-pressure arterial baroreceptors respectively, decreases the tonic inhibitory traffic in the afferent glossopharyngeal pathways to the central nervous system. Consequently, an increase in the efferent sympathetic activity occurs in a trial to preserve cardiovascular stability (Schrier 1988). The peripheral effects of this increase in SNS activity include: a) constriction of the arterial resistance vessels, b) an increase in the SVR, c) stimulation of renin release from the kidney leading to the activation of the RAS, and d) positive chronotropic and inotropic effects on the heart. The resultant increase in ANG II production will further increase the tone of the arterial resistance vessels. In addition, the SNS activity is also important in controlling the nonosmotic release of ADH (Schrier 1988).

Adrenergic pathways that run from the glossopharyngeal baroreceptor nuclei to the paraventricular and supraoptic nuclei in the hypothalamus terminate on neurons in these nuclei, which synthesise the ADH (Renud et al 1985; Brooks et al 1986; Sladek 1985). Electrical activity of the supraoptic neurons correlates with vasopressin release and is altered by changes in systemic blood pressure suggesting a role of vasopressin in blood pressure control (Renud et al 1985). Thus, a decrease in cardiac preload or arterial pressure inhibits baroreceptor activity and results in a simultaneous activation of the vasoconstrictor systems, namely, the SNS, the RAS and the nonosmotic release of ADH. Activation of these three systems is interdependent, and has additive and synergistic effects on the VSMC (Ishikawa et al

1984). The immediate response to this integrated system involves the vascular effects of the vasopressor hormones, but maintenance of the response is assisted by vasopressin-induced water retention, and by SNS and ANG II-mediated increase in the proximal renal tubular reabsorption of sodium (Schrier 1988).

Activity of SNS in humans can be assessed either by recording the sympathetic action potentials with micro-electrodes applied to the peripheral nerves (Wallin et al 1981; Christensen et al 1986), or by measuring the plasma concentration of the sympathetic neurotransmitters, which are secreted from the synaptic cleft into the plasma (Christensen 1973; Cryer 1976; Hjemdal 1984; Christensen et al 1986). Indeed, plasma concentrations of Nad in venous blood in the human forearm correlated with muscle sympathetic nervous activity (Wallen et al 1981).

2.2.2. The SNS in cirrhosis:

Since the first report by Shaldon et al in 1961, evidence of increased plasma concentrations of catecholeamines, in patients with cirrhosis and portal hypertension, is accumulating (Henriksen et al 1981; Arroyo et al 1983; Bichet et al 1982a; Bernardi et al 1983; Burghardt et al 1982; Nicholls et al 1985; Bendtsen et al 1990; Henriksen et al 1991; MacGilchrist et al 1991; Henriksen & RingLarsen 1994; Newby et al 1998a). Because the clearance of Nad is not significantly altered in patients with cirrhosis, the increased plasma concentration of Nad is a reflection of an increased release, rather than a decreased clearance (Zambraski & DiBona 1988). The highest plasma concentrations of Nad and adrenaline occur in patients with a high Child score, more pronounced portal hypertension, avid fluid and sodium retention, and hepatorenal syndrome (Arroyo et al 1983; Burghardt et al 1982; Gaudin et al 1991; Blends 1993; Fernandez-Rodriguez et al 1995), and shown further increase during β-adrenergic blockade with propranolol (Bendtsen et al 1990). Moreover, the plasma concentration of Nad is an independent prognostic factor in cirrhosis (Tage-Jensen et al 1988; Gines et al 1993). Also, the sympathetic burst frequencies recorded from skeletal muscle nerve fibres were enhanced in these patients and correlate positively with the plasma concentrations of Nad (Floras et al 1991).

Activation of the SNS in patients with cirrhosis is a homeostatic mechanism, which maintains basal peripheral vascular tone, as demonstrated by the reduction of arterial pressure following intravenous clonidine infusion (Willet et al 1986). However, activation of the SNS plays an important role in the pathogenesis of sodium and water retention. Indeed, a significant negative correlation was observed between plasma Nad concentrations and renal blood flow, glomerular filtration rate, urinary sodium and water excretion (Zambraski & DiBona 1988). Moreover, patients with cirrhosis and ascites submitted to head-out water immersion showed a significant increase in creatinine clearance (Zambraski & DiBona 1988).

Studies using selective catheterisation and tracer kinetic methods showed that SNS overactivity and Nad spillover occur in many organs, including the kidney, the liver, the prehepatic splanchnic areas, the heart, muscles and skin (Henriksen et al 1981; Henriksen et al 1984; Willet et al 1985; Henriksen et al 1986; Henriksen et al 1987; Henriksen et al 1991, Henriksen et al 1991a). Overactivity of the SNS in patients with cirrhosis may be triggered centrally by mechanisms such as encephalopathy and hypoxia, or peripherally by mechanisms such as low-pressure baroreceptor unloading, volume receptor stimulation, hepatocellular dysfunction and metabolic derangements (Henriksen et al 1998).

2.2.3. Lower body negative pressure (LBNP):

The technique of LBNP was first described to study responses to gravitational change (Stevens & Lamb 1965; Brown et al 1966). LBNP causes blood pooling in lower limbs, buttocks and pelvis, as demonstrated by pooling of plasma protein-bound ^{131}I (Wolthuis et al 1974), leading to a decrease in central venous pressure (CVP) and an increase in forearm vascular resistance at all degrees of LBNP.

Lower degrees of LBNP act through unloading the low-pressure cardiopulmonary receptors resulting in reflex sympathetic vasoconstriction in forearm resistance vessels. Higher degrees of LBNP unload both the low-pressure cardiopulmonary receptors and the carotid baroreceptors (Wolthuis et al 1974). Low degrees of LBNP

(<20 mm Hg) cause marked reduction in forearm blood flow (FBF) without affecting arterial blood pressure or HR (Zoller et al 1972; Johnson et al 1974). In contrast, higher degrees of negative pressure (20 - 80 mm Hg) increase HR, reduce the systolic and diastolic blood pressure (Steven & Lamb 1965; Brown et al 1966; Ardill et al 1967; Johnson et al 1974; Abboud et al 1979), decrease stroke volume (SV) and CO (Stevens & Lamb 1965; Murray et al 1968), and increase renin release (Baylis et al 1978; Mark et al 1978). These responses were blocked by propranolol (Mark et al 1978). Moreover, plasma concentrations of ADH increase only in subjects with LBNP-induced syncope (Baylis et al 1978; Goldsmith et al 1982) that usually develops during sustained LBNP of 30 mmHg or more (Murray et al 1968).

Sustained application of LBNP increases both forearm muscle sympathetic nerve activity (Sundlof & Wallin 1978) and plasma concentrations of Nad, which correlate with the fall in CVP (Goldsmith et al 1982; Grassi et al 1985). The LBNP-induced decrease in FBF can be abolished following sympathectomy (Ardill et al 1967) and is reduced by intra-brachial infusion of bethanidine or beryllium tosylate infusion (Brown et al 1966). These findings indicate that the SNS mediates the vasoconstriction observed during application of LBNP.

Patients with cirrhosis and ascites have reduced vasoconstrictor responses to the application of ice (Lunzer et al 1975) and to LBNP (Ryan et al 1993; Newby et al 1998a). The vasoconstrictor response to LBNP do not appear to be mediated by increased ANG II release, as they are not affected by concomitant infusion of losartan, an ANG II type 1 receptor antagonist (Newby et al 1998a). This indicates either impairment in the vascular smooth muscle cell contractile response especially when accompanied by impaired responses to Nad infusion (Lunzer et al 1975; Ryan et al 1993), or impairment in the low-pressure baroreceptor reflex when the vascular responses to Nad infusion are normal (Newby et al 1998a). The latter could be due to receptor down-regulation and/or defective neuronal transmission. In patients with pre-ascitic cirrhosis, however, LBNP application reduces the CVP and FBF, and increases plasma Nad concentrations and the renal sodium retention (Wong et al 1995, Wong et al 1998). These responses were similar in both patients and healthy

controls (Wong et al 1995, Wong et al 1998). In addition, LBNP increased renal concentrations of renin and ANG II in both groups, but this increase was significantly higher in the cirrhotic group (Wong et al 1998).

2.2.4. Autonomic dysfunction in cirrhosis:

Evidence of autonomic dysfunction in patients with cirrhosis has emerged from various studies utilising various tests, such as breath-holding, isometric exercise, atropine blockade, heart rate variability, orthostatic, and Valsalva manoeuvres (MacGilchrist & Reid 1990; Hendrickse et al 1992; Dillon et al 1994; Laffi et al 1996). These abnormalities are associated with the presence of liver failure, and are markers of an adverse prognosis (Dillon et al 1994; Hendrickse & Tiger 1992; Fleckenstein et al 1996). This autonomic dysfunction does not depend on disease aetiology (Hendrickse & Tiger 1992), and also occurs in patients with portal vein thrombosis (Voiget et al 1997). In addition, the autonomic dysfunction in cirrhosis involves vagal neuropathy (Hendrickse et al 1992; Hendrickse & Triger 1994), and is reversible after liver transplantation (Mohammed et al 1996). Also, blood pressure responses to orthostasis are impaired, probably due to blunted baroreceptor function (Bernardi et al 1982; Bernardi et al 1983; Iwao et al 1993). Moreover, sympathetic responses to isometric exercise are impaired in patients with cirrhosis (Bernardi et al 1982; MacGilchrist & Reid 1990). ANG II-mediated neuromodulation may be involved in the pathogenesis of the autonomic dysfunction in patients with cirrhosis, as evidenced by the correction of this dysfunction by captopril, an angiotensin converting enzyme inhibitor (Dillon et al 1997). The pathophysiological basis of the autonomic dysfunction is currently unknown, but it could occur within the central nervous system, within the peripheral nerves, or at the level of the post-receptor signal transduction pathways. On the basis of the available data, a multifactorial aetiology for the hyporesponsiveness in cirrhosis is most likely.

2.3. The renin-angiotensin system (RAS):

2.3.1. The RAS in health:

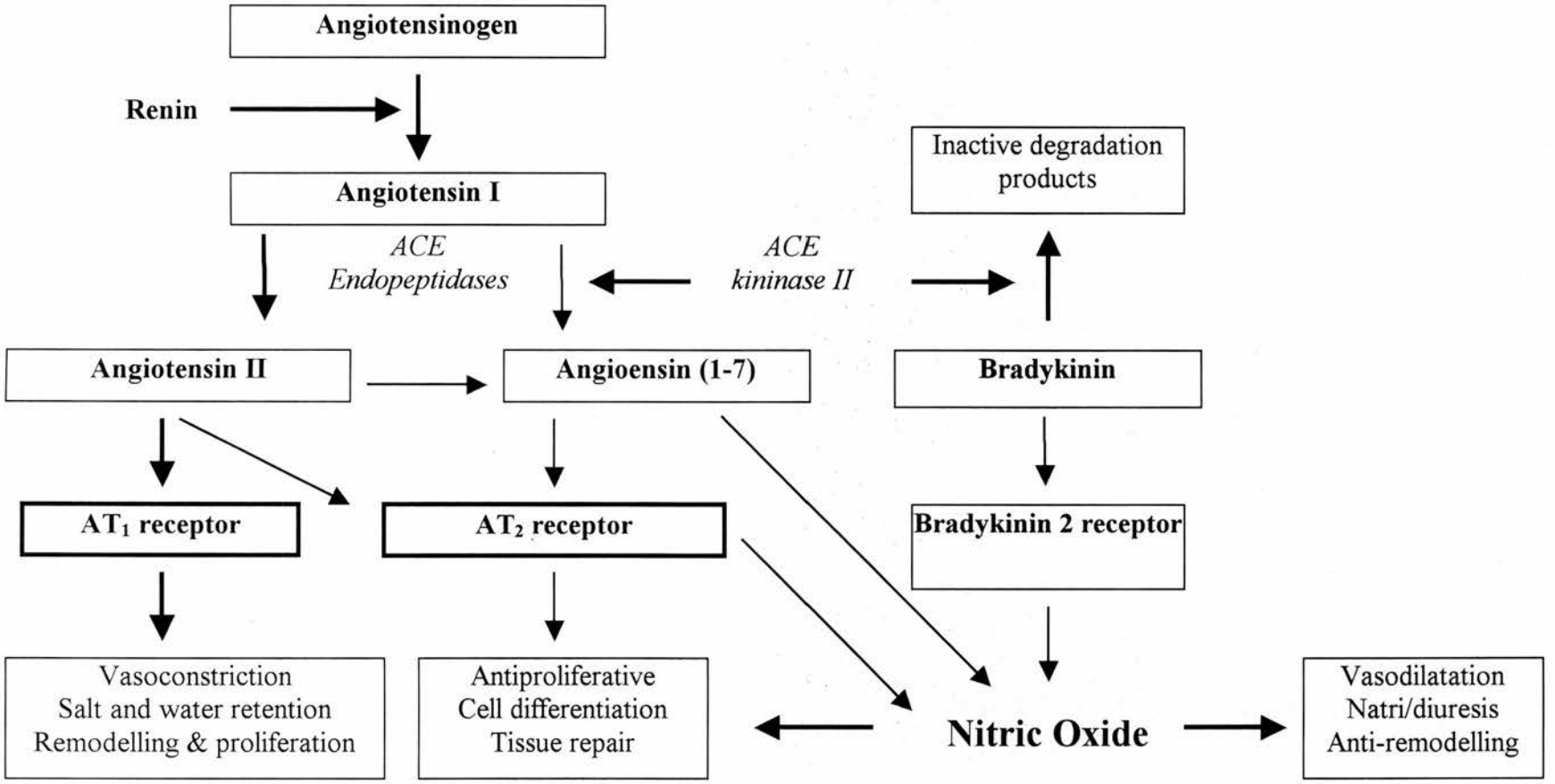
Through the direct actions of ANG II, the RAS plays a fundamental role in regulating blood pressure and body sodium and water balance. A reduction in arterial

pressure or effective arterial blood volume activates the RAS (Schrier & Caramelo 1988; Badalamenti et al 1993). Plasma concentrations of ANG II depend on the activity of renin, which is the rate-limiting step in converting angiotensinogen to angiotensin I, and on the activity of the angiotensin converting enzyme activity. In addition, the RAS can be manipulated at many levels including using enzyme inhibitors and receptor antagonists. The enzymatic cascade leading to the generation of ANG II is shown in Figure 1-6.

At least two main ANG II receptor subtypes have been identified: the AT_1 and AT_2 receptors. The majority of the physiological actions of ANG II, including vasoconstriction and aldosterone release, are mediated through the AT-1 receptor, whereas the role of the AT_2 receptor remains unclear (Matsubara et al 1998; Matsubara 1998). ANG II stimulates both endothelial NOS messenger RNA expression (Hennington et al 1998) and endothelial NOS activity (Olson et al 1997). Moreover, ANG II has been shown to increase NO bioavailability in isolated rings from rat carotid artery under static conditions (Boulanger et al 1995). Indeed, although low in large vessels such as the aorta, surface AT_2 receptor expression is high in the microvasculature (Nora et al 1998). Genetic murine AT_2 receptor knockout models are associated with increased systemic blood pressure and enhanced vasoconstrictor responses to ANG II (Hein et al 1995) whereas targeted overexpression of AT_2 receptor leads to an increased ANG II mediated cGMP production and reduced ANG II mediated vasoconstriction (Tsutsumi et al 1999).

In addition to the regulation of renin release, the SNS has synergistic effects with ANG II (Peach 1977). The magnitude of ANG II-induced vasoconstriction is enhanced during SNS inactivation, such as with sympathectomy or adrenergic blockade. This interaction may be produced through ANG II-mediated facilitation of Nad release or inhibition of its reuptake (Hjemdahl et al 1996). In addition, ANG II augments sympathetically mediated arterioconstriction (Seidelin et al 1991) and venoconstriction (Benjamin et al 1988) even when given at doses insufficient to cause vasoconstrictor or vasopressor responses.

FIGURE 1-6: THE GENERATION, RECEPTORS, ACTIONS, AND INTERACTIONS OF ANGIOTENSIN II.



2.3.2. The RAS in cirrhosis:

The RAS is activated in patients with cirrhosis with evidence of avid sodium retention (Epstein & Norsk 1988). This was demonstrated by increased plasma renin activity (PRA; Schroeder et al 1976; Bernardi et al 1982; Iwao et al 1994), and by raised plasma concentrations of ANG II (Hayes et al 1992; Newby et al 1998a) and aldosterone (Schroeder et al 1976). The elevated PRA in patients with cirrhosis is mainly due to an augmented renal release of renin, although reduced hepatic extraction may contribute (Arroyo et al 1979). Plasma ANG II and aldosterone concentrations and PRA correlate directly with the severity of cirrhosis, and inversely with both the MAP and the SVR (Aliaga et al 1995). In addition, PRA and plasma ANG II concentrations have been shown to correlate directly with the wedged hepatic venous pressure in patients with cirrhosis (Bosch et al 1980). This suggests a role for the increased intrahepatic pressure in facilitating renin release, and a role for ANG II in the altered hepatic haemodynamics. The former is supported by the presence of a hepatorenal reflex (Jalan et al 1997), and the latter is supported by several studies in animal and man, which showed an increase in the wedged hepatic venous pressure and a decrease in hepatic blood flow following systemic or portal infusion of ANG II (Segel et al 1963; Chiandussi et al 1963; Bashour et al 1963; Kelly & Nyhus 1966).

Despite the increased activity of the RAS, it retains its ability to respond to haemodynamic alterations (Iwao et al 1994; Bernardi et al 1983). Indeed, the response of the RAS may even be enhanced in patients with cirrhosis (Iwao et al 1994), but remains insufficient to restore normal haemodynamics in decompensated subjects (Schroeder et al 1976). Two weeks following orthotopic liver transplantation, the activity of the RAS returns to normal (Navasa et al 1993).

Reduced renal perfusion, secondary to activation of the SNS, may represent the primary stimulus for activation of the RAS in patients with cirrhosis. However, direct stimulation of the RAS may occur due to the reduction in the effective arterial blood volume as evidenced by the experiments of head-out water immersion (Epstein & Norsk 1988). In these patients, plasma aldosterone concentrations correlated

inversely with urinary sodium excretion (Wilkinson et al 1979). This suggests a permissive role for aldosterone in the pathogenesis in sodium retention in patients with cirrhosis (Arroyo et al 1988), as the majority of sodium retention, according to Epstein 1988, is due to impaired distal delivery of the filtrate. However, the co-existence of normal concentrations of aldosterone and sodium retention may be explained by the presence of high tubular sensitivity to aldosterone. This is evidenced by the ability of spironolactone, an aldosterone antagonist, to reverse sodium retention in these patients (Wilkinson et al 1979; Arroyo et al 1988).

Administration of saralasin (an ANG II-antagonist with partial agonist activity) or captopril (an angiotensin enzyme; ACE inhibitor) causes marked hypotension in patients with cirrhosis and ascites (Schroeder et al 1976; Pariente et al 1985; Wood et al 1985). Also, intra-brachial infusion of losartan, a selective AT_1 receptor antagonist, increases FBF in a similar group of patients (Newby et al 1998a). These results indicate that endogenous ANG II is essential for the maintenance of basal vascular tone in patients with cirrhosis and ascites. In addition, activation of the RAS is a determinant for the maintenance of glomerular filtration rate (GFR) as evidenced by the reduction of GFR by low dose captopril in patients with cirrhosis and ascites, without affecting arterial pressure (Gentilini et al 1993).

2.4. The endothelin system:

2.4.1. The endothelin system in health:

Endothelin-1 (ET-1), a potent vasoconstrictor, belongs to a 21-amino acid peptide family (ET-1, ET-2, ET-3) with a range of biological effects (Yanagisawa et al 1988; Spokes et al 1989; Masaki et al 1992). ET-1 was originally been identified in the culture medium of porcine aortic endothelial cells (Yanagisawa et al 1988; Masaki et al 1992), and is derived from a larger pre-pro-endothelin-1 (212 amino acids) which is cleaved by endopeptidases to produce big endothelin-1 (38 amino acids), which is then converted by specific endothelin converting enzymes to ET-1 (Figure 1-7; Yanagisawa et al 1988; Masaki et al 1992).

Molecular studies have, so far, identified two endothelin receptor subtypes in mammalian species (Figure 1-8): endothelin-A (ET_A) and endothelin-B (ET_B). In vascular smooth muscle cells, both receptors are expressed (Spokes et al 1989; Davenport et al 1995), and these mediate vasoconstriction (Spokes et al 1989; Tschudi & Luscher 1994; Davenport et al 1995; Haynes et al 1995). Only the ET_B receptors are found on endothelial cells, where they cause vasodilatation through the release of endothelium-derived vasodilators, such as nitric oxide (NO; Takayanagi et al 1991). ET-1 induced vasoconstriction is predominantly mediated by the ET_A receptor although ET_B receptors may contribute under some circumstances (Davenport & Maguire 1994).

2.4.2. The endothelin system in cirrhosis:

Patients with advanced cirrhosis and fluid retention have elevated plasma ET-1 concentrations (Moore et al; 1992; Uchihara et al 1992; Isobe et al 1993; Asbert et al 1993; Moller et al 1993; Matsumuto et al 1994; Gerbes et al 1995; Moller et al 1995; Salo et al 1995a; Kitano et al 1996; Newby et al 1998a; Nolte et al 2000), which are particularly high in patients with hepatorenal syndrome (Moore et al 1992; Bachmann-Brandt et al 2000). The elevated plasma concentrations of ET-1 normalise after liver transplantation (Bachmann-Brandt et al 2000), but remains elevated for up to 15 months following TIPSS (Nolte et al 2000). However, normal levels were shown in two earlier studies (Lerman et al 1991; Textor et al 1992). These two studies are different possibly because they involved assays, which lack sensitivity and specificity, and patients with early cirrhosis. The mechanisms underlying the elevated ET-1 concentrations are unclear, and do not appear to be related to hypovolemia or endotoxemia (Salo et al 1995a; Salo et al 1995b). Recent studies have demonstrated that high plasma concentrations of ET-1 are due to both decreased clearance by hepatocytes, and increased production by the gut and spleen (Kuddus et al 2000; Nagasue et al 2000). Moreover, the plasma concentrations of ET-1 correlate positively with the severity of liver disease as measured by Child-Pugh score (Isobe et al 1993; Moller et al 1995; Gerbes et al 1995), the hepatic blood flow as measured by D-sorbitol infusion, and the hepatic venous pressure gradient (Moller et al 1995), and correlate negatively with liver cell mass as measured by

galactose elimination capacity (Gerbes et al 1995), creatinine clearance (Uchihara et al 1992; Moller et al 1993; Moller et al 1995), and the ability to excrete a given water load (Hocher et al 1999). However, due to its paracrine and autocrine mode of action, the majority of ET-1 is released abluminally to act on adjacent vascular smooth muscle and endothelial cells and, as a consequence, plasma ET-1 concentrations may not truly reflect the underlying activity of the endothelin system (Frelin & Guedin 1994). It should be noted that ET-1 might act as an endocrine hormone when its plasma concentrations reach a large level (Haynes & Webb 1994). The increase in plasma ET-1 concentrations correspond with up-regulation of preproendothelin-1 mRNA and elevated plasma concentrations of big ET-1 (Newby et al 1998a) indicating that ET-l levels reflect an increased production, rather than reduced clearance, of the peptide.

A role for ET-1 in the vascular abnormalities of cirrhosis is indicated by the demonstration that low concentration of bacterial lipopolysaccharide promote ET-1 release from endothelial cells (Ros et al 1997). Furthermore, the increased expression of both ET-1 and ET_A-receptors reported in the hepatic sinusoidal endothelial and stellate cells of cirrhotic patients (Pinzani et al 1996) indicate that increased release of ET-1 by the cirrhotic liver may account for the raised plasma ET-1 concentrations, and contributes to the increased intra-hepatic resistance to flow. Interestingly, whilst the increase in circulating ET-1 concentrations is expected to cause receptor down-regulation (Rubanyi & Polokoff 1994), a preliminary investigation of intra-hepatic vessels indicated that the ET-1-mediated contraction is not altered in cirrhosis (Battaglia et al 1996). No studies have addressed the vascular responsiveness to ET-1 *in vivo*, or the role of endogenous ET-1 in the maintenance of basal peripheral vascular tone, in patients with cirrhosis.

Experimental studies in the rat model of cirrhosis and portal hypertension have shown reductions in splanchnic blood flow and portal venous pressure by the non-selective endothelin antagonists TAK-044 (Gandhi et al 1998), bosentan (Reichen et al 1998; Sogni et al 1998) and SP206970 (Kojima et al 2000), and by the selective ET_B receptor antagonist, IRL1038, while selective ET_A receptor antagonism

2.5. The anti-diuretic hormone (ADH; vasopressin):

The reduction in both arterial pressure and effective arterial blood volume increase the non-osmotic baroreceptor-mediated secretion of vasopressin (Schrier & Caramelo 1988; Badalamenti et al 1993). Plasma concentrations of ADH are increased in patients with decompensated cirrhosis (Bichet et al 1982; Arroyo et al 1988). This increase is not related to plasma osmolarity as evidenced by the inability of a water load to suppress ADH release in patients with cirrhosis and ascites (Bichet et al 1982). Also, plasma concentrations of ADH correlate directly with disease severity and inversely with both the MAP and SVR (Aliaga et al 1995), and return to normal within two weeks of orthotopic liver transplantation (Navasa et al 1993). Expansion of plasma volume by the insertion of a Le Veen shunt led to normalisation of the circulating ADH levels (Reznick et al 1983). Administration of demeclocycline, which interferes with the tubular actions of ADH, increases urine flow and decreases urine osmolarity in patients with cirrhosis and ascites (Arroyo et al 1988). Despite the increased activity of ADH, it responds to haemodynamic changes (Iwao et al 1994; Bernardi et al 1983), although this response is insufficient especially in advanced cirrhosis (Schroeder et al 1976). To our knowledge, the contribution of vasopressin to maintenance of basal vascular tone *in vivo* has not been investigated in patients with cirrhosis. However, V_1 receptor antagonists cause hypotension, and normalise the impaired water excretion in an experimental model of cirrhosis (Claria et al 1991) suggesting that vasopressin may contribute to the maintenance of arterial pressure in patients with cirrhosis.

3. Responses to the vasoconstrictor systems in cirrhosis:

In patients with cirrhosis, the increase in plasma concentrations of vasopressor hormones is due to an increased release, rather than reduced clearance. In addition, the vasoconstrictor systems are unable to prevent the development of peripheral vasodilatation despite their activation, and their significant contribution to vascular tone, especially as the disease worsens. Therefore, many *in vitro* and *in vivo* studies have been performed to address the possibility of impaired responses to vasoconstrictor agents.

3.1. In vitro studies:

Studies on isolated vessels permit the assessment of vascular responses to various stimuli in the absence of systemic hormonal and neuronal reflex effects. Therefore, these studies can determine whether an impaired vasopressor response is related to an alteration within the vascular wall. Studies using vessels from the experimental models of cirrhosis have confirmed the presence of impaired responses to α-adrenoceptor agonists, ET-1, and ANG II (Castro et al 1993; Hartleb et al 1994a; Hadoke & Hayes 1997). These studies have also shown normalization of the vascular responses to vasopressor agonists after endothelial denudation and NOS inhibition (Castro et al 1993; Hartleb et al 1994a; Sieber 1995) suggesting an endothelial role in regulating these responses.

The difficulties in obtaining viable human vessels with intact endothelium from patients with cirrhosis have restricted the use of *in vitro* techniques. However, in the absence of endothelium, responses to selective α_1-adrenoceptor agonists were reduced in studies using isolated hepatic arteries (Smith et al 1993, Heller et al 1999), portal veins (Heller et al 1999), and radial veins (Ryan et al 1996) obtained from patients with cirrhosis. Conversely, responses to α_1-adrenoceptor agonists in denuded hepatic arteries from cirrhotic patients and control subjects were similar in another study (Hadoke et al 1998). Using isoprenaline to study the β_2-adrenoceptor-mediated vasodilatation showed enhanced relaxation of portal veins, but impaired relaxation of hepatic arteries from patients with cirrhosis (Heller et al 1999). While the latter is consistent with receptor down-regulation, secondary to the increased plasma concentrations of Nad, the former suggests enhanced receptor function, and indicates that the functional effects of cirrhosis differs from one vessel to another.

We are unaware of any studies of ANG II-mediated contraction in arteries isolated from patients with cirrhosis. Also, vasopressor responses to the ADH are significantly impaired in denuded hepatic arteries from cirrhotic patients, whilst the maximum response to 5-HT is slightly enhanced (Islam et al 2000). This indicates a specific impairment of the V_1-receptor pathway, as the vasopressin-induced contraction seems to be mediated by V_1-receptor (Islam et al 2000).

Initial studies using isolated hepatic arteries from patients with cirrhosis support a role for the vasodilator NO in mediating the impaired responses to vasoconstrictor systems (Smith et al 1993; Robinson et al 1995). Also, NO release was proposed as the cause of impaired response to Nad in human radial veins (Ryan et al 1996). However, other studies did not confirm this role (Schepke et al 1997). Despite the fact that all isolated vessel studies share the limitation that all vessels had lost their endothelium, they indicate that both endothelium-independent and endothelium-dependent functional abnormalities develop in the vessels of patients with cirrhosis.

3.2. In vivo studies:

3.2.1. Systemic infusion of vasoconstrictors:

Most systemic studies have shown that the vasopressor response to ANG II (as assessed by changes in BP, CO and/or SVR) is significantly reduced in patients with cirrhosis with one exception (Lenz et al 1985). In contrast, responses to Nad are reduced, unaltered, or enhanced in patients with cirrhosis (Table 1-2). Inter-study variations are mainly due to methodological differences and variations in the patient population, including their sodium status, menstrual status, alcohol intake, concomitant diuretic therapy, severity of liver disease, and aetiology of liver disease. For example, the patient group was significantly older than controls in one study (Lenz et al 1985). Also, agonists were infused as a single bolus in some studies (Johnston & Jose 1963; Pinzani et al 1991), whereas infusion was continuous in others (Ames et al 1965; Laragh et al 1963; Lenz et al 1985; MacGilchrist et al 1991).

In addition, the dose of agonist infused and the parameters measured were variable between studies. Concomitant diuretic therapy has been shown to reduce vascular responses to Nad infusion (Oka & Manku 1981), effects, which may persist for months (Alexsandrow et al 1959). Similarly, alcohol intake can reduce vascular responses to Nad, but not to ANG II (Howes & Reid 1985). Therefore, the more recent systemic studies ensured that patients had abstained from alcohol for a minimum of 4 weeks (MacGilchrist et al 1991; Pinzani et al 1991). Furthermore, the increase in total blood volume in patients with cirrhosis (Eisenberg 1956; Lieberman

et al 1969; Wong et al 1994) with subsequent dilution of the infused doses of agonists may reduce the responses to these vasopressor agents. However, this hypervolemia does not influence FBF studies especially when comparing groups with similar baseline FBF. These variations make interpretation of the results of the systemic studies extremely difficult.

Although contributing to our understanding of the pathophysiology of portal hypertension, systemic studies have the disadvantage of invoking neurohumoral counter-regulatory mechanisms and having effects on a wide range of organs, including the heart, brain and kidneys (Benjamin et al 1995; Webb 1995). Moreover, previous studies investigating the role of the RAS in the maintenance of vascular tone in patients with cirrhosis (Schroeder et al 1976; Arroyo et al 1981) are weakened by the use of antagonists, such as saralasin, with partial agonist activity (Case et al 1976; Anderson et al 1977; Anderson et al 1980), or the use of angiotensin converting enzyme inhibitors (Pariente et al 1985), such as captopril, which also inhibits the breakdown of bradykinin, which causes vasodilatation through the release of NO (Figure 1-6). The general conclusion from all systemic studies is that an impaired response to exogenous vasoconstrictors does occur in patients with cirrhosis. Whether this impairment is specific to ANG II, or also true for Nad, ADH, and ET-1 requires further investigation. Also, from systemic studies alone, the nature and mechanisms underlying the altered vascular responses can't be clarified.

Table 1-2: Summary of the studies assessing vascular responses using systemic infusion of agonists.

Reference	Parameters	Nad	ANG II	Others
Mashford et al 1962*	MAP, CO, TPR	Enhanced	-	Impaired responses to tyramine.
Johnston & Jose 1963*	MAP, CO, SVR	Normal	Impaired	-
Laragh et al 1963	MAP	Normal	Impaired	-
Kaplan & Silah 1964	BP	Normal	Impaired	-
Ames et al 1965	BP	-	Impaired	-
Lunzer et al 1975	HR, FBF	Impaired	Normal	Normal response to adrenaline, impaired responses to reflex autonomic stimuli. **
Lenz et al 1985#	HR, MAP	Enhanced	Normal	Impaired responses to isoprenaline. Enhanced response to phenylephrine.
Ramond et al 1986	HR	-	-	Impaired responses to isoprenaline.
MacGilchrist et al 1991	HR, BP	Impaired	Impaired	Impaired responses to methyl Nad, phenylephrine, and isoprenaline.
Pinzani et al 1991*	HR, BP	Enhanced	Impaired	-

BP, blood pressure. HR, heart rate. FBF, forearm blood flow. MAP, mean arterial pressure. SVR, systemic vascular resistance. TPR, total peripheral resistance. *, indicates bolus infusion. #, all patients had liver encephalopay. ** autonomic stimuli tested were lower body negative pressure, application of ice, and the Valsalva manoeuvre.

3.2.2. Local infusion of vasoconstrictors:

In the last decade, studies using local infusions in the isolated forearm resistance vessels and dorsal hand veins are increasing, and those systemic infusions are decreasing. The main reason for this shift towards local infusions *in vivo* is to avoid invoking systemic neurohumoral effects or causing side effects. (Benjamin et al 1995; Webb 1995; Petrie et al 1998).

3.2.2.1. STUDIES USING DORSAL HAND VEIN COMPLIANCE:

Measurement of dorsal hand vein (DHV) compliance using the Aellig vein technique is a method designed to assess vascular reactivity by measuring the alterations in the distension capacity of a superficial dorsal hand vein held at a constant pressure (Aellig 1981; Aellig 1994a; Aellig 1994b). According to this technique, changes in vein diameter reflect changes in venous tone. This relatively non-invasive technique involves intravenous infusion of at locally active and sub-systemic doses of agonists and antagonists.

To my knowledge, only two controlled studies have utilized this technique in patients with cirrhosis, and their results are contradictory (Table 1-3). Again, the use of diuretics and the amount of salt intake may account for this variation. Indeed, frusemide causes dilatation of the DHV, which may be mediated by the release of PGI_2 from the venous endothelium (Pickkers et al 1997). Also, the enhanced response to Nad infusion occurred only in patients on a high salt diet (Wong et al 1995). In the non-controlled study (Calver et al 1994), infusion of L-NMMA, a specific NO synthase (NOS) inhibitor, did not affect the DHV diameter. Also, Nad induced venoconstriction was unaffected by co-infusion of L-NMMA.

Table 1-3: Dorsal hand veins response to vasopressor agents in patients with cirrhosis.

Reference	Parameter	Main results
Bierbrier et al 1994	DHV diameter	• Unaffected basal venous tone. • Reduced sensitivity to PE infusion, but normal maximum contraction.
Wong et al 1995	DHV diameter	• Reduced basal venous tone. • Enhanced response to Nad infusion, only during high sodium diet.
Calver et al 1994	DHV diameter	• Unaffected basal venous tone. • Unaffected response to Nad infusion.

DHV, dorsal hand vein. PE, phenylephrine. Nad, noradrenaline.

3.2.2.2. STUDIES USING VENOUS OCCLUSION PLETHYSMOGRAPHY:

The combination of sub-systemic and locally active intra-brachial artery infusions with FBF measurements using venous occlusion plethysmography represents a powerful and reproducible method of assessing *in vivo* vascular responses in an isolated circulation without invoking systemic effects (Benjamin et al 1995; Webb 1995; Petrie et al 1998). This technique is described in detail in Chapter 3.

- *Basal FBF:*

A precise conclusion about basal FBF in patients with cirrhosis cannot be made from previous studies, as some studies (Rodriguez-Perez et al 1993; Calver et al 1994; Albillos et al 1995; Wong et al 1996) have shown increased basal FBF in patients, and a further increase with disease progression (Campillo et al 1995; Albillos et al 1995), while others reported similar basal FBF in both patients and controls (Lunzer et al 1975; Ryan et al 1993; Wong et al 1995; Ryan et al 1996; Newby et al 1998a).

- *FBF responses to exogenous vasopressor agents:*

ANG II and Nad are the most frequently studied vasopressor agents in the forearm circulation (Table 1-4). Conflicting results have been reported. For example, the

responses to Nad were either reduced (Ryan et al 1993; Ryan et al 1996; Campillo et al 1995; Albillos et al 1995) or unaltered (Calver et al 1994; Newby et al 1998a) in patients with cirrhosis. However, two of theses studies (Ryan et al 1993; Newby et al 1998a) have shown that the vasopressor responses to ANG II are reduced. One study has combined the use of venous occlusion plethysmography to measure FBF and the systemic infusion of Nad (Lunzer et al 1975). However, it is difficult to compare the results with recent studies for two reasons: 1) FBF was measured without exclusion of the hand despite the fact that circulation in the hand occurs mainly through cutaneous vessels and contains a high proportion of arterio-venous shunts, and gives variable FBF; 2) systemic infusion of agonists have the disadvantage of invoking neurohumoral counter-regulatory mechanisms and having effects on a wide range of organs, including the heart, brain and kidneys (Benjamin et al 1995; Webb 1995). In deed, systemic infusion of ANG II produced systemic vasodilatation, and increased calf blood flow (Motwani & Struthers 1992).

- *Reasons of the conflicting results:*

The conflicting results obtained by these investigations may be related to the differences either in the disease severity, disease aetiology, methodology, or in a combination of all. Therefore, standardisation of future studies is needed. As discussed in Chapter 3, several precautions should be taken when performing a study using venous occlusion plethysmography in order to avoid its limitations. Only three studies have controlled their experiments by measuring blood flow in both arms. As a consequence, two of these studies reported similar results (Calver et al 1994; Newby et al 1998a), whilst the third did not include a healthy control group (Campillo et al 1995). In addition, variations in diuretic therapy, dietary sodium intake, and alcohol intake may also affect the results. For example, some studies have included patients who were not on any diuretic therapy (Ryan et al 1993; Wong et al 1995), others selected groups containing some patients receiving diuretics (Lunzer et al 1975; Campillo et al 1995), and others stopped diuretics for 1 day (Newby et al 1998a), 5 days (Ryan et al 1996), or 14 days (Albillos et al 1995) before performing the investigation. It is generally advised that if the effects of

diuretic therapy on systemic haemodynamics are to be avoided, diuretics should be stopped for at least 5 times the half-life of the drug-used.

- *Endogenous vasopressors and basal forearm vascular tone:*

Infusion of losartan, a selective AT_1 receptor antagonist caused a significant increased in FBF in patients with diuretic-responsive ascites but not in controls (Newby et al 1998a). This is consistent with responses to saralasin infusion in whole body studies (Schroeder et al 1976). These findings indicate that although the vasopressor responses to ANG II are impaired, endogenous ANG II contributes significantly to the maintenance of basal vascular tone in patients with cirrhosis and ascites. However, this role needs to be assessed in the earlier stages of cirrhosis, and in patients who were not on diuretic therapy.

- *Nitric oxide and basal forearm vascular tone:*

Compared to healthy controls, the contribution of NO to the maintenance of basal vascular tone in patients with early (Calver et al 1994; Ryan et al 1996) and advanced (Newby et al 1998a) cirrhosis was studied using L-NMMA. All these studies have shown that L-NMMA produces a similar reduction in FBF in both patients and controls. Therefore, increased basal NO production is not responsible for the impaired vasopressor responses in these patients, at least in the forearm bed. The possibility that increased agonist stimulated NO release in this territory has never been investigated before, and is tested in Chapter 4. To date, it is not clear whether the impaired response to the vasopressor agonists is a generalised phenomenon or specific to ANG II-mediated vasoconstriction. To our knowledge, the reactivity to ET-1 infusion has not been examined in patients with cirrhosis before, and will be tested in Chapter 5.

Table 1-4: Summary of the studies assessing vascular responses using intra-brachial infusion of agonists.

Reference	Parameters measured	Patients No (% with ascites) / controls	Nad	ANG II	Dose infused	Dose-response curve
Ryan et al 1993	BP, FBF	10 (0%) / 10	Impaired	Impaired	Nad: 25-100 ng/min.	Yes
		10 (0%) / 10			ANG II: 8-32 ng/min.	Yes
Calver et al 1994	FBF	7 (0%) / 6	Normal	-	Nad: 60-340 pmol/min.	No
Albillos et al 1995	FBF, MAP	12 (50%) / 10	Impaired	-	Nad: 0.3-3 μg/min.	Yes
Campillo et al 1995	CO, FBF	20 (50%) / none	Impaired	-	Nad: 60-200 pmol/min.	Yes
Ryan et al 1996	FBF	10 (0%) / 10	Impaired	-	Nad: 25-100 ng/min.	No
Newby et al 1998a	FBF, MAP	11 (100%) / 8	Normal	Impaired	Nad: 20-540 pmol/min.	Yes
		11 (100%) / 8			ANG II: 1-30 pmol/min.	Yes

ANG II, angiotensin II. BP, blood pressure. CO, cardiac output. FBF, forearm blood flow. MAP, mean arterial pressure. Nad, noradrenaline.

4. Mechanisms of vascular hyporesponsiveness

In patients with cirrhosis, the balance between vasodilators and vasoconstrictors tends to be towards vasodilatation especially as the disease progresses (Figure 1-9).

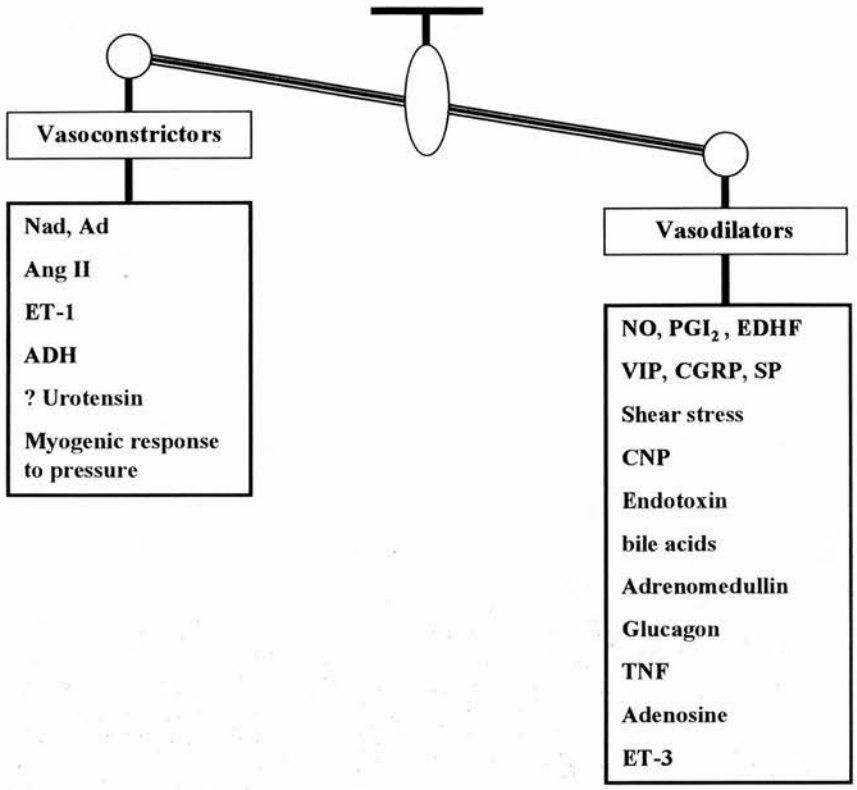


FIGURE 1-9: THE BALANCE OF VASCULAR MEDIATORS IN CIRRHOSIS - PREDOMINANT VASODILATATION.

The exact mechanism underlying the impaired responses to vasoconstrictor mediators is still under debate. However, the reduced responsiveness could be explained by either vascular smooth muscle (receptor and/or post-receptor) defects or the presence of excess vasodilators or both. Many studies have shown that reduced α-adrenergic responsiveness or sensitivity (Gerbes et al 1986; Bierbrier et al 1994; Karatapanis et al 1994), while others suggest that the decrease in vascular contractility is mainly a post-receptor defect (Murray et al 1985; Gopalakrishna et al 1993; Masuda et al 1993; Jeremy et al 1994; Groszmann 1994; Moreau et al 1994).

5. The vasodilator systems in cirrhosis:

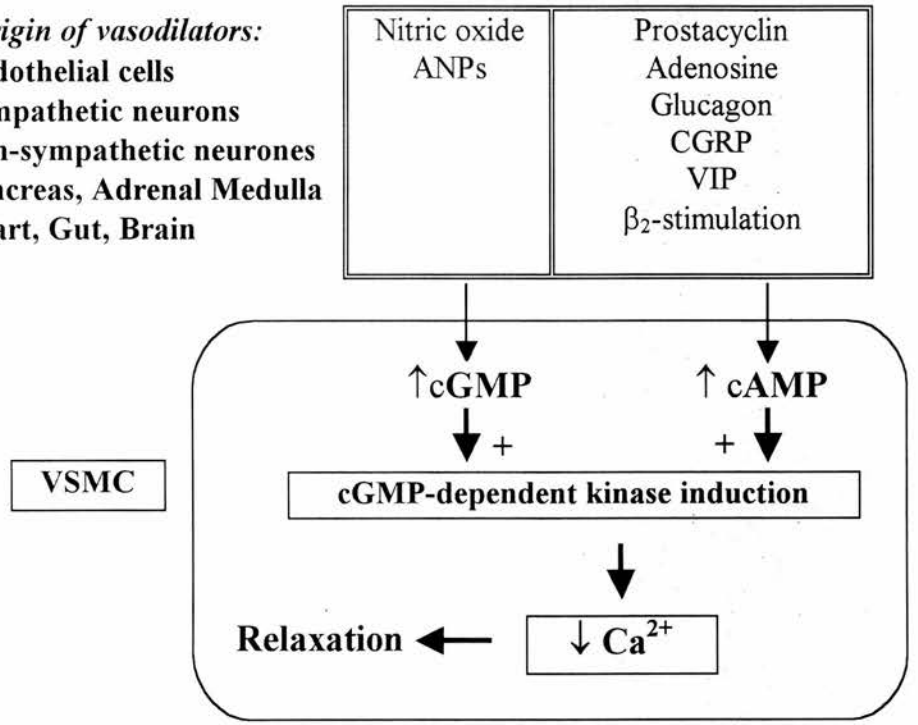
5.1. Mechanism of action of vasodilators:

Vasodilatation is induced by endothelium-derived factors such as NO, prostacyclin (PGI_2) and the endothelium-derived hyperpolarizing factor (EDHF). In addition, endocrine hormones, such as glucagon, and peptides released from non-sympathetic neurones, such as the calcitonin-gene-related peptide (CGRP) and the vasoactive intestinal peptide (VIP). Many, but not all, vascular territories have a dilator basal tone (Collier & Vallance 1991; Pickkers et al 1997). Vasodilator factors oppose the effects of vasoconstrictor mediators and reduce intracellular calcium influx via their action on cGMP and cAMP, which are formed in the smooth muscle cytosol from the actions of guanylate cyclase and adenylate cyclase on guanosine triphosphate and adenosine triphosphate respectively (Figure 1-10). Indeed, studies on vascular smooth muscle have shown that cGMP-dependent kinases can depress the rise in cytosolic calcium that occurs in response to ANG II (Lincolin et al 1996). The second postulated action of cGMP is stimulation of calcium pumps leading to calcium outflux from the cell through activation of calcium-ATP-ase activity (Lincolin et al 1996). The other major route, whereby vascular smooth muscle tone is modulated, is the transmembrane potassium channel. To date, 4 classes of potassium channels have been identified on the VSMC membrane (Brayden & Nelson 1992). Opening of potassium channels hyperpolarises the VSMC membrane, opposes calcium influx, and decreases the vascular tone. The yet un-identified EDHF is thought to produce vasodilatation through this mechanism (Barrier et al 2000). Studies into the relationship between potassium channels, calcium, and the regulation of vascular tone in patients with cirrhosis are warranted.

FIGURE 1-10: MECHANISMS OF ACTION OF VASODILATORS.

Cellular origin of vasodilators:

- **Endothelial cells**
- **Sympathetic neurons**
- **Non-sympathetic neurones**
- **Pancreas, Adrenal Medulla**
- **Heart, Gut, Brain**



ANPs, atrial natriuretic peptides. cAMP, cyclic adenosine monophosphate. cGMP, cyclic guanosine monophosphate. CGRP, Calcitonin-gene-related peptide. VIP, vasoactive intestinal peptide. VSMC, vascular smooth muscle cell.

5.2.-Nitric oxide (NO).

5.2.1. NO in health:

Twenty years ago, Furchgott & Zawadzki (1980) demonstrated that an endothelium-derived relaxing factor (EDRF) mediated the acetylcholine-induced vasodilatation. Many studies have subsequently shown that this EDRF is NO (Khan & Furchgott 1987; Ignarro et al 1987; Palmar et al 1987). NO is a gas with a short half-life which regulates many pathophysiological processes, including smooth muscle relaxation, central and peripheral neuronal transmission, platelet function, cytotoxic activity of the immune cells, septic shock and atherosclerosis (Moncada et al 1991; Moncada & Higgs 1993; Hobbs et al 1999). NO is released from the amino-acid L-arginine, by the enzyme NO synthase (NOS; Palmar et al 1988) by different stimuli including the increase in blood flow, shear stress, endotoxins, and endogenous vasodilators such as bradykinin, TNF-α, and adrenomedullin (Khoruts et al 1991; Richards et al 1996).

