VASOACTIVE EFFECTS OF LYSOPHOSPHATIDYLCHOLINE IN SMALL ARTERIES

by

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ABSTRACT

Lysophosphatidylcholine (1-acyl-*sn*-glycero-3-phosphocholine, LPC) is the most abundant glycerol-based lysophospholipid present in cell membranes and oxidized lipoproteins. It has been proposed that LPC contributes to the altered vaso-reactivity associated with various cardiovascular diseases in which elevated LPC levels were identified. However, the contribution of LPC in regulating vascular resistance has not been completely elucidated, as the majority of previous studies have used either large blood vessels or isolated cells. Therefore, our study aimed to investigate the vasoactive effects and the underlying mechanisms of LPC in small arteries/arterioles that are crucial in the determination of vascular resistance and the maintenance of organ function.

The unique finding of our investigation is that LPC possesses biphasic effects on both peripheral arterial resistance and coronary circulation, and even ventricular function. Specifically, in the isolated perfused rat mesenteric arterial bed, both endothelium-derived relaxing factors and thromboxane A_2 (TxA_2, a vasoconstricor) are diminished by LPC perfusion. However, LPC washout stimulates a rebound overproduction of TxA_2, which results in an enhanced contractile response to α_1-adrenoceptor stimulation.

Our study next found that sustained perfusion of hearts with LPC augmented coronary perfusion pressure and reduced left ventricular developed pressure. These effects were exaggerated when LPC was removed from the perfusate. Furthermore, LPC selectively potentiated the receptor-coupled vasoconstrictor response of isolated rat septal coronary artery to U-46619, a TxA_2 mimetic. Interestingly, when LPC was washed out, the potentiation to U-46619 was even more pronounced. Both the immediate and residual effects of LPC were endothelium-dependent. Endothelium-derived

hyperpolarizing factor was likely the sole mediator responsible for the direct effects of LPC on U-46619-vasoconstriction, whereas the augmented vasoconstrictor responses following LPC washout may in part be related to an increase in endothelin-1, and a striking reduction in the bioavailability of nitric oxide.

Our data suggest that simply reducing LPC levels to normal may not be sufficient to reverse the adverse consequences of this lysolipid accumulation in vasculature. Further understanding of the residual effects of LPC will enable the identification of more effective treatment targets for LPC-related diseases.

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AA	arachidonic acid
AC	adenylate cyclase
Ach	acetylcholine
Ag-II	angiotensin II
ATX	autotaxin
cAMP	cyclic adenylate monophosphate
cGMP	cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
COX	cyclooxygenase
$cPLA_2$	cytosolic PLA_2
CPP	coronary perfusion pressure
CRC	concentration-response curve
CYP	Cytochrome P-450
DAG	diacylglycerol
DRC	dose-response curve
EC	endothelial cell
EDCF	endothelium-derived constricting factor
EDHF	endothelium-derived hyperpolarizing factor
EDR	endothelium-dependent relaxation
EDRF	endothelium-derived relaxing factor
EETs	epoxyeicosatrienoic acids
ET-1	endothelin-1
GC	guanylyl cyclase
GPCR	G-protein coupled receptor
H_2O_2	hydrogen peroxide
HDL	high density lipoprotein
HETEs	hydroxyeicosatetraenoic acids
HR	heart rate

I/R	ischemia-reperfusion
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon- γ
IL-1β	interleukin-1 β
IL-2	interleukin-2
IL-8	interleukin-8
IP3	inositol triphosphate
$iPLA_2$	Ca^{2+}-independent intracellular PLA_2
K_{Ca}	Ca^{2+}-sensitive K^+ channels
KH	Krebs-Henseleit
LCAT	lecithin-cholesterol acyltransferase
LDL	low density lipoprotein
$_L$-NMMA	N^G-monomethyl-L-arginine methyl ester
LPA	lysophosphatidic acid
LPC	lysophosphatidylcholine
LPLD	lysophospholipase D
$Lp\text{-}PLA_2$	lipoprotein-associated PLA_2
LVDP	left ventricular developed pressure
MAB	mesenteric arterial bed
MCP-1	monocyte chemoattractant protein
NO	nitric oxide
NOS	nitric-oxide synthase
O^{2-}	superoxide
OH·	hydroxyl radicals
$ONOO^-$	peroxynitrite
oxLDL	oxidized low density lipoprotein
PAF-AH	platelet activating factor acetylhydrolases
PC	phosphatidylcholine
PE	phenylephrine

PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin F_2
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGI_2	prostacyclin
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLA_2	phosphalipase A_2
PLC	phospholipase C
PLD	phospholipase D
ROS	reactive oxygen species
SMC	smooth muscle cell
SNP	sodium nitroprusside
SOD	superoxide dismutases
$sPLA_2$	secretory phospholipase A_2
SQ-29548	[1S-[1a,2a(Z),3a,4a]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino] methyl]-7-oxabicyclo [2.2.1]hept-2-yl]-5-heptanoic acid
TP	thromboxane A_2 receptor
TxA_2	thromboxane A_2
TxAS	thromboxane synthase
TxB_2	thromboxane B_2
UK14,304	5-bromo-N-(2-imidazolin-2-yl)-6-quinoxalinamine
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor

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1. INTRODUCTION

1.1 Physiological and pathophysiological roles of lysophosphatidylcholine

Lysophosphatidylcholine (1-acyl-*sn*-glycero-3-phosphocholine, LPC) is the most abundant glycerol-based lysophospholipid present in cell membranes and oxidized lipoproteins. Although the cellular content of LPC is difficult to measure, plasma LPC levels have been reported to reach up to 20% of total phospholipids (Xu, 2002). LPC plays important physiological and pathophysiological roles in both humans and animals. For instance, it is responsible for the delivery of fatty acids and choline to tissues (Croset et al., 2000). On the other hand, elevated levels of LPC have been linked to the cardiovascular complications associated with atherosclerosis, ischemia, and diabetes (Takahara et al., 1997; Shi et al., 1999; Sonoki et al., 2003). The roles of LPC in atherosclerosis and myocardial ischemia are relatively well established.

1.1.1 Role of LPC in atherosclerosis

At present, LPC has been recognized as an important cell-signaling molecule that initiates and even amplifies several steps in the development of atherosclerosis. Atherosclerosis refers to the development of fibrofatty plaques within the arterial intima, with inflammation being its essential feature. The pathophysiological importance of LPC was initially noticed based on the finding that LPC is one of the major components of oxidized low density lipoprotein (oxLDL), which is abundant in atherosclerotic lesions and has been considered highly atherogenic. The amount of LPC in oxLDL was reported to be 40% higher than that in native LDL (Stiko et al., 1996; Chen et al., 1997). Some of the proatherothrombotic effects of oxLDL have been attributed to the multiple inflammatory effects that LPC exerts on almost all the cell types involved in

atherosclerosis, including endothelial cells (ECs), smooth muscle cells (SMCs), monocytes, macrophages, and T-cells (summarized in Table 1.1). These events could contribute to all stages of atherosclerosis. In addition to these *in vitro* studies, an *in vivo* study also confirmed the critical role of LPC in this pathological process by showing that the atherosclerotic lesion progression was significantly inhibited in LPC-receptor deficient mice compared to control (Parks et al., 2006).

Table 1.1 LPC-mediated biological effects on putative inflammatory cells

TARGET CELLS	EFFECTS
Neutrophils	• ROS generaration: NAD(P)H oxidase activation and myeloperosidase release • Functional responses: increased chemotaxis, elastase release
Monocytes/Macrophages	• Formation of inflammatory mediators: upregulation of cytokines (IL-1β, IL-8, VEGF, HB-EGF), Ca^{2+}-dependent PLA_2 enzymes, and arachidonic acid release • Functional responses: increased chemotaxis • Cytotoxicity: increased apoptosis
T-lymphocytes	• Formation of inflammatory mediators: upregulation of cytokines (IL-2, IFN-γ, and ROS) • Functional responses: increased chemotaxis • Cytotoxicity: increased apoptosis
Endothelial cells	• Homing of inflammatory cells: upregulation of adhesion molecules (ICAM-1/VCAM-1), P-selectin, and MCP-1 • Formation of inflammatory mediators: activation of Ca^{2+}-dependent PLA_2 enzymes, upregulation of COX-2, and arachidonic acid release • Functional responses: impaired proliferation/migration and reduced NO- and EDHF-mediated vasodilation • Cytotoxicity: apoptosis
Smooth muscle cells	• Homing of inflammatory cells: upregulation of MCP-1 • Oxidative stress: NAD(P)H oxidase activation • Functional responses: upregulation of growth factors and increased proliferation and migration • Cytotoxicity: apoptosis

ROS: reactive oxygen species, IL-1β: interleukin-1 β, IL-8: interleukin-8, VEGF: vascular endothelial growth factor, IL-2: interleukin-2, IFN-γ: interferon- γ, ICAM-1: intercellular adhesion molecule-1, VCAM-1: vascular cell adhesion molecule-1, MCP-1: monocyte chemoattractant protein, COX-2: cyclooxygenase-2, NO: nitric oxide, EDHF: endothelium-derived hyperpolarizing factor. Adapted from Matsumoto et al., 2007.

1.1.2 LPC as a biomarker of myocardium ischemia

It is well known that under cardiac ischemic conditions, LPC, a hydrolysis product of phospholipid degradation, greatly accumulates in ischemic myocardium in different animal species (Sobel et al., 1978; Shaikh and Downar, 1981; Snyder et al., 1981; Otani et al., 1989) as well as in human hearts (Sedlis et al., 1990; Sedlis et al., 1993; Sedlis et al., 1997). Due to the fact that the time course of LPC accumulation parallels the time kinetics of early ischemic ventricular arrythmias (Corr and Yamada, 1995; Sedlis et al., 1997), considerable efforts have been devoted to testing the hypothesis that LPC contributes to cardiac ischemia-reperfusion (I/R) injury. It is now clear that LPC possesses arrythmogenic properties (Man, 1988; Corr and Yamada, 1995; Bai et al., 2007). However, I/R injury is not limited to cardiomyocytes, but also extends to coronary endothelial cells, which have been suggested to be even more vulnerable (Schmiedl et al., 2001; Staat et al., 2005). Coronary endothelial dysfunction may be responsible for the lowered coronary flow/increased coronary resistance (Mehta et al., 1989; Hashimoto et al., 1991; Maulik et al., 1996; Tiefenbacher et al., 1996; Toufektsian et al., 2001) under I/R conditions (Figure 1.1). Interestingly, although there is no direct causal linkage available between LPC and this endothelial dysfunction, exogenous LPC has been shown to produce impaired ventricular function and elevated coronary resistance in isolated perfused rat hearts (Sargent et al., 1993; Hoque et al., 1997; Watanabe and Okada, 2003), and to selectively impair endothelium-dependent relaxation (EDR) induced by either nitric oxide (NO) or endothelium-derived hyperpolarizing factor (EDHF) (Eizawa et al., 1995; Leung et al., 1997) in isolated porcine large coronary

arteries. Collectively, these data suggest that the coronary endothelial dysfunction occurring following I/R may be related to the accumulation of LPC during this process.

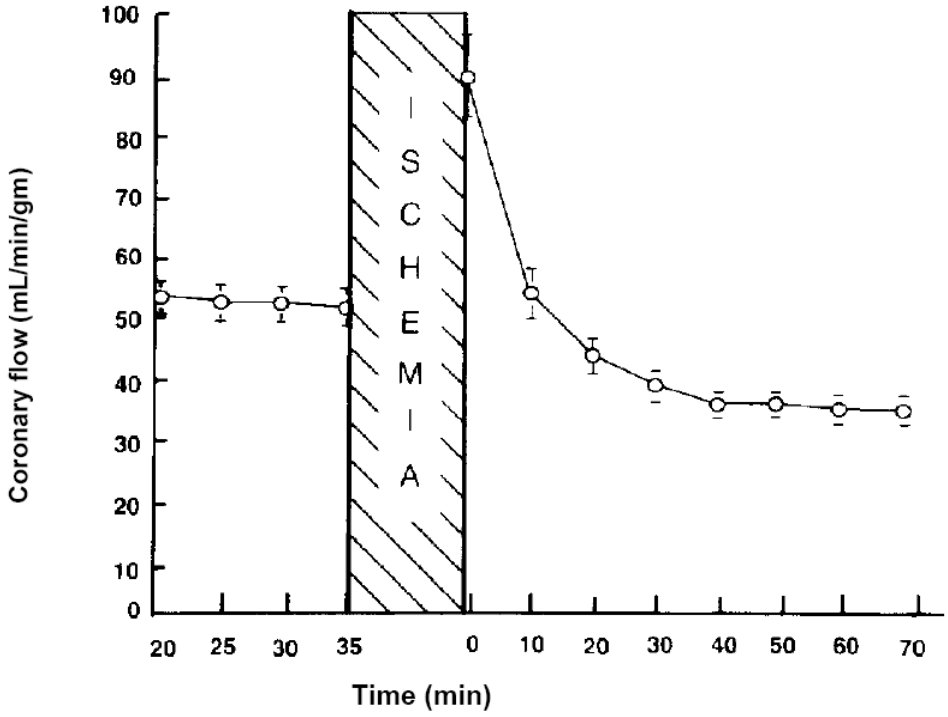

Figure 1.1 Influence of ischemia and reperfusion on coronary flow in isolated perfused rabbit heart. Figure was adapted from Hashimoto et al., 1991.

1.2 LPC metabolism

LPC is not a single homogenous entity but is composed of many molecular species with different acyl groups (Poulos et al., 1973) (Figure 1.2). LPC is a well-known intermediate in the metabolism of phosphatidylcholine (PC), which is the major lipid component of plasma but a minor phospholipid in cells and tissues (Croset et al., 2000). The major metabolic pathway of LPC is shown in Figure 1.2. LPC is produced as a result of PC hydrolysis by several isoforms of phospholipase A_2 (PLA_2). During the oxidative modification of LDL, there is extensive conversion of PC to LPC by this pathway (Sonoki et al., 2003). LPC is also generated in the reaction mediated by lecithin-cholesterol acyltransferase (LCAT) that transfers the fatty acid residue from PC to cholesterol (Subbaiah et al., 1992). Direct hepatic secretion is another important source of plasma LPC (Sekas et al., 1985). Once produced, LPC is either converted back to PC or further metabolized by the action of lysophospholipase D (LPLD) to form lysophosphatidic acid (LPA), which is also a bioactive phopholipid controlling numerous cellular responses through the activation of specific G-protein coupled receptors (GPCRs) (Tigyi and Parrill, 2003).

In addition to the LPC that exists in cell plasma, the other LPC physiological forms include free LPC, micellar LPC or LPC bound to lipoproteins, albumin, and immune complexes. The total physiological concentrations of LPC in body fluids were reported to vary between 5 and 180 μmol/L (Okajima et al., 1998; Sasagawa et al., 1998). However, LPC present in plasma is largely reversibly bounded to albumin and lipoprotein complexes, and this proportion is incapable of eliciting LPC-dependent biological responses (Vuong et al., 2001). If fact, if all the LPC at physiological

concentrations was actively mediating receptor-operated functions, the LPC receptors would be saturated and/or downregulated. The exact concentrations of free LPC, which is considered active in regulating the signaling effects of LPC, are unclear. Although the accumulation of LPC during various pathological conditions has been confirmed by a number of studies, the magnitude of the increase varies from study to study. In most previous *in vitro* biological studies, 1 – 30 μmol/L of LPC without carriers were used. Higher concentrations of LPC (> 30 μmol/L) may lyse cells due to its detergent-like properties (Jalink et al., 1990).

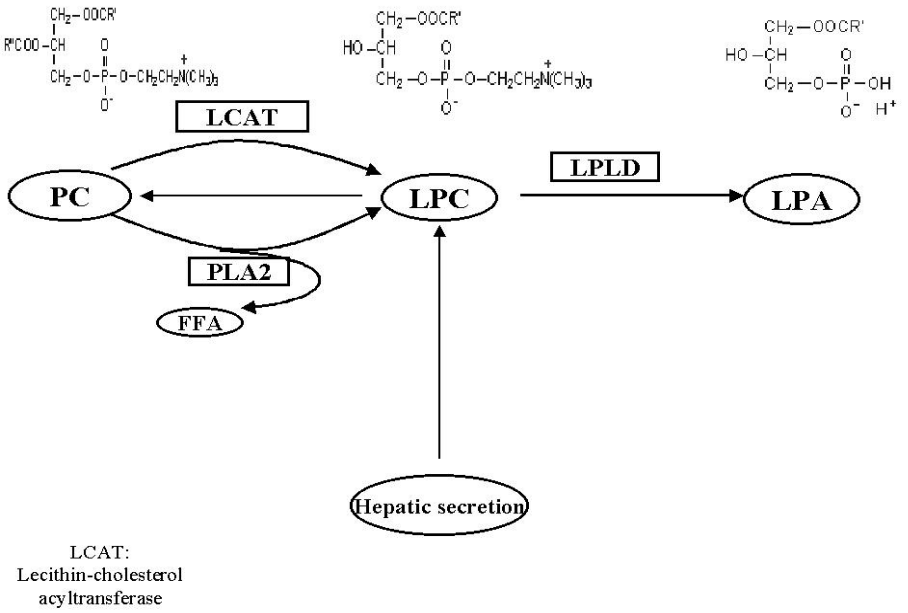

Figure 1.2 The major metabolism pathway of lysophosphatidylcholine. PC: phosphatidylcholine, LCAT: lecithin-cholesterol acyltransferase, PLA_2: phospholipase A_2, LPA: lysophosphatidic acid, LPLD: lysophospholipase D.

1.2.1 LPC-generating enzyme: PLA_2

PLA_2 controls the initial and rate-limiting step of the biosynthetic cascade from membrane phospholipids to lysolipids, arachidonic acid (AA) and the subsequent prostanoids (Figure 1.3). LPC is one of the by-products of this cascade. The other products, including prostaglandins, prostacyclin, and thromboxanes, also represent precursors of signaling molecules that can exert a variety of biological functions. The PLA_2 superfamily constitutes a heterogeneous group of enzymes, usually classified into four major groups (summarized in Table 1.2): cytosolic PLA_2 ($cPLA_2$), Ca^{2+}-dependent secretory PLA_2 ($sPLA_2$), and Ca^{2+}-independent intracellular PLA_2 ($iPLA_2$), and the platelet activating factor acetylhydrolases (PAF-AH) (Kougias et al., 2006). Each of these groups contains several members. One of the PAF-AH enzymes is GVIIA PLA_2, also known as lipoprotein-associated PLA_2 (Lp-PLA_2).

Given the tight control over these bioactive eicosanoids, PLA_2 is considered an independent risk factor for cardiovascular diseases, including atherosclerosis and coronary artery disease (Chakraborti, 2003). Among isoforms, $cPLA_2$ plays a central role in the agonist stimulated AA liberation, and the following generation of lipid mediators (Chakraborti, 2003; Leslie, 2004). On the other hand, $sPLA_2$ and Lp-PLA_2 are especially important in mediating the proinflammatory properties of LPC.

Table 1.2 PLA_2 classification according to biochemical properties

Family	Calcium requirement	Cellular localization
$sPLA_2$	Yes	Intracellular
$cPLA_2$	Yes	Extracellular
$iPLA_2$	No	Intracellular
PAF-AH(Lp- PLA_2)	No	Extracellular

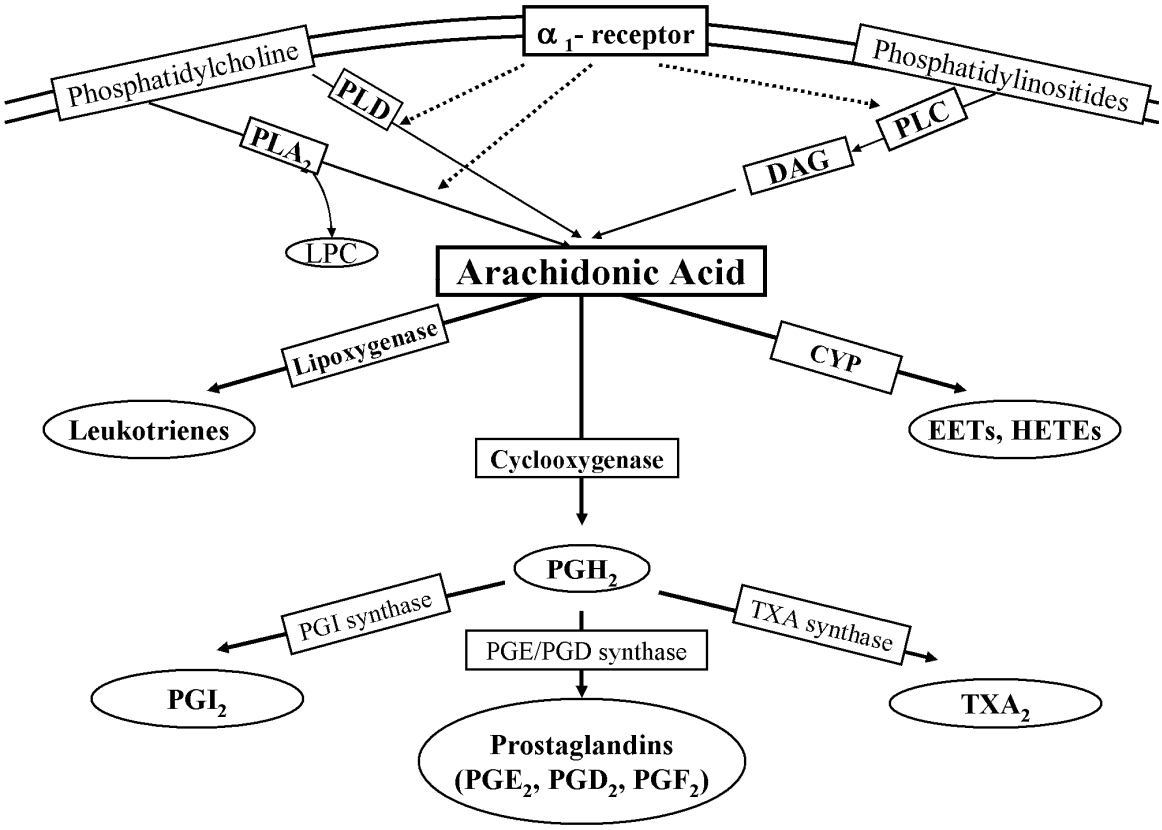

Figure 1.3 Pathways for the metabolism of arachidonic acid (AA). Within resting cells, AA resides predominantly at the C-2 position of membrane phospholipids and is released from there upon the hydrolysis of phospholipase A_2 (PLA_2) directly, or by phospholipase C (PLC) / phospholipase D (PLD) indirectly (Piomelli et al., 2000). PLA_2 - catalyzed reaction is the major source of AA and a rise in intracellular Ca^{2+} is believed to be the initial event leading to activation of PLA_2 and liberation of AA. The released AA can then be metabolized via several different pathways. In the cyclic pathway, initially AA is irreversibly converted to prostaglandin endoperoxide H_2 (PGH_2) by the enzymatic action of cyclooxygenase (COX-1 and COX-2). This is probably the rate-limiting step under most conditions. The final step is the isomerization of PGH_2 to various end-products, including TxA_2, PGI_2 and prostaglandins (PGE_2, $PGF_{2\alpha}$, and PGD_2). The lipoxygenase pathway synthesize leukotrienes. AA is also metabolized by cytochrome P450 (CYP) to epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acids (HETEs)

To date, at least 10 active mammalian $sPLA_2$ have been identified (IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XIIA) (Murakami et al., 2001; Kudo and Murakami, 2002). Of these, $sPLA_2$-II is expressed in many cell types such as ECs, SMCs, platelets, mast cells, neutrophils, macrophages, as well as in normal and atherosclerotic artery specimens (Kougias et al., 2006). Although it is reasonable to assume that $sPLA_2$-II is involved in atherosclerosis via LPC production, there has been no direct evidence to support this hypothesis. Instead, $sPLA_2$-II contributes to the pathogenesis of atherosclerosis through its non-catalytic functions, such as enhancing 15-lipoxygenase-induced LDL oxidation (Neuzil et al., 1998) and activating macrophages(Webb et al., 2003), etc.

Recently, systemic Lp-PLA_2 emerged as an independent biomarker for cardiovascular diseases (Lerman and McConnell, 2008). The majority of the circulating Lp-PLA_2 is found in the LDL lipid fraction and only a small percentage in high density lipoprotein (HDL) (Caslake et al., 2000). In fact, *in vivo*, Lp-PLA_2 is almost exclusively produced by the macrophages and foam cells from atherosclerotic plaques, and is subsequently released into the circulation, where it binds to lipoprotein (Sudhir, 2005). Therefore, Lp-PLA_2 is particularly engaged in vascular inflammation rather than systemic inflammation. Lp-PLA_2 specifically hydrolyzes the sn-2 fatty acids of oxidized phospholipids, mainly oxLDL (MacPhee et al., 1999). Macphee, et al. strongly suggested that Lp-PLA_2 was solely responsible for LPC-accumulation in oxLDL, using a selective inhibitor of Lp-PLA_2 (Macphee et al., 2005). This finding was further confirmed in an *in vivo* study showing that atherosclerotic plaque in human coronary arteries was associated with higher local coronary production of both Lp-PLA_2 and LPC, which was correlated to coronary endothelial dysfunction (Lavi et al., 2007). In summary, Lp-PLA_2 plays a

crucial role in generating LPC in plasma and stimulating the development of atherosclerosis.

1.2.2 LPC-degrading enzyme: LPLD

LPLD was found to convert LPC into LPA more than two decades ago, and later on, autotaxin (ATX) was revealed to be identical to LPLD (Tokumura et al., 2002; Umezu-Goto et al., 2002), and to function as a LPLD (van Meeteren and Moolenaar, 2007). The importance of LPLD relies more on its role being LPA-generating rather than LPC-degrading. LPA is produced and degraded mainly extracellularly through the actions of lysoPLD/ATX and lipid phosphatases, respectively (Figure 1.4). Like LPC, LPA refers to a family of lipid metabolites. As a pluripotent signaling molecule, it induces a wide range of cellular responses in multiple tissues and in diverse physiological or pathological situations. In the cardiovascular system, LPA is an activator of platelets (Gerrard and Robinson, 1984), a modulator promoting the differentiation, proliferation and migration of SMCs that are required for the development of intimal hyperplasia (Hayashi et al., 2001), and a bioactive mediator of atherothrombotic disease (Smyth et al., 2008). Exogenous administration of LPA results in acute and systemic increases in blood pressure in different animal species (Tokumura et al., 1978; Tigyi et al., 1995), suggesting a role for LPA in hypertension. The close relationship between circulating LPA levels and circulating LPLD levels suggests that LPLD expression, localization, or activity may be important in regulating bulk levels of LPA in the blood (Smyth et al., 2008).

Some of the effects of LPC have been proposed to be mediated through the formation of LPA. For instance, in rat coronary endothelial cells, the lipoprotein lipase-

augmenting property of LPC likely requires the formation of LPA (Pulinilkunnil et al., 2004). It was also suggested that LPC induces neuropathic pain through the generation of LPA and the activation of LPA receptors in a study using mice lacking a LPA receptor gene (Inoue et al., 2008). However, this hypothesis needs to be further investigated. Future studies using ATX knock out and transgenic mice or pharmacological inhibition of this enzyme would be useful to evaluate the potential relationship between the effects of LPC and LPA.

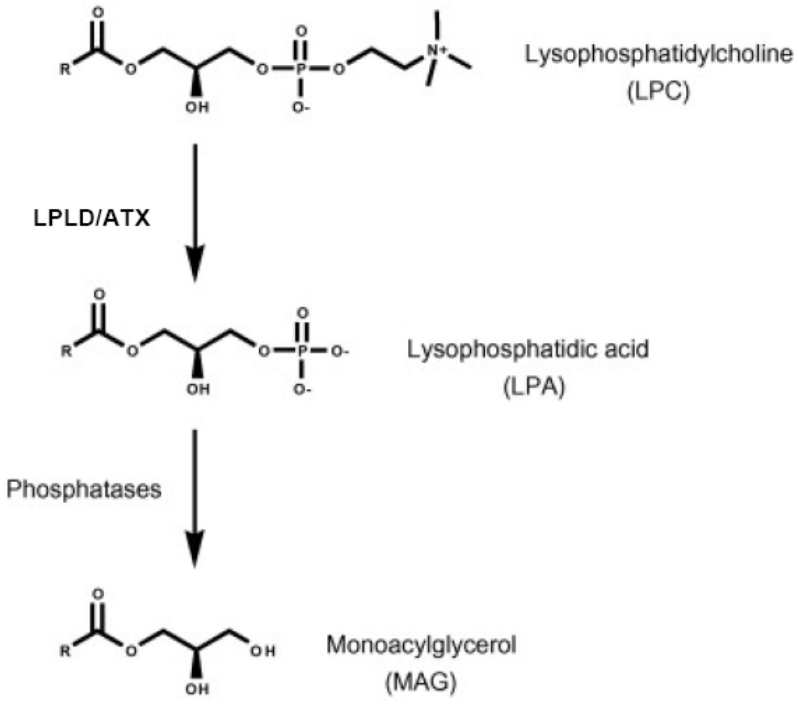

Figure 1.4 Regulation of LPA levels in the extracellular environment. LPA levels are determined by the balance between synthesis and degradation. ATX/lysoPLD is the major biosynthetic exo-enzyme, while degradation is mediated by lipid phosphatases, both membrane-associated and soluble. Figure was adapted from van Meeteran and Moolenaar, 2007.

1.3 Structure-activity relationship of LPC

As mentioned above, LPC is composed of many molecular species bearing different acyl chains. It has been realized that it is the length and saturation of these acyl chains (R_1 in Figure 1.5) that fundamentally determine the biological functions of LPC.

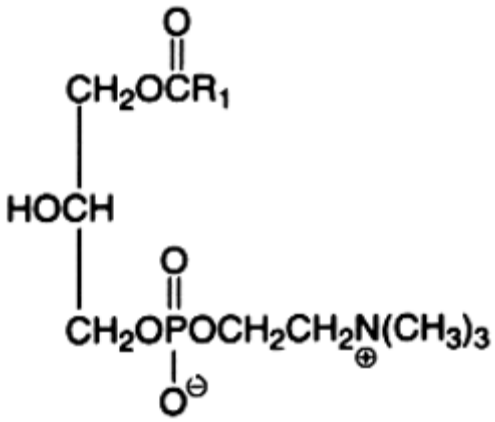

Figure 1.5 Structure of LPC. R_1 represents an acyl chain.

It was suggested that only LPC with more than 14 carbon acyl group elicits pathological effects (Fukao et al., 1996; Choy et al., 2004). In hyperlipidemic patients, it was found that LPC samples contained a higher proportion of long-chain acyl groups (>= C16:0), and it was this fraction of LPC that induced the impaired endothelium-dependent relaxation in patients (Chen et al., 1997). Acyl chain-dependent differences in the effects of LPC on neutrophils *in vitro* was also observed (Ojala et al., 2007). In this study, unsaturated LPC species produced a long-lasting superoxide generation. In contrast, saturated LPC species induced much less superoxide production, but are more potent in increasing cytoplasmic calcium and plasma membrane permeability. In human coronary artery smooth muscle cells, long and saturated chain containing LPC was reported to induce AA release more pronouncedly than the shorter and unsaturated chain containing LPC (Aiyar et al., 2007). Specifically, LPC (C16:0) produced the optimal stimulation.

Similarly, Lee et al. found that 16:0 LPC produced the most dramatic Ca^{2+} increase in HL-60 human leukemia cells compared to 14:0 and 18:0 LPCs (Lee et al., 2004). This is interesting as palmitoyl (C16:0) is the most abundant saturated LPC *in vivo* in humans and rats (Vesterqvist et al., 1992; Croset et al., 2000; Cunningham et al., 2008). More importantly, following LDL oxidation, 16:0 LPC was still the prevalent fraction present in atherosclerotic aorta compared with normal human aorta (Thukkani et al., 2003) (Table 1.3). These data suggest that LPC species containing long and saturated acyl chain (especially 16:0) are particularly important under both physiological and pathological conditions.

Regarding the mechanisms of this acyl chain-dependence of the effects of LPC, it was suggested that different critical micelle concentrations of the various species can barely explain this structure-activity relationship (Ojala et al., 2007). In another study, it was shown that LPC species with longer acyl chain have higher binding affinity for their receptor G2A (Kabarowski et al., 2001). However, the physiological significance of this selective binding remains to be defined.

Table 1.3 Accumulation of LPC molecular species in human atherosclerotic aorta.

LPC molecular species	Normal Aorta		Atherosclerotic Aorta	
	Average ± SEM	No. of patients	Average ± SEM	No. of patients
16:0	0.83 ±0.45	3	30.1 ±9.6	10*
18:0	0.62 ±0.31	3	22.0 ±7.6	10*
18:2	0.23 ±0.14	4	8.0 ±2.2	10†
20:4	0.22 ±0.15	4	4.4 ±1.1	10†
22:6	ND	4	0.9 ±0.2	10

Lipids from normal and atherosclerotic human aorta samples (50 mg) were extracted into chloroform in the presence of 14:0 lysophosphatidylcholine and quantitatively analyzed by ESI-MS for lysophosphatidylcholine molecular species. Values are expressed as picomolar of each lysophosphatidylcholine molecular species per nanomole of inorganic phosphate. ND indicates not detectable. *P<0.01 and †P<0.005, respectively, for comparisons between normal and atherosclerotic aorta for each molecular species using the Student t test. Adapted from (Thukkani et al., 2003).

