CARDIOVASCULAR ACTIVITIES OF *VERNONIA AMYGDALINA*

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CARDIOVASCULAR ACTIVITIES OF *VERNONIA AMYGDALINA*

by

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TABLE OF CONTENTS

ACKNOWLEDGEMENT ii

TABLE OF CONTENTS iii

LIST OF TABLES xi

LIST OF FIGURES xiii

LIST OF SYMBOLS xviii

LIST OF ABREVIATIONS xx

ABSTRAK xxiii

ABSTRACT xxv

CHAPTER I - INTRODUCTION

1.1 Background 1

1.2 Research Objectives 5

1.2.1 General Objective 5

1.2.2 Specific Objectives 5

CHAPTER II - LITERATURE REVIEW

2.1 The Cardiovascular System 6

2.1.1 Arteries and Arterioles 6

2.1.2 Vascular Endothelium 8

2.1.2(a) Ion Channels 8

2.1.2(a)(i) Ca^{2+}-conducting Transient Receptor Potential Channels 9

2.1.2(a)(ii) Calcium Activated K^+ Channels (K_{Ca}) 11

2.1.2(b) The Discovery that Endothelium Modulates Vascular Tone 11

2.1.2(b)(i) Nitric oxide (NO) 12

2.1.2(b)(ii)Endothelium-derived Hyperpolarizing Factors (EDHF) 15

2.1.2(b)(iii) Prostacyclin (PGI_2) 17

2.1.2(b)(iv) Endothelins 17

2.1.3 Vascular Smooth Muscle Cells 18

2.1.3(a) Molecular mechanism regulating Ca^{2+}-dependent contraction in smooth muscle 19

2.1.3(b) Two Phases of Vasoconstriction 21

2.1.3(b)(i) The Initial Phase 21

2.1.3(b)(ii) The Second Phase 22

2.1.3(c) Mechanisms for relaxation 23

2.1.3(c)(i) Hyperpolarization 24

2.1.3(c)(ii) cAMP –PKA-mediated vasodilation 25

2.1.3(c)(iii) cGMP-PKG-mediated vasodilation 26

2.1.3(c)(iv) Desensitization to Ca^{2+} 27

2.1.3(d) Vascular Ion Channels and Membrane Potential 28

2.1.3(d)(i) Inward-rectifier K^+ Channel (K_{ir}) 30

2.1.3(d)(ii) ATP-dependent K^+ Channel (K_{ATP}) 30

2.1.3(d)(iii) Voltage-dependent K^+ Channel (K_V) and Ca^{2+}-dependent K^+ Channels K_{Ca} (BK) 31

2.1.3(d)(iv) Voltage-sensitive Ca^{2+} Channels (VSCCs) 32

2.1.3(d)(v) Ca^{2+}-conducting Transient Receptor Potential Channels 33

2.1.3(d)(vi) Chloride Channels 35

2.2 Arterial Blood Pressure 35

2.2.1 Mean Arterial Pressure (MAP) 38

2.3 Hypertension 39

2.3.1 Essential (Primary) Hypertension 40

2.3.2 Secondary Hypertension 41

2.3.3 Physiological Basis for Therapeutic Intervention 42

2.4 Neurohumoral Control of the Heart and Circulation 44

2.4.1 Autonomic Neural Control 44

2.4.1(a) Parasympathetic Innervation 45

2.4.1(b) Sympathetic Innervation 46

2.4.1(c) Cardiac and Vascular Autonomic Receptors 47

2.4.2 Humoral Control 50

2.4.2(a) Circulating Catecholamines 51

2.4.2(b) Renin-Angiotensin-Aldosterone System 52

2.4.2(c) Natriuretic Peptide 53

2.4.2(d) Vasopressin (Antidiuretic Hormone) 53

2.5 Medicinal Plants for the Treatment of Hypertension 54

2.6 *Vernonia amygdalina* 58

2.6.1 Taxonomy of *Vernonia amygdalina* 63

CHAPTER III - METHODOLOGY

3.1 List of Tools and Equipments 64

3.2 List of Chemicals 65

3.3 Plant Materials 66

3.4 Preparation of Crude Extracts 66

3.5 Experimental Animals 67

3.6 Cardiovascular Activities of *Vernonia amygdalina* 69

3.6.1 Vasorelaxant Activity of *Vernonia amygdalina* in Rat Thoracic Aortic Rings 69

3.6.1(a) Preparation of Rat Thoracic Aorta Rings *In Vitro* and Recording of Isometric Vascular Tone 69

3.6.1(b) Preliminary Screening of the Effects of *Vernonia amygdalina* Crude Extracts on Rat Thoracic Aortic Rings 70

3.6.2 Hypotensive Activity of *Vernonia amygdalina* in Anesthetized Rats 71

3.6.2(a) Surgical Procedures for Hemodynamic Measurements in Anesthetized Rats 71

3.6.2(b) Hemodynamic Screening of Crude Extracts of *Vernonia amygdalina* in Anesthetized Rats 72

3.6.3 Antihypertensive Activity of *Vernonia amygdalina* in Conscious Spontaneous Hypertensive Rats (SHRs) 72

3.6.3(a) The Effect of Single Dose of *Vernonia amygdalina* Extracts on Blood Pressure of Conscious Spontaneous Hypertensive Rats 74

3.6.3(b) The Effect of Daily Oral Administration of *Vernonia amygdalina* Extracts on Blood Pressure of Conscious Spontaneous Hypertensive Rats 74

3.6.3(c) The Effect of Daily Oral Administration of *Vernonia amygdalina* Extracts on Pulse Wave Velocity (PWV) of Spontaneous Hypertensive Rats 75

3.6.3(d) Biochemical Analysis 76

3.6.4 Fractionation of VACE and VAPE 77

3.6.5 Mechanism Studies 78

3.6.5(a) Preliminary Screening of the Effects of *Vernonia amygdalina* Fractions on Rat Thoracic Aortic Rings 78

3.6.5(a)(i) Effects of HI-VACE on Extracellular Ca^{2+}-induced Contraction Activated by KCl 79

3.6.5(a)(ii) Possible Action of HI-VACE on Intracellular Sarcoplasmic Reticulum Ca^{2+} release Induced by PE 80

3.6.5(a)(iii) Effects of HI-VACE on the Vascular Tone: Dependence on Endothelium 81

3.6.5(a)(iv) Effects of Endothelial Mediators on HI-VACE -induced Relaxation 81

3.6.5(a)(v) Roles of Muscarinic and Adrenergic Receptors on HI-VACE-induced Vasorelaxation 82

3.6.5(b) Hemodynamic Screening of Fractions of *Vernonia amygdalina* in Anesthetized Rats 83

3.6.5(b)(i) Elucidation of Hypotensive Mechanism(s) of VAPE in Anesthetized Rats 83

3.6.6 Statistical Analysis 84

3.7 Phytochemical Analysis of *Vernonia amygdalina* 85

3.7.1 Gas Chromatography–Mass Spectrometry (GC-MS) 85

3.7.2 High Performance Liquid Chromatography (HPLC) 86

3.7.2(a) Preparation of Stock Solutions for HPLC Analysis 86

3.7.2(b) Chromatographic Instruments and Conditions 86

3.7.2(c) Selection of Detection Wavelength 87

3.7.2(d) Linearity 87

3.7.2(e) Accuracy 87

3.7.2(f) Precision 88

3.7.2(g) Limit of Detection (LOD) and Limit of Quantification (LOQ) 88

3.7.2(h) Quantification of LA and OA Compounds in a Mixture and Extracts 88

CHAPTER IV - RESULTS

4.1 Extraction and Fractionation of *Vernonia amygdalina* Leaves 90

4.2 Vasorelaxant Activity of *Vernonia amygdalina* 91

4.2.1 Preliminary Screening of the Effects of *Vernonia amygdalina* on Rat Thoracic Aortic Rings 91

4.2.2 Effects of HI-VACE on Extracellular Ca^{2+}-induced Contraction Activated by KCl 95

4.2.3 Possible Actions of HI-VACE on Intracellular Sarcoplasmic Reticulum Ca^{2+} Release Induced by PE 99

4.2.4 Effects of HI-VACE on the Vascular Tone: Dependence on Endothelium 100

4.2.5 Effects of Endothelial Mediators on HI-VACE-induced Relaxation 101

4.2.6 Roles of Muscarinic and Adrenergic Receptors on HI-VACE-induced Vasorelaxation 105

4.3 Hypotensive Activity of *Vernonia amygdalina* in Anesthetized Rats 109

4.3.1 Hemodynamic Screening of *Vernonia amgydalina* in Anesthetized Rats 109

4.3.2 Hypotensive Mechanism(s) of VAPE in Anesthetized Rats 113

4.4 Antihypertensive Effect of *Vernonia amygdalina* in Conscious Spontaneous Hypertensive Rats (SHRs) 121

4.4.1 Body weights 121

4.4.2 The Effect of Single Dose of *Vernonia amygdalina* Extracts on Blood Pressure of Conscious Spontaneous Hypertensive Rats 122

4.4.3 The Effect of Daily Oral Administration of *Vernonia amygdalina* Extracts on Blood Pressure of Conscious Spontaneous Hypertensive Rats 124

