Statin Mediated Vasodilation in the Vasculature

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Dedicated to the memory of John McEachern

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Signed,

Scott Copley

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THESIS ABSTRACT

Clinical trials have established the efficacy and safety of the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) in lowering cardiovascular morbidity and mortality in patients with and without coronary artery disease. Traditionally, the beneficial effects of statins have been ascribed entirely to their ability to lower serum cholesterol. Evidence indicates, however, that statins may exert cholesterol-independent or "pleiotropic" effects.

Statins may modulate vascular reactivity via the inhibition the RhoA/Rho-kinase pathway in both the vascular endothelium and the underlying vascular smooth muscle. To examine this hypothesis, we coupled the measurement of isometric force in isolated rat caudal artery segments with molecular analysis of the downstream targets of Rho-kinase in both the endothelium and smooth muscle.

We report that clinical concentrations of pravastatin inhibit α_1-adrenoreceptor mediated vascular contraction through an endothelial-dependent mechanism. Our results suggest that this is mediated by an increase in P[Ser1177]eNOS phosphorylation, consistent with increased eNOS activation, increased nitric oxide production and the inhibition of the RhoA/Rho-kinase pathway in the vascular endothelium.

In the context of ThromboxaneA_2 (TxA_2) receptor-mediated contraction we report that acute high dose simvastatin administration causes a robust reduction in contraction. We describe a concomitant increase eNOS Ser1177 phosphorylation, suggesting activation of eNOS and increased NO production, however, experiments in which

eNOS was inhibited suggest that this mechanism does not account for the majority of relaxation. Perhaps more importantly, we report the increased activation of smooth muscle myosin phosphatase that may account for simvastatin-mediated relaxation in this preparation.

Extending these results to a chronic setting we examined the consequence of 7-day statin administration on rats. Using non-invasive tail cuff we demonstrate reductions in the systolic blood pressure of healthy rats treated with clinically relevant doses of simvastatin for 7 days. Using a perfused isolated heart model we report reduced TxA_2-receptor mediated coronary perfusion pressure in hearts isolated from these animals and a reduction in TxA_2-receptor mediated contraction in isolated blood vessels. Western blot analysis revealed an increase in the expression of endothelial nitric oxide synthase (eNOS) that was concomitant with these effects. Additional administration of high dose simvastatin further reduced TxA_2-receptor mediated contraction via disinhibition of smooth muscle myosin phosphatase.

These results suggest that statins may be a viable treatment option to effect acute vasodilatation in patients with normal cholesterol levels but with abnormal vasomotor reactivity and/or endothelial dysfunction.

COMMON ABREVIATIONS

$[Ca^{2+}]_{cyt}$	cytosolic Ca^{2+} concentration
5-HT	serotonin
AA	arachidonic acid
ACh	acetylcholine
BP	blood pressure
cAMP	cyclic guanosine monophosphate
cGMP	cyclic adenosine monophospate
CAD	coronary artery disease
CHD	coronary heart disease
COX	cyclooxygenase
CPI-17	PKC-potentiated inhibitory protein of 17 kDa
CRP	C-reactive protein
CSFP	coronary slow flow phenomenon
DAG	diacylglycerol
EC	effective concentration
ECL	enhanced chemiluminescence
EDHR	endothelial derived hyperpolarizing
EET	epoxyeicosatrienoic acid
Emax	maximal contraction factor
EDRF	endothelial derived relaxing factor
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA reductase
eNOS	endothelial nitric oxide synthase
ET-1	endothelin-1
FH	familial hypercholesterolemia
FPP	farnesyl pyrophosphate
GGPP	geranylgeranyl pyrophosphate
GSNO	S-nitrosoglutathione
GTP	guanosine triphosphate
HDL	high density lipoprotein
HR	heart rate
HUVEC	human umbilical vein endothelial cell
IgG	immunoglobulin G

ILK	integrin linked kinase
IP_3	inositol trisphosphate
L-NAME	N_ω-Nitro-L-arginine methyl ester
LC_{20}	20 kDa light chains of myosin
LDL	low density lipoprotein
MAP	mean arterial pressure
MI	myocardial infarction
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MS	multiple sclerosis
MYPT1	myosin phosphatase targeting subunit
NO	nitric oxide
NOS	nitric oxide synthase
OD	optical density
P	statistical probability
PCI	percutaneous coronary intervention
PGI_2	prostacyclin
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C
PP1c	protein phosphatase type 1
RA	rheumatoid arthritis
ROK	Rho associated kinase (Rho-kinase)
ROS	reactive oxygen species
S.E.M	standard error of the mean
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	serine
SR	sarcoplasmic reticulum
TBS	tris buffered saline
TBS-T	tris buffered saline – Tween 20
TCA	trichloroacetic acid

Thr	threonine
TPR	total peripheral resistance
TRPC	transient receptor potential channel
TxA_2	thromboxane A_2
VSM	vascular smooth muscle
ZIPK	zipper interacting protein kinase

A. Literature Review

A.1 The Statins

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, better known as statins, are the most widely prescribed class of drug in the world. The following section will review the history, clinical effects, and mechanism by which statins lower cholesterol.

A.1.1 A Brief History

It was the Framingham study, orchestrated in the 1950s, that established the link between raised plasma cholesterol levels and increased risk of mortality from coronary heart disease (Castelli 1984). This association was subsequently attributed to low-density lipoprotein (LDL) cholesterol, opposed to high-density lipoprotein (HDL) cholesterol which has been inversely correlated with CHD mortality (Gordon, Castelli et al. 1977; Manninen, Tenkanen et al. 1992). Subsequent population studies found that lower levels of LDL were associated with decreased risk of CHD, with no bottom limit to the level of LDL that afforded risk reduction (Downs, Clearfield et al. 1998).

Prior to 1980 the pharmacological options for treating hypercholesterolemia were limited. Bile acid binding resins, niacin, fibrates and probucol were effective, however, had limited tolerability in humans (Ast and Frishman 1990; Steinberg and Gotto Jr 1999). It was not until the development of a class of drugs collectively known as the statins that pharmacological reduction of cholesterol became substantially effective.

The first statin was isolated and characterized in Japan by Akira Endo in 1976. ML236B, later named mevastatin, was isolated from *Penicillum citrinum* (Endo, Kuroda et al. 1976; Endo 1992). Mevastatin lowered plasma LDL cholesterol in both normal and hypercholesterolemic rabbits without significantly altering HDL levels (Watanabe, Ito et al. 1981), with similar results seen in monkeys (Kuroda, Tsujita et al. 1979). In dogs, a significant reduction in serum cholesterol was seen at dosages of 10 mg/kg per day with a 44-45% reduction seen at 100-400 mg/kg per day, with no side effects reported, even at the highest concentrations used (Tsujita, Kuroda et al. 1979).

Lovastatin, which was isolated from cultures of *Aspergillus terreus* by Alberts and Chen at Merck Research Labs in 1978 (Alberts, Chen et al. 1980) was the first statin available for prescription. Simvastatin, a synthetic derivative of lovastatin was developed by Merck in 1988 and is a more potent HMG CoA reductase inhibitor than lovastatin. This was followed by fluvastatin in 1991, atorvastatin in 1997, cerivastatin in 1998 and rosuvastatin in 2003 (Tobert 2003).

A.1.2 Clinical Evidence of Cholesterol Lowering

During the early 1980s Merck undertook small-scale clinical trials in healthy volunteers with very positive results. A double-blind study of 22 healthy subjects reported reductions in serum cholesterol of 14%, 25% and 24% with 5, 15 and 50 mg of lovastatin, respectively (Tobert, Hitzenberger et al. 1982). A similar study conducted over a four-week period confirmed these results (Tobert, Bell et al. 1982). In both studies HDL cholesterol, VLDL cholesterol and triglycerides were not affected.

Early clinical trials in patients with severe heterozygous familial hypercholesterolemia were also positive. Those with familial hypercholesterolemia (FH) have one mutant gene encoding the plasma membrane LDL receptor, which is responsible for the uptake and the degradation of plasma LDL. Consequently, patients have half the LDL receptors as normal and remove LDL at a lower rate than those with wildtype receptors (Goldstein, Hobbs et al. 1989). Subjects with FH received mevastatin for 4-8 weeks at a dose of 50-150 mg/day and showed a 22-28% reduction in total serum cholesterol (Bilheimer, Grundy et al. 1983).

Randomized, double blind phase II studies took place in 1984 and lovastatin again proved to be effective in patients with FH. Patients received 40 mg of lovastatin twice a day and displayed reductions in LDL of 39% (Lovastatin Study Group 1986). Larger phase III trials showed lovastatin reduced LDL cholesterol more than the bile acid sequestrant cholestyramine (Lovastatin Study Group III 1988) and the antilipidemic probucol (Lovastatin Study Group IV 1990), again, with very few reported adverse effects. In these trials modest reductions in plasma triglycerides were reported, along with small increases in HDL cholesterol.

The Scandinavian Simvastatin Survival Study (4S) was the first large-scale double blind clinical outcome trial of statins. Patients with coronary heart disease were assigned 20-40 mg of simvastatin or a placebo for five years. In the simvastatin treated group a 42% reduction in coronary related deaths and a 30% reduction in all cause mortality was noted, along with a 34% reduction in major coronary events such as myocardial infarction. These positive cardiovascular outcomes persisted in the

presence of risk factors such as hypertension, diabetes and smoking. This was a landmark study, not only confirming the efficacy of statins in secondary prevention of coronary events, but also supporting a central role for cholesterol in the development of cardiovascular morbidity (4S Group 1994).

Further clinical trials strengthened the evidence of secondary prevention of coronary heart disease with statins. The Cholesterol and Recurrent Events (CARE) study found similar outcomes to the 4S trial in subjects with average cholesterol levels (Sacks, Pfeffer et al. 1996). The Long-term Intervention with Pravastatin in Ischemic Disease (LIPID) study extended these finding further by including patients with unstable angina, in which pravastatin (40 mg daily) afforded a 24% reduction in CHD death (LIPID Study Group 1998).

Subsequently, the clinical effects of statins were extended to primary prevention of cardiovascular events. The West of Scotland Coronary Prevention Study (WOSCOPS) found a significant reduction in the primary end point of coronary death and nonfatal myocardial infarction after 5 years (Shepherd, Cobbe et al. 1995). While the Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS) reported a reduction in first acute major coronary events in subjects with average LDL but low HDL cholesterol. Interestingly, those with the lowest HDL levels benefited the most from statin treatment (Downs, Clearfield et al. 1998).

The Heart Protection Study, one of the largest lipid-lowering trials ever embarked upon, further demonstrated the benefit and safety of simvastatin (40 mg daily) in

patients with CHD, diabetes, and peripheral artery disease. This included subjects with LDL levels well below the population average. This study was the first to confirm beneficial effects in a large cohort of woman, and showed a significant reduction in non-CHD related events such as stroke (Heart Protection Study Collaborative Group 2002).

A.1.3 Safety

Although generally very well tolerated, the statins are associated with a number of side effects. These range in severity from creatine kinase elevation through to rhabdomyolysis, a severe form of skeletal muscle myopathy (Law and Rudnicka 2006). These side effects vary between statin agents and can be exacerbated when statins are used in conjunction with other drugs (Bottorff 2006).

The most common side effect of statin therapy is myalgia, or muscle pain. This condition affects 1% to 5% of patients but is usually mild and reversible with drug discontinuation (Thompson, Clarkson et al. 2003). More serious is the occurrence of rhabdomyolysis, which is the rapid breakdown of skeletal muscle. A meta-analysis examined the frequency of rhabdomyolysis in 252460 patients treated with both statins alone and statins in combination with other lipid lowering drugs. The authors noted that the average incidence per 10 000 person years for treatment with atorvastatin, pravastatin, or simvastatin was 0.44, although this was higher for cerivastatin (5.34). The incidence increased to 5.98 when atorvastatin, pravastatin, or simvastatin were administered in conjunction with a fibrate (Graham, Staffa et al. 2004).

There have been numerous reports of both increased and decreased risk of cancer in statin treated patients (Rose, Blackburn et al. 1974; Williams, Sorlie et al. 1981; Friis, Poulsen et al. 2005). A meta-analyses of 26 randomized controlled trials of statins in which either cancer incidence or cancer death were reported found that statins did not increase or decrease cancer incidence or cancer death and this null effect persisted regardless of statin type (Dale, Coleman et al. 2006).

A.1.4 Mechanism of Action

Cholesterol is an important component of cell membranes and a precursor to steroid hormones, bile acids and vitamin D. Most cell types in the human body produce cholesterol endogenously. Cholesterol is synthesized via the mevalonate pathway, a complex biosynthetic pathway containing over 30 enzymes (Goldstein and Brown 1990). Figure A1 provides a simplified overview of cholesterol synthesis.

Statins bind competitively and reversibly to HMG-CoA reductase, the rate-limiting enzyme of the mevalonate pathway that catalyzes the conversion of HMG-CoA to mevalonate (Corsini, Maggi et al. 1995). Statins inhibit HMG-CoA reductase by blocking substrate access through the interaction of hydrophobic side chains with the active site (Istvan and Deisenhofer 2001). The natural substrate of HMG-CoA reductase binds at micromolar concentrations, while statins bind at nanomolar concentrations, effectively displacing the natural substrate (Moghadasian 1999).

By blocking the production of mevalonate, statins inhibit intracellular cholesterol production, however, this is unlikely to account for clinically observed reductions in serum cholesterol. Low intracellular mevalonate results in the activation of sterol-

responsive-element-binding proteins that increase LDL receptor gene expression and consequently, LDL receptor expression at the plasma membrane. Indeed, increased LDL receptor mRNA and protein expression has been reported in response to statin treatment in dogs (Kovanen, Bilheimer et al. 1981), rabbits and hamsters (Ma, Gil et al. 1986). In humans administered simvastatin at 40 mg a day for three weeks, liver specimens showed a doubling in LDL receptor expression (Reihner, Rudling et al. 1990). In their noble prize winning work, Brown and Goldstein, determined the central role of the LDL receptor in regulating serum LDL cholesterol (Goldstein and Brown 1973). Thus, current dogma indicates that statins lower serum cholesterol by upregulating the LDL receptor resulting in increased LDL clearance from the serum.

Although LDL receptor upregulation is likely the primary mechanism by which statins reduce serum cholesterol, a number of studies have provided evidence that statins decrease the production of apolipoprotein-B-containing lipoproteins by the liver (Ginsberg, Le et al. 1987). Consistent with this mechanism is the observation that high doses of atorvastatin and simvastatin produce small reductions of LDL cholesterol in patients with homozygous FH, who lack the LDL receptor entirely (Raal, Pilcher et al. 1997).

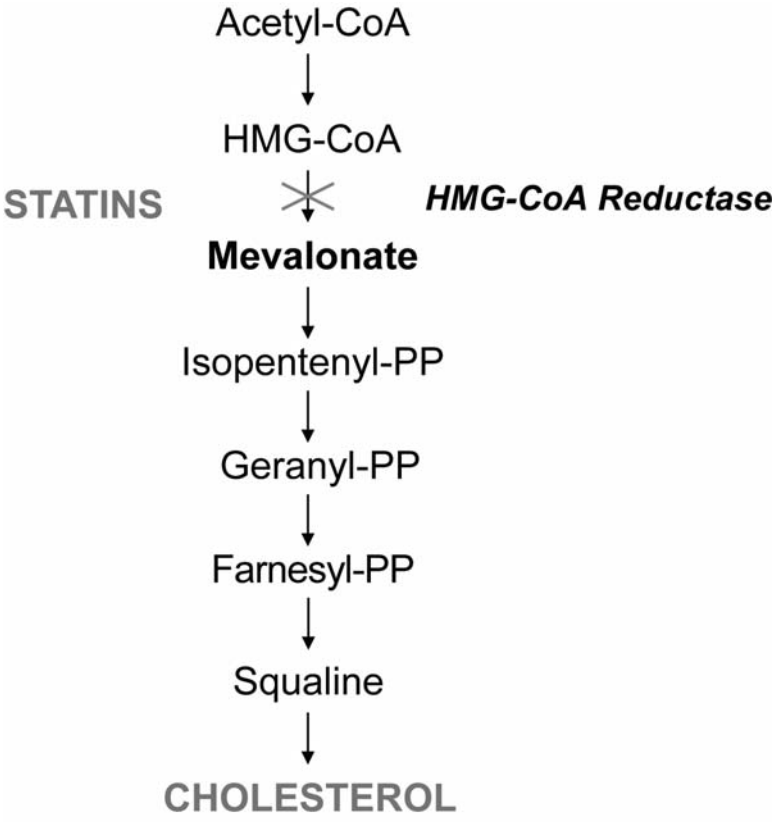

Figure A.1 Cholesterol Biosynthesis and the Mechanism of Action of Statins. Statins inhibit the conversion of HMG-CoA to mevalonate via competitive inhibition of the rate-limiting enzyme HMG-CoA reductase. This results in reduced downstream cholesterol production.

A.1.5 Statin Pharmacology

While all statins inhibit HMG-CoA reductase, there are differences between their pharmacology. Pravastatin and simvastatin are semi-synthetic derivatives of lovastatin, a natural fungal metabolite. The three aforementioned statins are structurally quite similar (Figure A2) (Hamelin and Turgeon 1998). Fluvastatin, atorvastatin and rosuvastatin are entirely synthetic and are more potent cholesterol lowering agents than the first and second-generation statins (Figure A2) (Igel, Sudhop et al. 2002). Both simvastatin and lovastatin are administered as a lactone form prodrug, which is transformed *in vivo* into an active form. Pravastatin, fluvastatin, atorvastatin are administered in an already active acid form (Igel, Sudhop et al. 2002).

Statins as a group have a high oral bioavailability, with absorption ranging from 33% for lovastatin to > 98% for fluvastatin (Duggan, Chen et al. 1989). All statins undergo extensive first-pass hepatic metabolism. The extent of this hepatic extraction varies between statins, evidence suggest that it may be greatest for simvastatin (>79%) and least for pravastatin (46%) (Vickers, Duncan et al. 1990). These differences may be due to differences in statin hydrophobicity or perhaps because of differential liver specificity between statin agents (Singhvi, Pan et al. 1990).

The majority of statins are lipophilic, however, pravastatin is strongly hydrophilic (Figure A3). This may confer differences in tissue permeability and perhaps account for differences in side effects between statins (Blum 1994). Hydrophobic statins such as simvastatin may be more likely to enter extrahepatic tissue such as smooth muscle and endothelial cells than hydrophilic statins such as pravastatin, that primarily enter

hepatocytes via organic anion transporters (Germershausen, Hunt et al. 1989; Hsiang, Zhu et al. 1999).

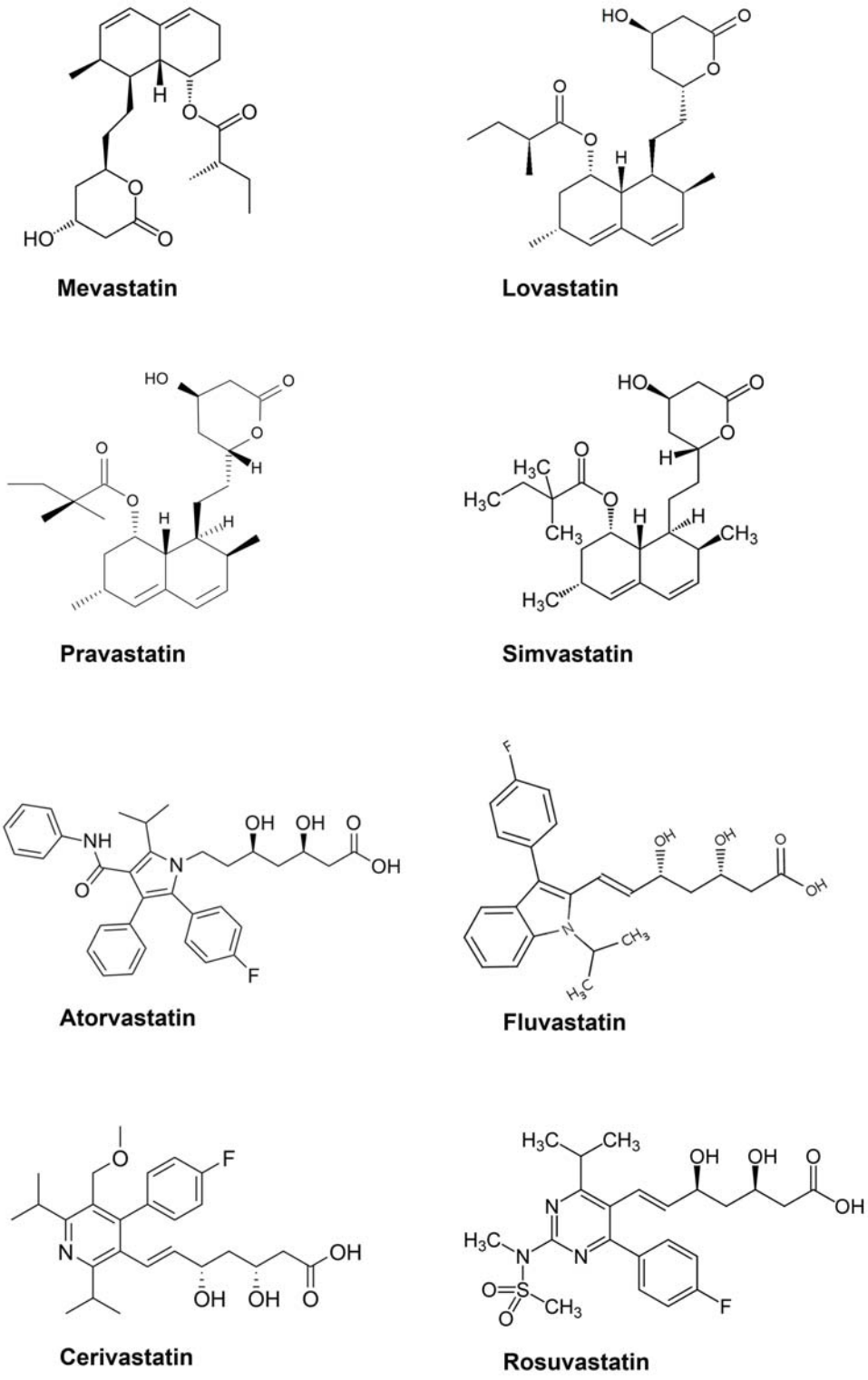

Figure A2 Statin Structure. Both mevastatin and lovastatin are devived from fungi. Pravastatin and simvastatin are biotransformed from mevastatin and lovastatin respectivly. All other depicted statins are entirly synthetic.

	Cerivastatin	Simvastatin	Fluvastatin	Atorvastatin	Rosuvastatin	Pravastatin
Log D Class	1.50 to 1.75	1.5 to 1.75	1.00 to 1.25	1.00 to 1.25	-0.25 to -0.05	-0.75 to -1.0
Comparative lipophilicity	******	*****	****	****	**	*

Figure A3 Comparison of Lipophilicity of the Statins. All log D values are at pH 7.4. For comparative lipophilicity: ****** = most lipophilic, * = least lipophilic. Mevastatin and lovastatin not tabulated due to lack of comparative data. Table adapted from White, 2002.

A.2 Pleiotropic Effects of Statins

Because of the strong link between elevated serum cholesterol and coronary heart disease, the benefits of statin therapy are often attributed solely to cholesterol lowering. Clinical and molecular data suggests, however, that statins have beneficial effects independent from cholesterol lowering, often referred to as "pleiotropic" effects. The following section will review clinical and molecular evidence for statin pleiotropy and outline several putative mechanisms.

A.2.1 Clinical Evidence for Cholesterol-Independent Effects.

Despite unequivocal clinical benefits of reducing plasma LDLs with statins, not all positive clinical outcomes may be attributed to cholesterol lowering. This was first reported in the WOSCOPS trial in which an overlap analysis compared cardiovascular event rates in subjects with LDL-cholesterol between 3.62 to 4.65 mmol/L, a range that occurred frequently in both the placebo and pravastatin treated groups. After adjustment for LDL and triglyceride levels a significant reduction in the cardiovascular event rate persisted (1998). This has also been demonstrated on a larger scale in a meta-analysis of approximately 2000 patients. The authors found that pravastatin lowered serum LDL by 28% and reduced the incidence of myocardial infarction by 66%. After adjustment for LDL cholesterol reduction, the treatment group effect was still statistically significant (Byington, Jukema et al. 1995). Furthermore, the COSMOS trial administered rosuvastatin (20 mg day) to subjects with ischemic heart disease and assessed the effect on coronary atherosclerosis using ultrasound. Rosuvastatin reduced plaque volume and increased lumen volume, however, the authors found no association between reduction in plaque volume and

reduction in plasma LDL (Takayama, Hiro et al. 2009), suggesting that statins may have antiatherogenic effects independent from cholesterol lowering.

Another line of evidence suggesting that statins have cholesterol independent effects is the rapid time course in which statins provide therapeutic benefit. In both the Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering (MIRACL) and the Pravastatin or Atorvastatin Evaluation and Infection Therapy (PROVE-IT) trials, reductions in cardiovascular events were noted after sixteen and four weeks, respectively. This is in stark contrast to trials with non-statin cholesterol lowering drugs, in which benefits occur after several years of treatment (Cannon, Braunwald et al. 2004; Kinlay, Schwartz et al. 2004). This rapid time course has been confirmed in a number of smaller trials that have assessed endothelial function. For example, in human subjects, 3 days of cerivastatin (40 mg) did not alter plasma LDL levels, however, flow-mediated dilatation was significantly increased, as were plasma nitrite/nitrate levels, both hallmarks of improved endothelial function. (Tsunekawa, Hayashi et al. 2001) Similar results were obtained in subjects with stable angina pectoris using 40 mg per day of pravastatin (Wassmann, Faul et al. 2003).

The existence of cholesterol inhibitors that work independent from HMG-CoA provides a good comparison to examine the existence of statin mediated cholesterol independent effects. Ezetimibe decreases plasma LDLs by inhibiting intestinal cholesterol absorption. In patients with stable CAD, both atorvastatin (40 mg day) and ezetimibe (10 mg day) reduced LDL to a similar extent. However, only the atorvastatin treated group showed improved endothelial function as assessed by ACh-mediated increase in forearm blood flow. (Wassmann, Faul et al. 2003; Fichtlscherer,

Schmidt-Lucke et al. 2006) The Ezetimibe and Simvastatin in Hypercholesterolemia Enhances Atherosclerosis Regression (ENHANCE) trial compared ezetimibe/simvastatin (10 mg / 80 mg) to 80 mg of simvastatin in subjects with familial hypercholesterolemia. Plasma LDL was lowered to 4.98 ± 1.56 mmol/L in the simvastatin group and 3.65 ± 1.36 mmol/L in the combined-therapy group. Despite greater cholesterol lowering in the combined group, the authors found no incremental reduction in intima-media thickness of the carotid artery, as assessed by ultrasound. This suggests that non-statin LDL-lowering agents do not have additional benefits in reducing atherosclerotic progression compared with statins alone (Kastelein, Akdim et al. 2008).

Clinical evidence also suggests that statins may have an immunomodulatory effects. The recent JUPITER trial extended statin treatment (20 mg rosuvastatin daily) to subjects with LDL levels below the current recommended level for statin treatment (<3.4 mmol/l) but with a C-reactive protein level of 2.0 mg/L. Subjects were free of existing CAD or diabetes. Although the study was stopped with only 1.9 years completed the authors reported a significant reduction in major cardiovascular events with 142 primary events in the statin group vs 251 in the placebo group. A sub analysis of the trial showed that individuals with the lowest achieved CRP levels benefited the most. Interestingly, there was no relationship between reduction in LDL and reduction in CRP. This study highlights the importance of inflammatory responses in the development of cardiovascular disease and suggests a possible statin mediated anti-inflammatory effect that may be independent from cholesterol lowering (Ridker, Danielson et al. 2009).

A number of studies have described statin mediated improvements in cardiovascular pathologies not associated with high cholesterol levels. Syndrome X is characterized by chest pain typical of angina, with no angiographic evidence of atherosclerosis of the coronary arteries. This disorder is thought to stem from microvascular dysfunction (Maseri, Crea et al. 1991). Kayikcioglu et al., randomized 40 syndrome X patients with exercise-induced ischaemic ST segment changes to either placebo or 40 mg pravastatin groups. The majority of patients included had cholesterol levels < 4.2 mmol/l placing them outside of the range usually considered for cholesterol lowering therapy. The authors found that statin therapy reduced ischemic episodes, prolonged exercise time and time to ST depression. The mechanism for this effect is uncertain but is unlikely to stem from reduced plasma cholesterol (Kayikcioglu, Payzin et al. 2003).

Providing further evidence of statin pleiotropy is the reduction in stroke occurrence seen in statin treated patients. Although still a subject of debate, a link between serum cholesterol and ischemic stroke has not been established. For example, in a meta-analysis of 45 prospective cohorts, containing a total of 450,000 individuals, no association between total serum cholesterol and stroke was found (Centres 1995). Despite this finding, numerous clinical trials have demonstrated that statins are efficacious in decreasing the incidence of ischemic stroke. Both the ASCOT-LLA and HPS trials provide strong evidence of a protective effect against stroke, independent of plasma LDL levels. (Heart Protection Study Collaborative Group 2002; Sever, Dahlöf et al. 2003). Furthermore, trials in which non-statin lipid lowering drugs were used do not report an altered risk for ischemic stroke (Klungel, Heckbert et al. 2002).

Finally, there is increasing evidence that suggests that statins may provide clinical benefits in pathologies unrelated to cardiovascular disease. Multiple sclerosis (MS) is a common inflammatory disease that results in the demyelination of neurons. One small study has found positive effects of statin treatment in MS patients. 28 patients with relapsing-remitting MS were administered simvastatin (80 mg) once a day for 6 months. Participants were examined for lesions using MRI at 4, 5 and 6 months. A 44% reduction in the number of lesions was documented along with a 41% reduction in the volume of new lesions (Vollmer, Key et al. 2004). Rheumatoid arthritis (RA) is another common debilitating inflammatory disorder in which statin treatment appears promising. For example, the administration of atorvastatin at 20 mg/day for 8 days to 5 patients with RA resulted in improvement in joint swelling, with a reduction in circulating C-reactive protein observed in 3 of the 5 patients (Abud-Mendoza, De La Fuente et al. 2003). A more recent study has examined data from a large observational cohort of RA patients. Among 4152 patients examined, 6.7% were taking statins and had lower C-reactive protein levels and lower swollen joint counts compared to patients not taking statins (Okamoto, Koizumi et al. 2007).

A.2.2 Evidence for Cholesterol-Independent Effects – Basic Science.

Animal studies have provided convincing evidence for cholesterol-independent effects of statins (Figure A4). These studies may also provide mechanistic insight into the clinically observed cholesterol independent effects of statins outlined in the previous section (Section A.2.1). Central to many of these effects may be a statin-mediated, improvement in endothelial function. Numerous cell culture experiments have provided evidence of a statin-mediated effect on endothelial nitric oxide synthase, a major source of nitric oxide in the vasculature. For example, chronically

exposing human umbilical vein endothelial cells (HUVEC) to 3% O_2, resulting in hypoxia, caused a 9-fold decrease in mRNA encoding for endothelial nitric oxide synthase (eNOS). Simvastatin (1 μM for 24 hours) prevented this down regulation and was associated with increased nitric oxide production (Laufs, Fata et al. 1997). A subsequent study using HUVEC cells found that simvastatin (1 μM for 30 minutes) increased the PKB-mediated phosphorylation of eNOS, resulting in increased enzyme activation and nitric oxide production (Kureishi, Luo et al. 2000).

Statins may also improve endothelial function by modulating vasoconstrictor production. The potent endothelial derived vasoconstrictor, endothelin-1 has a circulating concentration of 1.4 pmol/L but rises to 3.2 pmol/L in patients with symptomatic atherosclerosis (Lerman, Edwards et al. 1991) suggesting a central role for endothelin-1 in endothelial dysfunction. In bovine aortic endothelial cells, incubation with atorvastatin or simvastatin (10 μM for 24 hours) reduced endothelin-1 mRNA expression by 60-70% with a concomitant reduction in endothelin-1 protein levels of 25%-50% (Hernández-Perera, Pérez-Sala et al. 1998). These results were confirmed by the same group using similar conditions with nuclear run-on experiments and transfection studies with the preproET-1 promoter that identified a statin-mediated transcriptional down regulation of the endothelin-1 gene (Hernández-Perera, Pérez-Sala et al. 2000).

Angiotensin II is another potent vasoconstrictor whose upregulation is associated with cardiovascular pathology (Ferrario and Strawn 2006). In cultured vascular smooth muscle cells both cerivastatin and fluvastatin (10 μM for 24 hours) reduced the angiotensin type I receptor (AT_1-R) mRNA and protein level, and reduced AT_1-R

promoter activity as assessed by luciferase assay. Consistent with receptor down regulation, treatment of VSMCs for 24 hours with cerivastatin reduced the fura 2-AM Ca^{2+} response to angiotensin II. Interestingly these results were not reproducible with the hydrophilic pravastatin suggesting the pleiotropic effects of statins may differ depending on the type of statin utilized (see Section A1.4) (Ichiki et al., 2001).

Also of interest in endothelial dysfunction is the production of reactive oxygen species (ROS) as elevated levels of ROS lead to impaired vasoconstriction, vascular damage and dysfunction (Ogita and Liao 2004). The inhibition of ROS production may account for some of the clinically noted cholesterol independent effects of statins. The exposure of vascular smooth muscle cells to angiotensin II increases ROS production via the activation of NAD(P)H oxidase (Castro, JimÈnez et al. 1993). One study reported that atorvastatin attenuated AT_1 receptor mediated ROS production. To address the mechanism of this effect AT_1 receptor mRNA levels were measured and were found to be reduced in the vasculature of the rat after 30 days of atorvastatin treatment (50 mg / kg per day) (Wassmann, Laufs et al. 2001). In cultured rat VSMC 12 hours of atorvastatin treatment (10 μM) down regulated mRNA expression of the NAD(P)H subunit nox1 as assessed by northern blot analysis. The membrane translocation of Rac1 GTPase, which is essential for the activation of NAD(P)H, was also inhibited. The same authors assessed ROS production using lucigenin chemiluminescence in spontaneously hypertensive rats and found ROS production was reduced in aortic segments from rats treated for 30 days with atorvastatin (50 mg / kg) (Wassmann, Laufs et al. 2002). (Section A2.1).

There is evidence to suggest that statins may reduce infarct size in experimental

models of ischemia/infarction. This infarct limiting effect may partially account for the positive clinical outcomes of statins in ischemic stroke, as discussed in Section A2.1. One study injected normocholesterolemic mice with simvastatin for 14 days before occlusion of the middle cerebral artery to induce ischemia. Cerebral infarct size was decreased by 18, 27 and 46% with doses of 0.2, 2.0 and 20 mg/kg of simvastatin respectively. Importantly, serum cholesterol levels were not altered in the statin treated group (Endres, Laufs et al. 1998). Another study used the Langendorff perfused heart technique (Section B.2) to assess coronary flow and cardiac contractile function in the presence of ischemia in normocholestoremic rats. Hearts were exposed to 20 minutes of ischemia by abolishing coronary flow, followed by 45 minutes of reperfusion. Pretreatment of rats with simvastatin (25 μg per rat) significantly improved coronary flow and developed left ventricular pressure after ischemia. The time course, the type of injury imposed, and the use of an excised heart model make it unlikely that changes in cholesterol had any bearing on these results (Lefer, Campbell et al. 1999). Numerous animal studies have linked inhibition or deletion of endothelial nitric oxide synthase (eNOS) with increased infarct size in response to ischemic challenge in both the brain and myocardium (Huang, Huang et al. 1994; Bolli 2001), thus the improvements noted in the aforementioned studies may be due to increased nitric oxide production.

Both animal and cell culture experiments have provided evidence of statin mediated immunomodulation that confirm clinical observations of anti inflammatory effects (Section A2.1). Simvastatin was administered to normocholesterolemic rats (25 μg per rat via IP) 18 hours before superfusion of the nitric oxide synthase inhibitor L-NAME into the externalised mesenteric artery. Pretreatment with simvastatin

attenuated the increased leukocyte rolling and adherence to the mesenteric endothelium associated with L-NAME treatment (Pruefer, Scalia et al. 1999). Within atherosclerotic lesions, smooth muscle and endothelial cells express increased levels of leukocyte adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1) (Ross 1993). Statins inhibit the tumor necrosis factor-α induced upregulation of ICAM-1 by inhibiting ERK kinase activity (Chung, Lee et al. 2002). Similarly, cultured HUVECs incubated in a high insulin medium with or without cerivastatin had increased ICAM-1 expression in response to high insulin that was attenuated with pretreatment with cerivastatin at 10 nM for 24 hours (Okouchi, Okayama et al. 2003). Finally, in hypercholesterolemic humans, 40 mg of simvastatin daily for 6 weeks reduced the expression of ICAM-1 on blood mononuclear cells. (Rezaie-Majd, Prager et al. 2003).