1.4 Endothelium-dependent regulation of vascular tone

Vascular tone is the major determinant of peripheral vascular resistance. Defective vascular tone is an essential feature of cardiovascular diseases such as hypertension, coronary heart disease and atherosclerosis. Vascular tone is regulated by various extrinsic and intrinsic factors acting on the blood vessel. These factors work in an integrated way to control systemic vascular resistance/blood pressure, and regulate local blood flow within organs. In general, these factors can be divided into vasoconstrictors and vasodilators that competitively determine the vascular tone. Many of these vasoactive factors are derived from the vascular endothelium.

The endothelium is the single layer of cells that lines the luminal surface of blood vessels and plays a primary autocrine/paracrine regulatory role in the regulation of cardiovascular homeostasis, including vascular tone control, immune and inflammatory responses, cellular adhesion processes, and vascular remodeling (Grover-Paez and Zavalza-Gomez, 2009). The endothelium functions by secreting various vasoconstricting and vasodilating factors in response to circulating substances that bind to specific endothelial receptors and to mechanical stimuli such as fluid shear stress and pulsatile stretch (Schalkwijk and Stehouwer, 2005).

The healthy endothelium has an intact balance between the release of endothelium-derived relaxing factors (EDRF) and endothelium-derived constricting factors (EDCF). Many cardiovascular risk factors, such as smoking, aging, diabetes, hypertension, shear stress, and oxidative stress, may trigger the development of endothelial dysfunction, resulting in vascular remodeling, loss of antithrombotic factors, increase in vasoconstrictor and prothrombotic products, in addition to abnormal

vasoreactivity (Figure 1.6) (Hadi et al., 2005). Importantly, endothelial dysfunction is associated with most forms of cardiovascular disease, such as hypertension, atherosclerosis, coronary artery disease, chronic heart failure, peripheral artery disease, diabetes, and chronic renal failure, and has been identified as a common link for all cardiovascular risk factors (Endemann and Schiffrin, 2004).

Since endothelial functions are multiple and involve a variety of tissues and organs, any alterations in endothelial function may influence one or more of them either concurrently or separately. In the context of this dissertation, endothelial dysfunction specifically refers to potentiated vasoconstriction and/or impaired endothelium-dependent relaxation (EDR), as a result of increased EDCF and/or reduced bioavailability of EDRF.

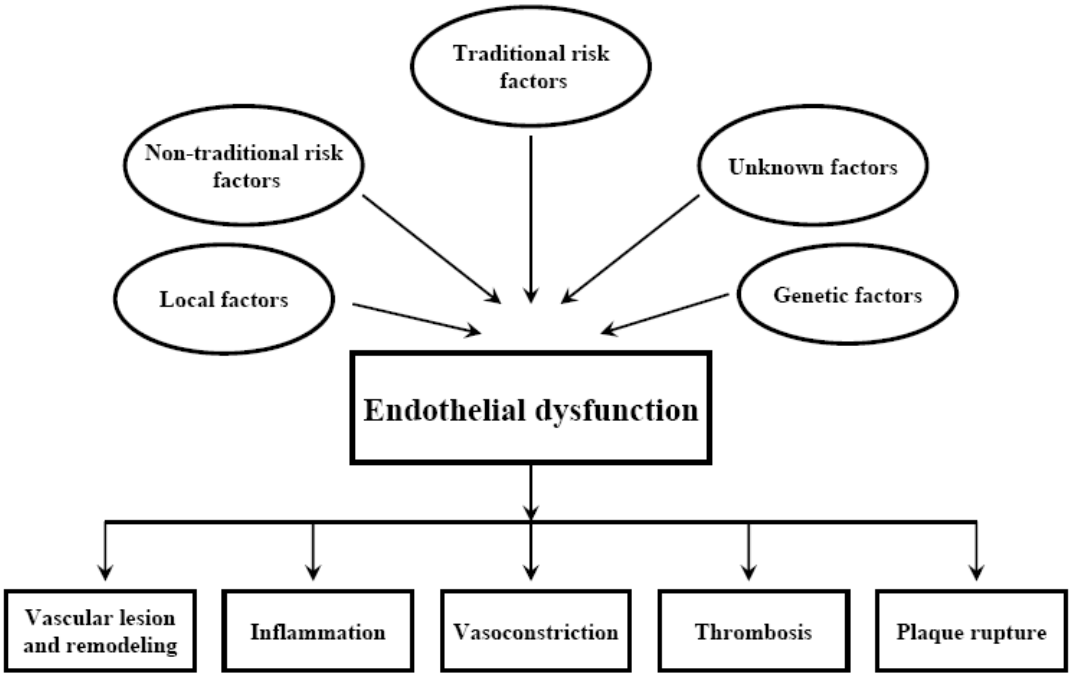

Figure 1.6 The various factors that affect the endothelium and the consequences of endothelial dysfunction (Hadi et al., 2005)

1.4.1 Endothelium-derived relaxing factors (EDRF)

The most important vasodilators released from endothelium are: nitric oxide (NO), prostacyclin (PGI_2), and endothelium-derived hyperpolarizing factor (EDHF). These substances act in a coordinated and interactive manner to maintain normal endothelial function and operate as support mechanisms when one pathway malfunctions. These three mediators are not always equally important – NO normally predominates in large arteries, whereas EDHF takes over in smaller blood vessels or in large arteries when the release of NO is impaired (McGuire et al., 2001).

NO is the best characterized and probably the most important EDRF. It originates from the transformation of the amino acid L-arginine into citrulline in the presence of NO synthase (NOS) that is present in endothelial cells (Figure 1.7). NO is produced and released either basally or under the influence of agonists, such as acetylcholine, bradykinin, substance P, serotonin and others, acting on specific endothelial receptors, and by mechanical forces (Luscher et al. 1990). After release from the endothelial cells, NO diffuses into the underlying smooth muscle cells and exerts its action. The mechanism is showed in Fig. 1.7. NO activates soluble guanylyl cyclase (GC), leading to the formation of cyclic guanosine monophosphate (cGMP), and the subsequent activation of cGMP-dependent protein kinase G (PKG). PKG either inhibits the Ca^{2+} influx or suppresses the sensitivity of contractile proteins to Ca^{2+}, causing the relaxation of smooth muscle (Stankevicius et al., 2003). Basal NO-release is known to occur in both conduit and resistance vessels. Under physiological conditions, two constitutive forms of NOS, ie. endothelial NOS (eNOS) and neuronal NOS (nNOS) play a role in NO production. The activation of another isoform of NOS, inducible NOS (iNOS), results in excess NO

production and is considered to contribute to many pathological conditions. In addition, NO can also influence the circulatory system through other mechanisms, including inhibition of leucocyte adhesion 1, antithrombic activity and as a modulator of growth factor signaling (Viljoen, 2008).

PGI_2 is one of the major AA metabolites. Briefly, AA is liberated by PLA_2 and subsequently converted to intermediates prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2) by cyclooxygenase (COX) isozymes. PGG_2 and PGH_2 are then further metabolized by individual synthases to form an array of eicosanoids, specifically, PGI_2 synthase for PGI_2 (Figure 1.3). Recently it was realized that PGI_2 is mainly a COX-2 catalyzed product (Arehart et al., 2007). PGI_2 elicits biological functions by activating its specific cell-surface receptors (IP) that are G-protein-coupled to adenylate cyclase (AC) and thereby elevate cyclic adenylate monophosphate (cAMP) levels (Figure 1.7). In addition to being a vasodilator, PGI_2 is also a potent inhibitor of platelet aggregation (Moncada et al., 1976; Moncada et al., 1977), and has been found to prevent atherosclerosis and thrombosis (Grosser et al., 2006; Fetalvero et al., 2007). PGI_2 analogues are currently being used to treat pulmonary hypertension (Humbert et al., 2004), and are likely to be effective in the management of atherothrombosis (Arehart et al., 2007).

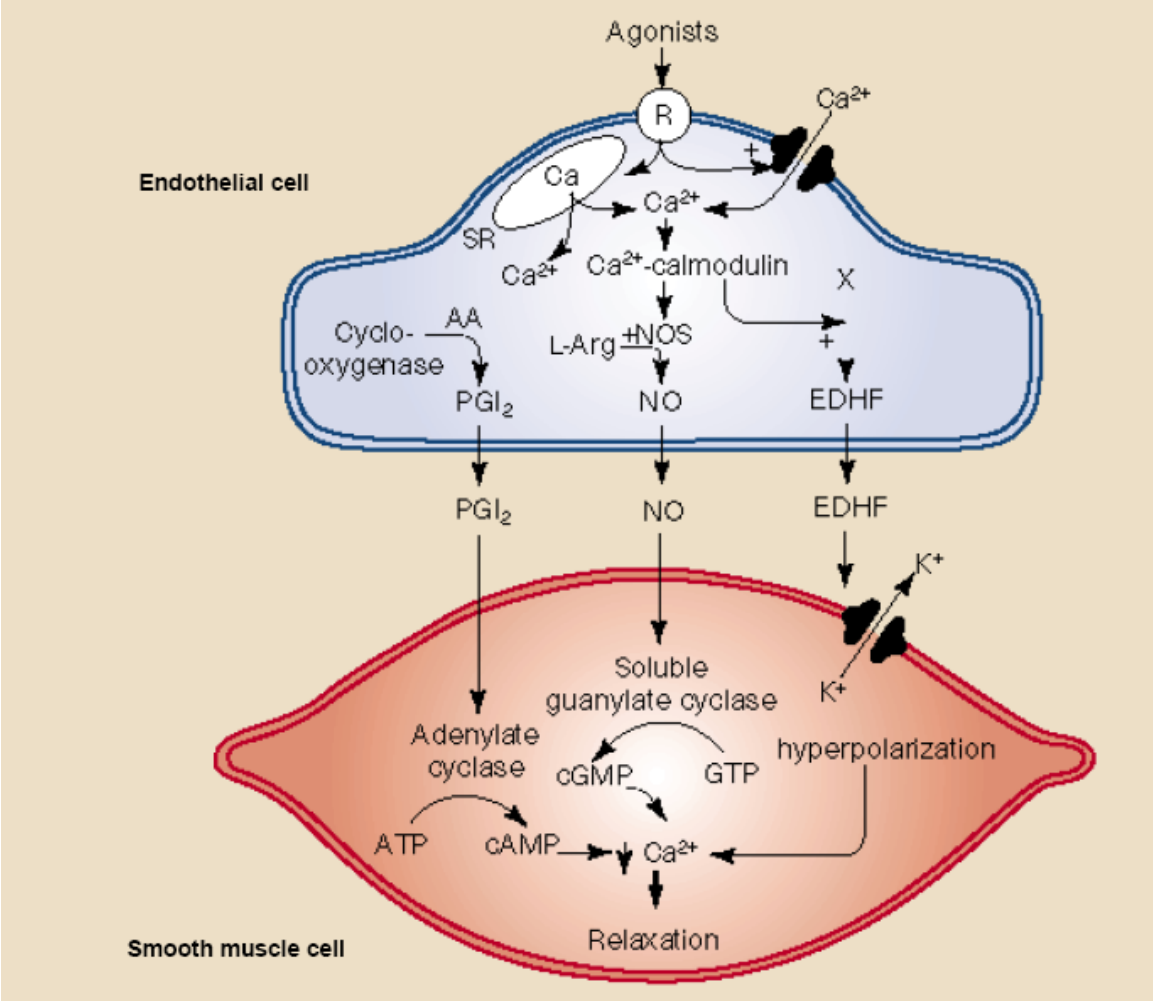

Figure 1.7 Relaxation of vascular smooth muscle cells by diffusable vasodilator substances from endothelial cells. Nitric oxide (NO) activates soluble guanylate cyclase, yielding increased levels of cyclic GMP (cGMP). Prostacyclin (PGI_2) activates adenylate cyclase, leading to increased production of cyclic AMP (cAMP). Endothelium-derived hyperpolarizing factor (EDHF) causes Ca^{2+}-dependent K^+ channels in vascular smooth muscle cells to open, leading to their hyperpolarization. AA, arachidonic acid; NOS, NO synthase; L-Arg, L-arginine; R, membrane receptor; SR, sarcoplasmic reticulum; X, unknown precursor. Aadapted from Vanhoutte, 1998.

EDHF refers to the endothelium-derived vasodilator that enables NO/PGI_2-independent relaxation. Despite its uncertain identity, EDHF has been shown to contribute to the regulation of vascular tone in various blood vessels from different species (Chen et al., 1988; Feletou and Vanhoutte, 1996; Ge et al., 2000). Based on the findings that EDHF is abolished by a combination of apamin and charybdotoxin, inhibitors of small- and medium-conductance K_{Ca} channels, respectively (Edwards and Weston, 1998; Fisslthaler et al., 1999), it is generally believed that the generation of EDHF within the ECs relies on an increase in the intracellular Ca^{2+} concentration, and the subsequent activation of Ca^{2+}-sensitive K^+ (K_{Ca}) channels. This results in the generation of EC hyperpolarization and/or the release of chemical factors, which are then transmitted to the SMCs to evoke hyperpolarization and vasodilation. There is a substantial species and tissue variation in the nature of EDHF. Leading candidate molecules for EDHF include: 1) epoxyeicosatrienoic acids (EETs), AA metabolites from P450 monooxygenase pathway (Figure 1.3) that hyperpolarizes SMCs by activation of large conductance K_{Ca} channels (Campbell and Gauthier, 2002; Fleming, 2007); 2) K^+ ions (McGuire et al., 2001; Bussemaker et al., 2002); 3) electrical communication through myoendothelial gap junctions (Chaytor et al., 1997; Fleming, 2000; Sandow and Hill, 2000); 4) hydrogen peroxide (H_2O_2) (Matoba et al., 2000; Miura et al., 2003; Shimokawa and Matoba, 2004); 5) C-type natriuretic peptide (CNP) (Ahluwalia and Hobbs, 2005). The putative mechanisms of some of these candidates are summarized in Figure 1.8.

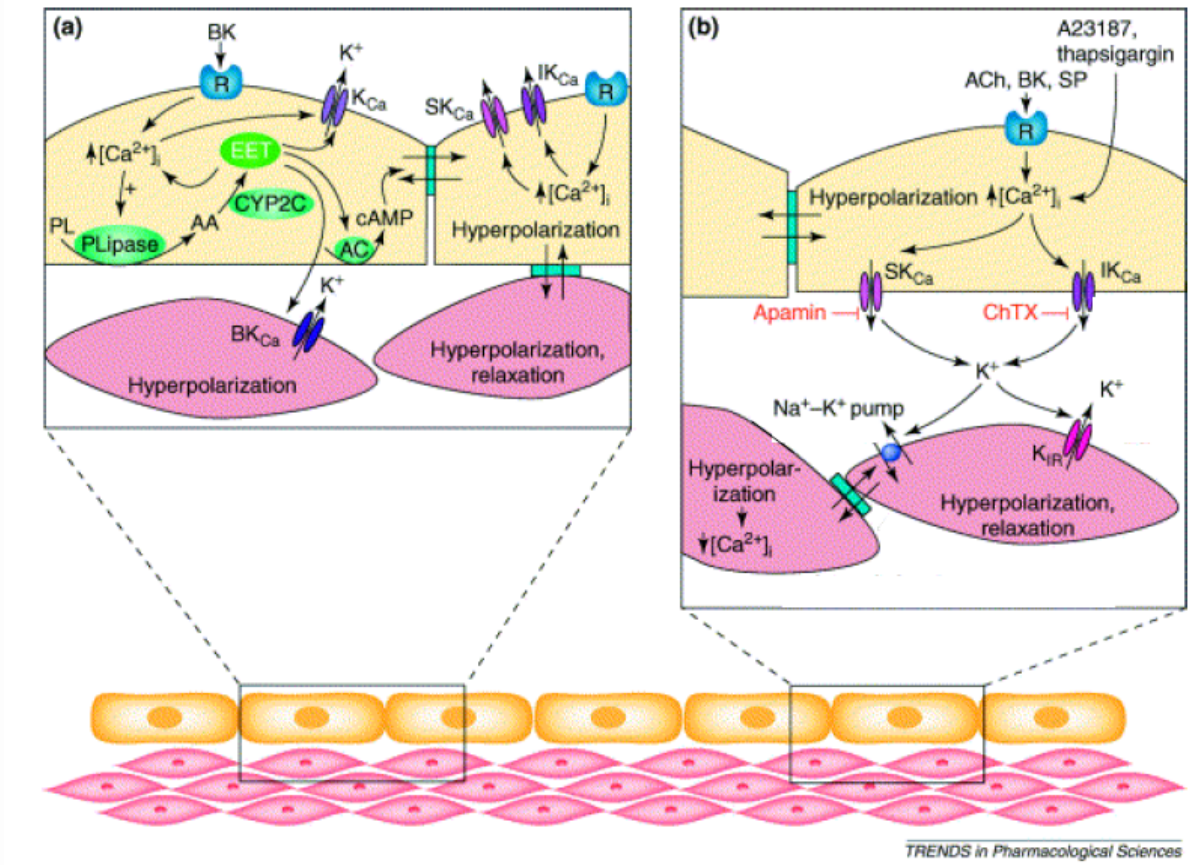

Figure 1.8 Proposed mechanisms of smooth muscle cell hyperpolarization. (a) Epoxyeicosatrienoic acids (EETs) might act as both intracellular and extracellular messengers. EETs affect Ca^{2+} signalling, the Ca^{2+} sensitivity of K_{Ca} channels and the generation of cAMP by AC, as well as gap junctional coupling in the endothelial cells. EETs and/or their metabolites can also diffuse to SMCs and activate large-conductance K_{Ca} channels (BK_{Ca} channels). (b) The role of K^+ ions. Endothelial cell stimulation by various agonists initiates endothelial cell hyperpolarization by activating small- and intermediate-conductance K_{Ca} channels (SK_{Ca} and IK_{Ca} channels). Subsequently, K^+ ions accumulate in the sub-endothelial space in concentrations sufficient to activate inwardly rectifying K^+ (K_{IR}) channels and/or the Na^+--K^+ pump. The hyperpolarization of the endothelial cells, following K_{Ca} channel activation, could also be transmitted along the monolayer of endothelial cells or towards the smooth muscle cells through gap junctions. Adapted from Busse et al., 2002.

1.4.2 Endothelium-derived constricting factors (EDCF)

Endothelin-1 (ET-1) secreted by the endothelium is one of the most potent vasoconstrictors. The release of ET-1 results in activation of two receptors: ET_A and ET_B. Activation of ET_A and ET_B receptors on SMCs results in a sustained constrictor action of ET-1. The ET_B receptors on the endothelium mediate the release of the dilators NO and PGI_2, as well as the rapid uptake of ET-1 (Haynes and Webb, 1998). Therefore, the endothelial ET_B receptor largely opposes the vascular effect of SMC-located $ET_{A/B}$ receptors. In addition to the direct vascular effects, ET-1 induces vascular smooth muscle cell proliferation and growth in a dose-dependent manner (Komuro et al., 1988).

Angiotensin II (Ag II) is one of the major systemic pressor hormones. Renin, originating from the kidney, acts on angiotensinogen to form angiotensin I, which is then cleaved by the angiotensin converting enzyme (located primarily in vascular endothelium) into Ag II, which binds to its specific receptors on the vascular wall. Two subtypes of the Ag II receptor, designated AT_1 and AT_2, have been identified. The smooth muscle cell-localized AT_1 receptor subtype mediates the predominant action of Ag II: vasoconstriction. These vasoactive actions are partly counteracted by the AT_2 receptor, which causes vasodilatation in some vascular beds from rats and human (Carey et al., 2001; Dimitropoulou et al., 2001; Batenburg et al., 2004). The renin-angiotensin system plays an important role in regulating blood volume and systemic vascular resistance. Excess circulating Ag II can cause myocardial infarction (Gavras and Gavras, 2002).

Thromboxane A_2 (TxA_2) is produced by thromboxane synthase on PGH_2 mainly in activated platelets and macrophages where this enzyme is highly expressed (Fig 1.3). In addition, the endothelium is also a site of origin (Ally and Horrobin, 1980). The

primary control of TxA_2 formation depends largely on the availability of thromboxane synthase (TxAS) and PGH_2 substrate. No endogenous factors have been shown to modulate TxAS activity. The physiological role of TXA_2 is platelet aggregation, vasoconstriction and bronchoconstriction (Oates et al., 1988b; Oates et al., 1988a). It is well documented that TxA_2 receptors (TPs) are able to activate a multitude of different signalling cascades depending on the specific organ systems where TPs are expressed. They are known to couple to at least nine G proteins, $G\alpha_q$, $G\alpha_{i2}$, $G\alpha_s$, $G\alpha_{11}$, $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_{15}$, $G\alpha_{16}$, and G_h (Halushka, 2000), which in turn activate numerous downstream effectors, including second messenger systems such as inositol trisphosphate (IP_3)/ diacylglycerol (DAG), cAMP, small G proteins (Ras, Rho), PI3 kinase, as well as PKC and PKA (Huang et al., 2004). Interestingly, as the precursor of TxA_2, PGH_2 can also act as a vasoconstrictor through the same receptors: TPs (Mais et al., 1985; Ogletree et al., 1985). An overproduction of TxA_2 has been detected in a series of diseases, such as myocardial infarction, hypertension, thrombosis, atherosclerosis and asthma, whereby this prostanoid is assumed to contribute to the underlying pathomechanisms by its potent stimulation of platelet aggregation and smooth muscle contraction (Dogne et al., 2004).

Reactive oxygen species (ROS) include free radicals, hydroxyl radicals ($OH\cdot$), superoxide (O_2^-), peroxynitrite ($ONOO^-$) and nonradical derivatives, such as hydrogen peroxide (H_2O_2) (Figure 1.9). All components of the vascular wall, including ECs, can produce ROS. In VSMCs and ECs, NADH/NADPH oxidases represent the most important source of O_2^- (Pagano et al., 1995; Griendling et al., 2000). NADPH oxidase catalyzes the NADPH-dependent reduction of oxygen to O_2^-, which in turn leads to the production of secondary derivatives such as $OH\cdot$ and H_2O_2. ROS production is

counterbalanced by antioxidant enzymes such as superoxide dismutases (SOD), catalase, glutathione peroxidase, thioredoxins, and peroxiredoxins. Low levels of ROS are necessary for normal vascular functions. Excess accumulation of ROS occurs in response to both mechanical and biochemical factors associated with many pathological conditions, such as atherosclerosis (Ohara et al., 1993), diabetes (Baynes and Thorpe, 1999), and cardiac I/R (Becker, 2004).

Importantly, the enhanced production of ROS in turn leads to multiple consequences for endothelial function. The reaction $O_2^{\cdot -} + NO \rightarrow ONOO^{\cdot -}$ is widely considered to be noxious in cardiovascular disease due to the depletion of endogenous NO, as well as decreased production of endothelial hyperpolarizing factor H_2O_2, and thus impaired vasodilation (Bauersachs et al., 1996; Wei et al., 1996). In addition, as a potent oxidant, $ONOO^-$ *per se* is intrinsically toxic. ROS also directly inhibit the main target of NO, soluble GC (Munzel et al., 2005), and inactivate PGI_2 synthase (Zou et al., 2004). On the other hand, ROS directly participates in vasoconstriction by releasing isoprostanes, which are a family of substances produced from AA via a free radical-catalyzed, and COX-independent mechanism (Patrono et al., 2005), and which are recognized as an indicator of oxidative stress (Morrow and Roberts, 1996). Isoprostanes act as partial agonists at the TxA_2 receptor and exert numerous biological effects, including vasoconstriction and platelet activation (Montuschi et al., 2004). In addition to the noted effects on vasomotor balance, ROS favor macrophage infiltration of the vessel wall, and SMC proliferation, migration, and apoptosis (Papaharalambus and Griendling, 2007).

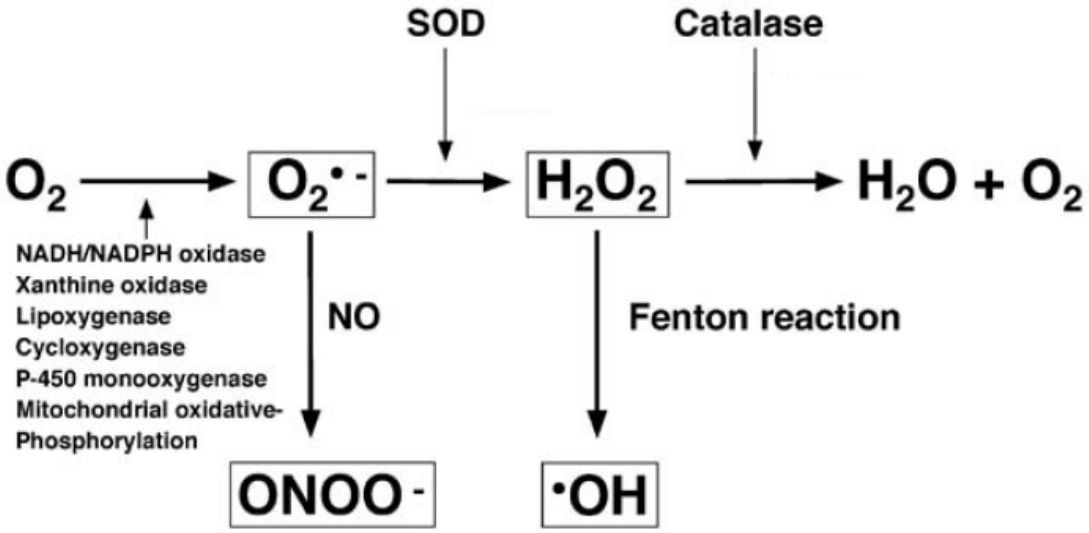

Figure 1.9 Reactive oxygen species in vascular wall. Multiple enzymes may induce ROS generation in cardiovascular cells which these include NADH/NADPH oxidase, xanthine oxidase, lipoxygenase, cyclooxygenase, P450 monooxygenase, and the enzymes of mitochondrial oxidative phosphorylation ($O_2^{-}\cdot$, superoxide anion radical; H_2O_2, hydrogen peroxide; ·OH, hydroxyl radical; ONOO-, peroxynitrite; SOD, superoxide dismutase). Figure was adapted from Yoshizumi et al., 2001.

1.5 Effects of LPC on intact blood vessels

The direct vasoactive effects of LPC have been studied in isolated vessel strips *ex vivo* and *in vivo*. Within the physiological and pathophysiological concentration range, LPC alone has no significant influence on vascular tone. In addition, LPC does not alter the vasoconstriction resulting from K^+-induced direct depolarization of vascular smooth muscle. The consensus from the majority of these studies is that LPC potentiates receptor-operated vasodilator and vasoconstrictor responsiveness in conduit arteries. For instance, in large artery ring preparations (from rats, rabbits, cattle, and pigs), LPC impairs EDR (Fukao et al., 1995; Froese et al., 1999; Vuong et al., 2001), probably by inhibiting EDHF and NO (Eizawa et al., 1995; Froese et al., 1999; Makino et al., 2000). Some agonist-induced contractions in isolated blood vessels were also potentiated by LPC, including contractile responses induced by UK14,304 (5-bromo-*N*-(2-imidazolin-2-yl)-6-quinoxalinamine, an α_2-adrenoceptor agonist) (Suenaga and Kamata, 2003), and Ag II (Galle et al., 2003; Suenaga and Kamata, 2003).

1.6 Effects of LPC on ECs nd SMCs

The effects of LPC in modulating vascular contractility may be partially explained by the results of studies in cultured endothelial and vascular smooth muscle cells, where LPC was shown to activate protein kinase C (PKC) (Kohno et al., 2001), increase intracellular Ca^{2+} (Terasawa et al., 2002), and activate MAP kinase in rat vascular SMCs (Yamakawa et al., 1998) or inhibit MAP kinase in bovine aortic ECs (Rikitake et al., 2000b). One of the inactivation mechanisms of vascular smooth muscle myosin phosphatase, the RhoA pathway, was reported to be stimulated by LPC and/or oxLDL as well (Galle et al., 2003). In addition, LPC-induced inhibition of the delayed rectifier K+ current in coronary SMCs may also be responsible for the enhanced vascular tone (Yeon et al., 2001).

LPC was found to promote the generation of ROS in intact blood vessels (Ohara et al., 1994), ECs (Kugiyama et al., 1999; Takeshita et al., 2000) and SMCs (Yamakawa et al., 2002). Interestingly, ROS are known to diminish vasodilation as discussed above, and enhance vasoconstrictor mechanisms, including the TxA_2 signaling pathway (Wilcox, 2002). Therefore, it would be reasonable to speculate that the effects of LPC are related to increased oxidative stress by LPC.

Both LPC and AA are the products of PC hydrolysis by PLA_2. Depending on the tissue, LPC has been shown to stimulate the release of AA and its metabolites by various mechanisms, such as increasing $[Ca^{2+}]_i$ and activating PKC (Rustenbeck and Lenzen, 1989; Wong et al., 1998; Golfman et al., 1999). Furthermore, LPC may also interfere with the individual production of AA metabolites, such as enhancing or inhibiting the synthesis of PGI_2 (Zembowicz et al., 1995b; Mahfouz and Kummerow, 2001).

1.7 Involvement of LPC receptors in the vasoactive effects of LPC

In recent years, two G-protein coupled receptors, G2A and GPR4, have been identified as the specific receptors for LPC (Kabarowski et al., 2001; Zhu et al., 2001; Xu, 2002). These receptors mediate intracellular responses to their ligands via heterotrimeric G proteins (Kabarowski et al., 2002; Xu, 2002). They use classic signaling pathways that have been examined in a variety of cell types, including: G_q → phopholipase C (PLC) → calcium mobilization; $G_{12/13}$ → Rho → actin rearrangement; G_i → extracellular signal-related kinase (ERK), phosphoinositide3kinase (PI3) and inhibition of adenylate cyclase (AC); G_s → AC → cAMP → protein kinase A (PKA). Some of the biological effects of LPC are suggested to be mediated by G2A and GPR4, but it is still unclear whether the vascular tone regulating effects are also through its specific GPCRs.

G2A and GPR4 were initially cloned in immune and tumor cells (Kabarowski et al., 2001; Zhu et al., 2001; Xu, 2002). Subsequently, Rikitake et al. found that G2A was expressed in human and murine monocytes/macrophages within atherosclerotic lesions, and may play a role in the LPC-initiated development of atherosclerotic lesions (Rikitake et al., 2002). This was further supported by the finding that in G2A-deficient LDL receptor knock out mice, the atherosclerotic lesion progression and intimal macrophage accumulation were significantly suppressed (Parks et al., 2006). The other studies in this area revealed that G2A is mainly involved in immune regulation (Radu et al., 2004; Ikeno et al., 2005; Yang et al., 2005).

On the other hand, GPR4, but not G2A, was detected in human microvascular endothelial cells (Lum et al., 2003; Kim et al., 2005). Furthermore, recent publications link GPR4 to endothelial function. It was found that tumor necrosis factor-α and H_2O_2

stimulation increased both GPR4 expression and $^{[3H]}$LPC binding to endothelial cells. Therefore, it was proposed that GPR4 may be responsible for LPC-induced proinflammatory activities (Lum et al., 2003). Subsequently, Lum's group provided strong evidence that LPC-mediated endothelial barrier dysfunction was regulated by endogenous GPR4 by showing that GPR4 knock-down prevented the endothelial stress fiber formation and RhoA activation in response to LPC (Qiao et al., 2006). It was also shown by another group that GPR4 over-expression in a rat endothelial cell line enhanced LPC-induced expression of adhesion molecules (Zou et al., 2007). The expression of GPR4 in smooth muscle cells has been investigated to a much less extent. To our knowledge, the only positive evidence is that the GPR4 mRNA level was found to be greater in aortic smooth muscle cells from type II diabetic rats than in those from control rats, but there was no direct link between this receptor upregulation and the LPC-induced aorta hyper-reactivity (Matsumoto et al., 2006).

In summary, LPC-specific receptors seem to be at least partially responsible for the proinflammatory and proatherogenic properties of LPC. Other physiological and pathophysiological roles of G2A and GPR4 in the vascular system remain unclear. Given that only GPR4 is found in vascular tissues, this subtype is more likely to be involved in the observed effects of LPC on vascular tone regulation. Unfortunately, nearly nothing has been done in this particular area. One of the major obstacles in LPC research is a lack of selective pharmacological inhibitors of LPC receptors. Nevertheless, antibodies of LPC receptors have been used to effectively block the actions of LPC in isolated mouse neutrophils, as well as *in vivo* in septic mouse models (Yan et al., 2004). This study has bought up a new opportunity to study the effects of LPC in the context of its

receptors. GPR4-specific antibodies would be a powerful tool to determine the contribution of GPR4 to the vasoactive effects of LPC. In addition, transgenic techniques have not been widely used in exploring the role of LPC in regulating vascular tone and blood pressure. In future studies, GPR4-deficient animal models as well as overexpression of LPC degrading enzyme PLD would be very valuable to fill in the gap in this field.

