# THE IMPACT OF HEPATIC CIRRHOSIS ON THE CONTRACTILE FUNCTION OF HUMAN HEPATIC ARTERIES

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## *DEDICATION*

*I would like to dedicate this thesis to my daughter, 'Zaima' whom I missed all the way whilst working on this thesis, when she had to spend her infancy thousands mile away; and to my wife for her extraordinary patience and sacrifice as a mother, in our daughter's absence and the valuable support she offered during this pain staking period; my mother and family members, who looked after my beloved child as well as giving support and encouragement to me, and in the memory of my late father who devoted his whole life for the well-being of his children. And finally, I like to dedicate all these efforts to the care of patients with liver disease.*

# ABSTRACT

**Background and Objectives:** The impaired pressor response in patients with cirrhosis of the liver may have implications for the pathogenesis and treatment of the hyperdynamic circulation associated with this condition. Studies in patients and animal models have not elucidated the cause(s) of this abnormality in the vasculature. A small number of studies have demonstrated impaired adrenoceptor-mediated contraction in hepatic arteries isolated from patients with cirrhosis. However, although use of animal models has suggested that responses to other vasoconstrictor agonists may also be altered in cirrhosis, this has not been addressed using the human hepatic artery. The aims of this thesis were (i) to assess the effect of cirrhosis on the responses of human hepatic artery to three vasoconstrictor agonists, arginine vasopressin (AVP), 5-hydroxytryptamine (5-HT) and endothelin-1 (ET-1), which may contribute to the development of the hyperdynamic circulation and (ii) to clarify the mechanism(s) of any abnormality detected.

**Methods:** Vascular responses were investigated *in vitro* using organ bath methodology. Method development was performed using porcine splanchnic arteries. The suitability of using hepatic arteries from liver donors as controls was also assessed by exposing porcine hepatic arteries to preservative solutions. Hepatic arteries from liver recipients (cirrhotic), and age and sex matched liver donors (non-cirrhotic), were studied. The presence of the endothelium was assessed functionally, histologically and immunohistochemically. Cumulative concentration-response curves to AVP, 5-HT, ET-1 and KCl were constructed in denuded hepatic arteries. The receptors responsible for agonist-mediated contraction were identified by performing concentration-response curves following exposure of the arteries to appropriate antagonists. Furthermore, the influence of nitric oxide synthase (NOS) activity was assessed using immunohistochemistry and functionally using an appropriate inhibitor.

**Results:** The endothelium was shown to be damaged considerably in isolated human and porcine hepatic arteries; hence, all subsequent studies used denuded vessels. The protocol developed using porcine hepatic arteries demonstrated that preservative solutions had no effect on contractile function but did alter endothelium-independent relaxation. Responses to vasoconstrictors were altered in arteries from patients with hepatic cirrhosis although the nature of this alteration was agonist-dependent: the maximum contraction to AVP was impaired whilst that to 5-HT was augmented. In contrast, the sensitivity of response to ET-1 was increased, whereas KCl-mediated contraction was unchanged. The contractile responses to AVP, 5-HT and KCl were unaffected by NOS inhibition. There was no immunoreactivity for iNOS in donor hepatic arteries nor in those from most patients: low levels of iNOS were only detected in arteries from patients with alcoholic liver disease. Use of antagonists demonstrated that AVP and ET-1 contracted human hepatic arteries via the $V_1$ and $ET_A$ receptors, respectively, whilst 5-HT acted predominantly via 5-$HT_1$-like receptors with a small contribution from the 5-$HT_{2A}$ subtype.

**Conclusions:** These studies demonstrated that cirrhosis of the liver is associated with an agonist-selective alteration of receptor-mediated contraction in denuded human hepatic arteries. Inducible nitric oxide synthase activity did not contribute to these alterations and unaltered responses to KCl suggest that these abnormalities are not due to structural changes in the vessel wall. These results indicate, therefore, that altered contractile functions in hepatic arteries from patients with cirrhosis are due to changes in receptor activity and/or post-receptor signal transduction pathways.

# DECLARATION

I hereby declare that the work presented in this thesis is my own and has not been submitted previously for any degree. This work was undertaken in the Liver Research Unit, Department of Medicine, and in collaboration with the Scottish Liver Transplant Unit, at Royal Infirmary, in the University of Edinburgh.

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# ABBREVIATIONS

| | |
|---|---|
| ACh | Acetylcholine |
| α-SMA | Alpha-smooth muscle actin |
| AH | Autoimmune hepatitis |
| AII | Angiotensin II |
| ALD | Alcoholic liver disease |
| ANOVA | Analysis of variance, |
| AVP | 8-Arginine vasopressin |
| BSA | Bovine serum albumin |
| $Ca^{2+}$ | Calcium |
| $CaCl_2$ | Calcium chloride |
| $[Ca^{2+}]_i$ | Intracellular calcium |
| CAH | Chronic active hepatitis |
| cAMP | 3' 5'-Cyclic adenosine monophosphate |
| CC | Cryptogenic cirrhosis |
| $CCl_4$ | Carbontetrachloride |
| CCRC | Cumulative concentration-response curve |
| $CO_2$ | Carbon-dioxide |
| 5-CT | 5-Carboxamidotryptamine |
| DAB | 3, 3'-Diaminobenzidine tetrahydrochloride |
| DAG | Diacylglycerol |
| DDAVP | Desmopressin |
| D-glucose | Dextrose glucose |

| | |
|---|---|
| DHA | Donor Hepatic Arteries |
| DIP | Dexamethasone, insulin and penicillin |
| D-NNA | $N^G$-nitro-D-arginine |
| E | Endothelium |
| EDCF | Endothelium-derived contracting factor |
| EDRF | Endothelium-derived relaxaing factor |
| Emax | Maximum contraction |
| eNOS | Endothelial cell derived nitric oxide synthase |
| ET-1 | Endothelin-1 |
| $ET_A$ | $Endothelin_A$ receptor |
| $ET_B$ | $Endothelin_B$ receptor |
| HCl | Hydrochloric acid |
| 5-HT | 5-Hydroxytryptamine |
| iNOS | Inducible nitric oxide synthase |
| $IP_3$ | Inositol 1,4,5-tris-phosphates |
| Ket | Ketanserin |
| $KH_2PO_4$ | Potassium dihydrogen phosphate |
| KHS | Krebs'-Henseleit solution, |
| L | Lumen |
| L-NNA | $N^G$-nitro-L-arginine |
| Meth | Methiothepin |
| $MgSO_4$ | Magnesium sulphate |
| mRNA | Messenger ribonucleic acid |
| μm | Micrometre |

| | |
|---|---|
| (n) | Number of subjects |
| $Na^+/K^+$ ATPase | Sodium-potassium adenosine triphosphatase |
| NaCl | Sodium chloride |
| $NaHCO_3$ | Sodium monohydrogen carbonate |
| NC | Non-contracted |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| ODQ | Oxadiazolol quinoxalin |
| PBC | Primary biliary cirrhosis |
| PBS | Phosphate buffer saline |
| PC | Partially pre-contracted |
| PE | Phenylephrine |
| PG | Prostaglandin |
| PKC | Protein kinase C |
| PLC | Phospholipase C |
| PRA | Pulmonary resistance arteries |
| RHA | Recipient hepatic arteries |
| SEM | Standard error of mean |
| SIN-1 | 3'-Morpholinosydnonimine |
| SLTU | Scottish liver transplant unit |
| SNP | Sodium nitroprusside |
| TA | Tunica adventitia |
| TBS | Tris-buffered saline |
| TI | Tunica intima |

| | |
|---|---|
| TM | Tunica media |
| UKTSSA | United Kingdom transplant support service Authority |
| UWS | University of Wisconsin solution |
| VSMCs | Vascular smooth muscle cells |

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# CHAPTER ONE

# GENERAL INTRODUCTION

## 1.1. Introduction

Cirrhosis of the liver is an important chronic health problem due to its profound impact on morbidity and mortality (Liach *et al.*, 1988). The outcome of this disease predominantly depends upon associated vascular complications: portal hypertension, ascites, variceal bleeding, hepatic encephalopathy and hepatorenal and hepatopulmonary syndromes (Sikuler *et al.*, 1985; Ryan *et al.*, 1995; Agusti *et al.*, 1990). Irrespective of the aetiology of cirrhosis, patients develop a characteristic cardiovascular dysfunction 'the hyperdynamic circulation' which leads directly to the development of these life-threatening complications. One component of the hyperdynamic circulation, consistently demonstrated over the last 50 years, is an impaired response to exogenous pressor agents. This suggests that altered contractile function in the vasculature may contribute to the hyperdynamic circulation. A complete understanding of the mechanism(s) responsible for this altered pressor response is crucial to understanding the pathogenesis of the hyperdynamic circulation and to the development of potential therapeutic and preventive interventions. The cause of this impaired pressor response remains unclear despite extensive research, using both *in vivo* and *in vitro* methodology, in patients and relevant animal models. This thesis describes studies performed, using isolated human hepatic arteries, in an attempt to both demonstrate the impact of hepatic cirrhosis on vascular function and to clarify the underlying mechanism(s) responsible for functional abnormalities.

## 1.2. Cirrhosis of The Liver

### *1.2.1. Liver Function and Structure*

The liver receives nutrient and toxin rich blood from the gastrointestinal tract. It performs a wide range of important processes that can be classified under five distinct headings: (i) excretory (producing e.g. bilirubin and urea), (ii) synthetic (producing specialised proteins such as albumin and the clotting factors), (iii) detoxification (of drugs and toxins), (iv) assisting digestion (supplying bile salts and bicarbonate) and (v) maintaining blood levels of amino acids and glucose.

In order to perform these functions, the liver receives oxygenated blood via the hepatic artery and blood from the gastrointestinal tract via the portal vein. It is drained via three main hepatic veins. This blood supply is used as a basis for dividing the liver into eight segments, in accordance with the subdivision of hepatic and portal veins. Each segment is made up of many smaller units (lobules), which are themselves formed of collections of the functional unit of the liver (the acinus; Figure 1.1). Blood enters the acinus from terminal branches of the portal vein and hepatic artery situated in the portal tract. It flows into the sinusoid (the capillary of the liver) and finally drains into the central vein. Bile flows in the opposite direction, from the hepatocytes, along bile canaliculi, and into the interlobular bile ducts located in the portal tract. The sinusoids are highly permeable, being lined with fenestrated endothelial cells and lacking basement membrane. This allows mixed blood (from portal vein and hepatic artery), at very low pressure, to gain access to the hepatocytes. Hepatocyte function varies depending upon position relative to the portal tract and this structure allows the liver to perform its necessary functional

objectives. The luminal surface of the sinusoidal endothelium is lined with Kupffer cells (which are involved in phagocytosis) (Wisse *et al.*, 1996) whilst hepatic stellate cells in the space of Disse (Figure 1.1) are smooth muscle type cells with contractile functional characteristics (Tanikawa, 1995). Several inflammatory cytokines and growth factors can activate these stellate cells and induce the synthesis of fibrous tissue (Friedman, 1990; Ramadori, 1991).

### *1.2.2. Blood Supply to the Liver*

Considering its size, the liver receives a disproportionate component (~25%) of the total cardiac output. Blood is supplied via the hepatic artery and portal vein which provide roughly 25% and 75% of the total hepatic blood flow (but 70% and 30% of the required oxygen), respectively (Greenway & Stark, 1971). Within the liver, these vessels divide to supply individual segments, continue into the sinusoids and terminate in the central vein. The central vein drains into the inferior vena cava via the hepatic veins. Intrahepatic pressure falls along the hepatic arterioles and portal veins, and reaches its lowest level in the hepatic veins. The hepatic venous system is valveless and is sensitive to elevations of central venous pressure (Lautt *et al.*, 1986a). Any such increase is transmitted directly to the sinusoids and causes an increased filtration of fluid and, thus, increased lymphatic drainage. This may lead to production of ascites. The main site of intrahepatic resistance has proved controversial with some investigations suggesting that blood pressure reduction occurs mainly in the sinusoids (Mitzner, 1974) (where it is controlled by endothelial Kupffer and stellate cells which can contract and swell to reduce patency of the sinusoid). More recently, however, it has been demonstrated that intrahepatic

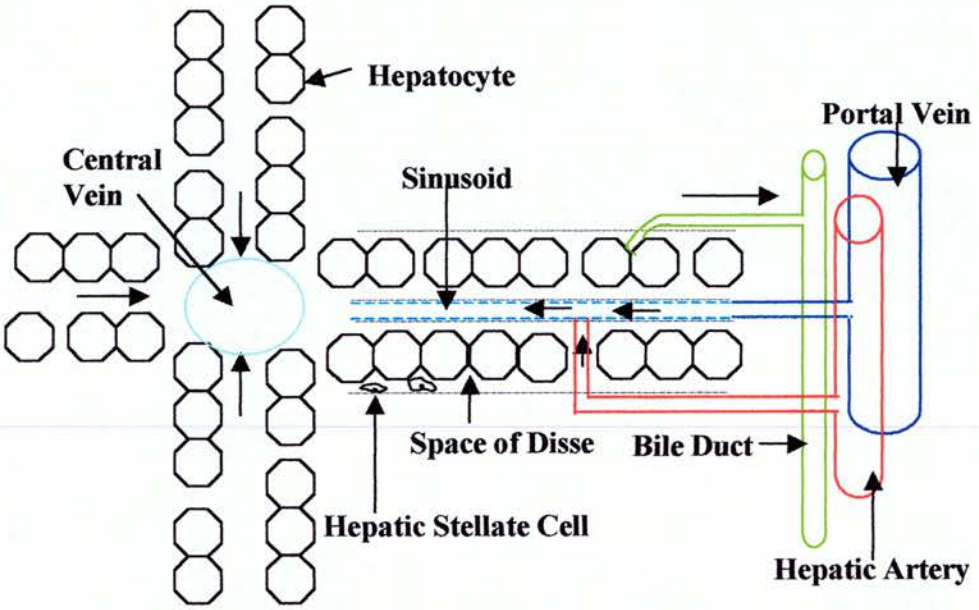



**Figure 1.1**. The Hepatic Acinus. This comprises the functional unit of the liver, in which blood from the hepatic artery and portal venous system merge and come into contact with hepatocytes before draining into the central hepatic vein. Hepatic Stellate cells located in the space of Disse, the space between sinusoids and hepatocytes.

resistance in this circuit originates in the post-sinusoidal network (Lautt *et al.*, 1986b).

Regulation of blood flow to the liver is complex with contributions from both intrinsic and extrinsic mechanisms (Lautt 1985; Lautt & Legare, 1987). Extrinsic mechanisms include sympathetic nervous activity and the action of humoural factors. An unusual component of nervous activity is "autoregulatory escape" in which arterioles (but not venules) fail to maintain contraction in the presence of continued nerve stimulation (Greenway, 1984). Humoral factors (including substances absorbed from the gut and released by splanchnic organs) which are supplied by both the hepatic artery and portal vein, gain access to the hepatic arterioles and can, thus, influence hepatic resistance. Intrinsic regulatory processes (which, by definition, are exclusive of nervous and blood-borne vasoactive factors and are also not dependent on metabolism) include the "hepatic artery buffer response" and "autoregulation". The hepatic artery buffer response regulates the intrahepatic resistance by maintaining a constant total hepatic blood flow (Lautt *et al.*, 1985); alterations in portal venous flow are balanced by an inverse response in the hepatic artery (although portal flow does not alter in response to changes in hepatic artery flow). This is thought to be mediated by washout of locally produced adenosine (although other possibilities include hepatic nerves, myogenic response, and systemic vasoactive factors) (Lautt 1985; Ezzat & Lautt, 1987). Autoregulation describes hepatic artery constriction in response to an increase in arterial perfusion pressure and is probably a myogenic response (although washout of adenosine could also explain this phenomenon) (Greenway, 1984; Hanson, 1973).

### *1.2.3. Development of Hepatic Cirrhosis*

The liver is susceptible to both acute (paracetamol poisoning; inherited syndromes; hepatitis (alcoholic, pyogenic, viral)) and chronic insults. Chronic hepatitis has several aetiologies and leads to the development of cirrhosis. Viral infection is the most common cause of cirrhosis worldwide, whereas excess alcohol intake over a long period is the most usual cause in the Western world. Other causes, however, include autoimmune disease, cholestatic disease (primary and secondary biliary cirrhosis, primary sclerosing cholangitis), hereditary conditions (haemochromatosis, Wilson's disease, $\alpha_1$-antitrypsin deficiency) metabolic disorders (glycogen storage disease, galactosaemia), drug toxicity (e.g. methotrexate) and some idiopathic (cryptogenic) conditions.

Hepatic cirrhosis is the end stage of chronic inflammatory processes. Irrespective of aetiology, persistent injury to the liver initiates a characteristic cascade of events; inflammatory reactions lead to the death of hepatocytes, stimulation of Kupffer cells and activation of stellate cells (Friedman *et al.*, 1993). The latter results in excess fibrous tissue formation, which blocks the fenestrations in the sinusoids and restricts blood supply to the hepatocytes. Consequently, these changes cause further impairment in the function of hepatocytes and considerably augment sinusoidal resistance and intrahepatic pressure (Rockey *et al.*, 1993).

### *1.2.4. Cardiovascular Complications of Cirrhosis*

The development of hepatic cirrhosis is accompanied by characteristic changes in cardiovascular function, which together are described as the "hyperdynamic"

circulation (Murray *et al.*, 1958; Kontos *et al.*, 1964). This consists of an increase in cardiac output and heart rate, accompanied by reduced mean arterial pressure and systemic vascular resistance. An important consequence of these changes is the development of portal hypertension, as this leads to the major life-threatening complications of cirrhosis (variceal haemorrhage, ascites, encephalopathy). Portal hypertension occurs as a consequence of increased blood flow into the portal system (Bosch *et al.*, 1988; Grose & Hayes, 1992) combined with obstruction of outflow through the liver (resulting from destruction of the hepatic architecture). It has been estimated that increased portal venous inflow contributes ~40%, and intrahepatic resistance ~60%, to portal hypertension (Benoit *et al.*, 1985).

*1.2.5. The Hyperdynamic Circulation*

The significance of the hyperdynamic circulation of cirrhosis is indicated by its role in the development of life-threatening complications (Liach *et al.*, 1988). It also has prognostic significance as the severity of the hyperdynamic circulation reflects hepatic dysfunction and disease progression (Gluud *et al.*, 1988). An understanding of the mechanism(s) that contribute to development of the hyperdynamic circulation is, therefore, essential in the development of therapeutic approaches to management of cirrhosis. Despite extensive investigation, however, the pathogenesis of these circulatory abnormalities remains controversial.

The increased cardiac output and systemic vasodilatation in the hyperdynamic circulation are evident despite activation of endogenous pressor systems (the sympathetic nervous systems (SNS) (Henriksen *et al.*, 1998; Arroyo *et al.*, 1983;

Bichet *et al.*, 1982a), renin-angiotensin-aldosterone (RAAS) (Schroeder *et al.*, 1976; Iwao *et al.*, 1994)), endothelin system (Gerbes *et al.*, 1995; Kitano *et al.*, 1996) and synthesis of vasopressin (AVP) (antidiuretic hormone, ADH) (Bichet *et al.*, 1982b; Badalamenti *et al.*, 1993). Indeed, increased pressor activity makes a significant contribution to maintenance of blood pressure as pharmacological inhibitors cause severe hypotension in these patients (Claria *et al.*, 1991; Esler *et al.*, 1992; Moreau & Lebrec, 1995)). Furthermore, plasma concentrations of endogenous vasoconstrictors are predictive of severity of cirrhosis and poor prognosis (Gines *et al.*, 1993; Arroyo *et al.*, 1988). The currently accepted explanation for the pathogenesis of the hyperdynamic circulation is the 'peripheral arterial vasodilatation hypothesis' (PAVH) (Schrier *et al.*, 1988). The PAVH integrates the major aspects of two previous models: the 'underfill' (Atkinson & Losowsky, 1961; Epstein, 1979) and 'overflow' (Lieberman *et al.*, 1970) hypotheses. The underfill hypothesis proposed that increased portal pressure leads to loss of fluid from the vascular compartment, thus activating pressor systems and renal sodium and water retention. In contrast, the overflow hypothesis suggested that hepatic dysfunction and/ or portal hypertension provided a stimulus for renal sodium and water retention with a reactive vasodilatation occurring to accommodate this extra volume. The PAVH unified components from these models with the proposal that peripheral arterial vasodilatation is the initial event in development of the hyperdynamic circulation. It is suggested that this dilatation occurs in response to changes in the liver, although the underlying mechanism for this response has not been identified. Peripheral vasodilatation leads to a reduction in peripheral vascular resistance (PVR) and central blood volume (effective hypovolemia). Consequently, compensatory

activation of endogenous vasopressor systems occurs in an attempt to restore normal PVR. In this way, the over active vasopressor and water retention systems attempt to restore blood volume and pressure (Schrier & Caramelo, 1988). This is effective in patients with mild-moderate cirrhosis but in advanced disease the pressor response and increase in blood volume are not sufficient to normalise the PVR, the pressor systems remain activated and renal fluid retention continues. As a consequence, portal hypertension becomes exacerbated and serious life-threatening complications (ascites, variceal bleeding hepatic encephalopathy and hepatorenal and hepatopulmonary syndromes) develop.

Despite its general acceptance, there are several problems with the PAVH. In particular, neither the mechanism of arterial vasodilatation nor the primary site of dilatation has been identified unequivocally. Several studies (in animals and humans) have demonstrated that the splanchnic circulation is the main site of initial vasodilatation (Vorobioff *et al.*, 1984; Maroto *et al.*, 1993; Iwao *et al.*, 1997a) but the exact mechanism has yet to be established. Furthermore, it has been suggested recently, that an increase in cardiac output, causing a reactive peripheral vasodilatation, is the initiating abnormality in the development of the hyperdynamic circulation (Bernardi & Trevisani, 1997). This is attributed to increased cardiac pre-load as cardiovascular abnormalities were shown to be evident in patients with mild cirrhosis only when seated (Lewis *et al.*, 1992; Bernardi & Trevisani, 1997; Wong *et al.* 1997; Iwao *et al.*, 1997b). This suggests that the PAVH does not fully explain the development of the hyperdynamic circulation and the exact cause(s) of this condition have yet to be understood.

*1.2.6. Impaired Pressor Response*

An unusual aspect of the hyperdynamic circulation of liver disease was identified in studies of secondary aldosteronism in the 1950s (Laragh, 1962; Ames *et al.*, 1965; Mashford *et al.*, 1962). These demonstrated an impaired pressor response to exogenous vasoconstrictors in patients with cirrhosis. More recent investigations, performed in the whole body (MacGilchrist *et al.*, 1991b) and also in isolated vascular territories such as in dorsal hand vein (DHV) (Wong *et al.*, 1995), fore arm blood flow (FBF) (Ryan *et al.*, 1993) or in conjunctival vessels (Morandini & Spanedda, 1966) have confirmed and supplemented these findings. In general, it has been shown that patients with advanced cirrhosis have an impaired pressor response to vasoconstrictors but several controversies remain. Angiotensin II (AII) and adrenoceptor-agonists (noradrenaline (NA), phenylephrine) are the agonists most commonly used but it has yet to be established whether impaired contraction is agonist selective or occurs with both AII and NA. Furthermore, the causes of the impaired pressor response have not been confirmed.

The impaired pressor response may be a significant component in the pathogenesis of the hyperdynamic circulation. Reduced contractile responsiveness in the vascular wall may contribute to the maintenance of systemic vasodilatation despite activation of endogenous pressor systems. Furthermore, as vasoconstrictor analogues of AVP are used to treat variceal haemorrhage, an impaired pressor response may have significant implications for patient survival. A large number of investigations, *in vivo* and *in vitro*, have been performed, using patients and animal models of cirrhosis (Bomzon, 1990; Hadoke & Hayes, 1997), in an effort to determine the causes of the

impaired pressor response. Interpretation of this work requires an understanding of the structure and function of the healthy vascular wall.

## 1.3. Vascular Structure and Function

### *1.3.1. Structure and Function of the Blood Vessels*

The cardiovascular system circulates blood throughout the body via veins and arteries which are interconnected by venules and capillaries. Alterations of tone, and hence lumen diameter, of arteries has an active role in ensuring optimum blood flow to organs whereas most veins return blood to the heart and provide little resistance. The vessel wall (with the exception of capillaries) is composed of three layers: the tunica intima, the tunica media and the tunica adventitia (Figure1.2). This structure is similar in arteries and veins but the latter tend to have thinner walls and larger cross sectional diameter than equivalent arteries (Rhodin, 1980).

#### 1.3.1.1. Tunica Intima

The intima is the innermost layer and in many blood vessels consists solely of a monolayer layer of endothelial cells and a basement membrane. In large elastic arteries and some (e.g. coronary) muscular arteries the intima includes a sub-endothelial layer composed of collagenous bundles, elastic fibrils, smooth muscle and perhaps, a small number of fibroblasts (Badimon *et al.*, 1993). This sub-endothelium however is scanty in most muscular arteries and absent from arterioles (Rhodin, 1980). The endothelium has a major role in the regulation of vascular tone and, consequently, blood flow (Searle & Sahab, 1992) and also modulates inflammation, haemostasis and vascular cell growth (Henderson, 1991; Furchgott &

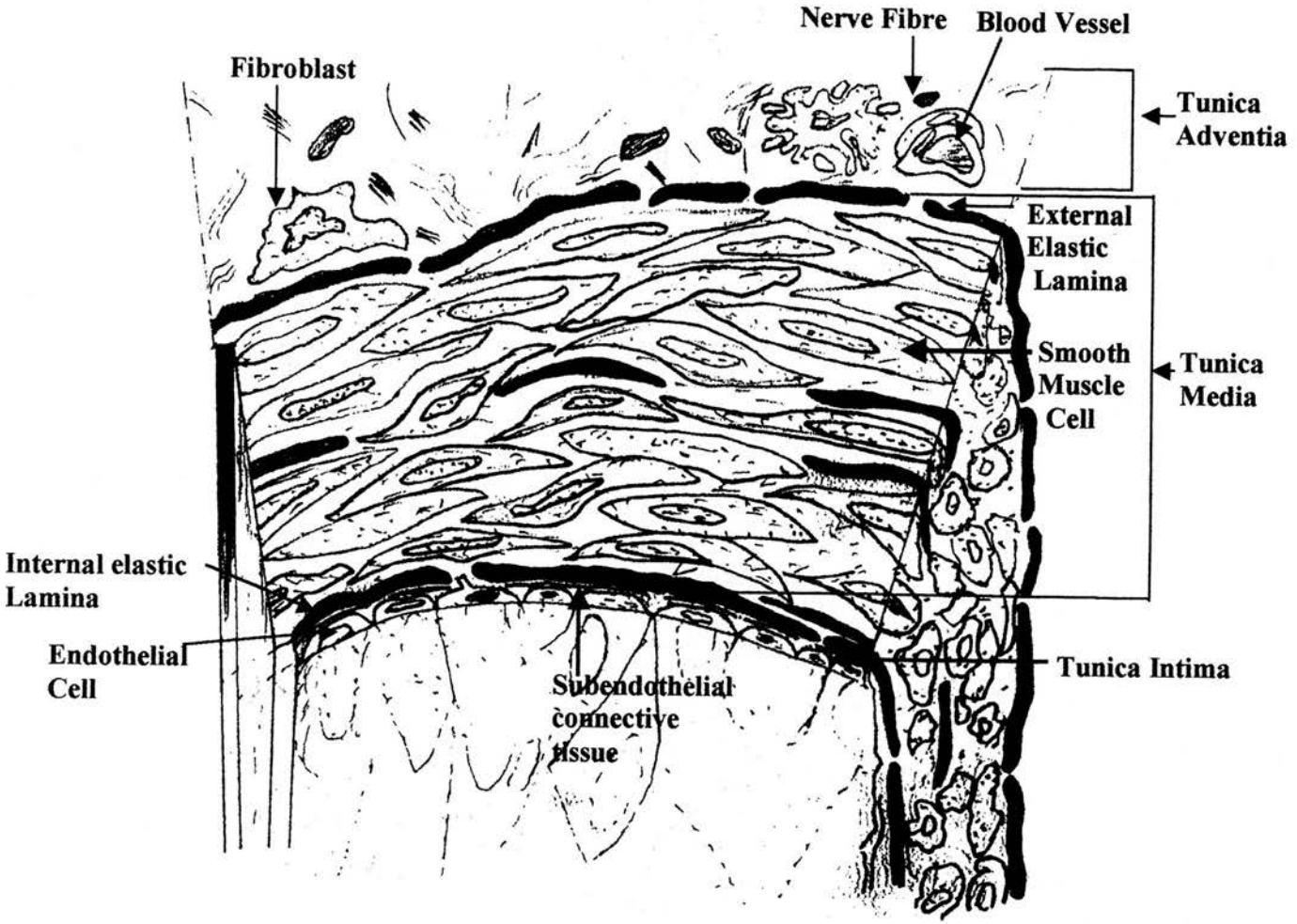



**Figure 1.2**. Cross section of a muscular artery showing the division into TI (Tunica Intima), TM (Tunica Media) and TA (Tunica Adventetia), and the major cellular components.

Vanhoutte, 1989; Marcus, 1990; Johns, 1991).

#### 1.3.1.2. Tunica Media

The middle layer, or tunica media, is composed of multiple layers of smooth muscle cells (SMCs) bounded on the luminal margin by the internal elastic lamina and on the adventitial margin by the external elastic lamina. The major components of the media are SMCs, which confer strength and enable the vessel to contract or relax in response to stimuli, and elastic laminae, which provide the major mechanical strength and resilience of the vessel wall. The relative proportions of these components vary in elastic and muscular arteries. Elastic laminae are usually thicker and well defined in large elastic (conducting) arteries, become gradually thinner in muscular arteries and may be ill defined in small arteries. In particular, the external elastic lamina gradually disappears from muscular arteries. In elastic arteries, the elastic laminae withstand most of the tension exerted by blood flow; whilst in muscular arteries this is done by SMCs (Bader, 1963). The arrangement of SMCs differs in elastic and muscular arteries but in general is arranged helically in concentric layers. The number of layers decreases towards the distal part of arterial system. The SMCs are sheathed by thin sheets of connective tissue but at certain points are electrically coupled in a syncytium (Beny & Connat, 1992). SMC tone is regulated by blood borne factors, endothelium-derived substances and transmitters released from the perivascular nerves (Moreau & Lebrec, 1995; Rembold, 1992; Searle & Sahab, 1992; Mulvany & Halpern, 1976).

#### 1.3.1.3. Tunica Adventitia

The outermost layer, the tunica adventitia, gives stability and strength, limits distension, and connects the blood vessel to its surrounding tissues. The thickness of the adventitia varies considerably depending on the type and location of a particular blood vessel. For example, it is almost totally absent from cerebral blood vessels, is thicker in large muscular arteries, than in elastic arteries and is thickest in veins (where it forms the major bulk of the vessel wall) than in arteries. It becomes gradually thinner towards the distal part of the vessels and is quite indistinct in arterioles and venules. The adventitia also carries nutrient vessels (vasa vasorum which supply the SMCs) and nerve fibres. The nerve fibres are usually unmyelinated and, in most vascular beds, the amount of innervation increases with decreasing vessel diameter (Nilsson *et al.*, 1986; Smeda *et al.*, 1988).

### *1.3.2. Regulation of Vascular Tone*

Vascular tone is determined by the contractile state of medial SMCs, which in turn, is regulated by a variety of factors: these include activity of the endothelial cells, nervous and hormonal stimuli, pressure, stretch and metabolic activity. In addition, smooth muscle in some vessels may be spontaneously active. In general, vascular smooth muscle cells contract when the intracellular calcium concentration ($[Ca^{2+}]_i$) increases. Elevation of $[Ca^{2+}]_i$ causes a calmodulin-mediated cycling of actin and myosin cross bridges which causes shortening of the cell and, thus, contraction (Adelstein & Eisenberg, 1980; Kuriyama *et al.*, 1982). Relaxation occurs when $[Ca^{2+}]_i$ falls or dilator agonists interfere with mechanisms linking $[Ca^{2+}]_i$ with the contractile machinery.

### 1.3.2.1. The Influence of the Endothelium on Vascular Tone

The endothelial cells can produce several vasodilators such as nitric oxide (NO), prostacyclin ($PGI_2$) and endothelium-derived hyperpolarising factor (EDHF), and vasoconstrictors (such as endothelin-1 (ET-1), prostanoids)) and plays a major role in coordination and control of the vascular tone (Searle & Sahab, 1992). Furthermore, the existence of certain important receptors for contractile agents (including $\alpha_2$- and β-adrenoceptors (Martinez-Cuesta *et al.*, 1996), as well as 5-$HT_{2C}$ (Cocks & Angus, 1983), $V_2$ receptors (Martinez *et al.*, 1994) and $ET_B$ (Schilling *et al.*, 1995) enables the endothelium to act as a local feedback control system. Stimulation of these endothelial receptors induces vasodilatation predominantly via release of NO as well as $PGI_2$ or EDHF. Moreover, flow-induced shear stress also releases NO from the endothelial cells and, therefore, plays an important role in modulating vascular tone. Similarly vascular tone can be modulated by the vasoconstrictors, which are released in response to anoxia, alteration of blood pressure or rapid stretching of the vessel wall (Searle & Sahab 1992). Among them ET-1 can have a significant role, as it has a potent vasoconstrictor effect. Therefore, the integrity of the endothelium is important in maintaining normal vascular tone and assessment of the endothelium is essential while investigating the vascular contractile function.

### 1.3.2.2. Regulation of Vascular Smooth Muscle Contraction

Processes that alter vascular tone can be separated into "Electromechanical" (those that involve changes in membrane potential) and "Pharmacomechanical" (those that involve receptor-mediated activation of $Ca^{2+}$ channels or generation of second messengers) coupling.

1.3.2.2.1. Electromechanical Coupling.

In the normal resting state, the resting potential of the SMCs (> -50mV) is determined primarily by a potassium ion ($K^+$) gradient and permeability to sodium ions. The $Ca^{2+}$ pump and a $Na^+$/ $Ca^{2+}$ exchanger in the cell membrane maintain $[Ca^{2+}]_i$ at a concentration much lower than the threshold level necessary for contraction. Depolarisation is dominated by $Ca^{2+}$, which enters through L-type (long acting) voltage-gated channels whereas repolarisation is mediated by efflux of $K^+$ ions through several channel types. Some types of vascular smooth muscle may also contain T-type (transient) calcium channels, which can contribute to the manifestation of spontaneous activity and maintenance of basal tone (Katz, 1996).

1.3.2.2.2. Pharmacomechanical Coupling.

Smooth muscle also contains receptor activated $Ca^{2+}$ channels, which are opened by the binding of hormones or neurotransmitters, without a change in membrane potential. In addition, activation of receptors results in generation of second messengers (such as $IP_3$), resulting in release of $Ca^{2+}$ from intracellular stores. Most vasoactive agonists stimulate pharmacomechanical coupling by activation of membrane receptors (mostly those from the 7 transmembrane domain G-protein coupled superfamily). This leads to a cascade of intracellular second messengers resulting in release of $Ca^{2+}$ from intracellular stores and influx from the extracellular environment (Walsh, 1994). Relaxation occurs in response to factors that stimulate production of cAMP or cGMP (by adenylate cyclase and guanylate cyclase, respectively), which mediate $Ca^{2+}$ efflux and reuptake by the intracellular stores (Walsh, 1994). Alternatively, relaxation can occur through activation of potassium

($K^+$) channels which hyperpolarises the cell membrane, blocking L-type calcium channels and, thus, reducing the influx of $Ca^{2+}$.

It is also worth noting that pharmacomechanical coupling can result in contraction and relaxation that are independent of changes in $[Ca^{2+}]_i$. These involve sensitisation or desensitisation of myosin phosphorylation, diacylglycerol (DAG)-mediated stimulation of protein kinase C (PKC) isozymes and direct regulation of cross-bridge cycling (Rembold, 1992; Walsh, 1994). This may be relevant to the vascular abnormalities in cirrhosis as several agonists (e.g. noradrenaline, arginine vasopressin and ET-1) can produce sustained contraction without further enhancement of $[Ca^{2+}]_i$ (Kubota *et al.*, 1992; Tansey *et al.*, 1992; Gong *et al.*, 1992). This process is called "$Ca^{2+}$ sensitisation" (Fujiwara *et al.*, 1989)

1.3.2.3. Second-Messenger Pathways.

Knowledge of the second messenger cascade has been exploited in an effort to determine whether impaired contractile response in cirrhosis is due to changes in the smooth muscle cell at a receptor or post-receptor level (Wu & Benoit, 1994; Huang *et al.*, 1997). Therefore, a clear understanding of these pathways is necessary for interpretation of these investigations. The second messenger pathways involved in pharmacomechanical contraction are illustrated in figure 1.3. Basically, receptor stimulation by an appropriate agonist causes a $G_q$-mediated cascade of phosphoinositide turnover. This results in production of inositol 1, 4, 5-trisphosphate ($IP_3$) and diacylglycerol (DAG), leading to $Ca^{2+}$ influx (Nelson *et al.*, 1990) and release release from intracellular stores (Berridge, 1993). Alternatively, some

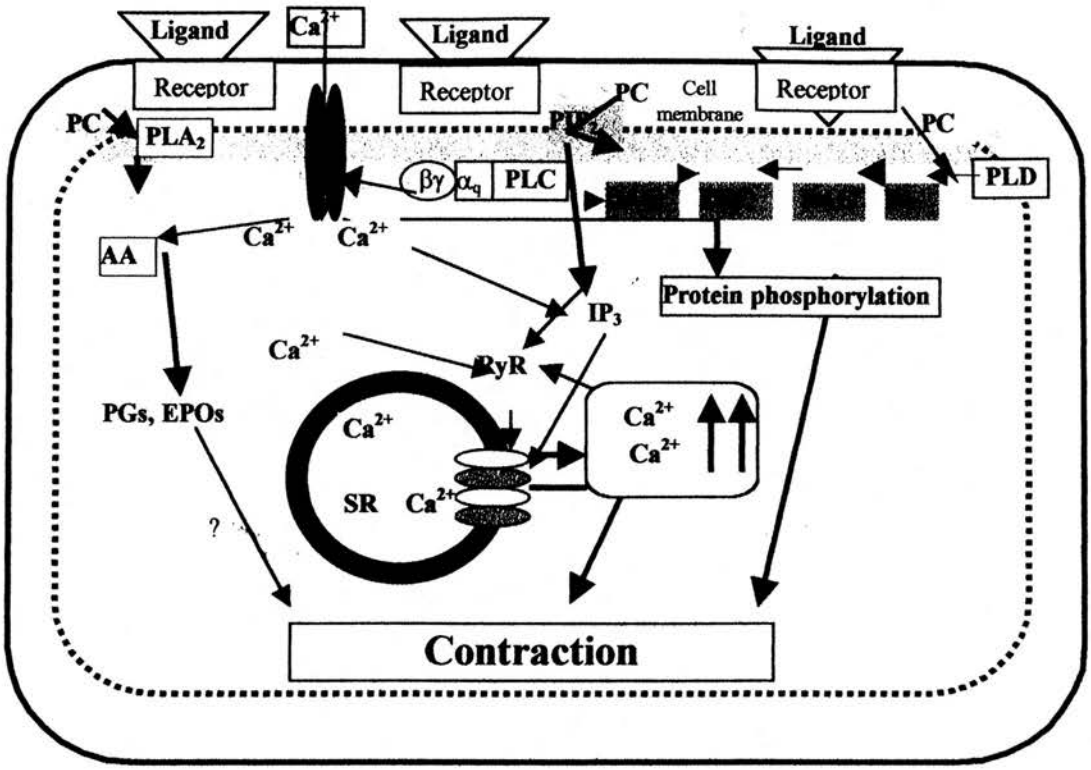



**Figure 1.3.** Mechanism of Agonist-Induced VSMC Contraction. Increased intracellular calcium is the hallmark of VSMC contraction, which combines with calmodulin to activate phosphorylation of myosin light chain kinase, resulting in actin-myosin cycling inducing contraction. Receptor (G-protein coupled receptor); $\alpha_q$, $\beta$ and $\gamma$ subunit of G-protein; PC, Phosphocholine; $PLA_2$, phospholipase $A_2$; PLC, phospholipase C; $PIP_2$, phosphoinositol 4, 5-biphosphate; $PIP_3$, inositol 1, 4, 5-triphosphate; PLD, phospholipase D; DAG, 1, 2-diacylglycerol; PKC, proteinkiase C; PA, phosphatidic acid; AA, arachidonic acid; PGs, prostaglandins; EPOs, epoxyeicosatrienoic acids; SR, sarcoplasmic reticulum; RyR, ryanodine receptor.

7-transmembrane-domain receptors are coupled to $G_i$ proteins. Stimulation of these receptors by an agonist produces a $G_i$-mediated inhibition of adenylate cyclase, leading to a reduction in cAMP formation. This inhibits the active reduction of $[Ca^{2+}]_i$ (by reducing both efflux and re-uptake of $Ca^{2+}$) and thus results in contraction (Yildiz *et al.*, 1998).

Pharmacomechanical relaxation occurs in response to stimulation of either adenylate or guanylate cyclase (Walsh, 1994) (Figure 1.4). Stimulation of 7 transmembrane domain receptors results in $G_s$-mediated activation of adenylate cyclase and, thus production of cAMP. In contrast, cGMP is formed by receptor-independent activation of soluble guanylate cyclase by, for example, nitric oxide (although some atrial natriuretic peptide receptors have particulate guanylate cyclase activity). cAMP and cGMP relax smooth muscle by a number of different mechanisms (Rembold, 1992) such as; (i) reducing $[Ca^{2+}]_i$ concentrations, (ii) diminishing $[Ca^{2+}]_i$ sensitivity to phosphorylating process, or (iii) uncoupling the force from myosin phosphorylation. These processes are mediated by cGMP-dependent (PKG) and cAMP-dependent (PKA) protein kinases (Walter *et al.*, 1988; Baltensperger *et al.*, 1990). In VSMCs, cAMP is more abundant than cGMP and can stimulate both PKA and PKG (Rembold, 1992). Protein kinase activation results in (i) $Ca^{2+}$ re-uptake by the SR and $Ca^{2+}$ extrusion across the sarcolemma (Furukawa *et al.*, 1988), (ii) inhibition of agonist-mediated $Ca^{2+}$ release from the SR (Hirata *et al.*, 1995) (iii) reduced sensitivity of contractile proteins to $Ca^{2+}$, (iv) activation of $K^+$ channels and (v) hyperpolarisation of the cell (Rembold, 1992).

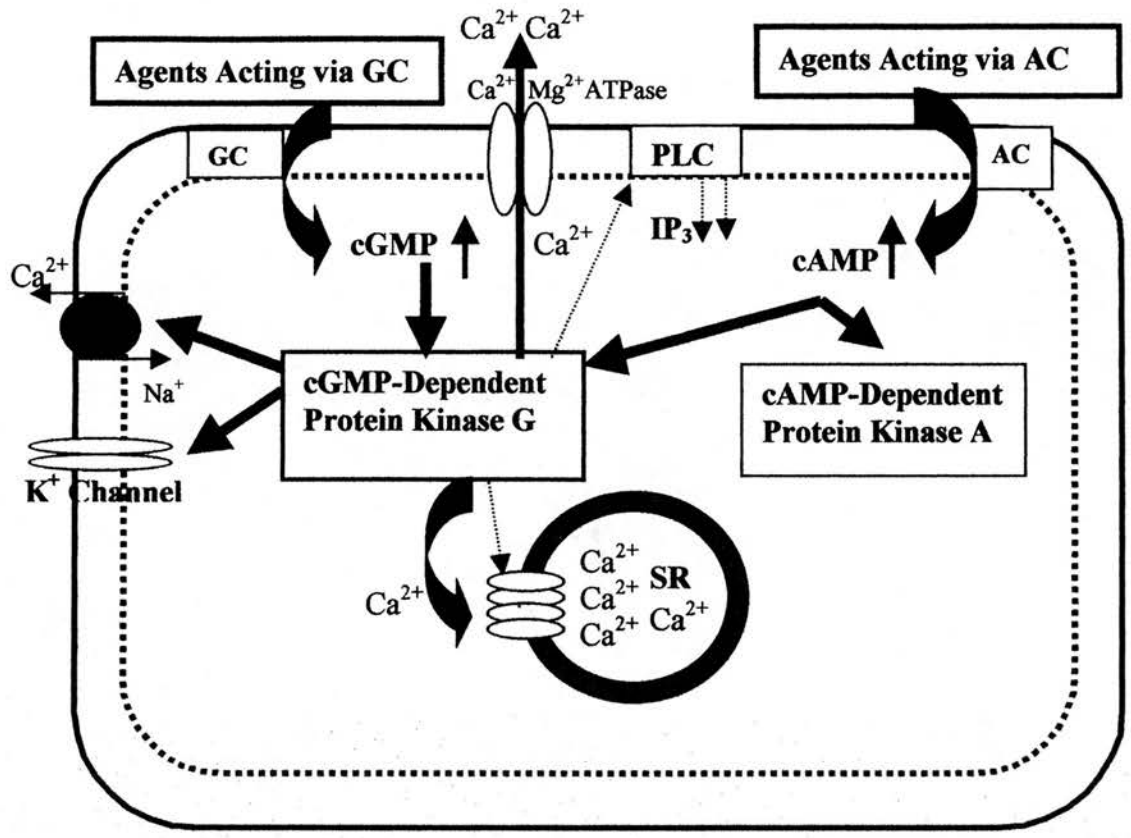



**Figure 1.4**. Mechanism of VSMC Relaxation. Reduction of intracellular below a threshold level induces VSMC relaxation. GC, guanylate cyclase; AC, adenine cyclase. Agents acting via GC are: NO, nitric oxide; ANP, atrial natriuretic peptide, and acting via AC are: PG, prostaglandin $I_2$ & $E_2$; CGRP, calcitonin-gene related peptide; VIP, vasoactive intestinal polypeptide. PLC, phospholipase C; $PIP_3$, inositol 1, 4, 5-triphosphate; cGMP, cyclic guanosine monophosphate; cAMP, cyclic adenosine monophosphate and SR, sarcoplasmic reticulum. Solid line, activation and broken line inhibition.

### 1.3.2.4. Regulation of Receptor-Mediated Contraction

Contractile responses mediated by G-protein coupled receptors can be regulated by limiting the access of a ligand to its receptor (Grady *et al.*, 1997). This can be achieved either by: (i) agonist degradation (e.g. breakdown of ACh by cholinesterase), (ii) agonist re-uptake (e.g. re-uptake of noradrenaline by neurones and other cells) or (iii) occupation of the receptor by a false transmitter. Repeated stimulation of a G-protein coupled receptor by an agonist can induce adaptive changes, which attenuate the response to this agonist. This is highly relevant to cirrhosis in which the plasma concentrations of many endogenous receptor-dependent vasoactive agents are chronically elevated (Hadoke, 2001). Adaptations include down regulation (depletion in the number of receptors) and desensitisation (uncoupling of a receptor from its second messengers). Receptor down-regulation occurs in response to prolonged (days-weeks) exposure of the receptor to its ligand (Lohse, 1995; Hadcock & Malbon, 1993). In this scenario, either internalisation and degradation of the receptor-ligand complex is increased or synthesis of new receptors is inhibited (Garland *et al.*, 1994). In contrast, desensitisation occurs within seconds or minutes of receptor activation, which diminishes sensitivity of response to the agonist (Grady *et al.*, 1997). This is mediated by phosphorylation of the receptor, which is, thus, uncoupled from its G-protein (Freedman & Lefkowitz, 1996).

The receptor-mediated response can also be altered by the activity of intracellular second messenger systems. The intracellular second messengers become depleted or uncoupled following repeated-prolonged activation of the post-receptor signalling pathway either by a receptor-dependent or independent stimulation. The altered level

and activity of the second messenger ($Ca^{2+}$, $IP_3$, DAG and PKC) therefore can also modulate VSMCs contraction (Huang *et al.*, 1995; Thrombino *et al.*, 1998).

### 1.4. Altered Vascular Function in Hepatic Cirrhosis

The demonstration of an impaired pressor response in cirrhosis was confirmed in a number of whole body experiments relatively soon (MacGilchrist *et al.*, 1991b; Pinzani *et al.*, 1991) after the initial description (Laragh, 1962; Ames *et al.*, 1965; Lunzer *et al.*, 1975). Some controversy remained, however, as to whether the impaired response was seen with NA alone (Lunzer *et al.*, 1975) or was evident with both NA and AII (MacGilchrist *et al.*, 1991a). Indeed, two Italian studies suggested an impaired response to NA and argued that results from the earlier investigations could be interpreted to support such impairments (Morandini & Spanedda, 1966; Morandini *et al.* 1967). It was not until the last few decades that a whole body investigation was performed using both AII and NA, plus a variety of selective adrenoceptor agonists, in an attempt to clarify this situation (Lenz *et al.*, 1985; MacGilchrist *et al.*, 1991a, b; Pinzani *et al.*, 1991). However, whilst MacGilchrist *et al.* (1991a) suggested a peripheral abnormality accounted for the impaired response to these agonists, none of these studies identified the cause of the impaired pressor response.

The lack of mechanistic detail obtained from whole body investigations *in vivo* highlighted the weakness of this type of study and indeed, this approach has now been largely superseded. The past decade has seen a rapid expansion in the use of measurements of regional blood flow and *in vitro* investigations using isolated

vessels (and also isolated organs and vascular territories), in an attempt to identify the cause(s) of the impaired pressor response in patients with cirrhosis.

### *1.4.1. In Vivo Studies*

Interpretation of whole body studies is hampered by the reflex changes induced by systemic changes in vascular tone. This problem has been overcome in recent years by the improvement of methods, such as venous occlusion plethysmography (Benjamin *et al.*, 1995) and dorsal hand vein compliance (Aellig, 1981), which allow local infusion of vasoconstrictors and measurement of blood flow in the forearm. These techniques use sub-systemic concentrations of an infused drug and, thus, do not induce systemic cardiovascular or central sympathetic responses. Over the past decade, these techniques have been used increasingly in an attempt to determine the causes of vascular dysfunction in cirrhosis. The use of isolated fore-arm and hand vasculatures have been criticised, however, for having little relevance to the splanchnic territory which may be the primary site of vasodilatation in cirrhosis.

As with whole body investigations, the majority of studies in isolated vascular territories have used α-adrenoceptor agonists or AII (although a recent report indicated a reduced response to ET-1 in cirrhosis) (Helmy *et al.*, 2001)). Many of these have supported the contention that contractile responses to these agonists are impaired in cirrhotic patients (Ryan *et al.*, 1993, 1996; Campillo *et al* 1995; Bierbrier *et al.*, 1994). However, this view is not unchallenged, with several reports that contractile responses are unaltered in cirrhosis (Calver *et al.*, 1994; Lunzer *et al.*, 1975). Furthermore, one study identified an impaired response to AII but found no

difference in response to exogenous NA or sympathetic stimulation, in cirrhotic individuals (Newby *et al.*, 1998b).

The reasons for these conflicting results are not clear, but the most likely explanations are variations in patient population (including age, disease aetiology and severity) and/ or methodology (Hadoke, 1999, 2001) between studies. For example, in one study the patient group was older than the control group (Lenz *et al.*, 1985) and, whilst some studies infused a single bolus of agonist (Pinzani *et al.*, 1991), others use a continuous infusion (Ames *et al.*, 1965; MacGilchrist *et al.*, 1991a,b). Methodological differences, however, may also be significant as difficulties are encountered with venous occlusion plethysmography when comparing two groups of patients with different basal forearm blood flow (Benjamin *et al.*, 1995; Rodriguez-Perez *et al.*, 1993; Calver *et al.*, 1994; Albillos *et al.*, 1995; Wong *et al.*, 1996). Similarly, aetiology, severity of the disease, treatment process such as, diuretic and steroid therapy, as well as alcohol intake, which can influence the investigations (Ryan *et al.*, 1993; Wong *et al.*, 1996; Pickkers *et al.*, 1997) are not controlled in a similar way in different studies.

