Effects of Transmural Distending Pressure on Integrated Venous Function in Normal Rat

by

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Guelph, Ontario, Canada

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ABSTRACT

EFFECTS OF TRANSMURAL DISTENDING PRESSURE ON INTEGRATED VENOUS FUNCTION IN NORMAL RAT

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Vasomotor tone is largely maintained by sympathetic nerves, myogenic reactivity and key local and circulating hormones. Acting together, these factors ensure moment-to-moment adjustments of net vascular tone required to maintain hemodynamic stability. In rat mesenteric small veins (MSV) and arteries (MSA), we investigated the contribution of the endothelium, L-type voltage operated calcium channels (L-VOCCs), PKC and Rho kinase to myogenic reactivity. The interaction of myogenic reactivity with norepinephrine (NE), endothelin-1 (ET-1), and sympathetic nerve activation was also investigated under conditions of changing transmural distending pressure. We also evaluated the relative contribution of alpha adrenergic (α-A) and endothelinergic receptors to NE and ET-1 contractile responses, respectively. Additionally, the effects of changing transmural pressure on endothelial dilator function of MSV were examined. Myogenic reactivity was not altered by nitric oxide synthase (NOS) inhibition or endothelium removal in both vessels. L-VOCCs blockade completely abolished arterial tone, while only partially reducing venous tone. PKC and Rho kinase inhibitors largely abolished venous and arterial myogenic reactivity. Increasing transmural pressure did not alter NE, ET-1, and bradykinin responses, but it significantly reduced neurogenic
contractions. MSV were more sensitive to NE, ET-1 and sympathetic nerve activation compared with corresponding arteries. $\alpha$-A and ET-1 receptor agonist and antagonist application revealed the participation of $\alpha_1$-A and ET$_A$ receptors in NE and ET-1 contractile responses, respectively. $\alpha_2$-A and ET$_B$ receptors appeared to mediate NE and ET-1 responses in MSV, respectively. Bradykinin induced-vasodilation was mainly reduced by NOS inhibition, and BK$_{Ca}$ and Sk$_{Ca}$ blockade. These results suggest that myogenic factors are important contributors to net venous tone in MSV; PKC and Rho kinase activation are important to myogenic reactivity in both vessels, while L-VOCCs play a limited role in the veins versus the arteries; mesenteric veins maintain an enhanced sensitivity to NE, ET-1 and sympathetic nerve activation compared to the arteries with neurogenic contractions being affected by transmural pressure elevations; $\alpha_1$-ARs and ET$_A$ are the predominant receptors mediating contractile responses to NE and ET-1, respectively, with functional evidence indicating the presence of $\alpha_2$-ARs and ET$_B$ receptors in MSV; and venous endothelial dilator function is not affected by an elevation of transmural pressure.
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DECLARATION OF WORK PERFORMED

I declare that with the exception of the items indicated below, all work reported in this thesis was performed by me.

Gabrielle Monteith wrote and ran the statistical programs for data analysis.
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* control, BQ-788 vs. BQ-610 and both; † BQ-610 vs. both at ET-1 \( 10^{-8} \) – \( 10^{-9} \) M. \( n \), number per group in parenthesis. Data are expressed as mean ± SEM (\( P < 0.05 \)).

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LIST OF ABBREVIATIONS

AR (s)                      Adrenergic receptor (s)
α-AR (s)                    Alpha adrenergic receptor (s)
ADMA                       Asymmetric dimethylarginine
ADP                        Adenosine diphosphate
AMP                        Adenosine monophosphate
ATP                        Adenosine 5′-triphosphate
ATP-ase                    Adenosine 5′-triphosphataes
BKCa                       Large conductance calcium-activated potassium channels
BK                          Bradykinin
Ca^2+                      Calcium ion
CaCl₂                      Calcium chloride
Cl                          Chloride ion
CO₂                        Carbon dioxide
COX                        Cyclooxygenase
CRCs                       Concentration response curves
cAMP                       Cyclic adenosine monophosphate
cGMP                       Cyclic guanosine monophosphate
DAG                        Diacylglycerol
DHPs                       Dihydropyridines
EC₅₀                       Half maximum contraction
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>$E_{\text{max}}$</td>
<td>Maximal contraction</td>
</tr>
<tr>
<td>EDHF</td>
<td>Endothelial-derived hyperpolarizing factor</td>
</tr>
<tr>
<td>EET$_S$</td>
<td>Epoxyeicosatrieionic acids</td>
</tr>
<tr>
<td>EFS</td>
<td>Electrical field stimulation</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetra-acetic acid</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial Na$^+$ channels</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>ECs</td>
</tr>
<tr>
<td>ET</td>
<td>Endothelin(s)</td>
</tr>
<tr>
<td>FRCs</td>
<td>Frequency response curves</td>
</tr>
<tr>
<td>GC</td>
<td>Guanylate cyclase</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IK$_{\text{Ca}}$</td>
<td>Intermediate conductance calcium-activated potassium channels</td>
</tr>
<tr>
<td>Indo</td>
<td>Indomethacin</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol 1, 4, 5-trisphosphate</td>
</tr>
<tr>
<td>K$^+$</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>K$_{\text{ATP}}$</td>
<td>ATP-sensitive K$^+$ channels</td>
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<tr>
<td>K$_{\text{Ca}}$</td>
<td>Calcium activated potassium channels</td>
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<tr>
<td>K$_{\text{IR}}$</td>
<td>Inwardly rectifying K$^+$ channels</td>
</tr>
<tr>
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<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>K\textsubscript{V}</td>
<td>Voltage-gated K\textsuperscript{+} channels</td>
</tr>
<tr>
<td>KCL</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>L-NAME</td>
<td>N\textsubscript{ω}-Nitro-L-arginine methyl ester hydrochloride</td>
</tr>
<tr>
<td>L-NNA</td>
<td>N\textsubscript{ω}-Nitro-L-arginine</td>
</tr>
<tr>
<td>MCFP</td>
<td>Mean circulatory filling pressure</td>
</tr>
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<td>Magnesium chloride</td>
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<tr>
<td>MLC</td>
<td>Myosin light chain</td>
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<td>MLCP</td>
<td>Myosin light chain phosphatase</td>
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<td>MSA</td>
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<td>Mesenteric small veins</td>
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<td>DMT</td>
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<td>NOS</td>
<td>Nitric oxide synthase</td>
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<tr>
<td>nNOS</td>
<td>Neural nitric oxide synthase</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<tr>
<td>eNOS</td>
<td>Endothelial (constitutive) nitric oxide synthase</td>
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<td>Full Name</td>
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<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
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<tr>
<td>PdBu</td>
<td>Phorbol 12,13-dibutyrae</td>
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<td>PGF2α</td>
<td>Prostaglandin F2 alpha</td>
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<td>PGI_{2}</td>
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<td>PKG</td>
<td>Protein kinase G</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<tr>
<td>PPADS</td>
<td>Pyridoxal-phosphate-6-azophenyl-2',4’-disulfonic acid</td>
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<tr>
<td>S_{50}</td>
<td>Half-maximal response</td>
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<td>Rho kinases</td>
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<td>Reactive oxygen species</td>
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<td>S-Nitroso-N-acetylpenicillamine</td>
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<td>SNP</td>
<td>Sodium nitroprusside</td>
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<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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<td>Description</td>
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</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
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<td>TRPC</td>
<td>Transient receptor potential channels</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
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<tr>
<td>VOCCs</td>
<td>Voltage-operated Ca(^{2+}) channels</td>
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1. **Function of the venous system**

1.1 **Venous function in vivo: Significance**

The major function of the venous system is to return blood from the periphery to the heart in order to maintain the filling of the heart. The venous system contains approximately 70% of the total blood volume with the majority being found in small veins and venules compared to only 18% in large arteries and 3% in terminal arteries and arterioles (Meissner et al., 2007; Pang 2000). This characteristic of the venous system is due to the large cross sectional areas of the veins compared to the arteries. Cross sectional areas of veins are approximately 4 times those of the corresponding arteries (Landis and Hortenstine, 1950). Thus the venous system is the major blood reservoir in the body. Within the venous circulation, small veins and venules hold approximately 30% and 45% of the blood volume respectively (Pang, 2000). Therefore, small veins and venules are the most substantial blood reservoir within the circulation.

The venous splanchnic vascular bed, including the mesenteric veins, is the largest venous bed in the body. Interestingly, this type of vasculature is well innervated, has a high population of alpha adrenergic receptors, and therefore it is highly sensitive to adrenergic stimulation (Xu et al., 2007). Another characteristic of this vascular bed is its high compliance being capable of holding approximately 30% of the total blood volume, thus playing a significant role in homeostatic responses to changes in intravascular volume and accompanying changes in distending pressure (Dhawan et al., 2005).
The compliance of blood vessels is a measure of their elasticity and it is defined as the ratio of a change in blood volume to the concomitant change in transmural distending pressures (Rothe, 1983). Since transmural distending pressure in the blood vessels is determined by the blood volume they contain, there is a close relationship between the blood volume and pressure. Thus when blood volume increases, there is an increase in systemic blood pressure. The large volume of blood that veins contain can also cause distending pressure which initiates many regional and systemic vascular responses. Pressurization of veins in the physiological range resulted in the development of myogenic reactivity suggesting that veins have the ability to increase their vascular tone appropriately and resist the increases in distending pressure (Brookes and Kaufman, 2003).

The large capacity of the venous vascular bed could compensate for the large increases in blood volume with minimal effects on cardiac output and blood pressure. However, minimal changes in venous tone can cause large shifts in blood volume toward the heart via venous return, with subsequent modification of cardiac output and blood pressure (Greenway, 1981; Shoukas and Sagawa, 1973). Therefore, distending pressure affects venous tone and net venous function. Regulation of the venous system is mediated primarily via the sympathetic nervous system. In fact, it has been estimated that baroreceptor-mediated changes in venous tone could alter cardiac output by up to 40% (Greene and Shoukas, 1986) and that autonomic ganglion blockade produces a significant drop in mean circulatory filling pressure (MCFP), an index of integrated venomotor tone (Fink et al., 2000; Martin et al., 1998).
Alterations in venous tone have been reported in several physiological and pathological conditions such as hypertension, heart failure, orthostatic hypotension, shock and acute stress (Fink et al., 2000; Gay et al., 1986; Martin et al., 1998; Martin et al., 1996). These conditions share the need for control of cardiac output to maintain filling of the heart and overall cardiovascular regulation reflecting the significant role of the venous function. However, the mechanisms responsible for regulating venous tone and subsequent hemodynamic events remain unclear. Taken together, it appears that venous function plays an essential role in cardiovascular homeostasis via modulation of venous return, cardiac output and arterial blood pressure in a number of health and disease states. Despite the important role of veins in overall cardiovascular regulation, there are limited studies that have evaluated their functional role in health and disease conditions.

2. Differences between veins and arteries

Studying the effects of physiology and pharmacological actions of chemicals on arteries and veins requires different techniques due to the differences in anatomy and physiology of these two components of the circulation. Therefore, experimental techniques used on the arteries may not be suitable for studies on veins, and for that reason most studies exploring cardiovascular pharmacology and physiology are conducted on arteries. However, data collected in studies using arterial tissue cannot necessarily be applied to the venous system. As mentioned above, veins contribute very little to total peripheral resistance; however, veins hold a significant amount of blood volume compared to arteries reflecting the importance of veins in controlling cardiovascular function.
2.1 Venous and arterial physiological functions

Arteries, which transport blood from the heart to tissues, are resistance vessels and are a part of the high pressure vascular system. Veins, which transport blood from the tissues back to the heart, are capacitance vessels and are a part of the low pressure vascular system. While arteries, with few exceptions, provide tissues with oxygen and nutrients, the veins, also with few exceptions, return blood to the heart for reoxygenation. In order to provide for vascular, ie. arterial versus venous, and regional specialization in the cardiovascular system, it is necessary for these differing vessel types to vary anatomically and with mechanisms regulating their overall level of tone. In general, arteries and veins consist of three layers, the tunica intima, tunica media and tunica adventitia. Endothelial cells are located within the tunica intima whereas smooth muscle cells are mainly located in the tunica media. Both arteries and veins receive nutrients via the vasa vasorum, which are located in the outermost vessel layer. Although arteries and veins contain similar layers, the tunica media of arteries is much thicker than that of veins. However, the tunica media layer of veins contains sufficient smooth muscle to contract or relax and therefore acts as a highly controllable reservoir for extra blood flow.

2.2 Venous and arterial innervation

In general, mesenteric arteries and veins are innervated by both the sympathetic division of the autonomic nervous system and spinal sensory nerves (Kreulen, 2003). Regarding neural controls of arteries and veins, sympathetic stimulation induces vasoconstriction while sensory neuron stimulation results in vasodilatation. The splanchnic bed, including the mesenteric circulation, receives about 60 % of the cardiac
output and holds about 30% of the total blood volume (Pang, 2000). About 60% of venous blood reserves can be mobilized by way of increases in peripheral vascular resistance caused by sympathetic nerve stimulation (Greenway, 1983).

Innervation density was found to be less in rat mesenteric small veins (MSV) compared to corresponding mesenteric small arteries (MSA) (Park et al., 2007). However, this does not necessarily mean that the nerve fibers of MSV are less than that of the MSA considering the thinner muscle layer MSV have and thus the number of axons per smooth muscle cell could be similar in both vessels. Moreover, MSA and MSV that are innervated by inferior mesenteric ganglion receive innervation from distinct neurons (Browning et al., 1999). Since this phenomenon is also observed in other vasculatures, it can be concluded that arteries and veins, including mesenteric vessels, are controlled separately (Brandao, 1976).

2.3 Venous and arterial responses to sympathetic neurogenic stimulation

It is well recognized that the sympathetic nervous system is a major effector system involved in the control of vasomotor tone. Mesenteric arteries and veins respond to sympathetic neurogenic stimulation in a frequency dependent manner with veins typically responding to lower frequencies (1-2 Hz) and arteries to higher frequencies (10-20 Hz) (Hottenstein and Kreulen, 1987). These observations suggest that a response to moderate increases in sympathetic tone would be constriction of the capacitance vessels, which would eject blood from the splanchnic vasculature into the systemic circulation, potentially without significant increases in systemic arterial resistance. In contrast, the response to larger increases in sympathetic tone would be both a decrease in venous
capacity with recruitment of blood volume from splanchnic vasculature and an increase in arterial tone and blood pressure.

The difference in responses to sympathetic activation in arteries and veins may be due to variations in the neurotransmitter content of sympathetic postganglionic neurons or postjunctional receptors populations on the vessels. In the rat mesentery, it has been demonstrated that the contractile responses to sympathetic neurogenic stimulation are mainly due to adenosine 5’-triphosphate (ATP) in arteries, whereas in veins NE makes a major contribution to sympathetic neurotransmission (Luo et al., 2003). Moreover, during nerve stimulation, the perivenous nerves release more NE compared with the periarterial nerves (Luo et al., 2004; Luo et al., 2003). These differences in sympathetic neurotransmission of mesenteric arteries versus veins underscores key functional differences of these vessel types in the mesentery, and could explain the higher sensitivity of the mesenteric veins to sympathetic nerve stimulation compared with arteries.

2.4 Venous and arterial responses to contractile agonists

The sensitivity of the venous contractile apparatus to either receptor-dependent agonists such as NE, phenylephrine (PE), ATP and endothelin-1 (ET-1), or receptor-independent agonists such potassium chloride (KCl) is greater than that of arteries (i.e. concentration of agonist producing half maximum contraction, EC$_{50}$, is smaller in the case of the veins) (Johnson et al., 2002; Thakali et al., 2004; Watts et al., 2002). The pattern of contraction seen in arteries and veins is also different. In general, a robust contraction in response to adrenergic agonist administration is not seen in mouse veins (Perez-Rivera et al., 2007; Sporkova et al., 2010). Recently, it has been shown that
mouse mesenteric veins do not desensitize to adrenergic stimuli to the same degree observed in arteries from the same vascular bed, suggesting that veins might have a complement of adrenergic receptors not present in arteries (Perez-Rivera et al., 2004). Moreover; in contrast to arteries, veins contract in response to sarafotoxin 6c (S6c), a selective ET<sub>B</sub> receptor agonist, (Johnson et al., 2002; Watts et al., 2002) and do not completely desensitize to ET-1 (Thakali et al., 2004). This may explain the higher sensitivity of veins to endothelinergic receptor stimulation compared to arteries and suggest the maintenance of ET-1 induced contraction overtime, a condition that may have a clinical relevance particularly in chronic disease conditions, associated with an altered ET receptor function, such as hypertension (Kirkby et al.; 2008).

3. Vascular smooth muscle cells

Smooth muscle cells are contained in the wall of various hallow organs and tissues including the blood vessels. Contraction of the veins and arteries is produced by smooth muscle cells which is initiated by calcium (Ca<sup>2+</sup>)-calmodulin to stimulate phosphorylation of myosin light chain (MLC) and can be regulated by sympathetic activation, circulating hormones, locally released substances, ions and mechanical stimuli such as stretch. Relaxation of the blood vessels, on the other hand, is initiated by the removal of Ca<sup>2+</sup> from the cytosol and activation of MLC phosphatase (MLCP). These physiological events in association with vascular endothelial functions regulate blood pressure and blood flow. Arterial and venous contractions are known to elevate blood pressure via increasing vascular resistance and venous return, respectively. An interesting characteristic of small resistance arteries and some veins is myogenic reactivity, as will be discussed, where vascular contractions in response to an increase in
transmural blood pressure cause shortening of vascular smooth muscle (VSM) cells, particularly of small arteries and arterioles. This decrease in blood vessel diameter allows blood flow and pressure to be maintained in many body organs.

3.1 Contraction of vascular smooth muscle

Vascular smooth muscle contraction is controlled principally by cell surface receptors (pharmacomechanical coupling), stretch and transmural pressure (electromechanical coupling). Activation of the contractile apparatus (myosin and actin) is caused by the propagation of action potentials, activation of stretch-dependent ion channels as well as the action of local and circulating hormones (Hill et al., 2001; Schubert et al., 2008; Webb, 2003). In general, activation of cell surface receptors by specific agonists such as NE, angiotensin II, and ET-1 causes increases in $\text{Ca}^{2+}$ concentrations inducing VSM contraction. The mechanisms involved with VSM contraction include activation of the phospholipase C-inositol 1, 4, 5-trisphosphate (PLC-IP$_3$) pathway with subsequent production of diacylglycerol (DAG) that activates protein kinase C (PKC) (Horowitz et al., 1996; Wier and Morgan, 2003). PKC inhibits the activity of MLCP. The latter is involved in the dephosphorylation of MLC by removing a high-energy phosphate from the light chain of myosin promoting VSM relaxation and thus balances the activity of MLCK (Schubert et al., 2008; Woodsome et al., 2001). As such, reduced MLCP activity enhances the phosphorylation of MLC causing contraction of VSM.

In the case of stretch and increases in transmural pressure, contraction of VSM is due to membrane depolarization and activation of stretch-dependent ion channels and
voltage-operated Ca\(^{2+}\) channels (VOCCs) in the plasma membrane, as well as intracellular second messengers such as PLC, DAG, PKC, and RhoA. This characteristic response to pressure elevation in small resistance arteries is called a myogenic response (Davis et al., 2001; Davis and Hill, 1999; Hill et al., 2001; Schubert et al., 2008). Contraction of VSM that is initiated by increases in intracellular Ca\(^{2+}\) concentration is named Ca\(^{2+}\)-dependent contraction of VSM. Sources of Ca\(^{2+}\) during contraction of VSM can be extracellular and/or intracellular (sarcoplasmic reticulum (SR)) via activation of VOCCs and IP\(_3\) receptors (IP\(_{3R}\)), respectively.

PKC and the small G protein RhoA, and its downstream target Rho kinase, play a crucial role in the regulation of MLCP activity. Protein kinase-C and the RhoA/ Rho kinase pathways can be activated by pressure increases leading to inactivation of MLCP, thus enhancing Ca\(^{2+}\) sensitivity of the contractile apparatus. This type of VSM contraction is named Ca\(^{2+}\)-independent (sensitization) contraction of VSM (Schubert et al., 2008).

3.2 Relaxation of vascular smooth muscle

Relaxation of VSM can be passive or active. Passive relaxation is caused, in vitro, by removal of the contractile agent (i.e. Ca\(^{2+}\)) whereas active relaxation is attributed to the activation of cyclic nucleotide-dependent signaling pathways in the presence of Ca\(^{2+}\) (Fukata et al., 2001). Two major signaling pathways are proposed; cyclic adenosine monophosphate-dependent protein kinase A (cAMP-PKA) and cyclic guanosine monophosphate-dependent protein kinase G (cGMP-PKG) (Hobbs and Ignarro, 1996). Relaxation of VSM depends on which pathway is being activated. In both conditions
however, there is a decrease in intracellular \(\text{Ca}^{2+}\) concentration (Surks et al., 1999). This can occur through a decrease in \(\text{Ca}^{2+}\) influx, an increase in \(\text{Ca}^{2+}\) outflux, or an increase in cytosolic \(\text{Ca}^{2+}\) uptake by the SR. These cellular events are due to an inhibition of receptor operated and voltage operated calcium channels and an activation of \(\text{Na}^+/	ext{Ca}^{2+}\) exchangers and \(\text{Ca}^{2+}\)-Mg-ATPase, respectively (Webb, 2003).

In addition to the aforementioned mechanisms involved in the relaxation of VSM, another pathway is associated with hyperpolarization of both endothelial and VSM cells initiated by the release of endothelial mediators. This can occur by opening of potassium (\(K^+\)) channels in response to the activation of endothelial receptors via specific vasodilator agents, and shear stress, as will be discussed. Finally, an increased activity of MLCP is also required to relax VSM, which provides an additional mechanism of VSM relaxation. The state of myosin phosphorylation is regulated by MLCP which phosphorylates the light chain of myosin causing it to disassociate from actin, thereby promoting VSM relaxation (Webb, 2003; Woodsome et al., 2001). In this regard, pharmacological inhibitors of PKC such as chelerythrine, and of Rho kinase such as Y-27632, block the activity of these kinases and thus induce relaxation of isolated blood vessels, abolishing tension and pressured-induced myogenic tone via an elevation of MLCP activity.

4. Vascular endothelial cells

Vascular endothelium is a highly active tissue that plays an important role in the regulation of vascular tone and cardiovascular homeostasis through the release of vasoactive substances. The endothelium and VSM cells communicate via myoendothelial gap junctions through which various molecules, such as \(\text{Ca}^{2+}\) and IP_3, and
electrical current (depolarization or hyperpolarization), are conducted from one cell to another (de Wit et al., 2008; Feletou and Vanhoutte, 2009). The regulation of vasomotor tone in the vasculature is determined by the balance between vasodilation and vasoconstriction. Endothelial-derived vasoactive substances, which exert opposing effects, contribute to moment-to-moment changes in vascular tone. An impairment of endothelium-dependent vasodilation is known as endothelial dysfunction. Vasodilators released by the vascular endothelium include nitric oxide (NO) and prostacyclin (prostaglandin I$_2$, PGI$_2$). However, in several blood vessels, particularly small resistance arteries, inhibition of the release of these two factors does not completely abolish vasodilation (Bellien et al., 2008). The remaining relaxation is explained by the release of an endothelial-derived hyperpolarizing factor (EDHF), the nature of which remains to be determined. The release of these substances is initiated not only by humoral influences but also by mechanical stimuli such as shear stress exerted by blood flow. On the other hand, vascular endothelial cells (ECs) produce several vasoconstrictors with ET-1 being the most potent.

4.1 Endothelium-derived vasodilators: Nitric oxide

Nitric oxide is a free radical gas synthesized in ECs from the amino acid L-arginine via the action of nitric oxide synthase (NOS) and in response to the activation of specific endothelial receptors by specific agonists (Figure 1) (Vanhoutte, 2001). Additionally, it is known that mechanical stimuli, particularly shear stress, significantly contribute to the release of NO (Figure 1). There are three isoforms of NOS including inducible NOS (iNOS), neuronal NOS (nNOS), and endothelial or constitutive NOS
Figure 1. Role of calcium activated K⁺ channels in endothelium-dependent vasodilation. Calcium activated K⁺ channels (\(K_{Ca}^{+}\)) including BK_{Ca}, IK_{Ca} and SK_{Ca} channels are expressed in the blood vessel with specific distribution. The IK_{Ca} and SK_{Ca} channels are present principally in the endothelial cells (EC) whereas BK_{Ca} channels are mainly found in the VSM cells (VSMC). An increase in endothelial intracellular Ca^{2+} levels via transient receptor potential vanilloid channel (TRPV4) subsequent to the activation of endothelial receptors via specific vasodilator agents (e.g. Ach, Acetylcholine; BK, bradykinin; SP, substance P) as well as shear stress, stimulates eNOS, IK_{Ca}, and SK_{Ca}. The resultant endothelial hyperpolarization is conducted to the VSMC via myoendothelial gap junctions (CX, Connexin family of proteins: isoforms of gap junction proteins). In addition, activation of VSM Na⁺/K⁺-ATPase and IK_{Ca} channels by increased levels of K⁺ ions causes hyperpolarization and relaxation of VSMC. Hyperpolarization of VSMC also counteracts the effect of vasoconstrictor agents such phenylephrine, PE. Moreover, after VSMC contraction, increased Ca^{2+} concentrations, following a stimulation of VSMC TRPV4 and transient receptor potential canonical channel (TRPC1), activates BK_{Ca} leading to VSMC hyperpolarization. Ryanodine receptor (RyR). Adapted from Feletou and Vanhoutte, 2009.
(eNOS). The eNOS is the most important NOS in vascular biology (Walford and Loscalzo, 2003). Nitric oxide has been implicated in a wide range of physiological functions including endothelium-dependent dilation. It is one of the main vasodilator factors modulating resting vascular tone in arteries, however there is great heterogeneity in vascular function across organs as will be discussed (Lagaud et al., 1999; Scotland et al., 2001). Moreover, the modulatory role of NO on vascular tone is thought to predominate in large arteries and reduced in small vessels (de Wit et al., 1998).

The activity of eNOS is controlled by the levels of intracellular Ca\(^{2+}\) (i.e. Ca\(^{2+}\)-dependent), although flow-mediated dilation could be Ca\(^{2+}\)-independent such as the eNOS activation of PKA pathways (Busse and Fleming, 2003). As mentioned above, ECs Ca\(^{2+}\) influx can be stimulated by either mechanical shear stress or chemical agonists. In the case of flow-induced shear stress there is Ca\(^{2+}\) influx through a number of different pathways including activation of mechanico-sensitive cation channels or modulation of cytoskeleton-mediated signal transduction pathways (Busse and Fleming, 2003). On the other hand, agonists such as bradykinin (BK) cause Ca\(^{2+}\) influx mainly through store-operated mechanisms triggering IP\(_3\) receptor-mediated intracellular Ca\(^{2+}\) release that stimulates Ca\(^{2+}\) influx across the plasma membrane (Parekh and Putney, 2005).

Once released, NO diffuses into adjacent VSM cells where it activates soluble guanylate cyclase (GC) with subsequent activation of PKG (cGMP-dependent protein kinase) (Hobbs and Ignarro, 1996; Pfeifer et al., 1998). This leads to inhibition of the activity of VOCCs and activation of MLCP which causes a decrease in intracellular Ca\(^{2+}\) concentration and disassociation of actin from MLC, thereby leading to relaxation of VSM (Bolotina et al., 1994; Cohen et al., 1999; Robertson et al., 1993; Somlyo and
Somlyo, 2003). Activation of K⁺ channels through NO-mediated activation of cGMP-dependent protein kinase may also elicit hyperpolarization of underlying VSM in some blood vessels. It has been reported that vasodilation resistant to GC inhibitors in rabbit aorta was blocked by charybdotoxin, a specific inhibitor of large conductance Ca²⁺ activated K⁺ channels (BKca), suggesting an activation of these ion channels by NO in the aorta (Bolotina et al., 1994). Nitric oxide also regulates production and function of ET-1 in the ECs, reduces superoxide anion, and has a central sympathoinhibitory action (Boulanger and Luscher, 1990). While the contribution of NO to endothelium-dependent vasodilation of VSM has been extensively studied using NOS inhibitors in various vascular beds, including the mesenteric circulation, the role of NO in the reactivity of MSV studied under conditions of changing transmural distending pressure has yet to be determined.

