Oxidative Stress and Cyclo-oxygenase-2 Mediate Endothelial Dysfunction in Diabetes and Hypertension

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DECLARATION

The experiments described in this dissertation were carried out in the Department of Physiology, the Chinese University of Hong Kong, between September 2005 and June 2009. This work is solely that of the author. No part of this dissertation is being concurrently submitted for any other degree, diploma or other qualification at this or any other institutions.

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Through gradual transformation from being a layman of cardiovascular research to an enthusiastic young researcher who always seeks to gain great knowledge about vascular biology and medicine, I have been fascinated deeply by a magic power of endothelial cells to maintain vascular homeostasis over the past four years during which I spared no effort to run several independent research projects with real self-satisfaction. While waiting for confirmation of the clinical relevance, a number of novel findings obtained from the present studies allow me to write up this dissertation. I have to say none of this could be made possible without a great help from people around me. My supervisor Yu Huang has been an amazing inspiration to me, with his serious and enthusiastic attitude towards science and seemingly unlimited energy. I am most grateful to his guidance and genuine interest in my personal development over the years. I indeed enjoy "hot" discussion and change views with him via thousands of e-mail communications and face-to-face conversation almost on a daily basis including many Sundays. The knowledge and experience acquired have certainly widened my horizon and readied myself to take challenges in my future research career.

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ABSTRACT

Vascular tone is locally regulated by endothelial cells through the synthesis and release of endothelium-dependent relaxing factors (EDRFs) and endothelium-dependent contracting factors (EDCFs). A delicate balance of EDRFs and EDCFs is essential for maintaining healthy vascular function. Endothelial dysfunction, characterized by a diminished nitric oxide (NO) bioavailability as a result of reduced release and/or production of NO, increased inactivation of NO, and elevated production of EDCFs and reactive oxygen species (ROS). Endothelial dysfunction set motion of a signaling cascade of intracellular events to the development of hypertension and atherosclerosis. The degree of endothelial dysfunction could predict the severity and perhaps pace of progression towards end organ damage related to vasculopathy.

ROS overproduction is intimately associated with the activated renin-angiotensin system (RAS) which causes oxidative stress. Although the RAS actively participates in the initiation and progression of vascular dysfunction under several pathological situations, the cellular links of the RAS components to other well-recognized and newly emerging pro-inflammatory biomarkers are not fully understood. In order to delineate clearer cellular mechanisms and potential novel prognostic markers involved in endothelial dysfunction, the present study aimed at investigating the contribution of oxidative stress, bone morphogenic protein 4 (BMP4), and cyclooxygenase-2 (COX-2) in the development of endothelial dysfunction in diabetes and hypertension. To achieve this main objective, a combination of experimental techniques together with the use of mouse and rat models of both diabetes and hypertension were used. Chronic drug treatment was initiated to verify some of the main findings in vitro, which helped to appreciate the pathological role of various harmful factors in vascular dysfunction. I also managed to link my new findings to the relevance of human diabetes and hypertension. This thesis consists of results obtained from four related projects.

Firstly, I employed type 2 diabetic db/db mice and examined in relative details an essential contribution of the RAS and associated oxidative stress to endothelial dysfunction in diabetes. Acetylcholine-induced endothelium-dependent dilatations (EDR) in both conduit arteries (aortas) and resistance-sized vessels (second-order small mesenteric arteries) were severely impaired in company with increased

expression of angiotensin-converting enzyme (ACE), angiotensin II, angiotensin type 1 (AT1) receptor, NAD(P)H oxidase subunits, ROS overproduction and nitrotyrosine (an index for oxidative stress). Acute treatment with AT1 receptor blocker, NAD(P)H oxidase inhibitor, and ROS scavengers significantly rescued the endothelial function in diabetes. My results indicate that ROS is a key contributor to the reduced NO bioavailability. Chronic treatment with enalapril (ACE inhibitor) and valsartan (AT1 receptor blocker) reversed endothelial dysfunction and normalized the up-regulation of the RAS and oxidative stress, thus justifying the clinical efficacy of RAS blockade in reducing the risk for the development of end organ dysfunction.

Secondly, I examined the effect of direct renin inhibition by aliskiren on endothelial dysfunction in spontaneously hypertensive rats (SHR). I was able to provide the first line of evidence supporting the vascular benefit of aliskiren by showing (1) that chronic daily oral administration of aliskiren to SHR improved EDR and attenuated endothelium-dependent contractions (EDC) in both aortas and renal arteries; the vascular benefits were related to favourable modulation of the RAS; (2) that aliskiren suppressed the RAS-dependent oxidative stress and up-regulated the expression in superoxide dismutases, anti-oxidant enzymes; (3) that aliskiren normalized the reduced phosphorylation of eNOS at serine-1177; and (4) that aliskiren reduced the overexpression of the two pro-inflammatory factors, BMP4 and COX-2. The results of the present project provide novel findings that that increase the clinical prospective for aliskiren and other more specific renin inhibitors in the prevention and treatment of hypertension-related vascular dysfunction.

Thirdly, I set to study the role of COX-2 in the generation of EDCFs and endothelial dysfunction in a two-kidney/two-clips (2K2C) rat model of renovascular hypertension. Although NO is known to be the major EDRF for over two decades, the chemical identity of EDCF is still unclear and heterogeneity of EDCFs is noted depending on vascular beds and species. Despite of earlier reports suggesting COX-1-derived arachidonic acid metabolites and probably also ROS as EDCFs, the role of pro-inflammatory COX-2 in EDCF responses is elusive, particularly in hypertension and it was unclear how ROS might be involved in the up-regulated activity and/or expression of COX-2 in blood vessels. Therefore, the present study was designed to identify the most likely EDCF that mediates EDC which oppose the beneficial effects of EDRFs in the vascular wall in renovascular hypertension. I delineated a sequence of cellular events leading to the release of a major EDCF and to elucidate a

pathological role of the up-regulated COX-2 in the generation of EDCFs. The present results clearly demonstrate that the ROS-stimulated COX-2-derived $PGF_{2\alpha}$ plays a principal role in endothelial dysfunction by acting as an EDCF. My results also offer some new insights into molecular and cellular mechanisms by which endothelial dysfunction is induced and may provide experimental basis for developing new strategies for drug intervention against renovascular hypertension.

Finally, I investigated BMP4 and COX-2 could couple together to play a pathological role in endothelial dysfunction in hypertension. BMP4 impaired EDR, elicited EDC, and exaggerated ROS production in mouse aortas. Noggin, apocynin and tempol exhibited similar efficacies in restoring EDR, preventing EDC, and eliminating ROS overproduction. BMP4 up-regulated the COX-2 expression, which was prevented by noggin, apocynin or tempol, and BMP4 failed to impair endothelial function nor to trigger EDC in COX-$2^{-/-}$ mouse aortas. Celecoxib blocked the vascular effect of BMP4 without affecting BMP4-induced ROS overproduction and COX-2 up-regulation. Renal arteries from hypertensive rats and humans had higher levels of COX-2 and BMP4 and impaired EDR. Noggin improved EDR and reduced the expression of both proteins. p38 MAPK inhibitor SB202190 prevented COX-2 up-regulation and EDC in BMP4-treated aortas. Taken together, I revealed that ROS serves as a pathological link between BMP4 stimulation and the downstream COX-2 up-regulation in endothelial cells, leading to endothelial dysfunction through p38 MAPK activation. The ensuing COX-2-derived $PGF_{2\alpha}$ acts to reduce EDR and to trigger thromboxane prostanoid receptor-mediated EDC as the major EDCF. This BMP4/COX-2 signaling pathway could be critical in the maintenance of endothelial dysfunction in hypertension.

The research experience of the past four years have aroused my awareness of the complicity of pathological processes resulting in endothelial dysfunction in diabetes and hypertension and inspired my enthusiasm in vascular biology and medicine research. The novel findings from the four related projects allow me to conclude with confidence that BMP4 and RAS-dependent oxidative stress is closely coupled to the pathological involvement of COX-2 as a critical downstream enzyme in the development of vascular dysfunction and these signaling components continue to represent targets for effective therapeutic intervention in diabetes and hypertension.

論文摘要

內皮細胞通過生成並釋放內皮依賴性舒張因子(EDRF)與內皮依賴性收縮因子(EDCF)來調節血管張力。EDRF 與 EDCF 之間的平衡對維持正常血管功能具有重要意義。 血管內皮功能障礙以一氧化氮(NO)產生與釋放的量減少，以及 EDCF 與氧自由基(ROS)的增加導致 NO 生物利用度減少為主要特徵。內皮細胞功能障礙引發一系列細胞內信號傳遞的改變，而與高血壓和動脈硬化密切相關。內皮功能障礙的程度能預測血管病變導致靶器官損害的進展與嚴重程度。

ROS 的增加與腎素-血管緊張素系統(RAS)的活化導致的氧化應激密切相關。雖然已知 RAS 參與了許多疾病中血管功能障礙的發生，RAS 系統中各組成部分與已知的或新發現的炎前因子之間的聯系未完全明了。為了更清楚闡明導致內皮細胞功能障礙的分子機制與可能的新預測指標，本研究的主要目的是：探討糖尿病與高血壓導致的內皮功能障礙中氧化應激，骨形態發生因子(BMP4)，與環氧化酶 2 (COX-2)的參與與作用。為此，本研究使用了大鼠與小鼠的高血壓與糖尿病的幾種實驗模型。為了驗證某些體外研究的重要發現，部份實驗中使用了慢性藥物治療。另外，我也成功的將一些新的發現與人類糖尿病與高血壓的血管病變聯系在一起。本論文包括四個相關實驗項目的結果。

第一，我用二型糖尿病小鼠 db/db 來研究 RAS 及其相關的氧化應激參與糖尿病內皮功能障礙的作用。在 db/db 小鼠的主動脈與腸系膜阻力血管中，乙酰膽鹼引起的內皮依賴性舒張(EDR)明顯減少，伴隨血管緊張素轉化酶(ACE)，血管緊張素(Ang II)，血管緊張素一型受體(AT_1R)，及 NAD(P)H 氧化酶的表達上調和硝基酪氨酸(nitrotyrosine)(氧化應激的主要指標之一)形成增加。AT_1 受體阻斷劑，NAD(P)H 氧化酶抑制劑及 ROS 清除劑可顯著改善糖尿病血管的內皮功能。以上實驗結果顯示，ROS 導致 NO 生物活性下降的主要原因。使用 ACE 抑制劑 enalapril 與 AT_1 受體阻斷劑 valsartan 的慢性治療可保護內皮功能，使 RAS 與氧化應激的上調正常化，從而支持了 RAS 阻斷劑可減輕糖尿病靶器官損害的臨床療效。

第二，我研究了 aliskiren 直接抑制腎素對自發性高血壓大鼠(SHR)的血管內皮功能的作用。此實驗初次驗證了 aliskiren 對血管的保護作用：1) aliskiren 慢性口服治療改善了 SHR 主動脈與腎內動脈的內皮依賴性舒張反應(EDR)並減少內皮依賴性收縮反應(EDC)，這些作用與 aliskiren 對 RAS 的調節有關；2) aliskiren 抑制 RAS 產生的氧化應激，使超氧化物歧化酶 SOD 表達上調；3) aliskiren 使 SHR 中減少的內皮性一氧化氮合成酶(eNOS)的磷酸化增加到正常水平；4) aliskiren 減少炎前因子 BMP4 與 COX-2 表達上調。本實驗為 aliskiren 及其他腎素抑制劑在高血壓相關血管功能失調的臨床效用提

供了新的證據。

第三，我研究了 COX-2 產生的 EDCF 在腎原性高血壓的雙腎雙夾(2K2C)大鼠模型中內皮功能障礙的角色。雖然 NO 與 EDRF 的研究較多，EDCF 的確切成份仍不清楚。雖然有研究顯示 COX-1 產生的花生四烯酸(arachidonic acid)代謝產物與 ROS 都可能起到 EDCF 的作用， COX-2 作為炎前因子引起的 EDCF 反應仍未被充分了解，尤其在高血壓中，ROS 可能促進 COX-2 在血管壁內的表達與活性增加的細胞機制也不清楚。因此，本實驗具體研究了腎原性高血壓中確定導致 EDC 並對抗 EDRF 作用的最有可能的 EDCF。為此，本實驗研究了一系列導致 EDCF 釋放的細胞內反應，並闡明了 COX-2 上調產生 EDCF 的病理角色，從而揭示了 ROS 刺激 COX-2 產生的前列腺素($PGF_{2\alpha}$)作為主要的 EDCF 而引起內皮功能障礙。這些結果為腎原性高血壓中內皮功能障礙的細胞與分子機制提供了新的證據，為發展新藥提供了實驗基礎。

最後，我詳細研究了 BMP4 與 COX-2 在高血壓中內皮功能障礙的共同作用。BMP4 可直接減少小鼠主動脈的 EDR，引起 EDC，以及增加 ROS 產生。BMP4 拮抗劑 noggin，NAD(P)H 氧化酶抑制劑 apocynin 和 ROS 清除劑 tempol 可改善 BMP4 處理後的 EDR，減低 EDC，抑制 ROS 形成。在 COX-2 基因敲除小鼠的主動脈中，BMP4 不起作用，而且 COX-2 抑制劑 celecoxib 可阻斷 BMP4 對血管的損害作用但不會影響 BMP4 引起的 ROS 增加與 COX-2 表達上調。SHR 大鼠的腎內動脈與高血壓病人的腎血管的 COX-2 與 BMP4 表達水平均上調，其血管舒張功能減弱，可被 noggin 處理而改善。Noggin 也可改善 BMP4 引起的血管內皮功能損傷並抑制 BMP4 與 COX-2 的上調。此外，p38 MAPK 抑制劑 SB202190 可改善 EDR，抑制 EDC 與 COX-2。這些結果顯示在內皮細胞中， BMP4 作為上游調控信號，通過 ROS 增加與激活 MAPK 而引起 COX-2 表達上調，導致內皮細胞功能障礙。其中 COX-2 產生的 $PGF_{2\alpha}$ 作為主要的 EDCF 作用於血栓素(thromboxane)受體引起 EDC 並減少 EDR。BMP4/COX-2 信號通路對產生與維持高血壓血管內皮功能障礙具有關鍵作用。

綜上所述，此論文的四項相關實驗研究清楚提示 BMP4 與 RAS 相關的氧化應激引起的 COX-2 在血管功能障礙的關鍵作用，這些信號傳遞為高血壓與糖尿病血管病變的治療途徑提供了新的作用機理與可能的藥物靶點。

ABBREVIATIONS

ACh	Acetylcholine
Ang I	Angiotensin I
Ang II	Angiotensin II
AT1R	Angiotensin II type 1 receptor
AT2R	Angiotensin II type 2 receptor
ACE	Angiotensin converting enzyme
ARB	Angiotensin receptor blocker
BMP	Bone morphogenic protein
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DETCA	Diethyldithiocarbamate acid
DHE	Dihydroethidium
DMSO	Dimethyl sulfoxide
EDC	Endothelium-dependent contractions
EDCF	Endothelium-derived contracting factors
EDHF	Endothelium-derived hyperpolarizing factors
EDR	Endothelium-dependent dilatations
EDRF	Endothelium-derived relaxing factors
eNOS	Endothlial nitric oxide synthase
EPR	Electron paramagnetic resonance
HX-XO	Hypoxanthine-xanthine oxidase
L-NAME	N^G-nitro-L-arginine methyl ester
MAPK	Mitogen activated protein kinase
NO	Nitric oxide
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGI_2	Prostacyclin
RAS	Renin-angiotensin system
RNAi	RNA interference
ROS	Reactive oxygen species
RHR	Renovascular hypertensive rats
SHR	Spontaneously hypertensive rats
SOD	Superoxide dismutase
SNP	Sodium nitroprusside
TGF-β	Transforming growth factor-β
TP receptor	Thromboxane prostanoid receptor
TXA_2	Thromboxane A_2
VSMCs	Vascular smooth muscle cells
VAS	Valeryl salicyclate
WKY	Wistar Kyoto rats

AWARDS AND PUBLICATIONS

1. ACADEMIC AWARDS AND TRAVEL GRANTS

1. 2nd Prize for Young Investigator Award Competition (Oral) at 11th Hong Kong Diabetes and Cardiovascular Risk Factors, East Meets West Symposium (30 Sept-1 October 2009)
2. Outstanding Award (Oral) of the 5th Hong Kong-Macau Postgraduate Symposium on Chinese Medicine (13 August 2009)
3. CUHK International Travel Grant for MOVD 2009 meeting in Japan (June 2009)
4. Young Excellent Paper Winner in "Pharmacology and Clinical Pharmacology Meeting between Three Parts of China on Both Sides of the Taiwan Straits: Mainland, Hong Kong and Taiwan", Shenyang, China (10th October 2008)
5. Travel grant from Hong Kong Pharmacology Society (December 2008)
6. 2nd Prize of Outstanding Oral Presentation for the Young Investigators' Award Competition at the 3rd Scientific Meeting of the Asian Society for Vascular Biology, Singapore (5th August 2008)
7. Outstanding Postgraduate Student Oral Presentation Award in the 10th Scientific Meeting of Hong Kong Pharmacology Society (15th December 2007)
8. 3rd Prize of Outstanding Poster Presentation at the 11th Annual Scientific Meeting of the Institute of Cardiovascular Science and Medicine, the University of Hong Kong (8th December 2007)
9. Travel grant from Hong Kong Pharmacology Society (November 2007)
10. Outstanding Award (Oral) of the 3rd Hong Kong-Macau Postgraduate Symposium on Chinese Medicine (16th August 2007)
11. 2nd Prize of Young Investigator Award Competition, 6th Scientific Conference on Cardiovascular Sciences Across the Strait, Urumqi, Xinjiang (29th July 2007)

2. SCIENTIFIC MEETINGS ATTENDED WITH ORAL PRESENTATIONS

1. 11th Hong Kong Diabetes and Cardiovascular Risk Factors, East Meets West Symposium. October 2009. *Oral Presentation for Young Investigator Award Competition*
2. 2009 Hong Kong-Macau Postgraduate Symposium on Chinese Medicine. Hong Kong, August 2009. *Oral Presentation Competition.*
3. 10th International Symposium on Mechanisms of Vasodilatation. Japan, June 2009. *Oral Presentation in "Endothelial Cells" Session.*
4. Annual Scientific Meeting of Hong Kong Society of Endocrinology, Metabolism and Reproduction. Hong Kong, November 2008. *Oral Presentation Competition.*
5. Pharmacology and Clinical Pharmacology Meeting between Three Parts of China on Both Sides of the Taiwan Straits: Mainland, Hong Kong and Taiwan. Shenyang, China, October 2008. *Oral Presentation Competition*
6. 3rd Scientific Meeting of the Asian Society for Vascular Biology. Singapore, August 2008. *Oral Presentation Competition.*
7. Faculty Research Day in Chinese University of Hong Kong. Hong Kong, July 2008. *Oral Presentation.*
8. International Symposium on Cardiovascular and Neurovascular Medicine (ISCNM) in conjunction with International Heart Failure Symposium. Hong Kong, February 2008. *Oral Presentation in "Hypertension and other cardiovascular risk factors" Session.*
9. 10th Annual Scientific Meeting of Hong Kong Pharmacology Society. Hong Kong,

December 2007. *Poster Presentation Competition.*

10. Tung Wah Group of Hospital Eddie Wang Symposium on Integrated Chinese and Western Medicine. Hong Kong, November 2007. *Oral presentation in Postgraduate Presentation Session.*
11. 2007 Hong Kong-Macau Postgraduate Symposium on Chinese Medicine. Hong Kong, August 2007. *Oral Presentation Competition.*
12. 6th Scientific Conference on Cardiovascular Sciences Across the Strait. Xinjiang, China, July 2007. *Oral Presentation Competition.*

3. PUBLICATIONS

Original research article

1. ***Wing WT**, Tian XY, Chen YC, Leung FP, Liu LM, Lee HK, Ng CF, Xu A, Yao X, Vanhoutte PM, Tipoe GL, Huang Y. Bone morphogenic protein-4 impairs endothelial function through oxidative stress-dependent cyclooxygenase-2 upregulation: implications on hypertension. ***Circulation Research*** (Accepted) (*Co-corresponding author)
2. **Wong WT**, Tian XY, Xu A, Ng CF, Lee HK, Chen ZY, Au, CL, Yao X, Huang Y (2010). Angiotensin II type 1 receptor-dependent oxidative stress mediates endothelial dysfunction in diabetic mice. ***Antioxidants & Redox Signaling*** *15;13(6):757-68.*
3. **Wong WT**, Wong SL, Tian XY & Huang Y (2010) Endothelial dysfunction: the common denominator in hypertension and diabetes. ***Journal of Cardiovascular Pharmacology.*** 55(4):300-7.
4. **Wong WT**, Ng CH, Tsang SY, Huang Y, Chen ZY (2009). Relative contribution of individual oxidized components in ox-LDL to inhibition on endothelium-dependent relaxation in rat aorta. ***Nutrition, Metabolism and Cardiovascular Diseases*** (In press)
5. **Wong WT**, Huang Y. Angiotensin AT2 receptor as a potential therapeutic target in hypertension (2009). ***Clinical and Experimental Pharmacology and Physiology*** **36**:3-4.
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8. Yang D, Luo Z, Ma S, **Wong WT**, Ma L, Feng X, Hao X, Cao T, Zhao Z, Liu D, Arendshorst WJ, Huang Y, Tepel M & Zhu Z (2010) Activation of TRPV1 by Dietary Capsaicin Improves Endothelium-Dependent Vasorelaxation and Prevents Hypertension. ***Cell Metabolism*** (*In Press*)
9. Han W, **Wong WT**, Tian XY, Huang Y, Wu L, Zhu D, & Gao PJ (2010) Contributory role of endothelium and voltage-gated potassium channels in apocynin-induced vasorelaxations. ***Journal of Hypertension*** (In press)

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11. Griffith JF, Wang YX, Zhou H, Kwong WH, **Wong WT**, Sun YL, Huang Y, Yeung DK, Qin L, Ahuja AT (2010). Likely causes of reduced bone perfusion in osteoporosis: novel findings in an ovariectomy rat model. ***Radiology*** 254(3):739-46.
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14. Yung LM, Leung FP, **Wong WT**, Tian XY, Yung LH, Chen ZY, Yao XQ, Huang Y (2008). Tea polyphenols benefit vascular function. ***Inflammopharmacology*** **16**:230-234.
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Manuscript recently submitted or under revision

19. **Wong WT,** Tian XY, Xu A, Yu J, Lau CW, Hoo RL, Wang Y, Lee VWY, Lam KS, Vanhoutte PM, Huang Y (2010) The obligatory role of adiponectin in the restoration of endothelial function in PPARγ agonist-treated diabetic mice. ***Cell Metabolism*** (Under revision)
20. Chang J, Li Y, Huang Y, Lam KS, Hoo RL, **Wong WT**, Cheng KY, Wang Y, Vanhoutte PM & Xu A. Adiponectin Prevents Diabetic Premature Senescence of Endothelial Progenitor Cells by Suppressing the p38 MAP kinase/p16INK4A Signaling Pathway. ***Diabetes*** (Resubmitted after revision)
21. **Wong WT**, Tian XY, Wang YX, Ng CF, Wang YX, Lee HK, Yao X, Au CL, Leung FP, Lau CW, Vanhoutte PM, Huang Y (2010) Oxidative Stress-dependent Cyclooxygenase-2-derived Prostaglandin F2α Impairs Endothelial Function in Renovascular Hypertensive Rats ***Hypertension*** (Submitted) (***Co-corresponding author**)
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CONTENTS

Declaration i
Acknowledgements ii
Abstract in English iii
Abstract in Chinese vi
Abbreviations viii
Publications and Awards ix
Contents xvii

CHAPTER I
INTRODUCTION

1.1 Endothelial cell function 1
1.1.1 Endothelium-derived relaxing factors 2
1.1.1.1 Nitric oxide (NO) 2
1.1.1.2 Prostacyclin (PGI_2) 2
1.1.1.3 Endothelium-derived hyperpolarizing factor (EDHFs) 3
1.1.2 Endothelium-derived contracting factors (EDCFs) 4
1.1.2.1 Reactive oxygen species (ROS) 4
1.1.2.1.1 Oxidant enzymes 5
1.1.2.1.2 Antioxidant enzymes 5
1.1.2.1.3 ROS and vascular tone 6
1.1.2.2 Endothelial prostaglandin synthase / cyclooxygenase (COX) 6
1.1.2.2.1 Endothelial COX-1 7
1.1.2.2.2 Endothelial COX-2 7
1.1.2.3 COX-derived prostanoids as EDCFs 7
1.1.2.4 Prostanoid receptors 9
1.1.3 Balance between EDRFs and EDCFs 9

1.2 Endothelial dysfunction 10
1.2.1 Endothelial dysfunction in diabetes 10
1.2.1.1 Oxidative stress in diabetic endothelial dysfunction 10
1.2.1.2 Reduced NO bioavailability in diabetes 11
1.2.1.3 Advanced glycation end products (AGEs) 12
1.2.2 Endothelial dysfunction in hypertension 12
1.2.2.1 Impaired endothelium dependent dilatations in hypertension 13
1.2.2.2 Endothelium-dependent contractions in hypertension 14

1.3 Renin-angiotensin system in diabetes and hypertension 15
1.3.1 Renin 17
1.3.1.1 Renin inhibitor 17
1.3.1.2 Renin inhibition and hypertension 17
1.3.1.3 Renin inhibition and diabetes 18
1.3.1.4 Renin inhibition and endothelial function 19
1.3.2 Angiotensin converting enzyme (ACE) 19
1.3.2.1 ACE inhibitors 19
1.3.2.2 ACE and hypertension 20

1.3.2.3 ACE and diabetes 20
1.3.3 Angiotensin receptors 20
1.3.3.1 Ang II type 1 receptor (AT_1R) 21
1.3.3.2 AT_1R inhibitors 21
1.3.3.3 AT_1R and hypertension 22
1.3.3.4 AT_1R and diabetes 22
1.3.3.5 AT_1R and NAD(P)H oxidases 22

