VASCULAR REACTIVITY, CHEMICAL PROFILE, TOXICOLOGICAL AND PHARMACOKINETIC STUDIES OF *ANDROGRAPHIS PANICULATA* NEES. EXTRACTS

BY

SRIRAMANENI RAGHAVA NAIDU

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DEDICATION

My Wife, Son (Anurag), Parents, Brother, Uncle, and Relatives for their love, support and encouragement

and

To Lord Saibaba

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Raghava Naidu Sriramaneni

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LIST OF ABBREVIATION & SYMBOLS

ACh	=	Acetylcholine
5-HT	=	5-hydroxytryptamine (Serotonin)
AC	=	Adenylyl cyclase
ACEI	=	Angiotensin converting enzyme inhibitors
ACN	=	Acetonitrile
ADP	=	Adenosine di-phosphate
AECB	=	Aqueous extract of *Caesalpinia benthamiana*
AhR	=	Aryl hydrocarbon receptor
ANG	=	Andrographolide
ANOVA	=	Analysis of variance
AP_1	=	AP1- (sub-fraction)
AP_3	=	14-deoxy-11, 12-didehydroandrographolide
AP_4	=	Neoandrographolide
AP	=	*Andrographis paniculata*
APCE	=	*Andrographis paniculata* chloroform extract
ATP	=	Adenosine triphosphate
AUC	=	Area under the curve
BCG	=	Bacillus Calmette-Guein
$CaCl_2$	=	Calcium chloride
cAMP	=	Cyclic Adenosine Monophosphate
cGMP	=	Cyclic Guanosine Phosphate
CK-MB	=	Creatine kinase
CO	=	Cardiac output
COX	=	Cyclooxygenase
CVD	=	Cardiovascular disease
CYPs	=	Cytochrome P450s
DA	=	14-deoxyandrographolide
DAG	=	Diacyl glycerol
DDA	=	14-deoxy-11, 12-didehydroandrographolide
DMSO	=	Dimethylsulphoxide

ECG	=	Electro cardio gram
EDNO	=	Endothelium-derived nitric oxide
EDRF	=	Endothelium-derived relaxant factors
EDTA	=	Ethylene diamine tetra acetic acid
EGCG-(-)	=	Epigallocatechin- 3-gallate
ELT	=	Euglobulin lysis time
eNOS	=	Endothelial nitric oxide synthase
FO	=	Fish Oil
GM-CSF	=	Granulocyte macrophage colony stimulating factor
GMP	=	Guanosine monophosphate
GSTP	=	Glutathione S-transferase
GTP	=	Guanosine 5-triphospahte
H_2O_2	=	Hydrogen peroxide
HCT	=	Haemotocrit
HGB	=	Haemoglobin
HIV	=	Human immuno deficiency virus
HMP	=	Herbal medicinal plants
HOCl	=	Hypochlorous acid
HPBLs	=	Human peripheral blood lymphocytes
HPLC	=	High performance liquid chromatography
IP_2	=	Inositol di phosphate
IP_3	=	Inositol triphosphate
IP_3	=	Inositol-1, 4, 5-trisphosphate
IP	=	Intraperitoneal
KCl	=	Potassium chloride
KH_2PO_4	=	Potassium dihydrogen phosphate
LAD	=	Left anterior descending artery
LDH	=	Lactate dehydrogenase
LDL	=	Low-density lipoprotein
L-NAME	=	Nitro-L-arginine methyl ester
LPS	=	Lipopolysaccharide
LVEDP	=	Left ventricular end diastolic pressure
MAP	=	Mean arterial blood pressure

MARDI	=	Malaysian Agriculture Development Institute
MBP	=	Mean blood pressure
MCH	=	Mean corpuscular hemoglobin
MCHC	=	Mean corpuscular hemoglobin concentration
MCV	=	Mean cell volume
MLCK	=	Myosin light chain kinase
NaCl	=	Sodium chloride,
$NaHCO_3$	=	Sodium bicarbonate
NE	=	Norepinephrine
NMR	=	Nuclear magnetic resonance
N	=	Nicotinic receptors
NO	=	Nitric oxide
NOS	=	Nitric oxide synthase
NSAID	=	Non-steroidal anti-inflammatory drugs
NSBP	=	Non-invasive systolic blood pressure
ODQ-1H	=	[1,2,4]oxadiazolo[4,2-α]quinoxalin-1-one
ODQ	=	Oxadiazole-[4,3-a]-quinoxalin-1-one
PAF	=	Platelet-activating factor
PBG	=	Peak blood glucose
PDE	=	Phosphodiesterase
PE	=	Phenylephrine
PGI_2	=	Prostacyclin
PKC	=	Protein kinase C
PLC	=	Phospholipase C
PLT	=	Platelets count
PMA	=	Phorbol 12-myristate 13-acetate
PMNL	=	Polymorph-nuclear leukocytes
PMNs	=	Polymorphonuclear neutrophils
PPH	=	Postprandial hyperglycemia
PTFE	=	Polytetrafluoroethylene
PTLC	=	Preparative thin-layer chromatography
QPCR	=	Quantitative polymerase chain reaction
RBC	=	Red blood count

ROS	=	Reactive oxygen species
ROS	=	Reactive oxygen species
SBP	=	Systolic Blood Pressure
SD	=	Sprague-Dawley
sGC	=	Soluble guanylyl cyclase
SHR	=	Spontaneously hypertensive rats
SNP	=	Sodium nitroprusside
SOD	=	Super oxide dismutase
SPE	=	Solid phase extraction
TBA	=	Thiobarbituric acid
TCM	=	Traditional Chinese medicine
TIMP-1	=	Tissue inhibitors of metalloproteinase-1
TLC	=	Thin-Layer Chromatography
VEGF	=	Vascular endothelial growth factor
v/v	=	Volume in volume
WBC	=	White blood count
WE	=	Water extract
WHO	=	World Health Organization

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LIST OF PUBLICATIONS

1 **Raghva Naidu**. S., Amirin Sadikun & Mohd. Zaini Asmawi (2009). The Effect of extracts of *Andrographis paniculata* aerial parts on rat thoracic aorta. Pharmacognosy Research [Phcog Res.] 1 (2); 54-59.

2 **Raghava Naidu**. S., Omar Z. Ameer, Ibrahim M. Salman, G. Venkatesh, Amirin Sadikun, and Mohd. Zaini Asmawi. (2009). Pharmacokinetic study of *Andrographis paniculata* on experimental animals. Pharmacologyonline, 1; 309-319.

LIST OF PRESENTATIONS

1 **Raghava Naidu.S,** Ibrahim M. Salman, Omar Z. Ameer, Amirin Sadikun, Mohd. Zaini Asmawi. Acute and Subacute Toxicological Study of Standardized Chloroform Extract of *Andrographis paniculata* on Experimental Animals. 13th Biological Graduate Conference. National University of Singapore, 15th – 18th December (2008).

2 **Raghava Naidu.S**, Asmawi. M.Z. and Amirin S. Chronic Treatment of Chloroform Extract of *Andrographis paniculata* Prevents the Endothelial Dysfunction in Spontaneously Hypertensive Rat Thoracic Aorta. Presented on Malaysian society for physiology and pharmacology, University Malaya, 6th April (2008).