4.2 Endothelium-derived vasodilators: Prostacyclin

Prostacyclin (PGI₂) is the major prostanoid produced by ECs. It is synthesized from arachidonic acid via the cyclooxygenase (COX) pathway by the aid of prostacyclin synthase enzyme. There are two distinct isoforms of COX, namely COX-1 (constitutive form) and COX-2 (inducible form) with COX-1 being associated with normal homeostatic mechanisms including PGI₂ production. Following its production, PGI₂ leaves ECs and binds to its receptor in the plasma membrane of VSM where it stimulates adenylyl cyclase (AC) with subsequent activation of PKA (cAMP-dependent protein kinase). Protein kinase A phosphorylates specific target proteins leading to a reduction in the intracellular Ca²⁺ levels and thus initiates VSM relaxation (Stankevicius et al., 2003).
This biological effect of PGI$_2$ can be blocked by use of indomethacin, a non-selective COX inhibitor. As with NO, PGI$_2$ is continuously released by ECs in response to physical stimuli and shear stress. However, despite the continuous release of PGI$_2$, its role in controlling vasomotor tone and mediating endothelium-dependent vasodilation may depend on the type of vascular bed and size of blood vessel studied. In skeletal muscle vasculature, blockade of COX pathway with indomethacin enhances myogenic reactivity in second but not third order arterioles (Hill et al., 1990a). In the mesenteric circulation, PGI$_2$ causes minimal (Cherry et al., 1982; Lagaud et al., 1999) or no changes (Tomioka et al., 1999) in the net vascular tone. These observations suggest regional differences in the modulatory role of PGI$_2$ in myogenic reactivity (Hill et al., 1990a) and that the reduction of PGI$_2$ production is compensated for by the enhanced release of another vasodilator such as NO and/or EDHF (Bolz and Pohl, 1997; Cherry et al., 1982; Parkington et al., 2004; Resende et al., 1998; Takamura et al., 1999). Moreover, it has recently been suggested that NOS inhibition can lead to a decrease in the synthesis of PGI$_2$ as a synergy exists between the level of NO and the rate of PGI$_2$ production in the ECs, suggesting a cross talk between the NOS and COX pathways (Laemmel et al., 2003). Reactive oxygen species (ROS) have been suggested to be involved in this interaction (Laemmel et al., 2003). Consequently, when NOS is inhibited, the level of ROS is increased leading to a decrease in the production of PGI$_2$ a condition which can be overcome by the mixed inhibition of NOS and ROS (Laemmel et al., 2003).
4.3 Endothelium-derived vasodilators: Endothelial-derived hyperpolarizing factor (EDHF)

Endothelial-derived hyperpolarizing factor-mediated responses are endothelium-dependent vasodilations that are resistant to NOS and COX inhibition. The chemical nature of EDHF or the mechanisms by which EDHF relaxes VSM is not clear, although there is evidence that hyperpolarization and subsequent relaxation induced by EDHF involve the activation of K⁺ channels in VSM (Bellien et al., 2008; Doughty et al., 1999; Edwards et al., 1998). Several chemical mediators produced by the endothelium function as EDHF in some vascular beds depending on species, type and size of the blood vessels studied (Bellien et al., 2008; Vanhoutte, 2009). At present, several candidates for an EDHF have been proposed, mainly in resistance arteries including the mesentery, but there is little agreement as to the nature of this factor. Endothelial-derived hyperpolarizing factor candidates include reactive oxygen species (hydrogen peroxide, H₂O₂), C-type natriuretic peptide (CNP), K⁺ ions, and arachidonic acid metabolites derived from cytochrome P-450 pathways such as epoxyeicosatrienoic acids (EET₅). Among these substances, cytochrome P-450 metabolites, H₂O₂ and activation of K⁺Ca channels have been shown to be important in flow- as well as agonist-induced dilation in different vascular preparations (Huang et al., 2004; Lagaud et al., 1999; Liu et al., 2011; Liu et al., 2006; Miura et al., 2003; Takamura et al., 1999). However, in the rat mesenteric vascular bed, cytochrome P-450 metabolites have a minor role in mediating endothelium-dependent vasodilation (Lagaud et al., 1999; Liu et al., 2006; Resende et al., 1998; Vanheel and Van de Voorde, 1997). Thus, in this thesis, molecules and
mechanisms that are likely to mediate EDHF-induced relaxations of mesenteric vessels will be studied.

The synthesis of EDHF occurs via endothelial stimulation by receptor-specific agonists as well as by shear stress, and its synthesis and release is mainly stimulated by intracellular Ca\(^{2+}\) elevation (Figure 1). The contribution of EDHF to the relaxation of VSM and thus to the modulation of vascular tone is most prominent in small resistance arteries and decreases inversely with vessel size (Nagao et al., 1992; Sandow and Hill, 2000; Shimokawa et al., 1996; Tomioka et al., 1999). This phenomenon may be explained by the finding that the number of gap junctions increases with the reduction of the size of the artery indicating a key role of the EDHF in endothelium-dependent vasodilation of small resistance arteries (Sandow and Hill, 2000). For example, in rat MSA, NO has a minor role in mediating agonist-induced vasodilation compared with flow-mediated vasodilation (Christensen et al., 2007; Lagaud et al., 1999; Liu et al., 2006) and flow- and acetylcholine-induced vasodilation are dependent mainly on H\(_2\)O\(_2\) and K\(_C\) channels as an EDHF, respectively (Edwards et al., 1998; Lagaud et al., 1999). While there have been several studies attempting to identify the identity of EDHF mediating endothelium-dependent dilation of arteries including the mesenteric circulation, the chemical nature of EDHF and its contribution to endothelium-dependent relaxation of mesenteric veins studied under near physiological conditions remains to be determined.

4.3.1 Reactive oxygen species as an EDHF: H\(_2\)O\(_2\)

The endothelium releases several kinds of ROS including superoxide, H\(_2\)O\(_2\), peroxynitrite, and hydroxyl radicals that can modulate vasomotor tone by several
mechanisms. Superoxide attenuates endothelium-dependent relaxation by scavenging NO forming peroxynitrite, a potent ROS, that inhibits the activity of $K_{Ca}$ in the VSM; therefore, impairing VSM relaxation (Huang et al., 1998). Hydrogen peroxide is formed from superoxide anion in the presence of superoxide dismutase (SOD) enzyme, a process called dismutation. Hydrogen peroxide has a direct hyperpolarizing effect on VSM of various arteries by opening $BK_{Ca}$ and activation of GC (Liu et al., 2011; Shimokawa, 2010). EDHF-mediated hyperpolarization and relaxation of VSM are inhibited by catalase, a specific inhibitor of $H_2O_2$, which proposes that $H_2O_2$ may be an EDHF mediating endothelium-dependent relaxation. This has been reported in several arteries from various vascular beds include mouse mesenteric and cerebral arteries (Drouin et al., 2007; Matoba et al., 2000), rat mesenteric arteries (Liu et al., 2006; Shimokawa et al., 1996), human mesenteric arteries (Matoba et al., 2002), human, canine and porcine coronary arteries (Matoba and Shimokawa, 2003; Liu et al., 2011; Yada et al., 2006).

4.3.2 Effects of transmural pressure increases on ROS production

Endothelium-dependent relaxation of VSM in response to increased blood flow as well as to activation of specific endothelial receptors, such as G protein-coupled acetylcholine muscarinic and BK receptors, is reduced in various forms of hypertension in human and animals, proposing the presence of endothelial dysfunction (Christensen et al., 2007; Gunduz et al., 2009; Gunduz et al., 2008). Findings of these studies suggest that an increased transmural pressure could be harmful to the physiological function of the vascular endothelium. However, the mechanisms for the impaired endothelial function following increases in transmural pressure remain to be elucidated. Proposed mechanisms that may be involved in pressure-induced endothelial dysfunction include...
down-regulation and up-regulation of VSM GC and superoxide anion pathways, respectively. Reduced levels of VSM GC causes a reduction in the activity of NO which proposes a reduced sensitivity of VSM for endogenous as well as NO donors associated with blood pressure elevations, considering that NO exerts its physiological effects via activation of GC (Martin et al., 2005; Preik et al., 1996). On the other hand, an increased level of superoxide reduces the bioavailability of endothelial-derived vasodilators including NO. Physiologically, superoxide anion is inactivated by SOD enzyme; however, accumulation of superoxide and the impairment of its enzymatic inhibition have resulted in endothelial dysfunction in arteries (Christensen et al., 2007).

4.3.3 Calcium activated K+ channels as EDHF-mediated vasodilation

Many electrophysiological and functional studies have been performed to identify and characterize K+ channels in blood vessels from different species (Jackson, 2005). To date, there are four different classes of K+ channels expressed on the endothelial and VSM cells. These include voltage-gated K+ channels (Kv), Ca2+-activated K+ channels (KCa), ATP-sensitive K+ channels (KATP), and inwardly rectifying K+ channels (Kir). It is known that K+ channels participate in the regulation of vascular tone and therefore blood flow via their important role in controlling the resting membrane potential (Lagaud et al., 1999; Wu et al., 2009).

Activation of K+ channels can be mediated by vasodilators released by the vascular endothelium causing K+ efflux out of the cell with subsequent decrease in membrane potential leading to membrane hyperpolarization, closure of VOCCs and vasodilation (Jackson, 2005). Of the various K+ channels identified, KCa channels have
been reported to function in EDHF-mediated responses in different vascular beds, including mesenteric circulation (Doughty et al., 1999; Edwards et al., 1998; Giachini et al., 2009; Lagaud et al., 1999). Three subtypes of $K_{Ca}$ channels have been identified in the vascular wall including large ($BK_{Ca}$), intermediate ($IK_{Ca}$), and small ($SK_{Ca}$) conductance $Ca^{2+}$-activated $K^{+}$ channels with specific cellular and subcellular localization (Jackson, 2005; Feletou and Vanhoutte, 2009). While $BK_{Ca}$ channels are well expressed in VSM cells, their expression in endothelial cells is low (Figure 1). On the other hand, $SK_{Ca}$ and $IK_{Ca}$ channels are principally expressed in ECs (Figure 1) (Jackson, 2005).

In pressurized rat mesenteric arteries, pharmacological inhibition of $K_{Ca}$ channels by use of specific blockers revealed that activation of endothelial $K_{Ca}$ channels by receptors-specific agonists (such as acetylcholine) may induce endothelium-dependent relaxation by opening endothelial $K^{+}$ channels that are sensitive to apamin (a selective $SK_{Ca}$ inhibitor) or to the combined application of charybdotoxin ($BK_{Ca}$ and $K_{IR}$ inhibitor) and apamin, but not to iberiotoxin (a selective $BK_{Ca}$ inhibitor) alone (Doughty et al., 1999; Lagaud et al., 1999). Additionally, in rat mesenteric arteries examined with isometric methods $SK_{Ca}$ channels importantly contribute to EDHF-mediated relaxation (Giachini et al., 2009). These results suggest that $BK_{Ca}$ channels are not involved in agonist (acetylcholine)-induced release of EDHF in small arteries. In contrast to small arteries, EDHF-induced relaxation in large veins examined under isometric tension has been associated with an activation of $BK_{Ca}$ channels (Martinez-Leon et al., 2003). Conversely, it has recently been reported that functional $BK_{Ca}$ channels are expressed in pressurized mouse MSA but not veins based on gene deletion of $\beta_1$-subunit of $BK_{Ca}$ and on responses to receptor-dependent (NE) and-independent (KCL) agonists in the presence
of paxilline, another selective $\text{BK}_{\text{Ca}}$ blocker (Xu et al., 2011). These differences between arteries and veins in the activity of $\text{BK}_{\text{Ca}}$ channels may be explained by the type, size of the blood vessels studied or species that may determine the activation of different channels or by differences in experimental methods, isobaric versus isometric.

4.4 Endothelium-derived vasoconstrictors: Endothelin

In addition to different vasodilators produced by vascular endothelium, vasoconstrictor substances, including ET, can be released from the vascular ECs (Johnson et al., 2002; Johnson et al., 2001). The ET family of peptides is composed of 3 isoforms (ET-1, ET-2, and ET-3) with ET-1 being the main isoform, and most potent, identified (Yanagisawa et al., 1988). Besides its direct vasoconstrictor effect, low concentrations of ET-1 have been reported to potentiate contractile responses to other vasoactive agents including NE and serotonin in various vascular beds, a possible physiological function of ET-1 in regulating vascular tone and reactivity (Garcia-Villalon et al., 2008; Thorin et al., 1998).

Since ET-1 has marked effects on the vascular tone (Brunner et al., 2006; Thorin et al., 1998), it has been implicated in the pathophysiology of a number of cardiovascular diseases such as hypertension, atherosclerosis, coronary artery disease, and congestive heart failure (Angerio 2005; Bohm et al., 2008; Gray et al., 2000b; Ivey et al., 2008; Kedzierski and Yanagisawa, 2001; Kirkby et al., 2008). These conditions are associated with upregulation of circulating ET-1 that has been reported to reduce endothelial dilator function by interfering with the release of vasodilators such NO and PGI$_2$ that cause ET-1 downregulation (Brunner et al., 2006; Halcox et al., 2001; Kedzierski and Yanagisawa, 2001). In this regard, it was shown that ET receptor inhibition improved endothelial-
induced dilation and increased coronary blood flow, thus improving endothelial
dysfunction in many disease conditions (Bauersachs et al., 2000; Best et al., 1999; Bohm
et al., 2008; Halcox et al., 2001).

ET-1 exerts its biological effects mainly through activation of two VSM G-
protein coupled ET-1 receptor subtypes, the ET$_A$ and ET$_B$ receptors with subsequent
stimulation of the PLC-IP3 pathway leading to increases in intracellular Ca$^{2+}$
concentrations (Motte et al., 2006). While stimulation of VSM ET$_B$ receptors by ET-1
contribute to vasoconstriction in some blood vessels, particularly veins, activation of ET$_B$
receptors on endothelial cells stimulates synthesis of NO and PGI$_2$ which causes
vasodilation that counteracts vasoconstriction (Johnson et al., 2002; Johnson et al.,
2001a; Motte et al., 2006; Perez-Rivera et al., 2005). The ET$_B$ receptors are also known
as clearance receptors based on their role in removing circulating ET-1 (Opgenorth et al.,
2000; Strachan et al., 1999). Accordingly, ET-1 receptors may function differently in
veins and arteries. Therefore, the functions of ET-1 receptors in health and disease have
been well characterized in isolated arteries and unpressurized veins of various vascular
beds including the splanchnic circulation by use of several selective ET-1 receptor
antagonists including BQ-123, BQ-610, selective ET$_A$ receptors antagonists; and BQ-
788, a selective ET$_B$ receptors antagonist (Johnson et al., 2002; Perez-Rivera et al., 2005;
Thakali et al., 2008). Results of these studies indicate that ET-1 causes concentration-
dependent contractions whose potency is greater in veins than in arteries. In addition,
ET-1-induced contractions are mediated mainly by VSM ET$_A$ receptors in veins and
arteries with some evidence that support the involvement of contractile ET$_B$ receptors in
ET-1-induced venoconstriction (Thakali et al., 2008). Therefore, the role of ET-1 in
controlling vasomotor tone may depend on the balance between ET-1 receptors mediating ET-1 effects and their distribution, as well as the prevailing integrity of the vascular endothelium. Investigating the possible contributions of $ET_A$ and $ET_B$ receptors to vascular contractions induced by ET-1 in mesenteric veins and arteries under conditions of changing transmural pressures has not been reported.

5. Myogenic reactivity

An increase in transmural distending pressure in the vasculature activates myogenic factors inherent to the VSM. Myogenic reactivity in blood vessels is comprised of two phenomena: “myogenic responses”, which involves alterations in vascular tone resulting in decreases in vessel diameter in response to a change in distending pressure and “myogenic tone” which involves maintaining vascular tone at a constant level of distending pressure (Schubert et al., 2008). Myogenic tone may be measured in pressurized vessels in vitro as the difference in vessel diameter under active conditions (presence of calcium in media) versus passive relaxed conditions (calcium free media) obtained at the same distending pressure (Brookes and Kaufman, 2003).

The first description of myogenic reactivity is generally accredited to Bayliss who studied the changes in the arterial wall that occurred in response to transmural pressure increases (Bayliss, 1902). Bayliss found that an increase in transmural pressure acts as a facilitating stimulus causing a shortening in VSM cells of blood vessels. In many organs, this reduction in vessel diameter has been suggested to maintain a constant blood flow and capillary hydrostatic pressure despite variations in blood pressure, a phenomenon known as autoregulation.
Since the discovery of Bayliss, and because of its physiological importance, myogenic reactivity has been characterized in small arteries and arterioles from a number of different vascular beds including the mesenteric, skeletal muscle, cerebral, renal, and coronary circulation (Gschwend et al., 2003; Lagaud et al., 2002; Lagaud et al., 1999; Ren et al., 2010; Sun et al., 1994; Sun et al., 1992). Venous myogenic tone, on the other hand, has been observed in many veins and venules including facial, saphenous, portal, skeletal muscle, bat wing, and mesenteric (Berczi et al., 1992; Brookes and Kaufman, 2003; Dornyei et al., 1996; Davis et al., 1992; Henrion et al., 1994; Johansson and Mellander, 1975). Unfortunately, in vitro studies exploring the origin as well as the mechanisms involved in pressure-induced venous myogenic reactivity of large capacitance beds such as the mesenteric circulation are lacking. This is likely due to the significant technical challenges associated with studying the more delicate and thinned walled small veins and venules under conditions of distending transmural pressure.

Myogenic reactivity develops independent of neural, endothelial, metabolic, and humoral influences, but it can be modulated by the endothelium. Accordingly, some studies have shown that inhibition of NOS or removal of the endothelium enhances the magnitude of arterial myogenic reactivity (Jarajapu and Knot, 2005; Nguyen et al., 1999; Szekeres et al., 2004) including a study in rat pressurized mesenteric arteries (Brookes and Kaufman, 2003). Conversely, other studies have shown no modulatory effects of endothelial-derived-NO on pressure-diameter curves in different vascular beds (Anschutz and Schubert, 2005; Matchkov et al., 2002; Murphy et al., 2007) including rat mesenteric arteries (Sun et al., 1992). Collectively, these findings indicate that the ability of the
endothelium to modulate myogenic reactivity is specific to the vascular bed and its function, and possibly differs between species (Nguyen et al., 1999; Sun et al., 1994).

5.1 Assessment of myogenic reactivity in vitro

Two principle in vitro techniques are used to study the physiological and pharmacological characteristics of isolated blood vessels. The first technique to be used was the wire myography (isometric) which involves passing two mounting wires through the lumen of a ring segment of a blood vessel and changes in isometric force are measured. The main disadvantage of this technique is that the attachment of the vessel rings to hooks causes endothelial damage. In addition, wire myography does not allow studying blood vessels under physiologically relevant conditions (i.e under transmural distending pressure and flow conditions). Thus, wire myography is ideal for analytical as well as electrophysiological studies. Another technique namely pressure myography (isobaric) has recently been introduced. This technique involves cannulating the two ends of the blood vessel in a closed system filled with physiological buffer solution and provides a wide range of transmural pressures. As such pressure myography is a useful technique to study the effect of changing transmural distending pressures as well as flow on the reactivity of blood vessels.

5.2 Significance of myogenic reactivity

Myogenic reactivity is important to the maintenance of basal tone, constant blood flow and capillary hydrostatic pressure in the face of changes in vascular blood pressure (Davis and Hill, 1999). Myogenic reactivity in resistance arteries is critical to autoregulation of tissue blood flow in virtually all vascular beds. Autoregulation describes
the functional capacity of blood vessels to respond to local metabolic changes and transmural forces generated by the vessel wall in response to alterations in distending pressure and blood flow. Moreover, at the systemic level, myogenic reactivity is thought to provide background basal tone upon which vasoconstrictors act. Thus myogenic reactivity can amplify the effects of vasoconstrictor agents on pressure (Metting et al., 1989). For example, an increase in transmural pressure in resistance arteries initiates myogenic reactivity which causes approximately two-thirds increase in total peripheral resistance and accompanying changes to systemic blood pressure (Metting et al., 1989). This aspect is referred to as a potential interaction of myogenic reactivity with vasoconstrictors.

Venous myogenic reactivity is also thought to be crucial in the overall control of cardiovascular homeostasis. Since more than 70 % of total blood volume lies in the venous system, only small changes in venous myogenic reactivity are needed in large capacitance beds to cause substantial shifts of blood towards the heart via venous return, with subsequent alterations in cardiac output and blood pressure (Greenway, 1981).

5.3 Signaling mechanisms involved in myogenic reactivity

Despite the importance of myogenic reactivity and overall vasomotor tone, the signaling mechanisms involved in the generation of myogenic reactivity are not completely understood. While it is agreed that vessel wall tension is the primary stimulus that activates myogenic factors in VSM, the sensors involved in recognizing and translating the stimulus to activation of myogenic factors are not clear. Proposed factors that sense the changes in vessel wall tension that subsequently lead to membrane depolarization and the activation of intracellular signaling events include stretch-
activated cation channels, mechano-sensitive enzymes, interactions between extracellular matrix proteins, cell surface integrins and the involvement of the cytoskeleton (Schubert et al., 2008; Schubert and Brayden, 2005). A limitation of some of these proposed sensors is the current absence of direct experimental evidence supporting their role in activation of myogenic factors.

A change in transmural pressure causes activation of multiple signaling pathways (Figure 2). Identifying these pathways has been studied mainly in arteries, while the mechanisms by which veins sense the changes in transmural pressure remain unclear. Myogenic reactivity in arteries is dependent upon Ca\(^{2+}\) entry into VSM cells from the extracellular space as removal of extracellular Ca\(^{2+}\) completely abolishes myogenic reactivity, whereas depletion of intracellular Ca\(^{2+}\) store via ryanodine receptor activation does not impact the myogenic reactivity (McCarron et al., 1997; Schubert et al., 2008). Other pathways involved in generation of myogenic reactivity including membrane depolarization (activation of VOCCs) and activation of second messengers have been investigated, with emphasis on arteries (Figure 2). In this thesis work, focus will be given to key mechanisms that are widely considered to be important pathways in the generation and the maintenance of myogenic reactivity including VSM depolarization and extra cellular Ca\(^{2+}\) influx via activation of VOCCs, as well as activation of intracellular signaling molecules, most notably PKC and Rho A/ Rho kinase (Figure 2).

As it is the case with many physiological mechanisms, a negative feedback is essential for the control of myogenic reactivity. Activation of BK\(_{Ca}\) channels provide negative feedback control of vascular tone by inducing hyperpolarization of VSM cells that shifts
membrane potential to become more negative, thereby closing VOCCs with the net result being VSM relaxation (Nelson et al., 1995; Xu et al., 2011; Yang et al., 2009).

5.3.1 Membrane depolarization

An increase in transmural pressure leads to VSM cell depolarization which has been identified in arteries and veins from a number of vascular beds (Rummery et al., 2007; Schubert et al., 1996; Thakali et al., 2010). Non-selective stretch-activated cation channels including transient receptor potential channels (TRPC) and epithelial Na\(^+\) channels (ENaC) are reported to mediate VSM depolarization, and to be involved in myogenic reactivity (Figure 2). Another non-selective stretch activated cation channel that can mediate pressure-induced membrane depolarization is chloride channels. However, understanding their potential role in the development of myogenic reactivity has been limited by the lack of specific inhibitors (Schubert et al., 2008).

Although the role of membrane depolarization of VSM cells in activating myogenic reactivity is appreciated, experimental work has shown that clamping membrane potential or blockade of VOCCs did not abolish pressure-induced myogenic reactivity and membrane depolarization in rat cerebral arteries, respectively (Knot and Nelson, 1998; McCarron et al., 1997). These findings suggest that VSM depolarization is not a mandatory step for pressure-induced myogenic reactivity and is probably not the only link between a change in transmural pressure and myogenic reactivity.

Cellular events that may be associated with membrane depolarization include activation of calcium channels, particularly the L-type VOCCs, as well as activation of some cellular enzymes that generate a number of second messengers involved in
Figure 2. Signaling mechanisms in VSM involved in the generation of pressure-induced myogenic reactivity. Vessel wall tension is the key stimulus that activates myogenic reactivity. Proposed factors that sense changes in vessel wall tension and lead to membrane depolarization and subsequent activation of intracellular signaling events include stretch-activated channels, mechano-sensitive enzymes, cell surface integrins and the involvement of the cytoskeleton. Consequently, there is an increase in intracellular Ca$^{2+}$ concentrations as well as an enhancement of its sensitivity by Ca$^{2+}$ entry via Ca$^{2+}$ channels and Ca$^{2+}$ release form intracellular stores; and activation of intracellular signaling pathways including IP3, inositol triphosphate; DAG, diacylglycerol; PKC, protein kinase C; and RhoA/ Rhokinase, respectively. The formed Ca$^{2+}$-calmodulin complex ((Ca$^{2+}$)$_4$CAM) stimulates myosin light chain kinase (MLCK) whereas PKC and Rho kinase inactivate myosin light chain phosphatase (MLCP) with the net results being VSM contraction. Abbreviations: TRP, transient receptor protein cation channel; ENaC, epithelial sodium cation channel; Cav1.2 voltage-operated Ca$^{2+}$ channel; CCE, capacitative calcium entry; MLC20, myosin light chain regulatory domain (20 kDa); Sk1, sphingosine kinase 1; PLC, phospholipase C; GPCR, G-protein-coupled receptor; IP3R, IP3 receptor; RyR, ryanodine receptor; SR – sarcoplasmic reticulum; CYP4A, cytochrome P450 4A; MLCP. Adapted from Shubert et al. 2008.
intracellular signaling pathways such as PLC-IP3, and RhoA/ Rho kinase pathways (Figure 2).

5.3.2 Voltage-operated calcium channels

It is established that VOCCs are important for VSM, cardiac myocyte and skeletal muscle contraction (Hill et al., 2006; Schubert et al., 2008). These calcium channels are divided into high voltage (L-, P/Q-, N-, and R-type) and low voltage (T-type) activated channels. Of these, the L-type channel is generally essential for arterial smooth muscle contraction including activation of myogenic factors (McCarron et al., 1997; Thakali et al., 2010; Wesselman et al., 1996; Zhang et al., 2007). Activation of L-type VOCCs is widely believed to be the initial event that leads to Ca\(^{2+}\) influx and eventually increases MLC phosphorylation and thus promotes contraction of VSM. Dihydropyridines (DHPs), such as nifedipine, have been shown to block the activity of L-type VOCCs and abolish myogenic reactivity, with minimal impact on membrane potential, in arteries from different vascular beds including mesenteric circulation (Chlopicki et al., 2001; McCarron et al., 1997; Potocnik et al., 2000; VanBavel et al., 1998; Wesselman et al., 1996; Zhang et al., 2007). These findings support Ca\(^{2+}\) influx through VOCCs having a key role in generation of myogenic reactivity. However, in some arteries, such as skeletal muscle, residual pressure-induced responses have been observed following blockade of L-type VOCCs (VanBavel et al., 2002; Kotecha and Hill, 2005). This supports the notion of heterogeneity between vessels of differing tissues with regards to the mechanisms underlying myogenic reactivity, which may have its basis in pressure differences experienced in these vessels or possibly the physiologic functions of the tissues they support.
In contrast to extensive arterial studies investigating the role of VOCCs, there has been limited work examining the role of these ion channels in mediating venous myogenic reactivity. In a study by Loufrani et al. (1999), it was reported that L-type VOCCs had a limited role in the development of venous tone in rabbit facial vein incubated with nifedipine and studied under isometric methods. In a recent study and in support of the findings of Loufrani et al., 1999, it has been shown in mesenteric veins examined under isobaric conditions that nifedipine was not effective in inhibiting KCL-induced contractions; considering that KCL causes Ca\(^{2+}\) influx mainly via L-type VOCCs (Thakali et al., 2010). Furthermore, it has recently been reported that selective activation of DHP Ca\(^{2+}\) channels by Bay K 8644 induced contraction of mouse MSA, but not veins, suggesting the lack of functional L-type VOCCs in veins (Xu et al., 2011). Collectively, these findings highlight an important difference with respect to the existence of functional L-type VOCCs in arteries compared with veins. Whether L-type VOCCs contribute to the development of pressure-induced myogenic reactivity in a large capacitance vascular bed, such as the splanchnic vascular beds, is unknown.

