VASCULAR EFFECTS OF TRYPTOPHAN

A Thesis Submitted to the

College of Graduate Studies and Research

In Partial Fulfillment of the Requirements for the

Degree of Master of Science in the Department of Pharmacology

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ABSTRACT

Previous studies have shown that L-tryptophan treatment has been known to reduce blood pressure (BP) in hypertensive rats. L-tryptophan is converted to serotonin (5-HT), a potent vasoconstrictor agonist. The direct vascular effects of L-tryptophan, an essential amino acid, and the mechanism that contributes to the fall in BP have not been fully explored. The present study aims to examine the direct vascular responses to both D- and L- tryptophan using perfused mesenteric vascular bed, an *ex-vivo* preparation that represents the resistance function of circulation. Perfusion was maintained at a constant flow rate (5 mL/min) with Krebs buffer (pH 7.4, 37˚C) after isolation from 12 to 14 week old male Sprague-Dawley rats. The basal perfusion pressure (PP) (mean ± SEM) was 27 ± 3 mmHg. Inclusion of D- and L-isomers in the perfusion medium led to concentration-dependent increase in PP. While the maximal response (E_{max}) was similar, D-tryptophan (EC_{50}: 0.25 ± 0.12* µmol; E_{max}: 128 ± 8 mmHg) was more potent (lower EC_{50} value; $^{*}p < 0.01$) than L-tryptophan (EC_{50}: 0.79 ± 0.30 µmol; E_{max}: 141 ± 7 mmHg). Inclusion of increasing concentrations (2, 5 and 10 nM) of the 5-HT_{2A} selective antagonist, ketanserin, led to parallel right-ward shifts in the concentration-response curves to D- and L-tryptophan with restoration of their E_{max}. In contrast, the α_1 selective agonist, methoxamine (30 µM), constricted preparations, both D- (IC_{50}: 0.94 ± 0.30* µmol; I_{max}: 96 ± 2%) and L-tryptophan (IC_{50}: 2.8 ± 1.0 µmol I_{max}: 88± 1%) evoked concentration-dependent vasodilatation, an effect that was resistant to blockade by either ketanserin or other 5-HT antagonists. Again, D-tryptophan was more potent than L-tryptophan in the presence of 5-HT antagonist ($^{*}p < 0.05$). Neither the removal of endothelium nor incubation with selective inhibitors of dilatory mediators released from the endothelium,

failed to alter the vasodilator responses to D- and L-tryptophan. In potassium chloride depolarized preparations, L-tryptophan evoked an additive vasoconstrictor response. The vasodilator responses to L-tryptophan persisted in the presence of glibenclamide, a K_{ATP} channel inhibitor, or tetraethyl ammonium, a BK_{Ca} channel inhibitor, or $BaCl_2$, a K_{ir} channel inhibitor, or ouabain, a Na^+-K^+-ATPase pump inhibitor. These data confirm that the essential amino acid, L-tryptophan, as well as its D-isomer, evoke a biphasic vasoconstrictor and vasodilator responses in the resistance type mesenteric vascular bed. While the vasoconstrictor responses are mediated by activation of vascular 5-HT receptors, the endothelium-independent vasodilator responses are not linked to activation of vascular 5-HT receptors, vascular potassium channels, Na^+-K^+-ATPase pump or via inhibition of voltage-operated Ca^{2+}-channels. Plasma concentration of L-tryptophan is about 90 - 120 µM. The endothelium/5-HT independent direct vasodilator responses characterized here for the first time could account for the antihypertensive/ BP lowering effect of L-tryptophan reported earlier by other laboratories.

ACKNOWLEDGEMENT

To begin with, I would like to thank the Almighty for giving physical and mental strength whenever required. I would honestly like to thank my supervisor, Dr. Venkat Gopalakrishnan for his constant motivation, support, and supervision for the entire duration of my masters program. It was his constant support, patience and thought provoking ideas that helped me achieve my Master of Science degree in 2 years. I certainly wish to convey gratitude to my committee members Drs. Peter Yu, Kaushik Desai, Steve Richardson and John Balsevich for their guidance and valuable suggestions. I would also like to thank Mrs. Cindy Wruck, Ms. Donna Dodge and Mr. Robert Wilcox for their help whenever required.

Special thanks to my grandparents, parents, brother and sister in-law for their support, sacrifice, constant motivation to achieve something, care and tons of love. I would also like to thank all members of my family and friends back in India and entire Department of Pharmacology at the University of Saskatchewan, who assisted me directly or indirectly towards the achievement of my degree.

DEDICATED TO MY GRANDPARENTS,

PARENTS, BROTHER & SISTER-IN-LAW.

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LIST OF ABBREVIATIONS

5-HT	Serotonin
5-HTP	5-Hydroxy tryptophan
5-HIAA	5-Hydroxyindole acetic acid
AA	Arachidonic acid
AC	Adenylyl cyclase
ATP	Adenosine triphosphate
$BaCl_2$	Barium chloride
BBB	Blood brain barrier
BH_4	Tetrahydroborate
BK	Bradykinin
$[Ca]_i$	Intracellular calcium concentration
cAMP	Cyclic adenosine monophosphate
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
COX	Cyclooxygenase
CSE	Cystathionine γ-lyase
DAG	Diacylglycerol
EC	Endothelial cells
EC_{50}	Half maximal effective concentration
ECE	Endothelin converting enzyme
EDRF	Endothelium-derived relaxing factor
EDHF	Endothelium-derived hyperpolarizing factor

E_{max}	Maximal response
Endo (-)	Endothelium denuded
Endo (+)	Endothelium intact
eNOS	Endothelial nitric oxide synthase
ET	Endothelin
GC	Guanylate cyclase
GPCR	G-protein coupled receptor
H_2S	Hydrogen sulphide
IC50	Half maximal inhibitory concentration
IK_{Ca}	Intermediate conductance calcium activated potassium channels
I_{max}	Maximal inhibitory concentration
IP_3	Inositol triphosphate
i.v.	Intravenous
i.p.	Intraperitoneally
Ket	Ketanserin tartarate
K_{Ca}	Calcium activated potassium channels
K_{ATP}	ATP dependent potassium channels
K_{ir}	Inward rectifying potassium channels
LAT	L-amino acid transporter
LNAA	Large neutral amino acids
L-NAME	N^G nitro L-arginine methyl ester
MAPK	Mitogen activated protein kinase
MEGJ	Myoendothelial gap junction

µmol	micro mole
mmHg	millimetre of mercury
NAD	Nicotine adenine dinucleotide
NEFAs	Non-esterified fatty acids
nmol	nano mole
NO	Nitric oxide
O_2^-	Superoxide anion/ Free radicals
OTC	Over-the-counter
PE	Phenylephrine hydrochloride
PDE	Phosphodiesterase
PGI_2	Prostacyclin
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PP	Perfusion pressure
ROS	Reactive oxygen species
SD	Sprague-Dawley
SEM	Standard error of the mean
sGC	Soluble guanylate cyclase
SHR	Spontaneously hypertensive rat
SK_{Ca}	Small conductance calcium activated potassium channels
SP	Substance P
TDO	Tryptophan dioxygenase

TPR	Total peripheral resistance
Trp	Tryptophan
TXA_2	Thromboxane A_2
TXS	Thromboxane synthase
VSMC	Vascular smooth muscle cell
W	Tryptophan

LIST OF CHEMICALS

Acetylcholine

Barium chloride

D-tryptophan methyl ester hydrochloride

Glibenclamide

Indomethacin

Ketanserin tartarate

L-tryptophan methyl ester hydrochloride

Methoxamine

N^G-nitro-1-arginine-methyl ester

Ouabain

Phenylephrine hydrochloride

Potassium chloride

Sodium deoxycholate

Tetraethylammonium

Thiopental sodium

All chemicals were obtained from Sigma-Aldrich Canada Ltd.

CHAPTER 1. INTRODUCTION

1.1 Introduction to blood vessels

The blood vessels are one of the major components of the circulatory system that transport blood throughout the body. Blood vessels mainly include: a) arteries, which carry oxygenated blood from the heart to various tissues (with the exception of pulmonary artery which carries deoxygenated blood); b) capillaries, which facilitate the exchange of oxygen, carbon dioxide, water and other chemicals between the blood and various tissues of the body and c) veins, which carry impure blood from the capillaries and returns back to the heart (with the exception of pulmonary vein which carries pure blood). However, arteries and veins can be further classified into aorta, arterioles, venules and venacava. This cycle of blood circulation from heart to peripheral organs and back to heart is maintained throughout one's life.

Anatomically, arteries and veins are composed of 3 layers namely *tunica intima* (innermost layer), *tunica media* (middle layer) and *tunica adventitia* (outermost layer). Major arteries like aorta and arterioles are also endowed with a thin inner lining of endothelial cells (EC). Occasionally, connective tissue is also found. The *tunica adventitia* layer is formed of connective tissue. It contains nerves that innervate the muscular layer, as well as nutrient capillaries in the larger blood vessels. *Tunica media* is composed of vascular smooth muscle cells (VSMC) along with few fibroblast cells. The contractile effect of VSMC contributes to the overall maintenance of the tone of the blood vessel. Major function of this layer is to maintain the tone of the blood vessel. The

innermost layer known as the *tunica intima* is directly in contact with the blood. It is composed of thin layer of EC sitting on the basement membrane which distinguishes *intima* from the *media*. EC of *tunica intima* perform critical functions in homeostasis, leukocyte trafficking into the vessel wall and in regulating vascular tone (Esper et al., 2006). Recent studies show that endothelial dysfunction has been allied with various pathological conditions such as atherosclerosis, diabetes, hypertension, hypercholesterolemia and inflammation (Grover-Pa´ez et al., 2009). Some of the major beneficial regulatory effects exerted in physiological states (with normal endothelium) and the deleterious effects encountered following dysfunctional endothelium is given in the Figure 1 below.

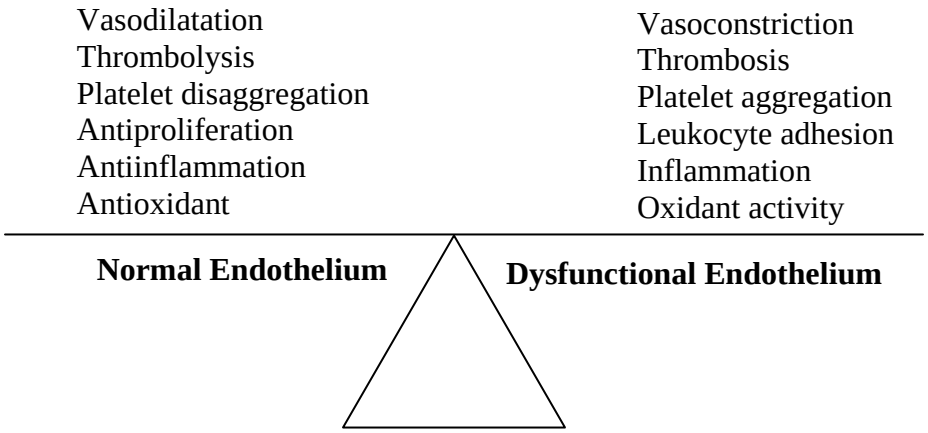


Figure 1: Regulatory functions of the endothelium. A comparison of the characteristics of normal *vs* dysfunctional endothelium (Figure modified from Esper et. al., 2004).

1.2 Endothelium and regulation of vascular tone

Analogous to other muscle cells, VSMC also contract when intracellular free calcium $[Ca^{2+}]_i$ level is increased. The relation between increase in $[Ca^{2+}]_i$ and contraction is much less in VSMC as compared to the striated or cardiac muscle. Vasoconstrictors and vasodilators operate by increasing or decreasing $[Ca^{2+}]_i$, and/or by varying the contractile sensitivity of the VSMC to elevated $[Ca^{2+}]_i$. Few examples of endogenous vasoconstrictors are norepinephrine, angiotensin II, vasopressin, endothelin-1 (ET-1) etc. Few examples of endogenous vasodilators are nitric oxide (NO), atrial natriuretic peptide (ANP), prostacyclin (PGI_2), calcitonin gene related peptide (CGRP) etc.

A new chapter in the regulation of vascular tone was opened with the discovery that endothelial layer not only acts as a physical barrier between plasma and extracellular fluid but it is an important source of numerous potent mediators (Furchgott et. al., 1989). Activation of endothelial receptors or mechanical forces exerted on EC releases factors that contract [thromboxane (TXA_2) and ET-1] or relax vascular smooth muscle (VSM) (Boulanger et al., 1994).

1.2.1 Contractile factors

Endothelin: In 1985, Hickey et al. showed that a vasoconstrictor factor was produced by cultured EC. This was later identified as endothelin, a 21-residue peptide, by Yanagisawa et al. in 1988, who isolated, analyzed and cloned the gene for this peptide. Three genes encode different sequences (ET-1, ET-2 and ET-3). Later, it was found that these isoforms are expressed in various tissues. Two receptors for ET-1 are known at present as

the ET_A and the ET_B receptor (Pollock et al., 1998). ET_A receptor has high affinity for both ET-1 and ET-2 but low affinity for ET-3, while ET_B receptor has equal affinity for all 3 isoforms of ET (Arai et. al., 1990; Sakurai et. al., 1990). Both ET_A and ET_B are expressed on VSMC but ET_A appears to be more predominant and is responsible for vasoconstriction. ET_B receptors are mainly found on endothelium and are also known as "clearance receptors" since it clears 80% of circulating endothelin from plasma and causes vasodilatation (Luscher and Barton, 2000; Kedzierski and Yanagisawa, 2001). The signal transduction of endothelin is shown in the Figure 3 on the next page.

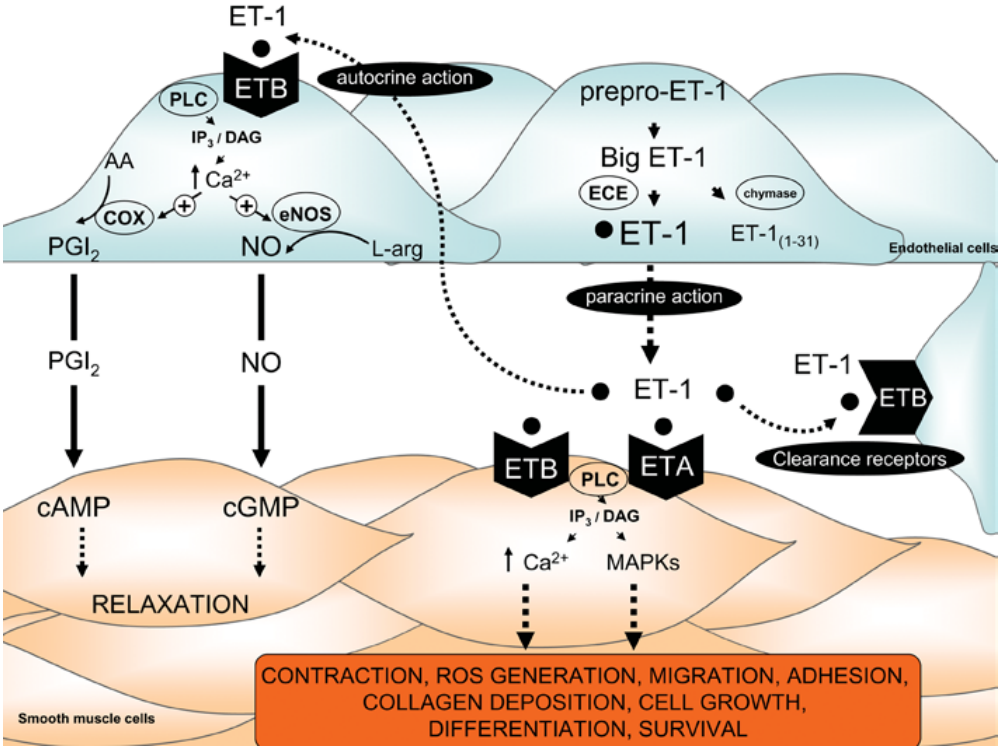


Figure 2: Signal transduction of endothelin. ET-1 synthesis is controlled via regulation of gene transcription and/or ECE activity and its release occurs mostly abluminally. ET-1 acts on ET_A and ET_B smooth muscle receptors to induce vasoconstrictor, pro-oxidant, pro-inflammatory and mitogenic responses. ET-1 also acts in an autocrine manner, at endothelial cell ET_B receptors, to stimulate the release of vasorelaxant agents such as NO

and PGI_2 (prostaglandin I_2). ET_B receptors present on kidney and lung endothelial cells also remove ET-1 from the circulation, functioning as clearance receptors. AA, arachidonic acid; eNOS, endothelial NOS. (Fig 3 is taken with permission from Tostes et. al., 2008 – See Appendix at the end).

Thromboxane (TXA_2): TXA_2 is produced during the catalysis of arachidonic acid by cyclooxygenase (COX) followed by thromboxane synthase (TXS). It is a prostaglandin (PGH_2) derivative. After the development of specific agonists and antagonists of TXA_2 in 1980s, human TXA_2 receptor protein was first purified by Narumiya's group (Ushikubi et al., 1989). On the other hand, platelets are well known to produce TXA_2 in response to various stimuli, via the actions of COX and TXS (Hamberg et al., 1975). In addition to platelets, many cells/tissues have been shown to produce TXA_2 via COX and TXS (Shen and Tai, 1986). Signal transduction of TXA_2 via G-protein coupling is shown in Figure 2.