1.4 Bone morphogenic protein-4 (BMP4) in hypertension and diabetes 23
1.4.1 BMP4 and shear stress 24
1.4.2 BMP4 and oxidative stress 24
1.4.3 BMP4 and hypertension 25
1.4.4 BMP4 and diabetes 25

1.5 Cyclo-oxygenase-2 (COX-2) in hypertension and diabetes 26
1.5.1 COX-2 and renovascular hypertension 27
1.5.2 COX-2 and diabetic vascular dysfunction 28
1.5.3 COX-2 inhibitors 29

1.6 Justifications, long-term impact and objectives of the present study 30

CHAPTER II
METHODS AND MATERIALS

2.1 Animals 32
2.1.1 Surgical procedures 32
2.1.2 Drug treatments in animal studies 32

2.2 Human artery specimen 33

2.3 Measurement of basic parameters 33
2.3.1 Blood pressure measurement 33
2.3.2 Oral glucose tolerance test 33
2.3.3 Plasma insulin 34
2.3.4 Wet weight for heart and kidney 34
2.3.5 Lipid profile 34
2.3.6 Plasma renin activity 34

2.4 Isometric force measurement 35
2.4.1 Experimental protocols 35

2.5 Western blotting 37

2.6 Immunohistochemistry 37

2.7 Detection of intracellular ROS by dihydroethidium fluorescence 38

2.8 Electron paramagnetic resonance (EPR) spectroscopy 39

2.9 Intracellular ROS measurement 40

2.10 Detection of prostanoids by enzyme immunoassay 40

2.11 Drugs, chemicals and other reagents 40
2.11.1 Chemicals 40
2.11.2 Composition of Krebs solution 42
2.11.3 Reagents for Western blot analysis 43
2.11.3.1 Reagents for sample preparation 43
2.11.3.2 Reagents for gel preparation (stacking and separating) 43
2.11.3.3 Buffers for electrophoresis, transfer, and washing 44
2.11.4 Primary antibodies 45

2.12 Statistical analysis 46

CHAPTER III
ANGIOTENSIN II TYPE 1 RECEPTOR-DEPENDENT OXIDATIVE STRESS MEDIATES DIABETIC ENDOTHELIAL DYSFUNCTION

3.1 Introduction 47

3.2 Methods and Materials 49
3.2.1 Animal model 49
3.2.2 Human renal arteries 49
3.2.3 Measurements of plasma lipid profile and insulin 49
3.2.4 Isometric force measurement 49
3.2.5 Detection of intracellular ROS by dihydroethidium (DHE) fluorescence 50
3.2.6 Immunohistochemical staining of Ang II 51
3.2.7 Western blotting 51
3.2.8 Organ culture of mouse arterial rings in high glucose medium 52
3.2.9 Spin trap and Electron paramagnetic resonance spectroscopy (EPR) .. 52
3.2.10 Drugs and solutions 53
3.2.11 Statistical Analysis 53

3.3 Results 54
3.3.1 Basic metabolic parameters 54
3.3.2 Effects of ARB and ROS scavengers on endothelium-dependent dilatations in conduit and resistance arteries 54
3.3.3 Improved endothelium-dependent dilatations in db/db mice by increasing NO bioavailability by chronic RAS blockade 55
3.3.4 Augmented ROS production in db/db mouse aortas mediated by AT_1R 55
3.3.5 Effects of RAS blockade on local production of Ang II in vascular wall .. 56
3.3.6 Western blot analysis of AT_1R, AT_2R, $p22^{phox}$, $p47^{phox}$, nitrotyrosine, eNOS and p-eNOS 56
3.3.7 Impaired endothelium-dependent relaxations in renal arteries from diabetic patients rescued by AT1R blockade 56
3.3.8 High glucose-induced endothelial dysfunction mediated by AT_1R 57

3.4 Discussion 80

CHAPTER IV
RENIN INHIBITION BY ALISKIREN AMELIORATES ENDOTHELIAL DYSFUNCTION IN SPONTANEOUSLY HYPERTENSIVE RATS

4.1 Introduction 85

4.2 Methods and Materials 88
4.2.1 Animals and drug treatment 88
4.2.2 Blood vessel preparation 88
4.2.3 Experimental protocol 89
4.2.4 Western blotting 89
4.2.5 Chemicals and drugs 90
4.2.6 Statistical analysis 90

4.3 Results 91
4.3.1 Antihypertensive effects of aliskiren in SHR 91
4.3.2 Improved endothelium-dependent dilatations in aortas from aliskiren-treated SHR 91
4.3.3 Effect of ROS scavengers on endothelium-dependent dilatations in aortas of aliskiren- and vehicle-treated rats 91
4.3.4 Effect of chronic aliskiren treatment on endothelium-dependent contractions in SHR aortas 92
4.3.5 Effect of aliskiren treatment on endothelium-dependent dilatations and contractions in intralobal renal arteries of SHR 92
4.3.6 Protein expression for RAS components, SOD, eNOS, COX and BMP4 93

4.4 Discussion 114

CHAPTER V
CYCLOOXYGENASE-2 DERIVED PROSTAGLANDIN $F_{2\alpha}$ MEDIATES ENDOTHELIUM-DEPENDENT CONTRACTIONS IN INTRALOBAL RENAL ARTERIES OF RENOVASCULAR HYPERTENSIVE RATS

5.1 Introduction 119

5.2 Methods and Materials 122
5.2.1 Animals and surgical procedure 122
5.2.2 Blood pressure measurement 122
5.2.3 Chronic treatment with celecoxib or valsartan 122
5.2.4 Wet weight of the heart and kidney 122
5.2.5 Blood vessel preparation 123
5.2.6 Vascular reactivity 123
5.2.6.1 Endothelium-dependent dilatations 123
5.2.6.2 Endothelium-dependent contractions (EDC) 124

5.2.6.2.1 Role of endothelium and NO 124
5.2.6.2.2 Involvement of non-selective cation channels and extracellular calcium ions 124
5.2.6.2.3 Involvement of COX and thromboxane-prostanoid (TP) receptor 124
5.2.6.2.4 Role of ROS 125
5.2.6.2.5 Specificity of inhibitors/antagonists 125
5.2.6.3 Responses to other vasoconstrictors 125
5.2.6.4 Hydrogen peroxide or HX-XO-induced vasoconstrictions 126
5.2.7 Western blot 126
5.2.8 Electron paramagnetic resonance (EPR) spectroscopy 127
5.2.9 Measurement of prostanoids by enzyme immunoassay 127
5.2.10 Chemicals and drugs 128
5.2.11 Statistical analysis 128

5.3 Results 129
5.3.1 Impaired endothelium-dependent dilatations in RHR 129
5.3.2 Augmented endothelium-dependent contractions (EDC) in RHR 129
5.3.2.1 Endothelium-dependent contractions require extracellular Ca^{2+} .. 130
5.3.2.2 COX-2 mediated endothelium-dependent contractions through acting on the TP receptor 130
5.3.2.3 ROS-mediated endothelium-dependent contractions 131
5.3.2.4 Agonist-induced vasoconstriction 131
5.3.3 Chronic treatment of celecoxib improves endothelial function in RHR renal arteries 131
5.3.3.1 Improvement of endothelium-dependent dilatations in celecoxib-treated RHR 131
5.3.3.2 Abolition of endothelium-dependent contractions in celecoxib-treated RHR 132
5.3.4 Increased COX-2, but not COX-1 expression in RHR renal arteries 132
5.3.5 Increased release of prostaglandins in RHR renal arteries 133
5.3.6 $PGF_{2\alpha}$ serves as an EDCF 133
5.3.7 Basic parameters and blood pressure alteration following drug treatments 134

5.4. Discussion 155

CHAPTER VI

COX-2 UP-REGULATION AND PROSTAGLANDIN $F_{2\alpha}$ MEDIATE BONE MORPHOGENIC PROTEIN 4-INDUCED ENDOTHELIAL DYSFUNCTION IN HYPERTENSION

6.1 Introduction 164

6.2 Methods and Materials 166
6.2.1 Blood vessel preparation 166
6.2.2 Functional studies 166
6.2.3 ROS detection by EPR spin trapping and H_2O_2-sensitive fluorescent

dye..... 167
6.2.4 Intracellular ROS measurement..... 168
6.2.5 Western blotting..... 168
6.2.6 Measurement of prostanoids by enzyme immunoassay..... 169
6.2.7 Constructs, lentivirus production and transduction..... 169
6.2.8 Quantitative reverse transcription PCR (q-RT-PCR)..... 170
6.2.9 Drugs..... 170
6.2.10 Statistical analysis..... 170

6.3 Results..... 172
6.3.1 BMP4 impairs endothelium-dependent dilatations..... 172
6.3.2 BMP-4-induced endothelial dysfunction and endothelium-dependent contractions are mediated by ROS and COX-2..... 172
6.3.3 BMP4 induces ROS overproduction detected by EPR spectroscopy and fluorescence imaging..... 173
6.3.4 BMP4 induces COX-2 up-regulation in mouse aortas..... 173
6.3.5 COX-2-derived $PGF_{2\alpha}$ mediates endothelium-dependent contractions 174
6.3.6 Association between BMP4 and COX-2 in hypertensive rats and human subjects..... 175

6.4 Discussion..... 199

References..... 204

CHAPTER I

INTRODUCTION

1.1 Endothelial cell function

The inner surface of the vascular wall is covered by a thin lining of cells known as endothelial cells. In 1976, Robert Francis Furchgott (June 4, 1916 – May 19, 2009) discovered chemical substances present in endothelial cells that can relax blood vessels in response to acetylcholine, a neurotransmitter of parasympathetic nerves and then named it as endothelium-derived relaxing factors (EDRFs) (Furchgott and Zawadzki, 1980). By 1986, he had worked out the nature of EDRF and its likely mechanism of action, and came to a conclusion that EDRF was in fact nitric oxide (NO), which was soon recognized as an important gaseous molecule in cardiovascular physiology. Apart from NO, endothelial cells are also capable of synthesizing and releasing prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factors (EDHFs) and both are also regarded as EDRFs, which reduce vascular tone. Besides EDRFs as beneficial regulators of vascular function, endothelial cells can produce and liberate vasoconstrictors (Luscher, 1990), termed as endothelium-derived constricting factors (EDCFs) that normally increase vascular tone and are generally considered as harmful modulators of vascular function. Under physiological conditions, EDRFs appear to dominant controllers of vascular tone by negating the unfavorable impact of EDCFs. However, several pathological states like diabetes and hypertension impair the release and function of EDRFs; this results in exaggerated release and function of EDCFs in the vascular wall. A delicate balance between EDRFs and EDCFs is important for vascular health and disturbance of this balance usually set a motion in a cascade of pathological events leading to functional abnormalities in the cardiovascular system.

1.1.1 Endothelium-derived relaxing factors

In response to shear stress (a physical stimulation in vivo) or chemical substances, such as circulating neurotransmitters, hormones, growth factors, and other vasoactive agents, endothelial cells are able to producing and releasing EDRFs, which readily diffuse and act on the adjacent vascular smooth muscle cells (VSMCs) to reduce vascular tension. NO, PGI_2 and EDHFs are among the best studied EDRFs in the vascular system.

1.1.1.1 Nitric oxide (NO)

In response to dilating ligands, such as acetylcholine (ACh) or bradykinin, stimulation of the respective G-protein coupled receptor activates endothelial nitric oxide synthase (eNOS) commonly through increasing intracellular calcium levels in endothelial cells. Stimulated eNOS catalyzes the enzymatic oxidation of L-arginine to form NO and L-citrulline (Palmer et al., 1988). Cofactors, like tetrahydrobiopterin (BH_4) contribute to the regulation of eNOS activity and thus the production of NO (Shinozaki et al., 1999; Tiefenbacher, 2001). Endothelial-derived NO is then released and travels towards VSMCs where it increases the activity of soluble guanylate cyclase, an enzyme that catalyzes the chemical conversion of guanylate triphosphate (GTP) into cyclic GMP (cGMP), an intermediate second messenger signal. Elevated intracellular cGMP level in VSMCs leads to vasodilatation through cGMP-dependent protein kinase-mediated mechanisms which normally lower $[Ca^{2+}]_i$ in VSMCs. Thus, NO plays one of key roles in the regulation of local vascular tone. Apart from regulating vascular reactivity, NO also actively participates in the control of several physiological processes including endothelial cell apoptosis and migration, VSMC proliferation and platelet aggregation (Garg and Hassid, 1989; Lopez-Farre et al., 1997; Radomski et al., 1990).

1.1.1.2 Prostacyclin (PGI_2)

Prostacyclin (PGI_2) is another EDRF and acts as a vasodilator. PGI_2 is derived from the chemical conversion of arachidonic acid in the plasma membrane by prostaglandin synthases from the precursor Prostacyclin H_2 (PGH_2). Prostaglandin synthases are also involved in generation of prostaglandin endoperoxides which

generally exert opposite effects of PGI_2 (Moncada et al., 1976). The vasodilating effect of PGI_2 was found in porcine coronary arteries (Shimokawa et al., 1988a) possibly through activation of adenylate cyclase and subsequent increase in VSMC intracellular cyclic AMP levels (Gorman et al., 1977). However, the vasodilator response of PGI_2 is somehow related to animal species and vascular beds chosen for examination as PGI_2 or prostacyclin analogs can not relax hamster aortas (Wong et al., 2009) or rat arteries (personal communication). Besides the vasodilator action, PGI_2 is a potent inhibitor of platelet activation and aggregation (Moncada et al., 1976). Thus, endothelial-derived NO and PGI_2 could act jointly or even synergistically to suppress platelet aggregation and platelet-induced contractions of VSMCs (Shimokawa et al., 1988b). Thus, the impaired production of PGI_2 is logically suggested to contribute to pathological changes in the vascular system, such as vasospasm, thrombosis and atherosclerosis.

1.1.1.3 Endothelium-derived hyperpolarizing factor (EDHFs)

Endothelium-derived hyperpolarizing factors (EDHFs) control local vascular tone particularly in small resistance-size blood vessels by regulating the membrane potential of VSMCs. It is now generally accepted that the initial event in most of the EDHF-mediated responses characterized to date is not only the hyperpolarization of VSMCs but also the hyperpolarization of endothelial cells due to the opening of small- and intermediate-conductance calcium-activated potassium (K_{Ca}) channels (SK_{Ca} and IK_{Ca}) (Busse et al., 2002). The relative contribution of EDHFs to endothelium-dependent dilatations increases with decreasing size of blood vessels (Shimokawa et al., 1996). The chemical identify for EDHF is still unknown but the chemical substance that produces a EDHF response can be different depending on the vessel size, vascular beds and species under study. A few of endogenous molecules have been frequently reported to act as EDHFs and they include (i) K^+ ions that are released along electrochemical gradient through the opened SK_{Ca} and IK_{Ca} channels in endothelial cells, activate either inwardly rectifying K^+ (K_{IR}) channel or Na^+/K^+ ATPase in VSMCs (Edwards et al., 1998), (ii) epoxyeicosatrienoic acid (EET) mediated by a cytochrome P450 (CYP) epoxygenase to activate VSMC large-conductance K_{Ca} channels (Campbell et al.,

1996; Fisslthaler et al., 1999); (iii) hydrogen peroxide (H_2O_2) to activate VSMC K_{Ca} channels (Matoba et al., 2002; Matoba et al., 2000); and (iv) the transmission of a hyperpolarizing signal through electrically coupled gap junctions from endothelial cells to VSMCs (Chaytor et al., 1998; Taylor et al., 1998) although gap junctions can readily conduct electrical signals between endothelial and endothelial cells or between VSMCs and VSMCs.

1.1.2 Endothelium-derived contracting factors (EDCFs)

In addition to EDRFs, endothelial cells also produce endothelium-derived contracting factors (EDCFs). While EDRFs, in particular NO, have received much attention over the past two decades, the physiological role of EDCF, its chemical features and mechanisms involved in EDCF-mediated vascular responses are still poorly understood. Results from previous studies suggest that PGH_2, thromboxane A_2 (TXA_2), leukotrienes, endothelin, and superoxide anions are the substances previously proposed for EDCFs. A multitude of EDCFs could be related to the heterogeneity in varied physiology of different vascular beds, species, and may also be associated with the experimental protocol adopted by various research groups. Prostaglandins are arachidonic acid metabolites which require enzymatic processing. Cyclooxygenase (COX) was identified as the major enzyme in the oxidative conversion of arachidonic acid into PGG_2 and PGH_2, which are further broken down to form PGE_2, PGD_2, PGI_2, $PGF_{2\alpha}$ and thromboxane A_2 by respective prostaglandin synthases (Wong et al., 2009). COX-derived EDCFs then diffuse to the adjacent VSMCs and bind to respective prostanoid receptors. Prostanoid receptors are grouped into five types based on their sensitivity to individual agonists, PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2, and TXA_2 and they are EP receptor, DP receptor, FP receptor, IP receptor and TP receptor (Coleman et al., 1994).

1.1.2.1 Reactive oxygen species (ROS)

ROS is a family of molecules consisting of molecular oxygen and the reducing derivatives generated in aerobic cells; they are short-lived but highly bioactive (Wassmann et al., 2004). ROS, such as superoxide anions ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$) and lipid radicals (L-$COO^{\cdot}$), have unpaired electrons and are classified as

free radicals. Hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and hypochlorous acid (HOCl), are not free radicals but possess oxidative activities. ROS formation and their elimination are kept delicately in balance within the vascular wall to ensure maintaining desirable levels of ROS required for normal physiological function of blood vessels. Either enhanced activities of oxidant enzymes or diminished activities of antioxidant enzymes disturb such equilibrium and cause oxidative stress. Vascular endothelial cell-derived ROS regulate vascular tone (Cai and Harrison, 2000), oxygen sensing (Kaelin, 2005), cell growth and proliferation (Felty and Roy, 2005), apoptosis (Cai et al., 2002), and inflammatory responses in blood vessels. Excessive ROS have been implicated in the pathogenesis of various forms of cardiovascular disorders, such as atherosclerosis, hypertension, diabetic cardiovascular complications, and ischemia-reperfusion injury (Li and Shah, 2004).

1.1.2.1.1 Oxidant enzymes

A variety of cellular enzymes serve as sources of ROS, including NADPH (nicotinamide adenine dinucleotide phosphate, reduced) oxidase, xanthine oxidase, uncoupled eNOS, arachidonic acid metabolizing enzymes including CYP epoxygenase, lipoxygenase and COX, and the mitochondrial respiratory chain (Li and Shah, 2004). The activities and expression of the oxidant enzymes can be up-regulated in blood vessels, thus increasing oxidative stress, for example, in hypertension and diabetes (Yung et al., 2006).

1.1.2.1.2 Antioxidant enzymes

Under physiological situations, the oxidant enzymes and antioxidant enzymes stay in equilibrium. The antioxidant enzymes are represented by superoxide dismutase (SOD) and catalase. SOD is the major cellular defense system to remove $O_2^{\cdot-}$ by converting $O_2^{\cdot-}$ into H_2O_2 in mammalian cells (Fridovich, 1975). Three isoforms are identified: (1) cytosolic copper/zinc-containing SOD [CuZn-SOD or SOD-1], (2) mitochondrial manganese-containing SOD [Mn-SOD or SOD-2], and (3) extracellular copper/zinc-SOD [ec-SOD or SOD-3] (Fridovich, 1975). The relative importance of each SOD isoform in the cellular protection against oxidative stress

may depend upon the type of cells under study. Catalase contains a molecule of ferric ion at its active site and is responsible for converting H_2O_2 into water and oxygen (Yang and Poovaiah, 2002). Catalase is likely involved in the generation of EDHF as endothelium-derived H_2O_2 is found to cause a membrane hyperpolarization of VSMCs in some resistance vessels (Matoba et al., 2002; Matoba et al., 2000).

1.1.2.1.3 ROS and vascular tone

Whether ROS can exert a direct effect on VSMC is still controversial. It is well established that $O_2^{\cdot-}$ regulates vasomotor tone by inactivating NO. ROS cause either vasoconstriction or vasodilatation depending on vascular beds and the amount of ROS present (Taniyama and Griendling, 2003). H_2O_2 causes endothelium-independent relaxations of mammalian arteries through hyperpolarizing the plasma membrane of VSMCs (Matoba et al., 2002). On the other hand, ROS generated by xanthine oxidase are vasoconstrictive and ROS-induced contractions are augmented in spontaneously hypertensive rats (Auch-Schwelk et al., 1989). In addition, ROS participate in vasoconstriction indirectly by releasing arachidonic acid derivatives and the latter activate endoperoxides-thromboxane A_2 prostanoid (TP) receptor in VSMCs.

1.1.2.2 Endothelial prostaglandin synthase / cyclooxygenase (COX)

Endothelial prostaglandin synthase or COX is the enzyme responsible for the formation of prostanoids from arachidonic acid located in the plasma membrane of endothelial cells. The level of EDCFs that account for endothelium-dependent contractions is controlled by the activity of COX. There are two major COX isoenzymes known as COX-1 and COX-2 although COX-3 has been recently identified. COX-1 is considered to be a constitutive enzyme, expressed in most mammalian cells, whereas COX-2 is generally believed to be an inducible enzyme with its expression being abundant in response to inflammatory insult. However, the relative role of COX-1 and COX-2 in determining the amount of individual prostanoids generated in different cell types is yet to be investigated.

1.1.2.2.1 Endothelial COX-1

COX-1-derived prostanoids generated in the endothelium appear to act as EDCFs in rat aortas, which diffuse to contract the underlying VSMCs by interaction with TP-receptors (Tang and Vanhoutte, 2009). The upregulated expression of COX-1, and the inhibitory effect of COX-1 inhibitors on the augmented endothelium-dependent contractions were observed in arteries from spontaneously hypertensive rats or from diabetic rats (Shi et al., 2007; Yang et al., 2002).

1.1.2.2.2 Endothelial COX-2

Besides being inducible, COX-2 is also expressed constitutively in the endothelium of rat pulmonary and human renal blood vessels (Baber et al., 2003; Therland et al., 2004) and in hamster aortas (Wong et al., 2009). The increase in the generation of COX-2-derived vasoconstrictive prostanoids was associated with the elevated arteriolar tone and blood pressure in diabetic mice (Bagi et al., 2005). The COX-2 expression is increased in kidneys and vascular walls during hypertension and diabetes (Bagi et al., 2006; Hartner et al., 2003), suggesting an inhibitory role of COX-2-derived prostanoids in impairing vasodilatations.

1.1.2.3 COX-derived prostanoids as EDCFs

Oxidative conversion of arachidonic acid by COX-2 in endothelial cells results in the formation of an array of prostanoids, which contributes to the occurrence of endothelium-dependent contractions. The exact identity of COX-dependent EDCFs remains controversial. The possible candidates of EDCFs reported include (i) endoperoxides (Auch-Schwelk et al., 1990; Ito et al., 1991), (ii) TXA_2 (Shirahase et al., 1988), (iii) PGI_2 (Gluais et al., 2005; Rapoport and Williams, 1996), (iv) isoprostane (Janssen and Tazzeo, 2002), and (v) $PGF_{2\alpha}$ (Wong et al., 2009). The vast heterogeneity in EDCFs depends on the experimental animal models used, the age of the animal, the vascular beds and the type of stimuli. Five prostanoids produced from the metabolism of arachidonic acid by the action of COX are PGI_2, TXA_2, PGD_2, PGE_2 and $PGF_{2\alpha}$ via their corresponding synthases as summarized in **Figure 1.1**. Isoprostane previously described as an EDCF can be generated via COX-independent pathways (Janssen and Tazzeo, 2002).

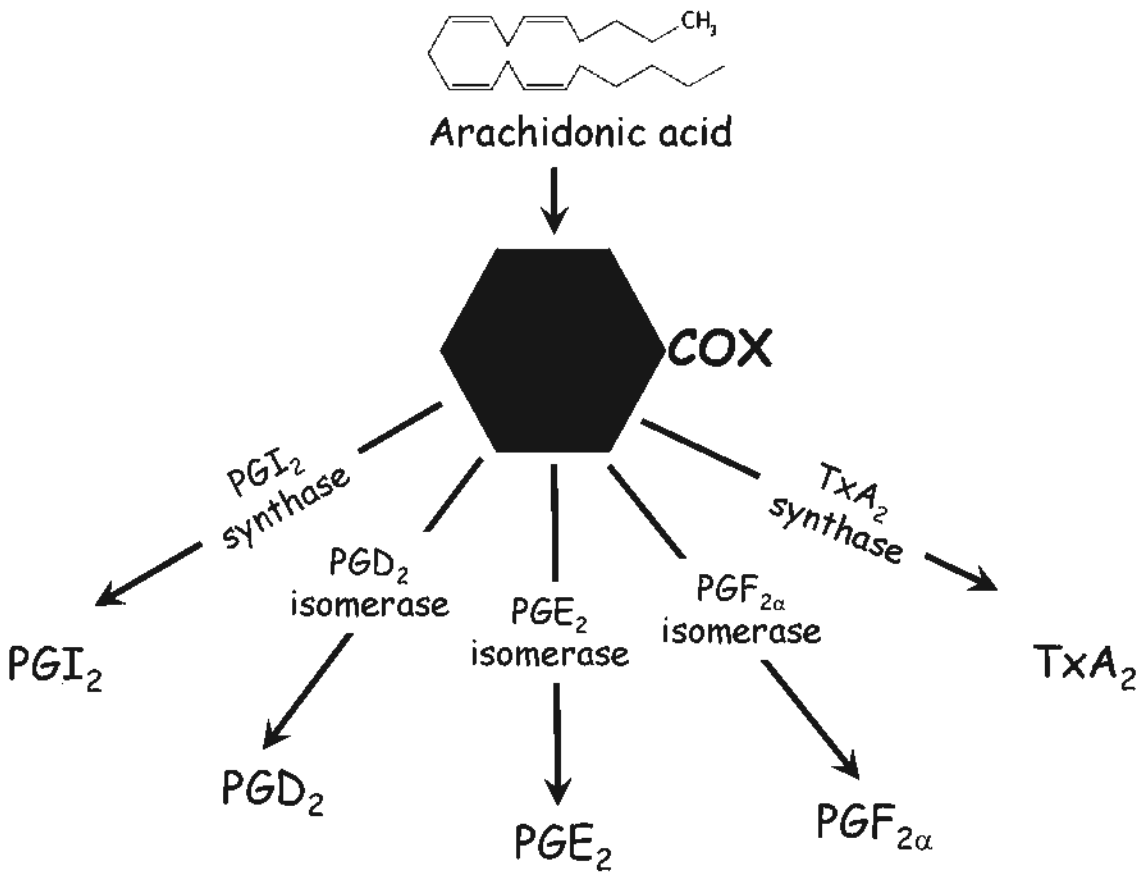


Figure 1.1
Enzymatic pathways involved in oxidative conversion of arachidonic acid into specific prostanoids.

