Hypotensive and Diuretic Effects of Camboginol and Morelloflavone from *Garcinia dulcis* in 2-Kidneys-1-Clip Renovascular Hypertensive Rat

Nattaya Thongsepee

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Physiology

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ชื่อวิทยานิพนธ์ ผลลดความดันเลือดและขับปัสสาวะของ camboginol และ morelloflavone จาก *Garcinia dulcis* ในหนู model 2-kidneys-1-clip renovascular hypertension

ผู้เขียน นางสาวนาตยา ทองเสภี

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ปีการศึกษา 2559

บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อทดสอบฤทธิ์ลดความดันเลือดแดงและขับปัสสาวะของ camboginol (isoprenylated bezophenone) และ morelloflavone (biflavonoid) ซึ่งเป็นสารต้านอนุมูลอิสระที่สกัดจากมะพูด (*Garcinia dulcis*) ในหนูความดันเลือดแดงสูง 2-kidneys-1-clip (2K1C) รวมทั้งทดสอบกลไกการคลายหลอดเลือด โดยการนำหนู Wistar rat เพศผู้ (น้ำหนักตัว 150-200 g, 6 ตัว/กลุ่ม) มาผ่าตัดหนีบหลอด renal artery ด้านซ้ายเพื่อชักนำให้เกิดภาวะความดันเลือดแดงสูงเป็นเวลา 4 สัปดาห์ หนูกลุ่มควบคุมจะได้รับการผ่าตัดเช่นเดียวกันแต่ไม่มีการหนีบหลอดเลือด เมื่อครบระยะเวลาจะนำหนูทั้งสองกลุ่มมาศึกษาแบบ *in vivo* โดยให้สารละลาย para-aminohippuric acid 0.5% และ inulin 1% ทาง jugular vein แก่หนูที่สลบลึก เพื่อใช้ในการประเมินค่า effective renal plasma flow (ERPF) และ glomerular filtration rate พร้อมทั้งให้ camboginol และ morelloflavone (ขนาด 0.1 mg/kg + 5 μg/min/kg BW) ควบคู่กัน ทำการบันทึกค่าความดันเลือดแดงและอัตราการเต้นของหัวใจตลอดการทดลองและเก็บตัวอย่างปัสสาวะและเลือดเพื่อใช้ในการวิเคราะห์ค่า osmolar และ free water clearance นอกจากนี้ยังประเมินค่า baroreflex sensitivity (BRS), ระดับของ malondialdehyde (MDA) ในเลือดและระดับของ endothelium nitric oxide synthase (eNOS) ในหลอดเลือดอีกด้วย การศึกษาแบบ in vitro จะทดสอบฤทธิ์และกลไกการออกฤทธิ์คลายตัวของสารทั้งสองที่ขนาดความเข้มข้น 10^{-13}-10^{-5} M ในหลอดเลือด thoracic artery โดยผลการทดลองพบว่า สารทั้งสองสามารถลดความดันเลือดแดง, เพิ่มปริมาณ ERPF, เพิ่มปริมาณปัสสาวะ, เพิ่มค่า BRS , ลดระดับ MDA ในเลือด และเพิ่มการแสดงออกของ eNOS ในหลอดเลือดของหนู 2K1C สารทั้งสองสามารถออกฤทธิ์คลายหลอดเลือดแดงของหนูกลุ่มควบคุมได้ดีกว่าหนูกลุ่ม 2K1C โดยกลไกคลายตัวผ่านทาง endothelium dependent vasorelaxtion pathway ทั้งนี้ฤทธิ์ในการขับปัสสาวะและลดความดันเลือดแดงของ camboginol และ morelloflavone น่าจะสัมพันธ์กับคุณสมบัติในการต่อต้านอนุมูลอิสระของสารทั้งสอง

Thesis Title	Hypotensive and Diuretic Effects of Camboginol and Morelloflavone from *Garcinia dulcis* in 2-Kidneys-1-Clip Renovascular Hypertensive Rat
Author	Ms. Nattaya Thongsepee
Major Program	Physiology
Academic Year	2016

Abstract

This study aimed to evaluate hypotensive and diuretic effects of camboginol (isoprenylated benzophenone) and morelloflavone (biflavonoid), a robust antioxidant which isolated from *Garcinia dulcis*, in 2-kidneys-1-clip (2K1C) renovascular hypertensive rats together with their vasorelaxant mechanisms. Male Wistar rats (150-200 g) were undergone either 2K1C or sham operation (SO) (n=6, each). Four weeks later, an *in vivo* study was performed by which clearance markers (0.5% para-aminohippuric acid and 1% inulin) were given via a jugular vein to estimate effective renal plasma flow (ERPF) and glomerular filtration rate in anesthetized rats. Camboginol or morelloflavone was given at the dose of 0.1 mg/kg + 5 μg/min/kg body weight, while arterial blood pressure (ABP) and heart rate were monitored via a carotid artery. Plasma and urine samples were collected for determination of osmolar and free water clearances. Baroreflex sensitivity (BRS), plasma malondialdehyde (MDA) level and an expression of endothelial nitric oxide synthase (eNOS) were also investigated. For an *in vitro* study, the relaxation of isolated aortic ring which phenylephrine-preconstriction was experimented using an organ bath technique by cumulative additions of either camboginol or morelloflavaone (10^{-13}-10^{-5} M) in the presence or absence of specific vasorelaxant inhibitors. It was found that camboginol and morelloflavone significantly decreased ABP, increased ERPF and increased urine flow rate in anesthetized 2K1C rats. Both also improved an impaired BRS, decreased plasma MDA levels and enhanced eNOS expression in vascular endothelium. The mechanisms of their vasorelaxant action mainly involved the different extent of endothelial dependent vasorelaxation pathway. The responsive mechanisms of diuretic and hypotensive effects of camboginol and morelloflavone are likely to associate with their scavenging activity.

Acknowledgements

I sincerely appreciate my major advisor Assoc. Prof. Dr. Siriphun Hiranyachattada for her guidance, suggestions and all supports. This thesis would not be completed and my Ph.D. course would not be achieved without her kindness.

I appreciate my co-advisor Assoc. Prof. Dr. Ekkasit Kumarnsit for his suggestions. I also appreciate Assoc. Prof. Dr. Wilawan Mahabusarakam for her provided substances, camboginol and morelloflavone, and for her suggestions in a part of isolation of the plant compound. I appreciate Dr. Wachiryah Thong-asa for her collaboration in a part of immunohistochemistry. In addition, I am grateful to Assoc. Prof. Dr. Kesorn Suwanprasert and all examining committees for their comments and suggestions.

I appreciate Prof. Dr. Andrew Allen for his guidance and suggestions when I deeply conducted a research at the Cardiovascular Regulation Laboratory, Department of Physiology, University of Melbourne, Australia.

I would like to thank Prince of Songkla University for the PSU-Ph.D scholarships and the Graduate School Research Support Funding for Thesis, Faculty of Science, Prince of Songkla University for the Overseas Research Scholarship, the Office of the Higher Education Commission, Thailand for financial support belong to the Higher Education Research Promotion and National Research University project of Thailand.

I would like to extent my thanks to all scientists and officers of Department of Physiology, Faculty of Science, Prince of Songkla University for their kindness and all supports along my Ph.D. courses.

Finally, I would like to express my sincere gratitude to my family and my friends for their encouragement and mental support.

Nattaya Thongsepee

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LIST OF ABBREVIATIONS AND SYMBOLS

Δ	Delta/Difference
μ	Micro
$\dot{V}$	Urine flow rate
1K1C	One-kidney-one-clip
2K1C	Two-kidneys-one-clip
2K2C	two-kidneys-two-clips
ABP	Arterial blood pressure
ACE	angiotensin-converting enzyme
ACh	Acetylcholine
ADH	Anti-diuretic hormone
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of varience
ATP	Adenosine triphosphate
BK_{Ca}	Large conductance potassium channel
BP	Blood pressure
BRS	Baroreflex sensitivity
BW	Body weight
C	Camboginol
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
C_{in}	Clearance of inulin
cNOS	Constitutive nitric oxide synthase
CO	Cardiac output
C_{Osm}	Clearance of osmolarity
COX	Cyclooxygenase
C_{PAH}	Clearance of para-aninohippurate
CVLM	Caudal ventrolateral medulla
DAG	Diacylglycerol
DBP	Diastolic blood pressure

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

DMSO	Dimethyl sulfoxide
DPPH	1, 1-diphenyl-2-picrylhydrazyl
EC_{50}	Half maximal effective concentration
EDHF	Endothelial-derived hyperpolarizing factor
ERBF	Effective renal blood flow
ERK	extracellular signal regulated kinase
ERPF	Effective renal plasma flow
ESC	The European Society of Cardiology
ESH	The European Society of Hypertension
ET-1	Endothelin-1
ET_B	Endothelin-B receptor
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
g	Gram
GFR	Glomerular filtration rate
HAT	Histone acetyltransferase
Hct	Hematocrit
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HR	Heart rate
IB	Immunobuffer
iNOS	Inducible nitric oxide synthase
IP_3	Inositol 1, 4, 5 triphosphate
JAK	Janus kinase
STAT	Signal transducers and activators of transcription
$K_{Ca^{2+}}$ channel	Calcium-activated potassium channel
K_{ATP} channel	Adenosine triphosphate-activated potassium channel
K_f	Glomerular ultrafiltration coefficient
Kg	Kilogram
KW	Kidney weight
L	Liter

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

LDL	Low density lipoprotein
L-NAME	Nω-Nitro-L-arginine methyl ester hydrochloride
LOX	Lipoxygenase
LPS	Lipopolysaccharide
M	Morrelloflavone
M	Molar
MABP	Mean arterial blood pressure
mL	Milliliter
MLCK	Myocin light chain kinase
MW	Molecular weight
NA	Nucleus ambiguous
NADH	Nicotinamide adenine dinucleotide + Hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate + Hydrogen
NFκB	Nuclear factor kappa B
NGS	Normal goat serun
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NTS	Nucleus of the solitary tract
O_2^-	Superoxide anion
PAH	Para-aminohippuric acid
pD_2	Negative logarithm of effective concentration
PE	Phenylephrine
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
PI	Phosphatidyl inositol
P_{in}	Arterial plasma concentration of inulin
PIP_2	Phosphatidylinositol 4, 5 bisphosphate
PKC	Phosphokinase C
PLA_2	Phosphilipase A_2

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

P_{Osm}	Plasma osmolality
P_{PAH}	Arterial plasma concentration of para-aminohippuric acid
PRA	Plasma renin activity
R	Resistance
RAS	Renin angiotensin system
RBF	Renal blood flow
RhoGEF	Guanine nucleotide exchange Rho kinase
ROCC	Receptor-operated Ca^{2+} channel
ROS	Reactive oxygen species
RPF	Renal Plasma flow
RU	Resistance unit
RVH	Renovascular hypertension
RVLM	Rostral ventrolateral medulla
RVR	renal vascular resistance
S.E.M.	Standard error of mean
IDM	Indomethacin
SBP	Systolic blood pressure
sGC	Soluble guanylate cyclase
SNP	Sodium nitroprusside
SO	Sham operation
SR	Sarcoplasmic reticulum
T	Tension
TBH_4	(6R) -5, 6, 7, 8-tetrahydrobiopterin
TC_{H_2O}	Nagative free water clearance
TEA	Tetraethyl ammonium
TPBS	Tris phosphate buffer solution
TPVR	Total peripheral resistance
TRPF	Total renal plasma flow
TXA_2	Thromboxane A_2
U_{in}	Urine concentration of inulin

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

U_{Osm}	Urine osmolality
U_{PAH}	Urine concentration of para-aminohippuric acid
V_1	Vasopressin receptor type 1
V_2	Vasopressin receptor type 2
VEGF	Vascular endothelial growth factor
VOCC	Voltage-operated Ca^{2+} channel
VR	Venous return
VSMC	Vascular smooth muscle cell
WHO	World health organization

CHAPTER 1
INTRODUCTION

1.1. Background and rationale

It is well established that hypertension is a major cause of morbidity and mortality since it is associated with coronary heart disease, cerebrovascular disease, peripheral artery disease, renal disease and heart failure. Genetic- and non-genetic animal models of hypertension have been used to study of pathophysiological changes and treatments. As the kidney is vital cardiovascular homeostasis, renal damage is a relatively minor cause of hypertension. Animal model of renovascular hypertension (RVH) involved restricting blood flow by clip on the renal artery caused hypertension in response to renal ischemia. The two-kidneys-one-clip (2K1C) model is most relevant characteristics to human RVH, which involves unilateral stenosis of the renal artery leading to a permanent reduction in renal perfusion pressure (RPP) and renal blood flow (RBF) in one kidney (Goldblatt et al. 1934). The induced hypertension is dependent upon activation of the renin angiotensin system (RAS), which plays an important role in the control of cardiovascular homeostasis affecting both arterial blood pressure (ABP) and fluid volume. Basically, the reduced RPP stimulates an increase in renin synthesis and release from the clipped kidney. Then renin cleaves angiotensin I from angiotensinogen. After that angiotensin-converting enzyme (ACE) acts on angiotensin I to produce angiotensin II (AII). Finally, direct vascular effects of circulating AII acutely increases total peripheral resistance (TPR) and raises ABP (Navar et al. 1998).

Renal function is altered in 2K1C model, stenosis of one renal artery results in an immediate fall in RBF and glomerular filtration rate (GFR) to that kidney. Three weeks after renal stenosis, the stenotic kidney shows reduced RBF and GFR, whereas the contralateral kidney has a tendency toward reduced GFR in spite of unchanged RBF (Oliveira-Sales et al. 2016). Four weeks after clipping, RBF and GFR of the non-clipped kidney are similar to that found in normotensive controls despite the higher ABP, renal vascular resistance (RVR) and AII level (Anderson et

al. 1985; Martinez-Maldonado 1991). The balance between AII and endothelial nitric oxide (NO), an effective endothelium-derived relaxing factor, is important in renal hemodynamics and in development of hypertension. The NO synthesis in the non-clipped increases within 4 weeks presumably related to an increase in vascular shear stress which counteracts the constrictor influence of elevated circulating AII (Sigmon & Beieirwaltes 1993). The natriuretic and diuretic effect of NO has been demonstrated in an *in vivo* experiment which may be due to the decreased sodium chloride and fluid reabsorption by the renal tubules (Ortiz & Garvin 2002).

Baroreflex modulation or baroreflex sensitivity (BRS) of heart rate (HR) is impaired in animals (Jones & Floras 1980) and patients (Gao et al. 2002) with RVH. The baroreflex is one of the body's homeostatic mechanisms to modulate ABP. Activation of arterial baroreceptors by a rise in systemic ABP leads to an increase in the discharge of vagal cardioinhibitory neurons and a decrease in the discharge of sympathetic neurons both to the heart and peripheral blood vessels resulting in bradycardia, decreased cardiac contractility and decreased TPR and venous return (VR) (Kirchheim 1976). It is reported that nicotinamide adenine dinucleotide phosphate (NADPH) oxidase seems to play an important role in the blunted BRS in the 2K1C model, through increased reactive oxygen species (ROS), mainly superoxide anion (O_2^-), production (Botelho-Ono et al. 2011).

Furthermore, impaired endothelium-dependent vasodilatation was observed in a number of experimental models of hypertension, including 2K1C hypertension (Lüscher et al. 1987; Dohi et al. 1996; Callera et al. 2000). The responsible mechanism is related to an increase in production of O_2^- by NADH/NADPH oxidase (Choi et al. 2014). The biological activity of NO is primary associated with endothelial NO synthase (eNOS) activity or it interaction with O_2^-, which is produces in the vascular wall by free radical-generating enzyme (Cai & Hariison 2000). The NO may be scavenged by O_2^-, causing reduced bioavailability of NO and diminishing vasorelaxation (Griendling et al. 1994). Therefore, it is well accepted that endothelial dysfunction in 2K1C hypertension is partially linked to the exaggerated production of O_2^- and that oxidative stress is responsible for impaired endothelial modulation (Toba et al. 2012; Arnalich-Montiel et al. 2014; Lerman et al. 2001; Oliveira-Sales et al. 2009).

A lot of intensive efforts have been conducted to research the local medical plants with hypotensive and/or antihypertensive potentials since the conventional antihypertensive drugs are usually associated with many side effects. *Garcinia dulcis* Kurz, a plant that belongs to the Guttiferae family, is widely distributed in Thailand and other Southeast Asian countries. *G. dulcis* is known as "maphuut" in Thailand and has been used in folk medicine. Its leaves and seeds are known to treat lymphatitis, parotitis, and struma (Kasahara & Henmi 1986) while the stem bark is used as antiseptic. The fruit juice possesses the properties as an anti-scurvy and expectorant for the relief of cough and sore throat. In addition, its root extract is also used as an antipyretic and antitoxin agent (Wuttidhammavej 1997).

It has been reported that *G. dulcis* contains at least four groups of chemical compounds including flavoniods (e.g. epicatechin, dulcisflavone, morelloflavone), benzophenone (e.g. garcinol and camboginol), xanthones (e.g. dulcisxanthone and garciniaxanthone) (Deachathai et al. 2005; Deachathai et al. 2006; Deachathai et al. 2008; Mahabusarakam et al. 2016) and benzophenone-xanthone dimer (e.g. garciduols A-C) (Iinuma et al. 1996). The amount of chemical constituents extracted from *G. dulcis* depended on the part of the plant specimens and the purified procedure. Some of isolated phenolic compounds from *G. dulcis* possess various biological activities. As previously reported, dulcisxanthone C-F and dulcinone from the flowers of *G. dulcis* showed a radical scavenging and antibacterial activity (Deachathai et al. 2006), dulcisxanthone G from the seed of *G. dulcis* showed an antibacterial and anti-oxidative activity (Deachathai et al. 2008). Moreover, cambogin, camboginol, dulcisflavone, epicatechin and morelloflavone from the fruits of *G. dulcis* demonstrated the radical trapping and antibacterial activity (Deachathai et al. 2005).

Camboginol (also called garcinol) is a plant benzophenone found in most *Garcinia* species. The chemical structure was firstly elucidated in 1980 by Rao et al. Camboginol exerts a wide range of physiological activities. The results from our laboratory indicated that camboginol could induce the dilatation of the isolated aorta rings from normotensive rats and its mechanism of action involved an endothelial-dependent NO signaling pathway (Lamai et al. 2013). The free radical, O_2^- scavenging activity of camboginol was shown to be as potent as that of gallic acid and

stronger than that of cathechin (Yamaguchi et al. 2000a). Camboginol also scavenges the free radical DPPH (1, 1-diphenyl-2-picrylhydrazyl) with three times greater potency than DL-α-tocopherol, a lipid-soluble natural anti-oxidant (Yamaguchi et al. 2000b). Camboginol exhibits an anti-inflammatory effect by interfering with various inflammatory cascades. First, camboginol suppressed inducible NO synthase (iNOS) synthesis by inhibiting nuclear factor kappa B (NFκB) activation leading to reduce NO generation (Liao et al. 2004). Second, camboginol inhibited the production of cyclooxygenase-2 (COX-2) and prostaglandins H_2 (PGH_2) in lipopolysaccharided (LPS)-activated macrophages (Hong et al. 2006). Third, camboginol inhibited the activation of 5-lipoxygenase (5-LOX) which is responsible for producing inflammatory molecules, leukotrienes (Kim et al. 2008). The other biological actions of camboginol such as anti-cancer (Tanaka et al. 2000; Yoshida et al. 2005), anti-HIV (Balasubramanyam et al. 2004; Mantelingu et al. 2007) and anti- ulcer (Das et al. 1997; Vaananen et al. 1991).

Morelloflavone, a biflavonoid comprising two covalently linked flavones, apigenin and luteolin, also found in most *Garcinia* species (Ansari et al. 1976; Verbeek et al. 2004). Several biological actions of morelloflavone have been reported. Recently, it is found that in normotensive rats, morelloflavone could induce the dilatation of the isolated aorta rings via an endothelial-dependent NO signaling pathway (Lamai et al. 2013). Morelloflavone inhibits secretory phospholipase A_2 from human synovial, bee and snake venom (Gil et al. 1997; Pereañez et al. 2014). In animal models it has anti-inflammatory effects, with a potent inhibition of 12-O-tetradecanoylphorbol 13-acetate-induced ear inflammation in mice after topical administration (Gil et al. 1997). Morelloflavone inhibits vascular smooth muscle cell migration, invasion and lamellipodium formation in culture through activation of multiple migration-related kinases, including focal adhesion kinase (FAK), Src, extracellular signal-regulated kinase (ERK) and RhoA (Pinkaew et al. 2009). The inhibition of RhoA and ERK pathways is also observed in vascular endothelial growth factor (VEGF)-stimulated human umbilical cord endothelial cells (Pang et al. 2009). Furthermore, morelloflavone inhibits neointimal proliferation in a mouse model of postangioplasty restenosis (Pinkaew et al. 2009). Morelloflavone inhibits 3-hydroxy-3-methylglutarlycoenzyme A (HMG-CoA) reductase leading to a decrease in *de novo*

cholesterol synthesis (Tuansulong et al. 2011). Oral morelloflavone therapy for 8 months significantly reduced the atherosclerotic areas of the mouse aortae (a 26% reduction), without changing plasma lipid profiles in $Ldlr^{-/-}$ $Apobec1^{-/-}$ mice (Pinkaew et al. 2012).

Against the background of increased ROS signaling and impaired NO function in 2K1C rat and the antioxidant and anti-inflammatory effects of camboginol and morelloflavone on vascular functions. The present study aimed to investigate the acute effects of camboginol and morelloflavone from *Garcinia dulcis* on ABP and HR, renal clearance, BRS, plasma malondialdehyde (MDA) and expression of aortic endothelial nitric oxide synthase (eNOS expression) in anesthetized 2K1C hypertensive and sham operative (SO) normotensive rats. The endothelium-dependent vasorelaxant signaling mechanisms of camboginol and morelloflavone action, including NO signaling pathway, prostacyclin signaling pathway, opening of ATP-activated potassium (K_{ATP}) channel and Ca^{2+}-activated potassium (K_{Ca}) channel, were also investigated in isolated thoracic aorta of both 2K1C and SO rats using specific inhibitors including Nω-Nitro-L-arginine methyl ester (L-NAME), indomethacin, glibenclamide and tetraethylammonium (TEA), respectively .

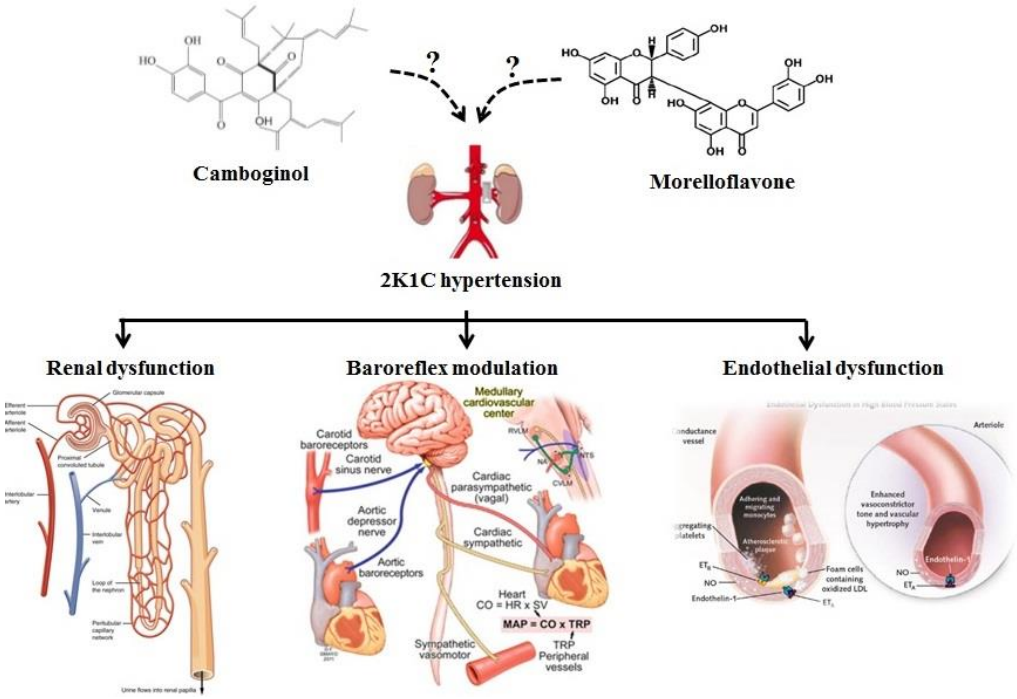

Figure 1.1. Conceptual framework of the study.

1.2. Literature review

1.2.1. Hypertension

1.2.1.1. Definition and classification of hypertension

Hypertension is a condition in which the blood vessels have persistently raised pressure. It is defined as values ≥140 mm Hg systolic blood pressure (SBP) and/or ≥90 mm Hg diastolic blood pressure (DBP). The recommended classification guideline from the European Society of Hypertension and the European Society of Cardiology (ESH/ESC) was shown in Table 1.1 (WHO 2013).

Table 1.1. Definition and classification of office blood pressure levels (mm Hg) (WHO 2013).

Category	**Systolic**		**Diastolic**
Optimal	<120	and	<80
Normal	120-129	and/or	80-84
High normal	130-139	and/or	85-89
Grade 1 Hypertension	140-159	and/or	90-99
Grade 2 Hypertension	160-179	and/or	100-109
Grade 3 Hypertension	≥180	and/or	≥110
Isolated systolic hypertension	≥140	and	<90

*The blood pressure (BP) category is defined by the highest level of BP, whether systolic or diastolic. Isolated systolic hypertension should be graded 1, 2, or 3 according to systolic BP values in the ranges indicated.

1.2.1.2. Causes of hypertension

Essential hypertension

Essential hypertension or primary hypertension is the most prevalent type of hypertension, affecting 90-95% of hypertension patients (Carretero & Oparil 2000). However, no direct cause has identified itself, there are many factors such as sedentary lifestyle, stress, visceral obesity, hypokalemia (Kytou et al. 2006), obesity (Wofford & Hall 2004), salt sensitivity (Lackland & Egan 2007), alcohol intake (Djousse & Mukamal 2009), vitamin D deficiency (Lee at al. 2008), aging (Touhimaa

2009), some inherited genetic mutations (Dickson & Sigmund 2006) and having a family history of hypertension (Luma & Spiotta 2006), elevation of renin, (Segura & Ruilope 2007), raised sympathetic nerve activity (Sorof & Daniels 2002), insulin resistance (Hwang et al. 1987) are thought to contribute to hypertension.

Secondary hypertension

Secondary hypertension is caused from an identifiable factor. This type is important to recognize because it has been treated differently from essential hypertension by which treating the underlying causes of the elevated ABP. Some are common and well-recognized causes such as Cushing's syndrome, which a condition where the adrenal grand overproduces the hormone cortisol. Other causes of this hypertension such as hyperthyroidism, hypothyroidism, adrenal gland cancer, kidney disease and metabolic syndrome (Dodt et al. 2009).

1.2.1.3. Prevalence of hypertension

Global burden

Hypertension is one of the most important causes of premature death worldwide killing near million people every year globally, and the problem is growing. Over 1 billion people are living with hypertension. As shown in Figure 1.2, the overall prevalence of hypertension globally in adult aged 25 and above was around 40% in 2008. The prevalence was highest in African Region (46.7%) while the lowest prevalence was found in the Americas (31.5%). In the Southeast Asia region, 36% of the adults have hypertension. Males had a slightly higher prevalence of hypertension than females. The prevalence of hypertension in low, lower-middle and upper-middle income countries is higher (40%) than in high-income countries (35%). In high-income countries, strong public health policies, effective preventive action and widely available diagnosis and treatment have led to a reduction in the prevalence of hypertension. In contrast, in many developing countries the disease burden caused by the hypertension had increased over the past decade.

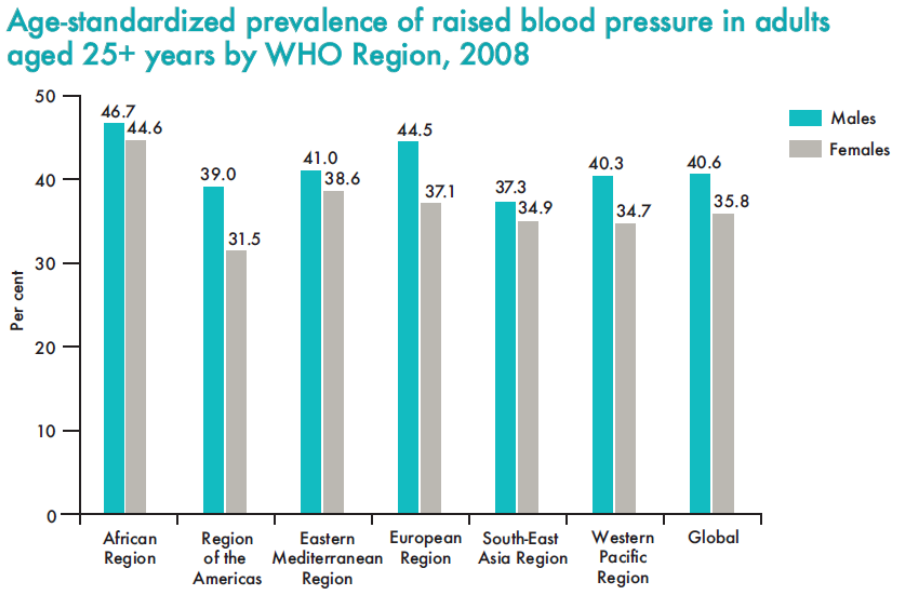

Figure 1.2. Age-standardized prevalence of raised blood pressure in adult aged 25^{+} years by WHO Region, 2008 (WHO 2013).

Burden in the Southeast Asia region

Hypertension is the leading risk factor for death claiming 1.5 million lives each year in this region. One in three adults has hypertension. Males have a slightly higher prevalence of hypertension than females. In the 10 countries from which data were available, the prevalence of hypertension range from 19% in Democratic People's Republic of Korea to 42% in Myanmar. Prevalence of hypertension is increasing in many countries. In India, raised BP increased from 5% in the 1960s to nearly 12% in 1990s, to more than 30% in 2008. In Indonesia, the percentage of adults with raised BP increased from 8% in 1995 to 32% in 2008. In Myanmar, the increased in hypertension prevalence from 18% to 31% in males and from 16% to 29% in females during 2004-2009 had been reported (WHO 2013). In Thailand, 37% of men and 31.6% of women age 25 years old and above were attached by hypertension as shown in Table 1.2 (Krishnan et al. 2013).

Table 1.2. Estimates of age-standardized prevalence (%) of raised blood pressure [a] in adults aged 25+ years in country of the SEA Region, 2008.

Country	Men	Women	Both
Bangladesh	39	38.1	38.6
	(28.1-49.8)[b]	(26.6-49.7)	(30.8-46.5
Bhutan	40.4	37.4	39.1
	(31.1-49.3)	(28.7-46.7)	(32.7-45.5)
Democratic People's	38.5	34.3	36.5
Republic of Korea	(27.0-49.8)	(22.3-46.2)	(27.9-44.8)
India	36	34.2	35.2
	(29.7-41.8)	(28.6-39.9)	(30.9-35.2)
Indonesia	42.7	39.2	41.0
	(35.3-49.9)	(32.5-46.0)	(35.9-45.8)
Maldives	41.5	35.1	38.4
	(30.3-52.7)	(23.0-47.1)	(30.1-46.6)
Myanmar	44.3	39.8	42.0
	(37.7-50.5)	(33.1-46.5)	(37.2-46.8)
Nepal	38.4	38.7	38.6
	(27.0-49.2)	(26.9-50.4)	(30.2-46.7)
Sri Lanka	41.9	37.0	39.4
	(34.0-38.2)	(29.4-44.6)	(33.8-44.6)
Thailand	37.0	31.6	34.2
	(31.3-42.5)	(26.0-37.1)	(30.0-38.1)
Timor-Leste	39.7	35.2	37.5
	(28.9-50.0)	(23.8-46.9)	(29.5-45.4)
SEA Region	37.6	35.4	36.6
	(32.6-42.4)	(30.9-39.8)	(33.1-39.8)
Global	40.8	36.0	38.4
	(37.7-43.7)	(33.3-38.6)	(36.3-40.5)

[a] Raised blood pressure defined as SBP ≥140 mm Hg or DBP ≥90 mm Hg or on medication.

[b] Figures in parentheses are 95% confidence intervals of the estimates.

1.2.1.4. Pathophysiology of hypertension

Cardiac output (CO) and peripheral vascular resistance (PVR)

Maintenance of a normal BP is dependent on the balance between two mainly factors; CO and PVR. It has been suggested that increased CO resulting from sympathetic dysfunction is the trigger for the development of hypertension. Moreover, increase in PVR is essentially the physiological response to accommodate change in pressure and maintain homeostasis. At the beginning of hypertension, PVR is not raised but the elevated BP is caused by an increased CO which is related to sympathetic over-activity. Therefore, the subsequent rise in PVR might develop in a compensatory manner to prevent the raised pressure being transmitted to the capillary bed where it would substantially affect cell homeostasis. In long-term hypertension, most patients with essential hypertension have a normal CO but a raised PVR. PVR is determined by small arterioles which the walls contain smooth muscle cells. Contraction of smooth muscle cells is through to be related to a rise in intracellular calcium concentration. Prolonged smooth constriction is through to induce structural changes with thickening of the arteriolar vessels walls possibly mediated by AII, leading to an irreversible rise in PVR (Beevers et al. 2001).

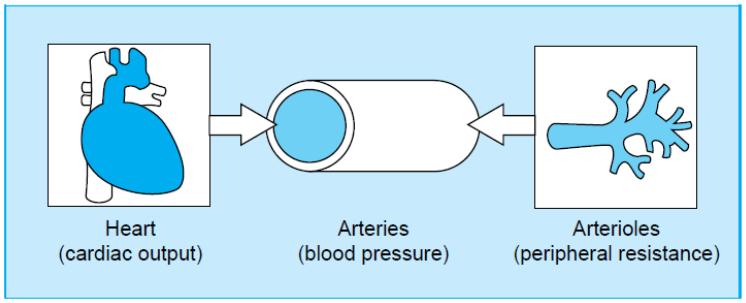

Figure 1.3. The heart, arteries and arteriole in regulation of blood pressure (Beevers et al. 2001).

Autonomic nervous system (ANS)

ANS has an important role in maintaining a normal ABP. Stimulation of ANS can cause both arteriolar constriction and dilatation. It is also important in the mediation of short term changes in ABP in response to stress and physical exercise. Over the last decade the role of sympathetic nervous system in the development and maintenance of ABP has been studied exhaustively. It has been identified that sympathetic stimulation of the heart, peripheral vasculature and kidneys resulting in increased CO and PVR (Beevers et al. 2001).

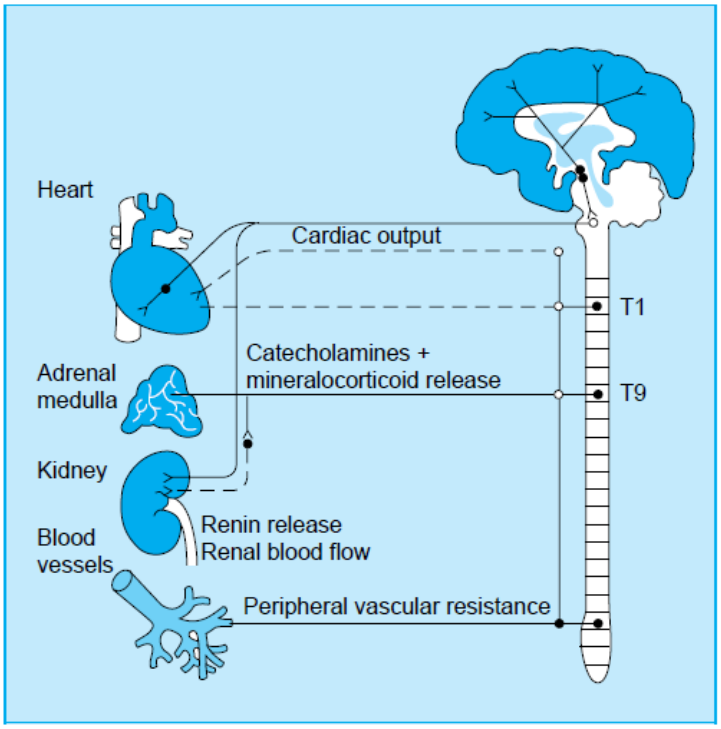

Figure 1.4. The autonomic nervous system and its control of blood pressure (Beevers et al. 2001).

Renin angiotensin system (RAS)

RAS may be the most important of the endocrine system that affect the control of ABP. Renin is secreted from the juxtaglomerular apparatus of the kidney in response to glomerular under perfusion or a reduced salt intake. It is also released in response to stimulation from the sympathetic nerve system. Renin is responsible for converting angiotensinogen to angiotensin I, a physiologically inactive substance which is rapidly converted to AII in the lungs by ACE. AII is a potent vasoconstrictor and thus causes a rise in ABP. AII also stimulates the release of aldosterone from the zona glomerulosa of the adrenal gland, which results in further rise in ABP related to sodium and water retention. The circulating RAS is not thought to be directly responsible for the rise in ABP in essential hypertension. In particular, many hypertensive patients have low levels of renin and AII especially elderly and black people.