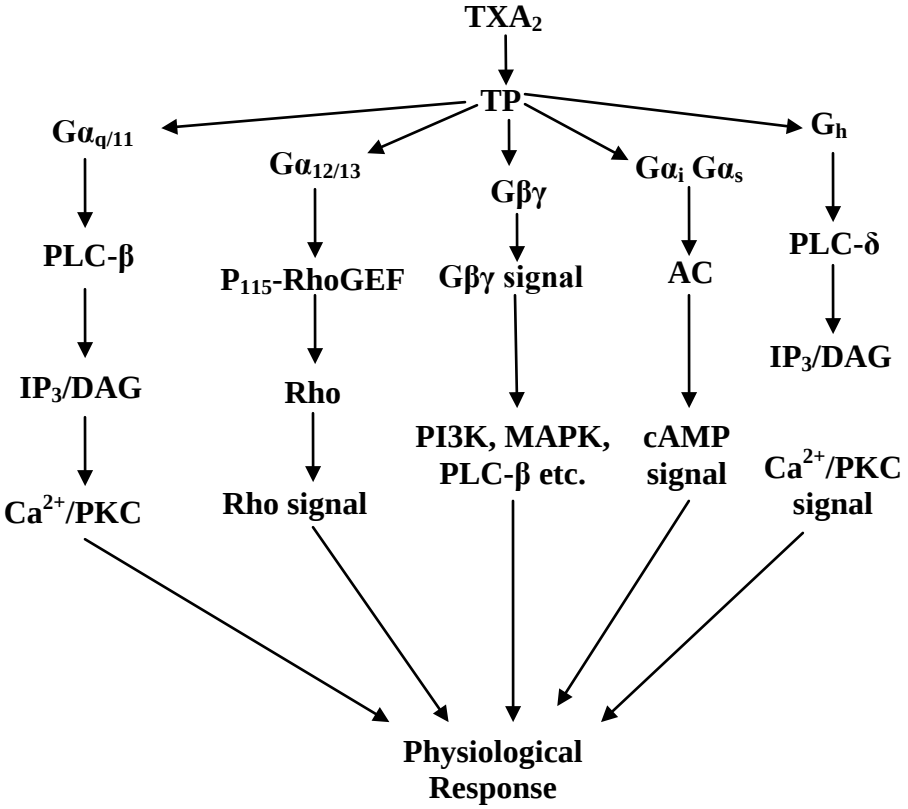


Figure 3: G-protein coupling of TXA_2 and signal transduction. AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; p115-RhoGEF, p115 guanine nucleotide exchange factor for Rho; PI3K, phosphatidylinositol 3- kinase; PLC-β, phospholipase C-β; PLC-δ, phospholipase C-δ;; MAPK, mitogen-activated kinase; PKC, protein kinase C (Diagram reproduced and modified from Nakahata N, 2008).

1.2.2 Relaxing factors

Some factors released from the endothelium lead to vascular smooth muscle relaxation. These factors include prostacyclin (PGI_2), Nitric oxide (NO) and as yet uncharacterized endothelium-derived hyperpolarizing factor (EDHF).

Nitric Oxide (NO): Factors which are both, produced and released by endothelium are known as endothelium-derived relaxing factor (EDRF). This factor released by endothelium which resulted in vascular smooth muscle relaxation was later found to be nitric oxide. EDRF was discovered and characterized by Robert F. Furchgott, a winner of the Nobel Prize in Medicine in 1998 along with his co-researchers Louis J. Ignarro and Ferid Murad. Signal transduction of NO is shown in Figure 4.

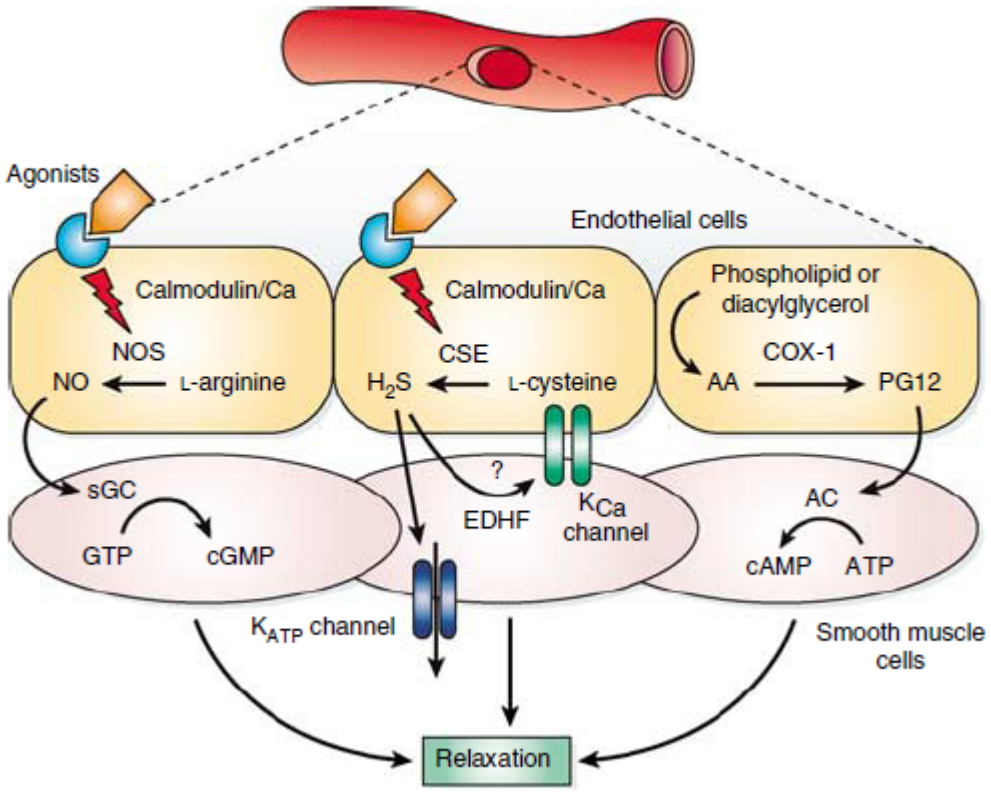


Figure 4: Molecular basis for endothelium-dependent vasorelaxation. NO, H_2S, and PGI_2 are produced from the endothelium by NOS, CSE, and COX-1, respectively. They are released from the endothelium and act on the adjacent smooth muscle cells to induce relaxation. NO and H_2S are also produced in smooth muscle cells. NOS, NO synthase; CSE, cystathionine γ-lyase; COX-1, cyclooxygenase-1; AA, arachidonic acid; EDHF, endothelium-derived hyperpolarizing factor (Taken with permission from Wang R, 2009 – See Appendix at the end).

Recent literature shows that there is a close association between endothelial dysfunction, NO production and the risks of cardiovascular diseases. (Drexler, 1997; Taddei and

Salvetti, 2002). Important functions of nitric oxide are as follows:

1) Reducing vascular permeability and synthesis of monocyte and lymphocyte adhesion molecules.
2) Decreases platelet aggregation, oxidation, inflammation, activation of thrombogenic mediators, cell growth, proliferation and migration. It inhibits proatherogenic and pro-inflammatory cytokines expression and favors fibrinolysis.
3) Nuclear factor kappa-B (NFkB) inhibitor (I-kB) is also expressed by NO. All these factors reduce atherogenesis and its complication. For this reason, NO is considered the antiatherogenic molecule (Cooke JP et al., 1994; Baldwin A, 2001; Tak P, Firestein G, 2001; Libby P, 2001; Thurberg B, Collins T, 1998; Marx N, 1999,2001).

Prostacyclin (PGI_2): Prostacyclin belongs to the superfamily of eicosanoids (20 carbon atoms in molecule). It is an arachidonic acid derivative produced by the endothelial cells. It has a very short half-life of 3 minutes (Moncada et al. 1976). By increasing the intracellular cyclic adenosine monophosphate (cAMP), it acts as a potent platelet aggregation inhibitor (Rubin et al. 1990; Moncada et al. 1976). It is a powerful systemic and pulmonary vasodilator and is being used for the management of pulmonary arterial hypertension since 1980s (Boutet et. al., 2008). Moreover, prostacyclin seems to play an important role as antiproliferative, cytoprotective, fibrinolytic mediator and it reduces leukocyte trafficking (Jones, 1996). Signal transduction pathway of PGI_2 is shown in Figure 4.

Endothelium-derived hyperpolarizing factor (EDHF): Besides NO and PGI_2, the existence of another factor released from endothelium has emerged. This factor is known as EDHF. Most vascular beds show vasodilator responses despite of selectively blocking of NO and PGI_2. Hyperpolarization of VSMC's is absolutely essential for this relaxation and hence, this factor has been termed endothelium-derived hyperpolarizing factor (Feletou et. al., 1988; Taylor et. al., 1988; Chen et. al., 1988; Garland et. al., 1995; Edwards et. al., 1998; Busse et. al., 2002). Various vascular beds of different animals show that several relaxing factors released from endothelium, can hyperpolarize the underlying VSM through different mechanisms shown in figure 5 (Bellien et. al., 2008).

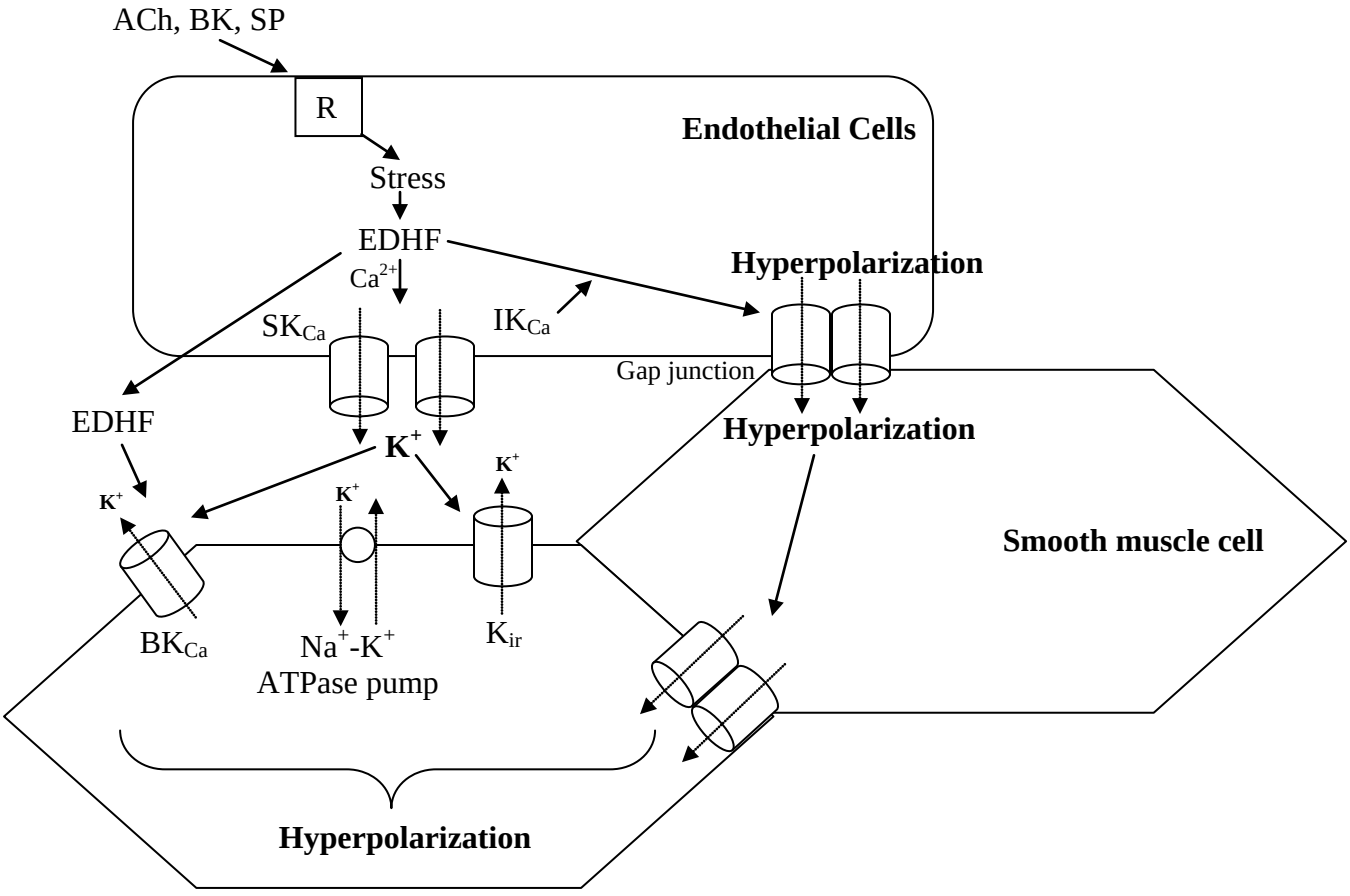


Figure 5: Schematic description of the main hyperpolarizing mechanisms mediated by EDHF. (Figure reproduced and modified from Bellien et. al., 2008)

Endothelial receptors when bound to acetylcholine (ACh), bradykinin (BK) and substance P (SP) or an increase in wall shear stress promotes the synthesis of NO and could also enhance the production of EDHF. EDHF can then hyperpolarize the VSMC by three major pathways.

1) EDHF can passively diffuse from the endothelium to activate calcium-activated potassium (K_{Ca}) channels of large conductance (BK_{Ca}) located on the smooth muscle cells thereby promoting the release of K^+ and membrane hyperpolarization.

2) EDHF can act in an autocrine manner facilitating the activation of small conductance (SK_{Ca}) and intermediate (IK_{Ca}) conductance directly mediated by Ca^{2+} activated K^+ channels present on the endothelium and thus promotes first the hyperpolarization of the EC. Then, this hyperpolarization is believed to be transmitted electronically through the myoendothelial gap junctions (MEGJ) thereby inducing hyperpolarization of the adjacent VSMC.

3) K^+ released from the endothelial SK_{Ca} and IK_{Ca} channels into the myoendothelial space activates the Na^+/K^+ ATPase and the inward rectifying potassium channels (K_{IR}) located on the smooth muscle cells promoting the release of K^+ and subsequent hyperpolarization of these cells. Finally, the smooth muscle cells hyperpolarization decreases the open-state of voltage-gate Ca^{2+} channels lowering cytosolic Ca^{2+} and thereby provoking vasorelaxation (Bellien et. al., 2008).

1.3 INTRODUCTION TO AMINO ACIDS, PEPTIDES & PROTEINS

Proteins are one of the most abundant biological macromolecules present in the cells and all parts of cells. They are present in great variety, have different molecular weights and have various biological functions. All proteins are composed of monomeric units of amino acids. Normally, all proteins are synthesized from 20 standard amino acids. Amino acids are defined as the molecules having both, an amino and carboxylic acid functional groups. They are very essential for life since they are also the building blocks of protein. In short, proteins are the polymers of amino acids with each amino acid residue joined to its neighbor by a strong covalent peptide bond. Here, the term residue means the loss of water molecules, when an amino acid is joined with another. Amino acids are important in being part of enzymes, coenzymes and also as a precursor for biosynthesis of various molecules of the body. (Wu, 2009)

1.3.1 History of amino acids

Asparagine was the first amino acid discovered when the French Chemists isolated it from asparagus (Vauquelin LN and Robiquet PJ, 1806). Cystine was discovered in 1810 (Wollaston WH, 1810), glycine and leucine in 1820 (Braconnot HM, 1820) and cysteine in 1884 (Baumann E, 1884). The last of the 20 standard amino acids to be discovered was threonine in 1938 (Simoni et. al., 2002).

1.3.2 General properties of amino acids

All the 20 standard amino acids are α-amino acids i.e. they have both the carboxyl and amino functional group attached to the same carbon. The general structure of amino

acid is shown in Fig 6. For all the standard amino acids except glycine, the α-carbon atom is bonded to 4 different functional groups namely: carboxylic acid, amino group, R group and a hydrogen atom. Thus, α-carbon atom is also a chiral center and 2 enantiomers are possible. All molecules with chiral center are also optically active which means that they can rotate the plane-polarized light (*l* or levorotatory and *d* or dextrorotatory). Based on the special nomenclature system for simple sugars and amino acids, amino acids are categorized into D and L system.

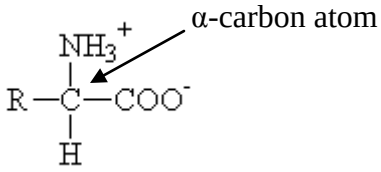


Figure 6: General structure of an amino acid. Above structure applies to all 20 standard amino acids with the exception of proline (cyclic amino acid). R-group attached to the α-carbon atom is different in all amino acids.

The 20 standard amino acids can also be classified on the basis of polarity. Amino acids with hydrophobic side groups include valine, leucine, isoleucine, methionine and phenylalanine. Asparagine, glutamic acid, glutamine, histidine, lysine, arginine and aspartic acid are the amino acids with hydrophilic side groups. Amino acids which are in between include glycine, alanine, serine, threonine, tyrosine, tryptophan, cysteine and proline.

Amino acids, when dissolved in water can act either as an acid or as a base. When in solution, amino acids exist as a dipolar ions or zwitterions. Zwitterions can behave

either as an acid by donating protons or as a base by accepting protons. Substances having this dual chemical property (amphoteric nature) are also known as ampholytes.

1.3.3 Cardiovascular effects of amino acids

Amino acids play a vital role in the functioning of central and peripheral nervous system. Some amino acids also have the capability to change the vascular tone (Katahira et. al., 1994). Intravenous (i.v.) administration of L-tryptophan, an essential amino acid has been shown to increase brain serotonin levels and can be used in several neuropsychiatric disorders (Wolf et al., 1984). Some amino acids serve as neurotransmitter or as a precursor for the synthesis of various neurotransmitters (Munro et al., 1986). Glycine, a non-essential amino acid derived from serine, acts as an inhibitory neurotransmitter in the CNS. Studies have revealed that glycine is protective against oxidative stress in several cardiovascular diseases (Hafidi et al., 2006). Antihypertensive action of L-serine has already been demonstrated by Mishra et al., 2008; Mishra et al., 2008. N-acetyl cysteine and taurine exert their beneficial effects by diminishing intracellular oxidative stress generation and glyco-oxidation (Anuradha et. al., 2009). Glycine and lysine prevent glycation. Recent studies also explore the beneficial effect of amino acids in diabetes and diabetic complications (Anuradha et al., 2009). Central administration of taurine, L-alanine and gamma amino butyric acid (GABA) exerts an antihypertensive effect (Sgaragli et al., 1972). Tyrosine, a precursor for the synthesis of adrenaline and noradrenaline, when orally administered lowers blood pressure (Sved et al., 1979). At lower concentrations (25 and 50 mg/kg, i.p.), L- and D-tryptophan both produced an increase in blood pressure (10-15mmHg). However, at

higher concentrations (100mg/kg, i.p.), L-tryptophan lowered the blood pressure of hypertensive rats by 30-35mmHg whereas D-tryptophan did not produce any fall in blood pressure (Wolf et al., 1984). They concluded that the lowering of blood pressure due to L-tryptophan in hypertensive rat models cannot be entirely the effect of change in brain serotonin levels. Despite the *in vivo* studies, there are no *in vitro* studies to establish the mechanisms of the vasodilator response to L-tryptophan. The present study is an attempt to address this issue.

1.4 INTRODUCTION TO TRYPTOPHAN

Tryptophan (Trp or W) is one of the 20 standard amino acids. Tryptophan was first isolated and reported in the beginning of last century (Hopkienns et al., 1901). It was isolated by hydrolysis of crude casein. It is also an essential or indispensible amino acid which means that mammals and humans cannot synthesize this amino acid *de novo*. Hence, it has to be obtained from the diet and is required by all living cells to synthesize protein (Leuchtenberger et al. 2005). As with other amino acids, only the L-stereoisomer of tryptophan is used in the synthesis of proteins and enzymes. However, D-tryptophan is at times found in natural peptides. Marine venom peptide, contryphan, contains D-tryptophan (Pallaghy et al., 1999).

