University of Alberta

CYTOMEGALOVIRUS AND VASCULAR FUNCTION DURING PREGNANCY

by

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Abstract

Human cytomegalovirus (HCMV) is implicated in several vascular diseases through endothelial dysfunction. Most of the research on CMV has focused on either congenital CMV infections or CMV disease in immunocompromised hosts; however, CMV has also been associated with vascular diseases in immunocompetent individuals, including atherosclerosis and the pregnancy-specific disorder preeclampsia. A direct connection between CMV and vascular dysfunction remains unknown. Therefore, in the presence or absence of an active CMV infection, I chose to study the systemic (mesenteric) and uterine vascular responses in intact, isolated arteries from non-pregnant (NP) and late pregnant (LP) mice. Furthermore, I investigated if a maternal CMV infection leads to poor fetal outcomes, independent of a congenital infection. Viral transmission to the fetus does not occur in the mouse, making it a useful model for studying maternal CMV infections. Sensitivity to the α_1-adrenergic receptor agonist, phenylephrine (PE), was decreased, sphingosine 1-phosphate (S1P)-induced vasodilation was decreased, and cholinergic (methacholine (ME)) vasodilation was increased in mesenteric arteries from CMV-infected NP and LP mice. Nitric oxide (NO) and prostanoid mediation of endothelium-dependent vasodilation were also increased in these arteries. In uterine arteries from CMV-infected NP mice, ME-induced vasodilation was increased with smooth muscle sensitivity to NO, similar to mesenteric arteries from CMV-infected NP mice. In addition, early interactions of CMV with endothelial cells increased sensitivity to ME in the absence of a fully systemic CMV infection. In contrast, PE-induced

vasoconstriction was increased and sensitivity to ME and ME-induced vasodilation were decreased in uterine arteries from CMV-infected LP mice. NO and prostanoid mediation were unaltered in the presence of a CMV infection; hence, decreased EDHF likely contributed to the reduced cholinergic sensitivity and vasodilation. Vascular dysfunction observed in these arteries was dependent on a systemic CMV infection. Finally, the pregnancy outcome was largely affected by genotypic susceptibility to CMV. C57Bl/6J mice were able to compensate for a CMV infection (normal fetal growth) whereas CMV-infected Balb/cJ mice (more susceptible) were infertile. Together, my findings provide evidence that an active CMV infection is a risk factor for vascular and fetal complications during pregnancy and potentially other cardiovascular diseases in the general population.

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Table of Contents

List of Tables

List of Figures

Abbreviations

BSA	Bovine albumin serum
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CMV	Cytomegalovirus
$cPLA_2$	Cytosolic phospholipase A_2
D18.5	Day 18.5
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
ddH_2O	Double-distilled water
E	Early viral gene
EDHF	Endothelium-derived hyperpolarizing factor
G protein	Guanine nucleotide-binding protein
gB	Glycoprotein B
gH	Glycoprotein H
gL	Glycoprotein L
gM	Glycoprotein M
gN	Glycoprotein N
gO	Glycoprotein O
GTP	Guanosine triphosphate
HCMV	Human Cytomegalovirus
ICAM	Intercellular adhesion molecule
IE	Immediate early viral gene
IFN-γ	Interferon-gamma
IP_3	Inositol triphosphate
IUGR	Intrauterine growth restriction
L	Late viral gene
L-NAME	N^G-nitro-L-arginine
LP	Late pregnant
LPP	Lipid phosphatase

MAP	Mitogen-activated serine/threonine protein
mCMV	Mouse Cytomegalovirus
ME	Methacholine
Meclo	Meclofenamate
MLC	Myosin light chain
MOI	Multiplicity of infection
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Normal goat serum
NO	Nitric oxide
NOS	Nitric oxide synthase
NOS-1	Neuronal nitric oxide synthase
NOS-2	Inducible nitric oxide synthase
NOS-3	Endothelial nitric oxide synthase
NP	Non-pregnant
PBS	Phosphate buffered saline
PE	Phenylephrine
PFU	Plaque-forming units
PGH_2	Prostaglandin H_2
PGHS	Prostaglandin H synthase
PGI_2	Prostacyclin
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PKG	cGMP-dependent protein kinase
PMCA	Plasma membrane calcium ATPase
PSS	Physiological saline solution
ROCK	Rho kinase
ROS	Reactive oxygen species
RT-qPCR	Reverse transcriptase real-time polymerase chain reaction
S1P	Sphingosine 1-phosphate
SERCA	Sarcoplasmic reticulum calcium ATPase

SK	Sphingosine kinase
SNP	Sodium nitroprusside
SPP	Sphingosine phosphatase
SR	Sarcoplasmic reticulum
TNF-α	Tumour necrosis factor-alpha
TxA_2	Thromboxane
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelium growth factor

Chapter 1
Introduction

1.1 PROLOGUE

Cardiovascular diseases are the main cause of death in developed countries (Mendez *et al.*, 2001) and are associated with several factors such as obesity, diabetes, and viral infections (Danesh *et al.*, 1997; Eckel *et al.*, 1998; Kannel *et al.*, 1979). Human Cytomegalovirus (HCMV) infections have been implicated in cardiovascular disorders including atherosclerosis, coronary heart disease, and cardiac transplant arteriopathy (Espinola-Klein *et al.*, 2002; Grahame-Clarke, 2005; Petrakopoulou *et al.*, 2004). In addition, HCMV has been associated with pregnancy-specific vascular complications, such as preeclampsia and intrauterine growth restriction (IUGR) (Grahame-Clarke, 2005; Ornoy *et al.*, 2006).

HCMV is the most common congenital viral infection of neonates (Stagno *et al.*, 1977). Consequently, fetal and neonatal complications associated with a congenital HCMV infection have been widely studied (Yinon *et al.*, 2010); however, few studies have investigated the maternal and fetal effects of a CMV infection in the absence of a congenital infection. Hence, I chose to investigate how an active CMV infection affects maternal vascular function during gestation in the absence of viral transmission to the fetus.

In this chapter I will first discuss vascular regulation in normal and pregnant states. I will also describe the differences in experimental model systems used for pregnancy-specific vascular studies. Finally, I will describe CMV infections in general and in relation to vascular disorders and pregnancy.

1.2 REGULATION OF VASCULAR TONE

The cardiovascular system is part of the circulatory system and is comprised of the heart, blood, and a network of blood vessels, including

capillaries and different orders of arteries and veins. It is critical for removing waste and transporting nutrients, gases, and hormones to all the organs in the body (West *et al.*, 1997). Both arteries and veins are composed of the tunica intima, tunica media, and tunica adventitia. The tunica intima is the inner lining of the vessel wall which includes the endothelial cells, basement membrane, and a thin layer of elastic tissue. The tunica media is composed of smooth muscle cells, elastic and collagen fibres, and proteoglycans. Connective tissue consisting of collagen and elastic fibres, fibroblasts, and some macrophages make up the tunica adventitia on the outside of the vessel wall (Cleaver *et al.*, 2003). Vascular tone, defined as the amount of constriction a blood vessel undergoes compared to its maximal dilated diameter (Orshal *et al.*, 2004), is induced by the vascular smooth muscle cells within the tunica media. Arteries have a thicker layer of smooth muscle cells than veins and are therefore capable of greater vascular tone than veins (Rhodin, 1980). In addition, arteries with a thick medial layer and a significant amount of elastic tissue have increased arterial distensibility or elasticity (Kaushal *et al.*, 2002).

Vascular tone is a major determinant of vascular resistance, or the resistance to blood flow within the cardiovascular system (Lautt, 1989). Constricted arteries with increased vascular tone (and vascular resistance) decrease blood flow whereas dilated or relaxed arteries with decreased vascular resistance increase blood flow. Changes in blood flow, regulated by arterial vascular tone/resistance, may cause subsequent changes in blood pressure to maintain homeostasis (Johnson, 1986). Small blood vessels that significantly contribute to peripheral and precapillary resistance and blood flow regulation are known as resistance arteries. They are defined as those arteries with diameters less than 500μm (Christensen *et al.*, 2001; Mulvany *et al.*, 1990).

Vascular tone is controlled by extrinsic and local (intrinsic) factors (Orshal *et al.*, 2004). Extrinsic factors include sympathetic and parasympathetic innervation of the vascular smooth muscle cells (Bevan, 1979; Hamel, 2006) and vasoactive hormones such as angiotensin II, vasopressin, and catecholamines

(Orshal *et al.*, 2004). Local factors that affect vascular tone include metabolic and temperature changes (Burton *et al.*, 1940; Wassink *et al.*, 2007), shear stress on the vessel wall (Dekker *et al.*, 2005), myogenic responses (Davis *et al.*, 1999), and vasoactive molecules released directly from the vasculature (Cardillo *et al.*, 2000). The importance of extrinsic and intrinsic factors to overall vascular tone varies among tissues and different types of climatic and/or physiological (ie. exercise and pregnancy) stimuli. Generally, extrinsic regulation ensures blood pressure is maintained throughout the body and that enough blood reaches the brain; therefore, extrinsic regulation can override local control of vascular tone. However, blood vessels in certain tissues, such as the brain, are less controlled by extrinsic factors and more responsive to local factors compared to other tissues, such as the skin and digestive tract (Brown *et al.*, 2006).

1.2.1 Vascular smooth muscle cell contractility

Vascular tone induced by the vascular smooth muscle cells is modulated by several intracellular signalling pathways. Smooth muscle cell contraction is caused by contractile filaments linked to a phosphorylated myosin light chain (MLC) complex. Intracellular calcium concentration and calcium sensitization in vascular smooth muscle cells are the principal mechanisms that mediate smooth muscle contraction. Increased intracellular calcium binds to the regulatory protein, calmodulin, which then associates with the catalytic subunit of MLC kinase to activate it (Adelstein *et al.*, 1981). Activated MLC kinase then phosphorylates the regulatory light chain of myosin. This allows myosin to interact with actin, thereby stimulating ATP hydrolysis via the myosin ATPase and causing the smooth muscle cell to contract (Sweeney *et al.*, 1994). Cytosolic calcium levels are increased due to an influx of extracellular calcium and/or release of intracellular stores of calcium from the sarcoplasmic reticulum (SR) (Nixon *et al.*, 1995; Somlyo *et al.*, 1991). There are several channels that span the smooth muscle cell plasma membrane which increase calcium influx when stimulated, including voltage-gated calcium channels (L-type and T-type) (Ertel *et al.*, 2000;

Friel *et al.*, 1989), ligand-gated non-selective cation channels (Loirand *et al.*, 1991), and store-operated calcium channels (Xu *et al.*, 2001). In addition, there are other channels or transporters on the plasma membrane that decrease intracellular calcium levels and/or induce hyperpolarization, such as the plasma membrane calcium ATPase (PMCA) (Gonzalez *et al.*, 1996), the sodium/calcium exchanger (dependent on a high extracellular sodium concentration) (Ashida *et al.*, 1987), and potassium channels (Nelson *et al.*, 1995). Channels on the SR that release calcium into the cytoplasm when stimulated include inositol triphosphate (IP_3)-sensitive calcium channels (Malysz *et al.*, 2001) and ryanodine receptors/calcium-release channels (Meissner, 1994); whereas, the SR calcium ATPase (SERCA) promotes calcium influx back into the SR (Martonosi, 1996) and the calcium-binding proteins calsequestrin and calreticulin prevent calcium release from the SR (Laporte *et al.*, 2004; MacLennan *et al.*, 1971; Milner *et al.*, 1991). Together, all of these plasma membrane and SR channels contribute to the regulation of smooth muscle cell contractility.

In addition to intracellular calcium concentration, smooth muscle cell contractility is also regulated by calcium sensitization. In contrast to MLC kinase, which phosphorylates MLC to induce smooth muscle contraction, the enzyme known as MLC phosphatase dephosphorylates MLC and inhibits smooth muscle contraction, even when calcium levels are maintained (Himpens *et al.*, 1989). This mechanism opposes MLC kinase activity and reduces the concentration of phosphorylated MLC (Gong *et al.*, 1992). Besides intracellular calcium levels, the activity of MLC phosphatase and MLC kinase are also controlled by several effector proteins in response to certain stimuli, such as agonists described in section 1.2.3 (Aburto *et al.*, 1993; Sward *et al.*, 2003). The guanosine triphosphate (GTP)-binding protein RhoA/Rho kinase (ROCK) signalling pathway plays a major role in calcium sensitization. RhoA GTPase activation stimulates ROCK which phosphorylates the myosin-binding subunit of MLC phosphatase, thereby inhibiting its activity and increasing calcium sensitization (Kawano *et al.*, 1999; Sakurada *et al.*, 2003). In addition, protein kinase C (PKC), a kinase stimulated by

increased intracellular calcium and diacylglycerol (DAG), phosphorylates an inhibitory protein of MLC phosphatase, CPI-17, which binds to the catalytic subunit of MLC phosphatase (Ito *et al.*, 2004; Niiro *et al.*, 2003; Toth *et al.*, 2000). Other protein kinases that lead to a reduction in calcium sensitization and intracellular calcium levels include cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG) and cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA). Interestingly, in smooth muscle, basal intracellular cAMP levels are higher than cGMP levels. At increased cAMP levels (approximately ten-fold higher than cGMP), cAMP can also activate PKG (Rembold, 1992). PKG and/or PKA phosphorylate and inhibit IP_3-sensitive calcium channels (Komalavilas *et al.*, 1996), RhoA binding to ROCK (Sauzeau *et al.*, 2000), and the phospholamban protein antagonist of SERCA (Cornwell *et al.*, 1991; Lincoln *et al.*, 1991). In addition, PKG and/or PKA have also been shown to phosphorylate and activate the PMCA pump (Gonzalez *et al.*, 1996; Marin *et al.*, 1999), potassium channels (Aiello *et al.*, 1998; Schubert *et al.*, 1996; Standen *et al.*, 1998; Taniguchi *et al.*, 1993; Wu *et al.*, 2007), and MLC phosphatase (Wooldridge *et al.*, 2004).

Different guanine nucleotide-binding heterotrimeric proteins (G proteins) are activated via G protein-coupled receptor stimulation. The α subunit of the G protein, bound to GTP, dissociates from the β and γ subunits at the plasma membrane and activates/deactivates specific downstream cytosolic proteins, enzymes, and molecules (depending on the α subunit type) that regulate cell function. There are four families of Gα proteins: $G\alpha_{i/o}$, $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ (Wettschureck *et al.*, 2005). Activated Gα proteins stimulate intracellular signal transduction pathways that regulate intracellular calcium concentrations and/or calcium sensitization in vascular smooth muscle. The GTP-bound $G\alpha_{i/o}$ protein interacts with and inhibits adenylyl cyclase activity, thereby reducing cAMP production and PKA stimulation and increasing intracellular calcium levels and calcium sensitization (constriction) (Aburto *et al.*, 1993; Heck *et al.*, 1998; Lee *et al.*, 1990; Morgado *et al.*, 2011). Conversely, the activated $G\alpha_s$ protein interacts

with and activates adenylyl cyclase which increases cAMP production, leading to a decrease in cytosolic calcium concentrations and calcium sensitization (relaxation) (McDaniel *et al.*, 1994). The activated $G\alpha_{q/11}$ stimulates phospholipase C which cleaves phosphatidylinositol 4,5-bisphosphate to IP_3 and DAG (Graham *et al.*, 1996; Smrcka *et al.*, 1991). IP_3 stimulates calcium release from the SR through IP_3-sensitive calcium channels, as previously mentioned (Malysz *et al.*, 2001). DAG, along with cytosolic calcium, activates PKC. PKC phosphorylates several downstream targets including CPI-17, resulting in the inhibition of MLC phosphatase (Niiro *et al.*, 2003; Toth *et al.*, 2000). As such, $G\alpha_{q/11}$ in the vascular smooth muscle is associated with constriction. $G\alpha_{12/13}$ in addition to $G\alpha_{q/11}$ can activate RhoA which stimulates ROCK. ROCK inhibits the MLC phosphatase thus increasing calcium sensitization in vascular smooth muscle cells (Table 1.1A) (Chikumi *et al.*, 2002; Sakurada *et al.*, 2003; Sward *et al.*, 2003; Zeng *et al.*, 2002). It is also worth mentioning that the β and γ subunits of G proteins can also interact with and regulate various ion channels (ie. potassium and calcium channels) (Kleuss *et al.*, 1993; Logothetis *et al.*, 1987) and certain isoforms of effector enzymes, such as adenylyl cyclase (Tang *et al.*, 1991) and phospholipase C (Camps *et al.*, 1992; Katz *et al.*, 1992; Murthy *et al.*, 1996; Wettschureck *et al.*, 2005); however, less research has been performed on these G protein subunits in association with vascular smooth muscle contractility regulation. G proteins stimulated in the vascular endothelium can also induce changes in vascular tone (Table 1.1B) which will be further described in section 1.2.2.

A

Gα protein in VSMC	Intracellular calcium response	Vascular response
$G\alpha_{i/o}$	↑Ca^{2+} ↑Ca^{2+} sensitization	Constriction
$G\alpha_{s}$	↓Ca^{2+} ↓Ca2+ sensitization	Relaxation
$G\alpha_{q/11}$	↑Ca^{2+} ↑Ca^{2+} sensitization	Constriction
$G\alpha_{12/13}$	↑Ca^{2+} sensitization	Constriction

B

Gα protein in EC	Intracellular calcium response	Vascular response
$G\alpha_{i/o}$	↑Akt-mediated NOS-3 phosphorylation (Ca^{2+}-independent)	Relaxation
$G\alpha_{s}$	No effect	No effect
$G\alpha_{q/11}$	↑Ca^{2+}	Relaxation
$G\alpha_{12/13}$	No effect	No effect

Table 1.1: Vascular responses following guanine nucleotide-binding protein (G protein) activation. Gα proteins in vascular smooth muscle cells (VSMC) lead to an increase or decrease calcium levels and/or calcium sensitization to stimulate vasoconstriction or vasorelaxation, respectively **(A)**. The $G\alpha_{q/11}$ and $G\alpha_{i/o}$ proteins in endothelial cells (EC) induce vasorelaxation in either a calcium-dependent or -independent manner, respectively (NOS activation) **(B)**.

1.2.2 Stimulated local vasoactive molecules released from the endothelium

Although there are several extrinsic and local factors that regulate vascular smooth muscle cell contractility and play an important role in blood flow regulation, I have focused on describing how certain intrinsic vasoactive substances affect vascular tone in isolated arteries. These include nitric oxide (NO), prostanoids, and endothelium-derived hyperpolarizing factors (EDHF) (Figure 1.1) (Cardillo *et al.*, 2000; Orshal *et al.*, 2004).

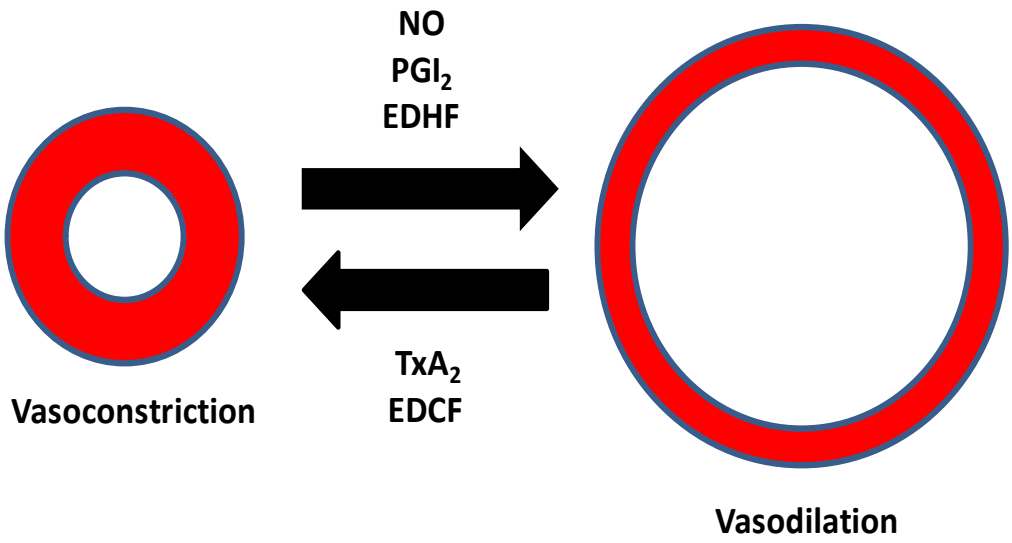


Figure 1.1: Local vasoactive substances that regulate vascular tone. Vasodilation is induced by nitric oxide (NO), vasodilatory prostanoids such as prostacyclin (PGI_2), and other endothelium-derived hyperpolarizing factors (EDHF). Vasoconstriction is induced by vasoconstrictory prostanoids, such as thromboxane (TxA_2), and other endothelium-derived contracting factors (EDCF), such as endothelins.

1.2.2.1 Nitric oxide

NO is primarily recognized as an endothelium-derived vasodilator but also functions as a neurotransmitter (Schuman *et al.*, 1991). In the vascular smooth muscle cells, NO interacts with soluble guanylyl cyclase (Davignon *et al.*, 2004).

As described previously, this enzyme converts GTP to cGMP (Potter, 2011), which activates PKG. Activated PKG leads to a decrease of intracellular calcium levels and calcium sensitization (Figure 1.2A) (Morgado *et al.*, 2011). Potential mechanisms affected by PKG-induced phosphorylation include increased calcium sequestration (Cornwell *et al.*, 1991; Komalavilas *et al.*, 1996; Schlossmann *et al.*, 2000), calcium efflux (Gonzalez *et al.*, 1996; Marin *et al.*, 1999; Rashatwar *et al.*, 1987), and MLC phosphatase activity (Lee *et al.*, 1997; Nakamura *et al.*, 2007), decreased calcium influx (Liu *et al.*, 1997; Taguchi *et al.*, 1997), and inhibition of RhoA-induced calcium sensitization (Morgado *et al.*, 2011; Sauzeau *et al.*, 2000).

In addition to vasodilation, NO also increases vascular permeability and angiogenesis (Fukumura *et al.*, 2001; Murohara *et al.*, 1998; Ziche *et al.*, 1997) and reduces endothelial leukocyte adhesion (Khan *et al.*, 1996; Kubes *et al.*, 1991) and platelet aggregation (Radomski *et al.*, 1990). As such, NO is extremely important for proper vascular endothelial function, control of vascular tone, and ultimately blood pressure regulation. Under pathological conditions, such as inflammatory vascular diseases, endothelial dysfunction occurs with increased reactive oxygen species (ROS) and subsequent oxidative stress and reduced NO bioavailability (Montezano *et al.*, 2011). NO formation may be reduced due to NO synthase (NOS) uncoupling, leading to further ROS production (Montezano *et al.*, 2011). In addition, excessive ROS production and oxidative stress may couple NO with superoxide to produce peroxynitrite, a cytotoxic ROS that can damage intracellular DNA and proteins (Griendling *et al.*, 2003; Szabo, 1996).

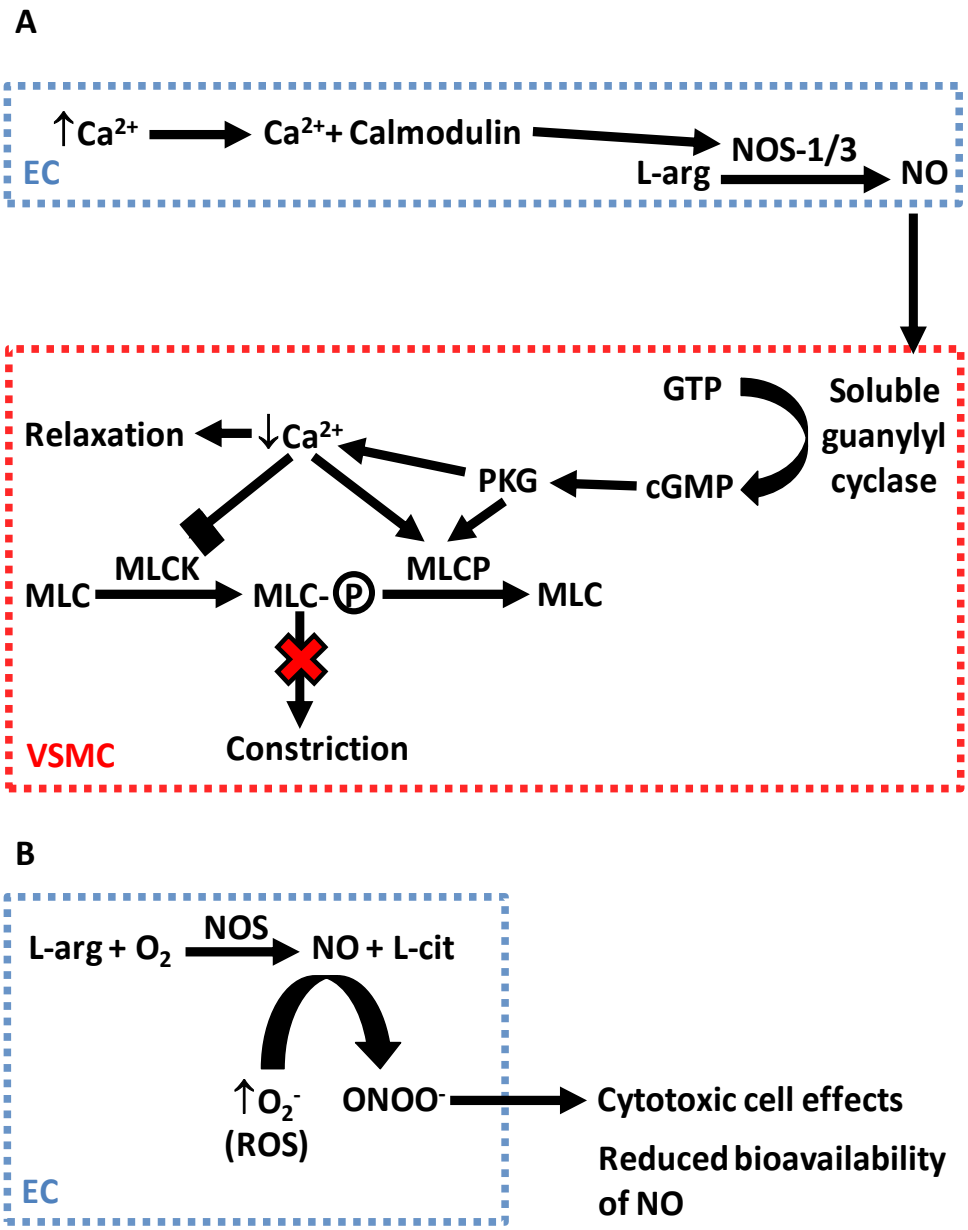

A
↑Ca2+
Ca2+ + Calmodulin
NOS-1/3
L-arg
NO
EC
GTP
Soluble guanylyl cyclase
Relaxation
↓Ca2+
PKG
cGMP
MLCK
MLCP
MLC
MLC-P
MLC
VSMC
Constriction
B
L-arg + O2
NOS
NO + L-cit
↑O2-
(ROS)
ONOO-
Cytotoxic cell effects
Reduced bioavailability of NO
EC

Figure 1.2: NO signalling from the endothelial cells across into the vascular smooth muscle cells in the presence or absence of reactive oxygen species (ROS). Vasorelaxation results from a decrease in both vascular smooth muscle cell calcium levels and calcium sensitization **(A)**. NO bioavailability is reduced with increased ROS **(B)**. L-arg: L-arginine; L-cit: L-citrulline; NOS: nitric oxide synthase; GTP: guanosine triphosphate; cGMP: cyclic guanosine; PKG: cGMP-dependent protein kinase; MLCK: myosin light chain kinase; MLCP: myosin light chain phosphatase; MLC-P: phosphorylated myosin light chain; O_2^-: superoxide; $ONOO^-$: peroxynitrite; EC: endothelial cell; VSMC: vascular smooth muscle cell.

NO and L-citrulline are produced from L-arginine and oxygen by three NOS enzymes (NOS-1/2/3) in the presence of several cofactors (ie. tetrahydrobiopterin and reduced nicotinamide adenine dinucleotide phosphate (NADPH)) (Korhonen *et al.*, 2005; Michel *et al.*, 1997; Palmer *et al.*, 1988). NOS-1 was first discovered in neuronal tissue whereas NOS-2 was originally purified from macrophages. NOS-3, the last to be identified, was initially isolated from vascular endothelial cells. Generally, NO-induced vasodilatory effects on the vascular smooth muscle are believed to come from rapidly diffusible endothelium-derived NO produced from NOS-3 (Furchgott *et al.*, 1980; Luscher *et al.*, 1997). However, in addition to NOS-3, NOS-1 and NOS-2 have also been identified in vascular endothelial and smooth muscle cells from specific arteries. The expression levels of each NOS enzyme depends on the origin of the blood vessel (Brophy *et al.*, 2000; Buchwalow *et al.*, 2002; Michel *et al.*, 1997). NOS-1 has been found in human umbilical vein endothelial cells (Lekontseva *et al.*, 2011) and endothelial and smooth muscle cells from large human renal, pancreatic, and coronary arteries (Brophy *et al.*, 2000; Buchwalow *et al.*, 2002). In addition, NOS-1/3 have been identified in endothelial and smooth muscle cells from rat mesenteric and uterine microvascular arteries (Scott *et al.*, 2007). NOS-2/3 have been identified in endothelial and smooth muscle cells from porcine carotid arteries and human pancreatic arteries (Buchwalow *et al.*, 2002). NO

produced in the vascular smooth muscle cells may interact directly with soluble guanylyl cyclase to activate PKG and induce smooth muscle cell relaxation. Nevertheless, it remains controversial if vascular smooth muscle cell-generated NO is sufficient to induce vasorelaxation independent of endothelium-provided NO (Brophy *et al.*, 2000; Buchwalow *et al.*, 2002; Schwarz *et al.*, 1999; Zehetgruber *et al.*, 1993).

All three NOS enzymes are activated when calcium-activated calmodulin binds to its specific domain on the NOS enzyme. NOS-3 can also be activated in a calcium-independent manner upon serine phosphorylation (Butt *et al.*, 2000; Ferro *et al.*, 2004; Fleming *et al.*, 1997). NOS-2 has a higher affinity for calmodulin than NOS-1/3 and as such, may not require increased intracellular calcium to bind calmodulin (Michel *et al.*, 1997; Stuehr, 1999). During inflammation, activated leukocytes (ie. macrophages) have increased NOS-2 activity and NO release (Coleman, 2001). Although NO is an important player in the immune system (decreases leukocyte adhesion and accumulation), increased NOS-2 activity and NO formation during inflammation also leads to an increase in peroxynitrite formation (Figure 1.2B) (Kubes *et al.*, 1991; Liu *et al.*, 1998). Therefore, NOS-2 activity may be beneficial or hazardous, depending on the types of cells it interacts with (infected versus normal) (Poon *et al.*, 2003).

1.2.2.2 Prostanoids

Eicosanoids include prostaglandins, prostacyclin (PGI_2), thromboxanes, and leukotrienes. They have several different physiological functions that include decreasing platelet aggregation (Moncada *et al.*, 1977), mediating inflammatory cytokine production (Williams *et al.*, 1997), and regulating vascular tone (Markwald *et al.*, 2011). The diverse roles of eicosanoids are mediated by a wide range of receptors (Halushka *et al.*, 1989). Prostaglandins, PGI_2, and thromboxanes fall into the subclass of eicosanoids known as prostanoids. Prostanoids are synthesized by prostaglandin H synthase (PGHS)-1/2 (Figure 1.3) (FitzGerald, 2002; Smith *et al.*, 1996). PGHS-1 is known as the constitutive

isoform whereas PGHS-2 expression is induced by the ubiquitous transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) (Smith *et al.*, 1996; Sugino *et al.*, 2004). NF-κB is a heterodimer composed of two subunits: p50 and p65. In its inactive form, NF-κB is bound to IκB and is localized to the cytoplasm. Phosphorylation of IκB leads to its release of NF-κB and subsequent IκB proteasomal degradation. This allows nuclear translocation and activation of NF-κB via the nuclear localization signal on the p65 subunit. NF-κB has a rapid response time since it does not require *de novo* protein synthesis to become functional (Baeuerle *et al.*, 1994; Janssen *et al.*, 1997; Kim *et al.*, 2004a). It is primarily stimulated in response to stress or pathogens (Schreck *et al.*, 1991; Tak *et al.*, 2001). Some examples of stimuli that activate NF-κB and induce PGHS-2 expression include inflammatory cytokines (ie. tumour necrosis factor (TNF)-α), lipoproteins, and hypoxia (Davidge, 2001; Ji *et al.*, 1998; Kniss *et al.*, 2001). Although both vascular endothelial and smooth muscle cells express PGHS-1/2, levels are much higher in endothelial cells (Armstrong *et al.*, 2004; DeWitt *et al.*, 1983; Schildknecht *et al.*, 2005).

Following an increase in intracellular calcium, phosphorylated cytosolic phospholipase A_2 ($cPLA_2$) is translocated to the perinuclear and/or endoplasmic reticular membrane where it breaks down glycerophospholipids into arachidonic acid and lysophospholipids (Bonventre, 1992; Chen *et al.*, 2008; Murakami *et al.*, 1998; Peters-Golden *et al.*, 1996; Schievella *et al.*, 1995; Smith *et al.*, 1991). Released arachidonic acid is converted to prostaglandin H_2 (PGH_2) by PGHS-1/2 at the perinuclear membrane and endoplasmic reticulum. PGH_2 can then be further converted by specific synthases into different prostaglandins as well as the vasodilator PGI_2 and the vasoconstrictor thromboxane (TxA_2) (Figure 1.3) (Davidge, 2001; Funk, 2001). Although several prostanoids can affect vascular tone, PGI_2 and TxA_2 have been thoroughly studied due to their association with cardiovascular diseases (Belton *et al.*, 2000; FitzGerald *et al.*, 1984; Tan *et al.*, 2007). PGI_2 synthase and TxA_2 synthase are both expressed in the vascular endothelium. PGI_2 and TxA_2 are released from the endothelium via facilitated

transport to interact with specific smooth muscle cell receptors. Therefore, the vascular response depends on the balance between their production and receptor activation (Camacho *et al.*, 2000; Funk, 2001; Tazawa *et al.*, 1996). In addition to endothelium-derived TxA_2, platelets and macrophages are other major sources of circulating TxA_2, which leads to an increase in vasoconstriction during an inflammatory response (Collins *et al.*, 2001; Kabashima *et al.*, 2003).

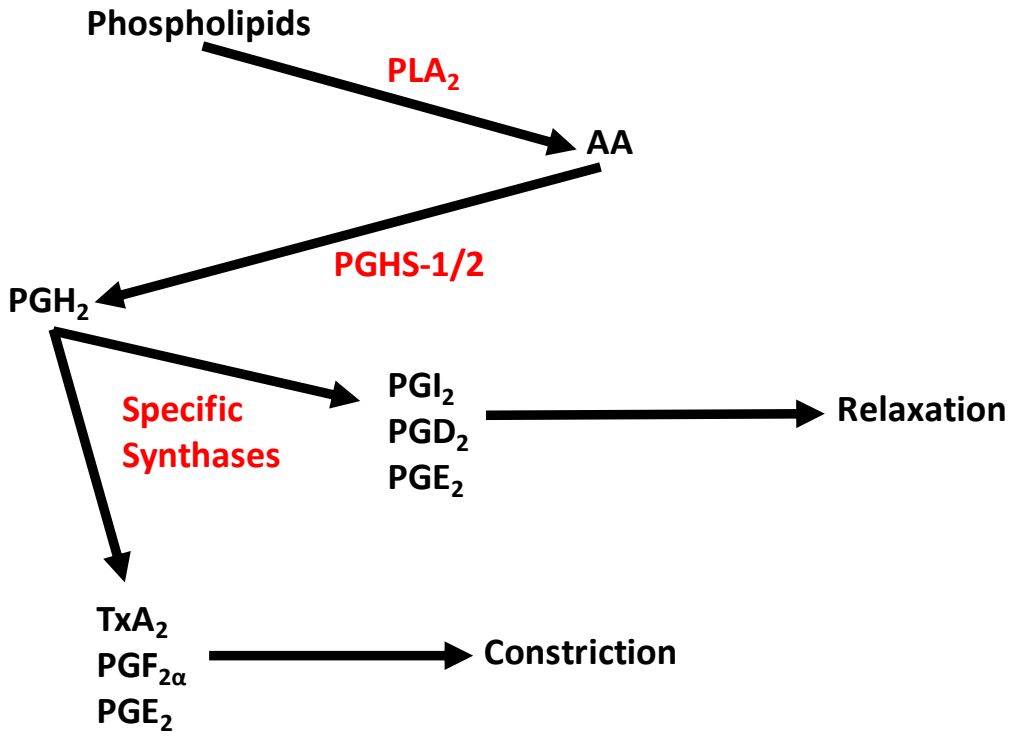


Figure 1.3: Synthesis of prostanoids. PLA_2: phospholipase A_2; AA: arachidonic acid; PGHS-1/2: prostaglandin H synthase-1/2; PGH_2: prostaglandin H_2; PGI_2: prostacyclin; PGD_2: prostaglandin D_2; PGE_2: prostaglandin E_2; TxA_2: thromboxane; $PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$.

The PGI_2 receptor is a G protein-coupled receptor predominantly associated with the G protein α subunit, $G\alpha_s$ (Nobles *et al.*, 2005). Stimulation of the PGI_2 receptor and the $G\alpha_s$ protein leads to the activation of adenylyl cyclase

which forms cAMP. cAMP activates PKA and PKG (at ten-fold-higher cAMP concentrations), which leads to a decrease in intracellular calcium levels and calcium sensitization in vascular smooth muscle cells (relaxation), as described previously (Table 1.1A) (McDaniel *et al.*, 1994; Morgado *et al.*, 2011; Rembold, 1992). The TxA_2 receptor is coupled to the G protein α subunits, $G\alpha_{q/11}$ and $G\alpha_{12/13}$. Activated $G\alpha_{q/11}$ stimulates phospholipase C which leads to downstream events including an increase in intracellular calcium release, calcium influx, and calcium sensitization (Graham *et al.*, 1996; Himpens *et al.*, 1990; Zelis *et al.*, 1989). $G\alpha_{q/11}$ and $G\alpha_{12/13}$ can also activate the GTP-binding protein RhoA which stimulates ROCK. As previously mentioned, ROCK inhibits the MLC phosphatase thus increasing calcium sensitization and maintaining calcium-induced vasoconstriction in vascular smooth muscle cells (Chikumi *et al.*, 2002; Sakurada *et al.*, 2003; Sward *et al.*, 2003; Zeng *et al.*, 2002). Consequently, TxA_2 acts as a potent vasoconstrictor (Table 1.1A) (Hanasaki *et al.*, 1990; Narumiya *et al.*, 1999; Sward *et al.*, 2003; Wilson *et al.*, 2005). Interestingly, TxA_2 binds to the same receptor as its precursor, PGH_2 (a vasoconstrictor also released from endothelial cells), and it is therefore difficult to differentiate between their vascular effects (Davidge, 2001; Gresele *et al.*, 1991; Hanasaki *et al.*, 1990; Karim *et al.*, 1996; Vezza *et al.*, 2002).

1.2.2.3 Endothelium-derived hyperpolarizing factors

Aside from NO and vasodilatory prostanoids (ie. PGI_2), other components of endothelium-derived relaxation include EDHF. EDHF are substances and/or electrical ions that are produced and released from the hyperpolarized endothelium to induce hyperpolarization of the underlying vascular smooth muscle cells. EDHF-mediated responses involve an increase in endothelial calcium levels and subsequent activation of calcium-dependent potassium channels. As such, EDHF is regulated by the intracellular calcium concentration. Endothelial hyperpolarization is electrically coupled to vascular smooth muscle cells via myoendothelial gap junctions (endothelial to smooth muscle cell gap

junctions) (Feletou, 2011; Feletou *et al.*, 2006; Fleming, 2000). Potassium channels are opened, the sodium/potassium-ATPase is activated, and voltage-gated calcium channels are closed in the vascular smooth muscle cells leading to vasorelaxation (Nagao *et al.*, 1993; Weston *et al.*, 2002).

In addition to NO, EDHF is largely involved in shear stress-induced vasodilation, particularly in smaller, resistance-sized arteries (Li *et al.*, 2003; Takamura *et al.*, 1999; Zhao *et al.*, 2005). Shear stress is the frictional or parallel force applied on the vascular wall, such as that induced by laminar blood flow (Li *et al.*, 2003; Traub *et al.*, 1998). Examples of EDHF include potassium ions (Beny *et al.*, 2000), hydrogen peroxide (Stankevicius *et al.*, 2003), and hydrogen sulphide (Li *et al.*, 2009), but there are several other potential candidates (Figure 1.4) (Mombouli *et al.*, 1997).

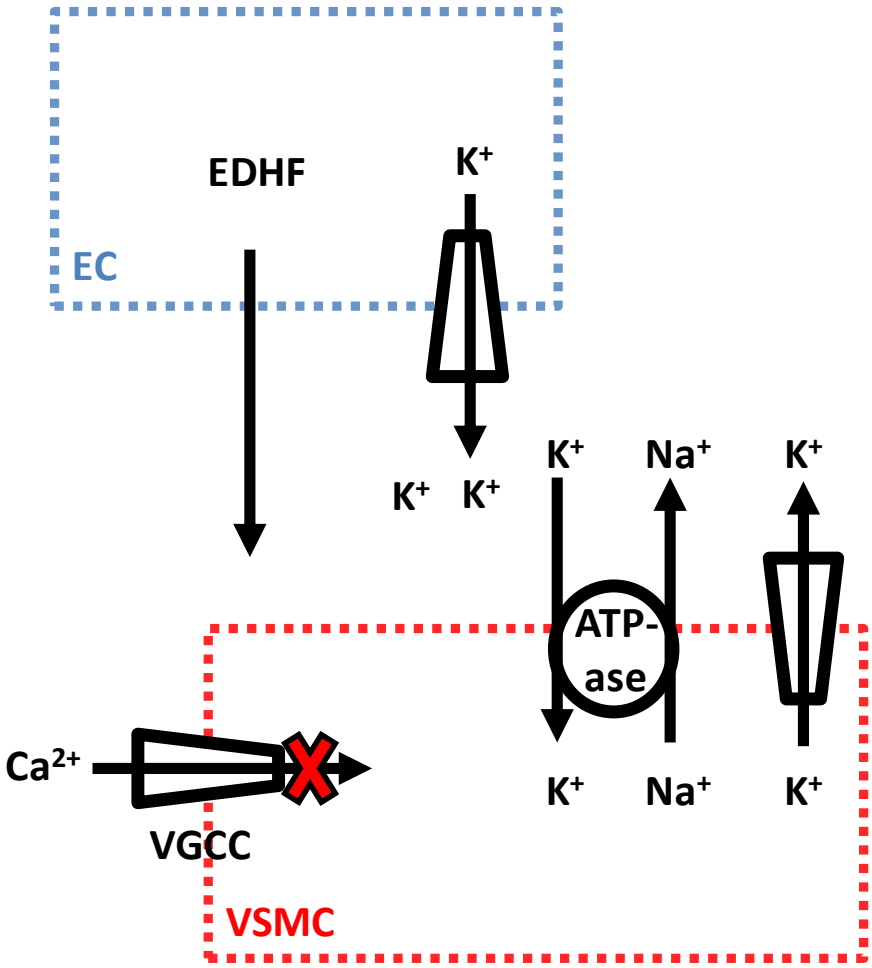


Figure 1.4: Endothelium-derived hyperpolarizing factor (EDHF)-induced vasorelaxation pathway. Endothelial hyperpolarization spreads to the vascular smooth muscle cells by activating sodium/potassium-ATPases, opening potassium channels, and closing VGCC (voltage-gated calcium channels).

1.2.3 Extracellular agonists that affect vascular intracellular signalling pathways

There are several vascular agonists that activate different signalling pathways via specific receptors on the vascular endothelial and smooth muscle cells. These include neurotransmitters such as adrenergic agonists (norepinephrine and epinephrine) and the cholinergic agonist acetylcholine, peptides, and bioactive phospholipids (sphingosine 1-phosphate; S1P). Some of these agonists,

such as acetylcholine and S1P, interact with functionally different receptors on the endothelial and/or smooth muscle cells which can have dual effects on vascular responses (Igarashi *et al.*, 2009).

1.2.3.1 Adrenergic agonists

The catecholamines, norepinephrine and epinephrine, are primarily secreted from the adrenal glands (Zhou *et al.*, 1995), postganglionic fibres throughout the sympathetic nervous system (Levy, 1971), and neurons within the brainstem (Talman *et al.*, 1980). They may act as neurotransmitters or as hormones in the circulation (Elenkov *et al.*, 2002; Roth *et al.*, 1982). Psychological or environmental stressors, such as the 'fight or flight' response, bright lights, or loud sounds, increase the production of catecholamines (Dimsdale *et al.*, 1980). This causes physiological changes including an increase in blood pressure and heart rate (Knight *et al.*, 2001). Norepinephrine and epinephrine have relatively short half-lives (few minutes) in circulation and are degraded by the catechol-O-methyltransferase and monoamine oxidase enzymes (Kvetnansky *et al.*, 1975; Mathew *et al.*, 1980; Roberts *et al.*, 1979).

Norepinephrine and epinephrine interact with α- and β-adrenergic receptors on vascular smooth muscle cells. Norepinephrine primarily binds to α-adrenergic receptors, particularly the α_1-adrenergic receptors, to induce vasoconstriction. The α_1-adrenergic receptors are predominantly coupled to the G protein α subunits, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ (Graham *et al.*, 1996; Sward *et al.*, 2003). As mentioned previously, activated $G\alpha_{q/11}$ and $G\alpha_{12/13}$ increase intracellular calcium release, calcium influx, and calcium sensitization (Graham *et al.*, 1996; Himpens *et al.*, 1990; Sward *et al.*, 2003; Zelis *et al.*, 1989; Zeng *et al.*, 2002). The α_2-adrenergic receptors are coupled to the $G\alpha_{i/o}$ protein which inhibits adenylyl cyclase activity and increases calcium influx and calcium sensitization (Aburto *et al.*, 1993; Heck *et al.*, 1998; Lee *et al.*, 1990; Morgado *et al.*, 2011). Although epinephrine also binds α-adrenergic receptors, it binds β-adrenergic receptors with greater affinity (Graham *et al.*, 1996; Hoffmann *et al.*, 2004). Activation of the β-

adrenergic receptor and $G\alpha_s$ stimulates adenylyl cyclase activity and cAMP production, activates PKA and PKG, and leads to a decrease in intracellular calcium levels and calcium sensitization in vascular smooth muscle cells (Table 1.1A) (Benovic *et al.*, 1988; McDaniel *et al.*, 1994; Rembold, 1992). Therefore, norepinephrine acts as a vasoconstrictor whereas epinephrine acts more as a vasodilator.

1.2.3.2 Cholinergic agonist

The neurotransmitter, acetylcholine, is produced in neurons from choline and acetyl-CoA via choline acetyltransferase and acts on the central, sympathetic, and parasympathetic nervous system (Arvidsson *et al.*, 1997; Blusztajn *et al.*, 1983; Hebb, 1972). In addition, acetylcholine and/or its synthesizing enzyme choline acetyltransferase are also found in non-neuronal cells including blood lymphocytes and leukocytes, epithelial cells (skin and airway), and vascular endothelial cells (Hecker *et al.*, 2009; Rinner *et al.*, 1998; Wessler *et al.*, 2003a; Wessler *et al.*, 1998; Wessler *et al.*, 2003b). Acetylcholine has a very short half-life (seconds) due to the efficiency of its ubiquitous degrading enzyme acetylcholinesterase (Hecker *et al.*, 2009; Wessler *et al.*, 1999). During inflammatory conditions, acetylcholine (released from the parasympathetic efferent vagus nerve and immune cells) has been implicated as an anti-inflammatory mediator that reduces pro-inflammatory cytokine production (Davis *et al.*, 1998; de Jonge *et al.*, 2007; Hecker *et al.*, 2009; Pavlov *et al.*, 2006; Pavlov *et al.*, 2003; Wessler *et al.*, 2003b).

Acetylcholine interacts with two different types of cholinergic receptors: nicotinic and muscarinic (M) receptors. Nicotinic receptors are ligand-gated ion channels that, in response to acetylcholine binding, become permeable to sodium, potassium, chloride and calcium ions (Bertrand *et al.*, 1993; Stroud *et al.*, 1990; Vernino *et al.*, 1992). These receptors are primarily present on neuronal cells, the postsynaptic end of the neuromuscular junctions, some epithelial cells, and leukocytes where they have been linked to reduced inflammatory cytokine

production as part of the cholinergic anti-inflammatory pathway (Borovikova *et al.*, 2000; de Jonge *et al.*, 2007; Elhusseiny *et al.*, 2000; Maus *et al.*, 1998; Razani-Boroujerdi *et al.*, 2008).

M receptors are G protein-coupled receptors. They are highly expressed at the neuromuscular junction in the parasympathetic and some sympathetic neuronal pathways but are also found on leukocytes and vascular endothelial and smooth muscle cells (McCorry, 2007; Razani-Boroujerdi *et al.*, 2008; Walch *et al.*, 2001). In vascular endothelial and smooth muscle cells, acetylcholine binds to specific G protein-coupled M receptors to regulate vascular tone which is not observed with nicotinic receptor stimulation (Elhusseiny *et al.*, 2000). There are five types of M receptors ($M_{1\text{-}5}$). $M_{1/3/5}$ signal through $G\alpha_{q/11}$ whereas $M_{2/4}$ signal through $G\alpha_{i/o}$. Of the five types of M receptors, M_3 is the main receptor expressed on vascular endothelial and smooth muscle cells (Walch *et al.*, 2001). Recently it has been shown that the ubiquitous transcription factor NF-κB regulates the transcription of the *M_3* gene through NF-κB-specific DNA-binding sites (Forsythe *et al.*, 2002; Paula *et al.*, 2010).

Ligand activation of M_3 releases $G\alpha_{q/11}$ and leads to increased intracellular calcium levels, similar to the α_1-adrenergic receptors (Caulfield *et al.*, 1998). At high acetylcholine concentrations, M_3 and $G\alpha_{q/11}$ on the vascular smooth muscle cells are stimulated leading to vasoconstriction. However, at lower physiological concentrations acetylcholine acts as a vasodilator. In contrast to smooth muscle cells, increased endothelial calcium levels following M_3 activation can increase the production and release of vasodilatory substances including NO, PGI_2, and/or EDHF (Table 1.1A,B) (Caulfield, 1993; Hammarstrom *et al.*, 1995; Norel *et al.*, 1996; Pesic *et al.*, 2009; Toda, 1983).

1.2.3.3 S1P

S1P, bound to lipoproteins and albumin in circulation, is a vasoactive sphingolipid that signals through five G protein-coupled receptors ($S1P_1$-$S1P_5$) (Kimura *et al.*, 2001; Murata *et al.*, 2000; Sanchez *et al.*, 2004). It regulates cell

proliferation and migration, angiogenesis, immune function, permeability, and vascular tone, depending on which S1P receptors circulating S1P interacts with (Hudson *et al.*, 2007; Igarashi *et al.*, 2009; Payne *et al.*, 2004; Saba *et al.*, 2004; Spiegel *et al.*, 2002). S1P is also suggested to act as an anti-apoptotic intracellular messenger (although many intracellular S1P targets have not been clearly defined) (Olivera *et al.*, 1999; Pyne *et al.*, 2010; Spiegel *et al.*, 2003a). Sphingosine is produced from ceramide and is phosphorylated by sphingosine kinase (SK) to form S1P. Two isoforms of SK exist which differ in tissue distribution, expression during development, and substrate affinity: SK-1 and SK-2. Regulation and function of SK-1 is better understood than SK-2 (Alemany *et al.*, 2007; Maceyka *et al.*, 2005; Melendez *et al.*, 2000). SK-1 localizes to subcellular domains including the plasma membrane, endoplasmic reticulum, and nucleus or may be excreted from the cell to produce extracellular S1P from circulating substrates. SK-2, on the other hand, localizes to the nucleus or cytosol, which varies among cell types. Most sphingosine is found within the membrane compartments and therefore this is where most of the SK-induced production of S1P occurs (Alemany *et al.*, 2007; Pyne *et al.*, 2009). S1P levels (and SK-1 activity) are increased by several stimuli including growth factors (ie. platelet-derived growth factor and nerve growth factor), other G protein-coupled receptor agonists (acetylcholine, bradykinin, and S1P), and inflammatory cytokines (ie. TNF-α and interleukins) (Alemany *et al.*, 2007; Meyer zu Heringdorf *et al.*, 1999; Pettus *et al.*, 2003; Pyne *et al.*, 1996; Rius *et al.*, 1997; Xia *et al.*, 1999). Pitson et al. showed that following stimulatory agonist exposure, SK-1 activity is increased due to mitogen-activated serine/threonine protein (MAP) kinase-mediated phosphorylation of SK-1 at a site-specific serine residue. This specific serine residue was determined using site-directed mutagenesis in human embryonic kidney cells *in vitro.* Pitson et al. further demonstrated that translocation of SK-1 to the cell membrane compartments is dependent on this site-specific phosphorylation of SK-1 (Pitson *et al.*, 2003).

Lipid phosphatases (LPP1, LPP2, and LPP3), S1P phosphatases (S1PP1 and S1PP2), and S1P lyase break down S1P and regulate its levels (Pyne *et al.*, 2000). LPPs are membrane proteins that act intracellularly or as ectoenzymes to dephosphorylate intracellular or circulating S1P, respectively (Brindley *et al.*, 2009; Jia *et al.*, 2003). S1PPs and S1P lyase are localized to the endoplasmic reticulum (Pyne *et al.*, 2009). Interestingly, platelets do not express S1P lyase and erythrocytes lack both S1P lyase and S1PPs. Hence, these cells are sources of high amounts of circulating S1P (Ito *et al.*, 2007; Tani *et al.*, 2005). It has also been recently indicated that vascular endothelial cells contribute to plasma S1P levels. Furthermore, in mouse embryonic endothelial cells, laminar shear stress decreased S1P lyase and S1PP1 mRNA expression (Venkataraman *et al.*, 2008).

S1P excreted from the cell (likely through ATP-binding cassette transporters) can interact with its receptors in an autocrine/paracrine fashion (Kim *et al.*, 2009; Pyne *et al.*, 2000; Spiegel *et al.*, 2003b; Venkataraman *et al.*, 2008). Circulating S1P in plasma measured using high-performance liquid chromatography in mice was shown to have a half-life of approximately 15 minutes (Igarashi *et al.*, 2008; Venkataraman *et al.*, 2008). Primary cellular sources of circulating S1P include platelets, erythrocytes, and vascular endothelial cells, as mentioned previously (Ito *et al.*, 2007; Tani *et al.*, 2005; Venkataraman *et al.*, 2008). The normal S1P plasma concentration is 0.1-1.2μM; however, with an activated immune response (platelet-stimulation) and/or increased vascular shear stress (endothelium-stimulation) this concentration can rise up to and above 5μM (Graeler *et al.*, 2002; Shikata *et al.*, 2003; Venkataraman *et al.*, 2008).

In the microvasculature, $S1P_1$ and $S1P_3$ are expressed on the vascular endothelial and smooth muscle cells whereas $S1P_2$ is found mainly on the smooth muscle cells (Peters *et al.*, 2007). $S1P_2$ expression is also present on endothelial cells from large, conduit arteries such as the aorta (Rizza *et al.*, 1999). $S1P_4$ and $S1P_5$ are generally not detectable in the vasculature (Peters *et al.*, 2007); however, $S1P_4$ mRNA has recently been found in human umbilical vein endothelial cells (Heo *et al.*, 2009). $S1P_2$ and $S1P_3$ are expressed on the vascular smooth muscle

cells, and interestingly, it has been demonstrated that both receptors couple to the $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ proteins (Table 1.2A) (Ancellin *et al.*, 1999; Sanchez *et al.*, 2004; Van Brocklyn *et al.*, 2002). Therefore, stimulation of these receptors increases intracellular calcium levels and activates ROCK (increases calcium sensitization) in smooth muscle cells (Murakami *et al.*, 2010; Sanchez *et al.*, 2004; Watterson *et al.*, 2005). $S1P_2$ is coupled more efficiently to $G\alpha_{12/13}$ whereas $S1P_3$ is coupled more efficiently to $G\alpha_{q/11}$ (Ancellin *et al.*, 1999; Okamoto *et al.*, 2000; Okamoto *et al.*, 2011; Sanchez *et al.*, 2007; Sugimoto *et al.*, 2003). On the vascular endothelium, stimulation of $S1P_1$ and $S1P_3$, coupled to the $G\alpha_{i/o}$ protein, activates NOS-3 via Akt kinase-mediated phosphorylation. Stimulation of $S1P_3$, coupled to $G\alpha_{q/11}$ on the endothelium, also increases intracellular calcium levels and NOS activity (Table 1.2B) (Datar *et al.*, 2010; Hemmings, 2006; Hemmings *et al.*, 2006; Hudson *et al.*, 2007; Kim *et al.*, 2004b; Kouretas *et al.*, 1998; Michell *et al.*, 1999). It has been suggested that in intact arteries, S1P induces vasoconstriction when interacting primarily with smooth muscle cells and vasodilation when it interacts mainly with the endothelium (Hemmings, 2006; Igarashi *et al.*, 2009); however, this has not been clearly shown (Figure 1.5). Furthermore, S1P-induced vascular responses differ among vascular beds which is likely attributed to differences in S1P receptor expression (Igarashi *et al.*, 2009).

A

S1P receptor on VSMC	Gα protein coupled to S1P receptor	Vascular response
$S1P_1$	$G\alpha_{i/o}$	Constriction
$S1P_2$	$G\alpha_{i/o}$ $G\alpha_{q/11}$ $G\alpha_{12/13}$	Constriction
$S1P_3$	$G\alpha_{i/o}$ $G\alpha_{q/11}$ $G\alpha_{12/13}$	Constriction

B

S1P receptor on EC	Gα protein coupled to S1P receptor	Vascular response
$S1P_1$	$G\alpha_{i/o}$	Relaxation
$S1P_3$	$G\alpha_{i/o}$ $G\alpha_{q/11}$ $G\alpha_{12/13}$	Relaxation

Table 1.2: Vascular responses following stimulation of vascular S1P receptors and G protein activation. Gα proteins associated with $S1P_{1-3}$ on the vascular smooth muscle cells stimulate vasoconstriction **(A)**. $S1P_{1/3}$ expressed on the vascular endothelial cells induce vasorelaxation primarily via the $G\alpha_{i/o}$ and $G\alpha_{q/11}$ proteins **(B)**.

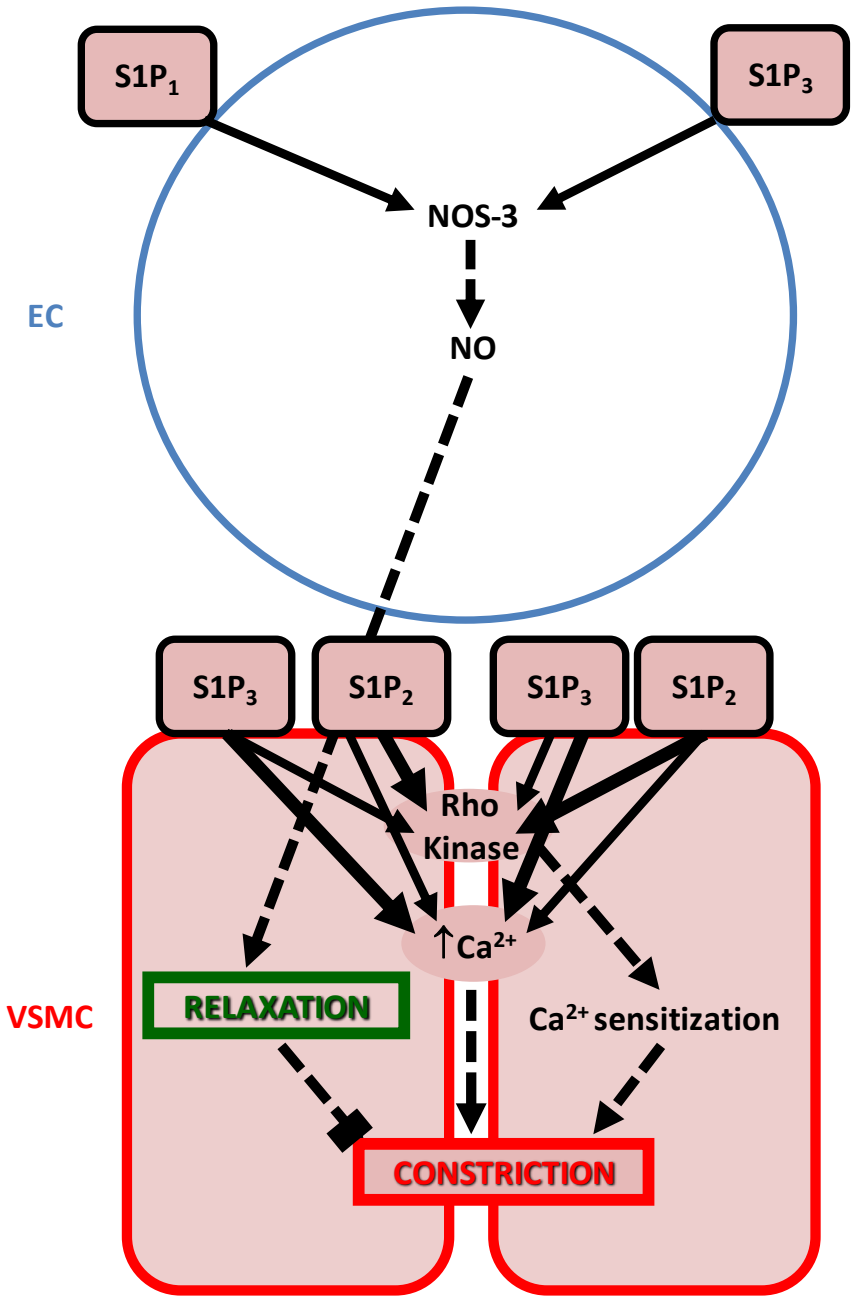


Figure 1.5: Vascular S1P receptors that can stimulate vasorelaxation and/or vasoconstriction responses. Vasoconstriction can be stimulated by $S1P_{2/3}$ on the vascular smooth muscle cells with increased calcium and calcium sensitization; whereas, vasorelaxation can be stimulated by $S1P_{1/3}$ on the endothelial cells with increased NO production.

At normal circulating S1P concentrations (0.1-1.2μM) (Graeler *et al.*, 2002; Venkataraman *et al.*, 2008), $S1P_1$ activation stimulates the endothelial barrier pathway (Wang *et al.*, 2009; Zhang *et al.*, 2010). Following $S1P_1$ stimulation, the small GTP-binding protein Rac1 is activated in a $G\alpha_{i/o}$-dependent manner leading to several events that reduce endothelial permeability, including endothelial junction and focal adhesion assembly and cortical actin formation (Lucke *et al.*, 2010; Wang *et al.*, 2009). In contrast, stimulation of $S1P_2$ and $S1P_3$ increases endothelial permeability by activating the RhoA/ROCK pathway and reducing Rac1 activity, thereby disrupting junction formation (Lucke *et al.*, 2010; Sanchez *et al.*, 2007; Singleton *et al.*, 2006). This increase in permeability occurs when circulating S1P levels approach or rise above 5μM, such as in times of inflammation and platelet activation (McVerry *et al.*, 2005; Shikata *et al.*, 2003).

1.3 PREGNANCY-INDUCED VASCULAR ADAPTATIONS

Extrinsic and intrinsic factors that regulate vascular tone as described previously also play a role in vascular adaptations that occur during pregnancy. These adaptations include vascular remodelling, increased capacity for vasodilation, and decreased sensitivity to vasoconstrictors. Pregnancy is associated with increased plasma volume and cardiac output and decreased systemic and uterine vascular resistance. Blood flow to the uterine vascular bed in human pregnancy increases 50- to 70-fold (Palmer *et al.*, 1992; Thornburg *et al.*, 2000; Zamudio *et al.*, 1995). Uterine vascular remodelling occurs in association with this increase in blood flow (Palmer *et al.*, 1992). Tissue cell culture and animal models (ie. sheep, guinea pigs, rats, mice) have been extensively used to delineate physiological changes that underlie vascular remodelling during pregnancy. Due to increased hypertrophy and hyperplasia of cells within the vascular wall illustrated with immunohistochemistry (Cipolla *et al.*, 1994; Hees *et al.*, 1987) and increased protein content (Annibale *et al.*, 1990), cell number (Keyes *et al.*, 1996), or DNA synthesis (Keyes *et al.*, 1997; Keyes *et al.*, 1996),

the lumen diameter and cross-sectional area of the main uterine artery and smaller arcuate and radial arteries are significantly increased (Osol *et al.*, 2009). During early pregnancy, fetal trophoblast cells migrate into resistance spiral arteries within the uterine wall proximal to the placenta where they replace much of the endothelium and smooth muscle to further reduce vascular resistance and increase blood flow (Adamson *et al.*, 2002; Blechner *et al.*, 1974; Caluwaerts *et al.*, 2005; Hees *et al.*, 1987). In addition, increased expression of growth factors within the uteroplacental unit (ie. vascular endothelium growth factor (VEGF)) (Cheung *et al.*, 1995; Reynolds *et al.*, 1995) and increased uterine arterial vasodilation mediated by NO and PGI_2 have been shown in pregnancy (Cooke *et al.*, 2003; Jovanovic *et al.*, 1997; Magness, 1991; Sladek *et al.*, 1997; Weiner *et al.*, 1989). In contrast, the sensivitity to several vasoconstrictors, including angiotensin II, norepinephrine, and neuropeptide Y, is decreased in uteroplacental arteries isolated from humans and animal models during pregnancy (Jovanovic *et al.*, 2000; Magness *et al.*, 1992; Paller *et al.*, 1989; Thaler *et al.*, 2005; Weiner *et al.*, 1991). Together, these changes either help accommodate increased uterine blood flow or contribute to it.

Along with these uterine vascular adaptations, the systemic vasculature also has to compensate for the increase in cardiac output and blood flow during pregnancy. Decreased systemic vascular resistance and increased systemic vasodilation occur in relation to reduced responsiveness to vasoconstrictors, such as angiotensin II, norepinephrine, and vasopressin (Harrison *et al.*, 1989; Landau *et al.*, 2002; Magness, 1998; Magness *et al.*, 1986; McLaughlin *et al.*, 1989; Paller, 1984; Thaler *et al.*, 2005), and increased endothelium-dependent vasodilation to vascular agonists including acetylcholine and bradykinin (Dantas *et al.*, 1999; Knock *et al.*, 1996). This change in vascular reactivity may be due to pregnancy-dependent changes in receptor expression, such as an increase in receptors that signal vasodilation responses and a decrease in receptors that cause vasoconstriction (Chanrachakul *et al.*, 2003; Ferreira *et al.*, 2009; Stennett *et al.*, 2009). However, blood pressure is only minimally reduced during pregnancy due

to sympathetic innervation of the systemic vascular smooth muscle cells and increased angiotensin II plasma levels (Conrad *et al.*, 1992; Pan *et al.*, 1990; Thaler *et al.*, 2005). These systemic and uterine vascular adaptations act together to maintain cardiovascular homeostasis during pregnancy.

