MYOGENIC AND ENDOTHELIAL PROPERTIES
OF THE MOUSE SEPTAL ARTERY
IN HEALTH AND DISEASE.

by
Amy Hoi-Mel Lui
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Department of **Pharmacology & Therapeutics**

The University of British Columbia
Vancouver, Canada

Date **April 27, 2001**
ABSTRACT

In this thesis I examined vascular responses of the mouse ventricular septal artery to increases in intravascular pressure and pertinent vasoactive mediators in two contrasting models: health and cardiac disease. This work is pertinent because the mouse has been extensively used as a cardiovascular and genetically modified animal model, with the differences between human and mouse genetics not as great as one would expect. Thus it is important to understand murine pharmacology in the context of its use as a tool to understand human pathology. In our first study, responses in the “normal”, “healthy” mouse coronary artery were characterized and quantified. Pressure-constriction and concentration-response curves were constructed and compared to published human coronary pharmacology. It was found that myogenic tone was higher in mice than in humans and rats, greatly contributed to by endothelin. Noradrenaline produced concentration-dependent vasodilation, mediated by endothelium-located $\alpha_2$-adrenoceptors and smooth muscle-located $\beta$-adrenoceptors. Acetylcholine also produced vasorelaxation, which was abolished with L-NAME. Interestingly, the endothelium-dependent vasodilators bradykinin and substance P produced no vasomotor effect on the coronary arteries, possibly indicating the absence of specific corresponding receptors. In the second study, a murine coxsackie B3-induced myocarditis was used as a representative model of cardiac disease. Again, myogenic and endothelial function was evaluated in this pathological condition. Although hemotoxylin and eosin immunostaining confirmed extensive myocyte damage due to infection, immunohistological methods did not reveal increased endothelin-1 levels near the coronary vasculature. Our study found no difference in coronary arterial pressure-induced
vasoconstriction between infected and uninfected/sham-infected mice in the physiological pressure range, as well as between time points in the infected group. However, at a lower pressure range (10-60 mmHg), data from the early time points (1, 3 and 7 days post-infection) indicated enhanced vessel tone which could be the result of increased release of vasoconstrictor factors. Endothelial dysfunction manifested as a diminished response to acetylcholine occurred at 28 days post-infection, which was recovered with pre-treatment using the mixed endothelin A/B receptor antagonist bosentan. The results in this thesis have allowed a further insight into the use of the mouse as a relevant human model of disease. In addition, information with regards to coronary microvascular function in myocarditis has been obtained and contributes to the overall understanding of the mechanism of pathogenesis.
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## DISCUSSION

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LIST OF ABBREVIATIONS

ACh    acetylcholine
ANOVA  analysis of variance
ATP    adenosine triphosphate
BHT 920 HCl  6-allyl-2-amino5,6,7,8-tetrahydro-4H-thiazolo[4,5-d]azepin dihydrochloride
BK     large conductance (potassium channel)
Ca$^{2+}$  calcium ion
CaCl$_2$  calcium chloride
cAMP    cyclic adenosine monophosphate
CAT    choline acetyltransferase
cGMP    cyclic guanine monophosphate
cNOS    constitutive nitric oxide synthase
Cl$^-$  chloride ion
CO$_2$  carbon dioxide
CTX    charybdotoxin
CVB3    coxsackievirus B3
DAG    diacylglycerol
DCM    dilated cardiomyopathy
DMEM    Delbecco’s Modified Eagle’s Media
ECE    endothelin-converting enzymes
EDTA   ethylenediaminetetraacetic acid
EGTA   ethyleneglycoltetraacetic acid
eNOS    endothelial nitric oxide synthase
ET-1    endothelin-1
ET-A    endothelin receptor type A
ET-B    endothelin receptor type B
IFN    interferon
IgM    immunoglobulin M
i.p.    intraperitoneal
IL     interleukin
iNOS   inducible nitric oxide synthase
IP$_3$  inositol trisphosphate
K$^+$   potassium ion
K$_{Ca}$  calcium-dependent potassium (channel)
KCl    potassium chloride
KH$_2$PO$_4$  potassium phosphate
L-NAME  $N^\omega$-nitro-$L$-arginine methyl ester
M$_3$    muscarinic “type 3” (receptor)
MgSO$_4$  magnesium sulfate
min    minute
mL     millilitre
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>mm Hg</td>
<td>millimeters of mercury</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NA</td>
<td>noradrenaline</td>
</tr>
<tr>
<td>Na⁺</td>
<td>sodium ion</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NAHCO₃</td>
<td>sodium bicarbonate</td>
</tr>
<tr>
<td>nM</td>
<td>nanomoles</td>
</tr>
<tr>
<td>Na₂PO₄</td>
<td>sodium phosphate</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>O₂</td>
<td>oxygen</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PSS</td>
<td>physiological salt solution</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SNP</td>
<td>sodium nitroprusside</td>
</tr>
<tr>
<td>TFN</td>
<td>transferrin</td>
</tr>
<tr>
<td>VGC</td>
<td>voltage-gated channel</td>
</tr>
<tr>
<td>VSM</td>
<td>vascular smooth muscle</td>
</tr>
<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
<tr>
<td>μM</td>
<td>micromole</td>
</tr>
</tbody>
</table>
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1. INTRODUCTION

1.1 PREFACE

The vascular myogenic response, inherent to smooth muscle of small resistance blood vessels, is independent of neural, metabolic and hormonal influences. Blood vessels, especially arterioles and small arteries, respond to increases in transmural pressure with constriction, and conversely, to pressure reduction with dilation. This myogenic response has two main physiological functions: to provide basal vascular tone and to autoregulate blood flow and capillary hydrostatic pressure. Basal vascular tone is required in order for the vessels to respond to dilator influences. This inherent state of constriction allows for the provision of a "regional blood flow reserve", upon which other mechanisms may produce a vasodilation or vasoconstriction. The myogenic response also maintains constant blood flow, thus minimizing changes in capillary hydrostatic pressure.

It is known that the vasculature comprises 35% of the volume of the myocardium (Weber et al., 1987), and that at the microvascular level shows a heterogeneous response to injury and disease (Hoffman, 1995). In a pathological state, the myogenic response may be altered or impaired, which may result in decreased blood flow causing ischemic conditions. For example, it is thought that in the case of acute viral myocarditis, there is generalized inflammation of the heart muscle accompanied by the over-constriction of the coronary microvasculature. This process is associated with both necrosis and degeneration of the myocytes, and incidence is associated with viral infection. Myocardial tissue damage may result from direct destruction by the organism, anti-heart immunity, and microvascular disease (Rossi and Mengel, 1992). Previous studies have reported that in the acute phase of viral myocarditis, physiological profiling of resistance
coronary vessels shows that disturbances of the coronary microcirculation occur, and are 
characterized by focal areas of microvascular spasm and vessel stiffness (Dong et al., 

This thesis characterizes the mouse coronary artery using various pharmacological agents 
as well as the myogenic response. In addition, a murine model of coxsackie B3-induced 
myocarditis will also be studied and compared to as a relevant and important model of 
cardiac disease.

1.2 MYOGENIC TONE

1.2.1 BACKGROUND

The discovery of the myogenic response was made in 1902 by Bayliss. He noticed a 
physiological response in which isolated carotid arteries constricted following sudden 
distension (Bayliss, 1902), and postulated that intravascular pressure contributed to 
vascular tone. Later, Folkow demonstrated that denervated tissue preparations developed 
pressure-dependent vascular tone, thus proving this response to be non-neural in origin 
(Folkow, 1949). This phenomenon was observed in both nonvascular and vascular 
smooth muscle, and the later development of isolated vessel techniques enabled 
quantification of the myogenic response in separation from other influences (Davis and 
Hill, 1999).

The myogenic response is a unique property of resistance vessels, which consist of small 
arteries (70 – 200 μm lumen diameter) through to distal arterioles (10 μm lumen 
diameter). In contrast, non-resistance arteries, which and are larger in size and differ in 
fraction, possess little tone. These arteries are conductance vessels and are mainly
responsible for the carrying and transport of blood. In addition, unlike resistance vessels, these conduit vessels contribute little to total peripheral resistance.

Myogenic tone is one component of the auto-regulation of flow during variations in systemic arterial pressure. Other factors controlling blood flow include metabolic, neural and hormonal. All these in synchronization produce the overall resistance in a given vascular bed. To study the myogenic reactivity in small blood vessels without the influence of extrinsic factors, two in vitro techniques can be used: the isometric ring preparations or the pressure myograph (Halpern et al., 1984).

1.2.2 ISOMETRIC RING PREPARATION VERSUS PRESSURE MYOGRAPHY

The isometric ring protocol, performed on the wire myograph, was developed in 1972 by Bevan and Osher (Bevan and Osher, 1972). This technique consists of the mounting of vessel ring segments onto two fine stainless steel wires that are clamped at each end to keep the vessel isometric. In addition, the apparatus is fastened to a force transducer and micrometer (Mulvany and Halpern, 1976). Thus using this myograph, the myogenic response can be investigated by using stretch of the vessel to assess force development.

The pressure myograph serves to mimic the physiological state more closely than the ring preparation wire myograph. Essentially, the equipment consists of a tissue bath where two axially aligned and apposed glass microcannulae (tip diameter = 30-50 μm) are immersed. Using fine silk thread, one end of the tissue segment is tied to the end of one cannula, and the other end to the other cannula. Upon clamping the distal cannula, the intraluminal pressure can be increased or decreased using a pressure-servo controlled peristaltic pump. In addition, the intraluminal pressure can also be detected using a flow-
through pressure transducer. Vessel diameter is measured using a video edge-detection system. The experimental protocol consists of changing the intraluminal pressure and observing the effect on vessel diameter.

The isometric ring preparation of quantitating the vascular myogenic response differs from pressure myography in several ways. Firstly, in the isometric preparation, stretch activation is represented by a lower, secondary increase in tension after stretch ("stretch-activation"). In contrast, using the pressure myography technique, an increase in intraluminal pressure results in a constriction that secondarily reduces total wall tension (i.e., passive wall tension > active wall tension). Secondly, isometric preparations show maximal stretch activation in response to large (unphysiological) changes in length, whereas isobaric preparations show maximal constrictions in response to much smaller length changes (Davis, 1993), even in the absence of detectable distension (Davis and Sikes, 1990). Finally, similar vessels may possess different agonist sensitivities (Dunn et al., 1994) and differences in magnitudes of agonist-induced vascular smooth muscle depolarization (Schubert et al., 1996) in different preparations.

