STUDY OF CARDIOVASCULAR ACTIVITIES OF *GYNURA PROCUMBENS* MERR. EXTRACT AND ITS FRACTIONS

NAVNEET KAUR

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STUDY OF CARDIOVASCULAR ACTIVITIES OF *GYNURA PROCUMBENS* MERR. EXTRACT AND ITS FRACTIONS

By

NAVNEET KAUR

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Dedicated

To my Family, Teachers and Friends

Whom I love the most

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LIST OF ABREVIATIONS

±	Plus minus
%	Percent
+ve	Positive
-ve	Negative
α	Alpha
β	Beta
γ	Gamma
Mg	Microgram
ºC	Degree Celsius
µm	Micrometer
5-MeU	5-methyl urapidil
ACE	Angiotensin converting enzyme
ACh	Acetylcholine
ADH	Antidiuretic hormone
AF-GPWE	Aqueous fraction of GPWE
ALP	Alkaline phosphatase
ALT	Alanine amine transferase
AngII	Angiotensin II
ANS	Autonomic nervous system
AST	Aspartate transaminase
AT	Angiotensin
ATP	Adenosine triphosphate
AUK^+	Absolute urine potassium
$AUNa^+$	Absolute urine sodium
AV	Atrioventricular
BK	Bradykinin
BP	Blood pressure
BPM	Beat per minute
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
CEC	Chlorethylclonidine
cGMP	Cyclic guanosine monophosphate
Cl^-	Chloride ion
CM	Cardiac muscle
CNS	Central nervous system
CO	Cardiac output
CO_2	Carbon dioxide

CPK	Creatine phosphate kinase
CVDs	Cardiovascular diseases
CVP	Central venous pressure
DCT	Distal convoluted tubule
DP	Diastolic blood pressure
ED	Endothelial dysfunction
EDHF	Endothelial derived hyperpolarizing factor
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
g	Gram
GFR	Glomerular filteration rate
GP25	*Gynura procumbens* 25% ethanol extract
GP50	*Gynura procumbens* 50% ethanol extract
GP75	*Gynura procumbens* 75% ethanol extract
GP95	*Gynura procumbens* 95% ethanol extract
GPWE	*Gynura procumbens* water extract
GPWE300	*Gynura procumbens* water extract 300 mg kg^{-1}
GPWE600	*Gynura procumbens* water extract 600 mg kg^{-1}
GTN	Glyceryl trinitrate
GTP	Guanosine triphosphate
h	Hour
H&E	Hematoxylin and eosin
HCl	Hydrochloric acid
HCO_3^-	Bicarbonate ion
HR	Heart rate
iNOS	Inducible isoform nitric oxide synthase
IP3	Inositol triphosphate
IsoP	Isoprenaline
IU	International unit
JG	Juxtaglomerular
K^+	Potassium ion
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogenphosphate
L	Litre
LCMS	Liquid chromatography–mass spectrometry
LDH	Lactate dehydrogenase
M	Muscarinic
M	Molar
MAP	Mean arterial pressure
Mg^{2+}	Magnesium ion

$MgSO_4$	Magnesium sulphate
min	Minute
ml	Milliliter
MLC	Myosin light chain
MLCK	Myosin light chain kinase
mM	Millimolar
mm	Millimeter
mmHg	Millimeter mercury
mmol	Millimoles
msec	Millisecond
Mtx	Methoxamine
N_1/N_M	Nicotinic muscle
N_2/N_N	Nicotinic neuronal
Na^+	Sodium ion
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NBF-GPWE	n-Butanol fraction of GPWE
NE	Norepinephrine
NFP	Net filteration pressure
ng	Nanogram
NH_4^+	Ammonium ion
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
O_2	Oxygen
PCT	Proximal convoluted tubule
PE	Phenylephrine
PGI_2	Prostacyclin
RAS	Renin angiotensin system
s	Second
SA	Sinoatrial
SD	Sprague Dawley
sGC	Guanyl cyclase
SH	Spontaneously hypertensive
SP	Systolic blood pressure
SR	Sarcoplasmic reticulum
SVR	Systemic vascular resistance
TPR	Total peripheral resistance
TxA_2	Thromboxane A_2
UFR	Urine flow rate

v/v	Volume by volume
Vera20	Verapamil 20 mg kg^{-1}
VSM	Vascular smooth muscle

KAJIAN AKTIVITI KARDIOVASKULAR EKSTRAK

***GYNURA PROCUMBENS* MERR.**

DAN FRAKSINYA

ABSTRAK

Gynura procumbens Merr. merupakan herba malar hijau yang digunakan secara tradisional untuk rawatan demam, ruam, penyakit buah pinggang, kencing manis dan hipertensi. Dalam kajian ini, kesan kardiovaskular berpandukan pengekstrakan dan fraksinasi *G. procumbens* telah dilakukan dalam usaha untuk mencari ekstrak aktif dan fraksi. Tumbuhan itu di keringkan, dikisar, diekstrak dengan kepekatan etanol : air yang berbeza (95%, 75%, 50%, 25% v/v) dan air. Ekstrak telah dikeringkan di bawah tekanan yang rendah dan kemudian dibeku-kering. Kesan ekstrak telah diperiksa pada cincin aorta tikus dan atrium kanan & kiri. Ekstrak air (GPWE) didapati paling aktif dalam pengenduran fenilefrin (PE) pra-kontraksi pada cincin aorta; kesan isoprenalina (IsoP) terhadap pengecutan maksimum dan degupan per minit atrium kiri dan kanan. Dengan cara pengekstrakan pelarut pelarut, larutan akueus GPWE difraksi menggunakan n-butanol. Kesan fraksi beku-kering kemudian dikaji secara '*in vitro*' pada cincin aorta terpencil dan sediaan atrium. Antara dua fraksi, tiada yang mengekalkan aktiviti kardiovaskular. Penyingkiran endothelium pada cincin aorta menghapuskan kesan pengenduran vaskular oleh GPWE. Ekstrak air (GPWE) juga didapati lebih aktif menurunkan tekanan darah dan kadar denyutan jantung pada tikus yang dibius tetapi sama dalam kajian '*in vitro*'; fraksi GPWE kurang berkesan dalam menurunkan tekanan darah dan kadar denyutan jantung tikus yang dibius. Dos tunggal GPWE, 1 g kg^{-1}, *p.o.*, menghasilkan pengurangan ketara

panjang berkekalan dalam tekanan arteri min (MAP) dan kadar denyutan jantung tikus sedar normotensi dan hipertensi pada 24 h. Dos yang berulang GPWE 600 mg kg^{-1}, *p.o.,* (GPWE600) mengurangkan MAP secara signifikan selepas 2 minggu, manakala 300 mg kg^{-1},p.o., (GPWE300) mengurangkan selepas 4 minggu dan 20 mg kg^{-1}, *p.o.,* verapamil (Vera20) menurunkan MAP dalam minggu pertama. Pengurangan dalam MAP pada semua kumpulan rawatan telah disertai dengan kejatuhan pada kadar jantung dari minggu pertama. Pengurangan yang signifikan pada berat badan, peningkatan dalam pengambilan air dan pengeluaran air kencing pada tikus hipertensi yang dirawat dengan GPWE dan verapamil telah diperhatikan. Dalam masa empat minggu tikus hipertensi dibius yang dirawat (GPWE600), kesan pengurangan MAP asetilkolina (ACh) & IsoP dan kesan pressor metoksamin (Mtx) telah dikurangkan secara signifikan. Sebaliknya, tiada perubahan signifikan dalam MAP telah dilihat dengan PE & angiotensin II (AngII). Perubahan dalam kadar jantung dengan ACh, PE, Mtx, AngII dan IsoP bagi GPWE600 tikus yang telah dibius direncatkan oleh GPWE 600 mg kg^{-1} secara signifikan. Peningkatan penanda bio buah pinggang dan hati telah dikurangkan secara signifikan pada tikus hipertensi dirawat dengan GPWE dan verapamil. Selain itu tiada keabnormalan utama melalui pemeriksaan histopatologi telah dilihat pada tikus hipertensi yang dirawat dengan GPWE dan verapamil. Hasil kajian mencadangkan bahawa *G. procumbens* menghalang kesan kardiovaskular secara tidak kompetitif. Analisis kimia GPWE menggunakan penyaringan fitokimia dan LCMS menunjukkan kehadiran flavonoid dan tanin. Ekstrak tersebut juga kaya dengan ion kalium.