It should be mentioned that another type of Ca\(^{2+}\) channels namely T-type VOCCs may also contribute to myogenic reactivity (Potocnik et al., 2000; VanBavel et al., 2002), however blocking these channels affects membrane potential, rather than blockade of Ca\(^{2+}\) entry (Potocnik et al., 2000). Therefore, these types of VOCCs may have a limited role in the development of myogenic reactivity, considering that pressure-induced myogenic reactivity can occur despite membrane depolarization inhibition (McCarron et al., 1997).
5.3.3 Protein kinase C

Since an increase in transmural pressure in cannulated vessels activates PLC and increases the production of DAG, which is the physiological activator of PKC, a contribution of PKC to the myogenic reactivity is possible (Slish et al., 2002). As mentioned above, an activation of PKC leads to inhibition of MLCP, an enzyme that is involved in the dephosphorylation of MLC. As such, PKC enhances Ca\(^{2+}\) sensitivity of the VSM contractile apparatus, which is important in the development of myogenic reactivity (Figure 2). The involvement of PKC in myogenic reactivity has been intensively studied in different vascular beds (Hill et al., 1990b; Korzick et al., 2004; Wesselman et al., 2001; VanBavel et al., 2001). Results of these studies indicate that PKC plays a key role in VSM contractions via modulation of Ca\(^{2+}\) sensitivity, particularly during alterations in transmural pressure. However, vascular variability in the expression, or distribution, of PKC may exist. In some arteries, such as rat tail and cerebral arteries, PKC inhibition does not affect myogenic reactivity (Jarajapu and Knot, 2005; Schubert et al., 2002). These studies are in agreement with other studies showing that PKC inhibition had no effect on contractions induced by PKC activation by phorbol 12, 13-dibutyrae (PdBu) and \(\alpha_1\)-adrenoceptor stimulation by PE in rat caudal (tail) artery compared with responses obtained in the aorta, suggesting that the PKC pathway has no role in the modulation of VSM contractile responses in the tail artery (Mueed et al., 2004).

Different isoforms of PKC are expressed in the VSM of various vascular beds. In rat MSA, five PKC isoforms have been identified by western blot analysis with isoforms specific antibodies to PKC (Ohanian et al., 1996). These include the Ca\(^{2+}\)-dependent
PKCs ($\alpha$ and $\gamma$), $\text{Ca}^{2+}$-independent PKCs ($\delta$ and $\epsilon$), and an atypical isoform ($\zeta$), with the majority being found in the cytoplasm of VSM. Only PKC\( \alpha \) and/or PKC\( \delta \) have been reported to mediate phorbol ester (PKC activator)-induced contractions of mesenteric arteries (Ohanian et al., 1996). Furthermore, the translocation of PKC\( \alpha \) isoform from the cytosol to plasma membrane has been observed in response to transmural pressure increases, as well as to stretch indicating its activation (Dessy et al., 2000; Yeon et al., 2002).

Inhibition of PKC can be achieved by use of various PKC inhibitors including H-7, calphostin C, chelerythrine, and staurosporine. In most vascular preparations, there have been no changes in pressure-induced elevation of intracellular $\text{Ca}^{2+}$ when PKC is inhibited (Bakker et al., 1999; Massett et al., 2002). However, the use of these inhibitors in determining the involvement of PKC in pressure-induced myogenic reactivity has been limited by the presence of different PKC isoforms (i.e the lack of isoform specific inhibitors), as well as by the belief that these inhibitors could interfere with other non-PKC mechanisms involved in VSM contraction (Schubert et al., 2008). Therefore, whether the contribution of PKC in upstream modulation of $\text{Ca}^{2+}$ sensitivity involved in pressure-induced myogenic reactivity is independent of other signaling mechanisms that underlie myogenic reactivity remains unclear. Despite these limitations, a great deal of research has been conducted to study the involvement of PKC in myogenic reactivity, particularly in arteries, by use of inhibitors that have substantial affinity to PKC such as chelerythrine. Although in limited studies PKC inhibition has been shown to abolish myogenic reactivity in rabbit facial veins studied under isometric methods (Loufrani et
al., 1999), understanding the contribution of PKC to myogenic reactivity in pressurized veins from a large capacitance bed like the mesentery has not been reported.

5.3.4 RhoA/ Rho kinase pathway

The small GTP-binding proteins belonging to the Rho family regulate various aspects of VSM cells functions including contractility. Rho kinases (ROCKs), downstream effectors of Rho, mediate RhoA-induced actin cytoskeleton changes via effects on MLC phosphorylation. ROCKs are protein serine/threonine kinases with two isoforms that have been identified in mammalian system including ROCK-I and ROCK-II, which have similar kinase domains.

RhoA/ Rho kinase signaling pathways play an important role in the development of myogenic reactivity in different vascular beds of different species (Bolz et al., 2003; Dubroca et al., 2007; Dubroca et al., 2005; Gokina et al., 2005; Schubert et al., 2002; VanBavel et al., 2001). Despite its role in $\text{Ca}^{2+}$ sensitivity modulation via the regulation of the activity of MLCP, the precise mechanism involved in the activation of RhoA/ Rho kinase pathway is not completely understood. Recently, the involvement of some enzymes such as sphingosine kinase-1 (Sk1) and sphingosine-1-phosphate phosphohydrolase-1 (SPP1) in the development and maintenance of myogenic reactivity in resistance arteries has been identified (Bolz et al., 2003; Peter et al., 2008). These enzymes stimulate the synthesis of sphingosine-1-phosphate (S1P) that in turn activates a small GTP RhoA and regulates ROCK with the net result being attenuation of the activity of MLCP that promotes vasoconstriction (Figure 2).

Since ROCKs are involved in various aspects of vascular function including myogenic reactivity, the development of selective inhibitors has gained considerable
interest in the pharmaceutical industry. Currently, Y27632, a non-isoform-selective ROCK, but with a higher affinity for Rho kinase compared with other kinases inhibitors, has been introduced, enabling the investigation of the relative contribution of ROCKs to myogenic reactivity. However, Y27632 cannot distinguish between the two isoforms of ROCK (Uehata et al., 1997). Furthermore, higher concentrations of Y27632 could also inhibit other serine-threonine kinases such as PKC, thereby producing non-specific effects (Mueed et al., 2004; Rikitake et al., 2005). Therefore, lower concentrations of ROCK inhibitors that abolish myogenic reactivity with minimal alterations on VSM contractility are recommended for use in order to avoid interfering with other signaling pathways underlying myogenic reactivity. In this regard, inactivation of RhoA/Rho kinase pathway by Y27632 would abolish myogenic reactivity without impact on pressure-induced intracellular Ca^{2+} elevation (Schubert et al., 2002; VanBavel et al., 2001). As with PKC, vascular variability with Rho kinase may also exist. For example Rho kinase inhibition by Y27632 partly attenuated myogenic reactivity in rat tail artery (Schubert et al., 2002). Consistently, findings of other studies have shown that Rho kinase inhibition by Y27632 had minimal inhibitory effects on contractile-response curves obtained with PdBu and PE in rat caudal (tail) artery compared with responses obtained in the aorta (Mueed et al., 2004). These findings suggested that the Rho kinase pathway has a minimal role in the modulation of VSM contractile responses in the tail artery. Studies investigating the involvement RhoA/Rho kinase pathway in regulating myogenic reactivity in veins are largely lacking. In limited work, Rho kinase inhibition has been shown to abolish stretch-activated myogenic tone in rabbit facial veins studied
with wire myography, but no pressure studies have yet been reported (Dubroca et al., 2005).

6. Sympathetic neurotransmission

The splanchnic vascular bed is innervated by sympathetic and spinal sensory nerves (Kreulen, 2003; Zheng et al., 1999). Sympathetic nerves are the most important vasomotor nerves supplying the splanchnic circulation that regulate the resistance and capacitance functions of arteries and veins, respectively. This control of VSM contraction is complex and involves multiple neurotransmitters, receptors and intracellular signaling mechanisms.

6.1 Mechanism of neurotransmitter release and action

At the sympathetic nerve terminals, sympathetic axons form varicosities which contain a significant number of neurotransmitters-containing vesicles. Sympathetic activation induces action potentials that cause release of neurotransmitters including NE, ATP and neuropeptide Y (NPY). Upon their release, these neurotransmitters bind to specific receptors on the VSM to produce their cellular effects. Both NE and ATP are co-stored in, and co-released from, sympathetic vesicles present in sympathetic varicosities formed by the nerve terminals (Burnstock and Kennedy, 1985; Kennedy, 1996; Westfall et al., 2002). While NE and ATP are the main neurotransmitters released by sympathetic nerves supplying mesenteric arteries (Bobalova and Mutafova-Yambolieva, 2001; Hottenstein and Kreulen, 1987; Luo et al., 2003; Westfall et al., 1995), NE is the major vasoconstrictor released by perivenous sympathetic nerves (Luo et al., 2004; Luo et al., 2003). The components of neurogenic contractions induced by
NE and ATP are often referred to as the adrenergic and purinergic component of neurogenic contraction, respectively. These variations in neuroeffector transmission may explain, in part, differences in hemodynamic function of arteries compared to veins. The likely action of neurally released NPY is to modulate the effects of NE and, possibly, ATP.

6.2 Norepinephrine

An increased venous tone is reported following reflex activation of the sympathetic nerves, direct sympathetic nerve activation, or exogenously applied NE (Bobalova and Mutafova-Yambolieva, 2001; Pang 2001; Perez-Rivera et al., 2004). NE is the main sympathetic neurotransmitter released from varicosities in the sympathetic perivascular nerves in rat mesentery. Shortly after its release, NE binds to alpha adrenergic receptors (α-ARs) to exert its physiological effects. Two types of vascular α-ARs have been identified on the VSM including α₁ and α₂ receptors (Guimaraes and Moura, 2001). Of these, α₁-ARs play a crucial role in the regulation of vascular tone. Further characterization of α₁ and α₂ receptors has revealed the presence of at least six adrenergic receptors subtypes on the vascular wall including α₁A, α₁B, α₁D, α₂A/D, α₂B, and α₂C (Guimaraes and Moura, 2001).

It is well known that NE activates α₁-ARs, which are mainly coupled to Gq/11-proteins, to stimulate PLC and promoting the hydrolysis of phosphatidylinositol bisphosphate to produce IP₃R and DAG. These molecules act as messengers mediating intracellular Ca²⁺ release from SR and activating PKC, respectively. Activation of VOCCs and receptor- and store-operated Ca²⁺ channels also participate in increasing
levels of intracellular Ca$^{2+}$ in response to the VSM effects of NE (McFadzean and Gibson, 2002).

Activation of postjunctional $\alpha_2$-ARs by NE is also coupled to GTP-binding proteins, which leads to the inhibition of the AC activity and VOCCs as well as stimulation of K$^+$ channels. $\alpha_2$-ARs may also couple to other intracellular pathways involving Na$^+$/K$^+$ exchange and the activation of PLA$_2$, PLC, and PLD, with the net result promoting VSM relaxation and constriction, respectively (Wier and Morgan, 2003). It should be mentioned that $\alpha_2$-ARs are also expressed in the vascular endothelium and their activation stimulates the release of NO, an action that could attenuate vasoconstriction produced by activation of postjunctional $\alpha_1$-ARs.

At the prejunctional level, activation of $\alpha_2$-ARs inhibits N-, P-, and Q-type of VOCCs, which results in a negative feed-back modulation of NE release (Guimaraes and Moura, 2001). Inhibition of these prejunctional receptors causes an increase in the amount of neurotransmitters released in response to sympathetic nerve activation. Accordingly in rat mesenteric vessels, yohimbine, an $\alpha_2$-AR antagonist, and UK14, 304, an $\alpha_2$-AR agonist, cause increases and decreases, respectively, in the release of NE, as well as contractions, induced by sympathetic nerve stimulation (Luo et al., 2004; Park et al., 2010). Approximately 95 % of the released NE is transported back into the nerve terminals by high affinity up-take mechanisms, terminating the action of NE. The action of NE can also be terminated, to a lesser extent, by postjunctional enzymes or diffusion of the neurotransmitter from the junctional region into nearby capillaries (Kreulen, 2003).

Pharmacological studies characterizing $\alpha$-ARs in isolated unpressurized mesenteric vessels revealed that exogenously applied NE produces its effects via
activation of $\alpha_1$.ARs in arteries (Hussain and Marshall, 2000; Yamamoto and Koike, 2001a, Yamamoto and Koike, 2001b), and both $\alpha_1$ and $\alpha_2$.ARs in veins (Perez-Rivera et al., 2007). Conversely, sympathetic neurogenic-induced contractions of mesenteric arteries were not changed in the presence of prazosin, an $\alpha_1$.AR antagonist, whereas prazosin abolished responses in the veins (Luo et al., 2003; Park et al., 2007). These results suggest that NE acting on $\alpha_1$.ARs is the predominant neurotransmitter modulating venous tone in the mesenteric circulation. Although $\alpha_2$.ARs contribute to exogenous NE-induced venoconstriction, activation of these receptors alone does not constrict isolated veins studied under no pressure conditions. This suggests that $\alpha_2$.ARs do not directly mediate NE-induced venoconstriction, but rather may sensitize the $\alpha_1$ receptor contractile mechanism (Perez-Rivera et al., 2007).

6.3 Adenosine 5’-triphosphate

The second major sympathetic neurotransmitter, ATP, also binds to specific postjunctional receptors to produce contraction of the VSM through activation of VOCCs (Gitterman and Evans, 2001). Two main isotypes of purinergic receptors have been identified including the P1 and P2 receptors, with P2 receptors mediating the physiological effects of ATP (Burnstock, 2004; Lagaud et al., 1996; Morato et al., 2008). Two subtypes of P2 receptors are now identified, the P2X and P2Y receptors which are ligand-gated cation channels and G-protein coupled receptors, respectively (Abbracchio and Burnstock, 1994; Burnstock, 2004; Burnstock and Kennedy, 1985; Lagaud et al., 1996; Morato et al., 2008). The P2X and P2Y receptors are also present on the vascular endothelium, with the P2Y receptor being the predominate endothelial purinergic receptor (Zang et al., 2006). Activation of endothelial P2Y receptors stimulates the
release of EDHF, which may attenuate vasoconstriction produced by activation of P2X receptors (Zang et al., 2006). The major mechanism for inactivation of released ATP is enzymatic degradation by a soluble nucleotidase that is also released with ATP from the nerve terminals (Todorov et al., 1997).

Functional studies characterizing P2 receptors in unpressurized rat mesenteric vessels have shown that both P2X and P2Y receptors mediate the contractile effects of exogenously applied ATP (Galligan et al., 2001; Lagaud et al., 1996). Further characterization of these receptors using selective purinergic blockers, such as Pyridoxal-phosphate-6-azophenyl-2’,4’-disulfonic acid (PPADS) and suramin, demonstrates that ATP-induced vasoconstriction is more specifically mediated by P2X₁ and P2Y₂ receptors in arteries and veins, respectively (Galligan et al., 2001). The absence of functional P2X₁ receptors in mesenteric veins was also confirmed by immunohistochemical analysis (Galligan et al., 2001; Hansen et al., 1999). Additionally, while both NE and ATP mediate neurogenic contractions in rat mesenteric arteries at low transmural pressures, ATP is the main neurotransmitter released at high transmural distending pressures based on response to selective α-adrenergic and purinergic receptor antagonists (Rummery et al., 2007). These observations indicate that increasing transmural pressure selectively enhances purinergic activity during sympathetic nerve activation which could contribute to pressure elevation seen in hypertension. Although these findings suggest that the physiological significance of ATP increases as transmural distending pressure elevates, experimental support from studies analyzing various neurotransmitters released during sympathetic activation stimulation is lacking.
6.4 Neuropeptide Y (NPY)

The third sympathetic neurotransmitter that can be released by peripheral sympathetic nerves, NPY, binds to specific postjunctional NPY receptors, which are G protein coupled receptors that mediate the multiple physiological effects of NPY. There are five different NPY receptor subtypes ie. Y1, Y2, Y3, Y4 and Y5, with Y1 receptors being the major postjunctional receptor mediating responses to endogenous NPY release, as well as exogenous application of NPY (Prieto et al., 2000; Zang et al., 2006). The actions and the mechanisms of action of endogenous NPY remain unclear. However, NPY is generally believed to have a modulatory role on the actions of NE and, possibly, ATP. In MSA, NPY receptor activation enhances noradrenergetic contractions via stimulation of non-selective cation channels, with subsequent Ca\(^{2+}\) influx through mainly L-type Ca\(^{2+}\) channels (Prieto et al., 2000; Prieto et al., 1997). NPY also interferes with AC-cAMP signaling pathways, and thus inhibits hyperpolarization (Prieto et al., 2000; Prieto et al., 1997). Pharmacological studies investigating the types of postjunctional NPY receptors involved in the potentiation of NE-induced vasoconstrictions in rat mesenteric arteries have suggested a role of both Y1 and Y2 receptors (McAuley and Westfall, 1992).
Rationale

The venous system plays a major role in overall cardiovascular homeostasis. Importantly, the venous splanchnic vascular bed, including the mesenteric veins, is the most significant venous bed in the body with major contributions to systemic vascular capacitance and blood pressure control. Major factors that regulate the function of veins in this segment of the circulation include the sympathetic nerves, myogenic reactivity, as well as local and circulating hormones. Mesenteric veins contain large volumes of blood which cause distending pressure. An increase in transmural distending pressure can alter net vascular tone by inducing myogenic reactivity and affecting the release of endothelial vasoactive mediators. Myogenic reactivity can also amplify responses to local and circulating vasoconstrictors by positive feedback on systemic blood pressure (Metting et al., 1989).

Alterations in venous myogenic reactivity have been reported in some physiological conditions such as exercise and pregnancy (Dhawan et al., 2005; Hohmann et al., 1990; Pang, 2001) and pathological conditions such as hypertension, heart failure, orthostatic hypotension, shock and acute stress (Fink et al., 2000; Gay et al., 1986; Martin et al., 1998; Martin et al., 1996). Despite the important role of veins in the homeostatic regulation of the cardiovascular system, there are limited studies evaluating their function in health and disease, with virtually no studies examining integrated venous function under conditions of changing physiological transmural pressures. In contrast, there is a substantial amount of work exploring mechanisms regulating integrated arterial function in a number of health and disease states.
Until recently, little has been known about the reactivity, and changes to integrated venous function, of mesenteric veins when studied at changing transmural pressures. Although, a recent study has shown that rat mesenteric veins are able to develop myogenic reactivity (Brookes and Kaufman, 2003), the origin and underlying mechanisms responsible for development of venous myogenic reactivity and its potential contribution to integrated venous function are still unclear. Additionally, the effects of changing transmural distending pressure on agonist and sympathetic nerve-induced contractions as well as the functioning of the vascular endothelium in veins has not been investigated. Thus, it is necessary to gain further understanding of these factors and the potential mechanisms involved in the regulation of venous function. Therefore, my doctoral thesis work will explore the effects of changing transmural distending pressure on key regulators of integrated venous function.

**Overall hypothesis:**

Increasing transmural distending pressure shifts net venomotor tone in isolated normal rat mesenteric small veins.

**Objectives:**

I will test the above hypothesis through experiments outlined in the following three main objectives:

1) Characterization of myogenic reactivity in isolated normal rat mesenteric small veins.

2) Investigation of the effects of transmural distending pressure on neurogenic-induced and agonist-induced contractions of isolated normal rat mesenteric small veins.
3) Investigation of the effects of transmural distending pressure on endothelial-dependent and endothelial-independent vasodilation responses of isolated normal rat mesenteric small veins.
GENERAL MATERIALS AND METHODS

Animals

Healthy male Sprague-Dawley rats (Charles River, Quebec, Canada) weighing 250-350 g were used in this study. Rats were housed in a humidity and temperature controlled room with a 12:12-h light-dark cycle and given standard chow and access to water ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Guelph, and conformed to standards set forth by the Canadian Council on Animal Care.

Preparation of mesenteric vessels

Rats were euthanized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg, CEVA Santé Animal, Libourne, France). The ileum (10 cm) and associated mesentery were removed and placed in cold (4°C) Krebs’ physiological salt solution (pH 7.35-7.40). Third order MSV or MSA approximately 2-3 mm in length were isolated by removing the adhering fat and connective tissue. The order of the mesenteric veins and arteries was identified according to branching order with the major vein or artery supplying the mesentery being the first order and subsequent branches being numbered accordingly.

Pressure myography

Following dissection, vessels were mounted on the inflow and outflow glass cannulas (tip diameter 100-150 µm) of a myograph vessel chamber (Danish Myotechnology, DMT; Aarhus, Denmark) and secured with 11-0 nylon suture. Cannulated veins or arteries were then placed on the stage of an inverted microscope.
(Nikon instruments Inc., NY, USA). To ensure that blood and metabolites were removed, mesenteric veins or arteries were perfused with warmed (37.0 ± 0.5°C), oxygenated (95% O₂- 5% CO₂) Krebs’ solution for 20 min at a physiological flow rate of 2 μL/min and 20 μL/min, respectively. Simultaneously, vessels were continually superfused with warmed and oxygenated Krebs’ solution at a rate of 7 mL/min using a peristaltic pump (Minipuls 3 peristaltic pump, Gilson, Inc. USA). Mesenteric vessels were visualized by a black and white camera attached to an inverted microscope for continuous monitoring of changes in vessel diameter, and data were obtained by Diamtrak® software (Adelaide, Australia) allowing for a resolution of 0.5 μm diameter changes. All experiments were performed in pressurized mesenteric veins and arteries under conditions of no intraluminal flow, with all drugs provided in the superfusing solution in known concentrations.

Solutions, drugs, and chemicals

Krebs’ physiological salt solution containing in mmol/L: sodium chloride 117, potassium chloride 4.7 (KCl), calcium chloride 2.5 (CaCl₂), magnesium chloride 1.2 (MgCl), sodium phosphate 1.2, sodium bicarbonate 25 and glucose 11; and gassed with 95% O₂ and 5% CO₂ (pH 7.35-7.40). NE, PE, clonidine, phentolamine, prazosin, yohimbine, acetylcholine chloride, BK acetate, Nω-Nitro-L-arginine (L-NNA, NOS inhibitor), indomethacin (COX inhibitor), ethylene glycol tetra-acetic acid (EGTA, chelating agent), nifedipine (L-VOCCs blocker), tetrodotoxin (TTX, voltage-dependent sodium channel blocker), sodium nitroprusside (SNP, NO donor), and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Oakville, Ontario, Canada); chelerythrine
(PKC inhibitor) was purchased from LC Laboratories (Woburn, MA, USA); Y-27632 (Rho kinase inhibitor) was purchased from Ascent Scientific (Princeton, NJ, USA); ET-1 (non-selective ET-1 receptor agonist, BQ-610 (selective ET<sub>A</sub> receptor antagonist) and BQ-788 (selective ET<sub>B</sub> receptor antagonist) were obtained from American Peptide Company, Inc (Sunnyvale, CA, USA); S-Nitroso-N-acetylpenicillamine (SNAP, NO donor) was obtained from Cayman chemical (Ann Arbor, Michigan, USA); paxilline and apamin were purchased from TOCRIS biosciences (Ellisville, Missouri, USA). L-NNA was prepared in 1M HCL and further diluted in water; prazosin was prepared in methanol in water; ET-1 and BQ-788 were prepared in 5 % ammonium hydroxide and BQ-610 in 20 % acetonitrile; indomethacin, nifedipine, chelerythrine, paxilline and SNAP were prepared in DMSO The final concentration of DMSO was \leq 0.01 \%. The other drugs were prepared in pure distilled water. All drug concentrations are expressed as final molar concentrations in the myograph chamber. (See Appendix III for a complete list of agonists, antagonists and inhibitors used in this thesis).

**Statistical analysis**

Changes in vessel inner (ID) and outer (OD) diameters were measured in \( \mu m \). Wall thickness was calculated by subtracting vessel OD from ID at low, intermediate and high transmural pressures. The wall-to-lumen ratio (\( h/R \)) was calculated from measurement of wall thickness and expressed as a ratio of the vessel diameter. Vascular compliance, the ratio of change in the vessels’ inner diameter (\( \Delta ID \)) to an associated change in transmural pressure (\( \Delta P \)), was calculated in MSV and MSA at physiological transmural pressure ranges of 4-8 mmHg and 40-80 mmHg using calcium-free Krebs’ solution, respectively. Agonist and neurogenic-induced contractions were expressed as a
percentage of the initial resting diameter (baseline) of the vessel. Agonist-induced relaxations were expressed as a percentage of the PGF2α-induced contractions. Agonist concentration producing half-maximal contraction (EC50) and maximum contraction (E_max) for NE, PE and ET-1 were determined for each concentration-response curve (CRC) fitted to four-parameter logistic equation (OriginLab 8.0, Northampton, MA, US). Agonist concentration producing half-maximal relaxation and maximum relaxation of BK, SNP, and SNAP were determined for each CRC fitted to a four-parameter logistic equation (OriginLab 8.0, Northampton, MA, US). The EC50 values are reported as pD2 values, which are the negative logarithm (-log) of the EC50 concentrations. Obtained frequency response curves (FRCs) from individual tissues were fitted to a three-parameter hill equation to determine half-maximal response (S50), and maximal response (S_max) in veins and arteries (OriginLab 8.0, Northampton, MA, US). Statistical analysis was performed by 2-way ANOVA for repeated measures followed by a post hoc Tukey’s or Dunnett’s test (SAS 9.1.3 institute Inc, Cary, NC, USA). The contractile responses to NE (10 µM) and KCL (120 mM) prior to incubation with chelerythrine and Y27632 and at the completion of the experiment in the continued presence of the inhibitors (Chapter 1) as well as clonidine contractile responses obtained in the absence and presence of yohimbine (Chapter 2) were analyzed by 1-way ANOVA for repeated measures. The assumptions of the ANOVA were assessed by comprehensive residual analysis. To meet the assumptions of the ANOVA and to handle percentage data, log or logit transforms were applied, when appropriate. A Shapiro-Wilk test was performed to assess normality of the data. At the end of the experiment where chelerythrine (2.5 µM) was applied (Chapter 1), the reduction in the contractile responses to NE (10 µM) was compared to
that of KCL (120 mM) by use of a paired t-test. Data are expressed as means ± SEM and
$P$ values < 0.05 were considered significant.
CHAPTER 1

CHARACTERISTICS OF MYOGENIC REACTIVITY IN ISOLATED RAT MESENTERIC VEINS

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Introduction

The prevailing level of vasomotor tone within a segment of the circulation is controlled primarily by sympathetic neurogenic influences, intrinsic myogenic properties of the VSM, and key local or circulating humoral factors (Monos et al., 1995; Murphy et al., 2002; Pang, 2001). Varying contributions from these factors, in part, provides functional specialization of the veins versus the arteries in a particular vascular bed while enabling the cardiovascular system to meet the changing demands of the body. Several studies conducted in veins including facial, saphenous, portal, skeletal muscle, and bat wing (Berczi et al., 1992; Davis et al., 1992; Dornyei et al., 1996; Henrion et al., 1994) have described myogenic tone in these vessels. However, the strength of myogenic tone varies between veins, possibly being dependent on the function of the vascular bed and its contribution to venous capacitance. Pressure-induced myogenic tone in rat saphenous vein is capable of controlling approximately 30% of its total capacity, being greatly affected by acute and chronic hemodynamic blood volume loads on the vessel wall (Monos et al., 1995; Monos et al., 1989). Brookes and Kaufman (2003) were able to demonstrate development of myogenic tone in fourth order rat mesenteric venules. The mesentery represents the largest capacitance bed in the body comprising about 25% of total circulating blood volume and therefore is capable of a wide range of transmural distending pressures (Pang, 2001; Pang, 2000). Importantly, only relatively small increases in active vascular tone of a major venous capacitance bed are required to significantly alter venous return and cardiovascular function (Monos et al., 1995). This underscores the potential importance of myogenic tone in overall venomotor tone in the low pressure venous system, as well as the hemodynamic consequences that may occur
from altered venous myogenic reactivity in cardiovascular diseases such as orthostatic hypotension (Monos et al., 2003). Currently, there remains a substantial paucity of work exploring the origin and mechanisms responsible for development of myogenic tone in veins, and the contribution of myogenic factors to integrated venous function. Undoubtedly this is due in no small part to the significant technical challenges associated with studying the more delicate and thinned walled small veins and venules under conditions of distending pressure. Failure to detect venous myogenic tone in most experiments may be the result of vulnerability of the wall of the small veins (Monos et al., 1995).