1.4.1 General properties of tryptophan

Aromatic amino acids include tryptophan, tyrosine and phenylalanine. The chemical formula of tryptophan is $C_{11}H_{12}N_2O_2$. It is a white crystalline solid, odourless and slightly bitter in taste with the molecular weight of 204 g/mole. Tryptophan is semi-polar in nature. Thus, it is not very soluble in water (11.4 g/L at 25°C). However, it is more soluble in hot water, hot alcohol and solutions of dilute acid or of alkali hydroxides. Tryptophan is unique since it has an indole functional group that is not present in other amino acids. The structure of L-tryptophan is shown in Figure 7.

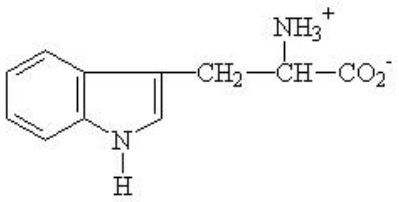


Figure 7: Structure of L-tryptophan

1.4.2 Nutritional source of L-tryptophan

Tryptophan is one of the least abundant amino acid found in dietary proteins. It can be obtained from chocolates, mangoes, dried dates, yogurt, cottage cheese, eggs, fish, poultry, sesame, chickpeas, sunflower seeds, pumpkin seeds, spirulina, and peanuts. Tryptophan constitutes only 1–1.5% of the amino acids present in casein, ovalbumin, and most meats (McCarthy et al., 1968). On the contrary, α-lactalbumin which is a minor milk protein is found to contain 6% tryptophan (Markus et al., 2002).

1.4.3 Biosynthesis of L-tryptophan in plants and microorganisms

Plants, fungi and microorganisms produce L-tryptophan by shikimic acid pathway (Herrmann and Weaver, 1999). Chorismate produced from shikimic acid pathway is condensed with glutamine to form anthranilate. The latter is then phosphoribosylated with phosphoribosylpyrophosphate (PRPP). Subsequent to ring opening of the ribose moiety and reductive decarboxylation, indole-3-glycerinephosphate is synthesized. The latter in turn is condensed with glyceraldehyde 3-phosphate to indole. In the final step, indole and serine are condensed in presence of tryptophan synthase to produce tryptophan (Radwanski et al., 1995). Large-scale industrial production of tryptophan is also

biosynthetic. It is based on the fermentation of serine and indole using *E. coli* in the presence of enzyme tryptophan synthase (Ikeda, 2002). The simplistic diagram of the biosynthetic pathway of L-tryptophan is shown below (Fig 8).

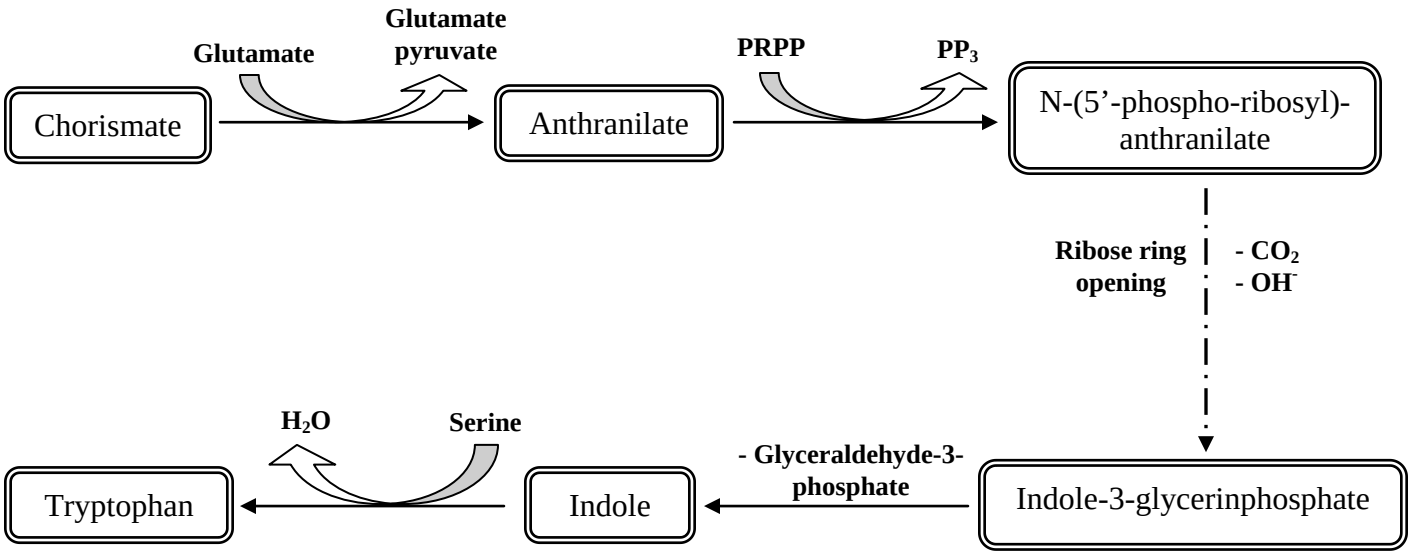


Figure 8: Simplistic diagram of the biosynthesis of tryptophan by shikimic acid pathway in plants and micro-organisms (Figure 8 reproduced and simplified from Radwanski et al., 1995).

1.4.4 Absorption of tryptophan from G.I.T

Humans derive L-tryptophan from their diet. It is required by the body for the synthesis of various proteins. Being semi-polar, tryptophan is easily absorbed from the stomach. Following absorption into the blood, tryptophan undergoes first-pass metabolism in liver by the enzyme tryptophan dioxygenase (TDO). Thus, only a part of

ingested tryptophan actually enters the systemic circulation. Diet rich in proteins increase the plasma concentration of tryptophan. Under fasting conditions, tryptophan is released to a certain extent from reservoirs in tissue or circulating proteins.

1.4.5 Plasma concentration of tryptophan

The plasma concentration of amino acids differs in various physiological and pathological states. Disease conditions, postprandial, postabsorptive, exercise, starvation and injury can cause variation in the total and free plasma amino acid levels in the body. The average total plasma concentration of tryptophan in normal volunteers (n = 100) after overnight fasting was found to be 44 ± 7 μmole/L (Cynober, 2002).

However, a significant difference in total plasma concentration of tryptophan in Sprague-Dawley rats (180 – 240 gm) was observed in control and fasting states as compared to humans. Plasma concentration of tryptophan in control and fasting states was found to be 90 – 117 μM and 76 – 125 μM respectively. Fasting states was defined as deprived of food but not water for 24 h (Knott et al., 1972). There was a significant increase in free plasma tryptophan levels in control and fasting states in Sprague-Dawley rats.

1.4.6 Regulation of free plasma tryptophan levels

Tryptophan is the only amino acid which exists in equilibrium between the albumin-free and albumin-bound state in the peripheral systemic circulation (McMenamy, 1965). Under resting conditions, tryptophan is almost 90% bound to

albumin which makes tryptophan-albumin complex almost impossible to cross the blood brain barrier (BBB). The remaining 10% of free tryptophan in plasma is actively transported across the BBB and enters the brain (Madras et al., 1974).

One of the factors which play a very important role while estimating free plasma concentration of tryptophan is the presence of non-esterfied fatty acids (NEFAs). NEFAs compete with tryptophan for binding to albumin, and high concentration of NEFAs displace tryptophan from albumin-tryptophan complex (Curzon et al., 1973) leading to an increase in plasma free concentration of tryptophan (Chaouloff et al., 1986; Blomstrand et al., 1989). An increase in the level of NEFAs can be due to activation of sympathetic nervous system activity resulting from sustained exercise (Fernstrom et al., 2006). Lipolysis induced by activation of β-adrenoceptors also increases the levels of NEFAs (Ranallo et al., 1998). Drugs such as benzodiazepines (Muller et al., 1975) and clofibrate can also increase the plasma levels of NEFA (Spano et al., 1974). One might expect that the rise in brain tryptophan levels is because of an increase in free plasma concentration of tryptophan following NEFA elevation. However, latest experiments have just yielded contradictory results (Fernstrom et al., 2006). Dissociation of tryptophan-albumin complex in the cerebral microvascular system is an important factor for the increase in free tryptophan levels in the brain (Pardridge, 1990;1998). It is still unclear whether total or free plasma tryptophan levels are important for the rate of uptake of tryptophan in brain.

1.4.7 Transport of tryptophan into the brain

For any substance to enter brain, it must cross the BBB. Since tryptophan is not highly lipophilic, passive diffusion of tryptophan through BBB is not possible. A competitive transporter that is shared with several large neutral amino acids (LNAAs) is responsible for the movement of tryptophan across BBB in rats (Fernstrom, 1983; Pardridge, 1977; Oldendorf et al., 1976) and humans (Hargreaves et al., 1988). This transporter is known as LAT1 or L -type amino acid transporter. LAT1 in BBB has more affinity for amino acids than L-type amino acid transporter in other tissues (Pardridge, 1983). Also LAT1 is expressed more in BBB than in other tissues (Boado et al., 1999). Thus, increase in plasma concentrations of LNAAs will result in competition of amino acids including tryptophan for LAT1. Since, tryptophan is low compared to other LNAAs, it will lead to decrease in rate of uptake of tryptophan into brain (Fernstrom, 2005). Mechanisms such as hemodynamics of cerebral microvasculature (Pardridge, 1990), kinetics of tryptophan transport into brain, alteration in protein metabolism in the brain and alteration in the rate of efflux of tryptophan from brain play an important role in the regulation of tryptophan levels in the brain (Kennett, 1986; O'Kane, 2003).

1.4.8 Metabolism of L-tryptophan

In humans, ingested tryptophan undergoes first-pass hepatic metabolism in the liver by enzyme TDO. This enzyme, found only in liver, has the capacity to irreversibly cleave tryptophan's indole ring to form N-formylkynurenine. Indoleamine 2,3-dioxygenase (IDO) is another enzyme which has the potential to break the indole ring. It is active at tryptophan concentrations present in the blood and has Km value (26 μM)

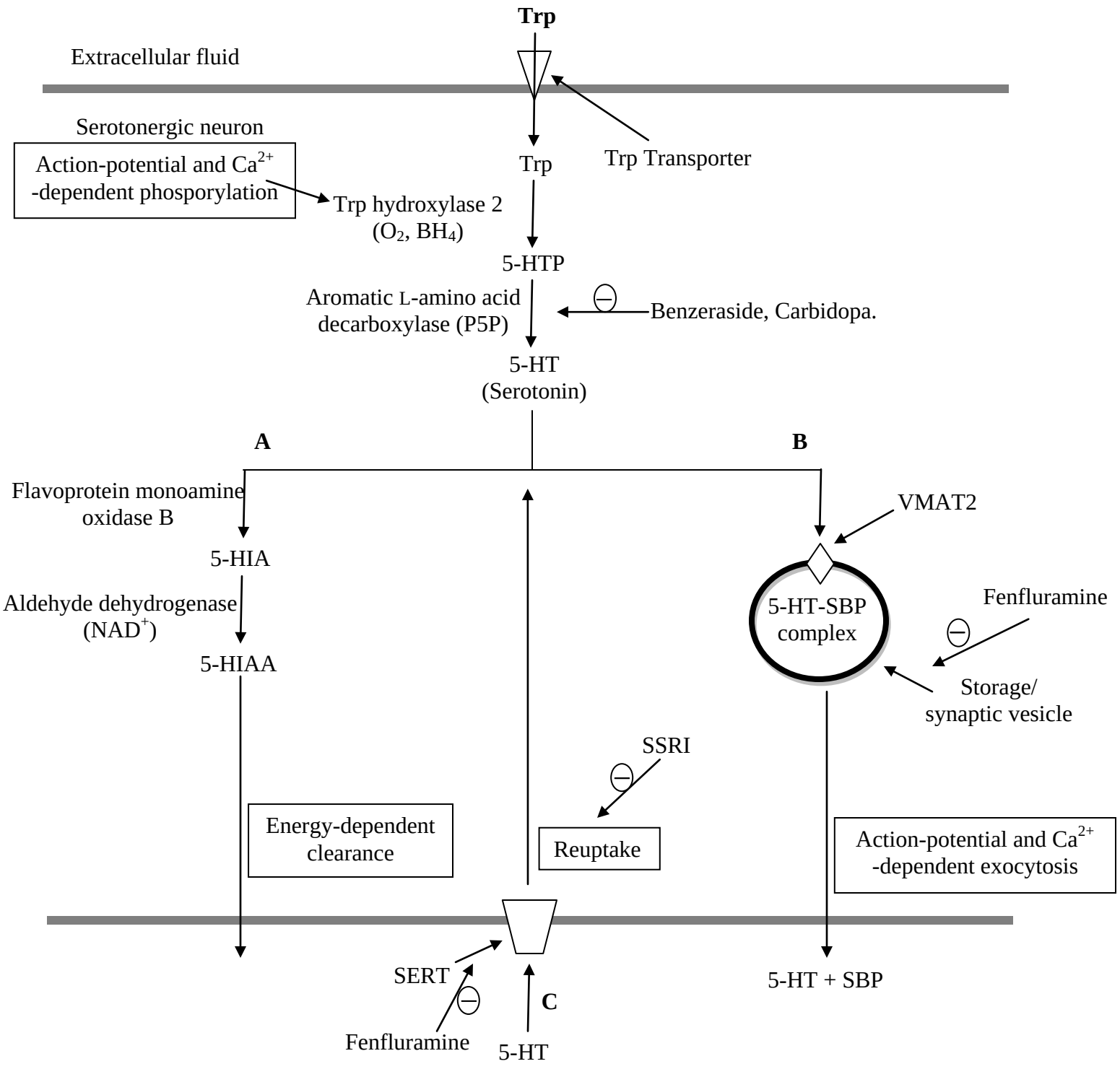
much lower than that of TDO (45 μM) (Yamazaki et al., 1985; Shimizu et al., 1978). TDO is specific for the L-isomer of tryptophan (Tanaka and Knox, 1959), while IDO metabolizes a wide variety of compounds (L- and D-tryptophan; serotonin; melatonin). The latter is also found in all extra-hepatic tissues (Yamazaki et al., 1985), including brain (Kwidzinski et al., 2005). The hepatic N-formylkynurenine generated by TDO is metabolized to L-kynurenine, kynurenic acid, xanthurenic acid, quinolinic acid, nicotinamide adenine dinucleotide (NAD), and, ultimately to carbon dioxide and water. A similar kynurenine pathway exists in brain (Guidetti et al., 1995) with its metabolites resulting in several neurological effects. Quinolinic acid, an N-methyl-D-aspartate (NMDA) agonist has neurodegenerative effects. (Stone and Perkins, 1981). Kynurenic acid, a glutamate receptor antagonist has shown to possess neuroprotective effects (Perkins and Stone, 1982). Kynurenic acid also results in the inhibition of striatal dopamine release (Rassoulpour et al., 2005). Kynurenine has an ability to stimulate neuronal growth and development (Dong-Ruyl et al., 1998). Nicotine adenine dinucleotide (NAD) formed from this pathway also acts as a cofactor in various enzymatic reactions. IDO uses molecular oxygen and superoxides (O_2^-) as substrates and thus acts as an anti-oxidant.

1.4.9 Metabolism of the tryptophan in CNS

As free tryptophan is transported across BBB and into the brain, several metabolites are formed from different metabolic pathways in the brain. Serotonergic neurons and mast cells convert tryptophan into serotonin and its metabolites. In astrocytes, microglial cells, macrophages and dendritic cells, tryptophan undergoes

kynurenine and kynurenic acid metabolism. In pinealocytes, tryptophan undergoes metabolism into melatonin (Ruddick et al., 2006). Tryptophan metabolism in serotonergic neurons is shown below (Fig 9).

Figure 9: Metabolism of tryptophan in serotonergic neurons. Tryptophan is transported from extracellular fluid into the neuron by the tryptophan transporter. Tryptophan hydroxylase 2, a rate-limiting enzyme located in the cytoplasm of the neuronal cell body, hydrolyses tryptophan to 5-HTP using O_2 and BH_4. Aromatic L-amino acid decarboxylase in presence of co-factor P5P, converts 5-HTP into 5-HT. Serotonin can either a) be converted into its metabolites 5-HIA and ultimately 5-HIAA in presence of the flavoprotein enzyme monoamine oxidase B and aldehyde dehydrogenase respectively, or b) be stored in synaptic vesicles with the help of VMAT2 and released on stimulation, or c) uptake of released serotonin into the neurons can take place with the help of SERT (O_2- Oxygen, BH_4- tetrahydrobiopterin, 5-HTP- 5-hydroxytryptophan, 5-HT- 5-hydroxytryptamine, P5P- Pyridoxal-5'-phosphate, 5-HIA- 5-hydroxyindole acetaldehyde, 5-HIAA- 5-hydroxyindoleacetic acid, SERT- Serotonin transporter, VMAT2- Vesicular monoamine transporter 2, SBP- serotonin-protein binding), SSRI- Selective serotonin reuptake inhibitor.


Trp
Extracellular fluid
Trp Transporter
Serotonergic neuron
Action-potential and Ca2+ -dependent phosporylation
Trp
Trp hydroxylase 2 (O2, BH4)
5-HTP
Aromatic L-amino acid decarboxylase (P5P)
Benzeraside, Carbidopa.
5-HT (Serotonin)
A
B
Flavoprotein monoamine oxidase B
VMAT2
5-HIA
Aldehyde dehydrogenase (NAD+)
5-HIAA
5-HT-SBP complex
Fenfluramine
Storage/ synaptic vesicle
SSRI
Energy-dependent clearance
Reuptake
Action-potential and Ca2+ -dependent exocytosis
SERT
C
5-HT + SBP
Fenfluramine
5-HT

1.5 Importance of L-tryptophan

L-tryptophan, being an essential amino acid cannot be synthesized by humans or by other mammals. It acts as a building block for protein synthesis, L-tryptophan is the precursor of serotonin, a neurotransmitter. Serotonin plays an important role in the motility of gut, control of appetite, mood and anger (Berger et al., 2009). Serotonin also aids in sleep and general metabolism. Aggression and depression appear to be related to an imbalance of serotonin or other neurotransmitters in brain (Ryding et al., 2008). Serotonin can be converted to melatonin, a neurohormone, which is important in the regulation of circadian rhythms of various biological functions (Altun et al., 2007). Melatonin is known to be a powerful antioxidant that protects mitochondrial and nuclear DNA (Hardeland, 2005; Reiter et al., 2001). Niacin is synthesized from tryptophan via the kynurenine pathway. Biosynthesis of kynurenines in the CNS has significant effects on the physiology and behavior. Altered kynurenine metabolism is associated with AIDS related dementia (Guillemin et al., 2005), Huntington's (Carlock et al., 1995) and Alzheimer's disease (Guillemin et al., 2002; 2003; 2005). Auxin, a plant growth hormone is also synthesized from tryptophan. L-tryptophan is available as an over-the-counter (OTC) drug in Canada for use as a brain calming agent.