4.4.4 The Effect of Daily Oral Administration of *Vernonia amygdalina* Extracts on Pulse Wave Velocity (PWV) of Spontaneous Hypertensive Rats 126

4.4.5 Biochemical Analysis 127

4.4.5(a) Effect of *Vernonia amgydalina* Extracts on Liver and Kidney Function 127

4.4.5(b) Effect of *Vernonia amgydalina* Extracts on Serum Cardiac Enzymes 127

4.5 Phytochemical Analysis of *Vernonia amygdalina* 129

4.5.1 Gas Chromatography–Mass Spectrometry (GC-MS) 129

4.5.2 High Performance Liquid Chromatography (HPLC) 135

4.5.2(a) Chromatographic analysis of two fatty acids 135

4.5.2(b) Quantification of Two Fatty Acids in Different Extracts of *Vernonia amygdalina* Plant Using HPLC Techniques 138

CHAPTER V - DISCUSSION

5.1 Vasorelaxant Activity of *Vernonia amygdalina* 140

5.2 Hypotensive Activity of *Vernonia amygdalina* in Anesthetized Rats 147

5.3 Antihypertensive Effect of *Vernonia amygdalina* in Spontaneous Hypertensive Rats (SHRs) 154

5.4 Phytochemical Analysis of *Vernonia amygdalina* 161

CHAPTER VI - CONCLUSION

6.1 Conclusion 164

6.2 Recommendations for Future Study 166

REFERENCES 167

APPENDICES

LIST OF TABLES

		Page
Table 2.1	Effects of sympathetic and parasympathetic stimulation on cardiac and vascular function.	47
Table 2.2	Nutritional composition of *Vernonia amygdalina* Del. leaves.	62
Table 4.1	Percentage of body weight gain of different groups in spontaneous hypertensive rats after 28 days of treatments.	121
Table 4.2	Average liver, kidney and cardiac function biomarkers in different groups after 28 days of treatment.	128
Table 4.3	Chemical profile of the chloroform crude extract of *Vernonia amygdalina* (VACE) identified by Gas Chromatography–Mass Spectrometry (GC-MS).	131
Table 4.4	Chemical profile of the hexane insoluble fraction of chloroform extract of *Vernonia amygdalina* (HI-VACE) identified by Gas Chromatography–Mass Spectrometry (GC-MS).	131
Table 4.5	Chemical profile of the hexane soluble fraction of chloroform crude extract of *Vernonia amygdalina* (HS-VACE) identified by Gas Chromatography–Mass Spectrometry (GC-MS).	132
Table 4.6	Chemical profile of the petroleum ether crude extract of *Vernonia amygdalina* (VAPE) identified by Gas Chromatography–Mass Spectrometry (GC-MS).	133

Table 4.7	Chemical profile of the fraction 3 of petroleum ether crude extract of *Vernonia amygdalina* identified by Gas Chromatography–Mass Spectrometry (GC-MS).	134
Table 4.8	Chemical profile of the fraction 4 of petroleum ether crude extract of *Vernonia amygdalina* identified by Gas Chromatography–Mass Spectrometry (GC-MS).	134
Table 4.9	Validation parameters for linoleic acid (LA) and oleic acid (OA).	136
Table 4.10	Analytical results of precision	137

LIST OF FIGURES

		Page
Figure 2.1	Structure of an artery	7
Figure 2.2	Regulation of vascular smooth muscle contraction by myosin light chain kinase (MLCK).	20
Figure 2.3	Receptors and signal transduction mechanisms that modulate intracellular calcium concentration and therefore the state of vascular tone.	27
Figure 2.4	Adrenergic and muscarinic receptors in the heart and blood vessels.	50
Figure 2.5	*Vernonia amygdalina* Del.	63
Figure 3.1	Schematic diagram of the serial extraction of *Vernonia amygdalina*.	68
Figure 3.2	Representation of the proximal and distal pressure wave forms.	76
Figure 4.1	The effects of the four crude extracts of *V. amygdalina*, petroleum ether extract (VAPE), chloroform extract (VACE), methanol extract (VAME) and water extract (VAWE) on the sustained contraction evoked by phenylephrine (1 μM) in isolated rat thoracic aortic rings.	93
Figure 4.2	The effects of the two fractions of VACE, hexane soluble (HS-VACE) and hexane-insoluble (HI-VACE) on the sustained contraction induced by phenylephrine (1 μM) in isolated rat thoracic aortic rings.	94

Figure 4.3	Vasodilation effect of HI-VACE on the sustained contraction induced by phenylephrine (PE) (1 µM) and KCl (80 mM) in isolated rat thoracic aortic rings.	96
Figure 4.4	The effect of 0.03 mg/ml and 0.06 mg/ml HI-VACE on $CaCl_2$-induced contraction in isolated rat thoracic aortic rings.	97
Figure 4.5	The effect of 0.1 µM and 1.0 µM of verapamil on $CaCl_2$-induced contraction in isolated rat thoracic aortic rings.	98
Figure 4.6	Effect of HI-VACE on intracellular calcium release induced by PE (1 µM) in isolated rat thoracic aortic rings bathed with Ca^{2+}-free solution.	99
Figure 4.7	Vasodilation effect of HI-VACE on the sustained contraction induced by PE (1 µM) in endothelium-intact and –denuded isolated rat thoracic aortic rings preparations.	100
Figure 4.8	Vasodilation effect of HI-VACE on the sustained contraction induced by PE (1 µM) in the presence and absence of 10 µM L-NAME in isolated rat thoracic aortic rings.	102
Figure 4.9	Vasodilation effect of HI-VACE on the sustained contraction induced by PE (1 µM) in the presence and absence of 10 µM methylene blue in isolated rat thoracic aortic rings.	103
Figure 4.10	Vasodilation effect of HI-VACE on the sustained contraction induced by PE (1 µM) in the presence and absence of 10 µM indomethacin in isolated rat thoracic aortic rings.	104
Figure 4.11	Vasodilation effect of HI-VACE on the sustained contraction induced by PE (1 µM) in the presence and absence of 1 µM atropine in isolated rat thoracic aortic rings.	106

Figure 4.12	Vasodilation effect of HI-VACE on the sustained contraction induced by PE (1 μM) in the presence and absence of 1 μM propranolol in isolated rat thoracic aortic rings.	107
Figure 4.13	Vasodilation effect of HI-VACE on the sustained contraction induced by KCl (80 mM) in the presence and absence of 1 μM prazosin in isolated rat thoracic aortic rings.	108
Figure 4.14	The hypotensive effects of the four crude extracts of *Vernonia amygdalina*, petroleum ether extract (VAPE), chloroform extract (VACE), methanol extract (VAME) and water extract (VAWE) in the anesthetized normotensive Sprague Dawley rats.	111
Figure 4.15	The hypotensive effects of the six fractions of VAPE, fraction 1 to fraction 6 in the anesthetized normotensive Sprague Dawley rats.	112
Figure 4.16	The effect of intravenous pre-treatment with (A) 50 μg/kg prazosin and (B) 25 mg/kg VAPE, on the NA-induced increment of mean arterial pressure (MAP) in the anesthetized normotensive Sprague Dawley rats.	114
Figure 4.17	The effect of intravenous pre-treatment with (A) 50 μg/kg prazosin and (B) 25 mg/kg VAPE, on the NA-induced increment of heart rate in the anesthetized normotensive Sprague Dawley rats.	115
Figure 4.18	The effect of intravenous pre-treatment with (A) 2 mg/kg propranolol and (B) 25 mg/kg VAPE, on the ISP-induced reduction of mean arterial pressure (MAP) in the anesthetized normotensive Sprague Dawley rats.	116

Figure 4.19	The effect of intravenous pre-treatment with (A) 2 mg/kg propranolol and (B) 25 mg/kg VAPE, on the ISP-induced increment of heart rate in the anesthetized normotensive Sprague Dawley rats.	117
Figure 4.20	The effect of intravenous pre-treatment with (A) 40 µg/kg neostigmine and (B) 25 mg/kg VAPE, on the ACh-induced reduction of mean arterial pressure (MAP) in the anesthetized normotensive Sprague Dawley rats.	118
Figure 4.21	The effect of intravenous pre-treatment with (A) 40 µg/kg neostigmine and (B) 25 mg/kg VAPE, on the ACh-induced reduction of heart rate in the anesthetized normotensive Sprague Dawley rats.	119
Figure 4.22	The effect of intravenous pre-treatment with 1 mg/kg atropine on the (A) ACh-induced reduction of mean arterial pressure (MAP) and (B) ACh-induced reduction of heart rate in the anesthetized normotensive Sprague Dawley rats.	120
Figure 4.23	Effect of a single oral administration of extract of *Vernonia amgydalina* on (A) mean arterial blood pressure (MAP) and (B) heart rate (HR) in spontaneous hypertensive rats.	123
Figure 4.24	Effect of repeated oral administration of extract of *Vernonia amgydalina* on (A) mean arterial blood pressure (MAP) and (B) heart rate (HR) in spontaneous hypertensive rats for 28 days.	125
Figure 4.25	Effect of daily oral administration (28 days) of extract of *Vernonia amgydalina* on pulse wave velocity in spontaneous hypertensive rats.	126