As well as effects on ICAM-1 statins may reduce the expression of the inducible endothelial cell adhesion molecules, vascular cell adhesion molecule (VCAM-1) and E-selectin (Rasmussen, Hansen et al. 2001). Statins also reduce CD40 expression in a dose dependent fashion (5 μM to 80 μM of simvastatin) in human endothelial cells, smooth muscle cells, and macrophages as assessed by western blot and flow cytometry (Mulhaupt, Matter et al. 2003). Furthermore, statin treated subjects display reduced staining for CD40 in atherosclerotic lesions compared to untreated patients (Mulhaupt, Matter et al. 2003). It is interesting to note that CD40 has been implicated in other inflammatory disorders such as rheumatoid arthritis and multiple sclerosis (Foy, Aruffo et al. 1996) and therefore may account for the positive effects of statins in these disease states mentioned in Section A.2.1.

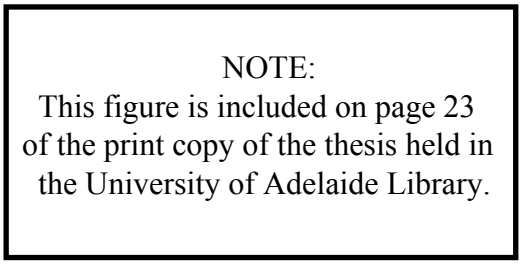

Figure A4. Pleiotropic Effects of Statins in the Cardiovascular System. Numerous cholesterol-independent effects of statins have been reported in the vasculature. Figure adapted from Mason, 2003.

A.2.3 General Mechanisms of Statin Cholesterol-Independent Effects.

A putative mechanism for many of the cholesterol-independent effects of statins involves the inhibition of isoprene formation. As mentioned in section A.1.3 statins inhibit the synthesis of mevalonate. Mevalonate is the primary precursor of isopentenyl pyrophosphate, which in turn is the precursor to the isoprenoids, specifically the 15-carbon farnesyl pyrophsphate (FPP) and the 20-carbon geranylgeranyl pyrophosphate (GGPP) (Figure A5) (Goldstein and Brown 1990). Both FPP and GGPP serve as hydrophobic post-translational modifications to a wide variety of proteins of which there are over 100 in the human genome (Takai, Sasaki et al. 2001). These include, G-proteins, nuclear lamins and small GTPases such as Rho, Rab, Rac and Ras (Zhang and Casey 1996). Once attached to a protein, these lipophilic isoprene groups facilitate interaction with the plasma membrane and lipophilic regions of other proteins. Translocation to the plasma membrane is an important step in the activation of G-proteins and small GTP-binding binding proteins.

RhoA is a monomeric GTPase that cycles between an inactive GDP-bound form found in the cytosol, and an active GTP-bound form localized to the plasma membrane (Zhang and Casey 1996; Hall 2005). The addition of a GGPP moiety is required to enable correct membrane targeting of RhoA and allows for the activation of its downstream effector, Rho-kinase. Statins, by inhibiting prenylation, and thus RhoA activation, may prevent the activation of Rho-kinase.

Although there is little direct evidence in the literature demonstrating statin mediated modulation of prenylation, there is a convincing body of indirect evidence. For

example, in cultured human anaplastic thyroid cancer cells, 48 hours of lovastatin (25 μM) treatment decreased the amount of RhoA and Rac1 present in the plasma membrane, as assessed by membrane/cytosol fractionation and western-blot analysis. Furthermore, the statin mediated reduction in RhoA/Rac1 membrane targeting was prevented by metabolic rescue involving the direct addition of GGPP (Zhong, Wang et al. 2003). More recently, in cultured human prostate cells, the addition of exogenous GGPP inhibited lovastatin-mediated G1 stage cell cycle arrest and cell senescence. Interestingly, transfection of these cells with constitutively active RhoA also inhibited these effects (Lee, Lee et al. 2006). More relevant to the cardiovascular system is the finding that incubation with GGPP abolished the mevastatin mediated increase in endothelial nitric oxide expression in cultured human endothelial cells, and that in this same cell line mevastatin decreased RhoA membrane translocation and GTP binding, which was again reversible with GGPP (Laufs and Liao 1998). Collectively these studies implicate the inhibition of protein prenylation, specifically GGPP attachment to RhoA, as an important component by which statins alter cellular function.

Figure A5 Statin Mediated Inhibition of Isoprenoid Production. As well as inhibiting cholesterol biosynthesis, statins inhibit the production of isoprenoid intermediates such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). FPP and GGPP are lipid moieties important in the posttranslational modification of signaling proteins, including Rho, Rac and Ras. Modified after Wilson, D.

A.3 Vascular Smooth Muscle

Vascular smooth muscle can contract and relax altering luminal diameter and thus is a major regulator of vasoconstriction and hense blood flow in the vascuature. The following section will review the mechanisms of vascular smooth muscle contraction and relaxation. A role for statins in the direct modulation of smooth muscle contraction will also be explored.

A.3.1 Vascular Smooth Muscle Contraction

A.3.1.1 The Contractile Apparatus

Smooth muscle contains ordered thick and thin filaments, however, not as ordered as skeletal and cardiac muscle and consequently, it lacks a distinctive striated pattern. Numerous studies using electron micrography and immunohistochemistryhave shown the presence of dense bodies, thought to be akin to Z-disks found in striated muscle (Bond and Somlyo 1982). Symmetrically aligned throughout the cytoplasm, dense bodies consist mainly of the actin binding protein α-actinin and serve as attachment sites for actin filaments (Stephens, Li et al. 1998). These thin actin filaments are arranged such that contraction by cross bridge cycling pulls these dense bodies towards one another, resulting in contraction and force generation.

The thick filaments are composed primarily of myosin hexamers containing two 200 kDa heavy chain subunits that form a coiled-tail region, and a pair of 20 kDa regulatory and 17 kDa essential light chains. The light chains form globular heads that contain both actin and Mg^{2+}ATP binding sites. The myosin heads protrude from the

thick filaments and interact with the actin thin fibers to form cross bridges (Hai and Murphy 1989).

A.3.1.2 Regulation of the Contractile Apparatus

Smooth muscle contraction is regulated by phosphorylation of the 20 kDa regulatory light chains of myosin (LC_{20}). Specifically, it is Ser19 of LC_{20} that is the major target of phosphorylation and thus regulation (Ikebe and Reardon 1990). In response to elevated cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$), Ca^{2+}/calmodulin regulated myosin light chain kinase (MLCK) phosphorylates Ser19 of LC_{20}, resulting in conformational changes in the myosin head that induce actin binding and a 100-fold increase in ATPase activity (Stull, Gallagher et al. 1991).

Although other LC_{20} residues are phosphorylated *in vitro* it is contentious as to whether these are biologically significant sites of regulation. Thr18 has been reported to be phosphorylated by protein kinase-C (PKC) and intergin-linked kinase (ILK) (Deng, Van Lierop et al. 2001). However, large quantities of these kinases were required to affect this phosphorylation, suggesting that the phosphorylation of this site may not occur in the context of contraction. In contrast proliferating vascular smooth muscle cells in culture have been shown to contain considerably more dual Ser19 / Thr18 phosphorylation (Gong, Gorenne et al. 2001), perhaps suggesting a role for Thr18 phosphorylation in cell division and proliferation.

Smooth muscle relaxation occurs through the dephosphorylation of Ser19 LC_{20} by myosin light chain phosphatase (MLCP), a specific type 1 protein phosphatase (PP1cδ) targeted to the myofilament by its specific 130 kDa myosin targeting subunit

(MYPT1). Therefore, it is the ratio of MLCK to MLCP1 activity that ultimately determines the contractile state of smooth muscle (Somlyo and Somlyo 2003). Figure A6 provides an outline of the determinates of smooth muscle contractility.

A.3.1.3 Ca^{2+} and Smooth Muscle Contraction

Free intracellular Ca^{2+} ($[Ca^{2+}]_{cyt}$), is the most important second messenger regulating smooth muscle contraction. Its importance is reflected in the exquisite regulation of $[Ca^{2+}]_{cyt}$, which is held at submicromolar concentrations at rest rising to 500 to 700 nmol in stimulated vascular smooth muscle cells (Himpens, Missiaen et al. 1995; Lee, Poburko et al. 2002). When Ca^{2+} enters the cytosol in smooth muscle cells it binds to myofilament-associated calmodulin that undergoes a Ca^{2+} dependent conformational change, causing the activation of actin tethered MLCK (Stull, Gallagher et al. 1991; Wilson, Sutherland et al. 2002) and consequently the phosphorylation of Ser19 LC_{20}.

A.3.1.4 Intracellular Ca^{2+} Entry

Neurotransmitters, hormones and locally released factors including norepinephrine, endothelin-1, angiotensin II and thromboxane A_2 bind to extracellular plasma membrane G-protein coupled receptors and activate associated intracellular G-proteins. Many of these G-protein coupled receptors activate phopholipase Cβ (PLCβ) (Ushio-Fukai, Griendling et al. 1998). PLCβ cleaves the membrane lipid, phosphatidylinositol 4,5-bisphosphate, generating insitol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates Ca^{2+} release from the SR by binding to SR IP_3 receptors that also act as Ca^{2+} channels. This results in increased Ca^{2+}/calmodulin dependent MLCK activation, increased LC_{20} phosphorylation and finally contraction (Taylor and Stull 1988).

A.3.1.5 Extracellular Ca^{2+} Entry

Voltage operated channels, predominately large conductance 25 pS L-type Ca^{2+} channels (also called $Ca_{V1.2}$) are an important source of extracellular Ca^{2+} entry. This realization has led to the development and clinical use of L-type Ca^{2+} channel blockers. In addition, several reports have indicated that the 7 pS voltage gated T-type Ca^{2+} channels, of the $Ca_{V3.X}$ family, may also regulate vascular smooth muscle contraction (Beltrame, Turner et al. 2004; Ball, Wilson et al. 2009).

The agonist-induced production of DAG activates protein kinase C (PKC). PKC has been documented to be involved in K^+ channel mediated membrane depolarization and activation of voltage gated L and T-type Ca^{2+} channels (Standen and Quayle 1998; Rainbow, Norman et al. 2009). There is also evidence that DAG can directly activate non-selective cation channels of the TRPC family independently from PKC and that this form of Ca^{2+} or Na^+ entry is of physiological importance in membrane depolarization and consequently the response to α_1-receptor activation (Helliwell and Large 1997; Hofmann, Obukhov et al. 1999; Inoue, Okada et al. 2001) Furthermore, PKC activation may cause phosphorylation of L-type Ca^{2+} channels and thus, may directly modulate extracellular Ca^{2+} entry (Ren, Zhang et al. ; Shistik, Ivanina et al. 1998). PKC also has Ca^{2+} independent effects, which may sensitize the contractile apparatus; this will be discussed in more detail below (Section A.3.2).

A.3.1.5 The Myogenic Response

The myogenic response, in which resistance arteries contract in response to increased pressure and relax due to decreased pressure, is essential in establishing basal tone. It

is also important in many physiological processes such as autoregulation of blood flow in response to altered local metabolism, reactive hyperemia and acute pressure changes, such as that occurring during orthostasis. The exact mechanism by which mechanical deformation is transduced is still equivocal but it is likely due to mechanically sensitive cation channels and chloride channels (Hill, Zou et al. 2001). Upon activation these channels depolarize the plasma membrane, opening voltage gated Ca^{2+} channels. This results in a rise in $[Ca^{2+}]_{cyt}$ and contraction (Hill, Davis et al. 2006). There is also recent evidence to suggest a Ca^{2+} independent mechanism of contraction is involved in regulating myogenic tone (Johnson, El-Yazbi et al. 2009).

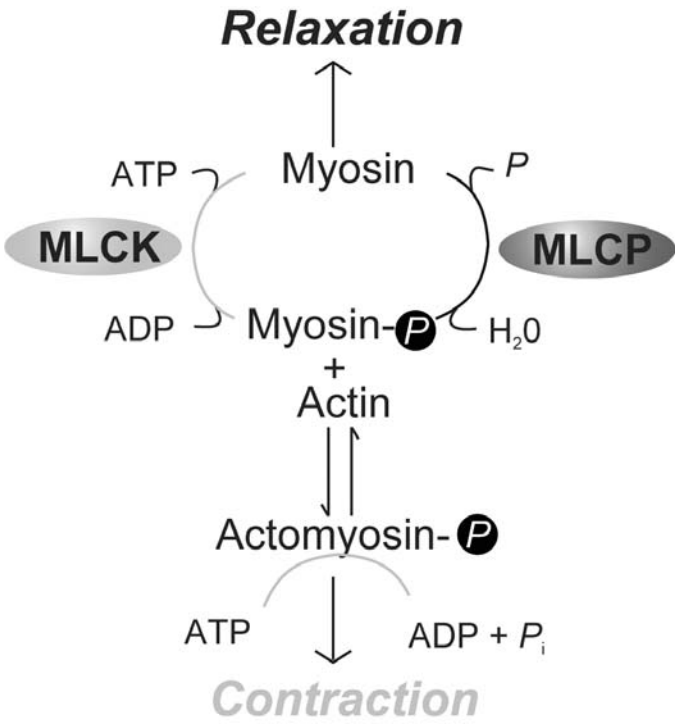

Figure A6. The Determinates of Vascular Smooth Muscle Contraction. Increased $[Ca^{2+}]_{cyt}$ results in the Ca^{2+}/calmodulin-mediated activation of MLCK which phosphorylates LC_{20} resulting in cross bridge cycling and force generation. The activation of MLCP decreases LC_{20} phosphorylation and favors relaxation. Thus, the extent of MLCK to MLCP activation determines the level of LC_{20} phosphorylation and force generation. Modified after Wilson, D.

A.3.2 Ca^{2+} Sensitization

Bradley and Morgan elucidated the concept of Ca^{2+} sensitization in 1987. They simultaneously measured isometric force and $[Ca^{2+}]_{cyt}$ using the Ca^{2+} sensor aequorin. Their results suggested that agonists such as the thromboxane A_2 mimetic, U-46619 and prostaglandin $F_{2\alpha}$ produced greater force at a given $[Ca^{2+}]_{cyt}$ compared to contractions induced by depolarizing the plasma membrane with K^+. The authors suggested that this may reflect changes in the sensitivity of the contractile apparatus to $[Ca^{2+}]_{cyt}$ in response to receptor mediated G-protein activation (Bradley and Morgan 1987).

Somlyo and Somlyo were the first to show that G-protein activation increases the sensitivity of the contractile apparatus to Ca^{2+} via the inhibition of myosin light chain phosphatase (MLCP). Rabbit portal vein strips were permeabilised using α-toxin and $[Ca^{2+}]_{cyt}$ fixed at a constant concentration. To remove any confounding effects MLCK was inhibited using ML-9. Both isometric tension and LC_{20} phosphorylation were measured. Incubating with the α_1-agonist, phenylephrine, decreased both the rate of relaxation and the rate of LC_{20} dephosphorylation, indicating that phenylephrine was inhibiting MLCP (Somlyo and Somlyo 1994).

MLCP is composed of a 38-kDa type I protein phosphatase, specific to Ser19 of LC_{20}, as well as a 130 kDa targeting subunit (MYPT1) and 20-kDa subunit about which little is known (Figure A7) (Hartshorne, Ito et al. 2004). In isolation PP1c demonstrates broad substrate specificity, however, Terrak *et al*., demonstrated that the addition of the MYPT1-targeting subunit conferred exquisite specificity to the PP1c for LC_{20} (Terrak, Kerff et al. 2004). In addition, the inhibition of MLCP has been

ascribed to the phosphorylation of the MYPT1 targeting subunit. Specifically, the original work documented that purified MLCP could be phosphorylated at Thr697 (numbering used throughout is from the rat isoform) by an unidentified co-purifying kinase and this phosphorylation inhibited phosphatase activity (Ichikawa, Ito et al. 1996). In another study, treating α-toxin permeabilised smooth muscle strips with ATPγS, which is used as substrate by protein kinases and results in phosphatase resistant thiophosphorylation, increased Ca^{2+} sensitivity and LC_{20} phosphorylation. The authors found a 5-fold decrease in MLCP activity when MYPT1 was thiophosphorylated, providing evidence that this phosphorylation has a biologically significant effect (Trinkle-Mulcahy, Ichikawa et al. 1995).

More recently it has been found that phosphorylation of the Thr855 (numbering used throughout is from the rat isoform) site also inhibits MLCP activity by dissociating MYPT1 from the PP1c (Murányi, MacDonald et al. 2002; Velasco, Armstrong et al. 2002; Muranyi, Derkach et al. 2005). Phosphorylation at Thr855 has been implicated in biologically significant processes such as regulation of the myogenic response (Johnson, El-Yazbi et al. 2009) and regulation of a_1-adrenoreceptor and thromboxane A_2 receptor-mediated contraction (Stevenson, Matthew et al. 2004; Wilson, Susnjar et al. 2005; Dimopoulos, Semba et al. 2007). Interestingly, in two of these studies there was apparently no role for Thr697 phosphorylation.

Figure A7. The Structure of Myosin Light Chain Phosphatase. Myosin phosphastase consists of three subunits: a PP1cδ catalytic subunit, a regulatory targeting subunit (MYPT1) and the M20 subunit. Phosphoylated CPI-17 binds directly to the PP1cδ subunit, inhibiting MLCP activity. Phosphorylation of Thr697 MYPT1 also inhibits PP1cδ activity, while phosphorylation of Thr855 MYPT1 dissociates the PP1cδ from MYPT1 reducing its specificity and activity towards LC_{20}. Figure adapted from Hartshorne, 2004.

A.3.3 RhoA / Rho-kinase and Ca^{2+} Sensitization

The small GTPase RhoA is abundantly expressed in smooth muscle (Hirata, Kikuchi et al. 1992; Worth, Campbell et al. 2004). Hirata and colleagues provided the first evidence that the small GTPase RhoA might be the link between G-coupled protein receptor activation and MYPT1 phosphorylation. In saponin skinned rabbit mesenteric artery, the addition of Ca^{2+} generated force in a dose-dependent fashion. However, the amount of force generated at a given Ca^{2+} concentration was increased by the addition of the non-hydrolysable GTP analogue, GTPγS. This sensitization was abolished after incubation with EDIN or C3, both bacterial exoenzymes that ADP-ribosylate and inhibit RhoA (Hirata, Kikuchi et al. 1992). Numerous studies have since provided evidence for the role of RhoA in inhibiting MLCP (Fujita, Takeuchi et al. 1995), furthermore, RhoA/Rho kinase mediated phosphorylation of MYPT1 has been associated with pathologies such as hypertension (Uehata, Ishizaki et al. 1997; Seko, Ito et al. 2003).

RhoA associated kinase (Rho-kinase), the downstream mediator of RhoA, was identified and cloned by Amano and colleagues (Amano, Ito et al. 1996). It was found that phosphorylation of MYPT1 by Rho-kinase inhibited phosphatase activity. Subsequently the substrates for Rho-kinase were identified as Thr697 and Thr855 (Kimura, Ito et al. 1996; Kawano, Fukata et al. 1999). In one study phospho specific antibodies to Thr697 were raised and the authors demonstrated that the stimulation of Swiss 3T3 cells by the RhoA activating lysophosphatidic acid increased phosphorylation of MYPT1 at Thr697 and that this effect could be inhibited by the Rho-kinase inhibitor Y27632 (Feng, Ito et al. 1999). Figure A8 provides a simplified overview of RhoA mediated Ca^{2+} sensitization.

Figure A8 Regulation of Smooth Muscle Contraction by the RhoA/Rho-kinase Pathway. In vascular smooth muscle the activation of RhoA leads to activation of Rho-kinase, which phosphorylates MYPT1 at Thr697 and Thr855 inhibiting MLCP activity and increasing LC_{20} phosphorylation and force generation. RhoA/Rho-kinase mediated Ca^{2+} sensitization can be inhibited by the Rho-kinase inhibitors fasudil and H1152. The ability of statins to inhibit RhoA membrane targeting and thus Rho-kinase activation suggests that these agents may have a similar effect to direct Rho-kinase inhibition. Modified after Wilson, D.

Despite a large body of evidence supporting a role for Rho-kinase in mediating Ca^{2+} sensitization other kinases may also phosphorylate MYPT1. Haystead and colleagues demonstrated that zipper interacting protein kinase (ZIPK) co-localizes with MLCP and that it has the capacity to phosphorylate MYPT1 resulting in Ca^{2+} sensitization and contraction (MacDonald, Borman et al. 2001; MacDonald, Eto et al. 2001). The authors suggested that ZIPK might represent the "governance" of MYPT1 phosphorylation (Haystead 2005). In addition, integrin linked kinase (ILK) also has the capacity to phosphorylate MYPT1 at Thr697 and Thr855 (Murányi, MacDonald et al. 2002). These results suggest that Rho-kinase may not directly phosphorylate MYPT1, instead, phosphorylation may be mediated by a cascade of kinases including Rho-kinase, ZIPK, and ILK. However, it is still unknown if ZIPK and ILK are upstream or downstream mediators between Rho-kinase and MYPT1 or if they represent alternative pathways.

A.3.4 MLCP Splice Variants

The existence of MLCP isoforms may provide another means by which Ca^{2+} sensitization of contraction may be regulated. To date four MLCP isoforms have been identified in mammals (Huang, Fisher et al. 2004).

The alternative splicing of a 3' exon results in the production of a lucine zipper positive or negative isoform of MLCP (Huang, Fisher et al. 2004). It has been suggested that MLCP may be activated by the interaction of PKG with this region resulting in reduced LC_{20} phosphorylation, and consequently, relaxation (Surks, Mochizuki et al. 1999). Brozovich's group has shown that the association of PKG with MLCP can be detected via immunoprecipitation only in tissues that expresses the

leucine zipper positive isoform of MYPT1. Furthermore, the sensitivity to cGMP-mediated smooth muscle relaxation correlates with the ratio of expression of lucine zipper positive / lucine zipper negative isoforms (Khatri, Joyce et al. 2001). This data, combined with evidence that suggests that this ratio is heterogeneous between tissue beds, species and between normal and diseased vessels (Huang, Fisher et al. 2004), suggests a mechanism that may partially account for alterations in vascular reactivity in these tissues/conditions.

Two other MLCP splice variants found in mammals appear to vary little between tissue types. The regulatory roles, if any, for these variants are not yet known, however, this splicing occurs close to the Thr695 residue (Dirksen, Vladic et al. 2000), which is involved in the regulation of MLCP, suggesting a possible mechanism by which these splice variants could affect contractility. Further studies will be required to determine if this is significant in the context of vascular regulation.

A.3.5 PKC/CPI-17 and Ca^{2+} Sensitization

Several groups have noted that activation of PKC by phorbol esters or by DAG results in increased LC_{20} phosphorylation and force generation independent of $[Ca^{2+}]_{cyt}$ (Rokolya, Barany et al. 1991; Gailly, Gong et al. 1997) and that this sensitization is insensitive to inhibition by RhoA and Rho-kinase inhibitors (Fu, Gong et al. 1998; Gong, Gorenne et al. 2001). These results suggest a RhoA independent mechanism of Ca^{2+} sensitization mediated by PKC activation.

Eto and colleagues discovered and cloned a phosphorylation dependent phosphatase type 1 inhibitor CPI-17 (C-kinase potentiated inhibitory protein of 17 kDa) (Eto,

Ohmori et al. 1995). They found that CPI-17 was highly expressed in smooth muscle tissue such as aorta and bladder, with modest expression found in striated muscle and in the brain (Eto, Senba et al. 1997). It is, however, noteworthy that all tissue is vascularised and will consequently contain smooth muscle and CPI-17. Using column chromatography two activators of CPI-17 were purified from aortic extracts. These were Ca^{2+} dependent PKCα and Ca^{2+} independent PKCδ (Eto, Kitazawa et al. 2001). In contrast, studies using affinity chromatography found interactions between CPI-17 and all isoforms of PKC (Zemlickova, Johannes et al. 2004). The phosphorylation of CPI-17 at Thr38 by PKC transforms the protein into a 6000-times more potent MLCP inhibitor (Senba, Eto et al. 1999).

Evidence suggests that when PKC is activated it induces CPI-17 dependent Ca^{2+} sensitization of contraction. Stimulation of femoral artery smooth muscle with histamine or phenylephrine results in Ca^{2+} sensitization accompanied by Thr38 CPI-17 phosphorylation. Furthermore, a component of this contraction is insensitive to Rho-kinase inhibitors (Kitazawa, Eto et al. 2000). Recently, Dimopoulos and colleagues found that phenylephrine induces CPI-17 phosphorylation within 7 seconds following stimulation and that the time course of phosphorylation mirrored the rapid increase in LC_{20} phosphorylation and force generation. They also found that part of this effect could be abolished with a PKC inhibitor. In contrast, MYPT1 phosphorylation occurred over a slower time course, with peak phosphorylation occurring after 1 minute. The MYPT1 component of sensitization could be abolished by a Rho-kinase inhibitor (Dimopoulos, Semba et al. 2007). This data suggests that PKC/CPI-17 may modulate the rapid onset of contraction while the RhoA/Rho-kinase pathway may modulate the slow development and maintenance of contraction.

CPI-17 may have varying importance depending on smooth muscle type and agonist. For example, Wilson and colleagues found that PKC inhibition had no effect on contraction mediated by activation of the thromboxane A_2 receptor and that no phosphorylation of CPI-17 could be detected by western blot (Wilson, Susnjar et al. 2005). However, in porcine coronary artery the activation of the same receptor caused contraction that was sensitive to PKC inhibition (Nobe and Paul 2001). A possible explanation for the differing vascular responses may be due to differences in the expression levels of MLCP and CPI-17 between vascular beds (Woodsome, Eto et al. 2001). There is also some evidence that Rho-kinase may directly phosphorylate CPI-17, suggesting cross talk between sensitization pathways. For example, in rabbit aortic smooth muscle cells the activation of Rho-kinase inhibited MLCP by causing the phosphorylation of Thr38 of CPI-17 (Pang and Guo, 2005). Recent evidence also suggests that CPI-17 may be activated by ZIPK, ILK, and p21 activated kinase (Deng, Sutherland et al. 2002; Eto 2009), however, although these kinases are biochemically capable of phosphorylating MYPT1 and CPI-17, the lack of selective pharmacological blockers has limited our understanding of the physiological relevance of these kinases in Ca^{2+} sensitization.

It is worth noting that dissecting Rho-kinase-mediated inhibition of MLCP from CPI-17 mediated inhibition is confounded by the finding that the common Rho-kinase inhibitors such as fasudil and Y-27632 have the capacity to inhibit certain isoforms of PKC (Walsh et al., in Moorhead 2007; Eto, Kitazawa et al. 2001). To date no information is available regarding the specificity of the new generation Rho-kinase inhibitor H1152.

A.3.6 Ca^{2+} Desensitization

In contrast to activation, inhibition of the RhoA/Rho-kinase pathway may lead to the phenomena of Ca^{2+} desensitization, in which reduced levels of contraction are developed for a given $[Ca^{2+}]_{cyt}$. This was first demonstrated in permeabilised rabbit ileum smooth muscle in which 8-bromo-cyclic guanosine monophosphate (8-br-cGMP) caused relaxation of GTPγS mediated contraction at a fixed Ca^{2+} level (Wu, Somlyo et al. 1996). The exact mechanism of Ca^{2+} desensitsation is still under debate. Sauzeau et al, characterized an inactivating phosphorylation site on Ser188 RhoA that could be phosphorylated by PKG (Sauzeau, Le Jeune et al. 2000). The inhibition of RhoA could potentially reduce Rho-kinase activation and thus, the inhibitory phosphoylation of MYPT1 at Thr855 and Thr697. Furthermore, mass spectrometry has provided evidence that PKG may phosphorylate MYPT1 at Ser696 and that this prevents the Rho-kinase mediated phosphorylation of the inhibitory Thr697 site, resulting in activation of MLCP and decreased contraction at a given level of Ca^{2+} (Wooldridge, MacDonald et al. 2004). In contrast, more recent work by Nakamura using antibody detection has suggested that PKG mediated Ser696 phosphorylation and Thr697 phosphorylation may not be mutually exclusive, however, Ser696 phosphorylation still leads Ca^{2+} desensitization (Nakamura, Koga et al. 2007).

A.3.7 Modulation of Smooth Muscle Function by Statins

There is evidence that statins may directly regulate $[Ca^{2+}]_{cyt}$ in vascular smooth muscle cells. Using the Ca^{2+}-sensitive fluorophore, fura 2-AM, to measure $[Ca^{2+}]_{cyt}$, a 24 hour simvastatin treatment (5 mg/L) reduced Ca^{2+} entry in response to vasopressin treatment in cultured rat vascular myocytes. The direct addition of mevalonate, the

downstream metabolite of HMG-CoA, restored Ca^{2+} entry to that of cells without simvastatin treatment (Ng, Davies et al. 1994). Although changes in plasma membrane cholesterol concentration have been shown to modulate Ca^{2+} entry, presumably via altered ion channel function (Bialecki, Tulenko et al. 1991), the authors found no associated changes in cellular cholesterol (Ng, Davies et al. 1994). However, inositol-1,4,5 trisphosphate production was reduced suggesting a reduction in G-protein coupled receptor activation. Similar to RhoA the hetrotrimeric G proteins G_o and G_q also undergo prenylation on their gamma subunits (Kisselev, Ermolaeva et al. 1995; Wedegaertner, Wilson et al. 1995). Statins may modulate the activity of these G proteins by reducing prenylation mediated targeting, thus reducing IP_3 and DAG production and thereby modulating TRPC channel mediated activation of voltage gated Ca^{2+} channels and reducing IP_3 mediated SR Ca^{2+} release. It is important to emphasize, however, that RhoA has an important role in cell division that is quite different from its role in modulating vascular contraction (Hall 2005). Consequently, caution must be taken in extrapolating the role of statins in modulating RhoA in cultured cells to quiescent contractile cells.

A number of studies have examined the effect of statin treatment on $[Ca^{2+}]_{cyt}$ in intact vessels. For example, isolated aortic rings were subject to a range of fixed extracellular Ca^{2+} concentrations prior to activation of voltage sensitive Ca^{2+} channels using 80 mM KCl. In the presence of simvastatin (3 μM) the force Ca^{2+} relationship was shifted to the right, possibly indicating reduced Ca^{2+} entry at a given concentration of extracellular Ca^{2+}. The authors found that this effect persisted after the endothelium was mechanically removed, indicating that this was a direct smooth muscle effect independent from endothelial derived factors (Alvarez de Sotomayor,

Pérez-Guerrero et al. 2001). A similar study found that vessels incubated with simvastatin or atorvastatin (50 μM) for 24 hours displayed decreased contraction in response to the α_1-adrenoreceptor agonist, phenylephrine (0.01-100 μM). These effects were reversed by the addition of mevalonate, the direct metabolite of HMG-CoA. Interestingly, vessels placed in Ca^{2+} free buffer and subsequently stimulated with phenylephrine displayed a transient contraction mediated by Ca^{2+} release, presumably from the SR, that could be abolished by treatment with simvastatin or atorvastatin (Tesfamariam, Frohlich et al. 1999).

There is also evidence that statins modulate some forms of agonist mediated smooth muscle contraction independent of the mevolanate pathway. Simvastatin and lovastatin (10 μM), relaxed segments of basilar artery without an endothelium but which had been preconstricted with serotonin or high-K^+ solution. Unlike the aforementioned studies, this effect was not abolished by the addition of mevalonate which indicates the involvement of a completely different mechanism or altered kinetics of mevalonate metabolism in this preparation. In the same study basilar artery strips were mounted in a pressure myograph and $[Ca^{2+}]_{cyt}$ levels measured using Fura 2-AM. The relaxation seen with statin treatment was associated with reduced $[Ca^{2+}]_{cyt}$ and a reduced Mn^{2+} quench rate. Using patch clamp techniques the authors measured Ca^{2+} current in isolated basilar artery cells and showed that statin treatment inhibited L-type Ca^{2+} current (Bergdahl, Persson et al. 2003). Unfortunately they did not report whether a nonselective cation channel current mediated by PLC/DAG was inhibited by statins, as this could account for the change in L-type Ca^{2+} current activation.

A.3.8 Statin Mediated Modulation of Myosin Phosphatase

Presently, there are no studies that have looked directly at a possible effect of statins on MLCP phosphorylation state, however, there is indirect pharmacological evidence of a statin mediated attenuation of the RhoA/Rho-kinase pathway. Specifically, mevastatin was chronically infused via mini-osmotic pump (20 mg/kg per day for 14 days) in eNOS knockout mice. Isolated thoracic aortic rings from these mice were mounted on a myograph and the response to serotonin and phenylephrine were recorded. Agonist mediated contraction was reduced in statin-treated mice, however, no effect was seen on KCl induced contraction, which suggests that mevastatin was not directly inhibiting Ca^{2+} entry. These data are consistent with mevastatin modulating the activation of RhoA/Rho-kinase because of the receptor dependence of this effect (Budzyn, Marley et al. 2004). To this authors knowledge there is currently no other literature that examines the possible modulation of myosin phosphatase by statins. This thesis will address this question in Section D.

A.4 The Endothelium

The vascular endothelium forms the inner lining of blood vessels. Originally thought to be an inert barrier, the endothelium is now recognized as a major autocrine/paracrine organ. The following section outlines the role of the endothelium in health and pathology, specifically in relation to vascular smooth muscle contractility. A role for statins in regulating the endothelium will be explored.

A.4.1 The Endothelial Regulation of Vascular Smooth Muscle

The endothelium influences vascular resistance by modulating the activity of the underlying vascular smooth muscle. The endothelium responds to changes in blood pressure, flow, oxygen tension and metabolite concentration by secreting vasodilators including nitric oxide and prostaglandins and vasoconstrictors such as endothelin-1. The endothelium may also modulate smooth muscle contraction by degrading vasoactive substances including serotonin and norepinephrine (Shepro and Dunham 1986) and by converting inactive precursors such angiotensin II into their active forms (Caldwell, Seegal et al. 1976)

The first evidence that the endothelium is a major regulator of smooth muscle contraction came from Furchgott and colleagues (Furchgott, Cherry et al. 1984). Their noble prize winning experiments explained why acetylcholine (ACh), a vasodilator *in vivo*, caused constriction in some isolated vessels. They found that carefully dissected rabbit aortic rings would relax to ACh and that removing the endothelium abolished this relaxation. They implicated a diffusible endothelial agent as the mediator of endothelial-dependent relaxation through a series of “sandwich” experiments. Strips of de-endothelised smooth muscle were mounted in a force transducer. These strips

were contracted and exposed to increasing concentrations of ACh. No relaxation was seen. When an additional strip, containing a functional endothelium, was placed in the same bath the de-endothelised strip regained the ability to relax to ACh. They termed this endothelial relaxing agent endothelium-dependent relaxing factor (EDRF) (Furchgott and Zawadzki 1980; Furchgott 1999).

Subsequently, EDRF was identified as the membrane permanent, labile gas, nitric oxide (Ignarro, Buga et al. 1987; Palmer, Ferrige et al. 1987). It was established that nitric oxide diffuses into the underlying smooth muscle where it activates soluble guanylate cyclase, increasing the formation of cyclic guanosine monophosphate (cGMP) (Arnold, Mittal et al. 1977). In turn, cGMP activates cGMP-dependent protein kinase (PKG), which opens K^+ channels, hyperpolarizes the plasma membrane and inhibits Ca^{2+} entry through voltage-gated channels (Bolotina, Najibi et al. 1994). Limiting extracellular Ca^{2+} entry reduces $[Ca^{2+}]_{cyt}$ and attenuates Ca^{2+}/calmodulin mediated activation of MLCK, LC_{20} phosphorylation, and thus contraction. More recent evidence suggests that there may be additional PKG mediated mechanisms, independent of extracellular Ca^{2+} entry, that increase MLCP activity and decrease contraction in a Ca^{2+} independent manner (Ca^{2+} desensitization, see Section A3) (Wooldridge, Macdonald et al. 2004; Nakamura, Koga et al. 2007).

A.4.2 Endothelial Dysfunction

In the clinic endothelial function is assessed by measuring blood flow or vessel diameter following occlusion of the brachial artery or after ACh administration. Typically, the term endothelial dysfunction is used to refer to the attenuation of endothelial dependent vasorelaxation, typically due to reduced synthesis or

bioavailability of nitric oxide, and is present in numerous cardiovascular disease states. For example, both reactive hyperemia and ACh mediated vasodilation are attenuated in hypertensive patients as indexed by strain gauge plethsmography (Panza, Casino et al. 1994; Panza, Garcia et al. 1995), suggesting significant endothelial dysfunction in this patient cohort.

The consequences of reduced nitric oxide production are wider than attenuated vascular relaxation. For example, stimulation of nitric oxide release with ACh inhibits platelet aggregation in normal humans, however, in patients with endothelial dysfunction ACh mediated inhibition of platelet aggregation is impaired, which may partly explain the increase of thrombotic events in patients with atherosclerosis (Diodati, Dakak et al. 1998). There is also evidence to suggest that endothelial dysfunction results in increased leukocyte adherence and diapedesis through the endothelium, characteristic of acute inflammation (Kubes, Suzuki et al. 1991).

Endothelial dysfunction may be central to the development of atherosclerosis. In rabbits fed a high cholesterol diet for 5 weeks the addition of the nitric oxide inhibitor L-NAME for 4 weeks reduced ACh relaxation of isolated aortic rings and cellular cyclic GMP levels. Furthermore, neointimal formation was increased in the ascending aorta in rats treated with L-NAME/high cholesterol compared to high cholesterol diet alone (Cayatte, Palacino et al. 1994). Endothelial dysfunction may be present in the very early stages of atherosclerosis, even before plaque generation occurs (McLenachan, Williams et al. 1991; Kinlay and Ganz 2000). For example, ACh was administered to both normal subjects and subjects with existing CAD. Coronary artery diameter was measured in angiographically normal vessels in both groups

before and after ACh infusion. Compared to normal subjects, patients with CAD had a significantly smaller luminal diameter, suggesting that endothelial dysfunction may be the first sign of impending atherosclerosis (Werns, Walton et al. 1989).

