VASODILATOR AND ANTIHYPERTENSIVE EFFECTS OF L-SERINE

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ABSTRACT

L-serine, a non-essential amino acid, plays a role in the biosynthesis of the amino acids, proteins, purine and pyrimidine nucleotides. It is important for the proper functioning of the nervous system. It has been considered in the treatment of patients with schizophrenia, depression, chronic fatigue syndrome and psychomotor retardation, and of the seizures encountered in patients with rare inborn errors of L-serine biosynthesis. However, there are no reports in the literature of the direct cardiovascular effects of L-serine. Using normotensive Sprague-Dawley rats, Sprague-Dawley rats rendered hypertensive by chronic treatment with the nitric oxide (NO) synthase inhibitior N^G nitro L-arginine methyl ester (L-NAME) and spontaneously hypertensive rats (SHR), the present study examined the *in vitro* and *in vivo* effects of L-serine. *In vitro* studies focused on L-serine induced changes in phenylephrine constricted third order branches of rat mesenteric arterioles while the *in vivo* studies examined the effects of intravenous infusion of L-serine on mean arterial pressure (MAP) and heart rate (HR) in intact anaesthetized rats. L-serine (10 to 200 μmol/L) evoked concentration-dependent vasodilatation in phenylephrine constricted endothelium-intact, but not in endothelium-denuded, rat mesenteric arterioles. The vasodilator responses to L-serine were absent in the combined presence of apamin, a calcium activated small conductance potassium (SK_{Ca}) channel inhibitor, and TRAM-34, a calcium activated intermediate conductance potassium (IK_{Ca}) channel inhibitor, or ouabain, a sodium pump inhibitor and barium (Ba^{2+}), an inward rectifying potassium (K_{ir}) channel inhibitor, or when the vessels were depolarized by potassium chloride. The maximal vasodilatation response (E_{max}) to L-serine was higher in vessels from L-NAME treated rats (40%) than from control rats (20%). In anesthetized rats, L-serine evoked a rapid, reversible, dose-dependent fall in MAP (without a significant change in HR), which was

more pronounced in L-NAME treated rats (> 60 mmHg) than in normotensive control rats (25 mmHg). The fall in MAP was inhibited ($p<0.01$) by apamin plus charybdotoxin pretreatment. Charybdotoxin was used in place of Tram-34 in *in vivo* studies since Tram-34 is not soluble in water or saline. In age matched Sprague-Dawley, Wistar-Kyoto (WKY) and SHR strains, D-serine had the same effects on MAP and HR as L-serine; however, L-serine evoked a greater maximal fall in MAP in all strains, and the effect was more pronounced in hypertensive rats. In contrast, the infusion of glycine, a metabolite of L-serine led to a dose-dependent fall in MAP in normotensive rats but a dose-dependent increase in MAP in both SHR and L-NAME treated hypertensive WKY rats. Both the depressor and pressor responses to glycine were abolished by pretreatment with the N-methyl D-aspartate receptor antagonist, MK-801. Regional hemodynamic studies performed using the fluorescent tagged microsphere distribution technique revealed that the fall in MAP and profound decrease in total peripheral resistance (TPR) evoked by acute L-serine infusion is due to increased blood flow in the splanchnic region and more particularly in the small intestinal vascular beds. This effect is blocked by the combined treatment with the K_{Ca} channel inhibitors, apamin plus charybdotoxin. Although resting MAP and TPR are higher, and cardiac output (CO) is lower both in SHR and in WKY rats rendered hypertensive by L-NAME treatment compared to normotensive WKY rats, L-serine infusion leads to a rapid fall in TPR and MAP, and an increase in CO in all models. This effect was more profound in the hypertensive rats. These findings suggest that L-serine could be helpful in overcoming splanchnic organ failure observed in patients with cardiopulmonary bypass. In addition, L-serine, either alone or in combination with other antihypertensive medications, could be considered in the management of endothelial dysfunctional states with reduced NO bioavailability such as hypertension and diabetes.

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LIST OF ABBREVIATIONS

$[Ca^{2+}]_i$:	Intracellular calcium
·OH:	Hydroxyl radicals
3-PG:	3-phosphoglycerate
3-PGDH	3-phosphoglycerate dehydrogenase
5-HT:	5-hydroxytryptamine
AC:	Adenylyl cyclase
ACE:	Angiotensin-converting-enzyme
ACh:	Acetylcholine
ADH:	Antidiuretic hormone
ADMA:	Asymmetric dimethylargine
Ang I:	Angiotensin I
Ang II:	Angiotensin II
Ang:	Angiotensin
ANP:	Atrial natriuretic peptide
ARA-S:	N-archidonoyl-L-serine
ATP:	Adenosine triphosphate
AVP:	Arginine vasopressin
Ba^{2+}:	Barium chloride
BK:	Bradykinin
BP:	Blood pressure
cAMP:	cyclic 3', 5' adenosine monophosphate
CBS:	Cystathionine ß-synthase

Celloslove:	2-ethoxy-ethyl acetate
cGMP:	cyclic 3', 5' guanosine monophosphate
ChTX:	Charybdotoxin
CI:	Cardiac index
CNP:	C-natriuretic peptide
CO:	Cardiac output
COX:	Cyclooxygenase
CR:	Concentration response curve
CSE:	Cystathionine γ-lyase
CSF:	Cerebrospinal fluid
cSHMT:	Cytosolic serine hydroxymethyltransferase
CYP:	Cytochrome P 450
DNA:	Deoxyribonucleic acid
DOCA:	Deoxycorticosterone acetate
DR:	Dose-response
EC:	Endothelial cells
EC_{50}:	Half maximal effective concentration
EDHF:	Endothelium-derived hyperpolarizing factor
EET:	Epoxyeicosatrienoic acid
E_{max}:	Maximal response
ENDO [-]:	Endothelium denuded
ENDO [+]:	Endothelium intact
eNOS:	Endothelial nitric oxide synthase
ET:	Endothelin

F_i:	Flow to individual sample
FDA:	Food and drug administration
GABA:	Gamma-aminobutyric acid
GC:	Guanylyl cyclase
GCS:	Glycine cleavage system
GHSR 1a:	Growth hormone secretagogue receptor 1a
GSH:	Glutathione
GTP:	Guanosine triphosphate
H_2O_2:	Hydrogen peroxide
H_2S:	Hydrogen sulfide
HO:	Heme oxygenase
HR:	Heart rate
HSP:	Heat -shock protein
IAF:	Intra abdominal fat
I_i:	Fluorescence intensity of the sample
$IK_{Ca:}$	Intermediate conductance calcium activated potassium channels
iNOS:	Inducible nitric oxide synthase
I_{ref}:	Fluorescence intensity of the reference blood sample
K_{Ca}:	Calcium activated potassium
KCl:	Potassium chloride
LAT:	L-amino acid transporter
L-NAME:	N^G nitro L-arginine methyl ester
MAP:	Mean arterial pressure
MEGJ:	Myoendothelial gap junction

mSHMT:	Mitochondrial serine hydroxymethyltransferase
MTHF:	Methyltetrahydrofolate
NAD^+:	Nicotinamide adenine dinucleotide
NADPH:	Nicotinamide adenine dinucleotide phosphate
NEFA:	Non-esterified fatty acid
NHANES:	National health and nutrition examination survey
nm:	Nanometer
NMDA:	N-methyl D-aspartate
nNOS:	Neuronal nitric oxide synthase
NO:	Nitric oxide
NOS:	Nitric oxide synthase
$O_2^{\bullet-}$:	Superoxide anions
OLF:	Oubain like factor
PDE:	Phosphodiesterase
PE:	Phenylephrine
PGI_2:	Prostacyclin
PKA:	Protein kinase A
PKC:	Protein kinase C
PLC:	Phospholipase C
PNS:	Parasympathetic nervous system
PSAT:	Phosphoserine aminotransferase
PSP:	Phosphoserine phosphatase
R:	Reference sample withdrawal rate (ml/min)
RAAS:	Renin angiotensin aldosterone system

RMP:	Resting membrane potential
RNA:	Ribonucleic acid
ROS:	Reactive oxygen species
SD:	Sprague-Dawley
SDH:	Serine dehydratase
SEM:	Standard error of the mean
sGC:	Soluble guanylate cyclase
SHMT:	Serine hydroxymethyltransferase
SHR:	Spontaneously hypertensive rat
$SK_{Ca:}$	Small conductance calcium activated potassium channels
SNP:	Sodium nitroprusside
SNS:	Sympathetic nervous system
SOD:	Superoxide dismutase
SPT/AGT:	Serine:pyruvate/alanine:glyoxylate aminotransferase
TG:	Triglycerides
THF:	Tetrahydrofolate
TPR:	Total peripheral resistance
TPRI:	Total peripheral resistance index
TXA_2:	Thromboxane
VSM:	Vascular smooth muscle
VSMC:	Vascular smooth muscle cell
WKY:	Wistar-Kyoto

LIST OF CHEMICALS

Acetylcholine

Apamin

Barium chloride

Cellosolve acetate

Charybdotoxin

D-serine

Fluorescent microspheres

Glycine

Indomethacin

L-serine

L-threonine

MK-801

N^G-nitro-l-arginine-methyl ester

Oubain

Phenylephrine

Potassium chloride

Sodium nitroprusside

Thiopental sodium

Tram-34

Tween® 20 and Tween®80

U4669

Chemicals were obtained from Sigma-Aldrich Canada Ltd, Abbot Laboratories Canada Ltd and EMD Bioscience Inc. USA.

CHAPTER 1

INTRODUCTION

1.1. Hypertension: An Overview

"Hypertension" is the medical term for blood pressure (BP) that remains high over time. High blood pressure is a very common disorder in which the pressure in the arteries is too high especially in middle-aged and elderly people. Once it has developed, it tends to last for life. High BP puts strain on the heart and the circulatory system, which can ultimately cause damage to several organs leading to heart failure, stroke and renal failure. As per medical guidelines, a BP reading of 120/80 (systolic/diastolic) millimeter of mercury (mmHg) is considered to be normal. Pre-hypertension is a condition where BP is elevated above normal but not to the level considered to be high BP. The latest classification of BP values proposed by the joint national committee (JNC 7) of the National Institutes of Health, USA, is given in the table below (Table 1). According to the JNC-7 classification a BP value of 140/90 mmHg or higher is considered as high blood pressure (Chobanian et al., 2003). High blood pressure is a 'risk factor' for developing cardiovascular diseases such as myocardial infarction, stroke, renal failure, transient ischemic attacks and peripheral vascular diseases (Lawes et al., 2008). Hypertension is the most common cardiovascular disease and is a major public health problem in both developed and developing countries (Kearney et al., 2005; Lawes et al., 2003). It produces a marked effect on patients, relatives and society, either because of hypertension *per se* or through its complications which can produce premature death or permanent disability.

Table 1. JNC-7 Classification of blood pressure for adults

BP Classification	**SBP* mmHg**	**DBP* mmHg**
Normal	<120	and <80
Prehypertension	120-139	or 80-89
Stage 1 Hypertension	140-159	or 90-99
Stage 2 Hypertension	>/=160	or >/=100

*DBP, diastolic blood pressure; *SBP, systolic blood pressure.

The risk of developing a cardiovascular complication is higher when the individual has hypertension combined with other risk factors such as diabetes, atherosclerosis, hyperlipidemia and insulin resistance state (Longo-Mbenza et al., 2002). Epidemiological studies show that there are significant geographical differences in the occurrence of hypertension and its complications both between and within countries. This geographical variation is considered to be influenced by the interaction of nutritional and environmental factors with the subject's genetic predisposition and susceptibility to develop hypertension. It is known that in people with hypertension more than 90 to 95 % have essential or idiopathic hypertension with unknown etiology. Only a small percentage of the hypertensive patients have an identifiable cause for secondary hypertension. Treating

hypertension involves lifestyle changes and pharmacotherapy with appropriate antihypertensive agents (Chobanian et al., 2003; Chockalingam et al., 2008).

1.1.1. Blood Pressure Regulatory Mechanisms

BP in the body is regulated at multiple levels through complicated interacting networks. These regulatory systems can be divided into extrinsic mechanisms and intrinsic mechanisms.

1.1.1.1. Extrinsic Mechanisms

The extrinsic control systems include the sympathetic nervous system (SNS), parasympathetic nervous system (PNS), and the circulating hormones which contribute to the regulation of BP and blood volume.

1.1.1.1.1. Nervous System

The nervous system plays an important role in the regulation of blood volume and BP. The BP regulatory center in the medulla plays a central role in regulation. Blood vessels are primarily innervated with sympathetic nerve fibers while the heart receives both sympathetic and parasympathetic innervations (Reis et al., 1984). Activation of the SNS leads to an increase in the vascular smooth muscle (VSM) tone resulting in vasoconstriction of the both resistance and capacitance vessels. This leads to an increase in total peripheral resistance (TPR). SNS activation also increases the heart rate (HR) and force of contraction, and causes renin release. Activation of the SNS also causes an increase in the

pre/post capillary resistance ratio, which leads to a decrease in capillary pressure and reabsorption of tissue fluid (Brunton LL, Parker KL, Lazo SJ. 2005. Goodman and Gillman's The Pharmacological Basis of Therapeutics. 11th Edition).

1.1.1.1.2. Humoral Systems

The humoral system regulates BP mainly through the renin angiotensin aldosterone system (RAAS), and other mediators described below.

1.1.1.1.2.1. Renin Angiotensin Aldosterone System

RAAS is a major endocrine system that contributes to the regulation of body fluid and BP. Decrease in perfusion of the juxtaglomerular apparatus in the kidney due to low BP results in the release of renin (Weber et al., 1975). Renin cleaves an inactive peptide angiotensinogen (alpha 2 globulin circulating in the blood), which promotes the formation angiotensin I (Ang I). Ang I is converted to angiotensin II (Ang II) by an enzyme found in lung capillaries called angiotensin-converting-enzyme (ACE). Ang II is a potent vasoconstrictor. Ang II acts as an agonist in an endocrine, paracrine, autocrine and intracrine fashion (Paul et al., 2006). Ang II stimulates the secretion of aldosterone from the adrenal cortex. Aldosterone causes the kidney tubules to retain sodium and water. This increases the blood volume which contributes to an increase in BP. RAAS hormonal system responds to low blood volume or a fall in BP (Solmon et al., 2005).

1.1.1.1.2.2. Arginine Vasopressin System

Arginine vasopressin (AVP) is a decapeptide which is also called antidiuretic hormone (ADH). AVP is derived from a prehormone which is synthesized in hypothalamus and stored in the posterior pituitary gland (Vander et al., 1995; Caldwell et al., 2006). AVP plays a major role in the reabsorption of water from the collecting ducts in the kidney *via* activation of V_2 receptors while at pharmacological doses it exerts a profound vasoconstriction by activating V_1 receptors found on vascular smooth muscle cells (VSMC) (McNeill, 1983; Gopalakrishnan et al., 1991).

1.1.1.1.2.3. Atrial Natriuretic Peptide

Atrial natriuretic peptide (ANP) is produced and released by cardiomyocytes of the atria of the heart. It is released in response to atrial stretch, stretching of the vessel walls, stimulation of beta receptors, hypervolemia, hypernatremia, elevation in Ang II and following exercise. It exerts both, a natriuretic and a vasodilatory effect. The overall effect of ANP is to counter RAAS mediated increase in BP (de Bold, 1985).

1.1.1.1.2.4. Oubain like Factor

An endogenous substance, an oubain like factor (OLF) that is indistinguishable from the digitalis glycoside, oubain, has been found circulating in blood (Hamlyn et al., 1991). This substance inhibits Na^+/K^+ activated ATPase and induces natriuresis and vasoconstriction. OLF is released in response to high sodium intake, hypervolemia and in conditions of cardiovascular disease state such as hypertension (Goto et. al., 1998).

1.1.1.2. Intrinsic Mechanisms

The intrinsic system regulates blood flow mainly within the tissue/organ or remains restricted to specific regional vascular beds. This mechanism includes myogenic mechanism which refers to the property of the muscle tissue to respond to stretch. It is likely that metabolic and humoral mechanisms contribute to the regulation of blood flow as an intrinsic control system (Shepherd et al., 1979; 1981 and Monos et al., 1995). In addition to the extrinsic and the intrinsic regulatory mechanisms, the endothelium is now widely recognized as a one of the major regulators of BP and blood flow. The endothelium can be considered as an endocrine organ in its own right and can be considered as a part of the humoral systems of regulation or as a separate system. It releases many vasodilatory and vasoconstrictor mediators and will be described in detail below.

1.1.1.2.1. Endothelium

The endothelium is the thin layer of cells that is present as an inner lining of the blood vessels. Thus, the endothelium is an interface between the lumen and the rest of the vessel wall. These cells are present in the circulatory system, beginning from the heart to the smallest capillaries. Endothelial cells (EC) are specialized epithelium called simple squamous epithelium. EC are very important for the regulation of various functions such as vasodilatation, vasoconstriction, thrombosis, fibrinolysis, angiogenesis, inflammation and edema formation. All of these factors contribute to the overall BP regulation by the endothelium. Transit of white blood cells into and out of the blood is controlled by the EC. In the brain capillaries the EC have tight junctions between them as a part of the blood brain barrier as a protective mechanism for the brain from blood borne substances. In the

renal glomerular capillaries the EC have gaps between them that allow the filtering function of the kidneys for urine formation. The endothelium releases a number of vasodilatory, vasoconstrictive and vasoactive mediators that play a role in the cardiovascular regulatory functions of endothelium. Thus, the endothelium plays an important role in the regulation of vascular tone (Dzau et. al., 1989). These endothelial mediators have been listed in the table below followed by a description of some of the more pertinent mediators (Table 2).

Table 2. Some of the most pertinent endothelial mediators and their function.

Mediator	Function
Nitric oxide (NO)	Vasodilator, inhibits platelet adhesion and aggregation
Prostacyclin (PGI_2)	Vasodilator, inhibits platelet adhesion and aggregation
Endothelium-derived hyperpolarizing factor (EDHF)	Vasodilator
Adenosine	Vasodilator
Atrial natriuretic peptide (ANP)	Vasodilator
Endothelin	Vasoconstrictor

Moreover, other blood borne mediators and mediators released from nerves act on the endothelium, platelets and vascular smooth muscle to regulate vascular tone, platelet function, capillary permeability and other cardiovascular functions. These mediators

include angiotensin II (Ang II), norepineprine, histamine, bradykinin, 5-hydroxytryptamine (5-HT) and thromboxane. In a physiological state there is a balance between these vasoconstrictor and vasodilator agents. In disease states such as hypertension and diabetes, this balance is disturbed due to altered bioavailability of one or more of the endothelial mediators such as NO (Levy et al., 1997).

1.1.1.2.2. Endothelium Derived Vasodilator Mediators

The endothelium produces a number of vasodilatory mediators such as EDHF, PGI_2 and NO (Moncada et al., 1979; Furchgott et al., 1980; Brandes et al., 2000). Carbon monoxide (Maines et al., 1997; Christian et al., 1999) and hydrogen sulfide (H_2S) (Yang G et al., 2008) have also been added to the list of vasodilatory mediators released from the endothelium.

1.1.1.2.2.1. Nitric Oxide

Furchgott described an endothelium-derived relaxing factor (EDRF) in 1980 that causes the vessel to relax. Endothelium-dependent agonists such as acetylcholine (ACh) and bradykinin stimulate EDRF production. EDRF was identified as NO in 1987 (Palmer et al., 1987; Ignarro et al., 1987). A lot of research has been done since in the role of NO in the cardiovascular and other body systems and functions. NO plays a pivotal role in the regulation of vascular tone and homeostasis (Ignarro et al., 1987; Palmer et al., 1987; Vane et al., 1990). eNOS knockout mice develop hypertension (Shesely et al., 1996). NO is synthesized from L-arginine by nitric oxide synthase (NOS) (Palmer et al, 1988). There are three isoforms of the NOS enzyme: neuronal (nNOSor type I NOS), inducible (iNOS or

type 2 NOS) and endothelial (eNOS or type 3 NOS) (Moncada et al., 1991). Each of the isoforms has separate locations. nNOS and eNOS are calcium-dependent and produce low levels of NO as a cell signaling molecule. iNOS is calcium-independent and produces large amounts of NO which can be cytotoxic or bactericidal (Bernardeau et al., 2001). NOS oxidizes the guanidino group of L-arginine in a process that consumes five electrons and results in the formation of NO with stoichiometric formation of L-citrulline. The process involves the oxidation of NADPH and the reduction of molecular oxygen. The transformation occurs at a catalytic site adjacent to a specific binding site of L-arginine. Tetrahydrobiopterin is required as a co-factor. The target of NO in the vascular smooth muscle cell is soluble guanylate cyclase. Stimulation of suoluble guanylate cyclase (sGC) causes an increase in cyclic guanosine monophosphate (cGMP) which ultimately leads to smooth muscle relaxation. NO directly causes hyperpolarization and relaxation of VSMC by activating calcium-dependent potassium channels which is independent of cGMP pathway (Bolotina et al., 1994). NO is an important regulator and mediator of numerous processes in the cardiovascular system, nervous system and the immune system. Thus, NO causes smooth muscle relaxation resulting in dilatation of the blood vessel and an increase in the blood flow. In the nervous system NO is involved in neurotransmission and has been associated with functions such as memory formation and avoidance learning. In the immune system production of large amounts of NO from iNOS acts as a bactericidal and cytotoxic agent against tumor cells. Acetylcholine (ACh), cytokines, platelet derived factors stimulate NO production by eNOS (Yuan, 2006). The vasodilator action of NO controls renal extracellular fluid homeostasis which is essential for the regulation of blood flow and BP (Yoon et al., 2000). Role of endogenous and exogenous NO on renin

secretion are mediated by a cGMP-induced inhibition of cAMP degradation and more exclusively mediated by phosphodiesterase (PDE)-3 (Kurtz et al., 1998).

1.1.1.2.2.2. Prostacyclin

Prostacyclin (PGI_2) is a prostanoid. Prostanoids are biologically active lipids, derived from twenty-carbon essential fatty acids (eicosanoids). They are further subdivided into three main groups, prostaglandins, PGI_2 and thromboxane (TXA_2), each one of them having distinct functions (Tohgi et al., 1992). PGI_2 is produced in the EC from cell membrane arachidonic acid. The enzyme prostacyclin synthase catabolizes the intermediate metabolite prostaglandin H_2 into PGI_2. PGI_2 a vasodilator and it inhibits platelet aggregation and VSMC proliferation. PGI_2 binds to G protein coupled receptor which activates adenylyl cyclase to produce cyclic 3', 5' adenosine monophosphate (cAMP). cAMP binds to specific locations on the regulatory units of the protein kinase, and causes dissociation between the regulatory and catalytic subunits, thus activating the catalytic units and enabling them to phosphorylate substrate proteins (Hanoune et al., 2001). cAMP activates protein kinase A (PKA). Activation of PKA phosphorylates number of proteins which inhibits myosin light-chain kinase and leads to smooth muscle relaxation. Increase in cAMP inhibits platelet activation and opposes increase in cytosolic free calcium levels in VSMC and promotes vasodilatation (Moncada et al., 1979). PGI_2 contributes substantially to myocardial protection (Utsunomiya et al., 1982; Csanyi et al., 2006).

1.1.1.2.2.3. Endothelium Derived Hyperpolarizing Factor

Endothelium derived hyperpolarizing factor (EDHF) is proposed to be a vasodilatory mediator whose identity is controversial. EDHF is released by EC and leads to NO/PGI_2 independent vasodilatation by relaxation of adjacent VSMC (Garland et al., 1996). The mechanism and nature of EDHF action is different among species, strain and between vascular beds (Mcguire et al., 2001). Several researchers have proposed that K^+ is the EDHF (Dong et al., 1997; McCulloch et al., 1997; Edwards et al., 1998). Epoxyeicosatrienoic acid (EET), a cytochrome P450 (CYP)-derived metabolite of arachidonic acid, produced a NO/PGI_2-independent relaxation in guinea-pig carotid artery which is pharmacologically distinct from the EDHF mediated response in rat mesenteric artery (Fleming et al., 2000; Mcguire et al., 2001). Hydrogen peroxide (H_2O_2) was proposed to be an EDHF in mouse, human mesenteric and porcine coronary arteries (Matoba et al., 2000; 2002; and 2003). C-natriuretic peptide (CNP) also has been proposed to act as an EDHF (Chauhan et al., 2003). L-NAME-insensitive NO was suggested to act as an EDHF in rabbit carotid artery and in rat small resistance arteries (Cohen et al., 1997; Chauhan et al., 2003). Myoendothelial gap junction (MEGJ) coupling between the endothelium and smooth muscle, has also been implicated in EDHF activity in many arteries, whereas EDHF activity may simply be due to the transfer of an endothelium derived hyperpolarization, and not to the activity of a factor *per se* (Coleman et al., 2001; Griffith , 2004). Thus there is no clear consensus with regard to the nature of EDHF. Based on experimental evidence it is agreed that there is an additional mechanism of vasodilatation that exists and it is NO/PGI_2 independent but mediates endothelium-

dependent hyperpolarization of both endothelium and VSMC and originates from the endothelium, called EDHF. There is some agreement that shear stress or receptor activation plays a critical role in the release of intracellular calcium which activates endothelial/non-endothelial potassium channels (Mcguire et al., 2001; Griffith et al., 2004). This rise in intracellular free calcium activates small (SK_{Ca}) and intermediate (IK_{Ca}) conductance calcium-activated potassium channels which are present on EC. This results in the release of an EDHF which hyperpolarizes the adjacent VSMC. The EDHF mediated response was abolished in the presence of depolarizing concentrations of potassium (Quilley et al., 1997). Inclusion of a combination of apamin (SK_{Ca} channel inhibitor), TRAM-34 (IK_{Ca} channel inhibitor), or charybdotoxin (IK_{Ca} and BK_{Ca} channel inhibitor) or ouabain (Na^+ pump inhibitor) and Ba^{2+} (K_{ir} channel inhibitor) blocked the EDHF mediated hyperpolarization of VSMC (Edwards et al., 1998; Parsons et al., 1994). EDHF compensates for the lack of NO and mediates ACh-induced vasodilation in chronic NOS-inhibited SD rats (Desai et al., 2006).

1.1.1.2.2.4. Carbon Monoxide

Carbon monoxide also relaxes vascular smooth muscle (Marks et al., 1991). Carbon monoxide is produced by the microsomal heme oxygenases (HO). HO catabolizes heme molecules into biliverdin, ferrous iron and carbon monoxide. There are three isoforms of HO. HO-1 is inducible, HO-2 and HO-3 are constitutive. HO-1 is a stress protein (also known as heat-shock protein HSP32) and it acts as a general cytoprotectant against increased stress such as a bacterial infection or an arthritic joint where the

expression and activity of HO increases along with an increase in carbon monoxide levels. Heme comes from the breakdown of hemoglobin, myoglobin and other heme containing proteins. The target of carbon monoxide is sGC causing an increase in cGMP. HO-3 is considered to be a sensor of oxygen (Maines et al., 1997; Christian et al., 1999).

1.1.1.2.2.5. Hydrogen sulfide

H_2S has been shown to play a role as a vasorelaxant and regulator of blood pressure in mice. Knockout mice lacking the enzyme cystathionine γ-lyase (CSE) have higher blood pressure (Yang et al., 2008). H_2S is synthesized from L-cysteine by cystathionine ß-synthase (CBS) and CSE. CSE is the main H_2S forming enzyme in the cardiovascular system whereas CBS is the main enzyme in the central nervous system (Wang et al., 2002). H_2S causes vasorelaxation by activating K_{ATP} channels in the vascular smooth muscle and causing hyperpolarization (Wang et al., 2002).

1.1.1.2.3. Endothelium Derived and Other Vasoconstrictor Factors

In normal physiological condition, the release of NO is predominant. NO modulates the release and resultant effect of vasoconstricting factors (Busse et al., 1985). In pathological conditions such as hypertension, atherosclerosis and diabetes, NO bioavailability and its vasodilator effect becomes impaired due to endothelial dysfunction, resulting in an imbalance between the endothelium derived relaxing and contracting factors. In such disease states, impairment of the release of NO augments the effects and release of endothelium derived vasoconstrictor factors, resulting in widespread vasoconstriction and an increase in BP (Vanhoutte et al., 1986).

1.1.1.2.3.1. Endothelin

ET is a 21 amino acid vasoconstrictor peptide and it is produced primarily in the endothelium (Yanagisawa et al., 1988). ET is one of the most potent vasoconstrictors and it is implicated in several vascular diseases. ET has three isoforms, namely ET-1, ET-2 and ET-3 and two receptors namely ET_A and ET_B (Hynynen et al., 2006). ET_1 released from the endothelium exerts vasoconstriction *via* activation of ET_A receptors present on adjacent VSMC (Schinelli et al., 2006). The ET_B subtype is predominantly expressed on EC and ET_B receptor activation on EC results in increased NO generation (Batra et al., 1993; Hopfner et al., 1999). In addition, in certain vascular beds, there are ET_B receptors present on VSMC which in concert with ET_A promote vasoconstriction (Batra et al., 1993; Hopfner et al., 1999). ET is rapidly metabolized when it passes through the lungs. Interaction of ET_1 with its ET_B receptors leads to its internalization and rapid breakdown in the lungs (Sokolovsky, 1995). With the exception of ET, all vasoactive substances have short half-lives and are involved in short term regulation of vascular tone (Yanagisawa et al., 1988; Agapitov et al., 2002). Both ET_A and ET_B receptors are G-protein coupled receptors that are linked to phospholipase C (PLC) mediated Ca^{2+} mobilization and protein kinase C (PKC) activation. ET_1 is a powerful long lasting vasoconstrictor agonist because it can promote Ca^{2+} independent effects subsequent to its effect mediated *via* activation of PKC and Rho-kinase (Christopher et al., 2003). Recently it has been suggested that both ET_A and ET_B receptors are found in the nervous system and play a critical role in neurotransmission. ET plays an important role in vascular function (Bernes et al., 1997).

1.1.1.2.3.2. Vasoconstrictor Prostanoids

TXA_2 in another prostanoid synthesized in platelets by thromboxane A synthase from endoperoxides produced by cyclooxygenase (COX) enzyme from arachidonic acid (Tohgi et al., 1992). PGI_2 and TXA_2 have important and opposing functions for the maintenance of physiological vascular tone. An optimum balance is maintained between PGI_2 and TXA_2 production in healthy states. TXA_2 binds to thromboxane receptors (G-protein coupled receptors coupled to the G protein G_q) and promotes platelet aggregation, vasoconstriction, and smooth muscle proliferation. TXA_2 and PGI_2 are therefore important mediators of pathological vascular events including thrombosis and atherogenesis.

1.1.1.2.3.3. Free Radicals

Free radicals are molecules or ions with unpaired electrons on an otherwise open shell configuration. These unpaired electrons are usually highly reactive. Free radicals include oxygen derived free radicals, which are commonly known as reactive oxygen species (ROS) (Halliwell, 1991). ROS include superoxide anions ($O_2^{\bullet-}$), hydroxyl radicals (·OH), as well as highly reactive non-radicals which do not have an unpaired electron in their orbit, such as hydrogen peroxide (H_2O_2) (Halliwell, 1991). Free radicals and ROS react with proteins, lipids and DNA to alter their structure and function. Oxidative stress is caused when there is an excess of free radicals and ROS production causing an imbalance with antioxidant defenses such as antioxidant enzymes. These free radicals are responsible for aging, tissue damage, and diseases. Superoxide anion can react with NO to form peroxynitrite (Gryglewski et al., 1986). Superoxide anion also stimulates the production of TXA_2 *via* activation of prostaglandin H synthase (de Artinano et al., 1999). In

cardiovascular disease state there is oxidative stress with a resultant increase in free radical generation. This in turn decreases superoxide dismutase (SOD) activity, vitamin E and NO production (Vijay et al., 1993). SODs are a class of enzymes that catalyzes the dismutation of superoxide to oxygen and hydrogen peroxide (McCord et al., 1969; McCord et al., 2005). They serve as an important antioxidant. SODs have three isoforms and they are found in different cell compartments. SOD-1 is found in the cytoplasm, SOD-2 in mitochondria and SOD-3 is extracellular. This excessive increase in free radical generation can inactivate PGI_2 synthase and decrease the expression of eNOS (Vidal et al., 1998; Davidge et al., 2001). It has been suggested that free radicals contribute to vasoconstriction, and free radical scavengers such as tempol successfully restore the generation of PGI_2 and NO, thereby augmenting vasodilatation (Katusic et al., 1989).