Figure 4.26 Representative HPLC chromatogram of 62.5 µg/mL standards mixture (Linoleic acid, LA and Oleic acid, OA). 136

Figure 4.27 Representative HPLC chromatogram of petroleum ether extract of *Vernonia amygdalina* (VAPE). 139

Figure 4.28 Representative HPLC chromatogram of hexane insoluble fraction of chloroform extract of *Vernonia amygdalina* (HI-VACE). 139

LIST OF SYMBOLS

%	percentage
<	less than
-	Negative/minus
=	equal
α	alpha
β	beta
γ	gamma
^{o}C	degree Celsius
BK_{Ca}	big conductance calcium activated potassium channel
Ca^{2+}	calcium ion
$[Ca^{2+}]$	calcium ion concentration
CO_2	carbon dioxide
g	gram
gCa^{2+}	ion conductance of calcium ion
gK^{+}	ion conductance of potassium ion
gNa^{+}	ion conductance of sodium ion
H^{+}	hydrogen ion
I_f	pacemaker current
IK_{Ca}	intermediate conductance calcium activated potassium channel
K^{+}	potassium ion
K_{Ca}	calcium activated potassium channel
K_{ir}	inward rectifier potassium channel
m^2	square meters

mL	milliliter
mm	millimeter
mM	millimolar
mV	millivolts
Na^+	sodium ion
nM	nanomolar
O_2	oxygen
SK_{Ca}	small conductance calcium activated potassium channel
µm	micrometer
µM	micromolar
US$	United States dollar

LIST OF ABBREVIATIONS

ACE	angiotensin converting enzyme
ACh	acetylcholine
ACN	acetonitrile
ADP	adenosine diphosphate
ANS	autonomic nervous system
ARP	absolute refractory period
ATP	adenosine triphosphate
AV	atrioventricular
CAD	coronary artery disease
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CRAC	calcium release activated channel
CVD	cardiovascular disease
DAG	diacyl glycerol
EDHF	endothelium-derived hyperpolarizing factor
EDRF	endothelium-derived relaxing factor
EDV	end diastolic volume
eNOS	endothelial nitric oxide synthase
ERP	effective refractory period
ESV	end systolic volume
ET-1	endothelin 1
FDA	Food and Drug Administration
GCMS	gas chromatography mass spectrometry

GPCR	G-protein coupled receptor
HDL	high-density lipoprotein
HI-VACE	hexane insoluble fraction of chloroform extract of *Vernonia amygdalina*
HPLC	high performance liquid chromatography
HS-VACE	hexane soluble fraction of chloroform extract of *Vernonia amygdalina*
iNOS	inducible nitric oxide synthase
IP_3	inositol triphosphate
ISP	isoprenaline
KCl	potassium chloride
LA	linoleic acid
L-NAME	N-ω-nitro-L-arginine methyl ester hydrochloride
MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phsophatase
mmHg	millimeter of mercury
NA	noradrenaline
NHMS	National Health and Morbidity Survey
NO	nitric oxide
nNOS	neural nitric oxide synthase
OA	oleic acid
PE	phenylephrine
PGI_2	prostaglandin I2 or prostacyclin
PIP_2	phosphatidyl inositol biphosphate
PKA	protein kinase A

PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C
ROC	receptor-operated channel
RyR	ryanodine receptor
SA	sinoatrial
SHR	spontaneous hypertensive rat
SL	semilunar
SOC	store-operated channel
SR	sarcoplasmic reticulum
SV	stroke volume
TCM	traditional and complementary medicine
TRPC	transient receptor potential cation channels
VA	*Vernonia amygdalina*
VACE	chloroform extract of *Vernonia amygdalina*
VAME	methanol extract of *Vernonia amygdalina*
VAPE	petroleum ether extract of *Vernonia amygdalina*
VAWE	water extract of *Vernonia amygdalina*
VCAM	vascular cell adhesion molecule
WHO	World Health Organization

AKTIVITI KARDIOVASKULAR *VERNONIA AMYGDALINA*

ABSTRAK

Beberapa ulasan mendokumenkan penggunaan etno-ubatan *Vernonia amygdalina* untuk rawatan hipertensi, tetapi penyiasatan secara saintifik masih terhad. Penyelidikan ini bertujuan untuk mengkaji kesan kardiovaskular *in vitro* dan *in vivo* dalam ekstrak daun *V. amygdalina*. Daun yang telah kering diekstrak dengan eter petroleum (VAPE), kloroform (VACE), metanol (VAME) dan air (VAWE) secara berturut-turut. Ekstrak tersebut dikaji dengan aktiviti vasorelaksan dalam gegelang aorta toraks tikus yang terpencil, aktiviti hipotensif terhadap tikus yang dibius, aktiviti anti-hipertensi, kekakuan arteri dan perubahan biokimia terhadap tikus hipertensi spontan (SHRs). Ekstrak yang paling berkesan dalam aktiviti vasorelaksan dan hipotensif seterusnya difraksinasi dan kajian mekanisme-mekanisme yang mungkin dijalani. Akhir sekali, analisis fitokimia *V. amygdalina* dijalankan dengan spektrometri jisim kromatografi gas dan kromatografi cecair prestasi tinggi. Hasil kajian menunjukkan bahawa semua ekstrak *V. amygdalina* mempunyai aktiviti vasorelaksan dan aktiviti hipotensif walaupun kepekatan berkesan untuk setiap ekstrak adalah berbeza; VACE menunjukkan aktiviti vasorelaksan yang paling berkesan manakala VAPE menunjukkan aktiviti hipotensf yang paling berkesan. Rawatan akut dan rawatan kronik (28 hari) oral 500 mg/kg ekstrak *V. amygdalina* menunjukkan kesan antihipertensi yang signifikan dan berkekalan. Fraksi heksana yang tidak larut daripada VACE (HI-VACE) menunjukkan kesan vasorelaksan yang tidak bersandar pada endotelium dan aktiviti antagonistik kalsium. Sebaliknya, 10 µM L-NAME, 10 µM metilena biru, 10 µM indomethacin, 1 µM atropina, 1 µM propranolol, 1 µM

prazosin tidak mengubah aktiviti vasorelaksan HI-VACE, menunjukkan bahawa laluan eNOS-NO-sGC-cGMP, pengeluaran prostanoid, reseptor muscarinic dan reseptor adrenergik tidak terlibat. Tiada fraksi VAPE menunjukkan kesan hipotensif yang ekuipoten atau lebih poten berbanding dengan asalnya. Kesan hipotensif daripada VAPE mungkin disebabkan oleh penghalang α-adrenergik dan β-adrenergik. Penurunan Ca^{2+} dalam sel yang disebabkan oleh penghalang saluran kalsium akan merencat tindakan yang teraruh oleh agonis α-adrenergik dan β-adrenergik. Oleh itu, VAPE berkemungkinan tinggi bertindak sebagai penghalang saluran kalsium. Sebatian yang terdapat dalam HI-VACE termasuk neofitadiena, asid linoleik dan asid oleik manakala dalam VAPE adalah 1-oktadekena, neophytadiene, asid palmitik, nonacosane, asid linoleik dan asid oleik. Kajian ini membekalkan asas farmakologi untuk penggunaan *V. amygdalina* dan mungkin lebih mendekati langkah fitoubat berasaskan bukti.

CARDIOVASCULAR ACTIVITIES OF *VERNONIA AMYGDALINA*

ABSTRACT

A few reviews on the ethno-medicinal use of *Vernonia amygdalina* for hypertension have been documented, but the scientific investigations are still very limited. The research was aimed to study the *in vitro* and *in vivo* cardiovascular effects of *V. amygdalina* leaf extracts. The dried leaf samples were successively extracted with petroleum ether (VAPE), chloroform (VACE), methanol (VAME) and water (VAWE). These extracts were examined for vasorelaxant activity in isolated rat thoracic aortic rings, hypotensive activity in anesthetized rats, anti-hypertensive activity, arterial stiffness and biochemical changes in spontaneous hypertensive rats (SHRs). The most effective vasorelaxant and hypotensive plant extracts were subjected for further fractionation and the possible mechanisms were elucidated. Lastly, phytochemical analysis of the most bioactive extract and fraction of *V. amygdalina* using gas chromatography mass spectrometry and high performance liquid chromatography were performed. The results showed that all *V. amygdalina* extracts evoke a vasodilating and hypotensive activity although the effective concentrations for each extract are different; VACE exhibit the most potent vasorelaxant activity whereas VAPE exhibit the most potent hypotensive activity. The acute and chronic (28 days) oral administration of 500 mg/kg *V. amygdalina* extracts induced a marked and sustained antihypertensive effects. Hexane-insoluble fraction of VACE (HI-VACE) exhibited an endothelium independent effect and calcium antagonistic activity. On the contrary, 10 µM L-NAME, 10 µM methylene blue, 10 µM indomethacin, 1 µM atropine, 1 µM propranolol, 1 µM prazosin did not alter HI-VACE-induced relaxation, indicating that eNOS-NO-sGC-cGMP pathways,

prostanoids production, muscarinic receptor and adrenergic receptor are not involved. None of the VAPE fraction showed equipotent or more potent hypotensive effect compared to their origin. The hypotensive effect of VAPE is possibly mediated as α-adrenergic and β-adrenergic blocker. Reduction of intracellular Ca^{2+} by calcium channel blocker would inhibit the response induced by both α-adrenoceptors and β-adrenoceptors agonists. Therefore, it is very likely that VAPE act as a calcium channel blocker. The compounds found in HI-VACE include neophytadiene, linoleic acid and oleic acid whereas in VAPE are 1-octadecene, neophytadiene, palmitic acid, nonacosane, linoleic acid and oleic acid. This study provides pharmacological basis for the use of *V. amygdalina* as anti-hypertensive agent and may be a step forward toward evidence-based phytomedicine.