3 **Raghava Naidu. S.,** Asmawi. M. Z. and Amirin. S. Vasorelaxant effect of chloroform extract of *Andrographis paniculata* on *in-vitro* rat thoracic aorta. Presented poster on workshop on the "Isolated Tissue Preparations and HPLC Training" June (2007) at IIUM & MSPP, Kuantan, Malaysia.

KAJIAN REAKTIVITI VASKULAR, PROFIL KIMIA, TOKSIKOLOGI DAN FARMAKOKINETIK EKSTRAK *ANDROGRAPHIS PANICULATA* (NEES.)

ABSTRAK

Tujuan kajian ini adalah untuk menilai reaktiviti vaskular, profil kimia, ketoksikan dan farmakokinetik ekstrak *Andrographis paniculata.* Ekstrak kloroform *Andrographis Paniculata* (APCE) didapati memberikan kesan vasorelaksasi poten keatas kontraksi aruhan norepinefrin (NE) pada aorta toraks tikus. Analisis HPLC dan ^{1}H-NMR APCE menunjukan kehadiran andrografolida (ANG), 14-deoksiandrografolida (DA) dan 14-deoksi-11,12-didehidrografolida (DDA). Rawatan kronik selama 4 minggu dengan APCE 25, 50 dan 100 mg/kg/hari pada tikus hipertensif spontan (SHR) menunjukkan peningkatan relaksasi tergantung-endotelium dan tidak tergantung-endotelium terhadap asetilkolina (ACh) dan natrium nitroprusida (SNP) mungkin kerana pengaktifan nitrik oksida (NO) sintase dan juga perangsangan pengeluaran NO dalam sel-sel endotelium yang membawa kepada perencatan bagi lintasan kontraksi aruhan-Ca^{2+}. Penurunan tekanan darah sistole (SBP) secara signifikan ($p<0.001$) mungkin disebabkan oleh tindakan vasodilatasi pada saluran darah.

APCE, sub-fraksi AP (AP_1), DA dan DDA bergantung dos merencat kedua-dua kontraksi tonik aruhan-NE dan kontraksi aruhan-K^+ berkepekatan tinggi (80mM), mencadangkan yang APCE, AP_1, DA dan DDA bertindak sebagai penghalang saluran Ca^{2+} kepada kedua-dua saluran kendalian reseptor dan saluran tergantung potensi. DA, DDA dan APCE juga merencat kontraksi fasik aruahan-NE menyarankan yang DA, DDA dan APCE merencat pembebasan Ca^{2+} daripada

retikulum sarkoplasma. Pengurangan kepekatan Ca^{2+} yang menyumbang kepada vasorelaksasi yang diaruhkan oleh DA, DDA dan APCE berkemungkinan melalui perencatan influks Ca^{2+} dan perencatan pembebasan Ca^{2+} dalam sel. Kesan perencatan DA, DDA dan APCE keatas kontrakasi fasik aruhan dos rendah NE mungkin bertindak melalui perencatan influks Ca^{2+} melalui lintasan bebas kalsium.

Akhirnya kajian ketoksikan akut dan ketoksikan kronik APCE 100, 300, 1,000 dan 2,000 mg/kg/hari tidak menunjukan tanda-tanda ketoksikan sehingga ke akhir 28 hari jangkamasa penyelidikan. Tiada perubahan peningkatan berat badan mingguan dan profil hematologi serta perubahan profil makroskopik dan histopatologi organ dalaman semasa postmortem. Oleh itu, keputusan yang diperolehi mencadangkan yang APCE adalah tidak toksik sehingga 2,000 mg/kg. Dalam kajian farmakokinetik APCE menggunakan ANG dan DDA sebagai penanda menunjukkan farmakokinetik tak linear pada dos 1,000 mg/kg pada tikus.

VASCULAR REACTIVITY, CHEMICAL PROFILE, TOXICOLOGICAL AND PHARMACOKINETIC STUDIES OF *ANDROGRAPHIS PANICULATA* NEES. EXTRACTS

ABSTRACT

The aims of the study were to evaluate the vascular reactivity, chemical profile, toxicity and pharmacokinetic of *Andrographis paniculata* (AP) extracts. *Andrographis paniculata* chloroform extract (APCE) was found to be a potent vasorelaxant against norepinephrine (NE)-induced contraction of rat thoracic aorta. The HPLC and ^{1}H-NMR analysis of APCE revealed the presence of andrographolide (ANG), 14-deoxyandrographolide (DA) and 14-deoxy-11, 12-didehydroandrographolide (DDA). Chronic treatment for four weeks of APCE 25, 50 and 100 mg/kg/day in spontaneously hypertensive rats (SHR) demonstrated that it enhances the endothelium-dependent and endothelium-independent relaxation to acetylcholine (ACh) and sodium nitroprusside (SNP) presumably due to the activation of nitric oxide (NO) synthase and stimulation of the NO production in endothelial cells which lead to inhibition of Ca^{2+}-induced contraction pathway. The systolic blood pressure (SBP) of SHR was significantly ($p<0.001$) reduced presumably due to its vasodilatory action on blood vessels.

APCE, sub-fraction of AP (AP_1), DA and DDA dose dependently inhibited both the NE-induced tonic contraction and high K^+ (80 mM)-induced contraction, suggesting that APCE, AP_1, DA and DDA act as a Ca^{2+} channel blocker of both receptor-operated and potential-dependent channels. DA, DDA and APCE also dose dependently inhibited the NE-induced phasic contraction, suggesting that DA, DDA and APCE inhibits the Ca^{2+} release from sarcoplasmic reticulum. The reduction in

intracellular Ca^{2+} concentration that contribute to vasorelaxation induced by DA, DDA and APCE may be through inhibition of Ca^{2+} influx and inhibitions of intracellular calcium released. The inhibitory effect of DA, DDA and APCE on lower dose of NE-induced phasic contraction may act by inhibiting calcium influx through calcium-independent pathway.

Finally the acute and chronic toxicity studies of APCE at 100, 300, 1000 and 2000 mg/kg/day showed there was no visible sign of toxicity until the end of the 28 days study period. There were no significant changes observed on the weekly body weight gain and hematological profile as well as macroscopic and histopathological profile of the internal organs on post mortem. Therefore, the results obtained suggest that APCE is nontoxic up to 2000 mg/kg body weight. In pharmacokinetic study of APCE using ANG and DDA as markers showed non-linear pharmacokinetics at a dose 1000 mg/kg in rats.