NO synthesized in the endothelial cells diffuses towards the underlying smooth muscle cells and mediates relaxation through the activation of the soluble guanylate cyclase enzyme which increases guanosine cyclic monophosphate (cGMP) concentrations (Arnold et al 1977). Three isoforms of NOS have been identified and shown, by molecular cloning, to share 50-60% homology (Hobbs et al 1999). The first is a constitutive isoform (nNOS or NOS I), whose activity is Ca^{2+}/calmodulin dependent and is mainly present in neuronal tissues. The second is a Ca^{2+} independent isoform (iNOS or NOS II) that has been isolated from different cells after induction with bacterial products or inflammatory mediators. The third is Ca^{2+}/calmodulin requiring constitutive isoform found in the endothelial cells (eNOS or NOS III), which plays an important role in vascular homeostasis (Hobbs et al 1999).

The genes encoding for NOS isoforms have been localized on human chromosomes 12, 17 and 7, respectively (Nathan & Xie 1994). Cytokines (mainly TNFα) and bacterial lipopolysaccharides can induce *de novo* synthesis of iNOS in both the

endothelium and the VSMC (Rees et al 1990; Radomski et al 1990). The activity of NOS in tissues or cells is determined by the conversion of ^{14}C- or ^{3}H-L-arginine to ^{14}C- or ^{3}H-L-citrulline respectively (Cahill et al 1993; Laffi et al 1993). Specific inhibitors of NOS have been developed, such as L-arginine analogues L-N^G-monomethyl-arginine (L-NMMA), N^G-nitro-L-arginine (L-NNA), and L-N^G-nitro-arginine methyl ester (L-NAME) (Moncada et al 1991; Hobbs et al 1999).

5.2.2. NO in cirrhosis:

A decade ago, it was hypothesized that NO may be have a role in he pathogenesis of the hyperdynamic circulatory abnormalities of patients with cirrhosis (Vallance & Moncada 1991). In support of this hypothesis, many studies in patients with cirrhosis have provided evidence of this role (Martin et al 1998). In some studies (Table 1-5), the hyperdynamic circulation has been reversed by blockade of the NO system, whereas others have concluded that NO can only be partially responsible for the vasodilatation in cirrhosis (Fernandez et al 1995; Kanwar et al 1996; Ryan et al 1996). The proposed haemodynamic consequences of NOS inhibition and the hypothetical role of NO in the pathogenesis of portal hypertension in patients with cirrhosis are shown in Figures 1-11 and 1-12.

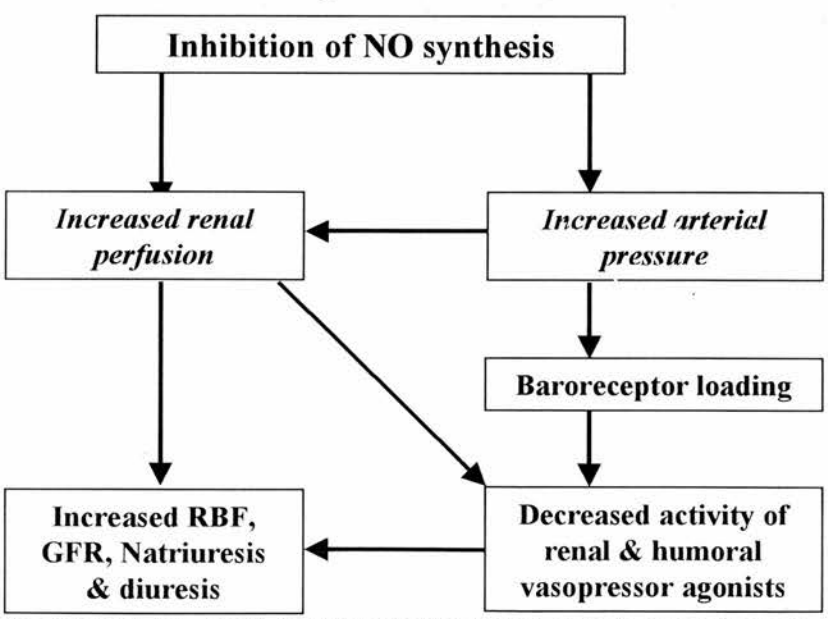


FIGURE 1-11: THE HAEMODYNAMIC CONSEQUENCES OF NITRIC OXIDE SYNTHASE INHIBITION.

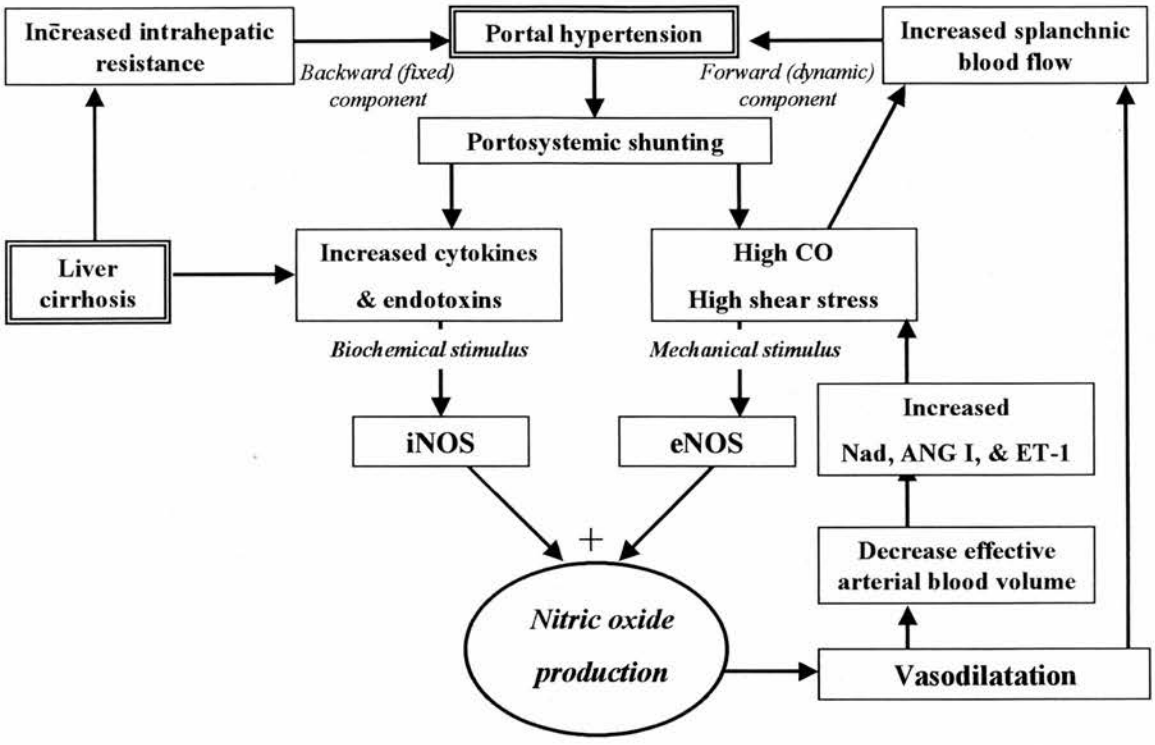


FIGURE 1-12: THE HYPOTHETICAL ROLE OF NITRIC OXIDE IN THE PATHOGENESIS OF PORTAL HYPERTENSION.

5.2.2.1. NITRIC OXIDE IN THE ANIMAL MODELS OF CIRRHOSIS:

While many studies in experimental cirrhosis indicate that increased vascular production of NO contributes to arterial vasodilatation, probably due to increased eNOS activity (Martin et al 1996; Morales-Ruiz et al 1996), others failed to do so (Sogni et al 1992; Karatapanis et al 1994). Therefore, the role of NO is still unclear. Up-regulation of eNOS precedes the onset of ascites formation in cirrhotic rats, as does increased NO production (Niederberger et al 1995; Niederberger et al 1995a; Ros et al 1995). Normalisation of NO synthesis produces beneficial effects on systemic haemodynamics (Pizcueta et al 1992), with suppression of the vasoconstrictor systems and improvement of renal function (Martin et al 1998).

5.2.2.2. NITRIC OXIDE IN PATIENTS WITH CIRRHOSIS:

In patients with cirrhosis, evidence of increased NO synthesis is also accumulating, suggesting a role in the pathogenesis of the hyperdynamic circulation (Table 1-5). However another study does not suggest so (Forrest et al 1995). Increased production NO starts before the onset of ascites formation, and contributes to the vasodilatation

observed in advanced cirrhosis by reducing the vascular responses to vasopressor agents (Martin et al 1998). The initial cause of NO overproduction is unknown. However, portal hypertension may contribute by increasing shear stress leading to up-regulation of eNOS. Also, splanchnic production of NO, mediated by either eNOS or iNOS, may also have a role. The role of NO in the pathogenesis of haemodynamic and renal abnormalities in patients with cirrhosis remains unclear.

Table 1-5: Evidence supporting increased nitric oxide synthesis in patients with cirrhosis and ascites.

Evidence	Reference
Increased plasma concentrations of NO metabolites.#	Guarner et al 1993. Barak et al 1999.
Increased exhaled NO in patients with hepato-pulmonary syndrome.	Mavoral et al 1994.
Normalisation of arterial responses by NOS inhibitors.	Campillo et al 1995.*
Increased NO in exhaled air. #	Matsumoto et al 1995; Sogni et al 1995a.
Increased NOS activity in neutrophils & monocytes.	Laffi et al 1995; Criado-Jimenez et al 1995.
Enhanced vasodilatation to NO-dependent vasodilators such as acetylcholine. #	Albillos et al 1995.
Increased plasma concentrations of NO #	Battista et al 1997.
The enhanced No production in the splanchnic vascular bed following L-arginine infusion.	Kakumitsu et al 1998

* This evidence is controversial to Ryan et al 1996 and Newby et al 1998a.
\# Also shown in pre-ascitic cirrhosis. * Does not include a healthy control group.

5.3. Other vasodilators in cirrhosis:

5.3.1. Atrial natriuretic peptides (ANPs):

The family of natriuretic peptides include at least three members: the atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and the weaker endothelial C-type natriuretic peptide (CNP), which play a role in volume homeostasis (De Bold 1985; Sudoh et al 1988; Suga et al 1992). ANPs produce vasodilatation, natriuresis, and diuresis via the guanylate cyclase and cGMP pathway (Luk et al 1994). Patients with cirrhosis and ascites showed elevated plasma concentrations of, and blunted renal responses to, ANP (Gines et al 1988; La Villa et al 1995; Wong et al 1995; Bandi et al 1997). Also, plasma concentrations of ANP and cGMP are increased in patients with pre-ascitic cirrhosis (Trevisani et al 1995; Iwao et al 1997, Iwao et al 2000). However, plasma levels of BNP have been shown to be normal in these patients (La Villa et al 1992a). The increase in ANP and cGMP correlated inversely with PVR (Iwao et al 1997), and is more evident in the supine position (Bernardi et al 1992; Iwao et al 1997). These controversial results of studies in patients with pre-ascitic cirrhosis may be related to variations in the sodium intake (Rector et al 1990), or the daily fluctuation in plasma ANP levels (Trevisani et al 1995). The cause of such high concentrations of ANP is unknown, because the atrial pressure is normal, and the central blood volume is reduced. A study in a rat model of cirrhosis showed that the ventricle is the main site of ANP synthesis (Poulos et al 1995). The subtle sodium retention, which precedes ascites formation, may be due to blunted renal responses to ANP (Wong et al 1995).

5.3.2. Calcitonin-gene-related peptide (CGRP):

CGRP, a 37-amino acid neuropeptide, is a neurotransmitter with powerful vasodilator effects (Rosenfeld et al 1983; Zaidi et al 1987; O'Halloran & Bloom 1991), which are important in the control of regional blood flow (Struthers et al 1986). Infusion of CGRP produces pronounced hypotension through a direct effect of CGRP rather than the release of other neurotransmitters (Struthers et al 1986; Zaidi et al 1987; O'Halloran & Bloom 1991). Plasma concentrations of CGRP are elevated in patients with cirrhosis (Bendtsen et al 1991; O'Halloran & Bloom 1991;

Gupta et al 1992; Moller et al 1995), and correlate directly with disease severity (Bendtsen et al 1991; Gupta et al 1992) and CO, and negatively with SVR (Moller et al 1996). These results support the hypothesis that CGRP may be implicated in the systemic vasodilatation in cirrhosis. Moreover, increased CGRP may also contribute to the abnormal distribution of the blood volume, in concert with other vasodilators like NO and ET-3. Better understanding of the role of CGRP in the pathogenesis of the haemodynamic alterations in cirrhosis requires use of its specific antagonists.

5.3.3. Glucagon:

Glucagon is 29-amino acid peptide secreted mainly by the α-cells of the islets of Langerhans in the pancreas and primarily inactivated by the liver. Glucagon promotes vasodilatation by direct, cAMP-mediated, VSMC relaxation or indirectly decreasing the sensitivity to vasopressor agents (Richardson & Withrington 1976; Pizcueta et al 1990; Nakahara et al 1997). Hyperglucagonemia occurs in portal hypertensive animals and humans, and results from portosystemic shunting, decreased hepatic clearance, and more importantly increased secretion (Sherwin et al 1974; Silva et al 1990; Lin et al 1996). Portal vein-stenosed rats with normal liver function also had marked hyperglucagonemia, suggesting that elevated glucagon levels do not depend on the presence of hepatocellular disease (Sherwin et al 1978). However, another study showed that hyperglucagonemia is mainly related to the liver function (Lewis et al 1991). Glucagon significantly decreases splanchnic vascular resistance (Benoit et al 1984) and increases splanchnic blood flow in patients with well-compensated cirrhosis (Lee et al 1988). In addition, an anti-serum directed against glucagon ameliorates the hyperdynamic circulation in portal hypertensive animals (Benoit et al 1986).

Somatostatin also constricts the splanchnic arterioles and reduces both the circulating glucagon levels and portal pressure, suggesting a role for glucagon in the modulating mesenteric blood flow (Kravetz et al 1988). In this study, somatostatin infusion decreased portal venous inflow, but not down to normal. In addition, plasma

glucagon levels did not correlate with portal venous inflow (Sikuler & Groszmann 1986), indicating that additional factors must also be involved.

5.3.4. Prostacyclin (PGI_2):

Prostacyclin is an endogenous vasodilator of an endothelial origin that relaxes the vascular smooth muscle cell by activating adenylate cyclase and increasing the intracellular cAMP levels (Smith 1986). The cyclo-oxygenase (COX) enzyme, which exists in a constitutive (COX-1) and an inducible (COX-2) isoforms, is involved in the biosynthesis of PGI_2 (Smith 1986; Masferrer et al 1990). Several studies have shown elevated plasma concentrations of prostacyclin in both human (Arroyo et al 1983; Rimola et al 1986; Guarner et al 1992) and experimental cirrhosis (Sitzmann et al 1989). The inhibition of prostaglandin biosynthesis by indomethacin has resulted in a decrease in prostacyclin levels and significant amelioration of the hyperdynamic circulation in patients with cirrhosis and portal hypertension (Bruix et al 1985), and in the rabbit model of portal hypertension (Sitzmann et al 1991), an effect, which has not been reported in the rat model (Blanchard et al 1985). Moreover, indomethacin infusion has been shown to normalise the increased gastric blood flow in portal hypertensive rats, but not in sham-operated controls (Pique et al 1988). This supports a role for prostaglandins in the pathogenesis of the hyperdynamic circulation of portal hypertension.

5.3.5. Adrenomedullin:

Adrenomedullin, a 52-amino acid peptide, is primarily released from the adrenal medulla and induces relaxation of the vascular smooth muscle via specific receptors (Eguchi et al 1994; Richards 1996). Plasma concentrations of adrenomedullin are elevated in patients with cardiac, renal, and liver failure (Cheung & Leung 1997; Fabrega et al 1997 Fernandez-Rodriguez et al 1998). In patients with cirrhosis, the circulating levels of adrenomedullin appear to be higher in decompensated patients, and correlate with the plasma concentrations of the vasopressor agents such as ET-1, vasopressin, and Nad (Guevara et al 1998). The potential role of this vasodilator peptide in the haemodynamic abnormalities in cirrhosis needs to be evaluated.

5.3.6. Bile acids:

Bile acids, which possess vasodilator properties, circulate in increased levels in patients with cirrhosis and portal hypertension and correlate with disease severity and the blood levels of liver function tests (Ohkubo et al 1984). Reduction of circulating bile acid levels to normal in portal vein-ligated rats did not ameliorate the hyperdynamic circulation (Genecin et al 1990). However, in a different model, bile acid depletion significantly decreased the splanchnic hyperaemia (Thomas et al 1991). Hence, the role of bile acids in mediating the hyperdynamic circulation is not well defined.

Many other vasodilators, including neuropeptides, bradykinin, histamine, adenosine and endotoxin have also been studied (Table 1-6). However, available evidence is scarce for most of them. These data suggest that the vasodilatation present in portal hypertension is multifactorial.

Table 1-6: Vasodilators agents that may be involved in the hyperdynamic circulation of cirrhosis.

Vasodilator	References
Adenosine	MacMathuna et al 1990; Moreau et al 1992.
Adrenomedullin	Fabrega et 1997; Cheung & Leung 1997; Guevara et al 1998.
Atrial Natriuretic peptide (ANP)	Gines et al 1988, LaVilla et al 1995; Wong et al 1995; Bandi et al 1997.
Bile acids	Ohkuba et al 1984.
Calcitonin-gene-related peptide (CGRP)	Bendtsen et al 1991; O'Halloran & Bloom 1991; Gupta et al 1992; Moller et al 1996a.
Cyclic adenosine mono-phosphate (cAMP)	Ishii et al 1979; Fabrega et al 1997.
Cyclic guanosine mono-phosphate (cGMP)	Miyase et al 1990; Fernandez-Rodriguez et al 1997; Lee et al 1997. Kirstetter et al 1997.
Endothelin-3 (ET-3)	Gulberg et al 1992.
Endotoxins	Triger 1991.
Encephalins	Thornton et al 1988; Thornton et al 1989.
Gamma-amino-butyric acid (GABA)	Minuk & MacCanell 1988.
Glucagon	Rodriguez-Perez et al 1993; Iwoa et al 1997, Lin et al 1996; Lewis et al 1991.
Kallikrein-Kinins	Perez-Ayuso et al 1984.
Nitric oxide (NO)	Guarner et al 1993; Mavoral et al 1994; Laffi et al 1995; Criado-Jimenez et al 1995; Battista et al 1997.
Platelet activating factor (PAF)	Caramelo et al 1987.
Prostacyclin (PGI_2)	Arroyo et al 1983; Rimola et al 1986; Guarner et al 1986.
Substance P (SP)	Hortnagl et al 1984; Fernandez-Rodriguez et al 1995; Lee et al 1997, Uemura et al 1998.
Tumour necrosis factor-alpha (TNF-α)	Lopez-Talavera et al 1995.
Vasoactive intestinal peptide (VIP)	Hunt et al 1979; Henriksen et al 1980a; Henriksen et al 1986a; Arsene et al 1987.

6. Aims of the thesis

We have concentrated upon the RAS and The SNS in patients with pre-ascitic cirrhosis, patients with diuretic-refractory ascites, and age- and sex-matched healthy controls. The aims of these studies, which are included in Chapter 3 were to:

1. evaluate the baseline FBF, PRA, and plasma concentrations of ANG II, Nad, and adrenaline, and their relation to disease severity.
2. determine the role of endogenous ANG II in the regulation of basal and sympathetically-stimulated forearm vascular tone.
3. assess the forearm vascular responses to exogenous ANG II and Nad, and whether these responses are dose-related.
4. assess the forearm vascular responses to reflex sympathetic stimulation by low-pressure baroreceptor unloading.
5. study the relationship between the vascular abnormalities, if any, and disease severity.

Because the most powerful vasoconstrictor peptide, ET-1, has not been studied before in patients with cirrhosis, we have expanded our studies to investigate the peripheral vascular reactivity to endogenous and exogenous ET-1 in cirrhosis in Chapter 4. The aims of these studies - in patients with pre-ascitic cirrhosis and matched healthy controls - were to:

1. measure the baseline plasma concentrations of ET-1 and big ET-1 in patients with cirrhosis and its relation to disease severity.
2. determine the forearm vascular responsiveness to exogenous ET-1, and
3. assess the role of endogenous ET-1 in the maintenance of basal forearm vascular tone using the selective ET_A and ET_B receptor antagonists, BQ-123 and BQ-788 respectively.

The most important finding while studying the RAS was the impaired vasoconstrictor responses to exogenous ANG II starting from the pre-ascitic stage of cirrhosis. However, the mechanism of this hyporesponsiveness is not clear. In addition, evidence of increased NO production in the animal models and in humans

with cirrhosis is accumulating. Therefore, the aims of the study presented in Chapter 5 - in patients with pre-ascitic cirrhosis and healthy control subjects - were to:

1. evaluate the contribution of endogenous NO release to the maintenance of basal peripheral vascular tone and,
2. assess the relationship between the vascular responses to ANG II and endogenous NO release.

The main findings of the studies presented in Chapter 4 were the impaired forearm vasoconstriction in responses to exogenous ET-1, and the enhanced vasodilatation in responses to the ET_A receptor antagonist, BQ-123. The mechanism underlying these responses are unknown Therefore, the aims of the study presented in Chapter 6, in patients with pre-ascitic cirrhosis and healthy controls, were to:

1. determine the relationship between the vascular responses to ET_A receptor blockade and endogenous NO release.

CHAPTER 2

SUBJECTS, MATERIALS, AND METHODS

1. Subjects

1.1. Subject recruitment:

All patients who participated in the studies were approached during their attendance at the Royal Infirmary of Edinburgh for further management of their chronic liver disease. All healthy control subjects were recruited from the community by advertisement or from the volunteer's data bank of the Clinical Research Centre at the Western General Hospital. None of the control subjects had clinically significant illness in their past history or on physical examination, and none was receiving regular medication. Details of characteristics of subjects included in each study are mentioned in the relevant chapter.

1.2. Ethics committee approval and consent:

All studies were conducted with the approval of the Health Volunteer and the Medicine and Clinical Oncology Sub-Committees of the Lothian Health Board Research Ethics Committee. Each subject was provided with a study information sheet to read at least one week before the first study day. After a verbal explanation, subjects then gave written, witnessed consent. All studies were undertaken in accordance with the Declaration of Helsinki (1989) of the World Medical Association.

1.3. Standard experimental conditions:

All studies were performed in the Clinical Research Centre at the Western General Hospital. Studies were performed in a quiet clinical laboratory maintained at a constant temperature between 22 and 24°C.

1.4. Patients inclusion criteria:

All patients were ambulant and had biopsy-proven cirrhosis, endoscopically-proven oesophageal varices, normal serum creatinine (<100 μmol/L), and no clinical evidence of systemic circulatory disturbances, apart from the group of patients who had diuretic-refractory ascites (Chapter 3). Patients were classified as having pre-ascitic cirrhosis if they did not have ascites, either at the time of the study or before, as assessed by both clinical and ultrasonographic examinations.

1.5. Exclusion criteria:

The main exclusion criteria included the presence of malignancy, encephalopathy, portosystemic shunt (surgical or TIPSS), previous gastrointestinal bleeding, or any significant cardiovascular disease, such as diabetes mellitus and hypertension.

1.6. Precautions:

To avoid the depressive effect of ethanol on vascular responses (Howes & Reid 1985), patients with alcoholic liver disease were abstinent from alcohol for at least one month as confirmed by history and random blood ethanol testing. In addition, all subjects were allowed a normal sodium diet of 150 mmol/day unless otherwise stated, in order to avoid the possibility of altering the activity of endogenous vasopressor systems (Rankin et al 1981; Stein et al 1995). None of the subjects received vasoactive or non-steroidal anti-inflammatory drugs in the week before, and all subjects abstained from food, tobacco and caffeine-containing drinks for a minimum of 4 hours prior to each study. All female subjects were post-menopausal, both for safety and to avoid the variability in vascular responses that may be associated with cyclic hormonal changes (Hashimoto et al 1995).

2. Pharmacological materials

2.1. Vehicle:

Physiological saline (0.9%; Baxter Ltd, Thetford, UK) was used as a vehicle to dissolve peptides and drugs for all studies, except where Nad was used (see below). All pre-dose measurements were made during infusion of this vehicle solution. Use of isotonic solution avoided problems with hyperosmolar solution, which can cause substantial vasodilatation (Overbeck & Grega 1970; Bank et al 1992).

2.2. Endothelin-1:

Human endothelin-1 (ET-1, MW 2492; $C_{109}H_{159}N_{25}O_{32}S_5$), a 21 amino acid peptide, is generated from big endothelin, the 38 amino acid precursor through the action of endothelin converting enzymes (ECE). It is one of the most potent vasoconstrictors yet identified. Among the three isoforms (ET-1, ET-2, and ET-3), ET-1 appears to be the major form produced by the vascular endothelial cells, with a wide variety of vascular and extra-vascular effects. Pharmaceutical grade, sterile, and pyrogen-free ET-1 (Clinalfa AG, Läufelfingen, Switzerland) was dissolved in saline 0.9%, filtered (Millipore 0.2 μm filter), and divided into aliquots under sterile conditions in the Royal Infirmary of Edinburgh, and then frozen at -20°C until used. All ET-1 vials used in the studies included in this thesis were from a single batch.

2.3. BQ-123 Sodium salt:

BQ-123 (MW 632.7; $C_{31}H_{41}N_6O.Na$) is synthetic cyclopentapeptide (Cyclo-[D-Asp-L-Pro-D-Val-Leu-D-Trp]-Na) with potent and highly selective ET_A-receptor antagonist properties (Ihara et al 1992). The specificity of BQ-123 as an endothelin receptor antagonist has been shown *in vivo* in animals, where it dose not affect vascular responses to potassium, Nad, phenylephrine, acetylcholine and nitro-glycerine (Ihara et al 1992; Douglas et al 1994; Bazil et al 1992). Pharmaceutical grade, sterile, pyrogen-free BQ-123 (Clinalfa) was dissolved in 0.9%saline, filtered (Millipore 0.2 μm filter), and divided into aliquots under sterile conditions in the Royal Infirmary of Edinburgh, and then frozen at -20°C until used. BQ-123 vials used in the studies included in this thesis were from a single batch.

2.4. BQ-788 Sodium salt:

BQ-788 (MW 663.7; $C_{34}H_{50}N_5O_7.Na$) is a synthetic derivative of a tripeptide (N-cis-2,6 dimethylpiperidino-carbonyl-γ-MeLeu-D-Trp-DNle-OH.Na). It is a potent and highly selective ET_B-receptor antagonist, with a selectivity ratio of 200 relative to the ET_A receptor. BQ-788 infusion produces forearm vasoconstriction and increases peripheral vascular resistance in healthy men (Strachan et al 1999). Pharmaceutical grade, sterile, and pyrogen-free BQ-788 (Clinalfa) was dissolved in 0.9% saline, filtered (Millipore 0.2 μm filter), and divided into aliquots under sterile conditions in the Royal Infirmary of Edinburgh, and then frozen at -20°C until used. All BQ-788 vials used in the studies included in this thesis were from a single batch.

2.5. Noradrenaline:

Noradrenaline (Nad; acid tartrate; MW337) is a catecholamine, derived from the amino acid L-tyrosine, released from sympathetic post-ganglionic nerve terminals. Nad is a potent agonist at α-adrenoceptors, causing constriction of vascular smooth muscle (Weiner & Taylor 1985). Parenteral Nad acid tartrate (Levophed, 1 mg/mL) was obtained from Sanofi-Winthrop Ltd, Guildford, UK. All vials of Nad used in the studies included in this thesis were from a single batch.

2.6. Losartan:

The first clinically available AT-1 receptor antagonist was losartan, a phenyl tetrazole substituted imidazole. Losartan has a 30 000-fold selectivity for the AT-1 receptor compared to the AT_2 receptor (Johnston 1995). Losartan (Dupont-Merck, Wilmington, USA) was dissolved in 0.9% saline, filtered (Millipore 0.2 μm filter), and divided into aliquots under sterile conditions in the Royal Infirmary of Edinburgh, and then frozen at -20°C until used. Pyrogen testing was negative. All losartan vials used in the studies included in this thesis were from a single batch.

2.7. Human angiotensin II:

Human angiotensin II (ANG II; MW 1046.2; $C_{50}H_{71}N_{13}O_{12}$), is an octapeptide derived from angiotensin I by the action of angiotensin converting enzyme (ACE). ANG II is a powerful vasoconstrictor that has other biological actions including

stimulation of aldosterone biosynthesis, vasopressin release, thirst, glucose activation and facilitation of sympathetic neurotransmission. ANG II (Clinalfa) was dissolved in 0.9% saline, filtered (Millipore 0.2 μm filter), divided into aliquots under sterile conditions in the Royal Infirmary of Edinburgh, and frozen at -20°C until being used. Pyrogen testing was also negative. ANG II vials used in the studies of Chapter 3 were from a single batch, while those used in Chapter 5 were from another patch.

2.8. Sodium nitroprusside dihydrate:

Pharmaceutical grade sodium nitroprusside (SNP) was obtained from David Bull Laboratories, Victoria, Australia, and was administered at titrated doses (80-600 ng/min) prepared using sterile 0.9% saline. Due to its light sensitivity, SNP was prepared and infused in syringes covered by opaque aluminium foil.

2.9. L-N^G monomethyl arginine acetate:

L-N^G monomethyl arginine (L-NMMA, $C_7H_{16}N_4O_2.CH_3COOH$; MW 188.2) is an endogenous methylated derivative of the amino acid L-arginine. L-NMMA is a specific and dose-dependent substrate analogue inhibitor of nitric oxide synthase in animals (Palmar et al 1988) and man (Vallance et al 1989a, Vallance et al 1989b). Because nitric oxide is continuously generated in the resistance vessels, L-NMMA causes peripheral vasoconstriction (Vallance et al 1989a), and increases blood pressure in both healthy humans (Haynes et al 1993) and patients with cirrhosis (Forrest et al 1995). Actions of L-NMMA can be reversed by L-arginine in a dose-dependent manner. Sterile and pyrogen-free L-NMMA (Clinalfa) was kept frozen at -20°C. Before each study, L-NMMA at a concentration of 4 μmol/min was prepared using sterile 0.9 % saline.

2.10. Vitamin C:

Vitamin C (Ascorbic acid, Evans Medical, Horsham, UK), at a final concentration of 10 μg/mL, was added to all diluent solutions for the studies using Nad to prevent its oxidation (Collier et al 1978).

Doses of all the previously mentioned pharmacological agents are mentioned in the relevant chapter. However, it should be emphasised that all were locally active (in the forearm circulation) and sub-systemic, and were chosen on the basis of previous studies as indicated in the corresponding chapters. Also, no local or systemic side effects were encountered with the use of any of these agents in any of the subject included.

3. Methods

3.1. Measurement of forearm blood flow:

This technique involves the combination of unilateral intra-brachial artery administration of vasoactive agents and measurement of FBF by strain gauge venous occlusion plethysmography (Figure 3-1). Using this technique, one can measure baseline FBF, study the function of blood vessels in health and disease, characterise the vascular properties of new drugs, and examine many physiological mechanisms using specific pharmacological probes (Webb 1995; Webb & Hand 1995). Over the last three decade, forearm venous occlusion plethysmography has been used extensively in physiological, pharmacological, and clinical studies on vascular smooth muscle and endothelial cell function and in the assessment of drug effects on the arterial vascular bed (Collier et al 1972; Collier & Robinson 1974; Collier et al 1978; Webb et al 1989; Webb et al 1988; Benjamin et al 1989; Vallance et al 1989a; Panza et al 1991; Cockcroft et al 1994, Verhaar et al 1998, Newby et al 1998, Strachan et al 1999).

In this thesis, we have used this technique to evaluate the response of the forearm resistance vessels to exogenous vasoactive agents such as ANG II, Nad, and ET-1, and study the contribution of endogenous ANG II, ET-1 and NO to the maintenance of basal vascular tone via infusing their selective antagonists such as losartan, BQ-123, BQ-788, and L-NMMA respectively. We have also combined this technique with the application of LBNP to assess the FBF responses to reflex sympathetic stimulation both in the basal state and during losartan infusion. In addition, we have also used venous occlusion plethysmography to measure FBF responses to ANG II and BQ-123 with and without the application of 'NO-clamp': a method of studying the contribution of endogenous NO release to the vascular responses of vasoactive agents.

In general, changes in local blood flow in response to vasoactive agents will reflect the changes in arteriolar tone if the arterial blood pressure remains constant, although effects on pre-capillary sphincters, or smaller arteries, may also contribute (Folkow et al 1971). In addition, responses to vasoactive agents in the

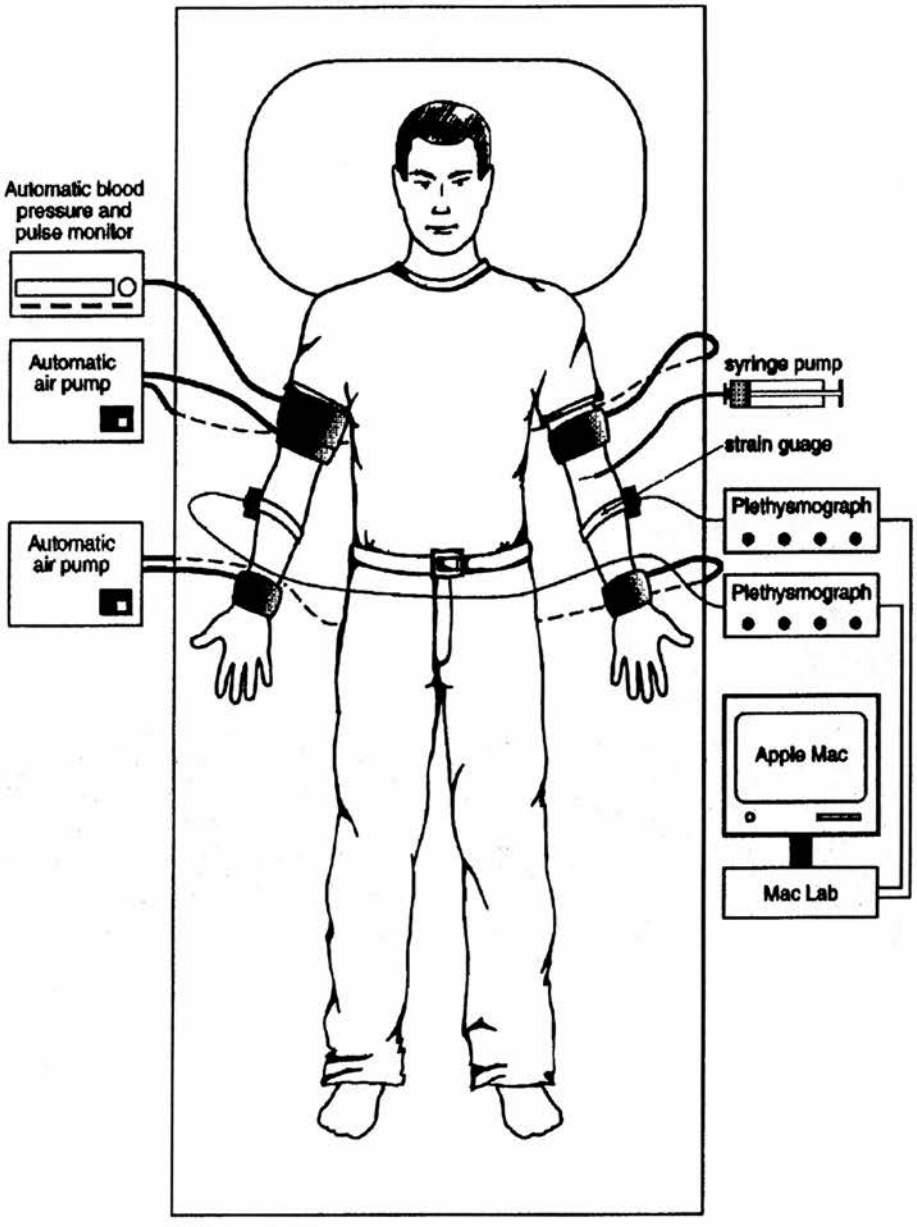


FIGURE 2-1: THE SET-UP OF BILATERAL FOREARM BLOOD FLOW MEASUREMENT USING VENOUS OCCLUSION PLETHYSMOGRAPHY AND UNILATERAL INTRA-BRACHIAL ARTERY INFUSION.

forearm parallel those in other resistance beds (Collier et al 1978), and are therefore applicable to most of the vessels that contribute to systemic vascular resistance and systemic blood pressure. Moreover, the changes in hepatic venous pressure gradient in response to propranolol treatment correlated with FBF in patients with cirrhosis (Albillos et al 1997).

3.1.1. Intra-brachial artery administration of drugs:

3.1.1.1. BACKGROUND:

The first description of arterial cannulation to assess responses of skeletal muscle to the local administration of drugs was described in 1946 (Allen et al 1946; Barcroft & Konzett 1949) in the form of adrenaline infusion into the femoral artery. However, most studies nowadays use the brachial artery, as it is easy to reach, and is more convenient. Also, femoral arteries can't be used in combination of LBNP.

3.1.1.2. TECHNIQUE:

Cannulation of brachial artery of the non-dominant arm was performed using a 27-standard wire gauge (SWG) steel needle (Cooper's Needle Works, Birmingham, UK) attached to a 16G epidural catheter (Portex Ltd, Hythe, Kent, UK) under 1% lignocaine (Xylocaine; Astra Pharmaceuticals Ltd, Kings Langley, UK) local anaesthesia. Patency was maintained by saline infusion at a constant rate of infusion (1 ml/min), via an IVAC P1000 syringe pump (IVAC Ltd., Basingstoke, UK), in order to prevent interpretation of the results being confounded by alterations in the flow rate causing changes in FBF; this is particularly important to avoid during infusion of vasoconstrictor substances.

3.1.1.3. SAFETY:

The extremely fine steel needle used to cannulate the brachial artery produces little local trauma and enables repeated studies in the same subjects. This technique has been in use at the Western General Hospital in Edinburgh for the last 10 years, with no serious adverse events associated with its use. This is consistent with the safety of long-term use of larger brachial and radial artery cannulae in clinical practice (Gardner et al 1974; Moran et al 1988). The fine gauge needle technique is also well

tolerated, allowing studies to be repeated up to 6 times in the same subjects at weekly intervals. Although some researchers use cannulae of a size sufficient to allow direct measurement of arterial blood pressure, we did not apply such a technique because these cannulae produce tissue trauma and are therefore less well tolerated and difficult to justify ethically. In addition, a recent study has shown that cannulation of the forearm has a small effect on local baseline FBF and on the vasoconstrictor responses to intravenously infused Nad (Kamper & Chang 1999). Alternatively, we have used a non-invasive method to measure blood pressure at the brachial artery of the non-infused arm for safety purposes and to exclude a systemic drug effect.

In studies that involve infusion of powerful vasoconstrictors or vasodilators, it is critical to consider carefully, and be guided by, the data from previous animal pharmacology and human studies especially when deciding the dose and duration of each vasoactive substance. The doses chosen in the studies described in this thesis have proved remarkably safe in previous forearm studies, avoiding the potential hazards that might be associated with studies undertaken in other vascular beds, such as the coronary circulation, or with systemic administration. Indeed, none of the subjects included in these studies have experienced any side effects related to the doses of the drugs. Theoretically, vessel occlusion is possible (Ashton et al 1963), but this has not been observed in practice even with major reductions in local blood flow caused by ANG II (Benjamin et al 1989) and ET-1 (Clarke et al 1989).

3.1.1.4. COMPLICATIONS:

A dull ache around the elbow or in the forearm may occur for up to 48 hours after cannulation, and is probably related to immobilisation. After each experiment, pressure is applied to the artery for at least 5 min to reduce the likelihood of bleeding or haematoma formation. Only two patients had localized bruises that resolved spontaneously. Occasionally the subject experiences pain in the hand if the median nerve, which is adjacent to the brachial artery in the anticubital fossa, is stimulated during cannulation. This is rare and immediately reversible when the needle is withdrawn.

3.1.2. Venous occlusion plethysmography:

3.1.2.1. HISTORICAL BACKGROUND:

Plethysmography, which means measurement of volume changes in a part of the body, was first used in humans as early as 1875 (Mosso 1875). It was then proposed as a method to measure blood flow in limbs and digits through the use of venous occlusion by Brodie & Russell in 1905. Venous occlusion water plethysmography was first shown to be suitable for measuring FBF and venous compliance by Hewlet & van Zwaluwenburg in 1909. In this technique, a proximal limb cuff is rapidly inflated to greater than the venous pressure but lower than the arterial pressure so that the increase in limb volume with time gives a measure of blood flow because the venous return is halted but arterial inflow continues unimpeded. In this system, the limb was sealed in a rigid jacket and volume changes of the limb following inflation of an upper arm venous occlusion cuff are measured by the displacement water or air in the jacket. The main difficulties related to this method were the establishment of fluid-tight seals, which did not impede blood flow or venous return, the bulk of the equipment, and the need of considerable preparation. Therefore, alternative air-filled plethysmographs have been used, but this was sensitive to temperature changes (Vanhuyse & Raman 1971). The venous occlusion water and air plethysmographs have been superseded by the introduction of strain gauges (Whitney in 1953), in which changes in limb circumference are detected as a change in electrical resistance of the gauge. Both the changes in limb circumference and electrical resistance were proportional with negligible error (Whitney 1953; Ensink & Hellige 1981). The original mercury-in-latex strain gauge was acting as a resistor in a Wheat stone bridge circuit, and FBF measured using such temperature compensated strain gauges was not only similar to that measured by the water displacement method, but also with a lower variance in results of repeated measurements (Clarke & Hellon 1957; Dahn & Hallbrook 1970; Paulev et al 1974). Recently, the mercury-in-latex gauges have been replaced by indium/gallium-in-silastic gauges, which are more rapidly applied and calibrated. The advantages of the strain gauge method include: 1) it eliminates the need for water and air seals, 2) the strain gauges are small and lightweight, 3) the strain gauges can be used for long periods, 4) subjects can move their limbs between recording periods without disturbing the equipment or

subsequent recordings, and 5) the time taken to apply and calibrate the gauges is short. Throughout the years, this method has been standardized and computerized, making it easier to carry out and reducing analysis bias (Chang & van Brumellen 1987; Chang et al 1988; Petrie et al 1998).

3.1.2.2. TECHNIQUE:

FBF measurements were made in both arms i.e. the infused and the control arms by venous occlusion plethysmography using mercury in-silastic strain gauges applied to the widest part of the forearm. Hands were excluded by rapid inflation of wrist cuffs to a pressure of 220 mmHg using E20 Rapid Cuff Inflators (D.E. Hokanson Inc, Washington DC, USA). Upper arm cuffs were inflated up to 40 mmHg for 10 seconds (s) in every 15 s to achieve venous occlusion and obtain blood flow measurements. Recordings of forearm blood flow were made every 3 min unless otherwise stated. Voltage output from a dual channel Vasculab SPG 16 strain gauge plethysmograph (Medasonics Inc, Mountain View, CA, USA) was transferred to a Macintosh personal computer (Classic II, Apple Computer Inc, Cupertion, CA) using a MacLab analogue-digital converter and Chart *v*4 software (both from AD Instruments, Castle Hill, Australia).

3.1.2.3. REPRODUCIBILITY:

Between-day intra-subject reproducibility of baseline FBF measurements had a coefficient of variation (CV) ranging from 7.8% to 15.6%. However reproducibility of responses to systemic exercise ranged from 13% to 29% (Roberts et al 1986). Another study of between-day intra-subject reproducibility of unilateral FBF showed a CV of 25% and concluded that unilateral FBF measurements are: "poorly suitable" for pharmacological trials (Altenkirch et al 1990). Also, poor within-day unilateral baseline FBF reproducibility was reported by a third study (Egan et al 1988). Simultaneous bilateral measurement of FBF as a method of reducing variability was first proposed over 40 years ago (Greenfield & Patterson 1954), and has been adopted thereafter by many investigators. In theory, measurements are adjusted for systemic changes unrelated to the local stimulus by expressing flow in the infused arm as a ratio of concurrent flow in the non-infused (control) arm (Webb 1995;

Benjamin et al 1995). In contrast, FBF ratios are more reproducible than unilateral FBF measurements both at rest (CV 19% *vs* 39%) and in responses to intra-arterial infusions of vasoconstrictor substances (Petrie et al 1998). Also, forearm vascular resistance may have a very small reproducibility advantage (CV 14%; Petrie et al 1998).

3.1.2.4. PRECAUTIONS:

The use of venous occlusion plethysmography has been shown to be both accurate (Norton et al 1982) and reproducible when used in clinical studies (Robertson et al 1986; Petrie et al 1998). However, some precautions need to be considered:

1. Non-experimental stimuli can cause significant and misleading changes in measured responses if unilateral measurements are used. Therefore, it is highly recommended that responses to intra-arterial infusions should be measured using bilateral forearm plethysmography with the results expressed as FBF ratios (Benjamin et al 1995; Webb 1995; Petrie et al 1998).
2. The pressure in the arteries distal to an upper arm compression cuff is not affected by inflation of the cuff to a sub-diastolic pressure (Wilkins & Bradley 1946). The amount of blood flow through bone (Edholm et al 1946) is small and is not detected by this technique. Therefore, it is easy to select a suitable upper arm collecting cuff pressure, which can temporarily stop venous return (apart from those in bone), but does not affect arterial inflow.
3. Compared with the forearm, the hand has less distension capacity (Greenfield et al 1963). Thus, inclusion of the hand may result in non-linear flow tracings, which are difficult to analyse. In addition, blood flow to the hand is predominantly through skin rather than muscle, and so is more sensitive to changes in temperature (Spearman 1945) and emotions (Blai et al 1959; Abramson & Ferris 1940). Moreover, skin blood flow has a different physiology and exhibits different responses to vasoactive drugs (Webb 1995). Therefore, the hand should be excluded during measurements by the application of a high-pressure occlusion cuff at the wrist.
4. The rate of blood flow into the forearm arteries is disturbed during the first minute after inflation of the high-pressure occlusion cuff at the wrist (Kerslake

1949). Therefore, FBF recordings should be made for 3 min and the recordings made in the first minute following wrist cuff inflation should never be used in the analysis.

5. Venous occlusion plethysmography measures the total blood flow through all tissues of a limb segment. The relative contribution of muscle to FBF at rest, as estimated using adrenaline iontophoresis to abolish skin blood flow, was ~75% of blood flow less than, and ~50% of blood flow higher than 6 mL/100 mL/min (Cooper et al 1955; Zelis et al 1960; Katz & Folz 1983).
6. Using the strain gauge technique, FBF is given in mL per 100 mL of tissue per min as calculated from the stretch of the gauge around one segment of the forearm during a period of venous occlusion. Values obtained by the strain gauge technique are similar to those obtained by volume methods (Whitney 1953; Paulev et al 1974). Measurement of the absolute flow requires knowledge of the forearm volume by water or air displacement. This introduces inaccuracy because the blood flow in bone is low, causing an underestimate of muscle blood flow.
7. FBF varies with temperature, and it is important to maintain a constant temperature to within ± 1°C throughout each study day.

3.1.2.5. STRENGTHS AND WEAKNESSES:

1. These studies enable the vascular responses to vasoactive mediators and drugs to be assessed *in vivo* in humans. Therefore, it has the advantages of an *in vivo* vascular technique especially when the vessels are exposed to physiological pressures, have physiological dimensions, have intact nervous mechanisms, bathed in the physiological medium of blood, exposed to local and circulating vasoactive and growth factors, and have not been exposed to general anaesthetic agent or to potentially significant vessel trauma or hypoxia.
2. Because the dose of drug administered is small, the forearm technique can be used early in the clinical evaluation of novel endogenous hormones or cardiovascular drugs.
3. Because the effects of infused drug are restricted to the forearm, it is possible to construct full dose-response relationships for the effects of drugs on FBF.

4. Baseline FBF and the forearm vascular response to intra-arterial infusion of vasoconstrictor agents, such as Nad, are not affected by left or right arm dominance (Kamper & Chang 1999).
5. Most *in vivo* human vascular studies use systemic doses, which can influence the neurohormonal reflexes and cause effects on other organs such as the brain, heart and kidneys through changes in systemic haemodynamic parameters. Thus, the direct vascular effects of systemic drug doses could not be readily attributed to the infused drug alone. In contrast, intra-brachial artery administration of sub-systemic and locally active doses of drugs together with measuring the FBF responses allows the direct vascular actions of the vasoactive drugs to be studied. Because FBF approximates to 20-50 $mL.min^{-1}$, and the CO is ~5000 mL/min, doses of 100-1000 fold lower than the systemically active doses can be used effectively within the forearm circulation. Therefore, the active amount of drug reaching the systemic circulation with brachial artery infusion of locally active doses is insignificant, unless the drug was given for very long periods or has a long half-life. In addition, vasoactive peptides are rapidly inactivated in the circulation.
6. Fluctuations in FBF occur in association with changing levels of alertness. However, because these fluctuations affect both arms similarly, blood flow in the control arm can serve as a contemporaneous control for drug effects in the infused arm, thereby reducing the variability of the measurement (Greenfield & patterson 1954).
7. The validity of the technique relies on some assumptions, such as arterial pressure remaining stable during the study period. There is much between-subjects variability in the forearm vascular response to changes in arterial pressure, and there is no simple way of distinguishing the autoregulatory response from the direct effect of a drug under these circumstances (Robinson 1990). However, provided that patients were comfortable and relaxed in a warm calm environment, were given sufficient time to settle, and were given sub-systemic doses, arterial pressure remains stable for considerably longer than is required to observe the response to a drug.