1.8 Rationale and hypothesis of the study

1.8.1 LPC control of vascular tone in isolated rat mesenteric arterial bed

Based on the current knowledge that LPC produces altered vascular reactivities, as well as the fact the LPC levels are elevated tremendously in various cardiovascular diseases, it is reasonable to assume that LPC is an important mediator that regulates the altered vascular tone that is associated with these diseases. Although it has been extensively studied, the contribution of LPC in regulating vascular resistance has not been completely elucidated, as the majority of previous studies have used either large blood vessels (aorta and superior mesenteric artery) or isolated cells. The mesenteric arterial bed (MAB) is an important effector organ regulating blood pressure, with small structural and functional changes eliciting significant alterations in peripheral resistance (Caveney et al., 1998), and it has never been used in the investigation of LPC. Therefore, the first stage of our study was undertaken to determine the vasoactive effects of LPC in the isolated perfused MAB and to investigate the underlying mechanisms of the changes it produced. *We hypothesized that LPC potentiates vasoconstrictor responses, and impairs endothelium-dependent relaxation in resistance vasculature.*

In the isolated perfused MAB, our specific objectives were to determine:

1) The effect of LPC and its washout on the basal perfusion pressure.
2) The effect of LPC and its washout on KCL-induced contractile responses.
3) The effects of LPC and its washout on PE-induced contractile responses and Ach-induced relaxant responses.
4) The involvement of major vasodilating and vasoconstricting factors in the effects of LPC.
5) The effect of LPA on perfused rat MAB, and whether the conversion from LPC to LPA is involved in the mechanisms of the effects of LPC.

1.8.2 LPC control of vascular tone in rat coronary arteries

It has been realized that coronary vasculature is a major target of cardiac I/R and of its treatment. In order to develop more effective clinical agents to prevent the "no-flow" phenomenon that happens at the microvascular level after coronary artery occlusion and reperfusion, studies are needed to investigate the mechanisms responsible for this endothelial injury. As mentioned above, prior studies suggest that changes in LPC levels during I/R could lead to impaired restoration of spontaneous circulation, and thus contribute to myocardial damage due to ischemia and reperfusion. Nevertheless, little data is available regarding the effects of LPC on the contractile responsiveness of small coronary arteries; and it is still unclear how LPC produces inhibition of coronary vascular relaxation. Consequently, how LPC contributes to the increased coronary resistance observed in I/R is not understood.

Our first study of LPC in isolated MAB demonstrated that in addition to its direct effect on the rat mesenteric arterial bed, LPC exerts residual effects on vascular reactivity long after washout of this lysolipid. Specifically, there was a robust potentiation of the

vasoconstrictor response to phenylephrine. Should this residual effect of LPC in modulating responses to vasoconstrictors also be evident in the coronary vasculature, cardiac injury would be expected to be accelerated when added onto the prevailing cardiomyocyte injury. Therefore, we next aimed to focus on the vasoactive effects of LPC on rat coronary arteries, including the coronary arterial bed in intact rat hearts, isolated rat septal coronary arteries, and cultured coronary arterial endothelial cells. *Our hypothesis is that in rat coronary arteries, LPC adversely alters both contractile and relaxant responses via modulating endothelium-dependent vasoactive factors.*

Our specific objectives were to determine:

1) The effects of I/R on coronary perfusion pressure and ventricular function in isolated perfused rat hearts.
2) The effects of LPC and its washout on coronary perfusion pressure and ventricular function in isolated perfused rat hearts.
3) The effects of LPC and its washout in isolated rat septal coronary arteries.
4) The effect of LPA on coronary arteries, and whether the conversion from LPC to LPA is involved in the mechanisms of the effects of LPC.
5) The involvement of endothelium in the observed effects of LPC.
6) The contribution of individual endothelium-dependent vasoactive factors by selective pharmacological inhibitors in intact coronary arteries.
7) The measurements of individual endothelium-dependent vasoactive factors in cultured coronary arterial endothelial cells treated with LPC and its washout.

2. MATERIALS AND METHODS

2.1 Animals

Male Wistar rats (300-400 g) used in the experiments were obtained from the Animal Care Center, UBC. Animals were pair-housed under a 12 h light:12 h dark regime, and given free access to standard rat chow and water. The investigation conforms to the guidelines for the care and use of laboratory animals published by the US NIH and the Canadian Council of Animal Care.

2.2 Methods

2.2.1 Isolated perfused rat mesenteric arterial bed

Male Wistar rats (300–400 g) were anesthetized with sodium pentobarbital (60 mg/kg i.p.), and the superior mesenteric arterial bed (MAB) was isolated as described previously (He and MacLeod, 2002). In brief, the abdominal cavity was opened, and the superior mesenteric artery was cannulated through an incision at its confluence with the dorsal aorta (Figure 2.1). The whole MAB was then separated by cutting close to the intestinal border. The MAB was flushed with heparinized warm Krebs bicarbonate buffer of the following composition: 113 m mol/L NaCl, 4.7 mM KCl, 11.5 mM glucose, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, and 25.0 mM $NaHCO_3$. The pH of the buffer following saturation with a 95% O_2, 5% CO_2 gas mixture was 7.4. Subsequent to flushing, the MAB was transferred into a jacketed organ chamber and perfused through the cannula with Krebs buffer maintained at 37°C and gassed with 95% O_2, 5% CO_2 (Figure 2.2). The perfusion rate was kept constant at 3 ml/min using a peristaltic pump (Buchler Instruments, Buchler Fort Lee, NJ). The perfusate flowed out through the cut ends of the mesenteric arterial bed. Vascular responses were detected as changes in

perfusion pressure, and this was continuously measured and recorded using a pressure transducer (PD23ID; Gould, Statham, CA) placed between the perfusate and the cannula and connected to a Grass polygraph (model 79D; Grass Instruments, Quincy, MA). A representative tracing is shown in Figure 2.3 to indicate the changes in perfusion pressure in the MAB induced by bolus injections of phenylehrine (PE, 0.9 – 300 nmol). Each injection was given after the perfusion pressure had returned to baseline. The distance from baseline to the peak of the rise in perfusion pressure was measured as the response to PE. The perfused MAB was allowed to stabilize for 40 min before four bolus injections of KCl (0.4 mmol), which was used to maximally activate the tissues. Perfusion pressure was allowed to return to baseline after each injection of KCl. After further equilibration for 40 min, dose-response curves (DRCs) to various agonists were performed.

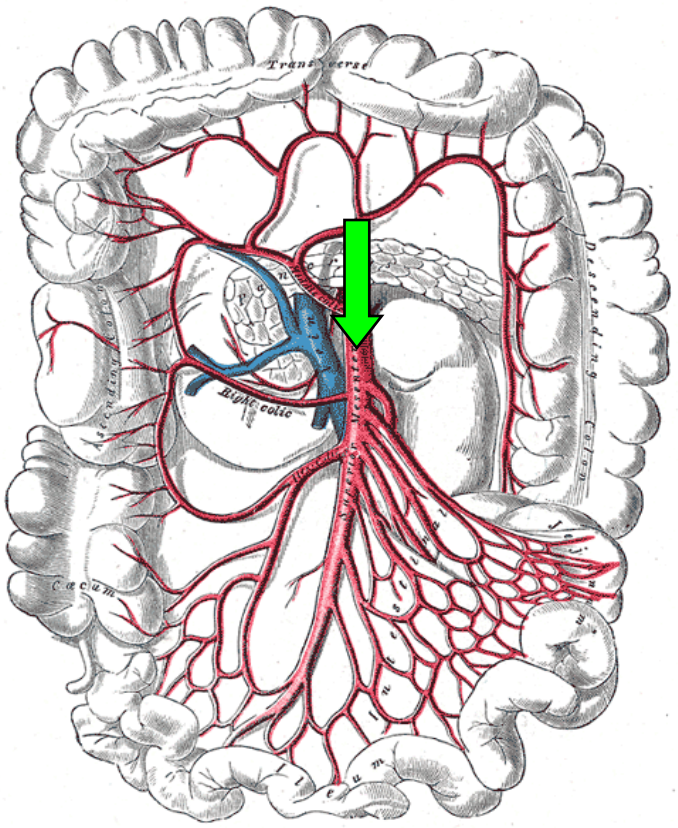

Figure 2.1 Ventral view of superior mesenteric arterial bed (MAB). The superior mesenteric artery arises from the anterior surface of the abdominal aorta. Together with its branches, it supplies the intestine from the lower part of the duodenum through two-thirds of the transverse colon, as well as the pancreas. The arrow depicts where the cannula goes.

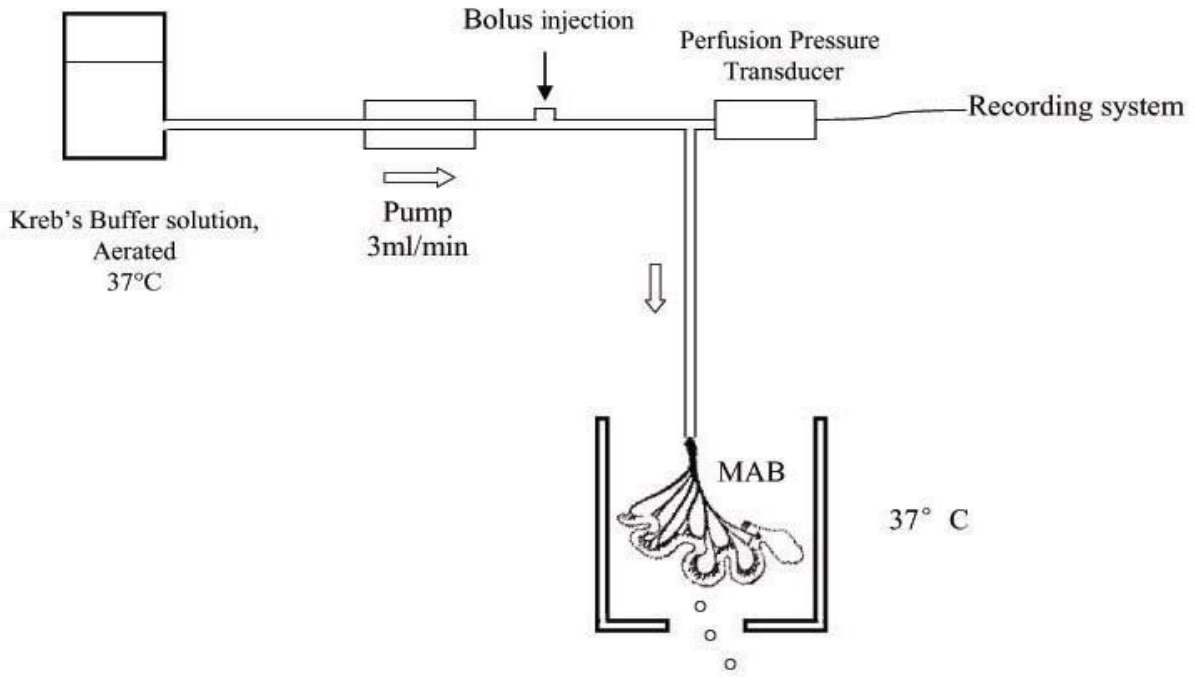

Figure 2.2 Schematic overview of isolated rat mesenteric arterial bed (MAB) perfused under constant flow rate. Perfusion solution was delivered at a constant flow rate to the superior mesenteric artery with the use of peristaltic pump from a water-jacketed reservoir. The reservoir was provided with oxygen 95% O_2, 5% CO_2. Perfusion pressure in the MAB was recorded by a pressure transducer placed in the inflow line.

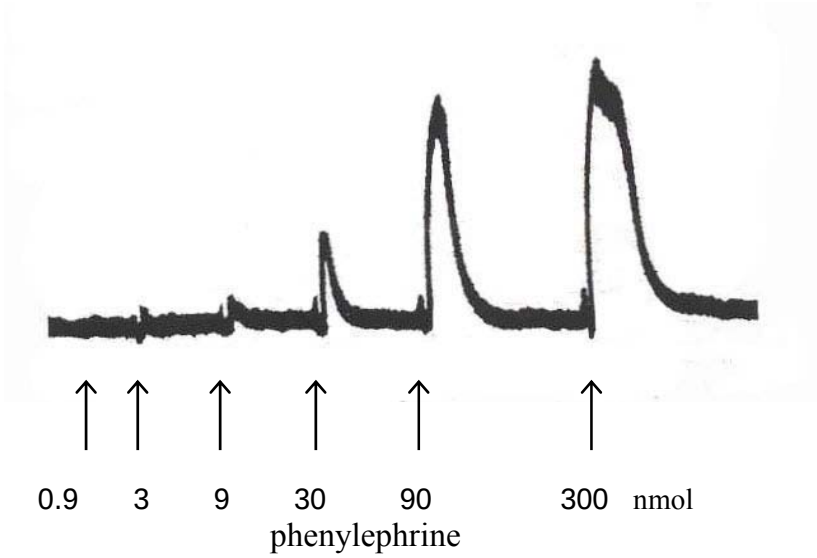

Figure 2.3 Representative tracing indicating the changes in perfusion pressure induced by bolus injections of phenylephrine (0.9 – 300 nmol) in mesenteric arterial bed (MAB).

2.2.1.1 Vasoconstrictor Responses

Palmitoyl-LPC (C 16:0), the most abundant LPC species in humans and rats (Vesterqvist et al., 1992; Croset et al., 2000; Cunningham et al., 2008), was used in all our experiments. To determine the effects of LPC on vasoconstrictor responses of the MAB, bolus injections of PE (0.9–300 nmol) or KCl (50–800 μmol) were given. These first DRCs to PE or KCl served as controls. Once perfusion pressure returned to baseline, palmitoyl-LPC (0.1–10 μmol/L) was added to the perfusion buffer. In preliminary experiments, none of these concentrations of LPC *per se* produced any observable change in contraction. In all of the subsequent experiments, 10 μmol/L LPC was used. After 40 min of perfusion with LPC, the second PE or KCl DRC was constructed in the presence of LPC. The tissues were then switched back to normal Krebs buffer and perfused for 1 h followed by construction of a third PE or KCl DRC. The 60-min washout was designed to test whether the expected effect of LPC was reversible or not. Perfusion pressure was allowed to return to baseline between each PE or KCl injection. To test whether the vascular responses were affected by time, a control experiment consisting of three consecutive DRCs to PE and KCl without the addition of LPC was done.

To determine the potential contribution of NO and thromboxane A_2 (TxA_2) to the observed modulatory effects of LPC on PE responses, the LPC perfusion and washout procedures described above were performed in the presence of a NOS inhibitor, $_L$-NMMA (N^G-monomethyl-L-arginine methyl ester; 300 μmol/L), a COX inhibitor (indomethacin; 20 μmol/L), or a TxA_2 receptor antagonist, SQ-29548 [[1*S*-[1*a*,2*a*(*Z*),3*a*,4*a*]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-

oxabicyclo[2.2.1]-hept-2-yl]-5-heptanoic acid; 0.3 μmol/L]. All inhibitors were added to the perfusate after the first DRC and kept present throughout the second and third DRCs.

In one set of the experiments, the tissues were treated with 10 μM LPA, and vasoconstrictor responses to PE were determined following the same protocol.

2.2.1.2 Vasodilatory Responses

To assess the effects of LPC on EDR, MABs were precontracted with submaximal concentrations of PE (1–3 μmol/L). Subsequently, increasing concentrations of acetylcholine (Ach; 3 nmol–0.3 μmol/L) were administered until maximal relaxation was attained. MABs were then perfused for 40 min with 10 μmol/L LPC, and the response to PE and Ach was repeated. A third Ach response was obtained after washout of LPC for 1 h. Control experiments consisted of three consecutive Ach-induced responses in the absence of LPC.

2.2.1.3 Enzyme Immunoassay of TxB_2

To measure the content of TxB_2, a stable metabolite of TxA_2, in the MAB effluent, samples were collected for 2 min on six different occasions as follows: before the first application of PE, during the first PE-DRC at each PE dose, after the 40-min perfusion with LPC, during the second PE-DRC at each PE dose, after the 60-min washout of LPC, and during the third PE-DRC at each PE dose. Samples were stored at −70°C until assayed.

The collected perfusates were extracted using Solid-Phase Extraction C-18 cartridges (Cayman Chemical, Ann Arbor, MI) after cartridge activation by 5 ml of methanol and 5 ml of ultrapure water. A 0.5-ml aliquot of each sample was acidified to ~ pH 4.0 and loaded onto the cartridge. The cartridge was washed with 5 ml of ultrapure

water followed by 5 ml of hexane. The TxB_2 fraction was eluted with 5 ml of ethyl acetate containing 1% methanol. The eluate was dried under a stream of nitrogen and reconstituted with 0.5 ml of enzyme immunoassay buffer. The concentration of TxB_2 in the eluate was determined by enzyme immunoassay, using a commercially available kit (Cayman Chemical, Ann Arbor, MI).

2.2.2 Isolated superior mesenteric artery

Male Wistar rats (300–400 g) were anesthetized with sodium pentobarbital (60 mg/kg i.p.), and the superior mesenteric artery (shown in Figure 2.1) was excised, placed in normal Krebs solution at room temperature and cleaned of fat and connective tissue. The artery was then cut into 4 mm segments. In some of the experiments, the endothelium was removed from each segment by rubbing the internal lumen of the vessel gently against a thin wire. The rings were then suspended in isolated tissue baths using triangular hooks (Figure 2.4), one of which was attached to a fixed tissue support, while the other was connected to a Grass FT.03 force displacement transducer which in turn was attached to a Grass polygraph (Model 7E, Grass Instruments Co., Quincy, MA, U.S.A.). The rings were equilibrated for 90 min under 1g resting tension in Krebs solution continuously aerated with 95% O_2/5% CO_2 and maintained at a temperature of 37°C. In intact or endothelium-denuded artery rings, the endothelial function was first confirmed by demonstrating their ability or inability, when precontracted with 3 μmol/L PE, to relax in response to 0.1 μmol/L Ach. The tissues were then washed for 60 min and cumulative concentration–response curves (CRCs) to PE were obtained before LPC addition, after 20-min equilibration and 40-min incubation with 10 μmol/L LPC, and than after washout of LPC for 60 min.

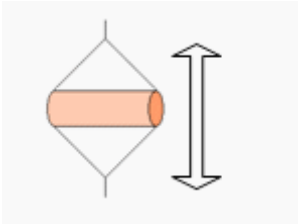

Figure 2.4 Schematic representation of an isolated superior mesenteric artery ring preparation.

2.2.3 Isolated Langendorff heart

2.2.3.1 Heart function measurements

Rats were anesthetized with 65 mg/kg sodium pentobarbital i.p., the thoracic cavity opened, and the heart carefully excised. The heart was immersed in cold (4°C) Krebs-Henseleit (KH) solution (pH 7.4). After the aorta was cannulated and tied below the innominate artery, hearts were perfused retrogradely by the nonrecirculating Langendorff technique as described previously (Pulinilkunnil and Rodrigues, 2006). Perfusion fluid was continuously gassed with 95% O_2/5% CO_2 in a double-walled, water-heated chamber maintained at 37°C with a temperature-controlled circulating water bath. The flow rate was controlled at 15 ml/min. Once a stable heart rate (HR) was reached, a water-filled plastic-film balloon attached to a metal cannula was inserted into the left ventricle, and connected to a pressure transducer. The heart was paced at a constant rate of 300 beats/min. Left ventricular developed pressure (LVDP) and coronary perfusion pressure (CPP) were monitored throughout the perfusion.

Following stabilization for 20 min, one set of hearts was subjected to global ischemia produced by stopping the perfusion for 32 min. After this, reperfusion was performed by restarting the flow of perfusate for another 60 min. In another group of

hearts, KH solution containing 3 μmol/L LPC was perfused for 40 min, followed by a further perfusion for 60 min with normal KH solution (LPC washout). These periods of ischemia and LPC perfusion were selected in preliminary experiments.

To determine the possible role of ET-1 in the effects of LPC, in some of the hearts perfused with LPC, 3 μmol/L bosentan was applied during the LPC perfusion and the following washout procedure.

2.2.3.2 Enzyme Immunoassay of TxB_2 from the coronary effluent

To measure the content of TxB_2, a stable metabolite of TxA_2, in the coronary effluent, samples were collected in I/R group on the following occasions: before ischemia and following reperfusion for 5 min, 10 min, 20 min, 30 min, and 40 min respectively. The coronary effluents in the LPC-treated group were collected as follows: before LPC addition, and following LPC perfusion for 20 min, and 40 min, and LPC washout for 10 min, 20 min, and 40 min, respectively. Samples were stored at –70°C until assayed.

The concentration of TxB_2 in the eluate was determined by enzyme immunoassay, using a commercially available kit (Cayman Chemical, Ann Arbor, MI) as described above in 2.2.1.3.

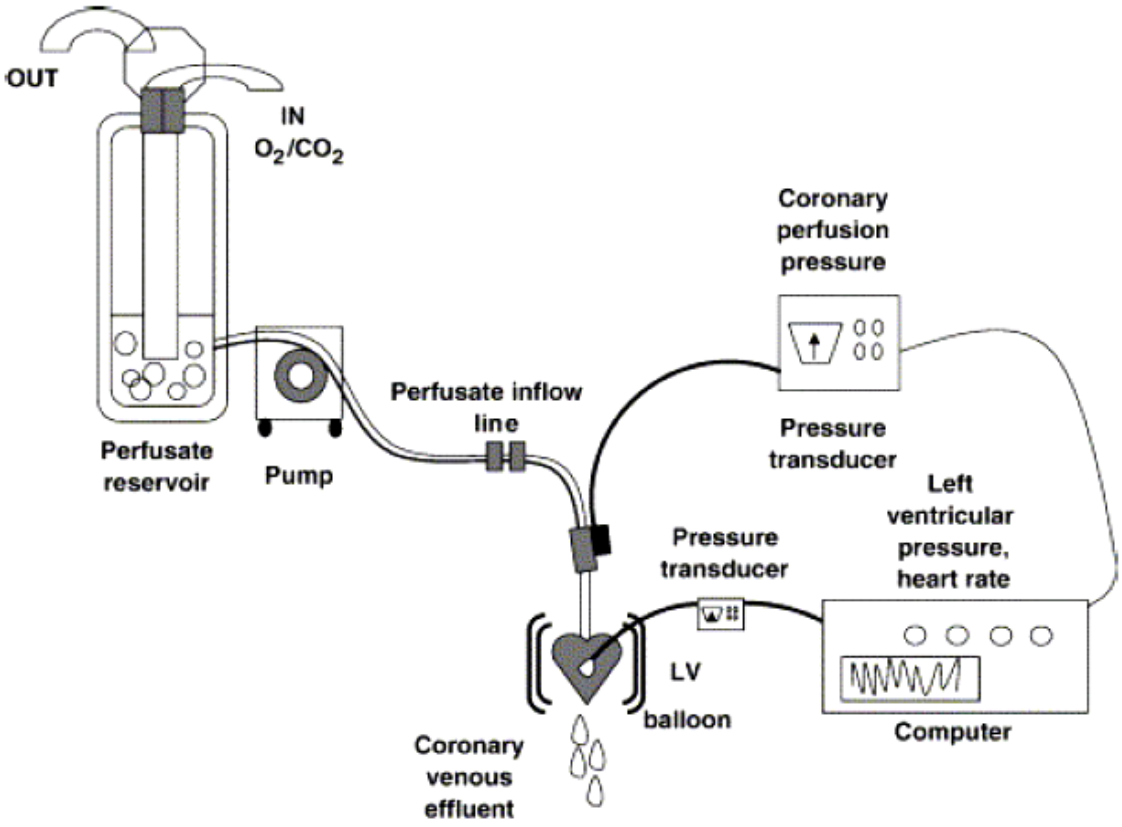

Figure 2.5 Schematic overview of isolated rat heart perfused at constant flow. Perfusion solution was delivered at a constant flow rate to the aorta with the use of a roller pump via the aortic cannula from a water-jacketed reservoir. The reservoir was provided with 95% O_2/5% CO_2. Perfusion solution delivery pressure, which reflects pressure in the coronary circulation, was recorded by a pressure transducer placed in the inflow line. Coronary perfusion pressure (CPP), left ventricular developed pressure (LVDP) and heart rate were recorded by pressure transducers connected to the aorta, and a small balloon placed in the left ventricle, respectively. Devices were connected to a computer which allowed recording and analysis of data. Adapted from Skrzpiec-Spring et al., 2007.

2.2.4 Isolated rat coronary septal artery in pressure myograph

2.2.4.1 Pressurized coronary septal artery

Coronary septal arteries were isolated as described previously (Moien-Afshari et al., 2004). Briefly, following careful removal of the right ventricle, the septal artery (shown in Figure 2.6) was dissected and cleaned of adherent cardiac muscle tissue. A 0.8-1.2 mm segment of the artery (inner diameter 150-200 μm) obtained at the level of the superior papillary muscle, was mounted at both ends onto glass cannulae in a pressure myograph chamber (Living Systems Instrumentation, Burlington, VT). As shown in Figure 2.7, both ends of the artery were tied using a single strand teased from 4-0 surgical silk thread, and the chamber then placed on an inverted microscope stage to measure arterial diameter. Arteries were initially pressurized to an intraluminal pressure of 20 mmHg (below the threshold for development of myogenic tone) using a pressure servo-control unit. The vessels were superfused continuously with aerated normal Krebs solution at 37°C. Arteries were allowed to equilibrate for 60 min before any chemical treatment. Throughout the experiments, lumen diameters were constantly measured using a video dimension analyzer, with data recorded on a computer. At the end of each experiment, arteries were superfused with calcium-free Krebs solution containing 2 mM EGTA for at least 15 min to obtain passive diameters for the calculation of relative contraction and relaxation.

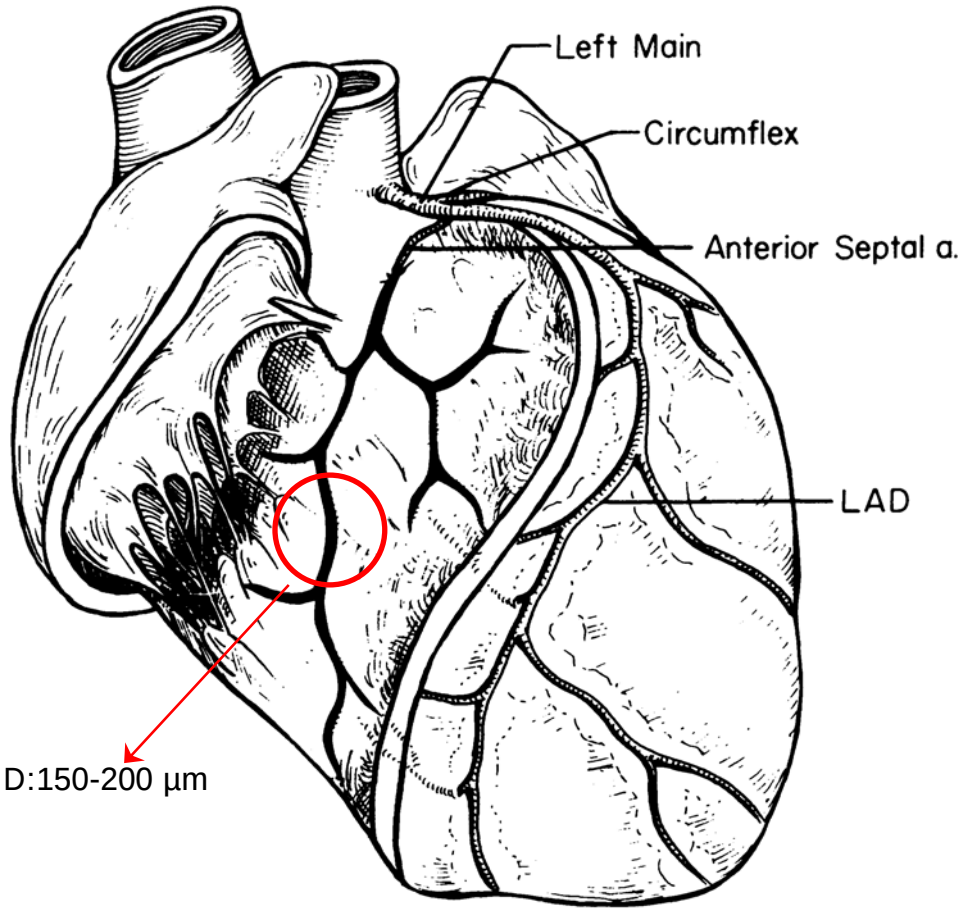

Figure 2.6 Illustration of a heart without the right ventricular wall. The septal coronary artery is located on the interventricular septum, and supplies 75% of the septum. The circle depicts the segment with an inner diameter between 150 to 200 µm that was used in experiments. Adapted from Tweddell et al., 1994.

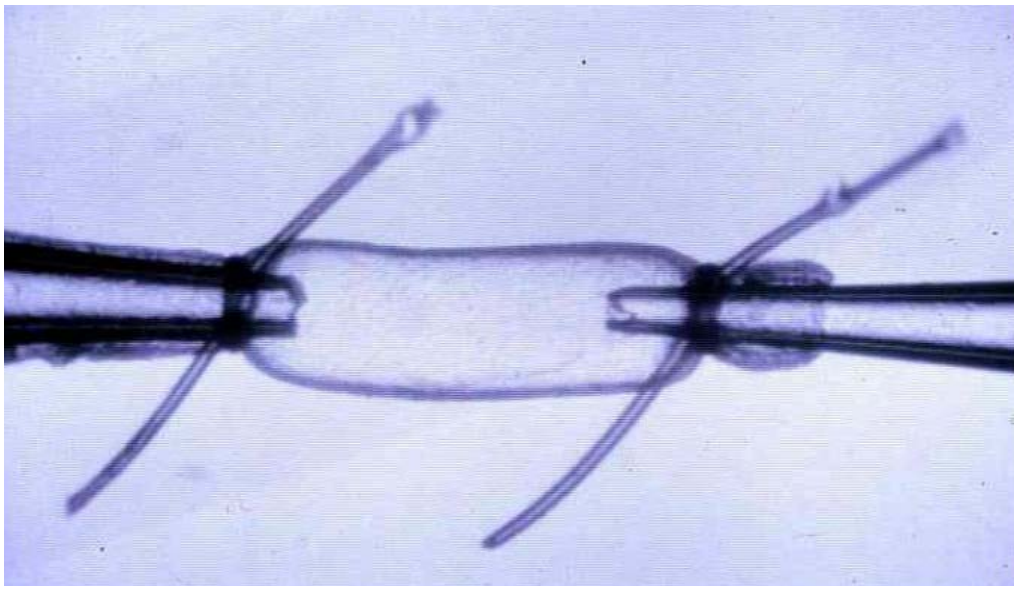

Figure 2.7 Microscope picture of a rat septal coronary artery mounted in an pressure myograph. The artery was cannulated at either end by glass micropipettes and secured by fine nylon strands teased from a nylon suture. The pressure inside the artery was regulated and the diameter was recorded by videometric means.

2.2.4.2 Vasoconstrictor and vasodilator responses before, during, and after washout of intraluminal LPC

Cumulative concentration response curves (CRCs) to the TxA_2 mimetic, U-46619, KCl, acetylcholine (Ach), or sodium nitroprusside (SNP) were determined in isolated coronary septal arteries in the absence of LPC, in the intraluminal presence of LPC (3 μmol/L) for 40 min, and after 60-min washout of LPC. To treat tissues with LPC intraluminally, one end of the cannula was opened transiently, and LPC-containing Krebs solution was allowed to flow through the arterial lumen using a peristaltic pump. Subsequently, the valve of the cannula was closed, and the tissues returned to a no-flow condition, after which the responses to various agonists were determined. To remove LPC from the lumen, the same process was repeated with normal Krebs solution replacing the LPC-containing buffer, and the responses to various agonists measured again. Where indicated, appropriate inhibitors were added to the perfusate surrounding the tissues prior to determination of agonist responses.

In one set of experiments, the tissues were treated with 3 μM LPA rather than LPC, and vasoconstrictor responses to U-46619 were determined following the same protocol.

2.2.4.3 Endothelium denudation

To examine the role of the endothelium, CRCs to U-46619 were also determined in endothelium-denuded septal coronary arteries. Prior to being mounted on the glass cannula, a piece of stainless steel wire (40 μm in diameter) was used to gently rub the intimal surface of the vessels. Functional denudation was confirmed by abolition of the dilatory response to Ach (1 μmol/L).