CHAPTER I

INTRODUCTION

1.1 BACKGROUND

The tide of hypertension is rising in Malaysia and all over the globe, thereby becoming an increasingly powerful threat to global health. According to the National Health and Morbidity Survey (NHMS) I, II and III in 1986, 1996 and 2006, the prevalence of hypertension in Malaysia was 14.4 %, 32.9 %, 42.6 % respectively for residents aged 30 years and above. Moreover, the most recent NHMS IV in 2011 has reported that the prevalence of hypertension in Malaysians aged 18 years and above was 32.7 % (5.8 million) and for aged 30 years and above was 43.5 %. These surveys show that the prevalence of hypertension in Malaysia has a relative increase of 30 % in 25 years from 1986 to the year 2011. Besides, only 35 % of Malaysian patients achieved blood pressure control (<140/90 mmHg) while on treatment (Clinical Practice Guideline on Management of Hypertension, 2013). Worldwide, approximately 40 % of adults aged 25 years and above had been diagnosed with hypertension in 2008. The number of people with the condition rose from 600 million in 1980 to 1 billion in 2008 (Danaei et al., 2011). In addition, it is estimated that by 2025, up to 1.56 billion adults worldwide will be hypertensive (Kearney et al., 2005).

The high prevalence of hypertension makes it a significant factor for mortality and morbidity. Hypertension is one of the most important contributors to heart disease and stroke – which together make up the world's number one cause of premature death and disability (WHO, 2013). Globally, cardiovascular disease accounts for

approximately 17 million deaths a year, nearly one third of the total global deaths (WHO, 2008). Of these, complications of hypertension account for 9.4 million deaths worldwide every year (Lim et al., 2012). Hypertension is responsible for at least 45% of deaths due to heart disease and 51% of deaths due to stroke (WHO, 2008). Individuals with hypertension are known to have a twofold higher risk of developing coronary artery disease (CAD), four times higher risk of congestive heart failure, and seven times higher risk of cerebrovascular disease and stroke compared to normotensive subjects. According to global estimates 62% of stroke, 49% of CAD, and 14% of other non-fatal cardiovascular disease (CVD) events are attributed to non-optimal blood pressure (Lawes et al., 2006). Hypertension also increases the risk of conditions such as kidney failure and blindness (WHO, 2013). For all these complications, hypertension contributes substantially to the escalating costs of health care. Even though it is easily diagnosed and treated, many people do not have access to basic health services, particularly in low- and middle-income countries, therefore, the disease burden caused by hypertension has increased over the past decade (Mendisi, 2013). Recognizing the public health importance of reducing the global burden of heart disease and stroke, World Health Organization (WHO) is calling for intensified efforts to prevent and control hypertension on the World Health Day of 2013.

In view of the growing number of hypertension and its life-threatening complications, coupled with the harsh side effects of costly modern pharmacological therapy resulting in patient non-compliance, the quest for alternatives which are relatively cost effective with minimal or no side effect is warranted. Natural products of plant origin have demonstrated promising potential. In fact, many ailments are known to be treated with herbal remedies throughout the history of mankind.

Representing an annual global market of US$ 60 billion every year, herbal medicines account for around 20 % of the overall drug market. According to WHO, up to 80 % of the population in Africa depends on traditional medicine for primary health care and in China, herbal medicines account for 30–50% of total medicinal consumption. In Europe, North America and other industrialized regions over 50% of the population have used complementary or alternative medicine at least once (WHO, 2004). Today, researches are focusing on the discovery of new therapeutic substances of natural origin with possible low or no toxicity to human, animal and environment, based on ethno-medical and ethno-veterinary practices. The WHO (1993) supports the use of effective and safe remedies and accepts traditional medicine as a valuable and readily available resource of health care. During the period 2000-2005, 23 new drugs derived from natural sources were approved by the Food and Drug Administration (FDA) and introduced to the market, for cancer, cardiovascular, neurological, metabolic and immunological diseases, and genetic disorders (Chin et al., 2006).

Several medicinal plants have been reported to have antihypertensive potential (Ameer, 2009; Globinmed, 2010; Kaur, 2013; Mashour et al., 1998; Tabassum and Ahmad, 2011; Yeap et al., 2010). An example of such plants is *Vernonia amygdalina*. A few studies have shown rationale in the use of *V. amygdalina* for the treatment of hypertension. Anti-hypertensive effect of *V. amygdalina* could be mediated through direct vasorelaxant mechanism from the findings of Taiwo and colleagues (2010). The inhibition of angiotensin converting enzyme (ACE), coupled with the antioxidant activities of *V. amygdalina* phenolic-rich extracts, could be another possible mechanism through which *V. amygdalina* exerts its anti-hypertensive property (Saliu et al., 2012). Furthermore, Ajibola and colleagues (2011) found that the polyphenolic fraction (chlorophyllic fraction) of the leaf extract of *V. amygdalina* displayed high

potency against both angiotensin converting enzyme (ACE) and renin as possible mechanisms of its antihypertensive potential.

V. amygdalina occurs wild in most countries of tropical Africa (Grubben, 2004) and had been recently introduced into the Malaysia herbal armament. The plant can be found along roadside or at home and commercial plantations in Malaysia (Atangwho et al., 2013; Globinmed, 2010; Yeap et al., 2010). This plant is well-known among Malaysian as a remedy for the management of diabetes mellitus and hypertension (Atangwho et al., 2013; Globinmed, 2010). Although *V. amygdalina* is commonly used ethno-medicinally for the management/treatment of hypertension (Gbolade, 2012; Karou et al., 2011; Lawal et al., 2010; Mensah et al., 2008; Saliu et al., 2012), the scientific investigations are still very few. The possible mechanism(s) of action of *V. amygdalina* extracts in alleviating hypertension in experimental animal models are lack of information. Moreover, the compound(s) in *V. amygdalina* responsible for the anti-hypertensive activity have not yet been identified. Hence, more scientific research such as mechanism study needs to be done to verify the effectiveness of *V. amygdalina* for its antihypertensive potential.

On the other hands, Malaysia, being a rich of biodiversity and multi-racial country, has a rich heritage of various traditional medicine practices, each origins from different ethnic groups and many forms of traditional health care still practicing in spite of a remarkably modern rural health service (Ariff and Khoo, 2006). In accordance with the concomitant use of herbal medicines in the management of various illness such as hypertension, understanding of the *V. amygdalina* underlying mechanism of action in control of hypertension responsible for the anti-hypertensive activity would help to improve the care and health outcome of hypertensive patients.

1.2 RESEARCH OBJECTIVES

1.2.1 General objective

To study the *in vitro* and *in vivo* cardiovascular effects of *Vernonia amygdalina* leaf extracts.

1.2.2 Specific objectives

1. To perform the vasorelaxant and hypotensive activities guided fractionation of *V. amygdalina* leaf extracts.
2. To elucidate the pharmacological mechanism(s) by which the most active plant extract exerts its proposed action by *in vitro* (vasorelaxant activity in isolated rat thoracic aortic rings) and *in vivo* (hypotensive activity in anesthetized Sprague Dawley rats) experimental methods.
3. To study the antihypertensive effect of *V. amygdalina* in conscious spontaneous hypertensive rats (SHRs).
4. To study the effect of *V. amygdalina* on arterial stiffness.
5. To study the biochemical changes (kidney function tests, liver function tests and cardiac enzymes) of *V. amygdalina* -treated spontaneous hypertensive rats (SHRs).
6. To perform a phytochemical analysis of the most bioactive extract and fraction of *V. amygdalina* using gas chromatography mass spectrometry (GCMS) and high performance liquid chromatography (HPLC).

CHAPTER II

LITERATURE REVIEW

2.1 THE CARDIOVASCULAR SYSTEM

The cardiovascular system (cardio- = heart; vascular = blood vessels) consists of three interrelated components: the heart, blood vessels, and blood. Arterial pressure is generated by the force of blood pushing against the walls of blood vessels (arteries) as it is pumped by the heart. This arterial pressure serves as the driving force for blood flow to all organ systems. The relative distribution of blood flow to organs is regulated by the vascular resistance of the individual organ. Appropriate systemic arterial pressure is perhaps the single most important requirement for proper operation of the cardiovascular system (Klabunde, 2012; Mohrman and Heller, 2010; Tortora and Derrickson, 2009).