1.2.3 ROLE OF ENDOTHELIAL

The endothelium, lining the inner surface of blood vessels, is the main regulator of vascular wall homeostasis. It secretes various factors in response to mechanical and hormonal stimuli, which, in turn, influence smooth muscle cell contractility, vascular structure, blood fluidity, and other cell-to-cell interactions (Thorin and Shreeve, 1998). Inclusively, the endothelium is important in regulating myogenic tone. Early studies
performed by Harder and co-workers indicated that the myogenic response to pressure
elevation was dependent on an intact endothelium. Removing the endothelial layer by
chemical reduced both pressure-induced depolarization and the myogenic response
(Harder, 1987). However, it was later shown in various tissue preparations that
mechanical removal of the endothelium did not abolish myogenic tone.
At present, it is believed that the myogenic response occurs as a result of mechanical
stimuli that act directly on the vascular smooth muscle cells to generate constriction. The
endothelium exerts its influence by releasing vasoactive factors, but the presence of an
intact endothelium is not a prerequisite for myogenic reactivity in blood vessels.

1.2.4 TRANSDUCTION MECHANISMS

1.2.4.1 DEPOLARIZATION OF VSM AND ELECTROMECHANICAL COUPLING

It has been shown extensively that membrane depolarization occurs as a response of the
smooth muscle cells to a stretch stimulus (Coburn, 1987; Knot and Nelson, 1998). This
depolarization is graded as pressure is increased. The result is always vessel constriction.
However, it has been difficult to establish a definitive cause-and-effect relationship
between membrane depolarization and myogenic responsiveness. Some hypotheses are
mentioned below.

1.2.4.1.1 STRETCH-ACTIVATED CHANNELS

Stretch-induced depolarization could be explained by activation of special
mechanosensitive non-selective ion channels on endothelial cells (Davis et al., 1992),
which are sensitive to membrane deformation. Cation-permeable mechanosensitive
channels were first described in cultured skeletal muscle cells (Guharay and Sachs, 1985), and have since been found in other cell types including smooth muscle cells. In vascular smooth muscle cells of freshly isolated porcine coronary arteries, Davis et al. found that patch pipette suction activated a non-selective cation channel that is permeable to K\(^+\), Na\(^+\) and Ca\(^{2+}\). Activation of these mechanosensitive channels initiates mechano-electric transduction, resulting in a membrane depolarization that remains elevated in spite of reduced vessel diameter—characteristic of myogenic response.

1.2.4.1.2 K\(^+\) CHANNELS

Inhibition of K\(^+\) channels is hypothesized to be a mechanism involved in stretch-induced depolarization. Although a direct role by K\(^+\) channels in initiating the myogenic response has not been shown, a membrane depolarization that inhibits potassium currents flowing through these channels may result in vascular tone. It has been observed that three types of K\(^+\) channels do not play a role in myogenic tone: the inward rectifier K\(^+\) channel, the ATP-sensitive K\(^+\) channel (Quayle et al., 1997), and the novel K\(^+\) channel (Evans et al., 1996). However, the voltage-dependent K\(^+\) channel and the Ca\(^{2+}\)-activated K\(^+\) channel types may be involved in membrane re-polarization. Blockers of K\(_{Ca}\) channels have been found to augment myogenic tone and further decrease luminal diameter (Nelson and Brayden, 1993). In particular, the involvement of BK channels is likely, since block of these channels by the specific BK channel inhibitor charybdotoxin (CTX) enhances vascular tone.
1.2.4.1.3 Cl\textsuperscript- CHANNELS

Chloride channel activation may be another mechanism by which stretch-activated membrane depolarization can be explained. It has been shown that Cl\textsuperscript- channel inhibitors DIDS and indanyloxyacetic acid inhibit tone of pressurized cerebral arteries (Nelson et al., 1997). However, the lack of specific Cl\textsuperscript- channel blockers makes the role of these channels in the myogenic response inconclusive.

1.2.4.1.4 VOLTAGE-GATED Ca\textsuperscript{2+} CHANNELS

Much evidence has confirmed the importance of VGC channels in the myogenic response. The possibility that activation of these channels may result in membrane depolarization has been suggested. Dihydropyridines have been shown to attenuate pressure- or stretch-induced intracellular calcium concentration, and VGC channel activators increase depolarization (Harder, 1984). However, it was shown that in rat cerebral arteries Ca\textsuperscript{2+} channel inhibitors (diltiazem and nisoldipine) blocked calcium influx and vasoconstriction but had no effect membrane depolarization (Knot and Nelson, 1998).

1.2.4.2 SECOND MESSENGERS

1.2.4.2.1 CALCIUM

The importance of the role of Ca\textsuperscript{2+} in arteriolar tone was first noted by Uchida and Bohr (Uchida and Bohr, 1969). They observed the abolishment of tone upon perfusion of the vessel with a Ca\textsuperscript{2+}-free solution. Laher et al. later reported that myogenic tone was dependent on Ca\textsuperscript{2+} entry (Laher et al., 1988), as demonstrated by Ca\textsuperscript{2+} influx (Cipolla and
Osol, 1998). At present, it is known that in vessels possessing myogenic tone, increases in transmural pressure lead to increases in intracellular calcium concentration ([Ca\(^{2+}\)](Boltz and Pohl, 1998; Knot and Nelson, 1998). This hypothesis has been confirmed by use of Ca\(^{2+}\)-sensitive fluorescent dyes in conjunction with video-based imaging and photometer techniques (Meininger et al., 1991). Although small arteries and arterioles possess intracellular Ca\(^{2+}\) stores, these vessels seem to show greater dependence on extracellular Ca\(^{2+}\) for contractile activity (Cauvin et al., 1984). Inhibitors of the sarcoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) has been shown to have no effect on amplitude of myogenic constriction in the rat cerebral arteries (Watanabe et al., 1993), although rate of development of tone is reduced.

1.2.4.2.2 G PROTEINS AND PHOSPHOLIPASE C

G proteins are activated in coronary smooth muscle cells in response to mechanical stimulation (Wierbitzky et al., 1994). In addition, a role for PLC in mechanotransduction has been suggested. Osol et al. observed the abolishment of myogenic tone in isolated cerebral arteries in the presence of the PLC inhibitor U-73122 (Osol et al., 1993), and Narayanan et al. measured production of inositol trisphosphate (IP\(_3\)) and diacylglycerol (DAG) in renal arterial smooth muscle cells in response to increased intraluminal pressure (Narayanan et al., 1994). The production of IP\(_3\) may contribute to the rise in [Ca\(^{2+}\)], by releasing stored Ca\(^{2+}\) from the sarcoplasmic reticulum, and DAG can directly activate protein kinase C (PKC).
1.2.4.2.3 PROTEIN KINASE C

Activation of PKC has been suggested to play a possible role in the myogenic response. It has been shown that inhibition of PKC by calphostin C attenuates tone in human coronary arteries (Miller et al., 1997). Staurosporine was shown to cause dose-dependent inhibition of myogenic tone in isolated rat cerebral arteries (Osol et al., 1993). PKC activators increase the level of arteriolar tone without an increase in $[Ca^{2+}]_i$ levels. In addition, agonists such as noradrenaline that act in part through PKC activation enhance myogenic responsiveness (Faber and Meininger, 1990). More specifically, PKC has been implicated to sensitize the contractile proteins to $Ca^{2+}$, and is important in the maintenance of constriction. However, the precise role of PKC in myogenic signaling has been made difficult due to its numerous isozymes.

1.2.4.3 CYTOSKELETON AND EXTRACELLULAR MATRIX

1.2.4.3.1 INTEGRINS

Integrins are a class of membrane-spanning heterodimeric receptors, found on most cells, including vascular smooth muscle cells (Platts et al, 1998). In vascular smooth muscle, at least 11 of the 22 known integrin subunit combinations occur. The predominance of specific integrin types suggest interactions with collagens and laminin in the basement membrane and the interstitial matrix (Glukhova et al, 1995). Integrins function as transducers of a variety of cellular signals, and thus may also interact with tyrosine kinases, phospholipase C, protein kinase C, as well as modulate membrane ion conductance (Clark and Brugge, 1995). Since integrins form an important mechanical link between the extracellular matrix and the vascular smooth muscle skeleton, and are
able to associate with both cytoskeletal (Shattil and Ginsberg, 1997) and signaling proteins (Hynes, 1992), they may play a role in regulating vasomotor function. More specifically, integrins may be responsible in the transduction of the physical stimulus of intraluminal pressure to result in contraction via the cellular contractile apparatus (Wang et al., 1993). It has been suggested that the integrin-dense plaque region of the vascular smooth muscle may be a likely site for a mechanosensitive element, consisting of an assemblage of proteins (Davis et al, 2001). This “mechanosensor” may also be involved in the sustenance of vascular tone after initial vasoconstrictor response.

1.2.4.3.2 CYTOSKELETON

Cytoskeletal stiffness increases in proportion to stress applied through integrin attachments (Muller et al., 1997). A potential role of the VSM cytoskeleton is in control of mechanosensitive ion channel gating, and both the microtubule system and the actin cytoskeleton may play a role in active force development.

1.3 VASOACTIVE MEDIATORS

Several vasoactive factors, released as either neural- or endothelial-derived mediators, are important in influencing the vascular state of resistance arteries. Below, the functional roles of noradrenaline, acetylcholine, endothelin-1 and nitric oxide in the coronary circulation are briefly summarized.
1.3.1 NORADRENALINE

1.3.1.1 SYNTHESIS, STORAGE AND RELEASE

Noradrenaline is synthesized as a result of a series of steps beginning from tyrosine, and immediately from dopamine by the enzyme dopamine β-hydroxylase. The hydroxylation of tyrosine is regarded as the rate-limiting step in the biosynthesis of catecholamines (Zigmond et al., 1989). Noradrenaline is co-stored in two types of regulated-release storage vesicles, namely the large dense core vesicles and small dense core vesicles. Release is from perivascular sympathetic nerves (Burnstock and Sneddon, 1985), and act on α- and β-adrenergic receptors in the membrane of the postsynaptic cell.

