University of Alberta

SPLENIC NEUROHORMONAL MODULATION OF RENAL AND MESENTERIC HEMODYNAMICS IN PORTAL HYPERTENSION

by

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DEDICATION

To my family:

my late father Mohamed Hamza

my mother Samia Hamza

my sister Deena Hamza

my husband Ramy Melhem

…and to my mentor and kindred spirit Dr. Susan Jacobs

for recognizing my potential before I had discovered it in myself.

ABSTRACT

Persistent elevation of portal venous pressure (portal hypertension- PH), is linked to chronic liver disease and invariably leads to multi-organ circulatory complications. Hallmarks of PH are renal dysfunction and a characteristic hemodynamic profile (hyperdynamic circulation), which synergistically cause the development of the fatal sequelae of PH. Despite extensive research, PH remains a serious clinical problem, with no effective treatment. In large part, this is due to lack of comprehensive knowledge regarding the initiation and early progression of renal dysfunction and the hyperdynamic circulation.

The spleen, which is actively engaged in cardiovascular regulation, is intimately connected with the portal venous system such that splenic venous pressure (SVP) is also elevated in PH. We therefore investigated the contribution of the spleen to PH-related cardiovascular dysregulation. Specifically, we employed an acute rat model to elucidate the existence of neurohormonal pathways activated in early PH.

It was known that PH-related renal dysfunction is *functional* and neurally mediated (via the hepato-renal reflex). We hypothesized that, in addition, selective elevation of splenic venous pressure (SVP) also increases renal vascular resistance and modulates renal vascular function, through reflex activation of splenic afferent and renal sympathetic nerves. Indeed, acutely elevated SVP by partial splenic vein occlusion (SVO) did increase splenic afferent nerve activity and reflexly increased renal sympathetic nerve activity (RSNA). Simultaneously, renal blood flow (RBF) and renal arterial conductance fell; this was α_1 adrenergic receptor-mediated and dependent on intact splenic and renal

nerves. Moreover, our data showed that, in the absence of increased SVP, PH did not affect RSNA or renal vascular function.

Although splanchnic vasodilation is characteristic of the hyperdynamic circulation in PH, its development is thought to be contingent upon an initial transient mesenteric vasoconstriction. Our data revealed that increased SVP reflexly activates mesenteric efferent nerves, and reduces mesenteric arterial blood flow, vascular conductance and resistance artery diameter; this was primarily mediated through angiotensin II release (spleno-renal reflex-, renal baroreceptor-, and mesenteric angiotensinergic nerve-mediated).

In conclusion, the spleen neurohormonally modulates renal and mesenteric circulations, thus contributing to the initiation of renal dysfunction and hyperdynamic circulation of PH.

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LIST OF ABBREVIATIONS

Ach	acetylcholine
ACE	angiotensin converting enzyme
ADH	anti-diuretic hormone
ANG I	angiotensin I
ANG II	angiotensin II
ANOVA	analysis of variance
ANS	Autonomic Nervous System
ATP	Adenosine Tri-Phosphate
AVP	arginine vasopressin
CG_x	celiac ganglionectomized
CGRP	Calcitonin Gene-Related Peptide
CNS	Central Nervous System
eNOS	endothelial nitric oxide synthase
GFR	glomerular filtration rate
HRS	hepato-renal syndrome
ID	inner diameter
IMV	inferior mesenteric vein
i.p	intra-peritoneal
i.v.	intra-venous
L-NMMA	N^G-monomethyl-L-arginine
LOS	Losartan-treated
LOS+SR49059	Losartan + SR49059-treated
MAP	mean arterial pressure
MD	mesenteric denervated
NA	nerve activity
NANC	Non-Adrenergic, Non-Cholinergic
NE	Norephinephrine
nNOS	neuronal nitric oxide synthase

NO	nitric oxide
NOS	nitric oxide synthase
NPY	Neuropeptide Y
NTS	nucleus of tractus solitarius
OD	outer diameter
PGI_2	prostacyclin
PH	portal hypertension
PO	phenoxybenzamine
PVL	portal vein ligation
PVP	portal venous pressure
RAAS	renin-angiotensin-aldosterone system
RBF	renal blood flow
RD	renal denervated
RSNA	renal sympathetic nerve activity
S_x	splenectomized
s.c.	subcutaneous
SD	splenic denervated
SEM	standard error of the mean
SMA	superior mesenteric artery
SMAC	superior mesenteric arterial conductance
SMAF	superior mesenteric arterial blood flow
SMV	superior mesenteric vein
SNS	sympathetic nervous system
SP	Substance P
SVL	splenic vein ligation
SVO	splenic vein occlusion
SVP	splenic venous pressure
TNFα	tumor necrosis factor alpha
TPR	Total Peripheral Resistance
VLM	ventrolateral medulla

CHAPTER 1:

INTRODUCTION

1. INTRODUCTION

The description of the circulation of the blood through the cardiovascular system by William Harvey in 1623 (55) motivated a great interest in the heart and blood vessels. Since then, there have been incredible advances in the field of cardiovascular physiology. The following study involves the investigation of aspects of cardiovascular *dysregulation* initiated by the spleen as it pertains to the portal hypertensive syndrome. However, before discussing the role of the spleen in this regulation and its emerging role in mediating the cardiovascular complications of portal hypertension, it is important to first progressively review some fundamental principles of cardiovascular regulation.

1.1 PRINCIPLES OF CARDIOVASCULAR REGULATION

1.1.1 The Cardiovascular System and Control of Vascular Tone

The mammalian cardiovascular system serves to distribute blood, oxygen and nutrients to all cells in the body, and to remove waste products. This system comprises the heart and a system of vessels supplying and draining blood from all organs and tissues. Modulation of vascular tone is an essential component of cardiovascular regulation as it is by this means that the body may control blood pressure, tissue perfusion and total body fluid homeostasis. The blood vessels are controlled by both intrinsic and extrinsic factors. Intrinsic vascular control concerns those mechanisms inherent to the blood vessels themselves, aside from any external influence; for example the myogenic response, endothelium-related reactivity and response to locally released tissue metabolites or vasoactive factors. In addition, the blood vessels are subject to extrinsic control, i.e. modulation by factors outside the vessels.

Extrinsic vascular control can be subdivided into two main systems: autonomic neural modulation and circulating hormonal control. While intrinsic mechanisms for regulation of vascular tone serve mainly local tissue needs, it is the extrinsic mechanisms of vascular control that definitively alter vascular tone to maintain cardiovascular homeostasis and serve the global needs of the organism. Aside from physiological contributions, altered extrinsic modulation of vascular tone also figures prominently in pathophysiology, such as in disorders of body fluid distribution and regulation. This investigation of the splenic contributions to complications of portal hypertension intimately relates to neurohormonal alterations in vascular tone, primarily in the renal and mesenteric vasculature. Thus a review of these key extrinsic vascular control mechanisms is vital.

1.2 AUTONOMIC NEURAL MODULATION OF VASCULAR TONE

Maintenance of blood pressure and the distribution of blood flow to different tissues of the body is mediated primarily by autonomic nervous system (ANS) control of the peripheral blood vessels. Our modern conception of the ANS was established by Gaskell in 1920 and Langley in 1921 (42). The two main branches of the ANS are the sympathetic and parasympathetic systems. With respect to regulation of vascular tone, it is the sympathetic arm of the ANS which plays the greater role.

1.2.1 Sympathetic Nervous System

The sympathetic nervous system, which originates with bulbospinal nerve fibres from the medulla oblongata of the brainstem, travels down the spinal cord to thoraco-lumbar

segments T1-L3 (thoraco-lumbar outflow). These nerve fibres then synapse with preganglionic neurons which, in turn, traverse the ventral roots of the spinal cord to the sympathetic paravertebral ganglia adjacent to the vertebral bodies. The preganglionic fibres either synapse directly with postganglionic neurons in these ganglia, or course past the sympathetic chain to eventually synapse with postganglionic neurons in the prevertebral ganglia innervating the abdominal and pelvic organs (celiac, superior/inferior mesenteric, hypogastric pelvic plexus). Axons of the sympathetic post-ganglionic neurons travel in the peripheral nerves to innervate organs and targets in the cardiovascular system. Of particular relevance to the current study is the fact that prevertebral ganglia are capable of mediating local reflexes limited to the peripheral organs and which can function independently of the CNS (85, 102). However, in the intact animal, CNS input to the prevertebral ganglia often modulates these reflexes. The concept of "vasomotor nerves" was established in the early 1850's based on independent observations of Bernard (1851) and Brown-Sequard (1852). Bernard showed that section of the cervical sympathetic nerves caused dilation of blood vessels and increased temperature of the rabbit ear (115). On the other hand, Brown-Sequard reported that direct stimulation of these nerves caused contraction of blood vessels in the face and ear of various animals; concluding that the cervical sympathetic chain sends motor nerves to the vessels of the head (115). These observations taken together demonstrated that blood vessels are controlled by nerves, which exhibit tonic resting activity (approx. 1-3 impulses/sec at rest (23)) and which generally produce vasoconstriction upon activation.

Sympathetic nerve fibres travel along the vessels initially in the outer adventitial layer (2) and often continue to penetrate right to the tunica media, the middle, mostly muscular

layer of arteries, thus providing these vessels with an extensive nerve plexus and a neural fibre network to vascular myocytes (2). Small arteries and terminal resistance arteries receive the most extensive sympathetic neural innervation. Sympathetic fibres even extend as far as innervating arteriovenous capillaries which, unlike true capillaries, are contractile (169). These vessels and intrinsic pre-capillary sphincters respond to sympathetic input with spontaneous changes in muscular tone (vasomotion) and hence regulate tissue perfusion (16, 169). Adrenergic nerve fibres in the tunica media are characterized by numerous varicosities (swellings) along their length which are in close contact with vascular smooth muscle cells (73); these comprise primarily norephinephrine-containing vesicles (156). Transmission between these varicosities and the adjacent vascular smooth muscle occurs "enpassant" as an action potential travels along the nerve axon to reach each varicosity (42). The action potential travelling along these nerves triggers the release of norepinephrine from the vesicles, which binds to α-adrenergic receptors located on the membrane of vascular myocytes. The α_1 subtype is the principal adrenergic receptor mediating blood vessel tone and generally produces vasoconstriction when activated. Although α_2 adrenergic receptors are also present post-junctionally in resistance vessels, these tend be more commonly located pre-junctionally on the sympathetic varicosities and to participate in modulation of neurotransmitter release, along with other locally released mediators (i.e. other neurotransmitters, circulating hormones, prostanoids, histamine) (28).

Sympathetic innervation of veins is neurohistologically similar to arterial innervation, including the presence of nerve fibres in the much thinner tunica media (2). However, innervation of the venous system is generally less extensive compared to the rich

sympathetic supply of the arteries. Although there is very little sympathetic innervation of skeletal muscle and conduit veins, the splanchnic venous bed is well served by the sympathetic nervous system. Indeed, sympathetic input to splanchnic veins is critical for the mobilization of blood from this area of the circulation (42).

As previously noted, sympathetic nerves innervating the vasculature are continually active, producing a resting vasoconstriction, or vascular tone. Consequently, any withdrawal of sympathetic drive to the vasculature as observed by Bernard above, will alone result in vasodilation, and is in fact the most common form of neurogenic vasodilatation (42). We now recognize that a key feature of the sympathetic nervous system is discrete localized regulation of organs and tissues. Thus, aside from severe situations (i.e. hemorrhage, fight or flight alarm response), increased sympathetic outflow does not translate into a global, whole body increase in sympathetic nerve activity. Independent modulation of sympathetic activity to different vascular beds ensures fine-tuned regulation of cardiovascular hemodynamics in various physiological and pathophysiological situations. This is possible as a result of: the non-uniform distribution of sympathetic fibres; unique reactivity of vascular smooth muscle of different vascular beds; local presence of vasodilator factors; and differing inherent levels of resting vascular tone in various tissues. This point is central to our understanding of the role of the spleen in portal hypertension, whereupon a discrete stimulus in the body can result in an equally discrete response mediated, at least in part, by the autonomic nerves.

The vascular effects of sympathetic activation are primarily vasoconstriction, which serves to reduce local blood flow and expel blood from the vascular bed, thus reducing blood volume of the organ in question. The reduction in capillary hydrostatic pressure

caused by this vasoconstriction additionally alters the Starling microvascular equilibrium and can lead to osmotic absorption of interstitial fluid into the intravascular compartment, contributing to the change in body fluid distribution and balance. In addition, sympathetic-mediated constriction of the peripheral vasculature causes a rise in total peripheral resistance (TPR) and, in turn, blood pressure. Alongside its contribution to homeostatic preservation of cardiac output and arterial pressure in hypovolemia, the sympathetic nervous system is also implicated in pathophysiological disorders such as hypertension, liver failure, obesity and congestive heart disease.

1.2.2 Parasympathetic Nervous System

The parasympathetic arm of the ANS begins with preganglionic fibres which emerge from the CNS, at cranial and sacral portions of the spinal cord (cranio-sacral outflow). Preganglionic fibres from cranial segments travel primarily in the vagus nerve, while preganglionic fibres from the sacral portion emerge in the sacral spinal nerves. These long fibres extend to parasympathetically innervated organs to synapse with short postganglionic fibres within the target tissue itself. It has been traditionally believed that there is no appreciable parasympathetic innervation of the blood vessels and that the sympathetic arm of the ANS is alone responsible for the vasomotor control of blood pressure (42). However, there is now evidence for limited parasympathetic innervation of resistance vessels, albeit not to the extent of sympathetic innervation (153). Muscarinic cholinergic receptors have been localized to many blood vessels, although there is not much evidence for direct cholinergic innervation of most vascular beds (153) with the exception of the coronary and cerebral circulations (40, 49). Parasympathetic fibres release acetylcholine (Ach), which binds to muscarinic receptors of multiple subtypes.

Binding of Ach to muscarinic M_3 receptors of vascular endothelial cells hyperpolarizes and relaxes vascular myocytes of resistance vessels in an endothelium dependent manner (48, 49). On the other hand, stimulation of muscarinic M_1, M_2 or M_3 receptors located on vascular smooth muscle cells generally results in vasoconstriction. Unlike the sympathetic nervous system, parasympathetic innervation of the vasculature does not exhibit a degree of resting tone i.e. there is no basal parasympathetic-mediated vascular tone. Rather, these nerves fire in response to increased organ function and a metabolic demand for augmented tissue blood flow.

1.2.3 Non-Adrenergic, Non-Cholinergic Innervation

Associated with the parasympathetic system are nerves which release neither norepinehrine nor acetylcholine: non-adrenergic non-cholinergic (NANC) nerves. These nerves are often sensory afferents in nature and have peripheral terminals which relay information to spinal ganglia and higher centers (4). However, these afferent nerves may also have efferent functions, which modulate vascular tone and, ultimately, cardiovascular hemodynamics. NANC nerves are described as a heterogeneous population containing different proportions of a variety of peptidergic and non-peptidergic neurotransmitters (4). Some examples of NANC transmitter substances are Substance P (SP), Calcitonin Gene-Related Peptide (CGRP), NO, ATP and Neuropeptide Y (NPY) (27). Innervation of the vasculature by NANC nerves has been demonstrated in both large and small arteries, as well as veins (90, 105). Similar to the pattern of innervation noted above for the sympathetic fibres, these nerves are located along vessels between the adventitial and medial layers, with nerves in some vessels penetrating into the tunica media. Most peripheral organs feature NANC sensory innervation, notably the

heart and rat mesenteric vasculature. NANC innervation of the vasculature elicits vasodilation or vasoconstrictive effects depending on the neurotransmitter released and the vascular bed under study. In addition, an interaction between NANC and noradrenergic neurotransmission has been observed, whereby the NANC nerves serve to modulate sympathetic modulation of vascular tone (5, 125). Of particular note are NANC nerves which primarily release NO, or nitrergic nerves (21). This nitrergic neurotransmission has been established in the vasculature of many autonomically innervated organs and participates in cardiovascular regulation by producing vasodilation or modification (even potential augmentation) of sympathetically-mediated vasoconstriction as a direct result of NO released from these nerve terminals (75, 106, 159, 164). Additionally, ANG II has also been identified as a neuropeptidergic transmitter and has been localized to neurons of sympathetic ganglia, with evidence of angiotensinergic innervation of mesenteric resistance vessels (123). In this case, ANG II is an endogenous neurotransmitter in addition to its role as a circulating hormonal neuromodulator in autonomic regulation of the vasculature. In summary, the establishment of NANC nerves and the diversity of neurotransmitters and interactions with sympathetic nerves add another layer of complexity to autonomic control of the vasculature and thus should be considered in any investigation involving vascular regulation.

1.2.4 Integrated Neural Control of Vascular Tone

Many biologic functions are controlled via negative feedback loops, where peripheral sensors continuously monitor a physiological variable (i.e. blood pressure) and are able to

transduce this information into a central signal that results in an appropriate effector response to normalize the variable.

The major pressure sensing areas in the cardiovascular system, are located in the carotid sinus and aortic arch. Upon activation by local vascular stretch in response to increased blood pressure, sensory information from these baroreceptors is carried in the sinus nerves and vagus to the brainstem vasomotor center. This input acts to inhibit the tonic activity of this center to ultimately reduce sympathetic vasoconstrictor tone in an effort to return blood pressure toward normal. Conversely, reduced blood pressure causes lower afferent discharge from the baroreceptors; reduced inhibition of the vasomotor center results in increased sympathetic outflow and increased total peripheral resistance. Once blood pressure is back to its normal set-point, baroreceptor activity will also return to normal (42). Other sensory elements also participate in the regulation of vascular tone and ultimately blood pressure. Low volume receptors are located at the junction of the vena cava and right atrium of the heart, pulmonary arteries and veins, left atrium and ventricles and in the main coronary arteries (72). Afferent fibres carrying information from these receptors travel in the vagus nerve (68).

The sympathetic nervous system is considered an efferent component of this total body control mechanism, alongside the peripheral sensors, the afferent nerve fibres and the integrative control centers in the brain. The principal central integrative center for vasoregulation is the vasomotor center of the medulla oblongata and mediates basic vasoregulatory reflexes. This center is tonically active and integrates information from cardiovascular baroreceptors, low volume receptors and chemoreceptors described above. In response to this information regarding the state of the body, sympathetic outflow is

altered to produce net peripheral vasoconstriction or vasodilation, or changes in regional tone and blood flow distribution. Sympathetic withdrawal produces vasodilation as this regulatory centre is not involved in control of vasodilatory fibres, which are instead influenced by centres in the motor cortex. It is important to note that sympathetic drive is not only influenced by input to the vasomotor center; sympathetic outflow is also modulated by input to cardiovascular regulatory centers in the hypothalamus and cortex as well as spinal reflex centers. This last factor is demonstrated by true spinal vasomotor reflexes that occur in normal physiology and are limited to a few segments of the spinal cord (71).

1.3 HORMONAL CONTROL OF VASCULAR TONE

Under physiological circumstances, neural regulation of vascular tone plays a prominent role in maintaining overall cardiovascular hemodynamic balance. Circulating hormones have a profound influence on vasomotor function, as is most evident during pathological events such as dehydration, hemorrhage or impairment of neural control mechanisms. However, circulating hormones may participate in physiological modulation of the vasculature, albeit at much lower plasma concentrations than observed in pathophysiology. In fact, alongside autonomic regulation, hormones provide additional fine-tuning of vascular tone which is often tissue specific. Three major hormonal mediators of vascular tone are epinephrine/norepinephrine, vasopressin (AVP) and ANG II.

1.3.1 Catecholamines

The catecholamines epinephrine and norepinephrine (NE) are secreted from the medulla of the adrenal glands, located on the upper end of each kidney, although most circulating NE is released from synaptic nerve clefts (31). Adrenal glands are innervated by preganglionic sympathetic fibres, coursing in the splanchnic nerve bundle. Increased sympathetic nerve activity results in increased catecholamine secretion from these glands. Typically secretion of these catecholamines is increased during exercise and in response to hypotension; they are also intimately involved in the fight-flight alarm response in the face of a massive increase in sympathetic outflow. While the metabolic functions of these catecholamines are most prominent (i.e. hepatic stimulation of glycogenolysis and glucose release), there are several important cardiovascular effects. Binding of epinephrine or NE to cardiac β_1 adrenergic receptors results in increased contractility and heart rate (31). There are also varied actions on the vasculature. Whereas epinephrine acts at vascular β_2 receptors in skeletal muscle to cause vasodilation, the more common response to epinephrine and NE in the rest of the vasculature is α_1 adrenergic receptor-mediated vasoconstriction (11, 31).

1.3.2 Vasopressin

Following its discovery in 1895 and its isolation in 1952 (121), AVP has been established as a significant regulator of body fluid balance, blood pressure and body temperature among several other functions. In its role in cardiovascular regulation, AVP is a critical mediator of vascular tone. AVP (also referred to as anti-diuretic hormone – ADH) is a peptide hormone of nine amino acids. It is synthesized as a larger prohormone

by the magnocellular neurons of the paraventricular and supraoptic nuclei located in the hypothalamus, cleaved to form the active nonapeptide, and transported along magnocellular neurons in the supraoptic-hypophyseal tract to the posterior lobe of the pituitary gland (155). The principle stimuli for release of AVP into the circulation are hypertonic conditions (15), hypotension and hypovolemia (139).

Increased blood osmolarity is detected by osmoreceptors in the hypothalamus, which then initiate stimulus-dependent increases in AVP synthesis and secretion (137). The AVP system is not as sensitive to reductions in arterial pressure as it is to changes in osmolarity. On the other hand, changes of arterial pressure in excess of 10% do result in larger increases in AVP release than are observed with osmolar stimuli (155). Just as baroreceptors in the aortic arch and carotid sinus respond to changes in blood pressure with decreased afferent signaling, mechanoreceptors in the left atrium and ventricle also respond to changes in blood volume and initiate increased AVP release. However, the balance between atrial receptor and arterial receptor-mediated AVP release varies among different species. In the dog (60) and sheep (88) reduction in blood volume in the absence of any change in arterial blood pressure increased plasma vasopressin concentration and reduced afferent impulses from atrial receptors (60, 88). In humans, reductions in central blood volume can increase vasopressin release in the absence of changes in arterial baroreceptor activity (139). However, in the rat, results are variable; arterial baroreceptors play a large role in AVP release, such that there is little or no increase in plasma AVP concentration unless there is an accompanying fall in mean arterial blood pressure (24, 26) (38). Although large reductions in blood pressure and volume are required to initiate massive release of AVP into the circulation, this does not detract from

the physiological contributions of lower levels of AVP to cardiovascular regulation via direct effects on the vasculature (6).

The actions of AVP are dependent on its actions with specific receptors. Several receptors have been identified that mediate the physiological effects of AVP, the main specific AVP receptors being V_1, V_2 and V_3 (74). Of these, the V_1 receptor subtype (specifically V_{1a} subtype) is located on vascular smooth muscle and mediates the vascular effects of AVP; the V_2 receptor mediates water retention at the kidney collecting ducts, while the V_3 receptor is located in the pituitary gland and mediates CNS actions of AVP, namely corticotropin secretion. The V_{1a} receptor is located in high density on vascular smooth muscle and generally results in vasoconstriction upon activation (29). This is also seen in the renal vasculature, where activation of V_{1a} receptors results in a reduction of blood flow to the inner medulla and selectively constricts efferent arterioles, there being no effect on the afferent arterioles (110). In fact, AVP causes vascoconstriction in most vascular beds, with the exception of cerebral and coronary vessels (139).

1.3.3 The Renin-Angiotensin-Aldosterone System

Although AVP is a very potent vasoactive hormone, the most critical hormonal contribution to moment-to-moment modulation of vascular tone is mediated by ANG II, originating from the renin-angiotensin-aldosterone system (RAAS).

The RAAS is the most powerful hormonal system for regulation of whole body sodium balance, body fluid as well as arterial pressure (61). Renin, discovered in 1898 (78), is a proteolytic enzyme and is the primary, rate limiting component of the RAAS (143). Renin is produced by juxtaglomerular granular cells present in afferent arteriolar walls of

the kidney and is secreted in response to several stimuli: a reduction in renal perfusion pressure (detected by intrarenal baroceptors of the afferent arteriolar vasculature) (168), a reduction in sodium chloride flowing past the macula densa of the renal tubule (tubuloglomerular feedback mechanism), and/or increased sympathetic outflow. In this last case, a reflex increase in RSNA, (induced, for example, by a reduction in blood pressure) stimulates renin release via activation of β_1 adrenoceptors located on juxtaglomerular cells. Generally these three mechanisms act together to synergistically coordinate renin release (145).

Once renin is released into the circulation, it acts on its substrate angiotensinogen. This α_2 globulin (plasma globular protein) is produced and released constitutively into the circulation by the liver. Renin acts on angiotensinogen to cleave off a decapeptide, angiotensin I (ANG I), which is subsequently cleaved to the active octapeptide ANG II by the angiotensin converting enzyme (ACE); this enzyme is located on the endothelial cell surface. Formation of ANG II is relatively rapid, and mostly occurs in a single pass of blood through the lungs, which is the first major area of endothelium encountered by circulating ANG I (143).

Subsequent to its definitive isolation in the early 1940's (20, 122), it has been established that circulating ANG II has many actions and together these ultimately act to preserve arterial pressure and salt and water balance (25). The principal actions of normal physiological levels of ANG II are vasoconstriction of vascular smooth muscle (directly or via interactions with the sympathetic nervous system); stimulation of aldosterone release from the adrenal cortex; and modulation of water and salt excretion through direct renal effects (34). Thus, in addition to supporting arterial pressure by facilitating sodium

and water reabsorption, ANG II directly and rapidly supports blood pressure by direct vascular effects. These multiple actions are manifested through specific intracellular signaling pathways that are activated upon binding of ANG II to its cell-surface receptors in various target tissues. The two identified ANG II receptors are defined as AT_1 and AT_2 (148). The classically described actions of ANG II are mediated by the AT_1 receptor subtype. While AT_1 has been established as the mediator of the cardiovascular effects of ANG II, the actions of the AT_2 receptor subtype have not been conclusively characterized. As substantially less is known about the AT_2 receptor subtype, its role in physiology and pathophysiological processes is largely unresolved. The AT_2 receptor is ubiquitously expressed in embryonic, fetal and neonatal tissues (124); it is only expressed in the adrenal medulla, uterus, ovary, vascular endothelium and a few brain regions of the adult. Much evidence supports a vasodilatory role for the AT_2 receptor in isolated arteries and effects which generally oppose the actions of AT_1 receptors in vivo (160). However, studies in mice with targeted deletion or over-expression of the AT_2 receptor have yielded varied results (124).

In addition to direct action of circulating ANG II on vascular AT_1 receptors causing vasoconstriction, ANG II also binds to AT_1 receptors on sympathetic ganglia and sympathetic nerve varicosities to increase NE release (neuromodulation) (127). This increased sympathetic vasoconstriction is augmented further by central actions of ANG II in the brainstem to increase sympathetic outflow (127). Thus ANG II plays a major role in control of vasomotor tone to maintain cardiovascular homeostasis and has long been implicated in several pathological disorders including hypertension.

1.4 THE SPLANCHNIC CIRCULATION

1.4.1 The Hepatic Portal Venous System

A portal venous system is defined as two capillary beds connected together via a system of veins, such that one drains into the next. The hepatic portal venous system was first described by Vesalius to be composed of the hepatic portal vein and all venous branches which drain into this main vessel (57, 133)(Figure 1.1). Blood from most of the splanchnic organs drains into the portal venous system; this includes the mesenteric, splenic and pancreatic circulations. A key distinction between the terms "splanchnic" and "mesenteric" must be made at this point: "splanchnic" refers to the entire abdominal portion of the digestive system (liver, spleen, pancreas, and intestines), whereas "mesenteric" refers specifically to the intestinal tract.

1.4.2 Mesenteric Circulation

The mesenteric circulation is a key component of the portal venous system and of the cardiovascular system as a whole. In addition to its vital role in maintaining cardiovascular homeostasis, this vascular bed is also recognized as a principal mediator of multiple organ dysfunction associated with disorders of fluid regulation and distribution (e.g. shock, heart failure, portal hypertension) (64). The mesentery is supplied by the superior mesenteric artery (SMA), which branches directly from the abdominal aorta and is, in fact, its largest branch. The blood supply to the mesentery is immense, with over 12% of cardiac output delivered through the SMA at rest (83) and ~30% of total blood volume held mainly in the venous capacitance vessels (41). Blood from the SMA supplies the entire small intestine, proximal colon and a portion of the pancreas. The inferior mesenteric artery supplies the distal colon and rectum (57). Once

blood has been distributed through the SMA to the mesentery and passes through the mesenteric microcirculation, it accumulates in the mesenteric venules. These venules converge to drain blood first into the inferior and superior mesenteric veins. The inferior mesenteric vein (IMV) drains into the superior mesenteric vein (SMV), which subsequently merges with the splenic vein to form the great hepatic portal vein. Blood from the portal vein flows directly to the liver and provides above 75% of its supply. Blood passes through the hepatic sinusoids (specialized vasculature of the liver) to eventually drain into the hepatic vein, which then empties into the inferior vena cava.

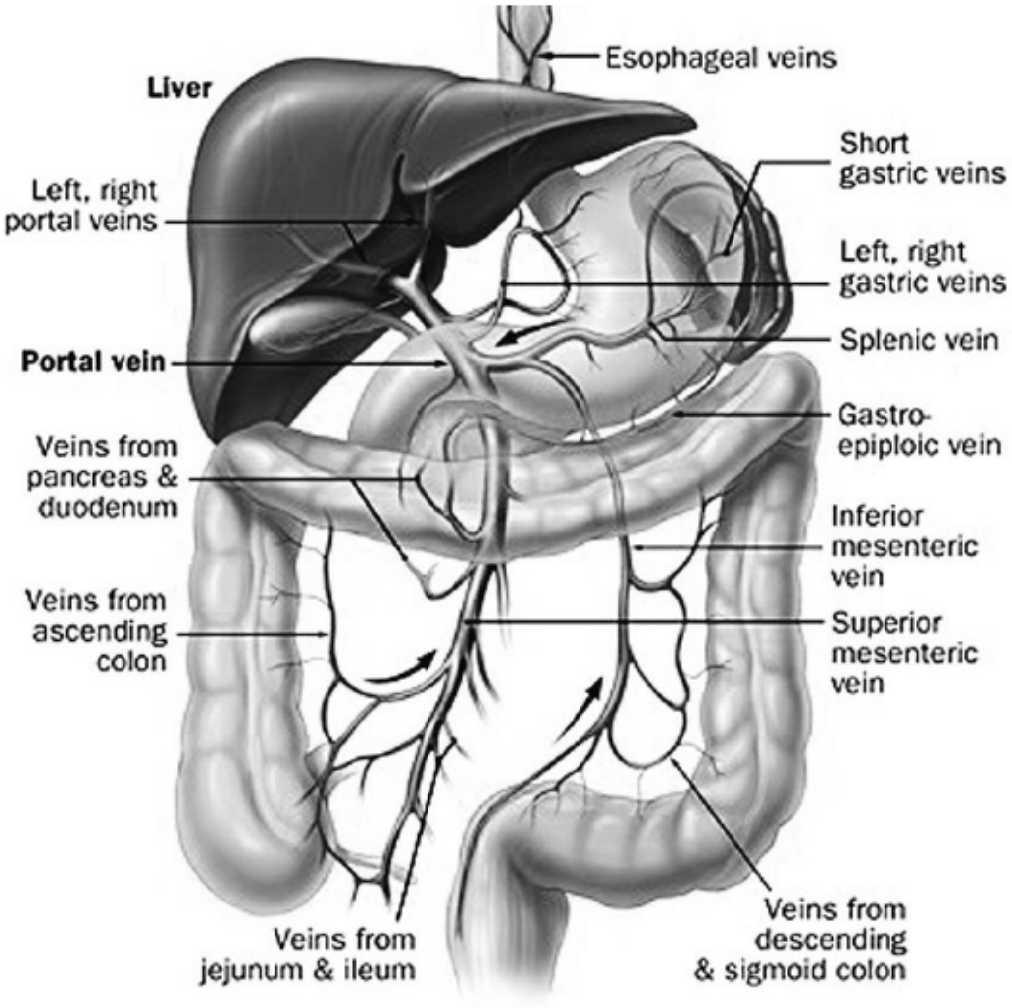

Figure 1.1 Schematic representation of the hepatic portal venous system. Illustration adapted from Cichoz-Lach et al, 2008 (32).

1.4.3 Mesenteric Microcirculation

Three major vascular components form the mesenteric microcirculation: small arteries and arterioles; capillaries; venules (Figure 1.2). With the exception of true capillaries, mesenteric vessels are included amongst the most reactive vessels in the body (130). The small arteries (>25 μm) and microscopic arterioles feature thick walls composed of vascular smooth muscle. These vessels form the largest section of resistance to blood flow across the complete mesenteric circulation, deeming them mesenteric "resistance vessels" (this term includes aforementioned vasoactive arteriovenous capillaries and pre-capillary sphincters). In general, were a drug or stimulus to produce a large drop in mesenteric blood flow, it would have to act on the walls of these resistance vessels. Blood flowing through the resistance vessels next encounters the true capillaries, which are not vasoactive and are the principal site for nutrient and oxygen exchange in the vascular bed – the "exchange vessels".

Microscopic veins (>40μm) which drain blood from the capillaries form the third critical component of the mesenteric microcirculation. Although these vessels (40 μm – 0.1mm diameter) have much thinner walls than the mesenteric resistance arteries, they also contain a significant portion of vascular smooth muscle and exhibit reactivity to extrinsic stimulation (131). In fact, contraction of these venules is a critical function of the mesenteric contribution to the maintenance of end diastolic volume, cardiac output and total peripheral resistance (158). Upon constriction of the mesenteric venules, blood stored in these vessels is forced back toward the heart. The ability to temporarily store blood in these vessels upon relaxation has led to their characterization as "capacitance vessels".

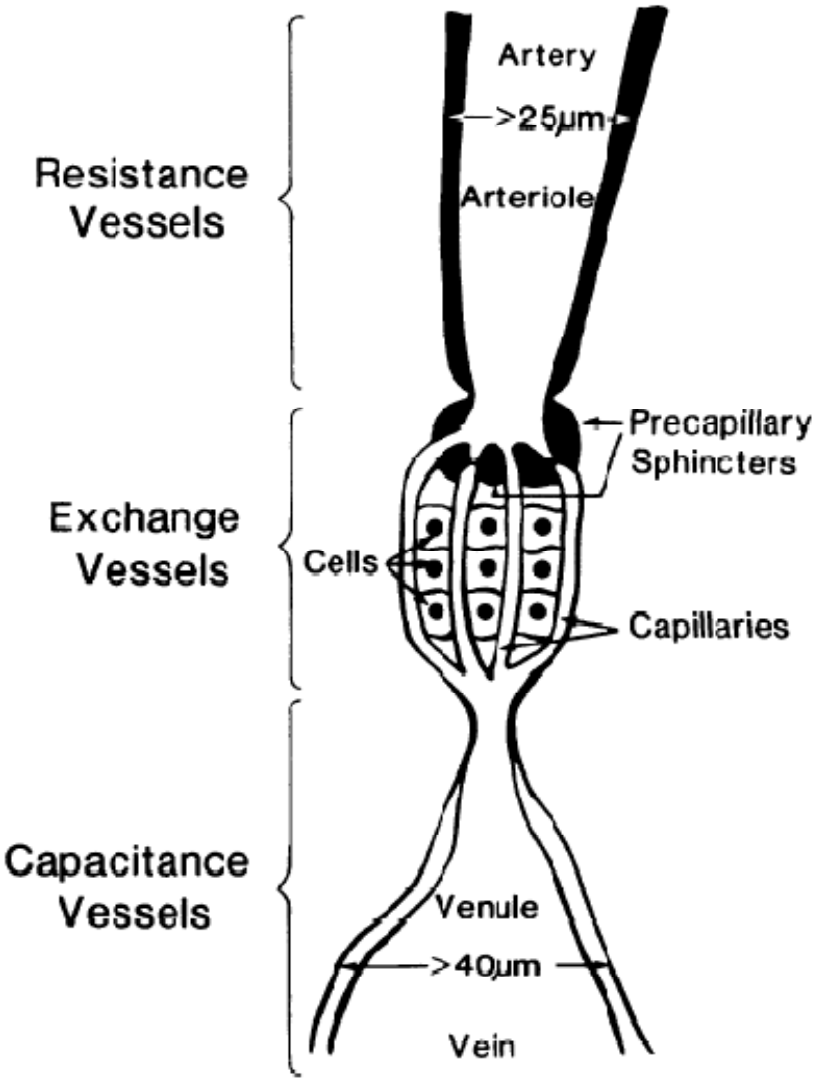

Figure 1.2 Schematic representation of the mesenteric microcirculation. Arterial vessels <25 μm in diameter are defined as arterioles. Venous vessels <20 μm in diameter are designated venules. Illustration adapted from Jacobson (1982) (83).