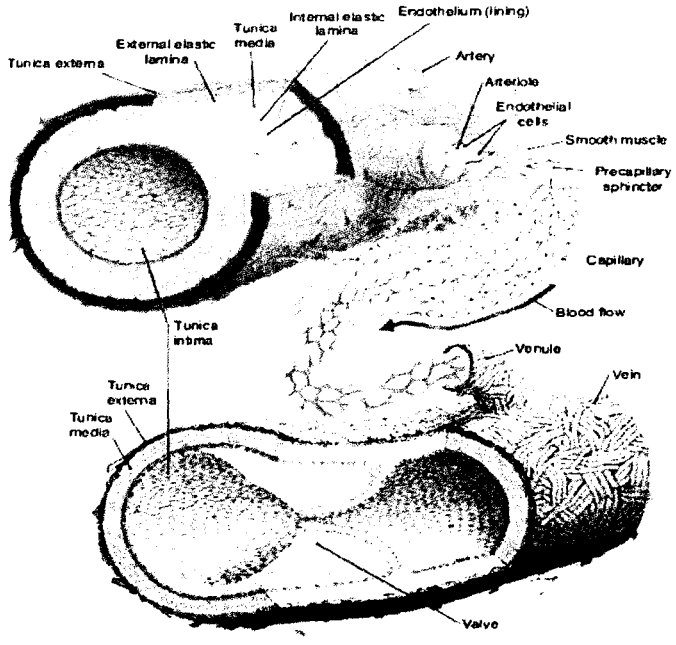
CHAPTER 1
INTRODUCTION

1.0 Introduction

In the treatment of cardiac diseases several synthetic, semi-synthetic and natural drug molecules have been used from the past decades. Among the series of emerging drug candidates, few of active principles were also isolated from the natural flora and have been tested and used in various conditions of cardiovascular malfunctions. The physiology and etiology of cardiovascular tissues are important in the screening and development of new drug candidates.

1.1 Vascular smooth muscle

The etiology of cardiovascular disease depends on the structural integrity and health of the blood vessels of the cardiovascular system. The wall of an artery consist of three distinct layers namely tunica intima, tunica media and tunica adventitia. Tunica intima, the inner most layer of the artery wall, consists of a single layer of endothelial cells and connective tissue. The amorphous mucopolysacharide ground substance containing elastin, collagen, and vascular smooth muscle cells is referred to as the tunica media layer. Tunica adventitia is the outermost layer surrounding the two inner layers and consists of strong fibrous tissue which maintains the shape of the vessel (Figure 1.1). Vascular smooth muscle is innervated primarily by the sympathetic nervous system through adrenergic receptors (adrenoceptors). Three types of adrenoceptors are present within vascular smooth muscle cells: alpha 1 (α_1), alpha 2 (α_2) and beta 2 (β_2). Norepinephrine is the main endogenous agonist for adrenoceptors.



Structure of an artery, arteriole, capillary network, venule, and vein.

Figure 1.1. Structure of artery and vein (Martini & Bartholomew, 2008).

1.2 Contraction of blood vessels

Smooth muscle contraction and the regulation of the contractile process has been the subject of intense studies for many years. Smooth muscle contraction is mainly composed of interlocked filaments held in place by a lattice work of fibres of dense bodies. The main contractile filaments are referred to as thick (contains myosin) and thin filaments (contains actin) owing to their microscopic appearance. Contraction occurs when these filaments slide over one another. This movement is mediated by the process of cross-bridge cycling. Variations in the free cytosolic calcium (Ca^{2+}) concentrations in vascular smooth muscle cells have been identified as the primary regulatory signal for smooth muscle contractions. The Ca^{2+} concentration in resting smooth muscle ranges between 80 to 270 mM while increase in the Ca^{2+} concentrations to 500 mM to 700 mM results in contraction (Webb, 2003).

Figure 1.2 (A) shows the presence of muscarinic (M_2) and muscarinic (M_3) receptors in most smooth muscle cells. Activation of M_3 receptors elicit contraction through stimulation of phospholipase C-β (PLC-β), which cleaves phosphatidylinositol-4, 5-bisphosphate into diacylglycerol (DAG) and inositol-1, 4, 5-trisphosphate (IP_3). The IP_3 mobilizes Ca^{2+} and triggers contraction. Beta (β)-adrenergic receptor activation stimulates adenylyl cyclase (AC) to generate cyclic adenosine-mono-phosphate (cAMP), which causes the relaxation of smooth muscle. M_2 receptors inhibit AC to prevent the relaxation effect on stimulation of the β-adrenergic receptor.

Figure 1.2 (B) illustrates that most peripheral blood vessels contain M_3 receptors on the endothelium, which trigger the synthesis of nitric oxide. Nitric oxide (NO) diffuses into the smooth muscle, where it mediates relaxation through the production of cyclic guanosine monophosphate (cGMP).

Figure 1.2 (C) shows the activation of M_1 receptors and nicotinic receptors (N) in parasympathetic ganglia causing the release of an inhibitory neurotransmitter (IN) from postganglionic neurons in gastrointestinal sphincters. This inhibitory neurotransmitter is usually adenosine triphosphate (ATP), NO or vasoactive intestinal peptide (VIP) and causes the sphincter smooth muscle to relax.

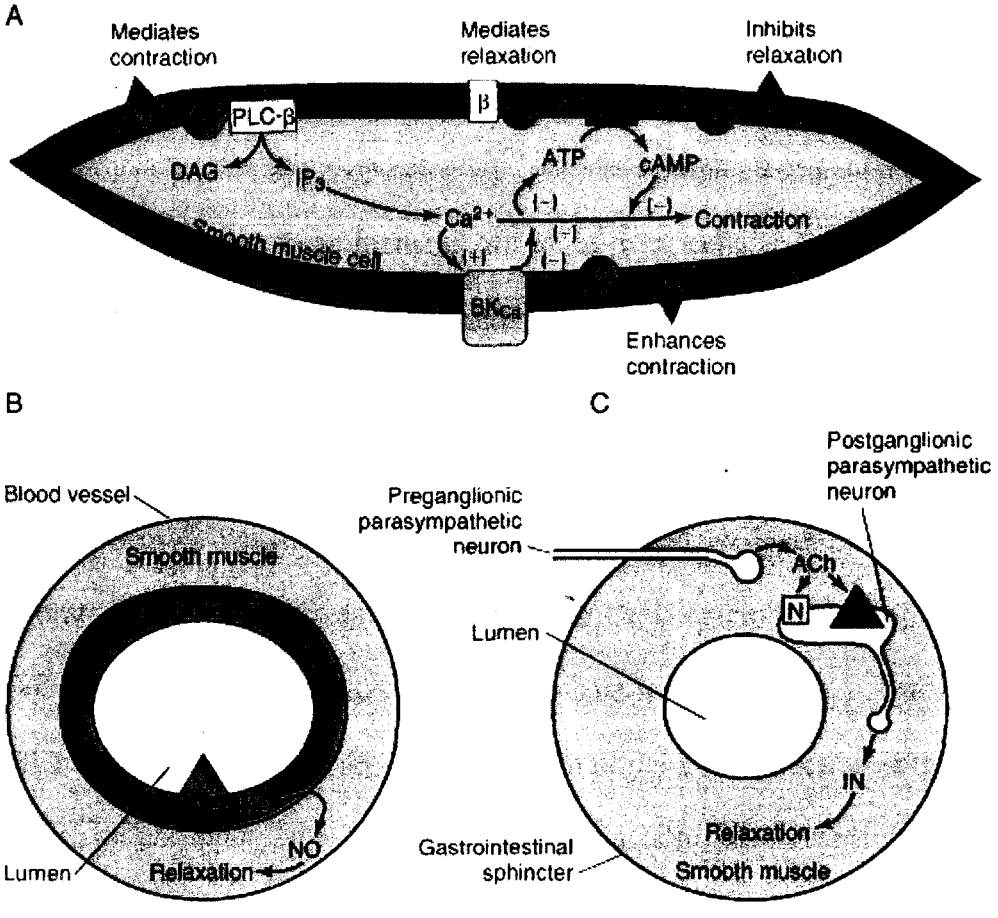


Figure 1.2. Receptor-mediated contraction and relaxation in different types of smooth muscle (Mineman and Wecker, 2004).