2.2.5 Isolated rat coronary septal artery in wire myograph

2.2.5.1 Isometric force measurements

The effects of LPC were also determined in a set of isometric experiments using a wire myograph, to compare to those obtained from isobaric experiments (pressure myograph). The septal artery (shown in Figure 2.6) was dissected and cleaned of adherent cardiac muscle tissue as described above. Each arterial ring was cut into 2-mm lengths, and threaded on two stainless wires (40 μm diameter), which were then attached to tissue holders of a 4-channel wire myograph (JP Trading, Aarhus, Denmark) containing Krebs solution aerated with 95 % O_2- 5 % CO_2. As shown in Figure 2.8, one of the tissue holders was connected to the lever of a force-displacement transducer to record the isometric force developed by the ring. Tissues were allowed to equilibrate for 60 min at 37 °C, during which time the Krebs solution was replaced at 20-30 min intervals. During the equilibration, the resting tension was gradually increased to 3 mN and kept at this level for 20-30 min. Each tissue was maximally activated with a solution of KCl (80 mM) that was prepared by equimolar substitution of NaCl in Krebs solution.

2.2.5.2 Vasoconstrictor responses before, during, and after washout of LPC

Following the equilibration, U-46619 was added to the wire myograph chamber containing 5 ml Krebs solution. CRCs to U-46619 were determined before, in the presence of LPC (3 μmol/L) for 40 min, and after 60-min washout of LPC, respectively.

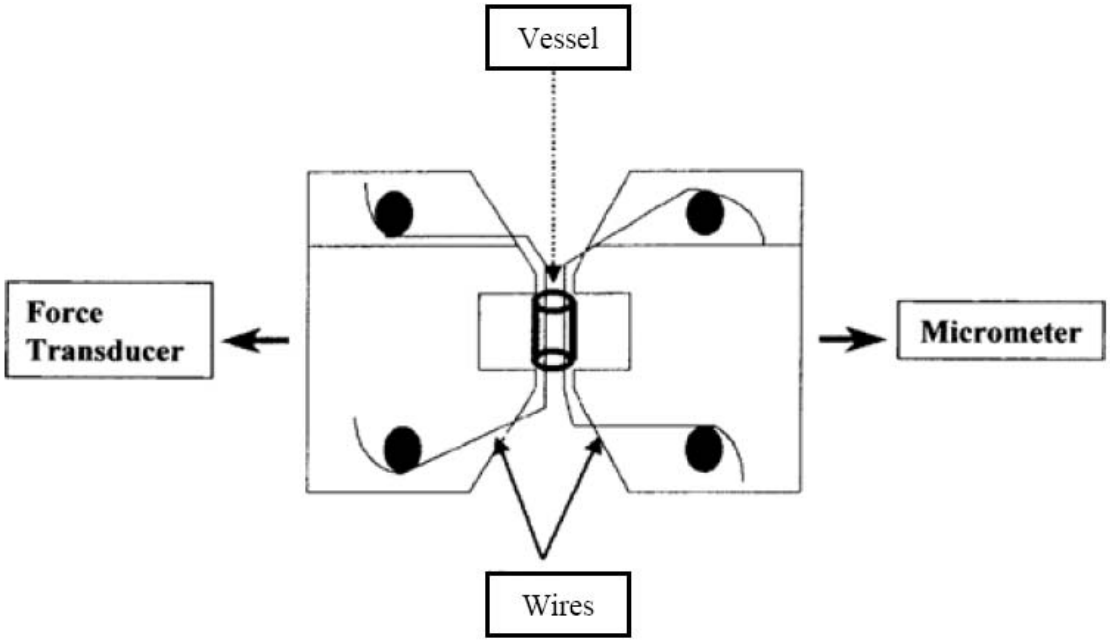

Figure 2.8 Illustration of an arterial ring mounted on a wire myograph. One of the stainless steel jaws (right) was driven by a micrometer, the other attached to a sensitive force transducer, which was connected to a specially designed myo-interface and thus to a chart recorder, allowing the force across the vessel wall to be recorded. The 2-mm long segment of coronary artery was mounted on 40-μm diameter stainless steel wires that were secured tight by the screws set into the jaws. The preparation was bathed in Krebs solution that could be extracted using a suction device. The myograph was kept at 37°C during the experiments by a built-in heater. Adapted from Methods in Molecular Medicine, Vol. 108: Hypertension: Methods and Protocols (Spiers and Padmanabhan, 2005).

2.2.6 Cultured bovine coronary arterial endothelial cells

2.2.6.1 Endothelial cell culture

Primary bovine coronary artery endothelial cells (BCAECs) were obtained from Clonetics (Walkersville, Md). BCAECs were recovered from cryopreservation by rapid thawing in a 37°C water bath and seeded at 2,000-3,000 cells/cm^2 in T-75 flasks. Cells were grown in endothelial growth medium (EGM) supplemented with EGM-MV Bulletkit (endothelial cell basic medium, 10 μg/ml hEGF, 1.0 mg/ml hydrocortisone, 50 mg/ml gentamicin, 50 μg/ml amphotericin-B, 3 mg/ml BBE, 2% v/v FBS) in a humidified atmosphere of 5% CO_2 at 37°C for 5-7 days, during which time fresh EGM was added every other day. Cells were then subcultured by exposure to 0.025% trypsin-0.01% EDTA and were seeded onto six-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ, USA) at passage 5-6 (100,000 cells/well).

2.2.6.2 LPC incubation and washout

Once BCAEC growth reached 80% to 90% confluence, they were co-cultured with Dulbecco's Modified Eagles Medium (DMEM, phenol red-free) containing 3 μmol/L LPC or with plain DMEM for 40 min. Supernatants were then collected and stored at -70°C. Cells were rinsed with DMEM 3 times, and then incubated with normal DMEM for another 60 min. Subsequently, the supernatants were harvested and all samples were stored at -70°C until assayed.

2.2.6.3 Enzyme Immunoassay of ET-1

The concentrations of ET-1 released from the cells to medium supernatant were determined by enzyme immunoassay, using a commercially available kit (Cayman Chemical, Ann Arbor, MI). Each kit consisted of a polystyrene 96-well immunoplate

pre-coated with a peptide antibody. Aliquots of the media samples were loaded in duplicate onto the wells, and the assay was carried out according to the manufacturer's protocol. Absorbance was measured at 405 nm in an automated plate reader. A standard curve was used to calculate ET-1 concentrations (pg ET-1/ml medium).

2.3 Drugs and chemicals

L-NMMA (N^G-monomethyl-L-arginine methyl ester), SQ-29548, and U-46619 were purchased from BIOMOL (Plymouth Meetings, PA). NS-398 (N-(2-Cyclohexyloxy-4-nitrophenyl)methanesulfonamide) was purchased from Calbiochem (La Jolla, CA). Bosentan was a generous gift from Actelion Ltd., Allschwil, Switzerland. L-NAME (N^{ω}-nitro-L-arginine methyl ester) and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Stock solutions of PE (0.01 mol/L), LPC (0.01 mol/L), and L-NMMA (0.1 mol/L) were prepared in distilled water. Indomethacin and SQ-29548 were dissolved in 100% ethanol and prepared as stock solutions of 0.1 mol/L and 0.1 mmol/L, respectively. Solutions of PE and indomethacin were made fresh. LPC and all of the inhibitors were further diluted to the required concentration in the perfusate reservoir. The final ethanol concentrations (0.03–0.3%, v/v) were without effect on contractile responses. Krebs buffer had the following composition (mmol/L): 113 NaCl, 4.7 KCl, 11.5 glucose, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 1.2 KH_2PO_4, and 25.0 $NaHCO_3$. Krebs-Henseleit solution contained (mmol/L): 117.4 NaCl, 4.7 KCl, 1.8 $CaCl_2$, 1.2 $MgSO_4$, 1.3 KH_2PO_4, 11.0 glucose, 25.0 $NaHCO_3$, and 0.5 EDTA.

2.4 Statistical analyses

All of the data are presented as mean ± S.E.M. In the MAB experiments, KCl-induced vasoconstrictor responses were expressed as the absolute increase in perfusion pressure. PE responses were expressed as a percentage of the maximal response of the first PE-DRC. The negative log of the PE concentration producing 50% of maximum response (pD_2) was obtained by nonlinear regression analysis of individual DRCs using GraphPad Prism, version 4.0 (GraphPad Software, Inc., San Diego, CA). Data were analyzed for significant differences using Number Cruncher Statistical Systems. Student's unpaired *t* test was used for comparisons between two means. One-way ANOVA followed by the Newman-Keuls test was used for comparison of more than two means. Two-way ANOVA, using the general linear model approach (repeated measures) followed by Newman-Keuls test, was used for comparisons between PE-DRCs and KCl-DRCs.

For the isolated septal coronary arteries, percent constriction was calculated using the equation 100%×(Db-Dd)/Db, whereas percent relaxation was calculated using the equation 100%×((Dd-Db)/($D_{Ca\text{-}free}$-Db)), where D is the diameter at baseline (b), upon stabilization after drug (d) addition, or following Ca^{2+} free solution. One vessel segment was obtained from each animal. The CRCs to U-46619 and Ach were analyzed by nonlinear regression using GraphPad Prism version 4.0 for calculation of pD_2 (-log EC_{50}) values and maximum responses (R_{max}). Statistical evaluation was done using analysis of variance (ANOVA) with multiple comparisons, and Bonferroni's test when appropriate. When CRCs were conducted using pharmacological inhibitors, the change in the shape of CRCs precluded the acquiring of R_{max}. In this circumstance, data were analyzed for

significant differences using two-way ANOVA and the general linear model approach (repeated measurements) followed by Newman–Keul's test for comparisons between CRCs. A $P<0.05$ was considered statistically significant in all analyses.

3. RESULTS

3.1 LPC potentiates phenylephrine responses in rat mesenteric arterial bed through modulation of thromboxane A_2

3.1.1 Effect of LPC on basal perfusion pressure

To examine the effect of LPC on basal perfusion pressure, isolated MABs were incubated with varying concentrations of LPC (0.1–10 μmol/L). Perfusion pressure was measured during a 40 min perfusion with LPC or 1 h after washout. In Figure 3.1(left), the effects of 10 μmol/L LPC on basal perfusion pressure are shown. LPC had no significant effect on perfusion pressure, either during incubation or following washout, suggesting that this lysophospholipid has no direct effect on the contractility of MAB. Basal MAB perfusion pressure in untreated control tissues remained unchanged throughout the experimental period (Figure 3.1, right).

3.1.2 KCL-induced contractile responses following LPC

Pressor responses of the MAB to bolus injections of KCl (50–800 μmol) were not significantly altered, either during perfusion with 10 μmol/L LPC or after LPC washout (Figure 3.2A). In untreated control tissues, KCl-DRCs remained constant over time (Figure 3.2B).

3.1.3 Effects of LPC on PE-induced contractile responses.

Bolus injections of PE (0.9–300 nmol) produced a concentration-dependent increase in perfusion pressure that did not significantly change over time (Figure 3.3B). The response to PE was unaffected by prior perfusion with LPC for either 40 (Figure 3.3A) or 150 min (Figure 3.3A inset). The PE CRC was even slightly but not significantly depressed following 150-min LPC perfusion. Interestingly, after washout of

LPC for 1 h, the PE response was markedly enhanced (Figure 3.3A). The maximal response to PE increased to 199 ± 24% of control ($P < 0.001$) after LPC washout, whereas the PE pD_2 value increased from 7.50 ± 0.04 to 8.13 ± 0.15 ($P < 0.001$). Further increasing the dose of PE above 90 nmol resulted in a decline in the contractile response.

3.1.4 Involvement of NO and TxA_2 in mediating the direct effects of LPC perfusion.

Before LPC perfusion, Ach (0.1–0.3 μmol) produced a maximal relaxation of 93 ± 5% of the response to PE (Figure 3.4, left). The Ach response remained unchanged over time (Figure 3.4, right). LPC perfusion almost completely abrogated the response to Ach, reducing the maximal relaxation to 7 ± 4% (Figure 3.4, left). In a separate experiment, we tested the effects of $_L$-NMMA on PE responses. $_L$-NMMA is a cell permeable and non-selective inhibitor of all three isoforms of NOS (K_i = 650 n mol/L for nNOS; K_i = 700 n mol/L for eNOS; K_i = 3.9 μ mol/L for iNOS) (Reif and McCreedy, 1995; O'kane et al., 1994). In vitro, it was shown to inhibit the relaxation of rat aortic rings induced by Ach (IC_{50}=9.5 μmol/L) (Sakuma et al., 1988). As expected, pretreatment with $_L$-NMMA significantly enhanced the PE response (Figure 3.5), with both an increase in the maximal response and pD_2 value (control, 7.03 ± 0.45; $_L$-NMMA, 7.49 ± 0.17; $P < 0.001$) being apparent. However, in the presence of LPC, the enhancement of the PE response by $_L$-NMMA was prevented (Figure 3.5). These data suggest that, in addition to suppressing NO, LPC may be exerting other actions to limit vasoconstrictor release or responsiveness to PE.

TxA_2 is a major vasoconstrictor prostanoid implicated in the regulation of vascular tone in response to α_1-adrenoceptor stimulation in the MAB. To investigate the involvement of TxA_2 in mediating PE responses, we measured levels of TxB_2, the stable

metabolite of TxA_2. Basal release of TxB_2 was stable over time. In untreated MAB, only the highest dose of PE tested (300 nmol) produced an increase in TxA_2 production (Figure 3.7). LPC perfusion for 40 min had no effect on basal levels of TxB_2 but suppressed its enhancement by PE (Figure 3.7), suggesting that LPC prevents the ability of PE to increase TxA_2 production.

3.1.5 Involvement of NO and TxA2 in mediating the indirect effects (after washout) of LPC.

Washout of LPC induced partial recovery of the Ach response (Figure 3.4). Despite this fractional recovery, contractile responses to PE were augmented (Figure 3.3A), suggesting that amplification of vasoconstrictor pathways, in addition to reduction of vasodilators such as NO, may be responsible for this effect. To investigate this possibility, after washout of LPC, a DRC to PE was performed in the presence of $_L$-NMMA (Figure 3.6). Interestingly, even in the presence of the NOS inhibitor, the response to PE was shifted to the left after LPC washout (pD_2 value in the presence of $_L$-NMMA alone, 7.54 ± 0.07; following LPC washout and in the presence of $_L$-NMMA, 8.16 ± 2.05; $P < 0.05$). These data suggest that the enhancement of the PE response after LPC washout is mediated by mechanisms in addition to NO inhibition.

To further investigate mechanisms facilitating the enhanced PE response after LPC washout, TxB_2 levels in the perfusion medium were measured. Interestingly, TxB_2 levels only increased after LPC washout (Figure 3.7), suggesting a role for this vasoconstrictor in the magnified response to PE. MABs were then pretreated with either indomethacin or SQ-29548. Indomethacin is a non-selective COX inhibitor (IC_{50}=740 nmol/L for COX-1 and IC_{50}=970 nmol/L for COX-2) (Stevenson and Lumbers, 1992;

Kalgutkar et al., 2000). SQ-29548 is a selective TxA_2 receptor antagonist with IC_{50}=12 nmol/L for inhibition of human platelet aggregation (Ogletree et al., 1985) and K_i=7.9 nmol/L for inhibition of U-46619 binding to human platelet membranes (Kattelman et al., 1986). Both indomethacin (pD_2 value after LPC washout, 8.24 ± 0.07; LPC washout plus indomethacin, 7.74 ± 0.25; $P < 0.05$) and SQ-29548 (pD_2 value after LPC washout, 8.24 ± 0.07; LPC washout plus SQ-29548, 7.73 ± 0.10; $P < 0.05$) completely prevented the enhancement of the PE DRC after LPC washout (Figure 3.8). Neither indomethacin nor SQ-29548 significantly changed PE-induced vasoconstriction by themselves (Figure 3.8 inset).

3.1.6 Exclusion of LPA in the indirect effects of LPC

To test if LPA, one of the metabolic products of LPC, mediates the delayed responses induced by LPC in the MAB, the responses of MAB to 10 μmol/L LPA perfusion and its washout were determined. However, neither LPA itself nor the following washout significantly modified the vasoconstriction to PE (Figure 3.9), suggesting that LPA is not involved in the effects of LPC.

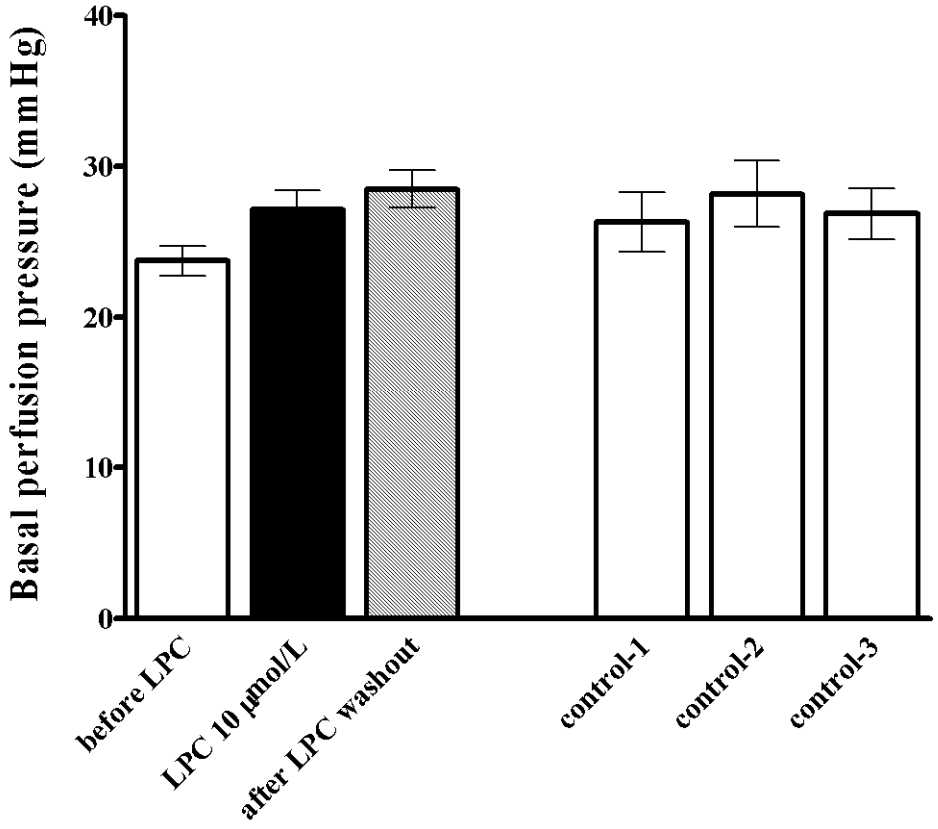

Figure 3.1 Effects of LPC on basal perfusion pressure in MAB. Basal perfusion pressure before, after 40-min perfusion with 10 µmol/L LPC, and after washout of LPC for 60 min (*n*=25, left). The right panel depicts basal perfusion pressure in untreated control tissues (*n*=7) at the same fixed time intervals. Data represent the mean ± S.E.M. Statistical evaluation was done using one-way ANOVA followed by Newman-Keuls test when appropriate.

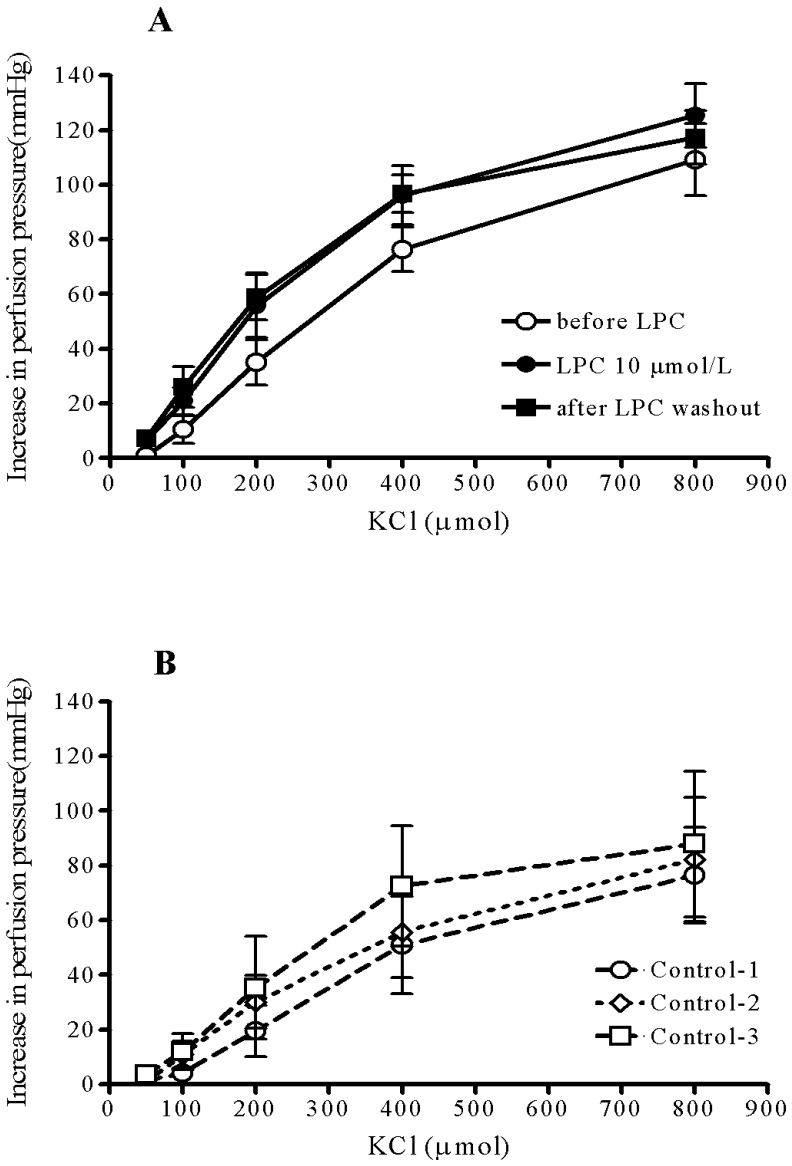

Figure 3.2 Effects of LPC on KCL response in MAB. A, dose-response curves of MABs to KCl before, after 40-min perfusion with 10 μmol/L LPC, and after washout of LPC for 60 min (*n*=6). B, dose-response curves to KCl in untreated control MABs (*n*=4) at the same fixed time intervals. Data represent the mean ± S.E.M. Data were analyzed for significant differences using NCSS. Two-way ANOVA using the general linear model approach (repeated measurements) followed by Newman–Keul's test was used

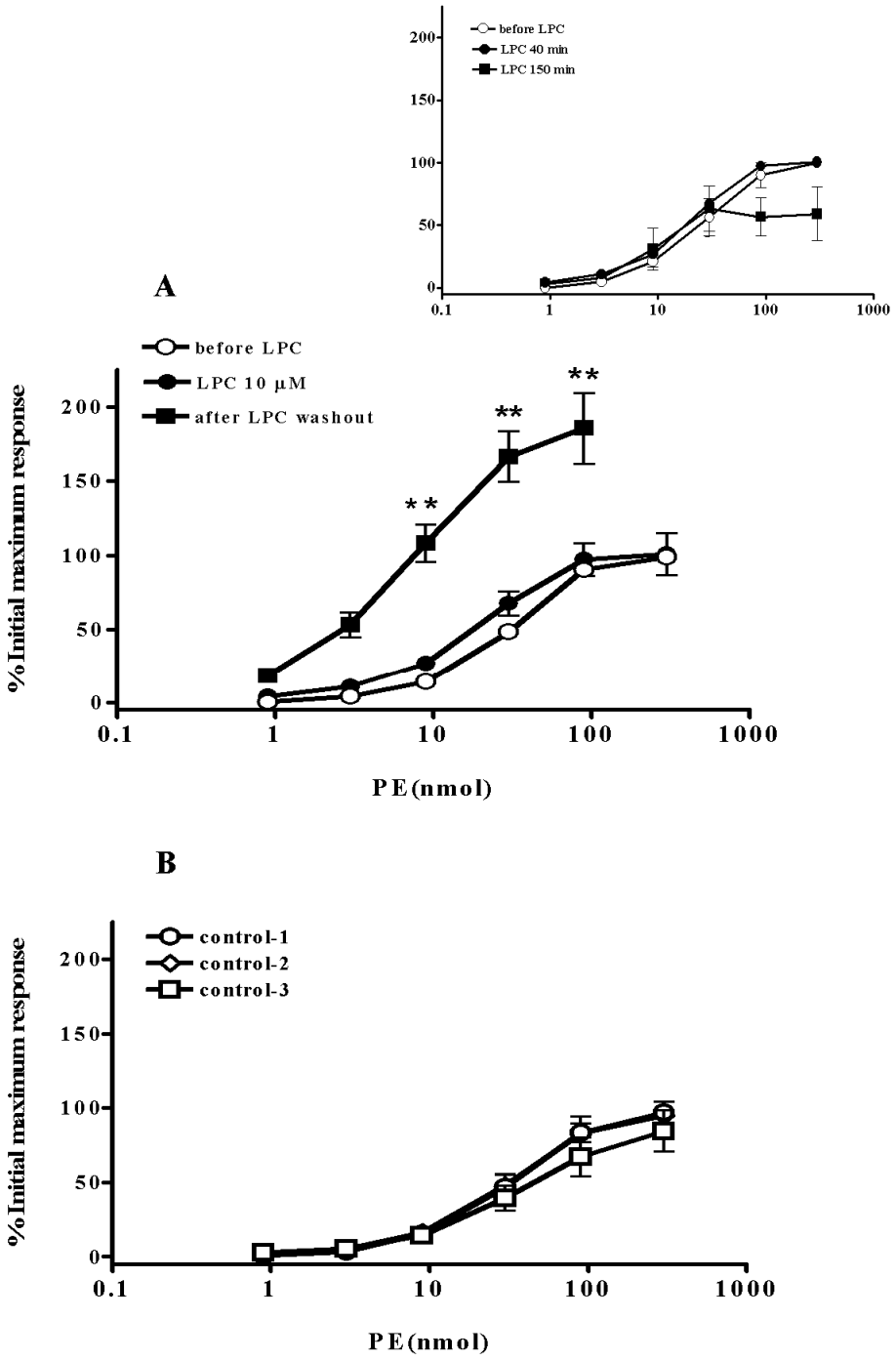

Figure 3.3 Effects of LPC on PE response in MAB. A, dose-response curves of MABs to PE before, after 40-min perfusion with 10 µmol/L LPC, and after washout of LPC for 60 min (n = 20). The inset depicts the effect of LPC perfusion for 150min (n = 3). B, dose-response curves to PE in untreated control MABs (n=11) at the same fixed time intervals. All data represent the mean ± S.E.M. **, $P < 0.01$ versus all other responses at the same dose (two-way ANOVA followed by Newman-Keuls test).

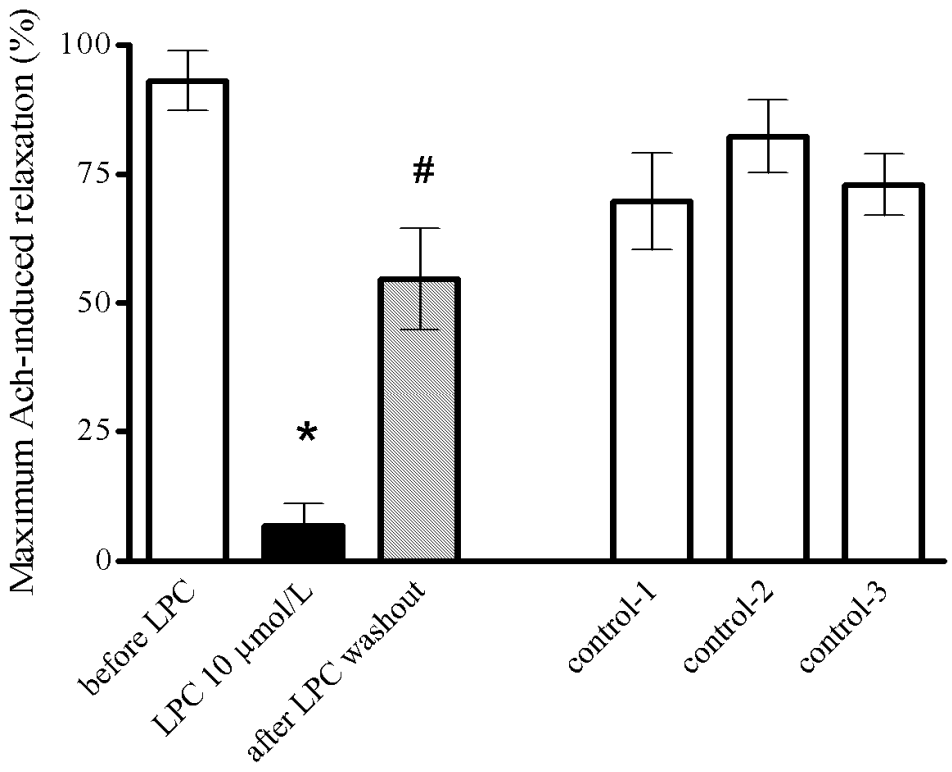

Figure 3.4 Effects of LPC on Ach responses in MAB. Maximal Ach-induced relaxation of perfused MABs precontracted with PE (1–3 µmol/L) before, after 40-min perfusion with 10 µmol/L LPC, and after washout of LPC for 60 min (n = 5, left). The right panel depicts maximal Ach-induced relaxation in untreated control tissues (n=5) at the same fixed time intervals. Data represent the mean ± S.E.M. Statistical evaluation was done using one-way ANOVA followed by Bonferroni's test when appropriate. *, P < 0.05 versus "before LPC" and "after LPC washout." #, P < 0.05 versus "before LPC" and "LPC 10 µmol/L" (one-way ANOVA followed by Newman-Keuls test).

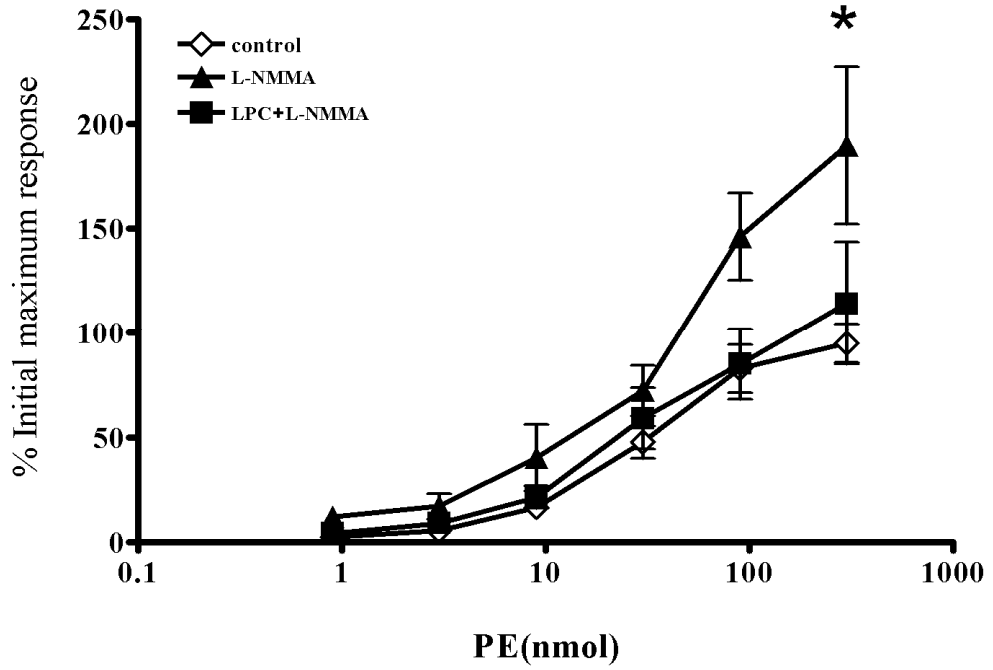

Figure 3.5 Effects of $_{L}$-NMMA on PE responses in LPC-treated MAB. Dose-response curves to PE in untreated MAB, in MAB treated with 300 μmol/L $_{L}$-NMMA alone for 40 min, and in MAB treated with 10 μmol/L LPC plus 300 μmol/L $_{L}$-NMMA for 40 min. Data represent the mean ± S.E.M. *n*=7-11. *, $P < 0.05$ versus all other responses at the same dose (two-way ANOVA followed by Newman-Keuls test).