Despite the conflicting data, studies on isolated vascular territories *in vivo* generally support the notion that an impaired response to exogenous vasoconstrictors does occur in patients with cirrhosis. The agonist-selectivity of this impairment, however, remains controversial and these studies have not identified the underlying alteration in the vessel wall responsible for hyporesponsiveness.

*1.4.2. In Vitro Use of Isolated Vessels*

Isolated vessel studies using *in vitro* methodology enable direct assessment of vascular responses in the absence both of systemic neuro-hormonal and reflex influences (Mulvany & Aalkjaer, 1990) and of circulating vasoactive factors. Moreover, these studies can investigate directly whether an impaired contractile response is due to an alteration in the contractile pathways mediated by the receptor or to remodelling of the vessel wall. Therefore, a large number of studies have been performed using this methodology to identify the mechanism(s) underlying altered responsiveness in cirrhosis.

1.4.2.1. Vessels Isolated from Animal Models of Cirrhosis

Due to the scarcity of suitable and viable human vascular tissue samples, most *in vitro* studies have used vessels from animal models of cirrhosis/ portal hypertension (Hadoke & Hayes, 1997). Three methods have been widely used to produce cirrhosis and/ or portal hypertension in animals (generally using rats, although rabbits (Cahill *et al.*, 1996; Sitzmann *et al.*, 1995) and dogs (Bomzon *et al.*, 1990) have also been used). These are: (i) treatment with carbon tetrachloride ($CCl_4$) and phenobarbitone, which provides a model of liver damage with portal hypertension but without jaundice, (ii) bile duct ligation (BDL), which causes acute jaundice followed by liver damage and portal hypertension, and (iii) portal vein ligation (PVL), producing portal hypertension without significant liver damage or ascites.

Vessels isolated from animals with experimental cirrhosis/ portal hypertension have been used to investigate the impaired pressor response to α-adrenoceptor agonists

and AII (Weigert *et al.*, 1995; Gadano *et al.*, 1997; Castro *et al.*, 1993). The use of AII has been restricted, however, by the demonstration that this agonist often produces only small, transient contractions in isolated vessel systems measuring isometric response. Indeed, in many vessels, sub-maximal pre-contraction with an alternative vasoconstrictor is required before AII will produce a sustained concentration-dependent contraction (Dunn *et al.*, 1994; Falloon *et al.*, 1995). Most studies have reported impaired contractile responses to α-adrenoceptor agonists and AII agonists (Lee, *et al.*, 1995; Karatapanis *et al.*, 1994; Claria *et al.*, 1994; Castro *et al.*, 1993), thus reinforcing the findings of *in vivo* studies of cirrhotic patients. Almost inevitably, however, some investigations have also reported no alteration in, or even an enhancement of, contractile responses to these agonists (Bomzon *et al.*, 1991; Cawley *et al.*, 1995; Moreno *et al.*, 1996).

Isolated vessel studies have also extended the *in vivo* observations by assessing vascular responses to a variety of other vasoconstrictor agonists, including AVP, 5-HT and ET-1 (Sieber & Groszman, 1992; Cummings *et al.*, 1986; Jacob *et al.*, 1991; Cahill *et al.*, 1998). These studies have demonstrated both that altered contractile function is not restricted to adrenoceptor agonists and AII and that impaired contractility may be agonist selective (Cummings *et al.*, 1986; Sieber & Groszman, 1992; Moreno *et al.*, 1996). However, similar to adrenergic agonists and AII, responses to these agonists were also varied widely (reviewed by Hadoke & Hayes 1997; Hadoke, 1999). For AVP, impaired contraction was reported in the perfused mesenteric bed or tail arteries of portal vein ligated rats (Sieber & Groszmann, 1992; Huang *et al.*, 1995) while, the response was unaltered when $CCl_4$

was used to induce cirrhosis (Ralevic *et al.*, 1996) or even enhanced in mesenteric veins of the portal vein ligated rats (Cummings *et al.*, 1986; Moreno *et al.*, 1996). Several other studies have demonstrated that in rats, 5-HT-mediated contraction was enhanced in aorta of $CCl_4$ induced cirrhosis (Jacob *et al.*, 1991) or in mesenteric vein after portal vein ligation (Cummings *et al.*, 1986; Kaumann *et al.*, 1988; Moreno *et al.*, 1996), but was reduced in aorta and portal vein of bile duct ligated rats (Jacob *et al.*, 1991). Similarly, it was reported that in rats ET-1-mediated contraction in aortae was enhanced following portal vein ligation (Cahill *et al.*, 1998) but reduced in $CCl_4$ induced-cirrhosis (Cailmail *et al.*, 1995) and also reduced in mesenteric vein after portal vein ligation (Moreno *et al.*, 1996). Investigations using animal models however, demonstrated that the several factors could contribute to conflicting findings between studies. In particular, these studies indicate that the effect of cirrhosis on vascular function may depend upon: the species of animal used, the technique used to induce cirrhosis/ portal hypertension (i.e. the aetiology of disease), the duration and severity of disease, the anatomical origin of the vessel, and the selection of a vein or an artery for study (Hadoke & Hayes, 1997).

In general, these experiments with vessels from animal models of cirrhosis indicate that cirrhosis/ portal hypertension produces an alteration in contractile function that is maintained *ex vivo*. Strikingly, these studies also indicate that alterations in contractile function may be agonist-dependent and may also depend upon the aetiology, duration and severity of cirrhosis. This literature also, however, serves to reinforce the limitations that must be acknowledged with isolated vessel techniques. In particular, that data obtained in a single isolated vessel cannot be extrapolated to

the entire vascular system. A major limitation, as ever, with animal models, however, is the question of relevance to the disease in humans. No animal model provides an ideal representation of human cirrhosis (Tsukamoto *et al.*, 1990). Therefore, it cannot be assumed that functional changes detected, or mechanisms of dysfunction identified, will apply to the condition in patients.

#### 1.4.2.2. Vessels Isolated from Patients with Cirrhosis

Relatively few studies have used isolated human vessels to investigate functional alterations in cirrhosis, mainly because of the many difficulties encountered in trying to obtain suitable, viable vessels. There is, however, an increasing body of literature in which functional investigations have been performed on vascular tissue (hepatic artery and portal vein) obtained at liver transplant (Smith *et al.*, 1997; Hadoke *et al.*, 1998; Heller *et al.*, 1999). In addition, one study used radial veins obtained by elective biopsy (Ryan *et al.*, 1996). Unfortunately, due to damage to the endothelium in vessels obtained from theatre (and deliberate removal of the endothelium from the radial vein), none of these studies has assessed the effects of cirrhosis on endothelium-dependent function (Ryan *et al.*, 1996; Hadoke *et al.*, 1998; Heller *et al.*, 1999). These vessels do, however, allow investigation of the effects of cirrhosis on VSMC function as well as the mechanism(s) responsible for any alteration.

As with *in vivo* studies, almost all *in vitro* studies using vessels from patients with cirrhosis have investigated adrenoceptor-mediated contraction. In most cases, this response has been found to be impaired in hepatic arteries (Smith *et al.*, 1997; Heller *et al.*, 1999), portal veins (Heller *et al.*, 1999) and radial (Ryan *et al.*, 1996) veins

from patients with cirrhosis. Only one study reported unaltered responses (to NA and PE) in common hepatic arteries (Hadoke *et al.*, 1998), although there is also a report of enhanced contractility in intrahepatic arteries isolated from cirrhotic patients (Battaglia *et al.*, 1996). None of these studies, however, have clearly identified the mechanism responsible for impaired contraction. Indeed, studies on the impact of cirrhosis on adrenoceptor function in humans (Gerbes *et al.*, 1986; MacGilchrist & Reid, 1990) have mostly been performed using blood cells (leukocytes, platelets) as a model for VSMCs. Similarly, although a wide range of vasopressor systems are activated in cirrhosis (and data from animal models suggests that contractile dysfunction is agonist selective) (Cummings *et al.*, 1986; Moreno *et al.*, 1996; Huang *et al.*, 1995) but the impact of cirrhosis on the function of human extrahepatic arteries has not been assessed using agonists other than adrenoceptor-agonists (e.g. AVP, 5-HT or ET-1).

### 1.5. Mechanism of Altered Vascular Function

Despite the extensive investigation detailed in previous sections, the exact causes of vascular dysfunction in cirrhosis remain unclear. Several potential mechanisms have been proposed, however, including: (i) increased vasodilator activity, (ii) impaired activity of contractile receptors, (iii) alterations in intracellular contractile signalling pathways, and (iv) structural changes in the vessel wall. Limited investigation of these processes has been performed using tissue from animal models (and, in some cases, patients) of cirrhosis.

*1.5.1. Altered Activity of Vasodilator Systems*

Given that individuals with cirrhosis exhibit peripheral vasodilatation, it seems logical that excessive activity of endogenous vasodilators may contribute to the vascular abnormalities in this condition (Groszmann, 1994a; Guarner *et al.*, 1993; Hayes *et* al., 1992). Potentially, both circulating vasodilators and those produced locally within the vascular wall could be responsible for altered contractility.

1.5.1.1. Blood-borne Vasodilators

The circulating concentrations of a considerable number of endogenous factors with vasodilator activity are increased in patients with cirrhosis (Abelmann, 1994; Hayes *et* al., 1992; Groszmann, 1994b). These include atrial natriuretic peptide (ANP), glucagon, prostaglandins $I_2$ and $E_2$, EDHF, kinins, lactate, adenosine, calcitonin-gene related peptide (CGRP), vasoactive intestinal polypeptide (VIP), substance P, adrenomedullin, and histamine (Iwao *et al.*, 1997a; Pizcueta *et al.*, 1990; Sitzmann *et al.*, 1991; O'Halloran & Bloom 1991; Lee *et al.*, 1997; Guevara *et al.*, 1998 ). Furthermore, endotoxins, bile acids and cytokines (e.g. tumour necrosis factor (TNF)), which can induce vasodilation are also elevated in cirrhosis (Khoruts *et al.*, 1991; Guldatana *et al.*, 1993). Plasma concentrations of these vasodilators often correlate with disease (cirrhosis) severity and haemodynamic alterations (Lavilla *et al.*, 1995; Benoit *et al.*, 1986; Gupta *et al.*, 1992). It is possible, therefore, that increased vasodilator activity in the blood contributes to the development of the hyperdynamic circulation and to contractile hyporesponsiveness. Indeed, kidneys from patients with cirrhosis function normally when transplanted into non-cirrhotic individuals and transfusion of blood from cirrhotic to non-cirrhotic animal induces

circulatory abnormalities in the latter.

Although the elevated plasma concentrations of so many vasodilators suggest that they all make some contribution to the hyperdynamic circulation in cirrhosis, this has yet to be established. Furthermore, whether such activation is the primary cause, or simply a consequence, of the hyperdynamic circulation is not clear. If, however, contractile hyporesponsiveness was solely due to the action of blood-borne factors, contractile function of vessels from cirrhotic individuals would be predicted to be normal *in vitro*. Studies with vessels from both patients and animal models have demonstrated that this is not the case, indicating that an alteration within the vessel wall itself contributes to contractile dysfunction.

#### 1.5.1.2. Nitric Oxide Activity

In the endothelial cells NO is synthesized from the amino-acid L-arginine, by the enzyme NO synthase (NOS) (Palmer *et al.*, 1988). Three isoforms of NOS have been identified by molecular cloning (Hoggs *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 neural tissues. The second is a $Ca^{2+}$ independent isoform (iNOS or NOS II), which has been isolated from different cells (e.g. VSMCs, monocytes) after induction with bacterial endotoxins 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 haemodynamics (Hoggs *et al.*, 1999). Specific inhibitors of NOS have been developed, such as L-arginine analogues $N^G$ -monomethyl-L-arginine (L-NMMA), $N^G$ -nitro-L-arginine (L-NNA),

and $N^G$ -nitro-L-arginine methyl ester (L-NAME) (Moncada *et al.*, 1991; Hoggs *et al.*, 1999) which can prevent NO production.

It was suggestion that in cirrhosis excessive nitric oxide (NO) production by the vessel wall was responsible for peripheral vasodilatation (Vallance and Moncada, 1991). This hypothesis mentioned that in cirrhosis, gut-derived endotoxins induced nitric oxide synthase (iNOS) activity in the vascular smooth muscle. This explanation remains possible but, despite extensive investigation, is still controversial. An alternative proposal is that, the activity of constitutive NO synthase (eNOS) is up-regulated in patients with cirrhosis as a consequence of the hyperdynamic circulation (Bomzon & Blendis, 1994). In this model, enhanced shear stress causes increased production of NO from eNOS, within the splanchnic circulation, one of the main sites of NO production. However, both enhanced eNOS activity (resulting from increasing shear stress) and translocation of gut-derived endotoxins are more likely to be significant in advanced, than in early, cirrhosis. A contribution of NO, from either source, to vascular abnormalities is not indicated in patients with mild cirrhosis (Forrest *et al.* 1996).

The role of NO in the vascular abnormalities of cirrhosis remains controversial. In particular, the degree of endotoxaemia in cirrhosis, especially in early cirrhosis, is debatable. Studies in animal models have provided conflicting data: many (although not all (Sogni *et al.*, 1992; Karatapanis *et al.*, 1994)) have demonstrated that vascular hyporesponsiveness is NO-dependent but the source of this NO has not been established. Endothelium-derived (eNOS) (Ros *et al.*, 1995; Martin *et al.*, 1996;

Niederberger *et al.*, 1996; Weigert *et al.*, 1995) and smooth muscle-derived (iNOS; endothelium-independent) (Michielsen *et al.*, 1995; Morales *et al.*, 1995) NO have both been shown to be responsible for contractile hyporesponsiveness in isolated vessels. Initial studies with human vessels although reported that the induction of iNOS was responsible for the impaired response to adrenergic agonists (Smith *et al.*, 1997; Ryan *et al.*, 1996), however, recent studies have challenged these findings (Heller *et al.*, 1999; Hadoke *et al.*, 1998).

### *1.5.2. Altered Contractile Activity in the Vessel Wall*

Alterations in either vascular structure or in contractile signalling pathways within the vessel wall could explain the altered contractile response demonstrated in vessels isolated from cirrhotic individuals. Remodelling is a possibility as persistent hypotension or hypertension can result in atrophy or hypertrophy, respectively, of the vessel wall (Pang & Scott, 1985). In cirrhosis both atrophy and hypertrophy can occur, as some vascular territories (splanchnic, pulmonary) become dilated (Iwao *et al.*, 1997a), whilst others (portal, cerebral, renal) are constricted (Arroyo *et al.*, 1988). An extensive remodelling in the portal venous territory was demonstrated following portal vein ligation in the rat (Jensen *et al.*, 1987). However, more recent studies suggest that remodelling does not account for changes in vascular contractility. In particular, the demonstration that contractile abnormalities are agonist-dependent suggests a selective alteration to particular agonists, rather than a general alteration in contractility.

Alterations in the contractile signalling pathways within the vascular wall may be

indicated: receptor-mediated contraction is sensitive to chronic exposure to an agonist (as described previously; section 1.3.2.3) and many endogenous vasoconstrictors are chronically elevated in cirrhotic patients. Therefore, down-regulation and/ or desensitisation of G-protein-coupled receptors could account for impaired contractility (section 1.3.2.3). Alternatively, abnormalities in function could result from changes induced in the receptor-signal transduction pathway, downstream from the receptor (section 1.3.2.3).

1.5.2.1. Altered Receptor Function.

Very few studies have investigated receptor number and expression in vessels from cirrhotic individuals. Those that are available, predominantly concerned with adrenoceptors or the $AT_1$ (AII) receptor. Some investigations have, however, assessed the influence of cirrhosis on receptors stimulated by ET-1 ($ET_A$ and $ET_B$) (Cahill *et al.*, 1998) and 5-HT ($5\text{-}HT_{2A}$) (Cummings *et al.*, 1986).

Altered receptor activity can be discounted in investigations where impaired contraction has been shown to be solely a consequence of enhanced NO production (Michielsen *et al.*, 1995; Morales *et al.*, 1995; Ros *et al.*, 1995; Kanwar *et al.*, 1996; Niederberger *et al.*, 1996; Smith *et al.*, 1997). There are many demonstrations, however, of NO-independent contractile hyporesponsiveness in vessels from animals (Liao *et al.*, 1994; Huang *et al.*, 1995; Karatapanis *et al.*, 1994; Huang *et al.*, 1997) and humans (Heller *et al.*, 1999) with cirrhosis.

#### 1.5.2.1.1. α-Adrenoceptors

Despite the high circulating levels of NA in cirrhosis, down-regulation of α-adrenoceptors do not appear to account for impaired vascular contraction. Reduced contractile responses to NA have been demonstrated in vessels isolated from animal models without any concomitant down-regulation or desensitisation of the $\alpha_1$-adrenoceptor (Liao *et al.*, 1994; Huang *et al.*, 1996)

#### 1.5.2.1.2. AII Receptor ($AT_1$-receptor)

It has been demonstrated that hyporesponsiveness of rat portal veins to AII (in cirrhotic/ portal hypertensive rats) was due to impaired activity of the $AT_1$ receptor (Huang *et al.*, 1997). In this study, phosphoinositide production was reduced in response to AII (receptor stimulation) but not in response to compounds (PLC, $NaF/AlCl_3$, guanosine-5'-O-(3-thiotriphosphate)) that stimulate the contractile cascade down-stream from the receptor. This may suggest receptor-desensitisation (where uncoupling of the receptor from its G-proteins prevents contraction). In contrast, the number of $AT_1$-receptors was reduced in the portal vein and mesenteric artery of portal hypertensive rabbits as well as in the aorta of bile duct-ligated rats, indicating receptor down-regulation (Sitzmann *et al.*, 1995; Tazi *et al.*, 1997). The situation is further complicated by the fact that $AT_1$-receptor number was increased in mesenteric arteries from $CCl_4$-induced cirrhotic rats but this was balanced by a reduction in receptor affinity. It is possible, therefore, that the role of receptor desensitisation/ down-regulation is dependent upon species, aetiology of cirrhosis and anatomical origin of the vessel studied.

#### 1.5.2.1.3. ET-1 Receptors

ET-1 acts via $ET_A$ and $ET_B$ receptors located in the vessel wall; in VSMCs both mediate vasoconstrictions, whilst the $ET_B$ receptor on the endothelium induces vasodilatation (Gray & Webb, 1996; Haynes *et al.*, 1995).

Up-regulation of ET-1 receptors has been reported in mesenteric arteries and aortae from portal vein-ligated rats (Cahill *et al.*, 1998). Similarly, $ET_A$ and $ET_B$ receptor populations or mRNA expression in the intrahepatic circulation are elevated in cirrhosis/ portal hypertension (Leivas *et al.*, 1998). These findings are surprising given that plasma ET-1 concentrations are elevated in this condition (Newby *et al.*, 1998b; Trevisani *et al.*, 1997). The mechanism of up-regulation is not clear but has been attributed to a compensatory hyper-activation resulting from increased production of NO (Redmond *et al.*, 1996).

#### 1.5.2.1.4. 5-HT Receptors

5-HT can induce vascular contraction via 5-$HT_{2A}$ and 5-$HT_1$-like receptors (Hoyer *et al.*, 1994; Yildiz *et al.*, 1998). A selective enhancement of 5-HT-mediated contraction has been demonstrated in mesenteric veins (Cummings *et al.*, 1986; Kaumann *et al.*, 1988; Moreno *et al.*, 1996) and aortae (Jacob *et al.*, 1991) from portal hypertensive/ cirrhotic rats. These results clearly suggest an up-regulation of 5-HT receptors is responsible for this alteration. However, it was found that hyperactivity of the 5-$HT_{2A}$ receptor was not responsible for augmented contraction as affinity of this receptor was not enhanced (Cummings *et al.*, 1986; Kaumann *et al.*, 1988). Moreover, a reduced response to 5-HT was demonstrated in portal veins

from portal vein or bile duct-ligated rats (Jacob *et al.*, 1991) indicating that the mechanism of altered response could depend upon aetiology and the anatomical origin of a particular vessel. At present, no studies appear to have assessed the activity of 5-$HT_1$-like receptors in cirrhosis. Therefore, the mechanism of altered response to 5-HT is uncertain.

### 1.5.2.2. Alterations in Intracellular Signalling Pathways

It is possible that reduced activity of intracellular second messenger systems accounts for contractile hyporesponsiveness in cirrhosis/ portal hypertension. For example, intracellular second messengers (such as $Ca^{2+}$, $IP_3$, DAG and PKC) may be depleted following sustained stimulation by the elevated plasma levels of the vasoactive agents in cirrhosis (Castro *et al.*, 1993; Huang *et al.*, 1997; Thrombino *et al.*, 1998). Therefore, assessment of second messengers following stimulation with receptor-dependent and -independent vasoconstrictors is necessary to identify the mechanism(s) of impaired contractile function in cirrhosis. Certainly, both altered activation of intracellular second messengers (Wu & Benoit, 1994; Huang *et al.*, 1996) and an impaired response to increases in $[Ca^{2+}]_i$ (Karatapanis *et al.*, 1994) have been implicated in vessels from cirrhotic animals. Only a limited number of studies, however, have directly investigated second messenger pathways in these tissues (Huang *et al.*, 1997; Wu & Benoit, 1994; Trombino *et al.*, 1998). It has been suggested that attenuated activity of the phosphoinositide messenger system is responsible for impaired contractile response in vessels from portal hypertensive rats (Huang *et al.*, 1995,1997) whilst decreased PKC activity has been implicated in aortic VSMCs from cirrhotic rats (Trombino *et al.*, 1998). Further studies are

required to confirm these results and to demonstrate their applicability to the condition in humans.

### *1.5.3. Summary*

Studies in both humans and animal models have largely confirmed that the development of cirrhosis/ portal hypertension is accompanied by an impairment in vascular contractile function. This impairment is maintained in isolated vessels *ex vivo* but the mechanisms involved remain unclear. Most data, however, have been obtained from animal models, with the few studies have used vessels from cirrhotic patients restricted to assessment of α-adrenoceptor-mediated contraction. It is unclear, therefore, whether altered contractile function in human arteries is agonist-selective, as demonstrated in vessels from cirrhotic/ portal hypertensive animals. Agonist selectivity could be clinically important given the fact that AVP and its analogues, as well as 5-HT- and ET-1-receptor antagonists, can reduce portal pressure (Freeman *et al.*, 1998; Nevens *et al.*, 1991, Sogni *et al.*, 1998).

## 1.6. Aims

This project investigated the hypothesis that the development of hepatic cirrhosis would produce an agonist-selective alteration in contractile function of the hepatic artery that would be maintained *ex vivo*.

The primary aim was to determine whether contractile responses elicited by AVP, 5-HT and ET-1 were altered in hepatic arteries isolated from patients undergoing liver transplant for cirrhosis. It was also intended to attempt to elucidate the mechanisms responsible for any alterations in contractile function identified in arteries from cirrhotic individuals. In particular, attempts would be made to identify the receptors mediating responses to each agonist and to clarify the role of nitric oxide in altered contractile function.

Initial studies were performed using porcine splanchnic arteries in order to develop and refine the protocols used for assessing function in (relatively scarce) human hepatic arteries. The use of porcine hepatic arteries as a model for the human vessels was extended to determine whether hepatic arteries from liver donors (which are stored in organ preservation solution during liver transplantation) were a suitable control for those taken from (cirrhotic) liver recipients (which were not exposed to preservation).

# CHAPTER TWO

# METHODS

## 2.1. Acquisition of Vessels

In order to study vascular reactivity in cirrhosis, hepatic arteries were obtained from patients with cirrhosis undergoing liver transplantation. Hepatic and mesenteric arteries were also collected from non-cirrhotic liver donors. In addition, investigations were performed using hepatic and mesenteric arteries from piglets and adult pigs to assess normal physiological vascular responses of these vessels.

### *2.1.1. Acquisition of Human Hepatic and Mesenteric Arteries*

Functional investigations using human blood vessels are largely restricted because of unavailability of viable tissue samples. However, the Scottish Liver Transplantation Unit (SLTU) at the Royal Infirmary of Edinburgh provided opportunities for obtaining viable human splanchnic blood vessels both from liver donors (non-cirrhotic) and recipients (cirrhotic). During retrieval of the healthy liver from an organ donor, the surgical team routinely collects the entire hepatic artery and the coeliac axis. These are stored with the donor liver, in a preservative (University of Wisconsin) solution (UWS; NPBI International BV, Emmer-Compascuum, The Netherlands and Du-Pont Pharmaceuticals Ltd., Herts, UK.) at 4°C. At the end of liver transplantation, after anastomoses of the graft, residual hepatic artery samples are discarded and so become available for functional analysis. Arrangements were also made with the retrieval team to collect small sections of the mesenteric artery from the liver donors. These were placed immediately into ice-cold oxygenated Krebs'-Henseleit solution (KHS) and transported to the Royal Infirmary.

The (cirrhotic) liver removed from the recipient is routinely fixed in formal saline for

histological analysis. Therefore, sections of hepatic artery were dissected directly from the explanted recipient livers immediately following removal from the patient and prior to fixing.

Donor and recipient hepatic, and donor mesenteric, arteries obtained from the transplant theatre were placed immediately in fresh, ice-cold, oxygenated KHS and transported to the laboratory for functional analysis. Sometimes, in these procedures donor hepatic arteries are kept in UWS and mesenteric arteries in KHS, respectively, up to 14 hours (maximal liver preservation time in the Scottish Liver Transplant Unit), while recipient hepatic arteries are obtained immediately following removal from the patients.

Ethical approval for use of human tissues was obtained from the Lothian Research Ethical Committee and the arrangements were approved by the United Kingdom Transplant Support Service Authority (UKTSSA).

*2.1.2. Acquisition of Hepatic and Mesenteric Arteries from Piglets*

Hepatic and mesenteric arteries were collected from piglets that were being killed in the animal house at Edinburgh University for the purposes of a separate project. Piglets (small white; 12-15 kg; aged 3-4 weeks) were obtained from the Roslin Institute (Roslin, Scotland, UK) on the appropriate day and given free access to food. Each piglet was anaesthetised with 30 mg/kg pentobarbitone sodium (Vet Drug, Middlefield Ind. Est., Falkirk, UK) via the auricular vein and then killed with an intravenous bolus of 150 mg/kg of the same drug. As soon as the heart stopped

beating, a deep, midline ventral incision was made and a section of the aorta with the coeliac axis, including the hepatic and mesenteric arteries, was removed. These vessels were placed in ice-cold, oxygenated KHS and transported to the laboratory. The piglets were treated in accordance with UK Home Office Guidelines (Scientific Procedures Act, 1986).

### *2.1.3. Acquisition of Hepatic and Mesenteric Arteries from Adult Pigs*

Hepatic and mesenteric arteries from male and female, large white pigs (6-8 months old, 50-60 kg weight) were obtained from a local abattoir after the animals had been killed. Following a ventral, midline incision the liver, stomach and intestines were removed and, in order to obtain the hepatic artery, a portion of the liver around the porta-hepatis was removed. Sections of superior mesenteric artery were also collected. Tissues were placed immediately into ice-cold, oxygenated KHS and transported to the laboratory.

## 2.2. Functional Analysis

### *2.2.1. Preparation of Arterial Rings*

The hepatic and mesenteric arteries were carefully cleaned of adherent connective tissue and fat, and if necessary, were stored in the refrigerator at 4°C in freshly prepared oxygenated KHS. Whenever possible, experiments were performed immediately after the retrieval of the arteries, no more studies were performed after 24 hours of collection in human hepatic arteries and 48 hours in porcine vessels, respectively.

Rings approximately 2mm in length were prepared from each artery. In some experiments, it was necessary to remove residual endothelial cells from the luminal surface. This was achieved by gently rubbing the luminal surface of the rings with a wire probe while the rings were kept completely immersed in KHS on a petri dish.

### *2.2.2. Mounting of Arterial Rings*

The arterial rings were mounted horizontally between two parallel, stainless steel wire hooks in 10 ml organ baths filled with KHS (Mulvany & Halpern, 1976). The lower hook was fixed to the base of the organ bath, whilst the upper hook was attached to a force displacement transducer (Grass FT-03) connected to a computerised data acquisition system (Dr H Brash, Dept. Medicine, Royal Infirmary of Edinburgh) for the measurement of isometric force. The organ baths were maintained at 37°C and continuously bubbled with a mixture of 95% $O_2$ - 5% $CO_2$.

### *2.2.3. Assessment of Viability and Reproducibility*

The rings were stretched gradually to their optimum resting force [previously shown to be 4g (Hadoke *et al.*, 1998)] and allowed to equilibrate for 45-60 minutes. During equilibration, the tension was regularly adjusted and the contents of the organ baths replaced at frequent intervals. After equilibration, the viability and reproducibility of contraction of each ring was tested by stimulating with a single concentration of KCl (100 mmol/L). Rings were washed with KHS once the contraction had stabilised. The procedure was repeated 3-4 times until reproducible, sustained contractions were obtained (Hadoke *et al.*, 1998).

*2.2.4. Test for the Endothelium*

The endothelium has a profound influence on vascular function (Furchgott & Zawadzki, 1980). Hepatic arteries obtained from the transplant theatre have usually lost a significant quantity of the endothelium (Hadoke *et al.*, 1997; Smith *et al.*, 1997). Therefore, it was necessary to assess the integrity of the endothelium in the arteries used for functional analyses. Arterial rings (2mm in length) were pre-contracted sub-maximally with phenylephrine (PE, $10^{-6}$-$3x10^{-5}$ mol/L). After achieving a stable contraction, the rings were exposed to cumulative concentrations of acetylcholine (ACh, $10^{-8}$-$3x10^{-6}$ mol/L) (Vanhoutte & Miller, 1985). ACh induces either relaxation or further contraction depending on the presence or absence of intact endothelial cells covering the arterial wall (Figure 2.1) (Jeng *et al.*, 1996a,b; Nardo *et al.*, 2000).

Endothelium-independent relaxation of these arteries was also assessed using 3'-morpholinosydnonimine (SIN-1, $10^{-10}$-$3x10^{-6}$ mol/L) after sub-maximal contraction with PE ($10^{-6}$-$3x10^{-5}$ mol/L) (Figure 2.2).

*2.2.5. Functional Studies*

Functional investigations were begun following equilibration of the arteries at their optimum resting force and assessment of viability and endothelial cell integrity. Contractile and relaxation functions of isolated arteries were assessed by producing cumulative concentration-response curves to a variety of agonists in the presence or absence of relevant antagonists. Experiments were performed according to the functional protocol indicated in individual chapters. Following completion of each

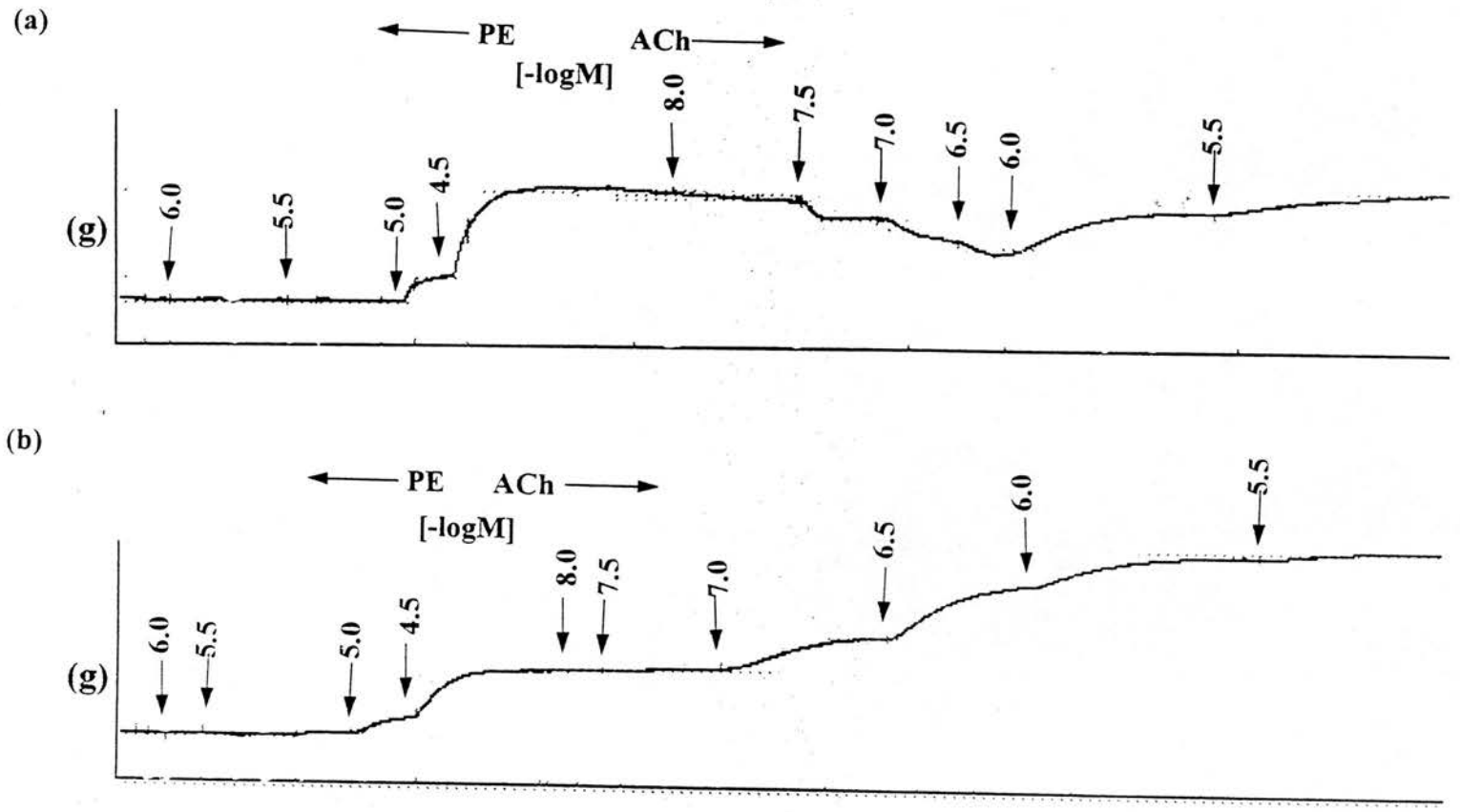



**Figure 2.1.** Traces demonstrating functional responses to acetylcholine (ACh, $10^{-8}$-$3x10^{-6}$ mol/L) after sub-maximal contraction with phenylephrine (PE, $10^{-6}$-$3x10^{-5}$ mol/L) in hepatic arteries obtained from piglet **(a)** with and **(b)** without a functional endothelium.

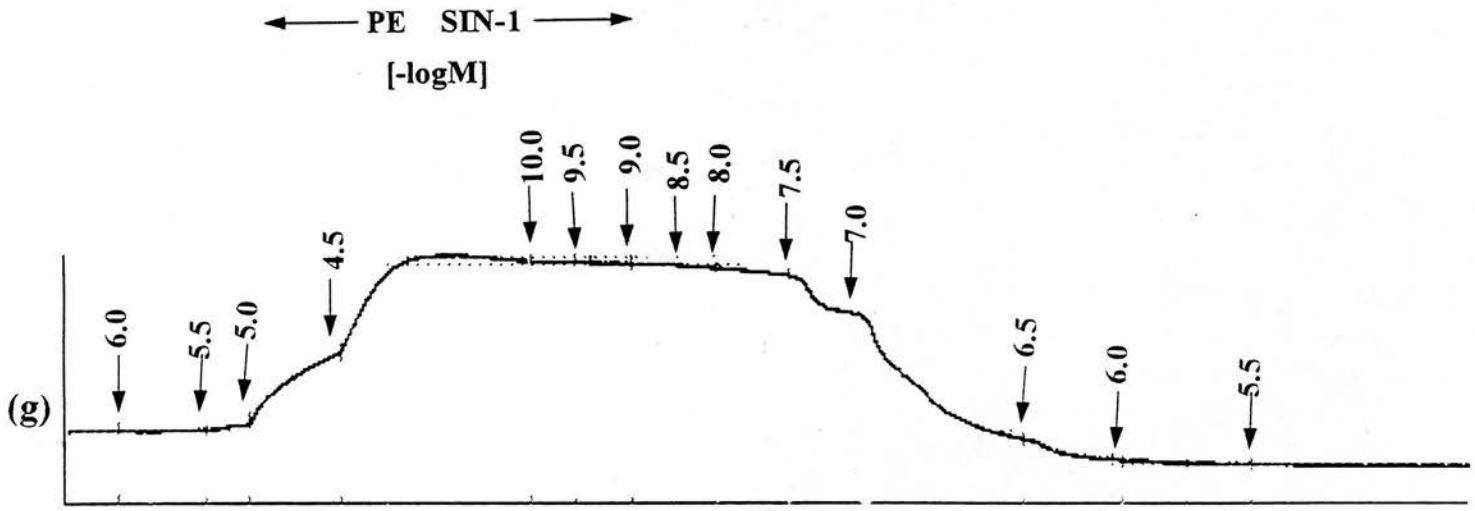



**Figure 2.2.** A trace showing the concentration-dependent relaxation produced in response to 3'-morpholinosydnonimine (SIN-1, $10^{-10}$-$3x10^{-6}$ mol/L) after sub-maximal contraction of denuded porcine hepatic artery with phenylephrine (PE, $10^{-6}$-$3x10^{-5}$ mol/L).

curve, the rings were washed repeatedly with KHS, allowed to return to baseline and equilibrated for 20-30 minutes before the next agonist was applied. Usually, after completion of investigations using receptor-dependent agonists, cumulative concentration-response curves were also produced using the receptor-independent vasoconstrictor, potassium chloride (KCl; 2.5-120 mmol/L).

### 2.3. Preparation of Working Solutions (Krebs'-Henseleit Solution)

Working solutions of Krebs'-Henseleit Solution (KHS) were prepared from stock solutions, 'A' and 'B', as required.

Stock Solution 'A' was prepared by dissolving NaCl, (138.4g); KCl, (7.0g) and $MgSO_4$. $7H_2O$, (5.8g) in 1 litre of deionised water. Similarly stock Solution 'B' was also prepared by dissolving $NaHCO_3$, (42.0g) and $KH_2PO_4$, (3.2g) in 1 litre deionised water.

Working solutions were prepared by mixing 100ml of each of solutions 'A' and 'B' in 1.8 litres of deionised water. D-glucose, (4.2g) and the $CaCl_2$. $6H_2O$, (1.1g) were dissolved in this solution to prepare a final working KHS of composition: (mmol/L) NaCl, 118.5; KCl, 4.7; $MgSO_4$, 1.2; $KH_2PO_4$, 1.2; $NaHCO_3$, 25; $CaCl_2$, 2.5; glucose, 11.6 and pH 7.4.

### 2.4. Histological Assessment of Endothelial Cell Integrity

Endothelial integrity was also assessed histologically in some vessels using the silver nitrate stain (Poole *et al.*, 1958). Rings, 8-10mm in length, were prepared from both

human and porcine hepatic arteries. Each ring was opened carefully, using a longitudinal incision, and then pinned out with the luminal surface exposed. Rings were washed for two minutes using a freshly prepared solution, of 4.6% weight/volume glucose and 2.4% weight/volume hepes (pH 7.4). The luminal surface was immersed in 0.4% silver nitrate solution for 1 minute and then exposed to light for 10 minutes to allow the stain to develop. Silver nitrate stains glycoproteins on the cell membrane but not the endothelial cell themselves (Figure 2.3). Therefore, edges of the cells stained dark brown whilst the cells themselves remained unstained, which allowed visual determination of the endothelial covering (Figure 2.3).

## 2.5. Immunohistochemistry

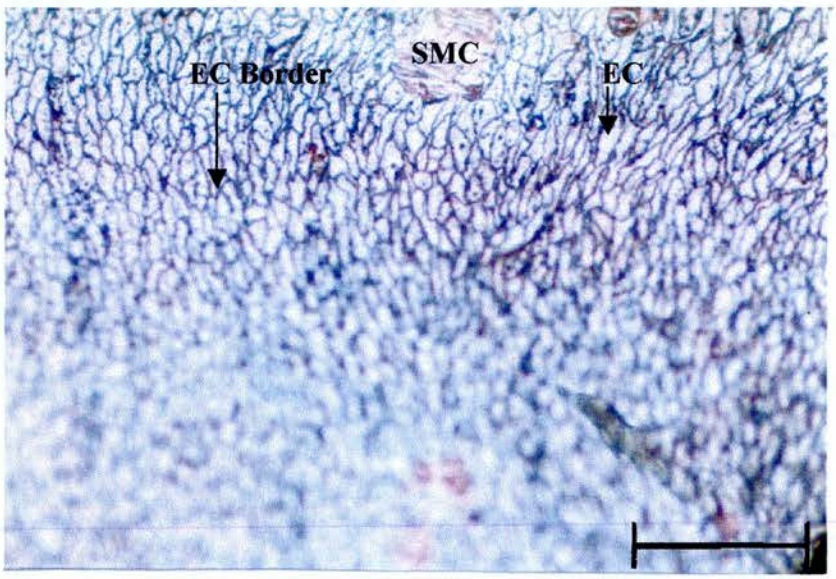
Immunohistochemistry was performed using both donor and recipient hepatic arteries for detection of immunoreactivity for inducible nitric oxide synthase (iNOS), α-smooth muscle actin (α-SMA) and endothelial cells.

### *2.5.1. Preparation of the Arteries*

After removing connective tissue and fat, arterial sections 1-2 cm in length were cut from each vessel and immediately fixed in neutral buffered formalin (10%) for paraffin embedding. Rings approximately 0.5cm in length were prepared from the formalin fixed arteries, processed using an enclosed tissue processor (by taking the arteries through graded alcohols into xylene) and finally embedded in paraffin wax.

Transverse arterial sections (3μm) were cut using a rotary microtome and floated in a water bath at 45°C. After 2-3 minutes, arterial sections were mounted onto tespa

**(a)** With Endothelium




**(b)** Without Endothelium

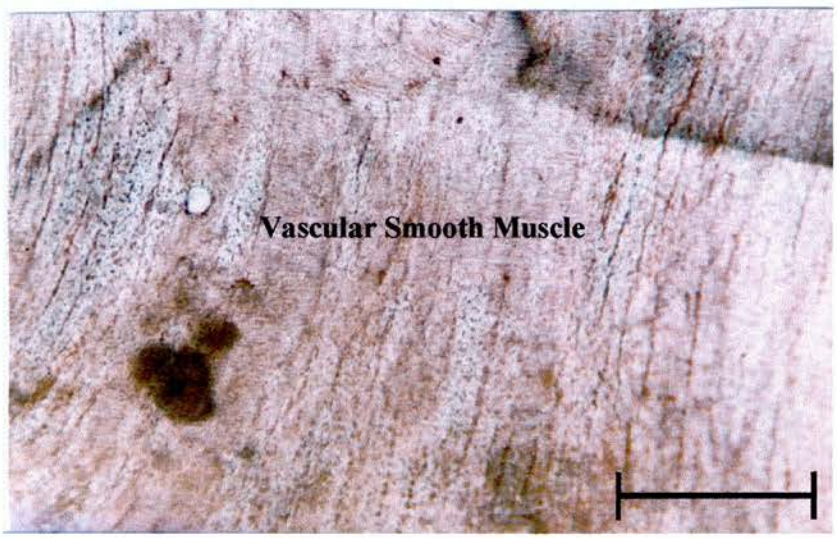



**Figure 2.3.** Photographs of longitudinal sections of hepatic arteries stained by silver nitrate **(a)** with and **(b)** without an intact endothelial covering. Endothelial cells are oriented in the direction of blood flow. EC, endothelial cells; SMC, smooth muscle cell. Scale=100μm.

(3-aminopropyltriethoxy-saline)-coated glass slides and dried overnight at 37°C.

*2.5.2. Immunohistochemical Analysis*

Immunohistochemistry is a technique, which can be used to identify cellular constituents (antigens) by eliciting antigen-antibody reactions. Antibodies (polyclonal or monoclonal) and lectins are used as a primary reagent in this technique. The sites of antibody binding (antigens) can be detected either by direct labelling of the bound primary antibody (or lectin) or additional amplification of the primary binding site using a secondary labelling procedure. For this study, immunohistochemical investigation of the vessel wall was performed using both direct and indirect antigen-detection techniques. In the direct method, a primary conjugated antibody is allowed to bind to the cellular antigen and this antibody-antigen complex is treated with an appropriate enzyme-tracer substance for proper visualisation. In the indirect method, a second amplifying conjugated antibody was applied, before being treated with an appropriate enzyme-tracer substance. The direct antigen-detection method was applied for detection of the endothelium, while the indirect method was used for identification of smooth muscle actin and inducible nitric oxide synthase (iNOS) in the vascular smooth muscle cells. Detection of binding sites can be achieved by interaction of tracer enzymes with a chromogen to produce a stable, coloured end product.

The two most common enzyme-labelled conjugate methods used to detect antigens are the peroxidase method and alkaline phosphatase method (Hsu *et al.*, 1981). In the peroxidase method, horseradish-peroxidase is combined with a chromogen, (3, 3'-

diaminobenzidine tetrahydrochloride [DAB]) to produce a dark brown insoluble coloured compound (Figure 2.4. a). In contrast, in the alkaline phosphatase method, a fuschin substrate is used as a tracer, which produces a permanent, insoluble red colour at the site of alkaline phosphatase activity (Figure 2.4. b).

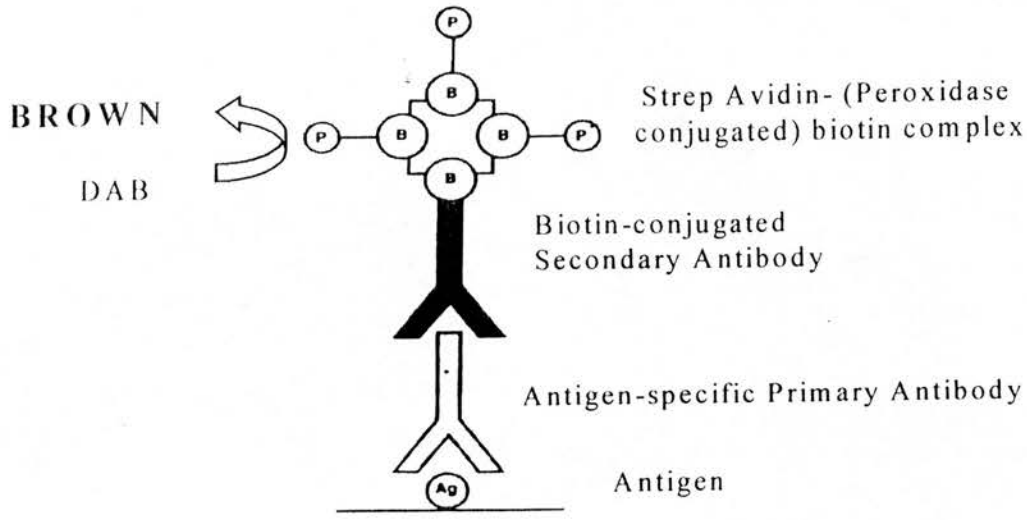
2.5.2.1. Peroxidase Method

The peroxidase method was used for immunohistochemical detection of vascular smooth muscle and endothelial cells in the vessel wall.

*Smooth Muscle Cells.* For the identification of α-actin in the vascular smooth muscle cells (Figure 2.5), transverse sections (3μm) of arteries were dewaxed in 100% xylene for 10 minutes, rehydrated through graded alcohols and washed in tap water for 15 minutes. The rings were treated with 1% hydrogen peroxide solution for 10 minutes at room temperature, to abolish the activity of endogenous peroxidase without affecting the immunoreactivity of antigens.

Thereafter, rings were washed for 15 minutes in water followed by 10 minutes in tris-buffered saline (TBS) at 37°C. The rings were treated with 0.1% trypsin (Sigma, UK) dissolved in TBS containing 0.1% calcium chloride (pH 7.8) at 37°C for 40 minutes to unmask antigen sites and then washed with phosphate buffer saline (PBS) for 10 minutes. The sections were incubated with goat serum, (1/1 dilution, in a solution made with 0.5ml of bovine serum albumin (BSA) + 14.5 ml of PBS and 6 drops of avidin/ml) for 30 minutes at room temperature to block non-specific staining.

(a)




(b)

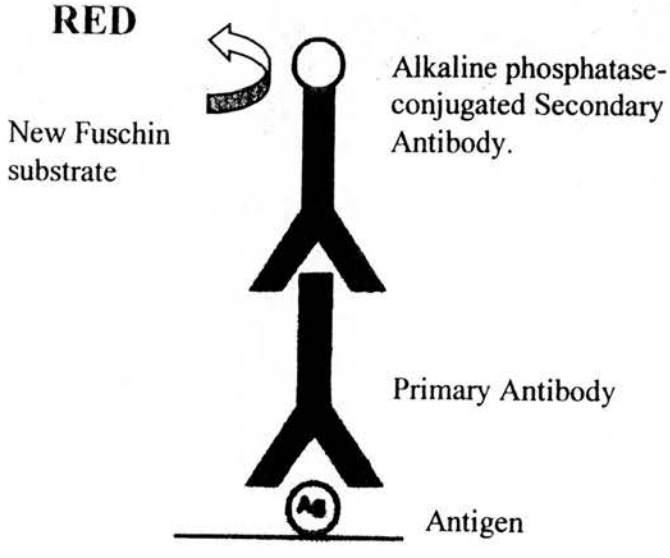



**Figure 2.4.** Diagram of the antibody binding cascades in (a) peroxidase method and (b) alkaline phosphatase method

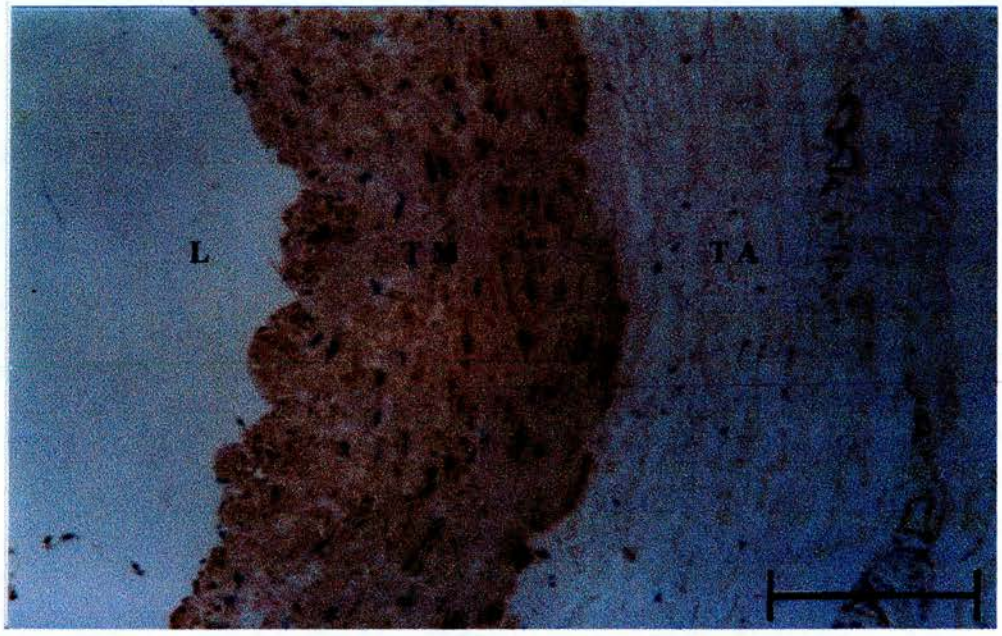


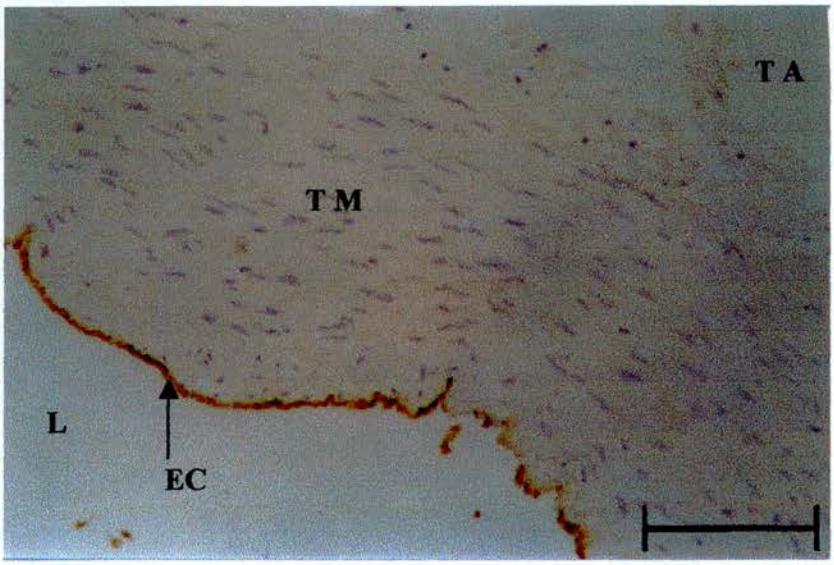

**Figure 2.5.** Photomicrograph of transverse section of hepatic artery stained with the α-SMA antibody of the vascular smooth muscle cell (VSMC) in the tunica media, using peroxidase method. L, lumen; TM, tunica media and TA, tunica adventitia. Scale=100μm.