1.3.1 Vascular complications during pregnancy

Failure of the uterine and systemic vasculature to adapt during pregnancy contributes to complications such as preeclampsia (Magness *et al.*, 1994; Ong *et al.*, 2005; Roberts *et al.*, 2005) and IUGR (Carbillon *et al.*, 2000; Ng *et al.*, 1992). Preeclampsia is diagnosed as the *de novo* onset of hypertension (blood pressure >140/90 mmHg) with proteinuria after 20 weeks gestation; however, other characteristics include an enhanced inflammatory state, platelet activation, generalized vasoconstriction, and reduced organ perfusion (Buhimschi *et al.*, 1995; Friedman *et al.*, 1991). IUGR occurs when fetuses do not reach their full growth potential (Danielian *et al.*, 1992). It is a major complication of pregnancy with both short-term and long-term consequences (Pallotto *et al.*, 2006). Reduced uterine blood flow can lead to IUGR in animal models (Anderson *et al.*, 2005; Sholook *et al.*, 2007) and in humans (Lang *et al.*, 2003). For example, even modest reductions of uterine blood flow in a sheep model (<25%) leads to IUGR (Lang *et al.*, 2003). These data underline the importance for normal vascular adaptations in both uterine and systemic arteries during pregnancy.

Infusion of the NOS inhibitor L-nitro-arginine methyl ester into pregnant rats increased mean arterial blood pressure and decreased pup weight, which also occur during preeclampsia and IUGR, respectively (Edwards *et al.*, 1996; Yallampalli *et al.*, 1993). Furthermore, Roggensack et al. illustrated that immunohistochemistry staining of nitrotyrosine (a marker for peroxynitrite activity) and NOS-3 is increased and the superoxide dismutase antioxidant enzyme is decreased in subcutaneous vessels obtained during a caesarean section from women with preeclampsia compared to normal pregnant women (Roggensack *et al.*, 1999). These results suggest that peroxynitrite formation is

increased and the bioavailability of NO is decreased in the systemic vasculature of women with preeclampsia. Further evidence of increased ROS during preeclampsia includes increased lipid peroxidation products (ie. malondialdehyde) in plasma taken from women with preeclampsia (Hubel, 1999; Hubel *et al.*, 1989; Walsh, 1994). In addition to preeclampsia, IUGR in the presence and absence of preeclampsia has also been associated with increased oxidative stress. Markers of lipid peroxidation and DNA damage caused by oxidative stress are increased in placental tissue from women with IUGR and/or IUGR with preeclampsia compared to normal pregnancies (Takagi *et al.*, 2004). It has previously been reported that increased oxidative stress causes vascular dysfunction in the placenta, such as with preeclampsia, and may reduce uteroplacental blood flow enough to significantly compromise fetal growth (Lang *et al.*, 2003; Myatt *et al.*, 2000; Trudinger *et al.*, 1985).

Imbalanced prostanoid production has also been linked to preeclampsia and IUGR. The ratio of TxB_2 (stable metabolite of TxA_2) to 6-keto-prostaglandin $F_{1\alpha}$ (stable metabolite of PGI_2) is increased in urine from women with preeclampsia (Kraayenbrink *et al.*, 1993; Mills *et al.*, 1999). A transgenic rat model of preeclampsia with an activated renin-angiotensin system and IUGR demonstrated an increased TxB_2 to 6-keto-prostaglandin $F_{1\alpha}$ ratio in the serum, increased vasoconstriction to an α_1-adrenergic agonist, and impaired acetylcholine-induced vasodilation compared to normal pregnant and non-pregnant rats (Verlohren *et al.*, 2008). Moreover, a rodent model of IUGR has been developed by infusing a synthetic TxA_2 analogue throughout the last half of pregnancy, suggesting that increased TxA_2 also has the ability to reduce uteroplacental blood flow and fetal growth (Hayakawa *et al.*, 2006). Decreased endothelium-dependent vasodilation to bradykinin (Knock *et al.*, 1996) and acetylcholine (McCarthy *et al.*, 1993) and increased angiotensin II-induced vasoconstriction (AbdAlla *et al.*, 2001) have also been shown in systemic arteries from women with preeclampsia (Zahumensky, 2009). This is likely due to impaired endothelium-dependent vasodilation mechanisms and/or increased

vascular smooth muscle vasoconstriction mechanisms (Khalil *et al.*, 2002; Roberts *et al.*, 2002). In addition, although little is known about how S1P mediates maternal vascular adaptations during pregnancy, increased S1P in plasma samples obtained in early human pregnancy has been identified as one of several biomarkers in a multivariate predictive model for preeclampsia and for babies born small for gestational age (Horgan *et al.*, 2011; Kenny *et al.*, 2010).

1.3.2 Experimental model systems

Many studies investigating vascular function, as mentioned above, use *in vivo* and/or *ex vivo* techniques in different experimental model systems. Common *in vivo* techniques include blood pressure (ie. the tail cuff method and implanted telemetry monitoring systems) or blood flow (ie. ultrasonic flow sensors) measurements alone or in combination with intra-arterial and intravenous injections of different vasoactive drugs (Bischoff *et al.*, 2000; Kurtz *et al.*, 2005; Salomone *et al.*, 2003). The two most common *ex vivo* methods used to measure vascular function are wire and pressure myography. The wire myograph system involves mounting a blood vessel ring segment onto wire hooks in an organ bath chamber and recording the tension of the blood vessel under isometric conditions in response to vasoactive drugs. Drugs being tested are administered directly to the bath where they interact with the vascular endothelium and smooth muscle cells simultaneously. The pressure myograph system, on the other hand, involves mounting a blood vessel onto glass cannulas in an organ bath chamber and measuring changes in lumen diameter under isobaric conditions in response to vasoactive drugs. It is a closed system and therefore drugs added to the bath directly interact only with the vascular smooth muscle cells initially. Drugs may also be infused into the vessel to interact directly with the endothelium using this system (Angus *et al.*, 2000; Halpern *et al.*, 1984).

Several different animal models have been used for pregnancy-specific vascular studies to help solve human physiological questions including non-human primates, sheep, guinea pigs, rats, and mice (Cooke *et al.*, 2003; Hennessy

et al., 1999; Magness *et al.*, 1986; Osol *et al.*, 1993; Weiner *et al.*, 1991). Each model has advantages and disadvantages for pregnancy research. Non-human primates, such as the baboon, have similar uterine biology, placentation, and gestational development compared to humans. At the same time, they have long gestational periods (184 days) and are very expensive to maintain (Carter, 2007; Powers *et al.*, 2008). Sheep, although genetically distinct from primates, are tolerant to invasive surgery during pregnancy and their fetuses have similar weights to humans. However, their gestational period is also long (144-150 days), their placentation is different from primates, and they are expensive to keep (Carter, 2007; Thatcher *et al.*, 1986). Of all the rodent models, guinea pigs share the most commonalities with human pregnancy. Their gestational period has three trimesters (66 days) and fetal and placental development undergo similar events to humans (Carter, 2007). In addition, guinea pigs normally deliver only 2-4 pups per litter, which is much closer to the human situation than the 6-12 pups delivered per litter by rats and mice (Eckstein *et al.*, 1955; Fraser *et al.*, 1951). Nevertheless, the guinea pig is very sensitive to stress and handling during pregnancy and since they take up more space, the costs to house guinea pigs versus other rodents is higher (Carter, 2007). Mice and rats both have short gestations (19-22 days) and can give birth to over 10 pups per litter (Fraser *et al.*, 1951; Kwong *et al.*, 2000). Similar to the guinea pig and primates, the mouse and rat share with humans what is known as hemochorial placentation, whereby maternal and fetal blood are separated by a single trophoblast cell layer at late gestation. Unlike the sheep with an epitheliochorial placenta (separation of maternal and fetal blood by several cell layers), hemochorial placentation supports a more efficient gas and nutrient exchange during pregnancy (Moll *et al.*, 1975; Osol *et al.*, 2009). The rodent uterus, although morphologically different from the human uterus, contains many of the same arteries (main uterine, arcuate, and radial arteries) present in the human uterus and also undergo similar vascular adaptations during pregnancy to support the significant increase in blood volume (Figure 1.6) (Osol *et al.*, 2009). The greatest gain in fetal weight in mice and rats

occurs in late pregnancy (within the 3-4 days prior to term) (Eisen, 1976). Placental development, however, differs between the mouse and rat model. Trophoblast invasion in the mouse is shallow and vascular remodelling is less extensive compared to both rats and humans (Carter, 2007; Georgiades *et al.*, 2002; Osol *et al.*, 2009). Therefore, vascular remodelling and trophoblast invasion studies are not favourable in the mouse model. Nevertheless, mice have a fast generation time, are small, easy to handle, and relatively inexpensive to house. In addition, the mouse genome contains many genes that are analogous to humans, such as those involved in placental development (Rossant *et al.*, 2001), and until recently, transgenic and knockout mice were much more readily available than knockout rat models (Zan *et al.*, 2003). In this way, the choice of animal model depends on what aspect of pregnancy one is interested in and the cost to do the study.

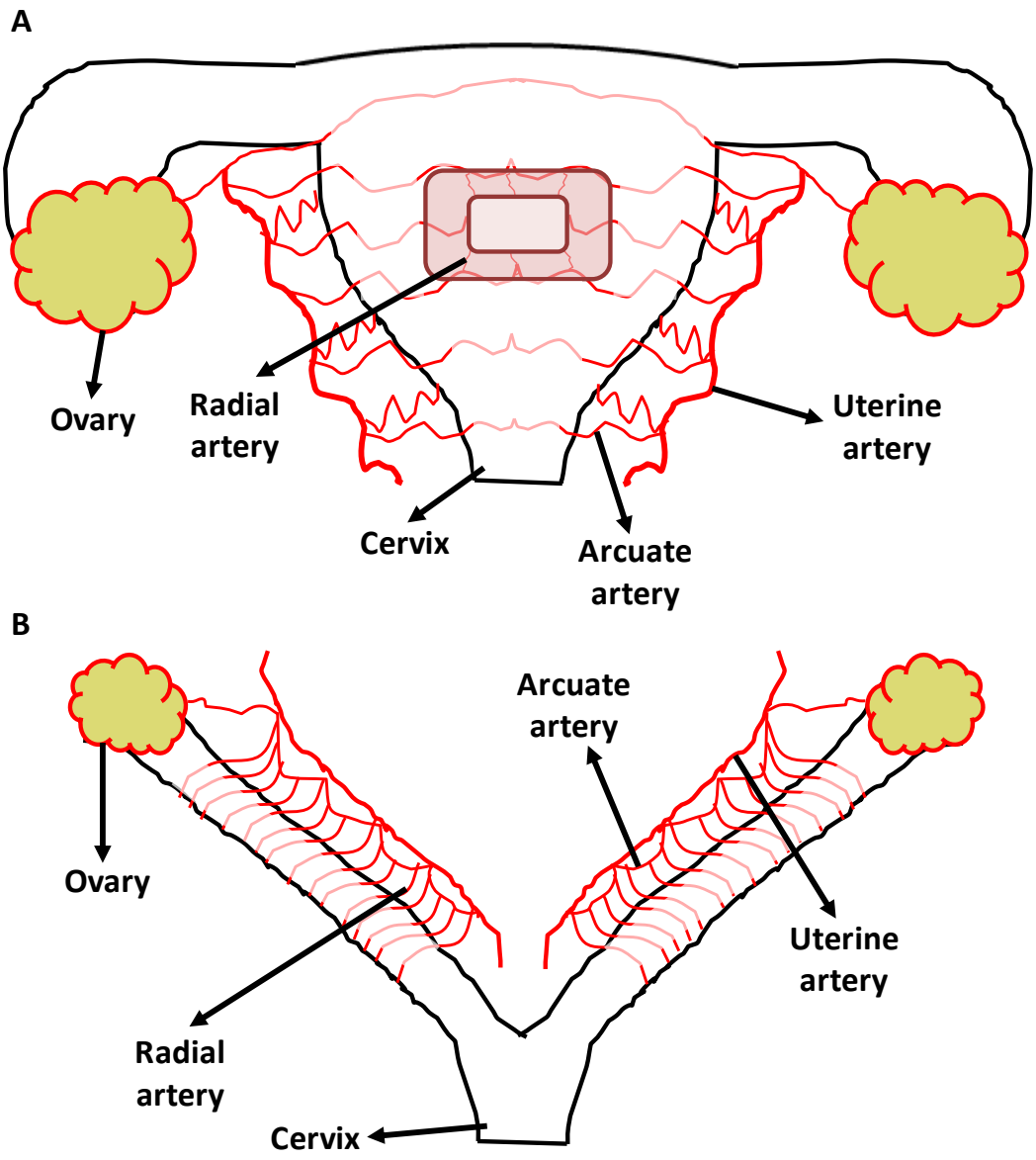


Figure 1.6: Uteri from the non-pregnant human and rodent. Both the human **(A)** and rodent **(B)** uterus contain uterine arteries, arcuate arteries, and radial arteries that supply blood to the uterus and undergo vascular adaptations to permit increased blood flow during pregnancy. Figure adapted from Osol *et al.*, 2009.

1.4 CYTOMEGALOVIRUS

Vascular diseases in both the non-pregnant and pregnant population have

been associated with specific bacterial and viral infections, including HCMV (Danesh *et al.*, 1997; Grahame-Clarke, 2005; Weis *et al.*, 2003). CMV is a genomically large, double-stranded DNA virus that belongs to the *Herpesviridae* family. It is species-specific and has been found in several mammals including mice, chimpanzees, and humans (Davison *et al.*, 2009). HCMV is widespread, infecting 40 to 80 percent of the human population. CMV attaches to, infects, and replicates within a wide range of cell types including epithelial, endothelial, and smooth muscle cells (Billstrom Schroeder *et al.*, 2002; Speir *et al.*, 1996). Although lymphocytes and neutrophils do not support full CMV replication, they may traffic CMV through the circulation (Pancholi *et al.*, 2004).

There are up to 12 different CMV envelope glycoproteins, six of which have been definitively implicated in CMV attachment to the cell surface and/or entry into the cell (Compton, 2004; Lopper *et al.*, 2002). Heparan sulfate proteoglycans are ubiquitously expressed on the cell surface and function in tethering the virus to the cell. They are required but not sufficient for CMV infection. All of the receptors expressed on the host cell surface that are required for CMV attachment and entry are still being established; however, epidermal growth factor receptor, toll-like receptors, and integrins are other potential cell-specific candidates (Compton, 2004; Isaacson *et al.*, 2007; Wang *et al.*, 2005; Wang *et al.*, 2003). Heparan sulphate proteoglycans form a complex with the glycoprotein M/N (gM/gN) heterodimer (Compton, 2004; Compton *et al.*, 1993). Membrane fusion/entry of CMV into the cell requires the gH/gL/gO heterotrimer (Compton, 2004). In addition, gB is also involved in viral attachment (Compton, 2004; Lopper *et al.*, 2002) and is required for entry of the virus into the cell (Bender *et al.*, 2005; Isaacson *et al.*, 2009; Laquerre *et al.*, 1998; Lopper *et al.*, 2002). By binding to cell surface receptors (ie. toll-like receptors), gB stimulates intracellular signalling pathways that prepare the cell for infection and activate the host early immune response (Boehme *et al.*, 2004; Simmen *et al.*, 2001). This includes the stimulation of kinases and transcription factors (ie. NF-κB), inflammatory cytokine secretion (ie. interferon (IFN)-γ and interleukin),

eicosanoid synthesis, and ROS production (Figure 1.7) (Akira, 2003; Boehme *et al.*, 2004; Boyle *et al.*, 1999; Compton *et al.*, 2003; Fortunato *et al.*, 2000; Lee *et al.*, 2011; Murayama *et al.*, 1998; Speir *et al.*, 1996; Speir *et al.*, 1998; Yurochko *et al.*, 1997).

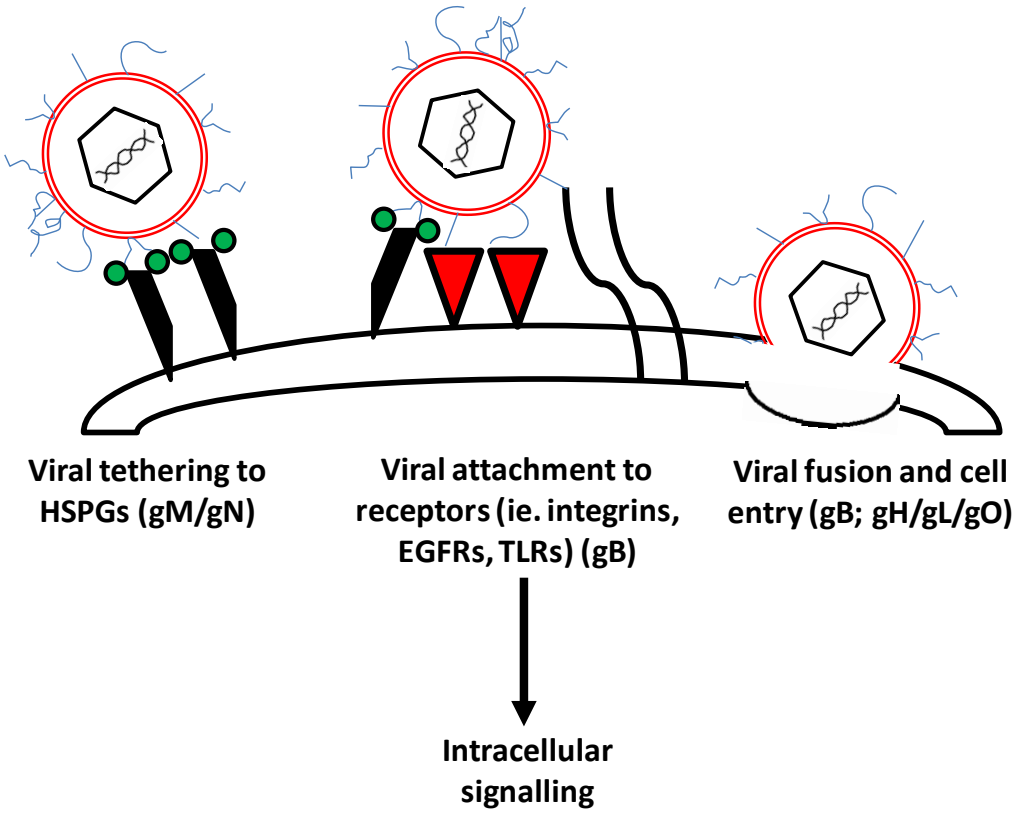


Figure 1.7: CMV glycoproteins and cell surface receptors involved in viral attachment and entry. The virus tethers to the cell surface via the gM/gN heterodimer binding to heparan sulphate proteoglycans (HSPGs) followed by secure viral attachment via gB and other cell receptors such as integrins, epidermal growth factor receptors (EGFRs), and toll-like receptors (TLRs). Viral fusion and entry into the cell requires gB and the gH/gL/gO heterotrimer.

Like other Herpes viruses, HCMV establishes a lifelong infection that cycles between periods of dormancy (latency) and reactivation (Sweet, 1999).

Viral latency is defined as the absence of infectious, replicating virus with the maintenance of the viral genome. Latent HCMV is primarily maintained in proliferative myeloid cells. Myeloid differentiation, as may occur with inflammatory stimuli, is associated with HCMV reactivation. In immunocompetent individuals, latency is established by the host's innate and adaptive immune response. The expression of antiviral cytokines and intracellular effector proteins are triggered, leading to the termination of viral gene expression by repressing the viral major immediate early promoter (Reddehase *et al.*, 2008; Reeves *et al.*, 2008). Hence, the immediate early (IE) viral genes are not expressed, preventing the expression of the early (E) and late (L) viral genes and egress of the virus that occur with an active/reactivated CMV infection (Figure 1.8) (Hummel *et al.*, 2002; Mocarski, 1996; Presti *et al.*, 1998). T lymphocytes help ensure that latency is generally maintained in the host (Reddehase *et al.*, 2008). There are two IE gene products that are required for activation of the CMV lytic cycle and viral replication. In HCMV they are known as IE1 and IE2, which correspond to IE1 and IE3 in mouse CMV (mCMV). Providing expression of these IE genes is inhibited, latency is sustained (Angulo *et al.*, 2000).

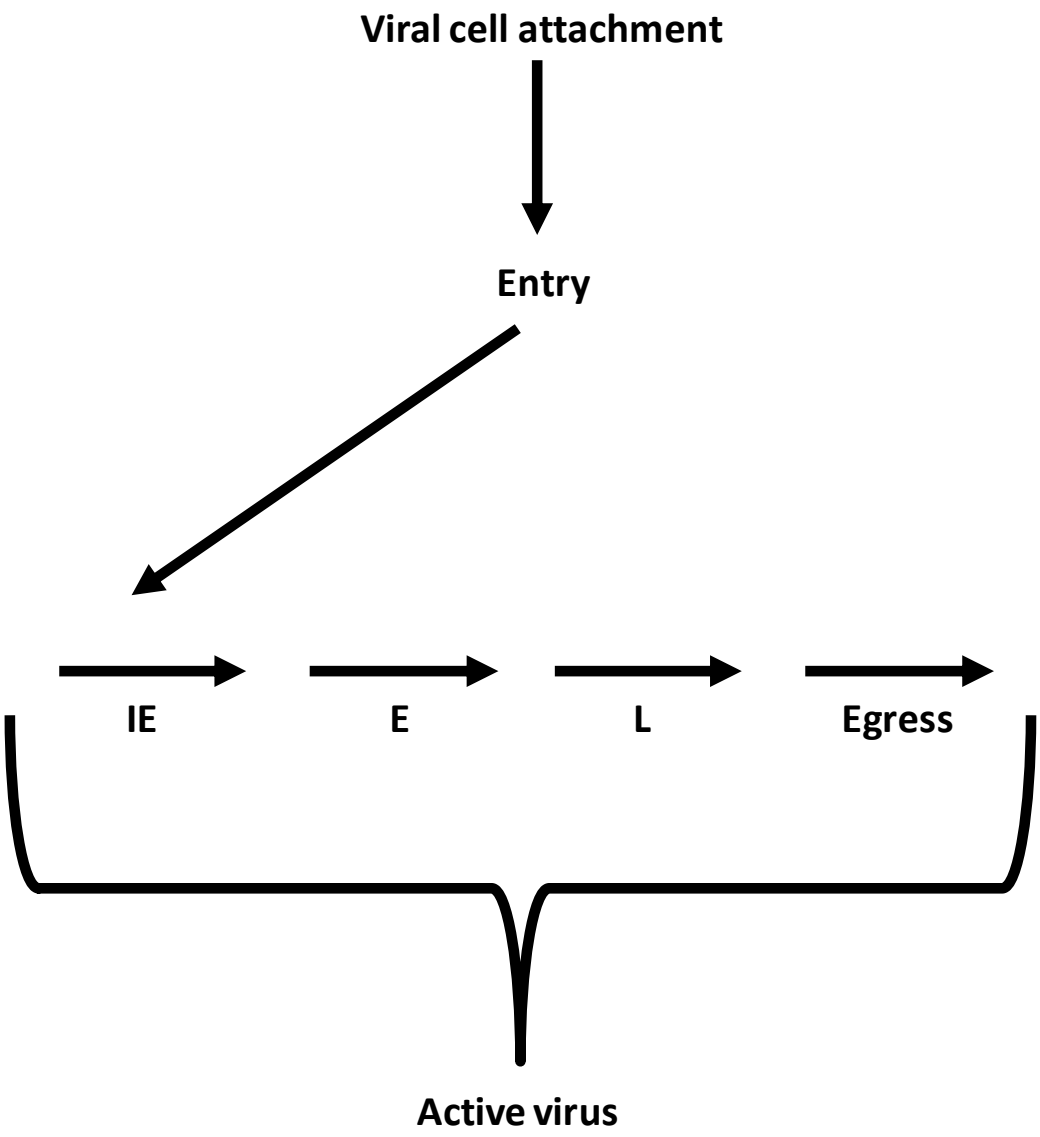


Figure 1.8: CMV lytic cycle. During an active CMV infection, immediate early (IE), early (E), and late (L) genes are expressed followed by egress of infectious enveloped viral capsids from the cell.

Most organs in the body can be sites of both active and latent CMV, where only lymphocytes and neutrophils do not support CMV replication (Pancholi *et al.*, 2004). During a lifelong, chronic CMV infection, periods of latency where infectious virus is undetectable is intermittent with periods of reactivation (Presti

et al., 1998). Reactivation of a CMV infection and thus production of infectious, replicating virus may occur in one organ or cell type, while latency is sustained in another organ or cell type within the same host (Balthesen *et al.*, 1993). In the human, the salivary gland is the main site where the viral genome load is high and reactivation is frequent whereas in the murine model, the lung is the predominant site (Balthesen *et al.*, 1993; Harari *et al.*, 2004; Landolfo *et al.*, 2003). Reactivation can occur in response to several different stimuli including stress, drug treatments, and pregnancy (Cheung *et al.*, 2007; Gould *et al.*, 1980; Prosch *et al.*, 2000; Tanaka *et al.*, 1983). Because there are many stimuli that likely contribute to viral reactivation in different tissues, the number of reactivation cycles an individual undergoes in a lifetime is difficult to estimate (Morita-Hoshi *et al.*, 2008). Viral replication and the subsequent immune response are likely necessary for the acceleration and/or establishment of vasculopathy (Lemstrom *et al.*, 1997; Tu *et al.*, 2006) and atherosclerosis (Grahame-Clarke, 2005). Whether or not vascular dysfunction persists when viral latency is established is unknown.

Components of the immune system, particularly CMV-specific T-cells, are important for establishing and also for maintaining a latent infection following an active CMV infection (Steffens *et al.*, 1998). Effectively resolving an active CMV infection, as occurs in fully immunocompetent hosts, reduces the copy number of viral genomes in tissues and particular organs within the host and reduces the incidence of reactivation (Reddehase *et al.*, 1994). Therefore, HCMV infections are generally asymptomatic in these individuals (Ahlfors *et al.*, 1978; Zanghellini *et al.*, 1999). In contrast, immunosuppressed or immunocompromised individuals who have reduced immune function (ie. transplant recipients and those with acquired immune deficiency syndrome) or an immature immune system (ie. developing fetuses) are more susceptible to the pathogenicity of HCMV (Einsele *et al.*, 2008; Freeman, 2009; Sweet, 1999). As such, HCMV vaccine development for those individuals at high risk of HCMV disease is a major focus for several research groups (Berencsi *et al.*, 2001; Bernstein *et al.*, 2002; Endresz *et al.*, 2001; Endresz *et al.*, 1999; Zhang *et al.*, 2006). Interestingly, the humoral immune

response to HCMV predominantly recognizes glycoproteins located on the outside of the virus and up to 70% of the antibodies from HCMV-seropositive individuals are gB-specific (Britt *et al.*, 1990). As such, gB is a prime candidate for HCMV vaccine development (Bernstein *et al.*, 2002; Endresz *et al.*, 1999; Zhang *et al.*, 2006).

1.4.1 CMV and vascular diseases

Although the general HCMV-infected population is thought to be asymptomatic, HCMV infections have been associated with tumourigenesis, vascular diseases including atherosclerosis and restenosis, and increased mortality (Abgueguen *et al.*, 2003; Cobbs *et al.*, 2002; Hirabayashi *et al.*, 2003; Kalil *et al.*, 2009; Maussang *et al.*, 2006; Melnychuk *et al.*, 2005). The initial cell binding and entry of CMV induces intracellular signals that lead to the activation of transcription factors and kinases and increase inflammatory cytokine gene expression, prostanoid synthesis, and reactive oxygen intermediates (Evers *et al.*, 2004; Fortunato *et al.*, 2000; Speir *et al.*, 1998). Production of these factors can be further amplified when a fully systemic, replicating, active HCMV infection develops (Compton *et al.*, 2003; Speir *et al.*, 1996; Zhu *et al.*, 1997). Viral-associated production of pro-inflammatory cytokines, such as TNF-α, IFN-γ, and interleukins (Compton *et al.*, 2003; Koskinen *et al.*, 1999; Rubin, 2001; Tang-Feldman *et al.*, 2006), release of catecholamines (Rubin, 2001), and increased expression of adhesion molecules on the surface of infected endothelial cells, such as vascular cell adhesion molecule (VCAM)-1 (Rahbar *et al.*, 2005), implicate HCMV and the stimulation of pro-inflammatory mediators in endothelial dysfunction and vascular diseases (Blankenberg *et al.*, 2001; Grahame-Clarke, 2005).

Furthermore, in a number of animal and human cell culture studies, an acute CMV infection has been associated with increased SK activity in vascular endothelial and fibroblast cells (Machesky *et al.*, 2008), increased angiotensin II levels in serum and arterial tissue (Cheng *et al.*, 2009), and both increased NOS-2

expression and serum nitrite and nitrate levels (Tanaka *et al.*, 1997; Tanaka *et al.*, 2001) and decreased NOS-3 activity in vascular endothelial and epithelial cells from different tissues (Shen *et al.*, 2006; Weis *et al.*, 2004). To facilitate CMV replication, PGHS-2 expression is also increased in fibroblasts and retinal epithelial cells (Hooks *et al.*, 2006; Zhu *et al.*, 2002). Together, this evidence indicates that an active CMV infection affects several vascular mediators that may have an effect on vascular function (Figure 1.9) (Cheng *et al.*, 2009; Eerdmans *et al.*, 1996; Hirabayashi *et al.*, 2003; Persoons *et al.*, 1998); however, direct links between HCMV, endothelial dysfunction, and vascular diseases remain undefined.

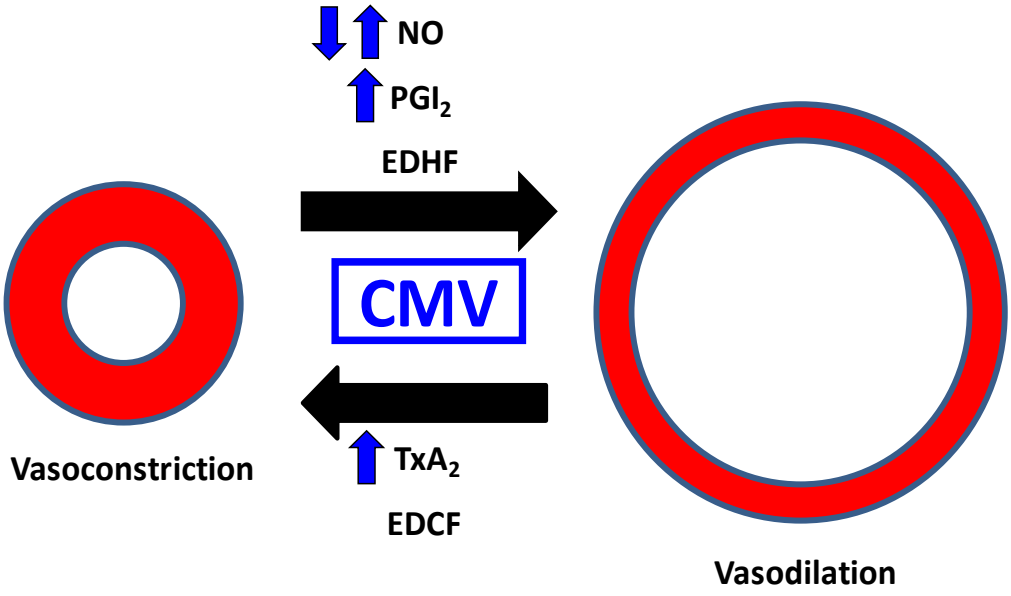


Figure 1.9: CMV-associated effects on some local vasoactive substances that regulate vascular tone.

1.4.2 CMV and pregnancy

In addition to the association between HCMV and vascular dysfunction in the non-pregnant adult population, HCMV can also cause severe fetal complications during pregnancy. It is the most common viral congenital infection

and occurs in 0.2%-2.2% of all live births (Adler, 1992; Demmler, 1991; Hanshaw, 1995; Yinon *et al.*, 2010). Congenital infections arise when HCMV crosses the placenta to infect the fetus (Hanshaw, 1995). Congenital CMV disease can cause intrauterine fetal death or symptoms including IUGR, jaundice, microcephaly, hepatosplenomegaly, sensorineural hearing loss, visual impairment, and cognitive delays (Lazzarotto *et al.*, 2000; Yinon *et al.*, 2010). Both primary and secondary maternal infections can lead to congenital infections. A primary or newly acquired (active) HCMV infection occurs in up to 4% of all pregnancies (Hanshaw, 1995; Yinon *et al.*, 2010). The likelihood of intrauterine transmission from a primary maternal infection during pregnancy is approximately 40% (Raynor, 1993; Stagno *et al.*, 1986; Yinon *et al.*, 2010). Secondary infections result from either reinfection with another strain of HCMV or reactivation of HCMV from latency (Ornoy *et al.*, 2006). Although the rate of transmission to the fetus during a secondary HCMV infection is only 1% (Raynor, 1993; Yinon *et al.*, 2010), more congenital infections arise from secondary infections because they are more frequent than primary infections during pregnancy (Ornoy *et al.*, 2006). It is generally accepted that primary maternal CMV infections cause more severe sequelae in congenitally-infected fetuses than secondary maternal infections; however, there is a significant amount of evidence demonstrating that secondary maternal CMV infections can also cause severe symptoms in the fetus and even intrauterine fetal death (Ahlfors *et al.*, 2001; Benshushan *et al.*, 1998; Boppana *et al.*, 1999; Ornoy *et al.*, 2006; Rousseau *et al.*, 2000).

Maternal HCMV screening is very controversial largely due to the lack of effective, well-established treatments for fetal HCMV infections (Nyholm *et al.*, 2010; Revello *et al.*, 2002). Nevertheless, the morbidity of congenital CMV disease is very severe and with effective treatment options HCMV screening is suggested to be cost effective (Cahill *et al.*, 2009). There are ongoing studies testing maternal therapies that may help reduce the potentially severe effects of a primary maternal HCMV infection. These include specific vaccines and passive

immunoglobulin therapy to reduce the likelihood of a maternal HCMV infection and/or prevent HCMV transmission to the fetus (Nigro *et al.*, 2005; Pass *et al.*, 2009; Schleiss, 2008; Schleiss *et al.*, 2003; Schleiss *et al.*, 2004).

Potential CMV transplacental transmission makes it difficult to determine if HCMV-induced fetal effects, such as IUGR, result solely from direct fetal infections or indirectly from maternal or placental vascular complications. Furthermore, HCMV is not regularly screened for in pregnant women in the absence of a congenital infection (Revello *et al.*, 2002); consequently, most studies investigating the gestational effects of a CMV infection have focused on congenital infections (Adler *et al.*, 2007; Yinon *et al.*, 2010). There are currently no studies aimed at investigating how an active maternal CMV infection affects pregnancy independent of a congenital infection. Although maternal HCMV seropositivity has been associated with maternal pregnancy-specific vascular complications (ie. preeclampsia) in several epidemiological studies, this association is somewhat controversial depending on the sample size and inclusion criteria used in each study (Conde-Agudelo *et al.*, 2008; Grahame-Clarke, 2005; Rustveld *et al.*, 2008; von Dadelszen *et al.*, 2003; Xie *et al.*, 2010). For example, two systematic reviews that examined the association between maternal infections and preeclampsia (Conde-Agudelo *et al.*, 2008; Rustveld *et al.*, 2008) and included the same three clinical CMV studies (Carreiras *et al.*, 2002; Trogstad *et al.*, 2001; von Dadelszen *et al.*, 2003) came to opposite conclusions regarding the association between CMV and preeclampsia based on the inclusion criteria of data sets collected from each of the studies.

1.4.3 CMV and animal models

Several confounding variables in the human population, such as socioeconomic status, make it difficult to study the direct pathogenesis caused by HCMV. Therefore, the use of animal models to study the effects of a CMV infection is necessary. Despite the fact that CMV is a species-specific virus, many viral genes are homologous among species. The choice of model largely depends

on what is being investigated. For instance, neonatal and placental effects of a congenital CMV infection and potential vaccines for congenital CMV infections are often studied in the guinea pig as transplacental transmission occurs in this rodent model (Bratcher *et al.*, 1995; Choi *et al.*, 1978; Griffith *et al.*, 1985; Schleiss *et al.*, 2004). In comparison, the rhesus monkey has been used more recently for vaccine and drug development and neurological developmental problems associated with CMV because its genetics and reproductive processes are more similar to humans. However, due to its high maintenance costs it is used less often than rodent models (Powers *et al.*, 2008; Tarantal *et al.*, 1998).

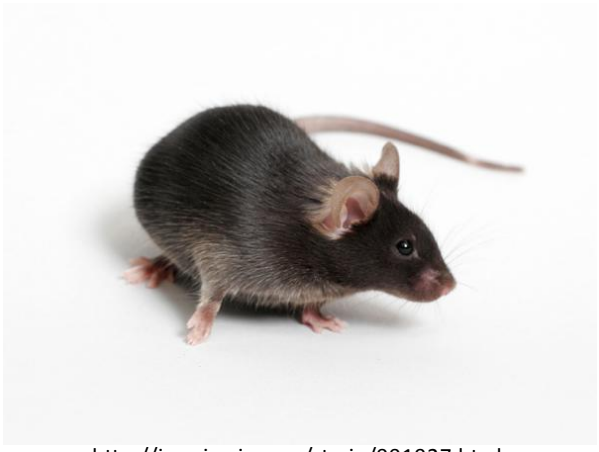
1.4.3.1 Mouse model

The mouse model is a popular experimental system for studying CMV pathogenesis (Hudson, 1979). This is due, in part, to the availability of mCMV for research purposes compared to other species-specific CMV, such as rat CMV. Although there are definite genetic differences between mCMV and HCMV, there is also significant homology (Rawlinson *et al.*, 1996). In addition, there are several biological characteristics both mCMV and HCMV infections share. For example, both have similar effects in immunocompromised hosts (Lenzo *et al.*, 2002), are susceptible to anti-viral drugs (Shanley *et al.*, 1985), and induce vascular diseases (Dal Canto *et al.*, 2000). One major distinction between mCMV and HCMV is that a maternal mCMV infection does not cross the placenta (Johnson, 1969). Hence, the fetus must be infected directly to study congenital mCMV infections (Kashiwai *et al.*, 1992). However, this lack of transplacental mCMV transmission makes the mouse model useful for studying how a maternal CMV infection affects vascular function during pregnancy, independent of a congenital infection. Moreover, the effects of an active maternal CMV infection on fetal growth/mortality can also be clearly assessed in the mouse (Gharavi *et al.*, 2004).

Different mouse strains have demonstrated different susceptibilities to CMV based on variations in immune response genes; specifically the Balb/c and

C57Bl/6 strains (Figure 1.10) (Chiodini *et al.*, 1993; Geist *et al.*, 2001; Mercer *et al.*, 1986). For example, the IL-10 anti-inflammatory cytokine that prevents the immune response from clearing an active CMV infection is significantly increased in Balb/c mice versus C57Bl/6 mCMV-infected mice (Geist *et al.*, 2001). Moreover, differences in the major histocompatibility complex loci on antigen-presenting cells (macrophages, dendritic cells, B cells) have also been linked to increased Balb/c susceptibility to mCMV compared to C57Bl/6 mice (Mercer *et al.*, 1986). Finally, C57Bl/6 mice express the CMV replication resistant allele $Cmv1^r$ whereas Balb/c strains express the CMV replication susceptibility allele $Cmv1^s$ (Lathbury *et al.*, 1996; Lee *et al.*, 2001; Scalzo *et al.*, 1990). The *Cmv1* gene regulates the natural killer cell-mediated control of CMV replication as part of the innate immune response. C57Bl/6 mice therefore use both the innate and adaptive (T lymphocytes) immune response to clear an active mCMV infection efficiently (Lathbury *et al.*, 1996; Lee *et al.*, 2001; Scalzo *et al.*, 1990; Scalzo *et al.*, 2005). Balb/c mice, with the $Cmv1^s$ allele, rely primarily on the slower adaptive immune response and are therefore much less effective at clearing CMV before it becomes fully established (Craighead *et al.*, 1992; Lathbury *et al.*, 1996). Together, this evidence suggests that the C57Bl/6 mouse strain is more representative of the immunocompetent human population than the Balb/c strain.

A



http://jaxmice.jax.org/strain/001927.html

B

http://www.sageresearchmodels.com/research-models/inbred-mice/balbc

Figure 1.10: C57Bl/6 and Balb/c laboratory mice strains. C57Bl/6 mice **(A)** are more resistant to mCMV infections than Balb/c mice **(B)**.

1.5 RATIONALE AND HYPOTHESIS

HCMV has been associated with several vascular diseases in the general population (Blankenberg *et al.*, 2001; Grahame-Clarke, 2005) including the

pregnancy-specific disorders preeclampsia (von Dadelszen *et al.*, 2003) and IUGR (Yinon *et al.*, 2010). Identifying if an active CMV infection is a true risk factor for vascular diseases and, if so, identifying potential therapies for CMV infections is critical for the cardiovascular health of both the pregnant and general population. However, it remains largely unknown how vascular function is affected by an active CMV infection.

In my project, I chose to use the mouse model to study the effect of an active CMV infection on vascular responses for a number of reasons. First, HCMV and mCMV have similar effects on vascular function (Dal Canto *et al.*, 2000). This makes it an appropriate model to study vascular mechanistic effects of a CMV infection under normal conditions. Secondly, mCMV does not cross the placenta in mice (Johnson, 1969). Therefore, the maternal consequences of a CMV infection can be elucidated in the absence of a congenital CMV infection. Lastly, the genotype variation among different mouse strains is advantageous to determine the vascular effects of CMV in genotypically resistant versus more susceptible hosts.

My general focus is to investigate the systemic (mesenteric) and uterine vascular endothelial and smooth muscle cell responses in intact, isolated arteries from mCMV-infected and uninfected non-pregnant (NP) and late pregnant (LP) mice. LP mice are also used to determine if a CMV infection significantly affects fetal growth, which primarily occurs near the end of mouse gestation (Eisen, 1976). I hypothesize that an active CMV infection will cause systemic and uterine vascular dysfunction in NP mice that will be exacerbated in LP mice leading to poor fetal outcomes.

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Chapter 2
Material and Methods

2.1 GENERAL MATERIAL AND METHODS

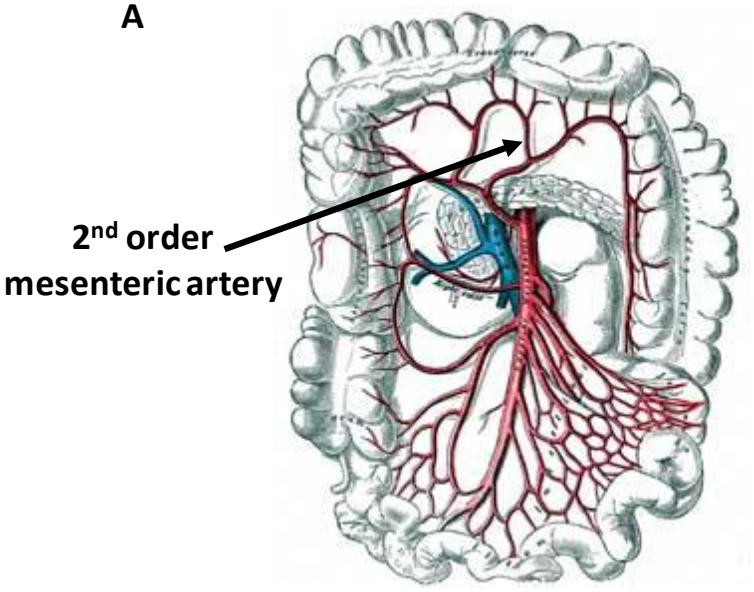
2.1.1 Animal model and tissue collection

Virgin C57B1/6J female mice were purchased from Jackson laboratories and housed in Health Sciences Laboratory Animal Services at the University of Alberta. Some of the mice (numbers indicated in each figure legend) were infected with 10^6 plaque-forming units (PFU) of mCMV (RM427+; gift from E. Mocarski, Stanford University, Stanford, CA) by intraperitoneal injection as described previously (Stoddart *et al.*, 1994). RM427+ contains a lacZ gene insertion in the nonessential immediate early 2 gene to allow detection of infectious virus by assessing β-galactosidase activity. Following a 5-day observational period, infected NP mice were either: a) euthanized by cervical dislocation 5-21 days after injection or b) bred 5-12 days after injection to male C57Bl/6J mice. This ensured that all infected mice were studied within one month of infection. Since infectious virus is still detectible in this time frame (Walton *et al.*, 2008), it is unlikely that the results of this study were influenced by differences in length of time between viral injection and euthanization. Breeding of both mCMV-infected and uninfected mice was done overnight and the presence of a vaginal plug was confirmed the next day (day 0.5 of gestation). Controls were age-matched uninfected NP or LP mice. LP mice were euthanized on day 18.5 of gestation (D18.5); delivery normally occurs on D19.5 in C57Bl/6J mice. In a few cases where two D18.5 pregnant mice arose on the same day one was euthanized on D17.5. Only five D17.5 LP mice were used in total and no differences were observed for any vascular response measured between D17.5 and D18.5 LP mice. These data were therefore combined.

The mesentery and uterine horns with associated vasculature were dissected from each mouse and immediately placed in cold HEPES-buffered

physiological saline solution (PSS; 10mM HEPES, 1.56mM $CaCl_2$, 142mM NaCl, 4.7mM KCl, 1.18mM KH_2PO_4, 1.17mM $MgSO_4$, and 5.5mM glucose at pH 7.5) (Figure 2.1). These animal protocols were approved by the University of Alberta Animal Welfare Committee and followed the guidelines outlined by the Canada Council of Animal Care.

A



http://embryology.med.unsw.edu.au/Notes/git13.htm

B

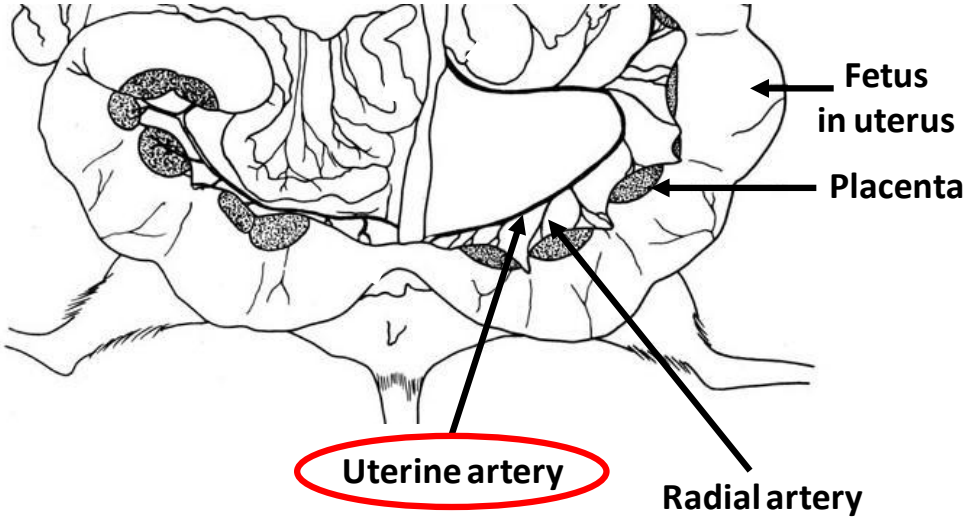


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Figure 2.1: The second-order mesenteric (A) and main uterine (B) artery were isolated and used for the vascular experiments. Both are small resistance-sized arteries (150-300μM). Note that the illustration in **(B)** is that of pregnant mouse.

2.1.2 Pressure myograph studies

Second-order mesenteric and main uterine arteries were dissected free of adipose and connective tissue (Figure 2.2). In some cases both artery types were isolated from the same animal; however, in other cases separate animals were used for each artery type. In an arteriograph (Living Systems Instrumentation, Burlington, VT.), one end of an artery was mounted and tied onto a glass cannula (80–100μm diameter) which was connected to a pressure transducer, to modulate the intraluminal pressure through a servo-controlled peristaltic pump, as previously described (Figure 2.3) (Halpern *et al.*, 1984; Hemmings *et al.*, 2004). Residual blood was flushed out of the artery with a low flow (15μL/min) of PSS. The opposite end of the artery was then mounted and tied onto a second cannula and closed off with a valve to hold pressure and prevent flow. The cannulated arteries were held in a 2.5mL bath containing PSS that was replaced every 10 minutes before and after the dose-response curves. The bath temperature was maintained at 37°C, and the arteries were pressurized to 60mmHg (mesenteric arteries) or 50mmHg (uterine arteries) for 30 minutes. These pressures have been previously reported as optimal for vascular responses in these arteries (Gros *et al.*, 2002; Osol *et al.*, 2008; Veerareddy *et al.*, 2002). Arteries unable to maintain pressure were discarded and new arteries from those same animals were isolated and mounted. Following equilibration, initial lumen diameter was measured via a CCD video camera module (Sony) connected to a compound microscope (Olympus). Lumen diameter was measured digitally using a video dimension analyzer (Halpern *et al.*, 1984).

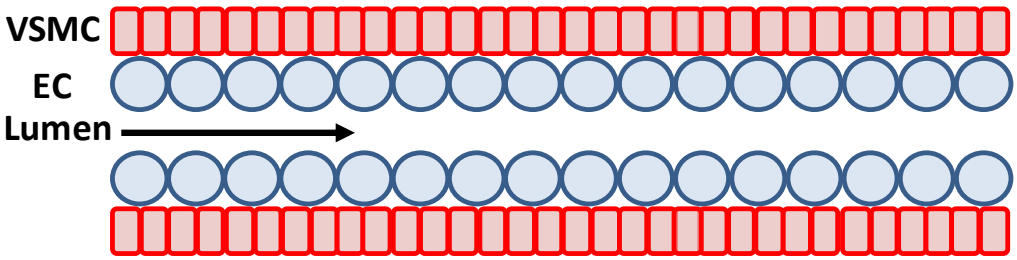

Figure 2.2: Diagram of a dissected artery free of adipose and connective tissue (tunica adventitia). The primary cell types within the vascular wall include the endothelial cells (EC) within the tunica intima and the vascular smooth muscle cells (VSMC) within the tunica media. Blood, containing agents that modulate vascular tone, passes through the lumen.

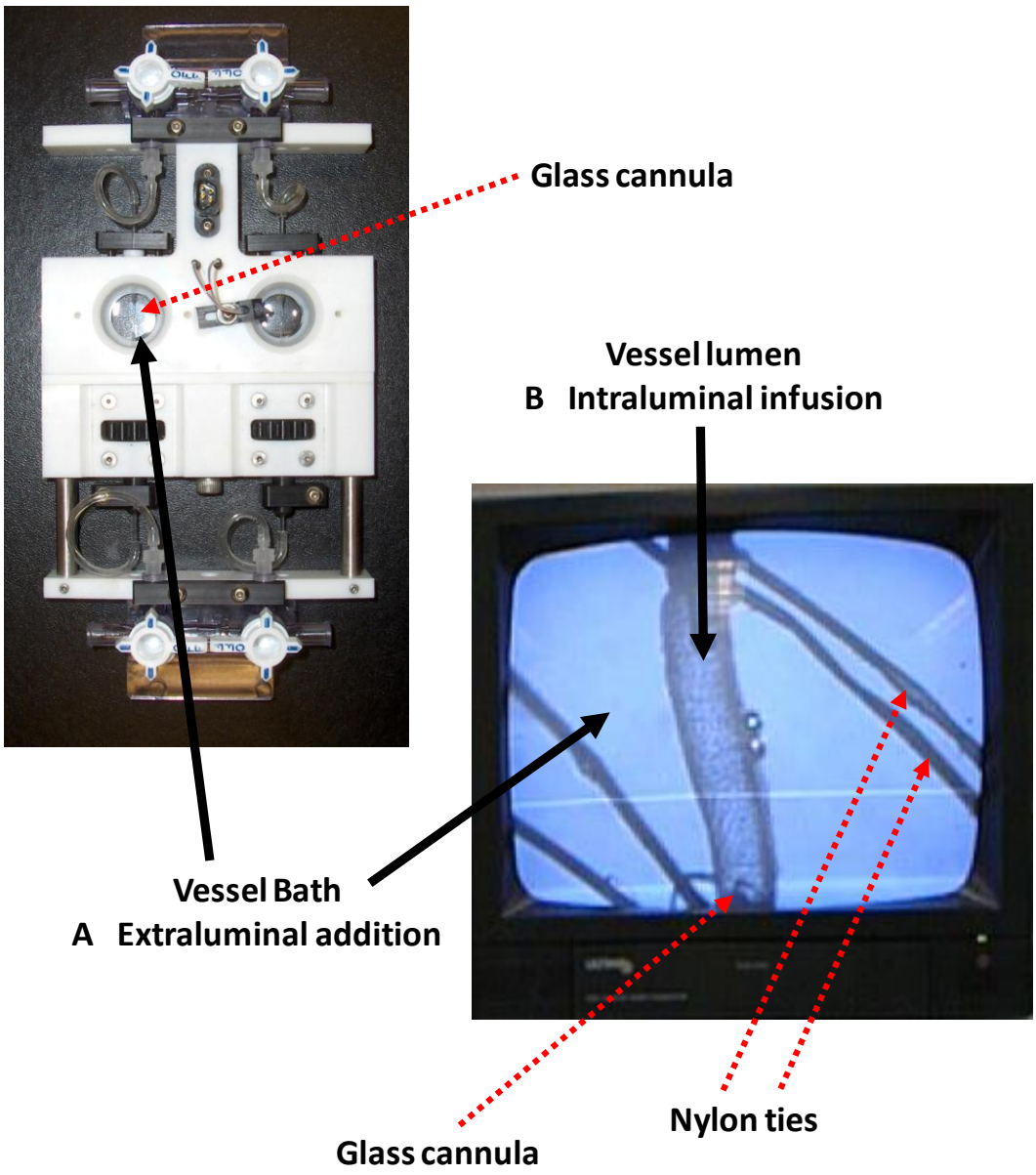

Figure 2.3: The pressure myograph system. After mounting and pressurizing an artery, drugs can be added to the bath to interact with the vascular smooth muscle directly **(extraluminal addition; A)** or infused into the artery to interact with the endothelium directly **(intraluminal addition; B)**.

2.2 MATERIAL AND METHODS SPECIFIC FOR CHAPTER 3

For this section, active mCMV-infected and uninfected NP and LP C57Bl/6J mice were used to determine if an active mCMV infection affects systemic and uterine vasoconstriction and/or vasodilation responses in NP and LP states.

2.2.1 Immunofluorescence

The heart, kidney, liver, lung, and spleen were isolated from three mCMV-infected and uninfected NP mice and tissues containing mesenteric and uterine arteries from three mCMV-infected and uninfected NP and LP mice were fixed in 4% formaldehyde overnight, washed with three 5-minute washes of phosphate buffered saline (PBS), and further incubated in 30% sucrose overnight. Tissues were then embedded into Tissue-Tek O.C.T. embedding medium (VWR), snap-frozen in liquid nitrogen, and stored at -80°C. The frozen tissues were sliced on a cryostat in 5-7μm sections, mounted onto glass slides, dried overnight and stored at -80°C. Prior to staining, slides were thawed and dried for 1-2 hours, and fixed in cold methanol for 10 minutes at -20°C. Slides were immediately rinsed with three 10-minute washes of PBS. Sections were then circled with a PAP pen and blocked with 50μL of 10% normal goat serum (NGS; Cedarlane) in PBS for 3 hours. After removing the blocking agent, 50μL of the primary antibody solution or blocking agent was added to each section, and incubated at 4°C overnight. The primary antibodies used for co-staining were rabbit anti-von Willebrand factor (1μg/mL; Sigma-Aldrich), as an endothelial cell marker (Wagner *et al.*, 1991), and chicken anti-β-galactosidase (1μg/mL; Abcam, Massachusetts, USA), as a marker of LacZ activity and an active CMV infection. Both antibodies were diluted in 10% NGS. Following three 5-minute washes with PBS, each section was co-stained with Alexa Fluor-594 goat anti-rabbit (10μg/mL) and Alexa Fluor-488 goat anti-chicken (4μg/mL) secondary antibodies (Molecular Probes, Oregon, USA) for 45 minutes in the dark. After three 5-minute washes with PBS, 4',6-diamidino-2-phenylindole (DAPI; 0.915mg/mL; Invitrogen) was added for 15

minutes in the dark to stain the nuclei. Slides were then washed again with three 5-minute washes of PBS and 45μL of Vectashield H:1000 (Vector Laboratories, Burlington, CA) was applied to each section. Coverslips were sealed to the slides which were then stored in the dark at 4°C. Stained sections were viewed with an Olympus IX81 fluorescent microscope (Olympus, Ontario, Canada) using Slidebook 2D, 3D Timelapse Imaging Software (Intelligent Imaging Innovations Inc., Colorado, USA).

2.2.2 LacZ expression

Salivary glands, fetuses and placentas from five mCMV-infected and uninfected LP mice were fixed and stained using the LacZ Detection Kit for Tissues (Invivogen, San Diego, CA) according to the manufacturer's instructions. The lacZ gene, inserted in the dispensable mCMV immediate early 2 gene, produces β-galactosidase when the virus is actively replicating, providing a reporter of a productive mCMV infection (Stoddart *et al.*, 1994). β-galactosidase activity was visualized by the conversion of its substrate, X-gal, to yield a blue-stained tissue. Photomicrographs were immediately taken.

2.2.3 Extraluminal pressure myograph experimental design

Vasoconstriction in equilibrated mesenteric and uterine arteries (section 2.1.3) was assessed using a dose-response to the following drugs: 10nM to 10μM of phenylephrine (PE; Sigma-Aldrich), an α_1-adrenergic agonist, or 0.01nM-1μM U46619, a TxA_2 mimetic (Calbiochem). Each drug was added directly to the bath of the dual-chamber arteriograph containing the mesenteric or uterine artery in increasing cumulative doses. Arteries which did not constrict to PE or U46619 were discarded and new arteries from the same animal were isolated and mounted. Vasodilation was assessed by preconstricting arteries to approximately 50% with U46619 (mesentery) or PE (uterine) (Table 2.1) followed by a dose-response to the endothelium-dependent vasodilator methacholine (ME; 1nM to 10μM; Sigma-Aldrich). Each dose of ME was incubated for 3 minutes before adding the next

and the bath was not replaced with fresh PSS between doses. I found that mesenteric arteries from LP mice, regardless of infection, did not maintain constriction to PE over the time required to complete a vasodilation experiment. Therefore, I used U46619 in mesenteric arteries and PE in uterine arteries for preconstriction. To confirm that the response to these two vasoconstrictors did not differ between mCMV-infected and uninfected mice, vasoconstriction responses to PE and U46619 in uterine arteries from a subset of animals (mCMV-infected and uninfected NP mice) were compared. PE- and U46619-induced vasoconstriction in uterine arteries showed consistent results between mCMV-infected and uninfected mice (Figure 2.4A,B). The passive lumen diameter was recorded after a thorough washout with Ca^{2+}-free EGTA-PSS (10mM HEPES, 142mM NaCl, 4.7mM KCl, 1.18mM KH_2PO_4, 1.17mM $MgSO_4$, 2mM EGTA) and a 10-minute incubation with 100μM of papaverine (Sigma-Aldrich). The percent constriction was calculated as 1-L2/L1 x 100; where L1 is the initial lumen diameter and L2 is the arterial lumen diameter post-drug addition. The percent dilation was calculated as L2-L1/L1 x 100 and normalized to the artery diameter when fully relaxed (passive lumen diameter). Distensibility of arteries was measured using 3-minute step-wise increases in intraluminal pressures from 4mmHg to 170mmHg. Distensibility was calculated as D2-D1/D1 x 100; where D1 is the initial lumen diameter at 4mmHg and D2 is the lumen diameter at each pressure step as previously described (Mateev *et al.*, 2006; Zhang *et al.*, 2001). Arteries tend to collapse at pressures less than 4mmHg and it was not possible to consistently measure the lumen diameter accurately; therefore the lumen diameter at 4mmHg was used as the initial starting diameter.

	Mesenteric arteries (U46619)	Uterine arteries (PE)
Uninfected NP: Avg. dose (μM)	0.736±0.484	2.01±0.661
Preconstriction (%)	48.9±1.80	52.2±2.09
mCMV-infected NP: Avg. dose (μM)	0.115±0.0441	2.46±1.02
Preconstriction (%)	50.7±1.78	53.8±3.74
Uninfected LP: Avg. dose (μM)	0.246±0.102	1.61±0.937
Preconstriction (%)	50.4±2.45	53.5±1.22
mCMV-infected LP: Avg. dose (μM)	0.209±0.0875	0.540±0.134
Preconstriction (%)	49.4±0.539	53.4±1.14

Table 2.1: Preconstriction values and average drug dose used for each vascular bed during extraluminal pressure myograph experiments. Preconstriction values using U46619 or PE were similar in mesenteric and uterine arteries from mCMV-infected and uninfected NP and LP mice. Data were expressed as the mean ± SEM. No significant differences in average dose used or preconstriction values in mesenteric arteries from mCMV-infected or uninfected NP and LP mice or in uterine arteries from mCMV-infected or uninfected NP and LP mice were determined by two-way ANOVA and the Bonferroni post-hoc analysis ($p>0.05$). It is important to note that these values were not used in the calculation for percent dilation.

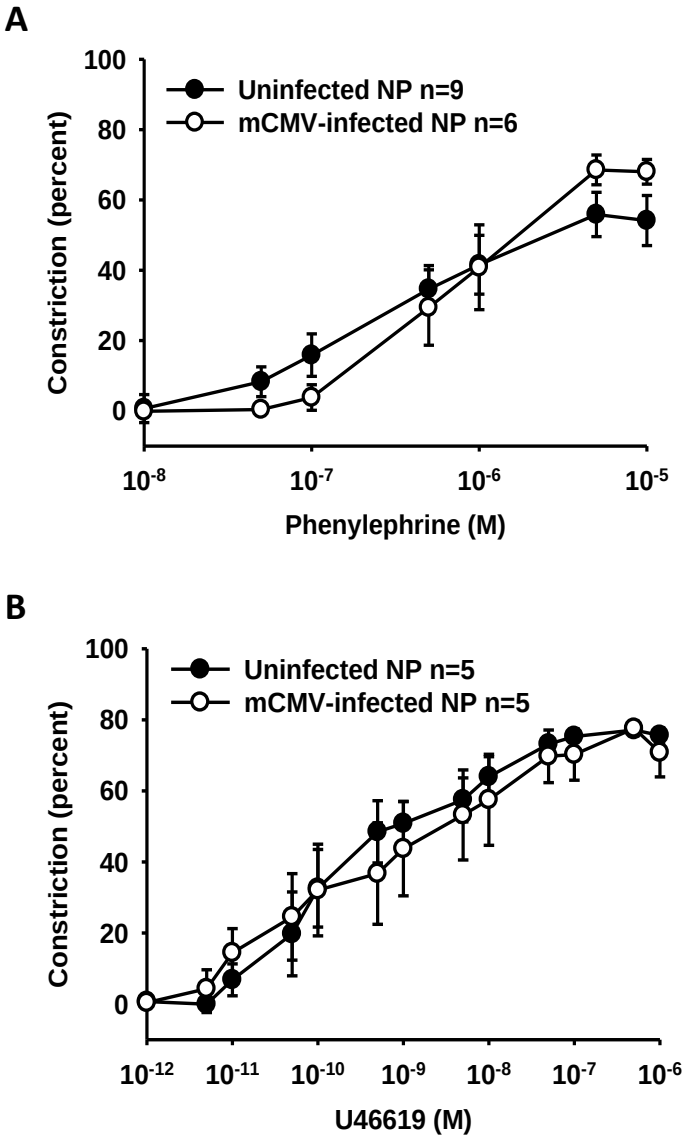

Figure 2.4: Vasoconstriction of uterine arteries from mCMV-infected and uninfected NP mice. No difference in vasoconstriction of uterine arteries from mCMV-infected and uninfected NP mice was detected after extraluminal addition of PE **(A)** or U46619 **(B)**. Data were expressed as the mean ± SEM percent decrease in arterial lumen diameter at each dose of PE or U46619 compared to the lumen diameter prior to drug addition. No significant difference between the curves was determined by Repeated Measures two-way ANOVA and no significant difference between specific points on the curves was determined with Holm Sidak's post-hoc analysis ($p>0.05$). n=number of animals.

2.2.4 Statistics

Data collected were averaged by group (mCMV-infected and uninfected; NP and LP; mesenteric and uterine) and treatment (PE; U46619; ME). Data which were at least two standard deviations from the mean were not included in the analysis (<5% of all values). Vascular responses were compared with Repeated Measures two-way ANOVA followed by Holm-Sidak's post-hoc analysis to determine significance. Differences in fetal and placental weights and ratios were determined by taking the mean of the average values from each litter, and comparing the two groups with a Student's t-test. The EC_{50} was calculated for each set of sigmoidal-shaped curves. The EC_{50} values for the ME dose-response curves for mesenteric arteries could not be calculated as these curves were not sigmoidal. EC_{50s} were compared using a Student's t-test. Significance was accepted at $p<0.05$.

2.3 MATERIAL AND METHODS SPECIFIC FOR CHAPTER 4

For this section, active mCMV-infected and uninfected NP and LP C57Bl/6J mice were used to determine the intracellular vasodilatory pathways in systemic and uterine arteries affected by an active mCMV infection.

2.3.1 Extraluminal pressure myograph experimental design

For each pair of mesenteric or uterine arteries mounted on the dual-chamber pressure arteriograph, one artery was pre-treated with 100μM of the NOS-1/2/3 inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; Calbiochem) (Cicala *et al.*, 2003; Williams *et al.*, 2005), 1μM of the PGHS-1/2 inhibitor meclofenamate (meclo; Sigma-Aldrich) (Williams *et al.*, 2005), 10μM of the TxA_2 receptor antagonist SQ29548 (Cayman Chemical) (Monshizadegan *et al.*, 1992; Pannirselvam *et al.*, 2005), or the combinations of L-NAME with meclo or L-NAME with SQ29548 (Figure 2.5A,B). For each inhibitor tested, a second untreated artery was always tested simultaneously to provide a direct comparison

of arteries taken from the same animal. Following measurement of the initial lumen diameter, one or a combination of inhibitors were incubated for 30 min in one of the two baths while the other artery remained untreated. I did not observe any differences in baseline vascular tone in response to the addition of any inhibitor or combination of inhibitors compared to untreated arteries. After recording the lumen diameters, both arteries were then preconstricted to approximately 50% with U46619 (mesenteric arteries) or PE (uterine arteries; mesenteric arteries with SQ29548) (Table 2.1). Endothelium-dependent vasodilation was then assessed in response to increasing concentrations of ME (1nM to 10μM). ME dose-response curves in the presence of SQ29548 following PE-induced preconstriction were completed within 20 minutes (2.5-minute incubation per dose of ME) to ensure PE-induced preconstriction was sustained. Endothelium-independent vasodilation was assessed in preconstricted arteries in response to sodium nitroprusside (SNP; 0.1nM to 10μM; Sigma-Aldrich) (Figure 2.5C). The percent dilation was calculated as described in section 2.2.3.