3.1.2.6. PITFALLS:

The forearm technique is most powerful when used for within-subject comparisons. However, between-group comparisons such as between patients and healthy controls are often performed. In this situation, several potential pitfalls may occur:

1. Patients and control subjects need to be well matched with respect to age and sex; blood pressure; FBF, forearm volume & length; renal function; serum cholesterol & glucose.
2. Results should be interpreted with caution if either the baseline blood pressures or FBF differ between the groups. Whether changes in absolute flow or calculated vascular resistance are used, normalising the results for initial differences between the groups is inherently unsound (Robinson 1990).
3. Differences in vessel wall geometry pose a major problem, and increases in wall : lumen ratio enhance the responses to both vasodilators and vasoconstrictors (Folkow 1982). Thus, enhanced responses to a wide range of vasoconstrictors (Doyle et al 1959; Egan et al 1987; Calver et al 1992) and vasodilators (Hulthen et al 1985) in patients with hypertension can be explained by differences in vessel geometry. This problem can be overcome by comparing dose-response relationships to different vasoactive drugs within each group separately. For example, by carefully selecting the dose of drugs, overlapping dose-response curves to a range of vasoactive drugs can be produced in the forearms of normal subjects and compared with responses in hypertensive subjects (Robinson et al 1984; Calver et al 1992). Based on the changes in vessel geometry, vasoconstrictors are likely to produce greater vasoconstriction in the patients with hypertension. However, if the effects of one agent among several tested was relatively much greater than for the other vasoconstrictors it might be reasonable to assume that there was a specific abnormality of this pathway in essential hypertension.
4. Finally, there is the question of whether the observed responses in the FBF (absolute or ratio or resistance) should be correlated with the doses or concentrations of the drugs infused. The majority of FBF studies have used the mass of drug delivered per unit time (min). However, a minority have suggested that a correction should be made for the drug induced increase or decrease in

blood flow (Angus & Lew 1992) or by limb volume (Chin-Dusting et al 1999). In reality, no correction is necessary (Benjamin et al 1995; Webb et al 1995).

3.2. The nitric oxide clamp 'NO-clamp' technique:

3.2.1. Definition:

'NO-clamp' is a balanced co-infusion of L-N^G-monomethyl arginine (a selective NO synthase inhibitor) and sodium nitroprusside (an exogenous NO donor) to block endogenous NO production and restore normal NO-mediated basal blood flow respectively.

3.2.2. Importance:

NO is continuously released by the endothelium, and relaxes the underlying vascular smooth muscle. Brachial artery administration of L-NMMA (a selective NOS inhibitor) causes forearm vasoconstriction and a reduction in basal blood flow (Vallance et al 1989a). When assessing the contribution of NO to the actions of a given vasoactive agent, these L-NMMA-induced changes in vessel geometry and blood flow may confound the interpretation of subsequent responses (Benjamin et al 1995; Webb 1995). However, co-infusion of sodium nitroprusside (SNP, an exogenous NO donor) with L-NMMA can be used to restore the baseline blood flow and vessel geometry by replacing endogenous NO with exogenous NO: the 'NO-clamp' (Stroes et al 1997). This technique establishes a stable baseline forearm blood flow (FBF) that can be maintained for at least 120 min (Verhaar et al 1998) and permits the assessment of vascular responses in the absence of endogenous NO synthesis (Stroes et al 1997; Verhaar et al 1998; Dijkhorst-Oei et al 1999).

3.2.3. Technique:

L-NMMA (Clinalfa AG, Läufelfingen, Switzerland) was continuously infused at a rate of 4 μmol/min for 20 min to achieve maximal inhibition of local NOS activity. This dose has previously been shown to produce a maximal vasoconstrictor response in the forearm circulation (Vallance et al 1989a; Calver et al 1994). Thereafter, SNP (David Bull Laboratories, Victoria, Australia) was co-infused at titrated doses (80-600 ng/min) until FBF had been restored to within 10% of baseline flow and was

sustained for at least 2 consecutive FBF measurements (variable duration). The drugs were dissolved in 0.9% saline (Baxter Healthcare Ltd., Thetford, England). Due to its light sensitivity, SNP was prepared and infused in syringes covered by opaque foil.

3.3. Lower body negative pressure (LBNP):

LBNP was applied using a previously described method (Brown et al 1966). Subjects rested supine within a plastic-covered steel cage, which enclosed the lower body from waist downwards and sealed above the level of the anterior superior iliac spines (Figure 2-3). A constant negative pressure of 15 mmHg was achieved using an industrial strength vacuum cleaner regulated by a pressure control unit (Medical Physics Department, Edinburgh University, Edinburgh, UK). Alterations to and from atmospheric pressure were attained within 1-2 seconds. This negative pressure causes selective forearm vasoconstriction, through pre-junctional release of Nad, without affecting systemic blood pressure or heart rate and without activating carotid baroreceptors (Seidelin et al 1991).

The combination of LBNP and intra-arterial infusion of local doses of drugs, agonists or selective receptor antagonists (such as the use of LBNP and losartan in Chapter 3) are usually used to study the potential interaction between these vasoactive agents and local sympathetic activity.

None of the subjects experienced any adverse effects during or after the application of LBNP apart from our observation of a transient increase in the lower limb oedema in two patients with advanced liver disease.

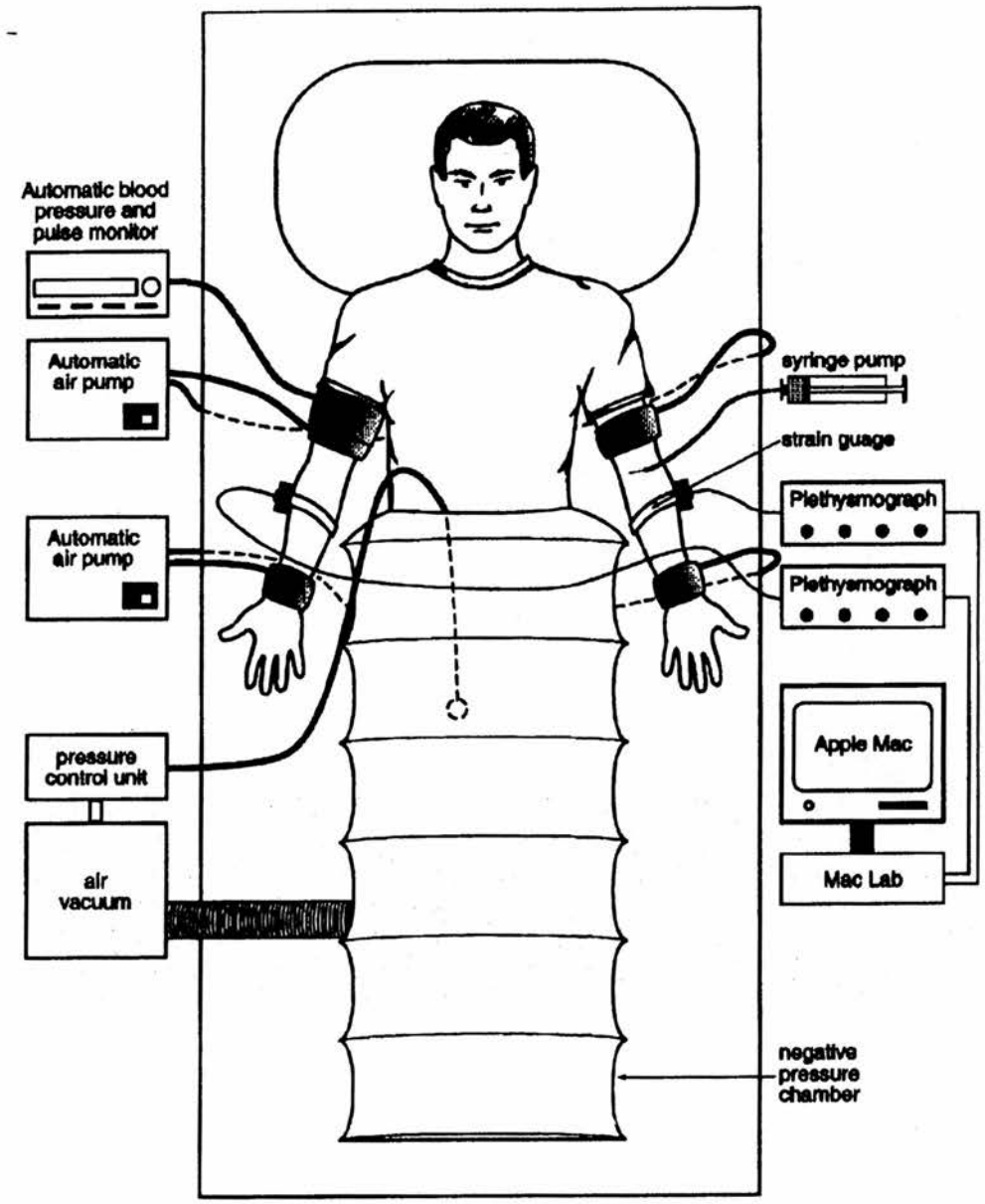


FIGURE 2-2: THE SET-UP OF BILATERAL FOREARM BLOOD FLOW MEASUREMENT AND THE APPLICATION OF LOWER BODY NEGATIVE PRESSURE.

3.4. Measurement of systemic haemodynamics:

3.4.1. Cardiac parameters & systemic vascular resistance (Electrical bioimpedance method):

Baseline cardiac functions including heart rate, stroke volume and cardiac output were measured using a non-invasive bioimpedance methodology (BoMed NC-COM3, BoMed Medical Manufacturer Ltd) as previously described (Apple et al 1986). Figures 2-3 and 2-4 show the sites of attachments of the electrodes and an example of the printouts of systemic haemodynamic results respectively.

Absolute cardiac output measured by electrical bioimpedance has been validated against thermodilution measurements with correlation coefficients ranging from 0.83 to 0.90 and differences ranging from 2% to 12% (Apple et al 1986; Salandin et al 1988; Northridge et al 1990; Thomas et al 1992). In addition, electrical bioimpedance measure of changes in cardiac output after drug intervention is not only in close agreement with simultaneous thermodilution measurements, but also has a lower within-subject coefficient of variation as well.

Cardiac index (CI), stroke index (SI), and total peripheral vascular resistive index (TPVRI) were calculated according to the formulae:

- CI ($mL.min^{-1}. m^{-2}$) = CO ($mL.min^{-1}$) / body surface area (BSA; m^2).
- SI = stroke volume (mL) / BSA (m^2).
- TPVRI (mm Hg. $mL^{-1}.min.m^2$) = MAP (mm Hg) / CI ($mL.min^{-1}. m^{-2}$).
- BSA (m^2) = Square root (Hight (cm) × Weight (kg) / 3600).

3.4.2. Blood pressure & heart rate: (Semiautomated oscillometric method):

Both blood pressure and HR were measured in the non-infused arm using a non-invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan) at intervals throughout each study. This methodology has been validated against intra-mercury sphygmomanometer (Wiinberg et al 1988) and the Hawkesley random-zero mercury sphygmomanometer (Evans et al 1989; Jamieson et al 1990).

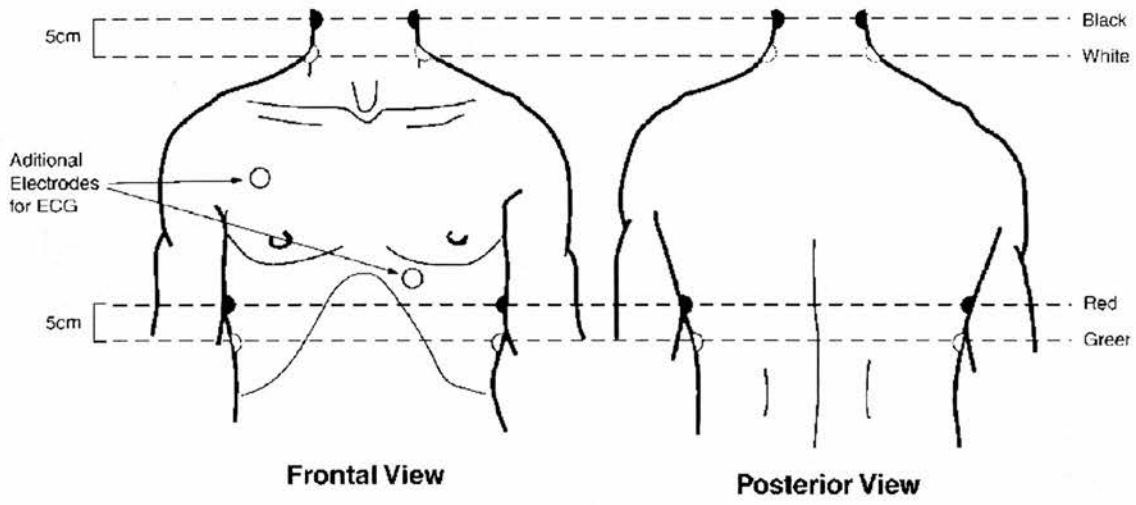


FIGURE 2-3: THE SITES OF ATTACHMENTS OF THE BOMED NC-COM3 ELECTRODES DURING MEASUREMENT OF SYSTEMIC HAEMODYNAMICS.

BoMed®
CARDIODYNAMIC MONITOR

Patient	**male**
ID #	**06 19 55**
height	**167 cm**
weight (1.13)	**082 kg**
VEPT	**06340 ml**
BSA	**1.89 msq**

CO	SV	EDV	PF	EF	HR	TFI	IC	ER	STR	ACI	TIME
:04.1	069	122	042	56	060	30.0	033	33	037	071	01:01
:04.1	069	121	042	56	060	30.0	033	33	037	070	
:04.1	069	122	042	56	060	30.0	033	33	037	070	
:04.1	069	121	042	56	060	30.0	033	33	037	070	

FIGURE 2-4: AN EXAMPLE OF SYSTEMIC HAEMODYNAMIC PARAMETERS RECORDED BY THE BOMED NC-COM3 ELECTRICAL BIOIMPEDANCE METHOD.

The MAP was calculated according to the formula: MAP (mm Hg) = diastolic arterial pressure + 1/3 pulse pressure (systolic arterial pressure - diastolic arterial pressure). Venous congestion due to pressures at or above mean diastolic pressure cause reflex vasoconstriction lasting ~1 minute (Patterson & Shepherd 1954). For this reason, blood pressure was measured at least one minute before any FBF measurements were made.

3.5. Blood assays:

3.5.1. Blood sampling:

After 30 min of supine rest, and before any drugs were administered, venous blood sample was withdrawn from the non-infused arm. Ten mL were admixed with each of the following: 1 mL of 5% disodium EDTA/ 2% sodium metabisulphite for measuring Nad and Ad concentrations; 0.5 mL of 0.45% *O*-phenanthroline/ 4.65% disodium EDTA for measuring ANG II concentration; and 1 mL of 1% disodium EDTA and 1000 KIU aprotinin (Bayer AG, Germany) for measuring plasma renin activity (PRA), ET-1 and big ET-1 concentrations. The samples were placed on ice and immediately centrifuged at 1500 *g* for 20 min. Plasma was then frozen and stored at -80°C until assayed. The relative centrifugal force (RCF) or *g* (gravity force) was calculated using the formula:

$$RCF = 1.118 \times 10^{-5} \times r \times n^2$$

Where r is the radius of the centrifuge in centimeter (cm), and n is the speed of rotation in round per minute (rpm).

3.5.2. Endothelin assay:

Baseline plasma concentrations of ET-1 (Peninsula Laboratories INC, Belmont, CA, USA) and big ET-1 (Peninsula Laboratories INC) concentrations were measured by radioimmunoassay (RIA) following extraction using Bond Elut® columns (Varian, Harbor City, CA, USA) as described previously (Rolinski et al 1994). Briefly, each 2.5 mL plasma was diluted with 2.5 mL of 20% acetic acid and loaded onto a column; these were washed, and eluted with 20% ammonium bicarbonate : 80% ethanol (2x1 mL). The eluates were evaporated under nitrogen in a waterbath at 37°C, and reconstituted in phosphate buffer of pH 7.4 (0.25 mL). Duplicate extracted

samples and standards containing 1-64 pg/ml endothelin-1 (each 100 μL) were incubated with rabbit polyclonal antibody raised against ET-1 (Peninsula Laboratories INC) for 24 hours at 4 °C and ^{125}I-ET-1 (NEN Life Science Products, Hounslow, UK). After vortexing, tubes were incubated for 18-24 hours at 4°C. Amerlex Donkey anti-rabbit gamma globulin (200 μL) bound on magnetic particles was added to all tubes, except the total-count tubes, and the tubes were then incubated for 30 min at room temperature. Tubes were, then, centrifuged for 20 min at 2000*g* at 4°C. The amount of radioactivity in the antibody-bound fraction was determined by gamma counting for 1 min. The recovery of added ET-1 was 84%. The intra-assay coefficients of variation for ET-1 and big ET-1 were 7.0% and 7.2% respectively, and the inter-assay coefficients of variation were 9.0% and 9.3% respectively. The cross reactivities of the ET-1 assay were ET-1 (100%), ET-2 (7%), ET-3 (7%), C-terminal fragment (0%), big ET-1 (10%), angiotensin I (ANG I; 0%), and ANG II (0%); and for the big ET-1 assay were ET-1 (0%), ET-2 (0%), ET-3 (0%), C-terminal fragment (100%), and big ET-1 (100%).

3.5.3. Angiotensin II assay:

Addition of EDTA/*O*-phenanthroline to the venous blood samples inhibits the converting and angiotensinase enzymes and therefore, inhibits the breakdown of plasma ANG II (Dusterdieck & MaElwee 1971). Plasma ANG II concentrations were measured by RIA following extraction using Bond Elut® columns (Varian, Harbor City, CA, USA) as previously described (Morton & Webb 1985). Briefly, the method of ANG II extraction was identical to that of ET-1, apart from the use of 4 mL of plasma. The extracts were then dried and re-dissolved in Tris buffer (50 mmol; PH 7.5) for the RIA (Peninsula Laboratories, Belmont, CA, USA). The intra-assay and the inter-assay coefficients of variation for ANG II were 7.2% and 9.3% respectively. The cross reactivities of the ANG II assay were ANG-II (100%), angiotensin I (0.5%), ANP (0%), and renin substrate (0.9%).

3.5.4. Plasma renin activity assay:

PRA was measured under standard conditions through the generation of ANG I using RIA as previously described (Haber et al 1969). PRA was estimated using RIA of

angiotensin I generated from plasma after a 1-hour incubation at pH 5.5 and at 37°C in conditions inhibiting the further conversion of angiotensin I (Rianen assay system Angiotensin I [^{125}I] kit; NEN Life Science Products). The intra-assay and the inter-assay coefficients of variation for PRA were 4% and 7% respectively. The cross reactivities of the angiotensin I assay were ANG I (100%), angiotensinogen (1%), ANG II (0.9%), and angiotensin III (0.1%).

3.5.5. Catecholamines assay:

Plasma catecholamines were measured by dual-electrode coulometric detection after separation on a reverse phase high performance liquid chromatography column as described previously (Goldstein et al 1981). A simple solvent extraction system was used for the selective and quantitative isolation of Nad and adrenaline from plasma. Data handling and acquisition was carried out using a JCL 9000 Chromatography Data System (Jones Chromatography Ltd, UK). The limits of detection were < 5 pg per injection for Nad and adrenaline, with inter-assay coefficients of variation of 1.6% for Nad, and 2.0% for adrenaline. The average recovery of dihydroxybenzylamine, which was used as the internal standard, was 88% (n=100).

3.5.6. Other assays:

- *Serum creatinine:*

Serum creatinine was assayed in the Department of Clinical Chemistry, Royal Infirmary of Edinburgh, using a Kodak Ectachem System E700 XRC analyser (Kodak Diagnostics Ltd, UK).

- *Urinary sodium excretion:*

Urine of the 24 h preceding the morning of the study were collected and Na^+ concentrations were determined by flame photometry.

3.6. Data management and statistical analysis:

3.6.1. Data extraction:

Plethysmographic data were extracted from the Chart™ (AD Instruments, Castle Hill, Australia) data files and forearm blood flows were calculated for individual venous occlusion cuff inflations using a template spreadsheet (Excel *v*5.0;

Microsoft). Recordings from the first 60 s following wrist cuff inflation were not included in the analysis because of the variability in blood flow this produces (Kerslake 1949). Usually, the last 5 flow recordings in each 3 min measurement period were calculated and averaged for each arm. Figures 2-5 & 2-6 shows examples of the plethysmographic recordings before and after infusions of a vasodilator and a vasoconstrictor respectively. In order to reduce the variability of blood flow data, the ratio of flows in the 2 arms was calculated for each time point, thereby using the non-infused arm as a contemporaneous control (Patterson & Shepherd 1954; Benjamin et al 1995; Webb 1995; Chin-Dusting et al 1999).

3.6.3. Calculations:

The percentage change in FBF was calculated as follows:

$$\textbf{\% change in FBF} = 100 \times [(I_t / NI_t) - (I_b / NI_b)] / (I_b / NI_b)$$

where I_t and NI_t are the blood flows in the infused and non-infused forearms respectively at a given time point (t) and I_b and NI_b are the blood flows in the infused and non-infused forearms respectively at baseline (b); time 0.

Unlike intra-arterial drug infusions, LBNP affects both arms, and therefore, the changes in FBF during LBNP and either saline or losartan infusions were calculated in the infused arm separately for every subject as % change from the flow during the same infusion without LBNP as follows:

$$\textbf{\% change in FBF during LBNP} = 100 \times [(FBF_{LBNP} - FBF_{S \text{ or } L}) / (FBF_{S \text{ or } L})]$$

where $FBF_{S \text{ or } L}$ is the blood in the infused arm during saline (S) or losartan (L) infusion without LBNP, and FBF_{LBNP} is the blood flow in the infused arm during the same infusion with LBNP, and then the results were averaged in each group.

To compare between the response curves without and during the application of the 'NO-clamp', a single-point data summary of the data set from each curve was obtained separately as area under the curve (AUC; Matthews et al 1990). The percentage change in area AUC was calculated according to the formula:

$$\textbf{\% change in AUC} = 100 \times [(AUC_{NOC} - AUC_{FNO}) / (AUC_{FNO})]$$

where NOC and FNO are the presence of the 'NO-clamp' and free NO respectively. FBF was expressed in mL.100 mL^{-1} of tissue.min^{-1} (Whitney 1953).

In Chapter 6, the % attenuation in AUC following application of the 'NO-clamp' was calculated according to the formula:

% change in AUC = 100 × [(% change$_{PC}$ - % change$_{AC}$) / (% change$_{AC}$)]

where PC and AC are the presence and absence of the 'NO-clamp' respectively.

3.6.2. Coefficient of variation:

The coefficient of variation in the ratio of basal infused to non-infused FBF was nearly two-fold less than that for basal absolute FBF (Table 2-1). This was true whether inter-subject, intra-subject (inter-study) or intra-subject (intra-study) coefficients were examined. The lower variability of the ratio data reflects the fact that small symmetrical variations in FBF occur continuously, most probably due to variations in sympathetic outflow.

Table 2-1: Mean intra-subject (inter-study), intra-subject (intra-study), and inter-subject coefficients of variation for basal forearm blood flow and basal ratio of FBF (infused arm / non-infused arm).

The coefficient of variation	Basal absolute FBF in the infused arm	Basal FBF ratio Infused / non-infused
Intra-subject (inter-study)	34% (Range 21-53%) (8 studies)	18% (Range 13-41%) (8 studies)
intra-subject (intra-study)	16% (Range 8-19%) (8 subjects)	9% (Range 6-16%) (8 subjects)
inter-subject	53% (Range 22-75%) (8 subjects)	21% (Range 11-38%) (8 subjects)

Each individual coefficient of variation was calculated from the mean and the standard deviation of 6-8 baseline observations obtained from studies presented in this thesis.

3.6.4. Data analysis:

Data were expressed as mean ± standard error of the mean (SEM) and examined by two-factor analysis of variance (ANOVA) with repeated measures (Chin-Dusting et al 1999), the Pearson's correlation coefficient and two-tailed paired and un-paired *t*-tests as appropriate. Statistical significance was taken at the 5% level.

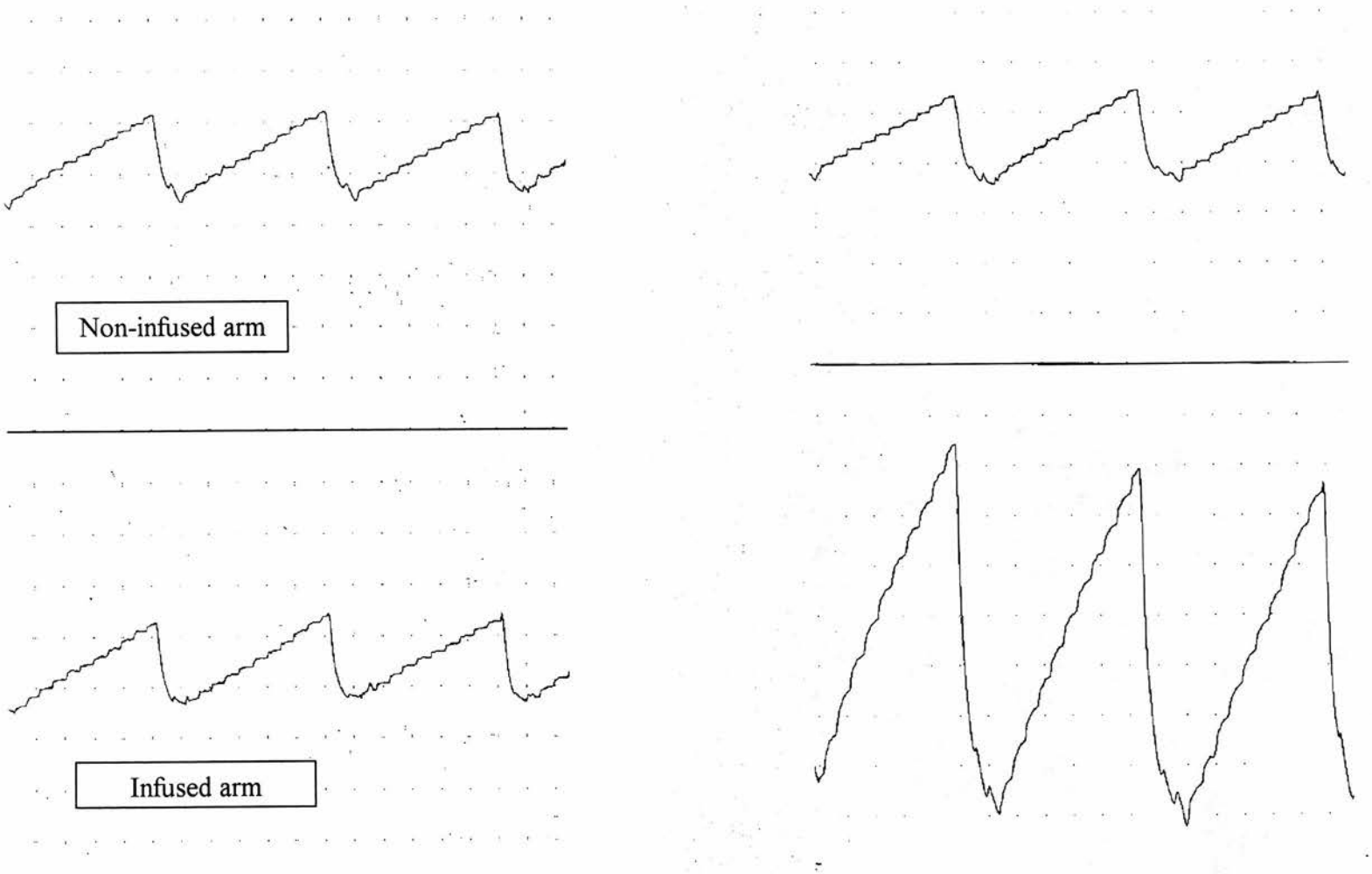


FIGURE 2-5: AN EXAMPLE OF THE PLETHYSMOGRAPHIC RECORDINGS BEFORE (LEFT) AND AFTER (RIGHT) INFUSION OF A VASODILATOR.

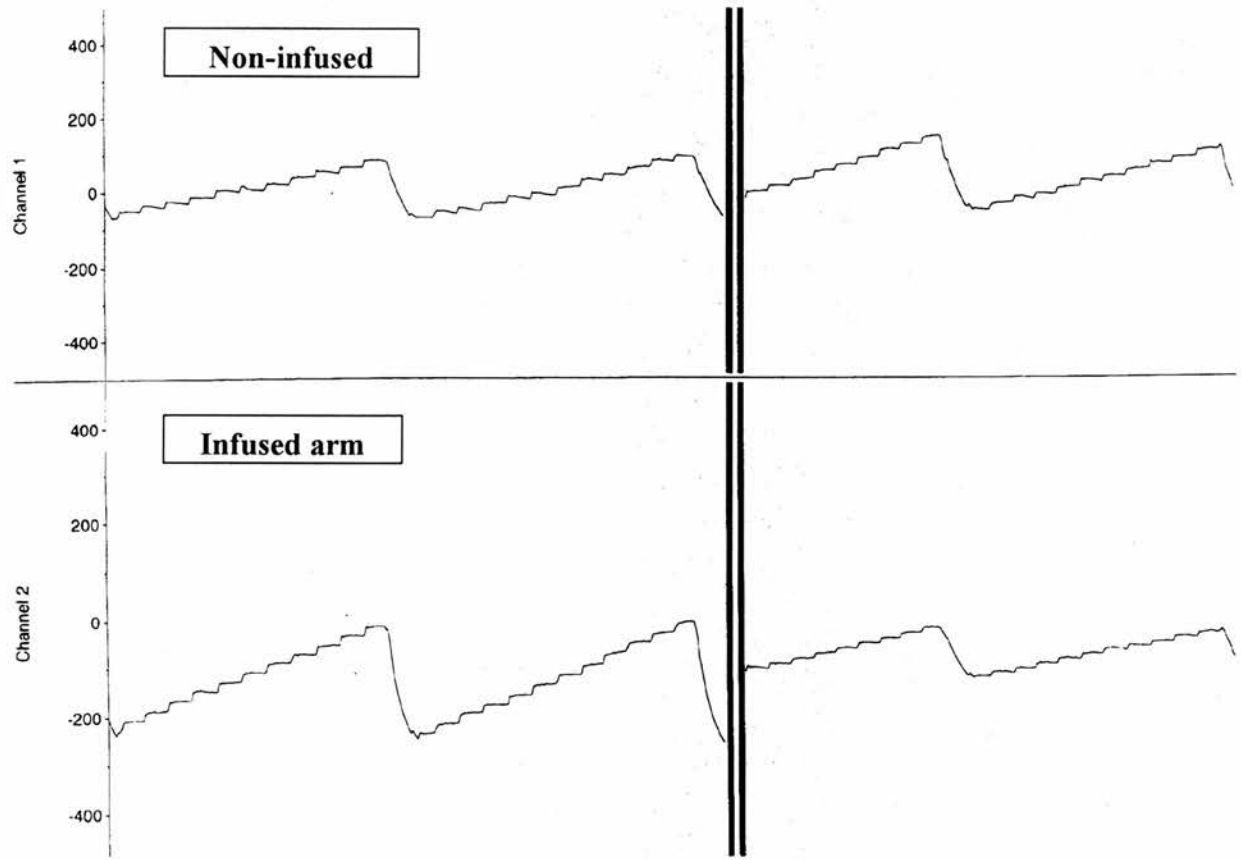


FIGURE 2-6: AN EXAMPLE OF THE PLETHYSMOGRAPHIC RECORDINGS BEFORE (LEFT) AND AFTER (RIGHT) INFUSION OF A VASOCONSTRICTOR.

CHAPTER 3

STUDIES ON THE RENIN-ANGIOTENSIN AND SYMPATHETIC NERVOUS SYSTEMS

Helmy A, Jalan R, Newby DE, Hayes PC, Webb DJ. Role of angiotensin II in regulation of basal and sympathetically-stimulated vascular tone in patients with early and advanced cirrhosis. ***Gastroenterology*** 2000;118:565-572.

1. Summary

Background/Aims: The renin-angiotensin and sympathetic nervous systems are activated in cirrhosis. In this chapter, we aimed to establish the role of angiotensin II in the regulation of basal and sympathetically-stimulated vascular tone in pre-ascitic cirrhotic patients, and diuretic-refractory ascites patients compared with age- and sex-matched healthy controls.

Methods: Forearm blood flow (FBF) responses to lower body negative pressure (LBNP) and to sub-systemic, intra-brachial infusions of losartan, an angiotensin II type-1 receptor antagonist, noradrenaline and angiotensin II were measured using venous occlusion plethysmography.

Results: In all groups, angiotensin II and noradrenaline caused dose-dependent reductions in FBF ($p<0.001$) and, whilst responses to noradrenaline were similar across the three groups, those to angiotensin II were less in both cirrhotic groups than in controls ($p<0.01$). Losartan caused a dose-dependent increase in FBF only in refractory ascites patients ($p<0.01$). LBNP caused less reduction in FBF in patients with refractory ascites than in both pre-ascitic patients and controls ($p<0.01$).

Conclusions: Despite hyporesponsiveness to exogenous angiotensin II in both early and advanced cirrhosis, endogenous angiotensin II contributes to the maintenance of basal vascular tone only in advanced cirrhosis. These findings suggest a role of angiotensin II in the pathogenesis of ascites. The forearm vascular responses to exogenous noradrenaline are unimpaired. However, attenuated LBNP responses occurred only in advanced cirrhosis, without apparent interaction with endogenous angiotensin II.

2. Introduction and aims

Liver cirrhosis is characterised by hyperdynamic circulatory changes, including high cardiac output and decreased systemic vascular resistance (Kowalski & Abelmann 1953; Murray et al 1958; Kontos et al 1964), that worsen with disease progression (Braillon et al 1986; Bendtsen et al 1990; Meng et al 1994). Although compensatory activation of vasopressin (Bichet et al 1982; Reznick et al 1983; Bichet et al 1983; Epstein et al 1984), and the renin-angiotensin (Schroeder et al 1976; Arroyo 1981, Bernardi et al 1982; Pariente et al 1985) and sympathetic nervous (Henriksen et al 1981; Bernardi et al 1982; Bichet et al 1982a; Ramond et al 1986; Flora et al 1991) systems occurs, impaired vascular reactivity has been proposed to contribute to the hyperdynamic circulation of cirrhosis and to the development of its complications, such as portal hypertension, ascites and hepatorenal syndrome (Schrier et al 1988).

Present evidence of this hyporesponsiveness has been provided by systemic studies and *in vitro* experiments. Although contributing to our understanding of the pathophysiology of portal hypertension, systemic studies have the disadvantage of invoking neurohumoral counter-regulatory mechanisms and having effects on a wide range of organs, including the heart, brain and kidneys (Benjamin et al 1995; Webb 1995). Moreover, previous studies investigating the role of the renin-angiotensin system in the maintenance of vascular tone in patients with cirrhosis (Schroeder et al 1976; Arroyo 1981) are weakened by the use of antagonists, such as saralasin, with partial agonist activity (Case et al 1976; Anderson et al 1977; Anderson et al 1980), or the use of angiotensin converting enzyme inhibitors (ACI; Pariente et al 1985), such as captopril, which also inhibits the breakdown of bradykinins. However, losartan, a selective angiotensin II type 1 (AT_1) receptor antagonist that is devoid of agonist activity, has recently become available for clinical use (Bauer et al 1995). Furthermore, results of previous studies addressing this issue in patients with advanced cirrhosis (MacGilchrist et al 1991; Newby et al 1998) have been confounded by the use of diuretics, which produce hypovolaemia (Shah et al 1978) with subsequent activation of the endogenous neurohumoral systems (Dwarakanathan et al 1975; Schrier et al 1988) In addition, the combination of sub-systemic and locally active intra-brachial artery infusions with bilateral forearm

blood flow (FBF) measurements using venous occlusion plethysmography represents a powerful and reproducible method of assessing *in vivo* vascular responses in an isolated circulation without invoking systemic effects (Benjamin et al 1995; Webb 1995; Petrie et al 1998). Therefore, the aims of the studies included in this chapter - in patients with pre-ascitic cirrhosis and those with diuretic-refractory ascites, and age- and sex-matched healthy controls – were to:

1. measure the baseline forearm blood flow and its relation to disease severity.
2. evaluate the baseline plasma concentrations of angiotensin II (ANG II), plasma renin activity (PRA), noradrenaline (Nad), and adrenaline, and their relation to disease severity.
3. determine the role of endogenous ANG II in regulation of basal and sympathetically-stimulated vascular tone in the forearm circulation;
4. assess the forearm vascular responses to exogenous ANG II and Nad, and whether these responses are dose-related.
5. assess the forearm vascular responses to reflex sympathetic stimulation by low-pressure baroreceptor unloading.
6. study the relation of vascular abnormalities, if any, and disease severity.

3. Subjects and methods

3.1. Subjects:

Eighteen patients with biopsy-proven cirrhosis, 8 in the pre-ascitic stage and 10 in the diuretic-refractory stage of the disease (Arroyo et al 1996) were recruited and compared with eight age- and sex-matched healthy volunteers (49 ± 6 years; Female: Male = 3: 5). After participation in the first study, one patient with refractory ascites underwent liver transplantation and another underwent transjugular intrahepatic portosystemic stent shunt (TIPSS) for variceal bleeding and the second study was performed in another two patients and so the sixteen studies, eight in each protocol, were completed in ten patients. All patients were ambulant and had normal serum creatinine (<100 μmol/L). Paracentesis-induced haemodynamic disturbances were shown to revert to the their basal levels within one week (Pozzi et al 1994). So, patients with diuretic-refractory ascites were studied one week after their regular therapeutic paracentesis, and were on a "no added salt diet" (100 mmol/day) and did not receive any diuretic therapy for at least two weeks before the study. Patients with alcoholic liver disease were abstinent from alcohol for at least one month (Howes & Reid 1985), confirmed by history and random blood ethanol testing. None of the subjects received vasoactive or non-steroidal anti-inflammatory drugs in the week before each phase of the study. All subjects abstained from food, tobacco, and caffeine containing drinks for at least 4 hours before each study.

To minimise between-subject variability, female control subjects who were pre-menopausal were studied between 7-12 days of their menstrual cycle (Hashimoto et al 1995). All female cirrhotic patients had amenorrhoea. Studies were undertaken with written informed consent from each subject, with the approval of the local research ethics committee, and in accordance with the Declaration of Helsinki (1989) of the World Medical Association.

3.2. Intra-arterial administration:

Cannulation of the brachial artery of the non-dominant arm was performed using a 27-standard wire gauge steel needle (Cooper's Needle Works, Birmingham, UK) under 1% lidocaine (Xylocaine, Astra Pharmaceuticals Ltd, Kings Langley, UK)

local anaesthesia. Patency was maintained by saline infusion at a constant rate of infusion (1 ml/min) via an IVAC P1000 syringe pump (IVAC Ltd., Basingstoke, UK).

3.3. Drugs:

Losartan (Dupont-Merck, Wilmington, USA) at doses of 30 and 90 μg/min; angiotensin II (Clinalfa AG, Läufelfingen, Switzerland) at doses of 1, 3, 10 and 30 pmol/min; and Nad (Levophed, Sanofi-Winthrop Ltd., Guildford, UK) at doses of 20, 60, 180, 540 pmol/min (Newby et al 1997; Newby et al 1998a), were dissolved in physiological saline (0.9% Baxter Healthcare Ltd., Thetford, UK) and administered intra-arterially. To prevent its oxidation, Nad was dissolved in saline containing 0.1% ascorbic acid (Evans Medical, Langhurst, UK).

3.4. Measurements:

All studies were performed in a quiet, temperature controlled room maintained at 22-24 °C. Blood pressure and pulse rate were measured in the control arm using a non-invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan) at intervals throughout the study (Wiinberg et al 1988). FBF blood flow was measured as previously described (Chapter 2).

3.5. Lower body negative pressure (LBNP):

Subjects rested supine in a plastic covered steel cage, which enclosed the lower body from the waist and downwards as previously described (Brown et al 1966; Seidelin et al 1991). A constant negative pressure of 15 mm Hg was achieved using an industrial strength vacuum cleaner regulated by a pressure control unit (Medical Physics Department, Edinburgh University, Edinburgh, UK). Alterations to and from atmospheric pressure were attained within 1-2 seconds. This negative pressure produces selective forearm vasoconstriction through low-pressure baroreceptor unloading without altering arterial pressure or heart rate, thereby eliminating the confounding effects of systemic hypotension (Seidelin et al 1991).

3.6. Study design:

Two protocols (Figure 1-1) were applied on two separate occasions one week apart and in random order. Subjects rested supine throughout each study. Strain gauges and cuffs were applied and the brachial artery of the non-dominant arm cannulated. FBF was measured in the last 3 minutes of each infusion period unless otherwise stated. Before participating in each of the protocols, saline was infused for 30 min to allow time for equilibration, with FBF measurements being made every 10 min and the final measure taken as the baseline FBF. In protocol 2, the rationale for combining LBNP with each of saline and losartan infusions was to assess the contribution of endogenous ANG II to the forearm vascular responses to low-pressure cardiopulmonary baroreceptors unloading.

3.7. Blood assays:

After 30 min of supine rest, and before any drugs were administered, venous blood was withdrawn from the non-infused arm. Ten mL were admixed with each of the following: 1 mL of 1% disodium EDTA/ 2% sodium metabisulphite for measuring adrenaline and Nad concentrations; 0.5 mL of 0.45% *O*-phenanthroline/ 4.65% disodium EDTA for measuring ANG II concentration; and 1 mL of 1% disodium EDTA and 1000 KIU aprotinin (Bayer AG, Leverkausen, Germany) for measuring plasma renin activity (PRA). Blood samples were placed on ice and immediately centrifuged at 1500 *g* for 20 min. Plasma was frozen and stored at -80°C until assayed. Adrenaline and Nad concentrations were determined using an electrochemical method following separation by reverse phase liquid chromatography (Goldstein et al 1981). Plasma ANG II concentrations were measured by radioimmunoassay (RIA) following extraction using Bond Elut® columns (Varian, Harbor City, CA, USA) as previously described (Morton & Webb 1985). PRA was measured under standard conditions through the generation of ANG I using RIA as previously described (Haber et al 1969).

FIGURE 3-1: SCHEMATIC DIAGRAM OF THE STUDY PROTOCOLS.

Protocol 1: (duration: 54 min)

(30 min)	*(each for 6 min)*			
Saline for equilibration	**Angiotensin II**			
	1	**3**	**10**	**30**
F **F** **F**	**F**	**F**	**F**	**F**

Protocol 2: (duration: 121 min)

(30 min)	*(each for 13 min)*					*(15 min)*	*(each for 6 min)*			
Saline for equilibration	**Saline**	**Losartan**		**Saline**		**Saline wash**	**Noradrenaline**			
		30 μg/min	**90 μg/min**				**20**	**60**	**180**	**540**
F **F** **F**	**F** **F**	**F** **F**	**F** **F**	**F** **F**		F	F	F	F	F
	L	**L**	**L**	**L**						

F, Bilateral forearm blood flow measurements each for 3 min. L, Lower body negative pressure (LBNP) application each for 3 min. Noradrenaline and Angiotensin II doses in pmol/min. Two protocols applies at random at least one week apart.

2.8. Data analysis and statistics:

Plethysmographic data were extracted from the Chart™ (AD Instruments, Castle Hill, Australia) data files and forearm blood flows were calculated for individual venous occlusion cuff inflations using a template spreadsheet (Excel *v*5.0; Microsoft). Recordings from the first 60 s following wrist cuff inflation were not included in the analysis because of the variability in blood flow this produces (Kerslake 1949). Usually, the last 5 flow recordings in each 3 min measurement period were calculated and averaged for each arm. In order to reduce the variability of blood flow data, the ratio of flows in the 2 arms was calculated for each time point, thereby using the non-infused arm as a contemporaneous control (Benjamin et al 1995; Webb 1995; Chin-Dusting et al 1999). The percentage change in FBF was calculated as follows:

$$\textbf{\% change in FBF} = 100 \times [(I_t / NI_t) - (I_b / NI_b)] / (I_b / NI_b);$$

where I_t and NI_t are the blood flows in the infused and non-infused forearms respectively at a given time point (t) and I_b and NI_b are the blood flows in the infused and non-infused forearms respectively at baseline (b); time 0.

Unlike intra-arterial drug infusions, LBNP affects both arms, and therefore, the changes in FBF during LBNP and either saline or losartan infusions were calculated in the infused arm separately for every subject as % change from the flow during the same infusion without LBNP as follows:

$$\textbf{\% change in FBF during LBNP} = 100 \times [(FBF_{LBNP} - FBF_{S \text{ or } L}) / (FBF_{S \text{ or } L})];$$

where $FBF_{S \text{ or } L}$ is the blood in the infused arm during saline (S) or losartan (L) infusion without LBNP, and FBF_{LBNP} is the blood flow in the infused arm during the same infusion with LBNP, and then the results were averaged in each group.

FBF was expressed in mL.100 mL^{-1} of tissue.min^{-1} (Whitney 1953). Data were expressed as mean ± SEM and examined by two-factor ANOVA with repeated measures, Pearson's correlation coefficient, and two-tailed paired and un-paired *t*-tests as appropriate. Statistical significance was taken at the 5% level.

4. Results

A summary of the patient characteristics is shown in Table 3-1. Patients with diuretic-refractory ascites had significantly higher Child-Pugh score (Child & Turcotte 1964; Pugh et al 1970) and prothrombin time, and significantly lower serum albumin, than the pre-ascitic patients ($p<0.01$).

Table 3-1: Patient characteristics.

Variable	Refractory ascites	Pre-ascitic cirrhosis
Age: (yr)	56 ± 4	48 ± 3
Sex: F:M	4:6	3:5
Child-Pugh score:	11.1 ± 0.5#	6.3 ± 0.4
Child grade: Grade A	0	6
Grade B	0	2
Grade C	10	0
Aetiology of cirrhosis:		
PBC	1	3
ALD	7	4
ALD+HBV	1	-
Cryptogenic	1	-
AICAH	-	1
Serum bilirubin: ($\mu mol.L^{-1}$)	52 ± 17	35 ± 12
Serum albumin: ($g.L^{-1}$)	27 ± 2#	38 ± 2
Prothrombin time: (s)	18 ± 1#	13 ± 1
Blood urea: ($mmol.L^{-1}$)	4 ± 1	4 ± 1
Serum creatinine: ($\mu mol.L^{-1}$)	89 ± 9	82 ± 4

Results are expressed as mean ± standard error of the mean.
# $p<0.01$ *vs* patients with pre-ascitic cirrhosis.
M, male. F, female. PBC, primary biliary cirrhosis. ALD, alcoholic liver disease. HBV, hepatitis B virus, AICAH, autoimmune chronic active hepatitis.

4.1. Baseline systemic haemodynamics:

There were no changes in heart rate or mean arterial pressure throughout the studies. Mean heart rate was significantly higher, and mean arterial pressure significantly

lower, in patients with diuretic-refractory ascites than in pre-ascitic patients and controls ($p<0.01$; Table 3-2). Heart rate correlated positively with PRA and basal ANG II concentrations ($r = 0.52$; $p<0.01$ and $r = 0.65$; $p<0.01$ respectively) and mean arterial pressure correlated negatively with PRA and the basal ANG II concentrations ($r = -0.62$; $p<0.01$ and $r = -0.61$; $p<0.01$ respectively).

4.2. Baseline forearm blood flow:

There were no significant differences between the baseline FBF in the infused and non-infused arms on each of the study days. The baseline FBF was significantly lower in patients with refractory ascites than in both the pre-ascitic patients and the control group (2.5 ± 0.2 *v* 4.8 ± 0.9 and 4.2 ± 0.8 mL.100 mL^{-1} of tissue.min^{-1} respectively; $p<0.05$; Figure 3-2). No significant change in the blood flow in the non-infused arm was observed during the protocols except during LBNP.

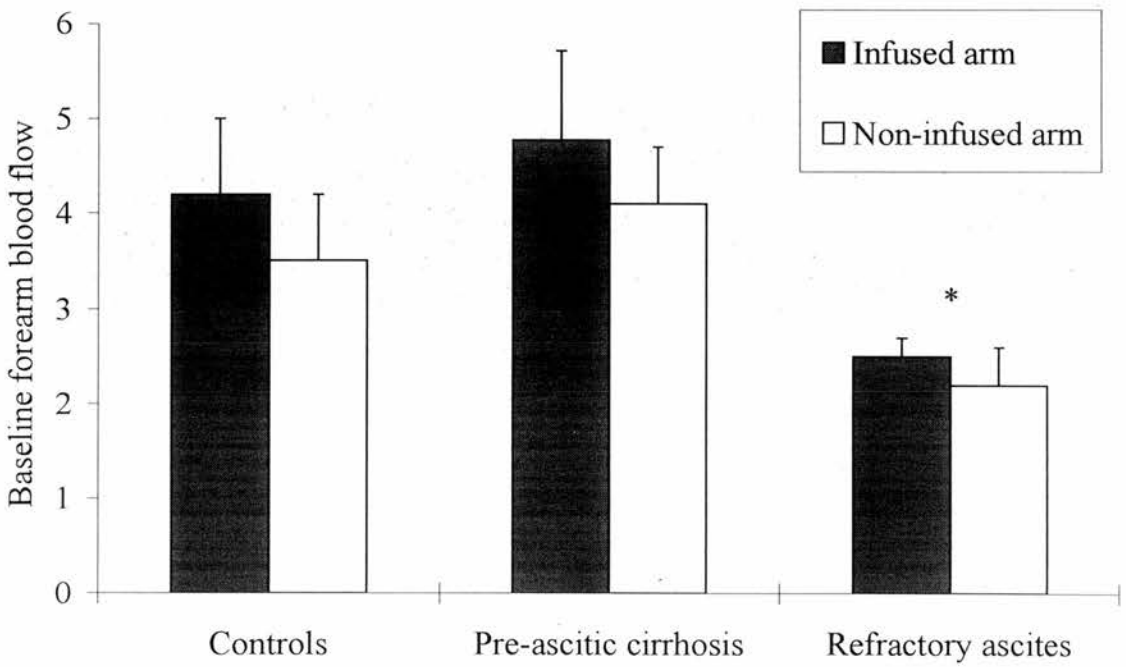


FIGURE 3-2: MEAN BASELINE BILATERAL FOREARM BLOOD FLOW.
* $p<0.05$ *vs* pre-ascitic cirrhosis and controls.
Blood flow is expressed as ml.100 mL tissue^{-1}.min^{-1}

Table 3-2: Baseline systemic haemodynamics and hormonal assays.