CHAPTER 2. HYPOTHESIS AND RATIONALE

2.1 Rationale for the present study

Ingestion of dietary proteins and gluconeogenic amino acids improves renal hemodynamics in several animal models and humans (Castellino et al., 1986; Daniels and Hostetter, 1990). The effects of these amino acids on other vascular beds have not been examined. N-octanoyl serine, a phospholipid intermediate isolated from bovine brain, evokes endothelium-dependent but nitric oxide (NO) and cycloxygenase (COX)-independent vasodilatation in isolated rat blood vessels (Milman et al., 2006). Recent studies from our laboratory have established that a non-essential amino acid, L-serine, *per se* evokes endothelium-dependent but NO and COX-independent vasodilatation in rat mesenteric arterioles via selective activation of EDHF (Mishra et al., 2008). In addition, acute L-serine infusion leads to a dramatic dose-dependent fall in BP. This effect of L-serine is much higher in both L-NAME-induced hypertensive rat and SHR models. In contrast to L-serine, infusion of glycine, a metabolite of L-serine, reduces BP in normotensive rats but paradoxically evokes pressor responses in both L-NAME-induced hypertensive and SHR strains (Mishra et al., 2008). The significance and importance of the cardiovascular protective and the antihypertensive effects of taurine, a sulphur containing amino acid, known for a long time, has been reinforced by recent reviews on this subject (Yamori et al., 1996; Xu et al., 2008). More recently, another amino acid, glutamic acid, rich in vegetable proteins, has received greater attention since it reduces BP in humans (Stamler et al., 2009). These recent studies led us to the conclusion that besides endogenous vasoactive peptides such as atrial natriuretic peptide (ANP),

bradykinin and CGRP, amino acids in circulation, could also contribute to the regulation of vascular smooth muscle (VSM) tone, total peripheral resistance (TPR) and BP. These observations have led us to examine the effects of different amino acids on vascular reactivity changes as major focus of our research.

While acute intravenous infusion of L-serine leads to dramatic fall in BP, the oral feeding of L-serine, being polar, is associated with less dramatic effect on BP because of its reduced absorption. To overcome this problem, we looked at options of coupling L-serine with other amino acids that would also promote fall in BP so these agents could be administered as orally active di or tripeptide to reduce cardiovascular disease (Erdmann et al., 2008). With this as the goal, other investigators have combined two amino acids, L-tryptophan (W) and histidine (H) as a dipeptide (W-H) and tested its effect on *in vitro* preparation such as rat aortic rings. W-H exerted endothelium-independent vasodilatation in rat aortic rings (Tanaka et al., 2008). This effect of W-H is dose-dependent and it is suggested to act as a Ca^{2+} channel antagonist since it potentiated the blockade of potassium chloride (KCl) evoked vasoconstrictor responses by verapamil in this preparation (Tanaka et al., 2008). It is possible that this effect of W-H could be due to its endogenous conversion of both L-tryptophan and histidine to serotonin (5-HT) and histamine respectively. Thus these biogenic amines in turn could mediate their vascular responses. In view of the potential bronchoconstrictor problems associated with conversion of histidine to histamine, a combination of L-serine and L-tryptophan is considered by our laboratory as a potential dipeptide that would provide cardiovascular protection. Moreover, such a combination we hypothesize would lead to endothelium-

dependent (L-serine-mediated) and endothelium-independent (L-tryptophan-induced blockade of Ca^{2+} channels) vasodilator/antihypertensive effect associated with the oral administration of such a serine-tryptophan combination treatment as an orally active dipeptide.

Before preparing and examining the effect of such a dipeptide, either as S-W, or W-S, it is important to examine the acute effects of L-tryptophan in parallel with L-serine. An earlier report suggested that L-tryptophan exerts an antihypertensive effect in SHR and this is suggested to occur by its conversion to serotonin in the brain (Sved et al., 1982). However, studies by others proposed that the effect seen with L- but not D-tryptophan cannot be explained simply by the changes in brain serotonin levels and that other peripheral mechanisms could contribute to its antihypertensive effect (Wolf et al., 1984**;** 1984). Others have concluded that the proposed antihypertensive effect of L-tryptophan is due to centrally mediated changes in several bioactive amines and angiotensin II (Fregly et al., 1988). However, this controversy remains to be settled. It should be pointed out that many of these BP measurement studies have been conducted using either the tail cuff methodology or by direct catheter recording from arteries cannulated in anaesthetized rats. Recent studies using radiotelemetry recording of BP measurement in conscious freely moving rats do not validate the changes reported by tail cuff or recording in anaesthetized rats as these methodologies provide false positive results for the changes in BP (Balakrishnan et al., 1998). While *in-vivo* studies with L-tryptophan would be important, first, it is necessary to demonstrate the direct vascular effects of L-tryptophan. It is known that serotonin which is biosynthesized from L-

tryptophan is a potent vasoconstrictor in several vascular beds (Watts, 2002). Most vascular beds are endowed with the enzymatic machinery to generate serotonin from L-tryptophan. It is considered that bioconversion to 5-HT is the major mechanism for L-tryptophan induced changes in BP in hypertensive rats. While elevated plasma 5-HT level and arterial hyper-responsiveness to 5-HT has been demonstrated in hypertensive rats, studies have also shown that administration of serotonin reduces BP in hypertensive rat models (Centurion et al., 2004; Terron et al., 2007; Diaz et al., 2008). In addition, vascular 5-HT_{2B} and 5-HT_7 receptor activation would promote vasodilatation to 5-HT in some arteries and veins have also been suggested (Ellis et al., 1995; Terron, 1997; Ishine et al., 2000). Given this complexity, it is important to examine the direct vascular effects of L- tryptophan and D-tryptophan and establish whether there are differences in vasoconstrictor and/or vasodilator responses to L- and D-tryptophan and the likely role played by endothelium in mediating these effects. In addition, whether these responses are mediated by its conversion to 5-HT would be an important issue to consider in the proposed experimental plan. Based on the above, the following working hypotheses have been formulated.

2.2 Working hypothesis

L-tryptophan but not its D-isomer, will evoke vasoconstrictor and vasodilator responses that are mediated by its conversion to serotonin. Serotonin antagonists will block these responses to L-tryptophan.

The vasoconstrictor responses to L-tryptophan are mediated by the activation of vascular 5-HT_{2A} receptors. The vasodilator responses are mediated via recruitment of both endothelium-derived relaxing factors (EDRF) and activation of processes on vascular smooth muscle cells that are endothelium-independent and this is via recruitment of another subtype of 5-HT receptors.

CHAPTER 3. MATERIALS AND METHODS

3.1 Animals

The protocol for the animal studies employed in the present study was approved by our University Review Committee and it 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, 1996. Male Sprague-Dawley rats at 11 weeks were obtained from Charles River, (St Constant, Quebec, Canada). The animals were allowed to recover for 1 week before the experimental protocol was initiated. All rats were kept under standard conditions with a light/dark cycle of 12 hrs and a constant temperature of 22 ± 1ºC. The rats were fed *ad libitum* with food pellets (Purina Rat Chow) and tap water. Experimental protocol was initiated only when the rats were 12-14 weeks old.

3.2 Materials

Chemicals and reagents used in these experiments are described as follows. Acetylcholine (ACh), barium chloride ($BaCl_2$), indomethacin, L-tryptophan methyl ester hydrochloride, D-tryptophan methyl ester hydrochloride, ouabain, phenylephrine hydrochloride (PE), N^G nitro L-arginine methyl ester (L-NAME), glibenclamide, ketanserin tartarate, tetraethylammonium bromide (TEA), sodium chloride, sodium bicarbonate, calcium chloride, magnesium chloride hexahydrate, dextrose, potassium dihydrogen phosphate and methoxamine hydrochloride were purchased from Sigma-Aldrich Chemical Co., Oakville, ON., Canada.

3.3 Methods

3.3.1 Choice of preparation

The perfused rat mesenteric vascular bed maintained at a constant flow rate (5mL/min) is a sensitive vascular preparation that consists of the changes in VSM tone arising from both conduit (superior mesenteric artery) artery and its primary, secondary, and tertiary branches arising in this arterial cascade. It is known that 25% of the cardiac output flows through the splanchnic vascular bed and the mesenteric vascular cascade is an important and major cardiovascular circuit. The vascular reactivity changes occurring in this vascular bed are considered to serve as a representative response for resistance type vessels (Mulvany, 1993). Our laboratory is well versed in setting up this *ex-vivo* preparation and repeated responses to both vasoconstrictor and vasodilator agonists could be determined in the same vascular bed (Moazed et al., 2009; Shastri et al., 2002; Misurski et al., 2001; 2001; 2002**)**. Therefore, it was decided to utilize this preparation to compare the responses to L- and D-tryptophan.

3.3.2 Surgical procedure for isolation of rat mesenteric vascular bed

Experiments were performed only when animals were 12 weeks old. Rats were anesthetized with thiopental sodium (100 mg/kg, i.p.), coeliotomy was performed by a mid-line incision and the heart was isolated. Without much delay, the superior mesenteric artery was identified and cleared of any adherent tissue using a cotton swab. Using curved forceps, two pieces of sutures were placed beneath the superior mesenteric artery at the level of the T-junction where the superior mesenteric artery branches off from the abdominal aorta. This was followed by a small incision made at the T-junction and the

superior mesenteric artery was cannulated with polyethylene tubing fitted on to a 20 gauge needle. A small amount of Krebs buffer was pushed into mesenteric vascular bed to remove blood clots. The mesenteric vascular bed was then cut carefully along the line of the intestinal tract in such a way that the bed was not stretched and there was minimal loss of arterioles. The entire bed was isolated from the gastrointestinal tract, and placed in a warm (37 ± 1ºC) Krebs solution. The mesenteric vascular bed was then transferred and connected to the perfusion apparatus (Misurski et al., 2000; Shastri et al., 2001).

3.3.3 Perfusion of mesenteric vascular bed

The mesenteric vascular bed was perfused via cannula with the Krebs buffer solution (pH 7.4) by means of a pump, at a constant flow rate of 5 ml/min. The composition of the Krebs buffer solution (in mM) was: NaCl 118, KCl 4.7, $MgCl_2.6H_2O$ 1.2, $CaCl_2.2H_2O$ 1.8, KH_2PO_4 1.2, $NaHCO_3$ 25 and Glucose 11.1. The buffer was continuously oxygenated using carbogen (95% O_2 oxygen + 5% CO_2) and maintained at a steady temperature 37ºC with the thermocirculator (from Harvard apparatus). An air pocket was incorporated in the route for preventing the air-bubbles from entering the mesenteric bed. Air bubbles are known to cause endothelium denudation and can result in variation of responses to pharmacological agents (Misurski et al., 2000; Shastri et al., 2001).

3.3.4 Recordings of variations in perfusion pressure to various agonists and antagonists

The variations in perfusion pressure in response to agonists and antagonists were measured using a strain gauge pressure transducer placed in the circuit. A chart program (Chart V5.5.6) using a PowerLab/8SP Series system (AD Instruments Pty. Ltd., Castlehill, NSW, Australia) was setup to visualize the changes in perfusion pressure on the computer. The pressure transducer, attached to the PowerLab was placed in the circuit of the flow of Krebs buffer to record the changes in perfusion pressure. The pressure transducer was calibrated each time using a sphygmomanometer before the start of the experiment.

3.3.5 Experimental procedure

3.3.5.1 Equilibrium period for mesenteric vascular bed

Once the mesenteric bed was hooked to the system, it was allowed to equilibrate for 1 h with normal Krebs buffer to attain steady basal perfusion pressure. Mesenteric vascular beds having basal perfusion pressure of above 20 mmHg and below 30 mmHg were used in the experiments. Lower basal perfusion pressure indicated that the mesenteric bed was not isolated properly and the level of resistance developed in the vascular bed was inadequate. Very high perfusion pressure indicated that either the suture was tied so firmly to the cannula that it obstructed the flow of Krebs buffer or that there was a blood clot in the mesenteric bed. Then, two bolus doses of PE (80 μmol/L), a non-selective α_1 agonist, were given in succession to ensure that equipotent constrictor responses were obtained and that the tissue had attained the required equilibrium to respond to exogenous agents.

3.3.5.2 Experimental procedure for concentration-response curves to vasoconstriction effects of tryptophan

Tryptophan, a semipolar compound is sparingly soluble in water (11.4 g/L at 25°C). To enhance the solubility of tryptophan, we decided to use its methyl ester hydrochloride salt. No differences were seen between the effects of the methyl ester hydrochloride salt form and the free amino acid (Wolf et al., 1984). From now onwards, L- and D-tryptophan methyl ester hydrochloride will be simply noted as L- and D-tryptophan respectively. The experiments were performed with two tissues isolated from two Sprague-Dawley rats and were perfused at the same time. Log dose of L- and D-tryptophan (0.01, 0.03, 0.1, 0.3, 1, 3 and10 µmol) within the pharmacological range were then injected as bolus dose of 0.5ml. Sufficient time (45 min-1 hr) was given for recovery of tissue after completion of the concentration-response curves to each agonist prior to constructing the second concentration-response curve in the same tissue. No desensitization of mesenteric bed was observed while taking the concentration-response curve to the next agonist. This was verified by repeating the concentration-response curves to the same agonist. Taking either D- or L- tryptophan panel first did not affect the results of the concentration-response curve. To elucidate the mechanism of contractile response, the responses to L-tryptophan were performed in the presence and absence of increasing concentration of ketanserin tartarate. Literature review did not reveal the dose of ketanserin used previously in mesenteric vascular bed. After performing a series of preliminary experiments, the dose of ketanserin was titrated to 2 nM, 5 nM and 10 nM. Ketanserin tartarate was dissolved in krebs buffer, adjusted to pH 7.4 and was perfused through mesenteric bed for 30 minutes. After perfusion of one concentration of

ketanserin tartarate, concentration-response curves to same doses of L-tryptophan (0.01, 0.03, 0.1, 0.3, 1, 3 and 10 µmol) were obtained. This procedure was repeated with different doses of ketanserin. Parallel studies were performed to establish the competitive blockade of 5-HT evoked responses by the same concentration of ketanserin.

3.3.5.3 Experimental procedure for concentration-response curves to vasodilatation effects of tryptophan

Methoxamine (30 µM), an α_1 specific agonist was perfused through mesenteric bed for 20 min, until a steady constriction level was obtained. Phenylephrine, another α_1 selective agonist was not used for constriction since a steady tonic response to this agonist was difficult to attain. Once the steady state tonic PP was attained, the presence of endothelium was always verified by a bolus dose of acetylcholine (ACh 300 pmol). The presence of intact endothelium produces 85-95% vasodilatation to ACh. Then, concentration-response curves to L- and D- tryptophan (0.01, 0.03, 0.1, 0.3, 1, 3, 10 and 30 µmol) were obtained. Sufficient recovery period (45 min to 1 hr) was given between the two concentration-response curves. Desensitization of tissue was verified by obtaining concentration-response curves to L- and D- tryptophan more than once in the same tissue. The order of conducting concentration-response curves to L- and D-tryptophan did not affect the results. Entire set of concentration-response curves to 5-HT was also taken in constricted mesenteric vascular bed.

With a view to characterize the mechanism of vasodilator response, ketanserin tartarate (5nM), a specific 5-HT_{2A} blocker, was added to Krebs buffer containing

methoxamine and perfused through mesenteric bed for 20 min. Concentration-response curves to L-tryptophan were compared in the presence and absence of ketanserin.

To determine whether vasodilator responses to L-tryptophan are mediated through endothelium, almost all relaxing factors derived from endothelium were blocked by employing a combination of L-NAME (100 µM), indomethacin (10 µM), $BaCl_2$ (50 µM) and ouabain (10 µM). These compounds were dissolved in Krebs buffer containing methoxamine and were perfused through mesenteric bed for 20 min after taking the control responses. Concentration-response curves to L-tryptophan were again taken in the presence of these inhibitors.

Individual experiments were carried out with each of TEA (non-selective potassium channel blocker) (0.5 mM), $BaCl_2$ (inward-rectifying potassium channel blocker) (50 µM), ouabain (Na^+-K^+ ATPase pump inhibitor) (5 µM) or glibenclamide (K_{ATP} channel inhibitor) (10 µM) after taking a control concentration-response curve to L-tryptophan. These compounds were dissolved in Krebs buffer and were perfused through mesenteric bed for 20 minutes. Concentration-response curves to L-tryptophan were again taken in the presence of these inhibitors.

To verify, whether the vasodilator responses to L-tryptophan were mediated by inhibition of voltage-operated calcium channels, a concentration-response curve to L-tryptophan was determined in the presence of KCl (100 mM) induced constriction. Instead of methoxamine, KCl (100mM) was perfused through mesenteric bed for a

minimum of 20 min or more until the elevated perfusion pressure reached a steady state. Then, concentration-response curves to L-tryptophan were determined.

3.3.5.4 Procedure for endothelium denudation in perfused mesenteric vascular bed.

Initially, the concentration-response for ACh-evoked vasodilatation was determined in methoxamine (30 μM) constricted mesenteric vascular beds. Subsequently, endothelium was denuded by perfusing sodium deoxycholate (1.8 mg/mL) for 15 seconds followed by injecting air bubbles in the perfusate for a period of 5 min. The mesenteric bed was perfused with normal Krebs buffer for 1 hr and was allowed to recover. After the recovery period, the absence of endothelium was ascertained by confirming the lack of vasodilator responses to ACh (dose: 0.1 to 300 pmol) (85-95% inhibition). Concentration-response curves to L-tryptophan were determined prior to and after endothelium denudation in methoxamine constricted state.

3.4 Statistical Analysis

The vasoconstrictor and vasodilator responses to L- and D- tryptophan were determined were determined in Sprague-Dawley rats. Data points are the mean ± Standard error of the mean (SEM) for $n \geq 4$. Graphpad prism (Version 5.0) was used to perform the statistics. Differences between the means of vasoconstriction and vasodilatation responses to L- and D- tryptophan were compared by t-test. Differences between the means of control and each of ketanserin (2nM, 5nM and 10nM) were compared by ANOVA test ($n = 5$). All other comparisons between control and various compounds ($n \geq 4$) used to block the vasodilator responses to L-tryptophan were also compared by t-test. The differences were considered significant (*) only when $p < 0.05$.