A.4.3 Endothelial Nitric Oxide Synthase

Nitric oxide is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS) (Palmer, Ashton et al. 1988). Currently, three mammalian isoforms of NOS have been characterized: neuronal nitric oxide synthase (nNOS or NOS I), inducible nitric oxide synthase (iNOS or NOS II), and endothelial nitric oxide synthase (eNOS or NOS III). Although discrete genes encode the three NOS isoforms, they share 50-60% sequence identity. Despite this similarity the three isoforms have distinctive expression patterns, catalytic, and regulatory properties (Dudzinski, Igarashi et al. 2006). Figure A9 illustrates the major differences between the three isoforms of NOS.

eNOS is expressed in the vascular endothelium, in platelets, and in cardiomyocytes and is classified as a Ca^{2+}/calmodulin-dependent enzyme. The activation of eNOS can be inhibited by calmodulin binding proteins including melittin, mastoparan, and calcineurin (Busse and Mülsch 1990). When no calmodulin is bound eNOS activity is greatly reduced. Furthermore, a 40-50 amino acid loop destabilizes interaction with calmodulin at low $[Ca^{2+}]_{cyt}$ levels, further inhibiting enzymatic activity (Fleming and Busse 1999). Consequently, it is the regulation of $[Ca^{2+}]_{cyt}$ that primarily controls eNOS activity. As in smooth muscle, endothelial $[Ca^{2+}]_{cyt}$ can be modulated by agonists functioning through G-protein coupled receptors, as well as in response to stretch (Naruse and Sokabe 1993).

Isoform	Gene	Location	Primary function
Neuronal NOS (nNOS or NOS1)	NOS1	Nervous system Skeletal muscle	Cellular communication
Inducible NOS (iNOS or NOS2)	NOS2A NOS2B NOS2C	Immune system	Immune defense against pathogens
Endothelial NOS (eNOS or NOS3)	NOS3	Endothelium Cardiac myocytes Platelets	Vasodilation

Figure A9 Distribution and Function of The Three Mammalian NOS Isoforms. nNOS was the first isoform discovered and is found in neuronal tissue, iNOS is inducible and is found in the immune system, however, may be present in a wide range of cells and tissues. eNOS is primarily found in vascular endothelial cells.

Although constitutively expressed in the vasculature, evidence suggests that eNOS protein expression may also be actively modulated in response to laminar shear stress (Dekker, van Soest et al. 2002), activation of the RhoA/Rho-kinase pathway, and in response to statin treatment (Laufs and Liao 1998). Evidence suggests that eNOS protein levels may be regulated by altered mRNA stability, with reduced eNOS stability reported in the presence of oxidized LDL, thrombin, inflammation and hypoxia (Eto, Barandiér et al. 2001; Tai, Robb et al. 2004)

The development of eNOS-deficient mutant mice has suggested role for eNOS in the development and maintenance of cardiovascular disease. Homozygous eNOS mutant mice are phenotypically and behaviorally indistinguishable from wild-type mice but display marked hypertension with a mean arterial pressure of 110 mmHg compared to 81 mmHg for wild type. Aortic rings from eNOS deficient mice no longer relax to acetylcholine and display reduced Ca^{2+} dependent eNOS activity, as assayed by 3H-arginine to citrulline conversion. (Huang, Huang et al. 1995). In addition, eNOS deficient mice that have undergone mechanical injury to the femoral artery display significantly more smooth muscle cell proliferation than in normal mice (Moroi, Zhang et al. 1998) indicating an anti-proliferative role for eNOS. eNOS mutant mice also display decreased bleeding time, suggesting hypercoagulability (Freedman, Sauter et al. 1999). Finally, these animals have increased susceptibility to diet-induced atherosclerosis (Chen, Kuhlencordt et al. 2001; Kuhlencordt, Gyurko et al. 2001) that may be explained by increased leukocyte rolling, leukocyte adherence and increased surface expression of P-selectin found in the vasculature (Lefer, Jones et al. 1999).

Although limited in scope, there is also evidence from humans that corroborates findings from this mouse model in suggesting a role for eNOS in cardiovascular disease states. For example, atherosclerotic carotid arteries from humans have reduced eNOS protein expression and display impaired nitric oxide production compared to normal control vessels (Oemar, Tschudi et al. 1998) and an eNOS gene polymorphism, specifically a Glu298Asp missense variant, has been associated with essential hypertension in two separate populations in Japan (Miyamoto, Saito et al. 1998).

A.4.4 Regulation of eNOS by Ser1177 Phosphorylation

As well as regulation by $[Ca^{2+}]_{cyt}$ and by changes in protein level eNOS may be regulated by phosphorylation. eNOS contains numerous sites that have the potential to become phosphorylated, however, current functional evidence suggests that Ser1177 is the primary site of regulation (Dudzinski, Igarashi et al. 2006). Using mass spectrometry, McCabe and colleagues demonstrated that eNOS could be phosphorylated by PKB in response to changes in flow and that that this phosphorylation increased the activity of the enzyme by 15-20 fold (Gallis, Corthals et al. 1999). The same group subsequently demonstrated that phosphorylation of eNOS at Ser1177, or mutation of Ser1177 with a negative aspartate residue, in order to mimic the phosphoryl group, increases eNOS activity and nitric oxide production and that compared to unphosphorylated wild type eNOS, mutant eNOS displayed a decreased rate of inactivation when Ca^{2+} was sequestered with EGTA. This suggests that Ser1177 phosphorylation may superactivate eNOS by reducing calmodulin dissociation, even a low $[Ca^{2+}]_i$ (McCabe, Fulton et al. 2000).

Evidence indicates that Ser1177 of eNOS is phosphorylated by the PI3Kinase regulated enzyme protein kinase-B (PKB). Sheer stress due to flowing blood against the endothelial layer is a major regulator of nitric oxide production (Buga, Gold et al. 1991). HUVECS exposed to shear stress showed increased phosphorylation of PKB and concomitant phosphorylation of eNOS with the level of phosphorylation dependent on the degree of shear. The phosphorylation of PKB and eNOS were inhibited after incubation with the PI3K inhibitor wortmannin. When HUVECS were transfected with an inactive form of PKB the ability to respond to shear with nitric oxide production and the phosphorylation of eNOS were abolished. (Dimmeler, Fleming et al. 1999). Another study measured [32_P] orthophosphate incorporation and demonstrated that shear stress induced phosphorylation of eNOS within 30 seconds. The same authors used mass spectrometry and confirmed that Ser1177 was the site of phosphorylation (Gallis, Corthals et al. 1999). Figure A10 illustrates the role of Ser1177 phosphorylation in eNOS regulation and smooth muscle contraction. Subsequent studies have also shown a role for protein kinase-A (PKA) and for AMP-activated kinase (Boo, Sorescu et al. 2002) (Chen, Mitchelhill et al. 1999) in phosphorylating and activating eNOS, suggesting further complexity in the regulation of the enzyme.

In animal models of diabetes initiated by steptozotocin/nicotinamide a reduction in PKB dependent eNOS phosphorylation and activation has been noted (Kobayashi, Taguchi et al. 2004). Agonists of peroxisome proliferator-activated receptor γ (PPARγ) have been reported to increase nitric oxide production in humans (Fujishima, Ohya et al. 1998). In bovine aortic endothelial cells the PPARγ agonist, trogliazone increased nitric oxide production in a dose and time-dependent manner but did not

alter eNOS protein levels. Further investigation using phospho-specific antibodies showed a significant increase in Ser1177 phosphorylation (Cho, Choi et al. 2004). In a similar study, using the same cell type treated with bezafibrate, increases in eNOS mRNA half-life, eNOS total protein and Ser1177 phosphorylation were reported (Wang, Wang et al. 2006), suggesting that eNOS may be regulated by changes in phosphorylation and protein level simultaneously.

Some vasoprotective signaling molecules may exert their effects through increased nitric oxide production. For example, estrogens have an inhibitory effect on the development of atherosclerosis (Chen, Li et al. 1996) and treatment of HUVECs with 17b-estradiol increased the conversion of L-arginine to L-citrulline, suggesting increased eNOS activity (Hisamoto, Ohmichi et al. 2001). The authors in this study also reported increased PKB activation along with increased phosphorylation of Ser1177 eNOS. Another study reported increased nitric oxide production in cultured cells treated with vascular endothelial endothelial growth factor (VEGF) or insulin. The authors examined the mechanism for this effect and reported increased PI3K and PKB activation, leading to increased phosphorylation of eNOS at Ser1177 (Fulton, Gratton et al. 1999). Similarly, increased nitric oxide production and Ser1177 phosphoylation has been reported in response to IGF-1, adiponectin and leptin treatment (Chen, Mitchelhill et al. 1999; Michell, Griffiths et al. 1999; Montagnani, Chen et al. 2001; Vecchione, Maffei et al. 2002). Thus, eNOS Ser1177 phosphorylation seems to be a crucial step in regulating eNOS activity and may be an important target for intervention to treat endothelial dysfunction.

A.4.5 Regulation of eNOS by Thr495 Phosphorylation

In addition to positive regulation of eNOS by Ser1177 phosphorylation, there is evidence of negative regulation by phosphorylation at Thr495. Chen et al, have shown that AMPK has the potential to phosphorylate eNOS at Thr495, located in the CaM-binding domain, resulting in reduced eNOS activity *in vitro* (Chen, Mitchelhill et al. 1999). It was subsequently demonstrated that this phosphorylation results in the disassociation of calmodulin from eNOS (Fleming, Fisslthaler et al. 2001).

Sugimoto et al, 2007 has provided evidence of a link between Rho-kinase and the inhibition of eNOS via Thr495 phosphorylation. The expression of constitutively active RhoA (RhoA CA) or Rho-kinase (Rho-K-cat) increased Thr495 phosphorylation as indexed by immunoblotting. This phosphorylation could be abolished with the Rho-kinase inhibitor, Y27632 (Sugimoto, Nakayama et al. 2007). The same group stimulated cultured COS-7 cells with thrombin (1 U/ml), resulting in MYPT1 Thr855 phosphorylation that could be abolished by Y27632 incubation (30 μM for 30 minutes), thus confirming the activation of the RhoA/Rho-kinase pathway. A concomitant phosphorylation of eNOS Thr495 was also noted which was partly abolished by the same inhibitor (Sugimoto, Nakayama et al. 2007). This study suggests that inhibition of the RhoA/ROK pathway may be an effective strategy to disinhibit eNOS. However, the failure of Y27632 to fully inhibit Thr495 phosphorylation suggests that other kinases may contribute to this phosphorylation event. Y27632 is known to inhibit isoforms of PKC (Walsh et al., in Moorhead 2007; Eto, Kitazawa et al. 2001) and indeed, a number of studies have suggested a role for PKC in Thr495 phosphorylation (Michell, Chen et al. 2001; Matsubara, Hayashi et al. 2003). Additional studies are required to determine the physiological role for eNOS

Thr495 phosphorylation in vascular function *in vivo* and whether the Rho-kinase pathway is a mediator.

A.4.6 Statins and eNOS

The possibility that statins modulate the endothelium by regulating eNOS has been suggested (Liao 2005). In HUVECS treated with mevastatin (10 μM for 48 hours), eNOS mRNA was increased by 3-fold and protein levels by 1.8-fold. This effect was reversible if HMG-CoA reducatase blockade was bypassed with L-mevalonate or GGPP (Laufs and Liao 1998). The authors examined the possible role of RhoA in mediating this effect and using radiolabled GTPδS identified that mevastatin treatment inhibited RhoA membrane translocation and RhoA GTP binding. Inhibition of RhoA with the bacterial exoenzyme C3 or over expression of a dominant negative form of RhoA mimicked the effects of mevastatin, while activation of RhoA with *Escherichia coli* cytotoxic necrotizing factor-1 decreased eNOS expression (Laufs and Liao 1998). These results are consistent with a statin mediated inhibition of RhoA prenylation, resulting in decreased Rho-kinase activation and reduced eNOS expression.

As well as modulating eNOS protein expression statins may regulate eNOS activity by increasing Ser1177 phosphorylation (Figure A10). In cultured endothelial cells (HUVEC) treatment with simvastatin (1 μM) for 30 minutes led to a dose dependent increase in PKB activation accompanied by increased ^{32}P eNOS. This effect was reversible after L-mevalonate treatment, the transfection of a dominant negative form of PBK, or inhibition of PI3K with wortmannin. The same authors treated bovine aortic endothelial cells with statins for 60 minutes and reported a 1.7 fold increase in

nitric oxide production after statin treatment that was also reversible with wortmannin (Kureishi, Luo et al. 2000). This effect may be due to inhibition of the RhoA/Rho-kinase pathway, as statin treated HUVECS transfected with constitutively active V14 RhoA or constitutively active Rho-kinase showed decreased PKB activity as assessed by immunoprecipitation-kinase assay (Ming, Viswambharan et al. 2002).

A number of studies using isolated vessels provide evidence that statins modulate vascular contraction by modulating endothelial function *in vivo*. In rat thoracic aorta incubation with simvastatin relaxed vessels preconstricted with endothelin-1 in a dose dependent fashion. Removing the endothelium or incubating with L-NAME, a broad-spectrum inhibitor of all NOS isoforms, reduced statin mediated relaxation, although the effect was not completely abolished, suggesting that some of the statin effect was due to additional endothelial-dependent mechanisms or direct modulation of smooth muscle (see Section A3) (Mraiche, Cena et al. 2005). More recent studies demonstrate that a 45 minute incubation with either simvastatin, pravastatin, atorvastatin or lovastatin (1 μM) reduce 5-HT developed tension in isolated swine coronary artery rings mounted on a force transducer. However, this group did not test for endothelial dependence or a biochemical mechanism (Crespo and Quidgley 2006).

Figure A10 The Regulation of Nitric Oxide Production by Ser1177 eNOS Phosphorylation. In endothelial cells, the activation of Rho-kinase by RhoA leads to the inhibition of PI3K/PKB mediated phosphorylation of Ser1177 and inhibition of eNOS, resulting in reduced nitric oxide production. By releasing Rho-kinase mediated inhibition of PI3K/PKB both direct Rho-kinase inhibitors and statins may increase Ser1177 eNOS phosphorylation leading to increased nitric oxide synthesis and smooth muscle relaxation. Modified after Wilsion, D.

A.4.7 The Prostanoids

The prostanoids are an important class of vasoconstrictors and vasodilators that form part of the eicosanoid family. The eicosanoids are 20 carbon fatty acids containing a central 5-carbon ring (Narumiya, Sugimoto et al. 1999). The prostanoid family consists of the prostaglandins: PGEH2, PGD_2, PGE_2, PGF_2, and PGI_2 (prostacyclin), and the thromboxanes (TxA_2). It has been established that prostanoids have a major role in regulation of vascular tone, in both physiology and pathology (Chan and Cervoni 1986; Mistry and Nasjletti 1988; Alfranca, Iniguez et al. 2006).

The synthesis of the prostanoids is complex and involves numerous converging metabolic pathways. Generally, activation of phospholipase-A_2 results in the release of arachidonic acid from membrane phospholipid stores (Lin, Lin et al. 1992). Arachidonic acid is then converted to the intermediate PGH_2 by cyclooxygenase (COX or PGH synthase) (Caughey, Cleland et al. 2001). Through the activity a number of enzymes PGH_2 is subsequently converted to the prostanoids. The type of prostanoid produced in a given tissue type is dependent of the enzymes present. Endothelial cells produce mainly prostacyclin (McAdam, Catella-Lawson et al. 1999), while platelets predominately produce thromboxane A_2 (Alfranca, Iniguez et al. 2006).

In the vasculature prostacyclin acts similar to nitric oxide in producing vasodilation (Rosolowsky and Campbell 1993) and inhibiting both platelet aggregation (Vane and Botting 1995; Cheng, Austin et al. 2002) and vascular smooth muscle proliferation (Todaka, Yokoyama et al. 1999). Stimulation of vascular endothelial cells with agonists including, acetylcholine, bradykinin or with shear stress results in

prostacyclin synthesis and release. Prostacyclin is unstable with a half-life of several minutes and acts in an autocrine or paracrine manner binding to G-coupled protein receptors (IP receptors) (Boie, Rushmore et al. 1994). In vascular smooth muscle the IP receptor activates the G_s subunit and causes relaxation by activating adenylyl cyclase which increases cAMP levels and activates cAMP-dependent protein kinase (PKA) (Gorman, Bunting et al. 1977; Lefkowitz, Pierce et al. 2002; Sprague, Bowles et al. 2008). Therefore, when examining endothelial mediated relaxation care must be taken to dissect the contribution of the prostaglandins from nitric oxide.

A.4.8 Endothelial-Derived Hyperpolarizing Factor

Nitric oxide and the prostaglandin PGI_2 have been recognized as the major mediators of endothelial mediated vascular relaxation. Evidence suggests, however, that inhibition of these factors does not always completely abolish endothelial-mediated relaxation, suggesting the existence of another endothelium-derived vasodilator (Feletou and Vanhoutte 1988; Taylor and Weston 1988). Because the relaxation caused by this factor is associated with hyperpolarisation of the plasma membrane and can be abolished by pharmacological inhibition of K^+ channels it has been termed endothelial-derived hyperpolarizing factor (EDHF) (Taylor and Weston 1988).

EDHF is most important in resistance vessels, opposed to large conduit vessels (Tomioka, Hattori et al. 2000) and has been suggested to play a role in blood pressure maintenance (Scotland, Madhani et al. 2005). A number of studies have suggested an inverse relationship with size of vessels and the magnitude of vasodilatation attributable to EDHF (Urakami-Harasawa, Shimokawa et al. 1997), although variations between vascular beds and species types have also been reported (Clark

and Fuchs 1997). Interestingly, evidence suggests that the generation of nitric oxide inhibits EDHF, suggesting that EDHF may act as a backup vasodilator and thus, may be important in states of nitric oxide deficient endothelial dysfunction (Shimokawa, Yasutake et al. 1996; Waldron, Ding et al. 1999). This notion is supported by evidence of altered EDHF response in hypertension (Mori, Ohyanagi et al. 2006), atherosclerosis (Moroe, Fujii et al. 2004) and diabetes (Fitzgerald, Kemp-Harper et al. 2005).

The identity of a specific EDHF is still in question. Indeed, it is likely that EDHF refers to a suite of factors including both diffusible molecules and direct electrical transmission from the endothelium to the vascular smooth muscle perhaps through myoendothelial gap junctions (Sandow 2004; Sokoya, Burns et al. 2006). There is considerable evidence to suggest that metabolites of arachadonic acid produced via epoxygenase CYP-450 may account for EDHF. Specifically, 14,15-epoxyeicosatrienoic acid (EETs) had been the most widely accepted candidate for EDHF. Indeed, hyperpolarization attributable to EDHF can be inhibited by phospholipase A_2 inhibitors that inhibit the production of EETs (Adeagbo and Henzel 1998) and a role for EETs mediated vasodilatation has been described in humans (Miura and Gutterman 1998; Archer, Gragasin et al. 2003). Evidence suggests, however, that EETs cannot account for EDHF effects in all vascular beds (Bussemaker, Popp et al. 2003). Furthermore, the use of CYP450 inhibitors, which has been the most common diagnostic tool implicating EETs as EDHF may be misleading as these inhibitors also inhibit K^+ channels that are involved in EDHF signal transduction (McGuire, Ding et al. 2001).

Other candidates for EDHF include hydrogen peroxide (Shimokawa and Morikawa 2005), C-type natriuretic peptide (Chauhan, Nilsson et al. 2003) and potassium ions (Bryan Jr, You et al. 2005). Due to the elusive nature of EDHF it is difficult to assess its importance in biological responses. In the current thesis mechanical removal of the endothelium is used to control for EDHF in myograph experiments.

A.5 Context of Thesis

The clinical use of statins is primarily based upon their cholesterol-lowering effects so that patients with hypercholesterolaemia and/or with high risk of cardiovascular events, such as those with established atherosclerotic disease, are prescribed these agents. Recent controversy has arisen as to the efficacy of statins in the primary prevention cardiovascular events (Ray, Seshasai et al. 2010)**,** raising the question as to whether selection of patients merely based upon plasma cholesterol levels is appropriate**.** Although these results may reflect a low risk of cardiovascular events in the primary prevention population, it may also suggest that the wrong therapeutic target has been focused upon and that alternate target populations should be considered; for example, those patients with evidence of inflammation (e.g. the JUPITER trial), endothelial dysfunction, or perhaps hypertension.

Given the evidence for the cholesterol independent effects of statins outlined in this review, consideration should also be given to the potential benefits of statins to improve cardiovascular symptoms, such as angina, in addition to the prevention of cardiac events (death and myocardial infarction). This may be relevant in conditions such as syndrome X, chronic stable angina and possibly aortic stenosis.

Therefore, there is a need to further understand the pleiotropic effects of statins. This thesis will focus on the vasomotor effects of statins. Specifically, this thesis will address the question as to whether statins can inhibit vascular smooth muscle contraction mediated by α_1-adrenoreceptor and thromboxane A_2 receptor activation because, as discussed further in this section, the downstream signaling pathways invoked by these receptors may be involved in numerous human disease states.

Furthermore, this thesis will examine the molecular mechanisms by which statins modulate vascular constriction as understanding the way statins interact with these signaling pathways will advance our understanding of the benefits of statins and may provide insights into expanded indications for their use. With this in mind the current section will also outline a potential novel therapeutic use for statins in the treatment of the debilitating and treatment resistant microvascular disorder, coronary slow flow.

A.5.1 Thromboxane A_2

Thromboxane A_2 (TxA_2) is a potent constrictor of blood vessels and is involved in platelet activation and blood clot formation (Palmer, Piper et al. 1970). TxA_2 is a member of the prostanoid subclass of eicosanoids and is produced predominantly in platelets. TxA_2 synthesis begins with the activation of phospholipase A_2, which results in the liberation of arachidonic acid (AA) from membrane bound lipids. AA is converted to prostaglandin H_2 by cyclooxygenases 1 and 2, which in turn is converted to TxA_2 by isomerization by thromboxane-A_2 synthase (Narumiya, Sugimoto et al. 1999). The half-life of TxA_2 has been reported as 30s (Hamberg, Svensson et al. 1975) and because of this the stable TxA_2 analogue U-46619 is often used for research purposes.

TxA_2 is an important regulator of many physiological processes including smooth-muscle contraction and proliferation, platelet-shape change and aggregation (Kinsella 2001). A role from TxA_2 in pathology has also long been suggested (Sellers and Stallone 2008). For example, administration of the thromboxane A_2 receptor antagonist AH23848 to genetically hypertensive rats results in normalization of systolic blood pressure (Li, Ferrario et al. 1998). Furthermore, in humans, increased

TxA_2 receptor expression (Liel, Nathan et al. 1993) and increased TxA_2 synthesis (Fitzgerald, Rocki et al. 1990) are seen in patients with pregnancy-induced hypertension. TxA_2 has also been implicated in the pathogenesis of myocardial ischemic episodes during unstable angina (Machado, Saavedra et al. 1994) and increases in TxA_2 synthesis have been reported in patients with ischemic heart disease (Serneri, Gastone et al. 1981).

In smooth muscle, it has been observed that TxA_2 causes a large increase in force in response to modest increases in cytosolic Ca^{2+}, implying that a large component of TxA_2 signaling is transuded via sensitization to Ca^{2+} (Himpens, Kitazawa et al. 1990). Wilson and colleagues have thoroughly described the mechanism of TxA_2 mediated contraction in the rat caudal artery (Figure A11). They identified that contraction is entirely dependent on nicardipine sensitive extracellular Ca^{2+} entry and that removal of sarcoplasmic reticulum Ca^{2+} has no effect on contraction, suggesting no role for IP_3 induced SR Ca^{2+} release. Furthermore, inhibiting the RhoA/Rho-kinase pathway in intact vessels completely abolished contraction, suggesting that Ca^{2+} sensitization plays an important role in TxA_2 mediated contractility. By analyzing the phosphorylation state of MYPT1 and CPI-17, the authors found that Ca^{2+} sensitization is mediated through the inhibition MLCP via phosphorlation of MYPT1 at Ser-855 and not Thr-697. TxA_2-dependent contraction was insensitive to PKC inhibition and TxA_2 receptor stimulation did not result in phosphorylation of CPI-17 (Wilson, Susnjar et al. 2005). These results suggest that in rat caudal artery TxA_2 causes contraction primarily through activation of L-Type Ca^{2+} channels and the RhoA/Rho-kinase pathway.

Therefore, as well as being a potential mediator of cardiovascular disease, and therefore an interesting therapeutic target, TxA_2 mediated constriction in the rat caudal artery is an excellent model in which to study the RhoA/Rho-kinase pathway. The TxA_2 analogue U-46619 is used to examine the effects of statins on the RhoA/Rho kinase pathway in this thesis.

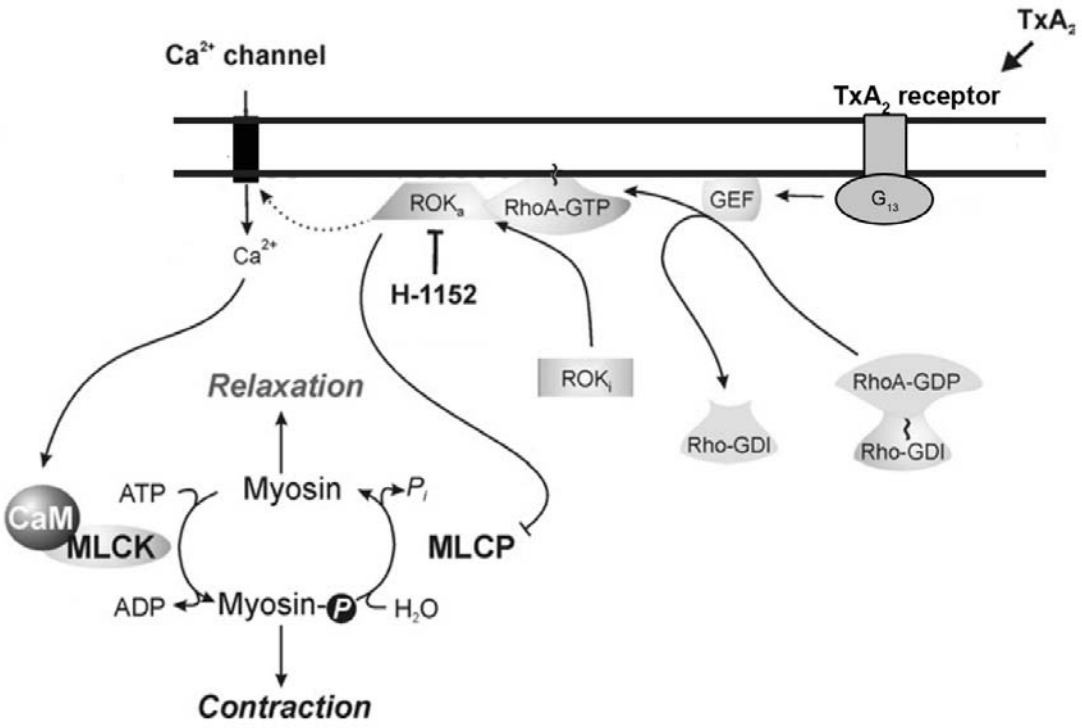

Figure A11 TxA_2 Receptor-Mediated Contraction in the Rat Caudal Artery. In rat caudal artery TxA_2 receptor activation induces contraction via extracellular Ca^{2+} entry and activation of RhoA/Rho-kinase leading to phosphorylation of MYPT1 at Thr-855, the inhibition of MLCP and Ca^{2+} sensitization of contraction. Figure adapted from Wilson, 2005.

A.5.2 α_1-Adrenergic Receptor Activation

The sympathetic nervous system is part of the autonomic nervous system and plays a key role in cardiovascular regulation. The sympathetic nervous system is central to the body's "flight or fight" response, causing an increase in heart rate, blood pressure, cardiac output, distribution of blood flow, dilatation of airways, increased pupil size, mobilization of fat and glycogen. Of particular interest in the context of this thesis is acute vasoconstriction. The sympathetic nervous system can alter vasoconstriction in blood vessels resulting in an increase or decrease in blood flow which alters organ function, total peripheral resistance and blood pressure.

A role for increased sympathetic drive in cardiovascular pathology has been established in both animal models and humans. Increased sympathetic activation is seen in humans during major ventricular arrhythmias and may be a causative factor (Meredith, Broughton et al. 1991) as well as in myocardial infarction and heart failure (Ciarka, van de Borne et al. 2008), stroke (Grassi, Arenare et al. 2009), hypertension (Julius and Majahalme 2000) and renal disease (Schlaich, Socratous et al. 2008).

There is evidence that sympathetic activity is increased in a large proportion of patients with hypertension (Anderson, Sinkey et al. 1989; Mancia, Grassi et al. 1999). Indeed elevated cardiac output and heart rate and alteration in total peripheral resistance are commonly reported in hypertension and may due to increased sympathetic activation (Guzzetti, Piccaluga et al. 1988; Anderson, Sinkey et al. 1989; Esler, Jennings et al. 1989). One point often disputed is whether sympathetic overdrive is consistently present 24 hours a day. However, this may be unimportant because repeated sympathetic activation, even if it occurs via associated repeated

oscillations in blood pressure from nomotensive to hypertensive are likely, in fact, to be harmful in the long term.

The α_1-adrenoreceptors are important mediators of the sympathetic nervous system responses in respect to vascular smooth muscle constriction and cardiac contractility (Docherty 2010). The sympathetic catacholamines epinephrine and norepinephrine bind to the α_1-adrenoreceptor, which is coupled to the G_q heterotrimeric G protein (Somlyo and Somlyo 2000). Activation of G_q results in the hydrolysis of phosphatidylinositol 4,5 bisphosphate to generate the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Graham, Perez et al. 1996). IP_3 induces intracellular Ca^{2+} release, whereas 2-diacylglycerol activates protein kinase C, both contributing to vascular smooth muscle contraction (Wang, Deng et al. 2008; Docherty 2010).

Increased α_1-receptor activation/expression has been reported in hypertensive humans and animals and may lead to constriction of blood vessels, accounting for the rise in total peripheral resistance often seen in essential hypertension (Malpas 2010). In one study, pharmacological inhibition of α_1-adrenergic receptors normalized blood pressure in approximately 30% of subjects. (Esler, Julius et al. 1977). There is also some evidence suggesting a role for α_1-adrenoreceptor activation during vasospasm in both the myocardium and in the cerebral circulation that may be due to hypercontractility of vessels mediated through Ca^{2+} sensitization.

In the present study the α_1-adrenoreceptor agonist phenylepherine is used to study the effects of statins on α_1-adrenoreceptor mediated contraction in blood vessels.

A.5.3 The Coronary Slow Flow Phenomenon

Arterioles and pre-arterioles are primarily responsible for vascular resistance within the circulatory system. Dysfunction in the resistance vessels may be responsible for a number of cardiovascular disorders including hypertension, coronary microvascular dysfunction and cerebral microvascular disease. Therapies directed towards the microvasculature are limited and thus advances are required if there are to be improvements in the disabling symptoms caused by these disorders.

The Coronary Slow Flow Phenomenon (CSFP) is a coronary microvascular disorder associated with recurrent, often disabling, chest pain. Originally thought to be an angiographic artifact, research conducted by our colleagues at the Queen Elizabeth Hospital has resulted in CSFP being recognized as a clinically significant disorder (Beltrame 2006).

CSFP is characterized by the delayed opacification of coronary vessels in the absence of obstructive coronary atherosclerotic disease. CSFP is diagnosed on angiography, and is usually found when patients undergo this procedure in the context of acute coronary syndrome. It would appear that CSFP accounts for at least 4% of acute coronary syndrome admissions (Beltrame, Limaye et al. 2000). The acute angina associated with CSFP does not resemble that seen in syndrome X, in which patients experience angina on exertion, but is more akin to the rest angina experienced in angina pectoris due to coronary artery spasm. (Beltrame, Limaye et al. 2000).

CSFP has been associated with coronary microvascular dysfunction. The involvement of impaired nitric oxide production has been implicated using pulse wave analysis to

non-invasively assess endothelial function. 15 CSFP patients and 15 healthy controls were used. Both groups showed a similar response to the endothelium dependent vasodilator, nitroglycerine. Patients in the CSFP group, however, displayed paradoxical vasoconstriction in response to salbutamol, an endothelial dependent vasodilator. (Beltrame, Limaye et al. 2003)

A role for endothelin-1 in the pathology of CSFP has also been established. Subcutaneous microvessels from 8 CSFP patients and 8 healthy controls were mounted on a myograph and concentration response curves were determined for endothelin-1. The CSFP group vessels were twice as sensitive to endothelin-1 (Turner and F. 2005). Furthermore, it has been noted that infusing endothelin-1 into the coronary arteries of animals reproduces the angiographic features of the CSFP (Larkin, Clarke et al. 1989; Hirata, Matsuda et al. 1990).

It has been demonstrated that the RhoA/Rho-kinase pathway is involved in endothelin-1 signaling in smooth muscle (Niiro, Koga et al. 2003). Interestingly, the Rho-kinase inhibitor, fasudil, is used clinically in Japan and studies indicate that it has positive clinical outcomes when used to treat microvascular disease (Mohri, Shimokawa et al. 2003). Currently, fausadil is not available in Australia. However, because statins may also inhibit the RhoA/Rho-kinase pathway (Sections A4 and A5) they may also be efficacious in the treatment of microvascular disorders. Therefore, it is of great interest to determine if statins have direct vascular effects and to determine the molecular mechanisms, as they may prove to be an effective treatment for CSFP.

B. General Methods

B.1 General Laboratory Reagents

Reagents were purchased from the following sources: acetylcholine, N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME), glycerol, HEPES sodium salt (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), complete protease inhibitor cocktail, sodium dodecyl sulfate, bromophenol blue, acrylamide, N,N'-methylene bisacrylamide, N,N,N',N'-tetramethylethylenediamine, acetone, glycerol, tricloroacetic acid (TCA), ammonium persulfate were from Sigma–Aldrich (Australia). Glycine and tris base were from Amresco (Solon, OH, USA). Tween-20 and 0.2 μm nitrocellulose membrane, were from Bio-Rad Laboratories (Australia). Prestained molecular weight markers (PageRuler™ Prestained Protein Ladder Plus) were from Fermentas Life Sciences (Canada). Dithiothreitol (DTT), ethylenediaminetetraacetic acid, acetic acid, hydrochloric acid, trichloroacetic acid, coomassie blue, ponceau-S, sodium chloride, potassium chloride, potassium dihydrogen phosphate, magnesium chloride, sodium bicarbonate, D-glucose and sucrose were from Merck & Co., Inc. (Australia).

Rabbit polyclonal antibodies specific for pan-MYPT1 (anti-MYPT1), MYPT1 Thr-855 (anti-[PThr855]-MYPT1), pan-eNOS (anti-eNOS) and eNOS Ser1177 (anti-[PSer1177]-eNOS) were from Upstate/Millipore USA, Inc. Polyclonal antibody specific for the light chains of myosin (anti-LC_{20}) was purchased from Santa Cruz Biotechnology (USA).

Secondary antibodies (Anti-Rabbit-HRP and anti-mouse-HRP) were purchased from Santa Cruz Biotechnology (USA). Medical grade X-Ray film was purchased from Agfa (Mortsel, Belgium).

B.2. Myography

B.2.1 Premise

The wire-myograph was developed in 1977 by Mulvany and Halpern and enables the direct measurement of isometric force in vessels smaller than 500μm (Mulvany and Halpern 1977). Previously, such measurements were restricted to large conduit vessels such as the aorta (Spector, Fleisch et al. 1969). By providing information about the response of isolated vessels to pharmacological and mechanical stimuli the wire-myograph has become a standard tool in basic cardiovascular research (Angus and Wright 2000; Spiers and Padmanabhan 2005). As opposed to whole organ perfusion techniques that are useful for providing information on the general response of an entire vascular bed, the wire myograph facilitates the analysis of specific segments of a given vascular bed. Wire myography provides an isometric measure of force and differs from perfusion myography that enables both isometric and isotonic measurement depending on how the system is configured.

Vessels are isolated and cut transversely into rings that are mounted by threading two stainless steel wires through the lumen of the vessel and subsequently attaching these wires to two supports. One support is attached to a micrometer that allows for the adjustment of resting vessel tension by altering vessel circumference. The second support is attached to a force transducer that measures tension. Vessels are kept at 37°C and are viable for up to 12 hours without a significant decline in contractile force (Mulvany 1988).

The major advantage of the preparation is the ease of set up, accuracy and reproducibility of results. The isolated nature of the technique facilitates force

measurement without interference from circulating humoral and neural factors. A significant advantage of the technique is the ability to snap freeze vessels during contraction, thus providing a snapshot of molecular activity at specific point in time.

B.2.2 Apparatus

The small vessel Mulvany wire myograph (Myograph model 610M, DMT, Denmark) consists of four detachable 6 ml organ baths. Each organ bath is fitted with a micrometer to alter vessel circumference and a force transducer with a detection range of 0.01mN – 1000mN. Figure B1 shows the configuration of the Mulvany wire myograph.