1.3.1.2 NORADRENALINE IN THE HUMAN CORONARY CIRCULATION

The sympathetic nervous system is of great importance in the regulation of coronary resistance. The heart possesses extensive sympathetic innervation, and noradrenaline mediates vasoconstriction of human coronary arteries via α-adrenoceptors (Corr and Burnstock, 1991). However, some studies have concluded that β-adrenoceptor mediated relaxation exceeds the constriction mediated by α-adrenoceptors (Ginsberg and Bristow, 1980). α-adrenoceptors on the smooth muscle mediate vasoconstriction via receptor-mediated events which increase IP$_3$ formation, and in turn increase intracellular calcium. β-adrenoceptors mediate vasorelaxation via increase in cAMP concentration. cAMP activates a protein kinase, which phosphorylates and inactivates myosin-light-chain kinase, thus inhibiting contraction (Rang and Dale, 1995).
1.3.2 ACETYLCHOLINE

1.3.2.1 SYNTHESIS, STORAGE AND RELEASE

Acetylcholine is synthesized from choline by the enzyme choline acetyltransferase (CAT). The rate-limiting process of acetylcholine synthesis is choline transport, the activity of which is regulated according to the rate at which acetylcholine is being released. After synthesis, acetylcholine is transported into and stored in synaptic vesicles, with release into the synaptic cleft coinciding with the arrival of the action potential at the motor-nerve terminal (Katz and Miledi, 1965). Post-synaptically, acetylcholine can act on nicotinic or muscarinic receptors. Acetylcholine is degraded by acetylcholinesterase.

1.3.2.2 ACETYLCHOLINE IN THE CORONARY CIRCULATION

Acetylcholine has action on both the endothelial and smooth muscle cells of the coronary vessels. Acetylcholine may act on muscarinic receptors located on the endothelial cells to initiate vasodilation. This initiates inositol trisphosphate production, and $\text{Ca}^{2+}$ release from the endoplasmic reticulum and activation of NO synthase by $\text{Ca}^{2+}$/calmodulin. In addition, acetylcholine can cause vasoconstriction of coronary arteries via direct action on smooth muscle cells, in the absence of an intact endothelium (Schipke et al., 1985). Correlatively, vascular damage and/or endothelium dysfunction may also result in acetylcholine-induced vasoconstriction, as a consequence of the involvement of vasoactive substances such as angiotensin II (Becker et al, 1991) and prostenoids (Shimizu et al, 1993) which likely increase calcium influx into smooth muscle cells.
1.3.3 ENDOTHELIN-1 (ET-1)

1.3.3.1 SYNTHESIS, STORAGE AND RELEASE

The potent vasoconstrictor peptide ET-1 is synthesized by cleavage of big ET-1 by endothelin-converting enzymes (ECE). In humans, big ET-1 and ECE-1 are primarily localized in secretory and storage granules in endothelial cells (Davenport et al., 1998). ET-1 is produced in these cells, but may also be expressed in other cell types including vascular smooth muscle cells (Liu et al., 1998). Under normal conditions, it is postulated that there is a low level of basal endothelin production and release, which mediates vascular tone in vivo. In addition, various stimuli such as hypoxia, ischemia or shear stress may induce transcription of ET-1 mRNA and subsequent synthesis and secretion of ET-1. Under these and other pathological conditions such as coronary artery disease and myocardial infarction, plasma levels of ET-1 are significantly elevated (Lerman et al., 1991; Tonnessen et al., 1995).

Receptors for endothelin have been isolated and their genes cloned (Arai et al., 1990). The receptors are classified on the basis of their affinity for the various endothelin isoforms. ET-1 may act on ET-A or ET-B receptors, with the receptor types in humans exhibiting significant sequence similarity with 63% amino acid identity (Sakamoto et al., 1991). However, each receptor type has differential distribution in various tissues. Binding of endothelin to its receptor is very tight with slow dissociation, resulting in prolonged pharmacological action. ET-A receptors are prevalent in the heart and vascular smooth muscle cells, whereas ET-B receptors are more widely distributed and predominate in the kidney, uterus, central nervous system and endothelial cells (Tamirisa et al., 1995).
Receptor activation causes stimulation of phospholipase C and subsequent hydrolysis of phosphatidylinositol. The result is two products: IP$_3$ and DAG. In addition to the stimulation of calcium release from the sarcoplasmic reticulum by IP$_3$, activated ET receptor also stimulates voltage-dependent calcium channels and probably the receptor-operated calcium channels. The global effect is increased intracellular calcium concentration and sensitization of the cellular contractile apparatus and contraction.

1.3.3.2 ENDOTHELIN-1 IN THE CORONARY CIRCULATION

In the human coronary circulation, ET-1 acts predominantly on ET-A receptors. It is unknown whether the mouse coronary circulation exhibits similar phenotypic distribution. ET-1 plays important short-term and long-term roles: short-term actions include contraction of vascular smooth muscle by mobilization of intracellular Ca$^{2+}$ and increased extracellular Ca$^{2+}$ influx (Levin, 1995). Under long-term (chronic) pathological conditions, endothelin concentrations are elevated, as is coronary artery tissue endothelin immunoreactivity (Lerman et al., 1995). It is hypothesized that ET-1 plays a significant role as a modulator of coronary vascular reactivity, both in coronary artery disease as well as in endothelial dysfunction. With respect to endothelial dysfunction, an imbalance between ET-1 and nitric oxide (NO) synthesis may be present; decreased NO and/or enhanced ET-1 production may lead to potentiation of vasoconstrictor effects and increased global vasoconstriction. Boulanger et al. demonstrated that ET-1 attenuated nitric oxide-induced cyclic GMP (cGMP) generation in vitro (Boulanger and Luscher, 1991), and Lerman et al. confirmed that coronary cGMP concentrations were suppressed
in humans with endothelial dysfunction and elevated endothelin concentrations (Lerman et al., 1995).

1.3.4 NITRIC OXIDE (NO)

1.3.4.1 SYNTHESIS, STORAGE AND RELEASE

Nitric oxide is synthesized by NO synthase (NOS) enzymes. There are several isoforms of NOS, including eNOS (endothelial), cNOS (constitutive) and iNOS (inducible). NOS catalyzes a reaction between molecular oxygen and L-arginine to form nitric oxide. Nitric oxide is produced in response to cytokine stimulation in macrophages, neutrophils, vascular smooth muscle and endothelial cells. In addition to the endothelium, vascular nitric oxide is also present in platelets (Rang and Dale, 1995).

1.3.4.2 NITRIC OXIDE IN THE CORONARY CIRCULATION

Nitric oxide in vascular regulation couples endothelial and smooth muscle cells. Nitric oxide diffuses to smooth muscle cells and activates guanylyl cyclase. cGMP activates cGMP-dependent protein kinase which phosphorylates myosin light chain and causes vascular relaxation. The physiological role of nitric oxide includes control of regional blood flow and blood pressure. Pathologically, nitric oxide may cause or contribute to atherogenesis, thrombosis and vasospasm. Nitric oxide also acts as a host defense mechanism against viruses and bacteria.
1.4 COXSACKIEVIRUSES

1.4.1 DISCOVERY, TAXONOMY AND PROPERTIES

Coxsackieviruses were first discovered approximately 50 years ago. Soon thereafter it was recognized that some of the coxsackievirus isolates caused generalized skeletal muscle destruction in the mouse, a histopathology distinct from other coxsackievirus isolates. Thus the different viruses were placed into two groups, A and B. The group A viruses cause generalized myositis, whereas the group B viruses cause infection of many tissues and organs which lead to a slower and more paralytic death with destruction of cardiac myocytes, pancreatic cells and hepatocytes. There are six serotypes of CVB viruses.

Genetically, these non-enveloped viruses have a single “plus” strand RNA genome with VPg-linked to the 5’ end of the genome and a poly A tail at the 3’ end, typical of picornaviruses. The genome, approximately 7000-7500 bases in length, is translated into a large polyprotein, which is cleaved into functional proteins by virus encoded proteases. Mutations in the genome result in antigenic variations in the virus proteins. The RNA of most CVB serotypes has been sequenced and cDNA molecules shown to be capable of replication (Kandolf and Hofschneider, 1985; Chapman et al, 1994). Recently it has been found that CVB RNA genomes can remain latent in cells, especially heart tissue, where no intact virus or viral proteins can be detected.
1.4.2 CVB TRANSMISSION AND INFECTION

Coxsackieviruses are ubiquitous and can spread rapidly within the community. The site of entry is believed to be the alimentary tract, but aerosol transmission may also be significant under sub-sanitary conditions. Incubation period is approximately one to two weeks, but may be longer. Infection is mediated by cell surface receptors, which facilitate binding and entry of CVB into susceptible host cells (Martino et al., 2000). Resistant to low pH, they replicate in the mucosal cells of the small intestine prior to entering the bloodstream and causing systemic infection.

1.4.3 CVB AND HUMAN DISEASE

The CVB are known to produce a variety of human diseases. A high proportion of infections with these viruses is subclinical, exhibited by a mild, undifferentiated febrile illness or upper respiratory tract infection. Infection in neonates can be both systematic and fatal. Clinical manifestations include myocarditis, meningitis, encephalitis and pulmonary disease (Chonmaitree and Mann, 1995; Grist et al., 1978). General symptoms include fever, severe unilateral chest pain, pleural inflammation, headache and sore throat. There may be abdominal tenderness associated with pancreatitis and hepatitis. In addition, virus-induced heart disease may develop necessitating heart transplantation. The role of CVB in virus-induced insulin-dependent diabetes melitus (IDDM) has also been suspected. Receptors may have a major role in the determination of viral tropism (Martino et al., 2000).
1.5 CVB3-INDUCED VIRAL MYOCARDITIS

1.5.1 INTRODUCTION

Viral myocarditis exhibits different clinical phenotypes depending on the age of patient. In infants, it can be manifested as acute heart failure and cardiogenic shock, and in older patients, it often presents as a chronic, slowly progressive heart failure and dilated cardiomyopathy (Liu and Opavsky, 2000). CVB3 produces myocarditis by direct myocyte damage and degeneration, as well as necrosis, with potential treatment uncertain. Myocardial and endothelial damage in the acute phase may progress to slow chronic myocyte loss and myocardial fibrosis, eventually resulting in dilated cardiomyopathy. A well-established animal model of CVB3-induced myocarditis is the mouse, from which much information has been gained. It has been demonstrated that early inflammatory infiltrates post-infection are associated with observed foci of necrosis, where direct infection of myocytes leads to cell death (McManus et al., 1993). In addition to viral infection and immune activation, coronary microvascular spasm is also thought to be involved (Silver and Kowalczyk, 1989). This abnormality may be secondary to endothelial cell damage (Dong et al., 1992), and may lead to potential sites of ischemia and reperfusion.