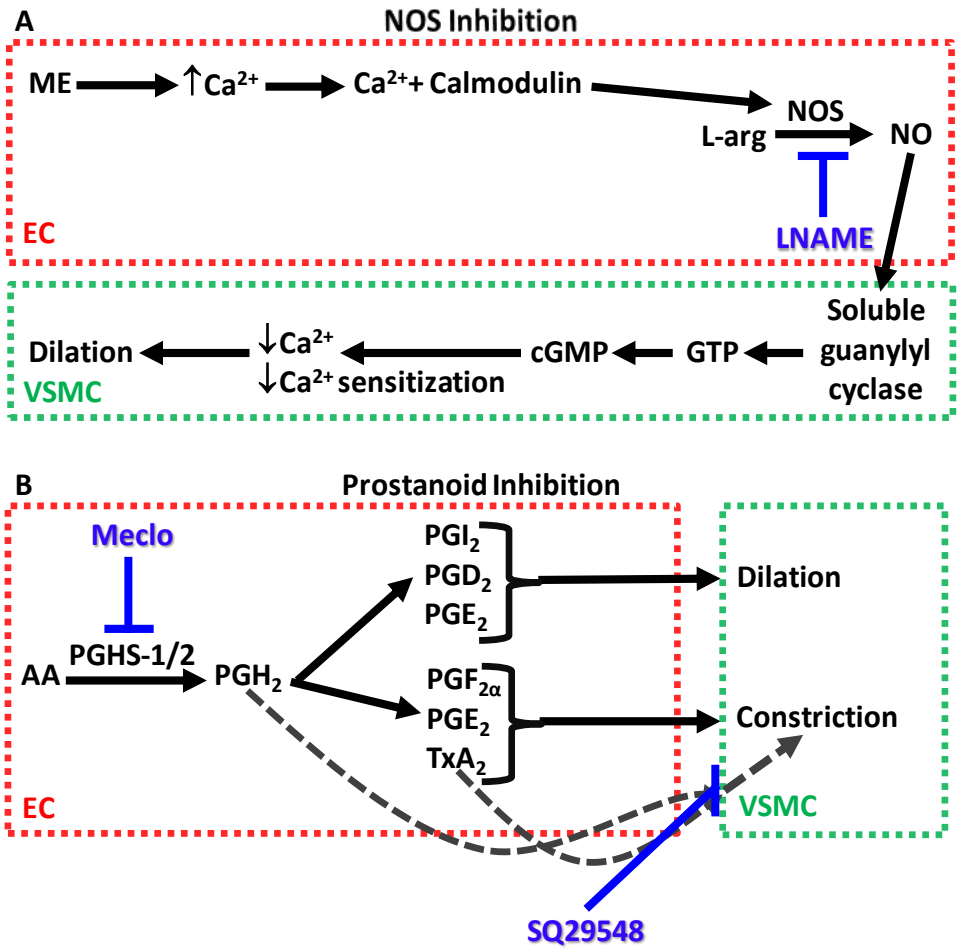
A
NOS Inhibition
ME
↑Ca2+
Ca2+ + Calmodulin
NOS
L-arg
NO
LNAME
EC
Soluble guanylyl cyclase
GTP
cGMP
↓Ca2+
↓Ca2+ sensitization
Dilation
VSMC
B
Prostanoid Inhibition
Meclo
PGHS-1/2
AA
PGH2
PGI2
PGD2
PGE2
PGF2α
PGE2
TxA2
Dilation
Constriction
EC
VSMC
SQ29548
C
Vascular Smooth Muscle Sensitivity to NO
SNP
NO
Soluble guanylyl cyclase
GTP
cGMP
↓Ca2+
↓Ca2+ sensitization
Dilation
VSMC

Figure 2.5: Diagram describing which signalling pathway each inhibitor affects. L-NAME is a competitive inhibitor for NOS in the vascular endothelium **(A)**. Meclo blocks PGHS-1/2 activity in the endothelium whereas SQ29548 is a specific TxA_2 receptor antagonist which can block vasoconstriction in the vascular smooth muscle cells **(B)**. SNP is a NO donor which induces vasodilation independent of the endothelium **(C)**. EC: endothelial cells; VSMC: vascular smooth muscle cells; ME: methacholine; L-arg: L-arginine; NOS: nitric oxide synthase; GTP: guanine triphosphate; cGMP: cyclic guanine monophosphate; PGHS-1/2: prostaglandin H synthase 1/2; PGH_2: prostaglandin H_2; PGI_2: prostacyclin; PGD_2: prostaglandin D_2; PGE_2: prostaglandin E_2; $PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$; TxA_2: thromboxane; SNP: sodium nitroprusside.

2.3.2. Quantitative Reverse Transcriptase (RT)-qPCR

Quantitative RT-qPCR was performed on dissected arteries immediately snap frozen following dissection and stored at -80°C. For each reaction, arteries were pooled from five to ten animals of the same status, and RNA was extracted with Trizol reagent. RNA was diluted in 10uL of RNAse-free sterile water and the concentration was determined with the Nanodrop (ND-1000 Spectrophotometer; Nanodrop Technologies Inc., Wilmington, DE) to ensure it was greater than or equal to 200ng/μL. RT was performed using the qScript cDNA supermix (Quanta Biosciences; Gaithersburg, MD) with 200ng/uL of extracted RNA. One tenth (20ng/μL) of cDNA samples were used for real-time PCR within 24 hours of the RT reaction. Real-time PCR was run using the Perfecta qPCR Fastmix (Quanta Biosciences), 20ng/μL of cDNA obtained from the RT reaction, and NOS-3-specific (Fagan *et al.*, 2001) and beta-actin (Maeda *et al.*, 2007) specific primers and probes (20μL reaction). The forward NOS-3 primer (5’ to 3’) is TCTGCGGCGATGTCACTATG, the reverse NOS-3 primer (5’ to 3’) is CATGCCGCCCTCTGTTG, and the NOS-3 probe (5’ to 3’) is CCAGCGTCCTGCAAACCGTGC. The beta-actin probe and primers came pre-

developed for TaqMan assays from Applied Biosystems (# 4352933E). The PCR conditions were 1 cycle at 45°C for 2 minutes, 1 cycle at 95°C for 30 seconds, and then 40 cycles of the amplification step (95°C for 10 seconds and 60°C (beta-actin) or 58°C (NOS-3) for 30 seconds) in the iCycler. To determine the amount of NOS-3 mRNA in each sample, levels were normalized to the positive control β-actin (did not differ between groups) as an mRNA ratio (Pfaffl *et al.*, 2002). PCR was repeated 3-4 times and samples that did not produce reliable β-actin results (cycle threshold > 29 cycles) were not included in the analysis.

2.3.3 Immunofluorescence

Mesenteric and uterine tissues were isolated, fixed and embedded in Tissue-Tek O.C.T. embedding medium and slides were prepared as described in section 2.2.1. The primary antibodies used were rabbit polyclonal NOS-2 (2μg/mL; Abcam) and rabbit polyclonal NOS-1 (1:800 dilution of whole serum; Abcam). Rabbit polyclonal NOS-3 (2μg/mL and 4μg/mL; Abcam) was also tried but following optimization and troubleshooting techniques, including antigen retrieval, specific staining was not successful on the fixed tissue. Following three 5-minute washes with PBS, each section was incubated with Alexa Fluor-488 goat anti-rabbit (10μg/mL) secondary antibody (Molecular Probes, Oregon, USA) for 45 minutes in the dark. After three 5-minute washes with PBS, DAPI (0.915mg/mL) was added for 15 minutes in the dark to stain the nuclei. Stained sections were prepared as described in section 2.2.1 for visualization on the fluorescent microscope.

2.3.4 Statistics

Data collected from the extraluminal pressure myograph experiments were averaged by group (mCMV-infected and uninfected; NP and LP; mesenteric and uterine) and treatment (ME ± L-NAME, meclo, or SQ29548; SNP) and then compared as described in section 2.2.4. The mean area under each dose-response curve was also calculated. The mean area under the inhibitor curve was subtracted

from the mean area under the control curve for each data set. The single value generated for the arteries from uninfected mice was then compared with that generated for the mCMV-infected mice using a Student's t-test. The EC_{50s} and IC_{50s} were calculated for each set of sigmoidal-shaped curves. The EC_{50}/IC_{50} values for the ME ± inhibitor dose-response curves for mesenteric arteries could not be calculated as these curves were not sigmoidal. EC_{50s}/IC_{50s} were compared using a Student's t-test. mRNA ratios from RT-qPCR were compared using a two-way ANOVA with Bonferroni's post-hoc analysis to determine significance. Significance was accepted at $p<0.05$.

2.4 MATERIAL AND METHODS SPECIFIC FOR CHAPTER 5

For this section, active mCMV-infected and uninfected NP and LP C57Bl/6J mice were used to determine how the bioactive sphingolipid S1P affects systemic and uterine vasconstriction and endothelium-dependent vasodilation responses during pregnancy, in the presence or absence of an active mCMV infection.

2.4.1 Extraluminal pressure myograph experimental design

For each pair of mesenteric or uterine arteries mounted on the dual-chamber pressure arteriograph, one artery was pre-treated with 100μM of L-NAME, 10μM of the $S1P_{1/3}$ antagonist VPC23019 (Avanti Polar Lipids) (Salomone *et al.*, 2008), or 1μM of the PGHS-1/2 inhibitor meclo for 30 minutes in PSS with 0.1% fatty acid-free Bovine Serum Albumin (BSA; Sigma-Aldrich), a carrier for S1P. The other artery was left untreated in 0.1% BSA-PSS for 30 minutes. To ensure the BSA used was fatty-acid free, as stated by the manufacturer, it was analyzed by high-pressure liquid chromatography to measure the absolute amount of fatty acid. The Sigma-Aldrich BSA was compared to fatty-acid free BSA stocks from two other companies. The fatty-acid free BSA from Sigma-Aldrich contained 0.0005% fatty acid, very comparable to that from Akron and significantly lower to that from Equitech. Increasing concentrations (100nM

to 10μM) of S1P (BioMol/Enzo Life Sciences) diluted in 0.1% BSA-PSS was added directly to the bath with or without the inhibitor/antagonist (extraluminal addition) and the corresponding change in lumen diameter was measured using a visual dimension analyzer as described in section 2.2.3. These results were calculated as percent constriction using 1-L2/L1 x 100; where L1 is the initial lumen diameter following the inhibitor addition in the 0.1% BSA-PSS bath and L2 is the arterial lumen diameter post-S1P addition as described in section 2.2.3. In some experiments, following the last dose of S1P (10μM) the ROCK kinase inhibitor Y27632 (10μM; Calbiochem) (Hemmings *et al.*, 2006; Wamhoff *et al.*, 2008) was added to the bath and incubated for 10 minutes. The reversal of constriction was calculated as S2-S1/S1 x 100; where S1 is the lumen diameter following the maximal dose of S1P (10μM) and S2 is the lumen diameter following the Y27632 incubation.

2.4.2 Intraluminal pressure myograph experimental design

I developed a novel technique to measure vascular responses to drugs that interact directly with the endothelium using the pressure myograph system. Prior to mounting a single artery, the tubing connected to one cannula was loaded with 290μL of PSS followed by 2cm of air and a second solution of 200μL of 0.1% BSA-PSS or 0.1% BSA-S1P. The 2cm air bubble loaded between each solution was too small to pass through the transducer and reach the artery, but was large enough to prevent mixing of solutions prior to the transducer. The reagents were pumped through the tubing until the PSS lead solution reached the end of the glass cannula (no air). One end of the artery was mounted onto the opposite cannula to flush out the residual blood and then the other end was tied onto the cannula attached to the loaded tubing. Following the 30-minute pressurization steps, the artery was incubated for three 10-minute washes in 0.1% BSA-PSS with or without 100μM of L-NAME or 10μM VPC23019. Arteries were then preconstricted to approximately 50% with U46619 (Table 2.2). A low-flow (30μL/min) was administered to infuse the PSS (9 minutes) followed by 0.1%

BSA-PSS, 0.1% BSA-0.1μM S1P or 0.1% BSA-1μM S1P (7 minutes) into the lumen of the pressurized artery. These S1P concentrations were chosen because they correspond to the physiological level of circulating S1P in the nanomolar to micromolar range (Igarashi *et al.*, 2009). After each flow step, the artery was allowed to equilibrate with no flow for 3 minutes and the lumen diameter was measured. In a separate group of arteries, the endothelium was removed by infusion of air prior to treatment with 0.1% BSA-S1P (0.1μM) to examine the endothelium-dependent effect. Addition of the endothelium-dependent vasodilator ME (10μM) to the bath was used to confirm the removal of the endothelium (no vasodilation response in constricted arteries). Percent dilation was calculated using L2-L1/L1 x 100; where L1 is the initial lumen diameter to infused PSS and L2 is the lumen diameter after infusion of 0.1% BSA-PSS or 0.1% BSA-S1P. The percent dilation was then normalized to the passive lumen diameter as described in section 2.2.3.

	Mesenteric arteries (U46619)	Uterine arteries (U46619)
Uninfected NP: Avg. dose (μM)	0.418±0.0856	0.309±0.0807
Preconstriction (%)	50.1±2.73	50.2±3.43
mCMV-infected NP: Avg. dose (μM)	0.827±0.311	0.755±0.334
Preconstriction (%)	50.5±2.55	50.6±2.38
Uninfected LP: Avg. dose (μM)	0.571±0.0485	0.787±0.198
Preconstriction (%)	49.8±2.25	62.9±1.74 *
mCMV-infected LP: Avg. dose (μM)	0.531±0.0313	0.505±0.0328
Preconstriction (%)	50.1±2.55	60.2±2.13 *

Table 2.2: Preconstriction values and average drug dose used for each vascular bed after flow-induced equilibration during intraluminal pressure myograph experiments. Preconstriction (~50%) following U46619 addition was maintained after flow in all groups except uterine arteries from mCMV-infected and uninfected LP mice (~60%). Data were expressed as the mean ± SEM. A significant difference between preconstriction values in mesenteric and uterine arteries from mCMV-infected and uninfected LP mice following U46619-induced preconstriction was determined by two-way ANOVA and the Bonferroni post-hoc analysis and symbolized with an * ($p<0.05$). It is important to note that these values were not used in the calculation for percent dilation.

2.4.3 Statistics

Data collected from the extraluminal and intraluminal pressure myograph experiments were averaged by group (mCMV-infected and uninfected; NP and LP; mesenteric and uterine) and treatment (S1P ± L-NAME or VPC23019, 0.1% BSA-PSS, and PSS; S1P concentration; S1P ± endothelium). Extraluminal S1P responses were compared as described in sections 2.2.4 and 2.3.4. Y27632-induced reversal of vasoconstriction induced by extraluminally applied S1P was compared between groups (mCMV-infected and uninfected) using a Student's t-test. Intraluminal S1P responses at 0.1μM and 1μM were also compared between groups (mCMV-infected and uninfected) using a Student's t-test. Intraluminal and extraluminal S1P responses in each group were compared between concentrations (0.1μM and 1μM S1P) using a two-way ANOVA followed by Bonferroni's *post hoc* analysis. Vascular responses in each group were compared between treatments (intraluminal and extraluminal PSS, 0.1% BSA-PSS, and 0.1% BSA-S1P) using a one-way ANOVA followed by Bonferroni's post-hoc analysis. Intraluminal vascular responses were compared by group (NP and LP; mesenteric and uterine) and treatment (S1P ± L-NAME or VPC23019, 0.1% BSA-PSS, and PSS; S1P ± endothelium) using a two-way ANOVA followed by Bonferroni's post-hoc analysis. Significance was accepted at $p<0.05$.

2.5 MATERIAL AND METHODS SPECIFIC FOR CHAPTER 6

For this section, active mCMV-infected and uninfected NP and LP C57Bl/6J mice were used to determine how direct virus to endothelium interactions may contribute to altered systemic and uterine endothelium-dependent vasodilation responses in comparison to effects of a systemic infection.

2.5.1 Intraluminal pressure myograph experimental design

The same infusion technique was used as described in section 2.4.2. For mesenteric and uterine arteries isolated from uninfected mice, the tubing

connected to one cannula was loaded with 290μL of PSS with or without gB (1μg/mL) or infectious mCMV (~10 multiplicity of infection (MOI)) followed by 2cm of air and 200μL of a single dose of ME diluted in PSS, PSS alone (control) or the fibroblast cell culture medium obtained by filtering out the virus with a 0.1μM filter (filtered virus control). These concentrations of gB and mCMV were chosen because it has been previously demonstrated that similar activation of intracellular signalling and IFN-responsive gene expression occur at these doses of gB and CMV (Boyle *et al.*, 1999). For mesenteric and uterine arteries isolated from mCMV-infected mice, the tubing was infused with 290μL of PSS followed by 2cm of air and 200μL of one dose of ME diluted in PSS or PSS alone (control). For all experiments, the artery was then mounted and following the 30-minute pressurization steps, a low-flow (30μL/min) was administered to arteries for 20 seconds every 10 minutes over a 30-minute to 1-hour period to allow viral attachment/early entry (no significant difference at either time point). CMV is the largest member of the *Herpesviridae* family and as such has a long replication cycle (up to 48-72 hours) (Britt, 2010; Mocarski, 1996). Therefore, a 30-minute to 1-hour incubation with infectious mCMV is enough time for viral attachment and possibly early entry, but is not sufficient for CMV viral gene expression, replication, or egress (Mocarski, 1996). Arteries were then preconstricted to approximately 50% with the addition of U46619 to the bath (Table 2.2). Flow was resumed for 9 minutes (PSS solution), stopped for 3 minutes, and the lumen diameter was recorded. Flow was then again resumed for approximately 7 minutes (until the single dose of ME was reached), stopped for 3 minutes, and lumen diameter was again recorded. Multiple arteries from each animal were used to obtain results for each dose of ME (1nM to 10μM). Because gB and infectious mCMV pre-treatment gave similar ME-induced vasodilation results in mesenteric and uterine arteries from uninfected NP and LP mice, the following inhibitor experiments were performed on arteries pre-treated with gB only. To determine the effect of vasoconstrictory prostanoids on vascular tone after ME infusion, some mesenteric arteries pre-treated with or without gB from uninfected mice or

some mesenteric arteries from mCMV-infected mice were incubated in the bath with the PGHS-1/2 inhibitor meclo (1μM) or the TxA_2 receptor antagonist SQ29548 (10μM). These pre-treatments were followed by preconstriction with U46619 (meclo) or PE (SQ29548) (Table 2.3) followed by infusion of the vasodilator ME (1μM and 10μM). To confirm that there were no differences in ME-induced vasodilation with U46619- and PE-induced preconstriction as used with meclo and SQ29548, respectively, ME responses were measured after each in the absence of inhibitors (ME response obtained within 20 minutes of PE-induced preconstriction). There was no significant difference in ME-induced vasodilation between U46619- and PE-induced preconstriction (Figure 2.6). To determine if the increased vasodilation to infused ME in uterine arteries could be seen with other vasodilators, bradykinin (1μM; Sigma-Aldrich) was also tested on uterine arteries from uninfected NP and LP mice infused with or without gB or uterine arteries from mCMV-infected mice. The percent dilation was calculated as L2-L1/L1 x 100; where L1 is the initial lumen diameter after flow into the PSS with or without gB or infectious mCMV pre-treatment and L2 is the lumen diameter after the ME, bradykinin, PSS control or filtered virus control. The percent dilation was then normalized to the passive lumen diameter as described in section 2.2.3.

	Mesenteric arteries (U46619)	Mesenteric arteries (PE)
Uninfected NP: Avg. dose (μM)	0.418±0.0856	25.8±6.48
Preconstriction (%)	50.1±2.73	35.0±4.52 *
mCMV-infected NP: Avg. dose (μM)	0.827±0.311	22.5±5.32
Preconstriction (%)	50.5±2.55	36.0±3.03 *
Uninfected LP: Avg. dose (μM)	0.571±0.0485	35.0±9.49
Preconstriction (%)	49.8±2.25	24.9±3.87 *
mCMV-infected LP: Avg. dose (μM)	0.531±0.0313	35.0±9.49
Preconstriction (%)	50.1±2.55	24.0±4.72 *

Table 2.3: Preconstriction values using U46619 versus PE (with SQ29548) and average drug dose used in mesenteric arteries after flow-induced equilibration during intraluminal pressure myograph experiments. Preconstriction values in mesenteric arteries from mCMV-infected or uninfected mice following U46619 or PE addition were compared in NP and LP groups. Preconstriction of mesenteric arteries with PE (maximal dose) was decreased compared to U46619-induced preconstriction in mCMV-infected and uninfected NP or LP mice. A significant difference was determined by two-way ANOVA and the Bonferroni post-hoc analysis and symbolized with an * ($p<0.05$). It is important to note that these values were not used in the calculation for percent dilation.

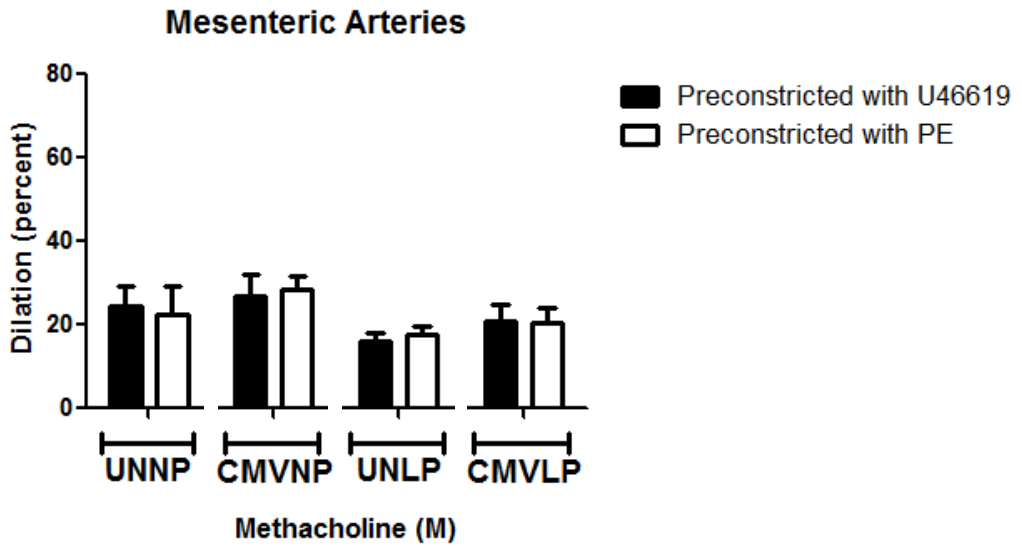

Figure 2.6: ME (1μM) responses for mesenteric arteries following U46619- or PE-induced preconstriction. Arteries were isolated and compared from mCMV-infected (CMV) and uninfected (UN) NP and LP mice. Data were expressed as the mean ± SEM percent increase in arterial lumen diameter compared to the passive lumen diameter in the presence of Ca^{2+}-free medium and papaverine. No significant difference between the bars in each animal group was determined by Student's t-test ($p>0.05$). n=4-7 animals.

2.5.2 Statistics

Intraluminal vascular responses were averaged and compared between groups (mCMV-infected and uninfected ± gB or mCMV) and treatment (ME concentration: 1nM to 10μM) using multiple two-way ANOVAs followed by Bonferroni's post-hoc analysis to determine significance**.** As described in section 2.4.2, intraluminal vascular responses with different treatments (ME ± meclo or SQ29548; bradykinin) were averaged and compared between groups (mCMV-infected and uninfected ± gB) using a one-way ANOVA followed by Bonferroni's post-hoc analysis to determine significance. Significance was accepted at $p<0.05$.

2.6 MATERIAL AND METHODS SPECIFIC FOR CHAPTER 7

For this section, active mCMV-infected and uninfected LP C57Bl/6J and NP and LP Balb/cJ mice were used to determine if there were any effects of a maternal CMV infection on fetal or placental development, independent of a congenital CMV infection, in mice genotypically resistant (C57Bl/6J) or susceptible (Balb/cJ) to CMV. Systemic and uterine cholinergic vasodilation responses were also measured in active mCMV-infected and uninfected NP Balb/cJ mice to compare with those responses observed in C57Bl/6J mice.

2.6.1 Fetal and placenta weights and fetal to placental ratios

The fetuses and placentas were isolated from pregnant mice only at D18.5. They were dissected free of fetal membranes and uterine tissue, blotted to remove excess liquid, and the wet weights were recorded for each fetus and placenta individually. The fetal to placental ratios were also determined.

2.6.2 Balb/cJ mouse strain

Virgin Balb/cJ mice were purchased from Jackson laboratories and housed in Health Sciences Laboratory Animal Services at the University of Alberta. Half of the mice were infected with 10^6PFU, $5x10^6$PFU, or 10^5PFU of mCMV RM427+ and half of the mice remained uninfected as described in section 2.1.1. Breeding was performed with 10 uninfected and 15 mCMV-infected (5 mice/dose) mice. All mice were tested for vaginal plugs and weighed at mid-gestation to confirm the pregnancies. The pregnancy success rate was defined as confirmed pregnancies following the presence of a vaginal plug. Intraluminal pressure myograph vascular studies were completed on 5 mCMV-infected and 5 uninfected NP Balb/cJ mice.

2.6.3 Intraluminal pressure myograph experimental design

Mesenteric and uterine arteries were isolated from NP Balb/cJ mice age-matched to the NP C57Bl/6J mice. The tubing was loaded with 290μL of PSS

followed by 2cm of air and 200μL of one dose of ME (10nM to 10μM) diluted in PSS or just PSS (control). Arteries were preconstricted with U46619 (Table 2.2) and reagents were infused, diameters were recorded, and the percent dilation was calculated as described in sections 2.4.2 and 2.5.1.

2.6.4 Statistics

Intraluminal vascular responses were averaged and compared between groups (mCMV-infected and uninfected) and treatment (ME concentration: 10nM to 10μM) using a two-way ANOVA followed by Bonferroni's post-hoc analysis to determine significance, as described in section 2.5.2. Significance was accepted at $p<0.05$.

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Chapter 3
Vascular Function in Mice with an Active Cytomegalovirus Infection

(The majority of data presented in this chapter was published in 2009 in *American Journal of Physiology: Heart and Circulatory Physiology* 296: H937-H945 in an article entitled "Impaired vascular function in mice with an active Cytomegalovirus infection," by R.B. Gombos, V. Wolan, K. McDonald, and D.G. Hemmings. Although the majority of results in each of the figures I performed, V. Wolan and K. McDonald contributed equally to some of the results in Figure 3.5.)

3.1 INTRODUCTION

HCMV is associated with several vascular diseases including atherosclerosis and vasculopathy, particularly in immunosuppressed individuals who have reduced immune function, such as transplant recipients (Einsele *et al.*, 2008; Freeman, 2009; Melnick *et al.*, 1995; Persoons *et al.*, 1998; Sweet, 1999). However, little is known about how a CMV infection affects vascular function under otherwise normal or mildly inflammatory conditions. It has been demonstrated that a fully active HCMV infection or viral attachment alone stimulates an inflammatory response involving cytokines, ROS, and prostanoids (Compton *et al.*, 2003; Speir *et al.*, 1996; Zhu *et al.*, 1997). Viral-induced production of inflammatory cytokines (Koskinen *et al.*, 1999) and increased expression of adhesion molecules on the surface of infected endothelial cells (Rahbar *et al.*, 2005) implicate HCMV in endothelial dysfunction and consequent vascular inflammatory diseases, such as atherosclerosis and coronary heart disease (Espinola-Klein *et al.*, 2002; Grahame-Clarke, 2005). Moreover, vascular dysfunction in the presence of a HCMV infection may also be attributed to altered NOS and/or PGHS activity (Hooks *et al.*, 2006; Shen *et al.*, 2006; Tanaka *et al.*, 2001) or increased vasoconstrictors, such as catecholamines (Rubin, 2001).

Nevertheless, direct connections between HCMV, endothelial dysfunction and vascular diseases remain unknown.

HCMV is also associated with pregnancy complications including preeclampsia (Grahame-Clarke, 2005; von Dadelszen *et al.*, 2003) and IUGR (Brown *et al.*, 1998; von Dadelszen *et al.*, 2003). Preeclampsia is diagnosed as the *de novo* onset of hypertension (blood pressure >140/90 mmHg) with proteinuria after 20 weeks gestation. Other characteristics of preeclampsia include an enhanced inflammatory state, platelet activation, generalized vasoconstriction and reduced organ perfusion (Friedman *et al.*, 1991). IUGR occurs when fetuses do not reach their full growth potential (Danielian *et al.*, 1992). It may result from reduced uterine blood flow as described in both animal models and humans (Anderson *et al.*, 2005; Lang *et al.*, 2003; Sholook *et al.*, 2007). Therefore, normal vascular adaptations of the systemic and uterine vasculature during pregnancy are very important for maintenance of pregnancy and proper fetal growth and development (Carbillon *et al.*, 2000; Magness *et al.*, 1994).

Due to potential placental transmission of HCMV to the fetus, it is difficult to determine if IUGR results from a direct fetal infection or indirectly through maternal or placental HCMV-associated vascular complications. To circumvent this problem, I used a mouse model of CMV infection (mCMV) which reportedly does not cross the placenta (Johnson, 1969). Based on the association between HCMV and vascular dysfunction, I hypothesize that an active *in vivo* CMV infection will increase vasoconstriction and decrease vasodilation in systemic and uterine arteries from NP female mice. I further hypothesize that these impaired vascular responses will be more evident in pregnancy Mesenteric arteries contribute to peripheral vascular resistance and have been used extensively in the literature to represent the systemic vasculature (Christensen *et al.*, 1993). Uterine arteries play an important role in reproduction. I show for the first time that, contrary to my hypothesis, an acute mCMV infection in both NP and LP mice leads to decreased vasoconstriction and increased vasodilation in mesenteric arteries. Vasodilation in uterine arteries from mCMV-infected NP

mice is also increased. In contrast and in agreement with my hypothesis, I show increased vasoconstriction and decreased vasodilation in uterine arteries from mCMV-infected LP mice.

3.2 RESULTS

3.2.1 LacZ expression in tissues from mCMV-infected and uninfected NP and LP mice

The heart, kidney, liver, lung and spleen were strongly positive for the β-galactosidase enzyme in mCMV-infected compared to uninfected NP mice (Figure 3.1A). In addition, the endothelial and vascular smooth muscle cells of mesenteric and uterine arteries from mCMV-infected NP and LP mice were also positive for the β-galactosidase enzyme compared to uninfected NP and LP mice (Figure 3.1B).

In LP mice sacrificed on D18.5, no β-galactosidase activity was detected in any of the fetuses examined (Figure 3.2). However, there was greater β-galactosidase activity detected on the maternal (Figure 3.2B) compared to fetal (inset) side of the placenta from mCMV-infected LP mice.

3.2.2 Passive lumen diameters of mesenteric and uterine arteries from mCMV-infected and uninfected mice

Passive lumen diameters in second-order mesenteric arteries from uninfected mice were significantly increased in LP compared to NP (LP: 223±9.7μm, NP: 189±5.1μm; $p<0.01$). This adaptive response to pregnancy in mesenteric arteries was absent in mCMV-infected mice (LP: 217±4.7μm, NP: 209±8.2μm; NS). In uterine arteries, passive lumen diameters were significantly increased in mCMV-infected LP versus NP mice (LP: 287±18.3μm, NP: 187±11.8μm; $p<0.01$) similarly to uninfected mice (LP: 283±13.0μm, NP: 173±5.9μm; $p<0.01$).

3.2.3 PE-induced vasoconstriction in mesenteric and uterine arteries from mCMV-infected and uninfected NP and LP mice

In uterine arteries from NP mice, there was no difference in vasoconstriction to the α_1-adrenergic agonist PE between mCMV-infected and uninfected mice (Figure 3.3A). However, maximal PE-induced vasoconstriction in uterine arteries from mCMV-infected LP mice was significantly greater ($p<0.05$) than in uterine arteries from uninfected LP mice (Figure 3.3B). In this animal model, the PE response was not significantly reduced in uterine arteries from LP compared to NP mice, regardless of mCMV infection status (compare Figure 3.3A,B).

In contrast to the uterine arteries from mCMV-infected mice, the sensitivity to PE was significantly decreased in mesenteric arteries from mCMV-infected NP (EC_{50}: 545±76.7nM) compared to uninfected NP (EC_{50}: 239±24.5nM) mice and in mesenteric arteries from mCMV-infected LP (EC_{50}: 460±83.5nM) compared to uninfected LP (EC_{50}: 77.5±14.2nM) mice ($p<0.05$; Figure 3.4A,B).

3.2.4 ME-induced vasodilation in mesenteric and uterine arteries from mCMV-infected and uninfected NP and LP mice

Although a dose-response to PE could be evaluated in mesenteric arteries, the vasoconstriction to a single dose was not stable for longer than 25 minutes and so could not be used for preconstriction in vasodilation studies. In addition, there was a significant difference in the PE dose required for 50% vasoconstriction in mesenteric arteries from mCMV-infected NP and LP mice (Figure 3.4A,B). The TxA_2/PGH_2 mimetic U46619 was therefore used as a preconstrictor for mesenteric arteries. To determine whether mCMV affects responses to this vasoconstrictor, a dose-response curve for U46619 was done on the mesenteric arteries. In both NP and LP mice, there were no significant differences in U46619-induced vasoconstriction in mesenteric arteries from mCMV-infected and uninfected mice (Figures 3.4C,D).

The vasodilation response to increasing concentrations of the cholinergic endothelium-dependent vasodilator ME was measured in mesenteric and uterine arteries isolated from mCMV-infected or uninfected NP and LP mice. Interestingly, in mesenteric arteries, there was a significant increase ($p<0.05$) in ME-induced vasodilation to ME in mCMV-infected compared to uninfected NP and LP mice (Figure 3.5A). In addition, an estimate of the concentration of ME causing 50% vasodilation in mesenteric arteries from mCMV-infected NP and LP mice was approximately 100nM whereas in mesenteric arteries from uninfected NP and LP mice it was approximately 750nM. This is indicative of an increase in sensitivity to ME in mesenteric arteries from mCMV-infected NP and LP mice. Mesenteric arteries from mCMV-infected NP mice showed the greatest ME-induced vasodilation ($p<0.05$). ME-induced vasodilation was lowest and not significantly different in mesenteric arteries from uninfected LP versus NP mice (Figure 3.5A).

Similarly, ME-induced vasodilation and sensitivity to ME in uterine arteries from mCMV-infected (EC_{50}: 52.8±1.36nM) compared to uninfected (EC_{50}: 102±11.1nM) NP mice was significantly increased ($p<0.05$; Figure 3.5B). In late pregnancy, sensitivity to ME was significantly decreased in uterine arteries from mCMV-infected (EC_{50}: 356±130nM) compared to uninfected (EC_{50}: 109±28.9nM) mice ($p<0.05$). There was no significant difference in the normal ME response in uterine arteries from uninfected LP versus NP mice (Figure 3.5B); however, there was a dramatic reduction in maximal ME-induced vasodilation and sensitivity to ME in uterine arteries from mCMV-infected LP (EC_{50}: 356±130nM) compared to NP (EC_{50}: 52.8±1.36nM) mice ($p<0.05$; Figure 3.5B).

3.3 SUMMARY OF RESULTS

3.3.1 A reporter of an active mCMV infection, β-galactosidase, was detected in heart, kidney, liver, lung, and spleen tissue from mCMV-infected NP mice and in

the endothelial and smooth muscle cells of mesenteric and uterine arteries isolated from mCMV-infected NP and LP mice (Figure 3.1).

3.3.2 mCMV did not infect the fetus in mCMV-infected pregnant mice (Figure 3.2).

3.3.3 Maximal PE-induced vasoconstriction was significantly increased in uterine arteries from mCMV-infected LP mice (Figure 3.3).

3.3.4 Sensitivity to PE was significantly decreased in mesenteric arteries from mCMV-infected NP and LP mice (Figure 3.4).

3.3.5 Maximal ME-induced vasodilation and sensitivity to ME was significantly increased in mesenteric arteries from mCMV-infected NP and LP mice and in uterine arteries from mCMV-infected NP mice; however, sensitivity to ME was significantly decreased in uterine arteries from mCMV-infected LP mice (Figure 3.5).

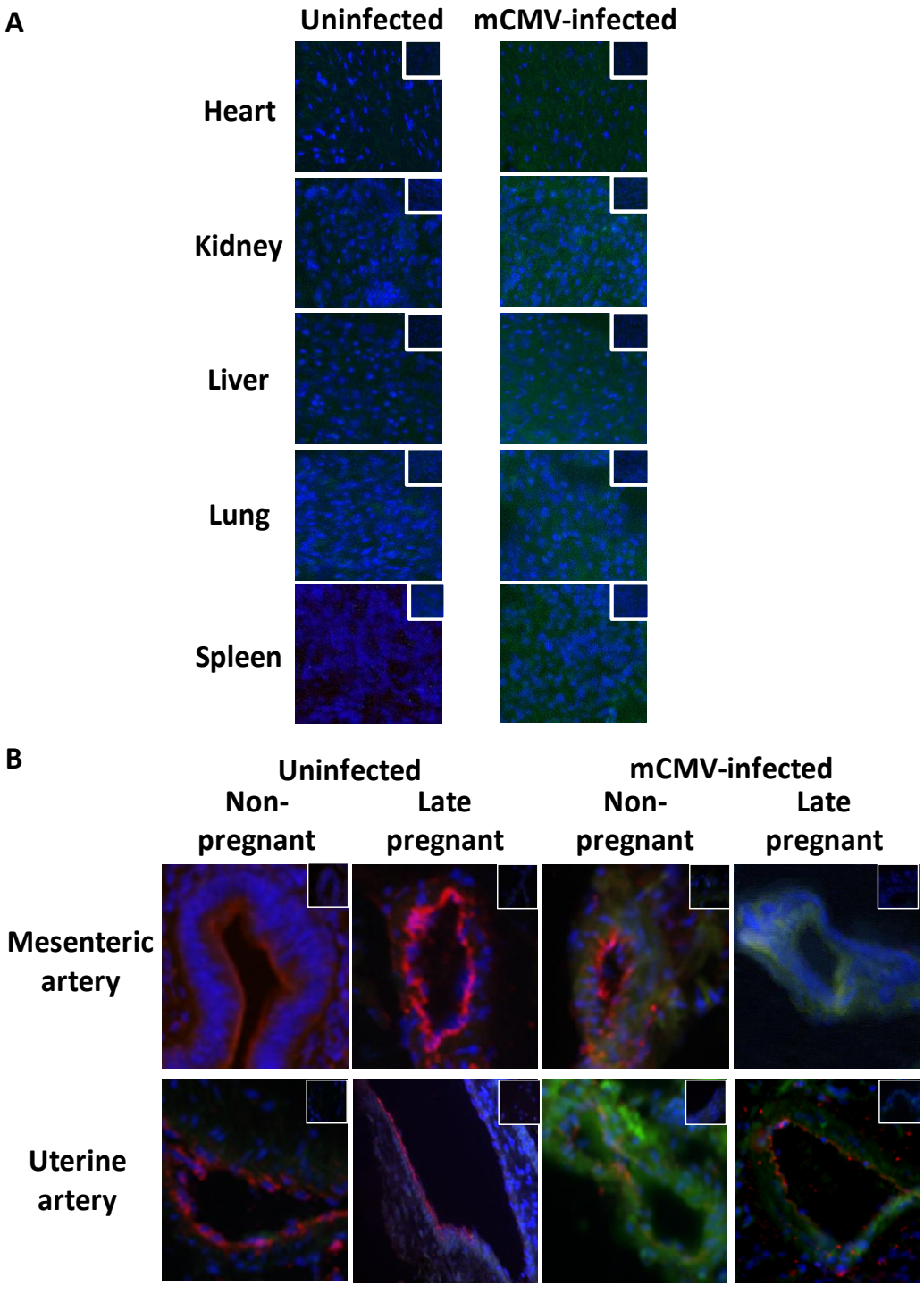

A
Uninfected
mCMV-infected
Heart
Kidney
Liver
Lung
Spleen
B
Uninfected
mCMV-infected
Non-pregnant
Late pregnant
Non-pregnant
Late pregnant
Mesenteric artery
Uterine artery

Figure 3.1: Reporter of an active mCMV infection present in tissues isolated from mCMV-infected mice. To confirm an active mCMV infection, the mouse heart, kidney, liver, lung and spleen tissue from mCMV-infected and uninfected NP mice were stained for β-galactosidase (green) **(A)**. The mesenteric and uterine arteries and surrounding tissue sections from mCMV-infected and uninfected NP and LP mice were also stained for β-galactosidase (green) and Von Willebrand factor to detect endothelium (red) **(B)**. All nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; blue). These images are representative of different sections taken from 3 mice in each group (n=3).

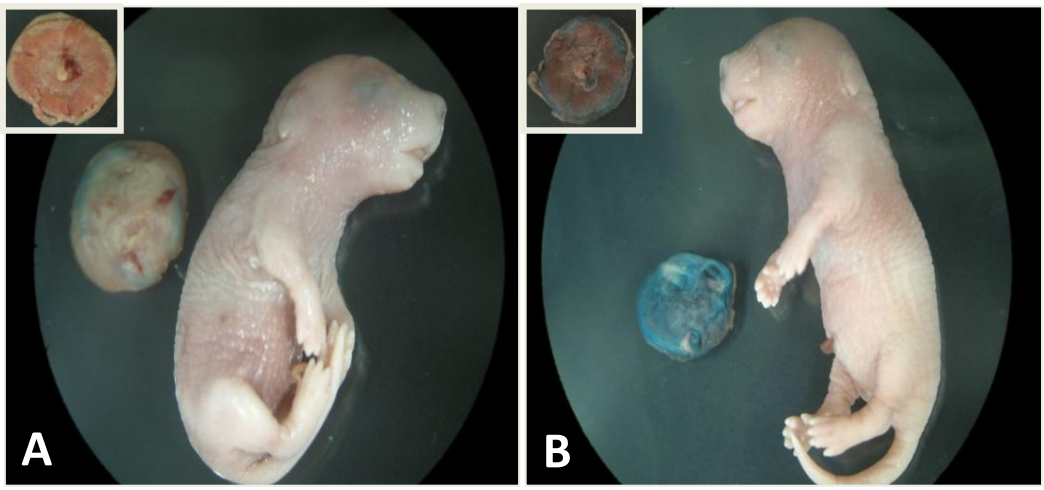


Figure 3.2: Reporter of an active mCMV infection present in placentas from mCMV-infected LP mice. LacZ expression detected through β-galactosidase activity (blue) was used to confirm an active mCMV infection. Staining was performed on the fetal (insets) and maternal side of the placentas in uninfected **(A)** and mCMV-infected **(B)** LP (D18.5) mice. These images are representative of different placentas and fetuses taken from 7 litters in each group (n=7).

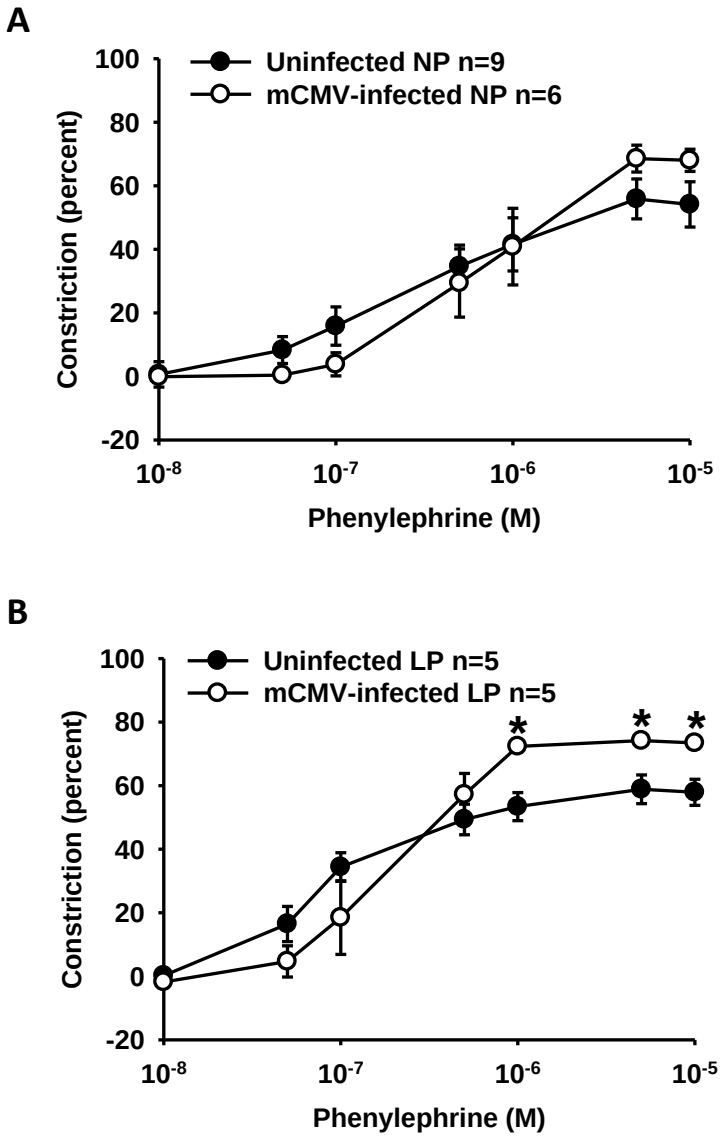


Figure 3.3: Vasoconstriction to PE in uterine arteries from mCMV-infected and uninfected NP and LP mice. Changes in lumen diameter were measured in response to increasing concentrations of PE in uterine arteries from mCMV-infected and uninfected NP **(A)** or LP **(B)** mice. Data were expressed as the mean ± SEM percent decrease in arterial lumen diameter at each dose of PE compared to the lumen diameter prior to drug addition. A significant difference between points on the curves was determined by Repeated Measures two-way ANOVA with Holm Sidak's post-hoc analysis and symbolized with an * ($p<0.05$). n=number of animals.

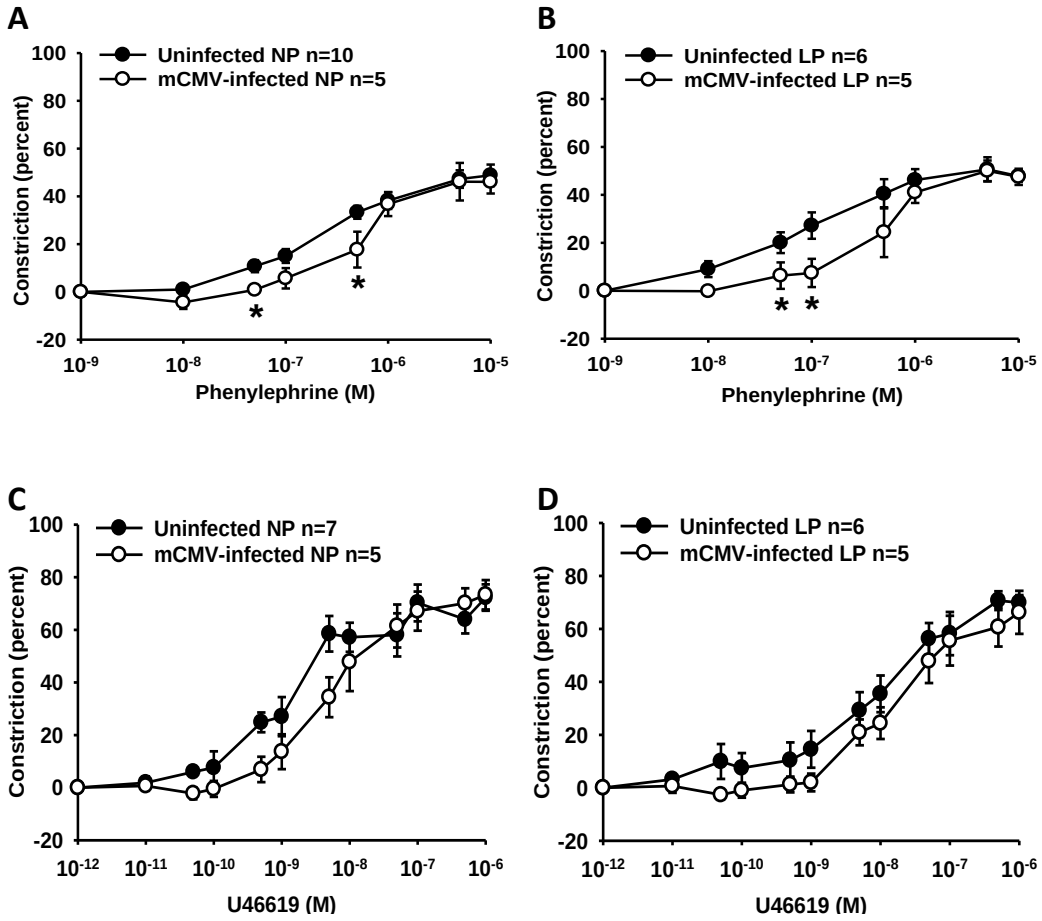
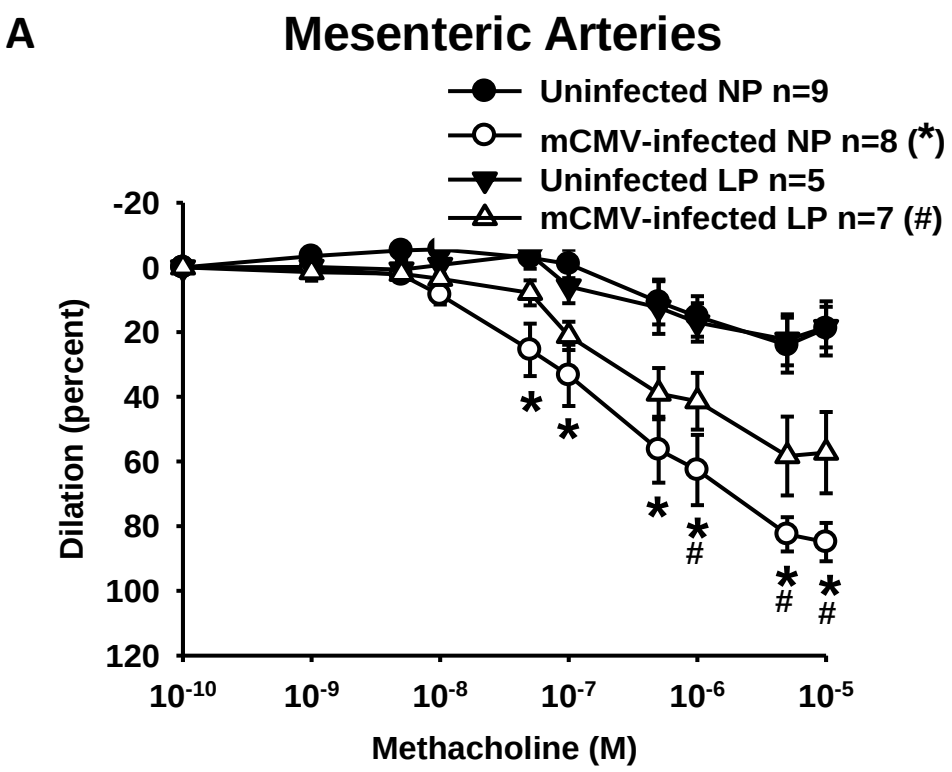


Figure 3.4: Vasoconstriction to PE and U46619 in mesenteric arteries from mCMV-infected and uninfected NP and LP mice. Changes in lumen diameter were measured in response to increasing concentrations of PE **(A, B)** or U46619 **(C, D)** in mesenteric arteries from mCMV-infected and uninfected NP **(A, C)** or LP **(B, D)** mice. Data were summarized and compared as in Figure 3.3. * = ($p<0.05$). n=number of animals.


A
Mesenteric Arteries
Uninfected NP n=9
mCMV-infected NP n=8 (*)
Uninfected LP n=5
mCMV-infected LP n=7 (#)
Dilation (percent)
-20
0
20
40
60
80
100
120
10-10
10-9
10-8
10-7
10-6
10-5
Methacholine (M)

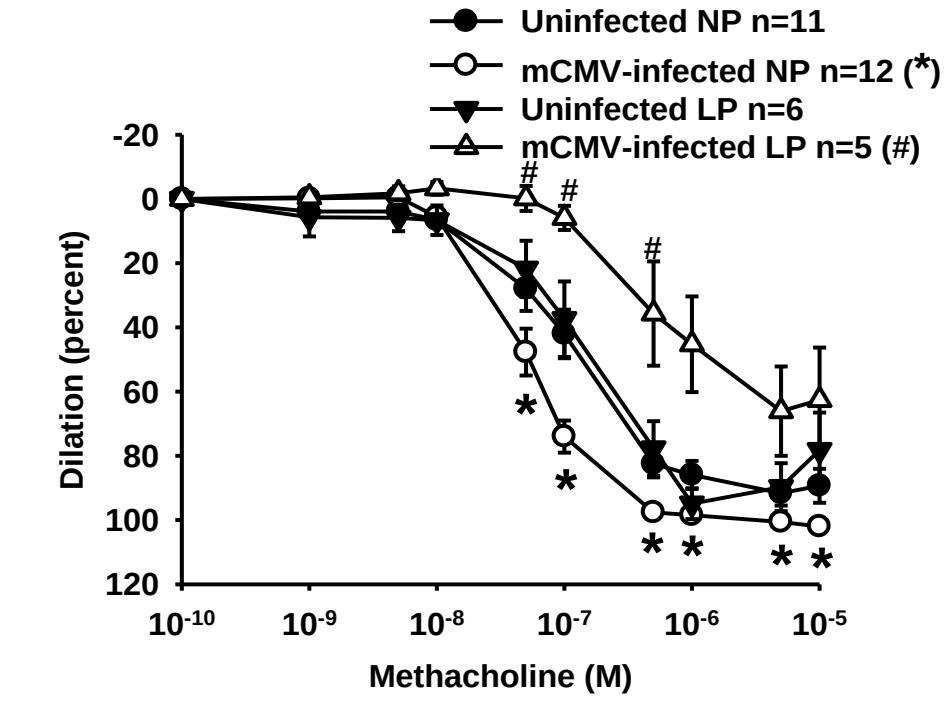

B
Uterine Arteries
Uninfected NP n=11
mCMV-infected NP n=12 (*)
Uninfected LP n=6
mCMV-infected LP n=5 (#)
Dilation (percent)
-20
0
20
40
60
80
100
120
10-10
10-9
10-8
10-7
10-6
10-5
Methacholine (M)

Figure 3.5: Vasodilation to ME in mesenteric and uterine arteries from mCMV-infected and uninfected NP and LP mice. Mesenteric arteries **(A)** were preconstricted with U46619. Uterine arteries **(B)** were preconstricted with PE. Changes in lumen diameter were measured in response to increasing concentrations of ME in arteries from NP and LP mCMV-infected and uninfected mice. Data were summarized and expressed as the mean ± SEM percent increase in arterial lumen diameter at each dose of ME compared to the lumen diameter following preconstriction in the absence of ME, and normalized to the arterial lumen diameter measured in the presence of calcium-free medium and papaverine. A significant difference between points on the curves was determined by Repeated Measures two-way ANOVA with Holm Sidak's post-hoc analysis and symbolized with an * (mCMV-infected versus uninfected NP), # (mCMV-infected versus uninfected LP), or & (mCMV-infected LP versus mCMV-infected NP) ($p<0.05$). n=number of animals.

3.4 DISCUSSION

The findings from this chapter are the first to illustrate that an active *in vivo* CMV infection results in vascular dysfunction in both NP and LP mice. As previously demonstrated (Johnson, 1969), I confirmed that mCMV does not infect the fetus, with an active mCMV infection more evident on the maternal compared to fetal side of the placenta. In addition, the reporter of an active mCMV infection, β-galactosidase, was found in the mesenteric and uterine arteries used in my *ex vivo* vascular response studies. Contrary to my hypothesis, mesenteric arteries, which are representative of systemic resistance arteries, showed decreased sensitivity to the α_1-adrenergic receptor agonist PE and increased cholinergic (ME)-induced vasodilation and sensitivity to ME when isolated from mCMV-infected compared to uninfected NP and LP mice. Similar ME results were found in uterine arteries from NP mice. In keeping with my hypothesis, uterine arteries from LP mice showed decreased sensitivity to ME and increased maximal vasoconstriction to PE. Thus, these data demonstrate that an active maternal CMV infection does affect maternal vascular function.

Much research has surrounded congenital CMV infections (Brown *et al.*, 1998); however, less is known about CMV-induced pregnancy complications independent of a congenital infection. An important aspect of this study in the mouse model is that it shows the effect of an active maternal infection without the influence of a congenital infection (Johnson, 1969). The lack of mCMV transmission to the fetus was confirmed by the absence of β-galactosidase expression in the fetus. The placental and fetal effects of a maternal mCMV infection are decribed in Chapter 7.

Mesenteric arteries from mCMV-infected NP and LP mice responded with greater vasodilation to ME than uninfected mice. It is interesting to contemplate a role for mCMV in hypotension since hypotension occurs in several diseases including diabetes (Maser *et al.*, 2007), autonomic neuropathy (Bouhanick *et al.*, 2007), septic shock (O'Brien *et al.*, 2007) and dementia in aging (Moretti *et al.*, 2008) in which CMV infections could play a part (Aiello *et*

al., 2006; Leung Ki *et al.*, 2008; von Muller *et al.*, 2008). In pregnancy, the consequences of chronic hypotension are reduced birth weight and preterm delivery likely as a result of reduced uteroplacental blood flow from reduced perfusion pressure (Grunberger *et al.*, 1979; Ng *et al.*, 1992). In support of my hypothesis, uterine arteries from mCMV-infected LP mice showed decreased sensitivity to ME and increased PE-induced vasoconstriction. This, in conjunction with the increased ME-induced vasodilation and decreased sensitivity to PE in mesenteric arteries and potential reduction in perfusion pressure, could lead to decreased blood flow to the placenta and fetus and IUGR. However, it is important to test other vascular mediators on arteries isolated from mCMV-infected NP and LP mice that may also contribute to overall systemic and/or uterine vascular resistance *in vivo* (Chapter 5).

The altered vascular responses observed in this study may be a direct effect of CMV infection in the vascular wall (Burnett *et al.*, 2004), or an indirect effect via a viral-induced inflammatory response (Zhou *et al.*, 1999). A fully productive infection or simply viral attachment alone (without internalization or viral replication) stimulates the cell to produce prostaglandins, ROS, and activates NF-κB leading to production of inflammatory cytokines, all of which can affect vascular responses (Compton *et al.*, 2003; Speir *et al.*, 1996; Zhu *et al.*, 1997). I show that both endothelial and vascular smooth muscle cells in mesenteric and uterine arteries are directly infected with mCMV. It is therefore likely that the impaired vascular responses in mCMV-infected mice involve direct viral-induced changes in endothelial function (Chapter 6). A seropositive status for HCMV is associated with endothelial dysfunction and an increased risk of adverse cardiovascular events (Espinola-Klein *et al.*, 2002; Grahame-Clarke, 2005; Petrakopoulou *et al.*, 2004). As well, CMV is associated with reduced phosphorylation activation of NOS-3 in aortic endothelial cells *in vitro* (Shen *et al.*, 2006) and an increase in the plasma levels of the NOS-3 inhibitor asymmetric dimethylarginine (Weis *et al.*, 2004). CMV also increases PGHS-2 activity which is important for viral replication (Zhu *et al.*, 2002); this can lead to production of

vasoconstrictory and/or vasodilatory prostanoids. It is important to note that the balance of factors contributing to endothelium-dependent vasodilation vary considerably among vascular beds and sometimes even within a vascular bed depending on the location (Campbell *et al.*, 2001; Hemmings *et al.*, 2004). Thus, it is essential to further investigate the relative roles of NO, prostanoids, and EDHF using inhibitors for these pathways to determine the mechanism by which mCMV is inducing differential vascular dysfunction in mesenteric compared to uterine arteries in this *ex vivo* model (Chapter 4).

Vascular adaptations to pregnancy include decreased sensitivity to vasoconstrictors (Hines *et al.*, 1992; Magness *et al.*, 1998; Paller *et al.*, 1989) and increased vasodilation to accomodate increased blood volume and maintain peripheral blood pressure (Magness *et al.*, 1998). In the mouse, Cooke and Davidge showed increased endothelium-dependent vasodilation in uterine and mesenteric arteries in pregnancy (Cooke *et al.*, 2003). In addition, several studies in rats and guinea pigs have also found a decreased sensitivity to PE and an increased sensitivity to ME in uterine and mesenteric arteries during pregnancy (Davidge *et al.*, 1992; Hermsteiner *et al.*, 2001; Kim *et al.*, 1994; Ni *et al.*, 1997; Parent *et al.*, 1990; Weiner *et al.*, 1992), although one study showed an increased sensitivity to PE in uterine arteries (Chirayath *et al.*, 2007). In my experiments in the uninfected mouse, however, no significant differences in PE or ME responses were found when LP were compared to NP mice in either mesenteric or uterine arteries. One explanation for these opposing results is the differing methodology to evaluate vascular function. Most studies used the wire myograph system (Cooke *et al.*, 2003; Davidge *et al.*, 1992; Kim *et al.*, 1994; Ni *et al.*, 1997; Parent *et al.*, 1990; Weiner *et al.*, 1992), which allows the drug to access both the endothelium and the vascular smooth muscle cells directly and simultaneously. In my study, the pressure myograph system was used in which drugs added to the bath must first pass through the vascular smooth muscle cells to interact with the endothelium (extraluminal addition). It is possible that extraluminally applied drugs in this system are less efficient at accessing the endothelium in arteries from

pregnant mice as readily as in the wire myograph system. Furthermore, contrasting vascular responses may be due to the stage of pregnancy vascular experiments were performed (ie. early versus late gestation (Hermsteiner *et al.*, 2001)), genetic and proteomic differences among rodent models, or differences in artery location (i.e. main compared to radial uterine arteries). For example, in the late pregnant rat, D'Angelo and Osol found increased sensitivity to PE in the main uterine artery (D'Angelo *et al.*, 1993) whereas Hermsteiner et al. found decreased sensitivity to PE in the radial uterine artery (Hermsteiner *et al.*, 2001). Although both studies used the pressure myograph system, different results were found.

Demonstration of a direct link between an active CMV infection and altered vascular responses suggests a new paradigm for understanding cardiovascular diseases stemming from vascular dysfunction. Nevertheless, further studies are essential to define the mechanisms by which CMV affects vascular function (Chapter 4). Determining which intracellular signalling pathway(s) a CMV infection affects and modulates vascular responses through will facilitate the development of valuable pharmacological and/or immunological agents for the effective treatment of HCMV-associated vascular dysfunction.

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Chapter 4

Differential Regulation of Endothelium-dependent and Endothelium-independent Vasodilation in Mesenteric and Uterine Arteries from Cytomegalovirus-infected Mice

(The majority of data presented in this chapter was published in 2010 in *American Journal of Physiology: Heart and Circulatory Physiology,* 299: H1124-1134 in an article entitled "Differential effects on nitric oxide-mediated vasodilation in mesenteric and uterine arteries from cytomegalovirus-infected mice," by R.B. Gombos and D.G. Hemmings.)

4.1 INTRODUCTION

In Chapter 3 I found increased cholinergic endothelium-dependent vasodilation in mesenteric and uterine arteries isolated from NP mice with an active mCMV infection. In late pregnancy, I continued to see increased endothelium-dependent vasodilation in mesenteric arteries from mCMV-infected compared to uninfected mice. In contrast, there was a dramatic decrease in sensitivity to the cholinergic agonist ME in the uterine arteries from mCMV-infected compared to uninfected LP mice (Gombos *et al.*, 2009). Thus, an active mCMV infection differentially affects cholinergic vascular responses in these two vascular beds and also in NP compared to LP mice. The intracellular vasodilatory pathways affected in mCMV-infected mice through which these differential effects occur are unknown and are the focus of this present chapter.

The balance of vasoactive factors contributing to vascular tone can vary greatly among or even within vascular beds (Campbell *et al.*, 2001; Hemmings *et al.*, 2004). In pregnancy, the uterine artery has greater capacity for vasodilation through increased NO and PGI_2 (Bird *et al.*, 2003) and also undergoes dramatic remodelling including hyperplasia and hypertrophy of the endothelial and/or smooth muscle cells within the vascular wall (Osol *et al.*, 2009). In addition, the

sensitivity to some vasoconstrictors, including angiotensin II and catecholamines, is decreased in uterine arteries during pregnancy (Thaler *et al.*, 2005; Weiner *et al.*, 1989b). Systemic arteries, such as those in the mesentery, do not increase in diameter to the same extent as uterine arteries during pregnancy (Osol *et al.*, 2009) but they do also show reduced sensitivity to vasoconstrictors and increased sensitivity to vasodilators (D'Angelo *et al.*, 1993; Dantas *et al.*, 1999; Harrison *et al.*, 1989). These vascular adaptations ensure sufficient blood flow reaches the placenta and fetus during gestation whilst simultaneously maintaining maternal blood pressure (Osol *et al.*, 2009).

A CMV infection affects the expression and activity of vasoactive factors such as NO and prostanoids. To date, these effects have only been shown in non-vascular tissues or in isolated endothelial cell cultures. A CMV infection can either increase (Tanaka *et al.*, 1997) or decrease (Shen *et al.*, 2006) NOS activity thereby affecting the levels of NO. In addition, PGHS-2 expression is increased during a CMV infection to facilitate viral replication in fibroblasts (Zhu *et al.*, 2002) and retinal epithelial cells (Hooks *et al.*, 2006). It is not yet known whether PGHS-2 activity is similarly affected in vascular cells. This is important since an increase in PGHS-2 activity can affect vascular tone through increased production of the vasodilator PGI_2 and/or the vasoconstrictors TxA_2 and PGH_2 (Davidge, 2001). However, the effects of an *in vivo* active CMV infection on the contribution of NO or prostanoids to endothelium-dependent vasodilation in isolated, intact blood vessels remain unknown.