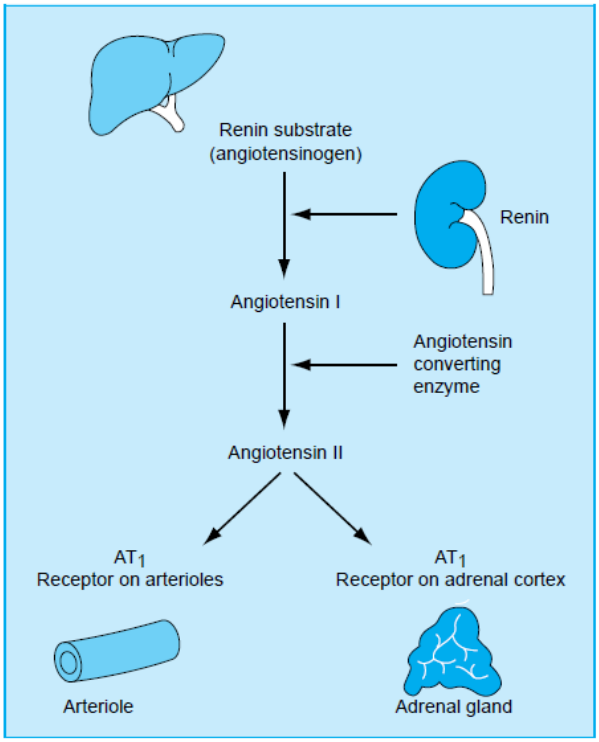

Figure 1.5. Renin angiotensin system and effected on blood pressure and aldosterone release (Beevers et al. 2001).

Endothelial dysfunction

Vascular endothelial cells play a key role in cardiovascular regulation by producing a number of potent local vasoactive agents, including the vasodilator and vasoconstrictor substances. Dysfunction of the endothelium has been implicated in human essential hypertension. Modulation of endothelial function is an attractive therapeutic option in attempting to minimize some of the important complications of hypertension. Clinically effective antihypertensive therapy appears to restore impaired production of NO, but does not seem to restore the impaired endothelium dependent vascular relaxation or vascular response to endothelial agonists. This indicates that such endothelial dysfunction is primary and becomes irreversible once the hypertensive process has become established (Beevers et al. 2001).

Vasoactive substances

Endothelin-1 (ET-1), a potent vasoconstrictor, is one of the major substances involved in maintaining vascular tone. It is secreted by endothelial cells and exerts its affects in paracrine and autocrine manner on vascular smooth muscle and counteracts the relaxing activity of NO (Hickey et al. 1985; Wagner et al. 1992). Both in animals and human infusion of ET-1 resulting in increased ABP (Vierhapper et al. 1990) and blocking the system using antagonists could restore the phenomenon (Krum et al. 1998). However, plasma levels of ET-1 are normal in patients with essential hypertension suggesting that activity of this system might not play a role in all types of hypertension but rather in specific disease states such as salt-sensitive hypertension and renal hypertension (Levin 1995).

Bradykinin is a vasodilatory peptide with autocrine and paracrine function has long had an indirect association with hypertension since apart from its direct vasodilatory effects. It stimulates release of other vasoactive substances like prostaglandins. This peptide from the kinin-kallikrein system is shown to reduce ABP by vasodilation as well as enhanced natriuresis and diuresis both achieved via increased RBF mediated by NO and prostaglandin release (Mattson & Cowley 1993; McGiff et al. 1975; Pasquié et al. 1999).

Atrial natriuretic peptide (ANP) belongs to a family of structurally and functionally related peptide hormones with cardio-renal functions. ANP mediates its

function via membrane-bound guanylatecyclase linked receptor, which further activates intracellular cGMP mediated processes. ANP releases from the atria in response to atrial distention stemming from hemodynamic overload. ANP causes natriuresis and diuresis resulting in modest reductions in ABP with concomitant decreases in plasma renin and aldosterone. Thus, the natriuretic peptide system by decreasing TPR balances the activity of the sympathetic nervous system and the RAS system in maintaining ABP (Brenner et al. 1990; Garcia et al. 1985).

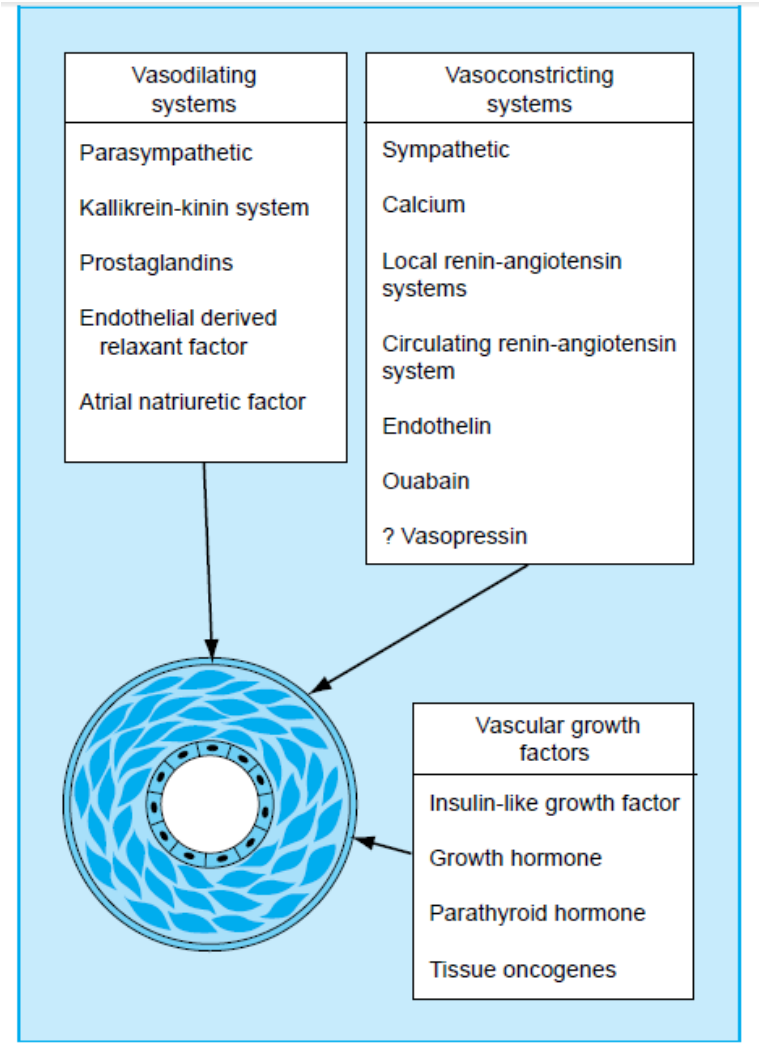

Figure 1.6. The control of peripheral arteriolar resistance (Beevers et al. 2001).

Genetic factors

Hypertension is about twice as common in subjects who have one or two hypertensive parents. Many epidemiological studies suggest that genetic factors account for approximately 30% of the variation in ABP in various populations. Some specific genetic mutations can rarely cause hypertension. Experimental models of genetic hypertension have shown that the inherited tendency to hypertension resides primarily in the kidney. For instance, animal and human studies show that a transplanted kidney from hypertensive donor raises the ABP and increases the requirement to use antihypertensive drugs in recipients coming from normotensive families. Conversely, a kidney from a normotensive donor does not raise the ABP in the recipient. Hypertension is rarely found in urban areas of Africa but it is very common in African cities and in black populations in Britain and the United State. Whereas the urban differences in Africa are clearly due to lifestyle and dietary factors. Hypertension is commoner in black people compared with white people may also have some genetic basis. There is some evidence from salt loading studies in medical students that black Americans are more susceptible to a given salt load than white Americans and may be more sensitive to the beneficial effects of salt restriction (Beevers et al. 2001).

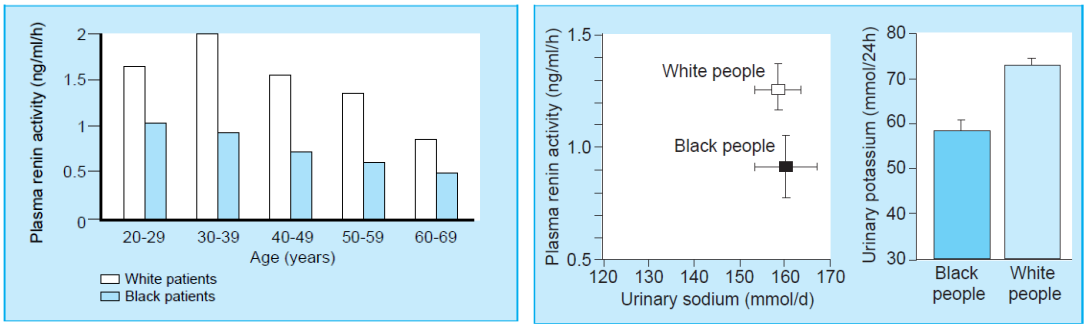

Figure 1.7. Genetic factor contributes hypertension. Plasma renin in black and white hypertensive patients (left). Renin and electrolytes in black and white people (right). (Beevers et al. 2001).

Diastolic dysfunction

In hypertension left ventricular hypertrophied, the ventricle cannot relax normally in diastole. Thus, to produce the necessary increase in ventricular input, especially during exercise, there is an increase in left arterial pressure rather than the normal reduction in ventricular pressure, which produces a suction effect. This can lead to an increase in pulmonary capillary pressure that is sufficient to induce pulmonary congestion. The raise in arterial pressure can also lead to atrial fibrillation, and in hypertrophied ventricles dependent on atrial systole the loss of atrial transport can result in a significant reduction in stroke volume and pulmonary edema (Beevers et al. 2001).

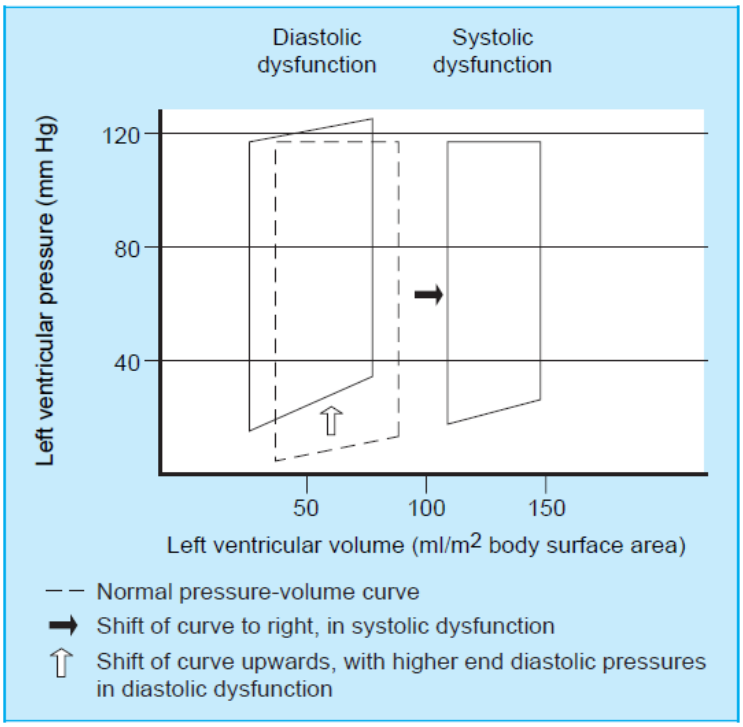

Figure 1.8. Pressure-volume curves demonstrating diastolic/systolic dysfunction Beevers et al. 2001).

1.2.2. Renovascular hypertension (RVH)

1.2.2.1. Epidemiology of RVH

RVH is a progressive condition characterized by the narrowing of one or both renal arteries, mainly due to atherosclerosis, and then cause to rise in ABP. Atherosclerotic disease is the predominant lesion detected in patients over 50 years of age. Population-based studies indicated that vascular stenosis (> 60% lumen occlusion base on Doppler flow) was found 6.8% of individual over 65 years of age and more common in men (9.1%) greater than in women (5.5%) (Hansen et al. 2002). Interestingly, renal artery stenosis resulting from atherosclerotic disease is common in individuals undergoing coronary angiography (18% to 20%) (Rihal et al. 2002) and in those undergoing peripheral vascular angiography for occlusive disease of the aorta and legs (35% to 50%) (Olin et al. 1990). Moreover, screening angiography in potential kidney donors indicates that renal arterial lesions can be asymptomatic and may be detected in up to 3% to 6% of normotensive individuals (Cragg et al. 1989; Neymark et al. 2000).

1.2.2.2 Establishment of RVH model

In 1934, Goldblatt and colleagues introduced the first animal model of hypertension in dogs evoked by unilateral constriction of the renal artery (Goldblatt et al. 1934). Later, partial occlusion of the renal artery resulted in hypertension was established in rats (Wilson et al. 1939), rabbits (Pickering 1945), dogs (Romero et al. 1981), monkeys (Panek et al. 1991), mice (Weisel et al. 1997) and pigs (Lerman et al. 1999), respectively.

1.2.2.3. Type of RVH model

The RVH can be classified into three type base on surgical procedure as shown in Figure 1.9.

1) One-kidney-one-clip (1K1C) model

In the 1K1C model, a clip is placed on the renal artery of the remaining kidney while the contralateral kidney is removed. There is an increase in ABP within a few hours after surgery resulting from RAS. Since there is a lacking of a functional kidney, a compensatory increasing in sodium and water excretion cannot occur so the

fluid volume is retained. Therefore, this model is particularly useful for studying the role of volume expansion in the development of hypertension (Gavras et al. 1975).

2) Two-kidneys-two-clips (2K2C) model

In the 2K2C model, both renal arteries are constricted and both kidneys are remained. It differs relatively little from the 1K1C model in hemodynamic and neurohumoral characteristics and parallels bilateral renal artery stenosis in humans.

3) *Two-kidneys-one-clip (2K1C) model*

In the 2K1C model, a constricting clip is placed on one side of renal artery while both kidneys are left intact. This model seems to be a counterpart for RVH in humans. Pathophysiology of hypertension in 2K1C RVH can be separated into 3 phases (Martinez-Maldonado 1991) as shown in Figure 1.10.

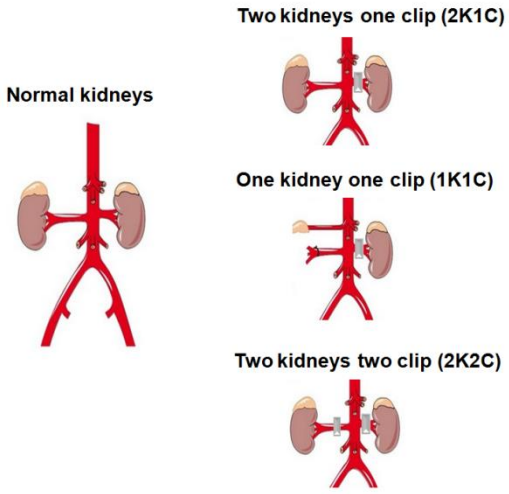

Figure 1.9. Three types of renovascular hypertension (RVH) model

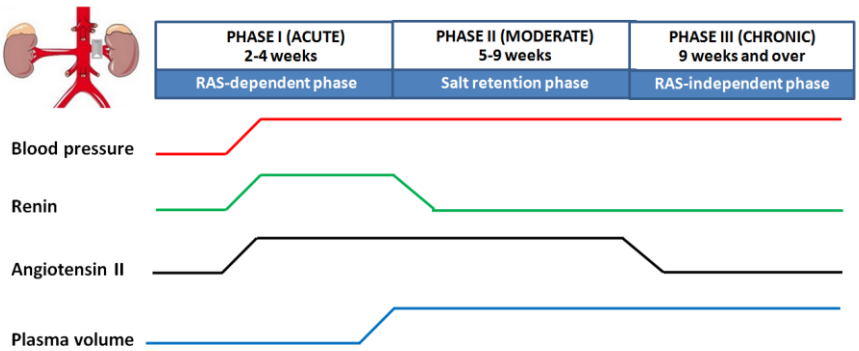

Figure 1.10. Three theoretical temporal phases of 2K1C model (applied from Martinez-Maldonado 1991).

Phase I: Renin-angiotensin-dependent phase

Phase I occurs within 4 weeks after unilateral stenosis of the renal artery. Development of hypertension in this phase is mediated by the RAS (Laragh et al. 1975; Swales et al. 1971; Brown et al. 1976). As shown in Figure 1.11, the reduced RPP stimulates an increase in renin synthesis and release from the clipped kidney. The renin cleaves angiotensin I from angiotensinogen, and then ACE acts on angiotensin I to produce AII. Direct vascular effects of circulating AII acutely increase TPR and raises ABP (Navar et al. 1998). Three weeks after renal stenosis, the stenotic kidney shows reduced RBF and GFR, whereas the contralateral kidney has a tendency toward reduced GFR in spite of unchanged RBF (Oliveira-Sales et al. 2016). However, RBF and GFR of the non-clipped kidney are similar to that found in normotensive controls by four weeks despite the higher ABP, RVR and AII level (Anderson et al. 1985; Martinez-Maldonado 1994).

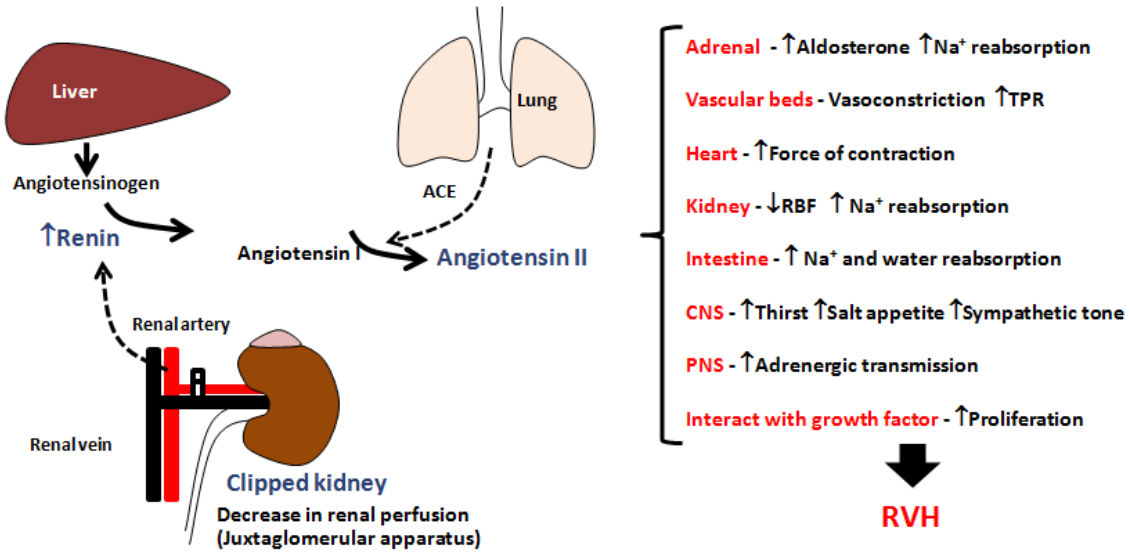

Figure 1.11. The role of renin angiotensin system in 2K1C RVH (applied from https://en.wikipedia.org/wiki/Renin_angiotensin_system).

Phase II: Salt retention phase

Phase II occurs 4-9 weeks after clipping the renal artery. The changes in this phase are explained as following;

Whole kidney effect of renal artery clipping

Whole kidney function between clipped and non-clipped kidney are obviously different. In conscious dogs and anesthetized rats, there is an immediate fall in RPF and GFR in the clipped kidney (Smith & Somova 1976). The unclipped kidney also undergoes changes in an attempt to protect its microenvironment. AII has a major vasoconstrictive action in the efferent arteriole of the glomerulus (Edwards 1983). Renal vascular resistance (RVR) rises as a result of an increase in both afferent and efferent arteriolar vasoconstriction. The ratio of afferent to efferent resistance falls in the clipped kidney but the ratio remains constant in unclipped kidney (Gdthberg et al. 1983). It is clear that variation in renal hemodynamic response in either kidney of 2K1C animals is dependent on the conditions under which the animals are studied, their species, the severity and the duration of hypertension.

Salt and water retention

Salt and water retention by the clipped kidney is initially balanced by the pressure diuresis induced by the elevated systemic ABP. The increase in non-clipped kidney RPF and GFR plays an important role in the attempt to maintain sodium and volume balance. As clipped kidney GFR falls to or below normal, circulating and local hormones influence salt and water reabsorption and lead to serious systemic hemodynamic and volume changes. The systemic and local effects may be triggered by the overproduction of AII (Hall 1986).

Role of AII and catecholamines

The systemic and local effects were triggered by the overproduction of AII (Hall et al. 1986). AII leads to renal sodium and water retention in a variety of ways because it is a major stimulus for the production of aldosterone by the zona glomerulosa of the adrenal glands, it may lead to salt retention through the effects of the mineralocorticoid (Hall et al. 1979). AII also is capable of central stimulation of

thirst and the secretion of anti-diuretic hormone (ADH), which will enhance water ingestion, retention and volume expansion (Schrier & Berl 1975).

AII can trigger the release of norepinephrine from the adrenal medulla and terminal nerve endings. Adrenergic receptor stimulation cause increased sodium reabsorption in the renal proximal tubule (Cogan 1986), a site that is also a target for the sodium-retentive effects of AII (Schuster 1986). Moreover, AII increases loop of Henle sodium reabsorption, perhaps through its capacity to reduce medullary blood flow (Faubert et al. 1987; Chou et al. 1886). Renal nerve stimulation is also known to enhance sodium reabsorption and it is possible that AII may also exert a local effect by afferent neural stimulation (DiBona 1978).

A vicious circle of further elevations of BP and structure damage of the kidneys and other organs will characterize the untreated second and third phases of 2K1C hypertension. Counterbalancing forces such as putative polar medullary lipids, ANF and prostaglandins are not sufficiently protective. The elevation of ANF does not bring plasma sodium levels back to normal, while prostaglandins are diminished or normal when they should be vigorously produced (Katayama et al. 1989).

Phase III: Systemic renin angiotensin independent phase

Phase III usually observed 9 weeks or more after clipping the renal artery. In dog, chronic 2K1C hypertension is eventually accompanied by a fall in PRA (Watkins et al. 1976). By contrast, PRA usually rises in the chronic model in the rat (more than 12 weeks) (Morton & Wallace 1983). Although PRA may be normal in humans with long-standing, predominantly unilateral RVH, its level is never low nor does it closely correlate with the degree of blood pressure elevation (Brown et al. 1965). Yet hypertension will respond to converting enzyme inhibition in this stage (Miller et al. 1972). The possibility must be considered that local renin-angiotensin action, rather than the circulating concentrations of these substances, is what determines the level of blood pressure and organ damage.

There is evidence that local vascular AII production may have an impact in the control of sympathetic neurotransmission and in smooth muscle hyperplasia (Naftilan et al. 1989). The presence of renin mRNA in the arterial wall, liver, adrenal gland, heart and brain were reported (Dzau et al. 1986; Lynch et al. 1986). This

extrarenal gene expression system does not appear to be under control of feedback regulation by either salt balance or the circulating renin-angiotensin levels (Swales & Samani 1989). The 2K1C hypertension of 4 week's duration in rat resulted in a 6-fold rise in renin mRNA levels in the clipped as compared with the control kidney. This was accompanied by an 8-fold reduction in the unclipped kidney. By the end of 20 weeks, the right kidney renin mRNA had fallen 16-fold as compared with age-matched, sham operated control rats, while the clipped kidney was only 4-fold higher than the control (Samani et al. 1989). In summary, the compartmentalized RAS may be responsible for persistent renal and perhaps peripheral vasoconstriction, increases sympathetic tone and perhaps alterations in cardiac muscle leading to hypertrophy (Pfeffer JM et al. 1982).

1.2.3. Structure and function of kidney

1.2.3.1. Structure of the kidney

According to Vander et al. (1991), the kidneys are paired organs that lie outside the peritoneal cavity in the posterior abdominal wall, one on each side of the vertebral column. The medial border of the kidney is indented by a deep fissure called the helium through which pass the renal vessels and nerves and in which lies the renal pelvis, the funnel-shaped continuation of the upper end of the ureter. The outer convex border of the renal pelvis is divided into major calyxes, each of which subdivides into several minor calyxes. Each of the latter is cupped around the projecting apex of a cone-shaped mass of tissue, a renal pyramid.

Longitudinal section of the kidney shows two major regions; an inner renal medulla and an outer renal cortex. The medulla is made up of a number of renal pyramids, the apexes calyxes. Each apical tip is called a papilla. Each pyramid of the medulla, topped by a region of renal cortex, forms a single lobe. Upon closer gross examination, additional features can be discerned: 1) the cortex has a highly granular appearance, missing from the medulla; 2) each medulla pyramid is divisible into an outer zone and an inner zone, including the papilla. All these distinctions reflect the arrangement of the various components of the microscopic subunits of the kidneys.

In human, each kidney is made up of approximately 1 million tiny unit terms nephrons. Each nephron consists of a filtering component, called the renal

corpuscle, and a tubule extending out from the renal corpuscle. The renal corpuscle consists of a compact tuft of interconnected capillary loops, the glomerulus and Bowman's capsule, into which the glomerulus protrudes.

The segment of the tubule that drains Bowman's capsule is the proximal tubule, which initially forms several coils follow by a straight segment which descends toward the medulla. The next segment, into which the proximal straight tubule drains, is the descending thin limb of Henle's loop. The descending thin limb ends at a hairpin loop, and the tubule then begins to ascending parallel to the descending limb. In long loops, the epithelium of the first portion of this ascending limb remains thin, although different from that of the descending limb, and this segment is called the ascending thin limb of Henle's loop. Beyond this segment, in this long loop, the epithelium thickens and this next segment is termed the thick ascending limb of Henle's loop. In short loops, there is no thin ascending limb and the thick ascending limb begins right at the hairpin loop.

Near the end of every thick ascending limb, the tubule passes between the arterioles supplying its renal corpuscle of origin. This very short segment is known as the macula densa. A little beyond the macula densa, the thick ascending limb ends and the distal convulated tubule begins. This is followed by the connecting tubule, which leads to a cortical collecting duct. All cortical collecting ducts run downward to enter the medulla and become outer medullary collecting ducts, and then inner medullary collecting ducts. The latter then merge to form several hundred large ducts, the last portions of which are called papillary collecting ducts, each of which empties into a calyx of renal pelvis. The pelvis is continuous with the ureter, which empties into the urinary bladder where urine is temporarily stored and from which it is intermittently eliminated.

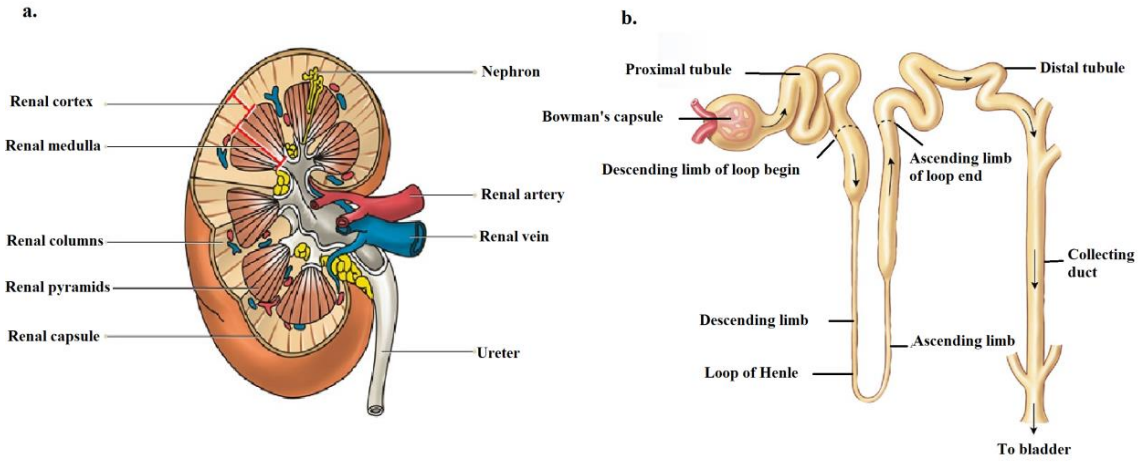

Figure 1.12. Anatomy of (a) kidney and (b) renal tubules (applied from http://www.newhealthadvisor.com/kidney-structure-and-function.html and http://tsbiomed.blogspot.com/2012/12/renal-physiology-fluid-and-electrolyte.html).

1.2.3.2. Renal functions

1) Regulation of water and electrolyte balance

The primary functions of the kidneys is to balance the body's waste and inorganic ions to maintain stable concentrations of these substances in the extracellular fluid, that is, the internal environment. Theoretically, a substance can appear in the body either as a result of ingestion or as a product of metabolism. If the quantity of any substance in the body is to be maintained at a constant level over a period of time, the total amounts ingested and produced must equal the total amounts excreted and consumed. This is a general statement of the balance concept. For water and hydrogen ion, balance is simpler for the mineral electrolytes. Since they are neither synthesized nor consumed by cells, their total-body balance reflects only ingestion versus excretion. Reflexes that alter urinary excretion constitute the major mechanisms that regulate the body balances of water and many of the inorganic ions determining the properties of the extracellular fluids. To appreciate the importance of these kidney regulations one need only make a partial list of the more important simple inorganic substances in the internal environment that are regulated, in large part, by the kidneys: water, sodium, potassium, chloride, calcium, magnesium, sulfate, phosphate and hydrogen ions.

2) Excretion of metabolic waste products and foreign chemicals

The regulatory role just described is obviously quite different from the popular conception of the kidneys as glorified garbage disposal units that rid the body of assorted wastes and poisons. It is true that some of the chemical reactions that occur within cells results ultimately in the end products that must be eliminated. These end products are called wasted products because they serve no known biological function in humans. For example, the catabolism of protein produces approximately 30 g of urea per day. Other end products produced in relatively large quantities are uric acid (from nucleic acids), creatinine (from muscle creatine), bilirulin and other end products of hemoglobin breakdown, and the metabolites of various hormones. Most of these substances are eliminated from the body as rapidly as they are produced, primarily by way of the kidneys. Moreover, the kidneys have another general excretory function, the elimination from the body of many foreign chemicals such as drugs, pesticides, food additives and so on.

3) Regulation of arterial blood pressure (ABP)

The kidneys are intimately involved in the regulation of ABP through two mechanisms. First, sodium balance is a critical determinant of cardiac output and, possibly, arteriolar resistance over any long time period, and the kidneys, as stated, regulate this balance. Second, the kidneys function as endocrine glands in the RAS, a hormonal complex of enzymes, protein and peptides that are important in the regulation of arterial pressure.

Renin is a proteolytic enzyme secreted into the blood by the kidneys, specifically by the granular cells of the juxtaglomerular apparatuses. Once in the blood stream, renin catalyzes the splitting of decapeptide, angiotensin I, from a plasma protein known as angiotensinogen, which is secreted by the liver and is always present in the plasma in high concentration. Under the influence of angiotensin converting enzyme, the terminal two amino acids are then split from the relatively inactive angiotensin I to yield the highly active octapeptide AII. Some converting enzyme is present in plasma, but most is on the endothelial surface of blood vessels, particularly the pulmonary capillaries. Accordingly, the conversion of angiotensin I to AII occurs mainly as blood flows through the lungs.

Thus, AII is a hormone in that it reaches its target organs, including the kidneys, via the arterial blood. However, because the kidneys produce renin and because renal tissue also contains both angiotensinogen and converting enzyme, it is likely that the reactions generating angiotensin I and, in turn, AII occur to some extent within the kidneys. Accordingly, the kidneys can probably be influenced not only by arterial AII but also by AII produced intrarenally. AII exerts a large number of effects on diverse tissues, but the end results of most of them are to increase ABP.

3) Secretion of erythropoietin and 1, 25-Dihydroxyvitamin D_3

The kidneys secrete another hormone, erythropoietin, which is involved in the control of erythrocyte production by the bone marrow. Erythropoietin stimulates the bone marrow to increase its production of erythrocytes. The renal disease may result in diminished erythropoietin secretion and the ensuring decrease in bone marrow activity is one important causal factor in the anemia of chronic renal disease. The kidneys also produce 1, 25 dihydroxyvitamin D_3, active form of vitamin D, which play a role in calcium metabolism.

4) Gluconeogenesis

During prolong fasting, the kidneys synthesize glucose from amino acids and other precursors and release it into the blood. Thus, like the liver, the kidneys are gluconeogenesis organs.

Table 1.3. Functions of the kidney (Vander et al. 1991).

1. Regulation of water and electrolyte balance
2. Excretion of metabolic waste products from the blood and their excretion in urine
3. Excretion of foreign chemicals from blood and their excretion in urine
4. Regulation of arterial blood pressure
5. Secretion of erythropoietin
6. Secretion of 1, 25-Dihydroxyvitamin D_3
7. Gluconeogenesis

1.2.3.3. Principle of renal clearance

The clearance of a substance is the volume of plasma from which that substance is completely cleared by the kidneys per unit time. Every substance in the blood has its own distinct clearance value and the units are always in volume of plasma per time. It should be evident that the basic clearance formula for any substance X is

$$C_X = \frac{\text{mass of X excrete/time}}{P_X}$$

$$C_X = \frac{U_X \dot{V}}{P_X}$$

When C_X = clearance of substance X

U_X = urine concentration of X

$\dot{V}$ = urine volume per time

P_X = arterial plasma concentration of X

Clearance of inulin (C_{in}) is a measure of GFR simply because the volume of plasma completely cleared of inulin. Inulin offers an excellent example. Since all excreted inulin must come from plasma, one can see that a certain volume of plasma loses its inulin while flowing through the kidney. This volume is obvious equal to the GFR since none of the inulin contained in the glomerular returns to the blood and since none of the plasma that escapes filtration loses any of its inulin. Therefore, a volume of plasma equal to the GFR has been completely cleared from inulin.

$$C_{in} = \frac{U_{in} \dot{V}}{P_{in}} = \text{GFR}$$

When C_{in} = clearance of inulin

U_{in} = urine concentration of inulin

$\dot{V}$ = urine volume per time

P_{in} = arterial plasma concentration of inulin

Another substance secreted by the proximal tubules is the organic anion para-aminohippuric acid (PAH). PAH is also filtered at the glomerulus, and, when its plasma concentration is fairly low, virtually all the PAH that escapes filtration is secreted. Since PAH is not reabsorbed, the net effect is that all the plasma supplying the nephrons is completely cleared of PAH. If PAH were completely clear from all the plasma flowing through the entire kidney, its clearance would measure the total renal plasma flow (TRPF). However, about 10 to 15 percent of the total renal plasma flow supplies nonsecreting portions of the kidneys, such as peripelvic fat and the medulla, and this plasma cannot, therefore, lose its PAH by secretion. Accordingly, the PAH clearance actually measures the so-called effective renal plasma flow (ERPF) and is approximately 85 to 90 percent of the TRPF. The clearance formula for PAH is, of course.

$$C_{PAH} = \frac{U_{PAH}\dot{V}}{P_{PAH}} = ERPF$$

When C_{PAH} = clearance of PAH

U_{PAH} = urine concentration of PAH

$\dot{V}$ = urine volume per time

P_{PAH} = arterial plasma concentration of PAH

Once we have measured the ERPF, we can calculate easily the effective renal blood flow (ERBF):

$$ERBF = \frac{ERPF}{1\text{-}Hct}$$

When Hct = hematocrit

The renal blood flow (RBF) in a typical adult is approximately 1.1 L/min. Thus, the kidneys receive 20 to 25 percent of the total cardiac output (5 L/min) even though their combined weight is less than 1 percent of total body weight. The basic equation for blood flow through any organ is

$$\text{Organ blood flow} = \Delta P/R$$

When ΔP = mean arterial pressure minus venous pressure for that organ

R = resistance to flow through that organ

From this equation, we can calculate the RVR:

$$RVR = MABP/ERPF$$

When RVR = renal vascular resistance

MABP = mean arterial blood pressure

ERPF = effective renal plasma flow

1.2.3.4. Alterations of renal function in 2K1C

1) Whole kidney function in 2K1C

It is clear that urine flow, sodium excretion, potassium excretion and functional sodium and potassium excretion of the non-clipped kidney increased to level greater than those observed for normal animals. Quantitatively, values for the same indexes of renal function for the clipped kidney are less than those for the non-clipped kidney or for kidneys of control rats (Huang et al. 1982; Ploth et al. 1944). The non-clipped kidney has a higher osmolar excretion rate and lower urine osmolality than the clipped kidney and it also absorbs larger quantities of free water (Kramer & Ochwadt 1972; Kramer & Ochwadt 1974). The non-clipped kidney excretes urine of lower osmolality than control kidneys and exhibits an impaired ability to elaborate a maximally concentrated urine that is not related to quantitative deficit of ADH, although it may be related to the lower medullary and papillary osmolalities observed in those tissue (Kramer & Ochwadt 1974). The non-clipped kidney also exhibits exaggerated increase of osmolar clearance and free water reabsorption in response to increase of ABP (Stumpe et al. 1969; Strumpe et al. 1970). The increased excretory function for the non-clipped kidney relative to the clipped side persists throughout the course of hypertension, although total excretory function for both kidneys may not be different from that for both kidneys of control animal (Kramer & Ochwadt 1972; Kramer & Ochwadt 1974).

2) Renal hemodynamic alterations in 2K1C

Stenosis of one renal artery results in a decrease of RBF to clipped kidney (Huang et al. 1982; Kramer & Ochwadt 1972) while RBF to the non-clipped kidney increases immediately in rat (Kramer & Ochwadt 1972). The RBF may remain greater than control (Huang et al. 1982; Ploth et al. 1981) or decrease slightly toward levels observed for control animals in the next few days (Kramer & Ochwadt 1972). Three weeks later, the clipped kidney shows reduced RBF and GFR, whereas the non-clipped kidney has a tendency toward reduced GFR in spite of unchanged RBF (Oliveira-Sale et al. 2016). However, the RBF for either the clipped or non-clipped kidney may not be different from values in control animal when factored for differences in kidneys weight (Folkow et al. 1977; Huang et al. 1982).

As ABP increases, the non-clipped kidney exhibits progressive increases of RVR as reflected by the maintenance of modestly increased levels of RBF at greatly elevated systemic BP. The increased RVR has been observed in *in vivo* (Huang et al. 1982; Ploth & Nava 1979) and *in vitro* preparation (Rostand et al. 1982), although RVR may not be disproportionately increased for the greater renal size (Folkow et al. 1977) or as compared with other vascular beds. Since the application of antagonists of the RAS reduced RVR in non-clipped kidney, it is accepted that this increased resistance is mediated by AII (Huang et al. 1982; Ploth et al. 1977).