2.1.1 Arteries and Arterioles

The wall of a blood vessel are made up of three layers or tunics of different tissues: tunica interna (an epithelial inner lining), tunica media (a middle layer consisting of a smooth muscle and elastic connective tissue), and tunica externa (a connective tissue outer covering) (Figure 2.1). Modifications of this basic design account for the structural and functional differences among the various vessel types (Tortora and Derrickson, 2009).

The wall of an artery has the three layers of a typical blood vessel, but has a thick muscular-to-elastic tunica media. Arteries are normally high compliance (stretch

easily) due to their plentiful elastic fibers and helps to conduct blood from the heart to various organ. Moreover, they act as a pressure reservoir for propelling blood onward during ventricular diastole.

Arterioles, literally meaning small arteries, play a key role in regulating the flow of blood from arteries into the capillary networks of the body's tissues by regulating resistance (opposition) to blood flow. Changes in the diameter of arterioles by sympathetic nerve supply in tunica externa, along with the actions of local chemical mediators, affect blood pressure. Vasoconstriction of arterioles increases blood pressure whereas vasodilation of arterioles decreases blood pressure (Mohrman and Heller, 2010; Tortora and Derrickson, 2009; Widmaier et al., 2014).

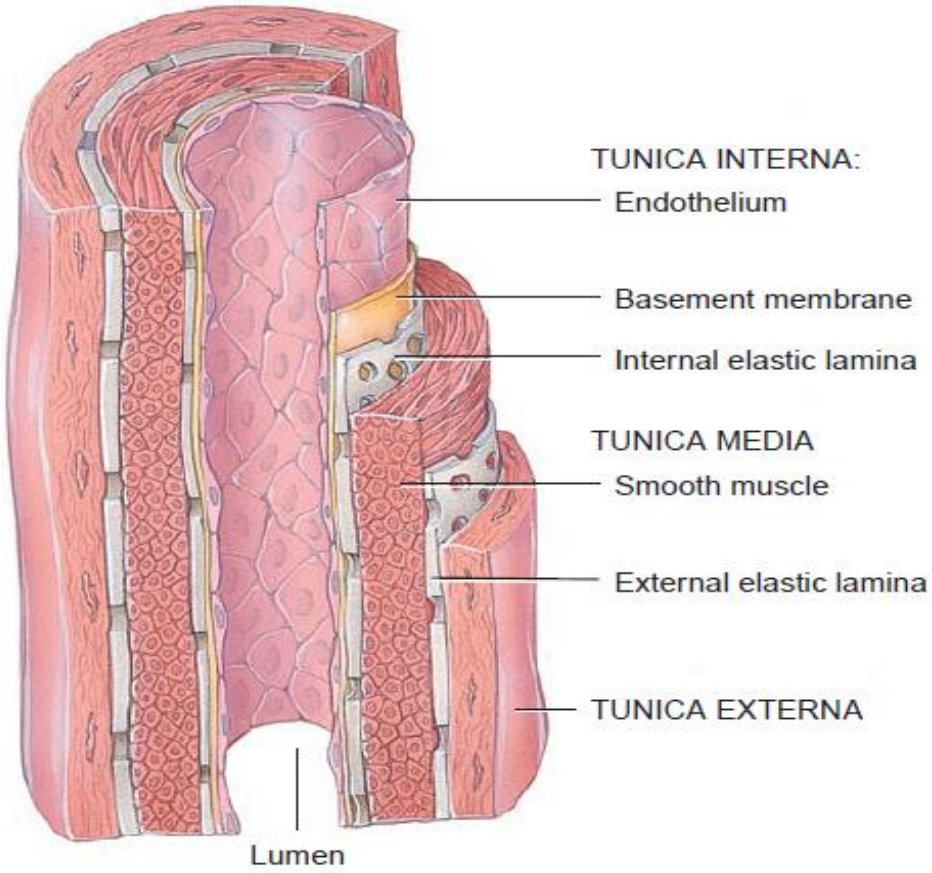

Figure 2.1 Structure of an artery (Tortora and Derrickson, 2009).

2.1.2 Vascular Endothelium

Endothelium is a monolayer of endothelial cells that lines the blood interface throughout the cardiovascular system, including the cardiac chambers. Endothelial cells play a wide variety of critical roles, including: (1) Regulating blood tissue exchange. (2) Paracrine secretions which include nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF), prostaglandin I2 (PGI_2), and endothelin-1 (ET-1) regulate vascular tone. (3) Inhibiting platelet aggregation (anti-thrombotic) primarily through biosynthesis of NO and PGI_2. (4) Modulating leukocyte adhesion and transendothelial migration through the biosynthesis of NO and the expression of surface adhesion molecules. (5) Endothelial surface enzymes (eg: angiotensin converting enzyme) modify vasoactive peptides in the bloodstream. (6) Endothelium initiates new blood vessel formation. The roles of endothelium are thus many, diverse and vitally important (Mohrman and Heller, 2010; Klabunde, 2012; Levick, 2010; Pappano and Wier, 2013).

2.1.2(a) Ion Channels

Endothelial cells express a variety of ion channels. Most of them influence intracellular Ca^{2+} ion concentration which is a key regulator of endothelial function (Tran and Watanabe, 2006) such as nitric oxide production and the hyperpermeability of inflammation. Basal cytosolic Ca^{2+} concentration, 30-100 nM, is much lower than extracellular Ca^{2+} concentration, 1 mM, and can be increased five- to ten-fold (e.g. by histamine), partly by the influx of extracellular Ca^{2+} through ion channels, and partly by release of Ca^{2+} stored in the endoplasmic reticulum (Levick, 2010; Socha et al., 2012).

Endothelial cells are not excitable, i.e. they cannot generate action potentials, because they do not express sufficient voltage-gated Na^+ and Ca^{2+} channels. They do, however, have a regulated negative intracellular potential of -30 to -68mV, which is generated mainly by the outward diffusion of K^+ ions through inward rectifier K^+ channels (K_{ir}) and calcium activated K^+ channels **(**K_{Ca}). Furthermore, the electrogenic $3Na^+$-$2K^+$ pump contributes about -8mV. Membrane potential magnitude is important because it affects the electrochemical force driving extracellular Ca^{2+} into the endothelial cell; abolition of the potential greatly attenuates Ca^{2+}-mediated responses (Levick, 2010). Besides that, changes in endothelial membrane potential can be conducted through gap junctions to serve as a signal, as in ascending vasodilation (Levick, 2010; Sandow et al., 2006).

2.1.2(a)(i) Ca^{2+}-conducting Transient Receptor Potential Channels

Although endothelium lacks voltage-gated Ca^{2+} channels, the surface membrane has two other types of Ca^{2+}-conducting channel: receptor-operated channels and store-operated channels. When the cell is stimulated by an agonist, such as histamine, these Ca^{2+} channels are activated and raise the intracellular free Ca^{2+} concentration five- to ten-fold (Levick, 2010).

Receptor-operated channels (ROCs) are cation-conducting channels that are activated via a biochemical cascade when an extracellular agent, the ‘agonist’, binds to its specific membrane receptor. Endothelial agonist include histamine, bradykinin, thrombin, serotonin, ATP and acetylcholine (Socha et al., 2012). The agonist receptor activated a G-protein, which activates the membrane-bound enzyme phospholipase C. Phospholipase C splits a phospholipid, phosphatidyl inositol biphosphate (PIP_2), into diacylglycerol (DAG) and inositol triphosphate (IP_3). The DAG activates the ROC ion

channel, and the IP_3 releases a small store of Ca^{2+} from the sarcoplasmic reticulum (Tran and Watanabe, 2006; Levick, 2010). ROCs are poorly selective cation channels that conduct Ca^{2+} and also some Na^+ and K^+. Since there is a large electrochemical gradient for Ca^{2+} influx, ROC activation raises cytosolic Ca^{2+} rapidly. The ROC constituent proteins were recently identified as members of the TRPC family (transient receptor potential, canonical subtype) (Levick, 2010).

Store-operated channels (SOCs) are also Ca^{2+}-conducting channels in the surface membrane, but their activation is associated with Ca^{2+} store release (Sinkins et al., 1998; Ong et al., 2002; Venkatachalam et al., 2002). There are two main types of SOC. Human endothelium expresses SOCs composed of TRPC1 protein (Groschner et al., 1998; Paria et al., 2003; Tiruppathi et al., 2002). These are low selectivity, cation-conducting channels that are activated by IP_3 as it releases the endoplasmic reticulum store. A second type of SOC is composed of Orai1 proteins. This channel is exquisitely Ca^{2+}-selective, and is activated by an endoplasmic reticulum proteins, ST1M1, following Ca^{2+} store release; so it is also called the CRAC channel (calcium-release activated channel) (Prakriya and Lewis, 2015). SOC activation leads to an influx of extracellular Ca^{2+}, called capacitative or store-operated Ca^{2+} entry (Prakriya and Lewis, 2015; Putney et al., 2001). This raises the free Ca^{2+} level, as well as restocking the SR store. SOCs are thought to be more abundant than ROCs in many types of endothelium (Levick, 2010).