To understand how an active CMV infection contributes to abnormal vascular responses shown in the mouse model (Chapter 3) and to cardiovascular disease in general, it is important to investigate the effects of infection on the vasoactive mediators that regulate vascular tone. I hypothesize that the increased endothelium-dependent vasodilation observed in mesenteric and uterine arteries from NP mice and mesenteric arteries of LP mice is mediated by an increase in NO contribution and/or an increase in the balance of vasodilator to vasoconstrictor prostanoids. I further hypothesize that the decreased sensitivity to

ME in uterine arteries of LP mice is mediated by a decrease in NO contribution and/or an increase in the balance of vasoconstrictor to vasodilator prostanoids. I show that increased ME-induced vasodilation in mesenteric arteries from mCMV-infected NP and LP mice and uterine arteries from mCMV-infected NP mice is mediated by increased smooth muscle cell sensitivity to NO and/or NO contribution. Decreased sensitivity to ME in uterine arteries from mCMV-infected LP mice cannot be explained by these mechanisms.

4.2 RESULTS

4.2.1 Contribution of NO and prostanoids to endothelium-dependent ME-induced vasodilation responses in mesenteric arteries

In agreement with my previous findings in Chapter 3 (Gombos *et al.*, 2009), there was increased endothelium-dependent vasodilation in mesenteric arteries from mCMV-infected compared to uninfected NP mice (Figure 4.1). I found that the contribution of NO to ME-induced vasodilation, as shown by NOS inhibition with L-NAME, was significantly greater ($p<0.05$) in mesenteric arteries from mCMV-infected compared to uninfected NP mice (Figure 4.1A,B). This is also demonstrated in Figure 4.1C where the difference between the two curves in Figure 4.1B was significantly greater than the difference between the curves from Figure 4.1A. Pre-treatment with the PGHS-1/2 inhibitor meclo (Figure 4.1D-F) or the TxA_2 receptor antagonist SQ29548 (Figure 4.1G-I) had no effect on ME-induced vasodilation. Combined pre-treatment with L-NAME and meclo in mesenteric arteries from uninfected NP mice (Figure 4.1J) inhibited vasodilation similarly to that seen with L-NAME pre-treatment alone (Figure 4.1A). This combined pre-treatment inhibited ME-induced vasodilation in mesenteric arteries from mCMV-infected NP mice (Figure 4.1K) to a significantly greater extent than in mesenteric arteries from uninfected NP mice ($p<0.05$, Figure 4.1J,L). Interestingly, however, the inhibition of endothelium-dependent vasodilation in the combined presence of L-NAME and meclo in mesenteric arteries from

mCMV-infected NP mice (Figure 4.1K) was significantly less ($p<0.05$) than that seen by pre-treatment with L-NAME alone in these arteries (Figure 4.1B). Thus, the remaining endothelium-dependent vasodilation due to EDHF in arteries from mCMV-infected NP mice was significantly increased compared to uninfected NP mice (Figure 4.1J,K).

To examine the possibility that the vasoconstrictors, TxA_2 or PGH_2 (they both act on the TxA_2 receptors (Vezza *et al.*, 2002)), were contributing to vascular responses in the mCMV-infected mice but were being masked by increased NO-mediated vasodilation, these results were repeated using SQ29548 in the presence or absence of L-NAME. As shown previously, SQ29548 pre-treatment alone had no effect on ME-induced vasodilation (Figure 4.1G-I) whereas the combination of SQ29548 and L-NAME mimicked the results found with the combined meclo and L-NAME pre-treatment (Figure 4.2A,B).

In LP mice, pre-treatment of mesenteric arteries with L-NAME significantly inhibited ME-induced vasodilation only in mCMV-infected mice, although comparison of differences in area under curve between mesenteric arteries from mCMV-infected and uninfected mice did not reach significance (Figure 4.3A-C). Similarly to NP mice, meclo (Figure 4.3D-F) or SQ29548 (Figure 4.3G-I) pre-treatment alone had no effect on arteries from either LP group. Maximal ME-induced vasodilation was significantly decreased ($p<0.05$) in the presence of both L-NAME and meclo in arteries from mCMV-infected LP mice (Figure 4.3J-L). However, there were no significant differences in the contribution of EDHF to endothelium-dependent vasodilation (Figure 4.3J,K).

4.2.2 Contribution of NO and prostanoids to endothelium-dependent ME-induced vasodilation responses in uterine arteries

Overall, there was increased vasodilation in uterine arteries from mCMV-infected NP mice and decreased sensitivity to ME in uterine arteries from mCMV-infected LP mice compared to uninfected mice, as previously shown (Chapter 3) (Gombos *et al.*, 2009). ME-induced vasodilation in uterine arteries

was significantly inhibited by L-NAME pre-treatment in all mCMV-infected NP (IC_{50}: 224±15.8nM) and LP (IC_{50}: 642±77.5nM) and uninfected NP (IC_{50}: 846±37.6nM) and LP (IC_{50}: 1014±269nM) groups (Figures 4.4A-C; 4.5A-C). Although the overall difference in area under the curve was not significantly different between mCMV-infected and uninfected NP or LP mice, the L-NAME IC_{50} was significantly decreased in uterine arteries from mCMV-infected (IC_{50}: 224±15.8nM) compared to uninfected (IC_{50}: 846±37.6nM) NP mice ($p<0.05$). This may be attributable to increased sensitivity to EDHF following ME addition in uterine arteries from mCMV-infected NP mice. Similarly to mesenteric arteries, meclo (Figures 4.4D-F; 4.5D-F) or SQ29548 (Figures 4.4G-I; 4.5G-I) pre-treatment alone had no significant effect on ME-induced vasodilation in uterine arteries from any NP or LP group. In contrast to results found in mesenteric arteries, pre-treatment with the combination of L-NAME and meclo (Figures 4.4J-L; 4.5J-L) or L-NAME and SQ29548 (Figure 4.6A,B) inhibited ME-induced vasodilation in uterine arteries from mCMV-infected and uninfected NP or LP mice to the same extent as that found with L-NAME pre-treatment alone (Figure 4.4A-C; 4.5A-C). L-NAME and meclo pre-treatment had an IC_{50} of 299±18.5nM in uterine arteries from mCMV-infected NP mice and 238±30.1nM in mCMV-infected LP mice and an IC_{50} of 440±30.8nM in uterine arteries from uninfected NP mice and 722±27.4nM in uninfected LP mice.

4.2.3 Sensitivity of the vascular smooth muscle to NO in mesenteric and uterine arteries

The sensitivity of vascular smooth muscle to NO was measured using SNP, a NO donor, in preconstricted arteries. Increased vasodilation in response to SNP was observed in both mesenteric and uterine arteries from mCMV-infected compared to uninfected NP mice (Figure 4.7A,C). In addition, an estimate of the concentration of SNP causing 50% vasodilation in mesenteric arteries from mCMV-infected NP mice was approximately 75nM whereas in mesenteric arteries from uninfected NP mice it was approximately 500nM. This is indicative

of an increase in sensitivity to SNP in mesenteric arteries from mCMV-infected NP mice (Figure 4.7A). However, the sensitivity to SNP was not significantly increased in uterine arteries from mCMV-infected (EC_{50}: 81.2±10.7nM) compared to uninfected (EC_{50}: 113±16.1nM) NP mice (Figure 4.7C). In contrast, in LP mice there were no significant differences in SNP-induced vasodilation between groups in either artery type (Figure 4.7B,D). Interestingly, in mCMV-infected NP mice, vasodilation to SNP in both mesenteric and uterine arteries was similar to SNP-induced vasodilation in mCMV-infected and uninfected LP mice.

4.2.4 Distensibility of mesenteric and uterine arteries

The distensibility of mesenteric arteries from mCMV-infected NP mice was not significantly different from uninfected NP mice (Figure 4.8A); however, it was significantly greater in mesenteric arteries from mCMV-infected compared to uninfected LP mice ($p<0.05$; Figure 4.8B). The distensibility of uterine arteries from both mCMV-infected NP and LP mice was significantly greater than in uterine arteries from uninfected NP and LP mice, respectively ($p<0.05$; Figure 4.8C,D).

4.2.5 Expression of NOS enzymes in mesenteric and uterine arteries

My functional studies were supported by evaluation of NOS enzyme expression by immunofluorescence and quantitative RT-qPCR. Given the limited quantity of vascular tissue available to study, I chose to quantitatively measure expression of the major endothelial NOS enzyme, NOS-3 by RT-qPCR (Figure 4.9). NOS-1 and NOS-2 expression was qualitatively assessed by immunofluorescence (Figure 4.10). NOS-3 mRNA was not significantly different in uterine arteries from mCMV-infected or uninfected NP and LP mice. In contrast, in mesenteric arteries, NOS-3 mRNA was significantly increased in mCMV-infected LP ($p<0.05$) and there was a trend towards an increase in mCMV-infected NP mice compared to the uninfected mice (Figure 4.9). NOS-2 but not NOS-1 expression was increased in mesenteric arteries from mCMV-

infected compared to uninfected NP and LP mice (Figure 4.10A,C). The expression of NOS-1 and NOS-2 were increased in the uterine arteries from both mCMV-infected and uninfected LP when compared to uterine arteries from NP mice (Figure 4.10B,D). An increase in the expression of NOS-1 was also observed when uterine arteries from mCMV-infected NP mice were compared to uterine arteries from uninfected NP mice (Figure 4.10B).

4.3 SUMMARY OF RESULTS

4.3.1 Increased ME-induced vasodilation in mesenteric arteries from mCMV-infected NP and LP mice was partially due to increased NO-mediated vasodilation, which overrode prostanoid-mediated vasoconstriction in mesenteric arteries from mCMV-infected NP mice (Figures 4.1, 4.3).

4.3.2 The vasoactive prostanoid that mediated the vascular responses to ME in mesenteric arteries from mCMV-infected NP mice acts on the TxA_2 receptor (Figure 4.2).

4.3.3 Increased ME-induced vasodilation in uterine arteries from mCMV-infected NP mice was not due to increased NO- or prostanoid-mediated vasodilation (Figures 4.4, 4.6).

4.3.4 Decreased ME-induced vasodilation in uterine arteries from mCMV-infected LP mice was not due to decreased NO- or prostanoid-mediated vasodilation or increased prostanoid-mediated vasoconstriction (Figure 4.5).

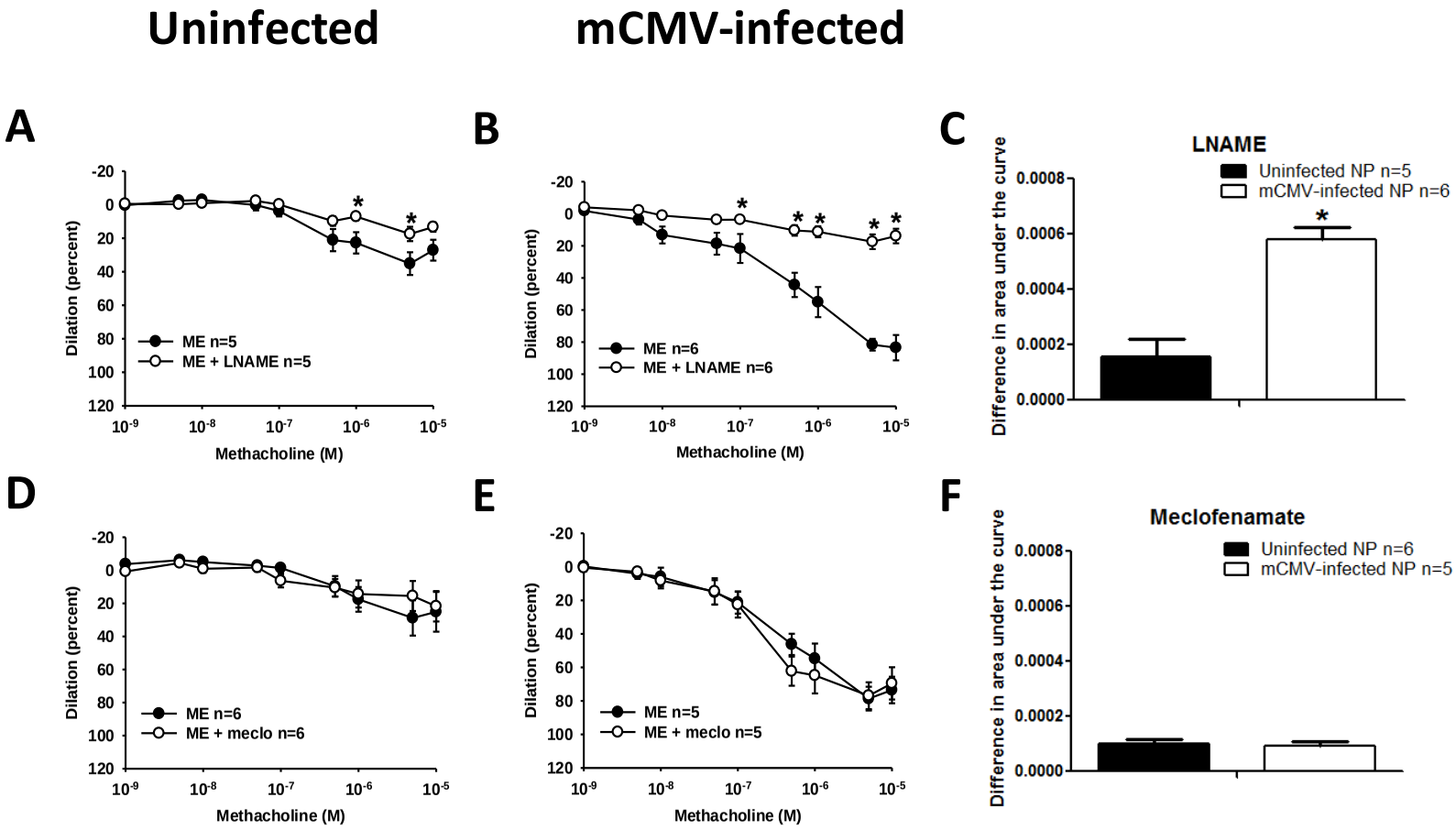
4.3.5 Increased ME-induced vasodilation in mesenteric and uterine arteries from mCMV-infected NP mice was also mediated by increased smooth muscle sensitivity to NO (Figure 4.7).

4.3.6 Distensibility of mesenteric arteries from mCMV-infected LP mice and uterine arteries from mCMV-infected NP and LP mice was increased compared to uninfected NP and LP mice (Figure 4.8).

4.3.7 NOS-3 mRNA expression was increased in mesenteric arteries from mCMV-infected mice (Figure 4.9).

4.3.8 NOS-1 protein expression was qualitatively increased in uterine arteries from mCMV-infected compared to uninfected NP mice (Figure 4.10).

4.3.9 NOS-2 protein expression was qualitatively increased in mesenteric arteries from mCMV-infected compared to uninfected NP and LP mice (Figure 4.10).


Uninfected
mCMV-infected
A
B
C
D
E
F
Dilation (percent)
Methacholine (M)
ME n=5
ME + LNAME n=5
ME n=6
ME + LNAME n=6
ME n=6
ME + meclo n=6
ME n=5
ME + meclo n=5
LNAME
Uninfected NP n=5
mCMV-infected NP n=6
Difference in area under the curve
Meclofenamate
Uninfected NP n=6
mCMV-infected NP n=5

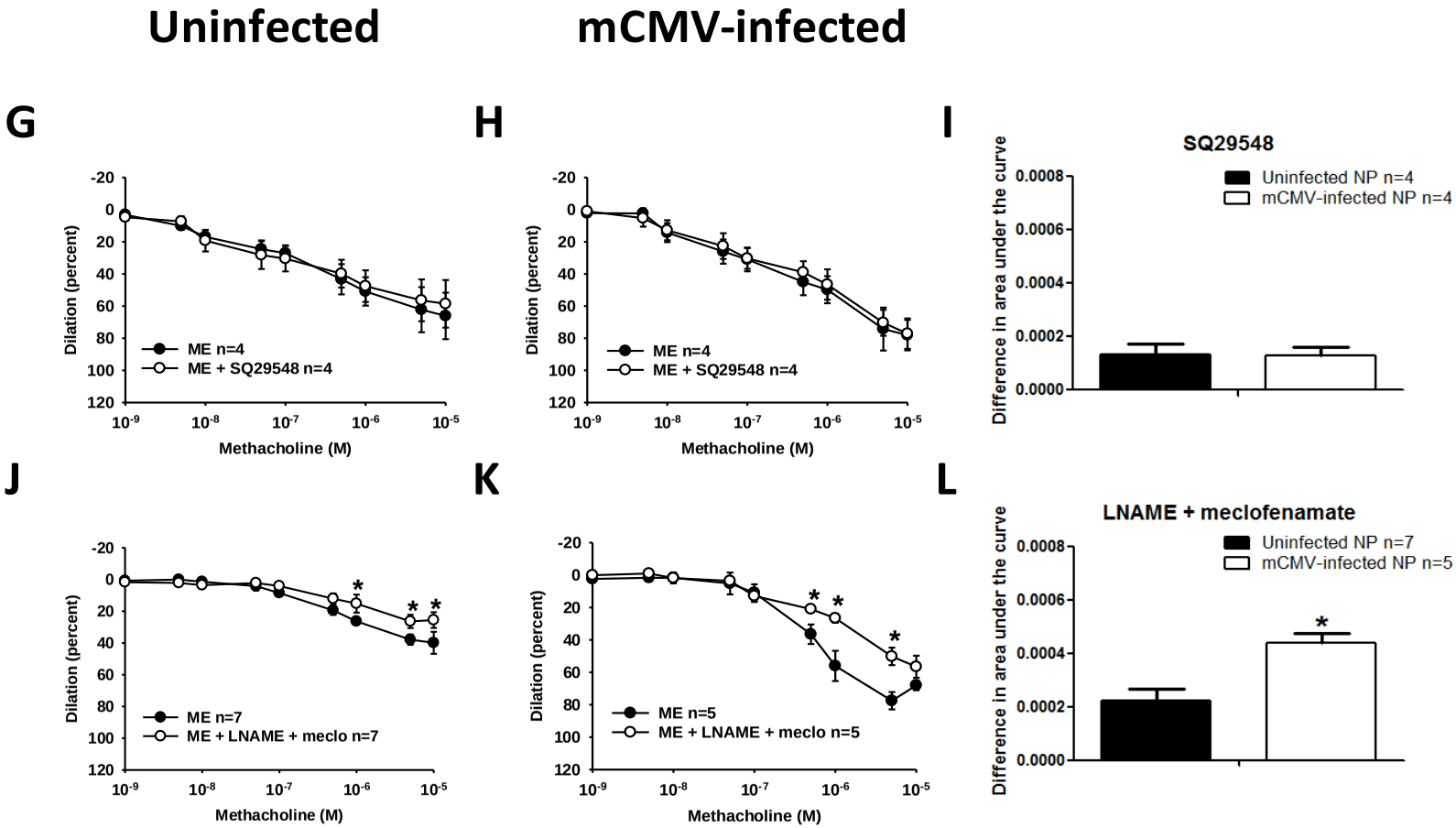

Uninfected
mCMV-infected
G
H
I
J
K
L
Dilation (percent)
Methacholine (M)
ME n=4
ME + SQ29548 n=4
SQ29548
Uninfected NP n=4
mCMV-infected NP n=4
Difference in area under the curve
ME n=7
ME + LNAME + meclo n=7
ME n=5
ME + LNAME + meclo n=5
LNAME + meclofenamate
Uninfected NP n=7
mCMV-infected NP n=5

Figure 4.1: Inhibition of ME-induced vasodilation in mesenteric arteries from mCMV-infected and uninfected NP mice. ME-induced vasodilation was measured in mesenteric arteries from uninfected **(A, D, G, J)** and mCMV-infected **(B, E, H, K)** NP mice after preconstriction of mesenteric arteries using U46619 in the presence or absence of L-NAME (100μM) **(A, B, C)**, meclofenamate (meclo; 1μM) **(D, E, F)**, SQ29548 (10μM) **(G, H, I)**, or L-NAME and meclo **(J, K, L)**. Results for each curve were summarized and expressed as the mean ± SEM percent increase in lumen diameter compared to the initial preconstricted diameter, and normalized to the passive lumen diameter. A significant difference between points on the curves was determined by Repeated Measures two-way ANOVA with Holm-Sidak's post-hoc analysis. For each graph, the area under the curve was calculated and the difference between two curves on the same graph was obtained **(C, F, I, L)**. These differences in area under the curve were compared between mCMV-infected and uninfected groups using a Student's t-test. Significant differences were symbolized with an * ($p<0.05$). n = number of animals.

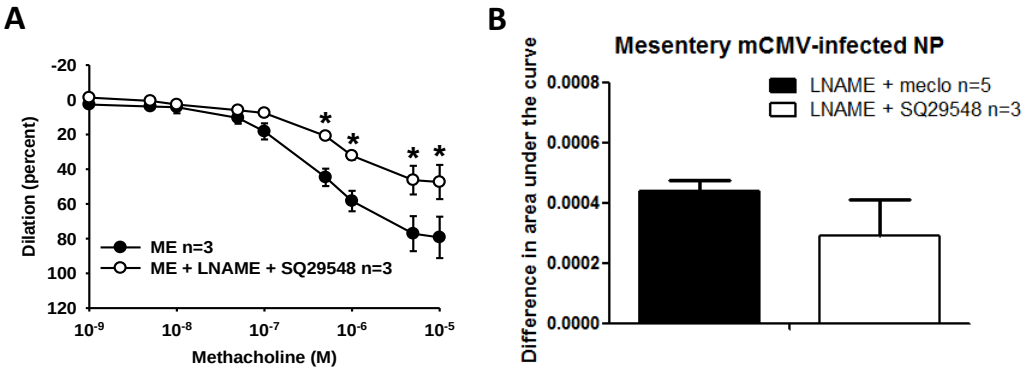
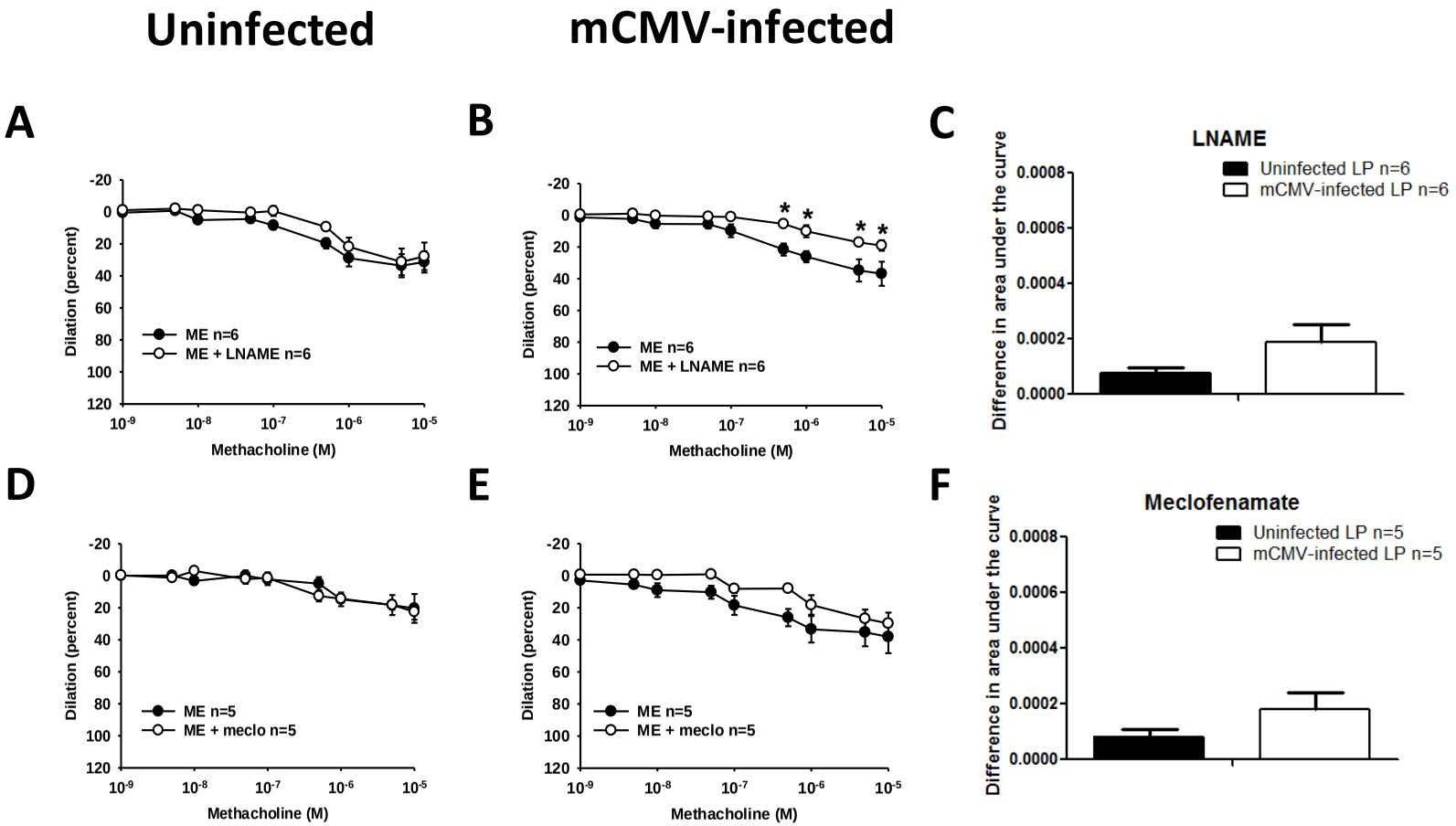


Figure 4.2: NO and TxA_2/PGH_2 contribution to ME-induced vasodilation in mesenteric arteries from mCMV-infected NP mice. ME-induced vasodilation was measured in mesenteric arteries from mCMV-infected NP mice after preconstriction of mesenteric arteries using U46619 in the presence or absence of L-NAME (100μM) and SQ29548 (10μM) **(A)**. Changes in lumen diameter were measured in response to increasing concentrations of ME following preconstriction of mesenteric arteries. The area under the curve was calculated and the difference between the two curves on panel **(A)** was obtained. For comparison purposes, the difference in the area under the curve for L-NAME with meclo was taken from Figure 4.1L **(B)**. Results were summarized and compared as in Figure 4.1. * = $p<0.05$. n=number of animals.


Uninfected
mCMV-infected
A
B
C
D
E
F
Dilation (percent)
Methacholine (M)
ME n=6
ME + LNAME n=6
ME n=5
ME + meclo n=5
LNAME
Meclofenamate
Uninfected LP n=6
mCMV-infected LP n=6
Uninfected LP n=5
mCMV-infected LP n=5
Difference in area under the curve

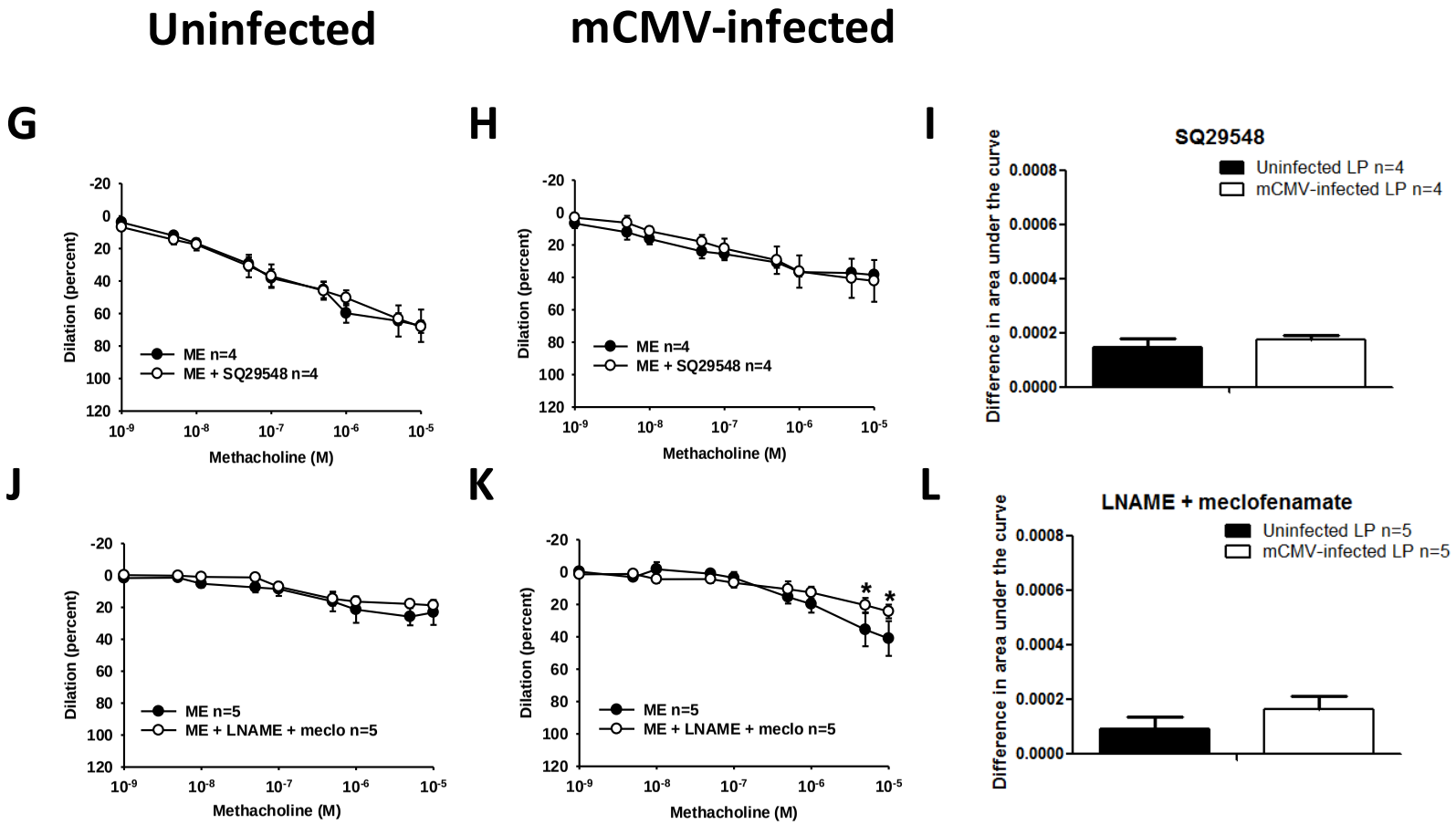
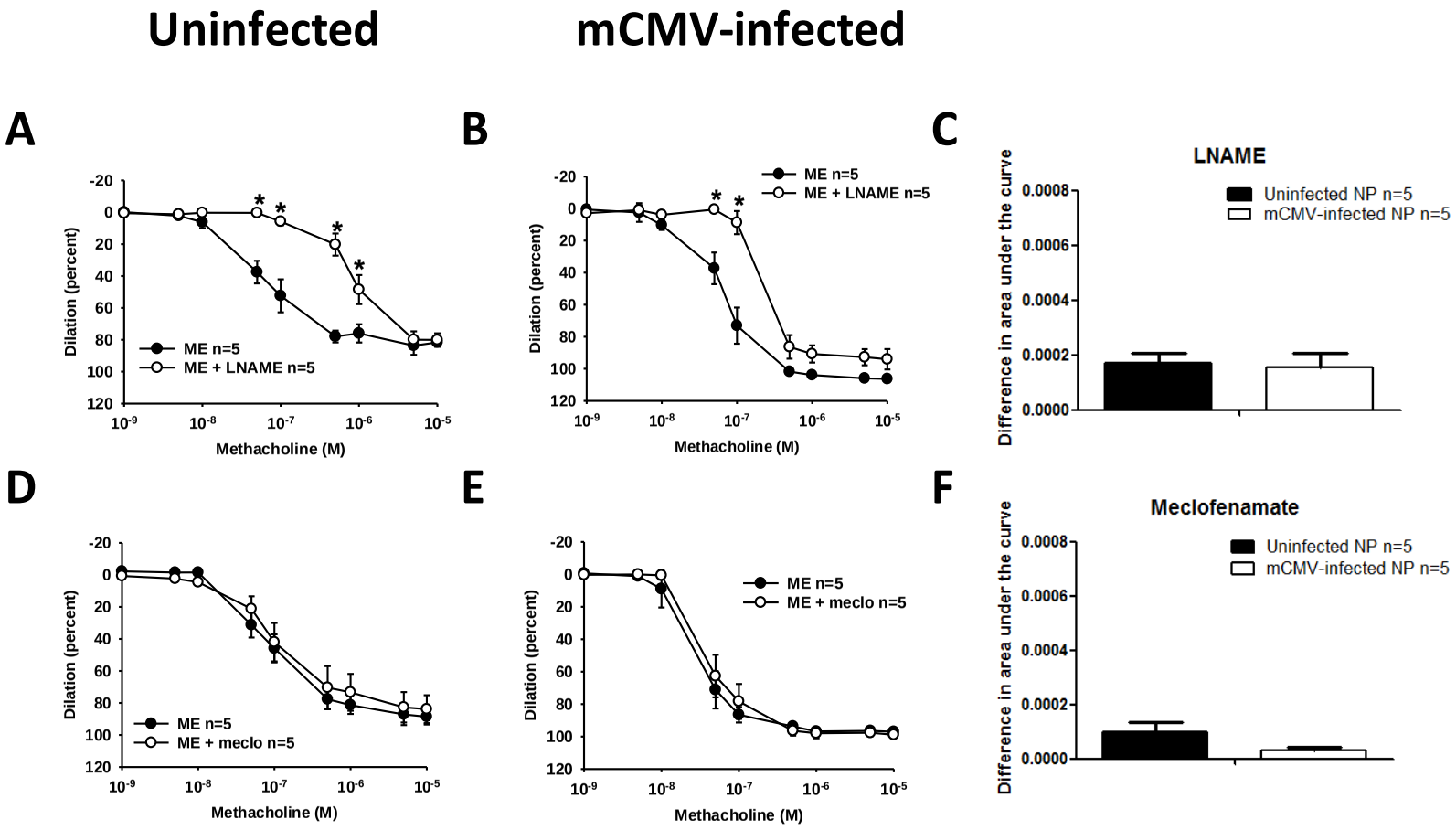

Uninfected
mCMV-infected
G
H
I
J
K
L
Dilation (percent)
Methacholine (M)
ME n=4
ME + SQ29548 n=4
ME n=5
ME + LNAME + meclo n=5
SQ29548
Uninfected LP n=4
mCMV-infected LP n=4
Difference in area under the curve
LNAME + meclofenamate
Uninfected LP n=5
mCMV-infected LP n=5

Figure 4.3: Inhibition of ME-induced vasodilation in mesenteric arteries from mCMV-infected and uninfected LP mice. ME-induced vasodilation was measured in mesenteric arteries from uninfected **(A, D, G, J)** and mCMV-infected **(B, E, H, K)** LP mice after preconstriction of mesenteric arteries using U46619 in the presence or absence of L-NAME (100μM) **(A, B, C)**, meclo (1μM) **(D, E, F)**, SQ29548 (10μM) **(G, H, I)**, or L-NAME and meclo **(J, K, L)**. For each graph, the area under the curve was calculated and the difference between two curves on the same graph was obtained **(C, F, I, L)**. Results were summarized and compared as in Figure 4.1. * = $p<0.05$. n=number of animals.


Uninfected
mCMV-infected
A
B
C
D
E
F
Dilation (percent)
Methacholine (M)
ME n=5
ME + LNAME n=5
ME + meclo n=5
LNAME
Meclofenamate
Uninfected NP n=5
mCMV-infected NP n=5
Difference in area under the curve

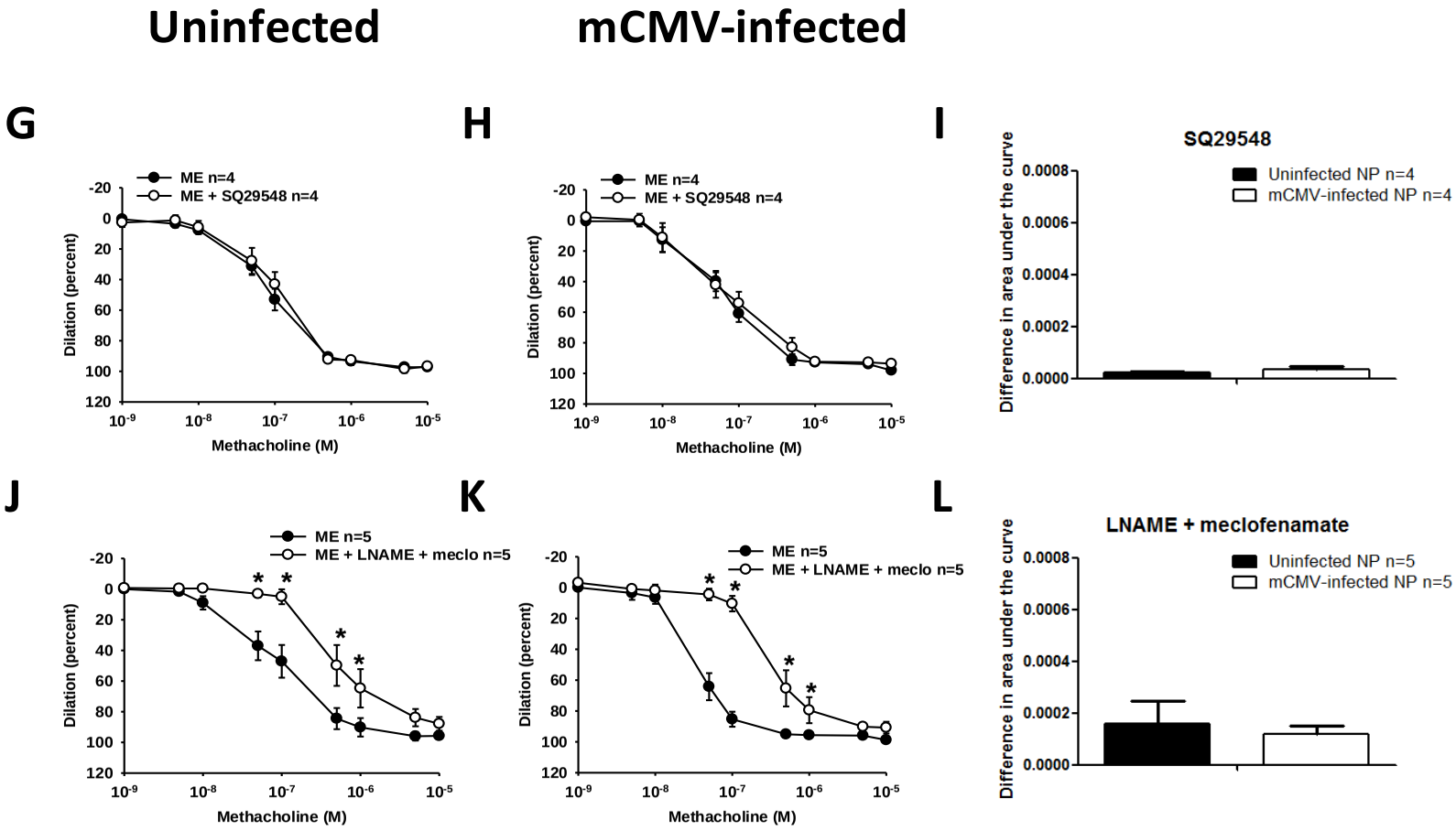
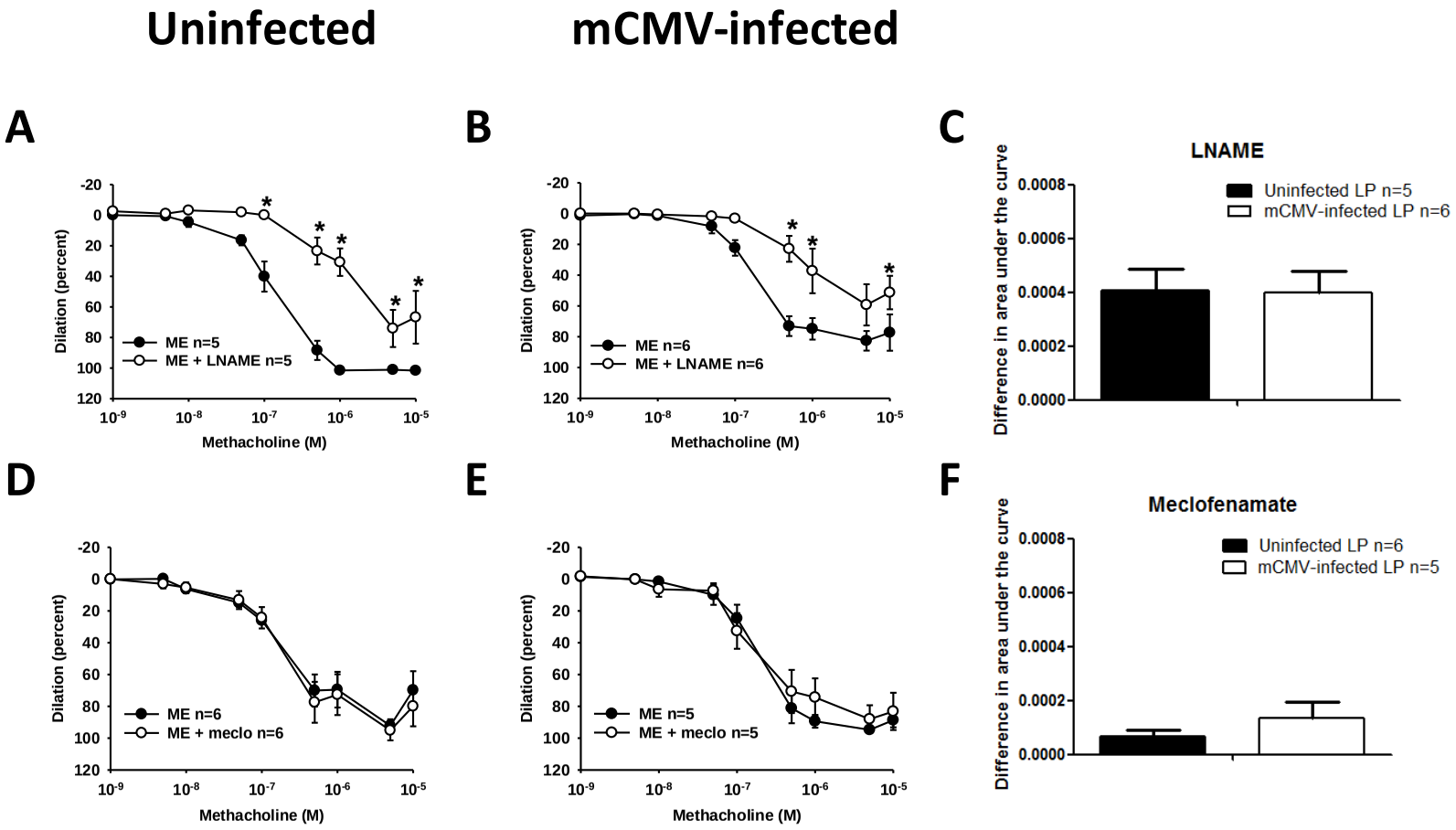

Uninfected
mCMV-infected
G
H
I
ME n=4
ME + SQ29548 n=4
Dilation (percent)
Methacholine (M)
SQ29548
Uninfected NP n=4
mCMV-infected NP n=4
Difference in area under the curve
J
K
L
ME n=5
ME + LNAME + meclo n=5
LNAME + meclofenamate
Uninfected NP n=5
mCMV-infected NP n=5

Figure 4.4: Inhibition of ME-induced vasodilation in uterine arteries from mCMV-infected and uninfected NP mice. ME-induced vasodilation was measured in uterine arteries from uninfected **(A, D, G, J)** and mCMV-infected **(B, E, H, K)** NP mice after preconstriction of uterine arteries using PE in the presence or absence of L-NAME (100μM) **(A, B, C)**, meclo (1μM) **(D, E, F)**, SQ29548 (10μM) **(G, H, I)**, or L-NAME and meclo **(J, K, L)**. For each graph, the area under the curve was calculated and the difference between two curves on the same graph was obtained **(C, F, I, L)**. Results were summarized and compared as in Figure 4.1. * = $p<0.05$. n=number of animals.


Uninfected
mCMV-infected
A
B
C
D
E
F
Dilation (percent)
Methacholine (M)
ME n=5
ME + LNAME n=5
ME n=6
ME + LNAME n=6
ME n=6
ME + meclo n=6
ME n=5
ME + meclo n=5
LNAME
Meclofenamate
Difference in area under the curve
Uninfected LP n=5
mCMV-infected LP n=6
Uninfected LP n=6
mCMV-infected LP n=5

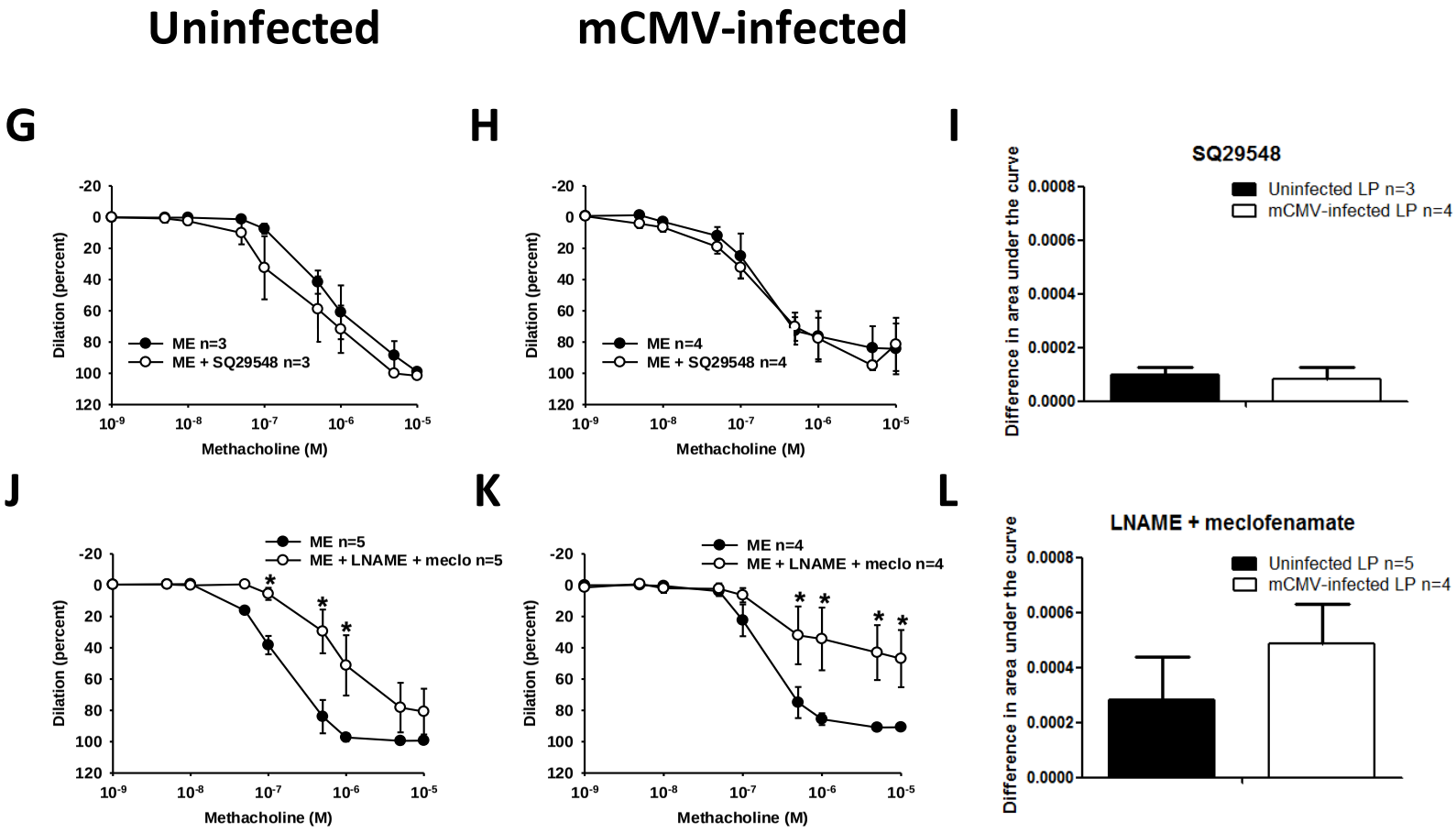

Uninfected
mCMV-infected
G
H
I
J
K
L
Dilation (percent)
Methacholine (M)
ME n=3
ME + SQ29548 n=3
ME n=4
ME + SQ29548 n=4
SQ29548
Uninfected LP n=3
mCMV-infected LP n=4
Difference in area under the curve
ME n=5
ME + LNAME + meclo n=5
ME n=4
ME + LNAME + meclo n=4
LNAME + meclofenamate
Uninfected LP n=5
mCMV-infected LP n=4

Figure 4.5: Inhibition of ME-induced vasodilation in uterine arteries from mCMV-infected and uninfected LP mice. ME-induced vasodilation was measured in uterine arteries from uninfected **(A, D, G, J)** and mCMV-infected **(B, E, H, K)** LP mice after preconstriction of uterine arteries using PE in the presence or absence of L-NAME (100μM) **(A, B, C)**, meclo (1μM) **(D, E, F)**, SQ29548 (10μM) **(G, H, I)**, or L-NAME and meclo **(J, K, L)**. For each graph, the area under the curve was calculated and the difference between two curves on the same graph was obtained **(C, F, I, L)**. Results were summarized and compared as in Figure 4.1. * = $p<0.05$. n=number of animals.

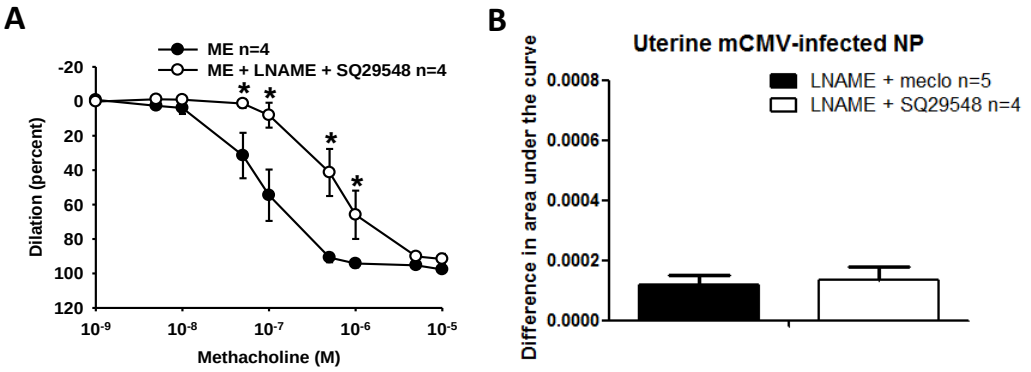


Figure 4.6: NO and TxA_2/PGH_2 contribution to ME-induced vasodilation in uterine arteries from mCMV-infected NP mice. ME-induced vasodilation was measured in uterine arteries mCMV-infected NP mice after preconstriction of uterine arteries using PE in the presence or absence of L-NAME (100μM) and SQ29548 (10μM) **(A)**. Changes in lumen diameter were measured in response to increasing concentrations of ME following preconstriction of mesenteric arteries. The area under the curve was calculated and the difference between the two curves on panel **(A)** was obtained. For comparison purposes, the difference in the area under the curve for L-NAME with meclo was taken from Figure 4.4L **(B)**. Results were summarized and compared as in Figure 4.1. * = $p<0.05$. n=number of animals.

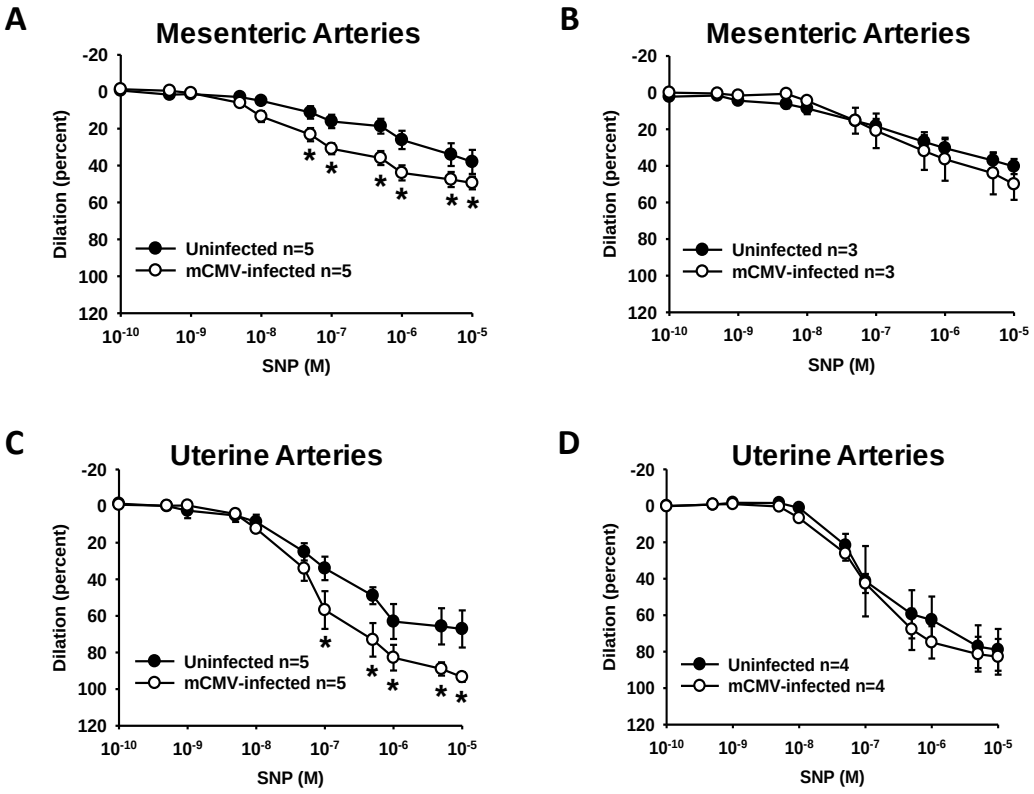


Figure 4.7: Endothelium-independent vasodilation to sodium nitroprusside (SNP) in mesenteric and uterine arteries from mCMV-infected and uninfected NP and LP mice. Changes in lumen diameter were measured in response to increasing concentrations of SNP following preconstriction with U46619 or PE of mesenteric **(A, B)** and uterine **(C, D)** arteries, respectively, from NP **(A, C)** and LP **(B, D)** mice. Results were summarized and expressed as the mean ± SEM percent increase in lumen diameter compared to the initial preconstricted diameter, and normalized to the passive lumen diameter. A significant difference between points on the curves was determined by Repeated Measures two-way ANOVA with Holm-Sidak's post-hoc analysis. A significant difference between points on the curves was symbolized with an * ($p<0.05$). n=number of animals.

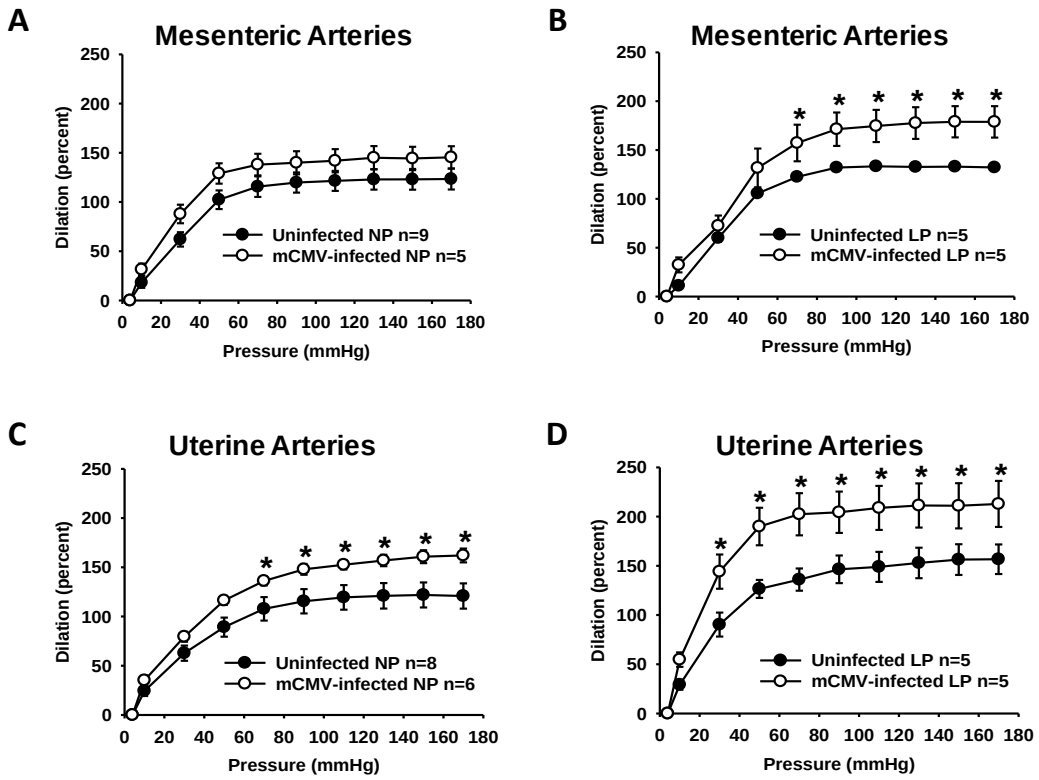


Figure 4.8: Distensibility curves for mesenteric and uterine arteries from mCMV-infected and uninfected NP and LP mice. Changes in lumen diameter were measured in mesenteric **(A, B)** and uterine **(C, D)** arteries from NP **(A, C)** and LP **(B, D)** mice in response to step-wise increases in intraluminal pressure in calcium-free PSS in the presence of papaverine. Results were summarized and expressed as the mean ± SEM percent increase in lumen diameter compared to the initial diameter, and normalized to the initial lumen diameter at 4mmHg. A significant difference between points on the curves was determined by Repeated Measures two-way ANOVA with Holm-Sidak's post-hoc analysis. Where error bars are not visible, errors were too small to be seen. A significant difference was symbolized with an * ($p<0.05$). n=number of animals.

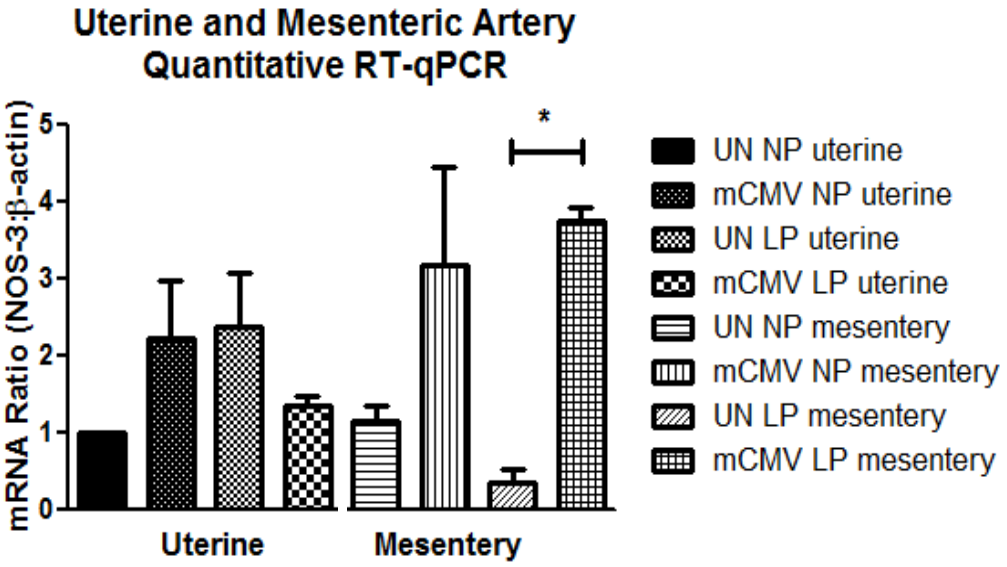


Figure 4.9: NOS-3 mRNA expression in mesenteric and uterine arteries from mCMV-infected and uninfected NP and LP mice. Following real-time RT-PCR, NOS-3 mRNA expression was normalized in each group to β-actin mRNA expression. Changes in the NOS-3:β-actin mRNA ratio was compared in mesenteric and uterine arteries from uninfected and infected NP and LP mice. A significant difference between points on the curves was determined by two-way ANOVA with Bonferroni's post-hoc analysis. A significant difference was symbolized with an * ($p<0.05$).

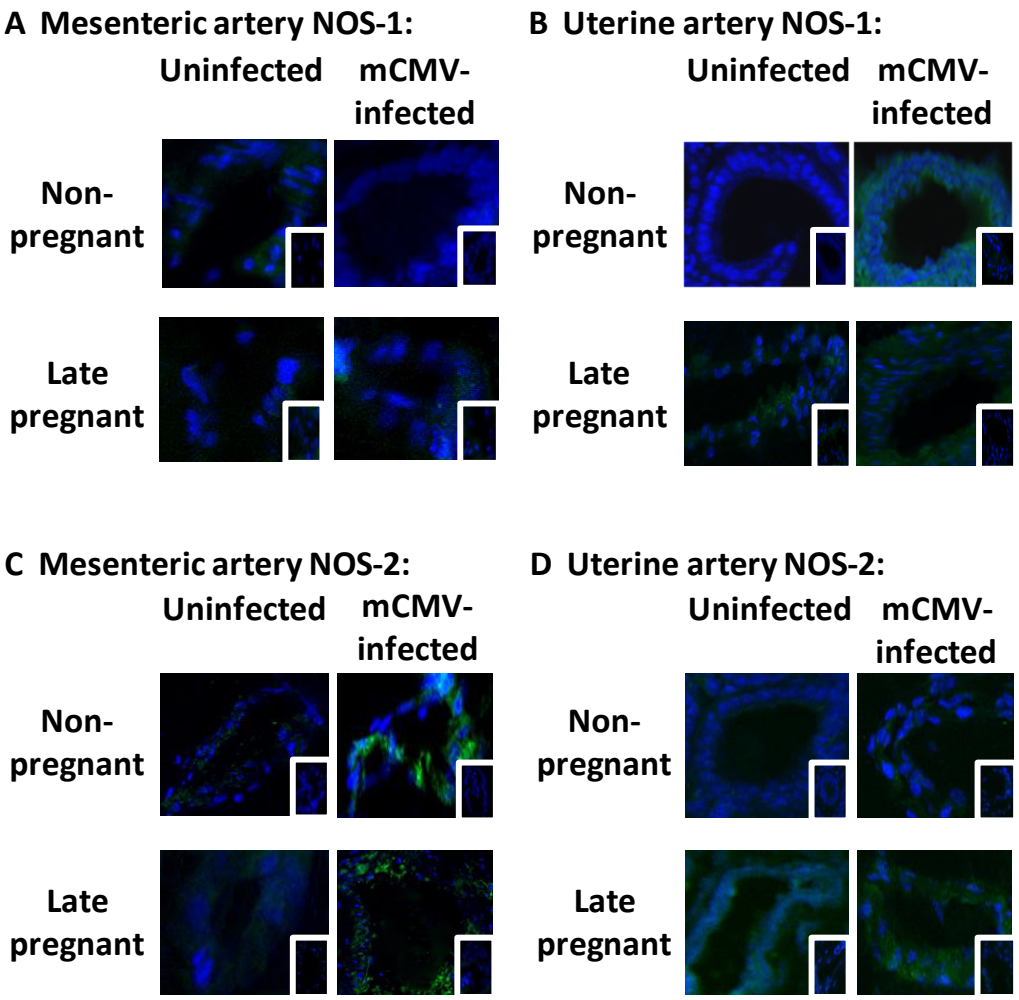


Figure 4.10: NOS-1 and NOS-2 expression in mesenteric and uterine arteries from mCMV-infected and uninfected NP and LP mice. NOS-1 **(A,B)** and NOS-2 **(C,D)** expression in mesenteric **(A,C)** and uterine **(B,D)** arteries were assessed by immunofluorescence in sections of tissues isolated from uninfected and mCMV-infected NP and LP mice. Sections were stained with NOS-1 or NOS-2 (green). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; blue). Negative controls are shown in the insets for each photomicrograph. These images are representative of different sections taken from 3 mice in each group (n=3).

4.4 DISCUSSION

To further investigate the differences in cholinergic endothelium-dependent vasodilation seen in mesenteric and uterine arteries from mCMV-infected and uninfected NP and LP mice (Chapter 3), I measured the contribution of NO and prostanoids to ME-induced vasodilation and smooth muscle cell sensitivity to NO in these arteries. My previous findings of increased vasodilation in mesenteric arteries from mCMV-infected NP mice (Chapter 3) (Gombos *et al.*, 2009) can now be explained by an increased contribution by NO, increased NOS-2 protein and NOS-3 mRNA expression, and increased vascular smooth muscle sensitivity to NO. In contrast, the increased vasodilation previously observed in uterine arteries from mCMV-infected NP mice (Chapter 3) (Gombos *et al.*, 2009) is likely in response to an increase in vascular smooth muscle sensitivity to NO. Although NOS-1 expression is increased in the arterial wall, NO production does not further contribute to this increased vasodilation in uterine arteries from mCMV-infected compared to uninfected NP mice. The situation in mCMV-infected LP mice differs from that of infected NP mice and again depends on the type of artery. In late pregnancy, an increase in NOS-2 protein and NOS-3 mRNA expression and an increase in the contribution of NO to vasodilation in mesenteric arteries from mCMV-infected mice were observed. In uterine arteries from mCMV-infected LP mice, neither the contribution of NO or prostanoids to ME-induced vasodilation nor the vascular smooth muscle sensitivity to NO was changed, although distensibility was greatly increased. NOS-1/2 protein and NOS-3 mRNA expression in uterine arteries from mCMV-infected LP mice was also similar to uninfected LP mice. Thus, the dramatic decrease in sensitivity to ME in these arteries (Chapter 3) (Gombos *et al.*, 2009) remains unexplained and is likely due to reduced EDHF. Taken together, these results provide novel evidence for differential effects of an active mCMV infection on the NO pathway that contribute to regulation of vascular tone.