1.1.1.2.4. Endothelial dysfunction

Endothelial dysfunction is a hall mark of cardiovascular diseases such as hypertension (Virdis et al., 2008) and diabetes and it is often an early finding in these conditions (Ulvi, 2005). Grossly speaking endothelial dysfunction can be defined as the dysfunction of one of more aspects of the regulatory function of the endothelium. Thus, vasodilation, vasoconstriction, coagulation/anticoagulation, fibrinolysis, angiogenesis etc may be affected by endothelial dysfunction (Le Brocq et al., 2008). However, endothelial dysfunction is more commonly measured as the ability of the blood vessel to relax in response to stimulation by endothelium-dependent agonists such as acetylcholine or bradykinin. Thus, endothelial dysfunction is commonly referred to as a reduced endothelium-dependent relaxation of the vessel, commonly an artery. NO is one of the

main vasodilatory mediators released by the endothelium in different vascular beds and reduced NO bioavailability is one of the main findings in endothelial dysfunction (Schulz et al., 2008; Davis et al., 1997). Patients with essential hypertension have reduced NO production compared to normal individuals (Forte et al., 1997). Supplementation with exogenous NO, novel NO donors or NO releasing compounds have been suggested to be beneficial for normalizing normal biological functions in disease states with reduced NO production (Hou et al., 1999). However, a more common reason for reduced NO bioavailability is an increase in oxidative stress with an excess of superoxide anion production that reacts with NO to form peroxynitrite (Darley-Usmar et al., 1995; Schulz et al., 2008; Pacher et al., 2007). Endothelial dysfunction and increase in vascular resistance in hypertensive patients is related to the imbalance of vasodilators (NO and PGI_2) and vasoconstrictors ET and TXA_2 and TXA_2 mediated vascular responses are exaggerated (Abe et al., 1995). In patients with pulmonary and essential hypertension, synthesis of endogenous PGI_2 was found to be reduced. Exogenous infusion of PGI_2 improved systemic circulation and decreased pulmonary artery pressure (Kadowitz et al., 1978). Often in some disease conditions such as end-stage renal failure, hypercholesterolemia and in some forms of hypertension, circulatory asymmetric dimethylargine (ADMA) level in blood is high and this interferes with the L-arginine stimulated NO synthesis (Moncada et al., 1991; Vallance et al., 1992). ADMA is an endogenous competitive inhibitor of NOS. In animal models, acute ADMA administration increases peripheral resistance and raises systemic blood pressure that is reversed by L-arginine. The L-arginine: ADMA ratio may be an endogenous determinant of arterial tone in some forms of hypertension. In addition, the

endothelium generates mediators that promote as well as inhibit vascular growth other besides being a source for contractile and vasodilatory mediators (Luscher et al., 1993).

1.2. Animal Models of Hypertension

Hypertension is a multifactorial and complex disease. It involves interactions between genetically determined homeostatic control mechanisms and environmental factors (Takahashi et al., 2004). The ideal animal model for hypertension research should have human-like physiology, cardiovascular anatomy, hemodynamics, and develop human disease characteristics and complications. No species or experimental model can answer all of these needs. So currently various experimental animal models of hypertension are used for hypertension research. The general categories of animal models of hypertension are divided into genetic and non genetic models. The genetic animal models of hypertension include those are phenotype driven with natural variation among inbred strains e.g. SHR and genotype-driven with genetic based manipulation of gene expression. The non genetic models are developed by renovascular surgery or injury, vasoactive intervenation, dietary and endocrine manipulation (Pinto et al., 1998). The non genetic models include two kidneys one-clip, one kidney one-clip, partial or total nephrectomy, NOS inhibition, deoxycorticosterone (DOCA) salt, dahl salt-sensitive and pregnancy induced (Lerman et al., 2005). Mice deficient with all or individual NOS isoforms (neuronal, inducible and endothelial) have been developed for metabolic and hypertension research (Yang et al., 1999; Tsutsui et al., 2006). Mice deficient in the CSE was developed to study cardiovascular effect of H_2S (Yang et al., 2008). In the present study we used two different animal models of hypertension; a genetic model and a chronic NOS inhibition model.

1.2.1. Spontaneously Hypertensive Rat (SHR)

SHR is an animal model genetically bred to display high BP. SHR strain was developed from a group of outbred normotensive Wistar-Kyoto (WKY) rats mated with female rats with high BP from the same strain. Subsequent brother and sister matings with continued selection for high BP from the same group led to the selection of a group of rats that developed high BP with increases in age (Okamoto et al., 1963). The SHR strain shows a functional increase in vascular resistance and this is attributed to centrally mediated sympathetic hyperactivity (Falckh et al., 1992). SHR shows structural changes in blood vessel wall and supersensitivity of the blood vessels to vasoconstrictor stimuli which is similar to humans with high BP (Bohr et al., 1984 and 1988). Initial studies for clinical efficacy of angiotensin receptor antagonists (ARB) in the management of essential human hypertension were primarily conducted in the experimental SHR model (Timmermans et al., 1993). SHR with suppressed NO synthesis very closely mimic the cardiac and renal outcomes seen in patients with essential hypertension. Thus, the SHR strain is considered one of the most useful models in hypertension research.

1.2.2. Rat Models with Chronic NOS Inhibition

Chronic inhibition of NO with N^G-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor increases BP in rats (Ribeiro et al., 1992). L-NAME induced hypertension in rats is characterized by changes in cardiovascular function with increases in cardiopulmonary reflex (Scrogin et al., 1998; Araujo et al., 1998). Chronic L-NAME treatment up regulates the EDHF system in rats (Desai et al., 2006). Chronic NO inhibition increases volume dependent hypertension and its pathophysiological characteristics are similar to essential

hypertension in humans (Lahera et al., 1992; Romeroet al., 1993). L-NAME treated hypertensive rats share similar pathophysiological findings as essential hypertension in humans with hypertensive heart disease and end stage renal disease (Zanchi et al., 1995). L-NAME treated hypertensive rat models have been used in a series of studies designed to investigate the effects of different classes of antihypertensive agents in the prevention of hypertension (Nakamura et al., 2001). Thus, the L-NAME treated hypertensive rat model is useful because it is foregoing, time saving and has good economic value.

1.3. Problems in Treating Hypertension

Reduction of BP to the target normotensive levels in patients with hypertension is not easily attained. The problem gets worse if the patients are older, obese or diabetic and remained untreated for a long time. Thus, early diagnosis and appropriate treatment is vital. Recently, National Health and Nutrition Examination Survey (NHANES, 2008) considered participants from all age groups for appropriate treatment of high BP (David et al, 2008). Among all participants considered, half of the participants showed a significant reduction in BP to less than 140/90 mmHg, the recommended level. Some participants did not show any improvement, and thus were considered as having uncontrolled hypertension, which may be due to poor compliance in taking the prescribed medication or having resistant hypertension. People with uncontrolled or resistant hypertension normally do not respond to antihypertensives. Many people with high BP need more than one medication to reduce their BP to the recommended level. In addition, people may be on several other medications for other conditions along with antihypertensive medication which often interact and reduce the efficacy of the antihypertensives. Simple reduction in BP is no

longer the ideal goal of hypertension treatment. Ideally, a new agent for the treatment of hypertension should have the following characteristics in order to represent a significant advancement over the present therapy: 1) a longer duration of action, absence of tachycardia and orthostatic hypotension, 2) the ability to promote sodium excretion, 3) absence of increased plasma renin activity, 4) a mechanism of action related to the cause of the hypertensive disease, and 5) fewer side effects and less toxicity than seen with presently available agents, 6) reversal and restoration of endothelial function, structural cardiovascular modification induced by hypertension and reduction in platelet aggregation. Various approaches within the pharmaceutical industry as well as in academic research laboratories can be taken in search of new antihypertensive agents.

1.3.1. Current Management of Hypertension

Treatment of hypertension involves lifestyle changes and drug therapy. if changes in life style are ineffective, drug therapy is initiated, often requiring more than one agent to reduce BP. All drug treatments have side effects. There are different drug classes that can be used to treat high BP.

1.3.2. Classification of Antihypertensive Drugs

Diuretics

1. Thiazides and related agents (hydrochlorothiazides, chlorothalidone, indapamide).

2. Loop diuretics (furosemide, bumetanide, torsemide).

3. K^+ sparing diuretics (amiloride, triameterene, spiranolactone)

Sympatholytic Drugs

1. Centrally acting agents (methyldopa, clonidine, guanfacine).

2. Ganglionic blocking agents (trimethaphan).

3. Adrenergic neuron blocking agents (guanethidine, reserpine)

4. Beta adrenergic antagonists (propranolol, metropolol)

5. Alpha adrenergic antagonists (prazosin, terazosin, phentolamine, phenoxybenzamine).

6. Mixed adrenergic antagonists (labetalol, carvedilol)

Vasodilators

1. Arterial (hydralazine, minoxidil, diazoxide)

2. Arterial and venous (nitroprusside)

Others

1. Ca^{2+} channel blockers (verapamil, diltiazem, nifedipine)

2. Angiotensin converting enzyme inhibitors (captopril, enalapril, lisinopril)

3. Angiotensin II receptor antagonists (losartan, valsartan, telmisartan)

Diuretics are usually recommended as the first line of therapy for most people who have hypertension. If one drug doesn't work, combination of antihypertensive agents often used.

Combination therapy is used to treat uncontrolled or resistant types of hypertension. Disadvantages of combination therapy are drug interactions, side effects and increased problems with compliance (Brunton LL, Parker KL, Lazo SJ. 2005. Goodman and Gillman's The Pharmacological Basis of Therapeutics. 11th Edition).

1.3.3. New Promising Drugs to Treat Hypertension

Renin inhibitor (Aliskiren) provides comprehensive Ang II suppression by blocking the largest step down the RAAS pathway which is rate-limiting (Schmieder et al, 2009). Aliskiren was approved in 2007 by the Food and Drug Administration (FDA) to treat primary hypertension. CYT006-AngQb is a vaccine designed to elicit production of anti-Ang II-specific antibodies has been showing promising outcomes in preclinical studies (Tissot et al., 2008).

1.4. Beneficial Effects of Amino Acids in Hypertension

Amino acids play an important role as neurotransmitters and serve as precursors to several neurotransmitters for proper functioning of the nervous system (Munro et al., 1986). As early as 1972 it was first demonstrated that central administration via intracerebroventricular infusion of taurine, gamma-aminobutyric acid (GABA), L-alanine and glycine produced a hypotensive response in anaesthetized rat models (Sgaragli et al., 1972). Dietary supplementation of tyrosine, which is a precursor for epinephrine and norepinephrine has the potential to decrease BP in hypertensive animals but has minimal

effect in normotensive animals (Sveda et al., 1979). Tryptophan a precursor for serotonin, injected intraperitoneally also has been shown to lower BP in both normotensive and hypertensive rats (Wolf et al., 1984). Taurine an amino acid neurotransmitter has been extensively studied for its potential as an antihypertensive agent (Nara et al., 1978). They concluded that supplementation of taurine in drinking water decreased BP in SHR as well as in stroke prone SHR. Some studies also proposed that the BP lowering effect of taurine is associated with increased salt excretion and decreased sympathetic discharge (Abe et al., 1987; Fujita et al., 1988; Militante et al., 2002; Oudit et al., 2004). Several studies show that another amino acid glycine protects against oxidative stress which is encountered in cardiovascular disease states including hypertension (Hafidi et al., 2006). These authors also proposed that glycine supplementation lowered BP probably by decreasing non-esterified fatty acid (NEFA), triglycerides (TG) and intra-abdominal fat (IAF). It has been suggested that glycine plays an important role in glutathione (GSH) synthesis. GSH scavenges free radicals which can reduce the availability of NO. Thus, GSH improves NO availability. Glycine rectifies endothelial dysfunction which contributes to lowering of BP (Lees et al., 1996). Glycine is thought to promote cardiovascular protection and exerts an antihypertensive effect via increased generation of S-nitrosoglutathione, a vasodilator intermediate generated from glycine which increases GSH synthesis (Lees et al., 1996). However, no systemic studies have been undertaken to examine the dose-dependent effects of glycine on BP regulation in both normotensive and hypertensive states. The present study aims to address these issues.

1.5. Serine

Serine is an organic compound with the molecular formula HO_2CCH (NH_2) CH_2OH. It is one of the 20 naturally occurring proteinogenic amino acids. Only the L-stereoisomer appears to be present in proteins naturally. Since it is synthesized in the body from other metabolites, it is not essential in human diet. However, it is important for cell growth and viability as it is always added and included in various cell culture media. Thus it is considered conditionally essential in cell culture (McCoy et al., 1956). Silk protein is a rich source of L-serine and it was first obtained from silk protein in 1865 (Cramer, 1865). In 1902, its structure was established (Fischer et al., 1902). In view of the presence of two hydroxyl groups in its structure, it was classified as a polar amino acid (Fischer et al., 1907).

1.5.1. Source of L-serine

L-serine is classified as a nutritionally non-essential amino acid. The main source of essential amino acids is from the dietary sources, whereas non-essential amino acids are normally synthesized by humans and other mammals from common intermediates (Ichihara et al., 1957). L-serine can be derived and biosynthesized from glucose metabolism as a glycolytic intermediate, 3-phosphoglycerate (3-PG), in a three-step process involving the enzymes: 3-phosphoglycerate dehydrogenase (3-PGDH), phosphoserine aminotransferase (PSAT), and phosphoserine phosphatase (PSP), dietary protein, glucose, protein phospholipid degradation, glycine, and threonine (Figure 1, Fischer et al., 1902; Stein et al., 1942; Greenstein et al. 1961; de Koning et al., 2003). L-serine synthesis and concentration

varies with species, tissues, and during different developmental stages (Narkewicz et al., 1996). The fasting plasma concentration of L-serine in a healthy human is 130 ± 30 μmol/L (de Koning et al., 2004).

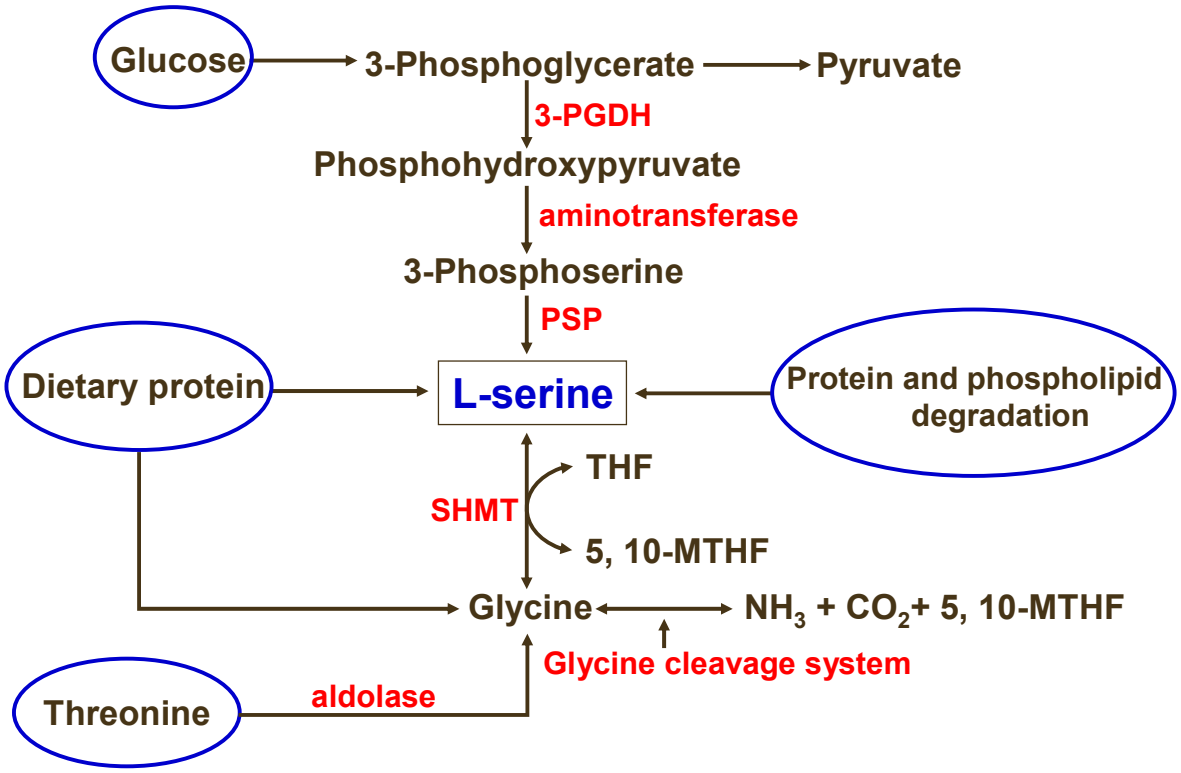

Figure 1. Various sources and biosynthetic pathways of L-serine.

3-PGDH, 3-phosphoglycerate dehydrogenase; PSP, phosphoserine phosphatase; SHMT, serine hydroxyl methyltransferase; THF, tetrahydrofolate.

1.5.2. Pathways of L-serine Formation

L-serine biosynthesis involves recruitment of two major pathways, namely phosphorylated and non-phosphorylated forms. The phosphorylated pathway is the primary pathway for L-serine synthesis involving 3-phosphoglycerate, L-phosphoserine, L-serine and phosphohydroxypyruvate, but it has no role in gluconeogenesis since it is responsible for dephosphorylation of phosphoserine in many tissues and enzymes for this pathway are abundant in many tissues e.g. brain, testis, spleen and kidney (Snell, 1986). The non-phosphorylated pathway involves D-glycerate, hydroxypyruvate and L-serine. This pathway is confined to the liver and the kidney since enzymes for this pathway are restricted selectively to these organs only (Rowsell et al., 1969; Cheung et al., 1969). Racemic serine can be prepared from methyl acrylate (Carter et al., 1955). The biosynthesis of L-serine starts with the oxidation of glycolytic intermediate, 3-phophoglycerate to 3- phosphohydroxypyruvate and nicotinamide adenine dinucleotide (NAD^+), which then is reduced to NADH. Reductive amination of this ketone by hydrolysis gives rise to L-serine. Serine hydroxymethyltransferase (SHMT) catalyzes the conversion of L-serine to glycine by retro-aldol cleavage and 5,6,7,8-tetrahydrofolate (THF) hydrolyzes to 5,10 methylenetetrhydrofolate (MTHF, Nelson et al., 2000). The Kidney synthesizes most of the L-serine from 3-phosphoglycerate whereas the glycine cleavage system interacts with SHMT and their combined action gives rise to L-serine in fetal liver (Lowry et al., 1987).

1.5.3. Metabolism of L-serine

Metabolism of L-serine involves three major enzymes namely, L-serine dehydratase (SDH), serine:pyruvate/alanine:glyoxylate aminotransferase (SPT/AGT), and SHMT. Most importantly, SDH and SPT/AGT participate in gluconeogenesis from L-serine (Xue et al., 1999). SHMT is a key enzyme that catalyzes the generation of 5,10 MTHF via formation of glycine from L-serine. SHMT has two isozymes, namely cytosolic (cSHMT) and mitochondrial (mSHMT). It promotes the interconversion between L-serine and glycine in a combined action with mitochondrial glycine cleavage system (GCS), when either amino acid is used or supplied (Snell, 1984). In rodents, SDH plays the major role in comparison to GCS, SPT/AGT and SHMT in the metabolism of L-serine (Xue et al., 1999).

1.5.4. Pathways of L-serine Utilization

There are various pathways of L-serine utilization (Figure 2). L-serine plays a key role in the formation of myelin, lipid messenger molecules, and it is required for normal functioning of the central nervous system. L-serine serves as a direct/indirect precursor for the synthesis of several neurotransmitters and amino acids (Mustafa et al., 2004). Moreover in cell growth and development, L-serine plays an essential role in the synthesis of purine and pyrimidine nucleotides being the source for the one carbon unit (Eagle., 1959; Snell et al., 1987).

1.5.4.1. L-serine and Gluconeogenesis

Gluconeogenesis from L-serine involves two enzymes, namely SDH and SPT/AGT. SDH is involved in the formation of pyruvate whereas SPT/AGT is involved in the

formation of hydroxypyruvate from L-serine (Figure 2). SPT/AGT enzyme distribution varies considerably between species (Xue et al., 1999). In carnivores, SPT/AGT is distributed predominantly in the mitochondria whereas in herbivores it is present in peroxisomes. In rodents, SDH is important while in humans, SPT/AGT enzyme plays a major role in gluconeogenesis (Xue et al., 1999). Recently it has been suggested that in brain, L-serine in the presence of D-/L-serine racemase, can generate pyruvate (De Miranda et al., 2002).

1.5.4.2. L-serine and Cystathionine Formation

Combined action of L-serine and homocysteine gives rise to cystathionine. Cystathionine is an intermediate in the synthesis of cysteine. It is generated from homocysteine and serine by cystathionine beta synthase, then subsequently cleaved to cysteine and alpha ketobutyrate by the enzymatic action of cystathionine gamma-lyase (Dudman et al., 1987). L-serine is required for the synthesis of cysteine, taurine and sulphate (Figure 2). Taurine and sulphate are formed in trans-sulphuration pathways. Taurine and sulphate are both important for the normal brain function. Taurine is considered to be an inhibitory neurotrasmitter, an osmoregulator and it is also responsible for migration of neurons (Schousboe et al., 1992). Deficiency of sulphate results in severe neurological problems since it plays an important role in the formation of steroid hormones and glucosphingolipids (Johnson et al., 2001). Both cysteine and glycine are metabolic products of L-serine and they serve as precursors for the biosynthesis of glutathione. Deficiency in glutathione is responsible for the cause of hemolytic anemia and neurological disorders (Wullner et al., 1999). In disease state like homocysteinuria, L-serine and glycine

levels are found to be low in the cerebrospinal fluid (CSF) but not in plasma; however, it is not clear whether in such a condition there is a decrease in the synthesis of L-serine and glycine or whether brain uses more L-serine and glycine (Surtees et al., 1997).

1.5.4.3. L-serine and Biosynthesis of Phospholipids

L-serine serves as a precursor for the biosynthesis of phosphoglycerols, sphingolipids, sphingosine, glycolipids. Sphingolipids are myelin constituents and they are essential for cellular proliferation and differentiation (Perry et al., 1998). L-serine derived phospholipids such as sphingosine play a central role in the production of myelin and they act as lipid messenger molecules (Hannun et al., 1989; Perry et al., 1998). Ceramide is converted from sphingosine, which is a precursor for gangliosides and spingomyelin (Hannun et al., 1989). Sphingolipids are abundant in the myelin sheath and are present in all membranes. Ceramide is critical for cell survival (Hannun., 1996). Deficiency of ceramide synthesis could result in apoptosis and neuronal cell death (Perry et al., 1998). Phosphoglycerol phosphatidylserine which is synthesized from L-serine can be converted to phosphatidylcholine through phophatidylethanolamine. Phosphatidylserine plays a role in apoptotic cell signaling (Tyurina et al., 2000). Impairment in phospholipid metabolism results in abnormalities in cerebral white matter (de Koning et al., 2000). Thus, L-serine has a key role in synthesizing complex macromolecules for normal functioning of the central nervous system (CNS) through different signaling pathways (Figure 2).

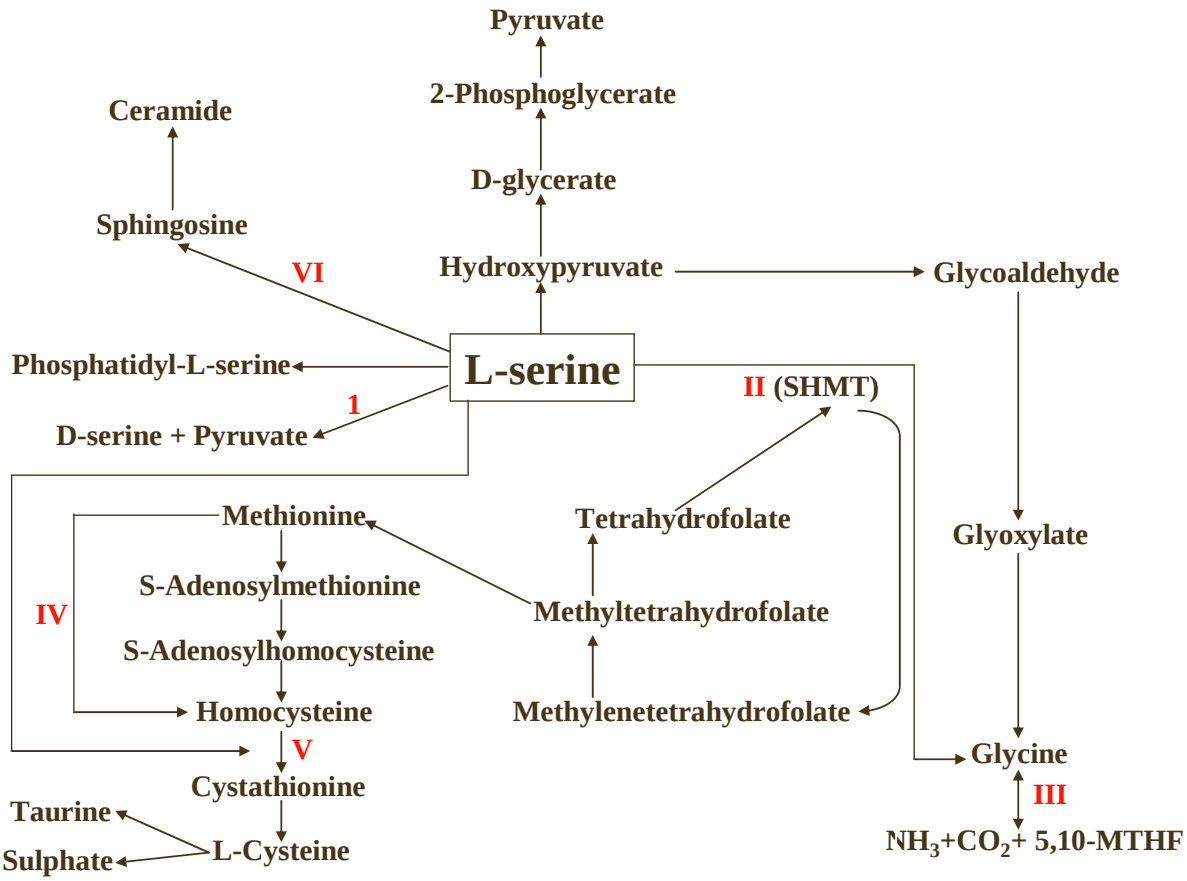

Figure 2. Involvement of L-serine in gluconeogenesis and major pathways for utilization I. D-/L-serine recemase, II. Serine hydroxymethyltransferase, III. Glycine cleavage system IV. Transmethylation of methionine, V. Trans-sulphuration of methionine, VI. Palmitoyl CoA.

1.5.4.4. Role of L-serine in the Formation of D-serine, Glycine, L-cysteine and Taurine

L-serine is a precursor for the neurotransmitters, D-serine, glycine and indirectly through L-cysteine, for the synthesis of inhibitory neurotransmitter, taurine. D-serine synthesized through serine racemase from L-serine, serves as a neuromodulator and endogenous agonist of the N-methyl-D-aspartate (NMDA) receptors in the brain (Jean-Pierre et al., 2000). Production of pyruvate and D-serine contributes to the energy production in astrocytes (Miranda et al., 2002). Supplementation with D-serine and glycine has been shown to reduce the occurrence of seizures in patients with 3-PGDH deficiency (de Koning et al., 1998). Glycine is synthesized from L-serine by the enzymatic action of SHMT. Glycine is an inhibitory neurotransmitter in the brain stem, spinal cord and retina (Bruun et al., 1972; Berger et al., 1999). Glycine binds to glycine receptors on the post-synaptic membrane, or together with glutamate, it can act as a co-agonist (Johnson et al., 1987). Glycine plays an important role for all purines. Glycine mediated inhibitory effects in the spinal cord maintain a delicate balance between glutamate and NMDA induced excitatory effect. Glycine acts as a co-agonist along with glutamate for NMDA receptors (Yang et al., 2008). It is involved in locomotor behavior (Legendre, 2001). Most proteins contain less glycine with the exception of collagens which contains more than 30% (Nelson et al., 2005). Glycine can be converted to glyoxylate by D-amino acid oxidase and subsequently oxidized by hepatic lactate dehydrogenase to oxalate (Nelson et al., 2005). In fetal brain development, L-serine regulates the neurotransmission by synthesizing neuromodulators and phospholipids (Mitoma et al., 1998). Alteration in L-serine biosynthesis might contribute to severe brain dysfunction in developmental stages. Taurine is beneficial in the management of cardiovascular ailments (Militante et al., 2000). Taurine

keeps potassium and magnesium inside the cell while keeping excessive sodium out, thus it works like a mild diuretic (Pop-Busui et al., 2001). Taurine treatment has been attempted for controlling seizures in epileptic patients exhibiting uncontrollable facial twitches (Kirk et al., 1994).

1.6. L-serine and Health Benefits

Role of L-serine in cell growth and development: conversion of L-serine to glycine by SHMT results in the formation of the one-carbon units required for the biosynthesis of purine bases, adenine and guanine. These bases are linked to the phosphate ester of pentose sugars that are essential components in the generation of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) and the end products of energy producing metabolic molecules such as adenosine triphosphate (ATP) and guanosine-5'-triphosphate (GTP). L-serine conversion to glycine via SHMT provides the same one-carbon units necessary for the production of pyrimidine nucleotide, deoxythymidine monophosphate, which is an essential component of DNA (McCoy et al., 1956). L-serine is an integral component of phospholipids and serves as a precursor for the biosynthesis of sphingolipids, glycolipids and phosphoglycerols. These are important constituents of cell membranes and they regulate signaling pathways for the cell survival and apoptosis (Eagle, 1959; Snell et al., 1987). L-serine is an important constituent for brain proteins and protective nerve coverings. L-serine contributes to the production of immunoglobulins and antibodies (Munro et al., 1986). L-serine has a pivotal role in proper functioning of the CNS. L-serine is a precursor for the production of several amino acids such as glycine, cysteine, phosphatidyl-L-serine, methionine, taurine, and tryptophan. Tryptophans act as a building

block in protein synthesis and serves as a biochemical precursor for serotonin biosynthesis, a neurotransmitter known to relieve stress, anxiety and depression (Schaechter et al., 1990). Some studies show that oral supplementation with L-serine reduces increased levels of methionine induced by homocysteine formation and γ-glutamyl transpeptidase activity in humans (Verhoef et al., 2004). Increased levels of plasma homocysteine and γ-glutamyl transpeptidase activity are associated with metabolic syndrome, cardiovascular diseases, and increased mortality risk (Lee et al., 2007). γ-Glutamyl transpeptidase plays an intermediary role in conversion of leukotriene D_4 from leukotriene C_4, which triggers the formation of P-selectin, thereby contributing to asthmatic and allergic reactions. Thus the inhibition of γ-glutamyl transpeptidase activity promoted by L-serine contributes to its anti-inflammatory effect (Kanwar et al., 1995).

1.7. L-serine Deficiency and Disease

L-serine deficiency leads to slow or delayed cognitive and physical skills, psychomotor retardation, seizures, microcephaly, and in some cases nystagmus, megaloblastic anemia, cataract and hypogonadism (de Koning et al., 2003). Microcephaly refers to health condition in which the head size is smaller than normal and is caused by underdevelopment of the brain (de Koning et al., 1998). Decrease in 3-PGDH activity results in low L-serine levels in both serum and CSF under a fasting state. Children with L-serine deficiency develop congenital microcephaly, psychomotor retardation and intractable seizures (Klomp et al., 2000). The gene encoding for 3-PGDH was found to be mutated on chromosome one (1p12). Due to this mutation, valine was substituted for methionine at

490 (V490M) position of the enzyme (Pind et al., 2002). The most common type of L-serine deficiency results in reduction of 3-PGDH enzyme, through a mutation in the gene (Klomp et al, 2000; Pind et al, 2002). Reduction in 3-PGDH contributes to hypomyelination and loss of white matter, characteristic of severe neurological disorder (Jaeken et al., 1996). Alteration in plasma SHMT activity alters L-serine and glycine levels in certain areas of the brain resulting in psychiatric disorder (Waziri et al., 1983; Waziri et al., 1984). Low levels of L-serine in CSF but not in plasma are the hall mark for 3-PGDH and 3-PSP deficiency (de Koning et al., 2004).

CHAPTER 2. HYPOTHESES AND RATIONALE

2.1. Background and Rationale for the Present Study

Recently, a study from our laboratory demonstrated that the vasodilator responses in the perfused mesenteric vascular bed evoked by ghrelin (a 28 amino acid peptide hormone released from the stomach) were not mediated via the classical action of ghrelin on the growth hormone secretagogue (GHS) receptor 1a (GHSR1a). Des-octanoyl ghrelin, a linear peptide and a precursor/metabolite of ghrelin with a very low affinity for the GHSR1a, evoked similar vasodilator responses as ghrelin. In addition, several N-terminal peptide fragments of des-acyl ghrelin gave a similar vasodilator response as full length des-acyl ghrelin in an *endothelium-dependent* manner (Moazed et al., 2009). The N-terminal sequence of des-acyl ghrelin is rich in serine residues (Kojima et al., 2001; Matsumoto et al., 2001). Moreover, it has been shown that N-arachidonoyl L-serine, an endocannabinoid-like brain constituent, produces endothelium-dependent vasodilatation of rat isolated mesenteric arteries and abdominal aorta (Milman et al., 2006). These authors did not examine whether L-serine *per se* would evoke endothelium-dependent vasodilatation. Currently, little is known about the cardiovascular effect of L-serine. The major goal of the present work is to address the cardiovascular effects of L-serine in a systematic fashion by carrying out a series of studies involving both *in vitro* and *in vivo* models.

2.2 Rationale for *in vitro* Studies with L-serine

The first phase of the work is to examine the effects of L-serine on phenylephrine constricted isolated rat mesenteric arteriole preparations. The third order branches of isolated mesenteric arterioles were chosen as the tissue preparation for the following reasons: **i)** it is easy to isolate, **ii)** it is a representative resistance type blood vessel that would reflect the resistance function of the circulation in the whole animal (Christensen and Mulvany, 1993), **iii)** ghrelin, des-acyl ghrelin and their N-terminal fragmented peptides, all evoke endothelium-dependent vasodilatation in the mesenteric vascular bed (Moazed et al., 2009). **iv)** Finally, Milman et al., (2006) employed mesenteric arteries to characterize the endothelium-dependent vasodilator responses to N-arachidonoyl-L-serine. Therefore, it is logical and rational to begin the first set of studies of the direct vasomotor effects of L-serine with rat isolated mesenteric arterioles.