The primary antibody, mouse anti-human α-smooth muscle actin (Novocastra laboratories Ltd., UK), was diluted to a concentration of 1/100 in BSA+PBS (as previously used in this laboratory) and 4 drops of biotin/ml of solution were added. This biotinylated primary antibody was applied to all arterial sections except those used as negative controls. Sections were incubated overnight (16-18 hours) at 4°C. Following incubation, the sections were equilibrated at room temperature for 30 minutes then given 3 washes for five minutes each with PBS. The biotinylated secondary antibody (1/100, goat anti-mouse antibody, Dako Ltd., UK; in BSA+PBS) was added to each section and, after 30 minutes, these were washed with PBS (3 washes for 5 minutes each). The streptavidin-biotin-horseradish peroxidase complex (Strep-ABC, Dako Ltd., UK) was then added to the sections and incubated for 30 minutes. Rings were then washed twice with PBS for 5 minutes. In order to visualise the site of positive reactivity, the DAB substrate (Sigma, UK) was applied, colour was allowed to develop (20-30 minutes) and then the sections were washed with water (Gramham & Karnovsky, 1966).

Nuclei were counter-stained using Harris Haematoxylin (BDH, UK) for 3 minutes. The sections were rinsed in water, differentiated in acid alcohol for 30 seconds and then washed with water for 5 minutes. Finally, sections were dehydrated through graded alcohols to xylene, mounted in Depex (Sigma, UK) and cover-slipped.

*Endothelial Cells.* For the identification of the endothelial cells (Figure 2.6), arterial sections were dewaxed, rehydrated, treated with hydrogen peroxide, trypsinised and then exposed to diluted goat serum as described previously. The endothelial cell

**(a)**




**(b)**

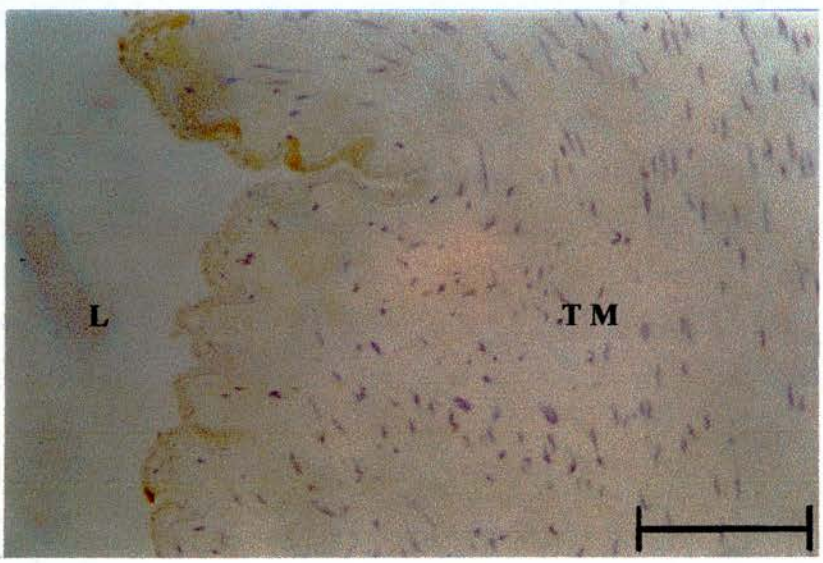



**Figure 2.6.** Photomicrographs of transverse sections of hepatic arteries stained with the Ulex antibody, using peroxidase method, **(a)** presence and **(b)** absence of the endothelial cells. L, lumen; EC, endothelial cell; TM, tunica media and TA, tunica adventitia. Scale=100μm.

antibody (Ulex, Vector Laboratories, UK; 1/200 dilution in 10% BSA/PBS) (as previously used in this laboratory) was applied for 30 minutes at room temperature for all sections except the negative controls. Following washing with PBS, sections were treated with streptavidin-biotin-horseradish peroxidase complex (Dako Ltd, UK.), exposed to DAB substrate and allowed to develop colour (30-40 minutes) for visualisation, as described above. After washing, sections were counter-stained with Harris-Haematoxylin, differentiated in acid alcohol and Depex mounted, as described previously.

#### 2.5.2.2. Alkaline Phosphatase Method

The alkaline phosphatase method was used for the detection of inducible nitric oxide synthase (iNOS) (Figure 2.7). In this technique, arterial sections were prepared and incubated with diluted goat serum (to reduce non-specific staining) as used previously in this laboratory.

The primary antibody (rabbit anti-iNOS antibody, Calbiochem, UK), dilution 1/150 (as previously used in this laboratory) was added to all sections except negative controls and incubated overnight (16-18 hours) at 4°C. The sections were washed with PBS and exposed to secondary antibody (goat anti-rabbit, Vector Laboratories, UK; dilution 1/100) for 30 minutes. The appropriate dilutions of antibodies and time of incubation were followed as determined previously in this laboratory. After washing with PBS, levamazole (10mM) (Sigma, UK) was applied to block endogenous phosphatase activity. In order to amplify the staining, fuschin (new fuschin kit, Dako Ltd., UK) was added to the sections and colour was allowed to

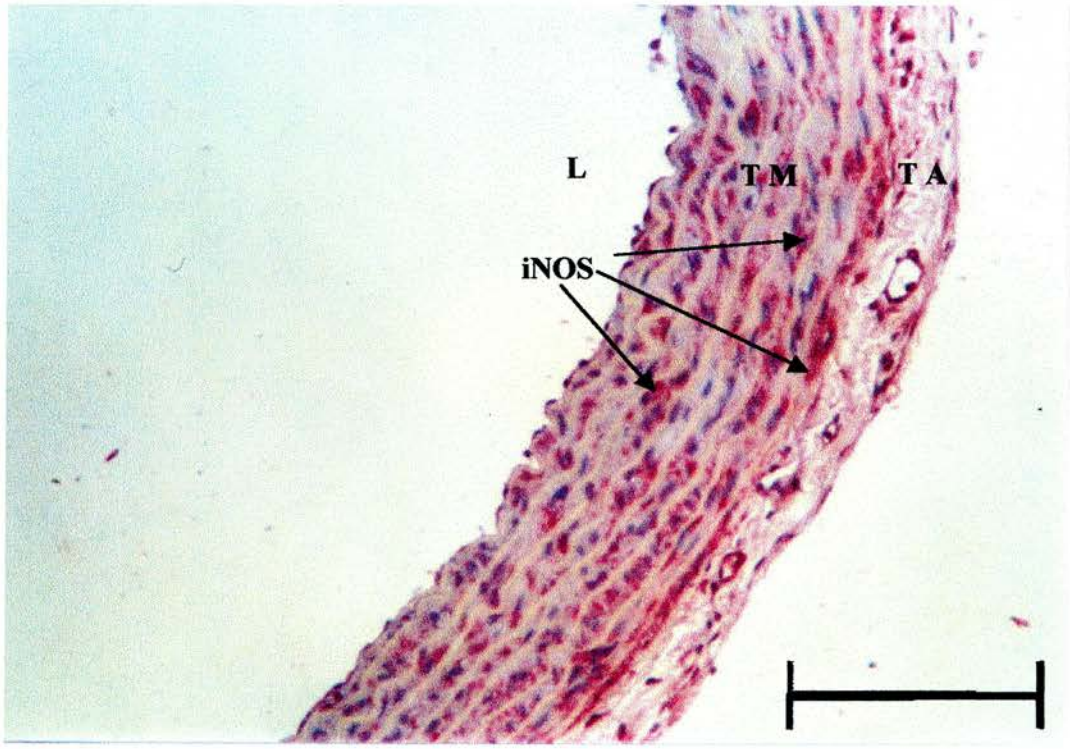



**Figure 2.7.** Photomicrograph of transverse section of rabbit thoracic aorta stained with the anti iNOS antibody using alkaline phosphatase method. L, lumen; TM, tunica media and TA, tunica adventitia. Scale=100μm.

develop (7-10 minutes). The sections were counter stained with Harris-Haematoxylin, differentiated in acid alcohol and aqua-mounted (BDH, UK) as described previously.

*2.5.3 Preparation of Working Solutions*

2.5.3.1. Tris Buffered Saline (0.05M, pH 7.6)

Tris base (6.04g) was dissolved in 1 litre deionised water and, after addition of thymol (1mg), the pH was adjusted at 7.6 by adding concentrated HCl solution. This tris buffered saline was suitable for use for up to 14 days.

2.5.3.2. Phosphate Buffered Saline (0.01M, pH 7.6)

Di-sodium hydrogen orthophosphate (63.5g) was added to 400 ml of deionised water and dissolved by microwaving for 30 seconds. Sodium di-hydrogen orthophosphate (8.5g) was also dissolved in 400 ml of deionised water, these two solutions were mixed and the volume increased to 5 litre using deionised water. The pH was adjusted to 7.6 by adding concentrated HCl.

**2.6. Materials**

8-Arginine vasopressin acetate, 5-hydroxytryptamine creatinine sulphate, prazosin hydrochloride, yohimbine hydrochloride, benzylpenicillin, $d(CH_2)_5Tyr(Me)AVP$, desmopressin, $N^G$-nitro-L-arginine (L-NNA), phenylephrine hydrochloride, acetylcholine chloride, indomethacin hydrochloride, hydrogen peroxide and trypsin were obtained from Sigma, Poole, Dorset, UK. University of Wisconsin solution (UWS) was obtained from NPBI International BV, Emmer-Compascuum, The

Netherlands and Du-Pont Pharmaceuticals Ltd., Herts, UK. Dexamethasone sodium phosphate from Faulding Pharmaceuticals, Warwick, UK. Insulin (velosulin) from NovoNordisk, Sussex, UK. 3'-Morpholinosydnonimine (SIN-1), ketanserin tartrate, $N^G$-nitro-D-arginine (D-NNA) and endothelin-1 were purchased from Alexis Corporation (UK) Ltd., Bingham, Nottingham, UK. 5-Carboxamidotryptamine maleate and methiothepin maleate were a gift from Glaxo-Wellcome Ltd. and Roche, Hertfordshire, UK., respectively. All salts were obtained from BDH, Poole, Dorset, UK. Stock solutions of drugs were prepared in deionised water except for indomethacin, which was prepared in ethanol (final bath concentration of ethanol did not exceed 0.1% v/v). As SIN-1 is sensitive to light, stock solutions were prepared in a strictly light controlled room and preserved in a light protective covering. Drugs were stored in 1.5 ml aliquots at -20° C, thawed as required and any residual amounts were discarded at the end of the experiments. Concentrations given are the final molar concentrations in the organ bath.

**2.7. Statistics**

Results are expressed as mean ± standard error of the mean (SEM), where 'n' represents the number of subjects. For each artery, mean values were calculated from the results of analysis of 2-4 rings where appropriate. The maximum contractile response of the receptor-dependent agonists was expressed both in grammes and as a percentage of the maximum contraction to KCl (to minimise any discrepancy in the sizes of the arterial rings (Lew & Angus, 1992)). The contractile responses to the receptor-independent vasoconstrictor, KCl, were expressed as a percentage maximum contraction and also in grammes. Sensitivity ($pD_2$ or $IC_{50}$) values were

determined for each concentration-response curve, using curve-fitting software (Fig. P., Biosoft, Cambridge, UK). For determining the relaxation effect, the initial sub-maximal contraction with PE was considered as 100% response and relaxation responses were calculated relative to this value. The sensitivity of relaxation responses to SIN-1 or acetylcholine is expressed as a negative log of the effective concentration (M, mol/L) of the drug required, producing 50% of the maximum response ($-\log IC_{50}$). Statistical analysis of entire curves was performed using two way analysis of variance (ANOVA) followed by the Tukey post-hoc test where appropriate. Maximum contraction and sensitivity ($pD_2$; $-\log IC_{50}$) values were compared using Student's unpaired or paired t-test, as appropriate, and values were considered significant when $P < 0.05$.

# CHAPTER THREE

# FUNCTIONAL RESPONSES OF PORCINE SPLANCHNIC (HEPATIC AND MESENTERIC) ARTERIES TO ARGININE VASOPRESSIN AND 5-HYDROXYTRYPTAMINE

### 3.1. Introduction

Vascular complications have a significant impact on the morbidity and mortality of patients with hepatic cirrhosis (Liach *et al.*, 1988). Splanchnic vessels, both from patients and from animal models of cirrhosis, have been used to investigate the manifestation and pathophysiology of abnormal vascular function associated with this condition. A complete understanding of normal vascular function is necessary to determine the alterations caused by the presence of disease. This is particularly important for agonists, such as 5-HT, because actions of this agonist can be mediated by several heterogeneous receptor sub-types (Martin & Humphrey, 1994) and the activity of some of these receptors may depend on the basal contractile state of the vessel (Yildiz *et al.*, 1998). For example 5-$HT_1$-like receptors only become active in some arteries *in vitro* following partial pre-contraction (Choppin & O'Connor, 1994, 1995).

One of the major limitations of *in vitro* studies of vascular dysfunction in cirrhosis is the unavailability of suitable human arteries. Whilst hepatic and mesenteric arteries, as well as portal veins, can be obtained from donors during liver transplantation (Smith *et al.*, 1997; Hadoke *et al.*, 1998; Heller *et al.*, 1999), these are still relatively scarce. Furthermore, it is possible that resuscitative procedures and liver preservation affect the normal physiological response of these vessels (Nataf *et al.*, 1995; Sorajja *et al.*, 1997). Therefore, it is necessary to investigate functional responses of splanchnic arteries from a suitable animal model as this will: (i) determine the normal physiological function of these vessels and (ii) provide data allowing development of a suitable protocol to investigate the effect of cirrhosis on these

responses. Vascular function has been studied in splanchnic arteries from rats (Cummings *et al.*, 1986; Ralevic *et al.*, 1996) and rabbits (Sitzman *et al.*, 1995; Cahill *et al.*, 1994) using a variety of vasoactive factors. However, the anatomy and physiology of the liver and splanchnic vessels in these small animals are different from those in humans (Vagianos *et al.*, 1990). In contrast, there is a close similarity in the liver and splanchnic vascular system in humans and pigs (Changani *et al.*, 1999; Swindle, 1984). Indeed, the function of porcine mesenteric arteries has been used as a model for responses of human vessels in previous studies of hepatic artery function in cirrhosis (Heller *et al.*, 1999). Therefore, porcine hepatic arteries provide a suitable model for developing the methodology required to assess the effect of cirrhosis on the responses of human hepatic arteries to AVP and 5-HT.

## 3.2. Aim

This study aimed to determine the normal functional responses of porcine splanchnic arteries *in vitro* and, thus, provide a basis for subsequent investigations using human hepatic arteries. Functional responses to be investigated included contractile responses to (i) the receptor-dependent agonists AVP and 5-HT and (ii) the receptor-independent vasoconstrictor, KCl, as well as (iii) relaxant responses to endothelium-dependent and endothelium-independent vasodilators.

## 3.3. Methods

### *3.3 1. Acquisition of Porcine Hepatic and Mesenteric Arteries*

Hepatic arteries and a section of the superior mesenteric artery were obtained from male and female, large white adult pigs (6-8 months old, 45-60 kg weight) from a

local abattoir. Arteries were placed immediately into ice-cold, oxygenated KHS and transported to the laboratory as described (Chapter 2.1). Hepatic and mesenteric arteries were also obtained from piglets (male and female; small white; 12-15 kg; aged 3-4 weeks), which had been killed for the purposes of another study (as described in Chapter 2.1).

### *3.3.2. Functional Analysis*

#### 3.3.2.1. Preparation of Arterial Rings

Adherent connective tissue and fat were cleaned from the arterial wall and rings, approximately 2mm in length, were cut from each vessel. Each ring was suspended in an organ bath containing freshly prepared KHS at 37°C and continuously gassed with a mixture of 95% $O_2$ - 5% $CO_2$, for the measurement of isometric force development (Chapter 2.2).

#### 3.3.2.2. Assessment of Viability and Endothelial Cell Function

The rings were stretched gradually to their optimum resting force, (4g; Hadoke *et al.*, 1998) and allowed to equilibrate for 55-60 minutes. Viability and reproducibility of contraction were tested using (100mmol/L) KCl (as described in Chapter 2.2). Initially, the presence of a functional endothelium was determined (adult pigs, n=7-8; piglets, n=4-5) by exposing the rings to ACh ($10^{-9}$-$3x10^{-5}$ mol/L), following precontraction with PE ($10^{-6}$-$3x10^{-5}$ mol/L) (Vanhoutte & Miller, 1985; Jeng *et al.*, 1996a,b). Endothelium-independent relaxation in the hepatic arteries was also assessed by treating the precontracted rings with SIN-1 ($10^{-9}$-$3x10^{-5}$ mol/L). At the end of the functional study, the rings were removed from the organ baths and

endothelial cell integrity was assessed histologically by silver nitrate staining (Chapter 2.4). For the rest of the study any residual endothelium was removed from the arteries as described (Chapter 2.2).

### *3.3.3. Assessment of Contractile Function in Porcine Hepatic and Mesenteric Arteries*

Cumulative concentration-response curves were produced using AVP ($10^{-11}$-$3x10^{-7}$ mol/L), 5-HT ($10^{-9}$-$3x10^{-5}$ mol/L) and KCl (2.5-120mM) in arteries from adult pigs (hepatic, n=28; mesenteric, n=24) and piglets (hepatic, n=9; mesenteric, n=7). The rings were washed repeatedly following completion of each curve and equilibrated for 40 minutes before the next concentration-response curve was obtained.

### *3.3.4. The Influence of Partial Pre-Contraction on 5-HT-Mediated Contraction of Porcine Hepatic Arteries*

#### 3.3.4.1. Preparation of Hepatic Arterial Rings

Four rings were prepared from each hepatic artery taken from adult pigs (n=11) or piglets (n=8), and prepared for measurement of isometric force generation, as described (Chapter 2.2). Any endothelium was removed from these arterial rings by rubbing the luminal surface (as described in Chapter 2.2). An $\alpha_1$-(prazosin; $10^{-6}$ mol/L) and an $\alpha_2$-(yohimbine; $10^{-6}$ mol/L) adrenoceptor antagonist were added to the KHS for the duration of the experiment. The viability and reproducibility of the contraction of each ring was tested using KCl as described (Chapter 2.1.3.3).

3.3.4.2. Functional Protocol

Two of the four rings were partially pre-contracted with KCl (20-40mM) to produce 25%-30% of the maximal response to KCl whilst the remaining two rings were not pre-contracted. One pre-contracted and one non-contracted ring were incubated with the 5-$HT_{2A}$-receptor antagonist, ketanserin ($10^{-6}$ mol/L) for 40 minutes. The remaining two rings were not treated with the antagonist and served as controls. Cumulative concentration-response curves were then constructed in control and incubated (in the continued presence of ketanserin) arterial rings using 5-HT ($10^{-9}$-$3x10^{-5}$ mol/L). Finally, the arteries were washed and allowed to equilibrate for 40mins, after which cumulative concentration-response curves were produced in the absence of ketanserin, using KCl (2.5-120mM).

## 3.4. Statistics

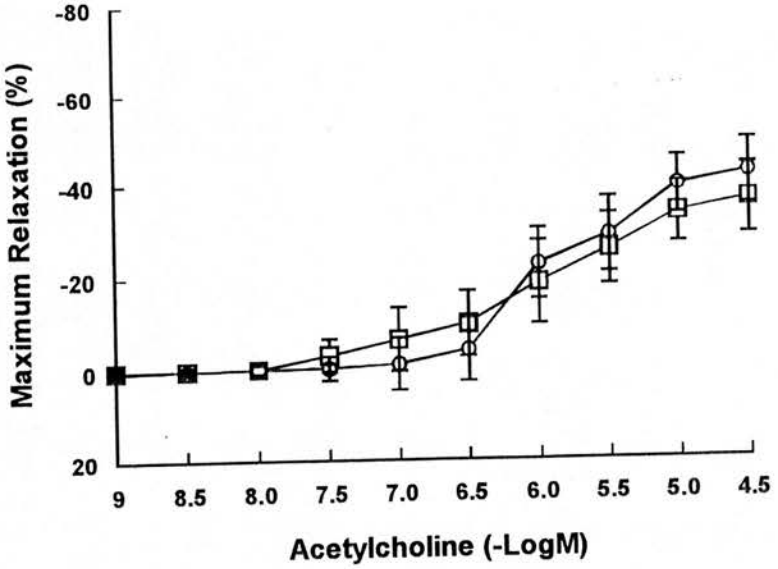
Statistics analysis was performed as described in Chapter 2.7.

## 3.5. Results

### *3.5.1. Assessment of Endothelium*

None of the arterial rings from adult pigs (8 hepatic, 7 mesenteric) tested for endothelial cell function relaxed in response to ACh (indicated by positive value). Instead, ACh produced a cumulative concentration-dependent contraction (indicated by negative value) in both hepatic (Emax., -35.82 ± 7.61%;) and mesenteric (Emax., -41.75 ± 7.15%) arteries (Figure 3.1a). In contrast, low concentration of ACh produced small relaxations in hepatic (Emax., 11.17 ± 6.02 %; n=5) and mesenteric (Emax., 17.26 ± 7.88 %; n=4) arteries from piglets (Figure 3.1b). However, at higher

**(a)**




**(b)**

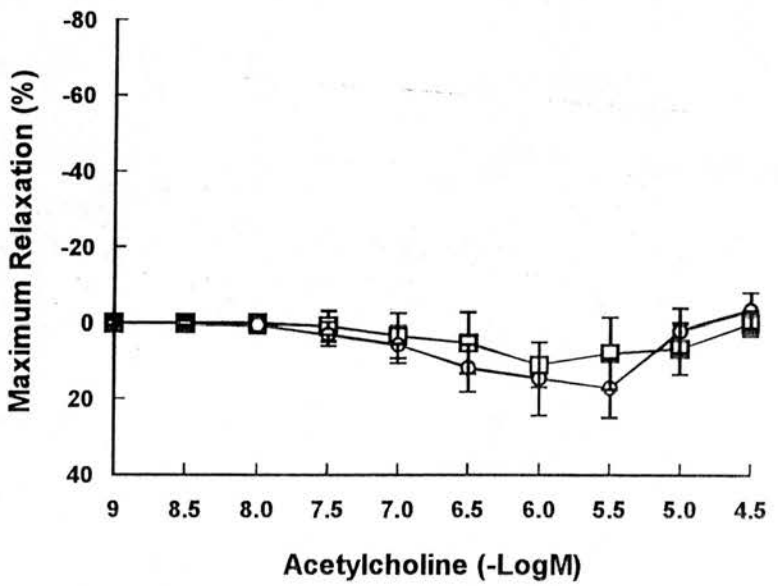



**Figure 3.1** Cumulative concentration-response curves to acetylcholine (ACh; $10^{-9}$-$3x10^{-5}$ mol/L) in hepatic (□) and mesenteric (○) arteries from **(a)** adult pigs (n=7-8) and **(b)** piglets (n=4-5), following pre-contraction with phenylephrine (PE; $10^{-6}$-$3x10^{-5}$ mol/L). Responses are mean ± s.e. mean for (n) individuals and expressed as a percentage of contraction induced by PE.

doses of ACh the response was reversed and the arteries were contracted (Figure 3.1b).

The hepatic arterial rings relaxed in response to SIN-1 (Figure 3.2) whether obtained from adult pigs (Emax., 112.04 ± 2.45%; -log$IC_{50}$, 6.48 ± 0.16) or piglets (Emax., 106.61 ± 3.21%; -log$IC_{50}$, 6.55 ± 0.18). The maximum relaxation and sensitivity of response to SIN-1 in these arteries were similar ($P$=0.20 and $P$=0.79, respectively).

Histological assessment with silver nitrate staining indicated that neither the hepatic nor the mesenteric arteries from adult pigs had an intact endothelium (Figure 3.3). In contrast, both the hepatic and mesenteric arteries from piglets displayed presence of residual endothelium (Figure 3.3).

*3.5.2. Contractile Responses of Porcine Hepatic and Mesenteric Arteries*

The receptor-dependent agonists AVP and 5-HT, and the receptor-independent vasoconstrictor, KCl, caused concentration-dependent contraction of denuded adult porcine hepatic and mesenteric arteries. The maximum contraction and sensitivity of the responses to these agonists were similar in hepatic and mesenteric arteries (Figure 3.4; Table 3.1a). The relative order of magnitude of maximum contraction (Emax) and potency ($pD_2$) of these contractile agents produced in hepatic (Emax., KCl>5-HT>AVP; $pD_2$, AVP>5-HT>KCl) and mesenteric (Emax., KCl>5-HT>AVP; $pD_2$, AVP>5-HT>KCl) arteries were similar (Table 3.1a).

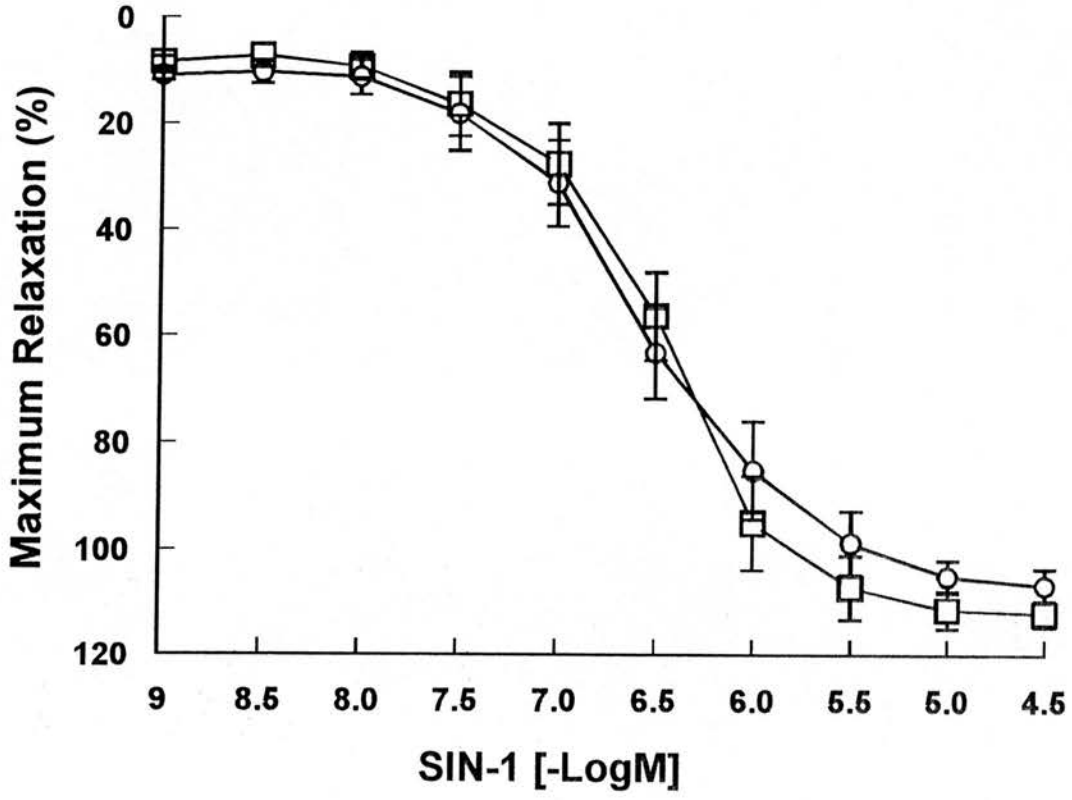
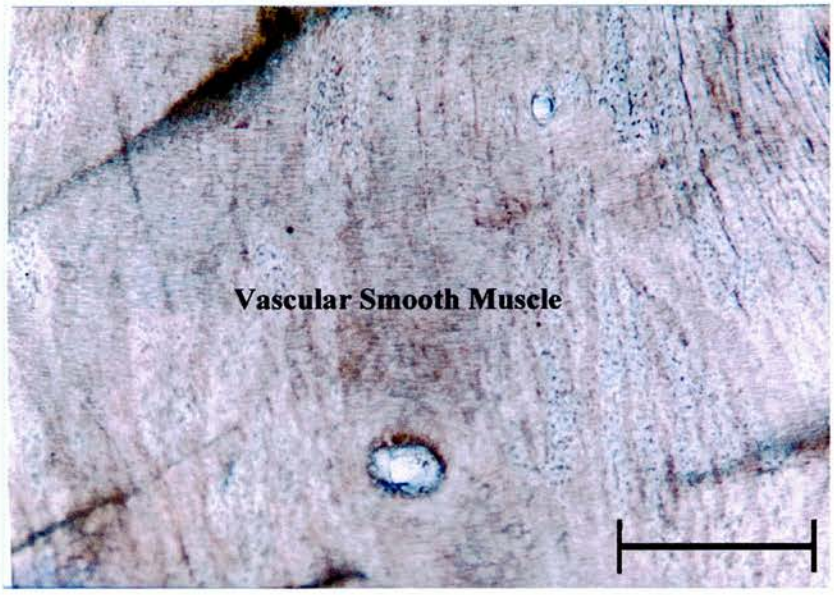



**Figure 3.2.** Cumulative concentration-response curves of hepatic arteries from adult pigs (□) and piglets (○), to 3'-morpholinosydnonimine (SIN-1; $10^{-9}$-$3x10^{-5}$ mol/L) following pre-contraction with phenylephrine (PE; $10^{-6}$-$3x10^{-5}$ mol/L). Responses are given as mean ± s.e. mean for (n) individuals, expressed as a percentage of the maximum contraction of PE.

**(a)** Adult Pig




**(b)** Piglet

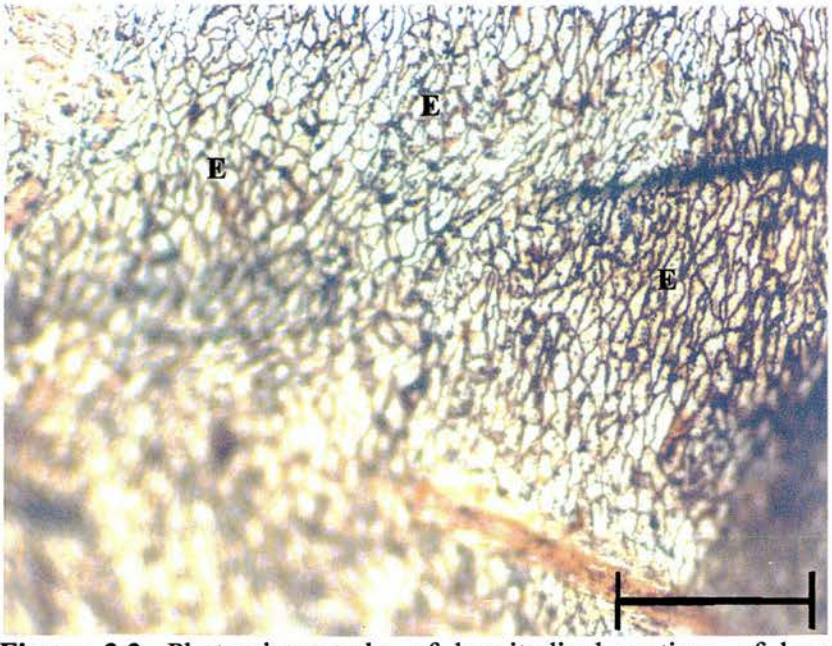



**Figure 3.3.** Photomicrographs of longitudinal sections of hepatic arteries from (a) adult pig and (b) piglet, stained with silver nitrate. The absence of endothelium (E) in adult porcine hepatic arteries is clearly evident from comparison with the piglet hepatic arteries. Scale=100μm.

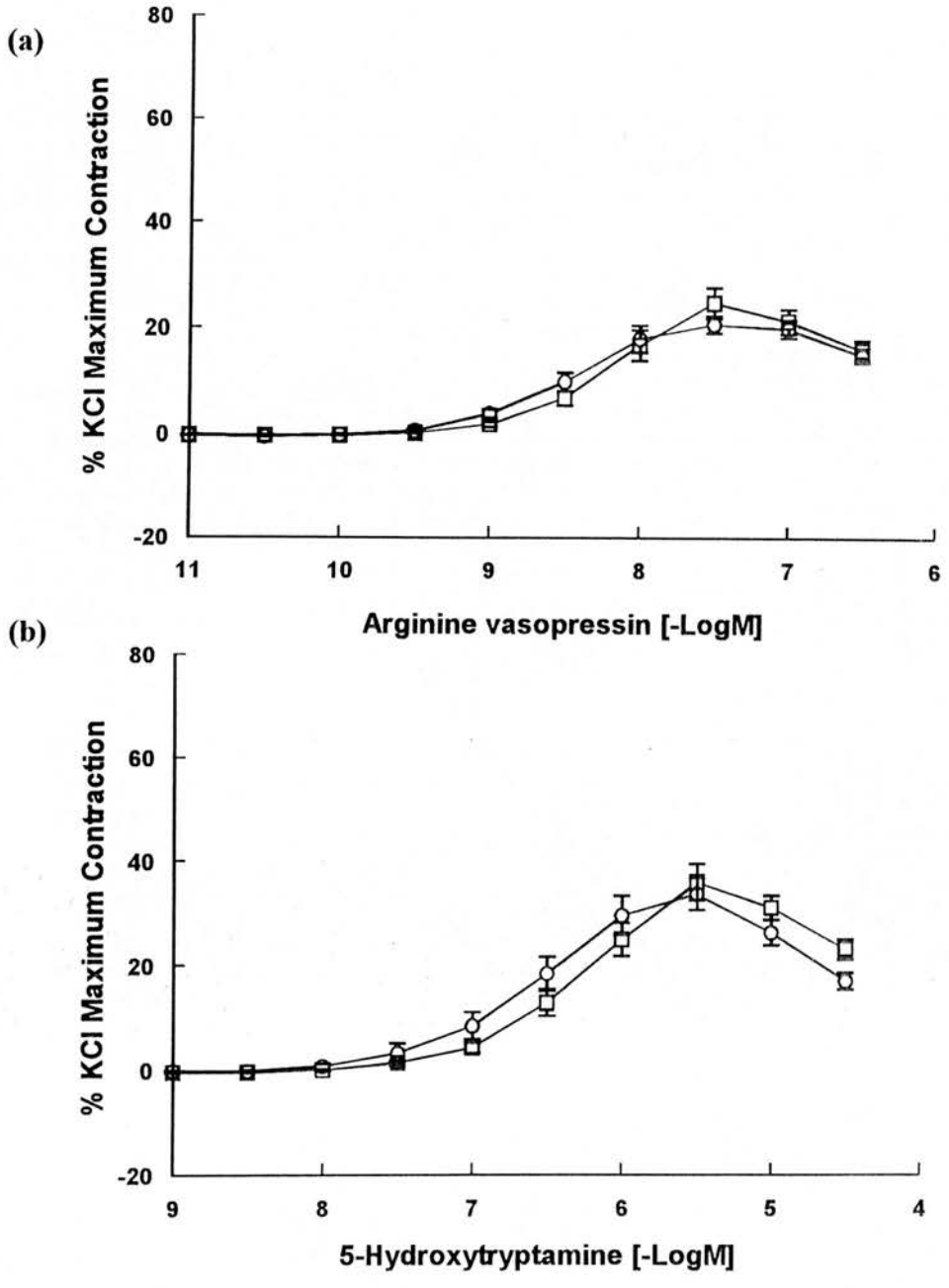



**Figure 3.4(a and b).** Cumulative concentration-response curves of hepatic (□) and mesenteric (○) arteries from adult pigs following exposure to **(a)** arginine vasopressin (AVP; n=18-21) and **(b)** 5-hydroxytryptamine (5-HT; n=23-28). Responses are mean ± s.e. mean for (n) individuals and expressed as a percentage of the maximum contraction induced by KCl.

(c)

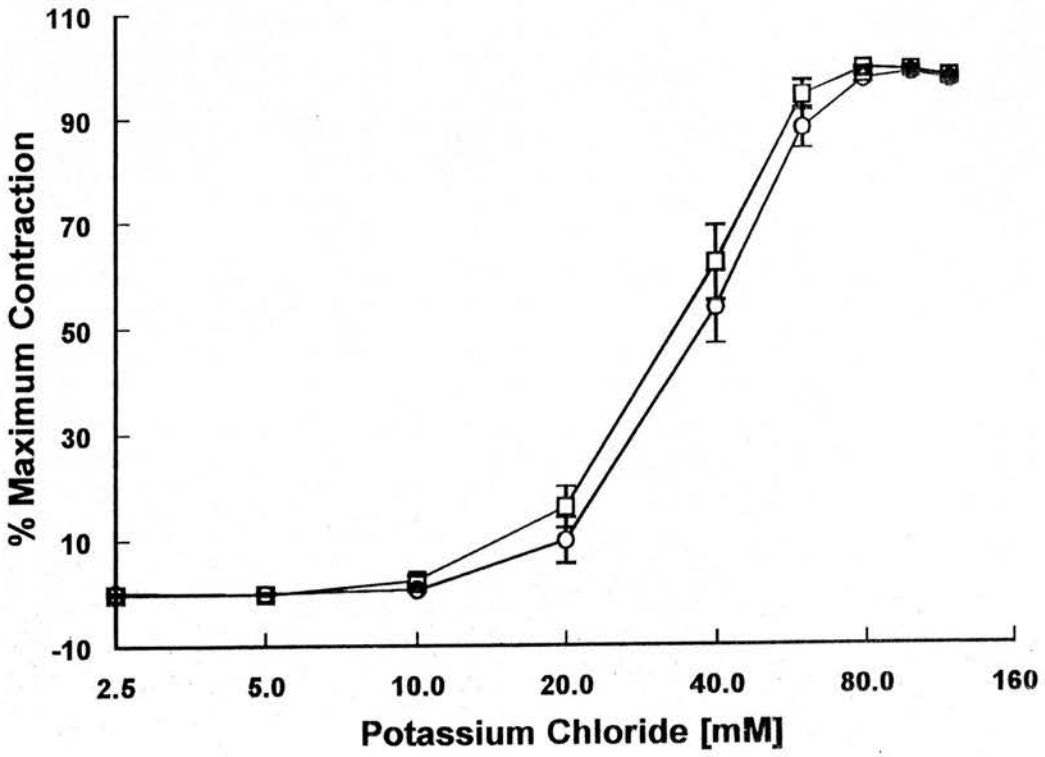



**Figure 3.4(c).** Cumulative concentration-response curves of hepatic (□) and mesenteric (○) arteries from adult pigs following exposure to potassium chloride (KCl; n=24-29). Responses are mean ± s.e. mean for (n) individuals and expressed as a percentage of the maximum contraction induced by KCl.

**Table 3.1** The maximum contraction (Emax) and sensitivity ($pD_2$) of porcine arteries to arginine vasopressin (AVP), 5-hydroxytryptamine (5-HT) and potassium chloride (KCl)

**(a) Adult Pigs**

| | Hepatic Artery | | | Mesenteric Artery | | |
|---|---|---|---|---|---|---|
| | Emax. | | $pD_2$ | Emax. | | $pD_2$ |
| | (g) | (% KCl) | | (g) | (%KCl) | |
| AVP | 2.83±0.20(21) | 26.30±2.27 | 8.12±0.07 | 3.06±0.25(18) | 25.58±1.87 | 8.14±0.09 |
| 5-HT | 4.06±0.28(28) | 40.25±3.12 | 6.10±0.08 | 4.16±0.37(23) | 37.68±3.23 | 6.23±0.07 |
| KCl | 11.32±0.68(29) | --------- | 1.47±0.03 | 11.48±0.87(24) | --------- | 1.45±0.03 |

**(b) Piglets**

| | Hepatic Artery (n=9) | | | Mesenteric Artery (n=7) | | |
|---|---|---|---|---|---|---|
| | Emax. | | $pD_2$ | Emax. | | $pD_2$ |
| | (g) | (% KCl) | | (g) | (% KCl) | |
| AVP | 2.44±0.36 | 21.60±2.83 | 8.04±0.12 | 2.71±0.26 | 24.71±2.52 | 8.16±0.12 |
| 5-HT | 0.47±0.14* | 4.43±1.43* | --------- | 0.52±0.11* | 4.95±1.20* | --------- |
| KCl | 11.34±1.06 | --------- | 1.46±0.05 | 11.08±0.56 | --------- | 1.48±0.06 |

Values are mean ± s.e. mean for (n) subjects. *$P$<0.0001, when compared with responses to 5-HT in adult porcine arteries, using Student's unpaired t-test.

Denuded hepatic and mesenteric arteries from piglets produced concentration-dependent contractions, similar to those produced in arteries from the adult pigs, when exposed to AVP and KCl (Figure 3.5; Table 3.1b). In contrast, 5-HT produced virtually no response or a very little response in these piglets arteries and the responses were not comparable with those produced in adult porcine hepatic and mesenteric arteries (Figure 3.5b; Table 3.1).

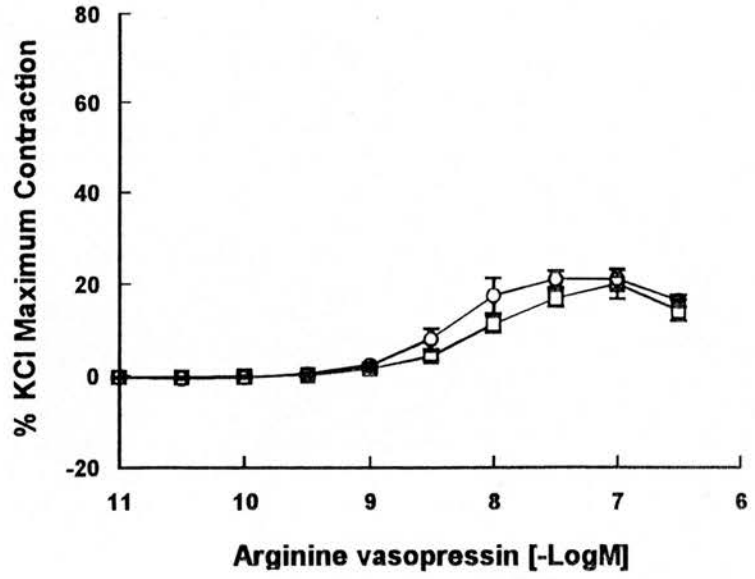
### *3.5.3. The Effect of Partial Pre-contraction on The Response of Porcine Hepatic Arteries to 5-HT.*

Following partial pre-contraction, the maximum contraction and sensitivity of the response to 5-HT in hepatic arteries either from adult pigs or piglets (Figure 3.6; Table 3.2) were increased, although in adult pigs the increased maximum contraction achieved only threshold significance ($P$=0.09; Table 3.2). More strikingly, in piglet hepatic arteries, after partial pre-contraction, the virtually inactive 5-HT produced a clear contraction similar to that produced in the adult porcine hepatic arteries (Table 3.2).

### *3.5.4. Effect of 5-HT Receptor Antagonists on the Contractile Response of Porcine Hepatic Arteries*

In non-contracted rings of hepatic artery from adult pigs the 5-$HT_2$-receptor antagonist, ketanserin, caused a rightward shift in the concentration-response curves; causing a significant reduction in sensitivity without altering the maximum contraction (Figure 3.7; Table 3.2). However, in the arterial rings either from adult pigs or piglets that had been partially pre-contracted, the effect of ketanserin was

(a)




(b)

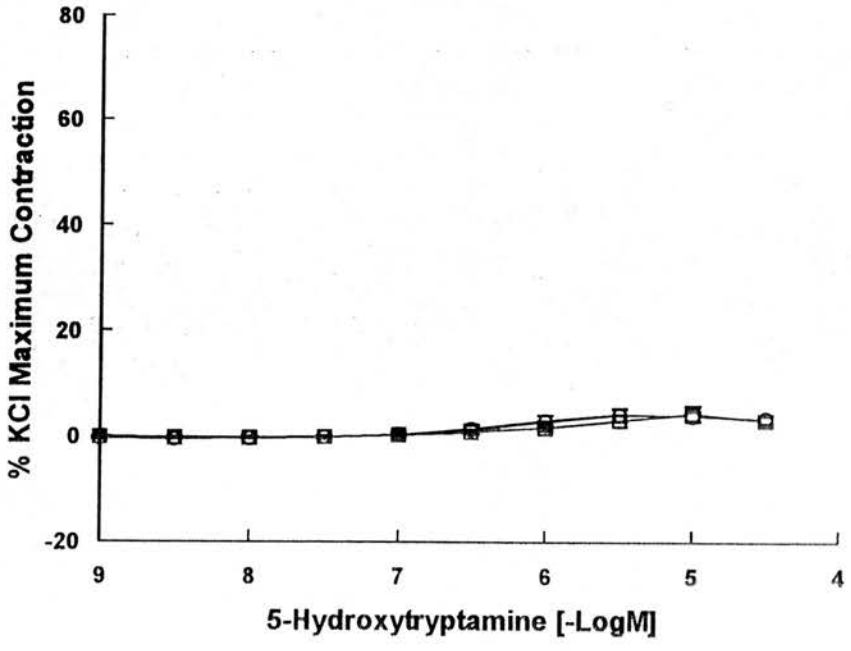



**Figure 3.5(a and b).** Comparison of cumulative concentration-response curves obtained in hepatic (□, n=9) and mesenteric (○, n=7) arteries from piglets in response to **(a)** arginine vasopressin (AVP) and **(b)** 5-hydroxytryptamine (5-HT). Responses are mean ± s.e. mean for (n) individuals and expressed as a percentage of the maximum contraction induced by KCl.

(c)

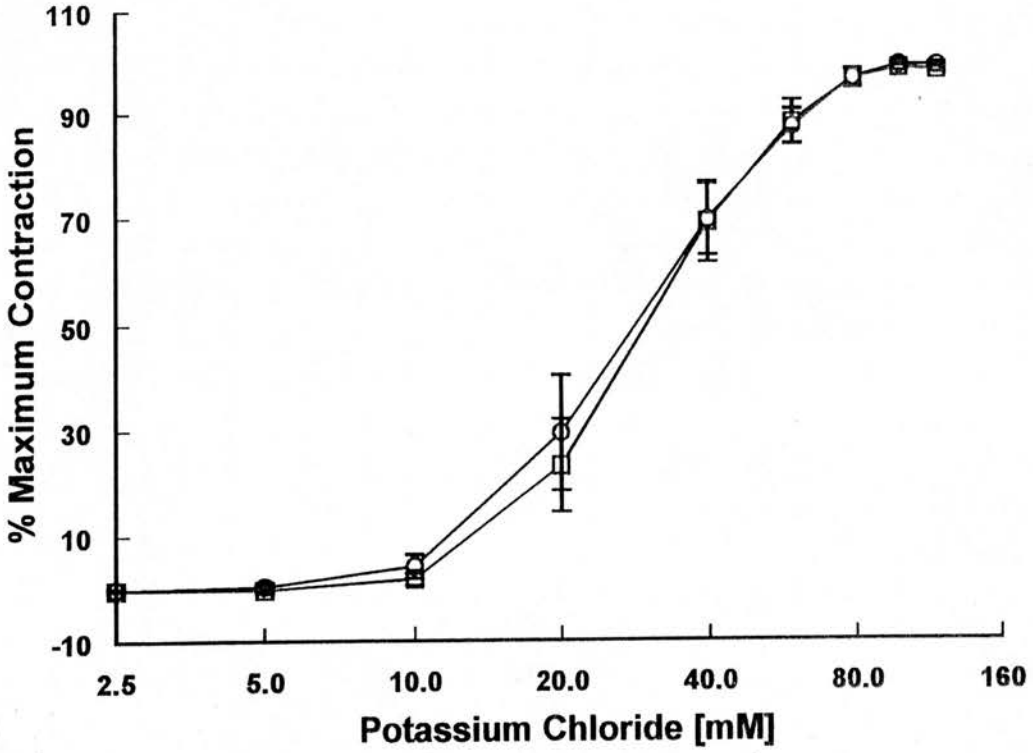



**Figure 3.5 (c).** Comparison of cumulative concentration-response curves obtained in hepatic (□, n=9) and mesenteric (○, n=7) arteries from piglets in response to potassium chloride (KCl). Responses are mean ± s.e. mean for (n) individuals and expressed as a percentage of the maximum contraction induced by KCl.

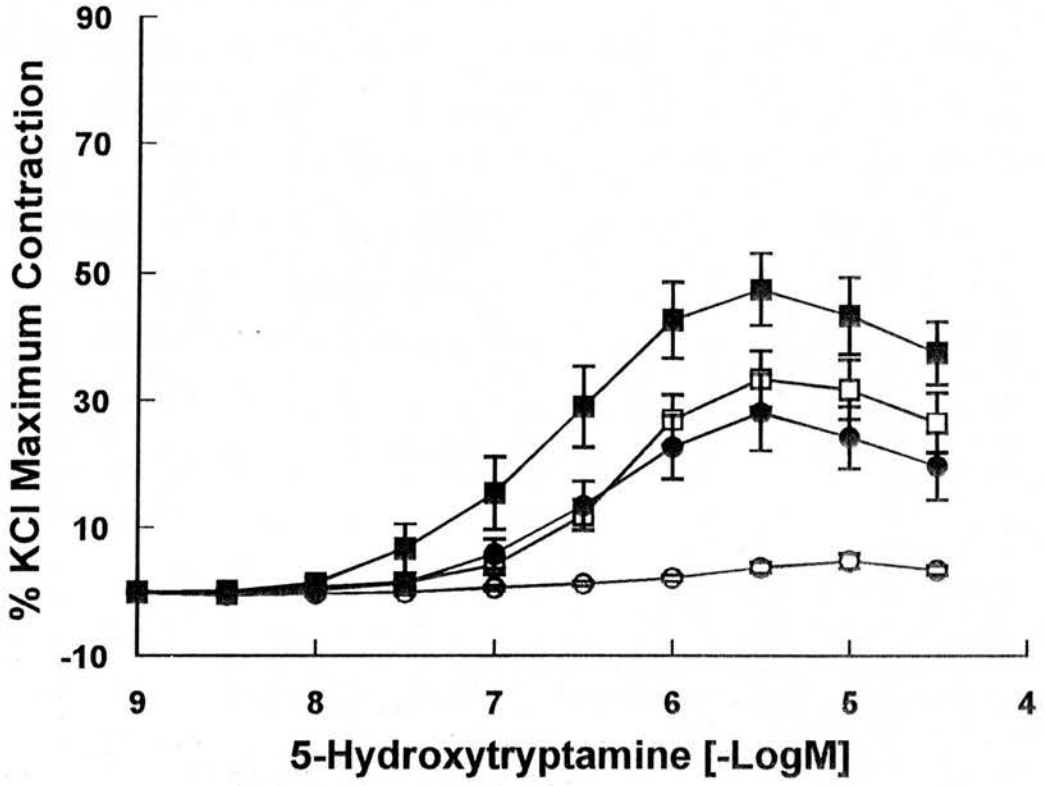



**Figure 3.6.** Cumulative concentration-response to 5-hydroxytryptamine (5-HT) in non-pre-contracted (□,○) and partially pre-contracted (■, ●) hepatic arteries obtained from adult pigs (squares; n=11), and piglets (circles; n=8). Responses are mean ± s.e. mean for (n) individuals and expressed as a percentage of the maximum contraction induced by KCl.