Interestingly, in contrast to my study using isolated intact arteries showing an increased contribution of NO to vasodilation and increased NOS-3 mRNA

expression, the majority of other studies that measure NOS-3 expression in cultured endothelial cells show that a CMV infection leads to decreased NOS-3 expression and activity (Bouwman *et al.*, 2005; Shen *et al.*, 2006). In addition, in the present study, the increased contribution by NO to vasodilation along with increased sensitivity of the vascular smooth muscle to NO and increased distensibility in the systemic vasculature are indicative of hypotension rather than hypertension; however, a recent study by Cheng et al. finds that an active CMV infection in young male and female mice increases arterial blood pressure and exacerbates atherosclerotic plaque formation in mice fed a high cholesterol diet (Cheng *et al.*, 2009). Although I previously found reduced sensitivity to PE in mesenteric arteries from mCMV-infected mice (Chapter 3) (Gombos *et al.*, 2009), Cheng et al. report increases in plasma and tissue expression of another vasoconstrictor, angiotensin II (Cheng *et al.*, 2009). Thus, it is possible that the responses observed in my study may be part of an adaptive response to counteract overall increased vasoconstriction in the mCMV-infected mice. These discrepancies strongly support the need to study responses in the vasculature further using different levels of molecular complexity, such as with isolated cells, other intact *ex vivo* arteries, and whole animals, to fully evaluate the physiological vascular responses during an active CMV infection.

Although NOS-3 is considered an important source of NO in the vasculature, both NOS-1 and NOS-2 also contribute to the production of NO. Most studies investigating the relationship between CMV infections and NOS regulation *in vivo* have focused on the inflammation-induced NOS-2 enzyme. NOS-2 activation in response to a mCMV infection has been shown to be part of an important protective innate immune response (Noda *et al.*, 2001). However, NOS-2 expression and activity increased with an mCMV infection and the resultant increase in NO production in the lungs and the brain have also been shown to contribute to the inflammatory disease, pneumonitis (Tanaka *et al.*, 1997), and developmental brain disorders (Kosugi *et al.*, 2002). Increased NOS-2 expression and NO are also detected in aqueous humor samples of AIDS patients

with CMV retinitis (Hsu *et al.*, 2003). It is therefore likely that a CMV infection in endothelial and vascular smooth muscle cells could lead to increased NO through increased expression and activity of the NOS-2 enzyme. Indeed, I found increased expression of NOS-2 in mesenteric arteries from both NP and LP mCMV-infected mice. There is no information about the effect of CMV infection on NOS-1 expression in the vasculature but I have now shown that NOS-1 is increased in uterine arteries from NP but not LP mCMV-infected mice. Importantly, in conditions of oxidative stress (i.e. infection and to some extent pregnancy), excess NO may combine with superoxide to produce peroxynitrite, a potent and damaging pro-oxidant, thereby reducing the bioavailability of NO (Miles *et al.*, 1996). It has been demonstrated that peroxynitrite nitrates several intracellular proteins and this can inactivate or enhance their catalytic activity (Myatt, 2010). Some of these proteins have important roles in vascular function. For example, nitration induces a loss of function in PGI_2 synthase and a gain of function in PGHS-2, which has been shown to reduce arterial vasodilation (Landino *et al.*, 1996; Zou *et al.*, 1999). Furthermore, reduced NO bioavailability has also been implicated in the development of cardiovascular diseases, such as atherosclerosis (Higashi *et al.*, 2009; Lubos *et al.*, 2008).

Different prostanoids stimulate either vasodilation or vasoconstriction and are produced by the PGHS-1/2 enzymes. In uninfected mice, inhibition of PGHS-1/2 alone in either mesenteric or uterine arteries did not affect ME-induced vasodilation in my study, demonstrating that prostanoids were not involved in endothelium-dependent vasodilation in these vascular beds (Morton *et al.*, 2010). Surprisingly, although CMV increases PGHS-2 expression and activity in fibroblasts and epithelial cells where one of its products, prostaglandin E_2, is important for viral replication (Hooks *et al.*, 2006; Zhu *et al.*, 2002), I found that there was still no contribution by prostanoids to endothelium-dependent vasodilation during a mCMV infection. Yet, when NOS and PGHS-1/2 or NOS and the TxA_2 receptor were simultaneously inhibited in mesenteric arteries only from mCMV-infected NP and LP mice, the residual vasodilation due to EDHF

was greater than that found with NOS inhibition alone. This suggests that during a CMV infection, PGHS-mediated production of vasoconstrictors, such as TxA_2 or PGH_2, is increased; however, when NOS remains active, it is able to compensate for and mask prostanoid-induced vasoconstriction (see also Chapter 6). Interestingly, NO itself has been shown to inhibit PGHS-2 and subsequent PGI_2 production, although the mechanism has not been defined (Onodera *et al.*, 2000). In addition, if oxidative stress is increased in mesenteric arteries from mCMV-infected mice, increased vasoconstrictory prostanoid effects may be attributed to a potential increase in peroxynitrite-induced inactivation of PGI_2 synthase (Zou *et al.*, 1997) and upregulation of PGHS-2 activity (Landino *et al.*, 1996), as mentioned previously. A reduction in PGI_2 synthase activity could shift the balance towards an increased production of TxA_2 or an accumulation of PGH_2 (Davidge, 2001; Zou *et al.*, 1999).

The increased sensitivity to NO in the vascular smooth muscle in mesenteric and uterine arteries from mCMV-infected NP mice may be caused by an increase in vascular smooth muscle mass since CMV is known to increase smooth muscle cell proliferation (Kloppenburg *et al.*, 2005; Yonemitsu *et al.*, 1997). In Chapter 3 I showed that both mesenteric and uterine artery diameters increase in pregnancy (Gombos *et al.*, 2009), reflecting other reports of pregnancy-induced remodelling and vascular smooth muscle cell hypertrophy and hyperplasia (Keyes *et al.*, 1996; Osol *et al.*, 2009; van der Heijden *et al.*, 2005). This evidence supports the idea that an increase in vascular smooth muscle mass causes an increase in sensitivity to NO in arteries from mCMV-infected NP mice comparable to that seen in arteries from LP mice. The increased sensitivity to NO in vascular smooth muscle in either type of artery from mCMV-infected NP mice was lost in mCMV-infected LP mice, demonstrating differences in the effects of viral infection in NP and LP conditions. Interestingly, the increased sensitivity to NO found in uterine arteries from mCMV-infected NP mice mirrored the increased sensitivity in uninfected LP compared to NP mice. Another explanation for the infection-induced increase in vascular smooth muscle sensitivity to NO in

arteries from mCMV-infected NP mice could be an increase in cGMP or soluble guanylyl cyclase protein expression and activity, which also normally increase in pregnancy (Conrad *et al.*, 1989; Itoh *et al.*, 1998). More specifically, a recent study provides evidence for increased cGMP in uterine vascular smooth muscle in ovine pregnancy (Rosenfeld *et al.*, 2009) and infection with CMV also increases cellular cGMP (Albrecht *et al.*, 1990).

The results of this study demonstrate novel ways in which a CMV infection affects the vasculature. CMV infections have been previously shown to impair cellular responses in single cell cultures (Bissinger *et al.*, 2002); however, the use of functional and molecular assays in isolated intact arteries from acute mCMV-infected mice to examine changes in vasoactive mediators and signalling pathways important to regulation of vascular responses is novel. Importantly, these results show dysregulation of the NO and prostanoid signalling pathways and suggest the importance for evaluation of the long-term effects of CMV infections. The differential effects of long-term infections on the vasoactive pathways that regulate vascular function in various vascular beds needs to be further investigated since it is clear that long-term CMV infections in humans are associated with endothelial dysfunction, impaired NO responses, and hypertensive vascular diseases (Grahame-Clarke *et al.*, 2003; Simmonds *et al.*, 2008; Streblow *et al.*, 2008). Chronic increases in NO production in response to CMV infection has important long-term implications for conditions of increased oxidative stress such as atherosclerosis, where NO bioavailability is reduced and increased levels of peroxynitrite are produced.

4.5 REFERENCES

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Chapter 5

Effect of Pregnancy and a Cytomegalovirus Infection on the Regulation of Vascular Tone by Sphingosine 1-Phosphate

(The majority of data presented in this chapter was submitted to the British Journal of Pharmacology in November 2011 (Manuscript ID: 2011-BJP-1491-RP) in an article entitled "Effect of pregnancy and cytomegalovirus infection on regulation of vascular tone by sphingosine 1-phosphate in isolated mouse arteries," by R.B. Gombos and D.G. Hemmings.)

5.1 INTRODUCTION

I have previously shown that vasodilation and vasoconstriction responses to ME and PE, respectively, are differentially altered in mesenteric and uterine arteries from mCMV-infected NP and LP mice (Chapter 3) (Gombos *et al.*, 2009). ME-induced vasodilation was increased in mesenteric and uterine arteries from mCMV-infected NP mice and in mesenteric arteries from mCMV-infected LP mice due to increased smooth muscle cell sensitivity to NO and/or NO contribution (Chapters 3,4) (Gombos *et al.*, 2010; Gombos *et al.*, 2009). In contrast, uterine arteries from LP mCMV-infected mice showed increased vasoconstriction to PE (Chapter 3) and decreased sensitivity to ME which was not attributed to changes in NO or prostanoid contribution or smooth muscle sensitivity to NO (Chapters 3,4) (Gombos *et al.*, 2010; Gombos *et al.*, 2009). Endothelium-dependent vasodilation may also be regulated differently in systemic and uterine arteries from NP and LP mice in the presence or absence of a CMV infection by other signalling molecules that affect vascular responses, such as the bioactive sphingolipid, S1P. Stimulation of muscarinic receptors activates SK-1 (Meyer zu Heringdorf *et al.*, 1998) and induces S1P-mediated NOS-3 activation (Mulders *et al.*, 2009), supporting the regulatory role of S1P in endothelium-dependent vasodilation. There is also evidence demonstrating that HCMV

increases SK-1 expression and activity in cultured fibroblasts and endothelial cells and increases sphingolipid synthesis in fibroblasts (Machesky *et al.*, 2008). As such, I chose to study how S1P affects mesenteric and uterine vascular responses from mCMV-infected and uninfected NP and LP mice.

S1P is a vasoactive sphingolipid that signals through five G-protein coupled receptors ($S1P_1$-$S1P_5$) (Sanchez *et al.*, 2004). In the microvasculature, $S1P_1$ and $S1P_3$ are expressed on the vascular endothelium and smooth muscle cells whereas $S1P_2$ is found mainly on the vascular smooth muscle cells. $S1P_4$ and $S1P_5$ are generally not detectable in the vasculature (Peters *et al.*, 2007); however, $S1P_4$ mRNA has recently been found in human umbilical vein endothelial cells (Heo *et al.*, 2009). S1P is synthesized inside the cell from the sphingomyelin metabolic pathway whereby ceramide is produced and broken down into sphingosine which is converted to S1P via SK-1/2 (Alewijnse *et al.*, 2004). S1P can then be transported out of the cell where it can act on the various S1P receptors in an autocrine or paracrine manner (Kim *et al.*, 2009). Intracellular and extracellular S1P are involved in several cellular functions including growth and proliferation, differentiation, migration, survival and vascular regulation (Hemmings, 2006; Igarashi *et al.*, 2009; Spiegel *et al.*, 2003a). In smooth muscle cells, S1P stimulates contraction through Ca^{2+} release and activation of ROCK via $S1P_2$ and $S1P_3$ (Sanchez *et al.*, 2004). In endothelial cells, S1P activates NOS-3 via $S1P_1$ and $S1P_3$ (Watterson *et al.*, 2005). This suggests that in intact arteries, S1P induces vasoconstriction when interacting with smooth muscle cells, and vasodilation when it interacts with the endothelium. Most studies investigating a vascular role for S1P have done so either *in vivo* in the context of many other vascular mediators (Bischoff *et al.*, 2000; Salomone *et al.*, 2003), *ex vivo* using the wire myograph technique where S1P is directly accessible to both the endothelium and the smooth muscle cells of intact arteries (Hedemann *et al.*, 2004; Salomone *et al.*, 2008; Salomone *et al.*, 2003), or *ex vivo* using the pressure myograph with S1P only added to the bath where it directly interacts with the smooth muscle cells of intact arteries (Hemmings *et al.*, 2004; Murakami *et al.*).

What is not yet known is the effect on overall vascular tone of S1P infused into isolated, pressurized arteries with an intact endothelium in normal physiological situations nor how this typical response is affected by normal pregnancy with or without the additional complication of an active viral infection.

During pregnancy, cardiac output increases and systemic vascular resistance decreases to maintain normal blood pressure in the face of increased plasma volume whereby blood flow to the uterine vascular bed in humans increases 50- to 70-fold over the gestational period (Assali *et al.*, 1960; Gant *et al.*, 1987; Magness *et al.*, 1986; Metcalfe *et al.*, 1974; Veille *et al.*, 1996). Endothelium-dependent vasodilation in the uterine artery is also increased during pregnancy by PGI_2, NO and EDHF (Carbillon *et al.*, 2000; Sladek *et al.*, 1997). Although several vascular mediators have been linked to this pregnancy-associated increase in vasodilation and decrease in vascular resistance in systemic and uterine arteries, little is known about the role of S1P (Dantas *et al.*, 1999; Harrison *et al.*, 1989; Hemmings *et al.*, 2006; Knock *et al.*, 1996; Thaler *et al.*, 2005).

Studies with S1P receptor knockout mice support the importance of S1P vascular responses related to pregnancy success. $S1P_1$ knockouts ($S1P_1^{-/-}$) are embryonically lethal due to incomplete cardiovascular development (Liu *et al.*, 1997a). Although $S1P_2^{-/-}$ and $S1P_3^{-/-}$ are viable with small but significant reductions of litter sizes (Ishii *et al.*, 2001; Ishii *et al.*, 2002), there are signs of vascular dysfunction in both. $S1P_3^{-/-}$ show complete loss of S1P-induced NO-dependent dilation in preconstricted aortas (Nofer *et al.*, 2004). These reports suggest that the normal increase in NO-mediated vasodilation in uterine arteries during pregnancy will be impaired in $S1P_3^{-/-}$, with potential effects on fetal development. Double null mice ($S1P_2^{-/-}/S1P_3^{-/-}$) show severe pregnancy complications with greatly reduced numbers of live-born pups that die shortly after birth (Ishii *et al.*, 2002). This suggests a late pregnancy complication possibly related to impaired NO responses. However, increased S1P in plasma samples obtained in early human pregnancy has been identified as one of several

biomarkers in a multivariate predictive model for the pregnancy-specific vascular disorder, preeclampsia and for babies born small for gestational age (Horgan *et al.*, 2011; Kenny *et al.*, 2010). Collectively, the vascular responses and abnormal pregnancy phenotypes in genetically engineered mice and association of increased S1P plasma levels with pregnancy-related vascular diseases suggest that an intact S1P signalling system and tight regulation of S1P concentrations are important for proper vascular function during pregnancy.

The objectives of the experiments in this chapter are as follows: first, to investigate the effects of S1P and its signalling pathway on overall vascular tone when infused inside intact pressurized mesenteric and uterine arteries from normal mice using the pressure myograph system; second, to determine if S1P contributes to the increased vasodilation responses that occur during normal pregnancy; and finally to examine S1P-mediated responses in arteries from active mCMV-infected NP and LP mice. I hypothesize that addition of S1P to the smooth muscle side of isolated, pressurized mesenteric and uterine arteries (extraluminal) will induce vasoconstriction that will be unchanged in pregnancy. I further hypothesize that intraluminal infusion of S1P into isolated, pressurized, preconstricted mesenteric and uterine arteries will induce vasodilation that will be increased in pregnancy. Since CMV infections can increase muscarinic-induced NO-mediated vasodilation (Chapter 4) (Gombos *et al.*, 2010) and potentially S1P receptor expression by activation of NF-κB (Liu *et al.*, 1997a; Yurochko *et al.*, 1995), I also hypothesize that an active mCMV infection will increase intraluminally infused S1P-induced vasodilation and vasoconstriction induced by extraluminally applied S1P in arteries from both LP and NP mice. In intact, pressurized arteries, I show that control of vascular responses by S1P interacting directly with endothelium is tightly regulated. Infusion of low concentrations leads to vasodilation that is abolished at higher concentrations. This is altered in pregnancy to favour increased vasodilatory capacity. Furthermore, a mCMV infection disrupts S1P-induced vasoconstriction and vasodilation responses in addition to S1P signalling pathways.

5.2 RESULTS

5.2.1 Vascular responses to extraluminal addition or intraluminal infusion of S1P in arteries from uninfected NP and LP mice

S1P added extraluminally to pressurized mesenteric and uterine arteries induced vasoconstriction in a dose-dependent manner. Uterine arteries from uninfected LP mice had significantly less S1P-induced vasoconstriction at 5μM of S1P than those from uninfected NP mice. Responses did not differ in mesenteric arteries (Figure 5.1A,B). Uterine arteries from uninfected NP mice had significantly higher S1P-induced vasoconstriction compared to mesenteric arteries ($p<0.05$; Figure 5.1A compared to Figure 5.1B); however, there was no significant difference in vasoconstriction induced by extraluminal application of S1P in uterine compared to mesenteric arteries from LP mice (Figure 5.1A compared to Figure 5.1B).

Infusion of 0.1μM of S1P with 0.1% BSA intraluminally in pressurized arteries preconstricted with U46619 led to vasodilation in both mesenteric and uterine arteries from uninfected NP mice (Figure 5.2A,B). To ensure the low flow used in these experiments did not induce a flow-mediated vasodilation response, infusion of either PSS alone or the carrier control 0.1% BSA was also done. Infusion of S1P at higher concentrations including 1μM (Figure 5.2A,B) and 10μM (mesenteric NP: 2.68±1.42%; uterine NP: -0.379±0.379%) produced no significant vasodilation response compared to either PSS (mesenteric NP: 0.102±0.771%; uterine NP: -1.41±0.597%) or BSA (mesenteric NP: 1.25±2.30%; uterine NP: 0.265±1.88%) controls in either artery from uninfected NP mice. In contrast, arteries from uninfected LP mice showed significant vasodilation with infusion of both 0.1μM and 1μM of S1P (Figure 5.2C,D) which was lost at 10μM (mesenteric LP: 2.94±2.94%; uterine LP: 2.57±0.435). There were also no vasodilation responses to infused PSS (mesenteric LP: -2.23±1.45%; uterine LP: 0.182±1.47%) or BSA (mesenteric LP: 0.516±0.348%; uterine LP: -0.638±1.65%) controls in arteries from uninfected LP mice.

The vascular responses to 0.1μM and 1μM S1P applied extraluminally as measured in the dose-response curves in Figure 5.1 are shown for comparison. In contrast to the vasodilation responses to intraluminal infusion of S1P, addition of 0.1μM S1P with 0.1% BSA to the bath where it interacted directly with uterine artery smooth muscle cells led to vasoconstriction which increased at 1μM in uterine arteries from both NP and LP mice (Figure 5.2B,D). Extraluminal addition of 0.1μM S1P to mesenteric arteries from either NP or LP mice had no significant effect; however, both constricted to extraluminal addition of 1μM (Figure 5.2A,C). As shown in Figure 5.1, uterine arteries from NP and LP mice were more sensitive to vasoconstriction at 1μM S1P than mesenteric arteries.

5.2.2 Role of the endothelium in vasodilation induced by S1P infusion

To determine if vasodilation induced by infusion of 0.1μM S1P was dependent on the endothelium, I measured the response to intraluminally infused S1P in mesenteric and uterine arteries from uninfected NP mice following endothelium removal. S1P-induced vasodilation in endothelium-denuded arteries was completely abolished and replaced with an S1P-induced vasoconstriction response that was significantly greater in uterine compared to mesenteric arteries (Figure 5.3).

5.2.3 Effect of an active mCMV infection on vascular responses to intraluminally infused S1P

Mesenteric arteries from mCMV-infected NP and LP mice had significantly decreased vasodilation induced by infused S1P compared to mesenteric arteries from uninfected mice. Uterine arteries from both mCMV-infected NP and LP mice showed similar responses to intraluminally infused S1P as uninfected NP and LP mice at 0.1μM and 1μM (Figure 5.4A,B). At 10μM S1P, similar to mesenteric and uterine arteries from uninfected mice, vasodilation was lost in all mCMV-infected NP and LP groups (mesenteric NP: -0.933±0.934%; mesenteric LP: 0.119±1.07%; uterine NP: 1.53±1.49%; uterine LP:

0.513±1.04%). In both mesenteric and uterine arteries from uninfected NP and LP mice, the vasodilation induced by intraluminal infusion of 0.1μM S1P was lost when vessels were pre-treated with the NOS inhibitor, L-NAME or with the $S1P_{1/3}$ antagonist, VPC23019 (Figure 5.5A,B). In mesenteric arteries from mCMV-infected NP and LP mice and in uterine arteries from mCMV-infected LP mice, pre-treatment with L-NAME or VPC23019 completely inhibited S1P-induced vasodilation similar to mesenteric and uterine arteries from uninfected NP and LP mice (Figure 5.5C,D). In contrast, while uterine arteries from mCMV-infected NP mice continued to show loss of S1P-induced vasodilation in the presence of L-NAME, this vasodilation was no longer blocked by pre-treatment with VPC23019 (Figure 5.5D).

5.2.4 Effect of an active mCMV infection on vascular responses to extraluminal addition of S1P

There was no significant difference in S1P-induced vasoconstriction in mesenteric arteries isolated from mCMV-infected NP compared to uninfected mice (Figure 5.6A). However, in uterine arteries from mCMV-infected versus uninfected NP mice, S1P-induced vasoconstriction to S1P was significantly reduced ($p<0.05$; Figure 5.6B) from 0.1μM to 10μM of S1P. In LP mice, there were no significant differences in S1P-induced vasoconstriction in mesenteric or uterine arteries from mCMV-infected compared to uninfected mice (Figure 5.6C,D).

Since extraluminally applied S1P binds directly to smooth muscle cell receptors but may also access and bind to endothelial receptors, I investigated the role of endothelium-dependent responses in modulating S1P-induced vasoconstriction using three agents: L-NAME, VPC23019, and meclo. L-NAME, which inhibits NOS, did not affect S1P-induced vasoconstriction in mesenteric or uterine arteries from any animal group (Figures 5.7A-D; 5.8A-D). When the arteries were pre-treated with the $S1P_{1/3}$ receptor antagonist VPC23019, sensitivity to S1P-induced vasoconstriction was significantly increased ($p<0.05$)

only in uterine arteries from uninfected NP mice (S1P EC_{50}: 1.29±0.412μM; VPC23019 IC_{50}: 0.366±0.0431μM) (Figure 5.9B). In mesenteric and uterine arteries from all other NP and LP groups, this antagonist had no effect on S1P-induced vasoconstriction (Figures 5.9A,C,D; 5.10A-D). Meclo, the PGHS-1/2 inhibitor, had no effect on S1P vasoconstriction responses in any group (Figures 5.11A-D; 5.12A-D).

5.2.5 Role of ROCK in vasoconstriction induced by extraluminally applied S1P in arteries from mCMV-infected and uninfected NP and LP mice

Since changes in endothelial function could not explain the reduced vasoconstriction observed in the uterine arteries from mCMV-infected NP mice, I investigated a role for altered ROCK activity. Following the final dose of extraluminal S1P (10μM), the ROCK inhibitor Y27632 was added to the bath. S1P-induced vasoconstriction was significantly reversed in all groups ($p<0.05$). Mesenteric arteries from mCMV-infected and uninfected NP or LP mice and uterine arteries from mCMV-infected and uninfected LP mice did not differ in Y27632-induced reversal of vasoconstriction. However, uterine arteries from mCMV-infected NP mice had significantly decreased Y27632-induced reversal of vasoconstriction compared to arteries from uninfected NP mice ($p<0.05$; Figure 5.13A,B).

5.3 SUMMARY OF RESULTS

5.3.1 Uterine arteries from NP mice were more sensitive to S1P-induced vasoconstriction than LP mice when S1P was applied extraluminally (Figure 5.1).

5.3.2 Vasoconstriction induced by extraluminally applied S1P was decreased in uterine arteries from mCMV-infected compared to uninfected NP mice (Figure 5.6).

5.3.3 NO did not mediate vasoconstriction induced by extraluminally applied S1P in mesenteric or uterine arteries from mCMV-infected and uninfected NP or LP mice (Figures 5.7, 5.8).

5.3.4 Inhibition of $S1P_{1/3}$ increased sensitivity to extraluminal S1P in uterine arteries from uninfected NP mice but did not affect S1P-induced vasoconstriction in uterine arteries from mCMV-infected NP mice or mesenteric arteries from mCMV-infected or uninfected NP mice (Figure 5.9).

5.3.5 $S1P_{1/3}$ did not mediate vasoconstriction induced by extraluminally applied S1P in mesenteric or uterine arteries from mCMV-infected and uninfected LP mice (Figure 5.10).

5.3.6 Prostanoids did not contribute to vasoconstriction induced by extraluminally applied S1P in mesenteric or uterine arteries from mCMV-infected and uninfected NP or LP mice (Figures 5.11, 5.12).

5.3.7 ROCK activity was reduced in uterine arteries from mCMV-infected compared to uninfected NP mice (Figure 5.13).

5.3.8 Extraluminal S1P application induced vasoconstriction at 0.1μM in uterine arteries from NP and LP mice and induced vasoconstriction at 1μM in uterine and mesenteric arteries from NP and LP mice (Figure 5.2).

5.3.9 Intraluminal infusion of S1P induced vasodilation at 0.1μM in uterine and mesenteric arteries from NP and LP mice whereas intraluminal vasodilation was maintained at 1μM only in uterine and mesenteric arteries from LP mice (Figure 5.2).

5.3.10 Vasodilation induced by infused S1P was endothelium-dependent (Figure 5.3).

5.3.11 Mesenteric arteries from mCMV-infected NP and LP mice had decreased vasodilation induced by infused S1P compared to uninfected NP and LP mice (Figure 5.4).

5.3.12 Vasodilation induced by infused S1P was NO-dependent and mediated by the $S1P_{1/3}$ receptors in mesenteric and uterine arteries from all groups except

uterine arteries from mCMV-infected NP mice, where NO-dependent vasodilation was not mediated by the $S1P_{1/3}$ receptors (Figure 5.5).

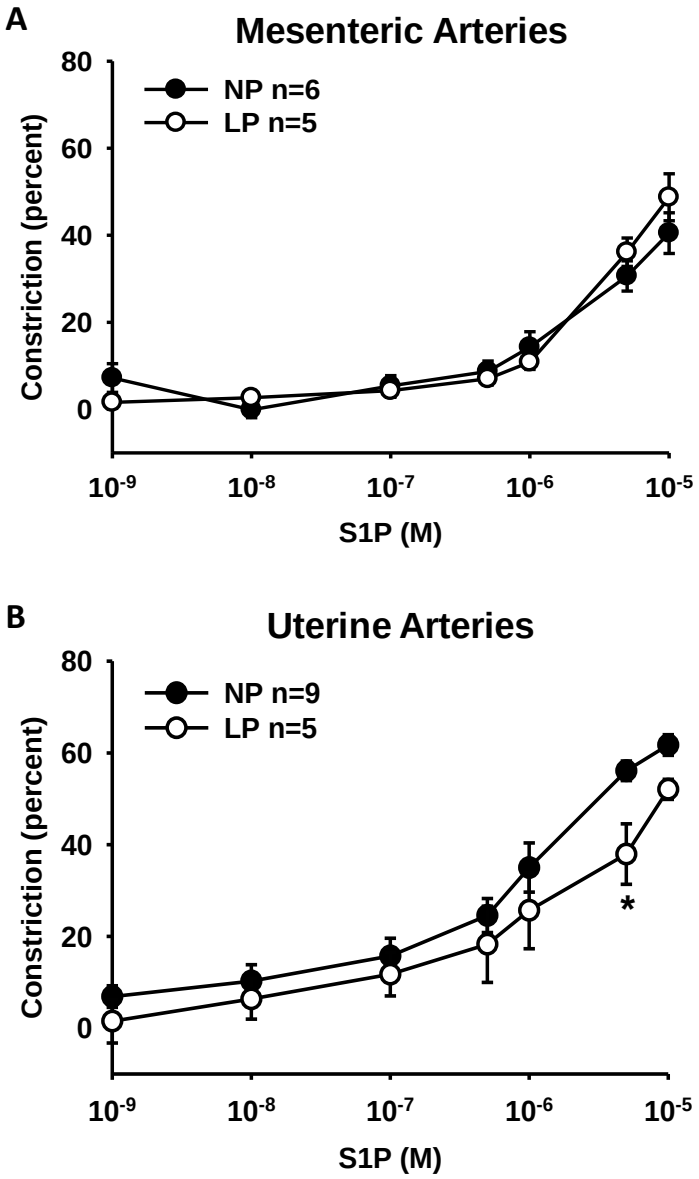

Figure 5.1: Extraluminal S1P dose-response curves for arteries from NP and LP mice. S1P was applied extraluminally to mesenteric **(A)** and uterine **(B)** arteries from uninfected NP and LP mice. Data were expressed as the mean ± SEM percent decrease in arterial lumen diameter compared to the initial equilibrated lumen diameter. A significant difference between points on the curves was determined by Repeated Measures two-way ANOVA with Holm Sidak's post-hoc analysis and symbolized with an * ($p<0.05$). n=number of animals.

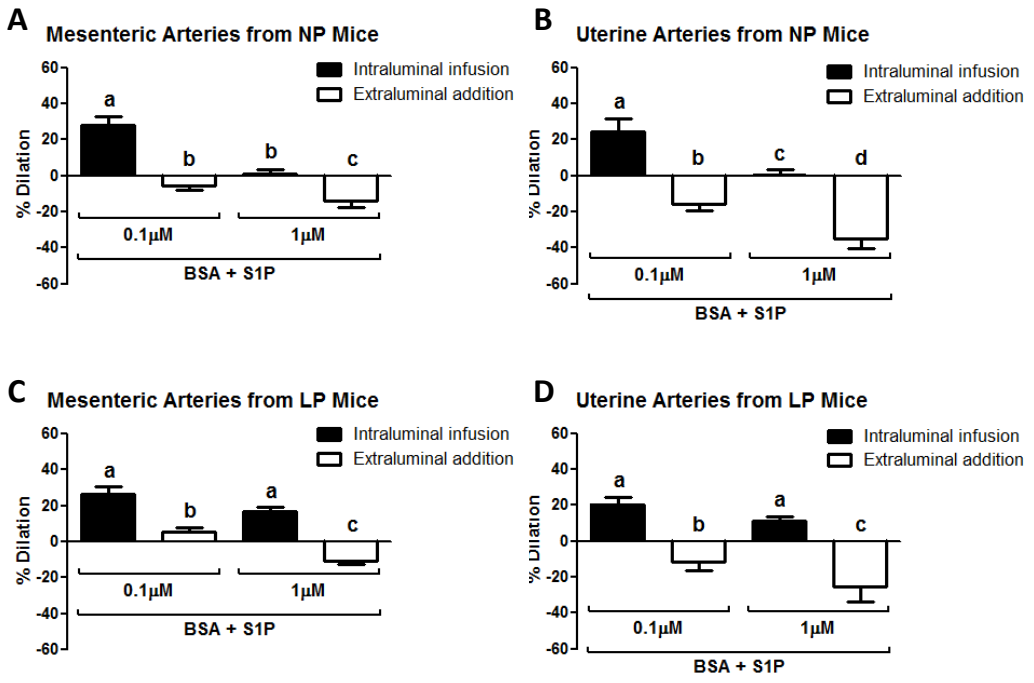

Figure 5.2: Intraluminal and extraluminal S1P responses at 0.1μM and 1μM. Mesenteric **(A,C)** and uterine **(B,D)** arteries from uninfected NP **(A,B)** and LP **(C,D)** mice were preconstricted to 50% with U46619 before intraluminal infusion of S1P. Intraluminal data were expressed as the mean ± SEM percent increase in arterial lumen diameter compared to the passive lumen diameter in the presence of Ca^{2+}-free medium and papaverine. Extraluminal data were taken from the curves in Figure 5.1 and expressed as the mean ± SEM percent decrease in arterial lumen diameter compared to the initial equilibrated lumen diameter. A significant difference between the bars was determined by two-way ANOVA and Bonferroni's post-hoc analysis and symbolized with an a, b, c, or d ($p<0.05$). n=5-9 animals.

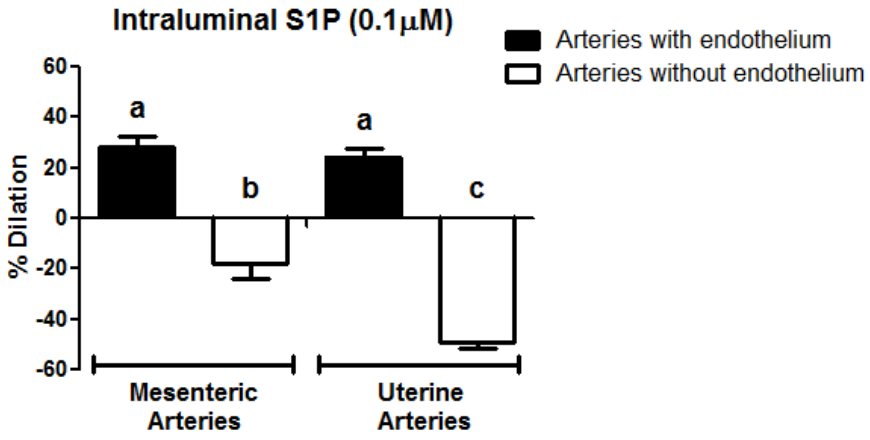

Figure 5.3: Vascular responses to intraluminally infused S1P at 0.1μM in arteries with or without the endothelium from uninfected NP mice. Percent dilation was measured for preconstricted mesenteric arteries with or without the endothelium and preconstricted uterine arteries with or without the endothelium. Data were expressed and summarized as in Figure 5.2. a, b, c = $p<0.05$. n=3-5 animals.

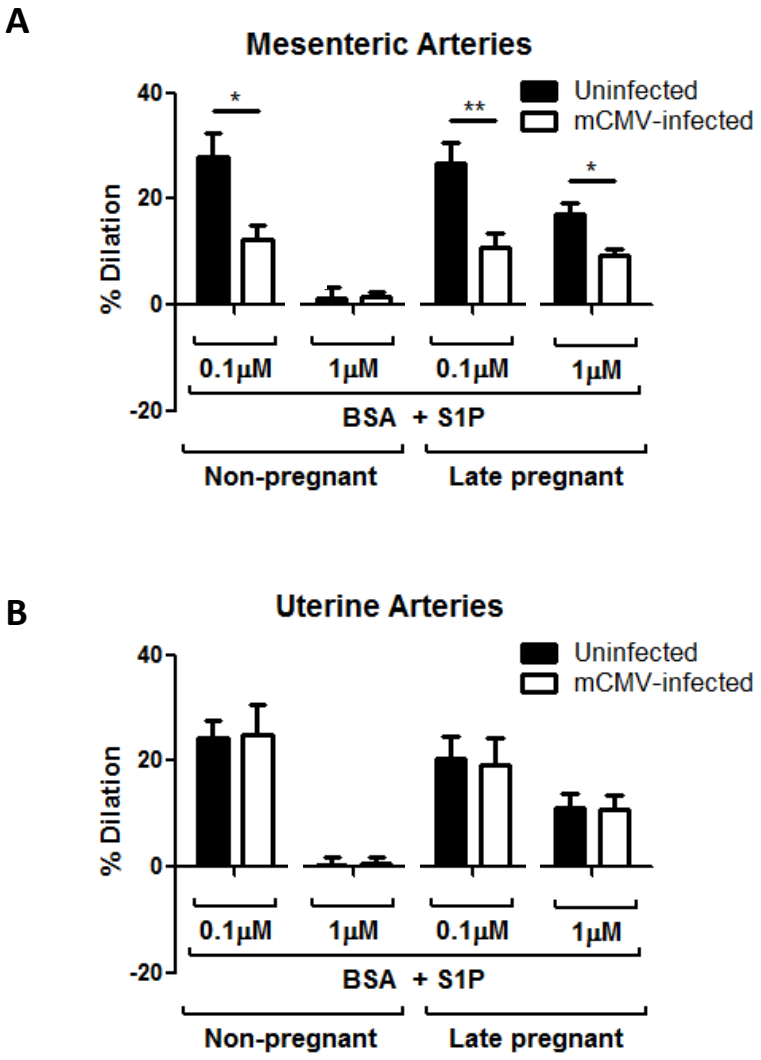

Figure 5.4: Vascular responses to intraluminally infused S1P at 0.1μM and 1μM. Responses were compared in mesenteric **(A)** and uterine **(B)** arteries from mCMV-infected and uninfected NP and LP mice. Data were expressed as in Figure 5.2, and at each S1P concentration the response of the mesenteric or uterine arteries from mCMV-infected mice was compared to that from uninfected mice with a Student's t-test ($p<0.05$). * = $p<0.05$; ** = $p<0.01$. n=5-7 animals.

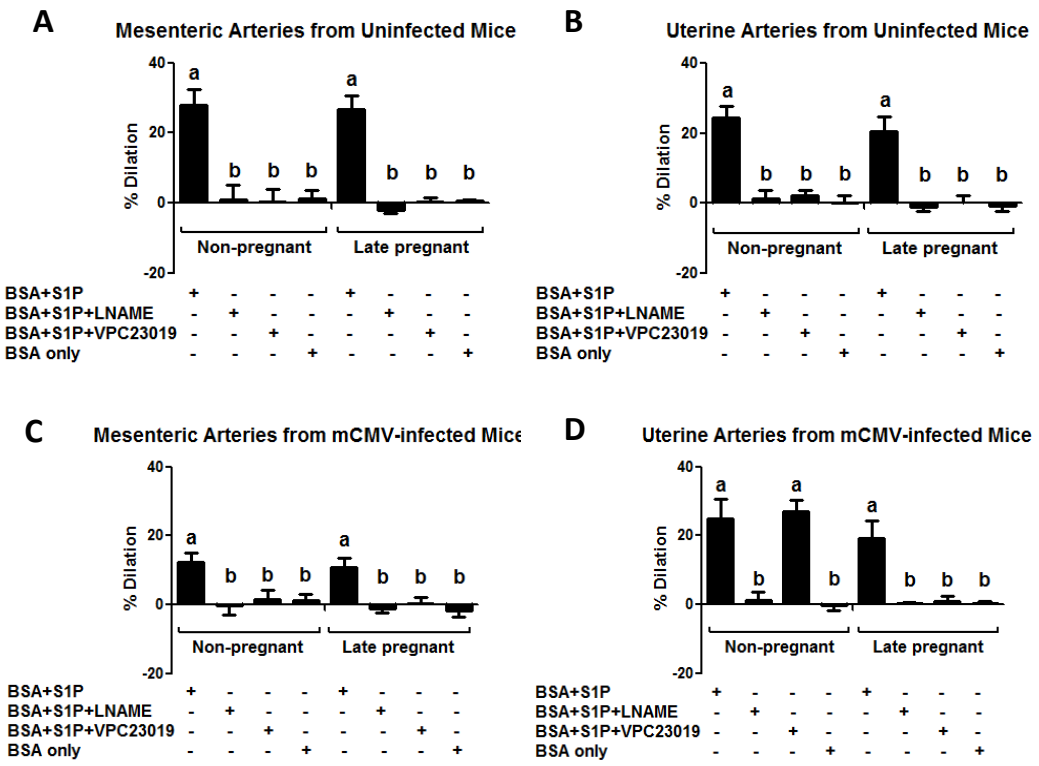

Figure 5.5: Vascular responses to intraluminally infused S1P at 0.1μM with or without pre-treatment with L-NAME (100μM) or VPC23019 (10μM). Intraluminal infusion of S1P induced vasodilation responses in preconstricted mesenteric **(A,C)** and uterine **(B,D)** arteries from uninfected **(A,B)** and mCMV-infected **(C,D)** NP and LP mice. Data were expressed and summarized as in Figure 5.2. a,b = $p<0.05$. n=5-9 animals.

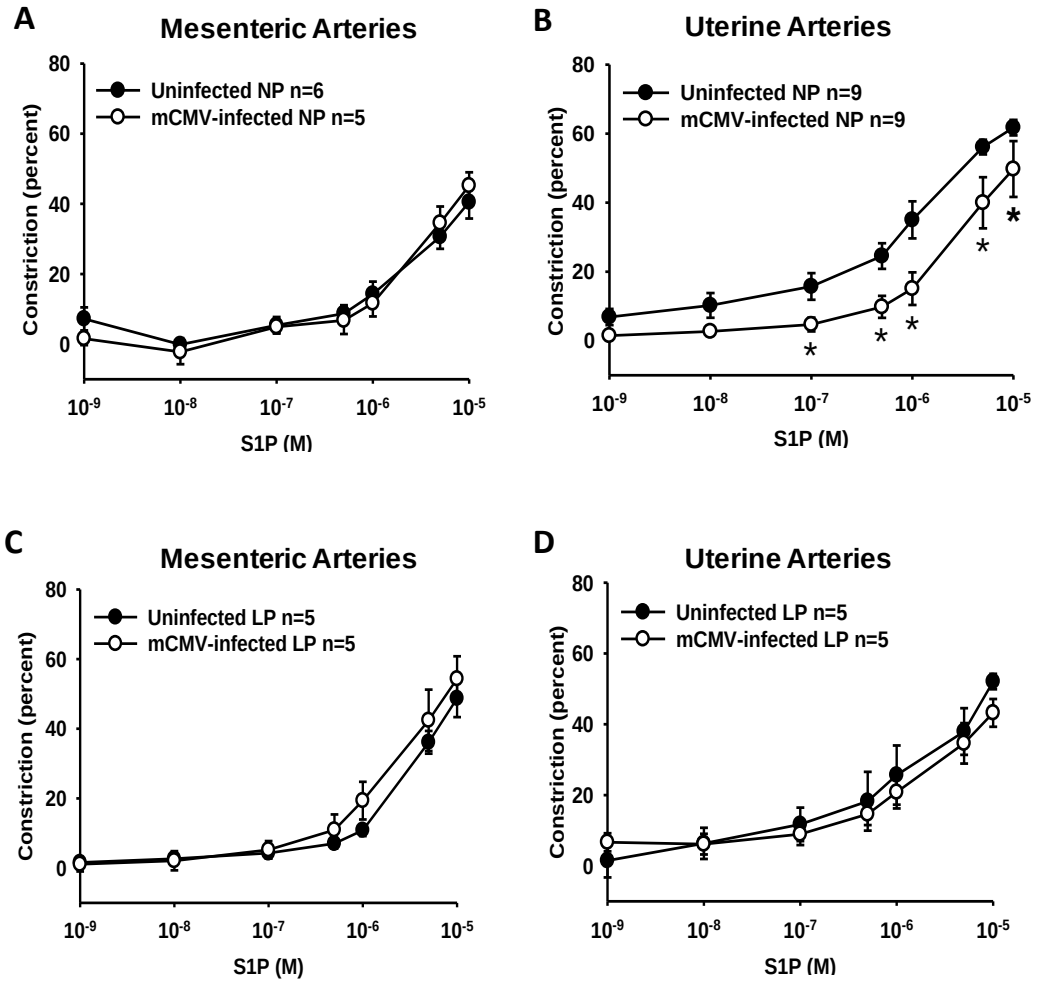

Figure 5.6: Dose-response curves for extraluminal application of S1P in arteries from mCMV-infected and uninfected NP and LP mice. Extraluminal application of S1P induced vasoconstriction in mesenteric **(A,C)** and uterine **(B,D)** arteries from mCMV-infected and uninfected NP **(A,B)** and LP **(C,D)** mice. Data were expressed as the mean ± SEM percent decrease in arterial lumen diameter compared to the initial equilibrated lumen diameter. A significant difference between the points on the curves was determined by Repeated Measures two-way ANOVA and Holm-Sidak's post-hoc analysis and symbolized with an * ($p<0.05$). n=number of animals.

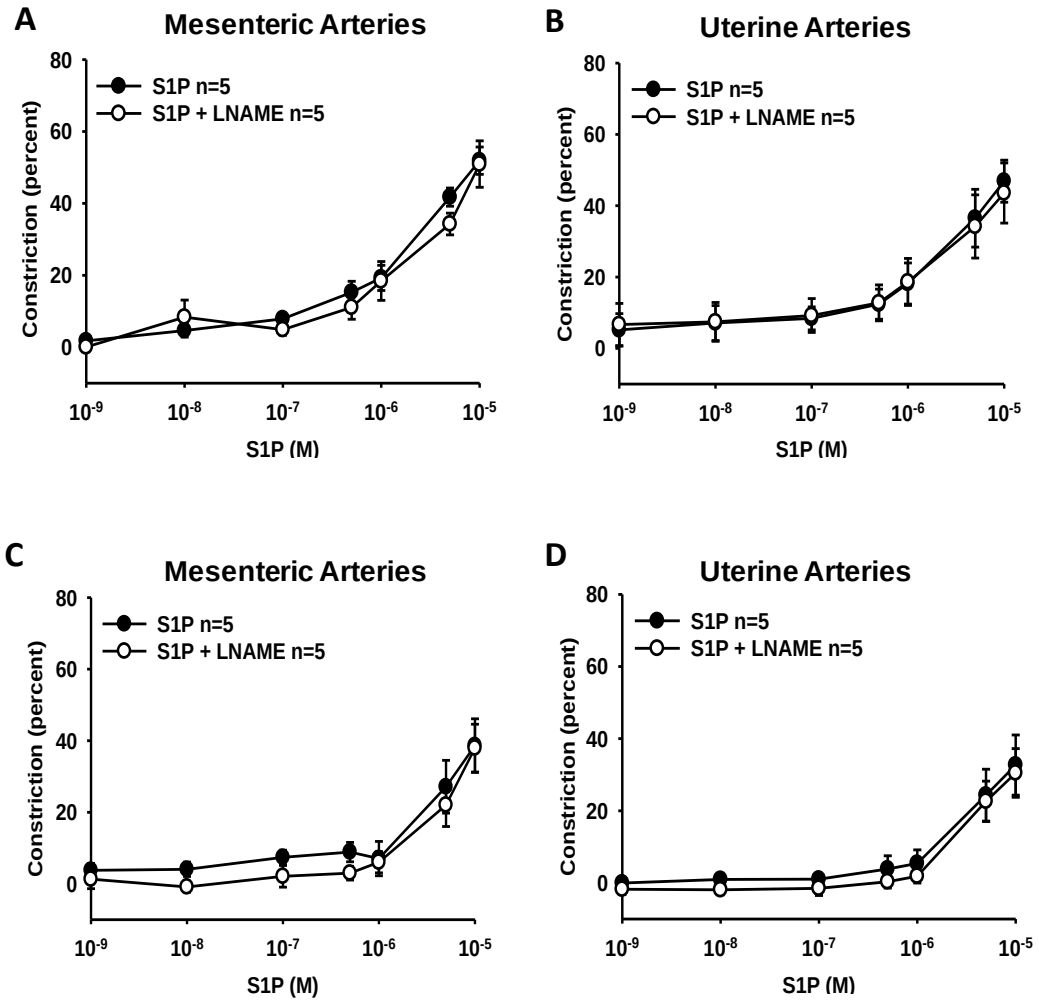

Figure 5.7: Dose-response curves for extraluminal application of S1P with or without pre-treatment with L-NAME (100μM) in arteries from mCMV-infected and uninfected NP mice. Extraluminal application of S1P induced vasoconstriction in mesenteric **(A,C)** and uterine **(B,D)** arteries from uninfected **(A,B)** and mCMV-infected **(C,D)** NP mice. Data were summarized and compared as in Figure 5.6. n=number of animals.

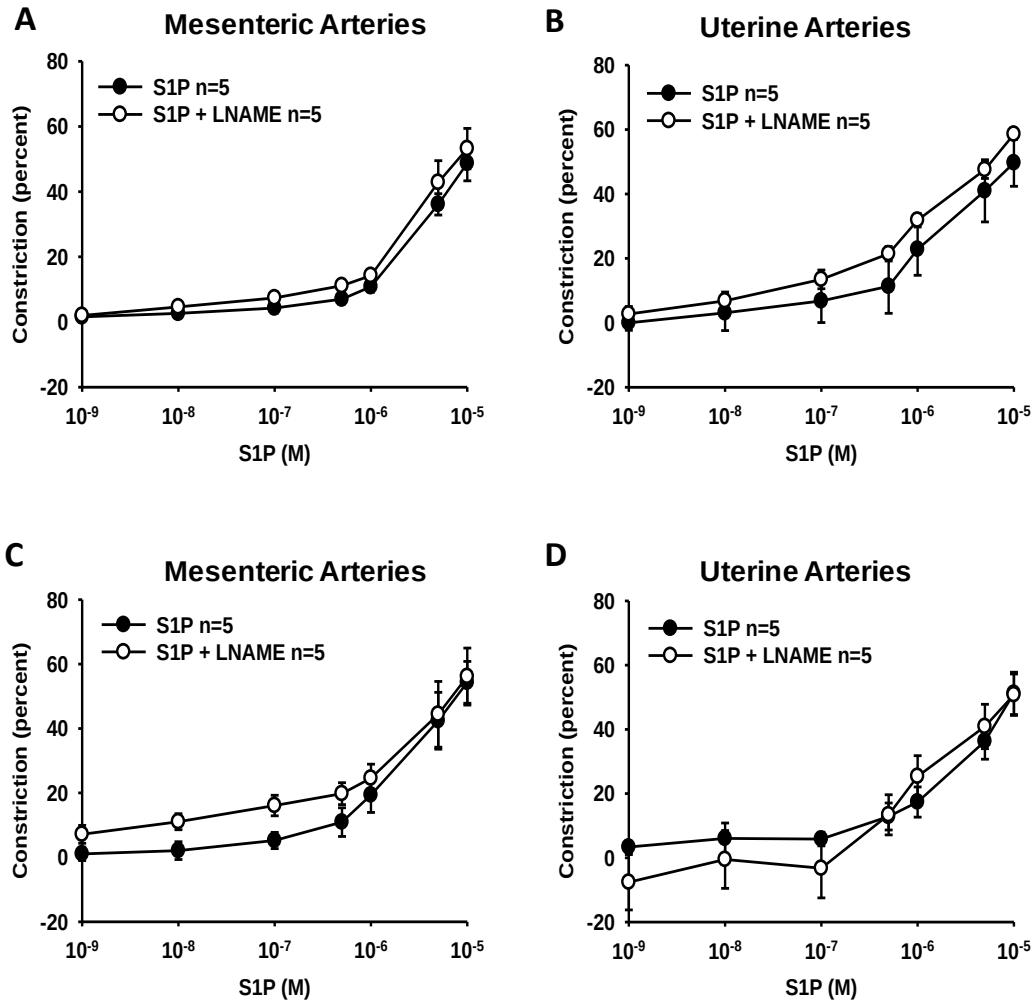

Figure 5.8: Dose-response curves for extraluminal application of S1P with or without pre-treatment with L-NAME (100μM) in arteries from mCMV-infected and uninfected LP mice. Extraluminal application of S1P induced vasoconstriction in mesenteric **(A,C)** and uterine **(B,D)** arteries from uninfected **(A,B)** and mCMV-infected **(C,D)** LP mice. Data were summarized and compared as in Figure 5.6. n=number of animals.

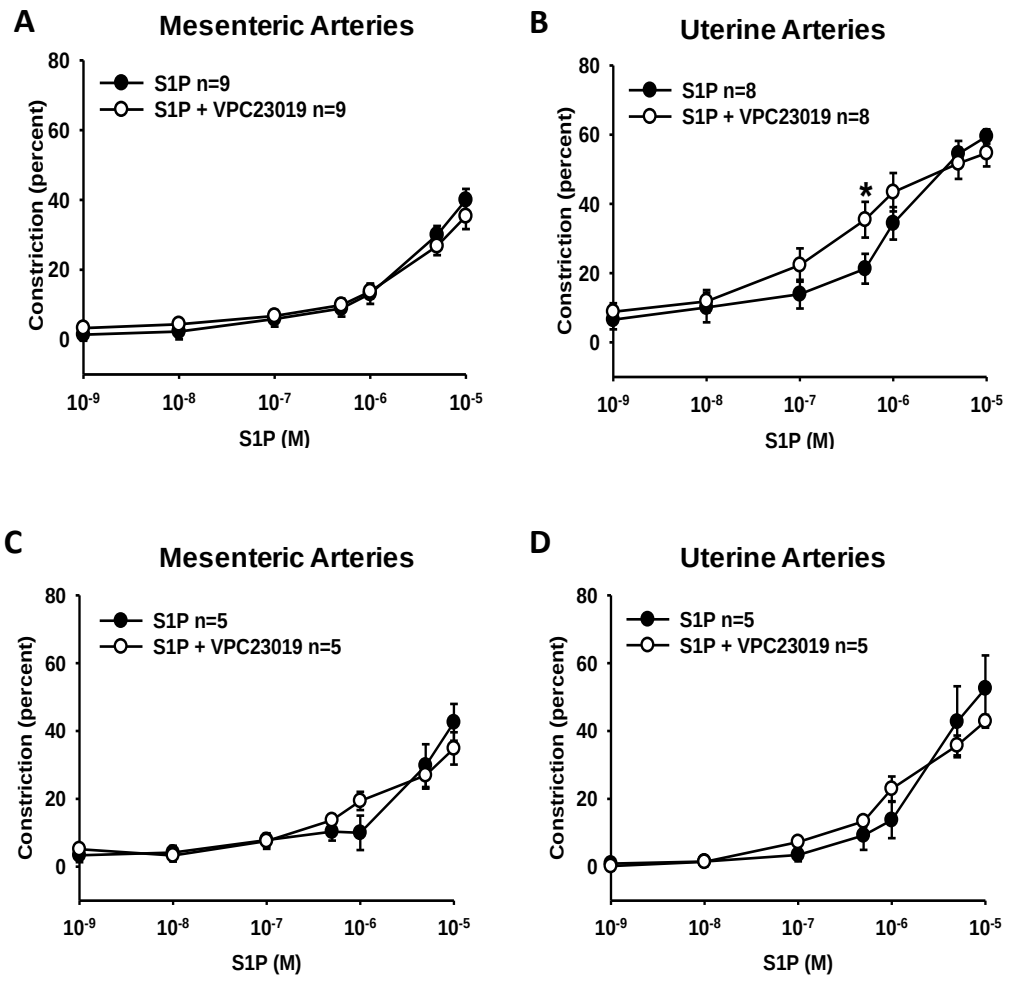

Figure 5.9: Dose-response curves for extraluminal application of S1P with or without pre-treatment with VPC23019 (10μM) in arteries from mCMV-infected and uninfected NP mice. Extraluminal application of S1P induced vasoconstriction in mesenteric **(A,C)** and uterine **(B,D)** arteries from uninfected **(A,B)** and mCMV-infected **(C,D)** NP mice. Data were summarized and compared as in Figure 5.6. * = $p<0.05$. n=number of animals.

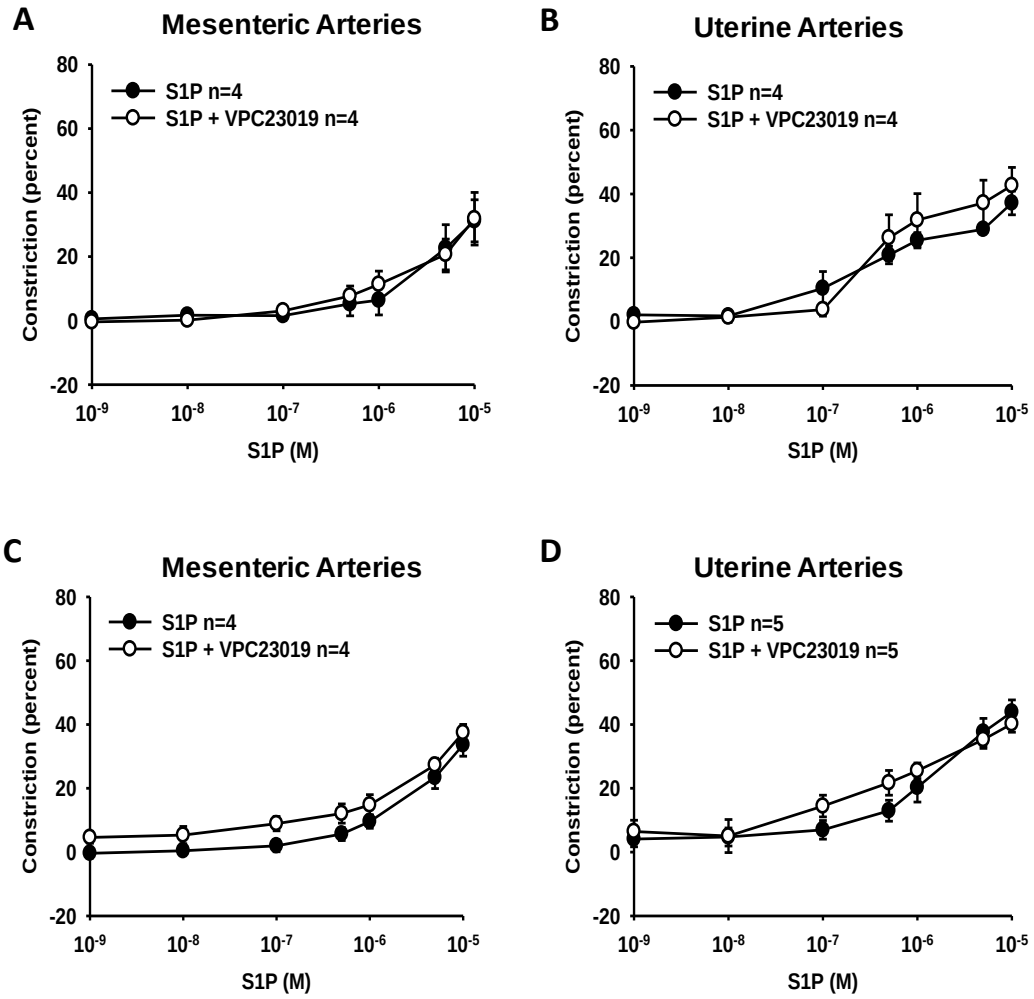

Figure 5.10: Dose-response curves for extraluminal application of S1P with or without pre-treatment with VPC23019 (10μM) in arteries from mCMV-infected and uninfected LP mice. Extraluminal application of S1P induced vasoconstriction in mesenteric **(A,C)** and uterine **(B,D)** arteries from uninfected **(A,B)** and mCMV-infected **(C,D)** LP mice. Data were summarized and compared as in Figure 5.6. n=number of animals.

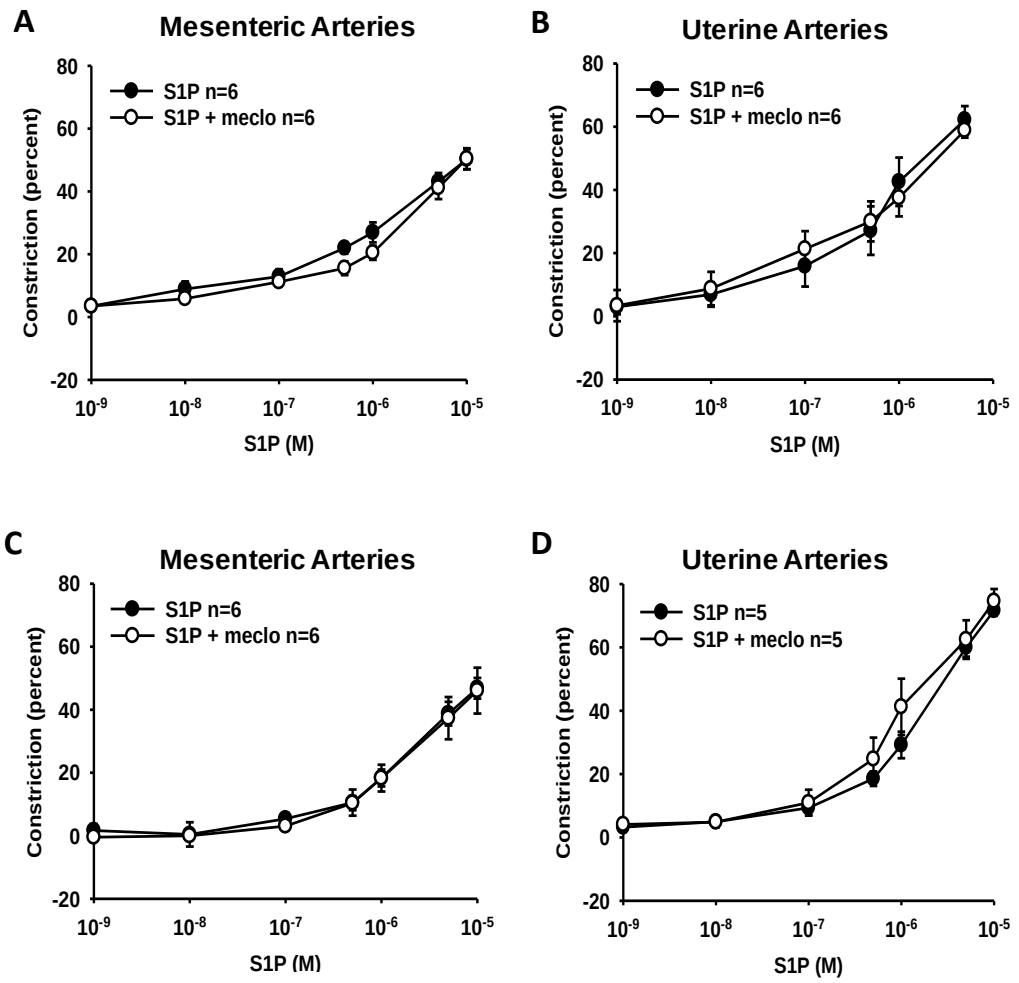

Figure 5.11: Dose-response curves for extraluminal application of S1P with or without pre-treatment with meclofenamate (meclo; 1μM) in arteries from mCMV-infected and uninfected NP mice. Extraluminal application of S1P induced vasoconstriction in mesenteric **(A,C)** and uterine **(B,D)** arteries from uninfected **(A,B)** and mCMV-infected **(C,D)** NP mice. Data were summarized and compared as in Figure 5.6. n=number of animals.

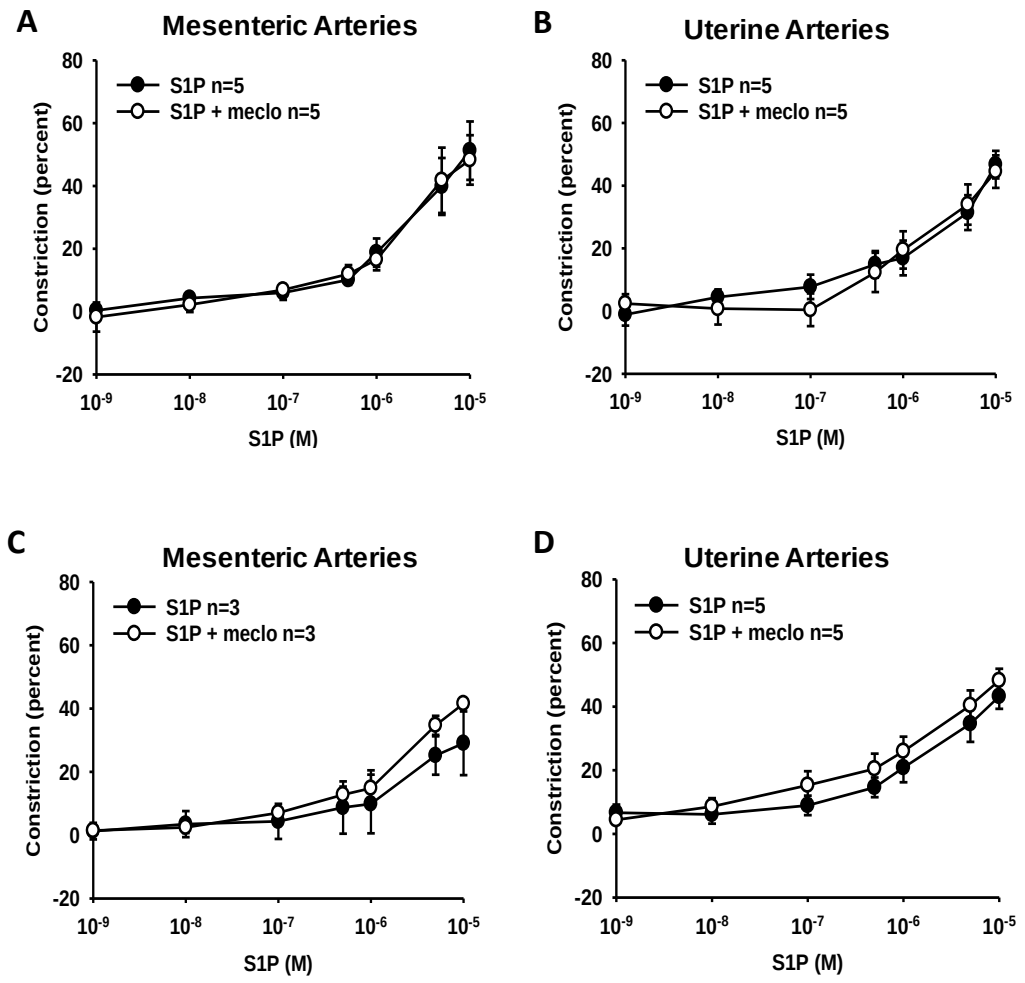

Figure 5.12: Dose-response curves for extraluminal application of S1P with or without pre-treatment with meclo (1μM) in arteries from mCMV-infected and uninfected LP mice. Extraluminal application of S1P induced vasoconstriction in mesenteric **(A,C)** and uterine **(B,D)** arteries from uninfected **(A,B)** and mCMV-infected **(C,D)** LP mice. Data were summarized and compared as in Figure 5.6. n=number of animals.

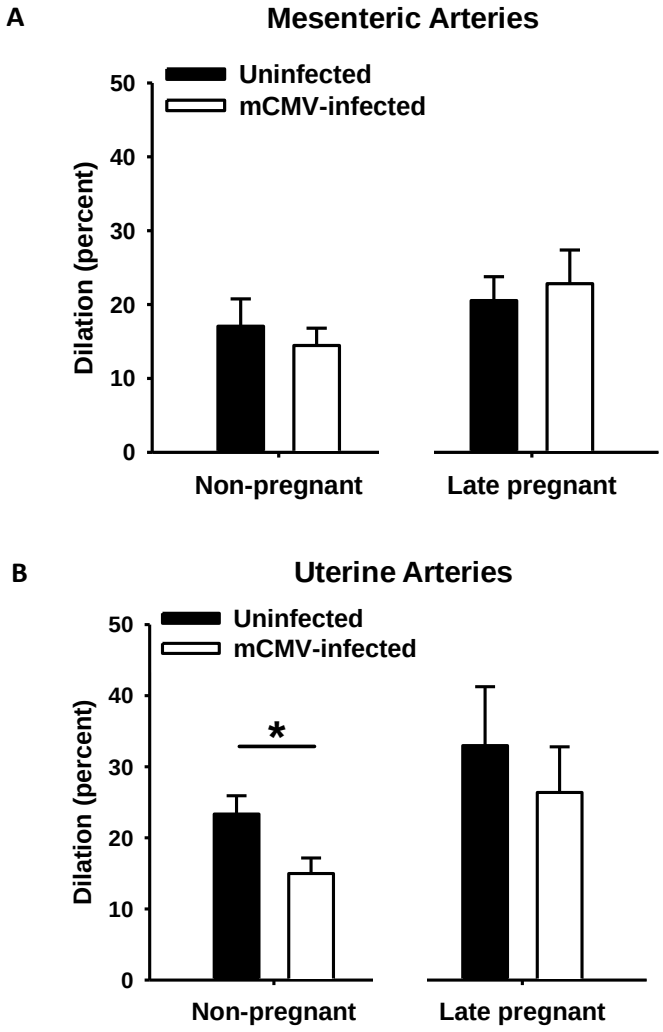

Figure 5.13: Reversal of vasoconstriction induced by extraluminal application of S1P by pre-treatment with Y27632 (10μM) in arteries from mCMV-infected and uninfected NP and LP mice. Y27632 reversed S1P-induced vasoconstriction in mesenteric **(A)** and uterine **(B)** arteries from mCMV-infected and uninfected NP and LP mice. Data were expressed as mean ± SEM percent increase in arterial lumen diameter compared to maximal S1P-induced vasoconstriction. A significant difference between mCMV-infected and uninfected mice in each group was determined using a Student's t-test and symbolized with an * ($p<0.05$). n=5-7 animals.