1.1.2.4 Prostanoid receptors

COX-derived prostanoids diffuse to the underlying VSMCs and stimulate prostanoid receptors. There are five prostanoid receptors, namely EP, DP, FP, IP and TP receptors (Coleman et al., 1994). Among these receptors, the TP-receptor mediates endothelium-dependent contractions as shown in various blood vessels of different species (Katusic and Vanhoutte, 1989; Wong et al., 2009; Yang et al., 2002). It is interesting to note although the TP-receptor is supposed to be specific to TXA_2 (Shirahase et al., 1988), PGI_2 or $PGF_{2\alpha}$ are also able to activate the TP-receptor to cause endothelium-dependent contractions (Gluais et al., 2005; Wong et al., 2009). Thus, the TP-receptor appears to be a target for multiple prostanoids. Therefore, the action of specific prostanoid on its corresponding prostanoid receptor in mediating vasoconstriction or vasodilatation deserves further in-depth investigation.

1.1.3 Balance between EDRFs and EDCFs

The endothelium is essential for the maintenance and regulation of vascular homeostasis, by releasing both EDRFs (NO in many conduit arteries) and EDCFs. In healthy blood vessels, the vascular tone is regulated by a fine balance between these two opposing forces. However, pathological conditions such as hypertension, diabetes mellitus or aging, impair endothelium-dependent dilatations associated with a declining impact of NO and other EDRFs due to the loss or decreased production of these beneficial factors and/or augments release of vaso-constricting prostanoids and ROS which can lower the bioavailability of EDRFs. Thus, the degree of reduced endothelium-derived NO or augmented EDCF responses predicts the severity of future vascular complications (Thomas et al., 2008). Treatment targeting the reversal of such disturbance between EDRFs and EDCFs under pathological conditions is therefore fundamentally important in reducing vascular mortality.

1.2 Endothelial dysfunction

Endothelial dysfunction, characterized by a diminished release of endothelium-derived NO and/or an augmented release of contracting prostanoids and ROS, is an important early event in the initiation and development of hypertension, diabetes and atherosclerosis.

1.2.1 Endothelial dysfunction in diabetes

Micro- and macrovascular dysfunctions are currently the major causes of morbidity and mortality in patients with diabetes mellitus. Endothelial dysfunction plays a critical role in the development of diabetic vasculopathy, which is associated with the reduced bioavailability of NO resulting from which overproduction of ROS, lipid peroxidation, and increased production of adhesion molecules (Mazzone et al., 2008). Impaired endothelium-dependent vasodilatations had been observed in type I and II diabetes from both clinical settings and animal studies. The forearm vasodilator response to the muscarinic acetylcholine receptor agonist, methacholine is impaired in patients with insulin-dependent diabetes (type I diabetes) (Johnstone et al., 1993) and non-insulin-dependent diabetes (type II diabetes) (Tan et al., 2002; Williams et al., 1996). Besides, the impaired endothelium-dependent vasodilatations could be also demonstrated in animal models of type I diabetes (Chang et al., 1993; Dai et al., 1993; Nassar et al., 2002) and type II diabetes (Elmi et al., 2008; Gao et al., 2007; Pannirselvam et al., 2002). The complex mechanisms by which hyperglycemia modifies the endothelial function include increased oxidative stress (Laight et al., 2000), glycation of proteins and lipids (Vlassara et al., 1992) and activation of protein kinase C (Hink et al., 2001).

1.2.1.1 Oxidative stress in diabetic endothelial dysfunction

Oxidative stress is caused by a disturbed balance between oxidant enzymes and antioxidant enzymes tilting towards an increase in ROS overproduction. Oxidative stress is a critical factor in diabetic endothelial dysfunction. Under a hyperglycemic condition the antioxidant enzymes like SOD and catalase were down-regulated,

resulting in an increased generation of oxygen-derived free radicals (Giugliano et al., 1996). Increases in ROS lead to impairment of endothelium-dependent vasodilatations through the reduction of NO bioavailability. It had been recently demonstrated that superoxide anion scavengers like superoxide dismutase improve the impaired endothelium-dependent vasodilatations in db/db diabetic mice (Elmi et al., 2008; Moien-Afshari et al., 2008). Chronic treatment with antioxidants including vitamin E and vitamin C were reported to prevent the development of endothelial dysfunction in diabetic patients and animals (Keegan et al., 1995; Ting et al., 1996). However, it remains controversial as to the beneficial effects of the use of antioxidants in the treatment of vascular dysfunction in diabetes because in clinical settings antioxidant treatment does not always yield a protective effect in reversing endothelial dysfunction in type II diabetic patients (Gazis et al., 1999).

1.2.1.2 Reduced NO bioavailability in diabetes

Bioavailability of endothelial-derived NO, as determined by the relative ratio of NO over ROS levels, is an important index for determining endothelial function. Any situation in which there is a reduced eNOS activity or elevated ROS production can normally lead to a reduced NO bioavailability and thus impairs endothelium-dependent vasodilatations. The phosphorylation of eNOS was found to be impaired in diabetic mouse aortas (Zhang et al., 2009; Zhong et al., 2007), renal arteries (Zhong et al., 2007a), and mesenteric resistance arteries (Su et al., 2008). Under hyperglycemic and enhanced oxidative stress states, phosphorylation of eNOS at Ser1177 site was also found to be diminished in human umbilical vein endothelial cells (Wang et al., 2009). As afore-mentioned, ROS production is increased in diabetic vascular tissues due to the imbalance between the oxidant and antioxidant enzymes. These highly reactive oxygen free radicals act quickly to remove NO, and thus further reduce NO bioavailability. Furthermore, the interaction between NO and superoxide radicals leads to the formation of peroxynitrite, another highly reactive free radical species that causes lipid peroxidation, DNA damage and protein nitration and collectively damages the vascular function (Bloodsworth et al., 2000).

1.2.1.3 Advanced glycation end products (AGEs)

The increased formation of advanced glycation end products (AGEs) has emerged to be another key mediator of the development of microvascular and macrovascular events of diabetes. AGEs generated from the non-enzymatic glycation of circulating and matrix proteins in a hyperglycemic environment is linked to vascular inflammation, dysfunction and injury through several proposed mechanisms including the elevated production of ROS (Goldin et al., 2006). The harmful effects of AGEs are initiated by binding to their receptors (RAGE). A raised plasma level of endogenous secretory RAGE was reported in type II diabetic patients with nephropathy (Gohda et al., 2008) and in atherosclerotic patients (Koyama et al., 2005). Recent animal studies support the clinical findings. In apoE$^{-/-}$ mice, RAGE contributes to the pathogenesis of endothelial dysfunction and atherosclerosis (Harja et al., 2008) and inhibition of AGE accumulation or RAGE deficiency retards the progression of diabetes-accelerated atherosclerosis (Forbes et al., 2004; Soro-Paavonen et al., 2008).

1.2.2 Endothelial dysfunction in hypertension

Impaired flow-mediated vasodilatations are common in hypertensive patients (Benndorf et al., 2007; Widlansky et al., 2003; Xu et al., 2009). Endothelium-dependent dilatations are reduced in isolated arteries from several animal models of hypertension including spontaneously hypertensive rats (Chan et al., 2007; Luscher et al., 1990), salt-induced hypertension (Hermann et al., 2003; Jimenez et al., 2007) and renovascular hypertension (Christensen et al., 2007; Garcia-Saura et al., 2005; Heitzer et al., 1999; Quaschning et al., 2006). Apart from the impairment of endothelium-dependent dilatations, the appearance of endothelium-dependent contractions also significantly contributes to the development of endothelial dysfunction in hypertension. Thus, any pharmacological agents that restore eNOS activity and NO production and/or suppress oxidative stress or endothelium-derived constricting factors are theoretically useful in slowing down the onset of endothelial dysfunction related to hypertension.

1.2.2.1 Impaired endothelium dependent dilatations in hypertension

In healthy vasculatures, endothelial cells respond to a number of physiological stimuli such as shear stress or vasodilators like thrombin or serotonin from aggregating platelets, by releasing NO which relaxes the underlying VSMCs (Vanhoutte et al., 2009). Although the impaired endothelium-dependent dilatations in hypertension have been clearly demonstrated in both clinical and animal studies, the precise cellular mechanisms are yet to be fully understood. Several studies including ours reported that the sensitivity of VSMCs to NO is not altered, thus excluding the role of decreased soluble guanylate cyclase activity and decreased cGMP-dependent protein kinase (PKG) activity in the impairment of endothelium-dependent dilatations related to hypertension. This suggests that the original culprit might be the failure of endothelial cells to generate adequate amount of NO from L-arginine or the rapid removal of NO , assuming its production is normal, by an exaggerated amount of superoxide anions in endothelial cells during hypertension. As a result, either the availability of NO surrounding target sites in VSMCs is effectively reduced or the half life for endothelium-derived NO is shorter. Of note, the vascular tissues express significantly less in both eNOS mRNA and protein levels in salt-induced hypertensive rats (Shah and Singh, 2007; Zhou et al., 2004), supporting a lowered production of endothelial-derived NO. In addition, $p22^{phox}$, a membrane-bound subunit of O_2^--generating NAD(P)H oxidase in aortas of salt-induced hypertensive rats is up-regulated (Shah and Singh, 2007). NAD(P)H oxidase is probably one of the major enzymes leading to oxidative stress during the onset of hypertension. Accordingly, apocynin, a commonly used inhibitor of NAD(P)H oxidase, is effective in preserving vascular function in hypertensive rats (Jimenez et al., 2007). Anti-hypertensive therapies, particularly with angiotensin-converting enzyme inhibitors and angiotensin receptor blockers, are proven to be associated with the improvement of endothelial function revealed in clinical trials (Higashi et al., 2005; Higashi et al., 1998; Perrone-Filardi et al., 2009). Nevertheless, the question whether endothelial dysfunction is a cause or consequence of hypertension remains a mystery and deserves further examination.

1.2.2.2 Endothelium-dependent contractions in hypertension

Apart from releasing EDRF/NO, endothelial cells can also produce EDCFs although their chemical identities remain elusive. Under physiological conditions, a balance between vasodilators and vasoconstrictors plays an important role in the regulation of vascular tone (Luscher et al., 1990; Vanhoutte, 1989). During hypertension, endothelial dysfunction is brought about by a reduction in endothelium-dependent dilatations and a concomitant release of endothelium-derived vasoconstrictor prostanoids (Vanhoutte, 2009). Non-selective inhibition of COX by indomethacin improves acetylcholine-mediated dilations of forearm resistance vessels in hypertensive patients (Taddei et al., 1997) and this improvement was attributable to the augmented NO bioavailability, suggesting that this certain COX-dependent prostanoids impairs endothelial function by antagonizing the NO action. Likewise, aspirin, a non-specific COX inhibitor can also enhance endothelium-dependent dilatations of forearm resistance vessels in hypertensive patients (Campia et al., 2002), though the effects were described to be independent of the prostanoid release. Furthermore, treatment with celecoxib, the selective COX-2 inhibitor leads to improvement of flow-mediated dilation of the brachial artery in patients with coronary artery disease (Chenevard et al., 2003) and hypertension (Widlansky et al., 2003), indicating an important role of COX-dependent prostanoids in vascular dysfunction. The endothelium-dependent contractions were augmented in aortas of spontaneously hypertensive rats (Luscher et al., 1990), partly due to increased production of COX-dependent prostanoids through acting on TP receptors. In most of published studies, the endothelium-dependent contractions can best be visualized by the presence of L-NAME, an inhibitor for NO production, suggesting that endogenous NO may be sufficient to counteract the contractile effect of EDCFs even under a oxidative state which lowers the bioavailability of NO. One exception is the recent observation that COX-2-dependent $PGF_{2\alpha}$ is able to cause endothelium-dependent contractions in the quiescent aortas of aged hamsters without pre-exposure to L-NAME (Wong et al., 2009). Taken together, EDCF-mediated responses play significant roles in the maintenance of endothelial dysfunction in hypertension and therapeutic strategies

targeting EDCF-generating pathways might be of potential relevance in retarding or even preventing hypertension-related vascular events.

1.3 Renin-angiotensin system in diabetes and hypertension

Evolving evidence suggests a critical role of a local renin-angiotensin system (RAS) in the vascular wall as a key negative regulator of endothelial function in diabetes and hypertension. Angiotensin II (Ang II) impairs endothelial function via AT_1 receptor by increasing NAD(P)H oxidase activity and production of superoxide anions (Higashi et al., 2003). Insulin resistance enhances NAD(P)H oxidase-mediated generation of ROS by up-regulating the AT_1 receptor (Henriksen et al., 2001; Shinozaki et al., 2004). RAS blockade with either angiotensin receptor blockers (ARB) or angiotensin converting enzyme (ACE) inhibitors is recommended as a first-line therapy for patients with type 2 diabetes complicated with hypertension or nephropathy. ARB (telmisartan) and ACE inhibitor (ramipril) are effective in alleviating symptoms or conditions in patients with vascular disease and high-risk diabetes (Yusuf et al., 2008). Animal studies support the significant role of RAS and the efficacy of ACE inhibitors and ARBs in diabetic nephropathy, retinopathy, and vascular complications and vascular benefits of RAS blockade might be mediated though (i) the up-regulation of eNOS (Kanno et al., 2001), (ii) reduction in oxidative stress (Yagi et al., 2004), and (iii) decrease in NAD(P)H oxidase activity (Ungvari et al., 2004). A schematic diagram of the renin-angiotensin-system (RAS) cascade is demonstrated in Figure 1.2.

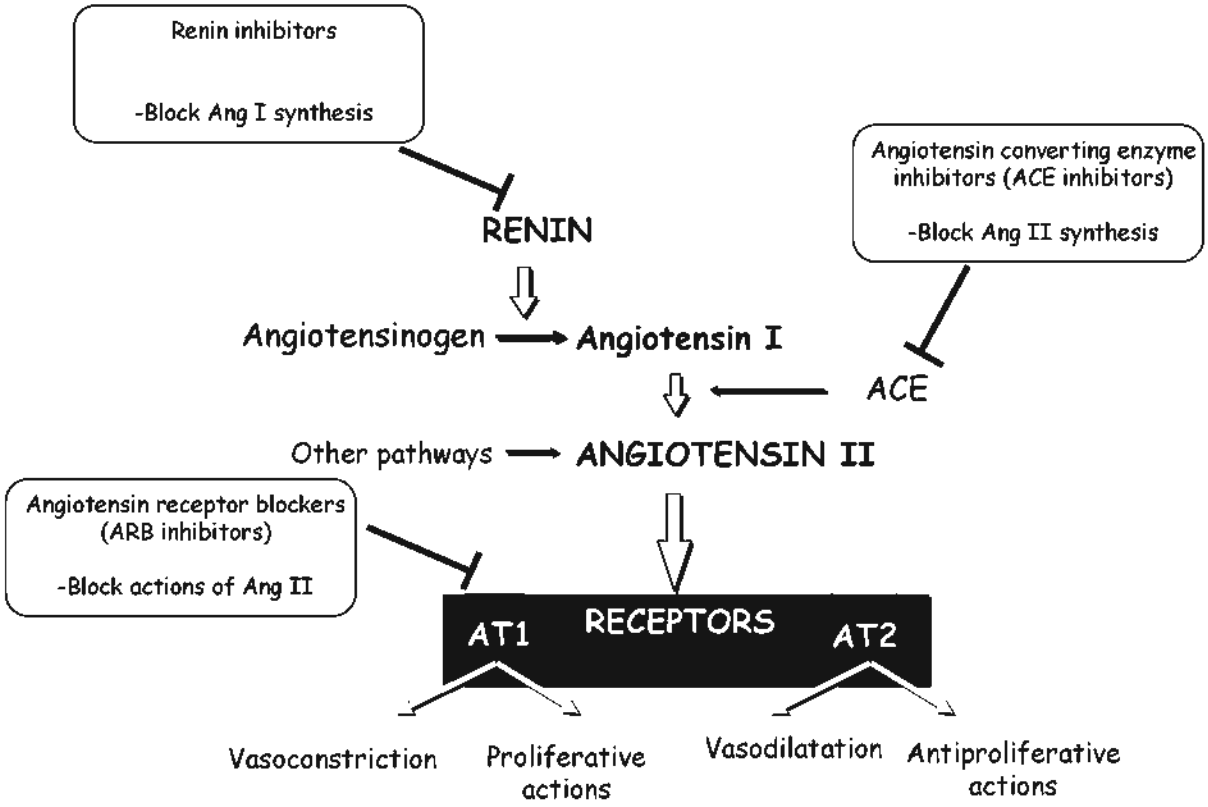


Figure 1.2.
The renin angiotensin system (RAS) starting from chemical conversion of angiotensinogen into angiotensin I (bio-inactive) catalyzed by renin. Angiotensin I is then converted into bioactive angiotensin II by angiotensin converting enzyme (ACE). Other pathways such as chymase may also mediate a local production of angiotensin II. Angiotensin binds to angiotensin type I (AT1) receptor to cause harmful actions such as vasoconstriction and VSMC proliferation while it can also activate the angiotensin type 2 (AT2) receptor to produce beneficial effects (vasodilatation and inhibition of VSMC proliferation) that oppose AT1 receptor-mediated action. Renin inhibitors can inhibit the production of angiotensin II; angiotensin receptor blockers (ARB) can block the AT1 receptor-mediated responses; and ACE inhibitors can reduce the production of angiotensin II.

1.3.1 Renin

Renin as the rate-limiting enzyme determines the amount of formation of vasoactive Ang II in the RAS. Renin is an aspartyl proteolytic enzyme whose substrate is angiotensinogen, a 60,000-dalton peptide formed within the liver and released into the circulation. Renin is synthesized, stored and released by the juxtaglomerular cells in the kidney. The decapeptide produced due to the action of renin from angiotensinogen is angiotensin I, which is in turn converted by ACE into Ang II. The renin activity generally serves as an index for the levels of Ang II formed naturally and reflects the importance of regulators that control the synthesis and release of renin.

1.3.1.1 Renin inhibitor

The first-generation renin inhibitors were peptide analogues of the pro-segment of renin or substrate analogues of the renin substrate, angiotensinogen. Studies of renin inhibitors (CGP 29287, remikiren and zankiren) show the inhibitors produce dose-dependent inhibition of the plasma renin activity (PRA) and lower blood pressure (Stanton, 2003). However, the development of renin inhibitors was hampered at earlier time mainly due to low bioavailability and high cost of these synthetic drugs. Recently, a newly synthesized renin inhibitor, aliskiren, is found to have an acceptable oral bioavailability. Aliskiren is a very potent and competitive renin inhibitor with an IC_{50} of 0.6 nmol/L measured by plasma renin activity assay (Vaidyanathan et al., 2008). Aliskiren is highly specific for rennin and may therefore represent a novel effective drug in the prevention and treatment of hypertension, when used alone or in combination with other antihypertensive agents (Muller and Luft, 2006).

1.3.1.2 Renin inhibition and hypertension

Aliskiren produces dose-dependent blood pressure-lowering effect and 24-h blood pressure is well controlled in hypertensive patients receiving a daily once dosage of approximately 300 mg. Its antihypertensive potency is equivalent to that of ARBs, ACE inhibitors, and diuretics. Persistent blood pressure reduction and prolonged suppression of the plasma renin activity are after aliskiren withdrawal

(Vaidyanathan et al., 2008) and this long-lasting effects implies that aliskiren might be superior to other RAS blockers. When given together with an ARB, aliskiren produces additional effect in reducing blood pressure, resulting in more complete RAS inhibition (Parving et al., 2008). In hypertensive mice by induced expression of human renin and angiotensinogen, acetylcholine-induced relaxations of the carotid artery were found to be impaired (Didion et al., 2000), further supporting that renin inhibition protects endothelial function in hypertension. Recent evidence shows that aliskiren also exerts both renoprotective and cardioprotective effects in patients with hypertension (Parving et al., 2008). Using rats transgenic for human renin and angiotensinogen genes, aliskiren had been demonstrated to ameliorate renal and cardiac damage (Pilz et al., 2005). Further investigations are required to reveal the end organ protection effects of renin inhibition by aliskiren during hypertension.

1.3.1.3 Renin inhibition and diabetes

Some evidence suggests that renin inhibition by aliskiren might provide an important therapeutic option for patients at high risk for cardiovascular diseases, especially for those with diabetes (Parving et al., 2008). Since renin acts on very upstream targets to reduce the RAS activity, beneficial effects of ACE inhibitors and ARBs could be attenuated through increased renin release resulting from inhibition of the negative feedback loop. It is thus logical to propose that direct renin inhibition would be a more effective approach to achieve a complete RAS blockade. Indeed, aliskiren is renoprotective that is independent of its blood pressure-lowering action in type 2 diabetic patients with nephropathy (Parving et al., 2008). Aliskiren protects against diabetes-associated production of superoxide anions and cardiac fibrosis (Singh et al., 2008). Furthermore, studies on streptozotocin-induced diabetic rats suggest that aliskiren may retard the development of diabetic nephropathy by suppressing the expression of genes that are thought to play central roles in the RAS activation and subsequent development of renal fibrosis (Feldman et al., 2008). Taken together, renin inhibition by aliskiren may well serve as an important therapeutic alternative for the treatment or even reversal of cardiovascular outcomes in diabetic patients.

However, the mechanisms underlying the vasoprotective effects of aliskiren and the role of renin as an agonistic ligand for the prorenin/renin receptor in the regulation of vascular function is still far from clear and need detailed investigation.

1.3.1.4 Renin inhibition and endothelial function

Limited data are available concerning the protective action of renin inhibition against endothelial dysfunction in hypertension or diabetes. Aliskiren has been recently shown to enhance the NO bioavailability and to protect against atherosclerotic changes in hyperlipidemic rabbits (Imanishi et al., 2008). It is yet to demonstrate whether aliskiren could protect endothelial function in animal models of hypertension and diabetes.

1.3.2 Angiotensin converting enzyme (ACE)

ACE is a membrane-bound enzyme expressed mainly in endothelial, epithelial and mesangial cells (Corvol et al., 1995; Wei et al., 1991). ACE converts the inactive decapeptide angiotensin I (Ang I) to the bioactive octapeptide Ang II. Actually, ACE is not only specific for Ang I, but it also cleaves other peptides including bradykinin, another peptide vasodilator (Linz et al., 1995). Some of the noted therapeutic benefits of ACE inhibitors are attributed to potentiating and prolonging the vasodilator action of bradykinin. ACE inhibitors are now widely used for managing hypertension. Many extensive studies have clearly documented that ACE inhibitors confer end organ protection in diabetes and hypertension (Ibrahim, 2006).

1.3.2.1 ACE inhibitors

ACE inhibitors are used primarily to treat hypertension and congestive heart failure. Based on the molecular structure, three groups of inhibitors have been developed and they are sulfhydryl-containing ACE inhibitors (e.g. captopril), dicarboxylate-containing ACE inhibitors (e.g. enalapril and ramipril), and phosphonate-containing ACE inhibitors (e.g. fosinopril). In general ACE inhibitors are able to lower arteriolar resistance, increase venous capacity, increase cardiac output and cardiac index and increase natriuresis.

1.3.2.2 ACE and hypertension

The ACE inhibitor, ramipril increases lifespan of spontaneously hypertensive rats to the similar level as in untreated normotensive Wistar-Kyoto rats (Linz et al., 1999). This lifespan extension is probably related to blood pressure reduction, a regression of left ventricular hypertrophy, and amelioration of cardiac and vascular dysfunction following normalization of eNOS expression and activity by ACE inhibitors. ACE inhibition by cilazapril or captopril can remarkably improve endothelial function in spontaneously hypertensive rats (Clozel, 1991) and long-term treatment with ramipril potentiates endothelium-dependent relaxations of rat aortas by enhancing the NO bioavailability (Berkenboom et al., 1995). Captopril can reduce the media cross-sectional area, wall-to-lumen ratio, and perivascular fibrosis in mesenteric arteries from ovariectomized spontaneously hypertensive rats (Garcia et al., 2006). Taken together, ACE inhibition prevents hypertension and protects hypertension-associated end organ damage.

1.3.2.3 ACE and diabetes

ACE inhibitors have been taken as the first line of choice for diabetic patients with kidney diseases as instructed by the American Diabetes Association. The ACE inhibitor prevents the development of microalbuminuria in type 2 diabetic patients (Ruggenenti et al., 2004). In a streptozotocin-induced diabetic rat model of nephropathy induced by, ACE inhibitors reduce the glomerular production of TGF-β and the progression of renal diseases (Hill et al., 2001). A combined use of ACE inhibitors and ARBs decreases renal damage in obese Zucker rats (Toblli et al., 2004). Acute administration of the ACE inhibitor, enalapril, improves endothelial function in type I diabetic patients (O'Driscoll et al., 1997) and ACE inhibition increases endothelium-dependent vasodilatations in the femoral artery of normotensive microalbuminuric type 1 diabetic patients (Arcaro et al., 1999), supporting the pathophysiological relevance of ACE in the development of diabetic vasculopathy.

1.3.3 Angiotensin receptors

There are 2 well-characterized receptors for Ang II, denoted as AT_1 and AT_2. Both

subtypes have strong affinities for Ang II and virtually none for Ang I. AT_1 receptors that mediate most of the classic effects of Ang II, are blocked by ARBs. Ang II acting on the AT_1 receptor results in vasoconstriction which reduces the capacity of the vascular tree, and aldosterone secretion, tubular sodium retention, and the release of antidiuretic hormone which conserves intravascular fluid volume. Ang II is also related to an increased sympathetic activity, increased thirst, intestinal fluid absorption and platelet agglutination, as well as to an increased cardiac contractility.