STUDY OF CARDIOVASCULAR ACTIVITIES OF

***GYNURA PROCUMBENS* MERR.**

EXTRACT AND ITS FRACTIONS

ABSTRACT

Gynura procumbens Merr. is an evergreen herb traditionally used for treatment of eruptive fevers, rash, kidney disease, diabetes and hypertension. In the present study, the cardiovascular effects-guided extraction and fractionation of *G. procumbens* was performed in an attempt to find the active extract and fraction. The plant was dried, milled and extracted with different concentrations of ethanol:water (95%, 75%, 50%, 25% v/v) and water. The extracts were dried under reduced pressure and later freeze-dried. The effect of the extracts was examined on isolated rat aortic ring and isolated right & left atrium. The water extract (GPWE) was found to be the most potent in relaxing phenylephrine (PE) precontracted aortic rings; isoprenaline (IsoP) induced maximum contractions and beats per minute for left & right atrium. By means of solvent-solvent extraction, the aqueous GPWE solution was fractionated using *n*-butanol. The effects of the freeze-dried fractions were then investigated *in vitro* on isolated aortic rings and atrium preparations. Among the two fractions, none retained the cardiovascular activity. Removal of the endothelium of the aortic ring completely abolished the vascular relaxing properties of GPWE also. The water extract (GPWE) also was found to be potent in lowering blood pressure and heart rate of anesthetized rats but alike '*in vitro'* studies; the fractions of GPWE were less effective in lowering blood pressure and heart rate of anaesthetized rats. Single dose of GPWE, 1 g kg^{-1}, *p.o*., produced significant long lasting reduction in mean arterial

pressure (MAP) and heart rate of conscious normotensive and hypertensive rats during 24 h. Repeated dose of GPWE 600 mg kg^{-1}, *p.o.,* (GPWE600) significantly reduced the MAP after 2 weeks, while 300 mg kg^{-1}, *p.o.* (GPWE300) reduced after 4 weeks and 20 mg kg^{-1}, *p.o*., by gastric gavage of verapamil (Vera20) lowered MAP in first week. The reduction in MAP in all treatment groups was accompanied by drop in heart rate from first week. Significant reduction in weight gain, increase in water intake and urine output in GPWE and verapamil treated hypertensive rats was observed. In anaesthetized four weeks treated hypertensive rats (GPWE600), the MAP lowering effect of acetylcholine (ACh) & IsoP and pressor effect of methoxamine (Mtx) were significantly reduced. Conversely, no significant changes in MAP were seen with PE & angiotensin II (AngII). The change in heart rate with ACh, PE, Mtx, AngII and IsoP in GPWE600 anaesthetized rats was significantly blocked. Increased renal and hepatic biomarkers were significantly reduced in GPWE and verapamil treated hypertensive rats, while no major histopathological abnormalities were seen in GPWE and verapamil treated hypertensive rats. The results suggest that *G. procumbens* induced its cardiovascular effects non-competitively. Chemical analysis of GPWE using phytochemical screening and LCMS indicated the presence of flavonoids and tannins. The extract was also found to be rich in potassium ions.

CHAPTER ONE

INTRODUCTION

The cardiovascular homeostasis is maintained by the balance amongst the volume of blood in the circulatory system, the efficiency of the heart to pump out blood, the regulation of arterial vascular resistance and the kidney function. The development of an elevated blood pressure (BP) or hypertension is presumed to be the consequence of inadequate functioning of any one component of this cardiovascular-renal control interaction. It's been reported that hypertension is affecting up to 26% of the adult population of most developed nations is affected (Kearney *et al*., 2005). The long term elevated BP results in stress on whole cardiovascular system, in particular on arteries, arterial resistance vessels, the cerebrovascular circulation, the heart and the kidneys, and leads to increased risk of target-organ damages, such as stroke, myocardial infarction, left ventricular hypertrophy, heart failure and end-stage renal failure (Chobanian *et al*., 2003). The blood pressure readings above the optimal level increase the risk associated with higher BP and is graded and continuous throughout the distribution. The blood pressure in the body depends on the following interrelated factors:

- The force of contraction of the **heart** -- how much the heart muscle gets stretched by the incoming blood.
- The degree to which the **arteries and arterioles** constrict -- increases the resistance to blood flow, thus requiring a higher blood pressure.
- The circulating **blood volume** -- the increase in the circulating blood volume, increases the stretching of heart muscle by the incoming blood.

The **kidney** influences blood pressure by:

- causing the arteries and veins to constrict.
- increasing the circulating blood volume.

1.1 The Cardiovascular system

The cardiovascular system is an organ system that transports nutrients (such as electrolytes, amino acids and lymph), blood cells, gases, hormones, etc. to and from cells in the body. This circulation helps to stabilize body temperature and pH, maintain homeostasis and fight diseases. This system is comprised of the heart and the circulatory system (blood vessels and blood).

1.1.1 Heart

1.1.1.1 Anatomy

The heart is a myogenic muscular organ found in all animals with a circulatory system, which pumps blood throughout the blood vessels by repeated, rhythmic contractions. It is enclosed in a double-walled fibrous sac called pericardium. This sac protects the heart, anchors its surrounding structures, and prevents overfilling of the heart with blood. The wall of the heart is composed of three layers. The outermost layer is, epicardium, the middle layer, which contracts, composed of cardiac muscle is known as myocardium, the innermost layer is endocardium, it merges with the inner lining (endothelium) of blood vessels and covers heart valves.

The heart is separated into right and left halves, each half consisting of an atrium and a ventricle. The two ventricles are separated by a muscular wall, the

interventricular septum. The atrium and ventricle, in each half of the heart are separated by the