**Table 3.2.** The maximum contraction (Emax) and sensitivity ($pD_2$) of non-contracted (NC) and partially pre-contracted (PC) hepatic arteries from adult pigs and piglets in response to 5-hydroxytryptamine (5-HT) in the presence or absence of ketanserin (Ket).

| | Adult Pigs (n=11) | | | Piglets (n=8) | | |
|---|---|---|---|---|---|---|
| | Emax. | | $pD_2$ | Emax. | | $pD_2$ |
| | (g) | (% KCl) | | (g) | (% KCl) | |
| NC | 3.58±0.46 | 33.60±4.14 | 6.12±0.11 | 0.57±0.12 | 5.12±1.19 | -------- |
| NC+Ket | 3.06±0.43 | 27.95±4.39 | 5.76±0.11* | 0.48±0.18 | 4.79±1.94 | -------- |
| PC | 4.97±0.63 | 45.36±5.67 | 6.49±0.14* | 3.09±0.08** | 28.42±5.86** | 6.45±0.10 |
| PC+Ket | 4.47±0.60 | 40.97±6.05 | 6.25±0.11 | 2.32±0.35 | 26.96±2.53 | 6.30±0.14 |

Values are mean ± s.e. mean for (n) subjects. *$P<0.05$ and **$P<0.02$, when compared with corresponding non-contracted arterial rings, using Student's unpaired t-test. NC, non-contracted, PC, partially pre-contracted rings and +Ket, with ketanserin.

(a)

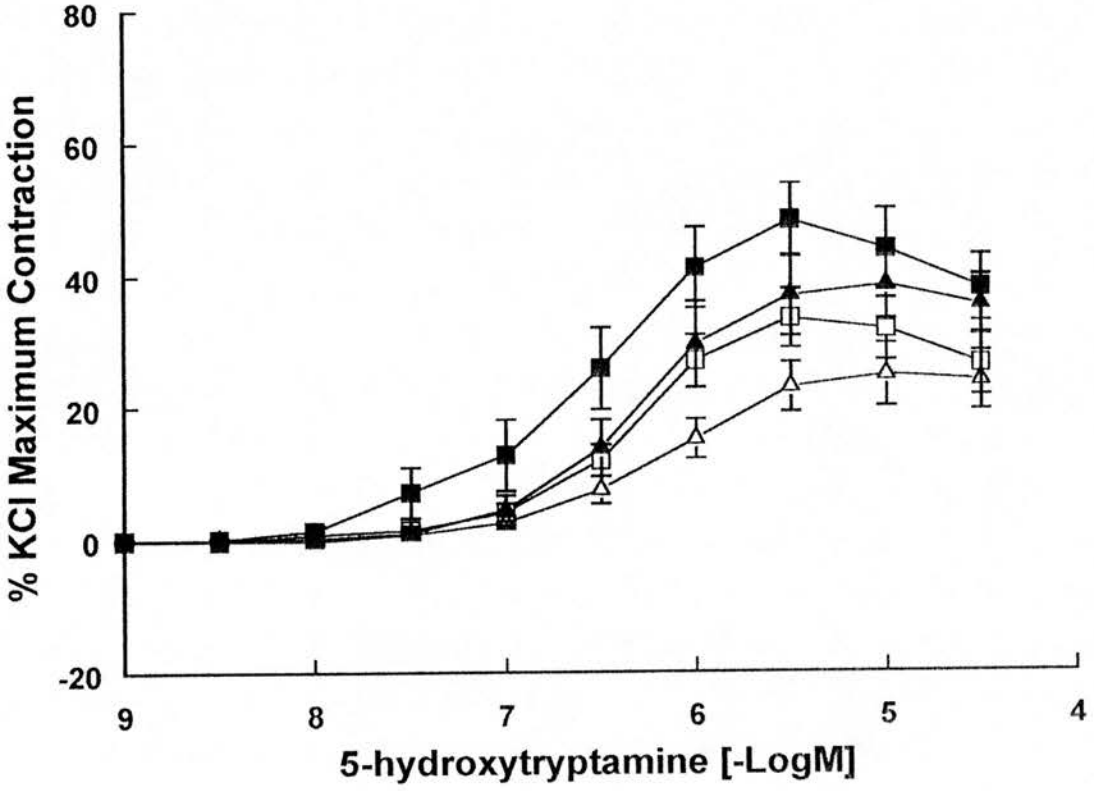



**Figure 3.7(a).** Cumulative concentration-response curves produced in response to 5-hydroxytryptamine (5-HT) in non pre-contracted (open symbol) or partially-precontracted (solid symbol) hepatic arterial rings from adult pigs (n=11) in the absence (control, □ and ■) or presence of ketanserin (Δ and ▲; $10^{-6}$ mol/L). Each point represents mean ± s.e. mean, expressed as a percentage of the maximum contraction induced by KCl.

**(b)**

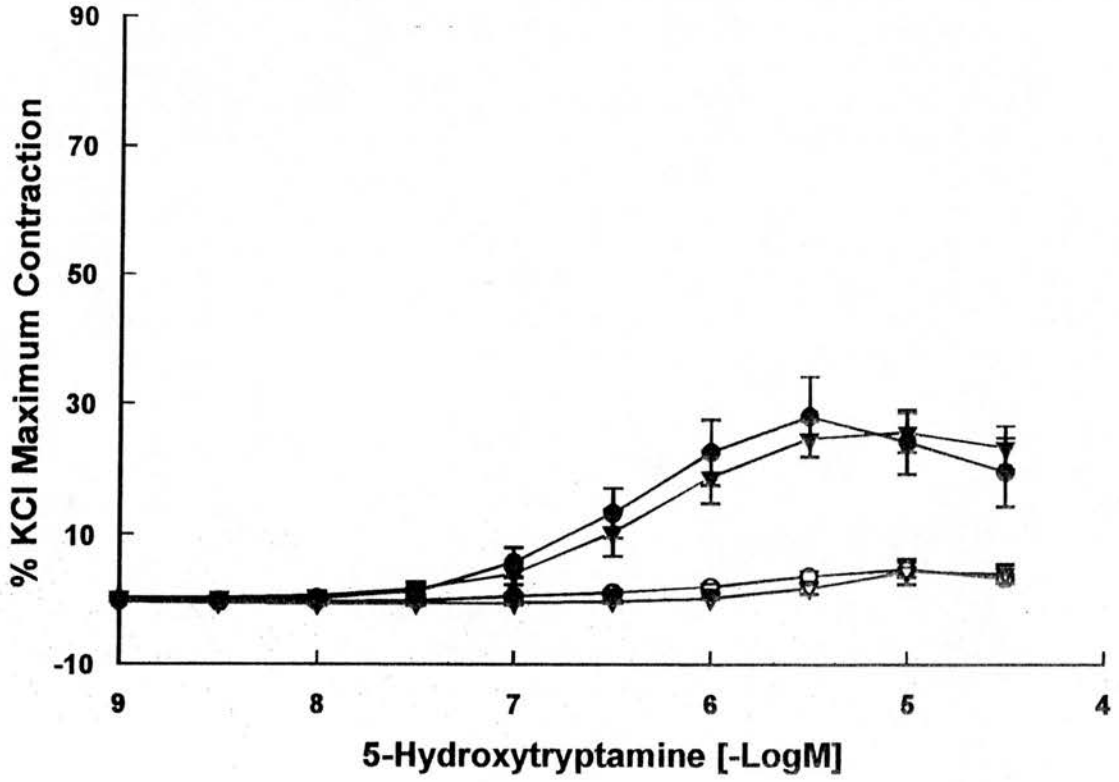



**Figure 3.7 (b).** Cumulative concentration-response curves produced in response to 5-hydroxytryptamine (5-HT) in non pre-contracted (open symbol) or partially-precontracted (solid symbol) hepatic arterial rings from piglets (n=8), in the absence (○ and ●) or presence of ketanserin (▽ and ▼; $10^{-6}$ mol/L). Each point represents mean ± s.e. mean, expressed as a percentage of the maximum contraction induced by KCl.

attenuated. In adult pig arteries, ketanserin produced a non-significant rightward shift in the concentration-response curve without altering maximum contraction ($P$=0.57) or sensitivity ($P$=0.18) (Figure 3.7a). In partially pre-contracted rings of piglet hepatic arteries, however, ketanserin had no effect on the concentration-response curve to 5-HT (Figure 3.7b; Table 3.2).

### *3.5.5. Potassium Chloride (KCl)-Mediated Response*

The maximum contraction and sensitivity of responses to KCl were similar in hepatic arteries from adult pigs or piglets, and previous partial pre-contraction and/ or exposure to antagonist did not affect this response (Table 3.3).

## 3.6. Discussion

This study was designed to develop a protocol to investigate the contractile responses of isolated human hepatic arteries to AVP and 5-HT, using porcine splanchnic arteries as a model. It was necessary to use an alternative to human hepatic arteries, as these are difficult to obtain for functional investigation. For this study, arteries were used without endothelium as this was consistently shown to be damaged during isolation of the vessels. However, the absence of endothelium was not considered to be an obstacle because (as described in previous studies of vascular abnormalities in cirrhosis (Hadoke *et al.*, 1998)) human hepatic arteries obtained from the operating theatre are likely to have no endothelium for similar reasons (Smith *et al.*, 1997; Heller *et al.*, 1999). It was demonstrated that both hepatic and mesenteric arteries from adult pigs responded to AVP and 5-HT in the concentration-range used. However, an unexpected observation was that 5-HT failed to contract arteries from

**Table 3.3.** The maximum contraction (Emax) and sensitivity ($pD_2$) of responses to potassium chloride (KCl) in hepatic arteries from adult pigs and piglets following cumulative concentration-response curves (CCRC) to 5-HT.

| | Adult Pigs (n=11) | | Piglets (n=8) | |
|---|---|---|---|---|
| | Emax. (g) | $pD_2$ | Emax. (g) | $pD_2$ |
| NC | 10.88±0.76 | 1.49±0.05 | 11.28±0.58 | 1.48±0.06 |
| NC+Ket | 11.24±0.80 | 1.48±0.06 | 10.91±1.90 | 1.52±0.07 |
| PC | 10.86±0.73 | 1.46±0.05 | 10.51±1.06 | 1.47±0.05 |
| PC+Ket | 11.59±0.88 | 1.50±0.05 | 10.88±1.12 | 1.49±0.04 |

Values are mean ± s.e. mean for (n) subjects. NC, non-contracted; PC, previously partially pre-contracted rings; +Ket, previously exposed to ketanserin. Values were compared using Student's unpaired t-test.

piglets, although these did contract in response to AVP and KCl. 5-HT-mediated contraction could be evoked in piglet hepatic arteries if they were initially contracted with a sub-maximal concentration of KCl; a similar technique has been used in other vessels to activate 5-$HT_1$-like-receptors (Choppin & O'Connor, 1994, 1995; Yildiz *et al.*, 1998).

Isolated hepatic arteries have been used almost exclusively for investigations of vascular function in patients with hepatic cirrhosis. The main reason for this is that hepatic arteries from both donor and recipient can be obtained during liver transplantation. However, this is a suitable choice for such investigations as the hepatic artery is a part of the splanchnic vascular territory, which has been shown to be the primary site of vasodilatation during the development of the hyperdynamic circulation (Vorobioff *et al.*, 1984; Iwao *et al.*, 1997a). These *ex vivo* studies have been restricted in measuring responses to α-adrenoceptor agonists (Hadoke *et al.*, 1998; Heller *et al.*, 1999) reflecting the fact that most of the studies of pressor response in cirrhotic patients have also used adrenoceptor agonists (Lunzer *et al.*, 1975; MacGilchrist & Reid, 1990). The other pressor agent used *in vivo*, AII, has been used to stimulate human hepatic arteries *in vitro* but was found to produce small, erratic contractile responses (Hadoke *et al.*, 1996). Similar problems with AII-mediated responses *in vitro* have been experienced in mesenteric arteries from experimental animals (Dunn *et al.*, 1994; Falloon *et al.*, 1995). In both cases, however, reproducible AII-mediated contraction was observed in an isobaric system (in which the vessels are subjected to transmural pressure) and could be induced in the isometric system by partially pre-contracting the vessel. Vessels in an isobaric

system become depolarised in response to transmural pressure and this depolarisation state is mimicked by partial pre-contraction in an isometric system (Harder *et al.*, 1987). This suggests, therefore, that a certain amount of depolarisation of the vessel wall is necessary before the vessel will contract in response to AII.

Few studies have assessed responses to AVP and 5-HT in splanchnic vessels; those that are available have used arteries from rats or rabbits (Cummings *et al.*, 1986; Kaumann *et al.*, 1988; Jacob *et al.*, 1991; Huang *et al.*, 1995) which may not be good models for human arteries. It was, therefore, necessary to determine the concentration ranges for AVP and 5-HT that would produce a complete concentration-response curve in human hepatic arteries. The present study used porcine splanchnic (hepatic and mesenteric) arteries as a model for human hepatic arteries, as the latter are difficult to obtain in sufficient quantity for extensive *in vitro* investigation. The porcine splanchnic arteries are a suitable model because: (i) pigs have similar splanchnic anatomy to humans (Swindle, 1984) and, (ii) previous *ex vivo* investigations have demonstrated that α-adrenoceptor-mediated contraction is similar in human and porcine hepatic arteries (Hadoke *et al.*, 1995). Both mesenteric and hepatic arteries were used in a previous study also showed that contractile responses to α-adrenoceptor agonists were similar in these arteries (Hadoke *et al.*, 1995).

This study highlighted the problems of damage to the endothelium that can occur during vessel retrieval, as the endothelium was completely absent from arteries isolated from adult pigs and only a small amount remained in those from piglets.

Failure to relax in response to ACh was not a result of inability of the smooth muscle to respond to a relaxant stimulus as these arteries retained the ability to relax when exposed to the endothelium-independent dilator, SIN-1. The absence of endothelium from these vessels is probably a consequence of the retrieval procedures. None of the pigs were sacrificed specifically for the purpose of this study; in order to reduce the number of experimental animals used, vessels were collected from a local abattoir (adult pigs) and from piglets being used for hepatic isolation (for the purposes of a separate project). The fact that these porcine vessels did not have a functional endothelium was not considered problematic for this investigation, as it is likely that hepatic arteries obtained from the transplant theatre would also not have an intact endothelium. Indeed, there are many descriptions of human vessels obtained from the operating theatre, which have lost their endothelium as a result of surgical manipulation (Smith *et al.*, 1997; Hadoke *et al.*, 1998; Heller *et al.*, 1999; Haudenschild *et al.*, 1981). In fact, intrahepatic arteries with an intact endothelium can be obtained (Lin *et al.*, 1994; Jeng *et al.*, 1997) which reinforces the suggestion that denudation results from vessel manipulation during surgery. Therefore, denuded porcine hepatic and mesenteric arteries are still a valid model for developing a functional protocol with AVP and 5-HT, and for this reason endothelial cell removal was ensured in all vessels.

Previous studies have demonstrated similar contractile responses to α-adrenoceptor agonists in human hepatic and mesenteric arteries (Hadoke *et al.*, 1995). This is consistent with the demonstration that contractile response to AVP, 5-HT or KCl is similar in porcine hepatic and mesenteric arteries. In both groups of arteries, AVP

had the highest potency and KCl the lowest. In contrast, KCl produced the largest maximum contraction and AVP the lowest. A similar pattern of response to AVP, 5-HT and KCl has also been reported in other studies in animal vessels (Moreno *et al.*, 1996; Cummings *et al.*, 1986). The concentration range of AVP, 5-HT or KCl used in these study produced a clear maximal contraction followed by a loss of tone at higher concentrations, enabling calculation of maximum contraction as well as sensitivity ($pD_2$) values for these agents in porcine arteries (Lew, 1995). In cirrhosis, both the maximum contractile response of the vessels and sensitivity to the agonist needs to be assessed, as some studies reported altered maximum contraction (Heller *et al.*, 1999; Cahill *et al.*, 1996) while in others the sensitivity of the response to vasoactive agents was affected (Cummings *et al.*, 1986; Jacob *et al.*, 1991). These results suggest, therefore, that the concentration ranges used in the porcine hepatic arteries would be suitable for functional analysis of isolated human hepatic arteries.

AVP acts via two receptor sub-types, $V_1$ and $V_2$, both of which are located in the vessel wall (Phillips *et al.*, 1991). Vascular responses to AVP could, therefore, vary depending on the distribution of these receptor sub-types in the wall of a particular vessel (Garcia-Villallon *et al.*, 1996). Previous studies have indicated that vascular $V_2$ receptors are primarily located in the endothelium and can mediate vasodilatation by release of nitric oxide (Aki *et al.*, 1994; Tagawa *et al.*, 1995). In contrast, the $V_1$ receptor is primarily located in the VSMCs and mediates vasoconstriction (Thibonnier, 1992). Therefore, in this study AVP-induced contraction of denuded porcine splanchnic arteries is probably mediated by $V_1$ receptors on the VSMCs. Many studies have demonstrated AVP-mediated contraction of isolated vessels and,

furthermore, confirmed that this response was mediated by $V_1$ receptors located on VSMCs. Indeed $V_1$-mediated contraction has been shown in human renal (Medina *et al.*, 1996) and gastric arteries (Calo *et al.*, 1997) as well as in rat mesenteric arteries (Stam *et al.*, 1998). In the present study, the effect of the $V_2$ receptor has been eliminated as the endothelium was removed. It is probable, therefore, that the $V_1$ receptor in VSMCs is responsible for mediating vasoconstriction in the porcine splanchnic arteries. However, further studies with selective $V_1$ receptor antagonists and $V_2$ receptor agonists would be necessary to confirm this. As the porcine hepatic arteries were not the primary interest for this project, performing such studies was not considered to be a priority.

An unexpected outcome of the present study was the demonstration of a significant functional difference between the arteries from adult and juvenile pigs. Whereas contractile responses to AVP or KCl were similar (Emax and potency) in arteries from adult pigs and piglets, 5-HT failed to produce any response in the piglet hepatic and mesenteric arteries. This striking difference of contractile response to 5-HT was not due to size differences as it was specific to one agonist, 5-HT, and contractile responses to the receptor-independent smooth muscle vasoconstrictor, KCl, were unchanged. This indicated that age has an effect on 5-HT receptor-mediated constriction of the porcine hepatic arteries and suggests that 5-HT receptors become active during progression from juvenile to adult. An alteration in 5-HT receptor-mediated vasoconstriction has also been reported in rabbit pulmonary resistance arteries (PRA) between neonate (4 and 7 days) and adult individuals (Morecroft & MacLean, 1998). In adult rabbit PRA, a co-existence of different vasoconstrictor

5-HT receptor sub-types (5-$HT_{2A}$ and 5-$HT_{1B/1D}$) was indicated, whereas in neonates, vasoconstriction was predominantly mediated by the 5-$HT_{2A}$ receptor subtype. However, at present, no other study is found investigating the maturation of 5-HT receptors with ageing in the vasculature. Development and ageing also have an effect on AII-mediated vascular contraction, although in contrast to 5-HT, contraction decreases with age (Cai *et al.*, 1994). In the rat aorta, AII-mediated vascular contraction and inositol phosphate accumulation in 6 and 24 month-old animals was significantly reduced when compared with 1 month-old rat. However, consistent with the present results, other studies also reported that ageing and development had no significant effect on responses to AVP, which were similar in juvenile and adult rats (De Novellis *et al.*, 1994).

5-HT mediates vasoconstriction via stimulation of classical 5-$HT_{2A}$ receptors (Martin & Humphrey, 1994). At present, accumulating evidence has suggested that, in some blood vessels, an alternative 5-HT-receptor sub-type can also mediate vascular contraction (Yildiz *et al.*, 1998; Ishida *et al.*, 1999). Under isometric conditions, no contraction usually occurs via this sub-type but partial pre-contraction can activate or amplify the response (Choppin & O'Connor, 1994; Movahedi & Purdy, 1997; Cocks *et al.*, 1993). This vasoconstrictor sub-type has been classified as the 5-$HT_1$-like receptor (Yildiz *et al.*, 1996; Movahedi & Purdy, 1997; Morecroft & Maclean, 1998). This study demonstrated that, in adult pig splanchnic arteries, similar to other studies (Kaumann *et al.*, 1988; MacLean *et al.*, 1996; Martin & Humphrey, 1994), 5-HT-induced vasoconstriction was mediated via 5-$HT_{2A}$ receptors, as this contraction was effectively antagonised by a relatively selective 5-$HT_{2A}$-receptor antagonist,

ketanserin. However, following partial precontraction, responses were predominantly mediated via another vasoconstrictor 5-HT-receptor sub-type, as ketanserin had only a non-significant effect. In contrast, the failure of 5-HT to induce vasoconstriction in non-contracted piglet splanchnic arteries suggested the absence of any activity of vasoconstrictor 5-HT receptors. In these arteries, noticeable contraction was only demonstrated after partial pre-contraction, on which ketanserin had no effect. These results indicated that, in piglet hepatic arteries, 5-HT-induced contraction was mediated by another vasoconstrictor 5-HT-receptor sub-type, probably the 5-$HT_1$-like receptor, which was inactive in non-precontracted condition. Although 5-HT can also produce contraction by stimulation of $\alpha$-adrenoceptors, this could not account for the 5-$HT_{2A}$ receptor-independent contraction seen in the present study, as in all experiments, $\alpha_1$ and $\alpha_2$-adrenoceptors had been blocked using prazosin and yohimbine, respectively (Choppin & O'Connor, 1994; Sahin-Erdemli *et al.*, 1991).

The mechanisms by which pre-contraction induces 5-HT-mediated contractile responses in porcine hepatic arteries are unclear and the presence of 5-$HT_1$-like receptors has not been studied in these arteries. The 5-$HT_{2A}$-receptor is a G-protein-coupled 7 transmembrane domain receptor, stimulation of which increases synthesis of inositol phosphates ($IP_3$) and diacylglycerol (DAG) leading to elevated intracellular $Ca^{2+}$ concentrations and thus, smooth muscle cell contraction (Martin & Humphrey, 1994; Saxena & Villalon, 1990). The 5-$HT_1$-like receptor is also a G-protein-coupled receptor but is inversely coupled with adenylate cyclase. Stimulation of this receptor reduces the levels of cytosolic cyclic adenosine monophosphate (cAMP) and leads to a rise in intracellular calcium (Hoyer e*t al.*, 1994). Additionally,

5-$HT_1$-like receptor can also enhance intracellular calcium by stimulating calcium influx (Movahedi & Purdy, 1997; Parsons & Whalley, 1989). As described, stimulation of 5-$HT_1$-like receptor does not result in contraction of some vessels mounted in an isometric system (Choppin & O'Connor, 1994, 1995). The reason for this is that cytosolic calcium levels are below the threshold concentration essential for contraction. However, following partial precontraction, using another non-5-HT agonist, intracellular calcium levels were elevated, while steady cytosolic calcium levels were maintained by subsequent increased of cytosolic cAMP activity (Yildiz *et al.*, 1998). In this situation, stimulation of the 5-$HT_1$-like receptor inhibits synthesis of cAMP as well as induces calcium influx. Consequently, cytosolic calcium levels are elevated and vasoconstriction is evoked. The current study suggests, therefore, that 5-$HT_1$-but not 5-$HT_{2A}$-receptors are present in piglet hepatic artery, whereas both receptor subtypes are active in similar arteries from adult pig as evidenced in PRA of adult rabbits.

### 3.7. Conclusions

This study demonstrated that porcine hepatic and mesenteric arteries could be used as a model to develop a functional protocol for the use of human hepatic arteries. It was shown that the arteries were devoid of endothelium and consequently neither the influence of the endothelium on contractile function nor endothelium-dependent relaxation could be assessed. A similar lack of endothelium is expected in human hepatic arteries. Using the porcine hepatic and mesenteric arteries as a model, concentration ranges of AVP and 5-HT were shown to produce clear maximum contraction enabling calculation of the $pD_2$ value, both of which will be necessary to

assess the effect of cirrhosis on human hepatic artery function. The demonstration of age-dependent changes in response to 5-HT in these arteries was unexpected and investigation of this phenomenon provided useful data on the mechanism of 5-HT-mediated contraction in these arteries. However, it is unlikely that age-dependent changes will be important in studying of the effect of cirrhosis in human hepatic arteries as it was anticipated that these studies would only include arteries from the adults.

# CHAPTER FOUR

# THE EFFECT OF ORGAN PRESERVATION ON FUNCTIONAL RESPONSES OF THE PORCINE HEPATIC ARTERY

## 4.1. Introduction.

The hyperdynamic circulation of cirrhosis exhibits characteristic haemodynamic disturbances in the peripheral vasculature. In particular, marked vasodilatation has been described in the splanchnic circulation (Iwao *et al.,* 1997a) but the cause of this abnormality has yet to be confirmed. Human hepatic arteries have been used to investigate the causes of altered function as they can be obtained during liver transplantation procedures (Heller *et al.,* 1999; Hadoke *et al.,* 1998; Smith *et al.,* 1997). Donor hepatic arteries are usually used as controls whilst the effects of liver cirrhosis on contractile function are assessed in hepatic arteries from (cirrhotic) liver recipients. The situation is complicated by the different treatment of donor and recipient hepatic arteries during the transplantation procedure, as hepatic arteries from the donor, but not those from the recipient, are exposed to preservative solutions while stored *ex vivo*. Conceivably, this exposure to preservative solution could affect the functional responsiveness of the donor arteries, limiting their use as controls for studying the effect of cirrhosis.

University of Wisconsin solution (UWS) is widely used for organ preservation and is recognised as the best available preservative solution for hepatocytes (Muhlbacher *et al.,* 1999; Belzer *et al.,* 1992). However, the structure and function of cells in blood vessels differ considerably from those of hepatocytes. It is, therefore, important to determine the effect of preservative solutions on the functional responses of the blood vessel. A small number of studies have investigated the effects of UWS on the functional response of blood vessels. These have consistently reported that contractile responses of denuded vessels were unaffected following incubation with

UWS (Jeng *et al.*, 1996b, 1997; Hadoke *et al.*, 1998). In contrast, there are conflicting reports on the effect of UWS on vessels with an intact endothelium (Lin *et al.*, 1994, 1995; Jeng *et al.*, 1997; Gryf-Lowczowski *et al.*, 1997; Nardo *et al.*, 2000). Another important consideration is that dexamethasone, insulin and penicillin are added to UWS for the preservation of liver (Sorajja *et al.*, 1997; Abebe *et al.*, 1993). Glucocorticoids may affect contractile and relaxant function of blood vessels (Changani *et al.*, 1999; Ullian, 1999) and insulin can enhance endothelium-mediated relaxation (Wamback & Liu, 1992). Therefore, the effects of preservative solutions on the functional responses of donor hepatic artery have to be assessed before these vessels are used as controls.

Normal human hepatic arteries, which have not been exposed to UWS, are difficult to obtain, and usually have a damaged endothelium (Heller *et al.*, 1999; Hadoke *et al.*, 1998; Smith *et al.*, 1997). Although, small intra-hepatic arteries with an intact functional endothelium can be obtained during hepatectomy, they are not ideal models for functional studies of extra-hepatic arteries. Furthermore, these arteries are obtained from patients with liver diseases and an influence of disease on normal function of blood vessels cannot be discounted. For assessment of the effect of preservative solutions on the normal functional response of hepatic arteries, it is necessary, therefore, to use a suitable animal model. The anatomy and physiology of the porcine liver and splanchnic vascular system closely resembles to those in humans (Swindle, 1984; Changani *et al.*, 1999), making porcine extra-hepatic arteries a useful model for assessing the effects of organ preservation on vascular function.

## 4.2. Aim

This study was designed to determine whether prolonged storage in preservative solution alters contractile function or endothelium-independent relaxation in denuded porcine hepatic arteries.

## 4.3. Methods

### *4.3.1. Acquisition of Hepatic Arteries*

Hepatic arteries from, healthy, adult large white pigs of either sex (age, 6-8 months; weight, 50-60kg; n=36) were collected from a local abattoir. Arteries were placed immediately into ice-cold oxygenated KHS, transported to the laboratory and prepared as described in Chapter 2.1.3.

### *4.3.2. Incubation of Hepatic Arteries with Preservative Solutions*

Hepatic arteries from 18 pigs were divided into two sections (3-4 cm in length): one section was stored in KHS whilst the other section was stored in UWS. The composition of UWS was (mmol/L): $K^+$, 125; $Na^+$, 28; $KH_2PO_4$, 25; $MgSO_4$, 5; raffinose, 30; lactobionate, 100; glutathione, 3; adenosine, 5; allopurinol, 1 and pentafraction (50g/L) (Southard & Belzer, 1995; Collins, 1997). Arteries from the remaining 18 pigs were divided in a similar way but stored in either KHS or in UWS containing dexamethasone, 16 mg/L ($3x10^{-5}$mol/L); insulin, 40 units/L and benzylpenicillin, 240 mg/L (DIP), according to the liver preservation protocol used by the Scottish Liver Transplant Unit. The arterial sections were stored at 4°C for 16 hours in 20 ml of the preservative solutions in a sterile universal container (as liver is

preserved maximum 14 hours in Scottish Liver Transplant Unit before transplantation).

*4.3.3. Functional Analysis of Hepatic Arteries*

Following incubation, two rings, 2mm in length, were prepared from each hepatic artery. Any residual endothelium was removed by gently rubbing the luminal surface with a wire probe and the rings were mounted on intraluminal wires in organ baths filled with KHS as described (Chapter 2.2.2).

4.3.3.1. Assessment of Viability and Reproducibility

After equilibration of the rings at their optimum resting force (4g), the viability and reproducibility of contractions were tested using (100mmol/L) KCl (Hadoke *et al.*, 1998).

4.3.3.2. Functional Protocol

For the investigation of contractile and relaxation functions of the hepatic arteries after storage either in KHS, UWS or UWS + DIP, cumulative concentration-response curves were constructed for the receptor-dependent agonists, AVP ($10^{-11}$-$3x10^{-7}$ mol/L) and 5-HT ($10^{-9}$-$3x10^{-5}$ mol/L), and the receptor-independent vasoconstrictor, KCl (2.5-120 mmol/L). At the end, endothelium-independent relaxation was assessed using the nitric oxide donor, SIN-1 ($10^{-9}$-$3x10^{-5}$ mol/L), following sub-maximal precontraction with PE ($10^{-6}$-$3x10^{-5}$ mol/L).

### 4.4. Statistics

Statistics analysis was performed as described in Chapter 2.7.

### 4.5. Results

*4.5.1. Effect of Preservative Solutions on Contractile Function of Arterial Rings*

The receptor-dependent agonists, AVP and 5-HT, as well as the receptor-independent vasoconstrictor, KCl, induced contraction in all arterial rings whether they were incubated with KHS, UWS or UWS + DIP.

The maximum contractile response and sensitivity to AVP, 5-HT and KCl were similar in arteries stored in KHS or UWS (Figure 4.1; Table 4.1). This situation was not altered by addition of DIP to the UWS, as responses of the vessels stored in UWS + DIP were also similar to controls (Table 4.1; Figure 4.2).

*4.5.2. Effect of Preservative Solutions on Relaxation of Arterial Rings*

SIN-1 produced concentration-dependent relaxation in all denuded arterial rings. Following incubation with UWS, maximum relaxation (Emax; 106.07 ± 0.82%) and sensitivity (-log $IC_{50}$; 6.23 ± 0.07) of the rings incubated in UWS were significantly reduced when compared with those stored in KHS (Emax; 110.19 ± 1.34%, $P$=0.01; -log $IC_{50}$; 6.47 ± 0.04, $P$ = 0.009) (Figure 4.3). Although, the sub-maximal contraction produced by PE was similar ($P$=0.55), following incubation with UWS (7.02 ± 0.52g) or KHS (7.57 ± 0.76g).

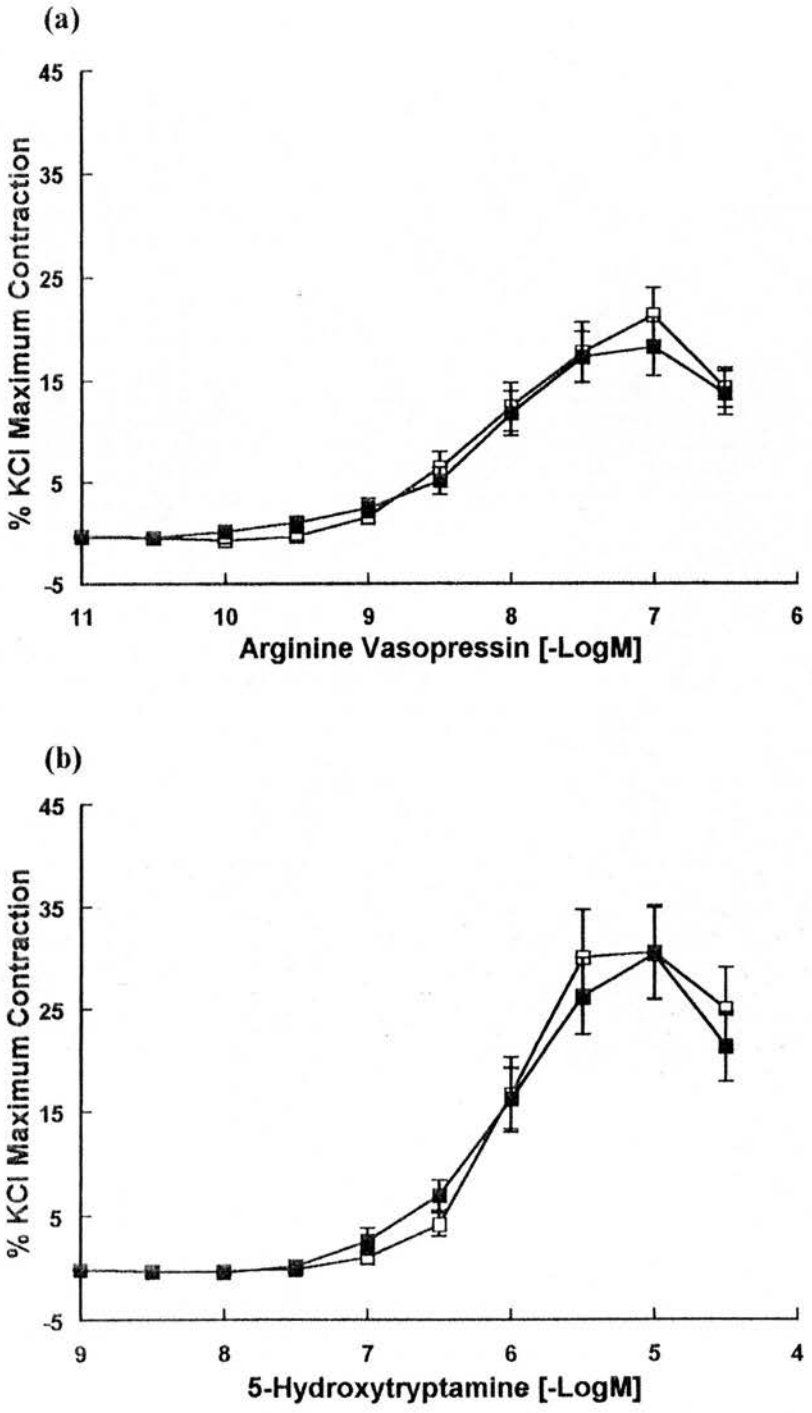



**Figure 4.1 (a, b).** Cumulative concentration-response curves produced in response to **(a)** arginine vasopressin (AVP) and **(b)** 5-hydroxytryptamine (5-HT) by porcine hepatic arteries, incubated in Krebs'-Henseleit solution, (□) or University of Wisconsin solution (■) , Responses are given as mean ± s.e. mean for (n) individuals and expressed as a percentage of the maximum contraction induced by KCl. In all cases n=18.

(c)

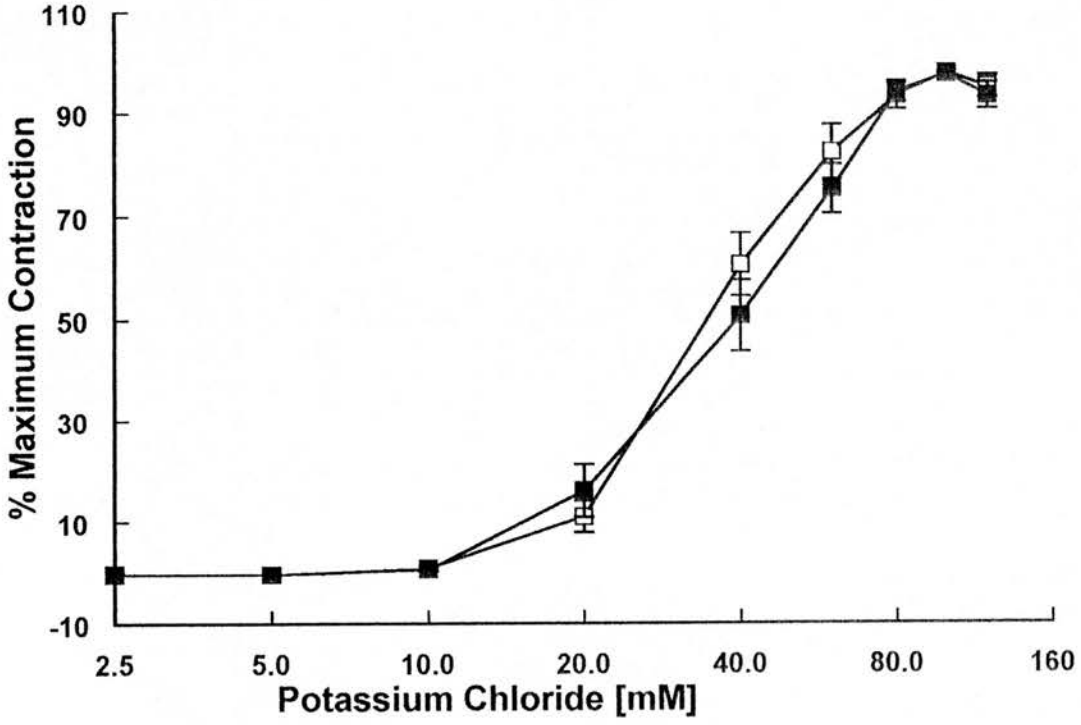



**Figure 4.1 (c).** Cumulative concentration-response curves produced in response to potassium chloride (KCl) by porcine hepatic arteries, incubated in Krebs'-Henseleit solution, (□) or University of Wisconsin solution (■), Responses are given as mean ± s.e. mean for (n) individuals and expressed as a percentage of the maximum contraction induced by KCl. In all cases n=18.

**Table 4.1.** Maximum contractile response (Emax) and sensitivity ($pD_2$) values of denuded porcine hepatic arteries (n=18) to AVP, 5-HT and KCl following incubation with:

**(a)** Krebs'-Henseleit Solution (KHS) or University of Wisconsin Solution (UWS).

| | **EMAX** | | | | | **$pD_2$** | | |
|---|---|---|---|---|---|---|---|---|
| | AVP | | 5-HT | | KCl | AVP | 5-HT | KCl |
| | (g) | (% KCl) | (g) | (% KCl) | (g) | | | |
| KHS (Control) | 2.65±0.30 | 23.20±2.92 | 3.69±0.44 | 33.25±4.75 | 12.92±1.01 | 8.03±0.09 | 5.95±0.07 | 1.41±0.03 |
| UWS | 2.42±0.32 | 19.79±2.66 | 3.86±0.44 | 32.29±4.39 | 13.22±1.25 | 8.08±0.09 | 5.98±0.08 | 1.41±0.04 |

**(b)** Krebs'-Henseleit Solution (KHS) or University of Wisconsin Solution (UWS) + Dexamethasone, Insulin and Penicillin (DIP)

| | **EMAX** | | | | | **$pD_2$** | | |
|---|---|---|---|---|---|---|---|---|
| | AVP | | 5-HT | | KCl | AVP | 5-HT | KCl |
| | (g) | (% KCl) | (g) | (% KCl) | (g) | | | |
| KHS (Control) | 2.47±0.27 | 20.21±2.07 | 4.11±0.34 | 34.31±3.13 | 12.49±0.74 | 7.99±0.07 | 5.95±0.10 | 1.46±0.03 |
| UWS+DIP | 2.62±0.25 | 22.50±2.45 | 4.20±0.40 | 33.39±2.26 | 12.36±0.82 | 8.10±0.07 | 6.04±0.08 | 1.46±0.02 |

Values are mean ± s.e. mean. Results were compared using Student's unpaired t-test and considered significant when $P<0.05$.

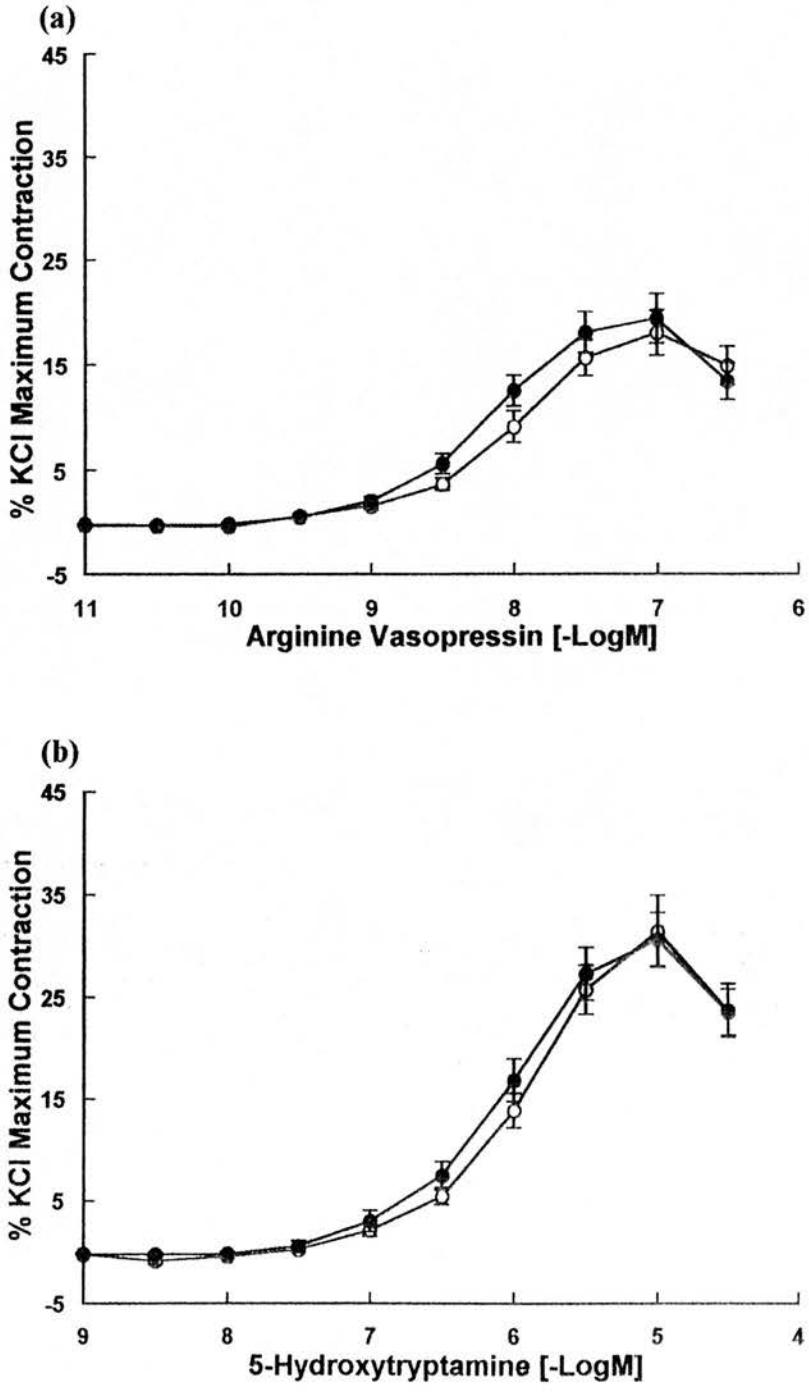



**Figure 4.2 (a, b).** Cumulative concentration-response curves produced in response to **(a)** arginine vasopressin (AVP) and **(b)** 5-hydroxytryptamine (5-HT by porcine hepatic arteries incubated in Krebs'-Henseleit solution, (○) or University of Wisconsin solution containing dexamethasone, insulin and penicillin (DIP), (●). Responses are given as mean ± s.e. mean for (n) individuals and expressed as a percentage of the maximum contraction induced by KCl. In all cases n=18.

(c)

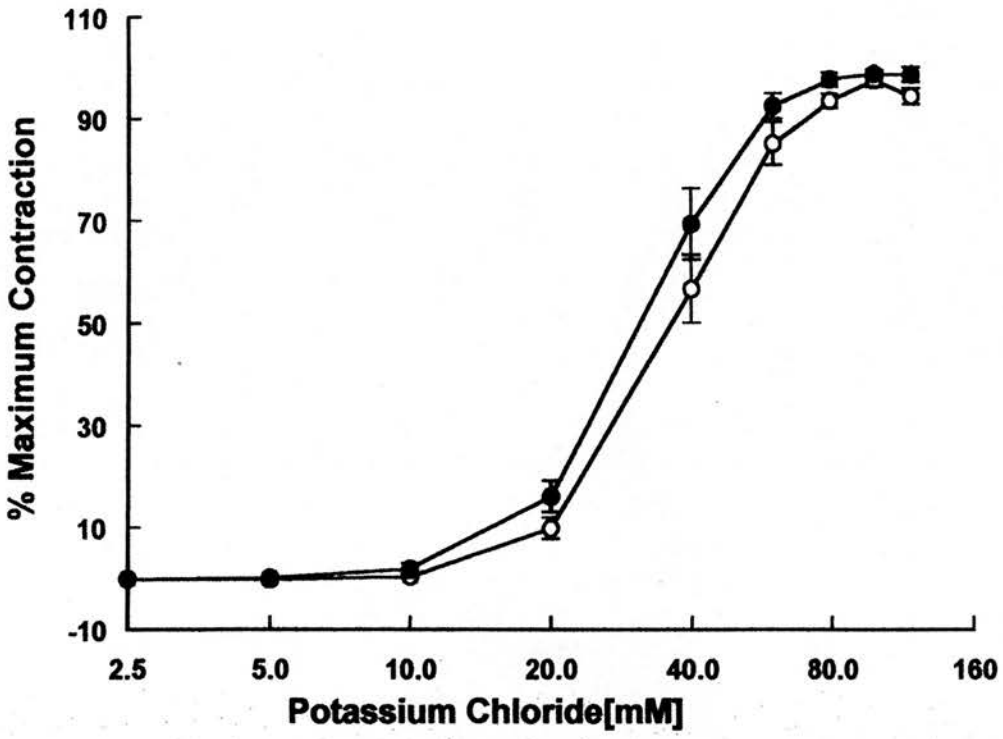



**Figure 4.2 (c)** Cumulative concentration-response curves produced in response to potassium chloride (KCl) by porcine hepatic arteries incubated in Krebs'-Henseleit solution, (○) or University of Wisconsin solution containing dexamethasone, insulin and penicillin (DIP), (●). Responses are given as mean ± s.e. mean for (n) individuals and expressed as a percentage of the maximum contraction induced by KCl. In all cases n=18.

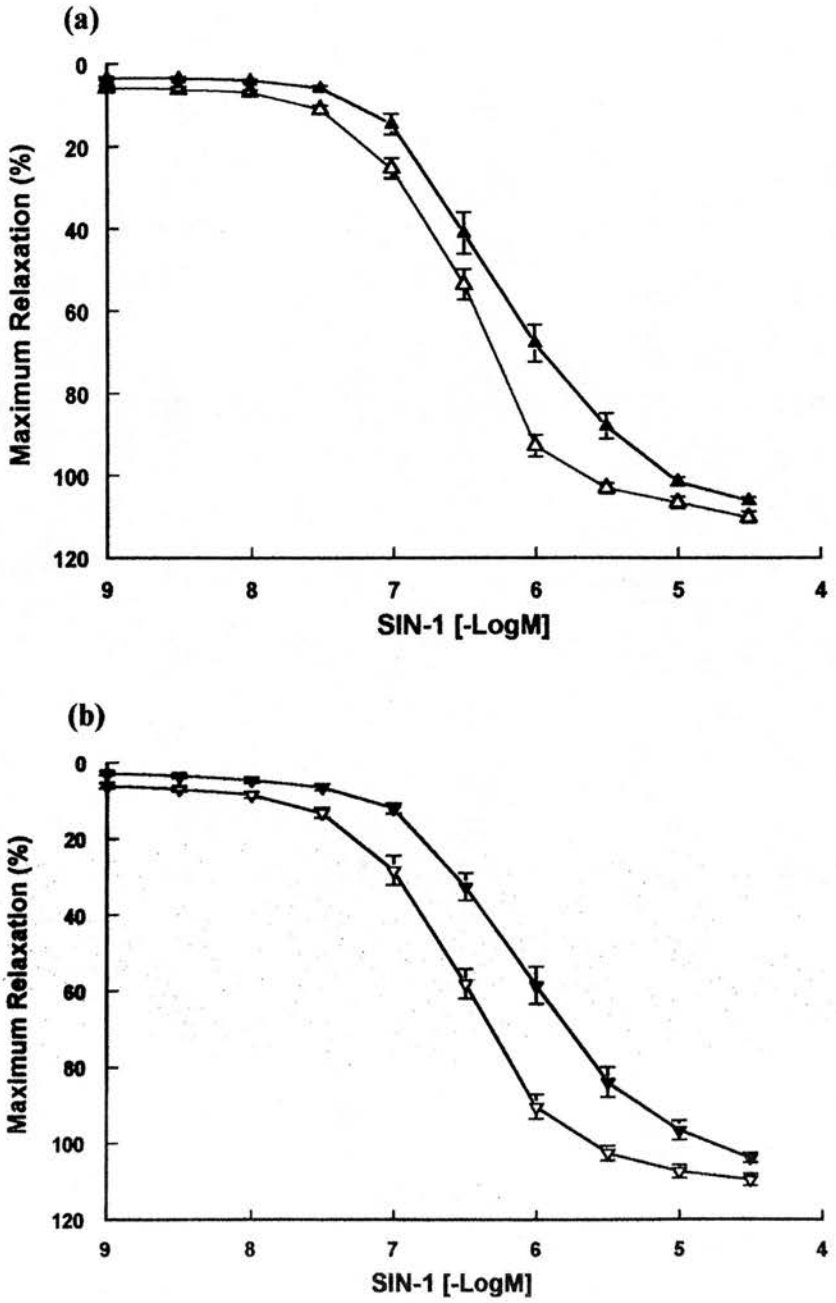



**Figure 4.3 (a, b).** Cumulative concentration-response curves produced by denuded porcine hepatic arteries in response to 3'-morpholinosydnonimine (SIN-1), following incubation with **(a)** Krebs'-Henseleit solution, (Δ) or University of Wisconsin solution (▲) and **(b)** with Krebs'-Henseleit solution, (∇) or University of Wisconsin solution containing dexamethasone, insulin and penicillin (DIP), (▼). Responses are given as mean ± s.e. mean for (n) subjects and expressed as a percentage of the maximum relaxation induced by phenylephrine. In all cases n=18.