Variable	Refractory ascites	Pre-ascitic cirrhosis	Controls
Pulse: (beat.min^{-1})			
before LBNP	88.0 ± 3.4*	72.4 ± 2.1	67.4 ± 2.6
after LBNP	89.0 ± 4.5*	74.7 ± 2.6	62.0 ± 2.3
MAP: (mm Hg)			
before LBNP	80.7 ± 1.2**	91.8 ± 3.2	93.7 ± 3.9
after LBNP	81.6 ± 2.1**	93.5 ± 3.2	94.9 ± 3.0
Basal FBF: (mL.100 mL $^{-1}$.min^{-1})			
infused arm	2.5 ± 0.2*	4.8 ± 0.9	4.2 ± 0.8
non-infused arm	2.2 ± 0.4*	4.2 ± 0.6	3.5 ± 0.7
Basal plasma:			
Adrenaline: (nmol.mL^{-1})	1.2 ± 0.3*	0.7 ± 0.2	0.4 ± 0.2
Noradrenaline: (nmol mL^{-1})	2.9 ± 0.3**	1.1 ± 0.1	1.4 ± 0.4
Angiotensin II: (pg mL^{-1})	237.7 ± 30.8*	57.3 ± 17.3$^{\#}$	3.2 ± 0.3
PRA: (ng.mL^{-1}.h^{-1})	15.4 ± 2.4 *	3.5 ± 1.0	1.7 ± 0.8

Results are expressed as mean ± SEM. bpm, beat per min.
# $p<0.01$ *vs* controls,
* $p<0.05$ *vs* patients with pre-ascitic cirrhosis & controls,
** $p<0.01$ *vs* patients with pre-ascitic cirrhosis & controls,

4.3. Forearm blood flow responses to angiotensin II and noradrenaline infusions:

Bilateral mean ± SEM absolute FBF (mL.100 mL^{-1} of tissue.min^{-1}) at baseline, and following ANG II and Nad infusions in all groups are shown in Tables 3-3 & 3-4. Both ANG II and Nad infusions produced significant dose dependent reductions in FBF in all subjects studied ($p<0.001$; Figure 3-3). The responses to ANG II were significantly greater in the control group than in patients with diuretic-refractory ascites ($p<0.001$) and patients with pre-ascitic cirrhosis ($p<0.01$). FBF responses to Nad were similar in all groups.

4.4. Forearm vascular responses to losartan infusions and LBNP:

Losartan caused dose-dependent increases in FBF in patients with refractory ascites ($p<0.001$) but not in patients with pre-ascitic cirrhosis or controls, $p<0.01$ by ANOVA (Figure 3-4). LBNP produced significantly less reduction in FBF in patients with refractory ascites than in either patients with pre-ascitic cirrhosis or controls ($p<0.001$ by ANOVA; Figure 3-5). In addition, responses to LBNP during saline and losartan infusions were similar within each group. Although there was a trend towards less reduction in FBF following LBNP during saline infusion compared with that during losartan infusions in the refractory ascites group, it did not reach statistical significance ($p=0.13$ & $p=0.42$ at doses of 30 & 90 μg/min respectively). Bilateral mean ± SEM absolute FBF (mL.100 mL^{-1} of tissue.min^{-1}) at baseline, and during saline, losartan and LBNP phases of the study in groups are shown in Figure 3-6.

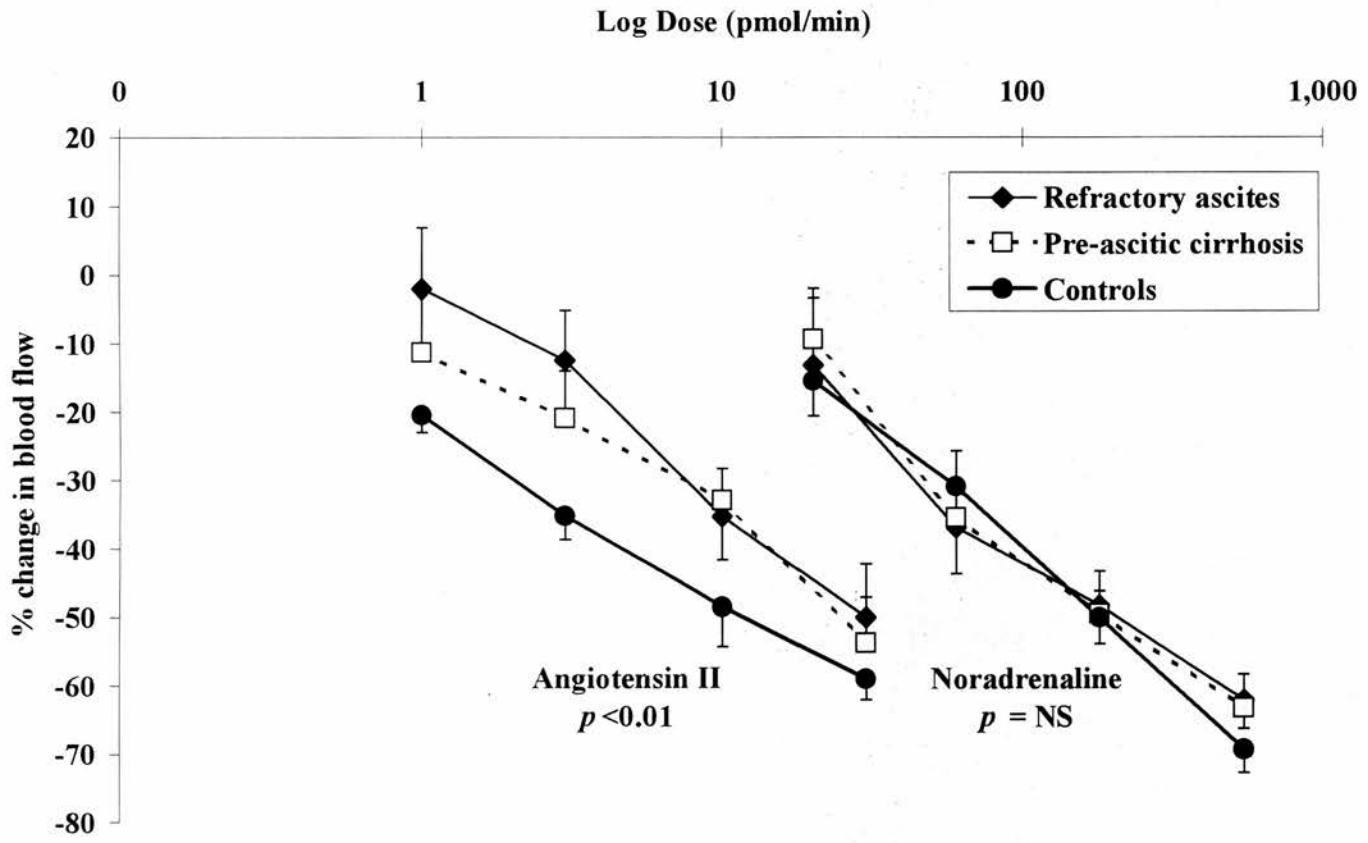


FIGURE 3-3: RESPONSES IN FOREARM BLOOD FLOW TO INCREMENTAL DOSES OF ANGIOTENSIN II AND NORADRENALINE.
NS, not significant. Results are shown as mean ± SEM.

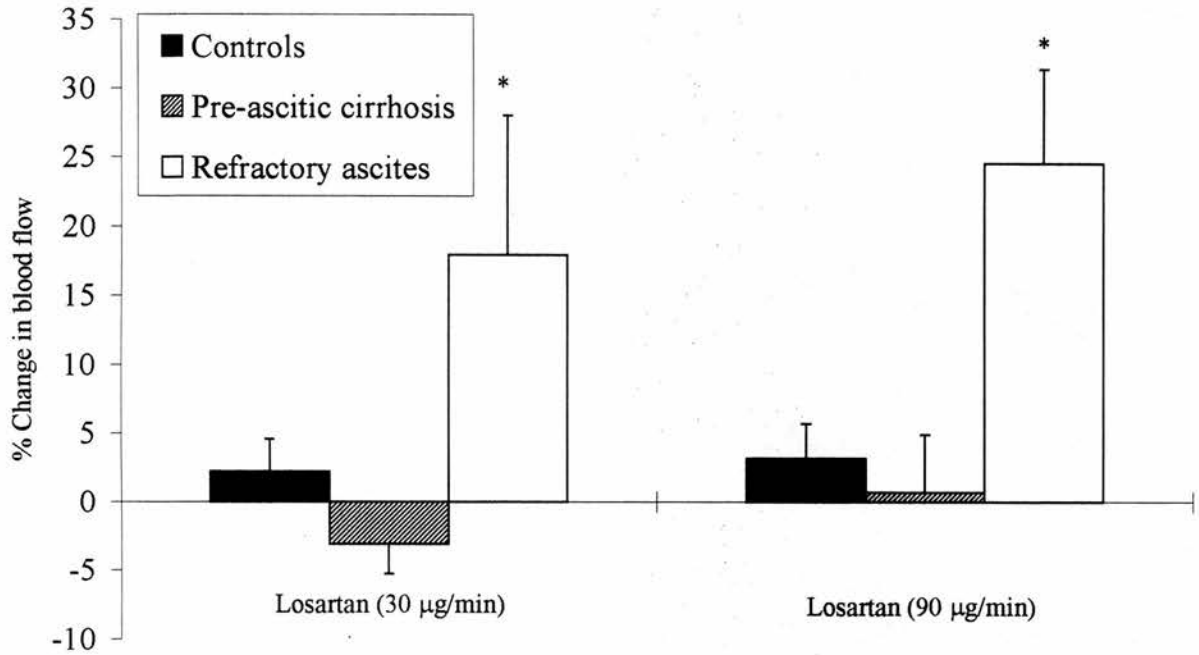


FIGURE 3-4: RESPONSES IN FOREARM BLOOD FLOW TO LOSARTAN INFUSIONS.
* $p<0.01$ by ANOVA *vs* pre-ascitic cirrhosis & controls. Results are shown as mean ± SEM.

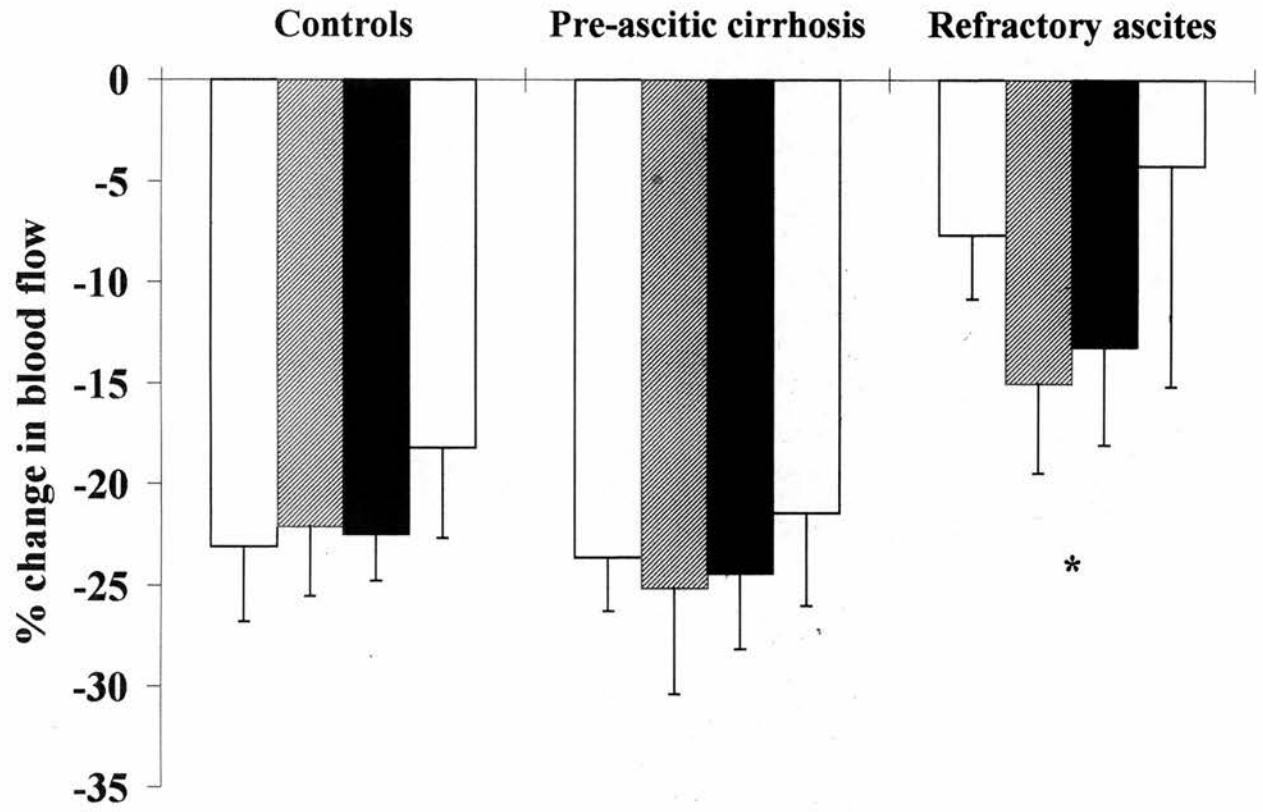


Figure 3-5: Responses in forearm blood flow to lower body negative pressure (LBNP) application.
During saline (white bars), losartan 30 mg/min (gray bars), and losartan 90 mg/min (black bars) infusions.
* $p<0.001$ by ANOVA *vs* pre-ascitic cirrhosis & controls. Results are shown as mean ± SEM.

Table 3-3: Absolute forearm blood flow during angiotensin II infusions.

Group	Controls		Pre-ascitic cirrhosis		Refractory ascites	
Infusion	Infused arm	Non-infused arm	Infused arm	Non-infused arm	Infused arm	Non-infused arm
Baseline	4.0 ± 1.0	3.5 ± 0.9	3.9 ± 0.5	3.3 ± 0.4	2.6 ± 0.5*	2.2 ± 0.5*
ANG II (1 pmol/min)	3.4 ± 1.0	3.7 ± 1.0	3.4 ± 0.6	3.2 ± 0.3	2.3 ± 0.4	2.0 ± 0.4
ANG II (3 pmol/min)	2.9 ± 1.1	3.7 ± 1.1	2.9 ± 0.5	3.1 ± 0.4	2.0 ± 0.3	2.0 ± 0.3
ANG II (10 pmol/min)	2.5 ± 0.9	3. 9 ± 1.0	2.6 ± 0.4	3.2 ± 0.4	1.6 ± 0.3	2.1 ± 0.4
ANG II (30 pmol/min)	1.7 ± 0.5	3.5 ± 0.7	1.7 ± 0.3	3.1 ± 0.4	1.2 ± 0.3	2.1 ± 0.3

Results are expressed as mean ± SEM.
* $p<0.01$ *vs* patients with pre-ascitic cirrhosis and controls.
Blood flow expressed in mL.100 mL^{-1} of tissue.min^{-1}.

Table 3-4: Absolute forearm blood flow during noradrenaline infusions.

Group	Controls		Pre-ascitic cirrhosis		Refractory ascites	
Infusion	Infused arm	Non-infused arm	Infused arm	Non-infused arm	Infused arm	Non-infused arm
Baseline Saline	6.5 ± 1.7	4.8 ± 1.2	6.2 ± 1.2	5.3 ± 0.9	3.0 ± 0.3*	2.5 ± 0.5*
Nad (20 pmol/min)	6.1 ± 2.0	4.7 ± 1.1	5.3 ± 0.9	5.1 ± 0.8	2.4 ± 0.4	2.4 ± 0.4
Nad (60 pmol/min)	5.4 ± 1.1	5.5 ± 1.0	4.2 ± 0.7	5.7 ± 0.9	1.8 ± 0.3	2.4 ± 0.4
Nad (180 pmol/min)	3.7 ± 0.9	5.2 ± 1.0	3.0 ± 0.5	5.3 ± 0.8	1.6 ± 0.2	2.7 ± 0.6
Nad (540 pmol/min)	2.4 ± 0.7	5.6 ± 1.2	2.4 ± 0.5	5.8 ± 1.0	1.3 ± 0.2	2.9 ± 0.6

Results are expressed as mean ± SEM.
* $p<0.01$ *vs* patients with pre-ascitic cirrhosis and controls.
Blood flow expressed in mL.100 mL^{-1} of tissue.min^{-1}.

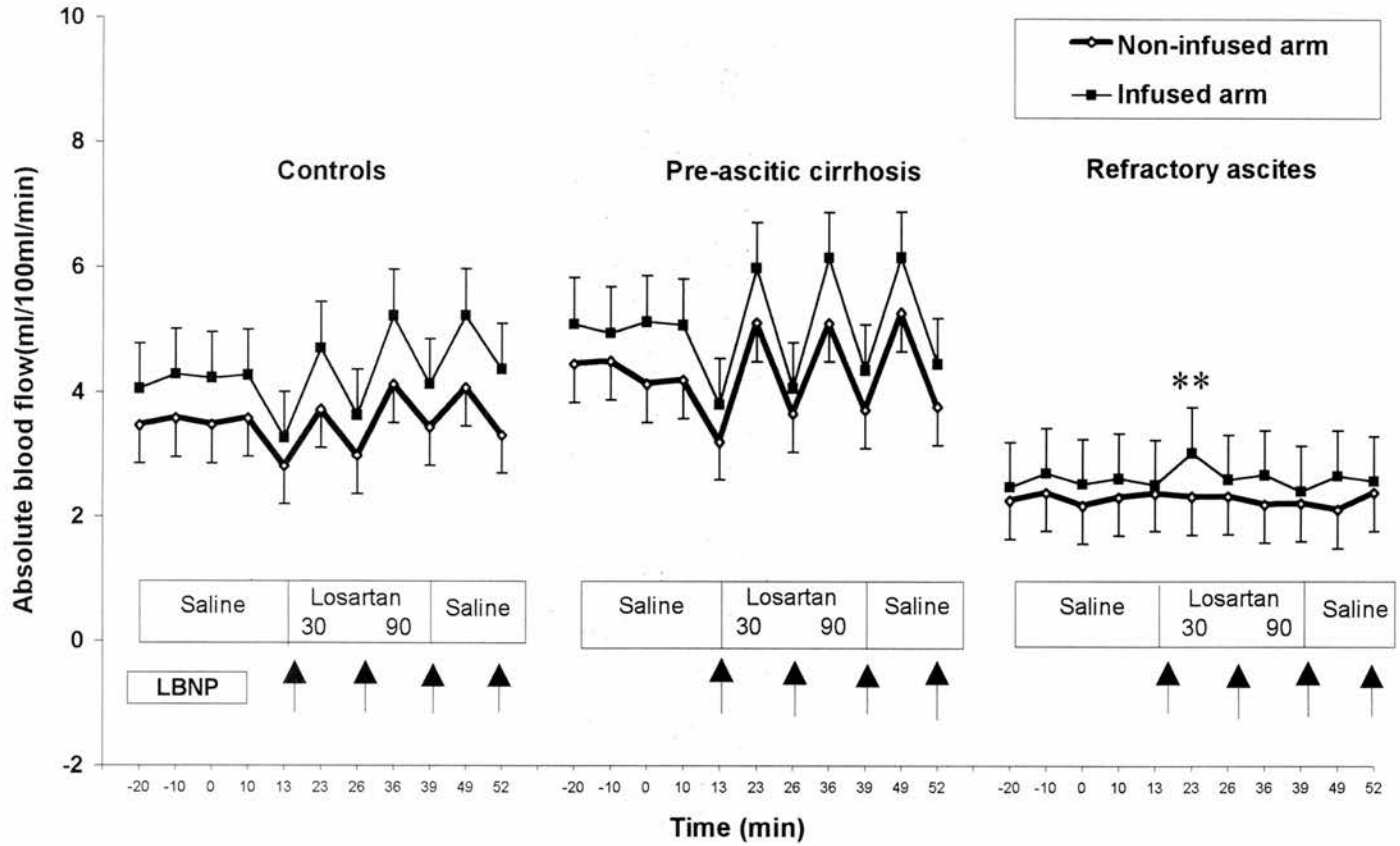


FIGURE 3-6: ABSOLUTE FOREARM BLOOD FLOW AT BASELINE, AND DURING SALINE, LOSARTAN AND LOWER BODY NEGATIVE PRESSURE (LBNP) PHASES OF THE STUDY.

Results are expressed as mean ± SEM. ** $p<0.01$ *vs* pre-ascitic cirrhosis and controls. FBF is expressed as mL.100 mL^{-1} of tissue.min^{-1}.

4.5. Assays:

As shown in Figure 3-7, basal plasma ANG II concentrations were significantly higher in patients with diuretic-refractory ascites than in pre-ascitic cirrhotics ($p<0.05$) and both were significantly higher than controls ($p<0.01$). Patients with refractory ascites had significantly higher basal PRA ($p<0.001$; Figure 3-8) and higher basal plasma Nad ($p<0.01$) and adrenaline concentrations ($p<0.05$) than the patients with pre-ascitic cirrhosis and controls (Figure 3-9). Child-Pugh score correlated positively with both PRA and plasma Nad concentrations ($r = +0.71$; $p<0.001$ and $r = +0.60$; $p<0.01$ respectively).

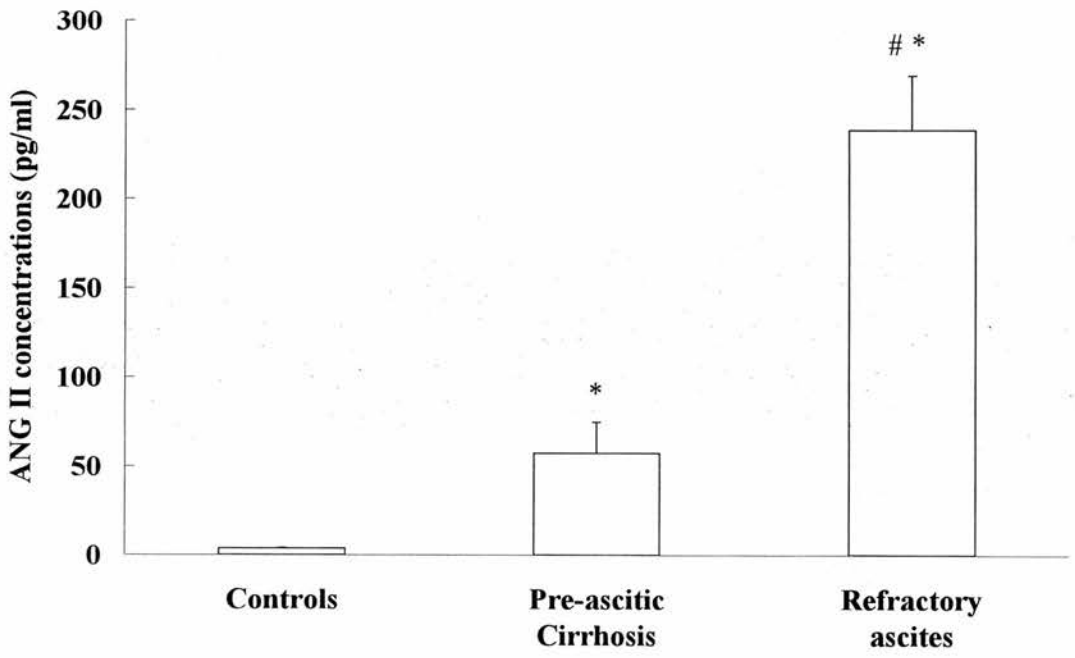


FIGURE 3-7: BASELINE PLASMA CONCENTRATIONS OF ANGIOTENSIN II. # $p<0.05$ *vs* pre-ascitic cirrhosis and controls. * $p<0.01$ *vs* controls.

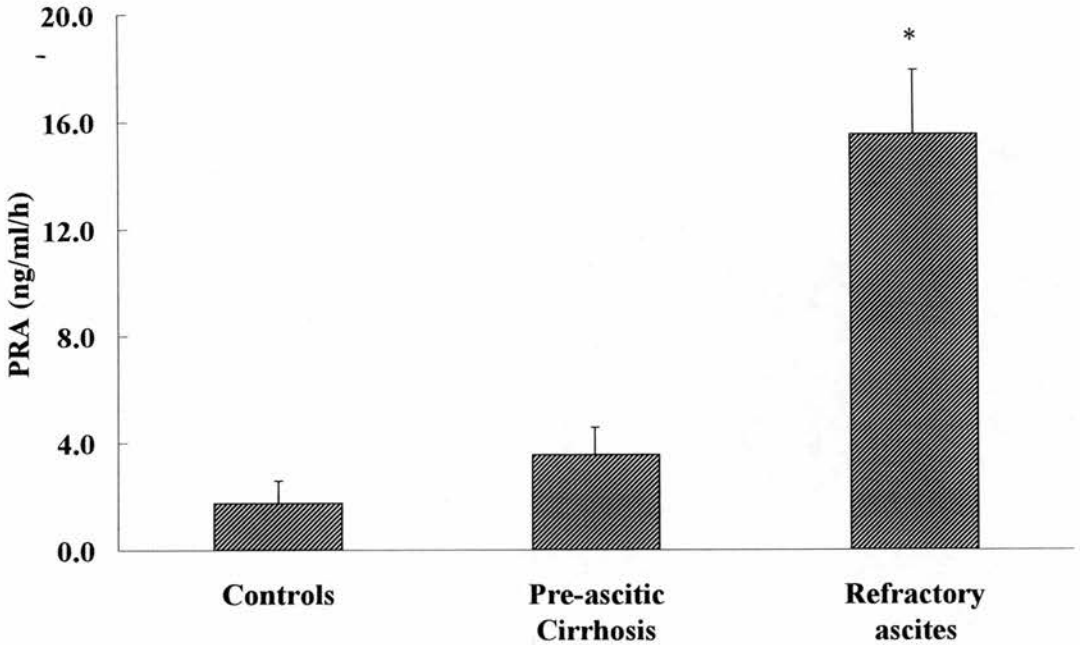
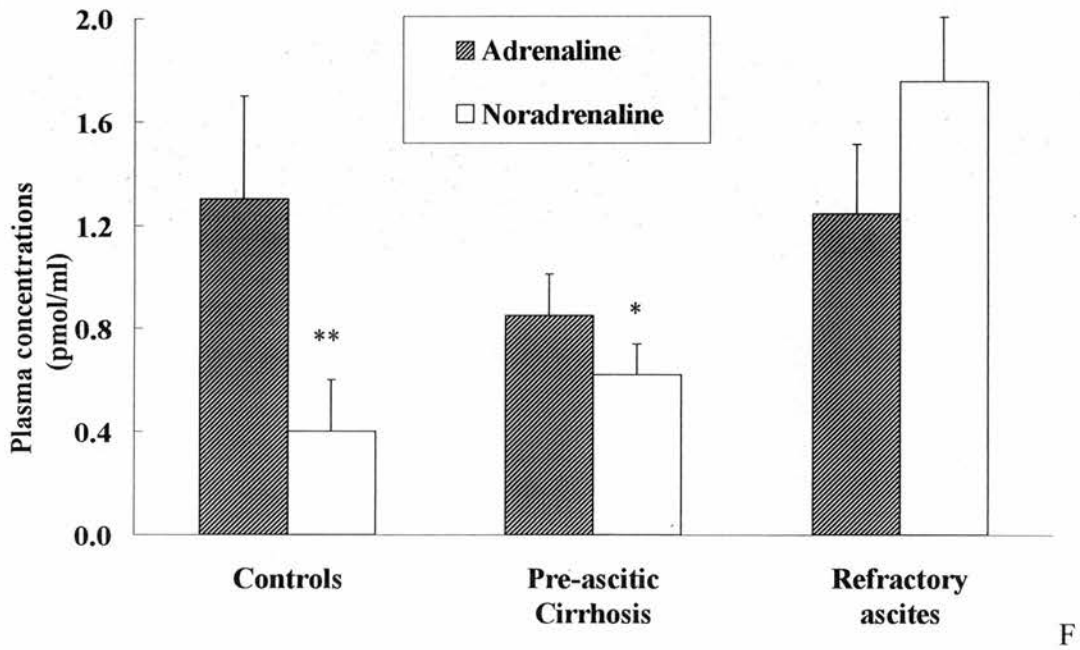


FIGURE 3-8: BASELINE PLASMA RENIN ACTIVITY.
* $p<0.001$ *vs* pre-ascitic cirrhosis and controls.



F

IGURE 3-9: BASELINE PLASMA CONCENTRATIONS OF ADRENALINE AND NORADRENALINE.
* $p<0.05$ *vs* patients with refractory ascites.
** $p<0.01$ *vs* patients with refractory ascites.

5. Discussion

In this study, we have shown that while patients with diuretic-refractory ascites and those with pre-ascitic cirrhosis were hyporesponsive to exogenous ANG II, endogenous ANG II contributed to the maintenance of basal vascular tone only in patients with diuretic-refractory ascites, as demonstrated by the increase in forearm blood flow with local intra-arterial losartan infusion. In addition, impaired peripheral vasoconstrictor responses to low-pressure baroreceptor unloading occurred in patients with refractory ascites although the vascular responses to exogenous Nad were similar in all groups.

Previous studies in patients with cirrhosis have produced contradictory results regarding the responsiveness to systemic administration of α-adrenergic agonists and ANG II. While a number of studies have shown that responses to Nad were not affected (Laragh et al 1963; Ames et al 1965; Lenz et al 1985; Hadoke et al 1998), others have shown an attenuated response (Lunzar et al 1975; MacGilchrist et al 1991). Moreover, responses to ANG II were reported to be impaired in one study (MacGilchrist et al 1991), whereas normal responses were reported in others (Laragh et al 1963; Lunzar et al 1975; Lenz et al 1985). However, systemic drug administration leads to concomitant effects on organs, such as the brain, kidney, and heart, and influences neurohumoral reflexes by affecting systemic haemodynamics. Consequently, vascular responses can't be wholly attributed to a direct effect of the drug (Benjamin et al 1995; Webb 1995). In contrast, the use of bilateral forearm blood flow measurements, with unilateral brachial artery infusions of vasoactive mediators at sub-systemic and locally active doses is a very powerful and reproducible tool for directly assessing vascular responses *in vivo* (Benjamin et al 1995; Webb 1995). However, previous studies using the forearm model in patients with liver cirrhosis are few and their results are also variable (Ryan et al 1993; Calver et al 1994; Campillo et al 1995; Ryan et al 1996; Newby et al 1998a). Because of the confounding influence of diuretic therapy and some methodological concerns, such as use of unilateral blood flow measurements and non-standardised infusion protocols, conflicting results have been reported, with the vasoconstrictor

responses to Nad either reduced (Ryan et al 1993; Campillo et al 1995; Ryan et al 1996), or unaltered (Calver et al 1994; Newby et al 1998a).

Using the bilateral forearm model, our group has previously shown that patients with cirrhosis and diuretic-responsive ascites have a significantly reduced vascular reactivity to exogenous ANG II and cardio-pulmonary baroreceptor unloading (Newby et al 1998a). However, maintenance of patients on regular diuretic therapy may have influenced these results by producing relative intravascular hypovolemia and associated neurohumoral activation (Dwarakanathan et al 1975). In the present study, we recruited patients with early and advanced cirrhosis who were not receiving maintenance diuretic therapy. Patients with refractory ascites were studied one week after their regular therapeutic paracentesis and albumin therapy and were haemodynamically stable.

In agreement with previous studies (Schroeder et al 1976; Arroyo et 1981; Bernardi et al 1982; Pariente et al 1985; Bendtsen et al 1990; Newby et al 1998a), we have shown that the renin angiotensin and sympathetic nervous systems are activated in patients with cirrhosis and this neurohumoral activation becomes more marked with disease progression. The observed reduction in baseline FBF in patients with diuretic-refractory ascites may, in part, be explained by this neurohumoral activation and supports the previously reported reduction in brachial, femoral and cerebral arterial blood flow patients with cirrhosis and ascites, using duplex-Doppler ultrasonography (Fernandez-Seara et al 1989; Maroto et al 1993; Dillon et al 1995). Interestingly, we observed a trend towards increased baseline FBF in patients with pre-ascitic cirrhosis. These observations support the hypothesis that the peripheral circulation behaves differently from the splanchnic one, especially in the advanced stages of the disease. This also indicates that the vasodilatation described in patients with advanced cirrhosis is mainly splanchnic (Schrier et al 1988; Rahman et al 1992).

The present study demonstrates that the forearm circulation in cirrhotic patients is hyporesponsive to ANG II even before the development of ascites, suggesting that this abnormality may contribute to the pathogenesis of the hyperdynamic circulatory

syndrome. The mechanism of this hyporesponsiveness is unclear but a previous study in the animal model of cirrhosis has suggested that it may be mediated through nitric oxide (Castro et al 1993). In addition, we have also demonstrated an increase in the plasma ANG II concentrations of patients with pre-ascitic cirrhosis. The mechanism underlying this increase is not clear, although the presence of an activated renin-angiotensin system is consistent with a compensated vasodilated state. Moreover, the activity of the renin-angiotensin system correlates with portal pressure gradient (Bosch et al 1980). In the present study, all patients with pre-ascitic cirrhosis had a high portal pressure as indicated by the presence of oesophageal varices, and this explains the observed high ANG II concentrations in this group. A reduction in the effective arterial blood volume contributes to the increased plasma concentration of vasopressor agents especially in patients with advanced cirrhosis, as evidenced by the acute suppression of their levels by both albumin and saline infusion (Wong et al 1979). It is a possibility that the increased activity of the endogenous vasopressor systems could have influenced the responses to the exogenously administered ANG II and Nad resulting in a shift of the dose-response curve. However, this is unlikely, at least regarding the responses to Nad, which were similar in the three studied groups despite the presence of activated sympathetic nervous system only in patients with diuretic-refractory ascites.

The finding that selective AT_1 receptor blockade with losartan increases FBF in patients with diuretic-refractory ascites but produces no change in FBF in healthy subjects or patients with pre-ascitic cirrhosis supports the concept that ANG II contributes to the maintenance of peripheral vascular tone only in patients with advanced liver disease but has no role in either healthy controls or early cirrhosis (Baan et al 1996; Newby et al 1997; Newby et al 1998a). Oral losartan and irbesartan have recently been shown to effectively reduce hepatic venous pressure gradient (HVPG) in patients with cirrhosis and portal hypertension (Schneider et al 1999, Klein et al 2000). The observed increase in plasma ANG II concentrations and the vascular dependence on ANG II in patients with cirrhosis indicate that ANG II contributes to the haemodynamic consequences of cirrhosis and, therefore, losartan may potentially be helpful in the treatment of portal hypertension. In addition, the

effects of the high plasma ANG II concentrations, observed in patients with pre-ascitic cirrhosis, on renal arteriolar blood flow may explain the subtle Na^+ retention, which is detected before the development of ascites (Wong et al 1995). Therefore, the use of AT_1 receptor blockers may also improve Na^+ excretion in patients with pre-ascitic cirrhosis (Girgrah et al 2000), and consequently prevent or delay the development of ascites.

LBNP has been used for many years to examine the neuro-circulatory reflex response to reduced venous return to the heart (Greenfield et al 1963). In healthy subjects, LBNP at intensities up to 20 mm Hg selectively unloads low-pressure cardiopulmonary baroreceptors without causing changes in heart rate or blood pressure. Such low intensity LBNP selectively reduces FBF (Zoller et al 1972; Johnson et al 1974; Hirsch et al 1989; Seidelin et al 1991) and increases forearm Nad spillover without affecting total peripheral vascular resistance or total body Nad spillover. In contrast, LBNP at intensities greater than 20 mm Hg also unloads arterial baroreceptors with concomitant increases in total body spillover of Nad, increases in heart rate, and decreases in blood pressure (Jacobs et al 1996). Previous studies in patients with decompensated cirrhosis have shown impaired vasoconstrictor responses to both LBNP and body tilting (Lunzer et al 1975; Bernardi et al 1982; Newby et al 1998a). In the present study, we document impaired vasoconstrictor responses to LBNP in patients with diuretic-refractory ascites despite normal responsiveness to exogenous Nad. This may be due to several mechanisms including baroreceptor down-regulation or defective sympathetic neurotransmission secondary to the autonomic dysfunction described with advanced cirrhosis (Oliver et al 1997). In earlier studies by Wong et al, central venous pressure (CVP) has been shown to be elevated in patients with pre-ascitic cirrhosis (Wong et al 1995; Wong et al 1998). One may, therefore, anticipate that higher intensities of LBNP are required to achieve similar reflex responses in these patients. Nevertheless, these studies have shown that, in response to LBNP of 15 mm Hg, patients with pre-ascitic cirrhosis have similar reductions in CVP and FBF as healthy subjects (Wong et al 1995; Wong et al 1998).

The observation that the vasoconstrictor responses to LBNP of 15 mm Hg, in the infused forearm, are unaffected by losartan infusions has been previously reported in both cirrhotic patients (Newby et al 1998a) and controls (Newby et al 1997; Bishop et al 1991; Duranteau et al 1995). This indicates that endogenous ANG II has little, if any, role in mediating these regional responses to low levels of LBNP in either situation. In addition, these findings suggest that influences of endogenous ANG II do not contribute to the impaired response to LBNP in patients with refractory ascites.

In conclusion, despite hyporesponsiveness to exogenous ANG II in both early and advanced cirrhosis, endogenous ANG II contributes to the maintenance of basal vascular tone only in advanced cirrhosis. These findings suggest a role of ANG II in the pathogenesis of ascites. The forearm vascular responses to exogenous Nad are unimpaired. However, attenuated LBNP responses occurred only in advanced cirrhosis, without apparent interaction with endogenous ANG II. Further studies are required to elucidate the mechanism of the observed hyporesponsiveness to ANG II. This will be shown in the Chapter 5.

CHAPTER 4

STUDIES ON THE ENDOTHELIN SYSTEM

Helmy A, Jalan R, Newby DE, NR Johnston, Hayes PC, Webb DJ. Altered peripheral vascular responses to exogenous and endogenous endothelin-1 in patients with well-compensated cirrhosis. ***Hepatology*** 2001;33:826-831.

1. Summary

Background/Aims: Plasma endothelin concentrations are elevated in cirrhosis and correlate with disease severity. This study assessed forearm vascular responses to exogenous endothelin-1 (ET-1), and evaluated the contribution of endogenous ET-1 to the maintenance of basal peripheral vascular tone in patients with well-compensated cirrhosis (n=11), and matched healthy controls (n=8).

Methods: Bilateral forearm blood flow (FBF) was measured at baseline, and following unilateral sub-systemic, intra-brachial artery infusions of ET-1 (2 and 6 pmol/min); BQ-123, a selective ET_A receptor antagonist (3 and 10 nmol/min), and BQ-788, a selective ET_B receptor antagonist (0.3 and 1 nmol/min), using venous occlusion plethysmography. Baseline systemic haemodynamics, and plasma ET-1 and big ET-1 concentrations, were measured using electrical bioimpedance and radioimmunoassay respectively.

Results: Patients and controls had similar baseline FBF, systemic haemodynamics, and plasma ET-1 and big ET-1 concentrations. In both groups, ET-1 and BQ-788 caused significant vasoconstriction ($p<0.001$) and BQ-123 caused significant vasodilatation ($p<0.001$). Compared with controls, cirrhotic patients had attenuated ET-1 responses ($p<0.001$), augmented BQ-123 responses ($p<0.001$), and similar BQ-788 responses ($p=0.62$).

Conclusions: Despite normal systemic haemodynamics and plasma ET-1 concentrations, forearm vascular responses to exogenous ET-1 are reduced in cirrhotic patients. The augmented vasodilatation response to BQ-123 in cirrhotic patients is consistent with a compensated vasodilated state, and a greater contribution of ET-1 to the maintenance of basal vascular tone acting through the ET_A receptor.

2. Introduction and aims

A hyperdynamic circulation characterized by low arterial pressure, high cardiac output, and low systemic vascular resistance is a feature of patients with advanced cirrhosis and portal hypertension (Kowalski & Abelmann 1953; Murray et al 1958; Kontos et al 1964). These circulatory disturbances worsen with disease progression (Braillon et al 1986; Bendtsen et al 1990; Meng et al 1994), and are accompanied by a reduced reactivity to vasopressor agents, including responses mediated by the renin-angiotensin and sympathetic nervous systems. These haemodynamic changes play a crucial role in the pathogenesis of portal hypertension and its complications, including variceal haemorrhage, ascites, and the hepatorenal syndrome (Schrier et al 1988; Groszmann et al 1994).

Endothelin-1 (ET-1) is the most potent vasoconstrictor known, belonging to a 21-amino acid peptide family with a range of biological effects (Yanagisawa et al 1988; Spokes et al 1989; Masaki et al 1992). ET-1 was originally identified in the culture medium of porcine aortic endothelial cells (Yanagisawa et al 1988; Masaki et al 1992), and is derived from a larger pre-pro-endothelin-1 (212 amino acids) which is cleaved by endopeptidases to produce big endothelin-1 (38 amino acids), which is then converted by specific endothelin converting enzymes (ECEs) to ET-1 (Yanagisawa et al 1988; Masaki et al 1992).

Molecular studies have, so far, identified two endothelin receptor subtypes in mammalian species: endothelin-A (ET_A) and endothelin-B (ET_B). In vascular smooth muscle cells, both receptors are expressed (Spokes et al 1989; Davenport et al 1995), and these mediate vasoconstriction (Spokes et al 1989; Tschudi & Luscher 1994; Davenport et al 1995; Haynes et al 1995). Only the ET_B receptors are found on endothelial cells, where these cause vasodilatation through the release of endothelium-derived vasodilators, such as nitric oxide (NO) (Takayanagi et al 1991). ET-1 induced vasoconstriction is predominantly mediated by the ET_A receptor but ET_B receptors may contribute under some circumstances (Davenport & Maguire 1994).

Since 1991, many studies have reported increased plasma concentrations of ET-1 in patients with decompensated liver cirrhosis (Moore et al 1992; Uchihara et al 1992; Asbert et al 1993; Isobe et al 1993; Moller et al 1993; Hartleb et al 1994; Matsumuto et al 1994; Gerbes et al 1995; Moller et al 1995; Salo et al 1995a; Tsai et al 1995; Kitano et al 1996; Newby et al 1998a), while normal levels were shown in two earlier studies (Lerman et al 1991; Textor et al 1992). The mechanisms underlying the elevated ET-1 concentrations are unclear, and do not appear to be related to hypovolemia or endotoxemia (Salo et al 1995a; Salo et al 1995b). However, there have been no studies to address the vascular responsiveness to ET-1 *in vivo*, or its role in the maintenance of basal peripheral vascular tone, in patients with cirrhosis. Because of the complications in decompensated cirrhosis associated with concomitant medications, and effects on baseline forearm and systemic haemodynamics, this study was conducted in patients with well-compensated cirrhosis and portal hypertension and in matched healthy controls.

The aims of these studies were to:

1. evaluate the plasma concentrations of ET-1 and its biological precursor big ET-1 in patients and its relation to disease severity.
2. determine the forearm vascular responsiveness to exogenous ET-1, and
3. assess the role of endogenous ET-1 in the maintenance of basal forearm vascular tone using the selective ET_A and ET_B receptor antagonists, BQ-123 and BQ-788 respectively.

3. Subjects and methods

3.1. Subjects:

Eleven patients with biopsy-proven and well-compensated cirrhosis (Child grade A; Table 4-1) and eight healthy control subjects were recruited. All patients were ambulant, and had endoscopically proven oesophageal varices, normal serum creatinine (<100 μmol/L), and no clinical evidence of systemic circulatory disturbance. Patients with alcoholic liver disease were abstinent from alcohol for at least one month, confirmed by history and random blood ethanol testing, to prevent the depression of vascular responses this causes (Howes & Reid 1985). To avoid the possibility of altering the endogenous vasopressor systems, all subjects in both groups were maintained on their normal sodium intake. None of the subjects received vasoactive or non-steroidal anti-inflammatory drugs in the week before, and all subjects abstained from food, tobacco and caffeine-containing drinks for at least 4 hours before, each study. All female subjects were post-menopausal, both for safety and to avoid the variability in vascular responses that may be associated with cyclic hormonal changes (Hashimoto et al 1995). Exclusion criteria included the presence of malignancy, encephalopathy, portosystemic shunt, previous gastrointestinal bleeding, or any significant cardiovascular disease such as diabetes mellitus and hypertension. Studies were undertaken in accordance with the Declaration of Helsinki (1989) of the World Medical Association, the approval of the local research ethics committee, and the written informed consent of each subject.

3.2. Drugs, intra-arterial administration and measurement of FBF:

All studies were performed in a quiet, temperature-controlled room maintained at 22 -24 °C. Cannulation of the brachial artery of the non-dominant arm was performed using a 27-standard wire gauge steel needle (Cooper's Needle Works, Birmingham, England) under 1% lidocaine (Xylocaine; Astra Pharmaceuticals Ltd, Kings Langley, England) local anaesthesia. Patency was maintained by saline infusion at a constant rate (1 mL/min) via an IVAC P1000 syringe pump (IVAC Ltd., Basingstoke, England). FBF was measured as previously described (Newby et al 1998a; Helmy et al 2000, Chapter 2).

ET-1 (Clinalfa AG, Läufelfingen, Switzerland) at doses of 2 and 6 pmol/mL, BQ-123 (a selective ET_A receptor antagonist; Clinalfa) at doses of 3 and 10 nmol/mL, and BQ-788 (a selective ET_B receptor antagonist; Clinalfa) at doses of 0.3 and 1 nmol/mL, were dissolved in physiological saline (0.9% Baxter Healthcare Ltd., Thetford, England) and administered intra-arterially.

On the basis of previous studies, we used doses that are sub-systemic and act locally to produce ~ 40 % reduction, ~ 30 % increase, and ~ 20 % reduction in FBF in healthy subjects after a 60 min infusion of ET-1 (5 pmol/min), BQ-123 (10 nmol/min), and BQ-788 (1 nmol/min) respectively (Haynes & Webb 1994; Newby et al 1998; Verhaar et al 1998). In such studies, by 60 min, these doses produce maximal and sustained responses (Haynes & Webb 1994; Newby et al 1998; Verhaar et al 1998). Pilot studies have suggested that higher doses may evoke systemic haemodynamic effects. Moreover, such studies have only used a single dose of ET-1, which has a maximal effect by 60 min because of the slow onset and offset of its action (Clarke et al 1989).

3.3. Study design:

Schematic diagrams of the three protocols applied in this study are shown in Figure 4-1. ET-1, BQ-123, and BQ-788 were administered on three separate occasions, in random order, at least one week apart. Subjects rested supine throughout each study. Strain gauges and cuffs were applied, and the brachial artery of the non-dominant arm cannulated. In all protocols, saline was infused for 30 min to allow time for equilibration, with FBF measurements being made every 10 min and the final measurement taken as the baseline FBF. Thereafter, ET-1, BQ-123, or BQ-788 was infused for 60 min at each of two incremental doses, and FBF measured every 10 min.

Protocol 1: (duration: 150 min)

(30 min)	*(60 min)*	*(60 min)*
Saline for equilibration	**ET-1 (2 pmol/min)**	**ET-1 (6 pmol/min)**

F * F # F F F F F F F F F F F F F F

Protocol 2: (duration: 150 min)

(30 min)	*(60 min)*	*(60 min)*
Saline for equilibration	**BQ-123 (3 nmol/min)**	**BQ-123 (10 nmol/min)**

F * F # F F F F F F F F F F F F F F

Protocol 3: (duration: 150 min)

(30 min)	*(60 min)*	*(60 min)*
Saline for equilibration	**BQ-788 (0.3 nmol/min)**	**BQ-788 (1 nmol/min)**

F * F # F F F F F F F F F F F F F F

FIGURE 4-1: SCHEMATIC DIAGRAM OF THE STUDY DESIGN.
Three protocols applied at random at least one week apart. F, Bilateral forearm blood flow measurements, each for 3 min. ET-1, Endothelin-1. *, time for blood sampling. #, time for assessment of systemic haemodynamics using electrical bioimpedance.

3.4. Measurement of systemic haemodynamics:

Arterial blood pressure and heart rate (HR) were measured in the non-infused arm using a non-invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan) at 20 min intervals throughout each study (Wiinberg et al 1988). The mean arterial pressure (MAP) was calculated according to the formula; MAP (mm Hg) = diastolic arterial pressure + 1/3 pulse pressure (systolic arterial pressure - diastolic arterial pressure).

Baseline cardiac functions, including HR, stroke volume and cardiac output, were measured using a non-invasive thoracic electrical bioimpedance method (BoMed NC-COM3, BoMed Medical Manufacturer Ltd, Irvine, CA, USA) as previously described (Apple et al 1986). Bioimpedance is a non-invasive and reproducible method for assessing systemic haemodynamic parameters, such as cardiac output and systemic vascular resistance (Apple et al 1986; Salandin et al 1988; Northridge et al 1990; Thomas et al 1992). Cardiac index (CI), stroke index (SI), and total peripheral vascular resistive index (TPVRI) were calculated according to the formulae: CI ($L.min^{-1}.m^{-2}$) = cardiac output ($L.min^{-1}$) / body surface area (m^2); SI ($mL.m^{-2}$) = stroke volume (mL) / body surface area (m^2); and TPVRI ($mm\ Hg.L^{-1}.min.m^2$) = MAP (mm Hg) / CI ($L.min^{-1}.m^{-2}$).

3.5. Blood assays:

Before drug administration and after 30 min of supine rest, 20 mL of venous blood was withdrawn and 10 mL were admixed with each of the following: 0.5 mL of 0.45% O-phenanthroline/ 4.65% disodium EDTA for plasma ANG II assay; and 1 mL of 1% disodium EDTA and 1000 KIU aprotinin (Bayer AG, Leverkusen, Germany) for plasma ET-1, Big ET-1 and plasma renin activity (PRA) assays. Blood samples were placed on ice and immediately centrifuged at 1500 g for 20 min. Plasma was frozen and stored at -80 °C until assayed. Plasma ANG II concentrations were measured by radioimmunoassay (RIA) following extraction using Bond Elut® columns (Varian, Harbor City, CA, USA) as previously described (Morton & Webb 1985). PRA was determined under standard conditions through the measurement of the generation of ANG I using RIA as previously described (Haber et al 1969).

Following extraction using Bond Elut® columns (Varian, Harbor City, CA, USA), the plasma concentrations of ET-1 (Peninsula Laboratories Europe Ltd., St. Helens, England) and big ET-1 (Peninsula Laboratories Europe Ltd.) were determined by RIA as previously described (Rolinski et al 1994).