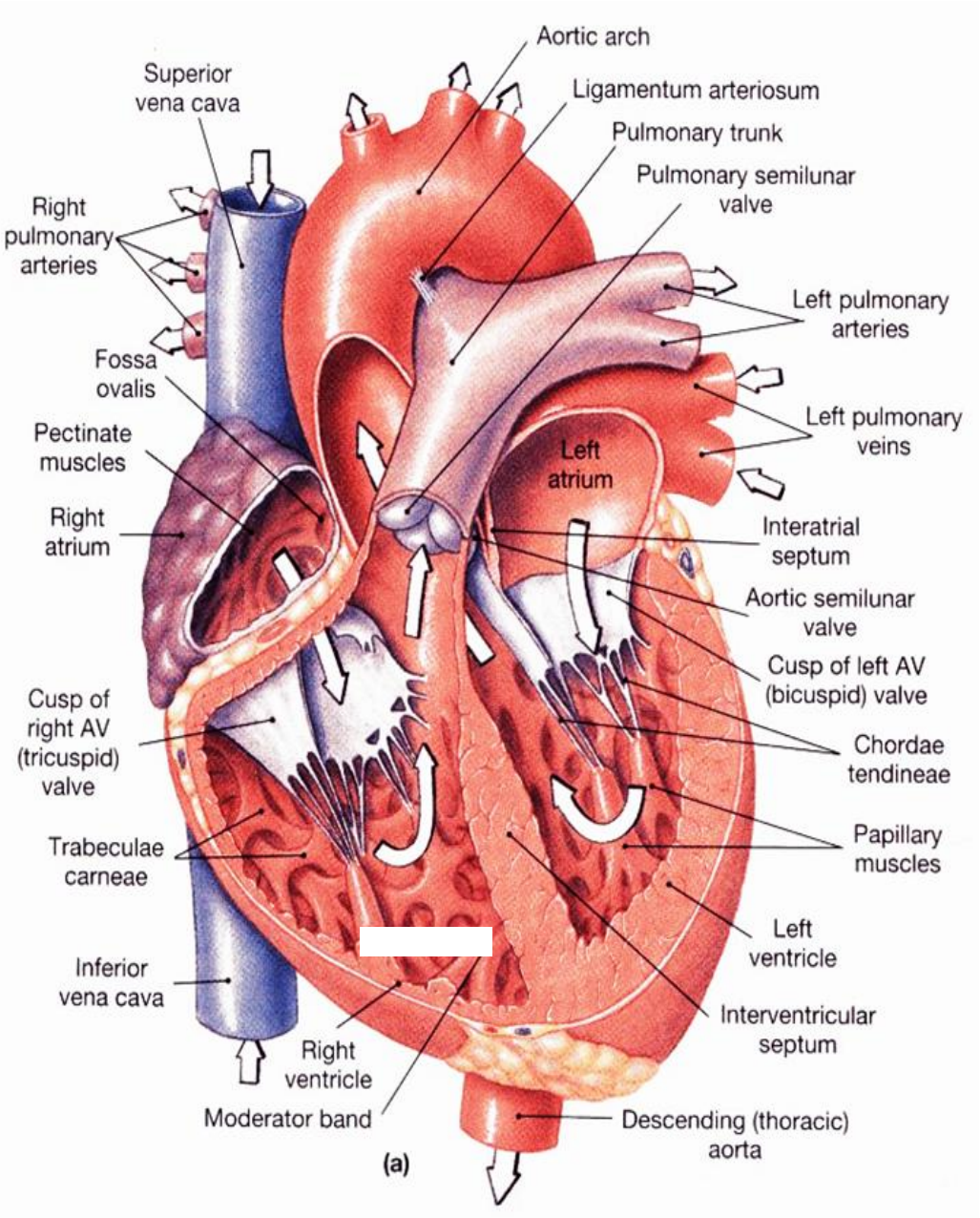

Figure 1.1 Diagrammatic section of the heart.
(Adapted from McKinley and O'Loughlin, 2007)

atrioventricular (AV) valves, which allow blood to flow from atrium to ventricle but not in the reverse direction. The right AV valve has three fibrous flaps or cusp and is known as tricuspid valve. The left AV valve has two flaps and is therefore called the bicuspid valve (Figure 1.1). The openings of the right ventricle into the pulmonary trunk and of the left ventricle into the aorta encompass semilunar valves, owing to the half-moon shape of the cusps and these are the pulmonary and aortic valves respectively. These valves permit blood to flow into the arteries during ventricular contraction but prevent blood from moving back into ventricles during relaxation. No valves are present at the entrances of the superior and inferior venae cavae into the right atrium, and of the pulmonary veins into the left atrium. However, atrial contraction pumps very little blood back into the veins as atrial contraction constricts their sides of entry into the atria, significantly increasing the resistance to backflow (Widmaier *et al.*, 2006).

1.1.1.2 Cardiac muscle

Cardiac muscle is a type of involuntary striated muscle found in the walls and histological foundation of the heart, specifically the myocardium. Cardiac muscle possesses properties of both skeletal and smooth muscle. The cardiac cells striated arrangement of thick myosin and thin actin filaments is similar to that of skeletal muscle. Cardiac muscle cells (cardiac myocytes) are significantly shorter than skeletal muscle fibers and have several branching processes. Adjacent cells are joined end to end at structures known as intercalated disks, within which the desmosomes are located. The later hold the muscle cells together and to which the myofibrils are attached. Adjacent to the intercalated disks, there are gap junctions, like those in many smooth muscles (Widmaier *et al.*, 2006). Cardiac muscle is

highly resistant to fatigue due to the presence of a large number of mitochondria, myoglobin and a good blood supply allowing continuous aerobic metabolism (Ganong, 2005).

1.1.1.3 Cardiac cycle

The cardiac contraction involves cyclic events, so called cardiac cycle. There are two phases of the cardiac cycle, diastole phase, the heart ventricles are relaxed and the heart fills with blood and systole phase, the ventricles contract and pump blood into the arteries. The frequency of the cardiac cycle is described by the heart rate.

The right and left atria contract simultaneously in the cardiac cycle, the events that are occurring on left side of the heart are parallel to the events occurring on the right side of the heart. The ventricles contract shortly after the atria. The sinoatrial node sends out electrical waves of excitation to both atria, and it is prevented from travelling down into the ventricles directly by strands of non-conducting fibrous tissue present laterally from the tricuspid/bicuspid valves to the septum. These waves of excitation travel towards the septum and into the atrioventricular (AV) node, where they are held for roughly 0.1 seconds. They are then discharged down the bundle of His, later down the Purkinje fibre, which are both situated inside the septum. The waves flow down towards the apex of the heart and are then discharged into the ventricles, causing them to contract (ventricular systole). This creates the beat of the heart (Guyton, 2006; Klabunde, 2007).

1.1.1.4 Physiology of cardiac muscle contraction

Cardiac myocytes can be divided into work cells and pacemaker cells. The work cells have a large stable resting membrane potential and display a prolonged action potential with a plateau phase. The pacemaker cells have smaller unstable resting potentials and spontaneously depolarize, generating the intrinsic electrical activity of the heart. Pacemaker cells are found in the sinoatrial (SA) and atrioventricular (AV) nodes. The cells of the bundle of His and some Purkinje cells are also capable of spontaneous firing (Pinnell and Turner, 2007).

On stimulation, myocardium membrane depolarizes, resulting in shortening of the contractile proteins followed by relaxation when the membrane potential returns to the resting state. The cells of the cardiac muscle are interconnected in groups that act in response to stimuli as a unit, contracting together whenever a single cell is stimulated (Howland and Mycek, 2006).

1.1.1.4.1 Cardiac action potential

The cells of cardiac muscle show spontaneous intrinsic rhythm produced by specialized "pace maker cells" located in the sinoatrial (SA), and atrioventricular (AV) nodes. Unlike nerve cells, the cardiac cells have long action potential which can be divided into five distinct phases (0-4) as shown in Figure 1.2. Phase (0): represents the fast depolarization, during which fast inward sodium (Na^+) current to the cells occurs. Na^+ channels close around +30 mv. Phase (1): is characterized by partial repolarization as result of outward potassium (K^+) current. Phase (2): is called the plateau phase in which calcium (Ca^{2+}) is exchanged to K^+ to aid in cardiac contraction process. The plateau phase of the action potential lasts about 250 msec. Phase (3): represents the repolarization phase of the cardiac action potential, as Ca^{2+}

channels close, and K^+ channels open and K^+ moves out of cell. Phase (4): is the last phase of the action potential. It is known as forward current or spontaneous depolarization and is characterized by gradual increase in cellular permeability to Na^+ (Howland and Mycek, 2006).