5.4 DISCUSSION

Although cholinergic stimulation plays a prominent role in the regulation of vascular tone (Chapters 3,4) (Gombos *et al.*, 2010; Gombos *et al.*, 2009), there are several other circulating mediators that also induce endothelium-dependent vasodilation and may be affected by an active CMV infection. S1P is a bioactive sphingolipid that acts on three different receptors on the vasculature to induce either vasodilation or vasoconstriction. Interestingly, stimulation of muscarinic receptors can activate SK-1 and induce S1P production (Meyer zu Heringdorf *et al.*, 1998). In pregnancy, the role S1P plays in the regulation of systemic and uterine vascular tone is unknown, although S1P has been demonstrated to induce vasoconstriction in human placental arteries (Hemmings *et al.*, 2006). In addition, increased S1P in plasma samples obtained in early pregnancy is one of 14 metabolites that comprise a consistent, predictive metabolic signature of preeclampsia (Kenny *et al.*, 2010). Furthermore, HCMV has also been shown to increase SK-1 expression and activity in cultured vascular endothelial cells (Machesky *et al.*, 2008). As such, I chose to study how S1P affects vascular responses during pregnancy in both the presence and absence of a CMV infection.

My approach to the investigation of S1P-mediated vascular responses in pregnancy complicated by infection was unique. I infused S1P into the mesenteric resistance and main uterine arteries isolated and pressurized on a pressure myograph system and showed that low concentrations induced endothelium-dependent vasodilation, which was lost at higher concentrations in arteries from uninfected NP mice. In contrast, direct interaction of S1P with smooth muscle cells of intact arteries led to vasoconstriction that was significantly higher in uterine compared to mesenteric arteries from uninfected NP mice. This highlights a more physiological approach to the study of S1P effects on the vascular system compared to previous techniques and demonstrates the likely importance of S1P-mediated permeability to vascular tone. In pregnancy, vasodilation induced by infused S1P was sustained at a 10-fold higher concentration in LP than in NP mice in both mesenteric and uterine arteries, suggesting an important role for S1P

in pregnancy-associated vascular adaptations. Finally, I showed how a CMV infection affects the S1P signalling pathway in an artery-specific manner. In previous studies, I illustrated that cholinergic endothelium-dependent vasodilation in mesenteric arteries from NP and LP mice and uterine arteries from NP mice was increased but sensitivity to the cholinergic agonist ME was decreased in uterine arteries from LP mice during a CMV infection (Chapter 3) (Gombos *et al.*, 2009). I have now shown that, contrary to my hypothesis, infusion of S1P induced significantly reduced vasodilation in mesenteric arteries from mCMV-infected compared to uninfected NP and LP mice. In addition, although infusion of S1P induced similar vasodilation in uterine arteries from mCMV-infected and uninfected NP or LP mice, the underlying regulation of this response by $S1P_1$ and/or $S1P_3$ in uterine arteries from mCMV-infected NP mice was lost and ROCK-dependent S1P-induced vasoconstriction was reduced.

The response of isolated arteries to vasoactive mediators has generally been studied using the wire or pressure myograph systems (previously described in Chapter 3). The wire myograph puts tension on the vessel and measures the force generated by the vessel in response to vasoactive drugs that have access to both the endothelium and smooth muscle simultaneously (Angus *et al.*, 2000). The pressure myograph system is a closed system that maintains the vessel at a physiological pressure and allows separate initial access to either endothelial or smooth muscle cells (Halpern *et al.*, 1984). This makes the pressure myograph system particularly useful to study several vasoactive drugs or circulating mediators that have different effects on smooth muscle cells versus the endothelium, including S1P. Previous use of this system has only involved addition of S1P directly to the smooth muscle side of the vessel (extraluminal addition) (Hemmings *et al.*, 2004; Murakami *et al.*, 2010). In this study, I used the pressure myograph system to infuse S1P directly inside the pressurized artery to interact with the endothelium (intraluminal) (also done in Chapters 6,7) and compared this to the vascular responses when S1P was only added to the bath to directly interact with the vascular smooth muscle cells (extraluminal), as done in

my previous studies (Chapters 3,4) (Gombos *et al.*, 2010; Gombos *et al.*, 2009).

S1P is one of several vascular mediators with differential effects depending on the expression level and distribution of receptors between endothelial and smooth muscle cells. Other important vascular mediators that act similarly to S1P include bradykinin (Tsutsui *et al.*, 2000), endothelin (Shetty *et al.*, 1993), and acetylcholine (Igarashi *et al.*, 2009). S1P also regulates endothelial permeability depending on concentration, receptor engagement and location (Igarashi *et al.*, 2009; Shikata *et al.*, 2003). Previous studies measuring the effects of S1P on vascular regulation have not differentiated the direct role of S1P on the vascular endothelium versus the smooth muscle cells in intact isolated arteries (Bischoff *et al.*, 2000; Hedemann *et al.*, 2004; Hemmings *et al.*, 2004; Murakami *et al.*, 2010; Salomone *et al.*, 2008; Salomone *et al.*, 2003). I have now shown that although direct interaction of S1P with smooth muscle cells of intact arteries led to dose-dependent vasoconstriction, infusion of S1P inside preconstricted arteries from NP mice to interact directly with endothelial cells led to vasodilation at low concentrations. This vasodilation was NO-dependent and could be inhibited with an $S1P_1$ and $S1P_3$ antagonist. Vasodilation was likely mediated through both receptors since both can lead to the activation of NOS (Igarashi *et al.*, 2009; Nofer *et al.*, 2004). In contrast, vasodilation did not occur with infusion of higher S1P concentrations (1μM and 10μM) into preconstricted arteries from uninfected NP mice. A likely explanation for the loss of vasodilation response with infusion of S1P at the higher concentrations is an increase in endothelial permeability. S1P concentrations approaching and greater than 5μM increase endothelial permeability (McVerry *et al.*, 2005; Shikata *et al.*, 2003) through increased engagement of $S1P_2$ and $S1P_3$ (Sanchez *et al.*, 2007; Singleton *et al.*, 2006). It is important to note that these permeability studies were all performed in cell culture. Endothelial cells may be less sensitive to S1P-induced permeability effects *in vitro* than in intact arteries used in my experiments. Increased endothelial permeability would allow greater access of circulating S1P to smooth muscle cells inducing vasoconstriction that balances the vasodilation induced at

both $S1P_1$ and $S1P_3$. At lower S1P concentrations (0.5 μM), S1P reduces permeability (Shikata *et al.,* 2003) via signalling through the $S1P_1$ receptor and the barrier-promoting pathway (Wang *et al.,* 2009; Zhang *et al.,* 2010), thus promoting a vasodilation response. In addition, I removed the endothelium from intact arteries and showed that an intact endothelial barrier was essential for the vasodilation response to 0.1 μM of infused S1P. In the absence of the endothelium, intraluminally infused S1P had direct access to the smooth muscle cells and induced a vasoconstriction response. Therefore, levels of S1P and S1P receptor expression are important determinants of vascular endothelial barrier function and tight regulation is important for control of vascular tone.

During pregnancy, the vasculature undergoes dramatic remodelling, particularly the uterine vasculature, with increased capacity for vasodilation (Carbillon *et al.*, 2000; Magness *et al.*, 1988; Sladek *et al.*, 1997) and reduced sensitivity to vasoconstrictors (Gant *et al.*, 1987; Magness *et al.*, 1986). Interestingly, the infusion of a 10-fold higher concentration of S1P (1μM) into arteries from LP mice continued to show vasodilation in contrast to the complete lack of response in arteries from NP mice, suggesting reduced endothelial permeability possibly through increased expression of $S1P_1$. In human pregnancies, $S1P_1$ mRNA expression was found in myometrial arteries taken from women at term (Hudson *et al.*, 2007) and the Hemmings lab has previously shown that $S1P_3$ protein expression is increased in term compared to preterm human decidua (Yamamoto *et al.*, 2010). Human placental arteries also express $S1P_1$ and $S1P_3$ mRNA at term (Hemmings *et al.*, 2006). Little else is known with respect to S1P levels, S1P receptor expression and vascular function in human pregnancy; however, increased $S1P_1$ mRNA expression was found in the gravid horn compared to the non-gravid horn of a pregnant sheep uterus early in pregnancy (Dunlap *et al.,* 2010). In addition, SK-1/2 expression and activity and S1P release are increased in the rat uterus during pregnancy (Jeng *et al.*, 2007; Serrano-Sanchez *et al.*, 2008). Therefore, S1P may play an important role in the vascular adaptations that occur in response to increased blood volume and cardiac output

during pregnancy, including a reduction in vascular resistance (Adamova *et al.*, 2009; Assali *et al.*, 1960) .

I previously showed that vascular dysfunction occurs during an active CMV infection in pregnancy (Chapters 3,4) (Gombos *et al.*, 2010; Gombos *et al.*, 2009). Like S1P, CMV has also been associated with preeclampsia, a pregnancy disorder characterized by vascular dysfunction (Grahame-Clarke, 2005; Kenny *et al.*, 2010; von Dadelszen *et al.*, 2003). CMV and S1P affect similar signalling pathways and have similar effects on cell function; however, only limited evidence exists for a direct effect of CMV on S1P signalling and there are no studies in pregnancy. S1P and CMV each promote cell proliferation and migration (Rahaman *et al.*, 2006; Spiegel *et al.*, 2003a) and endothelial permeability (Bentz *et al.*, 2006; Sanchez *et al.*, 2007; Wang *et al.*, 2009). More specifically, a CMV infection increases permeability in cultured microvascular endothelial cells (Bentz *et al.*, 2006), as found in response to other acute inflammatory conditions (Wilhelm, 1973). Similarly, high concentrations of S1P stimulate the $S1P_{2/3}$ receptors and decrease barrier function (Sanchez *et al.*, 2007; Singleton *et al.*, 2006). In addition, in fibroblasts a CMV infection also leads to an increased production of the transcription factor NF-κB (Yurochko *et al.*, 1995). The $S1P_1$ receptor (and potentially other homologous S1P receptors) contains a NF-κB response element (Liu *et al.*, 1997a), suggesting that expression of S1P receptors is upregulated with a CMV infection. S1P and CMV also both regulate vasodilation via NO and prostanoid activity; S1P stimulates NOS-3 and PGHS-2 activation in the endothelium and/or vascular smooth muscle (Hemmings *et al.*, 2006; Nodai *et al.*, 2007; Pettus *et al.*, 2003; Roviezzo *et al.*, 2006) while CMV activates PGHS-2 and may up or down-regulate the expression and activity of NOS-2/3, respectively, depending on the vessel type studied (Chapter 4) (Gombos *et al.*, 2010; Shen *et al.*, 2006; Tanaka *et al.*, 1997; Tanaka *et al.*, 2001; Zhu *et al.*, 2002). With such interconnecting signalling pathways, it is likely that a direct relationship between a CMV infection and S1P activity exists in the vascular system but has not yet been investigated.

I have now demonstrated that an active mCMV infection affects S1P-induced vasodilation and S1P signalling pathways. Mesenteric arteries from mCMV-infected mice had reduced NO-dependent S1P-induced vasodilation mediated by $S1P_1$ and/or $S1P_3$ and compared to arteries from uninfected mice. This suggests that the basal permeability of these arteries is increased during a mCMV infection. Furthermore, a CMV infection may reduce systemic vascular tone via the S1P pathway. Systemic vascular dysfunction suggests that CMV infections contribute to vascular diseases such as hypertension and preeclampsia via the S1P pathway.

Uterine arteries from mCMV-infected NP mice demonstrated decreased ROCK activity, which contributed to the decreased S1P-induced vasoconstriction. Moreover, my results suggest that S1P-induced vasoconstriction in mesenteric and uterine arteries from mCMV-infected and uninfected NP and LP mice is mediated by $S1P_2$, not $S1P_1$ or $S1P_3$. Although there was no overall effect on responses to infused S1P in uterine arteries from mCMV-infected NP mice, the loss of dependence on $S1P_1$ and/or $S1P_3$ illustrates a change in regulation of the response. Signalling through $S1P_1$ and $S1P_3$ inhibits apoptosis, reduces vascular inflammation (Keul *et al.*, 2007; Waeber *et al.*, 2004), and increases PGHS-2 activation and PGI_2 production (Nodai *et al.*, 2007). Therefore, loss of $S1P_1$ and $S1P_3$ on this vascular bed may increase apoptosis and vascular inflammation thereby increasing the spread of infection (Chan *et al.*, 2002). One explanation for the lack of overall effect on infused S1P-induced vasodilation in uterine arteries from mCMV-infected NP mice is that S1P is acting through $S1P_2$ on the endothelium to stimulate Ca^{2+} release (Skoura *et al.*, 2009), leading to the activation of NOS-3 and NO production (Balligand *et al.*, 2009). Moreover, $S1P_2$ expression in isolated vascular endothelial cells is variable in several different studies and may also account for differences in S1P-induced vascular responses in mesenteric and uterine arteries from mCMV-infected and uninfected NP and LP mice (Kimura *et al.*, 2000; Lee *et al.*, 2000; Morales-Ruiz *et al.*, 2001; Rizza *et al.*, 1999).

Infection with CMV during pregnancy did not appear to affect the vasodilation observed at the 10-fold higher concentration (1μM) of infused S1P. This suggests that an increase in $S1P_1$ expression during late pregnancy may override the effects of a CMV infection in uterine arteries from NP mice. The study of vascular responses in early pregnancy of mCMV-infected mice will begin to elucidate its importance in early-onset diseases in pregnancy such as preeclampsia (Gaugler-Senden *et al.*, 2008).

Using the pressure myograph system, I showed that the effects of S1P on vascular responses in intact pressurized arteries are highly dependent on the compartment to which S1P is applied and on S1P concentration. Infusion of low concentrations led to an overall vasodilatory response, whereas higher S1P concentrations likely increased endothelial permeability which increased access of S1P to vascular smooth muscle cells and therefore increased vasoconstriction (Shikata *et al.*, 2003). My results demonstrate the usefulness and reliability of the pressure myograph system to separately investigate the response of the endothelium versus smooth muscle cells in intact isolated arteries and begin to delineate the importance of permeability regulation on vascular tone. In pregnancy, however, infusion of S1P continued to induce vasodilation at a 10-fold higher S1P concentration demonstrating an important role for S1P in pregnancy-induced vascular adaptations. Finally, I illustrated that S1P-induced vasodilation and S1P-signalling pathways were affected by a CMV infection. A reduction in S1P-induced vasodilation in mesenteric arteries and loss in $S1P_1$ and/or $S1P_3$ mediation specifically in the uterine artery from CMV-infected NP mice implies that CMV infections affect vascular responses via the S1P pathway. Therefore, I showed that S1P is an important vascular regulator not only in normal physiological conditions, but also during normal pregnancy and viral infections.

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Chapter 6

Impact of Local Endothelial Challenge with Cytomegalovirus or Glycoprotein B on Vasodilation in Intact Pressurized Arteries from Non-pregnant and Pregnant Mice

(The majority of data presented in this chapter was submitted to the British Journal of Pharmacology in February 2012 (Manuscript ID: BIOLREPROD-2012-099168) in an article entitled "Impact of local endothelial challenge with cytomegalovirus or glycoprotein B on vasodilation in intact pressurized arteries from non-pregnant and pregnant mice," by R.B. Gombos, A. Lee, J. Teefy, and D.G. Hemmings. Although the majority of results in each of the figures I performed, A. Lee and J. Teefy contributed equally to some of the results in Figures 6.1A and 6.3A.)

6.1 INTRODUCTION

I previously found differences in vasodilation and vasoconstriction responses to the α_1-adrenergic receptor agonist PE, the cholinergic endothelium-dependent vasodilator ME, the endothelium-independent NO donor SNP, and the bioactive sphingolipid S1P in isolated, pressurized mesenteric and uterine arteries from mCMV-infected compared to uninfected NP and LP mice (Chapters 3-5) (Gombos *et al.*, 2010; Gombos *et al.*, 2009). The sensitivity to PE was decreased in mesenteric arteries from mCMV-infected NP and LP mice and PE-induced vasoconstriction was increased in uterine arteries from mCMV-infected LP mice (Chapter 3). In addition, ME-induced vasodilation was increased in mesenteric arteries from mCMV-infected NP and LP mice and in uterine arteries from mCMV-infected NP mice, whereas the sensitivity to ME was dramatically decreased in uterine arteries from mCMV-infected LP mice (Chapters 3,4). SNP-induced vasodilation was also increased in mesenteric and uterine arteries from mCMV-infected NP mice (Chapter 4) (Gombos *et al.*, 2010; Gombos *et al.*,

2009). In contrast to these experiments, where the reagent was applied extraluminally to the artery, vascular responses to S1P were measured following both extraluminal and intraluminal S1P application. Vascular responses to extraluminally applied S1P did not differ in mesenteric arteries from mCMV-infected compared to uninfected NP or LP mice or in uterine arteries from mCMV-infected compared to uninfected LP mice; however, extraluminally applied S1P-induced vasoconstriction was significantly decreased in uterine arteries from mCMV-infected compared to uninfected NP mice. Infused S1P induced vasodilation that was decreased only in mesenteric arteries from mCMV-infected compared to uninfected NP and LP mice (Chapter 5). It remains yet to be shown if ME-induced vasodilation responses will also be different in isolated, intact arteries from mCMV-infected and uninfected NP and LP mice following intraluminal infusion of ME compared to extraluminal addition of ME (Chapters 3,4) (Gombos *et al.*, 2010; Gombos *et al.*, 2009).

In addition, the vascular effects that others and I have observed during a systemic infection may differ from those following direct viral attachment and/or early entry into vascular endothelial cells (Cheng *et al.*, 2009; Compton *et al.*, 2003; Gombos *et al.*, 2010; Gombos *et al.*, 2009; Melnick *et al.*, 1995; Sprague *et al.*, 2009). In Chapter 3 (Gombos *et al.*, 2009), I demonstrated that the vascular endothelial and smooth muscle cells in mesenteric and uterine arteries are directly infected with mCMV during an active mCMV infection. Therefore, it is important to determine how direct virus to endothelium interactions may contribute to altered vascular responses in comparison to the overall effects of a systemic infection including inflammatory mediators.

The initial binding of CMV to the cell surface alone induces intracellular signals leading to activation of transcription factors and kinases and increased inflammatory cytokine gene expression, prostanoid synthesis, and ROS; all of which can affect vascular responses (Evers *et al.*, 2004; Fortunato *et al.*, 2000; Speir *et al.*, 1998). There are numerous viral envelope glycoproteins that have been implicated in CMV attachment and entry into the cell (Compton, 2004;

Lopper *et al.*, 2002). gB is required for the entry of the virus into the cell (Bender *et al.*, 2005; Isaacson *et al.*, 2009; Lopper *et al.*, 2002) and is specifically involved in viral attachment and fusion to the cell surface (Compton, 2004; Lopper *et al.*, 2002). Upon attachment, gB also triggers cellular transcription to prepare the cell for CMV infection (Lopper *et al.*, 2002; Simmen *et al.*, 2001). This includes an increase in NF-κB activity leading to an increase in inflammatory cytokine expression (Compton *et al.*, 2003; Fortunato *et al.*, 2000). With CMV replication and cell-to-cell spreading, the host immune response is activated. Several cytokines and inflammatory mediators are produced and released, such as TNF-α and IFN-γ, which can affect vascular responses and have been associated with vascular diseases (Sprague *et al.*, 2009; Tesauro *et al.*, 2008). Hence, it is still unknown if altered vascular responses in the presence of a CMV infection result directly from viral attachment/entry, indirectly from mediators of the immune response following a viral infection, or from a combination.

In this study, my objective is to investigate the vascular effects of viral attachment/early entry by measuring vasodilation induced by infusion of ME after a 1-hour incubation with either gB or infectious mCMV inside isolated, pressurized arteries from uninfected NP and LP mice. These responses will be compared to vasodilation responses to infused ME in arteries isolated from NP and LP mice actively infected *in vivo* with mCMV. Previous ME vascular studies (Chapters 3, 4) were done on the pressure myograph system with the addition of ME to the outside of the vessel (extraluminal addition). The current study is measuring ME-induced dilation after addition of the drug to the inside of the vessel (intraluminal addition), allowing interaction with the endothelium directly, as previously described (Chapter 5). This intraluminal technique will help deduce vascular response differences of direct or indirect viral interactions with the endothelium versus vascular smooth muscle cells of intact pressurized arteries. I hypothesize that exposure of the endothelium of pressurized, isolated, and intact arteries from uninfected NP mice to either gB protein or infectious virus will increase vasodilation to infused ME, which will account at least in part for my

previous findings in arteries from *in vivo* mCMV-infected mice (Chapters 3,4) (Gombos *et al.*, 2010; Gombos *et al.*, 2009). In addition, pre-treatments with gB or infectious virus in isolated, pressurized uterine arteries from uninfected LP mice will decrease sensitivity to infused ME similar to my previous findings in uterine arteries from mCMV-infected LP mice (Chapters 3,4) (Gombos *et al.*, 2010; Gombos *et al.*, 2009). I show that extraluminal and intraluminal addition of ME induce different vascular responses in mesenteric and uterine arteries. In addition, I demonstrate that CMV attachment/entry alone increases sensitivity to infused ME in NP mice, but does not affect vasodilation in LP mice. Although a full mCMV infection exacerbates vasodilation in uterine arteries from NP mice, vasodilation to infused ME is significantly reduced in mCMV-infected LP mice.

6.2 RESULTS

6.2.1 Vasodilation induced by infused ME in mesenteric arteries from uninfected mice (pre-treated with/without gB or infectious mCMV) compared to mesenteric arteries from mice with an *in vivo* mCMV infection

In NP and LP mice, there were no significant differences in vasodilation induced by infused ME (0.1μM to 10μM) among arteries from uninfected, uninfected with gB pre-treatment, uninfected with mCMV pre-treatment, or *in vivo* mCMV-infected mice. Vasodilation was absent in preconstricted arteries infused with PSS alone or filtered virus control (data not shown) at the same flow rate as ME infusion (Figures 6.1A,B). Because gB and infectious mCMV pre-treatment gave similar ME-induced vasodilation results in mesenteric arteries from uninfected NP and LP mice, the following experiments were performed only on mesenteric arteries treated with gB.

6.2.2 Contribution of TxA_2/PGH_2 to vascular responses induced by infused ME in mesenteric arteries

My previously published results demonstrated an increase in vasodilation

induced by extraluminal treatment with ME on mesenteric arteries from mCMV-infected compared to uninfected NP and LP mice (Gombos *et al.*, 2010). However, in the current study when ME was infused inside the arteries, I did not see a difference among gB-treated, mCMV-treated or untreated arteries from uninfected mice compared to arteries from *in vivo* mCMV-infected NP and LP mice (Figure 6.1A,B).

To determine if the lack of increased vasodilation to infused ME compared to extraluminal ME treatment of mesenteric arteries from mCMV-infected mice was due to an increased production of TxA_2/PGH_2, I inhibited PGHS-1/2 or the TxA_2 receptor with meclo or SQ29548, respectively. Both meclo and SQ29548 pre-treatments led to increased ME-induced vasodilation in mesenteric arteries from uninfected mice treated with gB and in arteries from mCMV-infected mice compared to untreated arteries from uninfected mice (Figure 6.2).

6.2.3 Vasodilation induced by infused ME in uterine arteries from uninfected mice (pre-treated with/without gB or infectious mCMV) compared to uterine arteries from mice with an *in vivo* mCMV infection

In uterine arteries from uninfected NP mice, pre-treatments with either gB or mCMV significantly increased vasodilation induced by infused ME at 0.1μM and 1μM by approximately 30% compared to no pre-treatment, indicative of increased sensitivity to ME. However, vasodilation at the maximum ME concentration did not differ among groups. Uterine arteries from mCMV-infected NP mice also showed increased vasodilation to infused ME at 0.1μM compared to controls, similarly to the gB- or mCMV-treated groups, but showed significantly greater vasodilation at 1μM and 10μM of infused ME compared to treated arteries from uninfected mice. Vasodilation was absent in the preconstricted uterine arteries where only PSS or filtered virus culture were infused (Figure 6.3A; data not shown).

In LP mice, there were no differences in ME responses in uterine arteries treated with gB or infectious mCMV compared to untreated uterine arteries from

uninfected mice. However, in uterine arteries from mCMV-infected LP mice, ME-induced vasodilation was significantly reduced from 0.1 to 10μM of ME. Vasodilation was absent with PSS alone or the filtered virus control (Figure 6.3B; data not shown).

6.2.4 Vasodilation to bradykinin in uterine arteries

To determine if the altered sensitivity to infused ME in response to gB pre-treatment of uterine arteries from uninfected mice or those from *in vivo* mCMV-infected mice is specific to muscarinic receptors or occurs with other vasodilators, bradykinin was infused into preconstricted arteries from uninfected NP and LP mice with or without gB pre-treatment or uterine arteries from mCMV-infected NP and LP mice. I found no increase in bradykinin-induced vasodilation in uterine arteries from uninfected mice with gB pre-treatment or in uterine arteries from mCMV-infected mice compared to untreated arteries from uninfected mice (Figure 6.4).

6.3 SUMMARY OF RESULTS

6.3.1 Vasodilation to infused ME was similar in mesenteric arteries treated with or without gB and mCMV from uninfected NP and LP mice and in arteries from mCMV-infected NP and LP mice (Figure 6.1).

6.3.2 Vasodilation to infused ME in mesenteric arteries from NP and LP mice did not differ between treatment groups (Figure 6.1). In contrast, increased vasodilation to extraluminally applied ME was shown previously in mesenteric arteries from mCMV-infected NP and LP mice (see Chapter 3: Figure 3.5).

6.3.3 PGHS-1/2 and TxA_2 receptor inhibition increased vasodilation to infused ME in gB-treated mesenteric arteries from uninfected mice and arteries from mCMV-infected mice (Figure 6.2).

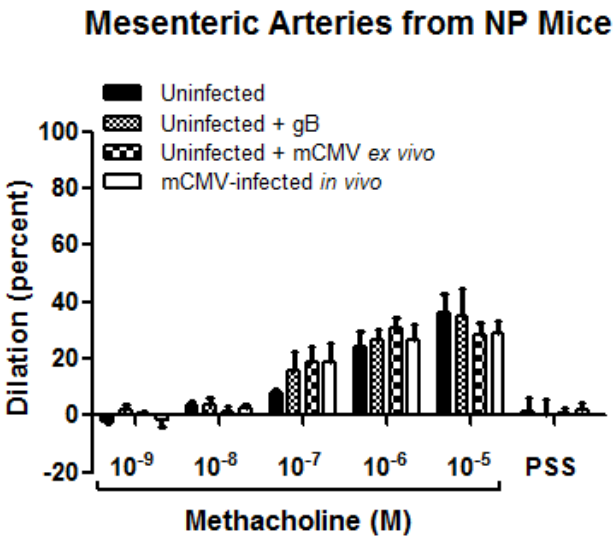
6.3.4 Sensitivity to infused ME was increased in gB- and mCMV-treated uterine arteries from uninfected NP mice and further increased along with increased

maximal vasodilation to infused ME in uterine arteries from mCMV-infected NP mice (Figure 6.3).

6.3.5 Vasodilation to infused ME was decreased in uterine arteries from mCMV-infected LP mice (Figure 6.3).

6.3.6 Altered vasodilation responses to infused ME in gB-treated uterine arteries from uninfected mice and in arteries from mCMV-infected mice did not occur in response to bradykinin (Figure 6.4).

A


Mesenteric Arteries from NP Mice
Uninfected
Uninfected + gB
Uninfected + mCMV ex vivo
mCMV-infected in vivo
Dilation (percent)
100
80
60
40
20
0
-20
10-9
10-8
10-7
10-6
10-5
PSS
Methacholine (M)

B

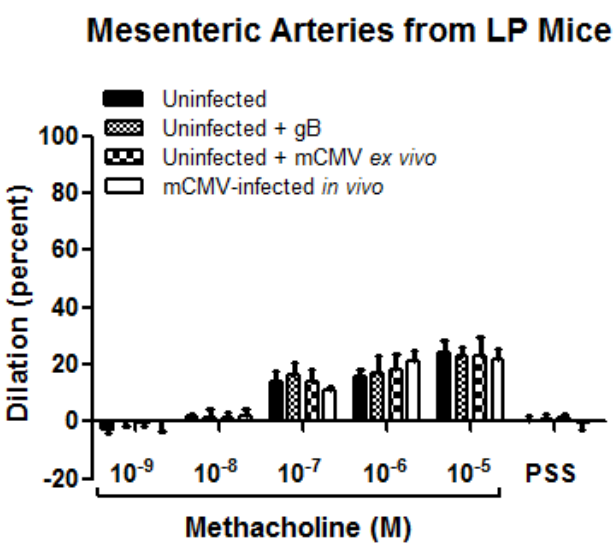

Mesenteric Arteries from LP Mice
Uninfected
Uninfected + gB
Uninfected + mCMV ex vivo
mCMV-infected in vivo
Dilation (percent)
100
80
60
40
20
0
-20
10-9
10-8
10-7
10-6
10-5
PSS
Methacholine (M)

Figure 6.1: Vasodilation induced by infusion of ME into preconstricted mesenteric arteries isolated from mCMV-infected and uninfected mice. Mesenteric arteries isolated from uninfected NP **(A)** and LP **(B)** mice were infused with PSS, 1μg/mL of gB, or infectious mCMV at an MOI of 10 and mesenteric arteries from mCMV-infected mice were infused with PSS. After a 30-minute to 1-hour incubation all arteries were preconstricted with U46619 followed by infusion of a single concentration of ME. This was repeated for each ME concentration from 10nM to 10μM. Data were expressed as the mean ± SEM percent increase in arterial lumen diameter compared to the passive lumen diameter in the presence of Ca^{2+}-free medium and papaverine. No significant difference among treatments at each dose was determined by two-way ANOVA and Bonferroni's post-hoc analysis. n=3-8 animals.

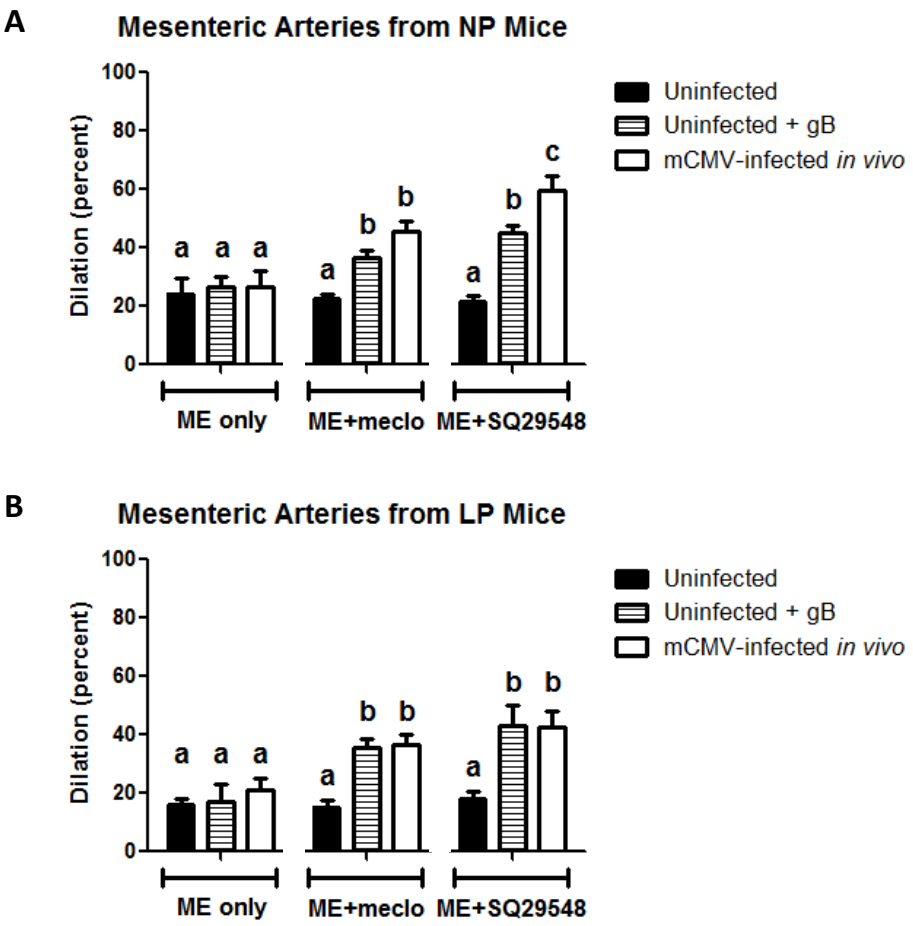


Figure 6.2: Vasodilation induced by infusion of 1μM ME into preconstricted mesenteric arteries in the presence or absence of meclofenamate (meclo; 1μM) or SQ29548 (10μM) for 30 minutes to 1 hour. Arteries were isolated from uninfected NP **(A)** and LP **(B)** mice and treated with or without 1μg/mL gB or were isolated from mCMV-infected NP and LP mice as described in Figure 6.1. ME data were taken from the curves in Figure 6.1. Data were expressed as the mean ± SEM percent increase in arterial lumen diameter compared to the passive lumen diameter in the presence of Ca^{2+}-free medium and papaverine. A significant difference between the bars in each pre-treatment group was determined by one-way ANOVA and Bonferroni's post-hoc analysis and symbolized with an a, b, or c ($p<0.05$). n=4-5 animals.

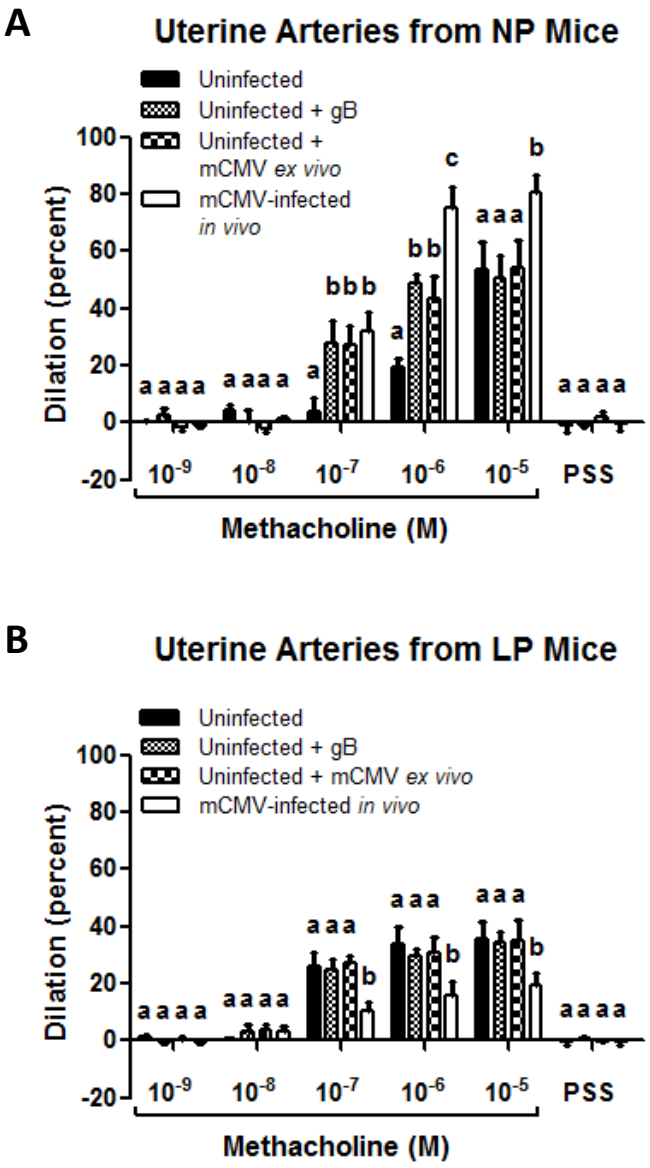

A
Uterine Arteries from NP Mice
Uninfected
Uninfected + gB
Uninfected + mCMV ex vivo
mCMV-infected in vivo
Dilation (percent)
100
80
60
40
20
0
-20
a a a a
a a a a
a
b b b
a
b b
c
a a a
b
a a a a
10-9
10-8
10-7
10-6
10-5
PSS
Methacholine (M)
B
Uterine Arteries from LP Mice
Uninfected
Uninfected + gB
Uninfected + mCMV ex vivo
mCMV-infected in vivo
Dilation (percent)
100
80
60
40
20
0
-20
a a a a
a a a a
a a a
b
a a a
b
a a a
b
a a a a
10-9
10-8
10-7
10-6
10-5
PSS
Methacholine (M)

Figure 6.3: Vasodilation induced by infusion of ME into preconstricted uterine arteries from mCMV-infected and uninfected mice. Uterine arteries isolated from NP **(A)** and LP **(B)** mice were treated as described in Figure 6.1. Data were expressed and summarized as in Figure 6.1. a; b; c = $p<0.05$. n=3-9 animals.

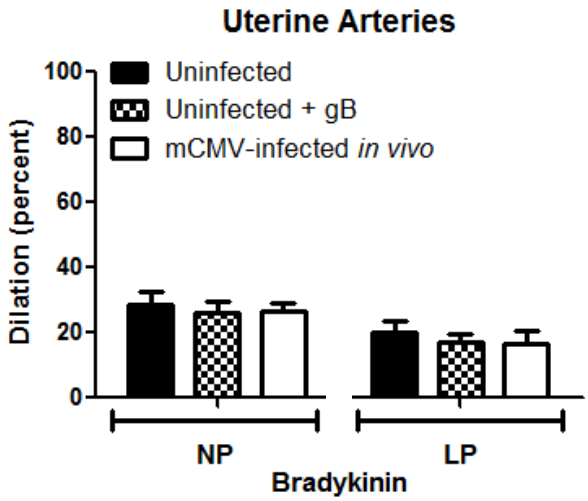


Figure 6.4: Vasodilation induced by infusion of 1μM bradykinin into preconstricted uterine arteries. Arteries were isolated from uninfected NP and LP mice and treated with or without 1μg/mL gB for 30 minutes to 1 hour or were isolated from mCMV-infected NP and LP mice as described in Figure 6.1. Data were expressed and summarized as in Figure 6.2. No significant difference between the bars in each NP and LP group was determined by one-way ANOVA and Bonferroni's post-hoc analysis.

6.4 DISCUSSION

Previously I have demonstrated that systemic and uterine vascular responses to several different vascular mediators are affected by an active CMV infection in both NP and LP states (Chapters 3-5) (Gombos *et al.*, 2010; Gombos *et al.*, 2009). However, it remains unknown whether direct virus to endothelium interactions contribute to altered vascular responses during a systemic *in vivo* infection (ie. inflammatory mediators). CMV is the largest member of the *Herpesviridae* family and as such has a long replication cycle (up to 48-72 hours) (Britt, 2010; Mocarski, 1996). CMV attachment alone to cell surface receptors induces intracellular signalling pathways that may affect vascular function (Fortunato *et al.*, 2000). My results from this chapter confirm that in addition to a fully systemic infection, a 1-hour exposure of endothelial cells to infectious CMV or gB protein also impacts vascular responses. This short exposure time likely leads to viral attachment and possibly early entry, but is not sufficient time for CMV viral gene expression, replication, or egress (Mocarski, 1996). Vasodilation responses to infused ME differed from vasodilation responses to extraluminally applied ME in mesenteric arteries from mCMV-infected NP and LP mice (Chapters 3,4) (Gombos *et al.*, 2010; Gombos *et al.*, 2009), suggesting diverse mechanistic effects following direct drug interactions with endothelial versus smooth muscle cells. However, although an *in vivo* mCMV infection or gB pre-treatment did not affect overall vasodilation to infused ME in mesenteric arteries, the contribution of vasconstrictory prostanoids to these responses was significantly increased in these arteries, as shown previously (Chapter 4) (Gombos *et al.*, 2010). In comparison, vasodilation to infused ME was favored in uterine arteries from mCMV-infected NP mice and in gB-treated uterine arteries from NP mice. These results mimic my previous findings using extraluminal ME application (Chapters 3,4) (Gombos *et al.*, 2010; Gombos *et al.*, 2009). Also in agreement with my extraluminal studies, cholinergic vascular dysfunction was much more apparent in uterine arteries from mCMV-infected LP mice. Together,

these findings demonstrate that viral attachment/early entry alone can differentially affect mesenteric and uterine arterial function in NP and LP mice through distinct mechanisms. As well, some vascular changes were only seen or were exacerbated with a full *in vivo* mCMV infection. This further suggests that although a fully systemic CMV infection may cause systemic and uterine vascular dysfunction, particularly during pregnancy, early viral attachment or gB-specific vaccinations may also have implications in hypertension.

In Chapters 3 and 4, where ME was added extraluminally, I showed an increase in ME-induced vasodilation in mesenteric arteries from mCMV-infected NP and LP mice. This increase was mediated in part through an increased contribution of NO which masked TxA_2/PGH_2-induced vasoconstriction that contributed to the vascular tone observed only in arteries from mCMV-infected mice (Chapter 4) (Gombos *et al.*, 2010). This suggests that mesenteric arterial endothelial cells from mCMV-infected mice may have increased PGHS-1/2 expression and/or activity compared to untreated mesenteric arterial endothelial cells from uninfected mice (Gombos *et al.*, 2010; Hooks *et al.*, 2006; Zhu *et al.*, 2002). In this current study, by blocking PGHS-1/2 or the TxA_2 receptor, I observed increased vasodilation to infused ME in gB-treated mesenteric arteries from uninfected mice and in arteries from *in vivo* mCMV-infected mice compared to controls. This suggests that during a CMV infection individuals may be predisposed to hypertension. Although an increase in PGHS-1/2 expression and/or activity may affect mesenteric arteries from *in vivo* mCMV-infected mice, it is unlikely to occur in gB-treated arteries within 30 minutes to 1 hour. Another explanation may be that gB pre-treatment increases the production of ROS and oxidative stress within the endothelial cell (Evers *et al.*, 2004; Fortunato *et al.*, 2000). In turn, peroxynitrite may be produced from NO and superoxide, leading to the nitration of PGHS-2 and prostacyclin (PGI_2) synthase. This nitration increases PGHS-2 activity and decreases PGI_2 synthase activity, increasing the PGH_2/TxA_2 to PGI_2 balance and, effectively, reducing vasodilation (Landino *et al.*, 1996; Zou *et al.*, 1997).

In contrast to my previous extraluminal results (Chapters 3,4) (Gombos *et al.*, 2010; Gombos *et al.*, 2009), my findings from this Chapter showed that vasodilation to infused ME was not different in mesenteric arteries from mCMV-infected compared to uninfected NP and LP mice. One explanation is that when ME is added extraluminally to the bath, there may be a decrease in muscarinic receptor interaction by ME on the endothelium (Figure 6.5). Therefore, intracellular calcium levels are reduced, activation of $cPLA_2$ is reduced, and secretion of arachidonic acid into the cytoplasm of endothelial cells is decreased, reducing prostanoid synthesis (Bonventre, 1992; Chen *et al.*, 2008). Although NOS is still activated with low intracellular calcium levels leading to increased levels of NO, less TxA_2/PGH_2 will be produced leading to greater vasodilation and reduced vascular tone. In comparison, intraluminal infusion of ME may activate more muscarinic receptors on the endothelium leading to increased intracellular calcium concentrations and maximal $cPLA_2$ activity (Figure 6.5) (Bonventre, 1992). Using the TxA_2 receptor antagonist and PGHS-1/2 inhibitor, my study confirmed that intraluminal ME infusion increased TxA_2/PGH_2 production and vascular tone in gB-treated mesenteric arteries from uninfected mice and in arteries from mCMV-infected mice. The levels of vasodilation reached after inhibition were similar to that observed in mesenteric arteries from mCMV-infected mice in previous studies where ME was applied extraluminally (Chapters 3-5) (Gombos *et al.*, 2010; Gombos *et al.*, 2009). This suggests that opposing vascular responses following intraluminal versus extraluminal drug addition reflect differences in ligand-receptor interactions on the apical and basal side of the endothelium, respectively, leading to differences in intracellular signalling and vascular tone (Figure 6.5).

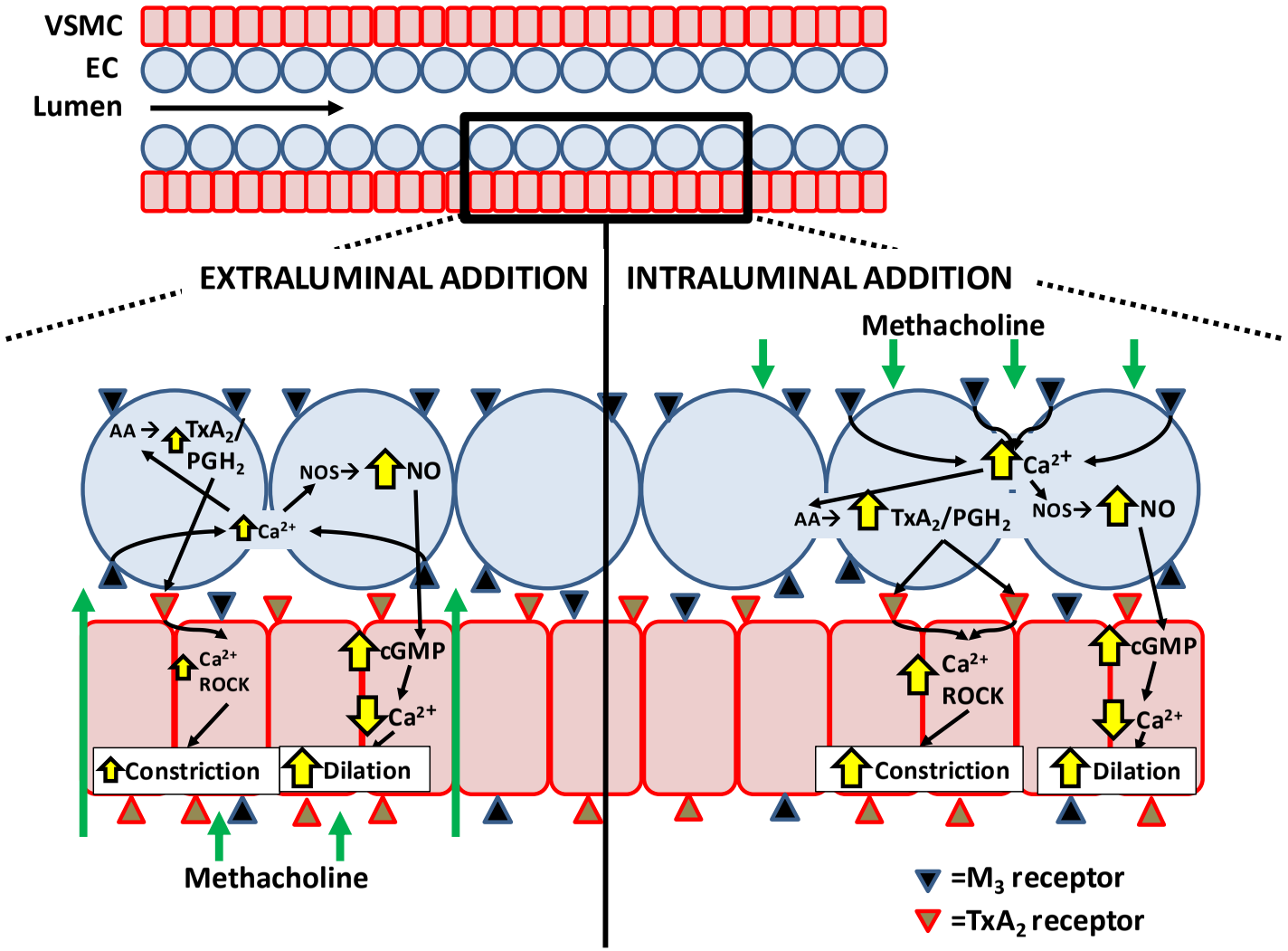

VSMC
EC
Lumen
EXTRALUMINAL ADDITION
INTRALUMINAL ADDITION
Methacholine
AA → TxA2/ PGH2
NOS → NO
Ca2+
Ca2+ ROCK
cGMP
Ca2+
Constriction
Dilation
Methacholine
=M3 receptor
=TxA2 receptor

Figure 6.5: Schematic representation of a mesenteric artery from a mCMV-infected mouse. Potential mechanism for extraluminal and intraluminal ME response differences in mesenteric arteries. VSMC: vascular smooth muscle cells; EC: endothelial cells; AA: arachidonic acid; TxA_2: thromboxane; NOS: nitric oxide synthase; NO: nitric oxide; cGMP: cyclic guanosine monophosphate.

Since the levels of vasodilation induced by infused ME in mesenteric arteries from mCMV-infected and uninfected mice is similar, the finding of increased mCMV-specific TxA_2/PGH_2 activity suggests that increased NO production must be compensating for this vasoconstrictory effect during ME stimulation, as demonstrated previously (Chapter 4) (Gombos *et al.*, 2010). Nevertheless, hypertensive conditions related to CMV infections (Cheng *et al.*, 2009; Melnick *et al.*, 1995; Persoons *et al.*, 1998; von Dadelszen *et al.*, 2003) may occur *in vivo* through increases in other mediators acting on vascular TxA_2 receptors, such as angiotensin II (Pfister *et al.*, 2011), or when NO production is unable to compensate for prostanoid-induced vasoconstriction, such as with increased oxidative stress and/or NOS uncoupling (Cai *et al.*, 2000; Szabo, 1996). Aspirin, an inhibitor of PGHS-1/2 activity, may be a potential treatment for prostanoid-mediated hypertension associated with a CMV infection. Speir et al. showed that treatment with aspirin decreases CMV infectivity and replication in coronary artery smooth muscle cells (Speir *et al.*, 1998) and it has been used as an antiviral agent to treat symptoms of an active CMV infection (Axelrod *et al.*, 2005). More commonly, aspirin also attenuates atherosclerosis, improves endothelium-dependent vasodilation (Husain *et al.*, 1998; Paul *et al.*, 2000) and, although controversial, low-dose aspirin is used by some clinicians to treat preeclampsia (Barth, 1998; Beilin, 1994). As CMV is associated with vascular diseases including atherosclerosis (Melnick *et al.*, 1995) and preeclampsia (von Dadelszen *et al.*, 2003), aspirin may be a possible treatment for CMV-induced vascular dysfunction.

Surprisingly, uterine arteries from uninfected NP mice treated for 1 hour with gB or infectious mCMV showed a significant increase in sensitivity to infused ME. This increase was, however, less than the increase observed in uterine arteries from mCMV-infected NP mice. This difference in vasodilation may be due to the infection of underlying vascular smooth muscle cells that occurs during a full *in vivo* mCMV infection. We previously demonstrated that the sensitivity to NO is significantly increased in vascular smooth muscle cells from uterine arteries in mCMV-infected NP mice, increasing ME-induced vasodilation (Chapter 4) (Gombos *et al.*, 2010). In contrast, vasodilation induced by infused ME was decreased only in uterine arteries from mCMV-infected LP mice, which may contribute to reduced placental perfusion and IUGR associated with a HCMV infection. These findings reflect those observed previously when ME was added extraluminally to isolated and pressurized uterine arteries from mCMV-infected NP mice (Chapters 3,4) (Gombos *et al.*, 2010; Gombos *et al.*, 2009). Together, this suggests that although viral attachment/early entry alone is enough to induce changes that affect cholinergic vascular responses in uterine arteries, it is not to the same extent as a full systemic infection.

Further investigation into the mechanistic differences in cholinergic vasodilation in gB-treated and untreated uterine arteries and uterine arteries from *in vivo* mCMV-infected mice is required to identify which signalling molecules are affected. To begin to delineate the vasodilatory signalling pathway affected by gB treatment and/or a mCMV infection in uterine arteries, I infused another vasodilator known as bradykinin, a potent vasodilatory peptide produced in both the nervous and cardiovascular system (Furchgott, 1984; Rabelo *et al.*, 2008; Sharma, 2006; Valdes *et al.*, 2008; Walker *et al.*, 1995). The production and release of bradykinin, a pro-inflammatory mediator, is significantly increased at sites of tissue injury and inflammation and signals the production of pain-stimulating cytokines (Hamza *et al.*, 2010; Walker *et al.*, 1995). Like the muscarinic receptor M_3, the constitutively expressed bradykinin receptor B_2 is present on both the vascular endothelial and smooth muscle cells and is also

coupled to the $G\alpha_{q/11}$ protein. I found that the effects of gB and a mCMV infection on uterine vasodilation were specific to the cholinergic system and were not observed with infusion of bradykinin, The lack of change in bradykinin-induced vasodilation suggests that the increased vasodilation in uterine arteries from mCMV-infected NP mice and decreased vasodilation in uterine arteries from mCMV-infected LP mice is specific to cholinergic agonists and muscarinic receptors. Further experiments investigating cholinergic vasodilation in gB-treated and untreated uterine arteries and uterine arteries from *in vivo* mCMV-infected mice are shown in Appendix A.

This study is the first to demonstrate that a short-term exposure of endothelium to infectious CMV or gB leading to direct cell attachment/early entry has effects on the vasculature independent of a full CMV infection. However, a systemic mCMV infection exacerbates these direct viral effects and can cause further endothelial dysfunction, such as the significantly reduced endothelium-dependent vasodilation seen in uterine arteries from mCMV-infected LP mice. Indirect effects of an *in vivo* mCMV infection, such as production of immune response mediators, must still be studied. It is important to note that vascular responses differ based on the timing of infection, vascular origin, and receptor expression. Therefore, this must be considered for the development of potential therapeutics for specific vascular diseases that may be caused by a CMV infection. Finally, my findings suggest that a CMV or gB vaccine which permits cell attachment/early entry but prevents viral replication and infection may have potentially positive vasodilatory effects without negatively affecting vascular function during pregnancy.

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Chapter 7

Pregnancy Outcome in Cytomegalovirus-infected Mice

7.1 INTRODUCTION

Following my studies on vascular function in systemic and uterine arteries from mCMV-infected and uninfected NP and LP mice (Chapters 3-6) (Gombos *et al.*, 2010; Gombos *et al.*, 2009), I wanted to determine if there were any effects of a maternal CMV infection on fetal or placental development independent of a congenital CMV infection. In pregnant mice Gharavi et al. showed that fetal loss is increased following the maternal antibody response to the phospholipid-binding CMV-derived peptide (Gharavi *et al.*, 2004). This occurs in the absence of CMV transmission to the fetus (Johnson, 1969). Furthermore, HCMV, in addition to preeclampsia, is also associated with IUGR (Brown *et al.*, 1998; Grahame-Clarke, 2005; von Dadelszen *et al.*, 2003). IUGR is a significant complication of pregnancy with both short-term and long-term consequences (Pallotto *et al.*, 2006). Reduced uterine blood flow leads to IUGR in animal models (Anderson *et al.*, 2005; Sholook *et al.*, 2007) and in humans (Lang *et al.*, 2003). Even modest reductions of uterine blood flow in a sheep model (<25%) leads to IUGR (Lang *et al.*, 2003). My previous findings have illustrated that cholinergic endothelium-dependent vasodilation is decreased and vasoconstriction is increased in uterine arteries from mCMV-infected compared to uninfected LP mice (Chapters 3,4,6) (Gombos *et al.*, 2010; Gombos *et al.*, 2009). This suggests that uterine blood flow is reduced in mCMV-infected LP mice which may lead to problems in fetal development, such as IUGR.

Interestingly, a clinical study performed by Carreiras et al. suggested that HCMV-infected pregnant women with certain major histocompatibility complex alleles are at higher risk of developing preeclampsia (Carreiras *et al.*, 2002); however, a direct connection between genotypic variation, CMV, and pregnancy complications remains unknown. To determine the effect of a maternal CMV

infection and genotypic susceptibility on pregnancy outcome, two different mouse strains have been used in this chapter. These strains represent the genotype heterogeneity in the human population and the variability in susceptibility to CMV infections. C57Bl/6J mice, which I have used for all of my previous experiments, are more resistant to CMV whereas Balb/cJ mice are genotypically more susceptible to CMV (Geist *et al.*, 2001; Mercer *et al.*, 1986). I hypothesize that a CMV infection in both strains of LP mice will lead to IUGR, with a greater effect in the Balb/cJ strain. This will be evident by decreased placental and fetal weights. Contrary to my hypothesis, I show that in the C57Bl/6J strain there is no change in fetal weights but there is an increase in the fetal to placental ratio in mCMV-infected LP mice, suggesting an increase in placental efficiency. In contrast, mCMV-infected LP Balb/cJ mice are unable to carry their fetuses to term at three different doses of mCMV (10^6, $5x10^5$, and 10^5 PFU).

Due to these findings, I further hypothesized that vasodilation induced by infused ME would be significantly more impaired in mCMV-infected NP Balb/cJ mice compared to uninfected Balb/cJ and mCMV-infected NP C75Bl/6J mice. However, again contrary to my hypothesis, endothelium-dependent vasodilation to infused ME in mesenteric and uterine arteries is increased in mCMV-infected NP Balb/cJ mice to a similar extent as that found in arteries from mCMV-infected NP C57Bl/6J mice.

7.2 RESULTS

7.2.1 Fetal and placental weights from mCMV-infected and uninfected LP C57Bl/6J mice

Litter size, fetal and placental weights were compared between mCMV-infected and uninfected LP (D18.5) C57Bl/6J mice. There was no significant difference in fetal or placental weights (Figure 7.1A,B); however, the fetal to placental ratio was significantly increased ($p<0.05$) in mCMV-infected LP mice (Figure 7.1C). There was no significant difference in litter size or maternal

weights throughout pregnancy between mCMV-infected and uninfected LP mice.

7.2.2 Fetal and placental weights from uninfected LP C57Bl/6J and Balb/cJ mice

Litter size, fetal and placental weights were compared between uninfected LP C57Bl/6J and Balb/cJ mice. There was no significant difference between fetal or placental weights (Figure 7.2A,B) or the fetal to placental ratio (Figure 7.2C). There was also no difference in litter size or maternal weights.

7.2.3 Pregnancy success rates in mCMV-infected and uninfected C57Bl/6J and Balb/cJ mice

The mCMV-infected and uninfected C57Bl/6J mice and uninfected Balb/cJ mice used in this study had a 100% pregnancy success rate. In contrast, none of the mCMV-infected Balb/cJ mice (10^6, $5x10^5$, 10^5 PFU) that were plugged were able to hold their pregnancy until term. One pregnant mouse sacrificed on D15 gestation already showed 7 out of 8 resorptions (Table 7.1).

7.2.4 Vasodilation induced by infused ME in mesenteric and uterine arteries from mCMV-infected and uninfected NP Balb/cJ mice

Similar to C57Bl/6J mice (Chapter 6), there was no significant difference in ME-induced vasodilation in mesenteric arteries from mCMV-infected compared to uninfected NP Balb/cJ mice (Figure 7.3A). In addition, uterine arteries from mCMV-infected NP Balb/cJ mice had significantly increased ME-induced vasodilation compared to uninfected NP mice (Figure 7.3B). Again, this increase was similar to uterine arteries from mCMV-infected NP C57Bl/6J mice (Chapter 6).

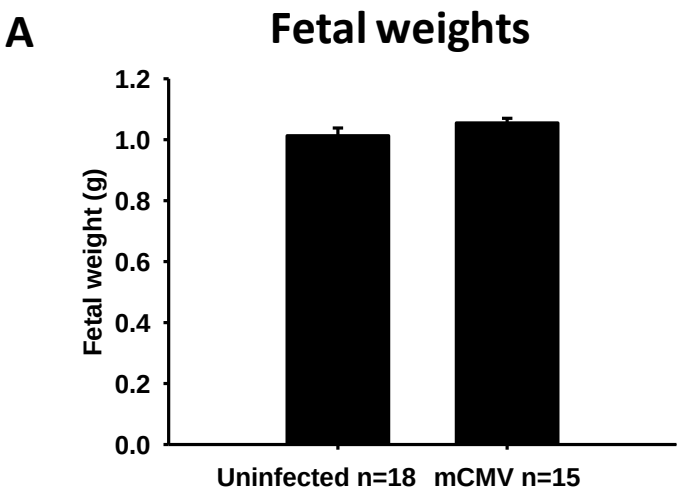
7.3 SUMMARY OF RESULTS

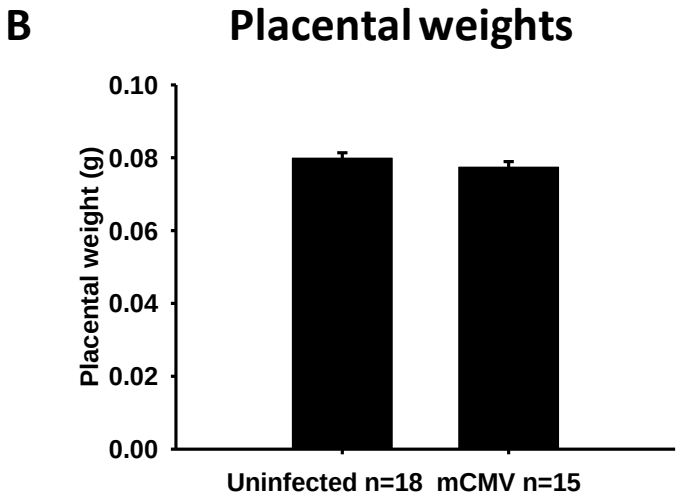
7.3.1 There were no differences in fetal or placental weights but the fetal to placental ratio was significantly increased in mCMV-infected compared to uninfected C57Bl/6J mice (Figure 7.1).

7.3.2 There were no differences in fetal or placental weights or the fetal to placental ratio between uninfected C57Bl/6J and Balb/cJ mice (Figure 7.2).

7.3.3 mCMV-infected Balb/cJ mice had a 0% pregnancy success rate compared to a 100% pregnancy success rate in mCMV-infected and uninfected C57Bl/6J mice and uninfected Balb/cJ mice (Table 7.1).

7.3.4 Vasodilation induced by infused ME was significantly increased in uterine arteries from mCMV-infected compared to uninfected NP Balb/cJ mice, similar to previous results in C57Bl/6J NP mice (Figure 7.3).


A
Fetal weights
Fetal weight (g)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Uninfected n=18
mCMV n=15


B
Placental weights
Placental weight (g)
0.10
0.08
0.06
0.04
0.02
0.00
Uninfected n=18
mCMV n=15

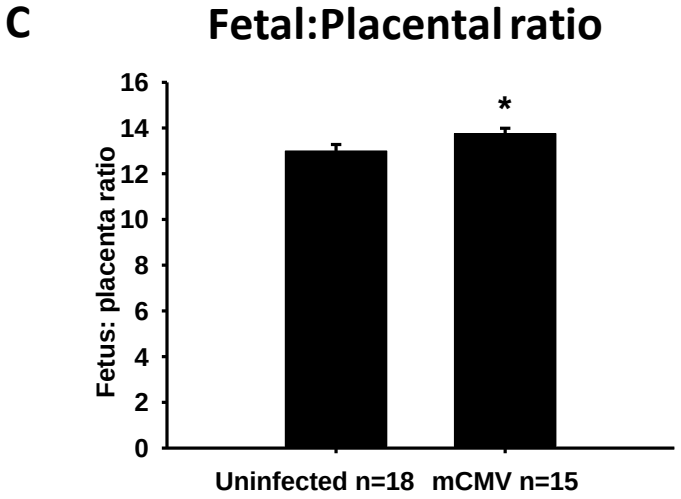
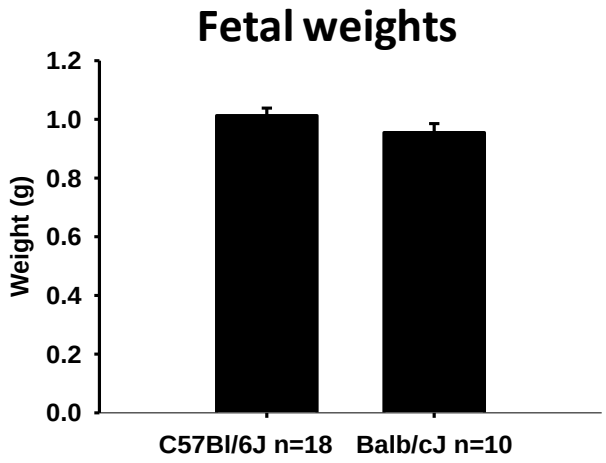
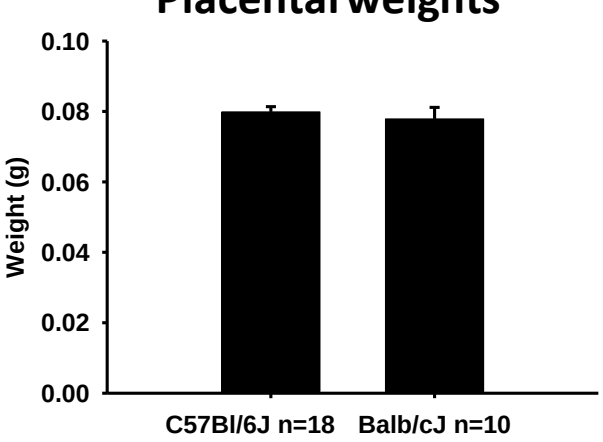

C
Fetal:Placental ratio
Fetus: placenta ratio
16
14
12
10
8
6
4
2
0
*
Uninfected n=18
mCMV n=15

Figure 7.1: Fetal weights, placental weights, and fetal to placental ratios from LP (D18.5) mCMV-infected and uninfected C57Bl/6J mice. Weights were recorded for fetuses **(A)** and placentas **(B)** from mCMV-infected (n=112, 15 litters) and uninfected (n=119, 18 litters) mice. The fetal to placental ratio **(C)** was measured directly from the paired fetal and placental weights. The fetal and placental weights and ratios were averaged for each litter. The means of all the litters were then averaged for the mCMV-infected and uninfected D18.5 pregnant mice and expressed as mean ± SEM. A significant difference between the groups was determined by Student's t-test and symbolized with an * ($p<0.05$).