1.3.3.1 Ang II type 1 receptor (AT_1R)

When Ang II binds to AT_1R in the plasma membrane, the G protein subunits in association with intracellular loops of the receptor protein dissociate and then activate phospholipase C, which cleaves phosphoinositides to form inositol trisphosphate (IP_3) and diacylglycerol. The G protein coupled to AT_1R belongs to the $G_{q/11}$ class (Kitos et al., 2006). IP_3 triggers mobilization of calcium ions from the sarcoplasmic reticulum. Activation of AT_1R may open plasma membrane calcium channels as diacylglycerol acts as an endogenous activator of protein kinase C which phosphorylates voltage-sensitive calcium channels through which calcium influx is augmented and vascular tone is increased. Ang II can also stimulate phospholipase A_2 and phospholipase D (Jung et al., 1998). Phospholipase A_2 cleaves phospholipids to form arachidonic acid, a substrate for various enzymes that mediate biosynthesis of eicosanoids. Activation of AT_1 receptors initiates a series of events that affect gene transcription and protein synthesis. Among the kinases affected by Ang II are those of the Src, JAK and STAT family.

1.3.3.2 AT_1R inhibitors

AT_1R inhibitors, also known as ARBs, AT_1-receptor antagonists or sartans, are a group of drugs which inhibits the effect of RAAS. They are commonly consumed by patients with hypertension, diabetic nephropathy and congestive heart failure. Valsartan, losartan, telmisartan, irbesartan, and olmesartan are clinically used AR_1T blockers.

1.3.3.3 AT_1R and hypertension

ARBs improve endothelial function in patients with essential hypertension. Endothelium-dependent dilatations in response to ACh in resistance arteries from subcutaneous biopsies are improved by one-year treatment with losartan in hypertensive patients, while endothelium-independent dilatations to sodium nitroprusside are unaffected (Schiffrin et al., 2000). Six-week valsartan treatment enhances ACh-induced vasodilator response with elevated level of basal NO production in hypertensive patients (Klingbeil et al., 2003). Hypertensive patients receiving valsartan treatment have better flow-mediated vasodilatation and their urinary excretion of 8-isoprostane and 8-hydroxy-2'-deoxyguanosine is significantly less, indicating a reduction in oxidative stress resulting from ARB treatment (Hirooka et al., 2008). Losartan treatment for 2 weeks lowers systolic blood pressure, reduces the expression of NADPH oxidase subunits of endothelial progenitor cells and promotes repair of vascular injuries in stroke-prone spontaneously hypertensive rats (Yao et al., 2007). On the other hand, blood pressure lowering effect is not necessary for the improved endothelial function in some studies using olmesartan (Ferrario, 2009). Collectively, these results suggest the ARBs are useful in preserving vascular function in hypertension.

1.3.3.4 AT_1R and diabetes

It has been reported that four-week treatment with losartan improved acetylcholine-induced vasodilatations in both conduit and resistance arteries in patients with type 2 diabetes (Cheetham et al., 2000; Cheetham et al., 2001). Clinical data suggest that the activation of RAS contributes to endothelial dysfunction in diabetes. However, precise mechanisms by which the RAS activation is linked to vascular dysfunction in diabetes is yet to be further delineated as diabetes is usually associated with other cardiovascular risk factors such as hypertension and hyperlipidemia.

1.3.3.5 AT_1R and NAD(P)H oxidases

Nonphagocytic NADPH oxidase, the primary physiologic enzymatic source of ROS in the vascular tissue, is a multi-subunit enzyme that catalyzes $O_2^{\cdot-}$ production by

the 1-electron reduction of O_2 using NADPH as the electron donor: $2O_2$+NAD(P)H $\rightarrow$ $2O_2^{\cdot-}$ + $NADP^+$ + H^+. The prototypical NAD(P)H oxidase is that found in neutrophils and consists of 5 subunits: $p40^{phox}$ ("phox" stands for phagocyte oxidase), $p47^{phox}$, $p67^{phox}$, $p22^{phox}$ and $gp91^{phox}$ (Babior, 1999). In un-stimulated cells, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ exist in the cytosol; whereas $p22^{phox}$ and gp91phox are present in the membrane where they occur as a heterodimeric flavoprotein, cytochrome b558. Upon stimulation, $p47^{phox}$ becomes phosphorylated, and the cytosolic subunits form a complex that translocates to the membrane where it associates with cytochrome b558 to assemble an active form of oxidase, which transfers electrons from the substrate to O_2 forming $O_2^{\cdot-}$ (Cai et al., 2003; Lassegue and Clempus, 2003). Ang II is a major regulator of vascular NAD(P)H oxidase and it stimulates NAD(P)H oxidase via AT_1 receptors. Ang II up-regulates the nox1 mRNA level in rat renal cortex (Chabrashvili et al., 2003) and in cultured VSMCs (Wingler et al., 2001). Increased expression of NAD(P)H oxidase and associated oxidative stress impair vascular function. Commonly used pharmacological inhibitors of NAD(P)H oxidase include apocynin and diphenylene iodonium (DPI). Apocynin possesses anti-inflammatory and anti-thrombic properties (Engels et al., 1992) and chronic apocynin treatment lowers blood pressure, attenuates oxidative stress, enhances the eNOS activity, and increases the NO production in blood vessels from spontaneously hypertensive rats (Baumer et al., 2007). These studies suggest that selective NAD(P)H oxidase inhibitors hold premise in improving endothelial dysfunction in pathological states and thus are potentially effective in treating hypertension and diabetes.

1.4 Bone morphogenic protein-4 (BMP4) in hypertension and diabetes

Bone morphogenic protein-4 (BMP4) belongs to the transforming growth factor-β superfamily, which was first discovered to mediate bone growth (Li and Wozney, 2001), and also serves as an essential signaling molecule for human embryonic development (Hogan, 1996). There exist three endogenous antagonist that counteract with BMP4, including noggin, chordin, and follistatin, and these antagonists bind to BMP 1a receptors with high affinity (Hogan, 1996).

1.4.1 BMP4 and shear stress

Several previous studies suggest a potential involvement of BMP4 in vascular inflammatory responses evolving into atherosclerosis. BMP4 and BMP2 were first identified in human atherosclerotic plaques (Dhore et al., 2001). Later studies showed that BMP4 can be produced in endothelial cells subjected to oscillatory shear stress and the released BMP4 stimulate ROS production in a NAD(P)H oxidases (p47phox)-dependent manner to trigger inflammatory reactions such as monocyte adhesion (Sorescu et al., 2004). BMP4 antagonist is found to be co-expressed with BMP4 in mouse endothelial cells in response to disturbed flow, or in human coronary atheromatous lesions (Chang et al., 2007). Therefore, BMP4 is postulated as a novel biomarker for atherosclerosis.

1.4.2 BMP4 and oxidative stress

A coupling between BMP4 and oxidative stress was first described in an in vitro shear stress model. Exposure to oscillatory shear stress in endothelial cells causes the release of BMP4 which in turn stimulates ROS generation from NOX1 (Sorescu et al., 2004). On the other hand, another member of the TGF-β family, BMP2 (but not BMP4) could be induced by tumor necrosis factor-α (TNF-α) and H_2O_2 in endothelial cells (Csiszar et al., 2006). Moreover, BMP2 also increased ROS production in intact arteries through NAD(P)H oxidases- and protein kinase C-dependent mechanisms, and subsequently activate mitogen-activated protein kinases (MAPK) p44/42 phosphorylation to aggregate inflammatory response in endothelial cells (Csiszar et al., 2006). Similarly, in VSMCs, BMP4 activated by thrombin, enhances the ROS production through $p47^{phox}$, which is important in regulating VSMC function during atherosclerosis and restenosis (Vendrov et al., 2006). BMP4 infusion in mice causes hypertension and the harmful effects of BMP4 on blood pressure, endothelial function and ROS production are reduced by NAD(P)H oxidase inhibitor or by genetic deletion of $p47^{phox}$ subunit (Miriyala et al., 2006). Most recently, BMP4 is found to stimulate ROS production from NOX4 in palmitate-mediated cellular inflammation in human endothelial cells, which is activated by toll like receptor-4 (TLR4) and NF-κB signals (Maloney et al., 2009). However, the pro-oxidant effects of BMP4 only exist in systemic arteries such as

aorta, carotid, or coronary arteries, while pulmonary arteries are resistant to BMP4-induced oxidative damage (Csiszar et al., 2008).

1.4.3 BMP4 and hypertension

BMP4 plays a positive role in the development of hypertension and chronic infusion of BMP4 induces significant and progressive hypertension (Miriyala et al., 2006). BMP4 infusion impairs endothelium-dependent dilatations of mouse arteries and increases the vascular NAD(P)H oxidases activity; both effects are abolished in $p47^{phox}$ knockout mouse. BMP4 and BMP2 directly impair endothelium-dependent vasodilatations of rat carotid and coronary arteries (Csiszar et al., 2006; Csiszar et al., 2008) and both are involved in vascular remodeling that promotes the development of pulmonary hypertension (Frank et al., 2005). Genetic analysis of patients indicates that BMP receptor BMPRII contributes to the pathogenesis of pulmonary arterial hypertension.(Yang et al., 2008). Later studies identify a differential role of BMP4 in systemic and pulmonary vasculature.

1.4.4 BMP4 and diabetes

The role of BMP4 in diabetes is unclear. Limited recent studies indicate that BMP4 and BMP receptors might be involved in glucose-stimulated insulin secretion (GSIS) in pancreatic β cells. Over-expression of BMP4 in β cells enhances GSIS, and *in vivo* BMP4 administration also improves glucose clearance (Goulley et al., 2007). Moreover, in heterozygous mutant mice for BMP 1a receptor, glucose metabolism is impaired and GSIS is reduced, suggesting that the presence of BMP4 might be beneficial in the β-cell function in diabetes (Scott et al., 2009). However, the BMP4 expression is increased in aortas from mice fed high fat diet (Maloney et al., 2009). Genetic analysis of human subjects shows a positive association between BMP 1a receptor expression in adipose tissue and obesity (Bottcher et al., 2009). Whether BMP/BMP receptor signaling is protective or harmful appears to depend on cell types used. It is becoming clear that BMP4 can damage vascular function.

1.5 Cyclo-oxygenase-2 (COX-2) in hypertension and diabetes

COX is the enzyme that converts arachidonic acids to prostaglandin H_2 (PGH_2), which is the precursor of several prostanoids, including PGE_2, PGD_2, $PGF_{2\alpha}$, TXA_2, prostacyclin (PGI_2) and 8-isoprostane. There are two isoforms of COX known as COX-1 and COX-2. It is widely accepted that COX-1 is constitutively expressed in most tissue including the vascular endothelial cells and smooth muscle cells, while the expression of COX-2 remains very low in physiological condition, which can be induced upon stimuli such as inflammation (Parente and Perretti, 2003). Of interest, recent studies also pointed out the constitutive expression of COX-2 in endothelial cells (Baber et al., 2003; Therland et al., 2004), which increases during aging (Wong et al., 2009).

The relationship between COX-2 and endothelial cell dysfunction have been extensively studied in various experimental settings and clinical studies demonstrating inflammatory response induced by COX-2 activation. For instance, COX-2 plays an important role in atherosclerotic events. COX-2 up-regulation was related to pro-inflammatory and pro-atherosclerotic effect of cigarette smoking on endothelial cell (Barbieri and Weksler, 2007). Increased expression of COX-2 in vasculature, but not COX-1, was associated with human coronary arterial disease and atherosclerosis, especially in lesion areas, accompanied by an up-regulation of PGE_2 synthase, which increased the level of PGE_2, in contrast, the endothelial generation of PGI_2 which was predominantly produced by COX-1 was decreased, which was not affected by COX-2 inhibitors (Flavahan, 2007). Moreover, oxidative stress, another important team player in endothelial dysfunction, can also regulate COX and prostanoid activity in endothelial cell dysfunction. After activation of endothelial cells induced by inflammatory stimuli, superoxide ($O_2^{\bullet-}$) was generated, which then interacted with nitric oxide to form peroxynitrite (ONOO-). Peroxynitrite caused nitration and inactivation of PGI_2 synthase, while activating PGH synthase, finally leading to a compensatory increase of TXA_2 (Zou et al., 2000; Zou et al., 2002).

1.5.1 COX-2 and renovascular hypertension

The involvement of prostanoids in renovascular hypertension was first demonstrated in renovascular hypertensive rats (RHR) as indomethacin, the non-specific COX inhibitor reduces the plasma renin activity (PRA) in both normotensive and hypertensive rats, and decreases blood pressure in RHR on low sodium diet (Stahl et al., 1981). In consistence with animal data, aspirin can lower blood pressure, reduce PRA, and decrease the level of plasma PGE_2 in patients with renovascular hypertension, although aspirin does not affect blood pressure and plasma renin level in patients with essential hypertension (Imanishi et al., 1989). Later studies showed the contribution of TXA_2 to the development of RHR. Chronic blockade of the TP receptor lowers blood pressure and decreases vascular responsiveness to vasopressin (Boussairi et al., 1994). COX-2 is of significant interest because it has been implicated in vascular inflammation and atherosclerosis. COX-2 is constitutively and abundantly expressed mainly in the healthy kidney, and plays a role in the regulation of renal function, such as maintaining basal renin release, and renal sodium and water handling. However, increasing evidence shows that COX-2 is clearly involved in the pathophysiology of renal diseases. In RHR, elevated expression of COX-2 and renin are detected in the macula densa and afferent arterioles of the ischemic kidney (2K1C model) (Hartner et al., 1998). In addition, COX-2 inhibitors also reduce the increased plasma renin activity, and renin mRNA expression, and blood pressure in RHR, but not in deoxycorticosterone acetate (DOCA)-salt induced hypertension (Okumura et al., 2002; Wang et al., 1999). This is explained by the possibility that certain stages during the development of hypertension may be related to the high COX-2 activity, thus COX inhibitors are effective in lowering blood pressure only during the COX-2-dependent stages. Long-term COX-2 inhibition has no effect on blood pressure despite of the protective effect against renal fibrosis (Richter et al., 2004). It is not fully resolved regarding the effect of COX-2 on blood pressure.

In addition to renal dysfunction and elevated blood pressure, vascular dysfunction is also associated with prostanoids in RHR. Indomethacin ameliorates the impaired endothelium-dependent vasodilatation in mesenteric resistance arterioles of RHR (Fortes et al., 1992). However, the clinical study shows

indomethacin treatment improves endothelial function only in patients with essential hypertension but not in patients with renovascular hypertension (Taddei et al., 1993). The contraction of rat aortas to serotonin and TXA_2 is augmented in RHR, which is related to a decreased NO bioavailability (Roson et al., 2001). Although the basal NO release is unaltered in the superior mesenteric arteries of RHR, the acetylcholine-induced NO-mediated dilatations are blunted (Stankevicius et al., 2002). It is suggested that EDHF may serve as an adaptive mechanism to compensate for the loss of NO in the 2K1C rat carotid artery (Sendao Oliveira and Bendhack, 2004).

1.5.2 COX-2 and diabetic vascular dysfunction

The role of COX-2 in diabetic vascular dysfunction is not thoroughly understood. There are only a few studies on the vascular COX-2 expression in diabetes. The *in vitro* study shows that high glucose exposure up-regulates the COX-2 expression in HUVECs, which is mediated by oxidative stress linked to the activation of protein kinase C and the NAD(P)H oxidase-dependent action, and also to a reduction of PGI_2 synthase activity (Cosentino et al., 2003). High glucose induces COX-2 up-regulation, increases PGE_2, and causes apoptosis of HUVECs in a PI3K/Akt -dependent pathway (Sheu et al., 2005). Animal studies on diabetic db/db mice also reveal a significant role of COX-2 up-regulation in aortic VSMCs that contributes to enhanced contractile responses in conduit and resistance arteries, associated with an increased TXA_2 release (Guo et al., 2005). TXA_2 derived from an enhanced COX-2 expression and activity is responsible for the increased arterial tone in skeletal muscle resistance arteries and for the increased blood pressure in db/db mice (Bagi et al., 2005). However, a recent study using human coronary arterioles shows that COX-2-derived prostanoids, most likely PGI_2, contribute to vasodilatations, which may serve as a compensatory mechanism to increase blood flow in coronary circulation (Szerafin et al., 2006). A reduced PGI_2 activity is also found in Zucker diabetic fatty rats, although the expressions of both COX-1 and COX-2 are not different from the lean control. The decreased PGI_2 synthase activity may contribute to the impaired activation by arachidonic acid of the large-conductance K_{Ca} channel in coronary arterial single myocytes isolated

from the Zucker diabetic rat coronary artery (Lu et al., 2005). In another animal model of type II diabetes, the Otsuka Long-Evans Tokushima Fatty (OLETF) rats, the enhanced expression of COX-1 and COX-2 with subsequent increase in TXA_2 and PGE_2 production triggered by acetylcholine accounts for disturbed balance between EDRFs and EDCFs, resulting in endothelial dysfunction (Matsumoto et al., 2007). Although the cellular mechanisms involved in COX-2 up-regulation in diabetes are only partially understood, oxidative stress appears to be an important detrimental factor for redox-sensitive signaling and transcriptional regulation involved in the initiation of vascular dysfunction. The involvement of inflammatory cytokines like TNF-α or nuclear receptors such as NF-κB impair vascular function probably also through up-regulating the COX-2 expression (Evans et al., 2002).

1.5.3 COX-2 inhibitors

Recent concerns over the safety of COX-2 inhibitors as severe cardiovascular adverse effects in COX-2 inhibitor users were reported (Hinz et al., 2007). It is conventionally accepted that COX-2 inhibitors increase the cardiovascular risk partly by increasing the susceptibility of platelet aggregation, vasoconstriction, and atherothrombosis, and partly by disturbing the balance between COX-1 generated TXA_2 and COX-2 generated PGI_2 as we generally believe that TXA_2 is pro-thrombotic while PGI_2 is anti-thrombotic. However, this notion still remains controversial depending on specific pathophysiologic conditions, animal models, and complexity of clinical settings. Indeed, some clinical studies do not report the side effects of COX-2 inhibitors as they do not increase the risk of platelet activation nor alter the level of TXA_2 and its metabolites in healthy subjects, patients with coronary artery disease, and in cigarette smokers (Chenevard et al., 2003; McAdam et al., 2005). By contrast, selective inhibition of COX-2 improves flow- and nitroglycerin-mediated vasodilatations in hypertensive patients (Widlansky et al., 2003). An advanced understanding of the more precise role of COX-2 in the pathogenesis of vascular and metabolic dysfunction will certainly help to provide novel cellular targets for the development of pharmacological intervention which are devoid of the obvious cardiovascular toxicity of the existing COX-2 inhibitors.

1.6 Justifications, long-term impact and objectives of the present project

Vascular tone is modulated by endothelial cells through the synthesis and release of both EDRFs and EDCFs. A balance between EDRFs and EDCFs is essential for maintaining normal vascular function. Endothelial dysfunction is characterized by a diminished NO bioavailability as a result of reduced release and/or production of NO, increased inactivation of NO, and increased production of EDCFs and ROS. The degree of endothelial dysfunction predicts the severity of cardiovascular risks.

Elevated ROS production in the vascular wall is one of the major initiators for endothelial dysfunction and ROS overproduction is often a result of activated RAS. Increased activity of the local RAS plays a key role in the induction of endothelial function in diabetes and hypertension. In view of critical participation of the RAS in the initiation and development of vascular dysfunction and associated events, the present study aimed to investigate the contribution of oxidative stress and intracellular events initiated by the RAS activation leading to endothelial dysfunction in diabetes and hypertension by using type 2 diabetic db/db mice and spontaneously hypertensive rats (SHR), to examine whether or not, chronic treatment with inhibitors/antagonists at different levels along the RAS axis including aliskiren (renin inhibitor), enalapril (ACE inhibitor) and valsartan (angiotensin receptor blocker), could ameliorate endothelial dysfunction. The results of the present study should provide novel experimental evidence in support of the clinical efficacy of RAS inhibitors in alleviating endothelial dysfunction in patients with diabetes and/or hypertension.

In addition to oxidative stress or ROS overproduction, elevated generation of EDCFs also occur in ageing, diabetes and hypertension. However, the chemical identity of EDCF is still poorly understood and the role of COX-2 in mediating endothelium-dependent contractions in hypertension remains largely unexplored. Of importance, it is basically unknown which COX-derived arachidonic acid derivative can be regarded as the most likely EDCF and whether EDCF and ROS could act independently of each other or they could actually couple together to

mediate endothelium-dependent contractions and whether such a mechanism is relevant to hypertension. Thus, the present study also aimed to identify the possible EDCFs accounting for endothelium-dependent contractions in the 2-kidney 2-clip rat model of renovascular hypertension and to delineate a sequence of cellular events leading to the release of a major EDCF and to elucidate a pathological role of COX-2 in the generation of EDCFs. Since the prevalence of end-stage renal disease secondary to hypertension multiplies rapidly and lack of effective treatment eventually leads to high mortality. The results from the present study shall help to provide new insights into molecular and cellular mechanisms by which endothelial dysfunction is induced and may provide experimental basis for developing new strategies for drug intervention against renovascular hypertension.

In addition, I also investigated the impact of bone-morphogenic protein-4 (BMP4) on vascular function in disease and whether BMP4 could act as a novel upstream activator for ROS overproduction and then trigger an increased expression and activity of COX-2 in endothelial cells as an important downstream target enzyme responsible for initiation and maintenance of endothelial dysfunction. The results may help better understanding of new pathological processes involved in vascular dysfunction in hypertension.

In order to achieve afore-mentioned objectives, the present project had investigated:

1. An essential role of the RAS activation and associated oxidative stress in the occurrence of endothelial dysfunction in diabetes and/or hypertension and the therapeutic impact of renin inhibitor, ARBs and ACE inhibitors in normalizing endothelial function in diseased states.
2. A major EDCF derived from increased activity and expression of COX-2 in blood vessels from rats with induced renovascular hypertension and cellular mechanisms involved
3. A novel role of BMP4 as an upstream activator of increased oxidative stress and up-regulation of COX-2 in the induction and maintenance of endothelial dysfunction and the relevance of BMP4 up-regulation to human hypertension.

CHAPTER II

METHODS AND MATERIALS

2.1 Animals

The use of animals for my experiments was approved by the Ethical Committee for Animal Research, Chinese University of Hong Kong (CUHK). Animals that were supplied by the CUHK Laboratory Animal Service Center including: male Sprague-Dawley rats, spontaneously hypertensive rats (SHR), with age matched normotensive Wistar-kyoto rats (WKY); leptin receptor deficient db/db (homozygous) and age/gender-matched db/m^{+} heterozygous mice generated from the C57BL/KsJ, and male C57BL/6J mice. COX-1$^{-/-}$, COX-2$^{-/-}$ generated from C57/BL6 background were supplied by the University of Hong Kong. All animals were housed at room temperature (25 °C) with alternating 12-hr light / 12-hr dark cycle and fed on standard rat chow and water *ad libitum*.

2.1.1 Surgical procedures

Renovascular hypertension rats (RHR) (2-kidney 2-clip, 2K2C) were induced as previously described (Zeng et al., 1998). Briefly, male Sprague-Dawley rats (80 to 100 g) were anaesthetized with a mixture of 35 mg/kg ketamine and 7 mg/kg xylazine. After a midline laparotomy, two silver clips with an internal diameter of 0.3 mm were placed around the root of left and right renal arteries. The sham-operated normotensive rats were subjected to laparotomy and renal artery separation procedure only.

2.1.2 Drug treatments in animal studies

1. Five weeks after surgery, RHR with blood pressure over 180 mmHg were randomly divided into several groups and administered orally with celecoxib (10 mg/kg/day; specific COX-2 inhibitor; Pfizer, NY, USA) or valsartan [10 mg/kg/day; angiotensin receptor blockers (ARB), Novartis Pharma, Basel, Switzerland] for 5

weeks. Some sham-operated rats were also treated by celecoxib for the same duration. Blood pressure was monitored weekly.

2. Male SHR aged at 6 months with average blood pressure over 180 mmHg, and age-matched normotensive WKY were both subjected to oral treatment of the renin inhibitor, aliskiren (10 mg/kg/day; Novartis Pharma, Basel, Switzerland) for 8 weeks. Meantime, two groups of SHR and WKY rats also receive oral gavage of vehicle control. Blood pressure was monitored weekly.

3. Male diabetic db/db mice aged 12 weeks with fasting blood glucose over 20 mmol/L were divided into several groups, and they received oral administration of valsartan (10mg/kg/day, ARB), enalapril [10 mg/kg/day, angiotensin converting enzyme (ACE) inhibitor, MSD, PA] and vehicle control for 6 weeks. Plasma glucose levels were determined using a blood glucose meter (Ascenia Elite XL, Bayer, IN).

2.2 Human artery specimen

Human renal arteries were obtained during surgery after informed consent from patients undergoing nephrectomy at ages between 45-80 years old. The group of diabetic patients had a fasting plasma glucose level ≥ 7.0 mmol/L (or 126 mg/dL) or 2–h plasma glucose ≥ 11.1 mmol/L (or 200 mg/dL). Patients were initially diagnosed as hypertensive by having systolic blood pressure over 140 mmHg and diastolic blood pressure over 90 mmHg.

2.3 Measurement of basic parameters

2.3.1 Blood pressure measurement

Systolic blood pressure was measured weekly by a tail-cuff sphygmomanometer (Harvard Rat Tail Blood Pressure Monitor System) in conscious rats or mice.

2.3.2 Oral glucose tolerance test

In db/db and db/m^{+} mice, after 6 hrs of fasting, glucose was loaded 1.2 g/kg with a 10% glucose solution via oral gavage, and the plasma glucose level was measured subsequently at 15, 30, 60 and 120 min.

2.3.3 Plasma insulin

Plasma insulin levels were assayed by enzyme immunoassay (Mercodia, Sweden) according to the manufacturer's instructions.

2.3.4 Wet weight for heart and kidney

Heart and kidney (from both sides) were dissected out and weighted for determination of a ratio of heart weight or kidney weight over body weight (HW/BW or KW/BW).