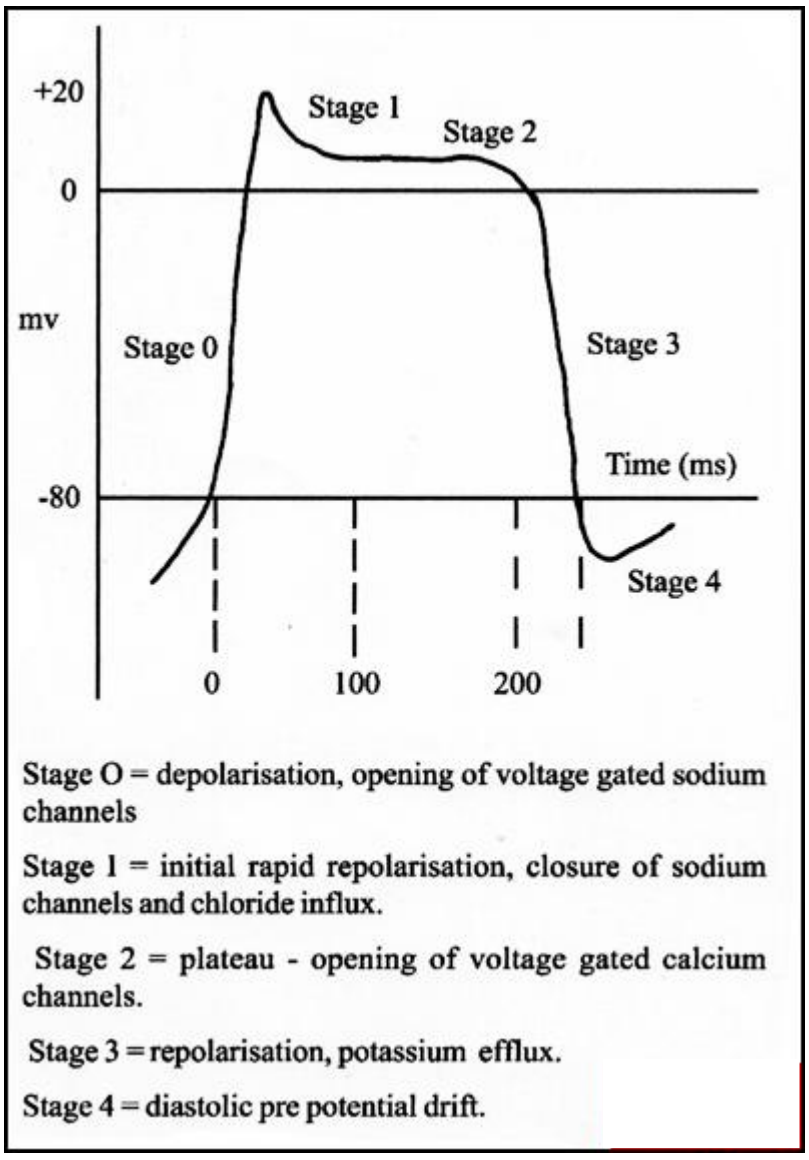

Figure 1.2 The cardiac action potential wave forms in adult human. (Adapted from Nerbonne and Kass, 2005)

1.1.1.4.2 Pacemaker action potential

The pacemaker potential is seen in cells in the SA and AV nodes. As shown in Figure 1.3, it differs from the action potential of cardiac myocytes, in that phases 1 and 2 are absent. Pacemaker cells unstable resting action potential and the spontaneous depolarization of the pacemaker potential gives the heart its auto-rhythmicity. The pacemaker potential is generated by a reduction in membrane permeability to K^+, because of Ca^{2+} influx, and increased Na^+ current because of Na^+- Ca^{2+} exchange. Once the threshold potential is reached, Ca^{2+} enters the cell, and depolarization occurs. In contrast to the cardiac myocyte action potential, there is no inward movement of Na^+ during depolarization. Repolarization (phase 3 of the action potential) occurs because of an increase in K^+ permeability. At the SA node, K^+ permeability can be further enhanced by vagal stimulation. This has the effect of hyperpolarizing the cell and reducing the rate of firing. Sympathetic stimulation has the opposite effect.

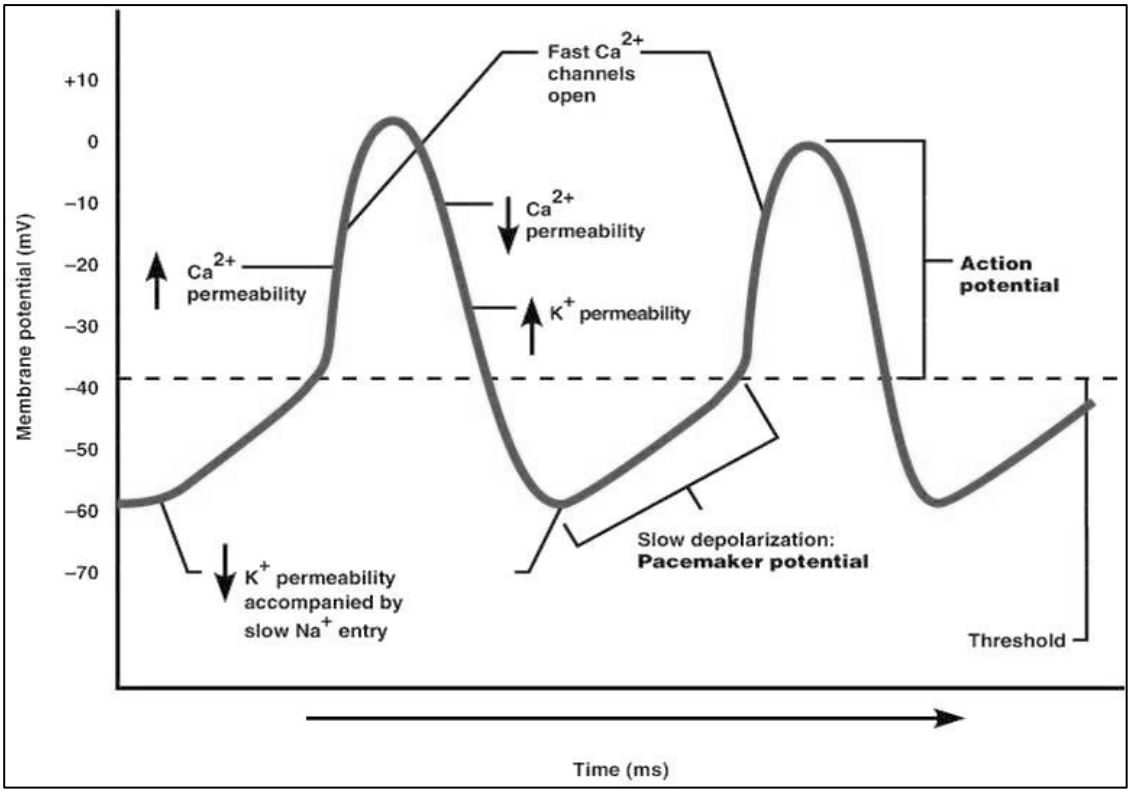

Figure 1.3 The pacemaker action potential waveforms.

1.1.1.4.3 Cardiac contraction

The contractile property of the myocardial cell is fundamentally similar to that of striated muscle. As action potential moves across cell membrane, and down T-tubules the voltage-gated Ca^{2+} channels open on cell surface. Ca^{2+} activates Ca^{2+} channels on endoplasmic reticulum. Ca^{2+} floods into the cytosol (contributes to plateau phase described above, as well as to prolonged contraction of cardiac muscle about 3 times longer than skeletal muscle contraction). Ca^{2+} binds to troponin, and tropomyosin then moves away from binding sites on actin, allowing cross bridge cycling just as in skeletal muscle. Ca^{2+} is eventually removed by active transport, and muscle relaxes. The contraction force of the cardiac muscle is directly proportional to the concentration of free cytosolic Ca^{2+}. Hence, agents that increase

Ca^{2+} levels (or increase the sensitivity of the contractile machinery) bring about an increase in the force of contraction (inotropic effect) (Howland and Mycek, 2006).