CHAPTER 4. RESULTS

4.1 Vasoconstrictor effect of tryptophan

Vasoconstrictor responses to L- and D- tryptophan were concentration-dependent at basal perfusion pressure in mesenteric vascular bed. L-tryptophan evoked vasoconstrictor responses were higher than those from D-tryptophan. The representative data from a single experiment that compares the response to L- and D- tryptophan were presented in Fig 10 and 11. Table 1 shows the changes in perfusion pressure for each dose of L- and D- tryptophan. Each data point was expressed as the mean ± SEM of 5 observations. E_{max} values of L- and D-tryptophan were observed to be 141 ± 7 and 127 ± 8 (in mmHg) respectively. EC_{50} values for L- and D-tryptophan were observed to be 0.79 ± 0.30 and 0.25 ± 0.12 (in μmol) respectively (shown in Table 2). The leftward shift of the concentration-response curves to D-tryptophan as compared to L-tryptophan was observed (Fig 12). D-tryptophan was found to be more potent (from the EC_{50} values) than L-tryptophan. There was no significant difference in E_{max} values between the two stereo-isomers of tryptophan. The rightward shift of the concentration-response curve to L-tryptophan in the presence of different concentrations of ketanserin was observed (Fig 13). This rightward shift indicates that ketanserin (at various concentrations) competitively inhibits the vasoconstriction effects of L-tryptophan. Table 3 contains data for each dose of L-tryptophan expressed as means ± SEM in presence of different concentrations of ketanserin. EC_{50} and E_{max} values of L-tryptophan in presence of varying doses of ketanserin are shown in Table 4.

Figure 10: A representative experiment that shows the concentration-dependent vasoconstrictor responses to L-tryptophan in the perfused rat mesenteric vascular bed. Numbers in graph indicate increasing concentration of L-tryptophan. (1-7 corresponds to 0.01, 0.03, 0.1, 0.3, 1, 3 and 10 µmol) Similar responses were reproduced in 7 other preparations.

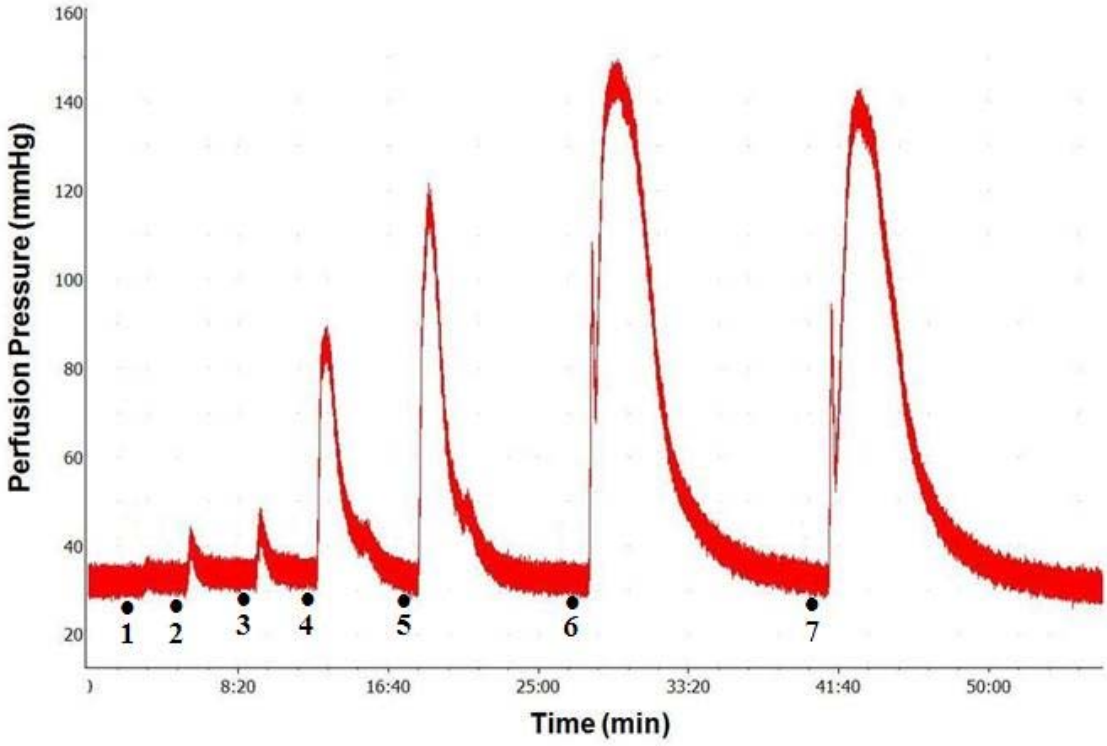


Figure 11: A representative experiment that shows the concentration-dependent vasoconstrictor responses to D-tryptophan in the perfused rat mesenteric vascular bed. Numbers in graph indicate increasing concentration of D-tryptophan. (1-7 corresponds to 0.01, 0.03, 0.1, 0.3, 1, 3 and 10 µmol) Similar responses were reproduced in 6 other preparations.

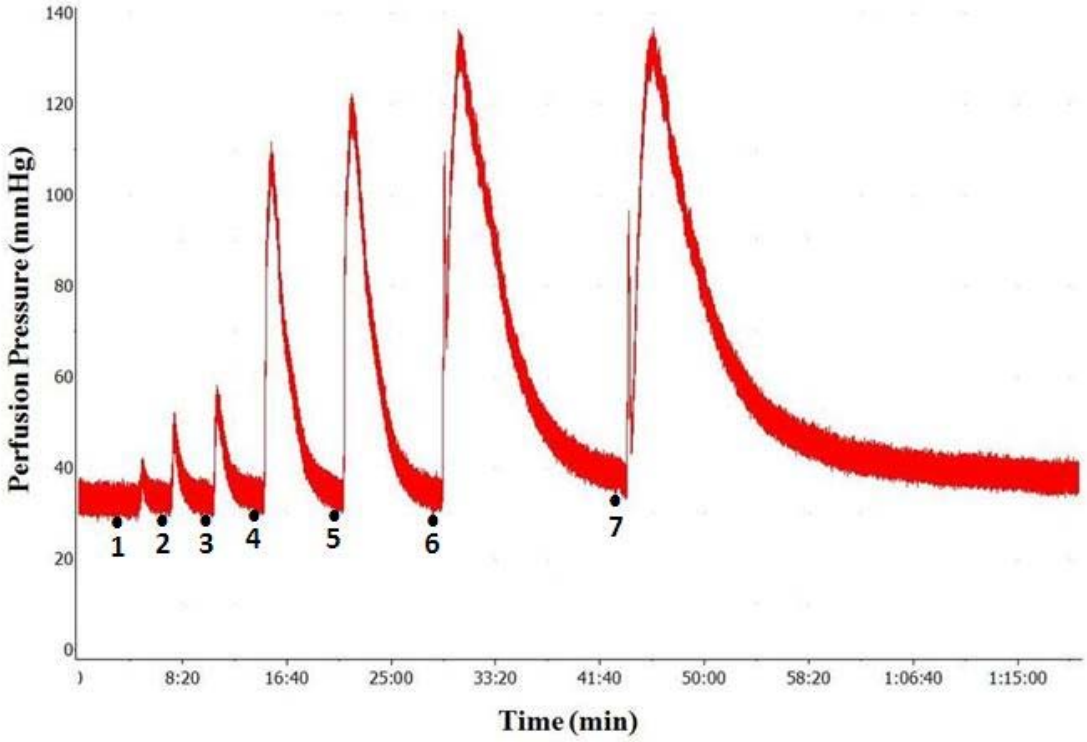


Table 1: Changes in perfusion pressure for each concentration of L- and D- tryptophan in isolated perfused rat mesenteric vascular bed. Data are expressed as mean ± SEM. N = 6.

Dose (µmol)	L-tryptophan response (mmHg)	D-tryptophan response (mmHg)
Baseline	29 ± 2	31 ± 1
0.01	31 ± 1	33 ± 1
0.03	35 ± 2	41 ± 2
0.1	40 ± 2	52 ± 2
0.3	64 ± 6	94 ± 6
1	99 ± 8	112 ± 5
3	132 ± 6	127 ± 8
10	141 ± 5	127 ± 9

Table 2: Comparison of EC_{50} and E_{max} values between L- and D- tryptophan in isolated perfused rat mesenteric vascular bed. Data are expressed as mean ± SEM. N = 6. *p < 0.05.

Drug	EC_{50} (µmol)	E_{max} (mmHg)
L-tryptophan	0.79 ± 0.30	141 ± 7
D-tryptophan	0.25 ± 0.12*	127 ± 8

Figure 12: The line graphs compare the concentration-response curves to L-tryptophan (○) and D-tryptophan (●) in isolated perfused rat mesenteric vascular bed. Each data point is expressed as mean ± SEM. N = 6. *$p < 0.05$.

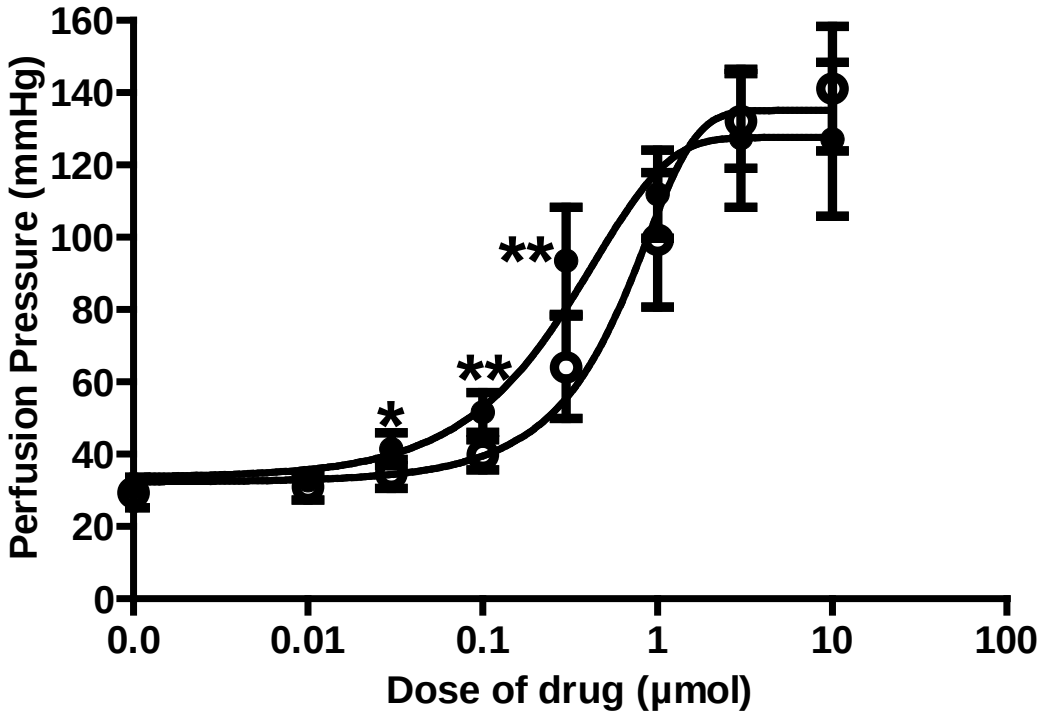


Figure 13: The following line graphs represent concentration-response curves to L-tryptophan in the presence of varying concentrations of ketanserin in isolated perfused rat mesenteric vascular bed. $N \geq 5$ for all graphs.

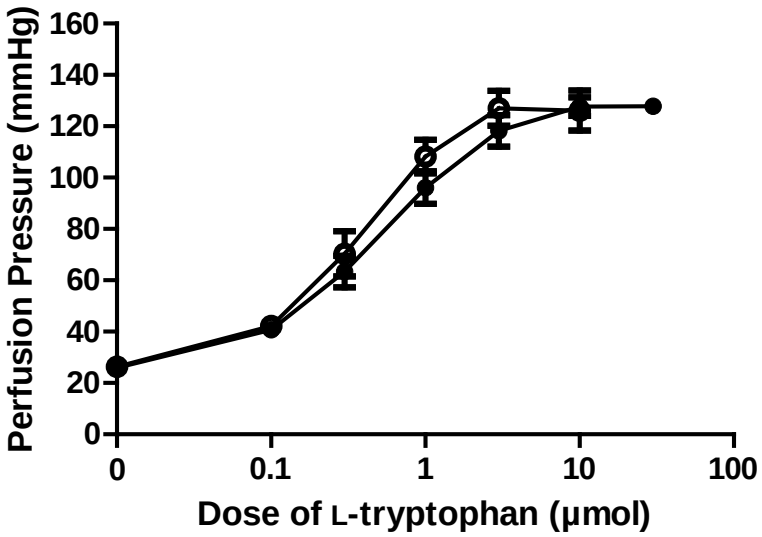


Figure 13a: Control (○) *vs* Ketanserin 2 nM (●)

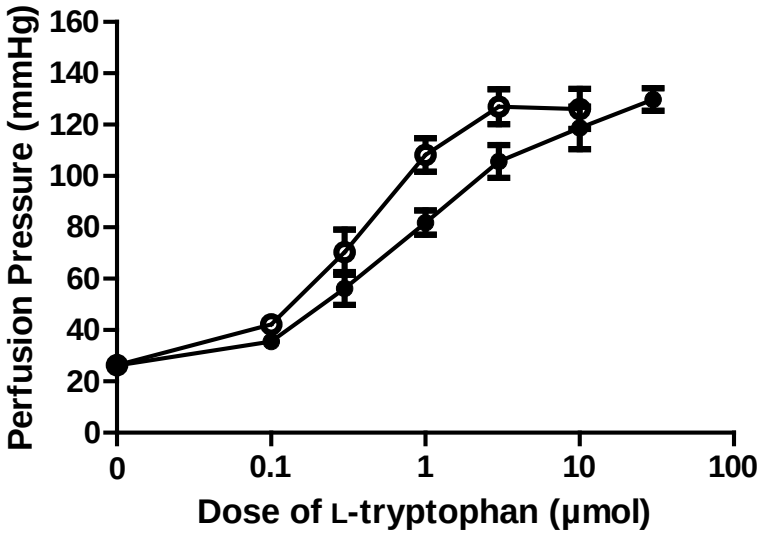


Figure 13b: Control (○) *vs* Ketanserin 5 nM (●)

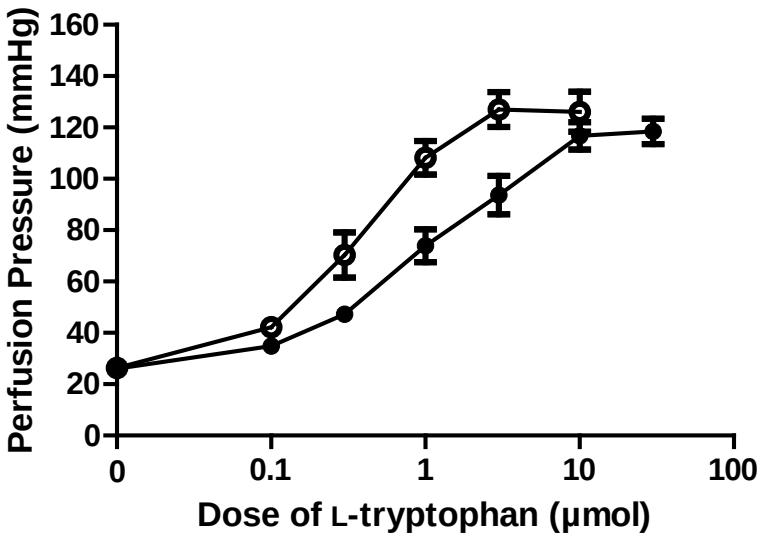


Figure 13c: Control (○) *vs* Ketanserin 10 nM (●)

Figure 14: The bar diagram compares the constrictor responses to L-tryptophan in the presence of varying concentrations of ketanserin 2 nM, 5 nM and 10 nM. Each data point expressed as mean ± SEM and N ≥ 5. (*$p < 0.05$).

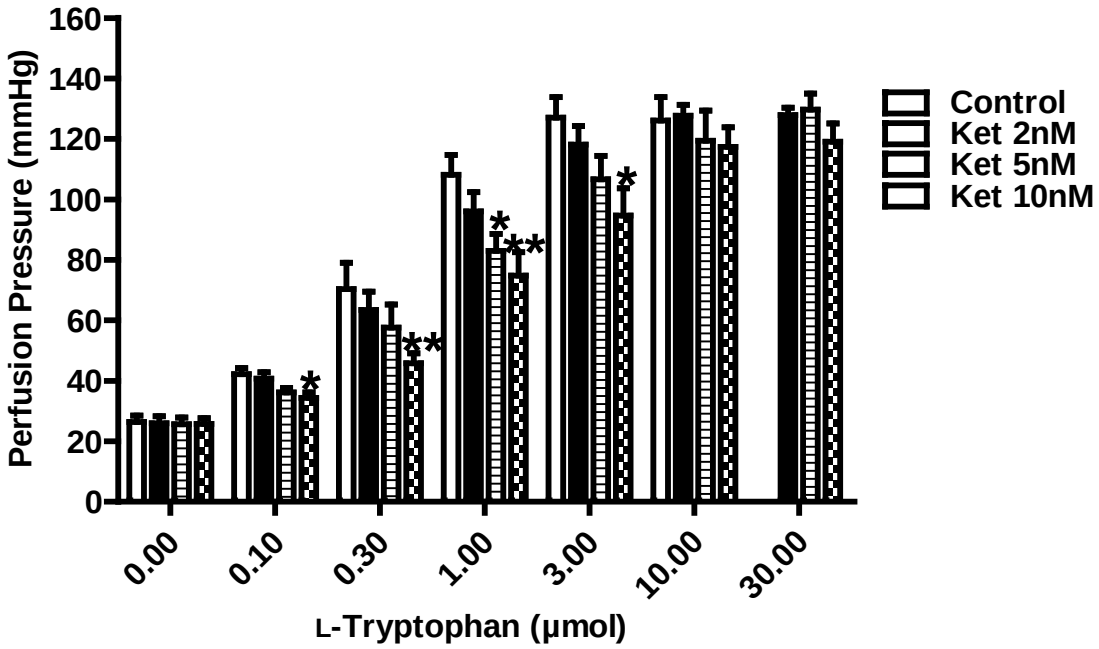


Table 3: Changes in perfusion pressure for each concentration of L-tryptophan in the presence of varying concentration of ketanserin. The data are expressed as mean ± SEM. $N \geq 5$.