2.3. Rationale for *in vivo* Studies

Although it is possible that the efficacy of L-serine as a vasodilator is similar to that reported for N-archidonoyl-L-serine in the mesenteric vascular bed, it could be higher or absent. The second stage of the project addresses whether the effect seen in the *in vitro* preparation in a resistance type arteriole would reflect as the likely change in the *in vivo* model following acute infusions of L-serine. Therefore, we examined the effects of an acute L-serine bolus infusion on the MAP and HR in normotensive and hypertensive rats. It is important that the study address the overall contribution and the effects of L-serine for the likely effects on BP particularly in the hypertensive rat models. These studies if successful would support the *in vitro* observations. It is also important to examine whether

the effects of L-serine is mimicked/shared by its D-isomer, D-serine, or its metabolite, glycine, or its precursor, L-threonine. This is in order to assess whether these amino acids *in fact* mediate the effects of L-serine subsequent to its bioconversion *in vivo*, or these related amino acids exert effects similar to L-serine via activating signaling mechanisms recruited by L-serine. If D-serine exhibits similar characteristics to L-serine, the cardiovascular effects of L-serine are not stereospecific. Thus, comparative studies with D-serine, glycine and threonine become vital to distinguish whether the effects of L-serine have selectivity and specificity.

2.4. Rationale for Regional Hemodynamic Studies

Once the effects of L-serine on MAP and HR are known, it is then important to determine the contribution of different vascular beds to the observed systemic effect. It is possible to monitor L-serine evoked changes in blood flow and vascular resistance in different vascular beds using the perfused microsphere technique. It is also feasible to calculate the changes in TPR before and after infusion of L-serine. Accordingly, this study will be the third in series of experiments assessing the *in vivo* effects of L-serine on different vascular beds besides the mesenteric vascular bed to account for the observed changes in systemic hemodynamic responses.

Finally and most importantly, the data from our laboratory and others have led to the conclusion that in the perfused mesenteric vascular bed, N-terminal peptide fragments of ghrelin evoke endothelium-dependent vasodilatation (Moazed et al., 2009). N-archidonoyl-L-serine also evokes endothelium-dependent vasodilatation in mesenteric arteries (Milman

et al., 2006). Interestingly, ghrelin-evoked hypotensive response was higher in L-NAME treated hypertensive rats. Pretreatment with a combination of apamin plus charybdotoxin, small and intermediate conductance K_{Ca} channel inhibitors, abolished the hypotensive responses to ghrelin (Shinde et al., 2005). There are no studies on the direct cardiovascular effects of L-serine either under *in vitro* or *in vivo* conditions. Based on the above, the following working hypotheses are formulated.

2.5. Working Hypothesis

L-serine evokes concentration-dependent changes in phenylephrine constricted mesenteric arterioles that is endothelium dependent. This action is mediated by the activation of Ca^{2+} dependent potassium channels present on the endothelium.

L-serine *per se* lowers mean arterial pressure (MAP) following its acute intravenous administration. The hypotensive effects of L-serine are greater in spontaneously hypertensive rats (SHR) and in rats rendered hypertensive by chronic inhibition of NOS by treatment with L-NAME, than in normotensive rats.

The fall in MAP in hypertensive rat is due to reduced total peripheral resistance (TPR), a summation of the fall in regional vascular resistance occurring in many vascular beds including the mesenteric vascular bed.

2.6. Experimental Strategy

2.6.1. Choice of Animal Models and Choice of Tissue:

All studies were performed in 12 to 14 week old male Sprague-Dawley rats, Wistar-Kyoto rats (WKY), SHR or Sprague-Dawley or WKY rats rendered hypertensive by L-NAME treatment for 5 days (Ribeiro et al., 1995). SHR is an ideal animal model, which mimics human essential hypertension (Okamoto et al., 1963). The origin of SHR is from mating with brother and sister in a continued selection fashion originated from outbred WKY male with elevated BP and female with slightly elevated BP. So it is appropriate to consider that WKY rats serve as normotensive control strain for SHR strain (Yukio et al., 1973). In SHR, the increase in BP is due to an increase in vascular resistance. Alteration in neurohormonal regulation also contributes to hypertension in these animals (Yukio et al., 1973). Since neuroendocrine dysregulation also appears to play a key role in the development of essential hypertension in humans, the SHR model is the closest animal model to human essential hypertension (Folkow et al., 1972). The SHR strain responds well to most of the antihypertensive agents used in human (Yamori, 1971). Thus, SHR is considered widely as a good laboratory model for screening antihypertensives (Okamoto, 1969). Previous studies have established that between 12 and 14 weeks of age, the elevation in MAP reaches a plateau and it would be the ideal age group to perform studies for lowering BP in the established phase of hypertension (Okamoto et al., 1973). The chronic L-NAME-induced hypertensive model Sprague-Dawley was chosen because ghrelin, showed an exaggerated hypotensive response in L-NAME treated rats (Shinde et al., 2005). The rationale for employing mesenteric arterioles for the *in vitro* studies was

highlighted earlier. Thus, the study design takes into consideration investigations at the following levels:

i) *In vitro Studies* – wire myography studies to examine the effects of L-serine on phenylephrine constricted third order branches of rat isolated mesenteric arterioles.

ii) *In vivo Studies* – The effects of acute intravenous infusion of L-serine in SD, WKY and SHR strains, SD and WKY rendered hypertensive by chronic treatment with L-NAME. Besides L-serine, the effects of comparable doses of D-serine, glycine and L-threonine will be determined.

iii) *Regional Hemodynamic Studies* – This will be performed in both hypertensive rat models as well as control normotensive strains to determine the vascular tissues targeted by L-serine. Besides changes in regional blood flow, perfused microsphere technique estimates changes in regional vascular resistance, TPR in the whole animal preparation.

CHAPTER 3. METHODS

3.1. Animals

The present study was approved by our University Review Committee and conformed to the Guide for the Care and Use of Laboratory Animals stipulated by the Canadian Council on Animal Care and the National Institute of Health (NIH) publication No. 85-23. Twelve to fourteen week-old male Sprague-Dawley rats (350 to 380g), Wistar-Kyoto (WKY) rats (300 to 350g) and SHR (250 to 270g) were obtained from Charles River (St. Constant, Quebec, Canada). One group of Sprague-Dawley and WKY rats received nitric oxide synthase (NOS) inhibitor, N^G nitro L-arginine methyl ester, L-NAME (0.7 mg/ml in drinking water ad libitum) for 5 to 7 days (Shinde et al., 2005; Desai et al., 2006). All other rats received plain water. In the present study, chronic L-NAME treated WKY and SHR served as hypertensive rat models, while untreated WKY and Sprague-Dawley rats served as normotensive control groups.

3.2. Materials

The chemicals used in these experiments and their sources are listed as follows: Acetylcholine (ACh), barium chloride (Ba^{2+}), indomethacin, L-serine, D-serine, glycine, L-threonine, N^G nitro L-arginine methyl ester (L-NAME) and (+)-5methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801), ouabain, phenylephrine hydrochloride (PE) were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON., Canada). Apamin and charybdotoxin (ChTX) were from EMD Biosciences Inc. (La Jolla,

CA, USA). 1-[(2-Chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34) was a gift from Dr. Heike Wulff, University of California at Davis. Thiopental sodium was obtained from Abbott Laboratories Ltd (Saint Laurent, Quebec, Canada). U46619 and another lot of L-serine were purchased from Calbiochem (San Diego, CA, USA). Fluorescent microspheres were purchased from Molecular Probes, Inc, (Eugene, Oregon, U.S.A.). Fluorescent microspheres are made up of polystyrene with a diameter of 10 to 15 µm. Fluorescent microspheres are available with different fluorescent dyes colors, whose fluorescence is well resolved from each other (Glenny et al., 1993).

Note: Charybdotoxin was used in place of TRAM-34 for *in vivo* studies since charybdotoxin is soluble in water where as TRAM-34 is insoluble in water, and soluble in dimethyl sulfoxide.

3.3. Experimental Procedures

3.3.1. In Vitro Study using Wire Myograph

The rats from normotensive Sprague-Dawley and Sprague-Dawley rats rendered hypertensive by pretreatment with L-NAME (0.7 mg/ml in drinking water for 5 to 7 days) were anaesthetized with an intraperitoneal injection of thiopental sodium (100 mg/kg, Shinde et al., 2005; Desai et al., 2006). Laparotomy was performed and the mesenteric bed was located. The rat mesentery was carefully excised, and cleaned of adherent tissues. A section of mesentery about 100 mm distal to the pylorus was tied with the help of two threads, rapidly removed and placed in oxygenated ice-cold Krebs buffer, the composition given below. With the aid of a dissection light microscope, the third order arterioles (inner

diameter ~120 μm) were carefully isolated. Six to eight rings (~2 mm length) were cut from the third order branches of the isolated blood vessels were mounted in wire myograph apparatus for isometric tension measurements. Rings were suspended between a micropositioner and force transducer with two stainless steel wires (40 μm diameter) in the myograph chamber (Model 610M Multi Wire Myograph System, Danish Myo technology, Denmark). Resting tension (1.5 mN) was maintained for an initial equilibration period of 1 hr in Krebs buffer of the following composition (in mmol/L): 120, NaCl; 1.8, $CaCl_2$; 4.8, KCl; 1.2, $MgCl_2$; 1.2, KH_2PO_4; 25, $NaHCO_3$; 10, glucose; pH 7.4 gassed with 95% O_2, 5% CO_2 and maintained at 37^0C (Buus et al., 1994). The rings were washed every 15 min in Krebs buffer. Isometric tension was recorded using a Powerlab data acquisition system (AD Instruments Pvt. Ltd., Sydney, Australia). Certain vessels were mounted without scratching the intimal surface while placing the hooks to ensure that the endothelium was not damaged. In some vessels endothelium was damaged by passing a thin wire /hair before they were mounted in the myograph. The endothelium was considered to be denuded if the vasodilator response to ACh was reduced to ≤ 10% of its original level (>90 %) with no change in the vasodilator response to sodium nitroprusside (SNP). The dose-response (DR) relationship for L-serine (1 μmol/L to 500 μmol/L) and ACh (1 pmol/L to 10 μmol/L) were determined in these vessels after sustained tonic vasoconstrictor response to a fixed concentration (≈ EC_{90}) of α_1 selective agonist, phenylephrine (PE, 10 μmol/L) was attained. In some experiments, instead of PE, vasoconstriction was attained using either the thromboxane analog, U46619 (1 μmol/L) or subjected to a depolarizing stimulus with potassium chloride (KCl, 80 mmol/L). In select experiments, mesenteric arterioles

isolated from L-NAME pretreated rats with intact endothelium that were preconstricted with PE, small increments in KCl were added to the organ baths such that the final concentration of KCl in the buffer would be 9.8, 12.8, 15.8 and 18.8 mmol/L and the changes in dilator responses were determined. When investigating the effects of various inhibitors such as a non-selective cyclooxygenase (COX) inhibitor, indomethacin (10 μmol/L), or apamin (SK_{Ca} inhibitor, 1 μmol/L) or TRAM-34 (IK_{Ca} inhibitor, 1 μmol/L), or ouabain (Na^+/K^+/ATPase inhibitor, 20 μmol/L), or Ba^{2+} (K_{ir} channel inhibitor, 50 μmol/L), they were added, either alone or in a combination as indicated, to the organ baths 20 min prior to the addition of PE. The concentrations of the above agents were chosen based on data established in an earlier study (Edwards et al., 1998). These agents were maintained until the responses to increasing concentrations of either L-serine or ACh were determined in vessels isolated from both normotensive control and chronic L-NAME treated rats.

3.3.2. In Vivo Studies

Rats from normotensive Sprague-Dawley, WKY, Sprague-Dawley and WKY rats rendered hypertensive by pretreatment with L-NAME (0.7 mg/ml in drinking water for 5 to 7 days) and SHR groups were considered for the present study. Rats were anaesthetized with an intraperitoneal injection of thiopental sodium and allowed to breathe spontaneously through a tracheal cannula. In the present study the femoral artery was cannulated and connected to a pressure transducer to record the changes in MAP and HR using the PowerLab data acquisition system (AD Instruments Pvt. Ltd. Sydney, Australia). Left or right femoral vein was cannulated to administer drugs as bolus injections in a limited

volume of 0.4 ml/kg of various agents prepared in saline and the pH was adjusted to 7.3. After ensuring that the MAP and HR had stabilized, the responses to bolus infusions of either L-serine (between 0.1 and 3.0 mmol/kg) or ACh (0.1 to 10 nmol/kg) were determined. During experiments sufficient time was given between each response for the MAP to recover to the resting baseline. To inhibit selectively the EDHF mediated responses *in vivo*, the animals were subjected to slow infusions of a combination of small conductance Ca^{2+}dependent K^{+} channel (SK_{Ca}) inhibitor apamin (75µg/kg) followed by an intermediate conductance Ca^{2+} dependent K^{+} channel (IK_{Ca}) inhibitor, charybdotoxin (ChTX, 75 µg/kg). The total infusion time period was maintained for 15 min with the help of an infusion pump (Harvard Apparatus, Quebec, Canada). We observed an elevation of MAP within 5 min following the slow infusion of apamin and ChTX, and it returned to a level slightly above the resting MAP level in about 30 min. After 45 min, we determined the dose-response (DR) curves to ACh and found to be optimal since the blockade of hypotensive responses to ACh was highly significant particularly in L-NAME treated rats. These data confirmed that combined infusion of apamin and ChTX at the dose level infused, blocked EDHF dependent fall in MAP evoked by ACh. Then, the effects of comparable doses (between 0.1 and 3.0 mmol/kg) of L-serine, D-serine, glycine, and L-threonine were determined. In subsequent experiments, the maximum dose of L-serine was limited to 2.0 mmol/kg in the chronic L-NAME treated rats, after finding that recovery to baseline was difficult following the profound fall in MAP attained with the 3.0 mmol/kg doses. The responses to either glycine or L-serine were also determined before and 30 min

after slow infusion over a period of 15 min of the NMDA receptor antagonist, MK-801 (75 mg/kg. i.p.).

Studies were also performed in a selected groups of normotensive Sprague-Dawley and WKY rats in which the effect of increasing doses (0.3 to 3.0 mmol/kg, iv.) of glycine infusion was examined both prior to and 1 hr after acute infusion of L-NAME (100 mg/kg, i.v. given over a period of 10 min) to compare the responses to glycine infusion in chronic L-NAME treated hypertensive WKY group vs. acute L-NAME treated hypertensive rats. The acute dose of L-NAME was determined from previous work (Rees et al., 1990).

3.3.3. Regional Hemodynamic Study

For this study twelve -week old male WKY rats (300 to 350g) and SHR (250 to 270g) were obtained from Charles River (St. Constant, Quebec, Canada). After one week acclimatization one group of WKY rats received L-NAME (0.7 mg/ml in drinking water given ad libitum) for 5 to 7 days and other rats received plain water. In this experiment, SHR and L-NAME pretreated rats served as hypertensive rat models and WKY rats served as the control group.

3.3.3.1. Surgical Procedure for Regional Hemodynamic Study

Rats were anesthetized with thiopental sodium (100 mg/kg, i.p.). Body temperature was maintained at 37^0C with the help of a controlled heating pad. The trachea was cannulated to allow spontaneous breathing. The right femoral artery was cannulated with polyethylene tubing (PE-20) and connected to a pressure transducer to record changes in

MAP and HR using a PowerLab data acquisition system (AD Instruments Inc., Sydney, Australia). The left femoral artery was cannulated and connected to a reciprocal syringe pump (Havard Apparatus, Quebec, Canada) for collecting the reference blood sample. The left femoral vein was cannulated to administer saline (vehicle) or L-serine (prepared in saline, pH adjusted to 7.3) either in the presence or absence of apamin plus ChTX. The total volume of infusion of all these agents was limited to maximum volume of 0.4 ml/kg. The right carotid artery was cannulated with saline filled PE-50 tubing connected to a pressure transducer. The carotid cannula was subsequently guided through the common carotid artery into the left ventricle, which was confirmed by the characteristic ventricular pressure wave form (Glenny et al., 1993; Gervais et al., 1999).

3.3.3.2. Hemodynamic Measurement using Fluorescent Microspheres

Hemodynamic measurements were performed using fluorescent microsphere distribution technique (Glenny et al., 1993; Gervais et al., 1999; Deveci et al., 1999). Four different colors (green, yellow, red-orange and carmine) of 15 μm diameter fluorescent microspheres (FluoSpheres®) were purchased and stored at 4^0C. The combination of microspheres used in a single study was determined on the basis of their absorption/emission maxima wavelength (nm) to avoid spill over (Hakkinen et al., 1995; De Angelis et al., 2005). To ensure steady MAP and HR, enough time (30 min) was given after all cannulation. Microspheres were then sonicated and vigorously vortexed for 2 to 3 min just before use to avoid sedimentation, then diluted to a final volume of 0.3 ml. 80,000 to 100,000 microspheres were suspended in 0.3 ml of 0.9% saline containing 0.01% w/v Tween 20. Microspheres were vortexed and immediately injected into the left ventricle

through the carotid artery cannula over a period of 20 seconds and flushed with 0.3 ml saline over a period of 20 seconds. Concurrently, for the reference blood sample, blood was withdrawn from femoral artery, downstream from the site of microsphere injection, at a rate of 0.5 ml/min, starting 10 seconds prior to simultaneous infusion of test drug and microspheres by injection and continued for a further 70 seconds. Reference blood sample was transferred to a tube with heparin (50 unit/ml), mixed well and kept on ice (Ishise et al., 1980). This procedure was carried out first for vehicle infusion, then L-serine (1 mmol/kg dose) after 10 min to confirm reproducibility, by employing a different colored microsphere (Glenny et al., 1993). The same procedure was employed to evaluate the effect of L-serine in presence of apamin plus ChTX (75 μg/kg) combination and they were infused slowly over a period of 15 min with the help of an infusion pump. After 30 min responses to L-serine infusion was determined. The experiment was terminated by giving an overdose of thiopental 10 min after the last microsphere injection. Then various organs/tissues such as heart, left as well as right kidney, liver, lungs, spleen, brain, stomach, small and large intestine, diaphragm, skeletal muscle and pancreas were collected in whole or part to quantify the fluorescence intensity of the dye from microspheres captured in each tissue. Briefly, the organs were removed and rinsed with cold saline, patted dry, weighed, and placed in 15 ml glass tubes. Both the reference blood samples and tissues were digested for 24 to 48 hr in 3 to 4 ml of potassium hydroxide (KOH, 4.0 M) solution per gram of tissue. The tubes were shaken thoroughly every few hours. After 48 hour, the microspheres were recovered by centrifuging for 20 min at a speed of 2000 x g (Precision, Durafuge 100, Winchester, VA, Van Oosterhout et al., 1995). Supernatants were carefully removed leaving < 1ml in each glass tube. The pellet with microspheres in each glass tube was

rinsed with 9 ml of 0.25% Tween 80 in demineralized water at 60^0C, vortexed, centrifuged again at a speed of 2000 x g for 20 min. A final rinse with demineralized water without Tween was carried out and the supernatant was carefully removed leaving 1ml. 3 ml of Cellosolve acetate (2-ethoxy-ethyl acetate), a solvent, was then added to the tubes, vortexed thoroughly and allowed to stand for ≥ 4 hours to dissolve the polystyrene coating of the microspheres, which were then centrifuged again at 2,000 x g for 10-15 min (Oosterhout et al., 1995). Tubes were vortexed after 4 hr, then centrifuged at 2000 x g for 10-15 minutes. The supernatant was removed and utilized for the measurement of fluorescence. To calculate the blood flow rate per tissue sample (ml/min/g), the following formula was used: $F_i = (I_i)(R)/I_{ref}$ where: F_i = flow to individual sample (ml/min), I_i = fluorescence intensity of the sample, R = reference sample withdrawal rate (ml/min), I_{ref} = fluorescence intensity of the reference blood sample. MAP (mmHg), HR (beats/min), CO (ml/min) = total number of injected microspheres * reference sample withdrawal rate/number of microspheres in the reference blood sample, cardiac index (CI, ml/min/kg) = CO/body surface area, TPR (mmHg/ml/min) = MAP/CO, TPR index (TPRI, mmHg/ml/min/kg) = MAP/CI, organ vascular resistance (mmHg/ml/min/g) = average MAP during microsphere infusion/local blood flow to individual organs were calculated as previously described (Granstam et al., 1998; Sampaio et al., 2003; De Angelis et al., 2006; Giancarla et al., 2007).

3.3.3.3. Measurement of Fluorescence

F-2500 fluorescence spectrometer (Hitachi, Tokyo, Japan) was used to measure fluorescence intensity at excitation and emission wavelengths between 350 to 750 nm using

5 nm slit width. Each individual sample was read in a 5 ml quartz cuvette in duplicate including reference blood samples (Oosterhout et al., 1995; Granstam et al., 1998; Sampaio et al., 2003; De Angelis et al., 2006; Giancarla et al., 2007).

3.4. Statistical Analysis

The vasodilator responses to L-serine and ACh were determined in both endothelium intact and denuded mesenteric arterioles isolated from normotensive control and L-NAME treated rats. Data represents the mean ± SEM of (n = 20 arterioles) from 8 rats. Difference between the means of both control and L-NAME treated vs. endothelium intact and denuded groups considered significant by a one-way analysis of variance followed by Tukey's post hoc test. The differences were considered significant when $p < 0.05$.

The change in MAP following infusion of each dose was plotted to generate the dose-response (DR) curves to L-serine, ACh and respective amino acids. The data are expressed as mean ± SEM (n = 5 to 7). Differences between the means of two groups were tested for significance by a one-way analysis of variance followed by Tukey's post hoc test. The differences were considered significant when $p < 0.05$.

The results obtained in hemodynamic studies are expressed as mean ± SEM of experiments performed in 5 to 7 rats for each group. The differences between mean values were analyzed by one-way analysis of variance for repeated measures followed by Bonferroni's multiple comparison tests and considered significant when the values were $p < 0.05$.

CHAPTER 4. RESULTS

4.1. In Vitro Studies

4.1.1. Vasodilator Effect of L-serine

L-serine evoked vasodilator effect was concentration-dependent in PE constricted endothelium intact arterioles isolated from normotensive and hypertensive rats. L-serine mediated vasodilator response was higher in mesenteric arterioles isolated from chronic L-NAME treated rats. A representative experiment shows the comparison of dose dependent vasodilator response to L-serine and ACh in endothelium-intact vessels (Figure 3 a and b). Their lack of response in endothelium-denuded vessels, isolated from a chronically L-NAME treated rat (Figure 3 c and d). L-serine mediated vasodilator response was slower and maximal (E_{max}) response was lower compared to ACh (Figure 3 a, b, c and d). In endothelium denuded mesenteric arterioles both ACh and L-serine failed to evoke vasodilatation, even at very high concentrations (Figure 3 c and d). In L-NAME treated group, the E_{max} for L-serine (40 ± 3 %) in endothelium-intact vessels was significantly shifted to the left ($p < 0.01$) compared to control group with E_{max} (20 ± 3%, Figure 4 a). In the same L-NAME treated group, the E_{max} for ACh (93 ± 2 %) in endothelium intact vessels was significantly shifted to the right compared to control group with E_{max} (79 ± 3 %, Figure 4 b). A graphical representation of the E_{max} values for L-serine and ACh are compared in endothelium intact and denuded mesenteric arterioles (Figure 5 a and b). In endothelium-intact arterioles isolated from L-NAME treated group, incubation with a

combination of either apamin plus TRAM-34 or ouabain (Na^+ pump inhibitor, 20 μmol/L) plus Ba^{2+} (K_{ir} channel inhibitor, 50 μmol/L) abolished the responses to both L-serine and ACh, whereas addition of either the non selective COX inhibitor, indomethacin (10 μmol/L), or apamin (SK_{Ca} inhibitor, 1 μmol/L) or TRAM-34 (IK_{Ca} inhibitor, 1 μmol/L) alone failed to affect the E_{max} (Figure 5 c and d). We observed similar responses to L-serine and ACh when another vasoconstrictor agonist, TXA_2 agonist, U46619 (1 μmol/L), was used to compare the Emax values for L-serine (40 %) and ACh (90 %) instead of PE; in contrast, L-serine failed to evoke vasodilatation in arterioles preconstricted with a depolarizing concentration of KCl (80 mmol/L), (Figure 6). Small increments of KCl between 10 and 20 mmol/L in PE constricted arterioles isolated from L-NAME treated rats evoked a concentration-dependent vasodilatation, and addition of L-serine failed to enhance further vasodilator response; incubation with a combination of Ba^{2+} (K_{ir} channel inhibitor) and ouabain (Na^+ pump inhibitor) abolished the responses to K^+ but not the combination of apamin plus TRAM-34 (Figures 7 and 8).

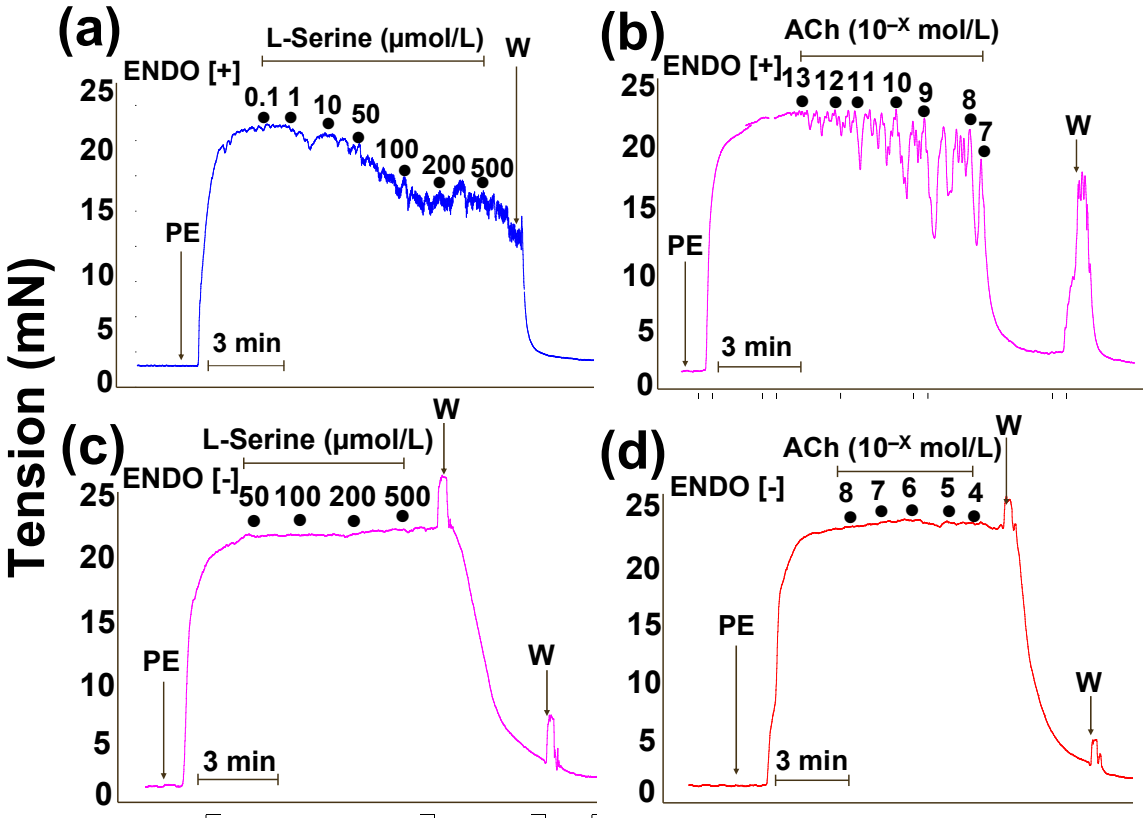

Figure 3. A representative tracing that demonstrates the pattern of vasodilator responses to cumulative addition of increasing concentrations of either L-serine (1 to 500 µmol/L) or ACh (0.1 pmol/L to 100 µmol/L) in endothelium-intact (ENDO [+] a and b) or endothelium-denuded (Endo [-] c and d) third order branches of mesenteric arterioles constricted with PE (10 µmol/L) *in vitro* after isolation from a 12 week old male Sprague-Dawley rat that received chronic L-NAME treatment (0.7 mg/ml, in drinking water) for five days. W - denotes when the tissues were washed in normal Krebs buffer to attain recovery. Similar responses were noted in vessels from 8 chronic L-NAME treated rats.

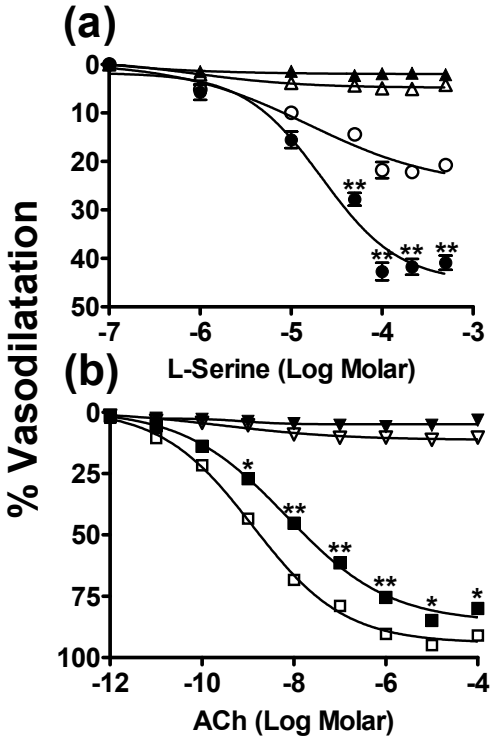

Figure 4. The line graphs compare the concentration curve (CR) curves to L-serine (a) determined in PE constricted mesenteric arterioles isolated from either normotensive control group (○) or chronic L-NAME treated hypertensive rats (●) with intact endothelium. The lack of responses to L-Serine in endothelium-denuded vessels in both control (△) and chronic L-NAME treated (▲) vessels are also shown. The CR curves to ACh (b) determined in either the control group (□) or chronic L-NAME treatment group (■) with intact endothelium or the lack of responses in endothelium-denuded vessels in control (▽) and chronic L-NAME treated (▼) vessels are shown. Each data point represents mean ± SEM of n > 20 arterioles isolated from > 8 rats from control and chronic L-NAME treatment groups. *$p < 0.05$ and **$p < 0.01$ compared to data point in the control group.

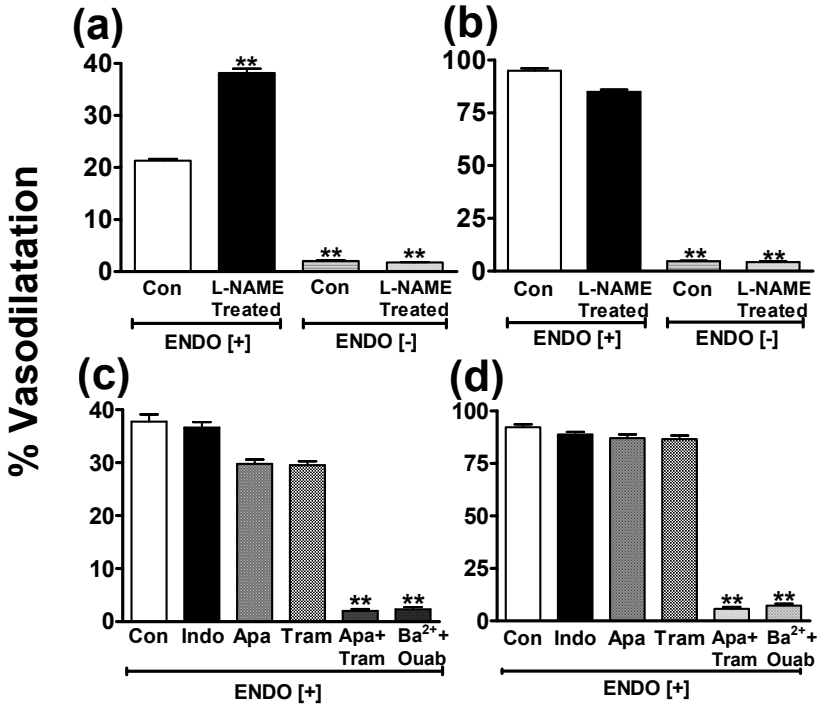

Figure 5. The bar diagram compares the maximal vasodilator response (E_{max}) to 200 µmol/L L-Serine (a) or 10 µmol/L ACh (b) in both control and chronic L-NAME treated rats in either endothelium-intact (ENDO [+]) or endothelium-denuded (Endo [-]) PE constricted mesenteric arterioles. The E_{max} values for L-serine (c) and ACh (d) attained in endothelium intact ([Endo [+]) arterioles isolated from chronic L-NAME treated rats either in the absence (Control - Con) or the presence of indomethacin (10 µmol/L, Indo), apamin (1 µmol/L, Apa) or TRAM-34 (1 µmol/L, Tram) or a combination of apamin plus TRAM-34 (1 µmol/L of each Apamin plus Tram-34) or a combination of barium chloride (Ba^{2+} 50 µmol/L) and ouabain (Ouab 20 µmol/L) are compared. Each data point is a pooled value of mean ± SEM from 20 preparations isolated from 8 different rats. **$p < 0.001$ compared to the respective data in endothelium-intact (Endo [+]) group.