Alterations in VSM membrane potential and intracellular Ca\(^{2+}\) levels cannot fully explain venous myogenic reactivity (Monos et al., 1989; Schubert et al., 2008; Thakali et al., 2010). In limited studies, inhibitors of PKC and Rho kinase, key regulatory molecules of VSM calcium sensitization, have been shown to abolish stretch-activated myogenic tone in rabbit facial veins using wire myography, but no studies have used pressurized vessels (Dubroca et al., 2005; Loufrani et al., 1999). Using isolated third order branches of mesenteric vessels and state-of-the-art pressure myography, the results of our study are the first to provide insight into the mechanisms of pressure induced myogenic tone in a large capacitance bed such as the mesentery, and to uncover important differences in mechanical mechanisms of vasomotor tone in veins compared to arteries. In the current study, we hypothesized that pressurized third order mesenteric veins would develop myogenic tone to increasing distending pressure and that Rho kinase and PKC would play a pivotal role in pressure induced vascular tone.
Splanchnic veins have a high population of adrenergic receptors and are therefore very sensitive to adrenergic stimulation compared to arteries. NE released by the peripheral sympathetic nervous system and local and systemic hormones including angiotensin II and endothelial derived NO, prostanoids, and ET-1 may interact with myogenic factors to affect overall vasomotor tone (Anschutz and Schubert, 2005; Bai et al., 2004; Gschwend et al., 2003; Liu et al., 1994; Szekeres et al., 2004). In order to evaluate interactions between myogenic factors and pharmacomechanical coupling, we also studied the effects of changing transmural distending pressure on contractile responses to NE and ET-1, two major vasoactive agents released from the sympathetic nerve terminals and endothelium, respectively. It was hypothesized that the MSV would show enhanced sensitivity to the contractile effects of ET-1 and NE compared to the MSA across the range of distending pressures studied. The site of modulation of myogenic reactivity by vasoactive agonists may occur at the level of the receptor (Ping and Johnson, 1992). However, angiotensin II, NE and ET-1 also share common cellular pathways in contracting VSM. Interactions between these vasoactive agents and myogenic factors may result from links to common signaling pathways, as well as changes in membrane potential that could modulate the level of vasomotor tone (Masset et al., 2002; Lombard et al., 1990; Liu et al., 1994). To our knowledge the effect of changing transmural distending pressure on NE or ET-1-induced contractions of MSV has not been studied.

Materials and Methods

See General Materials and Methods (pages 45 - 49).
Experimental protocols

Third order MSV (ID, 213.7 ± 6.4 µm at 4 mmHg, n = 57) or MSA (ID, 220.7 ± 4.5 µm at 60 mmHg, n = 55) were used in the present study. In all experiments, the viability of the vessel for study was assessed by a contractile response to NE (10 µM). The integrity of the endothelium was assessed by a dilator response to acetylcholine (1 µM) and BK (0.1 µM) in NE preconstricted MSV and MSA, respectively. Vessels that were unable to react adequately to NE, acetylcholine, BK or demonstrated pressure leaks were discarded. In total, 139 vessels we isolated for study, with 8 veins and 19 arteries being discarded either for failing viability testing with NE, or also for arteries not developing spontaneous myogenic responses during equilibration. Before each experiment, all vessels were allowed to equilibrate for a period of 30 min at a transmural pressure of 4 mmHg and 60 mmHg for MSV and MSA, respectively. MSA that did not develop spontaneous myogenic responses during equilibration were also not studied. No significant difference was noted with the baseline ID of MSV across experiments. Similarly, vessel size was also not different between MSA across each experiment. It should also be mentioned that only one vessel per rat was used for each experimental protocol throughout the study.

In the first set of experiments, following stabilization, mesenteric vessels were pressurized from 2 to 12 mmHg (increments of 2 mmHg) in MSV and 20 to 140 mmHg (increments of 20 mmHg) in MSA. Each pressure step was maintained for 10 min in MSV and 5 min in MSA to allow the vessel to reach a steady-state diameter and data were continuously recorded by use of Diamtrak® software. Control experiments were conducted in mesenteric vessels with an intact endothelium using calcium containing
Krebs’ solution. In order to confirm that the endothelium remained viable for the duration of the control experiment, we retested endothelial integrity at its completion and found no differences in vasodilatory responses to BK or acetylcholine before or after control experiments in either MSV or MSA, respectively. In a subset of experiments, the endothelium was either pharmacologically blocked by use of L-NNA (100 µM) (Murphy et al., 2007) or mechanically removed by perfusing the MSA with 1 ml of air (Sun et al., 1992) or passing a piece of hair of appropriate size through the lumen of MSV (preliminary work in our laboratory). The absence of the endothelium was confirmed by loss of the dilator response to acetylcholine or BK in NE preconstricted MSV and MSA, respectively. Additionally, to ensure that this procedure did not damage smooth muscle cell function, vessel responses to NE were evaluated before and after removal of the endothelium, and showed no significant differences.

In a second set of experiments, nifedipine (1 µM) (Thakali et al., 2010, Zhang et al., 2002), chelerythrine (2.5 µM) (Shirasawa et al., 2003) or Y-27632 (3 µM) (Gokina et al., 2005; Jarajapu and Knot, 2005; Schubert et al., 2002; VanBavel et al., 2001) was applied to the superfusing solution. These drug concentrations were chosen based on previous studies and initial preliminary work done in our laboratory. Vessel responses to increasing transmural pressure were evaluated following a 30 min incubation period for each inhibitor, and in the continued presence of the inhibitors. Following each experiment, passive pressure-response curves were generated by changing the superfusing medium to a calcium-free Krebs’ solution, which was prepared by replacing CaCl₂ with EGTA (2 mM). Mesenteric vessels were incubated with calcium-free Krebs’ solution for 20 min to obtain the passive diameter of each vessel. In order to confirm
selectivity of PKC and Rho kinase inhibitors on myogenic reactivity, we compared contractile responses to NE (10 µM) and KCl (120 mM) prior to incubation with inhibitors to contractile responses obtained at the end of the experiment in the continued presence of the inhibitors (Table 1). No significant differences were noted, in either MSV or MSA, with contractile responses to NE (10 µM) or KCl (120 mM) prior to application of Y27632 at 3 µM compared to responses obtained at the end of the experiment in the continued presence of the inhibitor. However, contractile responses to NE (10 µM) and KCl (120 mM) were significantly reduced following application of chelerythrine at 2.5 µM in both MSV and MSA, with responses to NE being more reduced than KCl responses ($P < 0.05$). Therefore, we completed an additional set of experiments in vessels with chelerythrine at 1 µM and found no significant differences with NE (10 µM) or KCl (120 mM) contractile responses before application of the inhibitor versus contractile responses obtained at the end of the experiment.

In the final set of experiments, following equilibration, NE ($10^{-11}$ M to $10^{-4}$ M) and ET-1 ($10^{-13}$ M to $10^{-8}$ M) CRCs were obtained in MSV with distending pressures fixed at 2, 6, and 12 mmHg; and MSA at 20, 60 and 120 mmHg. These pressure levels were chosen as representative of low, intermediate and high physiologic transmural pressures based on previous work in mesenteric vessels by others (Johnson et al., 2001b; Fenger-Gron et al., 1995). NE and ET-1 were applied in increasing and known concentrations to the superfusing solution. Each agonist concentration was applied for 5 min to allow adequate time to reach a steady-state response. A 20 min washout period was observed between successive applications of each NE concentration, whereas ET-1 was applied in a cumulative manner due to its pseudo-irreversible receptor binding
characteristic which results in long lasting constrictor effects and prolonged washout periods (Waggoner et al., 1992).

Results

Role of the endothelium in pressure-induced activation of myogenic reactivity

Third order MSV did not demonstrate myogenic responses; however, these vessels were able to develop myogenic tone with vessel diameters being significantly reduced in control vessels compared to diameters of vessels under passive conditions over the range of 2-6 mmHg (Figure 3-A). Compared with MSV, MSA of the same branching order developed both myogenic tone and myogenic responses following stepwise increases in transmural pressure, with myogenic responses beginning at 80 mmHg and persisting to the highest pressure tested (Figures 3-B - 8-B). Inhibition of NOS with L-NNA (100 µM) did not significantly affect the magnitude of myogenic reactivity in either MSV or MSA with increasing pressure when compared to control responses (Figure 3-A, B). However, vascular tone tended to be greater in L-NNA treated MSV and MSA compared with controls, and vascular tone was significantly greater in L-NNA treated MSV at 8-12 mmHg when compared to passive responses while comparable responses in control veins were not different from passive responses. Myogenic reactivity was also not different in denuded MSV or MSA compared to intact vessels (Figure 4-A, B). Additionally, there was no significant difference in responses obtained in L-NNA treated MSV and MSA compared with denuded MSV and MSA, respectively.
Role of extracellular calcium in pressure-induced activation of myogenic reactivity

In order to investigate the contribution of extracellular Ca\(^{2+}\) influx through L-VOCCs to myogenic reactivity, mesenteric vessels were pre-treated with nifedipine and responses to stepped increases in transmural pressure were obtained. Nifedipine (1 µM) resulted in a significant, but incomplete, loss of venous myogenic tone; however responses to pressure increases in MSV under passive conditions were significantly different from responses obtained in nifedipine treated MSV (Figure 5-A). By contrast, nifedipine completely abolished all myogenic reactivity in MSA, with no differences noted between responses obtained under passive conditions versus responses in the presence of nifedipine (Figure 5-B).

Role of PKC and Rho kinase in pressure-induced activation of myogenic reactivity

In order to study the contribution of VSM PKC and Rho kinase activation to myogenic reactivity, the PKC inhibitor, chelerythrine, and the Rho kinase inhibitor, Y27632, were individually applied to MSV and MSA. Chelerythrine (2.5 µM) and Y27632 (3 µM) completely abolished all myogenic reactivity that developed subsequent to increased transmural pressure in MSA (Figures 6-B & 8-B). Similar findings were noted in the MSV treated with Y27632 (Figure 8-A). However, some myogenic tone remained in chelerythrine treated MSV at a distending pressure of 6 mmHg, as evidenced by a significant difference between responses obtained under passive conditions (ID, 401.1 ± 9.1) versus responses in the presence of chelerythrine (ID, 361.8 ± 11.1) \( (P = 0.0165) \) (Figure 6-A). Myogenic reactivity was not significantly affected by 1 µM chelerythrine in either MSA or MSV at any of the pressure steps (Figure 7-A, B).
Total wall thickness, wall-to-lumen ratio (h/R) and vascular compliance

There was a significant reduction in wall thickness and h/R values as transmural pressure increased in both MSV and MSA (Table 2). MSV and MSA had similar wall thickness and h/R values at low and intermediate transmural pressures. However at high transmural pressures, both wall thickness and h/R were greater in MSA compared with corresponding MSV. The compliance (ΔID/ ΔP) of MSV was higher (17.45 ± 1.38 µm/mmHg, n= 7) than the corresponding MSA (0.90 ± 0.14 µm/mmHg, n= 5) (P < 0.05).

Effects of transmural distending pressure on contractile responses to NE and ET-1

Changes in mesenteric vessels’ ID as a function of NE and ET-1 contractile-responses were obtained at 3 levels of transmural pressure: low, intermediate and high pressures (Figures 9 & 10). NE and ET-1 caused dose-dependent decreases in mesenteric vessel diameter. There was no significant difference in the sensitivity to NE or ET-1 contractile responses in MSV or MSA at the different pressure levels studied (Table 3). However, as transmural pressure increased, there was a trend of enhanced contractile responses to NE and ET-1 in mesenteric veins, and to ET-1 in MSA, particularly with higher pressures. MSV were more sensitive to NE and ET-1 when compared with corresponding MSA at low, intermediate and high transmural pressures. Additionally, E_{max} values attained with NE did not differ between MSV and MSA whereas E_{max} values for ET-1 CRCs were greater in MSV compared with those obtained in MSA.
Discussion

Myogenic reactivity in mesenteric vessels

Mesenteric small veins and arteries used in our study had similar baseline internal diameters following equilibration. Consistent with previous studies (Brookes and Kaufman, 2003; Sun et al., 1992), mesenteric arteries demonstrated myogenic responses which began at 60 mmHg and increased in magnitude to the highest pressure tested (140 mmHg) in most experiments. In the present study, the MSA also showed evidence of myogenic tone prior to the 60 mmHg pressure step. When the contractile apparatus was left intact, ie. under active conditions, the lesser increase in the diameter in the MSV at increasing pressures compared to passive conditions demonstrates that these vessels increased their tone with increasing distending pressure. By contrast to the MSA, MSV did not demonstrate myogenic responses. Instead, these vessels showed myogenic tone being evident at the lowest distending pressure (2 mmHg) and continuing through to the highest pressure tested (12 mmHg).

Both mesenteric vessels had similar wall thickness and wall-to-lumen ratios \((h/R)\) at low and intermediate transmural pressures, although they tended to be higher in arteries. However at higher transmural pressures, wall thickness and \(h/R\) values were significantly greater in MSA compared with corresponding MSV. Veins experience lower distending pressures than comparable arteries. Veins also generally demonstrate smaller wall thickness and \(h/R\) values compared with arteries as the latter are under much greater distending pressures. Collectively, our results are consistent with the concept that increased \(h/R\) values in MSA is associated with a mechanism to normalize wall tension when blood pressure (distending pressure) is raised whereas decreasing \(h/R\) values in
MSV supports their capacitance function (Brookes and Kaufman, 2003; Bund, 2000; Dhawan et al., 2005). The change in wall morphology with increasing distending pressure highlights differences between these vessel types that contribute to their different function in the circulation. In keeping with their capacitance function, we also found that the MSV demonstrated significantly greater compliance than the MSA.

**Modulation of myogenic reactivity in mesenteric vessels by the endothelium**

The origin of myogenic reactivity resides in the VSM, being independent of the endothelium (Dornyei et al., 1996; Murphy et al., 2007). However, there is great heterogeneity in the function of arteries and veins across organs. As such, myogenic reactivity may be modulated by the endothelium in some vascular beds. Endothelium-derived vasoactive substances including NO, prostacyclin, EDHF, and ET-1 and thromboxane-A$_2$ have been suggested to affect pressure-induced myogenic reactivity in various vessels (Bai et al., 2004; Bolla et al., 2002; Gschwend et al., 2003; Nguyen et al., 1999). It is agreed that an increase in wall shear stress due to high flow conditions results in the release of endothelial derived vasodilators (Monos et al., 1995). In order to eliminate the effects of flow related changes on vascular diameter, we designed the current study to observe the effects of transmural distending pressure in the absence of vascular flow. This stable in vitro environment also contributes to a steady-state of metabolism in the vessels (Sun et al., 1992).

In the present study, removal of the endothelium from the mesenteric vessels did not affect the characteristics (ie. shape and slope) of the pressure-diameter curves supporting the notion that the endothelium is not involved in the origin of myogenic reactivity in the MSV or MSA. Removal of the endothelium also produced no significant
effect on the magnitude of the pressure-diameter curves suggesting that it also has no overall modulatory role on myogenic reactivity in the mesenteric vessels under conditions of no flow. Therefore, our findings instead suggest limited contributions from basal release of endothelial dilators in pressurized MSV, and comparable MSA, under the experimental conditions of the current study.

Because it is has been suggested that distending pressure may evoke endothelial release of a combination of constrictors and dilators that produce no net effect on myogenic reactivity following endothelium removal (Nguyen et al., 1999), we also evaluated the contribution of pressure-induced release of endothelial-derived NO to vasomotor tone. We found no significant effect of inhibition of NOS with L-NNA on pressure-diameter responses in either mesenteric vessel when compared to control responses. Similar findings have also been reported in pressurized fourth order rat mesenteric venules (Brookes and Kaufman, 2003).

Our results in MSA showing no modulatory effects of the endothelium on pressure-diameter curves are in agreement with previous studies conducted in pressurized arteries (Anschutz and Schubert, 2005; Murphy et al., 2007) including rat mesenteric arteries (Sun et al., 1992). However, some studies have shown that inhibition of NOS or removal of the endothelium enhances the magnitude of arterial myogenic reactivity (Jarajapu and Knot, 2005; Nguyen et al., 1999; Szekeres et al., 2004) including a study in rat pressurized mesenteric arteries (Brookes and Kaufman, 2003).

One explanation for the discrepancy between results of our study and the Brookes and Kaufman (2003) study in rat mesenteric vessels may be the choice of NOS inhibitor (L-NAME) used in their study. Murphy et al. (2007) have demonstrated an endothelium-
independent constrictor effect of L-NAME (100 μM), and not L-NNA (100 μM), on pressurized rat cremaster arterioles by an unidentified mechanism. These authors concluded that constrictor actions of L-NAME and possibly other NOS inhibitors in pressurized vessels should not automatically be interpreted as indicating a modulatory role for endogenous NO production in regulating myogenic reactivity.

**Signaling mechanisms responsible for myogenic reactivity in mesenteric vessels**

It is established that VOCCs are important for cardiac myocyte and skeletal muscle contraction, with the L-type channel generally essential for arterial smooth muscle contraction (Hill et al., 2006; McCarron et al., 1997; Schubert et al., 2008; Thakali et al., 2010; Wesselman et al., 1996; Zhang et al., 2007). We found that activation of L-VOCCs accounts, in part, for the development of myogenic tone in mesenteric veins subjected to increasing transmural pressure. A weak role of these ion channels in the development of venous myogenic tone was also reported in rabbit facial veins suggesting that this may be a general finding in veins (Loufrani et al., 1999). Recent work by Thakali et al. (2010) also supports the notion that pressurized mesenteric veins are resistant to calcium channel blocker-induced dilation. However, this group did not specifically exam myogenic reactivity or changing pressure conditions. Therefore, venous myogenic tone may be developed, in part, independent of L-VOCCs, suggesting that under physiologic conditions mesenteric veins may rely on other calcium sources including other calcium channels or intracellular Ca\(^{2+}\).

In contrast to the MSV, in the MSA and in agreement with other studies (Chlopicki et al., 2001; McCarron et al., 1997; Potocnik et al., 2000; VanBavel et al., 1998; Wesselman et al., 1996; Zhang et al., 2007; Zhang et al., 2002), we found that L-
VOCCs play a major role in the development of arterial myogenic responses with their blockade resulting in abolishment of all myogenic activity. However, in some arteries, such as skeletal muscle (Kotecha and Hill, 2005; VanBavel et al., 2002) and coronary (Pyles et al., 1997), residual pressure-induced responses have been observed following blockade of L-VOCCs. This supports the notion of heterogeneity between vessels of differing tissues with regards to the mechanisms underlying myogenic reactivity, which may have its basis in pressure differences experienced in these vessels or possibly the physiologic functions of the tissues they support. Collectively, these findings also highlight another important difference with the development of myogenic reactivity between MSA and MSV and suggest that other signaling pathways may be more involved with development of venous myogenic tone.

Calcium sensitization of the VSM contractile apparatus via activation of PKC and Rho kinase molecules represents another key signaling pathway used to modify vasomotor tone (Lagaud et al., 2002). PKC and Rho kinase are thought to act via inhibition of MLCP, an enzyme involved in dephosphorylation of MLC (Zou et al., 2000; Zou et al., 1995). While Rho kinase appears to phosphorylate MLCP directly, PKC has been suggested to act indirectly, by increasing the activity of CPI-17 (PKC-activated protein phosphatase inhibitor of 17 kDa) (Schubert et al., 2008). We found that inhibition of Rho kinase by Y27632 (3 μM) abolished myogenic reactivity in both mesenteric vessels at all transmural pressure steps. Additionally, we confirmed specificity of action of Y27632 at 3 μM by demonstrating no significant effects of the inhibitor on NE or KCl responses in either MSV or MSA. The results obtained with the PKC inhibitor, chelerythrine, were less conclusive. While chelerythrine at 2.5 μM
largely abolished myogenic reactivity in both vessels at most pressure steps, it also attenuated NE and KCl responses in both MSV and MSA, suggesting it may have acted in a non-specific manner, in part, to affect vascular tone. However, it should be emphasized that in the presence of chelerythrine at 2.5 µM, significant KCl and NE induced constriction still remained, while myogenic activity was absent as evidenced by the fully distended vessels.

In a separate set of experiments we also showed that chelerythrine at 1 µM did not significantly affect contractile responses to NE or KCl, but also did not significantly alter myogenic reactivity in either vessel. Additionally, we showed that reductions in NE responses in the presence of chelerythrine at 2.5 µM were significantly greater than corresponding reductions with KCl responses. These findings are in agreement with a demonstrated link of PKC to activation of the adrenergic pathway in VSM, while inhibition of K⁺ depolarized VSM would imply a more general non-specific effect on the vessels (Mueed et al., 2004). We therefore conclude that the absence of myogenic reactivity was not explained by the complete suppression of VSM contractility, rather is due, in part, to the effects of PKC inhibition (Wesselman et al., 2001).

Our results show a significant role for Rho kinase in myogenic reactivity in both vessels, while PKC may play a more widespread role in vascular tone development including myogenic reactivity. The work of others definitely supports the preservation of a key role for these molecules in pressure induced tone in most vascular preparations including pressurized rat mesenteric arteries and rabbit facial veins (Dubroca et al., 2005; Loufrani et al., 1999; Massett et al., 2002; Shirasawa et al., 2003; VanBavel et al., 2001; Wesselman et al., 2001; Yeon et al., 2002). This highlights the important role played by
calcium sensitization in myogenic reactivity of veins, which enables vessels to maintain a range of pressure induced vascular tone without large changes in intracellular Ca$^{2+}$ (Wesselman et al., 2001). Although most studies suggest that PKC and RhoA/Rho kinase pathways are activated independently to cause VSM contraction, cross talk between them has been suggested (Mueed et al., 2004). It is therefore conceivable that inhibition of each of these pathways ie. PKC or Rho kinase could produce substantial reductions in myogenic reactivity. One common link between the two pathways is CPI-17, which serves as a substrate for both Rho kinase and PKC, and whose actions prolong VSM contraction (Schubert et al., 2008).

Our results in MSA regarding the contribution of PKC and Rho kinase to myogenic reactivity revealed comparable findings to those reported in other studies in mesenteric arteries (Dubroca et al., 2007; VanBavel et al., 2001). This consistency with the involvement of PKC and Rho kinase in the development of pressure-induced vascular tone confirms the important role of these intracellular signaling mechanisms and thus calcium sensitization (calcium-independent mechanisms) in arterial myogenic reactivity, which can now also be extended to venous myogenic reactivity. However, in some arteries, such as rat tail and cerebral arteries, PKC inhibition did not affect myogenic reactivity, whereas Rho kinase inhibition only partly reduced myogenic reactivity (Gokina et al., 2005; Jarajapu and Knot, 2005; Schubert et al., 2002). In these studies and ours, comparable concentrations of PKC and Rho kinase inhibitors were used, suggesting vascular variability in the expression or distribution of these kinases.
Contractile responses to NE and ET-1 in pressurized mesenteric vessels

The sensitivity of small arteries and arterioles to exogenous NE induced contractions or endogenous NE released by sympathetic nerve stimulation can depend on the transmural pressure at which the vessels are studied. This supports the existence of an interplay or interaction between vasoactive agonists and myogenic reactivity on overall vasomotor tone in some vascular beds (Anschütz and Schubert, 2005; Dunn et al., 1994; Lombard et al., 1990; Rummery et al., 2007). Virtually no studies have been performed comparing interactions of vasoactive agonists with changing transmural pressure in veins making comparisons with findings in our study challenging. Both NE and ET-1 are important humoral factors regulating vasomotor tone in the mesentery. Dornyei et al. (1996) reported that pressure-induced contractions were enhanced in the low pressure range (0.5-2.0 mmHg) in rat skeletal (gracilis) muscle venules that were first preconstricted with NE then followed by increasing pressure steps (0.5-17.5 mmHg). Our results obtained by first fixing distending pressure then generating concentration responses to agonists showed no significant difference in the sensitivity of mesenteric vessels to NE or ET-1 when compared across increasing transmural pressure; albeit a tendency of enhanced responses to NE and ET-1 was observed in the veins. It is possible that the different methodological approaches accounts for the inconsistent findings between ours and the former study.

We also found that contractile responses to ET-1 and NE in MSV were significantly more sensitive than findings in comparable MSA across the range of low to high physiologic pressures studied. The higher sensitivity of MSV to NE may due to contributions from larger numbers of α1-ARs compared with MSA (Perez-Rivera et al.,
or to the involvement of α₂-ARs in NE-induced venous constriction (Perez-Rivera et al., 2007). Contractile responses to ET-1 in MSA occurs via activation of VSM ETₐ receptors while in veins activation of both VSM ETₐ and ET₆ receptors is possible (Johnson et al., 2002; Johnson et al., 2001a). Taken together, these findings indicate that the increased sensitivity of MSV compared with MSA to either agonist in the present study is maintained when these vessels are pressurized across a range of physiologic distending pressures. This finding has obvious relevance in vivo where vessels are subjected to a range of transmural distending pressures.

In summary, the present study shows that MSV develop myogenic reactivity in the form of myogenic tone that appeared to be greatest over the low to intermediate range of physiologic pressures studied. These findings highlight the potential importance of enhanced myogenic tone to overall venomotor tone at low-to-intermediate physiological distending pressures and their maintenance of adequate venous return in conjunction with input from other neurohormones, such NE and ET-1. The reduced myogenic tone at higher distending pressures may conceivably aid the capacitance function of these vessels and prevent large shifts in blood to the heart. Our findings also demonstrate a lack of interactions between neurohormones and myogenic reactivity to enhance vascular tone in mesenteric vessels. This finding in a large capacitance bed like the mesentery is understandable given the strong potential for detrimental shifts in blood toward the heart. Furthermore our results provide evidence for the contribution of PKC and Rho kinase to myogenic reactivity in MSV and confirm earlier studies conducted in MSA. Our study has provided a limited evaluation of the role of these molecules in the development of
venous myogenic reactivity. However, a greater understanding of the role of these molecules is warranted, which will require additional studies.
Figure 3. Effect of nitric oxide synthase (NOS) inhibition by Nω-nitro-L-arginine (L-NNA, 100 µM) on the development of myogenic reactivity in mesenteric small veins (A, \(n = 8\)) and arteries (B, \(n = 5\)). \(^{†}\) control vs. passive; \(^{‡}\) L-NNA vs. passive. Data are expressed as mean ± SEM \((P < 0.05)\).
Figure 4. Effect of the removal of the endothelium on the development of myogenic reactivity in mesenteric small veins (A, \( n = 5 \)) and arteries (B, \( n = 5 \)). Data are expressed as mean ± SEM.
A

- Control
- (-) Endothelium

Vessel inner diameter (μm)

Pressure (mmHg)

B

- Control
- (-) Endothelium

Vessel inner diameter (μm)

Pressure (mmHg)
Figure 5. Effect of L-type calcium channel blockade by nifedipine (1 µM) on the development of myogenic reactivity in mesenteric small veins (A, n = 7) and arteries (B, n = 5). * control vs. nifedipine; † control vs. passive; ‡ nifedipine vs. passive. Data are expressed as mean ± SEM (P < 0.05).
Figure 6. Effect of PKC inhibition by chelerythrine (2.5 µM) on the development of myogenic reactivity in mesenteric small veins (A, n = 5) and arteries (B, n = 5). * control vs. chelerythrine; † control vs. passive; ‡ chelerythrine vs. passive. Data are expressed as mean ± SEM (P < 0.05).
Figure 7. Effect of PKC inhibition by chelerythrine (1 µM) on the development of myogenic reactivity in mesenteric small veins (A, n = 4) and arteries (B, n = 4). † control vs. passive; ‡ chelerythrine vs. passive. Data are expressed as mean ± SEM (P < 0.05).
Figure 8. Effect of Rho-kinase inhibition by Y27632 (3 µM) on the development of myogenic reactivity in mesenteric small veins (A, n = 4) and arteries (B, n = 5). * control vs. Y27632; † control vs. passive. Data are expressed as mean ± SEM (P < 0.05).
Figure 9. Concentration-response curves of norepinephrine (NE) obtained in mesenteric small veins (closed circles) and arteries (open circles); at low (A), intermediate (B) and high (C) transmural pressures. $n$ refers to the number of vessels used in each protocol.