Dose of L-tryptophan (µmol)	Control (mmHg)	Ketanserin 2nM (mmHg)	Ketanserin 5nM (mmHg)	Ketanserin 10nM (mmHg)
0.0	26 ± 2	26 ± 2	26 ± 2	26 ± 2
0.1	42 ± 2	41 ± 2	36 ± 1	35 ± 2
0.3	70 ± 9	63 ± 6	56 ± 6	47 ± 3
1	108 ± 7	96 ± 6	82 ± 5	74 ± 6
3	127 ± 7	118 ± 6	106 ± 6	94 ± 8
10	126 ± 7	128 ± 4	119 ± 8	117 ± 5
30		128 ± 3	130 ± 4	119 ± 5

Table 4: Comparison of EC_{50} and E_{max} values (expressed as mean ± SEM) of L-tryptophan in presence of varying concentrations of ketanserin (Ket). $N \geq 5$. $^{*}p < 0.05$, $^{**}p < 0.01$.

Drug	Control	Ket 2nM	Ket 5nM	Ket 10nM
EC_{50} (µmol)	0.43 ± 0.2	0.53 ± 0.2	$0.91 \pm 0.4^{*}$	$1.20 \pm 0.5^{**}$
E_{max} (mmHg)	141 ± 6	128 ± 4	130 ± 4	119 ± 5

4.2 Vasodilator effects of tryptophan

L- and D-tryptophan evoked concentration-dependent vasodilatation in methoxamine constricted perfused mesenteric vascular beds. A representative experiment that shows concentration-dependent vasodilator responses to L- and D- tryptophan are shown (Figure 15 and 16). Table 5 shows % vasodilatation for each dose of L- and D-tryptophan. Maximum vasodilatation (% I_{max}) values for both L- and D- tryptophan was found to be 87.5 ± 1.2 and 96 ± 1.5 (Table 6). IC_{50} values (in µmol) for L- and D-tryptophan were found to be 2.78 ± 0.9 and 0.94 ± 0.3 respectively. The leftward shift of the concentration-response curves to D- tryptophan (as compared to L-tryptophan) was shown in Fig 17. Maximum vasodilatation responses to D-tryptophan were statistically significant as compared to L-tryptophan.

The concentration-vasodilator response curves to L-tryptophan were also determined in the presence and absence of ketanserin (Fig 18). Table 6 shows the IC_{50} and I_{max} values in the presence and absence of ketanserin (5nM). Figure 18 and table 6 clearly suggest that 5-HT_{2A} were not involved in the vasodilator response to L-tryptophan. Fig 19a and Fig 19b compare the vascular effects of L-tryptophan and 5-hydroxytryptamine (5-HT). L-tryptophan produced a dose-dependent fall in perfusion pressure while 5-HT evoked a vasoconstrictor effect as seen with an increase in perfusion pressure. Fig 20 shows the vascular effects of L-tryptophan in KCl (100 mM) induced constriction. A dose-dependent increase in perfusion pressure was observed.

The likely role of the relaxing factors from the endothelium contribute to the vasodilator effects of L-tryptophan were assessed by including a combination of inhibitors such as L-NAME (100 μM) + indomethacin (10 μM) + $BaCl_2$ (50 μM) + ouabain (10 μM). Endothelium denudation was verified by the attenuation of vasodilatation responses to acetylcholine as shown in Fig 21. Fig 22 compares the concentration-response curve of L-tryptophan in the presence and absence of inhibitors of endothelium dependent relaxing factors. Apparently, there was no blockade of the vasodilatation response to L-tryptophan. To verify the above data, endothelium was denuded with an alternative technique using air and sodium deoxycholate. Fig 23 shows and compares the vasodilator responses to L-tryptophan in the presence and absence of endothelium. There was no significant block in the vasodilator responses to L-tryptophan in the absence of endothelium.

Inclusion of the appropriate concentrations of either glibenclamide, a K_{ATP} blocker (10 μM); tetraethyl ammonium, a non-selective potassium channel blocker (0.5 mM); ouabain, a Na^+-K^+ ATPase pump inhibitor (10 μM) or a K_{ir} channel inhibitor, $BaCl_2$ (50 μM) failed to affect the vasodilator responses to increasing concentration of L-tryptophan in methoxamine preconstricted perfused mesenteric vascular bed (See Fig 24, 25, 26 and 27). Our results clearly indicate that there was no block in the vasodilator response to L-tryptophan.

Figure 15: A representative experiment that shows concentration-dependent vasodilator responses to L-tryptophan in methoxamine constricted perfused rat mesenteric vascular bed. Numbers in graph indicate increasing concentration of L-tryptophan (1-8 corresponds to 0.01, 0.03, 0.1, 0.3, 1, 3, 10 and 30 µmol). Similar responses were reproduced in 12 other preparations.

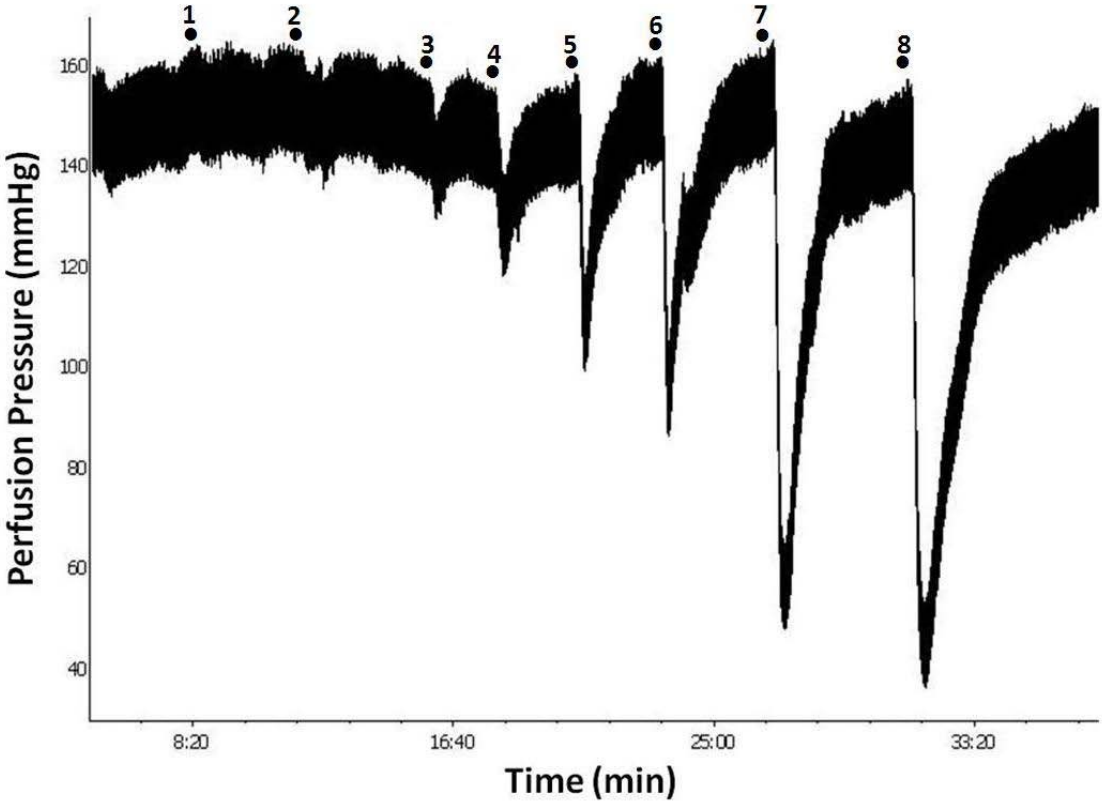


Figure 16: A representative experiment that shows concentration-dependent vasodilator responses to D-tryptophan in methoxamine constricted perfused rat mesenteric vascular bed. Numbers in graph indicate increasing concentration of D-tryptophan (1-8 corresponds to 0.01, 0.03, 0.1, 0.3, 1, 3, 10 and 30 µmol). Similar responses were reproduced in 3 other preparations.

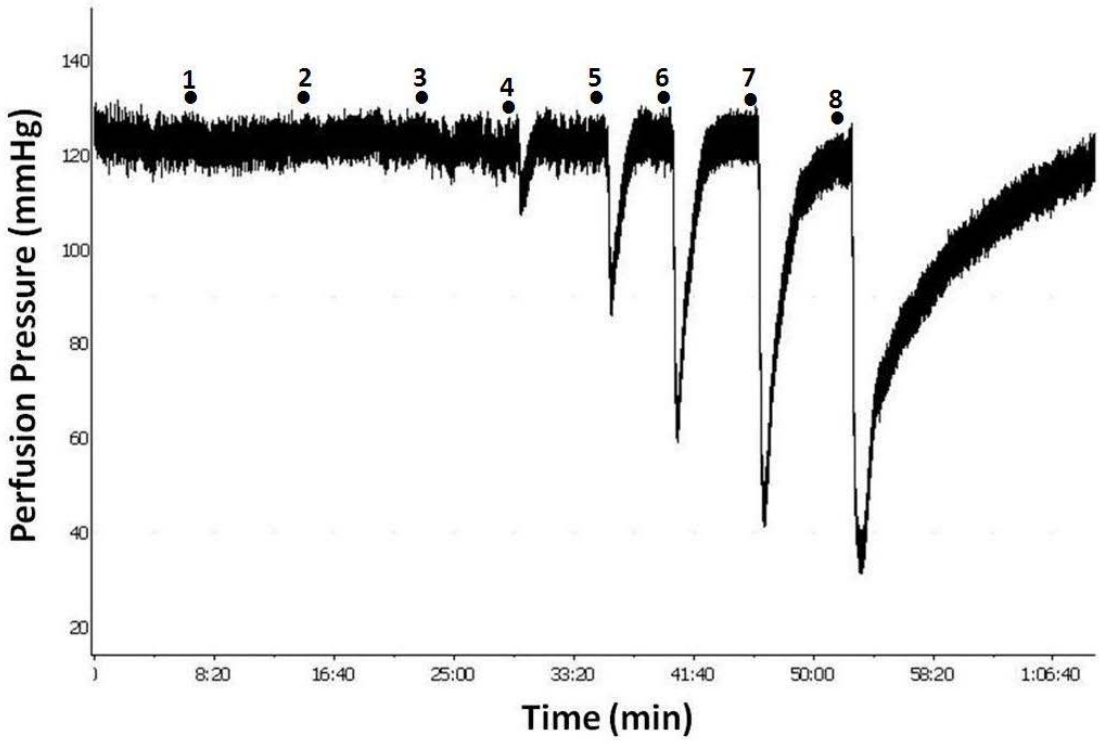


Figure 17: The line graphs compare concentration-vasodilator responses to L-tryptophan (○) and D-tryptophan (●) in isolated rat mesenteric vascular bed. Each data point is the mean ± SEM. N = 4. *$p < 0.05$.

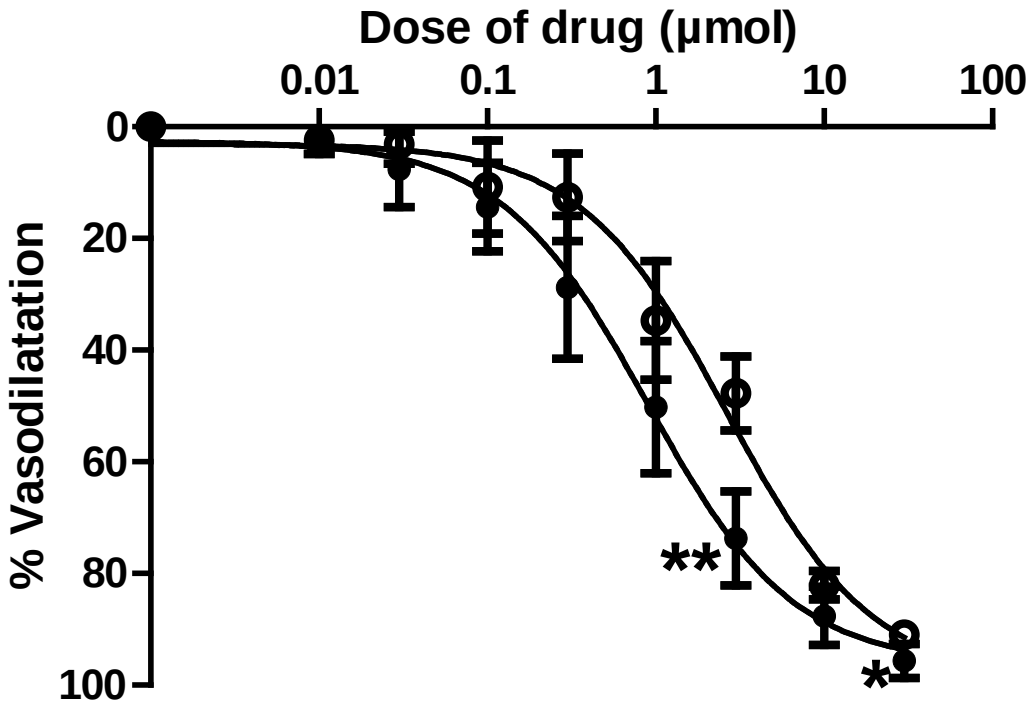


Table 5: % Vasodilatation for each concentration of L- and D- tryptophan. Each entry is the mean ± SEM. N = 4.

Dose	% Vasodilatation to L-tryptophan	% Vasodilatation to D-tryptophan
0.01	2 ± 1	2 ± 1
0.03	3 ± 2	8 ± 3
0.10	11 ± 4	15 ± 4
0.30	13 ± 4	29 ± 6
1.00	35 ± 5	50 ± 6
3.00	48 ± 3	74 ± 4
10.00	82 ± 1	88 ± 3
30.00	91 ± 1	96 ± 2

Table 6: IC_{50} and I_{max} values of L- and D-tryptophan. Comparison of IC50 values between L- and D-tryptophan. Each data is expressed as mean ± SEM. ¶N = 4 and ¶¶N=16. $^{*}p < 0.05$.

Drug	IC_{50} (µmol)	I_{max} (% Vasodilatation)
L-tryptophan¶¶	2.78 ± 0.9	87.5 ± 1.2
D-tryptophan¶	0.94 ± 0.3*	96 ± 1.5

Figure 18: Effect of (■) ketanserin (5 nM) on the vasodilator responses of (□) L-tryptophan in methoxamine constricted isolated perfused rat mesenteric vascular bed with each data point is the mean ± SEM and N = 4. (*$p < 0.05$; **$p < 0.01$).

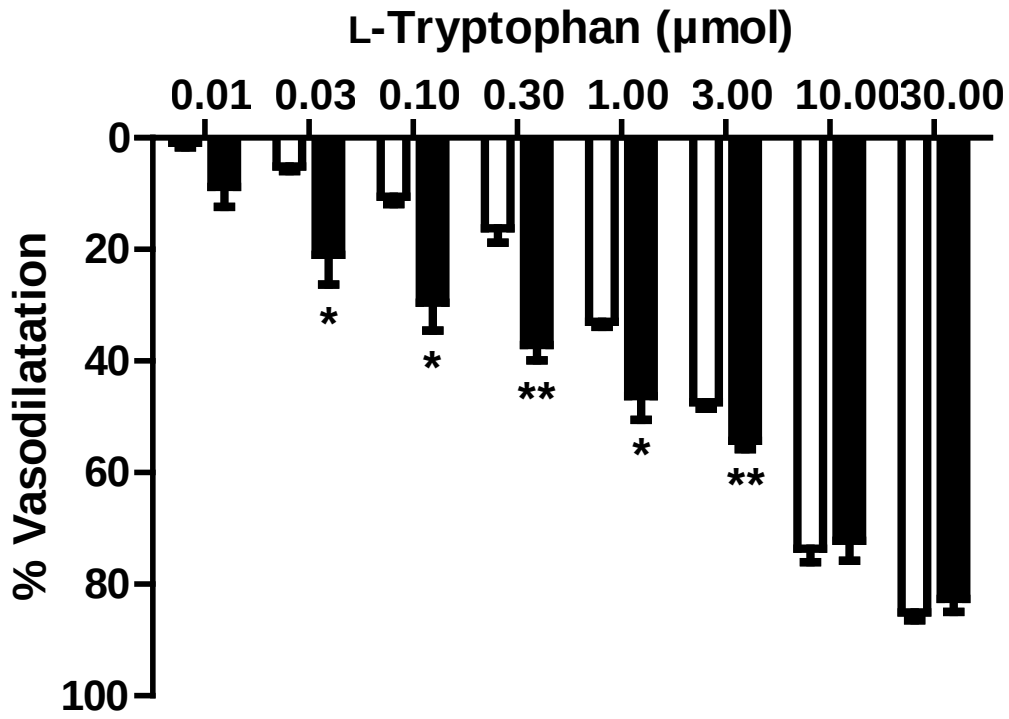


Table 7: IC_{50} and I_{max} values of L-tryptophan in presence and absence of ketanserin (5 nM). N = 4.

Drug	IC_{50} (µmol)	I_{max} (% Vasodilatation)
Control	2.54 ± 0.6	85 ± 1
Ketanserin (5nM)	1.59 ± 0.9	82 ± 2

Figure 19: Comparison of the vascular effects of L-tryptophan and 5-hydroxytryptamine (5-HT). Each data point is the mean ± SEM.

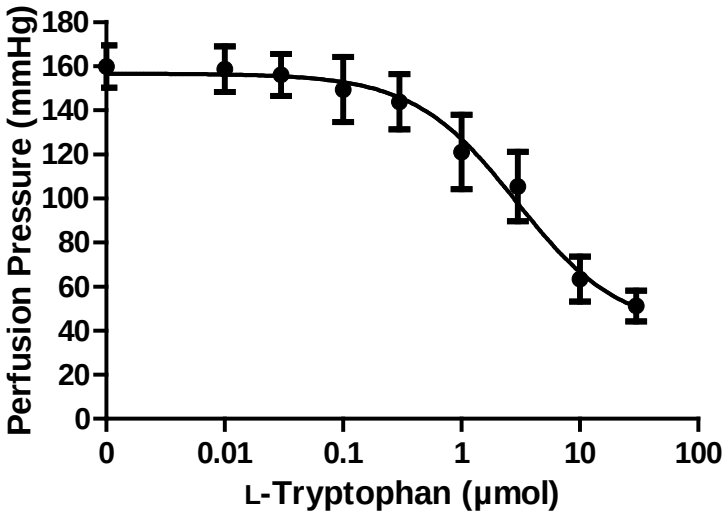


Figure 19a) L-tryptophan concentration-response curve in methoxamine induced constriction. L-tryptophan evokes concentration-dependent vasodilatation in MTX constricted isolated perfused rat mesenteric vascular bed. N = 4.