Control of mesenteric blood flow falls to the mesenteric arterioles and venules described above and follow much the same pattern as control of the vasculature elsewhere in the body. Mesenteric microvessels feature intrinsic regulation through local release of metabolic factors and myogenic mechanisms. Germane to the present work is extrinsic neural and hormonal regulation of mesenteric tone and, in turn, blood flow. Sympathetic drive to the mesenteric circulation originates with sympathetic preganglionic input to the celiac ganglion, forming synapses with postganglionic fibres which innervate the mesentery via splanchnic nerves. Once these fibres leave the celiac ganglion, they course along the SMA and continue to distribute along all subsequent branches of this vessel with nerves terminating on the vascular smooth muscle of the small arteries, arterioles, venules and microscopic veins. This dense sympathetic innervation of all vasoactively functional segments of the mesentery, *including* the venules and small veins (85), is a vital and critical consideration in any investigation probing neural regulation of this circulation. These nerves primarily release NE (8), which binds to abundantly present α-adrenergic receptors (95) to produce mesenteric vasoconstriction, a transient reduction in blood flow (11) and mobilization of blood from the capacitance vessels. Conversely, stimulation of β adrenergic receptors by epinephrine in this vascular bed increases splanchnic perfusion (19). As in other vascular beds, it is the relative proportions of epinephrine and NE as well as the proportion and distribution of adrenergic receptor subtypes which determines the net response of the mesenteric circulation. Ultimately, sympathetic neural outflow is critical for control of mesenteric blood flow (87). However, we now understand that the mesenteric innervation in particular is diverse in nerve type (i.e. NANC) and includes a plethora of substances released as neurotransmitters as

discussed earlier. This point alone must also be taken into account concerning mesenteric neural control. Although parasympathetic fibres from the vagus nerve do innervate the intestine, these fibres do not appear to greatly modulate mesenteric vascular tone (54). Rather, parasympathetic drive to this region appears to regulate visceral muscle and secretion, a role beyond the scope of this study.

In addition to sympathetic and parasympathetic innervation, the mesentery also features extensive innervation by the enteric nervous system. The enteric nervous system is considered part of the ANS and is extensive of itself, featuring over 100 million neurons (37) that are involved in the regulation of basic gastro-intestinal functions. The enteric system comprises: the myenteric plexus, which is primarily involved in gut motility, and the submucosal plexus, which controls secretion and reabsorptive functions (85). These functions of the enteric nervous system are integrated to whole body homoeostasis by CNS input through the sympathetic and parasympathetic nerves (85). Generally, the enteric nervous system is not involved in the regulation of the mesenteric vasculature, which is essentially under the influence of the sympathetic and NANC nerves.

Hormonal vascular control plays a major role in controlling the mesenteric microcirculation. Hormonal factors, including catecholamines, AVP and ANG II, generally produce an increase in mesenteric vascular resistance and a reduction in blood flow. Activation of release of these hormonal mediators was discussed in section 1.3. The mesenteric circulation is also exposed to blood-borne gastro-intestinal hormones released under physiological conditions of digestion and absorption, although, normally, these mediators do not affect the resistance vessels. On the other hand, several of these

mediators have been implicated in the splanchnic hemodynamic complications of portal hypertension; however they are beyond the scope of this study.

1.5 THE SPLEEN

Historically, the spleen has been viewed as an important organ for blood filtration and immunologic functions. As it is not critical for survival, the significance of the spleen in cardiovascular regulation has been largely overlooked. However, over the past several years, the role of the spleen as a key regulator of body fluid homeostasis under both normal and pathological conditions has been examined. We now know that the spleen has a significant role in mediating cardiovascular hemodynamics, and has been extensively reviewed (62).

1.5.1 Structure & Function of The Spleen

The notion of the spleen acting as a regulator of intravascular volume is not new. Stukely, in 1722, first suggested that the spleen was a "diverticulum of the systemic circulation, filling and emptying with blood and acting as a controller of blood volume"(147). This view was supported by several other investigators, notably Gray who concluded in 1854 that the function of the spleen "is to regulate the quantity and quality of blood"(56). These descriptions suggest that in addition to its hematological and immunologic functions, the spleen is a blood reservoir which can contract to expel significant volumes of blood when needed. Although this is an established phenomenon in some species such as the cat and horse, it is important to note that there is a great deal of interspecies variability in splenic structure and function. Thus, humans have only a minimal capacity to store and expel blood(147). And in the rat, physiological storage and expulsion of

significant amounts of blood does not occur at all, as evidenced by the virtual lack of smooth muscle in the connective tissue capsule (128). The rat is thus an optimal model for investigating the contribution of intrasplenic filtration to fluid volume and blood pressure homeostasis.

The splenic microcirculation is rather complex. The splenic artery branches to enter the hilum of the spleen. Inside the body of the spleen, arteries branch further to pass along the trabeculae (fibrous structure of the spleen) and, eventually, the white and red splenic pulp ("slow pathway"). Vascular arterio-venous shunts are present; these allow blood to bypass the red pulp ("fast pathway") and are under neurohormonal control. Venous drainage occurs in the red pulp where small venules converge, pass along the trabeculae, and ultimately emerge at the hilum. These hilar veins then converge to form the splenic vein.

The spleen is also richly innervated. Vasomotor sympathetic nerves, originating from the celiac plexus, form the splenic neural plexus which follows along the splenic vessels as they branch into the body of the spleen (151). These nerve fibres have been observed to extend between the vascular muscle cells in the tunica media of splenic arterioles (2). The venous side of the splenic circulation also exhibits extensive innervation, with nerve fibres travelling along the trabecular veins in the adventitial layer (33, 70); rootlets of these nerve fibres have also been observed to penetrate into the tunica media of these splenic veins (70). Although afferent innervation of the spleen has been questioned (117), afferent fibres have been demonstrated in the splenic nerve and are observed to enter the spinal cord at the 5^{th}-8^{th} thoracic segments via left dorsal nerve roots (33). Similarly, although parasympathetic innervation of the spleen has not been unequivocally

determined, vagal nerve fibres have been identified entering the spleen. However, they are thought to be mainly sensory in nature.

Owing to its extensive innervation, the spleen has been described as a reflexogenic area. Stimulation of splenic afferent fibres has been shown to elicit visceromotor reflexes – namely the contraction of abdominal muscles (33). Electrical stimulation of splenic afferent fibres has also been shown to produce viscero-visceral reflexes, with increases in splenic afferent nerve activity subsequently causing reflex increases in cardiopulmonary and renal sympathetic nerve activity (69). This increase in efferent nerve activity has been shown to augment ventricular contractile force, heart rate and blood pressure (69). These splenic viscero-visceral reflexes are spinal in nature (33, 69) with activity of splenic nerve fibres shown to persist after high cervical spinal cord transection (111). Thus supraspinal neural pathways are not vital for these reflexes to occur. Splenic afferent nerve activity has also increases in response to elevated splenic venous pressure, which is thought to be mediated by baroreceptors demonstrated in the muscular wall of the splenic vein (69, 70).

An important aspect of the splenic anatomy is the presence of a well developed lymphatic system. Indeed, this is one of the most important characteristics of splenic structure, aside from its extensive autonomic innervation, which allows the organ to participate in cardiovascular regulation. Several lymphatic collecting ducts converge to form the splenic lymphatic duct, which drains lymph from the spleen into the intestinal lymphatic duct. The existence of this pathway has been directly demonstrated in the rat by injecting methylene blue dye into the splenic artery and observing its passage into the splenic duct (89). After occlusion, the duct becomes visibly turgid, thus demonstrating the significant

pressure driving lymphatic drainage from the spleen i.e. the translocation of fluid from the intravascular to the extravascular compartment is an active process. It is no coincidence that the spleen, like the kidney, receives a significant portion of cardiac output (30, 44, 165) . The spleen, which comprises an integral part of the splanchnic vasculature, receives about 10% of cardiac output (8mL/min) in the conscious rat (30, 101), which stands it in an optimal position to regulate intravascular volume.

1.5.2 Physiological Regulation of Arterial Pressure by the Spleen

In addition to regulating intravascular volume (and indirectly blood pressure) through intrasplenic fluid extravasation, the spleen also participates directly in the control of arterial pressure via reflex modulation of renal function. During selective infusion of very low doses of the NOS- inhibitor L-NMMA into the splenic artery, an increase in mean arterial pressure is observed. In contrast, a similar infusion of an NO-donor reduced systemic blood pressure. These effects are spleen specific, since systemic infusion of either drug at these doses causes no such change in blood pressure. Interestingly, the pressor response to intrasplenic NO inhibition can be abolished by both splenic and renal denervation, or by blocking the renin-angiotensin system (39). Under normal physiological conditions, a decrease in systemic blood pressure would be accompanied by parallel falls in splenic blood flow. This would reduce flow-induced NO biosynthesis, as simulated with the infusion of a NOS-inhibitor. It is then proposed that this reduction in endothelial NO then triggers increased splenic afferent nerve activity; although the specific mechanism by which this would occur is yet to be defined, there is evidence that NO may suppress neurotransmitter release and thus nerve activity. Thus, it has been shown that, in the canine ileum and guinea pig myenteric plexus, NOS inhibition

increases acetylcholine release from enteric neurons (77, 93). Conversely, physiological release of NO has been shown to reduce sympathetic and parasympathetic excitability in the brainstem, while in the heart, NO inhibits the release of noradrenaline from sympathetic nerves (167). NO released from non-adrenergic, non-cholinergic (NANC) nerves also mediate the inhibition of sensory nerves in the rat mesentery (166). As such, it is conceivable that a reduction of NO in the splenic vasculature could attenuate the inhibition of splenic sensory nerves, resulting in an increase in splenic afferent nerve activity. Such an increase in splenic afferent nerve activity induces a reflex increase in renal efferent nerve activity – the splenorenal neural reflex (63). Splenorenal reflex mediated-stimulation of renin release and activation of the renin-angiotensin cascade could then potentially lead to increased generation of angiotensin II and, ultimately, an increase in arterial pressure. Furthermore, we have established that such a reflex increase in renal sympathetic nerve activity also results in increased renal arteriolar resistance; the ensuing reduction in renal blood flow would further stimulate renin release and angiotensin II production (63). Thus, reflexes arising from the spleen in response to a reduction in blood pressure act via renal sympathetic nerves and the renal microvasculature to maintain a normal blood pressure. While this reflex beneficially participates in physiological regulation of blood pressure, it may be an initiating factor in the development of cardiovascular dysregulation in conditions such as septic shock and as will be presented, portal hypertension.

1.6 PORTAL HYPERTENSION

Although the term “portal hypertension” was introduced in 1902, investigation of the portal venous system and of portal hypertension-related deaths was recorded as early as

1543 by Vesalius (133). Portal Hypertension (PH) is defined as pressure in the portal vein exceeding 10-12mmHg and is most commonly, but not exclusively, associated with chronic liver diseases such as alcoholic cirrhosis, viral hepatitis and schistosomiasis parasitic infection. Although liver disease and its associated complications have been studied over several centuries and despite great advances in our knowledge of the syndrome over time, PH has nevertheless remained an enigma. That PH and its complications are a high mortality clinical problem, with no FDA approved treatments in 2009 (138), demands the continued experimental investigation of this syndrome.

1.6.1 Etiology of Portal Hypertension

Any hindrance to portal venous blood flow will result in elevated portal venous pressure (normal 5-6mmHg), which is deemed PH if this value exceeds 10-12mmHg (66). A prehepatic blockage of the portal vein itself, such as a portal vein thrombosis, will elevate portal venous pressure, outside of any influence from the pathological changes in the liver tissue (52). Portal venous blood flow will be impeded and portal venous pressure also increased by an obstruction in the hepatic vein draining the liver; this is known as a post-hepatic blockage. In contrast to PH of pre-hepatic origin, obstruction of hepatic venous outflow, even if originating in the extrahepatic vessels as far from the liver as the inferior vena cava or right atrium, often results in hepatic tissue damage and liver failure in addition to PH (152). An intrahepatic blockage to portal venous blood flow is related to disease within the hepatic tissue itself and involves an initial increase in intrahepatic microvascular resistance. The microvasculature of the liver is composed of: portal venules, sinusoids (which are similar to other peripheral capillary beds) and hepatic venules (47). Normally, the hepatic vascular bed exhibits relatively low vascular

resistance. Essentially, destruction or fibrosis of the portal/hepatic venules and sinusoids modifies the hepatic circulation such that intrahepatic vascular resistance is increased. PH as a result of this destruction of the hepatic microvascular architecture is most commonly attributed to liver cirrhosis; cirrhosis is not only alcoholic in origin, but includes cirrhosis due to hepatitis B or C viral infections and schistosomiasis parasitic infection. More recently, non-alcoholic fatty liver disease has been added to the list of intrahepatic causes of PH (126).

1.6.2 Complications of Portal Hypertension

The manifestations of PH are widespread and certainly not limited to the liver. In fact, PH is now considered a multi-organ disease due to the diversity of clinical problems arising from elevated portal venous pressure and reduced portal venous blood flow. A quick listing of these clinical complications include: potentially fatal gastrointestinal bleeding; ascites, or an accumulation of fluid in the abdomen; hepatic encephalopathy, confusion and forgetfulness related to poor liver function; splenomegaly; renal dysfunction; hemodynamic disturbances (hyperdynamic circulation) (103). The development of renal dysfunction and the hyperdynamic circulation are critical in the progression of PH and are inextricably linked to the development of all other listed complications. Thus, these two complications of PH will be a major focus of the ensuing discussion and experimental investigation.

1.6.3 Renal Dysfunction in Portal Hypertension – The Hepato-Renal Syndrome

The earliest and most commonly observed alteration in renal function in cirrhosis is sodium retention (53). This increased sodium retention has been shown to occur before

the development of water retention and renal failure; it is also established as a key factor in the pathological formation of ascites. Following increased sodium retention, there is usually a decreased ability of the kidneys to excrete water (140). Gradually, this contributes to the increased total body water characteristic of PH and may progress to cause dilutional hyponatremia, increasing patient morbidity and mortality. Renal vasoconstriction is also a classic presentation in PH and tends to be more acute in the renal cortex (53). This vasoconstriction leads to reduced renal blood flow (RBF) and glomerular filtration rate (GFR). In severe cases, this can ultimately precipitate renal failure.

The deterioration of renal function has long been noted in conjunction with liver disease. Flint, in 1863, was perhaps the first to propose that there was some sort of connection between the liver and the kidney based on his observations that people who have succumbed to overt liver disease almost never showed any abnormalities of the kidneys upon autopsy (45). Frerichs subsequently documented oliguria, or severely decreased urine production, in patients with diseases of the liver (46). However, it wasn't until decades later that Hecker and Sherlock showed the presence of normal kidneys of hepatitis and cirrhosis patients who exhibited extreme oliguria before death (65) and revived interest in the link between the liver and the kidney in the setting of liver disease.

One of the most interesting and telling investigations into this link was the transplantation of kidneys from deceased liver disease patients into subjects with normally functioning livers completed by Koppel et al in 1969 (96). It was striking that these kidneys, which appeared to be in end-stage renal failure in the donor subjects, functioned normally in the absence of liver disease, and added tangible evidence that renal failure in liver disease

was *functional* in nature. The reversal of apparent renal failure in cirrhosis patients upon receipt of an orthotopic liver transplant by Iwatsuki et al in 1973 solidified this notion of functional renal failure (82) and raised curiosity about the mechanistic and physiologic basis of a hepato-renal link. At the time of these landmark transplantation studies, the presence of renal dysfunction of worsening degrees in acute or chronic liver disease became established as the "hepato-renal" syndrome (162), the severity of which soon became associated with poor prognosis and almost certain death. The "hepatorenal syndrome" is a condition which is characterized by intense renal vasoconstriction and impaired renal function in the setting of chronic liver disease with advanced hepatic failure including portal hypertension (10). Clinically, the diagnosis of hepatorenal syndrome is founded on exclusion of other causes of renal failure (59) and presents as a profound reduction of urine output in patients with severe liver failure (132). Chronic or acute liver disease must be present and is one of the major diagnostic criteria for this syndrome (10).

Although portal hypertension is virtually always present in the hepatorenal syndrome and is included amongst the major diagnostic criteria, elevated portal venous pressure alone (as in prehepatic portal hypertension with preserved liver function) does not give rise to the classic definition of hepatorenal syndrome. It must be noted, however, that portal hypertension alone is associated with renal vasoconstriction and impairment of renal function (107).

1.6.4 The Hepato-Renal Reflex

Early investigation into the mechanisms behind the hepato-renal syndrome (HRS) indicated that the connection between liver dysfunction and altered renal function was mediated, at least in part, by the sympathetic nervous system. Epstein et al (1970) noted renal hemodynamic instability in human cirrhosis patients, which suggested that, reduced renal perfusion in these patients was secondary to active renal vasoconstriction, rather than a stable mechanical obstruction. Further observation of these patients with angiography showed significant tortuosity and beading of renal interlobar and arcuate arteries, reflecting active vasoconstriction of the renal vessels. This hypothesis was further supported by the disappearance of apparent renal vascular constriction postmortem (43). Direct studies in dogs revealed that increases in portal venous pressure resulted in renal antidiuresis, an effect which was not altered by vagotomy or by pharmacological blockage of the sympathetic chain, but which was abolished by application of topical anesthetic to the renal sympathetic nerves (107). This last result was strongly suggestive of a visceral reflex, involving prevertebral ganglia, excluding influences dependent on vagal afferent nerves and the sympathetic paravertebral ganglia.

Following these studies, it was thought that increased portal and hepatic venous pressure was the initiating stimulus for this neutrally mediated antidiuresis. Increasing portal venous perfusion pressure was observed to increase hepatic afferent nerve activity and provided evidence for the existence of hepatic pressoreceptors sensitive to changes in portal venous pressure (119). The possibility of neural reflexes arising from the liver is supported by the extensive innervation of the hepatic vasculature, with the hepatic nerve plexus receiving fibres from the celiac plexus, vagi and phrenic nerves. These nerve

bundles have been located to the intrahepatic branches of the portal vein, with adrenergic nerve fibres extending along the hepatic vascular tree (58). Indirect elevation of portal venous pressure by inferior vena cava occlusion was subsequently shown to stimulate hepatic baroreceptors (7, 135), which again resulted in increases in hepatic afferent nerve discharge (97). Interestingly, reflex increases in renal and cardiopulmonary sympathetic efferent nerve activity were simultaneously recorded; hepatic denervation abolished the increase in renal efferent nerve activity, and thus established the hepato-renal neural reflex.

These early findings were supported by successive studies showing that direct elevation of portal venous pressure by portal vein distension results in reductions in blood pressure, matched with reflex increases in renal vascular resistance and renal sympathetic nerve activity (99, 100). In line with Liang's observations above (107), this reflex increase in renal vascular resistance and nerve activity is sympathetically mediated and additionally relies on intact hepatic nerves. This hepato-renal reflex could function in the absence of carotid sinus nerves and vagal afferents, however appeared to be augmented by both neural afferent systems in the intact animal (99, 100). These studies also importantly excluded the reliance of this reflex increase in renal nerve activity and changes in renal vascular resistance on any reduction of blood pressure as a result of elevated portal venous pressure. Sino-aortic denervation or cervical vagotomy, which *augmented* the reduction of blood pressure during increased portal pressure, did not correspondingly augment the reflex increase in renal nerve activity and renal vascular resistance, but in fact, reduced it compared to control (99, 100).

That liver disease is characterized by increased sympathetic outflow and, specifically, increased renal sympathetic nerve activity with associated sodium and water retention related to the activation of hepatic afferent nerves corroborates the contribution of the hepatorenal reflex to complications of PH (94, 129).

More recently, focus has shifted away from increased portal venous pressure as the trigger for initiating the hepato-renal reflex. Based on observations by Ming et al (2001) that intrahepatic blood flow is substantially decreased in cirrhosis and that all previous experimental elevations of portal venous pressure invariably also reduced portal venous flow, reduced intrahepatic blood flow was proposed as the critical initiating factor of the hepato-renal reflex (114). This initiating factor makes sense, as not all forms of PH involve increased intrahepatic pressure and stimulation of hepatic baroreceptors (i.e. pre-hepatic PH) (120), however these forms of PH all show reduced intrahepatic blood flow and characteristic renal dysfunction. Increased portal venous pressure as the initiator of the hepato-renal reflex implies a positive feedback mechanism whereby increased renal sodium and water retention leads to further increases in portal pressure and continual activation of hepatic afferent nerve mediated increases in renal sodium and water retention (114). However, decreased intrahepatic blood flow as the trigger for the hepato-renal reflex represents a negative feedback mechanism and makes teleological sense for the physiological regulation of renal function by this reflex. Ming et al (2001) subsequently determined that intraportal administration of adenosine, a flow-related bioactive substance known to activate sensory nerves (154), inhibited renal sodium and water excretion and that this was mediated via the hepato-renal reflex (114). Similarly, direct reduction of portal venous blood flow was later shown to initiate the hepato-renal

reflex via activation of intrahepatic adenosine A_1 receptors (112, 113). Thus, reduced intrahepatic blood flow as opposed to increased intrahepatic pressure appears to mediate hepato-renal reflex mediated renal dysfunction characteristic of PH.

1.6.5 Hemodynamic Complications of Portal Hypertension – The Hyperdynamic Circulation

Regardless of the etiology, as PH progresses (17), a characteristic circulatory picture first described in 1953 and termed the "hyperdynamic circulation" develops (98). In general, the hallmarks of the hyperdynamic circulation are: increased cardiac output, heart rate and total plasma volume; decreased arterial blood pressure (relative arterial underfilling) and systemic vascular resistance.

There have been a few theories proposed to explain this hemodynamic situation. The first, proposed in the early 1960's (12), was termed the "underfilling" theory and suggested that PH initially caused the formation of ascites. As fluid (containing sodium and water) leaves the intravascular space to accumulate in the abdomen, the subsequent decrease in intravascular volume results in the characteristic hypovolemia of PH and stimulates increased renal sodium and water retention. In principle, this cycle would persist as long as the instigating formation of ascites in response to PH continued. However, this explanation was weakened because patients with PH and ascites, in fact have *increased*, not decreased total intravascular blood volume (108). The second "overflow" hypothesis was then proposed (108) which, by contrast, suggested that ascites resulted from primary renal sodium and water retention, which was triggered not by a

reduction in intravascular blood volume, but rather, by some mechanism overriding the normal body fluid regulation systems (i.e. via the hepato-renal reflex). Thus, this initial sodium and water retention would overfill the intravascular space and lead to the formation of "overflow ascites" (136). The most recent and now most supported hypothesis was proposed almost two decades later and suggested that peripheral arterial vasodilation, leading to the perception of reduced effective arterial blood volume, is the triggering event for sodium and water retention in PH – the "Peripheral Arterial Vasodilation Hypothesis" (136) (Figure 1.3).

Much work has since focused on the development and perpetuation of the hyperdynamic circulation based on this hypothesis; since it has become evident that progressive vasodilation in most peripheral vascular beds plays a major role in the pathogenesis of the hyperdynamic circulation. Furthermore, the hyperdynamic circulation contributes to the characteristic and often lethal complications of PH (gastroesophageal varices; spontaneous bacterial peritonitis; hepatic encephalopathy) (149), and has been shown to be a good prognostic indicator. It is, however, important to note that some individual vascular beds may exhibit relative vasoconstriction and hypoperfusion (i.e. kidney) or may be normally perfused despite the presence of an *overall* reduction in systemic vascular resistance (118). Over the past few decades, it has become clear that there is not one factor or unifying mechanism which can explain the entire syndrome.

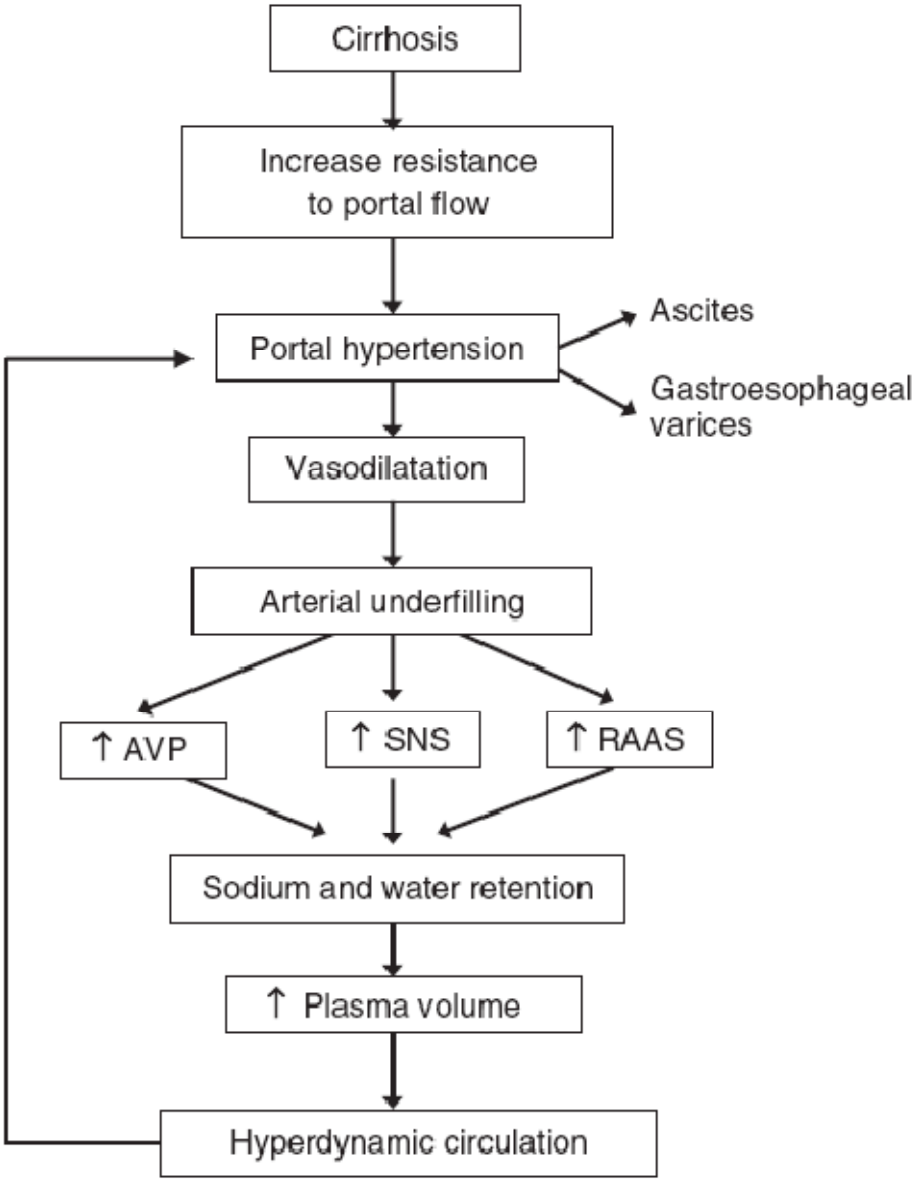

Figure 1.3 Schematic representation of the development of the hyperdynamic circulation in portal hypertension. AVP: arginine vasopressin; SNS: sympathetic nervous system; RAAS: renin-angiotensin-aldosterone system. Illustration adapted from Tsai (2007) (149).

Initially, the focus of investigation was on the delineation of circulating vasodilatory factors responsible for initiating the peripheral vasodilation of PH; either increased production of vasodilatory factors or diminished breakdown due to liver dysfunction or bypass of the liver altogether through porto-systemic collaterals. Several factors have been proposed and include carbon monoxide, prostacyclin (PGI_2), endocannabinoids, tumor necrosis factor alpha (TNFα), adrenomedullin and nitric oxide (NO) (81).Of these, the role of NO in the hyperdynamic circulation has been the most extensively studied and is now recognized as the most important vasodilator molecule mediating the excessive vasodilatation of PH; several other molecules of interest also appear to mediate vasodilation via NO release (79). The derangement of NO production within the liver has been implicated in the exacerbation of rising intrahepatic resistance and PH, while increased shear stress in the vasodilated splanchnic circulation has indicated increased NO production; this augments vasodilatation in the splanchnic vascular bed, increasing portal venous inflow and worsening PH. In PH, NO production is enhanced systemically, especially in the splanchnic arteries. This has been attributed to upregulation of endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) in mesenteric arteries (79). However, NO does not entirely account for the splanchnic vasodilatation and hyperdynamic circulation, since chronic administration of NOS inhibitors only partially corrects the mesenteric vasodilatation (9, 18) and targeted deletion of eNOS genes in mice does not prevent the development of the hyperdynamic circulation (80). Other vasoactive systems likely contribute to the reduced mesenteric resistance in PH. Exacerbating the effect of vasodilatory factors in the mesenteric vascular bed, is the *hyporesponsiveness* of this vasculature to vasoconstrictors (50).

Endothelial factors are also implicated in this hyporesponsiveness, which is completely reversed by removal of the endothelium (14). This suggests a role for prostanoids and indeed, vasodilatory prostanoids such as prostaglandin I2 (PGI2) have been shown to contribute to this vasodilatation in experimental PH (76, 142). However, the contribution of prostanoids to splanchnic vasodilatation in PH remains to be clarified given contradictory results in the literature (86).

Components of the nervous system have been shown to be involved in the maintenance of the hyperdynamic circulation. There is evidence for a link between increased sympathetic outflow and enhanced sympathetic nervous tone, which has been attributed to increased release of NE from organs such as the kidney (51, 67, 116). This increased sympathetic drive may be key in the avid sodium and water retention contributing to the blood volume expansion, which is necessary along with peripheral vasodilatation for the perpetuation of the hyperdynamic circulation (161). However, increased sympathetic neural signaling to organs such as the kidney may not wholly account for the altered systemic hemodynamics characteristic of the hyperdynamic circulation, since complete removal of sympathetic nervous input does not eliminate the hyperdynamic circulation in all forms of PH (1). This is suggestive of non-neurogenic factors contributing to the development and maintenance of the hyperdynamic circulation, the underlying details of which have not been fully elucidated.

In addition to possible peripheral mechanisms, central neural pathways have been compellingly shown to contribute to and be necessary for the development of the hyperdynamic circulation. Primary afferent neurons travelling in the vagus nerve are essential for the development of the hyperdynamic circulation, as demonstrated by the

absence of this hemodynamic profile in portal hypertensive rats in which these afferent nerves were destroyed by capsaicin (104, 109). Enhanced resting activation of central cardiovascular regulatory centres (nucleus of tractus solitarius – NTS; ventrolateral medulla – VLM) observed in portal hypertensive rats (22) is also completely dependent on primary afferent nerve signaling (144). That these animals were not able to effectively mount a normal central cardiovascular response to such stimuli as hemorrhage or volume loading revealed that central mechanisms of cardiovascular control in PH are disordered (22). Such reflex dysregulation may then contribute to the hemodynamic derangements of the hyperdynamic circulation. Ultimately, it has been proposed that central neural contributions to the hyperdynamic circulation involve an initial peripheral signal, carried to brain cardiovascular regulatory centres, such as the NTS, via vagal afferent neurons. Within the central cardiovascular centres, this signal is likely integrated to produce altered neural outflow and subsequent modulation of the peripheral vasculature. The nature of the initiating peripheral signal and the resulting peripheral effects following central integration are not known, however, it has been speculated that the initial signal may stem from increased activity of splanchnic afferent nerves activated by mesenteric vascular congestion (91). It has been shown, however, that whatever the nature of this afferent signal, it does not involve signaling through the prevertebral celiac ganglion (109).

As previously intimated, the detailed mechanisms behind the initial development of the hyperdynamic circulation have not been unequivocally established, although it has been proposed that elevated portal venous pressure in PH leads first to splanchnic vasodilatation, which consequently precipitates peripheral vasodilatation and descent into

the hyperdynamic circulation. Not excluding, and most likely in conjunction with central neural mediation of the hyperdynamic circulation, are recently proposed peripheral mechanisms pairing neural signaling with production of vasodilatory mediators. These mechanisms and the latter portion of this present work strive to directly delineate the crucial link between PH and the splanchnic vasodilatation which is so critical to its most puzzling hemodynamic complications.

In an effort to expose the mechanism underlying augmented NO production in the mesenteric circulation (evident as soon as 1 day following induction of PH by portal vein ligation (PVL) in rats), Tsai et al (2003) referred to the documented initial mesenteric vasoconstriction observed in response to acutely increased portal venous pressure in PVL animals (36, 141) to hypothesize that superior mesenteric arterial (SMA) vasoconstriction triggers eNOS upregulation and mesenteric overproduction of NO (150). These investigators revealed that SMA vasoconstriction, outside of the presence of PH, was the factor leading to increased mesenteric eNOS activity; conversely, PH in the absence of mesenteric vasoconstriction did not result in upregulation of eNOS (150). Myogenic reflex-mediated vasoconstriction in response to increased portal venous pressure was proposed as a component of this mechanism. However Coll et al (2008) showed early upregulation of adrenergic system-related genes in the initial hour after portal vein ligation in rats (35), implicating increased sympathetic drive in this initial mesenteric vasoconstriction. Both studies leave the possible factors stimulating this initial mesenteric vasoconstriction and increased mesenteric adrenergic tone open to question.

1.7 THE SPLEEN IN PORTAL HYPERTENSION

Splenic venous outflow contributes to portal venous blood flow (163), with blood from the spleen entering the portal vein via the splenic vein (Figure 1.1). Thus, it has been established that increases in portal venous pressure, invariably lead to corresponding increases in splenic venous pressure (SVP) (13, 146). As we have previously demonstrated a role for the spleen in cardiovascular regulation, we propose that in response to acute changes in splenic hemodynamics (increased SVP; decreased splenic venous blood flow); the spleen is also involved in the renal and hemodynamic *dysregulation* characteristic of PH. In this present study, functional evidence showing the existence of neural reflexes originating from the spleen, which trigger neurohormonal control of the renal and mesenteric vasculature, is presented in the context of PH.

1.7.1 Experimental Model

The current studies were designed to investigate the existence of PH-induced cardiovascular regulatory pathways. The studies were acute and carried out on anesthetized Male Long-Evans rats. In order to isolate effects independently originating from the spleen, the splenic vein was partially occluded to selectively increase splenic venous pressure to the degree present in PH. We measured splenic venous pressure via direct cannulation of the gastric vein in this model, advancing the tip of the cannula to rest at the junction of this vessel with the splenic vein. The values for splenic venous pressure measured by our gastric vein cannulation technique at rest and during portal hypertension are consistent with reported values in the literature. Portal venous pressure is frequently gauged in humans and experimental animals by splenic punction, whereby a

thin needle is inserted directly into the body of the spleen to rest in the splenic red pulp (13, 157). This splenic pulp communicates freely with intrasplenic branches of the portal vein such that splenic pulp pressure correlates directly with portal venous pressure (13, 84). Average splenic pulp pressure in healthy humans is 10 mmHg (13). Resting splenic pulp pressure in non-portal hypertensive rats has been reported by several investigators and ranges from ~ 8 mmHg (157) to 10 mmHg (3, 92, 141), which corresponds to baseline values for splenic venous pressure we observe via direct gastric vein cannulation.

The splenic venous effluent travels from the spleen to the portal vein via the splenic vein. The splenic vein offers resistance to blood flow, such that estimation of portal venous pressure upstream of this vessel will ultimately yield higher values than direct downstream cannulation of the portal vein. Indeed, intrasplenic pulp pressure is slightly higher than portal venous pressure by 2 – 6 mmHg at rest; this discrepancy increases further with increasing pressure as in portal hypertension (13). This is exemplified by a comparison of splenic pulp pressures of portal hypertensive humans, which ranges from 24-29 mmHg (13, 134) to portal venous pressure in these subjects which ranges from 7-16 mmHg (13, 134).