As a result of these multiple pathways, an agonist usually evokes a biphasic change in endothelial Ca^{2+} concentration. There is an initial high spike in free Ca^{2+} concentration, brought about by IP_3-mediated store release and DAG-mediated ROC activation. This is followed by a lesser but more sustained Ca^{2+} elevation due to SOC activation (Levick, 2010). Endothelial cells may also possess stretch-activated

channels, which allow Ca^{2+} entry in response to shear stress (Kwan et al., 2003; Levick, 2010; Resnick et al., 2003; Tseng et al., 1995).

2.1.2(a)(ii) Calcium Activated K^+ Channels (K_{Ca})

A rise in endothelial free Ca^{2+} activates a special type of K^+ channels, the calcium-activated potassium channels K_{Ca}. This increases the membrane permeability to K^+, leading to hyperpolarization. Hyperpolarization serve two functions. It increases the electrochemical force drawing extracellular Ca^{2+} into the cell; and it can be transmitted through myoendothelial gap junctions to induce arteriolar relaxation (Félétou and Vanhoutte, 2009; Levick, 2010).

There are three subtypes of K_{Ca} channel: small conductance (SK_{Ca}), intermediate conductance (IK_{Ca}) and big conductance (BK_{Ca}). SK_{Ca} and IK_{Ca} expressed predominantly in endothelium, whereas BK_{Ca} occurs in vascular smooth muscle (Levick, 2010; Yang et al., 2012). SK_{Ca} and IK_{Ca} in the endothelium were found to be important for nitric oxide release (Absi et al., 2007; Brähler et al., 2009; Doughty et al., 1999; McNeish et al., 2006; Stankevicius et al., 2006). The channels are pharmacologically distinguishable: SK_{Ca} is blocked by apamin, a constituent of bee stings; IK_{Ca} and BK_{Ca} are blocked by charybdotoxin, a constituent of scorpion stings; and BK_{Ca} is blocked by iberiotoxin (Levick, 2010).

2.1.2(b) The Discovery that Endothelium Modulates Vascular Tone

The discovery that endothelium secretes vasoactive agents was reported relatively late. The role of the endothelium as a regulator of vascular tone began to emerge when Vane and colleagues discovered in 1976 that blood vessels secrete a vasodilator substance, prostacyclin (PGI_2). A second endothelium-derived

vasodilating agent was discovered in 1980 by Furchgott and Zawadski, who noted that the vasodilation of large arteries by an acetylcholine analogue, carbachol, changed into vasoconstriction when the endothelial lining was rubbed away. It merged that agonists, such as acetylcholine, stimulate endothelium to secrete a vasodilator substance, nitric oxide. The vasodilator action of the endothelium-derived nitric oxides overrides the direct vasoconstrictor action of acetylcholine on arterial muscle. A third vasodilator, endothelium-derived hyperpolarizing factor (EDHF), was discovered in 1987 when it was realized that endothelium-dependent vasodilation continue to exist in small vessels after blocking nitric oxide and prostacyclin production. The response involves smooth muscle hyperpolarization. A fourth endothelial secretion, a vasoconstrictor peptide called endothelin, was discovered in 1989 by a Japanese group using molecular biology techniques. All four substances are released as they are produced, rather than being stored for later secretion (Levick, 2010).

2.1.2(b)(i) Nitric oxide (NO)

Nitric oxide is produced constitutively at a low basal rate which inhibits vascular tone and survives only seconds before degradation (Tousoulis et al., 2012). It is produced by a constitutively expressed enzyme, endothelial nitric oxide synthase, eNOS (NOS-III) which cleaves the nitrogen group from the amino acid L-arginine and combines it with oxygen to form NO (Cylwik et al., 2005; Moncada, 1993). Inactive analogues of arginine, such as nitroarginine methyl ester (NAME), compete with normal arginine for the eNOS binding site, and therefore act as eNOS blockers (Sainz et al., 2004). If NO synthesis is inhibited pharmacologically using eNOS blocker, vasoconstriction occurs in most vascular beds. NO is inactivated within seconds by two mechanisms: (1) NO reacts with a by-product of oxidative metabolism, the

superoxide anion O_2^-, to form peroxynitrite, $ONOO^-$. Peroxynitrite is then converted into ordinary nitrite (NO_2^-) and nitrate (NO_3^-) for excretion in the urine. (2) Some NO diffuses into the bloodstream, where its similarity to oxygen (O-O) causes it to bind to red cell haemoglobin (Levick, 2010).

On the other hand, eNOS can be transiently stimulated to produce high levels of NO by environmental stimuli or agonists. Basal shear stress provides an important, tonic drive for NO production (Förstermann and Sessa, 2012; Joannides et al., 1995; Rubani et al., 1986). During exercise, increased blood flow in the arteries feeding the active skeletal muscles raises the shear stress and consequently NO production. This results in flow-induced vasodilation in large, conduit arteries supplying active muscle groups. The shear stress is probably transduced into a biochemical signal by the glycocalyx, since glycocalyx-degrading enzymes reduce NO production. Transduction activates the enzyme phosphatidyl inositol-3 kinase (PI3 kinase), leading to the activation of protein kinase B (akt) (Datta et al., 1999; Toker and Newton, 2000), which phosphorylates eNOS (Dimmeler et al., 1999; Futon et al., 1999). Phosphorylation render eNOS more sensitive to background Ca^{2+}-calmodulin, the intracellular activator of eNOS (Levick, 2010).

Besides that, eNOS activity can be enhanced by agonists such as acetylcholine and inflammatory mediators. Acetylcholine raise endothelial free Ca^{2+} concentration via the PLC-ROC/SOC pathway. Some of the Ca^{2+} binds to an intracellular Ca^{2+}-binding protein, calmodulin. The Ca^{2+}-calmodulin complex enhances eNOS activity and hence NO production. Agonists that act in this way include bradykinin, thrombin, substance P, ATP, ADP, acetylcholine (via muscarinic M_3 receptors), vasoactive intestinal polypeptide, insulin and in some tissues/species histamine (Tousoulis et al., 2012; Levick, 2010). Several of these agents are released during inflammation, so NO

contributes to the characteristic redness (vasodilation) of inflamed tissue. NO causes vasodilation by two mechanisms: (1) Endothelial NO diffuses rapidly into neighboring vascular smooth muscle cells, where it binds to the haem group of a soluble enzyme, guanylyl cyclase. N-O is chemical cousin to O-O, oxygen, hence its high affinity for haem. The activated guanylyl cyclase catalyzes the production of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate. The cGMP activates kinases (enzymes that phosphorylate other proteins to alter their activity) that produce vascular relaxation (Giles et al., 2012). (2) High concentrations of NO directly activate big conductance K_{Ca} channels (BK_{Ca}) in the smooth muscle membrane. This hyperpolarizes the smooth muscle, leading to vascular relaxation. The drug nitroprusside also increases cGMP, causing vasodilation. Nitroprusside acts directly on the vascular smooth muscle; its action is not endothelially mediated (Levick, 2010).

Nitric oxide (NO) is soluble in both lipid and water, so it diffuses freely from the endothelium into the neighboring vascular smooth muscle and bloodstream, with multiple local effects (Förstermann and Sessa, 2012; Hermann et al., 2006; Levick, 2010; Klabunde, 2012):

1) NO lowers vascular tone in veins, and in large muscular arteries, such as the coronary artery. NO also dilates small resistance vessels, though endothelial derived hyperpolarizing factor (EDHF) is relatively more important in these vessels.

2) NO contributes to gap formation in venules during inflammation.

3) NO inhibits vascular myocytes proliferation, a component of atheroma.

4) NO inhibits platelet aggregation and thus protects blood vessels from thrombosis,

5) NO inhibits the transcription of leukocyte-binding adhesion molecules, such as endothelial vascular cell adhesion molecule (VCAM), involved in attaching leukocytes to the endothelial surface.

The inhibition of smooth muscle proliferation, platelet activation and leukocytes adhesion by NO are all anti-atheroma actions, and reduced NO availability is thought to contribute to atheroma formation. Plasma-derived cholesterol and fibrin trapped between the endothelium and tunica media, leading to the formation of atheromatous plaque (Levick, 2010; Klabunde, 2012).

2.1.2(b)(ii) Endothelium-derived Hyperpolarizing Factors (EDHF)

NO exerts its main physiological effects in large vessels, where eNOS is abundant. If NO and prostacyclin production are blocked in small arteries and arterioles, agonists such as acetylcholine and bradykinin still evoke endothelium-dependent dilatation, which is mediated by smooth muscle hyperpolarization (Edwards et al., 2010; Félétou and Vanhoutte, 2009). Also, in some tissues saline washed through agonist-stimulated vessels picks up a chemical that can hyperpolarize isolated vascular muscle – an 'endothelium-derived hyperpolarizing factor', EDHF.

The stimuli for EDHF production include the classical parasympathetic transmitter acetylcholine, inflammatory agents such as bradykinin, and possibly shear stress. The roles of EDHF are thought to be: (1) To help increase blood flow to exercising muscle, by dilating small, feed arteries. (2) To contribute to the cholinergic vasodilation of small resistance vessels in certain tissues with a cholinergic autonomic innervation. (3) To contribute to the vasodilation of inflammation (Levick, 2010).