1.5.2 ENDOTHELIN-1 (ET-1) IN VIRAL MYOCARDITIS

In the acute phase of CVB3 myocarditis, prior to the development of inflammatory infiltrates or significant hemodynamic compromise, infected myocytes develop increased de novo synthesis of ET-1. It has been shown that in encephalomyocarditic mice, peak plasma concentrations of ET-1 occurred at 5 days post-infection, and peak heart
concentrations at 7 days post-infection (Ono et al., 1999). It is hypothesized that this elevated level of circulating ET-1, contributed to by myocytes, interstitial and endothelial cells, may cause microvascular spasm and focal vasoconstriction, resulting in altered myocardial blood flow. The later introduction of inflammatory infiltrates such as macrophages may also contribute to increased ET-1. The result is a condition which may be conducive of impaired systolic and diastolic performance (Pandey et al., 1998), left ventricle failure, myocardial injury and ischemia (Hasdai et al., 1994), silent infarction (Tonnessen et al., 1995) and ultimately heart failure (Stewart et al., 1992).

1.5.3 HOST DEFENCE: CELL-MEDIATED IMMUNE PATHOGENICITY

The first wave of infiltrating cells in the mouse heart, consisting mainly of natural killer (NK) cells, occurs about 4-14 days post-infection (subacute phase). Various cytokines, including IL-1 beta, TNF-alpha, IFN-gamma and IL-2 are produced at this stage (Kawai, 1999). In addition, at this time, nitric oxide (NO) is thought to play both beneficial and detrimental roles (Beckman et al., 1990; Martino et al., 1995). Animal models with IFN element-1 gene and iNOS knockouts show a marked increase in mortality, with minimal ability to attenuate viral proliferation (Liu et al., 1996). Although NO is a modulator of immunological self-defense mechanisms and kills infectious agents, it is also associated with detrimental effects on the myocardial tissue (Ishiyama et al., 1997).

The second wave of infiltrating cells consists of T lymphocytes, which coincides with the most severe acute pathological damage. B lymphocyte infiltrates also increase at a later time point, with levels showing reciprocal changes of less intensity than those of the T lymphocytes (Kawai, 1999).
1.5.4 VIRUS DETECTION AND SEROLOGICAL MARKERS FOR DIAGNOSIS

Coxsackie B viruses are present in the pharyngeal washings of infected individuals for 1-2 weeks after infection and for a prolonged period of several weeks in the feces. This, however, is not of diagnostic significance in relation to heart disease, since detection of virus in the affected target organ is important.

In contrast, serological markers are commonly used to examine and diagnose viral involvement in heart disease. Patients with myocarditis show a fourfold rise in neutralizing antibodies to CVB3 (Abelmann, 1971). CVB-IgM antibody titres are elevated for up to six months in some myocarditic patients (McCartney et al., 1986), and is also a prevalent symptom in patients with dilated cardiomyopathy (Kitaura, 1981).

1.5.5 PERSISTANCE OF VIRAL RNA

The viral mechanism not only contributes to the acute phase of myocarditis, but also to the evolution of ongoing heart disease. Persisting virus may interact with the immune system and play a role in the transition from myocarditis to dilated cardiomyopathy. It is hypothesized that in the chronic phase of myocarditis, T lymphocytes infiltrate the myocardium in response to viral RNA in myocytes. Viral RNA may therefore generate new antigenic molecules with can trigger the immune inflammatory reaction (Martino et al., 1994). There is evidence that viral RNA is capable of replication (Klingel et al., 1992).
1.5.6 FROM MYOCARDITIS TO DILATED CARDIOMYOPATHY

Dilated cardiomyopathy (DCM), defined by the enlargement of cardiac chambers, thinning of ventricular walls and reduced contractility (Fenoglio et al., 1983; Keating and Sanguinetti, 1996), is a prevalent cause of heart disease and sudden death (Penninger et al., 1997). Most patients with DCM have a history of myocarditis. In addition, CVB picornaviruses can be detected in as many as 50% of these patients. The progression from acute viral myocarditis to DCM has been investigated, and it is hypothesized that the virus not only initiates the disease by infection and invasion, but persists as a molecular pathogen, causing cardiac damage, degeneration and dysfunction (Martino et al., 1994). Phenotypically, myocardial fibrosis is also prominent, as are cardiac lesions. The exact transition from viral myocarditis to DCM is unknown, but since culturable virus and viral capsid proteins are absent after the initial phase of myocarditis, it has been suggested that a cell-mediated autoimmune mechanism(s) may play a role in the pathogenesis (Kawai, 1999).

1.5.7 INITIAL SUPPORTIVE MEASURES AND FUTURE THERAPEUTIC IMPLICATIONS

Bed rest and avoidance of vigorous exercise is important for patients with severe heart failure. $\alpha_1$- and $\beta$-adrenoceptor blockers, including prazosin or doxazosin (Sole and Liu, 1993), have shown to be effective in the treatment of viral myocarditis and DCM. Angiotensin-converting enzyme (ACE) inhibitors such as captopril are also effective in reducing myocardial injury, at least in part by abolishing microvascular spasm. Calcium channel blockers, for example verapamil (Dong et al., 1992) and amlodipine, have been
observed to prolong survival and reduce myocardial damage. This may be due to altered inflammatory responses or the immunomodulating effect by inhibiting NO production (Matsumori, 1997). Endothelin receptor antagonists such as bosentan have been reported to have a cardioprotective effect by increasing cardiac output as well as decreasing left ventricular dysfunction and myocardial injury (Ono, K. et al, 1999). In addition, antiviral and immunosuppressive agents, as well as immunomodulating therapy may be an important approach to successful long-term therapy.
Schematic Diagram Depicting Pathophysiology of Myocarditis

Fig. 1.5.1 Schematic timeline showing the pathophysiological progression of myocarditis. Adapted from Ono et al., 1999. Kawai, C., 1999.
Vasomediators as Contributors to the Progression of Myocarditis

Fig. 1.5.2 Schematic diagram showing the involvement of the vasoactive mediators of the coronary microcirculation in the progression of myocarditis.
2. STUDY 1 - MYOGENIC AND ENDOTHELIAL REGULATION OF TONE IN THE HEALTHY MOUSE SEPTAL ARTERY

2.1 HYPOTHESES AND OBJECTIVES

HYPOTHESIS 1: The septal artery of the mouse is similar to that in the human with respect to basal myogenic tone and to drug-induced responses.

OBJECTIVE 1: To characterize the septal artery of the CD-1 mouse with respect to myogenic tone as well as responses to pertinent pharmacological agents and compare responses to published results from human data.

2.2 MATERIALS AND METHODS

2.2.1 TISSUE ISOLATION, PREPARATION AND DIAMETER MEASUREMENT

Male CD-1 mice (5-6 weeks of age) were anesthetized using sodium pentobarbital (Somnotol, 0.1mL/mouse i.p.) and heparin sulfate (Hepalean, 0.1mL/mouse i.p.). The heart was removed and placed into ice-cold physiological salt solution (PSS) following decapitation. The right ventricle was cut open and the septal artery dissected and transferred to a pressure arteriograph vessel chamber (Living Systems, Vt., USA) containing oxygenated (95% O₂ / 5% CO₂) PSS at room temperature. The superfusing solution was recirculated from a 100 mL external bath at a flow rate of 12.5 mL/ min.

One vessel end was tied to one borosilicate cannula (tip diameter 50-80 µm) using a single 4-0 nylon thread filament. The other vessel end was tied in a similar manner to the distal cannula. Under no flow conditions, the intraluminal pressure was incrementally increased from 0 mmHg to 40 mmHg using an electronic pressure servo system (Living
Systems, Vt., U.S.A.). The bath temperature was then heated to 37°C using an external heat exchanger. Upon reaching this bath temperature, the intraluminal pressure was again incrementally raised to 70 mmHg. The artery was equilibrated for 60 min at this pressure during which time spontaneous myogenic tone developed.

The vessel chamber was placed under an inverted video microscope, where the vessel image could be observed on a monitor. Vessel diameter was measured using a monochromatic video edge-detection system (Living Systems, Vt., U.S.A.). Intraluminal pressure and diameter were continuously recorded onto a personal computer using the software program DATAQ.

2.2.2 EXPERIMENTAL PROTOCOL

In experiments designed to measure the spontaneous myogenic response, pressure-diameter relationships were determined by increasing the intraluminal pressure from 10 to 20 mmHg, and in 20 mmHg increments to 120 mmHg thereafter. At each pressure, a 5 min stabilization period was allowed during which time the vessel typically achieved a steady state diameter. The same protocol was repeated using vessels pre-incubated with 1 μM or 10 μM bosentan for 30 min. In separate experiments, the concentration dependence of noradrenaline-induced relaxation was determined. Following the development of tone, cumulative additions of noradrenaline (1 nM-10 μM) were made to the PSS.

To investigate adrenoceptor activity, the α1-adrenoceptor selective agonists phenylephrine and methoxamine were applied to the coronary arteries. The α2-
adrenoceptor selective agonists BHT 920 and clonidine were used in separate experiments.

To elucidate which receptors mediate noradrenaline-induced relaxation, arteries were incubated with the $\alpha_2$-adrenoceptor selective antagonist, yohimbine (10 $\mu$M), and/or the nonselective $\beta$-adrenoceptor antagonist, propranolol (20 $\mu$M) for 30 min prior to a noradrenaline (10 $\mu$M) challenge. Phenylephrine (10 $\mu$M), the $\alpha_2$-adrenoceptor selective agonist 6-allyl-2-amino5,6,7,8-tetrahydro-4H-thiazolo-[4,5-d]azepin-dihydrochloride (BHT 920, 3 $\mu$M), and isoprenaline (10 $\mu$M) were used as controls before and after the application of an antagonist. BHT 920 (3 $\mu$M) and isoprenaline (10 $\mu$M) were also applied separately to the endothelium-denuded coronary artery. To determine whether nitric oxide is a possible effector of $\alpha_2$-adrenoceptor-mediated vasodilation, arteries were incubated with N$^G$-nitro-L-arginine methyl ester (L-NAME, 200 $\mu$M, 30 min) followed by a BHT 920 (1 $\mu$M) or noradrenaline (10 $\mu$M) challenge. L-NAME (200 $\mu$M) and propranolol (20 $\mu$M) were also applied in combination.