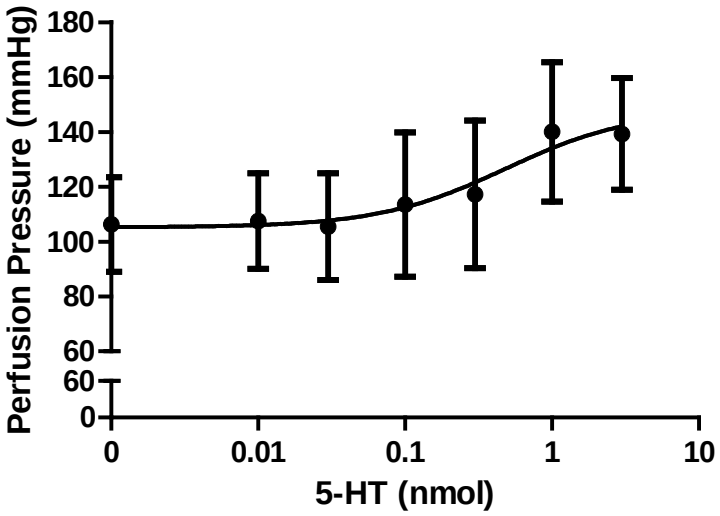


Figure 19b) 5-Hydroxytryptamine (5-HT) concentration-response curve in methoxamine induced constriction. 5-HT evokes concentration-dependent vasoconstriction in MTX constricted isolated perfused rat mesenteric vascular bed. N = 4.

Figure 20: Concentration-response curve to L-tryptophan in KCl (100mM) induced constriction in isolated perfused rat mesenteric vascular bed. Each data point is the mean ± SEM and N = 4.

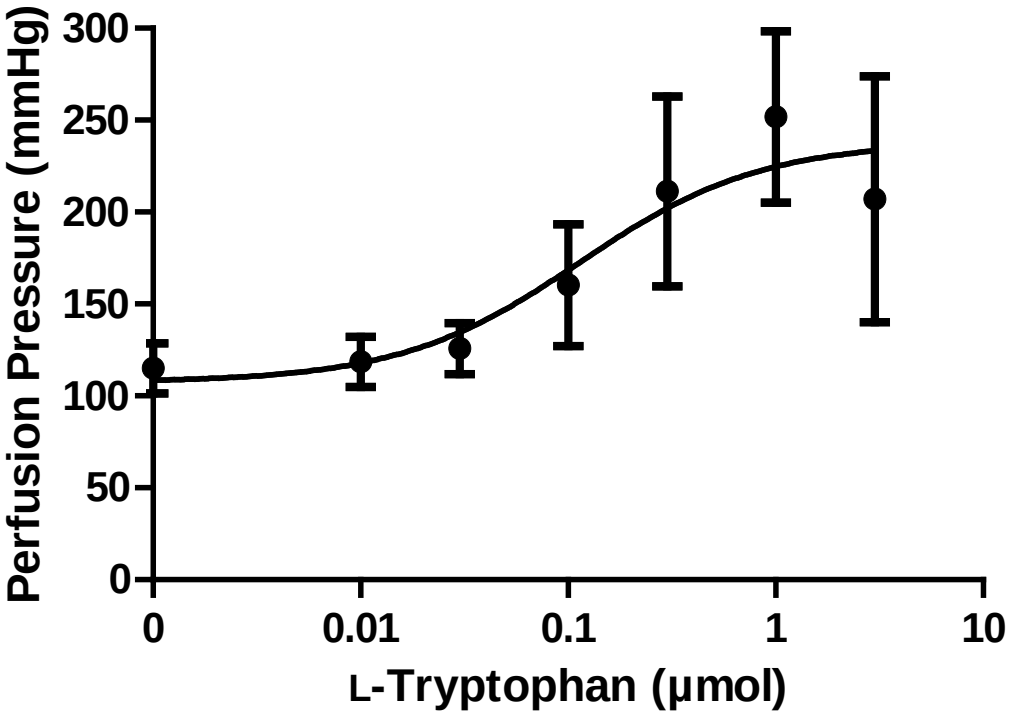


Figure 21: A line graph showing the attenuation of vasodilator responses to ACh after endothelium denudation in methoxamine constricted isolated perfused rat mesenteric vascular bed. Each data point is the mean ± SEM and N = 4.

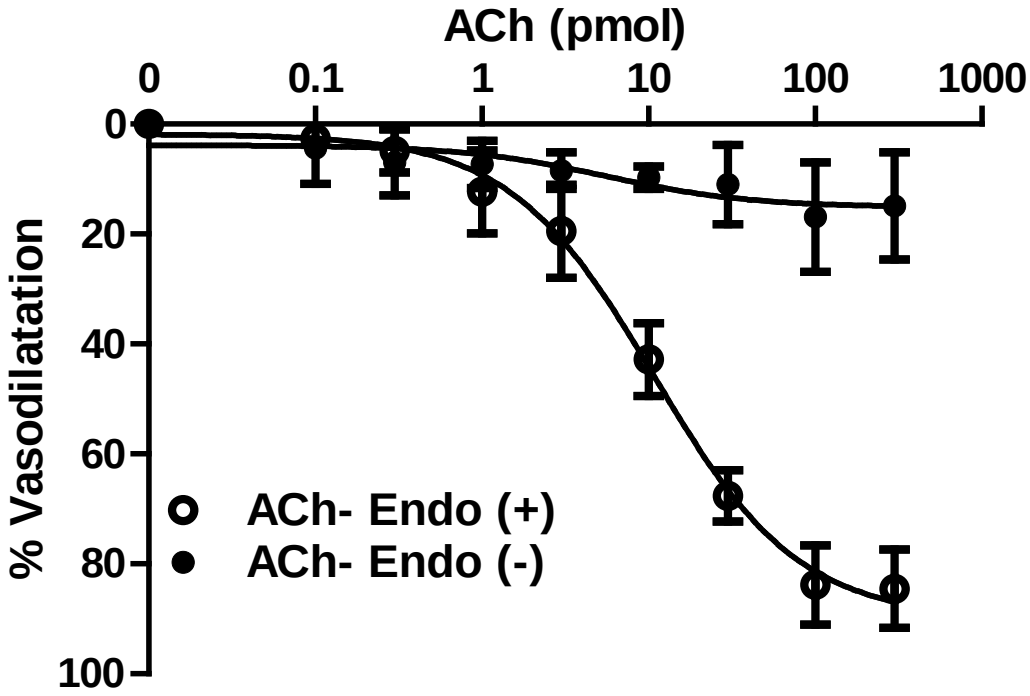


Figure 22: Vasodilator effects of L-tryptophan in absence (○) and presence (●) of combination of L-NAME (100 µM), indomethacin (10 µM), $BaCl_2$ (50 µM) and ouabain (10 µM) in methoxamine constricted isolated perfused rat mesenteric vascular bed. Each data point is the mean ± SEM and N = 4.

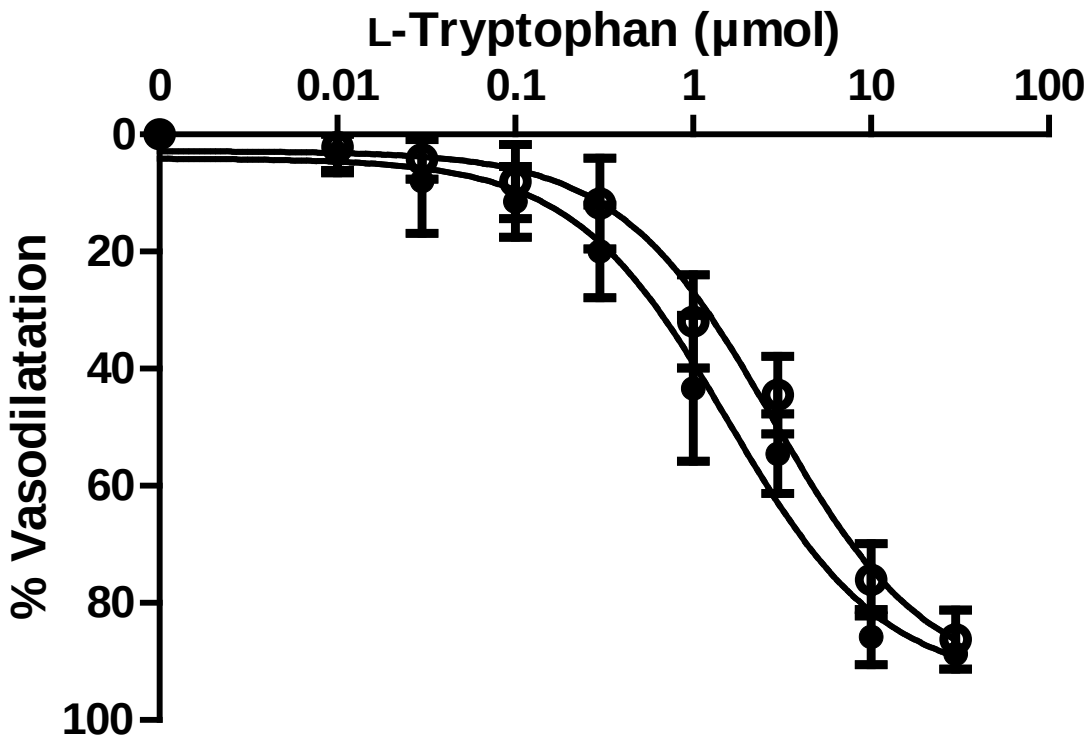


Figure 23: Vasodilator responses to L-tryptophan in presence and absence of endothelium in methoxamine constricted isolated rat perfused mesenteric vascular bed. Each data point is the mean ± SEM and N = 4. *$p < 0.05$.

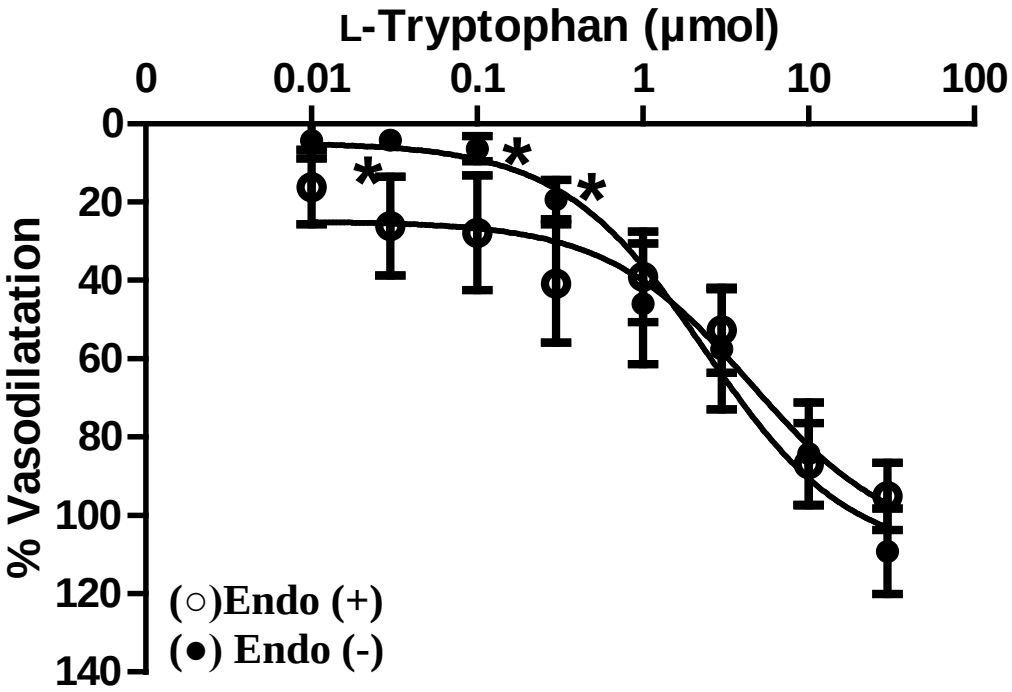


Figure 24: Effect of glibenclamide (10µM) on vasodilator response to L-tryptophan in methoxamine constricted perfused mesenteric vascular bed. Each data point is the mean ± SEM and N = 4. *$p < 0.05$.

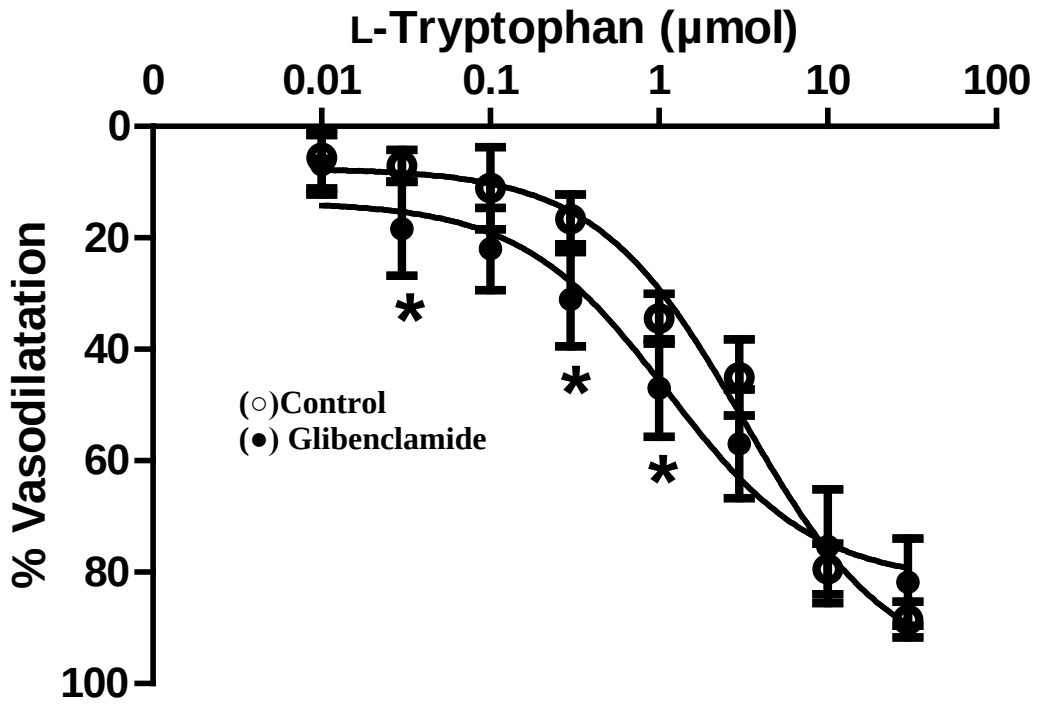


Figure 25: Effect of TEA (0.5 mM) on vasodilator response to L-tryptophan in methoxamine constricted isolated perfused rat mesenteric vascular bed. Each data point is the mean ± SEM and N = 4. *$p < 0.05$.

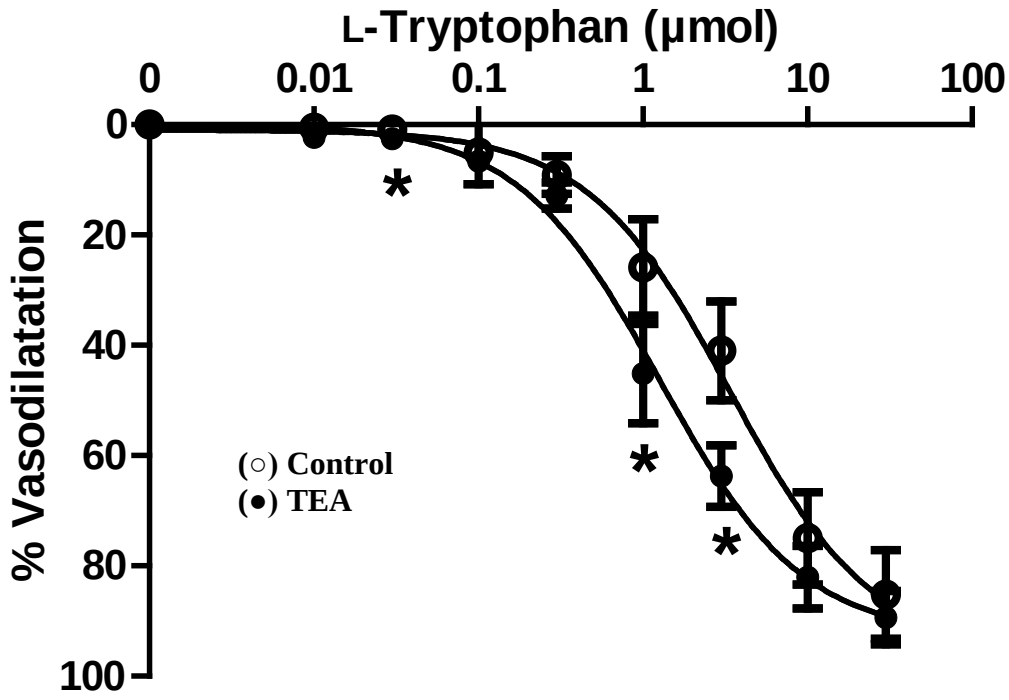


Figure 26: Effect of $BaCl_2$ (50 µM) on vasodilator response to L-tryptophan in methoxamine constricted isolated perfused rat mesenteric vascular bed. Each data point is the mean ± SEM and N = 4. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