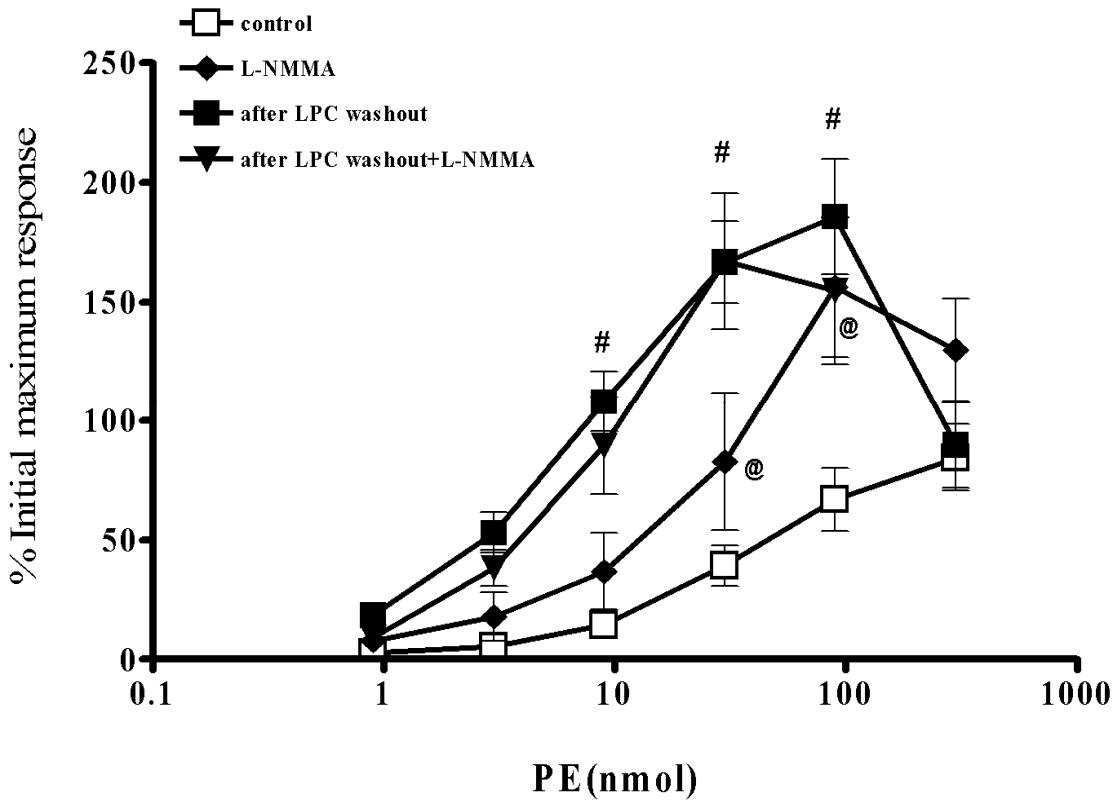

Figure 3.6 Effects of $_{L}$-NMMA on PE responses in MAB following LPC washout. Dose-response curves to PE in untreated MAB and in MAB pretreated with 300 μmol/L $_{L}$-NMMA alone for 140 min, after the washout of LPC for 60 min, and after the washout of LPC in the presence of 300 μmol/L $_{L}$-NMMA. Data represent the mean ± S.E.M. *n*=7-11. #, $P < 0.05$ versus all other responses at the same dose except "after LPC washout + $_{L}$-NMMA" @, $P < 0.05$ versus all other responses at the same dose (two-way ANOVA followed by Newman-Keuls test).

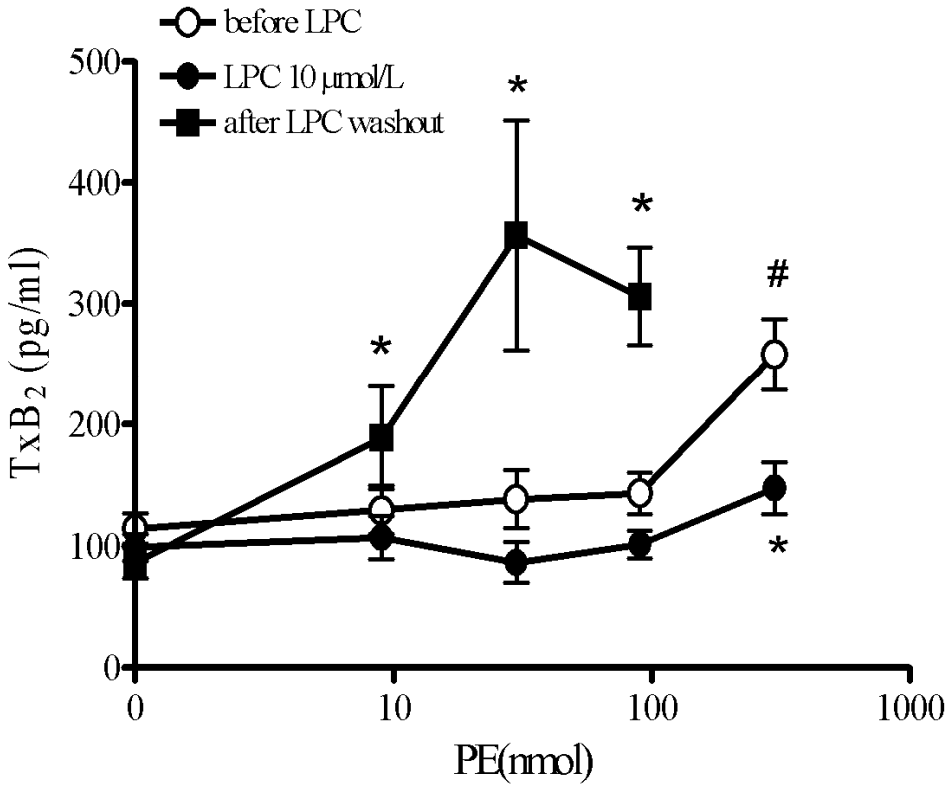

Figure 3.7 TxB_2 release from MAB. TxB_2 release from MAB in response to PE before, after 40-min perfusion with 10 µmol/L LPC, and after washout of LPC for 60 min (n = 8). Data represent the mean ± S.E.M. #, $P < 0.05$ versus all other PE doses in "before LPC" (one-way ANOVA followed by Newman-Keuls test). *, $P < 0.05$ versus all of the other groups at the same dose (two-way ANOVA followed by Newman-Keuls test).

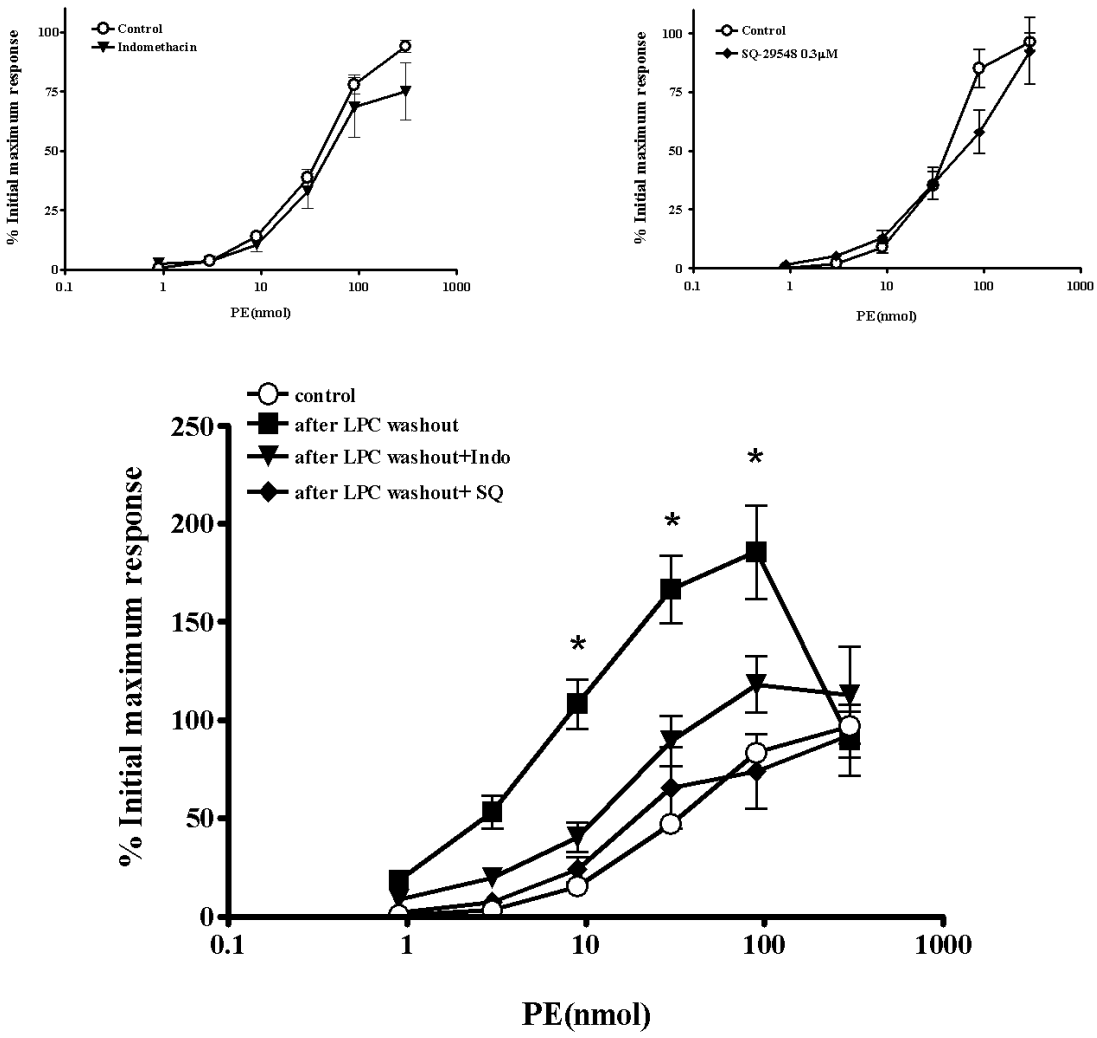

Figure 3.8 Effects of indomethacin and SQ-29548 on the potentiated PE response induced by LPC washout. Dose-response curves to PE in untreated MABs and in MAB after the washout of LPC for 60 min in the absence (n=12) and presence (*n*=4) of inhibitors (20 μmol/L indomethacin or 0.3 μmol/L SQ-29548. The insets depict the effects of indomethacin alone (left, *n*=3) and SQ-29548 alone (right, *n*=3). Data represent the mean ± S.E.M. *, $P < 0.05$ versus all other responses at the same dose (two-way ANOVA followed by Newman-Keuls test).

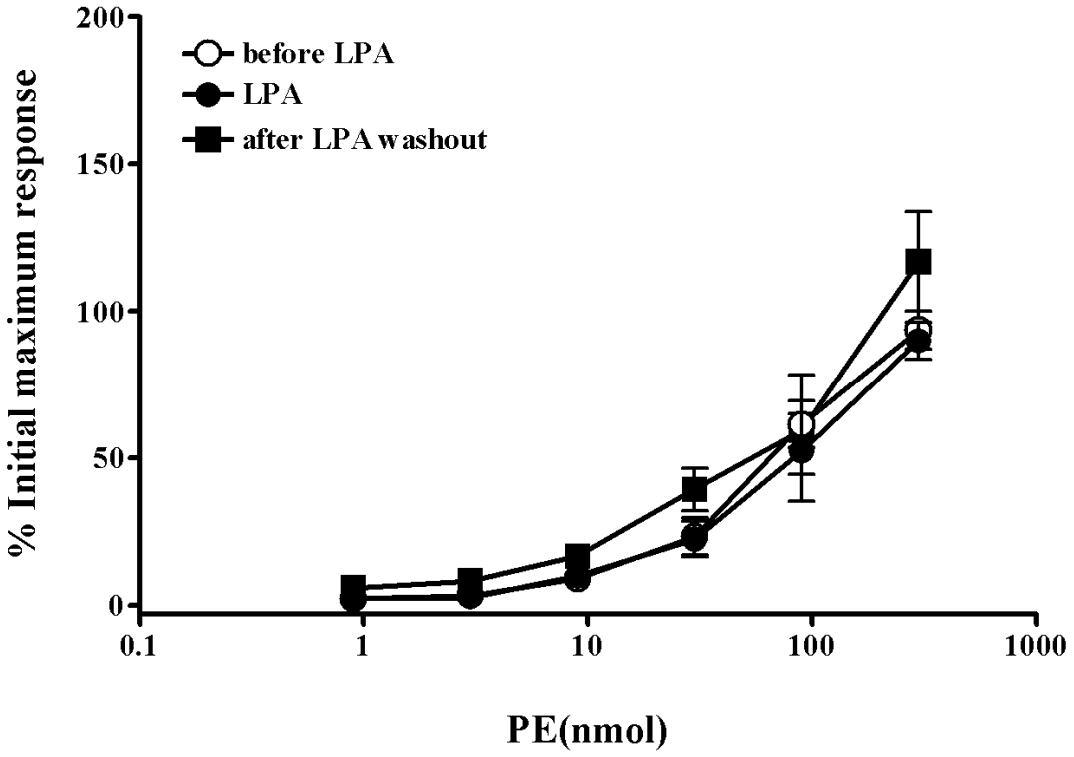

Figure 3.9 Effects of LPA on PE responses in MAB. Dose-response curves of MABs to PE before, after 40-min perfusion with 10 μmol/L LPA, and after washout of LPA for 60 min (n = 4). All data represent the mean ± S.E.M. There were no significant differences (two-way ANOVA followed by Newman-Keuls test).

3.2 Effects of LPC on phenylephrine responses in isolated rat superior mesenteric artery

It has been consistently observed in our laboratory that PE-induced contractile responses in superior mesenteric arteries tended to increase over time, as shown in Figure 3.10B (endothelium-intact) and Figure 3.11B (endothelium-removed). In the endothelium-denuded tissues, the increase in the third PE-R_{max} was significant (Figure 3.12B). When 10 μmol/L LPC was added to the Krebs solution and kept there for 40 min, CRCs to PE were unchanged in either preparation (Figure 3.10A, Figure 3.11A). Following the LPC washout for 60 min, there was a slight enhancement of the PE response in the endothelium-intact tissues (Figure 3.10A), with significantly increased R_{max} (Figure 3.12A) but unchanged pD_2 values (Figure 3.12C). However, this potentiation was completely abolished in the endothelium-denuded tissues (Figure 3.11A, Figure 3.12B), indicating an endothelium-dependent mechanism. In summary, LPC washout potentiated the PE induced contractile responses in superior mesenteric arteries, but to a much smaller magnitude than in the MAB.

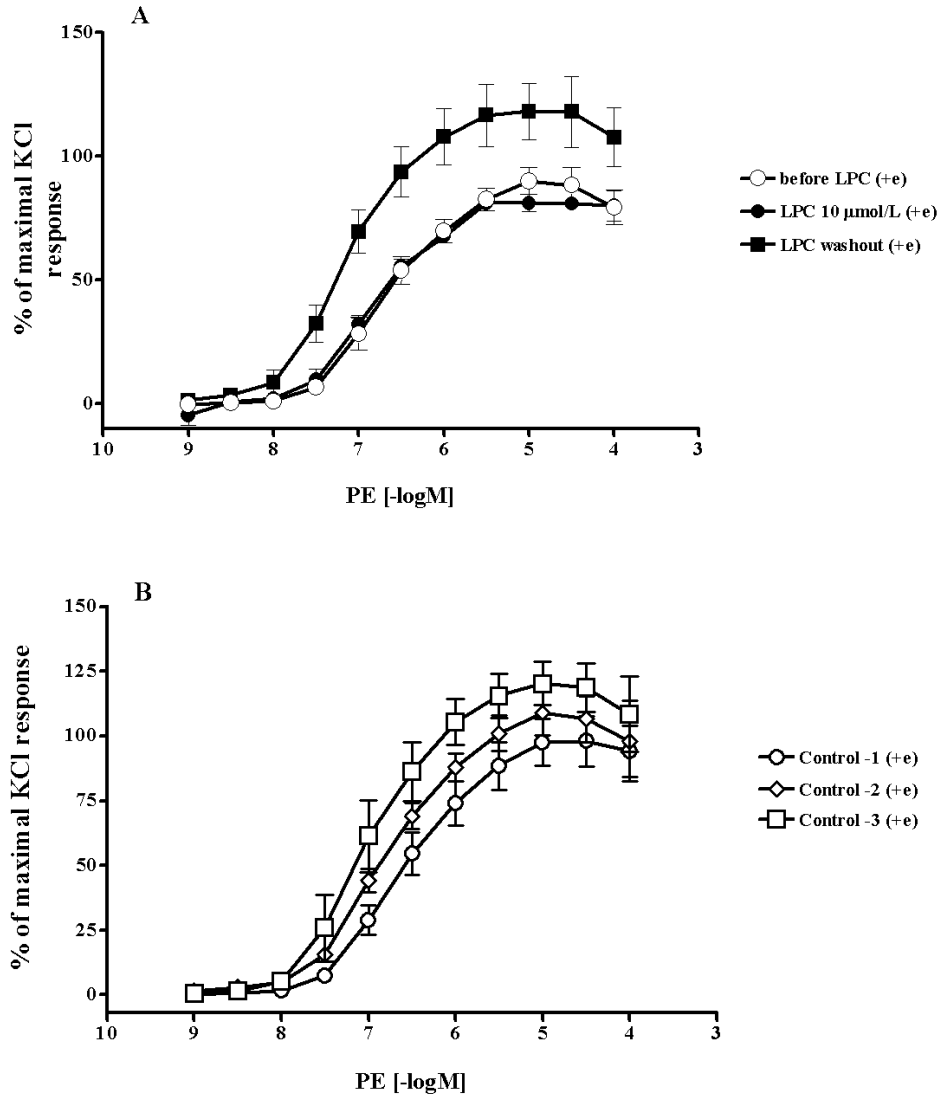

Figure 3.10 Effects of LPC on PE responses in endothelium-intact superior mesenteric arteries. A, cumulative concentration-response curves of endothelium-intact superior mesenteric arteries to PE before, after 40-min incubation with 10 µmol/L LPC, and after washout of LPC for 60 min (n = 4). B, cumulative concentration-response curves of untreated endothelium-intact superior mesenteric arteries to PE at the same fixed time intervals. (n = 4). All data represent the mean ± S.E.M. +e: endothelium-intact.

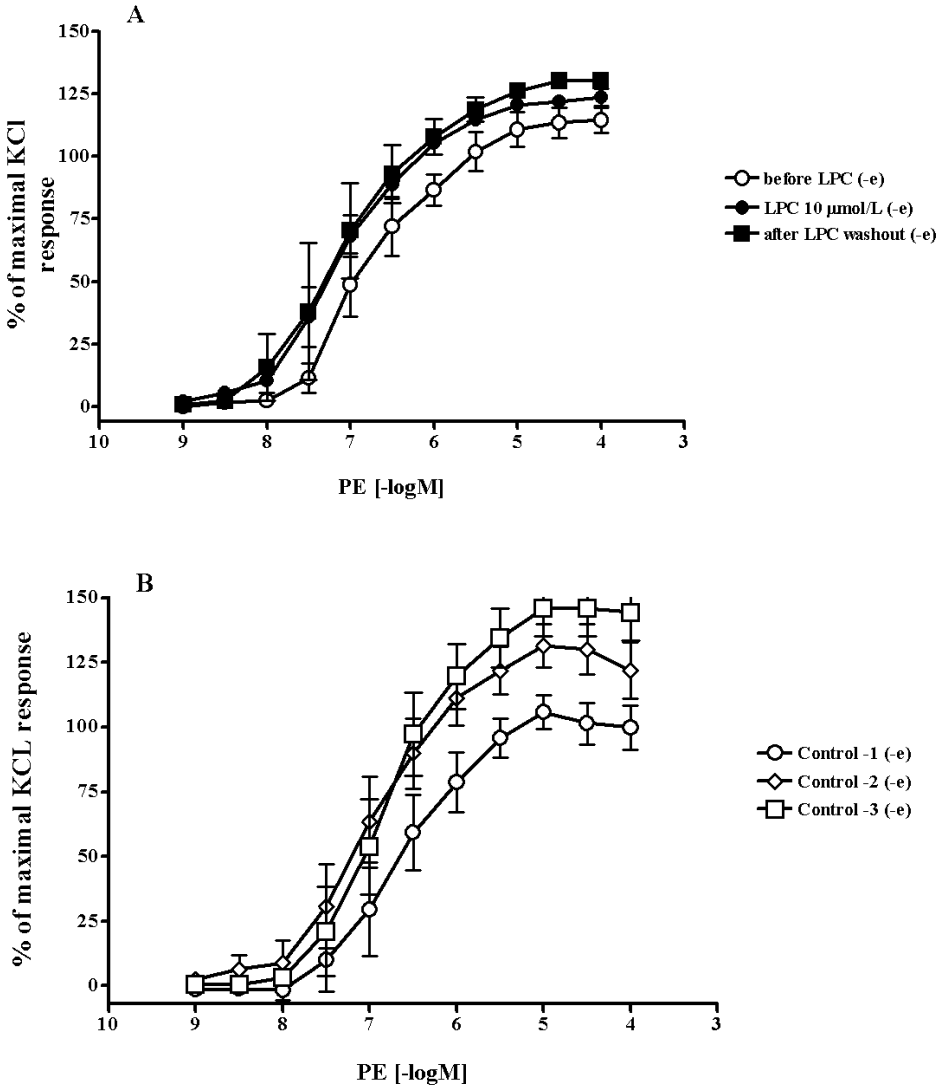

Figure 3.11 Effects of LPC on PE responses in endothelium-denuded superior mesenteric arteries. A, cumulative concentration-response curves of endothelium-denuded superior mesenteric arteries to PE before, after 40-min incubation with 10 µmol/L LPC, and after washout of LPC for 60 min (n = 4). B, cumulative concentration-response curves of untreated endothelium-denuded superior mesenteric arteries to PE at the same fixed time intervals. (n = 4). All data represent the mean ± S.E.M. -e: endothelium-denuded.

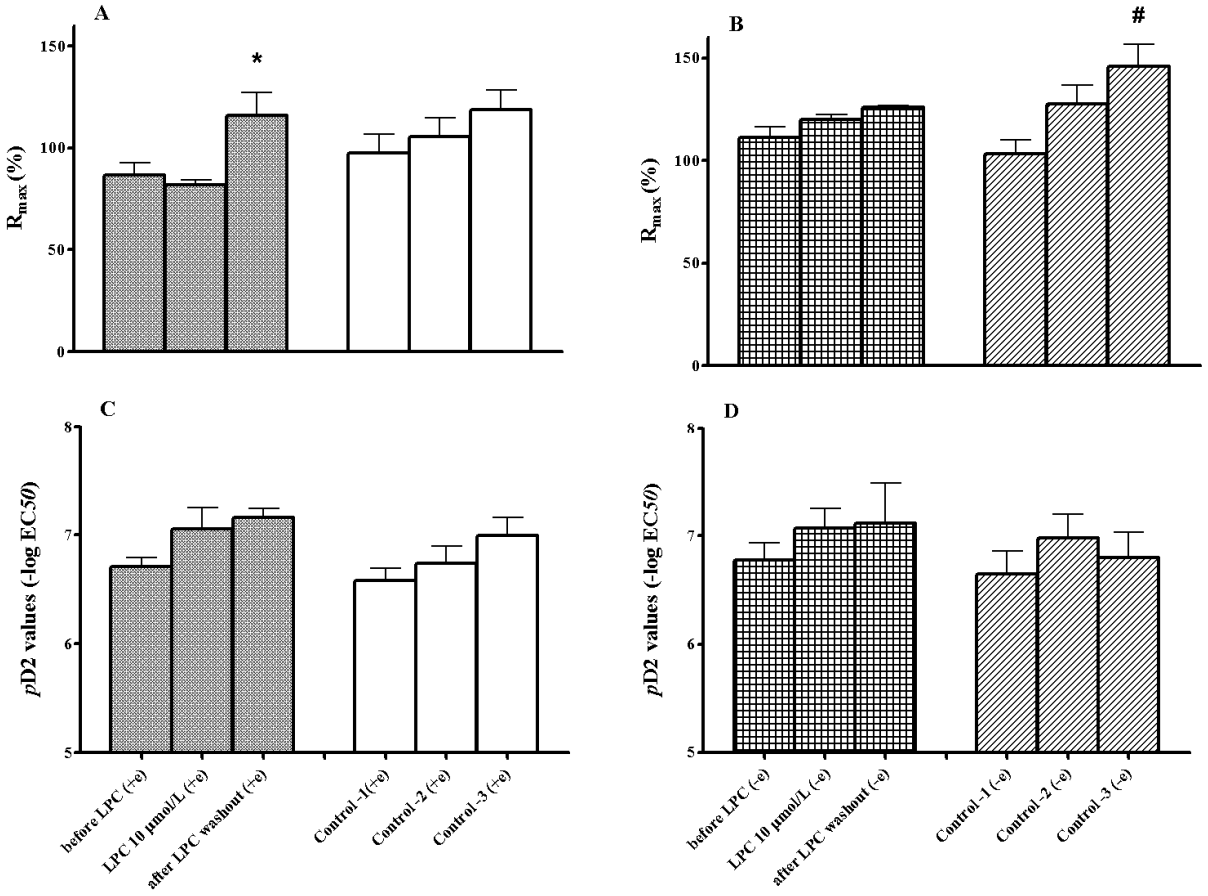

Figure 3.12 PE R_{max} and pD_2 values in superior mesenteric arteries. A, PE R_{max} values in LPC-treated or untreated endothelium-intact superior mesenteric arteries. B, PE R_{max} values in LPC-treated or untreated endothelium-denuded superior mesenteric arteries. C, PE pD_2 values in LPC-treated or untreated endothelium-intact superior mesenteric arteries. D, PE pD_2 values in LPC-treated or untreated endothelium-denuded superior mesenteric arteries. +e: endothelium-intact, -e: endothelium-denuded. All data represent the mean ± S.E.M. n=4. *, $P < 0.05$ versus "before LPC (+e)" and "LPC 10 μmol/L." $^{\#}$, $P < 0.05$ versus "Control-1 (-e)" (one-way ANOVA followed by Newman-Keuls test).

3.3 LPC endothelium-dependently modulates rat coronary arterial tone

3.3.1 LPC mimics the effects of I/R on coronary perfusion pressure and ventricular function in isolated perfused Langendorff hearts

In isolated hearts, ischemia followed by reperfusion produced a significant increase in CPP (Figure 3.13A). In addition, during reperfusion, heart function (LVDP) was not restored to pre-ischemic values, and demonstrated significant impairment (Figure 3.13B). On the other hand, sustained perfusion of isolated hearts with 3 μmol/L LPC for 40 min (minimal concentration that produced significant changes) tended to augment CPP (Figure 3.14A) and reduce LVDP (Figure 3.14B). More interestingly, these changes in CPP and LVDP were exaggerated when LPC was removed from the perfusate, suggesting that the detrimental effects of LPC are not only irreversible, but also enhanced following its removal. Thus, our data confirm the previously reported influence of LPC on coronary vascular resistance, but more importantly, illustrate a novel residual effect following washout of this lysolipid.

3.3.2 Effects of LPC on coronary resistance and heart function are not likely to be dependent on TxA_2 or ET-1.

To investigate the involvement of TxA_2 in the effects of LPC, TxB_2 contents in the coronary effluent during I/R and LPC-treatment were measured. In the I/R hearts, a significant increase in TxB_2 levels was only observed around the first 5 min of reperfusion (Figure 3.15A). This burst in TxA_2 release could be a consequence of the various mechanisms activated during the 32-min period of ischemia, possibly including the excess generation of LPC. However, during the following reperfusion period, TxA_2 production recovered to the basal level (Figure 3.15A), despite the concurrent increase in

coronary resistance (Figure 3.13). Similarly, in those hearts subjected to LPC perfusion and its following washout, TxA_2 release was only markedly enhanced at the end of 40-min LPC perfusion (Figure 3.15B), not the subsequent washout period. The discrepancy between the release of TxA_2 and effects of LPC on heart function suggests that TxA_2 is not likely to be responsible for the vasoconstricting effects of LPC.

To examine the possible role of ET-1 in mediating the direct and washout effects of LPC, bosentan (3 μmol/L), a dual endothelin receptor antagonist, was used during the LPC perfusion and washout procedure. However, neither the potentiation in CPP nor the deterioration in LVDP was prevented by this ET-1 blocker (Figure 3.16). Thus, ET-1 is unlikely to be the mediator that produces the effects of LPC in isolated hearts.

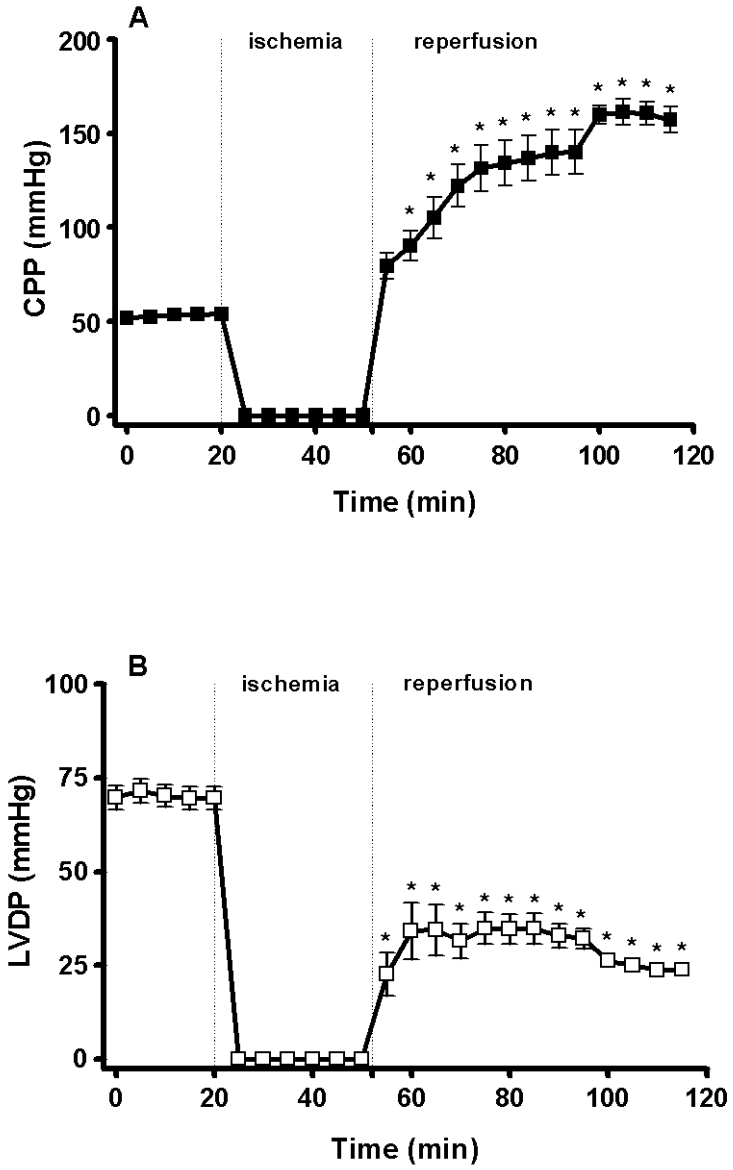

Figure 3.13 Ischemia/reperfusion alters isolated heart function. Langendorff-perfused rat hearts were subjected to 32 min global ischemia by arresting the flow of buffer solution. This was followed by 60 min of reperfusion. At the indicated times, coronary perfusion pressure (CPP, A) and left ventricular developed pressure (LVDP, B) were determined. Values are means ± SE, n=8-9. Statistical analysis was performed using one-way ANOVA. *Significantly different from pre-treatment, $P<0.05$.

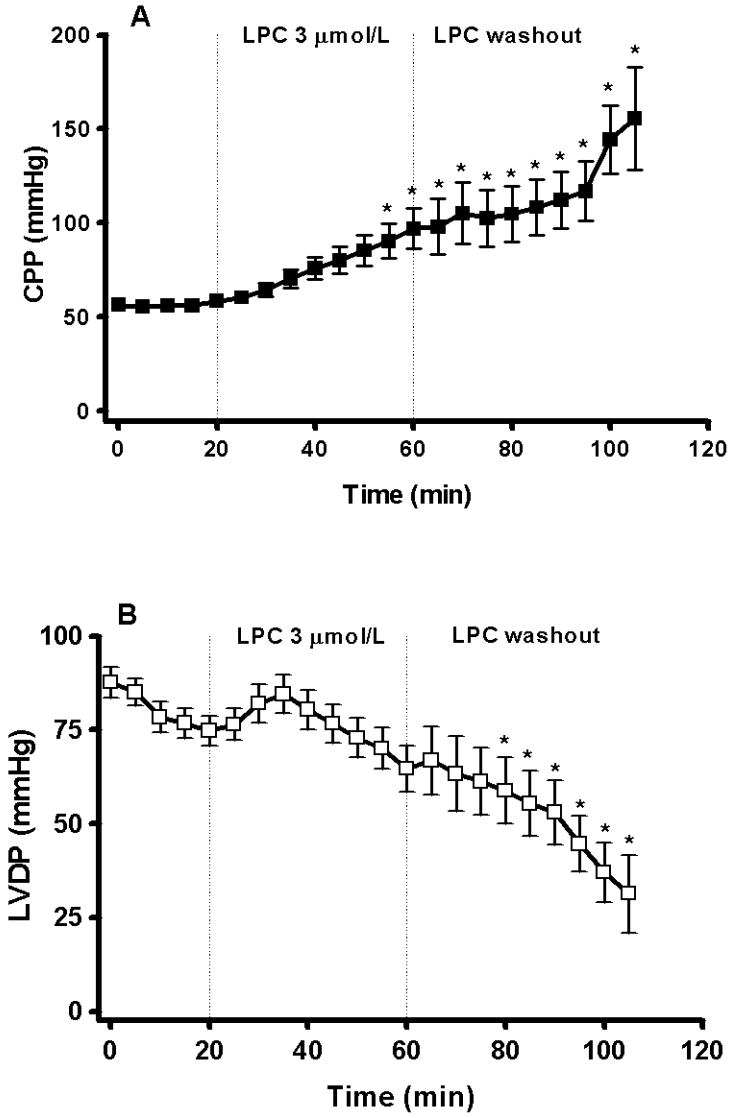

Figure 3.14 LPC alters isolated heart function. Isolated rat hearts were perfused with Krebs-Henseleit buffer containing LPC (3 μmol/L) for 40 min in the nonrecirculating Langendorff mode. Subsequently, the buffer solution was changed to normal Krebs-Henseleit buffer, and the perfusion maintained for another 60 min. Changes in coronary perfusion pressure (CPP, A) and left ventricular developed pressure (LVDP, B) were determined at the indicated times. Values are means ± SE, n=8-9. Statistical analysis was performed using one-way ANOVA. *Significantly different from pre-treatment, $P<0.05$.