Additionally, acetylcholine (1 nM-10 $\mu$M), both in the presence and absence of L-NAME, was applied cumulatively to the PSS and arterial luminal diameter recorded. This protocol was repeated using endothelium-denuded coronary vessels.

To study endothelium-dependent vasodilation, bradykinin and substance P were also applied to the septal arteries.

At the end of each experiment, normal $\text{Ca}^{2+}$-containing PSS was substituted with $\text{Ca}^{2+}$-free PSS to determine passive luminal diameter.
2.2.3 EXPRESSION OF RESULTS AND STATISTICAL ANALYSIS

Myogenic tone at each pressure was expressed as a percent constriction = \( 100\% \times \frac{(D_{Ca-free} - D_{PSS})}{D_{Ca-free}} \), where D is the diameter in calcium free (D_{Ca-free}) or Ca\(^{2+}\)-containing PSS (D_{PSS}). Percent relaxation was calculated using the equation \( 100\% \times \frac{(D_d - D_b)}{(D_{Ca-free} - D_b)} \), where D is the diameter upon stabilization after drug addition (d), baseline (b) or calcium free.

All results are expressed as mean ± S.E.M. of \( n \) animals. One vessel segment was obtained from each animal. Statistical evaluation was made using analysis of variance (ANOVA), and means were considered significantly different when \( P < 0.05 \).

2.2.4 SOLUTIONS AND CHEMICALS

The composition of the PSS was (in mM): NaCl 119, KCl 4.7, KH\(_2\)PO\(_4\) 1.18, NaHCO\(_3\) 24, MgSO\(_4\)7H\(_2\)O 1.17, CaCl\(_2\) 1.6, glucose 5.5 and EDTA 0.026. Ca\(^{2+}\)-free solution was a PSS solution containing no CaCl\(_2\) and 2.0 mM EGTA. The composition of 80mM high K\(^+\) solution was: NaCl 2.279, KCl 5.965, KH\(_2\)PO\(_4\) 0.1606, MgSO\(_4\) 0.1409, NaHCO\(_3\) 2.092, and 0.006 EDTA. BHT 920 HCl (6-allyl-2-amino5,6,7,8-tetrahydro-4H-thiazolo-[4,5-d]azepin-dihydrochloride) was supplied by Boehringer Ingelheim Canada, Ltd. (Ontario). Bosentan (4-tert-Butyl-N- [6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2'-bipyrimidin-4-yl] benzene sulfonamide monohydrate) was from Actelion Ltd., Switzerland. All other drugs were purchased from Sigma Chemical Company (St. Louis, MO).
2.3 RESULTS

2.3.1 BASAL TONE

Septal arteries (150-180 \( \mu \)m in diameter) developed basal tone in normal PSS. Spontaneous tone developed upon equilibration at 70 mmHg for 1 h. Step increases in pressure from 10 mmHg to 20 mmHg, then from 20-120 mmHg in 20 mmHg increments led to the development of myogenic tone at 60 mmHg (\( n=4 \)), manifested by a pressure-induced decrease in luminal diameter (Fig. 2.3.1A). Thus increases in pressure leads to constriction of the arteries in normal PSS (Fig. 2.3.1B). Vessel tone was present at pressures as low as 20 mmHg. Between 60-120 mmHg, vessel constriction was between 61-65% (\( n=4 \)). Spontaneous rhythmic activity was not seen in any of the arteries. In the presence of 1 \( \mu \)M bosentan, vessel tone decreased and vasoconstriction was calculated to be between 40-43% (\( n=3-4 \), Fig. 2.3.2). With incubation of the vessels with 10 \( \mu \)M bosentan, myogenic tone was further decreased, with vessel constriction between 29-33% at 60-120 mmHg (\( n=4-5 \), Fig. 2.3.2). In endothelium-denuded vessels, incubation with 10 \( \mu \)M bosentan resulted in similar vessel constriction of 20-26% at 80-120 mmHg (\( n=4 \)) as in control tissues (19-26%, \( n=4 \), Fig. 2.3.3). Vasoconstriction due to 80 mM K\(^+\) solution was used as a control for any non-specific effect of bosentan (10 \( \mu \)M); constriction to K\(^+\) was unaffected by bosentan (unpublished data). Tone was abolished when normal Ca\(^{2+}\)-containing PSS was substituted with Ca\(^{2+}\)-free PSS, whence step increases in intraluminal pressure only dilated the vessels (\( n=4 \), Fig.2.3.1A, B).
Trace Recording of Active and Passive Arterial Diameter in the Pressurized Mouse Septal Artery

Pressure-diameter relationship in Pressurized Mouse Septal Arteries Possessing Myogenic Tone

**Fig. 2.3.1A** Trace recording of arterial diameter in normal Ca\(^{2+}\)-containing PSS and Ca\(^{2+}\)-free PSS. In normal PSS, spontaneous myogenic tone develops at 60 mmHg. In Ca\(^{2+}\)-free PSS, tone is absent and the vessel displays passive behavior. **3.3.1B** The relationship between intraluminal pressure and vessel diameter in normal PSS and Ca\(^{2+}\)-free PSS. In normal PSS, increases in pressure lead to a decrease in vessel diameter. Between 60-120 mmHg, luminal diameter measured between 79.3±6.2 μm - 81.5±3.2 μm ( ■ , n=4). In Ca\(^{2+}\)-free PSS, increases in pressure lead to vasodilation. Between 60-120 mmHg, luminal diameter measured between 217.9±3.3 μm – 233.5±3.2 μm ( ▲ , n=4).
Bosentan-induced Inhibition of Myogenic Tone in Pressurized Septal Arteries of Pentobarbital-Anesthetized Mice

Fig. 2.3.2 Development of spontaneous myogenic tone in mouse septal arteries. Intraluminal pressure was increased from 10 mmHg to 20 mmHg, then from 20 mmHg to 120 mmHg in 20 mmHg increments. The vessel was allowed to equilibrate for 5 min at each pressure. Between 60-120 mmHg, vessel constriction in normal PSS was 61-65% (■, n=4). In the presence of 1 μM bosentan (▼), vessel constriction within the same pressure range was 40-43% (n=3-4). Pre-treatment with 10 μM bosentan (▲) resulted in 29-33% constriction (n=4).
Myogenic Tone in Endothelium-denuded Arteries in the Absence and Presence of Bosentan

Fig. 2.3.3 Development of spontaneous myogenic tone in endothelium-denuded mouse septal arteries. Between 60-120 mmHg, vessel constriction in normal PSS was 19-26% (■, n=4). In the presence of 10μM bosentan (▲), vessel constriction within the same pressure range was 20-26% (n=4). Tone was abolished when normal Ca$^{2+}$-containing PSS was substituted with Ca$^{2+}$-free PSS, whence step increases in intraluminal pressure only dilated the vessels (n=4, Fig. 2.3.1A, B).
2.3.2 EFFECT OF ACETYLCHOLINE

Acetylcholine (1 nM-10 μM) caused concentration-dependent vasodilation in septal arteries possessing basal tone (n=3-9, Fig. 2.3.4). At the threshold concentration of 1 nM acetylcholine, there was a detectable relaxation of 0.2±0.1% (% maximum response, n=5). At the highest studied concentration (10 μM), acetylcholine produced 65.3±10.3% relaxation (n=3). Relaxation induced by 10 μM acetylcholine was abolished by pretreatment with 200 μM L-NAME (n=5). In endothelium-denuded arteries, acetylcholine (10 μM) elicited no vasodilatatory response.

2.3.3 ENDOTHELIAL α2-ADRENOCEPTORS AND NORADRENALINE

There was no vascular response to either of the α1-adrenoceptor selective agonists phenylephrine or methoxamine (unpublished data). In comparison, both agents caused concentration-dependent vasoconstriction in size-matched mouse mesenteric arteries (unpublished data).

The α2-adrenoceptor agonist clonidine (1 μM and 10 μM, respectively) caused 22.1±3.1% and 33.5±3.3% relaxation of arteries possessing basal tone (n=4, Fig. 2.3.5). Another α2-adrenoceptor agonist, BHT 920 (1 μM), chosen near its EC50 in rabbit femoral arteries (Garcia-Villalon et al., 1992), caused 25.8±3.7% relaxation of arteries (n=5, Fig. 2.3.5). This response to BHT 920 was abolished after incubation with either 10 μM yohimbine (n=7, Fig. 2.3.5) or 200 μM L-NAME (n=5).
Acetylcholine Concentration-Response Curve

Fig. 2.3.4 Acetylcholine (ACh)-induced concentration-dependent relaxation of septal arteries pressurized at 70 mmHg. Acetylcholine was cumulatively applied to the bath. The vessel was allowed to reach steady-state within a 5 min period prior to subsequent acetylcholine addition.
Table Showing the Effects of Various α- and β-adrenoceptor Ligands on Septal Arteries Pressurized at 70 mmHg

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mechanism of Action</th>
<th>Relaxation (% maximum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clonidine (1 µM)</td>
<td>α2-specific agonist</td>
<td>22.1±3.1 (n=4)</td>
</tr>
<tr>
<td>Clonidine (10 µM)</td>
<td>α2-specific agonist</td>
<td>30.5±3.3 (n=3)</td>
</tr>
<tr>
<td>BHT 920</td>
<td>α2-specific agonist</td>
<td>25.8±3.7 (n=5)</td>
</tr>
<tr>
<td>BHT 920 + yohimbine</td>
<td>α2 + α,β agonist</td>
<td>No response</td>
</tr>
<tr>
<td>BHT 920 + L-NAME</td>
<td>α2 + NOS inhibitor</td>
<td>No response</td>
</tr>
<tr>
<td>Isoprenaline</td>
<td>β agonist</td>
<td>82.2±1.0 (n=5)</td>
</tr>
<tr>
<td>Isoprenaline + propranolol</td>
<td>β agonist</td>
<td>No response</td>
</tr>
<tr>
<td>Isoprenaline + yohimbine</td>
<td>β agonist</td>
<td>77.3±10.2 (n=4)</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>α,β agonist</td>
<td>86.5±3.0 (n=5)</td>
</tr>
<tr>
<td>Noradrenaline + yohimbine</td>
<td>α,β agonist + α antagonist</td>
<td>37.5±3.0 (n=5)</td>
</tr>
<tr>
<td>Noradrenaline + propranolol</td>
<td>α,β agonist + β antagonist</td>
<td>83.1±8.4 (n=5)</td>
</tr>
<tr>
<td>Noradrenaline + prazosin + propranolol</td>
<td>α,β agonist + α1 antagonist + β antagonist</td>
<td>45.0 (n=2)</td>
</tr>
<tr>
<td>Noradrenaline + L-NAME + propranolol</td>
<td>α,β agonist + NOS inhibitor + β antagonist</td>
<td>No response</td>
</tr>
<tr>
<td>Noradrenaline + yohimbine + propranolol</td>
<td>α,β agonist + α antagonist + β antagonist</td>
<td>No response</td>
</tr>
</tbody>
</table>