The identity of EDHF is controversial. Although endothelium-derived hyperpolarization can indeed be brought about by release, chemical factor(s) in some tissues, it can also be brought about by direct, electrical coupling between the endothelium and vascular muscle. Electrical transmission may be the primary hyperpolarizing mechanism, with released, soluble EDHFs serving to boost the

myocyte hyperpolarization (Edwards et al., 2010; Félétou and Vanhoutte, 2009; Giles et al., 2012).

Direct electrical transmission of endothelial hyperpolarization to vascular myocyte has been demonstrated in arterioles and small arteries (diameter ~ 100 μm), where myoendothelial gap junctions are abundant. When an agonist raises the endothelial free Ca^{2+} concentration, the activated small conductance K_{Ca} channel (SK_{Ca}) and intermediate conductance K_{Ca} channel (IK_{Ca}) hyperpolarize the endothelial cell. The endothelial hyperpolarization then spreads through the myoendothelial (heterocellular) gap junctions to hyperpolarize and relax the vascular myocytes (Edwards et al., 2010; Félétou and Vanhoutte, 2009). This form of 'EDHF' is inhibited by gap junctions blockers, and by apamin and charybotoxin, the blockers of endothelial SK_{Ca} and IK_{Ca} channels (Levick, 2010).

The endothelial cell also releases soluble factors (NO, prostacyclin, H_2O_2, and epoxyeicosatrienoic acid) that cause hyperpolarization of vascular smooth muscle by activation of large conductance K_{Ca} channels (BK_{Ca}). This action moves the membrane potential farther from the threshold at which Ca^{2+} entry occurs. In small coronary, renal and skeletal muscle arteries, epoxyeicosatrienoic acid (EET) may be an additional EDHF. In these vessels, the agonist bradykinin activates endothelial phospholipase A_2, which generates arachidonic acid. Arachidonic acid is converted by endothelial cytochrome P450 epoxygenase to diffusible EET. Myocyte EET receptors trigger a pathway that activates myocyte BK_{Ca} channels, leading to hyperpolarization and vasodilation. Additional candidate EDHF factors are hydrogen peroxide and c-natriuretic peptide. Also, activated endothelial SK_{Ca} and IK_{Ca} channels release K^+ ions into the interstitial spaces around neighboring vascular myocytes. The rise in extracellular K^+ reinforces myocyte hyperpolarization, by stimulating the myocytes

$3Na^+$-$2K^+$ pump and by activating myocyte K_{ir} channels. EDHF becomes an increasingly important as a vasodilator in the microcirculation as vessel radius decreases (Edwards et al., 2010; Félétou and Vanhoutte, 2009; Giles et al., 2012; Levick, 2010; Pappano and Wier, 2013).

2.1.2(b)(iii) Prostacyclin (PGI_2)

Like NO, prostacyclin (prostaglandin I_2, PGI_2) causes vasodilation and inhibits platelet aggregation, both of which are induced by the formation of cAMP (Kawabe et al., 2010; Majed and Khalil, 2012). It is produced by endothelium constitutively and also in response to agonists, such as thrombin. Phospholipase A_2 converts membrane phospholipids into the unsaturated fatty acid, arachidonic acid. The arachidonic acid is then converted by cyclo-oxygenases into prostacyclin (Kawabe et al., 2010; Majed and Khalil, 2012). Prostacyclin production is greatly increased in inflammation and contributes to the associated vasodilation. It also contributes to the cutaneous vasodilation associated with sweating (Klabunde, 2012; Levick, 2010; Pappano and Wier, 2013).

2.1.2(b)(iv) Endothelins

The endothelins are a family of peptides related to the snake venom sarafotoxin. Endothelin-1 (ET-1), the main isoform secreted by the endothelium (Battistini et al., 1993), causes a powerful, unusually sustained vasoconstriction, lasting 2-3 hours. ET-l is a potent vasoconstrictor for substance that is synthesized from an intracellular precursor by endothelin-converting enzyme (ECE) found on the endothelial cell membrane (Xu et al., 1994). ET-1 leaves the endothelial cell and can bind primarily to receptors (ET_A) on vascular smooth muscle (Huggins et al., 1993), which are

coupled via a G_q-protein to the myocyte phospholipase C-IP_3 system (Simonson and Dunn, 1990). The resulting rise in myocyte Ca^{2+} triggers vasoconstriction (Wagner et al., 1992). Over a longer time scale, endothelin also stimulates vascular and cardiac myocyte proliferation. ET-1 can also bind to a second type of receptor (ET_B) located on the vascular endothelium (Huggins et al., 1993) that stimulates nitric oxide and prostacyclin synthesis and release (Sakurai et al., 1990), which act as negative feedback mechanisms to counteract the ET_A-mediated vasoconstrictor effects of ET-1.

Endothelin is produced continuously and makes a small contribution to basal vascular tone in humans. Its production can be increased by hypoxia, angiotensin II, vasopressin, thrombin, cytokines, reactive oxygen species, and shearing forces. ET-1 release is inhibited by nitric oxide, as well as by prostacyclin and atrial natriuretic peptide. Plasma endothelin levels are raised in pre-eclamptic toxaemia (the hypertension of pregnancy) (Ajne et al., 2003) and heart failure (Murphy et al., 2010; Neuhold et al., 2010). Endothelin also contributes to cerebral artery vasospasm in patients with haemorrhagic strokes. Some forms of hypertension (e.g., pulmonary artery hypertension) appear to involve ET-1 and are treated with ET-1 receptor blockers (Klabunde, 2012; Levick, 2010; Pappano and Wier, 2013).

2.1.3 Vascular Smooth Muscle Cells

The tunica media of arteries, arterioles, venules and veins consists mainly of vascular smooth muscle cells (vascular myocytes). They are responsible for the control of total peripheral resistance, arterial and venous tone, and the distribution of blood flow throughout the body (Levick, 2010).

2.1.3(a) Molecular mechanism regulating Ca^{2+}-dependent contraction in smooth muscle

In smooth muscle, the interaction between myosin and actin, which leads to vascular contraction depends primarily on cytoplasmic Ca^{2+} concentration (Berridge, 2008). A rise in cystosolic Ca^{2+} concentration causes the formation of Ca^{2+}-calmodulin complex; calmodulin is a cytoplasmic protein presents in high abundance in smooth muscle cells, which binds 4 Ca^{2+} (Vogel, 1994). The Ca^{2+}-calmodulin complex activates enzymes, myosin-light chain kinase (MLCK) (Kamm and Stull, 2001). The 20-kDa regulatory light chain (MLC_{20}) is a component of the myosin heads involved in cross-bridge formation with actin filaments, and vascular myosin only forms cross-bridges when the MLC_{20} is phosphorylates. MLCK transfers a phosphate groups from ATP to the MLC_{20}, enabling myosin head to form a cross-bridge with actin (Figure 2.2) (Allen and Walsh, 1994; Hirano, 2007; Somlyo and Somlyo, 2003).

The phosphate group can be removed by the enzyme, myosin light chain phosphatase (MLCP). When intracellular Ca^{2+} concentration falls, MLCK activity declines and the competing MLCP dominates, dephosphorylating the myosin. Since dephosphorylated myosin cannot form new cross-bridges, the MLCP in effect turns off the myosin motor. As existing cross-bridges detach, new ones cannot forms, so the myocytes relax, leading to vasodilation (Hirano, 2007; Somlyo and Somlyo, 2003). Increased MLCP activation may explain cases of vascular relaxation with little fall on cytosolic Ca^{2+} concentration, e.g. hypoxic vasodilation.

The cytoplasmic Ca^{2+} concentration, and thus MLCK activity, is determined by the summation of the Ca^{2+} that enters the cytosol (influx) and that leaving the cytosol (efflux). Ca^{2+} enters the cytosol in two ways: (1) from the extracellular space, via influx through voltage-operated calcium channels (typically L-type, activated by

depolarization (Walsh, 2011). The voltage–sensitive Ca^{2+} channels have a low but finite open-state probability under basal conditions, allowing small extracellular Ca^{2+} influx that contributes to basal tone), receptor-operated calcium channels (ROCs, activated after the action of agonists on membrane receptors), and store-operated calcium channels (activated after depletion of sarcoplasmic reticulum Ca^{2+} stores), and (2) stored Ca^{2+} is released from the sarcoplasmic reticulum (SR) via activation of either ryanodine receptor channels, RyRs or inositol-1,4,5 triphosphate (IP_3) receptor channels (IP_3-stimulated) located on the SR (Hill-Eubanks et al., 2011). Ca^{2+} ions leave the cytosol via ATP-driven calcium transporters (i.e., Ca^{2+} pumps) located on both the SR (termed SERCAs) and plasma membrane (termed PMCAs) as well as activation of the Na^{+}/Ca^{2+} exchangers on the plasma membrane as in cardiac muscle. SR uptake is called Ca^{2+} sequestration and extracellular transfer is called Ca^{2+} expulsion. Due to the small but continuous influx of Ca^{2+} through Ca^{2+} channels in the basal state, the sarcolemmal Ca^{2+} pumps have to expel Ca^{2+} continuously, otherwise, Ca^{2+} would accumulate in the cell (Klabunde, 2012; Levick, 2010; Pappano and Wier, 2013).