* higher sensitivity of veins to NE compared to arteries. Data are expressed as mean ± SEM ($P < 0.05$).
Figure 10. Concentration-response curves of endothelin-1 (ET-1) obtained in mesenteric small veins (closed circles) and arteries (open circles); at low (A), intermediate (B) and high (C) transmural pressures. n refers to the number of vessels used in each protocol. * higher sensitivity and † maximum responses of veins to ET-1 compared to arteries.

Data are expressed as mean ± SEM (P < 0.05).
Table 1. Contractile responses of rat mesenteric small veins and arteries to norepinephrine (NE, 10 µM) and potassium chloride (KCl, 120 mM) prior to antagonist application (control) and at end of experiment in the presence of the inhibitors.

<table>
<thead>
<tr>
<th></th>
<th>% Contraction (ID)</th>
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<tbody>
<tr>
<td></td>
<td>Small vein</td>
</tr>
<tr>
<td><strong>NE</strong></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>70.3 ± 4.9</td>
</tr>
<tr>
<td>Chelerythrine, 1 µM</td>
<td>71.8 ± 4.4</td>
</tr>
<tr>
<td>Control</td>
<td>66.1 ± 1.0</td>
</tr>
<tr>
<td>Chelerythrine, 2.5 µM</td>
<td>22.1 ± 9.8 *† (4)</td>
</tr>
<tr>
<td>Control</td>
<td>71.4 ± 5.8</td>
</tr>
<tr>
<td>Y-27632, 3 µM</td>
<td>64.1 ± 8.6</td>
</tr>
</tbody>
</table>

|                  |              |              |
| **KCl**          |              |              |
| Control          | 81.1 ± 3.8   | 79.7 ± 2.3   |
| Chelerythrine, 1 µM | 84.1 ± 1.9  | 82.9 ± 0.8   |
| Control          | 85.5 ± 1.6   | 81.9 ± 0.9   |
| Chelerythrine, 2.5 µM | 59.2 ± 7.9 † (4) | 62.1 ± 4.7 * (3) |
| Control          | 76.4 ± 5.5   | 78.1 ± 3.6   |
| Y-27632, 3 µM    | 81.8 ± 1.3   | 67.9 ± 5.1   |

Values are expressed as mean ± SEM; n = number per group in parenthesis. 
P < 0.05, *compared with control; † compared with corresponding KCl value in presence of chelerythrine, 2.5 µM.
Table 2. Wall thickness and Wall-to-lumen ratio ($h/R$) values obtained from control pressure curves at low, intermediate and high transmural pressures in mesenteric small veins and arteries isolated from normal male rats.

<table>
<thead>
<tr>
<th></th>
<th>Wall Thickness, µm</th>
<th>$h/R$</th>
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<tbody>
<tr>
<td><strong>Small vein</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mmHg (29)</td>
<td>50.8 ± 2.8 †</td>
<td>0.24 ± 0.01 †</td>
</tr>
<tr>
<td>6 mmHg (29)</td>
<td>38.5 ± 3.1 ‡</td>
<td>0.14 ± 0.01 ‡</td>
</tr>
<tr>
<td>12 mmHg (29)</td>
<td>31.7 ± 2.4 *</td>
<td>0.10 ± 0.1 *</td>
</tr>
<tr>
<td><strong>Small artery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 mmHg (25)</td>
<td>50.5 ± 2.5 †</td>
<td>0.24 ± 0.002 †</td>
</tr>
<tr>
<td>60 mmHg (25)</td>
<td>40.5 ± 2.3</td>
<td>0.16 ± 0.004</td>
</tr>
<tr>
<td>120 mmHg (25)</td>
<td>42.4 ± 2.2</td>
<td>0.18 ± 0.01</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM; $n$ = number per group in parenthesis. $P < 0.05$, † compared with intermediate and high transmural pressures within a vessel; ‡ compared with high transmural pressure within a vessel; * compared with corresponding small artery.
Table 3. $E_{\text{max}}$ and $pD_2$ values obtained from norepinephrine (NE) and endothelin-1 (ET-1) concentration-response curves at low, intermediate and high transmural pressures in mesenteric small veins and arteries isolated from normal male rats.

<table>
<thead>
<tr>
<th></th>
<th>$pD_2$</th>
<th>$E_{\text{max}}$ (%)</th>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Small vein</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td></td>
<td></td>
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<tr>
<td>2 mmHg (4)</td>
<td>8.1 ± 0.1 $^*$</td>
<td>79.2 ± 2.0</td>
</tr>
<tr>
<td>6 mmHg (5)</td>
<td>8.3 ± 0.1 $^*$</td>
<td>71.4 ± 2.3</td>
</tr>
<tr>
<td>12 mmHg (6)</td>
<td>8.4 ± 0.1 $^*$</td>
<td>68.2 ± 3.7</td>
</tr>
<tr>
<td>ET-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mmHg (5)</td>
<td>9.9 ± 0.3 $^*$</td>
<td>92.3 ± 2.4 $^\dagger$</td>
</tr>
<tr>
<td>6 mmHg (4)</td>
<td>10.1 ± 0.1 $^*$</td>
<td>90.8 ± 0.8 $^\dagger$</td>
</tr>
<tr>
<td>12 mmHg (4)</td>
<td>10.6 ± 0.3 $^*$</td>
<td>90.1 ± 2.8 $^\dagger$</td>
</tr>
<tr>
<td><strong>Small artery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 mmHg (5)</td>
<td>6.4 ± 0.1</td>
<td>79.2 ± 2.1</td>
</tr>
<tr>
<td>60 mmHg (6)</td>
<td>6.6 ± 0.1</td>
<td>74.2 ± 2.6</td>
</tr>
<tr>
<td>120 mmHg (5)</td>
<td>6.9 ± 0.1</td>
<td>69.4 ± 3.3</td>
</tr>
<tr>
<td>ET-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 mmHg (5)</td>
<td>9.4 ± 0.1</td>
<td>79.8 ± 1.8</td>
</tr>
<tr>
<td>60 mmHg (4)</td>
<td>9.5 ± 0.04</td>
<td>76.4 ± 4.1</td>
</tr>
<tr>
<td>120 mmHg (5)</td>
<td>9.2 ± 0.1</td>
<td>75.0 ± 1.7</td>
</tr>
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</table>

Values are expressed as mean ± SEM; $n =$ number per group in parenthesis. $P < 0.05$, $^*$, $^\dagger$ compared with corresponding small artery. $pD_2$, negative logarithm of the agonist producing half-maximal constriction; $E_{\text{max}}$, maximum constriction.
CHAPTER 2

FUNCTIONAL CHARACTERISTICS OF ALPHA ADRENERGIC
RECEPTORS, ENDOTHELINERGIC RECEPTORS AND
SYMPATHETIC NEUROGENIC CONTRACTIONS IN ISOLATED
PRESSURIZED RAT MESENTERIC VEINS
Introduction

Vascular tone is regulated largely by the sympathetic nervous system, in which the main endogenous α-AR agonist, NE, is released from sympathetic nerves within the vessel wall. The vascular endothelium, also releases vasoconstrictors that can interact with neurohormones and other factors to maintain vascular homeostasis. ET-1 is the most potent endogenous vasoconstrictor released by the endothelium that contributes to the regulation of vascular tone and thus to overall cardiovascular regulation (Gray et al., 2000a; Serneri et al., 1995). Mesenteric veins are more sensitive to the contractile effects of sympathetic nerve stimulation (Hottenstein and Kreulen, 1987; Luo et al., 2003; Park et al., 2007), and exogenous NE (Perez-Rivera et al., 2007; Perez-Rivera et al., 2004; Sporkova et al., 2010) and ET-1 (Johnson et al., 2002; Perez-Rivera et al., 2005) compared to arteries. This may contribute to distinct functions (i.e resistance vs. capacitance) between these vessel types in the peripheral circulation. Differences in vascular reactivity between vessels may be due to variations in neurotransmitters release and/or post-junctional receptor populations, including α-ARs and ET-1 receptors.

In general, two α-adrenergic and two ET-1 receptor subtypes are expressed in VSM, the α1 and α2 receptors and the ET_A and ET_B receptors, respectively. Both receptors types are G-coupled proteins. The α2 and ET_B receptors are expressed in the endothelial cells and their activation can evoke vasodilation that counteracts vasoconstriction induced by NE and ET-1, respectively (Gray et al., 2000b; Motte et al., 2006). The α1 and ET_A receptors are important in regulating peripheral vascular resistance and systemic arterial blood pressure (Cotecchia, 2010; Motte et al., 2006). To date, data from studies employing non-pressurized arteries and veins exposed to different
\(\alpha\)-adrenergic and ET-1 receptor agonists and antagonists indicate that the contractile effects of \(\alpha\)-adrenergic and ET-1 receptor agonists are mediated mainly by the \(\alpha_1\)-ARs and ET\textsubscript{A} receptors, respectively (Daly et al., 1988; Gavin and Docherty, 1996; Gornemann et al., 2008; Perez-Rivera et al., 2005; Sporkova et al., 2010; Thakali et al., 2008). Moreover, there is evidence that support the involvement of contractile \(\alpha_2\)-adrenergic (Daly et al., 1988; Perez-Rivera et al., 2007; Sporkova et al., 2010) and ET\textsubscript{B} receptors (Johnson et al., 2002; Perez-Rivera et al., 2005) in venous constriction.

In Chapter 1, we have shown at physiological transmural distending pressures an increased sensitivity of MSV to NE and ET-1 when compared with corresponding MSA. However, functional \(\alpha\)-adrenergic and ET-1 receptors which may contribute to this difference between MSA and MSV studied under conditions of changing transmural pressure have not been determined.

Sympathetic nerve activation and subsequent release of neurotransmitters may also differ in MSV compared with corresponding MSA considering that neurogenic contractions of intact rat MSA contain components attributable to both ATP and NE (Gitterman and Evans, 2001; Luo et al., 2003; Rummery et al., 2007) while in MSV neurogenic contractions are mediated mainly by NE (Luo et al., 2003). Sympathetic nerve activation has been extensively investigated in unpressurized mesenteric veins and arteries from different species (Bobalova and Mutafova-Yambolieva, 2001; Luo et al., 2004; Mutafova-Yambolieva et al., 2003; Park et al., 2010; Park et al., 2007; Smyth et al., 2000) whereas only one study, that we are aware of, has investigated the effects of increasing transmural distending pressure on neurogenic contractions in MSA (Rummery et al., 2007). Rummery et al (2007), using second order mesenteric arteries, showed
enhanced neurogenic contractions at high transmural pressure (90 mmHg) compared with those obtained at low pressure (30 mmHg), and that ATP was found to be the main neurotransmitter released by sympathetic nerves supplying these arteries at high levels of transmural pressure. To the best of our knowledge, the effects of transmural pressure on sympathetic neurogenic contractions in MSV have not been investigated.

In vitro studies have demonstrated that pressurization of mesenteric arteries enhances vessel sensitivity to vasoactive agents compared to isometric methods (Buus et al., 1994; Dunn et al., 1994) and that increasing transmural distending pressure selectively reveals differences in postjunctional receptors mediating neurogenic contractions (Rummery et al., 2007). Given that the splanchnic vascular bed, including the mesenteric circulation, is a major vascular bed providing significant contributions to vascular capacitance and arterial resistance (Pang, 2001), it is important to understand the effect of changing transmural pressures on sympathetic neurotransmission as well as the relative contributions of α-adrenergic and ET-1 receptor subtypes to the contractile responses of key vasoactive agents, NE and ET-1, respectively.

In this study, we attempted for the first time under conditions of changing transmural pressure to investigate sympathetic neurogenic activity and to characterize postjunctional α-adrenergic and ET-1 receptors mediating NE-and ET-1-induced contractions in isolated rat MSV and MSA by use of different α-adrenergic and ET-1 receptors selective agonists and antagonists, respectively. We tested the hypothesis that the contractile effects of sympathetic nerve activation will change with increasing transmural pressure and that elevating transmural pressure will reveal differences in the
subtype of α-adrenergic and ET-1 receptors mediating NE-and ET-1-induced contractions of mesenteric vessels, respectively.

**Materials and Methods**

See General Materials and Methods (pages 45 - 49).

**Electrical field stimulation (EFS)**

The effects of sympathetic nerve stimulation on the MSV and MSA were studied using EFS. Cannulated pressurized mesenteric vessels were stimulated electrically via two platinum wire electrodes (contained within the cover of the myograph chamber) that ran parallel to the long axis of the vessel while being connected to a Grass S88X electrical stimulator (Astro-Med, West Warwick, U.S.A). MSV and MSA were stimulated by a train of 30 stimuli, and voltage of 60 V, over a frequency range of 0.2-30 Hz with duration and delay of 0.5 ms and 0.3 ms of each stimulus, respectively (Luo et al., 2004). TTX was used to confirm that the responses produced were neurogenic in origin, and not due to direct electrical stimulation of VSM cells. This was done by stimulating mesenteric vessels with a frequency of 20 Hz in the absence and presence of TTX. To ensure the return of neurogenic responses to values similar to those obtained before the application of TTX, mesenteric vessels were retested with the same parameters after a 20 min washout period from TTX.

**Experimental protocols**

In all experiments, the viability of the vessel for study was also assessed by a contractile response to NE (10 µM). The integrity of the endothelium was assessed by a
dilator response to BK (0.1 µM) and acetylcholine (1 µM) in NE preconstricted veins and arteries, respectively. Vessels that were unable to react adequately to NE, acetylcholine, BK or demonstrated pressure leaks were discarded. In the total of 281 vessels we isolated for study, 18 veins and 12 arteries were discarded either for failing viability testing with NE or the arteries did not develop spontaneous myogenic responses during equilibration.

In Chapter 1, we studied NE and ET-1 contractile responses at low pressure levels in arteries (20 mmHg) and veins (2 mmHg) and found no differences in these responses when compared with those obtained at intermediate transmural pressures, i.e. 60 mmHg in MSA and 6 mmHg in MSV, respectively. As such, we decided to study the effects of intermediate and high transmural pressures on postjunctional receptors contributing to vasoconstriction induced by NE and ET-1. Frequency response curves were generated in MSV and MSA pressurized at transmural pressures of 2, 6 and 12 mmHg and 20, 60, and 120 mmHg respectively. No significant difference was noted with the baseline ID of MSV across experiments. Similarly, vessel size was also not different between MSA across each experiment. It should also be mentioned that only one vessel per rat was used for each experimental protocol and all experiments were conducted in mesenteric vessels with an intact endothelium.

**Characterization of α-ARs mediating contractile responses to exogenous NE at increasing transmural pressures**

Third order MSV (ID, 290.3 ± 6.5 µm at 6 mmHg, n = 58) or MSA (ID, 203.4 ± 3.8 µm at 60 mmHg, n = 53) were studied. Following an appropriate equilibration period, NE (10^{-12} M to 10^{-4} M) and PE (10^{-10} M to 10^{-4} M) CRCs were obtained in
mesenteric vessels with transmural pressures fixed at 6, and 12 mmHg in MSV and 60 and 120 mmHg in MSA. NE and PE were applied in increasing and known concentrations to the superfusing solution. Each concentration was applied for 5 min to allow adequate time to reach a steady-state response and data were continuously recorded by use of Diamtrak® software (Adelaide, Australia). The effects of the nonselective α<sub>1</sub>-AR / α<sub>2</sub>-AR antagonist, phentolamine (10 µM) (Jensen et al., 2004; Sadeghi and Eikenburg, 1993), the selective α<sub>1</sub>-AR antagonist, prazosin (100 nM) (Luo et al., 2003; Park et al., 2010; Rummery et al., 2007; Xu et al., 2007), and the selective α<sub>2</sub>-AR antagonist, yohimbine (100 nM) (Faber and Meininger, 1990) on NE-induced contractions in mesenteric small vessels were evaluated following a 20 min pre-incubation period for each antagonist during agonist application and in the continued presence of the antagonists. To verify the selectivity of yohimbine to α<sub>2</sub>-ARs, PE CRCs were also generated in MSV and MSA at different transmural pressures in the presence of yohimbine. In a separate set of experiments, clonidine (a selective α<sub>2</sub>-AR agonist) -induced contractions in mesenteric vessels were also studied at intermediate transmural pressures in the absence and presence of yohimbine.

NE, PE and clonidine were applied cumulatively in MSA and non-cumulatively in MSV. Cumulative time control experiments in MSA revealed no significant changes in sensitivity or maximum responses for the two successive NE CRCs attained at intermediate or high transmural pressure (30 min between each CRC). Therefore, two NE CRCs were obtained in each individual MSA preparation. The first CRC was used as control and the subsequent curve was obtained after incubating the vessels with an α-AR antagonist as mentioned above. In MSV, reproducible cumulative CRCs were not
possible at 12 mmHg with MSV desensitizing to adrenergic agonists. Therefore, a non-cumulative approach was followed in MSV and only one agonist CRC was obtained in each individual MSV in the absence or presence of an α-AR antagonist. A 15 min washout period was observed between successive applications of each agonist concentration in mesenteric vein studies.

**Characterization of ET-1 receptors mediating contractile responses to ET-1 at increasing transmural pressures**

Third order MSV (ID, 292.6 ± 7.0 µm at 6 mmHg, n = 62) or MSA (ID, 196.0 ± 3.8 µm at 60 mmHg, n = 50) were examined. Following equilibration, ET-1 CRCs were obtained in vessels in the presence and absence of ET-1 receptor antagonists with transmural pressures fixed at 6, and 12 mmHg in MSV and 60 and 120 mmHg in MSA. Control ET-1 CRCs were obtained in mesenteric vessels superfused with normal Krebs’ solution. ET-1 was applied in increasing and known concentrations to the superfusing solution. Each concentration was applied for 5 min to allow adequate time to reach a steady-state response and data were continuously recorded by use of Diamtrak® software. Cumulative application of ET-1 was followed due to its pseudo-irreversible receptor binding characteristic (Waggoner et al., 1992). Thus, one ET-1 CRC was generated in each vessel. The effects of the ET\textsubscript{A} receptor selective antagonist, BQ-610 (100 nM) (Johnson et al., 2002; Perez-Rivera et al., 2005), the ET\textsubscript{B} receptor selective antagonist, BQ-788 (100 nM) (Johnson et al., 2002; Perez-Rivera et al., 2005; Sharifi and Schiffrin 1996) or both antagonists (i.e. BQ-610 + BQ-788) on ET-1-induced contractions in mesenteric vessels were evaluated following a 20 min pre-incubation period for each antagonist, and in the continued presence of the antagonists during ET-1 application.
Characterization of increasing transmural distending pressure on sympathetic neurogenic contractions

Third order MSV (ID, 268.9 ± 15.6 µm at 6 mmHg, n = 13) or MSA (ID, 204.9 ± 11.3 µm at 60 mmHg, n = 15) were examined. Following vessel stabilization, FRCs were generated in both MSV and MSA at transmural pressures of 2, 6 and 12 mmHg and 20, 60 and 120 mmHg, respectively. To ensure response stability, MSV and MSA were stimulated at 10 min intervals at a rate of 10 Hz for three times after which full FRCs were obtained with a washout period of 10 min between successive stimulations. Consistent with a previous study (Rummery et al., 2007), preliminary work accomplished in our laboratory indicated no differences in the amplitude of vasoconstrictions obtained from three consecutive nerve stimulation FRCs generated at 30 min intervals (data not shown).

In all experiments, the neurogenic origin of constrictions caused by EFS was verified by application of TTX at a concentration of 0.3 µM (Luo et al., 2004; Luo et al., 2003; Rummery et al., 2007; Smyth et al., 2000). TTX prevented sympathetic nerve activation by 85 ± 2.7 % of the maximum control responses. The residual neurogenic responses suggested a direct action of EFS on endothelial and/ or VSM cells. An ideal 100 % block of sympathetic nerve activation by TTX is required to further study neurogenic-induced contractions. This percentage of block was only obtained when the endothelium was removed. A new technology has recently been developed for measuring ATP and NPY overflow. In pressurized rat first order skeletal muscle arterioles, ATP and NPY overflow was measured by use of microelectrode biosensors and a peptide enzyme immunoassay, respectively (Evanson et al., 2011; Kuless et al.,
To the best of our knowledge, studies investigating neurotransmitters release in mesenteric circulation under conditions of changing transmural distending pressure are lacking. We were not able to consistently measure neurotransmitters released by sympathetic nerve activation using high performance liquid chromatography system (HPLC), which limited our further research with this objective. Thus we only studied the effects of changing transmural pressure on the contractile responses of sympathetic nerve activation in mesenteric vessels with an intact endothelium, and without further characterization of post-junctional receptors involved in sympathetic neurogenic contractions due to the difficulty of studying denuded mesenteric vessels.

Results

Contractile responses to NE, PE, ET-1 and EFS in pressurized rat mesenteric small veins and arteries

Changes in mesenteric vessel ID as a function of NE, PE, ET-1 or neurogenic contractile responses were obtained at various levels of transmural pressure (Figures 11-14 & 16-18). NE, PE, and ET-1 caused concentration-dependent decreases in mesenteric vessel diameter. In control experiments, there were no significant differences in the sensitivity to NE, PE and ET-1 within MSV or MSA studied across pressure levels (Tables 4-6). However, MSV were more sensitive to NE ($P < 0.0001$), PE ($P = 0.0003$), and ET-1 ($P < 0.05$) when compared with corresponding MSA across the range of physiologic transmural pressures studied. MSV were more sensitive to NE than PE ($P < 0.0001$) at intermediate and high transmural pressures (Table 4). By contrast, MSA showed similar responses to NE compared to those produced by PE.
Electric field stimulation caused frequency-dependent contractions of MSA and MSV examined, particularly at low and intermediate transmural pressures (Figure 18). In contrast to findings obtained with various agonists including NE, PE and ET-1 applied exogenously, increasing transmural pressure caused a significant rightward shift in FRCs obtained in both MSV and MSA (Figure 18 & Table 7). While high transmural pressure greatly reduced neurogenic contractions in MSV, neurogenic responses in MSA studied at high transmural pressure were almost absent. Therefore, it was not possible to determine $S_{\text{max}}$ and $S_{50}$ values reliably in MSA examined at high transmural pressure. It should be also mentioned that NE responses obtained in mesenteric vessels before and after the completion of FRCs experiment were not affected by transmural pressure increases. Comparisons of FRCs obtained in MSV with corresponding MSA at similar transmural pressure levels revealed that veins were more sensitive to EFS as indicated by the lower $S_{50}$ and greater $S_{\text{max}}$ values ($P < 0.05$).

**Effect of $\alpha_1$-AR and $\alpha_2$-AR blockade on the contractile responses to NE in pressurized rat mesenteric small veins and arteries**

In order to evaluate the relative contributions of $\alpha_1$ and $\alpha_2$-ARs to the contractile responses of mesenteric vessels to NE, mesenteric vessels were pre-treated with phentolamine, prazosin or yohimbine, and NE CRCs subsequently generated. To verify the selectivity of yohimbine to $\alpha_2$-ARs, PE CRCs were also generated in MSV and MSA in the presence of yohimbine. Antagonist application did not change the resting diameter of any vessels. Incubation with phentolamine (10 µM) or prazosin (100 nM) caused a complete abolishment of NE-induced contractions in MSV and MSA at both levels of transmural pressures studied (Figures 11 & 12). Thus, the EC$_{50}$ and E$_{\text{max}}$ values were not
determined in phentolamine or prazosin pre-treated vessels. Neither sensitivity nor the maximum contractile responses to NE were affected by yohimbine (100 nM) in MSA ($P > 0.05$) (Table 5). Similar findings were noted with PE in yohimbine pre-treated MSV and MSA (Table 4, 5). However, yohimbine caused a significant rightward shift in the NE-CRC obtained in MSV at transmural pressure of 6 mmHg ($P = 0.0005$) without affecting the maximum responses (Figure 11-A). These effects of yohimbine were not significant in MSV pressurized at 12 mmHg.

**Effect of $\alpha_2$-AR blockade on the contractile responses to clonidine in pressurized rat mesenteric small veins and arteries**

Since yohimbine antagonized NE but not PE contractile responses in MSV at intermediate transmural pressures, which suggests contribution from $\alpha_2$-ARs, additional experiments were performed with $\alpha_2$-receptor selective agonist, clonidine. Clonidine caused concentration-dependent contractions of MSV with the magnitude of contractions being reduced in the presence of yohimbine (Figure 15-A). To determine whether clonidine contracted arteries, MSA were also exposed to clonidine at intermediate transmural pressure. Interestingly, clonidine also caused concentration-dependent contractions of MSA which were not affected by yohimbine (Figure 15-B).

**Effect of ETA and ETB receptor blockade on ET-1-induced contractions of mesenteric small veins and arteries**

The contribution of ET$_A$ and ET$_B$ receptors to ET-1-induced contractions were studied in MSA and MSV pre-treated with an ET$_A$ (BQ-610, 100 nM) or an ET$_B$ (BQ-788, 100 nM) receptor antagonist, or both, and results were compared with their corresponding controls (Figures 16 & 17, Table 6). Pre-incubation with BQ-788 did not
alter ET-1 CRCs obtained in MSV or MSA. ET-1 contractile responses obtained in BQ-610 treated MSV and MSA were significantly lower than those obtained in corresponding control and BQ-788 treated vessels. Co-incubation of MSV and MSA with BQ-610 (100 nM) and BQ-788 (100 nM) resulted in a significant rightward shift in ET-1 CRCs (Table 6). In MSV, concurrent application of BQ-610 and BQ-788 caused further rightward shift in ET-1 CRCs as indicated by further decreases in $pD_2$ values compared with the values from BQ-610 treated MSV (Table 6, $P < 0.0001$). In contrast to MSV, combined application of BQ-610 and BQ-788 in MSA did not cause further changes in $pD_2$ values compared with the values obtained in the presence of BQ-610 alone. However, it is noted that at high transmural pressures (ie. 120 mmHg), responses obtained with the concentration range of $10^{-9}$ – $10^{-8}$ M ET-1 were significantly lower in MSA incubated with combined ET-1 receptor antagonists compared with values obtained in the presence of BQ-610 alone.

**Discussion**

Vascular tone is regulated by several factors including catecholamines as well as other local and circulating vasoactive substances. NE and ET-1 are considered the most important endogenous vasoconstrictors contributing to overall control of the cardiovascular system. Mesenteric veins are more sensitive to the contractile effects of exogenously applied NE (Perez-Rivera et al., 2007; Perez-Rivera et al., 2004; Sporkova et al., 2010), ET-1 (Johnson et al., 2002; Perez-Rivera et al., 2005), and sympathetic nerve stimulation (Hottenstein and Kreulen, 1987; Luo et al., 2003; Park et al., 2007) compared to arteries. The higher sensitivity of MSV to NE, ET-1 and sympathetic nerve activation compared to MSA was confirmed in the present study in pressurized
mesenteric vessels. Our study is the first to investigate postjunctional α-ARs and ET-1 receptor subtypes that contribute to the enhanced adrenergic and endothelinergic activity in rat MSV compared to MSA under conditions of changing transmural distending pressure. Additionally, our study is the first to assess the effects of elevating transmural pressure on sympathetic neurogenic contractions in cannulated pressurized mesenteric vessels. We used a functional pharmacological approach to identify α-adrenergic and ET-1 receptor subtypes involved in exogenous NE and ET-1-induced contractions of mesenteric small vessels. Our results suggest that α₁-AR and ETₐ receptors are the predominant postjunctional receptors mediating NE and ET-1-induced vasoconstriction in MSA and MSV, respectively. While α₂-adrenergic and ETₐ receptors appeared not to be involved in the contractile responses of NE and ET-1 in the artery, they contribute, in part, to NE-and ET-1-induced contractions in the vein respectively. Additionally, our findings suggest that increasing transmural pressure may impair neurotransmitters release by sympathetic nerves associated with mesenteric vessels.