3) Autoregulation of renal function in 2K1C

The normal kidney exhibits intrinsic mechanisms that precisely control RVR so that RBF and GFR are regulated with near constancy in spite of physiologic alterations of perfusion pressure. Many evidences indicated that altered autoregulatory function may be found in the non-clipped kidney of 2K1C hypertension (Ploth et al. 1981; Ploth et al. 1977; Rostand 1982). Previous studies extended to examine the efficiency of auroregulation of RBF in non-clipped kidneys (Ploth et al. 1982; Ploth et al. 1979). These observations indicated that baseline RVR in the non-clipped kidney is greater than that in control animals at similar perfusion pressures (Ploth et al. 1982). Further, compared with the reductions of RVR that are typically observed in normal kidneys when they are subjected to acute reductions of perfusion pressure, the reduction of RVR in the non-clipped hypertensive kidneys

during the same maneuver are greatly attenuated (Ploth et al. 1982). These data indicated that the capability of theses kidneys to autoregulate kidney RBF is also impaired (Ploth et al. 1982).

4) Characteristics of glomerular hemodynamics in 2K1C

Schwietzer and Gertz investigated the glomerular hemodynamic behavior for each kidney of 2K1C rats for 3-4 weeks after clipping when SBP was 180 mmHg. They found that during condition of systemic hypertension perfusion pressure to the clipped kidney distal to the stenotic clip was normal (124 mmHg). It must be presumed that cumulative resistance of the vasculature distal to the clip but proximal to the myoepithelial juxtaglomerular cells of the afferent arteriole results in exaggerated dissipation of the normal pressure distal to the clip, which results in stimulation of baroreceptor or other elements of the juxtaglomerular apparatus (Schwietzer & Gertz 1979). This reduced perfusion pressure at the afferent arteriole resulting in ongoing stimulation or activation of the RAS, which contributes to the development and the early maintenance phase of the elevated SBP (Ledingham 1975; Swales 1979).

5) Tubule absorptive behavior in non-clipped kidneys of 2K1C

Several micropuncture technique studies indicated that absorptive function of the single nephron is altered in non-clipped kidneys of 2K1C hypertension. Stumpe et al. (1969 & 1970) reported that absorption of sodium and water from proximal tubules of non-clipped kidneys was not altered in the early maintenance phase of RVH. However, they observed that decreased fractions of the filtered loads of sodium and water were absorbed in the loop of Henle and that the reduced fractional absorption was quantitatively grater in animals with higher BP (Stumpe et al. 1969; Stumpe et al. 1970). These data indicated that increases of medullary interstitial pressure following increases of systemic BP may reduce absorptive function in loop of Henle (Stumpe et al. 1970). Ploth et al. suggested that greater fractional water absorption in segments upstream from early distal tubule micropuncture sites in non-clipped kidneys at hypertensive BP (180-200 mm Hg) than

were observed for similar micropuncture sites of normal animals at normotensive BP (129-137 mm Hg) (Ploth et al. 1977).

1.2.4. Structure and function of vascular smooth muscle

1.2.4.1. Architecture of blood vessel wall

The wall of blood vessels consists of three layers; tunica intima, tunica media and tunica adventitia as shown in Figure 1.13.

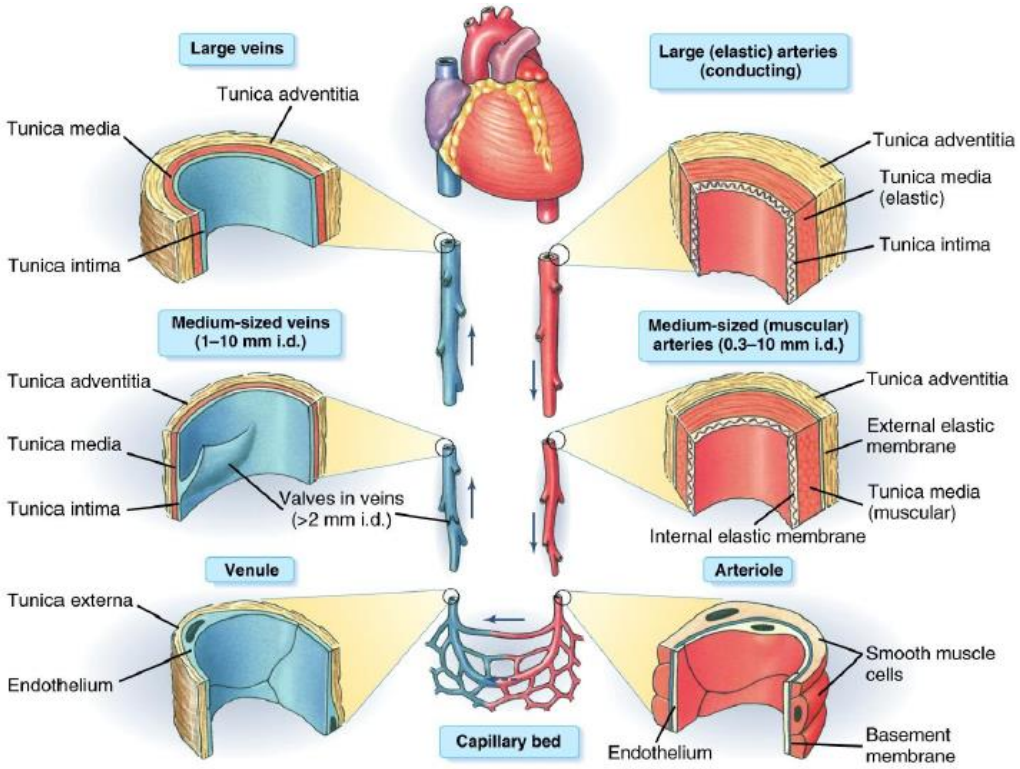

Figure 1.13. Structure of blood vessel wall (from http://websupport1.citytech.cuny.edu/Faculty/ibarjis/Teaching/Anatomy%20and%20Physiology/lecture21/Lecture/Lecture1.htm).

1) Tunica intima

The intimal layer is the innermost layer of all blood vessels which composes of 1) endothelial cells, a single layer of squamous cells separated from the subendothelial space by a thin basal lamina, constitute a selective barrier against plasma lipids and lipoproteins. The endothelial cells are flat and elongated with their

long axis parallel to that of the blood vessels. They have a thickness of about 0.2-0.5 μm, except in the area of nucleus, which makes the endothelial cell protrude slightly, 2) a thin basal lamina with about 80 nm thick and 3) subendothelial layer composes of collagenous bundles, elastic fibrils, smooth muscle cells and perhaps some fibroblasts. Such layer is usually present only in the large elastic arteries such as the human aorta, whereas in the majority of other blood vessels the intima consists of only endothelial cells and basal lamina.

2) Tunica media

The tunica media is the middle layer of the vascular wall. It is made up of VSMCs, a varied number of elastic sheets, bundles of collagenous fibrils and a network of elastic fibrils. This layer is thicker in arteries than in veins; smooth muscle cells may even be absent in large veins such as the vena cava. In the aorta, they may reach a thickness of 500 μm, whereas it is about 20-50 μm in medium-sized veins. In arterioles, it averages 2-3 layers of smooth muscle cells with an often incomplete elastic lamina. In venule, it consists of only 1-2 layers of smooth muscle cells.

3) Tunica adventitia

The tunica adventitia is the outermost layer of the vascular wall. Its thickness varies considerably depending on the type and location of the blood vessel. For instance, cerebral blood vessels almost entirely lack an adventitia. In all arteries and most veins, the adventitia consists of dense fibroelastic tissue without smooth muscle cells, except in large veins such as the vena cava where there occur bundles of longitudinally arranged smooth muscle cells. It also harbors the nutrient vessels of the vascular wall: arterioles, venules, capillaries and lymphatic vessels, collectively referred to as vasa vasorum. Nerves are also present in this layer. Many of these nerves are part of the peripheral nerve trunks for sensory organs, muscles and visceral organs, but some are vasomotor and sensory nerves for the vascular wall itself.

1.2.4.2. Vascular smooth muscle constraction

Contraction mechanism

In the intact body, the process of smooth muscle cell contraction is regulated principally by receptor and stretch activation of the contractile protein myosin and actin. A change in membrane potential, brought on by the firing of action potentials or by activation of stretch-dependent ion channels in the cell membrane, can also trigger contraction. For contraction to occur, myosin light chain kinase (MLCK) must phosphorylate the 20-kDa light chain of myosin, enabling the molecular interaction of myosin with actin. Energy released from ATP by myosin ATPase activity results in the cycling of the myosin cross-bridges with actin for contraction. Thus contractile activity in smooth muscle is determined primarily by the phosphorylation state of the light chain of myosin.

Ca^{2+}-dependent contraction of vascular smooth muscle

Contractile of smooth muscle is initiated by a Ca^{2+}-mediated change in the thick filaments, whereas in striated muscle Ca^{2+} mediates contraction by changes in the thin filaments. In response to specific stimuli in smooth muscle, the $[Ca^{2+}]_i$ increases and this activator Ca^{2+} combines with the acidic protein calmodulin (CaM). Ca^{2+}-CaM complex activates MLC kinase to phosphorylate the light chain of myocin. Cytosolic Ca^{2+} is increased through Ca^{2+} release from sarcoplasmic reticulum (SR) as well as entry from the extracellular space through receptor-operated Ca^{2+} channels. Agonists (norepinephrine, AII, ET-1, etc.) binding to serpentine receptors, coupled to a heterotrimeric G protein, stimulates phospholipase C activity. This enzyme is specific for the membrane lipid phosphatidylinositol 4, 5-bisphophate to catalyze the formation of two potent second messengers; inositol triphosphate (IP_3) and diacylglycerol (DAG). The binding of IP3 to receptors on the SR results in the release of Ca^{2+} into the cytosol. DAG, along with Ca^{2+}, activates protein kinase C (PKC), which phosphorylates specific target proteins. PKC has contraction-promoting effects such as phosphorylation of L-type Ca^{2+} channels or other proteins that regulate cross-bridge cycling. Finally, L-type Ca^{2+} channel (voltage-operated Ca^{2+} channels) in the membrane also open in response to membrane depolarization brought on by stretch of smooth muscle cell.

Ca^{2+} sensitization mechanism and contraction of vascular smooth muscle

In addition to the Ca^{2+}-dependent activation of MLC kinase, the state of myosin light chain phosphorylation is further regulated by MLC phosphatase which removed the high-energy phosphate from the light chain of myosin to promote smooth muscle relaxation. There are three subunits of MLC phosphatase; a 37-kDa catalytic subunit, a 20-kDa variable subunit and a 110- to 130-kDa myosin-binding subunit. The myosin-binding subunit, when phosphorylated, inhibits the enzymatic activity of MLC phosphatase, allowing the light chain of myosin to remain phosphorylated, thereby promoting contraction. The small G protein RhoA and its downstream target Rho kinase play an important role in the regulation of MLC phosphatase activity. Rho kinase, a serine/threonine kinase, phosphorylates the myosin-binding subunit of MLC phosphatase, inhibiting its activity and thus promoting the phosphorylated state of the myosin light chain.

Moreover, it is known that receptors activate a heterotrimeric G protein that is coupled to RhoA/Rho kinase signaling via guanine nucleotide exchange factors (RhoGEFs). Because RhoGEFs facilitate activation of RhoA, they regulate the duration and intensity of signaling via heterotrimeric G protein receptor coupling. There are about 70 RhoGEFs in the human genome and three RhoGEFs have been identified in smooth muscle; PDZ-RhoGEF, LARG (leukemia-associate RhoGEF) and p115-RhoGEF. Several recent studies suggest a role for additional regulations of MLC kinase and MLC phosphatase. Calmodulin-dependent protein kinase II promotes smooth muscle relaxation by decreasing the sensitivity of MLC kinase for Ca^{2+}.

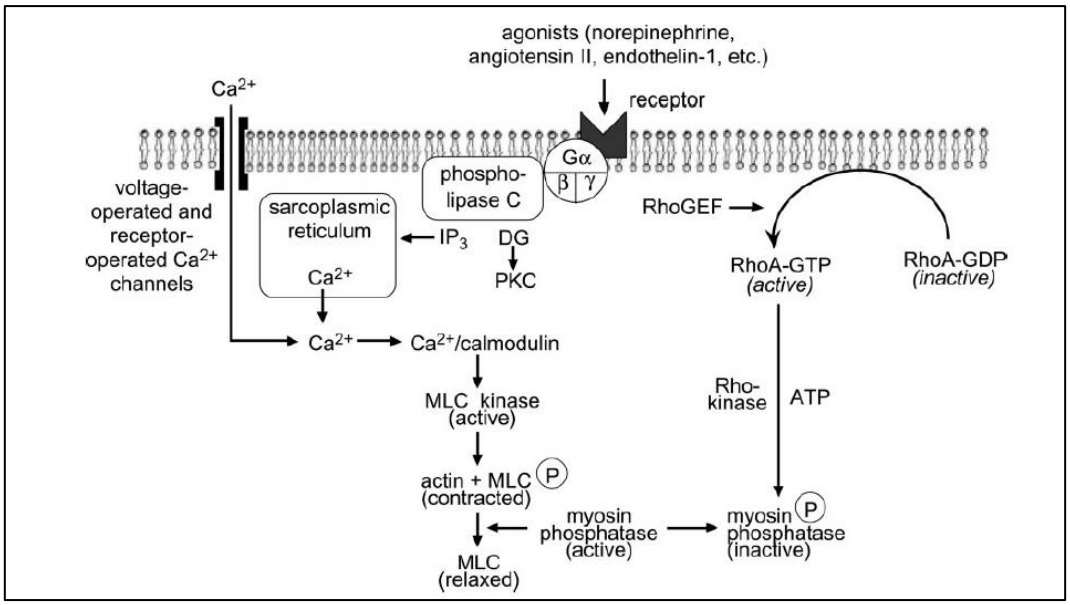

Figure 1.14. Mechanism of vascular smooth muscle contraction. Various agonists bind to specific receptors, subsequently, the response of the cell is to increase PLC activity via coupling through a G protein. The PLC produces two potent secondary messengers from the membrane lipid PIP_2; DAG and IP_3. The IP_3 binds to specific receptors on the SR, ryanodine receptor (RyR), cause a releasing of activator Ca^{2+}. The DAG along with Ca^{2+} activated PKC, which phosphorylates specific target proteins. The PKC has contractile-promoting effects such as phosphorylation of Ca^{2+} channels or other proteins that regulate cross-bridge cycling. Ca^{2+} binds to CaM leading to activation of MLCK. This kinase phosphorylates the light chain of myocin in connection with actin and cross-bridge cycling occurs. However, the elevation in Ca^{2+} concentration within the cell is transient, and the contractile response is maintained by a Ca^{2+} -sensitizing mechanism brought about by the initiation of myosin phosphatase activity by Rho kinase. This Ca^{2+} -sensitizing mechanism is initiated at the same time that PLC is activates and it involves the activation of the RhoA-GTP. The activation of RhoA by the G protein-couple receptor is not clear but involves a guanine nucleotide exchange Rho kinase (RhoGEF) and migration of RhoA to the plasma membrane. Upon activation, RhoA increases Rho kinase activity leading to inhibition of myosin phosphatase. This promotes the contractile state, since the light chain of myocin cannot be dephosphorylated (Webb 2003).

1.2.4.3. Vascular smooth muscle relaxation

Smooth muscle relaxation occurs either as a result of removal of the contractile stimulus or by the direct action of a substance that stimulates inhibitions of the contractile mechanism. The process of relaxation requires a decreased intracellular Ca^{2+} concentration and increased MLC phosphatase activity (Morgan 1990; Somlyo et al. 1999). The mechanisms that remove intracellular Ca^{2+} and/or increase MLC phosphatase activity may become altered, contributing to abnormal smooth muscle responsiveness.

A decrease in the intracellular concentration of activator Ca^{2+} elicits smooth muscle cell relaxation. Several mechanisms are implicated in the removal of cytosolic Ca^{2+} and involve the SR and the cell membrane. Ca^{2+} uptake into the SR is dependent on ATP hydrolysis. The SR Ca-Mg-ATPase, when phosphorylated, binds to Ca^{2+} ions, which are then translocated to the luminal side of the SR and released. Mg^{2+} is necessary for the activity of the enzyme, it binds to the catalytic site of the ATPase to mediate the reaction.

The cell membrane also contains Ca-Mg-ATPase, providing an additional mechanism for reducing the concentration of activator Ca^{2+} in the cell. This enzyme differs from the SR protein in that it has an autoinhibitory domain that can be bound by CaM, causing stimulation of the cell membrane Ca^{2+} pump. Na^{+}/Ca^{2+} exchangers are also located on the cell membrane and aid in decreasing intracellular Ca^{2+}. ROCC and VOCC located in the cell membrane are important in Ca^{2+} influx and smooth muscle contraction (Webb 2003).

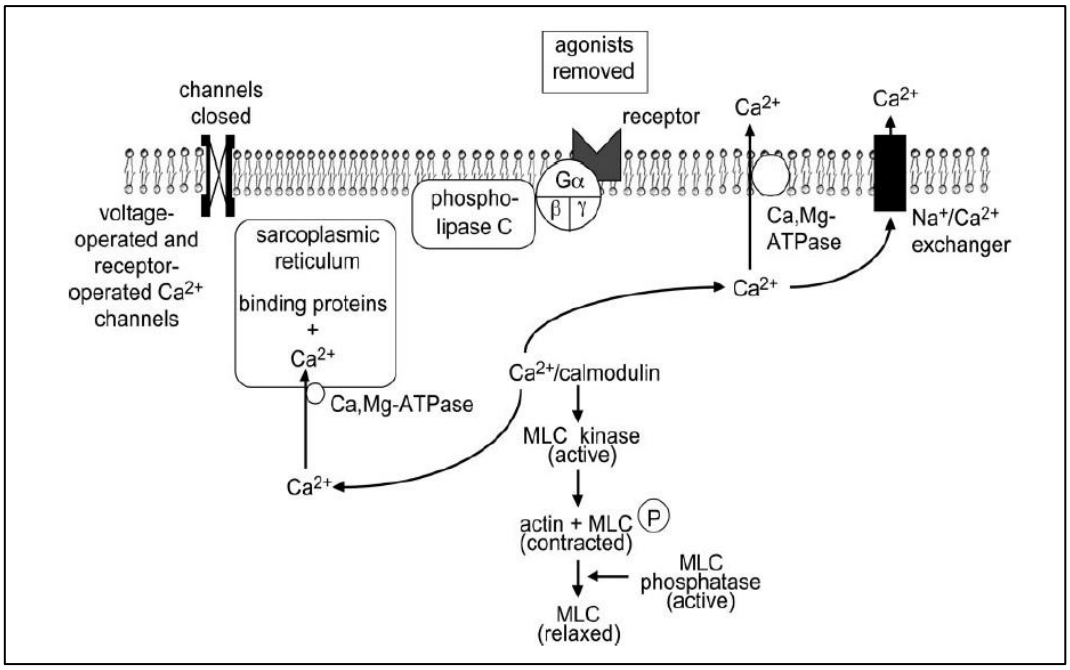

Figure 1.15. Mechanism of vascular smooth muscle relaxation. Smooth muscle relaxation occurs either as a result of the contractile stimuli or by direct action of substance that stimulates the contractile mechanism. The relaxation requires a decreased intracellular Ca^{2+} concentration and increased MLC phosphatase activity. The SR and cell membrane contain Ca, Mg-ATPases that remove Ca^{2+} from the cytosol. Na^+/Ca^{2+} exchangers are also located on the cell membrane and aid in decreasing intracellular Ca^{2+}. During relaxation, ROCC and VOCC in the cell membrane close resulting in a reduced Ca^{2+} entry into the cell (Webb 2003).

1.2.4.4. Role of endothelium on vascular tone

Endothelial cell is a thin layer of the cell line covers the inner surface of the blood vessels of the entire circulatory system (about 5,000 m^2), from the heart to the smallest capillary. It comprises about 2% of the mass of the human body (Eisenberg 1991). The endothelial cells secrete vasodilator mediators in response to substances released from autonomic and sensory nerves (e.g. ACh, NE, ATP, substance P), circulating hormones (e.g. catecholamine, vasopressin, AII, insulin), coagulation derivatives and platelet products (serotonin, ADP, thrombin) or autacoids produced by endothelial and vascular smooth muscle cells themselves (bradykinin, ADP/ATP/UTP, AII, endothelin) (Furchgott & Zawadzki 1980; Furchgott 1983; Vanhoutte 1988; Bassenge & Heuch 1990; Lucher & Vanhoutte 1990). Changes in shear stress elicit endothelium- and flow-derived vasodilatation. In healthy arteries, the endothelium presents a non-adhesive anti-thrombotic surface that is critical to homeostasis (Luscher & Vanhoutte 1990; Vane 1994). Endothelium-derived vasodilator substances also exert a profound regulatory influence on the thrombolytic and anti-proliferative functions of the endothelium.

Endothelium-derived vasodilators

Nitric oxide

The endothelium releases a factor that relaxes the underlying VSMC and this was later shown to be NO (Furchgott & Zawadzki 1980). Endothelial cells constitutively express a NO synthase (eNOS) that generates NO using L-arginine as a substrate. NO synthesis is enhanced after Ca^{2+}-CaM binding. eNOS is an NADPH-dependent oxygenase that requires tetrahydrobiopterin, FAD and FMN as co-factors (Moncada et al. 1991). In endothelial cells, the enzyme is localized preferentially in caveolae following post-translational acylation. eNOS is negatively regulated by caveolin. Stimulation of endothelial cells by agonists such as bradykinin dissociates the caveolin/NOS complex and allows binding and activation of NO systhesis by Ca^{2+}-CaM (Michel et al. 1997; Feron et al. 1998). Shear stress-induced activation of eNOS involves a Ca^{2+}-independent, protein tyrosine kinase-dependent mechanism (Ayajiki et al. 1996).

The relaxation of vascular smooth muscle by NO involves the stimulation of soluble guanylate cyclase and consequently the increased formation of cyclic GMP (Rapoport & Murad 1983). The latter activates cyclic GMP-dependent protein kinase which leads to an increased extrusion of Ca^{2+} from the cytosol in vascular smooth muscle and to the inhibition of contractile machinery. Cyclic GMP-dependent protein kinase phosphorylates K^+ channels to induce hyperpolarization and thereby inhibits vasoconstriction (Lincoln et al. 1994). In arteries, NO activates K^+ channels independently of cyclic GMP (Bolotina et al. 1985). Inhibition of eNOS using hydrolysis-resistant competitive analogs of L-arginine drastically reduces endothelium-dependent relaxation (Moncada et al. 1991). NO contributes to the balance between vasodilator and vasoconstrictor influences that determine vascular tone.

Prostacyclin (PGI_2)

PGI_2 is produced by endothelial cells in the vascular wall and acts as vasodilator (Moncada & Vane 1979). Its vasodilator activity is determined by the expression of specific receptors in the VSMC (Coleman et al. 1994). Its receptors are coupled to adenylate cyclase to elevate cAMP levels in VSMC (Kukovetz et al. 1979). Then the cAMP stimulates K_{ATP} channels to cause hyperpolarization of the cell membrane and inhibit the development of contraction (Parkington et al. 1995). The cAMP also increases the extrusion of Ca^{2+} from the cytosol in VSMC and inhibits the contractile machinery (Bukoski et al. 1989; Abe & Karaki 1992). In addition, PGI_2 facilitates the release of NO by endothelial cells (Shimokawa et al. 1988) and the action of PGI_2 in VSMC is potentiated by NO (Delpy et al. 1996). Indeed, increase in cGMP in target cells inhibits a phosphodiesterase that breaks down cAMP (Weigert et al. 1994; Delpy et al. 1996). Therefore, NO indirectly prolongs the half-life of the second messenger of PGI_2.

Endothelial-derived hyperpolarizing factors (EDHF)

In most medium- to resistance-sized arteries, electrophysiological studies have established that endothelium-dependent hyperpolarization of vascular smooth muscle is resistant to the combined inhibition of both NOS and cyclooxygenases. A component of the endothelium-dependent relaxation in these arteries is mediated by a

substance different from NO and prostacyclin. This component of endothelium-dependent vasodilatation has been ascribed to a diffusible endothelium-derived hyperpolarizing factor (EDHF), the identity of which is still controversial (Feletou & Vanhoutte 1996; Mombouli & Vanhoutte 1997). Epoxyeicosatrienoic acids, a metabolite of arachidonic acid, have been shown to act on large conductance BK_{Ca} channel (Gebremedhin et al. 1992; Hu & Kim 1993; Campbell et al. 1996). EDHF plays a significant role in the regulation of peripheral vascular resistance and local hemodynamics by activation of various K^+ channels in VSMC (Feletou & Vanhuotte 1988). A combination of charybdotoxin and apamin, a blocker of K_{Ca2+} channels, is necessary to inhibit the responses attributed to EDHF in rat hepatic and mesenteric arteries (Corriu et al. 1996) and in guinea-pig carotid arteries (Zygmunt & Hogestatt 1996) whereas apamin alone can inhibit EDHF-induced hyperpolarization in rabbit mesenteric arteries (Murphy & Brayden 1995). In addition, the synthetic K_{ATP} channel openers also inhibits by closing VOCC (Nelson et al. 1990), impairing the receptor-dependent activation of PLC and the subsequent release of Ca^{2+} from intracellular stores by reducing the Ca^{2+} sensitivity of the contractile proteins (Itoh et al. 1992; Itoh et al. 1994). These data suggested that K_{Ca2+} and K_{ATP} channels are possibly involved in activation pathway of the EDHF.

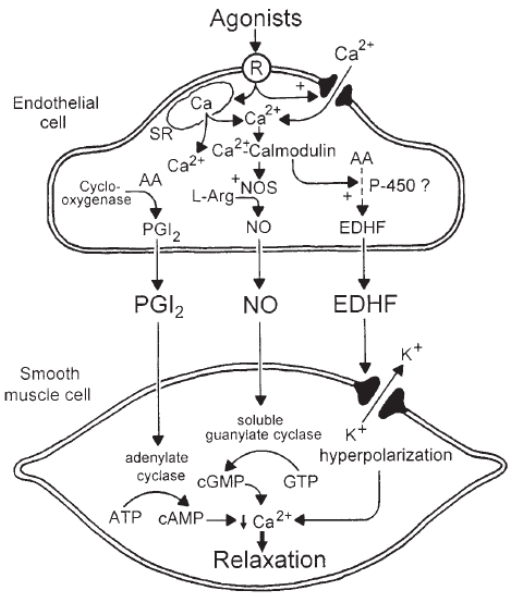

Figure 1.16. Schematic summarizing the release of relaxing factors from endothelial cells and their effect on vascular smooth muscle cell. The broken line indicates the action of an inhibitor or antagonist (Mombouli & Vanhoutte 1999)**.**

Endothelium-derived constriction factors

Vasoconstrictor prostaglandins

The metabolism of arachidonic acid by COX in endothelial cells may lead to the secretion of prostaglandin H_2 (PGH_2), the precursor of all prostanoids including thromboxane A_2 (TXA_2) (Moncada & Vane 1979). PGH_2 and TXA_2 act on endoperoxide/thromboxane receptors on VSMC to induce vasoconstriction (Halushka et al. 1989; Coleman et al. 1994). These substances attenuate endothelium-dependent relaxation induced by ACh in isolated human renal arteries (Luscher et al. 1987a & b). However, prostacyclin is the major endothelial metabolite of arachidonic acids that is generated through the COX pathway (Moncada & Vane 1979; Luscher & Banhoutte 1990). Thus, under normal circumstances, the influence of the small amounts of TXA_2 released by endothelial cells is masked by the production of prostacyclin, NO and EDHF.

Reactive oxygen species (ROS)

Endothelial cells secrete ROS and hydrogen peroxide (H_2O_2) in response to shear stress and endothelial agonists, such as bradykinin (Shimizu et al. 1994). O_2^- inactivates NO resulting in vasoconstriction in arteries (Rubanyi & Vanhoutte 1986). For instance, O_2^- mediates stretch- or aginist- induced endothelium-dependent contractions in canine cerebral arteries (Katusic & Vanhoutte 1989). ROS also facilitate the mobilization of cytosolic Ca^{2+} in VSMC (Suzuki & Ford 1992) or promote Ca^{2+} sensitization of the contractile element (Jin et al. 1991). Since endothelium-derived O_2^- is primarily a chemical antagonist of NO, the scavenger of the free-radical O_2^- dismutase potentiates endothelium-dependent vasodilatation (Rubanyi & Vanhoutte 1986).

Endothelin

Endothelial cells synthesize the prohormone big endothelin and express endothelin-converting enzymes to generate endothelin-1 (ET-1), which is the most potent vasoconstrictor (Yanagisawa et al. 1988; Masaki. 1995). The secretion of ET-1 can be induced by thrombin, interleukin-1, transforming growth factor-β1, platelet products and neurohormones such as vasopressin and cathecholamines (Luscher et al.

1996). However, the synthesis of ET-1 by endothelial cells is initiated by basal and stimulated production of NO (Masaki. 1995). The VSMCs express ET_B endothelin receptors type B (ET_B) (Masaki 1995). Thus, the direct vasoconstrictors action of ET-1 is modulated by the stimulation of NO and EDHF release at low concentrations of the peptide (DeNucci et al. 1988; Nakashima & Vanhoutte. 1993; Masaki 1995).

Endothelial Dysfunction in 2K1C hypertension

Impaired endothelium-dependent vasodilatation was observed in a number of experimental models of hypertension, including 2K1C hypertension (Lüscher et al. 1987; Dohi et al. 1996; Callera et al. 2000). The biological activity of nitric oxide (NO), an effective endothelium-derived relaxing factor, is primary associated with endothelial NO synthase (eNOS) activity or it interaction with superoxide anion, which is produces in the vascular wall by free radical-generating enzyme (Cai & Hariison 2000). Endothelium-derived NO may be scavenged by superoxide anion, causing reduced bioavailability of NO and diminishing vasorelaxation (Griendling et al. 1994). It is now well established that endothelial dysfunction in hypertension is partially linked to the exaggerated production of superoxide anion and that oxidative stress is responsible for impaired endothelial modulation in 2K1C hypertension (Toba et al. 2012; Arnalich-Montiel et al. 2014; Lerman et al. 2001; Oliveira-Sales et al. 2009).

1.2.5. Arterial baroreceptor reflex

1.2.5.1. Principle of the arterial baroreceptor reflex

As first described by Corneille Heymans, who won the Nobel Prize for Physiology in 1938, the sensors in the arterial baroreceptor reflex are the mechanosensitive afferent nerve endings called baroreceptors. These receptors are located in the carotic artery sinus and aortic arch where they provide instantaneous feedback on arterial stretch proportional to changes in pressure in arterial lumen.

When the artery is distended due to elevated pressure, the embedded barosensitive afferent nerve endings are activated. The baroreceptor sensors a stretch of the vessel wall and changes into electrical signal in the afferent nerves that is relayed to an integrating center in the brain through baroreceptor afferents from the

carotid sinus nerve and aortic depressor nerve synapse in the nucleus of the solitary tract (NTS). The parasympathetic (vagus nerve) baroreflex is processed through the nucleus ambiguous (NA) *en route* to the sinoatrial node of the heart. The sympathetic baroreceptor reflex pathway continues from the NTS through the caudal ventrolateral medulla (CLVM) and then to the rostral ventrolateral medulla (RVLM). The sympathetic outflow then goes to the interomediolateral cell column in the spinal cord and onto its effector organs (heart and blood vessels) (Wehrwein & Joyner 2013) (Wehrwein & Joyner 2013).

1.2.5.2. The arterial baroreceptor reflex arc

The baroreceptor reflex arc composes of following components;

1) Baroreceptors and sensory transduction

Baroreceptors are mechanosensitive afferent nerve endings that are interspersed in the arterial elastic layers. Baroreceptors detect mechanical deformation of vascular wall stretch due to changes in intraluminal pressure. There are two primary sites that contain clusters of arterial baroreceptors: the internal carotid artery at the carotid sinus and the aortic arch as shown in Figure 1.17. A unique feature of these baroreceptive regions is that the arterial wall has limited collagen and is highly compliant to allow distention of the wall each phase (Wehrwein & Joyner 2013).

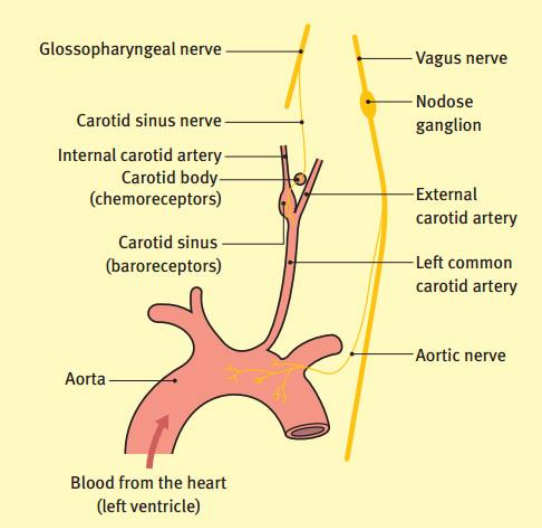

Figure 1.17. The location of the aortic and carotid baroreceptors (Kirkman & Sawdon 2010).

2) Afferent pathways

The electrical response from the baroreceptor is carried centrally to the medulla via afferent fiber. Information from the carotid baroreceptors towards via afferent fibers in the carotid sinus nerve (a branch of cranial nerve IX; glossopharyngeal) and the associated sensory cell bodies exist in the petrosal ganglia. Information from the aortic arch baroreceptors travels in the aortic depressor nerve (a branch of cranial nerve X; vagus nerve) with sensory cell bodies located primary in the nodose ganglia (Wehrwein & Joyner 2013).

3) Central integration

The medulla oblongata is the primary site of cardiovascular and baroreflex integration. In this region, the NTS serves as the primary site for the first synapse of the baroreceptor afferents and is the key integrating site for all baroreceptor. The NTS is a bilateral structure that receives monosynaptic inputs from afferents using glutamate as the primary neurotransmitter. The NTS integrates and relays baroreceptor afferent information via a polysynaptic pathway to other important medullary centers to control parasympathetic and sympathetic pathways to the heart and blood vessels (Wehrwein & Joyner 2013).

The baroreflex mediated parasympathetic pathways to the heart are regulated by a simple autonomic reflex arc. Excitatory pathways from the NTS project onto the cardioinhibitory area containing the NA and dorsal motor nucleus of the vagus. The cardiac component of the baroreflex is largely mediated in these regions where activation of vagal preganglionic efferent fibers results in vagal stimulation to the heart and associated bradycardia (Wehrwein & Joyner 2013).

The sympathetic pathways are more complex, with important control points in the NTS, CVLM and RVLM. The NTS sends projections to inhibitory interneurons in the CVLM and also directly to the RVLM. Activation of GABAergic fibers in the CVLM results in inhibition of sympathetiic excitatory neurons in the RVLM resulting in a reduction in MABP and sympathetic tone. The RVLM contains presympathetic neurons that project to the thoracic spinal cord and control sympathetic preganglionic fibers. Neurons in the rostral RVLM display spontaneous

firing with each cardiac cycle and exhibit a variable firing frequency with each pulse of arterial pressure (Wehrwein & Joyner 2013).

4) Efferent pathway

In the parasympathetic pathway, information from the NA and dorsal motor nucleus of the vagus control preganglionic fibers that exit the spinal column and synapse in the parasympathetic ganglia near the heart to activate postganglionic fibers. The ganglionic synapse and the cardiac postganglionic neuroeffector junction both rely on ACh as the neurotransmitter acting on α_4, β_2 nicotinic cholinergic receptors. Of particular importance relating to the baroreflex is the parasympathetic vagus nerve which has strong influence on the heart (Wehrwein & Joyner 2013).

In the sympathetic partway, information from the RVLM is passed to the interomediolateral cell column, sympathetic preganglionic fibers and then into sympathetic ganglia. ACh acting on α_7 nicotinic cholinergic receptors is the neurotransmitter in sympathetic ganglionic synapses. Sympathetic postganglionic fibers exit the ganglia and travel to the key baroreflex-associated sites of the heart and blood vessels. Norepinephrine is the key neurotransmitter released that acts on both α and β subtypes of adrenergic receptors on these target organs (Wehrwein & Joyner 2013).

5) Peripheral actions of effector mechanisms

Angiotensin II

In the short-term, the main cardiovascular effects of angiotensin II are the mediated by the activation of AT_1 receptors in the membranes of VSMC. Such activation elevates levels of intracellular calcium ions and leads to vasoconstriction. Angiotensin II also increases renal retention of sodium ions and water by acting on receptors in the proximal and late distal convoluted tubule of the nephron. Moreover, angiotensin II promotes the synthesis and release of the salt-retaining hormone aldosterone from adrenal cortical zona glomerulosa cells (Wehrwein & Joyner 2013) (Wehrwein & Joyner 2013).

Activity of parasympathetic nerves

Efferent fibres of the vagus nerve innervate the cardiac pacemaker cell and the atrial myocytes and conductive tissue of the cardiac ventricles. Action potentials result in the release of ACh from the terminal butons and subsequent activation of M_2 muscarinic receptors, which leads mainly to a decrease in HR (Wehrwein & Joyner 2013).

Activity of sympathetic nerves

Action potential is translated into release of adrenaline from chromaffin cells in the adrenal medulla, renin release from renal juxtaglomerular cells and noradrenaline release at peripheral synapses, which are found in cardiac myocytes and the vascular smooth muscle that surrounds blood vessels. Subsequent cardiovascular effects of adrenaline and noradrenaline are initiates when those catecholamines activate α and β adrenergic receptors that are found in cell membranes of the target tissues. The final effects of increased sympathetic nervous activity are increased heart rate (β_1 activation), increased cardiac contractility (β_1 activation), and increased vascular resistance (α_1 activation) (Wehrwein & Joyner 2013) (Wehrwein & Joyner 2013).