Vasoconstricting neurotransmitters and hormones bind to their receptors on the cell surface and initiate a series of processes leading to the contraction of vascular smooth muscle. Most receptors activate various types of guanosine 5-triphosphate (GTP) binding proteins (G-proteins), which are coupled to different ion channels and enzymes, and modulate their activities. These enzymes include both phospholipase C (PLC), which metabolises inositol diphosphate (IP_2) to produce inositol triphosphate (IP_3), diacylglycerol (DAG). Adenylate cyclase metabolises

ATP to produce cAMP. IP_3 releases Ca^{2+} from intracellular stores whereas DAG activates protein kinase C (PKC), which phosphorylates a number of proteins. In addition to the activation of the IP_3 metabolism, vasoconstrictors such as norepinephrine have been shown to depolarize the smooth muscle cells and consequently activate voltage operated Ca^{2+} channels in the plasma membrane of the smooth muscle, leading to an increased influx of extra cellular Ca^{2+}. Moreover, the existence of receptor operated Ca^{2+} channels have been proposed in smooth muscle cells (Webb, 2003). The activation of this mechanism increases intracellular Ca^{2+}, which is the primary signal for smooth muscle contraction (Bolton, 1979; Karaki *et al.*, 1984; Allen & Walsh, 1994). As a consequence of elevated intracellular Ca^{2+} concentration, Ca^{2+} binds to calmodulin to form Ca^{2+}- calmodulin complex, which removes the auto inhibition of myosin light chain kinase (MLCK). The activated MLCK phosphorylates reversibly the light chain of myosin and activates the myosin ATPase. The phosphorylated myosin cyclically binds to actin filaments producing force or the shortening of the smooth muscle (Figure 1.3). The contractile force does not, however, depend directly on intracellular Ca^{2+}, since the contractile force may be enhanced by increasing the responsiveness of the contractile machinery or the sensitivity of the myofilaments to intracellular Ca^{2+} (Webb, 2003). These modulatory mechanisms for changing the Ca^{2+} metabolism, serve an important role in the regulation of vascular smooth muscle tone (Somlyo *et al.*, 1999; Webb, 2003).

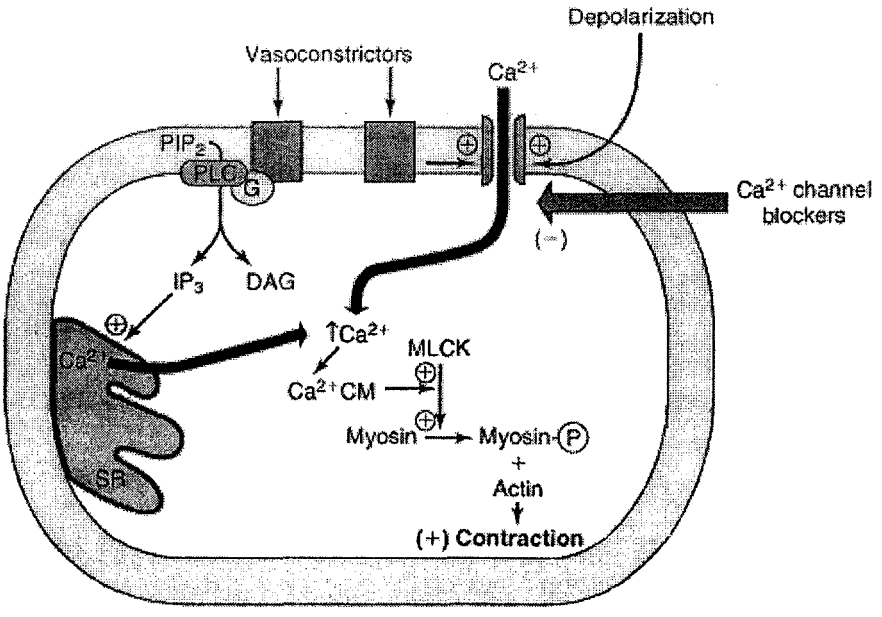


Figure 1.3. Mechanisms of contraction of vascular smooth muscle cells (Mineman and Wecker, 2004).

1.3 Smooth muscle relaxation

Relaxation of smooth muscle requires a fall in intracellular Ca^{2+} levels to resting levels and dephosphorylation of myosin. Smooth muscle relaxation occurs either as a result of removal of the contractile stimulus or by direct action of a substance that stimulates the inhibition of the contractile mechanism. This process is catalyzed by a specific myosin light chain phosphatase (MLCP) (Somlyo *et al.*, 1999). Alterations in the mechanisms that lead to reduction in intracellular Ca^{2+} levels and/or increase in MLCP activity may contribute to alterations in responsiveness of smooth muscle cells. Several mechanisms have been implicated in the sequestration or removal of cytosolic Ca^{2+}. For an instance, the inhibition of sarcoplasmic reticular Ca^{2+} and Mg^{2+}-ATPase activity which mediates the release of intracellular Ca^{2+} leads to reduction in cytosolic Ca^{2+} concentrations and hence causes relaxation of smooth muscle cells (Webb, 2003). In addition, the inhibition of receptor-operated and voltage-operated Ca^{2+} channels located in the plasma

membrane which are important in the Ca^{2+} influx and smooth muscle contraction, leads to the reduction in intracellular Ca^{2+} concentrations and hence causes smooth muscle relaxation (Webb, 2003).

1.4 Endothelium

1.4.1 Physiology of endothelium

The vascular endothelium is the largest endocrine organ in the body. It is approximately 14,000 square feet in surface area with a size of 6.5 tennis courts in area and five times the heart size in mass with a total weight of about 2 kg (Amudha *et al.*, 2002). The vascular endothelium under normal, healthy physiological conditions forms a continuous sheet of organized monolayer polyhedral cells. The endothelial cells are tightly interlocked so that passage of products from the blood occurs through the endothelial cell. These cells are both a passive filter and a metabolically active organ that synthesizes and release several vasoactive substances into the blood and into the underlying vascular smooth muscle cells, which regulates the vascular homeostasis.

The endothelium-derived vasoactive substances include vasodilators, such as NO, prostacyclin and yet unidentified endothelium-derived hyperpolarizing factor and also vasoconstrictors, such as free radicals, cyclooxygenase products and endothelin-I. Endothelium-derived vasoactive substances alter the vascular tone of the artery through myogenic mechanisms, local hormones or chemical substances, and/or metabolic by-products. Consequently, signal transmission causes muscle contraction or muscle relaxation and can occur through numerous pathways involving nerve signals, blood borne substances and locally generated substances.

For example, endothelium-derived vasoconstrictors typically bind to receptors on the smooth muscle cells and can elicit a contraction through enhancing intracellular Ca^{2+} concentrations. On the other hand, endothelium-derived vasodilators act in several ways to protect the integrity of the artery, chief among which are induction of vascular smooth muscle cell relaxation, inhibition of vascular smooth muscle cell growth, inhibition of platelet aggregation and thrombosis and inhibition of monocyte adhesion.