This principle also applies to experimental portal hypertension in rats, however the time at which portal venous pressure is measured after induction of portal hypertension is critical. The majority of estimates of portal venous pressure by splenic punction are reported in a rat model of chronic portal hypertension (portal vein ligation). Immediately after portal vein ligation (1 day) intrasplenic pressure rises to 17.7 ± 0.9 mmHg and further increases to 21.3 ± 1.0 mmHg by day 2 (141). However, as porto-systemic

collaterals begin to open/develop in the rat, intrasplenic pressure progressively decreases and stabilizes at ~ 14 mmHg by 8 days post-surgery (141). Several groups which have reported intrasplenic pressure values of ~14 mmHg in chronic portal hypertensive rats have completed these measurements more than 10 days after induction of portal hypertension (3, 92, 157).

Thus, our observation that splenic venous pressure (as measured by direct gastric vein cannulation) rises to 23-25 mmHg as portal venous pressure rises to 12-15 mmHg during acute partial portal vein occlusion is valid. That our values of splenic venous pressure at rest and during portal hypertension are consistent with the established literature, demonstrates that gastric vein cannulation alone does not physically alter splenic venous pressure. Moreover, measurement of splenic venous pressure via gastric venous cannulation during portal hypertension directly mirrors intrasplenic pressure simultaneously measured by splenic punction (S. Hamza, unpublished observation).

1.7.2 Splenic Mediation of Renal Dysfunction

We have shown that partial splenic vein occlusion increases splenic afferent nerve activity and causes a reflex increase in renal sympathetic nerve activity. The reduction in renal blood flow and renal arterial conductance is mediated by this splenorenal reflex and is abolished by splenic denervation, renal denervation and selective intrarenal arterial blockade of α_1-adrenergic receptors. Data presented also reveal independent modulation of renal vascular function by the spleen whereby PH, in the absence of changes in splenic venous outflow, does not result in activation of renal sympathetic nerves or reduction in renal blood flow or vascular conductance. Indeed, absence of the spleen itself precludes

any changes in renal hemodynamics in the face of PH. Furthermore, we demonstrate that splenic reflex modulation of renal vascular tone is mediated via integration at the prevertebral celiac ganglion; surgical excision of this ganglion abolishes the spleno-renal reflex.

1.7.3 Splenic Modulation of Mesenteric Vascular Tone and Hemodynamics – Implications for the Hyperdynamic Circulation

In addition to modulating renal vascular tone in PH, we present evidence supporting the contribution of the spleen to the initiation of the hyperdynamic circulation via mesenteric arterial vasoconstriction. Partial splenic vein occlusion is shown to reflexly activate mesenteric efferent nerve activity. This manipulation simultaneously causes a reduction in superior mesenteric arterial blood flow and conductance, a response which is dependent on splenic nerves, and involves (but is not wholly dependent upon) mesenteric nerves. We report that splenic control of mesenteric tone also heavily involves ANG II release mediated by an intact spleno-renal reflex, but also appears to include ANG II released by renal baroreceptor stimulation and mesenteric angiotensinergic nerves. Direct visualization of a reduction in diameter of mesenteric arterial resistance vessels upon partial splenic vein occlusion corroborates these hemodynamic observations and is also mediated by splenic nerves and ANG II.

In addition to physiological cardiovascular regulation, we conclude that the spleen initiates critical events in response to PH, events which play a prominent role in the development of such complications as renal dysfunction and the hyperdynamic circulation.

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CHAPTER 2:

SPLENO-RENAL REFLEX MODULATION OF RENAL VASCULAR TONE[1]

[1]*A version of this chapter has been published:*

Hamza, S.M. and S. Kaufman (2004). ***Splenorenal reflex modulates renal blood flow in the rat.*** J.Physiol 558.1: 277-282.

2.1 INTRODUCTION

Portal hypertension is defined as a pathological increase in portal venous pressure to above 10 mmHg (10, 13). Cirrhotic/portal hypertensive patients have elevated levels of norepinephrine in the renal venous blood (14), as well as significantly lower baseline mean renal blood flow levels compared to control subjects; this is indicative of increased renal sympathetic tone (9). These changes in renal hemodynamics in cirrhotic patients appear to be functional i.e. there is no intrinsic renal disease (20). Several studies have investigated the hepato-renal reflex whereby elevation of portal venous pressure causes increased hepatic afferent nerve activity, reflex increases in renal and cardiopulmonary sympathetic efferent nerve activity (8, 21), and decreased renal blood flow (16). In addition, acute superior mesenteric vein occlusion has also been shown to reduce renal blood flow, suggesting possible involvement of an intestinal-renal neural reflex (23). Thus, it has been proposed that altered neural reflexes mediate the abnormal renal hemodynamics observed in portal hypertension.

Blood from the splenic vein drains into the portal vein. Thus an increase in portal venous pressure causes a concomitant rise in splenic venous pressure. We wished to investigate whether changes in intrasplenic hemodynamics also alter renal function. There is both structural and functional evidence for a neural reflex pathway between the spleen and the kidneys (5, 7, 15, 22). We proposed therefore that derangement of renal function in portal hypertension may be mediated by a splenorenal neurogenic reflex, whereby elevated portal and splenic venous pressure would increase splenic afferent nerve activity, which would induce an increase in efferent renal sympathetic nerve activity and decrease renal blood flow. In order to isolate the spleen as the initiator of the splenorenal reflex, we

partially occluded the splenic vein to elevate splenic venous pressure to the degree observed in portal hypertension; this did not cause any obstruction to portal venous flow. The effects of renal denervation, splenic denervation, and pharmacological blockade of intrarenal α_1 adrenergic receptors were studied. In addition, we measured the changes in splenic afferent and renal efferent nerve activity elicited by splenic venous occlusion.

2.2 METHODS

The experimental procedures were approved by the local Animal Welfare Committee in accordance with the guidelines issued by the Canada Council on Animal Care. All animals were euthanized with an anaesthetic overdose of pentobarbital sodium (96 mg, IV, MTC Pharmaceuticals, Canada) at the completion of each experiment. All data were recorded online (DATAQ Instruments, USA) and analyzed using WINDAQ software (DATAQ Instruments).

2.2.1 Animals

Male Long-Evans rats (350-600g, Charles River, Canada) were housed in the University of Alberta Animal Facility for at least one week before experiments started. They were exposed to a 12/12hr light/dark cycle in a temperature and humidity controlled room. All rats were fed standard 0.3% sodium rat chow and water *ad libitum.* There were four experimental groups: Intact control rats (n=7), renal denervated rats (n=6), splenic denervated rats (n=6) and phenoxybenzamine-treated rats (n=6).

2.2.2 Surgery

Anaesthesia was induced with pentobarbital sodium (60mg/kg b.wt., IP), followed one hour later by Inactin (Sigma, Canada; ethyl-(methyl-propyl)-malonyl-thio-urea, 80mg/kg b.wt., SC). Body temperature was maintained at 37°C with a homeothermic blanket (Harvard Apparatus, Canada).

The femoral vein and artery were cannulated with Silastic (Dow Corning, USA, 0.51mm ID, 0.94 mm OD) and polyethylene tubing (VWR International, Canada, PE 50, 0.58mm ID, 0.97mm OD) for administration of isotonic saline (3mL/hr) and monitoring of systemic blood pressure respectively. Through a midline laparotomy, the gastric and renal vessels were exposed. A Silastic cannula (Dow Corning, USA; 0.30mm ID, 0.64mm OD) was inserted occlusively into the gastric vein, advanced until the tip lay at the junction with the splenic vein, and secured with a drop of Vetbond tissue glue (3M Animal Care Products, USA). This cannula was connected to a pressure transducer for online monitoring of splenic venous pressure.

A Prolene 6-0 loop (Ethicon, USA) was placed loosely around the splenic vein at its junction with the portal vein; this allowed for controlled partial occlusion of the splenic vein. Similarly, a loose ligature (Prolene 4-0) was placed around the hepatic portal vein (rostral to the junction with the splenic vein), to induce portal hypertension; in this case, a cannula (polyethylene, 0.58mm ID, 0.97mm OD) was inserted non-occlusively into the portal vein, and secured with tissue adhesive (3M Vetbond, Animal Care Products, St. Paul, MN, USA) to monitor portal venous pressure.

In renal denervation experiments, the renal nerves were stripped from the left renal artery and vein as previously described (18). Similarly, in splenic denervation experiments, the splenic nerves were stripped from the splenic artery and vein, distal to branching of the vessels toward the spleen (3).

2.2.3 Renal Blood Flow

A factory calibrated flow probe (1RB series, Transonic Systems, Ithaca, NY, USA) was placed around the left renal artery and covered in conducting jelly. A 35-40 minute stabilization time was allowed, during which time body temperature, mean arterial pressure (MAP) and renal blood flow were monitored. Baseline values of renal blood flow, MAP and splenic venous pressure were then recorded for 20 min, after which tension was applied to the splenic venous ligature to raise splenic venous pressure to 20-24mmHg. Splenic venous pressure, MAP and renal blood flow were recorded for a further 20 min.

Phenoxybenzamine (12.5µg in 150µL, Sigma, Canada) was administered over 30 sec into the renal artery with application of vibration to ensure even distribution throughout the kidney (Appendix A). After a stabilization period of 20-25 min, the effect of splenic venous occlusion on renal blood flow was measured as described above. At the end of each experiment, it was confirmed that the phenoxybenzamine had completely blocked intrarenal α_1 adrenergic receptors; the α_1- adrenergic agonist phenylephrine (0.15µg in 150µL, Sabex Canada) did not cause any change in renal blood flow.

2.2.4 Whole Fiber Nerve Recording

Separate groups of rats were used for these experiments. Nerve recordings were performed as previously described (17). The abdominal cavity was filled with mineral oil, and the splenic nerve isolated and cut. Splenic sensory afferent nerve activity was measured by placing the distal end of a small nerve fibre (closer to midline) onto bipolar platinum recording electrodes, and covering them with Kwik-Cast (WPI, Sarasota, FL). The nerve signal was amplified and filtered between 100 and 1000 Hz (Leaf Electronics Ltd. QT-B; WPI LPF-30, Sarasota, FL). Output from the amplifier was fed to a loudspeaker, and displayed on a PC (sampling rate between 3 and 10 kHz, Windaq, Dataq Instruments, Akron OH). After stabilization (20 min), afferent nerve activity was recorded on-line. Twenty minutes later, the splenic venous ligature was tightened until splenic venous pressure measured between 20-24mmHg. Nerve recording continued for a further 20 min. A similar procedure was used to measure renal efferent nerve activity, except that, in this case, the *proximal* end (closer to the spinal cord) of a small severed nerve fibre was placed on the recording electrodes. The analysis of nerve discharge was based on average discharge rate (spikes/sec) of visually identified action potentials in the raw filtered recordings.

2.2.5 Drugs

Phenoxybenzamine (Sigma, Canada) was dissolved in heparinized isotonic saline (heparin 10 000I.U./L) at a concentration of 12.5µg in 150µL. This dose of phenoxybenzamine has previously been shown to block intrarenal α-adrenergic receptors (26). Phenylephrine hydrochloride (Sabex, Canada; 10 mg/mL) was diluted with

heparinized isotonic saline (heparin 10,000 I.U./L) to a final concentration of 0.15μg in a 150μL volume.

2.2.6 Data Analysis

Results are based on the last ten minutes of each twenty-minute recording period described above. The data were analyzed using Student's t-test for paired data, or ANOVA followed by Student-Newman-Keuls post hoc test. Significance was accepted at $P<0.05$.

2.3 RESULTS

2.3.1 Splenic Venous Pressure

Preliminary experiments revealed that, when portal venous pressure was increased from 3.9 ± 0.4 mmHg to 13.6 ± 0.3 mmHg, there was an accompanying increase in splenic venous pressure from 10.1 ± 0.8 mmHg to 23.0 ± 0.7 mmHg (n=5). In the subsequent series of experiments, mean baseline splenic venous pressure for all the animals was 7.9±0.6 mmHg (n=25). This was increased to 20-24mmHg (Mean: 21.6±0.3 mmHg, n=25) by partial occlusion of the splenic vein i.e. to that pressure which would be associated with a portal venous pressure of 12-15 mmHg. There were no significant differences between the four experimental groups with regard to the baseline or experimental splenic venous pressures.

2.3.2 Renal Blood Flow

Mean baseline renal blood flow for all the animals was 8.9±0.4 $mL \bullet min^{-1}$ (n=25) . There were no significant differences between the baseline renal blood flows for any of the four

experimental groups (Fig 2.1). When splenic venous pressure was increased, there was an immediate decrease in renal blood flow in the intact animals (-2.1±0.2 mL•min^{-1}, n=7) (Fig 2.1). This response was greatly attenuated after renal denervation (-0.7±0.1 mL•min^{-1}, n=6), after splenic denervation (-0.8±0.1 mL•min^{-1}, n=6), and after intrarenal administration of phenoxybenzamine (-0.8±0.1mL•min^{-1}, n=6) (Fig 2.1).

2.3.3 Mean Arterial Pressure

Mean baseline MAP for all the animals was 98.7±2.5 mmHg (n=25). There were no significant differences between the baseline values for any of the four experimental groups (Fig 2.2). Moreover, the fall in MAP during splenic vein occlusion did not differ significantly between the intact (-12.4±2.8 mmHg, n=7), renal denervated (-8.3±1.9 mmHg, n=6), splenic denervated (-8.2±1.5 mmHg, n=6), and phenoxybenzamine-treated (-5.0±0.33 mmHg, n=6) animals (Fig 2.2). Although the baseline MAP and the fall in pressure tended to be lower in the phenoxybenzamine-treated animals, this did not reach significance (baseline MAP: P=0.306; change in MAP: P=0.133).

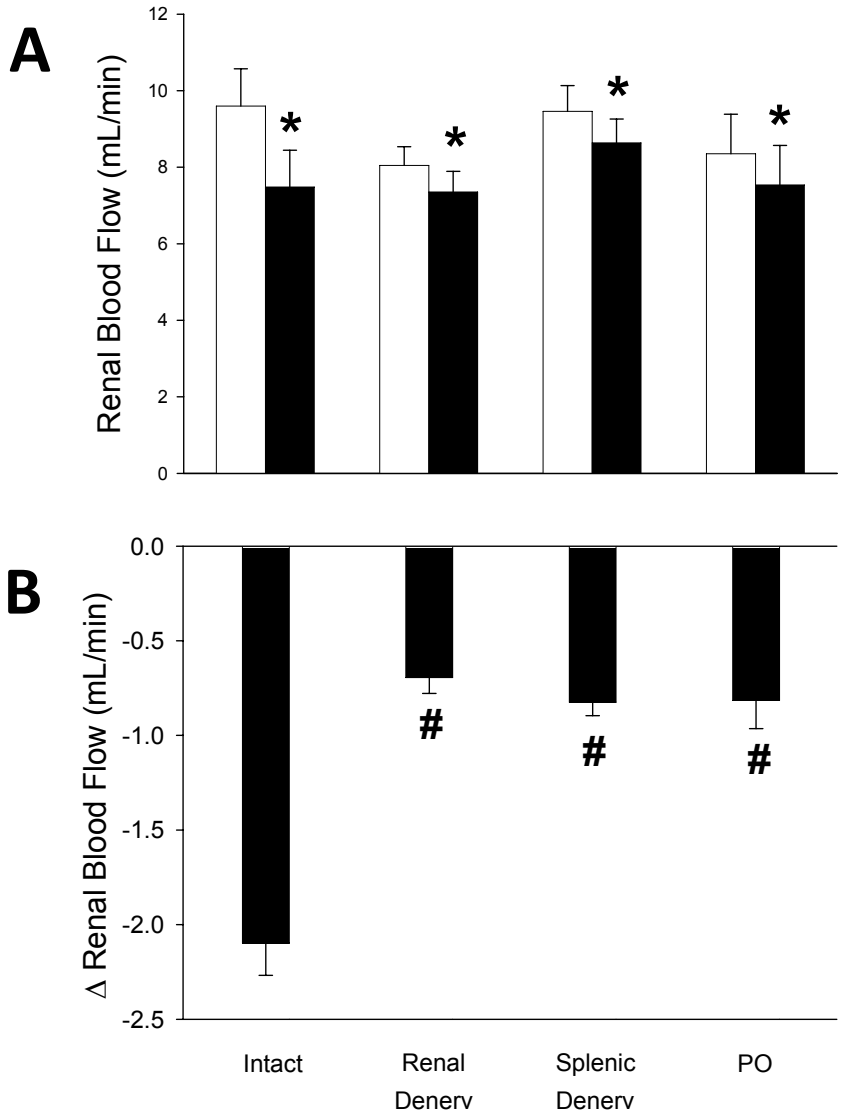

Figure 2.1: Effect of partial splenic vein ligation on renal blood flow of intact (n=7), renal denervated (Renal Denerv, n=6), splenic denervated (Splenic Denerv, n=6), and intrarenal phenoxybenzamine-treated (PO, 12.5µg, n=6) rats. A: Renal blood flow before (black bars) and after (open bars) partial splenic venous occlusion. B: Change in renal blood flow during partial splenic venous occlusion. Data presented as means ± SEM. *Significant change in renal blood flow, $P<0.05$. #Significant difference compared with intact group, $P<0.05$.

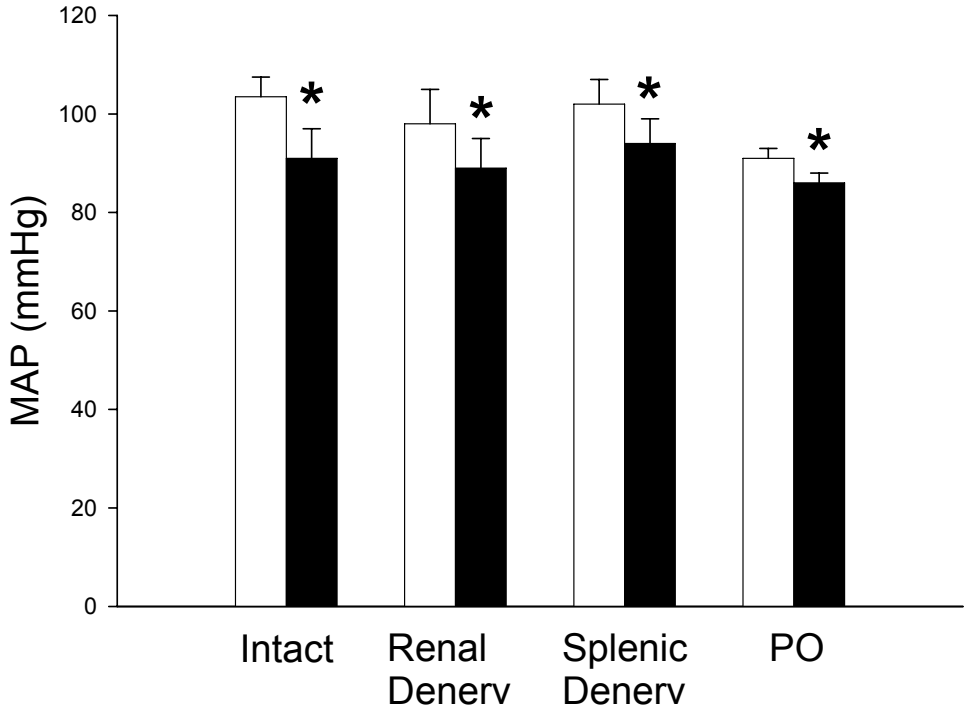

Figure 2.2: Effect of partial splenic vein ligation on MAP of intact (n=7), renal denervated (Renal Denerv, n=6), splenic denervated (Splenic Denerv, n=6) and intrarenal phenoxybenzamine-treated (PO, 12.5µg, intrarenal, n=6) rats. The open bars show baseline values. The black bars show values during partial splenic venous occlusion. The data are presented as mean ± SEM. *Significant difference between baseline MAP and MAP during partial splenic vein ligation, $P<0.05$.

2.3.4 Renal Arterial Conductance

Renal conductance was calculated as the ratio of flow to renal perfusion pressure (K = Q/P). There was a significant fall in renal conductance (0.088±0.011 to 0.079±0.014 mL• min^{-1} •$mmHg^{-1}$, n=7) in intact animals during splenic vein occlusion. However, there was no such change in the renal denervated (0.086±0.011 to 0.086±0.012 mL• min^{-1} •$mmHg^{-1}$, n=6), splenic denervated (0.094±0.008 to 0.094±0.009 mmHg/mL• min^{-1} n=6), or phenoxybenzamine-treated (0.092±0.012 to 0.088±0.013 mL• min^{-1} •$mmHg^{-1}$ n=6) animals.

2.3.5 Nerve Activity

Increased splenic venous pressure (partial splenic vein occlusion) caused an immediate and significant increase both in splenic afferent (3.0±0.3 to 6.6±0.6 spikes•sec^{-1}, n=5) and renal efferent (24.8±2.0 to 50.2±4.9 spikes•sec^{-1}, n=9) nerve activity (Fig. 2.3).

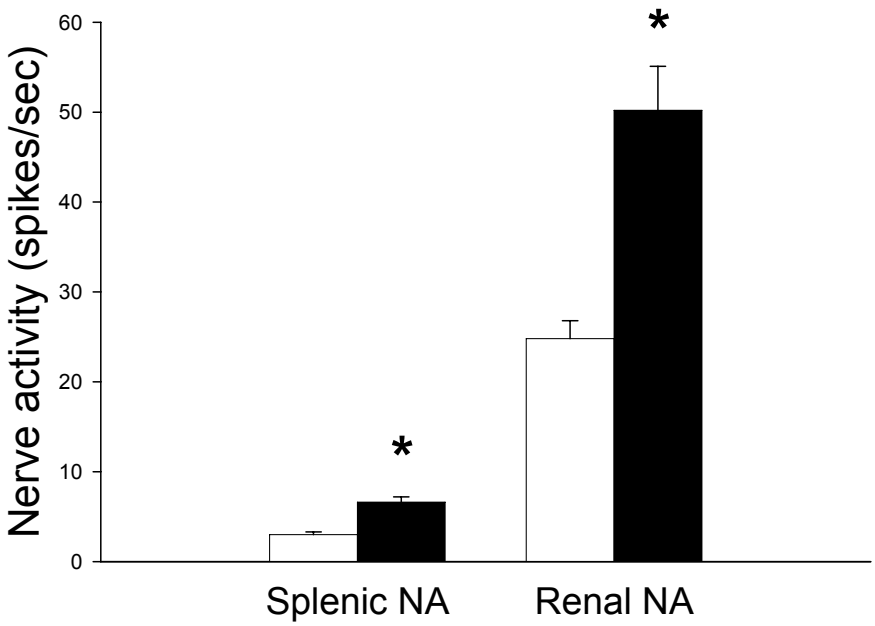

Figure 2.3: Effect of partial splenic vein ligation on splenic afferent (Splenic NA, n=5) and renal efferent (Renal NA, n=9) nerve activity. The open bars show baseline values. The black bars show values during partial splenic venous occlusion. Data are presented as means ± SEM. *Significant difference between baseline nerve activity and nerve activity during partial splenic vein ligation, $P<0.05$.

2.4 DISCUSSION

Splenic venous occlusion caused an immediate fall in renal blood flow which was attenuated by renal denervation, splenic denervation, and close renal arterial injection of the α_1 adrenergic blocker phenoxybenzamine. This was accompanied, in all groups, by a fall in MAP. Renal conductance fell only in the intact group. We also showed that splenic vein occlusion increased both splenic sensory afferent and renal efferent nerve activity. We conclude that obstruction to splenic venous outflow, such as would occur in portal hypertension, increases splenic sensory afferent nerve activity and renal sympathetic nerve activity through the *splenorenal* reflex. This causes renal vasoconstriction and a fall in renal blood flow. There is also a fall in systemic blood pressure.

Portal hypertension is associated with perturbations of renal function which are mediated, at least in part, through the renal sympathetic nerves (2). There is evidence that several neurogenic pathways are involved. The *hepatorenal* reflex has been shown to regulate renal blood flow (16, 19); portal vein occlusion increases hepatic sensory afferent nerve activity, and induces reflex increases in renal sympathetic nerve activity (21). It has also been shown that in dogs, acute occlusion of the superior mesenteric vein causes a reduction in renal arterial blood flow (23). The authors concluded that increased mesenteric outflow pressure, as would be associated with portal hypertension, initiates an *intestinal-renal* neural reflex. The model used in the current study, ligation of the splenic vein, does not cause any changes in portal venous pressure or mesenteric vascular pressure. The reflex changes we observed in renal blood flow were thus initiated by the spleen, there being no direct contribution from the liver or intestines.

α_1- adrenergic receptors mediate renal vasoconstriction (25). The dose of phenoxybenzamine (12.5μg), an irreversibly binding receptor antagonist, was chosen to completely block renal α-adrenergic receptors, but to have minimal systemic effects (12, 26). We verified that the intrarenal α_1- adrenergic receptors were indeed blocked by administering intrarenal phenylephrine at a dose (0.15μg) known to cause significant renal vasoconstriction (11). However, there was probably some phenoxybenzamine spillover into the systemic circulation. Although baseline MAP tended to be lower in the phenoxybenzamine-treated animals, this did not reach significance (which may reflect the limited sample size rather than lack of any systemic effect). This does not however detract from our finding that, in the phenoxybenzamine-treated animals, there was still a significant fall in blood pressure in response to splenic vein ligation.

We had hypothesized that phenoxybenzamine would block the effects of the renal sympathetic nerves on the renal arterioles, and prevent the drop in renal blood flow seen after splenic venous occlusion. Whereas it has previously been shown that phenoxybenzamine administration completely inhibits renal nerve-stimulated vasoconstriction (6), administration of phenoxybenzamine in our study attenuated, but did not abolish the decrease in renal blood flow during splenic venous occlusion. However, given that MAP fell during splenic venous occlusion, we reasoned that the residual drop in renal blood flow seen in the denervated and phenoxybenzamine-treated animals might have been secondary to the fall in blood pressure. Calculation of renal arterial conductance confirmed that, whereas conductance fell in the intact animals, there was no such change in the denervated and phenoxybenzamine-treated animals i.e. the residual decrease in renal blood flow after interruption of the splenorenal reflex could

indeed be accounted for by the fall in renal perfusion pressure. One might question why autoregulation did not prevent the fall in renal perfusion pressure from inducing a fall in renal blood flow, given that MAP was within the normal autoregulatory range of the kidney. However, it has been shown that moderate sympathetic activation, such as would have been induced by splenic vein occlusion, raises the lower limit of autoregulatory control of renal blood flow (24). As such, the fall in MAP could indeed have reduced renal blood flow, without changing renal vascular conductance.

Portal hypertension, induced both by cirrhosis and by portal vein ligation, is associated with a fall in MAP (1, 4, 17), which is not blocked by renal denervation (2). This is consistent with our observation that the fall in MAP induced by partial splenic vein ligation was likewise not blocked by splenic or renal denervation.

In conclusion, we have demonstrated that obstruction to splenic venous outflow, such as would be associated with portal hypertension, causes a reduction in renal blood flow, and a fall in MAP. Whereas the change in renal blood flow is neurally mediated through the splenorenal reflex, the fall in systemic blood pressure is unaltered by either splenic or renal denervation. It has previously been established that, in portal hypertension, renal function is influenced by both hepatorenal and intestinal-renal reflex pathways (16, 23). We now propose that, in addition, concomitant changes in intrasplenic hemodynamics also initiate splenorenal reflex pathways which contribute to the perturbations in renal and cardiovascular function associated with the condition.

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CHAPTER 3:

MESENTERIC CONGESTION ALONE DOES NOT MODULATE RENAL VASCULAR TONE; SIGNIFICANCE OF THE SPLEEN[2]

[2]*A version of this chapter has been published:*

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3.1 INTRODUCTION

Portal Hypertension (PH), which is often present in chronic liver disease, is characterized by a pathological elevation in portal venous pressure (>10 mmHg). Although end stage renal failure is common in chronic liver disease, there is no intrinsic renal disease (16). There is evidence however that increased sympathetic nervous activity contributes to PH-induced renal dysfunction (1, 11). Indeed, it has been established that renal function may be controlled by a hepato-renal reflex, whereby elevated portal venous pressure/reduced portal venous flow triggers increased hepatic afferent/renal efferent sympathetic nerve activity (8, 17-19, 22).

Given that the splenic vein drains into the portal vein, any increase in portal venous pressure is associated with a parallel increase in splenic venous pressure (26). Increased intrasplenic pressure induces an increase in splenic afferent nerve activity; which induces an increase in systemic blood pressure through two distinct pathways. It activates a spinal splenorenal reflex to increase renal sympathetic nerve activity and stimulate renin release (6), and it alters central neural control of sympathetic outflow (23). In this latter study, we showed that elevated splenic venous pressure induces activation of paraventricular and supraoptic nuclei of the hypothalamus, both of which are known to be important in cardiovascular homeostasis (25). In addition, the PH-induced obstruction of splenic venous outflow induces a fall in renal blood flow (RBF) which is mediated through the splenorenal reflex increase in renal sympathetic nerve activity(12).

Increased portal pressure also impedes blood draining from the gut. Thus it has been proposed that mesenteric congestion may contribute to renal (dys)function through

activation of an intestinal-renal reflex (3, 10, 13, 21, 28). Previous studies led us to believe that selective mesenteric congestion could alter splenic function, i.e. an intestinal-splenic reflex (14). In the present study we investigated whether such an increase in mesenteric venous pressure could also influence renal blood flow either directly to the kidney (intestinal-renal reflex) or indirectly through the spleen (intestinal-spleno-renal reflex).

We used acute partial portal vein ligation (PVL) in rats to observe the effect of elevated portal venous pressure on renal blood flow. As previously described, the portal vein was partially occluded above (PVLA) or immediately below (PVLB) the junction with the splenic vein (porto-splenic junction)(14). Occluding below (caudal to) the porto-splenic junction results in selective elevation of mesenteric venous pressure, without influencing the splenic circulation; thus any effect arising from this occlusion implicates the intestine. We measured the consequences of these maneuvers on portal venous pressure (PVP), mean arterial blood pressure (MAP) and renal blood flow (RBF). The effects of renal denervation, splenic denervation, celiac ganglionectomy (i.e. combined functional splenic, mesenteric and renal denervation) and splenectomy were studied. In a separate group of animals, we recorded the effects of occlusion (PVLA and PVLB) on splenic afferent and renal efferent nerve activity. Contrary to our hypothesis, selective mesenteric congestion did not modulate renal sympathetic nerve activity or RBF, either directly (intestinal-renal) or indirectly (intestinal-spleno-renal reflex). We conclude that the mesenteric vascular bed does not play a critical role in regulating RBF in PH.

3.2 METHODS

All experimental procedures were approved by the local Animal Welfare Committee in accordance with the guidelines issued by the Canada Council on Animal Care. All animals were euthanized with an anesthetic overdose of sodium pentobarbital (96 mg, I.P., Vetoquinol, Lavaltrie, Quebec, Canada) at the end of each experiment. Data were recorded online (DATAQ instruments, Akron, Ohio, USA) and analyzed using WINDAQ software (DATAQ Instruments), except for nerve activity data which were recorded with PowerLab equipment (ADInstruments, Castle Hill, Australia) and analyzed with Chart 5 software with Spike Histogram Module (ADInstruments).

3.2.1 Animals

Male Long-Evans rats (350-600g, Charles River, Montreal, Quebec, Canada) were housed in the University of Alberta Animal Facility for one week before experiments commenced. Animals were exposed to a 12h-12h light-dark cycle in a temperature and humidity controlled room. All rats were fed standard 0.3% sodium rat chow and water *ad libitum*.

3.2.2 Surgery

Anesthesia was induced with sodium pentobarbital (65 mg (kg body weight)$^{-1}$, I.P.), followed by Inactin (Sigma, Canada; ethyl-(methyl-propyl)-malonyl-thio-urea, 80-100mg (kg body weight)$^{-1}$, S.C.) to maintain anesthesia after the animal reached surgical plane. Body temperature was maintained at 37ºC with a homoeothermic blanket (Harvard Apparatus, Canada) or, for the nerve recording experiments, a Deltaphase™ thermal heating pad (Braintree Scientific, Braintree, MA, USA) (to reduce electrical interference).

The femoral vein and artery were cannulated with Silastic™ (0.51 mm i.d., 0.94 mm o.d.; Dow Corning, Midland, MI, USA) and polyethylene tubing (PE 50, 0.58 mm i.d., 0.97 mm o.d.; VWR International, Mississauga, Ontario, Canada) for administration of isotonic saline (3 mL h^{-1}) and monitoring of systemic blood pressure, respectively. Through a midline laparotomy, the stomach was reflected onto the thorax and the spleen was cleared from its connective tissue attachments to the stomach. Splenic vessels were kept intact. The portal vein was gently exposed down to the level of the superior mesenteric vein. A loose ligature (Prolene 1.5, Ethicon, USA) was placed around the hepatic portal vein, either *rostral* (PVLA) or *caudal* (PVLB) to the porto-splenic junction. A cannula (PE 50, 0.58 mm i.d., 0.97 mm o.d.; VWR International, Mississauga, Ontario, Canada) was inserted non-occlusively into the superior mesenteric vein, advanced to the level of the portal vein below the ligature and secured with tissue adhesive (3M Vetbond; Animal Care Products, St.Paul, MN, USA). This cannula was used to monitor portal venous pressure (PVP).

3.2.3 Renal/Splenic Denervation and Celiac Ganglionectomy

The renal nerves were stripped from the left renal artery and vein, which were subsequently painted with 5% phenol to destroy remaining fibres, as previously described(15). Similarly, the splenic nerves were stripped from the splenic artery and vein, distal to the branching of vessels towards the spleen. We have shown this procedure significantly reduces splenic tissue catecholamine levels (2). The celiac ganglion supplies nerve fibres to splenic, renal and mesenteric vascular beds excision of this ganglion thus interrupts splenic, renal and mesenteric nerve activity (4, 5). Celiac ganglionectomy was achieved by blunt dissection of the abdominal aorta at the level of the superior mesenteric

and celiac arteries. The nerves along the celiac artery and trunk of the superior mesenteric artery were stripped from their respective vessels. The unpaired celiac ganglion was then excised from surrounding tissues.

3.2.4 Splenectomy

Portal vein ligation not only increases intraportal venous pressure, but also decreases portal venous blood flow, which may induce a hepatorenal reflex-mediated change in renal function (22). The fall in portal venous blood flow is smaller after PVLB (-3.2±0.6mL/min) than after PVLA (-9.6±2.4mL/min)(14), since the former does not impede splenic venous outflow into the portal vein. In order to control for this, a group of animals was splenectomized, thus ensuring that there was no difference in the fall in portal venous blood flow into the liver following PVLA or PVL B. Branches of vessels leading directly into the spleen were tied off individually with 4-0 silk suture and completely ligated with fine tissue scissors. The spleen could thus be completely removed while maintaining the splenic vascular arcade intact.

3.2.5 Renal Blood Flow and Portal Venous Blood Flow

A factory calibrated flow probe (1RB series, Transonic Systems, Ithaca, NY, USA) was positioned around the left renal artery and covered in conducting gel. Zero-flow reading was confirmed before use by placing the probe in a non-turbulent water bath. A 30-35 min stabilization period was allowed, during which time, body temperature, MAP and RBF were monitored. Portal venous blood flow was similarly measured using a 3RB Transonic flow probe.

3.2.6 Experimental Protocol (Renal Blood Flow)

There were five experimental groups which were subjected to either PVLA (portal vein occlusion rostral to the junction with the splenic vein) or PVLB (portal vein occlusion caudal to the junction with the splenic vein): intact control rats (PVLA, $n = 9$; PVLB, $n = 9$), renal denervated (PVLA, $n = 6$; PVLB, $n = 6$), splenic denervated (PVLA, $n = 11$; PVLB, $n = 7$), celiac ganglionectomized (PVLA, $n = 9$; PVLB, $n = 8$) and splenectomized rats (PVLA, $n = 6$; PVLB, $n = 7$). Following the stabilization period, baseline RBF, MAP and PVP were recorded for 20 minutes, after which, the portal ligature was tightened to effect partial portal vein occlusion and elevation of PVP to 12-15 mmHg (i.e. experimental portal hypertension). PVP, MAP and RBF were then recorded for a further 10 minutes.