**Fig. 2.3.5** Effect of various α- and β-adrenoceptor ligands on septal arteries pressurized at 70 mmHg. Concentrations used: BHT 920 = 1 µM; isoprenaline; noradrenaline; prazosin; yohimbine = 10 µM; propranolol = 20 µM; L-NAME = 200 µM.
In septal arteries with basal tone, noradrenaline caused concentration-dependent (1 nM-10 μM) relaxation (n=5, Fig. 2.3.6), with a maximum response of 86.5±3.0% (n=5). In contrast, noradrenaline caused concentration-dependent vasoconstriction of mouse mesenteric arteries (n=5, Fig. 2.3.7), with a maximum response of 100.0±0.0% (n=4). In the coronary arteries, 10 μM yohimbine was partially effective in reducing the response to a single noradrenaline (10 μM) challenge (37.5±3.0%, n=5). Arterial incubation with L-NAME (200 μM) resulted in a slight inhibition of noradrenaline (10 μM)-induced vasodilation (79.7±8.7%, n=3). In arteries pre-incubated with both prazosin (10 μM) and propranolol (20 μM), noradrenaline (10 μM) produced 45.0±2.7% relaxation (n=2, Fig. 2.3.5).

In pressurized endothelium-denuded arteries, neither acetylcholine (10 μM) nor BHT 920 (3 μM) elicited vasodilatory responses (n=4, Fig. 2.3.8).

2.3.4 SMOOTH MUSCLE β-ADRENOCEPTORS AND NORADRENALINE

A 10 μM isoprenaline caused vasodilation (82.2±1.0%, n=5), which was antagonized by 20 μM propranolol (n=5), but unaffected by 10 μM yohimbine (77.3±10.2%, n=4, Fig. 2.3.5). In endothelial-denuded arteries, isoprenaline (10 μM) also induced vasorelaxation (Fig. 2.3.5). In septal arteries pressurized at 70 mmHg, propranolol (20 μM) did not significantly reduce the response to noradrenaline (10 μM, 83.1±8.4%, n=5). Combination of propranolol (20 μM) and yohimbine (10 μM) abolished the response to 10 μM noradrenaline (n=6, Fig. 2.3.5). In addition, the combination of propranolol (20 μM) and L-NAME (200 μM) abolished the response to 10 μM noradrenaline (0.2±1.2%, n=4).
Noradrenaline Concentration-Response Curve in Septal Arteries

Fig. 2.3.6 Noradrenaline (NA)-induced concentration-dependent relaxation of septal arteries pressurized at 70 mmHg (n=5). Noradrenaline was cumulatively applied to the bath. The vessel was allowed to reach steady-state within a 5 min period prior to subsequent noradrenaline addition.
Fig. 2.3.7 Noradrenaline (NA)-induced concentration-dependent constriction of mouse mesenteric arteries pressurized at 70 mmHg (n=4). Noradrenaline was cumulatively applied to the bath. The vessel was allowed to reach steady-state within a 5 min period prior to subsequent noradrenaline addition.
Trace Recording of the Effects of Acetylcholine, BHT 920 and Isoprenaline on Endothelium-denuded Septal Arterial Diameter

Fig. 2.3.8 Trace recording of endothelium-denuded septal arterial diameter. The vessel was pressurized at 70 mmHg. Both a 10 μM acetylcholine challenge and a 3 μM BHT 920 challenge produced no effect. A 10 μM isoprenaline caused vasodilation.
2.3.5 ENDOTHELIUM-DEPENDENT VASODILATORS

The endothelium-dependent vasodilators bradykinin and Substance P were applied on the pressurized arteries. Neither bradykinin (10 μM) nor Substance P (0.1 μM) produced any effect.

3. STUDY 2 - MYOGENIC AND ENDOTHELIAL REGULATION OF TONE IN THE SEPTAL ARTERY OF THE CVB3-INFECTED MYOCARDITIC MOUSE

3.1 HYPOTHESES AND OBJECTIVES

HYPOTHESIS 1: In CVB3-induced murine myocarditis, pressure-induced vascular tone is augmented compared to sham-infected and uninfected mice.

OBJECTIVE 1: To compare pressure-induced constriction (myogenic response) in CVB3-infected versus sham-infected and uninfected mice.

HYPOTHESIS 2: In CVB3-induced murine myocarditis, endothelium-dependent agonist-induced relaxation responses are impaired.

OBJECTIVE 2: To compare the effect of acetylcholine in coronary vessels from CVB3-infected versus sham-infected and uninfected mice.

HYPOTHESIS 3: Inhibition of ET receptors with bosentan will result in greater reduction of myogenic tone in CVB3-infected versus sham-infected and uninfected mice.

OBJECTIVE 3: To compare the degree of inhibition of tone by bosentan in CVB3-infected versus sham-infected and uninfected mice.
3.2 MATERIALS AND METHODS

3.2.1 TISSUE ISOLATION, CANNULATION AND DIAMETER MEASUREMENT

All methods were performed similar to study 1 (see section 2.2.1. above) except that the mice were anesthetized using 100% CO₂ gas. Each mouse to be sacrificed was placed into a plastic chamber with a gas line running into it. The chamber was then closed off, and the gas was turned on, filling the chamber with CO₂. It was turned off when the animal was no longer conscious.

3.2.2 STUDY PROTOCOL

3.2.2.1 INOCULATION

Three groups of male CD-1 mice (6-7 weeks of age) were purchased from Charles River (Quebec, Canada). The sham inoculated treatment group was inoculated intraperitoneally with 2 mL sterile phosphate buffered saline (PBS) and sacrificed at 1, 3, 7 and 28 days post-inoculation. The CVB3-infected treatment group was inoculated intraperitoneally with 1 mL of the Charles Guantt strain of CVB3 (University of Nebraska Medical Centre, Nebraska, USA) diluted in Delbecco’s Modified Eagle’s Media (DMEM) to 1.75 x 10^{10} plaque-forming units (pfu)/mL, and 1 mL sterile PBS. These mice were also sacrificed at the same four time points as above. The third treatment group was a non-treatment (uninfected) that was sacrificed at age-matched time points.
3.2.2.2 EXPERIMENTAL PROTOCOL

In experiments designed to measure the spontaneous myogenic response, pressure-diameter relationships were determined by increasing the intraluminal pressure from 5 to 10 to 20 mmHg, and in 20 mmHg increments to 120 mmHg thereafter. At each pressure, a 5 min stabilization period was allowed during which time the vessel typically achieved a steady state diameter. The same protocol was repeated using vessels pre-incubated with 10 µM bosentan for 30 min.

To investigate agonist-induced vasodilation, acetylcholine (1 nM - 10 µM) was applied cumulatively to the bath at 5 min intervals. A diameter reading was taken after each 5 min incubation period. In experiments using the endothelin receptor antagonist, the vessels were pre-incubated for 30 min with 10 µM bosentan prior to adding acetylcholine cumulatively.

To establish smooth muscle responsiveness to nitric oxide, a single challenge of 10 µM sodium nitroprusside (SNP, a donor of NO) was added to arteries taken from either uninfected or virus-infected mice and relaxation response was quantified.

At the end of each experiment, normal Ca\(^{2+}\)-containing PSS was substituted with Ca\(^{2+}\)-free PSS to determine passive luminal diameter.

3.2.3. EXPRESSION OF RESULTS AND STATISTICAL ANALYSIS

Similar to the previous study, myogenic tone at each pressure was expressed as a percent constriction = 100% x \([(D_{\text{Ca-free}} - D_{\text{PSS}}) / D_{\text{Ca-free}}]\), where D is the diameter in calcium free (D_{\text{Ca-free}}) or Ca\(^{2+}\)-containing PSS (D_{\text{PSS}}). Percent relaxation was calculated using the
equation 100% x \[ ((D_d - D_b)(D_{Ca-free} - D_b)) \], where D is the diameter upon stabilization after drug addition (d), baseline (b) or calcium free.

All results are expressed as mean ± S.E.M. of n animals. One vessel segment was obtained from each animal. Statistical evaluation was made using analysis of variance (ANOVA), and means were considered significantly different when P< 0.05.

3.2.4 SOLUTIONS AND CHEMICALS

The composition of the PBS was (in mM): NaCl 137, KCl 3.1, Na$_2$PO$_4$ 8.51, KH$_2$PO$_4$ 1.76. The PBS was made sterile by passing the solution through a 0.22 μm filter into a sterile 1 L bottle. The composition of the PSS and Ca$^{2+}$-free solutions were made similar to that in study 1. Sodium nitroprusside was purchased from Sigma Chemical Company (St. Louis, MO).