A similar but more pronounced reduction of SIN-1-mediated relaxation was detected in arteries incubated with UWS + DIP (Emax; 103.87 ± 1.23%; -log $IC_{50}$; 6.09 ± 0.07) when compared with the arteries incubated in KHS (Emax; 109.60 ± 1.57%, $P$ = 0.007; -log $IC_{50}$; 6.52 ± 0.05, $P$=0.0001) (Figure 4.3). These arteries also produced similar type ($P$=0.77) sub-maximal contraction in response to PE following incubation with UWS (7.72 ± 0.64g) or KHS (7.47 ± 0.54g).

**4.6. Discussion**

The present study investigated the effect of UWS, with or without the addition of dexamethasone, insulin and penicillin, on functional responses of denuded porcine hepatic arteries. It was demonstrated that exposure to preservative solutions had no effect on the maximum contraction and sensitivity of responses to the receptor-dependent agonists, AVP and 5-HT, or the receptor-independent vasoconstrictor, KCl. In contrast, the relaxation response to SIN-1 was reduced following incubation in preservative solutions.

Donor hepatic arteries, which are preserved along with the liver in UWS, have been used as controls in investigations studying the effect of cirrhosis on the functional responses of hepatic arteries from liver recipients. Although arteries can be stored in physiological salt solutions over short periods (up to approximately 2 days) without affecting functional responsiveness (Cartier *et al.*, 1993; McIntyre *et al.*, 1998), UWS differs from physiological salt solutions in several respects. In particular, the higher concentrations of potassium (125 mmol/L), lower concentrations of sodium (29 mmol/L), the absence of calcium, and the presence of potential anti-oxidant

glutathione as well as a higher osmolality (320 mOsmol/L). Raised osmolality does not appear to influence contractile function (Goto & Aimoto, 1991) but intracellular calcium ($[Ca^{2+}]_i$), which is the primary regulator of VSMC contraction, can be modulated by the variations in extracellular concentration of potassium, sodium as well as calcium (Gurney, 1994; McDonald *et al.*, 1994). Furthermore, UWS also contains adenosine, which is itself a vasodilator (Searle & Sahab, 1992).

*4.6.1. Effect of Preservative Solutions on Contraction*

The demonstration in this study, that contractile function of denuded porcine hepatic arteries is unaffected by exposure to UWS, is consistent with investigations using denuded porcine mesenteric (Heller *et al.*, 1999) or canine coronary (Lin *et al.*, 1995) arteries. Similarly, exposure to UWS had no effect on contraction in denuded hepatic arteries from patients with cirrhosis (Hadoke *et al.*, 1998) or hepatocellular carcinoma (Jeng *et al.*, 1996, 1997). In contrast, augmented contractility (to both a thromboxane analogue [U46619] and KCl) has been demonstrated in human saphenous veins with an intact endothelium after incubation with UWS (Anastasious *et al.*, 1997). The mechanism of the augmented response was unclear but the presence of the endothelium might be significant. Alternatively, structural and functional differences between veins and arteries may result in exposure to UWS having a more significant impact on the former (Buckley *et al.*, 1997; Garcia-Villalon *et al.*, 1996). These results confirm that exposure to UWS (for up to 16 hours) does not alter contractile function of denuded arteries.

The clearest evidence of altered contractile function in arteries exposed to UWS is seen under hypoxic conditions. Hypoxia-induced contraction is enhanced in arteries, from animals (Lin *et al.*, 1995) or humans (Jeng *et al.*, 1997), after storage in UWS. This enhancement, however, requires the presence of an intact endothelium, indicating that exposure to UWS is altering the function of endothelial, rather than smooth muscle, cells. This could be a result of either impaired activity of endothelium-derived vasodilators or, more probably, release of an endothelium-derived contracting factor (EDCF; e.g. endothelin-1 or a contractile prostaglandin), under hypoxic conditions. Therefore, when using human hepatic arteries to study the effects of cirrhosis on contractile function, it is likely that the influence of exposure to UWS on control arteries would only become significant if the arteries have an intact endothelium and are studied under hypoxic conditions.

Dexamethasone, insulin and penicillin are added routinely to UWS for preservation of liver during transplantation procedures. Few studies, however, have included these compounds when assessing the effects of UWS on vascular responsiveness. This addition may be significant as these additives may also have vasoactive properties. Corticosteroids, for example, can potentiate the activity of several vasoactive mediators by up-regulating receptors in the VSMCs (Ullian, 1999), modulating post-receptor signal transduction pathway (Calderone *et al.*, 1994) or even attenuating the activity of endothelium-derived vasodilators (Lockette *et al.*, 1986). Glucocorticoids can also directly increase the capacity of trans-membrane ion transporters (Kornel, 1993). Insulin, on the other hand, may inhibit contractile responses to various vasoactive substances (Cleland *et al.*, 1998a; Kahn *et al.*, 1998; McNally *et al.*, 1995)

although the mechanisms of this action remain unclear. Possibilities include enhanced NO release from the endothelium (Baron, 1999) or inhibition of extracellular calcium influx (Goud *et al.*, 1998). It has also been reported that insulin directly modulates transmembrane cation exchange through stimulation of the $Na^+/K^+$ ATPase and $Na^+/K^+$ pump in VSMCs (Moore, 1983). Activation of the $Na^+/K^+$ pump induces hyperpolarisation of the cell membrane and thereby impaired calcium influx via voltage-operated channels (Izhar *et al.*, 2000; Kahn *et al.*, 1993). Incubation of the arteries in non-sterile conditions possibly could induce iNOS activation. This activation should have been prevented by penicillin and also by dexamethasone. These results suggested that iNOS is not activated, as activation of iNOS would lead to impaired response to contractile functions of the agonists.

The present study demonstrated that the inclusion of dexamethasone, insulin and penicillin in the UWS had no effect on the contractile responses of denuded porcine hepatic arteries. These results are consistent with studies using canine coronary arteries (Sorajja *et al.,* 1997; Abebe *et al.,* 1993). It is unclear why these additives did not affect vascular function although several possible explanations may be proposed. The concentration of the dexamethasone or insulin may be significant as the effect of these compounds is concentration-dependent (Altura & Altura, 1974; McNally *et al.*, 1995). The very high concentration of dexamethasone ($3x10^{-5}$mol/L) used in this study, could be a factor, as corticosteroids may *inhibit* vasoconstriction at this concentration (Altura & Altura, 1974). Similarly, insulin reduces contractile responses in a concentration-dependent manner (0.1mU/ml-10mU/ml) (McNally *et al.*, 1995), however, at very high concentration (40mU/ml) its effect is unclear.

Similarly, the duration of exposure may be an important consideration. The vascular effect of steroids *in vivo* can be observed within seconds or minutes due to non-receptor-mediated direct cell membrane effects, but long-term exposure (e.g.16 hours) can produce both classical cytosolic receptor as well as direct non-receptor-mediated effects (Walker & Williams, 1992). However, such time limit for incubation *in vitro* is not well defined. In the case of insulin, inhibition of contractile function could be observed within minutes or hours but long-term exposure *in vivo* or in tissue culture could induce growth and proliferation of the vascular smooth muscle cells (Pfeiffer & Ditschuneit, 1981) which is unexpected in isolated vessel studies. Finally, most studies have reported altered contractile responses following exposure of vessels to these agents at either normal room temperature or body temperature. The lower incubation temperature (4°C) in the present study may have prevented dexamethasone and insulin, exerting an effect on vascular function.

*4.7.2. Effect of Preservative Solutions on Relaxation*

SIN-1, the active metabolite of molsidomine, which generates peroxynitrite and NO (Singh *et. al.*, 1999), produced an endothelium-independent relaxation of denuded porcine hepatic arteries. Although this relaxation remained evident in arteries exposed to UWS, there was a small but significant reduction in maximum relaxation and impairment of sensitivity compared with vessels incubated in KHS. This reduction was more pronounced after addition of dexamethasone, insulin and penicillin. Impaired relaxation responses to another nitro-vasodilator, sodium nitroprusside (SNP), have been reported in rat coronary arteries (Cartier *et al.*, 1993) and rabbit thoracic aorta (Gryf-Lowczowski *et al.*, 1997) following incubation with

UWS. In contrast, other studies have reported that response of denuded human hepatic arteries to SNP (Jeng *et al.*, 1996a,b) or SIN-1 (Hadoke *et al.*, 1998) were unaltered after such incubation. Similarly, unaltered responses to SNP have also been reported in canine coronary arteries (Lin *et al.*, 1994) and porcine hepatic arteries (Flanders *et al.*, 1996), even after addition of dexamethasone, insulin and penicillin (Sorajja *et al.*, 1997; Abebe *et al.*, 1993). These differences from the present study are difficult to explain. Methodological variations, however, including use of vessels from a variety of species and anatomical territories, use of different agonists and, particularly in humans, the effect of disease and treatments (Jeng *et al.*, 1996a,b; Hadoke *et al.*, 1998), might be implicated. Alternatively, the inclusion of glutathione in UWS may account for altered responses to SIN-1. Glutathione may undergo auto-oxidation during storage, leading to the generation of oxidative radicals (Gnaiger *et al.*, 2000; Astier & Paul, 1989). These radicals act as pro-oxidant and could potentially alter the balance between NO and peroxynitrite generation, resulting in a higher proportion of peroxynitrite to NO from SIN-1 (Singh *et al.*, 1999). This would not explain, however, the greater impairment in SIN-1-mediated relaxation in vessels exposed to UWS containing dexamethasone, insulin and penicillin.

The mechanism by which SIN-1 and other NO donors mediate relaxation may account for the differences between some studies (Tseng *et al.*, 2000; Van de Voorde *et al.*, 1991). Nitrovasodilators such as SNP or nitroglycerin produce vasodilatation primarily via the nitric oxide-guanylate cyclase pathway (Walter *et al.*, 1988; Vanhoutte, 1989). The vasodilatation mechanisms of SIN-1 could differ from other nitrovasodilators (Van de Voorde *et al.*, 1991). In rat aorta (Van de Voorde *et al.*,

1991), mesenteric (Plane et al., 1996) or pulmonary (Homer et al., 1999) arteries, inhibition of guanylate cyclase by oxadiazolol quinoxalin (ODQ) reduced responses to SNP or nitroglycerin but not SIN-1. The SIN-1-mdiated relaxation however was significantly inhibited by potassium channel blockers. Therefore, in addition to the nitric oxide-guanylate cyclase pathway, SIN-1 can also mediate vasodilatation via potassium channels in the VSMCs (Magnon *et al.*, 1998, Onoue & Katusic, 1997). Opening of $K^+$ channels induces VSMC hyperpolarisation, which reduces calcium influx (by closing voltage-gated calcium channels) resulting in vasodilatation (Gurney, 1994). Alteration of extracellular $K^+$ can affect this mechanism (Quayle *et al.*, 1997). In particular, very high extracellular $K^+$ prevents efflux of $K^+$ and, thus, prevents hyperpolarisation (Cook & Quast, 1990). Therefore, the high potassium (125 mmol/L) in UWS might be a factor in the reduction in SIN-1-mediated vasorelaxation.

The presence of dexamethasone, insulin and penicillin with UWS, further attenuated the relaxation response of the arteries elicited by SIN-1. It has been suggested that corticosteroids can have a direct effect on VSMC function (Ullian, 1999). Dexamethasone can increase extracellular calcium influx (Hayashi *et al.*, 1991) and release from intracellular stores (Perry & Webb, 1991), enhance sodium transport (Stern et al., 1994) and thus increase vascular tone. As a whole, corticosteroids can act on many individual sites (such as receptor-ligand binding, membrane G-protein, phospholipase C, inositol triphosphate, contractile proteins as well as protein kinase C) in the contractile pathway of VSMCs (Ullian, 1999). Furthermore, it can inhibit synthesis of vasodilator prostaglandins (Handa *et al.*, 1984) and inducible NO

synthase (Rees *et al.*, 1990; Niwa *et al.*, 1996) in the VSMCs. The reduced relaxation response in this experiment therefore could be the outcome of an integrated effect of dexamethasone on the vessels. Additionally, penicillin in UWS could prevent induction of NO synthesis resulting from non-aseptic conditions (Guarner *et al.*, 1993). Insulin could produce vasodilatation but this effect is mainly mediated by endothelial cells (Wamback & Liu, 1992) or activation of potassium channels (Izhar *et. al.*, 2000). Absence of the endothelium in the vessels as well as higher potassium in UWS might inhibit the response of insulin. Furthermore, it has been reported that the vasodilatation effect of insulin can be suppressed by dexamethasone (Scherrer *et al.*, 1993). However, the complex interaction between various components of UWS and the additives dexamethasone, insulin and penicillin with SIN-1 is still unclear. More studies therefore are required if these interactions are to be clarified.

### 4.8. Conclusions

The present study demonstrated that preservation of denuded porcine hepatic arteries in UWS, with or without dexamethasone, insulin and penicillin, had no effect on contractile function. These results suggest that, although donor hepatic arteries are exposed to UWS during liver transplant, they are suitable controls for investigating the effect of cirrhosis on the contractile response of denuded hepatic arteries.

# CHAPTER FIVE

# THE EFFECT OF CIRRHOSIS OF THE LIVER ON THE CONTRACTILE FUNCTION OF HUMAN HEPATIC ARTERIES

## 5.1. Introduction

The development of a hyperdynamic circulation (Groszman, 1996; Murray *et al.*, 1958) in patients with cirrhosis of the liver is associated with systemic (Schrier *et al.*, 1988a; Kontos *et al.*, 1964) and splanchnic vasodilatation (Vorobioff *et al.*, 1984; Iwao *et al.*, 1997). Individuals with cirrhosis also exhibit an impaired pressor response to vasoconstrictors (MacGilchrist, *et al.*, 1991a, b; Badalamenti *et al.*, 1993) but, despite many studies, the cause of this abnormality remains obscure. Impaired contraction remains in evidence in *ex vivo* preparations in which vessels are isolated from circulating vasoactive factors (Smith *et al.*, 1997; Heller *et al.*, 1999). This suggests, therefore, that an abnormality in the vessel wall accounts for altered contractile function in isolated arteries. Possible explanations of contractile hyporesponsiveness include receptor down-regulation (Gerbes *et al.*, 1986), a defect in the post-receptor signal transduction pathway (Laffi *et al.*, 1988) and/ or excessive synthesis of vasodilators by the vessel wall itself (Vallance & Moncada, 1991).

Studies of contractility in vessels isolated from individuals with cirrhosis and/ or portal hypertension have yielded conflicting results. These differences possibly reflect methodological variation, with many different blood vessels, from distinct vascular territories of several different species employed (Hadoke & Hayes, 1997). It is apparent, however, that the impact of cirrhosis on vascular contractility is dependent upon both the agonist studied (Liao *et al.*, 1994; Sogni *et al.*, 1996; Newby *et al.*, 1998b) and the source of the vessel under investigation. Although few studies have assessed contractile function in human splanchnic arteries, there is some evidence of an endothelium-independent impairment of α-adrenoceptor mediated

contraction in hepatic arteries from patients with cirrhosis (Smith *et al.*, 1997; Heller *et al.*, 1999) although a study in our own laboratory could not confirm this observation (Hadoke *et al.*, 1998). No studies, however, have reported the impact of hepatic cirrhosis on contractile responses of human hepatic arteries to agonists other than those which act on adrenoceptors. Given that the effect of cirrhosis on contractile function is likely to be agonist-dependent, this investigation was designed to assess the effect of cirrhosis on responses to AVP, 5-HT and ET-1 in human hepatic arteries. These agonists are of particular interest as their activity *in vivo* may be altered in patients with cirrhosis (Shah *et al.*, 1998) and their manipulation may be therapeutically beneficial (Freeman *et al.*, 1998; Vorobioff *et al.*, 1989; Nevens *et al.*, 1991; Gandhi *et al.*, 1998; Reichen *et al.*, 1998). Furthermore, studies using animal models have suggested that contractile responses to AVP (Huang *et al.*, 1995; Moreno *et al.*, 1996), 5-HT (Jacob *et al.*, 1991; Cummings *et al.*, 1986) and ET-1 (Cahill *et al.*, 1998; Cailmail *et al.*, 1995) may all be altered in cirrhosis/portal hypertension.

### 5.2. Aim

The study aimed (1) to determine whether the presence of hepatic cirrhosis was associated with alterations in contractile responses to AVP, 5-HT and ET-1 in isolated human hepatic artery and (2) to clarify the mechanisms responsible for any functional variation identified.

## 5.3. Methods

### *5.3.1. Acquisition and Preparation of Hepatic Artery*

Hepatic arteries from liver donors and recipients were obtained from the Scottish Liver Transplant Unit, during liver transplantation. Donor hepatic arteries were obtained from surplus material after the transplantation procedure, whilst recipient hepatic arteries (4-6cm) were dissected directly from the freshly explanted liver. Following retrieval, arteries were immediately placed in ice-cold, oxygenated Krebs'-Henseleit solution (KHS) and transported to the laboratory as described (Chapter 2.1.1). Functional investigations were usually performed immediately after the retrieval of the arteries and all experiments were performed within 24 hours of collection. Systemic haemodynamic parameters, recorded in liver recipients before transplantation, were obtained from anaesthetic notes.

### *5.3.2. Functional Analysis*

#### 5.3.2.1. Preparation of Arterial Rings

Hepatic arteries were cleaned of adherent connective tissue and cut into 2-4 rings (1.5-2mm in length). In appropriate cases, endothelium was removed if necessary (Chapter 2.1.1.) The rings were mounted in organ baths filled with KHS, at 37°C, and continuously bubbled with 95% $O_2$ - 5% $CO_2$ for the measurement of isometric force development (Chapter 2.2.2). All analyses were performed in the presence of indomethacin ($10^{-5}$ mol/L).

#### 5.3.2.2. Assessment of Vessel Viability and Endothelial Cell Function

The arterial rings were equilibrated at their optimum resting force (4g) (Hadoke *et*

*al.*, 1998) for 50-60 minutes and the viability and reproducibility of contractions of each ring was tested using at least 3 consecutive applications of (100 mmol/L) KCl (Chapter 2.2.3). Existence of functional endothelium was assessed in six donor and six recipient hepatic arteries, by adding the endothelium-dependent vasodilator, ACh ($10^{-7}$-$3x10^{-5}$ mol/L) (Vanhoutte & Miller, 1985; Jeng *et al.*, 1996a, b), following precontraction with PE ($10^{-6}$-$3x10^{-5}$ mol/L). Endothelium-independent relaxation was also assessed by exposing precontracted rings to SIN-1 ($10^{-9}$-$3x10^{-5}$ mol/L). At the end of the functional assessment, the rings were removed from the organ baths and endothelial cell integrity was assessed histologically by staining the luminal surface with silver nitrate (Chapter 2.4). In the subsequent experiments, any residual endothelium was removed by gently rubbing the luminal surface of the rings as described (Chapter 2.2.1).

#### 5.3.2.3. Immunohistochemical Assessment of Endothelial Cell Integrity

The presence of endothelium both in the donor and recipient hepatic arteries was also studied using an immunohistochemical technique. Transverse sections from donor (n=6) and recipient (n=6) hepatic arteries were stained using the Ulex antibody as described in Chapter 2.5.2.1.

### *5.3.3. AVP and 5-HT-Mediated Contraction*

#### 5.3.3.1. Subjects

Hepatic arteries were obtained from 42 liver donors (non-cirrhotic; 22 male, 20 female) and 36 recipients (cirrhotic; 14 male, 22 female). The donors (47 ± 2 yrs.) and recipients (51 ± 2 yrs.) were similar in age ($P$=0.19) and the mean duration of

liver disease in the recipients was 9 ± 1 yrs. Causes of death in the donors and of hepatic cirrhosis in the recipients are given in Table 5.1.

#### 5.3.3.2. Effect of Cirrhosis on AVP and 5-HT-Mediated Contraction

Cumulative concentration-response curves were constructed in denuded hepatic arteries using receptor-dependent agonists, AVP ($10^{-11}$-$3x10^{-7}$ mol/L) and 5-HT ($10^{9}$-$3x10^{-5}$ mol/L) and the receptor-independent vasoconstrictor, KCl (2.5-120mmol/L). The rings were washed repeatedly after completion of each curve and equilibrated for 30 minutes before the next agonist was applied.

#### 5.3.3.3. Analysis of Nitric Oxide Synthase Activity in the Hepatic Arteries

##### 5.3.3.3.1. Influence of Nitric Oxide Synthase Activity on Contractility.

Four rings were prepared from each donor and recipient hepatic artery and mounted in organ baths. Two rings were incubated with the nitric oxide synthase (NOS) inhibitor, $N^{G}$-nitro-L-arginine (L-NNA; $10^{-4}$ mol/L) and the remaining two rings with its inactive isomer $N^{G}$-nitro-D-arginine (D-NNA; $10^{-4}$ mol/L) for 45 minutes. Cumulative concentration-response curves were then constructed using AVP ($10^{-11}$-$3x10^{-7}$ mol/L), 5-HT ($10^{-9}$-$3x10^{-5}$ mol/L) and KCl (2.5-120mM) in the continued presence of L-NNA or D-NNA. Finally, endothelium-independent relaxation was assessed using SIN-1 ($10^{-9}$-$3x10^{-5}$ mol/L) following sub-maximal precontraction with PE ($10^{-6}$-$3x10^{-5}$ mol/L).

**Table 5.1.** Causes of death in liver donors and of cirrhosis in recipients undergoing liver transplantation. Hepatic arteries from these individuals were isolated for studies of AVP and 5-HT-mediated contraction.

| Cause of Death in Donor | No. | Cause of Cirrhosis in Recipient | No. |
|---|---|---|---|
| Sub-arachnoid Haemorrhage | 24 | Primary Biliary Cirrhosis | 14 |
| Intra-cerebral Bleeding | 9 | Alcoholic Liver Disease | 8 |
| Head Injury | 5 | Cryptogenic Cirrhosis | 7 |
| Hypoxia | 3 | Primary Sclerosing Cholangitis | 5 |
| Bleeding Aneurysm | 1 | Secondary Biliary Cirrhosis | 1 |
| | | Cirrhosis in Wilson's Disease | 1 |
| Total: | 42 | Total: | 36 |

5.3.3.3.2. Immunohistochemical Assessment of Nitric Oxide Synthase in Human Hepatic Artery

The presence of inducible nitric oxide synthase in the donor and recipient hepatic arteries was assessed immunohistochemically using the alkaline phosphatase method (Chapter 2.4). Recipient hepatic arteries were obtained from patients with cirrhosis caused by: alcoholic liver disease (ALD; n=3), primary biliary cirrhosis (PBC; n=3), cryptogenic cirrhosis (CC; n=2) or chronic active hepatitis (CAH; n=1). In this study, arterial rings prepared from the rabbit thoracic aorta for the purpose of a different investigation were used as a positive control. Induction of nitric oxide synthase activity was accomplished by intraperitoneal injection of lipopolysaccharide (E. coli, 055:B5; Sigma Ltd, UK), 30mg/kg body weight and the rabbit was sacrificed after 5 hours.

5.3.3.4. Identification of the Receptors Mediating AVP-Induced Contraction of Human Hepatic Artery

5.3.3.4.1. The Role of $V_2$-receptors

Arterial rings from either donor (n=6) or recipient (n=6) hepatic arteries were mounted in organ baths. After equilibration and confirmation of viability, cumulative concentration-response curves were constructed using a selective $V_2$-receptor agonist, desmopressin (DDAVP, $10^{-11}$-$3x10^{-6}$ mol/L) followed by AVP ($10^{-11}$-$3x10^{-7}$ mol/L) and KCl (2.5-120 mmol/L).

5.3.3.4.2. The Role of $V_1$-receptors

The Role of $V_1$-receptors was assessed in separate arterial rings from these

individuals. Four rings from either donor (n=10) or recipient (n=11) hepatic arteries were freshly mounted in organ baths. Three rings were incubated for 15 minutes with different concentrations ($10^{-8}$ mol/L, $10^{-7}$ mol/L and $10^{-6}$ mol/L) of a selective $V_1$-receptor antagonist, $d(CH_2)_5$ Tyr(Me)AVP. The fourth ring was not exposed to the antagonist and served as a control. Following the incubation, cumulative-concentration response curves were constructed using AVP ($10^{-11}$-$3x10^{-6}$ mol/L). Subsequently, the agonist and antagonist were washed from the organ baths and, following 30 minutes equilibration, cumulative concentration-response curves were constructed using KCl (2.5-120 mmol/L).

### *5.3.4. ET-1 Mediated Contraction of Human Hepatic artery*

#### 5.3.4.1. Subjects

Hepatic arteries were collected from 8 non-cirrhotic donors (3 male, 5 female) and 9 cirrhotic recipients (5 male, 4 female) of a similar age (43 ± 6 yrs. and 52 ± 4 yrs. respectively; $P$=0.20) at the time of liver transplantation. The mean duration of liver disease in recipients was 11 ± 2 yrs. Causes of death in the donors and of hepatic cirrhosis in the recipients are given in Table 5.2. Systemic haemodynamic parameters recorded in liver recipients before transplantation were obtained from anaesthetic notes. Arteries were collected, stored and transported to the laboratory as described (Chapter 2.1.1).

#### 5.3.4.2. Functional Protocol

Connective tissue was cleaned from the hepatic arteries and 4 rings (approximately 1.5mm in length) were prepared from each artery. Any residual endothelium was

**Table 5.2.** Causes of death in liver donors and of cirrhosis in recipients undergoing liver transplantation. Hepatic arteries from these individuals were isolated for studies of ET-1-mediated contraction.

| Cause of Death in Donor | No. | Cause of Cirrhosis in Recipient | No. |
|---|---|---|---|
| Sub-arachnoid Haemorrhage | 3 | Alcoholic Liver Disease | 4 |
| Intra-cerebral Bleeding | 2 | Primary Biliary Cirrhosis | 3 |
| Head Injury | 1 | Cryptogenic Cirrhosis | 1 |
| Intra-cranial Haemorrhage | 1 | Cirrhosis from Autoimmune Hepatitis | 1 |
| Road Traffic Accident | 1 | | |
| Total: | 8 | Total: | 9 |

removed and rings were mounted in organ baths for the measurement of isometric force development. After equilibration of the arterial rings, the viability and reproducibility of contractions were tested, using (100 mmol/L) KCl as described (Chapter 2.2) and then cumulative concentration-response curves were constructed using KCl (2.5-120 mmol/L). The rings were washed repeatedly and, after equilibrating for 30 minutes, three rings were incubated for 40 minutes with an $ET_A$ receptor antagonist, BQ-123, at different concentrations ($3x10^{-7}$ mol/L, $10^{-6}$ mol/L and $3x10^{-6}$ mol/L). The fourth ring was not exposed to the antagonist and served as a control. Following the incubation, cumulative-concentration response curves were constructed using ET-1 ($10^{-11}$-$3x10^{-6}$ mol/L).

## 5.4. Statistics

Statistical analysis was performed as described in Chapter 2.7.

## 5.5. Results

### *5.5.1. Test for Endothelium*

None of the hepatic arterial rings (6 donor, 6 recipient) tested for endothelial cell function relaxed in response to ACh. Indeed, ACh produced contractions in both donor and recipient hepatic arteries, which became more pronounced as the concentration of ACh was increased (Figure 5.1a,b). In contrast, all of these rings relaxed in response to SIN-1 (maximum relaxation, 103.21 ± 1.85 % and 106.80 ± 1.93 %, $P$=0.21; sensitivity (-log $IC_{50}$), 6.72 ± 0.04. and 6.68 ± 0.02, $P$=0.78, in donor and recipient hepatic arteries respectively) (Figure 5.1c), following sub-maximal contraction with PE (6.72 ± 0.04. and 6.68 ± 0.02 for donor and recipient

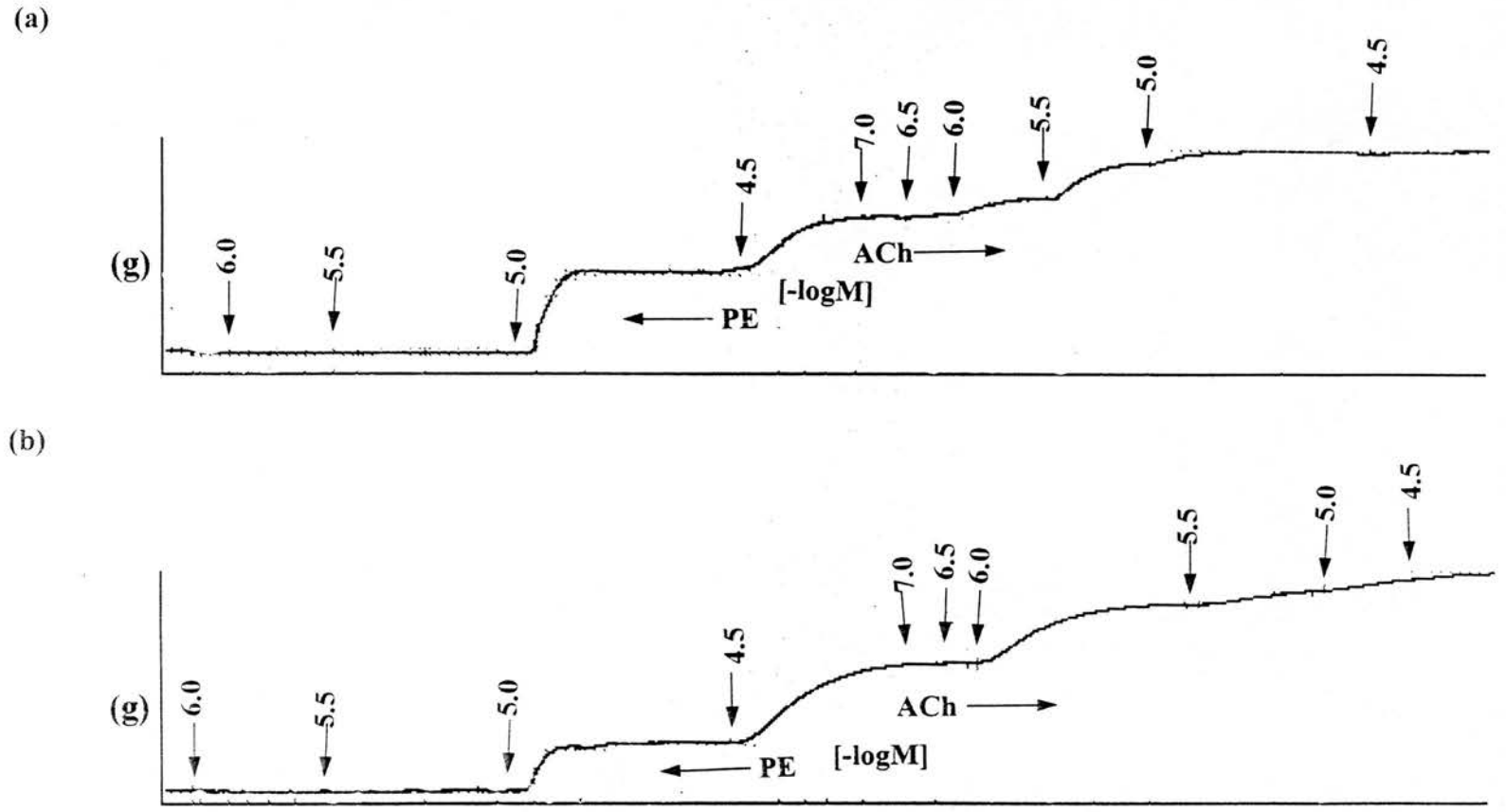



**Figure 5.1(a,b).** Test for endothelial cell function. Traces showing the response of human **(a)** recipient and **(b)** donor hepatic arteries to the endothelium-dependent vasodilator, acetylcholine (ACh, $10^{-7}$-$3x10^{-5}$ mol/L) after sub-maximal contraction with phenylephrine (PE, $10^{-6}$-$3x10^{-5}$ mol/L). The contractile response to ACh indicated an absence of functional endothelium in the arteries from both groups.

(c)

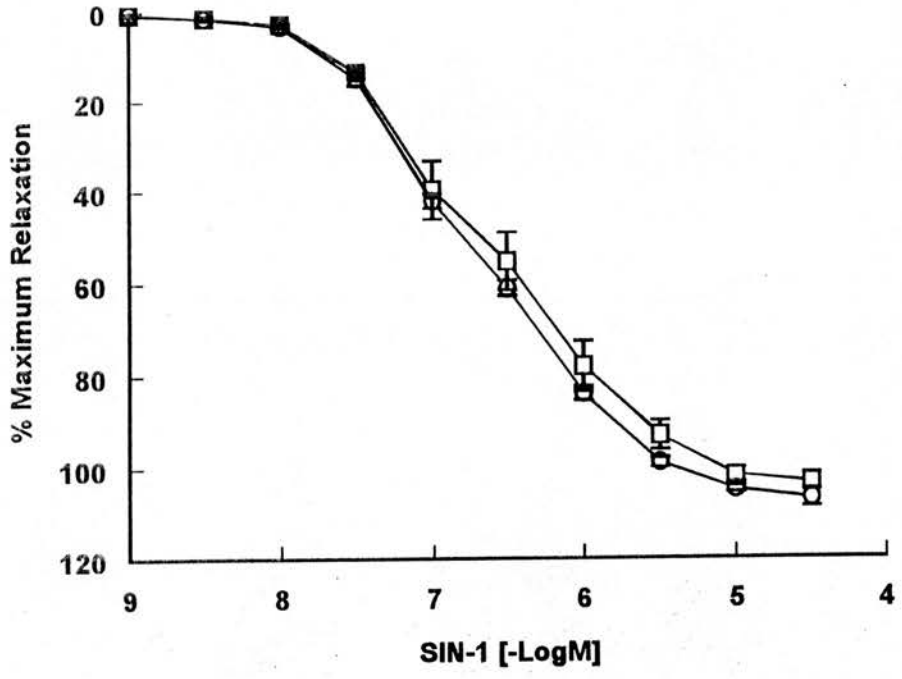



**Figure 5.1(c).** Test for endothelium-independent vasodilatation. Cumulative concentration-response curves produced by 3'-morpholinosydnonimine (SIN-1, $10^{-9}$-$3x10^{-5}$ mol/L) in recipient (O) and donor (❑) hepatic arteries after sub-maximal contraction with phenylephrine (PE, $10^{-6}$-$3x10^{-5}$ mol/L). Responses are given as mean ± s.e. mean for (n) individuals, expressed as a percentage of the maximum contraction to phenylephrine (PE).

hepatic arteries, respectively).

Similarly, histological assessment with silver nitrate indicated that none of the donor hepatic arteries had an intact endothelium, whilst, in a small number of recipient hepatic arteries, only a tiny patch of endothelial cells was observed (Figure 5.2).

Immunohistochemical staining with the Ulex antibody confirmed that none of the donor arterial rings had an intact endothelial cell layer, whilst rings from recipient arteries had only small patches of endothelium (Figure 5.3).

Therefore, in all subsequent experiments the absence of any functional endothelium was ensured by rubbing the luminal surface of the vessels, as described (Chapter 2.2.1).

### *5.5.2. Analysis of AVP and 5-HT-Mediated Contraction*

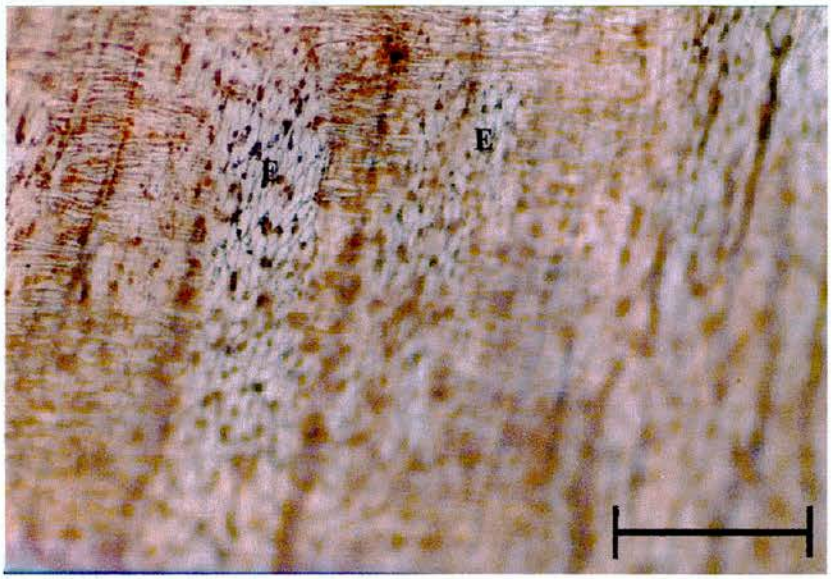
#### 5.5.2.1. Haemodynamic Profile

The cirrhotic liver recipients had elevated heart rate (100 ± 3 b/min, n=36; normal range 60-90 b/min) and cardiac output (10.5 ± 0.6 L/min, n=33; normal range 4-8 L/min) with low systemic vascular resistance (699 ± 35 dyne.sec/cm$^5$, n=31; normal range 1200-1500 dyne.sec/cm$^5$). These haemodynamic parameters indicated the presence of a hyperdynamic circulation.

#### 5.5.2.2. Effect of Cirrhosis on AVP and 5-HT-Mediated Contraction

Endothelium-denuded hepatic arteries from non-cirrhotic donors and cirrhotic

**(a)** Recipient Hepatic Artery




**(b)** Donor Hepatic Artery

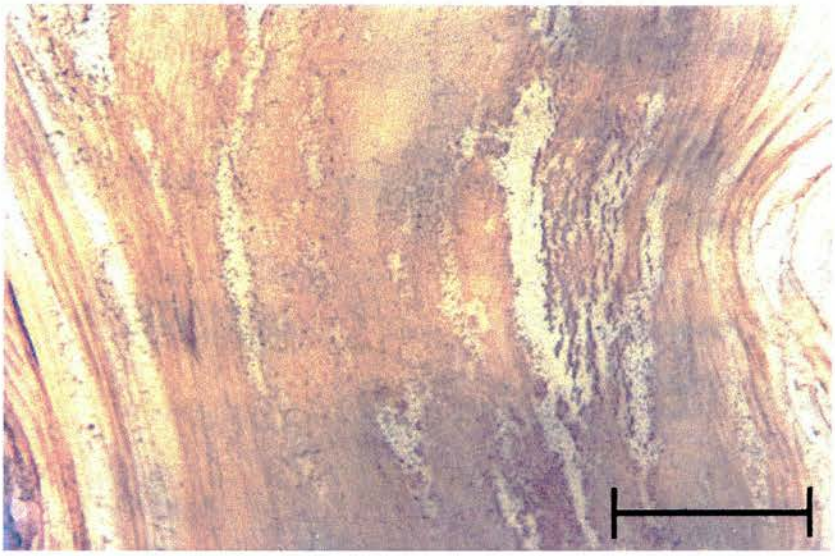
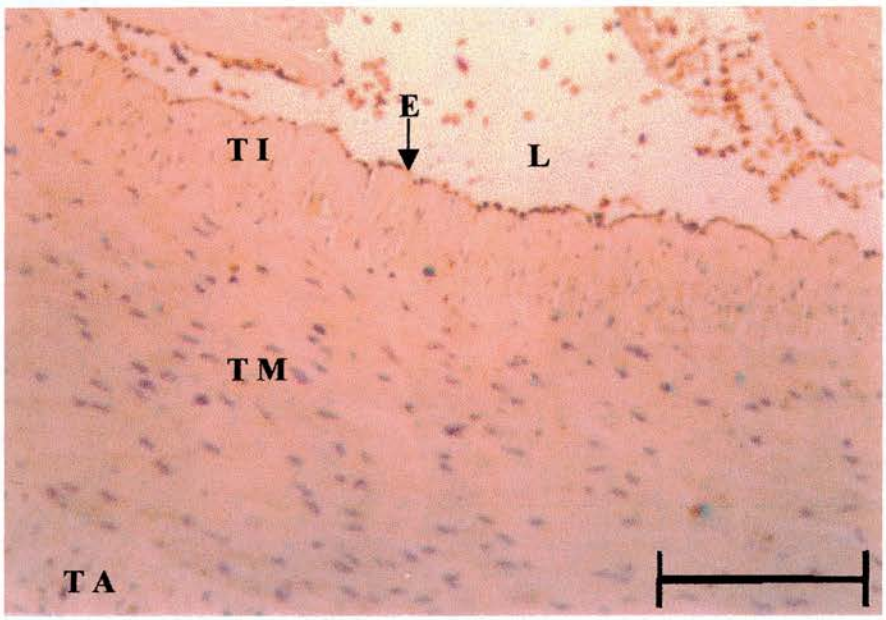


**Figure 5.2.** Photomicrographs of longitudinal sections of **(a)** recipient and **(b)** donor hepatic arteries, stained with silver nitrate. The presence of small residual endothelium (E) observed in recipient but not in donor and hepatic arteries. Scale=100μm.

**(a)** Recipient Hepatic Artery




**(b)** Donor Hepatic Artery

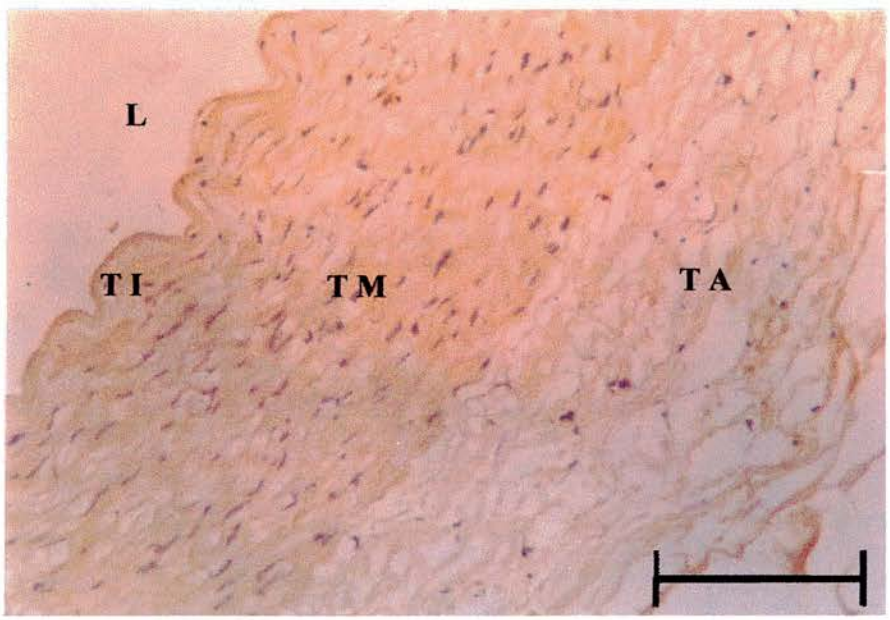



**Figure 5.3.** Photomicrographs of immunohistochemical staining using the antibody to demonstrate the presence of endothelial cells (E) in transverse sections of **(a)** recipient and **(b)** donor hepatic arteries. L, lumen; TI, tunica intima; TM, tunica media and TA, tunica adventitia. Scale=100μm.

recipients contracted, in a cumulative, concentration-dependent way, when exposed to receptor-dependent agonists, AVP and 5-HT, and the receptor-independent vasoconstrictor, KCl.

The maximum contractile response to AVP in recipient hepatic arteries (Emax, 3.53 ± 0.35g, n=22) was significantly smaller ($P$=0.003) than that produced in donor hepatic arteries (Emax, 5.73 ± 0.61g, n=23). This difference was also evident ($P$=0.0002) when the results were expressed as a percentage of maximum contractile response to KCl (Emax, 34.03 ± 3.42% and 60.69 ± 5.56%, respectively) (Figure 5.4.a). However, the sensitivity ($pD_2$) to this agonist was similar ($P$=0.86) in recipient (8.21 ± 0.08) and donor (8.16 ± 0.07) hepatic arteries.

In contrast, the maximum contractile response to 5-HT in recipient hepatic arteries (Emax, 8.60 ± 0.46g, n=25) was greater than the donor hepatic arteries (Emax, 7.12 ± 0.58g, n=26), but achieved only threshold significance ($P$=0.06). However, significance was achieved ($P$=0.01) when the results were expressed as a percentage of maximum contraction to KCl (Emax, 88.81 ± 5.43% and 71.63 ± 4.55%, respectively) (Figure 5.4.b). The sensitivity ($pD_2$) of the response to 5-HT was similar in recipient (6.60 ± 0.08) and donor (6.45 ± 0.08) hepatic arteries ($P$=0.20). In spite of altered maximum contractile responses to the receptor-dependent agonists, AVP and 5-HT, the maximum contractile response to the receptor-independent vasoconstrictor, KCl, was similar ($P$=0.81) in recipient (Emax, 10.32 ± 0.70g, n=25) and in donor (Emax, 10.09 ± 0.71g, n=26) hepatic arteries (Figure 5.4.c). The sensitivity to KCl, in recipient (1.63 ± 0.04) and donor (1.70 ± 0.05) hepatic arteries

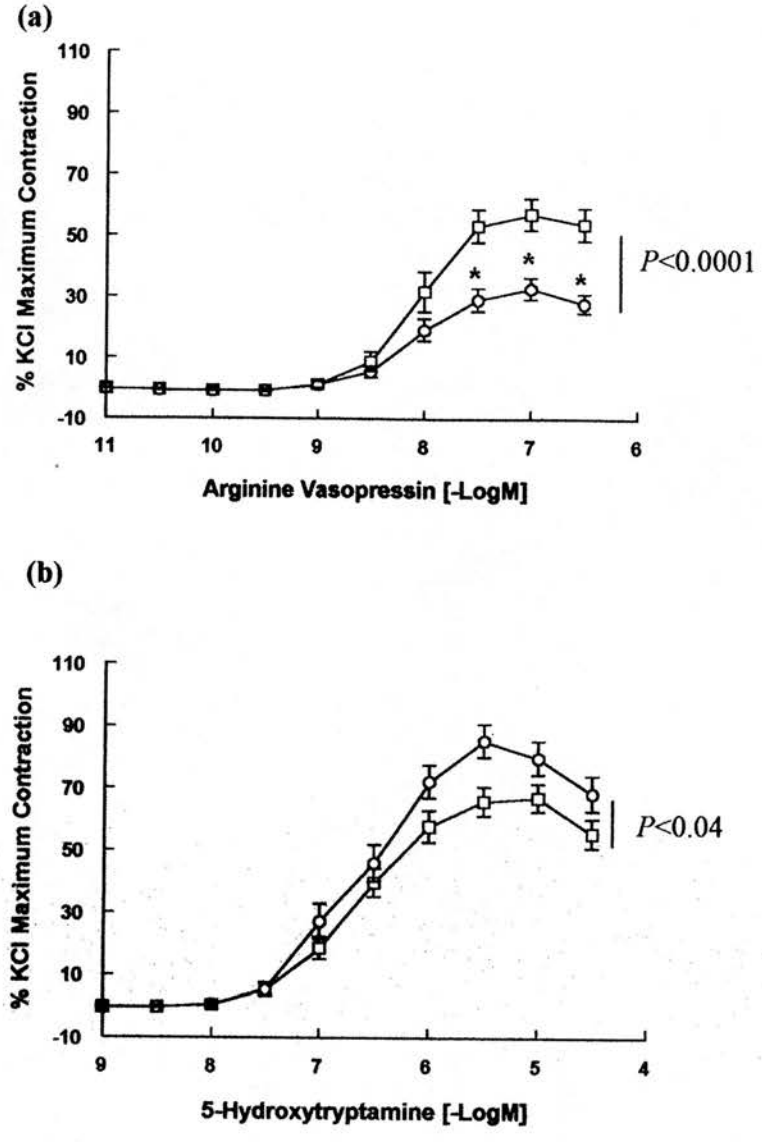



**Figure 5.4 (a,b).** Cumulative concentration-response curves comparing contractile responses of recipient (cirrhotic, O) and donor (non-cirrhotic, ❑) hepatic arteries following exposure to **(a)** arginine vasopressin (AVP; n=22-23) and **(b)** 5-hydroxytryptamine (5-HT; n=25-26). Responses are mean ± s.e. mean for (n) individuals and expressed as a percentage of the maximum contraction induced by KCl. Differences between curves were detected using two-way analysis of variance. $*P < 0.0001$ when individual points were compared using the Tukey post-hoc test.

(c)

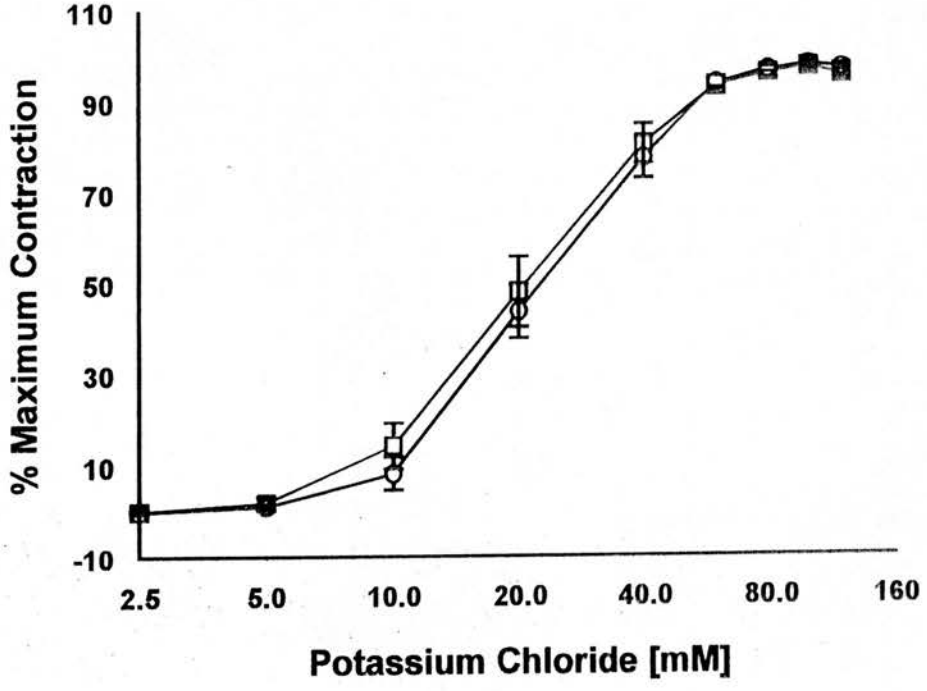



**Figure 5.4(c).** Cumulative concentration-response curves comparing contractile responses of recipient (cirrhotic, O) with donor (non-cirrhotic, ❑) hepatic arteries following exposure to potassium chloride (KCl; n=26). Responses are mean ± s.e. mean for (n) individuals and expressed as a percentage of the maximum contraction induced by KCl.

was also not different ($P$=0.34).