1.5 Endothelium and cardiovascular disease

Endothelial cells produce several biologically active substances that play key role in local regulation of blood flow, blood pressure and vascular tone (Furchgott & Vanhoutte, 1989; Luscher *et al.*, 1995; Gewaltig *et al.*, 2002; Endemann & Schiffrin, 2004). It is not surprising that alterations in physiological functions of endothelial cells have the potential to contribute directly to the impaired vascular homeostasis and hence to pathogenesis of cardiovascular disease. In support of this hypothesis, data from clinical and experimental studies have demonstrated that impairment of endothelial function either initiates or associates with the development and progression of cardiovascular disease (Endemann & Schiffrin, 2004). Endothelial dysfunction can be determined by the reduction in the activity of endothelium-derived vasodilators, mainly NO by local increases in antagonists (endothelium-derived contractile factors) to these vasodilators, or by other associates of these two factors. On the other hand, considerable evidence suggests that the manifestation of endothelial dysfunction occur before the development of cardiovascular disease (CVD) and endothelial function has been reported to be impaired even in the offspring of CVD parents (Taddei *et al.*, 1992; Taddei *et al.*, 1996; Amudha *et al.*,

2002). This shows that the onset of endothelial dysfunction may be an important pathogenic event preceding the development of clinically evident vascular disease.

Over the last decade, extensive research has focused on determining the presence and nature of endothelial dysfunction in experimental models of CVD and in patients with CVD (Amudha *et al.*, 2002). Investigations into the mechanisms of endothelial dysfunction both in hypertension and diabetes mellitus have demonstrated that reduced bioactivity and/or bioavailability of endothelium-derived nitric oxide plays an important role (Luscher & Noll, 1995; Endemann & Schiffrin, 2004). Several factors including low-density lipoprotein (LDL) cholesterol oxidation, increased production of reactive oxygen species (ROS) and decreased production of nitric oxide via endothelial nitric oxide synthase (eNOS) have been identified as important etiological factors in the reduced bioavailability and/or bioactivity of endothelium-derived nitric oxide and subsequently, in the development of endothelial dysfunction (Amudha *et al.*, 2002; Endemann & Schiffrin, 2004; Kalinowski & Malinski, 2004; Forestermann, 2005). An important implication of the free radical-oxidative stress hypothesis of endothelial dysfunction is that a wide range of antioxidants, including ascorbic acid and α-tocopherol, may inhibit ROS and reestablish endothelial function (Taddie *et al.*, 1998; Endemann & Schiffrin, 2004). Up to date most interventions attempting to improve endothelial dysfunction have targeted one or more of the numerous risk factors that can cause endothelial damage (Amudha *et al.*, 2002).

Many pharmacological agents have been suggested to achieve vascular protection through various mechanisms such as reduction in the blood pressure, inhibition of hyperglycemia and reduction in oxidative stress. Beneficial changes to the endothelium from these interventions might result from promotion of vascular relaxation, inhibition of vasoconstriction, reduction in the production of free radicals or other mechanisms that protect the endothelium from injury (Amudha *et al.*, 2002). The plants showing nitric oxide production can be a promising cadidates for vasorelaxation, which may have potential lead molecule for preventing and treating cardiovascular diseases such as hypertension and atherosclerosis (Park *et al.*, 2009).

1.6 Vascular mediators from the endothelium: Endothelium-derived relaxant factors (EDRF)

The discovery of endothelium-derived relaxing factor and its identification as nitric oxide, a highly reactive free radical gas, is one of the most exiting discoveries of biomedical research in the last two decades (Furchgott & Zawadzki, 1980; Plamer *et al.*, 1987). Over the past few years, NO has become established as a universal intercellular messenger that serves a variety of biomodulatory functions in physiological as well as pathological conditions. NO is synthesized from L-arginine by the enzymatic action of nitric oxide synthase (NOS), i.e., endothelial, neuronal and inducible, exist in mammalian cells. The endothelial subtype accounts for the majority of the basal and stimulated NO synthesis in endothelial cells throughout the vasculature (Figure 1.4). The average half-life of NO in tissue is about 3 to 6 seconds whereas in blood it is 1 to 2 seconds.

The synthesis of NO can be stimulated by an increase in endothelial Ca^{2+} concentration following physical and chemical stimuli such as shear stress and hypoxia, activation of cell surface receptors by a variety of endogenous substances like acetylcholine (ACh) and bradykinin, or application of Ca^{2+} channel agonists (Vanhoutte *et al.*, 1995; Luscher & Noll, 1995; Hansen & Nedergaard, 1999). After endothelial NOS is turned on by Ca^{2+} flux, its biosynthesizes NO in bursts for about a minute and it is turned off by phospharylation. On the other hand, it has also been (Schulz & Triggle, 1994) reported that there is a continuous and spontaneous basal release of NO from the endothelium, the amount of which regulates the arterial tone. Thus, impaired mechanism and/or inhibition of the NO production from endothelium causes dramatic decrease in blood flow and can certainly induce profound and sustained hypertension. This view is supported by the findings that chronic inhibition of NOS activity leads to a present of hypertension in experimental animals (Pechanova *et al.*, 2004).

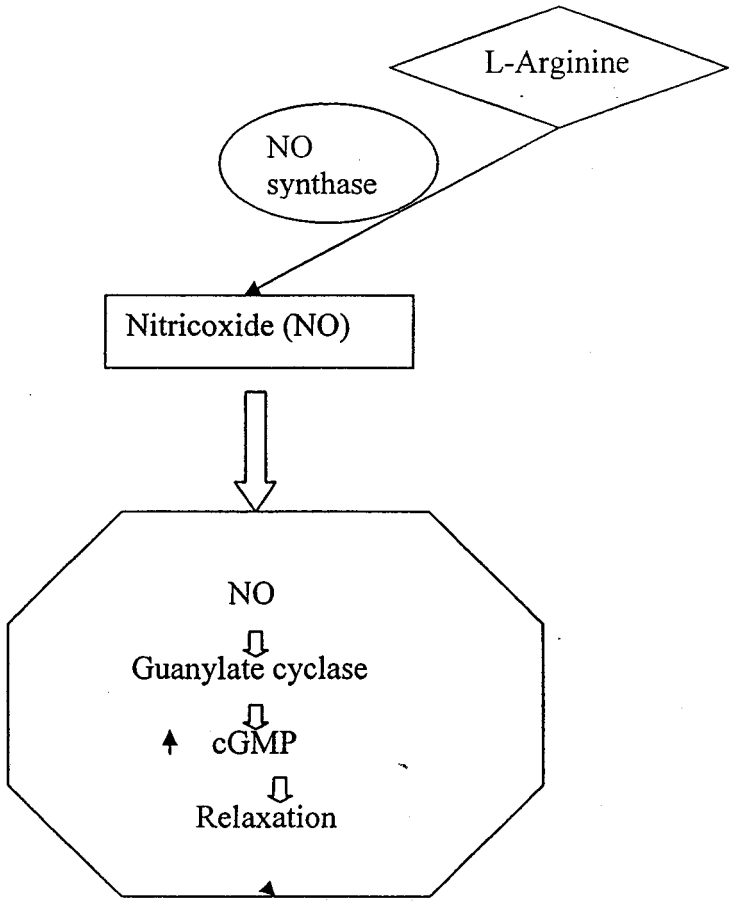


Figure 1.4. Biosynthesis of L-Arginine – nitricoxide (NO) pathway.