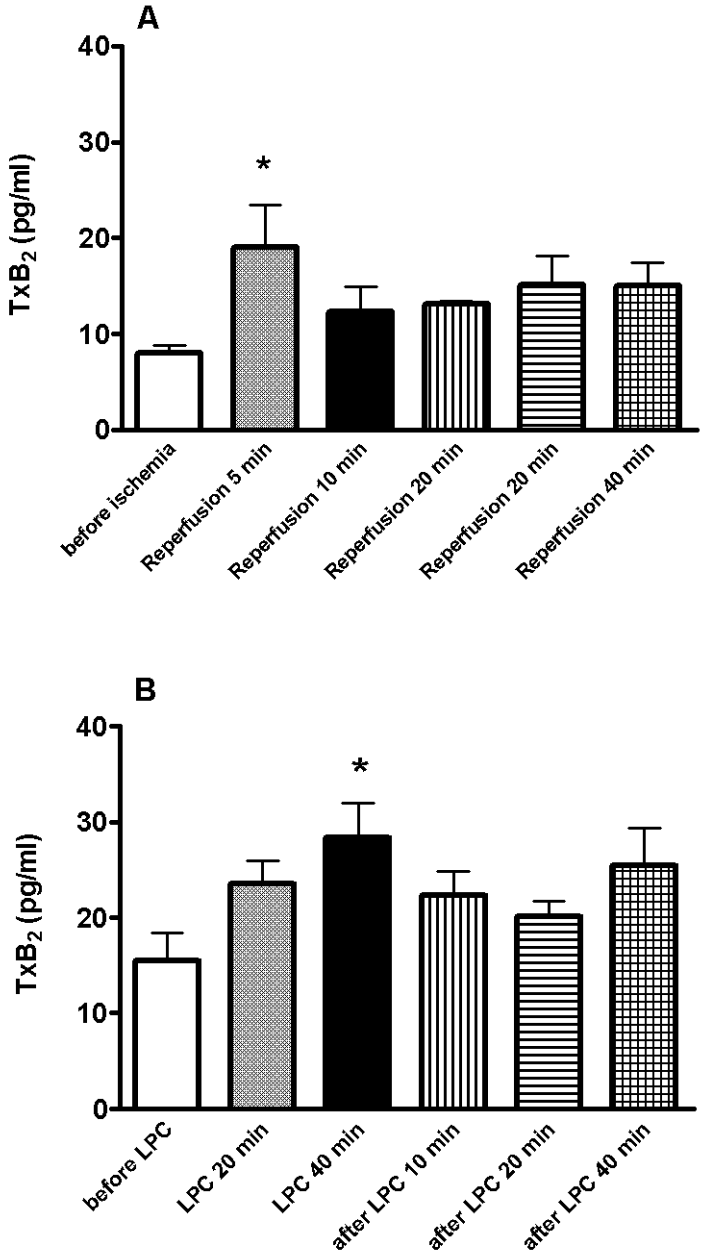

Figure 3.15 TxB_2 release from Langendorff hearts subjected to ischemia/reperfusion (A), or 3 μmol/L LPC perfusion (B) at the indicated times. Data represent the mean ± S.E.M. *n*=3. *, $P < 0.05$ versus the pretreatment level (one-way ANOVA followed by Newman-Keuls test).

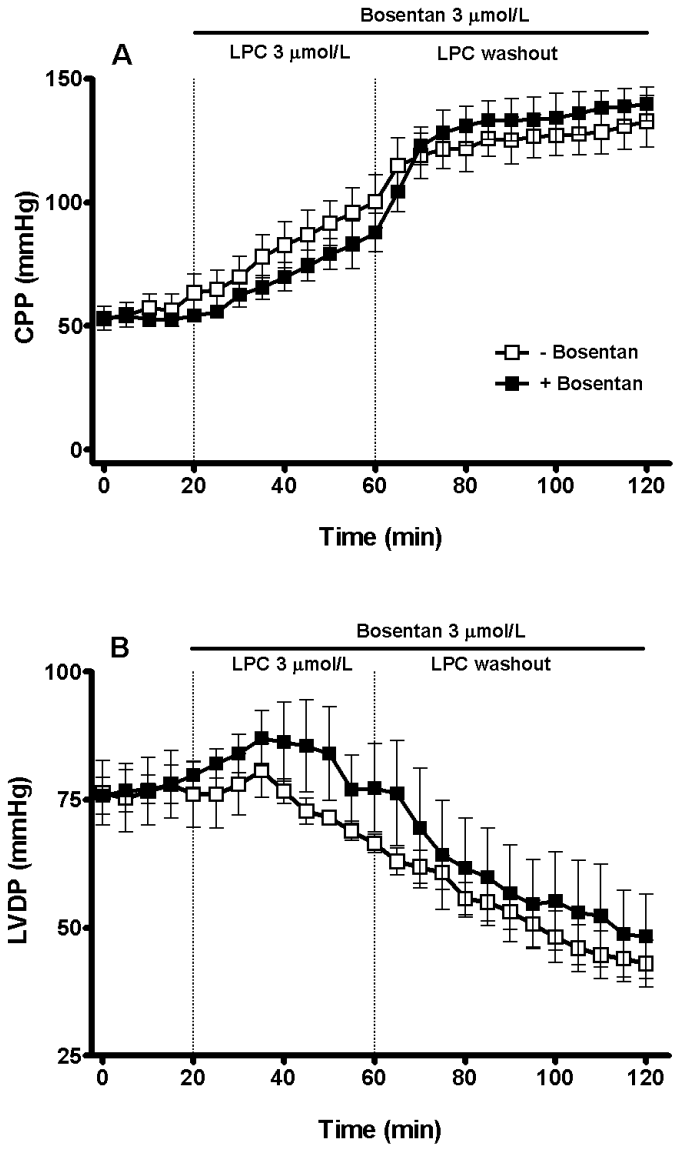

Figure 3.16 Bosentan in LPC-treated isolated perfused hearts. Isolated rat hearts were perfused with Krebs-Henseleit buffer containing LPC (3 μmol/L) for 40 min in nonrecirculating Langendorff mode. Subsequently, the buffer solution was changed to normal Krebs-Henseleit buffer, and the perfusion maintained for another 60 min. Bosentan (3 μmol/L) were added to the buffer during the indicated period. Changes in CPP (A) and LVDP (B) were determined. Values are means ± S.E.M, *n*=4. Statistical analysis between bosentan –treated and –untreated was performed using two-way ANOVA with the general linear model approach (repeated measurements) followed by Newman–Keul's test. Bosentan group was not significantly different from untreated group.

3.3.3 Effects of LPC on the vasoconstrictor responses to U-46619 in isolated coronary septal arteries using the pressure myograph

Unlike the isolated heart, intraluminal LPC had no direct contractile effect on isolated pressurized coronary arteries (Figure 3.17). The contractile responses to KCl were not significantly affected by LPC or its washout either (Figure 3.18). Nevertheless, LPC selectively altered the receptor-coupled vasoconstrictor response of this artery to U-46619, which is a TxA_2 mimetic, mediates vascular smooth muscle contraction through a signaling cascade that involves phosphatidylinositol turnover, activation of the small GTPase Rho, Rho kinase, myosin light chain kinase, and PKC (Fu et al., 1998; Sakurada et al., 2001; Nobe et al., 2004). As shown in Figure 3.19A, LPC significantly shifted the U-46619 CRC to the left (pD_2 value increased from 6.72 ± 0.15 to 7.41 ± 0.17, $P<0.05$, Figure 3.19, inset). Interestingly, when LPC was washed out and replaced with control Krebs buffer, the leftward shift was even more pronounced (pD_2 value increased to 7.81 ± 0.22, $P<0.05$, Figure 3.19, inset). The observation that this potentiating effect to U-46619 was only apparent after washout of LPC was confirmed by prolonged incubation with LPC (100 min, Figure 3.20). Under this condition and in the protracted presence of LPC, the potentiation to U-46619 (pD_2 7.43 ± 0.22) was similar to that found after 40 min incubation (pD_2 7.41 ± 0.17). However, LPC-washout following this prolonged incubation was able to generate the additional potentiation (pD_2 value increased to 7.95 ± 0.25, $P<0.05$). Appropriate time control experiments were also conducted for U-46619, and the three consecutive CRCs remained unchanged over the duration of the experiment (Figure 3.21, pD_2 values: 6.85 ± 0.17, 6.84 ± 0.20, 7.07 ± 0.22, $P>0.05$). Overall, these results in the coronary septal arteries are consistent with those obtained in the isolated

perfused hearts which showed continued augmentation of coronary resistance even upon removal of LPC.

To test if LPA mediates the delayed responses induced by LPC in the coronary artery, the U-46619 responses to 3 μmol/L LPA present intraluminally were determined. Similar to what was found in the MAB, LPA did not significantly modify the vasoconstriction to U-46619 (Figure 3.22), suggesting that LPA may not be involved in the effects of LPC.

It is important to note that in the wire myograph, using the same protocol, 3 μmol/L LPC did not produce significant changes to the vasoconstrictor responses to U-46619 (Figure 3.23). Taken together, these results imply that even the different ways in which blood vessels develop tension, as well as the location where the stimuli occur may markedly influence the behavior of the same kind of tissues.

3.3.4 Involvement of endothelium in affecting the response to U-46619 after LPC

When the CRCs to U-46619 were performed using endothelium-denuded coronary arteries (Figure 3.19B), a leftward shift was observed compared to endothelium-intact vessels (Figure 3.19A), suggesting that under normal conditions, U-46619-induced contraction is partly regulated by the endothelium. Interestingly, in these denuded arteries, no effect of LPC was observed, either during LPC perfusion or after LPC washout (Figure 3.19B), indicating that both the immediate and residual effects of LPC are endothelium-dependent.

3.3.5 LPC washout impairs the vasodilator response to Ach

The endothelium-dependent vasodilator responses to Ach were stable in isolated coronary septal arteries over time (Figure 3.24A). It is known that Ach-induced

relaxation is generally mediated by three components: NO, PGI_2 and EDHF. However, as shown in Figure 3.24B, compared to control, pre-treatment with $_L$-NAME almost completely attenuated the relaxant response of coronary artery to Ach. This result suggests that in Wistar rat septal coronary arteries, NO is the predominant mediator of Ach-induced vasorelaxation.

In the presence of LPC, there was no impairment in the Ach-induced vasodilator responses (Figure 3.25). Remarkably, following LPC removal from the lumen, there was a robust inhibition of the CRC to Ach (Figure 3.25), with significant reduction in both the pD_2 value (Figure 3.25, left inset) and R_{max} (Figure 3.25, right inset), suggesting that decreased release of endothelium-dependent relaxing factor (EDRF), mainly NO, is likely to be involved in the residual effects of LPC. Thus, it appears that compared to the direct effects of LPC, its washout recruits additional or even completely different mechanisms to alter the vasoconstrictor response to U-46619. It should be noted that CRCs to SNP were not impaired during LPC or after its washout (Figure 3.26), further confirming the selective involvement of endothelium in the effects of LPC.

To test if the impaired endothelial function is related to ROS, TEMPOL, a stable membrane-permeable superoxide scavenger, was applied during LPC incubation and its washout. TEMPOL has been shown to attenuate the inflammation induced by superoxide and peroxynitrite (Sayed-Ahmed et al., 2001) and lower blood pressure in a variety of models (Xu et al., 2004). However, in our study, 100 μmol/L TEMPOL did not improve the impaired Ach-relaxation following LPC washout (Figure 3.27), implying that superoxide is not likely to be responsible for the residual effects of LPC.

3.3.6 Involvement of ET-1 in mediating the residual effects of LPC

To examine the possible role of ET-1 in mediating the direct and washout effects of LPC, bosentan (1 μmol/L) was used during the LPC perfusion and washout procedure. Bosentan is a dual endothelin receptor antagonist, with a K_i of 4.7 nmol/L on human ET_A receptors and a K_i of 95 nmol/L on ET_B receptors (Clozel et al., 1994), and is used in the treatment of pulmonary arterial hypertension. The direct potentiating effect of U-46619 in the presence of LPC was unchanged by bosentan (Figure 3.28A). Interestingly, the additional potentiation to U-46619 following LPC washout was prevented by bosentan (Figure 3.28B). Bosentan exerted no significant effect on the responses to U-46619 alone (Figure 3.28 right inset). These data suggest that the augmented vasoconstrictor responses following LPC washout are related to an increase in ET-1.

3.3.7 The direct effects of LPC are dependent on EDRFs

To elucidate the underlying mechanism of the immediate effects of LPC on U-46619 induced vasoconstriction, an inhibitor cocktail (ACIL) consisting of 0.5 μmol/L apamin and 25 μmol/L charybdotoxin (to block endothelium-derived hyperpolarizing factor, EDHF), 10 μmol/L indomethacin (to block prostacyclin, PGI_2), and 10 μmol/L $_L$-NAME (to block NOS) was used during the intraluminal LPC perfusion. ACIL was used to exclude the involvement of EDRFs in rat coronary artery previously (Gustafsson et al., 1992). Among these inhibitors, the combination of apamin and charybdotoxin has been commonly employed to achieve a complete block of EDHF-mediated relaxation in various blood vessels by inhibiting small-conductance and large-conductance Ca^{2+}-activated potassium channels (Murphy and Brayden, 1995; Corriu et al., 1996; Waldron and Cole, 1999). Although the individual perfusion with ACIL or LPC significantly

potentiated the vasoconstrictor responses (Figure 3.29), the combined effects of LPC and ACIL were not greater than that of ACIL alone (Figure 3.29). These data suggest that the direct effects of LPC on U-46619-responses are dependent on one or more of the following EDRFs: EDHF, PGI_2 and/or NO.

3.3.8 EDHF is the major contributor to the direct effects of LPC

In order to investigate which EDRFs might contribute to the direct effects of LPC, inhibitors of NO (10 μmol/L $_L$-NAME), PGI_2 (10 μmol/L NS-398) and EDHF (0.5 μmol/L apamin + 25 μmol/L charybdotoxin) were applied individually during the intraluminal LPC perfusion. NS-398 is a cell-permeable and selective inhibitor of COX-2 over COX-1 (Gierse et al., 1995), and was successfully utilized as a selective PGI_2 synthesis inhibitor in arterial tissues previousely (Guo, 2005; Qi et al., 2007; Santhanam et al., 2007; Meziani et al., 2008). The vasoconstriction to U-46619 was significantly potentiated by each of these inhibitors as well as 3 μmol/L LPC alone (Figure 3.30-32). The co-incubation with LPC and $_L$-NAME had an additive effect in enhancing the responses to U-46619 (Figure 3.30), suggesting that the direct potentiation induced by LPC is independent of NO. Similarly, when NS-398 was combined with LPC, an additive effect was also observed at higher concentrations of U-46619 (Figure 3.31), which implies that PGI_2 is not likely involved in the direct effects of LPC. However, the combination of LPC with apamin and charybdotoxin did not exhibit any additional enhancement to U-46619 responses compared to apamin and charybdotoxin alone (Figure 3.32), suggesting that the immediate effects of LPC are dependent on attenuated release of EDHF. In summary, EDHF is likely the sole mediator responsible for the direct effects of LPC.

3.4 ET-1 levels in cultured bovine coronary arterial endothelial cells

In order to further investigate the role of ET-1 as one of the mediators contributing to the residual effects of LPC, ET-1 release from BCAECs treated with 3 μmol/L LPC and the following washout was measured. Figure 3.33 demonstrates the ET-1 release in response to U-46619 in cultured BCAECs treated with 3 μmol/L LPC for 40 min followed by washout for 60 min. Under basal conditions, BCAECs produced ET-1 and released it into the media at 41 ± 5 pg/ml. U-46619 (0.1 and 1 μmol/L) alone did not significantly elevate this basal level. In those cells incubated with 3 μmol/L LPC, only the ET-1 production stimulated by 0.1 μmol/L U-46619 was increased to 79 ± 20 pg/ml. However, following LPC washout, this level declined to 36 ± 5 pg/ml. Therefore, although LPC increased the production induced by 0.1 μmol/L U-46619, this effect was not sustained after LPC washout, which is different from the residual effect we observed in the intact coronary arteries.

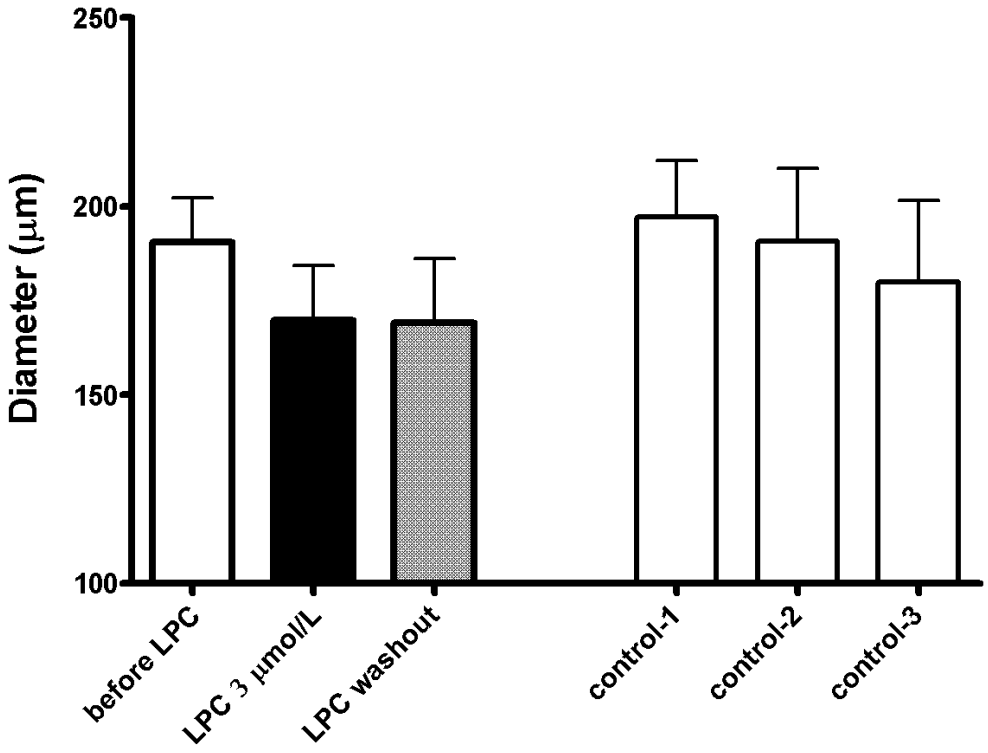

Figure 3.17 Resting internal diameter of isolated rat septal coronary arteries exposed to LPC perfusion for 40 min and the following 60-min washout (left), and under control conditions (right) using a pressure myograph. Values are means ± S.E.M. *n*=6-8. Statistical evaluation was done using one-way ANOVA followed by Bonferroni's test when appropriate.

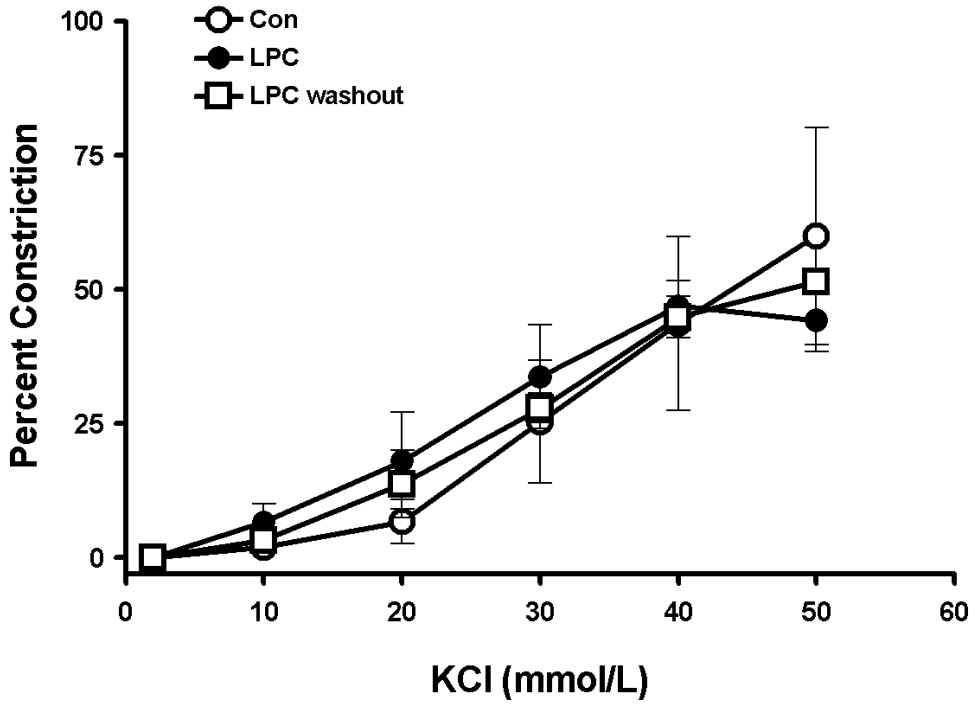

Figure 3.18 Effects of LPC on contractile responses to KCl (2-50 m mol/L) in isolated intact rat septal coronary arteries using a pressure myograph. The responses to KCl were determined in the absence, presence (40 min), and following washout (60 min) of LPC (3 μ mol/L) that was delivered intraluminally. Values are means ± S.E.M. *n*=4. Data were analyzed for significant differences using NCSS. Two-way ANOVA using the general linear model approach (repeated measurements) followed by Newman–Keul's test was used for comparisons between CRCs.

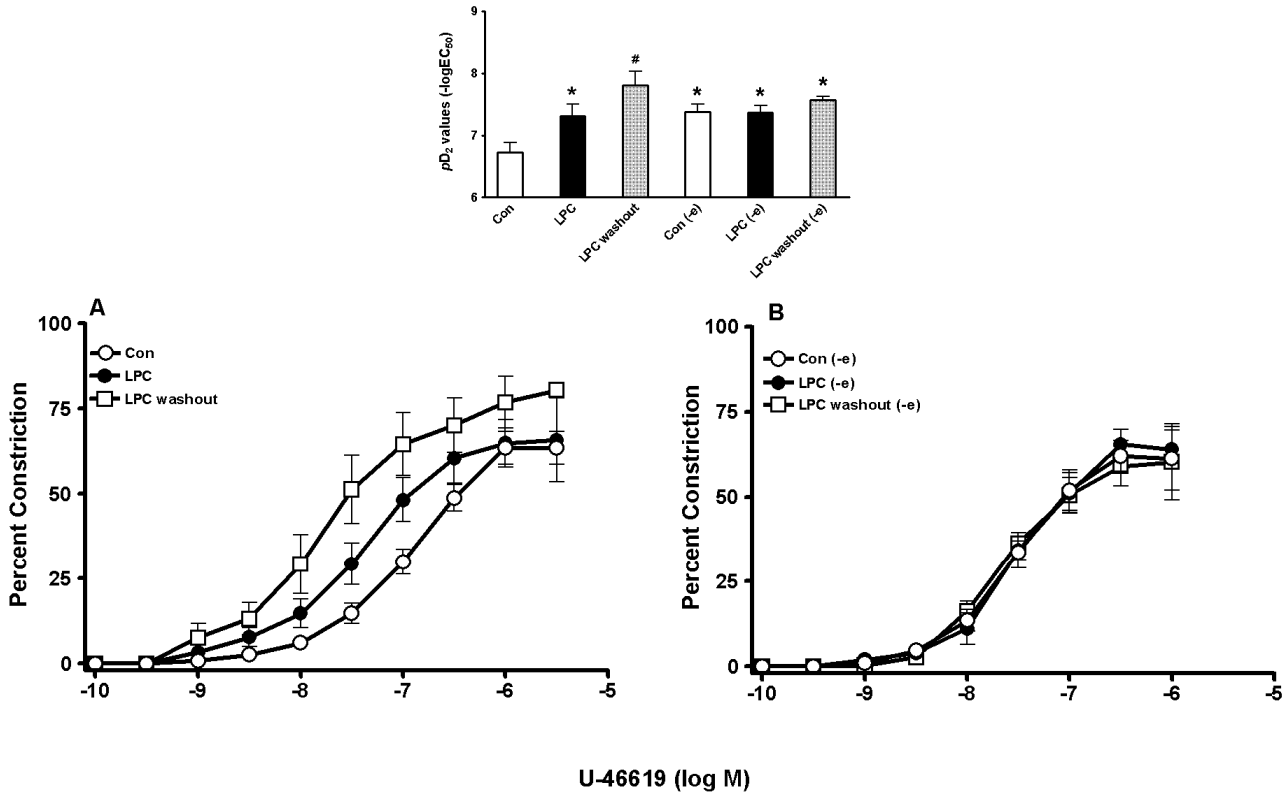

Figure 3.19 Effects of LPC on cumulative concentration-response curves (CRCs) to U-46619 in isolated intact and endothelium-denuded (-e) rat septal coronary arteries using a pressure myograph. The responses to U-46619 were determined in the absence, presence (40 min), and following washout (60 min) of LPC (3 μ mol/L) that was delivered intraluminally in intact (A) (n=13) and endothelium-denuded coronary arteries (B) (n=7). The inset depicts U-46619 pD_2 values in rat septal coronary arteries exposed to LPC. Values are means ± S.E.M. CRCs to U-46619 were analyzed by nonlinear regression for calculation of pD_2 (-log EC_{50}) values and maximum responses (R_{max}). Statistical evaluation was done using one-way ANOVA followed by Bonferroni's test when appropriate. *Significantly different from control, #Significantly different from all other groups, $P<0.05$.

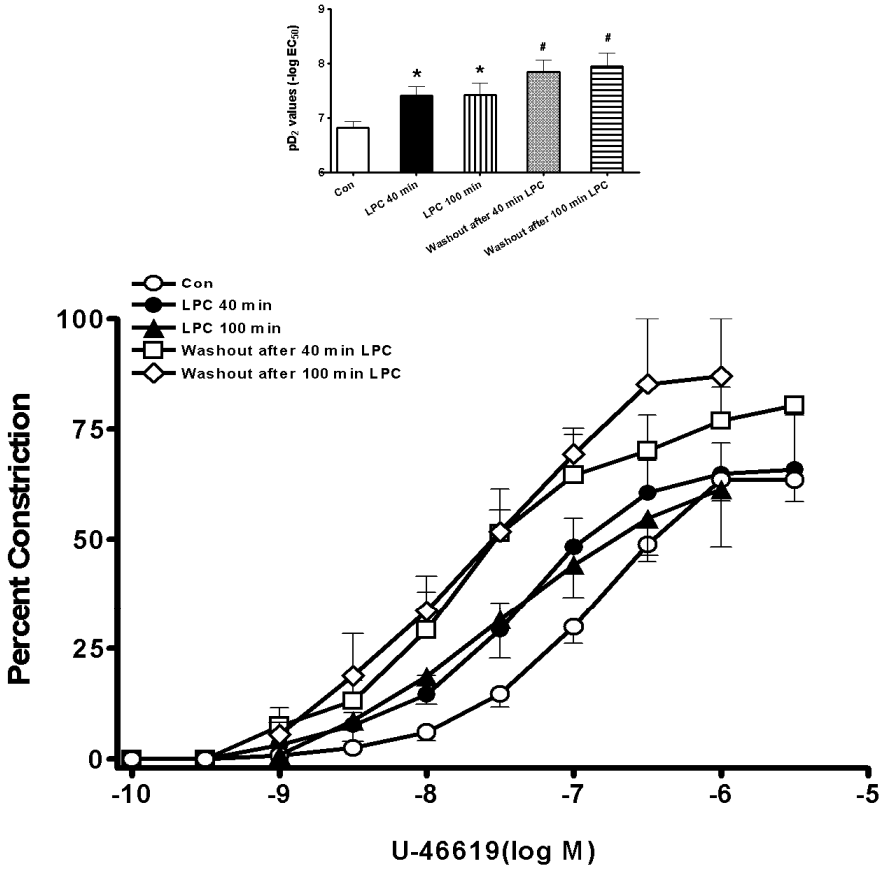

Figure 3.20 Effects of prolonged incubation with LPC on cumulative concentration-response curves (CRCs) to U-46619 in isolated intact rat septal coronary arteries using a pressure myograph. The responses to U-46619 were determined in the absence, presence (40 and 100 min), and following washout (60 min) of LPC (3 μ mol/L) that was delivered intraluminally in intact coronary arteries (n=6-13). The inset depicts U-46619 pD_2 values in rat septal coronary arteries exposed to LPC. Values are means ± S.E.M. CRCs to U-46619 were analyzed by nonlinear regression for calculation of pD_2 (-log EC_{50}) values and maximum responses (R_{max}). Statistical evaluation was done using one-way ANOVA followed by Bonferroni's test when appropriate. *Significantly different from control, #Significantly different from all other groups, $P<0.05$.

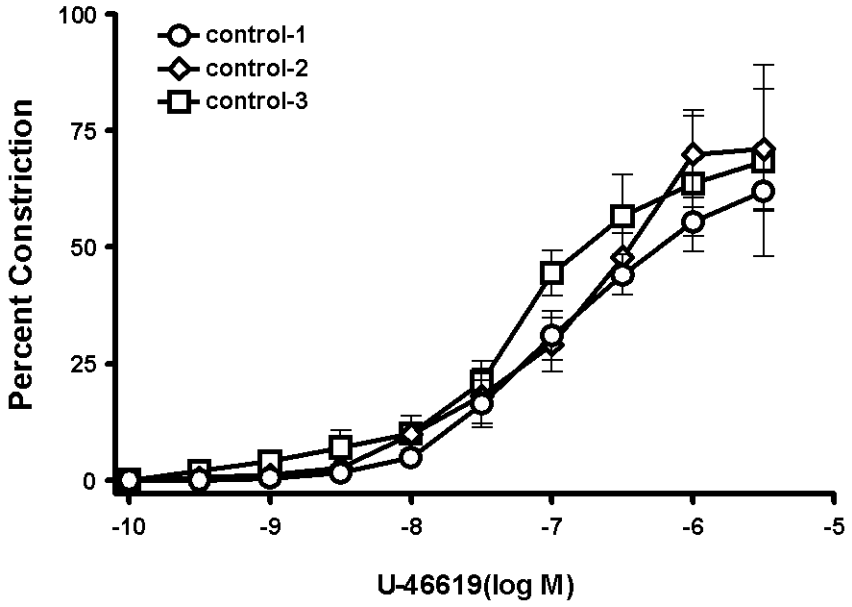

Figure 3.21 Cumulative concentration-response curves (CRCs) to U-46619 in untreated intact rat septal coronary arteries using a pressure myograph. The responses to U-46619 were determined at the same fixed time intervals as those in LPC-treated tissues. Values are means ± S.E.M. n=6. CRCs to U-46619 were analyzed by nonlinear regression for calculation of pD_2 (-log EC_{50}) values and maximum responses (R_{max}). Statistical evaluation was done using one-way ANOVA followed by Bonferroni's test when appropriate. There were no significant differences found between pD_2 or R_{max} values.