A


Fetal weights
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Weight (g)
C57Bl/6J n=18
Balb/cJ n=10

B


Placental weights
0.10
0.08
0.06
0.04
0.02
0.00
Weight (g)
C57Bl/6J n=18
Balb/cJ n=10

C

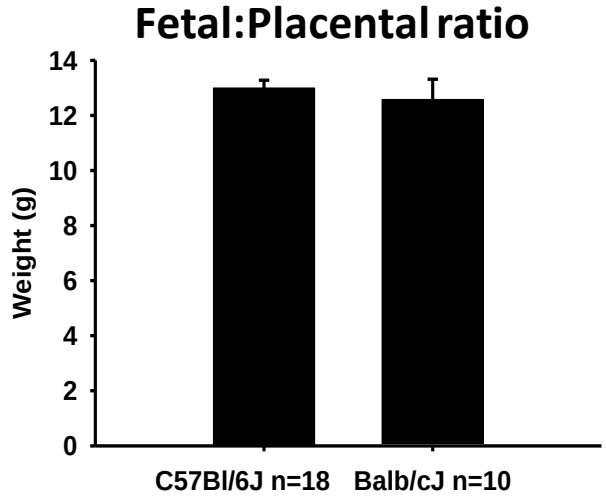

Fetal:Placental ratio
14
12
10
8
6
4
2
0
Weight (g)
C57Bl/6J n=18
Balb/cJ n=10

Figure 7.2: Fetal weights, placental weights, and fetal to placental ratios from LP (D18.5) C57Bl/6J and Balb/cJ uninfected mice. Weights were recorded for fetuses **(A)** and placentas **(B)** from C57Bl/6J (n=119, 18 litters) and Balb/cJ (n=69, 10 litters) uninfected mice. The fetal to placental ratio **(C)** was measured directly from the paired fetal and placental weights. Data were summarized and compared as in Figure 7.1.

	Uninfected C57Bl/6J	mCMV-infected C57Bl/6J	Uninfected Balb/cJ	mCMV-infected Balb/cJ *
Successful pregnancies (>D18.5)	10 / 10	10 / 10	10 / 10	0 / 15

*** One presumed pregnant was sacrificed on D15 of gestation with 7 resorptions out of 8 fetuses.**

Table 7.1: Number of successful pregnancies from mCMV-infected and uninfected C57Bl/6J and Balb/cJ mice following a vaginal plug. Although mCMV-infected Balb/cJ mice could be plugged, no pregnancies were successfully carried to term. In one presumed pregnant mouse sacrificed at D15, 7 of 8 fetuses had been resorbed (*). C57Bl/6J mice were infected with 10^6 PFU of mCMV. Balb/cJ mice were infected with 10^6 PFU (5 animals), $5x10^5$ PFU (5 animals), and 10^5 PFU (5 animals) of mCMV.

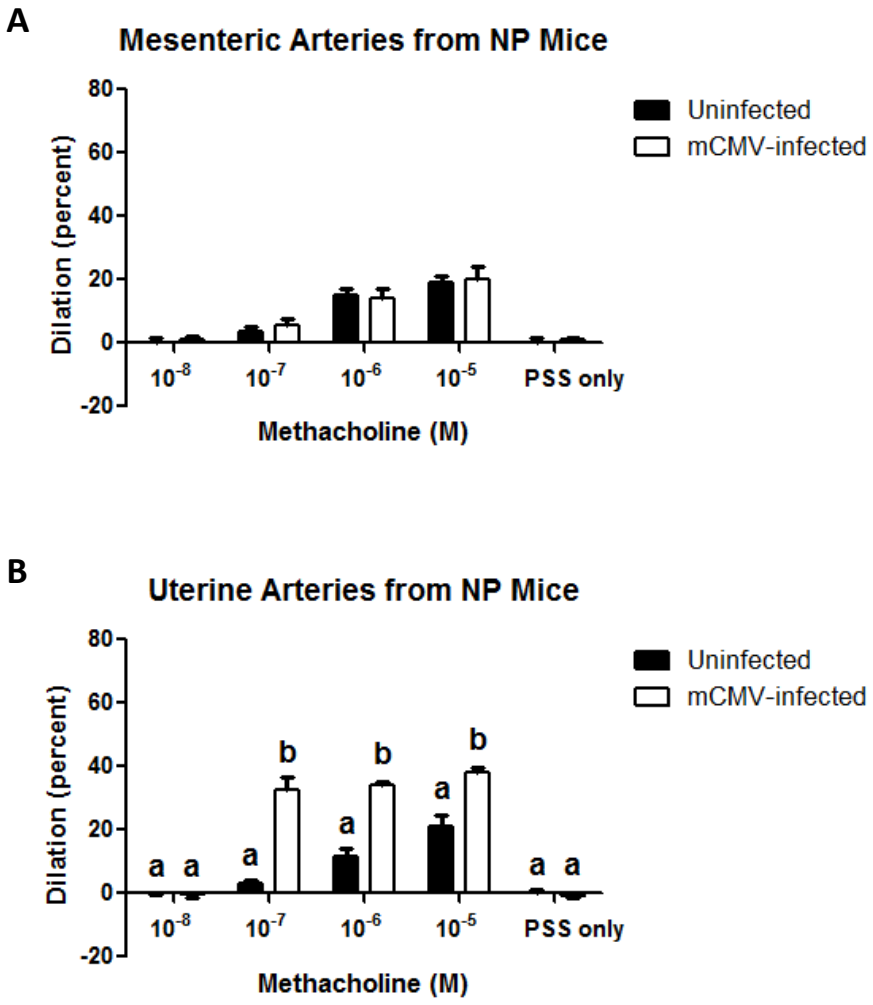


Figure 7.3: Vasodilation induced by infused ME in NP Balb/cJ mice. Mesenteric **(A)** and uterine **(B)** arteries were isolated from mCMV-infected (10^5 PFU) and uninfected mice. Data were expressed as the mean ± SEM percent increase in arterial lumen diameter compared to the passive lumen diameter in the presence of Ca^{2+}-free medium and papaverine. A significant difference between groups at each dose was determined by two-way ANOVA and Bonferroni's post-hoc analysis and symbolized with an a or b ($p<0.05$). n=3-4 animals.

7.4 DISCUSSION

To determine if the vascular dysfunction seen during an active maternal mCMV infection in pregnancy is associated with any adverse pregnancy outcomes, I measured the pregnancy success rates and placental and fetal weights in two different mouse strains. The C57Bl/6J strain was used for my previous vascular experiments (Chapters 3-6) (Gombos *et al.*, 2010; Gombos *et al.*, 2009) and is genotypically more resistant to CMV infections. The Balb/cJ strain, on the other hand, is genotypically more susceptible to CMV infections. Both strains were used to assess and compare the pregnancy outcomes caused by a maternal CMV infection in hosts that vary in genotype (Geist *et al.*, 2001; Mercer *et al.*, 1986). Genotype variations that determine susceptibility to CMV include major histocompatibility complex loci differences (Kekik *et al.*, 2009; Mercer *et al.*, 1986), cytokine expression (Geist *et al.*, 2001), and allelic differences in innate immune response genes (Lathbury *et al.*, 1996; Lee *et al.*, 2001; Scalzo *et al.*, 2007; Scalzo *et al.*, 2005).

My results in Chapter 7 demonstrate that the maternal immune genotype susceptibility to a CMV infection affects the outcome of pregnancy. There was no effect on fetal weights in these mCMV-infected LP C57Bl/6J mice; however, the fetal to placental ratio was increased suggesting increased placental efficiency in nutrient and oxygen transport. Differences in placenta structure were further investigated in Appendix B. In contrast, mCMV-infected LP Balb/cJ mice, which are more susceptible to a mCMV infection, were unable to carry their pregnancies to term. These differences between mouse strains may reflect genotypic disparity in the human population as observed in opposing studies on CMV-associated pregnancy complications (Conde-Agudelo *et al.*, 2008; Gambarotto *et al.*, 1997; Grahame-Clarke, 2005; von Dadelszen *et al.*, 2003). Pregnant women genotypically more resistant to CMV infections may be able to compensate for maternal vascular dysfunction and prevent potential fetal complications, whereas other more susceptible women may be prone to more severe pregnancy complications, such as preeclampsia, IUGR, and intrauterine fetal death.

Previous increased ME-induced vasodilation and decreased PE-induced vasoconstriction responses in mesenteric arteries from mCMV-infected LP C57Bl/6J mice (Chapter 3) (Gombos *et al.*, 2009) suggested that perfusion pressure was reduced in these mice, which may result in IUGR. Conversely, in this study I showed that the fetal weights are unaffected in mCMV-infected LP C57Bl/6J mice. Although IUGR is not always seen with reduced placental perfusion during gestational hypertension in humans (Xiong *et al.*, 2002), it is important to note that I have not assessed uterine artery blood flow *in vivo* to directly measure uteroplacental perfusion in this mouse model. In addition, I have only measured vascular reponses to a limited number of individual vasoactive agents. It is possible that the overall vascular responses to several circulating vascular mediators in systemic and uterine vascular beds *in vivo* may actually not affect uteroplacental perfusion in mCMV-infected LP C57Bl/6J mice.

It remains unknown what causes infertility in the mCMV-infected Balb/cJ strain. HCMV infections have been associated with infertility (Kapranos *et al.*, 2003; Tanaka *et al.*, 2006; Yang *et al.*, 1995); however, direct links between CMV and infertility have not been drawn. Severity of viral-associated diseases and/or viral titre can be significantly increased during pregnancy, including CMV (Gould *et al.*, 1980; Weinberg, 1984). In pregnant CMV-infected guinea pigs, there is a higher death rate and more severe CMV-associated symptoms, such as splenomegaly, as compared to NP due to a delayed immune response (Griffith *et al.*, 1983). Impaired pregnancy-associated vascular adaptations and reduced placental perfusion are also clearly associated with pregnancy loss (Norwitz *et al.*, 2001). None of the mCMV-infected Balb/cJ mice were able to carry their fetuses to D17.5/18.5, and therefore measuring vascular function in arteries isolated from mCMV-infected LP Balb/cJ mice was not possible. Although there were no vasodilation response differences between mCMV-infected NP C57Bl/6J and Balb/cJ mice (see also Chapter 6), major CMV susceptibility differences between these strains, including exacerbated vascular dysfunction, may be more apparent

during early or mid-pregnancy. Therefore it is important to determine at which stage pregnancies fail in mCMV-infected Balb/cJ mice.

Although impaired vascular function early in gestation may contribute to infertility and loss of pregnancy, there may be several other causes for infection-associated pregnancy problems that depend on the stage that pregnancy is lost (Roupa *et al.*, 2009; Weinberg, 1984). I observed one mouse at D15 with 7 of 8 resorptions. Another four mice I observed at mid-gestation following a vaginal plug had no evidence of a pregnancy. Therefore, problems with pregnancy may occur as early as fertilization or implantation or during later stages of placental and fetal development (Roupa *et al.*, 2009). In humans, diagnosis of a congenital infection has been associated with inadequate fetal development and fetal demise (Gaytant *et al.*, 2003; Yinon *et al.*, 2010). In mCMV-infected pregnant mice where CMV does not cross the placenta, congenital infections are not possible (Johnson, 1969). Gharavi et al. showed that the antibody response to a phospholipid-binding CMV-derived peptide during early to mid-pregnancy caused endothelial thrombus formation and fetal loss in Balb/c mice (Gharavi *et al.*, 2004). These data along with my results indicate that fetal developmental problems may arise with a maternal CMV infection in the absence of a congenital infection in early to mid-gestation following implantation.

In addition to maternal vascular dysfunction, my results show that an active maternal CMV infection has placental and fetal effects. Differences in pregnancy outcomes were clearly demonstrated between the C57Bl/6J and Balb/cJ mouse strains. Each strain has different variations of specific immune response genes that determine how susceptible they are to a CMV infection. Therefore, disparities in pregnancy outcomes in the presence of a HCMV infection (independent of a congenital infection) may occur based on a woman's immune genotype. Further research into these CMV-associated placental and fetal effects is required to determine how a maternal CMV infection may cause such pregnancy complications.

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Chapter 8
General Discussion

8.1 PROLOGUE

CMV infections have been associated with vascular diseases in both the general and pregnant population (Espinola-Klein *et al.*, 2002; Grahame-Clarke, 2005; Petrakopoulou *et al.*, 2004); however, a causal relationship between HCMV and vascular dysfunction has yet to be drawn. Most of the research that has studied the effects of CMV in pregnancy has focused on congenital infections that directly cause fetal and postnatal developmental complications, such as microcephaly and sensorineural hearing loss (Yinon *et al.*, 2010). Nevertheless, due to the lack of routine maternal screening for CMV, the effects of a maternal CMV infection on the maternal vascular function and the potential impact of this on fetal development, independent of a congenital infection, remain unknown (Hagay *et al.*, 1996; Munro *et al.*, 2005; Revello *et al.*, 2002).

My results have demonstrated that in agreement with my overall hypothesis, an active CMV infection affects systemic and uterine vascular function prior to and during pregnancy. Placental and/or fetal development are also negatively affected by a CMV infection, particularly in a mouse strain more genotypically susceptible to CMV. This chapter will summarize the most significant findings in all five data chapters (Chapters 3-7), discuss the clinical relevance of these findings, and outline my recommended future directions.

8.2 OVERVIEW AND SUMMARY

In Chapter 3 (Gombos *et al.*, 2009), my results were somewhat surprising based on my initial hypothesis that an active *in vivo* CMV infection would reduce vasodilation of isolated arteries to the endothelium-dependent vasodilator ME and increase vasoconstriction to the α_1-adrenergic receptor agonist PE added

extraluminally, and that these CMV-induced changes would be accentuated in late pregnancy. Contrary to my hypothesis, decreased sensitivity to PE and increased endothelium-dependent vasodilation were found in mesenteric arteries from mCMV-infected compared to uninfected NP and LP mice. Similarly, increased vasodilation was found in uterine arteries from infected versus uninfected NP mice. However, in agreement with my hypothesis, this was completely reversed in mCMV-infected compared to uninfected LP mice where sensitivity to ME in uterine arteries was significantly reduced. Uterine arteries from infected LP mice also showed increased vasoconstriction to PE. These novel data demonstrate abnormal systemic and uterine vascular responses during an active CMV infection in both NP and LP states and suggest that there may be compensatory mechanisms in arteries from CMV-infected NP mice to counteract reported increases in blood pressure (Cheng *et al.*, 2009) that are lacking in LP mice.

In an attempt to understand the mechanisms for these vascular changes in cholinergic endothelium-dependent vasodilation seen in mesenteric and uterine arteries from mCMV-infected NP and LP mice (Chapter 3), I measured the contribution of NO, prostanoids, and smooth muscle cell sensitivity to NO to vasodilation induced by extraluminally applied ME in these arteries in Chapter 4 (Gombos *et al.*, 2010). I found that the contribution by NO to ME-induced vasodilation was significantly increased in mesenteric, but not uterine, arteries isolated from mCMV-infected NP and LP mice. Prostanoid inhibition did not affect endothelium-dependent vasodilation in any group. Vasodilation responses to the NO donor SNP were increased in mesenteric and uterine arteries isolated only from mCMV-infected NP mice. These results explain the increased vasodilation responses observed in mesenteric arteries from mCMV-infected mice; however, the decreased sensitivity to ME in uterine arteries from LP mice could not be explained by these mechanisms. Thus, CMV infection affects the contribution of NO differently in endothelium-dependent vasodilation in LP compared to NP mice and also in the mesenteric compared to the uterine vascular bed.

Cholinergic stimulation plays an important role in the regulation of vascular tone; however, I was also interested in how other circulating mediators that induce endothelium-dependent vasodilation may be affected by pregnancy, in the presence or absence of an active CMV infection. In Chapter 5, I studied the vascular effects of S1P, a bioactive sphingolipid that can induce either vasodilation or vasoconstriction depending on which vascular cells it interacts with. To do this, I developed a technique using the pressure myograph system that enabled me to assess vascular responses to reagents that directly interact with the endothelium. In intact pressurized arteries, I showed that infusion of low S1P concentrations led to vasodilation in uterine and mesenteric arteries that was abolished at higher concentrations. This was altered in pregnancy to favour increased vasodilatory capacity, suggesting an important role for S1P in pregnancy-induced vascular adaptations. Infection with mCMV disrupted S1P signaling in uterine arteries and reduced vasodilation in mesenteric arteries from NP and LP mice. Therefore, while cholinergic endothelium-dependent vasodilation was increased in mesenteric and uterine arteries in the presence of a CMV infection (Chapters 3,4) (Gombos *et al.*, 2010; Gombos *et al.*, 2009), other vascular mediators such as S1P can reduce endothelium-dependent vasodilation during a CMV infection.

Although I showed that mesenteric and uterine vascular function was affected by an active CMV infection in NP and LP mice (Chapters 3-5) (Gombos *et al.*, 2010; Gombos *et al.*, 2009), it remained unknown how direct virus to endothelium interactions contributed to altered vascular responses in comparison to a systemic *in vivo* infection (Chapter 6). In Chapter 6, I measured the vascular responses in arteries isolated from mice infected with mCMV *in vivo* and compared them to responses in arteries isolated from uninfected mice but treated with gB, a necessary glycoprotein involved in CMV attachment and entry (Lopper *et al.*, 2002). To do this, I infused gB or CMV inside arteries to directly interact with the endothelium. Following this pre-treatment, I infused ME into the arteries rather than added it extraluminally as was done in Chapters 3 and 4 (Gombos *et*

al., 2010; Gombos *et al.*, 2009). In mesenteric arteries, neither gB pre-treatment nor an *in vivo* CMV infection significantly affected vasodilation to infused ME, unlike the increased vasodilation responses to extraluminally added ME in mesenteric arteries from mCMV-infected mice observed in Chapter 3. These opposing results are likely due to different drug and receptor interactions with extraluminal versus intraluminal addition of ME (see Figure 6.5). Nevertheless, I demonstrated increased TXA_2/PGH_2-mediated vasoconstriction in the presence of CMV attachment alone or in the presence of a fully systemic infection that reduced ME-induced vasodilation, in accordance with Chapter 4. In uterine arteries from uninfected NP mice, the sensitivity to infused ME was significantly increased by both gB and mCMV pre-treatment but this increase was less than that found in uterine arteries from *in vivo* mCMV-infected NP mice. Maximal vasodilation to infused ME was also significantly increased in uterine arteries from *in vivo* mCMV-infected NP mice not seen with gB or mCMV pre-treatment of uterine arteries from uninfected NP mice. However, sensitivity and maximal vasodilation to infused ME was significantly reduced only in uterine arteries from *in vivo* mCMV-infected LP mice. Together, these results demonstrate that attachment or early entry of CMV in endothelial cells affects endothelium-dependent vasodilation in the absence of a fully systemic CMV infection. Nevertheless, at least in uterine arteries, a full infection exacerbates these vascular responses, possibly through inflammatory mediators generated during an activated inflammatory response.

In summary of Chapters 3-6, mesenteric arteries have a reduced sensitivity to α_1-adrenergic receptor agonists and a decreased vasodilation response to infused S1P. Furthermore, NOS-2 expression, NO contribution, and smooth muscle cell sensitivity to NO are increased upon cholinergic stimulation, likely as a compensatory mechanism for increased vasoconstrictory prostanoid synthesis in these mesenteric arteries from mCMV-infected NP mice (Figure 8.1). This increase in vasoconstrictory prostanoid modulation of cholinergic vasodilation, also seen in mesenteric arteries from mCMV-infected LP mice, occurs as early as

CMV/gB cell attachment or early entry. Although mesenteric arteries from mCMV-infected LP mice respond to agonists similarly to mCMV-infected NP mice for the most part, there are a few differences. In addition to NOS-2 protein expression, NOS-3 mRNA expression is increased in mesenteric arteries from mCMV-infected LP mice whereas smooth muscle sensitivity to NO is unaffected compared to uninfected LP mice (Figure 8.1).

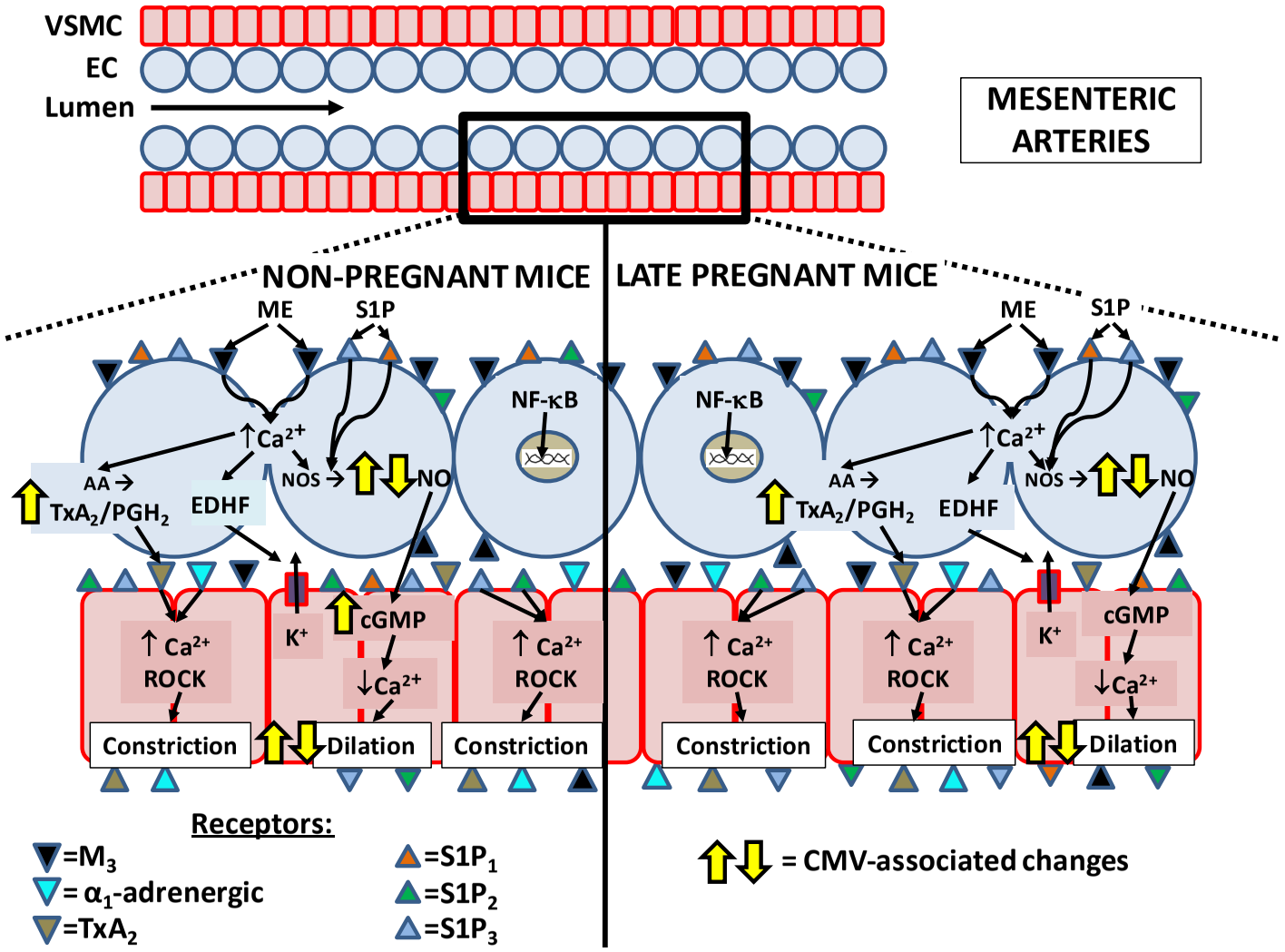
VSMC
EC
Lumen
MESENTERIC ARTERIES
NON-PREGNANT MICE
LATE PREGNANT MICE
ME
S1P
NF-κB
↑Ca2+
AA →
TxA2/PGH2
EDHF
NOS →
NO
↑ Ca2+ ROCK
K+
cGMP
↓Ca2+
Constriction
Dilation
Receptors:
=M3
= α1-adrenergic
=TxA2
=S1P1
=S1P2
=S1P3
= CMV-associated changes

Figure 8.1: Schematic representation of affected signalling pathways in a mesenteric artery from a mCMV-infected NP or LP mouse. S1P-induced vasodilation mediated by $S1P_{1/3}$ and NO is decreased in mesenteric arteries from mCMV-infected compared to uninfected NP and LP mice. In contrast, regulation of vasodilation induced by extraluminally or intraluminally applied ME showed that NO and TxA_2/PGH_2 production is increased in mesenteric arteries from mCMV-infected NP and LP mice. Further, vasodilation induced by extraluminally applied ME is increased in mesenteric arteries from mCMV-infected mice whereby NO-mediated vasodilation masks the TxA_2/PGH_2-mediated constriction effects. All enzymes/pathways affected by a CMV infection are illustrated by a yellow arrow. The receptors on the endothelial cells (EC) and vascular smooth muscle cells (VSMC) involved in the signalling pathways I studied are also shown. ME: methacholine; S1P: sphingosine 1-phosphate; AA: arachidonic acid; TxA_2: thromboxane; PGH_2: prostaglandin H_2; NOS: nitric oxide synthase; NO: nitric oxide; EDHF: endothelium-derived hyperpolarizing factor; cGMP: cyclic guanosine monophosphate; ROCK: Rho kinase; M_3: muscarinic 3 receptor.

The impact of a mCMV infection on vascular responses in uterine arteries differs from that found in mesenteric arteries. In mCMV-infected NP mice, vasodilation is increased in response to cholinergic stimulation which is likely due to an increase in smooth muscle cell sensitivity to NO. Although NOS-1 expression is also increased in these arteries, increased NO or prostanoid synthesis does not contribute to this increased cholinergic endothelium-dependent vasodilation. This suggests a reduction in the bioavailability of NO, possibly due to increased oxidative stress and peroxynitrite formation (see section 8.4). CMV/gB cell attachment or early entry increases sensitivity to the cholinergic agonist ME; however, a full infection and/or indirect mediators such as an activated inflammatory response exacerbate this response. S1P-induced vasoconstriction is also decreased in these arteries from mCMV-infected NP mice whereby ROCK activity is decreased and signalling through $S1P_1$ and/or $S1P_3$ is

downregulated (Figure 8.2). In uterine arteries from mCMV-infected LP mice, on the other hand, S1P-induced vascular responses are unaffected whereas cholinergic vasodilation is reduced and vasoconstriction to α_1-adrenergic receptor agonists is increased. However, NO and prostanoid contribution to cholinergic vasodilation and smooth muscle sensitivity to NO are unaffected by a mCMV infection in uterine arteries from LP mice; therefore, this change in vasodilation is likely due to EDHF (Figure 8.2). Unlike the increased sensitivity to the cholinergic agonist ME in uterine arteries from NP mice, gB cell attachment/early entry does not affect cholinergic vasodilation in uterine arteries from uninfected LP mice. Hence in LP mice, a full infection is required for endothelium-dependent vascular dysfunction to occur.

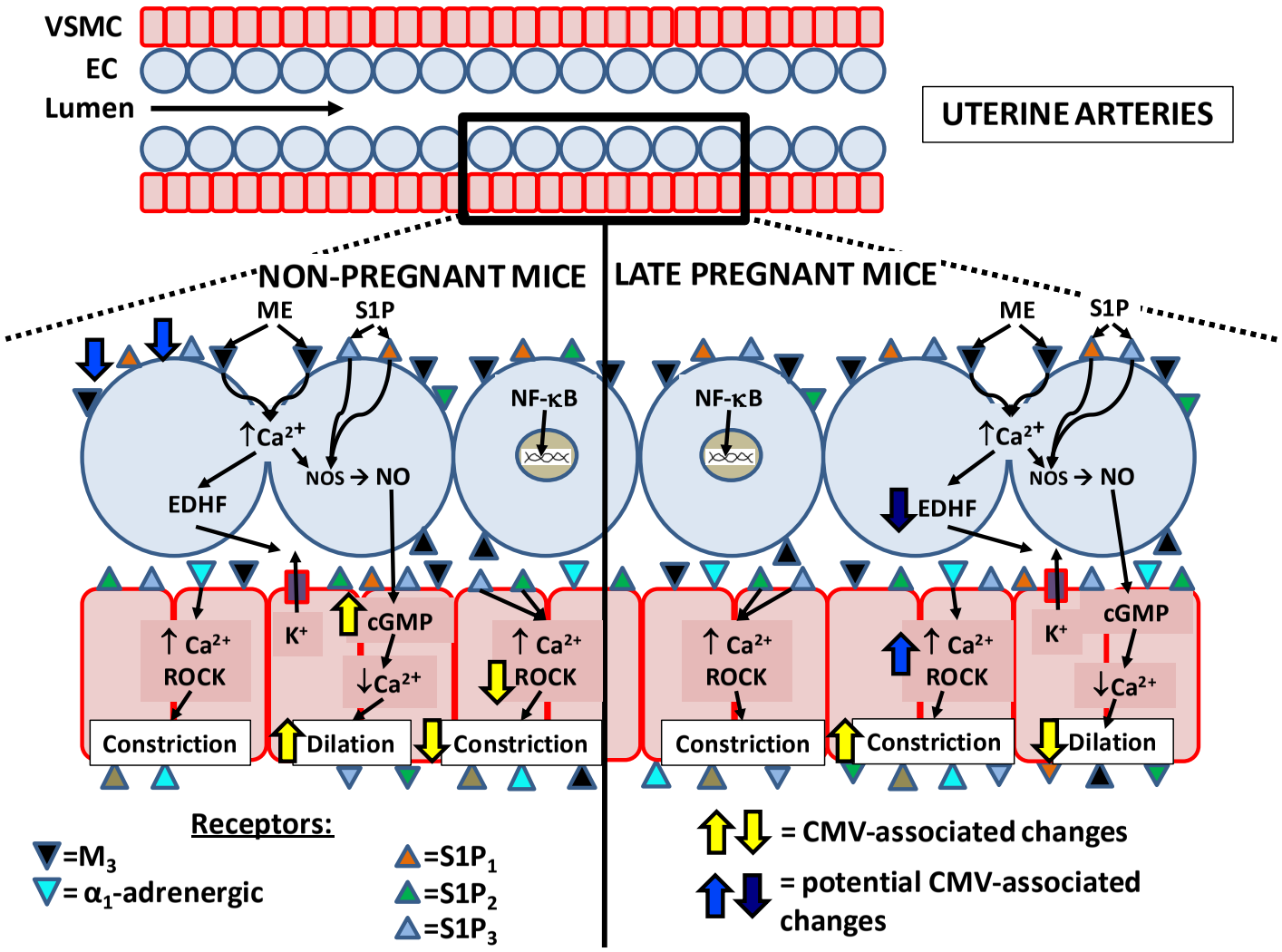
VSMC
EC
Lumen
UTERINE ARTERIES
NON-PREGNANT MICE
LATE PREGNANT MICE
ME
S1P
NF-κB
↑Ca2+
NOS → NO
EDHF
↑ Ca2+
ROCK
K+
cGMP
↓Ca2+
Constriction
Dilation
Receptors:
=M3
= α1-adrenergic
=S1P1
=S1P2
=S1P3
= CMV-associated changes
= potential CMV-associated changes

Figure 8.2: Schematic representation of affected signalling pathways in a uterine artery from a mCMV-infected NP or LP mouse. $S1P_{1/3}$ regulation of S1P-induced vasodilation is lost in uterine arteries from mCMV-infected compared to uninfected NP mice. S1P-induced vasoconstriction, partially mediated by Rho kinase (ROCK), is decreased in uterine arteries from mCMV-infected mice. ME-induced vasodilation and sensitivity to ME (muscarinic receptor-specific) is also increased in uterine arteries from mCMV-infected NP mice. It is partially mediated by increased vascular smooth muscle cell (VSMC) sensitivity to NO. ME-induced vasodilation is decreased in uterine arteries from mCMV-infected LP mice likely by reduced EDHF. PE-induced vasoconstriction is also increased in uterine arteries from mCMV-infected LP mice. All enzymes/pathways affected by a CMV infection are illustrated by a yellow arrow. The receptors on the ECs and VSMCs involved in the signalling pathways I studied are also listed. ME: methacholine; S1P: sphingosine 1-phosphate; NOS: nitric oxide synthase; NO: nitric oxide; EDHF: endothelium-derived hyperpolarizing factor; cGMP: cyclic guanosine monophosphate; M_3: muscarinic 3 receptor.

Finally, my findings in Chapter 7 demonstrate that although fetal growth is unaffected in LP mice more immunologically resistant to CMV, mice more genotypically susceptible to a CMV infection are infertile. The fetal to placental ratio, and likely placental efficiency, is increased in mCMV-infected mice that are more resistant to CMV, suggesting that they are able to compensate for adverse effects associated with a CMV infection better than those genotypically more susceptible to CMV.

8.3 CONCLUSIONS AND CLINICAL RELEVANCE

In conclusion, I have shown that an active CMV infection affects systemic and uterine vascular function in both LP and NP states and pregnancy outcome, independent of a congenital infection. Although my studies have been performed in the mouse model, vascular dysfunction seen during an active mCMV infection has also been associated with HCMV pathogenesis (Dal Canto *et al.*, 2000). If an active HCMV infection affects normal vascular function during human pregnancy, as I have demonstrated in mice, it is a potential risk factor for vascular complications such as preeclampsia and IUGR, independent of direct fetal effects such as microcephaly, hepatosplenomegaly (Lazzarotto *et al.*, 2000). Genotypically susceptible HCMV-infected women may also be at a higher risk of negative placental and fetal developmental effects, as previously suggested (Carreiras *et al.*, 2002) and corroborated by my findings (Chapter 7). Consequently, my research supports the notion that routine maternal screening for CMV in early and mid-pregnancy should be implemented.

Screening for an active CMV infection, prior to or in early pregnancy, as a risk factor for vascular and fetal complications may enable doctors to prevent and/or treat them. Treatments may include low-dose aspirin (Garovic, 2000), ganciclovir (Spector *et al.*, 1996), and/or gB (vasodilatory effects demonstrated in Chapter 6). Although the efficacy of low-dose aspirin for pregnancy-related vascular complications is controversial, it may be more effective with CMV-associated vascular dysfunction during pregnancy (Barth, 1998; Beilin, 1994; Speir *et al.*, 1998). Further research into the precise mechanisms affected by a CMV infection during pregnancy will also help identify other potential therapeutic targets and treatments.

A CMV-specific vaccine given prior to pregnancy may also reduce the prevalence of acquiring an active CMV infection during pregnancy. Nevertheless, development of an effective CMV vaccine is still in clinical trials. The viral coat protein, gB, is a prime candidate for HCMV vaccine development studies and trials (Bernstein *et al.*, 2002; Pass, 2009; Pass *et al.*, 2002; Pass *et al.*, 2009). My

results support its use as a vaccine given that I demonstrated that gB pre-treatment does not negatively affect mesenteric or uterine cholinergic endothelium-dependent vasodilation in pregnancy and further, may actually be beneficial in NP individuals as it leads to increased sensitivity to cholinergic stimulation. However, these results were obtained using the mouse model and, therefore, must be repeated with human arteries to confirm the vascular effects and potential benefits of gB pre-treatment (section 8.4).

Lastly, using the pressure myograph system I was also able to differentiate between agonist-induced vascular effects caused by direct endothelial versus smooth muscle cell interactions. My findings highlight the important roles these agonists play in vascular function and generate novel insight into how vascular tone is regulated in LP compared to NP states. Understanding how this regulation is disrupted under pathological conditions in humans will contribute to the development of new therapeutic tools targeted toward CMV-associated vascular complications, not only in the pregnant but also in the general population.

8.4 LIMITATIONS AND FUTURE DIRECTIONS

My vascular function experiments have focused on only some vascular mediators including the α_1-adrenergic agonist PE, TxA_2/PGH_2 mimetic U46619, cholinergic agonist ME, NO donor SNP, peptide bradykinin, and the bioactive sphingolipid S1P. Furthermore, agonists that did not have CMV-induced differential vascular responses in murine mesenteric and/or uterine arteries from my study (ie. bradykinin) may affect responses in other blood vessel types, including veins (Inacio *et al.*, 1997), or vascular beds, such as the placenta (Kenny *et al.*, 2002). Cheng et al. have recently shown that the expression of angiotensin II, a vasoconstrictor, is significantly increased in aortic tissue from mCMV-infected mice (Cheng *et al.*, 2009). Grahame-Clarke et al. have also demonstrated that vasodilation responses to both the endothelium-independent vasodilator glyceryl trinitrate and peptide bradykinin are reduced in forearm

arteries from CMV-seropositive individuals (Grahame-Clarke *et al.*, 2003). In addition, in my studies the contribution of NO and prostanoids to the vascular responses stimulated by the cholinergic agonist ME or S1P were investigated in mesenteric and uterine arteries from mCMV-infected and uninfected mice. Mechanistic analysis has yet to be completed for other vascular agonists. Determining the intracellular signalling pathways for each vascular agonist affected by an active mCMV infection may deduce common players as therapeutic targets for CMV-associated diseases. Therefore, further vascular function studies that determine the mechanistic responses for a wider range of agonists in blood vessels isolated from both humans and animal models are necessary.

The inhibitors used in my experiments were used to determine the general signal transduction pathways affected by an active CMV infection. As such, several of them were not specific for a single protein. L-NAME blocks NOS activity as an L-arginine analogue but does not distinguish between NOS-1/2/3 (Moncada *et al.*, 1997). Meclo is a competitive inhibitor for both PGHS-1/2 (Laneuville *et al.*, 1994; Smith *et al.*, 1994). VPC23019 is a competitive antagonist for both $S1P_1$ and $S1P_3$ (Davis *et al.*, 2005). To determine which isoforms and intracellular signalling pathways are mainly responsible for the vascular effects, vascular function studies should now be repeated with specific isoform inhibitors that are available, such as the PGHS-2 selective inhibitor NS398 (Futaki *et al.*, 1994), the SK-1 inhibitor SKI-II (Yan *et al.*, 2008), the soluble guanylyl cyclase inhibitor ODQ (Hwang *et al.*, 1998), or specific potassium channel blockers apamin and TRAM-34 (Morton *et al.*, 2010). In my experiments, SQ29548, the TxA_2 receptor antagonist, was used in addition to meclo to help deduce the specific prostanoids involved in vascular responses; however, this antagonist is unable to differentiate between TxA_2 versus PGH_2 agonistic effects (Vezza *et al.*, 2002). Addition of a specific TxA_2 synthase inhibitor, such as ozagrel (Nakazawa *et al.*, 1994; Virdis *et al.*, 2007), in combination with the TxA_2 receptor antagonist will be very useful in

differentiating between TxA_2- or PGH_2-induced vasoconstriction (Gresele *et al.*, 1991). Specific NOS isoform inhibitors, such as with the competitive inhibitors 7-nitroindazole for NOS-1 and 1400W for NOS-2, have also been used in some studies; however, their specificity has been questioned, particularly when used at higher concentrations (Arias-Salvatierra *et al.*, 2011; Babbedge *et al.*, 1993; Garvey *et al.*, 1997; Moore *et al.*, 1996; Rickard *et al.*, 1999). Moreover, although specific S1P receptor antagonists have become more commercially-available over the last few years (Jongsma *et al.*, 2006), knockout animals will be another useful resource. Studying vascular responses in CMV-infected and uninfected NP and LP knockout animals, including NOS-$1^{-/-}$, NOS-$2^{-/-}$, NOS-$3^{-/-}$, $S1P_2^{-/-}$, and $S1P_3^{-/-}$ mice ($S1P_1^{-/-}$ is lethal (Kono *et al.*, 2004)), in combination with specific inhibitors will help establish the specific effects of these enzymes and receptors on systemic and uterine vascular function.

Active CMV infections and cardiovascular diseases, such as atherosclerosis and hypertension, have been associated with increased ROS production within the vascular wall, potentially via NOS uncoupling (Cai *et al.*, 2000; Landmesser *et al.*, 2003; Miller *et al.*, 1998; Speir *et al.*, 1996; Speir *et al.*, 1998). Increased ROS, specifically superoxide, may interact with NO released from NOS-1/2/3 to produce peroxynitrite, thereby reducing the bioavailability of NO (Szabo, 1996). As such, even if similar amounts of NO are being produced in CMV-infected and uninfected individuals, the amount of NO available to interact with the vascular smooth muscle cells to induce vasodilation may be significantly reduced in CMV-infected individuals. My immunofluorescence results demonstrated that an active mCMV infection causes an increase in NOS-1/2 protein and NOS-3 mRNA expression in mesenteric and/or uterine arteries. In uterine arteries, I also showed that modulation of vasodilation by changes in NO production did not significantly differ from mCMV-infected and uninfected mice, even in the presence of increased NOS-1 expression. This suggests there is reduced NO bioavailability in uterine arteries from mCMV-infected mice. Although I have shown that increased NO production contributes to increased

ME-induced vasodilation in mesenteric arteries from mCMV-infected mice, this does not rule out the possibility that basal NO bioavailability is reduced in the presence of a mCMV infection. Davidge et al. previously showed that the antioxidant superoxide dismutase (reduces superoxide levels) increases NO-dependent vasorelaxation in mesenteric arteries from vitamin E-deficient compared to control rats albeit ME-induced vasodilation was not different between these groups. Thus, superoxide-induced NO inactivation (and peroxynitrite formation), though overcome by agonist-induced NO release, is still very apparent (Davidge *et al.*, 1998). Similar to my results in mesenteric arteries from mCMV-infected mice, Davidge et al. also found that in mesenteric arteries from vitamin E-deficient rats PGHS-dependent production of the vasoconstrictory prostanoid(s) that binds the PGH_2/TxA_2 receptor is increased following ME stimulation (Davidge *et al.*, 1993). Therefore, PGH_2/TxA_2 production may be favoured in the presence of peroxynitrite in mesenteric arteries from both vitamin-E deficient and CMV-infected rodent models. This is supported by the fact that peroxynitrite can increase PGHS-1/2 activity (Landino *et al.*, 1996) and inhibit PGI_2 synthase activity (Zou *et al.*, 1997; Zou *et al.*, 1999). Together, this evidence illustrates the importance of measuring NO bioavailability and the level of oxidative and nitrative stress in arteries isolated in mCMV-infected mice as ROS play an important role in vascular function. This may include measuring peroxynitrite, nitrotyrosine (formed by peroxynitrite), or other ROS with specific markers (Carter *et al.*, 1994; Collins, 2005; Roggensack *et al.*, 1999; Roychowdhury *et al.*, 2002; Wang *et al.*, 1996), measuring tetrahydrobiopterin levels (necessary NOS cofactor that prevents uncoupling) (Landmesser *et al.*, 2003), or using an antioxidant such as superoxide dismutase to determine its reversal effect on vasodilation in arteries from mCMV-infected mice (Davidge *et al.*, 1998).

My intraluminal results suggest that muscarinic receptors are increased and $S1P_1$ and/or $S1P_3$ are decreased on endothelial cells in uterine arteries from mCMV-infected NP mice. In addition, my results demonstrating different

vascular responses following extraluminal versus intraluminal ME or S1P addition suggest differential receptor expression on the apical and basolateral membranes of endothelial cells (ie. muscarinic receptors) or between endothelial and smooth muscle cells (ie. $S1P_{1-3}$ receptors) in arteries from mCMV-infected mice. It will be very important to measure the expression and localization of these receptors in these vascular cells to confirm these functional findings. Due to the differences between vascular beds, this technique requires one to isolate enough of the endothelial and smooth muscle cells specifically from murine mesenteric and uterine arteries to quantify receptor expression and localization differences for each cell type. Therefore, an appropriate isolation technique must be established with a high yield of these cells, such as laser capture microdissection (Frye *et al.*, 2002; Harris *et al.*, 2008; Moss *et al.*, 2007). This will also be useful to determine the receptor effects of a CMV infection on other arteries not specific to the murine model.

Most of my studies have focused on vascular responses in the presence of a CMV infection. This highlights some important questions: Does CMV have direct vascular effects? How big a role does the immune response play in these vascular responses? Are these vascular effects specific to a CMV infection or can they be attributed to other Herpes virus infections? Although I have measured some of the direct attachment/entry effects of CMV on the vasculature, the direct vascular effects of CMV replication and/or infection within endothelial and vascular smooth muscle cells remain undefined. Moreover, which immune response mediators affect vascular responses during a CMV infection are also unknown. TNF-α and IFN-γ are two such mediators with known vascular effects that are upregulated during an active CMV infection (Boyle *et al.*, 1999; Gamadia *et al.*, 2003; Geist *et al.*, 1994; Sprague *et al.*, 2009; Tesauro *et al.*, 2008). It will be very interesting to compare cytokine-induced vascular responses in arteries from uninfected hosts to the vascular responses observed in arteries from a CMV-infected host and determine how much of the vascular dysfunction can be attributed to these cytokines. Finally, though CMV is the major Herpes virus

associated with vascular diseases, other Herpes viruses with conserved, homologous proteins, such as gB, may also contribute to vascular dysfunction (Norais *et al.*, 1996). Herpes Simplex virus (HSV) increases platelet and peripheral blood mononuclear cell adhesion to vascular endothelial cells (Scheglovitova *et al.*, 2001; Visser *et al.*, 1988), suggesting a role for HSV in endothelial dysfunction. In addition, vascular diseases such as cardiac allograft vasculopathy (Bowles *et al.*, 2001; Shirali *et al.*, 2001; Valantine, 2004) and atherosclerosis (Alber *et al.*, 2000) have also been associated with the presence of Herpes viruses other than CMV. This suggests that other viruses may also affect vascular responses; however, direct evidence of this has not yet been demonstrated.

Placental function and fetal development are broad research areas and my thesis has only begun to exhibit how a CMV infection affects them. Based on an increased fetal to placental ratio in mCMV-infected C57Bl/6J mice, preliminary results suggest that placental efficiency is increased. Although I showed that fetal weights were unaffected in these resistant mCMV-infected LP mice (likely due to increased placental efficiency), placental viral infection and over-compensation can increase maternal susceptibility to a second hit (ie. bacterial infection). This second hit can lead to more severe pregnancy complications (Cardenas *et al.*, 2011). A technique has been developed and is now being widely employed that measures solute transport across murine placentas into the fetus as a direct measurement of placental efficiency (Angiolini *et al.*, 2006; Constancia *et al.*, 2005). This technique will be extremely useful as a confirmation of my findings and will help determine if placental function should be investigated further. Additional perinatal studies on mCMV-infected Balb/cJ mice are also required. They are much more susceptible to mCMV infections and understanding how and when a mCMV infection causes pregnancy loss will be very important for understanding potential placental and/or fetal effects of a maternal CMV infection. This should include studying groups of mCMV-infected Balb/cJ mice at each day of pregnancy following vaginal plugging to determine at what stage

pregnancy is affected. Establishing the time-frame for when mCMV-infected LP Balb/cJ mice are affected will help deduce potential mechanisms disrupted by an active maternal mCMV infection, such as implantation, placentation, or angiogenesis (Norwitz *et al.*, 2001), and may highlight therapeutic areas to target.

Though I have shown that vascular responses are affected by a CMV infection, I have not yet shown how this may pertain to vascular function *in vivo*. Cheng et al. showed that blood pressure in mice infected with a mCMV infection is increased with angiotensin II levels (Cheng *et al.*, 2009); however, I found that endothelium-dependent vasodilation is increased in mesenteric and uterine arteries from mCMV-infected NP mice. This may be explained as a compensatory mechanism for increased vasoconstriction to mediators, such as TxA_2, PGH_2, or angiotensin II *in vivo*. It remains unknown how a CMV infection affects blood pressure and/or blood flow regulation *in vivo* during pregnancy. I demonstrated that endothelium-dependent vasodilation in uterine arteries is decreased while TxA_2/PGH_2 modulation of vasodilation in mesenteric arteries is increased in mCMV-infected LP mice. This suggests that mCMV-infected LP mice are more susceptible to increased blood pressure and hypertension than NP mice but has yet to be confirmed *in vivo*. Moreover, in addition to evaluating the vascular effects of an acute, active CMV infection in mice at the reproductive stage, the mouse is a good model to study how a chronic CMV infection affects the vascular function over time (Appendix C). As such, *in vivo* vascular experiments will also be important to determine if and how a mCMV infection may contribute to vascular dysfunction and diseases with age, such as hypertension. Techniques that may be used to measure blood pressure in mice include the tail cuff method or implanted telemetry monitoring systems (Kurtz *et al.*, 2005). The blood pressure measurements achieved with the tail cuff method are highly variable due to the effects of stress induced by confining mice in small cylinders to obtain a blood pressure reading. In addition, the equipment required to measure blood pressure by tail cuff cannot be taken into and used in the biocontainment animal facility where the mCMV-infected mice are housed. Therefore, telemetry is the preferred

method of choice. However, implanting telemetry systems into mice is a very expensive surgical technique which may affect the ability of these animals to become pregnant or maintain their pregnancies (Braga *et al.*, 2009; Kurtz *et al.*, 2005). Blood flow measurements will also be important to determine how the vascular tone of peripheral blood vessels is affected *in vivo*, such as with Doppler ultrasonic velocity measurements (Hartley *et al.*, 2011). Understanding what happens in animal models *in vivo* is critical to further elucidate how a CMV infection contributes to vascular diseases in humans.

Lastly, basic research with clinical application should always lead back to the human situation. In addition to my studies in the mouse model, there are several human studies that should be performed to establish the relevance of my findings to the human population. Human studies may include *ex vivo* and *in vivo* vascular experiments performed on a sample population of women who are positive or negative for an active HCMV infection. An active infection can be confirmed by the presence of CMV DNA in the saliva and urine in addition to seropositivity for IgG and IgM CMV antibodies. Similar to the vascular studies performed in mice, vascular *ex vivo* experiments using different agonists and inhibitors on human blood vessels obtained during surgery (ie. Caesarean sections, cardiac surgeries, or abdominal surgeries), such as placental and myometrial arteries (Hudson *et al.*, 2007), mammary arteries (Henrion *et al.*, 1998), omental arteries (Vedernikov *et al.*, 1999), and mesenteric arteries (Dimitrova *et al.*, 2010), will be important to determine how vascular tone and intracellular signalling pathways are affected by an active HCMV infection. This is not only important in pregnant women but also in NP HCMV-infected individuals who are at risk of HCMV-associated vascular diseases (ie. immunocompromised or aged populations; see Appendix C). Furthermore, blood pressure (ie. sphygmomanometry) and blood flow measurements (ie. Doppler ultrasonography) in active HCMV-infected compared to uninfected NP and LP women will be important to determine the risk of vascular dysfunction and disease in a genotypically diverse population (Naslund *et al.*, 2006; Pickering *et*

al., 2005). Plethysmography is an additional non-invasive technique used to measure changes in arterial blood volume and vascular function among human sample groups that will be useful in establishing the *in vivo* vascular effects of an HCMV infection (Grahame-Clarke *et al.*, 2003; Naslund *et al.*, 2006). Results obtained from *in vivo* animal and human studies in addition to my *ex vivo* functional experiments will help identify important similarities to HCMV-associated effects and potential ways of treating HCMV-associated diseases.

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Appendix A

Role of NF-κB in Cholinergic Vasodilation in Uterine Arteries from Cytomegalovirus-infected Mice

A1.1 INTRODUCTION

NF-κB is a transcription factor that is stimulated upon gB attachment to the cell (Compton *et al.*, 2003). Previously, I observed an increase in sensitivity to infused intraluminal ME in gB- and infectious mCMV-treated uterine arteries from uninfected NP mice and a further increase in arteries from mCMV-infected NP mice. However, in uninfected LP mice, sensitivity to ME is similar in gB-treated and untreated uterine arteries. In contrast, ME-induced vasodilation is significantly decreased in uterine arteries from mCMV-infected LP mice (Chapter 6). It is unknown if NF-κB plays a role in vascular responses in gB-treated or untreated uterine arteries from uninfected NP and LP mice or in uterine arteries from mCMV-infected NP and LP mice. I show that blocking NF-κB translocation decreases vasodilation to infused ME in uterine arteries from all NP and LP groups except uterine arteries from mCMV-infected LP mice (remains unchanged). However, it remains unknown how NF-κB translocation affects the cholinergic pathway.

A1.2 MATERIAL AND METHODS

A1.2.1 Intraluminal pressure myograph experimental design

The same infusion technique was used as described in Chapter 2 (section 2.5.1). To determine the role of NF-κB activation in vasodilation to infused ME, some uterine arteries pre-treated or untreated with gB from uninfected NP and LP mice and some uterine arteries from mCMV-infected NP and LP mice were incubated in the bath with the cell-permeable, selective NF-κB inhibitor JSH-23 (20μM; EMD Biosciences) for 30 minutes (Ogawa *et al.*, 2008; Sandberg *et al.*,

2009). It has been clearly demonstrated previously that JSH-23 (IC_{50}: 7.1μM) inhibits the nuclear translocation and thus transcriptional activity of NF-κB without affecting degradation of IκB, the endogenous NF-κB inhibitor (Shin *et al.*, 2004). The percent dilation was calculated as L2-L1/L1 x 100; where L1 is the initial lumen diameter after flow into the PSS with or without gB pre-treatment and L2 is the lumen diameter after ME infusion. The percent dilation was then normalized to the passive lumen diameter as described in section 2.2.3.

A1.2.2 Statistics

As described in section 2.4.2, intraluminal pressure myograph results with ME ± JSH-23 were averaged and compared between groups (mCMV-infected and uninfected ± gB) using a one-way ANOVA followed by Bonferroni's *post-hoc* analysis to determine significance ($p<0.05$).

A1.3 RESULTS

A1.3.1 Vasodilation to infused ME and the role of NF-κB in uterine arteries

Increased ME-induced vasodilation in uterine arteries from mCMV-infected NP mice and gB-treated uterine arteries from uninfected NP mice was blocked by pre-treatment with the NF-κB inhibitor JSH-23 (Figure A1.1A). JSH-23 inhibits nuclear translocation of NF-κB without affecting degradation of IκB, the endogenous NF-κB inhibitor (Shin *et al.*, 2004). In contrast, reduced ME-induced vasodilation in uterine arteries from mCMV-infected LP mice was not changed in the presence of JSH-23; however, vasodilation in gB-treated and untreated uterine arteries from uninfected LP mice was partially reduced by JSH-23 (Figure A1.1B).

A1.4 SUMMARY OF RESULTS

A1.4.1 Blocking NF-κB translocation inhibited increased ME-induced vasodilation in gB-treated uterine arteries from uninfected NP mice and in uterine arteries from mCMV-infected NP mice (Figure A1.1A).

A1.4.2 Blocking NF-κB translocation also decreased ME-induced vasodilation in gB-treated and untreated uterine arteries from uninfected LP mice (Figure A1.1B).

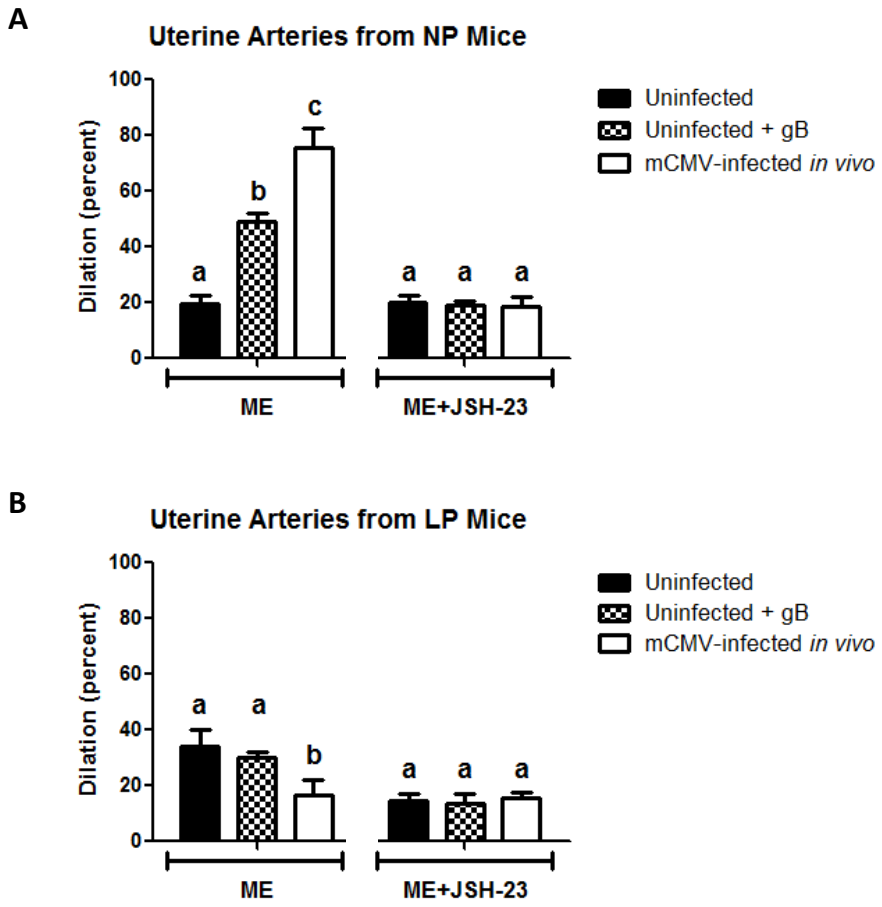

Figure A1.1: Vasodilation of uterine arteries induced by infusion of ME (1μM) or ME (1μM) with JSH-23 (20μM). Arteries were isolated from uninfected NP **(A)** and LP **(B)** mice and treated with or without 1μg/mL gB for 30 minutes to 1 hour or were isolated from mCMV-infected NP and LP mice. ME data were taken from the curves in Figure 6.3. Data were expressed as the mean ± SEM percent increase in arterial lumen diameter compared to the passive lumen diameter in the presence of Ca^{2+}-free medium and papaverine. A significant difference between the bars in each pre-treatment group was determined by one-way ANOVA and Bonferroni's post-hoc analysis and symbolized with an a or b ($p<0.05$). n=4-9 animals.

A1.5 DISCUSSION

NF-κB, a transcription factor that promotes the expression of several pro-inflammatory and some anti-inflammatory cytokines, such as TNF-α, IFN-γ, interleukin-2, interleukin-10, and the CMV immediate early genes (Cao *et al.*, 2006; Cookson *et al.*, 2010; Lee *et al.*, 2004; McCracken *et al.*, 2004; Prosch *et al.*, 1995), is activated upon attachment of CMV to toll-like receptors on the cell surface (Compton *et al.*, 2003; Fortunato *et al.*, 2000; Murayama *et al.*, 1997). In addition, NF-κB translocation occurs following activation of the cholinergic muscarinic 3 (M_3) receptor (Choi *et al.*, 2006; Guizzetti *et al.*, 2003). In uterine arteries, M_3 is the primary cholinergic receptor responsible for vasodilation (Jovanovic *et al.*, 1994; Pesic *et al.*, 2009). My results demonstrate that an increase in ME-induced vasodilation in uterine arteries from mCMV-infected NP mice is blocked by inhibiting NF-κB translocation. I also showed that the increased ME sensitivity seen with gB pre-treatment of uterine arteries from uninfected mice is blocked by inhibiting NF-κB translocation. It has recently been illustrated that NF-κB is necessary for the expression of the M_3 in pancreatic islets (Paula *et al.*, 2010) and a specific NF-κB DNA-binding site has been found on the human M_3 gene (Forsythe *et al.*, 2002). However, the short time period (~1 hour) for the increased vasodilation I see following ME infusion argues against an NF-κB-mediated increase in M_3 expression. Alternatively in the presence of a CMV infection and/or viral attachment, the cholinergic pathway (identified as a host-defence mechanism in immune cells to prevent excessive inflammation), may signal through NF-κB in uterine endothelial cells and indirectly affect other intracellular mediators of vascular tone (Pavlov *et al.*, 2006; Saeed *et al.*, 2005; Tracey, 2002). For instance, Arias-Salvatierra et al. have shown that NF-κB activation following LPS treatment in neurons induces iNOS-mediated NO production (Arias-Salvatierra *et al.*, 2011). This may also explain the increased uterine artery vasodilation seen in my study whereby a mCMV infection or gB pre-treatment in addition to ME addition may lead to NF-κB translocation and

iNOS stimulation. Further studies must be performed to determine how NF-κB translocation inhibition affects other signalling molecules that mediate vascular responses.

Throughout pregnancy, it has been demonstrated that NF-κB expression, and the levels of the inflammatory mediators it promotes, are suppressed prior to term as a means of regulating the maternal immune system (Hadfield *et al.*, 2011; McCracken *et al.*, 2004). As the uterus and myometrium prepare for labour at term, inflammation and contraction-associated proteins are significantly increased. In association, NF-κB activity and inflammatory gene expression are upregulated in the myometrium prior to the onset of labour at term (Choi *et al.*, 2007; Khanjani *et al.*, 2011). I observed that inhibition of NF-κB activity blocked the increased ME-induced vasodilation in gB-treated and untreated uterine arteries from LP uninfected mice, suggesting the increased vasodilation is dependent on NF-κB activity. Surprisingly, in the presence of an active mCMV infection during late pregnancy, ME-induced vasodilation was significantly reduced in the uterine artery and no longer affected by blocking NF-κB. This suggests that unlike NP mice, LP mCMV-infected mice are unable to increase cholinergic vasodilation during gestation. Consequently, an active maternal mCMV infection may cause uterine-specific vascular complications during pregnancy that may contribute to IUGR (Lang *et al.*, 2003).

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Appendix B

Placental Structural Differences in Cytomegalovirus-infected Compared to Uninfected Late Pregnant Mice

B2.1 INTRODUCTION

To determine if there are any obvious structural differences in placentas taken from mCMV-infected compared to uninfected LP C57Bl/6J mice, frozen placentas are stained with hematoxylin and eosin. I do not see any obvious structural or pathological differences; however, these results are preliminary and further studies must be performed to confirm these findings.

B2.2 MATERIAL AND METHODS

B2.2.1 Placenta structure

Cross-sections of fixed, frozen placentas taken from CMV-infected and uninfected D18.5 pregnant mice were sliced on a cryostat and mounted onto microscope slides as described in Chapter 2 (section 2.2.1). Slides were fixed in 10% formalin for 10 minutes in the fume hood. Slides were then placed in Gill-3 hematoxylin (Sigma-Aldrich) for 2 minutes, rinsed with double-distilled (dd)H_2O and washed in warm tap water for 3-5 minutes. Slides were then placed in 1% Eosin Alcohol (Sigma-Aldrich) for 5 minutes and washed in ddH_2O. Slides were dipped 20 times each in 70% EtOH, 95% EtOH, 100% EtOH followed by 3 times in Xylene. Permount (Sigma-Aldrich) was used to mount the cover slip. Slides were sealed with a clear nail polish. Qualitative observations of the placenta structure and general physiology were performed using the light microscope.

B2.3 RESULTS

B2.3.1 Placenta structural/pathological differences from mCMV-infected and uninfected C57Bl/6J mice

Using hematoxylin and eosin to stain for DNA and protein, respectively, I observed no obvious structural or pathological differences in mouse placenta cross-sections. There were more arteries in the placenta cross-sections from mCMV-infected mice (Figure B2.1); however, differences in vascularization can only be concluded after full placenta casts and appropriate analyses have been performed (Whiteley *et al.*, 2006).

B2.4 SUMMARY OF RESULTS

B2.4.1 There were no obvious structural or pathological differences in placentas from mCMV-infected and uninfected C57Bl/6J mice (Figure B2.1).

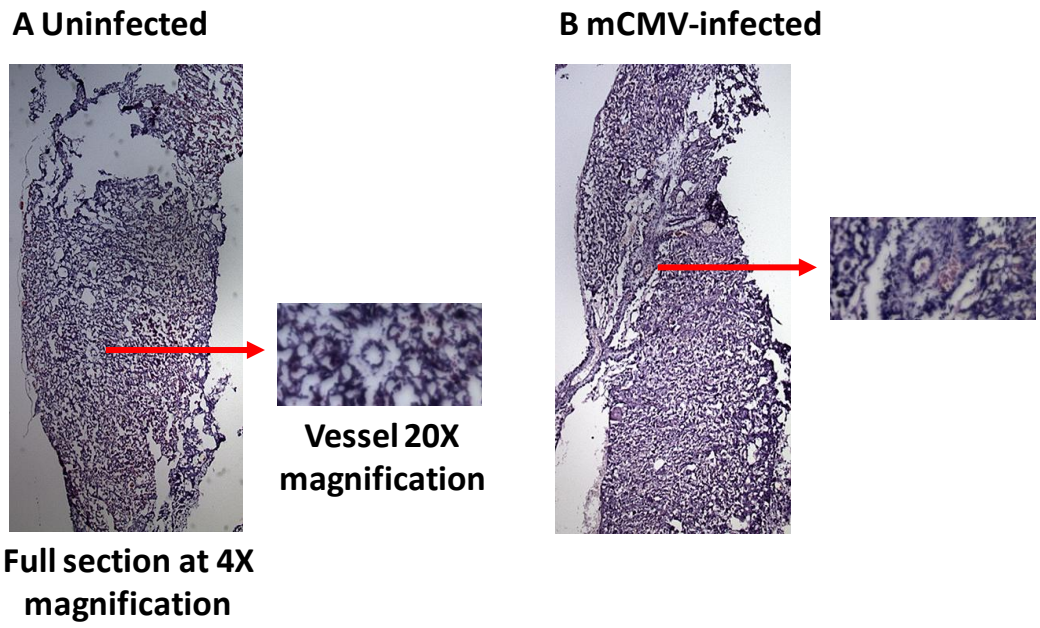

Figure B2.1: Hematoxylin and eosin staining of placental sections from mCMV-infected and uninfected C57Bl/6J mice. Red staining is protein and blue staining is DNA. No differences in physiology or structure. These images are representative of different sections taken from 3 mice in each group (n=3).

B2.5 REFERENCES

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Appendix C

Vascular Dysfunction in Young, Mid-aged and Aged Mice with Latent Cytomegalovirus Infections

(The majority of data presented in this appendix will be submitted for publication in December 2011 in an article entitled "Vascular Dysfunction in Young, Mid-aged and Aged Mice with Latent Cytomegalovirus Infections," by R.B. Gombos, J. Brown, J. Teefy, R.L. Gibeault, K.L. Conn, L.M. Schang, D.G. Hemmings. The majority of results in each of the figures I performed.)