2.3.5 Lipid profile

After animals were sacrificed, blood was drawn from the inferior vena cava and collected in heparin coated test tube. Plasma was separated by centrifugation and stored at -80 ºC until further assay. Plasma levels of total cholesterol and triglyceride were determined using enzymatic methods (Stanbio, Boerne, TX, USA). The total cholesterol level was measured as described before (Pasin et al., 1998). A blank was prepared by substituting 0.01 mL of distilled water from the cholesterol sample. Samples were mixed and incubated for 15 min at 37 °C. Absorbance was read at 500 nm using a spectrophotometer. The triglycerides level was assayed based on a modified method (Bucolo and David, 1973). Briefly, triglycerides are converted to glycerol and fatty acids, and then into NADH. Finally, the formation of colored formazan took place in response to the addition of 2-(p-iodophenyl)-3-p-nitrophenyl-5-phenyltetrazolium. Absorbance at 500 nm was recorded and the reading was directly proportional to the concentration of triglycerides in the sample. For the measurement of the level of high-density lipoprotein (HDL), the low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) portions were removed by the addition of HDL cholesterol assay reagent (Sigma, kit number 352-4) (Guan et al., 2006). The remaining level of cholesterol, that is HDL, is obtained.

2.3.6 Plasma renin activity

The plasma renin activity was measured as the activity by determining the production of angiotensin I from endogenous angiotensinogen using a radioimmunoassay kit (plasma renin activity kit; SRL, Tokyo, Japan).

2.4 Isometric force measurement

After animals were sacrificed by CO_2 inhalation, the thoracic aorta, kidney, or small intestine with mesentery were rapidly removed and placed in oxygenated ice-cold Krebs-Henseleit solution. Segments of blood vessels including aortas, intralobal renal arteries, or mesenteric resistant arteries were carefully dissected free from adjacent connective tissues. Large-size aortas were suspended between two hooks in organ baths. One hook was connected to a Grass force transducer and other one was fixed to the bottom. Changes in isometric tension of aortas were continually recorded. A baseline optimal tone of 25 mN was applied to all aortic rings. In some aortas, the endothelial layer was mechanically disrupted by rubbing the luminal surface of a ring segment with a fine forceps tip. Functional removal of the endothelium was verified if the relaxant effect of acetylcholine (1 μmol/L) was absent.

Changes in isometric tension of rat intralobal renal arteries, mouse aortas or mesenteric resistance arteries were recorded in a Multi Myograph System (Danish Myo Technology A/S, Denmark) as previously described (Leung et al., 2007). Renal arteries were stretched to an optimal baseline tension of 2.5 mN, mouse aortas of 3 mN and mesenteric resistance arteries of 1 mN and then allowed to equilibrate for 60 min before the start of the experiment. Each experiment was performed on rings prepared from different animals.

2.4.1 Experimental protocols

Each ring was first contracted by 60 mmol/L KCl and rinsed several times in Krebs solution, and after washout, phenylephrine (1 μmol/L, α_1-adrenoceptor agonist) was used to produce a steady contraction and subsequently relaxed by cumulative addition of acetylcholine, the muscarinic acetylcholine receptor agonist.

To test the acute effect of pharmacological inhibitors on endothelium-dependent relaxations, acetylcholine (1 nmol/L to 10 μmol/L)-induced relaxing effects in phenylephrine-precontracted arterial rings were assessed in control and in the presence of each of the following drugs: 3 μmol/L losartan (ARB), 100 μmol/L apocynin [NAD(P)H oxidases inhibitor], 100 μmol/L tempol [superoxide

dismutase (SOD) mimetic], 1 mmol/L tiron (SOD mimetic) plus 100 µmol/L DETCA (ROS scavengers), 3 µmol/L celecoxib or NS398, or DuP697 (selective COX-2 inhibitor), 0.1 µmol/L sc-560 or 0.3 mmol/L VAS (selective COX-1 inhibitor), 0.1 µmol/L S18886 (specific TP receptor antagonist), 10 µmol/L cycloheximide (protein synthesis inhibitor).

To study the endothelium-dependent contractions, 100 µmol/L N^G-nitro-L-arginine methyl ester [L-NAME, non-selective nitric oxide synthase (NOS) inhibitor] were present in the entire duration of the experiment for clear visualization of the contractile responses. Acetylcholine (0.1 - 10 µmol/L)-induced contractions were tested in control (L-NAME only) or in the presence of L-NAME plus 3 µmol/L celecoxib, or NS398, or DuP697 (COX-2 inhibitor), 0.3 µmol/L SC560 or 0.3 mmol/L VAS (COX-1 inhibitor), 0.1 µmol/L S18886 (TP receptor antagonist), 100 µmol/L apocynin [NAD(P)H oxidases inhibitor], 100 µmol/L tempol [superoxide dismutase (SOD) mimetic], 1 mmol/L tiron (SOD mimetic) plus 100 µmol/L DETCA (ROS scavenger), 50 µmol/L 2-aminoethoxydiphenyl borate (2-APB, non-selective cation channel blocker).

In some rings, 100 nmol/L angiotensin II was added to bathing solution to induce a transient contraction. 100 µmol/L H_2O_2 was also used to evoke a contraction in some rings. In addition, the contraction to endothelin-1 (1 - 50 nmol/L) was also tested and compared in arteries from both normotensive and hypertensive rats.

To test the responsiveness and sensitivity of blood vessels in response to stimulation of different prostanoid receptors, 11-deoxy-$PGF_{2\alpha}$ (10^{-10} to 10^{-7} g/L, FP receptor agonist), U46619 (1 nmol/L - 1 µmol/L, TP receptor agonist), PGE_2 (1 nmol/L - 1 µmol/L, EP receptor agonist), PGI_2 (1 nmol/L - 10 µmol/L, IP receptor agonist) were tested in the presence of L-NAME.

Endothelium-independent relaxations to sodium nitroprusside (SNP) (1 nmol/L - 1 µmol/L) were studied in rings without endothelium.

Each human renal artery was cut into 2-3 ring segments (2-3 mm in length). Rings were suspended in organ baths. Individual ring was initially stretched to an optimal tension of 10 mN and then allowed to equilibrate for 90 min before the commencement of the experiment.

2.5 Western blotting

Blood vessels (aortas and renal arteries) were snap frozen in liquid nitrogen and subsequently homogenized in ice-cold RIPA lysis buffer that contained 1 µmol/L leupetin, 5 µmol/L aprotonin, 100 µmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L sodium fluoride, and 2 mg/mL β-glycerolphosphate. The lysates were incubated for 30 min on ice and then centrifuged for 20 min at 20,000 g. The supernatant was collected and analyzed for protein concentration using the Lowry method (Bio-Rad, Hercules, CA, USA). Sample buffer containing 5% β-mercaptoethanol was added to the sample, and then denatured by boiling for 10 min. For each sample, 50 µg of protein was separated with 7.5% - 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), together with the prestained and biotinylated size marker. The resolved proteins were electrophorectically transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA) using wet transfer (Bio-Rad) at 100 V for 60 min at 4 °C. The membranes were blocked with 5% non-fat milk or 1% bovine serum albumin (BSA) dissolved in phosphate buffer saline with 0.1% Tween-20 (PBST) for 1 hour at room temperature. Primary antibodies against target proteins (information summarized in Section 2.13.4) were incubated at 4 °C overnight, while the corresponding secondary antibodies conjugated to horseradish peroxidase (HRP) (DakoCytomation, Carpinteria, CA) were used at a dilution of 1:3000 and incubated for 1 hour at room temperature. The membranes were developed with enhanced chemiluminescence detection solutions (ECL reagents; Amersham Pharmacia, Pittsburgh, PA, USA) and exposed on X-ray films. Densitometry was performed using a documentation program (Flurochem, Alpha Innotech Corp. San Leandro, CA, USA). GAPDH or β-actin was selected as housekeeping protein for checking equal loading of each sample. Summarized data represented the mean of 4-5 separate experiments.

2.6 Immunohistochemistry

Aortic rings were fixed in 4% paraformaldehyde at 4°C overnight, dehydrated, processed and embedded in paraffin. Cross sections at 5 µm were cut on

microtome (Leica Microsystems, Germany). After rehydrated to water, sections were microwave boiled in 0.01 mol/L citrate buffer (pH 6.0) for 10 min for antigen retrieval, then incubated for 15 min with 3% H_2O_2 at room temperature to block endogenous peroxidase activity. After washed with phosphate buffer saline (PBS), sections were blocked in 5% normal goat or donkey serum according to the host species (Jackson Immunoresearch, West Grove, PA) for 1 hour at room temperature. Primary antibodies (anti-Ang II, 1:500, Peninsula laboratory, Belmont CA) diluted in normal serum were incubated overnight at 4ºC. The slides were washed with PBS three times (5 min each). Biotin-SP conjugated goat anti-rabbit secondary antibodies (1:500, Jackson Immunoresearch) diluted in PBS were added and incubated for 1 hr at room temperature. Slides were washed with PBS three times (5 min each) and incubated for 30 min with streptavidin-HRP conjugate (1:500, Zymed laboratory, San Francisco, CA) at room temperature, and washed. Positive staining was developed as brown precipitate by 3,3'-diamonobenzidine tetrachloride (DAB) chromogen substrate (Vector laboratory, Burlingame, CA). Slides were rinsed with water and counterstained with hematoxylin. Pictures were taken under Leica DMRBE microscope with a SPOT-RT digital camera and SPOT Advanced software (Diagnostic Instruments, Sertling Heights, MI) and intensities of signals were analyzed by ImageJ (National Institute of Health, USA).

2.7 Detection of intracellular ROS by dihydroethidium fluorescence

The amount of intracellular ROS production was determined using dihydroethidium (DHE) (Molecular Probes, Eugene, OR), which binds to DNA when oxidized to emit fluorescence (Robinson et al., 2006). Aortic rings from db/m^+ and db/db mice were obtained as described above and treated with or without acetylcholine stimulation. To investigate the inhibitory effect of the RAS blockade on the ROS production, aortas were exposed for 30 min to each of the following inhibitors: losartan, apocynin or tempol before the application of acetylcholine, as to mimic the procedure in the functional study. To verify the endothelial origin of the ROS production, the endothelial layer was removed by rolling the luminal surface with fine forceps tips before loading DHE dye. To examine the role of extracellular

calcium ions in the generation of ROS, aortic rings were bathed in a calcium-free Krebs solution for 30 min before the addition of acetylcholine. Frozen sections of the aortic ring were prepared in 10-µm thickness using a cryostat (Shandon, Pittsburgh, PA, USA) and incubated in Krebs solution containing DHE (5 µmol/L) for 10 min at 37 ºC. Fluorescent intensity was measured by confocal microscope (FV1000, Olympus, Tokyo, Japan) at excitation/emission of 488/605 nm to visualize the fluorescence signal.

2.8 Electron paramagnetic resonance (EPR) spectroscopy

The formation of ROS was measured by a reaction with the spin trap TEMPONE-H using EPR spectroscopy, using the method adopted from Dikalov et al., (1998). Diethylenetriaminepentaacetic acid (DTPA) was used to decrease the self-oxidation of hydroxylamine catalyzed by traces of transition metal ions. Rat renal arteries or mouse aortas were dissected and incubated in DTPA (100 µmol/L) and TEMPONE-H (80 µmol/L) in oxygenated Krebs solution at 37 ºC for 30 min. Acetylcholine was added for 5 min to trigger the release of ROS in the presence of 100 µmol/L L-NAME. Arterial tissues together with 200 µL of incubation medium were collected, homogenized, and stored in glass Pasteur pipettes (tip inner diameter: 1 mm) at -80°C. The samples were thawed in room temperature before the start of the measurement. EPR spectra were recorded at room temperature using a Bruker EMX EPR spectrometer (Bruker, Karlsruhe, Germany). The EPR settings were as follows: field center, 3475 G; field sweep, 60 G; microwave frequency, 9.72 GHz; microwave power, 20 mW; magnetic field modulation, 100 kHz; modulation amplitude, 2.0 G; conversion time, 655 msec; detector time constant, 1024 msec; and acquisition of ten scans. The generation of ROS by chemical interaction between xanthine oxidase (XO, 0.01 U/mL) and hypoxanthine (HX, 100 µmol/L) was taken as the positive control. A mixture of Krebs solution containing DTPA and TEMPONE-H served as the negative control. TEMPONE-H were dissolved in oxygen-free (Argon bubbled) double-distilled water.

2.9 Intracellular ROS measurement

After mouse thoracic aorta was dissected out and cut into several ring segments, rings were fluorescently labeled with 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H_2DFDA, Molecular Probes) together with 0.02% (v/v) pluronic acid in Krebs solution. Extracellular CM-H_2DFDA was washed away by rinsing arterial tissues three times in Krebs solution after each ring was cut open longitudinally and mounted on a block of silicone elastomer fixed on the base of the flow chamber as described previously (Hercule et al., 2009). The chamber was filled with Krebs solution, sealed with a cover glass smeared with high vacuum grease (Dow Corning, Midland, MI, USA) and then fixed by screws. The flow chamber was perfused with Krebs solution at 37 ºC for 20 min at a rate of 1 mL/min, maintained by a six-channel perfusion pump. The loaded aortic strips were examined under a laser scanning confocal microscope, with an excitation wavelength of 480 nm and an emission wavelength at 505-530 nm.

2.10 Detection of prostanoids by enzyme immunoassay

The levels of arachidonic acid-derived prostanoids were measured by EIA kits (Cayman Chemical, Ann Arbor, MI, USA) according to the instruction of the manufacturer. Rat renal arteries, or mouse aortas were exposed to 100 µmol/L acetylcholine for 5 min in a 500-µL bathing Krebs solution with and without acute drug treatment and the solution was then collected and kept at -80 ºC until later processing; The six prostanoids or their metabolites, $PGF_{2\alpha}$, PGE_2, PGD_2, 6-keto $PGF_{1\alpha}$ (for PGI_2) and TXB_2 (for TXA_2) and 8-isoprostane, were assayed according to the manufacturer's instructions.

2.11 Drugs, chemicals and other reagents

2.11.1 Chemicals

Chemicals	Description	Solvent	Source
Acetylcholine hydrochloride (ACh)	Muscarinic acetylcholine receptor agonist	H_2O	Sigma, St. Louis, MO
Actinomycin D	Transcription inhibitor	DMSO	Sigma

Aliskiren	Renin inhibitor	H_2O	Novartis Pharma, Basel, Switzerland
Angiotensin II	Angiotensin II type 1 receptor (AT_1R) agonist, vasoconstrictor	H_2O	Tocris, Bristol, UK
2-Aminoethoxydiphenyl Borate (2-APB)	Non-selective cation channel blocker	DMSO	Calbiochem, San Diego, CA
Apocynin	NAD(P)H oxidases inhibitor	DMSO	Calbiochem
BMP4	Bone morphogenic protein-4	BSA-HCl	Sigma
Celecoxib	specific COX-2 inhibitor	DMSO	Pfizer, NY
Cycloheximide	Translation inhibitor	DMSO	Calbiochem
Diethyldithiocarbamate (DETCA)	Cu/Zn-superoxide dismutase (Cu/Zn-SOD) inhibitor	H_2O	Sigma
Diethylenetriaminepentaacetic acid (DTPA)	Spin trap reagent	H_2O	Sigma
DuP697	COX-2 inhibitor	DMSO	Calbiochem
Enalapril	Angiotensin converting enzyme inhibitor (ACEI)	H_2O	MSD, PA
Endothelin-1	Endothelin-1 receptor agonist	H_2O	Tocris
Hypoxanthine	Substrate for xanthine oxidase	H_2O	Sigma
Indomethacin	Non-selective COX inhibitor	DMSO	Sigma
Losartan	AT_1R blocker	DMSO	Cayman, Ann Arbor, MI, USA
N^G-nitro-L-arginine methyl ester (L-NAME)	Nitric oxide synthase (NOS) inhibitor	H_2O	Sigma
Noggin	BMP4 antagonist	PBS	Sigma

NS398	COX-2 inhibitor	DMSO	Tocris
PD98059	MAPK P44/42 inhibitor	DMSO	Tocris
Phenylephrine	α-adrenergic receptor	H_2O	Sigma/RBI
PGE_2	EP receptor agonist	H_2O	Calbiochem
$PGF_{2\alpha}$	FP receptor agonist	DMSO	Cayman
PGI_2	IP receptor agonist	H_2O	Cayman
SB202190	MAPK p38 inhibitor	DMSO	Tocris
SC560	COX-1 inhibitor	DMSO	Sigma
Sodium nitroprusside	Exogenous NO donor	H_2O	Sigma
SP600125	JNK/SAPK inhibitor	DMSO	Tocris
S18886	TP receptor blocker	DMSO	Servier, Suresnes, France
Tempol	SOD mimetic	H_2O	Sigma
TEMPONE-H	Spin trap reagent	H_2O	Alexis, San Diego, CA
Tiron	Reactive oxygen species (ROS) scavengers	H_2O	Sigma
U46619	TP receptor agonist	DMSO	Sigma
VAS	COX-1 inhibitor	DMSO	Cayman
Valsartan	AT_1R blocker (ARB)	H_2O	Novartis
Xanthine oxidase	Oxidant producing enzyme	H_2O	Sigma

2.11.2 Composition of Krebs solution

The solution was freshly prepared before the experiments. The pH value was maintained at 7.4 by continuously bubbling with 95% O_2 plus 5% CO_2 at 37 ºC.

Chemicals	**Final concentration (mmol/L)**
NaCl	119
$HaHCO_3$	25
$MgCl_2$·6H2O	1

KCl	4.7
KH_2PO_4	1.2
$CaCl_2$	2.5
D-glucose	11.1

2.11.3 Reagents for Western blot analysis

2.11.3.1 Reagents for sample preparation		
RIPA buffer		
	NaCl	8 g
	KCl	0.2 mmol/L
	Na_2PO_4	1.44 mmol/L
	KH_2PO_4	0.24 mmol/L
	NP-40	1%
	Sodium dodecyl sulfate (SDS)	0.1%
	Sodium deoxycholate	0.5%
Protease inhibitors		
	5 mg/mL aprotonin	5 µg/mL
	200 mmol/L EDTA	1 mmol/L
	200 mmol/L EGTA	1 mmol/L
	259 mg/mL β-glycerolphosphate	2 mg/mL
	10 mg/mL leupetin	1 µg/mL
	100 mmol/L phenylmethylsulfonyl fluoride (PMSF)	1 mmol/L
	125 mmol/L sodium fluoride	1 mmol/L
	100 mmol/L sodium orthovanadate	1 mmol/L
2.11.3.2 Reagents for gel preparation (stacking and separating)		
30% acrylamide		made up to 100 mL

	Acrylamide	29.2 g
	Methylene bis-acrylamide	0.8 g
1.5 M Lower Tris-base buffer (pH 8.8)		made up to 100 mL
	Tris base	18.17 g
	10% SDS	4 mL
0.5 M Upper Tris-base buffer (pH 6.8)		made up to 100 mL
	Tris base	6.047 g
	10% SDS	4 mL
Others		
	Tetramethylethylene diamide (TEMED)	2% in final solution
	Ammonium persulphate (freshly prepared)	0.1% in final solution
2.11.3.3 Buffers for electrophoresis, transfer, and washing		
SDS gel loading buffer (2X)		
	Tris (from 1M Tris-HCl, pH 6.8)	125 mmol/L
	SDS	4%
	Glycerol	20%
	Bromophenol blue	0.06%
	β-mecaptoethanol	10% freshly add
Electrophoresis running buffer	Adjust pH to 8.3	
	Tris	25 mmol/L
	Glycine	250 mmol/L
	SDS	0.1%
Transfer buffer		
	Tris base	48 mmol/L
	Glycine	39 mmol/L
	SDS	0.037%
	Methanol	20%

Phosphate buffered saline with Tween-20 (PBST) Adjust pH to 7.4		
	NaCl	135 mmol/L
	$NaHPO_4$	3.2 mmol/L
	KH_2PO_4	0.5 mmol/L
	KCl	1.3 mmol/L
	Tween 20	0.05%

2.11.4 Primary antibodies

Primary antibodies for target proteins were diluted in 1% bovine serum albumin (dissolved in PBST) or 5 % non-fat milk for western blotting or in 5 % host serum for immunostaining and incubated overnight at 4 °C.

Antigen	**Host species**	**Type**	**Application**	**Company**
Angiotensin II (Ang II)	rabbit	polyclonal	IHC (1:500)	Peninsula laboratory
Angiotensin II type 1 receptor (AT_1R)	mouse	monoclonal	WB (1:1000)	Abcam
Angiotensin II type 2 receptor (AT_2R)	rabbit	polyclonal	WB (1:1000)	Abcam
Angiotensin converting enzyme ACE (N-20)	goat	polyclonal	WB (1:1000) IHC (I:200)	Santa Cruz
β-actin	mouse	monoclonal	WB (1:2000)	Abcam
BMP-4	mouse	monoclonal	WB (1:1000)	Sigma
Chymase	mouse	monoclonal	WB (1:500)	Abcam
COX-1	mouse	Monoclonal	WB (1:1000)	Cayman
COX-2	goat	polyclonal	WB (1:1000) IHC (1:100)	Santa Cruz
Endothelial nitric	rabbit	monoclonal	WB (1:500)	BD

oxide synthase (eNOS)				Transduction Laboratories
eNOS phosphorylated at Ser-1177 (p-eNOS)	rabbit	polyclonal	WB (1:1000)	Upstate
GAPDH (6C5)	mouse	monoclonal	WB (1:5000)	Ambion Inc.
Nitrotyrosine (1A6)	mouse	monoclonal	WB (1:1000)	Upstate
$p47^{phox}$ (H-195)	rabbit	polyclonal	WB (1:500)	Santa Cruz
$p22^{phox}$ (FL-195)	rabbit	polyclonal	WB (1:500)	Santa Cruz
p38 MAPK	rabbit	polyclonal	WB (1:1000)	Cell Signaling
Phosphor-p38 MAPK	mouse	monoclonal	WB (1:500)	Cell Signaling
Renin	mouse	monoclonal	WB (1:1000)	Swant, Switzerland
Renin receptor (ATP6IP2)	rabbit	polyclonal	WB (1:1000)	Abcam
SOD-1	rabbit	polyclonal	WB (1:2000)	Santa Cruz
SOD-2	goat	polyclonal	WB (1:2000)	Santa Cruz

2.12 Statistical analysis

Results were mean ± SEM on n blood vessels from separate animals. The cumulative concentration-response relationship was analyzed with a non-linear curve fitting (GraphPad Prism, Version 4.0). The pD_2 was calculated as the negative logarithm of the dilator concentration that induced 50% of the maximal relaxation (E_{max}). Protein expression analysis were normalized to GAPDH and then expressed relative to control. Student's *t*-test (unpaired two-tailed) was used and concentration-response curves were analyzed by two-way ANOVA followed by Bonferroni post-tests. Levels of probabilities of less than 0.05 were regarded as significant.

CHAPTER III

ANGIOTENSIN II TYPE 1 RECEPTOR-DEPENDENT OXIDATIVE STRESS MEDIATES DIABETIC ENDOTHELIAL DYSFUNCTION

3.1 Introduction

Type 2 diabetes mellitus is associated with an increased risk of cardiovascular complications (Malmberg et al., 2000). Although the exact mechanisms are incompletely understood, endothelial dysfunction plays a critical role in the initiation and progression of diabetic vascular diseases (De Vriese et al., 2000). The endothelium is essential for the maintenance and regulation of vascular homeostasis, by releasing both endothelium-derived relaxing factors such as nitric oxide (NO) and contracting factors such as reactive oxygen species (ROS). Endothelial dysfunction, characterized by a diminished release of endothelium-derived NO and/or an augmented release of contracting prostanoids and ROS (Wong et al., 2009), is an important early event in the development of hypertension, diabetes and atherosclerosis. The degree of reduced endothelium-derived NO predicts the severity of future vascular complications (Thomas et al., 2008).

Elevated ROS production in the vascular wall has been implicated as one of the major initiators for endothelial dysfunction (Cai and Harrison, 2000; Thomas et al., 2003) by direct inactivation of endothelium-derived NO. It is thus of great importance to define and explore oxidative mechanisms involved in endothelial dysfunction in type 2 diabetes as its prevalence has progressively and drastically increased, causing high morbidity and mortality (Hadi and Suwaidi, 2007). Sources of endogenous ROS that cause endothelial dysfunction include NAD(P)H oxidases (Cai et al., 2003) and endothelial nitric oxide synthase (eNOS) uncoupling (Oak and Cai, 2007). Increased ROS production and oxidative stress have been demonstrated in hypertension (Li et al., 2006; Vaziri et al., 2000), diabetes (Gao and Mann, 2009; Thomas et al., 2003), and atherosclerosis (Vendrov et al., 2006;

Xiong et al., 2009). Among these pathological changes, the role of the renin-angiotensin system (RAS) had been best defined in hypertension due to the wide application of RAS blockers for lowering blood pressure. Of importance, more evidence suggests a significant role of a local RAS in the vascular wall as a key negative regulator of endothelial function in diabetes. Diabetic patients have higher levels of plasma angiotensin II (Ang II) (Burnier and Zanchi, 2006). Ang II binds to both Ang II type 1 (AT_1R) and type 2 receptor (AT_2R) (Touyz and Schiffrin, 2000). Most known detrimental effects of Ang II are attributed to AT_1R which is linked to NAD(P)H oxidase activation and ROS production (Higashi et al., 2003). Exposure to high glucose up-regulates the AT_1R expression in cultured vascular smooth muscle cells (Sodhi et al., 2003). However, the precise intracellular mechanisms by which hyperglycaemia may increase the expression and function of AT_1R, and subsequent oxidative stress that in turn impairs endothelial function are not thoroughly understood.

In the present study, I examined the hypotheses that the upregulation of AT_1R together with oxidative stress plays a critical role in the induction and maintenance of endothelial dysfunction in aortas and mesenteric resistance arteries of type 2 diabetic *db/db* mice and more significantly, in renal arteries from diabetic patients. I intended to investigate whether AT_1R blockers could reverse diabetic endothelial dysfunction through direct actions on the endothelium.