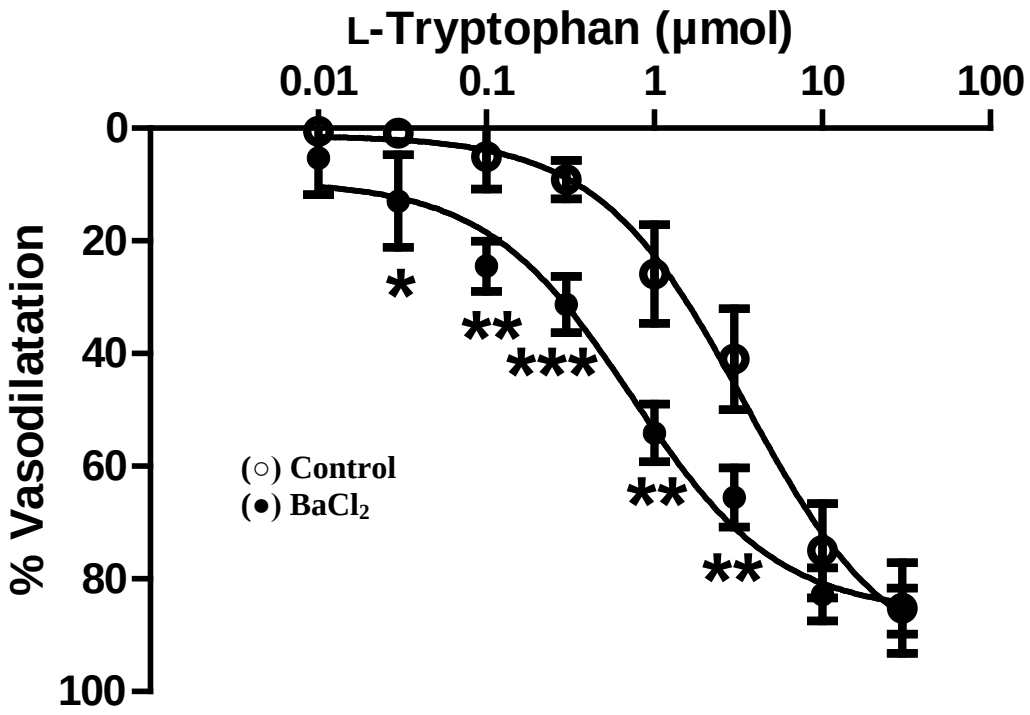
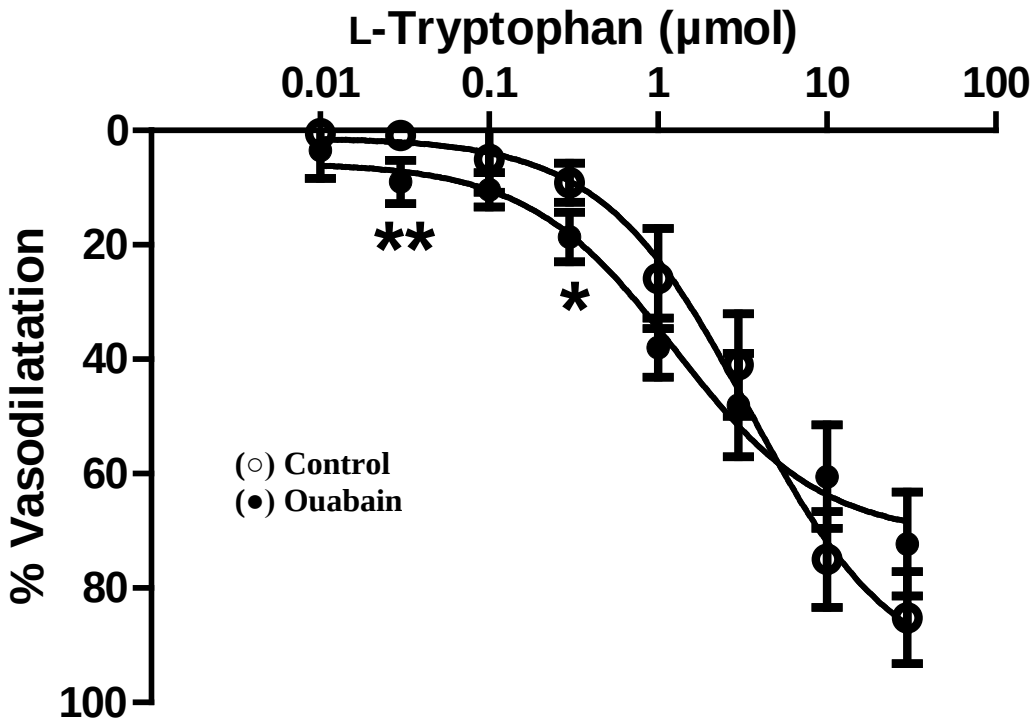


Figure 27: Effect of ouabain (10 µM) on vasodilator response to L-tryptophan in methoxamine constricted isolated perfused rat mesenteric vascular bed. Each data point is the mean ± SEM and N = 4. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.



CHAPTER 5. DISCUSSION

5.1 Vasoconstrictor response to tryptophan

The present investigation demonstrates that both L- and D-tryptophan evoke concentration-dependent (0.01 to 10µmol) increases in perfusion pressure of the mesenteric vascular bed. Most of higher organisms such as mammals recognize only the L-stereoisomer of amino-acid for protein synthesis and other functions. Experiments showed that L-tryptophan evoked slightly higher contractile response than D-tryptophan. Although, there was no significant difference in the maximum constrictor responses between the two isomers, EC_{50} values clearly indicate that D-tryptophan was more potent that L-tryptophan.

To characterize the mechanism of contractile response, our first target was to block 5-HT receptors. As of now, 5-HT receptors have been classified into seven major subtypes (5-HT_{1-7}) (Linder et al., 2007). From the literature, we found that 5-HT_{2A} and 5-HT_{2B} receptors are the main contractile receptors in the rat renal artery. The 5-HT_{2B} receptor is the primary contractile receptor in aorta. The 5-HT_{2A} subtype is the predominant vasoconstrictor receptor activated by 5-HT in mesenteric vascular bed (Watts, 2002). Ketanserin, a specific 5-HT_{2A} antagonist was used to attenuate vasoconstrictor responses. It is known that ketanserin has more affinity towards 5-HT_{2A} and it also binds less potently to other 5-HT receptor subtypes, alpha-adrenergic and dopamine receptors (Nippon Yakurigaku Zasshi, 1989). At doses of ketanserin greater than 10nM, non-competitive blockade of vasoconstriction responses to L-tryptophan was

observed. This means that at higher doses, ketanserin is non-specific. At appropriate doses of ketanserin (2nM, 5nM and 10nM), vasoconstrictor effects of L-tryptophan were competitively blocked. A rightward shift in the concentration-response curve was observed with increasing in concentrations of ketanserin. EC_{50} values in the presence of varying concentrations of ketanserin are an indication for the rightward shift in the concentration-response curve to L-tryptophan. However, it is important to note that the E_{max} values in the presence of different concentrations of ketanserin were not statistically significant. This proves that ketanserin (2 nM, 5 nM and 10 nM) competitively blocks the vasoconstrictor responses to L-tryptophan. Since there is no significant variation in the E_{max} values between D- and L-stereoisomers of tryptophan, we believe that D-tryptophan also has the same mechanism of action.

5.2 Vasodilator response to tryptophan

The current investigation also demonstrates the vasodilator effects of tryptophan when the perfusion pressure was elevated with methoxamine (α_1 specific agonist) in perfused rat mesenteric vascular bed. Both L- and D-tryptophan evoked a concentration-dependent decrease in perfusion pressure at pharmacological doses. However, due to unknown reasons, the vasodilator effect of L-tryptophan (I_{max}: 87.49 ± 1.21 %) (n=16) was lower than that of D-tryptophan (I_{max}: 95.74 ± 1.54 %) (n=4). The IC_{50} value for D-tryptophan was also found to be lower than L-tryptophan.

5.2.1 Role of 5-HT receptors

As 5-HT_2 receptors are involved in the vasoconstrictor effect of L-tryptophan, we thought of the former may also play an important role in mediating the vasodilator responses. So ketanserin (5 nM) was used to block 5-HT_{2A} receptors. Ketanserin (10nM) was not ideal because it significantly decreased perfusion pressure which was elevated by methoxamine (α_1-receptors). Ketanserin (2nM) did not have much effect on the elevated perfusion pressure but the extent of 5-HT_{2A} receptor blockade with that dose was unclear. Ketanserin (5nM) was chosen, since it did not decrease elevated perfusion pressure significantly (<10 mmHg). Results show that vasodilator effects of L-tryptophan (at lower doses) were actually potentiated as compared to control. The most probable explanation for this result is the fact that 5-HT_{2A} receptors are involved mostly in vasoconstriction responses in mesenteric vascular bed. Since vasoconstriction effect of this receptor is blocked by ketanserin, we actually see more vasodilatation response to L-tryptophan. Although the I_{max} values in presence and absence of ketanserin did not change

significantly, vasodilator effect of L-tryptophan at lower doses were significantly potentiated in the presence of ketanserin.

The possibility that other 5-HT receptors might be responsible for vasodilator responses to tryptophan cannot be ruled out. Literature studies reveal that 5-HT_{2B}, 5-$HT_{1B/1D}$ and 5-HT_7 are involved in the vasodilator responses to 5-HT in different vascular preparations (Ellis et al., 1995; Centurión D et al., 2004; Terrón JA et al., 2007). Concentration-response curves to L-tryptophan and 5-HT were taken in presence of methoxamine induced constriction. The results clearly show that 5-HT evoked a concentration-dependent increase in perfusion pressure while the effect of L-tryptophan was exactly the opposite.

The following conclusions can be derived from these results:

A) L-tryptophan does not exert vasodilator effects by converting into 5-HT. If L-tryptophan would have been converted to 5-HT and then exerted its effect, then vasoconstrictor effect of L-tryptophan would have been observed which is not the case. Thus the vasodilator effect of tryptophan is independent of its conversion to 5-HT.

B) 5-HT receptors are not involved in the vasodilator response to tryptophan. We know that 5-HT itself is an agonist and will obviously have more affinity and intrinsic activity than L-tryptophan for 5-HT receptors. If any of the 5-HT receptors had been involved in evoking vasodilatation to L-tryptophan, then 5-HT would have evoked vasodilatation by

activating that receptor, which is not the case. Thus, 5-HT receptors are not responsible for the vasodilator effect of tryptophan.

5.2.2 Role of calcium channels

The possibility that L-tryptophan acts as a calcium channel antagonist cannot be ignored. Potassium chloride (KCl 100 mM) was used to constrict perfused mesenteric bed instead of methoxamine. KCl evoked constriction is mediated through opening of calcium channels. L-tryptophan responses were determined in presence of KCl induced constriction in mesenteric vascular bed. From the results, it is clear that L-tryptophan evoked a concentration-dependent vasoconstriction in perfused mesenteric bed. Thus, this suggests that L-tryptophan is not a calcium channel antagonist.

5.2.3 Role of endothelium

Relaxing factors released from the endothelium might play a role in mediating the vasodilator responses to L-tryptophan was considered. ACh was used as a test drug to ascertain the presence of endothelium. Endothelium denudation/removal was characterized by attenuation of vasodilator responses to ACh. Most of the vasodilator factors released from endothelium were blocked by using a combination of L-NAME, indomethacin (a non-selective COX inhibitor), $BaCl_2$ (K_{ir} inhibitor) and ouabain (Na^+-K^+ ATPase pump blocker). Responses to L-tryptophan were taken in presence and absence of these blockers. Results showed that there is no significant difference between responses in the two groups. This proves that endothelium is not involved in the vasodilator responses to L-tryptophan.

To eliminate the possibility of other vasodilatation factors released from endothelium, the latter was denuded with the help of sodium deoxycholate and air. Results indicate that there is no significant change in the I_{max} following endothelium removal. Thus for the second time, we confirm that endothelium does not play any role in vasodilatation response to L-tryptophan.

5.2.4 Role of potassium channels

The possibility that potassium channels located on VSMC could be targeted by L-tryptophan to promote vasodilatation was considered. In one of the experiments, glibenclamide was used to block ATP-dependent potassium channels. Another set of experiments, we included tetraethylammonium (TEA) which is a non-selective potassium channel blocker. Experiments were also performed in the presence of $BaCl_2$, which is an inward-rectifying potassium channel blocker (K_{ir} blocker). Experiments aimed at blocking Na+-K+ ATPase pump were also carried out in the presence of ouabain.

Results showed that inclusion of glibenclamide, TEA, $BaCl_2$, L-NAME and ouabain did not produce any appreciable blockade of vasodilator response to L-tryptophan. However, a significant potentiation of vasodilatation at low doses of L-tryptophan was observed in presence of all the blockers mentioned previously. The reason for potentiation is currently unknown.

CHAPTER 6. CONCLUSION

6.1 Summary of major findings for vasoconstriction responses to tryptophan

- Addition of tryptophan evoked a concentration-dependent vasoconstriction in perfused rat mesenteric vascular bed.
- There were no significant differences between the maximum vasoconstriction effects (E_{max}) evoked by L- and D-tryptophan.
- EC_{50} values for D- and L-tryptophan were $0.25 \pm 0.12^{*}$ and 0.79 ± 0.30 μmol respectively ($^{*}p < 0.05$).
- Vasoconstrictor effects to L-tryptophan were competitively blocked by ketanserin, a 5-HT_{2A} selective antagonist, since there was a concentration-dependent rightward shift for L-tryptophan in the presence of increasing concentration of ketanserin.
- There was no significant difference in E_{max} values, which proves that ketanserin at doses (5 nM & 10 nM) competitively blocks vasoconstrictor effects of L-tryptophan.
- Thus tryptophan produces 5-HT dependent vasoconstrictor responses in perfused mesenteric vascular bed.
- Since there was no significant change in the E_{max} of both L- and D-tryptophan, we believe it is highly likely that ketanserin blocks D-tryptophan in the same way.

6.2 Summary of major findings for vasodilator responses to tryptophan

- Tryptophan produced a dose- dependent vasodilatation in methoxamine (α_1- specific agonist) constricted perfused mesenteric vascular bed.
- Inclusion of optimal concentration of ketanserin, a 5-HT_{2A} selective antagonist, failed to affect the vasodilator responses to L-tryptophan as there were no significant changes in IC_{50} and I_{max} values of L-tryptophan. These data confirm that the vasodilator responses to L-tryptophan is not mediated by its conversion to 5-HT.
- Contrary to vasodilator responses to L-tryptophan, addition of 5-HT produced a dose-dependent increase in perfusion pressure when perfused mesenteric vascular bed was constricted with methoxamine.
- Thus vasodilator effect of L-tryptophan is independent of its conversion to 5-HT.
- When KCl (opens Ca^{2+} channels) was used to constrict perfused mesenteric vascular bed, L-tryptophan produced a dose-dependent increase in perfusion pressure. Thus, the vasodilator response to L-tryptophan is not due to blockade a Ca^{2+} channels.
- TEA (tetraethylammonium), which is a non-selective Ca^{2+} activated potassium channel blocker (K_{Ca}) failed to affect the vasodilator responses to L-tryptophan.
- Ouabain, a Na^{+}-K^{+} ATPase inhibitor failed to affect the vasodilator responses to L-tryptophan.
- $BaCl_2$, which is a K_{ir} (inward rectifying potassium channel blocker) also failed to affect the vasodilator affect of L-tryptophan.
- Glibenclamide (K_{ATP} channel blocker) did not have any effect on vasodilator response to L-tryptophan.

- L-NAME, indomethacin, $BaCl_2$ and ouabain in combination failed to affect the vasodilator responses to L-tryptophan, an observation similar to that seen in the absence of endothelium.
- Despite the above mentioned innovating and interesting findings, the likely mechanism that mediates vasodilator responses to L- and D-tryptophan remains to be established.
- The data from the present study has ruled out the potential role of endothelium-dependent vasodilator mechanisms.
- In addition, the likely participation of either blockade of Ca^{2+} channels and activation of different vascular K^+ channels has been ruled out. It is therefore likely that L-tryptophan could affect events at the level of Ca^{2+}-independent mechanisms such as Rho-Kinase that regulates VSM tone to promote endothelium-independent vasodilatation.

6.3 Significance of tryptophan study

Tryptophan is one of the essential amino acids. It has been known for years that high protein diet increases the plasma concentration of amino acids. To our understanding, literature does not reveal any *in-vitro* study to explain the vascular effects of tryptophan. *In-vivo* studies using tryptophan were carried out in 1980s. Antihypertensive action of L-tryptophan (*in-vivo*) in spontaneously hypertensive rats (SHR) rats observed earlier were attributed to activation of serotonergic mechanism (Sved et al., 1982). In 1983, Wolf et al., proved that antihypertensive action of L-tryptophan is not mediated through brain serotonin or serotonergic neurons. Again in 1984, Wolf et al., proved that the antihypertensive effects of L-tryptophan in normotensive and SHR rats cannot be entirely explained through changes in brain serotonin levels. Antihypertensive action of indolepyruvic acid- a keto analogue of tryptophan was believed to be correlated with cerebral serotonin metabolism (Squadrito et al., 1989). Studies on chronic dietary treatment of L-tryptophan on SHR reduced the mean blood pressure but the exact mechanism of action was not established (Fregly et al., 1988).

To our knowledge, this is the first report showing the anti-hypertensive effect of L-tryptophan in an *ex-vivo* model of perfused mesenteric vascular bed. The data from the present study suggests that the significant vasodilatation seen in the perfused mesenteric vascular bed could contribute to the possible antihypertensive action reported earlier. We also believe that vasodilatation of 85-90% in resistance vessels of perfused mesenteric vascular bed might be the reason of fall in blood pressure as seen with SHR in previous

studies. (Wolf et al.,1984; Sved et al.,1982). We also confirm the presence of serotonergic system in the blood vessels as suggested earlier (Linder et al., 2007).

6.4 Limitations of the present study

1) The present study concludes that the vasoconstrictor responses to both L- and D-tryptophan were mediated by their likely conversion to serotonin (5-HT). This was based on the dose-dependent competitive shift characteristics that both isomers show in the presence of ketanserin. At this time, it is not known whether the level of shift attained in the dose-response curves to 5-HT in the presence of the same concentrations of ketanserin were similar or higher. Moreover, further studies of determination of the comparison of pA_2 values for ketanserin against L- and D-tryptophan *vs* 5-HT-evoked vasoconstrictor responses would settle whether all of the vasoconstrictor responses to both D- and L-tryptophan are indeed mediated by their conversion to 5-HT.

2) In methoxamine constricted but not KCl depolarized tissues, both L- and D-tryptophan evoked vasodilatation and this is not mediated by 5-HT since addition of 5-HT enhanced the vasoconstrictor responses further in both methoxamine constricted and KCl depolarized states. The mechanism that contributes to the vasodilator effect has not been elucidated in the present study although the likely role for endothelial vasodilator mediators, as well as vascular K_{ATP} and BK_{Ca} channel as well as voltage operated Ca^{2+} channel have been ruled out as possible candidates. Further studies are warranted to resolve the mechanism underlying endothelium-independent direct vasodilator responses to L-tryptophan.

6.5 Future directions

1) While the data from the present study provides evidence that both L-tryptophan and D-tryptophan exert a biphasic effect [vasoconstrictor and vasodilator responses] in the perfused mesenteric vascular bed, it is unclear whether this phenomenon exists in other vascular beds. Instead of repeating or extending the study to other vascular beds and using isolated blood vessels, it would be appropriate to establish first the regional hemodynamic effects of L- and D-tryptophan using microsphere distribution methodology. This will give us an estimate of the vascular beds that are sensitive to the actions of these amino acids in an *in vivo* model. This will also give an estimate of the changes in total peripheral resistance, cardiac output evoked by D- and L-tryptophan. Based on the above, more detailed mechanistic studies could be performed using isolated blood vessels/ perfused vascular bed preparations that are sensitive to tryptophan.

2) Previous studies have shown L-tryptophan reduces blood pressure in SHR (Wolf et al., 1984; 1984; Sved et al.,1982; Squadrito et al., 1989; Fregly et al., 1988). However, these studies were performed using either the tail cuff methodology or direct catheter recording of BP in anaesthetized rats. Both methods have serious limitations. It is now feasible to examine the chronic / long term effects of infusion of L- and D- tryptophan in conscious, freely moving rats using radiotelemetry methodology. Such studies, a logical extension will enhance the significance of this study.

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