3.3 RESULTS

3.3.1 BASAL TONE

In the uninfected group, pressure-induced myogenic constriction was found to be between 27-31% in the 80-120 mmHg intravascular pressure range (n=5, Fig. 3.3.1). The sham-inoculated group also constricted to a similar extent over this pressure range (n=3-5, Fig. 3.3.2). In addition, there was no significant difference found between sham-infected and uninfected groups (10-120 mmHg). In the CVB3-infected group, myogenic tone was found to be between 22-35% constriction between 80-120 mmHg (n=3-5, Fig. 3.3.3). Thus there were no significant differences in pressure-constriction curves between
the uninfected, sham-infected and CVB3-infected groups in the higher pressure (physiological) range.
Myogenic Tone in Pressurized Septal Arteries of Uninfected Mice

Anesthetized with Carbon Dioxide

Fig. 3.3.1 Development of spontaneous myogenic tone in septal arteries of mice anesthetized with 100% CO$_2$. Intraluminal pressure was increased from 10 mmHg to 20 mmHg, then from 20 mmHg to 120 mmHg in 20 mmHg increments. The vessel was allowed to equilibrate for 5 min at each pressure. Between 80-120 mmHg, vessel constriction in normal PSS was 27-32% (■, n=5).
Fig. 3.3.2 Development of spontaneous myogenic tone in septal arteries of the sham-inoculated mice at timepoints 1 (■, n=5), 3 (▲, n=3), 7 (▼, n=5) and 28 (●, n=4) days post-infection. There was no significant difference at all pressures among all time points.
Myogenic Tone in Pressurized Septal Arteries of CVB3-infected Mice at 1, 3, 7 and 28 days Post-infection

Fig. 3.3.3 Development of spontaneous myogenic tone in septal arteries of CVB3-infected mice at timepoints 1 ( ■, n=4), 3 ( ▲, n=4), 7 ( ▼, n=5) and 28 ( ◆, n=4) days post-infection. There was no significant difference at all pressures among all time points.
Pre-treatment of coronary arteries with 10 µM bosentan reduces pressure-induced constriction of uninfected animals to 22-24% (over 80-120 mmHg, n=3). In comparison, vessels taken from infected mice possessed lesser myogenic tone (10-18% constriction, n=4-5) in the presence of the ET-1 receptor antagonist. This difference was not found statistically significant.

3.3.2 AGONIST-INDUCED RELAXATION RESPONSES

In the uninfected group, maximum relaxation response to acetylcholine (ACh) was 51.3±6.0% relaxation (n=5, Fig. 3.3.4). There were no significant differences in relaxation response curves between uninfected and sham-infected groups (1 nM- 10 µM). There were also no significant differences in acetylcholine concentration-relaxation response curves between uninfected and virus-infected groups at 1, 3 and 7 days post-infection (1 nM- 10 µM). There was, however, a significant difference in relaxation response between the uninfected and virus-infected group at 28 days post-infection. At this time point, responses to all concentrations of acetylcholine were attenuated compared to controls. Acetylcholine (1-10 µM) induced a statistically significant diminished vasodilatory response (n=3-5). Maximum response of these vessels to the highest acetylcholine concentration was only 29.1±8.0% relaxation (n=4, Fig. 3.3.5).

However, relaxation response to acetylcholine fully recovered with pre-incubation of these vessels with 10 µM bosentan, with maximum response to 10 µM acetylcholine producing 72.4±10.3% relaxation (10 µM, n=4, Fig. 3.3.5).

SNP (10 µM) produced 77.7±4.8% relaxation in uninfected mouse septal arteries (n=3). In the infected counterparts, SNP (10 µM) produced 84.7±5.0% relaxation (n=5).
Acetylcholine Concentration-response Curve of 
Arteries Isolated from Uninfected Mice

**Fig. 3.3.4** Acetylcholine (ACh)-induced concentration-dependent relaxation of septal arteries pressurized at 70 mmHg, retrieved from non-infected mice anesthetized with 100% CO₂. Acetylcholine was cumulatively applied to the bath. The vessel was allowed to reach steady-state within a 5 min period prior to subsequent acetylcholine addition. Maximum dilation achieved with 10 μM acetylcholine was 51.2±6.0% (% maximum, n=5).
Effect of Bosentan on the Acetylcholine Concentration-response Curve of Arteries from CVB3-infected Mice Sacrificed at 28 Days Post-infection

Fig. 3.3.5 Acetylcholine (ACh)-induced concentration-dependent relaxation of septal arteries of CVB3-infected mice sacrificed 28 days post-infection, in the absence (■, n=4) and presence (▽, n=4) of 10 μM bosentan. Arteries were pressurized at 70 mmHg.
4. DISCUSSION

4.1 STUDY 1 - MYOGENIC AND ENDOTHELIAL REGULATION OF TONE IN THE HEALTHY MOUSE SEPTAL ARTERY

The mouse is routinely used as a model system to assess cardiovascular diseases, such as atherogenesis (Keidar et al., 1999), thrombosis (Fujihira et al., 1993), myocardial infarction (Harada et al., 1999), chronic heart transplant rejection (Koglin and Russell, 1999), myocarditis (Carthy et al., 1998) and cardiotoxicity (Rosenoff et al., 1975). The first successful genetic manipulation in the mouse in the early 1980’s and the eventual ease of recombinant DNA technology naturally made it an animal of choice for modeling of human disease. However, the validity of the mouse model in assessing human pathophysiology has not been evaluated. Although mice and humans share highly conserved genes that regulate fundamental aspects of cardiovascular morphogenesis (Chien, 1996), there may still be important variability in physiological regulation and responses. For example, it has been reported that the coronary circulation of small animals may not be optimal in the study of coronary angiogenesis and revascularization, due to extensive collateralization (Chien, 1996). Uncertainties about the vasoregulatory states of murine intramural coronary arteries and the applicability of the mouse for modeling abnormalities hypothesized in human hearts, served as a foundation for our observations.

The blood pressure of the mouse is reported to be in the vicinity of 90-100 mmHg (Wang et al., 1997). Our study demonstrated that the mouse septal artery possessed a relatively high degree of myogenic tone within this pressure range. In comparison to the male rat septal artery where maximal tone is between 30-40 % constriction (Wellman et
al., 1996), basal tone in the mouse septal artery is approximately two-fold greater. The blockade of endothelin receptors by bosentan significantly decreased the extent of basal tone, indicating that in the mouse coronary circulation, endothelin is an important regulator of diameter and hence coronary flow. Addition of bosentan to deendothelialized arteries did not alter the extent of myogenic tone (compared to control vessels untreated with bosentan), indicating that basal release of endothelin occurs from endothelial cells. The target for endothelin is endothelin receptors located in smooth muscle cells of the media.

Noradrenaline produced a $\alpha_2$- and $\beta$-adrenoceptor-mediated, concentration-dependent relaxation in the mouse coronary resistance artery. Inhibition of nitric oxide synthase with L-NAME attenuated the vasodilatory response to the selective $\alpha_2$-adrenoceptor agonist BHT 920, thus suggesting that this response is mediated by nitric oxide. These results are in agreement with Thorin et al. (1998) who reported that $\alpha_2$-adrenoceptor mediated relaxation in mesenteric arteries of mice could be antagonized by nitric oxide synthase inhibition. Furthermore, since BHT 920 elicited no response in the endothelium-denuded artery, it was concluded that the $\alpha_2$-adrenoceptors of the mouse coronary artery were endothelium-located.

Interestingly, propranolol alone did not significantly inhibit noradrenaline-induced relaxation. This suggested the presence of a possible convergence of intracellular signaling between the $\alpha_2$- and $\beta$-adrenoceptor-mediated vasodilation pathways, where the inhibition of $\alpha_2$-adrenoceptors would potentially affect the signaling pathway and thus response to noradrenaline by $\beta$-adrenoceptors, but not vice versa.
The concentration of noradrenaline in the coronary circulation at rest is approximately 1.2 nM (Hjemdahl et al., 1983). This same concentration of noradrenaline is reported to cause $\alpha_1$- and $\alpha_2$- adrenoceptor mediated constriction in the isolated human coronary artery, even in the absence of $\beta$-blockade (Saetrum Opgaard and Edvinsson, 1997)(Fig. 4.4.1). It should be noted that a direct comparison of the effects of noradrenaline on the same septal artery of different species using the same technique could not be made, thus we presented, as accurately as possible, the effects of this drug on different coronary arteries. However, we demonstrated that in the isolated mouse septal artery, not only is there is a lack of $\alpha_1$-adrenoceptor response, but noradrenaline (1 nM- 10 $\mu$M) consistently caused vasodilation; both mark important phenotypic species differences in the pharmacology of the coronary circulation.
Table Showing Species Comparison of the Effect of Noradrenaline Applied on Coronary Arteries

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>CORONARY ARTERY</th>
<th>TISSUE PREPARATION</th>
<th>EFFECT OF NA</th>
<th>MEDIATED VIA RECEPTOR TYPE(S):</th>
<th>REFERENCE(S)</th>
</tr>
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<tbody>
<tr>
<td>HUMAN</td>
<td>EPICARDIAL</td>
<td>ISOLATED SEGMENTS</td>
<td>VASOCONSTRICTION AND VASODILATION</td>
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<tr>
<td>RAT</td>
<td>INTRAMYOCARDIAL</td>
<td>ISOLATED SEGMENTS</td>
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<tr>
<td>PIG</td>
<td>RIGHT CORONARY ARTERY</td>
<td>ISOLATED RINGS</td>
<td>VASODILATION</td>
<td>BETA</td>
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</tr>
<tr>
<td>DOG</td>
<td>LARGE CORONARY ARTERIES</td>
<td>ISOLATED RINGS</td>
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<td>ALPHA-1, 2</td>
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</tr>
<tr>
<td>HORSE</td>
<td>UNSPECIFIED</td>
<td>ISOLATED RINGS</td>
<td>VASOCONSTRICTION</td>
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</tr>
<tr>
<td>RABBIT</td>
<td>LEFT ANTERIOR DESCENDING</td>
<td>ISOLATED RINGS</td>
<td>VASODILATION; VASOCONSTRICTION AT HIGH [NA]</td>
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<tr>
<td>MOUSE</td>
<td>SEPTAL</td>
<td>ISOLATED SEGMENTS</td>
<td>VASODILATION</td>
<td>ALPHA-2, BETA</td>
<td>LUI ET AL.</td>
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Fig. 4.4.1 Species comparison of the effect of noradrenaline on various coronary arteries.
Our study also demonstrated that the mediation of acetylcholine-induced vasodilation in the mouse coronary. In comparison to the rat coronary artery, that of the mouse did not fully dilate to 10 μM acetylcholine (unpublished data). However, the relaxation response to acetylcholine in both species occurred via endothelium-derived nitric oxide, as confirmed by the lack of response to acetylcholine in the presence of L-NAME or absence of endothelium. This mechanism of acetylcholine action is similar to that in the isolated human coronary artery. Paradoxically, in human coronary microvessels (Angus et al., 1991) (non-septal) and epicardial vessels (Kalsner, 1985), variable populations of acetylcholine receptors may also exist on the medial smooth muscle, mediating vasoconstriction, countering the role of those on the endothelium which mediate vasorelaxation.