3.6. Data analysis and statistics:

Plethysmographic data were extracted from the Chart™ (AD Instruments, Castle Hill, Australia) data files and forearm blood flows were calculated for individual venous occlusion cuff inflations using a template spreadsheet (Excel v5.0; Microsoft). Recordings from the first 60 s following inflation of the wrist cuff were not included in the analysis because of the variability in blood flow this produces (Kerslake 1949). Usually, the last 5 flow recordings in each 3 min period were calculated and averaged for each arm. To reduce the variability of blood flow data, the ratio of flows in both arms was calculated for each time point, thereby using the non-infused arm as a contemporaneous control (Benjamin et al 1995; Webb 1995). The percentage change in FBF was calculated as follows:

$$\textbf{\% change in FBF} = 100 \times [(I_t / NI_t) - (I_b / NI_b)] / (I_b / NI_b);$$

where I_t and NI_t are the blood flows in the infused and non-infused forearms respectively at a given time point (t) and I_b and NI_b are the blood flows in the infused and non-infused forearms respectively at baseline (b); time 0.

FBF was expressed in mL.100 mL tissue^{-1}.min^{-1} (Whitney 1953). Data were expressed as mean ± standard error of the mean (SEM) and examined by two-factor analysis of variance (ANOVA) with repeated measures, regression analysis and two-tailed paired and un-paired Student's t-tests as appropriate. Statistical significance was taken at the 5% level.

4. Results

A summary of baseline characteristics of the subjects is shown in Table 4-1. Patients with cirrhosis were well matched to the control subjects for age, sex and body mass index (BMI). Six patients (5 males and 1 female) completed all 3 protocols; one female patient completed two protocols, and the remaining 4 studies were performed in 4 female patients. Therefore, the 24 studies were completed in 11 patients. No adverse effects were observed in either patients or controls during or after the studies. The 24 h urinary Na^+ excretion was similar in both groups (Table 4-2).

Table 4-1: Subjects characteristics.

Variable	Cirrhotics (n=11)	Controls (n=8)
Age (years)	52.1 ± 3.3	49.0 ± 4.9
Sex (male:female)	5:6	5:3
BMI ($kg.m^{-2}$)	24.3 ± 0.8	24.8 ± 1.3
BSA (m^2)	1.8 ± 0.1	1.8 ± 0.1
Liver disease aetiology		
ALD	4	-
PBC	5	-
ALD+HCV	1	-
Cryptogenic	1	-
Child-Pugh Score	5.7 ± 0.2	-
Child grade A	11	-
Oesophageal varices	11	-
24 h Urinary Na^+ ($mmol.L^{-1}$)	147.5 ± 20.8	173.9 ± 20.1

Results are expressed as mean ± SEM. ALD, alcoholic liver disease. BMI, body mass index. BSA, body surface area. HCV, hepatitis C virus. PBC, primary biliary cirrhosis.

4.1. Baseline systemic haemodynamics:

As shown in Table 4-2, both cirrhotic patients and healthy controls had similar baseline systemic haemodynamics including HR, MAP, SI, CI and TPVRI. There was no significant difference in baseline FBF (mL.100 mL tissue^{-1}. min^{-1}) in the infused arm between patients with cirrhosis and controls subjects (2.8 ± 0.3 and 2.5 ± 0.3 respectively; Table 4-2). Throughout all protocols, there were no significant changes in MAP, HR, or FBF in the non-infused arm.

Table 4-2: Baseline systemic haemodynamics and hormonal assays.

Variable	Cirrhotics	Controls
Heart rate (beat.min^{-1})	72.3 ± 2.7	65.8 ± 2.8
Mean arterial pressure (mm Hg)	93.9 ± 3.2	95.2 ± 4.2
Cardiac index (L.min^{-1}.m^{-2})	3.7 ± 0.2	3.7 ± 0.4
Stroke index (mL.m^{-2})	54.3 ± 4.8	56.8 ± 4.7
TPVRI (mm Hg.L^{-1}.min.m^{2})	25.8 ± 1.2	28.3 ± 4.3
FBF infused arm **	2.8 ± 0.3	2.5 ± 0.3
FBF non-infused arm **	2.9 ± 0.3	2.3 ± 0.3
Plasma ANG II concentrations (pg.mL^{-1})	31.8 ± 7.2*	10.9 ± 2.0
Plasma renin activity (ng.mL^{-1}.h^{-1})	1.7 ± 0.3#	1.1 ± 0.1
Plasma ET-1 (fmol.mL^{-1})	1.2 ± 0.1	1.2 ± 0.2
Plasma big ET-1 (fmol.mL^{-1})	7.6 ± 0.8	6.8 ± 1.8

Results expressed as mean ± SEM. * $p<0.05$. # $p = 0.08$. ** expressed in mL.100 mL tissue^{-1}.min^{-1}

4.2. Forearm blood flow responses to ET-1 infusions:

ET-1 infusion caused a significant dose-dependent reduction in FBF both in cirrhotic patients and in controls ($p<0.001$, ANOVA for each group). However, the vasoconstriction was significantly less in the patients than in the controls ($p<0.001$, two-way ANOVA with repeated measures; Figure 4-2). The maximum reduction in FBF following infusion of the first dose of ET-1 (2 pmol/mL) was 11.5 ± 5.3% and 25.4 ± 3.6% in the cirrhotic patients and controls respectively, and reached 25.0 ± 3.6% and 36.3 ± 5.2% respectively following infusion of the second dose (6

pmol/mL; Figure 4-2). Absolute FBF in both arms before and during infusion of ET-1 is shown in Table 4-3.

4.3. Forearm blood flow responses to BQ-123 infusions:

ET_A receptor antagonism, using BQ-123 infusion, produced a dose-dependent increase in FBF in both cirrhotic patients and controls ($p<0.001$, ANOVA for each group). This vasodilator response was significantly greater in the patients than the controls ($p<0.001$, two-way ANOVA with repeated measures). The maximum increase in FBF following infusion of the first dose of BQ-123 (3 nmol/min) was 30.0 ± 5.2% and 10.0 ± 2.2% in cirrhotic patients and controls respectively, and reached 47.7 ± 6.1% and 37.1 ± 12.5% respectively following infusion of the second dose (10 nmol/min; Figure 4-3). Absolute FBF in both arms before and during infusion of BQ-123 is shown in Table 4-4.

4.4. Forearm blood flow responses to BQ-788 infusions:

ET_B receptor antagonism, using BQ-788 infusion, produced a significant dose-dependent reduction in FBF in both the cirrhotic patients and controls ($p<0.001$, ANOVA for each group). This vasoconstrictor response was similar in the two groups ($p=0.62$). The maximum reduction in FBF following infusion of the first dose of BQ-788 (0.3 nmol/mL) was 11.4 ± 5.8% and 14.2 ± 6.2% in cirrhotic patients and controls respectively, and reached 22.2 ± 7.5% and 26.4 ± 9.2% respectively following infusion of the second dose (1 nmol/mL; Figure 4-4). Absolute FBF in both arms before and during infusion of BQ-788 is shown in Table 4-5.

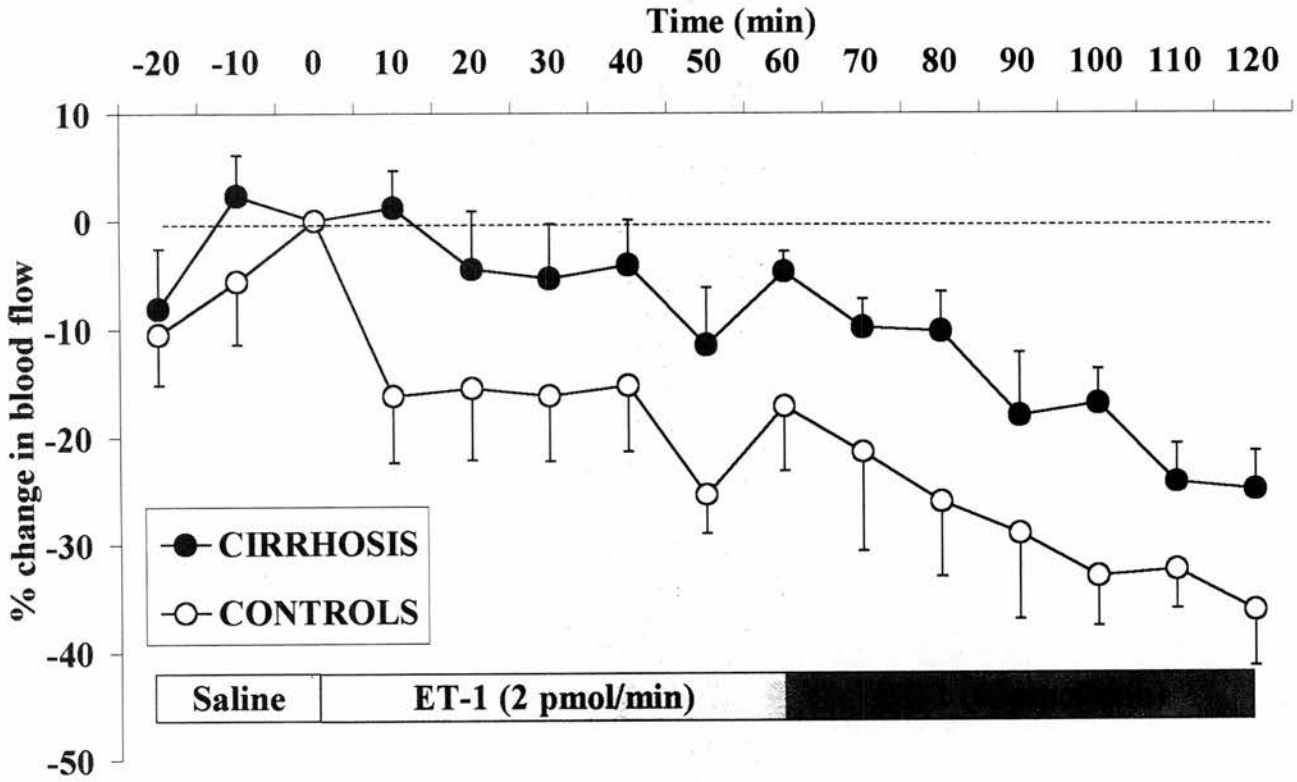


FIGURE 4-2: PERCENTAGE CHANGE IN FOREARM BLOOD FLOW IN RESPONSE TO ENDOTHELIN-1 INFUSION. Mean ± SEM. $p<0.001$ dose-response, and cirrhotics *vs* controls, two-way ANOVA with repeated measures.

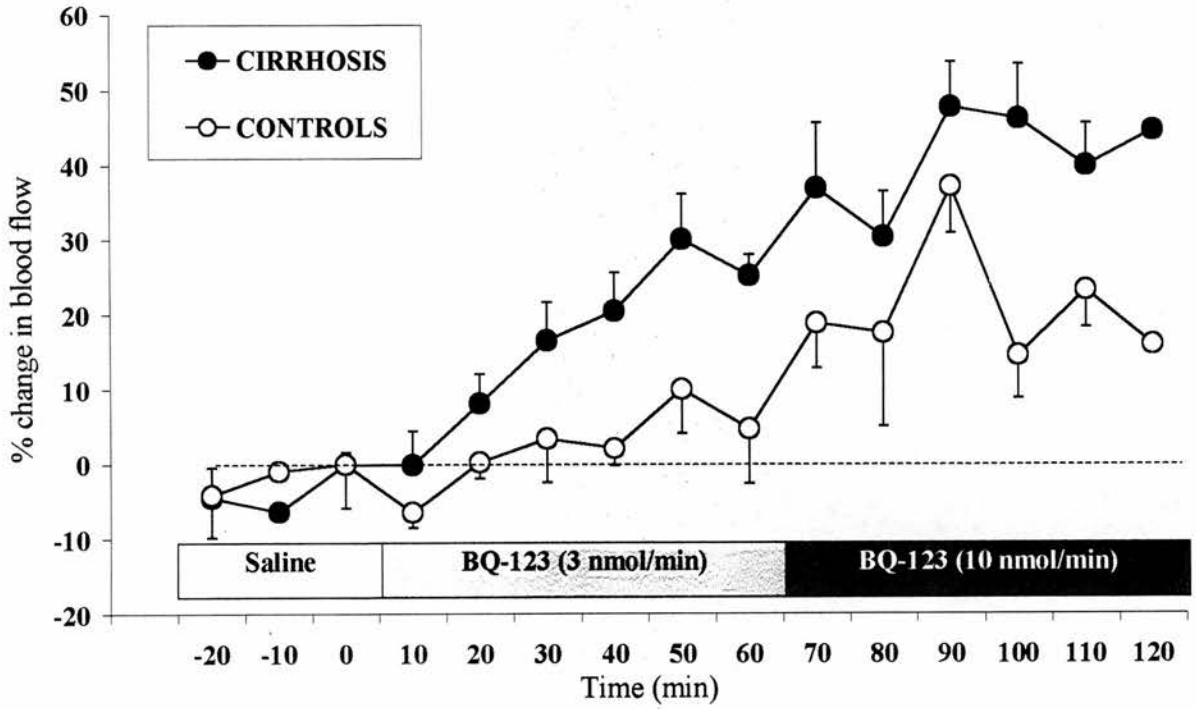


FIGURE 4-3: PERCENTAGE CHANGE IN FOREARM BLOOD FLOW IN RESPONSE TO BQ-123 INFUSION. Mean ± SEM. $p<0.001$ dose-response, and cirrhotics *vs* controls, two-way ANOVA with repeated measures.

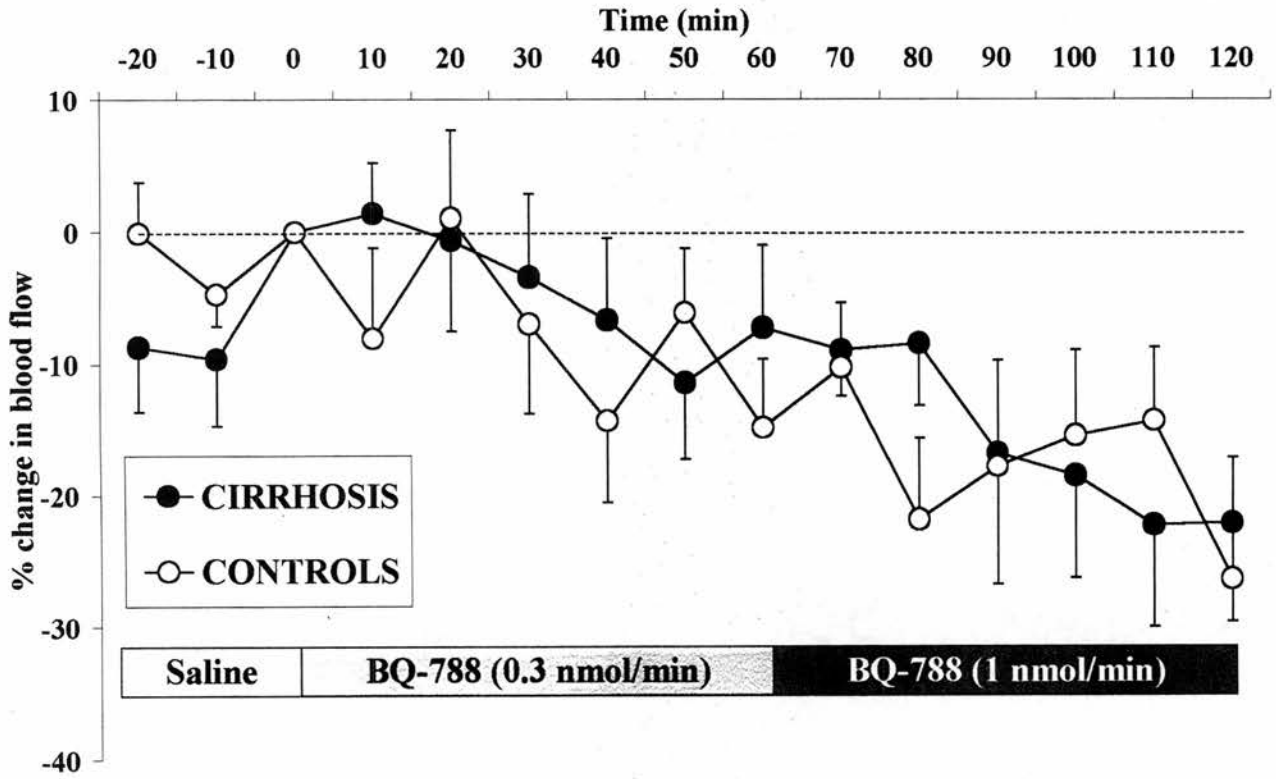


FIGURE 4-4: PERCENTAGE CHANGE IN FOREARM BLOOD FLOW IN RESPONSE TO BQ-788 INFUSION. Mean ± SEM. $p<0.001$ dose-response, and $p= 0.62$ cirrhotics *vs* controls, two-way ANOVA with repeated measures.

Table 4-3: Absolute forearm blood flow during endothelin-1 infusion.

	Controls		Cirrhotics	
Time	Infused arm	Non-infused arm	Infused arm	Non-infused arm
Baseline 0	2.5 ± 0.3	2.3 ± 0.3	2.8 ± 0.3	2.9 ± 0.3
10 min	2.2 ± 0.3	2.4 ± 0.3	2.8 ± 0.3	2.9 ± 0.2
20 min	2.1 ± 0.2	2.4 ± 0.3	2.6 ± 0.3	2.9 ± 0.3
30 min	2.3 ± 0.2	2.6 ± 0.3	2.5 ± 0.3	2.8 ± 0.2
40 min	2.1 ± 0.3	2.4 ± 0.3	2.6 ± 0.3	2.9 ± 0.3
50 min	2.2 ± 0.3	2.8 ± 0.3	2.5 ± 0.3	3.0 ± 0.3
60 min	2.2 ± 0.3	2.5 ± 0.3	2.6 ± 0.3	3.0 ± 0.3
70 min	1.9 ± 0.2	2.4 ± 0.3	2.5 ± 0.2	3.0 ± 0.3
80 min	1.9 ± 0.3	2.6 ± 0.4	2.4 ± 0.3	2.8 ± 0.2
90 min	2.1 ± 0.3	3.1 ± 0.6	2.4 ± 0.4	3.0 ± 0.3
100 min	2.0 ± 0.3	2.8 ± 0.4	2.3 ± 0.4	2.8 ± 0.3
110 min	2.0 ± 0.3	2.9 ± 0.5	2.4 ± 0.5	3.1 ± 0.4
120 min	1.9 ± 0.3	2.8 ± 0.4	2.4 ± 0.5	3.1 ± 0.4

Results are expressed as mean ± SEM.
Blood flow is expressed in mL.100 mL^{-1} of tissue.min^{-1}.

Table 4-4: Absolute forearm blood flow during BQ-123 infusion.

	Controls		Cirrhotics	
Time	Infused arm	Non-infused arm	Infused arm	Non-infused arm
Baseline 0	2.7 ± 0.3	2.3 ± 0.3	3.8 ± 0.4	2.6 ± 0.2
10 min	2.7 ± 0.4	2.5 ± 0.3	3.8 ± 0.4	2.7 ± 0.2
20 min	2.7 ± 0.2	2.3 ± 0.2	3.9 ± 0.5	2.5 ± 0.2
30 min	2.6 ± 0.3	2.2 ± 0.3	4.2 ± 0.6	2.4 ± 0.2
40 min	2.8 ± 0.3	2.3 ± 0.2	4.6 ± 0.6	2.6 ± 0.1
50 min	2.9 ± 0.3	2.2 ± 0.2	4.6 ± 0.8	2.4 ± 0.2
60 min	2.8 ± 0.3	2.3 ± 0.2	4.6 ± 0.8	2.5 ± 0.4
70 min	3.0 ± 0.2	2.2 ± 0.2	4.6 ± 0.7	2.3 ± 0.2
80 min	3.0 ± 0.4	2.2 ± 0.2	4.2 ± 0.7	2.2 ± 0.2
90 min	3.2 ± 0.4	2.1 ± 0.3	5.1 ± 0.7	2.4 ± 0.2
100 min	2.8 ± 0.3	2.1 ± 0.2	5.0 ± 0.8	2.3 ± 0.2
110 min	3.3 ± 0.3	2.3 ± 0.2	4.9 ± 0.8	2.4 ± 0.2
120 min	3.2 ± 0.3	2.4 ± 0.2	5.0 ± 0.7	2.4 ± 0.2

Results are expressed as mean ± SEM.
Blood flow is expressed in mL.100 mL^{-1} of tissue.min^{-1}.

Table 4-5: Absolute forearm blood flow during BO-788 infusion.

-	Controls		Cirrhotics	
Time	Infused arm	Non-infused arm	Infused arm	Non-infused arm
Baseline 0	3.7 ± 0.5	3.4 ± 0.9	4.4 ± 0.7	3.3 ± 0.5
10 min	3.8 ± 0.6	3.9 ± 1.2	4.5 ± 0.8	3.3 ± 0.4
20 min	3.6 ± 0.5	3.5 ± 1.1	4.4 ± 0.8	3.4 ± 0.5
30 min	3.4 ± 0.6	3.7 ± 1.4	4.1 ± 0.6	3.3 ± 0.5
40 min	3.1 ± 0.5	3.8 ± 1.5	4.2 ± 0.6	3.6 ± 0.6
50 min	3.4 ± 0.6	3.4 ± 1.0	3.9 ± 0.6	3.5 ± 0.5
60 min	3.0 ± 0.4	3.4 ± 1.0	4.5 ± 0.7	3.7 ± 0.5
70 min	3.2 ± 0.6	3.4 ± 1.2	4.3 ± 0.7	3.5 ± 0.4
80 min	2.9 ± 0.5	4.3 ± 2.1	4.4 ± 0.7	3.8 ± 0.6
90 min	3.0 ± 0.5	3.9 ± 1.6	3.7 ± 0.5	3.9 ± 0.6
100 min	3.3 ± 0.5	4.1 ± 1.6	4.1 ± 0.6	4.1 ± 0.5
110 min	3.6 ± 1.1	4.5 ± 2.3	4.2 ± 0.7	4.4 ± 0.6
120 min	3.8 ± 1.3	4.4 ± 0.9	3.9 ± 0.5	4.1 ± 0.4

Results are expressed as mean ± SEM.
Blood flow is expressed in mL.100 mL^{-1} of tissue.min^{-1}.

4.5. Blood assays:

Basal plasma ANG II concentrations were higher ($p<0.05$) and PRA tended to be higher ($p= 0.08$) in patients with cirrhosis. However, basal plasma concentrations of ET-1 and big ET-1 were similar in pre-ascitic cirrhotic patients and controls (Table 4-2) and correlated positively with each other ($r= 0.82$; $p<0.001$). In addition, plasma concentrations of ET-1 and Big ET-1 were significantly higher in patients with advanced cirrhosis compared with patients with pre-ascitic cirrhosis and controls ($p<0.01$ for each; Figure 4-5)

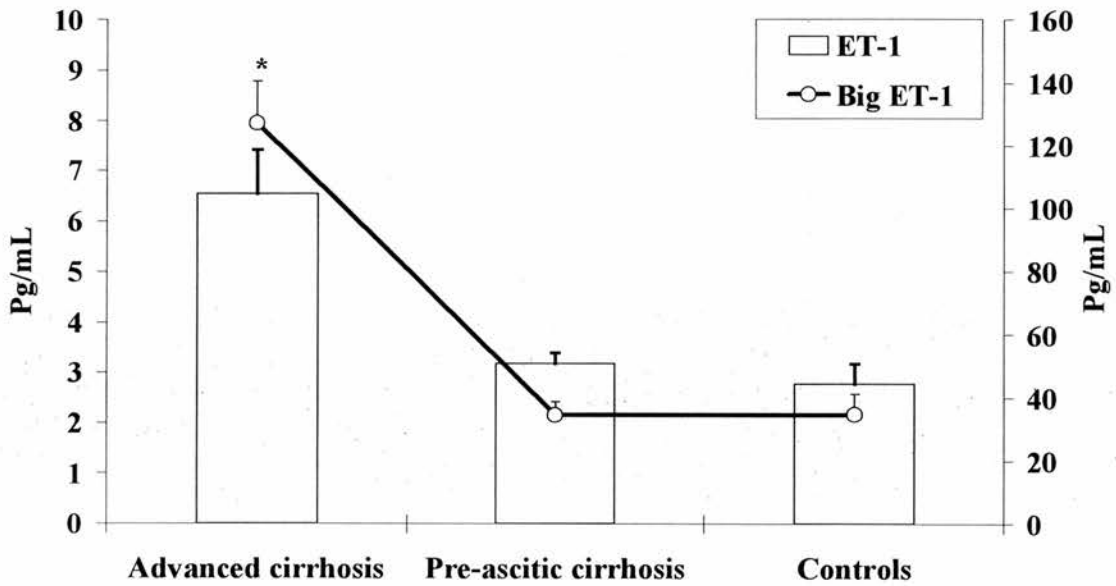


FIGURE 4-5: BASELINE PLASMA CONCENTRATIONS OF ENDOTHELIN-1 AND BIG ENDOTHELIN-1.
* $p<0.001$ vs pre-ascitic cirrhotic patients and controls for both.

5. Discussion

In patients with well-compensated cirrhosis, portal hypertension and normal plasma ET-1 concentrations, we have shown peripheral vascular hyporesponsiveness to exogenous ET-1 before the development of any systemic haemodynamic abnormality. Moreover, these patients have an augmented response to ET_A receptor antagonism, whilst retaining a normal response to ET_B receptor antagonism. These findings are consistent with an activated endothelin system in cirrhosis leading to downregulation of the responses to exogenous ET-1, and a greater contribution of endogenous ET-1 to basal forearm vascular tone acting through the ET_A receptor. It also suggests a potential counter-regulatory role for ET-1 in the pathogenesis of the circulatory disturbances of cirrhosis.

Patients with advanced cirrhosis and fluid retention have elevated plasma ET-1 concentrations (Moore et al; 1992; Uchihara et al 1992; Isobe et al 1993; Asbert et al 1993; Moller et al 1993; Matsumuto et al 1994; Gerbes et al 1995; Moller et al 1995; Salo et al 1995a), which are particularly high in those patients with hepatorenal syndrome (Moore et al 1992). Moreover, plasma ET-1 concentrations correlate positively with the severity of liver disease as measured by Child-Pugh score (Isobe et al 1993; Moller et al 1995; Gerbes et al 1995), the hepatic blood flow (measured by D-sorbitol infusion) and the hepatic venous pressure gradient (Moller et al 1995), and inversely with the liver cell mass as measured by galactose elimination capacity (Gerbes et al 1995) and creatinine clearance (Uchihara et al 1992; Moller et al 1993; Moller et al 1995). However, due to its paracrine mode of action, the majority of ET-1 is released abluminally to act on adjacent vascular smooth muscle and endothelial cells and, as a consequence, plasma ET-1 concentrations may not truly reflect the underlying activity of the endothelin system (Frelin & Guedin 1994). In the present study, we have confirmed the previous findings of normal plasma ET-1 and big ET-1 concentrations in patients with well-compensated cirrhosis (Lerman et al 1991; Textor et al 1992; Uchihara et al 1992) but have additionally demonstrated evidence of functional abnormalities in the tonic activity of the endothelin system. This is consistent with evidence suggesting that modest activation of the endothelin system can occur without a change in plasma ET-1 concentrations.

For the first time, we report an impaired *in vivo* vasoconstrictor response to exogenous ET-1 in patients with well-compensated cirrhosis in the absence of a measurable abnormality in baseline FBF, cardiac function or systemic haemodynamics. This reduced responsiveness to ET-1 may, in part, reflect a greater basal ET-1 activity as suggested by the enhanced vasodilator response to BQ-123 infusion. Using the same forearm model we have also shown impaired vasoconstrictor response to ANG II in patients with early (Helmy et al 2000) and advanced cirrhosis (Newby et al 1998a; Helmy et al 2000). This observation is in keeping with the impaired vasopressor responses to both ET-1 (Hartleb et al 1994a) and ANG II (Castro et al 1993) in the rat model of secondary biliary cirrhosis and portal hypertension. The mechanisms underlying the reduced responsiveness to ET-1 remain unclear, but downregulation of the ET_A receptor secondary to activation of the endothelin system may be implicated. In addition, NO, and possibly other mediators, may be involved (Castro et al 1993; Sieber 1995; Sogni et al 1995; Martin et al 1998). This is supported by the observations of increased NO production, and nitric oxide synthase (NOS) activity, in portal hypertension[49] and by the reversal of the ET-1 (Hartleb et al 1994a) and ANG II (Castroe et al 1993) hyporesponsiveness observed in the rat model of cirrhosis following NOS inhibition.

Several studies have suggested that alterations in post-receptor signal transduction pathways may be involved in the hyporesponsiveness to vasopressor agents in cirrhosis (Murray & Paller 1985; Lee et al 1986; Mahl & Groszmann 1991; Lee & Severson 1994; Moreau & Lebrec 1995; Huang et al 1996; Levy 1997; Hadoke 1999). Protein kinase C (PKC) is one of the main intracellular messengers involved in the signal transduction of protein-G coupled receptors, including ET-1 and ANG II receptors (Lee & Severson 1994), and through phosphorylation, has additional actions to reduce the catalytic activity of NOS in vascular endothelial cells (Hirata et al 1995). Thus, altered PKC activity may contribute to the observed reduction in the ET-1 vasoconstrictor response. This is supported by the observed abnormalities in PKC activity in the lymphocytes of patients with cirrhosis (Spinozzi et al 1991), in the intestinal microcirculation of portal hypertensive rats (Wu & Benoit 1994), in cultured aortic vascular smooth muscle cells obtained from cirrhotic rats (Tazi et al

1997), and in cirrhotic rat aortae (Trombino et al 1998). Another possible explanation for the reduced vasoconstrictor response to ET-1 is down-regulation of endothelin receptors by the increase in transforming growth factor beta-1 (TGFβ1), which has been reported to decrease ET-1 receptor density in cirrhosis (Gabriel et al 1999). Further studies directly assessing the role of these mechanisms in the impaired vasoconstrictor responses of patients with cirrhosis are needed.

This study has also demonstrated, for the first time, that patients with well-compensated cirrhosis exhibit an enhanced forearm vasodilatation response to the infusion of the ET_A receptor antagonist, BQ-123. This observation is consistent with an activated endothelin system, and a greater contribution of ET-1 to the maintenance of basal forearm vascular tone acting through the ET_A receptor in these patients. Interestingly, this forearm vasodilator response to BQ-123 infusion has been shown to be largely reversed by inhibiting endogenous NO production, but not by cyclo-oxygenase inhibition, in healthy subjects (Verhaar et al 1998). Whether the enhanced forearm vasodilatation observed in the present study, in patients with well-compensated cirrhosis, is mediated through a higher endogenous NO production is currently unknown.

In the present study, we have shown that patients with well-compensated cirrhosis and healthy controls have similar forearm vasoconstrictor responses to the infusion of the ET_B receptor antagonist BQ-788. The interpretation of this observation is complex for several reasons. First, the relative predominance of ET_B receptor-mediated vasoconstriction or vasodilatation (Takayanagi et al 1991; Davenport & Maguire 1994; Tschudi & Luscher 1994; Davenport et al 1995; Haynes et al 1995) will depend largely on the proportionate expression of these receptors in the vascular smooth muscle and endothelial cells. Selective cell-specific blockade of the ET_B receptors would be valuable to dissect out these relative contributions but the tools needed to address this issue do not currently exist (Gray & Webb 1996). Second, since the ET_B receptor appears to function as a clearance receptor (Gray & Webb 1996; Strachan et al 1999), the responses to BQ-788 could possibly represent the effect of ET-1 displacement on to the ET_A receptor. Thus, the vasoconstriction

following ET_B receptor antagonism, by BQ-788, is the sum of the unopposed vasoconstrictor action of endogenous ET-1 at the ET_A receptors, and the absence of its vasodilator action at the endothelial ET_B receptors. Third, it is unlikely that stimulation of ET_B receptors is the sole mechanism for the increased NO production in cirrhosis, in which case one would have anticipated greater vasoconstriction in such patients following selective ET_B receptor antagonism. Further studies are now needed to address the relationship between NO production and ET_B receptor stimulation, in patients with cirrhosis.

The findings of the present study apply directly to the forearm circulation, but add to our understanding of the role of ET-1 in regulation of peripheral vascular tone in cirrhosis, and are probably representative of the systemic resistance vascular beds (Webb 1995). As yet, there are no reports of systemic studies assessing the actions of either exogenous or endogenous ET-1 in the systemic and splanchnic circulations in patients with cirrhosis, apart from a preliminary report of infusing the ET_A receptor antagonist, BQ-123, in 3 patients with advanced cirrhosis and hepatorenal syndrome. Interestingly, this study showed an improvement in renal blood flow and creatinine clearance (Soper et al 1996). However, on the basis of our study, and the peripheral vasodilatation hypothesis (Schrier et al 1988), if selective ET_A receptor antagonists were to exert their major effect on the splanchnic circulation, then they might further exacerbate portal hypertension and systemic hypotension. Therefore, we believe that systemic studies are clearly needed to assess the clinical role of ET-1 antagonists in patients with cirrhosis and portal hypertension.

Patients with pre-ascitic cirrhosis often appear, at some stage, to have normal systemic haemodynamics (Wong et al 1994, Wong et al 1996; Henriksen et al 1999) due to a compensated vasodilated state. Our findings are in agreement with this since, in these patients, we have demonstrated a normal cardiac output and peripheral vascular resistance with an evidence of activation of the renin-angiotensin system. In addition, the observed forearm vascular hyporesponsiveness to ET-1 and enhanced dilatation following BQ-123 infusion indicates that these altered vascular responses

precede the appearance of systemic haemodynamic derangements, or ascites formation, and may, therefore, have a role in their pathogenesis.

Experimental studies in the rat model of cirrhosis and portal hypertension have shown reductions in splanchnic blood flow and portal venous pressure by the non-selective endothelin receptor antagonists TAK-044 (Gandhi et al 1998), bosentan (Reichen et al 1998; Sogni et al 1998), and SP206970 (Kojima et al 2000), and by the selective ET_B receptor antagonist, IRL1038, while selective ET_A receptor antagonism produced no clear effects (Cahill et al 1998): a response which has recently been shown in a preliminary report in patients with cirrhosis (Siegerstetter et al 2000). Also, ET_B receptor overexpressin in the splanchnic vasculature was demonstrated in the same rat model (Cahill et al 1998) suggesting a role of ET-1 in the pathogenesis of splanchnic vasodilatation, mediated through the ET_B receptor, which is central in the pathogenesis of portal hypertension and its complications. When these experimental data are viewed in concert with the findings of the present study, we may speculate that the use of selective ET_B receptor antagonists or non-selective ET antagonists may have a therapeutic role in patients with portal hypertension through reducing splanchnic blood flow without producing derangement in systemic haemodynamic.

One of the potential limitations of the present study is that four out of the eleven patients had alcoholic liver disease. Indeed, excessive and regular alcohol intake may alter the vascular responses to exogenous vasopressor agents such as noradrenaline (Howes & Reid 1985). In each phase of the present study, only two or three out of eight patients had alcoholic cirrhosis. All subjects were abstinent from alcohol for a minimum of one month as determined by both clinical history and repeated random blood ethanol testing. In our previous studies (Newby et al 1998a; Helmy et al 2000) incorporating similar patients, we have shown normal vascular responses to noradrenaline infusion. In addition, we have re-evaluated the present results following exclusion of patients with alcoholic cirrhosis and have found consistent results without alteration in the statistical significance or in the magnitude of the effects of the study findings

In conclusion, the forearm circulation in patients with well-compensated cirrhosis is hyporesponsive to exogenous ET-1 despite the absence of any significant differences in basal systemic haemodynamic characteristics and normal circulating ET-1 concentrations in these patients. The enhanced vasodilatation in response to BQ-123 infusion in cirrhotic patients suggests a compensated vasodilated state, an activated endothelin system, and a greater contribution of ET-1 to the maintenance of forearm basal vascular tone acting through the ET_A receptor. The mechanism underlying this hyporesponsiveness to ET-1 warrants further investigation. The role of NO in mediating the enhanced forearm vasodilatation in response to ETA receptor blockade will be addressed with in Chapter 6.

CHAPTER 5

NITRIC OXIDE AND THE IMPAIRED VASCULAR RESPONSES TO ANGIOTENSIN II

Helmy A, Newby DE, Jalan R, NR Johnston, Hayes PC, Webb DJ. Reduced Vasoconstrictor response to angiotensin II in patients with pre-Ascitic cirrhosis is mediated by nitric oxide. Submitted to ***Hepatology***.

1. Summary

Background/Aims: Altered vascular responses to vasopressor agents contribute to the pathogenesis of the circulatory dysfunction in cirrhosis. The aim of this study was to assess the role of endogenous nitric oxide (NO) in the reduced vascular responsiveness to angiotensin II (ANG II) in 8 patients with pre-ascitic cirrhosis compared with 8 age- and sex-matched healthy controls.

Methods: Forearm blood flow (FBF) responses to sub-systemic, locally-active intra-brachial infusions of ANG II were measured using venous occlusion plethysmography before and during the application of an 'NO-clamp': a balanced co-infusion of L-N^G-monomethyl arginine (a selective NO synthase inhibitor) and sodium nitroprusside (an exogenous NO donor) to block endogenous NO production and restore normal NO-mediated basal blood flow respectively.

Results: Before applying the 'NO-clamp', ANG II caused dose-dependent reductions of FBF in both groups ($p<0.001$) that were significantly attenuated in the cirrhotic patients ($p= 0.012$). In the presence of the 'NO-clamp', the ANG II mediated vasoconstriction was enhanced in cirrhotic patients ($p<0.01$), unchanged in controls, and was now similar in both groups.

Conclusions: This study confirms that vasoconstriction to ANG II is reduced in patients with pre-ascitic cirrhosis, and suggests that this is principally due to enhanced NO generation mediated by ANG II.

2. Introduction

Patients with cirrhosis commonly exhibit a hyperdynamic circulation, characterized by a high cardiac output, hypervolemia, a low systemic vascular resistance and an increased portal pressure (Kowalski & Ablemann 1953; Murray et al 1958; Kontos 1964), that worsens with disease severity (Braillon et al 1986; Bendtsen et al 1990; Meng et al 1994). This hyperdynamic circulation is associated with impaired responses to vasopressor and neurohumoral systems, and is believed to be responsible for the development of the complications of cirrhosis such as ascites, oesophageal varices and the hepatorenal syndrome (Schrier et al 1988; Groszmann 1994).

We have previously demonstrated impaired peripheral vasoconstriction to exogenous angiotensin II (ANG II) in patients with pre-ascitic cirrhosis (Helmy et al 2000 & Chapter 3), diuretic-responsive ascites (Newby et al 1998a), and diuretic-refractory ascites (Helmy et al 2000 & Chapter 3). The potential mechanisms underlying these impaired responses include receptor down-regulation (Hadoke 1999), post-receptor signal transduction defects (Murray & Paller 1985; Huang et al 1996), and increased circulating levels of vasodilators (Genecin & Groszmann 1994). Animal models of cirrhosis and portal hypertension have shown that the vascular hyporesponsiveness to ANG II and endothelin-1 (ET-1) can be corrected by endothelial denudation or inhibition of nitric oxide (NO) synthesis (Castro et al 1993; Hartleb et al 1994a; Sieber 1995). Indeed, many workers have demonstrated an increase in endothelium-derived NO synthesis in patients with cirrhosis (Sogni et al 1995; Martin et al 1998), as suggested by increased plasma concentrations of NO and its metabolites (Guarner et al 1993; Albillos et al 1995; Matsumuto et al 1995; Battista et al 1997), enhanced responses to NO-dependent vasodilators (Albillos et al 1995), increased NO in exhaled air (Matsumuto et al 1995; Sogni et al 1995a), and increased nitric oxide synthase (NOS) activity in both monocytes (Laffi et al 1995) and polymorphonuclear cells (Criado-Jimenez et al 1995) However, at present, there have been no clinical studies to assess the contribution of endogenous NO production to the impaired vasoconstriction to ANG II in patients with cirrhosis.

L-N^G-monomethyl arginine (L-NMMA) is a selective and competitive NOS inhibitor that can block NO synthesis (Vallance et al 1989a). Because NO is continuously released by the endothelium, and tonically relaxes the underlying vascular smooth muscle, brachial artery administration of L-NMMA causes forearm vasoconstriction and a reduction in basal blood flow (Vallance et al 1989a). When assessing the contribution of NO to the actions of a given vasoactive agent, these L-NMMA-induced changes in vessel geometry and blood flow may confound the interpretation of subsequent responses (Benjamin et al 1995; Webb 1995). However, co-infusion of sodium nitroprusside (SNP, an exogenous NO donor) with L-NMMA can be used to restore the baseline blood flow and vessel geometry by replacing endogenous NO with exogenous NO - the 'NO-clamp' (Stroes et al 1997). This technique establishes a stable baseline forearm blood flow (FBF) that can be maintained for at least 120 min (Verhaar et al 1998) and permits the assessment of vascular responses in the absence of endogenous NO synthesis (Stroes et al 1997; Verhaar et al 1998).

The aims of the study presented in this chapter - in patients with pre-ascitic cirrhosis and healthy control subjects – were to:

1. assess the relationship between the vascular reactivity to ANG II and endogenous NO release using the 'NO-clamp' technique.
2. evaluate the contribution of endogenous NO release to the maintenance of basal peripheral vascular tone.

3. Subjects and methods

3.1. Subjects:

Eight patients with biopsy-proven cirrhosis in the pre-ascitic stage were recruited and compared with eight age- and sex-matched healthy volunteers. All patients were ambulant and had normal serum creatinine (<100 μmol/L), endoscopically proven oesophageal varices and no clinical evidence of systemic circulatory disturbance. Patients with alcoholic liver disease were abstinent from alcohol for at least one month, confirmed by history and random blood ethanol testing, to prevent the depression of vascular responses this causes (Howes & Reid 1985). To avoid the possibility of altering the activity of endogenous vasopressor systems, all subjects in both groups were maintained on their normal sodium intake Rankin et al 1981; Stein et al 1995). None of the subjects received vasoactive or non-steroidal anti-inflammatory drugs in the week before, and all subjects abstained from food, tobacco and caffeine-containing drinks for at least 4 hours prior to, each study. All female subjects were post-menopausal, both for safety and to avoid the variability in vascular responses that may be associated with cyclic hormonal changes (Hashimoto et al 1995). Exclusion criteria included the presence of malignancy, encephalopathy, portosystemic shunt, previous gastrointestinal bleeding, or any significant cardiovascular disease such as diabetes mellitus and hypertension. Studies were undertaken with the approval of the local research ethics committee, written informed consent of each subject, and in accordance with the Declaration of Helsinki (1989) of the World Medical Association.

3.2. Intra-arterial administration and drugs:

The brachial artery of the non-dominant arm was cannulated using a 27-standard wire gauge steel needle (Cooper's Needle Works, Birmingham, England) under local anaesthesia by 1% lidocaine (Xylocaine, Astra Pharmaceuticals Ltd, Kings Langley, England). Needle patency was maintained by saline infusion via an IVAC P1000 syringe pump (IVAC Ltd., Basingstoke, England) at a constant rate of infusion (1 mL/min).

Pharmaceutical grade synthetic ANG II peptide (Clinalfa AG, Läufelfingen, Switzerland) was infused at locally active and sub-systemic incremental doses of 3, 10 and 30 pmol/min (Benjamin et al 1989; Newby et al 1997; Newby et al 1998a; Helmy et al 2000). L-NMMA (Clinalfa) was given at a dose of 4 μmol/min. This dose has previously been shown to produce a maximal vasoconstrictor response in the forearm circulation (Vallance et al 1989a; Calver et al 1994). SNP (David Bull Laboratories, Victoria, Australia), was administered at titrated doses (80-600 ng/min), see below. The drugs were dissolved in physiological saline (0.9%, Baxter Healthcare Ltd., Thetford, England). Due to its light sensitivity, SNP was prepared and infused in syringes covered by opaque foil.

3.3. Measurement of systemic haemodynamics:

Blood pressure and pulse rate were measured in the control arm using a non-invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan) at intervals throughout the study (Wiinberg et al 1988). The mean arterial pressure (MAP) was calculated according to the formula; MAP (mm Hg) = diastolic arterial pressure + 1/3 pulse pressure (systolic arterial pressure - diastolic arterial pressure). Baseline cardiac functions, including heart rate, stroke volume and cardiac output, were measured using a non-invasive thoracic electrical bioimpedance method (BoMed NC-COM3, BoMed Medical Manufacturer Ltd) as described previously (Apple et al 1986; Northridge et al 1990). Arterial compliance (AC) was calculated according to the formula: AC (mL. mm Hg^{-1}) = stroke volume (mL) / pulse pressure (mmHg). Cardiac index (CI), stroke index (SI), and total peripheral vascular resistive index (TPVRI) were calculated according to the formulae: CI (mL. min^{-1}. m^{-2}) = cardiac output (mL. min^{-1}) / body surface area (m^2); SI (mL.m^{-2}) = stroke volume (mL) / body surface area (m^2); and TPVRI (mm Hg. mL^{-1}. min. m^2) = MAP (mm Hg) / CI (mL. min^{-1}. m^{-2}).

3.4. Measurement of forearm blood flow:

All studies were performed in a quiet, temperature controlled room maintained at 22-24 °C. FBF was measured as previously described (Newby et al 1997; Newby et al 1988a, Helmy et al 2000; and Chapter 2).

3.5. Study design:

A schematic diagram of the study protocol is shown in Figure 5-1. Following baseline infusion of saline for 30 min (phase 1), ANG II was infused at incremental doses of 3, 10 and 30 pmol/min, each for 6 min (phase 2). After a saline washout period of 20 min (phase 3), endogenous NO synthesis in the infused forearm was inhibited by the 'NO-clamp' as described previously (Stroes 1997; Verhaar et al 1998; Dijkhorst-Oei et al 1999). Briefly, L-NMMA was continuously infused at a rate of 4 μmol/min for 20 min to achieve maximal inhibition of local NOS activity (phase 4). Thereafter, SNP was co-infused at titrated doses until FBF had been restored to within 10% of baseline flow and was sustained for at least 2 consecutive FBF measurements (phase 5; variable duration). The same doses of ANG II were re-infused, each for 6 min, during the 'NO-clamp' (phase 6). ANG II does not undergo tachyphylaxis on repeated infusion in the human forearm (Benjamin et al 1989; Dijkhorst-Oei et al 1999).

3.6. Blood assays:

After 30 min of supine rest, and before any drugs were administered, venous blood was withdrawn from the non-infused arm. Ten mL were admixed with each of the following: 1 mL of 1% disodium EDTA and 1000 KIU aprotinin (Bayer AG, Leverkusen, Germany) for measuring plasma renin activity (PRA); and 0.5 mL of 0.45% *O*-phenanthrolene/ 4.65% disodium EDTA for measuring ANG II concentration. The samples were placed on ice and immediately centrifuged at 1500 *g* for 20 min. Plasma was frozen and stored at -80°C until assayed. Plasma ANG II concentrations were measured by radioimmunoassay (RIA) following extraction using Bond Elut® columns (Varian, Harbor City, CA, USA) as described previously (Morton & Webb 1985). PRA was measured under standard conditions through the generation of ANG I using RIA as described previously (Haber et al 1969).

Phase	I	IIa	IIb	IIc	III	IV	V	VIa	VIb	VIc
Duration (min)	30	6	6	6	15	20	Variable	6	6	6
Pump 1 **	Saline	ANG II 10	ANG II 30	ANG II 90	Saline	Saline	Saline	ANG II 10	ANG II 30	ANG II 90
Pump 2 **	Saline	Saline			Saline	Saline	SNP	SNP		
Pump 3 **	Saline	Saline			Saline	L-NMMA	L-NMMA	L-NMMA		
	F * F # F	F	F	F	F	F F	F F F	F	F	F

FIGURE 5-1: SCHEMATIC DIAGRAM OF THE STUDY PROTOCOL.
F, Bilateral FBF measurements each for 3 min. ANG II doses are 3, 10 and 30 pmol/min. L-NMMA dose is 4 μmol/min.
SNP at titrated doses ranging from 80-600 ng/min. *, time for blood sampling. #, time for assessment of systemic haemodynamics using electrical bioimpedance. **, infusion at a rate of 20 mL/h.

3.7. Statistical analysis:

FBF was expressed in mL. 100 mL tissue^{-1}. Min^{-1} (Whitney 1953) Recordings from the first 60 s following wrist cuff inflation were not included in the analysis because of the variability in blood flow this produces (Kerslake 1949). Usually, the final 5 flow recordings in each 3 min measurement period were calculated and averaged for each arm. In order to reduce the variability of blood flow data, the ratio of flows in the 2 arms was calculated for each time point, thus using the non-infused arm as a contemporaneous control (Benjamin et al 1995; Webb 1995). The percentage change in FBF was calculated according to the formula:

$$\textbf{\% change in FBF} = 100 \times [(I_t / NI_t) - (I_b / NI_b)] / (I_b / NI_b);$$

where I_t and NI_t are the blood flows in the infused and non-infused forearms respectively at a given time point (t) and I_b and NI_b are the blood flows in the infused and non-infused forearms respectively at baseline (b); time 0.

Data were expressed as mean ± standard error of the mean (SEM) and examined by 2-way analysis of variance (ANOVA) for repeated measures, the Pearson's correlation coefficient or two-tailed paired and un-paired Student's *t*-tests as appropriate. A probability value of $p<0.05$ was taken to represent a statistically significant difference.

4. Results

A summary of baseline characteristics of the subjects is shown in Table 5-1. Patients with cirrhosis were well matched to the control subjects for age, sex and body mass index (BMI). As shown in Table 5-2, cirrhotic patients and healthy controls also had similar HR and MAP. However, patients with pre-ascitic cirrhosis had higher SI ($p<0.05$), higher CI ($p<0.01$) and lower TPVRI ($p<0.05$). Throughout the study there were no significant changes in MAP, HR, or FBF in the non-infused arm.

Table 5-1: Subjects characteristics.