It has been suggested that there is a diminished basal NO synthesis in the vasculature of patients with hypertension and diabetes as well as in experimental hypertension and diabetes (Endemann & Schiffrin, 2004). On the other hand, considerable number of studies suggests that the synthesis and release of NO is unaffected or it may even be enhanced, but excessive production of super oxide anions leads to increased incapacitation of NO and subsequently, attenuates endothelium-dependent vasodilatation in hypertensive and diabetic arteries (Tschudi *et al.*, 1996; Maffei *et al.*, 2002). In this perspective, it is suggested that other than synthesis/release of NO, activation of eNOS may also lead to release of superoxide anions in higher quantities in hypertensive and diabetic arteries (Milstein & Katuski,

1999; Forstermann, 2005). In physiological conditions, the concentration of super oxide radicals remains low within the organism as a result of its reaction with super oxide dismutase (SOD) enzyme. However, in hypertension and diabetes mellitus, there may be an increase in the production of these radicals or deficiency of SOD (Gewaltig & Kodja, 2002; Endemann & Schiffrin, 2004).

NO plays different roles depending on the site of its production. NO synthesized by eNOS diffuses out in all directions. About 10% to 30% of NO diffuses to the wall of blood vessels and triggers a cascade of events leading to smooth muscle relaxation. NO relaxes vascular smooth muscle via the activation of soluble guanylate cyclase that converts guanosine monophosphate (GMP) to cyclic GMP (Moncada *et al.*, 1991; Hansen & Nedergaard, 1999). In smooth muscle cells, cGMP has been reported to activate the cGMP-dependent protein kinase that regulates several pathways involved in Ca^{2+} homeostasis with the end result being a reduction in the concentration of intracellular Ca^{2+} available for contraction and a decrease in the sensitivity of contractile proteins to Ca^{2+} (Moncada & Higgs, 1993; Hansen & Nedergaard, 1999). In addition, NO has been reported to hyperpolarize vascular smooth muscle via cGMP-dependent mechanisms as well as directly activating Ca^{2+} activated K^+ channels (Kca) and Na^+/K^+ adenosine 5'-triphosphate (ATPase) activity (Gupta *et al.*, 1994; Cohen *et al.*, 1995).

1.6.1 Prostacyclin

Prostacyclin (PGI_2) is the major relaxant prostanoid produced by the vascular endothelial cells. The formation of PGs begins with the liberation of arachidonic acid from cell membrane phospholipids by phospholipase A_2. Arachidonic acid is

then converted into PGG_2 and PGH_2 by the enzyme cyclooxygenase (COX). Finally, PGH_2 is converted to PGI_2 by the action of PGI_2 synthase (Gryglewski, 1995). Although other PGs (PGE_2, $PGF_{2\alpha}$ and PGD_2) are also synthesized in the endothelial cells, PGI_2 is the major vasodilator prostanoid in all the vascular cells. Physiologically, PGI_2 is a local rather than circulating hormone, because its blood levels are too low to have any general effects. The production of PGs can be blocked by non-steroidal anti-inflammatory drugs, such as indomethacin and aspirin, which inhibit both isoforms i.e. COX 1 & COX 2 - activity (Akpaffiong & Taylor, 1998; Nascimento *et al.*, 2003). Recently another catalytically active COX variant, COX-3, derived from an alternative splicing of the COX-1 gene, has been identified in the brain (Chandrasekharan et al., 2002).

Endothelial cells release PGI_2 in response to shear stress, hypoxia, and stimulation of various receptors on endothelial membrane (Luscher & Noll, 1995). Moreover, it is suggested from *in vitro* experiments that NO activates PGI_2 synthesis by activation of prostaglandin-H synthase and possibly by increasing cyclooxygenase activity by a cGMP-independent pathway (Gryglewski, 1995). PGI_2 exerts its vasodilatory actions by binding to membrane receptors on the smooth muscle, which activate adenylate cyclase and subsequently increase the intracellular concentration of cAMP. The elevation of intracellular cAMP leads to the reduction of intracellular Ca^{2+} concentration and to decrease in the sensitivity of contractile proteins to Ca^{2+} (Cohen & Vanhoutte, 1995). When compared with the inhibition of eNOS, the blockade of COX has negligible impact on blood pressure. On the other hand, the inhibition of COX has been found to enhance endothelium-mediated vasodilatation in hypertensive and diabetic rat arteries (Taddei *et al.*, 1997;

Akpaffiong & Taylor, 1998; Nascimento *et al.*, 2003). Therefore, hypertension and diabetes appear to be associated with an imbalance in endothelial production of COX-derived vasodilator and vasoconstrictor factors (figure 1.5).

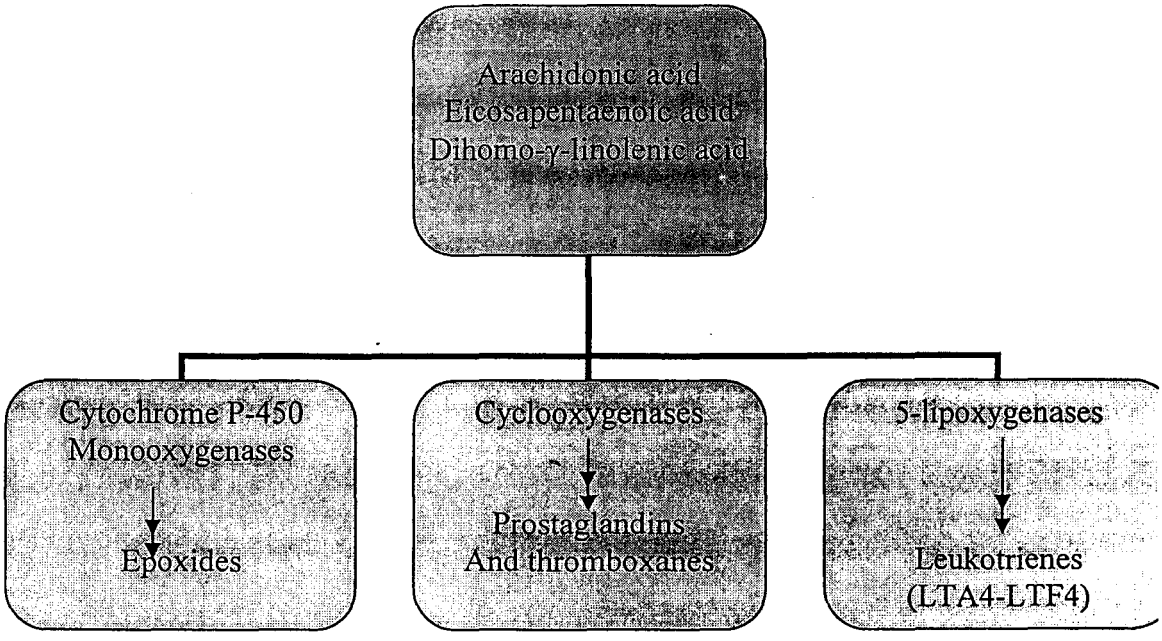


Figure 1.5. Biosynthesis of prostaglandins, lipoxygenase and cytochrome P- 450 pathway.