1.1.2 Systemic and pulmonary circulation

As reported by British physiologist, William Harvey in 1628, the cardiovascular system is formed of two, closed circuit of vessels. The blood is pumped out of the heart through one set of vessels and returns to the heart by another set. These two circulatory circuits (Figure 1.4) originate and terminate in the heart. The heart is also divided longitudinally into two functional halves, each containing two chambers: an upper chamber, the atrium, and a lower chamber, the ventricle. The atrium of each side pumps blood into the ventricle on that side. No direct flow between the two atria or the two ventricles in adult heart is found (Widmaier *et al.*, 2006).

In thirteenth century, Ibn Al-Nafis, an eminent Arab physician, defined the pulmonary circulation. Based on his report, the blood pumped from the right ventricle pass through lungs and returns to the left atrium is the pulmonary circulation. While the blood pumped from the left ventricle, run through all the organs and tissues of the body except the lungs, and returns to the right atrium, is systemic circulation. The vessels carrying blood away from the heart are called arteries, and those bringing back blood from body organs and tissues to the heart are called veins (Al-Ghazal, 2007).

In the systemic circuit, blood leaves the left ventricle by a single artery, the aorta (Figure 1.4). From the aorta, the arteries of the systemic circulation branch off, dividing into smaller vessels, the smallest arteries branch into arterioles, which in turn branch into a large number (estimated at 10 billion) of exceedingly small

vessels called capillaries, which fuse to form larger-diameter vessels, the venules. The arterioles, capillaries, and venules together comprise the microcirculation. The venules in the systemic circulation join to form larger vessels, the veins. Two large veins formed, the inferior vena cava which accumulates blood from organs and tissues below the heart, and the superior vena cava collects blood from organs and tissues above the heart. These two main veins return the blood to the right atrium.

In pulmonary circulation, blood leaves the right ventricle through a single large artery known as the pulmonary trunk. This large artery divides into two pulmonary arteries, supplying the right and left lung. In the lungs, the arteries continue to divide, eventually forming capillaries that join into venules and then veins. Four pulmonary veins carry the blood from lungs and empty into the left atrium.

While blood circulates through the lung capillaries, it exchange carbon dioxide with oxygen supplied to the lungs through breathing process. Therefore, high oxygen contents are found in the blood in the pulmonary veins, left side of the heart, and systemic arteries. After this oxygenated blood circulates through peripheral tissues and organs capillaries, some of this oxygen is exchanged with carbon dioxide from the blood, resulting in the lower oxygen content of blood in the venous side of the systemic circulation.

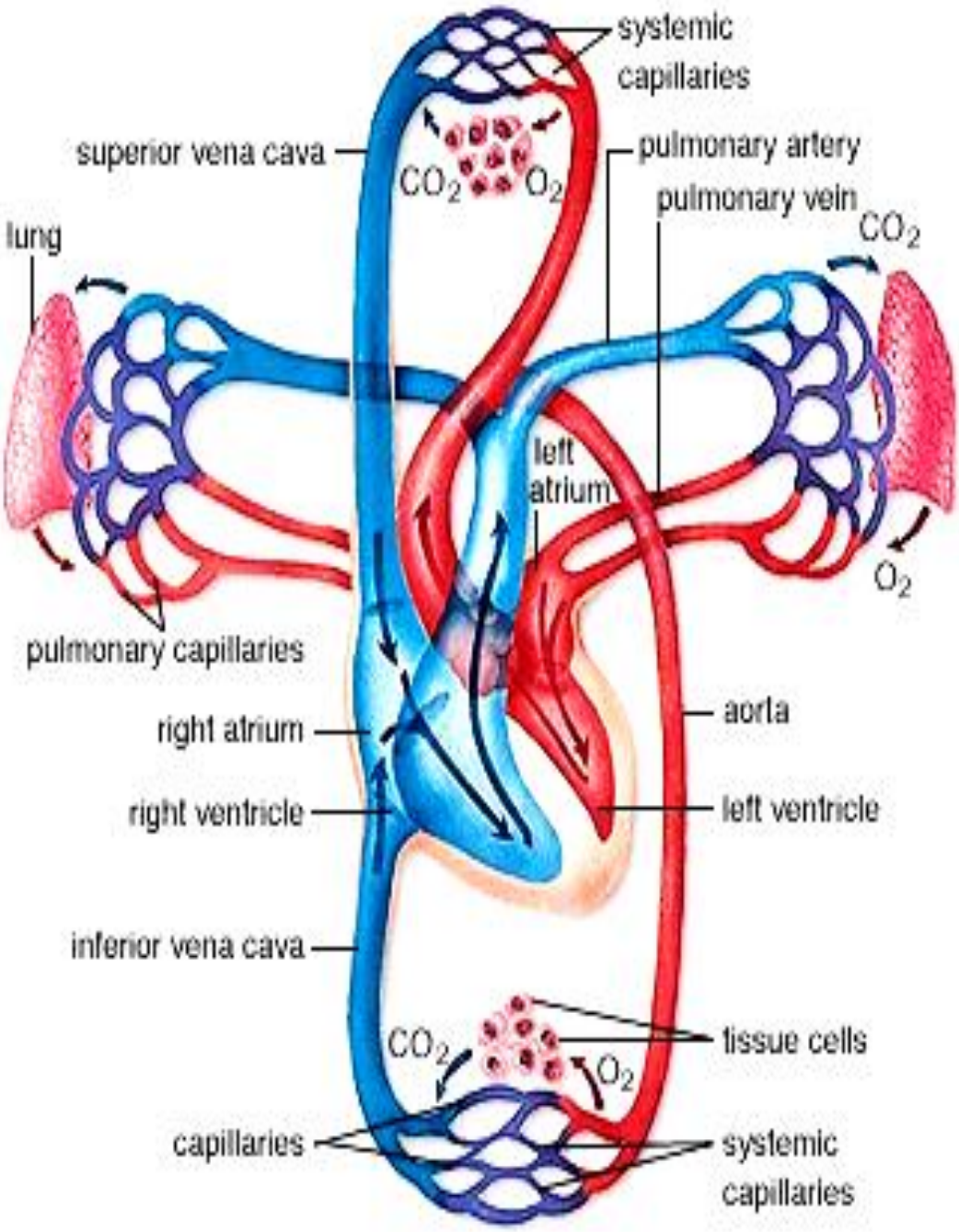

Figure 1.4 The systemic and pulmonary circulation.
(Adapted from McKinley and O'Loughlin, 2007)

1.1.2.1 Arteries and arterioles

One of the root causes responsible for the essential hypertension is the increase of peripheral resistance to the flow of blood in circulatory system. Small arteries (with a diameter less than 300μm) and the arterioles, constituting the distal part of arterial vasculature (the resistance vessels), mainly contribute in determination of peripheral resistance (Christensen and Mulvany, 2001). The wall of an artery comprises of three distinct layers namely tunica intima, tunica media and tunica adventitia (Figure 1.5). Tunica intima, the inner most layer of the artery wall, consists of a single layer of longitudinally arranged endothelial cells, and is separated from the circumferentially-arranged smooth muscle cells and connective tissue of the tunica media by an internal elastic lamina (Ganong, 1999). The outermost, tunica adventitia consists of collagen, fibroblasts and sympathetic nerves. It is the lumen diameter, which determines the resistance caused by the arteries.

The aorta and other systemic arteries are low-resistance conducting tubes. Due to presence of large amount of elastic tissues in vessel walls, they act as a "pressure reservoir" for blood flow through the tissues during diastole (Widmaier *et al.*, 2006).