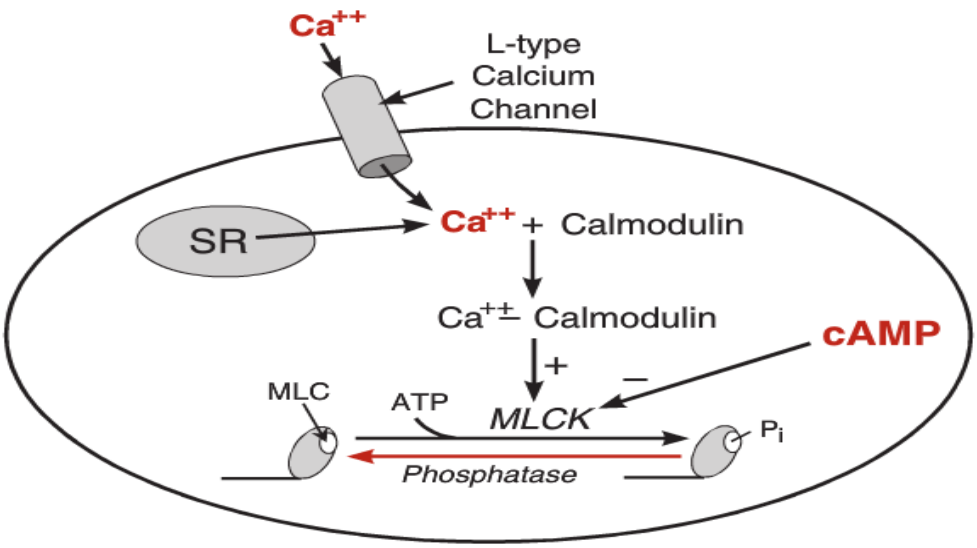

Figure 2.2 Regulation of vascular smooth muscle contraction by myosin light chain kinase (MLCK). ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; MLC, myosin light chains; P_i, phosphate group; SR, sarcoplasmic reticulum (Klabunde, 2012).

2.1.3(b) Two Phases of Vasoconstriction

If the vessel wall is loaded with a Ca^{2+}-sensitive fluorescent dye, it is found that the cytosolic free Ca^{2+} concentration exhibits two distinct phases following stimulation. In the initial, transient phase, there is a rapid increase in cytosolic Ca^{2+} concentration, from ~0.1 µM to ~ 1 µM. This occurs synchronously in all the myocytes throughout the vessel wall, and is followed almost immediately by a rise in tension. In the second, tonic phase, starting 30-60 s later, the average cytosolic [Ca^{2+}] in the vessel wall retreats to a lower but still suprabasal level; yet the force of contraction is well maintained. During the second phase, individual myocytes may exhibit intermittent asynchronous Ca^{2+} waves, especially in small artery myocytes with a low contractile tone; second phase Ca^{2+} tend to be more stable in vessels with a high basal tone (Levick, 2010).

2.1.3(b)(i)The Initial Phase

The initial large increase in myocyte free Ca^{2+} results from the release of stored Ca^{2+} from the SR, particularly in large arteries, aided by an influx of extracellular Ca^{2+}, particularly in small arteries and arterioles. In large arteries, Ca^{2+} store release accounts for most of the initial cytosolic Ca^{2+} transient, because the SR is well developed. Store discharge is triggered chiefly by receptor-induced IP_3 production. Store replenishment is brought about by an influx of extracellular Ca^{2+} ions through the store-operated TRP channels in the adjacent sarcolemma (capacitative Ca^{2+} entry). In smaller, resistance arteries/arterioles, extracellular Ca^{2+} influx is more important, because small resistance vessels tend to have scanty SR but abundant VSCCs. The VSCCs are activated by depolarization, which is brought about by i_{cat} and $i_{Cl(Ca)}$. Extracellular Ca^{2+} influx

through the activated VSCCs, and also through ROCs, raises the cytosolic Ca^{2+} concentration.

To summarize the initial 30-60 s phase of vasoconstriction, the agonist-receptor complex activates the PLCβ-IP_3-DAG pathway (Figure 2.3). IP_3 releases stored Ca^{2+}, which raises cytosolic Ca^{2+} and activates depolarizing current $i_{Cl(Ca)}$. The DAG activates depolarizing current i_{cat}. The depolarization by i_{cat} and $i_{Cl(Ca)}$ increases the open-state probability of sarcolemmal VSCCs, admitting extracellular Ca^{2+}. DAG also activates sarcolemmal ROCs, that admit extracellular Ca^{2+}. A 4 Ca^{2+}-calmodulin complex then activates MLCK, which phosphorylates the myosin light chains, enabling the myosin heads to form crossbridges with the actin filament. Crossbridge flexion then generates shortening and tension (Levick, 2010).

2.1.3(b)(ii) The Second Phase

During the tonic phase, the mean cytosolic Ca^{2+} concentration, averaged across the whole tunica media, drops below the transient peak value; and in some vessels the individual myocytes develop intermittent Ca^{2+} waves. Each Ca^{2+} wave is generated by SR store discharge by IP_3. Since the sarcolemmal pumps expel some Ca^{2+} during each wave, extracellular Ca^{2+} influx through VSCCs and cat-SOCs is needed to recharge the stores. The importance of extracellular Ca^{2+} influx during tonic contraction is demonstrated by the relaxation induced by nifedipine and cat-SOC blockers.

During the tonic phase, vasoconstriction is well maintained despite a fall in mean cytosolic [Ca^{2+}]. Therefore, some additional mechanism must come into play. This mechanism is Ca^{2+} sensitization - a given level of Ca^{2+} has an increased contractile effect during the tonic phase. Ca^{2+} sensitization is brought about by kinases that are activated by the G protein-coupled receptors. Kinases are a large family of enzymes

that phosphorylate other proteins to change their activity. The kinase chiefly responsible for Ca^{2+} sensitization is rhoA kinase. Agonist-receptor complexes coupled to G_{12} protein activate rhoA, a monomeric GTPase that activates rhoA kinase. RhoA kinase inhibits myosin light chain phosphatase, the enzyme responsible for dephosphorylating the myosin head and turning off the myosin motor. This shifts the dynamic balance between MLC kinase and MLC phosphatase in favour of phosphorylation, despite a fall in Ca^{2+}-calmodulin.

Ca^{2+} sensitization is also promoted in some vessels by protein kinase C-α (PKCα), which is activated by DAG and cytosolic Ca^{2+}. PKC activates CPI-17 protein, which, like rhoA kinase, inhibits myosin light chain phosphatase. PKC also triggers a MAP kinase pathway that phosphorylates caldesmon, a regulatory protein on the actin filament facilitating crossbridge formation. This may explain why adrenergic-stimulated tone is maintained in some vessels despite a fall in myosin light chain phosphorylation (Levick, 2010).

2.1.3(c) Mechanisms for relaxation

A major function of blood vessels, especially resistance vessels, is to dilate, so as to raise blood flow to exercising muscle, myocardium, etc. Vasodilation is not an active process; it is simply a reduction of tonic contractile tension, i.e. a relaxation, and it can be brought about by four mechanisms: (1) hyperpolarization, (2) the adenylyl cyclase-cAMP-protein kinase A (PKA) pathway, (3) the guanylyl cyclase-cGMP-protein kinase G (PKG) pathway, and (4) desensitization to Ca^{2+}.

The first three mechanisms produce vasodilation by reducing the cytosolic Ca^{2+} concentration, which reduces MLC kinase activity. This allows the constitutive background MLC phosphatase activity to predominate and turn off the myosin motor.

The fourth vasodilator mechanism, desensitization to Ca^{2+}, produces vascular relaxation despite little fall in cytosolic Ca^{2+} concentration (Levick, 2010; Mohrman and Heller, 2010).

2.1.3(c)(i) Hyperpolarization

Hyperpolarization closes VSCCs, leading to a fall in cytosolic free [Ca^{2+}] and vasodilation. Examples are as follows: (1) Skeletal muscle contraction, myocardial contraction and brain activity raise the K^+ ion concentration in the local interstitial fluid. Extravascular K^+ increases vascular K_{ir} activity, leading to hyperpolarization and vasodilation. This is one of several mechanisms that match blood flow to tissue metabolic activity (Félétou, 2011). (2) Endothelium-derived hyperpolarization factors (EDHF) elicit vasodilation through myocyte hyperpolarization. In feed arteries, conducted vasodilation is due to the conduction of endothelial hyperpolarization into myocytes through myoendothelial gap junctions (Edwards et al., 2010; Félétou and Vanhoutte, 2009). (3) Hypoxia, if severe, can activate myocyte K_{ATP} channels, leading to hyperpolarization, reduced cytosolic [Ca^{2+}] and hypoxic vasodilation (Kalsner, 1995). In many arteries, however, Ca^{2+} desensitization is the main mechanism underlying hypoxic vasodilation, and there is little reduction in cytosolic [Ca^{2+}]. K_{ATP}-activating drugs, such as diazoxide, pinacidil and cromakalim, elicit vasodilation through myocyte hyperpolarization. (4) Sensory nerve neuropeptides, such as calcitonin gene-related peptide and vasoactive intestinal polypeptide, are released during inflammation, and contribute to the vasodilation of inflammation by causing hyperpolarization (Levick, 2010; Mohrman and Heller, 2010).