The myograph surface, upon which the individual organ baths are placed, is heated. A thermometer placed into one organ bath ensures that the bath solution is maintained at 37°C. All solutions used during experiments were kept at 37°C in a heat bath. A 5ml pipette was used to fill organ baths with solution. A working bath volume of 4ml was used in all experiments.

Following from the manufacturers instructions the force transducers were calibrated using a 2 g weight once every month or upon moving the unit.

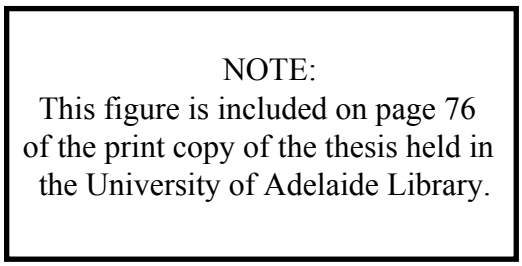

Figure B1 The Mulvany Wire Myograph. Vessels, 2 mm in length are threaded with two steel wires and attached to a force transducer by securing wires with screws. Figures adapted from Danish Myo Technology, 2008.

B.2.3 Vessel Dissection and Mounting

Rats 300-350g were sacrificed by CO_2 inhalation (Sections C, D) or by removal of heart from an anesthetized animal (Section E). Using a scalpel, an incision was made around the circumference of the base of the tail then down the length of tail, enabling the removal of the surrounding skin. The caudal artery rests in a ventral groove in the tail and was removed by cutting under the artery along the groove. Great care was taken not to stretch the vessel during this procedure as stretch of greater than 25% resulted in functional damage and impaired response to agonists.

Immediately following dissection vessels were placed in a Silguard-lined petri dish containing Ca^{2+} free HEPES-TYRODE (containing in mM [135.5 NaCl 5.9 KCl 1.2 $MgCl_2$ 11.6 HEPES 11.6 glucose pH 7.4]) at 4°C. Using a dissecting microscope and sharp dissecting scissors adipose and connective tissue were gently removed from the length of the vessel before cutting arteries into 2 mm segments.

Arterial segments, 2 mm in length, were mounted into the myograph using 40 μm wires as outlined by the manufacturer (DMT, Denmark), (Figure B1). Once all vessels were mounted the bath solution was replaced with normal HEPES-Tyrode solution (for Sections C and D) containing in mM: NaCl (135.5) KCl (5.9) $MgCl_2$ (1.2) $CaCl_2$ (2.5) HEPES (11.6) glucose (11.6), pH 7.4. or KREBS (for section E) containing in mM: NaCl (118), KH_2PO_4 (1.18), $NaHCO_3$ (25), $MgCl_2$ (1.05), $CaCl_2$ (2.34), EDTA (0.01), and glucose (5.56), pH 7.4, warmed to 37°C. For experiments using KREBS, the organ baths were bubbled with carbogen (95% O_2 and 5% CO_2) to maintain the correct oxygen tension and pH (7.4). Control experiments indicated there was no difference in vascular responses conducted in HEPES-Tyrode vs KREBS,

however, myocardial tissue is not viable in HEPES-Tyrode consequently KREBS buffer was chosen for all experiments involving myocardial coronary perfusion or subsequent isolated vessel work from the same animals in which Langendorff experiments were conducted (Section E).

During the equilibration period vessels were adjusted to resting tension by adjusting the micrometer. The optimal resting tension of 10 mN for caudal artery segments was established by generating length-tension curves to 87 mM KCl.

B.2.4 Vessel Normalisation With KCl

After equilibration vessels were stimulated with a depolarising 87 mM HEPES-Tyrode solution, to keep solutions isoosmotic an equimolar concentration of KCl was substituted for NaCl. In sections C and D this was 87mM HEPES-Tyrode, in section E this was high K^+ KREBS. This was done to ensure the contractile integrity of vessels and to ensure all vessels were capable of generating similar responses. Importantly, KCl stimulation provides a force measurement independent of agonist stimulation that subsequent responses can be normalized to. This approach enabled us to account for slight variation in responses caused by small differences in vessel sizes. KCl stimulation was repeated 3 times with a 20-minute intervening washout period between each stimulation. The final KCl stimulation was used for normalizing subsequent responses.

B.2.5 Endothelial Integrity

As discussed in Section A5 the endothelium is a major regulator of smooth muscle contraction and thus the integrity of the endothelium has the potential to alter vascular

responses.

Vessels were contracted using the selective α_1-adrenergic agonist phenylephrine (10 μM, EC_{90}), and tested for relaxation to acetylcholine (1 μM). The presence of a functional endothelium was considered confirmed by an acetylcholine-mediated relaxation to 90% of resting tension (Figure B2). For experiments in which the removal of the endothelium was required vessels were contracted with phenylephrine (10 μM) and the absence of an endothelium confirmed by lack of acetylcholine (1 μM) mediated relaxation (Figure B2).

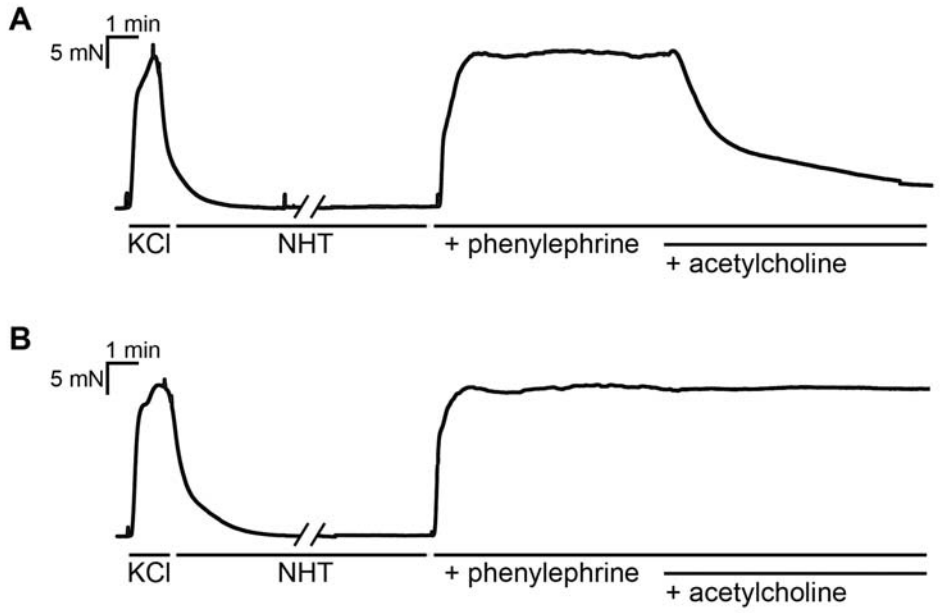

Figure B2 Endothelial Removal Abolishes Acetylcholine Mediated Relaxation. Rat caudal artey was mounted in a wire myograph in the presence (**A**) or absense (**B**) of an endothelium. Following normalising KCl contractions vessels were relaxed with normal HEPES-Tyrode (NHT) and stimulated with the α_1-adrenoreceptor agonist, phenylepherine (10 μM) for 10 minutes followed by the addition of acetylcholine (1 μM). Mechanical removal of the endothelium abolished acetylcholine mediated relaxation.

B.2.6 Experimental Protocol

B.2.6.1 Dose Response Curves

Following the final KCl contraction, vessels were equilibrated in HEPES-Tyrode or Krebs for 20 minutes. Increasing concentrations of agonist (phenylephrine or U-46619) were added cumulatively directly to bath. At each concentration of agonist, vessels were allowed to reach plateau response before the next concentration was added.

B.2.6.2 Fixed Dose Experiments

As with dose response curves, vessels were equilibrated in HEPES-Tyrode or Krebs for 20 minutes following KCl stimulation. Vessels were then pre-treated with vehicle control, statin or appropriate inhibitors for a 60-minute period. Following this pre-treatment, vessels were stimulated with agonist (10 μM phenylephrine for section C, 1 μM U-46619 for section D and E) in the presence of statins and/or inhibitors. Vessels were stimulated for 10 minutes before vessels were snap frozen.

B.2.6.3 Snap Freezing of Vessels

In order to facilitate molecular analysis of phosphorylation state vessels were snap frozen using a dry ice cold 10% trichloroacetic acid (TCA)/acetone solution. This procedure has the advantage of inactivating enzymatic activity by the following three mechanisms: 1) thermal inactivation, 2) irreversible acidic precipitation and 3) dehydration. Specifically, using a Pasteur pipette dry ice cold 10% TCA/Acetone solution was perfused over the vessel. This was followed by a 1-minute rinse with dry ice cold acetone in order to avoid acid hydrolysis of the sample. Vessels were quickly

removed from the myograph, placed into a dry ice cold microfuge tube prior to storage at -80^{o}C.

B.2.7 Data Acquisition and Analysis

The myograph was connected to a four-channel Powerlab unit (ADinstruments, Australia) attached to an Apple Mini-Mac (Apple, California). Myograph output was recorded using Powerlab Chart v6.00 (ADInstruments Pty Ltd., Australia).

All recordings were in mN. Developed tension was determined by subtracting resting tension from maximal stable tension. All developed tension readings were expressed as a percentage of the third response to successive stimulations with KCl solution.

B.3 Langendorff Perfused Heart

B.3.1 Premise

The isolated perfused mammalian heart preparation was developed in 1897 by Oscar Langendorff (Langendorff 1898). The basic principal of the technique is to maintain cardiac activity by perfusing the heart through the coronary arteries via a cannula inserted into the aorta. Perfusate flows retrograde from the aorta forcing shut the aortic valves and preventing perfusate from entering the left ventricle. Flow is directed through the ostia into the coronary arteries. After flowing through the coronary vascular network the perfusate enters the right atrium via the coronary sinus and exits the heart through the pulmonary artery (Zimmer 2000).

Due to its simplicity, accuracy, and high reproducibility the Langendorff technique is widely used to measure coronary vascular resistance, myocardial contractility and myocardial metabolism. The absence of basal hormonal and central neural regulation, make this technique well suited for studying the direct effect of pharmacological agents on the coronary arteries. One disadvantage of the technique is the requirement for high coronary flow and the typical use of a protein/colloid free perfusate that over time leads to myocardial oedema. The short time frame of experiments conducted in the current study has enabled us to avoid this shortcoming (Skrzypiec-Spring, Grotthus et al. 2007).

Of primary interest to vascular researchers is arterial flow, which is proportional to the mean radius of coronary arteries. A change in vessel diameter by vasoconstriction or vasodilation results in a change in resistance that is portional to $1/r^4$ where r =

mean radius of artery. The flow in coronary circulation can be described by Poiseuille's law:

$$F = \frac{\pi \Delta P \, r^4}{8 \eta L}$$

Where R = resistance, ΔP = pressure difference and F = coronary flow, η=viscosity, L= length and r= radius.

One realizes this can be expressed as a more simple equation similar to Ohm's law:

$$\text{Flow} = \frac{(\Delta P)}{\text{Resistance}}$$

As a consequence of this relationship both variables must be measured to obtain resistance. However, if one value is kept constant, only one value need be recorded. With this in mind, two versions of the Langendorff technique have been developed in which the delivery of perfusate can occur either at constant flow or constant hydrostatic pressure. In the constant flow model coronary perfusion is measured and is proportional to coronary resistance. In the constant pressure model coronary flow is measured and is inversely proportional to coronary resistance (Dhein, Wilhelm Mohr et al. 2005).

In the current study perfusate was delivered at constant flow (10ml / min). Delivering perfusate at a constant flow effectively overrides autoregulatory mechanisms (Skrzypiec-Spring, Grotthus et al. 2007). This is advantageous when examining the effects of vasoactive substances (in the case of this work, thromboxane A_2 receptor activation) that may also modulate contractility or heart rate and thus have confounding effects on flow.

B.3.2 Apparatus and Solutions

Because of the strong relationship between cardiac contractile function and temperature (Döring 1990; Sutherland and Hearse 2000) Krebs solution containing in mM: NaCl (118), KH_2PO_4 (1.18), $NaHCO_3$ (25), $MgCl_2$ (1.05), $CaCl_2$ (2.34), EDTA (0.01), and glucose (5.56), pH 7.4 was maintained at 37°C by immersion of perfusate in a water bath. Warmed water from the water bath was circulated via gear pump around a subsystem into a heat exchanger (a double walled cylinder) through which perfusate was passed and through a movable chamber designed to surround the heart and maintain cardiac temperature at 37°C.

Oxygen was provided to cardiac tissue by gassing the perfusion solution with carbogen (95% O_2 and 5% CO_2). The 5% carbon dioxide is required to maintain a pH of 7.4 at 37 °C.

Perfusate (Krebs) was freshly prepared for each experiment and was filtered through a 0.45 μm polyether sulfone membrane (Millipore, USA). Perfusate was delivered at a constant flow rate (10 ml / min) by a peristaltic pump. The pressure of the circulating perfusion solution was measured upstream of the aortic cannula using a pressure transducer attached by a side arm. Prior to starting each experiment all tubing and the cannula were carefully checked and air bubbles removed as these can damage the vascular endothelium or cause ischemia if they become trapped in the coronary circulation. Left ventricular systolic and diastolic pressures were recorded using a pressure transducer connected to a small balloon placed in the left ventricle (Figure B3).

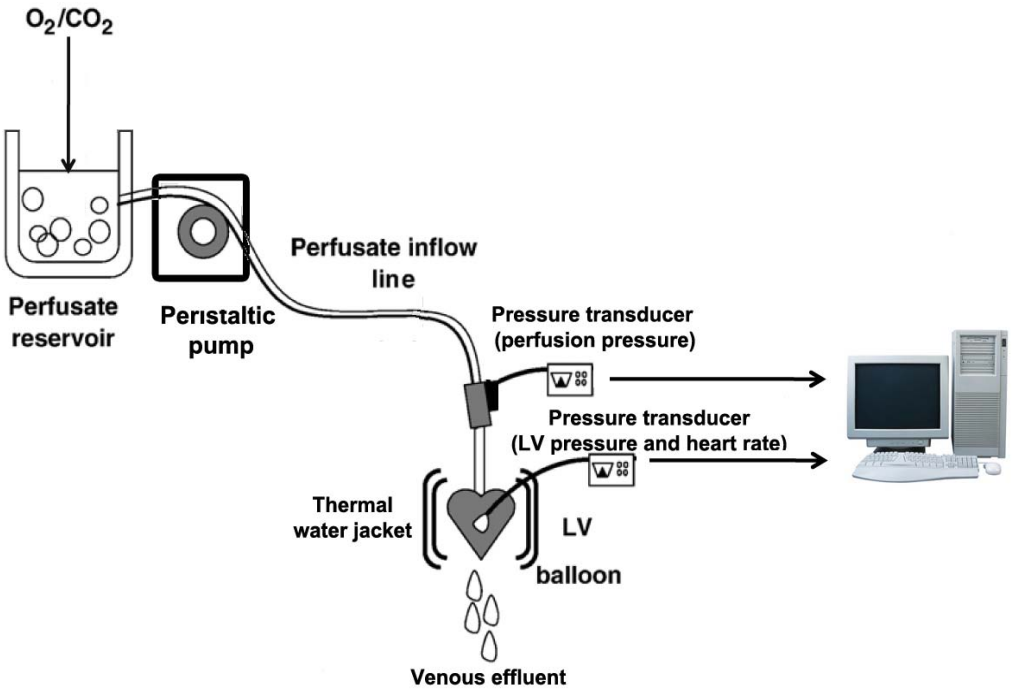

Figure B3 The Langendorff Perfused Heart Preparation. Isolated rat hearts were perfused with carbogen bubbled perfusate at a constant flow rate of 10 ml/min. Coronary perfusion pressure was measured using a force transducer attached via side arm and left ventricular pressure was measured using a latex balloon inserted into the left ventricle, also attached to a force transducer. Figure adapted from Skrzypiec-Spring, 2007 .

B.3.3 Preparation of Animals

Rats weighing 300-350g were anesthetized in an airtight bell jar containing 1 ml of volatile Forthane (isoflurane), and anesthesia was maintained during surgical procedure using nose cone delivery of Forthane. Rats were placed in the supine position and a median laparotomy performed. The abdominal cavity was opened and the mesentery pushed aside to reveal the abdominal vein. Heparin (500 IU) was delivered directly into the vein using a 21G (0.8 x 40mm) needle, followed by the withdrawal of 5 ml of blood for experiments not described in this thesis. The diaphragm was then cut by a transabdominal incision to expose the thoracic cavity and the thoracic cavity opened by cutting along the ribs on both sides. The beating heart was gently raised with the fingers and the aorta, vena cava and pulmonary vessels cut 5 mm distal to the base of the heart.

The heart was immediately placed in ice-cold Ca^{2+} free KREBS containing in mM: NaCl (118), KH_2PO_4 (1.18), $NaHCO_3$ (25), $MgCl_2$ (1.05), EDTA (0.01), and glucose (5.56), pH 7.4. This was done in order to arrest beating and to protect the myocardium from ischemic injury prior to reperfusion.

B.3.4 Reperfusion of Isolated Heart

The isolated heart was rinsed in Ca^{2+} free KREBS and held by forceps by the wall of the aortic root. The aorta was then slipped over a metal cannula and firmly fixed in place with a length of surgical suture. Care was taken not to insert the cannula too far into the aorta, as this can occlude the ostia of the coronary arteries or cause aortic valve incompetence. Coronary perfusion was commenced by starting the peristaltic pump. At this point the heart turned a light pink colour as blood was removed from

small vessels and capillaries. After practice this entire procedure, from extraction of heart to reperfusion, could be performed within 2 minutes. Regular cardiac rhythm returned within 1-10 minutes. During this time remnants of superficial fat, the lungs, and connective tissue were removed from the beating heart.

To undertake intraventricular pressure measurement a 4 mm fluid-filled balloon (AD instruments, Australia) connected to a pressure transducer was inserted into the left ventricle. Specifically, the atrial appendage was opened and the balloon inserted into the left ventricle through the mitral valve. Once inserted the balloon was inflated to 10 mmHg. Hearts were allowed to equilibrate for 10 minutes at 37°C, after which heart rate was paced by a stimulator 10% above the spontaneously occurring heart rate. The right atrium was paced using a bipolar $AgCl_2$ electrode (with two tips) that was placed close to the insertion of the upper vena cava. The stimulator was set to 1 V with a duration of 1 ms, with frequency adjusted to alter heart rate. Following establishment of pacing, hearts were allowed to equilibrate for a further 30 minutes before the dose-response to U-46619 was performed.

B.3.5 Dose Response to U-46619

Prior to commencement of each experiment 300 ml of carbogen bubbled Krebs was placed into the water bath at 37°C. After the equilibration period the perfusion source was switched to the Krebs solution and U-46619 added in a cumulative stepwise fashion (1 nM – 3000 nM). Perfusion pressure was allowed to plateau before the next concentration was added. Due to the onset of arrhythmia at high concentrations of U-46619 dose-response curves were terminated at 3 μM U-46619.

B.3.6 Data Acquisition and Analysis

Pressure transducers were attached to a PowerLab/bridge amp (AD Instruments, Australia), with recordings monitored and stored on a computer. Data was recorded and analyzed using Chart 6.0 (AD Instruments, Australia). The following data was recorded directly: perfusion pressure (PP) and left ventricular pressure (LVP). Heart rate was derived from the cyclic rate of LVP and the rate of pressure change (dP/dT) was also derived from LVP.

In order to construct dose response curves perfusion pressure was determined at the point of plateau for each concentration of U-46619. This data was expressed as developed perfusion pressure, which was determined by subtracting perfusion pressure at the point of interest from baseline perfusion pressure. Developed perfusion pressure was reported in mmHg. For the purpose of this thesis LVP and dP/dT recordings were not analysed, as they will be presented in another thesis.

B.4 Separation of Proteins Using SDS-PAGE

B.4.1 Premise

Electrophoresis is the motion of particles relative to fluid under the influence of an electric field. When applied to biological samples it refers to the separation of molecules according to their electrophoretic mobility, which is a function of molecule shape and molecular weight. The first reproducible example of electrophoresis was published in 1937 by Arne Tiselius who separated the protein constituents of blood serum (Tiselius 1937). Subsequently, Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has become one of the most frequently used techniques in biological research.

SDS-PAGE is the separation of proteins in the presence of the ionic detergent sodium dodecyl sulfate (SDS). Proteins are coated with detergent and become negatively charged by their attachment to the SDS anions. When an electrical field is applied to the gel, negative charge flows from negative cathode into the upper buffer chamber, through the gel and into the lower buffer chamber that is attached to the positive anode. Negatively charged molecules move from negative to positive and thus proteins are fractionated by size (Laemmli 1970).

B.4.2 Acrylamide Gel Composition and Preparation

Polyacrylamide gels are prepared by polymerizing acrylamide and N,N-methylene-bis-acrylamide (bisacrylamide) which acts as a crosslinker. The polymerization is initiated by the addition of a free radical source - ammonium persulfate or riboflavin along with a catalyst N,N,N,N-tetramethylethylenediamine (TEMED). Due to the

relatively uniform negative charge on all proteins incubated with SDS, separation of most globular proteins is largely dependent on the size of the pores formed by the crosslinked acrylamide. Pore size is dependent on the total amount of acrylamide (including bisacrylamide) (%T) and the amount of cross-linker, which is expressed as a ratio of crosslinker to acrylamide (%C). 5%C provides the smallest pore size with any increase or decrease in %C increasing the pore size. 2.67%C is traditionally used for most preparations, as is the case here. Gels are usually referred to as total acrylamide given as a percentage (w/v). Generally, pore size decreases with increasing %T. Proteins with low molecular weights separate best on high % acrylamide gels, while higher molecular weight proteins require a lower acrylamide concentration to affect efficient separation.

Gels were cast using a Mini-PROTEAN casting module (BioRad). Gels were 8.3 x 7.3 cm and were 1.5mm thick. For both eNOS and MYPT 7.5% acrylamide gels were utilized. Stacking gels had the following composition: 7.3% (w/v) acrylamide, 0.2% (w/v) bisacrylamide, 375 mM Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.025% (v/v) TEMED, 0.025% (w/v) ammonium persulfate. The stacking gel solution was gently mixed to avoid the introduction of air bubbles which inhibit polymerization, and cast between glass plates unit. To ensure a uniformly smooth separating gel interface the gel air interface was overlaid with a thin layer of water-saturated butanol. Gels were allowed to polymerize for 1 hour. Following removal of butanol and several rinses to remove residual water saturated butanol a stacking gel was cast which was composed of: 4.87% (w/v) acrylamide, 0.13% (w/v) bisacrylamide, 375 mM Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.025% (v/v) TEMED, 0.025% (w/v) ammonium persulfate. Typically gels were cast using 1.5mm x 15 well combs.

B.4.3 Sample Preparation

Following myography or isolation of arteries, enzymatic activity was stopped by snap freezing vessels in dry ice cold 10 % trichloroacetic acid (TCA) / acetone followed by 3 rinses in acetone to remove residual TCA. Samples were stored at -80°C prior to protein extraction using a modified 1X SDS sample buffer (Laemmli 1970). For eNOS SDS sample buffer was composed of: 50 mM Tris/HCl pH 6.8, 1% (w/v) SDS, 10% (v/v) complete protease inhibitor cocktail (Sigma Aldrich), 20 mM dithiothreitol, 15% (v/v) glycerol and 0.1 % (w/v) bromophenol blue. For MYPT1 analysis sample buffer was composed of: 50 mM Tris-HCl, pH 6.8, 1% SDS, 10% (v/v) complete protease inhibitor cocktail, 20 mM dithiothreitol, 100 μM di-isopropylfluorophosphate, 10% sucrose and 0.1 % (w/v) bromophenol blue.

In studies represented by the first two chapters of this thesis 80 μl of SDS buffer was added to each 2 mm segment of caudal artery. For later western blot analysis empirical evaluation identified that protein extraction could be optimized using 120 μl of sample buffer for each 2 mm caudal artery segment, and 250 μl for a 1 cm arterial segment used in the analysis of total eNOS. Immediately following the addition of sample buffer samples were heated to 95°C for 5 minutes. Samples were then vortexed for 1 minute and were agitated on a shaker for 1 hour. Samples were then centrifuged for 5 minutes at 4000 RPM to sediment residual insoluble particulate material. Samples were immediately loaded into the gels.

B.4.4 SDS-PAGE Loading and Running

Gels were inserted into a Biorad Mini-PROTEAN electrophoresis module. Wells were filled with cold SDS electrophoresis buffer composed of: 25 mM Tris-HCl, 192 mM glycine and 1% (w/v) SDS. Using a 20 μl pipette 4 μl of molecular mass markers were loaded into the first lane of each gel. The optimal sample loading volume was determined for eNOS and MYPT by preliminary experiments. Figure B4 indicates the liner loading range for each antibody used. An equal volume of SDS sample buffer was loaded into empty wells to ensure uniformity of protein migration. The inner buffer chamber, followed by the outer chamber was gently filled with SDS electrophoresis buffer. The gel unit was then connected to a power supply and electrophoresis conducted at a constant voltage of 200V for one hour, or until the bromphenol blue dye front reached the bottom of the separating gel.

Because this study examines myofilament-associated proteins that are not easily solubilized in detergents compatible with protein assays, variations in protein load could not be account for in this fashion. Alternatively, this study accounts for variations in protein load using four techniques: 1) vessel size similarity confirmed by KCl-mediated contractions in the wire myograph, 2) validation that all study antibodies detect in the linear range when loading 2 mm segments of caudal artery (Figures B4 and B5), 3) for eNOS total protein experiments in section E, the normalization of samples to corresponding myosin heavy chain bands on coomassie stained gels and, 4) for experiments indexing phosphorylation, the use of ratiometric analysis, that presents phosphorylation data as a ratio of OD values for phosphorylated protein and total pan-protein ie. P[Thr855]-MYPT1 / Pan-MYPT1.

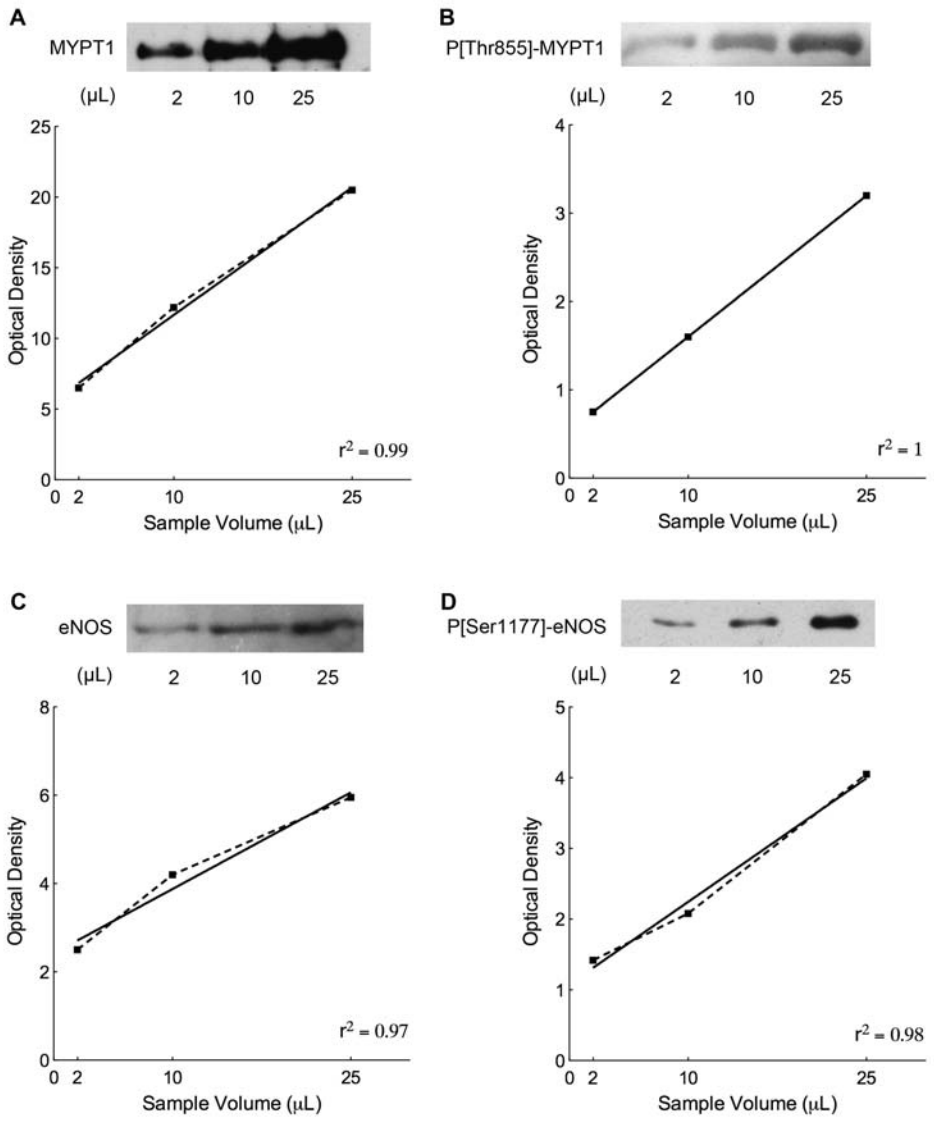

Figure B4 Western Blot Analysis was Conducted Within the Linear Range of Detection for Each Protein/Antibody Pair. To ensure that western blot detection was within the linear range of detection 2 μL, 10 μL and 25 μLs of protein extracts from rat caudal arteries stimulated for 10 minutes with U46619 (1μ) were loaded into acrylamide gels. Following protein transfer nitrocellulose membranes were probed with the following antibodies: (**A**) pan-MYPT1, (**B**) P[Thr855]-MYPT1, (**C**) pan-eNOS, and (**D**) P[Ser1177]-eNOS. Linear regression analysis of the OD for each antibody/protein load was conducted and provided the following r^2 values as an index of linearity: Pan-MYPT1, 0.99, P[Thr855]-MYPT1, 1.0, Pan-eNOS, 0.97 and P[Ser1177]-eNOS, 0.98, validating that detection was within the linear range.

B.5 Separation of P-LC_{20} Using Urea Glycerol Electrophoresis

B.5.1 Premise

The separation of unphosphorylated, monophosphorylated and diphosphorylated forms of myosin LC_{20} using urea/glycerol gel electrophoresis was first demonstrated by Sobiezek and Jertschin in 1986. In this system proteins are solubilized using a combination of the non-ionic chaotropic agents urea and thiourea, enabling the low molecular weight myosin LC_{20} to be electrophoretically separated by charge. The negative charge imparted by a phosphoryl group makes it possible to separate unphosphorylated from single and diphosphorylated myosin LC_{20} (Sobieszek and Jertschin 1986).

B.5.2 Sample Preparation

Following myography or arterial isolation of arteries, enzymatic activity was stopped by snap freezing in dry ice cold in 10% trichloroacetic acid (TCA) and samples rinsed 3 times in acetone to remove residual TCA. Samples were stored at -80°C prior to protein extraction. Rat caudal artery samples were extracted in 100 μL of thio-urea sample buffer containing 4 M urea, 2 M thiourea, 200 mM Tris, 220mM glycine, 10 mM dithiothreitol 0.01% (w/v) bromophenol blue, 10 mM EDTA (pH 7), 600 mM potassium iodide and 1% (w/v) mixed bed resin (20-50 mesh). Samples were vortexed for 2 minutes and were then agitated for 60 minutes at room temperature. Following extraction samples were immediately loaded onto gels.

B.5.3 Preparation and Running of Urea Glycerol Gels.

Separating and stacking gels were made as per section B.3.2, however, a modified stacking and separating gel composition was used. Separating gel was composed of: 10 % (w/v) acrylamide, 0.27 % (w/v)*N,N'*-methylene bisacrylamide (Bis), 0.375 M Tris-HCl (pH 8.8), 40 % (v/v) glycerol, 0.5 % (w/v) ammonium persulphate and 0.044 % (w/v) *N,N,N',N'*-tetramethylethylenediamine (TEMED). Stacking gel was composed of: 2.25 % (w/v) acrylamide, 0.12 % (w/v) Bis, 0.12 M Tris-HCl (pH 6.8), 8.5 M urea, 0.6 % (w/v) ammonium persulphate and 0.2 % (w/v) TEMED.

Preliminary experiments determined the linear range for the relationship between resulting signal intensity and protein amount (Figure B5). 10 μl of each sample was loaded. Inner and outer chambers were filled with running buffer (50 mM tris, 0.1 M glycine (pH 8.8)) and the gel tanked attached to a power supply. Electrophoresis was carried out at 6 mA for 5 h. Samples were transferred to nitrocellulose membrane (Section B5.2) and phosphorylated and non-phosphorylated LC_{20} visualized using western blot analysis (Section B5).

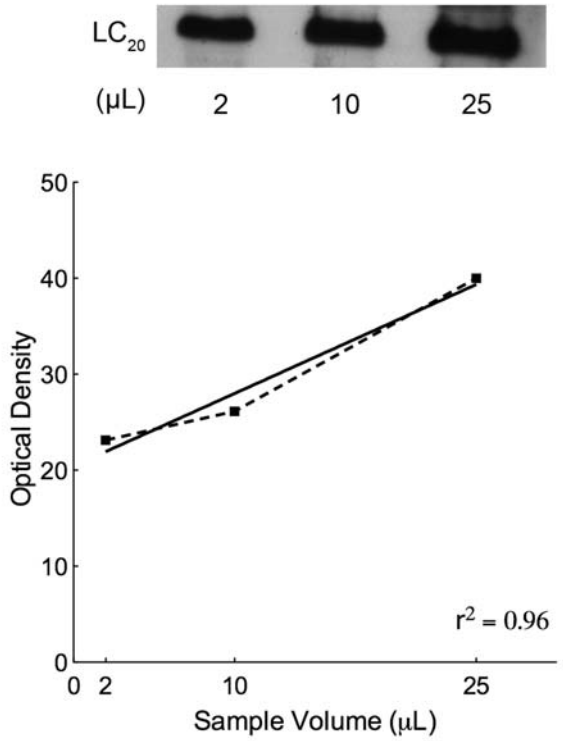

Figure B5 Western Blot Analysis was Conducted within the Linear Range of Detection for LC_{20}. To ensure that western blot detection was within the linear range of detection 2 μL, 10 μL and 25μLs of protein extracts from rat caudal arteries stimulated for 10 minutes with U46619 (1 μM) were loaded into urea glycerol gels. Following protein transfer the nitrocellulose membranes were probed with LC_{20} antibody. Linear regression analysis of the optical density was conducted and provided an r^2 value of 0.96 validating that detection was within the linear range.

B.6 Western Blot Analysis

B.6.1 Premise

Western blotting (or immunoblotting), is used to identify specific proteins that have previously been separated using SDS-PAGE then transferred and immobilized onto a nitrocellulose membrane (Towbin, Staehelin et al. 1979). Proteins are visualized using polyclonal or monoclonal antibodies specific to the protein of interest. Western blotting is a highly sensitive technique, with reports of detection of antigen down to 1 fg reported (Walker, Feather et al. 1995). A particular strength of the western blot technique is the ability to employ antibodies that are specific for phosphoryated amino acids on a given protein along with being able to detect the total amount of a given protein. This approach facilitates the estimate of relative changes in the phosphorylation state of a protein.

B.6.2 Transfer of Separated Proteins to Nitrocellulose

Following separation of proteins using SDS-PAGE samples were transferred from acrylamide gels to nitrocellulose. Transfer was performed using a vertical tank Mini-Protean II transfer system (BIO-RAD, Australia).

Because of significant variability in the transfer process it can be difficult to compare blots transferred in multiple units. To overcome this limitation, where possible, gels were transferred on a single transfer unit in a single run. To facilitate this, gels were placed on a light table and trimmed using a metal ruler. For both eNOS and MYPT1, gels were trimmed below the 90 kDa prestained molecular weight marker and above the 200 kDa molecular weight marker.

Transfer buffer was prepared and refrigerated two hours prior to transferring gels to ensure it was at 4°C. The composition of transfer buffer for eNOS and MYPT was: 25 mM tris, 192 mM glycine, 1% (w/v) SDS and 20% (v/v) methanol. Transfer buffer was similar for LC_{20}, transfer, with the exception that SDS was excluded. Prior to transfer acrylamide gels were equilibrated for 10 minutes at room temperature in transfer buffer. A piece of filter paper was laid on top of a Scotch-Brite pad. Acrylamide gels were placed on top of the filter paper. A sheet of nitrocellulose membrane was then placed on top of gels, special care was taken to avoid the introduction of air bubbles between the gel/membrane interface. Finally, another layer of filter paper followed by another Scotch-Brite pad were placed on top. This preparation was then placed into a transfer cassette (BIO-RAD) and the entire assembly inserted into a transfer unit and tank (BIO-RAD). The cassette was inserted with the membrane orientated towards the negative anode side of the transfer unit.

The transfer tank was gently filled with transfer buffer; to maintain constant temperature a block of ice in a sealed container was added to tank. A magnetic stir bar was added and tank was placed on a stir block and was gently stirred for the entire transfer period. Tank was connected to a power source and a voltage of 100 V applied. Optimum transfer times have been established previously in our laboratory. For eNOS and LC_{20} detection transfer was 45 minutes and for MYPT1 detection 30 minutes.