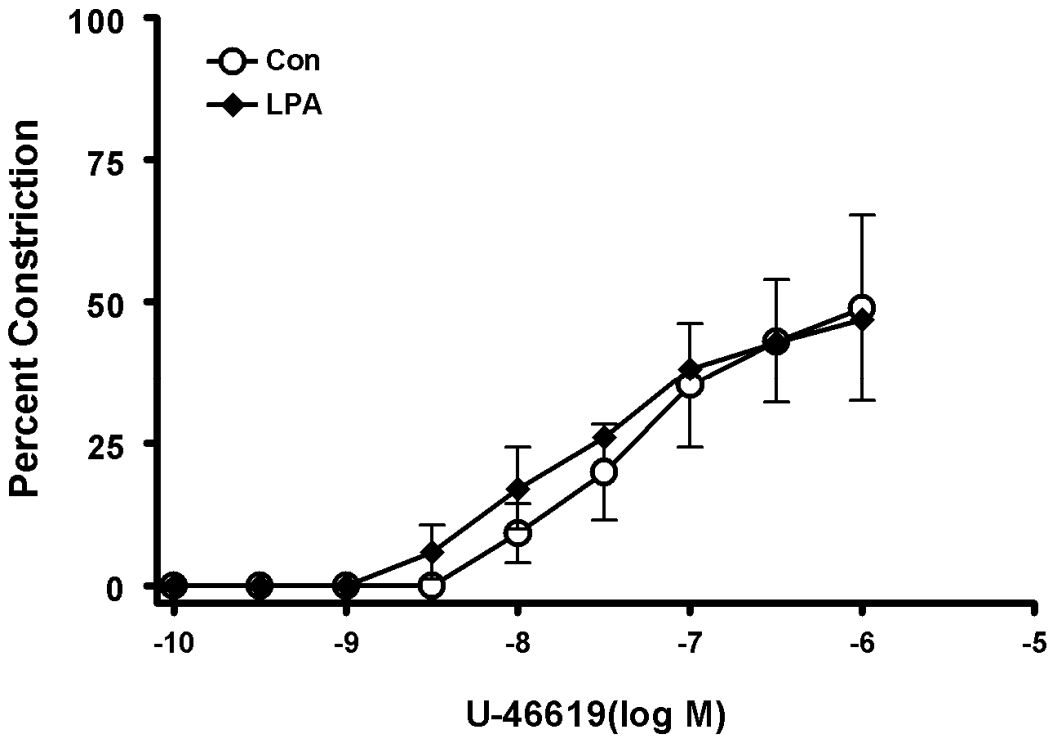

Figure 3.22 Effects of LPA on cumulative concentration-response curves (CRCs) to U-46619 in isolated intact rat septal coronary arteries using a pressure myograph. The responses to U-46619 were determined in the absence and presence (40 min) of LPA (3 μmol/L) that was delivered intraluminally. Values are means ± S.E.M. n=3. CRCs to U-46619 were analyzed by nonlinear regression for calculation of pD_2 (-log EC_{50}) values and maximum responses (R_{max}). Statistical evaluation was done using one-way ANOVA followed by Bonferroni's test when appropriate. There was no significant difference found between pD_2 or R_{max} values.

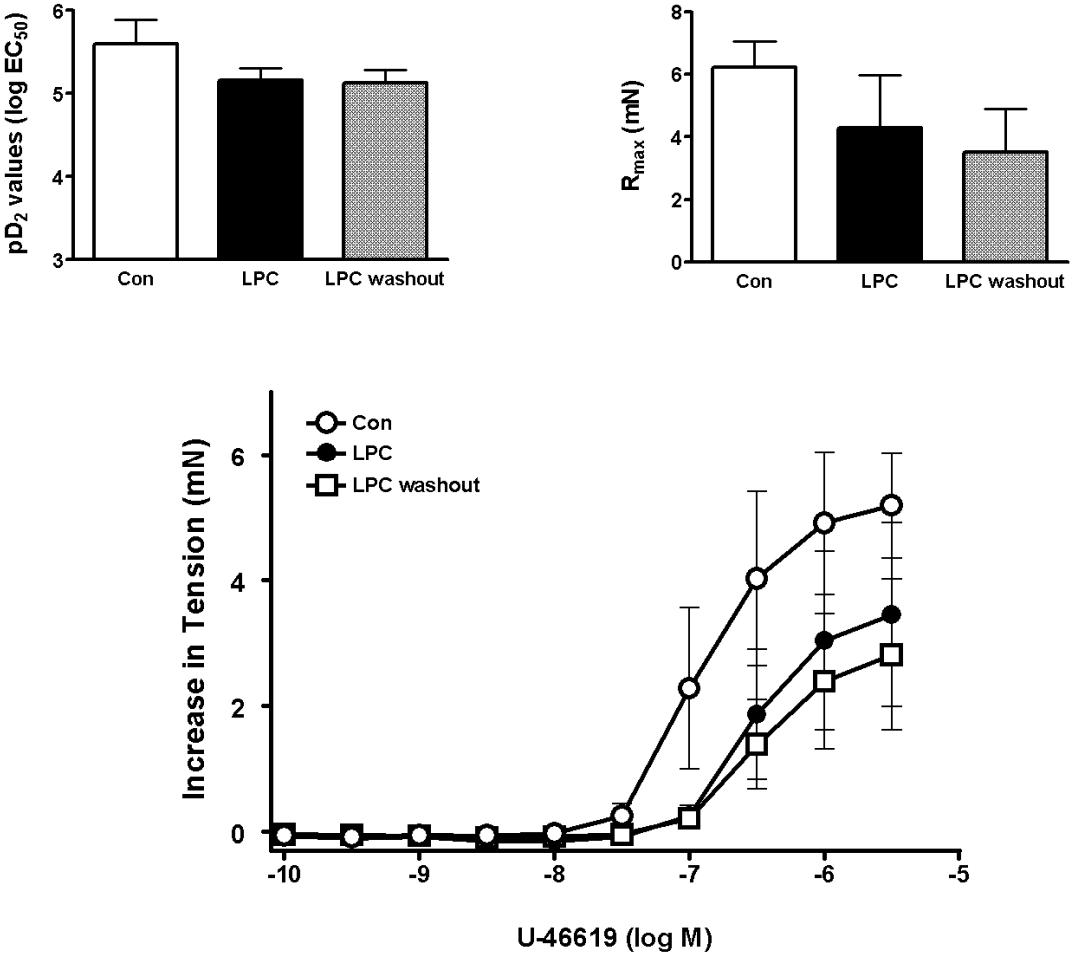

Figure 3.23 Effects of LPC on cumulative concentration-response curves (CRCs) to U-46619 in isolated intact rat septal coronary arteries using a wire myograph. The responses to U-46619 were determined in the absence, presence (40 min), and following washout (60 min) of LPC (3 μ mol/L) (n=4). The insets depict U-46619 $p\mathrm{D}_2$ (left) and R_{max} values (right) in intact rat septal coronary arteries exposed to LPC. Values are means ± S.E.M. CRCs to U-46619 were analyzed by nonlinear regression for calculation of $p\mathrm{D}_2$ (-log EC_{50}) values and maximum responses (R_{max}). Statistical evaluation was done using one-way ANOVA followed by Bonferroni's test when appropriate. There were no significant differences between pD2 or R_{max} values

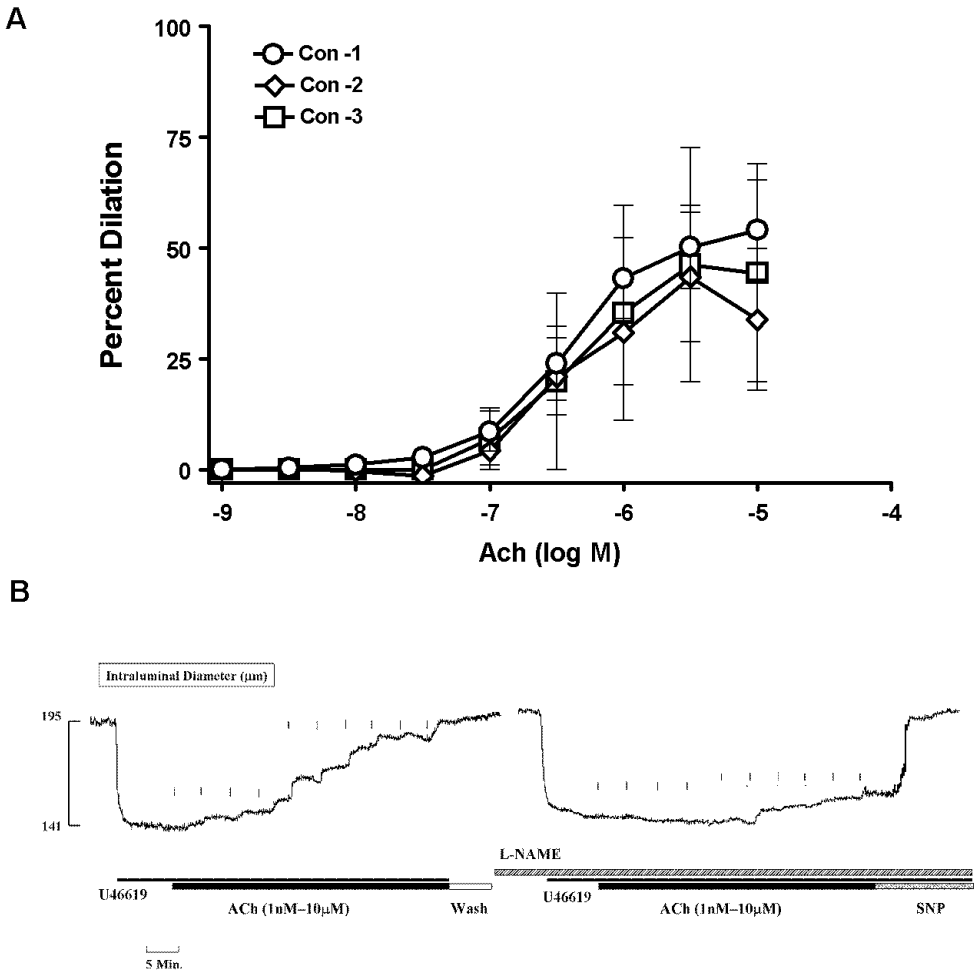

Figure 3.24 A, cumulative concentration-response curves (CRCs) to Ach in untreated isolated intact rat septal coronary arteries using a pressure myograph. The responses to Ach were determined at the same fixed time intervals as those in LPC-treated tissues. Values are means ± S.E.M. *n*=3. CRCs to Ach were analyzed by nonlinear regression for calculation of pD_2 (-log EC_{50}) values and maximum responses (R_{max}). Statistical evaluation was done using one-way ANOVA followed by Bonferroni's test when appropriate. There were no significant differences between *p*D2 or R_{max} values. B, representative recording shows the inhibitory action of $_L$-NAME (100 µ mol/L) on the dilation evoked by Ach (1 nmol/L–10 µmol/L) after pre-constriction with U-46619 (0.1 µ mol/L) using a pressure myograph. In the presence of $_L$-NAME, Ach-induced vasodilation was greatly attenuated. Maximal vasodilation occurred with SNP (1 µ mol/L), a directly acting vasodilator that is an NO donor.

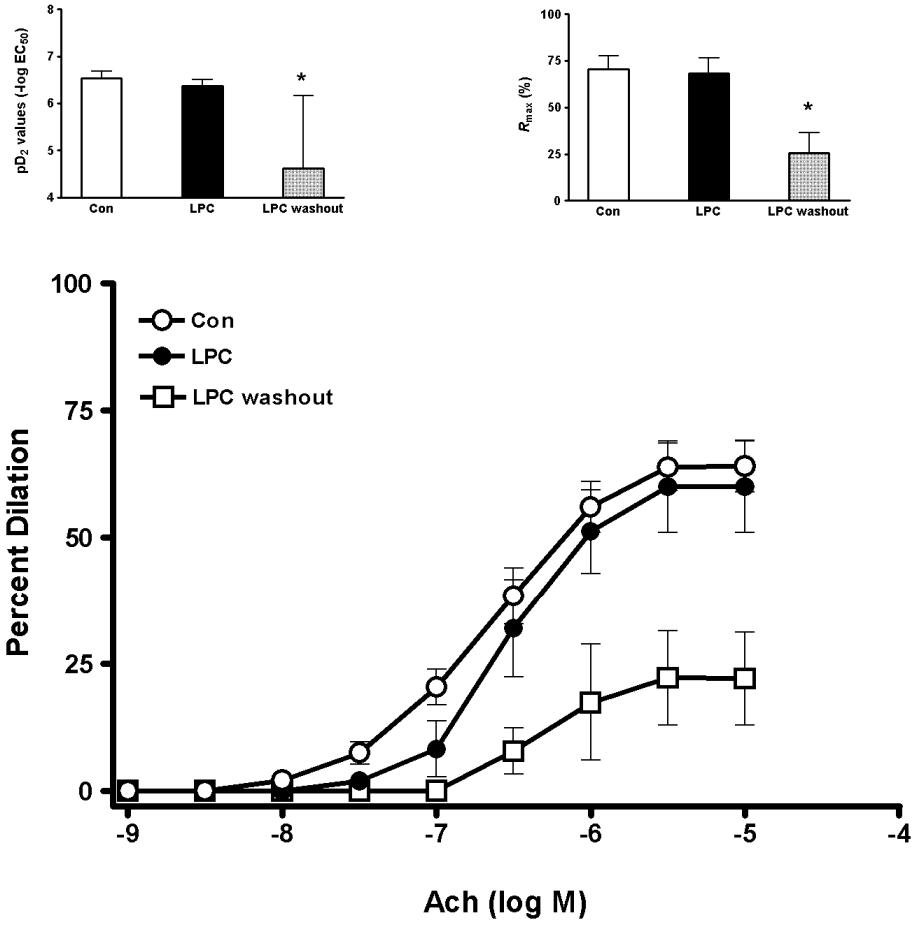

Figure 3.25 Effects of LPC on cumulative concentration-response curves (CRCs) to Ach in isolated intact rat septal coronary arteries using a pressure myograph. The responses to SNP were determined in the absence, presence (40 min), and following washout (60 min) of LPC (3 μmol/L) that was delivered intraluminally (n=6). The insets depict Ach pD_2 (left) and R_{max} values (right) in intact rat septal coronary arteries exposed to LPC. Values are means ± S.E.M. CRCs to Ach were analyzed by nonlinear regression for calculation of pD_2 (-log EC_{50}) values and maximum responses (R_{max}). Statistical evaluation was done using one-way ANOVA followed by Bonferroni's test when appropriate. *Significantly different from control, P<0.05.

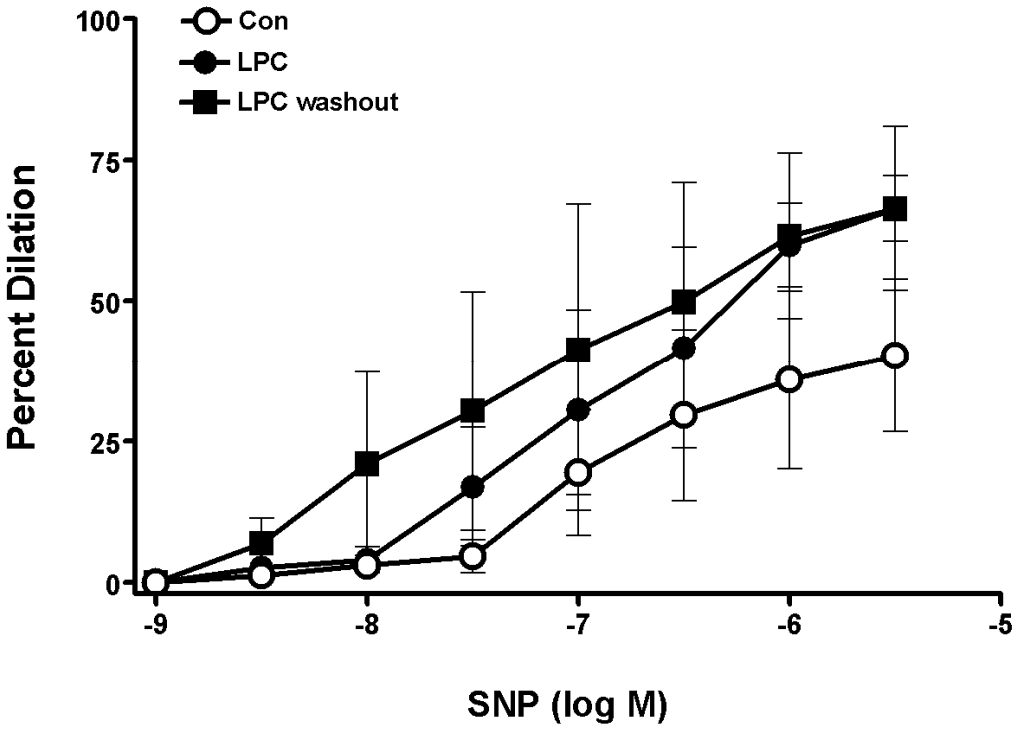

Figure 3.26 Effects of LPC on cumulative concentration-response curves (CRCs) to SNP in isolated intact rat septal coronary arteries using a pressure myograph. The responses to SNP were determined in the absence, presence (40 min), and following washout (60 min) of LPC (3 μmol/L) that was delivered intraluminally. Values are means ± S.E.M. n=3. CRCs to SNP were analyzed by nonlinear regression for calculation of pD_2 (-log EC_{50}) values and maximum responses (R_{max}). Statistical evaluation was done using one-way ANOVA followed by Bonferroni's test when appropriate. There were no significant differences between pD_2 or R_{max} values.

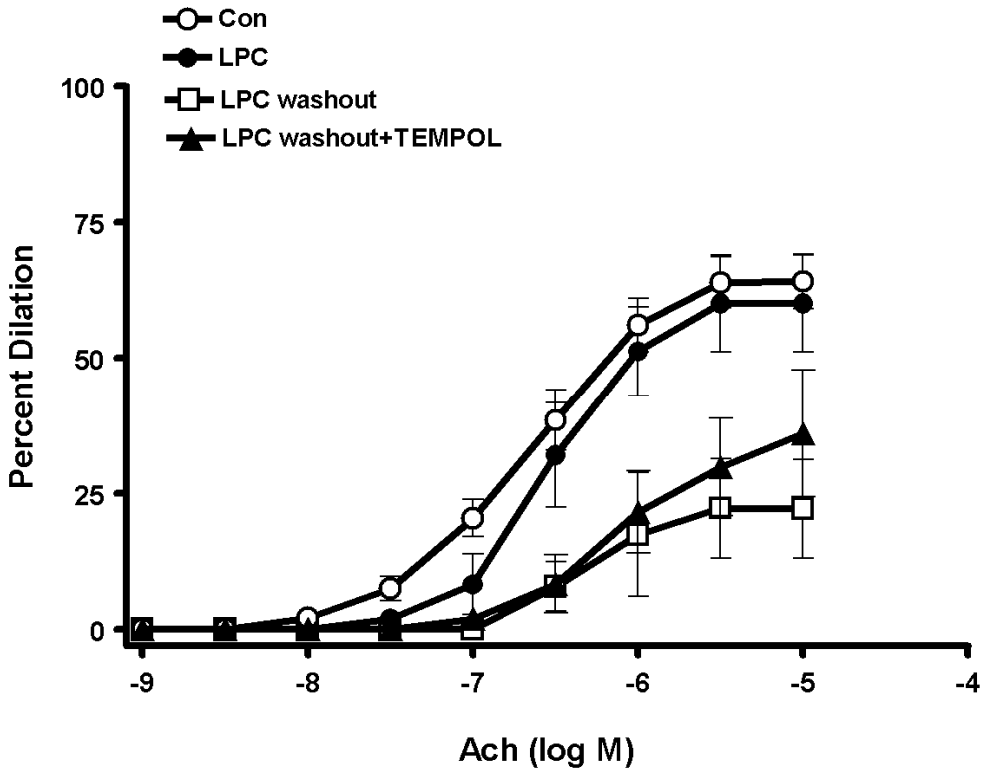

Figure 3.27 The effects of TEMPOL (100 μmol/L) to the impaired Ach-response following LPC washout using a pressure myograph. Values are means ± S.E.M. n=4. CRCs to Ach were analyzed by nonlinear regression for calculation of pD_2 (-log EC_{50}) values and maximum responses (R_{max}). Statistical evaluation was done using one-way ANOVA followed by Bonferroni's test when appropriate. There was no significant difference between the CRCs to "LPC washout" and "LPC washout+TEMPOL"

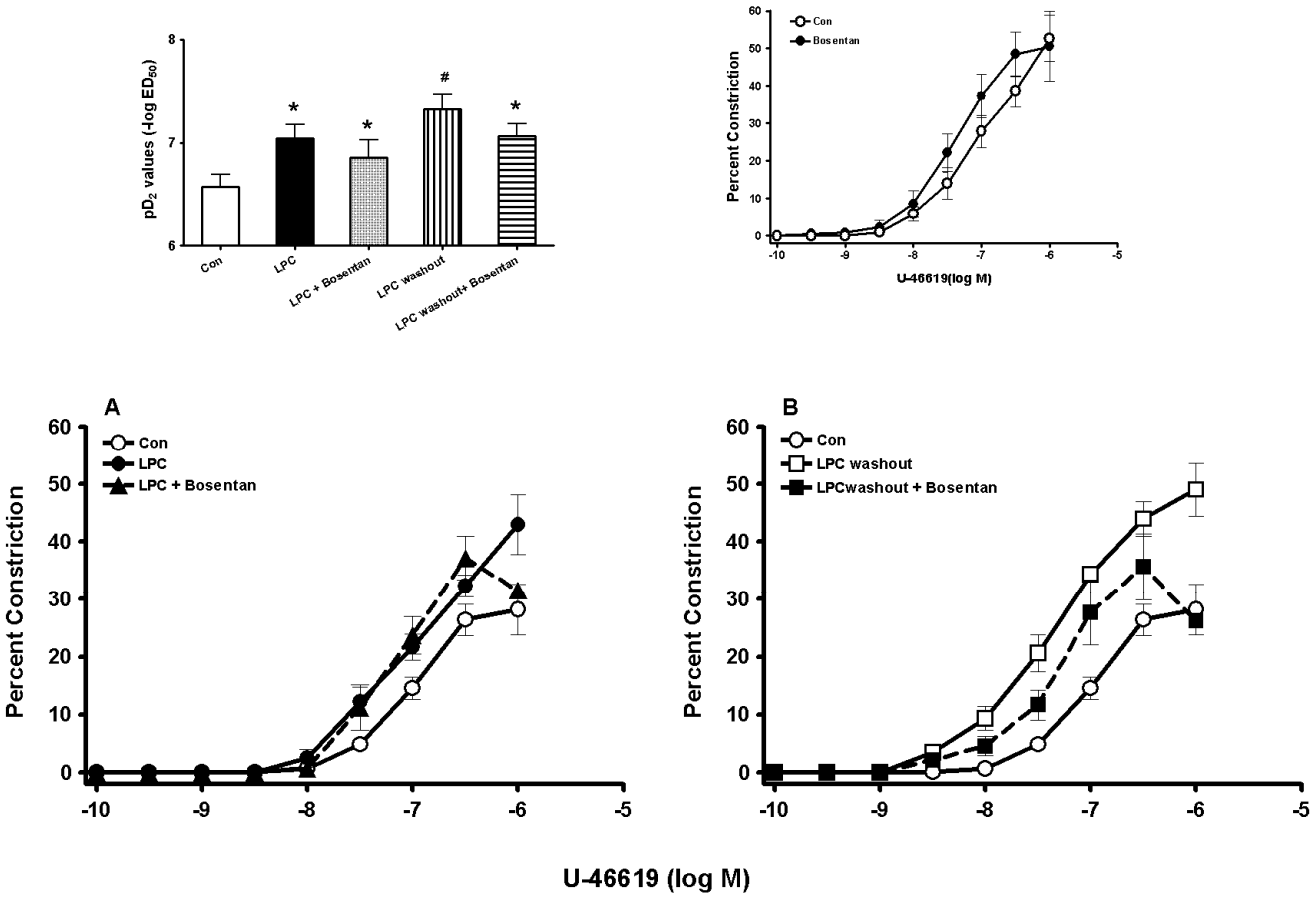

Figure 3.28 Bosentan moderates LPC-induced potentiation in cumulative concentration-response curves (CRCs) to U-46619 in isolated intact rat septal coronary arteries using a pressure myograph. The responses to U-46619 were determined in the absence and presence (40 min) of LPC (3 μmol/L) that was delivered intraluminally with or without 1 μmol/L bosentan (A, *n*=6). The responses to U-46619 were also determined after washout of LPC for 60 min in the absence or presence of 1 μmol/L bosentan (B, *n*=6). The left inset depicts U-46619 pD_2 values in intact rat septal coronary arteries. The right inset shows the effects of 1 μmol/L bosentan alone on U-46619-CRC (*n*=6). Values are means ± S.E.M. CRCs to U-46619 were analyzed by nonlinear regression for calculation of *p*D2 (-log EC50) values and maximum responses (R_{max}). Statistical evaluation was done using one-way ANOVA followed by Bonferroni's test when appropriate. *Significantly different from control, #Significantly different from all other groups, $P<0.05$.

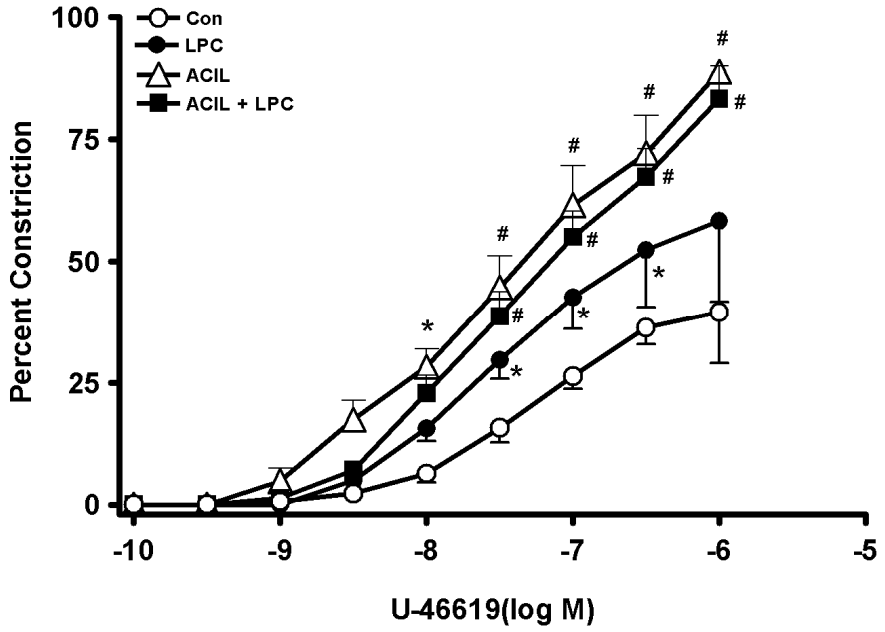

Figure 3.29 Endothelium-dependent relaxing factors are involved in the direct effects of LPC on cumulative concentration-response curves (CRCs) to U-46619 in isolated intact rat septal coronary arteries using a pressure myograph. The responses to U-46619 were determined in the absence, presence of LPC or the vasodilator inhibitor cocktail (ACIL, 40 min) that were delivered intraluminally. ACIL=apamin (0.5 μmol/L), charybdotoxin (25 μmol/L), indomethacin (10 μmol/L), and $_{L}$-NAME (10 μmol/L). Values are means ± S.E.M. n=5-7. Data were analyzed for significant differences using NCSS. Two-way ANOVA using the general linear model approach (repeated measurements) followed by Newman–Keul's test was used for comparisons between CRCs. *Significantly different from control, #Significantly different from both control and LPC groups, $P<0.05$.

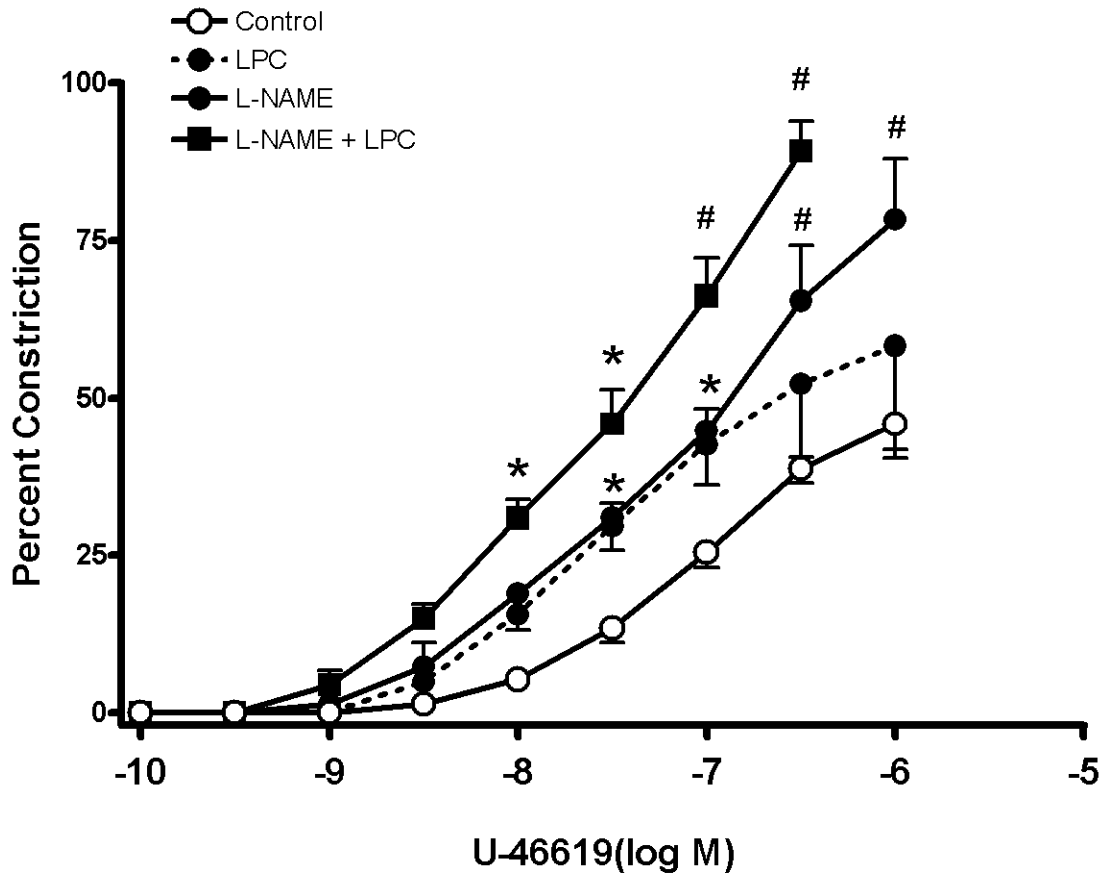

Figure 3.30 Involvement of NO in the direct effects of LPC on cumulative concentration-response curves (CRCs) to U-46619 in isolated intact rat septal coronary arteries using a pressure myograph. The responses to U-46619 were determined in the absence or presence of LPC, or LPC with 10 μmol/L $_{L}$-NAME, Values are means ± S.E.M. *n*=3-7. Data were analyzed for significant differences using NCSS. Two-way ANOVA using the general linear model approach (repeated measurements) followed by Newman–Keul's test was used for comparisons between CRCs. *Significantly different from control, #Significantly different from both control and LPC groups, $P<0.05$.

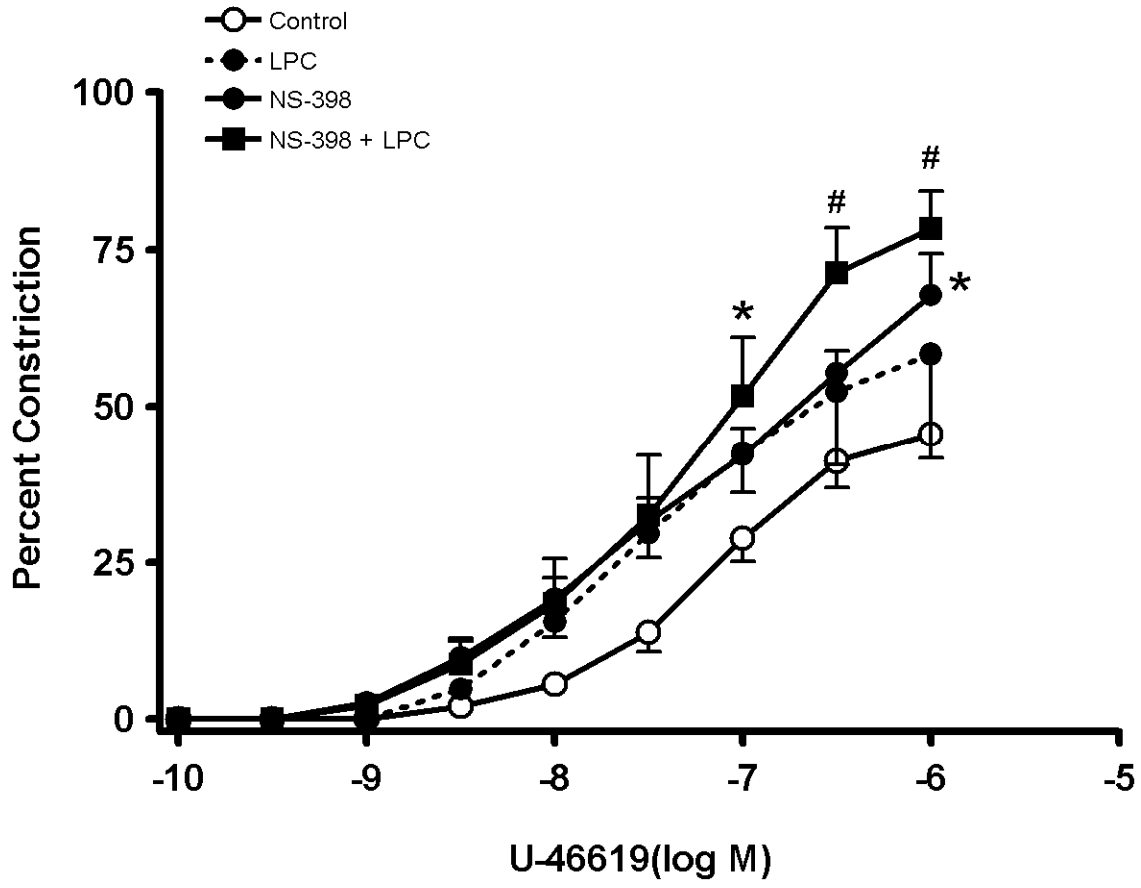

Figure 3.31 Involvement of PGI_2 in the direct effects of LPC on cumulative concentration-response curves (CRCs) to U-46619 in isolated intact rat septal coronary arteries using a pressure myograph. The responses to U-46619 were determined in the absence or presence of LPC, or LPC with 10 μmol/L NS-398. Values are means ± S.E.M. *n*=5-7. Data were analyzed for significant differences using NCSS. Two-way ANOVA using the general linear model approach (repeated measurements) followed by Newman–Keul's test was used for comparisons between CRCs. *Significantly different from control, #Significantly different from both control and LPC groups, $P<0.05$.