3.2 Methods and Materials

3.2.1 Animal model

All animal experiments were performed on type 2 diabetic mice (C57BL/KSJ) lacking the gene encoding for leptin receptor (*db/db*) and heterozygote (*db/m*$^{+}$) control which were supplied by Chinese University of Hong Kong (CUHK) Laboratory Animal Service Center after an approval was obtained from the Animal Experimentation Ethics Committee, CUHK. Mice were kept in a temperature-controlled holding room (22–24°C) with a 12-h light/dark cycle, and fed a standard diet and water ad libitum. At the age of 12 weeks, adult male *db/db* mice were treated for 6 weeks with valsartan or enalapril at 10 mg/kg body weight/day or vehicle via oral gavage. Plasma glucose levels were determined using a blood glucose meter (Ascenia Elite XL, Bayer, IN). Systolic blood pressure was measured by a tail-cuff method.

3.2.2 Human renal arteries

Human renal arteries were obtained during surgery after informed consent from patients undergoing nephrectomy at ages between 56-82 years old. The group of diabetic patients had a fasting plasma glucose level ≥ 7.0 mmol/L (126 mg/dL) or 2–h plasma glucose ≥ 11.1 mmol/L (200 mg/dl).

3.2.3 Measurements of plasma lipid profile and insulin

Plasma levels of total cholesterol and triglyceride were determined using enzymatic methods (Stanbio, Boerne, TX, USA) and plasma insulin level was assayed by enzyme immunoassay (Mercodia, Sweden).

3.2.4 Isometric force measurement

After mice were sacrificed by CO_2 inhalation, the thoracic aortas and small intestines with mesentery were rapidly removed and placed in oxygenated ice-cold Krebs-Henseleit solution. Segments of blood vessels including aortas and mesenteric resistance arteries were carefully dissected free from adjacent connective tissues. Changes in isometric tension of vessels were recorded in a

Multi Myograph System (Danish Myo Technology A/S, Denmark) as previously described (Huang et al., 2003) and changes in isometric tension were recorded. The aortic ring was stretched to an optimal baseline tension of 3 mN and mesenteric resistance arteries of 1 mN, and then allowed to equilibrate for 60 min before the start of the experiment. Each ring was first contracted by 60 mmol/L KCl and rinsed in Krebs solution, and after wash out, phenylephrine (1 μmol/L) was used to produce a steady contraction and relaxed by cumulative additions of acetylcholine (ACh) (10^{-8} to 10^{-5} mol/L) in control or in the presence of 3 μmol/L losartan (ARB), 100 μmol/L apocynin [NAD(P)H oxidases inhibitor], 100 μmol/L tempol [superoxide dismutase (SOD) mimetic] or combination of 1 mmol/L tiron (SOD mimetic) and 100 μmol/L diethyldithiocarbamate (DETCA, ROS scavenger). Endothelium-independent relaxations to sodium nitroprusside (SNP) (10^{-9} to 10^{-6} mol/L) were studied in rings without endothelium. Each experiment was performed on rings prepared from different mice.

Each human renal artery was cut into 2-3 ring segments (2-3 mm in length). Rings were suspended in organ baths as described previously (Leung et al., 2006). Each ring was initially stretched to an optimal tension of 25 mN and then allowed to equilibrate for 90 min before the start of the experiment.

3.2.5 Detection of intracellular ROS by dihydroethidium (DHE) fluorescence

The amount of intracellular ROS production was determined using DHE (Molecular Probes, Eugene, OR), which binds to DNA when oxidized to emit fluorescence (Robinson et al., 2006). Aortic rings from *db/m*$^{+}$ and *db/db* mice were obtained as described above and treated with or without acetylcholine. To investigate the inhibitory effects of the RAS on ROS production, aortas were exposed for 30 min to one of the inhibitors including 3 μmol/L losartan, 100 μmol/L apocynin, or 100 μmol/L tempol before the addition of 10 μmol/L acetylcholine, as to mimic the conditions in the functional study. To verify the contribution of ROS production from endothelium, the endothelial layer was removed by rolling the luminal surface with fine forceps tips. To examine the role of extracellular calcium ions on the generation of ROS, calcium-free Krebs solution was prepared to incubate the aortic

rings for 30 min before the addition of acetylcholine. Frozen sections of the aortic ring were cut in 10-μm thickness using cryostat and incubated for 10 min at 37°C in Krebs solution containing 5 μmol/L DHE. Fluorescent intensity was measured by confocal microscope (FV1000, Olympus, Tokyo, Japan) at excitation/emission of 488/605 nm to visualize the signal.

3.2.6 Immunohistochemical staining of Ang II

Aortic rings were fixed in 4% paraformaldehyde at 4ºC overnight, dehydrated, processed and embedded in paraffin. Cross sections at 5 μm were cut on microtome (Leica Microsystems, Germany). After rehydrated to water, sections were microwave boiled in 0.01 mol/L citrate buffer (pH 6.0) for 10 min for antigen retrieval, then incubated for 15 min with 3% H_2O_2 at room temperature to block endogenous peroxidase activity. After washed with phosphate buffer saline (PBS), sections were blocked in 5% normal goat or donkey serum according to the host species (Jackson Immunoresearch, West Grove, PA) for 1 hour at room temperature. Primary antibody (anti-Ang II, 1:500, Peninsula laboratory, Belmont CA) diluted in normal serum were incubated overnight at 4ºC. The slides were washed with PBS three times (5 min each). Biotin-SP conjugated goat anti-rabbit secondary antibodies (1:500, Jackson Immunoresearch) diluted in PBS were added and incubated for 1 hr at room temperature. Slides were washed with PBS three times (5 min each) and incubated for 30 min with streptavidin-HRP conjugate (1:500, Zymed laboratory, San Francisco, CA) at room temperature, and washed. Positive staining was developed as brown precipitate by 3,3'-diamonobenzidine tetrachloride (DAB) chromogen substrate (Vector laboratory, Burlingame, CA). Slides were rinsed with water and counterstained with hematoxylin. Pictures were taken under Leica DMRBE microscope with a SPOT-RT digital camera and SPOT Advanced software (Diagnostic Instruments, Sertling Heights, MI) and intensities of signals were analyzed by ImageJ (National Institute of Health, USA).

3.2.7 Western blotting

Protein samples prepared from aorta homogenates were electrophoresed through a 10 % SDS-poly-acrylamide gel, transferred onto an immobilon-P polyvinylidene

difluoride membrane (Millipore Corp., Bedford, MA). Nonspecific binding sites were blocked with 5 % non-fat milk or 1 % BSA in 0.05 % Tween-20 phosphate-buffered saline. The blots were incubated overnight at 4°C with the primary antibodies: monoclonal anti-AT_1R, polyclonal anti-AT_2R (1:1000, Abcam, Cambridge, UK); monoclonal anti-nitrotyrosine (1:2000, Abcam, Cambridge, UK), polyclonal anti-phospho-eNOS Ser^{1177} (1:1000, Upstate Biotechnology, Lake Placid, NY); polyclonal anti-ACE, anti-eNOS, anti-$p22^{phox}$ and anti-$p47^{phox}$ (1:1000, Santa Cruz, CA); followed by HRP-conjugated secondary antibody (DakoCytomation, Carpinteria, CA). Monoclonal anti-β-actin (1:5000, Abcam) was used as a housekeeping protein.

3.2.8 Organ culture of mouse arterial rings in high glucose medium

High glucose (30 mmol/L) and mannitol (osmotic control) solutions were prepared in Dulbeco's Modified Eagle's Media (DMEM, Gibco, Gaithersberg, MD) culture media supplemented with 10% fetal bovine serum (FBS, Gibco), plus 100 IU/ml penicillin and 100 µg/ml streptomycin. Mouse thoracic aortic rings (2 mm in length) were then incubated in five groups including 5 mmol/L glucose alone (NG), 5 mmol/L glucose plus 25 mmol/L mannitol (M), 30 mmol/L glucose (HG), 30 mmol/L glucose plus 3 µmol/L losartan (HG + losartan) for 36 hours in an incubator kept at 37°C. After the incubation period, the segments were transferred to fresh Krebs solution, mounted in a myograph, and changes in arterial tone were recorded.

3.2.9 Spin trap and Electron paramagnetic resonance spectroscopy (EPR)

Hypoxanthine (100 µmol/L) plus xanthine oxidase (0.1 U/mL) were incubated in Krebs solution at 37°C bubbled with 95% O_2 and 5% CO_2. Spin trap, 100 µmol/L TEMPONE-H (1-Hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine HCl, Alexis), was added to the medium solution to trap superoxide radicals and peroxynitrite for 30 min. In order to inhibit reactions catalyzed by transition metals, 100 µmol/L diethylenetriaminepentaacetate (DTPA, Sigma) was added. The medium were then transferred to a glass tube and put in liquid nitrogen immediately before EPR

measurement. All inhibitors were added 10 min prior to TEMPONE-H. The EPR measurements were performed at room temperature using an EMX-A ESR spectrometer (Bruker, Germany). The EPR-settings were as follows: field swept: 3444-3504 G; microwave power: 200 mW; magnetic field sweep time: 671s.

3.2.10 Drugs and solutions

Acetylcholine, N^G-nitro-L-arginine methyl ester (L-NAME), phenylephrine, angiotensin II, sodium nitroprusside (SNP), tempol, tiron, and DETCA were purchased from Sigma-Aldrich Chemical (St Louis, MO). Apocynin was from Calbiochem (San Diego, CA). Losartan was purchased from Cayman (Ann Arbor, MI). Besides losartan was dissolved in DMSO (Sigma-Aldrich), all other drugs were dissolved in double distilled water. Krebs solution contained (mmol/L): 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11 D-glucose. A Ca^{2+}-free solution was identical to Krebs solution with exclusion of Ca^{2+} and addition of 2 mmol/L EGTA.

3.2.11 Statistical Analysis

Results were mean ± SEM from different mice. Concentration-response curves were analyzed by non-linear regression curve fitting using GraphPad Prism software (Version 4.0, San Diego, CA) to approximate E_{max} as the maximal response and pIC_{50} as the negative logarithm of the drug concentration that produced 50% of E_{max}. These values are summarized in for relaxant responses in both mouse and human arteries. The protein expression was quantified by densitometer (FluorChem, Alpha Innotech, San Leandro, CA) and normalized to β-actin and then compared with control. Images of DHE fluorescence were analyzed by ImageJ (NIH). Statistical significance was determined by two-tailed Student's t-test or one-way ANOVA followed by Bonferroni post-tests when more than two treatments were compared. $P<0.05$ was regarded as significantly different.

3.3 Results

3.3.1 Basic metabolic parameters

Body weight gain in *db/db* mice increased gradually from 4 to 16 weeks when compared to age-matched *db/m+* lean control mice (**Figure 3.1A**). Body weight of 18-week old *db/db* mice (55.7 ± 1.7 g, $p<0.05$ vs *db/m*$^+$) was greater compared to non-diabetic aged-matched *db/m*$^+$ mice (26.6 ± 1.5 g). Valsartan or enalapril treatment for 6 weeks did not alter body weight of *db/db* mice. Oral glucose tolerance test revealed a progressive impairment in glucose sensitivity **(Figure 3.1B)** comparing to lean control. The levels of fasting blood glucose and plasma insulin were higher in *db/db* mice than *db/m*$^+$ mice and these values were unaffected by valsartan or enalapril treatment **(Table 3.1)**. However, valsartan and enalapril treatment both improved glucose tolerance **(Figure 3.2A-C)**. Blood pressure of *db/db* mice (127.3 ± 3.9 mmHg, $p<0.05$ vs *db/m*$^+$) was higher than that of *db/m*$^+$ mice (92.6 ± 1.6 mmHg) which was reduced by valsartan (102.6 ± 4.3 mmHg, $p<0.05$ vs *db/db*) or enalapril (93.0 ± 1.9 mmHg, $p < 0.05$ vs *db/db*) treatment **(Figure 3.2D)**. Besides, the elevated levels of plasma triglyceride in *db/db* mice were insensitive to valsartan or enalapril treatment. By contrast, valsartan or enalapril treatment reversed the increased level of total cholesterol in *db/db* mice **(Table 1)**.

3.3.2 Effects of ARB and ROS scavengers on endothelium-dependent dilatations in conduit and resistance arteries

Acetylcholine (ACh)-induced endothelium-dependent dilatations were impaired in *db/db* mouse aortas and mesenteric resistance arteries (MRA) as compared with those of non-diabetic *db/m*$^+$ mice **(Figure 3.3A&B** and **3.4A&B)**, whilst sodium nitroprusside (SNP)-induced endothelium-independent dilatations were comparable between the two groups **(Figure 3.3C&3.4C)**. AT_1R blockade by 3 μmol/L losartan (30-min incubation) **(Figure 3.5A&3.6A)** and inhibition of NAD(P)H oxidases by 100 μmol/L apocynin **(Figure 3.5B&3.6B)** significantly improved, although not normalized, ACh-induced vasodilatations; in addition, no further improvement could be obtained by using the combination of losartan and apocynin

(Figure 3.5C&3.6C). Furthermore, SOD mimetic 100 µmol/L tempol **(Figure 3.5D&3.6D)** also enhanced the blunted dilatations to ACh in *db/db* mouse aortas to a similar extent as apocynin.

3.3.3 Improved endothelium-dependent dilatations in db/db mice by increasing NO bioavailability by chronic RAS blockade

Six week chronic treatment of valsartan or enalapril significantly improved endothelium dependent dilatations in *db/db* mouse aortas as shown in representative tracings **(Figure 3.7A)** and summarized data **(Figure 3.7B&C)**. Likewise, impaired acetylcholine-induced relaxations in MRA from *db/db* mice were also improved by RAS blockade **(Figure 3.8A&B)**. In MRA from *db/m*$^{+}$, L-NAME inhibited about 50% of ACh-induced vasodilatation **(Figure 3.9A)**; however, in *db/db* mouse MRA, L-NAME abolished ACh-induced relaxation **(Figure 3.9B)**. In addition, L-NAME also abolished the improved relaxation to ACh in valsartan or enalapril-treated *db/db* mice to the same extent as *db/db* **(Figure 3.9C&D)**. Similarly, ROS scavenging agents Tiron plus DETCA acutely normalized ACh-induced dilatations in *db/db* MRA **(Figure 3.10A)** which were also reversed by co-incubation with L-NAME **(Figure 3.10B)**.

3.3.4 Augmented ROS production in db/db mouse aortas mediated by AT_1R

The basal level of ROS reflected by the intensity of dihydroethidium (DHE) fluorescence was much higher in the wall of *db/db* mouse aortas **(Figure 3.11)**. ROS signal markedly increased in response to 10 µmol/L ACh, but to a greater extent in *db/db* mouse aortas. Acute exposure of *db/db* mouse aortas to100 µmol/L L-NAME attenuated ACh-stimulated rises in ROS. The increased ROS generation was eliminated by 30-min treatment with 3 µmol/L losartan, 100 µmol/L apocynin or 100 µmol/L tempol. Furthermore, the ACh-stimulated ROS increase was greatly diminished in the absence of extracellular Ca^{2+} ions or in aortas without endothelium **(Figure 3.11)**. Increased DHE fluorescence intensity in *db/db* mouse aortas was also abolished by chronic valsartan or enalapril treatment **(Figure 3.12)**.

3.3.5 Effects of RAS blockade on local production of Ang II in vascular wall

Increased Ang II staining was observed in aortic vascular wall from *db/db* mice compared to lean *db/m*$^{+}$ control **(Figure 3.13A&B)**, accompanied by angiotensin converting enzyme (ACE) upregulation **(Figure 3.13C)**. Chronic RAS blockade normalized ACE expression and tissue Ang II levels **(Figure 3.13A-C)**.

3.3.6 Western blot analysis of AT_1R, AT_2R, $p22^{phox}$, $p47^{phox}$, nitrotyrosine, eNOS and p-eNOS

Immunoblotting showed that a significantly increased expression of AT_1R in *db/db* mouse aortas was normalized by valsartan or enalapril treatment **(Figure 3.14A)** while AT_2R expression remained unaffected **(Figure 3.14B)**. In addition, chronic therapy with valsartan or enalapril reduced the increased level of NAD(P)H oxidase subunits $p22^{phox}$ **(Figure 3.15A)** and $p47^{phox}$ **(Figure 3.15B)**. The elevated nitrotyrosine levels in *db/db* mouse aortas were also reversed by the treatment with valsartan or enalapril **(Figure 3.15C)**. Phosphorylation of eNOS at ser1177 reduced in *db/db* mouse aortas but could not be reversed by RAS blockade, while total eNOS protein expression remained unchanged **(Figure 3.16)**.

3.3.7 Impaired endothelium-dependent relaxations in renal arteries from diabetic patients rescued by AT1R blockade

Renal arteries obtained from diabetic patients relaxed significantly less in response to ACh than those from non-diabetic subjects as shown in representative tracings **(Figure 3.17A&B)** and summarized data **(Figure 3.18A)**. Acute exposure to 3 μmol/L losartan for 30 min markedly enhanced the ACh-induced relaxations in diabetic renal arteries **(Figure 3.17C&3.18B)** without affecting relaxations in non-diabetic human renal arteries **(Figure 3.18C)**.

3.3.8 High glucose-induced endothelial dysfunction mediated by AT_1R

Chronic exposure (36-hour) of non-diabetic mouse aortas to high glucose (30 mmol/L), but not to mannitol resulted in impaired ACh-induced dilatations **(Figure 3.19A)** whilst SNP-induced endothelium-independent relaxations were unaffected **(Figure 3.19B)**. The presence of 3 µmol/L losartan prevented the impairment of ACh-induced dilatations in high glucose treated aortic rings **(Figure 3.19C)**. Likewise, losartan inhibited high glucose-stimulated increase in ROS production in the mouse aortic wall as demonstrated by DHE fluorescence **(Figure 3.20)**. To further define the principal role of AT_1R in endothelial dysfunction, we demonstrated that losartan restored ACh-induced dilatations which were impaired by 12-hour incubation with Ang II (100 nmol/L) in non-diabetic mouse aortas **(Figure 3.19D)**. By contrast, 3 µmol/L losartan used in the functional study did not scavenge ROS as indicated by electron paramagnetic resonance spectra **(Figure 3.21)**.

Table 3.1

Basic parameters in *db/m*$^+$ control, *db/db*, and *db/db* mice chronically treated with valsartan or enalapril

Parameter	*db/m*$^+$	*db/db*	*db/db* + Valsartan	*db/db* + Enalapril
Body weight, g	26.6 ± 1.5	55.7 ± 1.7*	52.8 ± 1.4	55.7 ± 2.8
Blood pressure, mmHg	92.6 ± 1.6	127.3 ± 3.9*	102.6 ± 4.3#	93.0 ± 1.9#
Plasma level of				
Glucose (fasting), mmol/L	5.2 ± 2.2	17.0 ± 3.7*	14.0 ± 1.6	15.1 ± 1.6
Insulin, ng/mL	1.4 ± 0.12	24.6 ± 3.5*	26.2 ± 4.4	25.8 ± 5.1
Total cholesterol, mg/dL	75.7 ± 2.4	133.1 ± 6.4*	97.5 ± 3.7#	113.9 ± 5.3#
Triglyceride, mg/dL	86.5 ± 5.2	184.3 ± 15*	174.7 ± 10	166.3 ± 13

Results are mean ± SEM of measurements from 6-8 different mice. *P<0.05 versus *db/m*$^+$ group; #P<0.05 versus *db/db* group.

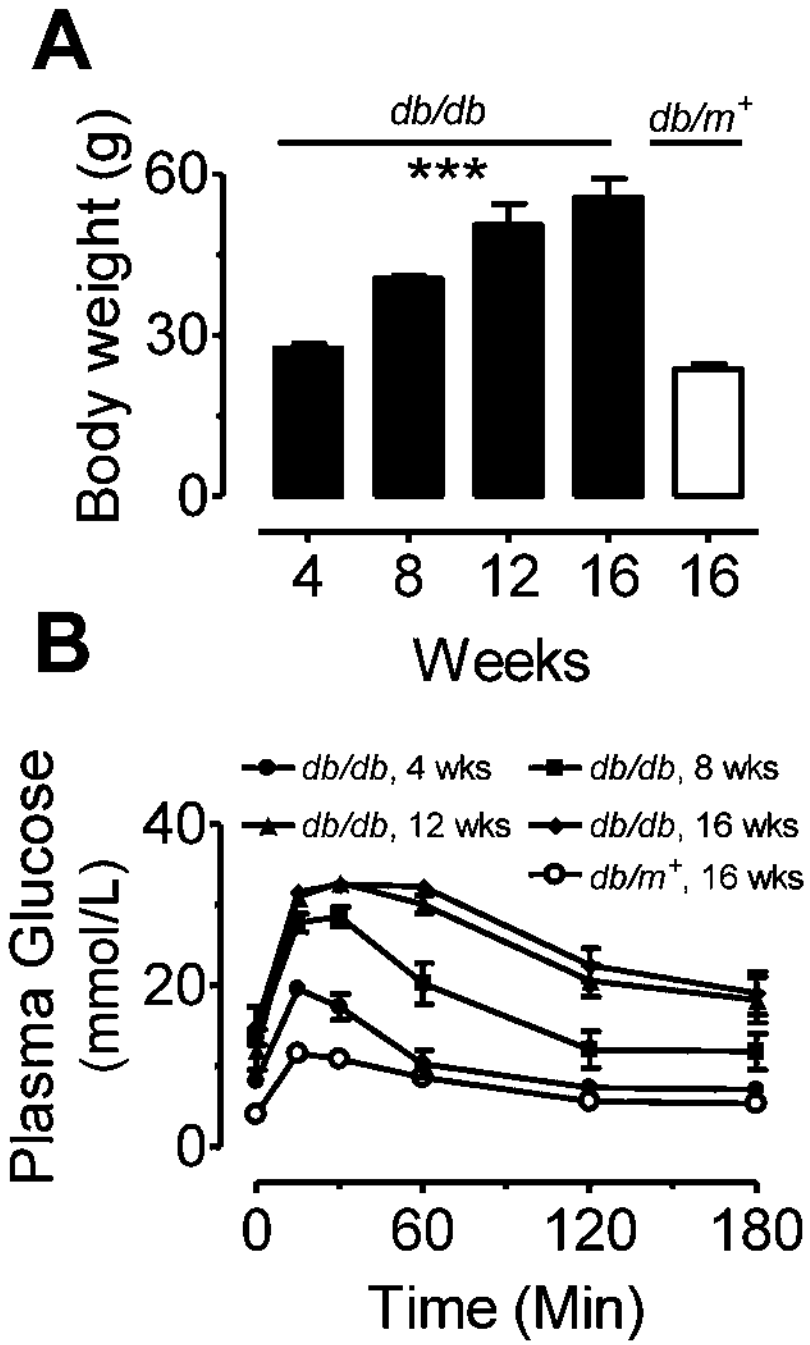


Figure 3.1.
(A) Gain in body weight of db/db mice from 4 to 16 weeks as compared with the *db/m+* lean mice. **(B)** Oral glucose tolerance test in *db/m+* and *db/db* mice at 4, 8, 12 and 16 weeks. Data are mean ± SEM of 5-7 experiments. Statistical significance between groups is indicated by ***p<0.001.

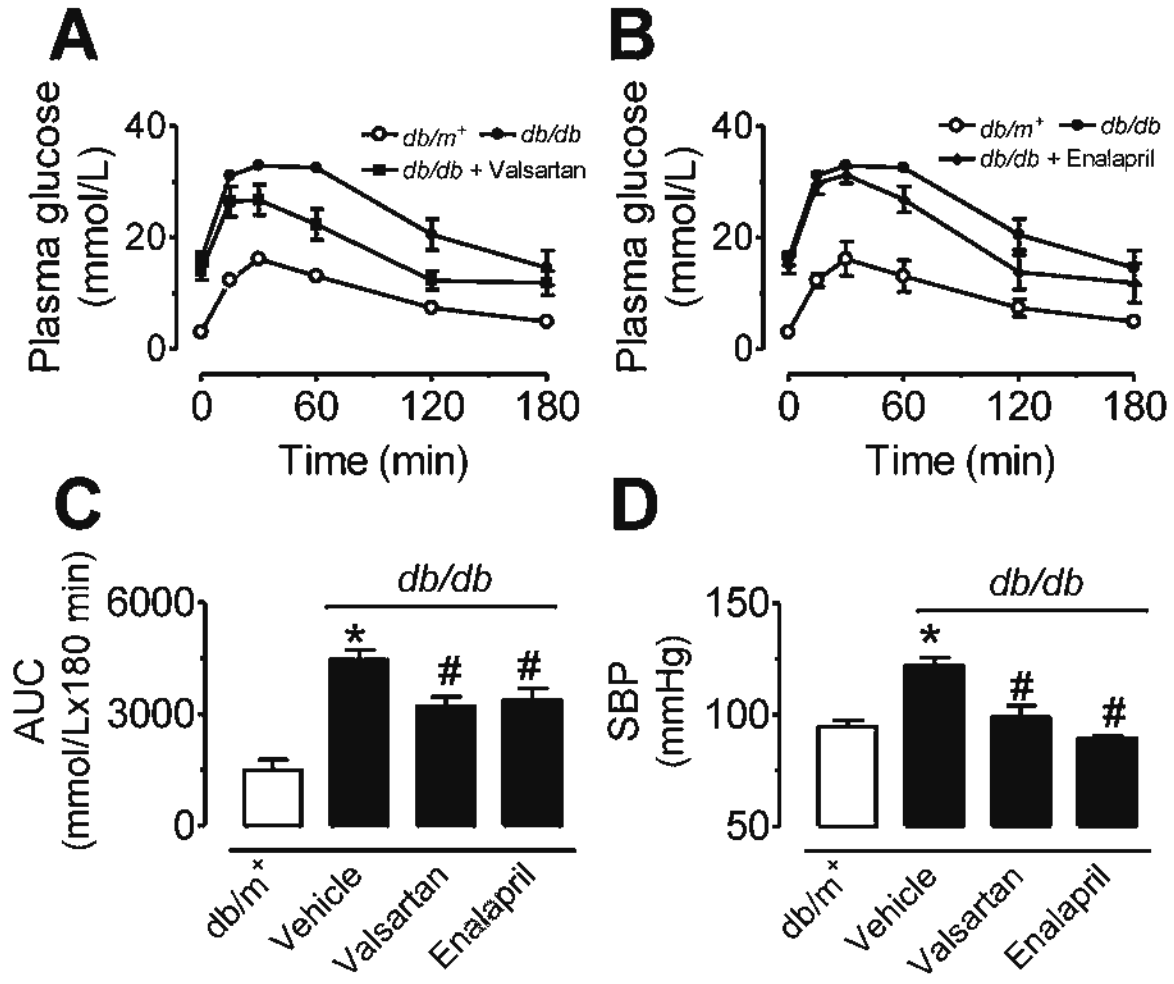


Figure 3.2.
Effects of chronic treatment of valsartan **(A)** and enalapril **(B)** on oral glucose tolerance test (OGTT) in *db/db* mice. **(C)** Summarized data showing area under curve (AUC) of OGTT in different treatment groups. **(D)** Systolic blood pressure (SBP) of *db/m*+, *db/db*, *db/db* treated with valsartan and *db/db* treated with enalapril. Results are mean ± SEM of 6-8 experiments. Statistical significances are indicated by *p<0.05 vs *db/m*+ and # p<0.05 vs *db/db*.