Lastly, the endothelium-dependent vasodilators bradykinin and substance P were found to be inactive in the mouse coronary arteries. In contrast, bradykinin has been reported to relax the coronary arteries of various species including human and dog (Regoli and Barabe, 1980), and is due to both the generation of nitric oxide as well as activation of phospholipase A2 with release of prostaglandin I2. The absence of a vasodilatory response to bradykinin in mouse septal arteries may be an important aspect under pathological conditions in which inflammation occurs. Substance P is also a coronary artery vasorelaxant in the human (Tousoulis et al., 1999), as well as in swine (Uchida et al., 1999). Like bradykinin, substance P causes vasodilation and increased permeability of smooth muscle cells. The role of substance P in the coronary circulation is not fully understood.
In conclusion, the present study elucidated responses of the pressure myograph-mounted mouse coronary artery to pharmacological agents of pertinence to those previously reported in the human coronary artery. Briefly, the isolated mouse septal artery possessed a high degree of myogenic tone to which endothelin contributed significantly. These coronary arteries relaxed to noradrenaline via (endothelium-located) $\alpha_2$- and (smooth muscle-located) $\beta$-adrenoceptors, as well as to acetylcholine via endothelium-derived nitric oxide. However, $\alpha_1$-adrenoceptor selective agonists as well as bradykinin and substance P were found to have no vasoactive effect. Differences between human and murine responses illustrated by these findings may impact on the relevance of the mouse coronary artery as a potential model of human coronary vessel diseases.

4.2 STUDY 2 - MYOGENIC AND ENDOTHELIAL REGULATION OF TONE IN THE MYOCARDITIC MOUSE SEPTAL ARTERY

Although previous studies have reported focal coronary arterial narrowing in myocarditis, our present study did not detect enhanced pressure-induced vasoconstriction in the physiological range at all studied time points. Time points were chosen accordingly to represent the phases and progression of myocarditis. Endothelial dysfunction was detected at 28 days post-infection, manifested by a blunted response to the endothelium-dependent agonist acetylcholine. Interestingly, this response fully recovered in the presence of the mixed endothelin A/B receptor antagonist bosentan.

Small vessel disease is thought to be an important aspect of myocardial injury; microvascular abnormalities exist and likely contribute to the progression of viral myocarditis. Myocarditis may later exacerbate into further complications associated with
congestive cardiomyopathy. Previous studies have shown that, in the acute phase, extensive damage to the aorta, smaller coronary arteries and capillaries could be identified (Burch, 1975). These vascular lesions appeared to be a result of direct viral damage, as viral crystals could be detected adjacent to areas of cytonecrosis. Although one study did not report significant stenosis in the coronary arteries of myocarditic patients (Hasumi et al, 1983), it is now generally accepted that the virus is able to invade the vascular endothelium leading to vascular spasm. In addition, studies have shown lumenal narrowing and endothelial cell swelling and damage, all of which appear noncontiguous and variable in degree. A marked increase in infiltrating mononuclear cells around the microvessels was also noted (Nakamura et al., 1996). A later study confirmed CVB-induced coronary microvascular narrowing (Silver et al., 1989) be treated and successfully reversed by verapamil (Dong et al., 1992). It has been hypothesized that this pattern of focal constriction may be the result of increased production and release of endothelin-1. As a result, this hyperreactive myocardial microcirculation has been suggested as the pathogenic mechanism responsible for ischemia and reperfusion injury, silent myocardial infarction, and ventricular dysfunction (heart failure).

In this study, histological staining with hematoxylin and eosin revealed extensive myocyte damage as well as damage of the spleen, liver and lung. Hematoxylin stains acidic structures, such as nuclei, rough endoplasmic reticulum and ribosomes, a purplish-blue. Eosin stains basic structures, like cytoplasmic proteins, red or pink. In general, when the H&E staining technique is applied to animal cells, nuclei stain blue and the cytoplasm pink or red. However, immunohistochemical methods did not reveal any
significant ET-1 localization around the infected coronary vessels at any studied time points (unpublished data). Septal vessels isolated from uninfected and sham-infected mice anesthetized with carbon dioxide (study 2) possessed lower basal tone than vessels from mice anesthetized with sodium pentobarbital (study 1). We hypothesized that this was likely due to the loss of autoregulation as a direct result of carbon dioxide. Arteries dissected from hearts of CVB3-infected mice did not possess significantly greater basal tone at any studied time point in the physiological blood pressure range as compared to its uninfected and sham infected counterparts. However, slight elevations in myogenic reactivity at the lower pressure points at 1, 3 and 7 days post-inoculation indicated the possible presence of increased levels of circulating vasoconstrictor(s).

In addition, in the presence of bosentan, there was no significant difference in myogenic tone in vessels from uninfected versus CVB3-infected mice (60-120 mmHg), although constriction was globally decreased as compared to untreated vessels. These results suggest that, using our technique of isolation and myography of cardiac resistance arteries, there was no physiological indication of increased production of ET-1 that effectively contributed to greater myogenic reactivity. Interestingly, this study found a significantly decreased vasodilation response to sub-maximal and maximal acetylcholine concentrations in vessels 28 days post-infection as compared to control and sham-infected groups. This diminution, estimated to be approximately 40%, was hypothesized to be the result of endothelial damage and dysfunction. This was confirmed with the use of the nitric oxide donor SNP; SNP-induced vasorelaxation was not impaired in the infected vessels, indicating no smooth muscle dysfunction with respect to signaling and initiation of vasodilation. This abnormal endothelial response is a likely mechanism
involved in and contributing to impaired coronary hemodynamics, and in combination with increased global ET-1 levels, contributes to ischemic myocyte injury and necrosis, eventually leading to cardiac hypertrophy and failure. ET-1 itself also regulates cardiomyocyte growth during hypertrophy (Seta et al., 2000), thus further potentiating the problem. Although this study does not examine acetylcholine response past 28 days post-infection, it would be interesting to continue monitoring endothelial dysfunction to see if responses are further diminished in the chronic stage of disease.

Interestingly, pre-treatment of these vessels with bosentan prior to acetylcholine challenge resulted in complete recovery of the vasodilatory response. ET-1 antagonists have been shown to reduce myocardial infarct size in the ischemic heart (Brunner, 1997). Increased ET-1 levels can lead to hypertrophy; thus, potential treatment using ET-1 antagonists may prove therapeutic. Ono et al. showed that treatment with bosentan resulted in the lowering of both heart weight/body weight ratio as well as histological scores for myocardial necrosis in myocarditic mice (Ono et al., 1999). This finding is in agreement with the hypothesis that ET-1 levels are elevated in myocarditis, likely near the vasculature, and partially contributed to by ET-1 released from endothelial cells, interstitial cells and nearby macrophages.

In conclusion, the present study evaluated the myocarditic coronary artery for enhanced myogenic tone or indication of hyperconstriction. No significant difference in pressure-induced constriction was found between arteries from infected and uninfected mice. With respect to endothelial function, the endothelium-dependent agonist acetylcholine was found to produce a diminished response in vessels isolated 28 days post-infection, reversed by pre-treatment with bosentan. More detailed studies, perhaps with the
inclusion of more immunohistochemical data, may prove beneficial in assessing this condition further.

5. SUMMARY AND MAJOR CONCLUSIONS

5.1 STUDY 1

This study focused on determining the relevance of the mouse model for assessing cardiac disease. This aim was achieved by quantifying myogenic and receptor-mediated responses using the mouse septal artery. These resistance vessels were observed to possess a high degree of pressure-induced constriction. Endothelin acting on endothelin receptors present on the smooth muscle cells contributed significantly to this basal tone. Noradrenaline produced a $\alpha_2$- and $\beta$-adrenoceptor mediated, concentration-dependent vasodilation. Furthermore, the $\alpha_2$-adrenoceptors were found to be located on the endothelium, with the $\beta$-adrenoceptors on smooth muscle cells. This response differs from the pharmacology in the human coronary artery, where noradrenaline induces $\alpha_1$- and $\alpha_2$-mediated vasoconstriction. However, similar to the human coronary artery, acetylcholine produced concentration-dependent relaxation of the coronary arteries, mediated by nitric oxide. Lastly, the endothelium-dependent vasodilators bradykinin and substance P were found to be inactive in this mouse model, indicating yet another significant difference in physiological responses between the human and mouse coronary vasculature.
5.2 STUDY 2

This study compared a model of cardiac disease, namely CVB3-induced myocarditis, to health. Focus was placed on determining and quantifying changes in vascular tone and endothelial function. Although it is hypothesized that increased endothelin-1 levels in myocarditis lead to microvascular narrowing and/or spasm, our technique of myography did not detect changes in myogenic tone in diseased vessels at 1, 3, 7 and 28 days post-infection in the physiological range. However, increased tone was present at early time points at lower pressures. In addition, although extensive myocyte damage was verified, immunohistochemical techniques did not detect high ET-1 levels near the coronary microvasculature. However, endothelial dysfunction was detected in the chronic stage of myocarditis (28 days post-infection). Response to the endothelium-dependent agonist acetylcholine was ameliorated by the endothelin receptor antagonist bosentan.

6. FUTURE DIRECTIONS

Future directions include extension of this study to include data past 28 days post-infection. Since this study only identified a difference at one time point for only one response, it would be interesting to observe a longer time course to see whether response to acetylcholine is further blunted, and whether bosentan proves beneficial in recovering the response. This work is worthwhile since the involvement of ET-1 in chronic heart failure is important, as endothelin receptor antagonists have been reported to increase cardiac output, and decrease left ventricular hypertrophy and dilatation as well as cardiac
fibrosis. Thus bosentan and other similar agents may prove cardioprotective and beneficial in the human heart resulting in an increase long-term survival.

In addition, further investigation to identify the mechanism(s) contributing to the increase in myogenic tone seen in the low pressure range (under 60 mmHg) in the early time points of myocarditis should be made, since this preliminary observation correlates with previous studies reporting peak ET-1 levels between 3-7 days post-infection.

Finally, the regulation of NO production in this disease should also be characterized, as the balance between NO and ET-1 is all-important in affecting and determining the vascular state. This may be achieved by studying NO production and release through each of two pathways, namely α-adrenoceptor and muscarinic receptor activation.
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