**Alpha adrenergic receptors mediating NE-induced contractions of mesenteric small veins and arteries**

In the current study, we reported that MSV were more sensitive to NE than corresponding MSA and that NE CRCs obtained at intermediate transmural pressure were not changed in MSV or MSA compared to responses observed at higher pressures. These findings confirm our results reported in Chapter 1. Moreover, removing the endothelium did not affect NE (10 µM) responses obtained in mesenteric vessels studied at various levels of transmural pressure, suggesting no dilator influence of the endothelial α₂-AR. In the present study, we also found that phentolamine, a non-selective α-AR
antagonist, and prazosin, a selective $\alpha_1$-AR antagonist, both abolished the contractile responses to NE in MSV and MSA. These observations, in addition to results obtained with selective $\alpha_1$ and $\alpha_2$-AR agonists, PE and clonidine respectively, suggest that in MSV both receptor types contribute to the contractile responses of NE, a non-selective $\alpha$-AR agonist. However in experiments where the contractile responses of these agonists were obtained in MSA, NE potency was similar to that of PE while clonidine and PE responses were not affected by yohimbine. Similar findings were also reported in previous in situ (Eikenburg, 1984) and in vitro studies (Kanagy, 1997; Park et al., 2007; Perez-Rivera et al., 2007) including studies in pressurized mesenteric arteries (Buus et al., 1994). Therefore, our findings in MSA indicate that NE and PE are acting via $\alpha_1$-ARs with clonidine possibly producing its contractile effects via activation of $\alpha_1$-ARs, rather than the $\alpha_2$-ARs.

The lack of functional $\alpha_2$-ARs mediating NE-induced contractions in MSA is supported by previous studies reporting that demonstration of functional $\alpha_2$-ARs in arteries has proved difficult or inconsistent (Jarajapu et al., 2001; Silva et al., 1996) including studies in MSA (Eikenburg, 1984; Perez-Rivera et al., 2007; Sporkova et al., 2010). In agreement with our results, previous studies have also shown that $\alpha_2$-AR activation did constrict arteries studied in situ or under isometric methods; however this effect was antagonized by an $\alpha_1$-AR antagonist supporting the notion that $\alpha_2$-AR agonists used could have activated $\alpha_1$-ARs (Castillo et al., 2006; Kanagy, 1997; Molin and Bendhack, 2004; Zacharia et al., 2004). Taken together, our results combined with those of other studies support a lack of functional contractile $\alpha_2$-ARs mediating contractions to $\alpha_2$-AR agonists in MSA.
In contrast to MSA, MSV showed higher sensitivity to NE compared to PE. These results are consistent with those obtained by Gornermann et al. (2007) in porcine pulmonary veins examined under isometric methods. These findings suggest activation of $\alpha_1$- and $\alpha_2$-ARs by NE in rat MSV. Additionally, PE responses were not affected by yohimbine at any pressure level whereas NE and clonidine contractile effects were reduced by yohimbine in MSV pressurized at intermediate transmural pressure (i.e 6 mmHg). While these results suggest that NE is a largely an $\alpha_1$-AR agonist with some agonist effects on $\alpha_2$-ARs, substantial activation of $\alpha_1$-ARs by clonidine is likely. This conclusion is supported by the failure of yohimbine to completely abolish clonidine contractile responses in MSV, which may reflect a loss of selectivity of clonidine in veins (Blochl-Daum et al., 1991; Flavahan, 2005). Alternatively, functional interaction i.e. cross-talk between $\alpha$-AR subtypes may also explain findings with clonidine and yohimbine (Daly et al., 1988). Accordingly, it has recently been suggested that although selective activation of $\alpha_2$-ARs by UK 14, 304 did not cause venous constriction, it caused an additive effect when applied simultaneously with PE (Sporkova et al., 2010). Similarly, an enhanced effect of PE was produced in the presence of UK 14, 304 (Perez-Rivera et al., 2007). Therefore, the findings of these studies suggest the presence of a synergistic receptor interactions mediating $\alpha$-AR activation in MSV in which $\alpha_2$-ARs facilitate $\alpha_1$-AR coupled $\text{Ca}^{2+}$ signaling (Sporkova et al., 2010). Collectively, our findings suggest that $\alpha_2$-ARs are involved in NE and clonidine contractile responses in MSV studied at intermediate transmural pressure.

Our findings with clonidine are not in agreement with previous studies demonstrating that selective $\alpha_2$-AR agonists did not produce substantial contraction of
arteries or veins (Jarajapu et al., 2001; Zacharia et al., 2004) including studies in mesenteric vessels (Luo et al., 2003; Perez-Rivera et al., 2007; Sporkova et al., 2010). This discrepancy may due to differences in methodology used in those studies. Vessels from these studies were not pressurized compared to vessels used in the present study. Therefore pressure may have sensitized $\alpha_2$-ARs to the effect of $\alpha$-AR agonists (Buus et al., 1994). However previous studies have shown that selective activation of $\alpha_2$-ARs did not cause contraction of pressurized mouse MSV (Flavahan, 2005). An alternative explanation is that MSV in mice may express fewer $\alpha_2$-ARs than those of other species with inefficient coupling to the intracellular signaling mechanisms mediating venous constriction, which suggests species variation in the expression of $\alpha_2$-ARs subtypes or post-receptor pathway (Flavahan, 2005; Perez-Rivera et al., 2007).

A potential limitation of the present study is that the experiments were performed in mesenteric vessels with an intact endothelium. Considering that endothelial $\alpha_2$-ARs could be activated and therefore evoke vasodilators release that could counteract the constrictor effects of $\alpha_2$-AR agonists (Vanhouette, 2001), it will be important to confirm whether $\alpha_2$-AR agonists activate endothelial $\alpha_2$-ARs in MSV. However, pharmacological blockade of the endothelium using indomethacin and L-NNA as well as removal of the endothelium did not affect constrictions to $\alpha_2$-AR agonist including clonidine (Flavahan, 2005; Jarajapu et al., 2001; Perez-Rivera et al., 2007). Moreover, the present study, as well as others, shows that removing of the endothelium did not affect NE responses (Anschutz and Schubert, 2005). Taken together, our findings combined with those of other studies conducted in mesenteric vessels suggest that $\alpha_2$-
ARs are functionally expressed in MSV, but not in MSA (Perez-Rivera et al., 2007; Sporkova et al., 2010).

In the present study, it should be mentioned that in MSA studied at high transmural pressure, prazosin seemed to not affect NE contractile responses at low NE concentrations compared to phentolamine (Figure 12-B). While this finding may be of limited physiologic significance, it may suggest that at higher levels of physiologic pressure, α₂-ARs might participate in vasoconstriction induced by low concentrations of NE in the MSA. However this assumption is limited by the lack of significant antagonizing effects of yohimbine on NE, as well as clonidine CRCs obtained in MSA. Furthermore, our finding that yohimbine was ineffective in reducing NE-induced contractions in MSV at transmural pressures of 12 mmHg was not expected. A possible explanation of this finding is that α₁-ARs may become the predominate adrenoceptors at high transmural pressure and, thus NE would only activate these receptors.

**Endothelin receptors mediating ET-1-induced contractions of mesenteric small veins and arteries**

In the present study, we reported that MSV were more sensitive to ET-1 than corresponding MSA and that ET-1 CRCs obtained at intermediate transmural pressure were not changed in MSA or MSV compared to responses observed at higher pressure. These findings also confirm our observations reported in Chapter 1. Additionally, removal of the endothelium did not affect ET-1 CRCs in mesenteric vessels studied at various levels of transmural pressure, ruling out any significant influence of endothelial ET$_{B}$ receptors on net tone (data not shown). We also found that BQ-610, a selective ET$_{A}$ receptor antagonist, significantly reduced the contractile responses of ET-1 in MSV and
Moreover, the selective ET$_B$ receptor antagonist, BQ-788, did not change the slope or shape of ET-1 CRCs obtained in MSV and MSA. In contrast to the MSA, the combined application of BQ-610 and BQ-788 in MSV caused a significantly further rightward shift in ET-1 CRCs compared to those obtained with BQ-610 alone. Collectively, these observations suggest that increasing transmural pressure does not affect VSM responses to ET-1 which are mediated mainly by the ET$_A$ receptors type. In addition to ET$_A$ receptors, ET$_B$ receptors may contribute to ET-1 contractile responses in MSV.

Vasoconstrictor responses to ET-1 are mainly mediated by ET$_A$ receptors (Johnson et al., 2002; Perez-Rivera et al., 2005). Results of our study with ET-1 confirm those reported previously by others. The functional expression of ET$_B$ receptors in MSV is supported by experimental data obtained with the highly selective ET$_B$ receptor agonist, S6c, which failed to contract arteries (Johnson et al., 2002; Perez-Rivera et al., 2005). In the present study our goal was to assess vascular reactivity of mesenteric vessels to ET-1. Therefore, we did not study responses of mesenteric vessels to S6c.

Since combined ET$_A$ and ET$_B$ receptor blockade in MSV caused significantly further rightward shifts in ET-1 CRCs compared with results of BQ-610 alone, these results imply that ET$_B$ receptors are unmasked by inactivation of ET$_A$ receptors and can contribute to ET-1 contractile responses (Mickley et al., 1997). These findings were observed at both intermediate and high transmural pressure. Interestingly, our data in MSV, with the coapplication of BQ-610 and BQ-788, are different from those reported previously by others who demonstrated no greater rightward shift in the ET-1 CRCs than that caused by BQ-610 alone (Johnson et al., 2002). While these data may suggest that
ET-1 contractile responses in MSV are mediated via direct and additive activation of venous ET-1 receptors, pressure may have enhanced the expression of ET \textsubscript{B} receptors (Lindstedt et al., 2009); however, this assumption is limited by the lack of the inhibitory effect of BQ-788 on ET-1 responses. An interplay between ET \textsubscript{A} and ET \textsubscript{B} receptors may explain differences with the findings of BQ-788 alone and when combined with BQ-610 in veins (Fukuroda et al., 1996). This pharmacological receptor interaction was not observed in ET \textsubscript{B} receptor deficient rats, suggesting this interaction may require the presence of functional ET \textsubscript{B} receptors (Thakali et al., 2008). The concept of “cross-talk” has recently been explained as a component of ET-1 receptor activity that functionally affects venous endothelin receptor pharmacology (Thakali et al., 2008). Specifically, it has been suggested that ET \textsubscript{A} mediated responses vary as a function of ET \textsubscript{B} receptor activity, and vice versa. Furthermore, ET \textsubscript{B} is known as a clearance receptor that removes plasma ET-1 from the circulation (Fukuroda et al., 1994). However, venous and arterial VSM cells express both ET \textsubscript{A} and ET \textsubscript{B} receptors. As such, pharmacologic endothelial receptor interaction may not account for the findings observed with simultaneous blockade of both ET \textsubscript{A} and ET \textsubscript{B} receptors suggesting the potential involvement of downstream signaling pathways mediated by functional ET \textsubscript{B} receptors (Fukuroda et al., 1996; Thakali et al., 2008). Further studies are required to elucidate endothelial receptor interactions, as well as signaling mechanisms mediating the apparent cross-talk.

As mentioned above, the activation of endothelial cell ET \textsubscript{B} receptors induces release of dilators. When these receptors are blocked, an enhancement of ET-1 contractile responses may be observed. However, in the present study we did not observe an enhanced activity of ET-1 in the presence of BQ-788 or when the vascular
endothelium was mechanically removed. These findings suggest minimal ET-1-mediated NO and/or other endothelial relaxing factors release in mesenteric vessels.

In contrast to MSV, and in agreement with other studies (Mickley et al., 1997; Perez-Rivera et al., 2005), experiments conducted in MSA revealed that simultaneous blockade of both ET\textsubscript{A} and ET\textsubscript{B} receptors by BQ-788 and BQ-610 did not cause further inhibition of ET-1 responses than that produced by the blockade of ET\textsubscript{A} receptors alone. This finding combined with the results obtained with BQ-788 suggests that functional ET\textsubscript{B} receptors may not be expressed in rat MSA (Gray et al., 2000b; Mickley et al., 1997). This assumption is further supported by previous studies conducted in unpressurized and pressurized MSA showing that S6c produces no, or limited, contractile responses, respectively (Gray et al., 2000b; Perez-Rivera et al., 2005; Sharifi and Schiffirin, 1996). Additionally, in the present study, although the “cross-talk” phenomenon is likely to occur in MSV, modification of ET\textsubscript{A} receptor-mediated mechanisms in MSA seems unlikely. Collectively, these observations suggest that under normal physiological conditions, vasoconstrictor responses to ET-1 in resistance vessels are mainly mediated by ET\textsubscript{A} receptors.

**Transmural pressure changes and neurogenic contractions induced by EFS**

Since the splanchnic vascular bed including the mesenteric circulation holds up to one third of total blood volume with the majority being found in veins, small changes in venomotor tone could result in a significant shift of blood toward the heart affecting both cardiac output and blood pressure (Pang, 2000). As such, in case of decreases in sympathetic nerve activity, the vascular-mediated increases in blood pressure could be due to an augmentation in venous tone. The present study in pressurized mesenteric
vessels showed that MSV are more sensitive than corresponding MSA to sympathetic nerve stimulation as FRCs in MSV were left shifted and greater than corresponding MSA. These findings are consistent with those reported previously by others who also demonstrated lower $S_{50}$ values in veins compared with those obtained in arteries, and suggested that an augmentation in sympathetic nerve activity in vivo would produce greater contraction of veins than arteries (Hottenstein and Kreulen, 1987; Luo et al., 2003; Park et al., 2007). Our study is the first to confirm these observations in mesenteric vessels studied under conditions of changing transmural pressures, and to report marked pressure effects on neurogenic responses.

In the present study, we observed that direct activation of sympathetic nerve terminals on mesenteric vessels by EFS were significantly impaired with an elevation of transmural pressure and almost blunted at higher pressures, especially in MSA. Interestingly these findings were opposite to what we observed in chapter 1, as well as with the exogenous application of NE in this chapter, where increasing pressure did not change NE CRCs obtained in MSV and MSA. Our results are not in agreement with another study showing that increasing transmural pressure enhanced neurogenic responses in MSA (Rummery et al., 2007). In the current study, VSM and endothelium functions were preserved in all vessels as indicated by responses to NE, acetylcholine and BK, suggesting increasing transmural pressure did not impair vascular function. Interpretation of our findings with EFS when transmural pressure is increased is difficult considering studies investigating EFS responses in both veins and arteries at physiological pressures are lacking. Pressure-induced myogenic tone and its associated changes may affect neurotransmitter release (Dr. WG Wier, personnel communication).
As such, when transmural pressure is low greater neurogenic contractile responses are observed, which may be due to an elevation in neurotransmitters release. In rabbit ear artery studied with isometric methods, sympathetic nerve-induced contractions as well as NE release decreased in segments that developed myogenic tone compared to those not shown myogenic tone (Owen et al., 1983). MSA used in the study of Rummery et al (2007) were of second order which did not decrease in diameter during equilibration period, indicating they did not possess myogenic tone. Thus the absence of myogenic tone may account for the discrepancy in the findings observed in MSA used in our study compared to those reported by others who showed that neurogenic contractions were not reduced in MSA. Interestingly, NE release is increased in unpressurized MSV and MSA from hypertensive rat compared with corresponding sham vessels, indicating an alteration in sympathetic neural function in hypertension (Luo et al., 2004; Luo et al., 2003).

In summary, the present study has shown that $\alpha_1$-ARs and ET$_A$ receptors are the predominant postjunctional receptors mediating contractions induced by exogenous NE and ET-1 in isolated pressurized mesenteric vessels, respectively. Activation of VSM $\alpha_2$-ARs and ET$_B$ receptors appeared to be involved in responses to NE and ET-1 in MSV, respectively but not in MSA, suggesting the lack of functional $\alpha_2$-ARs and ET$_B$ receptors in MSA. The existence of a specific subtype of $\alpha$-ARs and ET-1 receptors that mediate constriction of MSV may be important in the selection of appropriate drugs for the management of altered venous function reported in some disease conditions such as hypertension or congestive heart failure (Bohm et al., 2008; Gay et al., 1986; Gray et al., 2000b; Martin et al., 1998). The current study has also shown that increasing transmural pressure significantly reduced neurogenic responses in mesenteric vessels. Further
studies evaluating α-ARs mediating constrictions to sympathetic neurogenic stimulation and examining neurotransmitters release in the mesenteric circulation under conditions of changing transmural distending pressure, which may more closely approach physiological conditions, are warranted.
Figure 11. Effect of phentolamine (10 µM), prazosin (100 nM) and yohimbine (100 nM) on the contractile responses to norepinephrine (NE) in mesenteric small veins pressurized at 6 mmHg (A) and 12 mmHg (B). * reduced sensitivity of veins to NE by yohimbine at 6 mmHg; phentolamine and prazosin abolished NE responses at both pressure levels. Data are expressed as mean ± SEM (\(P < 0.05\)).
Figure 12. Effect of phentolamine (10 µM), prazosin (100 nM) and yohimbine (100 nM) on the contractile responses to norepinephrine (NE) in mesenteric small arteries pressurized at 60 mmHg (A) and 120 mmHg (B). $n$, number per group in parenthesis. Phentolamine and prazosin abolished NE responses at both pressure levels. Data are expressed as mean ± SEM.
A

% Contraction

\[ \text{NE (M)} \]

Control \((n = 15)\)
Prazosin \((n = 5)\)
Phentolamine \((n = 5)\)
Yohimbine \((n = 5)\)

B

% Contraction

\[ \text{NE (M)} \]

Control \((n = 15)\)
Prazosin \((n = 6)\)
Phentolamine \((n = 5)\)
Yohimbine \((n = 4)\)
Figure 13. Effect of yohimbine (100 nM) on phenylephrine (PE)-induced contractions of mesenteric small veins pressurized at 6 mmHg (A) and 12 mmHg (B) and mesenteric small arteries pressurized at 60 mmHg (C) and 120 mmHg (D). $n$, number per group in parenthesis. Data are expressed as mean ± SEM.
Figure 14. Comparison of norepinephrine (NE) and phenylephrine (PE) concentration response curves obtained in rat mesenteric small veins at 6 mmHg (A) and 12 mmHg (B); and small arteries at 60 mmHg (C) and 120 mmHg (D). * higher sensitivity of veins to NE compared to PE. $n$, number per group in parenthesis. Data are expressed as mean ± SEM ($P < 0.05$).
Figure 15. Effect of yohimbine (1 µM) on clonidine contractile responses of mesenteric small veins and arteries pressurized at 6 mmHg (A) and 60 mmHg (B), respectively.

* control vs. yohimbine. $n$, number per group in parenthesis. Data are expressed as mean ± SEM ($P < 0.05$).
A

[Graph showing the effect of Clonidine on contraction with Control and Yohimbine groups.]

B

[Graph showing the effect of Clonidine on contraction with Control and Yohimbine groups.]
Figure 16. Concentration-response curves of endothelin-1 (ET-1) obtained in mesenteric small veins pressurized at 6 mmHg (A) and 12 mmHg (B) in the absence (closed circles) and presence of ET<sub>A</sub> receptor selective antagonist (BQ-610, 100 nM, open circles), or ET<sub>B</sub> receptor selective antagonist (BQ-788, 100 nM, closed triangles) or both (open triangles). * control, BQ-788 vs. BQ-610 and both; † BQ-610 vs. both. n, number per group in parenthesis. Data are expressed as mean ± SEM (P < 0.05).
Figure 17. Concentration-response curves of endothelin-1 (ET-1) obtained in mesenteric small arteries pressurized at 60 mmHg (A) and 120 mmHg (B) in the absence (closed circles) and presence of ET\textsubscript{A} receptor selective antagonist (BQ-610, 100 nM, open circles), or ET\textsubscript{B} receptor selective antagonist (BQ-788, 100 nM, closed triangles) or both (open triangles). * control, BQ-788 vs. BQ-610 and both; † BQ-610 vs. both at ET-110\textsuperscript{-8} – 10\textsuperscript{-9} M. n, number per group in parenthesis. Data are expressed as mean ± SEM (P < 0.05).
Figure 18. Comparison of frequency response curves for neurogenic contractions of mesenteric small veins (A) and arteries (B) obtained at low (closed circles), intermediate (open circles) and high (closed triangles) transmural pressures. Data are expressed as mean ± SEM. * neurogenic contractions at low vs. intermediate and high transmural pressures. Data are expressed as mean ± SEM ($P < 0.05$).
Table 4. $E_{\text{max}}$ and $pD_2$ values obtained from norepinephrine (NE) and phenylephrine (PE) concentration-response curves at intermediate and high transmural pressures in the absence and presence of $\alpha_1$ and $\alpha_2$-AR antagonists in mesenteric small veins isolated from normal male rats.

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<th>Small vein (6 mmHg)</th>
<th></th>
<th>Small vein (12 mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$pD_2$</td>
<td>$E_{\text{max}}$ (%)</td>
<td>$pD_2$</td>
</tr>
<tr>
<td>NE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.3 ± 0.1 *† (5)</td>
<td>66.0 ± 2.7 (5)</td>
<td>8.2 ± 0.1 * (5)</td>
</tr>
<tr>
<td>Phentolamine, 10 µM</td>
<td>ND (4)</td>
<td>ND (4)</td>
<td>ND (4)</td>
</tr>
<tr>
<td>Prazosin, 100 nM</td>
<td>ND (4)</td>
<td>ND (4)</td>
<td>ND (4)</td>
</tr>
<tr>
<td>Yohimbine, 100 nM</td>
<td>7.6 ± 0.1 (5)</td>
<td>57.6 ± 4.6 (5)</td>
<td>8.1 ± 0.2 (5)</td>
</tr>
<tr>
<td>PE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.3 ± 0.3 (4)</td>
<td>64.8 ± 2.9 (4)</td>
<td>7.6 ± 0.1 (4)</td>
</tr>
<tr>
<td>Yohimbine, 100 nM</td>
<td>7.4 ± 0.2 (4)</td>
<td>65.6 ± 1.6 (4)</td>
<td>7.4 ± 0.2 (4)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SE; $n$ = number per group in parenthesis. * compared with PE control at corresponding transmural pressure, † compared with NE / Yohimbine at 6 mmHg. $pD_2$, negative logarithm of the agonist producing half- maximal constriction; $E_{\text{max}}$, maximum constriction; ND, not determined.
Table 5. $E_{\text{max}}$ and $pD_2$ values obtained from norepinephrine (NE) and phenylephrine (PE) concentration-response curves at intermediate and high transmural pressures in the absence and presence of $\alpha_1$ and $\alpha_2$-AR antagonists in mesenteric small arteries isolated from normal male rats.

<table>
<thead>
<tr>
<th></th>
<th>Small Artery (60 mmHg)</th>
<th>Small artery (120 mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$pD_2$</td>
<td>$E_{\text{max}}$ (%)</td>
</tr>
<tr>
<td>NE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.8 ± 0.1 (15)</td>
<td>75.8 ± 1.9 (15)</td>
</tr>
<tr>
<td>Phentolamine, 10 µM</td>
<td>ND (5)</td>
<td>ND (5)</td>
</tr>
<tr>
<td>Prazosin, 100 nM</td>
<td>ND (5)</td>
<td>ND (5)</td>
</tr>
<tr>
<td>Yohimbine, 100 nM</td>
<td>6.5 ± 0.2 (5)</td>
<td>73.4 ± 2.8 (5)</td>
</tr>
<tr>
<td>PE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.9 ± 0.02 (4)</td>
<td>78.1 ± 1.3 (4)</td>
</tr>
<tr>
<td>Yohimbine, 100 nM</td>
<td>6.7 ± 0.2 (4)</td>
<td>72.3 ± 1.8 (4)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM; $n$, number per group in parenthesis. $pD_2$, negative logarithm of the agonist producing half-maximal constriction; $E_{\text{max}}$, maximum constriction; ND, not determined.
Table 6. $E_{\text{max}}$ and $pD_2$ values obtained from endothelin-1 (ET-1) concentration-response curves at intermediate and high transmural pressures in the absence and presence of ET_A (BQ-610, 100 nM) and ET_B (BQ-788, 100 nM) receptor antagonists in mesenteric small veins and arteries isolated from normal male rats.

<table>
<thead>
<tr>
<th></th>
<th>Small vein</th>
<th>Small artery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$pD_2$</td>
<td>$E_{\text{max}}$ (%)</td>
</tr>
<tr>
<td><strong>6 mmHg</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (11)</td>
<td>10.0 ± 0.1</td>
<td>89.1 ± 1.2</td>
</tr>
<tr>
<td>BQ-610 (8)</td>
<td>8.9 ± 0.3 *</td>
<td>80.1 ± 2.4 *</td>
</tr>
<tr>
<td>BQ-788 (7)</td>
<td>9.8 ± 0.2 †</td>
<td>89.2 ± 1.6 †</td>
</tr>
<tr>
<td>Both (7)</td>
<td>8.2 ± 0.1 *†¥</td>
<td>83.8 ± 2.7</td>
</tr>
<tr>
<td><strong>12 mmHg</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (10)</td>
<td>9.9 ± 0.1</td>
<td>90.5 ± 0.9</td>
</tr>
<tr>
<td>BQ-610 (6)</td>
<td>9.1 ± 0.1 *</td>
<td>86.0 ± 2.4 *</td>
</tr>
<tr>
<td>BQ-788 (7)</td>
<td>9.5 ± 0.1 †</td>
<td>89.6 ± 0.8 †</td>
</tr>
<tr>
<td>Both (6)</td>
<td>8.2 ± 0.2 *‡¥</td>
<td>87.1 ± 1.1</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM; n, number per group in parenthesis. $P < 0.05$, within vessel and pressure * compared with control, † compared with BQ-610, ‡ compared with BQ-788; †¥ compared with corresponding value within vessel and between pressures. $pD_2$, negative logarithm of the agonist producing half-maximal constriction; $E_{\text{max}}$, maximum constriction.
Table 7. \( S_{\text{max}} \) and \( S_{50} \) values obtained from frequency-response curves at low, intermediate and high transmural pressures in mesenteric small veins and arteries isolated from normal male rats.

<table>
<thead>
<tr>
<th></th>
<th>Small vein</th>
<th>Small artery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( S_{50} ) (Hz)</td>
<td>( S_{\text{max}} ) (%)</td>
</tr>
<tr>
<td>2 mmHg</td>
<td>0.8 ± 0.3 †</td>
<td>78.9 ± 2.2 †</td>
</tr>
<tr>
<td>6 mmHg</td>
<td>0.5 ± 0.2 †</td>
<td>53.8 ± 4.4 †</td>
</tr>
<tr>
<td>12 mmHg</td>
<td>0.3 ± 0.1</td>
<td>20.8 ± 1.3 *</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM; \( n \), number per group in parenthesis. \( P < 0.05 \), * compared with values within vessel and between pressures, † compared with corresponding values in small artery; \( S_{50} \), half-maximal response; \( S_{\text{max}} \), maximal response; ND, not determined.
CHAPTER 3

EFFECTS OF TRANSMURAL PRESSURE ELEVATION ON ENDOTHELium-DEPENDENT AND ENDOTHELium-INDEPENDENT VASODILATION IN ISOLATED RAT MESENTERIC VEINS
Introduction

The basal vascular tone of MSV in vivo is under the control of neural, humoral, metabolic and myogenic factors. Accordingly, changes in vessel diameter by these factors, subsequent to flow or pressure changes, play an essential role in the regulation of vasomotor tone. The vascular endothelium releases vasoactive factors including NO, prostacyclin, and EDHF. These factors cause endothelium-dependent vasodilation and has been the subject of reviews (Martinez-Leon et al., 2003; Matrougui et al.; 1997; Lagaud et al.; 1999; Gunduz et al.; 2008, Christensen et al.; 2007; Thorsgaard et al., 2003). The production of these relaxing factors is triggered by the shear stress of blood flow and receptor-specific activation by agonists such as acetylcholine and BK.

Endothelium-dependent vasodilation has been widely investigated in the high pressure arm of the cardiovascular system, the arteries, with some studies showing the presence of endothelium dysfunction in mesenteric arteries of healthy rats studied in vitro at a high level of transmural pressure (Christensen et al., 2007; Gunduz et al., 2009; Gunduz et al., 2008). An impaired endothelial function has also been reported in some disease conditions in arteries, such as hypertension (Huang et al., 1998; Koller and Huang, 1994; Lockette et al., 1986; Schnackenberg et al., 1998) including studies in rat mesentery (Gunduz et al., 2009; Matrougui et al., 1997). Findings of these in vitro studies suggest that an increased level of transmural distending pressures may be harmful to the vascular regulatory function of the endothelium via mainly reducing NO function, which has been considered as a major factor in the pathogenesis of hypertension.