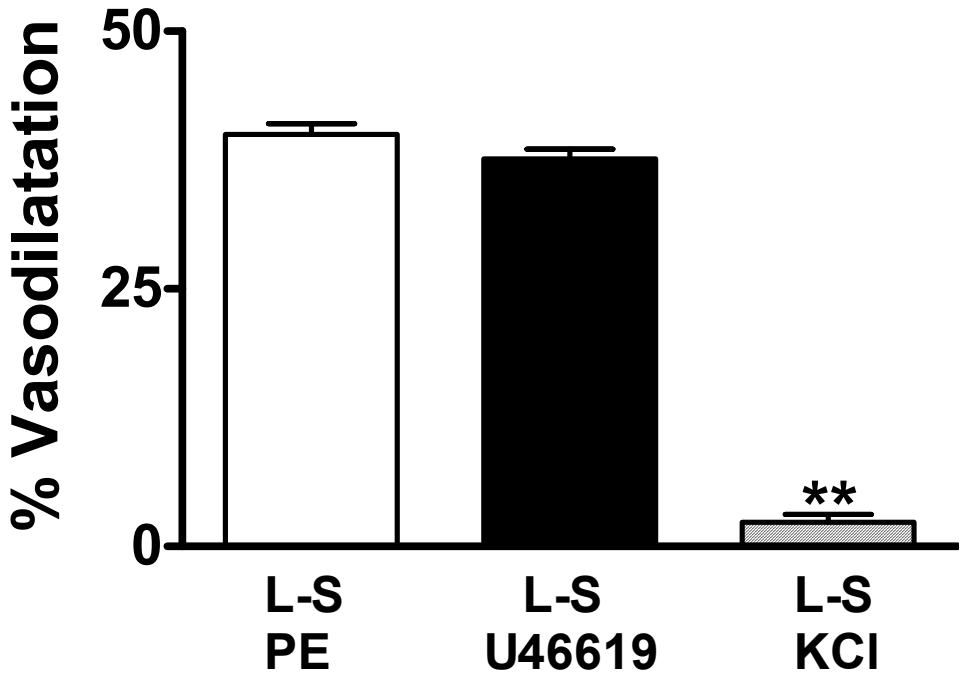

Figure 6. The maximal vasodilator responses (Emax) to L-serine (L-S, 500 μmol/L) attained in either phenylephrine (PE 10 μmol/L) or thromboxane analog, U46619 (1 μmol/L) or high potassium chloride (KCl, 80 mmol/L) constricted third order branches of mesenteric arterioles maintained in wire myograph after isolation from chronic L-NAME treated rats. Each data point is mean ± SEM of 5 separate experiments using vessels isolated from four L-NAME treated rats. **$p < 0.01$ compared to the responses to L-S data in the presence of either PE or U46619.

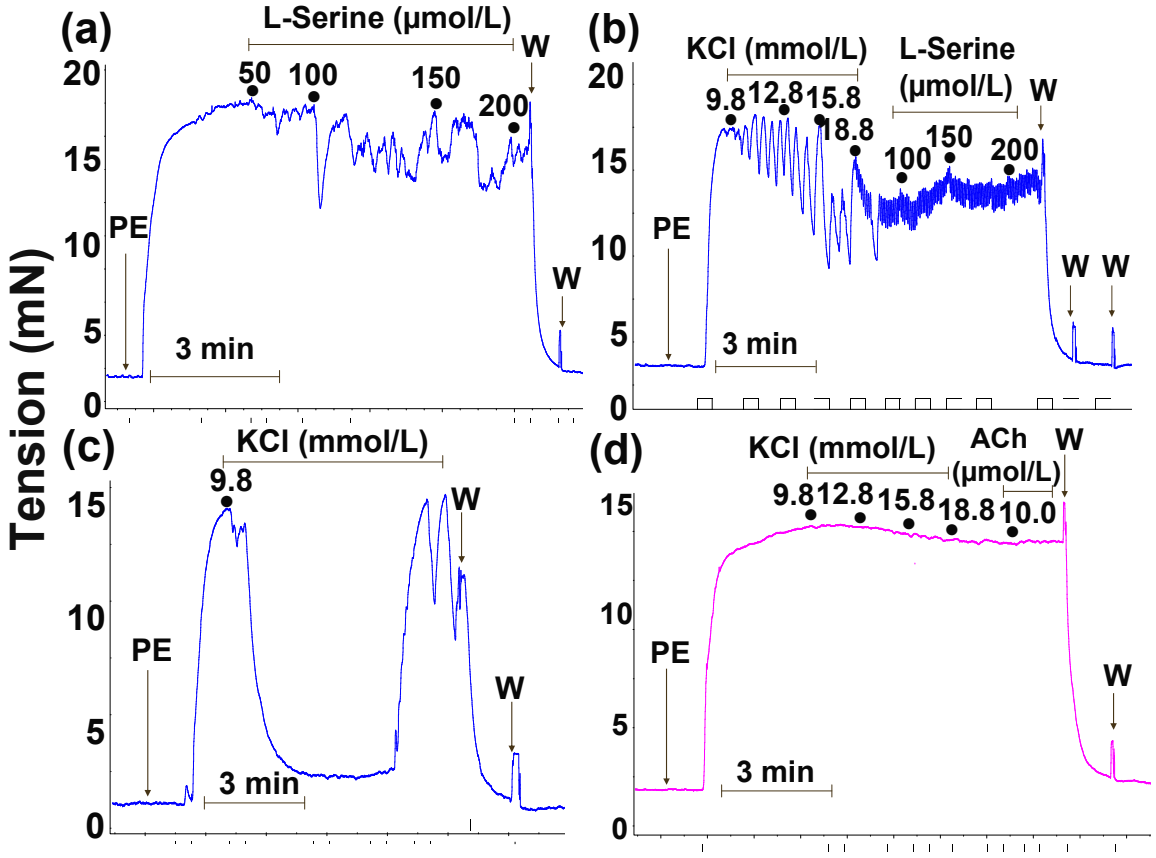

Figure 7. The data of a typical experiment of concentration dependent vasodilator responses to cumulative increases in either L-serine (Panel a: L-Serine 50 to 200 µmol/L) or KCl (Panel b: KCl 9.7 to 18.7 µmol/L followed by L-serine 100 to 500 µmol/L) in PE (10 µmol/L) constricted endothelium-intact mesenteric arterioles isolated from L-NAME treated rat. Panel c shows that in the same vessel, after pretreatment with apamin plus TRAM-34 (1 µmol/L) addition of the first dose of KCl (9.7 mmol/L) evoked vasodilatation whereas when the tissues were washed and incubated with a combination of Ba^{2+} (50 µmol/L) and ouabain (20 µmol/L), either the addition of KCl (9.7 to 18.7 mmol/L) or ACh (10 µmol/L) failed to elicit vasodilatation.

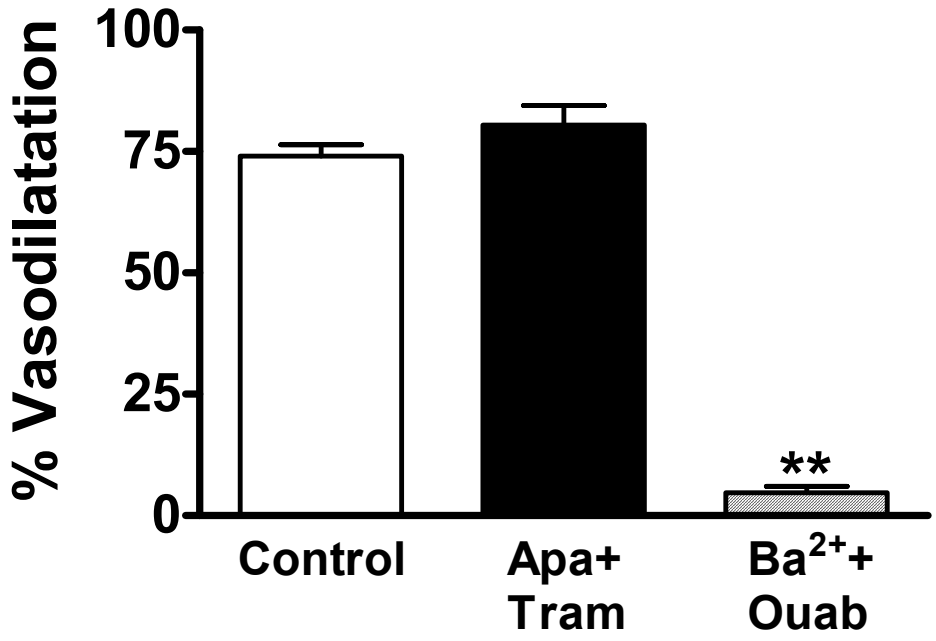

Figure 8. The bar diagram shows the pooled mean ± SEM data from 4 separate experiments comparing the maximal vasodilator responses to KCl (18.7 mmol/L) attained in PE (10 μmol/L) constricted arterioles isolated from L-NAME treated rats either before (control □) or after apamin plus TRAM-34 (■) or ouabain plus Ba^{2+} (□) pretreatments *in vitro*. **$p < 0.01$ compared to the responses to KCl either in the absence or presence of apamin plus TRAM-34.

4.2. In vivo Studies

4.2.1. Acute Hemodynamic Effect of L-serine

As a follow up our *in vitro* observation, the acute effect of a bolus dose of L-serine infusion was examined in normotensive and L-NAME treated hypertensive rats. In the L-NAME treatment group, basal MAP (135 ± 6 mmHg; n = 12) was significantly higher ($P < 0.01$) compared to the normotensive control group (93 ± 8 mmHg; n = 12, Table 3). L-serine evoked a dose-dependent fall in MAP in both control and L-NAME treatment groups, but the effect was abrupt and more profound in the L-NAME treatment group (Figure 9). A typical experiment shows a combination of apamin plus ChTX infusion abolished the hypotensive response to L-serine in control rat (Figure 9 b). Saline infusion alone failed to affect BP in either group of animals. The entire experiment was reproducible in both the control and L-NAME treated group. In chronic L-NAME treatment rats, the fall in MAP was observed at a concentration of 0.3 mmol/kg and the maximal fall attained at 3.0 mmol/kg which was much higher (140 mmHg to 50 mmHg) in this group of rats than control rats (Figure 9 c). A combination of apamin plus ChTX infusion abolished the responses to L-serine up to 2.0 mmol/kg but in higher concentration (3.0 mmol/kg) we observed a fall in MAP which was much lower compared to control response (Figure 9 c and d). ACh (0.1 to 10.0 nmol/kg) infusion evoked a dose-dependent fall in MAP in both control and L-NAME treatment groups (Figure 10 a and c). The dose-dependent fall in MAP evoked by ACh was abolished only in L-NAME treatment but not in control rats that received apamin plus ChTX pretreatment since NO pathway is blocked by inhibition of NOS by L-NAME pretreatment (Figure 10 b and d). Data from several

experiments revealed that the fall in MAP evoked by L-serine was much lower in the normotensive control group of rats compared to ACh, with a minimal rightward shift in the DR curve after pretreatment with apamin plus ChTX combination (Figure 11 a and b). Pretreatment with apamin plus ChTX abolished the responses to L-serine at concentrations range up to 2.0 mmol/kg, and the rightward shift in the DR curve to L-serine was much higher compared to ACh in L-NAME pretreated rats (Figure 11 c and d). L-serine and ACh evoked fall in MAP were accompanied by a non-significant increase in HR in both normotensive control and L-NAME pretreated hypertensive rats (Table 3).

Table 3. Comparison of mean arterial pressure (MAP, mmHg) and heart rate (HR, beats per minute) recorded before and 30 seconds after infusion of L-serine (2 to 3 mmol/kg) in 12 week old male Sprague-Dawley and L-NAME pretreated Sprague-Dawley rats.

Strain	**Baseline**		**L-serine**	
	MAP	**HR**	**MAP**	**HR**
Sprague-Dawley	108 ± 5	352 ± 9	79 ± 3†	376 ± 8
Sprague-Dawley (L-NAME treated)	139 ± 4*	330 ± 6	45 ± 3††	380 ± 7

Each data point is mean ± SEM of n = 7 to 9 rats/group.

*$p < 0.05$ vs. Sprague-Dawley (Control) group; $^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$ vs. respective baseline value in the same group prior to infusion of L-serine.

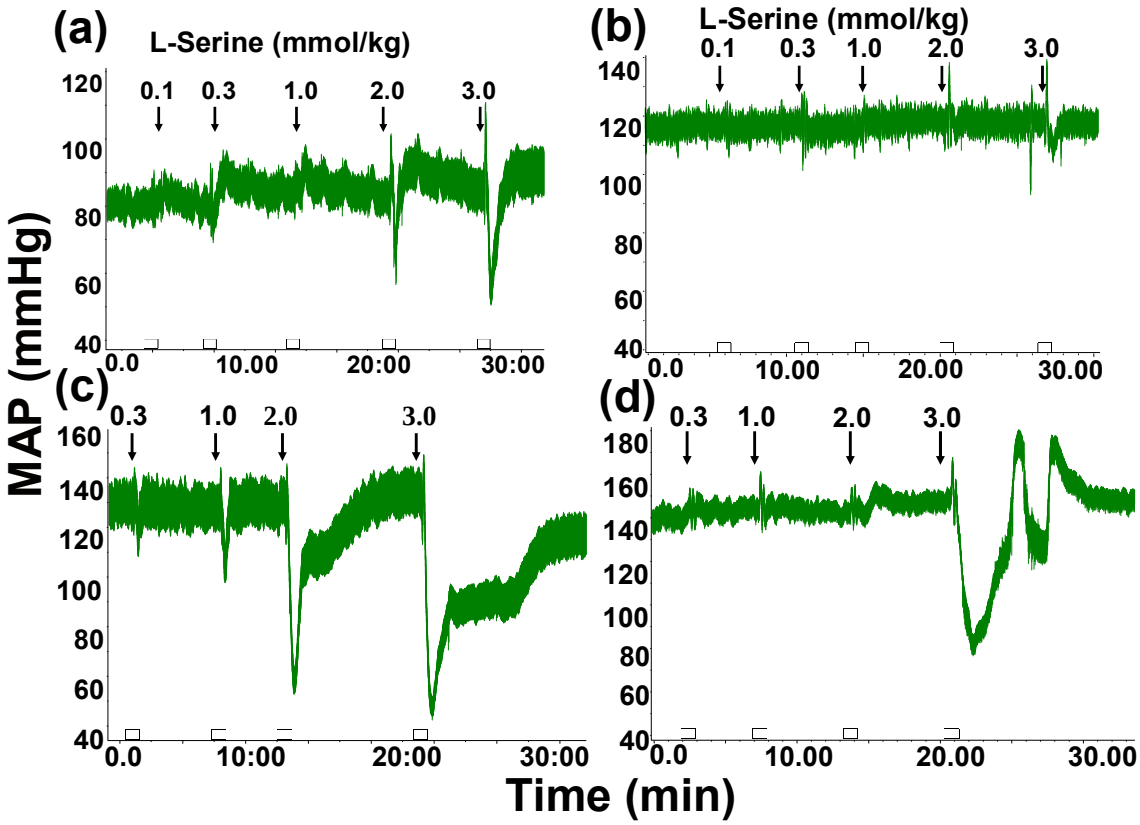

Figure 9. A typical experiment compares the fall in MAP to acute i.v. infusion of various doses of L-serine (0.1 to 3.0 mmol/kg) in a control rat before (a) and 45 min after (b) and in a chronic L-NAME (0.7 mg/ml, in drinking water for five days) treated rat before (c) and 45 min after (d) the infusion of apamin plus ChTX (75 µg/kg each).

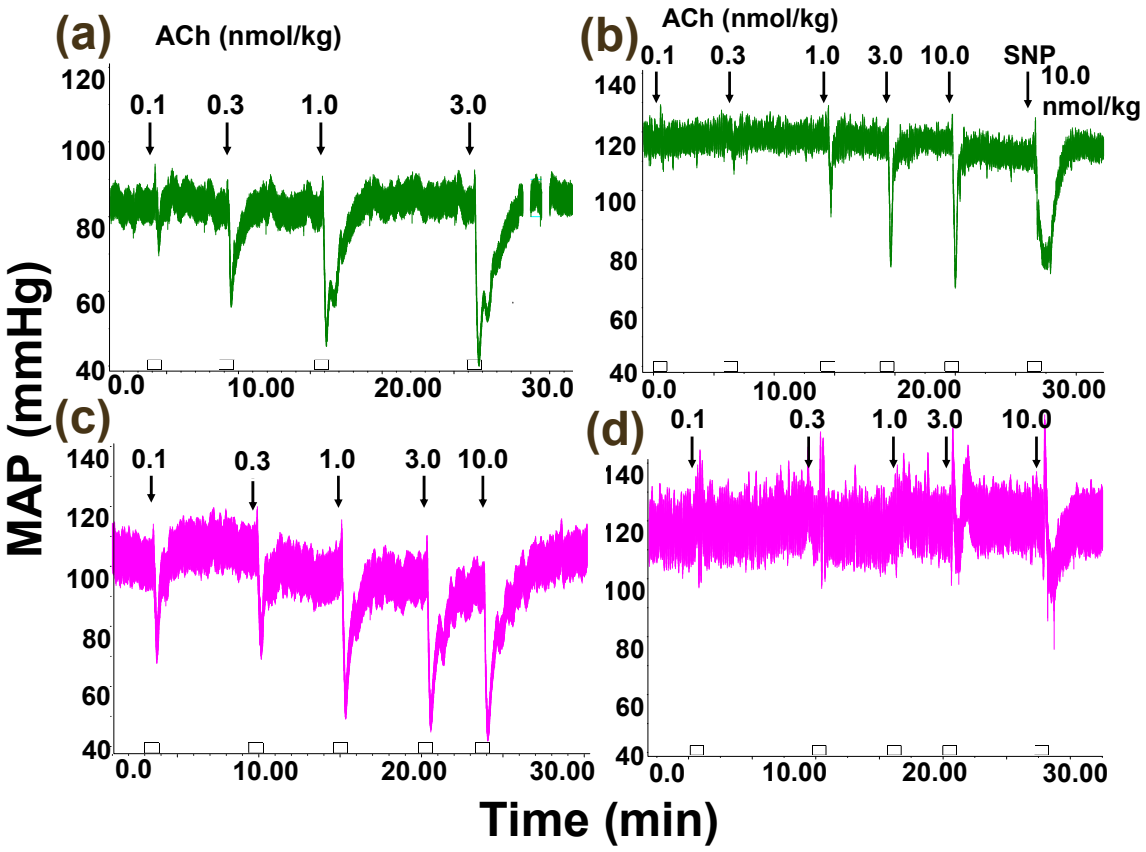

Figure 10. A typical experiment compares the fall in MAP to acute i.v. infusion of various doses of ACh (0.1 to 10.0 nmol/kg) in a control rat before (a) and 45 min after (b) and in a chronic L-NAME treated rat before (c) and 45 min after (d) the infusion of apamin plus ChTX (75 µg/kg each).

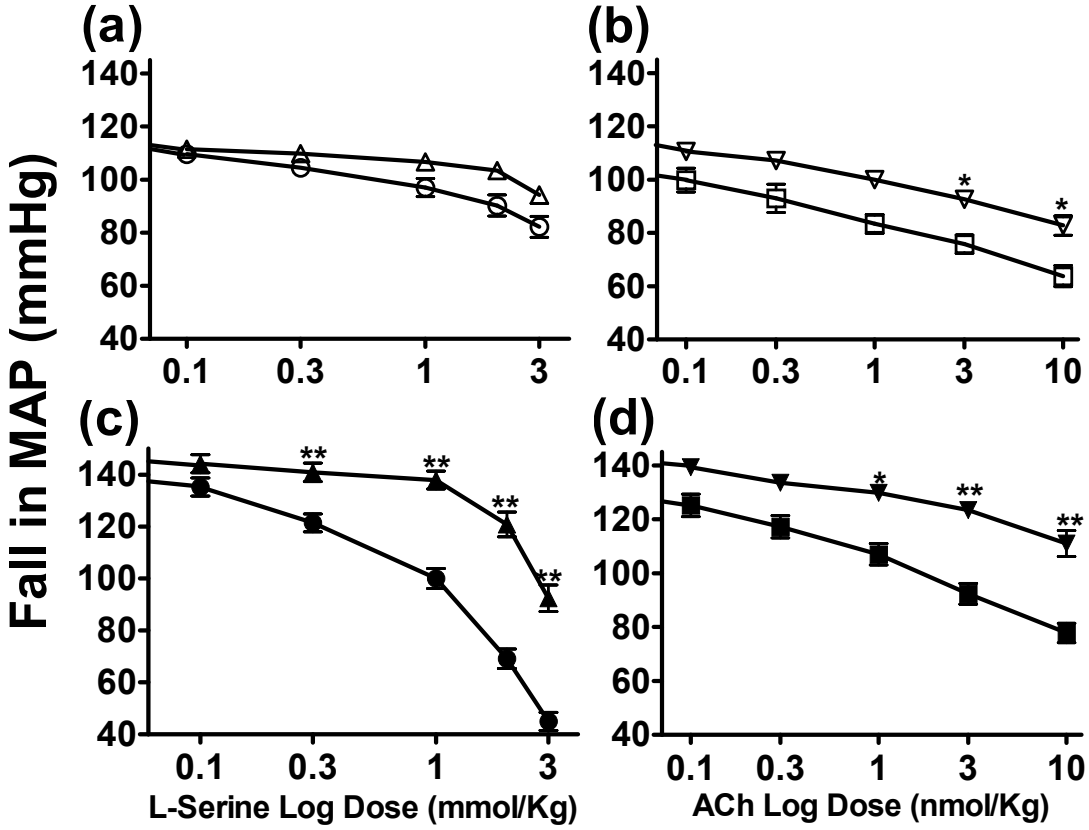

Figure 11. The dose response relationship between L-serine (a - before [○] and after [△] apamin plus ChTX) or ACh (b - before [□] and after [▽] apamin plus ChTX) infusion and the maximal fall in MAP attained in the control group. The DR relationship between L-Serine (c - before [●] and after [▲] apamin plus ChTX) or ACh (d - before [■] and after [▼] apamin plus ChTX) and the maximal fall in MAP in L-NAME treated hypertensive rats. Each data point is mean ± SEM of $n > 8$ rats per each group. *$p < 0.05$ and **$p < 0.01$ compared to the data point in the control group.

4.2.2. Acute Hemodynamic Effect of L-serine, D-serine, Glycine and L-threonine

After establishing the acute hemodynamic effects of L-serine, it was important to examine whether the precursor of L-serine, L-threonine or its metabolites such as D-serine and glycine, would evoke effects like L-serine in normotensive and hypertensive rat models. For this study we considered two normotensive rat models, Sprague-Dawley and WKY rats, and two hypertensive rat models namely, L-NAME pretreated hypertensive Sprague-Dawley and WKY rats, and SHR strains. In normotensive Sprague-Dawley and WKY strains, the basal MAP and HR were similar. In L-NAME pretreated hypertensive Sprague-Dawley and WKY rats and SHR MAP was higher (142 ± 3 mmHg, 149 ± 4 mmHg and169 ± 4 mmHg, Table 4). Pooled data from several experiments revealed that acute infusion of comparable doses of D-and L-serine evoked reversible, dose dependent fall in MAP in both normotensive and hypertensive rat models. A representative experiment performed in parallel to demonstrate the fall in MAP evoked by D-and L-serine in a normotensive WKY rat (Figure 12 a), chronic L-NAME pretreated hypertensive WKY rat (Figure 12 b) and SHR strain (Figure 13 a). It is evident from all these experiments, the responses to L-serine was much higher and more pronounced at each incremental doses compared to D-serine (Figure 12 a, b and 13 a). The profound fall in MAP after infusion of 3.0 mmol/kg required that the dose be limited to 2.0 mmol/kg in L-NAME pretreated rats. This was because the return of MAP to baseline was difficult after administering this dose in hypertensive rats (Figure 12 b and Figure 14 c). Although the baseline MAP was higher in the SHR strains (169 ± 4 mmHg) compared to L-NAME pretreated hypertensive WKY rats (149 ± 4 mmHg) the fall in MAP evoked by D-and L-serine was relatively lower in SHR strain in comparison to L-NAME pretreated hypertensive WKY rats (Figure 12, 13

and Table 4). Data gathered from several D-and L-serine DR curves confirmed that the BP lowering effect of L-serine was more potent and efficacious at each incremental dose compared to D-serine. The maximal BP lowering effect of L-serine occurred in the following rank order in different strains: chronic L-NAME treated WKY > SHR > WKY ≥ Sprague-Dawley rats (Figure 14). The fall in MAP evoked by L-serine in all groups was accompanied by a marginal increase in HR but it failed to attain statistical significance in all groups (Table 4). The estimated EC_{50} values for D-and L-serine were 1.4-2.3 and 0.8-1.1 mmol/kg assuming the maximal effect occurred at 3.0 mmol/kg in all groups of animals. Concentration higher than 3.0 mmol/kg was not infused since it was difficult to record the MAP to basal level. D-and L-serine evoked a fall in MAP that was significantly reduced by pretreatment with apamin and ChTX in both normotensive and hypertensive rat models but the degree of inhibition to L-serine was relatively higher compared to D-serine in SHR strain (Figure 13 b and Figure 15). Glycine is a metabolite or precursor of L-serine (Snell, 1984 and 1986). When comparable doses of glycine were infused (0.3 to 3.0 mmol/kg), it evoked a fall in MAP with no significant increase in HR in normotensive Sprague-Dawley and WKY strains. However, the degree of fall in MAP attained with glycine was relatively lower than the responses attained with comparable doses of L-serine (Figure 16 and Table 4). Glycine evoked a fall in MAP that was abolished by pretreatment with NMDA receptor selective antagonist, MK-801 (75 mg/kg). Pretreatment with MK-801 did not affect the basal as well as L-serine evoked fall in MAP (Figure 16). Paradoxically, and unlike L-serine, glycine evoked dose-dependent pressor responses in both SHR and L-NAME treated rats (Figure 17 a and b left panel). The maximal pressor responses to glycine attained at 3.0 mmol/kg (29 ± 3 mmHg, $p < 0.01$) and (39 ± 5 mmHg, $p < 0.01$) in

SHR and L-NAME treated rats. Pretreatment with MK-801 a selective NMDA receptor antagonist, abolished the pressor response induced by glycine in both L-NAME pretreated WKY and SHR strains. It was confirmed that MK-801 did not affect the responses to L-serine (Figure 17 a and b, right panel). Glycine evoked a fall in MAP in normotensive WKY rats and elevation in MAP in both the hypertensive rat models. These responses blocked after pretreatment with NMDA receptor selective antagonist MK-801 are graphically represented (Figure 18). The data from a representative experiment demonstrates the hypotensive response to glycine in normotensive rats as well as the pressor response in hypertensive rats including the selective blockade of either response to glycine by NMDA antagonist, MK-801 (Figure 18). In order to ensure that an acute elevation in MAP following L-NAME (100 mg/kg) administration did not contribute to the exaggerated pressor response to glycine, experiments were performed in normotensive WKY rats. Glycine infusion evoked a dose dependent fall in MAP in this group of rats. Glycine neither elevated nor reduced MAP in this group of rats following acute intravenous administration of L-NAME, where as L-serine still produced a dose dependent fall in MAP (Figure 19). Comparable doses of L-threonine, a precursor of L-serine, had no effect on MAP in normotensive and hypertensive rat models (Figure 20). The dose dependent responses to all the amino acids were reproducible irrespective of the order in which they were infused in all the strains.

Table 4. Comparison of mean arterial pressure (MAP, mmHg) and heart rate (HR, beats per minute) recorded continuously before and after infusion of either L-serine or glycine (2 to 3 mmol/kg) in 12 to14 week old male Sprague-Dawley, L-NAME pretreated Sprague-Dawley, Wistar Kyoto (WKY), L-NAME pretreated WKY and spontaneously hypertensive (SHR) rats.

Strain	Baseline		L-Serine		Glycine	
	MAP	**HR**	**MAP**	**HR**	**MAP**	**HR**
Sprague-Dawley (Control)	111 ± 4	364 ± 11	83 ± 5 †	386 ± 10	91 ± 3	381 ± 12
Sprague-Dawley (L-NAME Treated)	142 ± 3 *	343 ± 8	47 ± 4 ††	391 ± 9	180 ± 5 †	321 ± 7
Wistar-Kyoto (Control)	114 ± 3	381 ± 10	69 ± 4 †	410 ± 8	89 ± 2 †	399 ± 9
Wistar-kyoto (L-NAME Treated)	149 ± 4 **	377 ± 12	52 ± 3 ††	427 ± 11	183 ± 4 †	354 ± 13
SHR	169 ± 4 **	401 ± 8	88 ± 4 ††	443 ± 10	198 ± 5 †	383 ± 8

Each data point is mean ± SEM of n = 5 to 7 rats per group.

*$p < 0.05$ vs. SD (control) group; **$p < 0.01$ vs. WKY (control) group; $^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$ vs. respective baseline value in the same group prior to infusion of either L-serine or glycine.

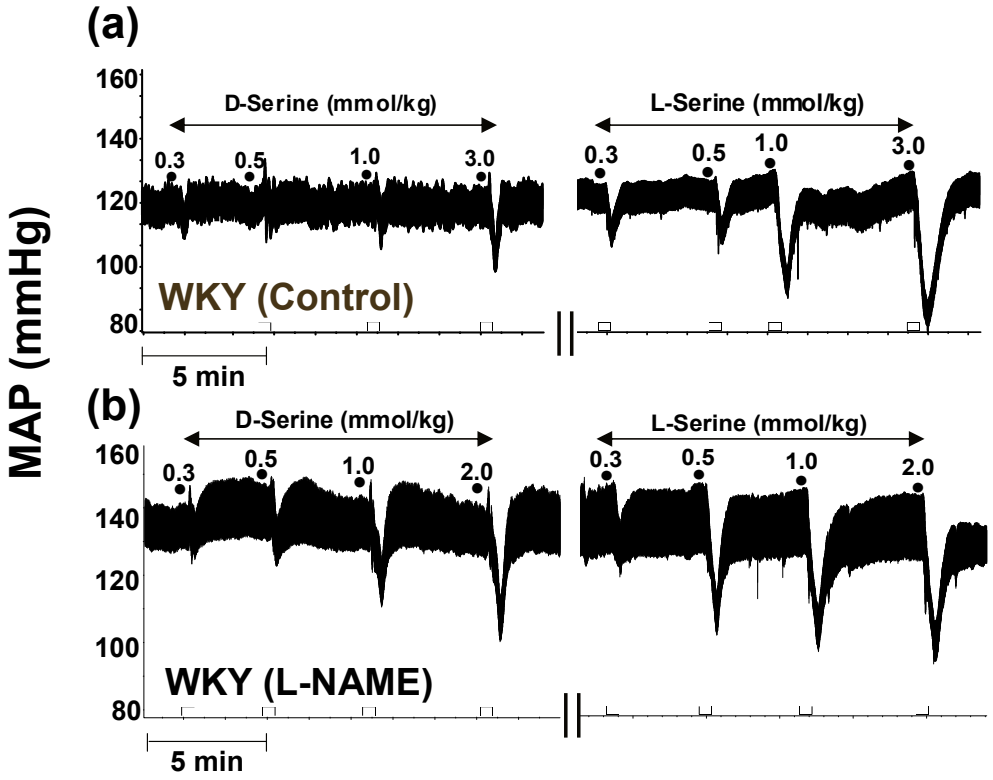

Figure 12. A typical experiment compares MAP to acute comparable doses (0.3 to 3.0 mmol/kg) of D-serine followed by L-serine (a) infusion in a 14 week old male normotensive Wistar-Kyoto [WKY (control)] rat. The bottom panels (b) show the changes in MAP to D-serine followed by L-Serine infusion (0.3 to 2.0 mmol/kg) in a hypertensive WKY [WKY (L-NAME)] rat subjected to chronic nitric oxide synthase (NOS) inhibition by pretreatment with L-NAME (0.7 mg/ml in drinking water) for 5 days. The maximal dose of D- or L- serine used was limited to 2.0 mmol/kg due to protracted hypotension at higher doses, when it was infused in L-NAME treated rats. Similar response patterns were seen for D- and L-serine in 6 WKY (control) and L-NAME treated WKY rats. The response patterns to both isomers were also comparable to that of WKY control group in 12 to 14 week old male Sprague-Dawley rats (n = 5 to 7 rats).