*5.5.3. Role of Nitric Oxide Synthase Activity in the Hepatic Arteries*

5.5.3.1. Influence of Nitric Oxide Synthase Activity on Contractile Function

Denuded hepatic arteries obtained from cirrhotic recipients (n=9) and non-cirrhotic donors (n=8), produced cumulative concentration-dependents contraction in response to AVP, 5-HT and KCl whether incubated with L-NNA or D-NNA. Neither L-NNA nor D-NNA had any effect on basal tone in recipient or donor hepatic arteries (Table 5.3). In arteries treated with D-NNA, responses (Emax but not $pD_2$) to AVP were smaller in recipient than donor, whereas responses (Emax but not $pD_2$) to 5-HT were larger in the recipient vessels (Table 5.3; Figure 5.5). This is consistent with results obtained using untreated arteries in the earlier part of this study.

In both recipient and donor hepatic arteries, responses to AVP, 5-HT and KCl were also unaffected by exposure to L-NNA producing contraction similar to those obtained using arteries incubated with the inactive isomer, D-NNA. (Figure 5.5; Table 5.3). However, the rings, from both group of arteries, following exposure to L-NNA, relaxed in response to SIN-1 (Emax, 108.13 ± 1.21 % and 105.22 ± 1.84 %, $P$=0.19 and -log $IC_{50}$, 6.67 ± 0.10 and 6.66 ± 0.09, $P$ = 0.92, in recipient and donor hepatic arteries respectively).

5.5.3.2. Nitric Oxide Synthase Immunoreactivity

Intense immunoreactivity in the rabbit aortic ring indicated the induction of iNOS in this vessel (positive control) following endotoxaemia (Chapter 2, Figure 2.7). In the

**Table 5.3.** Maximum contractile response (Emax) and sensitivity ($pD_2$) to arginine vasopressin (AVP), 5-hydroxytryptamine (5-HT) and potassium chloride (KCl) obtained in recipient and donor hepatic arteries following incubation with D-NNA or L-NNA

**(a)** Recipient Hepatic Artery (n=9)

| | **D-NNA** | | | **L-NNA** | | |
|---|---|---|---|---|---|---|
| | Emax | | $pD_2$ | Emax | | $pD_2$ |
| | (g) | (%KCl) | | (g) | (%KCl) | |
| AVP | 4.21±0.56 | 41.05±4.48 | 7.91±0.17 | 4.58±0.63 | 47.06±6.39 | 7.90±0.07 |
| 5-HT | 8.38±0.98 | 85.58±4.71 | 6.73±0.14 | 8.96±0.97 | 89.02±5.03 | 6.61±0.16 |
| KCl | 10.18±1.00 | --------- | 1.67±0.06 | 10.38±1.19 | --------- | 1.65±0.08 |

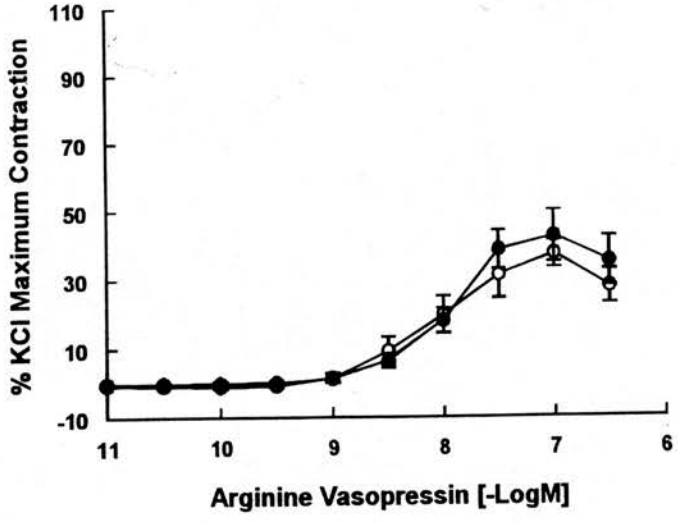
**(b)** Donor Hepatic Artery (N=8)

| | **D-NNA** | | | **L-NNA** | | |
|---|---|---|---|---|---|---|
| | Emax | | $pD_2$ | Emax | | $pD_2$ |
| | (g) | (%KCl) | | (g) | (%KCl) | |
| AVP | 6.82±0.81* | 71.55±9.26** | 8.07±0.07 | 6.75±0.73 | 68.80±8.78 | 7.90±0.14 |
| 5-HT | 6.90±0.81 | 67.04±8.06 | 6.55±0.16 | 7.05±0.88 | 69.48±8.32 | 6.51±0.19 |
| KCl | 10.74±1.10 | --------- | 1.60±0.06 | 10.21±0.88 | --------- | 1.58±0.07 |

Values are mean ± s.e. mean for (n) subjects. *P<0.01 and **P<0.005, when compared with the recipient hepatic arteries, using Student's unpaired t-test.

**(a)**

**(i)**




**(ii)**

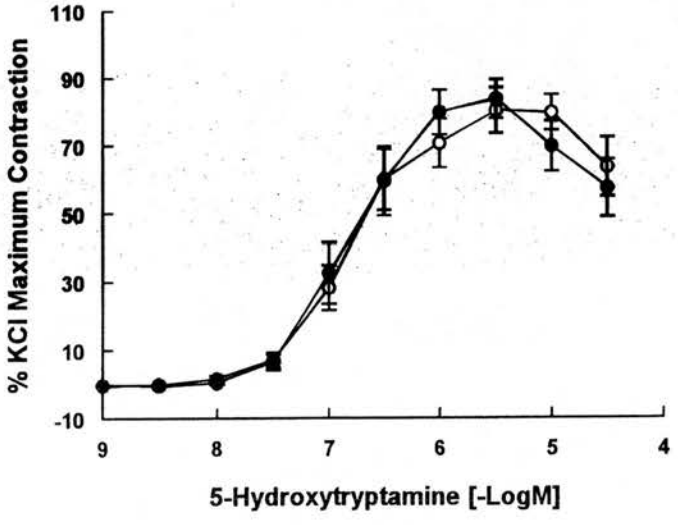
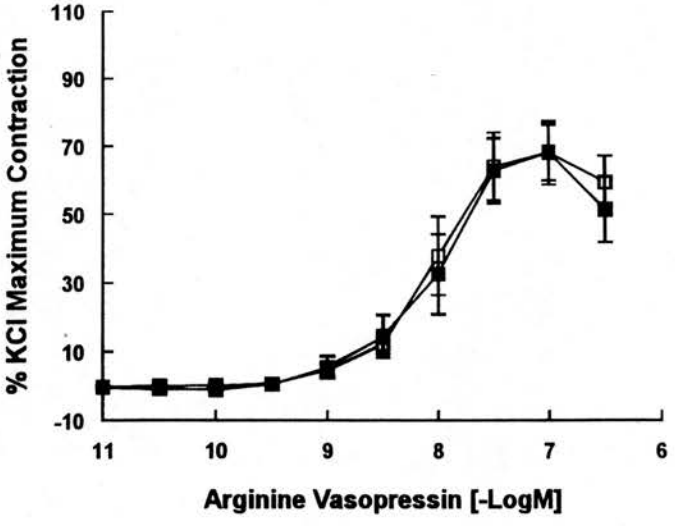



**Figure 5.5(a).** The effect of Nitric Oxide Synthase (NOS) inhibition on the contractile responses of recipient (O, n=9) hepatic arteries. Cumulative concentration-response curves produced by these arteries after incubation with D-NNA (open symbol) and L-NNA (solid symbol), in response to **(i)** arginine vasopressin (AVP) and **(ii)** 5-hydroxytryptamine (5-HT). Responses are given as mean ± s.e.mean for (n) individuals and expressed as a percentage of the maximum contraction induced by KCl.

**(b)**

**(i)**




**(ii)**

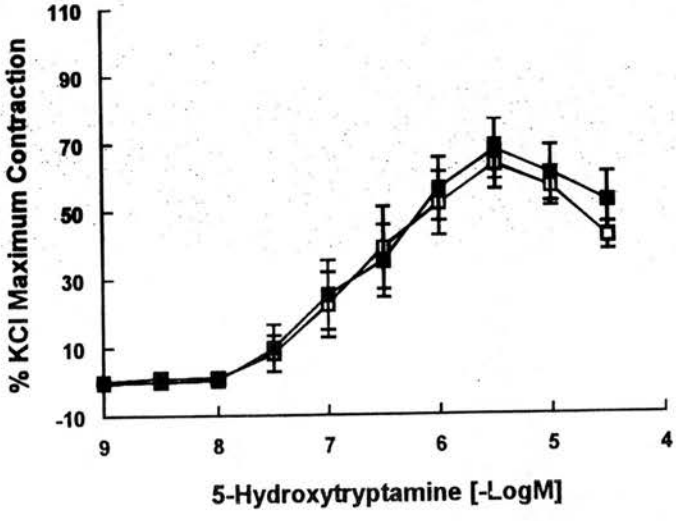



**Figure 5.5(b).** The effect of Nitric Oxide Synthase (NOS) inhibition on the contractile responses of donor (❑, n=8) hepatic arteries. Cumulative concentration-response curves produced by these arteries after incubation with D-NNA (open symbol) and L-NNA (solid symbol), in response to **(i)** arginine vasopressin (AVP) and **(ii)** 5-hydroxytryptamine (5-HT). Responses are given as mean ± s.e.mean for (n) individuals and expressed as a percentage of the maximum contraction induced by KCl.

recipient group (Figure 5.6a), only hepatic arterial rings from patients with alcoholic liver diseases (n=3) had mild staining for iNOS (Figure 5.6a(i)). iNOS was not detected in arteries from patients with primary biliary cirrhosis (n=3), cirrhosis from chronic active hepatitis (n=1) or cryptogenic cirrhosis (n=2) (Figure 5.6a(ii, iii or iv)). None of the arterial rings obtained from the donor hepatic arteries demonstrated any staining for iNOS (Figure 5.6b).

### *5.5.4. Identification of the Receptors Mediating AVP-induced Contraction of Human Hepatic Arteries*

#### 5.5.4.1. The Role of $V_2$-receptors

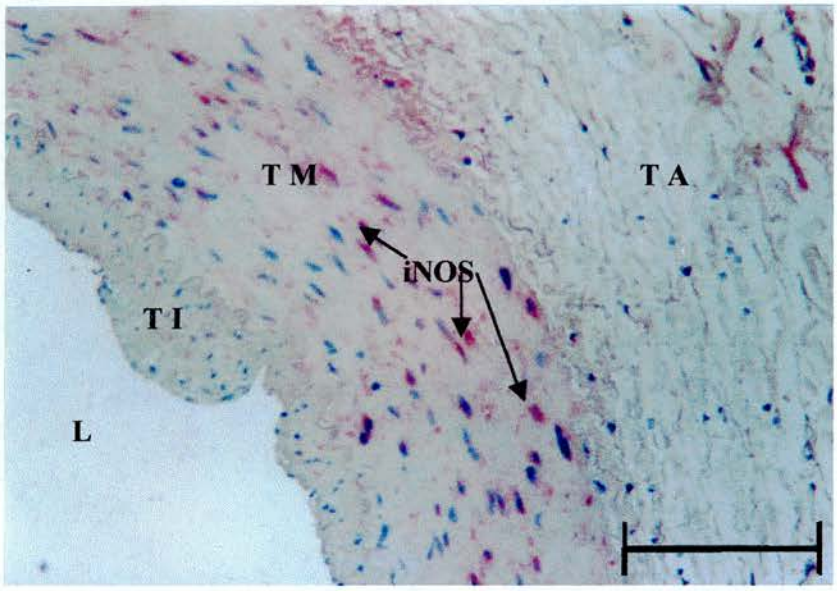
Hepatic arterial rings either from the recipient (n=6) or from the donor (n=6) groups failed to contract in response to the selective $V_2$-receptor agonist, DDAVP (Figure 5.7a). These arteries did, however, contract in response to AVP (Figure 5.7b) and KCl (Figure 5.7c).

As demonstrated previously, the AVP-mediated contraction was smaller in recipient (Emax, 4.35 ± 0.50g) than in donor hepatic arteries (Emax, 6.75 ± 0.45g; $P$=0.005), whereas, the sensitivity of response was unchanged ($pD_2$, 7.97 ± 0.19 and 8.04 ± 0.12, in recipient and donor hepatic arteries respectively; $P$=0.71). KCl-mediated responses of recipient (Emax, 10.32 ± 1.13g; $pD_2$, 1.63 ± 0.08) and donor (Emax, 10.16 ± 0.1.23g; $pD_2$, 1.64 ± 0.05) hepatic arteries were similar.

#### 5.5.4.2. The Role of $V_1$-receptors

Incubation of recipient and donor hepatic arterial rings, with a $V_1$-receptor antagonist

**(i)** Hepatic Artery from Patient with Alcoholic Liver Disease




**(ii)** Hepatic Artery from Patient with Primary Biliary Cirrhosis

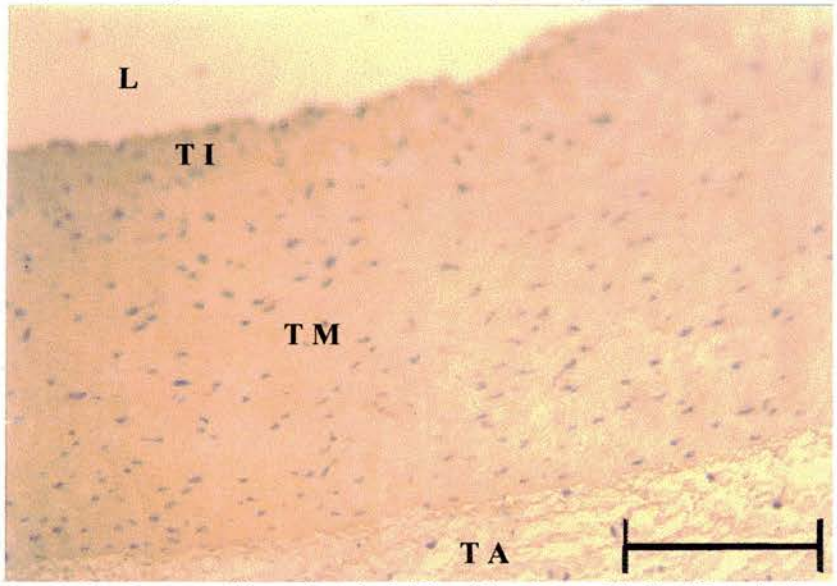
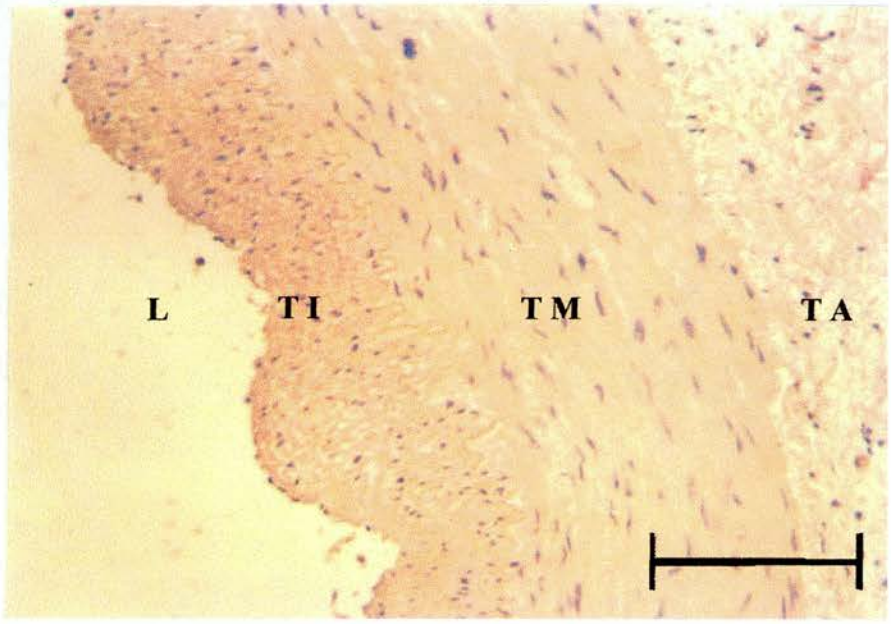



**Figure 5.6a.** Immunohistochemical identification of inducible nitric oxide synthase (iNOS) in the vessel wall (VSMCs). Photomicrographs demonstrating immunoreactivity for iNOS (red colouration) in transverse sections of hepatic arteries from patient with **(i)** alcoholic liver disease but not in that from patient with **(ii)** primary biliary cirrhosis. L, lumen; TI, tunica intima; TM, tunica media and TA, tunica adventitia. Scale=100μm.

**(iii)** Hepatic Artery from Patient with Cryptogenic Cirrhosis




**(iv)** Hepatic Artery from Patient with Chronic Active Hepatitis

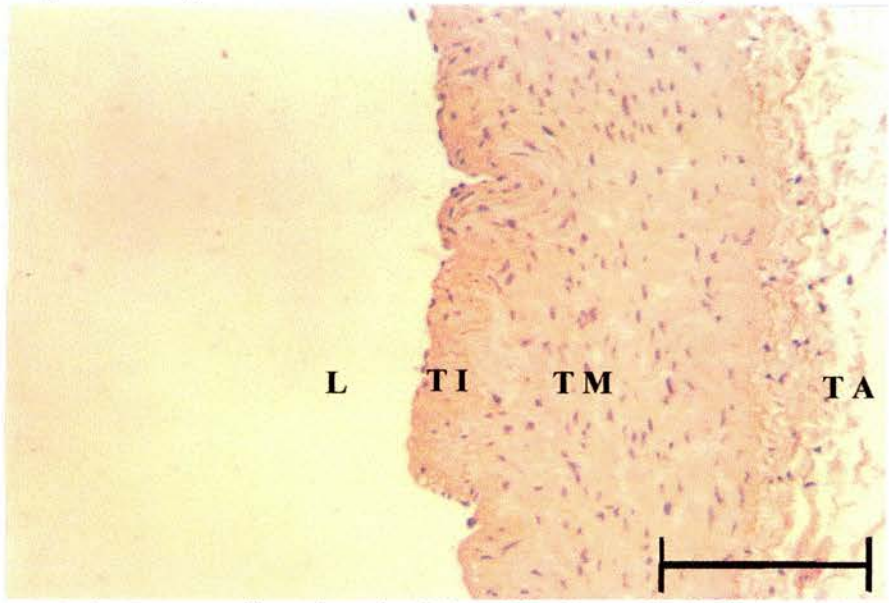



**Figure 5.6a.** Immunohistochemical identification of inducible nitric oxide synthase (iNOS) in the vessel wall. Photomicrographs demonstrating immunoreactivity for iNOS (red colouration) in transverse sections of hepatic arteries from patient with **(i)** alcoholic liver disease but not in those from patients with **(iii)** cryptogenic cirrhosis and **(iv)** chronic active hepatitis. L, lumen; TI, tunica intima; TM, tunica media and TA, tunica adventitia. Scale=100μm.

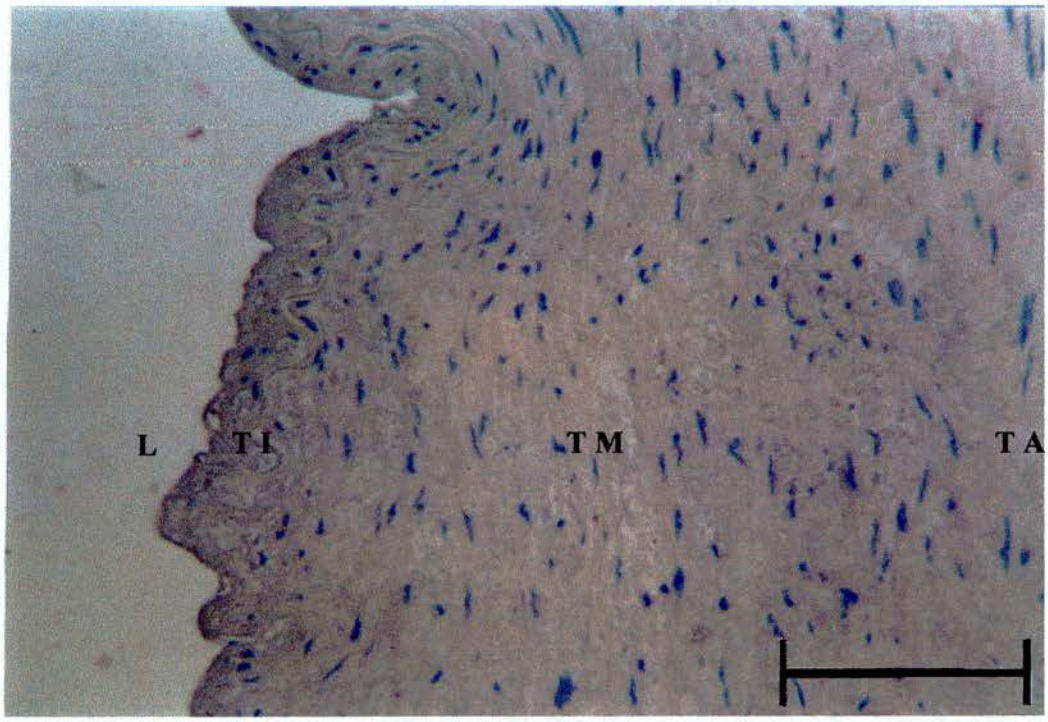



**Figure 5.6b.** Immunohistochemical identification of inducible nitric oxide synthase (iNOS) in the vessel wall (VSMCs). Photomicrograph demonstrating the lack of immunoreactivity for iNOS in a transverse section of donor hepatic artery. L, lumen; TI, tunica intima; TM, tunica media and TA, tunica adventitia. Scale=100μm.

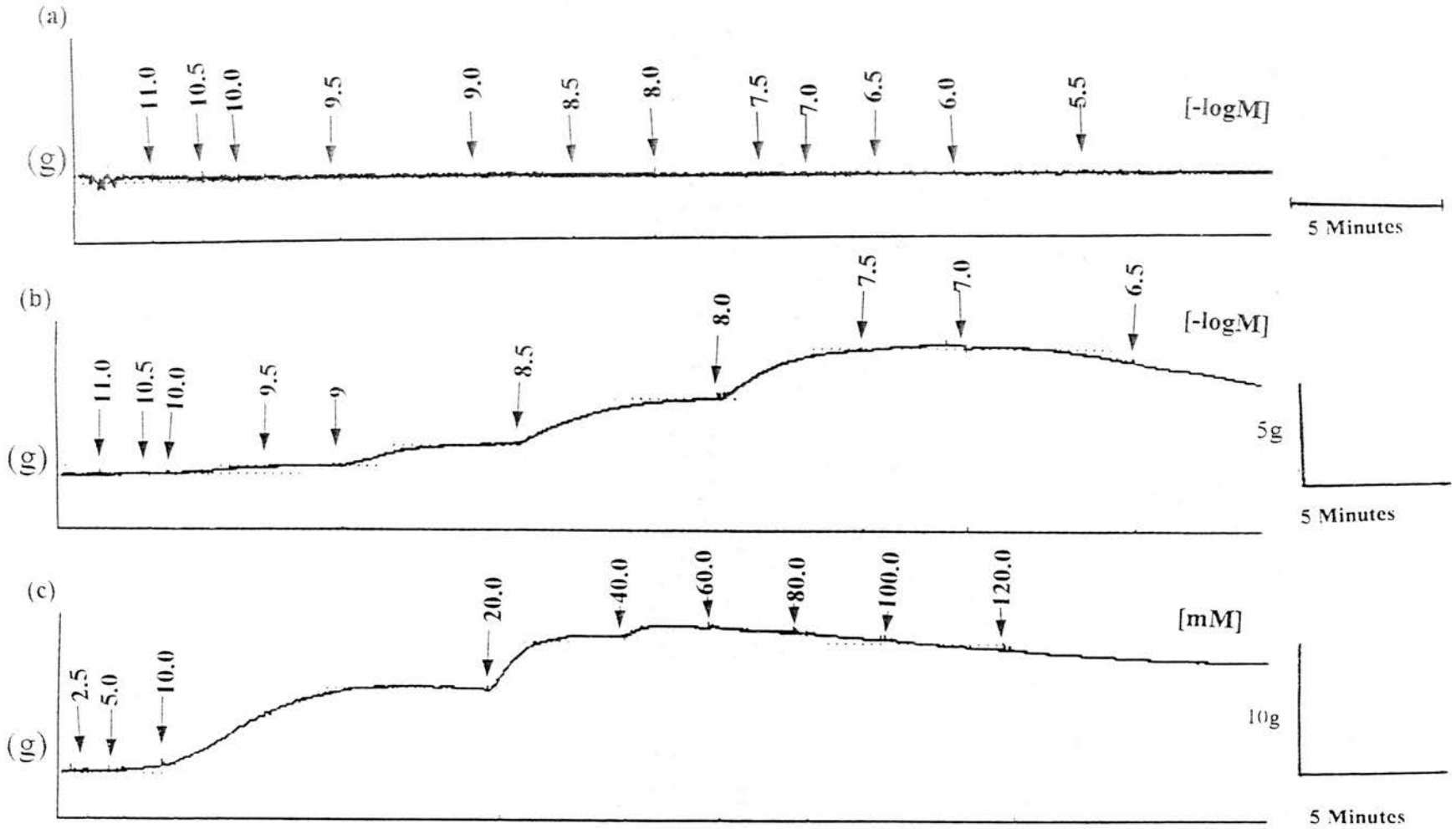



**Figure 5.7.** Assessment of the role of $V_2$-receptors in mediating contractile response of hepatic arteries to AVP. Traces showing cumulative concentration-response curves in donor hepatic arteries to **(a)** desmopressin (DDAVP, $10^{-11}$-$3x10^{-6}$ mol/L) and **(b)** arginine vasopressin (AVP, $10^{-11}$-$3x10^{-7}$ mol/L).

$d(CH_2)_5Tyr(Me)AVP$, produced a concentration-dependent rightward shift in the response to AVP with a significant reduction in sensitivity ($pD_2$) (Table 5.4; Figure 5.8). Exposure to the $V_1$-receptor antagonist had no effect on the maximum contractile responses to AVP in either recipient or donor hepatic arteries when compared with their corresponding controls (Table 5.4). The maximum contractile response and sensitivity to KCl in recipient and donor hepatic arteries were also similar following exposure to the $V_1$-receptor antagonist (Table 5.4).

### *5.5.5. Effect of Hepatic Cirrhosis on ET-1-Mediated Contraction of the Hepatic Arteries*

#### 5.5.5.1 Haemodynamic Profile

As with the previous group, recipients with hepatic cirrhosis had elevated heart rate (119 ± 9 b/min, n=9; normal range 60-90 b/min) and cardiac output (9.19 ± 1.02 L/min, n=8; normal range 4-8 L/min), combined with low systemic vascular resistance (648 ± 68 dyne.sec/cm$^5$, n=8; normal range 1200-1500 dyne.sec/cm$^5$).

#### 5.5.3.2. ET-1-mediated Contraction of Hepatic Arteries

Both recipient and donor hepatic arteries contracted in response to ET-1 (Figure 5.9). The maximum contraction in recipient arteries, whether expressed in grammes or as a percentage of maximum contractile response to KCl was similar to the response obtained in donor hepatic arteries (Table 5.5). Strikingly, however, the concentration-response curve produced by ET-1 in recipient hepatic arteries was shifted to the left, compared with donor hepatic arteries, reflecting a significant increase in sensitivity.

**Table 5.4.** The maximum contractile response (Emax) and sensitivity ($pD_2$) of responses to arginine vasopressin (AVP) in the absence (control) and presence of the $V_1$-receptor antagonist, $d(CH_2)5Tyr(Me)AVP$ at a concentration $10^{-8}$ mol/L, $10^{-7}$ mol/L or $10^{-6}$ mol/L and to potassium chloride (KCl).

**(a)** Arginine Vasopressin

| | Recipient Hepatic Arteries (n=9-11) | | | Donor Hepatic Arteries (n=8-10) | | |
|---|---|---|---|---|---|---|
| | Emax(g) | Emax (KCl%) | $pD_2$ | Emax (g) | Emax (KCl%) | $pD_2$ |
| Control | 4.18±0.59 | 39.81±5.75 | 8.03 ± 0.12 | 6.93±0.77* | 70.38±9.87* | 8.07 ± 0.16 |
| $10^{-8}$mol/L | 3.89±0.73 | 41.89±7.69 | 7.25 ± 0.14[a] | 6.37±0.60 | 64.73±8.32 | 7.27 ± 0.14[d] |
| $10^{-7}$mol/L | 4.15±0.88 | 39.47±8.59 | 6.56 ± 0.11[b] | 6.34±0.78 | 63.16±9.84 | 6.71 ± 0.12[b] |
| $10^{-6}$mol/L | 4.30±0.82 | 36.47±4.21 | 6.15 ± 0.14[c] | 5.99±0.68 | 56.22±9.67 | 6.07 ± 0.10[c] |

**(b)** Potassium Chloride.

| | Recipient Hepatic Arteries (n=9-11) | | Donor Hepatic Arteries (n=8-10) | |
|---|---|---|---|---|
| | Emax(g) | $pD_2$ | Emax(g) | $pD_2$ |
| Control | 11.34±0.92 | 1.67 ± 0.04 | 11.34±1.52 | 1.64 ± 0.07 |
| Ring-1 | 10.37±0.87 | 1.69 ± 0.05 | 11.05±1.44 | 1.63 ± 0.08 |
| Ring-2 | 11.42±1.36 | 1.61 ± 0.05 | 11.33±1.36 | 1.60 ± 0.06 |
| Ring-3 | 10.93±1.13 | 1.63 ± 0.07 | 11.88±1.61 | 1.61 ± 0.08 |

Values are mean ± s.e. mean for (n) subjects. *$P<0.02$, when compared with the control response of the recipient hepatic arteries, [a]$P<0.0002$, [b]$P<0.0001$ and [c]$P<0.00001$, when compared with the relevant control, using Student's unpaired t-test.

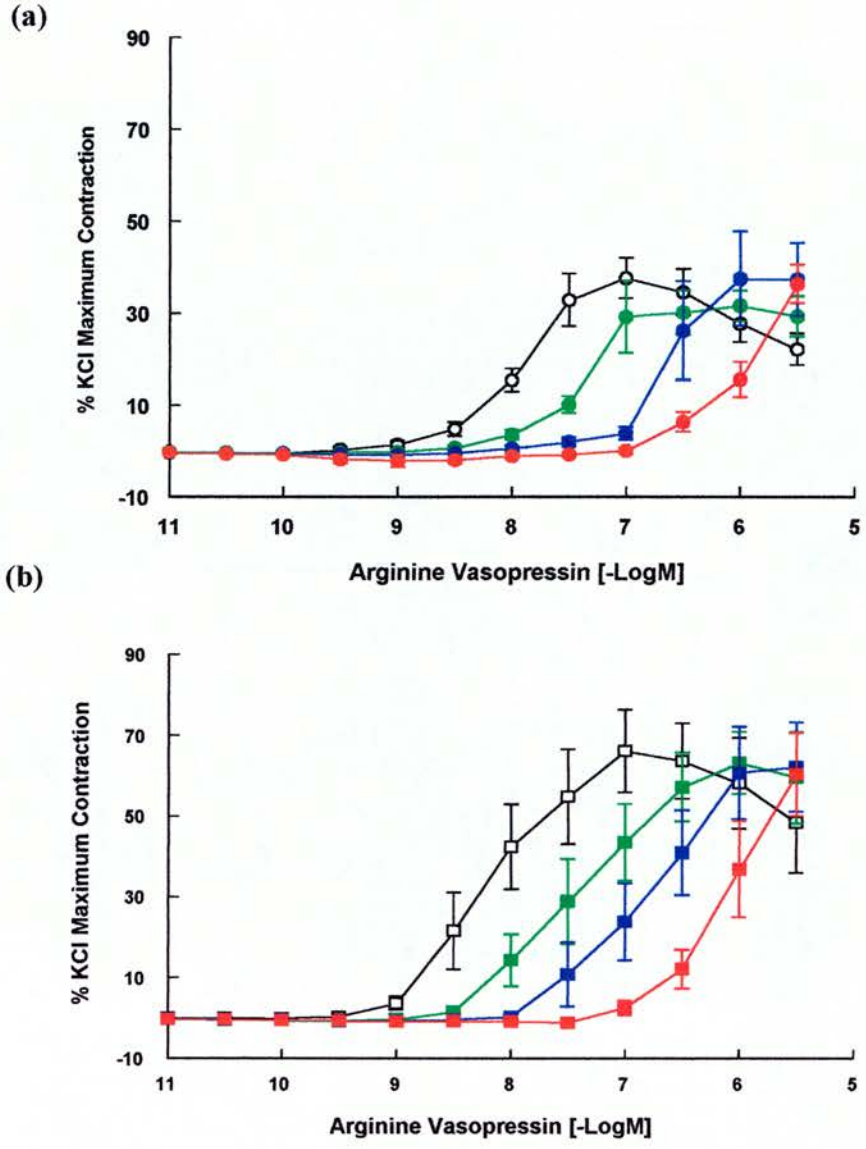



**Figure 5.8.** The effect of incubation with the $V_1$-receptor antagonist, $d(CH_2)_5Tyr(Me)AVP$ on the contractile response of the hepatic arteries. Cumulative concentration-response curves produced in response to arginine vasopressin (AVP) in the absence (control; black, n=10-11) and presence of $V_1$-receptor antagonist ($10^{-8}$ mol/L, green, n=10-11; $10^{-7}$ mol/L, blue; n=8-10 and $10^{-6}$ mol/L, red; n=8-9) obtained in **(a)** recipient (○) and **(b)** donor (□) hepatic arteries. Values are mean ± s.e. mean for (n) individuals and are expressed as a percentage of the maximum contraction induced by KCl.

(a)

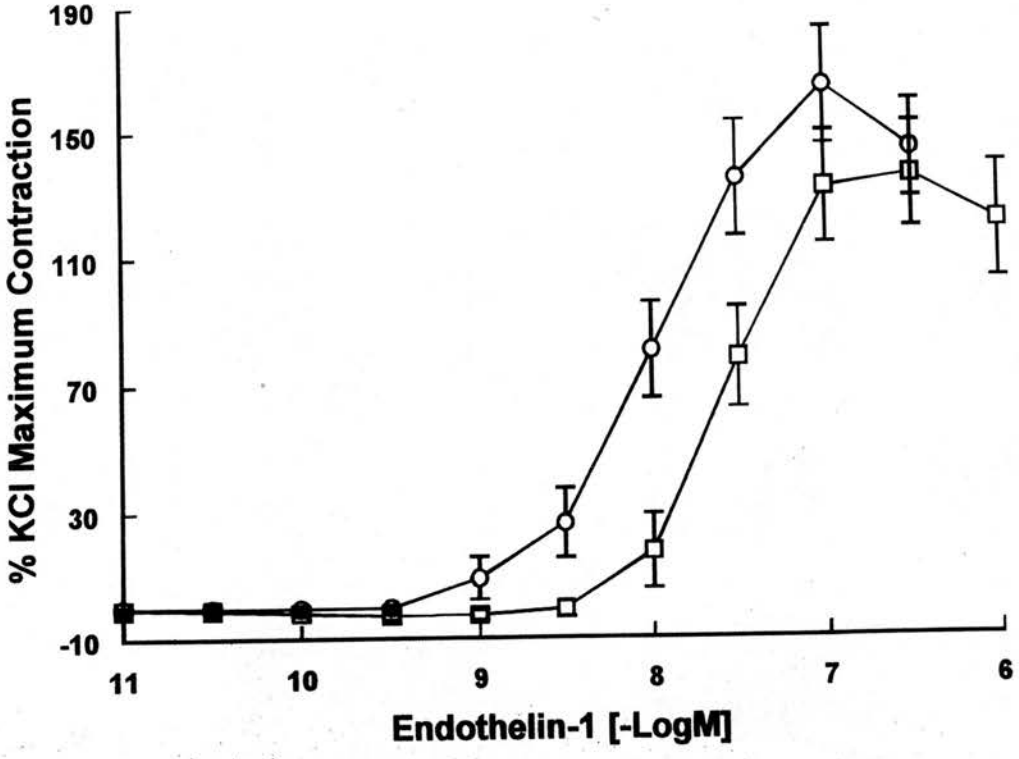



**Figure 5.9a.** The effect of cirrhosis on endothelin-1-mediated contraction of human hepatic arteries. Cumulative concentration-response curves to endothelin-1 were compared in recipient (cirrhotic, O; n=9) and donor (non-cirrhotic, □; n=8) hepatic arteries. Values are mean ± s.e. mean for (n) individuals and are expressed as a percentage of the maximum contraction induced by KCl.

**Table 5.5.** The maximum contraction (Emax) and sensitivity ($pD_2$) of the responses to potassium chloride (KCl) and endothelin-1 (ET-1) in the absence (control) and presence of the endothelin$_A$ ($ET_A$) receptor antagonist, BQ-123 at a concentration of $3x10^{-7}$ mol/L, $10^{-6}$ mol/L or $3x10^{-6}$ mol/L.

**(a)** Response to Potassium Chloride

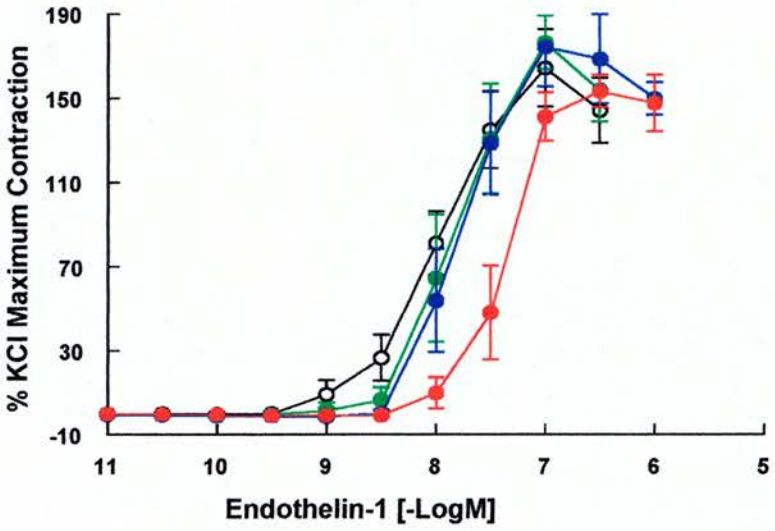
| | Recipient Hepatic Arteries (n=9) | | Donor Hepatic Arteries (n=8) | |
|---|---|---|---|---|
| | Emax(g) | $pD_2$ | Emax(g) | $pD_2$ |
| Control | 7.68±1.10 | 1.59±0.05 | 7.95±1.71 | 1.63±0.08 |
| Ring-1 | 8.21±1.35 | 1.62±0.08 | 8.16±1.48 | 1.65±0.10 |
| Ring-2 | 7.41±0.95 | 1.63±0.09 | 9.40±0.98 | 1.66±0.11 |
| Ring-3 | 9.81±0.94 | 1.63±0.08 | 8.78±1.58 | 1.64±0.12 |

**(b)** Response to Endothelin-1

| | Recipient Hepatic Arteries (n=9) | | | Donor Hepatic Arteries (n=8) | | |
|---|---|---|---|---|---|---|
| | Emax | | $pD_2$ | Emax | | $pD_2$ |
| | (g) | (%KCl) | | (g) | (%KCl) | |
| Control | 12.5±1.7 | 171.2±17.3 | 7.94±0.08 | 9.8±1.7 | 142.3±16.1 | 7.55±0.09* |
| $3x10^{-7}$mol/L | 13.8±1.6 | 176.4±12.6 | 7.83±0.14 | 10.5±1.4 | 135.4±9.6 | 7.36±0.07 |
| $1x10^{-6}$mol/L | 13.5±1.6 | 186.3±17.8 | 7.74±0.12 | 11.8±1.5 | 146.2±17.9 | 7.20±0.06[a] |
| $3x10^{-6}$mol/L | 16.1±1.9 | 162.1±7.7 | 7.36±0.09[b] | 10.8±1.6 | 137.8±20.3 | 6.83±0.15[b] |

Values are mean ± s.e.mean for (n) subjects. *$P$<0.01 when compared with the control response of the recipient hepatic arteries, [a]$P$<0.01 and [b]$P$<0.001, when compared with their own corresponding control in each group, using Student's unpaired t-test.

**(b)**




**(c)**

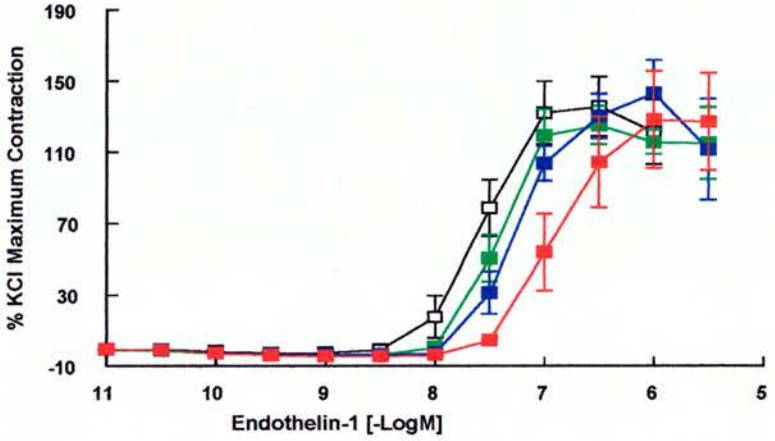



**Figure 5.9(b & c).** The effect of incubation with the endothelin$_A$ ($ET_A$)-receptor antagonist, BQ-123 on the contractile response of the hepatic arteries. Cumulative concentration-response curves produced in response to endothelin-1 (ET-1) in the absence (control, black) and presence of $ET_A$-receptor antagonist ($3x10^{-7}$ mol/L, green; $10^{-7}$ mol/L, blue and $10^{-6}$ mol/L, red) obtained in **(b)** recipient (○, n=9) and **(c)** donor (□, n=8) hepatic arteries. Values are mean ± s.e. mean for (n) individuals and are expressed as a percentage of the maximum contraction induced by KCl.

Incubation with the $ET_A$ receptor antagonist, BQ-123, produced a concentration-dependent rightward shift of the contractile-response curve to ET-1 along with a significant reduction of sensitivity, in both recipient (Figure 5.9b) and donor (Figure 5.9c) hepatic arteries, when compared with their corresponding controls (Table 5.5). This reduction of sensitivity in donor hepatic arteries was significant at $10^{-6}$ mol/L and $3x10^{-6}$ mol/L BQ-123, whilst, in recipient hepatic arteries the reduction only became significant at the higher concentration ($3x10^{-6}$ mol/L) (Table 5.5). However, exposure to the $ET_A$ receptor antagonist, BQ-123, had no effect on the ET-1-mediated maximum contractile responses in either group of arteries (Table 5.5).

In this study, the concentration-response curve to KCl was constructed before determination of ET-1-mediated contractile response. As in previous studies, the magnitude of KCl-mediated contraction and sensitivity of response were similar in rings from donor and recipient hepatic arteries (Table 5.5).

**5.6. Discussion**

This study investigated the effect of hepatic cirrhosis on contractile responses of human hepatic arteries to AVP, 5-HT and ET-1. It demonstrated that, in patients with hepatic cirrhosis, contractile responses of the hepatic arteries were altered in an agonist-dependent manner. The mechanisms responsible for these changes were investigated and it was shown that nitric oxide activity had no role in functional alterations. Similarly, structural alterations of the arterial wall were not implicated, as the contraction produced by KCl was similar in donor and recipient hepatic arteries. As predicted (Chapter 3), the arteries used in this study did not have a functional

endothelium, indicating that functional changes are due to abnormalities in the vascular smooth muscle. The lack of endothelium on these arteries, however, means that a role for endothelium-mediated changes contributing to the functional abnormalities in patients with cirrhosis cannot be discounted. One possible explanation for the altered responses described in this study is selective alteration in the activity of individual receptor types. Therefore, the receptors that mediate the contractile responses to AVP and ET-1 were identified pharmacologically. Due to the complexity of the 5-HT receptor system, the mechanisms of the 5-HT-mediated contraction were investigated separately (in Chapter 6).

### *5.6.1. Effect of Cirrhosis on Contractile Function*

Impaired pressor response to vasoconstrictors is a consistent finding in patients with hepatic cirrhosis (Lunzer *et al.*, 1975; MacGilchrist *et al.*, 1991a, b; Newby *et al.*, 1998a, b). Studies using isolated vessels from the patients with cirrhosis (Ryan *et al.*, 1996; Smith *et al.*, 1997; Heller *et al.*, 1999), or from animal models of this condition (Seiber & Groszmann, 1992; Karatapanis *et al.*, 1994; Huang *et al.*, 1995), have also reported impaired contractile responses. The present study revealed for the first time in isolated human hepatic arteries that the manifestation of the impaired response in cirrhotic individuals is not the same for all vasoconstrictors. Strikingly, the contraction of human hepatic arteries induced by AVP was reduced in the cirrhotic group, whereas responses to 5-HT and ET-1 were increased. Furthermore, these results confirmed that alterations were only evident in receptor-mediated vasoconstriction as responses to KCl were unchanged. Consistent with these data, studies with vessels from animal models have also shown that abnormalities in

function are agonist-dependent, as responses to 5-HT (Cummings *et al.*, 1988; Silva *et al.*, 1998) and ET-1 (Cahill *et al.*, 1998) have been shown to be significantly increased whereas responses to adrenoceptor agonists are reduced (Bomzon *et al.*, 1991; Liao *et al.*, 1994). The increased activity of 5-HT and ET-1 constrictor systems is also supported by *in vivo* studies, in which portal pressure was reduced (in patients or in animal models) using 5-$HT_{2A}$ and a mixed ($ET_A$ and $ET_B$) ET-1-receptor antagonist (ketanserin and bosentan, respectively) (Vorobioff *et al.*, 1989; Sogni *et al.*, 1998). Therefore, it is evident that the effect of hepatic cirrhosis will vary depending upon the agonist used to induce contraction.

### *5.6.2. Validation of Donor Hepatic Arteries as Control Vessel*

The altered contractile response in recipient (cirrhotic) hepatic arteries was detected in this study while using donor hepatic arteries as controls. The donor hepatic arteries are routinely exposed to preservative solution (University of Wisconsin solution [UWS] to which dexamethasone, insulin and penicillin have been added) for a considerable period of time (up to 14 hours). It is conceivable that exposure to preservative solution could alter contractile responses in the donor hepatic arteries. Indeed, preservation in UWS increased contraction to KCl and U46619 in human saphenous vein (Anastasious *et al.*, 1997) and augmented hypoxia-induced contraction in arteries (human intrahepatic and canine coronary arteries) with intact endothelium (Jeng *et al.*, 1997; Lin *et al.*, 1995). However, the previous study (Chapter 4), and references in the literature, suggested that exposure to UWS is unlikely to have altered function in denuded human hepatic arteries. Several studies have also reported that UWS has no significant effect on the contractile response of

denuded vessels either from humans (Hadoke *et al.*, 1998; Jeng *et al.*, 1996b, 1997) or animals (Heller *et al.*, 1999; Lin *et al.*, 1995), even after the inclusion of dexamethasone, insulin and penicillin (Sorajja *et al.*, 1997; Abebe *et al.*, 1993). Consequently, donor hepatic arteries have consistently been used as controls by many investigators, despite previous exposure to preservative solutions (Heller *et al.*, 1999; Hadoke *et al.*, 1998; Smith *et al.*, 1997). Therefore, use of donor hepatic arteries as a control in this study appears justified and contractile abnormalities detected are unlikely to be due to the effects of UWS in the control.

### *5.6.3. Mechanism of Altered Contractile Function*

#### 5.6.3.1. Role of vascular remodelling, endothelium-derived and humoral factors

It has been suggested that altered vascular responses in hepatic cirrhosis may be due to the increased activity of circulating humoral factors and/or synthesis of vasoactive substances by the endothelium (Benoit *et al.*, 1984; Atucha *et al.*, 1996). However, in this study, altered contractile function was detected in vessels without an endothelium and which were isolated from the effect of humoral factors. This indicated that the altered contractile responses to AVP, 5-HT and ET-1 must be a consequence of alterations induced in the VSMCs. Factors, which could contribute to such alterations, are (a) vascular remodelling, (b) production of vasoactive substances and (c) altered receptor-mediated contraction in the VSMCs of the arterial wall. Remodelling of the vessel wall could be an important cause of altered contractile response in cirrhosis. This is particularly important while investigating the functional responses of vessels from the splanchnic vascular territory, the sustained

increase of portal pressure in cirrhosis could lead to alterations in the structure of the vascular wall. It has been suggested that prolonged alteration of blood pressure such as chronic hypotension and hypertension can induce medial atrophy and hyperplasia/hypertrophy, respectively (Pang & Scott, 1985). Moreover, studies in animal models of portal hypertension have shown that extensive remodelling occurs in the portal vein and its tributaries (Johansson, 1976). However, in the present study, the altered contractile responses in recipient hepatic arteries were not the consequence of vascular remodelling, as the response to KCl in these arteries was similar to that of the donor hepatic arteries. Moreover, the contractile response to AVP was reduced whereas that to 5-HT and ET-1 was increased. If any structural change had occurred within the vessel wall, it would be expected that contractile responses of all three vasoconstrictors would be altered in a similar way.

#### 5.6.3.2. Role of iNOS Activation

It has been suggested that increased NO synthesis accounts for the impaired pressor response and hyperdynamic circulation in cirrhosis. Excessive NO production in cirrhotic patients is indicated by increased plasma levels of the second messenger of NO stimulation (cGMP) (Schneider *et al.*, 1994) and metabolites of NO (nitrite and nitrate) (Guarner *et al.*, 1993) as well as by direct measurement of NO in the circulation (Battista *et al.*, 1997). Furthermore, it has been shown that inhibition of NO synthesis can reverse systemic and splanchnic hypotension in cirrhosis (Pizcueta *et al.*, 1992; Sieber & groszmann, 1992). Similarly, studies with isolated vessels from animal models of cirrhosis suggested that inhibition of NO activity could also reverse hyporesponsiveness to several vasoconstrictors (Pateron *et al.*, 1999; Gadano *et al.*,

1997). However, whether increased NO synthesis in cirrhosis is a cause or consequence of the hyperdynamic circulation is yet not clear. Two major alterations in the hepatic and splanchnic circulations could lead to increase NO production: (i) a combination of enhanced back pressure (resulting from intrahepatic obstruction) and increased blood flow in the splanchnic arteries results in excessive synthesis of NO by the endothelial cells (via eNOS) (Sogni *et al.*, 1995; Bomzon & Blendis, 1994; Shah *et al.*, 1998; Battista *et al.*, 1997), and (ii) this increased intrahepatic pressure also leads to opening of the porto-systemic shunts and thus, allows various gut-derived substances, including bacterial endotoxins, to enter into the systemic circulation. These substances, particularly the endotoxins, can increase NO production via inducing iNOS in the VSMCs (Vallance & Moncada, 1991; Busse & Mulsch, 1990). However, whether increased NO synthesis in cirrhosis is the result of over activity of eNOS (Muruganandam & Mutus, 1994; Forrest *et al.*, 1996) or iNOS (Laffi *et al.*, 1993; Chen & Metha, 1996) remains controversial (Bomzon & Blendis, 1994).