3.2.7 Whole Fiber Nerve Recording

In the case of splenic afferent nerve activity, the intestines were placed gently back into the abdominal cavity after portal vein cannulation and covered with moist gauze. The splenic arcade was then isolated over this gauze and the edges of the abdominal wall were sutured to a pre-made steel support ring to create an abdominal "well" (3.0 Cotton, Davis-Geck, American Cyanamid Co., N.Y., USA). This was then filled with heavy mineral oil (Laboratoire Atlas, Inc., Montreal, Quebec, Canada). The splenic vessels were gently exposed using blunt dissection under the mineral oil. Great care was taken to ensure that the nerves were at no time exposed to air. An approximately 1 cm length of splenic nerve was exposed and carefully dissected from the vessels and surrounding tissues with fine forceps (#5, Dumont, 0.05 x 0.01 mm, Fine Science Tools, Vancouver,

British Columbia, Canada). The proximal end (close to midline) of the nerve was cut with fine tissue scissors for afferent nerve activity. The protocol for renal nerve isolation was similar, except the left renal vessels were exposed by gently retracting the intestines to the animal's right with moist gauze. This essentially formed a space adequate to fill with mineral oil. A branch of the renal nerve was then isolated as with the splenic nerve above; however, the nerve was cut distally (i.e. closer to the kidney) to allow for recording of efferent nerve activity. The ends of cut nerves were then placed onto bipolar silver-platinum electrodes and the nerve signal was amplified (pre-amplifier, Gould) and filtered between 100 and 10,000Hz. Output from the amplifier was fed to a loudspeaker and displayed on a PC (Sampling rates Renal efferent: 4 kHz; Splenic afferent: 10 kHz, PowerLab, ADInstruments, Chapel Hill, Australia).

3.2.8 Experimental Protocol (Nerve Activity)

Separate groups of rats were used for these experiments (Splenic Afferent: PVLA, $n = 7$, PVLB, $n = 9$; Renal Efferent: PVLA, $n = 10$, PVLB, $n = 9$). After a 30-35 min stabilization period, either splenic afferent or renal efferent nerve activity was recorded online for 20 minutes, after which the portal venous ligature was tightened to elevate PVP to 12-15 mmHg. Nerve activity was recorded for a further 10 min. Analysis of nerve activity was based on average firing rate (spikes/sec) of identified action potentials in the raw, filtered recordings (Chart 5 Software, Spike Histogram Module, ADInstruments)(12). Initially, background noise was determined by recording post-mortem signals at the end of each experiment. As this was not different from determining background noise directly from the recorded nerve trace (as recommended by ADInstruments), this method was used instead for subsequent experiments.

3.2.9 Data Analysis

Results are based on the first 10 minutes of each 20 minute recording period. Data were analyzed using One Way ANOVA (Figures 3.1-3.3, or Student's t-test (Figure 3.5)). Two Way ANOVA was used for comparing data between PVLA and PVLB treatments. Significance was accepted at $p < 0.05$.

3.3 RESULTS

3.3.1 Portal Venous Pressure and Flow

Mean baseline portal venous pressure for all animals in the RBF study was 6.5±0.1mmHg (*n*=78). This was elevated to 12-15 mmHg (mean: 13.2±0.1mmHg) by partial occlusion of the portal vein. There were no significant differences between the experimental groups with respect to the baseline or experimental portal venous pressure values. In the splenectomized animals, there was no difference between the fall in portal venous blood flow subsequent to PVLA (-9.9±1.9mL/min, n=4) and PVLB (-11.9±2.7mL/min, n=3, p=0.553)

3.3.2 Mean Arterial Pressure

Baseline MAP was similar in the intact, renal and splenic denervated animals, but lower in the celiac ganglionectomized animals (Figure 3.1). MAP fell or tended to fall from baseline in all groups following PVLA and PVLB (Figure 3.1).

3.3.3 Renal Blood Flow

Baseline RBF was similar in the intact, renal/splenic denervated and splenectomized animals, but lower in the celiac ganglionectomized animals (Figure 3.2). When portal venous pressure was increased by rostral occlusion (PVLA), there was an immediate drop in RBF in the intact animals (-1.2±0.2mL/min, Figure 3.2), which was significantly attenuated or abolished in the denervated and splenectomized animals (Figure 3.2, *top*), Elevation of portal venous pressure by caudal occlusion (PVLB) in the intact animals resulted in a significantly smaller drop in RBF (-0.5±0.1mL/min) compared with that observed after PVLA (Figure 3.2, *bottom*). There were no changes in RBF in the denervated and splenectomized animals following PVLB (Figure 3.2, *bottom*).

3.3.4 Renal Arterial Conductance

Renal conductance (*K*) was calculated as the ratio of flow (*Q*) to renal perfusion pressure (*P*): $K=Q/P$. During PVLA, renal arterial conductance dropped significantly from baseline in the intact animals (-0.007±0.002mL·min^{-1}·mmHg^{-1}, Figure 3.3, *left*). This change was completely abolished after renal denervation, splenic denervation, celiac ganglionectomy and splenectomy (Figure 3.3, *left*). Renal conductance did not change in any of the groups subjected to PVLB (Figure 3.3, *right*).

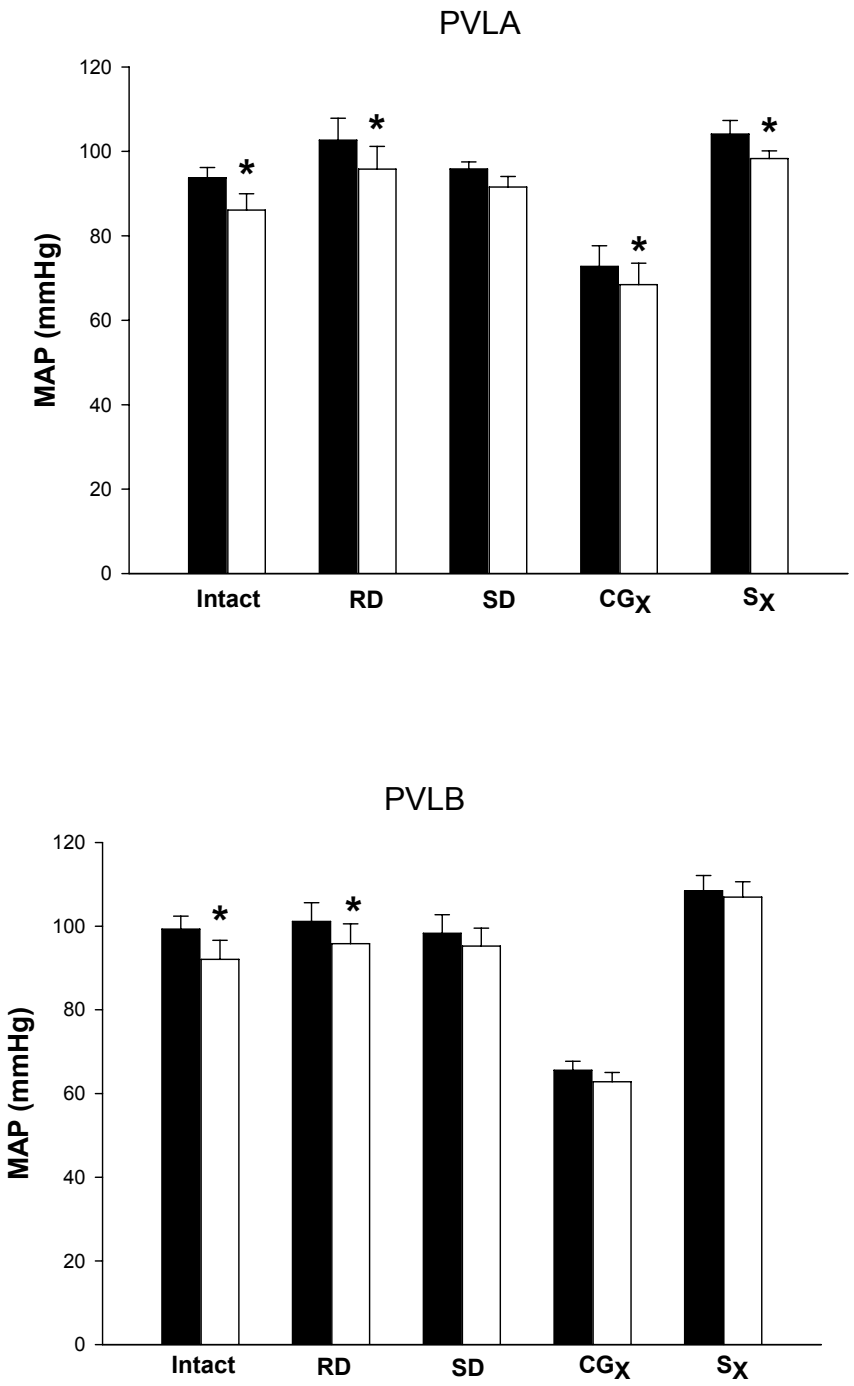

Figure 3.1: Effect of partial portal vein ligation on change of MAP in intact (PVLA: *n* = 9; PVLB: *n* = 9), renal denervated (RD; PVLA: *n* = 6; PVLB: *n* = 5), splenic denervated (SD; PVLA: *n* = 11; PVLB: *n* = 7), celiac ganglionectomized (CG_X; PVLA: *n* = 9; PVLB: *n* = 8) and splenectomized (S_X; PVLA: *n* = *6*; PVLB: *n* = *7*) rats. Data are presented as means ± SEM, * Significant difference from baseline MAP; # significant difference between baseline MAP of intact and celiac ganglionectomized animals; $p<0.05$.

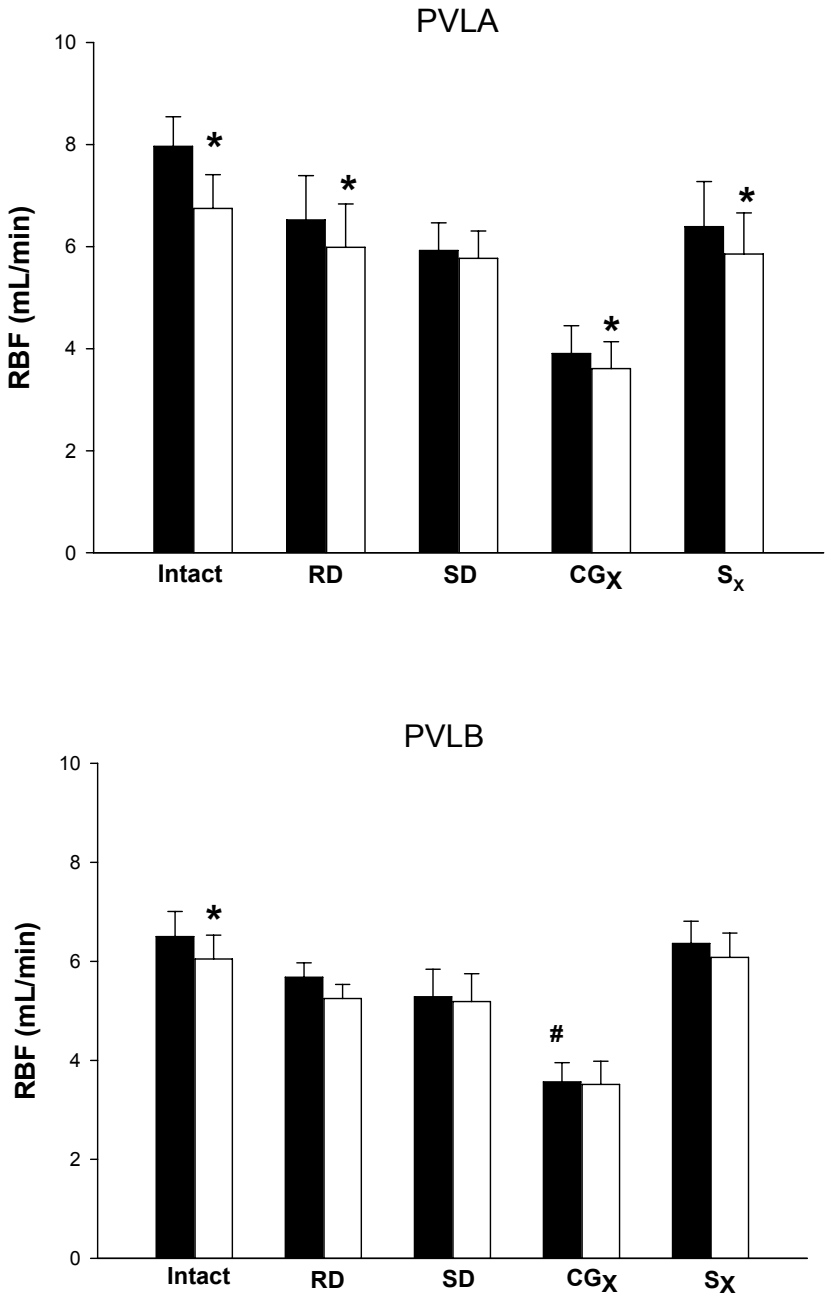

Figure 3.2: Effect of partial portal vein ligation on Renal Blood Flow of intact (PVLA: *n* = 9; PVLB: *n* = 9), renal denervated (RD; PVLA: *n* = 6; PVLB: *n* = 5), splenic denervated (SD; PVLA: *n* = 11; PVLB: *n* = 7), celiac ganglionectomized (CG_X; PVLA: *n* = 9; PVLB: *n* = 8) and splenectomized (S_X;PVLA: *n* =6; PVLB: *n* = 7) rats. Data are presented as means ± SEM, * Significant difference from baseline; # Significant difference between baseline RBF of intact and celiac ganglionectomized animals $p< 0.05$.

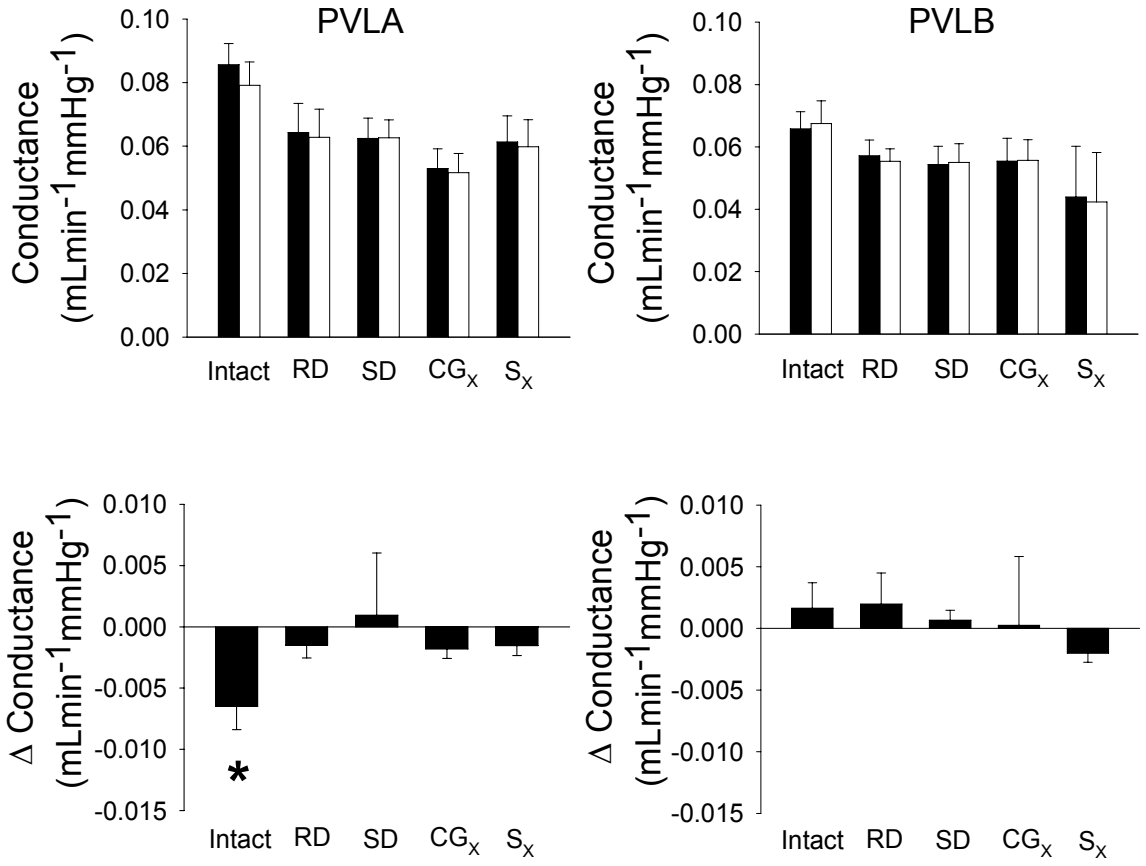

Figure 3.3: Effect of partial portal vein ligation on renal arterial conductance (upper panels) and change in renal arterial conductance (lower panels) of intact (PVLA: *n* = 9; PVLB: *n* = 9), renal denervated (RD; PVLA: *n* = 6; PVLB: *n* = 5), splenic denervated (SD; PVLA: *n* = 11; PVLB: *n* = 7), celiac ganglionectomized (CG_X; PVLA: *n* = 9; PVLB: *n* = 8) and splenectomized (S_X; PVLA: *n* = *6*; PVLB: *n* = *7*) rats. Data are presented as means ± SEM, * Significant difference from baseline; $p< 0.05$.

3.3.5 Nerve Activity

Mean baseline PVP for all animals in this section was 6.6±0.2 mmHg (*n*=35). There was no statistical difference between baseline values of either splenic afferent or renal efferent nerve activity (p=0.064). As in the RBF study above, PVP was elevated to 12-15mmHg (mean: 13.7±0.3 mmHg, *n* = 35). Mean baseline MAP for all animals was 96.2±1.9 mmHg, which fell to 90.4±1.2 during PVLA ($p<0.05$) or to 88.6±5.5 mmHg during PVLB. PVLA caused a significant increase in activity in both splenic afferent (Figure 3.4A, 3.5A) and renal efferent nerves (Figure 3.4C,3.5B). This was not observed following PVLB (Figure 3.4B, D and Figure 3.5).

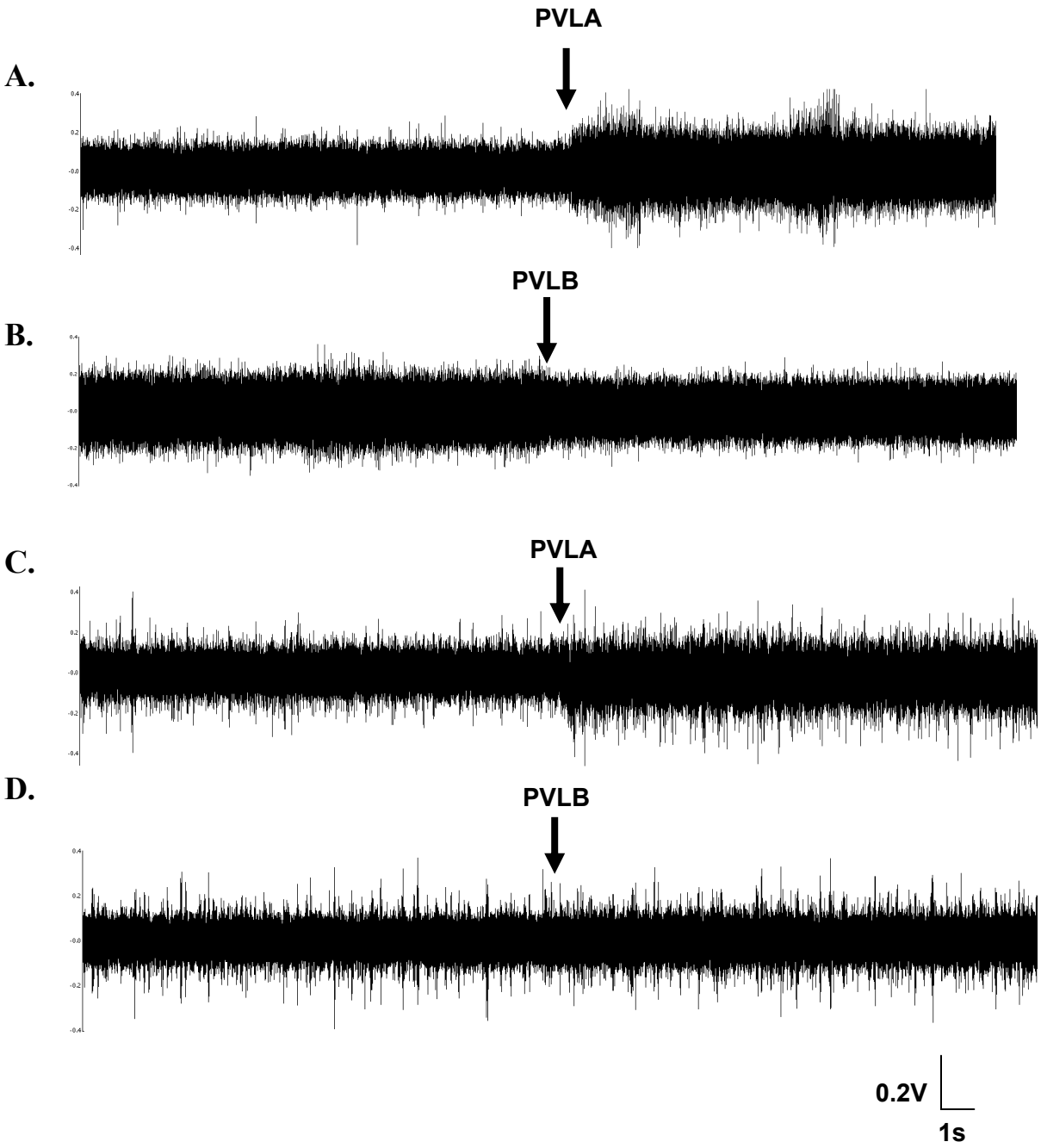

Figure 3.4: Representative nerve tracings. Effect of partial portal vein ligation on splenic afferent (panels A-B) and renal efferent (panels C-D) nerve activity. 20 second samples of nerve activity during baseline and PVL are shown.

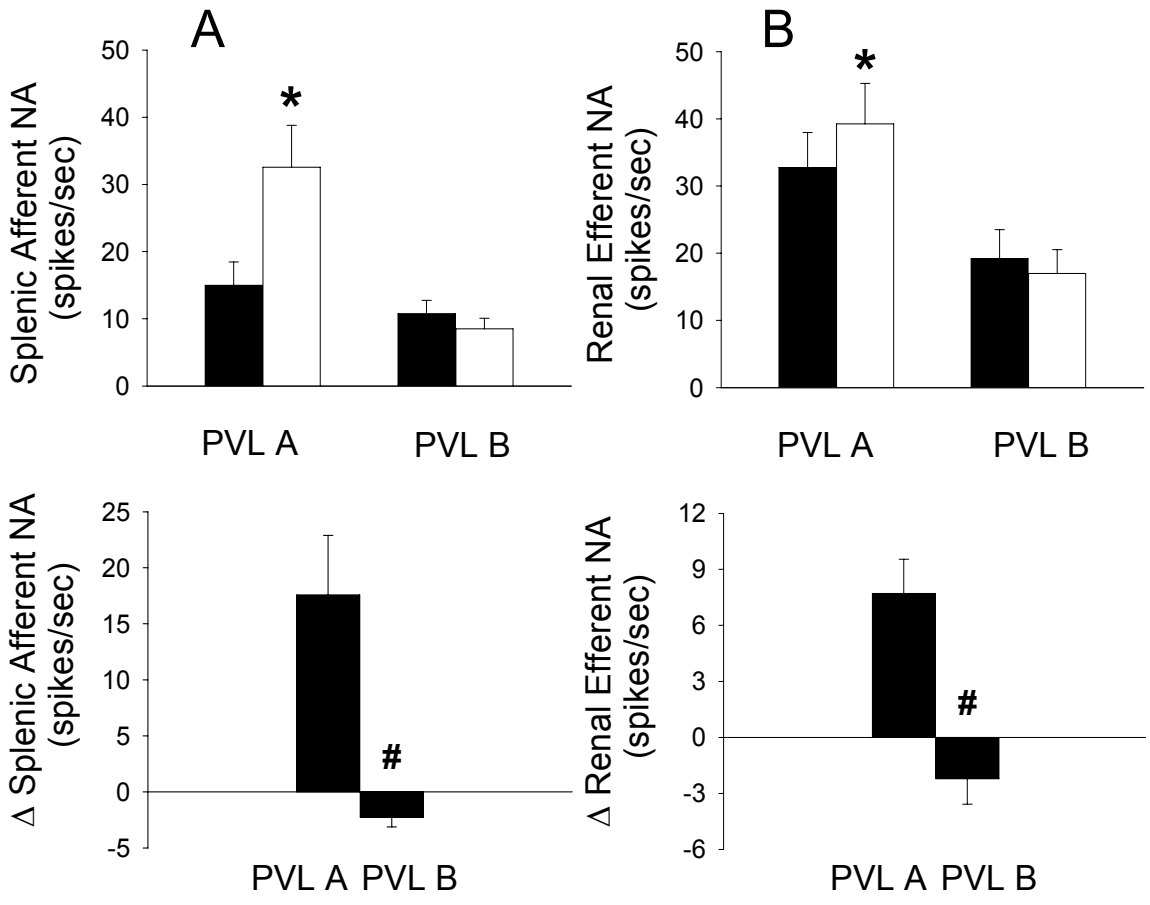

Figure 3.5: Splenic afferent (A) and Renal efferent (B) nerve activity after partial portal vein ligation either rostral (PVLA: Splenic Afferent: *n* = 7; Renal Efferent: *n* = 10) or caudal (PVLB: Splenic Afferent: *n* = 9; Renal Efferent: *n* = 9) to the porto-splenic junction. Data presented as absolute nerve activity (upper panels) and change in nerve activity (lower panels). Data are presented as means ± SEM, * Significant difference from baseline; # Significant difference between PVLA and PVLB, $p < 0.05$.

3.4 DISCUSSION

The fall in renal arterial conductance observed in intact animals after PVLA was completely abolished by renal denervation, by splenic denervation, by celiac ganglionectomy and by splenectomy. Renal conductance did not change in any of the groups (intact, denervated or splenectomized) during PVLB. Moreover, although both splenic afferent and renal efferent nerve activity increased during PVLA, this increase was not observed during PVLB. Had mesenteric congestion triggered a direct neural reflex (i.e. intestinal-renal reflex), we would have observed an increase in renal efferent nerve activity and a fall in renal conductance during PVLB. Similarly, had mesenteric congestion triggered an indirect neural reflex through the spleen (i.e. intestinal-spleno-renal reflex), we would have observed increases in *both* splenic afferent and renal efferent nerve activity during PVLB. We did not observe *any* changes in nerve activity or renal arterial conductance with PVLB. It appears therefore that, after PVL, the intestine does not initiate either direct or indirect neural reflexes to control RBF. The residual fall in RBF observed in the denervated animals subjected to PVLA or PVLB, may be attributed to the fall in MAP, as there was no change in renal arterial conductance. Based on these observations, we conclude that selective mesenteric congestion alone does not play a role in regulating RBF. By contrast, the fall in renal conductance in the intact animals after PVLA confirms our previous findings that there is neural modulation of RBF mediated through the spleen.

It is known that the hepatorenal reflex may be elicited by changes in intrahepatic blood flow(22). Thus PVLA could potentially, by reducing portal venous blood flow, have initiated the change in renal vascular conductance through the hepatorenal reflex.

PVLB does not cause such a marked fall in intrahepatic blood flow as PVLA because blood continues to flow unimpeded from the spleen into the portal vein and liver. The failure of PVLB to increase renal vascular conductance could then have been attributed to the smaller fall in intrahepatic blood flow. We eliminated the contribution of the spleen to changes in intrahepatic blood flow by splenectomizing the animals. Despite the fact that the fall in blood flow (-11.9±2.7mL/min) was then just as great as that observed after PVLA in the intact animals (-9.6±2.4mL/min) (14), PVLB still failed to elicit a change in renal vascular conductance. We conclude therefore that the fall in renal vascular conductance elicited by PVLA was mediated primarily through the splenorenal reflex, rather than through the hepatorenal reflex.

The role of the mesenteric vascular bed as a reflexogenic region had been investigated by others (3, 10, 13, 28). The most extensive study to date of intestinal-renal reflex regulation of RBF in PH was done by Miller et al. (1983) (21). They found that occlusion of the superior mesenteric vein in dogs (equivalent to PVLB in our experiments) caused a profound reduction in cardiac filling pressure and output, and a fall in RBF. Normalization of cardiac hemodynamics by intravenous fluid resuscitation did not restore RBF. Although splanchnic ganglionectomy did not prevent the fall in RBF, normalization of cardiac indices in these animals did partially restore RBF towards normal. The authors concluded that the renal perturbations observed in portal hypertension are due to an intestinal-renal reflex initiated by intestinal venous congestion (21). There are a few points to be considered as to why our results are at variance with this conclusion. First, in the absence of measures of systemic blood pressure or renal vascular conductance in the studies of Miller et al, it is impossible to conclude whether

there was any change in renal vascular tone. Secondly, it was not noted by those investigators whether or not their dogs had been splenectomized, so it is probable that the spleen was intact. This is critical, as the dog spleen differs in structure and function from that of the human and rat (20, 27). Sympathetic nerve stimulation in the dog has been shown to result in active expulsion of a large volume of blood (splenic contraction) (9)which would greatly complicate interpretation of the results of Miller et al's study. Thirdly, their experimental protocol was very different from our own. The superior mesenteric vein of the dogs was *completely occluded* (21)for two minutes. By contrast, we only partially occluded the portal/superior mesenteric vein for 10 minutes, while measuring PVP throughout to ensure that there was the same degree of PH in all animals. Complete occlusion of the superior mesenteric vein would have deprived the liver of its main blood supply, thus potentially causing ischemia of the hepatic tissues and subsequent metabolic derangement, which could ultimately have affected RBF. Fourthly, they did not measure nerve activity, which makes it difficult to conclusively establish the presence of a functioning neural reflex.

3.4.1 Perspectives and Significance

Pathophysiologically, we have evidence that the splenorenal reflex can contribute to PH-induced renal dysfunction. However, this reflex may also have an important role in normal physiology. We have shown that increased splenic venous pressure (in the absence of changes in blood flow) elevates splenic afferent nerve activity (24). However, a previous study from this laboratory (6) prompted us to consider that changes in splenic venous blood flow and intrasplenic NO biosynthesis may also be important, and may be responsible for activating the splenorenal reflex. While we acknowledge that this requires

further investigation, we propose that whereas increased splenic pressure initiates changes in central control of systemic blood pressure (23), the splenorenal reflex may be initiated by a reduction in splenic venous blood *flow*. The concept that there might be different types of nerve signaling within a single nerve is not without precedence; DiBona has shown that within the renal sympathetic nerves, specific subgroups of nerve fibres convey differential information encoded in the frequency domain of the firing (7).

Normally the changes in splenic blood flow and pressure are congruent. Thus, in hypervolemia, there is increased intrasplenic and mesenteric pressure and flow. The rise in intrasplenic pressure would increase signalling from the intrasplenic pressoreceptors and initiate a reflex reduction in systemic blood pressure. In the absence of any fall in splenic blood flow, there would be no increase in renal efferent nerve activity. There would also be no reason physiologically for the increased mesenteric blood volume to initiate a reflex to *conserve* renal salt and water. By contrast, in hypovolemia, there would be reduced intrasplenic and mesenteric pressure and flow. The reduction in splenic blood flow would, through reduced intrasplenic NO biosynthesis, induce a splenorenal reflex increase in renal sympathetic nerve activity. This would restore systemic blood pressure/volume both by increasing renin release and by reducing renal blood flow and increasing renal salt and water retention.

We propose that, in PH, there is a unique combination of increased splenic and mesenteric venous pressure and *reduced* splenic venous flow. It is this latter phenomenon which initiates the increase in renal sympathetic nerve activity and renal dysfunction. Under these circumstances, there would be no physiological basis for suggesting that either the increased intrasplenic or mesenteric venous pressure should initiate reflexes to

reduce renal salt and water excretion, as indeed our data show. We did not find any evidence that the mesenteric vascular congestion associated with PH contributes to increasing renal vascular resistance.

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CHAPTER 4:

SPLENIC NEUROHORMONAL MODULATION OF MESENTERIC ARTERIAL DIAMETER AND TONE

4.1 INTRODUCTION

Patients suffering from chronic liver diseases such as cirrhosis and viral hepatitis often develop portal hypertension (PH). This condition is defined by a portal venous pressure exceeding 10mmHg. PH, regardless of etiology (i.e. pre-, intra- or post-hepatic obstruction of portal venous blood flow), results in the most confounding and devastating complication of liver disease - the hyperdynamic circulation (8).

The hyperdynamic circulatory profile is characterized by increased cardiac output and heart rate with decreased arterial blood pressure despite the presence of an expanded plasma volume. A key feature of this syndrome is an overall decrease in systemic vascular resistance (9). This hemodynamic picture was first described over five decades ago (25) and, in spite of an immense amount of clinical and experimental investigation, it remains a serious and often fatal complication of PH, precipitating as it does the deadly sequelae of this syndrome (formation of ascites; esophageal variceal hemorrhage; hepatopulmonary syndrome; hepatic coma; hepato-renal syndrome and renal failure (19)). No clear delineation of the pathophysiological mechanisms underlying its development has been firmly established to date.

The currently accepted theory regarding the general development of the hyperdynamic circulation in PH was introduced as the “Peripheral Arterial Vasodilation Hypothesis” by Schrier et al in 1988 (31). In this theory, PH eventually induces peripheral arterial vasodilatation, which subsequently initiates the profound sodium and water retention characteristic of this syndrome. Uninhibited sodium and water retention then contributes to expansion of plasma blood volume which, coupled with peripheral vasodilatation,

fuels the descent into the hyperdynamic circulation. More recently, it has since been noted that vasodilation occurs soon after induction of portal hypertension, and is particularly marked in the splanchnic vascular beds (36). Thus, it has been postulated that PH first induces splanchnic vasodilatation which, through reflex peripheral and central mechanisms (26, 27, 29, 31, 32), establishes a generalized systemic arterial vasodilation.

Previous studies have suggested that reduced splanchnic vascular tone in PH involves a combination of (i) increased vasorelaxation as a result of enhanced release/levels of circulating vasodilatory factors, and (ii) hyporeactivity of these vessels to vasoconstrictors due to impaired vascular smooth muscle signaling (36). However, it is the vital link between PH and splanchnic vasodilatation entailing the above factors which has not yet been elucidated and has become the focus of recent inquiry.

The splanchnic arterial vasculature has been physiologically established as the chief site for changes in vascular resistance (36). Attention is beginning to focus on the superior mesenteric artery (SMA); this artery supplies the entire mesenteric arterial vascular bed, and is thus an ideal vessel of study in PH-induced hyperdynamic circulation (36). Close investigation of a chronic portal vein ligated (PVL) rat model of PH has revealed that for the first two days following induction of PH, SMA blood flow is significantly decreased, a result of a significant fall in mesenteric vascular conductance (12). The increased mesenteric arterial resistance has been attributed to a myogenic reflex vasoconstriction in response to the acute increase in portal venous pressure initially induced by PVL. However, by day 4 of PVL, the rats exhibited *increased* SMA flow and *reduced* total peripheral resistance, changes characteristic of a hyperdynamic circulation (12). This pattern of a transient mesenteric vasoconstriction preceding the establishment of the

hyperdynamic hemodynamic profile has been observed by other investigators, and recently has been shown to elicit an increase in endothelial nitric oxide synthase (eNOS) activity in the SMA as early as 10 hours after induction of PH by PVL in rats (1, 34). Interestingly, PH in the absence of mesenteric vasoconstriction (PVL inducing an attenuated increase in portal venous pressure) is not associated with mesenteric arterial upregulation of eNOS or any changes in mesenteric vascular responsiveness (34). Thus, it is now believed that the initial mesenteric vasoconstriction leads to a series of events preceding the development of splanchnic vasodilatation, which, in turn, goes on to establish peripheral arterial vasodilation and a hyperdynamic circulation.