Variable	Controls (n=8)	Cirrhotics (n=8)
Age (years)	51.1 ± 4.3	52.9 ± 3.5
Sex (male:female)	4:4	4:4
Weight (kg)	75.4 ± 4.7	69.3 ± 6.8
Height (cm)	167 ± 4	170 ± 4
BSA (m^2)	1.8 ± 0.1	1.8 ± 0.1
BMI ($kg.m^{-2}$)	26.9 ± 1.1	23.5 ± 1.1
Liver disease aetiology		
Primary biliary cirrhosis	5	-
Hepatitis C virus	2	-
Alcoholic liver disease	1	-
Child-Pugh Score	6.4	-
Child Grade A	6	-
B	2	
Oesophageal varices	8	-

Results are expressed as mean ± SEM.

4.1. Baseline forearm blood flow:

As shown in Table 5-3, there were no significant differences between the baseline FBF in the infused and non-infused arms in each of the study groups. In addition, FBF after the application of the 'NO-clamp' was unchanged from baseline, and was similar in both groups (Figure 5-2). No significant change in the FBF in the non-infused arm was observed during the study in either group.

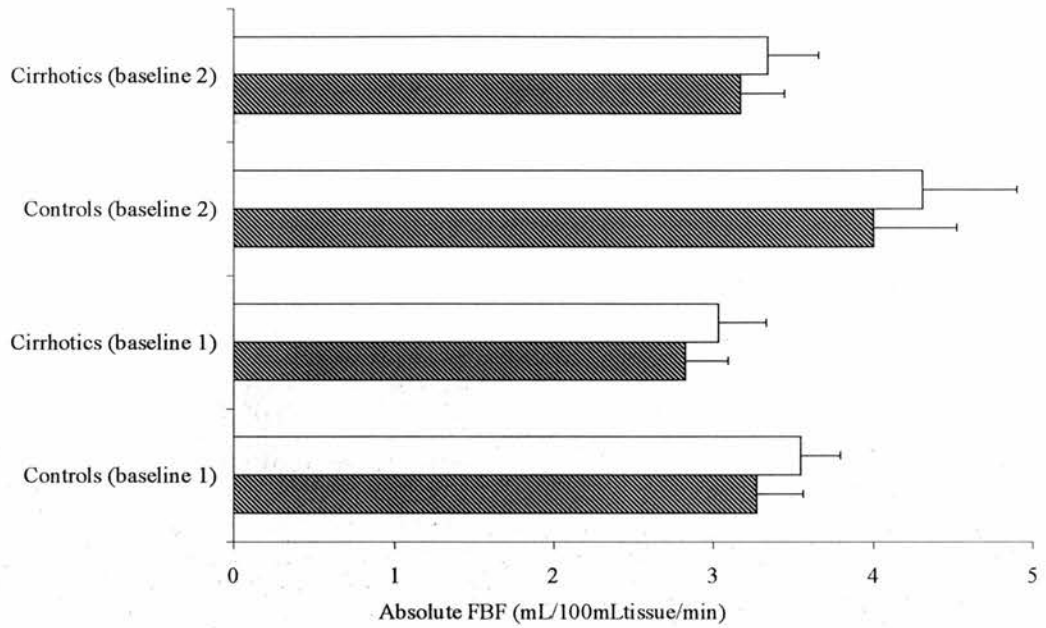


FIGURE 5-2: BASELINE BILATERAL FOREARM BLOOD FLOW.
White bars, the infused arm. Hatched bars, the non-infused arms.
Baseline 1, baseline at the end of phase 1 of the study protocol (saline infusion).
Baseline 2, baseline after adjustment of the 'NO-clamp' and before re-infusion of ANG II (end of phase 5 of the study protocol).
$p>0.05$ infused *vs* non-infused, by un-paired *t*-tests.
$p>0.05$ baseline 1 *vs* baseline 2 in each forearm, by paired *t*-tests.

4.2. Responses to L-NMMA infusion:

As shown in Table 5-3, L-NMMA infusion significantly reduced FBF in both patients with pre-ascitic cirrhosis and healthy controls ($p<0.001$, one-way ANOVA *vs* baseline 1). This response reached a plateau after 10 min of infusion, and was similar in both groups at 10 and 20 min infusion of L-NMMA. Blood flow in the infused arm was reduced by 34.7 ± 2.4 % and 31.0 ± 5.4 % in the cirrhotic patients, and by 26.6 ± 8.3 % and 25.6 ± 7.6 % in the control group respectively (Figure 5-3).

In addition, the dose of SNP required to restore baseline FBF was similar in patients and controls (970 ± 59 ng/min and 1110 ± 44 ng/min respectively, $p>0.05$).

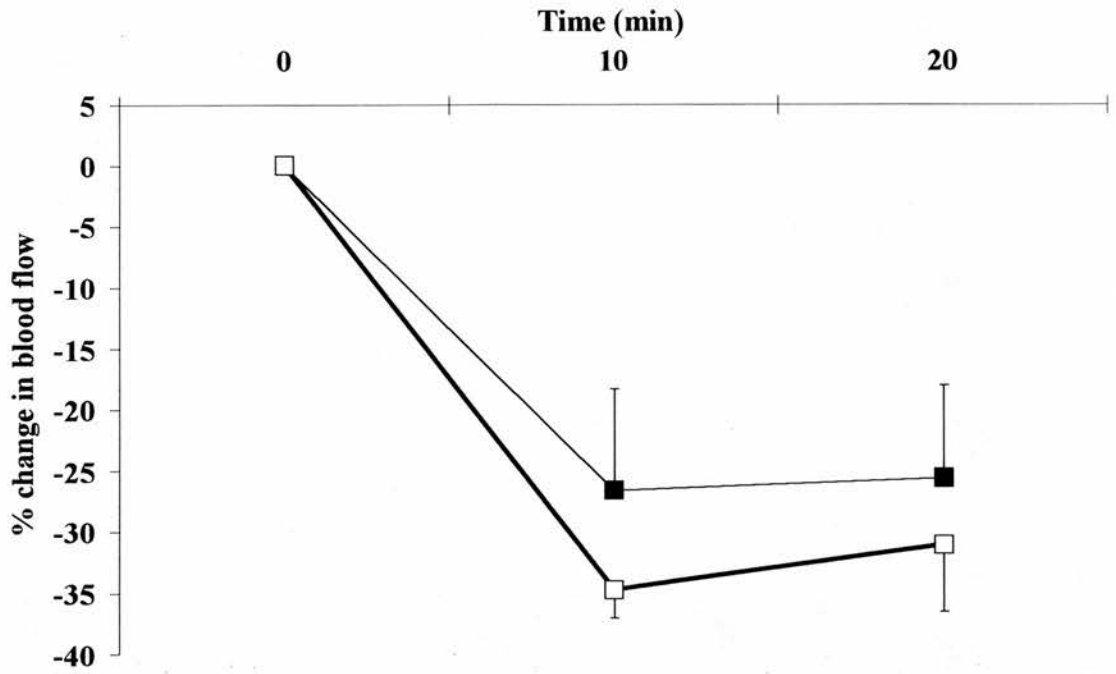


FIGURE 5-3: PERCENTAGE CHANGE IN FOREARM BLOOD FLOW IN RESPONSE TO L-NMMA INFUSION
L-NMMA dose is 4 μmol/min. Cirrhotic patients, (□). Controls (■).
$p<0.001$ from baseline 1 in both groups.
$p>0.05$ cirrhotic patients *vs* controls, two-way ANOVA with replication.

4.3. Responses to ANG II infusion before the 'NO-clamp':

Before applying the 'NO-clamp', ANG II infusion dose-dependently reduced FBF ($p<0.001$, one-way ANOVA) in both groups. However, responses were significantly greater in the control group than in patients with pre-ascitic cirrhosis ($p = 0.012$, two-way ANOVA with repeated measures; Figure 5-4 and 5-5).

4.4. Responses to ANG II infusion during the 'NO-clamp':

ANG II infusion also dose-dependently reduced FBF during the 'NO-clamp' ($p<0.001$, one-way ANOVA). Now, responses were similar in both groups ($p= 0.75$, 2-way ANOVA with repeated measures; Figure 5-4). Compared with the responses to ANG II infusion before the application of the 'NO-clamp', patients with pre-

ascitic cirrhosis had greater reductions in FBF with ANG II infusion ($p<0.001$, 2-way ANOVA with repeated measures), while controls showed similar responses ($p= 0.57$, 2-way ANOVA with repeated measures; Figure 5-5).

4.5. Blood assays:

As shown in Table 5-2, basal plasma ANG II concentrations were higher in patients with pre-ascitic cirrhotics than in controls ($p= 0.04$) and there was a trend towards higher basal PRA in the cirrhotic group ($p= 0.08$). Basal plasma ANG II concentrations correlated positively with basal PRA ($p<0.0001$; $r = 0.87$).

Table 5-2: Basal systemic haemodynamics and hormonal assays.

Variable	Controls (n=8)	Cirrhotics (n=8)	Unit
Cardiac index	3.6 ± 0.2	5.3 ± 0.5#	$L.min^{-1}.m^{-2}$
Stroke index	57.6 ± 3.9	76.6 ± 7.0*	$mL.m^{-2}$
Heart rate	64.3 ± 3.2	69.7 ± 3.8	beat/min
MAP	90.0 ± 2.4	87.4 ± 4.6	mm Hg
TPVRI	25.3 ± 1.7	17.7 ± 2.1*	$mm\ Hg.L^{-1}.min.m^2$
ANG II	10.6 ± 1.6	25.1 ± 6.0*	pg/mL
PRA	1.1 ± 0.1	1.6 ± 0.3**	$ng.mL^{-1}.h^{-1}$

Results are expressed as mean ± SEM. # $p<0.01$. * $p<0.05$. ** $p= 0.08$.
MAP, mean arterial pressure. TPVRI, total peripheral vascular resistive index.

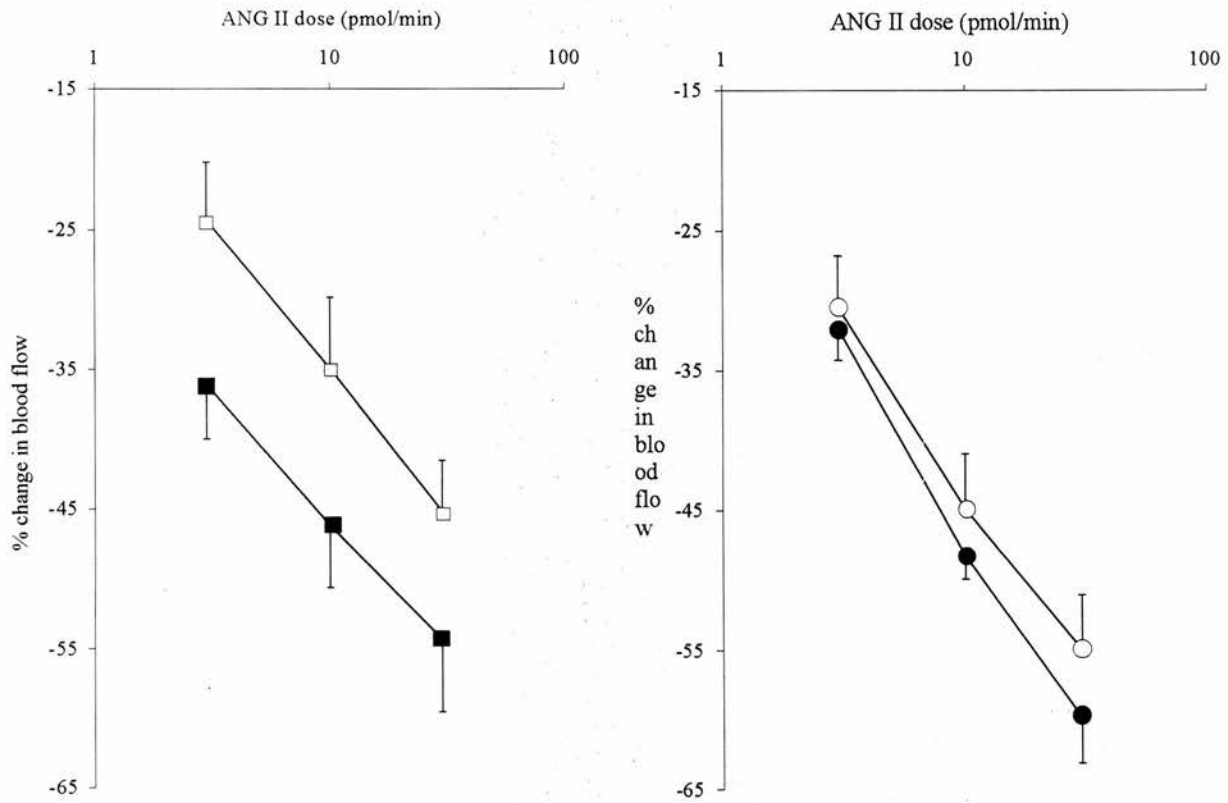


FIGURE 5-4: FOREARM BLOOD FLOW RESPONSES TO ANGIOTENSIN II INFUSIONS BEFORE AND DURING APPLICATION OF THE 'NO-CLAMP'.

Left: in cirrhotic patients ($p<0.001$, two-way ANOVA with repeated measures). Right: in controls ($p= 0.75$, two-way ANOVA with repeated measures). $p<0.001$ dose-response in each group, one-way ANOVA. Open symbols, before the 'NO-clamp'. Closed symbols, during the 'NO-clamp'. Angiotensin II doses in pmol/min.

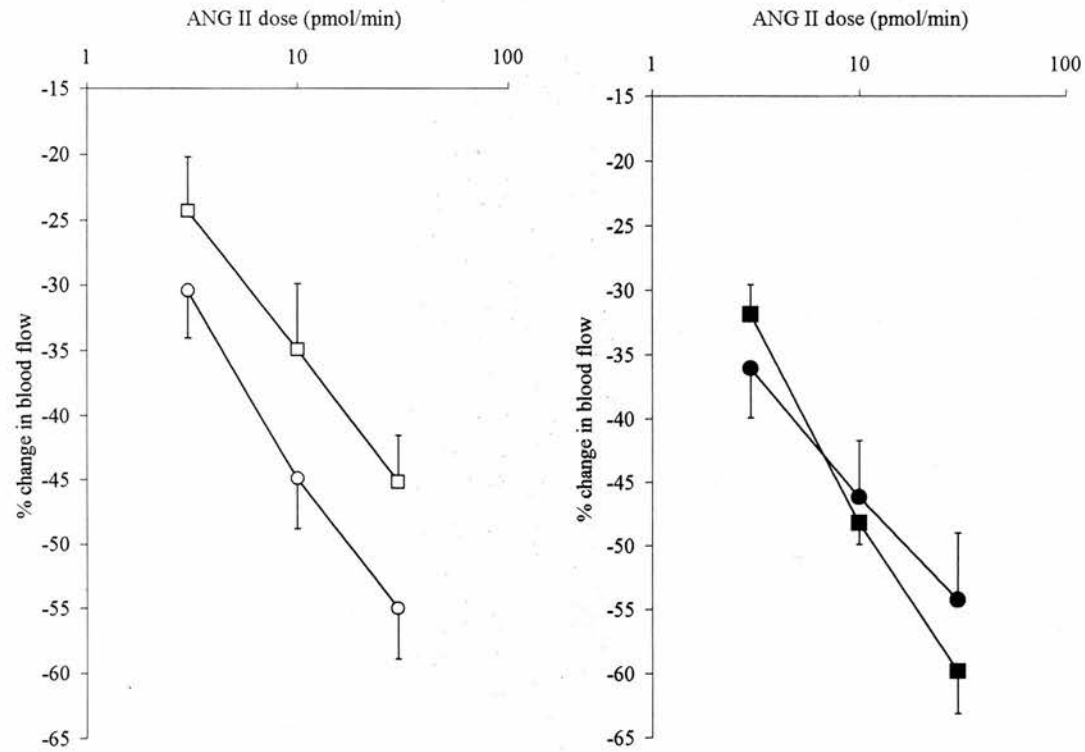


FIGURE 5-5: FOREARM BLOOD FLOW RESPONSES ANGIOTENSIN II INFUSIONS IN PATIENTS WITH CIRRHOSIS AND CONTROLS.

Squares, patients with cirrhosis. Circles, controls. Left: before the 'NO-clamp' (open symbols; p= 0.012, two-way ANOVA with repeated measures). Right: during the 'NO-clamp' (solid symbols; p= 0. 57, two-way ANOVA with repeated measures). p<0.001 dose-response in each phase, one-way ANOVA. Angiotensin II doses in pmol/min.

Table 5-3: Absolute forearm blood flow during all phases of the study.

	Controls		Cirrhotics	
Phase	Infused arm	Non-infused arm	Infused arm	Non-infused arm
Saline (Baseline 1)	3.0 ± 0.3	2.8 ± 0.3	3.6 ± 0.3	3.3 ± 0.3
ANG II (3 pmol/min + saline)	2.3 ± 0.4	3.0 ± 0.4	2.7 ± 0.2	3.3 ± 0.3
ANG II (10 pmol/min + saline)	1.7 ± 0.2	3.0 ± 0.3	2.2 ± 0.2	3.2 ± 0.4
ANG II (30 pmol/min + saline)	1.3 ± 0.2	2.8 ± 0.3	1.8 ± 0.2	2.9 ± 0.3
Saline wash	3.0 ± 0.4	3.1 ± 0.5	2.6 ± 0.3	3.2 ± 0.5
L-NMMA at 10 min	1.9 ± 0.3	2.8 ± 0.3	1.9 ± 0.2	3.7 ± 0.6
L-NMMA at 20 min	2.3 ± 0.3	3.5 ± 0.4	1.8 ± 0.1	3.4 ± 0.6
Adjusted 'NO-clamp' (Baseline 2)	3.3 ± 0.3	3.2 ± 0.3	4.3 ± 0.6	4.0 ± 0.5
ANG II (3 pmol/min + clamp)	2.3 ± 0.3	3.5 ± 0.4	2.6 ± 0.2	3.7 ± 0.5
ANG II (10 pmol/min + clamp)	2.0 ± 0.2	3.6 ± 0.3	2.2 ± 0.3	4.0 ± 0.5
ANG II (30 pmol/min + clamp)	1.9 ± 0.2	4.2 ± 0.4	1.7 ± 0.3	3.8 ± 0.5

Results are expressed as means ± SEM. 'NO-clamp' is a balanced co-infusion of L-N^G-monomethyl arginine (a selective NO synthase inhibitor) and sodium nitroprusside (an exogenous NO donor) to block endogenous NO production without affecting basal blood flow. Blood flow expressed in mL.100 mL tissue^{-1}. min^{-1}.

5. Discussion

Consistent with our previous work (Chapter 3; Helmy et al 2000), we have demonstrated impaired forearm vasoconstrictor responses to exogenous ANG II in patients with pre-ascitic cirrhosis, despite normal vasoconstriction to L-NMMA. These attenuated responses to ANG II were augmented and normalized by the application of 'NO-clamp'. These findings suggest that the impairment of ANG II responses in patients with pre-ascitic cirrhosis is principally due to an enhanced ANG II-mediated NO release.

At least two main ANG II receptor subtypes have been identified: the AT_1 and AT_2 receptors. The majority of the physiological actions of ANG II, including vasoconstriction and aldosterone release, are mediated through the AT-1 receptor, whereas the role of the AT_2 receptor remains unclear (Matsubara et al 1998; Matsubara 1998). ANG II stimulates both endothelial NOS messenger RNA expression (Hennington et al 1998) and endothelial NOS activity (Olson et al 1997). Moreover, ANG II has been shown to increase NO bioavailability in isolated rings from rat carotid artery under static conditions, an effect that is abolished by losartan, a selective AT-1 receptor antagonist, or L-NMMA (Boulanger et al 1995). Thus, ANG II- mediated NO release may be enhanced or mediated by the AT-1 receptor but this does not preclude an interaction with the AT_2 receptor. Indeed, although low in large vessels such as the aorta, surface AT_2 receptor expression is high in the microvasculature (Nora et al 1998). Genetic murine AT_2 receptor knockout models are associated with an increase in both systemic blood pressure and vasoconstrictor responses to ANG II (Hein et al 1995), whereas targeted overexpression of AT_2 receptor leads to an increased ANG II mediated cGMP production and reduced ANG II mediated vasoconstriction (Tsutsumi et al 1999). The vascular effects of the AT_2 receptor have been shown to be mediated, at least in part, by NO release in rat endothelial cells (Wiemer et al 1993), isolated rat carotid arteries (Boulanger et al 1995), and canine coronary arteries (Seyedi et al 1995). Our findings of an impaired vasoconstrictor response to ANG II, mediated by enhanced release of NO, may indicate relative overexpression and activity of the AT_2 receptor in patients with cirrhosis. However, this needs to be tested using a selective AT_2 receptor antagonist.

Finally, It has also been suggested that ANG II infusion can induce NO synthesis and activity through its vasoconstriction-induced increase in vessel wall shear stress (Dijkhorst-Oei et al 1999) as shear stress is a major stimulus for NO synthesis (Nishida et al 1992; Hecker et al 1993), but this needs to be confirmed.

We (Newby et al 1998a; Helmy et al 2000), and others (Schroeder et al 1976; Arroyo et al 1981; Bernardi et al 1982; Pariente et al 1985) have previously shown an increased activity of the RAS in patients with cirrhosis that correlates with both disease severity (Helmy et al 2000) and portal pressure (Bosch et al 1980). The elevated basal plasma ANG II concentrations of patients in the present study are consistent with our previous findings in a similar group of patients (Helmy et al 2000), and may be related to the high portal pressure in these patients (Bosch et al 1980), as indicated by the presence of oesophageal varices. Activation of the RAS, together with the altered systemic haemodynamic parameters in patients with pre-ascitic cirrhosis, is consistent with a compensated vasodilated state. In addition, higher basal plasma ANG II concentrations may contribute to the subtle sodium retention reported in these patients (Wong et al 1995), and consequently to the pathogenesis of ascites (Schrier et al 1988). Moreover, down-regulation of the AT_1 receptor and/or over-expression of the AT_2 receptor may result from the increased activity of the RAS in cirrhotic patients, and lead to the impaired vasoconstriction in response to exogenous ANG II.

The 'NO-clamp' technique has been successfully used in previous studies in healthy volunteers (Stroes et al 1997; Verhaar et al 1998; Dijkhorst-Oei et al 1999). Using this *in vivo* technique, we have demonstrated, for the first time, normalisation of ANG II-mediated vasoconstriction in patients with pre-ascitic cirrhosis. This finding is consistent with the previously demonstrated improvement in the vascular responses to ANG II in the rat model of cirrhosis following endothelial denudation or NOS inhibition (Castro et al 1993). These data suggest a direct ANG II-mediated release of endothelial NO, which may account for the attenuated vasoconstrictor response to ANG II infusion in these patients. It remains to be investigated whether

the effects of ANG II on NO synthesis are agonist-specific or can be elicited by other vasopressor agents such as ET-1.

ANG II-induced vasoconstriction was unaltered in the healthy controls during 'NO-clamp', a finding at variance with a previous study (Dijkhorst-Oei et al 1999). This apparent disparity may be explained by differences in the study populations and study design. Unlike the study of Dijkhorst-Oei et al 1999, our study was performed on a single occasion and FBF responses are known to be much less variable when comparisons are made within rather than between days (Newby et al 1997a).

The significant vasoconstriction produced by L-NMMA, a specific inhibitor of NOS, infusion in the studied groups confirms the role of endogenous NO production in the maintenance of basal peripheral vascular tone in both healthy humans (Vallance et al 1989a; Calver et al 1994; Campillo et al 1995; Ryan et al 1996; Newby et al 1998a) and patients with early (Calver et al 1994; Campillo et al 1995; Ryan et al 1996) and advanced cirrhosis (Campillo et al 1995; Newby et al 1998a). The similarity of the L-NMMA-mediated vasoconstrictor response in both groups is in keeping with the results of previous studies in a similar group of patients (Calver et al 1994; Campillo et al 1995; Ryan et al 1996), and argues against a greater basal NOS activation in the peripheral arterioles of patients with well-compensated cirrhosis.

The 'NO-clamp' has been shown to be able to maintain a stable FBF for at least 120 min (Verhaar et al 1998) and it is, therefore, unlikely that the augmented vasoconstriction to ANG II was the result of tachyphylaxis to SNP. Moreover, the mean SNP dose required to restore baseline FBF was similar in both groups and there was no change of ANG II-mediated vasoconstriction in control subjects. From the present study, we are unable to determine whether the ANG II-mediated NO release is due to stimulation of constitutive NOS (cNOS) and/or inducible NOS (iNOS) because L-NMMA inhibits both cNOS and iNOS unselectively. NOS inhibitors that can selectively inhibit each of these enzymes would be required to address this issue, but are currently not available for clinical studies.

Impaired responses to vasopressor systems, including the RAS, in addition to increased NO release may be responsible for the development of splanchnic vasodilatation, and consequently the pathogenesis of portal hypertension and its complications (Schrier et al 1988, Groszmann 1994, Hadoke 1999). Given that 'NO-clamp' can not be applied in the splanchnic circulation, the potential therapeutic role of NOS inhibition have been studied both in the rat model (Pizcueta et al 1992; Niederberger et al 1995; Pilette et al 1996), and patients with cirrhosis through the systemic infusion of the NOS inhibitor, L-NMMA (Forrest et al 1995). Interestingly, these studies showed a significant improvement in systemic haemodynamics, but no change in the portal venous pressure or portal collateral shunting (Pizcueta et al 1992; Niederberger et al 1995; Pilette et al 1996). The reason for this improvement in systemic, but not portal haemodynamics is unclear. However, the study of Forrest et al 1995, on patients with cirrhosis, did not measure the hepatic blood flow, the activity of the vasopressor systems, or the renal function. As shown by the animal studies (Pizcueta et al 1992; Niederberger et al 1995), the absence of changes in portal venous pressure may be explained by a concomitant reduction in splanchnic arterial blood flow and an increase in the intrahepatic vascular resistance. Factors related to the severity of cirrhosis and portal hypertension, the dose of L-NMMA, duration and type of the NOS inhibitor used, and the concomitant use of vasoactive medications, such as diuretics, should also be considered.

In conclusion, this study confirms that patients with pre-ascitic cirrhosis have impaired forearm vasoconstrictor responses to exogenous ANG II and normal vasoconstrictor responses to L-NMMA. The restoration of normal vasoconstrictor responses to ANG II during the 'NO-clamp' suggests a greater ANG II-induced NO release in these patients, and that NO mediates the peripheral hyporesponsiveness to ANG II seen in the cirrhotic patients.

CHAPTER 6

NITRIC OXIDE AND THE ENHANCED VASODILATATION IN RESPONSE TO ENDOTHELIN TYPE-A RECEPTOR BLOCKADE

Helmy A, Newby DE, Jalan R, Hayes PC, Webb DJ. Enhanced forearm vasodilation after endothelin-A receptor antagonism in patients with pre-ascitic cirrhosis: role of nitric oxide. Underpreparation.

1. Summary

Background/Aims: Patients with cirrhosis have systemic vasodilatation and increased nitric oxide (NO) production despite activated vasopressor systems, including the endothelin system. The aims of this study were to assess the contribution of endogenous ET-1 and NO to the maintenance of basal forearm vascular tone in patients with pre-ascitic cirrhosis (n=7) and age- and sex-matched healthy controls (n=7).

Methods: Using venous occlusion plethysmography, forearm blood flow (FBF) responses to sub-systemic, locally-active intra-arterial infusion of BQ-123 (a selective ET_A receptor antagonist; 10 nmol/min) were measured before, and during the application of an 'NO-clamp': a balanced co-infusion of L-N^G-monomethyl arginine (a selective NO synthase inhibitor) and sodium nitroprusside (an exogenous NO donor) to block endogenous NO production and restore normal NO-mediated basal FBF respectively.

Results: L-NMMA infusion produced a reduction in FBF ($p<0.001$), which was similar in both groups. Before applying the 'NO-clamp', BQ-123 caused an increase in FBF in both groups ($p<0.001$) that was greater in patients with cirrhosis ($p<0.01$). During the 'NO-clamp', the BQ-123-induced vasodilatation was abolished in controls and attenuated in patients ($p<0.001$), but remained significantly greater in patients with cirrhosis ($p<0.01$).

Conclusions: These findings indicate a greater contribution of endogenous ET-1 to the maintenance of basal forearm vascular tone in patients with pre-ascitic cirrhosis. In addition, the enhanced vasodilatation to BQ-123 in these patients can 't be entirely attributed to NO release, but is likely to be related to direct ET-1 mediated tone.

2. Introduction

Patients with cirrhosis exhibit a hyperdynamic circulation characterized by a high cardiac output, hypervolemia, a low systemic vascular resistance and an increased portal pressure (Kowalski & Abelmann 1953; Murray et al 1958; Kontos et al 1964) that worsen with disease severity (Schrier et al 1988; Bendtsen et al 1990). This hyperdynamic circulation is due to systemic arteriolar dilatation, which occurs despite the presence of activated vasopressor systems including the endothelin system, and is believed to be responsible for the development of the complications of cirrhosis such as ascites, oesophageal varices, and hepatorenal syndrome (Schrier et al 1988; Groszmann 1994). Therefore, a better understanding of the mechanism(s) underlying the systemic vasodilatation associated with cirrhosis is essential for the development of therapeutic, and possibly preventive, interventions.

Endothelin-1 (ET-1) is one of the most potent vasoconstrictor known, belonging to a 21-amino acid peptide family with a range of biological effects (Yanagisawa et al 1988; Arai et al 1990; Masaki and Yanagisawa 1992). Molecular studies have identified, so far, two endothelin receptor subtypes in mammalian species: endothelin-A (ET_A; Arai et al 1990) and endothelin-B (ET_B; Sakurai et al 1990). In vascular smooth muscle cells, both receptors are expressed (Arai et al 1990; Sakurai et al 1990) and mediate vasoconstriction (Spokes et al 1989; Tschudi et al 1994; Davenport et al 1995; Haynes et al 1995). The vasoconstriction induced by ET-1 is predominantly mediated by the ET_A receptor, but the ET_B receptors may contribute under some circumstances (Davenport & Maguire 1994). The ET_B receptors are also found on endothelial cells where they cause vasodilatation through the release of endothelium-derived vasodilators, such as NO (Takayanagi et al 1991). Therefore, the ET-1 mediated vascular tone is a result of a balance between the constrictive and relaxive effects on these two receptors.

We have demonstrated that selective ET_A receptor antagonism, using BQ-123, causes forearm vasodilatation in healthy humans (Verhaar et al 1998; Helmy et al 2001), and this vasodilatation response is principally mediated through NO generation (Verhaar et al 1998). We have also shown that BQ-123 infusion produces an

enhanced forearm vasodilatation in patients with pre-ascitic cirrhosis (Helmy et al 2001). The exact mechanism underlying this enhanced vasodilatation is unknown. However, evidence of increased nitric oxide (NO) production in patients with cirrhosis is accumulating (Sogni et al 1995; Martin et al 1998). These were in the form of increased plasma concentrations of NO and its metabolites (Guarner et al 1993; Albillos et al 1995; Matsumuto et al 1995; Battista et al 1997; Barak et al 1999), enhanced responses to NO-dependent vasodilators (Albillos et al 1995), increased NO in exhaled air (Matsumuto et al 1995; Sogni et al 1995a), and increased nitric oxide synthase (NOS) activity in both monocytes (Laffi et al 1995) and polymorphonuclear cells (Criado-Jimenez et al 1995). However, at present, there have been no clinical studies to assess the contribution of endogenous NO production to the enhanced vasodilatation response to ET_A receptor blockade in patients with cirrhosis.

Therefore, the aims of the present study, in patients with pre-ascitic cirrhosis and healthy controls, were to:

1. assess the contribution of endogenous ET-1 and NO to the maintenance of basal peripheral vascular tone, and
2. determine the relationship between AT_A receptor antagonism and endogenous NO release.

3. Subjects and methods

3.1 Subjects:

Seven patients with biopsy-proven cirrhosis in the pre-ascitic stage and eight age- and sex-matched healthy volunteers were recruited. All patients were ambulant, had normal serum creatinine (<100 μmol/L), and endoscopically proven oesophageal varices. To avoid the depressive effect of ethanol on vascular responses (Howes & Reid 1985), patients with alcoholic liver disease were abstinent from alcohol for at least one month as confirmed by history and random blood ethanol testing. In addition, all subjects in both groups were maintained on their normal sodium diet to avoid the possibility of altering the activity of endogenous vasopressor systems (Rankin et al 1981; Stein et al 1995). None of the subjects received vasoactive or non-steroidal anti-inflammatory drugs in the week before, and all subjects abstained from food, tobacco and caffeine-containing drinks for at least 4 hours prior to each study. All female subjects were post-menopausal, both for safety and to avoid the variability in vascular responses that may be associated with cyclic hormonal changes (Hashimoto et al 1995). Exclusion criteria included the presence of malignancy, encephalopathy, surgical or interventional radiological portosystemic shunt, or any significant cardiovascular disease such as diabetes mellitus and hypertension. Studies were undertaken with the approval of the local research ethics committee, written informed consent of each subject, and in accordance with the Declaration of Helsinki (1989) of the World Medical Association.

3.2. Intra-arterial administration and drugs:

The brachial artery of the non-dominant arm was cannulated using a 27-standard wire gauge steel needle (Cooper's Needle Works, Birmingham, England) under local anaesthesia using 1% lidocaine (Xylocaine, Astra Pharmaceuticals Ltd, Kings Langley, England). Needle patency was maintained by saline infusion via an IVAC P1000 syringe pump (IVAC Ltd., Basingstoke, England) at a constant rate of infusion (1 mL/min).

All drugs were dissolved in physiological saline (0.9%, Baxter Healthcare Ltd., Thetford, England). Pharmaceutical grade BQ-123 (Clinalfa AG, Läufelfingen,

Switzerland), a selective ET_A receptor antagonist (Ihara et al 1992); was infused at the locally active and sub-systemic dose of 10 nmol/mL. With this dose, FBF has been shown to plateau after 60 min of infusion (Verhaar et al 1998). For the 'NO-clamp' (see below), the selective nitric oxide synthase (NOS) inhibitor L-N^G-monomethyl arginine (L-NMMA, Clinalfa) at a dose of 4 μmol/min, and the exogenous NO donor sodium nitroprusside (SNP, David Bull Laboratories, Victoria, Australia) at titrated doses (80-600 ng/min) were used. This dose of L-NMMA has previously been shown to produce a maximal vasoconstrictor response in the forearm circulation (Vallance et al 1989a; Calver et al 1994). Due to the light sensitivity of SNP, it was prepared and infused in syringes covered by opaque foil.

3.3. Measurement of systemic haemodynamics:

Baseline cardiac function, including heart rate, stroke volume and cardiac output, was measured using a non-invasive thoracic electrical bioimpedance method (BoMed NC-COM3, BoMed Medical Manufacturer Ltd) as previously described (Appel et al 1986; Northridge et al 1990). Cardiac index (CI), stroke index (SI), and total peripheral vascular resistive index (TPVRI) were calculated according to the formulae: CI (mL. min^{-1}. m^{-2}) = cardiac output (mL. min^{-1}) / body surface area (m^2); SI (mL. m^{-2}) = stroke volume (mL) / body surface area (m^2); and TPVRI (mm Hg. mL^{-1}. min. m^2) = MAP (mm Hg) / CI (mL. min^{-1}. m^{-2}).

Blood pressure and pulse rate were measured in the control arm using a non-invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan) at intervals throughout the study (Wiinberg et al 1988). The mean arterial pressure (MAP) was calculated according to the formula; MAP (mm Hg) = diastolic arterial pressure + 1/3 pulse pressure.

3.4. Measurement of forearm blood flow (FBF):

All studies were performed in a quiet, temperature controlled room maintained at 22-24 °C. FBF was measured as previously described (Verhaar et al 1998; Newby et al 1998a; Helmy et al 2000). Briefly, FBF measurements were made in both the infused and non-infused arms by venous occlusion plethysmography using mercury-in-

Silastic strain gauges applied to the widest part of the forearm. Hands were excluded by rapid inflation of wrist cuffs to a pressure of 220 mm Hg using E20 Rapid Cuff Inflators (D.E. Hokanson Inc, Washington DC, USA). Upper arm cuffs were inflated to 40 mm Hg for 10 seconds in every 15 seconds to achieve venous occlusion and obtain blood flow measurements.

3.5. Study design:

Two protocols (Figure 6-1) were applied on two separate occasions one week apart and in random order. Before participating in each of the protocols, saline was infused for 30 min to allow time for equilibration, with FBF measurements being made every 10 min and the final measure taken as the baseline FBF. In protocol 1, BQ-123 was infused at a dose of 10 nmol/min for 60 min during which FBF was measured every 10 min. In protocol 2, to assess the contribution of endogenous NO production to the forearm vascular responses to ET_A receptor blockade, endogenous NO synthesis was inhibited by the 'NO-clamp' as previously described (Stroes et al 1997, Verhaar et al 1998; Dijkhorst-oei et al 1999). Briefly, L-NMMA was continuously infused at a rate of 4 μmol/min for 20 min to achieve maximal inhibition of local NOS activity. Thereafter, SNP was co-infused at titrated doses until FBF had been restored to within 10% of baseline flow and was sustained for at least 2 consecutive FBF measurements. Once a stable baseline FBF was obtained, the same dose of BQ-123 was co-infused with the 'NO-clamp' for 60 min.

3.6. Blood assays:

After 30 min of supine rest, and before any drugs were administered, venous blood was withdrawn from the non-infused arm. Ten mL were admixed with each of the following: 1 mL of 1% disodium EDTA and 1000 KIU aprotinin (Bayer AG, Leverkusen, Germany) for measuring plasma ET-1, big-ET-1, and plasma renin activity (PRA); and 0.5 mL of 0.45% *O*-phenanthrolene/ 4.65% disodium EDTA for measuring plasma ANG II concentration. The samples were placed on ice and immediately centrifuged at 1500 *g* for 20 min. Plasma was frozen and stored at -80°C until assayed. Following extraction using Bond Elut® columns (Varian, Harbor City,

Protocol 1: (duration: 90 min)

Phase	1	2
Duration	(30 min)	(60 min)
Pump 1 (60 mL/h)	Saline	BQ-123 (10 nmol/min)
Measures	F * F # F	F F F F F F

Protocol 2: (duration: variable)

Phase	1	2	3	4
Duration	(30 min)	(20 min)	variable	(60 min)
Pump 1 (20 mL/h)	Saline	L-NMMA	L-NMMA	L-NMMA
Pump 2 (20 mL/h)	Saline	Saline	SNP	SNP
Pump 3 (20 mL/h)	Saline	Saline	Saline	BQ-123 (30 nmol/min)
Measures	F F F	F F	F F F F	F F F F F F

FIGURE 6-1: SCHEMATIC DIAGRAM OF THE STUDY PROTOCOLS.

F = Bilateral FBF measurements each for 3 min. BQ-123 dose is 10 nmol/min. L-NMMA dose is 4 μmol/min. SNP at titrated doses ranging from 80-600 ng/min. *, time for blood sampling. #, time for measuring systemic haemodynamics using electrical bioimpedance.

CA, USA), the plasma concentrations of ET-1 (Peninsula Laboratories Europe Ltd., St. Helens, England) and big ET-1 (Peninsula Laboratories Europe Ltd.) were determined by radioimmunoassay as described previously (Rolinski et al 1994). PRA was measured under standard conditions through the generation of ANG I using RIA as described previously (Haber et al 1969). Plasma ANG II concentrations were measured by RIA following extraction using Bond Elut® columns (Varian) as described previously (Morton & Webb 1985).

3.7. Statistical analysis:

FBF was expressed in mL. 100 mL tissue^{-1}. min^{-1} (Whitney et al 1953) Recordings from the first 60 s following wrist cuff inflation were not included in the analysis because of the variability in blood flow this produces (Kerslake 1949). Usually, the final 5 flow recordings in each 3 min measurement period were calculated and averaged for each arm. In order to reduce the variability of blood flow data, the ratio of flows in the 2 arms was calculated for each time point, thus using the non-infused arm as a contemporaneous control (Benjamin et al 1995; Webb 1995). The percentage change in FBF was calculated according to the formula:

$$\textbf{\% change in FBF} = 100 \times [(I_t / NI_t) - (I_b / NI_b)] / (I_b / NI_b);$$

where I_t and NI_t are the blood flows in the infused and non-infused forearms respectively at a given time point (t) and I_b and NI_b are the blood flows at baseline (b); time 0.

The % attenuation in area under the curve was calculated according to the formula:

$$\textbf{\% change in AUC} = 100 \times (\%change_{PC} - \%change_{AC}) / (\%change_{AC});$$

where PC and AC are the presence and absence of the 'NO-clamp' respectively.

Data were expressed as mean ± standard error of the mean (SEM) and examined by 2-way analysis of variance (ANOVA) for repeated measures, the Pearson's correlation coefficient or two-tailed paired and un-paired Student's *t*-tests as appropriate. A probability value of $p<0.05$ was taken to represent a statistically significant difference.

4. Results

A summary of baseline subject's characteristics is shown in Table 6-1. Patients with cirrhosis were well matched to the control subjects for age, sex and body surface area (BSA) although weight and, consequently, body mass index (BMI) were significantly less in the cirrhotic group ($p<0.05$). In comparison to healthy controls, patients with cirrhosis had similar HR and MAP, but higher SI ($p<0.05$), higher CI ($p<0.01$) and lower TPVRI ($p<0.01$, Table 6-2). Throughout each study there were no significant changes in MAP, HR, or FBF in the non-infused arm.

Table 6-1: Subjects characteristics.

Variable	Cirrhotics (n=7)	Controls (n=7)
Age (years)	53 ± 5	52 ± 5
Sex (male: female)	3:4	3:4
Weight (kg)	61 ± 4*	75 ± 4
Height (cm)	167 ± 4	167 ± 4
BSA (m^2)	1.7 ± 0.1	1.8 ± 0.1
BMI ($kg.m^{-2}$)	22.0 ± 0.9*	26.9 ± 1.1
Liver disease aetiology		
Primary biliary cirrhosis	3	-
Hepatitis C virus	1	-
Alcoholic liver disease	2	-
Autoimmune CAH	1	-
Child-Pugh Score	6.3 ± 0.5	-
Child Grade A	4	-
B	3	-
Oesophageal varices	7	-

Results are expressed as mean ± SEM. * $p < 0.05$ *vs* controls.
BMA, body mass index. BSA, body surface area. CAH, chronic active hepatitis.

Table 6-2: Baseline systemic haemodynamics and hormonal assays.

Variable	cirrhotics (n= 7)	Controls (n= 7)	Unit
HR	67 ± 2	61 ± 2	$beat.min^{-1}$
MAP	85 ± 5	90 ± 2	mm Hg
CI	5.2 ± 0.4#	3.6 ± 0.2	$L.min^{-1}.m^{-2}$
SI	77 ± 6*	58 ± 4	$mL.m^{-2}$
SV	128 ± 11	105 ± 8	mL
TPVRI	17 ± 2#	25 ± 2	$mm\ Hg.L^{-1}.min.m^2$
FBF (infused)	2.9 ± 0.4	3.1 ± 0.2	$mL.100\ mL\ tissue^{-1}.\ min^{-1}$
FBF (non-infused)	2.7 ± 0.4	3.0 ± 0.3	$mL.100\ mL\ tisue^{-1}.\ min^{-1}$
ET-1	3.3 ± 0.2	4.0 ± 0.6	$pg.mL^{-1}$
Big ET-1	78 ± 26	67 ± 21	$pg.mL^{-1}$
ANG II	33 ± 8*	11 ± 2	$pg.mL^{-1}$
PRA	1.4 ± 0.3	1.1 ± 0.1	$ng.mL^{-1}.h^{-1}$

Results are expressed as mean ± SEM. * $p<0.05$. # $p<0.01$.
CI, cardiac index. FBF, forearm blood flow. HR, heart rate. MAP, mean arterial pressure. SI, stroke index. TPVRI, total peripheral vascular resistive index.

4.1. Baseline forearm blood flow:

There was no significant difference between the baseline FBF in the infused and non-infused arms in each of the study groups (Table 6-2 and Figure 6-2). In addition, FBF after the application of the 'NO-clamp' was unchanged from baseline, and similar in both groups.

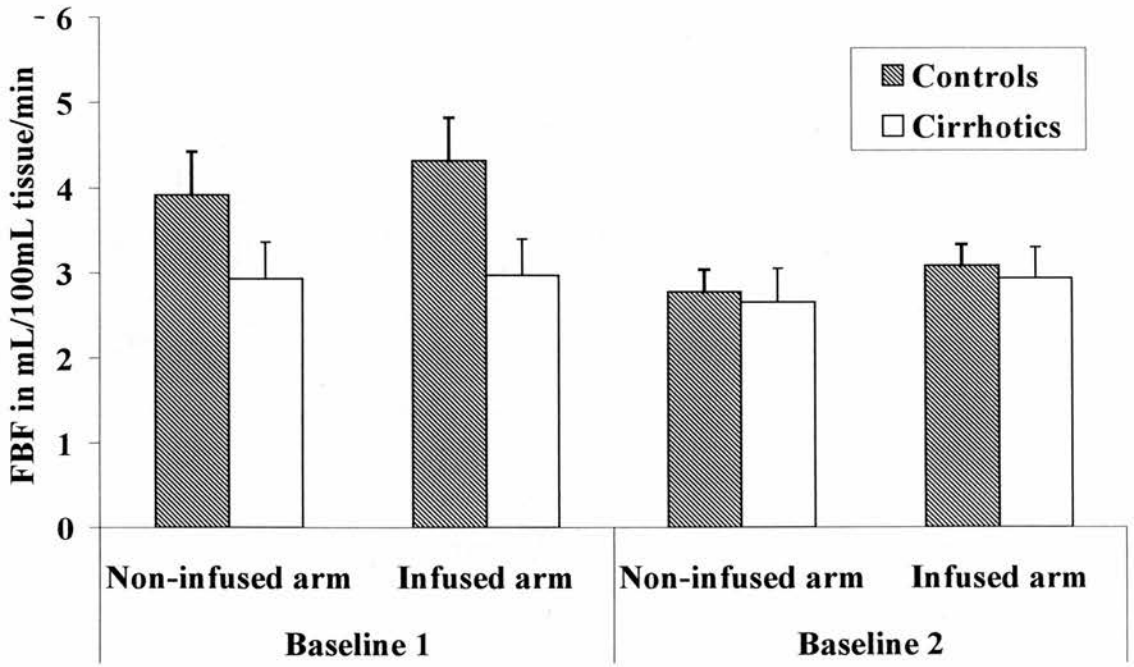


FIGURE 6-2: BASELINE FOREARM BLOOD FLOW.
Baseline 1 = baseline at the end of phase 1 of the study protocol (saline infusion).
Baseline 2 = baseline after adjustment of the 'NO-clamp' and before the re-infusion of ANG II (end of phase 5 of the study protocol).
$p>0.05$ infused *vs* non-infused, by the un-paired *t*-tests.
$p>0.05$ baseline 1 *vs* Baseline 2 in each forearm, by the paired *t*-tests.

4.2. Responses to L-NMMA infusion:

L-NMMA infusion significantly reduced FBF in both patients with pre-ascitic cirrhosis and healthy controls ($p<0.001$, one-way ANOVA *vs* baseline). This response reached a plateau after 10 min of infusion, and was similar in both groups at both 10 and 20 min. Blood flow in the infused arm was reduced by 33.5 ± 5 % and 35.2 ± 4 % in the cirrhotic patients, and by 30.1 ± 2 % and 28.5 ± 4 % in the healthy controls respectively (Figure 6-3). The dose of SNP required to restore baseline FBF was similar in the cirrhotic patients and controls (806 ± 140 ng/min and 811 ± 109 ng/min respectively, p = NS).

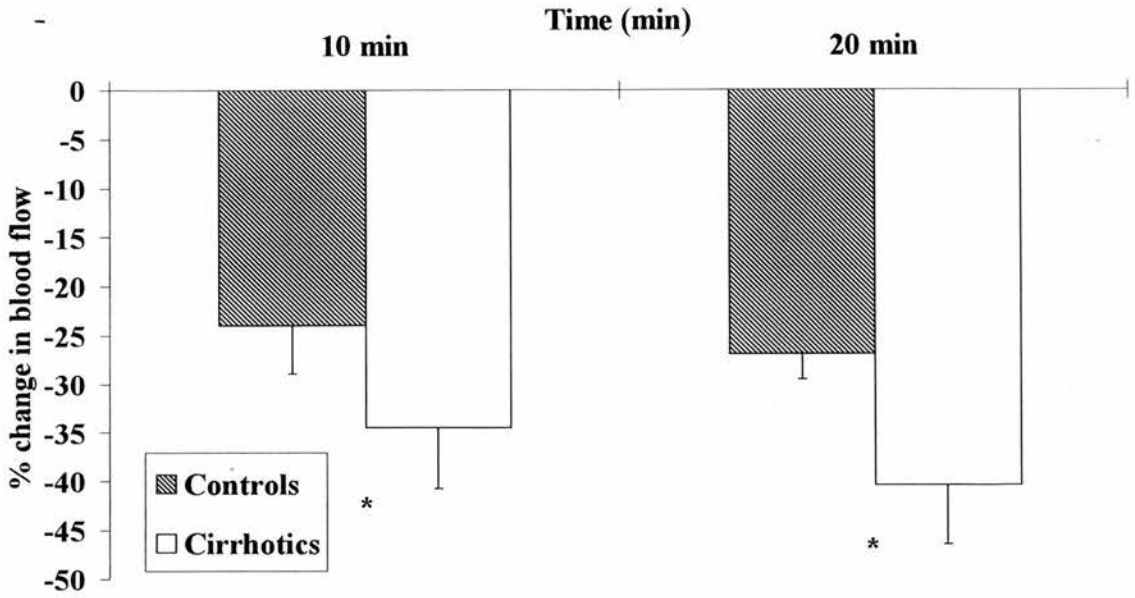


FIGURE 6-3: PERCENTAGE CHANGE IN FOREARM BLOOD FLOW AFTER L-NMMA INFUSION.
L-NMMa dose is 4 μmol/min.
p= 0.75, two-way ANOVA with repeated measures;
p<0.001 from baseline in each group, one-way ANOVA.