They are stretched during systole and recoil during diastole. The arteriole walls have less elastic tissue but much more smooth muscle. The muscle is innervated by noradrenergic nerve fibers, which are constrictor in their function and in some cases by cholinergic fibers (e.g., coronary vessels), which act by dilating the vessels. The arterioles are the main site of the resistance to blood flow, and little changes in their diameter result in large changes in the total peripheral resistance (TPR) (Ganong, 1999).

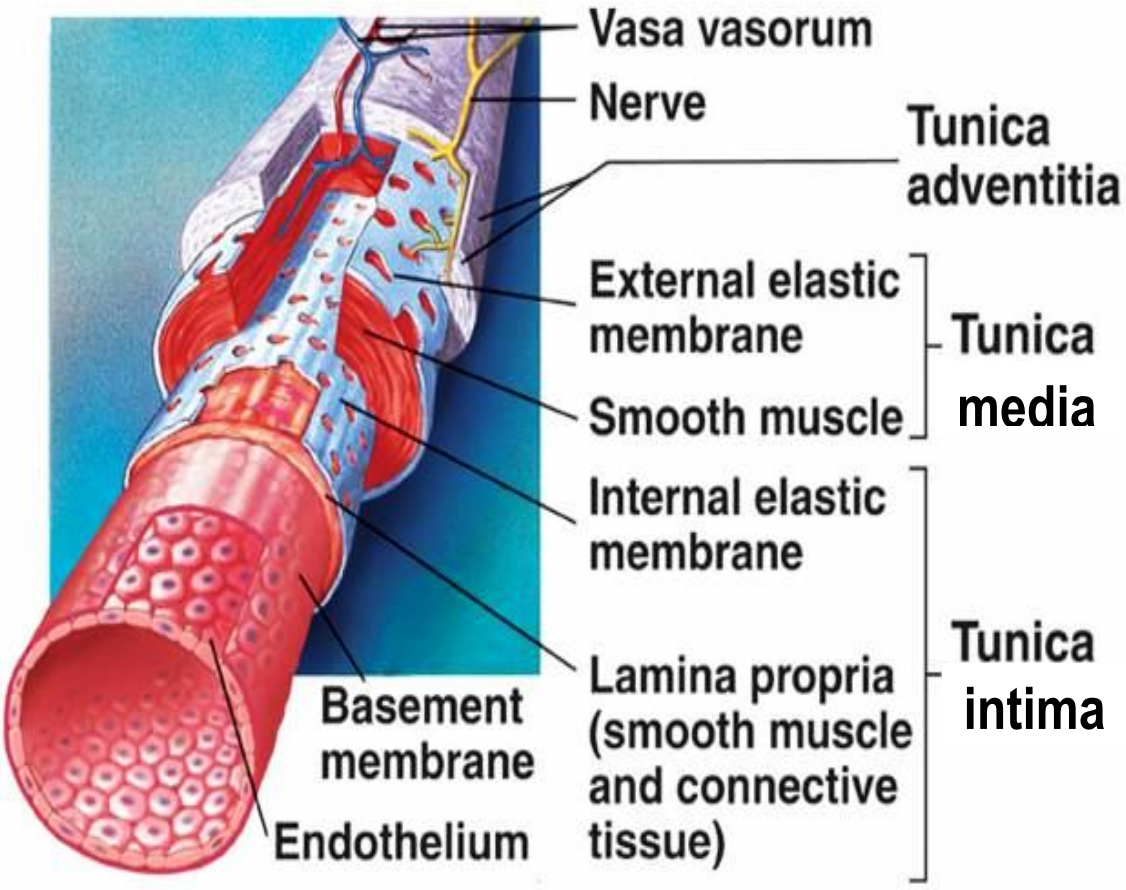

Figure 1.5 Diagrammatic section of the blood vessel.
(Adapted from Guyton, 1991a)

1.1.2.1.1 Contraction of vascular smooth muscle

The vascular smooth muscle (VSM) undergoes slow, persistent and tonic contractions. Vascular smooth muscle, actin and myosin filament are appropriately organized for maintaining tonic contractions and reducing lumen diameter. Contraction results with the filaments slide over on one another. This movement is facilitated by the process of cross-bridge cycling. Contraction in vascular smooth muscle can be triggered by mechanical, electrical, and chemical stimuli. Contraction can result from the passive stretching of vascular smooth muscle and is known as myogenic response. Electrical depolarization of the vascular smooth muscle cell membrane also stimulates contraction, probably by opening voltage dependent calcium channels (L-type calcium channels), which causes an increase in the intracellular concentration of calcium. Finally, a number of chemical stimuli such as norepinephrine, angiotensin II, vasopressin, endothelin-1, and thromboxane A_2 can cause contraction. These substances bind to specific receptors on the vascular smooth muscle cell (or to receptors on the endothelium adjacent to the VSM), resulting in vascular smooth muscle contraction.

All the mechanisms of contraction involves different signal transduction pathways, however result in increased intracellular calcium, which is the primary signal for smooth muscle contraction (Bolton, 1979; Karaki and Weiss, 1984). In resting smooth muscle, increase in Ca^{2+} concentration from 80 to 270 mM to 500 mM to 700 mM results in contraction (Webb, 2003). The mechanism by which an increase in intracellular calcium stimulates vascular smooth muscle contraction is explained in the Figure 1.6. An increase of free intracellular calcium results, either with increased influx of calcium through calcium channels or by discharge of calcium from internal stores (sarcoplasmic reticulum). The free calcium binds with

the special calcium binding protein called calmodulin. Calcium-calmodulin activates myosin light chain kinase (MLCK), an enzyme that results in phosphorylation of myosin light chains (MLC) in the presence of ATP. Myosin light chains are 20-kD regulatory subunits found on the myosin heads. Phosphorylated MLC cause formation of cross-bridge between the myosin heads and the actin filaments, and hence, smooth muscle contraction. Intracellular calcium concentrations, therefore, are very important in regulating smooth muscle contraction. The concentration of intracellular calcium depends upon the balance between the calcium that enters the cells, the calcium that is discharged by intracellular storage sites and moving of calcium either back into storage sites or out of the cell. Calcium is restored in the sarcoplasmic reticulum by an ATP-dependent calcium pump. Calcium is removed from the cell to the external environment by either an ATP-dependent calcium pump or by the sodium-calcium exchanger.

The contractile force, however, does not depend directly on intracellular Ca^{2+}, as the contractile force can 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).

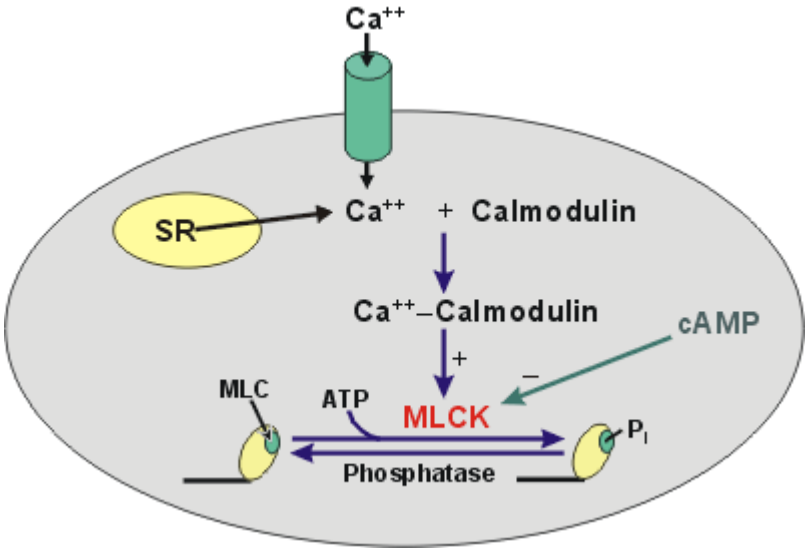

Figure 1.6 Mechanisms of contraction of vascular smooth muscle cells. (Adapted from Klabunde, 2011)

Abbreviations

SR - sarcoplasmic reticulum, Ca^{2+} - calcium ion, cAMP - cyclic adenosine monophosphate, ATP - adenosine triphosphate, MLC – myosin light chain, MLCK – myosin light chain kinase.