We investigated the mechanism by which PH initiated the initial mesenteric arterial vasoconstriction. We have long been interested in the role of the spleen in cardiovascular regulation and have demonstrated the contributions of this organ to control of blood pressure (14, 15) and intravascular volume (5, 20, 21, 33). In addition to physiological regulation, the spleen is implicated in PH; given that splenic venous effluent flows into the portal vein, any increase in portal venous pressure ultimately results in increased splenic microvascular pressure (6, 33). Recently, we have established that the spleen contributes to PH-induced renal dysfunction as a result of modulation of renal vascular tone via the spleno-renal reflex (16, 17). In addition, a previous study suggested the possibility of an intestinal-splenic reflex pathway (22). Based on this information, and the knowledge that PH triggers increased splenic afferent nerve activity (17, 22), we hypothesized that the rise in splenic venous pressure associated with PH, may trigger a spleno-mesenteric neural reflex mediated by splenic afferent and mesenteric efferent nerves. We postulated that this putative reflex contributes to the increased mesenteric

arterial tone observed after an acute increase in portal venous pressure. Our studies reveal that partial splenic vein occlusion (SVO) induces a significant increase in mesenteric efferent nerve activity, which is abolished by prior splenic denervation. We subsequently studied the potential mesenteric hemodynamic consequences of this reflex by simultaneous measurement of mean arterial pressure (MAP) and superior mesenteric arterial blood flow (SMAF), from which we calculated mesenteric arterial conductance (SMAC). We found that SVO causes a significant reduction in SMAC, which is attenuated by splenic denervation and relies, in part on ANG II release. Although this reduction in SMAC is not affected by complete mesenteric denervation, it is also partially mediated by α_1 adrenergic receptors.

As the true site of mesenteric arterial resistance lies in the microvasculature (vessel diameter 10 -150 μm (36)), we sought to verify our hemodynamic observations by direct visualization of mesenteric resistance arteries and veins by intravital microscopy. In accordance with the above findings, SVO caused a modest fall in mesenteric arterial diameter, which was absent in splenic denervated, renal denervated animals or animals in which vascular AT_1 receptors were pharmacologically blocked. Virtually no change was observed in mesenteric veins during SVO.

We conclude that the spleen is involved in the early mesenteric arterial vasoconstriction of PH in part via (a) release of ANG II and (b) activation of α_1 adrenergic receptors. Release of ANG II is primarily mediated by the spleno-renal reflex, with potential contributions from intrarenal baroreceptors and spleno-mesenteric reflex activation of mesenteric perivascular angiotensinergic nerves.

4.2 METHODS

All experimental procedures were approved by the local Animal Welfare Committee in accordance with the guidelines issued by the Canada Council on Animal Care. All animals were euthanized with an anesthetic overdose of pentobarbital sodium at the completion of each experiment (96mg i.v.; Vetoquinol, Lavaltrie, QC, Canada).

4.2.1 Animals and Housing

Seventy-five male Long-Evans rats (300-550g) were obtained from Charles River Canada (Quebec, Canada) and housed for one week in the University of Alberta Animal Facility before commencement of experiments. They were fed a standard 0.3% sodium rat chow maintenance diet (to prevent obesity) and water ad libitum (24). Animals were maintained in a temperature- and humidity-controlled environment with a12:12hr light-dark cycle.

4.2.2 Anesthesia and Surgery

Anesthesia was induced with pentobarbital sodium (65 mg/kg body wt i.p.; Vetoquinol, QC, Canada), followed by maintenance with Inactin [ethyl-(methylpropyl)-malonyl-thio-urea, 100mg/kg body wt s.c.; Sigma]. Body temperature was monitored and maintained at 36-37˚C with a Deltaphase thermal heating pad (Braintree Scientific, Braintree, MA) for nerve recording experiments to minimize electrical interference; a homeothermic blanket (Harvard Apparatus) was used for hemodynamic and intravital studies. The femoral vein and artery were cannulated with Silastic [0.51mm inner diameter (ID), 0.94mm outer diameter (OD); Dow Corning, Midland, MI] and polyethylene (PE-50, 0.58mm ID, 0.97mm OD; VWR International, Mississauga, ON Canada) tubing for administration of

isotonic saline (3mL/hr) and monitoring of systemic arterial pressure, respectively. Via a midline abdominal laparotomy, the stomach was reflected onto the thorax and the spleen was cleared from its connective tissue attachments to the stomach and large intestine. The gastric vessels were exposed and a Silastic™ cannula (0.30 mm ID, 0.64mm OD, Dow Corning) was inserted occlusively into the gastric vein and advanced until the tip lay at the junction of the splenic vein. For intravital studies, the gastric vein was similarly cannulated with Micro-Renethane® tubing (MRE-025; Braintree Scientific Inc., Braintree, MA). In all cases, this cannula was connected to a pressure transducer for online measurement of splenic venous pressure.

A Prolene 6-0 loop (Ethicon, USA) was placed loosely around the splenic vein at its junction with the portal vein in all experiments except those of the intravital study. In these experiments, an inflatable vascular occluder balloon (#18080-01; Fine Science Tools, BC, Canada) was sutured around the splenic vein at the described location to minimize movement of the intravital image during elevation of splenic venous pressure. Both methods allowed for controlled partial occlusion of the splenic vein.

For hemodynamic experiments involving selective mesenteric blockade of ANG II AT_1-, AVP V_{1a} -, and/or α_1 adrenergic receptors, one of the earliest branches of the SMA (inferior pancreatico-duodenal) was cannulated with slightly stretched PE-10 tubing (0.28mm ID, 0.61mm OD; VWR International, Mississauga, ON, Canada). The cannula tip was retrogradely advanced to the main trunk of the SMA, such that the drug infusion entered the vessel and perfused the entire mesenteric vascular bed. The cannula was held in place with 4-0 silk sutures and a small drop of surgical tissue adhesive (3M VetBond; Animal Care Products, St. Paul, MN). The efficacy of this cannulation for mesenteric

perfusion was verified post-mortem by Evans-Blue dye infusion, which revealed the entire mesenteric circulation including the proximal colon to be dyed. The caecum and distal colon were not perfused with dye (unpublished observation, S. Hamza). The cannula position did not hamper extravascular flow probe placement around the SMA or interfere with the blood flow signal.

4.2.3 Denervations

In splenic denervation experiments, the splenic nerves were stripped from the main trunks of the splenic artery and vein, distal to branching of the vessels toward the spleen (4). In bilateral renal denervation experiments, the renal nerves were stripped from the right and left renal vessels, which were subsequently painted with 5% phenol solution to destroy any remaining nerve fibres (23). For mesenteric denervation experiments, all nerves were carefully stripped from the main trunk of the SMA, close to its origin from the abdominal aorta. Great care was taken to complete this without damaging the intestinal lymph duct running alongside the vessel at this point. This procedure results in effective denervation of the entire mesentery (2).

4.2.4 Drugs

For experiments involving blockade of vasoconstrictor systems, we opted to selectively infuse pharmacological agents directly into the mesenteric circulation to prevent excessive disruption of resting systemic hemodynamic parameters; thus avoiding mis-interpretation of results. This entailed cannulation of a branch of the SMA as outlined in section 4.2.2.

Pharmacological blockade of angiotensin II AT_1 receptors was achieved by infusion of Losartan potassium (Fluka). Losartan was dissolved in double distilled water for administration of 0.5mg/kg (~0.1 mL bolus injection into the mesenteric arterial cannula) following completion of the surgical protocol and before commencement of the pre-experiment stabilization period. This dose was determined to result in minimal systemic effects in pilot experiments (unpublished observation, S. Hamza). The efficacy of mesenteric AT_1 receptor blockade was verified at experiment end by: (a) mesenteric infusion of 0.0099 μg/kg ANG II (0.1mL bolus) with the subsequent absence of any reduction in mesenteric blood flow and/or (b) elevated superior mesenteric arterial conductance relative to pre-infusion baseline values.

The AVP V_{1a} receptor blocker SR49059 (gift from Sanofi-Aventis) was initially solubilized in dimethyl sulfoxide (DMSO; Sigma), then subsequently diluted in 10% DMSO to create a 2 mg/mL solution. 0.033mg/kg SR49059 (~0.1mL bolus) was similarly injected into the mesenteric cannula, minutes before or after administration of Losartan. We have determined that 10% DMSO alone does not result in any physiological effects (unpublished observation, S.Hamza, Appendix C). The efficacy of V_{1a} receptor blockade was verified at experiment end by mesenteric infusion of 14.8ng/kg AVP (0.1mL bolus) with subsequent absence of any drop in mesenteric blood flow.

The α_1 adrenergic receptor blocker Prazosin hydrachloride (Sigma) was dissolved in warmed distilled water to create a 1 mg/mL solution. 0.3 mg/kg Prazosin (~0.1mL bolus) was injected into the mesenteric cannula, minutes before or after administration of Losartan. The efficacy of α_1 adrenergic blockade was verified at experiment end by (a)

mesenteric infusion of 1µg/kg phenylephrine in distilled water and/or (b) elevated superior mesenteric arterial conductance relative to pre-infusion baseline values.

4.2.5 Whole Fiber Nerve Recording

For determination of mesenteric efferent nerve activity, a loop of jejunum/ileum consisting of 3-4 mesenteric arcades (range corresponding to mesenteric arcades 9-15) was gently arranged over a small black plastic support plate, which had been placed in the abdomen (black background intended to help visualize mesenteric nerves) (Figure 4.1). The edges of the abdominal incision, were then sutured (3-0 cotton, Davis-Geck, American Cyanamid) to a pre-made steel support ring, which was subsequently raised to create an abdominal "well". The abdomen was filled with heavy mineral oil (Laboratoire Atlas, Montreal, QC, Canada) to submerge the mesenteric loop. A mesenteric nerve bundle was located running along a first order mesenteric neurovascular bundle, with the SMA designated zero order. An ~1-2 cm length of the mesenteric nerve was gently isolated from the vessels and surrounding connective tissue under the oil by blunt dissection with fine forceps (No. 5 Dumont, 0.05 x 0.01mm; Fine Science Tools, BC, Canada). The distal end (closest to the intestinal loop) was cut with fine tissue scissors for recording of efferent nerve signals. The cut end of the nerve was gently wrapped around bipolar silver-platinum electrodes, and the nerve signal was amplified (preamplifier, Gould) and filtered between 100 and 10,000 Hz. Output from the amplifier was fed to a loudspeaker and displayed on a PC for online recording of nerve activity at a sampling rate of 4 kHz (Chart 5 acquisition software with Spike Histogram Module, PowerLab, ADInstruments, Castle Hill, Australia).

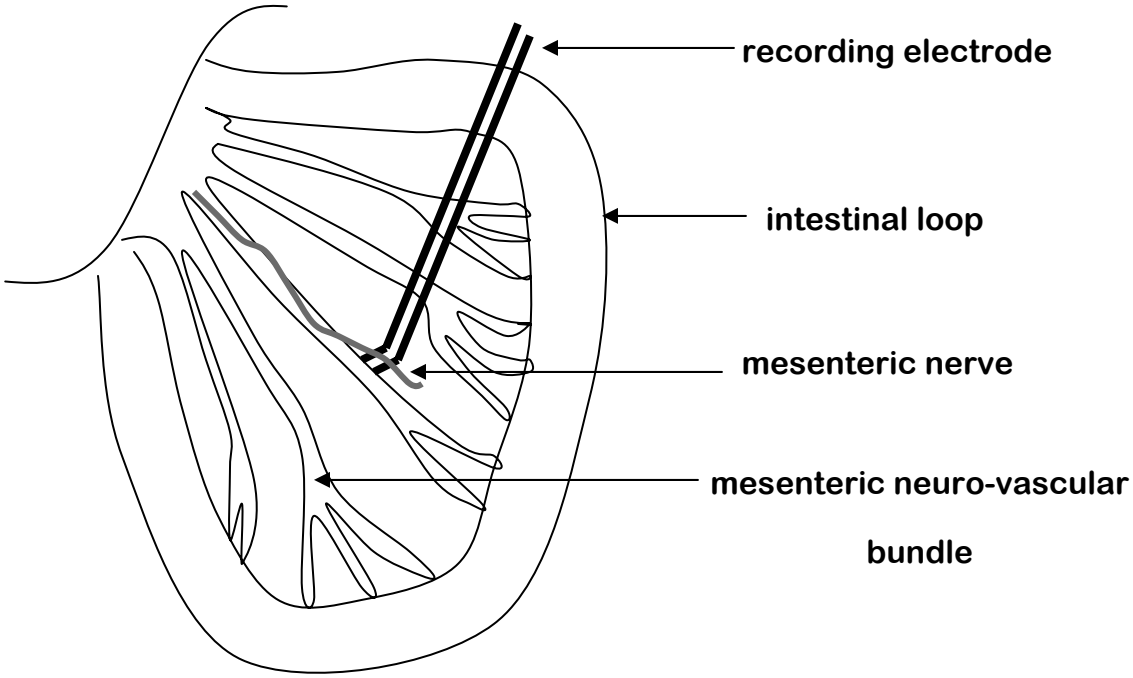

Figure 4.1 Schematic representation of acute mesenteric efferent nerve preparation. A loop of jejunum/ileum corresponding to mesenteric vascular arcades ~ 9-15 was gently arranged over a black plastic support plate placed into the abdomen of the animal. Under mineral oil, a mesenteric nerve was identified along a first order mesenteric vascular bundle (superior mesenteric artery designated zero order) and isolated from surrounding tissues. The nerve was cut distally and wrapped around a bipolar silver-platinum recording electrode.

4.2.5.1 Experimental Protocol

Following surgery, intact (n=11) or splenic denervated (SD; n=7) animals were stabilized for 30 minutes before online recording of baseline mesenteric efferent nerve activity for 20 minutes. At this point, the splenic venous ligature was tightened to increase splenic venous pressure to 20-25mmHg (the degree observed during PH (17)). Nerve activity was recorded for a further 10 minutes. Analysis of nerve activity was based on average firing rate (spikes/sec) of action potentials identified in the raw recordings. Background noise was determined directly from the recorded nerve trace (as recommended by ADInstruments) as this method does not differ from recording of postmortem signals at experiment end (S. Hamza, unpublished observation).

4.2.6 Hemodynamic Study – SMA Blood Flow and Vascular Conductance

Following the outlined surgical preparation, the SMA was gently cleared at a point close to its origin from the abdominal aorta. A factory calibrated flow probe (1RB series, Transonic Systems, Ithaca, NY) was positioned around the SMA and covered in conducting gel. Prior to use, zero-flow reading was confirmed by placing the probe in a non-turbulent water bath. Animals were stabilized for 30 minutes, during which, temperature, MAP and SMAF were continuously monitored.

4.2.6.1 Experimental Protocol

Following stabilization, baseline MAP, SMAF and splenic venous pressure were recorded online in Intact (n=10), SD (n=8), mesenteric denervated (MD; n=6), bilateral renal denervated (RD; n=5), Losartan treated (LOS; n=5), Losartan + SR49059 treated (LOS+SR49059; n=4) or Losartan + Prazosin treated (LOS + Prazosin; n=6) animals.

The splenic venous ligature was then tightened to selectively increase splenic venous pressure to 20-25mmHg and hemodynamic parameters were recorded for a further 3 min.

4.2.7 Intravital Microscopy

Following cannulation of the gastric vein and implantation of the vascular occluder balloon around the splenic vein as outlined above, the stomach was gently reflected back into the abdomen and the gastric venous cannula and balloon actuator tube were drawn caudally and loosely sutured to the right edge of the abdominal incision. The top half of the abdominal incision was then loosely sutured closed (4-0 silk, Ethicon, USA). The animal was prepared for direct visualization of mesenteric microvessels by intravital microscopy as previously described (10). Briefly, the animal was transferred to a plexiglass platform equipped with a stage and heating pad. A loop of jejunum/ileum (corresponding to mesenteric arcades ~9-15) was gently exteriorized onto moist gauze, extreme care taken to gently handle the intestinal portion of the loops only with moist cotton swabs. At no time was tension applied to the intestinal loop or was the clear mesenteric tissue touched directly with the cotton swabs during this procedure. The gut loop was wrapped in the warm, moist gauze and the animal carefully turned onto its left side. The animal's position was adjusted such that the abdomen was flush against the stage portion of the plexiglass platform, without compression of the exteriorized mesenteric loop (Figure 4.2). The gauze was unwrapped from the intestinal loop and removed. The loop was then carefully arranged over a glass microscope slide mounted onto the plexiglass stage and gently held in place with microclamps. This arrangement displayed 3-4 mesenteric windows. During this time, the tissue was kept moist by repeated application of warm saline. The mesentery was quickly covered with Saran wrap

to keep the tissues moist and preserve the viability of the microvessels. The plexiglass platform holding the animal preparation was transferred to the stage of an inverted microscope (Leica). Under 20X optical magnification, paired mesenteric arterioles (40-70 μm diameter) and venules (70-110 μm) were visualized in the clear mesenteric windows. This image was displayed on a high-resolution monitor (RadioShack, 49-2511, Canada) using a black and white CCD camera (Hitachi KP-M2 S1, USA).

Stabilized images were recorded throughout the experiment by VCR (Panasonic PV-V4023-K, USA) for off-line computerized image analysis at experiment end (Capiscope, KK Technology, Colyton, Devon, UK) using frame grabber analysis software (Matrox Meteor II, USA).

4.2.7.1 Experimental Protocol

Following 30 min stabilization, baseline MAP, splenic venous pressure and the mesenteric microvessel image were recorded for 5-10 minutes in Intact (n=13), SD (n=10), RD (n=4) and systemic Losartan treated (3 mg/kg i.v., LOS; n=6) animals. The vascular occluder balloon was inflated to increase splenic venous pressure to 20-25mmHg and all parameters were recorded for a further 5 min. At experiment end, external wall diameter of vessels was measured using the image analysis software (Capiscope), which was previously calibrated with a micrometer custom designed for the camera and monitor used. Three consecutive measurements were taken at each time point and averaged to give a final value.

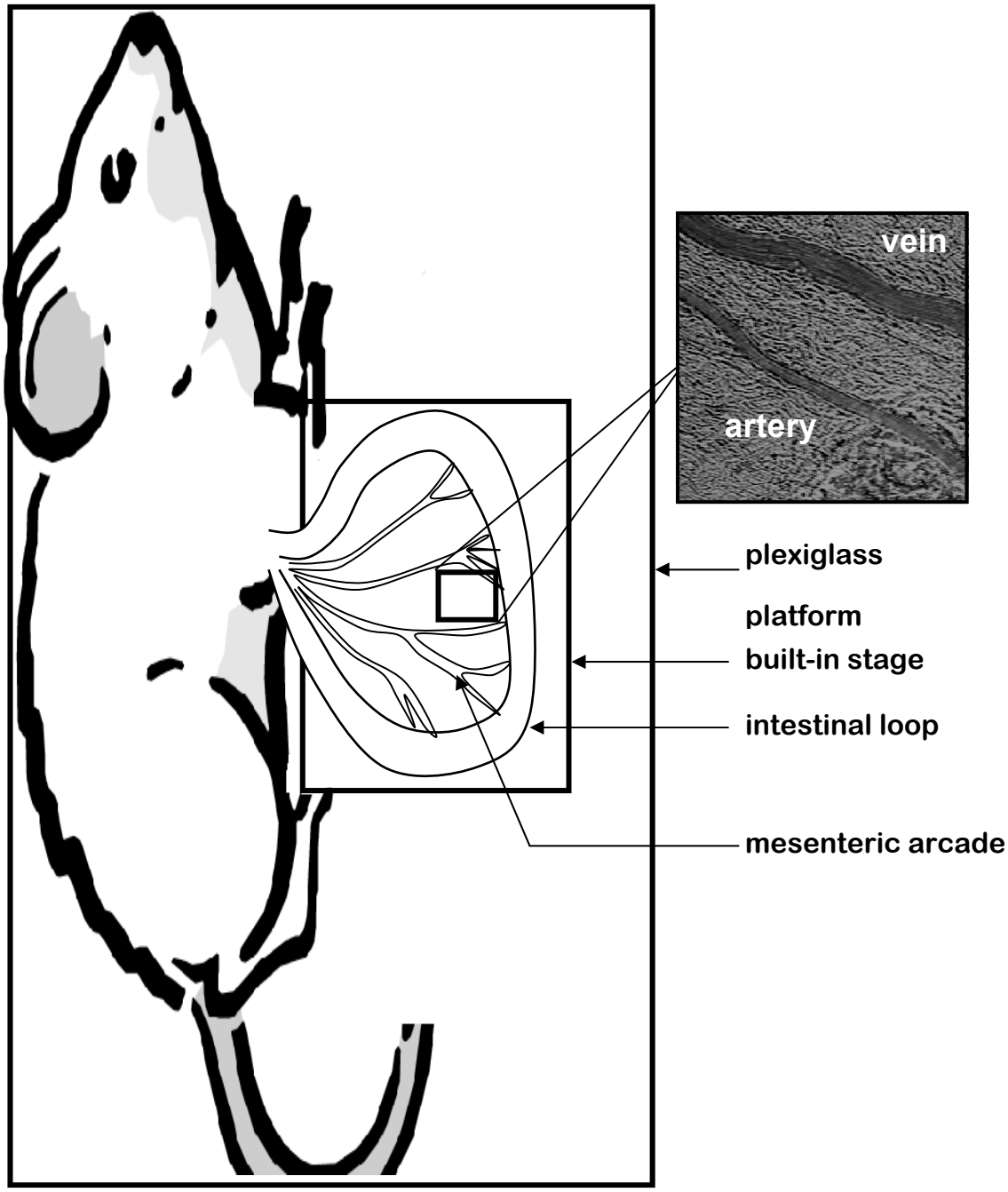

Figure 4.2 Schematic representation of intravital microscopy experimental preparation. Following instrumentation, the animal is laid onto its left side on a plexiglass platform equipped with a raised stage. The intestinal loop is gently arranged over a glass slide placed onto the stage such that clear mesenteric windows are exposed; the loop is gently held in place by small rubberized retractors. An inverted microscope is focused in the mesenteric windows to visualize resistance vessels (inset).

4.2.8 Data Analysis

Unless otherwise stated, data were analyzed with One Way Repeated Measures ANOVA with Student-Newman Keuls post-hoc test (Figures 4.3, 4.4, 4.5, 4.6, 4.7). Two Way Repeated Measures ANOVA was used for comparing data between treatment groups for hemodynamic and vessel diameter studies (Figures 4.4, 4.5, 4.6, 4.7). Correlation between data sets was analyzed with the Spearman Correlation test. Significance was accepted at $p < 0.05$.

4.3 RESULTS

4.3.1 Mesenteric Efferent Nerve Activity

Mean baseline splenic venous pressure for all animals in the mesenteric nerve activity study was 4.8 ± 0.4 mmHg (n=18) and was elevated to 20-25mmHg (mean 23.4 ± 0.3 mmHg) by partial SVO. Baseline MAP was similar in intact and splenic denervated animals. Upon SVO, MAP fell significantly in intact animals (98.8 ± 3.0 to 91.5 ± 3.1 mmHg, $p < 0.05$; n=11), however, this fall was virtually absent in animals which had been splenic denervated (106.3 ± 5.1 to 103.3 ± 5.1 mmHg; n=7).

In response to SVO, mesenteric efferent nerve activity rose (Figure 4.3 A), an effect which was abolished by prior splenic denervation (Figure 4.3 B) and was not correlated to changes in MAP (correlation coefficient: -0.101, $p > 0.05$). Of note, resting mesenteric efferent nerve activity was significantly higher in SD animals ($p < 0.05$, Fig 4.3).

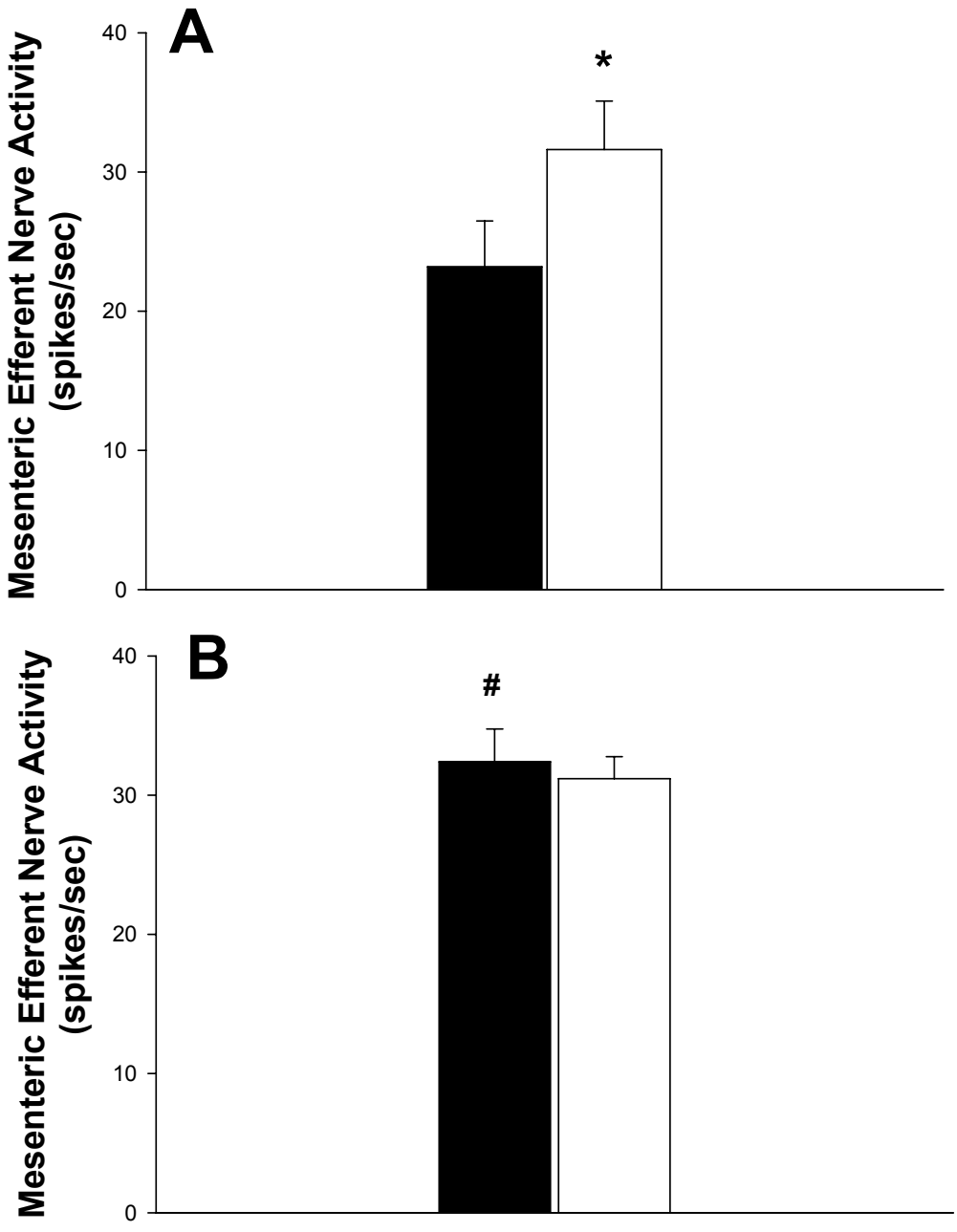

Figure 4.3 Effect of partial splenic vein occlusion (SVO) on mesenteric efferent nerve activity in *A:* intact (n= 11) and *B:* splenic denervated (n = 7) rats. Black bars represent baseline values; open bars represent nerve activity during partial SVO. Data are presented as means ± SEM. *Significant difference from baseline. #Significant difference from intact baseline nerve activity; $p< 0.05$.

4.3.2 Hemodynamic Effects– Superior Mesenteric Arterial Blood Flow and Conductance

Mean baseline splenic venous pressure for all animals in this study was 5.9 ± 0.3 mmHg and this was raised to 20-25 mmHg (mean: 24.3 ± 0.4 mmHg, n=44) by partial SVO.

Mean arterial pressure: Baseline MAP of intact animals and all experimental groups was similar (Table 4.1), although this was lower in animals in which components of both the renin-angiotensin and sympathetic nervous systems have been compromised. In response to SVO, MAP fell significantly ($p < 0.05$, Figure 4.4). There was no such change in the splenic denervated animals (-1.8 ± 1.7 mmHg at 10 seconds $p < 0.05$, Figure 4.4 A). In the mesenteric denervated animals, the SVO-induced drop in MAP mirrored that seen in the intact animals ($p< 0.05$, Figure 4.4 B). The SVO-induced drop in MAP was similarly unaffected by bilateral renal denervation, Losartan, Losartan + SR49059 or Losartan + Prazosin treatment (Figure 4.4 C, D, E, F).

Table 4.1 Baseline hemodynamic values for rats included in the measurement of mesenteric hemodynamic responses to partial splenic vein occlusion.

Group	MAP (mmHg)	Superior Mesenteric Arterial Flow (mL/min)	Superior Mesenteric Arterial Conductance ($mLmin^{-1}mmHg^{-1}$)
Intact (n=10)	97.0 ± 3.8	10.9 ± 1.2	0.114 ± 0.01
SD (n=8)	98.2 ± 5.5	10.4 ± 1.2	0.107 ± 0.01
MD (n=6)	98.2 ± 4.5	10.8 ± 1.1	0.111 ± 0.01
RD (n=5)	87.7 ±2.3	8.9 ± 0.7	0.101 ± 0.01
LOS (n=5)	96.6 ± 3.1	8.3 ± 1.1	0.086 ± 0.01
LOS+SR49059 (n=4)	84.2 ± 1.8	8.5 ± 0.5	0.109 ± 0.01
LOS+Prazosin (n=6)	70.2 ± 2.5*	6.1 ± 0.4*	0.087 ± 0.01

*Significant difference from Intact group: $p<0.05$ One Way ANOVA.

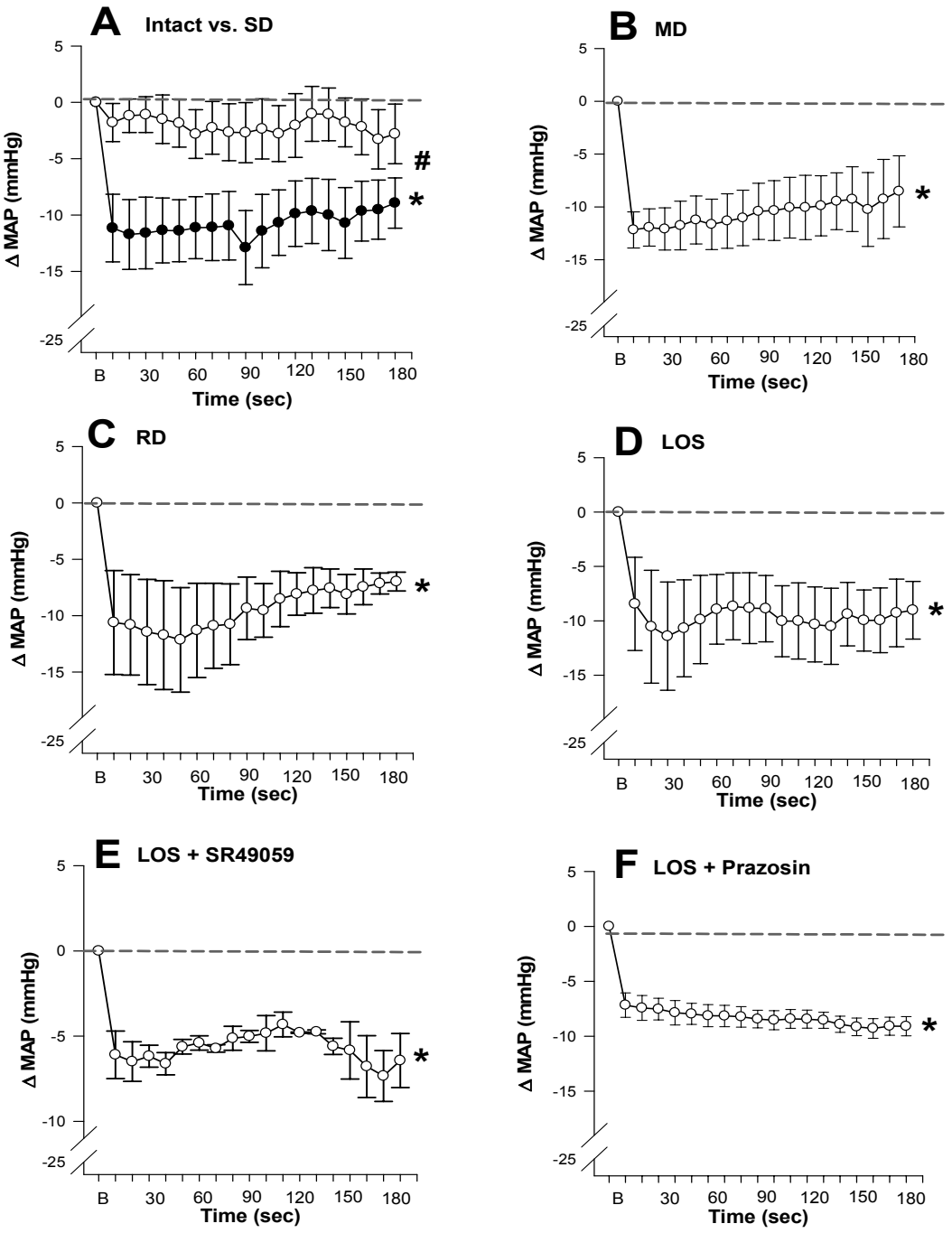

Figure 4.4 Effect of partial splenic vein occlusion on change of mean arterial pressure (MAP) from baseline in *A*: Intact (n=10); splenic denervated (SD; n=8), *B*: mesenteric denervated (MD; n=6), *C*: renal denervated (RD; n=5), *D*: Losartan treated (LOS; n=5), *E*: Losartan & SR49059 treated (LOS + SR49059; n=4) and *F*: Losartan & Prazosin treated (LOS+Prazosin; n=6) rats. Closed circles: Intact; Open circles: experimental groups. Data are presented as means ± SEM. *Significant difference from own baseline; #Significant difference from Intact group, $p < 0.05$.

Superior mesenteric artery blood flow: There was no difference in baseline SMAF across all groups with the exception of animals in which both renin-angiotensin and sympathetic nervous systems have been pharmacologically compromised (Table 4.1). When splenic venous pressure was increased by partial SVO, there was a significant decrease in SMAF from baseline in intact animals ($p < 0.05$, Figure 4.5 A). Although this response was also observed in splenic denervated animals ($p < 0.05$, Figure 4.5 A), the magnitude of the reduction in SMAF from baseline was significantly reduced ($p < 0.05$, Figure 4.5 A). After mesenteric denervation, renal denervation, or after administration of Losartan, SMAF dropped in response to increased splenic venous pressure to a similar degree to that seen in the intact animals (Figure 4.5 B, C, D). Although combined mesenteric AT_1 and V_{1a} receptor blockade did not prevent the SVO-induced fall in SMAF, the drop was significantly smaller than that observed in intact animals ($p < 0.05$, Figure 4.5 E). Similarly, combined mesenteric AT_1 and α_1 adrenergic receptor blockade significantly attenuated, but did not completely prevent the fall in SMAF upon SVO ($p<0.05$, Figure 4.5 F).