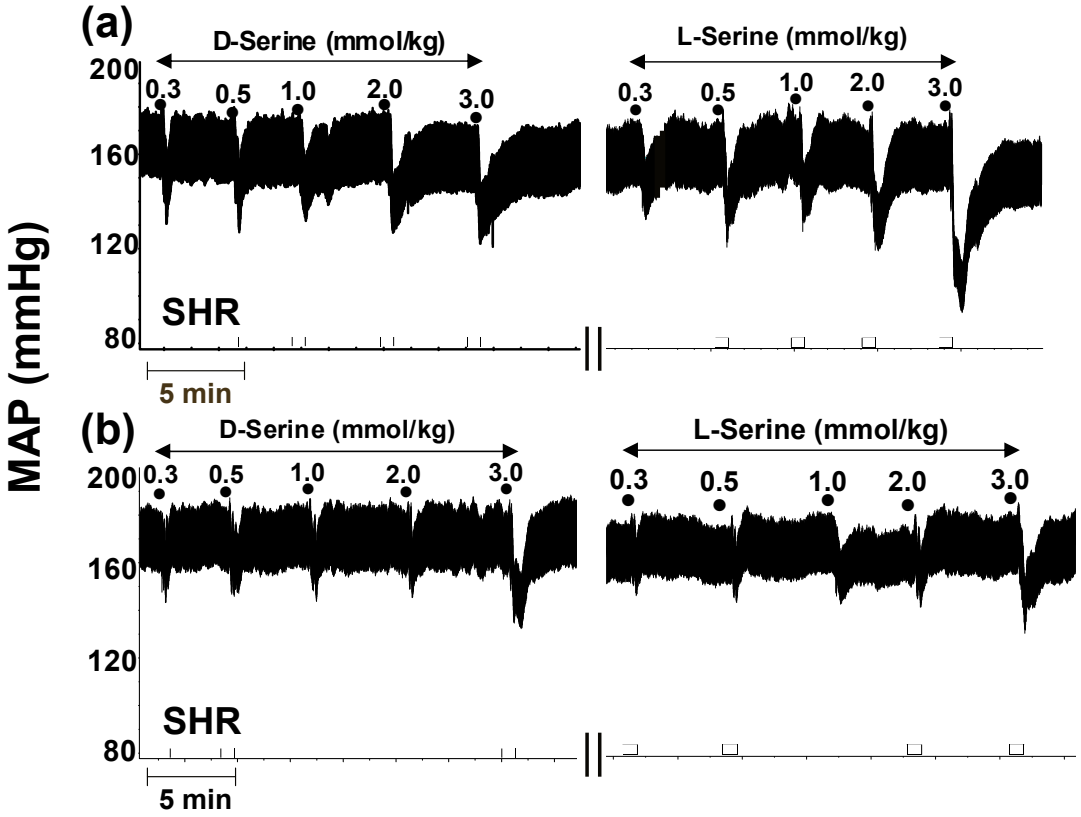

Figure 13. This is a typical experiment that compares MAP to acute i.v. infusion of increasing doses (0.3 to 3.0 mmol/kg) of D-Serine followed by L-serine in a 14 week old male SHR in the upper panel (a). The lower panel (b) depicts the responses to similar doses of D-serine followed by L-Serine infused in the same animal after pretreatment with apamin and charybdotoxin combination (75 μg/kg of each given i.v. by slow infusion over a 15 min period). Similar results were obtained in 6 age-matched male SHR.

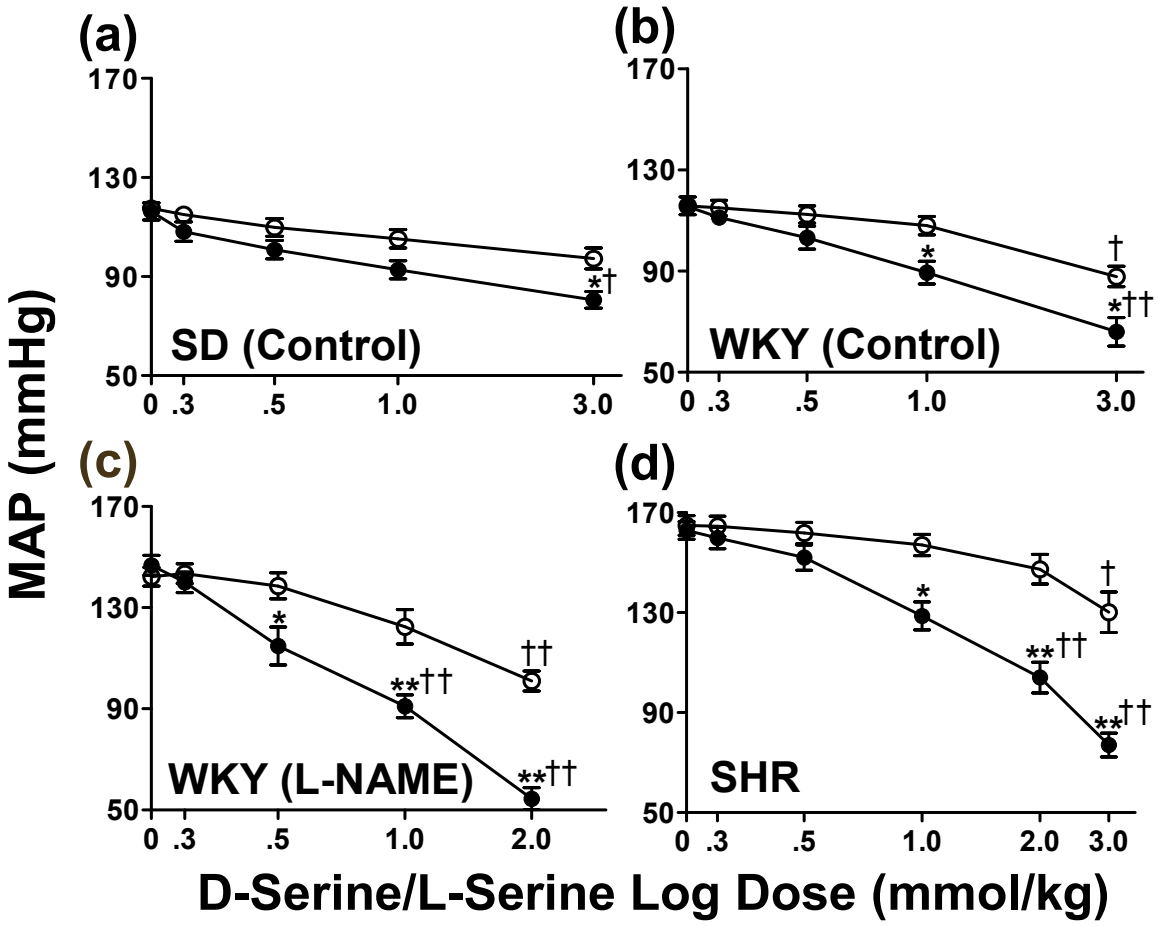

Figure 14. Dose response curves comparing mean arterial pressure (MAP) evoked by D-serine (○) and L-serine (●) in 14 week old male normotensive Sprague-Dawley (a) or WKY (b) vs. hypertensive WKY that received L-NAME (0.7mg/ml in drinking water for five days) treatment (c) or SHR (d) strains. Each data point represents mean ± SEM (n = 5 to 7 rats/group). *$p < 0.05$ and **$p < 0.01$ vs. data point for D-serine dose in the same group. $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ vs. data for basal MAP prior to infusion of D- or L-serine in the same group. The maximal dose of D- or L- serine used was limited to 2 mmol/kg due to protracted hypotension at higher doses, when it was infused in L-NAME treated rats.

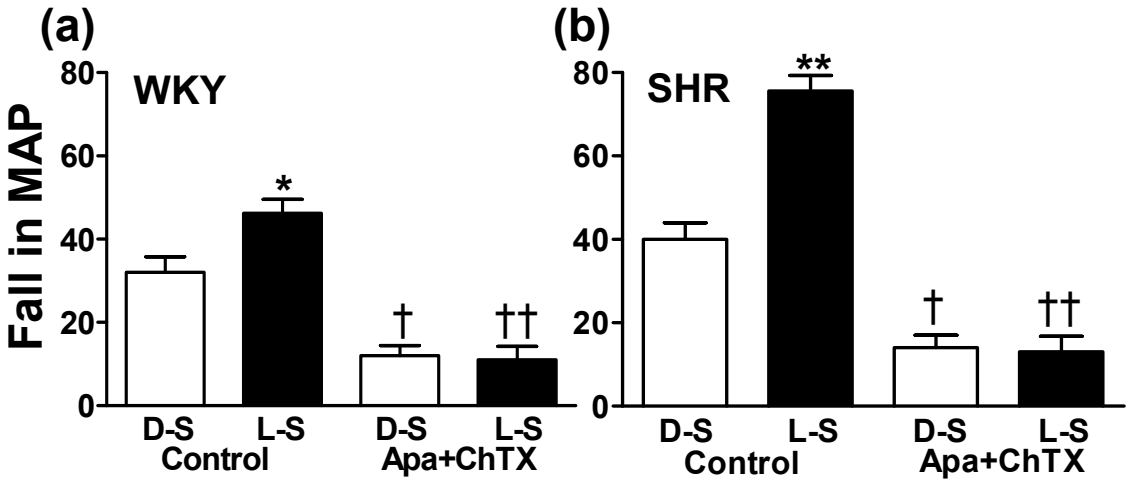

Figure 15. Each bar represents mean ± SEM maximum fall in MAP determined in 5 to 7 rats for each group. *$p < 0.05$, **$p < 0.01$ vs. data for D-serine (D-S) in the same strain. $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ vs. respective data for D-S or L-serine (L-S) values prior to slow infusion of a combination of apamin + charybdotoxin (75 µg/kg, i.v.) in the same group.

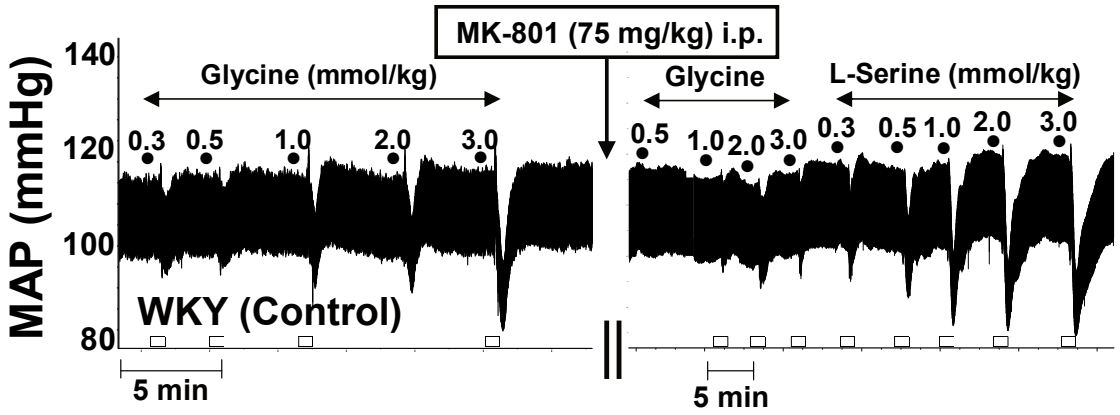

Figure 16. A typical experiment shows the fall in MAP evoked by increasing doses (0.3 to 3.0 mmol/kg, i.v.) of glycine before and 30 minutes after the infusion of NMDA selective antagonist, MK-801 (75 mg/kg, i.p.), given over a period of 15 minutes. MK-801 blocked the responses to glycine but not to L-serine. The time scale on the X-axis is reduced for the right hand side panel MK-801 treatment as the data was compressed to accommodate data for glycine followed by responses to L-serine (a total of 9 responses). Similar results were reproduced in seven age-matched male normotensive Sprague-Dawley and WKY rats.

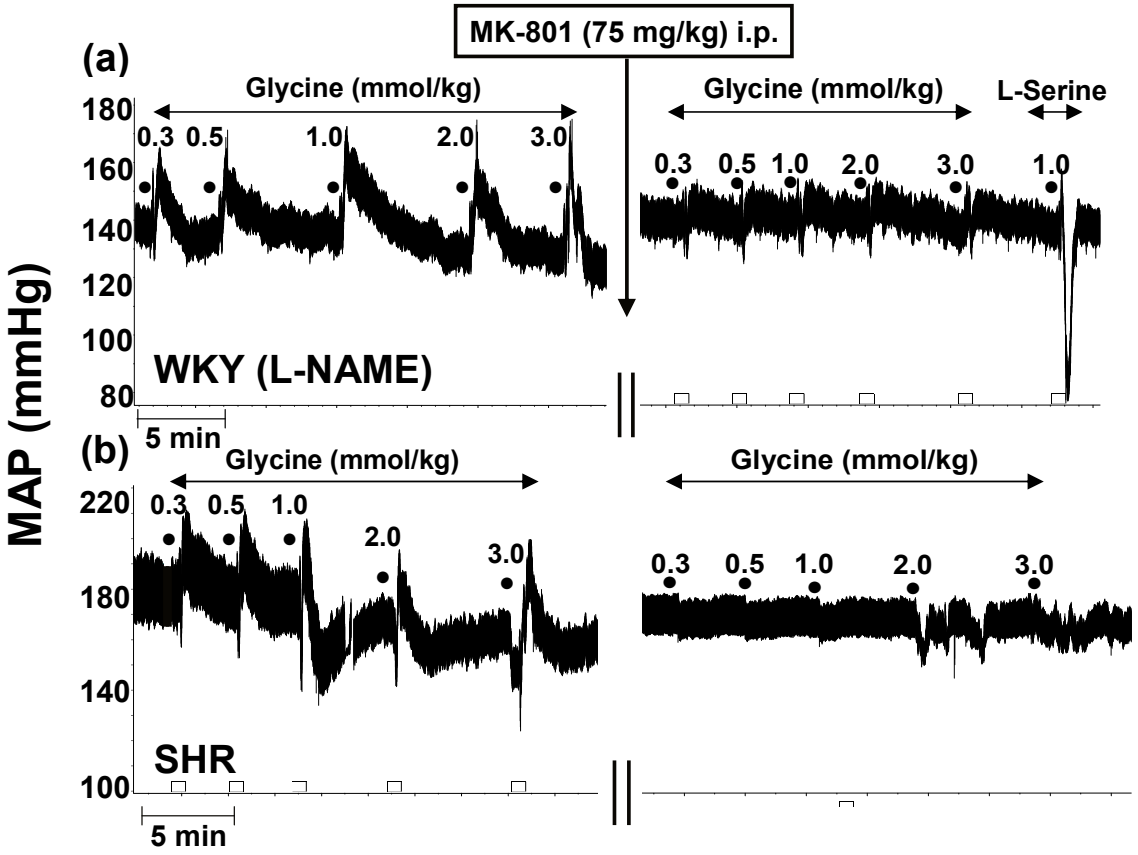

Figure 17. A typical experiment compares MAP with increasing doses of glycine (0.3 to 3.0 mmol/kg, i.v.) infusion in L-NAME treated WKY rat (a) before (left upper panel) and after (right upper panel) the administration of NMDA antagonist, MK-801. The pressor responses to glycine were absent after MK-801 treatment, but the response to L-Serine persisted. The lower panel (b) shows the pressor responses to increasing doses of glycine infusion and the lack of pressor responses to the same doses of glycine infusion after the administration of MK-801.

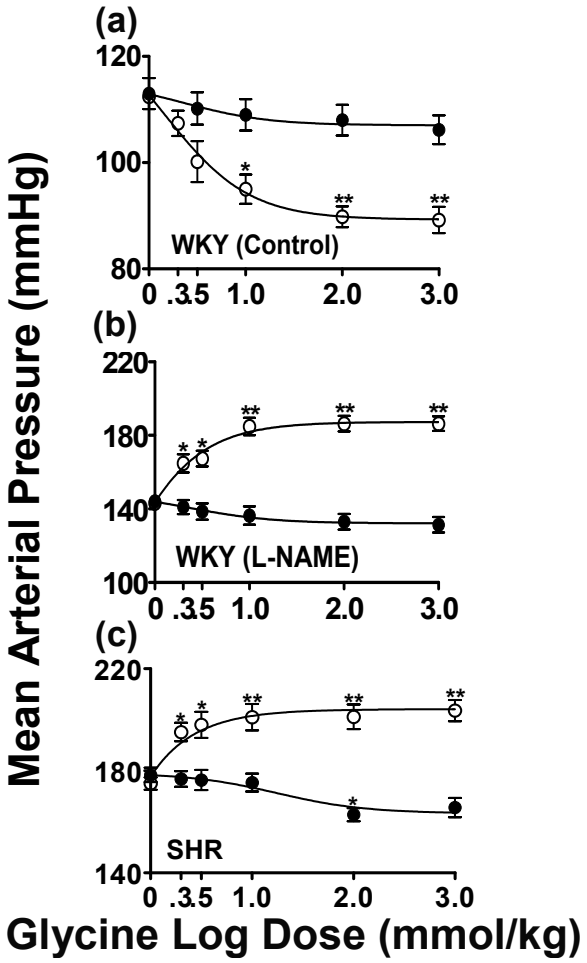

Figure 18. The dose-response relationships for glycine evoked changes in MAP either before (○) and after MK-801 (●) infusion in WKY (a), L-NAME pretreated hypertensive WKY (b) or SHR (c) strains. Each data point is mean ± SEM (n = 5 to 7 rats/group). *$p < 0.05$ and **$p < 0.01$ vs. data of basal MAP prior to infusion of glycine in each group.

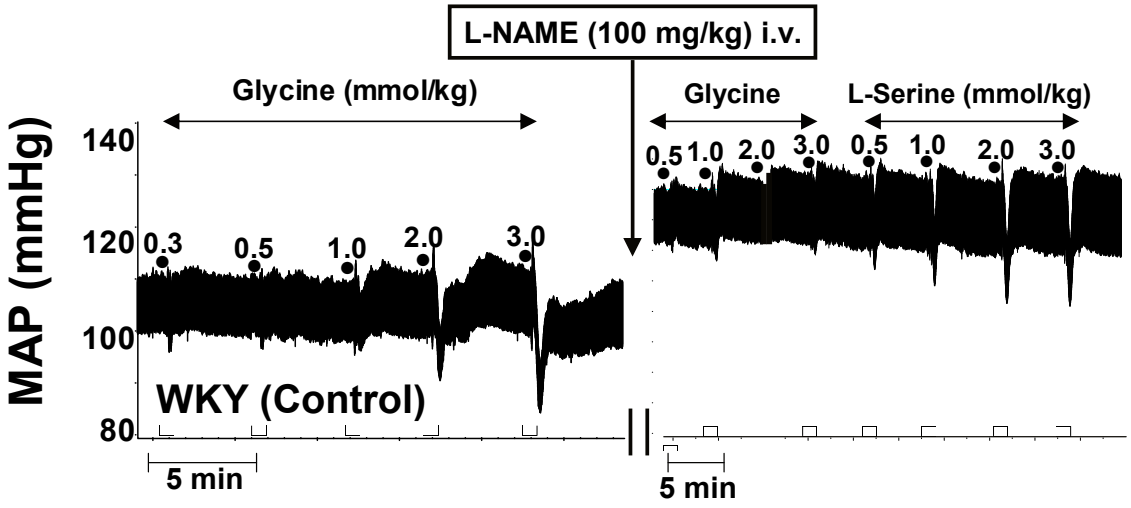

Figure 19. A typical experiment compares MAP to increasing doses of glycine (0.3 to 3.0 mmol/kg, i.v.) before (left hand panel) and the lack of responses to the same doses of glycine 1 hour after L-NAME (100 mg/kg, i.v.) infusion in a normotensive WKY rat. While glycine responses were blunted L-serine responses persisted following acute L-NAME infusion. The X-axis time scale was compressed for the right hand side panel to accommodate 8 responses. Similar data were reproduced in 5 WKY rats.

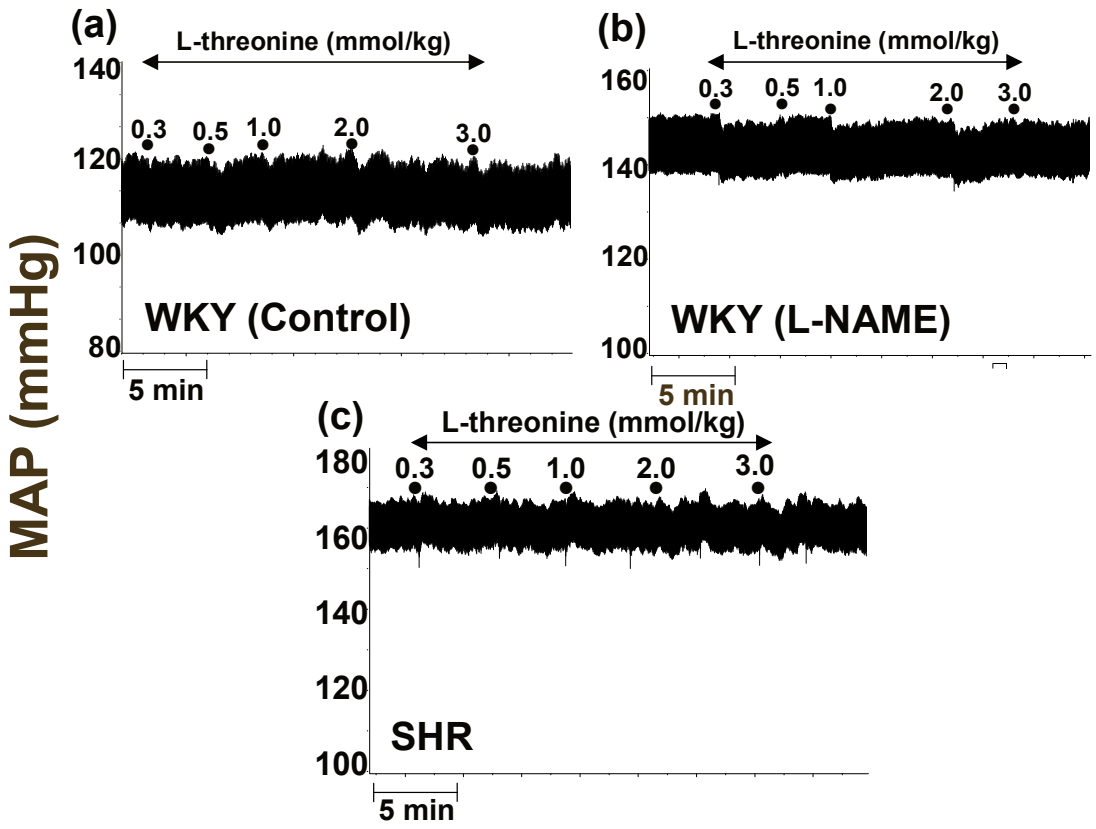

Figure 20. A representative experiment depicts that infusion of L-threonine (0.3 to 3.0 mmol/kg) failed to alter MAP recording in a normotensive WKY rat (a) and chronic L-NAME treated hypertensive WKY rat (b) and a SHR (c) strain. These results were reproduced in 5 to 7 different rats from each group.

4.2.3. Regional Hemodynamic Study

4.2.3.1. Comparison of Basal Regional Blood Flow

We observed significant reductions in basal blood flow to several organs in hypertensive rat models compared to normotensive rats. A subtle difference exists in basal flow to different organs between L-NAME pretreated hypertensive WKY and SHR strains.

4.2.3.1.1. Normotensive vs. Hypertensive Rat Models

We compared the basal regional blood flow to 13 different organs/tissues after vehicle (physiological saline) infusion (0.4 ml/kg) in normotensive WKY, L-NAME pretreated hypertensive WKY and SHR strains. Basal blood flow to left and right kidney was found to be similar with a variation that was <5% in the respective normotensive or hypertensive rats. A significant difference in blood flow was observed in both L-NAME pretreated hypertensive WKY and SHR strains compared to normotensive WKY rats. In normotensive WKY rats, kidney received the highest blood flow (5.4 to 6.0 ml/min/g) followed by heart (3.4 to 3.7 ml/min/g). Except liver, all other organs received blood flow between 1.0 to 1.5 ml/min/kg (Table 5, Figure 22, 23 and 24). On the other hand in chronic L-NAME treated WKY rats, a decrease in blood flow occurred in most organs studied except brain and the heart. The decrease in blood flow to spleen was significant ($p < 0.01$) followed by small intestine and kidney ($p < 0.05$) compared to what was observed in normotensive control rats (Table 5, Figure 22, 23 and 24). In SHR strain, there was a decrease in blood flow to most organs studied compared to normotensive control WKY rats. The decrease in blood flow was noted in spleen and kidney ($p < 0.01$), pancreas, small

and large intestine ($p < 0.05$) while blood flow values were not significantly lower in the brain and heart compared to the values noted in their normotensive strains (Table 5, Figure 22, 23 and 24). In SHR strain there was a significant increase in blood flow to heart ($p < 0.01$) and brain ($p < 0.05$) compared to normotensive control strain of WKY rats (Table 5, Figure 22, 23 and 24).

4.2.3.1.2. L-NAME Pretreated Hypertensive WKY vs. SHR

In SHR strain, there was an increase in blood flow to heart ($p < 0.01$) and a decrease in blood flow to kidney ($p < 0.05$) compared to the response noted in L-NAME pretreated hypertensive rats. On the other hand, there is an overall decrease in blood flow in all other organs studied in SHR compared to L-NAME pretreated hypertensive WKY rats (Table 5, Figure 22, 23 and 24).

4.2.3.2. Acetylcholine Induced Changes in Regional Blood Flow

ACh is a well known endothelium-dependent vasodilator that promotes a rapid, reversible, but robust dose-dependent fall in MAP both in normotensive and hypertensive rats (Desai et al., 2006). L-serine is also an endothelium-dependent vasodilator and it decreases MAP in normotensive and hypertensive rat models but L-serine is less potent compared to ACh. Therefore it was necessary to examine ACh-evoked increases in blood flow to different organs as a known reference sample, as a positive control for appropriate comparison to L-serine. A submaximal dose of ACh (10 nmol/kg) increased blood flow significantly only to heart and kidney ($p < 0.05$) compared to vehicle infusion in the same group of animals. ACh infusion increased the blood flow to all organ studied but the

increase observed did not attain statistical significance (Table 6 and Figure 21). Moreover the sequence of vehicle and ACh infusion did not alter the selective and significant in crease in blood flow to heart and kidney evoked by ACh infusion in all strains.

4.2.3.3. L-serine Induced Changes in Regional Blood Flow

4.2.3.3.1. Normotensive Control WKY vs. Hypertensive Rat Models

L-serine induced increase in blood flow in normotensive control WKY, L-NAME pretreated WKY and SHR stains. In normotensive WKY group L-serine (1 mmol/kg) infusion increase in blood flow was significant in small intestine and heart (74% and 39%, $p < 0.05$) compared to vehicle infusion (Table 10, Figure 22, 23 and 24). In L-NAME treated hypertensive WKY rats, L-serine induced increase in blood flow was significant in most organ studied compared to vehicle infusion in the same group. It followed the following rank order small intestine (392%, $p < 0.001$, Table 11), spleen, diaphragm, and heart (155%, 111%, and 65%, $p < 0.01$, Table 11), liver, large intestine and kidney (103%, 85% and 30%, $p < 0.05$, Table 11). The increase in blood flow evoked by L-serine was much less in stomach (12%), lungs (11%), pancreas (7%) and brain (3%), (Figure 22, 23, and 24, Table 11). In SHR group there was an increase in blood flow occurring in brain and heart prior to infusion of L-serine (Table 5). After L-serine infusion, a significant increase in blood flow was noted in small intestine (265%), spleen (177%) and kidney (82%), liver (160%), diaphragm (143%), large intestine (124%), pancreas (121%), brain (27%) and heart (21%). The increase in blood flow evoked by L-serine infusion was

minimal in the case of lungs and stomach and it was not significant compared to vehicle infusion in the same group of rats (Figure 22, 23 and 24, Table 5 and Table 12).

4.2.3.3.2. L-NAME Pretreated Hypertensive WKY vs. SHR

In L-NAME pretreated WKY rats, L-serine induced increase in blood flow was significantly higher in small intestine and heart ($p < 0.05$) compared to SHR stains. There was no statistical difference in all other organ studied between these two hypertensive rat models (Table 5, Figure 22, 23 and 24).

4.2.3.4. Comparison of Basal Peripheral Vascular Resistance

4.2.3.4.1. Normotensive WKY vs. Hypertensive Rat Models

A wide spread increase in organ vascular resistance in both L-NAME treated hypertensive WKY and SHR rats was noted (Table 7). Data from several experiment revealed that increase in vascular resistance was more pronounced in SHR compared to L-NAME induced hypertensive rats (Table 7). In L-NAME treated rats, peripheral vascular resistance significantly increased in spleen, small intestine, large intestine ($p < 0.01$), kidney, liver, stomach, lungs, pancreas and diaphragm ($p < 0.05$) compared to the peripheral vascular resistance seen in the normotensive control WKY rats receiving vehicle infusion except heart and brain (Table 7, Figure 25, 26, and 27). In the SHR group, peripheral organ vascular resistance increased significantly in most organs studied such as large intestine, spleen, kidney, pancreas, small intestine, stomach, liver, lungs and

diaphragm ($p < 0.01$) compared to the peripheral vascular resistance encountered in normotensive control rats receiving vehicle infusion (Table 7, Figure 25, 26 and 27).

4.2.3.4.2. L-NAME Pretreated Hypertensive WKY vs. SHR

In the SHR group, basal peripheral vascular resistance was significantly higher in most organs studied in comparison to L-NAME pretreated hypertensive WKY rats. The following rank order of response was noted: pancreas ($p < 0.01$), kidney, liver, spleen, brain, lungs, stomach, small intestine, large intestine and diaphragm ($p < 0.05$) while it was negligible or absent in heart (Table 7, Figure 25, 26, and 27).

4.2.3.5. L-serine Induced Changes in Peripheral Vascular Resistance

L-serine infusion reduced organ vascular resistance in both normotensive and hypertensive rat models. L-serine evoked decrease in organ vascular resistance occurred in multiple organs in both hypertensive models (L-NAME treated and SHR) compared to normotensive WKY rats.

4.2.3.5.1. Normotensive Control WKY vs. Hypertensive Rats

L-serine infusion in normotensive control WKY rat produced a significant decrease in peripheral vascular resistance only in small intestine compared to vehicle infusion given in same group of rats (42%, $p < 0.05$, Table 13). In L-NAME pretreated hypertensive rats, L-serine infusion significantly reduced the peripheral vascular resistance in small intestine, spleen and liver (79%, 61% and 51%, $p < 0.001$), diaphragm and large intestine (52% and 46%, $p < 0.01$, Table 14), heart and kidney (40% and 24%, $p < 0.05$) compared to vehicle

infusion in same group of rats (Table 14). Decrease in the organ vascular resistance after L-serine infusion in lungs (11%), stomach (10%), pancreas (7%), brain (3%) and did not attain statistical significance compared to vehicle infusion in same group of rats (Table 14, Figure 25, 26 and 27). Pooled data from several experiment showed that in L-NAME pretreated hypertensive rats the profound reduction in vascular resistance was observed in small intestine, spleen and liver after acute L-serine infusion. This reduction in vascular resistance was even lower compared to vehicle infusion in the normotensive WKY rats (Table 7, Figure 25, 26 and 27). In SHR group, L-serine infusion reduced organ vascular resistance significantly in spleen (64%) and liver (61%) ($p < 0.001$, Table 15), diaphragm (58%), large intestine (55%) and pancreas (54%) ($p < 0.01$, Table 15), kidney (46%) ($p < 0.05$, Table 15) compared to vehicle infusion in same animals. However, the reduction in vascular resistance was either minimal or negligible in the heart (19%), brain (32%), lungs (19%) and stomach (Table 15, Figure 25, 26, and 27).

4.2.3.5.2. L-NAME Pretreated Hypertensive WKY vs. SHR

L-serine infusion led to a decrease in peripheral vascular resistance in several organs of SHR and L-NAME treated hypertensive rats. In L-NAME treated hypertensive WKY rats, L-serine infusion lowered vascular resistance in heart ($p < 0.05$, Table 7) but this was not the case in the SHR strain. In contrast, it reduced vascular resistance in pancreas ($p < 0.01$, Table 7). The reduction in vascular resistance attained was also significantly higher ($p < 0.05$) in small intestine after L-serine infusion in L-NAME pretreated rats compared to SHR strain (Table 7, Table 14 and Table 15).

4.2.3.6. Comparison of Basal Systemic Hemodynamics

Pooled data from several experiments showed that both MAP and TPR values were higher in L-NAME pretreated hypertensive WKY (142 ± 7 and 1.79 ± 0.02) and SHR strains (166 ± 6 and 1.96 ± 0.05) compared to normotensive WKY rats (108 ± 4 and 1.05 ± 0.04). In SHR strain, we found both MAP and CI were significantly higher ($p < 0.05$) compared to L-NAME pretreated hypertensive WKY rats (166 ± 6 vs. 142 ± 7 and 274 ± 5 vs. 232 ± 6, Table 8). HR was higher ($p < 0.05$) in SHR strains compared to L-NAME pretreated hypertensive WKY and normotensive WKY rats (398 ± 12 vs. 368 ± 11 and 357 ± 8, Table 8). There was a significant decrease in CO both in L-NAME pretreated hypertensive WKY and SHR (79 ± 5, 84 ± 4 vs. 103 ± 3, $p < 0.05$) compared to normotensive control WKY rats (Table 8).

4.2.3.6.1. L-serine Evoked Changes in Systemic Hemodynamics

4.2.3.6.1.1. Normotensive Control WKY Rats

Acute L-serine infusion significantly reduced MAP, TPR and TPRI (22%, 24% and 26%, $p < 0.05$, Table 16), and increased CO (11%, $p < 0.05$, Table 16) compared to vehicle infusion in WKY rats (Table 8 and Table 16). The changes in HR (6%), CI (7%) after L-serine infusion did not attain statistical significance compared to vehicle infusion in WKY rats (Table 8 and Table 16).

4.2.3.6.1.2. L-NAME Pretreated Hypertensive WKY Rats

In L-NAME treated hypertensive WKY rats, acute L-serine infusion significantly decreased MAP, TPR and TPRI (46%, 68% and 67%, $p < 0.001$, Table 17) and increased CO and CI (75% and 70%, $P < 0.001$, Table 17) compared to vehicle infusion in the same group of rats. The increase in HR (7%) evoked by L-serine infusion did not attain statistical significance compared to vehicle infusion in the same group (Table 8 and Table 17).

4.2.3.6.1.3. SHR

In SHR strain acute L-serine infusion significantly reduced the MAP, TPR and TPRI (34%, 53% and 48%, $p < 0.01$, Table 18) and increased in CO and CI (41% and 42%, $p < 0.01$, Table 18) compared to vehicle infusion in the same group of rats. The increase in HR (8%) after L-serine infusion did not attain statistical significance compared to vehicle infusion in the same group of rats (Table 8 and Table 18).