1.1.2.1.2 Relaxation of vascular smooth muscle

Relaxation of vascular smooth muscle involves drop in intracellular Ca^{2+} levels to resting levels and dephosphorylation of myosin light chain. Vascular smooth muscle relaxation follows either removal of the contractile stimulus or by direct inhibition of the contractile mechanism. This process is catalyzed by a specific enzyme myosin light chain phosphatase (MLCP) (Somlyo *et al.*, 1999). Changes leading to reduction in intracellular Ca^{2+} levels and/or increase in MLCP activity add to alterations in responsiveness of smooth muscle cells. Several mechanisms have been implicated in the removal of cytosolic Ca^{2+}. The inhibition of

sarcoplasmic reticular Ca^{2+} /Mg^{2+}- ATPase activity facilitates the translocation of intracellular Ca^{2+} to sarcoplasmic reticulum, result in reduction in cytosolic Ca^{2+} concentrations and hence causes relaxation of smooth muscle cells (Webb, 2003). Furthermore, the inhibition of receptor-operated and voltage-operated Ca^{2+} channels in the plasma membrane, which help 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).

1.1.2.2 Endothelium

Until recently, it was thought that the vascular endothelial cells simply acted as an inert barrier between the vessel wall and lumen. Recent studies have shown, the endothelium is a dynamic endocrine organ which lines the entire vascular system. The endothelium controls vascular function by responding to various hormones, neurotransmitters and vasoactive factors (Galley and Webster, 2004). The balanced formation of these vasoactive factors is atheroprotective, whereas imbalance of these factors leads to endothelial dysfunction (ED), which is an early indicator of atherosclerosis (Lerman and Zeiher, 2005). Endothelial cells are present at the interior surface of all vessels, with different structures and phenotypes in different vessel (Ghitescu and Robert, 2002). Endothelial cells in arteries and veins are continuous and thicker, while in capillaries are fenestrated and thinner to allow for exchange of metabolites and gases (Rhodin, 1980). In addition, endothelial cells can display heterogeneous responses to stimulation in different vascular beds, and even in different sections of the same vascular bed (Ferrer *et al*., 1995; Thorin *et al.,* 1997; Hill *et al*., 2001). This suggests that ED may occur in selective vascular beds too (Hill *et al*., 2001).

The endothelium releases various vasoactive substances, which can be vasodilating such as nitric oxide (NO), prostacyclin (PGI_2) and endothelium derived hyperpolarizing factor (EDHF) or vasoconstrictive such as thromboxane (TXA_2) and endothelin-1 (ET-1). These endothelium-derived vasoactive substances alter the vascular tone through local hormones, chemical substances or myogenic mechanisms.

Furchgott and Zawadzki were the first to identify nitric oxide (NO), an endothelium-dependent vasodilator of the underlying smooth muscle (Furchgott and Zawadzki, 1980). Nitric oxide plays an important role in the maintenance of basal vasodilator tone of the blood vessels (Vallance *et al.*, 1989). NO is formed from the amino acid L-arginine under the influence of the enzyme nitric oxide synthase (NOS) (Palmer *et al*., 1988). There are three isoforms of NOS: neuronal nitric oxide synthase (nNOS) which produces NO to regulate release of synaptic neurotransmitter (Prast and Philippu, 2001), macrophage or inducible isoform (iNOS) which is only expressed in cells that have been exposed to inflammatory mediators or other injurious stimuli that activate the macrophages (Michel and Feron, 1997), and endothelial NOS (eNOS) which produces nitric oxide in the vasculature (Lamas *et al*., 1992). Considering that the ability of a blood vessel to dilate is largely dependent upon the activity of eNOS, the present discussion will focus on this isoform.

Endothelial nitric oxide synthase (eNOS) is located in small invaginations of the cell membrane (caveolae) as an inactive eNOS bound to the protein caveolin (Bucci *et al*., 2000). Increase of intracellular Ca^{2+} levels, separates eNOS from caveolin and is activated (Bucci *et al*., 2000). NO agonists by acting on calcium channel release Ca^{2+} from the endoplasmic reticulum or influx of calcium can

influence the detachment of eNOS from caveolin (Hutcheson and Griffith, 1997; Berkels *et al*., 1999; Bae *et al*., 2003). Examples of such NO agonists include bradykinin (BK), acetylcholine (ACh), adenosine triphosphate (ATP), adenosine di-phosphate (ADP), substance P and thrombin (Moncada and Higgs, 2006). On depletion of intracellular Ca^{2+} stores an unknown signal is sent to the membrane receptors to open Ca^{2+} channels inflow of extracellular Ca^{2+} in the cell (Lambert *et al*., 1986; Schilling *et al*., 1992; Schilling and Elliott, 1992). Ca^{2+} attaches to the protein cytoplasmic calmodulin, which undergoes structural changes and activate eNOS (Fleming and Busse, 1999). eNOS then converts L-arginine into NO (Palmer *et al*., 1988). This mechanism of NO production works at higher levels of intra- or extracellular Ca^{2+}. A reduction in Ca^{2+} causes the calcium calmodulin complex to dissociate from eNOS, which in turn binds with caveolin and becomes inactivated (Fleming and Busse, 1999).

Another mechanism of eNOS activation is the phosphorylation of eNOS (Butt *et al*., 2000). Phosphorylation of eNOS occurs via protein kinases (Michel and Feron, 1997), such as protein kinase A (Bae *et al*., 2003) and cyclic guanosine-3', 5-monophosphate (cGMP) protein kinase dependent II (Butt *et al*., 2000). Shear stress initiates eNOS phosphorylation by the actions of protein kinase B (Akt) (Boo *et al*., 2002).