Vasopressin

The cardiovascular effects of vasopressin result from the activation of V_1 receptors in VSMC and such activation causes vasoconstriction. Vasopressin also acts on V_2 receptors in the collecting duct of the nephron. Such actions results mostly in the insertion of aquaporins into the luminal membrane and leads to increase reabsorption of water from the nephrons (Wehrwein & Joyner 2013).

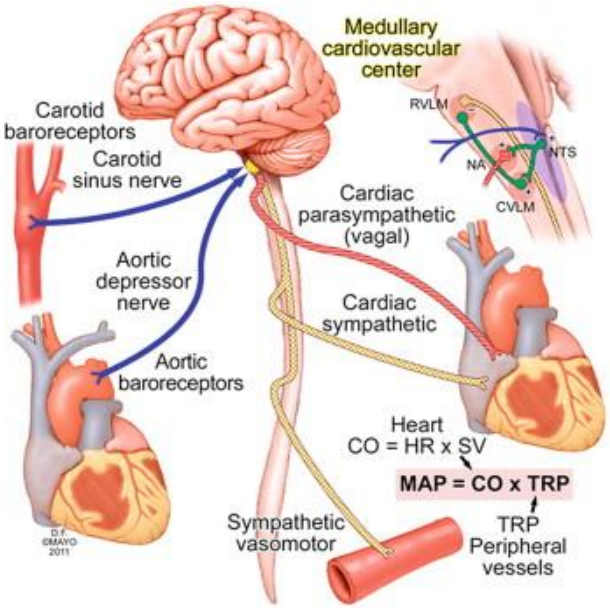

Figure 1.18. The arterial baroreceptor reflex arc (Wehrwein & Joyner 2013).

1.2.5.3. Baroreceptor reflex sensitivity (BRS)

BRS, also known as baroreflex gain, is used as a measurement of the autonomic effector response to a given change in ABP. As an example, when ABP is changed pharmacologically using the vasoconstrictor phenylephrine (PE) and vasodilator sodium nitroprusside (SNP), a large range of pressure can be observed. The changes in pressure are associated with the baroreceptor reflex counterregulation in HR.

As shown in Figure 1.19, BRS is determined from the slope of the relationship between MABP (systolic and diastolic arterial pressures or carotid sinus pressures can also be used) and HR (or sympathetic nerve activity). The HR over a range of MABP is plotted and analyzed by linear regression. The slope of this regression line obtained from the linear part of the curve is the BRS. The BRS is high when the slope is steep (solid line) such that there is a large heart rate response to a given change in MABP. The BRS is low when the slope is flat (dashed line) such that there is a minimal HR response to BP change (Wehrwein & Joyner 2013).

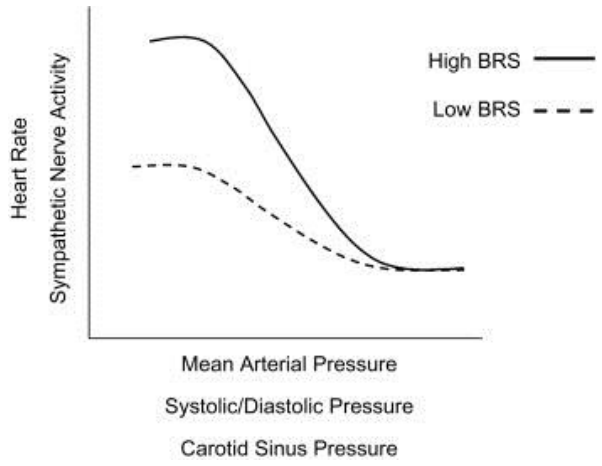

Figure 1.19. Baroreceptor reflex sensitivity (Wehrwein & Joyner 2013).

1.2.5.4. Baroreceptor reflex resetting in hypertension

An Increase in MABP results in activation of baroreceptors as vessel wall stretch increases, however, baroreceptor activation declines rapidly within seconds of an increase in pressure resulting in less afferent activity to the same pressure. When MABP is acutely or chronically elevated for more that several seconds, there is peripheral baroreceptor 'resetting'. Resetting refers to a shift in the threshold for baroreceptor activation leading to a decline in afferent activation observed to a given elevation in pressure.

As shown in Figure 1.20, the pressure increases from point 1 to point 2 and there is an initial rise in afferent activity. This is followed by a reduction in the afferent traffic (point 3) that is generated in response to the same elevated pressure. This blunting of afferent responses is the consequence of resetting. Notably, this new level of afferent activity after resetting is still higher that at baseline, suggesting that there is incomplete resetting during this acute rise in pressure. At point 2 on the graph, the afferent response is nearing the flatter portion of the sigmiodal curve and this would limit further afferent activation to increasing pressure. The resetting response allows the baroreceptor reflex to continue to function over a dynamic range on the linear portion of the curve rather than to have the prevailing pressure shifted to the flat part of the sigmoidal relationship (Wehrwein & Joyner 2013).

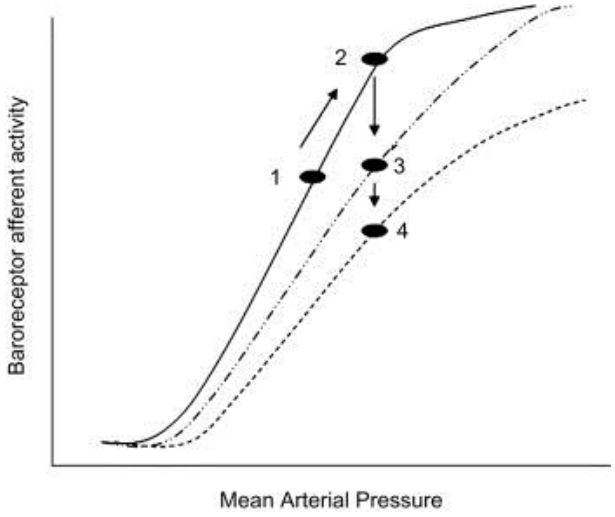

Figure 1.20. Acute and chronic resetting of the baroreceptor reflex. Solid line (left curve): normal response; dashed-dotted line (middle curve): acute resetting; dashed line (right curve): chronic resetting (Chapleau et al. 1989).

1.2.6. Treatment of hypertension

1.2.6.1. Non-pharmacologic treatment

Life style modification is the first step in the treatment of hypertension; it includes weight reduction, dietary sodium restriction, regular aerobic exercise, smoking cessation and reduction in alcohol consumption. In pre-hypertensive patients with SBP between 120-139 mm Hg and DBP between 80-89 mm Hg simply making lifestyle changes would delay and possible halt progression to hypertension. In stage I hypertensive patient, life style changes for 6-12 months might prevent the necessity for drug therapies and should be encouraged in the absence of cardiovascular and renal risk factors (Foëx 2004).

1.2.6.2. Pharmacologic treatment

It is difficult to control the ABP at the optimal level in hypertensive patients through lifestyle changes alone. So treatment with anti-hypertensive drugs becomes necessary. The classes of anti-hypertensive drugs are discussed following;

ACE inhibitors

ACE inhibitors are increasingly being used as first line therapy. They have a dual mode of action; 1) prevent the production of AII and 2) decrease the metabolism of vasodilator bradykinin (Delacroix et al. 2014). They have relatively few side effects and contraindications except bilateral renal artery stenoses. Although ACE inhibitors are effective in unilateral renovascular hypertension, there is risk of ischaemic atrophy. Therefore, angioplasty or surgical renal artery reconstruction are preferable to long-term purely medical therapy. ACE inhibitors are first choice agents in diabetic and heart failure hypertensive patients. The HOPE trial has shown that ramipril reduced the risk of cardiovascular events even in the absence of hypertension suggesting that this ACE inhibitor may exert a protective effect by mechanisms other than the reduction in blood pressure (Foëx 2004).

AII receptors blockers (ARBs)

ARBs possess a hypotensive effect by specifically binding to AII type 1 receptors and inhibiting the vasoconstriction, fluid retention and sympathomimetic action mediated by AII. The decrease in ABP induced by ARBs is correlated to an extent with the renin activity in individual patients. ARBs are used alone or in combination with diuretics and Ca^{2+} channel blockers for the treatment of grade I-III hypertension. ARBs exhibit the cardioprotective effects by inhibiting cardiac hypertrophy and improving the outcome of heart failure. In kidneys, ARBs dilate efferent arterioles, reduce the intraglomerular pressure, improve proteinuria and prevent exacerbation of the renal function in the long term. Moreover, ARBs also improve the regulation of cerebral blood flow, prevent atherosclerosis, inhibit occurrence of atrial fibrillation, improve insulin sensitivity and prevent the new occurrence of diabetes mellitus. Therefore, ARBs are used as the first choice for patients with complication of the heart, kidney or brain and those with diabetes mellitus. However, administration of ARBs to pregnant or breast-feeding women or to patients with severe liver damage is contraindicated, and measures such as reducing the dose are necessary if the creatinine level is ≥ 2 mg per 100 ml. ARBs must not be used in patients with bilateral renal artery stenosis or patients with one kidney and unilateral renal artery stenosis, in principle, because of the risk of a rapid decrease in

renal function. A decrease in body fluid volume and Na^+ deficiency are also quasi-contraindications. Attention to hyperkalemia is necessary while using ARBs with a potassium-sparing diuretic (JSH 2009).

β-blockers

High sympathetic tone, angina, and previous myocardial infarction are good reasons for using β-blockers. However, β-blockers therapy is associated with symptoms of depression, fatigue, and sexual dysfunction. The β-blockers also exert adverse effects on glucose and lipid metabolism when used alone or in combination with diuretics. Therefore, they are not the first choice of treatment in elderly patients or when hypertension is complicated by other diseases such as diabetes mellitus and abnormal glucose tolerance. These side-effects have to be taken into consideration in the evaluation of the benefits of treatment (Foëx 2004).

Calcium channel blockers

Calcium channel blockers produce hypotensive effects by inhibiting the L-type Ca^{2+} channel involved in the influx of extracellular Ca^{2+} ions, thus relaxing the vascular smooth muscle and reducing TPR. Calcium channel blockers can be divided into dihydropyridines (DHPs; nifedipine, nimodipine,amlodipine) and non-DHP (verapamil, diltiazem). DHPs rapidly and potently reduce ABP and show little cardioinhibitory effect while non-DHP calcium channel blockers have slower and milder hypotensive effects accompanied by a cardioinhibitory effect. Calcium channel blockers are effective in the elderly and may be selected as monotherapy for patients with Raynaud's phenomenon, peripheral vascular disease, or asthma, as such patients do not tolerate β-blockers. Diltiazem and verapamil are contraindicated in heart failure. Nifedipine is effective in severe hypertension and can be used sublingually; there is need for caution because of the risk of excessive hypotension. Calcium channel blockers are often associated with b-blockers, diuretics and/or ACE inhibitors (Foëx 2004, JSH 2009).

Diuretics

Thiazide diuretics are primarily used as antihypertensive drugs. They produce hypotensive effects by decreasing TPR in the long term, while they reduce circulating blood volume in the short term by inhibiting Na^+ reabsorption by the distal convoluted tubules. Diuretics are expected to be effective in patients with increased salt sensitivity, low renin hypertension, kidney disorders, diabetes mellitus and insulin resistance. While diuretic affect metabolism and may cause hypokalemia, impaired glucose tolerance and hyperuricemia.

Loop diuretics cause diuresis by inhibiting NaCl reabsorption in the ascending limbs of the loop of Henle. They have stronger diuretic effects but weaker hypotensive effects with a shorter duration than thiazide diuretics. As they are also effective in patients with a reduced renal function, they are used for the treatment of patients with hypertension and congestive heart failure (JSH 2009).

α-adrenergic blockers

α-blocker selectively block α_1-receptors on the smooth muscle side of the sympathetic nerve terminal. They do not inhibit suppressive α_2-receptors on the sympathetic nerve terminal side and rarely cause tachycardia, especially when they are the long-acting type. Prazosin is shorter acting than doxazosin, indoramin and terazosin. They have favorable effects on lipid metabolism, such as decrease in the total cholesterol and triglyceride levels and increase in the high-density lipoprotein-cholesterol level. They may cause dizziness, palpitation and syncope due to orthostatic hypotension (Foëx 2004, JSH 2009).

Classic vasodilators

Classic vasodilators dilate blood vessels by acting directly on the vascular smooth muscle. Hydralazine and minoxidil are directly acting vasodilators. The usage of these drugs has declined because of the potential for serious side-effects such as lupus syndrome with hydralazine and hirsutism with minoxidil. Other adverse effects are headache, palpitation, tachycardia, angina pectoris and edema. Moreover, fulminant hepatitis has been reported and hence liver disorder is a contraindication (Foëx 2004, JSH 2009).

Central adrenergic inhibitors

Methyldopa is both a false neurotransmitter and α_2-adrenoceptor agonist. Clonidine and dexmedetomidine, agonists at centrally located α_2-adrenoceptors, make the circulation more stable, reduce the release of catecholamines in response to stress and cause sedation such that dexmedetomidine is now use for sedation in intensive care units. Moxonidine is representative of a new class of antihypertensive agents acting on immidazoline$_1$ receptors (I_1). Moxonidine reduces sympathetic activity by acting on centers in RVLM, thereby reducing TPR (Foëx 2004).

Aldosterone antagonists and potassium-sparing diuretics

These drugs promote Na^+ excretion without the loss of K^+ by acting on the distal convoluted tubules and common collecting ducts. Triamterene produces a similar effect independently of aldosterone by suppressing the amiloride-sensitive epithelial Na channel. Aldosterone antagonists are used to treat the low renin hypertension and to protect the organ damage due to toxic effect of aldosterone. Clinical studies have shown that the outcome of heart failure or myocardial infarction is improved by aldosterone antagonists. Aldosterone antagonists may cause hyperkalemia when used with an RAS inhibitor or in patient with kidney dysfunction. Spironolactone has adverse effects such as erectile dysfunction, gynecomastia and menorrhalgia (JSH 2009).

1.2.7. Camboginol and morelloflavone

1.2.7.1. *Garcinia dulcis*

Garcinia dulcis Kurz, a folk medical plant that belongs to the Guttiferae family, is widely distributed Southeast Asian countries. It is known as “Maphuut” (Thailand). It has a medium-size, evergreen, much branched perennial tree, 10-13 m high, with an upright growth habit. Bark is dark brown and rough and the branches are green, sparsely hairy angled and orthotropic or horizontal and have a white latex. The leaves are opposite, simple, large, ovate, oval to oblong up to 10-30 cm long by 3-15 cm wide with acute apex and truncate base, entire margin, glossy green above and dull pale green below and often sparsely hairy, but reddishpink when young. Petioles are 1-2 cm long. Flowers are in small densely packed, axillary fascicle of 6-

12. Flowers are pentamerous with 5 sepals and 5 yellowish-green or yellowish-white petals. Male flowers are smaller, 6 mm across with 5 adelphous stamens, yellow lobed central disk and a rudimentary pistil. Female flower has thick disk lobes, 5-lobed stigma, sessile and larger than 12 mm across. The fruits are globose to oblate, 5-8 cm in diameter, smooth, green when immature tuning yellow or deep yellow when ripe. The fruit is thin-skinned with a yellow, fibrous but soft pulp and 1-5 brown seeds, each 2.5 cm long (Lim 2012).

Figure 1.21. Tree, flower and fruit of *Garcinia dulcis*.

1.2.6.2. Camboginol

1.2.6.2.1. Chemical structure of camboginol

Camboginol ($C_{38}H_{50}O_{16}$; molecular weight 602), also known as garcinol, is a plant benzophenone with a yellow crystalline compound. Rao and colleagues extracted camboginol from latex of *Garcinia cambogia* and elucidated its chemical structure in 1980. Camboginal was also extracted from the rind of the fruit of *Garcinia indica* (Yamaguchi et al. 2000 a&b) and the fruits of *Garcinia dulcis* (Deachathai et al. 2005). The C-3 ketonic group and the phenolic ring bearing hydroxyl group (-OH) are the principle oxidation sites of camboginol generating it oxidized products during metabolic transformations some of which are also biologically active (Sang et al. 2001; Sang et al. 2002). The 1, 2 carbon-carbon double bond of α, β-unsaturated ketone is an important for apoptosis-inducing

activity and cytotoxicity of camboginol (Pan et al. 2001). The isoprenyl chain of camboginol consists of hydrophobic faces which are important for its binging to biological targets (Roux et al. 2000).

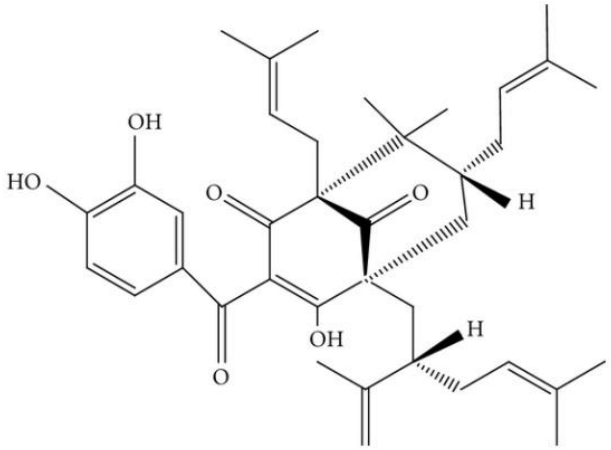

Figure 1.22. Chemical structure of camboginol or garcinol (Rao et al. 1980).

1.2.6.2.2 Biological activities of camboginol

1) Antioxidant activity

Yamaguchi et al. (2000a) reported that camboginol had a property to retard O_2^- anion to nearly the same amount as DL-alpha-tocopherol, an established anti-oxidant using a hypoxanthine/xanthine oxidase system, while its ability to quell hydroxyl radicals in the Fenton reaction system was even better than that of alpha-tocopherol. Sang et al. (2001 & 2002) proposed the antioxidant mechanism of camboginol according to which the compound reacts with peroxyl radicals by a single electron transfer followed by deprotonation of the hydroxyl group from the enolized 1, 3-diketone to form a resonance pair. Depending on the position of hydroxyl group (C-3 or C-5) which initiates the reaction, different compounds are formed.

2) Anti-inflammatory activity

Liao et al. (2004) reported that camboginol inhibits the expression of iNOS and COX-2 in lipopolysaccharide (LPS)-activated macrophages. Camboginol also lowers the LPS-induced increase of intracellular ROS which contribute to the activation of NF-κB. Hong et al. (2006) found that camboginol modulated arachidonic acid metabolism by retarding the phosphorylation of cytosolic PLA2 through the inhibition of extracellular ERK1/2 kinase activation and suppression of iNOS expression through modulation of the JAK/STAT-1 signaling pathway. When added prior to LPS, cambiginol suppressed NF-κB activation and COX-2 expression through the interruption of LPS binding to toll-like receptors. Koeberle et al. (2009) reported that camboginol selectively suppressed PGE_2 synthesis and 5-LOX product formations provides molecular basis for its anti-inflammatory and anti-carcinogenic effects.

3) Anti-cancer activity

Tanaka et al. (2000) reported that 0.01% and 0.05% dietary camboginol significantly reduced the formation of azoxymethane-induced colonic aberrant crypt foci, precursors for colon cancer, in male F344 rats in a dose-dependent manner. In addition, camboginol also increased liver glutathione S-transferase and quinone reductase levels, both detoxifying enzymes associated with cancer suppression. Yoshida et al. (2005) found that camboginol decreased COX-2 expression in tongue lesions induced with 4-nitroquinoline 1-oxide (4-NQO) in male F344 rats. Hong et al. (2006) showed that camboginol exerted a much high potency of inhibition against the colon cancer which was accompanied by increase in Caspase 3 activity, indicative of an apoptotic pathway. Camboginol also inhibited breast, prostate and pancreatic cancer cell growth by induction of apoptosis which was mediated by caspase-3 followed by down regulation of the NF-κB pathway (Ahmad et al. 2010, Ahmad et al. 2011).

4) Anti-HIV activity

Balasubramanyam et al. (2004) reported that camboginol is a potent non-specific inhibitor of histone acetyltransferases (HAT) p300 which strongly inhibited HAT activity-dependent chromatin transcription. Since, dysfunction of HAT is known to cause several diseases including cancer, neurodegenaration, asthma, diabetes, AIDS and cardiac hypertrophy and p300 protein plays a critical role in cell growth, differentiation, and death. Mantelingu et al. (2007) shown that camboginol inhibited histone acetylation of HIV infected cells and consequently inhibited the multiplication of HIV.

5) Anti-ulcer activity

Yamaguchi et al. (2000a) reported that camboginol has potent free radical scavenging activity against hydroxyl radical which stronger than that of α-Tocopherol. Since hydroxyl radical is regarded as the most damaging ROS, camboginol is expected to be useful for preventing diseases caused by the hydroxyl radical damages such as stress-induced gastric ulcer (Das et al. 1997; Das et al. 1998) and NSAID drug-induced gastric ulcers (Vaananen et al. 1991; Yoshikawa et al. 1993).

1.2.6.3. Morelloflavone

1.2.6.3.1. Chemical structure of morelloflavone

Morelloflavone, a plant biflavonoid comprising two covalently linked flavones; apigenin and luteolin, occurs in most *Garcinia* species (Verbeek et al 2004). Its chemical structure was firstly elucidated in 1976 by Ansari et al. from the leaves of *Garcinia dulcis* (Roxb.) Kurz. The configuration, substitution and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity such as radical scavenging and metal ion chelation ability. The hydroxyl configuration is the most significant determinant of scavenging of ROS because it donates hydrogen and an electron to hydroxyl, peroxyl and peroxynitrite radicals, stabilizing them and giving rise to a relatively stable flavonoids radical.

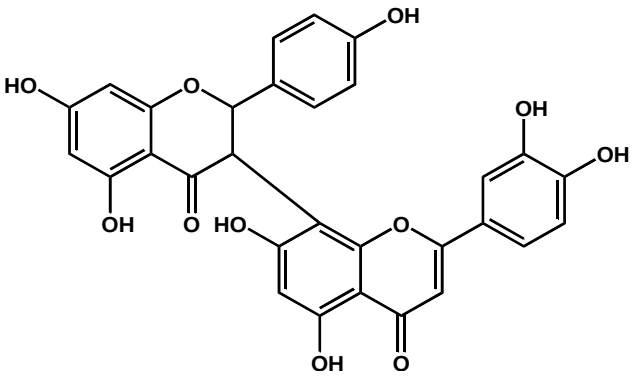

Figure 1.23. Chemical structure of morelloflavone ($C_{30}H_{20}O_{11}$, MW 556), a biflavonoid comprising two colavently linked flavones; apigenin and luteolin (Ansari et al. 1976).

1.2.6.3.2. Biological activity of morelloflavone

1) Anti-atherosclerosis activity

Pinkaew et al. (2009) reported that morelloflavone inhibited VSMC migration, invasion and lamellipodium formation in a cell culture system through activation of multiple migration-related kinases, including FAK, c-Src, ERK and RhoA in VSMCs. The inhibition of RhoA and ERK pathways is also observed in VEGF-stimulated human umbilical cord endothelial cells (Pang et al. 2009). Oral morelloflavone therapy for 8 months significantly reduced the atherosclerotic areas of the mouse aortas (a 26% reduction), without changing plasma lipid profiles or weights in $Ldlr^{-/-}$ $Apobec1^{-/-}$ mice (Pinkaew et al. 2012). The immunohistochemistry study showed that morelloflavone reduced the number of VSMC in the atherosclerotic lesion while it did not change the density of macrophages in the lesion or the percentages of proliferating and apoptotic cells (Pinkaew et al. 2012).

2) Anti-cancer activity

Pang et al. (2009) shown that morelloflavone inhibited VEGF-induced cell proliferation, migration, invasion and capillary-like tube formation of primary cultured human umbilical vascular endothelial cells in a dose-dependent fashion. Morelloflavone also inhibited tumour growth and tumour angiogenesis of prostate cancer cells in xenograft mouse tumor model, suggesting that morelloflavone

inhibited tumorigenesis by targeting angiogenesis. The antiangiogenic action of morelloflavone was established by activation of Rho-GTPases and ERK signalling pathways.

3) Anti-HIV activity

Lin et al. (1997) found that morelloflavone demonstrated significant antiviral activity by inhibiting both HIV-1 reverse transcriptase (HIV-1 RT) and HIV-1 (strain LAV-1) in phytohemagglutinin-stimulated primary human peripheral blood mononuclear cells.

4) Antioxidant and anti-inflammatory activities

Gil et al. (1997) reported that morelloflavone inhibited secretory phosphalipase A_2 (PLA_2) with a high potency on the human recombinant synovial and bee venom enzymes and its anti-inflammatory activity apparently not related to the synthesis of eicosanoids but likely dependent the scavenging of ROS. Hutadilok-Towatana et al. (2007) also found that morelloflavone exhibited strong antioxidation effects in both Fe^{2+}-mediated and non-metal induced human low-density lipoprotein (LDL) oxidations.

5) Hypocholesterolemic activity

Tuansulong et al. (2011) reported that morelloflavone show to have hypocholesterolemic activity by which affected the HMG-CoA reductase, the rate-limiting enzyme of the cholesterol biosynthetic pathway. Morelloflavone inhibited the enzyme activity by competing with the HMG-CoA whereas it was non-competitive towards NADPH.

6) Vasorelaxant activities

Lamai et al. (2013) reported that morelloflavone induce the dilatation of thoracic vessels from normotensive rat and the machanisms of action is endothelium-dependent which involved nitric oxide signaling pathway and partially K_{ATP} and $K_{Ca^{2+}}$ channels.

1.3. Objectives

1.3.1. To investigate effect of camboginol and morelloflavone on ABP, HR, renal clearance, BRS, plasma malondialdehyde (MDA) level and endothelium eNOS expression in anesthetized 2K1C hypertensive and sham operative (SO) normotensive rats.

1.3.2. To study vasorelaxant effect of camboginol and morelloflavone on isolated thoracic aortic ring from 2K1C and SO rats. The vasorelaxant mechanisms of camboginol including 1) NO generation, 2) PGI_2 synthesis, 3) activation of K_{ATP} channel and 4) activation of K_{Ca} channel were also elucidated using specific vasorelaxant inhibitors; Nω-Nitro-L-arginine methyl ester (L-NAME), indomethacin, glibenclamide and tetraethylammonium, respectively.

1.4. Hypotheses

1.4.1. Camboginol and/or morelloflavone treatment exert hypotensive and/or diuretic effect in anesthetized SO normotensive and/or 2K1C hypertensive rats. They may improve the BRS, decrease plasma MDA levels and increase eNOS expression on vascular endothelium in both or some group of rats.

1.4.2. Camboginol and/or morelloflavone treatment express vasodilatation effect in isolated aortic ring from SO and/or 2K1C rats and mechanism of their action may associated endothelial dependent vasorelaxant pathways.

CHAPTER 2
MATERIALS AND METHODS

2.1. Extraction of camboginol and morelloflavone

The extraction of camboginol and morelloflavone was performed as previously described (Deachathai et al. 2005). Briefly, the fresh ripe fruits of *G. dulcis* were collected from Songkhla province, Thailand. The voucher specimen has been deposited at the Herbarium of Prince of Songkla University, Thailand. The fruits were washed and chopped in to small pieces and then immersed in acetone (Me_2CO) for 5 days at room temperature. Acetone was removed by evaporation to give a liquid extract that was further partitioned with hexane to yield a solid extract.

To purify camboginol, the solid extract was subjected to column chromatography (CC) and eluted with a gradient of dichloromethane (CH_2Cl_2) and methanol (MeOH). The collected fractions were combined according to their thin layer chromatography (TLC) characteristics and fractionated by CC using CH_2Cl_2 and CH_2Cl_2-Me_2CO as eluents to yield camboginol (MW 602).

In case of morelloflavone, the solid was further fractionated by dissolving in CH_2Cl_2 to give CH_2Cl_2 soluble fraction. This fraction was fractionated by quick CC and was subjected to CC using CH_2Cl_2- MeOH to yield morelloflavone (MW 552).

2.2. Animals and experimental design

2.2.1. Animals

Male Wistar rats (body weight 150-200 g, n=96) were obtained from the Southern Laboratory Animal Facility (Prince of Songkla University, Thailand). Rats were housed under control conditions (temperature 23-24°C; humidity 50-55%; lighting 0600-1800 h), fed a laboratory diet containing 34.2 mmol sodium chloride/kg dry weight food (S.W.T., Thailand) and were allow free access to reverse osmosis

water. All experiments were approved by the Prince of Songkla University Animal Ethics Committee (Reference No. 31/2014).

There are two part of experiment in this study, the *in vivo* (n = 36) and *in vitro* (n =60). Each part consist of six groups of rats (n = 6, each), namely SO + Vehicle (SO), SO + camboginol (SO+C), SO + morelloflavone (SO+M), 2K1C + Vehicle (2K1C), 2K1C + camboginol (2K1C+C) and 2K1C + morelloflavone (2K1C+M).

In a part of immunohistochemistry, the animals (n=10) were purchased from the National Laboratory Animal Center (Mahidol University, Thailand). The animals were divided into 4 groups; control (n=2), 2K1C (n=2), 2K1C+C (n=3) and 2K1C+M (n=3). Rats were induced hypertension and housed under control conditions as mentioned above at the Animal Facility of Department of Zoology, Kasetsart University. Collection of thoracic aorta was performed four weeks after the induction of hypertension.

2.2.2. Establishment of 2K1C hypertensive rat

Rats were anaesthetized with a single dose of pentobarbital sodium (50 mg/kg BW i.p.). Only the deep sedated animals were gone through the following surgical protocol; the left kidney was exposed through a 1 cm retroperitoneal incision the left renal artery was then exposed and cleared from surrounding connective tissues, and a U-shaped silver clip with a 0.20 mm gap was placed around it close to the junction with the abdominal aorta. The free edges of the clip were compressed firmly to prevent dislodging. At the end of surgery, the muscle and skin layer were sutured separately with catgut and silk No. 4/0, respectively. The SO included the entire surgery with the exception of renal artery clipping. At the end of the surgery, all animals received a single dose of ampicillin (50 mg/kg BW, i.m.) injection and were allowed to recover in separate cages for 2-3 h under an angle poise lamp and remained untouched for 4 weeks afterward in order to develop hypertension.

2.3. Chemical reagents

- ABC peroxidase staining kit, Thermo Scientific, Rockford, IL, USA
- Acetic acid (CH_3COOH), Merck, Darmstadt, Germany
- Acetylcholine (ACh) chloride, Sigma Chemical Co., St. Louis, MO, USA
- Ammonium sulfate ($H_6N_2O_3S$), Fluka, Buchs, Switzerland
- Anthrone ($C_{14}H_{10}O$), Fluka, Buchs, Switzerland
- Calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$), Merck, Darmstadt, Germany
- D(+)-Glucose anhydrous ($C_6H_{12}O_6$), Fluka, Buchs, Switzerland
- Dimethylsulfoxide (DMSO), Sigma Chemical Co., St. Louis, MO, USA
- Di-sodium hydrogen phosphate anhydrous (Na_2HPO_4), Catlo Erba Reagenti, Milan Italy
- eNOS antibody, Thermo Fisher, Rockford, IL, USA
- Glibenclamide, Sigma Chemical Co., St. Louis, MO, USA
- Goat-anti-Rabbit IgG (H+L) Secondary Antibody, Biotin, Thermo Fisher, Rockford, IL, USA
- Heparin, Leo pharmaceutical, Ballerup, Denmark
- Hydrochloric acid (HCl), Merck, Darmstadt, Germany
- Indomethacin, Sigma Chemical Co., St. Louis, MO, USA
- Inulin, Sigma Chemical Co., St. Louis, MO, USA
- Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), Merck, Darmstadt, Germany
- Metal Enhanced DAB Substrate Kit, Thermo Scientific, Rockford, IL, USA
- n-Butanol ($CH_3(CH_2)_3OH$), Lab-Scan Analytical Science, Bangkok, Thailand
- N-(1-naphthyl)-ethylenediamine dihydrochloride ($C_{12}H_{16}Cl_2N_2$), Merck, Darmstadt, Germany
- Normal goat serum (NGS), Novex® by Life Technologies Corporation, Frederick, MD, USA
- Nω-Nitro-L-arginine methyl ester (L-NAME) , Sigma Chemical Co., St. Louis, MO, USA
- Para-aminohippuric acid (PAH), Sigma Chemical Co., St. Louis, MO, USA
- Pentobarbital sodium, CEVA Santé Animal, Brussels, Belgium
- Phenylephrine (PE), Sigma Chemical Co., St. Louis, MO, USA
- Potassium chloride (KCl), Merck, Darmstadt, Germany

- Sodium chloride (NaCl), Ajax Finechem, Seven Hill, New South Wales, Australia
- Sodium dodecyl sulfate (SDS, $C_{12}H_{25}NaO_4S$), Fluka, Buchs, Switzerland
- Sodium dihydrogen phosphate dehydrate ($NaH_2PO_4 \cdot 2H_2O$), Merck, Darmstadt, Germany
- Sodium hydrogen carbonate ($NaHCO_3$), Riedel-de, Seelze, Denmark
- Sodium hydroxide (NaOH), Carlo Erba Reagenti, Milan, Italy
- Sodium nitrite ($NaNO_2$), Carlo Erba Reagenti, Milan, Italy
- Sodium nitroprusside (SNP), Sigma Chemical Co., St. Louis, MO, USA
- Sulfuric acid (H_2SO_4), Merck, Darmstadt, Germany
- Tetraethyl ammonium (TEA), Sigma Chemical Co., St. Louis, MO, USA
- Thiobarbituric acid (TBA, $C_4H_4N_2O_2S$), Sigma, Deisenhofen, Germany
- Trichloroacetic acid (CCl_3COOH), Carlo Erba Reagenti, Milan, Italy

2.4. Equipment

- Automatic pipettes, Eppendorf, Geritebau, Hamburg, Germany
- Balance, Model CC023D10ADBAAA, Avery Barkel, United Kingdom
- Centrifuge, Model 4232, A.L.C., Italy
- Digital infusion syringe pump, Model SP101i, World Precision Instruments, Sarasota, Florida, USA
- Force transducer, Model FT03, Grass, USA
- Homeothermic blanket control unit, Harvard, Southnatick, Massachusetts, USA
- IEC Micro-Hematocrit centrifuge, Model MB, Needham Heights, Massachusetts, USA
- Microscope, Olympus DP 73, Shinjuku, Tokyo, Japan
- Osmometer, Fiske Model 210, USA
- pH meter, Model UB-5, Denver Instruments, Denver, USA
- Polyethylene tube, Clay Adams, Parsippany, New Jersey, USA
- PowerLab, ADInstruments, USA
- Spectrophotometer, Model Spectro SC, Labomed, Culver City, California, USA

2.5. The *in vivo* study of renal clearance and BRS

Four weeks after the operation, renal clearance and BRS were studied in four group of animals (n=6, ea). Rats were anesthetized with pentobarbital sodium (60 mg/kg BW i.p.; an additional dose was given when necessary) and placed on a thermostatically-controlled heated table to maintain body temperature at 37ºC. A tracheotomy was performed and the left carotid artery was cannulated, using polyethelene tube-50 (PE-50) filled with heparinized 0.9% NaCl, and connected to a pressure transducer, coupled to a PowerLab system, to measure ABP and HR, and for blood sampling. The right jugular vein was cannulated, using PE-50, and infused with clearance markers containing 1% inulin and 0.5% PAH dissolved in 0.9% NaCl at a rate of 1.6 mL/h/100g BW. Via a suprapubic midline incision, the left and right ureters were cannulated, using PE-10 connected to PE-50 for urine sample collection. Camboginol or morelloflavone dissolved in DMSO was given via the jugular vein as a bolus injection (0.1 mg/kg BW; volume of injection was 0.05 mL/100g BW) and then followed by continuous infusion at the rate of 5 μg/min/kg BW along with the clearance markers. This experimental dose of camboginol and morelloflavone was chosen base on the minimal effective dose of a specific AII receptor antagonist, candesartan (Hiranyachattada et al. 2005). The same amount of DMSO was given as vehicle in both SO and 2K1C groups.

After 45 min infusion, the renal clearances of particular marker and osmolar clearance were determined. Three consecutive 20 min urine collections were made during the 60 min of experimental period. An arterial blood sample (400 μL) was taken at the midpoint of the first and third urine collecting period. A small amount of the blood sample was used to determine the hematocrit by the microcapillary method, and the remainder was centrifuged and the plasma was collected for determination of the concentrations of inulin and PAH. Blood cells were resuspended in 200 μL 0.9% NaCl and returned to the animal via the right jugular vein. Urine flow rate ($\dot{V}$) was determined gravimetrically by collecting urine into pre-weighed tubes and assuming a density of 1 g/mL. The PAH and inulin concentrations, either in plasma or urine samples, were determined by a spectrophotometric method according to Smith et al. (1954) and Davidson and Sackner (1963), respectively. Plasma and urine osmolality (P_{Osm} and U_{Osm}) were

measured by using Micro-Osmometer, and then osmolar and negative free water clearance (C_{Osm} and TC_{H_2O}) were calculated.

The BRS determination was performed after the 60 min of renal clearance study by measuring HR and ABP before and after acute injection of the two vasoactive drugs, PE (a specific α_1 receptor agonist) and SNP (a NO donor) at the same doses of 1, 2, 4, 8, 16 and 32 μg/kg BW. The maximal response in MABP and HR were then determined.

At the end of experiment, a large blood sample was collected via carotid artery catheter in heparinized tube and was centrifuged at 4000 rpm for 10 min then plasma was stored at -20°C until analysis for plasma MDA level. Then, rats were killed by intravenous injection of saturated $MgSO_4$. Both kidneys were removed, decapsulated, dried on blotting paper and weighed. The heart was also removed and weighed after carefully clearing away blood vessels, fat and connective tissues. The organ weight to total body weight ratio was calculated.