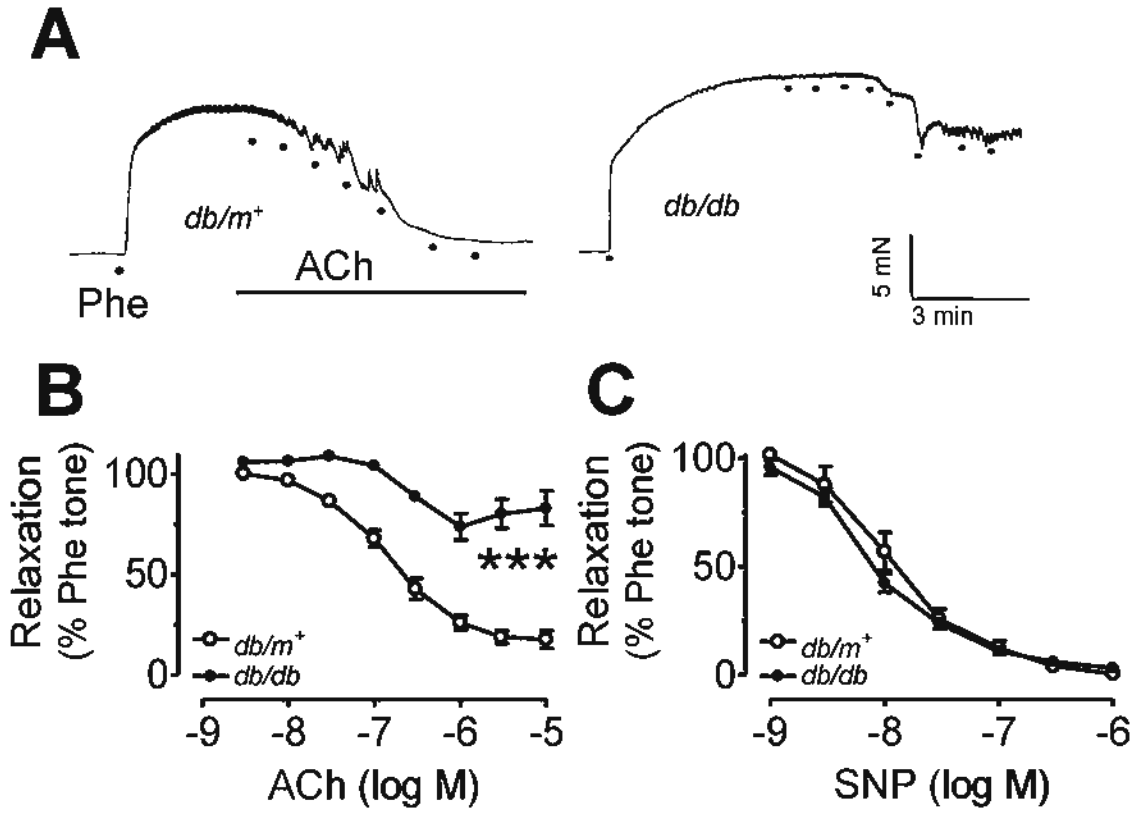


Figure 3.3.
(A) Representative traces showing acetylcholine (ACh)-induced endothelium-dependent dilatations were blunted in aortas of *db/db* mice as compared with those of *db/m+* mice. **(B)** Concentration-response curves for ACh-induced dilatations in *db/db* and *db/m+* mouse aortas. **(C)** Concentration-response curves for endothelium-independent dilatations to sodium nitroprusside (SNP) in *db/m+* and db/db mouse aortas. Data are mean ± SEM of 8-12 experiments. Statistical significance between *db/m+* and db/db mice is indicated by ***p<0.001.

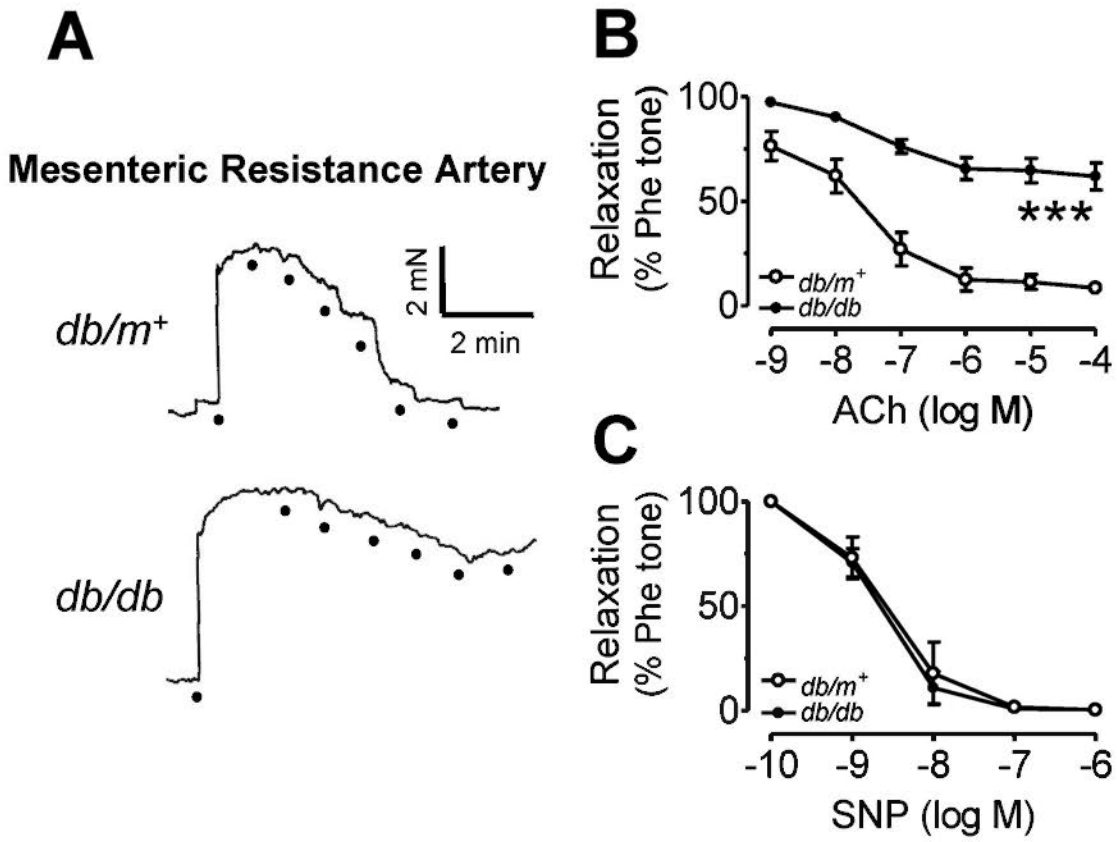


Figure 3.4.
(A) Representative traces showing that ACh-induced endothelium-dependent dilatations were blunted in second-order mesenteric resistance arteries (MRA) from *db/db* mice as compared with those of *db/m+* control. **(B)** Concentration-response curves for ACh-induced dilatations in *db/db* and *db/m+* mouse SMA. **(C)** SNP-induced endothelium-independent dilatations were unaltered in *db/db* and *db/m+* MRA. Results are mean ± SEM of 6-8 experiments. Statistical significance between *db/m+* and *db/db* groups is indicated by ***p<0.01.

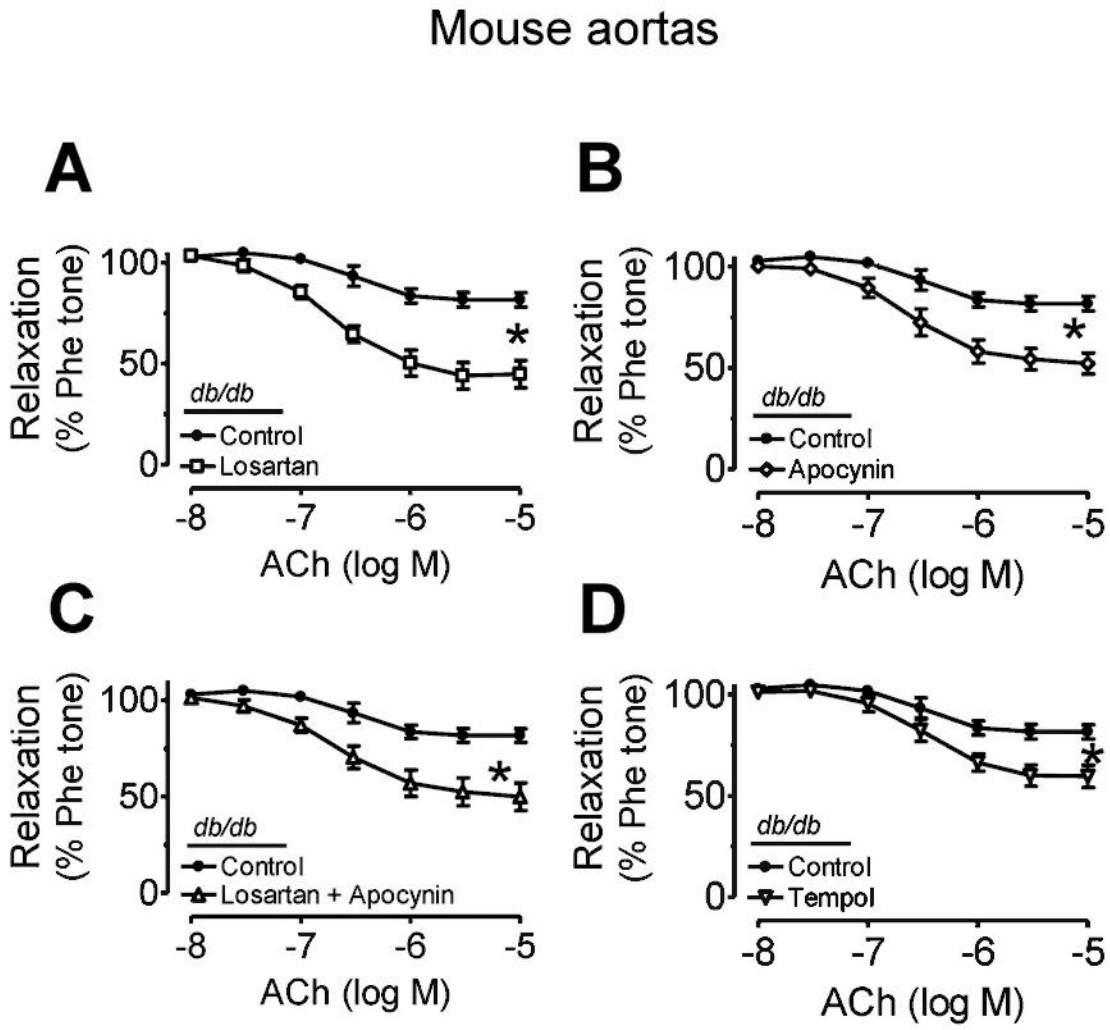


Figure 3.5.

Effects of acute 30-min exposure of 3 µmol/L losartan **(A)**, 100 µmol/L apocynin **(B)**, combination of 3 µmol/L losartan and 100 µmol/L apocynin **(C)**, and 100 µmol/L tempol **(D)** on ACh-induced endothelium-dependent dilatations in *db/db* mouse aortas. Results are mean ± SEM of 6-8 experiments. Statistical significances between control and treatment groups are indicated by *$p<0.05$

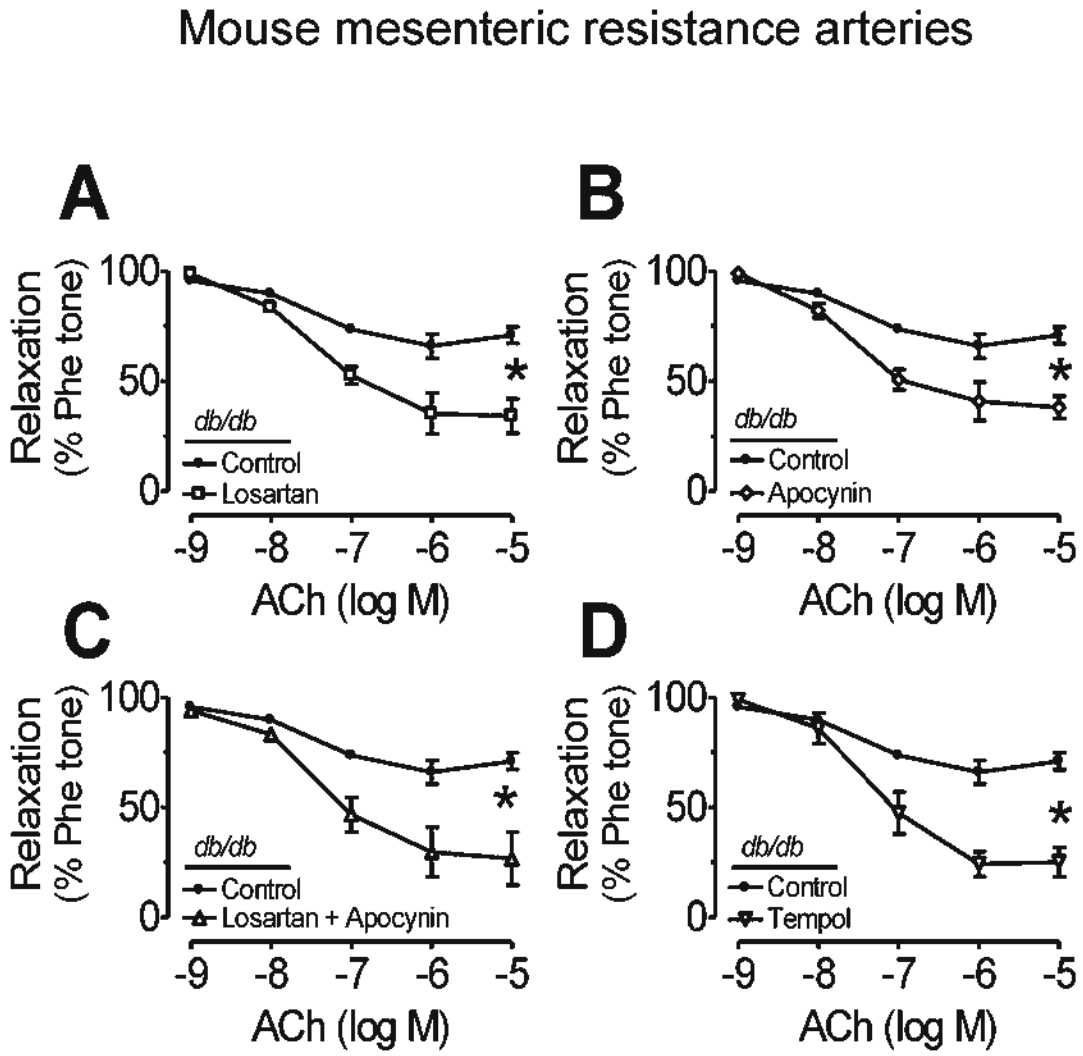


Figure 3.6.
Effects of acute 30-min exposure of 3 µmol/L losartan **(A)**, 100 µmol/L apocynin **(B)**, combination of 3 µmol/L losartan and 100 µmol/L apocynin **(C)**, and 100 µmol/L tempol **(D)** on ACh-induced endothelium-dependent dilatations in *db/db* mouse MRA. Results are mean ± SEM of 6-8 experiments. Statistical significances between control and treatment groups are indicated by *$p<0.05$.

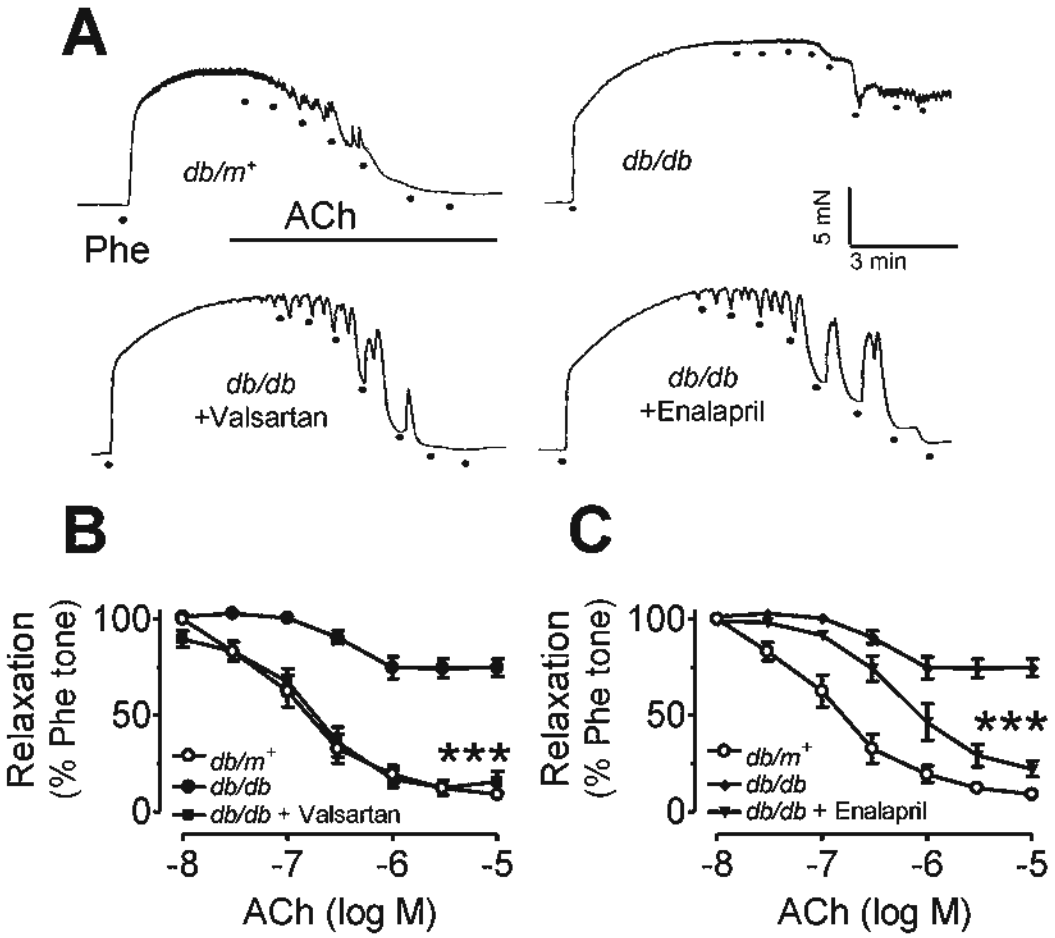


Figure 3.7.
(A) Representative tracings showing that the impaired ACh-induced endothelium-dependent dilatations in *db/db* mouse aortas were rescued by chronic treatment with valsartan or enalapril. Concentration-response curves for dilatations in db/db mice treated chronically with valsartan **(B)** or enalapril **(C)**. Results are mean ± SEM of 6-8 experiments. Statistical significances between control and treatment groups are indicated by ***p<0.001.

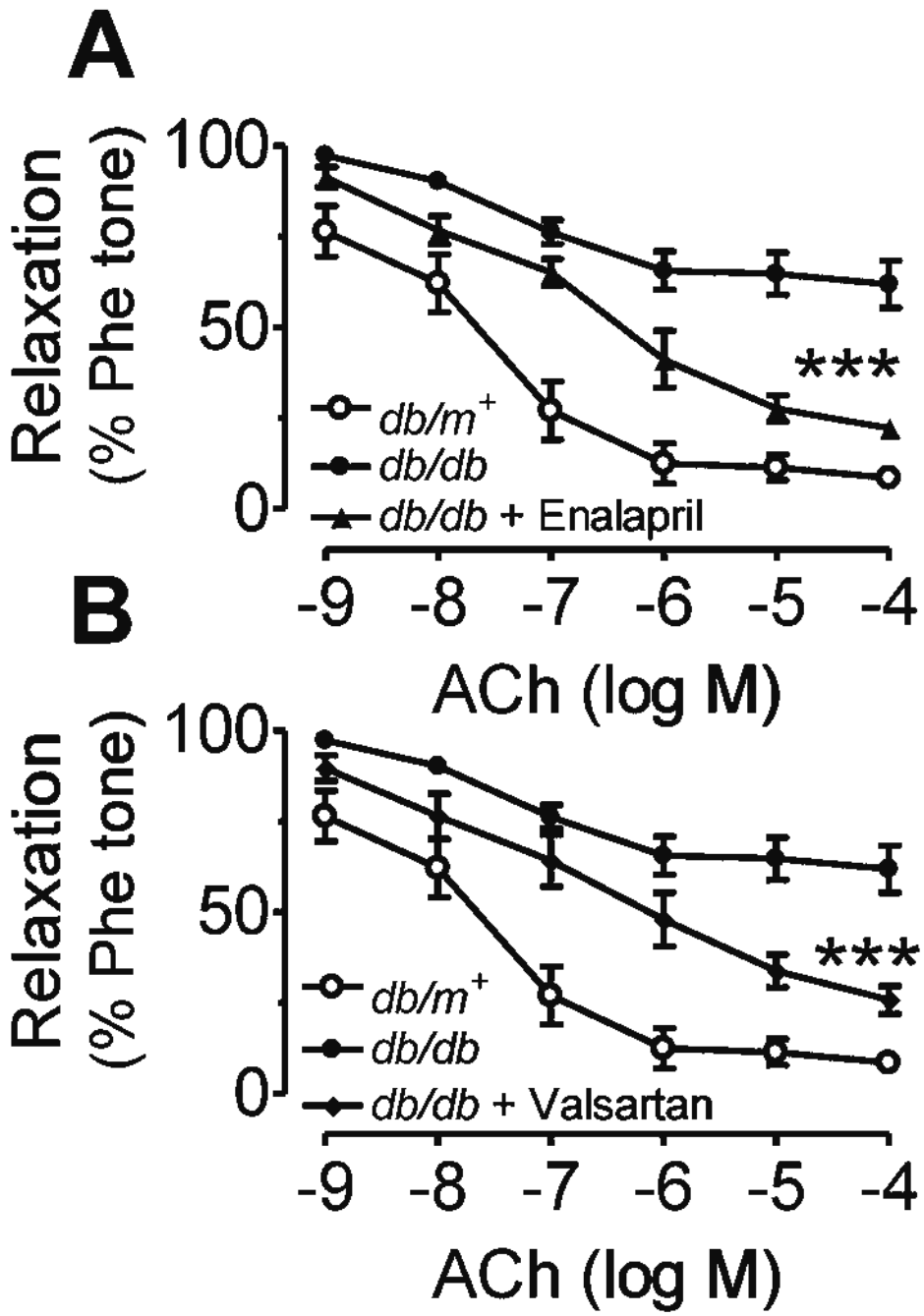


Figure 3.8.
The impaired ACh-induced endothelium-dependent dilatations in MRA of *db/db* mice were partially restored by chronic treatment with valsartan **(A)** and enalapril **(B)**. Results are mean ± SEM of 6-8 experiments. Statistical significances between control and treatment groups are indicated by ***p<0.001.

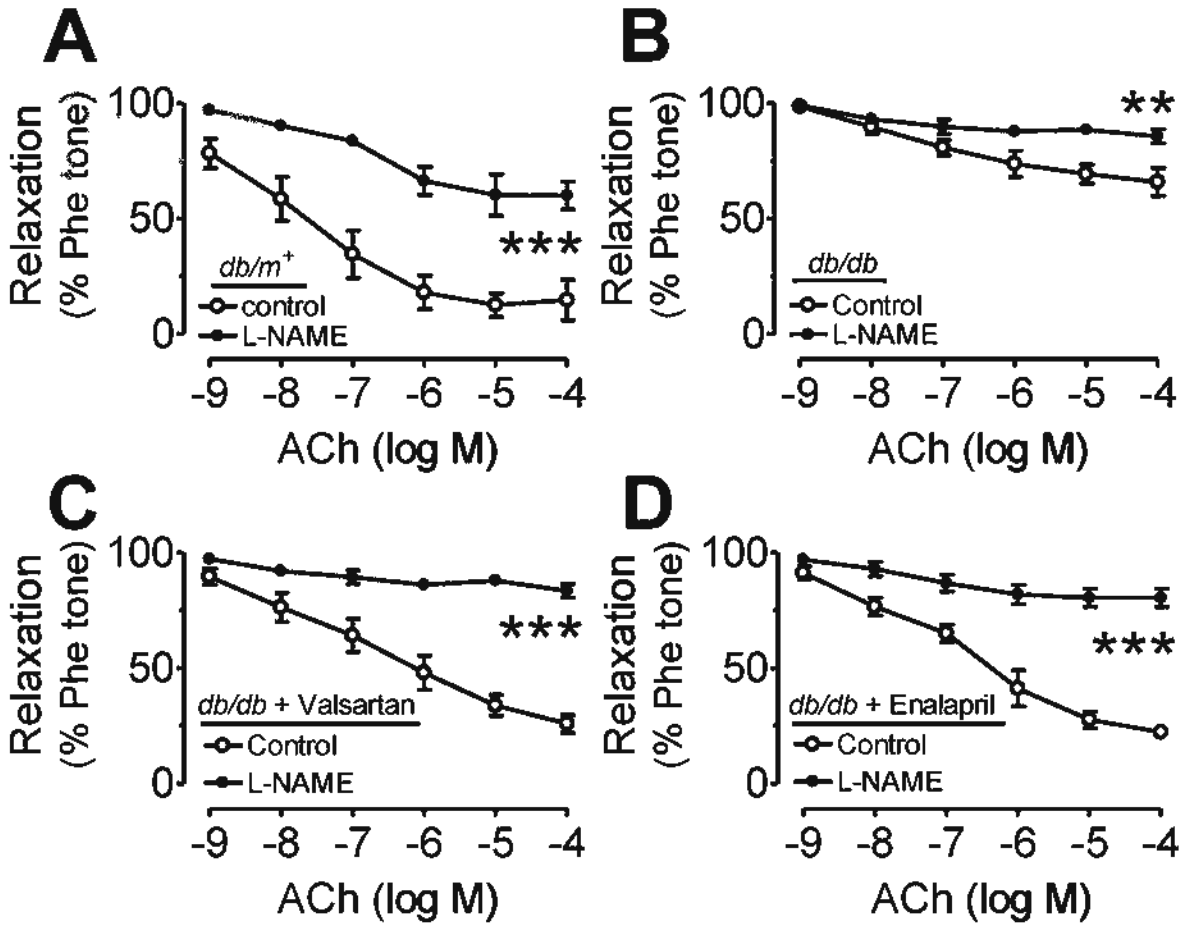


Figure 3.9.
Effects of 100 µmol/L L-NAME (nitric oxide synthase inhibitor) on ACh-induced endothelium-dependent dilatations in MRA from *db/m*+ **(A)**; *db/db* **(B)**; *db/db* treated with valsartan **(C)**; and *db/db* treated with enalapril **(D)**. Results are mean ± SEM of 6-8 experiments. Statistical significances between control and treatment groups are indicated by ***p<0.001 and **p<0.01.