4.2.3.6.1.4. Comparison in L-NAME Pretreated Hypertensive Rats and SHR

The basal MAP and TPR values observed were significantly higher ($p < 0.05$) in SHR strain (166 ± 6 mmHg, 1.96 ± 0.05 mmHg/ml/min) compared to level encountered in L-NAME treated rats (142 ± 7 mmHg, 1.79 ± 0.02 mmHg/ml/min, Table 8). However the reduction in there systemic hemodynamic parameters following L-serine infusion was significantly higher ($p < 0.05$) in L-NAME treated hypertensive rats (MAP: 46%, TPR: 68%, $p < 0.001$, Table 17) in comparison to SHR strains (MAP: 34% and TPR: 53%, $p < 0.01$, Table 18). Moreover the level of increase in CO and CI was higher ($p < 0.05$) in L-

NAME pretreated hypertensive WKY rats (CO: 75% and CI: 70%, Table 17) than what was noted in SHR groups (CO: 41% and CI: 42%, Table 18). The increase in HR in both L-NAME treated hypertensive WKY rats (8%) and SHR groups (7%) did not attain statistical significance (Table 8, Table 17 and Table 18).

4.2.3.7. Effect of L-serine in Presence of Apamin and Charybdotoxin

Pretreatment with apamin and ChTX for 45 min, significantly attenuated the increase in blood flow to small intestine in normotensive control WKY rats and several organs of L-NAME treated hypertensive rats and SHR groups (Table 9, Table 19, Table 20, Table 21, Figure 28, 29, 30, 31 and 32).

4.2.3.7.1. Normotensive Control WKY Rats

Pretreatment with apamin and ChTX for 45 min significantly reduced the L-serine induced increase in blood flow to small intestine ($p < 0.05$) in normotensive WKY compared to the values obtained after L-serine infusion in the same group of rats (Table 9, Table 19, and Figure 30).

4.2.3.7.2. L-NAME Pretreated Hypertensive WKY Rats

L-serine induced increases in blood flow to small intestine, large intestine, heart, both left and right kidney, spleen, skeletal muscle and diaphragm were significantly reduced ($p < 0.01$) by pretreatment with apamin and ChTX combination while blood flow to liver, brain, spleen, lungs, stomach and pancreas were not reduced to a significant extent. (Table 9, Table 20, Figure 28, 29, 30, 31 and 32).

4.2.3.7.3. SHR

Pretreatment with apamin and ChTX combination significantly reduced ($p < 0.05$) the increase in blood flow induced by L-serine to small intestine, spleen, the kidneys, large intestine, skeletal muscle, and diaphragm in SHR. While blood flow to heart, liver, stomach, lungs, pancreas and brain were not reduced to a significant extent (Table 9, Table 21, Figure 28, 29, 30, 31 and 32).

4.2.3.7.4. Comparison between L-NAME Pretreated Hypertensive Rats and SHR

In L-NAME treated hypertensive rats, pretreatment with apamin and ChTX combination was significantly reduced the increase in blood flow to L-serine in small intestine ($p < 0.01$) and heart, spleen, diaphragm and skeletal muscle ($p < 0.05$). While in SHR group, pretreatment with apamin and ChTX combination was significantly reduced ($p < 0.05$) the increase in blood flow to L-serine in spleen, small intestine, lungs, large intestine and diaphragm (Table 9, Table 20, Table 21, Figure 28, 29, 30, 31 and 32).

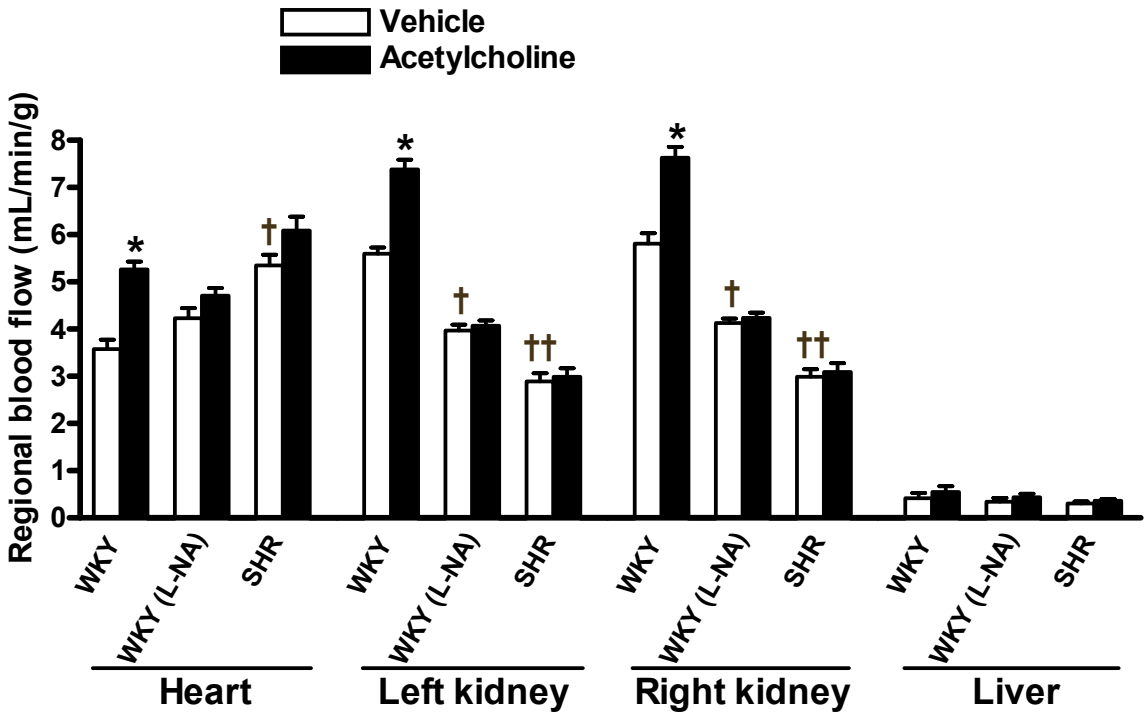

Figure 21. Regional blood flow in heart, kidney and liver of 14 weeks old male normotensive Wistar-Kyoto (WKY) rats, L-NAME-induced hypertensive WKY [WKY (L-NA)] rats and spontaneously hypertensive rats (SHR) following a single intravenous bolus dose of vehicle (saline, 0. 4ml/kg) followed by acetylcholine (ACh, 10 nmol/kg) infusion. Each bar is a mean ± SEM of 5 to 7 rats. *$p < 0.05$ compared to vehicle treatment in the same group. $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ compared to normotensive control WKY group.

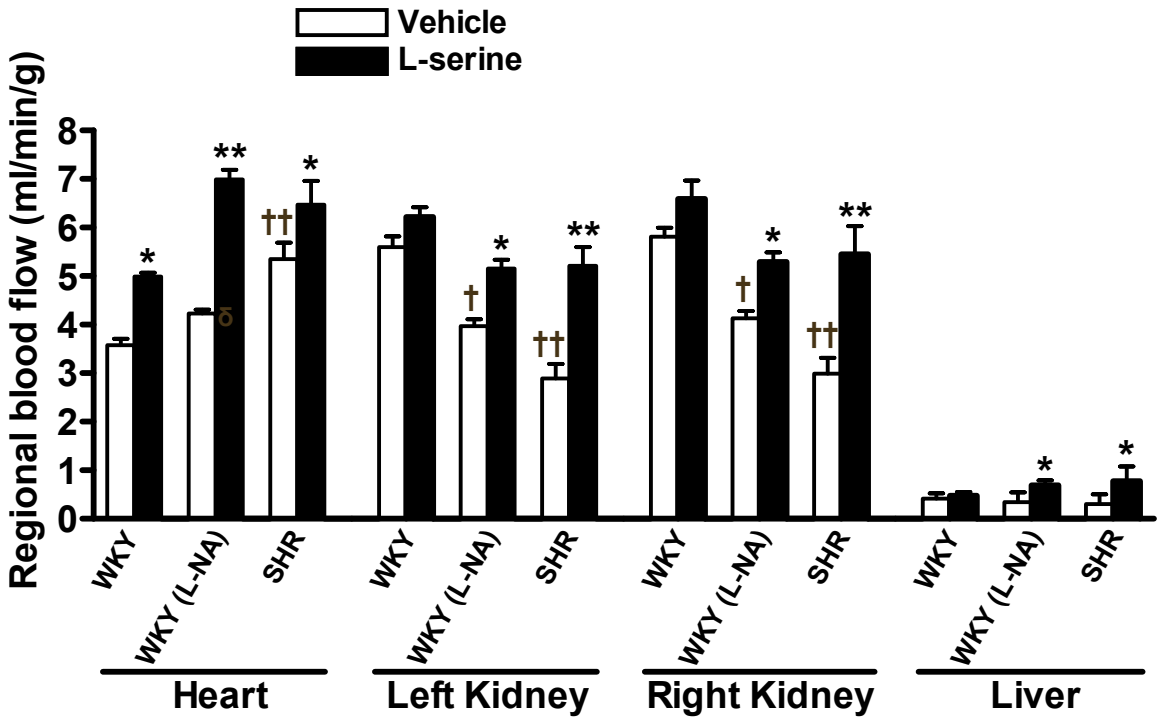

Figure 22. Changes in regional blood flow to heart, left kidney, right kidney and liver following a single bolus dose of vehicle followed by L-serine (1 mmol/kg, i.v.) in 14 week old male WKY rats, L-NAME pretreated hypertensive WKY [WKY (L-NA)] rats and SHR strains. Each bar is a mean ± SEM of 5 to 7 rats. *$p < 0.05$ and **$p < 0.01$ compared to vehicle treatment in the same group. $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ compared to normotensive control WKY group.

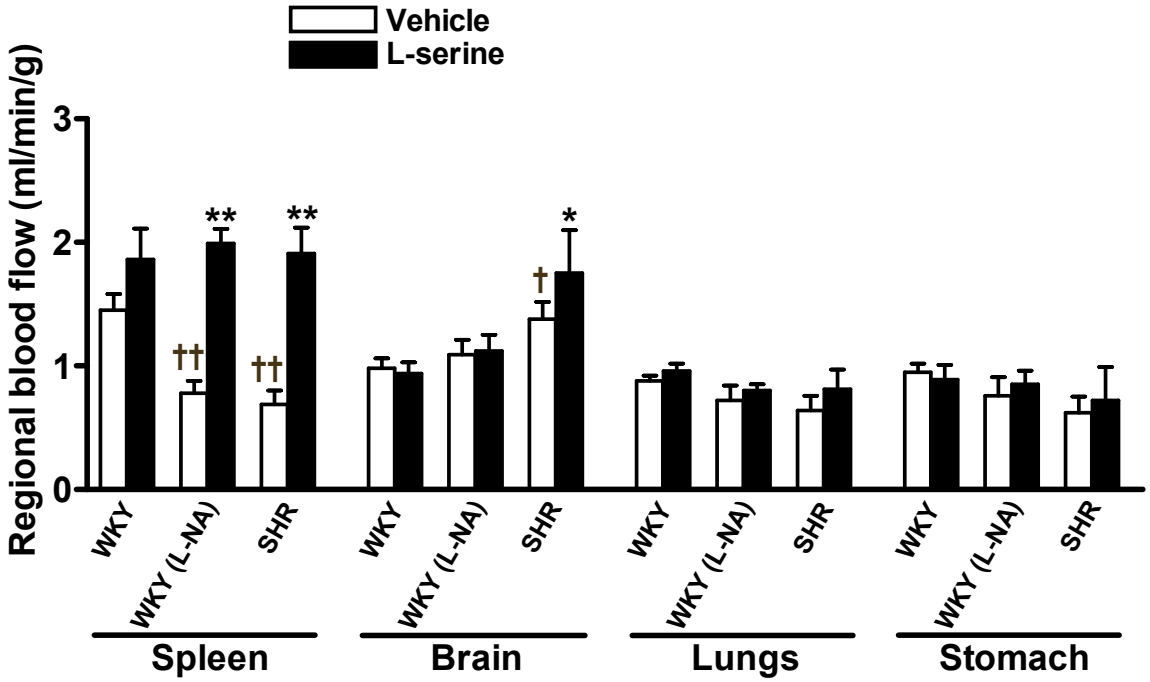

Figure 23. Changes in regional blood flow to spleen, brain, lungs and stomach following a single bolus dose of vehicle followed by L-serine (1 mmol/kg, i.v.) in 14 week old male WKY rats, L-NAME pretreated hypertensive WKY [WKY (L-NA)] rats and SHR strains. Each bar is a mean ± SEM of 5-7 rats. *$p < 0.05$ and **$p < 0.01$ compared to vehicle treatment in the same group. $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ compared to normotensive control WKY group.

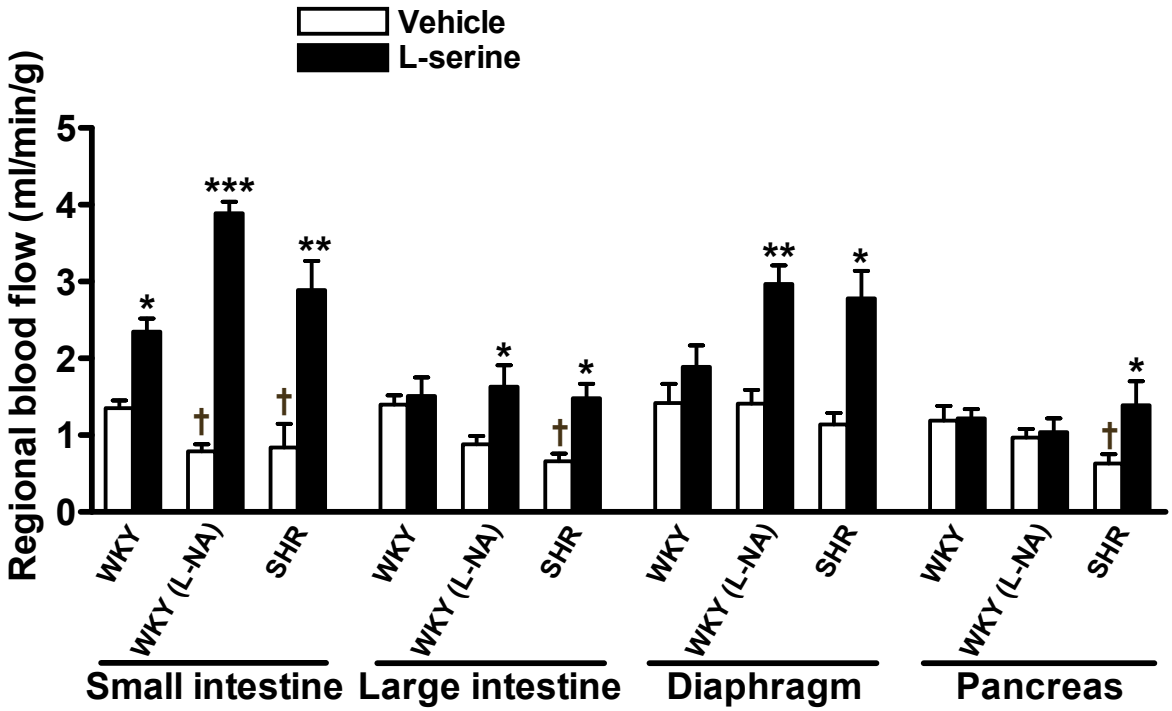

Figure 24. Changes in regional blood flow to small intestine, large intestine, diaphragm and pancreas following a single bolus dose of vehicle followed by L-Serine (1 mmol/kg, i.v.) in 14 week old male WKY rats, L-NAME pretreated hypertensive WKY [WKY (L-NA)] rats and SHR strains. Each bar is a mean ± SEM of 5-7 rats. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to vehicle treatment in the same group. $^{\dagger}p < 0.05$ compared to normotensive control WKY group.

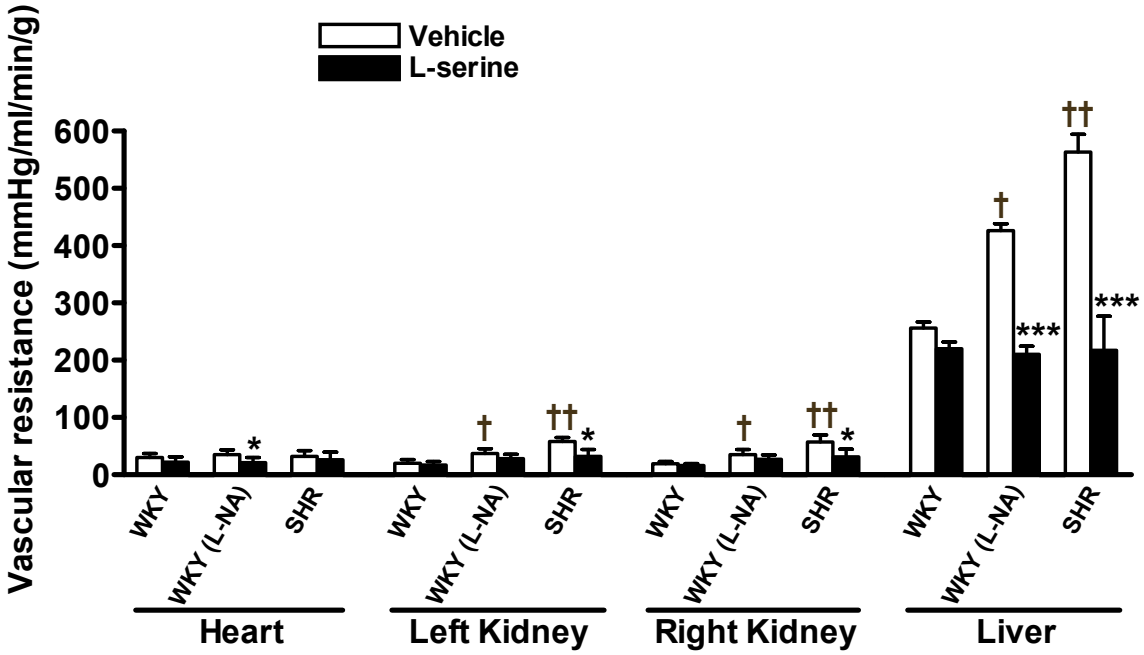

Figure 25. Changes in peripheral vascular resistance following a single bolus dose of vehicle followed by L-serine (1 mmol/kg, i.v.) in heart, left kidney, right kidney and liver of 14 week old male WKY rats, L-NAME pretreated hypertensive WKY [WKY (L-NA)] rats and SHR strains. Each bar is a mean ± SEM of 5 to 7 rats. *$p < 0.05$ and ***$p < 0.001$ compared to vehicle treatment in the same group. $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ compared to normotensive control WKY group.

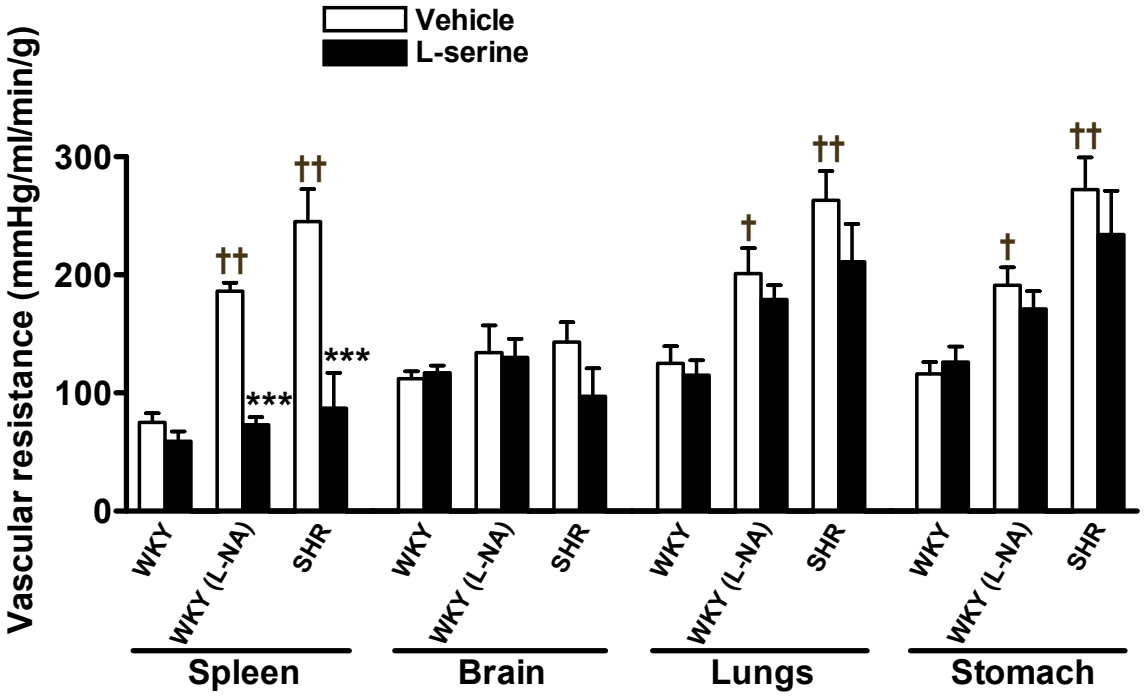

Figure 26. Changes in peripheral vascular resistance following a single bolus dose of vehicle followed by L-serine (1 mmol/kg, i.v.) in spleen, brain, lungs and stomach of 14 week old male WKY rats, L-NAME pretreated hypertensive WKY [WKY (L-NA)] rats and SHR strains. Each bar is a mean ± SEM of 5 to 7 rats. ***$p < 0.001$ compared to vehicle treatment in the same group. $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ compared to normotensive control WKY group.

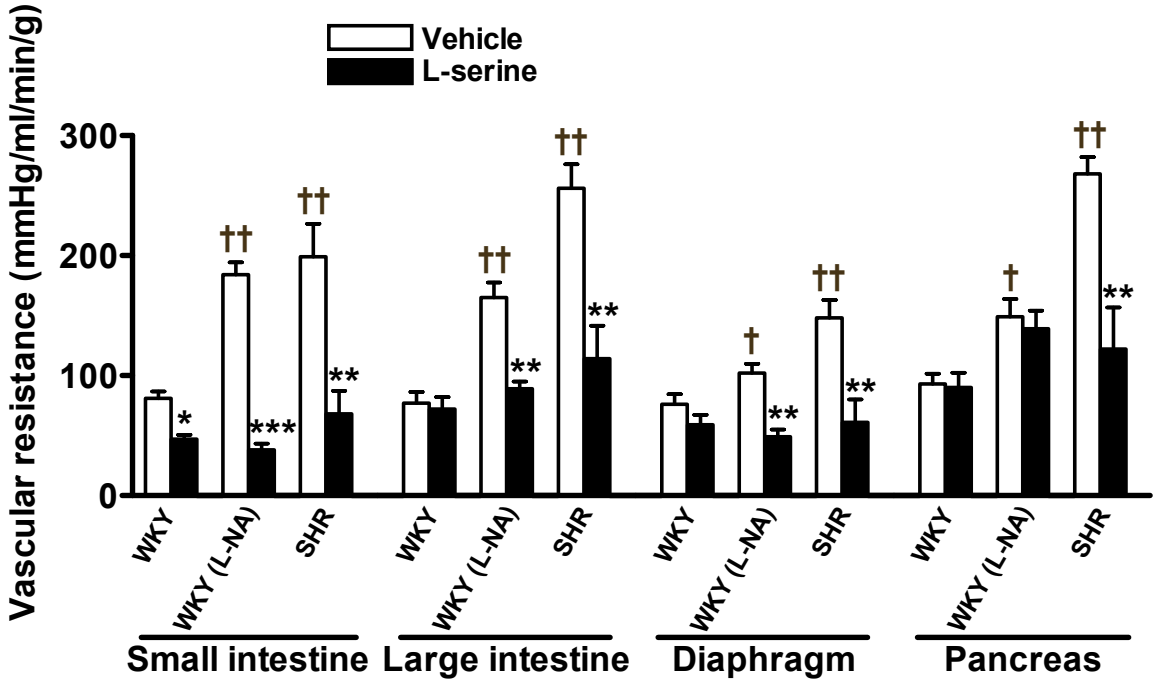

Figure 27. Peripheral vascular resistance following a single bolus dose of intravenous infusion of vehicle followed by L-serine (1 mmol/kg)in small intestine, large intestine, diaphragm and pancreas of 14 week old male WKY rats, L-NAME pretreated hypertensive WKY [WKY (L-NA)] rats and SHR strains. Each bar is a mean ± SEM of 5 to 7 rats. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to vehicle treatment in the same group. $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ compared to normotensive control WKY group.

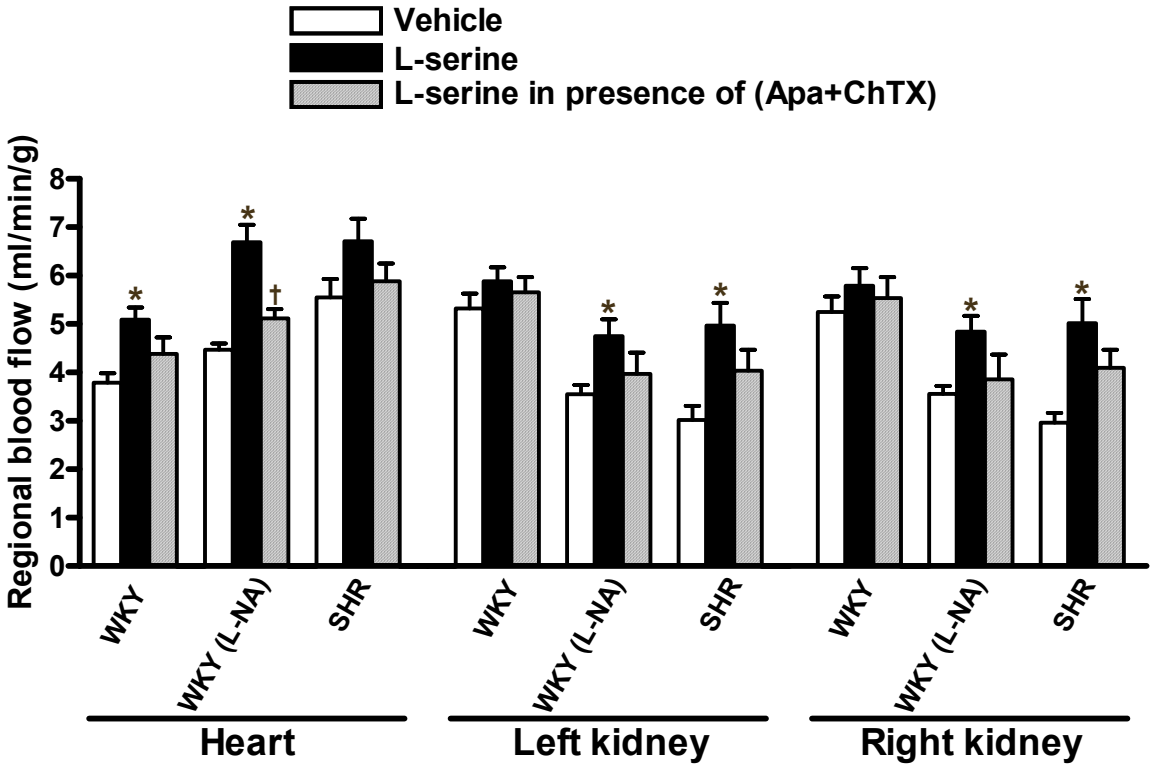

Figure 28. Changes in regional blood flow following acute infusion of vehicle, L-serine and followed by L-serine (1 mmol/kg, i.v.) in presence of apamin and charybdotoxin (75 μg/kg) in heart, left and right kidney of 14 week old male WKY, L-NAME pretreated hypertensive WKY [WKY (L-NA)] rats and SHR strains. Each bar is a mean ± SEM of 2 to 3 rats. *$p < 0.05$ and **$p < 0.01$ compared to vehicle treatment in the same group and $^{\dagger}p < 0.05$ compared to L-serine treatment in the same group.

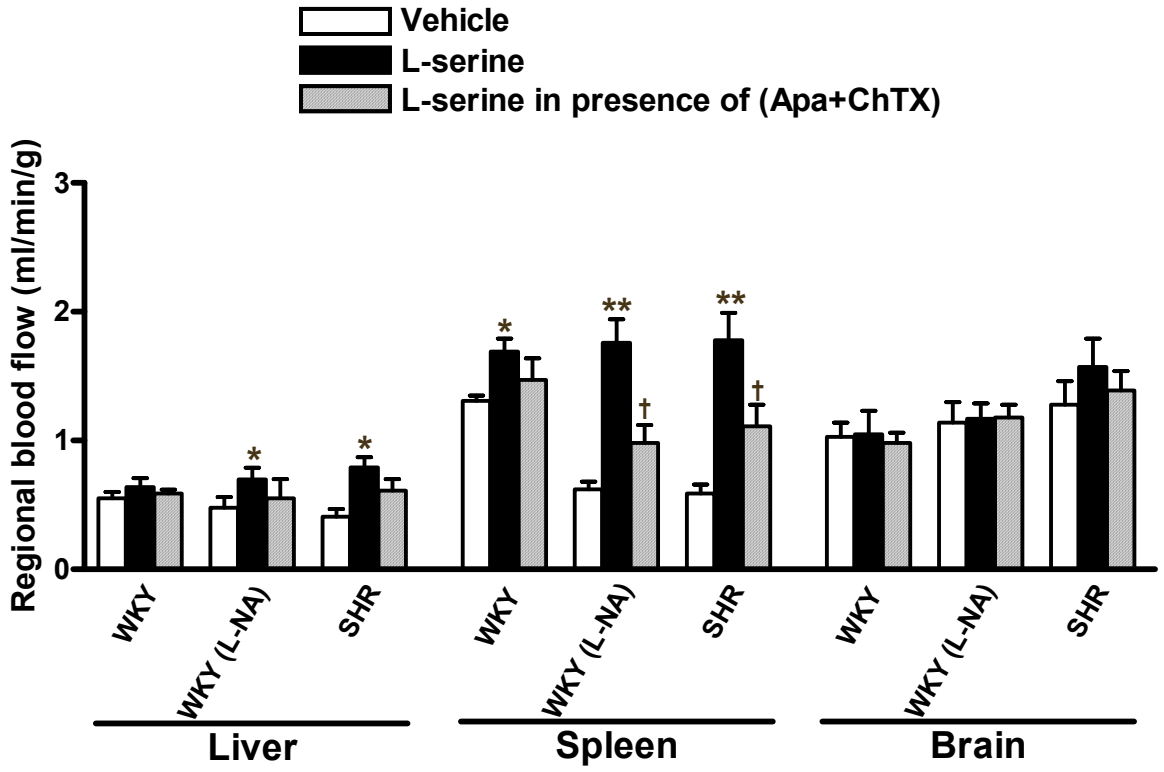

Figure 29. Changes in regional blood flow following acute infusion of vehicle, L-serine and followed by L-serine (1 mmol/kg, i.v.) in presence of apamin and charybdotoxin (75 μg/kg) in liver, spleen and brain of 14 week old male WKY, L-NAME pretreated hypertensive WKY [WKY (L-NA)] rats and SHR strains. *$p < 0.05$ and **$p < 0.01$ compared to vehicle treatment in the same group and $^{\dagger}p < 0.05$ compared to L-serine treatment in the same group.

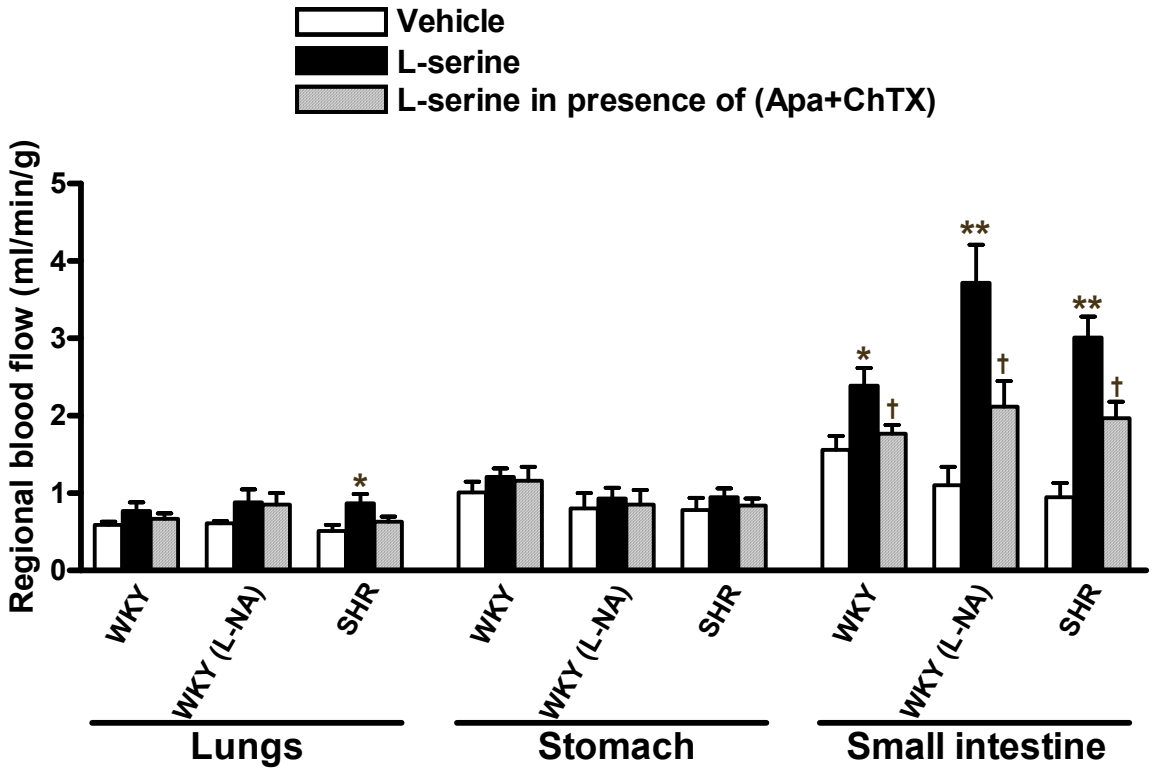

Figure 30. Changes in regional blood flow following acute infusion of vehicle, L-serine and followed by L-serine (1 mmol/kg, i.v.) in presence of apamin and charybdotoxin (75 μg/kg) in lungs, stomach and small intestine of 14 week old male WKY, L-NAME pretreated hypertensive WKY [WKY (L-NA)] rats and SHR strains. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.01$ compared to vehicle treatment in the same group and $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ compared to L-serine treatment in the same group.