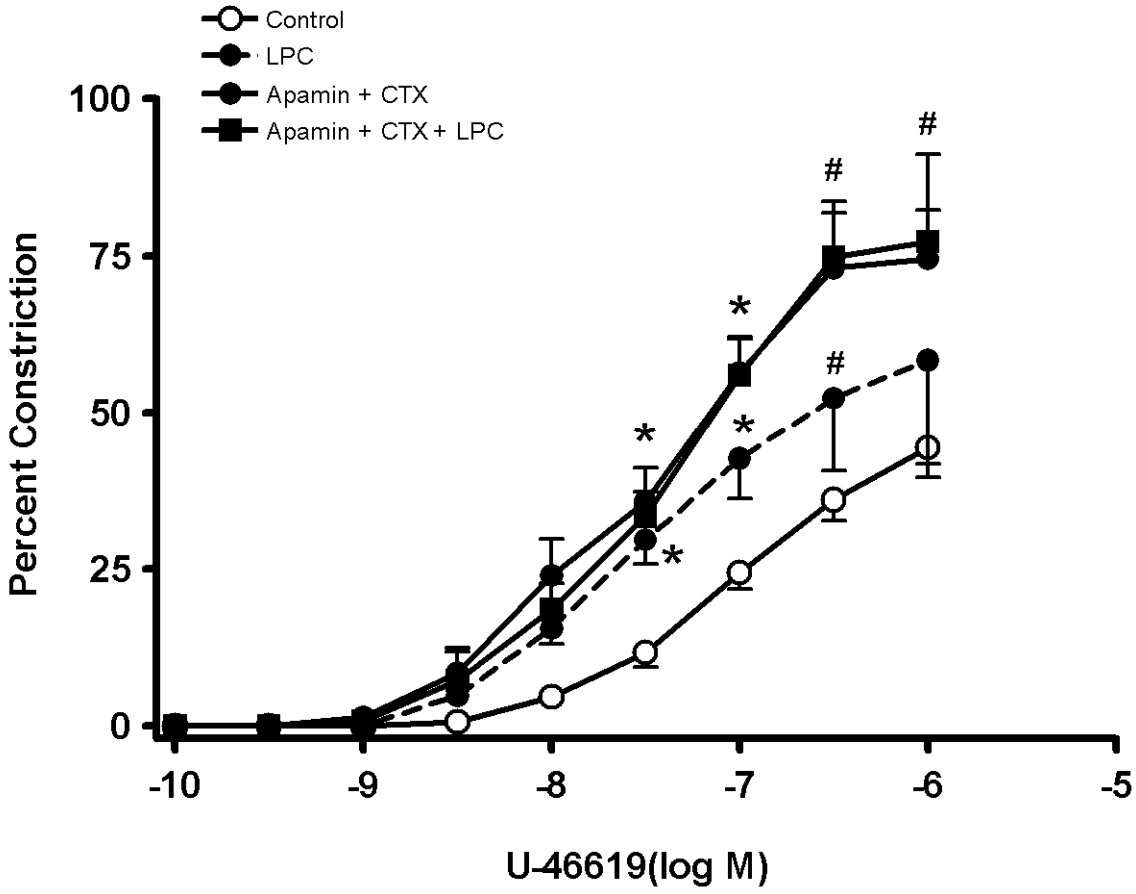

Figure 3.32 Involvement of EDHF in the direct effects of LPC on cumulative concentration-response curves (CRCs) to U-46619 in isolated intact rat septal coronary arteries using a pressure myograph. The responses to U-46619 were determined in the absence or presence of LPC, or LPC with 0.5 μmol/L apamin + 25 μmol/L charybdotoxin. Values are means ± S.E.M. *n*=4-7. Data were analyzed for significant differences using NCSS. Two-way ANOVA using the general linear model approach (repeated measurements) followed by Newman–Keul's test was used for comparisons between CRCs. *Significantly different from control, #Significantly different from both control and LPC groups, $P<0.05$.

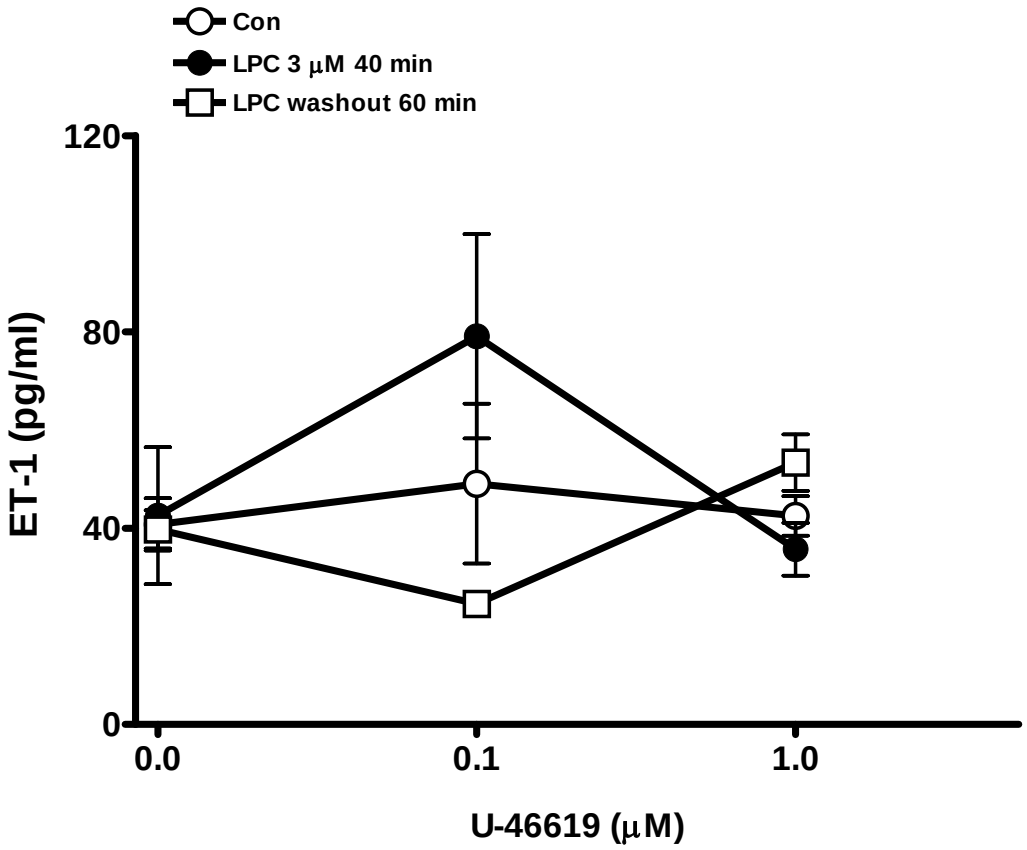

Figure 3.33 ET-1 release from BCAECs in response to U-46619 before, after 40-min incubation with 3 μmol/L LPC, and after washout of LPC for 60 min. Data represent the mean ± S.E.M. $n = 4$. Data were analyzed for significant differences using NCSS. Two-way ANOVA using the general linear model approach (repeated measurements) followed by Newman–Keul's test was used for comparisons between CRCs. No significant difference was observed.

4. DISCUSSION

4.1 Importance of using small arteries and arterioles

Ever since its proatherogenic role was recognized, LPC has been extensively investigated in the cardiovascular system. However, the evidence in terms of its role in modulating vascular tone is still limited, largely due to the fact most previous studies utilized large conduit arteries, such as aorta and superior mesenteric arteries. *In vivo* studies are even more rare. Using blood vessel preparations consisting of small arteries and/or arterioles enabled us to obtain intriguing and novel findings about LPC in relation to its ability to control vascular reactivity.

It is well known that there are numerous structural and functional distinctions exist between large conduit arteries and small arteries/arterioles. Compared to large elastic arteries, small arteries (diameter of 100 μm –500 μm) and arterioles (diameter < 100 μm) are characterized by a larger ratio of vascular wall to lumen size with more smooth muscle and less elastic material (Mulvany and Aalkjaer, 1990). These muscular arteries are the primary site of vascular resistance. Even a small change in internal diameter causes a large change in vascular resistance to blood flow. They adapt their diameter actively by constriction and dilation of vascular SMCs, thereby actively regulating blood flow to the individual demands of the organ that they supply. In addition, small arteries/ arterioles also contribute to the regulation of arterial blood pressure (mean arterial blood pressure = cardiac output × total peripheral resistance). Thus, they are important for the maintenance of organ function, the development of organ diseases, and pathological conditions related to vascular resistance, such as hypertension.

Conduit arteries and small arteries respond to pathological stimuli differently. For instance, the pharmacological properties of small pulmonary arteries (100-300 μm) and the left main pulmonary arteries (1-2 mm) from normal rats were compared *in vitro* (Leach et al., 1992). It was shown that noradrenaline was a potent vasoconstrictor in large but not small pulmonary arteries. Ach-induced EDR was remarkably more evident in large arteries (88%) than in the smaller arteries (25%) even with the endothelium intact (Priest et al., 1997). In a separate study, maximum responses of conduit (~ 500 μm), but not resistance pulmonary arteries (< 250 μm) from rats with streptozotocin-induced diabetes to PE and serotonin were significantly reduced (Gurney and Howarth, 2009). The underlying mechanisms for this greater resistance of the smaller pulmonary arteries to streptozotocin-induced chronic diabetes, however, remains unknown. More interestingly, ischemia followed by reperfusion markedly and selectively impaired EDR in coronary microvessels (110-220 μm) but not in large conduit coronary vessels from dogs (Quillen et al., 1990).

Indeed, our results demonstrate that contractile responses to PE were dramatically changed in the whole MAB that contains larger arteries as well as first-, second-, and third-generation arterioles, but only slightly affected by LPC in the single superior mesenteric artery. In addition to structural differences, other factor such as the manner by which tissues develop tension and the location in which LPC is present, may also account for the observed distinct pharmacological behaviors in these two preparations.

Among the small arteries/arterioles, resistance arteries are defined as "precapillary vessels that contribute significantly both passively to the resting resistance and actively to the blood flow control during altered demands" (Christensen and Mulvany, 2001). Rat

MAB is believed to be truly a group of resistance arteries (Christensen and Mulvany, 2001), receiving one fifth of the total cardiac output (Caveney et al., 1998), thus largely determining the arterial blood pressure. Whether or not the rat coronary septal artery (< 200 μm) is a true resistance artery is still controversial. Nevertheless, this artery supplies approximately 75% of the inter-ventricular septum (Blair, 1961). Consequently, the dysfunction of septal coronary artery could have a great impact on coronary flow and contribute to ischemic heart disease.

Regional heterogeneity of vasoregulation is also evident in different vascular beds consisting of small arteries/arterioles. Therefore, it is not surprising to find that the vasoactive properties of LPC are not exactly the same in the two different preparations we used. Nevertheless, the unique finding of our investigation is that LPC possesses biphasic effects on the contractile and relaxant responses of these small arteries/arterioles, which has never been reported before.

4.2 LPC potentiates PE responses in rat MAB through modulation of TxA_2

To our knowledge, our study is the first to investigate the effects of LPC on the reactivity of a perfused resistance artery bed. As reported previously in conduit blood vessels, LPC perfusion of MAB caused impairment in Ach-induced maximum relaxation. The novel finding in the present study was the potentiation of PE-induced contractile responses after washout of LPC, an effect likely related to increased production of TxA_2.

4.2.1 Direct effects of LPC in isolated perfused rat MAB

The balance between vasodilators (NO, EDHF, and PGI_2) and vasoconstrictors (TxA_2 and ET-1) is pivotal in the regulation of vascular tone. Because most of these vasoactive factors originate from vascular endothelium, we examined the effects of LPC

on endothelial function. Measurement of EDR supported the established view that LPC inhibits EDR, probably through inactivation of NO and EDHF (Cowan and Steffen, 1995; Froese et al., 1999; Rikitake et al., 2000a). Impaired EDR is normally linked to potentiated vasoconstrictor responses. However, the impaired Ach-induced EDR caused by LPC perfusion was not accompanied by an increased response to PE. Other studies using aortic rings from normal rats have also demonstrated similar effects of LPC, i.e., reduced EDR with no significant change in PE responses (Ceylan et al., 2004). These data suggest that, even in the presence of NO/EDHF inhibition by LPC, vasoconstrictor responsiveness or release may also be inhibited by LPC, thus preventing augmentation in responses to PE. This possibility is supported by the observation that LPC prevented amplification of PE responses in the presence of $_{L}$-NMMA and reduced the PE-induced production of TxA_2.

4.2.2 Residual effects of LPC in isolated perfused rat MAB

Unexpectedly, the response to PE was dramatically potentiated only after the removal of LPC. A time-dependent mechanism can be ruled out, as prolonged perfusion with LPC (150 min) did not produce any potentiation of PE responses. The enhancement of the PE response could be blocked by both indomethacin and SQ-29548, suggesting an important contribution of vasoconstrictor prostanoids, which was confirmed by the finding of potentiation of PE-induced TxA_2 production. At present, the mechanism for this rebound production of excessive TxA_2 is not known.

The contribution of time in producing the potentiated contractile responses and TxA_2 production in response to PE can be ruled out, as these were stable over time in the

absence of LPC. These effects were unlikely to be due solely to continued endothelial impairment, because Ach-induced relaxation had partially recovered.

As a potent inducer of platelet aggregation, vasoconstriction, and bronchoconstriction, TxA_2 has been implicated in vascular pathogenesis (Dogne et al., 2004). Although the major source of TxA_2 is platelets, this vasoconstrictor can also be produced within the vascular walls by both endothelial (Ally and Horrobin, 1980) and smooth muscle cells (Shiokoshi et al., 2002). The mechanism of TxA_2 synthesis includes phospholipid hydrolysis by PLA_2, release of arachidonic acid, and metabolism to TxA_2 by the COX-TxA_2 synthase pathway. α_1-Receptor activation initiates phospholipid hydrolysis and release of TxA_2 (Terzic et al., 1993; Nishio et al., 1996; Ruan et al., 1998; Bolla et al., 2002; Parmentier et al., 2004). To our knowledge, an effect of LPC on α_1-adrenoceptor-mediated TxA_2 production in the vasculature has not previously been reported. However, our observation that LPC abrogated the increase in TxA_2 production produced by PE in the MAB is consistent with a previous study in platelets, in which an inhibitory effect of LPC on agonist-induced TxA_2 production was observed (Yuan et al., 1996). Although we are not aware of any studies showing potentiation of TxA_2 production by LPC, an interaction between these two agents has previously been suggested. For instance, LPC was found to enhance the ability of TxA_2 to increase vascular smooth muscle cell proliferation (Koba et al., 2000). Taken together, these findings strongly suggest that LPC is able to alter the biosynthesis of TxA_2, as well as its downstream signaling. Details of the mechanism of these modulatory effects need to be further investigated. Irrespective of the mechanism(s), the increased release of TxA_2

could be a major contributor toward hypertensive (Seeger et al., 1989), thrombotic, and ischemic diseases (Ally and Horrobin, 1980; Muller, 1991).

It is tempting to speculate that conditions that evoke an increase in LPC levels initially trigger compensatory mechanisms to neutralize its injurious effects. Thus, the suppression of PE-induced TxA_2 production could compensate for the endothelium dysfunction. Wu and co-workers (Zembowicz et al., 1995a; Zembowicz et al., 1995b) have also suggested that LPC is a two-faced molecule, which possesses both vasoprotective and proatherogenic properties. An additional caveat is the suggestion that residual effects of LPC after washout could arise from its metabolism to LPA (Tokumura, 2004). However, the same concentration of LPA had no effect on the vasoconstrictor response of the MAB to PE, suggesting that LPA is not likely mediating the effects of LPC in this preparation.

In conclusion, LPC modulates the vascular tone of the rat resistance arterial bed, even after washout. The modifications include potentiated PE responses and enhanced TxA_2 production. Thus, when evaluating the effects of LPC in the vasculature, both its immediate and residual effects need to be considered. Especially in situations where LPC release increases acutely and then falls, such as during cardiac I/R, the observed immediate and residual effects of LPC on the vasculature could contribute to the pathogenesis of this disease.

4.3 LPC endothelium-dependently modulates rat coronary arterial tone

Following I/R, accumulation of LPC may increase coronary vascular resistance (Maulik et al., 1996; Tiefenbacher et al., 1996; Toufektsian et al., 2001). This was confirmed by our finding that LPC directly induced a persistent enhancement in CPP in isolated perfused hearts. In addition, exposure of isolated coronary septal arteries to LPC augmented the vasoconstriction to U-46619 as well. More importantly, these deleterious modulations of coronary vascular contractility by LPC persisted and even increased long after the lysolipid was removed. Our results further imply that the mechanisms responsible for the direct and residual effects of LPC in modulating vascular tone are distinct, and are dependent on reduced EDHF, and increased ET-1/decreased NO respectively.

In various isolated blood vessels, LPC has been consistently shown to selectively alter the agonist-mediated vasoconstrictor and vasodilator responses (Galle et al., 2003; Suenaga and Kamata, 2003). Resting vascular tone and the K^+-induced smooth muscle cell depolarization were not significantly affected by LPC in most cases. Our results obtained from isolated rat coronary artery are in agreement with all these findings. Intriguingly, we also found that in intact perfused heart, LPC exhibited a direct vasoconstricting effect in the absence of any agonist. Furthermore, bosentan failed to block the potentiating effect of LPC and its washout on CPP in isolated perfused hearts, whereas it successfully prevented the additional enhancement in U-46619 response in isolated pressurized coronary artery. All these inconsistencies suggest that although LPC provoked increased vascular resistance in both perfused hearts and isolated coronary arteries, the underlying mechanisms are not identical.

There is currently no direct evidence to explain these inter-tissue variations. Nonetheless, we speculate that one of the mechanisms could be the complex cross-talk between coronary vasculature and cardiac myocytes via multiple chemical and mechanical mechanisms, which has been comprehensively discussed in a recent review article (Westerhof et al., 2006). As a specific example, Chilian and colleagues found that cardiac myocytes have a requisite role in the constriction of coronary resistance vessels to α_1-adrenergic stimuli, which may be mediated by ET-1 and other unidentified myocyte-derived vasoconstrictors (Tiefenbacher et al., 1998). Therefore, it is very possible that in our study, LPC perfusion of isolated hearts stimulates certain mechanism(s) other than ET-1 outside the coronary lumen, which in turn act on coronary vasculature and enhance coronary resistance.

4.3.1 Direct effects of LPC in isolated pressurized rat coronary septal arteries

TxA_2 has been suggested to participate in the pathogenesis of ischemic heart disease (Tokumura, 2004). The other reason that we chose to study the vasoconstrictor response of coronary artery to U-46619, a TxA_2 mimetic, is that we and other researchers (Tune et al., 2002) found that the α_1-adrenoceptor agonist PE does not produce vasoconstriction in all sections of coronary arteries. Particularly, our preliminary experiments showed that in Wistar rat coronary arteries, α-$_1$ adrenoceptor mediated vasoconstriction seemed to be confined to vessels larger than 200 μm. A possible explanation is that α-$_1$ adrenoceptors are unevenly distributed along the coronary artery trees (Chilian, 1991). In contrast, a stable TxA_2 mimetic, U-46619, produces potent and consistent vasoconstriction throughout rat coronary arteries.

Given the observation that the U-46619-induced contractile responses were markedly potentiated in endothelium-denuded rat coronary septal arteries, our data suggest that EDRFs also contribute to regulating the overall vasoconstriction observed to this TxA_2 mimetic. More importantly, as endothelium-removal completely abolished the direct and exaggerated responses to U-46619 following LPC washout, our data suggest that the potentiating effects of LPC on contractile responses to U-46619 are entirely endothelium-dependent. Consequently, we examined the endothelium-derived vasoactive factors that could participate in mediating the direct responses to LPC. Interestingly, in the presence of LPC, the relaxant response to Ach, which has been shown result primarily from the release of NO in rat coronary arteries by our as well as other studies (Tschudi et al., 1994; Parker et al., 1997), remained unchanged, suggesting that LPC has limited direct influence on NO. This was corroborated using the NO-blocker, $_L$-NAME, which induced an additive potentiation with LPC of U-46619 responses. In the absence of any involvement of NO in these direct effects of LPC, we tested the contribution of EDHF using blockers that inhibit endothelial potassium conductance. Our data suggest that in the presence of LPC, the direct potentiation of contractile responses to U-46619 is likely dependent on reduced release of EDHF (Figure 4.1). EDHF, as one of the vasodilators that contribute to EDR, has been reported to be inhibited by LPC previously (Cowan and Steffen, 1995; Eizawa et al., 1995; Fukao et al., 1995; Froese et al., 1999). In these *in vitro* artery ring preparations, EDHF was considered to be K^+ ions, but the further inhibitory mechanisms of LPC on EDHF have not been explored. It is interesting to find that in our study, EDHF is present in the isolated rat coronary artery as a mediator

regulating the contractile response to U-46619, but not a major contributor to the Ach response.

The observation that LPC did not immediately impaire EDR in the isolated pressurized coronary artery is different from those obtained using isometric tension measurements, in which exogenous LPC exhibited direct inhibition of EDR in coronary arteries (VanBenthuysen et al., 1987; Quillen et al., 1990; Tiefenbacher et al., 1996). This discrepancy may arise from different experimental methodologies including species, type of coronary arteries used, and methods for measuring vascular reactivity. Particularly, several lines of evidence have suggested that pressurized and wire-mounted preparations, the most common *in vitro* techniques employed in small artery studies, could exhibit substantially different results even when using the same kind of tissue. For instance, the same small arteries under isobaric condition (pressurized) were either more sensitive or less sensitive to the same vasoconstrictor than those under isometric condition (wire myograph) (Dunn et al., 1994; Falloon et al., 1995; Schubert et al., 1996). The underlying mechanisms are still obscure. However, one of the possible explanations is the less negative resting membrane potential of isobaric preparation (Schubert et al., 1996). More importantly, it is generally believed that pressure myograph has the advantage of closely mimicking *in vivo* physiology due to the intraluminal pressure it provides (Schubert et al., 1996). In our study, the U-46619-response *per se* was not compared between pressure and wire myograph. Nevertheless, the responses of coronary arteries mounted on a wire myograph to U-46619 were not significantly influenced by LPC at the same concentration used in the pressure myograph. It is important to note that in addition to the different vessel wall tension and membrane potential, the ways by

which LPC interacts with the tissues may also contribute to this discrepancy. Specifically, in the pressure myograph, LPC was present only in the arterial lumen and was surrounded by endothelial cells under no-flow conditions. Whereas, the LPC added in the wire myograph was communicating with not only the endothelium, but also the SMCs. Considering the possible coronary origin of LPC accumulated during I/R (Sedlis et al., 1990; Sedlis et al., 1993; Sedlis et al., 1997), LPC is unlikely to directly interact with SMCs in the *in vivo* situation. Taken together, we believe that the results obtained from the pressure myograph are more likely to reflect the pathophysiological role of LPC.

4.3.2 Residual effects of LPC in isolated pressurized rat coronary septal arteries

Residual effects of LPC were also observed following its washout, including a reduction in response to Ach, and a further potentiation of the vasoconstrictor effects of U-46619. These residual effects were different from those seen in the presence of LPC, and appear to include multiple mechanisms, one of them being an increase in the release of ET-1 (Figure 4.1). Interestingly, it was previously reported that LPC enhanced rabbit cerebral arterial myogenic tone by stimulating the production of ET-1, although this was a direct effect of LPC (Xie and Bevan, 1999). In addition, based on a recent theory that there is a negative cross-talk between ET-1 and NO (Marasciulo et al., 2006), it is reasonable to speculate that the enhanced release of ET-1 might be associated with impaired production and/or activity of NO. This speculation is supported by our observation that after LPC washout, there was a striking attenuation in the relaxant response to Ach. The underlying mechanisms of this inhibited biovailability of NO remained unclear. However, superoxide anion is unlikely to be involved, as the superoxide scavenger did not improve the diminished Ach response by LPC.

LPC did not elicit a residual effect on ET-1 generation in cultured BCAECs, which implies that the unique effects of LPC detected in intact coronary arteries need the integral layer of endothelium cells, and even the participation of the underlying vascular smooth muscle. Alternatively, species differences could be another reason that LPC produced different effects in cultured BCAECs. Whatever the explanation, the data obtained from BCAECs cannot be used to explain the mechanisms occurring in intact arteries in our study.

It is generally accepted that excessive LPC is a proatherogenic and proinflammatory mediator that contributes to the pathogenesis of diabetes (Shi et al., 1999), atherosclerosis (Matsumoto et al., 2007), and cardiac I/R (Hashizume and Abiko, 1999). It does so largely by inducing coronary endothelial dysfunction (Eizawa et al., 1995; Leung et al., 1997). Our study for the first time reports that this endothelial dysfunction with LPC, in the form of an enhancement in coronary tone and delayed attenuation in EDR, is evident long after its elimination from the perfusate. As the latter effect is consistent with previous reports that EDR was only attenuated following I/R , and not ischemia alone (Lefer et al., 1991), when the elevated level of LPC returned to normal, our data suggest that this delayed coronary effect of LPC, when added on to the immediate noxious effects of this lysolipid, could exacerbate heart dysfunction following I/R. The involvement of the endothelium in these delayed effects supports the suggestion that the coronary endothelium may be a drug target for minimizing I/R injury (Laude et al., 2004).

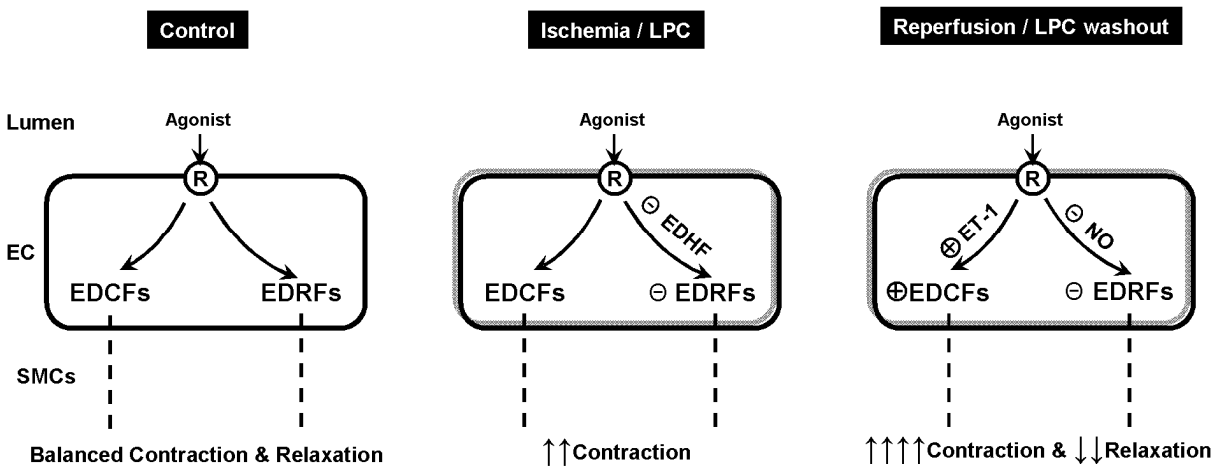

Figure 4.1 Proposed mechanism illustrating the direct and residual effects of LPC on endothelium-dependent vasoactive factors and regulation of smooth muscle contractility. EDHF is the likely contributor for the direct effects of LPC (middle panel), whereas a balance between increased ET-1 and decreased NO produces the residual effects of LPC (right panel). EC = endothelial cell; EDCFs = endothelium-dependent contracting factors; EDRFs = endothelium-dependent relaxing factors; EDHF = endothelium-derived hyperpolarizing factor; ET-1 = endothelin-1; NO = nitric oxide; SMCs = smooth muscle cells.

4.4 Limitations of present study and direction of future studies

At present, the mechanism(s) underlying the residual effects of LPC are unclear. We tested LPA, an important vasoactive compound and a key metabolite derived from LPC, in both MAB and the pressure myograph using the same protocol. As LPA had no significant influence on vasoconstriction to PE and U-46619, it is likely that these effects of LPC are direct, and unrelated to its metabolic by-products.

Due to the nature of the isolated rat coronary septal arteries, our study using this preparation only relied on the use of pharmacological tools. Each of these artery segments was only 1-2 mm long, with diameters less than 200 μm, and surrounded with relatively large volume (30 ml) of buffer solution. It is not technically feasible to measure the expression and regulation of the possible moderators (such as iNOS and ET-1) in them. Neither is it possible to detect the concentrations of vasoactive compounds released from these tissues. Alternative means could be the direct measurement of ET-1 production from LPC-treated endothelial cells and/or isolated perfused hearts. Unfortunately, it turned out that LPC either did not produce the same effects or acts via different mechanisms (as discussed above) in these tissues. On the other hand, these limitations also reflect the strict tissue-dependence of the effects of LPC, and it is wise to be cautious when using evidence obtained from related but different tissues to explain the phenomena occurred in intact blood vessels.

It is unknown whether the observed effects of LPC in our study are dependent on LPC-specific receptors (G2A and GPR4) or not. As a matter of fact, this is a gap that needs to be filled in LPC research (as addressed in the Introduction). The majority of previous work has been focused on G2A and its involvement in LPC-induced

proinflammatory and proatherogenic activities. So far, it appears that GPR4 is more likely to be involved in the effects of LPC in regulating vascular tone. It would be useful to selectively block GPR4 and then examine the changes in response to LPC. Unfortunately, the only available selective blockers to LPC, LPC receptor antibodies, are problematic when applied to the pressure myograph apparatus due to their affinity for the plastic tubing. In future studies, the development of selective pharmacological antagonists of GPR4, GPR4-deficient animal models, as well as small interfering RNAs techniques could possibly lead to a breakthrough in LPC research.

Another limitation that exists in LPC research is that all the current studies have been conducted using exogenous LPC, making it difficult to draw conclusions on the role of endogenous LPC sources in the homoeostatic regulation of vascular tone. The improvement in this area will depend on the availability of more selective and effective modulators of the LPC-generating enzyme (PLA_2) and LPC-degrading enzyme (LPLD).

5. SUMMARY AND CONCLUSION

Our findings imply that when evaluating the regulation of vascular tone, small arteries (including resistance arteries) and conduit arteries can present discrete or even contradictory results, and the former is a much more reliable tool considering their role in determining peripheral resistance *in vivo*.

Our results for the first time demonstrate that the detrimental effects of LPC in both rat MAB and coronary septal arteries are evident in the presence and even after washout of the lysolipid. However, distinct mediators are responsible for the biphasic consequences of LPC in the two discrete preparations.

In rat mesenteric resistance arterial bed, both EDRFs and TxA_2 are diminished, resulting in an overall unchanged vasoconstrictor response to α_1-adrenoceptor stimulation. LPC washout stimulates a rebound overproduction of TxA_2, which is the major contributor to the enhanced contractile response to PE.

In rat coronary septal arteries, reduced release of EDHF is the likely contributor to the direct effects of LPC in potentiating the response to U-46619, whereas increased ET-1 associated with a decrease in NO bioavailability appear to be responsible for the residual effects of LPC, including the further enhancement in vasoconstrictor response and remarkable impairment in endothelium-dependent relaxation (Figure 4.1).

In conclusion, LPC exerts substantial influence on both peripheral arterial resistance regulation and coronary circulation, and even ventricular function. Particularly, our data suggest that in addition to reducing the accumulation of LPC, efforts targeting an improvement in endothelium-dependent regulation of vascular tone together with the

development of specific LPC-inhibitors would be required to limit the vascular and cardiac damage induced by LPC and its residual consequences.

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THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A08-0763

Investigator or Course Director: Brian B. Rodrigues

Department: Pharmaceutical Sciences

Animals:

Rats Wistar 300

Start Date: **July 1, 2007** **Approval Date:** **January 12, 2009**

Funding Sources:

Funding Agency:	Heart and Stroke Foundation of British Columbia and Yukon
Funding Title:	High fat induced changes in cardiac metabolism and its consequences
Unfunded title:	N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

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