Following transfer, gels were stained with coomassie brilliant blue (R-250) to validate transfer. Under the transfer conditions used the high molecular weight myosin heavy

chains did not transfer completely which provided means to normalize for any variation in protein load. Gels were covered in coomassie blue (0.2% coomassie blue (w/v), 10% acetic acid (v/v), 50% ethanol) (v/v)) and were agitated slowly for 4 hours. Gels were then incubated with destain solution (10% acetic acid (v/v)) overnight to remove excess coomassie. Gels were scanned using a BIO-RAD GS-710 densitometer.

To further verify transfer efficiency nitrocellulose bound proteins were visualized using Ponceau S. Nitrocellulose membranes were placed in Ponceau S solution (0.1% (w/v) Ponceau S, 5% (v/v) glacial acetic acid) for 5 minutes at room temperature and were gently agitated. Western blots were destained for 2 minutes in deionized H_2O. After visualization membrane was destained by agitating in TBS (50 mM Tris-HCl, pH 7.4 and 150 mM NaCl) for 5 minutes.

B.6.3 Blocking

Nonspecific binding of primary and secondary antibodies to nitrocellulose were blocked by incubating western blots in 100 ml of blocking solution (50mM Tric HCl pH 7.4, 150 mM NaCl, 0.05% Tween (v/v), 5% nonfat milk powder (w/v)) for one hour with gentle agitation.

B.6.4 Incubation with Primary Antibody

Following the blocking procedure membranes were transferred into a sealable roller tube containing primary antibodies diluted in a solution containing: 50mM Tric HCl pH 7.4, 150 mM NaCl, 0.05% Tween (v/v), 1% nonfat milk powder (w/v)). Primary antibody dilutions were: anti-MYPT (mouse) 1:1000, anti-P[Thr855]MYPT (rabbit)

1:1000, anti-eNOS (rabbit) 1:1000, anti-P[Ser1177]eNOS (rabbit) 1:1000 dilution, anti-LC20 (rabbit) 1:1000 dilution.

Following a 60-minute incubation with primary antibodies membranes were rinsed three times with 20 ml TBS-T (50mM Tric HCl pH 7.4, 150 mM NaCl, 0.05% Tween (v/v)) for 5 minutes to remove unbound primary antibody.

B.6.5 Incubation with Secondary Antibody

In order to visualize bound primary antibodies horseradish peroxidase (HRP) conjugated secondary antibodies specific for either rabbit or mouse IgG were used. For use with west femto (Pierce/Thermo Scientific, USA) secondary antibodies were diluted 1:50,000 and for use with standard ECL reagents (GE Healthcare, UK) secondary antibodies were diluted 1:10,000. Secondary antibodies were diluted in 10 ml of TBS-T. Membranes were incubated in tubes with secondary antibody for 1 hour at room temperature with gentle rotation.

Following incubation membranes were rinsed three times with 20 ml TBS-T for 5 minutes to remove unbound secondary antibody.

B.6.6 Enhanced Chemiluminescence Detection of Specific Proteins

Two ECL reagents were used in the described experiments. Standard ECL reagent (GE Healthcare, UK) and the ultra sensitive West Femto (Pierce/Thermo Scientific, USA). Both reagents are delivered in two separate solutions that are mixed 1:1 to create an active substrate for HRP-chemiluminescence detection. For standard ECL

reagent 500 μl of final solution was used per blot. For West Femto 250 μl was used per blot.

Using a 1 ml pipette ECL reagent was applied to the nitrocellulose membrane. Excess reagent was removed and membrane covered with commercial plastic wrap. Western blots were exposed to autoradiographic film for 5 minutes, 2 minutes, 1 minute, 30 seconds, 10 seconds and 2 seconds. Film was developed on an X-Ray developer (Agfa), using standard developer chemicals (Agfa).

B.6.7 Reprobe for MYPT1

Both pan-MYPT1 (mouse) and [PThr855]-MYPT1 (rabbit) were visualized on the same blot. This had the advantage of greatly reducing interblot variability. Preliminary experiments and previously published reports (Wilson, Susnjar et al. 2005) indicated that there was no cross reactivity between mouse IgG containing primary antibody and rabbit IgG detecting secondary antibody and vice versa.

Blots were probed for pan-MYPT1 as described above. Following blot development ECL regent was removed with three rinses in 20 ml TBT-T. This was followed by a 10-minute incubation in 10% acetic acid to denature and deactivate bound mouse secondary antibody. In order to validate this was successful blots were reincubated with ECL reagents and were redeveloped. No signal was present after this treatment.

Blots were then reprobed for [PThr855]-MYPT1 using the procedure described above (Section B5.4).

B.6.8 Data Analysis

Both developed film and coomassie stained gels were scanned on a densiometric scanner (GS-710, BIO-RAD) and optical density analysed using Quantity One software (BIO-RAD).

Optical density (OD) is the result of the darkness of a given pixel in a digital image. Quantity One detects the average OD of a selected area times its area (INT*mm^2), referred to a volume. A bounding box was drawn around the largest band on the image. Bounding boxes with the same dimensions were then placed around each band of interest. Background subtraction was performed by selecting an area of film/gel that did not contain signal. OD volume was assessed for each bounding box.

To account for variability imparted by extraction of sample and/or loading of sample onto the gel, phosphorylation data is expressed as a ratio of OD values for phos/pan (ie. P[Thr855]-MYPT1/Pan-MYPT1). For total protein experiments in section E, each band of interest was normalized to the corresponding sample on the coomassie stained gel. Specifically a distinctive high molecular weight protein (myosin) was used for this process. In cases where samples spanned multiple blots each sample was normalized to an internal control that was standard across all blots (10 μl of rat caudal artery). Normalized values are presented as arbitrary OD units.

C. Acute Statin Administration Inhibits α_1-Adrenoreceptor Mediated Constriction in Rat Caudal Artery via Activation of Endothelial Nitric Oxide Synthase.

C.1 Introduction

The 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase inhibitors, better known as statins, are commonly prescribed for the treatment of hypercholesterolemia (Maron, Fazio et al. 2000; Pasternak, Smith Jr et al. 2002). By inhibiting the rate-limiting step of the mevalonate pathway statins inhibit intracellular cholesterol production. This results in the upregulation of hepatic plasma membrane LDL receptors and removal of serum LDL (Goldstein and Brown 1973; Reihner, Rudling et al. 1990). Numerous clinical trials have documented the efficacy of statins particularly in secondary prevention of coronary artery disease, myocardial infarction and stroke (Shepherd, Cobbe et al. 1995; Sacks, Pfeffer et al. 1996; Baigent, Keech et al. 2005; Collaborators 2005).

Endothelial dysfunction is characterized by impaired endothelial-dependent vasorelaxation and is a robust predictor of long-term cardiovascular outcome. (Schachinger, Britten et al. 2000). Hypercholesterolemia is associated with impaired endothelial function and statins, by lowing plasma LDLs, improve endothelial-dependent vasodilation (Tamai, Matsuoka et al. 1997). In human subjects treated with statins, however, improvements in endothelial function are seen before cholesterol lowering occurs while non-statin lipid lowering drugs do not afford such improvements, suggesting that statins may have cholesterol independent effects (Tsunekawa, Hayashi et al. 2001).

The cholesterol independent, or “pleiotropic” effects of statins may be mediated by their ability to inhibit the synthesis of isoprenoid groups that, like cholesterol, are produced downstream from mevalonate (Goldstein and Brown 1990). Isoprenoids

such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) are important posttranslational modifications and by inhibiting their production statins inhibit the membrane targeting of the small GTPase, RhoA, limiting the activation of its downstream effector, Rho-kinase (Wang, Liu et al. 2008).

Evidence from cell culture has demonstrated that endothelial nitric oxide synthase (eNOS) is regulated by the RhoA/Rho-kinase pathway and that statins are able to increase the synthesis of nitric oxide via two mechanisms. Firstly, by increasing endothelial nitric oxide synthase (eNOS) total protein (Laufs, La Fata et al. 1998; Laufs, Gertz et al. 2002), and secondly, by increasing PI3K/PKB mediated activation of eNOS through phosphorylation of Ser1177 of eNOS (Laufs, La Fata et al. 1998; Harris, Blackstone et al. 2004). Because the dysregulation of nitric oxide has been demonstrated to contribute to impaired vasorelaxation, increased nitric oxide production by statins may account for the reported cholesterol independent improvements in endothelial function. This also suggests that statins may be useful in conditions involving increased vascular contraction, such as hypertension and vasospasm.

The vascular α_1-adrenergic receptor is important in regulating smooth muscle contraction. A role for increased sympathetic drive and increased vascular α_1-adrenergic receptor activation/expression has been described in several cardiovascular disease states, including vasospasm and essential hypertension (Malpas 2010). The catecholamines bind to this heterotrimeric G-protein coupled receptor with the subsequent activation of the G_q subunit resulting in the phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5 bisphosphate to generate the second

messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 induces intracellular Ca^{2+} release, whereas DAG activates protein kinase C and TRPC channels, both contributing to vascular smooth muscle contraction (Wang, Deng et al. 2008; Docherty 2010).

Our laboratory (Dunn, Copley et al. 2008) and others (Budzyn, Marley et al. 2004) have described an inhibitory effect of statins on contractions elicited by α_1-adrenoreceptor activation. The following series of studies describe results obtained using an isolated rat caudal artery model designed to identify the mechanism(s) involved in statin-mediated inhibition of α_1-adrenoreceptor mediated contraction. Experiments were designed to determine the role of the vascular smooth muscle, endothelium, and nitric oxide, specifically, whether statin mediated attenuation of contraction was due to uprgeulation of eNOS protein synthesis and/or increased eNOS activation mediated by phosphorylation of Ser1177 eNOS. Furthermore, experiments were designed to determine if this effect is specific to the hydrophilic pravastatin or hydrophobic simvastatin. This study indicates that both the hydrophilic pravastatin and hydrophobic simvastatin relax α_1-adrenoreceptor mediated constriction via increased phosphorylation of eNOS at Ser1177.

C.2 Methods

C.2.1 Materials

General laboratory reagents are listed in Section B1. Reagents specific to this paper were purchased from the following sources: pravastatin, simvastatin, phenylephrine, indomethacin, GSNO were from Sigma–Aldrich (Australia). Rabbit polyclonal antibodies specific for pan-eNOS (anti-eNOS) and eNOS Ser1177 (anti-P[Ser1177]-eNOS) were purchased from Upstate/Millipore USA, Inc. Anti-Rabbit-HRP were purchased from Santa Cruz Biotechnology (USA). Enhanced chemiluminescent (ECL) reagents were from GE Healthcare (Australia).

C.2.2 Preparation of Isolated Arteries

All animal experiments were approved by the University of Adelaide animal ethics committee. Male Sprague Dawley rats, 300-350g, were killed using CO_2 inhalation. Caudal artery was dissected and placed into cold Ca^{2+}-free, HEPES-Tyrode buffer containing in mM [135.5 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 11.6 HEPES, 11.6 glucose, pH 7.4]. Where appropriate the endothelium was removed by rubbing vessel segments 30 times over a wire 0.34 mm in diameter. Each arterial segment was cleaned of adventitia and cut into 2 mm segments.

C.2.3 Vascular Myography

Caudal artery segments were mounted on a Mulvany wire-myograph (610M, Danish myotechnologies, Denmark) coupled to a computer (Apple, USA) and Chart 6.0 software (AD Instruments, Australia) enabling quantification and recording of isometric force. Vessels were equilibrated in normal HEPES-Tyrode buffer

containing in mM [135.5 NaCl 5.9 KCl 1.2 $MgCl_2$, 2.5 mM $CaCl_2$, 11.6 HEPES 11.6 glucose pH 7.4] and set at an optimal resting tension of 10 mN. Arteries were allowed to equilibrate for 30 minutes before being stimulated three times with 87 mM K^+-HEPES-Tyrode buffer containing in mM [54.4 NaCl, 87 KCl, 1.2 $MgCl_2$, 2.5 mM $CaCl_2$,11.6 HEPES 11.6 glucose pH 7.4]. The presence of a functional endothelium was confirmed by acetylecholine (1 μM) mediated relaxation in response to a phenylepherine (10μM) challenge. Following relaxation in HEPES-Tyrode solution, arteries were incubated with therapeutic plasma concentrations of pravastatin (112 μM), and/or the NOS blocker N^G-monomethyl-L-arginine (L-NAME) (10 μM) for one hour. Following a 10 minute stimulation with phenylephrine (10 μM) in the presence or absence of inhibitors and/or pravastatin, tissues were snap frozen in dry ice cold 10% trichloroacetic acid/acetone, followed by 3 washes with dry ice-cold acetone to remove residual TCA, and stored at -80°C prior to SDS-PAGE/western blotting.

C.2.4 Western Blotting of eNOS

Protein was extracted from snap frozen caudal artery segments using 80 μl SDS-PAGE buffer containing 50 mM Tris/HCl, pH 6.8, 1% (w/v) SDS, 10% (w/v) complete protease inhibitor cocktail, 20 mM DTT and 15% (v/v) glycerol. Samples were heated to 95°C for 5 min, then agitated for 60 minutes prior to SDS/PAGE using a BioRad mini protean II unit at 200 V for 60 minutes. Proteins were transferred onto 0.22 μm nitrocellulose at 100 V for 60 minutes in transfer buffer containing 25 mM tris, 192 mM glycine, and 20% (v/v) methanol.

Following transfer, non specific antibody binding sites on the nitrocellulose membranes were blocked using a solution containing 5% non-fat dried milk in Tris buffered saline – Tween (TBS-T) (20 mM, Tris, 500 mM NaCl, 0.05% (v/v) Tween-20) for 60 minutes, followed by incubation with 1% non-fat dried milk/TBS-T containing anti-eNOS (1:1000) or anti-P[Ser1177]eNOS (1:1000) for 60 minutes. Membranes were washed 3 times in TBS-T prior to incubation in secondary antibody solution containing a 1-10,000 dilution of horseradish peroxidase (HRP) conjugated secondary antibodies (anti-rabbit IgG-HRP). HRP-mediated chemiluminescence signal detection used ECL reagents and standard X-Ray film (Afga). Optical density of protein bands were analyzed with Quantity One software (BioRad).

C.2.5 Statistical Analysis

All results are presented as the mean ± S.E.M where *n* indicates the number of independent experiments for each treatment. Student's *t* test was used to determine statistical significance. Asterisks indicate statistically significant differences from control.

C.3 Results

C.3.1 Acute Pravastatin Administration Inhibits α_1-Adrenoreceptor Mediated Contraction Through an Endothelial / Nitric Oxide Mediated Mechanism in Rat Caudal Artery

The α_1-adrenoreceptor-agonist phenylephrine causes contraction of rat caudal artery in a concentration-dependent manner. Figure C1 depicts a typical dose-response curve to phenylephrine. Half-maximal activation occurred at 3 μM and maximal contraction at 30 μM. EC_{90} was 10 μM, and was selected as the working concentration of agonist for all subsequent experiments. Following a series of 87 mM KCl stimulations/washouts arteries were incubated for 60 minutes in the presence or absence of the human therapeutic plasma equivalent level of pravastatin (112 nM) followed by stimulation with phenylephrine (10 μM). A 60-minute pravastatin pre-treatment significantly attenuated phenylephrine-mediated constriction compared to control (Figure C2, control = 101.1 ± 2.6%, pravastatin = 94 ± 3.2%, $P < 0.05$, $n = 8$).

In order to establish the endothelial dependence of the statin mediated attenuation of α_1-adrenoreceptor-mediated contraction the endothelium was mechanically removed and the absence of endothelium validated by a lack of acetylcholine-mediated vasodilatation. In the absence of endothelium the pravastatin-mediated inhibition of α_1-adrenoreceptor-mediated contraction was abolished (Figure C2, control = 101.1, ± 2.6%, endothelium removed + pravastatin = 101 ± 3.4%, $P = NS$, $n = 8$).

The role of endothelial nitric oxide synthase (eNOS), in the statin mediated attenuation of α_1-adrenoreceptor-mediated contraction was examined by pretreating

endothelium-intact vessels with pravastatin in the presence or absence of the NOS inhibitor L-Nitro Arginine Methyl Ester (L-NAME, 100 μM). Similar to endothelial removal, the co incubation of L-NAME with pravastatin abolished the pravastatin-mediated reduction in phenylephrine-developed tension (Figure C2, control 101 ± 2.6%, L-NAME + pravastatin = 99.9 ± 3.68, P = NS, n = 8).

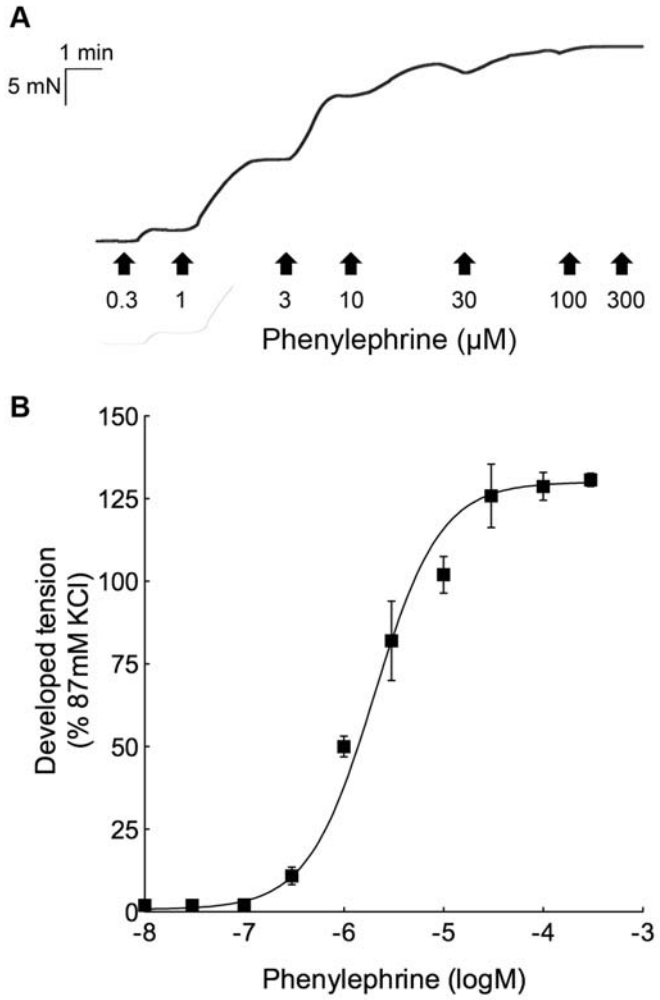

Figure C1 Phenylepherine-Mediated Vasoconstriction of Rat Caudal Artery is Concentration Dependent. Caudal artery segments were mounted in a wire myograph and developed tension recorded in response to increasing concentrations of the α_1-adrenoreceptor agonist, phenylepherine. **A)** Representative trace of phenylepherine-mediated dose response **(B)** Culmative dose response data. EC_{50} occurred at 3 μM and EC_{100} at 30 μM (n = 3).

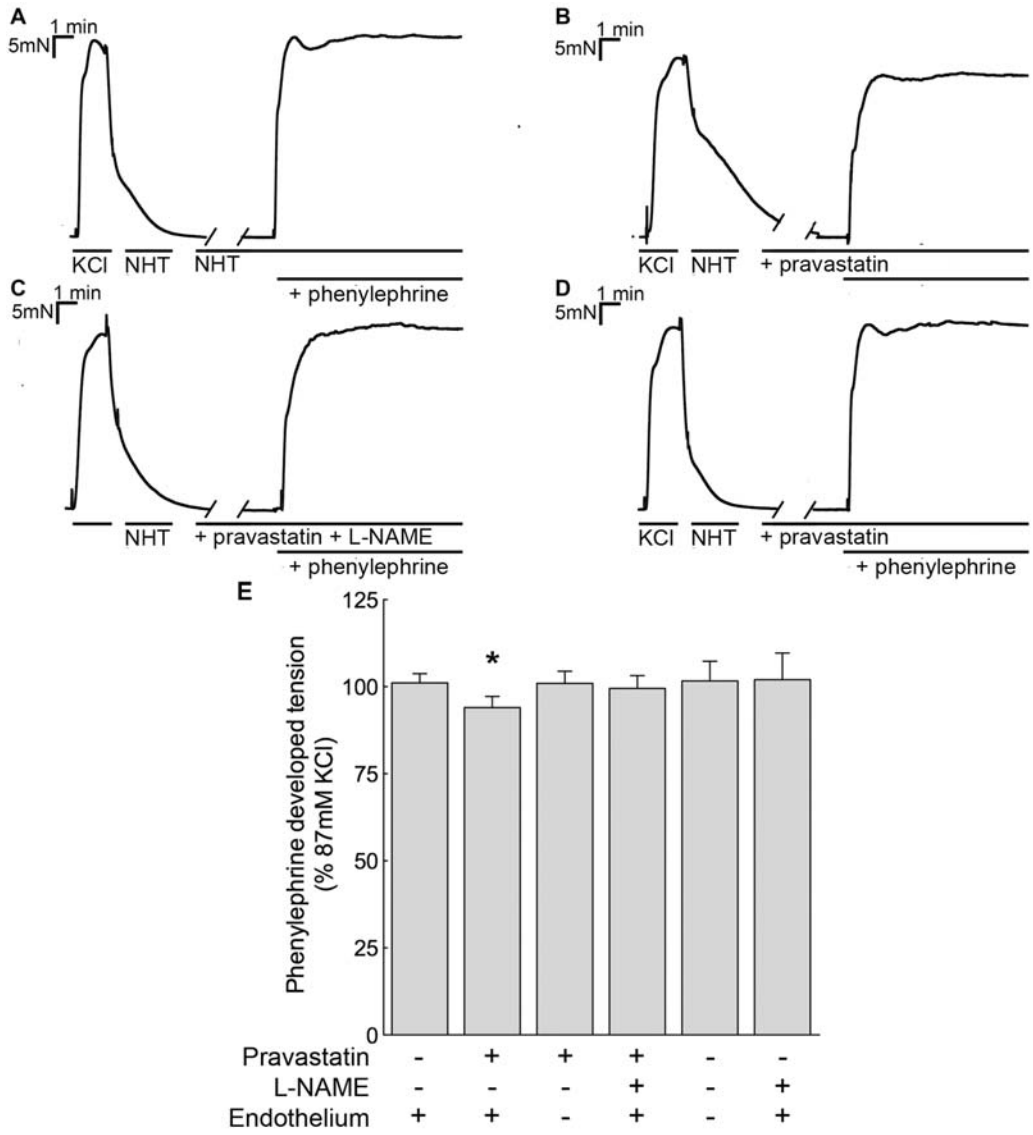

Figure C2 Pravastatin Inhibits α_1-adrenoreceptor Mediated Contraction Through an Endothelial and Nitric Oxide Synthase-Dependent Mechanism. Rat caudal artery in the presence and absence of an endothelium was mounted in a myograph and incubated with pravastatin (112 nM) and/or L-NAME (100 μM) for 60 minutes prior to stimulation with phenylephrine (10 μM). (**A-D)** illustrates representative data for individual experimental treatments. (**E)** represents pooled data. Pravastatin inhibited phenylephrine-mediated contraction **(B)** compared to control **(A)** (* $P < 0.5$, n=8). Coincubation with L-NAME (**C**), or removal of the endothelium (**D**) abolished pravastatin-mediated attenuation of phenylephrine-mediated developed tension (P = NS, n = 8).

C.3.2 Inhibition of Prostaglandin Synthesis Attenuates α_1-Adrenoreceptor Mediated Contraction.

Because PGI_2 is a well-described endothelial derived vasodilator (Frangos, Eskin et al. 1985; Vane and Botting 1995), it was necessary to examine a role for the prostanoid in pravastatin-mediated inhibition of α_1-adrenoreceptor contraction. The non-specific cyclooxygenase inhibitor, indomethacin, was used to block prostanoid synthesis. Rat caudal artery was incubated with pravastatin (112 μM) in the presence or absence of indomethacin (10 μM) for 60 minutes prior to α_1-adrenoreceptor stimulation with phenylephrine (10 μM). Pre-treatment with either pravastatin or indomethacin inhibited α_1-adrenoreceptor-mediated developed tension compared to control (Figure C3, control = 100.9 ± 2.6%, pravastatin 95.48 ± 3.8%, indomethacin = 91.5 ± 5.9%, $P < 0.5$, $n = 6$). Coincubation with pravastatin and indomethacin did not alter phenylephrine developed tension compared to indomethacin alone (Figure C3, indomethacin = 91.5 ± 5.=5.9%, indomethacin + pravastatin = 89.5 ± 3.6%, $n = 6$).

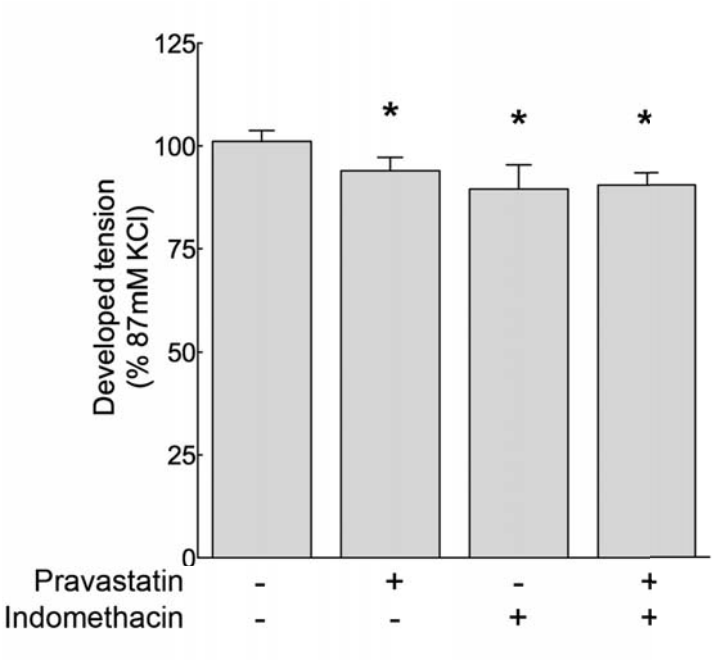

Figure C3 Inhibition of Prostaglandin Synthesis Attenuates α_1-Adrenoreceptor Mediated Contraction. Rat caudal artery was incubated with pravastatin (112 μM), in the presence or absence of indomethacin (10 μM) for 60 minutes prior to stimulation with phenylephrine. Independently, the administration of pravastatin or indomehtacin reduced α_1-adrenoreceptor mediated contraction compared to control (* $P < 0.5$, $n = 6$). However, coincubation with indomethacin and pravastatin was not additive compared with pravastatin or indomethacin alone (P = NS, $n = 6$).

C.3.3 Pravastatin Does Not Alter The Contractile Response to Plasma Membrane Depolarization with KCl Solution

A number of studies have reported that statins alter intracellular Ca^{2+} entry in smooth muscle cells (Ng, Davies et al. 1994; Bergdahl, Persson et al. 2003). In order to determine whether acute treatment with plasma therapeutic equivalent concentrations of pravastatin alters the activation state of plasma membrane associated voltage-dependent Ca^{2+} channels, arteries were incubated in the presence or absence of pravastatin (112 nM) followed by stimulation with a depolarizing solution containing 87 mM KCl. Incubation with pravastatin did not alter KCl developed tension compared to control (Figure C4, control = 95.80 ± 2.1%, pravastatin = 93.25 ± 3.35, $P < 0.5$, $n = 5$).

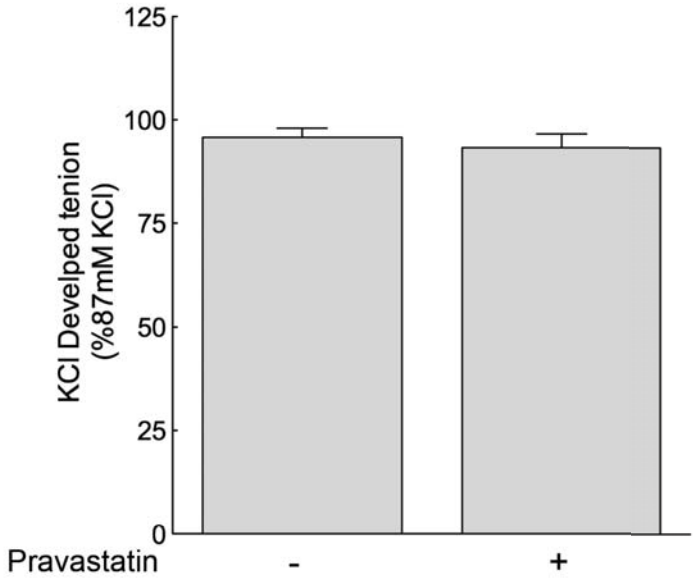

Figure C4 Pravastatin does Not Alter Developed Tension Induced by Plasma Membrane Depolarization. Rat caudal artery was incubated in the presence or absence of 112 mM pravastatin for 60 minutes prior to stimulation with 87 mM KCl. Incubation with pravastatin did not alter contraction compared to control (P < NS, n = 8).

C.3.4 Acute Pravastatin Treatment Does Not Alter Smooth Muscle Sensitivity to GSNO

To determine if pravastatin inhibits α_1-adrenoreceptor mediated contraction by sensitizing the smooth muscle to endothelial derived nitric oxide, endothelium denuded vessels were incubated for 60 minutes in the presence or absence of pravastatin (112 nM). Vessels were stimulated with phenylephrine and following stable contraction were subsequently relaxed using the nitric oxide donor GSNO. Pretreatment with pravastatin did not alter the GSNO mediated relaxation of α_1-adrenoreceptor induced contraction (Figure C5, P = NS, n = 3).

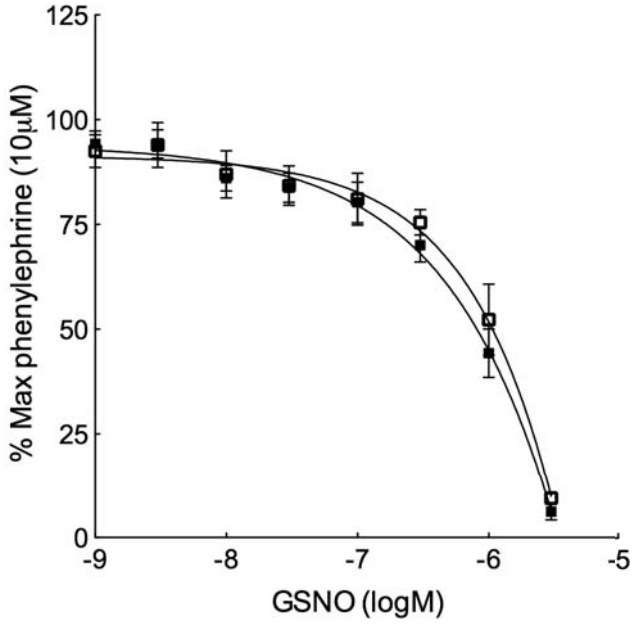

Figure C5 Pravastatin Does Not Alter Smooth Muscle Sensitivity to GSNO. Rat caudal artery without an endothelium was incubated with or without 112 mM pravastatin for 60 minutes prior to stimulation with 10 μM phenylephrine. After a stable contraction was reached vessels were relaxed with increasing concentrations of GSNO. Incubation with pravastatin did not alter dose-dependant GSNO-mediated relaxation compared to control (P = NS, n = 3).

C.3.5 Acute Pravastatin Administration Inhibits α_1-Adrenoreceptor Mediated Contraction by Activation of Nitric Oxide Synthase

Studies using cultured cells have documented that statin treatment leads to increased eNOS mRNA and protein expression, with others reporting an increased activating phosphorylation of Ser1177 of eNOS (Laufs, Fata et al. 1997; Kureishi, Luo et al. 2000). In order to test whether acute pravastatin administration inhibits α_1-adrenoreceptor mediated contraction by up regulating eNOS protein synthesis and/or by activating eNOS through phosphorylation of Ser1177, endothelium intact vessels were preincubated in the presence or absence of pravastatin (112 nM) for 60 minutes followed by stimulation with the α_1-adrenoreceptor agonist, phenylephrine. Vessels were snap frozen once phenylephrine induced contraction reached plateau. Western blot analysis of total eNOS protein identified there was no change in total eNOS protein levels between control and pravastatin groups (Figure C6, control = 2.61 ± 0.41, pravastatin = 2.49 ± 0.48, P = NS, n = 8). In contrast, western blot analysis of the ratio of P[Ser1177]eNOS/Pan eNOS revealed that a 60 minute incubation with pravastatin caused a significant increase in phosphorylation of Ser1177 eNOS (Figure C7, control = 1.02 ± 0.19, pravastatin = 1.66 ± 0.22, $P < 0.05$, n = 8).

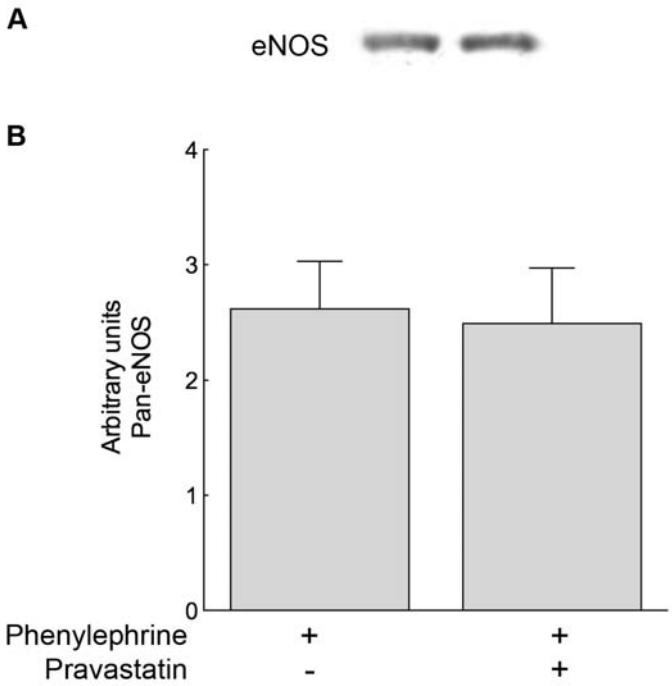

Figure C6 Acute 60-Minute Pravastatin Treatment Does Not Alter Total eNOS Protein Expression Levels. Rat caudal artery was incubated in the presence or absence of pravastatin (112 μM) for 60 minutes before stimulation with phenylephrine. Vessels were snap frozen after 10 minutes stimulation and samples separated using SDS page. eNOS total protein was visualized using western blot analysis. Pravastatin did not alter total eNOS compared to control (P = NS, n = 8).

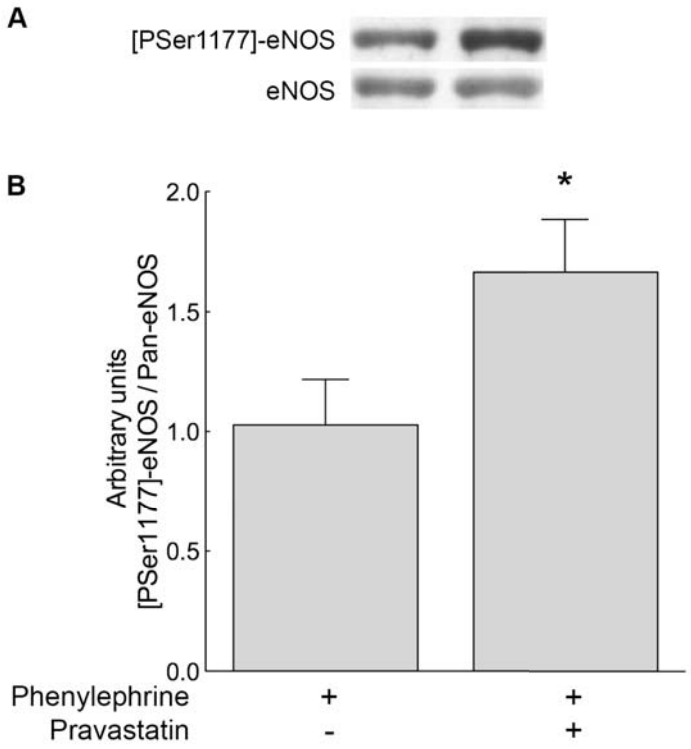

Figure C7 Acute Pravastatin Treatment Activates eNOS via Increased Phosphorylation of P[Ser1177]-eNOS. Rat caudal artery was incubated with pravastatin (112 μM) for 60 minutes before stimulation with phenylephrine. eNOS Ser1177 phosphorylation was determined using ratiometric western blot analysis of P[Ser1177]eNOS / total eNOS. Incubation with pravastatin significantly increased P[Ser1177]eNOS phosphorylation compared to control (* $P < 0.05$, $n = 8$).

C.3.6 Statin Mediated Inhibition of α_1-Adrenoreceptor Mediated Contraction is Independent from Statin Solubility

Pravastatin is highly hydrophilic compared to lipophilic simvastatin. In order to test whether the effects of pravastatin described herein are dependent on lipophilicity arteries were incubated in the presence or absence of simvastatin (10 μM). As with pravastatin, simvastatin reduced α_1-adrenoreceptor mediated contraction compared to control (Figure C8, control = 113.2 ± 2.66%, simvastatin = 102.08 ± 3.29, $P < 0.5$, $n = 5$). Co-incubation with L-NAME abolished the effect of simvastain (Figure C8, control = 113.2 ± 2.66%, simvastain + L-NAME = 122.1 ± 8%, P = NS, $n = 5$).