2.6. Determination of plasma malondialdehyde (MDA)

The MDA content was assayed in the form of TBA reacting substances (modified from Ohkawa et al. 1979. Briefly, 0.8 mL of plasma sample, distilled water, and MDA standard was added into tubes followed by 0.2 mL of 8.1% SDS and 1.5 mL of 20% acetic acid. The mixture was mixed well and was adjusted the pH to 3.5 with 1.5 N NaOH, then 1.5 mL of 0.8% TBA was added, made up to 4.0 mL with distilled water and mixed well. The mixture was heated in 95°C water bath for 60 min and was cooled down in ice-bath. Distilled water (1.0 mL) and n-butanol (5.0 mL) were added into the mixture. After shake vigorously, the mixture was centrifuged at 4,000 rpm for 10 min. The absorbance of the upper organic layer was measured at 532 nm using spectrophotometer. The amount of TBA reactive substances was determined from standard curve generation by MDA from acid hydrolysis of 1, 1, 3, 3-tetramethoxypropane. The values of plasma MDA were expressed as μmol/L.

2.7. The *in vitro* study of thoracic aortic ring relaxation

2.7.1. Preparation of isolated thoracic aortic ring

Separate groups of 2K1C and SO rats (n=6, ea) were anesthetized with pentobarbital sodium (60 mg/kg, i.p.) and sacrificed by decapitation. The thoracic aorta was dissected and cut into four ring segments approximately 5 mm in length each (two intact- and two denuded-endothelial rings). The denuded rings were performed by mechanical removal of endothelium. The rings were mounted in 20 mL organ baths containing 37°C Krebs Henseleit solution which was composed of (mM) 118.41 NaCl, 4.6 KCl, 1.12 $MgSO_4.7H_2O$, 1.18 KH_2PO_4, 1.9 $CaCl_2$, 25.0 $NaHCO_3$ and 11.66 D-glucose. The pH of solution was maintained at 7.4 by continuous aeration with 95% O_2 and 5% CO_2. The resting tension of 1 g was set and the tension changes during the courses of experiment were recorded using force displacement transducer connected to PowerLab system. Endothelial function of aortic rings was tested by the addition of 10^{-5} M ACh into the 10^{-7} M PE precontracted rings. The 80% relaxation was accepted and considered as an intact endothelium and the disappearance of relaxation was considered denuded endothelium (Molina et al. 1992).

2.7.2. Concentration-response curve of camboginol and morelloflavone

After 45 min equilibration, aortic rings (both intact and denuded endothelium) from 2K1C and SO rats were precontracted by addition of 10^{-7} M PE. When the maximal contraction response developed, the tension was recorded. Camboginol, morelloflavone or vehicle (0.1% DMSO) was added cumulatively, allowing the final concentration to be 10^{-13}-10^{-5} M. Subsequent concentrations were added after the maximal response by the previous concentration developed and recorded.

2.7.3. Effect of specific inhibitors on vasorelaxation response of camboginol or morelloflavone

After 15 min equilibration, the intact-endothelial aortic rings from 2K1C and SO rats were incubated with each specific inhibitor for 30 min before

precontraction with 10^{-7} M PE. The doses of each inhibitor were 10^{-4} M L-NAME, 10^{-6} M indomethacin, 10^{-5} M glibenclamide and 10^{-3} M TEA. Then, camboginol (10^{-13}-10^{-5} M), morelloflavone (10^{-13}-10^{-5} M) or vehicle (0.1% DMSO) was added cumulatively after the maximal contraction was developed and 5-7 min sustained. Subsequent concentrations were added after the maximal response by the previous concentration developed and recorded.

2.8. Immunohistochemistry

One hour after camboginol or morelloflavone (0.1 mg/kg + 5 µg/min/kg) was given via jugular vein of anesthetized 2K1C rat, it was then terminated by overdose injection of pentobarbital sodium and thoracic aorta was removed, dissected and cut into segment (5 mm each). The segment was fixed in 4% buffered formaldehyde overnight and then was transferred to store in cryoprotectant solution at -20°C until analysis. The segment was embedded in Tissue Freezing Medium® and the sections of 10 µm were cut on a cryostat. The eNOS expression in aortic sections was evaluated using floating-technique immunohistochemistry. The sections were washed by allowing the section to swirl in the immunobuffer (IB; 0.1 M Tris phosphate buffer solution (TPBS) + 0.3% Triton-X) for 10 min (3 times). These sections were transferred to incubate in 3% peroxidase blocking solution for 15 minutes with gentle agitation and were washed with IB for 10 min (3 times). The sections were blocked with 10% NGS for 30 minutes and then were incubated with rabbit anti-eNOS antibody (dilute 1:500, Lot # QJ214409) at 4°C for 24 h on the shaker. The sections were washed with TPBS for 10 min (3 times). Next, sections were incubated with biotinylated goat anti-rabbit IgG (dilute 1:2000, Lot # QE215187) for 1 h at room temperature and were protected from light. The sections were washed with TPBS for 10 min (3 times), and thereafter the sections were stained using ABC peroxidase staining kit and metal enhanced DAB substrate kit, respectively, and ensured these sections were covered from light. Finally, the sections were mounted onto slide, allowed to dry, protected from light, and covered slip section with Per Mount. Sections were digitized using an Olympus DP 73 microscope.

2.9. Calculations

2.9.1. Mean arterial blood pressure (MABP)

MABP is calculated by the following equation.

$$MABP = DBP + \frac{1}{3}(SBP-DBP) = DBP + \frac{1}{3}PP$$

When MABP = Mean arterial blood pressure (mm Hg)

DBP = Diastolic blood pressure (mm Hg)

SBP = Systolic blood pressure (mm Hg)

PP = Pulse pressure (mm Hg)

2.9.2. Baroreflex sensitivity (BRS)

BRS is calculated by the following equation.

$$BRS = \frac{\Delta HR}{\Delta MABP}$$

When ΔHR = Changed heart rate (bpm)

$\Delta MABP$ = Changed mean arterial blood pressure (mm Hg)

2.9.3. Clearance of PAH (C_{PAH})

C_{PAH} was used to estimate renal plasma flow (RPF). Theoretically, if the substance X is completely cleared from the plasma the amount of substance X delivered to kidneys in blood (RPF x P_X) were equaled to the amount extracted in urine (U_X x $\dot{V}$) and therefore the clearance rate of that substance is equal to the RPF (Guyton and Hall, 2006). However, no one known substance completely cleared from plasma. PAH is about 90% cleared from the plasma via either glomerular filtration of tubular secretion. Therefore, the clearance of PAH can be used as an approximation of RPF. The reasons that PAH is not completely cleared from the plasma may result from the fact that some blood flows through areas of the kidney that do not remove

PAH (e.g. renal capsule, renal pelvis, and perirenal fat), the circulation in some areas such as the outer medulla does not come into intact with the tubular epithelium and some tubular epithelium does not remove PAH completely. Therefore, the C_{PAH} is measure from the flow in areas that effective removes PAH from plasma, thus determined is called the effective renal plasma flow (ERPF) (Valtin and Schafer, 1995). PAH clearance is calculated according to the following equation.

$$C_{PAH} = ERPF = \frac{U_{PAH} \times \dot{V}}{P_{PAH}}$$

ERPF was estimated by C_{PAH} and is not exact RPF. However, RPF can be calculated from the knowledge that only about 90% of PAH is cleared from the plasma. It is refer that ERPF is lower than RPF by 10%, and therefore RPF can calculated by the following equation.

$$RPF = ERPF + 0.1ERPF$$

When RPF = Renal plasma flow (mL/min)

ERPF = Effective renal plasma flow (mL/min)

2.9.4. Ranal vascular resistance (RVR)

RVR is calculated by following equation.

$$RVR = \frac{MABP}{ERPF}$$

When RVR = Renal vascular resistance (mm Hg/mL/min)

MABP = Mean arterial blood pressure (mm Hg)

EPPF = Effective renal plasma flow (mL/min)

2.9.5. Clearance of inulin (C_{in})

C_{in} is used to estimate the GFR. Inulin is a substance that freely filtrated via glomerulus and is not reabsorbed or secreted by renal tubules. Therefore, the rate of inulin excreted in urine (U_{in} x $\dot{V}$) is equal to the filtration rate of inulin by kidney (GFR x P_{in}) and thus, GFR is equal to C_{in} (Guyton and Hall, 2006) as shown in below equation.

$$C_{in} = GFR = \frac{U_{in} \times \dot{V}}{P_{in}}$$

When C_{in} = clearance of inulin (mL/min)

GFR = Glomerular filtration rate (mL/min)

U_{in} = concentration of inulin in urine (mg%)

P_{in} = concentration of inulin in plasma (mg%)

$\dot{V}$ = urine flow rate (mL/min)

2.9.6. Urine flow rate ($\dot{V}$)

$\dot{V}$ was determined gravimetrically by collecting urine into pre-weighed tube and assuming a density of 1 g/mL. The $\dot{V}$ is calculated by the following equation.

$$\dot{V} = \frac{\text{Weight of urine in x minute}}{\text{x minute}}$$

When $\dot{V}$ = Urine flow rate (mL/min)

2.9.7. Filtration fraction (FF)

FF is the ratio of the GRF to the RPF. The FF represents the proportion of the fluid reaching the kidneys which passes into the renal tubules. The FF is calculated by the following equation.

$$FF = \frac{GFR}{RPF}$$

When FF = Filtration fraction

GFR = Glomerular filtration rate (mL/min)

RPF = Renal plasma flow (mL/min)

2.9.8. Osmolar clearance (C_{Osm})

C_{Osm} is the theoretical volume if plasma per unit time that the kidney has to filter in order to extract all the solutes out of that volume of plasma. The C_{Osm} is calculated by following equation.

$$C_{Osm} = \frac{U_{Osm} \times \dot{V}}{P_{Osm}}$$

When C_{Osm} = Osmolar clearance (mL/min)

U_{in} = urine osmolarity (mOsm)

P_{in} = Plasma osmolarity (mOsm)

$\dot{V}$ = urine flow rate (mL/min)

Moreover, fractional of salt excretion is also calculated by the following equation.

$$\text{Fractional of salt excretion} = \frac{C_{Osm}}{GFR}$$

When F_{Osm} = Fractional of osmolality

C_{Osm} = Osmolar clearance (mL/min)

GFR = Glomerular filtration rate (mL/min)

2.9.9. Free water clearance (C_{H_2O})

C_{H_2O} is the theoretical volume of plasma that the kidney has to filter in a certain amount of time in order to extract all the free water from that volume of plasma. The C_{H_2O} is calculated by following equation.

$$C_{H_2O} = \dot{V} - C_{Osm}$$

When C_{Osm} = Osmolar clearance (mL/min)

C_{H_2O} = Free water clearance (mL/min)

$\dot{V}$ = urine flow rate (mL/min)

2.9.10. Contraction responses of thoracic aorta

Contraction response of thoracic aortic ring to PE is calculated by the following equation.

$$\% \text{ Contraction to PE} = \left(\frac{T_{PE} - T_r}{T_r}\right) \times 100$$

When T_{PE} = Tension in response to PE (g)

T_r = resting tension (g)

2.9.11. Relaxation responses of thoracic aorta

Relaxation response of PE-preconstricted thoracic aortic ring to either camboginol (C), morelloflavone (M), vehicle (DMSO), acethylcholine (ACh), or sodium nitro prusside (SNP) is calculated by the following equation.

$$\%\text{Relaxation to x} = \frac{(T_{PE} - T_r) - (T_{PE} - T_x)}{T_{PE} - T_r} \times 100$$

When T_{PE} = Tension in response to PE (g)

T_r = resting tension (g)

T_x = Tension in response to X (g)

2.10. Statistical analyses

For *in vivo* study, all data were expressed as the mean±S.E.M. Comparison between the mean values were performed with one-way analysis of variance (ANOVA) (GraphPad Prism 5, San Diego, CA, USA) followed by Student-Newman Keuls post hoc test or Student t-test. A $p < 0.05$ was considered to be statistically significant difference.

For *in vitro* study, the degree of vasorelaxation in each experiment were expressed as a %relaxation from PE (10^{-7} M) precontraction tension. All values were expressed as mean±S.E.M. Significant difference between the group means was determined using ANOVA followed by Student-Newman Keuls post hoc test or Student t-test. The negative logarithm (pD_2) and half maximal effective concentration (EC_{50}) values were calculated using GraphPad Prism 5. Statistical significance of the mean differences were accepted when $p < 0.05$.

CHAPTER 3
RESULTS

3.1. Effects of camboginol

3.1.1. Effects of camboginol on ABP and HR

As shown in Figure 3.1 and Figure 3.2, four weeks after experimental renal stenosis, the basal SBP, DBP, PP and MABP in 2K1C rats were significantly higher than in those of SO rats (SBP; 208±8 vs. 155±4, DBP; 143±4 vs. 123±4, PP; 64±7 vs. 32±3 and MABP; 165±5 vs. 134±4 mm Hg, respectively, $p < 0.05$). The HR of 2K1C rats was not different from SO rats (212±5 vs. 210±5 bpm). The data suggested the successful development of 2K1C hypertensive model.

When 0.1 mg/kg BW camboginol was injected in 2K1C rats, the SBP, DBP, MABP and HR decreased significantly when compared to vehicle injection (SBP; 118±9 vs. 208±9, DBP; 62±5 vs. 144±5, MABP; 81±4 vs. 165±5 mm Hg and HR; 178± 3 vs. 212 ± 5 bpm, respectively, $p < 0.05$, Figure 3.2). These data suggested that camboginol possess an acute bradycardic and hypotensive effect in 2K1C rats.

The bolus injection of 0.1 mg/kg BW camboginol in SO rats also showed a significantly decrease in SBP, DBP, MABP and HR in comparison to the vehicle injection (SBP; 104±3 vs. 153±4, DBP; 51±4 vs. 120±3, MABP; 69±3 vs.131±3 mm Hg and HR 154± 9 vs. 210 ± 5 bpm, respectively, $p < 0.05$, Figure 3.2). These data suggested that camboginol also possess an acute bradycardic and hypotensive effect in SO rats.

However, the MABP between camboginol- (0.1 mg/kg BW + 5 μg/min/kg BW) and vehicle-treated 2K1C rats during the renal clearance study did not different (152±4 vs. 152±2 mm Hg, respectively; Figure 3.3a).

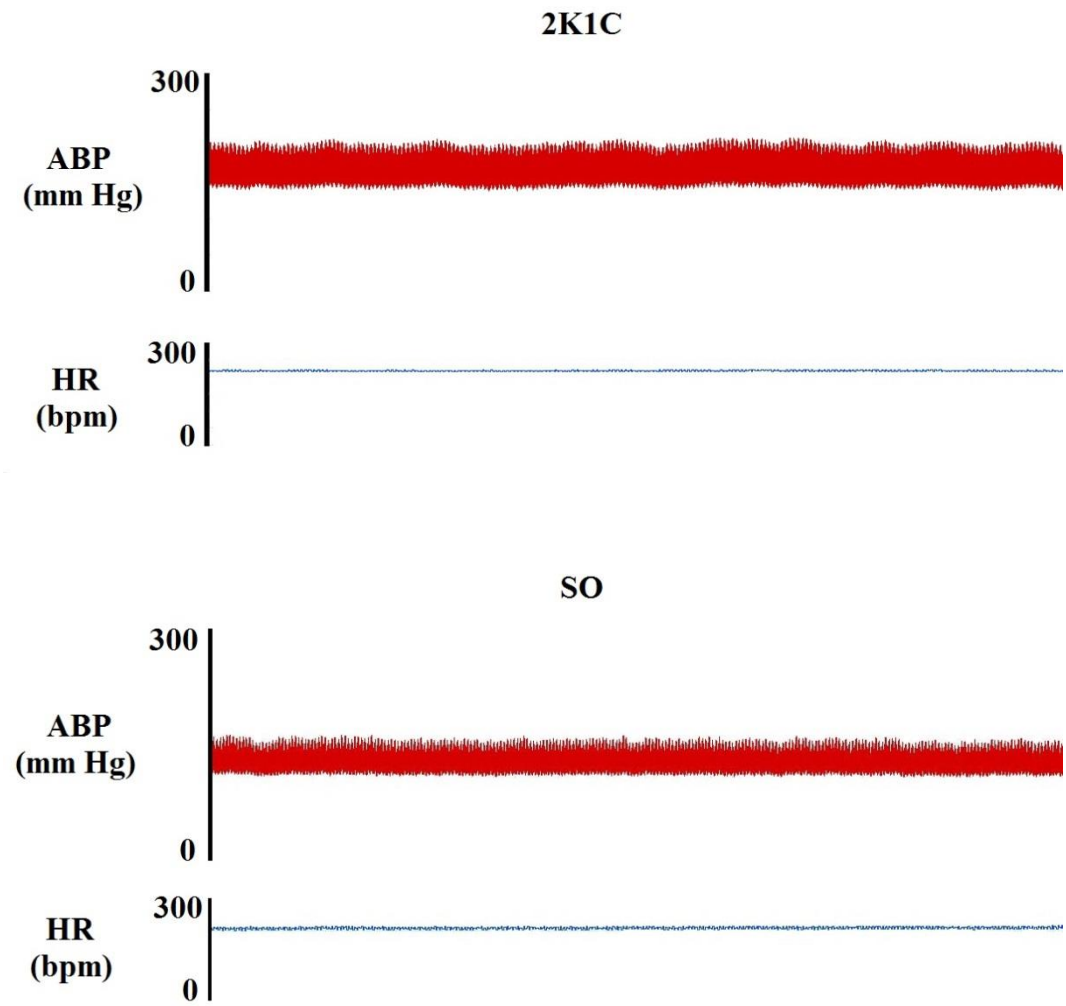

Figure 3.1 Tracing of recorded arterial blood pressure (ABP, red) and heart rate (HR, blue) of 2-kidneys-1-clip (2K1C; upper panel) and sham operative (SO; lower panel) rat.

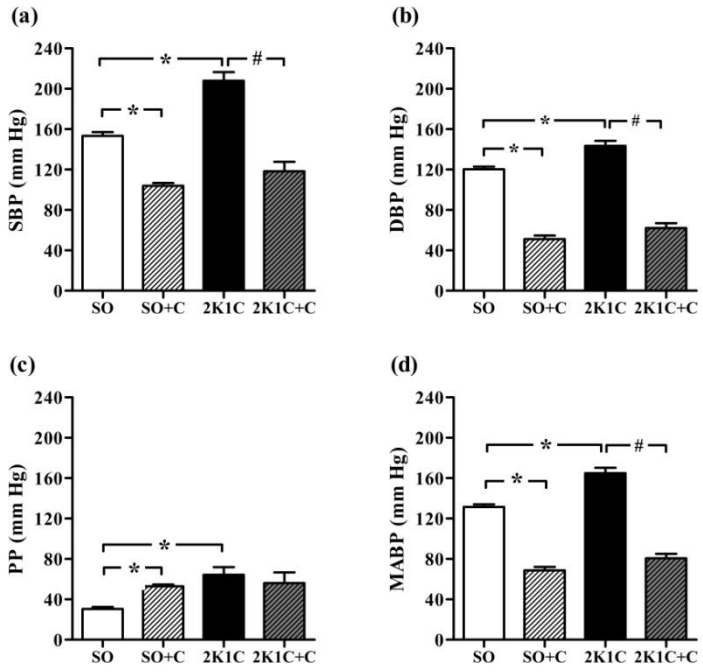

Figure 3.2. Effects of camboginol (C; 0.1 mg/kg BW) on systolic blood pressure (SBP; a), diastolic blood pressure (DBP; b), pulse pressure (PP; c) and mean arterial blood pressure (MABP; d) in the 2-kidneys-1-clip (2K1C) and sham operation (SO) groups.

Data are mean ± S.E.M. $^{*,\#}$ $p < 0.05$ compared with SO and 2K1C group, respectively (one-way ANOVA with Newman-Keuls post hoc test).

3.1.2. Effects of camboginol on renal clearance

As shown in Figure 3.3 and Table suppl. 1, 2K1C rats had significantly higher RVR, FF, $\dot{V}$, C_{Osm}, C_{Osm}/GFR and TC_{H_2O} when compared to SO rats (RVR; 70±6 vs. 39±4 RU, FF; 0.63±0.10 vs. 0.36±0.02, $\dot{V}$; 11.5±0.7 vs. 8.8±1.0 µL/min/g KW, C_{Osm}; 75.6±6.2 vs. 51.1±4.7 µL/min/g KW, C_{Osm}/GFR; 6.25±0.93 vs. 3.79±0.48% and TC_{H_2O}; 56.2±3.5 vs. 41.0±4.1 µL/min/g KW, respectively, $p < 0.05$) but had a significantly lower ERPF (2.29±0.25 vs. 3.63±0.36 mL/min/g KW, $p < 0.05$). However, there was no significant difference of GFR, U_{Osm} and P_{Osm} between 2K1C and SO rats (GFR; 1.52±0.20 vs. 1.43±0.12 mL/min/g KW, U_{Osm}; 1240±49 vs. 1425±46 mOsm/kg H_2O and P_{Osm}; 330±4 vs. 326±3 mOsm/kg H_2O, respectively).

When camboginol (0.1mg/kg + 5 µg/min/kg BW) was given in 2K1C rats, the RVR, U_{Osm} and P_{Osm} significantly decreased in comparison to vehicle-treated 2K1C (RVR; 41±3 vs. 70±6 RU, U_{Osm}; 558±30 vs. 1240±49 mOsm/kg H_2O and P_{Osm}; 310±2 vs. 330±4 mOsm/kg H_2O, respectively, $p < 0.05$). However, the ERPF, GFR, $\dot{V}$, C_{Osm}, C_{Osm}/GFR and TC_{H_2O} were found to be significantly higher (ERPF; 3.88±0.37 vs. 2.29±0.25 mL/min/g KW, GFR; 2.03±0.05 vs. 1.52±0.20 mL/min/g KW; $\dot{V}$; 68.1±5.1 vs. 11.5±0.7 µL/min/g KW, C_{Osm}; 154.0±6.3 vs. 75.6±6.2 µL/min/g KW, C_{Osm}/GFR: 9.28±0.48 vs. 6.25±0.93% and TC_{H_2O}; 65.9±2.2 vs. 56.2±3.5 µL/min/g KW, respectively, $p < 0.05$).

The administration of camboginol (0.1mg/kg + 5 µg/min/kg BW) in SO rats resulting in a significantlt increased in GFR, FF, $\dot{V}$ and C_{Osm} (GFR; 2.14±0.07 vs. 1.43±0.12 mL/min/g KW, FF; 0.55±0.05 vs. 0.36±0.02, $\dot{V}$; 42.5±5.4 vs. 8.8±1.0 µL/min/g KW and C_{Osm}; 78.3±7.5 vs. 51.1±4.7 µL/min/g KW, respectively, $p < 0.05$) but a significantly decreased in U_{Osm} and P_{Osm} (U_{Osm}; 593±29 vs. 1425±46 mOsm/kg H_2O and P_{Osm}; 311±3 vs. 326±3 mOsm/kg H_2O, $p < 0.05$) when compared to vehicle-treated SO rats.

The data suggested that the decreased RBF of 2K1C rats was due to the renal vasoconstriction. Renal tubular water and electrolytes excretion of 2K1C rats were also impaired. Camboginol treatment can corrected this impairment of both RBF and renal electrolytes excretion in 2K1C rats. The diuretic effect of camboginol was also observed in SO rats.

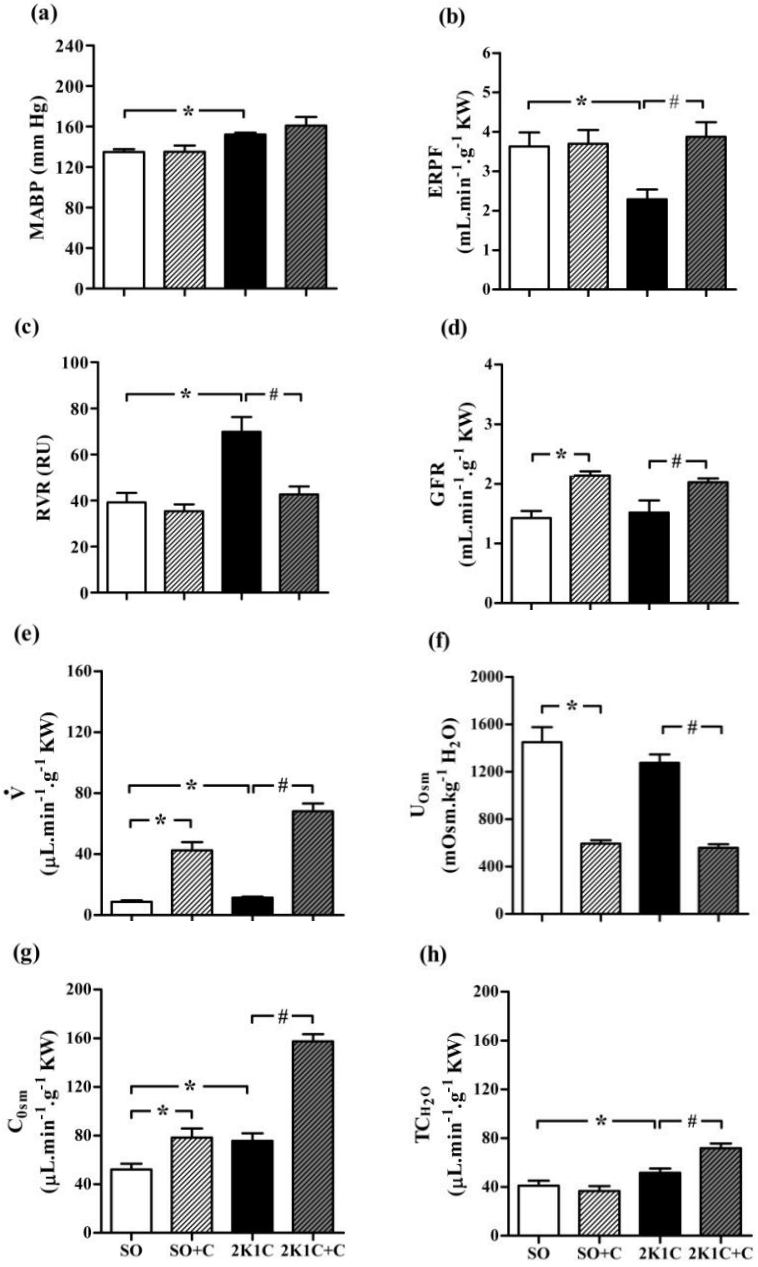

Figure 3.3. Effects of camboginol (C; 0.1mg/kg + 5 µg/min/kg BW) on mean arterial blood pressure (MABP; a), effective renal plasma flow (ERPF; b), renal vascular resistance (RVR; c), glomerular filtration rate (GFR; d), urine flow rate ($\dot{V}$; e), urine osmolality (U_{Osm}; f), osmolar clearance (C_{Osm}; g) and negative free water clearance (TC_{H_2O}; h), in 2-kidneys-1-clip (2K1C) and sham operative (SO) group during clearance study.

Values are mean ± S.E.M. $^{*, \#}$ $p < 0.05$ compared with SO and 2K1C group, respectively (one-way ANOVA with Newman-Keuls post hoc test).

3.1.3. Effects of camboginol on BRS

As shown in Figure 3.4a, c and e, the BRS responses to the six doses of PE (1, 2, 4, 8, 16 and 32 μg/kg BW) intravenous injection in 2K1C were significantly lowered when compared to those of SO rats (-0.11±0.04 vs. -0.61±0.13, -0.16±0.04 vs. -0.55±0.10, -0.22±0.05 vs. -0.60±0.08, 0.21±0.03 vs. -0.63±0.05, -0.20±0.03 vs. 0.78±0.08 and -0.23±0.04 vs. -1.03±0.09 bpm/mm Hg, respectively, $p < 0.05$). There was no significantly difference in respective BRS to PE between camboginol- and vehicle-treated SO rats. Camboginol treatment resulting in a significantly higher BRS in 2K1C compared with vehicle-treated rats at the respective doses of PE injection (-0.40±0.04 vs. -0.11±0.04, -0.41±0.07 vs. -0.16±0.04, -0.49±0.04 vs. -0.22±0.05, -0.71±0.17 vs. 0.21±0.03, -1.26±0.14 vs. -0.20±0.03 and -1.29±0.21 vs. -0.23±0.04 bpm/mm Hg, respectively, $p < 0.05$). The data suggested that the BRS were blunted in 2K1C rats and camboginol treatment can restore this BRS impairment.

As shown in Figure 3.4b, d and f, the BRS responses to the six doses of SNP (1, 2, 4, 8, 16 and 32 μg/kg BW) were significantly lowered when compared to those of SO rats (-0.04±0.02 vs. -0.32±0.05, -0.10±0.04 vs. -0.34±0.08, -0.09±0.04 vs. -0.28±0.04, -0.11±0.03 vs. -0.28±0.03, -0.10±0.02 vs. -0.29±0.04 and -0.12±0.02 vs. -0.34±0.06 bpm/mm Hg, respectively, $p < 0.05$). There was no significantly difference in respective BRS to SNP between camboginol- and vehicle-treated SO rats. Camboginol treatment resulting in a higher significantly BRS in 2K1C compared with vehicle-treated rats at respective doses of SNP (-0.22±0.03 vs. -0.04±0.02, -0.36±0.04 vs. -0.10±0.04, -0.36±0.03 vs. -0.09±0.04, -0.39±0.03 vs. -0.11±0.03, -0.42±0.03 vs. -0.10±0.02 and -0.43±0.02 vs. -0.12±0.02 bpm/mm Hg, respectively, $p < 0.05$). The data suggested that camboginol treatment had no effect on BRS in normotensive rat.

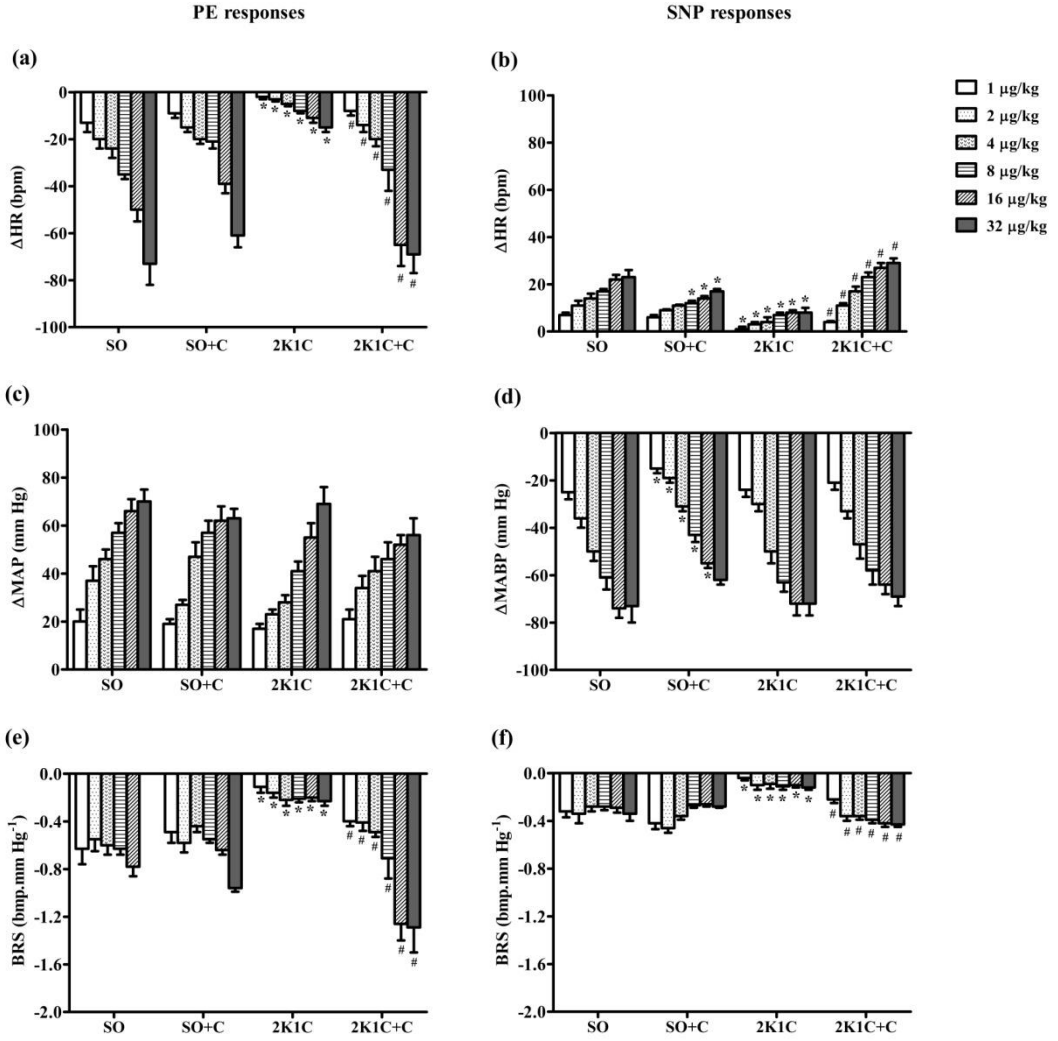

Figure 3.4. Baroreflex sensitivity (BRS) in response to either phenylephrine (PE; left panel) or sodium nitroprusside (SNP; right panel) in 2-kidneys-1-clip (2K1C) and sham operation (SO) group during treatment with camboginol (C; 0.1 mg/kg + 5 µg/min/kg BW). BRS = ΔHR/ΔMABP.

Data are mean ± S.E.M. $^{*, \#}$ $p < 0.05$ compared with the SO and 2K1C group at the respective concentrations of PE or SNP, respectively (one-way ANOVA with Newman-Keuls post hoc test).

3.1.4. Effect of camboginol on plasma MDA levels

As shown in Figure 3.5, levels of plasma MDA in 2K1C rats were significantly higher than SO rats (64.3±8.9 vs. 30.0±2.5 μmol/L, $p < 0.05$, respectively). Camboginol treatment significantly lower the plasma MDA level in 2K1C rats compared with vehicle-treated 2K1C rats (31.3±11.4 vs. 64.3±8.9 μmol/L, $p < 0.05$, respectively). The data suggested that oxidative stress status was observed in 2K1C rats and camboginol exert *in vivo* free radical scavenging activity.

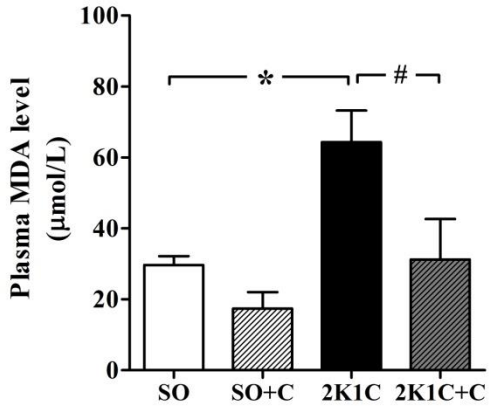

Figure 3.5. Effect of camboginol (0.1 mg/kg + 5 μg/min/kg BW) on plasma malondialdehyde (MDA) levels in 2-kidneys-1-clip (2K1C) hypertensive and sham operative (SO) normotensive rats.
Data are mean ± S.E.M. $^{*,\#}$ $p < 0.05$ compared with SO and 2K1C group, respectively (one-way ANOVA with Newman-Keuls post hoc test).

3.1.5. Body weight, kidney weight and cardiac mass

As shown in Table 3.1, the body weight changed between SO and 2K1C rats after four week of experimental renal stenosis were not significantly different. The left clipped and the right non-clipped kidney weight of 2K1C rat decreased by 73% and increased by 24% when compared to the respective ipsilateral kidneys of SO rat. The left atrophied kidney was due to the reduction in renal blood flow while the right hypertrophied kidney was due to functional compensation. The cardiac mass of 2K1C groups significantly increased in comparison to those SO groups. This finding suggested the increased afterload in this rat model. There was no significantly different in hematocrit between these rats.

Table 3.1. Comparison of body weight at the beginning of experiment (Pre-BW), 4 weeks after induction of hypertension by experimental renal stenosis (Post-BW) and body weight changes (△BW). Left and right kidney weight (KW), cardiac mass and hematocrit of 2-kidneys-1-clip (2K1C) and sham operation (SO) rats were determined and compared after either camboginol (C) (0.1 mg/kg + 5 µg/min/kg BW) or vehicle (V or DMSO) treatment.

Parameters	SO+V	SO+C	2K1C+V	2K1C+C
Number of rats	6	6	6	6
Pre-BW (g)	206 ± 4	186 ± 14	194 ± 9	183 ± 14
Post-BW (g)	388 ± 3	350 ± 17	379 ± 20	349 ± 10
△BW (g)	182 ± 6	160 ± 21	185 ± 19	161 ± 22
Left KW (g)	1.05 ± 0.02	0.96 ± 0.03	0.28 ± 0.07*	0.24 ± 0.03*
Left KW/BW (%)	0.27 ± 0.01	0.28 ± 0.01	0.07 ± 0.01*	0.08 ± 0.01*
Right KW (g)	1.18 ± 0.02	0.98 ± 0.03	1.46 ± 0.07*	1.30 ± 0.10*
Right KW/BW (%)	0.30 ± 0.01	0.28 ± 0.01	0.39 ± 0.02*	0.36 ± 0.02*
Cardiac mass (g)	1.06 ± 0.02	1.01 ± 0.01	1.23 ± 0.12*	1.17 ± 0.03*
Cardiac mass/BW (%)	0.27 ± 0.01	0.29 ± 0.01	0.32 ± 0.02*	0.34 ± 0.01*
Hematocrit (%)	49.2 ± 0.9	51.7 ± 0.6	51.6 ± 0.8	49.8 ± 0.8

Data are mean ± S.E.M.

*p value < 0.05 compared with respective SO groups (Student t-test).

3.1.6. Effect of camboginol on isolated aortic relaxation

3.1.6.1. Vasorelaxant effect of camboginol

As shown in Figure 3.6a-f, the addition of the cumulative doses of camboginol (10^{-13}-10^{-5} M) significantly relaxed the PE-preconstracted endothelium-intact aortic rings in a concentration-dependent manner with the pD_2 = 8.01 ± 0.66 (EC_{50} = 9.77 nM) of 2K1C and the pD_2 = 9.67 ± 0.19 (EC_{50} = 0.21 nM) of SO rats ($p < 0.05$). Denudation of the functional endothelium completely abolished camboginol-induced vasorelaxation in both 2K1C and SO rats. These data indicated the vasorelaxation effect of camboginol involved in endothelium-dependent mechanism.