4.3. Responses to BQ-123 infusion without the 'NO-clamp':

ET_A receptor antagonism, with BQ-123 infusion, increased FBF in both patients with cirrhosis and controls (p<0.001, one-way ANOVA for each group). This vasodilatation was significantly greater in the patients than the controls (p<0.001, two-way ANOVA with repeated measures). The maximum increase in FBF was 52.1 ± 6.4% and 37.1 ± 5.6% in cirrhotic patients and controls respectively (Figure 6-4). The Mean absolute FBF in both arms during this protocol is shown in Table 6-3.

4.4. Responses to BQ-123 infusion during the 'NO-clamp':

Application of the 'NO-clamp' significantly attenuated the BQ-123-induced vasodilatation in both groups (p<0.001, 2-way ANOVA for both groups). However, FBF remained greater in the patients with cirrhosis than controls (p<0.01, 2-way ANOVA). Whilst significant vasodilatation persisted during 'NO-clamp' application in patients with cirrhosis (p<0.01, one-way ANOVA), BQ-123-induced vasodilatation was nearly abolished, and FBF did not change from baseline in the control group (p>0.05, one-way ANOVA). The maximum increase in FBF was 21.6

± 6.2% and 8.6 ± 5.2% in the cirrhotic patients and controls respectively (Figure 6-4). The Mean absolute FBF in both arms during this protocol is shown in Table 6-4.

The mean ± SEM area under the curve in both groups before and during the application of the 'NO-clamp' are shown in Figure 6-5. The percentage attenuation in the area under the curve for BQ-123-induced vasodilatation due to application of the 'NO-clamp' was 66 ± 9% and 82 ± 24% for patients and controls respectively ($p>0.05$, two-way ANOVA with repeated measures). Also, the absolute change in the calculated area under the curve following application of the 'NO-clamp' was 152 ± 31 and 116 ± 35 in the patients with cirrhosis and controls respectively ($p>0.05$, unpaired *t*-test).

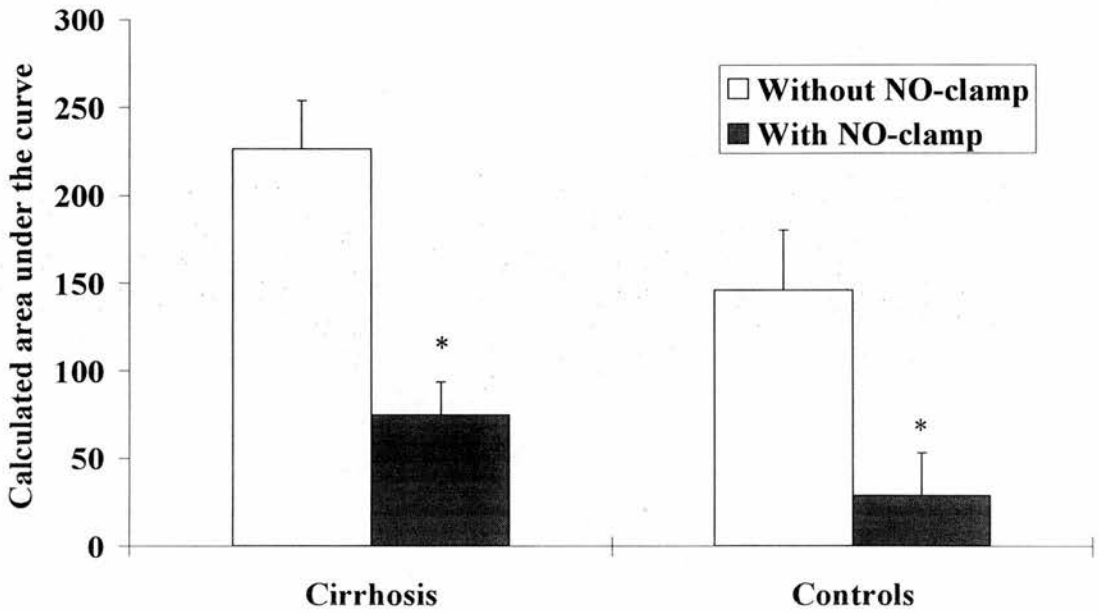


FIGURE 6-5: CALCULATED AREA UNDER THE BQ-123 INFUSION CURVE WITHOUT AND WITH THE APPLICATION OF THE 'NO-CLAMP'.
* $p<0.001$ with clamp *vs* without clamp in each groups.

4.5. Blood assays:

As shown in Table 2, basal plasma ANG II concentrations were higher in patients than controls ($p<0.05$) but basal PRA was similar in both groups ($p>0.05$). In addition, basal plasma ET-1 and big ET-1 concentrations were similar in both groups.

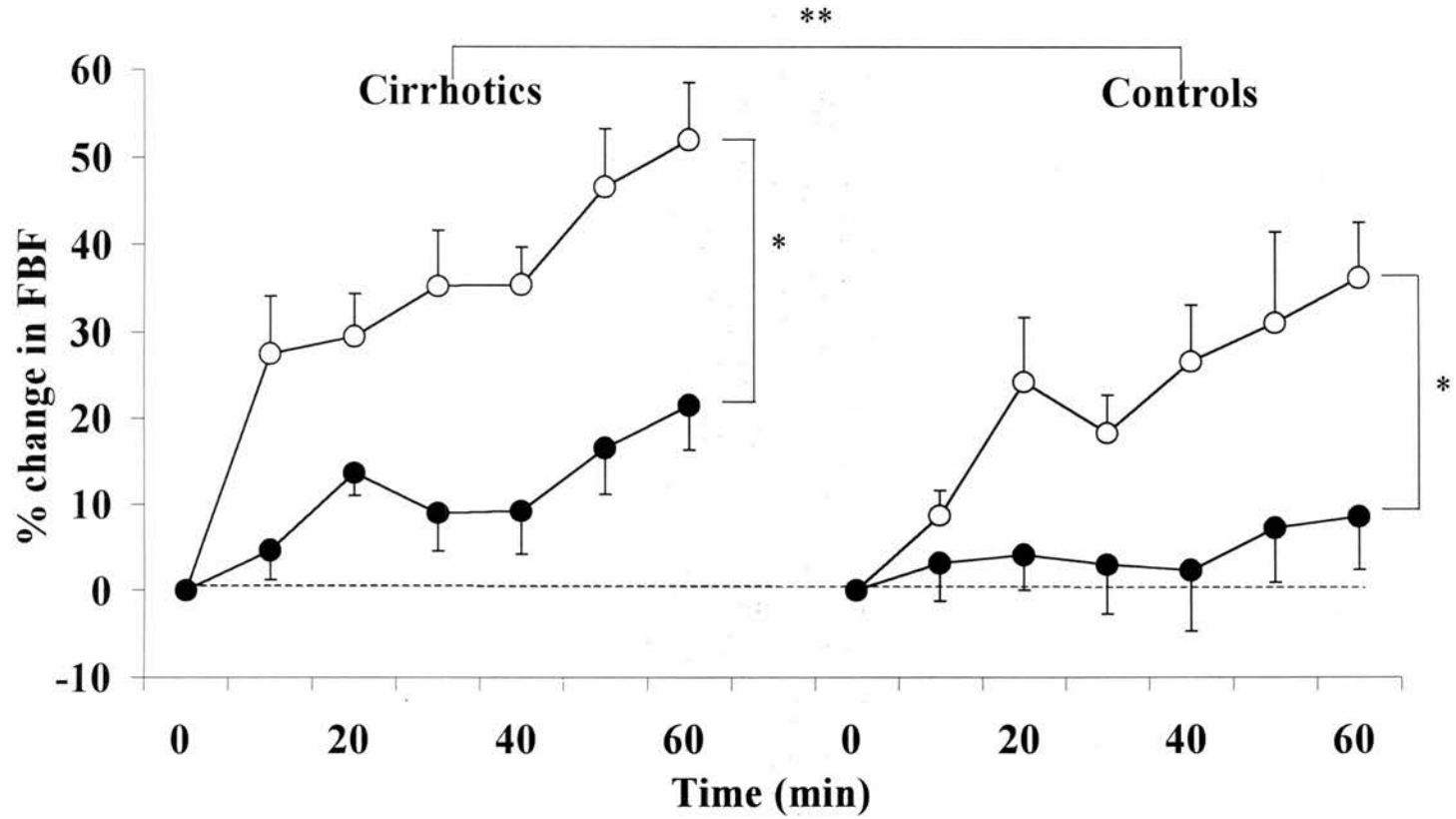


FIGURE 6-4: PERCENTAGE CHANGE IN FOREARM BLOOD FLOW IN RESPONSE TO BQ-123 INFUSION.

BQ-123 dose is 10 nmol/min. Left, Patients with cirrhosis. Right, controls. ●, with 'NO-clamp'. ○,without the application of the 'NO-clamp'. $p<0.001$ form baseline in each response except responses with 'NO-clamp' in controls, one-way ANOVA. * $p<0.001$ without 'NO-clamp' *vs* with 'NO-clamp' in patients with cirrhosis and controls, two-way ANOVA with repeated measures. ** $p<0.01$ patients *vs* controls with or without 'NO-clamp', two-way ANOVA with repeated measures.

Table 6-3: Absolute forearm blood flow during protocol 1.

	Cirrhotics		Controls	
Infusion (time)	Non-infused arm	Infused arm	Non-infused arm	Infused arm
Saline (Baseline 0)	2.9 ± 0.4	3.0 ± 0.4	3.8 ± 0.5	4.1 ± 0.5
BQ-123 (10 min)	2.6 ± 0.3	3.4 ± 0.4	3.5 ± 0.4	4.1 ± 0.4
BQ-123 (20 min)	2.6 ± 0.3	3.5 ± 0.5	3.3 ± 0.4	4.4 ± 0.4
BQ-123 (30 min)	2.7 ± 0.3	3.8 ± 0.5	3.7 ± 0.4	4.7 ± 0.5
BQ-123 (40 min)	2.7 ± 0.4	3.8 ± 0.6	3.4 ± 0.3	4.6 ± 0.4
BQ-123 (50 min)	2.7 ± 0.4	4.1 ± 0.7	3.4 ± 0.4	4.8 ± 0.5
BQ-123 (60 min)	2.8 ± 0.4	4.3 ± 0.7	3.3 ± 0.4	4.9 ± 0.4

Results are expressed as mean ± SEM. Blood flow is expressed as mL.100 mL $tissue^{-1}$. min^{-1}.

Table 6-4: Absolute forearm bloof flow before and during protocol 2.

	Cirrhotics		Controls	
Infusion (time)	Non-infused arm	Infused arm	Non-infused arm	Infused arm
Saline (Baseline 1)	2.7 ± 0.4	2.9 ± 0.4	3.0 ± 0.3	3.1 ± 0.2
L-NMMA (10 min)	2.8 ± 0.4	2.0 ± 0.3	3.1 ± 0.3	2.2 ± 0.1
L-NMMA (20 min)	3.2 ± 0.6	2.1 ± 0.3	3.2 ± 0.2	2.4 ± 0.1
Adjusted 'NO-clamp'* (Baseline 2)	3.1 ± 0.5	3.7 ± 0.5	3.3 ± 0.3	3.7 ± 0.4
BQ-123 (10 min)	3.4 ± 0.5	4.3 ± 0.6	3.3 ± 0.4	3.9 ± 0.5
BQ-123 (20 min)	3.1 ± 0.4	4.2 ± 0.5	3.0 ± 0.3	3.6 ± 0.5
BQ-123 (30 min)	3.2 ± 0.4	4.3 ± 0.5	3.2 ± 0.3	3.8 ± 0.6
BQ-123 (40 min)	3.0 ± 0.3	4.0 ± 0.4	3.4 ± 0.3	4.0 ± 0.7
BQ-123 (50 min)	3.1 ± 0.4	4.4 ± 0.5	3.5 ± 0.4	4.4 ± 0.7
BQ-123 (60 min)	3.1 ± 0.4	4.5 ± 0.4	3.4 ± 0.5	4.2 ± 0.7

Results are expressed as mean ± SEM. Blood flow is expressed as mL.100 mL $tissue^{-1}$. min^{-1}. * 'NO-clamp' is a balanced co-infusion of L-N^G-monomethyl arginine (a selective NO synthase inhibitor) and sodium nitroprusside (an exogenous NO donor) to block endogenous NO production without affecting basal blood flow.

5. Discussion

Consistent with our previous findings in Chapter 4, we have demonstrated an enhanced forearm vasodilatation response to exogenous BQ-123 in patients with pre-ascitic cirrhosis. Application of the 'NO-clamp' attenuated this vasodilatation in both groups, but the vasodilatation persisted in the patient group. These findings suggest that although NO substantially contributes to the BQ-123-induced vasodilatation, it is unlikely to be the mediator of the enhanced vasodilatation in patients with cirrhosis.

The use of BQ-123 selective inhibits the actions of endogenously produced ET-1 on the ET_A receptor while allowing it to act unopposed on the non-isopeptide-selective ET_B receptor. Stimulation of the endothelial ET_B receptor produces dilatation and the vascular smooth muscle ET_B receptor causes constriction. Therefore, the overall response to ET_B receptor stimulation will depend on the balance between these two actions. Pharmacological agents that can distinguish between the endothelial and smooth muscle ET_B receptors are, so far, not available for clinical use. Nevertheless, previous studies in healthy controls using ET_B receptor subtype-specific antagonist, BQ-788, have shown that the balance of ET-1 effects on the ET_B receptor favors vasodilatation, probably through endothelial production of NO (Haynes et al 1995; Verhaar et al 1998). Indeed, we have previously shown that infusion of BQ-788, a selective ET_B receptor antagonist, constricts the forearm arteries (Haynes et al 1995; Verhaar et al 1998; Helmy et al 2001), and increases systemic vascular resistance (Strachan et al 1999).

In ageement with a previous study in healthy controls (Verhaar et al 1998), we have shown that selective ET_A receptor antagonism causes dilatation of the forearm resistance vessels. This response appears, to a large extent, to be due to NO production as demonstrated by the marked reduction of BQ-123-induced vasodilatation with the concomitant use of the 'NO-clamp' (Verhaar et al 1998). We have also shown, in this study and previously (Helmy et al 2001), an enhanced forearm vasodilatation following selective ET_A receptor antagonism in patients with pre-ascitic cirrhosis. Together with the observed systemic haemodynamic derangements, this enhanced vasodilatory responses to BQ-123 infusion suggests an

activated endothelin system, a compensated vasodilated state, and greater dependence of basal vascular tone on ET-1 in these patients, mediated through the ET_A receptor.

The exact mechanism underlying this enhanced vasodilatation in the cirrhotic patients is not clear. It could be either due to an increased ET_B receptor expression with subsequent greater NO release, or increased production of NO-unrelated vasodilators or both. The detection of ET_B receptor overexpression in an animal model of cirrhosis (Cahill et al 1998) favours the first possibility. However, the absence of enhanced forearm vasoconstriction during selective ET_B receptor antagonism (Helmy et al 2001) or NOS inhibition (Calver et al 1994; Ryan et al 1996) in a similar group of patients argues against this possibility. In addition, the concomitant application of the 'NO-clamp' with BQ-123 infusion did not reduce FBF in the cirrhotic patients to baseline as in the healthy controls. This indicates that the enhanced dilatation observed in these patients is largely, but not exclusively, mediated through endothelial NO release. Further studies to address the relative expression of the ET_B receptor in various vascular beds and its relationship to NO production, in patients with cirrhosis, are now needed.

Endothelins have been shown to interact with the endothelial cells, in autocrine and paracrine manners, to produce not only NO but also prostacyclin (Suzuki et al 1991), and atrial natriuretic peptide (ANP) (Fukuda et al 1989; Stasch et al 1989), probably mediated through its endothelial ET_B receptor. In addition, cyclo-oxygenase inhibitors potentiated the ET-1 induced vasoconstriction (De Nucci et al 1988). Moreover, pretreatment of rats with antiserum to ANP potentiated the vasopressor responses to ET-1 (Valentin et al 1991). High plasma concentrations of ANP and cyclic guanosine monophosphate (cGMP) have recently been confirmed in patients with pre-ascitic cirrhosis (Iwao et al 1997; Iwao et al 2000) and inversely correlate with peripheral vascular resistance (Iwao et al 1997). Also, prostacyclin production increases in patients with cirrhosis (Arroyo et al 1983; Guarner et al 1992), and its inhibition reduced COP and increase systemic vascular resistance (Bruix et al 1985).

Indeed, patients included in the present study had significantly lower systemic vascular resistance. Because we did not measure the concentration of these vasodilators, we can't answer from the present study whether the enhanced FBF in following BQ-123 infusion is due to greater ET_B receptor-mediated production of these vasodilators in patients with pre-ascitic cirrhosis. However, we postulate that selective inhibition of the ET_A receptor allows the unopposed action of endogenous ET-1 on the ET_B receptor with subsequent generation of endothelium-derived vasodilators.

It has also been suggested that endothelin may produce vasodilatation by activating the K^+ channels causing membrane hyperpolarization (Lippton et al 1991). The relationship between this effect and the production of the endothelial derived hyperpolarizing factor (EDHP) is unknown (Hadoke 1999). In addition, whether endothelin produces these actions through the ET_B receptor, as the case with NO, or through another yet un-identified receptor, that can't be blocked by BQ-123, needs to be investigated. In addition to the role of the ET_B receptor in mediating the vasodilatory responses, it appears to function as a clearance receptor (Fukuroda et al 1994). Whether an increase in ET_B receptor-mediated clearance of endothelin contributes to the enhanced vasodilatation following ET_A receptor antagonism is currently unknown.

If NO is the sole mediator of vasodilation in cirrhosis, one might expect that systemic infusion of a selective NOS inhibiton, such as L-NMMA, would reduce the splanchnic vasodilatation in cirrhosis, and consequently reduce portal pressure. However, this was not the case in either the rat model of portal hypetension (Pizcueta et al 1992; Niederberger et al 1995; Pilette et al 1996), or in patients with portal hypertension (Forrest et al 1995). Interestingly, L-NMMA infusion in all these studies improved the systemic haemodynamics without affecting portal pressure.

The significant vasoconstriction produced by L-NMMA, a specific inhibitor of NOS, infusion in the studied groups confirms the role of endogenous NO production in the maintenance of basal peripheral vascular tone in both healthy humans (Vallance et al

1989a; Calver et al 1994; Ryan et al 1996) and patients with early (Calver et al 1994; Ryan et al 1996) and advanced cirrhosis (Newby et al 1998a). The similarity of the L-NMMA-mediated vasoconstrictor response in both groups is in keeping with the results of previous studies in a similar group of patients (Calver et al 1994; Ryan et al 1996), and argues against a greater basal NOS activation in the peripheral arterioles of patients with well-compensated cirrhosis.

In conclusion, this study confirms that patients with pre-ascitic cirrhosis have enhanced forearm vasodilator responses to selective ET_A receptor antagonism and normal vasoconstrictor responses to L-NMMA. The persistance of greater FBF in these patients during the concomittant application of the 'NO-clamp' and BQ-123 infusion, despite being attenuated in both groups, indicates that although NO substantially contributes to the enhanced vasodilatation induced by BQ-123 in these patients, other vasodilators are likely to have a role.

CHAPTER 7

GENERAL DISCUSSION & FUTURE DIRECTIONS

1. General discussion

1.1. Overview of the Main results:

The main findings of the series of studies included in this thesis indicate that the forearm resistance vessels in patients with cirrhosis and portal hypertension are hyporesponsive to both exogenous ANG II and ET-1, but not Nad. These abnormalities start from the pre-ascitic stage of the disease, suggesting a role for ANG II and ET-1 in ascites pathogenesis. However, hyporesponsiveness to reflex sympathetic stimulation using LBNP occurs only in the advanced stages of the disease, suggesting a role of cardiopulmonary baroreceptors in ascites perpetuation. In addition, the observation of forearm vasodilatation in response to losartan infusion only in patients with advanced liver disease suggests that endogenous ANG II does not contribute to the maintenance of basal peripheral vascular tone except under conditions of marked RAS activation. In contrast, NO and ET-1 contribute to basal vascular tone in both the healthy state and early cirrhosis, although ET-1 contributes more in patients with pre-ascitic cirrhosis than healthy controls. Moreover, there is an important interaction between the endogenous tonic vasopressor and vasodepressor systems since NO generation largely mediates the vasodilatation response to ET_A receptor antagonism, and, in patients with cirrhosis, the impairment of ANG II vasoconstriction. However, the abnormalities in endothelin mediated vascular tone cannot be entirely attributed to an increase in NO release, and is likely to reflect the direct vascular effects of ET-1.

We have demonstrated an increase in plasma ANG II concentrations of patients with pre-ascitic cirrhosis. The mechanism underlying this increase is not clear, although the presence of an activated RAS is consistent with a compensated vasodilated state, and correlated with portal pressure gradient (Bosch et al 1980). In addition, we have demonstrated that the responses to LBNP are not affected by losartan infusion, indicating that endogenous ANG II has little, if any, role in mediating the forearm vascular responses to low levels of LBNP, and that endogenous ANG II does not contribute to the impaired response to LBNP in patients with advanced cirrhosis.

Basal NO release does not appear to be enhanced in patients with pre-ascitic cirrhosis. This is demonstrated by their normal forearm vasoconstriction in response to L-NMMA infusion. However, the ANG II-stimulated NO release is greater in these patients as shown by normalisation of their impaired responses to exogenous ANG II during the application of the 'NO-clamp'.

The enhanced vasodilatation in response to BQ-123 in patients with pre-ascitic cirrhosis is consistent with a compensated vasodilated state, an activated endothelin system, a greater contribution of ET-1 to basal peripheral vascular tone. Although NO largely mediates the forearm vasodilatation in response to ET_A receptor antagonism in both patients with cirrhosis and healthy controls, the enhanced vasodilatation to BQ-123 infusion in the cirrhotic group cannot be attributed to NO release alone, but is also related to a direct ET-1 mediated tone.

1.2. Selection of patients:

Variations in the characteristics of the patients involved in previous studies make the interpretation of their results very difficult (Hadoke 1999). These criteria include the severity of liver disease, the concomitant use of diuretics, alcohol intake, the presence or absence of portal hypertension, the amount of dietary salt, and to a lesser extent the aetiology of cirrhosis. All these variables may affect the endogenous neurohumoral systems as well as the vascular reactivity to exogenous vasoactive mediators. Therefore, most of the studies included in this thesis were performed in patients with biopsy-proven cirrhosis, endoscopically proven portal hypertension, who were abstinent from alcohol for a minimum of one month, on the same salt intake (150 mmol/day) as the controls, and were not receiving diuretics or any vasoactive medications. In addition, patients in the pre-ascitic stage of cirrhosis have mild, or negligible activation of endogenous vasopressor systems and consequently less effect of these systems was seen on the responses to exogenous mediators. In addition, we concentrated on the pre-ascitic stage of cirrhosis because we believe that better understanding of the neurohumoral abnormalities occurring in this early stage is crucial for the development of better therapeutic and/or preventive options. However, it should be noted that the pre-ascitic stage of cirrhosis is the longest in the

natural history of the disease, during which subtle abnormalities occur either progressively or intermittently. For example, urinary sodium excretion has been shown to be normal or impaired, systemic haemodynamics are temporarily altered by postural changes, and PRA can be low, normal or high.

All patients with pre-ascitic cirrhosis included in this thesis had portal hypertension as indicated by the presence of endoscopically proven oesophageal varices. This may explain the demonstrated increase in the activity of the RAS and the endothelin system in patients with pre-ascitic cirrhosis. Indeed, the activity of the RAS correlates with portal pressure gradient (Bosch et al 1980). This factor needs to be considered when comparing our results with those in patients with early cirrhosis without any evidence of portal hypertension. Patients with diuretic-refractory ascites were not on diuretic therapy and were studies 7 days after their regular paracentesis. By this time, all the circulatory and humoral effects of paracentesis return to the pre-procedure levels (Pozzi et al 1994).

1.3. Effects of bile acids and vascular responses:

Bile acids have vasodilator properties and circulate in increased levels in patients with primary biliary cirrhosis. Therefore, one may assume that the observed vascular hyporesponsiveness to ANG II or ET-1 may be related to high circulating bile acids in these patients. Indeed, plasma concentrations of bile acids correlate with disease severity and with the blood levels of liver function tests (Ohkubo et al 1984). However, most patients with pre-ascitic cirrhosis included in this thesis were not jaundiced, their plasma bilirubin levels were < 30 μmol/L, and were Child grade A. In addition, patients with pre-ascitic cirrhosis had normal vasoconstrictor responses to exogenous Nad. Also, the effects of reducing the circulating levels of bile acids on the hyperdynamic circulation in experimental cirrhosis are contradictory (Genecin et al 1990; Thomas et al 1991). Furthermore, we have observed that patients with pre-ascitic primary biliary cirrhosis have a statistically similar systemic vascular resistive index to those with cirrhosis of other aetiologies.

1.4. Effects of alcohol intake:

Excēssive and regular alcohol intake can affect vascular responses to exogenous vasopressor agents as previously shown with Nad (Howes & Reid 1985). Therefore, all patients with alcoholic liver disease included in this thesis were abstinent from alcohol for a minimum of one month as determined by both clinical history and repeated random blood ethanol estimations. In our previous studies (Newby et al 1998a; Helmy et al 2000), we have shown normal vascular responses to Nad infusion. In addition, we have re-evaluated the results following exclusion of patients with alcoholic cirrhosis. In this post-hoc analysis, exclusion of patients with alcoholic cirrhosis did not affect the statistical significance or the magnitude of the study findings. Therefore, it is very unlikely that inclusion of such patients had an effect on the validity of the results of the whole groups.

1.6. Effects of salt intake:

Sodium status may alter the peripheral vascular responses to vasoactive substances. For example, vasopressor responses to exogenous Nad are affected only by extremes of sodium intake: differing from 10 to 80 fold in both healthy subjects (Rankin et al 1981; Stein et al 1995) and patients with cirrhosis (Wong et al 1995; Wong et al 1996). One of the potential limitations of the studies presented in Chapter 3 in patients with pre-ascitic cirrhosis and diuretic-refractory ascites is that patients in the diuretic-refractory group were on a "no added-salt diet" (100 mmol/day), while patients with pre-ascitic cirrhosis and controls were allowed normal sodium intake (150 mmol/day). The variation in sodium intake is modest (1.5 fold) and such small differences, around the usual western diet, have not previously been shown to influence subsequent vascular responses. Indeed, we found similar reductions in FBF to Nad infusion in patients and controls.

1.7. Selection of the forearm circulation:

It is generally accepted that the systemic vasodilatation in patients with cirrhosis mainly affects the splanchnic circulation. In this thesis, we have concentrated on the reactivity of local forearm resistance vessels. The changes in FBF mainly reflect the changes in arteriolar resistance tone, although effects on pre-capillary sphincters or

smaller arteries, may also contribute (Folkow et al 1971). Responses to vasoactive agents in the forearm circulation tend to parallel those in the major resistance beds (Collier et al 1978; Webb & Hand 1995), and are therefore applicable to most of the vessels that contribute to systemic vascular resistance. Also, the changes in hepatic venous pressure gradient in response to propranolol treatment have been shown to correlate with FBF in patients with cirrhosis (Albillos et al 1997). We have shown that FBF becomes less with disease severity, a finding, which is consistent with what have previously been shown in renal, brachial and cerebral blood flows (Fernandez-Seara et al 1989; Maroto et al 1993; Dillon et al 1995). Therefore, the forearm represents an accessible model to study the systemic vascular responses to different vasoactive drugs and mediators. The ideal drug treatment of portal hypertension should not adversely affect the systemic haemodynamics, and hence, knowledge of the effects of vasopressor systems on the peripheral circulation is essential before testing their effects on the whole body. In addition, some of the studies, which are designed to examine a pathophysiologic mechanism, such as those using the 'NO-clamp' technique, are difficult to apply in the splanchnic circulation.

1.8. Selection of the plethysmography methodology:

The combination of unilateral brachial artery infusion of locally active doses of vasoactive agents and bilateral FBF measurements using mercury-in-silastic strain-gauge venous occlusion plethysmography is a commonly used, powerful, and reproducible, method of assessing vascular responses *in vivo* without invoking systemic effects (Benjamin et al 1995; Webb 1995). We have used this method putting into considerations, while designing and performing the studies included in this thesis, all the strengths, weaknesses, pitfalls, and precautions of this technique, which are mentioned in Chapter 2. We have also used selective receptor antagonists, which are devoid of agonist activity when assessing the role of endogenous mediators.

1.9. Data analysis:

The correct statistical method to analyse data, which represent serial measurements of a variable, such as blood flow, is still debatable. However, it is generally accepted

that the use of ANOVA with replication followed by *post-hoc-tests* is the most suitable and correct way for this type of experiments (Chin-Dusting et al 1999). The second best method is to obtain a single-point summary of the data set from both groups i.e., patients *vs* controls or during 'NO-clamp' *vs* without 'NO-clamp' as area under the curve (Matthews et al 1990). The third method of analysing serial measures is the use of the maximal response point only. This method is the least sensitive, as it does not take into account the full data set available (Chin-Dusting et al 1999). In this thesis we have mainly used ANOVA with replication and AUC in some situations. It can also be noticed that we did not use the calculated FVR (perfusion pressure divided by FBF). This is because this formula is driven through a distensible system by a pulsatile pressure rather than a fixed resistance under a steady driving pressure (Benjamin et al 1995; Chin-Dusting et al 1999).

1.10. The renin-angiotensin and sympathetic nervous systems:

We have confirmed the presence of increased activity of the RAS and SNS in patients with cirrhosis and its correlation with disease severity. Despite the forearm vascular hyporesponsiveness to exogenous ANG II in both early and advanced cirrhosis, endogenous ANG II contributes to the maintenance of basal forearm vascular tone only in advanced cirrhosis. These findings suggest a role for ANG II in the pathogenesis of ascites and the subtitle sodium retention in patients with pre-ascitic cirrhosis (Wong et al 1995; Wong et al 1998). The impaired vasoconstriction in response to ANG II was augmented and normalized by the application of 'NO-clamp'. These findings indicate that the attenuated ANG II responses in patients with pre-ascitic cirrhosis are principally due to an enhanced ANG II-mediated NO release. The possible overexpression of the AT_2 receptors in these patients needs to be examined.

The forearm vascular responses to exogenous Nad are unimpaired. However, attenuated LBNP responses occurred only in advanced cirrhosis, without apparent interaction with endogenous ANG II, suggesting low-pressure baroreceptor down-regulation or defective neuronal transmission. With advancing liver disease, further activation of the neurohumoral systems occurs, and the peripheral circulation is

progressively constricts. AT_1 receptor blockade may provide a useful strategy for preventing the development of ascites in pre-ascitic patients but must be used with caution in patients with advanced liver disease (Binder 1999).

1.11. The endothelin system:

We have shown, for the first time in patients with cirrhosis, that the forearm resistance vessels exhibit an impaired vasoconstriction in responses to exogenous ET-1, a normal response to ET_B receptor blockade, and an enhanced vasodilatation in response to ET_A receptor blockade. These findings suggest an activated endothelin system, and a greater contribution of endogenous ET-1 to the maintenance of basal peripheral vascular tone in patients with pre-ascitic cirrhosis. In addition, these findings may suggest a role for the endothelin system in ascites pathogenesis and the subtle sodium retention observed in this stage if the disease (Wong et al 1995).

The mechanism of the observed hyporesponsiveness to ET-1 is still unclear. However, ET_A receptor down-regulation and/or a post-receptor defect may have a role. This needs to be evaluated in future studies. Also, a possible role for endogenous vasodilators, such as NO, cannot be excluded. The role of the ET_B receptor is complex because of: 1) its existence in both the vascular smooth muscle and vascular endothelial cells mediating both vasoconstriction and vasodilatation respectively; 2) the unavailability of antagonists, which can selectively block the ET_B receptor on each of these cells; 3) its possible involvement in the clearance of ET-1. In the presence of the 'NO-clamp', the BQ-123-induced vasodilatation was abolished in controls and attenuated in patients with cirrhosis, with FBF remaining significantly greater in the patients' group. Therefore, the enhanced vasodilatation abnormalities in endothelin mediated vascular tone cannot be entirely attributed to an increase in NO release, and is likely to reflect the direct vascular effects of ET-1.

A recent preliminary report has shown forearm vasodilatation in response to ET-1 infusion in patients with decompensated cirrhosis (Vaughan et al 2000). However, the results of this report are questionable due to major methodological problems, such as the inclusion of 5 patients only who had advanced cirrhosis, the

measurement of FBF in one arm only, and it did not show what medications these patients were on.

2. Future directions

Although the work included in this thesis has broadened our understanding of the peripheral vascular reactivity in patients with cirrhosis and portal hypertension, it has generated many questions, which need to be addressed in the future.

New and powerful vasopressor mediators, such as urotensin II, continue to be recognized, and their role in homeostasis and in the pathogenesis of the haemodynamic abnormalities will need to be addressed. Moreover, the vascular reactivity to exogenous vasopressin, and its contribution to the maintenance of basal vascular tone have, to date, not been studied in humans *in vivo*, despite the characterisation of its receptors and the availability of their selective antagonists.

The effects of systemic doses of losartan and the endothelin receptor antagonists on systemic, renal, hepatic haemodynamics need to be evaluated. Also, clinical studies on the potential therapeutic role of these antagonists in reducing portal pressure in patients with cirrhosis are needed, which may hopefully pave the way towards their use in future clinical trials.

Extension of the current studies to examine the relative expression of ANG II receptors (AT_1 & AT_2) and the ET-1 receptors (ET_A & ET_B) in different vascular bed in patients with cirrhosis needs to be examined. Doing so will improve the current understanding of the altered vascular responses to exogenous and endogenous ANG II and ET-1 respectively. In addition, when selective AT_2 receptor antagonists become available for clinical use, their contribution to the impaired responses to ANG II, and to the release of NO can be determined. Moreover, the ET_B receptor exists on both the vascular smooth muscle and vascular endothelial cells, and mediates both vasoconstriction and vasodilatation respectively. The lack of availability of antagonists, which can selectively block this receptor on either cell limits the definition of the exact ET_B receptor-mediated actions of ET-1. Also, the

'NO-clamp' technique can be used to assess the contribution of endogenously produced NO to the observed forearm vascular responses to ET-1 and BQ-788.

Several studies have suggested that alterations in post-receptor signal transduction pathways may be involved in the hyporesponsiveness to vasoconstrictors in cirrhosis (Murray et al 1985; Lee & Severson 1994; Haung et al 1996), but this has not yet been confirmed. For example, defective PKC, which is one of the main transducers of the vasoconstrictor effects (Lee & Severson 1994), has been shown in both human and experimental cirrhosis (Spinozzi et al 1991; Wu & Benoit 1994; Tazi et al 1997; Trombino et al 1998; Lahaye et al 1998). Therefore, studies, which compare PKC activity, expression and subcellular distributions of PKC isoforms in VSMC obtained from the hepatic arteries of healthy controls and cirrhotic patients (liver transplant donor and recipient respectively) are required. Also, the involvement of PKC isoforms in agonist-induced vasoconstriction can be determined using phorbol ester, which down-regulates PKC. Moreover, whether PKC isoforms are differentially activated by different vasoconstrictors in control and cirrhotic human VSMC needs to be identified.

The responses of the splanchnic and renal vessels to low-pressure baroreceptor unloading and the role of these receptors in the pathogenesis of splanchnic vasodilatation can be examined using LBNP in patients with cirrhosis who have TIPSS *in situ* during their follow up portogram. In addition, the hepatic arterial blood flow responses to low-pressure baroreceptor unloading can be measured in both patients with cirrhosis and healthy controls before and after LBNP using colour Doppler ultrasonography. When combining these experiments with simultaneous FBF measurements, one can clearly demonstrate the interactions between the blood flow changes in the renal, hepatic or splanchnic territories and those in the forearm circulation.

In patients with advanced cirrhosis, we have shown that losartan infusion produces vasodilatation in the forearm circulation. This effect may, at least in

part, be due to a decrease in the ANG II-mediated activation of the SNS. To obtain a direct evidence of such an effect, muscle sympathetic nerve activity can be measured using microneurography before and after losartan administration. The same needs to be done to assess the activity of the SNS before and after BQ-123 infusion.

In patients with cirrhosis and portal hypertension, the potential therapeutic role of NOS inhibitors, such as L-NMMA, in reducing portal pressure has only been examined in one clinical study (Forrest et al 1995). Interestingly, this study showed a significant improvement in systemic haemodynamics but not the wedged hepatic pressure. The reason for this improvement in systemic but not portal haemodynamics is unclear. Therefore, the study of Forrest et al 1995 needs to be reproduced with measurement of the hepatic blood flow, the activity of the vasopressor systems and the renal function (Martin et al 1998). As shown in the experimental animal studies (Pizcueta et al 1992; Niederberger et al 1995), the absence of changes in portal venous pressure may be explained by a concomitant reduction in splanchnic arterial blood flow and an increase in the intrahepatic vascular resistance. Factors related to the severity of cirrhosis and portal hypertension, the dose of L-NMMA, duration and type of the NOS inhibitor used, and the concomitant use of vasoactive medications such as diuretics should also be considered when designing this study.

CHAPTER 8

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APPENDICES

1. Abstracts originating from this work, which were published and/or presented in scientific meetings:

1. **A Helmy,** R Jalan, DE Newby, PC Hayes, DJ Webb. Contribution of angiotensin II (AII) and angiotensin receptor (AT_1) in the pathogenesis of ascites. ***Gut*** 1998;42 (suppl 1):A20.

2. **A Helmy,** DE Newby, R Jalan, PC Hayes, DJ Webb. Forearm blood flow (FBF) responses to exogenous and endogenous sympathetic stimulation in patients with cirrhosis. ***Gut*** 1998;42 (suppl 1):A28.

3. **A Helmy,** R Jalan, DE Newby, PC Hayes, DJ Webb. Impaired peripheral vascular response to angiotensin II in patients with pre-ascitic cirrhosis. ***Hepatology*** 1998;28(4) Pt 2:449A.

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12. **A Helmy**, DE Newby, R Jalan, DJ Webb, PC Hayes. Altered peripheral vascular responses to vasoactive mediators in patients with pre-ascitic cirrhosis: role of nitric oxide. ***J Hepatol*** 2001;34 (suppl 1):57.

2. Papers originating from this work:

1. **Helmy A**, Jalan R, Newby DE, Hayes PC, Webb DJ. Role of angiotensin II in regulation of basal and sympathetically stimulated vascular tone in patients with early and advanced cirrhosis. Gastroenterology 2000;118:565-572.

2. **Helmy A**, Jalan R, Newby DE, NR Johnston, Hayes PC, Webb DJ. Altered peripheral vascular responses to exogenous and endogenous endothelin-1 in patients with well-compensated cirrhosis. Hepatology 2001;33:826-831.

3. **Helmy A**, Newby DE, Jalan R, NR Johnston, Hayes PC, Webb DJ. Reduced forearm vascular responses to angiotensin II in patients with pre-ascitic cirrhosis is mediated by nitric oxide. Submitted to Hepatology.

4. **Helmy A**, Newby DE, Jalan R, Hayes PC, Webb DJ. Enhanced forearm vasodilation after endothelin-A receptor antagonism in patients with pre-ascitic cirrhosis: role of nitric oxide. Awaiting submission.

5. **Helmy A,** Newby DE, Hayes PC, Webb DJ. Measuring forearm blood flow and interpreting the responses to drugs and vasoactive mediators in patients with cirrhosis and portal hypertension. Under preparation.

6. **Helmy A,** Newby DE, Hayes PC. Aspects of the hyperdynamic circulation in patients with cirrhosis and the role of vasopressor systems and nitric oxide. Underpreparation.

3. Other bibliography:

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3.2. Papers:

1. CL King, A Medhat, I Malhotra, M Nafeh, **A Helmy**, J Kahaudry, S Ibrahim, M El-Sherbiny, S Zaky, R Stupi, K Brustoski, M Shehata and M T Shata. Cytokine control of parasite-specific anergy in human urinary schistosomiasis: IL-4 and IL-10 modulate lymphocyte reactivity. ***J Immunol*** 1996;156:715-721.

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Role of Angiotensin II in Regulation of Basal and Sympathetically Stimulated Vascular Tone in Early and Advanced Cirrhosis

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Background & Aims: **The renin-angiotensin and sympathetic nervous systems are activated in cirrhosis. This study aimed to establish the role of angiotensin II (ANG II) in the regulation of basal and sympathetically stimulated vascular tone in preascitic cirrhotic patients and patients with diuretic-refractory ascites compared with age- and sex-matched healthy controls.** ***Methods:*** **Forearm blood flow (FBF) responses to lower body negative pressure (LBNP) and to subsystemic, intrabrachial infusions of losartan, an angiotensin II type 1 (AT_1) receptor antagonist, norepinephrine, and ANG II were measured using venous occlusion plethysmography.** ***Results:*** **In all groups, ANG II and norepinephrine caused dose-dependent reductions in FBF ($P < 0.001$); responses to norepinephrine were similar across the 3 groups but those to ANG II were less in both cirrhotic groups than in controls ($P < 0.01$). Losartan caused a dose-dependent increase in FBF only in patients with refractory ascites ($P < 0.01$). LBNP caused less reduction in FBF in refractory ascites patients than in both preascitic patients and controls ($P < 0.01$).** ***Conclusions:*** **Despite hyporesponsiveness to exogenous ANG II in both early and advanced cirrhosis, endogenous ANG II contributes to the maintenance of basal vascular tone only in advanced cirrhosis. These findings suggest a role of ANG II in the pathogenesis of ascites. Attenuated LBNP responses occurred only in advanced cirrhosis, without apparent interaction with endogenous ANG II.**

Cirrhosis is characterized by hyperdynamic circulatory changes, including high cardiac output and decreased systemic vascular resistance,[1–3] that worsen with disease progression.[4] Although compensatory activation of vasopressin[5–8] and the renin-angiotensin [9–12] and sympathetic nervous[9,13–16] systems occurs, impaired vascular reactivity has been proposed to contribute to the hyperdynamic circulation of cirrhosis and to the development of its complications, such as portal hypertension, ascites, and hepatorenal syndrome.[17]

Present evidence of this hyporesponsiveness has been provided by systemic studies and in vitro experiments. Although contributing to our understanding of the pathophysiology of portal hypertension, systemic studies have the disadvantage of invoking neurohumoral, counterregulatory mechanisms and having effects on a wide range of organs, including the heart, brain, and kidneys.[18,19] Also, previous studies investigating the role of the renin-angiotensin system[6,8] in the maintenance of vascular tone in patients with cirrhosis were weakened by the use of antagonists, such as saralasin, with partial agonist activity.[20–22] However, losartan, a selective angiotensin II type 1 (AT_1) receptor antagonist that is devoid of agonist activity, has recently become available for clinical use.[23] In addition, the combination of subsystemic and locally active intrabrachial artery infusions with bilateral forearm blood flow (FBF) measurements using venous occlusion plethysmography represents a powerful and reproducible method of assessing in vivo vascular responses in a single circulation without invoking systemic effects.[18,19]

Therefore, the aims of this study were to evaluate, in patients with preascitic cirrhosis and those with diuretic-refractory ascites, the role of endogenous angiotensin II (ANG II) in the maintenance of basal and sympathetically stimulated vascular tone in the forearm circulation; to assess the forearm vascular responses to exogenous norepinephrine (NE) and ANG II; and to determine whether changes in reactivity, if any, are related to disease severity.

Abbreviations used in this paper: ANG II, angiotensin II; AT_1, angiotensin II type 1; EP, epinephrine; FBF, forearm blood flow; LBNP, lower body negative pressure; MAP, mean arterial pressure; NE, norepinephrine; PRA, plasma renin activity.

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Patients and Methods

Subjects

Eighteen patients with biopsy-proven cirrhosis, 8 in the preascitic stage and 10 in the diuretic-refractory stage of the disease,[24] were recruited and compared with 8 age- and sex-matched healthy volunteers (age, 49 ± 6 years; 3 women and 5 men). All patients were ambulant and had normal serum creatinine levels (<100 μmol/L). Patients with diuretic-refractory ascites were studied during a no-added-salt diet (100 mmol/day) 1 week after their regular therapeutic paracentesis and did not receive diuretic therapy for at least 2 weeks before the study. Patients with alcoholic liver disease were abstinent from alcohol for at least 1 month,[25] confirmed by history and random blood ethanol testing. None of the subjects received vasoactive or nonsteroidal anti-inflammatory drugs in the week before each phase of the study. All subjects abstained from food, tobacco, and caffeine-containing drinks for at least 4 hours before each study. To minimize between-subject variability, female control subjects who were premenopausal were studied between day 7 and day 12 of their menstrual cycle.[26] All female cirrhotic patients were amenorrheic. Studies were undertaken with written informed consent from each subject, with the approval of the local research ethics committee, and in accordance with the Declaration of Helsinki (1989) of the World Medical Association.

Drugs and Intra-arterial Administration

Cannulation of the brachial artery of the nondominant arm was performed using a 27-standard wire gauge steel needle (Cooper's Needle Works, Birmingham, England) under 1% lidocaine (Xylocaine; Astra Pharmaceuticals Ltd., Kings Langley, England) local anesthesia. Patency was maintained by saline infusion at a constant rate of infusion (1 mL/min) via an IVAC P1000 syringe pump (IVAC Ltd., Basingstoke, England).

Losartan (Dupont-Merck, Wilmington, DE) at doses of 30 and 90 μg/min; ANG II (Clinalfa, AG Läufelfingen, Switzerland) at doses of 1, 3, 10, and 30 pmol/min; and NE (Levophed; Sanofi-Winthrop Ltd., Guildford, England) at doses of 20, 60, 180, and 540 pmol/min were dissolved in physiological saline (0.9% Baxter Healthcare Ltd., Thetford, England) and administered intra-arterially at these locally active doses.[27,28] To prevent its oxidation, NE was dissolved in saline containing 0.1% ascorbic acid (Evans Medical, Langhurst, England).

Measurements

All studies were performed in a quiet, temperature-controlled room maintained at 22°C–24°C. Blood pressure and pulse rate were measured in the control arm using a noninvasive oscillometric sphygmomanometer (Takeda UA 751; Takeda Medical Inc., Tokyo, Japan) at intervals throughout the study.[29] FBF was measured as described previously.[28,29] Briefly, FBF measurements were made in both arms (i.e., the infused and the noninfused arms) by venous occlusion plethysmography using mercury-in-Silastic strain gauges applied to the widest part of the forearm. The hands were excluded from measurements by rapid inflation of wrist cuffs to a pressure of 220 mm Hg using E20 Rapid Cuff Inflators (D. E. Hokanson Inc., Washington, DC). Upper-arm cuffs were inflated to 40 mm Hg for 10 seconds at every 15 seconds to achieve venous occlusion and obtain blood flow measurements.

Lower Body Negative Pressure

Subjects were rested supine in a plastic-covered steel cage that enclosed the lower body from the waist downward, as described previously.[30,31] A constant negative pressure of 15 mm Hg was achieved using an industrial-strength vacuum cleaner regulated by a pressure control unit (Medical Physics Department, Edinburgh University, Edinburgh, Scotland). Alterations to and from atmospheric pressure were attained within 1–2 seconds. This negative pressure produces selective forearm vasoconstriction through low-pressure baroreceptor unloading without altering arterial pressure or heart rate, thereby eliminating the confounding effects of systemic hypotension.[31]

Study Design

Two protocols (Figure 1) were applied on 2 separate occasions 1 week apart and in random order. Subjects rested supine throughout each study. Strain gauges and cuffs were applied, and the brachial artery of the nondominant arm was cannulated. FBF was measured in the last 3 minutes of each infusion period unless otherwise stated. Before participating in each of the protocols, saline was infused for 30 minutes to allow time for equilibration, with FBF measurements made every 10 minutes, and the final measure taken as the baseline FBF. In protocol 2, the rationale for combining lower body negative pressure (LBNP) with each of the saline and losartan infusions was to assess the contribution of endogenous ANG II to the

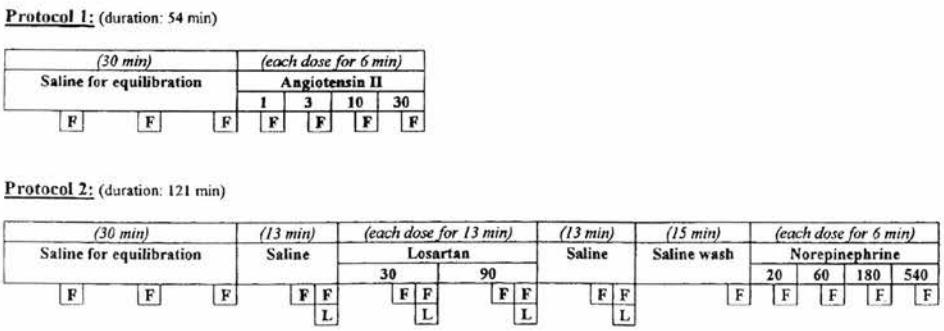

Figure 1. Schematic diagram of the 2 study protocols 1 week apart. F, Bilateral FBF measurements each for 3 minutes; L, LBNP application each for 3 minutes. NE doses are in picomoles per minute, ANG II doses in picomoles per minute, and losartan doses in micrograms per minute.

forearm vascular responses during low-pressure cardiopulmonary baroreceptor unloading.

Blood Assays

After 30 minutes of supine rest, and before any drugs were administered, venous blood was withdrawn from the noninfused arm. Ten milliliters was admixed with each of the following: 1 mL of 1% disodium EDTA/2% sodium metabisulfite for measuring epinephrine (EP) and NE concentrations; 0.5 mL of 0.45% *O*-phenanthroline/4.65% disodium EDTA for measuring ANG II concentration; and 1 mL of 1% disodium EDTA and 1000 KIU aprotinin (Bayer AG, Leverkusen, Germany) for measuring plasma renin activity (PRA). The samples were placed on ice and immediately centrifuged at 1500*g* for 20 minutes. Plasma was frozen and stored at −80°C until assayed. EP and NE concentrations were determined using an electrochemical method following separation by reverse-phase liquid chromatography.[32] Plasma ANG II concentrations were measured by radioimmunoassay following extraction using Bond Elut columns (Varian, Harber City, CA) as previously described.[33] PRA was measured under standard conditions through the generation of ANG I using radioimmunoassay as previously described.[34]

Data Analysis and Statistics

Plethysmographic data were extracted from the Chart (AD Instruments, Castle Hill, Australia) data files, and FBFs were calculated for individual venous occlusion cuff inflations using a template spreadsheet (Excel v. 5.0; Microsoft). Recordings from the first 60 seconds after wrist-cuff inflation were not included in the analysis because of the variability in blood flow this produces.[35] Usually, the last 5 flow recordings in each 3-minute measurement period were calculated and averaged for each arm.