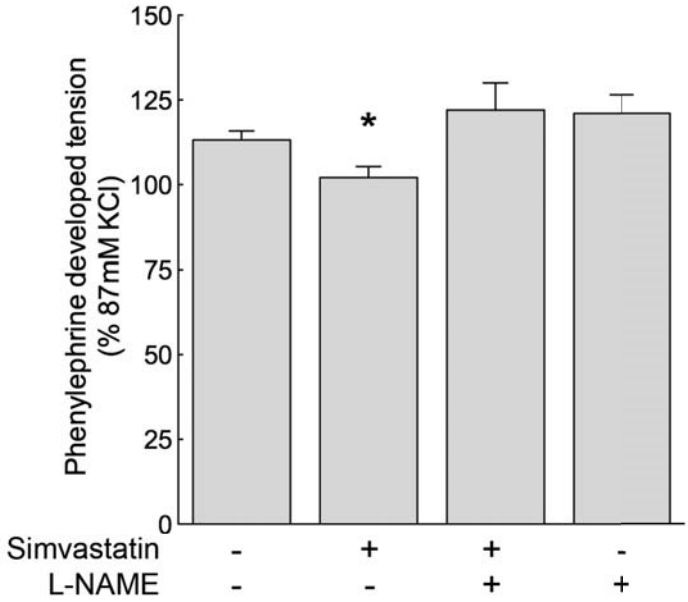

Figure C8 Simvastatin Inhibits α_1-Adrenoreceptor Mediated Contraction Through an Endothelial / Nitric Oxide Synthase-Dependent Mechanism. Rat caudal artery was mounted in a myograph and incubated with simvastatin (10 μM), and/or L-NAME (100 μM) for 60 minutes before stimulation with the α_1-adrenoreceptor agonist, phenylephrine. Simvastatin reduced α_1-adrenoreceptor mediated developed tension compared to control ($P < 0.05$, $n = 6$). Co-incubation with L-NAME abolished this effect (P = NS, $n = 6$).

C.4 Discussion

In the present study incubating rat caudal artery for 60 minutes with pravastatin at the human therapeutic equivalent concentration (112 nM) attenuated vascular smooth muscle contraction in response to stimulation with the specific α_1-adrenoreceptor agonist, phenylephrine (Figure C2). This effect was abolished in the absence of an endothelium and in the presence of the inhibitor of nitric oxide synthase, L-NAME, suggesting that this acute effect is mediated through an endothelial/nitric oxide dependent mechanism (Figure C2).

Several studies using cultured cells have demonstrated that statins are able to increase the production of nitric oxide independent from lipid lowering (Kureishi, Luo et al. 2000; Laufs, Gertz et al. 2002). Furthermore, it has been demonstrated that increased nitric oxide production can be caused by increased expression of eNOS mRNA and protein (Laufs, La Fata et al. 1998) and by increased phosphorylation of Ser1177-eNOS that increases enzymatic activity (Kureishi, Luo et al. 2000; Harris, Blackstone et al. 2004). In the present study incubation with pravastatin for 60 minutes did not alter eNOS protein levels (Figure C6), however, a significant increase in the phosphorylation state of Ser1177 eNOS was identified (Figure C7). Taken together these data suggest that NOS-dependent inhibition of α_1-adrenoreceptor mediated contraction is due to increased nitric oxide production via P[Ser1177] mediated activation of eNOS. The lack of change in eNOS total protein is likely due to the short 60-minute timeframe in which vessels were incubated with pravastatin. In contrast, previous studies using proliferating cells in culture have seen statin-mediated increases in eNOS mRNA expression after 48 hours of incubation (Laufs, La Fata et al. 1998).

The likely mechanism by which pravastatin increases the phosphorylation of eNOS may be via inhibition of isoprenoid synthesis resulting in reduced activation of the RhoA/Rho kinase pathway. The incubation of cultured cells with statins has been shown to reduce the synthesis of both farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) (Zhong, Liang et al. 2005). The addition of a GGPP group is required for correct membrane targeting of RhoA and the subsequent activation of Rho-kinase. Indeed, incubating statin treated cultured endothelial cells with exogenous GGPP abolishes the effect of statins on eNOS phosphorylation in culture (Laufs and Liao 1998). Wolfrum et al., has demonstrated that direct Rho-kinase inhibition results in increased activation of phosphatidylinositol-kinase (PI3K) resulting in activation of protein kinase B (PKB) (Wolfrum, Dendorfer et al. 2004). Numerous studies have demonstrated the capacity of PKB to activate eNOS through Ser1177 phosphorylation (Dimmeler, Fleming et al. 1999; Fulton, Gratton et al. 1999; Kureishi, Luo et al. 2000; Wolfrum, Dendorfer et al. 2004).

The present study found that inhibiting all isoforms of cyclooxygenase with indomethacin decreased contraction in response to α_1-adrenoreceptor simulation (Figure C3). This may be due to reduced autocrine/paracrine release of endogenous $PGF_{2\alpha}$ or thromboxane A_2, both of which are vasoconstrictors produced by the endothelium (Ali, Barrett et al. 1980; Ingerman-Wojenski, Silver et al. 1981). This result suggests that in the rat caudal artery, in the context of α_1-adrenoreceptor simulation, prostacyclin does not play a significant role in modulating contraction. Interestingly, pravastatin and indomethacin mediated attenuation of contraction were

not additive, suggesting a common pathway, perhaps membrane hyperpolarization and/or the increased production of cGMP or cAMP.

It has been reported that statins are able to directly relax smooth muscle (Budzyn, Marley et al. 2004). Several studies have reported a direct effect of statins on smooth muscle extracellular Ca^{2+} entry (Tesfamariam, Frohlich et al. 1999; Pérez-Guerrero, Márquez-Martín et al. 2005), perhaps due to modulation of L-type Ca^{2+} channels (Bergdahl, Persson et al. 2003). Another potential smooth muscle mechanism is via Ca^{2+} desensitization. In vascular smooth muscle RhoA is activated by agonists binding to G-protein coupled receptors resulting in the activation of Rho-kinase. In turn, Rho-kinase phosphorylates the targeting subunit of myosin light chain phosphatase (MYPT1) at Thr697 and Thr855 (Feng, Ito et al. 1999; Velasco, Armstrong et al. 2002). The inhibition of MLCP results in increased contraction at a given cytoplasmic Ca^{2+} concentration, a phenomena that has been extensively reviewed by Somlyo and Somlyo (Somlyo and Somlyo 2003). By reducing the activation of the RhoA/Rho-kinase pathway statins may reduce the inhibitory phosphorylation of MYPT1, thus favoring relaxation. In the context of α1-adrenoreceptor-mediated contraction this study rules out a direct smooth muscle effect through a number of lines of evidence. Firstly, removal of the endothelium abolished the effects of pravastatin (Figure C2). Secondly, no change in the smooth muscle sensitivity to the nitric oxide donor GSNO were seen (Figure C5), suggesting that the relaxation seen was not due to hypersensitivity to existing levels of nitric oxide or alterations in nitric oxide bioavailability. Thirdly, pravastatin did not alter developed tension in response to KCl mediated membrane depolarization (Figure C4),

suggesting that K^+ or Ca^{2+} channel activation may not play a significant role in the pravastatin effect.

The absence of a direct smooth muscle effect in the current study may be due to the use of the specific α_1-agonist, phenylephrine (Figure C2). It is well established that phenylephrine causes contraction in vascular smooth muscle by binding to α_1-receptors coupled to G_q (Somlyo and Somlyo 2000). Activation of this G-protein stimulates the phospholipase Cβ mediated production of IP_3, that in turn stimulates sarcoplasmic reticulum IP_3 receptors resulting in Ca^{2+} release from the sarcoplasmic reticulum and contraction (Graham, Perez et al. 1996). Although Kitazawa's group has used large femoral arteries and documented a role for RhoA, Rho-kinase and myosin phosphatase throughout phenylephrine mediated contraction (Kitazawa, Eto et al. 2003), there is evidence that the role of Rho/Rho-kinase in smooth muscle contraction varies across vascular beds (Asano and Nomura 2003). For example, direct Rho-kinase inhibition abolished almost all phenylephrine contraction in aorta, with less effect seen in superior mesenteric artery and with no effect in second order branches of mesenteric artery (Budzyn, Paull et al. 2006). Thus, the results of the present study suggest that the RhoA/Rho-kinase pathway in smooth muscle may not contribute to α_1-adrenoreceptor mediated constriction in the rat caudal artery.

It has been reported that the distinct physiochemical properties of statins may influence their pleiotropic effects. For example, pravastatin is strongly hydrophilic compared to simvastatin and atorvastain both of which are highly lipophilic. This may confer differences in tissue permeability and may account for differences in side effects observed between statin agents (Hamelin and Turgeon 1998). Hydrophobic

statins such as simvastatin may be more likely to enter extrahepatic tissue such as smooth muscle and endothelial cells than are hydrophilic statins such as pravastatin, which primarily enter the liver via an organic cation transporter (Blum 1994). Despite these chemical differences, the strongly hydrophobic simvastatin reduced α_1-adrenorceptor mediated contraction to a similar magnitude as pravastatin and with the same endothelial dependence (Figure C8). These data suggest that in the context of α_1-adrenoreceptor-mediated contraction in the rat caudal artery statin mediated attenuation of contraction is independent of statin hydrophobicity.

A role for increased sympathetic drive in cardiovascular pathology has been established in animal models and humans in states such as cardiac arrhythmia (Meredith, Broughton et al. 1991), myocardial infarction, heart failure (Ciarka, van de Borne et al. 2008), tako-tsubo cardiomyopathy, stroke (Grassi, Arenare et al. 2009), hypertension (Julius and Majahalme 2000) and renal disease (Schlaich, Socratous et al. 2008). The α_1-adrenoreceptors are important mediators of the sympathetic nervous system responses, specifically, of vascular smooth muscle constriction and cardiac contractility (Docherty 2010). The present study suggests that the administration of pravastatin or simvastatin may be beneficial in abrogating the effect of pathological α1-adrenoreceptor activation. Furthermore, the increased activation of nitric oxide synthase may provide clinical benefit in cases of endothelial dysfunction and may contribute an explanation to evidence that statins inhibit platelet thrombus formation and reduce MI and stroke beyond the levels predicted by LDL reduction.

C.5 Conclusion

Clinical concentrations of pravastatin inhibit α_1-adrenoreceptor mediated constriction through an endothelial/nitric oxide-dependent mechanism without increasing total eNOS but consistent with an increase in P[Ser1177]eNOS and inhibition of RhoA signaling.

D. Simvastatin Attenuates Thromboxane A_2 Receptor Mediated Contraction in an Endothelial and Smooth Muscle Dependent Manner

D.1 Introduction

Thromboxane A_2 (TxA_2) is a metabolite of arachidonic acid and a powerful vasoconstrictor and platelet activator (Kinsella 2001). The half-life of TxA_2 has been reported as 30 seconds (Hamberg, Svensson et al. 1975) and thus, the stable TxA_2 analogue U-46619 is used for research purposes.

The signal transduction of TxA_2-mediated contraction has been examined in a number of vascular smooth muscle preparations (Himpens, Kitazawa et al. 1990; Tosun, Paul et al. 1998; Cogolludo, Moreno et al. 2003). In the rat caudal artery, TxA_2 causes contraction by stimulating the TxA_2 receptor, resulting in Ca^{2+} entry via L-type Ca^{2+} channels, activation of MLCK, LC_{20} phosphorylation and contraction (Wilson, Susnjar et al. 2005). In this same preparation TxA_2 receptor activation also results in Ca^{2+} sensitization of contraction, in which force generation is increased for a given cytosolic Ca^{2+} concentration (Somlyo and Somlyo 2003). This involves activation of the small GTPase, RhoA, leading to activation of Rho-kinase, which phosphorylates the myosin-targeting subunit of MLCP (MYPT1) at Thr-855 (Wilson, Susnjar et al. 2005). Phosphorylation at this site inhibits MLCP which results in increased LC_{20} phosphorylation and contraction (Feng, Ito et al. 1999; Velasco, Armstrong et al. 2002; Muranyi, Derkach et al. 2005).

Because TxA_2 has been implicated in cardiovascular, renal and respiratory diseases (Sellers and Stallone 2008), the pharmacological modulation of TxA_2 receptor-mediated signaling is of great clinical interest. The statins (HMG-CoA reductase inhibitors) may represent a novel therapeutic strategy to target TxA_2 receptor activation. Traditionally prescribed for the treatment of hypercholesterolemia, statins

are effective in the secondary prevention of cardiovascular morbitiy and mortality (4S Group 1994; Shepherd, Cobbe et al. 1995; Sacks, Pfeffer et al. 1996; Baigent, Keech et al. 2005), however, statins also exert beneficial cardiovascular effects independent from cholesterol lowering (Liao and Laufs 2005; Wang, Liu et al. 2008). These cholesterol independent effects may arise from their ability to inhibit the mevalonate pathway, and thereby block the production of lipid isoprene groups including farnesyl pyrophosphate and geranylgeranyl pyrophosphate (Goldstein and Brown 1990). Along with being intermediates along the path to cholesterol synthesis, isoprene moieties are important posttranslational modifications required for correct targeting of cellular proteins involved in a variety of signaling processes, including cell migration, proliferation and contraction (Zhang and Casey 1996; Hall 2005).

Of particular interest in the context of TxA_2 signaling is the potential of statins to inhibit RhoA mediated activation of Rho-kinase. Because RhoA requires the post-translational addition of geranylgeranyl pyrophosphate for normal function and correct membrane targeting (Tanaka, Tatsuno et al. 1998; Terano, Shiina et al. 1998), statins, by reducing RhoA prenylation, may relax smooth muscle by releasing the TxA_2 receptor-mediated RhoA/Rho-kinase-dependent inhibition of smooth muscle MLCP thereby favoring LC_{20} dephosphorylation and relaxation. Furthermore, RhoA may also regulate vascular tone through vascular endothelium-dependant mechanisms. In the endothelium, it has been demonstrated that RhoA-mediated Rho-kinase activation inhibits PKB, which results in a reduction in phosphorylation of Ser1177 of endothelial nitric oxide (eNOS), decreasing its activity and reducing nitric oxide synthesis (Ming, Viswambharan et al. 2002). Therefore, statins may also reduce TxA_2 receptor-mediated contraction by increasing nitric oxide production due to the

release of Rho-kinase-mediated inhibition of PI3K/PKB-mediated eNOS phosphorylation (Rikitake and Liao 2005).

In the previous chapter pravastatin was shown to reduce α_1-adrenoreceptor-mediated developed tension in rat caudal artery (Section C). This effect was mediated through the endothelium via increased activation of endothelial nitric oxide synthase. In contrast, pilot studies in our laboratory indicate that high dose simvastatin opposed to pravastatin pretreatment in rat caudal artery inhibits contraction mediated by the TxA_2 receptor independent from the endothelium through a smooth muscle effect.

The present study makes use of an isolated rat caudal artery model with the aim of determining the importance of endothelial nitric oxide and smooth muscle mechanisms responsible for statin mediated attenuation of TxA_2 receptor-mediated contraction. Experiments were designed to examine molecular substrates downstream from RhoA in the vascular endothelium and smooth muscle. Both the phosphorylation status of Ser1177 eNOS and phosphorylation status of Thr855 of the MYPT1 subunit of myosin phosphatase were examined.

D.2 Methods

D.2.1 Materials

General laboratory reagents are listed in Section B1. Reagents specific to this paper were purchased from the following sources: Simvastatin, 9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U-46619) and diisopropyl flurophosphate were from Sigma–Aldrich (Australia). Glycyl; (S)-(+)-2-Methyl-4-glycyl-1-(4-methylisoquinolinyl-5-sulfonyl) homopiperazine (H-1152) was from Calbiochem-Novabiochem Corp. (San Diego, CA, U.S.A.). Mixed bed resin was from BIO-RAD (Australia). Urea and thiourea were from Merk (Australia). Polyclonal antibodies specific for pan-MYPT1 (anti-MYPT1), MYPT1 Thr-855 (anti-[PThr855]-MYPT1), pan-eNOS (anti-eNOS), and eNOS Ser1177 (anti-[PSer1177]-eNOS) were purchased from Upstate/Millipore USA, Inc. Polyclonal antibody specific for the light chains of myosin (anti-LC_{20}) was purchased from Santa Cruz Biotechnology (USA). West Femto™ was purchased from Pierce/Thermo Scientific (USA). ECL reagents were from GE Healthcare (Australia).

D.2.2 Preparation of Isolated Arteries

All animal experiments were approved by the University of Adelaide animal ethics committee. Male Sprague Dawley rats, weighing 350 - 400 g, were terminated using CO_2 inhalation. Caudal artery was dissected and placed into cold Ca^{2+}-free HEPES-Tyrode buffer containing in mM [135.5 NaCl 5.9 KCl 1.2 $MgCl_2$ 11.6 HEPES 11.6 glucose pH 7.4]. Where appropriate the endothelium was removed by rubbing vessel segments 30 times over a wire 0.34 mm in diameter. Each arterial segment was cleaned of adventitia and cut into 2 mm segments.

D.2.3 Vascular Myography

Arterial segments were mounted on a Mulvany wire-myograph (610M, Danish myotechnologies, Denmark) to facilitate quantification of isometric developed tension following stimulation. Vessels were equilibrated in normal HEPES-Tyrode (NHT) buffer and set at an optimal resting tension of 10 mN, which length tension curves identified produced a maximal contractile response to 87 mM K^+. Arteries were equilibrated for 30 minutes before being stimulated three times with 87mM K^+-HEPES-Tyrode buffer, in which NaCl was substituted with KCl to maintain an isoosmolar solution, containing in mM (54.4 NaCl, 87 KCl, 1.2 $MgCl_2$, 2.5 mM $CaCl_2$,11.6 HEPES, 11.6 glucose pH 7.4). Following relaxation in NHT buffer, tissues were incubated with simvastatin (10 μM), H1152 (1 μM) and/or N^G-monomethyl-L-arginine (L-NAME) (10 μM) for 60 minutes. Following a 10 minute stimulation with U-46619 (1 μM) in the presence or absence of inhibitors / simvastatin, tissues were snap frozen in dry ice cold 10% trichloroacetic acid/acetone, followed by washing with dry ice-cold acetone, and stored at -80°C prior to SDS-PAGE / western blotting.

D.2.4 Western Blot Analysis of eNOS and MYPT-1

For analysis of total eNOS and P[Ser1177]eNOS protein was extracted from snap frozen caudal artery segments using 80 μl SDS-PAGE buffer containing: 50 mM Tris/HCl, pH 6.8, 1% (w/v) SDS, 1x Protease Inhibitor Cocktail, 10 mM DTT, 15% glycerol, and 0.1% (w/v) bromophenol blue. For analysis of MYPT1 and P[Thr855]MYPT1 proteins were extracted using 80 μl of glycerol free sample buffer containing 50 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 1x Protease Inhibitor Cocktail,

100 μM di-isopropylfluorophosphate, 10 mM DTT, 10% (w/v) sucrose, and 0.1% (w/v) bromophenol blue. Samples were heated to 95°C for 5 min, then agitated for 60 min prior to SDS/PAGE using a BioRad mini protean II unit at 200 V for 60 minutes. For analysis of MYPT1 proteins were transferred onto 0.22 μm nitrocellulose (BIO-RAD) at 100 V for 30 minutes in transfer buffer containing 25 mM Tris/HCl, 192 mM glycine, 1% (w/v) SDS and 20% (v/v) methanol. For analysis of eNOS, proteins were transferred onto 0.22 μm nitrocellulose at 100 V for 60 minutes in transfer buffer containing 25 mM Tris/HCl, 192 mM glycine, and 20% (v/v) methanol.

Western blots were blocked with 5% non-fat dried milk in Tris buffered saline – Tween (TBS-T) (20 mM, Tris, 500 mM NaCl, 0.05% (v/v) Tween-20) for 60 minutes, followed by incubation with 1% non-fat dried milk/TBS-T containing either anti-MYPT1 (1:1000), anti-P[Thr855]MYPT1 (1:1000), anti-eNOS (1:1000) or anti-P[Ser1177]eNOS (1:1000) for 60 minutes. Nitrocellulose membranes were washed 3 times in TBS-T and incubated with a 1-10,000 dilution (in TBS-T) of horseradish peroxidase (HRP) conjugated secondary antibodies, anti-rabbit IgG-HRP for P[Thr855]MYPT1, eNOS, P[Ser1177]eNOS) or anti-mouse IgG-HRP for MYPT1 (1:10,000). HRP-mediated chemiluminescence signal detection used ECL reagents and standard X-Ray film. Optical density of protein bands were analyzed with Quantity One software (BioRad).

D.2.5 LC_{20} Phosphorylation

Protein was extracted from snap frozen caudal artery segments using urea/glycerol extraction buffer containing 4 M urea, 2 M thiourea, 200 mM Tris, 220 mM glycine, 10 mM DTT, 0.01% (w/v) bromophenol blue, 10 mM EDTA (pH 7), 600 mM

potassium iodide, 1% (w/v) mixed bed resin (20-50 mesh). Samples were extracted by agitation for 60 minutes at room temperature then run on a mini-gel unit (BIO-RAD) for 4 hours at 5 mA. Proteins were transferred to nitrocellulose using SDS free transfer buffer (25 mM Tris-HCl, 192 mM glycine and 20% (v/v) methanol) at 100 V for 60 minutes. LC_{20} phosphorylation was detected using anti-LC_{20} (1:10,000 dilution) antibody, followed by anti Rb-HRP conjugated secondary antibody (1:100,000 dilution). Chemiluminescence signal detection used west femto (Pierce) ECL reagents and standard X-Ray film.

D.2.6 Statistical Analysis

All results are presented as the mean ± S.E.M where *n* indicates the number of independent experiments for each treatment. Student's *t* test was used to determine statistical significance where $P < 0.05$ was considered statistically significant. Asterisks indicate statistically significant differences from control.

D.3 Results

D.3.1 Simvastatin Attenuates TxA_2 Receptor-Mediated Developed Tension, Primarily Through a Smooth Muscle Mediated Mechanism.

Caudal artery segments had intact endothelium-dependent vasodilator responses as judged by maximal ACh relaxation following phenylephrine challenge. Previous work indicated that U-46619 causes sustained contraction of rat caudal artery smooth muscle through the specific activation of the TxA_2 receptor (Wilson, Susnjar et al. 2005). Figure D1 depicts a typical dose response curve to U-46619 treatment in rat caudal artery. EC_{50} was 30nM, with peak isometric contraction achieved at 1 μM. Similar to previous studies, TxA_2 receptor activation with 1 μM U-46619 caused maximal contraction after 30 seconds, which was maintained through the 10 minutes of stimulation. Treating vessels with the nitric oxide synthase inhibitor, L-NAME, caused an increase in U-46619 developed tension consistent with blocking tonic nitric oxide release, and the presence of an intact endothelium (Figure D2, control = 94.01 ± 2.97%, L-NAME = 102.91 ± 5.1%, P = NS, n = 8). Incubation of vessels with simvastatin (10 μM) for 60 minutes prior to TxA_2 receptor stimulation reduced developed tension by 50% (Figure D2, control = 94.01 ± 2.97%, simvastatin = 53.78 ± 8.83%, $P < 0.5$, n = 8). A similar 10 μM simvastatin treatment followed by U46619 challenge conducted in the presence of L-NAME caused a slight increase in developed tension, consistent with the blockade of tonic NO release. (Figure D2, simvastatin = 53.78 ± 8.83% simvastatin + L-NAME = 63.05 ± 11.40, P = NS, n = 8). Physical removal of the endothelium followed by 60 minute incubation with 10 μM simvastatin and subsequent U-46619 stimulation again revealed a 50% reduction in developed tension (Figure D2, control = 94.01 ± 2.97%, no endothelium + simvastatin

= 47.96 ± 3.82%, $P < 0.5$, $n = 8$), consistent with the notion that a large part of acute high dose simvastatin-mediated attenuation of force was independent from the endothelium or nitric oxide. Incubation with the direct and specific inhibitor of Rho-kinase (a downstream effector of RhoA), H1152 (1 μM), and activator of myosin phosphatase attenuated almost all U-46619 induced developed tension (Figure D2, control 94.01 ± 2.97%, H1152 = 3.8 ± 3.68, $P < 0.5$, $n = 8$).

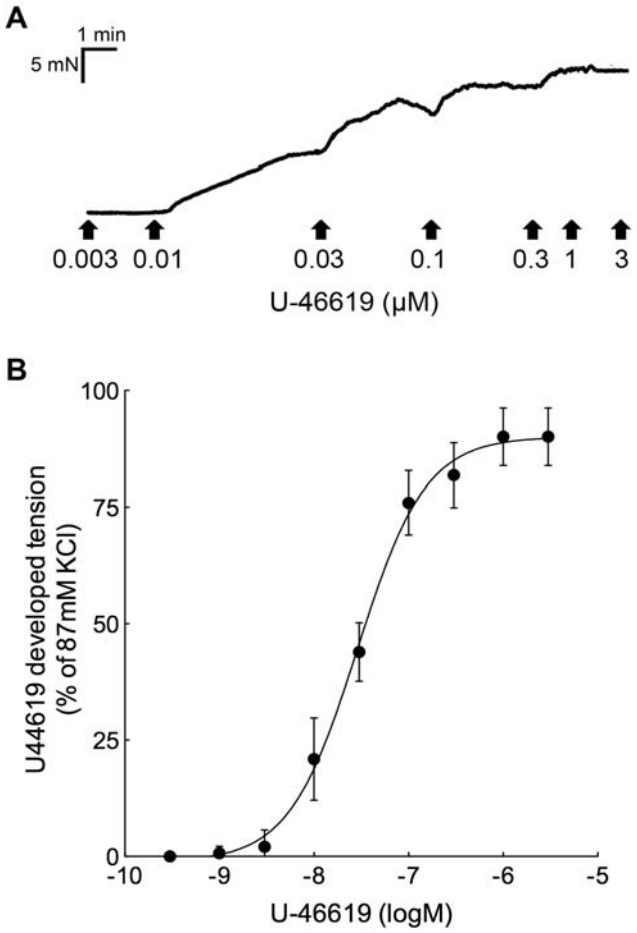

Figure D.1 Concentration-Dependent Contractile Response of Isolated Rat Caudal Arteries to the TxA_2 Mimetic, U-46619. Caudal artery was mounted in a wire myograph and developed tension recorded in response to increasing concentrations of U-46619. **(A)** Representative U-46619 dose-response curve. **(B)** Culmative dose response data. EC_{50} was at 30nM, and EC_{100} at 1 μM (*n*=3).

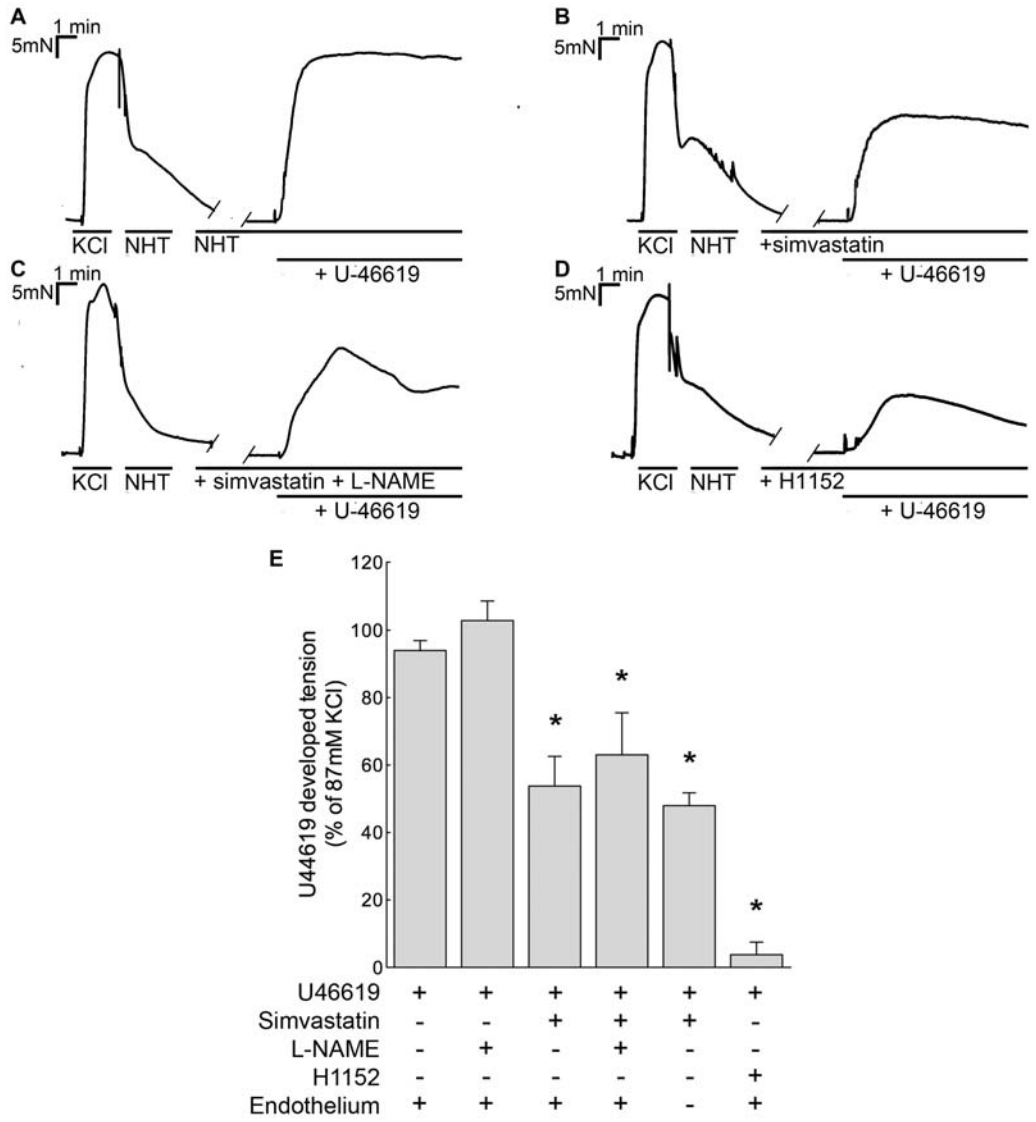

Figure D.2 Acute High Dose Simvastatin Incubation Reduces TxA_2 Receptor-Mediated Vascular Contraction. Following initial KCl-induced contractions vessels were incubated in the presence or absence of simvastatin (10 μM) for 60 minutes, followed by stimulation of the TxA_2 receptor with U-46619 (1 μM). (**A-D**) illustrates representative traces of raw data for individual experiments. (**E**) represents pooled data. Acute simvastatin treatment (**B**) attenuated TxA_2 receptor-mediated developed tension compared to control (**A**) (* $P < 0.5$, $n = 8$). Following endothelial removal or in the presence of the nitric oxide synthase inhibitor L-NAME (**C**, 100 μM) simvastatin maintained its vasodilator properties (* $P < 0.5$, $n = 8$). The inhibitor of Rho-kinase, H1152 (**D**), also reduced TxA_2 receptor-mediated developed tension compared to control (* $P < 0.5$, $n = 8$).

D.3.2 Acute High Dose Simvastatin and Direct Rho-Kinase Inhibition Reduce TxA_2 Receptor-Mediated Myosin Light Chain Phosphorylation

Following stimulation of the TxA_2 receptor, with the stable thromboxane A_2 analogue U-46619, developed tension was both maximal and stable prior to freezing of arterial segments (Figure D2). Consistent with this increase in developed tension, urea-glycerol gel analysis revealed an increase in the phosphorylation state of LC_{20} (Figure D3, control = 26.58 ± 5.27, U-46619 = 77.02 ± 6.1, $P < 0.5$, $n = 4$). Arteries treated with simvastatin (10 μM) for 60 minutes prior to the administration of U-46619 for 10 minutes had a 50 % reduction in developed tension (Figure D2) and a corresponding reduction in LC_{20} phosphorylation (Figure D3, U-46619 = 77.02 ± 6.1, U-46619 + simvastatin = 60.42 ± 1.24, $P < 0.5$, $n = 4$). The administration of the direct Rho-kinase inhibitor H1152 (1 μM) also significantly attenuated U-46619-mediated light chain phosphorylation (Figure D3, U-46619 = 77.02 ± 6.1, U-46619 + H1152 = 31.39 ± 3.39, $P < 05$, $n = 4$).

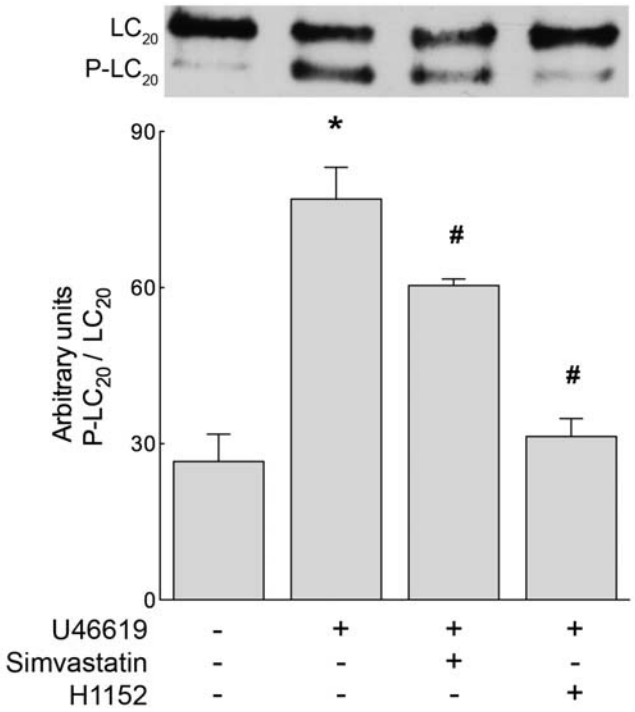

Figure D.3 High Dose Simvastatin Reduces TxA_2 Receptor-Mediated Light Chain Phosphorylation. Vessels were mounted on a wire myograph and were incubated for 60 minutes in the presence or absence of simvastatin (10 μM) or H1152 (1 μM) prior to stimulation of the TxA_2 receptor for 10 minutes. Vessels were snap frozen and LC_{20} phosphorylation detected using urea glycerol protein separation and western blot analysis. TxA_2 receptor stimulation increased LC_{20} phosphorylation compared to control (* $P < 0.05$, $n = 4$). Treatment with simvastatin or H1152 for 60 minutes significantly reduced LC_{20} phosphorylation compared to U-46619 alone (# $P < 0.05$, $n = 4$).

D.3.3 Direct Rho-Kinase Inhibition or Simvastatin Both Increase the Phosphorylation of Ser 1177 on Endothelial Nitric Oxide Synthase

A 10-minute stimulation of arteries with U-46619 alone did not alter the Ser1177 phosphorylation state of endothelial nitric oxide synthase (Figure D4, control = 81.62 ± 6.5, U-46619 = 81.7 ± 10.14, n = 8). In order to test whether statins increased Ser1177 eNOS phosphorylation in the rat caudal artery preparation, we preincubated isolated vessels for 60 minutes with simvastatin (10 μM) followed by stimulation of TxA_2 receptor with U-46619. Western blot analysis of the ratio of P[Ser1177]eNOS/Pan-eNOS revealed that simvastatin caused a significant increase phosphorylation of Ser1177 eNOS (Figure D4, control = 81.62 ± 6.5, simvastatin = 152.23 ± 18.16, $P < 0.05$, n = 8). A similar result was identified when arteries were incubated for 60 minutes with the Rho-kinase inhibitor H1152 (1 μM) prior to TxA_2 receptor activation (Figure D4, control = 81.62 ± 6.5, H1152 = 158.43 ± 24.29, $P < 0.05$, n = 8).

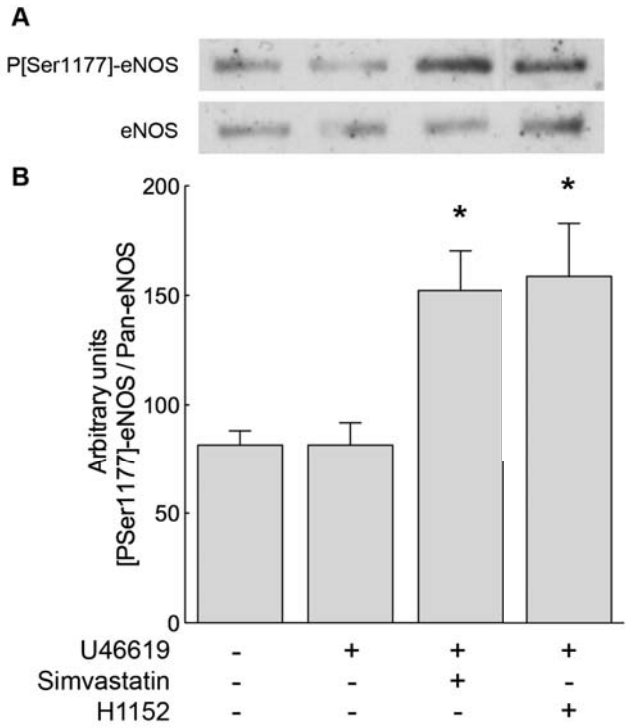

Figure D.4 Acute High Dose Simvastatin Treatment Activates eNOS via Increased Ser1177 Phosphorylation. Rat caudal artery was incubated with simvastatin (10 μM) or H1152 (1 μM) for 60 minutes before stimulation of the TxA_2 receptor. Vessels were snap frozen after 10 minutes stimulation and samples separated with SDS/ PAGE. eNOS Ser1177 phosphorylation was determined using western blot combined with the ratiometric analysis of P[Ser1177]eNOS / total eNOS. TxA_2 receptor stimulation did not increase P[Ser1177] eNOS phosphorylation above control (P = NS, n = 8). Incubation with simvastatin or the direct Rho-kinase inhibitor H1152 significantly increased P[Ser1177] eNOS phosphorylation above control ($P < 0.05$, n=8). * = $P < 0.5$ vs control.