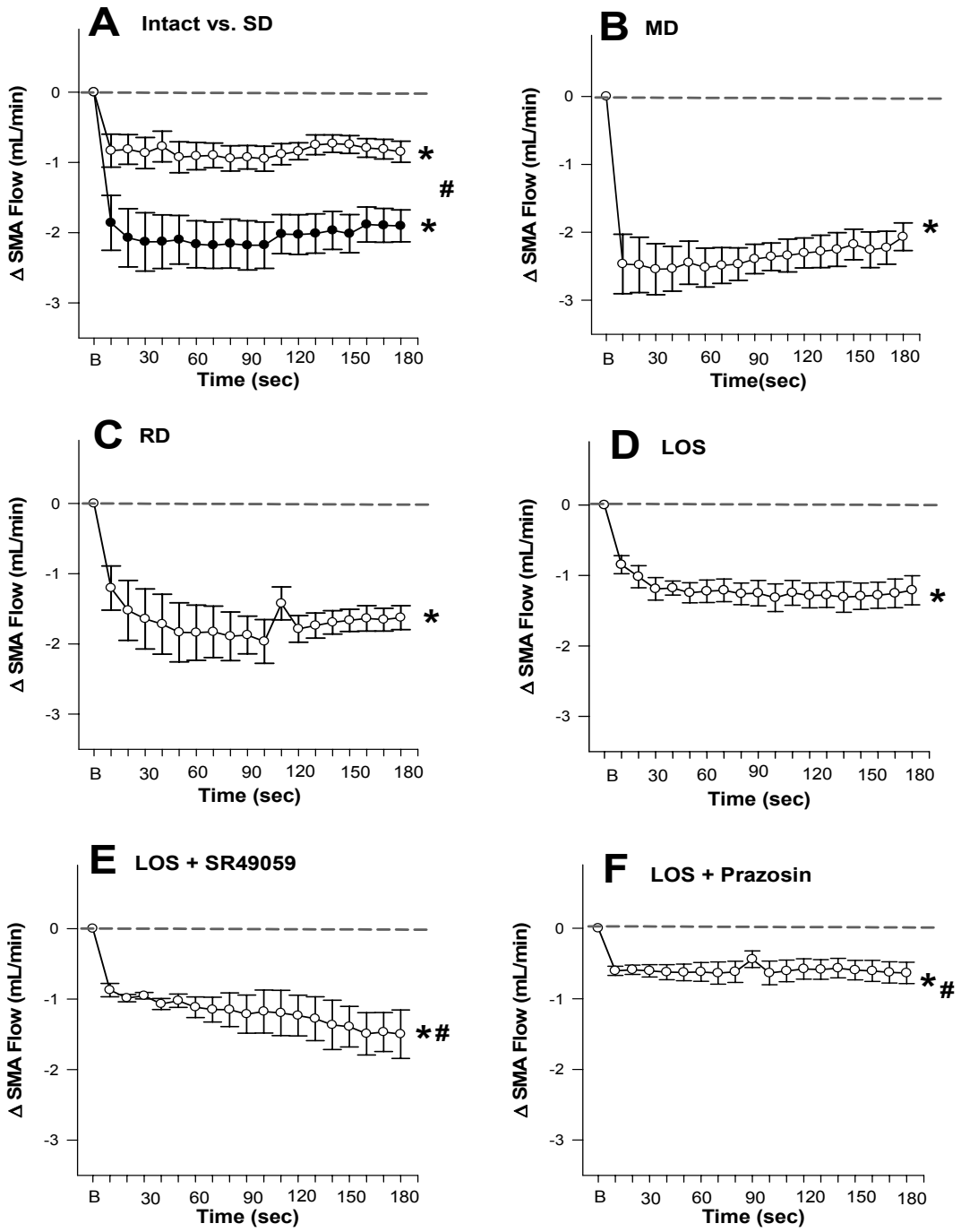

Figure 4.5 Effect of partial splenic vein occlusion on change in superior mesenteric arterial (SMA) blood flow from baseline of *A*: Intact (n=10); splenic denervated (SD; n=8), *B*: mesenteric denervated (MD; n=6), *C*: renal denervated (RD; n=5), *D*: Losartan treated (LOS; n=5), *E*: Losartan + SR49059 treated (LOS + SR49059; n=4) and *F*: Losartan + Prazosin treated (LOS + Prazosin; n=6) rats. Closed circles: Intact; Open circles: experimental groups. Data are presented as means ± SEM. *Significant difference from own baseline; #Significant difference from Intact group, $p < 0.05$.

Mesenteric arterial conductance: Mesenteric arterial conductance (*K*) was calculated as the ratio of superior mesenteric arterial blood flow (*Q*) to mesenteric perfusion pressure (*P*): $K = Q/P$. Baseline superior mesenteric arterial conductance (SMAC) was similar across all groups (Table 4.1). Upon SVO, SMAC dropped immediately and significantly from baseline in intact animals ($p < 0.05$, Figure 4.6 A) and remained depressed for the duration of SVO. In splenic denervated animals, there was also a significant SVO-induced drop in SMAC (Figure 4.6 A), although the response was significantly reduced compared to intact animals ($p < 0.05$, Figure 4.6 A). In mesenteric denervated animals, SMAC fell significantly from baseline in response to SVO ($p < 0.05$, Figure 4.6 B), and with a magnitude no different from that observed in intact animals (Figure 4.6 B). Bilateral renal denervation abolished the initial SVO-induced drop in SMAC (at 10 seconds) although, over time, SMAC progressively declined until it had fallen to the same degree observed in intact animals (Figure 4.6 C). Losartan treatment did not abolish the SVO-induced drop in SMAC, however this was significantly reduced compared to Intact animals ($p<0.05$, Figure 4.6 D). Losartan + SR49059 blocked the SVO-induced fall in SMAC compared to intact animals ($p<0.05$, Figure 4.6 E). Interestingly, mesenteric infusion of Losartan + Prazosin completely prevented any fall in SMAC in response to SVO ($p<0.05$, Figure 4.6 F). SMAC was not correlated with MAP during SVO in intact (correlation coefficient: 0.282, $p > 0.05$); splenic denervated (correlation coefficient:-0.424, $p > 0.05$); mesenteric denervated (correlation coefficient: -0.0468, $p > 0.05$); Losartan treated (correlation coefficient: -0.460, $p > 0.05$); Losartan + SR 49059 treated (correlation coefficient: -0.0294, $p > 0.05$) animals. On the other hand, SMAC was negatively correlated with MAP in bilateral renal denervated (correlation coefficient: -

0.840, $p < 0.05$) and Losartan + Prazosin treated (correlation coefficient: -0.975, $p<0.05$) animals.

4.3.3 In Vivo Vascular Effects – Visualization of Mesenteric Resistance Vessels With Intravital Microscopy

Partial SVO raised splenic venous pressure to 20-25mmHg (mean: 24.6 ± 0.6 mmHg; n=29). Resting MAP and average baseline mesenteric vessel diameter of animals included in this study was similar to all other groups investigated (Table 4.2). Immediately upon SVO (at 15 seconds after initiation of SVO), mesenteric arterial diameter dropped in intact animals (-3.1 ± 1.1 μm; Figure 4.7 A, 4.8), but did not reach significance from baseline. There was no such change in arterial diameter after splenic denervation (+0.32 ± 0.5 μm; Figure 4.7 A, 4.8), bilateral renal denervation (+1.4 ± 0.6 μm, Figure 4.7 C, 4.8) or systemic Losartan treatment (+0.45 ± 0.3 μm, Figure 4.7 E, 4.8); these changes were all significantly different from intact animals ($p < 0.05$, Figure 4.7 A, C, E, Fig 4.8). SVO induced no change in mesenteric vein diameter in any of the groups (Figure 4.7 B, D, F).

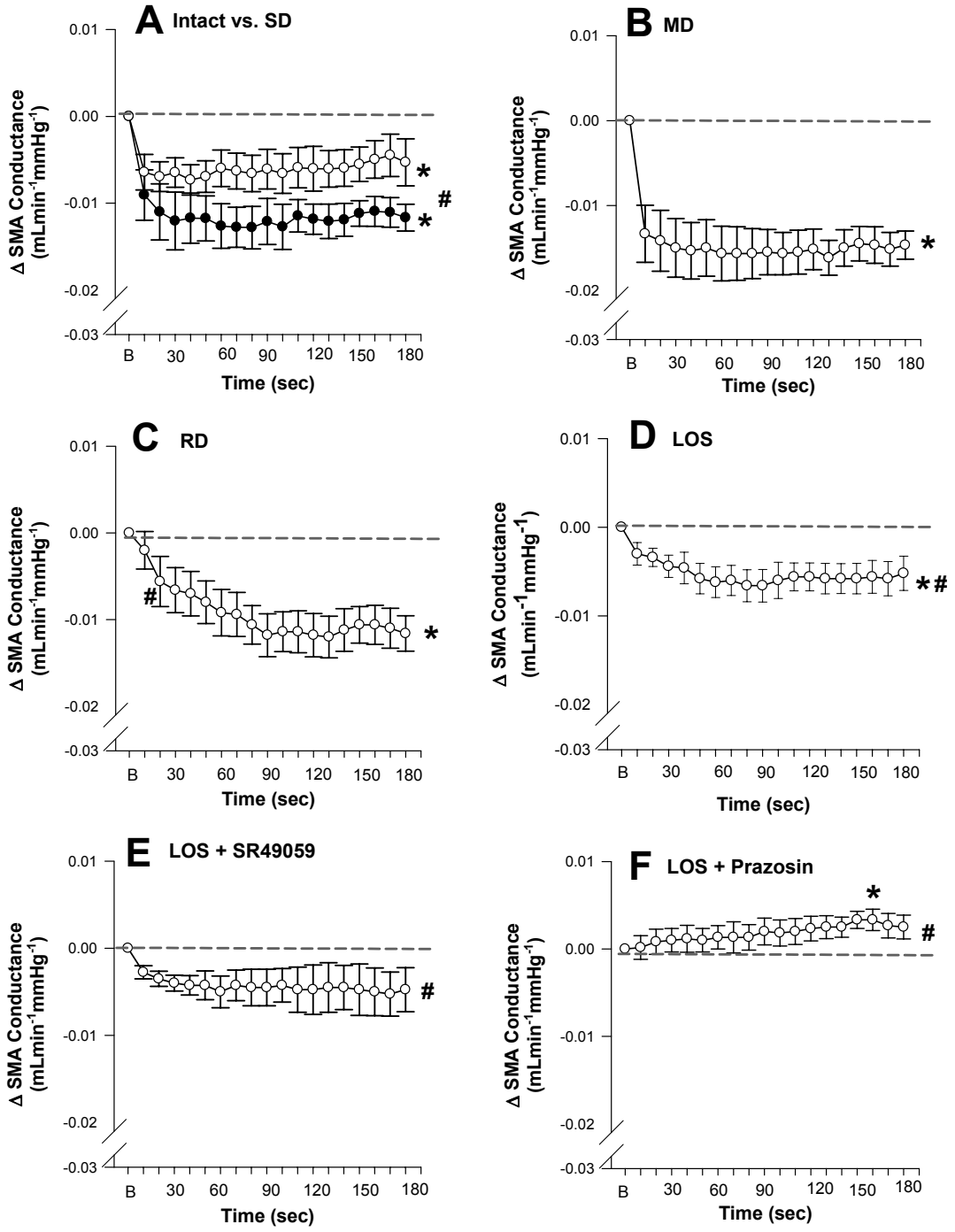

Figure 4.6 Effect of partial splenic vein occlusion on change in superior mesenteric arterial (SMA) conductance from baseline of *A*: Intact (n=10); splenic denervated (SD; n=8), *B*: mesenteric denervated (MD; n=6), *C*: bilateral renal denervated (RD; n=5), *D*: Losartan-treated (LOS; n=5), *E*: Losartan+SR49059 treated (LOS+SR49059; n=4) and *F*: Losartan + Prazosin treated (LOS+Prazosin; n=6) rats. Closed circles: Intact; Open circles: experimental groups. Data are presented as means ± SEM. *Significant difference from own baseline; #Significant difference from Intact group; $p < 0.05$.

Table 4.2 Average baseline hemodynamic parameters of rats included in direct observation of mesenteric vessels during partial splenic vein occlusion.

Group	MAP (mmHg)	Arterial Diameter (µm)	Venous Diameter (µm)
Intact (n=13)	96.1 ± 3.0	55.7 ± 2.9	97.7 ± 6.2
SD (n=10)	93.3 ± 3.2	54.0 ± 1.9	104.1 ± 6.1
RD (n=4)	92.0 ± 3.6	52.7 ± 10.2	93.9 ± 19.0
LOS (n=6)	79.2 ± 8.1	63.7 ± 4.8	92.9 ± 3.5

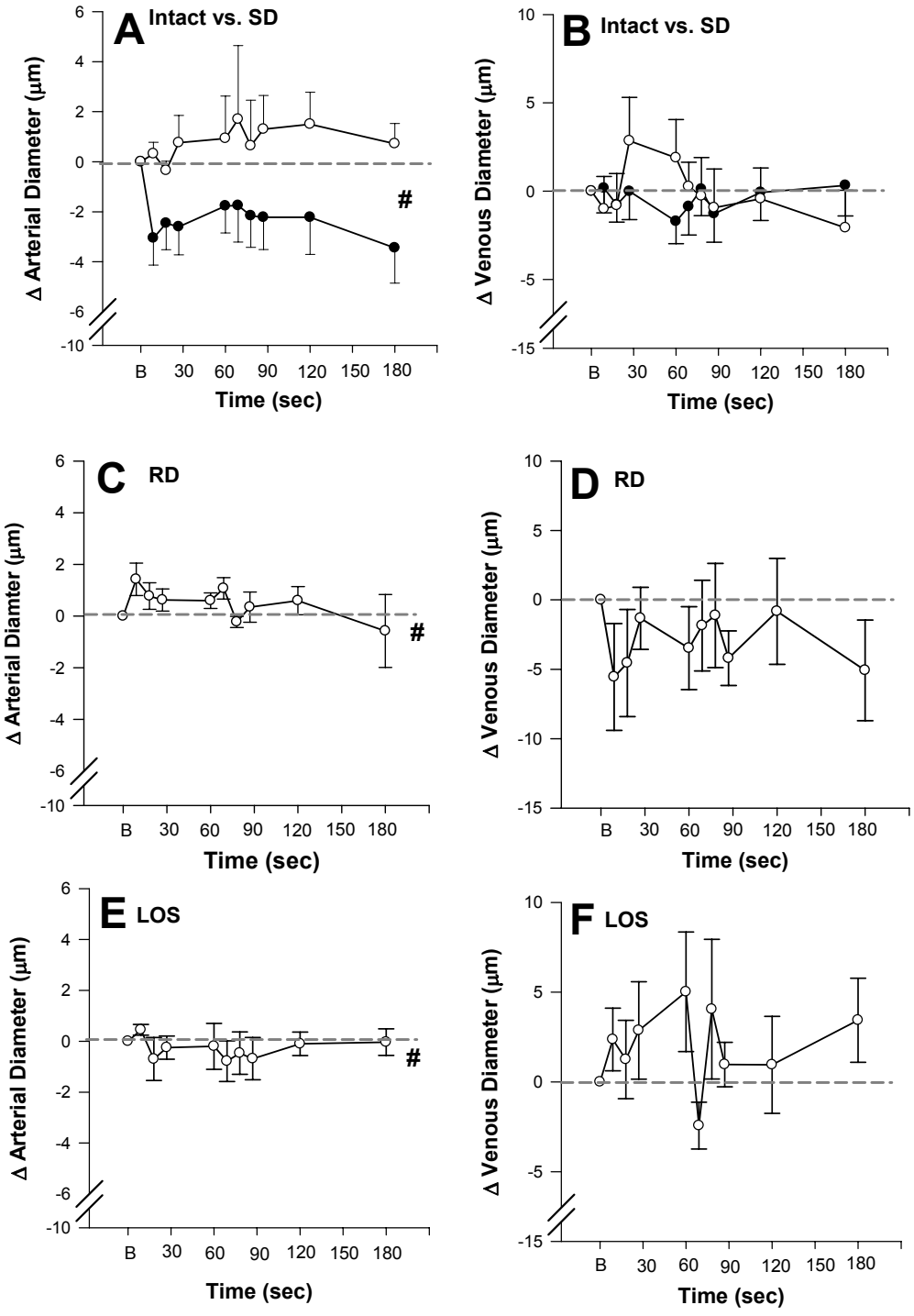

Figure 4.7 Effect of partial splenic vein occlusion on change in mesenteric resistance vessel diameter in *A, B:* Intact, splenic denervated (SD); *C, D:* renal denervated (RD) and *E, F:* Losartan treated (LOS) rats. *A, C, E*: Change in arterial diameter; *B, D, F*: Change in venous diameter. Closed circles: intact; Open circles: experimental group. Data presented as means ± SEM. #Significant difference from Intact; $p < 0.05$.

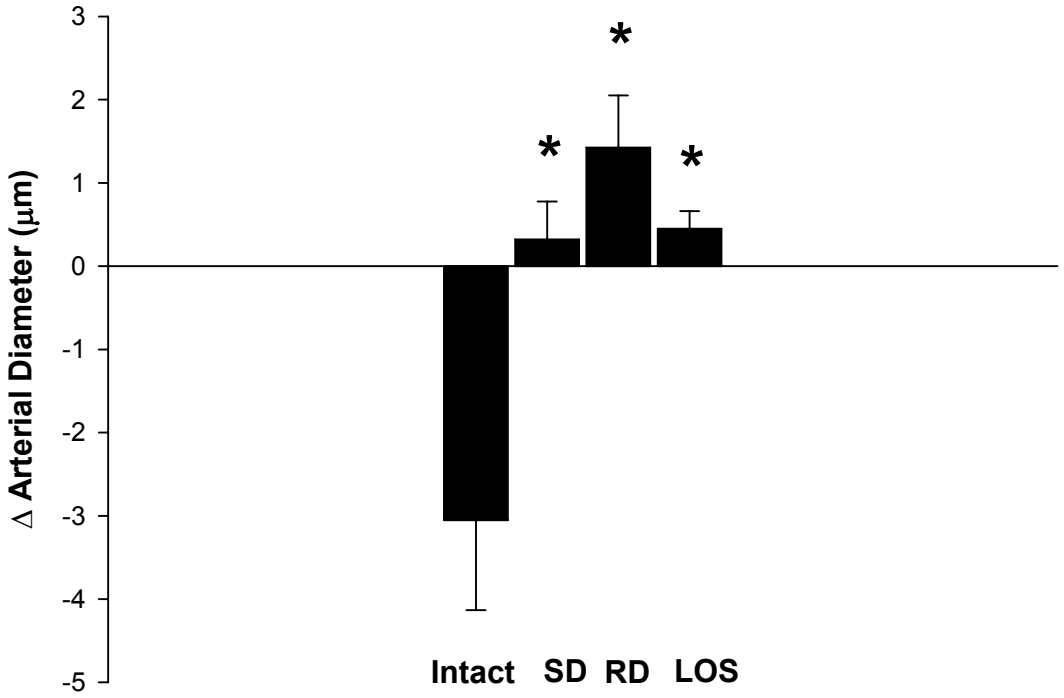

Figure 4.8 Change in mesenteric arterial diameter from baseline at 15 seconds during partial splenic vein occlusion of intact (n= 13); splenic denervated (SD; n=10); bilateral renal denervated (RD; n=4); and Losartan treated (LOS; n=6) animals. Data are presented as means ± SEM. *Significant difference from Intact animals (One Way ANOVA); $p < 0.05$.

4.4 DISCUSSION

Elevated splenic venous pressure, as occurs in PH, triggers increased splenic afferent nerve activity (17), which initiates a reflex increase in mesenteric efferent nerve activity; this response is abolished in the absence of splenic nerves. Increased splenic venous pressure also induces changes in mesenteric hemodynamics. Upon partial SVO, mesenteric arterial conductance immediately falls, a response which is significantly attenuated following splenic denervation. The SVO-induced reduction in mesenteric arterial conductance in intact animals is not correlated with MAP, indicating that this effect is mediated by active vasoconstriction as opposed to being secondary to the fall in MAP and SMAF.

Interestingly, this modulation of mesenteric hemodynamics by the spleen is not exclusively neurally mediated by the splenic afferent and mesenteric efferent nerves, as demonstrated by the failure of either splenic or mesenteric denervation to abolish the SVO-induced fall in mesenteric arterial conductance. The change in mesenteric conductance was similarly independent of MAP in both the splenic and mesenteric denervated groups, suggesting active constriction of mesenteric vessels was mediated, at least in part, by a humoral factor. Bilateral renal denervation to eliminate spleno-renal reflex-mediated release of angiotensin II prevented the immediate drop in mesenteric arterial conductance, but did not prevent a gradual fall in conductance to the degree observed in intact animals. In contrast, blockade of angiotensin II AT_1 receptors significantly reduced the magnitude of the SVO-induced fall in mesenteric conductance. Thus, it appears that although splenic modulation of mesenteric hemodynamics is mediated, at least in part, by angiotensin II, the source of this hormone is not completely

accounted for by renal sympathetic nerve- mediated release. We propose that reduced renal perfusion pressure, as a consequence of the SVO-induced fall in renal arterial conductance (17) stimulates renal baroreceptor-mediated release of angiotensin II (37), which then contributes to modulation of mesenteric arterial tone.

Given the reflex activation of mesenteric efferent nerves by SVO, there also is a potential role for ANG II released directly from mesenteric angiotensinergic nerves; such nerves have recently been shown to extensively innervate rat and human celiac ganglia and mesenteric resistance vessels (30). Angiotensinergic nerve fibres have been particularly localized to mesenteric resistance arteries and are observed to form *en passant* synapses with vascular smooth muscle cells in the tunica media (30). Due to the complexity of mesenteric autonomic innervation, we cannot differentiate the type of mesenteric efferent nerves which were reflexively activated during SVO in this study. It is quite probable that mesenteric angiotensinogenic nerves were activated by SVO and that, in conjunction renal baroreceptor release of ANG II, they contributed to the significant reduction in mesenteric arterial conductance; such a response would of course be completely prevented by AT_1 receptor blockade.

It is possible that non-adrenergic, non-cholinergic (NANC) nerves, which are well established in the mesentery and many of which have dilatory effects (3), were activated during SVO. In the absence of the influence of ANG II, we would have expected to unmask the effects of these nerves; this would have been observed as an increase in mesenteric vascular conductance and resistance vessel diameter. Since we did not see this, we further investigated the possibility that splenic modulation of mesenteric hemodynamics involved maintenance of vascular tone by other humoral mediators. We

have previously shown that acute elevation of splenic venous pressure in conscious rats results in activation of magnocellular cells in the paraventricular nucleus of the hypothalamus (28), which subsequently secrete AVP. We therefore tested the possibility that AVP mediates the reduction in mesenteric arterial conductance in conjunction with ANG II by selectively blocking both V_{1a} and AT_1 receptors. This treatment did not uncover any potential effects of mesenteric dilatory nerves, and in fact, was no different from blockade of AT_1 receptors alone. Our data do not therefore support a role for AVP in splenic neurohormonal modulation of mesenteric hemodynamics. This leaves open the question of what prevents mesenteric vasodilation in the absence of ANG II. One possibility could be the mesenteric adrenergic nerves. Indeed, combined mesenteric blockade of angiotensin II AT_1 and α_1 adrenergic receptors completely prevented the SVO-induced fall in mesenteric arterial conductance, with values tending to increase above baseline over time. This finding, in conjunction with our measured increase in mesenteric efferent nerve activity upon SVO, suggests splenic reflex activation of mesenteric sympathetic nerve fibres. However, our data do not indicate such a direct splenic reflex activation of mesenteric adrenergic nerves since splenic denervation did not prevent the fall in mesenteric conductance. We have previously reported a modest, but statistically significant reduction in right atrial pressure upon partial SVO in intact anesthetized rats (28). It is probable that this reduction in right atrial pressure resulted in centrally-mediated activation of mesenteric sympathetic outflow of corresponding magnitude, which contributed to the maintenance of mesenteric arterial tone (13) in the absence of splenic nerves.

Direct visualization of mesenteric resistance vessels revealed that, immediately upon SVO, mesenteric arterial diameter fell in the intact animals. No changes in mesenteric arterial diameter were observed in splenic denervated, bilateral renal denervated or Losartan treated animals. The absence of any reduction in arterial diameter in splenic denervated animals does not correlate with the significant, albeit attenuated, reduction in mesenteric arterial conductance. It is possible that, if splenic denervation attenuated the reduction in arterial diameter in the same manner as we saw with mesenteric arterial conductance, the actual change in arterial diameter was too small to be detected. This inconsistency requires further investigation. Similarly, the absence of any change in arterial diameter in bilateral renal denervated animals is incongruent with the observed gradual reduction in mesenteric arterial conductance in this group. It is interesting to note however that mesenteric venous diameter was not stable in these animals compared to the intact group. Given that mesenteric venous tone may contribute to increased mesenteric resistance (35), a possible change in venous resistance, downstream of the SMA, could have reduced measured values of mesenteric arterial blood flow, and that this was subsequently reflected in our calculation of mesenteric conductance.

It is widely accepted that the hyperdynamic circulation of PH is preceded by the development of splanchnic vasodilatation (31). Many mechanisms underlying this development of splanchnic vasodilatation have been proposed and have tended to focus on overproduction or reduced metabolism of vasodilatory factors and reduced sensitivity of the mesenteric vasculature to vasoconstrictors (18). However, transient mesenteric vasoconstriction was recently demonstrated to occur in the early stages of PH (12). It has been suggested that mesenteric arterial vasoconstriction stimulates increased catalytic

activity of eNOS in the superior mesenteric artery (34) and precedes development of splanchnic vasodilation and the hyperdynamic circulation (12).

These findings however, leave open to question the mechanism underlying the initiation of this transient mesenteric vasoconstriction. It has been proposed that the increase in mesenteric arterial resistance can be attributed to a myogenic reflex caused by the sudden increase in portal pressure (7, 12). However, as we have shown in our own studies, we now know that splenic venous pressure is also elevated in PH. Based on the findings presented here, we propose that the initial reduction in SMA flow and increase in SMA resistance is mediated by splenic afferent nerve activation of (a) ANG II release and (b) mesenteric efferent nerves (Figure 4.9). PH-induced increases in splenic afferent nerve activity (16) would cause reflex activation of both renal efferent (17) and mesenteric efferent nerves. Consequent spleno-renal reflex release of ANG II would then act on mesenteric resistance vessels to increase mesenteric vascular tone and decrease vascular conductance, as demonstrated in these studies. Simultaneously, ANG II release from mesenteric angiotensinergic nerve terminals would contribute to this ANG II mediated increase in mesenteric vascular tone. Additionally, it has been shown that genes involved in adrenergic neuronal transmission are upregulated in the SMA of rats in the early stages of PH induced by PVL (11). This is evident just one hour after induction of PH, and corresponds to the timeline of mesenteric vasoconstriction, thus suggesting a role for adrenergic nerve signaling. While our data do not support direct splenic reflex activation of mesenteric adrenergic nerves, putative central activation of mesenteric sympathetic outflow upon elevation of splenic venous pressure may additionally contribute to the mesenteric vasoconstriction by release of NE, which would act on mesenteric vascular α_1

adrenergic receptors. As ANG II is known to modulate sympathetic nerves, potentiation of NE release from mesenteric sympathetic nerves by this hormone may also be involved, indirectly augmenting mesenteric vasoconstriction.

We have presented evidence linking the spleen with the initial mesenteric vasoconstriction of early PH. This finding has implications for the instigation of NO overproduction in the mesentery, which is now known to precede the development of splanchnic vasodilatation that underlies establishment of the hyperdynamic circulation. Thus, in addition to mediating renal dysfunction of PH, the spleen may also be critically involved in the initiation of the hemodynamic complications of this syndrome, which remains a serious and lethal clinical problem.

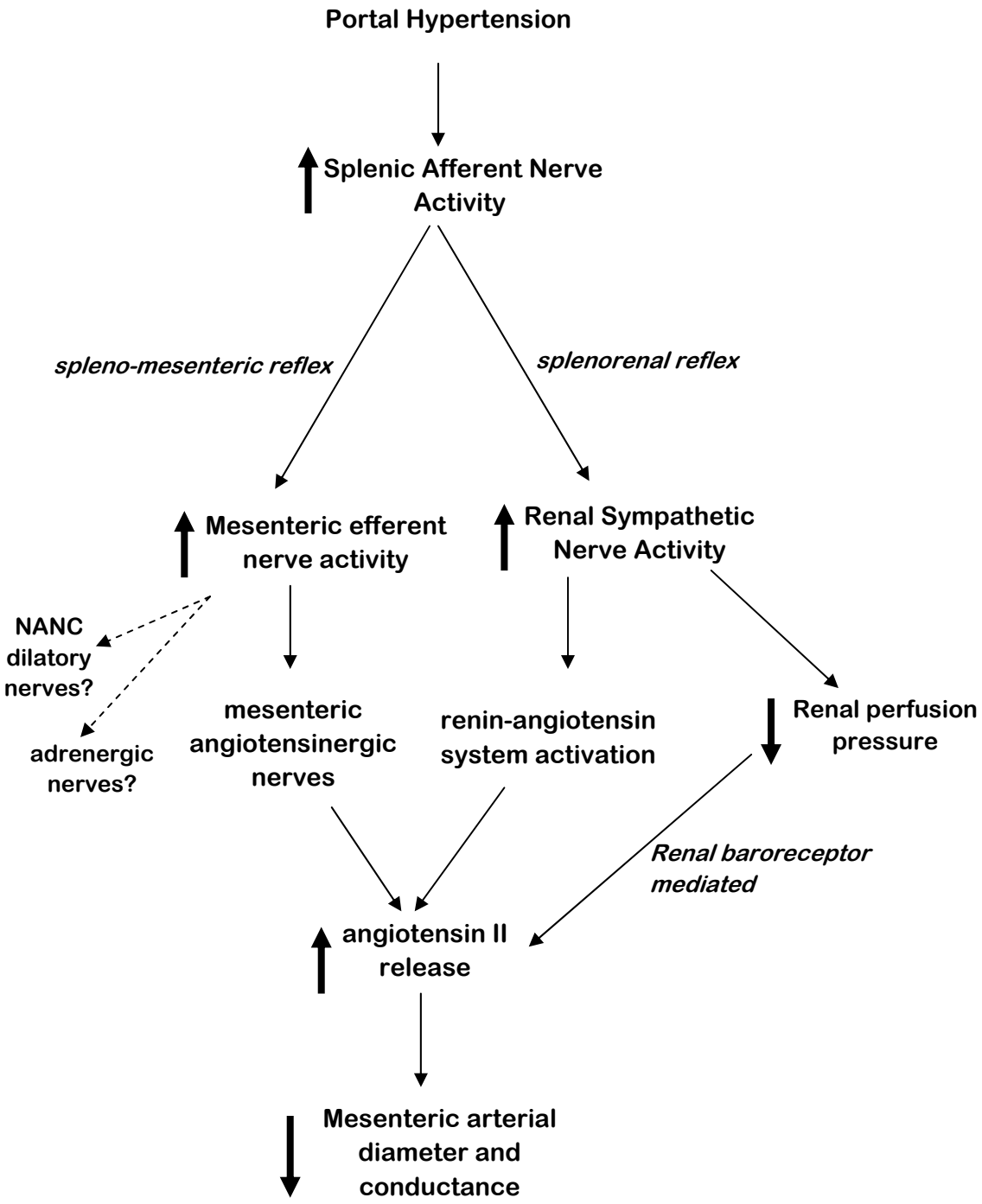

Figure 4.9 Schematic representation of proposed splenic neurohormonal modulation of mesenteric vascular tone in portal hypertension.

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CHAPTER 5:

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

In spite of extensive scientific and clinical research spanning several decades PH, regardless of etiology, remains a serious and generally lethal condition with no definitive treatment (38). From a seemingly simple elevation in portal venous pressure, many interconnected complications may arise : esophageal varices; hepatic encephalopathy/coma; renal dysfunction; hyperdynamic circulation; and ascites. While each of these complications alone are potentially life threatening, it is renal dysfunction and the hyperdynamic circulation which arguably "set the stage" for the development of all other complications and thus can be considered the earliest and most hemodynamically critical consequences of PH.

Renal dysfunction and the hyperdynamic circulation are not mutually exclusive and, in fact, are intimately engaged in initiating and perpetuating the cardiovascular devastation of PH. However, historically, the link between PH and either renal dysfunction or the development of the hyperdynamic circulation has not been clearly understood, although it has more recently become the focus of investigation. It has been shown that in response to elevated portal venous pressure or reduced portal venous blood flow, a neural hepato-renal reflex alters renal function such that renal blood flow and vascular conductance are reduced and sodium and water reabsorption is increased (9, 28, 30, 31). This demonstration of hepato-renal reflex control of renal function helped further clarify the functional nature of renal failure in PH, which had been observed, but unexplained up to this point (10, 11, 21, 25). The extreme manifestation of this reflex in the setting of hepatocellular dysfunction, also formed our understanding of the hepato-renal syndrome, a fatal condition characterized by profound sodium and water retention and end-stage

renal failure which can only be definitively reversed by liver transplantation (12). Concurrent to these events, PH has also been shown to induce early splanchnic hemodynamic changes (6) which are associated with augmented release of NO in the mesenteric circulation (47). Indeed, eNOS upregulation and increased NO production are also implicated in mediating the PH-associated reduction in sensitivity of the vasculature to vasoconstrictor agents (46). However, until recently, it was not clear if this overproduction of NO was a cause or a consequence of the hyperdynamic circulation, since chronically increased blood flow, a hallmark of this state, is known to increase endothelial NO release in many vascular beds (4, 34). Wiest et al (1999) demonstrated that endothelial NO overproduction in the mesenteric arteries of rats was evident in the early stages of PH, before the establishment of the hyperdynamic circulation (48). This was supported by the observation that eNOS derived NO overproduction in the superior mesenteric artery of rats is apparent as early as one day after induction of PH by partial portal vein ligation (PVL) (20). Investigation of the mechanisms behind this increase in NOS activity and excessive NO production brought focus to a key step in the initial hemodynamic changes of PH, namely a significant *decrease* in superior mesenteric arterial blood flow paired with significantly elevated superior mesenteric arterial resistance (6). This was apparent for the first two days after PVL in rats and specific to the mesenteric vascular bed; increases in superior mesenteric blood flow and establishment of the hyperdynamic circulation was not observed until four days later (6). Vasoconstriction is known to trigger eNOS upregulation and indeed, this initial mesenteric arterial constriction was shown to increase NOS activity in rats with chronic PH (43). Interestingly, superior mesenteric arterial constriction of itself, in the absence of

PH, increased NOS activity; conversely, PH in the absence of SMA constriction was not associated with upregulation of eNOS (43). Paired with mesenteric downregulation of genes involved in adrenergic signaling (5), splanchnic vasodilatation ensues and goes on to establish peripheral arterial vasodilatation (37).

It is at this juncture (the cusp of establishing the hyperdynamic circulation) that a synergy between PH-induced renal dysfunction and alteration of cardiovascular hemodynamics, is most apparent. In addition to direct stimulation of renal sodium and water retention by the hepato-renal reflex, the reduction in effective arterial blood pressure as a result of peripheral arterial vasodilatation and accumulation of blood volume on the venous side of the circulation reduces central cardiovascular input from arterial baroreceptors and cardiopulmonary receptors, thus reflexively increasing renal sympathetic outflow and further augmenting renal sodium and water retention. This series of events continues to contribute to the development of the classic hemodynamic profile of the hyperdynamic circulation: expanded plasma volume, arterial hypotension, increased cardiac output and heart rate. These circulatory changes then go on to initiate and exacerbate the well-known complications of PH. Thus renal dysfunction and the hyperdynamic circulation, although independently initiated, act in concert to gradually create the multi-organ cardiovascular syndrome of PH.

This work directly addresses the *initiation* of these two key complications and further illuminates their link to PH. Splenic venous pressure is increased in PH (2). Given that the spleen plays a critical role in cardiovascular regulation (15), it is not unexpected that this organ is involved in the aforementioned cardiovascular *dysregulation*. Indeed, we have now shown independent modulation of renal hemodynamics and vascular tone by

the spleen during selective elevation of splenic venous pressure of a magnitude correspondent to PH (Figure 5.1). We did not directly measure changes in renal sodium and urine excretion. However, renal sympathetic nerves, which extensively innervate the renal vasculature and major segments of the tubular nephron (3), are known to modulate urinary sodium and water excretion by altering renal hemodynamics (via changes in renal vascular resistance) in addition to having direct effects on the renal tubular reabsorption (1, 18, 22). Thus, the spleno-renal reflex-mediated increase in renal sympathetic nerve activity and reduction in renal arterial conductance and blood flow (16) likely contribute to the renal sodium and water retention characteristic of PH. In the renal vasculature, activation of AT_1 receptors by ANG II constricts post-glomerular (efferent) arterioles, which decreases renal blood flow. As this dominant post-glomerular constriction increases intraglomerular capillary pressure, glomerular filtration rate is subsequently increased. In our studies, activation of the splenorenal reflex resulted in a reduction in renal blood flow, which is not likely mediated by intrarenal actions of ANG II as the reduction in renal blood flow and renal vascular conductance which we observed was completely abolished by selective renal blockade of α_1 adrenergic receptors (16); thus implicating norepinephrine released from renal sympathetic nerve terminals. Since the reduction in renal vascular conductance upon activation of the splenorenal reflex is mediated via activation of these α_1 adrenergic receptors (which primarily constricts renal afferent arterioles (8)), this reflex would be expected to result in a reduction of intraglomerular capillary pressure and a reduction in GFR. Via its renal actions, the spleen thus propagates the hyperdynamic circulation by expansion of plasma volume. Akin to prolonged activation of the hepato-renal reflex in PH, chronic stimulation of the

spleno-renal reflex in the setting of hepatocellular dysfunction may alone lead to development of the hepato-renal syndrome and eventual renal failure.