1.6.2 Endothelium-derived hyperpolarizing factor (EDHF)

Defined as a group of yet unidentified substances which produce vascular smooth muscle hyperpolarization and relaxation. The nature of the responses attributed to EDHF is still unresolved, but the evidence from several sources suggests that there are multiple EDHFs, and that the chemical mediators of the EDHF response may vary with the vascular bed (Edwards & Weston, 1998; McGuire *et al.*, 2001). In recent years, the most popular candidates for EDHF have been the non-prostanoid products of the metabolism of arachidonic acid, namely epoxyeicosatrienoic acids (Campbell *et al.*, 2003; Archer *et al.*, 2003; Gauthier *et al.*, 2005). EDHF has been reported to be diffusible factor, which causes the opening of

K^+ channels in the smooth muscle membrane (Quilley *et al.*, 1997; Edwards & Weston, 1998; McGuire *et al.*, 2001; Triggle *et al.*, 2003). Involvement of adenosine 5'-triphosphate (ATP)-sensitive K^+ channels (K_{ATP}) and Na^+/ K^+-ATPase has been reported in some vessels, but in the majority of the studies EDHF has been suggested to act through Ca^{2+} activated K^+ channels (McGuire *et al.*, 2001). The action of EDHF can be inhibited by K^+ channel blockers or by depolarizing the smooth muscle with increasing extra cellular high K^+ concentrations (Adeagbo *et al.*, 1993; Ueda *et al.*, 2005).

The release of EDHF is as similar as that of NO and PGI_2 which is initiated by an increase in intracellular free Ca^{2+} concentration in the endothelial cell (Cohen & Vanhoutte, 1995; Luscher & Noll, 1995). Consequently, many autocoids and hormones that release NO and PGI_2 have also been shown to release EDHF (Cohen & Vanhoutte 1995). The mechanisms whereby hyperpolarization causes relaxation remain controversial (Triggle *et al.*, 2003). Most likely, the hyperpolarization of the smooth muscle cell membrane reduces Ca^{2+} influx through voltage dependent Ca^{2+} channels, which allows the Ca^{2+} sequestration and removal of lower intracellular free Ca^{2+} concentration. The importance of EDHF in endothelium-dependent relaxations has been reported to increase as the artery size decreases (McGuire *et al.*, 2001; Triggle *et al.*, 2003). It is also suggested that EDHF-mediated relaxation is upregulated in the pathophysiological states, such as hypertension and diabetes, which are associated with reduced bioavailability of NO (Ding *et al.*, 2000; McGuire *et al.*, 2001; Endemann & Schiffrin, 2004). Decreased endothelium-mediated hyperpolarization has been observed in many forms of experimental hypertension

and diabetes (McGuire *et al.*, 2001; Triggle *et al.*, 2003; Endemann & Schiffrin, 2004).

Hydrogen sulphide (H2S) is increasingly being recognized as an important signalling molecule in the cardiovascular and nervous systems (Csaba Szabó (2007). The production of H2S from L-cysteine is catalysed primarily by two enzymes, cystathionine γ-lyase and cystathionine β-synthase. Evidence is accumulating to demonstrate that inhibitors of H2S production or therapeutic H2S donor compounds exert significant effects in various animal models of inflammation, reperfusion injury and circulatory shock. H2S can also induce a reversible state of hypothermia and suspended-animation-like state in rodents (Csaba Szabó (2007). Intravenous bolus injection of H2S transiently decreased the blood pressure of the rats by 12 ± 30 mmHg which was antagonized by prior blockade of K_{ATP} channels (Zhao et al., 2001). H2S also relaxed rat aortic tissues in vitro in a K_{ATP} channels-dependent manner (Zhao et al., 2001).

Over the last decade, studies have unraveled many aspects of endogenous production and physiological functions of carbon monoxide (CO). The majority of endogenous CO is produced in a reaction catalyzed by the enzyme heme oxygenase (HO). Inducible HO (HO-1) and constitutive HO (HO-2) are mostly recognized for their roles in the oxidation of heme and production of CO and biliverdin, whereas the biological function of the third HO isoform, HO-3, is still unclear (Wu & Wang, 2005). The interaction of CO and ion channels constitutes an important mechanism for the biological effect of CO. Most noticeable among the CO-targeted ion channels are K^+ channels. This superfamily is composed of voltage- dependent Kv, ATP-

sensitive KATP, and calcium activated KCa channels (Wu & Wang, 2005). The interaction of CO and K^+ channels may be the dominant force in driving the CO-induced vasorelaxation in specific types of blood vessels, especially peripheral resistance and cerebral arterioles. In other types of blood vessels, CO effects on K^+ channels may become less important compared with activation of the cGMP pathway by CO (Wu & Wang, 2005).

Barkoudah et al. (2004) recently showed that application of a CO-releasing compound dilated arteriolar branches of the middle cerebral artery from piglets by activating BKCa channels in vascular smooth muscle cells. However, removal of endothelium or blocking the sGC-cGMP pathway abolished CO-induced vasorelaxation. Release of NO from endothelium or activation of sGC-cGMP pathway in vascular SMC allowed CO to cause vascular dilation (Wu & Wang, 2005).

1.7 Hypertension

Hypertension is one of the leading causes of cardiovascular and cerebrovascular complications, the most common reason to visit physician offices, and number one reason for drug prescriptions all over the world (Kearney *et al.*, 2005). Hypertension is characterized by a normal cardiac output and elevated arterial pressure. The etiology of hypertension is multifactorial and the precise mechanisms are not completely understood. Hypertension is a consequence of the interaction between genetics and the internal environment of the body.

Two forms of hypertension have been described:

1. Primary or essential hypertension
2. Secondary hypertension

Essential hypertension is a far more common condition and accounts for 95% of all cases of hypertension whereas secondary hypertension accounts for only 5%. Endothelial dysfunction and vascular smooth muscle dysfunction are initiating and perpetuating factors in essential hypertension (Luscher & Noll, 1990; Amudha *et al.*, 2002; Endemann & Schiffrin, 2004). In hypertension, the cardiovascular system exhibits several important physiological and morphological changes, chief among which are increase in the blood pressure, cardiac hypertrophy (increased cardiac muscle mass of blood vessels), impairment in vascular contraction and relaxation (Taddie *et al.*, 1998; Ludwig *et al.*, 2002; Sabbatini *et al.*, 2002; Endeman & Schiffrin, 2004). However, inspite of several studies on animal models of hypertension as well as in hypertensive human subjects, the exact mechanisms responsible for these pathological changes remain uncertain. It is suggested that the narrowing of the blood vessels contributed to increased peripheral vascular resistance and consequently to elevated blood pressure. This view is supported by the regression observed in the abnormal structure of blood vessels in hypertension towards physiological value with antihypertensive treatments (Ludwig *et al.*, 2002; Sabbatini *et al.*, 2002).