Functional responses of the endothelium of MSV may also differ when examined at various levels of transmural pressure. Interestingly, cultured endothelial cells prepared
from human umbilical cord veins produce less NO when exposed to high transmural pressure (Hishikawa et al., 1995). However, to date no study has investigated the effects of changing transmural distending pressure on endothelial function of a large capacitance MSV or the nature of endothelium-derived vasodilators released in response to an increased blood flow or receptor-selective agonists such as BK. Endothelium-dependent vasodilation induced by agonists and flow may be mediated by distinct endothelium-derived vasodilator factors. In this regard, it has been suggested that in MSA flow could activate another vasodilation mechanism (NO-mediated vasodilation) compared to those activated by agonists such as acetylcholine (EDHF-mediated vasodilation) (Chang et al., 2002; Takamura et al., 1999; Thorsgaard et al., 2003). However, it is not known how endothelium-dependent dilation is being activated in MSV.

It is thought that agonist-induced endothelium-dependent vasodilation is lower in veins than that in arteries, and that NO release in response to agonists as well as shear stress is lower in veins compared to arteries (Hamilton et al., 1997; Muir et al., 2010; Rubira et al., 2007; Yang et al., 1991). Whether this speculation is acceptable at the level of splanchnic vasculature is unclear. The identity of EDHF in MSV remains elusive. However, it is believed that in resistance arteries EDHF acts via activation of K\(^+\) channels in VSM, particularly Ca\(^{2+}\)-activated K\(^+\) channels (K\(_{Ca}\)) (Doughty et al., 1999; Edwards et al., 1998; Giachini et al., 2009; Lagaud et al., 1999). Three subtypes of K\(_{Ca}\) channels have been identified in the vascular wall including large (BK\(_{Ca}\)), intermediate (IK\(_{Ca}\)), and small (SK\(_{Ca}\)) conductance Ca\(^{2+}\)-activated K\(^+\) channels with specific cellular and subcellular localization (Feletou and Vanhoutte, 2009; Jackson, 2005). The inhibition of BK\(_{Ca}\) and SK\(_{Ca}\) channels located in the VSM and endothelial cells, respectively, would be
considered as a unique characteristic of the non-NO and non-prostanoid-type of vasodilation and hyperpolarization induced by receptor-specific agonists such as acetylcholine (Doughty et al., 1999; Edwards et al., 1998; Giachini et al., 2009; Lagaud et al., 1999). EDHF-induced relaxation in human large veins examined under isometric tension has been associated with activation of BK_{Ca} channels (Martinez-Leon et al., 2003). Conversely, it has been reported that BK_{Ca} channels play no role in vasodilation induced by BK in pig large conduit pulmonary veins studied with isometric methods (Aschner et al., 2002). Additionally, functional BK_{Ca} channels are not expressed in pressurized mouse MSV (Xu et al., 2011). These differences in the activity of BK_{Ca} channels may be explained by vascular differences as well as species variation (Aschner et al., 2002).

Elucidation of the signaling mechanisms responsible for vasoactive responses at the level of the splanchnic capacitance vasculature is essential to our understanding of small vein physiology. Investigating endothelium-dependent vasodilation in MSV is critical for understanding the regulatory mechanisms of the mesenteric circulation, considering that small veins hold a significant amount of the total blood volume (Pang, 2001; Pang, 2000). Until recently, most studies investigating venous vascular reactivity have been conducted in large conduit veins with virtually no studies examining vasoreactivity in pressurized MSV. In the present study, we investigated the effects of increasing transmural distending pressure on endothelial function in cannulated pressurized MSV. We only evaluated endothelium-dependent vasodilation evoked by BK in MSV. We also attempted to investigate the key vasodilator substances involved in endothelium-dependent vasodilation induced by BK in MSV. In order to assess the
reliance of BK on the endothelium, we also tested the effect of the removal of the endothelium on BK-induced vasodilation.

**Materials and Methods**

See General Materials and Methods (pages 45 - 49).

**Experimental protocols**

Third order MSV (ID, 341.9 ± 8.1 µm at 6 mmHg, n = 90) were used in this study. In all experiments, the viability of the MSV for study was assessed by contractile responses to NE (1 µM), while the integrity of the endothelium was assessed by dilator responses to BK (0.1 µM) in PGF2α (1 µM) preconstricted veins. Vessels that were unable to react adequately to NE, PGF2α, and BK or demonstrated pressure leaks were discarded. In total, 120 veins we isolated for study, with 30 veins being discarded either for failing viability testing with NE, or responding weakly to PGF2α and BK.

Before each experiment, all vessels were allowed to equilibrate for a period of 30 min at the desired transmural pressure (i.e. 6 mmHg or 12 mmHg). No significant difference was noted with the baseline ID of small veins across experiments. It should also be mentioned that only one vessel per rat was used for each experimental protocol throughout the study.

Following an appropriate equilibration period, BK (10^{-10} - 10^{-6} M) SNP (10^{-10} - 10^{-4} M), and SNAP (10^{-9} - 10^{-4} M) CRCs were obtained in MSV with transmural pressures fixed at 6 and 12 mmHg. BK, SNP, and SNAP were applied in increasing and known concentrations to the superfusing solution. Each concentration was applied for at least 3
min to allow adequate time to reach a steady-state response and data were continuously recorded by use of Diamtrak® software. A non-cumulative application of the agonists was used with a 10 min washout period observed between applications of each agonist. BK CRCs (10^{-10} - 10^{-6} M) in the absence and presence of L-NNA (100 µM), indomethacin (1 µM) (Johnson et al., 2002) or both; and responses to BK (10^{-7} M) in the absence and presence of paxilline (BK_{Ca} blocker, 500 nM) (Xu et al., 2011), apamin (SK_{Ca} blocker, 300 nM) (Lagaud et al., 1999) or both were obtained in MSV preconstricted with PGF2α (1 µM). Further experiments were conducted in the presence of L-NNA plus paxilline, and L-NNA plus paxilline plus apamin. L-NNA and indomethacin were added individually, or combined, to the bath for at least 20 min prior to generating BK CRCs. Paxilline and apamin were also added individually, or combined, to the bath for at least 20 min prior to testing vasodilation-induced by BK (10^{-7} M). At the end of all experiments, the superfusion solution was changed to a calcium free kreb’s solution that also contained 2 mM EGTA. Mesenteric veins were incubated with calcium free kreb’s solution for 20 min to obtain the passive diameter of each vein at the levels of transmural pressures studied (i.e 6 and 12 mmHg). In a separate set of experiments, the effect of the removal of the endothelium on BK-induced vasodilation of MSV was also evaluated.

Results

Contractile responses of mesenteric small veins to PGF2α

Time control experiments showed that application of PGF2α (1 µM) for 30 min resulted in series of contractions and relaxations of MSV particularly at higher transmural
pressures, making generation of cumulative CRCs to BK difficult. Therefore, a non-cumulative approach was followed and only one BK CRC was obtained in each individual vein in the absence and presence of the different inhibitors. PGF2α (1 μM) applied for 5 to 10 min caused sustained and steady contractions that persisted for an additional 5 min. A washout period of 10 min was allowed to attain the initial base line diameter. The percentage of contraction induced by PGF2α at 6 mmHg (41.9 ± 2.9 %) was not significantly different from corresponding values obtained at 12 mmHg (34.3 % ± 2.7) (Table 8). Additionally, incubation of MSV with different inhibitors did not affect the contractile responses of MSV to PGF2α at any level of transmural pressure examined.

Relaxation responses of mesenteric small veins to BK and NO donor compounds

SNP and SNAP

The endothelium-dependent vasodilator, BK, induced concentration-dependent (10^{-10} - 10^{-6} M) dilations in MSV (Figure 19-A). The control E_{max} and pD_{2} values obtained with BK at intermediate transmural pressure were not significantly different compared with corresponding values attained at high transmural pressures (Table 9). Likewise, direct stimulation of VSM with SNP (10^{-10} - 10^{-4} M) and SNAP (10^{-9} - 10^{-4} M) dilated PGF2α-preconstricted MSV in a concentration-dependent manner (Figure 19-B, C). The E_{max} and pD_{2} values attained with SNP were similar in MSV studied at intermediate and high transmural pressures (Table 9). Similar results were observed with SNAP. Comparison of SNP and SNAP-induced vasodilation of MSV studied at various levels of transmural pressure showed that SNP was more potent than SNAP (Table 9, P = 0.0022).
Effects of NOS, COX inhibition and endothelium removal on BK-induced relaxation of mesenteric small veins

Incubation with L-NNA (100 µM) and/ or indomethacin (1 µM) did not change the resting diameter of mesenteric veins. The $E_{\text{max}}$ and $pD_2$ values obtained with BK in the presence of L-NNA and/ or indomethacin at intermediate transmural pressure were not significantly different from corresponding values obtained at high pressure (Table 9). The $pD_2$ values for BK CRCs obtained at intermediate transmural pressure were significantly reduced by indomethacin (Table 9, $P = 0.0027$). However, indomethacin did not significantly change $pD_2$ values obtained with BK at high transmural pressure. There were no differences in the $E_{\text{max}}$ values of BK obtained in the presence of indomethacin at any level of transmural pressure compared with their control values (Table 9). On the other hand, incubation with L-NNA significantly reduced vasodilation evoked by BK at intermediate transmural pressure (Figures 20-A & 22-A). The combination of L-NNA plus indomethacin did not cause greater inhibition of BK-induced relaxation than did L-NNA alone (Table 9). Endothelium removal completely abolished BK-induced vasodilation (Figure 23), without impairing NE-induced contractions of MSV. At high transmural pressure, L-NNA alone or combined with indomethacin significantly decreased the $E_{\text{max}}$ in response to BK application in MSV ($P = 0.0054$) (Table 9). In the presence of L-NNA, there was a rightward shift of BK CRCs obtained at high transmural pressure, but it did not reach the significance level. Point to point analysis showed that L-NNA applied alone ($P = 0.0007$) or with indomethacin ($P = < 0.0001$) at high pressure significantly reduced BK responses obtained with all concentrations (Figure 21-A, C).
Effects of large and small conductance \(K_{Ca}\) channels blockade on BK-induced relaxation of mesenteric small veins

As demonstrated above, neither L-NNA nor the combined application of L-NNA and indomethacin completely abolished BK-induced relaxation (Figures 20-A, C & 21-A, C). These L-NNA and indomethacin resistant components of BK could be due to the release of other endothelial relaxing factors such as EDHF. The \(K_{Ca}\) channels have been suggested to mediate EDHF-induced relaxation of blood vessels. Since increasing transmural pressure did not affect responses of MSV to BK obtained in the absence or presence of different inhibitors, we decided to investigate the possible contribution of key \(K_{Ca}\) channels to vasodilation evoked by BK at intermediate transmural pressure (i.e 6 mmHg). Incubation with \(BK_{Ca}\) blocker, paxilline (500 nM), \(SK_{Ca}\) blocker, apamin (300 nM), or both for a period of 20 min did not change the resting diameter of mesenteric veins. Similarly, no change in mesenteric veins diameter was observed after incubation with L-NNA combined with either apamin or paxilline or both. Blockade of \(SK_{Ca}\) or \(BK_{Ca}\) with apamin \(P = 0.0009\) and paxilline \(P = 0.0001\) significantly reduced BK-induced vasodilation of MSV, respectively (Figure 23-B). We further examined the inhibitory effect of the combination of apamin plus paxilline on BK-induced vasodilation. The combined application of apamin and paxilline did reduce BK vasodilatory responses \(P = 0.0004\), but it did not cause greater inhibition of vasodilation compared with responses obtained in the presence of either blocker (Figure 23-B). Since L-NNA, apamin, and paxilline applied individually resulted in a significant inhibition of vasodilation evoked by BK (Figure 23-A, B) and in order to investigate whether NOS inhibition along with \(SK_{Ca}\) and \(BK_{Ca}\) channels blockade would abolish BK-induced relaxation...
vasodilation, we examined the BK-induced vasodilation response in MSV incubated with these three inhibitors. Accordingly, further reduction in BK-induced vasodilation was observed with this combination compared with responses obtained when apamin and/or paxilline were applied (Figure 23-B).

**Discussion**

Our study is the first to explore the effects of BK, and NO donors (SNP and SNAP) in cannulated, pressurized MSV. Our findings indicate that BK, SNP and SNAP cause marked dilation in preconstricted MSV that was not changed as transmural distending pressure is increased. Our study also demonstrates that mechanisms responsible for vasodilation responses in MSV involve the activation of eNOS, BK\(_{Ca}\) and SK\(_{Ca}\) channels. However, there was a residual effect of BK in vessels treated with NOS inhibitor, SK\(_{Ca}\), or BK\(_{Ca}\) channels blocker. The remaining vasodilation was also observed with the combined application of apamin, paxilline and L-NNA despite causing further reduction in endothelium-dependent dilations induced by BK. On the other hand the involvement of the COX pathway was noticeable only at intermediate transmural pressures with some variations in its effect. However when combined with L-NNA, indomethacin did not further change BK CRCs than did L-NNA alone at any pressure level. Finally, endothelium removal abolished BK-induced dilation confirming the endothelium-dependent action of BK in rat mesentery.
Modulation of endothelium-dependent and -independent vasodilation by transmural pressure elevation

The present study has shown that in MSV exposed to high transmural pressure, endothelium-dependent and endothelium-independent vasodilation evoked by BK and SNP respectively, were not impaired. I also attempted to assess another commonly used endothelium-dependent vasodilator such as acetylcholine, but acetylcholine did not dilate MSV. However, it was possible to evaluate the effect of increasing transmural distending pressure on vasodilation induced by another type of NO donor, SNAP. Similarly, an elevated level of transmural pressure did not alter SNAP CRCs compared with responses obtained at intermediate pressure confirming that endothelium-independent vasodilation did not change with transmural pressure increases. To the best of our knowledge, no study has evaluated endothelium-dependent and endothelium-independent vasodilation under conditions of changing transmural pressures in MSV making comparisons of our results with others challenging. Moreover, there are few available vascular reactivity data collected in large conduit veins to which we can compare our findings in small capacitance veins (Muir et al., 2010).

Our results provide a new physiologic difference regarding the reactivity of veins compared with arteries. Previous in vitro studies conducted in corresponding pressurized MSA (Christensen et al., 2007; Gunduz et al., 2009; Gunduz et al., 2008; Matrougui et al., 1997) as well as other vasculatures (Huang et al., 1998; Koller and Huang, 1994; Paniagua et al., 2000; Schnackenberg et al., 1998) have shown that endothelium-dependent vasodilation evoked by flow as well as by receptor-selective agonists is significantly reduced with increases of transmural distending pressure. These studies
have also reported that endothelium-independent vasodilation induced by the NO donor, SNP or SNAP, were reduced by transmural distending pressure elevation. The possible mechanisms involved in the inhibition of flow-evoked vasodilation in MSA examined at high transmural pressure are the activation of endothelin receptors and an increased formation of superoxide (Christensen et al., 2007). However, in the present study, the lack of pressure effect on BK as well as NO donor evoked vasodilation suggests that in PGF2α-preconstricted MSV the activity of endothelial NOS and VSM guanylyl cyclase is not altered respectively. Similarly, a recent study examined large conduit human saphenous veins under isometric tension and found that endothelium-dependent and endothelium-independent vasodilation to acetylcholine and SNP, respectively, in veins preconstricted with a sub-maximal dose of PE, were not reduced in hypertensive subjects (Muir et al., 2010). Our findings with BK-induced vasodilation at high transmural distending pressure did not enable us to make further investigations regarding potential mechanisms associated with increasing transmural pressure that could inhibit vasodilation (Christensen et al., 2007; Huang et al., 1998). Alternatively, we further investigated possible endothelium-derived factors participating in vasodilation induced by BK in MSV.

**Contribution of NO and prostacyclin to endothelium-dependent vasodilation evoked by BK**

Cyclooxygenase inhibition by indomethacin did not reduce vasodilation obtained with any BK concentrations applied at high transmural pressure whereas at intermediate transmural pressure indomethacin application caused a rightward shift in BK CRCs
without affecting the maximal response. The failure of indomethacin alone to reduce vasodilation induced by BK at high transmural pressure was not likely to be due to their inability to block prostacyclin production as revealed by experiments conducted at intermediate pressure. It should also be noted that at intermediate transmural pressure although indomethacin increased $EC_{50}$ of BK, responses observed at high concentrations of BK (i.e $E_{max}$) were not changed. This may due to differences in the nature of vasodilators released by the endothelium in response to various BK concentrations (Bolz et al., 1999). At lower concentrations, BK may evoke the release of several dilators including NO, prostacyclin, and EDHF whereas at higher concentrations it may stimulate the release of relaxing factors other than prostacyclin such as NO and EDHF. While this assumption may explain the vasodilation observed at intermediate pressure, the lack of indomethacin effect at high transmural pressure may not involve the same mechanisms. Alternatively, at higher transmural pressure factors other than prostacyclin are possibly involved in vasodilation induced by BK and COX inhibition may have led to enhanced release of NO and/ or EDHF. Accordingly, it has been suggested in human saphenous vein the existence of a reciprocally inhibitory interaction between NO and prostacyclin where in the absence of either NO or prostacyclin, levels of the other vasodilator mediator could be raised sufficiently to cause vasodilation (Barker et al., 1996).

In contrast to indomethacin, NOS inhibition by L-NNA reduced BK CRCs at both levels of transmural pressure studied, although changes in $EC_{50}$ did not reach the significant level at high pressure. The reduction in BK responses by L-NNA was observed at all BK concentrations. Simultaneous inhibition of the activity of both NOS and COX may enhance the reduction in endothelium-dependent vasodilation. However,
the combination of L-NNA and indomethacin caused no further changes in BK CRCs compared to those obtained with L-NNA alone. These findings suggest that endothelium-dependent vasodilation evoked by BK is mediated largely by NO. However, NO did not fully account for the dilation induced by BK since it was not completely inhibited by L-NNA, suggesting a contribution from other endothelial relaxing factors. It should be mentioned that the combination of L-NNA and indomethacin seems to cause greater inhibition of vasodilation induced by low concentrations of BK compared to responses obtained with L-NNA alone. Collectively, these results indicate that in MSV vasodilation-induced by BK is mediated in part by prostacyclin and NO, and NO, at intermediate and high transmural pressures, respectively. The dilator response to BK which persisted in the presence of L-NNA and indomethacin may be attributable to EDHF (Lagaud et al., 1999).

Our findings with L-NNA and indomethacin applied separately, or together, in MSV at intermediate transmural pressure are comparable with other studies conducted in MSA pressurized at 80 mmHg which showed that acetylcholine-induced vasodilation was reduced but not completely abolished by L-NAME and/or indomethacin (Lagaud et al., 1999). However in recent studies conducted in MSA kept at 50 or 80 mmHg, acetylcholine-induced vasodilation was not affected by treatment with either asymmetric dimethylarginine (ADMA) or indomethacin (Christensen et al., 2007; Thorsgaard et al., 2003). This discrepancy in NOS inhibition in MSA in the above studies may be explained by the differing choice of the NOS inhibitors. However, ADMA was able to significantly reduce flow-mediated vasodilation of MSA suggesting that ADMA is effective in inhibiting NOS activity (Christensen et al., 2007; Thorsgaard et al., 2003).
Therefore, the observed discrepancy in small arteries may be related to differences in the vasodilator factors released by the endothelium in response to either flow or receptor-specific agonists with NO and EDHF mediating mainly flow- and agonist-induced vasodilation, respectively (Christensen et al., 2007; Thorsgaard et al., 2003).

**Contribution of K\textsubscript{Ca} to endothelium-dependent vasodilation evoked by BK**

The vasodilator responses to BK which persisted in the presence of L-NNA and indomethacin indicate the involvement of another endothelium-derived vasodilator factors, most likely EDHF (Martinez-Leon et al., 2003). Although the identity of EDHF in MSV remains unidentified; it is believed that EDHF acts via activation of K\textsuperscript{+} channels in VSM, particularly K\textsubscript{Ca} channels. Two main K\textsubscript{Ca}, the VSM BK\textsubscript{Ca} and endothelial cells SK\textsubscript{Ca} channels, have been suggested to mediate endothelium-dependent vasodilation in several vascular preparations but whether these K\textsubscript{Ca} channels are involved in BK-induced dilations of MSV has not previously been reported.

To investigate the effects of VSM BK\textsubscript{Ca} and endothelial cells SK\textsubscript{Ca} channels in mediating vasodilation responses to BK, MSV were individually treated with paxilline and apamin, respectively. Apamin and paxilline significantly reduced the maximum dilation in response to BK by 41.9 % and 47.9 %, respectively. The co-application of apamin and paxilline caused a reduction of 46.9 % in BK-induced dilation which did not differ from corresponding values obtained with either blocker alone. These results indicate that stimulation of endothelial cells by BK activates apamin-sensitive K\textsuperscript{+} channels (ie. SK\textsubscript{Ca} channels) and the resultant K\textsuperscript{+} efflux hyperpolarizes the adjacent VSM cells possibly via activation of BK\textsubscript{Ca}, suggesting that EDHF to be endothelium-derived
K⁺ ions, and that paxilline-sensitive K⁺ channels (ie. VSM BK<sub>Ca</sub>) channels are involved in EDHF-mediated hyperpolarization and subsequent vasodilation induced by BK. Moreover, our experiments provide evidence that apamin- and paxilline-sensitive K⁺ channels appear to contribute equally to BK-induced dilations of MSV. These findings are not in agreement with other venous studies demonstrating that SK<sub>Ca</sub> had no role in endothelium-dependent dilation induced by BK (Martinez-Leon et al., 2003). With regard to BK<sub>Ca</sub> channels, our results confirm earlier findings in human large conduit veins studied with isometric tension showing that BK<sub>Ca</sub> blockade resulted in significant inhibition of vasodilation of human large conduit veins (Gruhn et al., 2002; Martinez-Leon et al., 2003).

The VSM BK<sub>Ca</sub> constitute a subgroup of K<sub>Ca</sub> channels that serve as a negative feedback mechanism restoring membrane potential and limiting intracellular Ca<sup>2+</sup> increases resulted from agonists-induced vasoconstriction, as well as myogenic reactivity, with the net result being membrane hyperpolarizing and vasodilation (Xu et al., 2011; Yang et al., 2009). Activation of BK<sub>Ca</sub> channels has been suggested to occur via the NO–cGMP pathway and thus BK<sub>Ca</sub> channels could modulate the vasodilator response to both exogenous and endogenous receptor-mediated NO release in isolated arteries (Bolotina et al., 1994; Gruhn et al., 2002). Accordingly, our results show that combined application of paxilline plus L-NNA caused further inhibition of BK-evoked vasodilation by 73.9 % suggesting a synergistic action of NOS inhibition and BK<sub>Ca</sub> blockade by L-NNA and paxilline on vasodilation in MSV, respectively. Conversely, it has been reported that in pig large conduit veins studied with isometric methods, BK<sub>Ca</sub> channels play no role in endothelium-dependent vasodilation induced by BK (Aschner et al., 2002). Additionally
in mouse MSV, functional BK$_{Ca}$ channels do not exist (Xu et al., 2011). Taken together, these findings indicate the crucial role of BK$_{Ca}$ and SK$_{Ca}$ channels in mediating BK-induced vasodilation of MSV, although vascular bed (skeletal vs. pulmonary vs. splanchnic) and species (human vs. pig vs. rat vs. mouse) variations may exist.

In the present study interpretation of the absence of further inhibition of vasodilation with combined application of apamin and paxilline in MSV is made more difficult by the lack of other studies using pressurized MSV. Our findings suggest that the role of apamin and paxilline in mediating BK-induced dilation may have been compensated for by the enhanced release of other vasodilators such as NO. Evidence of compensatory vasodilator mechanisms has been proposed in some studies (Martinez-Leon et al., 2003). At the level of the mesenteric circulation the increased release of NO, when EDHF is inhibited, may represent a mechanism that maintains the control of vascular tone by the endothelium. Accordingly when L-NNA was included, combined application of apamin and paxilline caused further reduction in the BK-induced dilation by up to 72.9 % compared to 46.9 % caused by the application of apamine and paxilline only. The residual effect of BK observed, when combined application of L-NNA, paxilline and apamin was tested, suggests an effect from other ion channels. It should be mentioned that there are other types of K$^+$ channels which may be involved in vasodilation including voltage-gated K$^+$ channels (K$_V$), ATP-sensitive K$^+$ channels (K$_{ATP}$), and inwardly rectifying K$^+$ channels (K$_{IR}$) that can be inhibited by 4-aminopyridine, glibenclamide, and barium, respectively. Of these, the VSM K$_{IR}$ and Na$^+$/K$^+$ ATP-ase channels, which are not affected by apamin or paxilline, have been shown to modulate endothelium-dependent vasodilation in large conduit veins (Martinez-Leon et
al., 2003). The effects of these ion channels in mediating endothelium-dependent vasodilation were not investigated in the present study.

In further contrast to our findings in pressurized MSV, experimental evidence in small arteries indicates that iberiotoxin-sensitive $K^+$ channels (ie. $BK_{Ca}$) are not involved in acetylcholine-induced dilation, which is completely dependent on the activation of $SK_{Ca}$ channels (Giachini et al., 2009; Lagaud et al., 1999). Furthermore in arteries, apamin and charybdotoxin applied simultaneously with a NOS inhibitor reduced dilation evoked by acetylcholine (Thorsgaard et al., 2003). In another study in the presence of indomethacin and L-NNA, the combined application of apamin and charybdotoxin, but not apamin and iberiotoxin, was effective in reducing vasodilation induced by acetylcholine (Edwards et al., 1998). These results in arteries suggest the involvement of $SK_{Ca}$ but not $BK_{Ca}$ channels in mediating vasodilation induced by acetylcholine; however, a contribution from other endothelial and VSM cell $K^+$ channels to vasodilation is possible (Edwards et al., 1998; Thorsgaard et al., 2003). A potential explanation of this discrepancy in the type of $K^+$ channels mediating agonist-evoked endothelium dependent dilation in arteries and veins is that the activation of different $K^+$ channels may be determined by the type of the blood vessels (vein vs. artery) which may add another difference in vascular reactivity of veins compared with arteries.

In summary, our results provide new insight regarding the reactivity of small veins. The present study suggests that in rat MSV, BK-evoked vasodilation is not affected by an elevation of transmural pressure and that NO and EDHF mediate endothelium-dependent vasodilation in MSV. The participation of prostacyclin in mediating vasodilation induced by BK may be important at intermediate pressure.
EDHF-induced vasodilation involves activation of $K_{Ca}$ channels sensitive to apamin and paxilline. Therefore, EDHF-mediated vasodilation of MSV by $K_{Ca}$ channels may provide a novel mechanism for the regulation of the endothelium-dependent vasodilation and consequently the physiological regulation of venous tone. In the current study, all experiments were carried out under no flow condition; however the function of the vascular endothelium is modulated by shear stress exerted by flowing blood. Flow-dependent vasodilation has been studied in several arteries, but not veins, including the mesenteric vasculature (Christensen et al., 2007; Gunduz et al., 2008; Thorsgaard et al., 2003). Further studied are needed to evaluate the effect of shear stress on vascular reactivity of MSV under conditions of changing transmural pressures.
Figure 19. Concentration-response curves of bradykinin (BK, A), SNP (B) and SNAP (C) obtained in mesenteric small veins preconstricted with PGF2α, 1 µM, at intermediate (6 mmHg, closed circles) and high transmural pressures (12 mmHg, open circles). n refers to the number of vessels used in each protocol. Data are expressed as mean ± SEM.
Figure 20. Concentration-response curves of bradykinin (BK) obtained in mesenteric small veins preconstricted with PGF2α, 1 µM, at intermediate transmural pressure (6 mmHg) in the absence (closed circles) and presence of L-NNA, 100 µM (A, open circles), indomethacin, 1 µM (B, indo, open circles) or both (C, open circles). * control vs. treatment. n refers to the number of vessels used in each protocol. Data are expressed as mean ± SEM (P < 0.05).
Figure 21. Concentration-response curves of bradykinin (BK) obtained in mesenteric small veins preconstricted with PGF2α, 1 µM, at high transmural pressure (12 mmHg) in the absence (closed circles) and presence of L-NNA, 100 µM (A, open circles), indomethacin, 1 µM (B, indo, open circles) or both (C, open circles). * control vs. treatment. n refers to the number of vessels used in each protocol. Data are expressed as mean ± SEM (P < 0.05).
Figure 22. Effects of L-NNA (100 µM), indomethacin (Indo, 1 µM) or both on endothelium-dependent dilation in response to bradykinin (BK) in mesenteric small veins preconstricted with PGF2α, 1 µM, at intermediate (A, 6 mmHg) or high (B, 12 mmHg) transmural pressure. *control vs. treatment. n refers to the number of vessels used in each protocol. Data are expressed as mean ± SEM (P < 0.05).
Figure 23. Effects of A) L-NNA, 100 µM, indomethacin (Indo, 1 µM) or both; B) apamin (300 nM), paxilline (500 nM), or both; paxilline + L-NNA; apamin + paxilline + L-NNA; and the removal of the endothelium on endothelium-dependent dilation in response to bradykinin (BK, 100 nM) in mesenteric small veins pressurized at intermediate transmural pressure (6 mmHg) and preconstricted with PGF2α (1 µM). $n$ refers to the number of vessels used in each protocol. * vs. control; † vs. paxilline; ‡ vs. apamin + paxilline. Data are expressed as mean ± SEM ($P < 0.05$).
Table 8. Contractile responses of rat mesenteric small veins to PGF2α, 1 µM, prior to application (control) and in the presence of various inhibitors.