C3.1 INTRODUCTION

Human cytomegalovirus (HCMV) is a member of the *β-Herpesviridae* family (Roizmann *et al.*, 1992). In the human population, it infects 40 to 80 percent of individuals (Demmler, 1991). Although in the general population it is thought to be mostly asymptomatic, infection has been associated with tumorigenesis, latent rejection following transplants, vascular diseases such as atherosclerosis and restenosis, and increased mortality (Cobbs *et al.*, 2002; Hirabayashi *et al.*, 2003; Kalil *et al.*, 2009; Maussang *et al.*, 2006; Melnychuk *et al.*, 2005). The mechanisms by which HCMV infections result in vasculopathy are still unclear.

The prevalence of systemic cardiovascular diseases increases dramatically with age, particularly after age 50 (2007; Chow *et al.*, 2005; Mittelmark *et al.*, 1993). Increased vascular tone, endothelial dysfunction, and hypertension occur with age (Cooper *et al.*, 1994). Moreover, vasodilation is decreased and vasoconstriction is increased. NO production, bioavailability and smooth muscle sensitivity to it typically decrease whereas PGHS-1/2 expression and production of vasoconstrictor prostanoids is increased with age (Karaki *et al.*, 1985; Matz *et al.*, 2000; Smith *et al.*, 2006a; Smith *et al.*, 2006b; Soucy *et al.*, 2006). Infertility also greatly increases as women age, where between 30 to 45 years of age the

fertility rate drops by greater than 30% (Heffner, 2004). Potential causes of reduced fertility prior to menopause include chromosomal abnormalities, hormonal imbalances, uterine and ovarian malformations and hypertensive complications; all of which increase with age (Heffner, 2004; Roupa Z, 2009). Hypertensive complications, such as increased vascular resistance (Greene *et al.*, 1989) and decreased endothelium-dependent vasodilation (Cardillo *et al.*, 1998) may be increased in HCMV-infected individuals (Grahame-Clarke, 2005).

Like other herpesviruses, CMV is never cleared from the host following primary infection. It rather maintains a lifelong infection through cycles of latency (dormancy) and reactivation (Presti *et al.*, 1998). Latency is defined as the presence of viral DNA in the absence of infectious, replicating virus (Presti *et al.*, 1998). Reactivation of a CMV infection, and thus production of infectious, replicating virus, occurs in different tissues in response to various stimuli including stress, drug treatments and pregnancy (Cheung *et al.*, 2007; Prosch *et al.*, 2000; Tanaka *et al.*, 1983). The number of reactivation cycles an individual undergoes in a lifetime is difficult to estimate because of the many stimuli that likely contribute to viral reactivation. Viral replication and the subsequent immune responses are likely necessary for the acceleration and/or establishment of vasculopathy (Lemstrom *et al.*, 1997; Tu *et al.*, 2006) and atherosclerosis (Grahame-Clarke, 2005). Previously, I have shown that an active mCMV infection impairs both mesenteric and uterine artery responses in both young NP and LP mice (Gombos *et al.*, 2009). What is not yet known is whether these vascular changes are maintained once the active infection becomes latent.

During a lifelong CMV infection, periods of latency where infectious virus is undetectable are intermittent with periods of activation (Presti *et al.*, 1998). Whether or not vascular dysfunction persists when infectious virus is undetectable is unknown. To evaluate this, I measured vasoconstriction and vasodilation responses in mesenteric and uterine arteries isolated from uninfected young, mid-aged and aged female mice compared to age-matched mice with a latent mCMV infection where infectious virus was undetectable but mCMV DNA was present.

My objectives were to investigate the effect of a latent CMV infection on vascular function and fertility with age. I therefore hypothesize that mesenteric and uterine arteries from uninfected aged mice will have a greater reduction in NO-mediated vasodilation compared to arteries from uninfected young or mid-aged mice. I further hypothesize that the oldest animals infected with CMV at a young age will experience the greatest vascular dysfunction compared to age-matched controls and all other animal groups. In addition, uterine arteries from mid-aged but not young latent infected mice will show decreased vasodilation compared to age-matched uninfected controls and this will be associated with increased infertility.

C3.2 MATERIAL AND METHODS

C3.2.1 Animal model and tissue collection

C57B1/6J female mice purchased from Jackson Laboratories were housed in Health Sciences Laboratory Services at the University of Alberta. They were infected with mCMV containing a lacZ insertion in the nonessential immediate early 2 gene (RM427+; gift from E. Mocarski, Stanford University, Stanford, CA) (Stoddart *et al.*, 1994). RM427+ was propagated in mouse fibroblasts as previously described (Gombos *et al.*, 2009). An active mCMV infection was detected by staining for β-galactosidase protein, expressed from the lacZ insertion. Young female mice were injected with 10^6 plaque forming units of RM427+ intraperitoneally and euthanized via cervical dislocation within 1-2 weeks post infection (acute mCMV infection) or 2-3 months post infection (latent mCMV infection). Young infected and uninfected mice ranged from 4-6 months of age. Latent mCMV-infected and uninfected mid-aged mice (7-10 months of age) were euthanized 5-8 months post infection and latent mCMV-infected and uninfected aged mice (18-30 months of age) were euthanized 16-28 months post infection. Some latent mCMV-infected and uninfected mid-aged female mice were bred to young uninfected males. Tissues including the heart, kidney, liver, lungs, spleen, uterus and the mesentery were collected from each animal. A latent

infection was defined as the presence of viral DNA in the lung, spleen, or kidney as assessed by PCR but the absence of detectable infectious mCMV as assessed by lack of detection of β-galactosidase protein established in each animal a minimum of two months after receiving 10^6 plaque forming units of mCMV intraperitoneally.

C3.2.2 Immunofluorescence

The heart, kidney, liver, lungs and spleen were immediately embedded in OCT (Tissue-Tek) and snap frozen. Tissues in OCT were cryo-sectioned into 7μm slices, mounted onto slides, dried overnight, and stored at -80°C. Slides were thawed for 1-2 hours at RT, fixed in cold methanol for 10 minutes at -20°C, and washed with phosphate-buffered saline (PBS) three times for 10 minutes each. Tissue sections on the slides were circled with a PAP pen and blocked with 10% normal goat serum (NGS; Cedarlane) for 1 hour. After blocking, chicken anti-β-galactosidase primary antibody (1μg/mL; AbCam, Massachusetts, USA) diluted in 10% NGS was added to two of the three sections on the slide, with the remaining section receiving only 10% NGS as the negative control. The slides were incubated at 4°C overnight. Following three 5-minute washes with PBS, Alexa Fluor-488 goat anti-chicken (4μg/mL) secondary antibody was added to each section and incubated for 45 minutes at room temperature. After three 10-minute washes in PBS, 4',6-diamidino-2-phenylindole (DAPI, 0.915mg/mL; Invitrogen) was added for 15 minutes at room temperature and washed again three times for 5 minutes each with PBS. Vectashield H:1000 (Vector Laboratories, Burlington, CA) was applied and the section was then sealed with a coverslip and stored in the dark at 4°C. Stained sections were viewed with an Olympus X81 fluorescent microscope (Olympus, Ontario, Canada) using Slidebook 2, 3D Timelapse Imaging Software to normalize the images (Intelligent Imaging Innovations Inc., Colorado, USA).

C3.2.3 Extraluminal pressure myograph experimental design

The second-order mesenteric and main uterine arteries were dissected free of adipose and connective tissue in PSS (10mM HEPES, 1.56mM $CaCl_2$, 142mM NaCl, 4.7mM KCl, 1.18mM KH_2PO_4, 1.17mM $MgSO_4$, and 5.5mM glucose at pH 7.5) and immediately used in myograph studies using a dual chamber arteriograph (Living Systems Instrumentation, Burlington, VT). In each chamber, one end of an artery was mounted and tied onto a glass cannula (80-100μm diameter) connected to a pressure transducer to modulate intraluminal pressure, as previously described (Halpern *et al.*, 1984; Hemmings *et al.*, 2004). Residual blood was removed from the artery with a low flow (10μL/min) of PSS and the other end of the vessel was mounted and tied onto the second cannula. Pressure was maintained by closing off the valve after pressurizing to 60mmHg (mesenteric arteries) or 50mmHg (uterine arteries) for 30 minutes (Gros *et al.*, 2002; Osol *et al.*, 2008; Veerareddy *et al.*, 2002). Arteries unable to maintain pressure were discarded and not included in the data sets. In this situation, new arteries from the same animal were mounted. Each 2.5mL PSS bath containing the vessel was kept at a constant temperature of 37°C.

Following equilibration, a CCD video camera module (Sony) connected to a compound microscope was used to measure the initial lumen diameter with a video dimension analyzer as previously described (Gombos *et al.*, 2010; Halpern *et al.*, 1984). Vasoconstriction in mesenteric and uterine arteries was assessed following increasing concentrations of the α1 adrenergic agonist PE (10nM to 10μM; Sigma). Vasodilation was assessed to increasing concentrations of ME (1nM to 10μM; Sigma) after 50% preconstriction to PE with or without pre-treatment with the NOS inhibitor L-NAME (100μM; Calbiochem) and/or the PGHS-1/2 inhibitor meclo (1μM; Sigma). Endothelium-independent dilation was assessed by adding the NO donor, SNP (0.1nM to 100μM, Sigma) to preconstricted arteries. The percent constriction was calculated as 1-L2/L1 x 100; where L1 is the initial lumen diameter and L2 is the arterial lumen diameter following drug addition. The percent dilation was calculated as L2-L1/L1 x 100

which was normalized to the artery diameter when fully relaxed. This was attained by measuring the passive lumen diameter following a thorough washout with Ca^{2+}-free EGTA PSS (10mM HEPES, 142mM NaCl, 4.7mM KCl, 1.18mM KH2PO4, 1.17mM MgSO4, 2mM EGTA) and a ten-minute incubation with 100μM of papaverine (Sigma). After incubation in Ca^{2+}-free EGTA PSS, a distensibility curve was performed on arteries with step increases in pressure from 4mmHg to 170mmHg. The lumen diameter was unable to be read at 0mmHg (collapsed vessel) and therefore pressure readings began at 4mmHg as previously described (Gombos *et al.*, 2010). The percent dilation for distensibility was calculated as D2-D1/D2 x 100; where D1 is the initial diameter and D2 is the lumen diameter post-pressure change.

C3.2.4 Statistics

Results collected were averaged by group (mCMV-infected, uninfected, mesentery and uterine) and treatment and reported as the mean and standard error. Values for the PE, ME and SNP dose-response curves were compared for arteries from mCMV-infected and uninfected mice with the Repeated Measures two-way ANOVA to determine significance between curves ($p<0.05$). This was followed by Holm-Sidak's post-hoc analysis to determine significance between points within the curves. The EC_{50} was calculated for each set of sigmoidal-shaped curves for the uterine arteries. The EC_{50} values for mesenteric arteries could not be calculated as these curves were not sigmoidal. EC_{50s} were compared using a Student's t-test ($p<0.001$; $p<0.05$).

C3.3 RESULTS

C3.3.1 Detection of a reporter of infectious mCMV, β-galactosidase, in young, mid-aged, and aged mice

To evaluate the status of infection in active versus latent infected mice, I assessed β-galactosidase protein in the heart, kidney, liver, lung and spleen.

Detection of β-galactosidase produced from the LacZ gene insertion into the non-essential IE2 gene in the mCMV RM427+ virus represents the presence of active, replicating virus. In contrast, a latent infection was defined as undetectable β-galactosidase protein expression in mCMV-infected mice. In the young, acute mCMV-infected mice, β-galactosidase was detected in all five tissues (active infection) (Figure C3.1A). In contrast, no tissues tested from the uninfected or mCMV-infected young mice 2 months post infection (latent infection) expressed β-galactosidase (Figure C3.1A). Similarly, tissues from mid-aged (Figure C3.1B) or aged (Figure C3.1C) uninfected or latent mCMV-infected mice did not express β-galactosidase.

C3.3.2 Latent mCMV-infected and uninfected mid-aged pregnancy success rate

Latent mCMV-infected and uninfected mid-aged mice were bred. Seven of fifteen uninfected mid-aged mice (46.7%) but only three of eighteen latent mCMV-infected mid-aged mice (16.7%) were plugged and carried their pregnancies to term. This is low in comparison to young active mCMV-infected mice with 90.0% completed pregnancies after plugging (nine of ten) and the young uninfected mice with an 83.3% completed pregnancies after plugging (ten of twelve).

C3.3.3 PE-induced vasoconstriction in arteries from young, mid-aged, and aged latent mCMV-infected and uninfected mice

Vasoconstriction to the α1-adrenergic agonist PE in mesenteric arteries significantly increased ($p<0.05$) in latent mCMV-infected young mice compared to uninfected mice (Figure C3.2A). No differences were seen in mesenteric arteries from latent infected mid-aged and aged mice compared to age-matched uninfected mice (Figure C3.2B,C). Similar to mesenteric arteries, PE-induced vasoconstriction of uterine arteries significantly increased ($p<0.05$) in latent mCMV-infected young mice compared to uninfected young mice (Figure C3.2D).

Overall, PE-induced vasoconstriction was significantly more sensitive ($p<0.001$) between curves in uterine arteries from latent mCMV-infected mid-aged mice (EC_{50}: 88.9±5.82nM) compared to uninfected mid-aged mice (EC_{50}: 706±79.1nM) (Figure C3.2E). PE-induced vasoconstriction did not differ between uterine arteries from latent mCMV-infected and uninfected aged mice (Figure C3.2F).

C3.3.4 Endothelium-dependent ME-induced vasodilation in arteries from young, mid-aged, and aged latent mCMV-infected and uninfected mice

Vasodilation curves significantly differed ($p<0.01$) in mesenteric arteries from young and aged mice; however, vasodilation at specific doses of the endothelium-dependent vasodilator ME in mesenteric arteries from latent mCMV-infected and uninfected mice significantly differed only in mid-aged and aged mice (Figure C3.3A-C). At the maximum ME concentration, vasodilation was significantly decreased ($p<0.05$) in mesenteric arteries from latent mCMV-infected compared to uninfected mid-aged mice. In contrast, maximal ME-induced vasodilation was significantly increased ($p<0.05$) in mesenteric arteries from latent mCMV-infected compared to uninfected aged mice (Figure C3.3B,C). Only uterine arteries from young latent mCMV-infected (EC_{50}: 74.0±2.77nM) compared to young uninfected mice (EC_{50}: 111±7.13nM) showed a significantly increased sensitivity to ME ($p<0.001$) (Figure C3.3D). Overall, ME-induced vasodilation was significantly more sensitive between curves in uterine arteries from young, mid-aged and aged mCMV-infected compared to uninfected mice ($p<0.05$) (Figure C3.3D,E,F); however, uterine arteries from latent mCMV-infected and uninfected mid-aged and aged mice did not differ at specific doses of ME (Figure C3.3E,F).

C3.3.5 Contribution of NO and prostanoids to endothelium-dependent ME-induced vasodilation in arteries from young, mid-aged, and aged latent mCMV-infected and uninfected mice

To determine if the contribution of NO and prostanoids to ME-induced vasodilation is altered with age and latent mCMV infection, I measured the difference in vasodilation with and without NO and/or PGHS inhibitors between latent mCMV-infected and uninfected mice. There were no differences in NO contribution to ME-induced vasodilation in mesenteric arteries from latent mCMV-infected compared to uninfected mice at any age (Figure C3.4A-C,G-I,M-O). In addition, PGHS inhibition did not alter ME-induced vasodilation in mesenteric arteries in any group (Figure C3.4D-F,J-L,P-R). Additionally, simultaneous inhibition of NOS and PGHS-1/2 did not alter ME-induced dilation differently than inhibition of NO alone in any group (data not shown). Similar results were found in uterine arteries (data not shown).

C3.3.6 Endothelium-independent SNP-induced vasodilation in arteries from young, mid-aged, and aged latent mCMV-infected and uninfected mice

Mesenteric arteries from latent mCMV-infected young mice had significantly increased vasodilation to the NO donor SNP at only one physiologically relevant dose, 0.5μM ($p<0.05$) compared to age-matched uninfected mice (Figure C3.5A). Mesenteric arteries from latent mCMV-infected mid-aged mice had significantly decreased SNP-induced vasodilation ($p<0.05$) compared to uninfected age-matched mice (Figure C3.5B). Mesenteric arteries from latent mCMV-infected and uninfected aged mice did not differ in response to SNP (Figure C3.5C). In contrast to mesenteric arteries, uterine arteries from latent mCMV-infected young and mid-aged mice did not differ in response to SNP compared to age-matched uninfected mice (Figure C3.5D,E). In uterine arteries from latent mCMV-infected aged mice, however, SNP-induced dilation was significantly decreased ($p<0.05$) compared to uninfected age-matched mice (Figure C3.5F).

C3.3.7 Distensibility of arteries from young, mid-aged, and aged latent mCMV-infected and uninfected mice

Mesenteric arteries from young latent mCMV-infected had significantly decreased passive distensibility ($p<0.05$) compared to young uninfected mice (Figure C3.6A). Mesenteric arteries from mid-aged and aged latent mCMV-infected mice (Figure C3.6B,C) and uterine arteries from young, mid-aged and aged latent mCMV-infected mice showed no differences in passive distensibility compared to age-matched controls (Figure C3.6D-F).

C3.3.8 Vascular responses in arteries from young, mid-aged, and aged uninfected mice independent of a mCMV infection

Mesenteric and uterine arteries from young, mid-aged and aged uninfected mice did not differ in response to PE-induced vasoconstriction (Figure C3.7A,D). Although mesenteric arteries also did not differ in response to ME (Figure C3.7B), uterine arteries from mid-aged uninfected mice (EC_{50}: 63.4±2.60nM) were significantly more sensitive ($p<0.001$) to ME than both young (EC_{50}: 110±7.13nM) and aged (EC_{50}: 153±16.6nM) uninfected mice (Figure C3.7E). Mesenteric arteries from mid-aged and aged uninfected mice had significantly increased endothelium-independent SNP-induced vasodilation ($p<0.05$) compared to young uninfected mice (Figure C3.7C). Uterine arteries from young, mid-aged and aged uninfected mice did not differ in response to SNP (Figure C3.7F). There were no differences in distensibility for either mesenteric or uterine arteries between any of these age groups (data not shown).

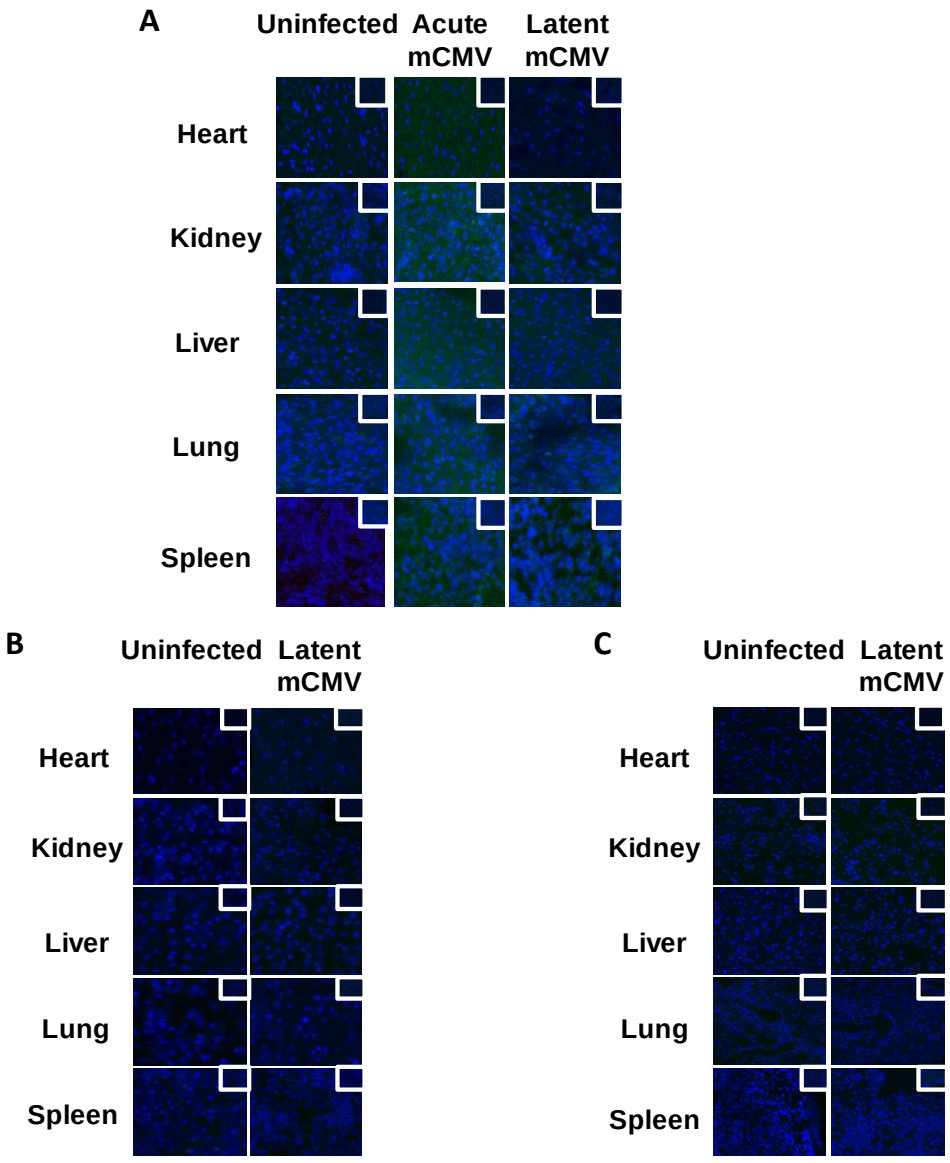


Figure C3.1: Immunofluorescence staining for β-galactosidase protein expression in tissues from mCMV-infected and uninfected young, mid-aged, and aged mice. Heart, kidney, liver, lung and spleen tissues from young **(A)**, mid-aged **(B)**, and aged **(C)** mice that were uninfected or infected at 1-2 months of age were collected 2 weeks post infection **(acute infection; A)** or >2 months post infection **(latent infection; A (young), B (mid-aged), C (aged))**. Tissues were stained for β-galactosidase protein expression (green) to test for an active infection. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue). Insets represent negative controls.

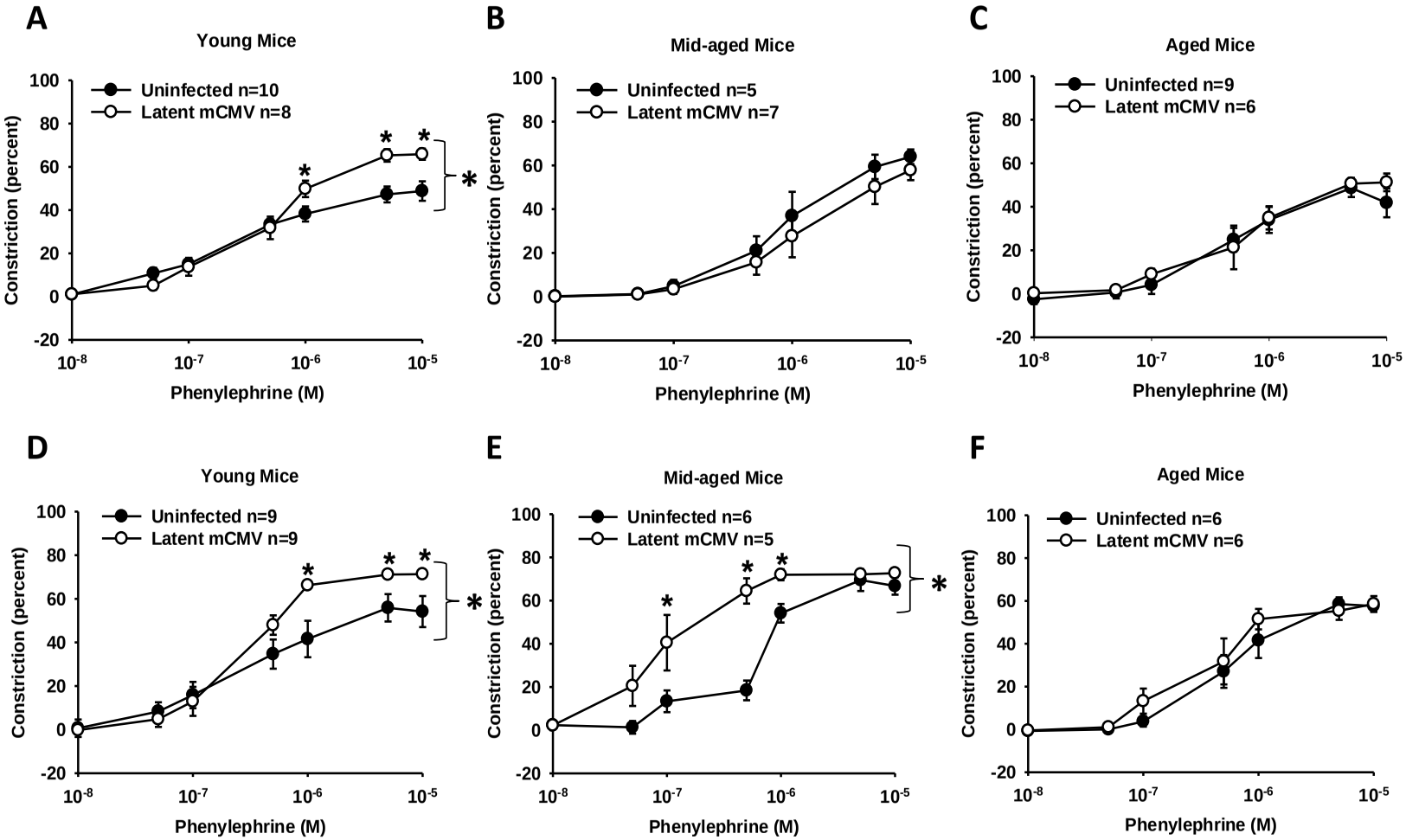

A
Young Mice
Uninfected n=10
Latent mCMV n=8
B
Mid-aged Mice
Uninfected n=5
Latent mCMV n=7
C
Aged Mice
Uninfected n=9
Latent mCMV n=6
D
Young Mice
Uninfected n=9
Latent mCMV n=9
E
Mid-aged Mice
Uninfected n=6
Latent mCMV n=5
F
Aged Mice
Uninfected n=6
Latent mCMV n=6
Constriction (percent)
Phenylephrine (M)
100
80
60
40
20
0
-20
10-8
10-7
10-6
10-5
*

Figure C3.2: Responses to PE by mesenteric and uterine arteries from latent mCMV-infected and uninfected young, mid-aged, and aged mice. PE-induced vasoconstriction was measured in mesenteric **(A,B,C)** and uterine **(D,E,F)** arteries from latent mCMV-infected and age-matched uninfected young **(A,D)**, mid-aged **(B,E)**, and aged **(C,F)** mice. Results for each curve were summarized and expressed as mean ± SEM percent decrease in lumen diameter compared with the initial equilibrated diameter at each PE concentration. A significant difference between points on the curves was calculated using a Repeated Measures two-way ANOVA ($p<0.05$). This was followed by Holm-Sidak's post-hoc analysis to determine significance between points within the curves ($p<0.05$). * = significant differences ($p<0.05$). n = number of animals.

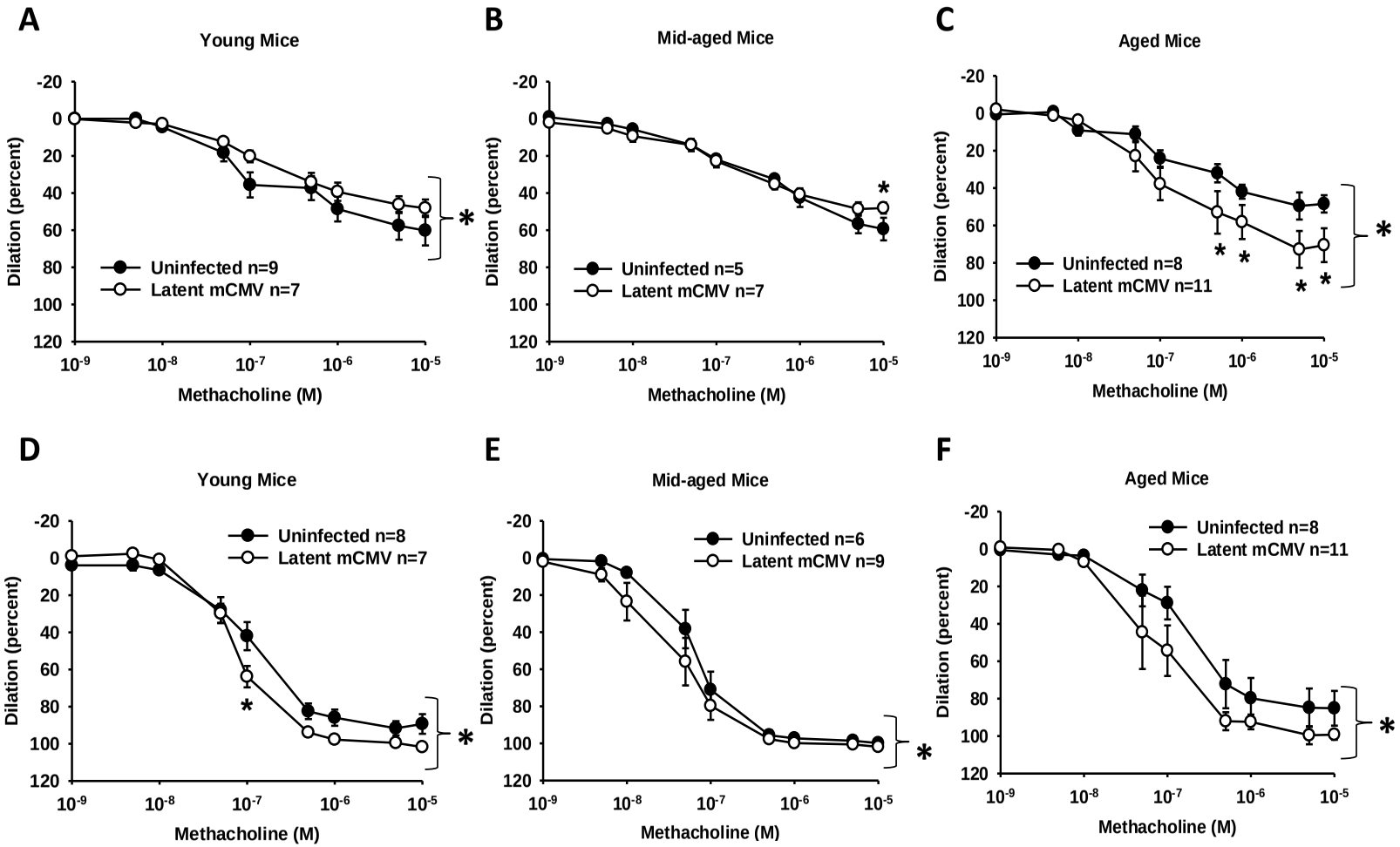
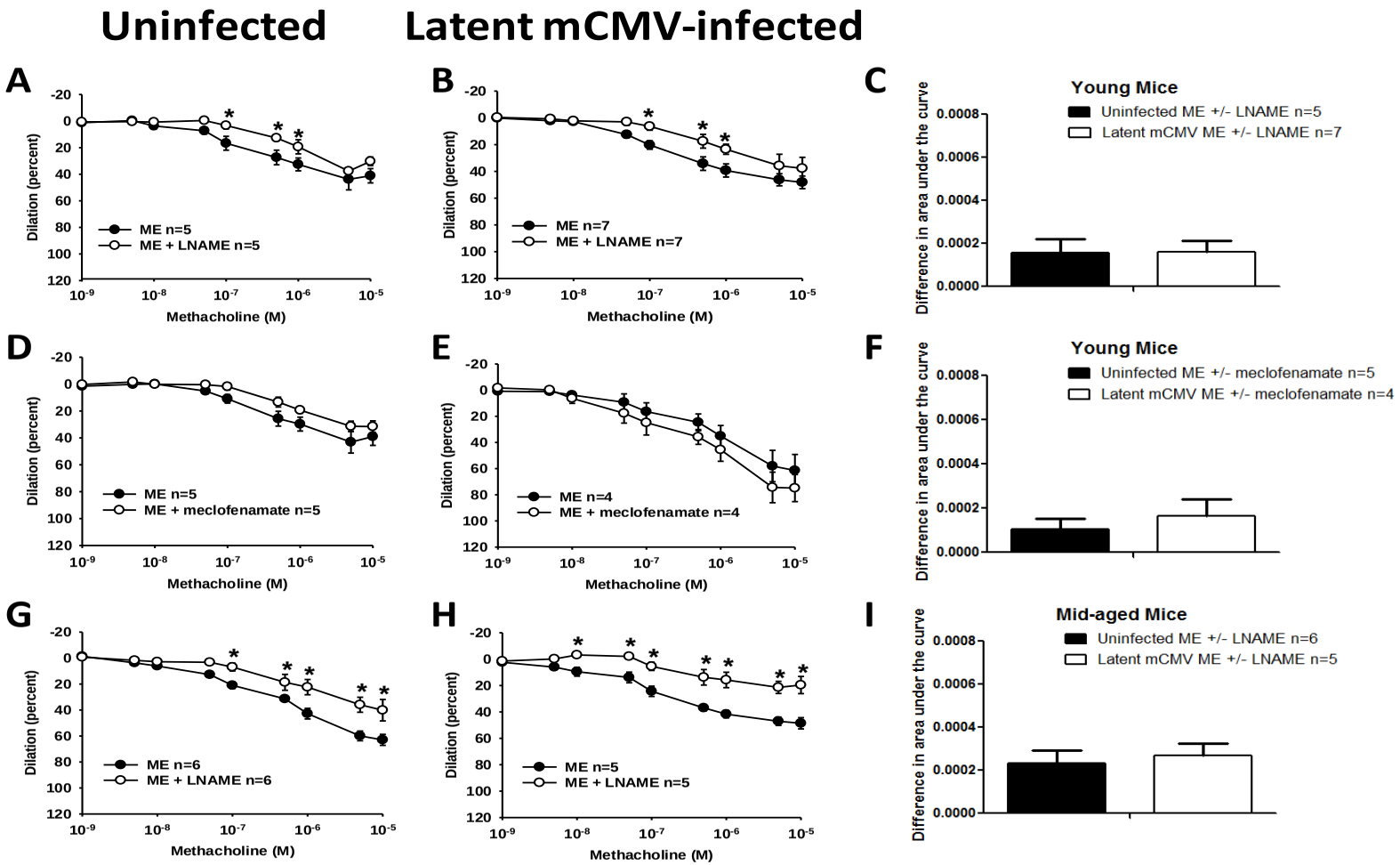

A
Young Mice
Dilation (percent)
Methacholine (M)
Uninfected n=9
Latent mCMV n=7
B
Mid-aged Mice
Uninfected n=5
Latent mCMV n=7
C
Aged Mice
Uninfected n=8
Latent mCMV n=11
D
Young Mice
Uninfected n=8
Latent mCMV n=7
E
Mid-aged Mice
Uninfected n=6
Latent mCMV n=9
F
Aged Mice
Uninfected n=8
Latent mCMV n=11

Figure C3.3: Responses to ME by mesenteric and uterine arteries from latent mCMV-infected and uninfected young, mid-aged, and aged mice. ME-induced vasodilation was measured in mesenteric **(A,B,C)** and uterine **(D,E,F)** arteries from latent mCMV-infected and age-matched uninfected young **(A,D)**, mid-aged **(B,E)**, and aged **(C,F)** mice. Results for each curve were summarized and expressed as mean ± SEM percent increase in lumen diameter compared with the initial preconstricted diameter and normalized to the passive lumen diameter. Significance was assessed as for Figure C3.2. * = significant differences ($p<0.05$). n = number of animals.


Uninfected
Latent mCMV-infected
A
B
C
D
E
F
G
H
I
Dilation (percent)
Methacholine (M)
ME n=5
ME + LNAME n=5
ME n=7
ME + LNAME n=7
Young Mice
Uninfected ME +/- LNAME n=5
Latent mCMV ME +/- LNAME n=7
Difference in area under the curve
ME n=5
ME + meclofenamate n=5
ME n=4
ME + meclofenamate n=4
Young Mice
Uninfected ME +/- meclofenamate n=5
Latent mCMV ME +/- meclofenamate n=4
ME n=6
ME + LNAME n=6
ME n=5
ME + LNAME n=5
Mid-aged Mice
Uninfected ME +/- LNAME n=6
Latent mCMV ME +/- LNAME n=5

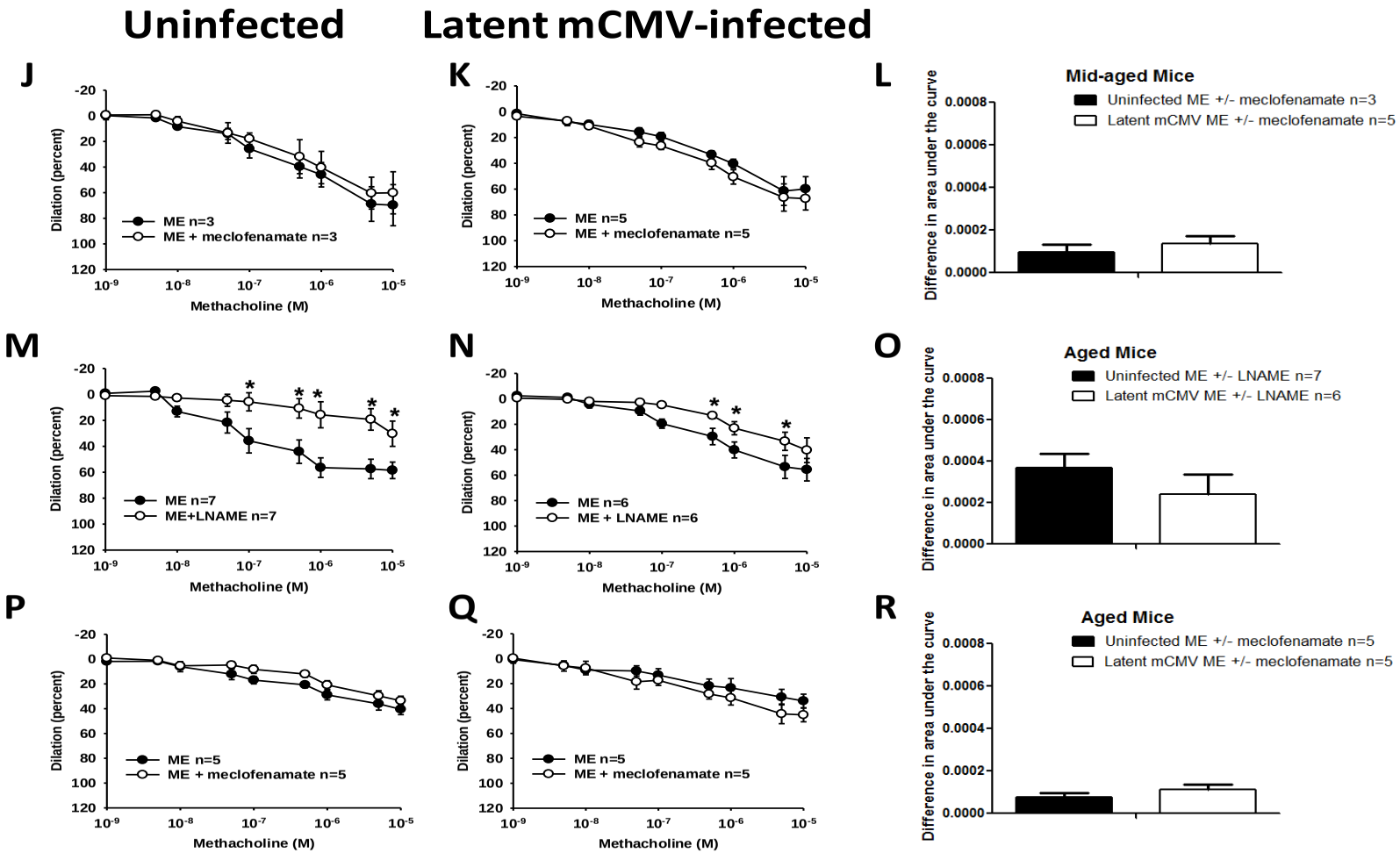

Uninfected
Latent mCMV-infected
J
K
L
M
N
O
P
Q
R
Dilation (percent)
Methacholine (M)
ME n=3
ME + meclofenamate n=3
ME n=5
ME + meclofenamate n=5
ME n=7
ME+LNAME n=7
ME n=6
ME + LNAME n=6
Mid-aged Mice
Uninfected ME +/- meclofenamate n=3
Latent mCMV ME +/- meclofenamate n=5
Aged Mice
Uninfected ME +/- LNAME n=7
Latent mCMV ME +/- LNAME n=6
Uninfected ME +/- meclofenamate n=5
Latent mCMV ME +/- meclofenamate n=5
Difference in area under the curve

Figure C3.4: NO and prostanoid contribution to ME-induced vasodilation in mesenteric arteries from latent mCMV-infected and uninfected young, mid-aged, and aged mice. ME-induced vasodilation was measured in mesenteric arteries from young **(A-F)**, mid-aged **(G-L)**, and aged **(M-R)** latent mCMV-infected **(B,E,H,K,N,Q)** and uninfected **(A,D,G,J,M,P)** mice in the presence and absence of L-NAME (100μM) **(A-C,G-I,M-O)** or meclo (1μM) **(D-F,J-L,P-R)**. Results for each curve were expressed as mean ± SEM and the dose-response curves were compared as in Figure C3.2. For the ME dose-response curves with L-NAME **(C,I,O)** or meclo **(F,L,R)** inhibition, the area under the curve was calculated and the difference between 2 curves on the same graph was obtained. These differences in area under the curve were compared between latent mCMV-infected and uninfected groups with a Student's t-test. * = significant differences ($p<0.05$). n = number of animals.

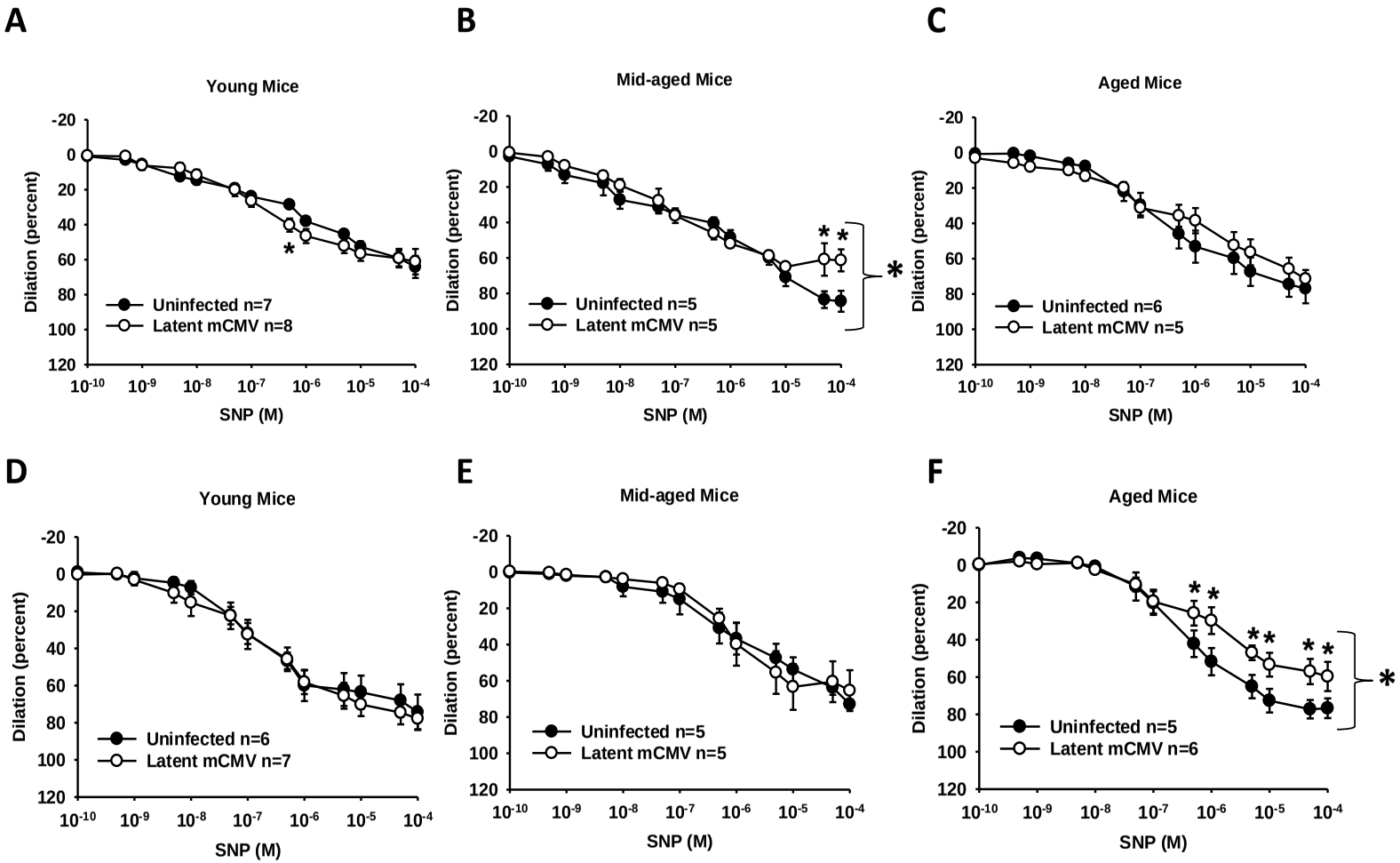

A
Young Mice
Dilation (percent)
SNP (M)
Uninfected n=7
Latent mCMV n=8
B
Mid-aged Mice
Uninfected n=5
Latent mCMV n=5
C
Aged Mice
Uninfected n=6
Latent mCMV n=5
D
Young Mice
Uninfected n=6
Latent mCMV n=7
E
Mid-aged Mice
Uninfected n=5
Latent mCMV n=5
F
Aged Mice
Uninfected n=5
Latent mCMV n=6

Figure C3.5: Responses to SNP by mesenteric and uterine arteries from latent mCMV-infected and uninfected young, mid-aged, and aged mice. SNP-induced vasodilation was measured in mesenteric **(A,B,C)** and uterine **(D,E,F)** arteries from latent mCMV-infected and age-matched uninfected young **(A,D)**, mid-aged **(B,E)**, and aged **(C,F)** mice. Results were summarized and presented as mean ± SEM and significant differences were assessed as for Figure C3.2. * = significant differences ($p<0.05$). n = number of animals.

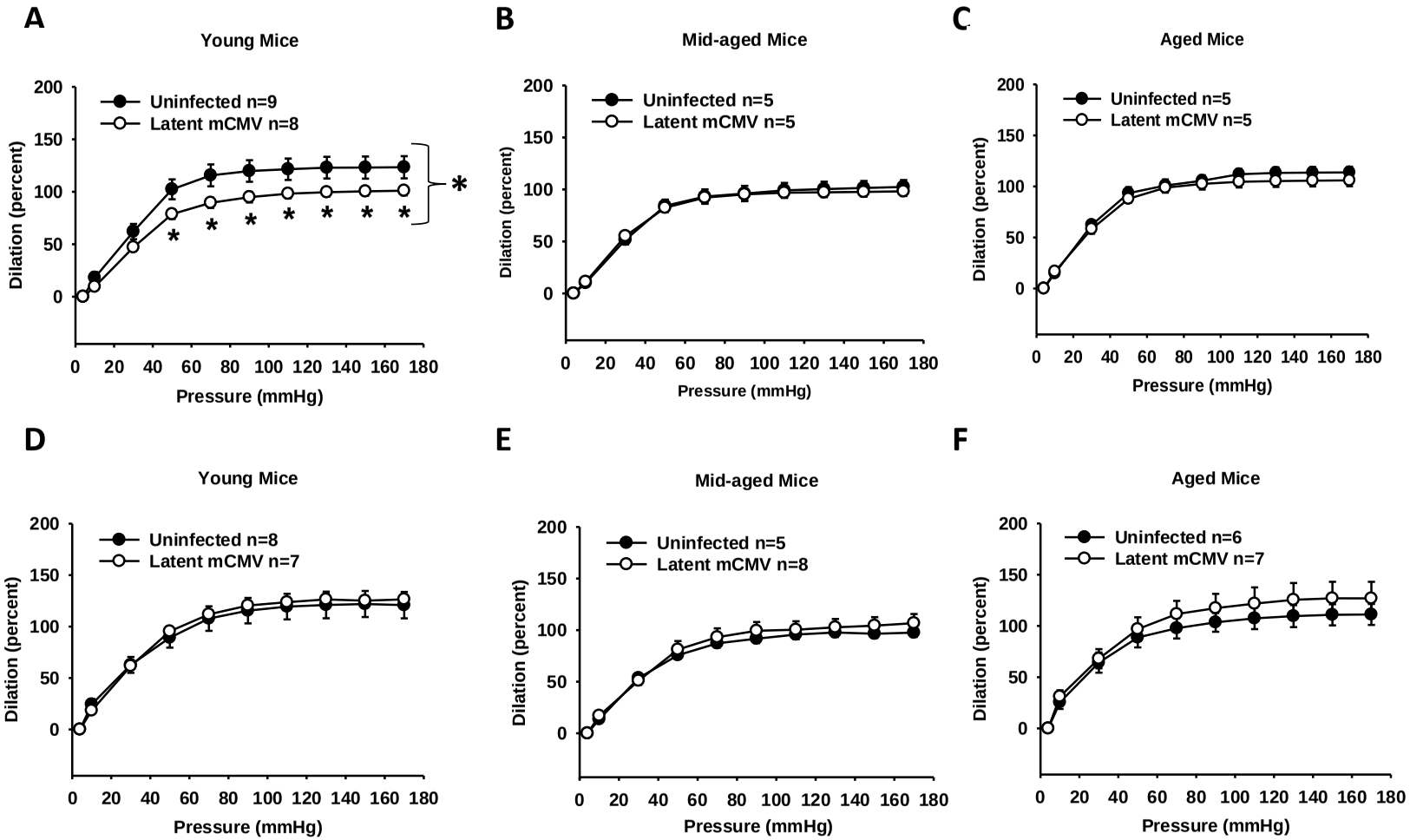

A
Young Mice
Uninfected n=9
Latent mCMV n=8
Dilation (percent)
Pressure (mmHg)
B
Mid-aged Mice
Uninfected n=5
Latent mCMV n=5
Dilation (percent)
Pressure (mmHg)
C
Aged Mice
Uninfected n=5
Latent mCMV n=5
Dilation (percent)
Pressure (mmHg)
D
Young Mice
Uninfected n=8
Latent mCMV n=7
Dilation (percent)
Pressure (mmHg)
E
Mid-aged Mice
Uninfected n=5
Latent mCMV n=8
Dilation (percent)
Pressure (mmHg)
F
Aged Mice
Uninfected n=6
Latent mCMV n=7
Dilation (percent)
Pressure (mmHg)

Figure C3.6: Distensibility curves for mesenteric and uterine arteries from latent mCMV-infected and uninfected young, mid-aged, and aged mice. Changes in lumen diameter were measured after stepwise increases in intraluminal pressure in Ca^{2+}-free EGTA PSS in the presence of papaverine, in mesenteric **(A,B,C)** and uterine **(D,E,F)** arteries from latent mCMV-infected and age-matched uninfected young **(A,D)**, mid-aged **(B,E)**, and aged **(C,F)** mice. Results were expressed as mean ± SEM percent increase in lumen diameter in comparison with the initial diameter at 4 mmHg. Significant differences were assessed as for Figure C3.2. Where error bars are not visible the errors were too small to be seen. * = significant differences ($p<0.05$). n = number of animals.

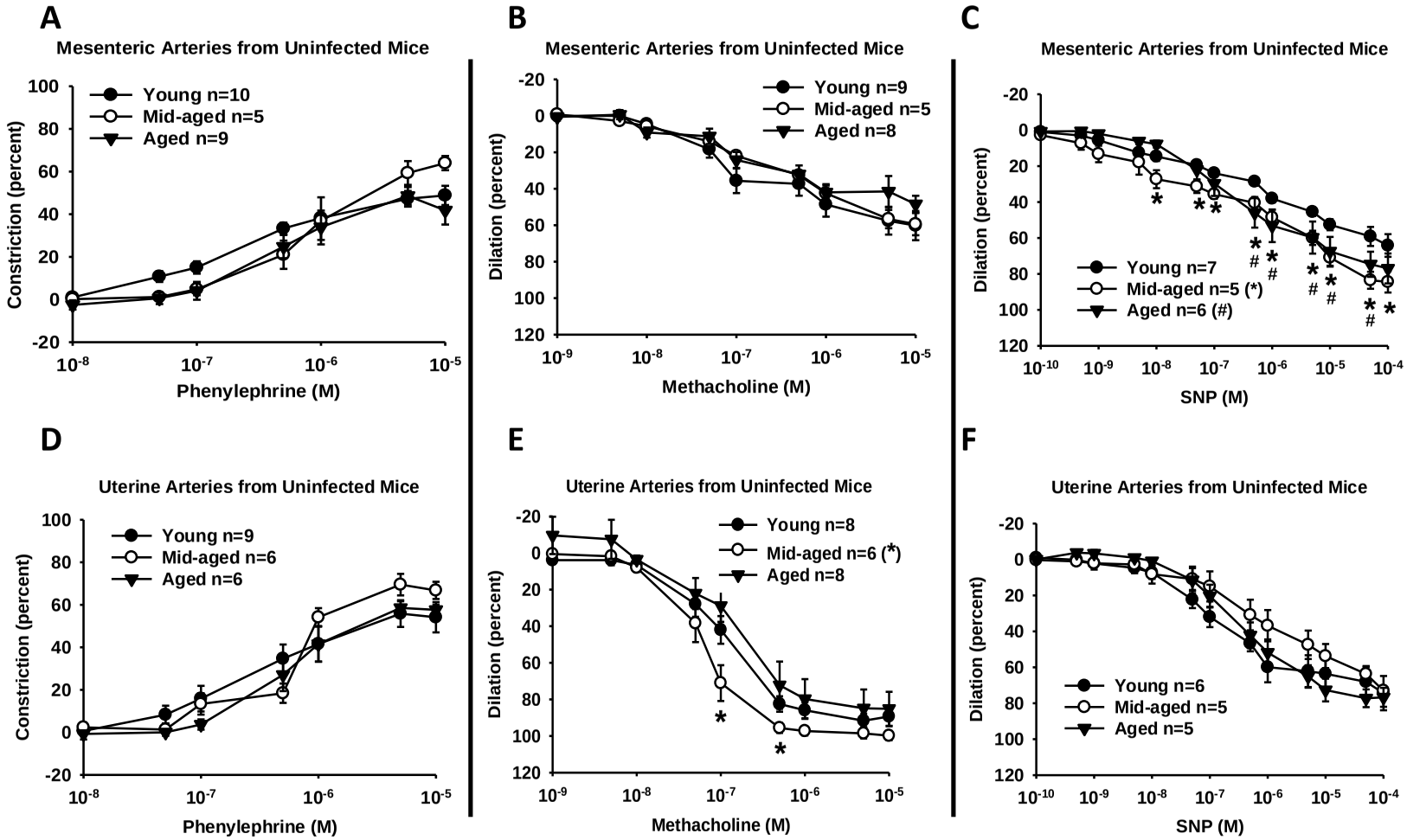

A
Mesenteric Arteries from Uninfected Mice
Young n=10
Mid-aged n=5
Aged n=9
Constriction (percent)
Phenylephrine (M)
B
Mesenteric Arteries from Uninfected Mice
Young n=9
Mid-aged n=5
Aged n=8
Dilation (percent)
Methacholine (M)
C
Mesenteric Arteries from Uninfected Mice
Young n=7
Mid-aged n=5 (*)
Aged n=6 (#)
Dilation (percent)
SNP (M)
D
Uterine Arteries from Uninfected Mice
Young n=9
Mid-aged n=6
Aged n=6
Constriction (percent)
Phenylephrine (M)
E
Uterine Arteries from Uninfected Mice
Young n=8
Mid-aged n=6 (*)
Aged n=8
Dilation (percent)
Methacholine (M)
F
Uterine Arteries from Uninfected Mice
Young n=6
Mid-aged n=5
Aged n=5
Dilation (percent)
SNP (M)

Figure C3.7: Comparison of vascular responses in mesenteric and uterine arteries from uninfected young, mid-aged, and aged mice. PE-induced vasoconstriction for mesenteric **(A)** and uterine **(D)** arteries, ME-induced vasodilation for mesenteric **(B)** and uterine **(E)** arteries, and SNP-induced vasodilation for mesenteric **(C)** and uterine **(F)** arteries were measured and compared among the three age groups. Results were summarized and presented as mean ± SEM and significant differences between points on the curves was calculated using a Repeated Measures two-way ANOVA with Holm-Sidak's post-hoc analysis ($p<0.05$). Significant differences ($p<0.05$): *, mid-aged compared to young; #, aged compared to young.

C3.4 DISCUSSION

Active infections by viruses from the *Herpesviridae* family, with detection of infectious virus and specific immune response mediators, have largely been associated with impaired physiological responses and several diseases (Compton *et al.*, 2003; Gombos *et al.*, 2009; Grefte *et al.*, 1993; Hislop *et al.*, 2007; O'Reilly *et al.*, 1977; Soldan *et al.*, 1997). My study shows that vascular dysfunction occurs in mice infected with mCMV, a *β-Herpesviridae* virus, even after the active infection has progressed to the latent stage with no detectable infectious virus. However, the specific vascular changes depend greatly on the origin of the vascular bed and in some cases worsen with age. In young mice, mesenteric and uterine arteries showed increased vasoconstriction and increased vasodilation in latent mCMV-infected compared to uninfected age-matched mice. In mid-aged latent infected mice, vasodilation was reduced in mesenteric arteries and vasoconstriction was increased in uterine arteries. Interestingly, infertility was increased in mid-aged latent mCMV-infected compared to uninfected mice, indicating that a mCMV infection also affects reproductive capacity. Finally, in aged mCMV-infected mice, mesenteric arteries showed increased vasodilation while uterine arteries showed decreased vasodilation.

Viral latency has been operationally defined as the absence of infectious, replicating virus with maintenance of the viral genome in the tissues of the host (Presti *et al.*, 1998). Most organs in the body can be sites of both active and latent CMV, where only lymphocytes and neutrophils do not support CMV replication (Pancholi *et al.*, 2004). While viral latency may be occurring in one organ or cell type, viral replication (ie. reactivation) may be occurring in another organ or cell type within the same host (Balthesen *et al.*, 1993). It is also difficult to predict when reactivation of a latent CMV infection will occur (Morita-Hoshi *et al.*, 2008). Components of the immune system, particularly CMV-specific T-cells, are important for resolving active infections and maintaining a latent infection (Steffens *et al.*, 1998). Effectively reducing the load of viral genomes within the host and particular organs, as occurs in fully immunocompetent hosts, accelerates

the establishment of latency and reduces the incidence of reactivation (Reddehase *et al.*, 1994). In the murine model, the lung is a predominant site where viral genome load is high and reactivation is frequent (Balthesen *et al.*, 1993). In my study, I detected mCMV DNA in lungs, spleens and kidneys and were unable to detect a reporter of productive infection, β-galactosidase, in any of the five different organs in my latent mCMV-infected mouse model, including the lung, in any of the three age groups with a minimum of two months post infection. Therefore, although viral replication could be occurring elsewhere in the latent infected mice, the presence of viral DNA with the lack of detectable reporter of active replication in five organs, together with evidence from many other studies, implies a latent infection (Yuhasz *et al.*, 1994).

Mesenteric arteries from young latent mCMV-infected mice showed increased PE-induced vasoconstriction, slightly increased SNP-induced vasodilation and no change in ME-induced vasodilation. These surprising results indicate that in young latent infected mice, vascular responses are dysfunctional even in the absence of detectable virus. Sensitivity of smooth muscle cells to NO is likely increased to compensate for increased vasoconstriction, similar to what I showed previously in NP active CMV-infected mice (Gombos *et al.*, 2010). In the mice from this previous study, mesenteric arteries showed increased vasodilation due to increased sensitivity of the vascular smooth muscle to NO and increased NOS activity. Given that Cheng at al. showed that acute CMV-infected mice had increased blood pressure (Cheng *et al.*, 2009), the increased dilation observed in my previous studies may also be compensatory for increased vasoconstriction activity. It is important to note that I have tested only three vasoactive mediators out of many available candidates. Other studies have shown that angiotensin II (Cheng *et al.*, 2009) and bradykinin (Grahame-Clarke *et al.*, 2003) are up- or downregulated with an active CMV infection, respectively, and induce a hypertensive phenotype. My results showing increased vasoconstriction in isolated mesenteric arteries with little change in dilation from latent mCMV-

infected mice suggest possible hypertension. These results suggest ongoing vascular effects are maintained when an active CMV infection is undetectable.

Inhibition of NOS significantly reduced ME-induced vasodilation in both mesenteric and uterine arteries from latent mCMV-infected young, mid-aged and aged mice. However, in contrast to the increased contribution of NO to ME-induced vasodilation in mesenteric arteries from young active mCMV-infected mice compared to uninfected controls (Gombos *et al.*, 2010), the contribution of NO to the ME responses in arteries from latent mCMV-infected young mice was not different from their uninfected controls. Mesenteric arteries from latent mCMV-infected young and mid-aged mice had minimally but significantly reduced ME-induced endothelium-dependent and SNP-induced endothelium-independent vasodilation. In contrast and contrary to my hypothesis, those from latent mCMV-infected aged mice showed increased ME-induced vasodilation. It is known that aging (Duprez, 2008; Soucy *et al.*, 2006) and a CMV infection (Cheng *et al.*, 2009; Grahame-Clarke *et al.*, 2003) are associated with increased blood pressure, vascular stiffness and vascular dysfunction. Thus the systemic vasculature may compensate for this combined phenotype with increased endothelium-dependent vasodilation, even in the presence of a latent, not active, mCMV infection.

Uterine arteries from latent mCMV-infected mice responded differently to PE, SNP, and ME than mesenteric arteries. In uterine arteries from young latent mCMV-infected compared to uninfected mice, the vasoconstriction induced by PE and the sensitivity to ME were increased. The increase in sensitivity, but not maximal vasodilation, to ME in uterine arteries from young latent mCMV-infected mice compared to uninfected mice may be explained by an increase in muscarinic receptor expression (Eglen *et al.*, 1990; Pesic *et al.*, 2009). The increase in vasodilation capacity to compensate for increased vasoconstriction of uterine arteries, as was observed in young and aged latent mCMV-infected mice, was reduced in uterine arteries from mid-aged latent mCMV-infected mice. The increase in sensitivity to PE-induced vasoconstriction in the absence of a

compensatory increase in vasodilation could lead to reduced uterine blood flow and contribute to the reduced number of completed pregnancies in latent CMV-infected compared to uninfected mid-aged mice (16.7% versus 46.7%, respectively). Although C57BL/6J mice are fertile until 12 months of age, the frequency of gestational complications, including embryo resorptions, increase with age (Holinka *et al.*, 1979). Knowing that hypertensive conditions increase pregnancy complications among women in their forties, reduced fertility in the uninfected mid-aged animals is not unexpected (Heffner, 2004). However, it is surprising that a latent mCMV infection with no detectable reporter for an active infection can exacerbate vascular dysfunction and infertility. Although aging alone reduces reproductive success, a latent CMV infection that increases vasoconstriction in uterine arteries may further contribute to reproductive complications in mid-aged women. In contrast, in aged mice, uterine arteries from latent mCMV-infected and uninfected mice did not differ in response to PE. However, smooth muscle sensitivity to NO was decreased in uterine arteries from latent mCMV-infected aged mice. This suggests that compensatory vasodilation mechanisms are reduced in uterine arteries compared to mesenteric arteries with age. As aged mice are no longer reproductively active (Holinka *et al.*, 1979), increased uterine artery blood flow is much less important than in young and mid-aged mice. In contrast, it is essential to compensate for vascular dysfunction in systemic (mesenteric) vascular beds that regulate blood pressure and cardiovascular function (Mohammed *et al.*, 2007).

CMV latency is established in the host as a life-long infection. Reactivated CMV is associated with several vascular diseases that occur with age, including atherosclerosis (Melnick *et al.*, 1995). During a CMV infection, several cytokines, such as IFN-γ, are produced by immune cells, which are important in clearing CMV and inhibiting lytic replication (Presti *et al.*, 1998). Cytokine production is reduced in immunosuppressed individuals who are highly susceptible to CMV disease (Gamadia *et al.*, 2003). IFN-γ also inhibits reactivation of mCMV and sustains latency (Presti *et al.*, 1998). Interestingly, IFN-γ causes vascular

dysfunction itself (Skaro *et al.*, 2005). It reduces sensitivity to NO, decreases endothelial NOS and increases inducible NOS expression in human coronary artery allografts (Koh *et al.*, 2004). Therefore, immune response mediators that increase during active and latent mCMV infections may contribute to the vascular dysfunction and vasculopathies. Measuring circulating levels and testing the effects of several inflammatory cytokines, including IFN-γ and TNF-α, on vascular function to compare the direct and indirect effects of a CMV infection will be important.

Finally, it is perhaps surprising that I found few differences in vascular responses with age, independent of infection status. Moreover, the changes involved increased rather than decreased vasodilation, as would perhaps have been predicted by previously observed decreases in NO production, bioavailability and smooth muscle sensitivity with age (Karaki *et al.*, 1985; Smith *et al.*, 2006a; Smith *et al.*, 2006b; Soucy *et al.*, 2006). SNP-induced vasodilation in mesenteric arteries was increased in uninfected mid-aged and aged compared to uninfected young mice and ME-induced vasodilation in uterine arteries was increased in uninfected mid-aged mice compared to uninfected young and aged mice. The increased mesenteric endothelium-independent dilation may be a compensatory mechanism for the increased vascular dysfunction that occurs with aging in the absence of a CMV-infection (Labinskyy *et al.*, 2006). Increased capacity for vasodilation could perhaps explain the finding that myogenic tone is reduced in mesenteric arteries from both male and female mid-aged and aged mice compared to young mice (Gros *et al.*, 2002). Although there were no differences observed in PE-induced vasoconstriction with aging alone in this study, in contrast to the increases seen using the rat model (Muller-Delp *et al.*, 2002), my findings are consistent with other C57BL/6 mouse studies (Gros *et al.*, 2002).

We are the first to show that both active and latent mCMV infections can cause vascular dysfunction in two different types of arteries at several different ages. Determining what mediators are involved in vascular dysfunction during a

CMV infection will be very important in targeting factors that contribute to vascular-specific complications that occur with age. Understanding CMV-induced vascular dysfunction will increase our knowledge on the development of cardiovascular diseases associated with a CMV infection, and the vascular complications that may contribute to infertility in CMV-infected mid-aged women.

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