D.3.4 Simvastatin and Direct Rho-Kinase Inhibition Attenuate the Inhibitory Thr855 Phosphorylation of the Targeting Subunit of Myosin Phosphatase

Previous work has shown that U-46619 causes Ca^{2+} sensitization of contraction via RhoA/Rho-kinase mediated inhibitory phosphorylation of Thr855 of the targeting subunit (MYPT1) of myosin phosphatase (Wilson et al., 2005). Consistent with this, TxA_2 increased the inhibitory phosphorylation of Thr855 of MYPT1 (Figure D5, control = 100 ± 4.5, U-46619 = 132.54 ± 6.50, $P < 0.5$, $n = 8$). The Rho-kinase inhibitor H-1152 (1 μM) significantly attenuated the TxA_2 receptor-mediated increase in P[Thr855]MYPT1/pan-MYPT1 compared with U-46619 treatment alone (Figure D5, U-46619 = 132.54 ± 6.50, U-46619 + H1152 = 63.09 ± 4.6, $P < 0.5$, $n = 8$). Incubation of isolated arteries for 60 minutes in the presence of simvastatin followed by a 10 minute challenge with U-46619 (1 μM) in the presence of simvastatin (10 μM) also caused a significant reduction the ratio of P[Thr-855]MYPT1 / pan-MYPT1 (Figure D5, U-46619 = 132.54 ± 6.50, U-46619 + simvastatin = 84.1 ± 6.5, $P < 0.5$, $n = 8$).

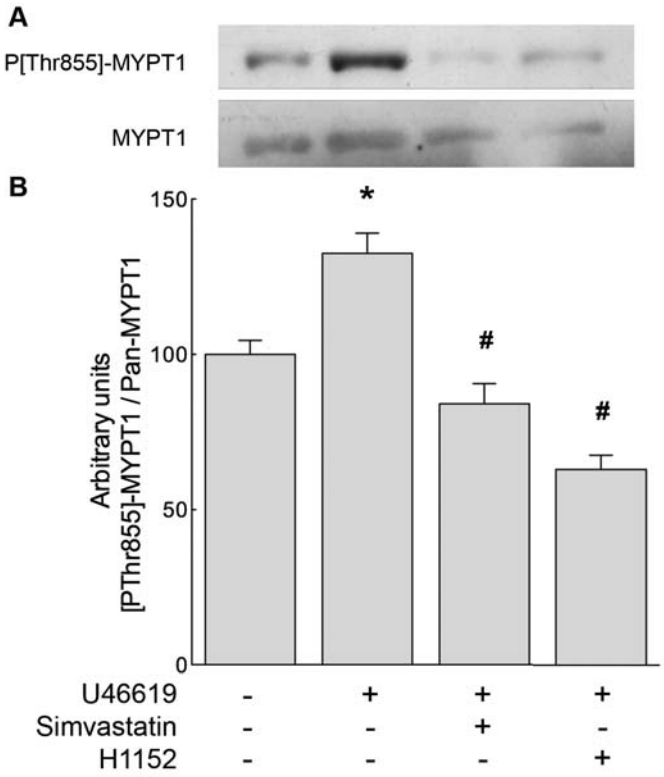

Figure D.5 Acute High Dose Simvastatin Treatment Reduces the TxA_2 Receptor Mediated Increase in MYPT1 Phosphorylation. Rat caudal artery was incubated with simvastatin (10 μM) or H1152 (1 μM) for 60 minutes before stimulation of the TxA_2 receptor. Vessels were snap frozen after 10 minutes stimulation and samples separated with SDS page. MYPT1 phosphorylation was determined using western blot combined with the ratiometric analysis of P[Thr855]MYPT1 / pan-MYPT1. Stimulation of the TxA_2 receptor with U46619 increased Thr855 phosphorylation compared to control (* $P < 0.05$, n=8). Incubation with simvastatin (10 μM) or the direct Rho kinase inhibitor, H1152 (1 μM), reduced Thr855 phosphorylation compared to U-46619 alone (# $P < 0.05$, n=8).

D.3.5 Acute Administration of High Dose Simvastatin Improves the Efficacy of the Direct Rho-Kinase Inhibitor H1152

Because of the possibility that both H1152 and simvastatin relax rat caudal artery by inhibiting Rho-kinase mediated phosphorylation of Thr855-MYPT (Figure 5) it was of interest to determine if pretreatment with simvastatin could increase the efficacy of H1152 in relaxing contraction mediated by the TxA_2 receptor. Treating vessels with simvastatin for 60-minutes reduced maximal contraction consistent with Figure 2. Simvastatin treatment also improved the efficacy of direct Rho-kinase inhibition with H1152 at 0.03, 0.01 and 1 μM (Figure D6, $P < 0.5$, $n = 8$).

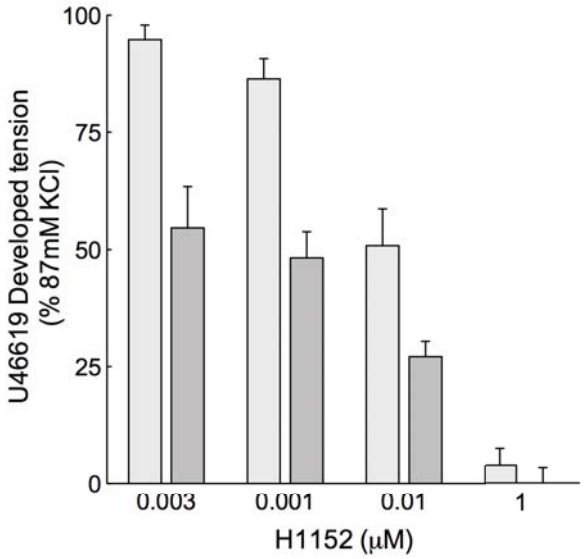

Figure D.6 Acute Simvastatin Treatment Enhances the Efficacy of Direct Rho-Kinase Inhibition with H1152. Arterial segments were incubated in the presence (■) or absence (□) of simvastatin (10 μM) for 60 minutes. Stimulating the TxA_2 receptor with U-46619 generated a stable contraction after which vessels were relaxed using H1152 (0.03 – 1μM). Preincubation with simvastatin increased H1152 mediated relaxation at 0.03 – 0.1 μM (* $P < 0.5$, $n = 8$).

D.4 Discussion

Thromboxane A_2 (TxA_2) is a metabolite of arachidonic acid produced predominantly in platelets. It is an important regulator of many physiological processes including smooth-muscle contraction and proliferation following vascular injury, and platelet activation and aggregation (Kinsella 2001). The actions of TxA_2 are mediated by the G protein-coupled TxA_2 receptor (Armstrong and Wilson 1995). Previous work in the rat caudal artery has shown that activation of the TP receptor causes contraction by simulating Ca^{2+} entry through L-type Ca^{2+} channels, with no contribution from the intracellular sarcoplasmic reticulum (Wilson, Susnjar et al. 2005). Importantly, a large component of TxA_2 receptor-mediated contraction is dependent on sensitization to Ca^{2+} via the RhoA/Rho-kinase mediated inhibition of myosin phosphatase. (Wilson, Susnjar et al. 2005). Consistent with this previous work, in the present study inhibition of Rho-kinase with H1152 almost completely abolished contraction (Figure D2) confirming that a large component TxA_2-receptor mediated contraction is mediated by Ca^{2+} sensitization. Furthermore, we confirm that TxA_2 stimulation caused contraction that was associated with the phosphorylation of Thr855-MYPT and this phosphorylation was sensitive to Rho-kinase inhibition (Figure D5).

A role for protein kinase C (PKC) in Ca^{2+} sensitization has been demonstrated. Activation of PKC by DAG results in the activation of C-kinase potentiated inhibitory protein of 17 kDa (CPI-17), which binds to MLCP and strongly inhibits the enzyme (Eto 2009). Although PKC/CPI-17 has been shown to be important in some tissue types it is not involved in TxA_2 receptor mediated contraction in the rat caudal artery (Wilson, Susnjar et al. 2005). Therefore, TxA_2 receptor mediated contraction in this

preparation is an ideal model in which to study the effects of RhoA/Rho-kinase mediated Ca^{2+} sensitization of contraction, without the confounding effects of PKC.

Previous data from our laboratory indicated that statins inhibit α_1-adrenoreceptor mediated contraction in rat caudal artery and that removing the endothelium or incubating with the NOS inhibitor L-NAME abolished the statin effect (Section D). This implies that in the context of α_1-adrenoreceptor stimulation, statins have vascular effects mediated entirely by the endothelium. The results of the present study indicate that in the context of acute high does simvastatin administration and TxA_2 receptor activation there is a predominant direct smooth muscle mediated attenuation of contraction. Furthermore, we have demonstrated that this smooth muscle mediated inhibition of contraction by simvastatin is associated with a reduction of the inhibitory phosphorylation of Thr855-MYPT1 (Figure D5) that favors LC_{20} dephosphorylation (Figure D3) and relaxation (Figure D2). The differing results between studies performed with α1-adrenoreceptor activation and TxA_2-receptor activation may be a reflection of the relative importance of the RhoA/Rho-kinase pathway in the signaling of these two receptor types.

Although there are numerous alternative smooth muscle mechanisms by which simvastatin may relax TxA_2 receptor mediated contraction, such as inhibition of Ca^{2+} entry, treatment with the specific inhibitor of Rho-kinase, H1152, reduced contraction to baseline and decreased inhibitory Thr855 MYPT1 phosphorylation. This result suggests that the modulation of MYPT1 has the potential to account for the entire statin effect, however, the current study cannot rule out other smooth muscle effects such as modulation of Ca^{2+} channels.

In the present study, ratiometric western blotting indicated that eNOS was being strongly activated after an acute 60 minute incubation with simvastatin (Figure D4). This is consistent with numerous studies that have found that statins activate eNOS by phosphorylating the Ser1177 (Kureishi, Luo et al. 2000; Ohkita, Sugii et al. 2006), including previous work by this laboratory (Section C). This suggests that similar to direct Rho-kinase inhibition simvastatin has the capacity to mediate an increase in phosphorylation of Ser1177 eNOS. In the present study, endothelial removal or incubation with L-NAME had little effect on the vasomotor properties of simvastatin subsequent to TxA_2 stimulation, indicating that this activation of eNOS has a relatively minor functional effect in the context of TxA_2-receptor mediated contraction. Previous work demonstrating inhibition of α_1-adrenoreceptor mediated contraction with statins has shown only a modest reduction in tension. This was not detected in this study, although a non significant increase in contraction was seen in the simvastatin treated group when L-NAME was added and there was a significant change in eNOS phosphorylation at Ser1177, suggesting that a nitric oxide effect is present but may indicate that the sample size was under powered to identify the small functional contribution made by the endothelium.

The lack of effect in response to endothelial removal or NOS blockade with L-NAME is somewhat surprising in light of work by Haysteads group and more recently Nakamura et al, which suggests that nitric oxide mediated activation of PKG and the subsequent production of cGMP results in phosphorylation of P[Ser854]MYPT which may prevent inhibitory Thr855 phosphorylation and thus promote relaxation (Wooldridge, MacDonald et al. 2004; Nakamura, Koga et al. 2007; Somlyo 2007).

The fact that simvastatin was able to attenuate TxA_2 mediated contraction in the absence of an endothelium indicates this is not a mechanism through which statins are functioning in this preparation and indicates that MLCP can be modulated independent from nitric oxide.

Preincubation with simvastatin for 60-minutes increased the efficacy of direct Rho-kinase inhibition. These results suggest that statins do not completely abolish Rho-kinase activation, because treatment with H1152 was able to further reduce developed tension. Currently the Rho-kinase inhibitors are used successfully to treat vasospasm in Japan (Masumoto, Mohri et al. 2002), however, these drugs are expensive and currently unavailable in Australia. The data presented here suggests benefits of combining low dose Rho-kinase inhibitor therapy with statin treatment and also the need to exercise caution when administering Rho-kinase inhibitor therapy to those already on statins.

A role from TxA_2 in pathology has been suggested (Sellers and Stallone 2008). TxA_2 synthesis is upregulated in a number of cardiovascular disease states, TP receptor receptor density is increased in atherosclerotic vessels. The TP receptor is upregulated in response to soluble particles present in cigarette smoke (Zhang, Zhang et al. 2008), this finding may explain the increased Rho-kinase activation seen in the forearm vasculature of smokers (Noma, Higashi et al. 2003). Evidence suggests a role for TxA_2 in the initiation and maintenance of hypertension (Katugampola and Davenport 2001; Francois, Athirakul et al. 2004). The results of the current study suggest that simvastatin may be an effective inhibitor of TxA_2 receptor mediated signaling, and

therefore may be of clinical benefit in the aforementioned pathologies. The present study indicates that simvastatin inhibits the RhoA/Rho-kinase pathway, downstream from the TxA_2 receptor. Because there is also biochemical evidence to support a role for the RhoA/Rho-kinase signaling during the activation of Et-1 and Ang-II receptors (Wynne, Chiao et al. 2009) and in numerous pathological states, the use of statins may also be effective in the management of the pathological activation of these pathways.

This study suggests that high dose statins may have a profound effect on vascular resistance. Therefore, a bolus dose of statins may be a viable treatment for vasospastic disorders non-responsive to Ca^{2+} channels blockers or nitrates and in patients with endothelial dysfunction. Current studies are underway aimed at investigating the therapeutic concentration required to manifest this response and the duration of the effect.

D.5 Conclusion

Using intact vessels, in an acute setting, we have documented a statin mediated reduction in TxA_2-receptor mediated developed tension and described two molecular mechanisms to account for this: 1) increased nitric oxide production via activation of endothelial nitric oxide synthase (eNOS), and 2) perhaps a more prominent role of increased activation of smooth muscle myosin phosphatase via inhibition of Rho-kinase.

E. Differential Effects of Chronic and Acute Simvastatin Treatment in Rats

E.1 Introduction

The statins may have clinically relevant vascular effects independent of their ability to lower cholesterol (Liao and Laufs 2005; Wang, Liu et al. 2008). By inhibiting the rate-limiting step in the mevalonate pathway statins inhibit the production of important lipid molecules including farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) (Goldstein and Brown 1990). The addition of a GGPP moiety is required to enable membrane targeting of the small GTPase, RhoA, a molecule that has a major role in cardiovascular signaling (Van Aelst and CD'Souza-Schorey 1997).

Through its downstream effector, Rho-kinase, RhoA modulates vascular contractility via both the endothelium and smooth muscle. In the endothelium, endothelial nitric oxide synthase (eNOS) can be regulated by the RhoA/Rho-kinase pathway at both a transcriptional and post-translational level (Wolfrum, Dendorfer et al. 2004). In the smooth muscle inhibition of MCLP by phosphorylation of its targeting subunit (MYPT1) by Rho-kinase increases phosphorylation of the regulatory light chains of myosin (LC_{20}) and results in contraction (Somlyo, 2003). Thus, statins, by inhibiting the activation of the RhoA/Rho-kinase pathway, may inhibit vascular contractility by increasing nitric oxide production and by activating smooth muscle MLCP.

Previous work from our laboratory has identified that statins inhibit agonist-induced contractility in isolated blood vessels after 60 minutes of incubation. Specifically, clinical doses of pravastatin inhibit contraction in the context of α1-adrenoreceptor-mediated contraction by increasing nitric oxide production via increased phosphorylation of Ser1177 eNOS, while in the context of TxA_2 receptor-mediated

contraction, high dose simvastatin treatment directly relaxes smooth muscle by activating smooth muscle myosin phosphatase via phosphorylation of MYPT1 at Thr855.

These findings suggest that statins are capable of inhibiting the RhoA/Rho-kinase pathway after acute treatment and therefore may be useful in the treatment of acute disorders involving increased vascular constriction, such as vasospasm. Because evidence suggests that the RhoA/Rho-kinase pathway also plays an important role in chronic cardiovascular pathologies such as atherosclerosis (Mallat, Gojova et al. 2003), restenosis (Sawada, Itoh et al. 2000), hypertension (Seko, Ito et al. 2003) and cardiac hypertrophy (Zhao and Rivkees 2003) it is of great clinical interest to determine if the effects of statins mediated by RhoA/Rho-kinase inhibition persist in a chronic setting, and thus whether the statins may be of use in chronic disorders.

With this in mind, the current study was designed to test the vascular effects of chronic simvastatin treatment in rats. The aims were threefold 1) To determine if chronic simvastatin treatment alters vascular reactivity 2) To determine if this altered vascular reactivity is mediated by the inhibition of RhoA/Rho-kinase signaling in the endothelium, or in the smooth muscle. 3) To determine if this altered vascular reactivity results in blood pressure modulation in healthy rats. Furthermore, this study addresses the question as to weather acute administration of high dose statins has incremental benefits combined with low dose chronic treatment. In order to address these aims this study makes use of the coronary vascular bed in isolated perfused hearts and isolated blood vessels coupled to molecular analysis to determine the roles of eNOS and smooth muscle MLCP.

E.2 Methods

E.2.1 Materials

Reagents were purchased from the following sources: Simvastatin, Methyl cellulose 9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U-46619) and diisopropyl flurophosphate were from Sigma–Aldrich (Australia). Glycyl; (S)-(+)-2-Methyl-4-glycyl-1-(4-methylisoquinolinyl-5-sulfonyl) homopiperazine (H-1152) from Calbiochem-Novabiochem Corp. (San Diego, CA, U.S.A.). Forthane (isoflurane) was from Abbott Australasis Pty Ltd (Australia). Rabbit polyclonal antibodies specific for pan-MYPT1 (anti-MYPT1), MYPT1 Thr-855 (anti-[PThr855]-MYPT1), pan-eNOS (anti-eNOS) and eNOS Ser1177 (anti-[PSer1177]-eNOS) were from Upstate/Millipore USA, Inc. West Femto™ was purchased from Pierce/Thermo Scientific (USA).

E.2.2 Simvastatin Administration and Blood Pressure Measurement

All animal work was subject to approval by The University of Adelaide Animal Ethics Committee and the Institute of Medical and Veterinary Science/Central Northern Adelaide Health Service Animal Ethics Committee.

Male Sprague Dawley rats weighing 400 g were anaesthetized using forthane (1.5% / O_2) inhalation. Simvastatin (5 mg / kg in 500 μl of 0.5% methylcellulose) was administered daily via oral gavage. The vehicle control group received 500 μl of 0.5% methylcellulose daily. Rats received simvastatin for 6 consecutive days and were sacrificed on day 7.

Blood pressure was recorded using a non-invasive blood pressure (NIBP) tail cuff (ADInstruments Pty Ltd., Australia). Rats were anesthetized and placed on a heated mat at 37°C. The blood pressure cuff was placed with the sensor on the ventral side of the tail and three consecutive readings were recorded using a Labchart system (AD instruments, Australia) attached to a computer (Mini-Mac, Apple Inc, California). Recordings were made and analyzed with Powerlab Chart v6.00 program (ADInstruments, Australia). Pressure readings were taken prior to simvastatin treatment (day 0) and following 7 days of treatment.

E.2.3 Preparation of the Perfused Rat Heart and Isolated Arteries

Following anesthesia rats were placed in the supine position and a transabdominal incision made to expose the thoracic cavity. The thorax was then opened by cutting along the ribs on both sides. The heart was raised with the fingers and the great vessels were cut 5 mm distal to the base of the heart. The heart was then placed in ice-cold Ca^{2+} free Krebs solution containing in mM: NaCl (118), KH_2PO_4 (1.18), $NaHCO_3$ (25), $MgCl_2$ (1.05), EDTA (0.01), and glucose (5.56), pH 7.4

Following removal of the heart, the caudal artery was dissected and placed into cold nominally free Ca^{2+} HEPES-Tyrode buffer containing in mM: NaCl (135.5), KCl (5.9), $MgCl_2$ (1.2), HEPES (11.6), glucose (11.6), pH 7.4]. Each caudal artery segment was cleaned of adventitia and cut into 2 mm segments for use in myograph experiments. Three 1 cm segments of caudal artery were cleaned of adventitia and adipose and were snap frozen in liquid nitrogen and samples stored at -80°C and used for biochemical analysis of total eNOS expression.

E.2.4 Measurement of Coronary Perfusion Pressure in the Perfused Langendorff Heart

Excised hearts were grasped with forceps by the aorta and were slipped over a metal cannula. Hearts were permanently attached to the cannula via ligation. Hearts were then perfused with fresh Krebs solution containing in mM: NaCl (118), KH_2PO_4 (1.18), $NaHCO_3$ (25), $MgCl_2$ (1.05), $CaCl_2$ (2.34), EDTA (0.01), and glucose (5.56), pH 7.4. Reperfusion occurred within two minutes of removing the heart from the animal. The Krebs solution was bubbled with carbogen (95% O_2 and 5% CO_2) and temperature controlled at 37°C. Perfusate was delivered at a constant flow rate at 5 ml / min. Perfusion pressure was measured by a pressure transducer (AD Instruments, Australia) attached by side arm. Left ventricular pressure was measured using an intraventricular fluid filled balloon attached to a pressure transducer. The balloon was inflated until a resting pressure of 10 mmHg was achieved. Hearts were paced using a stimulator at 10% above resting heart rate. Data was recorded using a Powerlab system with Labchart software (AD Instruments).

E.2.5 Vascular Myography

Caudal artery segments 2 mm in length were mounted on a Mulvany wire-myograph (610M, Danish myotechnologies, Denmark). Vessels were equilibrated in normal Krebs solution and set at an optimal resting tension of 10 mN. Organ baths were bubbled with carbogen throughout the experiment. Arterial segments were allowed to equilibrate for 30 minutes before being stimulated three times with high K^+-Krebs buffer, made by replacing the NaCl in Krebs buffer with isomolar KCl. Following relaxation in normal Krebs solution, tissues were incubated with simvastatin (10 μM), H1152 (1 μM) and/or N^G-monomethyl-L-arginine (L-NAME) (10 μM) for one hour.

Following a 10 minute stimulation with U-46619 (1 μM) in the presence or absence of inhibitors, tissues were snap frozen in dry ice cold 10% trichloroacetic acid/acetone, washed with dry ice-cold acetone, and stored at -80°C prior to biochemical analysis using SDS-PAGE/western blotting.

E.2.6 Western Blotting of eNOS and MYPT-1

For analysis of total eNOS and P[Ser1177]eNOS protein was extracted from 1 cm snap frozen caudal artery segments using 200 μl SDS-PAGE extraction buffer containing 50 mM Tris/HCl, pH 6.8, 1% SDS, 1x Protease Inhibitor Cocktail, 100 μM di-isopropylfluorophosphate, 20 mM dithiothreitol and 10% sucrose. For analysis of MYPT1 and P[Thr855]MYPT1 the 2 mm caudal artery segments snap frozen on a myograph were extracted using 80 μl of SDS-PAGE extraction buffer. Samples were heated to 95°C for 5 min, then agitated for 60 min prior to SDS-PAGE at 200 V for 60 minutes (BioRad mini protean II). In order to improve consistency in transfer of both MYPT1 and eNOS multiple gels were transferred on a single transfer unit by cutting gels at the appropriate prestained lane markers. For MYPT1 analysis proteins were transferred onto 0.2 μm nitrocellulose (BIO-RAD) at 100 V for 30 min in transfer buffer containing 25 mM tris, 192 mM glycine, 1% SDS and 20% methanol. For eNOS, proteins were transferred onto 0.2 μm nitrocellulose at 100 V for 1 hour in transfer buffer containing 25 mM tris, 192 mM glycine, and 20% methanol.

Following transfer of proteins to nitrocellulose, non-specific antibody binding sites were blocked using 5% non-fat dried milk in Tris buffered saline – Tween (TBS-T) (20 mM, Tris, 500 mM NaCl, 0.05% Tween-20) for 60 minutes, followed by incubation with 1% non-fat dried milk/TBST containing either anti-MYPT1 (1:5000

dilution), anti-P[Thr855]MYPT1 (1:5000 dilution), anti-eNOS (1:5000 dilution) or anti-P[Ser1177]eNOS (1:5000 dilution) for 60 minutes. Nitrocelluose membranes were washed 3 times in TBS-T and incubated with a 1-50,000 dilution (in TBS-T) of horse radish peroxidase (HRP) conjugated secondary antibodies, anti-rabbit IgG-HRP for P[Thr855]MYPT1, eNOS, P[Ser1177]eNOS) or anti-mouse IgG-HRP for MYPT1 (1:50,000). HRP-mediated chemiluminescence signal detection used the high sensitivity West Femto™ chemiluminescent detection reagent (Pierce) and standard X-Ray film (AGFA). Optical density of protein bands on film Western blots were analyzed with Quantity One software (Bio Rad, Australia). Variations in protein load were accounted for by normalizing signal to bands representing myosin heavy chain on the relevant coomassie stained gel.

E.2.7 Data Analysis

For Langandorff perfusion pressure responses, sigmoid curves were fit to data using nonlinear regression (GraphPad Prism, version 5.0) and the EC_{50} and the concentration for maximal response (E_{max}) determined. All results are presented as the mean ± S.E.M where *n* indicates the number of independent experiments for each treatment. Student's *t* test was used to determine statistical significance where $P < 0.05$ was considered statistically significant. Asterisks indicate statistically significant differences from control.

E.3 Results

E.3.1 Chronic Simvastatin Treatment Lowers Systolic Blood Pressure in Healthy Rats

In order to determine whether chronic simvastatin has blood pressure modulating effects rats were administered either vehicle (0.5% methyl cellulose) or 5 mg / kg of simvastatin per day for 7 days. Rats were lightly anesthetized and blood pressure recorded prior to simvastin administration (Day 0) and one day following final administration (Day 7). At day 0 the vehicle control group had a mean blood pressure of 119.91 ± 4.99 mmHg with no significant difference between the day 0 simvastatin group (122.65 ± 3.37 mmHg, P = NS, n=5). Treatment for 7 days with vehicle did not significantly alter systolic blood pressure from day 0 (vehicle day 0 = 119.91 ± 4.49 mmHg, vehicle day 7 = 112.65 ± 5.37 mmHg, $P < 0.5$, n=5). Chronic simvastatin treatment, however, did significantly lower blood pressure compared to day 0 (simvastatin day 0 = 122.65 ± 3.37 mmHg, simvastatin day 7 = 101mmHG ± 6.03 mmHg, $P < 0.5$, n=5).

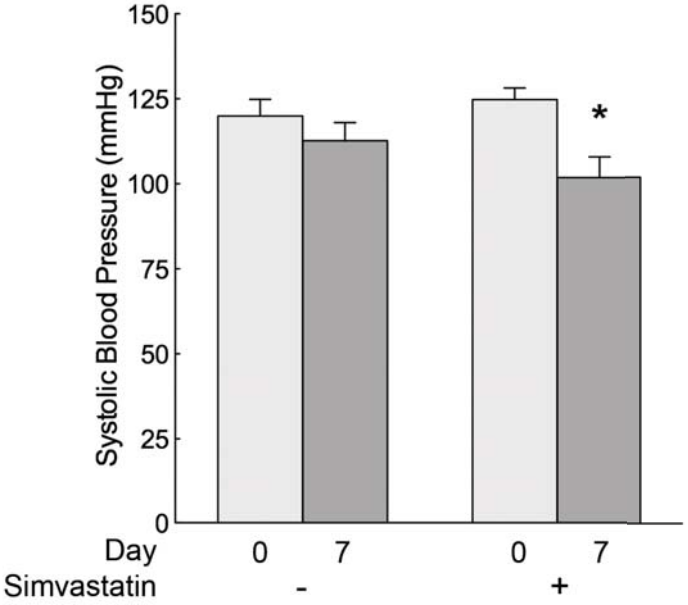

Figure E.1 Chronic Simvastatin Treatment Decreases Systolic Blood Pressure in Lightly Anesthetized Rats. Rats were lightly anesthetized and blood pressure measured using tail cuff before and after a 7-day treatment with either simvastatin (5 mg / kg) or vehicle (0.5% methylcellulose). Following 7 days treatment with vehicle systolic blood pressure was not significantly altered compared to day 0 (P = NS, n=5). After 7 days treatment with simvastatin (5 mg / kg), blood pressure was significantly decreased compared to day 0 (* $P < 0.5$, n=6).

E.3.2 Chronic Simvastatin Administration Decreases TxA_2 Receptor-Mediated Perfusion Pressure.

In order to assess the effect of 7 days of simvastatin treatment on coronary microvascular reactivity rats were administered either vehicle or simvastatin (5 mg / kg) for 7 days and were sacrificed on day 7. Hearts were cannulated on a Langendorff isolated heart apparatus and perfusion pressure measured. In hearts from vehicle treated rat TxA_2 receptor activation with U-46619 increased perfusion pressure in a concentration-dependent manner, with half-maximal perfusion pressure at 34 nM and maximal perfusion pressure at 1 μM (Figure E2). Hearts from simvastatin treated rats were less sensitive to U-46619 (Figure E2, EC_{50} : control = 34.09, 32.57 – 35.68 nM (range) simvastatin = 120, 101.8 – 143.2 (range) nM, $P < 0.05$, $n = 8$) and had decreased maximal perfusion pressure compared to control animals (Figure E2, E_{max} : vehicle = 103.5 ± 7.65 mmHg, simvastatin = 73.86 ± 12.08 mmHg, $P < 0.5$, $n = 8$).

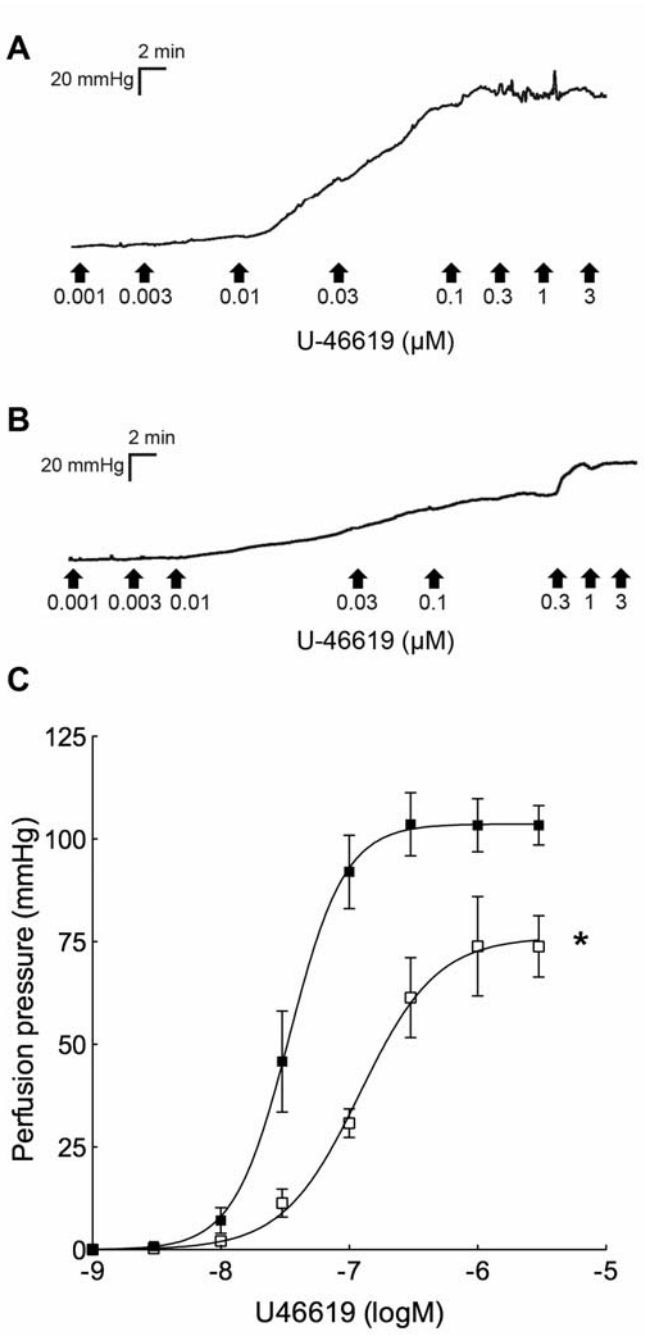

Figure E.2 Chronic Statin Treatment Decreases Coronary Vascular Sensitivity to TxA_2 Receptor Activation in Isolated Rat Hearts. Representative traces of dose-response to TxA_2 receptor activation with U-46619 (1 μM) from Langendorff perfused hearts treated for seven days with vehicle (**A**) or simvastatin (5 mg / kg, **B**). (**C**) Combined Langendorff data. Chronic treatment with simvastatin (□) decreased EC_{50} ($P < 0.5$, $n = 8$) and E_{max} (* $P < 0.5$, $n = 8$) compared to control (■).

E.3.3 Chronic Simvastatin Administration Up-Regulates eNOS Total Protein Expression but Does Not Modulate the Phosphorylation State of Ser1177 eNOS.

Work from our laboratory suggests a role for Ser1177 phospohrylation in acute statin mediated inhibition of TxA_2 receptor-mediated developed tension (Figure C5). To examine whether eNOS is modulated in the context of chronic simvastatin administration caudal artery segments from rats treated with vehicle or simvastatin for 7 days were snap frozen and proteins separated using SDS-PAGE. Total eNOS and P[Ser1177]eNOS phosphorylation were detected with western blot analysis. A significant increase in eNOS total protein was observed in the simvastatin treated group compared to vehicle control (Figure E3, simvastatin = 1.08 ± 0.07, vehicle = 0.85 ± 0.05, $P < 0.5$, $n = 8$). The ratio of P[Ser1177]eNOS / Pan eNOS revealed that chronic simvastatin treatment did not alter eNOS phosphorylation (Figure E4, vehicle = 0.35 ± 0.051, simvastatin 0.36 ± 0.054, P = NS, $n = 8$).

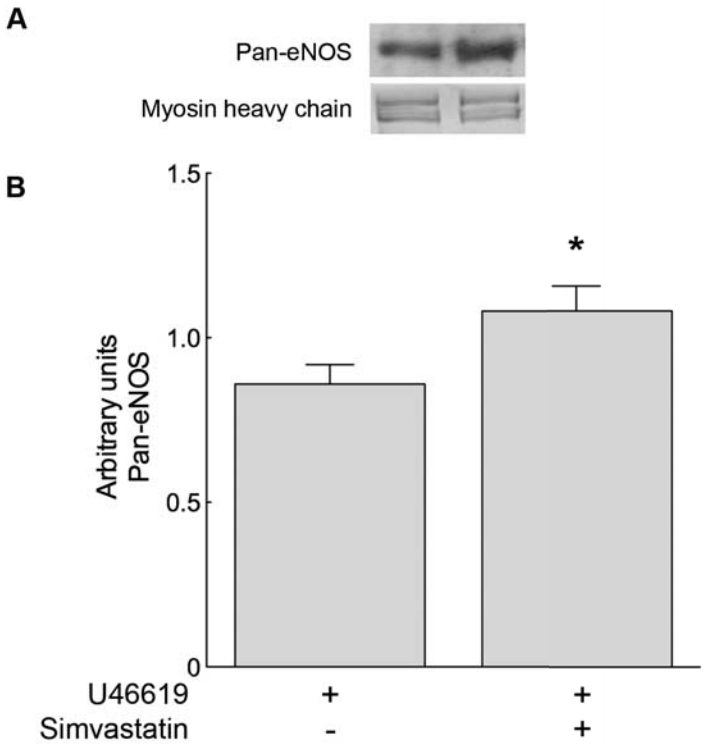

Figure E.3 Chronic Simvastatin Treatment Increases eNOS Expression in Rat Caudal Artery. Caudal artery from rats treated for 7 days with vehicle or simvastatin (5 mg / kg) were snap frozen on a myograph and separated using SDS-PAGE. Total eNOS was visualized using western blot analysis. In rats treated for 7 days with simvastatin there was a significant increase in total eNOS compared to vehicle control (* $P < 0.5$, $n = 8$). Slight differences in protein load were accounted for by normalizing samples to protein bands representing myosin heavy chain on coomassie gels.