3.1.6.2. Effect of L-NAME on camboginol-induced vasorelaxation

As shown in Figure 3.7a-f, pretreatment of the intact endothelium aortic rings with 10^{-4} M L-NAME significantly and completely abolished camboginol-induced vasorelaxation in both SO and 2K1C rats. Pretreatment of L-NAME had no effect on the endothelium-denuded aortic rings from SO and 2K1C rats. The data suggested that vasorelaxation effect of camboginol might involve in endothelial NO signaling pathway.

3.1.6.3. Effect of indomethacin on camboginol-induced vasorelaxation

As shown in Figure 3.8a-f, the %relaxation of intact endothelium aortic rings from SO rats in response to 10^{-11}-10^{-5} M camboginol in the presence of 10^{-6} M indomethacin were significantly decreased in comparison to in the absence of inhibitor but this inhibition effect did not observe in endothelium-denuded rings. Pretreatment of indomethacin in endothelium-intact and -denuded aortic rings from 2K1C rats did not change the %relaxation response to camboginol. The data suggested that the vasorelaxation effect of camboginol might involve PGI_2 signaling pathway and this mechanism might be impaired in 2K1C rats.

3.1.6.4. Effect of glibenclamide on camboginol-induced vasorelaxation

As shown in Figure 3.9a-f, the %relaxation of endothelium-intact aortic rings from 2K1C and SO rats in response to 10^{-11}-10^{-5} M camboginol in the presence of 10^{-5} M glibencamide were significantly decreased in comparison to in the absence of inhibitor. Pretreatment of glibendlamide in endothelium-denuded aortic rings from 2K1C and SO rats did not change the %relaxation response to camboginol. The data suggested that the vasorelaxation effect of camboginol in 2K1C and SO rats might involve EDHF-activated K_{ATP} channel signaling pathway.

3.1.6.5. Effect of tetraethyl ammoniun on camboginol-induced vasorelaxation

As shown in Figure 3.10a-f, pretreatment of the endothelium-intact and -denuded aortic rings from 2K1C and SO rats with 10^{-3} M tetraethyl ammonium did not affect the %relaxation response to camboginol. The data suggested that the vasorelaxation effect of camboginol did not involve K_{Ca} channel signaling pathway.

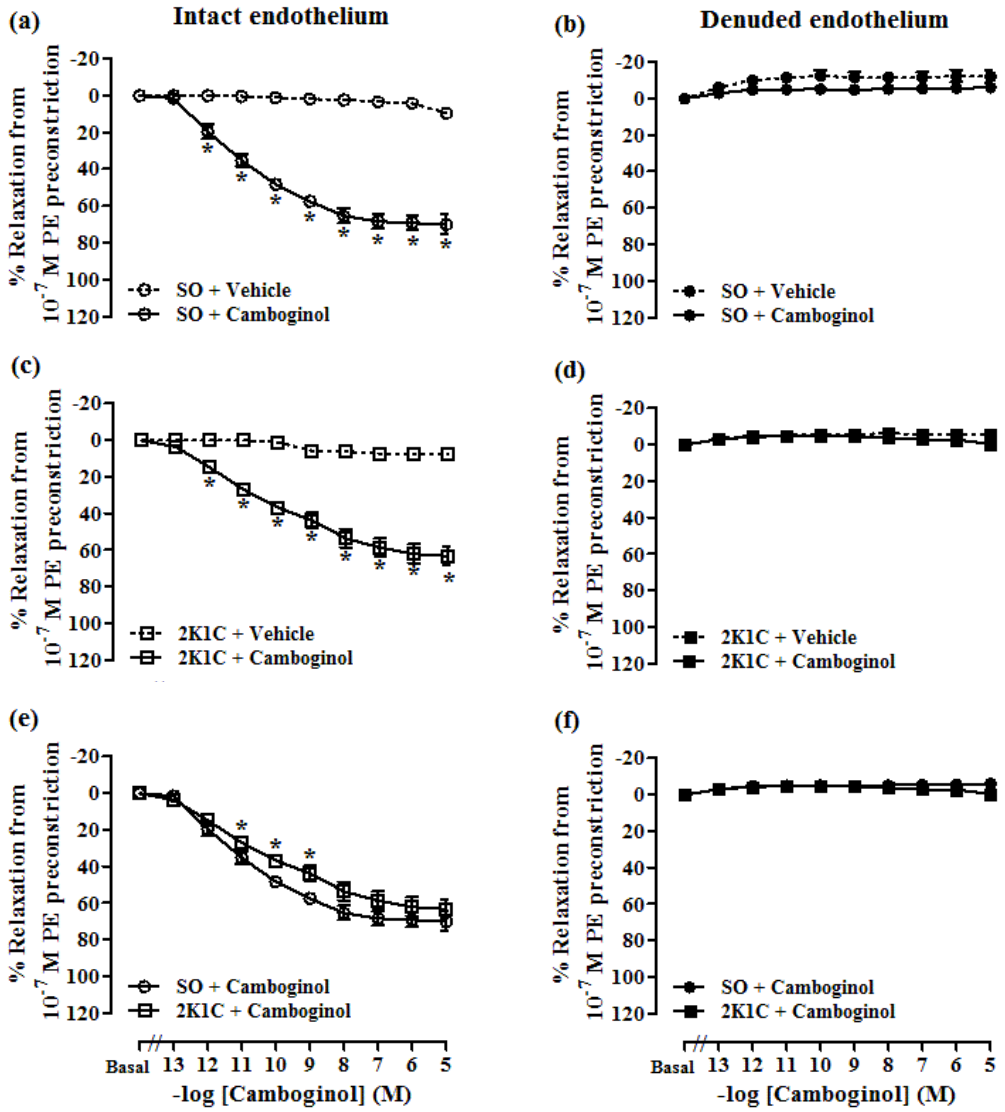

Figure 3.6. Effects of camboginol or vehicle (DMSO) on vasorelaxation of endothelium-intact (left panel; a, c and e) or -denuded (right panel; b, d and f) aortic rings from 2-kidneys-1-clip (2K1C) or sham operative (SO) group.
Values are mean ± S.E.M of the %relaxation from 10^{-7} M phenylephrine (PE) precontraction. * $p < 0.05$ compared with respective control group (one-way ANOVA with Newman-Keuls post hoc test).

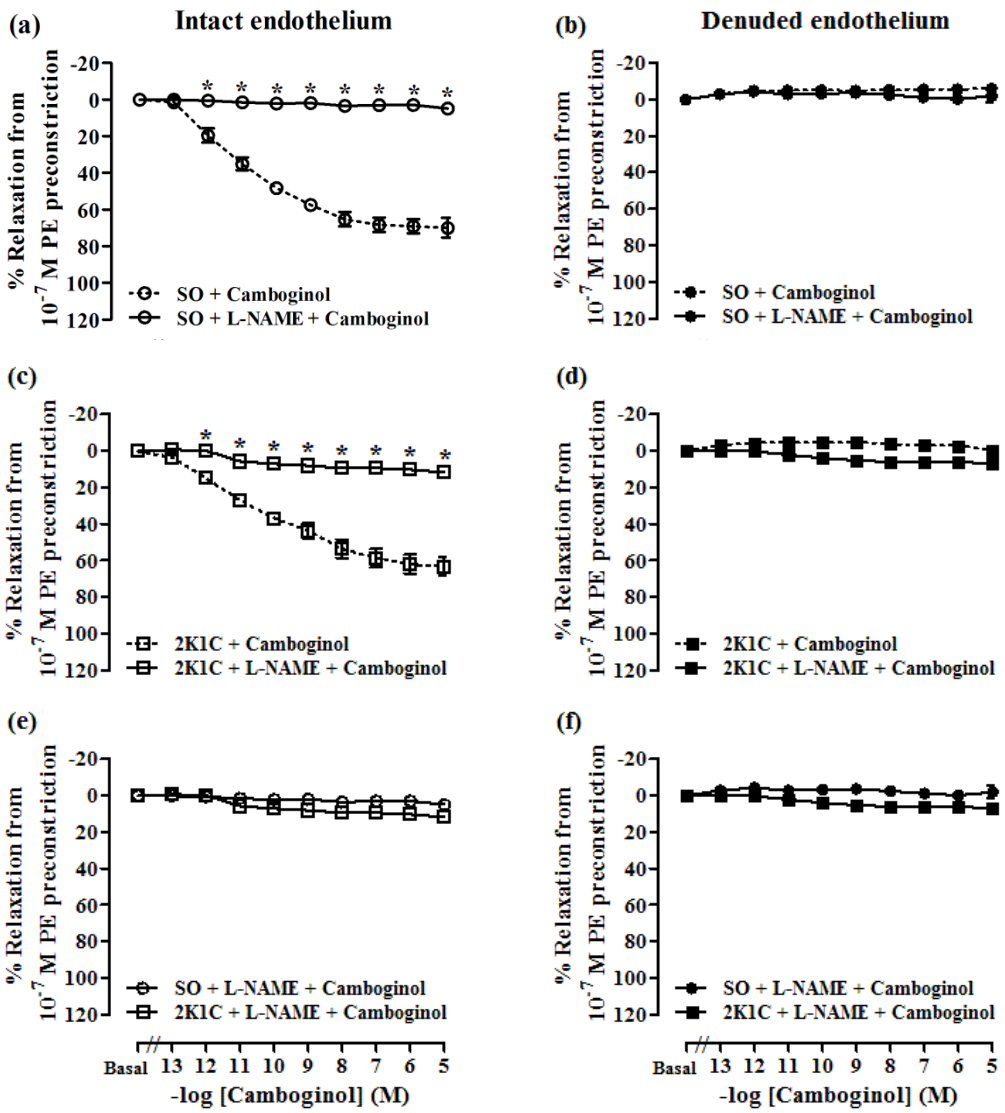

Figure 3.7. Effects of camboginol on vasorelaxation of endothelium-intact (left panel; a, c and e) or -denuded (right panel; b, d and f) aortic rings from 2-kidneys-1-clip (2K1C) or sham operative (SO) group in the presence of 10^{-4} M L-NAME. Values are mean ± S.E.M of the %relaxation from 10^{-7} M phenylephrine (PE) precontraction. * $p < 0.05$ compared with respective control group (one-way ANOVA with Newman-Keuls post hoc test).

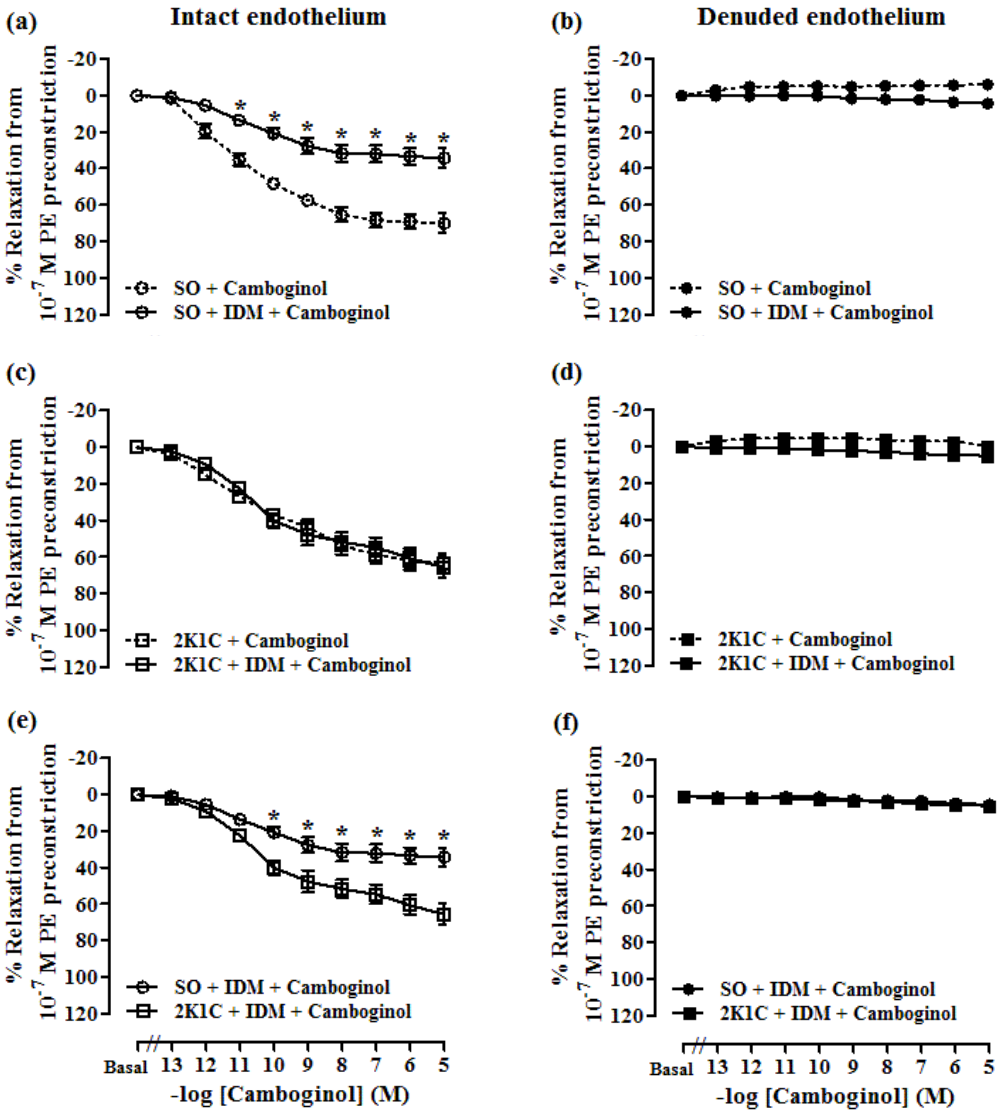

Figure 3.8. Effects of camboginol on vasorelaxation of endothelium-intact (left panel; a, c and e) or -denuded (right panel; b, d and f) aortic rings from 2-kidneys-1-clip (2K1C) or sham operative (SO) group in the presence of 10^{-6} M indomethacin (IDM).

Values are mean ± S.E.M of the %relaxation from 10^{-7} M phenylephrine (PE) precontraction. * $p < 0.05$ compared with respective control group (one-way ANOVA with Newman-Keuls post hoc test).

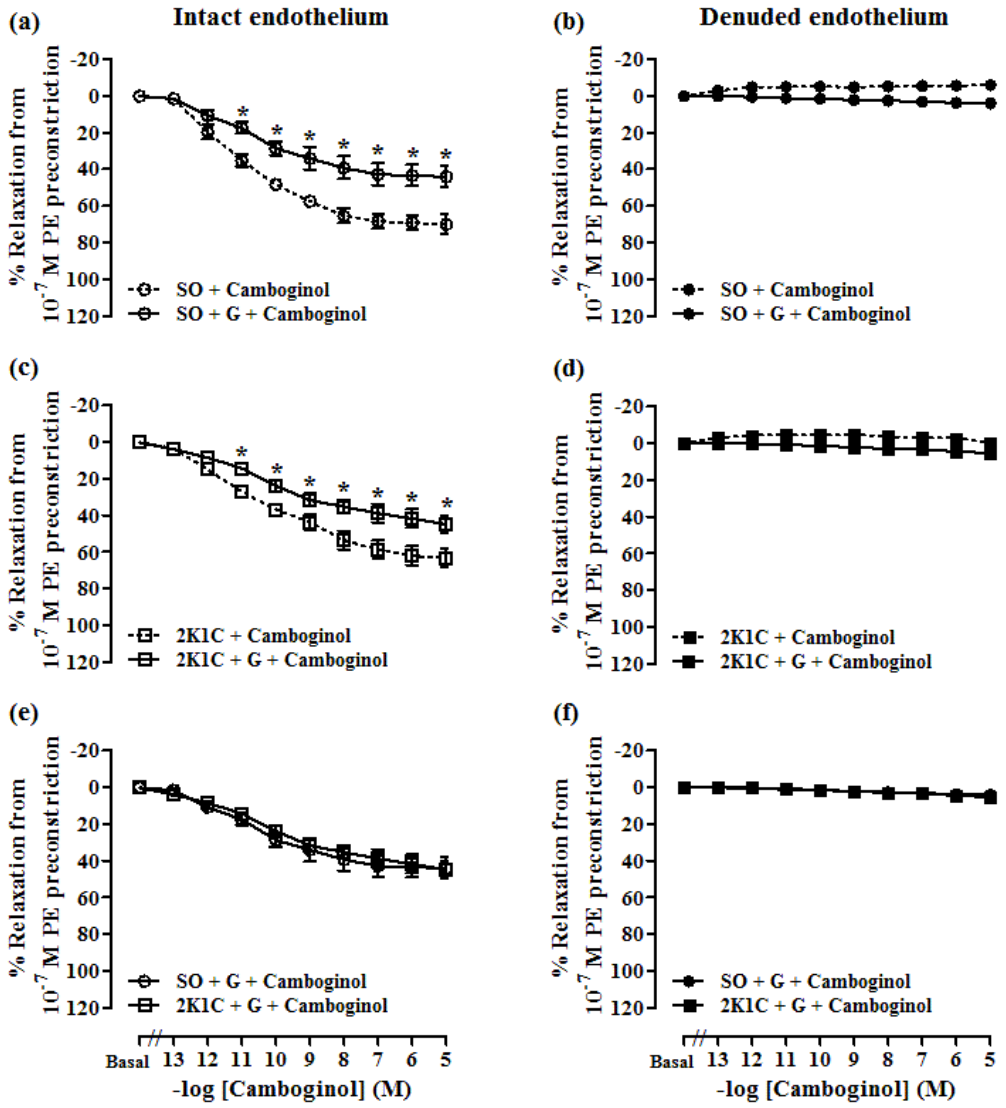

Figure 3.9. Effects of camboginol on vasorelaxation of endothelium- intact (left panel; a, c and e) or -denuded (right panel; b, d and f) aortic rings from 2-kidneys-1-clip (2K1C) or sham operative (SO) group in the presence of 10^{-6} M glibenclamide (G).

Values are mean ± S.E.M of the %relaxation from 10^{-7} M phenylephrine (PE) precontraction. * $p < 0.05$ compared with respective control group (one-way ANOVA with Newman-Keuls post hoc test).

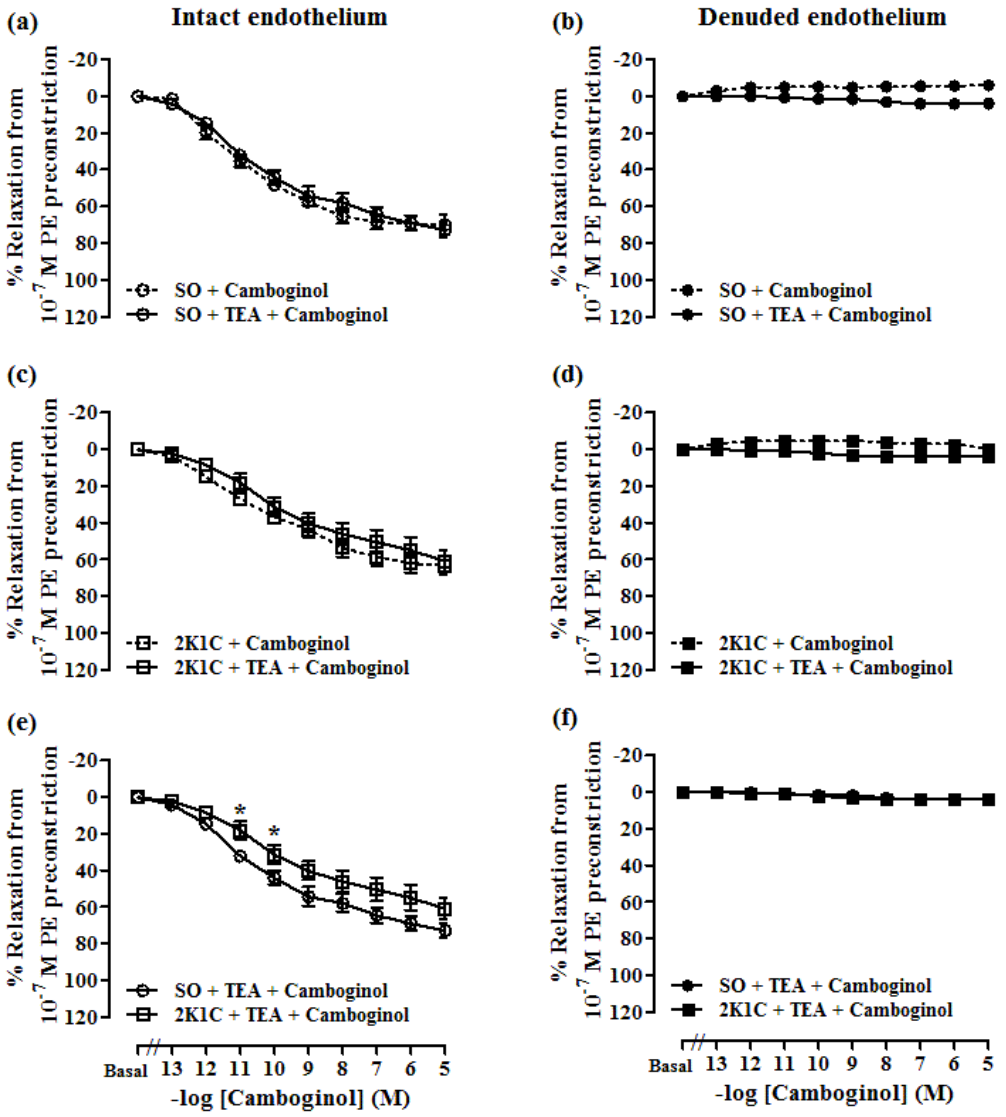

Figure 3.10. Effects of camboginol or vehicle (DMSO) on vasorelaxation of endothelium-intact (left panel; a, c and e) or -denuded (right panel; b, d and f) aortic rings from 2-kidneys-1-clip (2K1C) or sham operative (SO) group in the presence of 10^{-3} M tetraethylammonium (TEA).

Values are mean ± S.E.M of the %relaxation from 10^{-7} M phenylephrine (PE) precontraction. * $p < 0.05$ compared with respective control group (one-way ANOVA with Newman-Keuls post hoc test).

3.1.7. Effect of camboginol on eNOS expression

As shown in Figure 3.11, 2K1C rats exhibited a significant reduction of eNOS expression in aortic endothelium when compared with SO rats. Treatment with camboginol was able to enhance protein expression of aortic eNOS in 2K1C rat. This finding further supported the mechanism of vasorelaxation by camboginol which involves the endothelial NO signaling pathway.

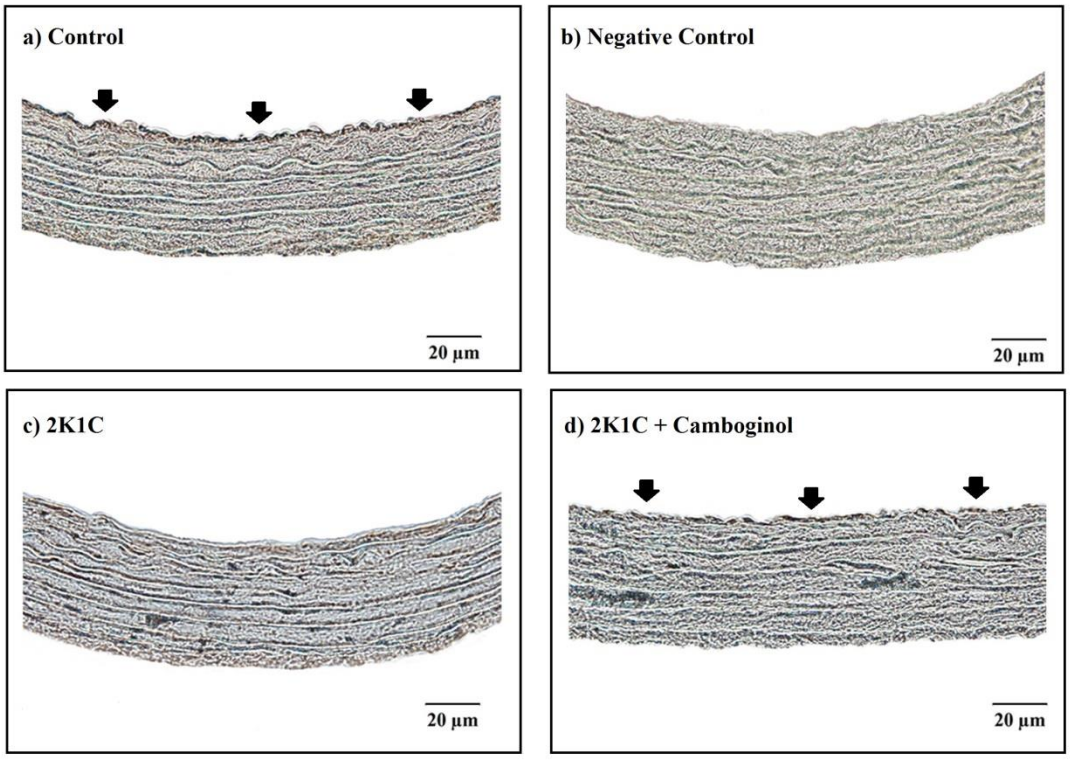

Figure 3.11. Endothelial nitric oxide synthase (eNOS) expression in aortic endothelium of control and 2-kidneys-1-clip (2K1C) rat; control (a), negative control (b), 2K1C (c) and after camboginol (0.1 mg/kg + 5µg/min/kg BW) treatment in 2K1C. Brown staining in aortic endothelium represents eNOS in the tunica intima layer of aortic wall. Black staining represents nucleus of vascular smooth muscle cells. Arrow indicates the eNOS location. Magnification 400X and scale bare = 20 µm.

3.2. Effects of morelloflavone

3.2.1 Effects of morelloflavone on ABP and HR

As shown in Figure 3.12, four weeks after experimental renal stenosis, the SBP, DBP, PP and MABP in 2K1C rats were significantly higher than in those of SO rats (SBP; 185±4 vs. 155±2, DBP; 136±1 vs. 125±4, PP; 49±4 vs. 30±2 and MABP; 152±1 vs. 135±3 mm Hg, respectively, $p < 0.05$). The resting HR of 2K1C group was not significantly different from SO (208±6 vs. 213±3 bpm). The data suggested that induction of 2K1C hypertensive rat was successful.

When morelloflavone (0.1 mg/kg + 5 μg/min/kg BW) was given 2K1C rats, the SBP, DBP and MABP decreased significantly when compared to vehicle-treated 2K1C rats (SBP; 165±4 vs. 185±4, DBP; 126±5 vs. 136±1 and MABP; 138±6 vs. 152±1 mm Hg, respectively, $p < 0.05$) whereas the PP and HR did not alter statistically (PP; 41±4 vs. 49±4 mm Hg and HR; 207± 5 vs. 208 ± 6 bpm, respectively). The data suggested that morelloflavone exerted a hypotensive potential in 2K1C rat.

However, the treatment of either morelloflavone or its vehicle in SO rats showed no significant alterations in SBP, DBP, PP, MABP and HR (SBP; 155±2 vs. 155±2, DBP; 121±2 vs. 125±4, PP; 35±2 vs. 30±2, MABP; 132±2 vs. 135±3 mm Hg and HR; 203±5 vs. 213±3 bpm, respectively). This finding suggested that morelloflavone might has no effect on ABP of normotensive rat.

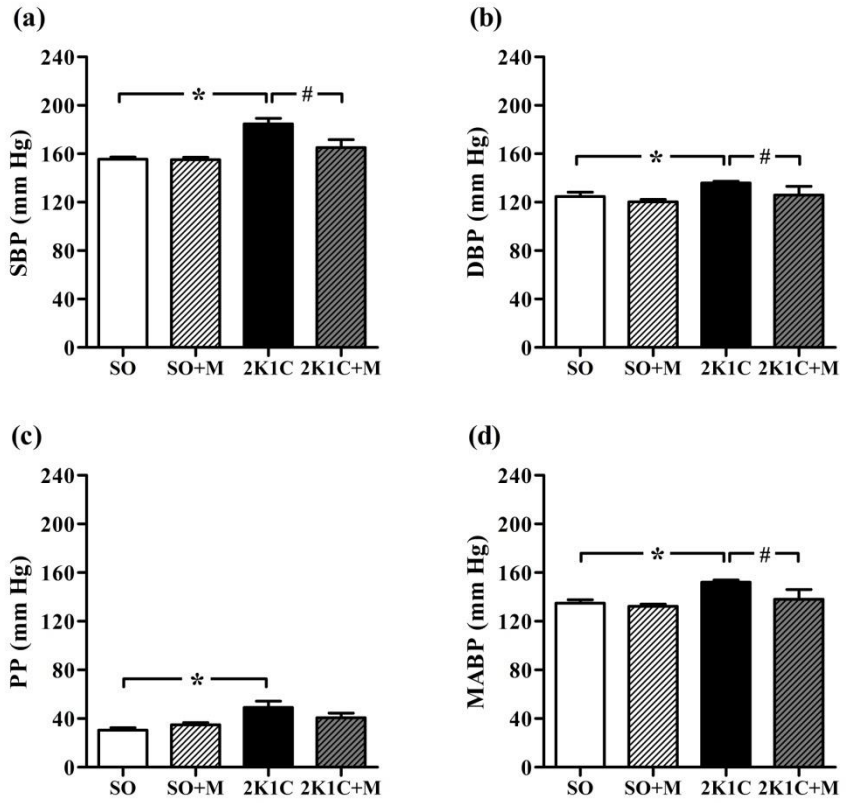

Figure 3.12. Effects of morelloflavone (M; 0.1 mg/kg BW + 5 μg/min/kg BW) on systolic blood pressure (SBP; a), diastolic blood pressure (DBP; b), pulse pressure (PP; c) and mean arterial pressure (MABP; d) in 2-kidneys-1-clip (2K1C) and sham operative (SO) group during clearance study.
Values are mean ± S.E.M. $^{*,\#}$ $p < 0.05$ compared with SO and 2K1C group, respectively (one-way ANOVA with Newman-Keuls post hoc test).

3.2.2. Effects of morelloflavone on renal clearance

As shown in Figure 3.13 and Table Suppl. 2, 2K1C rats had significantly higher RVR, FF, $\dot{V}$, C_{Osm}, C_{Osm}/GFR and TC_{H_2O} when compared to SO rats (RVR; 70±6 vs. 39±4 RU, FF; 0.63±0.10 vs. 0.36±0.02, $\dot{V}$; 18.2±3.9 vs. 9.6±0.9 μL/min/g KW, C_{Osm}; 75.6±5.8 vs. 52.1±3.8 μL/min/g KW, C_{Osm}/GFR; 6.25±0.93 vs. 3.79±0.48% and TC_{H_2O}; 56.2±5.4 vs. 41.0±3.6 μL/min/g KW, respectively, $p < 0.05$) but had a significantly lower ERPF (2.29±0.25 vs.3.63±0.36 mL/min/g KW, $p <$

0.05). However, there was no significant difference of GFR, U_{Osm} and P_{Osm} between 2K1C and SO rats (GFR; 1.52±0.20 vs. 1.43±0.12 mL/min/g KW, U_{Osm}; 1240±49 vs. 1425±46 mOsm/kg H_2O and P_{Osm}; 330±4 vs. 326±3 mOsm/kg H_2O, respectively).

When morelloflavone (0.1 mg/kg + 5 μg/min/kg BW) was given in 2K1C rats, a significant decrease in RVR, FF, U_{Osm} and TC_{H_2O} were observed when compared to vehicle-treated 2K1C rats (RVR; 45±6 vs. 70±6 RU, FF: 0.42±0.06 vs. 0.63±0.10, U_{Osm} 670±84 vs. 1240±49 mOsm/kg H_2O and TC_{H_2O}; 40.4±3.8 vs. 56.2±5.4 μL/min/g KW, respectively, $p < 0.05$). However, the ERPF, $\dot{V}$ and C_{Osm} were found to be significantly higher (ERPF; 3.44±0.49 vs. 2.29±0.25 mL/min/g KW, $\dot{V}$; 42.0±9.4 vs. 11.5±0.7 μL/min/g KW and C_{Osm}; 106.3±11.4 vs. 75.6±6.2 μL/min/g KW, respectively, $p < 0.05$). The GFR and P_{Osm} were not significantly different between the two groups (GFR; 1.44±0.08 vs. 1.52±0.20 mL/min/g KW and P_{Osm}; 317±4 vs. 330±4 mOsm/kg H_2O, respectively).

The administration of morelloflavone (0.1 mg/kg + 5 μg/min/kg BW) in SO rats resulted in a significant increase in GFR, FF, $\dot{V}$ and C_{Osm} but a decrease in U_{Osm} when compared to vehicle-treated SO rats (GFR; 1.72±0.14 vs. 1.43±0.12 mL/min/g KW, FF; 0.45±0.05 vs. 0.36±0.02, $\dot{V}$; 44.7±4.3 vs. 8.79±0.97 μL/min/g KW, C_{Osm}; 87.5±7.2 vs. 51.1±4.7 μL/min/g KW and U_{Osm}; 613±41 vs. 1426±46 mOsm/kg H_2O, respectively, $p < 0.05$) while the ERPF, RVR, P_{Osm} and TC_{H_2O} remained unaltered (ERPF; 3.96±0.28 vs. 3.63±0.36 mL/min/g KW, RVR; 35±2 vs. 39±4 RU, P_{Osm}; 321±2 vs. 326±3 mOsm/kg H_2O and TC_{H_2O}; 31.7±3.8 vs. 41.0±4.1 μL/min/g KW, respectively).

The data suggested that the impairment of both RBF and renal electrolytes excretion were observed in 2K1C rats. Morelloflavone treatment can correct the impairment of both RBF and renal electrolytes excretion in 2K1C rats. The diuretic effect of morelloflavone was also observed in SO rats.

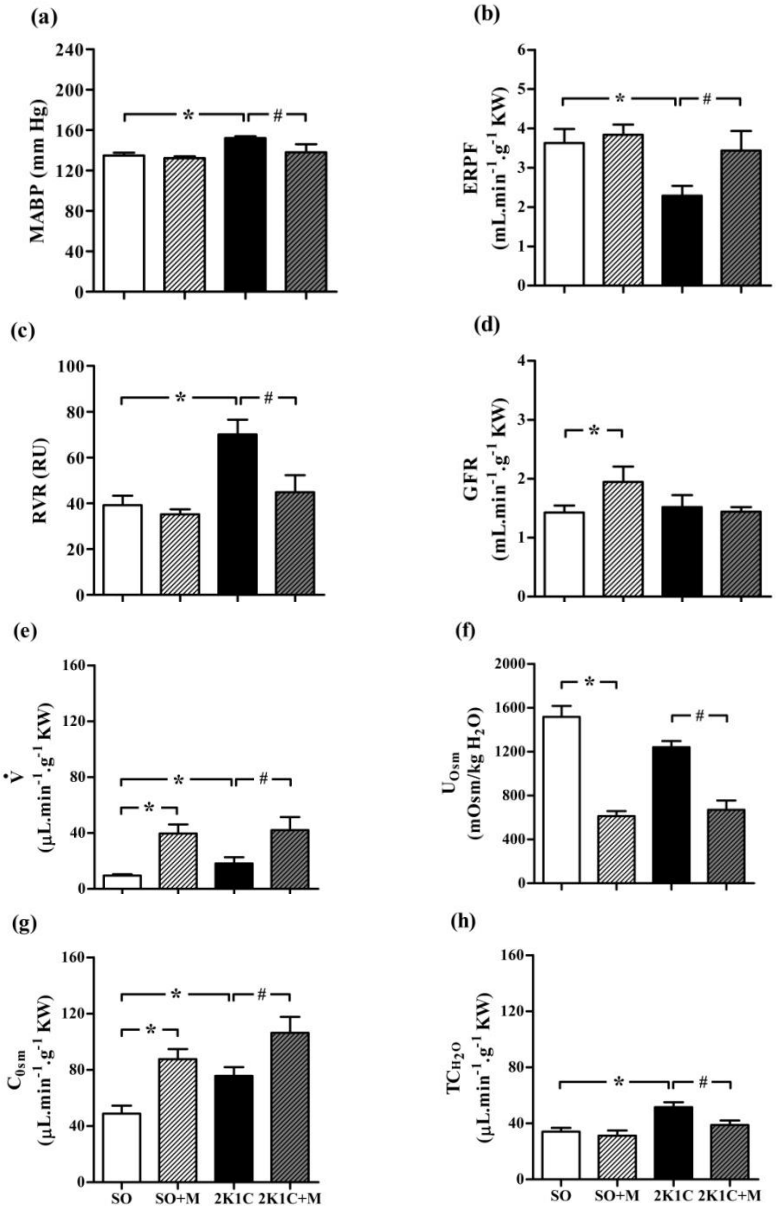

Figure 3.13. Effects of morelloflavone (M; 0.1mg/kg + 5 µg/min/kg BW) on mean arterial blood pressure (MABP; a), effective renal plasma flow (ERPF; b), renal vascular resistance (RVR; c), glomerular filtration rate (GFR; d), urine flow rate ($\dot{V}$; e), urine osmolality (U_{Osm}; f), osmolar clearance (C_{Osm}; g) and negative free water clearance (TC_{H_2O}; h), in 2-kidneys-1-clip (2K1C) and sham operative (SO) group during clearance study.

Values are mean ± S.E.M. $^{*, \#}$ $p < 0.05$ compared with SO and 2K1C group, respectively (one-way ANOVA with Newman-Keuls post hoc test).