The importance of the splenorenal reflex in mediating changes in renal function in PH is further highlighted by our demonstration that mesenteric congestion, which was initially thought to alter renal function via an intestinal-renal reflex (29), does not independently result in any changes in renal blood flow or renal vascular tone (14). Only when the increases in portal venous pressure additionally elevate splenic venous pressure, is there a reduction in renal blood flow and arterial conductance. Furthermore, this is completely dependent on splenic and renal nerves, and involves integration at the celiac ganglion (14). That this reflex regulation of renal vascular function does not rely on changes in blood flow through the liver and is abolished in the absence of the spleen provides further evidence supporting this organ's critical role in PH. This last result additionally sheds light on the possible mechanism of activation of splenic afferent nerves. In the absence of the spleen, but with an intact splenic neuro-vascular arcade, the spleno-renal neural reflex is abolished (14). This suggests that a reduction in splenic venous blood flow rather than signaling from venous pressoreceptors – as would occur during partial splenic vein occlusion – is the primary stimulus for activation of splenic afferent nerves and consequent reflex activation of renal sympathetic nerves. This proposal is corroborated by previous findings demonstrating that intrasplenic blockade of NO synthesis induces an increase in systemic blood pressure (23), via spleno-renal reflex-mediated release of ANG II (7). NO has been shown to inhibit nerve activity (42). Therefore, reduced bioavailability of NO upon a reduction in splenic venous blood flow may remove resting inhibition of splenic afferent nerves and result in initiation of the spleno-renal reflex. It is

important to bear in mind that splenic afferent nerves are also stimulated by increased splenic venous pressure, in the absence of changes in splenic venous blood flow (32)(S. Hamza, unpublished observation, Appendix B). However, this does not trigger the spleno-renal reflex and may invoke other neural reflexes involving the spleen as will be discussed.

In addition to independently initiating changes in renal vascular tone and indirect perpetuation of the hyperdynamic circulation via probable renal sodium and water retention, we have also demonstrated that the spleen is directly involved in mediating mesenteric vasoconstriction, a development which is now thought to precede the critical development of splanchnic vasodilatation. Partial splenic vein occlusion (SVO) to elevate splenic venous pressure (and reduce splenic venous blood flow) causes a reflex increase in mesenteric efferent nerve activity, which is abolished by splenic denervation. Partial SVO also decreases mesenteric arterial conductance, which is mirrored by a reduction in mesenteric arteriolar diameter, with no observed change in venous diameter; this is supported by measurement of superior mesenteric venous flow, which essentially reflects upstream changes in arterial conductance (S. Hamza, unpublished observation; Appendix C). This fall in mesenteric arterial conductance is significantly attenuated by splenic denervation and is in great part dependent on release of ANG II from the kidney (both renal sympathetic nerve- and renal baroreceptor-mediated) and mesenteric angiotensinergic nerves. Although it is as yet unclear whether splenic afferent nerve activity causes direct reflex activation of mesenteric adrenergic fibres, contributions from these nerves cannot be unequivocally dismissed and, in fact, are clearly involved in the SVO-induced reduction in mesenteric conductance. In the natural history of PH,

splanchnic blood flow is not increased until the hyperdynamic circulation is established (6). As such, it is entirely possible that early decreases in splenic venous blood flow in the initial stages of PH (24), *before* splanchnic blood flow is increased, would trigger the mesenteric vasoconstriction which is now known to upregulate eNOS and cause excessive NO production (43).

Reflex control of the mesenteric vasculature by the spleen via ANG II release and/or mesenteric efferent nerve activation (angiotensinergic or sympathetic) also has physiological relevance. Activation of these mechanisms in response to reduced splenic venous blood flow would make teleological sense in a low volume, low pressure state; release of ANG II/NE mediated mesenteric vasoconstriction would mobilize splanchnic blood stores and help support blood pressure and perfusion of key organs. In a high volume, high blood flow state, increased bioavailability of NO in the splenic vein would preclude activation of the spleno-renal reflex, preventing inappropriate renal sodium and water retention.

Alternatively, although we have shown that PH-induced increases in splenic venous pressure alone do not trigger the spleno-renal reflex, this stimulus may be involved in reflex activation of mesenteric efferent nerves – particularly mesenteric dilatory nerves (NANC). That splenic congestion, as reflected by an increase in splenic venous pressure, would result in reflex activation of mesenteric efferent dilatory nerves and subsequent splanchnic dilatation also makes teleological sense; a high volume signal from the spleen would trigger mesenteric dilatation to accommodate the additional volume and maintain cardiovascular homeostasis.

Normally, changes in splenic venous pressure and flow occur in parallel. However, in the case of PH, a unique situation arises where splenic blood flow is initially decreased due to portal obstruction while splenic venous pressure is increased. We propose that it is the balance between these two stimuli which determines the net effect on mesenteric vascular tone over the course of development of PH. As such, while the response to increased splenic venous pressure may not be important in the *initiation* of the hyperdynamic circulation via mesenteric vasoconstriction, it may potentially be involved in maintaining the profound systemic vasodilatation once the hyperdynamic circulation is established and splenic venous pressure *and* flow continue to rise (50). This possibility requires further investigation to first determine if (a) partial SVO does indeed reflexively increase activity of mesenteric vasodilatory nerves and (b) if this is mediated by an increase in splenic venous pressure alone.

The possibility of physiological regulation of mesenteric vascular tone by splenic afferent nerves, modulated by a balance between changes in splenic venous flow and pressure, is further supported by the unexpected observation that resting mesenteric efferent nerve activity is significantly higher in splenic denervated animals compared to their intact counterparts. Thus it appears that basal mesenteric neurovascular tone is "fine tuned" by splenic afferent outflow according to the hemodynamic status of the animal as sensed by splenic venous baroreceptors (17) and/or NO bioavailability. This ultimately provides additional evidence of cardiovascular regulation by the spleen.

Given that the spleen independently initiates and perpetuates the two key complications of PH, and that splenic blood flow contributes over 50% of portal inflow in PH (33), splenectomy may appear to be an ideal treatment. However, in reality this is far from the

case. Splenectomy and diversion of splenic venous blood flow away from the portal circulation yield inconsistent results and are generally deemed ineffective. Historically, it has been reported as early as the 1930's that attempts to reduce portal inflow and, in turn, portal venous pressure by splenectomy only results in temporary relief (36). Walker in 1952 concluded that in PH "splenectomy alone should never be done; it does not reduce the risk of fatal bleeding and may destroy the only vein which is available for anastomosis" (45). This sentiment has since been corroborated by several other studies. Surgical clamping of the splenic vein to eliminate splenic outflow does not reduce portal venous pressure (13). Complete splenectomy results in insubstantial decreases in portal venous pressure (26, 27) or none at all (35), and does not improve esophageal varices (39). Additionally, surgical spleno-renal shunts, which divert splenic venous outflow away from the portal circulation directly to the renal vein, do not consistently decrease portal venous pressure (40, 41).

Splenectomy may not solve the complications of human PH for several reasons. First, the splenic vasculature is extensively involved in the formation of porto-systemic collaterals over time (44). Elimination of many of these shunts by splenectomy reduces the available pathways for diversion of blood away from the congested portal venous system, serving only to further increase portal venous pressure and exacerbate PH. Secondly, in the clinical setting; splenectomy is generally performed as a treatment for the complications of PH once the syndrome has been long established. Based on the results from this work, the spleen contributes to the initiation of renal dysfunction and events which lead to the establishment of the hyperdynamic circulation. Once these cardiovascular changes are set in motion, it appears that removal of the spleen does little to reverse or even alleviate

established complications. In other words, if splenectomy were to be considered as a treatment for PH, it may be a more relevant target in the very early stages of PH as a prophylactic measure, before the changes in renal function and splanchnic vasodilatation take firm hold. Third, it is interesting to note that in splenectomy procedures, a portion of the splenic vein, of varying lengths is often left behind (49). This splenic vein "stump" would likely include a segment of the splenic nerve, which is known to dip into the muscular wall of the splenic vein (19). Although splenic venous blood flow would be eliminated, the possibility remains that splenectomy fails to reduce portal venous pressure. Thus splenic baroreceptors in the remaining portion of the splenic vein would still be able to respond to increases in pressure. This suggests that perhaps splenic neural modulation of mesenteric vasodilatory nerves may not be completely eliminated, thus maintaining splanchnic vasodilatation and advancing the descent toward the intractable sequelae of the hyperdynamic circulation. Further experimental studies focused on chronic PH are necessary to determine if early intervention of splenic reflex pathways prevents the initiation and progression of renal dysfunction and the hyperdynamic circulation.

5.2 CONCLUSION

In conclusion, we have demonstrated that the spleen is involved in the initiation of two critical complications of PH: renal dysfunction and the hyperdynamic circulation (Figure 5.1). We have examined splenic reflex control of renal and mesenteric vascular tone in the setting of pathophysiology. However, the spleno-renal reflex, splenic modulation of mesenteric tone via spleno-renal reflex and mesenteric angiotensinergic nerve- mediated ANG II release, and putative splenic activation of mesenteric vasodilatory nerves

additionally have relevance in physiologic cardiovascular control. The demonstration of the existence of these splenic reflex pathways now demands direct investigation of their contribution in a model of chronic PH, an experimental situation which more closely mirrors the human condition.

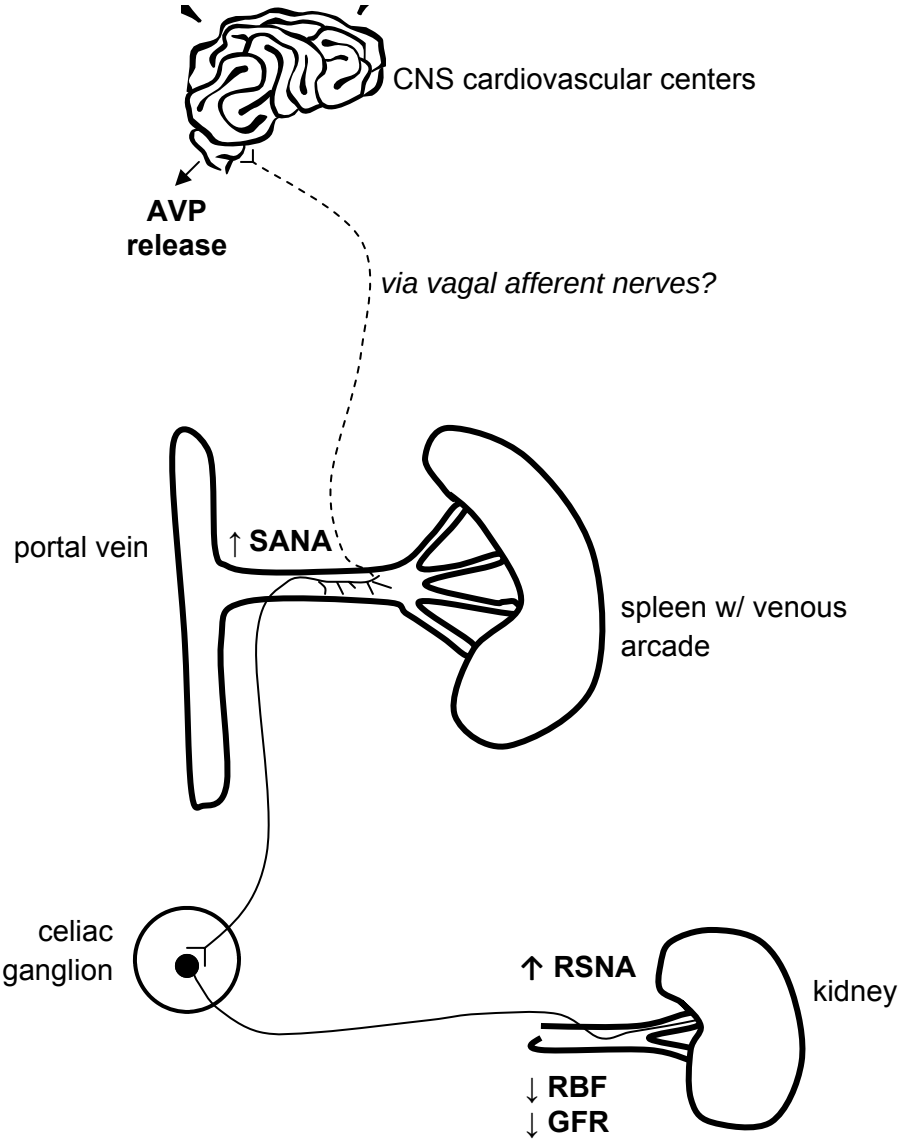

Figure 5.1 Schematic representation of proposed neural pathway of the spleno-renal reflex.

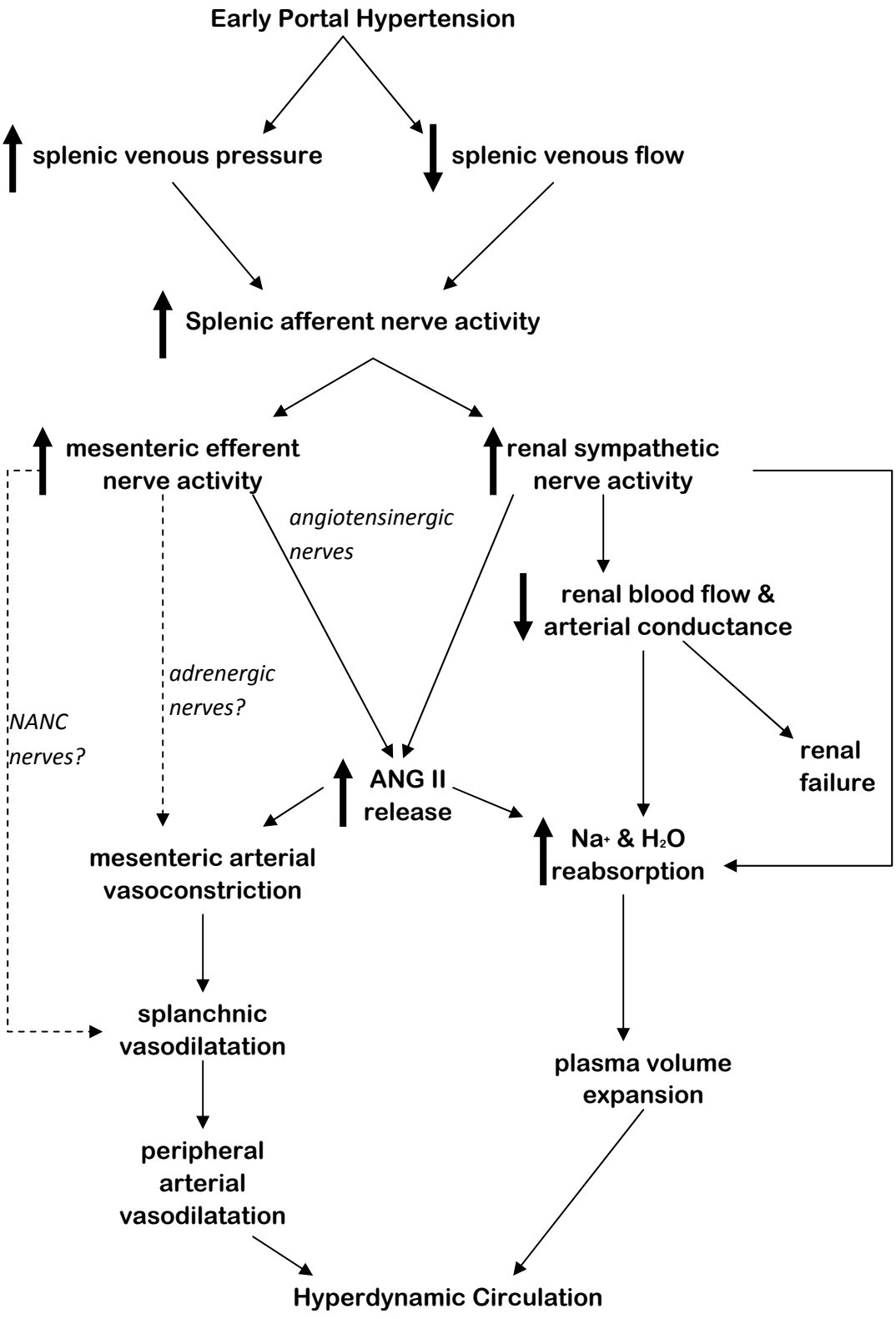

Figure 5.2 Schematic representation of splenic neurohormonal initiation of the renal dysfunction and hyperdynamic circulation of portal hypertension.

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CHAPTER 6:

APPENDICES

APPENDIX A:

METHODOLOGY FOR ACUTE INTRARENAL ARTERIAL INFUSION[3]

[3]*A version of this section has been published:*

Hamza, S.M. and S. Kaufman. (2004). ***A vibrator prevents streaming during close arterial infusion into the kidney.*** *Am J Physiol. Renal Physiol.* 286: F643-F646.

A.1 INTRODUCTION

Pharmacological agents are commonly administered during the course of investigating renal function. However, intravenous infusion may cause systemic effects which indirectly influence the kidney. To avoid this problem, the drugs are often infused directly into the renal artery. Unfortunately, laminar flow patterns within arteries cause streaming and uneven distribution of infusate within the organ, with the result that the drug may only partially, if at all, perfuse the vascular beds of interest (4, 18, 20). This can lead to variability of data and potential misinterpretation of the results. The general problem of streaming during intra-arterial infusion is also of clinical significance because therapeutic agents, particularly for chemotherapy, may be delivered in this manner in order to achieve high local concentrations without accompanying systemic toxicity(2, 6, 11-14).

Several investigators have described protocols to improve mixing during intra-arterial injection of drugs. These have involved introduction of balloon cannulae to cause turbulence (1), pulsed infusion (22), and extracorporeal blood circuits (4). In 1995, Parekh devised a multiple catheter system with a magnetic pump which could draw back blood to premix with the drug to be infused (18). Although this technique does improve uniform delivery, it is expensive and requires extensive preparation. We describe a simple, inexpensive method to improve drug delivery to the kidney. We have shown that this system improves spacial dye distribution, as well the functional response to phenylephrine.

A.2 METHODS

The experimental procedures were approved by the local Animal Welfare Committee in accordance with the guidelines issued by the Canada Council on Animal Care. All animals were killed with an anesthetic overdose of pentobarbital sodium at the completion of the each experiment.

A.2.1 Animals and Housing

Male Long Evans rats (350-550g, Charles River, Canada) were housed for at least one week in the University of Alberta Animal Facility. They were maintained on a 0.3% sodium diet and tap water ad libitum in a temperature and humidity controlled environment with a 12/12hr light/dark cycle.

A.2.2 Anaesthesia and Surgery

The animals were injected with sodium pentobarbital (50mg/kg body weight, i.p.), followed by Inactin (Byck, ethyl- (1-methyl-propyl)-malonyl-thio-urea, 80mg/kg body weight, sc) one hour later. Body temperature was maintained at 37°C (Homeothermic blanket, Harvard Apparatus, Canada).

The left femoral vein was cannulated with Silastic tubing (0.51mmID, 0.94mm OD, Dow Corning, U.S.A.), and infused with isotonic saline (3mL/h). The left femoral artery was cannulated with polyethylene tubing (PE 50 0.58mm ID, 0.97mm OD, VWR, Mississauga, Ontario), which was connected to a pressure transducer for online recording of mean arterial pressure (MAP). After a midline laparotomy, the intestines were reflected to the right side of the animal. The stomach was reflected onto the chest and

held in position with hemostats. The spleen was detached from the stomach and gently retracted to the left side of the animal. All exteriorized organs were covered with moistened gauze and plastic wrap. The renal artery was carefully separated from the renal vein, ensuring the adventitia was left intact to preserve the renal nerves. A transit-time flow probe (Transonic Systems, Ithaca, NY) was placed around the renal artery distal to its origin from the descending aorta.

A.2.3 Preparation and Insertion of Renal Arterial Catheter

A 33 gauge stainless steel needle (Hamilton Company, Nevada) was inserted into one end of a 10 cm length of Microline tubing (I.D.0.25mm, O.D. 0.76mm, Cole-Palmer, Ontario, Canada) which had been stretched to provide a tight seal around the needle. The other end of this tubing was attached to a syringe. With curved forceps, the renal artery wall was gently held up, while the 33-gauge needle was inserted at the junction of the renal artery and descending aorta (Fig A.1). The needle was advanced ~ 2mm into the artery and held in place with a small drop of Vet Bond tissue adhesive (3M Animal Care Products, St. Paul, Minnesota, U.S.A.) at its point of entry into the vessel. An immediate flow of blood into the line upon insertion indicated patency. It was not necessary at any time to interrupt blood flow to the kidney. The animal was left to stabilize for 40 min while MAP and renal blood flow were monitored.

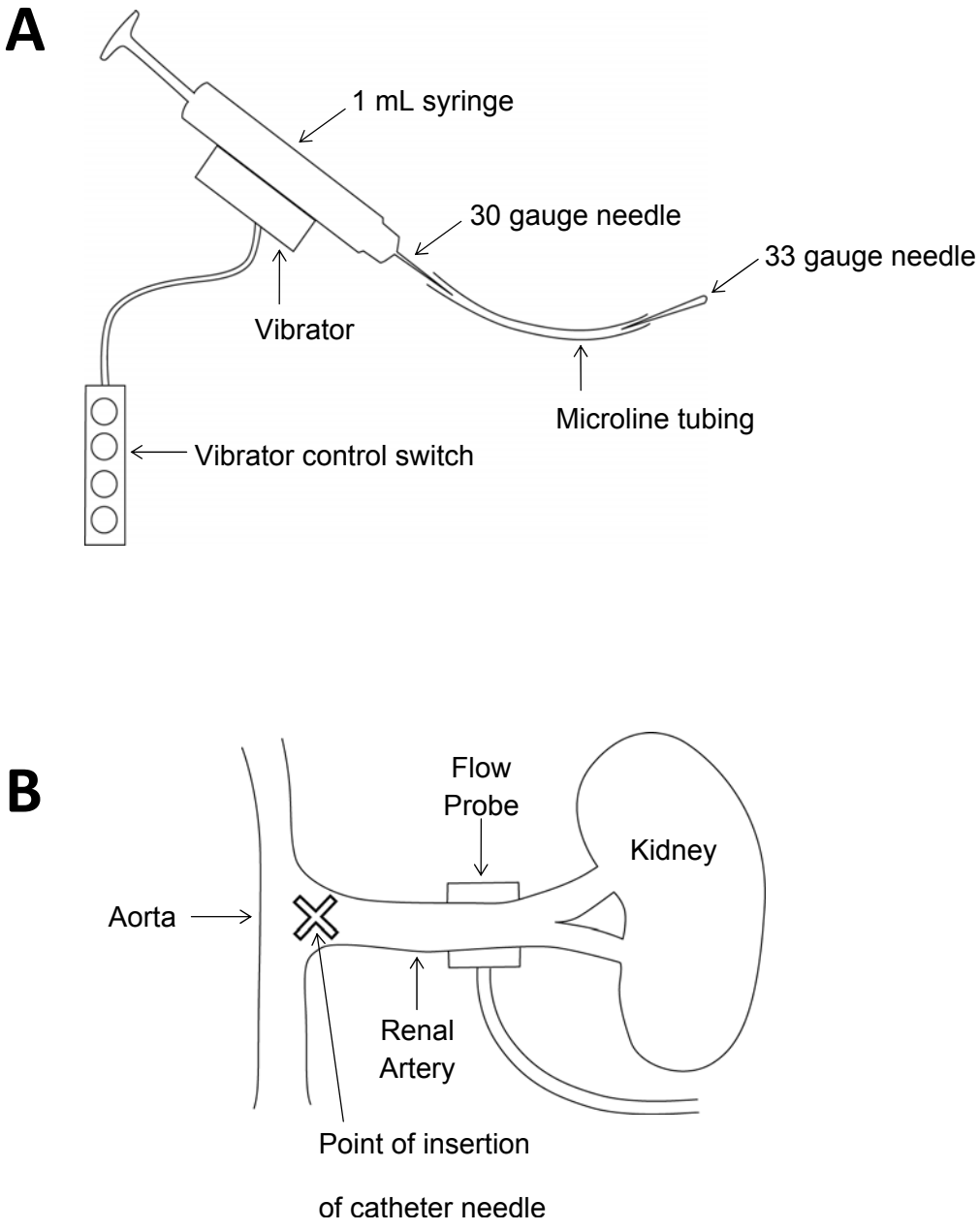

Figure A1: Schematic representation of apparatus (A) and surgical preparation (B) for close-arterial injection into the kidney and measurement of renal blood flow.

A.2.4 Experimental Protocol

The vibrator (i-Vibe egg, Doc Johnson Enterprises, CA, U.S.A.) was attached, with plastic tape (3M Colourflex, Ontario, Canada), to the syringe to be used for dye infusion. Vibration was turned on at the lowest setting ~30 sec before the dye was injected. The dye solution (1mL) was then slowly infused over a second period of 30 sec. In the control group, the dye was infused in exactly the same manner, except that vibration was not applied to the syringe. Separate animals were used for the experiments with/without vibration.

A similar procedure was followed for the injection of phenylephrine, except that MAP and renal blood flow values were recorded continuously online (DI-151RS, DATAQ Instruments, Adron, OH, U.S.A.) using WINDAQ software for analysis (DATAQ Instruments, Adron, OH, U.S.A.). Following stabilization (40 min), baseline measurements were made over 10 min. The phenylephrine was then infused over a period of 30 sec at doses of 0, 0.05, 0.15, and 0.5μg dissolved in 150μL isotonic saline. There was a 10 min recovery period between each dose. Each animal was used for both with/without vibration experiments, with the order of testing being alternated between consecutive experiments.

A.2.5 Drugs and Solutions

Evans Blue (Fisher Scientific, Ontario, Canada) was dissolved in isotonic saline (0.5% w/v). Solutions of phenylephrine (Sabex, Quebec, Canada) were made by serial dilution in heparinized isotonic saline (10,000IU/L).

A.2.6 Data Analysis and Statistics

The maximal decrease in renal blood flow following infusion of each dose of phenylephrine was measured. Two way repeated measures ANOVA, followed by the Student-Newman-Keuls Method for post-hoc analysis, was used to determine the statistical significance of the change in renal blood flow between injection with/without vibration. Statistical significance was accepted at $p < 0.05$. Means ± SEM are reported in the figures and in the text.

A.3 RESULTS

Injection of dye directly into the renal artery, with no vibration, resulted in uneven patches of dye accumulation over the surface of the kidney (Fig A.2A). With application of vibration to the syringe during injection, the kidney showed even mottled distribution of color over both dorsal and ventral surfaces (Figure A.2B).

There was no difference between the two groups with respect to baseline renal blood flow (With vibration: 6.3±0.6mL/min, n=8; without vibration: 6.5±0.6mL/min, n=8). Intra-arterial infusion of the α_1-adrenergic agonist phenylephrine induced a dose-dependent reduction in renal blood flow. Vibration caused a leftward shift of the dose-response curve relative to the response in the absence of vibration i.e. there was a greater decrease in renal blood flow when the phenylephrine injection was accompanied by vibration (Fig A.3). Repeated measures two-way ANOVA confirmed that there was a significant effect of "treatment" (With vibration vs. without vibration: $p<0.001$). Although systemic blood pressure rose during the phenylephrine infusion, there was never any significant difference between the groups (with/without vibration), even at the highest dose (With

vibration: 92.4±2.5mmHg to 101.6±3.2mmHg, n=8; without vibration: 93.2±3.7mmHg to 101±3.3mmHg, n=8).

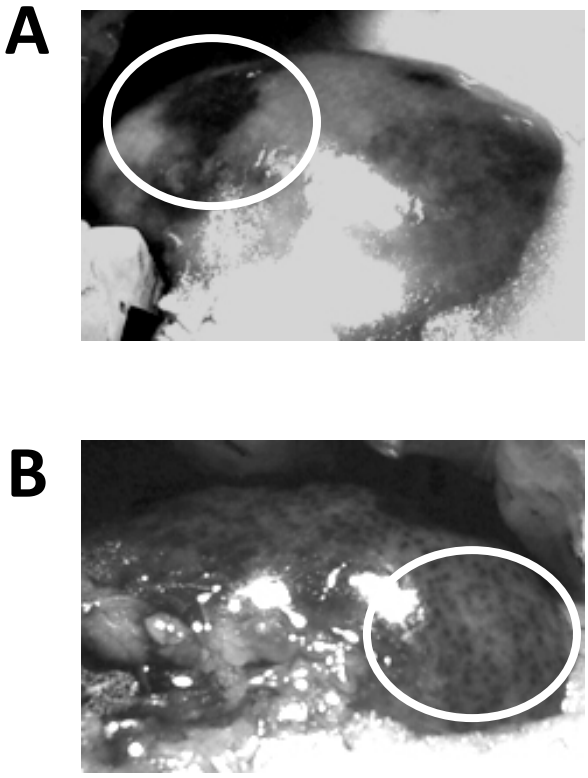

Figure A2: Intrarenal arterial injection of Evans Blue Dye. 1mL bolus of Evans Blue dye was injected with or without vibration. A. External appearance of the kidney after direct injection of dye with no vibration. B. External appearance of the kidney after injection with applied vibration. Outlines delineate regions of intense coloration (A) or uniform mottling (B).

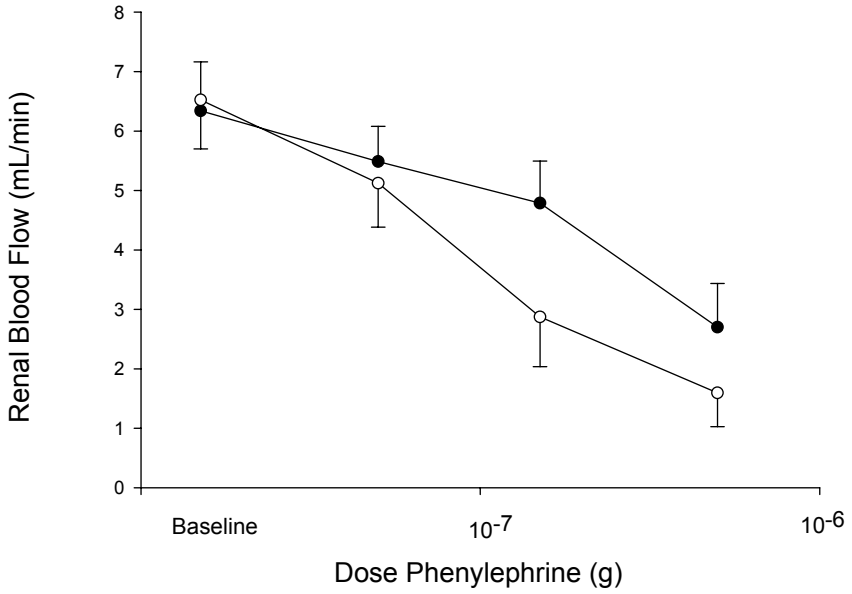

Figure A3. Effect of vibration on change in renal blood flow induced by close-arterial infusion of phenylephrine. Open circles: Vibration applied to syringe. Closed circles: No vibration. n=8. Vertical lines delineate standard error of mean. There was a significant effect of treatment (vibration) on renal blood flow ($P<0.001$, repeated measures two-way ANOVA).

A.4 DISCUSSION

Direct infusion of Evans Blue into the renal artery resulted in uneven delivery of dye to the renal tissue. Application of vibration during the infusion improved dye distribution, so there was uniform coloration on both dorsal and ventral faces of the kidney. There was also functional improvement of drug distribution during vibration. The vascular response to phenylephrine, as reflected by the decrease in renal blood flow, was significantly greater when vibration was applied to the syringe during infusion. We suggest that transmission of vibration, both along the microline tubing and within the fluid column, causes turbulence and mixing as the infusate enters the blood stream. This ensures that as the blood flows through all downstream branches of the renal artery into the kidney, there is homogeneous delivery of drug to the renal tissue. The greatest effect of vibration on renal blood flow (55% reduction vs. 25% reduction) occurred after infusion of phenylephrine at $1.5x10^{-7}$g (infused over 30 sec, at a flow rate of about 5mL/min). Significantly, this is the concentration of phenylephrine ($7.5x10^{-7}$M) which consistently induces vasoconstriction in the isolated perfused kidney (17, 19).

The issue of streaming in arterial flow has been extensively studied, at least in part because of the clinical importance of delivering chemotherapeutic drugs to target organs where treatment may be complicated by uneven distribution and focal toxicity (2, 3, 5, 6, 14). Variable delivery of tracer due to streaming has also been demonstrated in life-sized glass models of the human hepatic artery (15), the human carotid artery (11), and the human iliofemoral/pelvic arteries (12), as well as during carotid artery infusion in rats (20). Our data were in agreement with those obtained by Parekh, that close-arterial

infusion of dye into the rat kidney normally results in extremely uneven distribution of coloration (18).

The cannulation technique and the use of the vibrator offer several advantages over previously reported methods of infusing drugs into the kidney of the rat. In contrast to most other techniques, renal blood flow does not have to be interrupted, even momentarily, during the cannulation procedure. Although Fine et al acknowledge that blood flow should not be stopped for more than 10 to 20 seconds (7)it is our experience that ligating the aorta or renal artery results in almost immediate blanching of the kidney. This will undoubtedly induce both direct (renin release) and indirect (renal afferent nerve activity) responses to alter both renal function and systemic hemodynamics (9, 16).

Another approach has been to cannulate the suprarenal artery (10, 21). Not only does this fail to address the problem of streaming and uneven distribution of infusate, but we found that ligation of the suprarenal artery induced lability of the MAP which directly affected renal blood flow (unpublished observation).

Cupples and Sonnenberg recognized the need to ensure adequate distribution of infused drug with blood before entering the kidney (4). To this end they used an extracorporeal circuit as devised by Fink & Brody (8). This circuit involved shunting blood from the carotid artery to an aortic pouch leading to the left renal artery. This enabled the test substances to be infused some distance upstream from the kidney, which ensured adequate mixing. The disadvantage of this method lies in the fact that, as admitted by the authors themselves, it is highly invasive.

It was in light of these previous attempts to address the problem of streaming that Parekh, in 1995, developed a multiple catheter system with a magnetic pump whereby blood could be drawn back and mixed with the test substance prior to being re-infused into the animal (18). Parekh showed convincingly that with this system, not only was injected dye evenly distributed in the kidney, but the renal responses to vasoactive drugs were augmented and the systemic responses reduced. The disadvantage of this system is that it is complicated to set up, involving as it does fused multiple cannulae and a magnetic membrane pump. By contrast, our method of simply applying steady vibration to the syringe with a commercial vibrator is economical, efficient and significantly improves drug distribution in the kidney.

We describe applying vibration directly to the infusion syringe. However, the system worked equally well when the vibrator was taped to the hard plastic male adapter on an intravenous infusion set (Abbott Laboratories, Illinois, USA); this would allow one to use a syringe pump or a peristaltic pump placed upstream of the vibrator to administer the solution. Moreover, one may use either a flank or a midline approach to the kidney, since the vibrator is applied several centimeters distal to the tip of the cannula (Figure A.1). The ability to ensure homogeneous drug distribution during close-arterial infusion is critical to ensuring meaningful, reproducible experimental data, not only in the kidney, but also in other target organs such as the brain and liver.