<table>
<thead>
<tr>
<th></th>
<th>6 mmHg</th>
<th>12 mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PGF2α</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>41.9 ± 2.9</td>
<td>34.3 ± 2.7</td>
</tr>
<tr>
<td>L-NNA</td>
<td>36.4 ± 6.9</td>
<td>37.5 ± 6.5</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>33.6 ± 5.5</td>
<td>25.1 ± 2.6</td>
</tr>
<tr>
<td>L-NN + Indomethacin</td>
<td>34.9 ± 8.4</td>
<td>38.6 ± 2.9</td>
</tr>
<tr>
<td><strong>PGF2α</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>51.4 ± 1.9</td>
<td>ND</td>
</tr>
<tr>
<td>Paxilline</td>
<td>53.2 ± 3.1</td>
<td>ND</td>
</tr>
<tr>
<td>Apamin</td>
<td>46.2 ± 6.6</td>
<td>ND</td>
</tr>
<tr>
<td>Apamin + Paxilline</td>
<td>50.7 ± 7.7</td>
<td>ND</td>
</tr>
<tr>
<td>Paxilline + L-NNA</td>
<td>49.8 ± 4.7</td>
<td>ND</td>
</tr>
<tr>
<td>Paxilline + Apamin + L-NNA</td>
<td>49.02 ± 10.5</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM; n = number in parenthesis; ND, not done.
Table 9. $E_{\text{max}}$ and $pD_2$ values for bradykinin (BK), SNP and SNAP in rat mesenteric small veins pressurized at intermediate and high transmural pressures.

<table>
<thead>
<tr>
<th></th>
<th>6 mmHg</th>
<th></th>
<th>12 mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$E_{\text{max}}$ (%)</td>
<td>$pD_2$</td>
<td>$E_{\text{max}}$ (%)</td>
</tr>
<tr>
<td><strong>BK</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>106.5 ± 7.1</td>
<td>8.4 ± 0.1</td>
<td>103.9 ± 9.0</td>
</tr>
<tr>
<td>L-NNA</td>
<td>76.9 ± 6.6 *</td>
<td>7.6 ± 0.1 *</td>
<td>71.9 ± 4.3 *</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>107.0 ± 9.9</td>
<td>7.5 ± 0.1 *</td>
<td>80.0 ± 11.8</td>
</tr>
<tr>
<td>L-NNA + Indomethacin</td>
<td>65.6 ± 5.9 *</td>
<td>7.3 ± 0.1 *</td>
<td>55.2 ± 8.3 *</td>
</tr>
<tr>
<td><strong>SNP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNP</td>
<td>96.9 ± 5.2</td>
<td>7.6 ± 0.3</td>
<td>100.5 ± 6.5</td>
</tr>
<tr>
<td><strong>SNAP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNAP</td>
<td>105.4 ± 2.0</td>
<td>6.2 ± 0.2 †</td>
<td>101.9 ± 2.0</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM; for “n” values see figure 19-22. $P < 0.05,*$ compared with corresponding control values; † compared with corresponding SNP values within a pressure. $pD_2$, negative logarithm of the agonist producing half-maximal relaxation; $E_{\text{max}}$, maximum relaxation.
General discussion

The physiological role of veins in the body is different from that of arteries. In general, the blood circulates continuously from and to the heart through large arteries, small arteries and arterioles; capillaries; venules, small veins and large veins, respectively. This movement of blood, by arteries and veins, is crucial to ensure tissue supply of oxygen and nutrients and the distribution of various substances including hormones, as well as the return of un-oxygenated blood to the heart. Various vascular beds are classified in the body including cerebral, pulmonary, coronary, splanchnic, skeletal and renal. The splanchnic vasculature represents the most important active capacitance bed in the body.

The net level of vasomotor tone within a segment of the circulation is controlled mainly, according to its physiological requirement, by neural, humoral and local intrinsic myogenic factors. Approximately 70 % of the total blood volume is contained in the veins (Pang, 2001). As such, changes in venous capacitance play a major role in the control of venous return and cardiac output. At anytime and under any hemodynamic conditions, cardiac output must equal venous return indicating the reliance of cardiac output on venous return (Greenway and Lautt, 1986). Changes in venous tone have important implications for the control of venous function and overall cardiovascular homeostasis. Reduced venous capacitance can alter venous return, cardiac output and peripheral resistance and thus blood pressure by way of autoregulation (Greenway and Lautt, 1986). Altered venous capacitance has been reported in some cardiovascular diseases in rats such as hypertension (Martin et al., 1998; Simon, 1976).
A better understanding of the factors that contribute to the regulation of venous function should provide insight into the relationship between changes in transmural distending pressure and vascular reactivity. This thesis investigated the role of transmural distending pressure changes on the vascular reactivity of MSV with additional experiments performed in corresponding MSA in order to gain a greater understanding of integrated venous function of capacitance vessels studied at physiological pressures. The experiments described in this thesis in third order mesenteric vessels were designed to evaluate (i) venous myogenic reactivity to increasing transmural distending pressure, and to obtain mechanistic information regarding the modulation of venous tone by the endothelium and the contribution of key cellular and intracellular events in pressure induced venomotor tone, (ii) the effects of changing transmural distending pressure on contractile responses to NE, ET-1 and vascular sympathetic nerve activation, and postjunctional α-adrenergic and ET-1 receptor subtypes mediating NE-and ET-1-induced contractions in pressurized vessels, (iii) whether increasing transmural distending pressure is associated with impairment of endothelial dilator function, and (iv) key vasodilator substances involved in agonist-induced endothelium-dependent vasodilation.

In chapter 1, I described the generation of pressure induced-myogenic reactivity in MSV and MSA and identified key mechanisms responsible for the development of myogenic reactivity. My results from Chapter 1 experiments indicate that MSV are able to develop myogenic reactivity. The presence of myogenic tone, in the MSV suggests that this mechanism participates in the regulation of venous return and cardiac output, when changes in transmural pressure occur. Venous myogenic reactivity was not modulated by the endothelium as indicated by the lack of significant changes in the
magnitude of myogenic reactivity following pharmacological blockade by L-NNA and mechanical removal of the endothelium. Therefore, these observations, supported by those reported by Brookes and Kaufman (2003), provide direct evidence that basal release of endothelial-derived dilators, in particular NO, appear not to contribute to myogenic reactivity in pressurized MSV. Local mesenteric blood pressure at the base of mesenteric venous arcade is 10 to 15 mmHg while at the base of mesenteric arterial arcade is 60 to 75 mmHg (Fenger-Gron et al., 1995). The absence of high distending pressures and pulsatile flow conditions in the physiologic pressure range of most veins could explain a reduced need for constitutive dilator release in these vessels. In contrast, arteries see high pressures and as such need greater dilator release to counteract myogenic reactivity. There is great vascular heterogeneity in the function of veins and arteries of various organs which, at a physiological level, may importantly reflect adaptive or protective mechanisms. Thus findings obtained in one vascular bed may not directly apply to another. For instance, skeletal muscle vessels experience large pressure changes and develop myogenic reactivity which is greater than that exhibited by other vessels, such as cerebral and mesenteric (Kotecha and Hill, 2005; Sun et al., 1994; Sun et al., 1992). Thus continuous endothelium-derived vasodilators release in skeletal muscle vascular bed may be required to counteract pressure-induced myogenic reactivity. In skeletal muscle venules, venous myogenic reactivity in response to transmural pressure elevation is modulated by endothelium-derived dilators, only in the range of 2 to 5 mmHg, ie. at lower levels of transmural pressure (Dornyei et al., 1996). In skeletal muscle arteries, the endothelium is also able to modulate pressure-induced myogenic reactivity (Sun et al., 1994). Additionally, relative to MSA, splenic small arteries do not
develop myogenic reactivity unless vessels are first pre-treated with NOS inhibitor, L-NAME, suggesting an increased release of NO when transmural pressure is increased (Brookes and Kaufman, 2003). Thus, NO release, in response to transmural pressure elevations, in splenic arteries would allow an increase in transmural pressure to be transmitted to the microvasculature, thus raising the driving force of blood extravasation. Taken together, these differences suggest that the endothelium appears to have an organ-specific function in the modulation of pressure-induced myogenic reactivity.

My results in third order MSA suggest no role of endothelium-derived vasodilators in the regulation of myogenic reactivity. While these findings extend to those reported in previous studies examining similar size MSA (Sun et al., 1992), they are also opposite to others reported in fourth order MSA (Brookes and Kaufman, 2003). This difference in MSA between our study and the study of Brookes and Kaufman (2003) may be due to differences in vessels size used in both studies. Thus, the ability of the endothelium to release vasodilators, when transmural pressure is increased, may vary between vessels within a given vascular bed, giving that the strength of the myogenic reactivity increases as the diameter of the vessels decreases (Sun et al., 1994, Sun et al., 1992).

It is also possible that high intravascular pressures could have attenuated dilator responses in the mesenteric vessels in our study (Gunduz et al., 2008). However, in our study the integrity of the endothelium to agonist evoked dilator release remained intact. Additionally, lack of modulatory effects of the endothelium on myogenic reactivity was noted at lower pressures in both vessels. Collectively, these mixed results suggest that the strength of endothelial modulation or counter-regulation of myogenic reactivity is
determined by the vascular bed, possibly being more important in those vessels that experience large pressure changes (Dornyei et al., 1996; Sun et al., 1994), and also possibly varying between species studied (Nguyen et al., 1999).

My results from experiments conducted in Chapter 1 also showed that mechanisms underlying myogenic reactivity involved activation of cell membrane L-type VOCCs and intracellular downstream molecules such as PKC and Rho kinase. While there was a large contribution of PKC and Rho kinase to venous myogenic reactivity, the role of L-type VOCCs was partial. Evidence for a weak role of L-type VOCCs in mediating venous myogenic reactivity was also reported in large conduit veins (Loufrani et al., 1999), suggesting that these ion channels may act similarly in all veins. Findings with L-type VOCCs in MSV were different to those obtained in corresponding MSA which showed that activation of these ion channels is largely responsible for the development of myogenic reactivity. These differences in the role of L-type VOCCs in myogenic reactivity in MSV and MSA may suggest differences in membrane potential ($E_m$) between these vessels or that MSV have fewer L-type VOCCs. However, MSV and MSA have similar resting $E_m$ and KCL-induced depolarization, as well as similar expression of L-type VOCCs (Thakali et al., 2010). A possible reason for this apparent functional discrepancy is that MSV may rely on other mechanisms for increasing intracellular Ca$^{2+}$ followed increases in transmural distending pressure. In fact, an increased intracellular Ca$^{2+}$ inactivates venous L-type VOCCs (Thakali et al., 2010). Taken together, these findings suggest that depolarization of venous smooth muscle cells, by pressure elevations, causes an increase in intracellular Ca$^{2+}$ level that is largely independent of L-type VOCCs activation. As such, pharmacological agents, such as
nifedipine, that are known to block L-type VOCCs in VSM may have a limited role in the regulation of venous tone and its subsequent hemodynamic changes. Our study did not compare the relationship of venous myogenic characteristics across differing sizes of mesenteric veins. It is unknown if pressure induced myogenic reactivity increases in mesenteric veins with the order of generation of the vessel as has been found in mesenteric arteries (Sun et al., 1992).

In Chapter 1, I also described the interaction effects of increasing transmural pressure on the reactivity of mesenteric vessels to NE and ET-1. Pressure-induced myogenic reactivity observed in MSA and MSV can enhance the reactivity of these vessels to contractile responses of NE (Dunn et al., 1994) and possibly ET-1. Such interactions were absent in the mesenteric vessels in our study, thus limiting any potential exaggerating effects of myogenic reactivity on the vasoconstrictor actions of NE and ET-1. Interactions between pressure-induced myogenic reactivity and vasoactive agents may be important in organs or tissues that have high metabolic demands such as skeletal muscles (Dornyei et al., 1996). Hindquarter skeletal muscle vasculature is characterized by steep concentration-resistance and pressure-diameter relationships in response to vasoactive agonists and transmural distending pressures, respectively (Bund, 2000; Dornyei et al., 1996; Sun et al., 1994). Therefore, a greater resistance in the venular network of this vascular bed would be required to maintain the integrity of the microvasculature and facilitate venous return in response to exercise, postural changes, and hormones (Metting et al., 1989). The degree of pressure changes experienced in vivo in the distal mesenteric arcade appears to be less, and the pressure gradient shallower, than those in the hindquarter skeletal muscle vasculature as it branches from the femoral...
artery (Bund, 2000; Fronek and Zweifach, 1975). Therefore, the presence of a less pronounced (magnitude and rate of gain) myogenic mechanism in the mesenteric arcade may be necessary to effectively autoregulate blood flow in this organ when compared to the hindquarter gracilis muscle vasculature (Dornyei et al., 1996; Fronek and Zweifach, 1975; Karibe et al., 1997; Sun et al., 1994). Additionally, the mesenteric bed has a high level of sympathetic innervation with nerve density being greater in the veins given their reduced layers of VSM (Li et al., 2010). As such, interactions between myogenic reactivity and neurogenic influences that produce enhanced venoconstrictor tone in the MSV may be detrimental to cardiovascular homeostasis.

Interestingly, in Chapter 2, I showed that elevations of transmural pressure caused a marked impairment in neurogenic contractions in mesenteric vessels. This finding provides evidence that neurotransmission may be dependent on the level of transmural pressure at which mesenteric vessels are studied. Sympathetic nerve activity has a tonic baseline level of activity that is adjusted up and down in response to a variety of afferent inputs (Malpas, 2010). These adjustments occur rapidly in response to changes in blood pressure and subsequent myogenic reactivity. Therefore, moment-to-moment adjustments of sympathetic input to mesenteric vessels operating against a background of myogenic tone could conceivably offer the best means to maintain tight regulatory control over the balance of venous return and capacitance function in a vascular bed where small changes in venoconstrictor tone have large hemodynamic consequences. At the moment in vitro studies have not been extensive enough to form any overall picture of the possible effects of increasing transmural pressures on neurogenic contractions in mesenteric vessels. Additionally, the effects of changing transmural distending pressure on neurotransmitters
release (an obvious area for future work) have not been explored. One potential reason for not being able to detect and/or measure neurotransmitters in isolated pressurized vessels is the small tissue size. Unlike pressure myography techniques which enabled us to only pressurize one vessel at a time, studies evaluating different kinds of neurotransmitters released by sympathetic nerve activation have been done mainly by stimulating a number of unpressurized vessels in the same testing bath in order to collect substantial amount of neurotransmitters that could be measured. Another explanation is the sample size for measurement of neurotransmitters. The capacity of the myograph tissue bath chamber is approximately 5 ml, which is approximately 5 times the size of the tissue bath used to study unpressurized vessels (Luo et al., 2004). In an attempt to overcome these obstacles, I tried to use vessels larger than 2 mm length as well as reduce the volume of the tissue bath by using silicon blocks designed in our laboratory. While it was possible to use larger vessels, it was not possible to reduce the volume of the tissue bath to smaller than 1.5 ml. Consequently, our laboratory was able to detect NE released by EFS using HPLC system; however, it was not able to reproduce these findings consistently. It is encouraged to figure out a way in which measurement of neurotransmitters could be possible in mesenteric vessels studied at physiological pressures.

In Chapter 2, I also addressed the effects of increasing transmural pressure on the activity of post-junctional α-ARs (α₁ and α₂) and ET-1 (ETₐ and ET₆) receptors mediating NE and ET-1 contractile responses, respectively. The majority of studies characterizing these receptor subtypes have been conducted in unpressurized vessels. Increases in transmural distending pressure can enhance the expression of ET₆ receptors.
and possibly other receptor subtypes in VSM (Lindstedt et al., 2009). Up-regulation of 
$ET_B$ receptors is also observed in some cardiovascular diseases such as hypertension 
(Fink et al., 2007). Had $ET_B$ receptors up-regulation occurred at high transmural 
pressures in our study, an enhanced contraction of mesenteric vessels to ET-1 would have 
been seen. However this was not the case. Mesenteric vessels used in our study were 
exposed to high transmural pressure for a maximum period of two hours. Thus responses 
to acute pressure elevations may not reflect its potential role in up-regulating post-
junctional receptor subtypes, such $ET_B$ (Lindstedt et al., 2009). Using a functional 
pharmacological approach, my results in Chapter 2 suggest that in contrast to MSA, MSV 
also express functional $\alpha_2$-adrenergic and $ET_B$ receptors.

To the best of our knowledge, there have been no studies examining the effects of 
changing transmural distending pressure on vasodilator responses in isolated pressurized 
MSV. As discussed earlier, dilators release may be more important in arteries compared 
to veins considering the high transmural pressure levels that arteries experience. 
However, my results from Chapter 3 experiments showed that BK, SNP and SNAP 
caused dilations of MSV comparable to those reported with acetylcholine, SNP and 
SNAP in MSA (Christensen et al., 2007; Gunduz et al., 2008). Although it has been 
suggested that acute increases in transmural pressure in MSA can impair endothelium-
dependent and endothelium-independent vasodilation (Christensen et al., 2007; Gunduz 
et al., 2008), my results in Chapter 3 in MSV indicate the opposite. Differences in the 
protocols used may account for this discrepancy. Gunduz et al. (2008) studied the 
responses to acetylcholine and SNP at three different transmural distending pressures, 
ie.50, 80, and 120 mmHg, on each vessel used. Christensen et al. (2007) evaluated the
effects of elevating transmural pressure from 50 mmHg to 120 mmHg for 1 hour before resetting the pressure to 50 mmHg on vasodilation induced by increased flow, acetylcholine and SNAP. Time related effects, therefore, cannot be ruled out as a cause of endothelial dysfunction in these studies. In our study, the effects of intermediate (ie. 6 mmHg) and high transmural pressure (ie. 12 mmHg) on vasodilation evoked by BK, SNP and SNAP were evaluated in separate vessels. My results from Chapter 3 suggest that endothelial dysfunction did not occur in normal rat MSV exposed to acute increases in high transmural pressure. Changes in cellular pathways occurred during chronic exposure to high levels of transmural pressure may indicate otherwise. In pathologic conditions, such as hypertension, there is evidence of increased production of superoxide anions, thus reducing NO bioavailability and impairing endothelium-dependent vasodilation in arteries (Versari et al., 2009).

In summary, the findings presented in this thesis have provided important insights into integrated venous function of MSV studied under conditions of changing transmural pressure. MSV develop myogenic tone that appeared to be largely mediated by downstream molecules such as PKC and Rho kinase, but in part, by the L-type VOCCS. Interactions between myogenic tone and physiologically relevant vasoactive agents such as NE and ET-1 did not occur in MSA and MSV. Our results provide evidence for the impairment of neurogenic contractions with increasing of transmural pressures. Furthermore, characterization of postjunctional receptor subtypes revealed a predominant involvement of α1 and ET\textsubscript{A} receptors in the contractile responses of NE and ET-1 respectively. Evidence for the expression of functional α2 receptors mediating NE and clonidine contractile responses was also observed in MSV. Interactions between ET\textsubscript{A} and
ET$_B$ receptors seem also to occur in pressurized MSV suggesting that ET$_B$ receptors might participate in contractile responses of ET-1. Finally, the present thesis suggests the lack of the effects of high pressure on endothelium-dependent and endothelium-independent vasodilation in MSV and that endothelium-dependent vasodilation responses induced by BK is mediated, in part, by NO, prostacyclin and EDHF. Future studies exploring differences in mechanisms of pressure-induced myogenic reactivity in veins compared with arteries as well as the relationship between changes in transmural distending pressure and vascular reactivity in certain cardiovascular diseases marked by altered venous function, such as hypertension, are of relevance for circulatory physiology and may reveal potential pharmacological targets that could be of therapeutic value.


Johansson B, Mellander S. Static and dynamic components in the vascular myogenic response to passive changes in length as revealed by electrical and mechanical recordings from the rat portal vein. Circ.Res. 1975; 36: 76-83.


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Park J, Galligan JJ, Fink GD, Swain GM. Differences in sympathetic neuroeffector transmission to rat mesenteric arteries and veins as probed by in vitro continuous amperometry and video imaging. J.Physiol. 2007; 584: 819-834.


Rummery NM, Brock JA, Pakdeechote P, Ralevic V, Dunn WR. ATP is the predominant sympathetic neurotransmitter in rat mesenteric arteries at high pressure. J.Physiol. 2007; 582: 745-754.


Waggoner WG, Genova SL, Rash VA. Kinetic analyses demonstrate that the equilibrium assumption does not apply to [125I] endothelin-1 binding data. Life Sci. 1992; 51: 1869-1876.


APPENDICES

APPENDIX- I SOURCES OF MATERIALS

Acetonitrile…………………………….Caledon laboratories chemicals, Georgetown, ON
Acetylcholine……………………………………..Sigma- Aldrich, Oakville, ON
Ammonium hydroxide…………………………..Sigma- Aldrich, Oakville, ON
Apamin…………………………………………….TOCRIS biosciences, Ellisville, Missouri
Bradykinin…………………………………………Sigma- Aldrich, Oakville, ON
BQ-610………………………………………….....American Peptide Company, Sunnyvale, CA
BQ-788…………………………………………...American Peptide Company, Sunnyvale, CA
Calcium Chloride…………………………Caledon laboratories chemicals, Georgetown, ON
Chelerythrine…………………………………….LC Laboratories, Woburn, MA
Clonidine………………………………………….Sigma- Aldrich, Oakville, ON
DMSO………………………………………………Sigma- Aldrich, Oakville, ON
EGTA………………………………………………Sigma- Aldrich, Oakville, ON
ET-1………………………………………………American Peptide Company, Sunnyvale, CA
Ethanol………………………………………………Caledon laboratories chemicals, Georgetown, ON
Gas tank (95% O₂ and 5% CO₂)……………………..Linde Canada Ltd, Guelph, ON
HCL (1M)………………………………………………Fisher scientific, Nepean, ON
Indomethacin……………………………………..Sigma- Aldrich, Oakville, ON
L-NNA………………………………………………Sigma- Aldrich, Oakville, ON
Magnesium chloride………………………….Caledon laboratories chemicals, Georgetown, ON
Nifedipine……………………………………….Sigma- Aldrich, Oakville, ON
Norepinephrine………………………………….Sigma- Aldrich, Oakville, ON
Paxilline………………………………………….TOCRIS biosciences, Ellisville, Missouri
Phentolamine…………………………………….Sigma- Aldrich, Oakville, ON
Phenylephrine…………………………………….Sigma- Aldrich, Oakville, ON
Potassium Chloride………………………….Caledon laboratories chemicals, Georgetown, ON
Prazosin………………………………………….Sigma- Aldrich, Oakville, ON
Sodium bicarbonate…………………………Caledon laboratories chemicals, Georgetown, ON
Sodium chloride……………………………..Caledon laboratories chemicals, Georgetown, ON
Sodium phosphate…………………………Caledon laboratories chemicals, Georgetown, ON
SNAP………………………………………………Cayman chemical, Ann Arbor, Michigan
SNP………………………………………………..Sigma- Aldrich, Oakville, ON
Y-27632………………………………………….Ascent scientific, Princeton, NJ
Yohimbine……………………………………….Sigma- Aldrich, Oakville, ON
**APPENDIX- II SOLUTION**

**Krebs’ Physiological Salt Solution**

**Calcium containing solution**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>117</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>4.7</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>2.5</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>1.2</td>
</tr>
<tr>
<td>Sodium phosphate</td>
<td>1.2</td>
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<tr>
<td>Sodium bicarbonate</td>
<td>25</td>
</tr>
<tr>
<td>Glucose</td>
<td>11</td>
</tr>
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</table>

pH to 7.35-7.40 with 95% O2 and 5% CO2

**Calcium free solution**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration (mmol/L)</th>
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</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>117</td>
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<tr>
<td>Potassium chloride</td>
<td>4.7</td>
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<td>Sodium phosphate</td>
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<tr>
<td>Sodium bicarbonate</td>
<td>25</td>
</tr>
<tr>
<td>Glucose</td>
<td>11</td>
</tr>
<tr>
<td>EGTA</td>
<td>2</td>
</tr>
</tbody>
</table>

pH to 7.35-7.40 with 95% O2 and 5% CO2
### APPENDIX- III SUMMARY OF AGONISTS, ANTAGONISTS AND INHIBITORS USED IN THIS THESIS

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration</th>
<th>Targeted pathway</th>
</tr>
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<tbody>
<tr>
<td>Acetylcholine</td>
<td>1.0 µM</td>
<td>Activates endothelial muscarinic receptor</td>
</tr>
<tr>
<td>Apamin</td>
<td>300 nM</td>
<td>Small conductance calcium-activated potassium channels inhibition</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>1.0 mM (Stock solution)</td>
<td>Activates endothelial bradykinin receptor</td>
</tr>
<tr>
<td>BQ-610</td>
<td>100 nM</td>
<td>Selective ET$_A$ receptors blockade</td>
</tr>
<tr>
<td>BQ-788</td>
<td>100 nM</td>
<td>Selective ET$_B$ receptors blockade</td>
</tr>
<tr>
<td>Chelerythrine</td>
<td>2.5 µM</td>
<td>Protein kinase C inhibition</td>
</tr>
<tr>
<td>Clonidine</td>
<td>10 µM (Stock solution)</td>
<td>$\alpha_2$-adrenergic activation</td>
</tr>
<tr>
<td>ET-1</td>
<td>100 µM (Stock solution)</td>
<td>Non-selective ET-1 receptors activation</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>1.0 µM</td>
<td>Non-selective cyclooxygenase inhibition</td>
</tr>
<tr>
<td>KCL</td>
<td>120 mM</td>
<td>Change membrane potential and evoke membrane depolarization</td>
</tr>
<tr>
<td>L-NNA</td>
<td>100 mM</td>
<td>Nitric oxide synthase inhibition</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>1.0 µM</td>
<td>L-type voltage-operated calcium channels blockade</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>10 µM</td>
<td>Alpha adrenergic receptors activation</td>
</tr>
<tr>
<td>Paxilline</td>
<td>500 nM</td>
<td>Large conductance calcium-activated potassium channels inhibition</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>10 µM</td>
<td>Non-selective alpha adrenergic receptors blockade</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>10 µM (Stock solution)</td>
<td>$\alpha_1$-adrenergic activation</td>
</tr>
<tr>
<td>Prazosin</td>
<td>0.1 µM</td>
<td>$\alpha_1$-adrenergic blockade</td>
</tr>
<tr>
<td>SNAP</td>
<td>10 µM (Stock solution)</td>
<td>Nitric oxide donor that activates guanylate cyclase</td>
</tr>
<tr>
<td>SNP</td>
<td>10 µM (Stock solution)</td>
<td>Nitric oxide donor that activates guanylate cyclase</td>
</tr>
<tr>
<td>Y-27632</td>
<td>3 µM</td>
<td>Rho kinase inhibition</td>
</tr>
<tr>
<td>Yohimbine</td>
<td>100 nM &amp; 1 µM</td>
<td>$\alpha_2$-adrenergic blockade</td>
</tr>
</tbody>
</table>