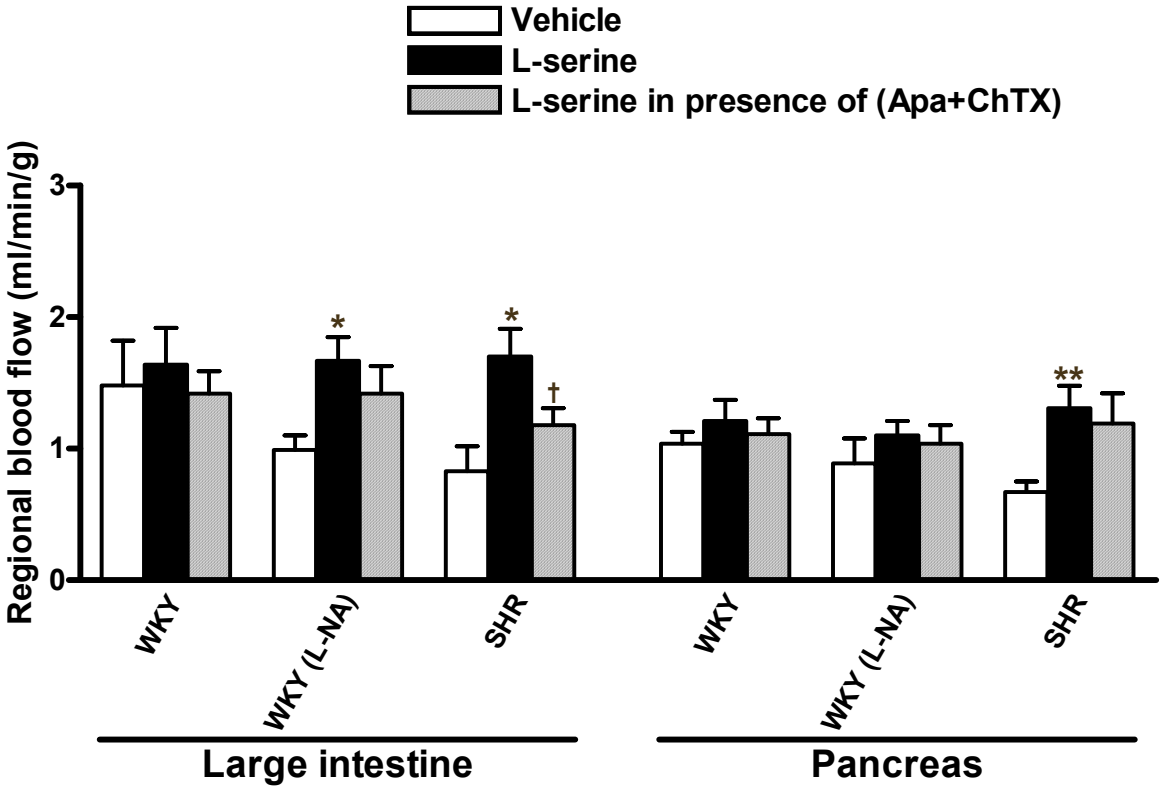

Figure 31. Changes in regional blood flow following acute infusion of vehicle, L-serine and followed by L-serine (1 mmol/kg, i.v.) in presence of apamin and charybdotoxin (75 μg/kg) in large intestine and pancreas of 14 week old male WKY, L-NAME pretreated hypertensive WKY [WKY (L-NA)] rats and SHR strains. *$p < 0.05$ and **$p < 0.01$ compared to vehicle treatment in the same group and $^{\dagger}p < 0.05$ compared to L-serine treatment in the same group.

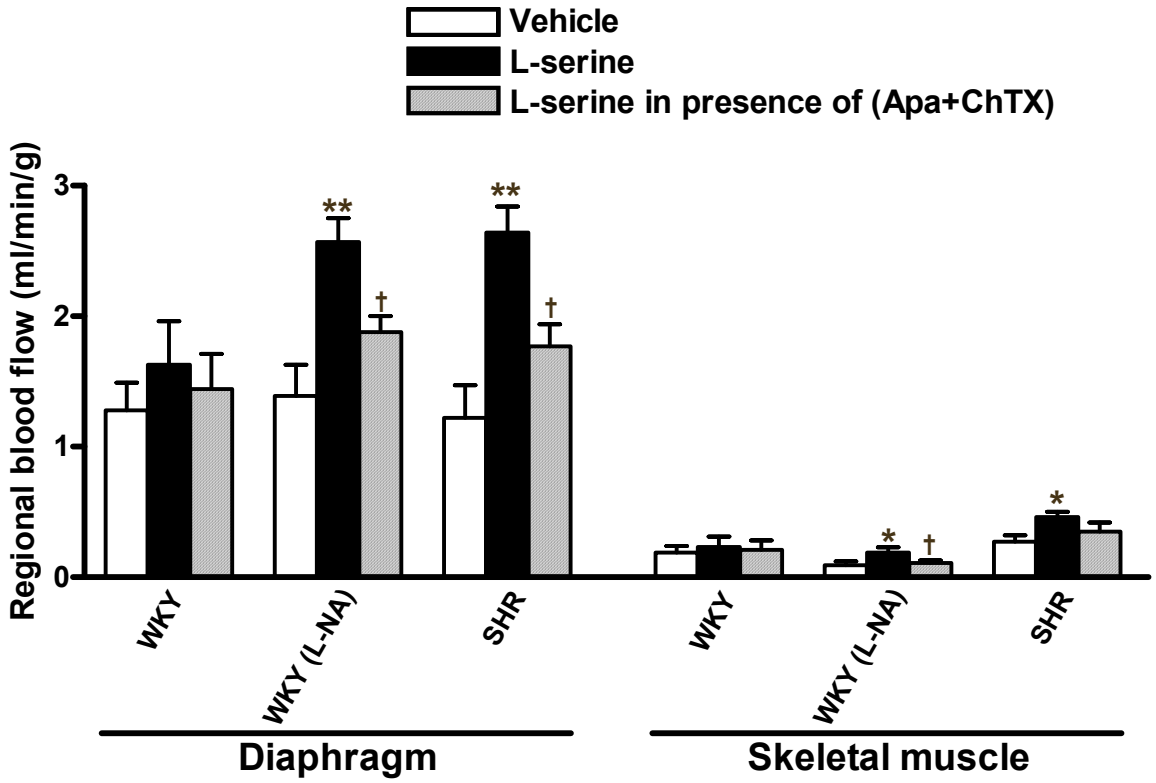

Figure 32. Changes in regional blood flow following acute infusion of vehicle, L-serine and followed by L-serine (1 mmol/kg, i.v.) in presence of apamin and charybdotoxin (75 μg/kg) in diaphragm and skeletal muscle of 14 week old male WKY, L-NAME pretreated hypertensive WKY [WKY (L-NA)] rats and SHR strains. *$p < 0.05$ and **$p < 0.01$ compared to vehicle treatment in the same group and $^{\dagger}p < 0.05$ compared to L-serine treatment in the same group.

Table 5. Changes in regional blood flow (ml/min/g) in different organs/tissues after acute infusion of L-serine (1 mmol/kg, i.v.) in 14 week old male normotensive WKY, L-NAME pretreated WKY (0.7 mg/ml L-NAME in drinking water for 5 to 7 days) and SHR strains.

Tissues	**Regional blood flow (ml/min/g)**					
	WKY (Control)		**WKY (L-NAME treated)**		**SHR**	
	(Vehicle)	**(L-serine)**	**(Vehicle)**	**(L-serine)**	**(Vehicle)**	**(L-serine)**
Heart	3.58 ±0.13	4.99±0.07*	4.23±0.05	6.99±0.12**	5.35±0.14††	6.47±0.19*
L. kidney	5.60±0.22	6.22±0.20	3.97±0.14†	5.16±0.18*	2.89±0.20††	5.21±0.19**
R. kidney	5.81±0.19	6.59±0.28	4.13±0.15†	5.29±0.20*	2.99±0.13††	5.45±0.18**
Liver	0.42±0.06	0.48±0.07	0.34±0.06	0.69±0.08*	0.30±0.05	0.78±0.07*
Spleen	1.45±0.13	1.86±0.25	0.78±0.10††	1.99±0.12**	0.69±0.11††	1.91±0.18**
Brain	0.98±0.08	0.94±0.09	1.09±0.12	1.12±0.13	1.38±0.14†	1.75±0.15*
Lungs	0.88±0.07	0.96±0.06	0.72±0.12	0.80±0.11	0.64±0.12	0.80±0.16
Stomach	0.95±0.14	0.89±0.12	0.76±0.15	0.85±0.18	0.62±0.13	0.72±0.17
S. intestine	1.35±0.18	2.35±0.17*	0.79±0.09†	3.89±0.15***	0.84±0.15†	2.89±0.32**
L. intestine	1.40±0.22	1.51±0.24	0.88±0.11	1.63±0.28*	0.66±0.10†	1.48±0.23*
Diaphragm	1.42±0.25	1.89±0.28	1.41±0.18	2.97±0.24**	1.14±0.15	2.78±0.26*
Pancreas	1.19±0.19	1.22±0.19	0.97±0.16	1.04±0.18	0.63±0.12†	1.39±0.13*

Data reported as mean ± SEM for 5 to 7 rats/group.

*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to respective control group.

$^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ compared to control group.

Table 6. Changes in regional blood flow (ml/min/g) in different organs/tissues after acute infusion of ACh (10 nmol/kg, i.v.) in 14 week old male normotensive WKY, L-NAME pretreated WKY (0.7 mg/ml L-NAME in drinking water for 5 to 7 days) and SHR strains.

Tissues	**Regional blood flow (ml/min/g)**					
	WKY (Control)		**WKY (L-NAME treated)**		**SHR**	
	(Vehicle)	**(ACh)**	**(Vehicle)**	**(ACh)**	**(Vehicle)**	**(ACh)**
Heart	3.58±0.19	5.26±0.17*	4.23±0.14†	4.71±0.16	5.35±0.28††	6.09±0.29
L. kidney	5.60±0.35	7.38±0.33*	3.97±0.13†	4.07±0.11	2.89±0.17††	2.99±0.18
R. kidney	5.81±0.22	7.63±0.23*	4.13±0.09†	4.24±0.11	2.99±0.16††	3.09±0.19
Liver	0.42±0.11	0.55±0.12	0.34±0.08	0.44±0.07	0.30±0.05	0.36±0.04
Spleen	1.45±0.11	1.56±0.12	0.82±0.14†	0.86±0.14	0.64±0.06††	0.77±0.05
Brain	0.98±0.06	1.11±0.07	1.09±0.12	1.18±0.11	1.38±0.13†	1.54±0.16
Lungs	0.88±0.12	0.95±0.12	0.72±0.07	0.81±0.08	0.64±0.09	0.74±0.13
Stomach	0.95±0.13	0.91±0.12	0.76±0.15	0.54±0.14	0.62±0.09	0.45±0.10
S. intestine	1.35±0.14	1.44±0.11	0.79±0.09†	0.87±0.09	0.84±0.10†	0.96±0.09
L. intestine	1.41±0.10	1.49±0.11	0.88±0.06†	0.93±0.068	0.66±0.06†	0.71±0.08
Pancreas	1.19±0.11	1.22±0.08	0.97±0.20	0.99±0.18	0.63±0.15†	0.68±0.11
Diaphragm	1.42±0.16	1.45±0.08	1.41±0.09	1.55±0.11	1.14±0.15	1.22±0.16
Skeletal muscle	0.15±0.02	0.22±0.03	0.11±0.02	0.13±0.04	0.22±0.05	0.27±0.07

Data reported as mean ± SEM for 5 to 7 rats/group.

*$p < 0.05$ compared to respective control group.

$^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ compared to control group before ACh infusion.

Table 7. Acute L-serine (1 mmol/kg, i.v.) infusion reduced peripheral vascular resistance (mmHg/ml/min/g) in different organs/tissues in 14 week old male normotensive WKY, L-NAME pretreated WKY (0.7 mg/ml L-NAME in drinking water for 5 to 7days) and SHR strains.

Tissues	**Regional vascular resistance (mm Hg/ml/min/g)**					
	WKY (Control)		**WKY (L-NAME treated)**		**SHR**	
	(Vehicle)	**(L-serine)**	**(Vehicle)**	**(L-serine)**	**(Vehicle)**	**(L-serine)**
Heart	30±4.4	22±4.0	35±4.4	21±3.4*	32±4.0	26±3.5
L. kidney	20±2.5	17±2.4	37±2.6†	28±2.7	58±3.1††	32±3.0*
R. kidney	19±1.4	16±1.4	35±3.3†	27±3.8	57±3.5††	31±3.5*
Liver	256±16.7	220±15.9	426±16.4†	210±14.8***	563±21.4††	217±18.3***
Spleen	75±7.9	59±8.3	186±7.3††	73±6.7***	245±27.6††	87±17.2***
Brain	112±6.4	117±6.1	134±21.2	130±19.9	143±16.9	97±16.8
Lungs	125±14.5	115±12.7	201±21.7†	179±22.2	263±24.9††	211±22.1
Stomach	116±10.2	126±13.3	191±15.3†	171±15.0	272±27.4††	234±23.2
S. intestine	81±5.9	47±3.5*	184±10.3††	38±5.4***	199±17.6††	68±16.3**
L. intestine	77±9.4	72±10.2	165±12.6††	89±14.1**	256±20.1††	114±17.7**
Diaphragm	76±8.7	59±8.4	102±7.8†	49±8.1**	148±12.2††	61±16.0**
Pancreas	93±8.5	90±12.3	149±14.8†	139±15.2	268±14.1††	122±17.8**

Data reported as mean ± SEM for 5 to 7 rats/group.

*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to respective control group.

$^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ compared to control group.

Table 8. Comparison of systemic hemodynamic changes evoked by L-serine (1 mmol/kg, i.v.) in 14 week old WKY, L-NAME pretreated WKY (0.7 mg/ml L-NAME in drinking water for 5 to 7 days), and SHR strains.

Group →	**WKY Control**		**WKY (L-NAME treated)**		**SHR**	
Parameter	**Vehicle**	**L-Serine**	**Vehicle**	**L-Serine**	**Vehicle**	**L-Serine**
MAP (mmHg)	108±4	84±5*	142±7††	77±6***	166±6††	109±7**
HR (beats/min)	357±3	381±9	368±11	391±10	398±12†	425±8
CO (ml/min)	103±3	114±4*	79±5††	138±4***	84±4†	119±8**
TPR (mmHg/ml/min)	1.05±0.04	0.80±0.03*	1.79±0.02††	0.58±0.04***	1.96±0.05††	0.93±0.05**
CI (ml/min/kg)	309±7	331±9	232±6††	394±7***	274±5†	390±16**
TPRI (mmHg/ml/min/kg)	0.35±0.09	0.26±0.07*	0.62±0.05†	0.20±0.07***	0.67±0.06†	0.35±0.06**

Data reported as mean ± SEM for 5 to 7 rats/group.

*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to respective control group.

†$p < 0.05$ and ††$p < 0.01$ compared to control group.

Table 9. L-serine (1 mmol/kg) induced increase in regional blood flow (ml/min/g) in different organs/tissues decreased in presence of apamin and charybdotoxin in normotensive WKY, L-NAME pretreated WKY (0.7 mg/ml L-NAME in drinking water for 5 to 7 days) and SHR strains

Tissues	**Regional blood flow (ml/min/g)**								
	WKY (Control) (Vehicle)	WKY (Control) (L-serine)	WKY (Control) (L-s-Apa+ChTX)	WKY (L-NAME) (Vehicle)	WKY (L-NAME) (L-s)	WKY (L-NAME) (L-s-Apa+ChTX)	SHR (Vehicle)	SHR (L-s)	SHR (L-s-Apa+ChTX)
Heart	3.79±0.19	5.09±0.25*	4.38±0.24†	4.47±0.19	6.69±0.36*	5.12±0.29†	5.55±0.38	6.71±0.41	5.88±0.37
L. kidney	5.32±0.31	5.88±0.29	5.66±0.31	3.55±0.14	4.75±0.25*	3.97±0.17	3.02±0.29	4.97±0.28*	4.04±0.31
R. kidney	5.25±0.32	5.79±0.27	5.54±0.36	3.56±0.18	4.84±0.23*	3.89±0.21	2.96±0.20	5.02±0.34*	4.10±0.37
Liver	0.55±0.05	0.64±0.07	0.59±0.03	0.48±0.03	0.79±0.09*	0.55±0.05	0.41±0.04	0.77±0.07*	0.61±0.07
Spleen	1.3±0.10	1.63±0.11	1.47±0.17	0.62±0.06	1.76±0.18**	0.98±0.11†	0.59±0.07	1.78±0.21**	1.03±0.17†
Brain	1.03±0.11	1.05±0.18	0.98±0.08	1.14±0.14	1.17±0.12	1.18±0.10	1.28±0.18	1.57±0.22	1.39±0.15
Lungs	0.69±0.04	0.77±0.11	0.71±0.07	0.61±0.03	0.88±0.07	0.85±0.05	0.51±0.05	0.87±0.10*	0.63±0.07
Stomach	1.01±0.14	1.21±0.11	1.16±0.18	0.80±0.15	0.93±0.17	0.86±0.16	0.78±0.06	0.95±0.11	0.84±0.09
S. intestine	1.56±0.11	2.37±0.19*	1.68±0.13†	1.10±0.24	3.72±0.39**	2.12±0.28†	0.95±0.18	3.01±0.27**	1.97±0.21†
L. intestine	1.35±0.34	1.64±0.28	1.42±0.17	0.99±0.11	1.67±0.18*	1.42±0.21	0.83±0.15	1.70±0.21*	1.18±0.17†
Pancreas	1.04±0.09	1.21±0.16	1.11 ±0.12	0.89±0.14	1.10±0.21	1.04±0.19	0.67±0.08	1.31±0.17**	1.19±0.14
Diaphragm	1.28±0.21	1.63±0.33	1.44±0.27	1.39±0.12	2.57±0.28**	1.65±0.14†	1.22±0.19	2.64±0.22**	1.77±0.17†
Skeletal muscle	0.19±0.05	0.23±0.08	0.21±0.07	0.09±0.03	0.17±0.05*	0.12±0.04	0.27±0.03	0.46±0.05*	0.35±0.04

Data reported as mean ± SEM for 3 to 4 rats/group.

*$p < 0.05$, and **$p < 0.01$ compared to vehicle infusion in the same group.

$^{\dagger}p < 0.05$ compared to L-serine treatment in the same group of rats.

Table 10. Percentage increase or decrease in regional blood flow to acute L-serine (1 mmol/kg, i.v.) infusion in 14 week old male normotensive control WKY rats.

Tissues	Regional blood flow (ml/min/g)		
	WKY (Control) (Vehicle)	**WKY (Control) (L-serine)**	**% (Increase) ↑**
Heart	3.58±0.13	4.99±0.07*	39 ↑
L. kidney	5.60±0.22	6.22±0.20	11 ↑
R. kidney	5.81±0.19	6.59±0.28	13 ↑
Liver	0.42±0.06	0.48±0.07	14 ↑
Spleen	1.45±0.13	1.86±0.25	28 ↑
Brain	0.98±0.08	0.94±0.09	- 4 ↓
Lungs	0.88±0.07	0.96±0.06	9 ↑
Stomach	0.95±0.14	0.89±0.12	- 6 ↓
S. intestine	1.35±0.18	2.35±0.17*	74 ↑
L. intestine	1.40±0.22	1.51±0.24	8 ↑
Diaphragm	1.42±0.25	1.89±0.28	33 ↑
Pancreas	1.19±0.19	1.22±0.19	3 ↑

Data reported as mean ± SEM for 5 to 7 rats/group.

*$p < 0.05$ compared to vehicle treatment in the same group of rats.

Table 11. Percentage increase or decrease in regional blood flow to acute L-serine (1 mmol/kg, i.v.) infusion in 14 week old male L-NAME pretreated WKY rats (0.7 mg/ml L-NAME in drinking water for 5 to 7 days).

Tissues	Regional blood flow (ml/min/g)		
	WKY (L-NAME) (Vehicle)	WKY (L-NAME) (L-serine)	% (Increase) ↑
Heart	4.23±0.05	6.99±0.12**	65 ↑
L. kidney	3.97±0.14	5.16±0.18*	30 ↑
R. kidney	4.13±0.15	5.29±0.20*	28 ↑
Liver	0.34±0.06	0.69±0.08*	103 ↑
Spleen	0.78±0.10	1.99±0.12**	155 ↑
Brain	1.09±0.12	1.12±0.13	3 ↑
Lungs	0.72±0.12	0.80±0.11	11 ↑
Stomach	0.76±0.15	0.85±0.18	12 ↑
S. intestine	0.79±0.09	3.89±0.15***	392 ↑
L. intestine	0.88±0.11	1.63±0.28*	85 ↑
Diaphragm	1.41±0.18	2.97±0.24*	111 ↑
Pancreas	0.97±0.16	1.04±0.18	7 ↑

Data reported as mean ± SEM for 5 to 7 rats/group.

*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to vehicle infusion in the same group.

Table 12. Percentage increase or decrease in regional blood flow to acute L-serine (1 mmol/kg, i.v.) infusion in 14 week old male SHR strains.

Tissues	Regional blood flow (ml/min/g)		
	SHR (Vehicle)	SHR (L-serine)	% (Increase) ↑
Heart	5.35±0.14	6.47±0.19*	21 ↑
L. kidney	2.89±0.20	5.21±0.19**	80 ↑
R. kidney	2.99±0.13	5.45±0.18**	82 ↑
Liver	0.30±0.05	0.78±0.07*	160 ↑
Spleen	0.69±0.11	1.91±0.18**	177 ↑
Brain	1.38±0.14	1.75±0.15*	27 ↑
Lungs	0.64±0.12	0.80±0.16	25 ↑
Stomach	0.62±0.13	0.72±0.17	16 ↑
S. intestine	0.84±0.15	2.89±0.32**	265 ↑
L. intestine	0.66±0.10	1.48±0.23*	124 ↑
Diaphragm	1.14±0.15	2.78±0.26*	143 ↑
Pancreas	0.63±0.12	1.39±0.13*	121 ↑

Data reported as mean ± SEM for 5 to 7 rats/group.

*$p < 0.05$ and **$p < 0.01$ compared to vehicle infusion in the same group.

Table 13. Percentage decrease or increase in regional organ vascular resistance to acute L-serine (1 mmol/kg, i.v.) infusion in 14 week old male normotensive control WKY rats.

Tissues	**Regional vascular resistance (mmHg/ml/min/g)**		
	WKY (Control) (Vehicle)	**WKY (Control) (L-serine)**	**% (Decrease) ↓**
Heart	30±4.4	22±4.0	27 ↓
L. kidney	20±2.5	17±2.4	15 ↓
R. kidney	19±1.4	16±1.4	16 ↓
Liver	256±16.7	220±15.9	14 ↓
Spleen	75±7.9	59±8.3	21 ↓
Brain	112±6.4	117±6.1	-4 ↑
Lungs	125±14.5	115±12.7	8 ↓
Stomach	116±10.2	126±13.3	-8 ↑
S. intestine	81±5.9	47±3.5*	42 ↓
L. intestine	77±9.4	72±10.2	6 ↓
Diaphragm	76±8.7	59±8.4	22 ↓
Pancreas	93±8.5	90±12.3	3 ↓

Data reported as mean ± SEM for 5 to 7 rats/group.

*$p < 0.05$ compared to vehicle treatment in the same group of rats.

Table 14. Percentage decrease or increase in regional organ vascular resistance to acute L-serine (1 mmol/kg, i.v.) infusion in 14 week old male L-NAME pretreated WKY rats (0.7 mg/ml L-NAME in drinking water for 5 to 7 days).

Tissues	Regional vascular resistance (mmHg/ml/min/g)		
	WKY (L-NAME) (Vehicle)	WKY (L-NAME) (L-serine)	% (Decrease) ↓
Heart	35±4.4	21±3.4*	40 ↓
L. kidney	37±2.6	28±2.7	24 ↓
R. kidney	35±3.3	27±3.8	23 ↓
Liver	426±16.4	210±14.8***	51 ↓
Spleen	186±7.3	73±6.7***	61 ↓
Brain	134±21.2	130±19.9	3 ↓
Lungs	201±21.7	179±22.2	11 ↓
Stomach	191±15.3	171±15.0	10 ↓
S. intestine	184±10.3	38±5.4***	79 ↓
L. intestine	165±12.6	89±14.1**	46 ↓
Diaphragm	102±7.8	49±8.1**	52 ↓
Pancreas	149±14.8	139±15.2	7 ↓

Data reported as mean ± SEM for 5 to 7 rats/group.

*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to vehicle infusion in the same group.

Table 15. Percentage decrease or increase in regional organ vascular resistance to acute L-serine (1 mmol/kg, i.v.) infusion in 14 week old male SHR strains.

Tissues	Regional vascular resistance (mmHg/ml/min/g)		
	SHR (Vehicle)	SHR (L-serine)	% (Decrease) ↓
Heart	32±4.0	26±3.5	19 ↓
L. kidney	58±3.1	32±3.0*	45 ↓
R. kidney	57±3.5	31±3.5*	46 ↓
Liver	563±21.4	217±18.3***	61 ↓
Spleen	245±27.6	87±17.2***	64 ↓
Brain	143±16.9	97±16.8	32 ↓
Lungs	263±24.9	211±22.1	19 ↓
Stomach	272±27.4	234±23.2	13 ↓
S. intestine	199±7.6	68±6.3**	70 ↓
L. intestine	256±20.1	114±17.7**	55 ↓
Diaphragm	148±12.2	61±16.0**	58 ↓
Pancreas	268±14.1	122±17.8**	54 ↓

Data reported as mean ± SEM for 5 to 7 rats/group.

*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to vehicle infusion in the same group.

Table 16. Percentage changes in MAP (mmHg), HR (beats/min), CO (ml/min) and TPR (mmHg/ml/min), CI (ml/min/kg) and TPRI (mmHg/ml/min/kg) to L-serine (1 mmol/kg, i.v.) infusion in 14 week old normotensive control WKY rats.

Group	**WKY (Control)**		
Parameter	**Vehicle**	**L-serine**	**(% Decrease/Increase) ↓↑**
MAP (mmHg)	108±4	84±5*	22 ↓
HR (beats/min)	357±3	381±9	6 ↑
CO (ml/min)	103±3	114±4*	11 ↑
TPR (mmHg/ml/min)	1.05±0.04	0.80±0.03*	24 ↓
CI (ml/min/kg)	309±7	331±9	7 ↑
TPRI (mmHg/ml/min/kg)	0.35±0.09	0.26±0.07*	26 ↓

Data reported as mean ± SEM for 5 to 7 rats/group.

*$p < 0.05$ compared to vehicle infusion in the same group.

Table 17. Percentage changes in MAP (mmHg), HR (beats/min), CO (ml/min), TPR (mmHg/ml/min), CI (ml/min/kg) and TPRI (mmHg/ml/min/kg) to L-serine (1 mmol/kg, i.v.) infusion in 14 week old male L-NAME pretreated WKY rats (0.7 mg/ml L-NAME in drinking water for 5 to 7 days).

Group	**WKY (Chronic L-NAME)**		
Parameter	**Vehicle**	**L-serine**	**(% Decrease/Increase) ↓↑**
MAP (mmHg)	142±7	77±6***	46 ↓
HR (beats/min)	368±11	399±10	8 ↑
CO (ml/min)	79±5	138±4***	75 ↑
TPR (mmHg/ml/min)	1.79±0.02	0.58±0.04***	68 ↓
CI (ml/min/kg)	232±6	394±7***	70 ↑
TPRI (mmHg/ml/min/kg)	0.62±0.05	0.20±0.07***	67 ↓

Data reported as mean ± SEM for 5 to 7 rats/group.

***$p < 0.001$ compared to vehicle infusion in the same group.

Table 18. Percentage changes in MAP (mmHg), HR (beats/min), CO (ml/min), TPR (mmHg/ml/min), CI (ml/min/kg) and TPRI (mmHg/ml/min/kg) to L-serine (1 mmol/kg, i.v.) infusion in 14 week old male SHR strains.

Group	**SHR**		
Parameter	**Vehicle**	**L-serine**	**(% Decrease/Increase) ↓↑**
MAP (mmHg)	166±6	109±7**	34 ↓
HR (beats/min)	398±12	425±8	7 ↑
CO (ml/min)	84±4	119±8**	41 ↑
TPR (mmHg/ml/min)	1.96±0.05	0.93±0.05**	53 ↓
CI (ml/min/kg)	274±5	390±16**	42 ↑
TPRI (mmHg/ml/min/kg)	0.67±0.06	0.35±0.06**	48 ↓

Data reported as mean ± SEM for 5 to 7 rats/group.

**$p < 0.01$ compared to vehicle infusion in the same group.

Table 19. Comparison of increase in regional blood flow to various tissues after acute L-serine (1 mmol/kg, i.v.) infusion and L-serine infusion in presence of apamin and charybdotoxin in 14 week old male normotensive control WKY rats.

Tissues	**Regional blood flow (ml/min/g)**				
	WKY Control (Vehicle)	WKY Control (L-serine)	% ↑	WKY Control (L-serine-Apa+ChTX)	% ↑
Heart	3.79±0.19	5.09±0.25*	34	4.38±0.24	16
L. kidney	5.32±0.31	5.88±0.29	11	5.66±0.31	6
R. kidney	5.25±0.32	5.79±0.27	10	5.54±0.36	6
Liver	0.55±0.05	0.64±0.07	16	0.59±0.03	7
Spleen	1.31±0.10	1.63±0.11	24	1.47±0.17	12
Brain	1.03±0.11	1.05±0.18	2	0.98±0.08	-5
Lungs	0.69±0.04	0.77±0.11	12	0.71±0.07	3
Stomach	1.01±0.14	1.21±0.11	20	1.16±0.18	15
S. intestine	1.56±0.11	2.37±0.19*	52	1.68±0.13†	8
L. intestine	1.35±0.34	1.64±0.28	21	1.42±0.17	5
Pancreas	1.04±0.09	1.21±0.16	16	1.11±0.12	7
Diaphragm	1.28±0.21	1.63±0.33	27	1.44±0.27	13
Skeletal muscle	0.19±0.05	0.23±0.08	21	0.21±0.07	11

Data reported as mean ± SEM for 3 to 4 rats/group.

*$p < 0.05$ compared to vehicle infusion in the same group.

$^{\dagger}p < 0.05$ compared to L-serine treatment in the same group of rats.

Table 20. Comparison of increase in regional blood flow to various tissues after acute L-serine (1 mmol/kg, i.v.) infusion and L-serine infusion in presence of apamin and charybdotoxin in 14 week old male L-NAME pretreated hypertensive WKY rats (0.7 g/ml L-NAME in drinking water for 5 to 7days).

Tissues	**Regional blood flow (ml/min/g)**				
	WKY (L-NAME) (Vehicle)	WKY (L-NAME) (L-serine)	% ↑	WKY (L-NAME) (L-serine-Apa+ChTX)	% ↑
Heart	4.47±0.19	6.69±0.36*	50	5.12±0.29†	15
L. kidney	3.55±0.14	4.75±0.25*	34	3.97±0.17	12
R. kidney	3.56±0.18	4.84±0.23*	36	3.89±0.21	9
Liver	0.48±0.03	0.79±0.09*	65	0.55±0.05	15
Spleen	0.62±0.06	1.76±0.18**	184	0.98±0.11†	58
Brain	1.14±0.14	1.17±0.12	3	1.18±0.10	4
Lungs	0.61±0.03	0.88±0.07	44	0.85±0.05	39
Stomach	0.80±0.15	0.93±0.17	16	0.86±0.16	6
S. intestine	1.10±0.24	3.72±0.39**	238	2.12±0.28†	93
L. intestine	0.99±0.11	1.67±0.18*	69	1.42±0.21	43
Pancreas	0.89±0.14	1.10±0.21	24	1.04±0.19	17
Diaphragm	1.39±0.12	2.57±0.28**	85	1.65±0.14†	19
Skeletal muscle	0.09±0.03	0.17±0.05*	77	0.12±0.04†	33

Data reported as mean ± SEM for 3 to 4 rats/group.

*$p < 0.05$, and **$p < 0.01$ compared to vehicle infusion in the same group.

$^{\dagger}p < 0.05$ compared to L-serine treatment in the same group of rats.

Table 21. Comparison of increase in regional blood flow to various tissues after acute L-serine (1 mmol/kg, i.v.) infusion and L-serine infusion in presence of apamin and charybdotoxin in 14 week old age matched male SHR.