A.5 BIBLIOGRAPHY

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APPENDIX B:

SPLENECTOMY AND PRESERVATION OF SPLENIC AFFERENT NERVE ACTIVITY

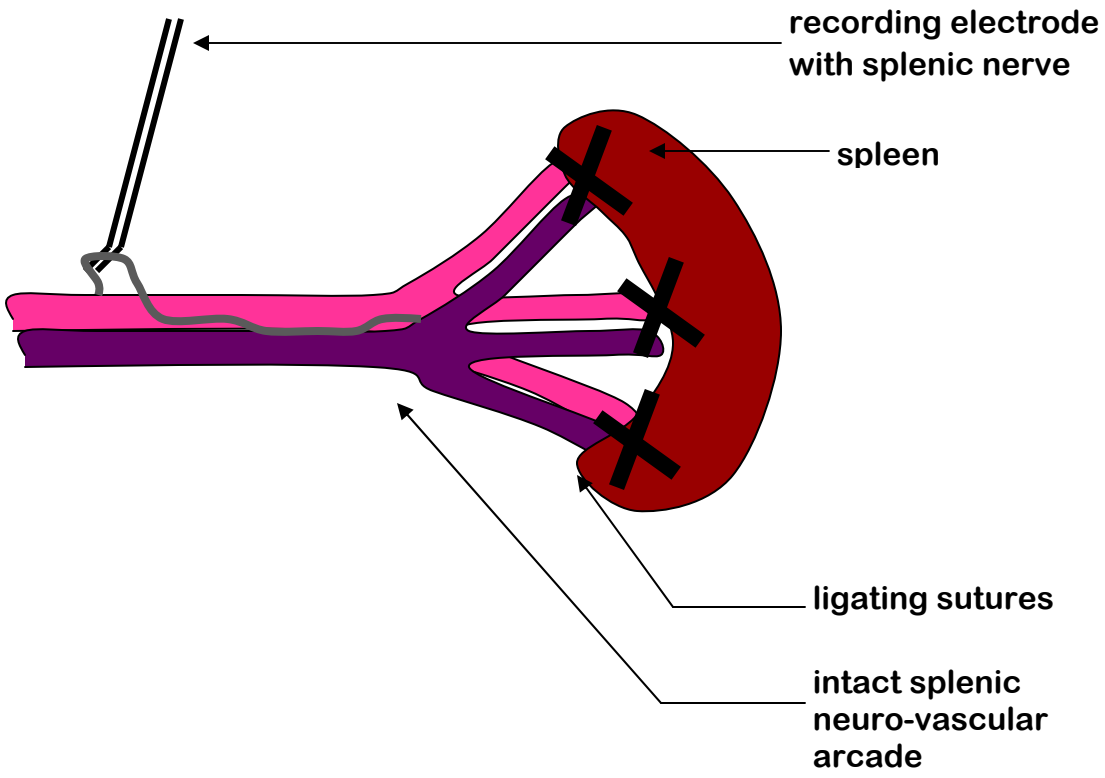

Figure B1 Schematic representation of in vivo splenic afferent nerve preparation after splenectomy. The spleen and associated arcade of vessels was laid over moist gauze in the abdomen of the anesthetized animal. 4-0 silk ligating sutures were tied firmly around each splenic vascular bundle at the hilum of the spleen. The spleen was then cut away and removed from the animal, leaving the splenic neurovascular arcade intact. A premade steel support ring was sutured to the edges of the abdominal incision and raised to create a "well" which was then filled with mineral oil. A splenic nerve was located alongside the main trunks of the splenic vessels and gently isolated from surrounding tissues. The nerve was cut proximally and gently wrapped around a recording electrode.

EFFECT OF SPLENECTOMY ON SPLENIC AFFERENT NERVE ACTIVITY

Anesthetized male rats (n = 7) were surgically prepared with a femoral arterial cannula for measurement of mean arterial pressure (MAP); a 4-0 Prolene loop was placed around the portal vein for elevation of portal venous pressure by PVL. Following splenectomy and isolation of the splenic nerve (Figure B.1), the animal was stabilized for 25 min and baseline parameters were recorded online. Splenic afferent nerve activity was detected at rest in the absence of the spleen. During PVL, portal venous pressure rose from 6.0 ± 0.2 to 13.8 ± 0.6 mmHg and MAP dropped from 95.6 ± 3.3 to 88.2 ± 3.0 mmHg. There was a significant increase in splenic afferent nerve activity (Figure B.2).

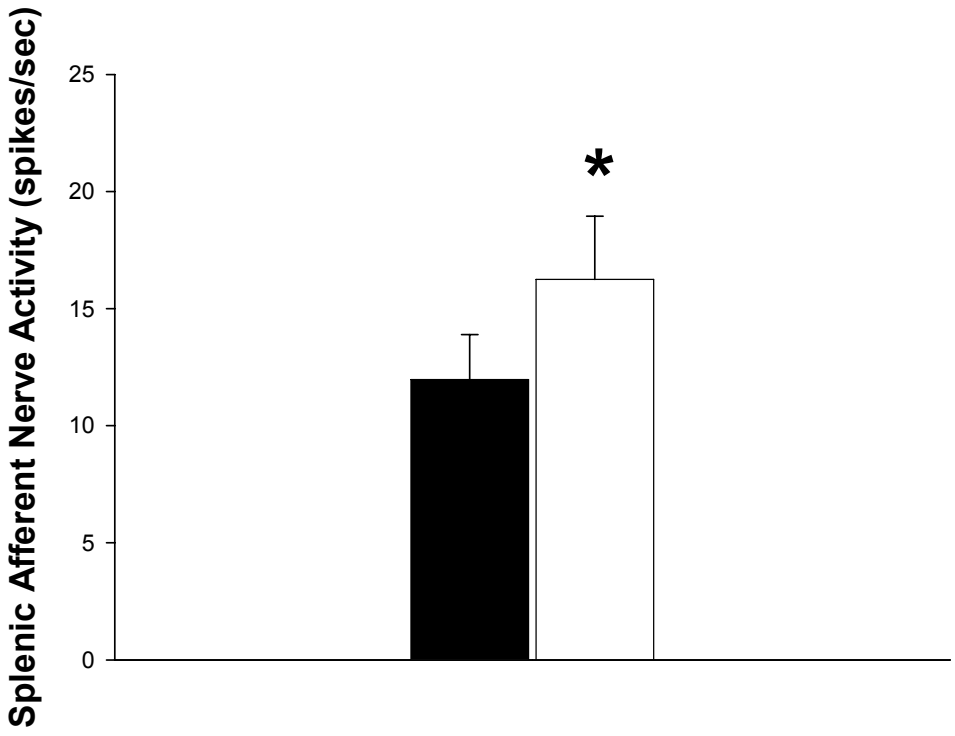

Figure B2 Effect of partial portal vein ligation (PVL) on splenic afferent nerve activity after splenectomy. Black bar: baseline; Open bar: during PVL. Data presented as mean ± SEM. *Significant difference from baseline (One Way RM ANOVA); significance accepted at $p < 0.05$.

APPENDIX C:

EFFECT OF PARTIAL SPLENIC VEIN OCCLUSION ON SUPERIOR MESENTERIC VENOUS FLOW

SURGICAL PREPARATION AND EXPERIMENTAL PROTOCOL

Anesthetized male rats (n=62) were prepared as outlined in section 4.2, with the exception of placement of a 1RB transit-time flow probe around the superior mesenteric artery. In this series of experiments a 3RB transit-time flow probe (Transonic System, Ithaca, NY) was positioned around the superior mesenteric vein (SMV), just below its junction with the splenic vein. Thus, excluding splenic venous outflow and any changes that may solely occur due to partial splenic vein occlusion (SVO). The experimental protocol outlined in section 4.2 was followed with measurement of mean arterial pressure (MAP) and SMV flow at rest and during partial SVO. A total of nine groups were studied: Intact (n=7); splenic denervated (n=7); mesenteric denervated (n=7); bilateral renal denervated (n=6); systemic angiotensin II (ANG II) AT_1 receptor blockade with Losartan (n=9); 10% DMSO vehicle control (n=5); systemic vasopressin (AVP) V_{1a} receptor blockade with SR49059 (n=8); SR49059 + mesenteric denervation (n=6); and SR49059 + bilateral renal denervation (n=7).

For systemic AT_1 blockade, animals were treated with 3mg/kg Losartan, which was given as a 0.1mL i.v. bolus just before the pre-experimental stabilization period. Efficacy of AT_1 receptor blockade was tested with i.v. infusion of 0.1 μg/kg angiotensin II. Systemic blockade of V_{1a} receptors, 1 mg/kg SR49059 dissolved in 10% DMSO was administered as a 0.1 mL i.v. bolus just prior to the pre-experimental stabilization period. Efficacy of V_{1a} receptor blockade was verified with i.v. infusion of 200ng/kg arginine vasopressin.

During partial SVO, splenic venous pressure was raised from 7.4 ± 0.4 to 22.3 ± 0.2 mmHg (n=62).

Table C1 Mean arterial pressure (MAP) at baseline and during partial splenic vein occlusion(SVO).

Group	Baseline MAP (mmHg)
Intact	96.8 ± 5.0
Splenic Denervated	90.1 ± 2.2
Mesenteric Denervated	79.6 ± 2.4
Bilateral Renal Denervated	87.8 ± 2.3
Losartan	76.4 ± 3.6
10% DMSO	103.8 ± 6.1*
SR49059	88.1 ± 3.1
SR49059+mesenteric denervation	90.7 ± 2.3
SR49059+bilateral renal denervation	76.5 ± 2.0*

*Significant difference from 10%DMSO group (One Way RM ANOVA); significance accepted at $p < 0.05$.

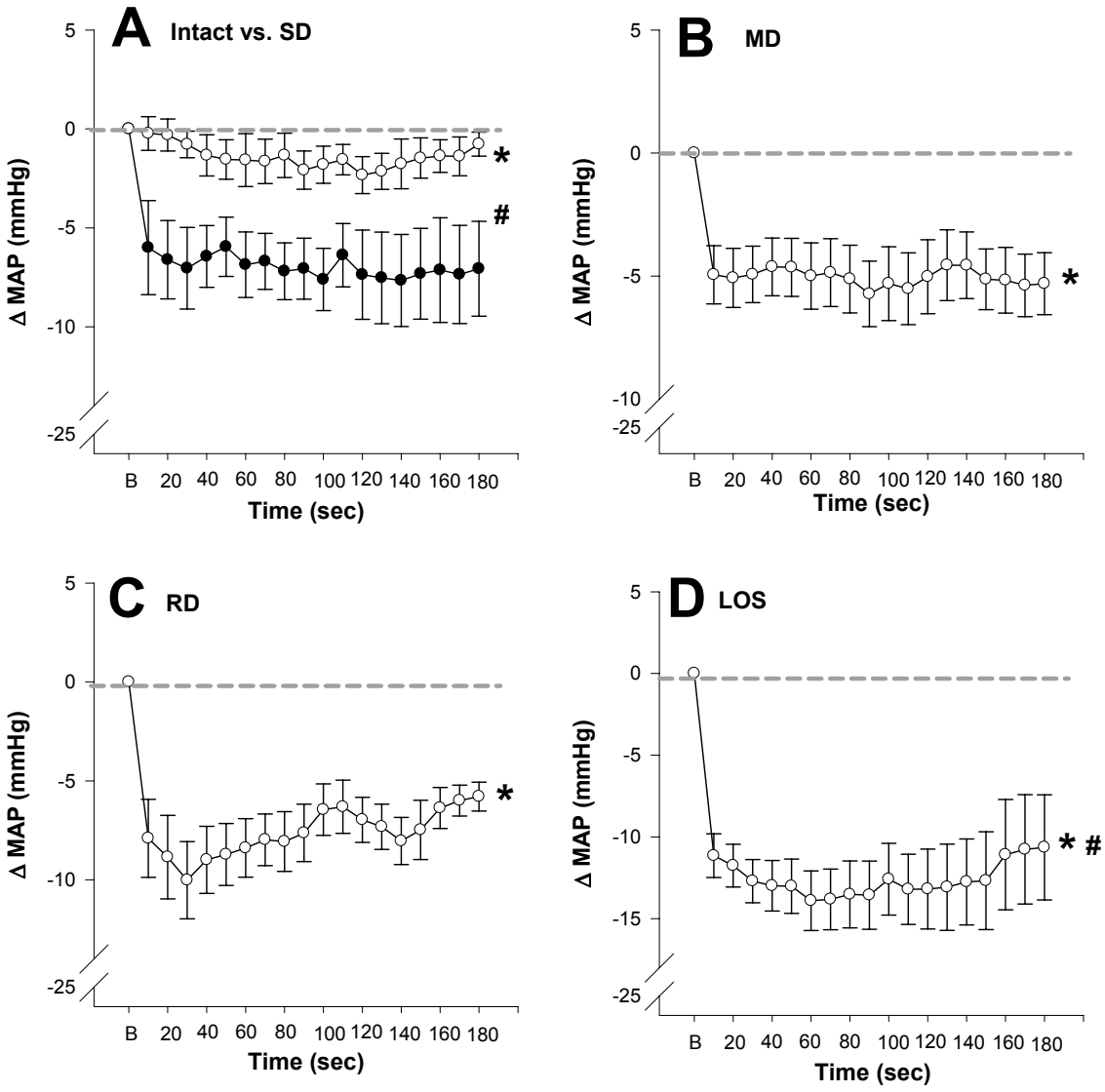

Figure C1: Effect of partial splenic vein occlusion (SVO) on change in mean arterial pressure (MAP) from baseline (B). *A*: Intact, splenic denervated (SD); *B*: mesenteric denervated (MD); *C*: bilateral renal denervated (RD); *D*: Losartan-treated (3mg/kg ; LOS). Losartan was given immediately before pre-experimental stabilization period. Closed circles: Intact; Open circles: experimental groups. Data presented as means ± SEM. *Significant difference from own baseline (One Way RM ANOVA). #Significant difference from Intact group (Two Way RM ANOVA); $p < 0.05$.

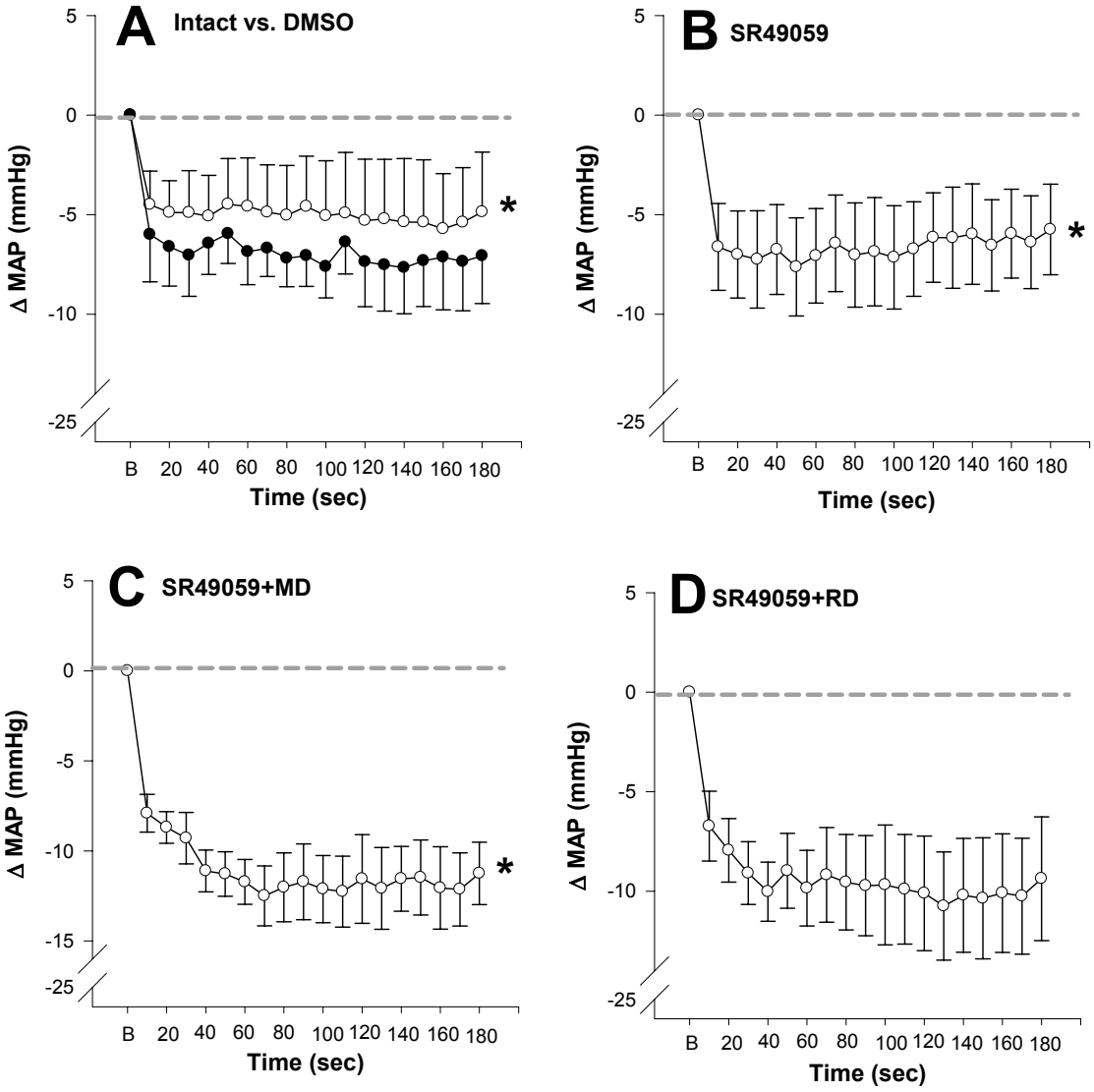

Figure C2: Effect of partial splenic vein occlusion (SVO) on change in mean arterial pressure (MAP) from baseline (B). *A*: Intact, 10% DMSO vehicle control (10% DMSO solution given i.v. as a 0.1 mL bolus – DMSO); *B*: SR49059-treated (1mg/mL ; SR49059); *C*: SR49059 + mesenteric denervation (SR49059+MD); *D*: SR49059 + bilateral renal denervation (SR49059+RD). SR49059 or vehicle was given immediately before pre-experimental stabilization period. Closed circles: Intact; Open circles: experimental groups. Data presented as means ± SEM. *Significant difference from own baseline (One Way RM ANOVA). #Significant difference from Intact group (Two Way RM ANOVA); $p < 0.05$.

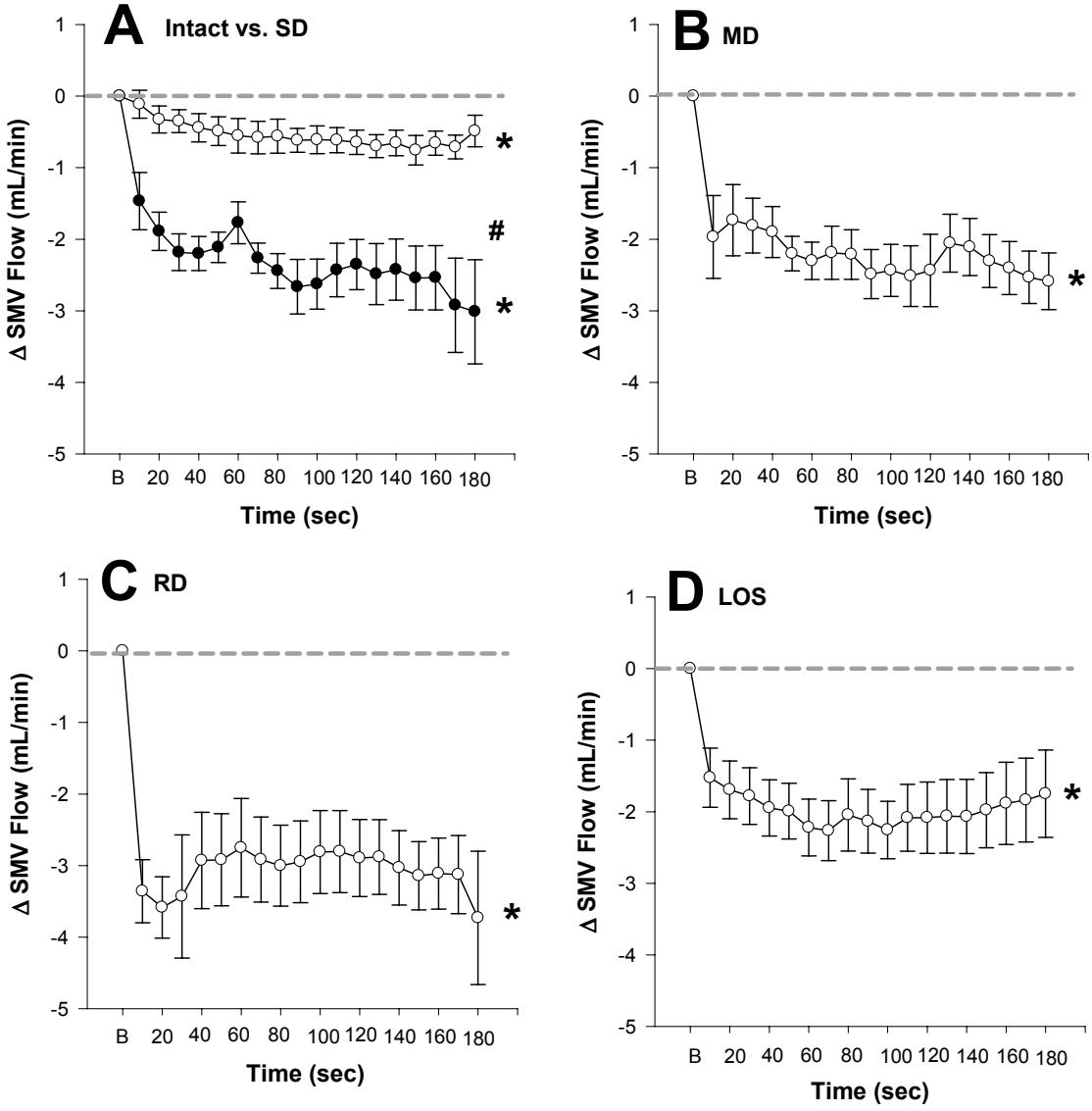

Figure C3: Effect of partial splenic vein occlusion (SVO) on change in superior mesenteric venous (SMV) blood flow from baseline (B). *A*: Intact, splenic denervated (SD); *B*: mesenteric denervated (MD); *C*: bilateral renal denervated (RD); *D*: Losartan-treated (3mg/kg ; LOS). Losartan was given immediately before pre-experimental stabilization period. Closed circles: Intact; Open circles: experimental groups. Data presented as means ± SEM. *Significant difference from own baseline (One Way RM ANOVA). #Significant difference from Intact group (Two Way RM ANOVA); $p < 0.05$.

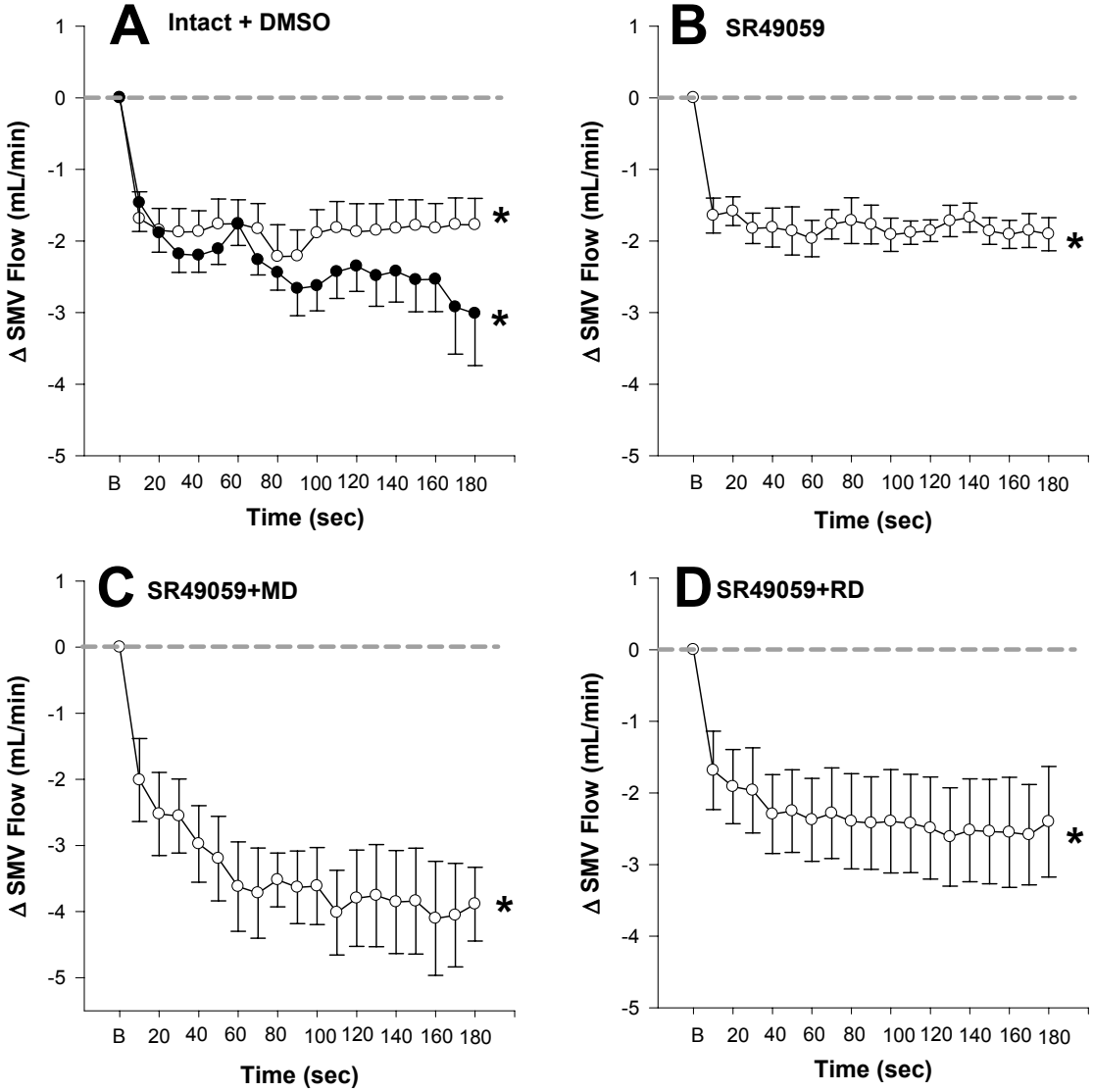

Figure C4 Effect of partial splenic vein occlusion on change in superior mesenteric venous (SMV) blood flow from baseline (B). *A*: Intact, 10% DMSO vehicle control (10% DMSO solution given i.v. as a 0.1 mL bolus – DMSO); *B*: SR49059-treated (1mg/mL ; SR49059); *C*: SR49059 + mesenteric denervation (SR49059+MD); *D*: SR49059 + bilateral renal denervation (SR49059+RD). SR49059 or vehicle was given immediately before pre-experimental stabilization period. Closed circles: Intact; Open circles: experimental groups. Data presented as means ± SEM. *Significant difference from own baseline (One Way RM ANOVA). #Significant difference from Intact group (Two Way RM ANOVA); $p < 0.05$.

APPENDIX D:

EFFECT OF PARTIAL SPLENIC VEIN OCCLUSION ON SUPERIOR MESENTERIC ARTERIAL FLOW AND CONDUCTANCE – SYSTEMIC LOSARTAN TREATMENT GROUP

SURGICAL PREPARATION AND EXPERIMENTAL PROTOCOL

Male rats (n=6) were anesthetized and surgically prepared as outlined in section 4.2. Losartan potassium (Fluka) was dissolved in double distilled water to give a 10mg/mL solution. Systemic pharmacological blockade of ANG II AT_1 receptors was achieved by administration of 3mg/kg Losartan i.v. (~0.1 mL bolus injection) following completion of the surgical protocol and before commencement of the pre-experiment stabilization period. This dose was determined to result in minimal systemic effects in pilot experiments (unpublished observation, S. Hamza). Experimental protocol as outlined in section 4.2. Efficacy of AT_1 receptor blockade was verified at experiment end by infusion of 0.1 μg/kg ANG II i.v. (0.1mL bolus) and subsequent absence of any pressor effect.

During partial SVO, splenic venous pressure was raised from 4.8 ± 0.9 mmHg to 22.1 ± 1.8 mmHg (n=6). Baseline parameters were as follows: Mean Arterial Pressure: 87.0 ± 5.1 mmHg; Superior Mesenteric Arterial Flow: 9.3 ± 0.7 mL/min; Superior Mesenteric arterial Conductance: 0.110 ± 0.01 $mLmin^{-1}mmHg^{-1}$. These values were no different from Intact baseline parameters (Chapter 4).

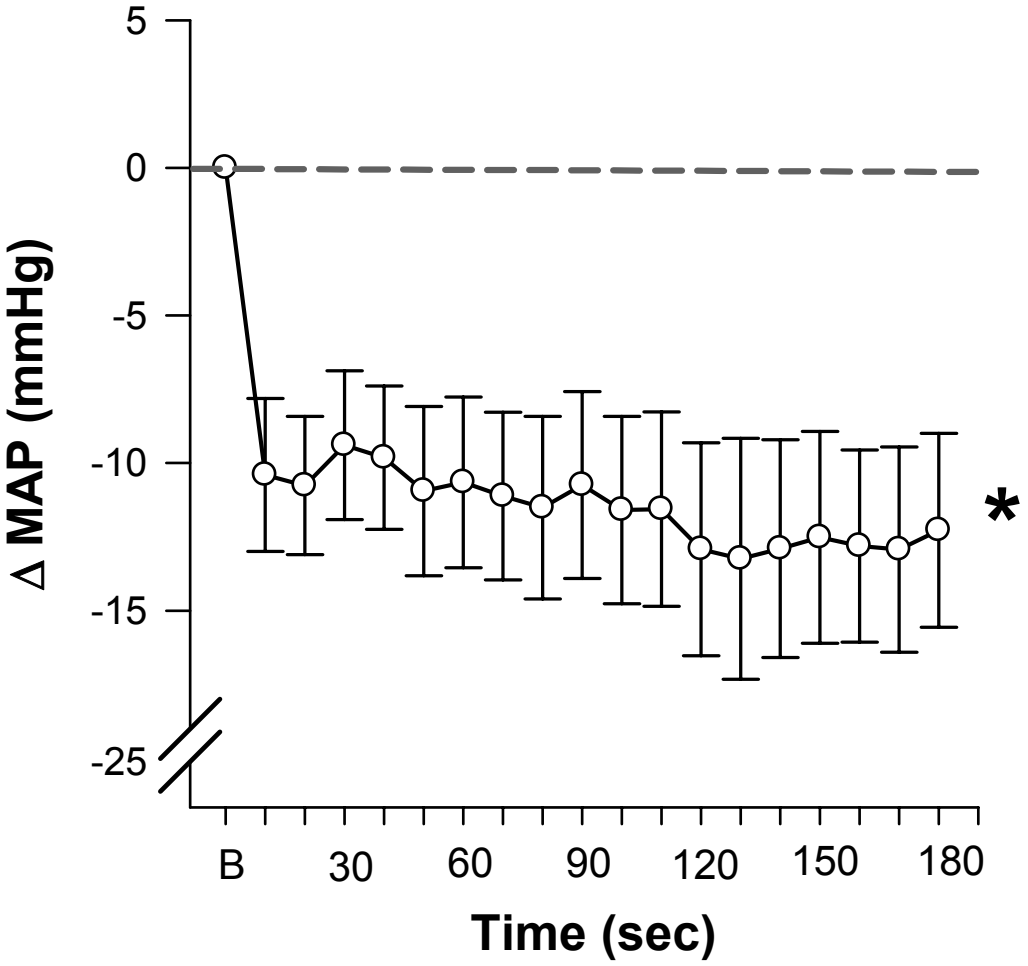

Figure D1 Effect of partial splenic vein occlusion on change of mean arterial pressure (MAP) from baseline in systemic Losartan-treated rats (n=6). Data are presented as means ± SEM. *Significant difference from own baseline (One Way RM ANOVA, $p<0.05$).

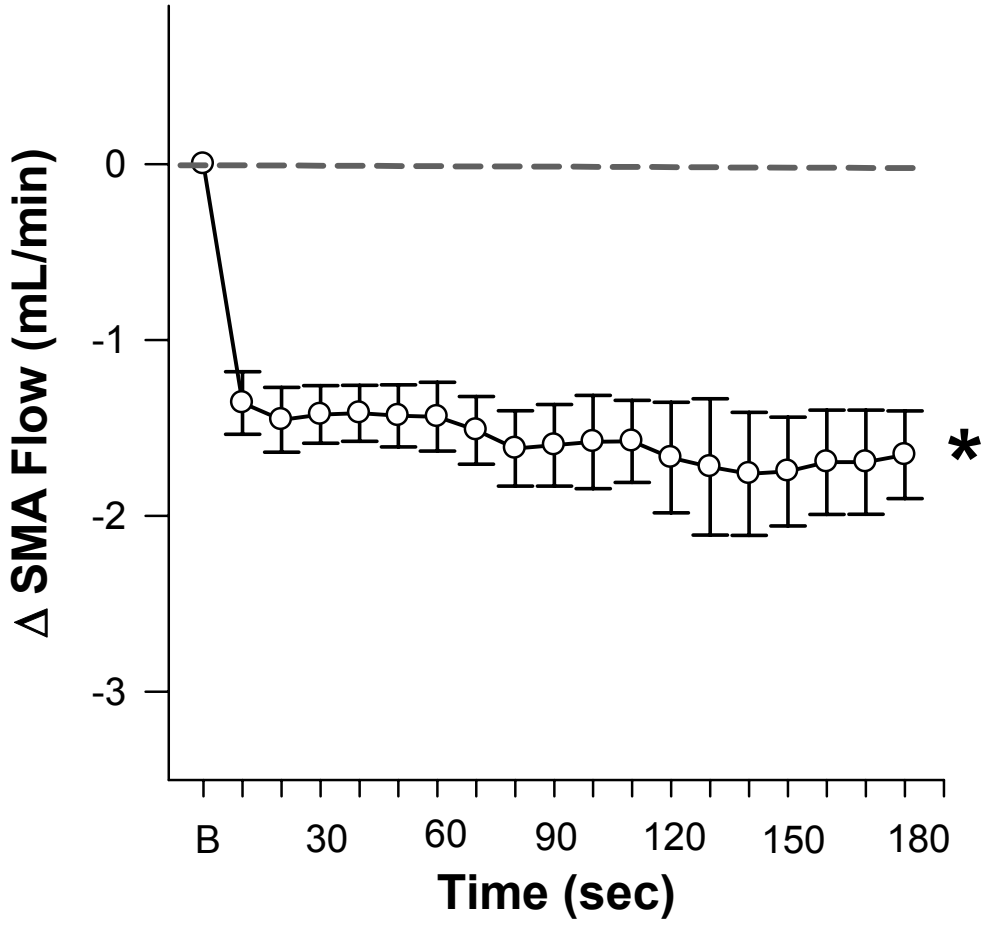

Figure D2 Effect of partial splenic vein occlusion on change in superior mesenteric arterial (SMA) blood flow from baseline of systemic Losartan-treated rats (n=6). Data are presented as means ± SEM. *Significant difference from own baseline (One Way RM ANOVA, $p<0.05$).

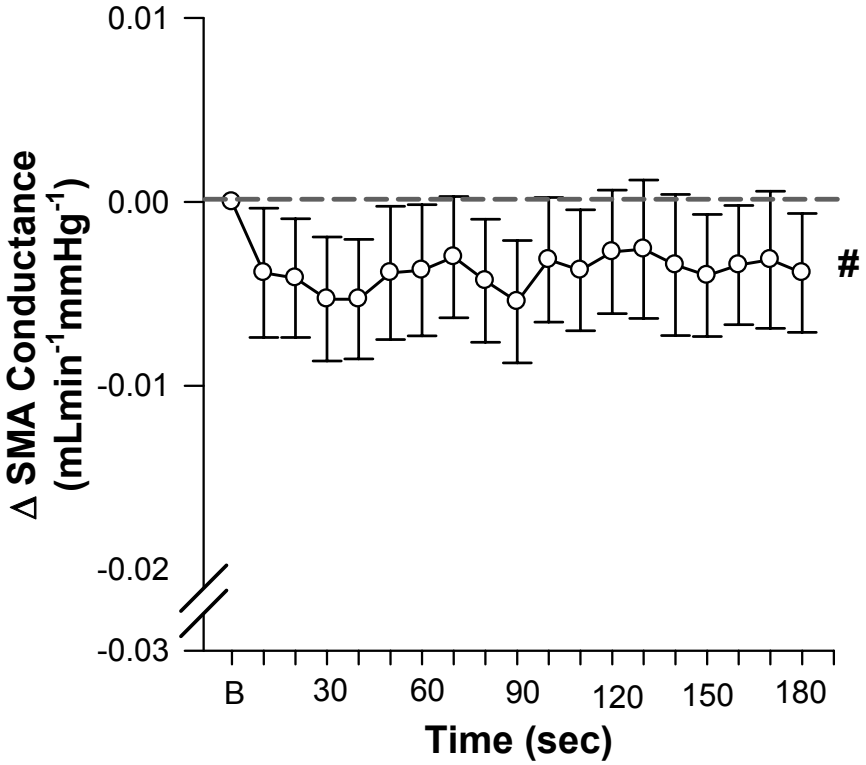

Figure D3 Effect of partial splenic vein occlusion on change in superior mesenteric arterial (SMA) conductance from baseline of systemic Losartan-treated rats (n=6). Data are presented as means ± SEM. #Significant difference from Intact group (Section 4.3.2; Two Way RM ANOVA, $p<0.05$).