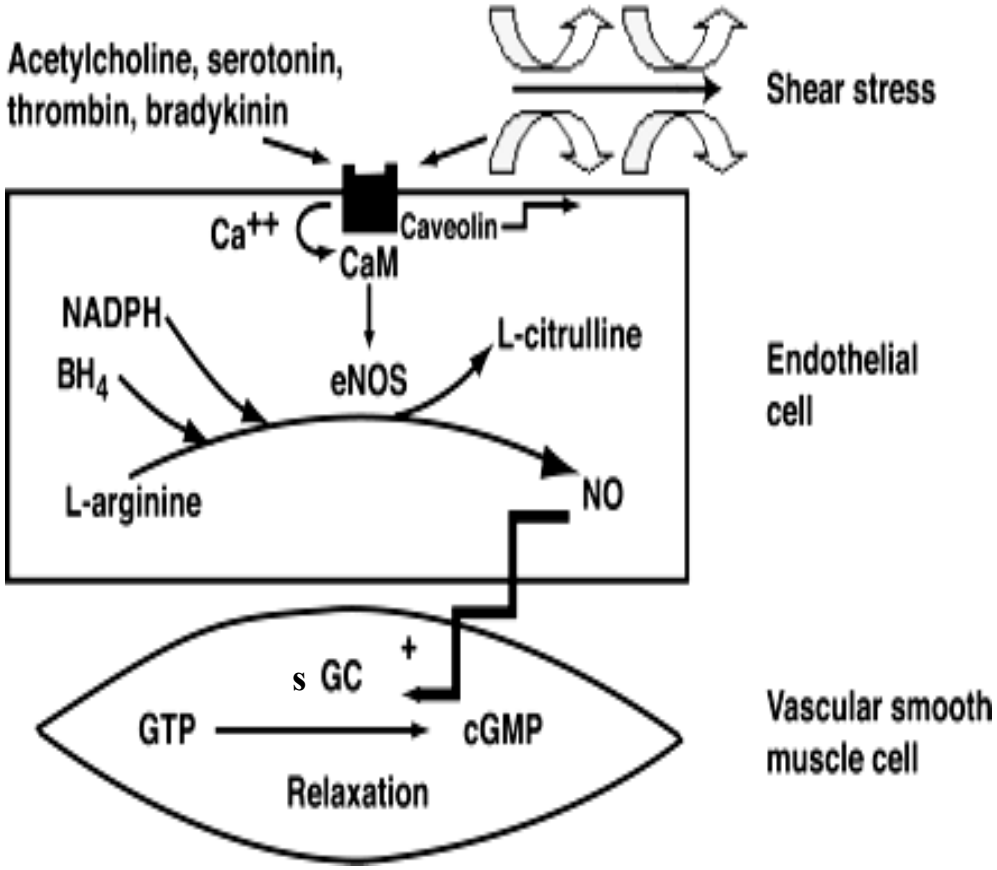

Figure 1.7 Production of nitric oxide (NO) by endothelial cells resulting in vascular smooth muscle cell relaxation.
(Adapted from Davignon and Ganz, 2004)

Abbreviations

NADPH - nicotinamide adenine dinucleotide phosphate, Ca^{++} - calcium ions, BH_4 - tetrahydrobiopterin, CaM - calmodulin, eNOS – endothelial nitric oxide synthase , NO – nitric oxide , GTP – guanosine triphosphate, cGMP - cyclic guanosine-3', 5-monophosphate, sGC – soluble guanylyl cyclase

Increased blood flow results shear stress in the vessel and increase NO production by eNOS phosphorylation and by increase of intracellular Ca^{2+} as a result of stimulation of endothelial cell receptors (Traub and Berk, 1998; Tran *et al*., 2000; Boo *et al*., 2002). The Ca^{2+} and eNOS phosphorylation induced NO production is dependent on the duration of the shear stress. During shorter duration of shear stress,

intracellular Ca^{2+} release (Kuchan and Frangos, 1994), while shear stress of longer duration (>30 min) NO production is dependent on eNOS phosphorylation (Pittner *et al*., 2005).

Synthesized NO diffuses across the endothelial cell into the adjacent smooth muscle (Ignarro *et al*., 1986) (Figure 1.7), where it binds and activates the enzyme soluble guanylyl cyclase (sGC) (Ignarro *et al*., 1986). The activated enzyme increases the conversion rate of guanosine triphosphate (GTP) to cGMP (Arnold *et al*., 1977), which decreases smooth muscle tension (Jones *et al*., 1999). Further, cGMP reduces Ca^{2+} release from the sarcoplasmic reticulum in the smooth muscle cell (Collins *et al*., 1986), and also helps to restore Ca^{2+} to the sarcoplasmic reticulum (Cornwell *et al*., 1991). Both actions reduce the contraction of smooth muscle cells.

Continuous active production of NO maintains the basal vasodilator tone (Gladwin *et al*., 2004). The vessels constriction resulting with NO inhibitor, N^G monomethyl-L-arginine (L-NMMA), gave a dose dependent increase in blood pressure (Rees *et al*., 1989; Shesely *et al*., 1996). Administration of NO reversed the constriction (Rees *et al*., 1989; Fagan *et al*., 1999). However, the vessel is capable of dilating in the absence of NO. After removal of or damage to the endothelium, administration of glyceryl trinitrate (GTN) can still result in vasodilation (Vallance *et al*., 1989). The mechanism by which GTN causes vasodilation is not clear. Several researchers have suggested that GTN undergoes bioconversion to NO (Ignarro *et al*., 1988; Feelisch *et al*., 1988; Vallance *et al*., 1989; Marks *et al*., 1991), but not all agree, as GTN has been found to cause vasodilation without increasing NO (Kearney *et al*., 1998; Hussain *et al*., 1999; Artz *et al*., 2002; Kleschyov *et al*., 2003; Nunez *et al*., 2005). Further, the breakdown products of GTN have been

shown to activate sGC (Fung *et al*., 1992; Tian *et al*.,2001). It is worth noting that other vasoactive agents such as calcium ionophore A23187 and isosorbide-dinitrate induce vasorelaxation without an increase in NO concentration (Moncada and Higgs, 2006). Therefore, NO does not seem to be the only agent that can activate the sGC-cGMP pathway. Further research is needed to identify the precise mechanism of the agents, in particular, more research is needed *in vivo* due to the differences in response between intact or a denuded endothelium (Galley and Webster, 2004).

Aside from vasodilation, NO is also involved in preventing platelet and leukocyte activation and adhesion to the vessel wall (Kubes *et al*., 1991; Nong *et al*., 1997; Gidday *et al*., 1998; Ahluwalia *et al*., 2004). When the endothelium is damaged, the subsequent inflammation causes an increase in leukocytes at the damaged site (Lefer *et al*., 1999). Inflammatory mediators such as TNF-α, interleukin-1 (IL-1) and chemokines stimulate the release of iNOS (Chauhan *et al*., 2003), which prevents leukocytes from adhering to the endothelium and reduces inflammatory mediators (Peng *et al*., 1998), as well as down-regulating and reducing the expression of adhesion molecules (Ahluwalia *et al*., 2004).

1.1.2.2.1 Endothelial dysfunction

Endothelial dysfunction or disruption of the normal endothelial function is one of the earliest steps in the progress of atherosclerosis (Drexler, 2001; Landmesser *et al*., 2004). The endothelium maintains the equilibrium between vasodilation and vasoconstriction, pro-thrombotic and anti-thrombotic, inflammatory and anti-inflammatory processes for health (Drexler, 2001; Landmesser *et al*., 2004). For these processes production of nitric oxide (NO) is one of the key mediators. Balanced production of NO (vasodilator) and endothelin

(vasoconstrictors) maintains vascular tone. In addition to vasodilation NO reduces platelet adhesion, leukocyte migration and proliferation of vascular smooth muscle cells (Cai and Harrison, 2000; Vallance and Chan, 2001).

Endothelial dysfunction refers to a pathological condition in which many of the regular functions of the endothelium are disrupted leading to a complex array of abnormalities including disruption of normal vaso-regulation, increased adhesion and migration of leukocytes, platelet aggregation and vascular smooth muscle cell proliferation and resulting in hypertension, atherosclerosis, platelet aggregation and ischemia (Landmesser *et al*., 2004). Reduced NO bioavailability is fundamental to the development of endothelial dysfunction (Cai and Harrison, 2000). The hallmark of endothelial dysfunction is therefore attenuated NO dependent vasodilation (Deanfield *et al*., 2007).

During endothelium NOS activation, cofactor NADPH catalyse uncoupling of enzyme from caveolin and electrons donated by NADPH are transferred to molecular oxygen (O_2) rather than L-arginine's amine group, producing superoxide (O^{2-}) rather than NO. Thus, endothelium NOS uncoupling not only produces ROS, but reduces NO generation, potential for vasorelaxation (Behrendt and Ganz, 2002) and ROS caused modification of proteins through oxidative mechanisms are amplified. This results in increased oxidative stress and further increased levels of ROS production.

It is been proposed that decrease in NO signaling, as result of reduced nitric oxide synthase (NOS) production, contribute to the endothelial dysfunction (Rees, *et al.,* 1989; Moncada, *et al.,* 1991; Caterina, 2000). Studies have shown, acetylcholine (ACh) mediated endothelial vasodilation and blood flow was reduced in