In recent past, the pharmacological research is focused on multidisciplinary drug discovery aaproach on isolation, synthesis and evaluation of emerging vasodilating drug molecules with maximum therapeutic effect with minimum or no

side effects. The plants which can enhance the NO-cGMP pathway and a direct action on the vascular smooth muscle through a dephosphorylation of myosin light chain kinase, resulting in vasodilation.

1.8 Some pharmacologically active moieties from natural flora used in cardiovascular malfunctions

Several coumarin derivatives have been shown to possess cardiovascular properties. Many of them are selective coronary vasodilators, an effect that may be related to a Ca^{2+}-antagonistic activity. Carbochromen (3-diethylaminoethyl-7-ethoxycarbonylmethoxy-4-methylcoumarin) is a potent specific coronary vasodilator which has been used for many years in the treatment of angina pectoris. Although the exact mechanism of action remains still unknown, it has been reported that carbochromen coronary effects could be mediated by an increased release of prostaglandins. Khellin (2-methyl-5, 8-dimethoxyfurochromone) is an active principle obtained from *Ammi visnaga* L. has strong vasodilator and spasmolytic activities. It probably decreases the availability of Ca^{2+} required for smooth muscle activation acting at multiple sites (Toimil *et al.*, 2002).

Vasorelaxing activity of the aqueous extract of *Caesalpinia benthamiana* (AECB) roots was tested using isolated rat aortic rings precontracted by phenylephrine (PE). Interaction of AECB with NO generation was also investigated by quantitative polymerase chain reaction (QPCR) analysis and its antioxidant properties were assessed by using human polymorphonuclear neutrophils (PMNs) in a cellular pathophysiological model of oxidative burst. Scavenging activities versus

superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl) were evaluated in the cell-free system (Alexis *et al.*, 2008).

The *in vitro* and *ex vivo* suppressive effects of *Andrographis paniculata* on nitric oxide production in mouse peritoneal macrophages is elicited by Bacillus Calmette-Guein (BCG) and stimulated by lipopolysaccharide (LPS). Incubation of BCG-induced macrophages with the methanol extract of *A. paniculata* reduced LPS stimulated NO production. The diterpene lactones andrographolide and neoandrographolide were isolated as active components from the extract. These compounds suppressed NO production in a concentration-dependent manner in the concentration range from 0.1 to 100 μM and their IC_{50} values were 7.9 and 35.5 μM. Neoandrographolide at doses of 5 and 25 mg/kg/day suppressed NO production by 35% and 40%. However, andrographolide did not reduce NO production on oral administration at the same doses. These results indicate that neoandrographolide, which inhibited NO production both *in vitro* and *ex vivo* may play an important role in the use of *A. paniculata* as an anti-inflammatory crude drug (Javzan *et al.*, 2002).

1.9. Conventional antihypertensive agents

Antihypertensive agents act at one or more of the four anatomic control sites and produce their effects by interfering with normal mechanisms of blood pressure regulation. A useful classification of these agents categorizes them according to the principal regulatory site or mechanism on which they act (Chobanian *et al.*, 2003).

1) Diuretics, which lowers blood pressure by depleting the body sodium ion concentration and reducing blood volume and by other mechanisms

Four basic types of diuretics are used in treatment of hypertension.

a) Thiazide diuretics: Hydrochlorthiazide, chlorthiazide, indapamide *etc*

b) High ceiling diuretics: Furosemide, bumetanide, ethacrynic acid

c) Aldosterone antagonist: Spironolactone

d) Angiotensin II receptor: Losartan, irbesartan, valsartan, telmisartan.

2) Drugs modifying sympathetic nervous system activity

a) Centrally acting drugs: Clonidine and methyldopa are believed to act upon vasomotor centers in the brain to decrease peripheral sympathetic nervous system tone. The drugs decrease cardiac output and peripheral resistance.

b) Inhibitors of neurotransmitter release and/or storage, guanethidine are actively transported into adrenergic nerve endings and inhibit norepinephrine release with nerve stimulation. Nerve uptake is necessary for the drug's antihypertensive actions.

c) Adrenergic receptor blockers

i) α-adrenergic receptor antagonists: Prazosin, doxazosin, terazosin

Prazosin's antihypertensive actions are mentioned via competitive blockade of α_1-receptor in peripheral arterioles, reducing vascular resistance. Prazocin is selective α_1-receptor blocker its first dose produce severe hypotension.

ii) β-adrenergic receptor antagonist: Propranolol, acebutolol, atenolol, metoprolol

β-adrenergic receptor antagonists reduce blood pressure by reducing heart rate and myocardial contractility (reduced cardiac output). These are considered as effective agents for mild to moderate hypertension.

3) Combination alpha/beta adrenergic receptor blockers: Labetalol, trandate

Labetalol exhibits both selective α_1-adrenoceptor blockade and non-selective β-adrenoceptor blockade. The antihypertensive action of the drugs is results from a decrease in peripheral resistance with little or no decrease on cardiac out put. The drug is most useful for the treatment of mild to moderate hypertension.

3) Vasodilators

a) Direct vasodilators: Hydralazine, minoxidil, diazoxide, sodium nitroprusside

Direct vasodilators act upon vascular smooth muscle to produce a relaxation of vascular tone and a decrease in peripheral resistance.

b) Calcium entry blockers: Isradipine, nifedipine, diltiazem, amlodipine, felodipine, lercanidipine and verapamil hydrochloride.

The calcium entry blockers inhibit calcium entry into myocardial and vascular smooth muscle cells. The drugs can decrease peripheral resistance and decrease cardiac output the relative sensitivity of vascular smooth muscle and cardiac tissue vary with the three prototype agents. They are used for treatment of mild or moderate hypertension.

4) Angiotensin converting enzyme inhibitors (ACE Inhibitors): Captorpil, esinopril, ramipril, perindopril.

CHAPTER 2
REVIEW OF LITERATURE

2.0 *Andrographis paniculata*

Malaysia is a known as one of the 12 mega-diversity centre harbouring a multitude of medicinal plant species each presumably studded with as yet unknown genetic and chemical variations of economic importance. Several medicinal plants occurring in Malaysia species are used over several centuries in the traditional systems of medicine. Nearly 75% of the herbal drugs and perfumery products used in the world are available in natural state. Therefore, the rich and varied plant diversity, especially the genetic diversity of medicinal and aromatic plants, is one of country important strengths and is the bedrock for all future bio-industrial developments. Unfortunately, the renowned medicinal plant wealth of Malaysia has seldom been subjected to genetic scrutiny keeping in mind the latent and patentable properties and economic utility of the selected plant types. As severe habitat losses and consequent endangerment and extinction of known and hitherto lesser known species of economic value are not uncommon in this country, it is imperative that heritable variations within the otherwise unimproved natural populations of prospective taxa are studied for selection, improvement and development of suitable cultivars. Otherwise called bio-prospecting, this line of research is essential to fish out useful genes and gene products for commercialisation in the now unfolded patent regime. Knowledge of the genetic diversity is also a prerequisite for any *in situ* and *ex situ* conservation schemes (Hamrick *et al.*, 1991) as it is not practical to conserve all genotypes of a given species against the mass extinction spasm projected for the 21st century (Raven, 1999).