Tissues	**Regional blood flow (ml/min/g)**				
	SHR (Vehicle)	SHR (L-serine)	% ↑	SHR (L-serine-Apa+ChTX)	% ↑
Heart	5.55±0.38	6.71±0.41	21	5.88±0.37	6
L. kidney	3.02±0.29	4.97±0.28*	65	4.04±0.31	54
R. kidney	2.96±0.20	5.02±0.34*	69	4.10±0.37	55
Liver	0.41±0.04	0.77±0.07*	88	0.61±0.07	46
Spleen	0.59±0.07	1.78±0.21**	201	1.03±0.17†	88
Brain	1.28±0.18	1.57±0.22	23	1.39±0.15	9
Lungs	0.51±0.05	0.87±0.10*	71	0.63±0.07	24
Stomach	0.78±0.06	0.95±0.11	22	0.84±0.09	8
S. intestine	0.95±0.18	3.01±0.27**	216	1.97±0.21†	107
L. intestine	0.83±0.15	1.70±0.21*	105	1.18±0.17†	42
Pancreas	0.67±0.08	1.31±0.17**	96	1.19±0.14	78
Diaphragm	1.22±0.19	2.64±0.22**	99	1.77±0.17†	48
Skeletal muscle	0.27±0.03	0.46±0.05*	68	0.35±0.04	30

Data reported as mean ± SEM for 2 to 3 rats/group.

*$p < 0.05$ and **$p < 0.01$ compared to vehicle infusion in the same group.

$^{\dagger}p < 0.05$ compared to L-serine treatment the same group of rats.

CHAPTER 5. DISCUSSION

5.1. Vasodilator Properties of L-serine

The present study demonstrates the vasodilator effect of L-serine (10 to 200 μmol/L) in rat mesenteric arteriole. L-serine evoked vasodilator effect is more pronounced in NO compromised state. L-serine evoked vasodilatation was concentration-dependent and it was observed only in endothelium-intact preparations. The maximal vasodilator responses to L-serine was observed in mesenteric arteriole isolated from L-NAME pretreated hypertensive rats compared to normotensive control rats (40 ± 3% vs. 20 ± 3%). The vasodilator responses to L-serine were similar when vessels were preconstricted with PE, or the thromboxane analog, U46619. The vasodilator responses to L-serine were insensitive to blockade by either NOS inhibitor, L-NAME, or the COX inhibitor, indomethacin. Pretreatment with either a combination of apamin plus TRAM-34 or ouabain plus Ba^{2+} significantly blocked the vasodilator responses to L-serine in these arterioles. L-serine mediated vasodilator effect was absent when mesenteric arterioles were depolarized with KCl (80 mmol/L). In PE constricted mesenteric arterioles, addition of KCl (9.8 to 18.8 mmol/L) evoked a vasodilator responses, this effect was abolished in vessels pretreated with a combination of ouabain plus Ba^{2+} but not in the combine presence of apamin plus TRAM-34. In this preparation, addition of L-serine failed to increase further the vasodilator responses to KCl. From this data it is evident that, L-serine promotes K^+ efflux by activating SK_{Ca} and IK_{Ca} channels present in the endothelium. The

efflux of K^+ from endothelium hyperpolarized the VSMC via activation of the Na^+ pump and subsequent efflux of K^+ through K_{ir} channels.

5.2. BP Lowering Effect of L-serine

Our *in vitro* observations were extended to explore the potential *in vivo* effects of L-serine. Acute L-serine infusion in normotensive and L-NAME pretreated hypertensive rats lowers BP with minimal increase in HR. The BP lowering effect of L-serine was dose dependent and more pronounced in L-NAME pretreated hypertensive rats compared to normotensive control rats. Pretreatment with combination of apamin (SK_{Ca} inhibitor) and ChTX (IK_{Ca} inhibitor) significantly abolished the BP lowering effect of L-serine (0.1 to 2.0 mmol/kg). L-serine infusion at 3.0 mmol/kg concentration elicited a profound fall in MAP which was >70 mmHg in L-NAME pretreated hypertensive rats and this effect was inhibited significantly but not completely blocked by apamin and ChTX. This effect suggests the involvement of additional mechanisms in reduction of MAP in L-NAME pretreated hypertensive rat models. L-serine evoked a fall in BP that was insensitive to indomethacin and acute L-NAME pretreatment. This observation confirms that the L-serine evoked fall in BP was NO- and COX-independent. Taken together all these data favor the conclusion that L-serine likely promotes selective activation of SK_{Ca} and IK_{Ca} channels present on the endothelium. The fall in MAP observed was lower in normotensive control rats (E_{max} > 25 mmHg) than in L-NAME pretreated hypertensive rats (E_{max} > 60 mmHg). Thus, L-serine mediated reduction in MAP was more profound in L-NAME pretreated hypertensive rats where NO production was compromised due to NOS

inhibition. Thus, when the NO system is blunted, the EDHF mechanism is overactive to compensate endothelium-dependent vasodilatation (Desai et al., 2006; Bellien et al., 2006).

5.2.1. NOS Inhibition Exaggerated L-serine Mediated Effect

EDHF mediated endothelium dependent vasodilatation is lower when NO system is active (Bauersachs et al., 1996; Busse et al., 2002). Some earlier reports suggested that L-serine modulates endothelium derived NO generation by increasing the efflux of L-arginine which serves as a precursor for NO (Kakoki et al., 2006). L-serine mediated vasodilatation in isolated mesenteric arterioles was inhibited by pretreatment with apamin and TRAM-34 combination in L-NAME pretreated hypertensive rats. Similarly, the BP lowering effect of L-serine was also attenuated by pretreatments with apamin and ChTX combination. L-serine did not evoke a vasodilator response in arterioles isolated from L-NAME pretreated hypertensive rat pretreated with either a combination of ouabain and Ba^{2+} or depolarized with KCl. These results suggest that L-serine increases K^+ conductance in myendothelial junctions by activating SK_{Ca} and IK_{Ca} channels present on the endothelium and the increase in K^+ conductance activates Na^+/K^+ ATPase in the adjacent VSMC. The subsequent efflux of K^+ through K_{ir} channels causes hyperpolariztion of the adjacent VSMC and contributes to the vasodilatation observed in resistant type mesenteric arterioles (Edwards et al., 1998). L-serine evoked profound fall in MAP in L-NAME pretreated hypertensive rats were not related to its elevated MAP since the fall in MAP in SHR was lower irrespective of its higher MAP compared to L-NAME pretreated hypertensive rats. From *in vitro* and *in vivo* data, it is evident that acute L-serine infusion evoked a dose dependent vasodilatation and

fall in MAP which was higher in L-NAME pretreated hypertensive rats when NO path way is compromised. Several candidates are suggested to play a role as mediator of EDHF in different vascular beds. They are K^+, EET, H_2O_2, CNP and MEGJ which are proposed to produce endothelium dependent vasodilatation. Their responses were resistant to inhibitors of COX and NOS (Edwards et al., 1998; Busse et al., 2002; Chauhan et al., 2003; Feletou et al., 2006). Although in NO compromised state EDHF plays a compensatory role and contributes to agonist mediated vasodilatation it does not appear to contribute to the maintenance of basal vascular tone (Desai et al., 2006; Parkington et al., 2002).

5.3. BP Lowering Effects of L-serine Compared to its Stereoisomer D-serine

L-serine is converted to D-serine *in vivo* (de Koning et al., 2003 and 2004). It is possible D-serine could contribute to the antihypertensive effect of L-serine. To address this issue, we compared the fall in MAP evoked by L-serine and its stereoisomer, D-serine, in normotensive WKY, L-NAME pretreated hypertensive and SHR strains. Data from the present study shows that acute infusion of D-serine also produced a reversible, dose dependent fall in MAP like L-serine without a significant increase in HR in all strains. However, the D-serine evoked fall in MAP was much lower compared to L-serine in all these rat models. This data is consistent with our *in vitro* data that L-serine evoked vasodilatation and BP lowering effect is more pronounced in NO compromised state. BP lowering effect of D-and L-serine was significantly inhibited by pretreatment with a combination of SK_{Ca} and IK_{Ca} channel inhibitors (apamin and ChTX) both in L-NAME pretreated hypertensive WKY and SHR strains. The degree of inhibition observed with L-

serine was higher compared to D-serine in SHR rats (Figure 11 b). These results are similar and consistent with the results obtained using age matched L-NAME pretreated hypertensive Sprague-Dalwey rats.

5.4. Comparison of L-serine and Glycine Effect in Blood Pressure Regulation

To maintain intracellular concentration of one carbon groups in different cellular compartments, reversible interconversion between of L-serine to glycine and glycine to L-serine occurs in eukaryotes (Appling et al., 1991). Studies have shown that glycine decreases blood pressure, fatty acids and rectifies vascular dysfunction by reducing oxidative stress (Matilla et al., 2002; Brawley et al., 2003; Hafidi et al., 2004). Therefore, it was important to examine and compare the responses to glycine along with D-and L-serine in the same rat models. In normotensive WKY rats, comparable doses of acute glycine infusion elicited a dose dependent fall in MAP. However, the degree of fall was comparable to D-serine but lower than L-serine. In L-NAME pretreated hypertensive WKY rats and SHR strains acute glycine infusion dose dependently elevated the MAP and the degree of elevation was higher in L-NAME pretreated hypertensive WKY rats compared to SHR strains. In the same L-NAME pretreated hypertensive WKY and SHR strains, L-serine evoked a dose dependent fall in MAP. Pretreatment with selective NMDA receptor antagonist, MK-801, significantly inhibited the glycine evoked fall in MAP in normotensive rats and elevated MAP in hypertensive rats. It failed to affect the hypotensive responses to L-serine. Either the elevation or the fall in MAP evoked by glycine was absent in WKY rats subjected to acute L-NAME infusion. These data suggest that glycine evokes a pressor response in rats that are hypertensive subsequent to either

chronic L-NAME treatment or in genetic model of SHR with prolonged phase of hypertension but not following the rapid elevation in MAP attained after acute infusion of L-NAME.

5.4.1. Glycine but not L-serine Elevates MAP in Hypertensive Rat Models

Glycine is proposed to mediate NO-dependent vasodilatation via activation of NMDA receptors. NMDA receptor activation promotes Ca^{2+} influx that results in increased NOS activity. This has been demonstrated in kidney and brain (Deng et al., 2002; Leung et al., 2002). NMDA receptor antagonist or NOS inhibitor significantly inhibited the vasodilatation and increase in glomerular and tubular filtration evoked by glycine (Deng et al., 2002; Slamowitz et al., 2004; Qui et al., 1995; De Nicola et al., 1992). Some studies have also reported that glycine mediated reduction in BP may be due to hyperpolarization of endothelial cells via activation of chloride channels (Wheeler et al., 2000). The dose-dependent fall in MAP following acute glycine (0.3 to 3.0 mmol/kg) infusion seen in the present study in normotensive WKY rats appears to be mediated by NO dependent NMDA receptor activation as reported earlier (Deng et al., 2002). This depressor response was blocked by pretreatment with either the NMDA receptor antagonist, MK-801, or NOS inhibitor L-NAME. Infusion of glycine in the same dose range as L-serine evoked a rapid reversible increase in MAP which was higher in L-NAME treated hypertensive WKY rats compared to SHR. Despite progressive endothelial dysfunction in SHR, NO mediated response is not inhibited completely whereas in chronic L-NAME treated hypertensive rats, NO mediated responses are completely inhibited. Thus, glycine mediated elevation in MAP was lower in SHR compared to L-NAME treated hypertensive rats. Pretreatment

with NMDA receptor antagonist, MK-801, abolished the pressor response seen in both L-NAME treated hypertensive and SHR models. The present study shows that glycine evoked a depressor response in normotensive and pressor response in hypertensive rat models. Both depressor and pressor response were sensitive to blockade by NMDA receptor antagonist, MK-801, or the NOS inhibitor L-NAME. It has been reported that glycine mediated contractile response in isolated rat pial arteriole was sensitive to NMDA receptor antagonist (Huang et al., 1994). In contrast to glycine, L-serine produced a dose dependent fall in MAP both in normotensive and hypertensive rat models. This depressor response to L-serine was higher and more profound in hypertensive rat models whereas glycine elicited pressor response, which was opposite to L-serine evoked depressor response. L-serine evoked profound fall in MAP was not sensitive to either NMDA receptor antagonist, MK-801, or NOS inhibitor L-NAME. Thus, these data confirm that glycine and L-serine regulate BP differently by different mechanisms in normotensive and hypertensive rat models.

5.5. Effect of L-threonine in Blood Pressure Regulation

L-threonine serves as a precursor to L-serine and it also promotes the subsequent formation of D-serine, glycine and other metabolites of L-serine. SHMT and L-threonine aldolase plays a role in the biosynthesis of L-serine *in vivo*. Studies show that dietary administration of L-threonine in rat models over a period of six weeks elevates systolic BP via an increase in acetaldehyde formation (Vasdev et al., 1995). In the present study comparable doses of acute L-threonine infusion did not affect either MAP or HR in all the rat models. Based on these data, it is reasonable to conclude that L-serine mediated BP

lowering effect is profound in hypertensive rat models. Compared to D-serine, L-serine is more potent and efficacious. Acute L-threonine infusion has no effect on MAP or HR of any group of rat models. Therefore, L-serine mediated response is unique, stereo-selective, not mimicked or shared by its precursor L-threonine, metabolites, D-serine and glycine.

5.6. Comparison of Antihypertensive Effect of L-serine in Hypertensive Rat Models

In the present study, L-serine (0.1 to 3.0 mmol/kg) infusion evoked a dose dependent hypotensive response in normotensive and two well established hypertensive rat models. The hypotensive response elicited by L-serine was higher and more pronounced in hypertensive rat models where NO pathway was compromised. The dose of L-serine used in these studies was higher than the physiological plasma L-serine concentration which is 130 ± 30 μmol (de Koning et al., 2004). L-serine evoked profound fall in MAP was more pronounced in L-NAME pretreated hypertensive rats (≥ 93 mmHg) compared to SHR (≥ 80 mmHg). L-serine evoked fall in MAP was associated with a marginal increase in HR that did not attain statistical significance. The higher initial baseline MAP in hypertensive rats was unrelated to the fall in MAP evoked by L-serine compared to normotensive control rats, since in SHR strains L-serine (3.0 mmol/kg) evoked fall in MAP was lower compared to L-NAME pretreated hypertensive rat model despite higher initial baseline MAP.

5.7. Regional and Systemic Hemodynamic Assessment to L-serine

L-serine infusion evoked significant changes in CO, CI, MAP, TPR and TPRI by increasing regional blood flow to various tissues, which was greater in hypertensive rats.

5.7.1. L-serine Targets Multiple Vascular Beds in Hypertensive Rats

Acute L-serine infusion evoked a rapid fall in MAP in normotensive WKY (24 mmHg), L-NAME treated WKY (65 mmHg) and SHR (57 mmHg) strains. Changes in HR attained were not statistically significant although the increase in HR was noticed in both normotensive as well as hypertensive rat models (Table 8). L-serine infusion led to a significant increase in blood flow to several organs that includes small intestine, spleen, diaphragm, skeletal muscle, large intestine, liver, kidney, heart and pancreas. L-serine evoked changes in blood flow to brain, lungs and stomach in both SHR and L-NAME treated rats were minimal or negligible. In contrast, in normotensive rats, L-serine induced increase in blood flow was significant only in small intestine (Table 5). The degree of increase in blood flow to several organs was significantly different between L-NAME treated hypertensive WKY and SHR. L-serine induced blood flow was higher in organs of hypertensive rat models compared to normotensive rats. The most common organ targeted by L-serine in all animal models was small intestine. The increase in blood flow occurred in the following rank order amongst the strains studied: small intestine of WKY (74%, $p < 0.05$), L-NAME treated hypertensive rat (392%, $p < 0.001$), SHR (265%, $p < 0.01$). Peripheral organ vascular resistance was higher in both hypertensive rat models (Table 7). Data collected from several experiments revealed that like regional blood flow, L-serine mediated decrease in organ vascular resistance in several organs were significantly different between L-NAME treated hypertensive WKY and SHR strains. In normotensive WKY rats, L-serine mediated decrease in vascular resistance was observed only in small intestine (42%, $p < 0.05$) which was lower compared to L-NAME pretreated hypertensive WKY (79%, $p < 0.001$) and SHR strains (67%, $p < 0.01$). L-serine infusion significantly

reduced the elevated vascular resistance in several tissues, small intestine, spleen, liver, large intestine, diaphragm, skeletal muscle and heart in L-NAME treated hypertensive WKY rat models. There were no significant reduction in vascular resistance in stomach, brain, lungs, kidney and pancreas. In SHR, acute L-serine infusion reduced vascular resistance in most organ studied but the degree of overall reduction in organ vascular resistance was lower in SHR than in L-NAME treated hypertensive rats. The present data suggests that that L-serine induced increase in blood flow and decrease in organ vascular resistance occurred in several organs and this contributes to the profound fall in MAP in hypertensive rats. L-serine mediated reduction in organ vascular resistance and increase in organ blood flow plays a critical role for its contribution to the systemic hemodynamic changes encountered in normotensive and hypertensive rat models. In L-NAME treated hypertensive rats and the SHR, the reduction in organ vascular resistance was more pronounced compared to normotensive rats. In hypertensive rat models basal MAP, TPR and TPRI were higher with reduced CO and CI (Table 8). L-serine infusion significantly reduces MAP, TPR and TPRI in both hypersensitive models with significant increase in CO and CI. This effect was more pronounced in L-NAME pretreated hypertensive WKY rats compared to SHR. L-serine infusion induced a fall in MAP, TPR and an increase in CO in all strains. However, the magnitude of fall in TPR, MAP and increase in CO encountered were significantly higher in the hypertensive rat models. Over all, the results are consistent with the suggestion that L-serine induced changes in hemodynamic effects are dramatic in NO compromised state. In both hypertensive rat models, L-serine induced profound fall in MAP. It is due to decrease in peripheral organ vascular resistance and increase in peripheral organ blood flow in multiple tissues. However, this effect is

predominant in splanchnic vascular beds. Thus, the profound fall in MAP is due to increased blood flow promoted by L-serine in the intestinal and abdominal region of hypertensive rat. Blood flow to the visceral organs is maintained by splanchnic vascular network which accounts for one fourth of the total CO (Isabelle et al., 2008; Rowell, 1973; Perko et al., 1998). L-serine induced increase in organ blood flow is sensitive to pretreatment with apamin and charybdtoxin in several tissues that includes heart, spleen, small intestine, diaphragm, and skeletal muscle in L-NAME treated hypertensive rats compared to recruitment of vascular beds of spleen, lungs, diaphragm, small and large intestine in SHR strain (Figure 28, 29, 30, 31 and 32).

5.8. Proposed Mechanisms for L-serine Mediated Vasodilatation

L-serine mediated vasodilatation is endothelium dependent and NO as well as COX independent. This response is sensitive to combined treatment with apamin and ChTX. This result suggests that L-serine promotes K^+ efflux by activating SK_{Ca} and IK_{Ca} channels present in the endothelium. The efflux of K^+ from endothelium hyperpolarizes the VSMC via activation of the Na^+ pump and subsequent efflux of K^+ through K_{ir} channels. L-serine could activate SK_{Ca} and IK_{Ca} channels present in endothelium in one of the following ways: 1) L-serine could activate its own receptor which may be present in the endothelium leading to an increase in Ca^{2+}, which then activate SK_{Ca} and IK_{Ca} channels. 2) L-serine can enter directly in to the endothelial cell via L-type amino acid transporter (LAT) and then activate SK_{Ca} and IK_{Ca} channels directly or it can activate EDHF directly which in turn

hyperpolarizes VSMC by decreasing resting membrane potential (RMP). A schematic representation of L-serine mediated vasodilatation is proposed as follows (Figure 33).

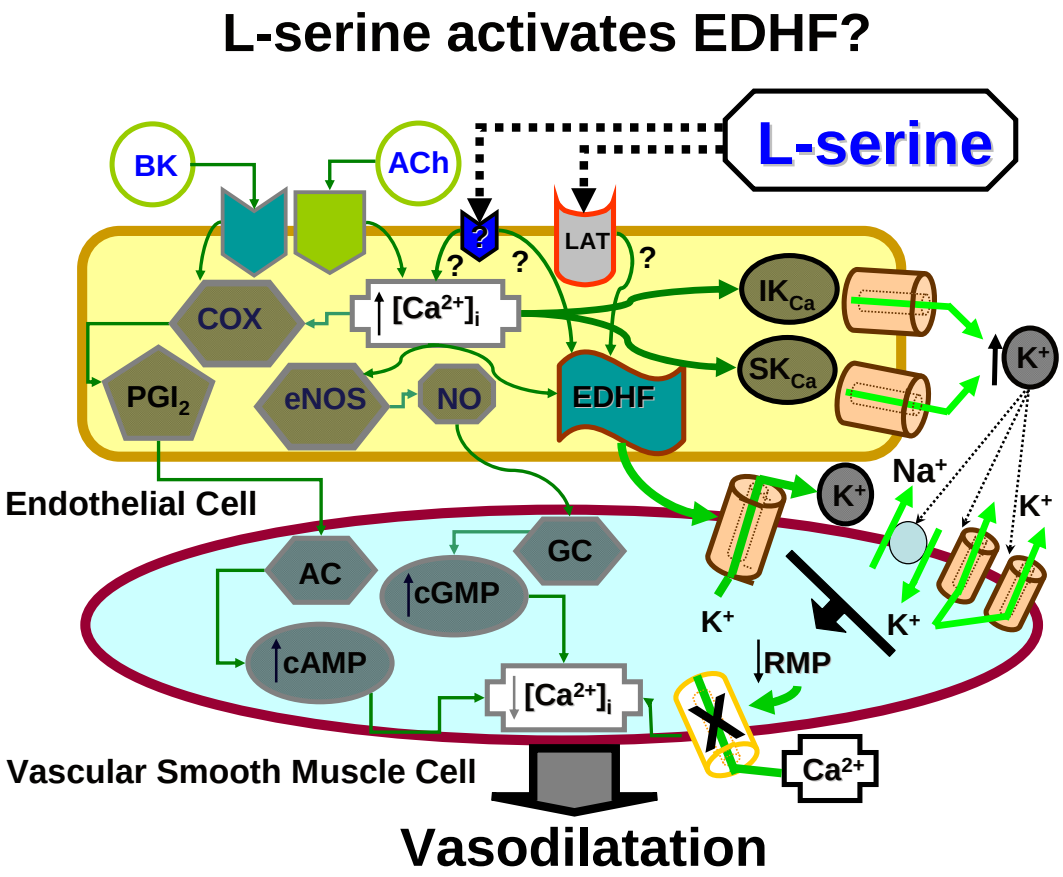

Figure 33. Possible pathways of L-serine mediated vasodilatation.

BK, bradykinin; ACh, acetylcholine; COX, cyclo-oxygenase; LAT, L-amino acid transporter; PGI_2, prostacyclin; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; EDHF, Endothelial hyperpolarizing factor; IK_{Ca}, calcium activated intermediate conductance potassium channel; SK_{Ca}, calcium activated small conductance potassium channel; K^+, potassium; Na^+, sodium; $[Ca^{2+}]_i$, intracellular calcium; AC, adenylyl cyclase; GC, guanylyl cyclase; cAMP, cyclic 3', 5' adenosine monophosphate; cGMP, cyclic 3', 5' guanosine monophosphate; RMP, resting membrane potential.

CHAPTER 6. CONCLUSIONS AND FUTURE WORK

6.1. Summary of Major Findings

The *in vitro* studies revealed that L-serine evokes concentration-dependent (10 to 200 μmol/L) vasodilatation in phenylephrine constricted endothelium-intact, but not in endothelium-denuded vessels. The vasodilator response to L-serine is unaffected by the inclusion of NOS inhibitor, L-NAME and COX inhibitor, indomethacin, but is abolished by the inclusion of a combination of apamin (SK_{Ca} channel inhibitor) and TRAM-34 (IK_{Ca} channel inhibitor) or ouabain (Na^+ pump inhibitor) and Ba^{2+} (K_{ir} channel inhibitor) when the vessels were constricted by KCl. The maximal response to L-serine was higher in the L-NAME treatment group (L-NAME 40% vs. control 20%) in contrast to the E_{max} to ACh (L-NAME 79% vs. control 93%).

The data from the *in vivo* studies are consistent with our *in vitro* observations. L-serine evokes a rapid, reversible, dose-dependent fall in MAP, which is more pronounced in L-NAME treated rats (>60 mmHg) than the control rats (25 mmHg). This was inhibited by apamin plus charybdotoxin pretreatment. D-serine evokes less vasodilatation than L-serine in normotensive rats as well as in L-NAME induced hypertensive rats, and in the SHR strain. While comparable doses of glycine also evoke a fall in MAP in normotensive rats, unlike L-serine, glycine increases MAP in both models of hypertension. The most profound elevation of MAP was observed in the L-NAME pretreated hypertensive rats. Both the hypotensive and hypertensive responses to glycine are blocked by pretreatment

with the NMDA receptor antagonist, MK-801. However, the responses to L-serine was not affected by MK-801. Thus, the BP changes evoked by glycine and L-serine are unrelated and work through different mechanisms. L-threonine, a precursor of L-serine, did not affect the MAP or HR in hypertensive rats. Based on these findings, it is reasonable to conclude that the antihypertensive effect of L-serine is not shared or mimicked by its isomer, metabolite or its precursor.

L-serine infusion promotes significant increase in blood flow and decrease in vascular resistance in several organs of hypertensive rats. This contributes to the profound BP lowering effect of acute L-serine infusion despite higher MAP and TPR observed in L-NAME pretreated hypertensive and in SHR strains. L-serine increases in blood flow predominantly in targets intestinal and splanchnic vascular beds, and this is higher in hypertensive rat models. Acute L-serine infusion led to a significant reduction in TPR, followed by substantial increase in CO which accounts for the profound fall in MAP in L-NAME pretreated hypertensive WKY and SHR strains. Apamin and ChTX pretreatment inhibited L-serine induced increase in regional blood flow to several organs in hypertensive rats with the maximal effect seen in the splanchinc region and intestinal vascular beds.

Taken together, the data from the present study suggest that the non-essential amino acid, L-serine, evokes endothelium-dependent, NO-independent, COX-independent, but apamin plus charybdotoxin sensitive, activation of endothelial SK_{Ca} and IK_{Ca} channels that lead to endothelium-dependent hyperpolarization of adjacent VSMC. This results in vasodilatation of rat mesenteric arterioles. The data from the regional hemodynamic studies confirmed

that this effect of L-serine is not wide spread but occurs mainly in the splanchnic region and more particularly in the small intestinal vascular beds. Moreover, the vasodilator responses evoked by L-serine in different vascular beds are greater in the hypertensive rat models. However, increased blood flow was seen predominantly in the splanchnic vascular beds with reduced regional vascular resistance which in turn contributes to reduced TPR and the fall in MAP, an effect that is exaggerated in hypertensive rats.

6.2. Significance and Clinical Perspective of the Work

L-serine evoked vasodilatation and BP lowering effect was more profound in the NO compromised state. Progressive endothelial dysfunction and reduced NO bioavailability with increased TPR and MAP values are the major manifestation and reflection of the underlying cardiovascular disease in condition like essential hypertension, atherosclerosis and diabetes. The present study shows that acute L-serine infusion has potential to reduce the elevated TPR and MAP with minimal increases in HR by increasing blood flow and decreasing vascular resistance in several organs of hypertensive rats. Oral treatment with L-serine (600 to 1400 mg/kg/day) has been shown to be beneficial in the management of neurological disorders and chronic fatigue syndrome without any adverse effect (Addington, 1999; Klomp et al., 2000; de Koning et al., 2004). A recent report suggested that oral supplementation of L-serine reduces high cholesterol induced fatty streak formation in hypercholesterolemic rabbits without affecting plasma lipid levels (Movahedian et al., 2006). High level of plasma homocysteine is a risk factor for cardiovascular disease since it reacts with reactive oxygen intermediates by auto-oxidation that damages endothelial cells and contributes to thrombosis (van Guldener et al., 2001).

Another study shows that elevated level of homocysteine increases bone fracture (McLean et al., 2004). Dietary supplementation of L-serine has homocysteine lowering effect in humans (Verhoef et al., 2004). These reports suggested that L-serine has some indirect cardiovascular protective effects by lowering plasma homocysteine level and fatty streak formation. The present study shows that L-serine has potential to reduce BP in hypertensive animals. Supplementation of L-serine alone or in combination with other antihypertensive agents could be considered in the management of hypertension. L-serine infusion improves blood flow and decreases vascular resistance in multiple organs. Most markedly in the splanchnic vascular beds. It has been shown that multiple organ failure occurs due to splanchnic ischemia, which reduces oxygen delivery to various tissues (Krack et al., 2005). Inadequate perfusion destroys the gastrointestinal barrier causing acidosis, which helps in releasing enteric bacterial toxins to systemic circulation, which in turn increases mortality in intensive care patients (Riddington et al., 1996; O' Dwyer et al., 1997). L-serine can be synthesized from glucose and other glycolytic intermediates (de Koning et al., 2003 and 2004). Thus, increased glucose absorption and its metabolism could elevate L-serine level in plasma, which in turn could enhance intestinal and splanchnic blood flow. Increase in perfusion to the splanchnic region provides adequate oxygen supply to various tissues and contributes to the absorption of nutrients. Thus, L-serine mediated increased blood flow in these vascular beds could play an important role not only in the absorption of nutrients but also help in the regulation of MAP and promote beneficial cardiovascular protective effect. A number of reports suggest the importance of postprandial elevation in blood flow in the intestinal and splanchnic region (Takagi et al., 1988; Eriksen et al., 1994). Therefore, L-serine could be evaluated for its therapeutic

benefit in overcoming conditions associated with reduced splanchnic perfusion in the future.

6.3. Limitations of the Study and Possible Avenues for Future Directions

i) Our *in vitro* studies show that L-serine evokes endothelium-dependent vasodilatation via activation of K_{Ca} channels on the endothelium and evokes hyperpolarization of the adjacent VSMC. It is unclear whether the L-serine effect is mediated via its putative cell surface receptor present on the EC or it occurs subsequent to L-serine movement through the neutral amino acid transporter, at the large neutral amino acid transporter, which is likely linked to activation of SK_{Ca} and IK_{Ca} channels present on the EC. Thus, cellular studies towards characterization of the signaling mechanism that would link L-serine in opening endothelial K_{Ca} channels are warranted.

ii) The present study suggests that L-serine, like ACh, bradykinin and ghrelins, promotes endothelium-dependent hyperpolarization (Edwards et al., 1998; Busse et al., 2002; Feletou and Vanhoutte, 2006; Moazed et al., 2009). While the pharmacological evidence strongly supports a role for L-serine in activating the endothelium-dependent hyperpolarization, direct membrane potential recordings have not been conducted to establish this phenomenon. This remains to be addressed.

iii) Both L-serine and glycine are often included in cell culture medium as an essential nutrient and the possible effects of L-serine on cell growth and cell survival in EC and VSMC in primary culture is worthy of consideration.

iv) The responses to L-serine have been performed following its acute i.v. infusion in bolus doses and it is unclear whether it would be feasible to demonstrate a similar effect of L-serine subsequent to oral administration of high doses. The possibility exists that L-serine, could remain ionized in the gastrointestinal tract and not be absorbed. This may pose problems concerning its oral bioavailability. This too needs to be examined.

v) The present study was conducted in anaesthetized rat models. It is important to track the changes in MAP and HR for a longer period following chronic administration of L-serine in conscious, freely moving rats using radiotelemetry recording.

vi) The study was remains focused on the examination of L-serine and glycine induced changes in MAP and HR in normotensive, in L-NAME treated hypertensive rats and in the SHR model. It is known that endothelin antagonists such as bosentan are not effective as antihypertensive agents in the SHR model, but do lower the BP of salt-sensitive DOCA-salt model of hypertension (Schiffrin, 1998; Yu, Gopalakrishnan and McNeill, 1999). Thus, the effects of L-serine on hemodynamic parameters should be examined in other animal models of hypertension and in diabetes.

vii) The present study confirms that glycine infusion enhances pressor responses and thus differs from L-serine. Glycine recruits NMDA receptors, and the pathways and mechanisms recruited by L-serine are different. Thus, examination of regional hemodynamic studies with glycine to investigate the vascular beds that contribute to vasoconstriction in hypertensive rat models assumes relevance.

viii) The L-serine evoked decrease in MAP is higher in L-NAME treated rats. This confirms that the antihypertensive effect of L-serine is greater under conditions of reduced NO bioavailability. It is important that the effects of L-serine infusion be compared in eNOS knockout mice and wild type mice to characterize the NO-independent effects of L-serine.

ix) L-serine infusion could be examined in other animals such as pigs and dogs. In this regard, my supervisor has started hemodynamic studies in newborn piglets in collaboration with another investigator. A similar dose-dependent fall in MAP and vascular resistance in the mesenteric vascular bed has been observed. L-serine works in piglets in the same dose ranges as employed in the present study.

x) Finally, a phase 1 clinical trial has been approved by the Human Ethics Board at the University of Saskatchewan to study the effects of the infusion of L-serine in normal human volunteers in an effort to establish the effects of L-serine on systemic hemodynamics.

The scope and future work steming from the current findings is indeed vast and wide-ranging. To summarize, the present work provides pivotal new information from *in vitro, in vivo* and regional hemodynamic studies. Thus, the present study provides the impetus and rationale for future studies along the lines enumerated above. This work lays the foundation and opens a new avenue in our understanding of the role of individual amino acids in the regulation of BP. Foods that high in L-serine are soybeans, lentils, mature

seeds, almonds, chickpeas, egg and yolk. L-serine has the potential to be a nutraceutical agent, perhaps as an adjunct, in the management of hypertension.

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