3.2.3. Effects of morelloflavone on BRS

As shown in Figure 3.14a, c and e, the BRS responses to the six doses of PE (1, 2, 4, 8, 16 and 32 μg/kg BW) intravenous injection in 2K1C were significantly lowered when compared to those of SO rats (-0.11±0.05 vs. -0.73±0.22, -0.13±0.04 vs -0.55±0.10, -0.22±0.05 vs. -0.55±0.10, -0.21±0.03 vs. -0.63±0.05, -0.20±0.03 vs. -0.78±0.08 and -0.23±0.04 vs. -1.03±0.08 bpm/mm Hg, respectively, $p < 0.05$). There was no significant difference in respective BRS to PE between SO with morelloflavone-treated and vehicle-treated rats. Morelloflavone treatment significantly resulted in a higher BRS in 2K1C in comparison to vehicle-treated rats at the respective doses of PE injection (-0.43±0.06 vs. -0.11±0.05, -0.42±0.09 vs. -0.13±0.04, -0.43±0.11 vs. -0.22±0.05, -0.37±0.04 vs. -0.21±0.03, -0.53±0.14 vs. -0.20±0.03 and -0.77±0.15 vs. -0.23±0.04 bpm/mm Hg, respectively, $p < 0.05$).

As shown in Figure 3.14b, d and f, The BRS to the six doses of SNP (1, 2, 4, 8, 16 and 32 μg/kg BW) intravenous injection in 2K1C rats were significantly lowered when compared to those of SO rats (-0.04±0.02 vs. -0.32±0.05, -0.10±0.04 vs. -0.34±0.08, -0.09±0.04 vs. -0.28±0.04, -0.11±0.03 vs. -0.28±0.03, -0.10±0.02 vs. -0.29±0.04 and -0.12±0.02 vs. -0.34±0.06 bpm/mm Hg, respectively, $p < 0.05$). There was no significant difference in BRS between SO with merelloflavone-treated and vehicle-treated rats at the respective doses of SNP. Morelloflavone treatment significantly induced a higher BRS in 2K1C in comparison to vehicle-treated rats at the respective doses of SNP injection (-0.44±0.07 vs. -0.04±0.02, -0.31±0.06 vs. -0.10±0.04, -0.21±0.03 vs. -0.09±0.04, -0.20±0.04 vs. -0.11±0.03, -0.21±0.03 vs. -0.10±0.02 and -0.23±0.03 vs. -0.12±0.02 bpm/mm Hg, respectively, $p < 0.05$.

The data suggested that the BRS were blunted in 2K1C rats and morelloflavone treatment can restore this BRS impairment.

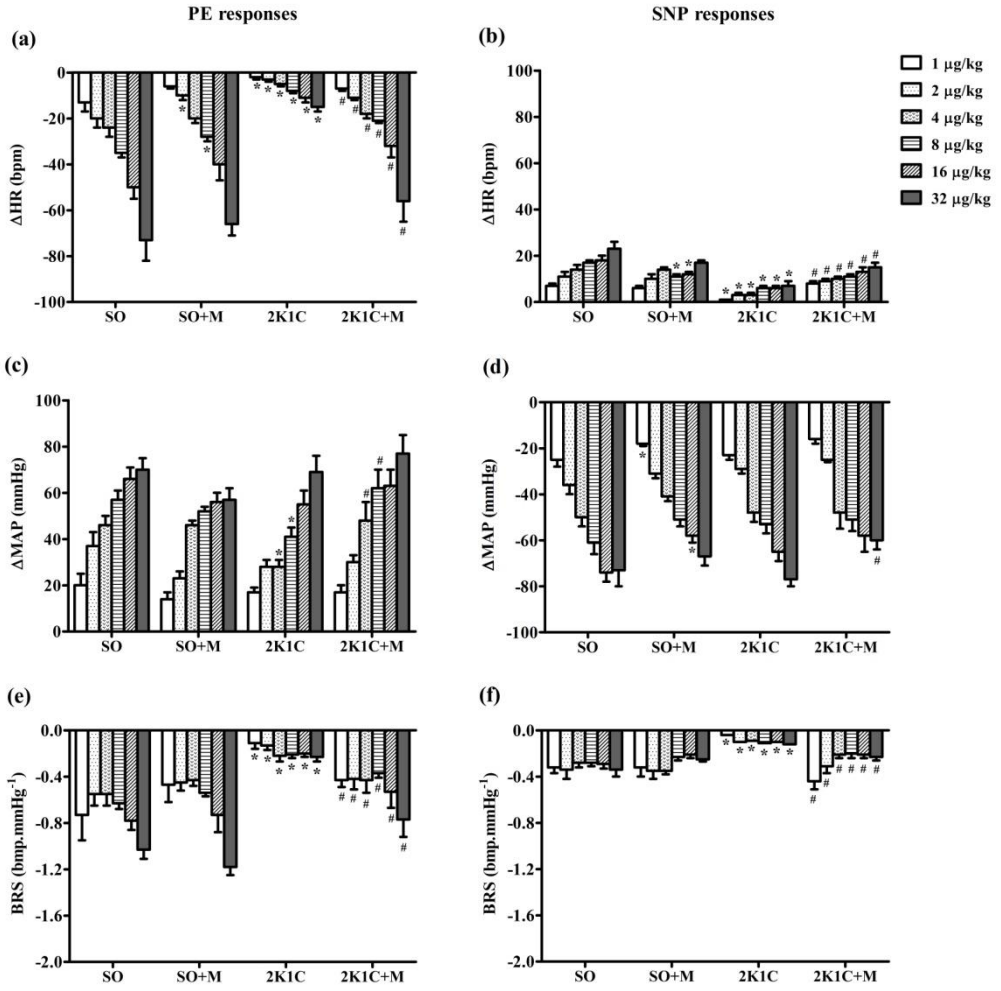

Figure 3.14. Baroreflex sensitivity (BRS) in response to either phenylephrine (PE; left panel) or sodium nitroprusside (SNP; right panel) in 2-kidneys-1-clip (2K1C) and sham operation (SO) group during treatment with morelloflavone (M; 0.1 mg/kg + 5 µg/min/kg BW). BRS = ΔHR/ΔMABP.

Data are mean ± S.E.M. $^{*, \#}$ $p < 0.05$ compared with the SO and 2K1C group at the respective concentrations of PE or SNP, respectively (one-way ANOVA with Newman-Keuls post hoc test).

3.2.4. Effect of morelloflavone on plasma MDA levels

As shown in Figure 3.15, the level of plasma MDA in 2K1C rats were significantly higher than SO rats (64.3±8.9 vs. 30.0±2.5 μmol/L, $p < 0.05$, respectively). Morelloflavone treatment significantly lower the plasma MDA level in 2K1C rats compared with vehicle-treated 2K1C rats (36.1±9.7 vs. 64.3±8.9 μmol/L, $p < 0.05$, respectively). These results demonstrated that an increase in plasma MDA content confirmed that oxidative damage has been induced in 2K1C rat and morelloflavone exerted an antioxidative activity.

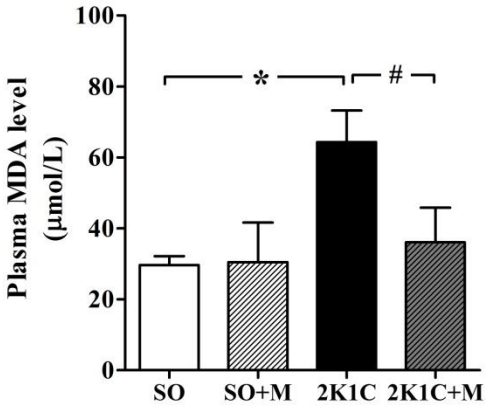

Figure 3.15. Effect of camboginol (0.1 mg/kg + 5 μg/min/kg BW) on plasma malondialdehyde (MDA) levels in 2-kidneys-1-clip (2K1C) hypertensive and sham operative (SO) normotensive rats.
Data are mean ± S.E.M. $^{*, \#}$ $p < 0.05$ compared with SO and 2K1C group, respectively (one-way ANOVA with Newman-Keuls post hoc test).

3.2.5. Body weight, kidney weight and cardiac mass

As shown in Table 3.2, the body weight changed between SO and 2K1C rats after four week of experimental renal stenosis were not significantly different. The left clipped and the right non-clipped kidney weight of 2K1C rat decreased by 73% and increased by 24% when compared to the respective ipsilateral kidneys of SO rat. The left atrophied kidney was due to the reduction in renal blood flow while the right hypertrophied kidney was due to functional compensation. The cardiac mass of 2K1C groups significantly increased in comparison to those SO groups. This finding suggested the increased afterload in this rat model. There was no significantly different in hematocrit between these rats.

Table 3.2. Comparison of body weight at the beginning of experiment (Pre-BW), 4 weeks after induction of hypertension by experimental renal stenosis (Post-BW) and body weight changes (△BW). Left and right kidney weight (KW), cardiac mass and hematocrit of 2-kidneys-1-clip (2K1C) and sham operation (SO) rats were determined and compared after either morelloflavone (M) (0.1 mg/kg + 5 µg/min/kg BW) or vehicle (V or DMSO) treatment.

Parameters	**SO+V**	**SO+M**	**2K1C+V**	**2K1C+M**
Number of rats	6	6	6	6
Pre-BW (g)	206 ± 4	203 ± 3	194 ± 9	186 ± 9
Post-BW (g)	388 ± 3	383 ± 10	379 ± 20	358 ± 13
△BW (g)	182 ± 6	181 ± 10	185 ± 19	172 ± 19
Left KW (g)	1.05 ± 0.02	0.99 ± 0.02	0.28 ± 0.07*	0.23 ± 0.02*
Left KW/BW (%)	0.27 ± 0.01	0.26 ± 0.01	0.07 ± 0.01*	0.06 ± 0.01*
Right KW (g)	1.18 ± 0.02	1.05 ± 0.03	1.46 ± 0.07*	1.25 ± 0.03*
Right KW/BW (%)	0.30 ± 0.01	0.27 ± 0.01	0.39 ± 0.02*	0.35 ± 0.01*
Cardiac mass (g)	1.06 ± 0.02	1.00 ± 0.03	1.23 ± 0.12*	1.22 ± 0.02*
Cardiac mass/BW (%)	0.27 ± 0.01	0.26 ± 0.01	0.32 ± 0.02*	0.34 ± 0.01*
Hematocrit (%)	49.2 ± 0.9	52.0 ± 0.4	51.6 ± 1.3	51.6 ± 0.8

Data are mean ± S.E.M.

* $p < 0.05$ compared with respective SO groups (Student t-test).

3.2.6. Effect of morelloflavone on isolated aortic relaxation

3.2.6.1. Vasorelaxant effect of morelloflavone

As shown in Figure 3.16a-f, the addition of the cumulative doses of morelloflavone (10^{-13}-10^{-5} M) significantly relaxed the PE-preconstracted intact endothelium aortic rings in a concentration-dependent manner with the pD_2 = 6.99 ± 0.91 (EC_{50} = 102.8 nM) of 2K1C and the pD_2 = 9.92 ±0.86 (EC_{50} = 0.52 nM) of SO rats ($p < 0.05$). Denudation of the functional endothelium completely abolished morelloflavone-induced vasorelaxation in both 2K1C and SO rats. The data indicated the vasorelaxation effect of morelloflavone which might be endothelium dependent.

3.2.6.2. Effect of L-NAME on morelloflavone-induced vasorelaxation

As shown in Figure 3.17a-f, pretreatment of the intact endothelium aortic rings with 10^{-4} M L-NAME significantly and completely abolished morelloflavone - induced vasorelaxation in both SO and 2K1C rats. Pretreatment of L-NAME had no effect on the endothelium-denuded aortic rings from SO and 2K1C rats. The data suggested that vasorelaxation effect of morelloflavone might involve in endothelial NO signaling pathway.

3.2.6.3. Effect of indomethacin on morelloflavone-induced vasorelaxation

As shown in Figure 3.18a-f, pretreatment of the intact endothelium aortic rings from SO rats with 10^{-6} M indomethacin showed biphasic effect; the %relaxation of these rings in response to 10^{-12}-10^{-11} M morelloflavone in the presence of inhibitor were significantly decreased in comparison to in the absence of inhibitor, in the contrast, the %relaxation of these rings in response to 10^{-8}-10^{-5} M morelloflavone in the presence of inhibitor were significantly increased in comparison to in the absence of inhibitor. Pretreatment of indomethacin had no effect on the denuded endothelium aortic rings from SO and both intact and denuded endothelium aortic rings from 2K1C rats. The data suggested that the vasorelaxation effect of morelloflavone might involve PGI_2 pathway and this mechanism might be impaired in 2K1C rats.

3.2.6.4. Effect of glibenclamide on morelloflavone-induced vasorelaxation

As shown in Figure 3.19a-f, the %relaxation of intact endothelium aortic rings from SO rats in response to 10^{-13}-10^{-12} M morelloflavone in the presence of 10^{-5} M glibenclamide were significantly decreased in comparison to in the absence of inhibitor, however these partially inhibition effect of this inhibitor was not observed in the endothelium-denuded rings. Pretreatment of glibenclamide in intact and denuded endothelium aortic rings from 2K1C rats did not change the %relaxation in responses to morelloflavone. The data suggested that the vasorelaxation effect of morelloflavone in SO rats might involve EDHF-activated K_{ATP} channel signaling pathway and this mechanism might be impaired in 2K1C rats.

3.2.6.5. Effect of tetraethylammonium on morelloflavone-induced vasorelaxation

As shown in Figure 3.20a-f, pretreatment of the intact endothelium aortic rings from SO rats with 10^{-3} M tetraethylammonium showed biphasic effect; the %relaxation of these rings in response to 10^{-13}-10^{-11} M morelloflavone in the presence of inhibitor were significantly decreased in comparison to in the absence of inhibitor, in the contrast, the %relaxation of these rings in response to 10^{-5} M morelloflavone in the presence of inhibitor were significantly increased in comparison to in the absence of inhibitor. Pretreatment of tetraethylammonium had no effect on the denuded endothelium aortic rings from SO and both intact and denuded endothelium aortic rings from 2K1C rats. The data suggested that the vasorelaxation effect of morelloflavone might involve K_{Ca} channel signaling pathway and this mechanism might be impaired in 2K1C rats.

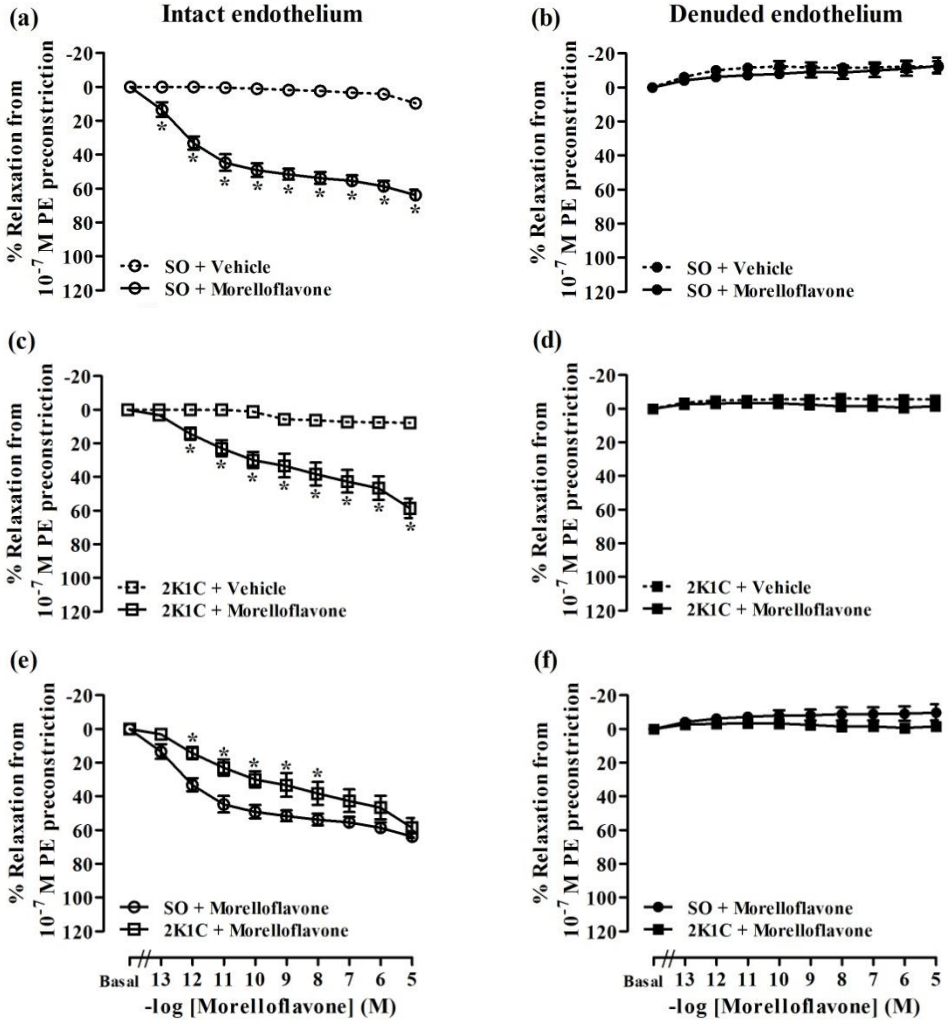

Figure 3.16. Effects of morelloflavone or vehicle (DMSO) on vasorelaxation of endothelium-intact (left panel; a, c and e) or -denuded (right panel; b, d and f) aorta rings from 2-kidneys-1-clip (2K1C) or sham operative (SO) group.
Values are mean ± S.E.M of the %relaxation from 10^{-7} M phenylephrine (PE) precontraction. * $p < 0.05$ compared with respective control group (one-way ANOVA with Newman-Keuls post hoc test).

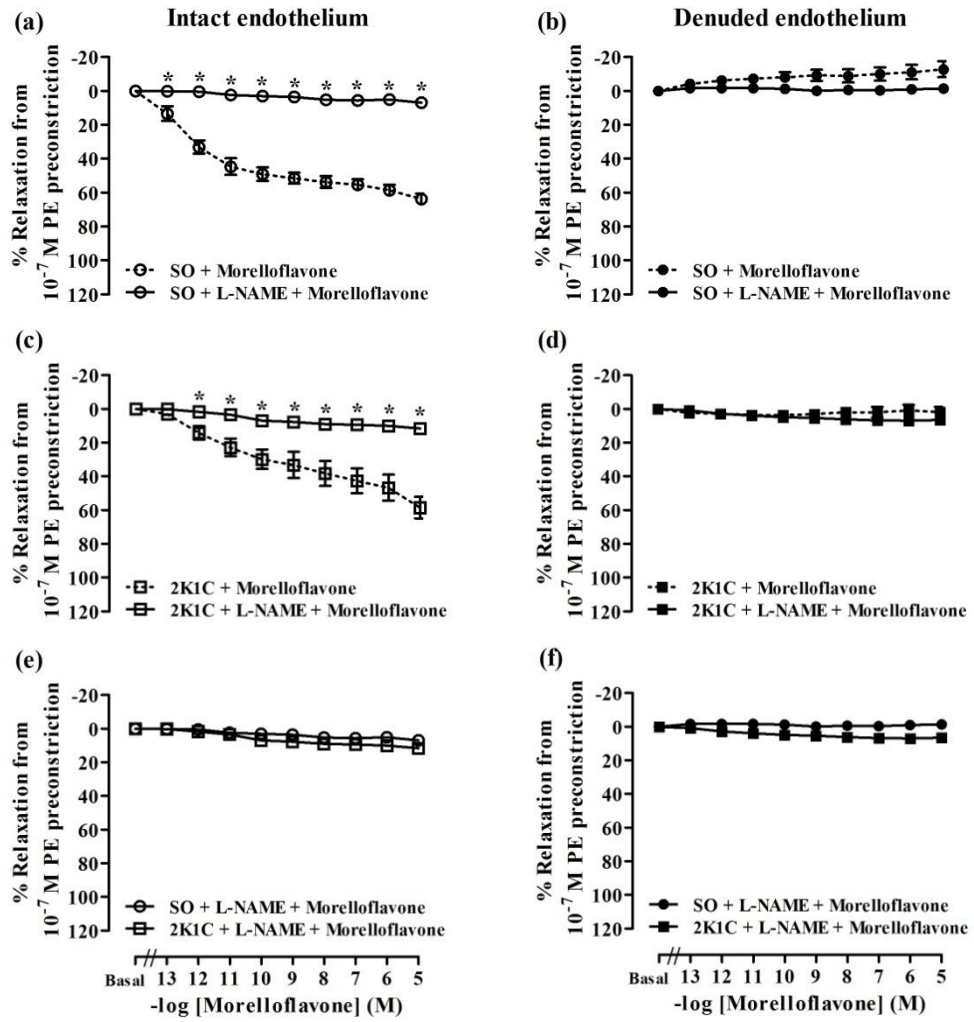

Figure 3.17. Effects of morelloflavone on vasorelaxation of endothelium-intact (left panel; a, c and e) and -denuded (right panel; b, d and f) aorta rings from 2-kidneys-1-clip (2K1C) and sham operative (SO) group in the presence of 10^{-4} M L-NAME. Values are mean ± S.E.M of the %relaxation from 10^{-7} M phenylephrine (PE) precontraction. * $p < 0.05$ compared with respective control group (one-way ANOVA with Newman-Keuls post hoc test).

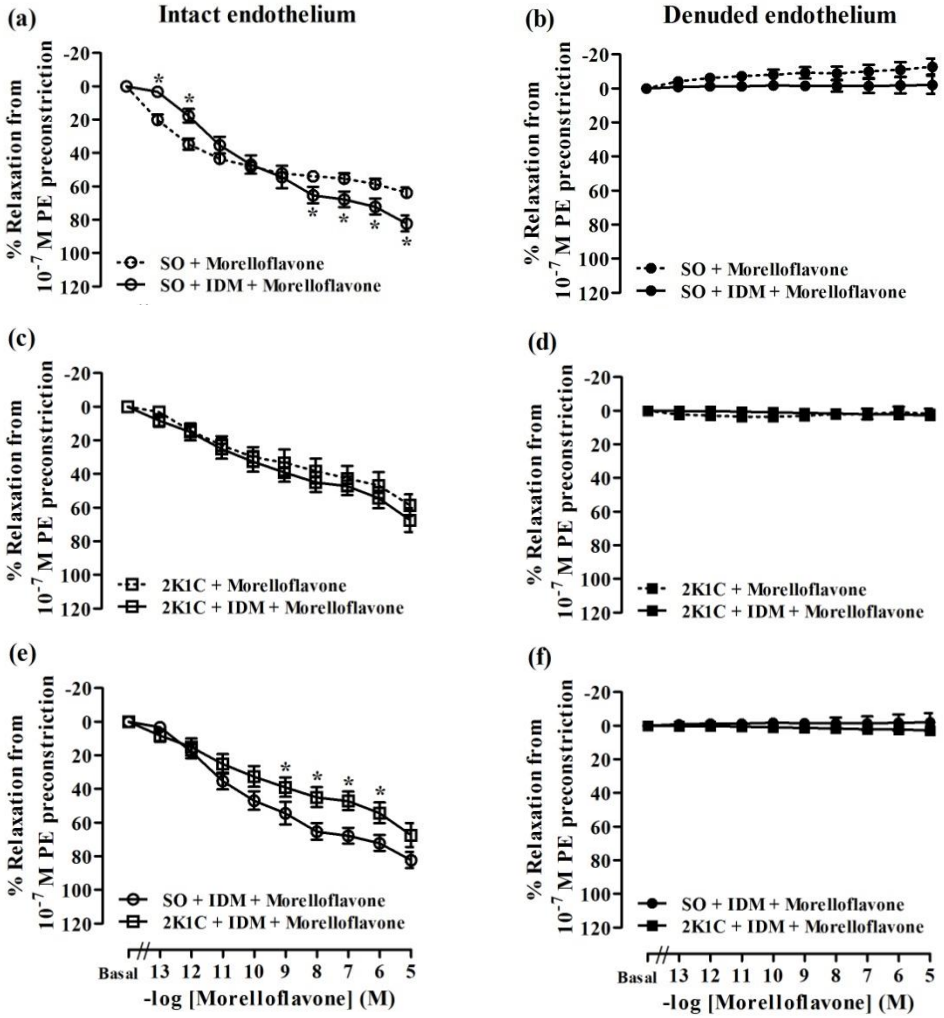

Figure 3.18. Effects of morelloflavone on vasorelaxation of endothelium-intact (left panel; a, c and e) and -denuded (right panel; b, d and f) aorta rings from 2-kidneys-1-clip (2K1C) and sham operative (SO) group in the presence of 10^{-6} M indomethacin (IDM).

Values are mean ± S.E.M of the %relaxation from 10^{-7} M phenylephrine (PE) precontraction. * $p < 0.05$ compared with respective control group (one-way ANOVA with Newman-Keuls post hoc test).

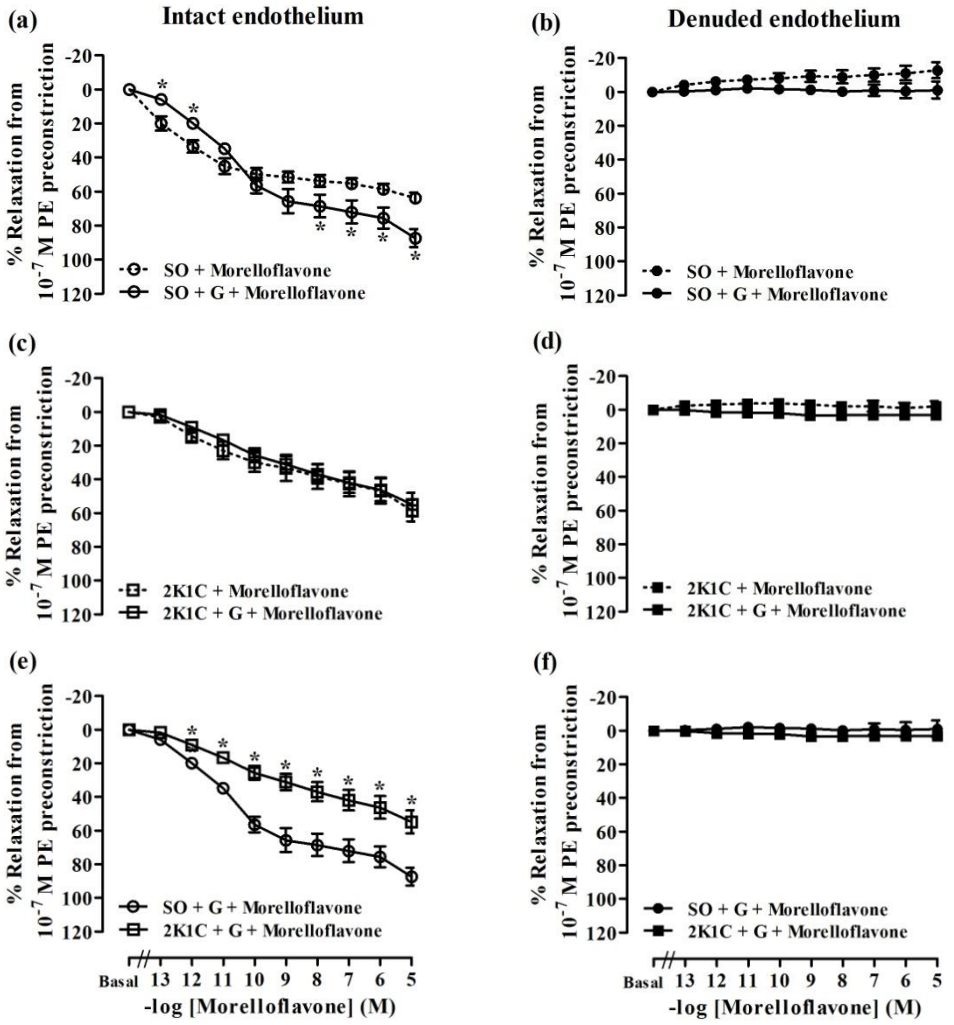

Figure 3.19. Effects of morelloflavone on vasorelaxation of endothelium-intact (left panel; a, c and e) and -denuded (right panel; b, d and f) aorta ring from 2-kidneys-1-clip (2K1C) and sham operative (SO) group in the presence of 10^{-6} M glibenclamide (G).

Values are mean ± S.E.M of the %relaxation from 10^{-7} M phenylephrine (PE) precontraction. * $p < 0.05$ compared with respective control group (one-way ANOVA with Newman-Keuls post hoc test).

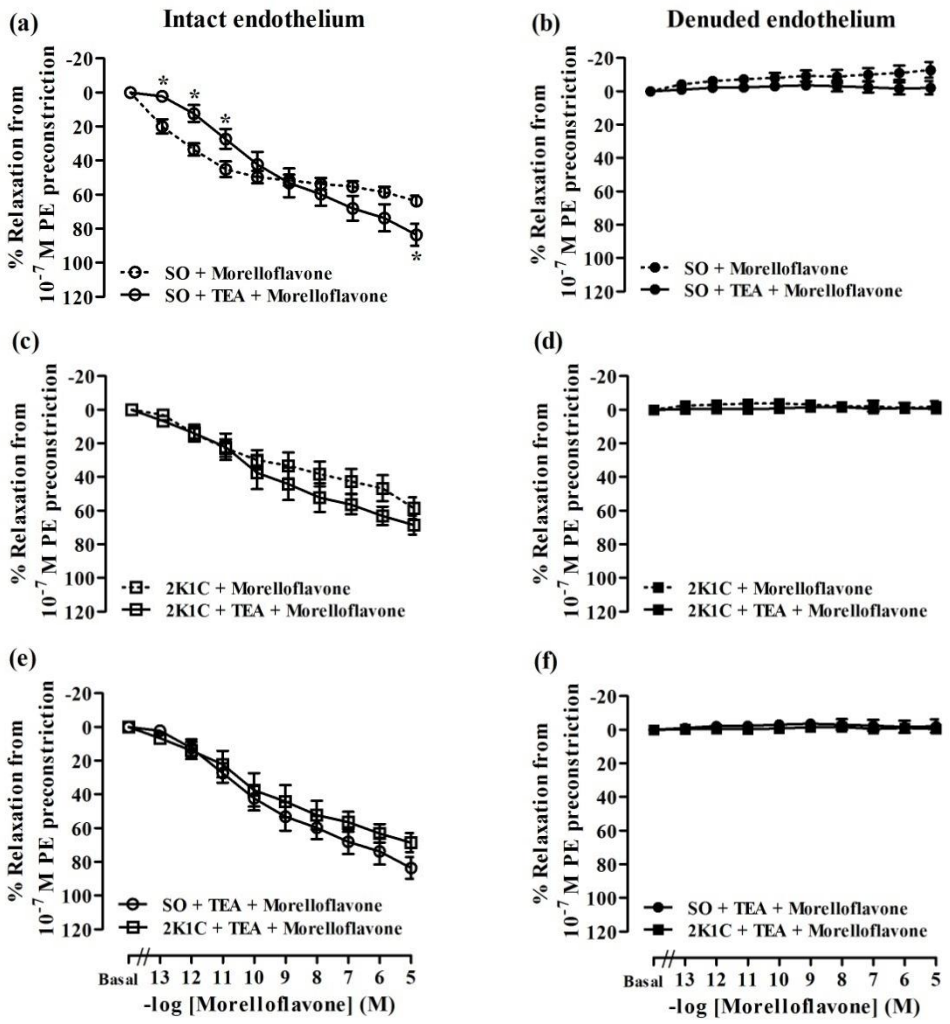

Figure 3.20. Effects of morelloflavone on vasorelaxation of endothelium- intact (left panel; a, c and e) and -denuded (right panel; b, d and f) aorta ring from 2-kidneys-1-clip (2K1C) and sham operative (SO) group in the presence of 10^{-3} M tetraethyl ammonium (TEA).

Values are mean ± S.E.M of the %relaxation from 10^{-7} M phenylephrine (PE) precontraction. * $p < 0.05$ compared with respective control group (one-way ANOVA with Newman-Keuls post hoc test).

3.2.7. Effect of morelloflavone on eNOS expression

As shown in Figure 3.21, 2K1C rats exhibited a significant reduction of eNOS expression in aortic endothelium when compared with SO rats. Treatment with morelloflavone was able to enhance protein expression of aortic eNOS. This finding further supported the mechanism of casorelaxation by camboginol which involves the endothelial NO signaling pathway.

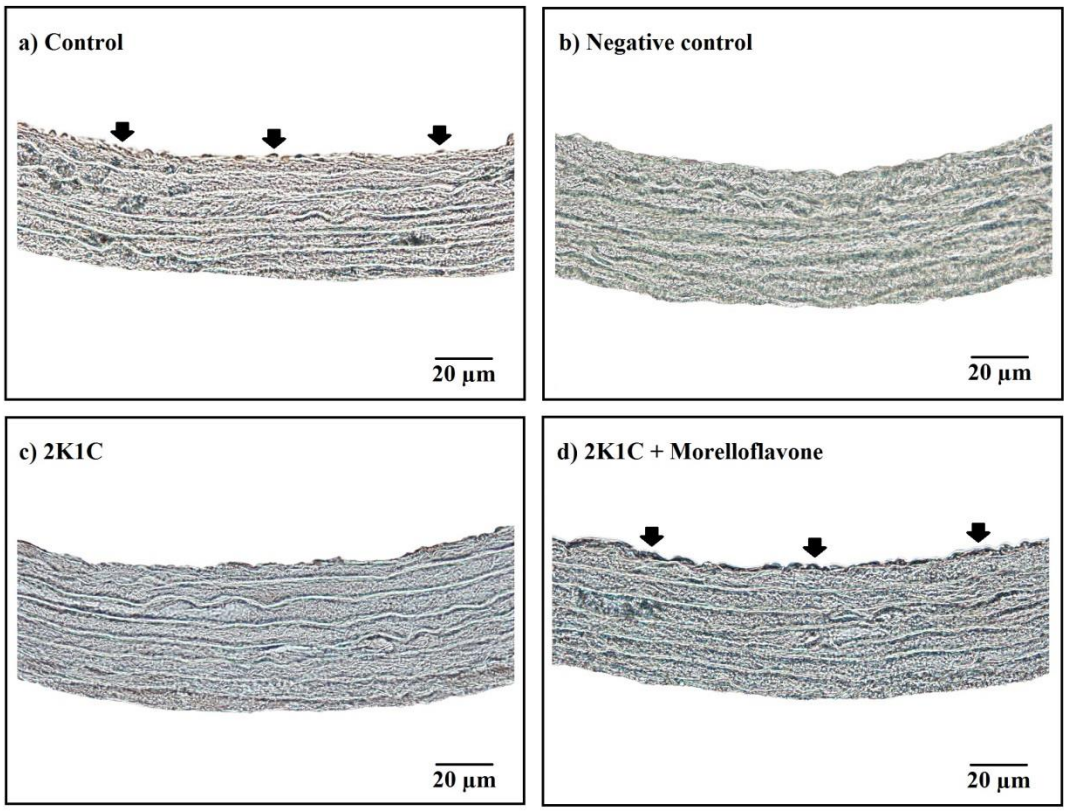

Figure 3.21. Endothelial nitric oxide synthase (eNOS) expression in aortic endothelial of control and 2-kidneys-1-clip (2K1C) rat; control (a), negative control (b), 2K1C (c) and after morelloflavone (0.1 mg/kg + 5 µg/min/kg BW) treatment in 2K1C. Brown staining in aortic endothelium represents eNOS in the in the tunica intima layer of aortic wall. Black staining represents nucleus of vascular smooth muscle cells. Arrow indicates the eNOS location. Magnification 400X and scale bar = 20 µm.

CHAPTER 4
DISCUSSION

4.1. Development of 2K1C hypertension

4.1.1. Cardiovascular function changes in 2K1C

2K1C is most relevant characteristic model to human RVH which involves unilateral stenosis of the renal artery leading to a permanent reduction in renal perfusion pressure in one kidney resulting in increased AII production (Goldblatt et al. 1934; Navar et al. 1998). In this study, 4 weeks after experimental renal stenosis, SBP, DBP, PP and MABP of 2K1C rat measuring under pentobarbitone sodium anesthetization were significantly higher than those of SO rat. It is also found that the RVR of 2K1C was significantly higher than that of SO rat suggesting an increase peripheral resistance in the kidney. These data suggested that the development of hypertension was successful. These results were also supported by the significant increase in the cardiac mass and atrophy of clipped left kidney with hypertrophy of non-clipped right kidney of 2K1C rat when compared to SO rat.

It is found that level of plasma MDA in 2K1C were significantly higher than on SO rat which refer the oxidative stress status in this hypertensive rat. It is accepted that an increase in circulating AII can activate the vascular smooth muscle NADPH oxidase which is an important cellular source of ROS, especially O_2^- (Griendling et al. 1994). The role of O_2^- is related to endothelial dysfunction by which NO can be scavenged by O_2^- to form peroxynitrite ($ONOO^-$) resulting in reduced NO bioavailability and diminishing vasorelaxation (Griendling et al. 1994; Cai & Harrison et al. 2000; Oliveira-Sale et al. 2008). In this study, the vasorelaxation in response to ACh in endothelium-intact aortic rings from 2K1C was significantly lower than those from SO rat (Figure Suppl. 2) while vasoconstriction response to PE in these rings was significantly higher (Figure Suppl. 1). The immunohistological study represented the obviously less eNOS expression in 2K1C when compared to control. This finding is similar to the previous reports by Sánchez

et al. (2006) and Ulker et al. (2003) that showed the eNOS expression significantly decreases in the dysfunction arteries from hypertensive rat. An overproduction of O_2^- reduced bioactive NO by promoting NO inactivation and by promoting eNOS uncoupling (Förstermann, 2010). Regarding baroreceptor reflex, 2K1C rats also exhibited the impairment of BRS which may due to the inability to increase HR in response to a decrease ABP and vice versa. It has been proved that an impairment of BRS in RVH was related to an overproduction of O_2^- by NADPH oxidase enzyme (Botelho-Ono et al., 2011; Queiroz et al., 2012). Acute administration of vitamin C or apocynin, a NADPH oxidase inhibitor prominently improved the blunt BRS in 2K1C rat (Botelho-One et al., 2011). These data suggested that cardiovascular dysfunction was observed in this animal model.