To reduce the variability of blood flow data, the ratio of flows in the 2 arms was calculated for each time point, thereby using the noninfused arm as a contemporaneous control.[18,19] The percentage change in FBF was calculated as follows:

$$\% \text{ Change in FBF} = 100 \times [(I_t/NI_T) - (I_b/NI_b)]/(I_b/NI_b),$$

where I_t and NI_t are the blood flows in the infused and noninfused forearms, respectively, at a given time point (t), and I_b and NI_b are the blood flows in the infused and noninfused forearms, respectively, at baseline (b); time 0.

The changes in FBF during LBNP were calculated in each forearm as % change from the flow immediately before the application of LBNP as follows:

$$\% \text{ Change in FBF During LBNP} = 100 \times [(FBF_{LBNP} - FBF_{PRE})/(FBF_{PRE})],$$

where FBF_{PRE} is the forearm blood flow immediately before LBNP, and FBF_{LBNP} is the forearm blood flow during LBNP.

Mean arterial pressure (MAP) was calculated according to the formula: MAP = Diastolic Arterial Pressure + 1/3 Pulse Pressure (Systolic Arterial Pressure − Diastolic Arterial Pressure). FBF was expressed in mL · 100 mL tissue^{-1} · min^{-1}.[36] Data are expressed as mean ± SEM and examined by analysis of variance (ANOVA) with multiple comparisons, the Pearson's correlation coefficient, and 2-tailed paired and unpaired Student t tests as appropriate. Statistical significance was taken at the 5% level.

Results

A summary of the patient characteristics is shown in Table 1. Patients with diuretic-refractory ascites had a significantly higher Child–Pugh score and prothrombin time and significantly lower serum albumin level than preascitic patients ($P < 0.01$). After participation in the first study, 1 patient with refractory ascites underwent liver transplantation and another underwent transjugular intrahepatic portosystemic stent shunt for variceal bleeding, and the second study was performed in another 2 patients. Therefore, the 16 studies, 8 in each protocol, were completed in 10 patients.

Baseline Hemodynamics

There were no changes in heart rate or MAP throughout the studies. Mean heart rate was significantly higher and MAP significantly lower in patients with diuretic-refractory ascites than in preascitic patients and controls ($P < 0.01$; Table 2). Heart rate correlated positively with PRA and basal ANG II concentrations ($r = +0.52$, $P < 0.01$; and $r = +0.65$, $P < 0.01$, respectively), and MAP correlated negatively with PRA and the basal ANG II concentrations ($r = -0.62$, $P < 0.01$; and $r = -0.61$, $P < 0.01$, respectively).

Baseline FBF

There were no significant differences between the baseline FBF in the infused and noninfused arms on each

Table 1. Patient Characteristics

Variable	Refractory ascites	Preascitic cirrhosis
Age (*yr*)	56 ± 4	48 ± 3
Sex (F:M)	4:6	3:5
Child–Pugh score	11.1 ± 0.5[a]	6.3 ± 0.4
Child grade		
A	0	6
B	0	2
C	10	0
Etiology of cirrhosis		
PBC	1	3
ALD	7	4
ALD + HBV	1	—
Cryptogenic	1	—
AICAH	—	1
Serum bilirubin (*μmol/L*)	52 ± 17	35 ± 12
Serum albumin (*g/L*)	27 ± 2[a]	38 ± 2
Prothrombin time (*s*)	18 ± 1[a]	13 ± 1
Blood urea (*mmol/L*)	4 ± 1	4 ± 1
Serum creatinine (*μmol/L*)	89 ± 9	82 ± 4

NOTE. Results are expressed as mean ± SEM.
PBC, primary biliary cirrhosis; ALD, alcoholic liver disease; HBV, hepatitis B virus; AICAH, autoimmune chronic active hepatitis.
[a]$P < 0.01$ vs. preascitic cirrhotic patients.

Table 2. Baseline Systemic Hemodynamics, FBF, and Humoral Mediators

Variable	Refractory ascites	Preascitic cirrhosis	Controls
Pulse (*bpm*)			
Before LBNP	88.0 ± 3.4[b]	72.4 ± 2.1	67.4 ± 2.6
After LBNP	89.0 ± 4.5[b]	74.7 ± 2.6	62.0 ± 2.3
MAP (*mm Hg*)			
Before LBNP	80.7 ± 1.2[c]	91.8 ± 3.2	93.7 ± 3.9
After LBNP	81.6 ± 2.1[c]	93.5 ± 3.2	94.9 ± 3.0
Baseline FBF ($mL \cdot 100\ mL^{-1} \cdot min^{-1}$)			
Infused arm	2.5 ± 0.2[d]	4.8 ± 0.9	4.2 ± 0.8
Noninfused arm	2.2 ± 0.4[d]	4.2 ± 0.6	3.5 ± 0.7
Basal plasma			
EP (*nmol/mL*)	1.2 ± 0.3[d]	0.7 ± 0.2	0.4 ± 0.2
NE (*nmol/mL*)	2.9 ± 0.3[c]	1.1 ± 0.1	1.4 ± 0.4
ANG II (*pg/mL*)	237.7 ± 30.8[b]	57.3 ± 17.3[a]	3.2 ± 0.3
PRA ($ng \cdot mL^{-1} \cdot h^{-1}$)	15.4 ± 2.4[b]	3.5 ± 1.0	1.7 ± 0.8

NOTE. Results are expressed as mean ± SEM.
bpm, beats per minute.
[a]$P < 0.01$ vs. controls.
[b]$P < 0.05$ vs. preascitic cirrhotics and controls.
[c]$P < 0.01$ vs. preascitic cirrhotics and controls.
[d]$P < 0.05$ vs. preascitic cirrhotics and controls.

study day. The baseline FBF was significantly lower in patients with refractory ascites than in both preascitic patients and the control group (2.5 ± 0.2 vs. 4.8 ± 0.9 and 4.2 ± 0.8 mL · 100 mL tissue^{-1} · min^{-1}, respectively; $P < 0.05$). No significant change in blood flow in the noninfused arm was observed during the protocols, except during LBNP.

Forearm Vascular Responses to ANG II and NE Infusions

Both ANG II and NE infusions produced significant dose-dependent reductions in FBF ($P < 0.001$) in all subjects studied (Figure 2). The responses to ANG II were significantly greater in the control group than in patients with diuretic-refractory ascites ($P < 0.001$) and patients with preascitic cirrhosis ($P < 0.01$). FBF responses to NE were similar in all groups.

Forearm Vascular Responses to Losartan Infusions and LBNP

Absolute values of FBF (mL · 100 mL tissue^{-1} · min^{-1}) for each arm at baseline and during saline, losartan, and LBNP phases of the study in all groups are shown in Table 3. Losartan caused dose-dependent increases in FBF in patients with refractory ascites ($P < 0.001$) but not in patients with preascitic cirrhosis or controls ($P < 0.01$ by 3-way ANOVA between groups; Figure 3). LBNP produced significantly less reduction in FBF in patients with refractory ascites than in either patients with preascitic cirrhosis or controls ($P < 0.01$ by 2-way ANOVA; Figure 4). In addition, responses to LBNP were similar during saline and losartan infusions within each group. Although LBNP responses appeared to be less during saline than during losartan infusions in the refractory ascites group, this did not reach statistical significance ($P = 0.13$ and $P = 0.42$ at doses of 30 and 90 μg/min, respectively).

Assays

Basal plasma ANG II concentrations were significantly higher in patients with diuretic-refractory ascites than in preascitic cirrhotics ($P < 0.05$), and both were significantly higher than in controls ($P < 0.01$). Patients with refractory ascites had significantly higher basal PRA ($P < 0.001$) and higher basal plasma NE ($P < 0.01$) and EP concentrations ($P < 0.05$) than patients with preascitic cirrhosis and controls. Child–Pugh score correlated positively with both PRA and plasma NE concentrations (r =

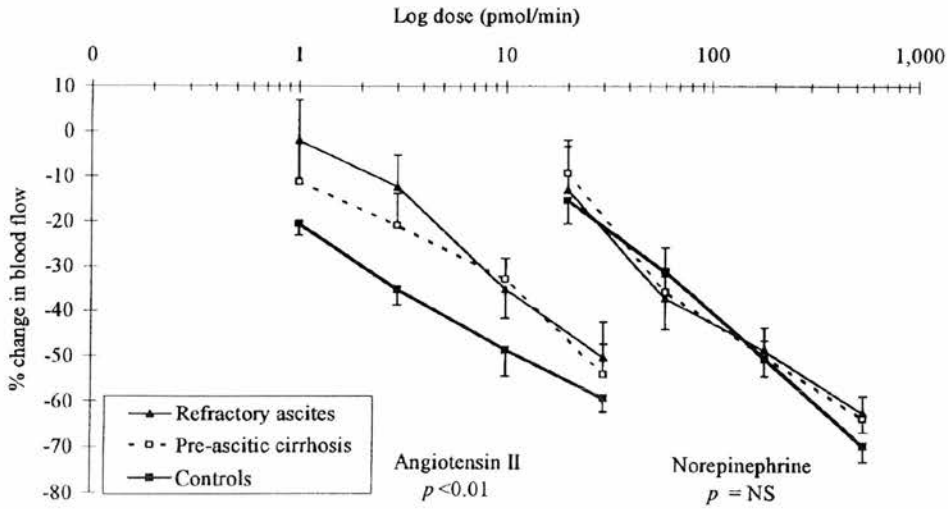

Figure 2. Responses in FBF to incremental doses of ANG II (pmol/min) and NE (pmol/min). NS, not significant. Results are mean ±SEM.

Table 3. Absolute FBF During Baseline, Saline, Losartan, and LBNP Phases of the Study

	Refractory ascites		Preascitic cirrhosis		Controls	
	Noninfused arm[a]	Infused arm[a]	Noninfused arm	Infused arm	Noninfused arm	Infused arm
Baseline	2.2 ± 0.4	2.5 ± 0.2	4.2 ± 0.6	4.8 ± 0.9	3.5 ± 0.7	4.2 ± 0.8
Saline (1)	2.3 ± 0.4	2.6 ± 0.3	4.2 ± 0.6	5.1 ± 1.0	3.6 ± 0.7	4.3 ± 0.8
Saline (1) + LBNP	2.4 ± 0.4	2.5 ± 0.3	3.2 ± 0.5	3.8 ± 0.7	2.8 ± 0.6	3.3 ± 0.7
Losartan 30	2.3 ± 0.5	3.1 ± 0.4	5.1 ± 0.8	6.0 ± 1.1	3.7 ± 1.0	4.7 ± 1.2
Losartan 30 + LBNP	2.4 ± 0.4	2.6 ± 0.3	3.7 ± 0.5	4.1 ± 0.5	3.0 ± 0.7	3.6 ± 0.6
Losartan 90	2.2 ± 0.4	3.0 ± 0.3	5.1 ± 0.8	6.2 ± 1.1	4.1 ± 0.9	5.2 ± 1.1
Losartan 90 + LBNP	2.2 ± 0.3	2.4 ± 0.3	3.7 ± 0.6	4.4 ± 0.6	3.5 ± 0.8	4.1 ± 0.9
Saline (2)	2.1 ± 0.5	2.7 ± 0.3	5.3 ± 0.9	6.2 ± 1.2	4.1 ± 0.9	5.2 ± 1.1
Saline (2) + LBNP	2.4 ± 0.5	2.6 ± 0.3	3.8 ± 0.5	4.5 ± 0.6	3.3 ± 0.9	4.4 ± 1.2

NOTE. Results are expressed as mean ± SEM. Data are reported in mL · 100 mL tissue^{-1} · min^{-1}.
[a]$P < 0.001$ by 3-way ANOVA; refractory ascites vs. preascitic cirrhotics and controls.

+0.71, $P < 0.001$; and $r = +0.60$, $P < 0.01$, respectively).

Discussion

In this study we show that although patients with diuretic-refractory ascites and those with preascitic cirrhosis are hyporesponsive to exogenous ANG II, endogenous ANG II contributes to the maintenance of basal vascular tone, as demonstrated by the response to losartan, only in patients with diuretic-refractory ascites. In addition, impaired peripheral vasoconstrictor responses to low-pressure baroreceptor unloading occurs in patients with refractory ascites, although the vascular responses to exogenous NE are similar in all groups.

Previous studies in patients with cirrhosis have produced contradictory results regarding the responsiveness to systemic administration of α-adrenergic agonists and ANG II. Many studies showed that responses to NE were not affected,[37–39] but others showed an attenuated response.[40,41] Responses to ANG II were reported to be impaired in one study,[41] whereas normal responses were reported in others.[38–40] However, systemic drug administration leads to concomitant effects on organs, such as the brain, kidney, and heart, and influences neurohumoral reflexes by affecting systemic hemodynamics. Consequently, vascular responses cannot be wholly attributed to a direct effect of the drug.[18,19] In contrast, the use of bilateral FBF measurements, with unilateral brachial artery infusions of vasoactive mediators at subsystemic and locally active doses, is a very powerful and reproducible tool for directly assessing vascular responses in vivo.[18,19] However, previous studies using the forearm model in patients with cirrhosis are few and their results are also variable.[28,42–45] Because of the confounding influence of diuretic therapy and some methodological concerns, such as use of unilateral blood flow measurements and nonstandardized infusion protocols, conflicting results have been reported, e.g., either reduced[42–44] or unaltered[28,45] vasoconstrictor responses to NE.

Using the bilateral forearm model, we have previously shown that patients with a combination of cirrhosis and diuretic-responsive ascites have a significantly reduced vascular reactivity to exogenous ANG II and cardiopulmonary baroreceptor unloading.[28] However, maintenance of patients on regular diuretic therapy may have influenced these results by producing relative intravascular hypovo-

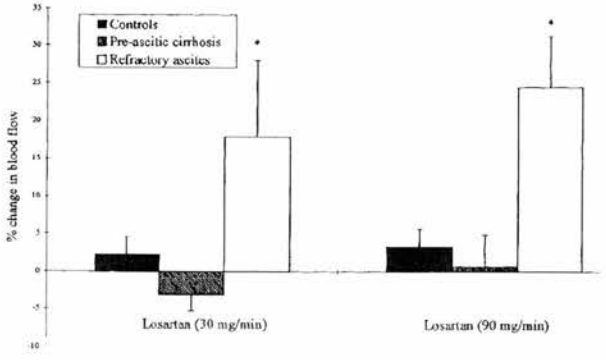

Figure 3. Responses in FBF to losartan infusions in the 3 studied groups. Results are mean ±SEM. *$P < 0.01$ by 3-way ANOVA; refractory ascites vs. preascitic cirrhosis and controls.

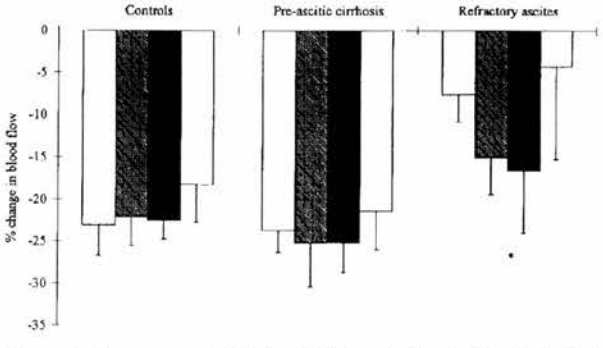

Figure 4. Responses in FBF to LBNP application in the 3 studied groups during saline (□), 30 μg/min losartan (■), and 90 μg/min losartan (■) infusions. Results are mean ±SEM. *$P < 0.01$ by 3-way ANOVA; refractory ascites vs. preascitic cirrhosis and controls.

lemia and associated neurohumoral activation. In the present study, we recruited patients with early and advanced cirrhosis who were not receiving maintenance diuretic therapy. Patients with refractory ascites were studied 1 week after their regular therapeutic paracentesis and albumin therapy and were hemodynamically stable.

In agreement with previous studies,[4,9–12,28] we have shown that the renin-angiotensin and sympathetic nervous systems are activated in patients with cirrhosis and this neurohumoral activation becomes more marked with disease progression. The observed reduction in baseline FBF in patients with diuretic-refractory ascites may, in part, be explained by this neurohumoral activation and supports the previously reported reduction in brachial, femoral, and cerebral arterial blood flow patients with cirrhosis and ascites, using duplex-Doppler ultrasonography.[46,47]

The present study demonstrates that the forearm circulation is hyporesponsive to ANG II in cirrhotic patients even before the development of ascites, suggesting that this abnormality may contribute to the pathogenesis of the hyperdynamic circulatory syndrome. The mechanism of this hyporesponsiveness is unclear, but a recent study has suggested that it may be mediated through nitric oxide.[48] In addition, we have shown an increase in plasma ANG II concentrations in patients with preascitic cirrhosis. The mechanism underlying this increase is not clear, although the presence of an activated renin-angiotensin system is consistent with a compensated vasodilated state. Moreover, the activity of the renin-angiotensin system correlates with portal pressure gradient[49]; in the present study, all patients with preascitic cirrhosis had a high portal pressure as indicated by the presence of esophageal varices. Increased activity of the endogenous vasopressor systems could influence responses to exogenously administered ANG II and NE, resulting in a right shift of the dose-response curve. However, this is unlikely, at least for responses to NE, which were similar in all groups despite the presence of an activated sympathetic nervous system only in patients with diuretic-refractory ascites.

The finding that selective AT_1-receptor blockade with losartan increases FBF in patients with diuretic-refractory ascites but produces no change in FBF in healthy subjects or patients with preascitic cirrhosis supports the concept that ANG II contributes to the maintenance of basal vascular tone only in patients with advanced liver disease, having no role in either healthy controls or early cirrhosis.[27,28,50] A recent finding that oral losartan effectively reduced hepatic venous pressure gradient in patients with cirrhosis and portal hypertension,[51] together with the observed increase in plasma ANG II concentrations and vascular dependence on ANG II in severe cirrhosis, indicates that ANG II contributes to the hemodynamic consequences of cirrhosis and, therefore, that losartan may potentially be helpful in the treatment of portal hypertension. Additionally, the effects of the high plasma ANG II concentrations observed in patients with preascitic cirrhosis on renal arteriolar blood flow and distribution help to explain the subtle Na^+ retention detected before the development of ascites[52] and suggest that use of AT_1-receptor blockers may improve Na^+ excretion in patients with preascitic cirrhosis, and thus prevent or delay the development of ascites.

LBNP has been used for many years to examine the neurocirculatory reflex response to reduced venous return to the heart.[53] In healthy subjects, LBNP at intensities up to 20 mm Hg selectively unloads low-pressure cardiopulmonary baroreceptors without causing changes in heart rate or blood pressure. Such low-intensity LBNP selectively reduces FBF[31,54–57] and increases forearm NE spillover without affecting total peripheral vascular resistance or total body NE spillover. In contrast, LBNP at intensities greater than 20 mm Hg also unloads arterial baroreceptors with concomitant increases in total body spillover of NE, increases in heart rate, and decreases in blood pressure.[57] Previous studies in patients with decompensated cirrhosis have shown impaired vasoconstrictor responses to both LBNP and body tilting.[9,28,40] In the present study, we document impaired vasoconstrictor responses to LBNP in patients with diuretic-refractory ascites despite normal responsiveness to exogenous NE. This may be the result of several mechanisms including baroreceptor down-regulation or defective sympathetic neurotransmission secondary to the autonomic dysfunction described with advanced cirrhosis.[58] In earlier studies by Wong et al.,[52,59] central venous pressure was elevated in patients with preascitic cirrhosis. One may therefore anticipate that higher intensities of LBNP are required to achieve similar reflex responses in these patients. Nevertheless, these studies have shown that, in response to LBNP of 15 mm Hg, patients with preascitic cirrhosis have reductions in central venous pressure and FBF similar to healthy subjects.[52,59]

The observation that the vasoconstrictor responses to LBNP of 15 mm Hg in the infused forearm are unaffected by losartan infusions has been reported in both cirrhotic patients[28] and controls,[27,60,61] and indicates that endogenous ANG II has little, if any, role in mediating these regional responses to low levels of LBNP in either situation. These findings also suggest that influences of endogenous ANG II does not contribute to the impaired response to LBNP in patients with refractory ascites.

One of the potential limitations of the present study is that patients in the diuretic-refractory group were on a

no-added-salt diet (100 mmol/day), whereas patients with preascitic cirrhosis and controls were allowed normal sodium intake (150 mmol/day). Sodium status may alter the peripheral vascular responses to vasoactive substances. However, pressor responses to exogenous NE, for example, in healthy subjects are affected only by extremes of sodium intake, differing from 10–80-fold.[52,62,63] In our study, the variation in sodium intake is modest (1.5-fold) and such small differences around the usual Western diet have not been shown to influence subsequent vascular responses. Indeed, we found similar reductions in FBF to NE infusion in patient and control groups.

In conclusion, despite hyporesponsiveness to exogenous ANG II in both early and advanced cirrhosis, endogenous ANG II contributes to the maintenance of basal forearm vascular tone only in advanced cirrhosis. These findings suggest a role for ANG II in the pathogenesis of ascites. The forearm vascular responses to exogenous NE are unimpaired. However, attenuated LBNP responses occur only in advanced cirrhosis, without apparent interaction with endogenous ANG II, suggesting low-pressure baroreceptor down-regulation or defective neuronal transmission. With advancing liver disease, further activation of the neurohumoral systems occurs, and the peripheral circulation is progressively vasoconstricted. AT_1-receptor blockade may provide a useful strategy for preventing the development of ascites in preascitic patients but must be used with caution in patients with advanced liver disease.[64] Further studies are required to elucidate the mechanism of the observed hyporesponsiveness to ANG II.

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Altered Peripheral Vascular Responses to Exogenous and Endogenous Endothelin-1 in Patients With Well-Compensated Cirrhosis

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Plasma endothelin concentrations are elevated in cirrhosis and correlate with disease severity. This study assessed forearm vascular responses to exogenous endothelin-1 (ET-1), and evaluated the contribution of endogenous ET-1 to the maintenance of basal peripheral vascular tone in patients with well-compensated cirrhosis (n = 11) and matched healthy controls (n = 8). Bilateral forearm blood flow (FBF) was measured at baseline and following unilateral, subsystemic, intrabrachial artery infusions of ET-1 (2 and 6 pmol/min); BQ-123, a selective ET_A receptor antagonist (3 and 10 nmol/min); and BQ-788, a selective ET_B receptor antagonist (0.3 and 1 nmol/min) using venous occlusion plethysmography. Baseline systemic hemodynamics and plasma ET-1 and big ET-1 concentrations were measured using electrical bioimpedance and radioimmunoassay, respectively. Patients and controls had similar baseline FBF, systemic hemodynamics, and plasma ET-1 and big ET-1 concentrations. In both groups, ET-1 and BQ-788 caused significant vasoconstriction ($P < .001$) and BQ-123 caused significant vasodilatation ($P < .001$). Compared with controls, cirrhotic patients had attenuated ET-1 responses ($P < .001$), augmented BQ-123 responses ($P < .001$), and similar BQ-788 responses ($P = .62$). Despite normal systemic hemodynamics and plasma ET-1 concentrations, forearm vascular responses to exogenous ET-1 are reduced in cirrhotic patients. The augmented vasodilatation to BQ-123 in cirrhotic patients is consistent with a compensated vasodilated state, and a greater contribution of ET-1 to the maintenance of basal vascular tone acting through the ET_A receptor. (HEPATOLOGY 2001;33:826-831.)

A hyperdynamic circulation characterized by low arterial pressure, high cardiac output, and low systemic vascular resistance is a feature of patients with advanced cirrhosis and portal hypertension.[1-3] This hyperdynamic circulation worsens with disease progression[4-6] and is accompanied by a reduced reactivity to vasopressor systems including the renin-angiotensin and sympathetic nervous systems. These hemodynamic changes play a crucial role in the pathogenesis of portal hypertension and its complications, including variceal hemorrhage, ascites, and the hepatorenal syndrome.[7,8]

Endothelin-1 (ET-1) is the most potent vasoconstrictor known, belonging to a 21-amino acid peptide family with a range of biological effects.[9-11] ET-1 was originally identified in the culture medium of porcine aortic endothelial cells[9,10] and is derived from a larger pre-pro-endothelin-1 (212 amino acids), which is cleaved by endopeptidases to produce big ET-1 (38 amino acids), which is then converted to ET-1 by specific endothelin-converting enzymes.[9-10]

Molecular studies have, so far, identified 2 endothelin receptor subtypes in mammalian species: endothelin-A (ET_A) and endothelin-B (ET_B). In vascular smooth muscle cells, both receptors are expressed[11,12] and mediate vasoconstriction.[11-14] Only the ET_B receptors are found on endothelial cells, where these cause vasodilatation through the release of endothelium-derived vasodilators, such as nitric oxide (NO).[15] ET-1–induced vasoconstriction is predominantly mediated by the ET_A receptor, but ET_B receptors may contribute under some circumstances.[16]

Since 1991, many studies have reported increased plasma concentrations of ET-1 in patients with cirrhosis.[17-26] The mechanisms underlying the elevated ET-1 concentrations are unclear and do not appear to be related to hypovolemia or endotoxemia.[25,27] However, there have been no studies to address the vascular responsiveness to ET-1 *in vivo*, or its role in the maintenance of basal peripheral vascular tone in patients with cirrhosis. Therefore, the aims of the present study—in patients with well-compensated cirrhosis and portal hypertension—were to determine the forearm vascular responsiveness to exogenous ET-1 and to assess the role of endogenous ET-1 in the maintenance of basal forearm vascular tone using the selective ET_A and ET_B receptor antagonists, BQ-123 and BQ-788, respectively.

PATIENTS AND METHODS

Patients. Eleven patients with biopsy-proven and well-compensated cirrhosis and 8 healthy control subjects were recruited. All patients were ambulant and had endoscopically proven esophageal varices, normal serum creatinine (<100 mol/L), and no clinical ev-

Abbreviations: ET-1, endothelin-1; ET_A, endothelin type A receptor; ET_B, endothelin type B receptor; NO, nitric oxide; FBF, forearm blood flow; HR, heart rate; MAP, mean arterial pressure.

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idence of systemic circulatory disturbance. Patients with alcohol-induced liver disease were abstinent from alcohol for at least 1 month, confirmed by history and repeated random blood ethanol testing, to prevent the depression of vascular responses that this causes.[28] To avoid the possibility of altering the endogenous vasopressor systems, all subjects in both groups were maintained on their normal sodium intake. None of the subjects received vasoactive or nonsteroidal anti-inflammatory drugs in the week before, and all subjects abstained from food, tobacco, and caffeine-containing drinks for at least 4 hours before each study. All female subjects were postmenopausal, both for safety and to avoid the variability in vascular responses that may be associated with cyclic hormonal changes.[29] Studies were undertaken in accordance with the Declaration of Helsinki (1989) of the World Medical Association, the approval of the local research ethics committee, and the written informed consent of each subject.

Drugs and Intra-arterial Administration. Cannulation of the brachial artery of the nondominant arm was performed using a 27-standard wire gauge steel needle (Cooper's Needle Works, Birmingham, UK) under 1% lidocaine (Xylocaine; Astra Pharmaceuticals Ltd., Kings Langley, UK) local anesthesia. Patency was maintained by saline infusion at a constant rate (1 mL/min) via an IVAC P1000 syringe pump (IVAC Ltd., Basingstoke, UK).

ET-1 (Clinalfa AG, Läufelfingen, Switzerland) at doses of 2 and 6 pmol/mL, BQ-123 (Clinalfa AG) at doses of 3 and 10 nmol/mL, and BQ-788 (Clinalfa AG) at doses of 0.3 and 1 nmol/mL, were dissolved in physiologic saline (0.9% Baxter Healthcare Ltd., Thetford, UK) and administered intra-arterially. On the basis of previous studies, we studied doses that are subsystemic and act locally to produce approximately 40% reduction, approximately 30% increase, and approximately 20% reduction in forearm blood flow (FBF) in healthy subjects after 60 minutes of infusion of ET-1 (5 pmol/min), BQ-123 (10 nmol/min), and BQ-788 (1 nmol/min), respectively.[30-32] In such studies, by 60 minutes these doses produce maximal and sustained responses.[30-32] Pilot studies have suggested that higher doses may evoke systemic hemodynamic effects.

Measurement of FBF. The combination of subsystemic and locally active intrabrachial artery infusions with bilateral FBF measurements using venous occlusion plethysmography is a powerful and reproducible method of directly assessing *in vivo* vascular responses to vasoactive mediators without invoking systemic effects.[33,34] All studies were performed in a quiet, temperature-controlled room maintained at 22 to 24°C. FBF was measured as previously described.[26,35]

Study Design. Schematic diagrams of the 3 protocols applied in this study are shown in Fig. 1. ET-1, BQ-123, and BQ-788 were administered on 3 separate occasions, in random order, at least 1 week apart. Subjects rested supine throughout each study. Strain gauges and cuffs were applied, and the brachial artery of the nondominant arm cannulated. In all protocols, saline was infused for 30 minutes to allow time for equilibration, with FBF measurements being made every 10 minutes and the final measurement taken as the baseline FBF. Thereafter, ET-1, BQ-123, or BQ-788 was infused for 60 minutes at each of 2 incremental doses, and FBF was measured every 10 minutes.

Measurement of Systemic Hemodynamics. Arterial blood pressure and heart rate (HR) were measured in the noninfused arm using a noninvasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan) at 20-minute intervals throughout each study.[36] The mean arterial pressure (MAP) was calculated according to the formula; MAP (mm Hg) = diastolic arterial pressure + 1/3 pulse pressure.

Baseline cardiac functions, including HR, stroke volume, and cardiac output, were measured using a noninvasive thoracic electrical bioimpedance method (BoMed NC-COM3; BoMed Medical Manufacturer Ltd., Irvine, CA) as previously described.[37] Bioimpedance is a noninvasive and reproducible method for assessing systemic hemodynamic parameters, including cardiac output and systemic vascular resistance.[38-40]

Blood Assays. After 30 minutes of supine rest, 10 mL venous blood was withdrawn from the control arm and admixed with 1 mL of 1% disodium EDTA and 1,000 KIU aprotinin (Bayer AG, Leverkusen, Germany). Blood samples were placed on ice and immediately centrifuged at 1,500g for 20 minutes. Plasma was frozen and stored at −80°C until assayed. Following extraction using Bond Elut columns (Varian, Harber City, CA), the plasma concentrations of ET-1 (Peninsula Laboratories Europe Ltd., St. Helens, UK) and big ET-1 (Peninsula Laboratories Europe Ltd.) were determined by radioimmunoassay as previously described.[41] The intra-assay coefficients of variation for ET-1 and big ET-1 were 7.0% and 7.2%, respectively, and the interassay coefficients of variation were 9.0% and 9.3%, respectively.

Measurement of Urinary Sodium Excretion. On the day before the study, urine was collected for 24 hours to measure total volume and Na^+ concentration using flame photometry.

Data Analysis and Statistics. Plethysmographic data were extracted from the Chart™ (AD Instruments, Castle Hill, Australia), and data files and FBFs were calculated for individual venous occlusion cuff inflations using a template spreadsheet (Excel v5.0; Microsoft, Redmond, WA). Recordings from the first 60 seconds following inflation of the wrist cuff were not included in the analysis because of the variability in blood flow that this produces.[42] Usually, the last 5 flow recordings in each 3-minute period were calculated and averaged for each arm. To reduce the variability of blood flow data, the ratio of flows in both arms was calculated for each time point, thereby using the noninfused arm as a contemporaneous control.[33,34] The percentage change in FBF was calculated as follows: % change in FBF = $100 \times [(I_t/NI_t) - (I_b/NI_b)]/(I_b/NI_b)$; where I_t and NI_t are the blood flows in the infused and noninfused forearms, respectively, at a given time point (t), and I_b and NI_b are the blood flows in the infused and noninfused forearms, respectively, at baseline (b); time 0. FBF was expressed in mL × 100 mL tissue^{-1} × min^{-1}.[43]

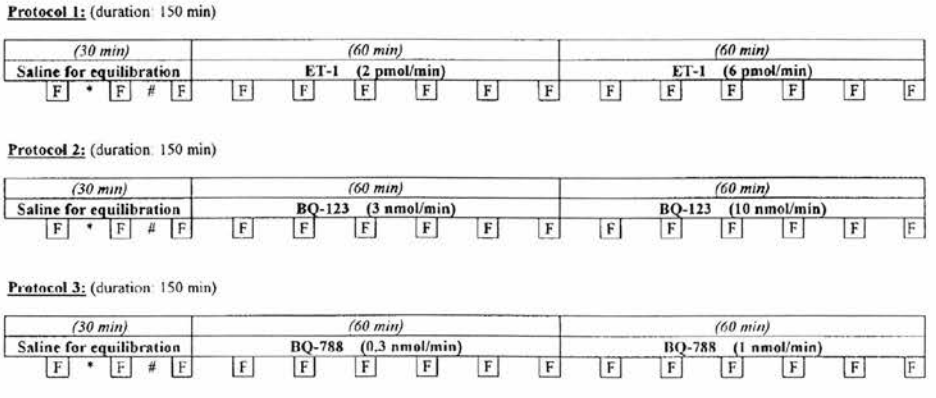

FIG. 1. Schematic diagram of the study design. Three protocols, at least 1 week apart, were undertaken in random order. F = bilateral FBF measurements, each for 3 minutes. *Blood sampling. #Assessment of electrical bioimpedance.

TABLE 1. Subject Characteristics

Variable	Cirrhotic Patients (n = 11)	Controls (n = 8)
Age (years)	52.1 ± 3.3	49.0 ± 4.9
Sex (male:female)	5:6	5:3
BMI (kg·m^{-2})	24.3 ± 0.8	24.8 ± 1.3
BSA (m^2)	1.8 ± 0.1	1.8 ± 0.1
Liver disease etiology		—
ALD	4	
PBC	5	
ALD + HCV	1	
Cryptogenic	1	
Child-Pugh score	5.7 ± 0.2	—
Child grade A	11	—
Esophageal varices	11	—
Urinary Na^+/24 h (mmol/L)	147.5 ± 20.8	173.9 ± 20.1

Results are expressed as mean ± SEM.

Abbreviations: BMI, body mass index; BSA, body surface area; ALD, alcohol-induced liver disease; PBC, primary biliary cirrhosis; HCV, hepatitis C virus.

Data were expressed as mean ± SEM and examined by 2-factor ANOVA with repeated measures, regression analysis, and 2-tailed paired and unpaired Student's t tests as appropriate. Statistical significance was taken at the 5% level.

RESULTS

A summary of baseline characteristics of the subjects is shown in Table 1. Patients with cirrhosis were well matched to the control subjects for age, sex, and body mass index. Six patients (5 men and 1 woman) completed all 3 protocols; 1 female patient completed 2 protocols, and the remaining 4 studies were performed in 4 female patients. Therefore, the 24 studies were completed in 11 patients. No adverse effects were observed in either patients or controls during or after the studies. The 24-hour urinary Na^+ excretion was similar in both groups (Table 2).

Baseline Hemodynamics. As shown in Table 2, both cirrhotic patients and healthy controls had similar baseline systemic hemodynamics including HR, MAP, stroke index, cardiac index, and total peripheral vascular resistive index. There was no significant difference in baseline FBF (mL × 100 mL tissue^{-1} × min^{-1}) in the infused arm between patients with cirrhosis and control subjects (2.8 ± 0.3 and 2.5 ± 0.3, respectively; Table 2). Throughout all protocols, there were no significant changes in MAP, HR, or FBF in the noninfused arm.

TABLE 2. Basal Forearm and Systemic Hemodynamics and Plasma ET-1 and Big ET-1 Concentrations in Both Groups

Variable	Cirrhotic Patients	Controls
Heart rate (beat/min)	72.3 ± 2.7	65.8 ± 2.8
MAP (mm Hg)	93.9 ± 3.2	95.2 ± 4.2
Cardiac index (L × min^{-1} × m^{-2})	3.7 ± 0.2	3.7 ± 0.4
Stroke index (mL × m^{-2})	54.3 ± 4.8	56.8 ± 4.7
TPVRI (mm Hg × L^{-1} × min × m^2)	25.8 ± 1.2	28.3 ± 4.3
FBF infused arm (mL × 100 mL tissue^{-1} × min^{-1})	2.8 ± 0.3	2.5 ± 0.3
FBF non-infused arm (mL × 100 mL tissue^{-1} × min^{-1})	2.9 ± 0.3	2.3 ± 0.3
Plasma ET-1 (fmol/mL)	1.2 ± 0.1	1.2 ± 0.2
Plasma big ET-1 (fmol/mL)	7.6 ± 0.8	6.8 ± 1.8

Results are expressed as mean ± SEM.

Abbreviation: TPVRI, total peripheral vascular resistive index.

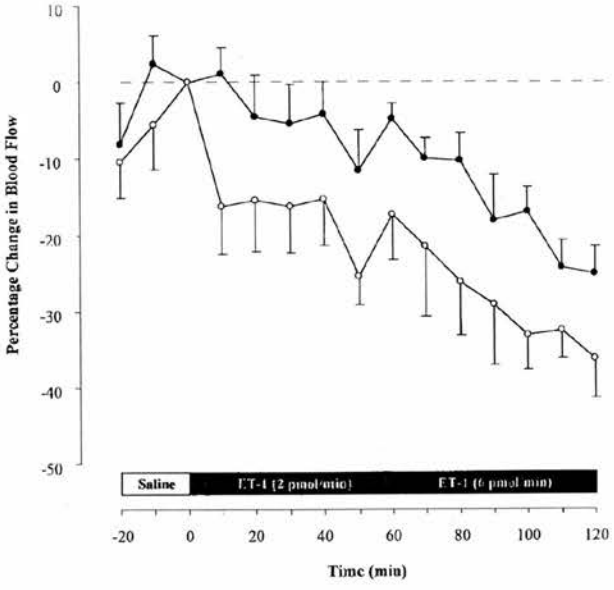

FIG. 2. Mean (SEM) percentage change in FBF in response to ET-1 infusions in cirrhotic patients (●) and healthy controls (○). $P < .001$ dose response and cirrhotics vs. controls by 2-way ANOVA with repeated measures.

FBF Responses to ET-1 Infusions. ET-1 infusion caused a significant dose-dependent reduction in FBF both in cirrhotic patients and in controls ($P < .001$, ANOVA for each group). However, the vasoconstriction was significantly less in the patients than in the controls ($P < .001$, 2-way ANOVA with repeated measures; Fig. 2). The maximum reduction in FBF after infusion of the first dose of ET-1 (2 pmol/mL) was 11.5 ± 5.3% and 25.4 ± 3.6% in the cirrhotic patients and controls, respectively, and reached 25.0 ± 3.6% and 36.3 ± 5.2%, respectively, after infusion of the second dose (6 pmol/mL; Fig. 2).

FBF Responses to BQ-123 Infusions. ET_A receptor antagonism, using BQ-123 infusion, produced a dose-dependent increase in FBF in both cirrhotic patients and controls ($P < .001$, ANOVA for each group). This vasodilator response was significantly greater in the patients than the controls ($P < .001$, 2-way ANOVA with repeated measures). The maximum increase in FBF after infusion of the first dose of BQ-123 (3 nmol/min) was 30.0 ± 5.2% and 10.0 ± 2.2% in cirrhotic patients and controls, respectively, and reached 47.7 ± 6.1% and 37.1 ± 12.5%, respectively, after infusion of the second dose (10 nmol/min; Fig. 3).

FBF Responses to BQ-788 Infusions. ET_B receptor antagonism, using BQ-788 infusion, produced a significant dose-dependent reduction in FBF in both the cirrhotic patients and controls ($P < .001$, ANOVA for each group). This vasoconstrictor response was similar in the 2 groups ($P = .62$). The maximum reduction in FBF after infusion of the first dose of BQ-788 (0.3 nmol/mL) was 11.4 ± 5.8% and 14.2 ± 6.2% in cirrhotic patients and controls, respectively, and reached 22.2 ± 7.5% and 26.4 ± 9.2%, respectively, after infusion of the second dose (1 nmol/mL; Fig. 4).

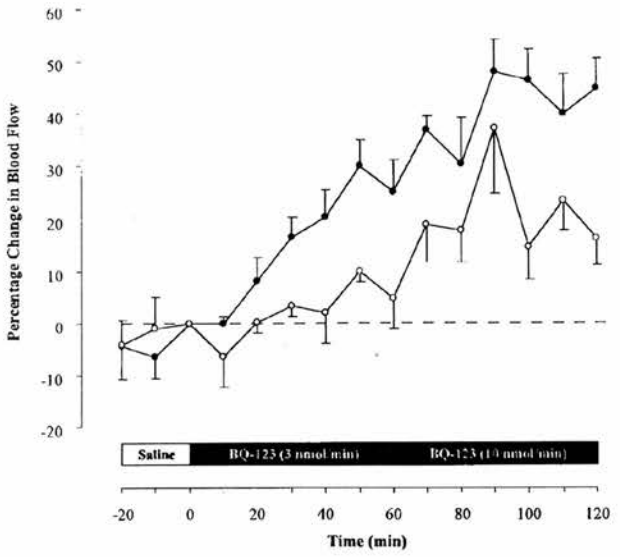

FIG. 3. Mean (SEM) percentage change in FBF in response to BQ-123 (ET_A receptor antagonist) infusions in cirrhotic patients (●) and healthy controls (○). $P < .001$ dose response and cirrhotics vs. controls by 2-way ANOVA with repeated measures.

Blood Assays. Basal plasma concentrations of ET-1 and big ET-1 were similar in cirrhotic patients and controls (Table 2) and correlated positively with each other ($r = 0.82$; $P < .001$).

DISCUSSION

In patients with well-compensated cirrhosis, portal hypertension, and normal plasma ET-1 concentrations, we have shown peripheral vascular hyporesponsiveness to exogenous ET-1 before the development of any systemic hemodynamic abnormality. Moreover, these patients have an augmented response to ET_A receptor antagonism, while retaining a normal response to ET_B receptor antagonism. These findings are consistent with an activated endothelin system in cirrhosis, a greater contribution of ET-1 to basal forearm vascular tone acting through the ET_A receptor, and a potential counterregulatory role for ET-1 in the pathogenesis of the circulatory disturbances of cirrhosis.

Patients with advanced cirrhosis and fluid retention[17-21,23-26] have elevated plasma ET-1 concentrations and are particularly high in those with hepatorenal syndrome.[17] Moreover, plasma ET-1 concentrations correlate positively with the severity of liver disease (measured by Child-Pugh score),[20,25,44] the hepatic blood flow (measured by D-sorbitol infusion), and the hepatic venous pressure gradient,[21] and inversely with the liver cell mass (measured by galactose elimination capacity)[25] and creatinine clearance.[16,18,19] However, because of its paracrine mode of action, the majority of ET-1 is released abluminally to act on adjacent vascular smooth muscle and endothelial cells, and as a consequence, plasma ET-1 concentrations may not truly reflect the underlying activity of the endothelin system.[45] In the present study, we have confirmed the previous findings of normal plasma ET-1 and big ET-1 concentrations in patients with well-compensated cirrhosis[18,46,47] but have additionally shown evidence of functional abnormalities in the tonic activity of the endothelin system. This suggests that perturbation of the endothelin system may precede the onset of detectable changes in plasma ET-1 concentrations.

We report an impaired *in vivo* vasoconstrictor response to exogenous ET-1 in patients with well-compensated cirrhosis in the absence of a measurable abnormality in baseline FBF, cardiac function, or systemic hemodynamics. This reduced responsiveness to ET-1 may, in part, reflect a greater basal ET-1 activity as suggested by the enhanced vasodilator response to BQ-123 infusion. Using the same forearm model we have also shown impaired vasoconstrictor response to angiotensin II in patients with early[35] and advanced cirrhosis.[26,35] This observation is in keeping with the impaired pressor responses to both ET-1[48] and angiotensin II[49] in the rat model of secondary biliary cirrhosis and portal hypertension. The mechanisms underlying the reduced responsiveness to ET-1 remain unclear, but NO, and possibly other mediators, may be involved.[49-51] This is supported by the observations of increased NO production, and nitric oxide synthase activity, in portal hypertension[50] and by the reversal of the ET-1[48] and angiotensin II[49] hyporesponsiveness observed in the rat model of cirrhosis after NO synthase inhibition.

This study has also shown, for the first time, that patients with well-compensated cirrhosis exhibit an enhanced forearm vasodilatation response to infusion of the ET_A receptor antagonist, BQ-123. This observation is consistent with a compensated vasodilated state, an activated endothelin system, and a greater contribution of ET-1 to the maintenance of basal forearm vascular tone acting through the ET_A receptor in these patients. Interestingly, this forearm vasodilator response to BQ-123 infusion has been shown to be largely reversed by inhibiting endogenous NO production, but not by cyclo-oxygenase inhibition, in healthy subjects.[32] Whether the enhanced forearm vasodilatation observed in the present study in patients with well-compensated cirrhosis is mediated through a higher endogenous NO production is currently unknown.

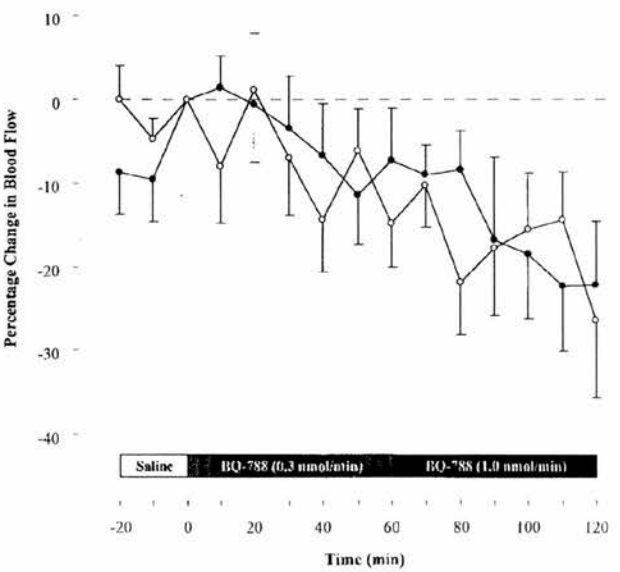

FIG. 4. Mean (SEM) percentage change in FBF in response to BQ-788 (ET_B receptor antagonist) infusions in cirrhotic patients (●) and healthy controls (○). $P < .001$ dose response and $P = .62$ cirrhotic patients vs. controls by 2-way ANOVA with repeated measures.

In the present study, we have shown that patients with well-compensated cirrhosis and healthy controls have similar forearm vasoconstrictor responses to the infusion of the ET_B receptor antagonist BQ-788. The interpretation of this observation is complex for several reasons. First, the relative predominance of ET_B receptor-mediated vasoconstriction or vasodilatation[12-16] will depend largely on the proportionate expression of these receptors in the vascular smooth muscle and endothelial cells. Selective cell-specific blockade of the ET_B receptors would be valuable to dissect out these relative contributions but the techniques to address this issue do not currently exist.[52] Second, since the ET_B receptor appears to function as a clearance receptor,[52,53] the responses to BQ-788 are likely, at least in part, to represent the effect of ET-1 displacement on the ET_A receptor. Thus, the vasoconstriction after ET_B receptor antagonism, by BQ-788, is the sum of the unopposed action of endogenous ET-1 on the ET_A receptors, and the absence of its action on the ET_B receptors. Third, it is unlikely that stimulation of ET_B receptors is the sole mechanism for the increased NO production in cirrhosis, since one would have anticipated greater vasoconstriction in such patients following selective ET_B receptor antagonism. Further studies are now needed to address the relationship between NO production and ET_B receptor stimulation, in patients with cirrhosis.

The findings of the present study apply directly to the forearm circulation, but add to our understanding of the role of ET-1 in regulation of peripheral vascular tone in cirrhosis and are probably representative of the systemic resistance vascular beds.[34] As yet, there are no reports of systemic studies assessing the actions of either exogenous or endogenous ET-1 in the systemic and splanchnic circulations in patients with cirrhosis, apart from a preliminary report of infusing the ET_A receptor antagonist, BQ-123, in 3 patients with advanced cirrhosis and hepatorenal syndrome. Interestingly, this study showed an improvement in renal blood flow and creatinine clearance.[54] However, on the basis of our study and the peripheral vasodilatation hypothesis,[7] if selective ET_A receptor antagonists were to exert their major effect on the splanchnic circulation, then they might further exacerbate portal hypertension and systemic hypotension. Therefore, we believe that systemic studies are clearly needed to assess the clinical role of ET-1 antagonists in patients with cirrhosis and portal hypertension.

One of the potential limitations of the present study is that 4 of the 11 patients had alcohol-induced liver disease. Indeed, excessive and regular alcohol intake may alter the vascular responses to exogenous vasopressor agents such as noradrenaline.[28] In each phase of the present study, only 2 or 3 of 8 patients had alcohol-induced cirrhosis. All subjects were abstinent from alcohol for a minimum of 1 month as determined by both clinical history and repeated random blood ethanol testing. In our previous studies[26,35] incorporating similar patients, we have shown normal vascular responses to noradrenaline infusion. In addition, we have reevaluated the present results following exclusion of patients with alcohol-induced cirrhosis and have found consistent results without altering the statistical significance or the magnitude of the effects of the study findings.

In conclusion, the forearm circulation in patients with well-compensated cirrhosis is hyporesponsive to exogenous ET-1, despite the absence of any significant differences in basal systemic hemodynamic characteristics and normal circulating ET-1 concentrations in these patients. The enhanced vasodilatory response to BQ-123 in cirrhotic patients suggests a compensated vasodilated state, an activated endothelin system, and a greater contribution of ET-1 to the maintenance of forearm basal vascular tone acting through the ET_A receptor. The mechanism underlying this hyporesponsiveness to ET-1 warrants further investigation.

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