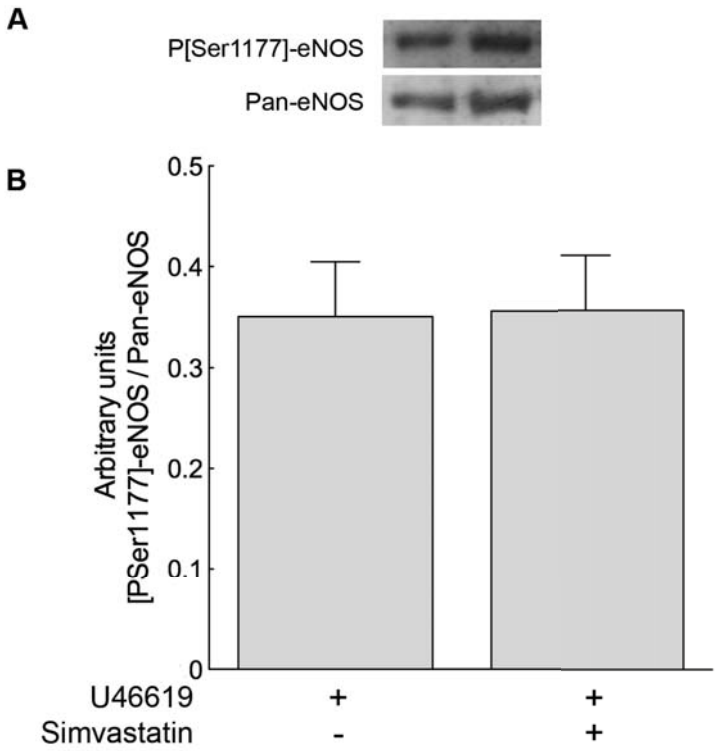

Figure E.4 Chronic Simvastatin Treatment Does not Alter the Phosphorylation State of Ser1177 eNOS. Seven days of simvastatin treatment (5 mg / kg) did not alter Ser1177 phosphorylation compared to control in rat caudal artery (P = NS, n = 8). Data expressed as a ratio of P[Ser1177-eNOS] / Pan-eNOS.

E.3.4 Chronic Statin Treatment Decreases TxA_2 Receptor-Mediated Developed Tension

In order to test the endothelial dependence of chronic statin treatment, and to determine if acute statin therapy may have incremental effects over chronic treatment alone, rat caudal artery from vehicle treated and simvastatin treated rats were mounted on a wire myograph. Vessels had intact endothelium-dependent vasodilator responses as judged by ACh relaxation following phenylephrine challenge. Consistent with data obtained in the Langendorff perfused heart model (Figure E2), TxA_2 receptor mediated developed tension was significantly lower in isolated vessels from the 7 day statin treated group compared to vehicle control (Figure E5, vehicle = 81.1 ± 2.5%, simvastatin = 69.7 ± 1.9%, $P < 0.5$, $n = 8$). Incubating isolated vessels for 60-minutes with the nitric oxide synthase inhibitor, L-NAME, caused an increase in U-46619 developed tension consistent with blocking tonic nitric oxide release in control animals, and abolished the difference in the statin mediated inhibition of developed tension in the chronic statin treated group (Figure E5, vehicle = 81.1 ± 2.5%, simvastatin + L-NAME = 85.2 ± 4.5%, P = NS, $n = 8$). However, acute incubation of isolated vessels with high dose simvastatin (10 μM) for 60-minutes prior to U-46619 stimulation reduced developed tension in both the vehicle treated and chronic simvastatin groups (Figure E5, $P < 0.5$, $n = 8$). Co incubation with L-NAME (100 μm) failed to inhibit this effect. Incubation with the direct inhibitor of Rho-kinase and activator of myosin phosphatase, H1152 (1 μM), attenuated almost all U-46619 induced developed tension in both the vehicle and simvastatin groups (Figure E5, $P < 0.5$, $n = 6$).

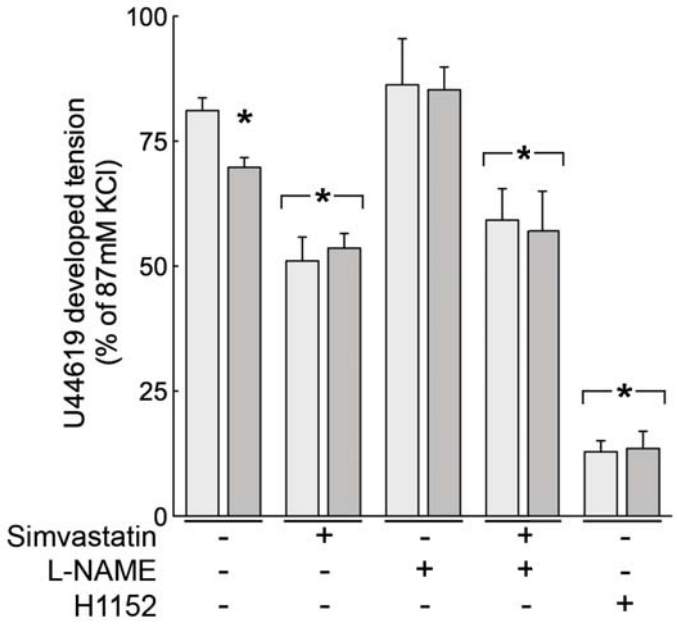

Figure E.5 Acute and Chronic Simvastatin Treatment Reduces TxA_2 Receptor Mediated Contraction in Isolated Rat Caudal Artery. Vessels from rats administered vehicle (□) or simvastatin (5 mg / kg, ■) for 7 days were mounted on a wire myograph and were either untreated or treated with simvastatin (10 μM) for 60-minutes in the presence or absence of L-NAME (100 μM), or treated with H1152 (1μM) for 60-minutes before stimulation with the TxA_2 receptor agonist, U-46619 (1 μM) for 10-minutes. Chronic 7 day simvastatin treatment decreased TxA_2 mediated contraction compared to vehicle control (* $P < 0.5$, $n = 8$), and this effect was abolished with L-NAME (P = NS, $n = 8$). Acute simvastatin treatment attenuated TxA_2 receptor mediated developed tension in both vehicle and chronic simvastatin treated rats (* $P < 0.5$, $n = 8$), L-NAME treatment did not abolish this effect (* $P > 0.5$, $n = 6$). Incubation with H1152 significantly reduced developed tension in response to TxA_2 receptor activation compared to control in both vehicle and simvastatin treated groups (* $P < 0.5$, $n = 6$).

E.3.5 Acute Simvastatin but Not Chronic Simvastatin Treatment Attenuates Inhibitory Thr855 Phosphorylation of the MYPT1 Targeting Subunit of Myosin Phosphatase

Previous work has shown that TxA_2 receptor stimulation leads to phosphorylation of the Thr855 of the targeting subunit of myosin phosphatase (MYPT1) and that this can be inhibited with acute administration of simvastatin (10 μM) (Figure D5). In the present study, administration of simvastatin (5 mg / kg) for 7 days did not alter Thr855 phosphorylation compared to the vehicle treated group (Figure E6, P = NS, n = 8). Stimulation of the TxA_2 receptor with U-46619 increased inhibitory phosophorylation at Thr855 in both vehicle treated and chronic simvastatin treated groups compared to control (Figure E6, $P < 0.5$, $n = 8$). Incubation for 60-minutes in the presence of simvastatin (10 μM) caused a significant reduction in the ratio of P[Thr855]MYPT1 / MYPT1 in both vehicle and chronically simvastatin treated vessels compared to untreated U-46619 stimulated vessels (Figure E6, $P < 0.5$, $n = 8$). Coincubation of simvastatin with L-NAME did not abolish this effect (Figure E6, $P < 0.5$, $n = 8$). The Rho-kinase inhibitor H-1152 (1 μM) significantly attenuated the U-46619-mediated increase in P[Thr-855]MYPT1/MYPT1 compared with U-46619 treatment alone in both 7 day treated vehicle and simvastatin groups (Figure E6, $P < 0.5$, $n = 8$).

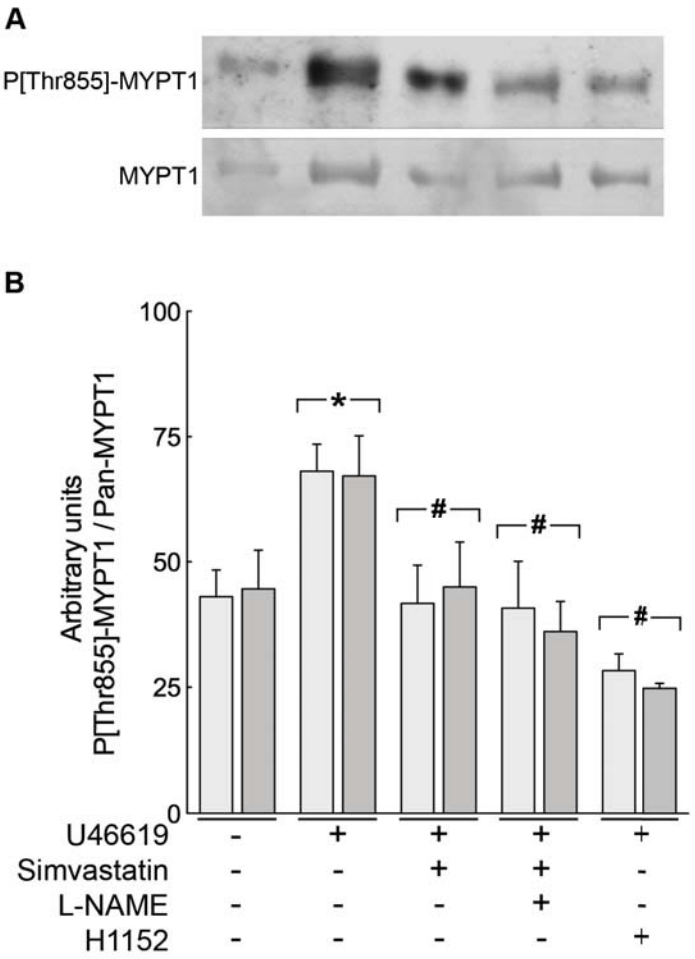

Figure E.6 Acute but Not Chronic Simvastatin Treatment Reduces Inhibitory Phosphorylation of MYPT1. Vessels from rats administered vehicle (□) or simvastatin (■) for 7 days were snap frozen after 60-minute incubation with either simvastatin (10 μM) or H1152 (1 μM). Stimulation of the TxA_2 receptor increased Thr855 phosphorylation compared to control in both vehicle and simvastatin treated rats (* $P < 0.5$, $n = 8$). There was no difference in Thr855 phosphorylation between the chronically treated simvastatin group vs vehicle control (P = NS, $n = 8$). Acute simvastatin or H1152 reduced TxA_2 receptor mediated Thr855 phosphorylation (# $P < 0.5$ vs U46619, $n = 8$). The presence of L-NAME (100 μM) did not alter simvastain-mediated reduction in Thr855 phosphorylation (P = NS, $n = 8$).

E.4 Discussion

A statin mediated reduction in blood pressure has been reported clinically in humans. In a meta-analysis of 20 separate statin trials, a reduction in systolic blood pressure was observed in statin treated subjects compared to those given a placebo or a non-statin lipid-lowering drug. This effect was greatest in those with the highest systolic blood pressure and was unrelated to differences in cholesterol, age, or the length of treatment. (Strazzullo, Kerry et al. 2007). A more recent large clinical trial has corroborated these results finding a small but significant decrease in both systolic and diastolic blood pressure (Golomb, Dimsdale et al. 2008). Consistent with these reports, we have shown a blood pressure lowering effect in healthy rats after 7 days of simvastatin treatment (Figure E1).

Although a number of groups have reported variable results with tail cuff measurements either with or without anesthesia (Bazil, Krulan et al. 1993; Van Vliet, Chafe et al. 2000; Ibrahim, Berk et al. 2006) our results taken under light anesthesia, with consistent heating, seem to have provided consistent results as evidenced by the fact that our control groups at day 0 and 7 and simvastatin group at day 0 were not significantly different (Figure E1).

The present study also demonstrates that blood vessels from rats treated for 7 days with simvastatin exhibit reduced contraction in response to TxA_2 stimulation (Figure E5). That this effect could be abolished with the inhibiter of NOS, L-NAME, suggests that this chronic effect of simvastatin was mediated by an endothelial/nitric oxide mediated mechanism (Figure E5). Furthermore, hearts from these animals displayed reduced perfusion pressure in response to TxA_2 receptor stimulation (Figure E2) that

may be attributable to the same mechanism. Chronic statin treatment was accompanied by the up-regulation of endothelial nitric oxide synthase, the main source of nitric oxide in the vasculature (Figure E3). These results confirm findings from a number of cell culture studies that have demonstrated increased eNOS expression in response to statin treatment (Laufs, La Fata et al. 1998; Laufs, Gertz et al. 2002). The chronic upregulation of eNOS may result in increased nitric oxide production and the subsequent hyperpolarization of the vascular smooth muscle plasma membrane, resulting in reduced L-Type Ca^{2+} entry. This mechanism could account for the reduction in TxA_2 receptor mediated smooth muscle contraction (Figure E5) and TxA_2-mediated coronary perfusion pressure (Figure E2), which in turn, may account for the blood pressure lowing effects described (Figure E1).

Although increased nitric oxide production may entirely account for the reduction in blood pressure, other groups have provided evidence that statin treatment may reduce circulating levels of endothelin-1 (Hernandez-Perera, Perez-Sala et al. 1998), angiotensin II , reduce angiotensin II type I receptor expression (Nickenig, Baumer et al. 1999), reduce free radical production (Rikitake, Kawashima et al. 2001; Wassmann, Laufs et al. 2001), and improve arterial compliance (Fukuta, Sane et al. 2005), all of which may contribute to reduced blood pressure. However, these phenomena could also be a consequence of reduced blood pressure due to increased nitric oxide production. Therefore, further studies will be required to determine the cause of statin mediated blood pressure lowering.

Previous data from our laboratory (Chapter C) indicates that statins relax smooth muscle through different pathways depending upon the agonist used. We have shown

that acute statin administration reduces α_1-adrenoreceptor contraction in rat caudal artery entirely through an endothelial/nitric oxide mechanism. Whereas acute statin mediated inhibition of TxA_2 receptor-mediated contraction involves a direct smooth muscle effect associated with reduced inhibitory phosphorylation of Thr855-MYPT1. In the present study chronic statin treatment relaxed TxA_2 receptor mediated contraction through a nitric oxide / endothelial dependent mechanism. The discrepancy between this and previous work using acute high does simvastatin (Section D) may be due to the concentration of statin used. However, this raises the question as to how simvastatin is able to inhibit endothelial RhoA but not smooth muscle RhoA. This may represent a difference in the kinetics of RhoA prenylation between these cell types, perhaps representing an altered half-life for GGPP groups or even altered activity of the gerenylgeranyl transferase. Alternatively, the lack of a smooth muscle effect may represent a shift in equilibrium in the vascular smooth muscle sensitivity to Ca^{2+}.

Importantly, a role for nitric oxide in smooth muscle Ca^{2+} desensitization has been suggested. Haystead and colleagues demonstrated that phosphorylation of Ser695 of the targeting subunit of myosin phosphatase (MYPT1), prevented phosphorylation of the adjacent Thr697 site in response to a constitutively activated form of Rho-kinase (Wooldridge, MacDonald et al. 2004). More recently, Nakamura et al, using diphospho antibodies specific to MYPT1 phosphorylated at Ser696 and Thr697, determined that cGMP treatment of α-toxin–permablised femoral arteries increases Ser696 phosphorylation concomitant with decreased Thr697 phosphorylation. However, a significant amount of diphosphorylated MYPT1 was present, indicating that phosphorylation at Ser696 does not necessarily preclude Thr-697 phosphorylation

(Nakamura, Koga et al. 2007). As Somylo pointed out in a recent editorial, the technique employed by Nakamura does not allow for the differentiation of phosphoylation on a single molecule of MYPT1 (Somlyo 2007), thus the exact mechanism of nitric oxide/cGMP/PKG mediated Ca^{2+} desensitization is still under debate. Nevertheless, the data in this paper shows little effect of endothelial removal or NOS blockade with L-NAME on the status of MYPT1 Thr855 phosphorylation which suggests that the phenomena reported by Heystead and Nakamura may be biochemically possible but physiologically inconsequential.

Nitric oxide, as well as being a potent vasodilator, inhibits platelet aggregation, smooth muscle proliferation, and leucocyte activation and attachment to endothelial cells. Deficient nitric oxide-dependent vasorelaxation is a hallmark of endothelial dysfunction (Quyyumi 1998; Lavi, Yang et al. 2008) and endothelial dysfunction is central to the development of coronary and peripheral ischemic diseases (Davignon and Ganz 2004). Raised plasma LDL has been associated with impaired endothelial dysfunction, therefore, the ability of statins to reduce plasma LDL is thought to be the major mechanism by which they limit atherosclerosis (Nicholls, Tuzcu et al. 2007). The increased nitric oxide expression noted in this study may provide an additional mechanism by which statins could protect against atherosclerosis. Furthermore, these results give an explanation for clinical findings that statins reduce cardiovascular morbidity and mortality before reductions in plaques occur (Centres 1994), in patients with normal cholesterol levels (Byington, Jukema et al. 1995; Sacks, Pfeffer et al. 1996), and restore endothelial function independent from serum cholesterol (Tsunekawa, Hayashi et al. 2001, Fichtlscherer, Schmidt-Lucke et al. 2006).

Therefore, this study suggests that statins may be beneficial strategy for improving endothelial function, even in patients with normal cholesterol profiles.

Despite the lack of a direct smooth muscle effect in chronically treated rats (Figure E5), the finding that incubating isolated vessels from these animals with high dose simvastatin for 60-minutes was still able to inhibit TxA_2 receptor mediated contraction through a smooth muscle MLCP-dependent mechanism (Figure E5) provides additional rational for a new therapeutic use of statins. The ability of acute high dose simvastatin to cause a robust attenuation of TxA_2 contraction in vessels from rats already treated with simvastatin suggests that that chronic treatment with statins does not desensitize blood vessels to the smooth muscle / Rho-kinase mediated effects of bolus doses. Thus patients already on statins may still benefit from the potential acute vasodilator properties of simvastatin. Indeed, in a recent study of 350 patients who were already on a statin, adding an 80 mg dose of atorvastatin 12 hours before their PCI procedure and a second, 40 mg dose 2 hours before halved the rate of in-hospital myocardial infarctions (Di Sciascio, Patti et al. 2009).

E.5 Conclusion

The present study has documented a reduction in systolic blood pressure in healthy rats treated with simvastatin for 7 days. Isolated hearts from these animals displayed reduced TxA_2-receptor mediated coronary perfusion pressure, while isolated blood vessels displayed a reduction in TxA_2-receptor mediated contraction. An increase in the expression of endothelial nitric oxide synthase (eNOS) was concomitant with these effects and suggests a likely mechanism. Furthermore, acute administration of high dose simvastatin further reduced TxA_2-receptor mediated contraction via activation of smooth muscle myosin phosphatase.

F. General Discussion: Advances in understanding statin therapy

F.1 Synopsis of results

This thesis has examined both the acute (60-minute) and chronic (7 days) effects of statin treatment on the generation of agonist-mediated contraction in rat caudal artery. In the acute phase (60 minutes of incubation) both pravastatin and simvastatin inhibit α_1-adrenoreceptor mediated constriction through an endothelial/nitric oxide-dependent mechanism. This effect was associated with increased phosphorylation of P[Ser1177]eNOS without an increase in total protein. Similarly with thromboxane A_2-receptor mediated developed tension, simvastatin also caused an endothelial-mediated attenuation of contraction evidenced by an increase in P[Ser1177]eNOS, however, NOS inhibition or endothelial removal did not completely abolish this effect, suggesting additional mechanisms. Indeed, there was an additional simvastatin-mediated attenuation of vascular smooth muscle contraction that was insensitive to inhibition with L-NAME/endothelium removal and was associated with activation of myosin light chain phosphatase as indexed by a decreased phosphorylation of P[Thr855]-MYPT1 and decreased P[LC_{20}].

Administering clinically relevant therapeutic concentrations of simvastatin to rats for 7 days lowered systolic blood pressure and reduced TxA_2-receptor mediated coronary perfusion pressure in hearts isolated from these animals. Furthermore, vessels isolated from chronic statin treated rats displayed reduced contraction to TxA_2-receptor stimulation that was entirely dependent on nitric oxide and the endothelium. This was associated with increased eNOS expression but not P[Ser1177]eNOS phosphorylation. No modulation of smooth muscle MLCP was detected in chronically treated animals, as assessed by MYPT1 Thr855 phosphorylation. However, treatment of vessels from chronically treated animals with an additional high dose of

simvastatin (60 minutes) further reduced contraction in a smooth muscle dependent manner and decreased phosphorylation of P[Thr855]-MYPT.

F.2 Advances in Mechanisms of Action

A possible mechanism for the effects documented herein includes the inhibition of the RhoA / Rho kinase pathway via inhibition of isoprenoid formation (Figure A3) (Liao 2005). RhoA is a monomeric GTPase that cycles between an inactive GDP-bound form found in the cytosol, and an active GTP-bound form localized to the plasma membrane (Zhang and Casey 1996). By inhibiting the mevolanate pathway statins inhibit the synthesis of farnesyl pyrophsphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (Goldstein and Brown 1990; Zhong, Liang et al. 2005). The addition of the GGPP moiety is required to enable correct membrane targeting of RhoA and allows for the activation of its downstream effector, Rho-kinase (Van Aelst and CD'Souza-Schorey 1997). Statins, by inhibiting prenylation, and thus RhoA activation, may prevent activation of Rho-kinase with the consequence of activating eNOS and MLCP (Figure F1).

F.2.1 The Endothelium and Nitric Oxide

Nitric oxide (NO) is a potent endothelial cell derived vasorelaxant and exerts its effect on the smooth muscle by hyperpolarizing the plasma membrane through guanyl cyclase/PKG-mediated activation of K^+ channels (Furchgott and Zawadzki 1980). Ultimately, this limits intracellular Ca^{2+} entry, the activation of calmodulin, the activation of MLCK, and contraction (Figure F1). In the endothelium NO is produced by the Ca^{2+}/calmodulin dependent enzyme eNOS, which is maximally activated by phosphorylation on the Ser1177 site (Dimmeler, Fleming et al. 1999; Fulton, Gratton et al. 1999). In the endothelium Rho-kinase inhibits Protein Kinase B (PKB) mediated phosphorylation of eNOS, resulting in reduced NO synthesis (Wolfrum, Dendorfer et

al. 2004). Statins, by blocking the prenylation and thus membrane targeting of RhoA may inhibit Rho-kinase activation, thereby increasing endothelial nitric oxide production through enhanced activation of eNOS, resulting in vasodilatation (Figure F1) (Kureishi, Luo et al. 2000).

The current study does not directly measure nitric oxide production. Indeed, direct measurement of nitric oxide has rarely been employed in intact arterial vessels due to the small amount of endothelium relative to smooth muscle, short half-life of nitric oxide, and thus small amounts of nitric oxide present (Archer 1993). A number of groups have used fluorescence probes combined with fluorescence microscopy in order to directly measure nitric-oxide, with the most common probe being 4,5-diaminofluorescein (DAF-2) (Lopez-Figueroa, Caamano et al. 2000; Navarro-Antolin, Lopez-Munoz et al. 2001). The accuracy of the measurements produced, however, have been seriously questioned, as the presence of divalent cations such as Ca^{2+} increase DAF-2 fluorescence (Broillet, Randin et al. 2001). Therefore, in the present study the role of nitric oxide in the inhibition of α_1-adrenoreceptor and TxA_2 receptor-mediated contraction has been inferred by the removal of the endothelium and by incubation with L-NAME, an arginine analogue and specific NOS inhibitor (Bogle, Moncada et al. 1992). However, there are studies using large monolayers of cultured endothelial cells that have measured nitric oxide and indicated that increased Ser1177 phosphorylation has the potential to increase nitric oxide production by 15-20 fold (Dimmeler, Fleming et al. 1999). Furthermore, there is ample evidence in the literature that indicates statins increase the $t_{½}$ of eNOS mRNA which leads to an associated increased eNOS expression with increased nitric oxide release (Laufs, La Fata et al. 1998; Laufs and Liao 1998).

F.2.2 Vascular Smooth Muscle

In the smooth muscle Rho-kinase regulates contractility by inhibiting MLCP by phosphorylating its myosin targeting subunit (MYPT1) at Thr855 and Thr697 (Velasco, Armstrong et al. 2002; Hartshorne, Ito et al. 2004; Wilson, Susnjar et al. 2005). Inhibition of MLCP favors LC_{20} phosphorylation, thus increased contraction at a given $[Ca^{2+}]_{cyt}$ level, a phenomenon referred to as Ca^{2+} sensitization (Somlyo and Somlyo 2003). Statins, by inhibiting RhoA membrane targeting, thus Rho-kinase activation, may favor relaxation by removing Rho-kinase mediated inhibition of MLCP, thus desensitizing the smooth muscle contractile apparatus to Ca^{2+} (Figure F1).

In the present study, we have not examined the phosphorylation state of the Thr697-MYPT1 site. Wilson *et al*., have previously shown that in the context of TxA_2 mediated contraction in the rat caudal artery the phosphorylation state of Thr697 is not significantly altered, furthermore, that pharmacological inhibition of Rho-kinase does not alter Thr697 (Wilson, Susnjar et al. 2005). The present study provides further evidence that the Thr855 site is the functionally important site as phosphorylation was increased with TxA_2 stimulation and phosphorylation could be reduced using simvastatin and the Rho-kinase inhibitor, H1152 (Section D, Figure 4).

The ability of the Rho-kinase inhibitor, H1152, to completely abolish vascular contraction suggests that inhibition of the RhoA/Rho-kinase pathway can lead to sufficient activation of MLCP which enables it to completely inhibit Ca^{2+}/calmodulin-dependent contraction. However, the present study design does not allow us to

quantify the degree to which statin mediated phosphorylation of Thr855 MYPT1 caused attenuation of vascular contraction. Further studies using direct Ca^{2+} imaging coupled with L-type Ca^{2+} channel blockade will further clarify the role of Ca^{2+} entry vs Ca^{2+} desensitization in statin mediated attenuation of TxA_2 mediated contraction.

The studies documented in this thesis examine molecular targets downstream from the RhoA/Rho-kinase pathway. This study does not directly measure the prenylation state of RhoA and thus cannot conclusively implicate modulation of RhoA prenylation in the effects described herein. To this authors knowledge, there have been no studies that have directly measured this in intact vessels. A number of studies using intact vessels have shown that the co-incubation with L-mevalonate or GGPP can restore contraction or decrease eNOS expression, providing indirect evidence of modulation of prenylation (Laufs and Liao 1998; Pérez-Guerrero, Márquez-Martín et al. 2005). Futhermore inhibitors of GGPP transferase, the enzyme responsible for attaching the GGPP group to its target protein, have been shown to have similar effects as statins (Guijarro, Blanco-Colio et al. 1998; Laufs, Kilter et al. 2002). However, the majority of these studies are in the context of proliferating cells in which the proportion of active RhoA is much higher (Hall 2005). This laboratory is currently developing a mass spectrometry based technique to measure RhoA prenylation in intact vessel preparations.

Figure F1 Mechanisms of Statin-Mediated Inhibition of Agonist Induced Contraction in the Rat Caudal Artery. Statins inhibit agonist-induced contraction by inhibiting RhoA prenylation and thus, Rho-kinase activation. This may result in relaxation through both (**A**) endothelial and (**B**) direct smooth muscle mechanisms. Figure modified after Wilson, D.

F.3 Advances is Clinical Understanding

F.3.1 Endothelial Dysfunction

Many clinical trials have reported that inhibitors of HMG-CoA reductase reduce cardiovascular morbidity and mortality in patients with and without coronary artery disease (4S Group 1994; Shepherd, Cobbe et al. 1995; Sacks, Pfeffer et al. 1996; Downs, Clearfield et al. 1998; Heart Protection Study Collaborative Group 2002). The studies reported in this thesis document statin mediated inhibition of contraction in both chronic and acute treated vessels, as well reduced coronary perfusion pressure that is attributable to increased nitric oxide production. Therefore, statins may have a general effect (ie. not agonist dependent) mediated by increased nitric oxide bioavailability at normal clinical concentrations. This suggests that increased nitric oxide production, independent of LDL lowering, may be responsible for some of the positive cardiovascular effects noted in these clinical trials. Furthermore, these results go some way in explaining the rapid statin-mediated improvement in endothelial function demonstrated not only in hypercholesterolemic patients (Wassmann, Faul et al. 2003) but also in normalcholesterolemic subjects (Beckman, Liao et al. 2004) and numerous animal models including spontaneously hypertensive rats (SHR) (Wassmann, Laufs et al. 2001).

F.3.2 Hypertension

The blood pressure lowering effect documented in this thesis is consistent with a number of clinical trials that describe decreased systolic blood pressure in statin treated humans (Strazzullo, Kerry et al. 2007; Golomb, Dimsdale et al. 2008). The effect of statins on blood pressure may be explained by increased nitric oxide production resulting improved endothelial function and improved vasodilatation

(Perticone, Ceravolo et al. 2001; Giansante and Fiotti 2005). However, it seems unlikely that this can explain the effect entirely, as changes in vascular total peripheral resistance (TPR) due to vasoconstriction/dilation are usually offset by alterations in blood volume mediated by the kidneys (Guyton 1987). Statins may modulate components of the renin-angiotensin-aldosterone system (Nickenig, Baumer et al. 1999), this combined with decreased TPR could account for the modest reduction in blood pressure reported herein. Another contributing factor may be reduced serum cholesterol levels, which have positive effects on arterial compliance and thus improve vasodilator capacity (Leibovitz, Hazanov et al. 2001), however, it seems unlikely that this effect would be relevant following short or even 7 day treatment.

These results suggest that statins may be a viable antihypertensive drug for individuals without systemic endothelial dysfunction. Clinical trials have shown on average a 3-5 mmHg reduction in systolic BP, however, this effect becomes larger the higher the baseline blood pressure (Strazzullo, Kerry et al. 2007). So far, there have been no studies examining statin mediated blood pressure modulation in patients with stage II hypertension, or in patients with endothelial dysfunction. The results of this thesis suggest that those with mild non-systemic endothelial dysfunction are especially likely to benefit. The combination of statins with drugs such as diuretics will also be of great interest.

F.3.3 Pathological Activation of RhoA/Rho-Kinase Signaling

The studies described in this thesis document that acute high dose simvastatin causes a largely vascular smooth muscle-dependent reduction in TxA_2 mediated contraction

via inhibition of RhoA/ROK signaling as indicated by the degree in MYPT1 Thr855 phosphorylation (Section D and E), suggesting that acute high dose simvastatin administration may be a novel treatment for disorders involving the dysregulation of TxA_2. A major role for TxA_2 in pulmonary hypertension has also been documented with studies reporting perturbation in the balance between protacyclin and TxA_2 synthesis (Frolich, Ogletree et al. 1980; Christman, McPherson et al. 1992). Interestingly, the administration of the TxA_2 synthase inhibitor and TP receptor antagonist, terbogrel, was efficacious in treating pulmonary hypertension, however, continued treatment was not viable due to side effects including leg pain (Langleben, Christman et al. 2002). Thus, statins may represent an already available and well-tolerated alternative to inhibit TxA_2 signaling in pulmonary hypertension. The smooth muscle effects of simvastatin were not present in chronic experiments at a dose of 5 mg/kg per day. This suggests that either desensitization is occurring during chronic treatment, or that smooth muscle effects may not be present at the lower dose used. Ongoing studies are underway to determine the time course of the vascular smooth muscle / MLCP effect and to determine the minimum dosage of statins required, as these results will determine the clinical potential of treating chronic disorders involving TxA_2 receptor activation with statins.

A role for RhoA / Rho-kinase has also been described in the signaling of vasoconstrictors such as angiotensin II (Seko, Ito et al. 2003), serotonin (Watanabe, Faraci et al. 2005) and endothelin-1 (Niiro, Koga et al. 2003). Furthermore, these agonists have been implicated in numerous cardiovascular disease states (Frishman, Huberfeld et al. 1995; Kedzierski and Yanagisawa 2001; Unger 2002). We have shown that high dose simvastatin can inhibit the RhoA/ROK pathway in the context

of TxA_2 receptor mediated signaling, it remains to be seen if high dose statins can alter the activation of vascular smooth muscle RhoA / Rho-kinase in the context of the aforementioned agonists but it is likely that the beneficial effect of bolus dose statin treatment will be directly related to the extent to which each of these agonists activates the RhoA / Rho-kinase / MYP1 axis of Ca^{2+} sensitization. A therapeutic drug with the ability to reduce the activation of the RhoA / Rho-kinase pathway regardless of the agonist involved in the activation of the pathway may prove exceedingly useful. Ongoing studies within our research group are aimed at determining the efficacy of statins in attenuating the general activation of RhoA / Rho-kinase in both blood vessels and platelets.

F.3.4 Vasospasm

Coronary artery spasm is an important contributor to a wide variety of ischemic diseases. A role for vasospasm has been described in variant angina (Miller, Waters et al. 1981), myocardial infarction (Fukai, Koyanagi et al. 1993) and stroke (Kassell, Sasaki et al. 1985). TxA_2 has been implicated in the pathogenesis of myocardial ischemic episodes during unstable angina (Machado, Saavedra et al. 1994) and increases in TxA_2 synthesis have been reported in patients with ischemic heart disease (Serneri, Gastone et al. 1981). Furthermore, TxA_2 may play a role in vasospasm that occurs after subarachnoid hemorrhage (Chan, Durity et al. 1984; Ansar, Larsen et al. 2009) a relatively rare cause of stroke with a high rate of morbidity and mortality.

The RhoA / Rho-kinase pathway has been implicated in the pathogenesis of coronary spasm, this is likely partly dependent on TxA_2 receptor activation in the vasculature and platelets (Sato, Tani et al. 2000). Administration of a Rho-kinase inhibitor limits

coronary spasm in a porcine model of vasospasm (Kandabashi, Shimokawa et al. 2000). Furthermore, in patients with vasospastic angina, administration of Rho-kinase inhibitors reduces ACh mediated coronary spasm and related myocardial ischemia (Masumoto, Mohri et al. 2002). Currently Rho-kinase inhibitors are available for clinical use only in Japan (Shimokawa, Hiramori et al. 2002). The results presented herein suggest that statins have the capacity to inhibit the same pathway, and hence high doses of lipophilic statins may prove equally useful in acute settings. Indeed, statin therapy reduces the risk for vasospasm after subarachnoid hemorrhage (Parra, Kreiter et al. 2005). In a meta-analysis a total of 158 patients were examined, 78 received a statin and 80 received placebo (Sillberg, Wells et al. 2008). The incidence of vasospasm was significantly decreased in the statin treated group. Further clinical trials are required to determine if statins reduce the incidence of other forms of vasospasm.

In addition, our results (Section C, Figure 5) suggest that simvastatin treatment can improve the efficacy of Rho-kinase inhibitors, making combined statin/Rho-kinase inhibitor therapy an attractive, economically viable, option to treat vasospasm.

F.3.5 Reducing Percutaneous Coronary Intervention (PCI) Complications.

Percutaneous coronary intervention (PCI) is a mechanical technique used to open obstructed blood vessels and is utilized in the treatment of coronary artery disease and occlusive peripheral vascular disorders (Smith Jr, Dove et al. 2001). Acute closure of blood vessels, termed "no reflow" can occur after successful PTCA in 2-10% of patients (Rezkalla and Kloner 2002). Furthermore, 20% of successful PCI procedures result in restenosis of the opened vessel (Holmes Jr, Vlietstra et al. 1984). Coronary vasospasm, independent from proliferative occlusion, has been implicated in vessel closure and has been documented in both animals (Leveen, Wolf et al. 1985) and in humans (Fischell, Derby et al. 1988). Although the mechanism by which this vasospasm occurs is largely unknown, the release of vasoconstrictors such as TxA_2 from platelets may play a major role.

The findings presented in this thesis suggests that statins may improve the outcome of PCI through a variety of mechanisms. A high-dose bolus administration may help prevent no reflow by inhibiting smooth muscle vasoconstriction and thus vasospasm by activating MLCP. Indeed, the administration of a bolus dose of atorvastatin to patients not already on a statin prior to percutaneous coronary intervention significantly reduced the risk of myocardial infarction by about a third (Briguori, Colombo et al. 2004). Chronic statin therapy may also improve the outcome of PCI by upregulating nitric oxide production, further contributing to vasorelaxation and inhibiting platelet activation. This upregulation of nitric oxide production may also be beneficial as protection against restenosis. Indeed, there is clinical evidence for a protective statin effect against both proliferative and vasospastic restenosis (Kamishirado, Inoue et al. 2007). Furthermore, our results suggest that patients on

chronic statin therapy can still benefit from the smooth muscle effects of acute high dose administration (Section E). This is also borne out clinically, as in a study of 350 patients who were already receiving statin therapy, adding an 80 mg dose of atorvastatin 12 hours before their PCI procedure and a second, 40 mg dose 2 hours before halved the rate of in-hospital myocardial infarctions (Di Sciascio, Patti et al. 2009).

F.3.6 Microvascular Disorders

The Coronary Slow Flow Phenomennon (CSFP) is a microvascular disorder of the coronary arteries characterized by the delayed opacification of coronary vessels in the absence of obstructive coronary atherosclerosis. (Beltrame, Limaye et al. 2000; Beltrame 2006). CSFP patients suffer acute angina with 80% of those diagnosed experiencing repeat episodes, with 30% re-presenting to the emergency department with severe angina and 20% requiring readmission to the coronary care unit (Beltrame, Limaye et al. 2000). There is currently no effective treatment available for CSFP patients.

CSFP is associated with coronary microvascular dysfunction and unpublished studies have suggested a role for endothelin-1 (Turner and F. 2005). The angina experienced by CSFP patients is insensitive to nitrate treatment, suggesting that the uprgeulation of nitric oxide by chronic low dose treatment with statins may not be useful. However, the acute vasodilator properties of high does simvastatin independent of the endothelium may be useful in the treatment of this disease. Clinical trials of high dose simvastatin in this patient group are currently in planning stage.

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