Many of the studies which implicated NO in altered contractile responses have demonstrated that increased NO activity was mediated by eNOS (Weigert *et al.*, 1995; Castro *et al.*, 1993; Sogni *et al.*, 1995). However, in the current study, it was not possible to assess the role of eNOS as the endothelium was denuded from the hepatic arteries. Investigation of iNOS in VSMCs of these arteries demonstrated that this isozyme is generally not present in hepatic arteries either from cirrhotic recipients and non-cirrhotic donors. It was also shown that NO activity did not modulate the functional responses to AVP or 5-HT. Therefore, it is apparent that

iNOS activity does not contribute to the contractile abnormalities determined in the present study. In contrast, with the present data, a small number of studies has suggested that iNOS in the VSMCs could have a role in increased NO production in cirrhosis (Vallance & Moncada, 1991; Smith *et al.*, 1997; Ryan *et al.*, 1996). The apparent similarity between the vascular abnormalities in endotoxic shock and cirrhosis suggested the responsibility of a common factor such as over activity of iNOS in the VSMCs induced by endotoxin (Vallance & Moncada, 1991; Lumsden *et al.*, 1988). However, exposure of human vessels to endotoxin *in vivo* (dorsal hand vein) (Bhagat & Vallance, 1996) or *in vitro* (internal mammary artery and saphenous vein) (Thorin-Trescases *et al.*, 1995), fails to activate iNOS. Similarly, studies indicating the existence of iNOS in recipient hepatic arteries (Smith *et al.*, 1997) or in forearm vessels (Ryan *et al.*, 1996), from patients with cirrhosis, have not been supported by other studies (Hadoke *et al.*, 1998; Shah *et al.*, 1998; Heller *et al.*, 1999).

An unexpected finding in the current study was the immunoreactivity to iNOS in hepatic arteries from patients with alcoholic liver disease (ALD), although inhibition with L-NNA did not produce enhanced contractile responsiveness. This indicated that iNOS has no impact on contractile function. This is consistent with a previous study, where iNOS mRNA was detected in donor hepatic arteries but had no effect on functional responses (Smith *et al.*, 1997). Furthermore, the hyporesponsiveness demonstrated in the current study was specific to AVP, whereas responses to 5-HT and ET-1 were enhanced. Over activity of iNOS, would be expected to effect all three agonists in a similar way and many also inhibit receptor-independent (KCl)

vasoconstriction (Sieber & Groszmann, 1992). Therefore, it is most unlikely that iNOS activation accounts for altered vascular functions in this study. However, the existence of iNOS only in arteries from patients with ALD has important implications for future research as it suggested that the mechanisms responsible for vascular hyporesponsiveness are dependent upon the aetiology of cirrhosis. This aetiological variation may explain the difference between the present results and the previous studies which have used a higher proportion of subjects with ALD (Ryan *et al.*, 1996). In particular, the influence of aetiology may also explain the detection of iNOS mRNA in the previous study using hepatic arteries from patients with viral hepatitis (Smith *et al.*, 1997).

### *5.6.4. Effect of Cirrhosis on Contractile Function*

#### 5.6.4.1. Effect of Cirrhosis on AVP-Mediated Contraction

In cirrhotic patients, AVP and its analogues can reduce portal pressure by decreasing splanchnic blood flow and are used in the treatment of acute variceal haemorrhage (Westaby *et al.*, 1988; Freeman *et al.*, 1998). These agents can also constrict splanchnic vessels (in patients with cirrhosis or in animal models) and, thus, reduce gastric and mesenteric hyperaemia (Panes *et al.*, 1994; Heinemann *et al.*, 1998). Therefore, the reduced responses to AVP demonstrated in this study could be important in the pathogenesis and treatment of the vascular abnormalities in cirrhosis. Unfortunately, no studies have investigated contractile responses to AVP (or its analogues) using splanchnic arteries from patients with this condition. Only a few studies are available in animal models and these represent conflicting results. Hyporesponsiveness to AVP has been reported in mesenteric (Sieber & Groszmann,

1992) and tail (Huang *et al.*, 1995) arteries from portal vein ligated rats. In contrast, another study, using carbon tetrachloride-induced cirrhotic rats, reported unaltered AVP-mediated response in the mesenteric arterial bed (Ralevic *et al.*, 1996), whilst an increased response to AVP has been reported in mesenteric veins from portal vein ligated rats (Cummings *et al.*, 1986; Moreno *et al.*, 1996). These results, therefore, reflect the heterogeneity of AVP-mediated contraction depending on the animal model and vascular territory used. Although one study suggested that excessive NO production was responsible for this impaired response (Sieber & Groszmann, 1992), most others did not agree with this mechanism (Heinemann *et al.*, 1998; Huang *et al.*, 1995). Similarly, the current study also demonstrated that NO was not responsible for reduced AVP-mediated contraction. The actual mechanism of impaired AVP-mediated response remains uncertain but could be the result of abnormalities either in the vasoconstrictor AVP-receptor or at a post receptor level in the VSMCs. It was, therefore, necessary to identify the receptor mediating AVP-induced contraction in human hepatic arteries.

As discussed previously (Chapter 3) it was likely that AVP-mediated vasoconstriction occurred via the $V_1$ receptor located in the VSMCs (Thibonnier, 1992). The $V_2$ receptor was not expected to have a significant role as it is found primarily in the endothelium and mediates vasodilatation (Tagawa *et al.*, 1995; Aki *et al.*, 1994). The failure of the selective $V_2$ receptor-agonist (DDAVP) to contract human hepatic arteries confirmed that the $V_2$ receptor did not contribute to AVP-mediated contraction of these vessels. The rightward shift in the AVP-mediated contraction in the presence of the selective $V_1$ receptor antagonist confirmed that, as

in human renal (Medina *et al.*, 1996) and gastric (Calo *et al.*, 1997) arteries, this was a $V_1$ receptor-mediated response. Therefore, the attenuated response to AVP in arteries from cirrhotic patients is probably due to a defect in the $V_1$ receptor-mediated contractile pathway. Whether this is due to receptor down regulation and/or a defect in the post receptor signal transduction mechanism has yet to be established.

There is considerable evidence of changes in the AVP system in patients with hepatic cirrhosis, which could result in receptor down-regulation. However, the evidence for receptor down-regulation in hepatic cirrhosis is sparse and often contradictory (Gerbes *et al.*, 1986; Liao *et al.*, 1994) and the activity of $V_1$ receptors has not been studied in cirrhotic patients. Plasma levels of AVP are not always elevated in cirrhosis (and are more dependent upon the function of the kidney than on the liver (Arroyo *et al.*, 1988)), but persistently high plasma levels of AVP are generally reported in advanced cirrhosis (as non-osmotic stimulation significantly enhances AVP release (Martin & Schrier, 1997)). It is known that repeated or constant exposure to AVP can reduce the response mediated by the $V_1$ receptor, as it becomes desensitised following receptor occupancy, resulting in a reduction of either specific binding sites or of inositol phosphate production (Thibonnier 1992; Caramelo *et al.*, 1989). In the present study, arteries are used from cirrhotic recipients with advanced liver disease. Presumably, elevated plasma levels of AVP could result in receptor down-regulation or desensitisation and, consequently, impaired AVP-mediated contraction. However, the plasma levels of AVP and expression of $V_1$-receptor in these vessels have not been assessed. Therefore, ligand binding studies and investigation of second messenger response of $V_1$-receptor-mediated contractile

pathway are required to identify the site of impaired AVP-mediated contraction.

#### 5.6.4.2. Effect of Cirrhosis on 5-HT-Mediated Contraction

The increased response to 5-HT seen in recipient hepatic arteries in this study is consistent with many other investigations using vessels from animal models of cirrhosis/portal hypertension (Cummings *et al.*, 1986; Nevens *et al.*, 1991; Kaumann *et al.*, 1988). Only one study presents a conflicting picture, showing reduced responsiveness to 5-HT in vessels from rat models (Jacob *et al.*, 1991). However, the mechanism responsible for altered 5-HT-mediated contraction (whether increased or impaired) has not been identified. 5-HT can activate several distinct 5-HT receptor sub-types of which 5-$HT_{2A}$ and 5-$HT_1$-like receptors mediate contraction of VSMCs (Hoyer *et al.*, 1994; Martin & Humphrey, 1994; Yildiz *et al.*, 1996). The enhanced response to 5-HT in cirrhosis could conceivably be the result of up-regulation of vasoconstrictor 5-HT-receptors, as in cirrhosis the plasma levels of 5-HT may be reduced (Beaudry *et al.*, 1994). Studies using mesenteric or portal veins from portal hypertensive rats, however, find no evidence of receptor up-regulation with the affinity of 5-$HT_{2A}$ receptor antagonists unaltered (Cummings *et al.*, 1986; Kaumann *et al.*, 1988). This suggested that alterations of receptors other than the 5-$HT_{2A}$ subtype, possibly 5-$HT_1$-like receptors, may be involved. The complexity of the 5-HT-mediated response (namely the existence of 'silent' receptors, a possible role for several 5-HT receptor subtypes and the need to precontract the vessels), necessitated a detailed investigation to determine the receptor subtype responsible. This study is described in a separate chapter (Chapter 6).

5.6.4.3. Effect of Cirrhosis on ET-1-Mediated Contraction

The current study indicated that the sensitivity of the response to ET-1 was increased in hepatic arteries from patients with cirrhosis. Similar results have been reported in studies using portal veins from cirrhotic rats (Petrowsky *et al.*, 1999) or mesenteric arteries and thoracic aortae from portal vein ligated rats (Cahill *et al.*, 1998). Several studies *in vivo* with animal models ($CCl_4$ induced, portal vein or bile duct ligated rats) also suggested enhanced activity of ET-1 as ET-1-receptors antagonist reduced portal pressure (Gandhi *et al.*, 1998; Reichen *et al.*, 1998; Sogni *et al.*, 1998). Although, the mechanism of enhanced response to ET-1 in cirrhosis is uncertain. However, it was suggested that *in vivo* persistent vasodilatation induced by NO in this condition could enhance activity of ET-1 (Cahill *et al.*, 1996, 1998) by increasing expression of ET-1 receptors ($ET_A$ and $ET_B$) in intra (Rockey *et al.*, 1998; Leivas *et al.*, 1998) and extra (Cahill *et al.*, 1998)-hepatic circulations. The ET-1 receptor expression has not been determined in the current study but it is likely that cirrhosis enhanced their expression in hepatic arteries as it did in aortae and superior mesenteric arteries from cirrhotic rats. The enhanced response had been contrasted by studies where *in vivo* impaired pressor responses to ET-1 were observed in forearm blood vessels of well-compensated cirrhotic patients (Helmy *et al.*, 2001) or in arterial pressure of bile duct ligated cirrhotic rats (Hartleb *et al.*, 1994b). The mechanism of impaired response was not explained, indeed both studies indicated that increased NO production was the most likely contributing factor which interacted ET-1 activity. Moreover, increased plasma levels of ET-1 in cirrhosis (Asbert *et al.*, 1993 Kitano *et al.*, 1996; Trevisani *et al.*, 1997), could conceivably lead to down-regulation of ET-1 receptors (Rubanyi & Polokoff, 1994) and thus

reduced ET-1-mediated response. However, in a previous study (Helmy *et al.*, 2001), plasma ET-1 levels were not elevated. Many studies have also reported unchanged (Battaglia *et al.*, 1996) or even decreased plasma levels of ET-1 (Veglio *et al.*, 1992) in cirrhosis. Furthermore, the plasma levels of ET-1 could not reflect the actual vasoactive function as this peptide can modulate the contractility of the vessels by its direct autocrine and paracrine actions. Therefore, the role of the plasma levels of ET-1 in the altered response remains uncertain.

ET-1 acts via two distinct receptors; $ET_A$ and $ET_B$. The $ET_A$-receptor is located on the VSMCs and mediates vasoconstriction, whereas the $ET_B$ receptor is predominantly located on the endothelial cells and induces NO and prostacyclin release (Gray & Webb, 1996). The $ET_B$ receptor is also present in VSMCs where it mediates vasoconstriction (Touyz *et al.*, 1995). ET-1 exhibits higher affinity for $ET_A$ than the $ET_B$ receptor (Gerbes *et al.*, 1995) and depending on vascular area the ratio of these receptors could vary (Davenport & Maguire, 1994). Therefore, in the current study an initial attempted was made to identify the ET-1 receptors in the VSMCs of human hepatic arteries. This study demonstrated that vasoconstrictor $ET_A$-receptors on the VSMCs of these arteries, mediated ET-1-induced contraction. However, it was noticeable that in the recipient hepatic arteries, a (5 fold) higher concentration of BQ-123, (a selective $ET_A$-receptor antagonist) was required to produce a response equivalent to that in the donor hepatic arteries. This suggests that the activity of $ET_A$-receptor in the recipient hepatic arteries may be up-regulated, and this could account for the enhanced ET-1-mediated contraction in cirrhosis. However, in this study, the contribution of $ET_B$-receptors in mediating ET-1-induced contraction in these vessels

has not been investigated. Further studies therefore, are required to determine the contribution of $ET_B$ receptors in ET-1-induced contraction of the human hepatic arteries.

### 5.7. Conclusions

This study indicated that, in patients with hepatic cirrhosis, contraction of hepatic arteries was altered in an agonist-dependent manner. Nitric oxide generation and structural remodelling of the arterial wall were shown not to account for these alterations. This study therefore suggested that these alterations in contractile function were the result of changes at the receptor level (up-or down-regulation), and/or in the post-receptor signal transduction pathways.

# CHAPTER SIX

# RECEPTORS RESPONSIBLE FOR THE 5-HYDROXYTRYPTAMINE-MEDIATED CONTRACTION OF HUMAN HEPATIC ARTERIES AND THE INFLUENCE OF LIVER CIRRHOSIS

## 6.1. Introduction

5-Hydroxytryptamine (5-HT) can modulate splanchnic blood flow (Shah *et al.*, 1998) and has been considered as a possible mediator in the pathophysiology of portal hypertension (Fernandez *et al.*, 1993). The enhanced 5-HT-mediated contraction demonstrated in hepatic arteries from patients with cirrhosis (Chapter 5) was consistent with studies using splanchnic vessels from animal models of portal hypertension and/ or cirrhosis (Cummings *et al.*, 1986; Kaumann *et al.*, 1988; Nevens *et al.*, 1991). This abnormal contractile response may contribute to the pathogenesis of vascular complications in portal hypertension and, thus, is a potential target for therapeutic intervention. It is possible that an alteration in 5-HT-receptor activity accounts for the enhanced contraction in cirrhotic individuals but this has yet to be confirmed.

Classically, 5-HT-mediated vasoconstriction occurs via stimulation of the 5-$HT_{2A}$ receptor (Martin & Humphrey, 1994) but there is increasing evidence that 5-$HT_1$-like receptors also mediate vascular contraction (Yildiz *et al.*, 1998; Ishida *et al.*, 1999). Previously (Chapter 3) it was demonstrated that 5-$HT_{2A}$ and another, unidentified, 5-HT receptor, (possibly the 5-$HT_1$-like receptor subtype) contributed to 5-HT-mediated vasoconstriction in porcine hepatic arteries. Many studies have indicated that the contribution of 5-$HT_1$-like receptors varies between different vessels and, in many cases, partial pre-contraction was necessary to observe contractile response mediated by these receptors (Choppin & O'Connor, 1994, 1995; Yildiz *et al.*, 1998). Furthermore, some investigations have demonstrated that the influence of 5-$HT_1$-like receptors is more significant in arteries isolated from individuals with cardiovascular

disease (e.g. angina) (Mcfadden *et al.*, 1992). The receptors responsible for mediating contraction of the human hepatic artery in response to 5-HT, and the effect of cirrhosis on these receptors, have not been identified.

## 6.2. Aim

This study aimed to determine (i) the contribution of 5-$HT_{2A}$ and 5-$HT_1$-like receptors towards the contractile response of 5-HT in human hepatic arteries and (ii) the effect of cirrhosis on the responses mediated by these receptors.

## 6.3. Methods

### *6.3.1. Acquisition of Hepatic Arteries*

Hepatic arteries were obtained from 16 non-cirrhotic liver donors (11 female, 5 male) and 15 cirrhotic recipients (9 female, 6 male). The donors (46 ± 4 yrs.) and recipients (49 ± 3 yrs.) were similar in age ($P = 0.47$). Causes of death in donors and cirrhosis in recipients are given in Table 1. The mean duration of liver disease in recipients was 7.4 ± 1.0 yrs. In recipients, systemic haemodynamic values were obtained from anaesthetic notes, which were recorded before the transplantation procedure. The Lothian Research Ethics Committee approved the use of human tissues.

### *6.3.2. Functional Studies*

After removing the adherent connective tissue, eight to sixteen rings (2mm in length) were taken from each artery. Following confirmation that the vessels had no endothelium (Chapter 2.2), the rings were mounted in organ baths containing KHS for the measurement of isometric force development (Chapter 2.2.3).

**Table 6.1.** Causes of death in liver donors and of cirrhosis in recipients undergoing liver transplantation. Hepatic arteries from these individuals were isolated for analysis of 5-HT-mediated contraction.

| Cause of Death in Donor | No. | Cause of Cirrhosis in Recipient | No. |
|---|---|---|---|
| Sub-arachnoid Haemorrhage | 6 | Primary Biliary Cirrhosis | 6 |
| Intra-cerebral Bleeding | 4 | Primary Sclerosing Cholangitis | 3 |
| Head Injury | 2 | Alcoholic Liver Disease | 2 |
| Road Traffic Accident | 2 | Cryptogenic Cirrhosis | 2 |
| Brain Stem Injury | 1 | Cirrhosis in Autoimmune Hepatitis | 1 |
| Cerebral Contution | 1 | Cirrhosis in Wilson's Disease | 1 |
| Total: | 16 | Total: | 15 |

For all experiments, prazosin ($\alpha_1$-antagonist, $10^{-6}$ mol/L) and yohimbine ($\alpha_2$-antagonist, $10^{-6}$ mol/L) were included in the KHS to eliminate the contribution of $\alpha$-adrenergic receptor stimulation. Similarly, indomethacin ($10^{-5}$ mol/L) was also included in the KHS to inhibit endogenous production of prostaglandins. The viability and reproducibility of the contractile response of each ring were assessed using KCl (100 mmol/L) as described (Chapter 2.2.3).

*6.3.3. Functional Protocol*

Four rings from each artery were suspended in parallel in separate organ baths. Two rings were partially pre-contracted with KCl (20-35 mmol/L) to produce 25-30% of the maximal response to KCl, but the two remaining rings were not pre-contracted. One pre-contracted and another non-precontracted rings were incubated, either with the 5-$HT_{2A}$ receptor antagonist, ketanserin ($10^{-6}$ mol/L), for 40 minutes or with the 5-$HT_1$-like receptor antagonist, methiothepin ($10^{-7}$mol/L) for 30 minutes. The two remaining rings were exposed to vehicle and acted as controls. Following incubation, cumulative concentration-response curves were constructed using either 5-HT ($10^{-9}$-$3x10^{-5}$ mol/L) or 5-carboxamidotryptamine (5-CT, $10^{-9}$-$3x10^{-5}$ mol/L, a relatively selective 5-$HT_1$-like receptor agonist). Thereafter, the agonist and antagonist were washed out of the rings using KHS, and cumulative concentration-response curves were produced using KCl (2.5-120 mmol/L).

**6.4. Statistics**

Statistics analysis was performed as described in Chapter 2.7.

## 6.5. Results

### *6.5.1. Haemodynamic Profile*

The presence of a hyperdynamic circulatory state in the liver recipients was demonstrated by elevated heart rate (105 ± 5 b/min, n=15; normal range 60-90 b/min) and cardiac output 9.78 ± 0.62 L/min, n=14; normal range 4-8 L/min), associated with low systemic vascular resistance (638 ± 59 dyne.sec/cm$^5$, n=14; normal range 1200-1500 dyn.sec/cm$^5$).

### *6.5.2. Contraction of Hepatic Arteries*

The vasoconstrictors 5-HT, 5-CT and KCl caused concentration-dependent contraction of all arterial rings used in this study.

#### 6.6.2.1. Contractile Response of Non-Contracted and Partially-Pre-contracted Rings

The maximum contractile response (Emax) to 5-HT was significantly larger ($P$=0.03) in recipient (8.92 ± 0.65g), than in donor (7.13 ± 0.61g) hepatic arteries (as reported in Chapter 5). The contractile response to 5-CT was identical to that produced with 5-HT, as the maximum contraction (Emax) in recipient hepatic arteries (8.61 ± 0.44g) was significantly greater ($P$=0.04) than that produced in arteries from liver donor (6.96 ± 0.62g). These differences were still evident (Figure 6.1) when the contractile responses were expressed as a percentage of maximum response to KCl (Table 6.2). In the recipient hepatic arteries there was a tendency towards an increased sensitivity of response to both 5-HT and 5-CT (Figure 6.1), but this achieved only threshold significance ($P$=0.07 and $P$=0.06, respectively) (Table 6.2).

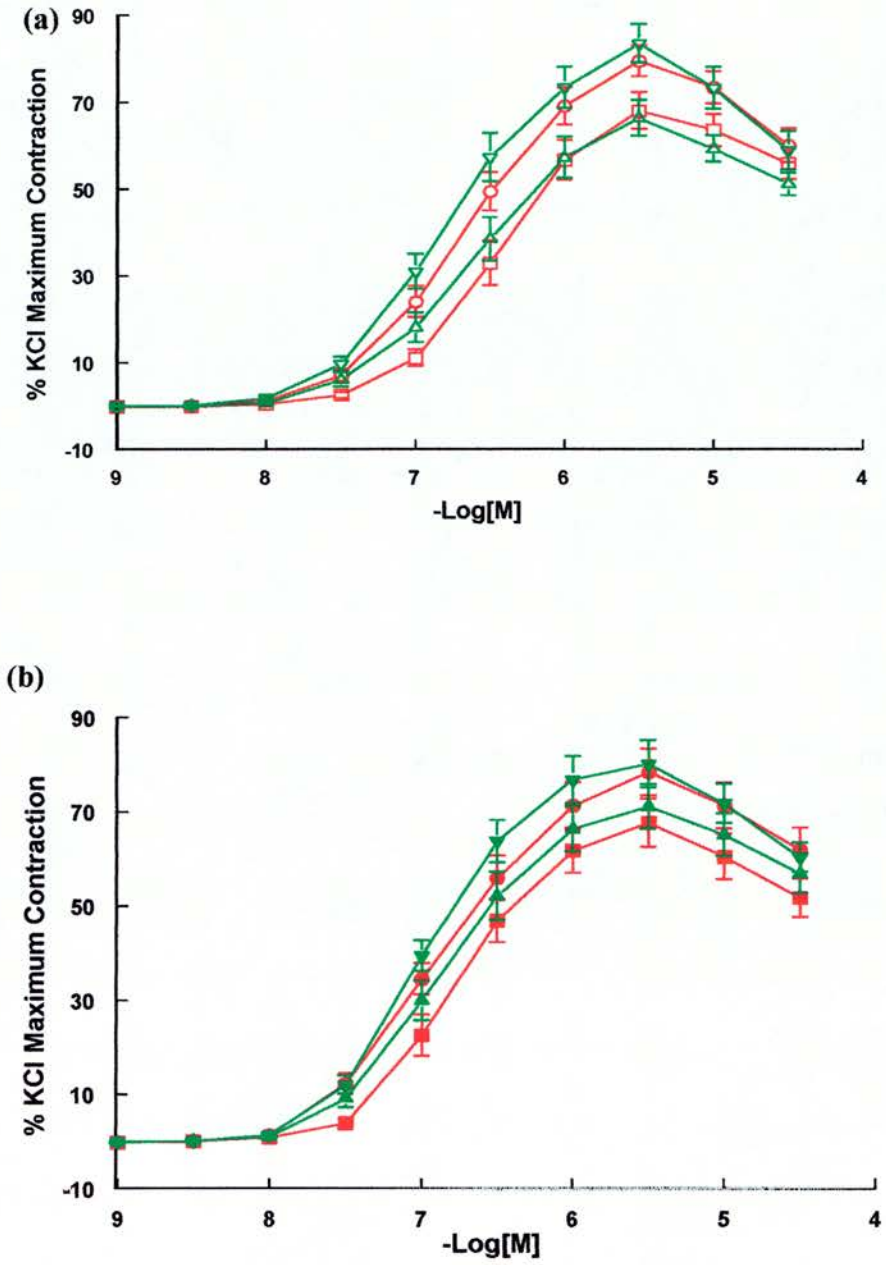



**FIGURE 6.1.** The effect of cirrhosis on the contractile responses induced by 5-hydroxytryptamine (5-HT; ❑, ○; n=14-16) and 5-carboxyamidotryptamine (5-CT; Δ, ∇; n=14-16). Cumulative concentration-response curves were produced in recipient (○and ∇) and donor (❑ and Δ) hepatic arteries using 5-HT and 5-CT in (a) non-contracted (open symbols) and (b) partially-precontracted (closed symbols) arterial rings. Each point represents mean ± s.e. mean, expressed as a percentage of the maximum contraction induced by KCl.

**Table 6.2.** Maximum contractile response (Emax) and sensitivity ($pD_2$) of non-contracted and partially-precontracted rings from recipient and donor hepatic artery to 5-hydroxytryptamine (5-HT) and 5-carboxyamidotryptamine (5-CT).

| | **Recipient Hepatic Artery (n=14-15)** | | | | **Donor Hepatic Artery (n=14-16)** | | | |
|---|---|---|---|---|---|---|---|---|
| | Non-Contracted | | Partially-Precontracted | | Non-Contracted | | Partially-Precontracted | |
| | Emax (% KCl) | $pD_2$ | Emax (% KCl) | $pD_2$ | Emax (% KCl) | $pD_2$ | Emax (% KCl) | $pD_2$ |
| 5-HT | 82.27 ± 3.31 | 6.66±0.07 | 80.86±4.83 | 6.85±0.06[a] | 70.88 ± 3.97* | 6.46±0.07 | 70.12±4.88 | 6.72±007[b] |
| 5-CT | 84.52 ± 4.32 | 6.75±0.06 | 83.59±5.03 | 6.92±0.04[a] | 69.32±4.10* | 6.58±0.06 | 70.35±4.67 | 6.84±0.06[c] |

Values are mean ± s.e. mean for (n) subjects. *$P < 0.05$, when compared with recipient hepatic artery. [a]$P < 0.05$, [b]$P < 0.02$ and [c]$P < 0.01$, when compared with corresponding non-contracted arterial rings, using Student's unpaired t-test.

Strikingly the sensitivities of responses to both 5-HT and 5-CT were enhanced in both recipient and donor hepatic arteries following partial precontration (Table 6.2). The difference between maximum contraction in recipient hepatic arteries to 5-HT (8.29 ± 0.52g) and 5-CT (8.31 ± 0.41g), when compared with responses in donor hepatic arteries (7.74 ± 0.61g and 7.15 ± 0.63g, respectively) was abolished. Although there was a trend towards enhanced maximum contraction these differences did not achieve significance ($P$=0.49 and $P$=0.14, respectively), even when expressed as a percentage of the maximum response to KCl ($P$=0.13 and $P$=0.11, respectively) (Table 6.2).

#### 6.5.2.2. Effect of 5-HT Receptor Antagonists on the Contractile Response of Non-Contracted Rings

The 5-$HT_1$-like receptor antagonist, methiothepin, caused a rightward shift in the concentration-response curves to both 5-HT (Figure 6.2) and 5-CT (Figure 6.3) in non-precontracted rings of donor or recipient hepatic artery. There was a corresponding reduction in the sensitivity of these responses but the amplitude of contraction (Emax) was unchanged (Table 6.3). In contrast, the 5-$HT_{2A}$-receptor antagonist, ketanserin had no effect on the response to 5-CT (Figure 6.3) in donor or recipient hepatic arteries. Ketanserin produced a rightward shift in responses to 5-HT (Figure 6.2); this achieved significance in the donor ($P$=0.04), but failed to achieve significance ($P$=0.11) in the recipient hepatic arteries (Table 6.3).

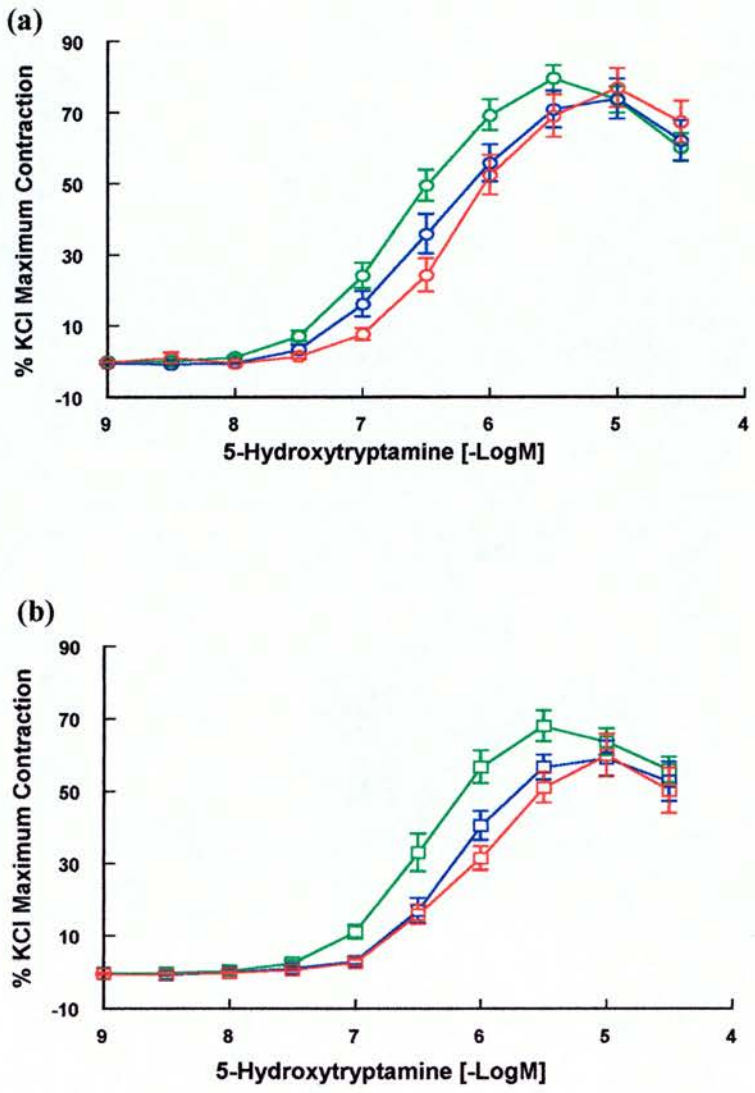



**FIGURE 6.2.** The effect of 5-HT-receptor antagonism on contractile responses to 5-HT in **(a)** recipient (○) and **(b)** donor (□) hepatic arteries. Cumulative concentration-response curves were produced in non-contracted arterial rings in the absence of antagonists (○, □; n=15) and following incubation with either $10^{-7}$ mol/L methiothepin (○, □, n=11-12) or $10^{-6}$ mol/L ketanserin (○, □; n=9-11). Each point represents mean ± s.e. mean expressed as a percentage of the maximum contraction induced by KCl.

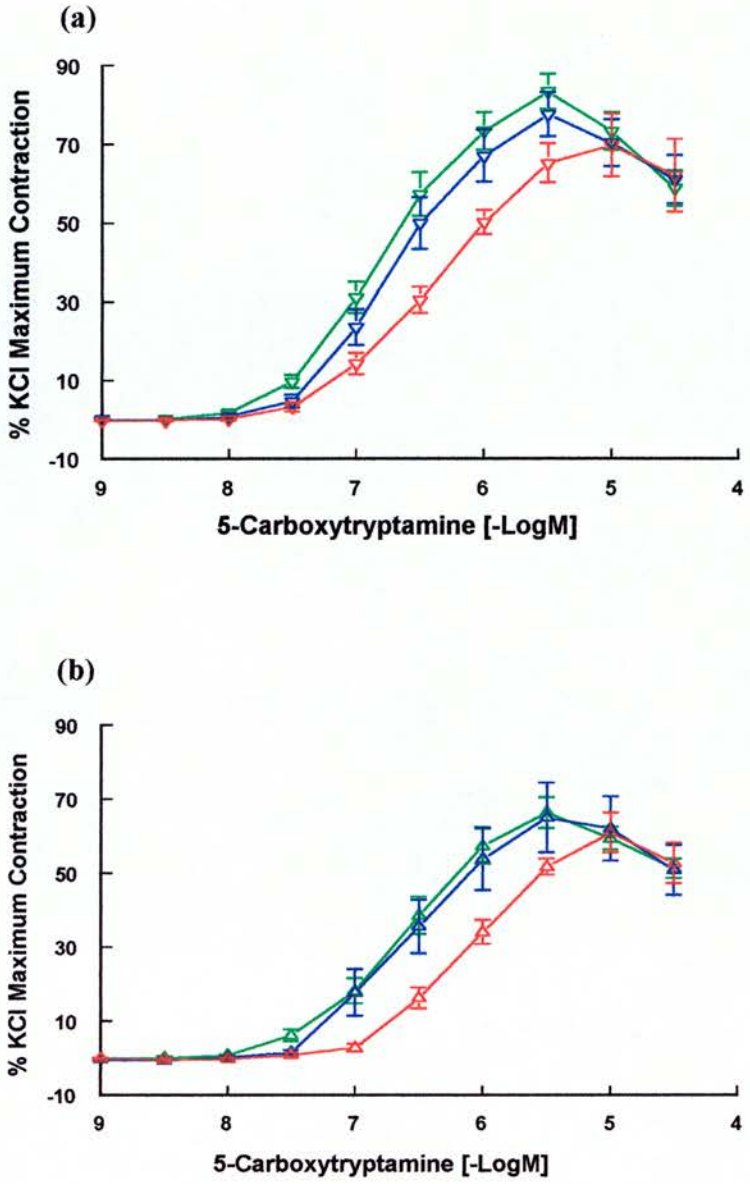



**FIGURE 6.3.** The effect of 5-HT-receptor antagonism on contractile responses to 5-carboxyamidotryptamine (5-CT) in **(a)** recipient (∇) and **(b)** donor (Δ) hepatic arteries. Cumulative concentration-response curves were produced in non-pre-contracted arterial rings in the absence of antagonists (Δ, ∇; n=15) and following incubation with either $10^{-7}$ mol/L methiothepin (Δ, ∇; n=11-12) or $10^{-6}$ mol/L ketanserin (Δ, ∇; n=9-11). Each point represents mean ± s.e. mean expressed as a percentage of the maximum contraction induced by KCl.

**Table 6.3.** The maximum contraction (Emax) and sensitivity ($pD_2$) of non-contracted and partially pre-contracted arterial rings from recipient and donor hepatic arteries to 5-hydroxytryptamine (5-HT) and 5-carboxyamidotryptamine (5-CT).

| | **Recipient Hepatic Artery (n=10-15)** | | | | **Donor Hepatic Artery (n=9-16)** | | | |
|---|---|---|---|---|---|---|---|---|
| | Non-Contracted | | Partially Pre-contracted | | Non-Contracted | | Partially Pre-contracted | |
| | Emax (g) | $pD_2$ | Emax (g) | $pD_2$ | Emax (g) | $pD_2$ | Emax (g) | $pD_2$ |
| 5-HT(control) | 8.92±0.65 | 6.65±0.07 | 8.29±0.52 | 6.85±0.06 | 7.13±0.61 | 6.46±0.07 | 7.74±0.54 | 6.72±0.07 |
| 5-HT+Meth | 7.83±0.62 | 6.23±0.07[e] | 7.62±0.74 | 6.51±0.09[d] | 6.94±0.64 | 6.10±0.08[c] | 7.32±0.97 | 6.29±0.10[e] |
| 5-HT+Ket | 8.02±0.88 | 6.45±0.13 | 8.54±0.50 | 6.80±0.11 | 6.93±0.52 | 6.22±0.10[a] | 7.86±0.48 | 6.57±0.13 |
| 5-CT(control) | 8.61±0.44 | 6.75±0.06 | 8.31±0.41 | 6.92±0.04 | 6.96±0.62 | 6.58±0.06 | 7.15±0.63 | 6.84±0.06 |
| 5-CT+Meth | 8.10±0.57 | 6.43±0.10[b] | 7.86±0.66 | 6.52±0.10[d] | 6.42±0.69 | 6.12±0.09[e] | 7.24±0.68 | 6.20±0.11[f] |
| 5-CT+Ket | 8.64±0.55 | 6.66±0.08 | 8.47±0.45 | 6.83±0.07 | 6.40±0.96 | 6.54±0.09 | 7.13±1.06 | 6.74±0.08 |

Values are mean ± s.e. mean for (n) subjects. Absence (control) and presence of either methiothepin (Meth, $10^{-7}$mol/L) or ketanserin (Ket, $10^{-6}$ mol/L). [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.002$, [d]$P < 0.005$,[e]$P < 0.001$ and [f]$P < 0.0001$, when compared with corresponding control using Student's unpaired t-test.

#### 6.5.2.3. Effect of 5-HT Receptor Antagonists on the Functional Response of Partially Pre-Contracted Rings.

In partially precontracted rings from both donor and recipient hepatic arteries, methiothepin caused a rightward shift in responses to 5-HT (Figure 6.4) and 5-CT (Figure 6.5) similar to that produced in non-precontracted arteries (Table 6.3). In contrast, ketanserin had no effect on responses to either 5-HT (Figure 6.4) or 5-CT (Figure 6.5) following partial pre-contraction (Table 6.3).

#### 6.5.2.4. Potassium Chloride (KCl)-Mediated Contraction

The contractile response produced by KCl was similar in arteries following exposure to either 5-HT or 5-CT. Responses to KCl were also unaltered in rings which had been partially precontracted or incubated with antagonists (methiothepin and ketanserin) before exposure to 5-HT or 5-CT (Table 6.4).

## *6.6. Discussion*

This study assessed the contributions of 5-$HT_{2A}$ and 5-$HT_1$-like receptor subtypes to 5-HT-mediated contraction of the human hepatic artery. The use of arteries from patients with hepatic cirrhosis also allowed an assessment of whether the presence of disease altered the contribution made by these receptors in hepatic arteries. It was shown that, as in the adult pig (Chapter 3), both 5-$HT_1$-like and 5-$HT_{2A}$ receptors mediate the contractile response to 5-HT in the human hepatic artery. However, selective stimulation of the 5-$HT_1$-like-receptor also produced a greater contraction in recipient, compared with donor, hepatic arteries. This indicates that functional changes in cirrhosis are not solely due to changes in the 5-$HT_{2A}$ receptor sub-type.

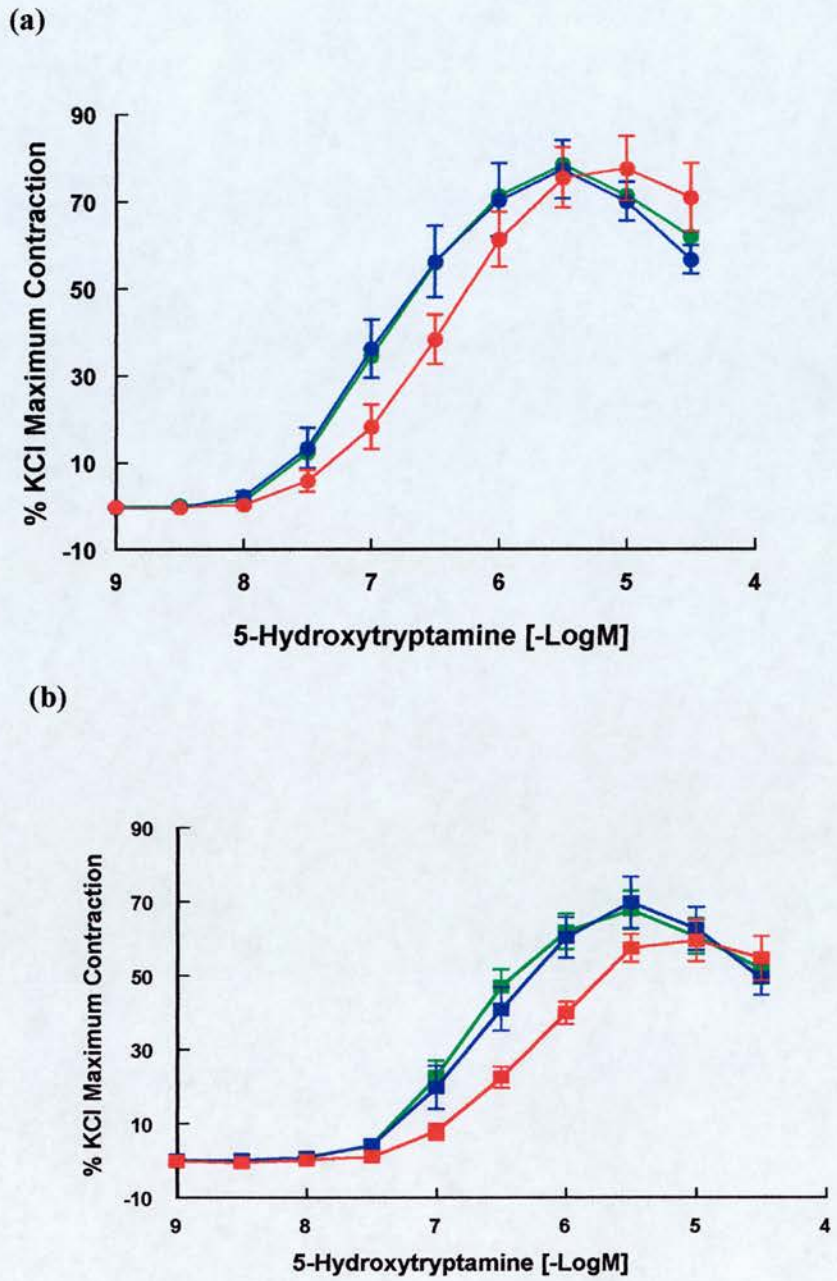



**FIGURE 6.4.** The effect of 5-HT-receptor antagonists on the contractile responses of **(a)** recipient (O) and **(b)** donor (❑) hepatic arteries to 5-hydroxytryptamine (5-HT). Cumulative concentration-response curves were produced in partially-precontracted arterial rings in the absence of antagonists (O,❑; n=15-16) and following incubation with either $10^{-7}$ mol/L methiothepin (O,❑; n=11-12) or $10^{-6}$ mol/L ketanserin (O,❑; n=9-11). Each point represents mean ± s.e. mean expressed as a percentage of the maximum contraction induced by KCl.

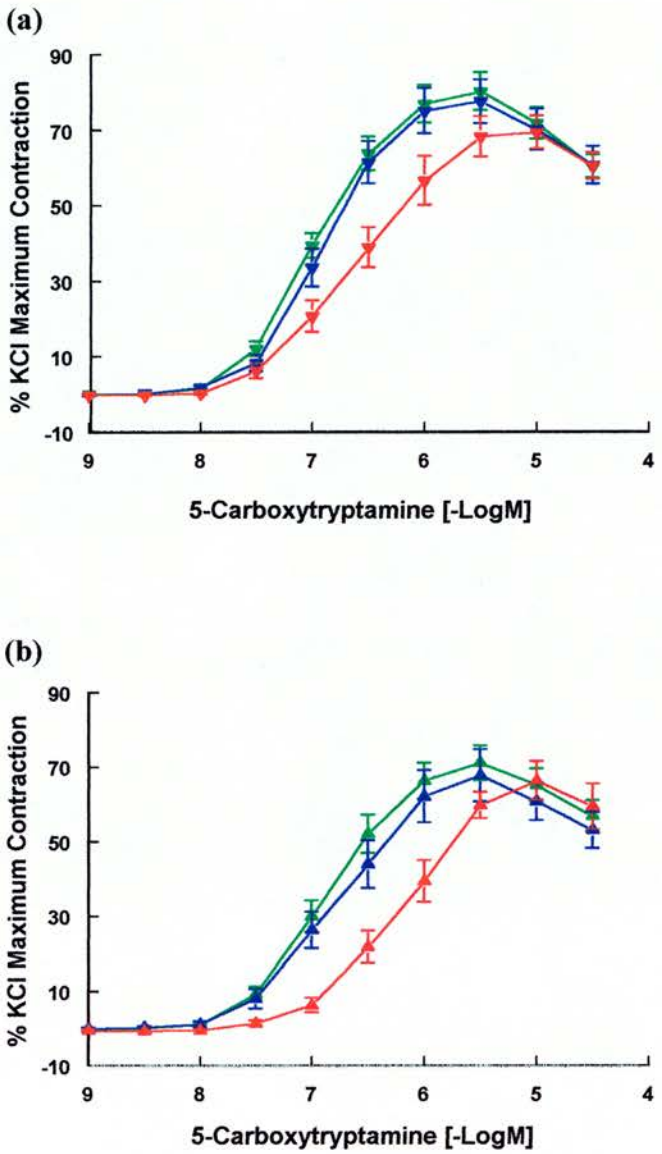



**FIGURE 6.5**. The effect of 5-HT-receptor antagonism on contractile responses to 5-carboxyamidotryptamine (5-CT) in **(a)** recipient (▼) and **(b)** donor (▲) hepatic arteries. Cumulative concentration-response curves were produced in partially-precontracted arterial rings in the absence of antagonist (▲, ▼; n=15-16) and following incubation with either $10^{-7}$ mol/L methiothepin (▲, ▼; n=11-12) or $10^{-6}$ mol/L ketanserin (▲, ▼; n=9-11). Each point represents mean ± s.e. mean expressed as a percentage of the maximum contraction induced by KCl.

**Table 6.4.** The maximum contraction (Emax) and sensitivity ($pD_2$) of recipient and donor hepatic arteries to potassium chloride (KCl).

**(a)** Arterial rings used for studies of 5-HT-mediated response

| | **Recipient Hepatic Artery (n=10-15)** | | | | **Donor Hepatic Artery (n=9-16)** | | | |
|---|---|---|---|---|---|---|---|---|
| | Non-contracted | | Partially Pre-contracted | | Non-contracted | | Partially Pre-contracted | |
| | Emax (g) | $pD_2$ | Emax (g) | $pD_2$ | Emax (g) | $pD_2$ | Emax (g) | $pD_2$ |
| Control | 11.28±0.93 | 1.60±0.04 | 10.98±0.95 | 1.60±0.03 | 10.36±1.00 | 1.63±0.03 | 11.58±0.82 | 1.62±0.03 |
| Meth | 10.51±1.08 | 1.66±0.04 | 10.03±1.01 | 1.64±0.06 | 11.54±0.89 | 1.60±0.04 | 11.68±1.43 | 1.65±0.05 |
| Ket | 10.81±1.41 | 1.64±0.05 | 11.21±1.04 | 1.67±0.06 | 11.50±1.34 | 1.61±0.04 | 11.46±1.32 | 1.64±0.04 |

**(b)** Arterial rings used for the studies of 5-CT-mediated response

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Control | 10.70±0.75 | 1.62±0.03 | 10.59±0.76 | 1.69±0.04 | 10.46±0.85 | 1.61±0.03 | 10.90±1.11 | 1.63±0.04 |
| Meth | 11.97±1.10 | 1.62±0.05 | 11.07±1.12 | 1.60±0.05 | 10.67±1.07 | 1.63±0.05 | 11.10±1.13 | 1.60±0.05 |
| Ket | 11.33±0.94 | 1.64±0.04 | 10.83±0.82 | 1.61±0.05 | 10.58±1.43 | 1.64±0.05 | 11.31±1.66 | 1.63±0.04 |

Values are mean ± s.e. mean for (n) subjects and results were compared using Student's unpaired t-test. Responses to KCl were obtained following completion of concentration-response curves to 5-HT and 5-CT in control arteries and in those, which had been incubated with 5-HT receptor antagonists. Exposure to KCl was performed in the absence of methiothepin (Meth) or ketanserin (Ket) in non-pre-contracted rings.

Furthermore, there was an indication that the contribution of the 5-$HT_{2A}$ receptor was diminished in arteries from patients with cirrhosis. This is reflected by the demonstration that the 5-$HT_{2A}$ receptor antagonist, ketanserin, failed to produce a significant inhibition of 5-HT-mediated contraction in the recipient hepatic artery.

*6.6.1. Contribution of 5-$HT_{2A}$ Receptor to 5-HT-Mediated Contraction in Cirrhosis*

As discussed previously (Chapter 5), several studies have reported enhanced 5-HT-mediated contraction in animals with portal hypertension and/or cirrhosis (Mastai *et al.*, 1990; Fernandez *et al.*, 1993; Nevens *et al.*, 1991; Kaumann *et al.*, 1988; Cummings *et al.*, 1986). No previous studies have assessed 5-HT-mediated contraction in vessels isolated from patients with this condition but enhanced 5-HT-mediated contraction has been demonstrated in the systemic and splanchnic circulations (Vorobioff *et al.*, 1989; Hadengue *et al.*, 1987). The use of 5-$HT_{2A}$ receptor antagonists in these patients significantly reduced portal pressure. These studies have also suggested that the increased responsiveness to 5-HT occurs primarily in the splanchnic, intrahepatic and portocollateral circulations (Fernandez *et al.*, 1993; Shah *et al.*, 1998; Mosca *et al.*, 1992). This is consistent with the enhanced responsiveness to 5-HT in hepatic arteries from cirrhotic patients demonstrated in the present study. Therefore, investigation of the mechanisms of 5-HT-mediated contraction in these arteries could provide information relevant to both the pathogenesis of this condition and the development of therapeutic interventions.

The underlying mechanisms of enhanced response to 5-HT in cirrhosis *in vitro* and *in*

*vivo* remain unclear. Although 5-$HT_{2A}$-receptor antagonists (ketanserin, ritanserin, ICI 169369) reduce portal pressure (Cummings *et al.*, 1986; Fernandez *et al.*, 1993; Kaumann *et al.*, 1988), it has not been established that this is the result of action upon the 5-$HT_{2A}$-receptor. In addition to blocking 5-$HT_{2A}$-receptors, these antagonists can also block responses mediated by α-adrenoceptors in the systemic and splanchnic circulations (Yildiz *et al.*, 1998; Xu *et al.*, 1990). In portal hypertensive subjects, 5-$HT_{2A}$-receptor antagonists reduced systemic vascular resistance, heart rate and cardiac output which consequently decreased portal venous inflow and reduced portal pressure (Vorobioff *et al.*, 1989; Hadengue *et al.*, 1987). This portal pressure reduction could be a combined effect due to 5-$HT_{2A}$ and α-adrenoceptor inhibition (Kaumann *et al.*, 1988). It has been suggested that ritanserin may slightly reduce resistance in both the porto-collateral and porto-hepatic circulation (Fernandez *et at.*, 1993; Mastai *et al.*, 1990). Small reductions of resistance in these territories may combine to produce a significant reduction in portal pressure (Fernandez *et al.*, 1993). The data obtained using antagonists have suggested that portal hypertension is due, in part, to over activity in the 5-HT system. This could be due to increased availability of 5-HT or up-regulation of 5-HT receptors. However, there was evidence that 5-$HT_{2A}$ receptor affinity was not enhanced in vessels from portal hypertensive animals (Cummings *et al.*, 1986; Kaumann *et al.*, 1988). Therefore, it seems likely that 5-$HT_{2A}$ receptors are not the key mediator of the enhanced responses to 5-HT in cirrhosis. This suggests a role for other 5-HT receptor sub-types, which would be consistent with the demonstration of both 5-$HT_{2A}$ and 5-$HT_1$-like receptors in the human hepatic artery.

*6.6.2. Contribution of the 5-HT$_1$-like Receptor to 5-HT-Mediated Contraction in Cirrhosis*

The current study extended observations obtained in porcine hepatic arteries (Chapter 3) to show that 5-HT$_{2A}$ receptor-independent responses to 5-HT are mediated by the 5-HT$_1$-like receptor sub-type in human hepatic arteries. This was achieved using two approaches: (i) use of a 5-HT$_1$-like receptor agonist, 5-CT, and (ii) partial pre-contraction of the arteries with KCl. It was shown in donor hepatic arteries that both 5-HT$_{2A}$ and 5-HT$_1$-like receptors mediated the response induced by 5-HT, whilst in the recipient this effect was predominantly mediated by the 5-HT$_1$-like receptor. This is demonstrated by the fact that both methiothepin and ketanserin significantly antagonised 5-HT-mediated responses in donor hepatic arteries but ketanserin failed to produce a significant effect in arteries from liver recipients. As with porcine hepatic arteries (Chapter 5), partial pre-contraction augmented the response to 5-HT and also to 5-CT in these arteries. A trend towards an increased response in recipient hepatic arteries was still evident but the significance of this difference was lost. The ability of methiothepin, but not ketanserin, to antagonise 5-HT-medediated contractions in partially pre-contracted arteries indicated that these responses were mediated by the 5-HT$_1$-like receptor (Saxena *et al.*, 1998; Bradley *et al.*, 1986). This confirms that partial pre-contraction inhibits the 5-HT$_{2A}$ receptor contribution to 5-HT-mediated contractions in these vessels. These results therefore indicated that, in cirrhosis the 5-HT$_1$-like receptor has a key role in mediating 5-HT-induced contraction in the hepatic arteries.