4.1.2. Renal function changes in 2K1C

It is found that 2K1C rats has significantly higher RVR, FF, $\dot{V}$, C_{Osm}, C_{Osm}/GFR and TC_{H_2O} but has a significantly lower ERPF when compared to SO rats. However, there was no significant different of GFR, U_{Osm} and P_{Osm} between 2K1C and SO rats. The pioneering study reported that RBF and GFR were not different in the non-clipped kidneys of the hypertensive rats compared to normotensive rats (Ploth et al. 1981). However, the non-clipped kidneys of 2K1C rats exhibited a significantly higher RVR, $\dot{V}$, absolute and fractional sodium excretion and absolute and fractional potassium excretion than in kidneys of normal rats (Ploth et al. 1981; Anderson et al. 1985; Martinez-Maldonado1991).

The higher $\dot{V}$ in 2K1C may be due to an abnormal of tubular electrolyte and water reabsorption in response to high circulating level of AII or NO similar to experimented in normal control. In normotensive rats, AII has been shown to exert biphasic effect in which low dose (10^{-12}-10^{-9} M) when applied to either luminal or peritubular side stimulates renal tubular fluid absorption while the higher dose (10^{-7}-10^{-6} M) causes inhibition (Harris and Young. 1997). Moreover, addition of SNP, a NO donor, showed an inhibition of renal proximal fluid reabsorption (Eitle et al. 1998). Thus, the inhibition of tubular electrolytes and water reabsorption due to higher levels of AII and perhaps NO may contribute to the high urine flow and osmolar clearance of 2K1C.

4.2. Diuretic and hypotensive effect of camboginol

4.2.1. Diuretic effect of camboginol

The diuretic effect of camboginol was not only observed in normotensive SO but also in hypertensive 2K1C with the similar degree, however, the mechanisms of diuretic action might be different in some extent. In camboginol-treated 2K1C rats, the RVR, U_{Osm} and P_{Osm} were decreased significantly while ERPF, GFR, $\dot{V}$, C_{Osm}, C_{Osm}/GFR and TC_{H_2O} were increased significantly when compared with the vehicle-treated 2K1C rats. After camboginol administration in SO rats, the GFR, FF, $\dot{V}$ and C_{Osm} were increased significantly while U_{Osm} and P_{Osm} decreased significantly in comparison to the vehicle-treated SO rats. Since camboginol relaxes the vascular smooth muscle of the thoracic aorta isolated from hypertensive and normotensive rats and the mechanism of vasorelaxation may be involve the endothelial NO signaling pathway, so we can assume that camboginol also relaxes the renal afferent arterioles resulting in an increased GFR. The diuretic effect of camboginol in normotension and hypertension may exert via both glomerular and tubular function.

4.2.2 Hypotensive effect of camboginol

It is found that an acute intravenous administration of camboginol showed a significant transient reduction in SBP, DBP, MABP and HR in 2K1C and SO rats suggesting the hypotensive potential of this plant compound. This hypotensive effect did not sustain during the infusion of our experimental dose (0.1 mg/kg + 5 µg/min/kg BW) in both 2K1C and SO. One of the explanations of this may be due to the well regulation of baroreflex in SO and the improved BRS in 2K1C caused by camboginol. The selected dose of camboginol in this in vivo study may not be high enough to maintain its hypotensive action comparable to candesartan, a specific AII receptor antagonist previously reported (Hiranyachattada et al., 2005).

However, the acute bradycardic effect of camboginol may involve NO action on sinoatrial node firing rate since NO has been shown to exert biphasic effect depending on its concentration. NO could increase HR by activating hyperpolarization-activated pacemaker current (I_f) at low concentration but it could potentially decrease HR by inhibition of L-type calcium current at high concentration

(Mani et al., 2002). Moreover, it is likely that camboginol may act directly on the NTS, the cardiovascular regulating center in medulla oblongata, to cause bradycardia since the overexpression of eNOS was reported in the NTS which consequent in the reduction in sympathetic nerve activity, HR and ABP in conscious rats (Sakai et al., 2000).

Camboginol treatment suppressed an elevated plasma MDA in 2K1C supporting the robust antioxidant property of camboginol which previously reported by Yamaguchi et al. (2000a & 2000b) and Hutadilok-Towatana et al. (2007). In addition, camboginol treatment could improve an impaired BRS in 2K1C rat. Likewise, it is possible that the antioxidant property of camboginol may improve the impairment of 2K1C autonomic function.

The % relaxation of thoracic aorta from 2K1C by cumulative addition of camboginol showed the higher pD_2 and EC_{50} when compared to SO rat suggesting the higher concentration of camboginol was required to induce the vasorelaxation to the similar degree as in SO. The mechanisms of camboginol action in SO aorta were found to be endothelial dependent involving mainly NO generation and partially both PGI_2 production and the operation of vascular K_{ATP} channel. However in 2K1C, the camboginol actions occurred mainly through NO generation and partially K_{ATP} channel with the lesser extent than in SO. This may be due to an impairment of endothelial function in 2K1C rat.

The immunohistochemical study also found that camboginol treatment was shown to augment eNOS expression in the vascular endothelium of 2K1C. This finding further supported the mechanism of camboginol action in 2K1C that might occur via eNOS expression in the vascular endothelium.

4.3. Diuretic and hypotensive effect of morelloflavone

4.3.1. Diuretic effect of morelloflavone

The diuretic effect of morelloflavone was observed both in SO normotensive and 2K1C hypertensive with the similar degree, however, the mechanisms of diuretic action might be different in some extent. After morelloflavone administration in SO rats, GFR, FF, $\dot{V}$ and C_{Osm} increased significantly when compared to the vehicle-treated SO rats while other renal function parameters remained unchanged. These finding suggested the diuretic effect of morelloflavone in normotension may exert via both glomerular and tubular function. Morelloflavone relaxes vascular smooth muscle of the thoracic aorta isolated from normotensive rat (Lamai et al. 2013), thus, it is likely that it may relax the renal afferent arterioles resulting in an increased GFR. It is also feasible that morelloflavone might exert its diuretic effect by inhibit epithelial electrolyte transport in either proximal or distal part of the nephron. Other plant flavonoids that elicited renal vasodilation and increased urine flow and urinary sodium and water excretion including the flavoniods extracted from *Clerodendron trichotomum* and *Spergularia purpurea* (Lu et al. 1994; Jouad et al. 2001).

When morelloflavone was given in 2K1C rats, a significant decrease in RVR, FF, U_{Osm} and TC_{H_2O} were observed when compared to vehicle-treated 2K1C rats. However, the ERPF, $\dot{V}$ and C_{Osm} were found to be significantly higher. These indicated the inhibition of renal tubular electrolyte reabsorption by morelloflavone rather than its increased filtered load action like those in SO rats. In addition, in SO rats, morelloflavone did not alter tubular free water reabsorption which contradicts to in 2K1C rats suggesting another different pathway of its diuretic action in hypertensive model. Distal tubular water reabsorption occurs mainly in response to ADH action, so it is possible that morelloflavone can improve water reabsorption by increase and expression of renal tubular water channel, aquaporin in this model.

4.3.2. Hypotensive effect of morelloflavone

Morelloflavone administration in 2K1C rats demonstrated a significant reduction in SBP, DBP and MABP which was not found in SO. These results suggested the pronounced hypotensive effect of morelloflavone in hypertension rather than in normotension. The marked hypotensive effect of morelloflavone seen in 2K1C rat may be due to its both antioxidant and NO-dependent vasorelaxant property. The reason that this hypotensive effect was not obviously seen in SO rats may be the well regulation of baroreceptor reflex in response to the chosen dose of morelloflavone (0.1 mg/kg + 5 μg/min/kg BW). This dose was the minimal hypotensive dose comparable to candesartan, an AII receptor blocker previously reported (Hiranyachattada et al. 2005). At this minimal hypotensive effective dose of morelloflavone selected in this study, taken together with the more impaired BRS of 2K1C than SO, the pronounced reduction in arterial blood pressure was then observed.

Morelloflavone treatment suppressed an elevated plasma MDA in 2K1C supporting the robust scavenging property of morelloflavone. Additionally, the BRS morelloflavone could restore an impaired BRS in 2K1C rats. These data suggested that the antioxidant property of morelloflavone may improve the impairment of autonomic function in 2K1C hypertension.

The % relaxation of thoracic aorta from 2K1C by cumulative addition of morelloflavone showed the higher pD_2 and EC_{50} when compared to SO rat suggesting the higher concentration of morelloflavone was required to induce the vasorelaxation to the similar degree as in SO. The vasorelaxant mechanism of of morelloflavone in 2K1C is likely to be endothelium-dependent and involve NO signaling pathway similar to those of SO rats even though the more endothelium damage occurred in 2K1C than SO. The immunohistochemical study also found that morelloflavone treatment was shown to augment eNOS expression in the vascular endothelium of 2K1C rats. This finding further supported the mechanism of morelloflavone action in 2K1C that might occur via eNOS expression in the vascular endothelium. The other signaling pathways which may contribute to the vasorelaxant mechanism of morelloflavone in normotension included the operation of K_{Ca}, K_{ATP} channels or EDHF pathway which was unlikely to occur in 2K1C hypertension.

CHAPTER 5
CONCLUSIONS

It is concluded that camboginol (an isoprenylated benzophenone) and morelloflavone (a biflavonoids) from *Garcinia dulcis* exerted various effects in 2K1C hypertensive rat including;

1. Camboginol and morelloflavone treatment increased RBF and increased urine flow in both anesthetized 2K1C hypertensive and SO normotensive rats suggesting theirs diuretic effect.

2. Camboginol treatment decreased ABP in both anesthetized 2K1C hypertensive and SO normotensive rats. This effect of morelloflavone also observed in 2K1C hypertension but not SO normotension. These data suggested theirs acute hypotensive effect.

3. Camboginol and morelloflavone treatment decreased plasma MDA levels in 2K1C hypertensive rats suggesting theirs scavenging activity.

4. Camboginol and morelloflavone treatment improved an impaired BRS in anesthetized 2K1C hypertensive rats.

5. Camboginol and morelloflavone treatment enhanced eNOS expression on vascular endothelium of 2K1C hypertensive rats.

6. Camboginol and morelloflavone treatment exerted an endothelial dependent vasorelaxant action in both 2K1C hypertensive and SO normotensive rats.

7. The mechanism of camboginol and morelloflavone actions is an endothelium dependent mechanism, mainly an endothelium NO signaling pathway.

These findings suggested that camboginol and morelloflavone exerted hypotensive and diuretic effects in 2K1C hypertensive rats. These effects associated with their antioxidant property. Camboginol and morelloflavone may have a potential as anti-hypertensive drug. However, an application of these plant bioactive compounds as an alternative treatment of vascular hypertension requires further pharmacological studies regarding its effects in resistant vessels and intentsive cellular mechanisms of its action.

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APPENDICES

APPENDIX A

Supplementary data

Supplementary 1. Effect of camboginol and morelloflavone on renal function.

Table Suppl. 1. Effect of camboginol on renal function in sham operative (SO) and 2-kidneys-1-clip (2K1C) rat which treated with either camboginol (C) or vehicle (V).

Paremeters	SO+V	SO+C	2K1C+V	2K1C+C
MABP (mm Hg)	135±3	135±6	152±2*	152±4
ERPF (mL/min/g KW)	3.63±0.36	3.69±0.35	2.29±0.25*	3.88±0.37$^{\#}$
RPF (mL/min/g KW)	3.99±0.39	4.06±0.39	2.52±0.28*	4.26±0.4$^{\#}$
RVR (RU)	39±4	35±2	70±6*	41±3$^{\#}$
GFR (mL/min/g KW)	1.43±0.12	2.14±0.07*	1.52±0.20	2.03±0.05$^{\#}$
FF (GFR/RPF)	0.36±0.02	0.55±0.05*	0.63±0.10*	0.50±0.04
$\dot{V}$ (μL/min/g KW)	8.79±0.97	42.52±5.41*	11.49±0.65*	68.08±5.10$^{\#}$
U_{Osm} (mOsm/kg H_2O)	1425±46	593±29*	1240±49	558±30$^{\#}$
P_{Osm} (mOsm/kg H_2O)	326±3	311±3*	330±4	310±2$^{\#}$
C_{Osm} (μL/min/g KW)	51.1±4.7	78.3±7.5*	75.6±6.2*	154.0±6.3$^{\#}$
C_{Osm}/GFR (%)	3.79±0.48	3.65±0.32	6.25±0.93*	9.28±0.48$^{\#}$
TC_{H_2O} (μL/min/g KW)	41.0±4.1	36.6±4.1	56.2±3.5*	65.9±2.2$^{\#}$

Data are mean±S.E.M.

$^{*, \#}p<0.05$ compared with SO+V and 2K1C+V group, respectively.

Table Suppl. 2. Effect of moreloflavone on renal function in sham operative (SO) and 2-kidneys-1-clip (2K1C) rat which treated with either camboginol (C) or vehicle (V).

Paremeters	**SO+V**	**SO+M**	**2K1C+V**	**2K1C+M**
MABP (mm Hg)	135±3	132±2	152±2*	138±6$^{\#}$
ERPF (mL/min/g KW)	3.63±0.36	3.96±0.28	2.29±0.25*	3.44±0.49$^{\#}$
RPF (mL/min/g KW)	3.99±0.39	4.36±0.31	2.52±0.28*	3.78±0.54$^{\#}$
RVR (RU)	39±4	35±2	70±6	45±6$^{\#}$
GFR (mL/min/g KW)	1.43±0.12	1.72±0.14*	1.52±0.20	1.44±0.08
FF (GFR/RPF)	0.36±0.02	0.45±0.05*	0.63±0.10*	0.42±0.06$^{\#}$
$\dot{V}$ (μL/min/g KW)	8.79±0.97	44.65±4.34*	11.49±0.65*	42.00±9.36$^{\#}$
U_{Osm} (mOsm/kg H_2O)	1426±46	613±41*	1240±49	670±84$^{\#}$
P_{Osm} (mOsm/kg H_2O)	326±3	321±2	330±4	317±4
C_{Osm} (μL/min/g KW)	51.1±4.7	87.5±7.2*	75.6±6.2*	106.3±11.4$^{\#}$
C_{Osm}/GFR (%)	3.79±0.48	4.97±1.06	6.25±0.93*	7.34±0.61
TC_{H_2O} (μL/min/g KW)	41.0±4.1	31.7±3.8	56.2±3.5*	40.36±3.76$^{\#}$

Data are mean±S.E.M.

$^{*, \#}p<0.05$ compared with SO+V and 2K1C+V group, respectively.

Supplementary 2. Contraction responses to phenylephrine (PE)

The contractile responses to cumulative dose of PE (10^{-8}-10^{-4} M) was significantly augmented in endothelium-denuded aortic rings (94.2±9.7-152.6±6.8%; pD_2, 8.61±0.15) compared with the endothelium-intact rings from SO rats (98.2±11.1-121.93±13.5%; pD_2, 8.38±0.17; $p<0.05$, Figure Suppl. 1a). In 2K1C, the contractile responses to PE at dose of 10^{-8} M was significantly augmented in endothelium-denuded aortic rings (87.4±10.4 %; pD_2, 8.60±0.13) when compared with the endothelium-intact from 2K1C rats (57.1±11.6%; pD_2, 8.19±0.13; $p<0.05$) (Figure Suppl. 1b). The contractile responses to PE (10^{-7}-10^{-5} M) was significantly augmented in the intact-endothelium aortic rings from 2K1C rats (134.8±8.2 - 165.2±9.1%; pD_2, 8.19±0.13) in comparison to the rings from 2K1C rats (98.2±11.1-126.1±11.4%; pD_2, 8.38±0.17; $p<0.05$, Figure Suppl. 1c). However, the contractile response to PE between the endothelium-denuded ring from 2K1C and SO rats were not different (Figure Suppl. 1d).

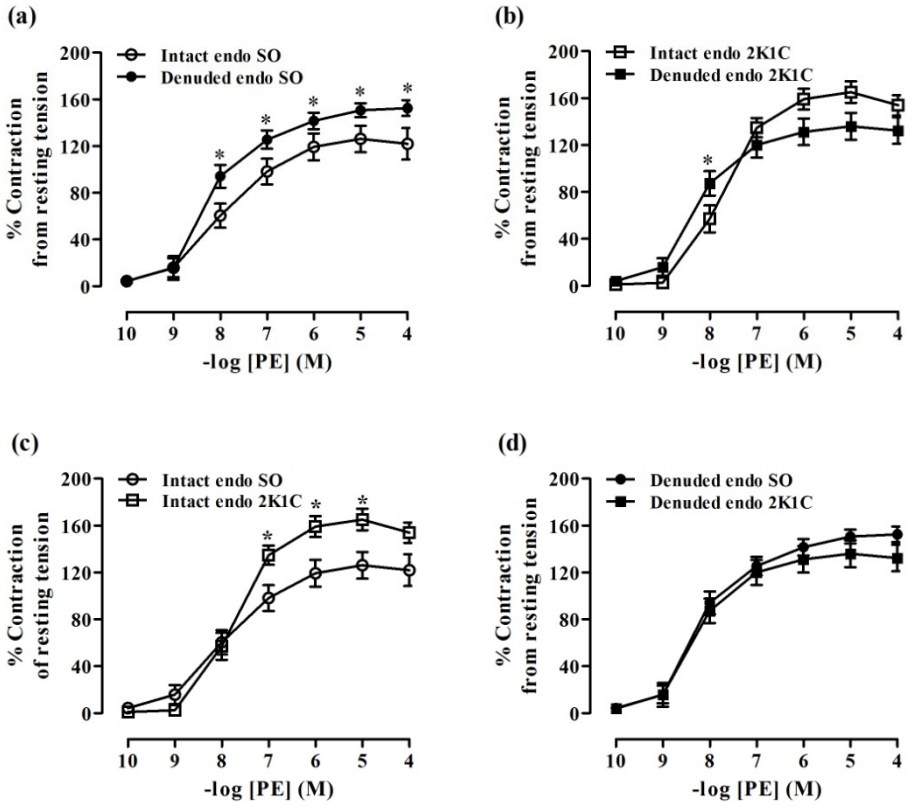

Figure Suppl. 1. Effects of phenylephrine (PE; 10^{-10}-10^{-4} M)) on vasoconstriction of endothelium-intact vs. -denuded aortic ring from sham operative (SO) group (a), endothelium-intact vs. -denuded aortic ring from 2-kidneys-1-clip (2K1C) group (b), endothelium-intact aortic rings between 2K1C and SO group (c) and endothelium-denuded aortic rings between 2K1C and SO group (d).

Data are mean ± S.E.M.

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

Supplementary 3. Vasorelaxation responses to acetylcholine (ACh)

The relaxation responses to cumulative dose of ACh (10^{-10}-10^{-4} M) were significantly higher in endothelium-intact aortic rings from SO group than those in response to vehicle (10.2±3.1-77.2±4.5 vs. 1.0±0.2-10.7±0.4%, respectively, $p<0.05$, Figure Suppl. 2a). There was not found the difference between the responses to ACh and vehicle in endothelium-denuded aortic rings from SO group (Figure Suppl. 2b).

The relaxation responses to cumulative dose of ACh (10^{-6}-10^{-4} M) were significantly higher in endothelium-intact aortic rings from 2K1C group than those in response to vehicle (55.9±6.5-66.4±8.1 vs. 12.3±4.0-13.8±4.7%, respectively, $p<0.05$, Figure Suppl. 2c). There was not found the difference between the responses to ACh and vehicle in endothelium-denuded aortic rings from 2K1C group (Figure Suppl. 2b).

The relaxation responses to cumulative dose of ACh (10^{-8}-10^{-7} M) in endothelium-intact rings from 2K1C group were significantly less than those of SO group (7.6±3.5-23.2±8.7%; pD_2, 5.71±0.47 vs. 14.5±4.1-53.3±1.3%; pD_2, 6.98±0.08, respectively, $p<0.05$, Figure Suppl. 2e). In contrast, the vasoconstriction responses to cumulative dose of ACh (10^{-11}-10^{-4} M) in endothelium-denuded aortic ring from SO group were significantly higher than those from 2K1C group (7.5±1.7-6.4±0.6 vs. 1.5±0.8-19.4±2.7%, respectively, $p<0.05$, Figure Suppl. 2f).

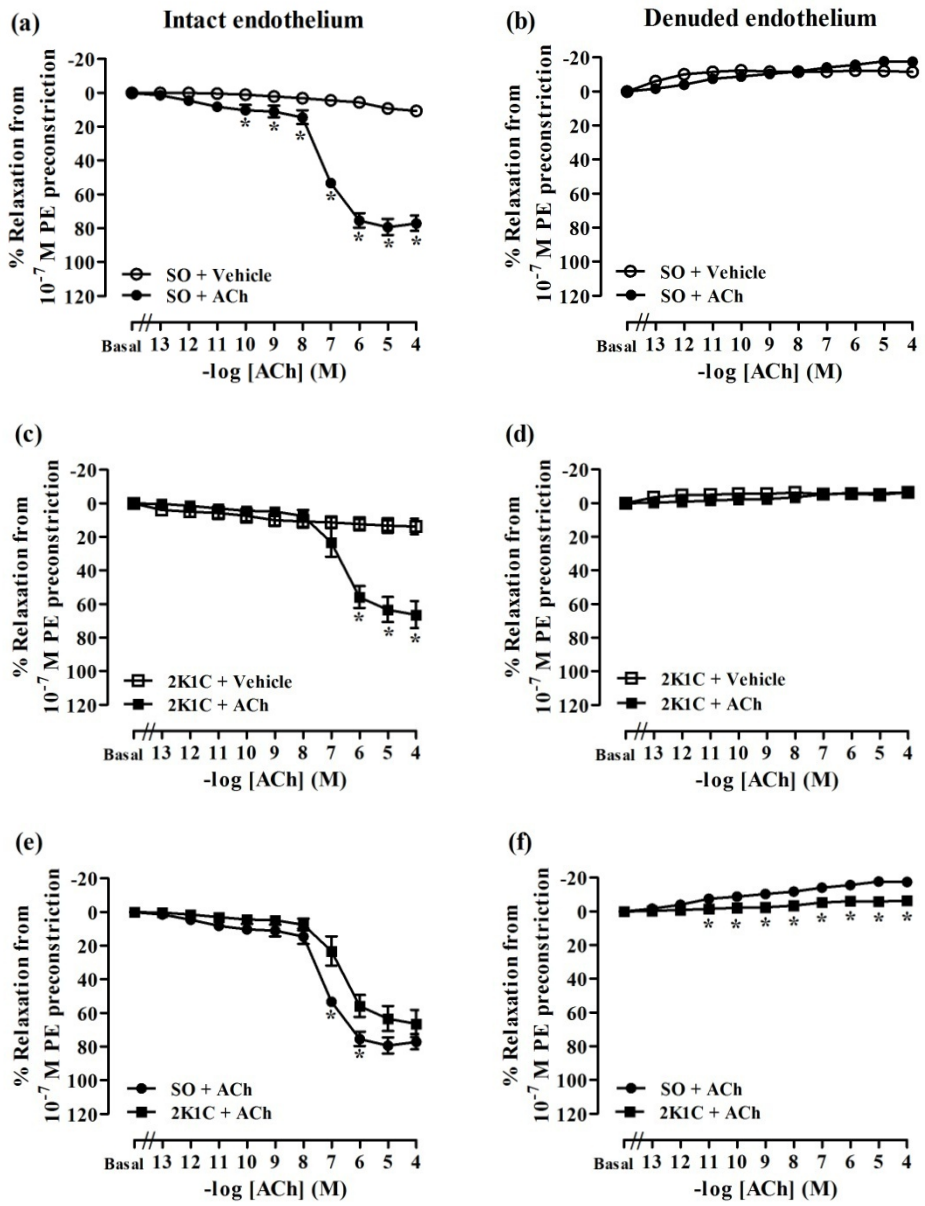

Figure Suppl. 2. Effects of acetylcholine (ACh) or vehicle on vasorelaxation of endothelium-intact (left panel; a, c, e) or -denuded (right panel, b, d, f) aorta rings from 2-kidneys-1-clip (2K1C) or sham operative (SO) groups.

Data are mean ± S.E.M.

*p<0.05 compared with respective control group.

Supplementary 4. Vasorelaxation responses to sodium nitroprusside (SNP)

The relaxation responses to cumulative dose of SNP (10^{-13}-10^{-4} M) were significantly higher in endothelium-intact aortic rings from SO group than those in response to vehicle (31.6±5.9-98.0±0.8 vs. 1.0±0.2-10.7±0.4%, respectively, $p<0.05$, Figure Suppl. 3a). The SNP (10^{-12}-10^{-4} M) also induced vasorelaxation in endothelium-denuded aortic rings from SO groups (1.04±1.03-102.9±3.0%; Figure Suppl. 3b).

The relaxation responses to cumulative dose of SNP (10^{-13}-10^{-4} M) were significantly higher in endothelium-intact aortic rings from SO group than those in response to vehicle (29.9±6.3-99.01±3.0 vs. 3.9±2.8-13.8±4.7%, respectively, $p<0.05$, Figure Suppl. 3c). The SNP (10^{-9}-10^{-4} M) also induced vasorelaxation in endothelium-denuded aortic rings from SO groups (7.8±2.2-105.4±3.1%; Figure Suppl. 3d).

The relaxation responses to cumulative dose of SNP (10^{-13}-10^{-4} M) between endothelium-intact aortic rings from 2K1C and SO group were no different (Figure Suppl. 3e). In endothelium-denuded aortic rings, the relaxation responses to SNP (10^{-9}-10^{-8} M) in 2K1C were significantly less than those in SO group (7.8±2.2-70.9±7.1%; pD_2, 8.27±0.12 vs. 30.0±8.8-87.3±1.2%; pD_2, 8.74±0.14, respectively, $p<0.05$, Figure Suppl. 3f).

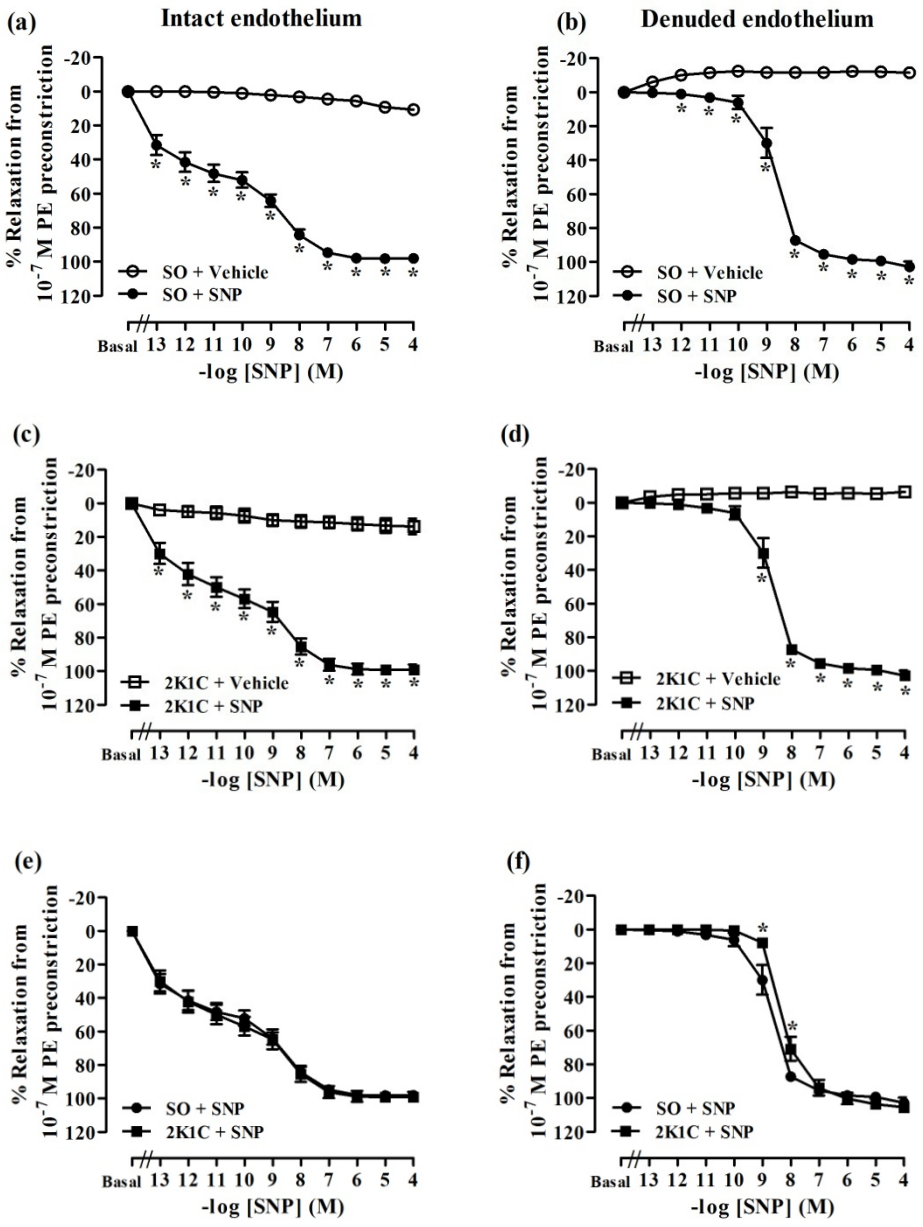

Figure Suppl. 3. Effects of sodium nitroprusside (SNP) or vehicle on vasorelaxation of endothelium-intact (left panel; a, c, e) or -denuded (right panel, b, d, f) aorta rings from 2-kidneys-1-clip (2K1C) or sham operative (SO) groups.

Data are mean ± S.E.M.

*p<0.05 compared with respective control group.

APPENDIX B

Determination of inulin concentration

(Davidson et al., 1963)

Preparation of anthrone reagent

1. Slowly added 100 mL of H_2SO_4 to 26 mL of iced-cooled DW in a flask immersed in an ice-bath. The mixture was allowed to cool.
2. Added 147 mg of anthrone.
3. Stir until the mixture completely dissolved.

Preparation of plasma protein

1. Mixed 30 mL of plasma with 1.5 mL of trichloroacrtic acid (CCl_3COOH).
2. Mixed well and stand for 10 minutes, shaking again at least once.
3. Centrifuge at 4,000 rpm for 20 minutes to precipitated protein.

Procedure

1. Mixed 500 µL of distilled water (use as Blank), inulin standard solution, plasma filtrate and diluted urine in to cold test tubes, which the bottom of tubes are completed immersed in ice-bath. All determinations were done in duplicated.
2. Added 300 µL of anthrone reagent, mix well. Cool in ice-bath before its place in water bath.
3. Placed in water bath at 38 $^{\circ}$C for 50 minutes.
4. The concentration of inulin is determined directly from standard curve for range of 1 to 6.8 mg% of fructose.

APPENDIX C

Determination of para-aminohippuric (PAH) acid concentration (Smith et al., 1945)

Reagents

1. 0.1% sodium nitrate ($NaNO_2$)
   Added 0.1 g of $NaNO_2$ into DW and diluted to make 100 mL solution.
2. O.5% ammonium sulfamate ($H_6N_2O_3S$)
   Added 0.5 g of $H_6N_2O_3S$ into DW and diluted to make 100 mL solution.
3. 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride ($C_{12}H_{16}Cl_2N_2$)
   Added 0.1 g of $C_{12}H_{16}Cl_2N_2$ into DW and diluted to make 100 mL solution.
4. 3.2% trichoroacetic acis (CCl_3COOH)
   Added 3.2 g of CCl_3COOH into DW and diluted to make 100 mL solution.
5. 0.2N hydrochloric acid (HCl)
   Diluted 1.67 mL of HCl with DW, made up to 100 mL solution.

Procedure

1. Added 50 µL of PAH standard solution, plasma and diluted urine into 1,100 µL of 3.2% trichloroacetic acid. Mixed well and after about 10 minutes, centrifuged plasma for 4 minutes at 4,000 rpm to precipitate protein. All determinations were done in duplicate.
2. Pipetted 1,000 µL of 3.2% trichoroacetic acid (blank) and solution from (1) into test tubes.
3. Added 200 µL of 0.2 N HCl and 0.1 mL of 0.1% $NaNO_2$. Mix thoroughly, let stand not less than 3 min or more than 5 minutes after $NaNO_2$.
4. Added 100 µL of 0.5% $H_6N_2O_3S$. Mixed and let stand not less than 3 min or more than 5 minutes later.
5. Added 100 µL of 0.1% $C_{12}H_{16}Cl_2N$ and Mixed well.
6. Let stand at room temperature for 15-60 min.
7. Measured the developed color by the spectrophotometer at 540 nm.
8. The concentration of PAH is determined directly from standard curve for the range of 1-10 mg% of PAH.

APPENDIX D

Method for the determination of malondialdehyde content plasma (Modified from Ohkawa et al. 1979)

Solutions

1. 8.1% sodium dodecyl sulfate ($C_{12}H_{25}NaO_4S$)
   Added 8.1 g of $C_{12}H_{25}NaO_4S$ into distilled water and made up to 100 mL.
2. 20% acetic acid
   Diluted 20 mL acetic acid with distilled water and made up to 100 mL.
3. 0.8% thiobarbituric acid (TBA)
   Added 0.8 g of TBA into distilled water, heated, stir and made up to 100 mL.
4. 1.5 N sodium hydroxide (NaOH)
   Added 6.122 g of NaOH into distilled water and made up to 100 mL.
5. N-nutanol

Procedure

1. Added 0.8 mL of distilled water, malonaldehyde bis-dimethyl acetal (MDA) solution and plasma sample into test tubes for blank, standard and sample, respectively. All determinations were performed in triplicate.
2. Added 0.2 mL of 8.1% $C_{12}H_{25}NaO_4S$ and 1.5 mL of 20% acetic acid, mixed well.
3. Adjusted the pH of mixture to 3.5 with NaOH.
4. Added 1.5 mL of 0.8% TBA and made up to 4.0 mL with distilled water, mixed well.
5. Heated in 95°C water bath for 60 min.
6. Cooled down in ice bath.
7. Added 1.0 mL of distilled water and 5.0 mL of n-butanol and mixed.
8. Centrifuged at 4,000 rpm for 10 min.
9. Read absorbance of upper organic layer at 532 nm.
10. The concentrations of MDA in plasma are determined from standard curve of MDA for 0.25, 0.50, 0.75, 1.00 and 1.25 x 10^{-5} M, respectively.

APPENDIX E

Immunohistochemistry of eNOS expression in rat aorta

(Floating technique)

Reagents

1. 4% buffered parafomaldehyde
2. Cryoprotectant buffer
3. Immunobuffer
4. 10% Normal goat serum (NGS)
5. Tris phosphate buffered solution (TPBS)
6. Rabbit anti-eNOS primary antibody
7. Biotinylated goat anti-rabbit IgG secondary antibody
8. Standard ABC peroxidase staining kits (ABC kit)
9. Metal enhanced DAB substrate kits (DAB kit)

Procedure

1. Fixed the thoracic aortic segments (5 mm) in 4% buffered parafomaldehyde overnight at room temperature.
2. Cut the sections of 10 μm on a cryostat and stored the segtions in cryoprotectant buffer at -20°C until analysis.
3. Washed the sections by allowing the section to swirl in the immunobuffer (IB) for 10 min x 3 times.
4. Incubated the sections with 3% peroxidase block for 15 minutes with gentle shaking and washed the sections with IB for 10 min x 3 times.
5. Blocked the section with 10% NGS in IB for 30 min with gentle shaking.
6. Incubated the sections with rabbit anti-eNOS primary antibody (1:500) for 24 h at 4°C with gentle shaking.
7. Washed the sections with TPBS for 10 min x 3 times and incubated the sections with biotinylated goat anti-rabbit IgG secondary antibody (1:500) for 1 h at room temperature with gentle shaking. Covered the section from light.

8. Washed the sections with TPBS for 10 min x 3 times and stained the section with ABC kit for 30 min at room temperature with gentle shaking. Covered the section from light.
9. Washed the sections with TPBS for 10 min x 3 times and counter stained the section with DAB kit for 10 min at room temperature with gentle shaking. Covered the section from light.
10. Washed the sections with TPBS for 10 min x 3 times and mounted the sections onto slide. Allowed the section dry and covered from light. Covered slip section with Per Mount.

VITAE

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Academic Background

Degree	Name of Institution	Year of Graduation
Bachelor of Nursing	Mahidol University	2004
Master degree of Sciences (Physiology)	Mahidol University	2010
Doctor of Philosophy (Physiology)	Prince of Songkla University	2016

Scholarship Awards During Enrolment

- PSU- Ph.D. Scholarships, Prince of Songkla University.
- PSU Graduated Scholarships for Research, Prince of Songkla University.
- The Scholarships for Oversea Reseaech, Faculty of Sciences, Prince of Songkla University.
- The Scholarships of Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission.

List of Publications

Nattaya Thongsepee, Wilawan Mahabusarakam, Siriphun Hirunyachattada. Diuretic and hypotensive effect of morelloflavone from *Garcinia dulcis* in two-kidneys-one-clip hypertensive rat. *Journal Sains Malaysiana* 2017 (accepted).

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hypertensive rat. Proceeding in Burapha University International Conference 2017. 3-4 August 2017, Pattaya, Thailand (submitted).

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List of Presentations

Abstract & Oral Presentation

Nattaya Thongsepee, Wilawan Mahabusarakam, Siriphun Hiranyachattada. Diuretic and hypotensive effect of morelloflavone from *Garcinia dulcis* in 2K1C renovascular hypertensive rat. 113th TRF Seminar Series in Basic Research: From Basic Research to Application. 25 September 2015. Faculty of Science, Prince of Songkla University, Thailand.

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Wilawan Mahabusarakam, Ilham Abdullah, Nattaya Thongsepee, Siriphun Hiranyachatada. Biflavonoids from the green branches of *Garcinia dulcis* and hypotensive effect of morelloflavone. 38th IUPS World Congress: Rhythms of Life (IUPS 2017), 1-5 August 2017, Rio De Janeiro, Brazil