In the vasculature, the 5-HT$_1$-like receptor is widely distributed and, depending on

species and vascular territory, can exist in functional or non-functional states (Martin & Humphrey, 1994; Choppin & O'Connor, 1995; Gerhardt & VanHeerikhuizen, 1997). The existence of a functional 5-$HT_1$-like receptor has also been reported in human pulmonary (MacLean *et al.*, 1996), internal mammary (Yildiz *et al.*, 1996) and coronary (Cocks *et al.*, 1993) arteries. However, many recent studies have indicated that the distribution of 5-$HT_1$-like receptors is more extensive than was anticipated (Hoyer *et al.*, 1994; Yildiz *et al.*, 1996; Gerhardt & VanHeerikhuizen, 1997). Their existence has also been suggested in arterio-venous anastomoses, collateral vessels and even in the hepatic stellate cells (Martin & Humphrey, 1994; Mosca *et al.*, 1992; Shah *et al.*, 1998). Additionally, the presence of disease can alter the expression of the 5-$HT_1$-like receptor. This is shown by the demonstration that the activity of this receptor is increased in the epicardial coronary artery of patients with angina (Jansen *et al.*, 1993) or in intra-hepatic and portocollateral vessels in portal hypertension (Mosca *et al.*, 1992; Shah *et al.*, 1998). Furthermore, the 5-$HT_1$-like receptor is more predominant in the distal vessels of a circulation, such as in the venular part of the splanchnic vascular territory (Mosca *et al.*, 1992; Blackshear *et al.*, 1985). Consequently, 5-$HT_1$-like receptors could be important in increasing resistance of the mesenteric, porto-collateral and intrahepatic circulations resulting in the development of abnormal vascular conditions associated with hepatic cirrhosis.

### *6.6.3. Mechanisms of Enhanced Response to 5-HT in Cirrhosis*

The results of this study indicated that over activity of 5-$HT_1$-like receptors in cirrhosis could be responsible for the enhanced 5-HT-mediated response in hepatic arteries from cirrhotic patients. However, the absence of any studies addressing the

activity of 5-$HT_1$-like receptors in these arteries either in patients with cirrhosis or in animal models has greatly restricted knowledge about the mechanisms of enhanced response. This study however, indicated that partial pre-contraction could induce alterations in the 5-$HT_1$-like receptor-mediated contractile pathway, resulting in amplified 5-HT-mediated contraction. It is possible that changes in patients with hepatic cirrhosis mimic the effect of partial precontraction. For instance, the homeostatic environment could be altered as a result of metabolic and circulatory change (Hayes *et al.*, 1992). Alternatively, the increased intrahepatic resistance and splanchnic blood flow enhanced pressure in the portal venous territory, which could cause depolarisation of the vessels in this system. These metabolic and mechanical changes could activate, or amplify the activity of 5-$HT_1$-like receptors. Activation or amplification of 5-$HT_1$-like receptors by a variety of neuro-humoral agents (angiotensin II, histamine, α-adrenoceptor agonists, prostaglandin $F_{2\alpha}$) has also been reported in several studies (Yildiz *et al.*, 1998; Delalande, 1992). However, the precise mechanism of amplification of 5-$HT_1$-like receptor-mediated vascular contraction is as yet unknown.

As described (Chapter 3) partial pre-contraction can activate silent 5-$HT_1$-like receptor but the mechanism of enhancing activity of 5-$HT_1$-like or blunting the response to 5-$HT_{2A}$ receptors following such precontraction is uncertain. Apart from initial sub-threshold increase of intracellular calcium levels, another potential mechanism is that the allosteric modulation of the receptors by pre-contraction, which can alter activity of the 5-$HT_{2A}$ and 5-$HT_1$-like receptors (Yildiz *et al.*, 1998; Purdy *et al.*, 1993; Kaumann & Frenken, 1985). Existence of such allosteric

modulating mechanism in the vascular 5-$HT_{2A}$ receptor has been described where normal activity of 5-$HT_{2A}$ receptor in rabbit aorta is reduced by the allosteric inhibition (Purdy *et al.*, 1993). Therefore, in the present study, allosteric modulation could be responsible for blunting the activity of 5-$HT_{2A}$ receptor in partially precontracted hepatic arteries. Alternatively, the allosteric mechanism can also modulate the receptor from a low to a high activity state (Purdy *et al.*, 1993) and can activate silent 5-$HT_1$-like receptor (Xu *et al.*, 1990; Purdy *et al.*, 1993). It can therefore, be suggested that this could be a mechanism, which modulated 5-$HT_1$-like receptors in human hepatic arteries following partial precontraction and consequently, enhanced 5-HT-mediated contraction. Therefore, in cirrhosis, altered plasma levels of 5-HT (described in Chapter 5) as well as alteration in the humoral environment could induce changes within the 5-$HT_1$-like receptor and/or in the post-receptor contractile pathway in hepatic arteries, which could be responsible for the enhanced 5-HT-mediated contraction.

### 6.7. Conclusions

The present study indicated the existence of functional vasoconstrictor 5-$HT_1$-like receptors in human hepatic arteries. In donor hepatic arteries, 5-HT-induced vasoconstriction was a cumulative action of 5-$HT_{2A}$ and 5-$HT_1$-like receptors, while in recipient, it was predominantly mediated via the 5-$HT_1$-like receptors. This study further indicated that in cirrhosis, the enhanced response to 5-HT in the hepatic arteries was mediated by 5-$HT_1$-like receptors. This enhanced response may be due to the alterations of receptor activity and/or within the post receptor signal-transduction pathway. It can therefore be suggested that over activity of 5-$HT_1$-like

receptor might have a substantial role in modulating splanchnic blood flow, subsequently generating portal hypertension or persistently maintaining this pathological condition.

# CHAPTER SEVEN

# GENERAL DISCUSSION

## 7.1. General Discussion

It has been demonstrated that cirrhosis of the liver is associated with an impaired pressor response to vasoconstrictors and altered contractile function in isolated vessels. The aim of this project was to use human hepatic arteries to assess the effect of cirrhosis on vascular contractile function. Attempts would also be made to identify the mechanisms responsible for any abnormality detected.

The agonists chosen for assessment using human hepatic arteries (AVP, 5-HT and ET-1) are all endogenous vasoconstrictors (Hadoke, 1999). Their relevance to the hyperdynamic circulation of cirrhosis is indicated by the fact that plasma concentrations of all three are altered in cirrhotic patients (Akriviadis *et al.*, 1997; Beaudry *et al.*, 1994; Trevisani *et al.*, 1997). In addition, there is clinical and experimental evidence to suggest that manipulation of these vasoconstrictor systems may have therapeutic benefit in patients with cirrhosis (Shah *et al.*, 1998; Hadoke, 2001).

## 7.2. Method Development

The difficulties associated with obtaining human hepatic arteries, together with the small amount of information available in the literature, made it necessary to perform a certain amount of method development using porcine splanchnic arteries as a model. In particular, it was necessary to determine concentration ranges of agonists to use and also to assess the effect of organ preservation on donor hepatic artery function.

*7.2.1. Agonist Response*

In porcine splanchnic arteries, AVP and 5-HT produced complete concentration-response curves in the ranges used, reaching an obvious maximum (Emax) and thus allowing calculation of the $pD_2$. Both these values were required as Emax and/or $pD_2$ may be altered in arteries from patients with cirrhosis (Lew, 1995; Lew & Angus, 1992). The concentration-ranges used were also consistent with the other studies using splanchnic vessels from small animals (Stam *et al.*, 1998; Choppin & O'Connor, 1995) and renal or coronary vessels from humans (Medina, *et al.*, 1996; Kaumann *et al.*, 1994). Therefore, it was inferred that these ranges would be suitable for use in human hepatic arteries. Arteries in this study were shown to be devoid of endothelium as a result of surgical and preservation procedures. Consequently, these results can only be extrapolated to denuded human hepatic arteries. This was not problematic, as it was expected that human hepatic arteries would also loose endothelium during surgery/retrieval and such denuded arteries were subsequently used by many studies (Smith *et al.*, 1997; Hadoke *et al.*, 1998; Heller *et al.*, 1999).

An unexpected result observed while using splanchnic arteries from juvenile pigs was that the response to 5-HT was age-dependent. Receptor studies showed this to be due to differences in 5-HT receptor activity and indicated the presence of at least two different 5-HT receptor subtypes in porcine hepatic arteries as evidenced in isolated rabbit mesenteric and renal (Choppin & O'Connor, 1995, 1994), porcine cerebral (Den-Boer *et al.*, 1991) and guinea-pig iliac (Sahin-Erdemli *et al.*, 1991) arteries. One of these was positively identified as the $5\text{-}HT_{2A}$ receptor, because it was sensitive to ketanserin (Hoyer *et al.*, 1994). This receptor was active in adult pig

hepatic arteries. The other receptor was tentatively identified as the 5-$HT_1$-like receptor because it was activated, in both adult and juvenile pig hepatic arteries, by partial pre-contraction: a procedure which activates 5-$HT_1$-like receptors in a variety of arteries from other species (Choppin & O'Connor, 1995, 1994). Furthermore, responses mediated by this receptor were not antagonised by ketanserin as, it has no effect on 5-$HT_1$-like receptor (Martin & Humphrey, 1994). Selective 5-$HT_1$-like receptor agonists and antagonists were not used to further identify this receptor in the porcine hepatic arteries, as this was not a primary goal of the project. Evidence obtained in the human hepatic arteries, however, supports the identification of this receptor.

*7.2.2. Effect of Organ Preservation*

It was demonstrated that exposure to preservative solution (UWS, with or without dexamethasone, insulin and penicillin) under conditions similar to those used during liver preservation before transplantation had no effect on the contractile responses to AVP and 5-HT in porcine hepatic arteries. This suggested that donor hepatic arteries (which are exposed to the organ preservation procedure) can be used as a control for recipient hepatic arteries (which are not) when assessing functional responses to AVP and 5-HT. This was consistent with the use of donor hepatic arteries from this source as controls in many studies of α-adrenoceptor-mediated contraction in cirrhosis (Smith *et al.*, 1997; Hadoke *et al.*, 1998; Heller *et al.*, 1999). However, damage to the endothelium in the porcine hepatic arteries prevented an assessment of the effects of organ preservation in endothelial cell function. This is important because previous studies suggested that most functional alterations following

preservation are due to changes in the endothelium, rather than the smooth muscle cells (Lin *et al.*, 1995; Anastasious *et al.*, 1997; Jeng *et al.*, 1997). The small but significant impairment of SIN-1-mediated response in arteries incubated with preservative solutions suggests that care must be taken if relaxation responses are compared in donor and recipient hepatic arteries.

### 7.3. Effect of Cirrhosis on Contractile Response of Human Hepatic Arteries

This study formed the centrepiece of the project. Method development with porcine hepatic arteries enabled optimisation of the conditions for *in vitro* assessment of human hepatic arteries. This allowed the most efficient use of arteries obtained from the transplant theatre, which were obviously a scarce resource. Furthermore, studies with UWS had confirmed that the donor hepatic artery was a valid control for recipient hepatic arteries when comparing responses to vasoconstrictors.

It was demonstrated for the first time that cirrhosis of the liver is associated with altered contractile responses to AVP, 5-HT and ET-1 in human hepatic arteries. Furthermore, this study extended previous work using human hepatic arteries by showing that these alterations were strictly agonist-selective (Cummings *et al.*, 1986; Silva *et al.*, 1998; Cahil *et al.*, 1998). It also demonstrated that, in hepatic arteries, cirrhosis could alter Emax and/or $pD_2$. The Emax to AVP was reduced whereas that to 5-HT was increased while, in both cases, the $pD_2$ were unaffected. In the case of ET-1, however, Emax was unchanged but $pD_2$ was increased, indicating an increased sensitivity of hepatic arteries to this agonist. These results further indicated that although cirrhosis, irrespective of species, is associated with a hyperdynamic

circulation, responses to distinct agonists are not altered in the same way. It is, therefore, essential to investigate individual pressor systems independently as they can be affected in different ways by the development of hepatic cirrhosis.

### 7.4. Mechanisms of Altered Vascular Contraction

Many studies have attempted to identify the pathophysiology of the vascular abnormalities of cirrhosis, but the underlying mechanism remains controversial (Hadoke, 2001). This study demonstrated that the altered function detected *in vitro* was due to abnormalities in the vascular smooth muscle cells. The absence of endothelial cells removed the possible effect of endothelium-derived relaxing or contracting factors (Searle & Sahab, 1992), whilst *in vitro* methodology excluded a role for circulating factors (Hayes *et al.*, 1992). It remains possible, however, that these factors may contribute to vascular dysfunction *in vivo.*

The similar contractile response to KCl in arteries from cirrhotic and non-cirrhotic individuals indicated that differences in arterial size did not account for variations in agonist-mediated contraction. Induction of nitric oxide production in the VSMCs was also unlikely to be responsible, as this would be expected to reduce the contraction produced by all vasoconstrictor agonists (Sieber & Groszmann, 1992; Karatapanis *et al.*, 1994; Hartleb *et al.*, 1994b). This was confirmed by the functional investigations which demonstrated that inhibition of NOS altered neither the basal tone nor the contractile responses to AVP and 5-HT in recipient hepatic arteries. Immunohistochemistry also confirmed that donor and most recipient hepatic arteries did not express iNOS. An intriguing observation was the demonstration of weak

iNOS immunoreactivity in hepatic arteries from patients with alcoholic liver disease. Use of L-NNA suggested that the iNOS was not implicated on the contractile function. However, this iNOS staining only in patients with ALD signified that the pathogenesis of vascular abnormalities could vary depending on the aetiology of liver cirrhosis. This variability suggested that altered contraction was due to changes in the activity of a particular receptor and/or in the post-receptor signal transduction pathways.

### 7.5. Receptor Involvement

The current study demonstrated that cirrhosis exerted selective effects on receptor-dependent contraction of recipient hepatic arteries. This effect was not dependent on the endothelial cell function and circulating humoral agents. Therefore, to clarify the mechanisms of these contractile abnormalities, it was necessary to identify the receptor(s) in the VSMCs mediating contractile function induced by these agonists. Use of porcine vessels (Chapter 3) demonstrated that the AVP and 5-HT caused vasoconstriction by stimulation of receptors located in the VSMCs of hepatic arteries. The receptors that mediated contraction to AVP, 5-HT and ET-1 were identified pharmacologically in human hepatic arteries.

It was demonstrated for AVP that similar to human renal (Medina *et al.*, 1996) and gastric (Calo *et al.*, 1997) arteries, the $V_1$ receptor was responsible for mediating vasoconstriction in the human hepatic arteries as responses were antagonised by the $V_1$ receptor antagonist. In contrast, a selective $V_2$ receptor-agonist (DDAVP) did not produce any contraction excluding the existence of $V_2$ receptor in these denuded

hepatic arteries. Many studies also demonstrated that the $V_2$ receptor was located primarily in the endothelium, which induced vasodilatation (Aki *et al.*, 1994; Tagawa *et al.*, 1995). It was therefore, apparent that abnormality in the $V_1$ receptor-mediated pathway was responsible for reduced AVP-mediated contraction.

In human hepatic arteries, 5-HT-mediated contraction was shown to be mediated by both 5-$HT_{2A}$ and 5-$HT_1$-like receptors as suggested by several studies (Martin & Humphrey, 1994; Yildiz *et al.*, 1996). In this study, the enhanced response to 5-HT in cirrhosis was contributed by both 5-$HT_{2A}$ and 5-$HT_1$-like receptors, because, in addition to 5-HT, the response to a relatively selective 5-$HT_1$-like receptor agonist (5-CT) was also increased. The hepatic cirrhosis appeared to alter the relative contribution of these receptors in mediating contractile function similar to the changes in the vessels induced by pre-contraction (MacLean, *et al.*, 1996; Yildiz *et al.*, 1996; Cocks *et al.*, 1993). The role of 5-$HT_1$-like receptors became more significant whilst the contribution of the 5-$HT_{2A}$ receptor was impaired, in hepatic arteries from patients with this disease. Certain other condition such as angina can also amplify the contribution of 5-$HT_1$-like receptors in 5-HT-mediated contraction (McFadden *et al.*, 1992). In hepatic cirrhosis, altered portal and systemic haemodynamics, metabolic functions and existence of humoral factors in abnormal amount could enhance the activity of 5-$HT_1$-like receptors (Hayes *et al.*, 1992; Yildiz *et al.*, 1998).

The study with ET-1 demonstrated the $ET_A$-receptor mediated vasoconstriction in human hepatic arteries. In cirrhosis, therefore, the altered activity of the $ET_A$-receptor

could be responsible for the enhanced sensitivity of response to ET-1. Increased sensitivity of response to ET-1 has also been reported in mesenteric arteries and thoracic aortae, or in portal veins from animal models of portal hypertension with or without cirrhosis (Cahill *et al.*, 1998; Petrowsky *et al.*, 1999). Moreover, in the animal models, the mixed $ET_A$ and $ET_B$ receptor antagonists, could reduced portal pressure indicating enhanced activity of the ET-1 system (Gandhi *et al.*, 1998; Reichen *et al.*, 1998; Sogni *et al.*, 1998). The mechanism of increased activity of the ET-1 system in cirrhosis has not been explained. It could be due to the compensatory hyperactivation of the ET-1 pressor system as a result of persistent vasodilatation in cirrhosis, where expression of ET-1 receptors ($ET_A$ and $ET_B$) had been enhanced (Rockey *et al.*, 1998; Leivas *et al.*, 1998; Cahill *et al.*, 1998). In contrast, it has also been reported that the ET-1-mediated pressor response in forearm blood vessels of well-compensated cirrhotic patients (Helmy *et al.*, 2001) or the arterial pressure in cirrhotic rat model (Hartleb *et al.*, 1994b) was impaired. Although, the mechanism of the attenuated pressor response *in vivo* was uncertain, it was suggested that increased endothelial NO production via the $ET_B$ receptor could possibly be responsible for this effect. Alternatively, it could be due to ET-1 receptor down-regulation (Rubanyi & Polokoff, 1994) as a result of increased plasma concentrations of ET-1 in cirrhosis (Asbert *et al.*, 1993; Kitano *et al.*, 1996; Trevisani *et al.*, 1997). However, this explanation appears unlikely, as previous studies found that the plasma ET-1 concentration was not increased (Helmy *et al.*, 2001; Battaglia *et al.*, 1996). Indeed, one study reported a decrease in plasma ET-1 concentration in patients with hepatic cirrhosis (Veglio *et al.*, 1992).

In the present study, a contribution from the $ET_B$ receptor to the altered contractile response can not be discounted, as this was not directly investigated. The $ET_B$ receptor mediates vasodilatation or vasoconstriction depending on its location in the endothelial cells or VSMCs, respectively (Gray & Webb, 1996; Touyz *et al.*, 1995). However, in this study, altered activity of the $ET_A$ receptor in VSMCs was most likely mediating the enhanced ET-1 response in cirrhosis. This is evidence by the fact that in cirrhotic recipient hepatic arteries, noticeably higher (5 fold) concentrations of $ET_A$-receptor antagonist (BQ-123) were required to antagonise the response to ET-1, as compared to donor hepatic arteries. Therefore, this study indicated that the up-regulation of the $ET_A$-receptor in the recipient hepatic arteries could account for the increased ET-1-mediated contraction in cirrhosis. However, the contribution of $ET_B$-receptors in ET-1-induced contraction of human hepatic arteries with and without cirrhosis requires to be evaluated.

### 7.6. Conclusions

This study investigated the effect of hepatic cirrhosis on functional responses of human hepatic arteries. Performing method development using porcine splanchnic arteries allowed optimum use of scarce human arteries and also demonstrated that organ preservation procedures were unlikely to alter contractile response of donor hepatic arteries.

The demonstration that vessel retrieval procedures (probably trauma during surgery) led to removal of the endothelium limited the investigation to an assessment of the effects of cirrhosis on VSMC contraction. For the first time, however, it was shown

that altered contractile function in hepatic arteries from patients with cirrhosis is agonist-dependent and represents more than a simple hyporesponsiveness to all vasoconstrictors. These results have profound implications for the investigation of the pathogenesis of the hyperdynamic circulation and for the development of potential therapeutic interventions for complications associated with these vascular abnormalities.

The mechanisms responsible for the alteration in contractile function remain to be clarified. The absence of an endothelium indicates that changes are restricted to the VSMCs but a role for endothelium-derived factors contributing *in vivo* cannot be excluded. In order to determine the impact of hepatic cirrhosis on the function of the endothelial cells it would be necessary to acquire vessels with an intact endothelium from both cirrhotic patients and non-cirrhotic controls. One possible way of achieving this would be to isolate sub-cutaeneous resistance arteries from elective fat biopsies. It is also unlikely that humoral factors contribute to the changes identified, as all functional work was performed *ex vivo* isolated from circulating factors. However, such factors may have a role *in vivo* and future research, such as performing functional investigations in the presence of plasma from cirrhotic patients or exposing cultured VSMCs to cirrhotic plasma is required. More precisely, to assess the role of both endothelium and humoral factors on the altered contractile response, further studies such as venous occlusion plethysmography (fore arm blood flow) and dorsal hand vein compliance can be performed using these agonists (AVP, 5-HT and ET-1) in patients with cirrhosis.

Given that the contractile abnormality is in the VSMCs, several possible mechanisms presented themselves. It was shown that neither NO release from the VSMCs, nor structural remodelling were responsible for the functional changes observed. This suggested, therefore, that changes in the receptor-mediated contractile pathways contributed these altered arterial contractions. The receptors responsible for the vascular contraction ($V_1$ for AVP; 5-$HT_{2A}$ and 5-$HT_1$-like for 5-HT and $ET_A$ for ET-1) were identified. These alterations could be either in these receptors or post-receptor signal transduction pathways. Combined pharmacological and ligand-binding studies could determine the function and expression of these receptors. Similarly, post-receptor signal transduction pathways can be assessed by measuring the intracellular second messengers such as inositol phosphates, calcium ions and protein kinase C, following stimulation of these receptors. Future research should endeavour to determine the effect of hepatic cirrhosis on expression and contractile activity of these receptors and also on intracellular contractile second messenger pathways.

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# Selective Alteration of Agonist-Mediated Contraction in Hepatic Arteries Isolated From Patients With Cirrhosis

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***Background & Aims:*** **Impaired pressor function in cirrhosis may be specific to certain agonists and vascular territories. This investigation determined whether responses to arginine vasopressin (AVP) and 5-hydroxytryptamine (5-HT) were impaired in hepatic arteries from cirrhotic patients.** ***Methods:*** **Cumulative concentration-response curves were produced for AVP ($10^{-11}$ to $3 \times 10^{-6}$ mol/L), 5-HT ($10^{-9}$ to $3 \times 10^{-5}$ mol/L), and potassium chloride (2.5–120 mmol/L) in hepatic arteries from liver donors (noncirrhotic) and recipients (cirrhotic). The receptor stimulated by AVP was identified using a $V_1$-receptor antagonist (d[$CH_2$]$_5$Tyr[Me]AVP) and a selective $V_2$-receptor agonist (desmopressin [DDAVP]).** ***Results:*** **Cirrhotic patients had a high heart rate (98 ± 4 beats/min) and cardiac output (9.87 ± 0.51 L/min) but low peripheral vascular resistance (711 ± 35 dyn · s/cm$^5$). None of the arteries had a functional endothelium. Maximal contraction (but not sensitivity) to AVP was smaller ($P = 0.0002$) in hepatic arteries from recipients (34.03% ± 3.42% KCl) than donors (60.69% ± 5.56% KCl). 5-HT–mediated contraction was enhanced in recipient hepatic arteries (88.81% ± 5.43% KCl vs. 71.63% ± 4.46% KCl; $P = 0.01$), but sensitivities were similar ($P = 0.20$). KCl-mediated contractions were similar ($P = 0.87$) in both groups. Arteries did not respond to DDAVP, but d($CH_2$)$_5$Tyr(Me)AVP produced a concentration-dependent rightward shift in the response to AVP.** ***Conclusions:*** **These results demonstrate a selective impairment of $V_1$ receptor–mediated contraction in denuded hepatic arteries from cirrhotic patients, suggesting an abnormality within the vascular smooth muscle.**

A hyperdynamic circulatory state,[1] consisting of arterial vasodilatation and increased cardiac output, is common in patients with cirrhosis.[2] The causes of these abnormalities remain obscure but may be linked to an impaired response to pressor hormones.[3] Indeed, there is evidence to suggest that the function of cells in the vascular wall becomes impaired in patients with cirrhosis,[4] although this remains controversial.[5]

Studies in cirrhotic patients have suggested that contractile hyporesponsiveness could be agonist dependent,[3,6] whereas our own study has shown that α-adrenoceptor–mediated contraction was unaltered in hepatic arteries isolated from cirrhotic patients.[7] The effect of cirrhosis on the pressor response to arginine vasopressin (AVP) and 5-hydroxytryptamine (5-HT) is of particular interest. AVP and its analogues reduce portal pressure and are used in the treatment of acute variceal hemorrhage in cirrhosis.[8] Similarly, clinical and experimental studies have shown that 5-$HT_2$–receptor antagonists reduce portal pressure in patients[9] and in animal models of portal hypertension.[10] Therefore, altered pressor responses to AVP and 5-HT may be important both in the pathogenesis and in the treatment of vascular complications in cirrhosis. Functional investigations of AVP- and 5-HT–mediated contraction in vessels isolated from animal models of cirrhosis have produced conflicting results[5] and are not directly applicable to the clinical situation in humans. This study aimed to determine whether the presence of cirrhosis was associated with alterations in the contractile responses to AVP and 5-HT of isolated hepatic arteries. Such studies in human hepatic arteries have not been reported. An additional aim was to characterize the AVP-receptor subtype present in human hepatic arteries by employing a $V_1$-receptor antagonist and a selective $V_2$-receptor agonist.

## Materials and Methods

### Effect of Cirrhosis on Hepatic Artery Contraction

Hepatic arteries were collected from 37 donors (noncirrhotic; 20 men and 17 women) and 33 recipients (cirrhotic; 14 men and 19 women) of similar age (46.7 ± 2.0 and 51.6 ± 1.9

*Abbreviations used in this paper:* ACh, acetylcholine; AVP, 8-arginine vasopressin; DDAVP, desmopressin; 5-HT, 5-hydroxytryptamine; NO, nitric oxide; PHE, phenylephrine; UW, University of Wisconsin.



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years, respectively; $P = 0.09$) at liver transplantation. Causes of cirrhosis (mean duration, 9.2 ± 1.2 years) were primary biliary cirrhosis (n = 13), alcoholic liver disease (n = 8), cryptogenic (n = 6), primary sclerosing cholangitis (n = 5), and secondary biliary cirrhosis (n = 1). Causes of death in liver donors were subarachnoid hemorrhage (n = 21), intracerebral hemorrhage (n = 8), head injury (n = 4), hypoxia (n = 3), and aneurysm (n = 1). Donor hepatic arteries were obtained after transplantation, having been stored (with the donor liver) in preservative (University of Wisconsin [UW]) solution for up to 16 hours. Recipient hepatic arteries (4–6 cm) were dissected from the freshly explanted liver and were never stored in UW solution. Upon retrieval, hepatic arteries were placed immediately in Krebs–Henseleit solution. The Lothian Research Ethics Committee approved the use of human tissues.

Arteries were cleaned of adherent connective tissue, and rings 2 mm in length were mounted between 2 parallel hooks in 10-mL organ baths containing Krebs–Henseleit solution (composition in mmol/L: NaCl, 118.5; KCl, 4.7; $MgSO_4$, 1.2; $KH_2PO_4$, 1.2; $NaHCO_3$, 25; $CaCl_2$, 2.5; and glucose, 11.6; pH 7.4). Indomethacin ($10^{-5}$ mol/L) was added to the Krebs' solution to inhibit the production of endogenous prostaglandins. The organ baths were maintained at 37°C and bubbled with 95% $O_2$–5% $CO_2$. The lower hook was fixed, and the upper hook was attached to an isometric force transducer (Grass FTO3) connected to a computerized data acquisition system. Whenever possible, functional investigations were performed immediately after retrieval of the hepatic arteries. However, the irregular timing of transplant operations necessitated storing some arteries in Krebs–Henseleit solution at 4°C for a maximum of 14 hours before functional investigation.

The rings were equilibrated at their optimal resting force (4*g*)[7] for 40–50 minutes. The viability and reproducibility of contractions were tested using 3–4 consecutive applications of KCl (100 mmol/L). Cumulative concentration-response curves were constructed using AVP ($10^{-11}$ to $3 \times 10^{-6}$ mol/L), 5-HT ($10^{-9}$ to $3 \times 10^{-5}$ mol/L), and KCl (2.5–120 mmol/L). The rings were washed repeatedly after completion of each curve and equilibrated for 30 minutes before the next agonist was applied.

Human hepatic arteries obtained during liver transplantation are generally devoid of endothelium.[4,7,11] Endothelial cell function was assessed by adding the endothelium-dependent vasodilator acetylcholine (ACh; $10^{-6}$ to $3 \times 10^{-5}$ mol/L)[12,13] to 6 donor and recipient hepatic arteries precontracted with phenylephrine (PHE) ($10^{-6}$ to $3 \times 10^{-5}$ mol/L). Endothelial cell integrity was also assessed by staining the luminal surface using silver nitrate.[14] In subsequent experiments, removal of the endothelium was ensured by rubbing the lumen with a wire probe.

## Identification of the Receptor Activated by AVP

Fresh rings were prepared and cumulative concentration-response curves obtained for the selective $V_2$ agonist desmopressin (DDAVP; $10^{-11}$ to $3 \times 10^{-6}$ mol/L) and KCl (2.5–120 mmol/L). Four more rings from each artery were then suspended in parallel: 3 were incubated with the $V_1$-receptor antagonist, $d(CH_2)_5Tyr(Me)AVP$ ($10^{-8}$, $10^{-7}$, and $10^{-6}$ mol/L), for 15 minutes, whereas the fourth (control) was not exposed to the antagonist. Cumulative concentration-response curves were constructed to AVP ($10^{-11}$ to $3 \times 10^{-6}$ mol/L) in the presence of the antagonist. Finally, cumulative concentration-response curves were obtained to KCl (2.5–120 mmol/L) in the absence of the antagonist.

## Effect of Organ Preservation on Hepatic Artery Function

Responses to α-adrenoceptor agonists and KCl are similar in denuded hepatic arteries from pigs and humans.[15] Porcine hepatic arteries were used to confirm that preservation of the donor liver using UW solution was not responsible for functional differences between donor and recipient hepatic arteries. Hepatic arteries from 8 adult pigs were obtained from the abattoir, and the endothelium was removed. Sections of each artery were incubated at 4°C for 14–16 hours in 20 mL of either Krebs' solution or UW solution containing dexamethasone (0.016 mg/mL), insulin (0.04 U/mL), and benzylpenicillin (0.24 mg/mL). Responses to 5-HT ($10^{-9}$ to $3 \times 10^{-5}$ mol/L), AVP ($10^{-11}$ to $3 \times 10^{-7}$ mol/L), and KCl (2.5–120 mmol/L) were then obtained in 2-mm rings of these arteries.

## Materials

5-HT creatinine sulfate, 8-AVP, DDAVP, $d(CH_2)_5Tyr(Me)AVP$, indomethacin HCl, PHE HCl, ACh chloride, and benzylpenicillin sodium were obtained from Sigma (Poole, Dorset, England). Dexamethasone sodium phosphate was from Faulding Pharmaceuticals (Warwick, England), and insulin (Velosulin) was from NovoNordisk Pharmaceuticals (Sussex, England). UW solution was obtained from NPBI International BV (Emmer-Compascuum, the Netherlands). All salts were obtained from BDH (Poole, Dorset, England). Stock solutions were prepared in deionized water, except indomethacin, which was prepared in ethanol (final bath concentration of ethanol, <1% vol/vol). Aliquots of 1.5 mL were stored at −20°C and thawed as required, and any residue was discarded at the end of the experiment. Concentrations given are the final molar concentrations in the organ bath.

## Statistics

Results are expressed as mean ± SEM; n represents the number of subjects. Maximal contractions are given in grams and as a percentage of maximum contraction to KCl to control for variations in the size of the arterial rings.[16] Responses to ACh are a percentage of the precontraction induced by PHE. Sensitivity ($pD_2$) values were determined using curve-fitting software (Fig.P.; Biosoft, Cambridge, England). Maximal contraction and sensitivity were compared using the Student unpaired *t* test. Concentration-response curves were compared using 2-way analysis of variance (ANOVA), followed by a

Tukey post hoc test. Differences were considered significant at $P < 0.05$.

## Results

Cirrhotic patients had a high heart rate (98 ± 4 beats/min; n = 33; normal range, 60–90 beats/min) and cardiac output (9.87 ± 0.51 L/min, n = 31; normal range, 4–8 L/min), combined with a low systemic vascular resistance (711 ± 35 dyn · s/cm$^5$, n = 29; normal range, 1200–1500 dyn · s/cm$^5$).

### Test for Endothelium

None of the arteries (6 donor, 6 recipient) relaxed in response to ACh. Indeed, ACh ($10^{-6}$ mol/L) contracted recipient (1.83% ± 0.47%) and donor (2.37% ± 0.35%) hepatic arteries, with further contractions as the ACh concentration was increased to $3 \times 10^{-6}$ mol/L (6.95% ± 1.49% and 5.46% ± 1.10%, respectively) and $3 \times 10^{-5}$ mol/L (9.30% ± 0.95% and 11.82% ± 0.68%, respectively). The absence of an endothelium was confirmed using the silver nitrate stain.

### Effect of Cirrhosis on Hepatic Artery Contraction

The maximal contractile response to AVP (Figure 1*A*) was significantly ($P = 0.003$) smaller in recipient (3.53 ± 0.35 g; n = 22) than in donor (5.73 ± 0.61 g; n = 23) arteries. This difference was maintained when contractions were expressed as a percentage of maximal response to KCl (34.03% ± 3.42% vs. 60.69% ± 5.56%; $P = 0.0002$). This impairment was also detected ($P < 0.0001$) when the entire curves were compared using 2-way ANOVA (Figure 1*A*). Sensitivity ($pD_2$) to this agonist, however, was similar ($P = 0.86$) in donor (8.19 ± 0.07) and recipient (8.21 ± 0.08) hepatic arteries.

5-HT produced a larger contraction in recipient (8.60 ± 0.46 g; n = 25) than in donor (7.12 ± 0.58 g; n = 26) hepatic arteries (Figure 1*B*). This difference was of borderline significance ($P = 0.06$), but significance was achieved when contractions were expressed as a percentage of the maximal contraction to KCl (88.81% ± 5.43% vs. 71.63% ± 4.55%; $P = 0.01$). The sensitivity ($pD_2$) of this response was similar in donor and recipient hepatic arteries (6.45 ± 0.08 and 6.60 ± 0.08, respectively; $P = 0.20$). The difference between the curves was confirmed by 2-way ANOVA (Figure 1*B*; $P = 0.04$).

The maximal contraction (10.09 ± 0.71 and 10.32 ± 0.70 g, respectively; $P = 0.81$) and sensitivity (1.70 ± 0.05 and 1.63 ± 0.04, respectively; $P = 0.34$) to KCl were similar in donor and recipient hepatic arteries.

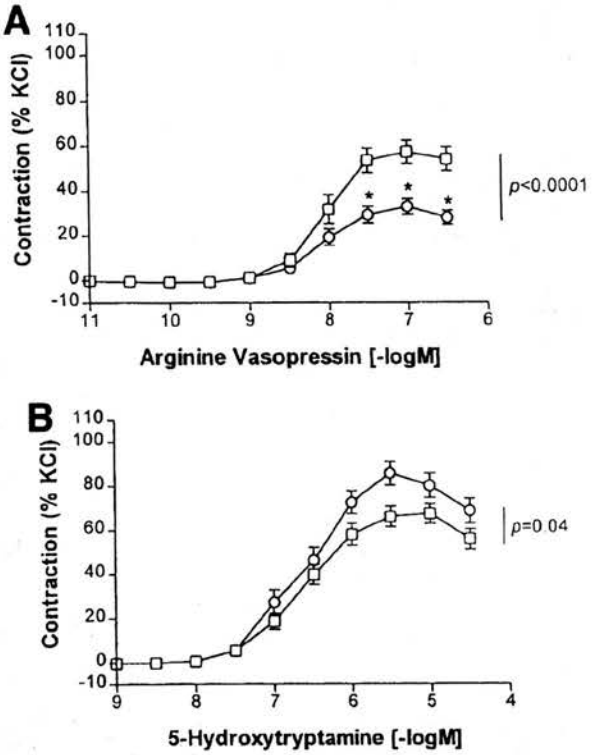


**Figure 1.** Cumulative concentration-response curves produced in response to (*A*) AVP (n = 22–23) and (*B*) 5-HT (n = 25–26) by donor (noncirrhotic, □) and recipient (cirrhotic, ○) hepatic arteries. Responses are given as mean ± SEM and expressed as a percentage of the maximal contraction induced by KCl. Differences between curves were obtained using 2-way ANOVA. *$P < 0.0001$ when individual points were compared using the Tukey post hoc test.

### Identification of the Receptor Activated by AVP

The selective $V_2$ agonist, DDAVP, did not contract hepatic arteries from donors or recipients, although these contracted in response to KCl (maximal contraction, 11.23 ± 1.29 and 10.85 ± 0.64 g, respectively; $P = 0.76$; $pD_2$, 1.65 ± 0.09 and 1.63 ± 0.04, respectively; $P = 0.74$). Incubation with the $V_1$ antagonist, d(CH$_2$)$_5$Tyr(Me)AVP, produced a concentration-dependent rightward shift in the response to AVP (Figure 2), reducing the sensitivity (but not the maximal contraction) to this agonist (Table 1). This effect was similar in arteries from recipients and donors. The sizes of the responses to KCl were unaltered after incubation with this antagonist (Table 1). Similarly, the sensitivities ($pD_2$) of donor (1.64 ± 0.07) and recipient (1.67 ± 0.04) hepatic arteries to KCl were unaltered by incubation with $10^{-8}$ mol/L (1.63 ± 0.08 and 1.69 ± 0.05, respectively), $10^{-7}$ mol/L (1.60 ± 0.06 and 1.61 ± 0.05, respectively), or $10^{-6}$ mol/L (1.61 ± 0.08 and 1.63 ± 0.07, respectively) d(CH$_2$)$_5$Tyr(Me)AVP.

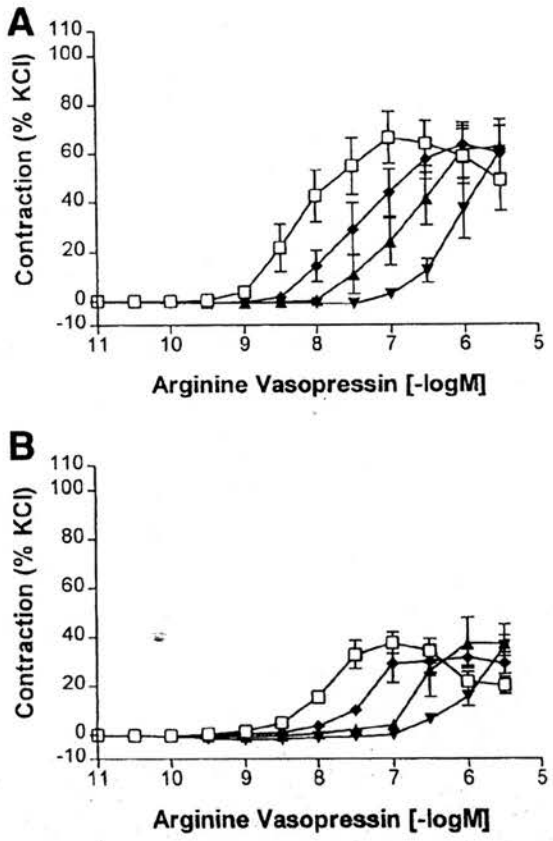


**Figure 2.** Effect of incubation in the absence (control, □) or presence of $10^{-8}$ mol/L (◆), $10^{-7}$ mol/L (▲), and $10^{-6}$ mol/L (▼) of the $V_1$ antagonist, d($CH_2$)5Tyr(Me)AVP, on responses to AVP in (*A*) donor (n = 11) and (*B*) recipient (n = 10) hepatic arteries. The sensitivities ($pD_2$) of donor (8.07 ± 0.16) and recipient (8.01 ± 0.11) hepatic arteries were reduced in a concentration-dependent manner by $10^{-8}$ mol/L (7.27 ± 0.14 and 7.25 ± 0.14, respectively; $P < 0.002$), $10^{-7}$ mol/L (6.71 ± 0.12 and 6.56 ± 0.11, respectively; $P < 0.001$), and $10^{-6}$ mol/L (6.07 ± 0.10 and 6.15 ± 0.14, respectively; $P < 0.001$) of d($CH_2$)5Tyr(Me)AVP. Values are mean ± SEM and are expressed as a percentage of the maximal contraction induced by KCl.

### Effect of Organ Preservation on Hepatic Artery Function

Maximal contractions of porcine hepatic arteries by 5-HT (4.15 ± 0.67 g), AVP (2.83 ± 0.41 g), and KCl (11.95 ± 1.27 g) after storage for 14–16 hours in UW solution (n = 8) were not different from responses produced after storage in Krebs' solution (n = 8) (3.79 ± 0.48 g, $P = 0.67$; 2.57 ± 0.48 g, $P = 0.68$; and 11.29 ± 1.12 g, $P = 0.70$, respectively). The sensitivities ($pD_2$) of arteries stored in UW solution to 5-HT (6.08 ± 0.10), AVP (8.19 ± 0.08), and KCl (1.45 ± 0.03) were also similar to values obtained with sections stored in Krebs' solution (5.98 ± 0.10, $P = 0.50$; 8.04 ± 0.13, $P = 0.33$; and 1.47 ± 0.03, $P = 0.57$, respectively).

## Discussion

This investigation demonstrates that receptor-dependent contraction of denuded human hepatic arteries is altered in patients with cirrhosis. This corresponds with recent reports[4,11] of attenuated $\alpha_1$-adrenoceptor–mediated contraction in hepatic arteries and portal veins from similar patients (although our previous study did not detect an impaired response to α-adrenoceptor agonists[7]). The present results show that the vascular abnormality in patients with cirrhosis is agonist dependent, because AVP-mediated contraction was reduced, whereas the response to 5-HT was augmented. In the absence of a functional endothelium, this suggests an alteration in the vascular smooth muscle cells. Because AVP and its analogues are used to treat acute variceal hemorrhage, and may act by reducing portal hypertension via contraction of splanchnic arteries,[17] this may have implications for clinical practice.

Investigation of hepatic artery function is relevant to the pathogenesis of vascular abnormalities in cirrhosis, because dilation of splanchnic arteries in early cirrhosis contributes significantly to the hyperdynamic circulation.[18] Donor hepatic arteries have been used previously, by ourselves[7] and others,[4,11] as controls in functional investigations. Their suitability is demonstrated by their functional similarity to porcine hepatic and mesenteric arteries.[15] This is reinforced by our confirmation that organ preservation in UW solution does not alter contractility of porcine hepatic arteries.[4] Trauma during surgery is a recognized cause of endothelial cell loss[19] and explains the absence of endothelium in the arteries used in the present, and previous,[4,7,11] studies. Consequently, the changes in AVP- and 5-HT–mediated contraction must

**Table 1.** Effect of the $V_1$ Antagonist d($CH_2$)5Tyr(Me)AVP on the Maximal Contractile Responses of Donor and Recipient Hepatic Arteries

| | AVP | | KCl |
|---|---|---|---|
| Antagonist | (*g*) | (*%KCl*) | (*g*) |
| Donor hepatic arteries | | | |
| None (10–11) | 6.93 ± 0.77[a] | 70.38 ± 9.87[a] | 11.34 ± 1.52 |
| $10^{-8}$ mol/L (10–11) | 6.37 ± 0.60 | 64.73 ± 8.32 | 11.05 ± 1.44 |
| $10^{-7}$ mol/L (8–10) | 6.34 ± 0.78 | 63.16 ± 9.84 | 11.33 ± 1.36 |
| $10^{-6}$ mol/L (8–9) | 5.99 ± 0.68 | 56.22 ± 9.67 | 11.88 ± 1.61 |
| Recipient hepatic arteries | | | |
| None (10) | 4.18 ± 0.59 | 39.81 ± 5.75 | 11.34 ± 0.92 |
| $10^{-8}$ mol/L (10) | 3.89 ± 0.73 | 41.89 ± 7.69 | 10.37 ± 0.87 |
| $10^{-7}$ mol/L (8) | 4.15 ± 0.88 | 39.47 ± 8.59 | 11.42 ± 1.36 |
| $10^{-6}$ mol/L (8) | 4.30 ± 0.82 | 36.47 ± 4.21 | 10.93 ± 1.13 |

NOTE. Values are mean ± SEM for (n) subjects.
[a]$P < 0.02$ compared with the control responses in the recipient group. All comparisons were made using the Student unpaired *t* test.

be the result of alterations in the arterial smooth muscle rather than release of vasoactive factors from the endothelium.

AVP and 5-HT were investigated because (1) circulating concentrations of these hormones become altered during the development of cirrhosis and (2) pharmacological manipulations of AVP and 5-HT systems can reduce portal hypertension. Nonosmotic secretion of AVP is increased in cirrhosis, resulting in elevated plasma AVP concentrations.[20] In contrast, 5-HT levels in whole blood, as well as unconjugated (active) and conjugated (metabolites) forms in the plasma, are reduced in advanced disease.[21] AVP and its analogues reduce hepatic blood flow and portal pressure, and are used to treat acute variceal hemorrhage,[8] while 5-$HT_2$ receptor antagonists reduce portal pressure in both animal models[22] and cirrhotic patients.[9]

Attenuated AVP-mediated contraction in recipient hepatic arteries is consistent with the impaired pressor response (to adrenoceptor agonists and angiotensin II) reported in patients with cirrhosis.[3,6] In contrast, the enhanced response to 5-HT was unexpected, although there are reports of increased pressor response (to adrenoceptor agonists) in cirrhotic patients.[23,24] Few studies of vascular function in animal models of cirrhosis have used AVP and 5-HT,[5] but portal hypertension in rats is associated with reduced arterial contraction (with unaltered sensitivity) to AVP.[25,26] This has been attributed to release of endothelium-derived vasodilators,[26] although a corresponding reduction in intracellular inositol phosphate accumulation suggests impaired excitation-contraction coupling within the smooth muscle.[25] Regional variations are evident with a selective enhancement of AVP-mediated contraction in mesenteric veins from portal-hypertensive rats,[22,27] which was not caused by increased smooth muscle mass. The effect of cirrhosis/portal hypertension on responses to 5-HT seems to depend on the animal model and vascular territory studied.[5] Our results are similar to those obtained using aortae from rats with $CCl_4$-induced cirrhosis,[28] mesenteric and portal veins from portal vein–ligated rats,[22,29] and portal veins from mice with schistosomiasis.[30] In rat vessels, 5-HT–induced contraction is mediated by 5-$HT_2$ receptors, and the augmented response was independent of smooth muscle mass and receptor affinity.[22,29] This suggests, therefore, an alteration in the post-receptor signal transduction mechanisms within the smooth muscle cell.

The agonist-selective alteration in contraction of hepatic arteries contrasted with the unaltered response to the (receptor-independent) direct-depolarizing agent, KCl. This is consistent with previous reports of human hepatic artery function in cirrhosis[4,11] and indicates that the altered responses to AVP and 5-HT are not the result of changes in smooth muscle mass or altered sensitivity of contractile proteins to intracellular calcium. This suggests, therefore, altered function of contractile receptors and/or the post-receptor second messenger cascade. AVP can stimulate both $V_1$ and $V_2$ receptors, with $V_1$ receptors usually mediating contraction of vascular smooth muscle. In the present study, the lack of response to the selective $V_2$-receptor agonist (DDAVP), combined with the inhibition of AVP-mediated contraction by the $V_1$-receptor antagonist, confirms that, as in human renal and gastric arteries,[31,32] this is a $V_1$ receptor–mediated response. 5-HT can activate several distinct 5-HT receptor subtypes,[24] with the 5-$HT_{2A}$– and 5-$HT_1$–like receptor subtypes mediating contraction of vascular smooth muscle.[29] In some territories, including the splanchnic vasculature,[33] partial precontraction of the artery is required to facilitate 5-HT–mediated contraction. We have preliminary data to confirm that the resting tone of the human hepatic artery influences the mechanism of 5-HT–mediated contraction,[34] and, consequently, identification of the receptor subtype responsible for 5-HT–mediated contraction of this artery was beyond the scope of the present investigation.

Receptor-mediated contraction rapidly becomes desensitized in response to receptor occupancy, resulting in reductions of either specific binding or inositol phosphate production.[35] Consequently, the elevated AVP concentrations in patients with advanced cirrhosis could cause receptor down-regulation, thus accounting for impaired contraction. Unfortunately, the evidence for receptor down-regulation in cirrhosis is sparse and often contradictory,[36] and expression of $V_1$ receptors has not been assessed in tissues from patients. Similarly, the increased response to 5-HT could be the result of receptor up-regulation in response to reduced circulating 5-HT, although this was not the cause of augmented contraction in rat mesenteric veins.[22] In the absence of ligand binding studies (which require more tissue than was available) and assessment of second messenger responses, the sites of impaired AVP-mediated contraction and enhanced response to 5-HT cannot be confirmed.

The release of nitric oxide (NO), after activation of inducible NO synthase in the vascular smooth muscle cells, has been suggested as the cause of impaired contractile function in cirrhosis.[11] This is unlikely in the present study, because NO release would impair responses to both AVP and 5-HT rather than selectively inhibit the response to AVP. Furthermore, Heller et al.[4]

reported that inhibition of NO synthesis did not alter contractile function in hepatic arteries from patients, which corresponds with our preliminary demonstration that NO inhibition did not affect responses to either AVP or 5-HT in these arteries.[37]

In conclusion, this study demonstrates selective alterations in receptor-mediated contraction of hepatic arteries from patients with cirrhosis. These are not the result of vascular remodeling or impaired sensitivity of contractile proteins, suggesting abnormalities at the receptor–second messenger level, possibly as a result of altered hormone concentrations in patients with cirrhosis. These changes may contribute to the hyperdynamic circulation and may also have important implications for the use of AVP analogues and 5-$HT_2$ antagonists for the pharmacological reduction of portal hypertension.

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## *Zollinger of the Zollinger–Ellison syndrome*

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Robert Milton Zollinger (1903–1993) was born in Millersport, Ohio, and received his M.D. degree from Ohio State University in 1927. His residency training in surgery was at the Peter Bent Brigham Hospital in Boston and at the Lakeside Hospital in Cleveland. After a stint on the faculty at Harvard Medical School, in 1946 he was appointed professor and chairman of the department of surgery at his alma mater, a post he held until retirement in 1974. Zollinger was an avid horticulturist and rose fancier.

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