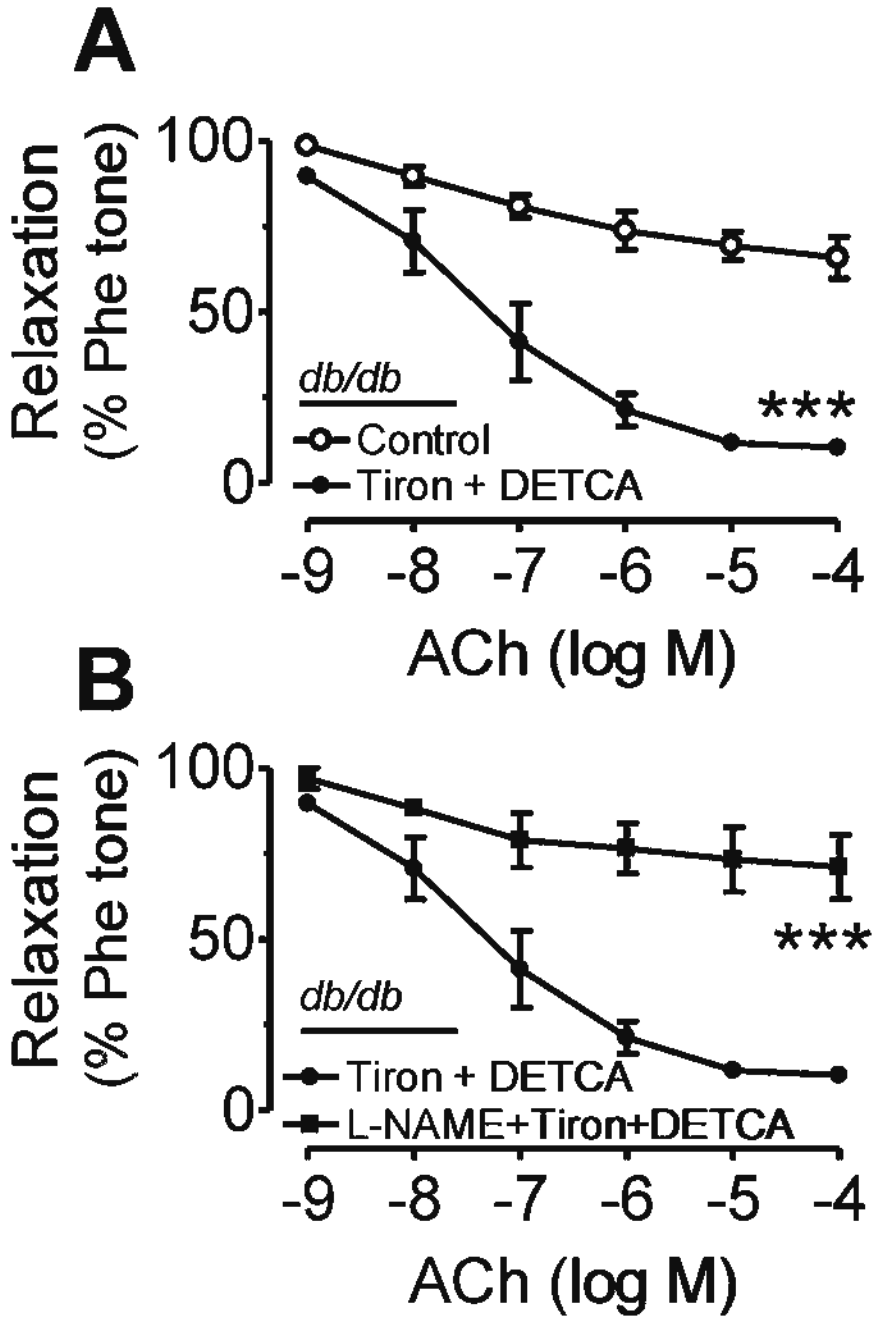


Figure 3.10.
(A) Effects of a combination of 1 mmol/L tiron plus 100 µmol/L DETCA on ACh-induced endothelium-dependent dilatations in MRA from *db/db* mice. **(B)** Reversal of the effect of tiron plus DETCA by co-treatment with 100 µmol/L L-NAME. Results are mean ± SEM of 6-8 experiments. Statistical significances between control and treatment groups are indicated by ***p<0.001.

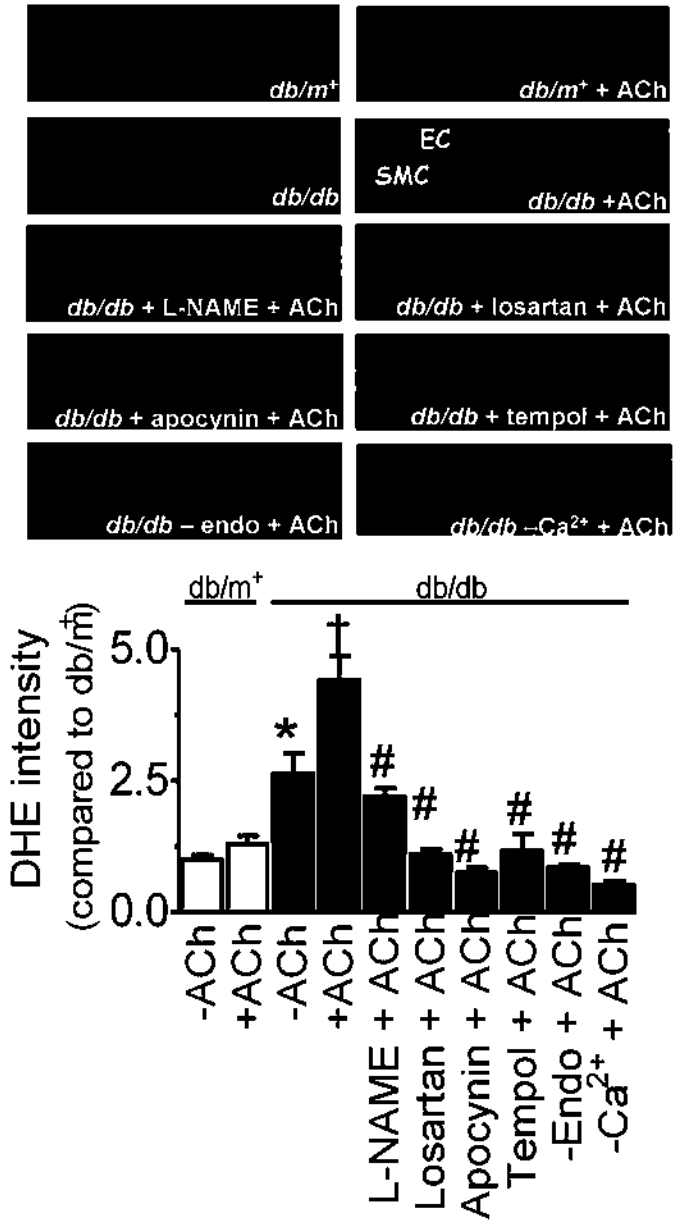


Figure 3.11.
(A) Representative images showing dihydroethidium (DHE) fluorescence intensity indicative of ROS production in *db/m*+ and *db/db* mouse aortas with and without ACh stimulation; and the effects of 100 µmol/L L-NAME, 3 µmol/L losartan, 100 µmol/L apocynin, 100 µmol/L tempol, removal of endothelium and absence of extracellular calcium on ROS generation under ACh stimulation in *db/db* mouse aortas. **(B)** Summarized values for the DHE fluorescent intensity. Results are mean ± SEM of 4-6 experiments. Statistical significance is indicated by **p*<0.05 vs *db/m*+ -ACh. †*p*<0.05 vs *db/db* -ACh. # *P*<0.05 vs *db/db* +ACh.

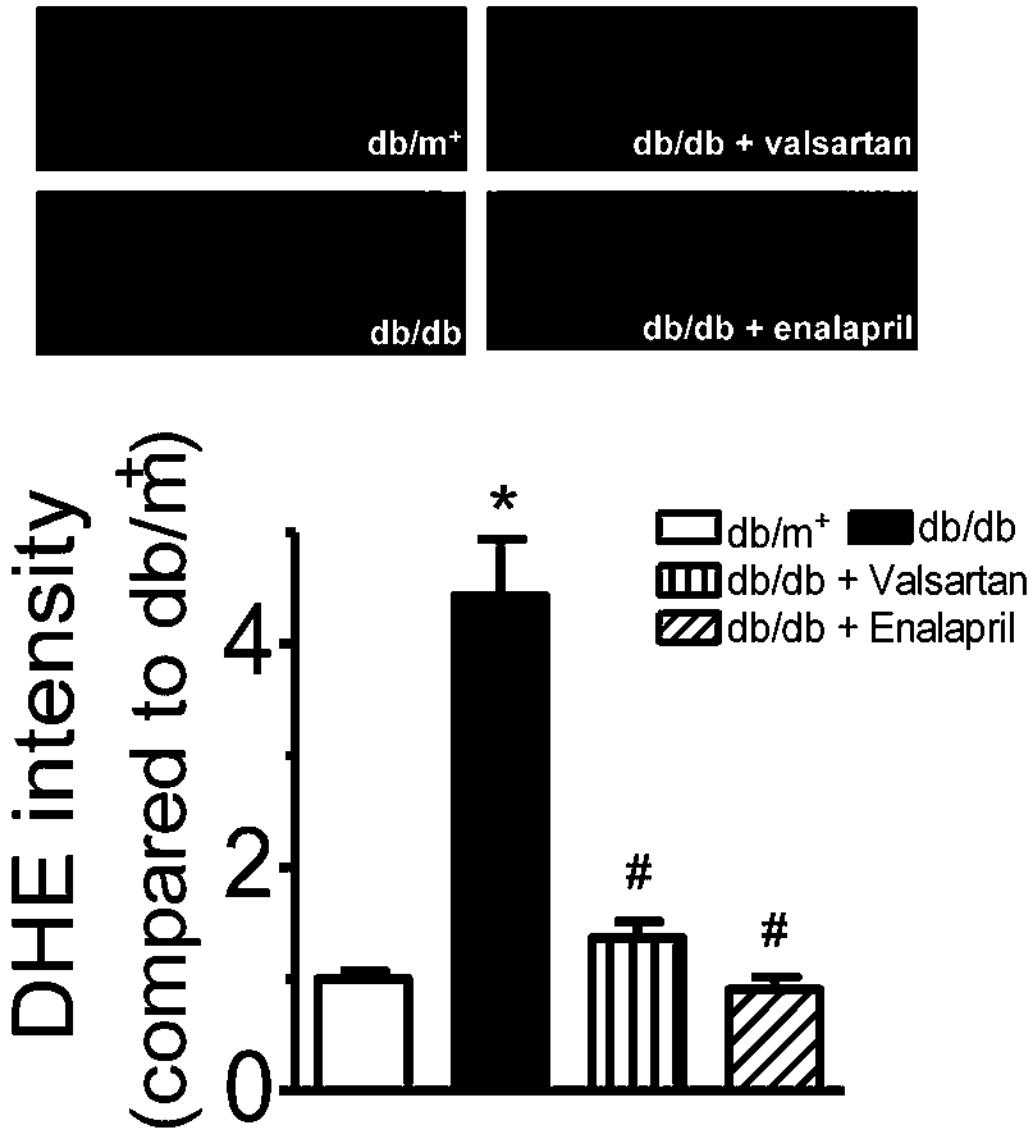


Figure 3.12.
DHE fluorescence images showing that an enhanced ROS intensity in *db/db* mouse aortas was prevented by chronic treatment with valsartan or enalapril. Results are mean ± SEM of 4 experiments. Statistical significance is indicated by *p<0.05 vs *db/m*+ and #p<0.05 vs *db/db*.

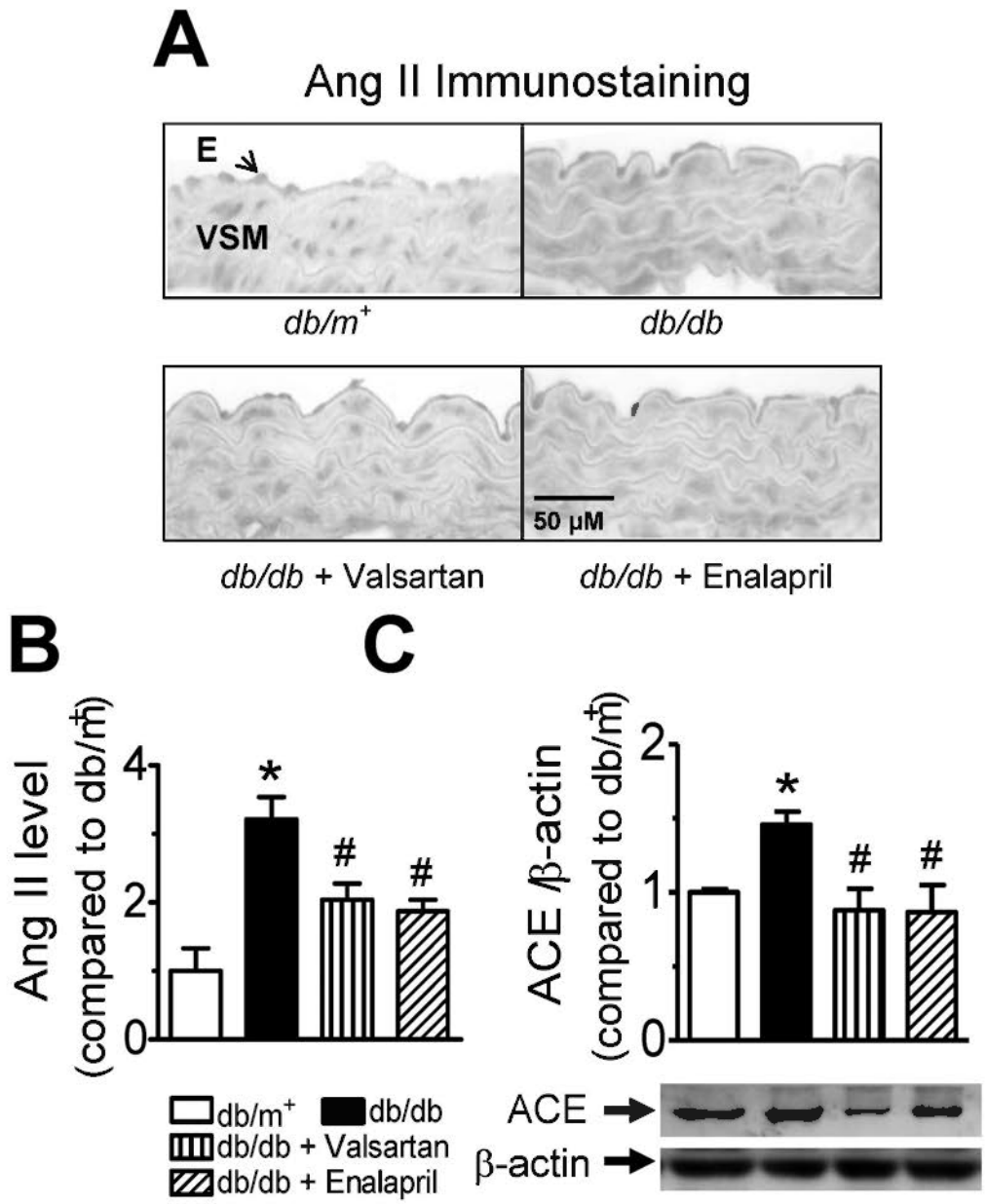


Figure 3.13.
(A) Representative images showing Ang II immunostaining in mouse aortas from *db/m+*, *db/db*, *db/db* treated with valsartan and *db/db* treated with enalapril. **(B)** Summarized data for Ang II-sensitive staining in different groups of mice. **(C)** Western blot analysis showing that increased expression of angiotensin converting enzyme (ACE) was attenuated by chronic valsartan or enalapril treatment. Results are mean ± SEM of 4 experiments. Statistical significance is indicated by *p<0.05 vs *db/m+* and #p<0.05 vs *db/db*.

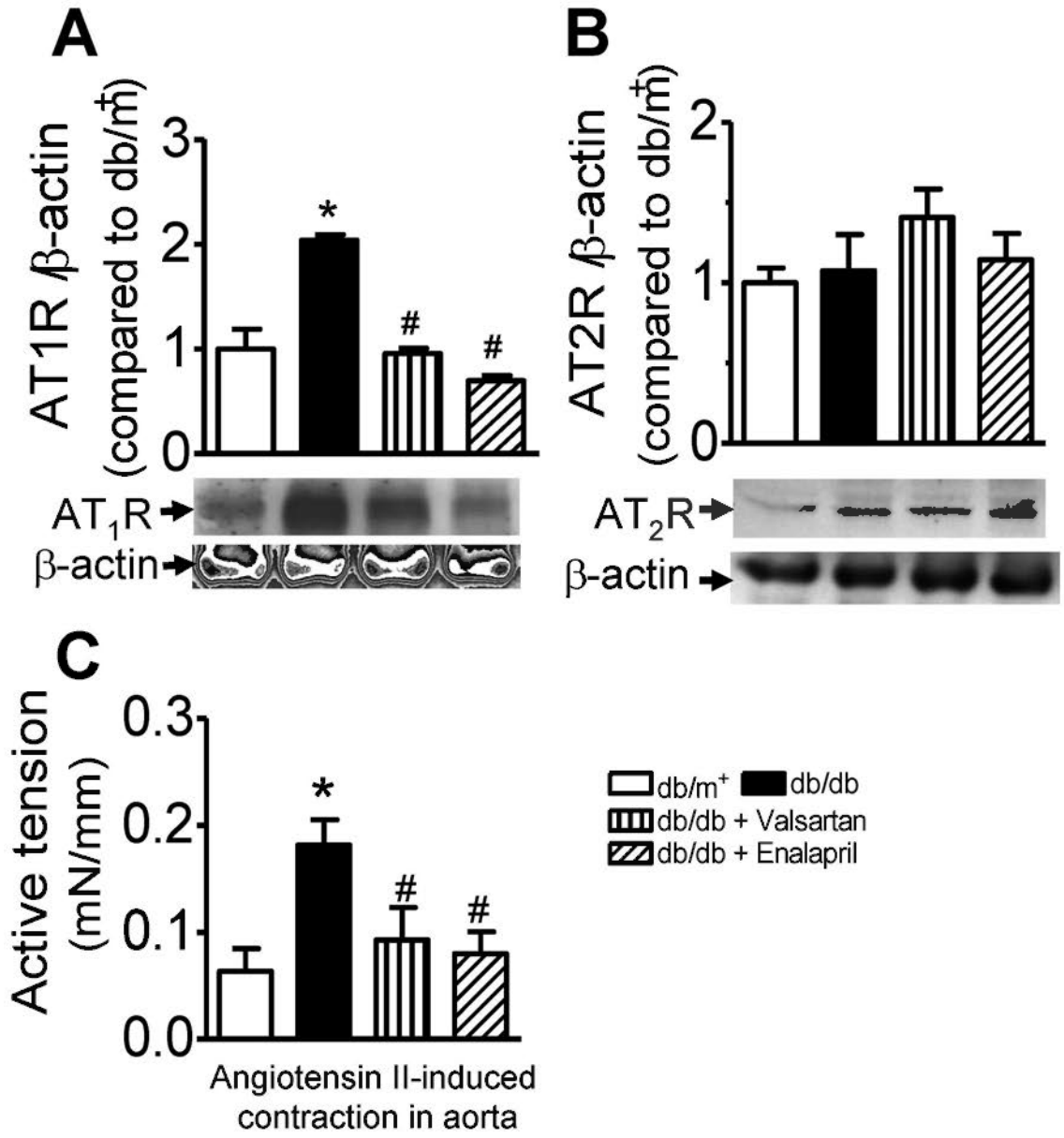


Figure 3.14.
Western blot analysis showing that the protein expression levels of AT1R **(A)** and AT2R **(B)** in *db/m*+, *db/db*, *db/db* treated with valsartan and *db/db* treated with enalapril. **(C)** Ang II (100 nmol/L)-induced contraction in mouse aortas from different groups of mice. Results are mean ± SEM of 4-6 experiments. Statistical significance is indicated by *p<0.05 vs *db/m*+ and #p<0.05 vs *db/db*.

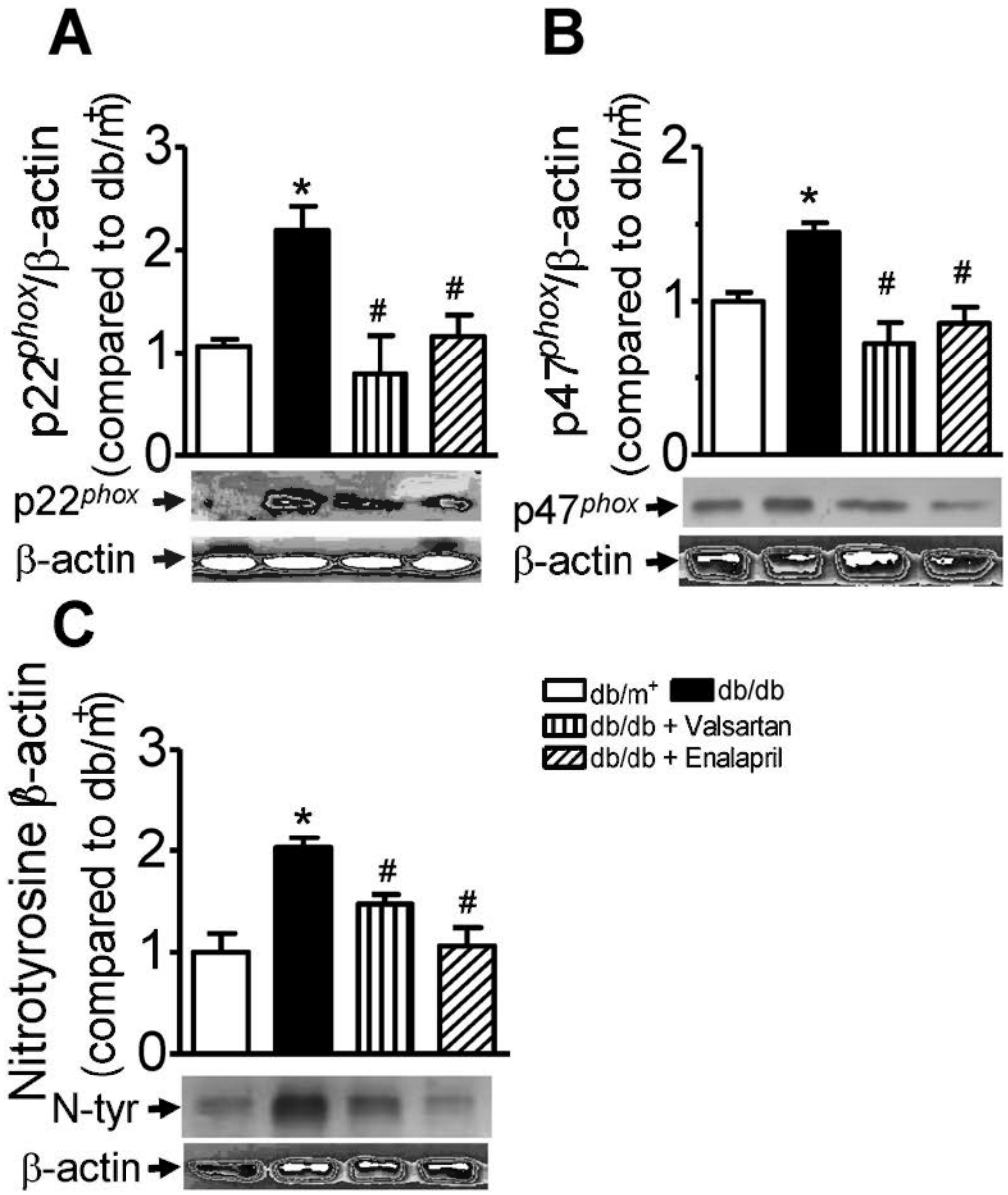


Figure 3.15.
Western blot analysis showing the protein expression levels of NAD(P)H oxidase subunit p22*phox* (membrane-bound subunit) **(A)** and p47*phox* (cytosolic subunit) **(B)** and nitrotyrosine **(C)** in *db/m+*, *db/db*, *db/db* treated with valsartan and *db/db* treated with enalapril. Results are mean ± SEM of 4 experiments. Statistical significance is indicated by *p<0.05 vs *db/m+* and #p<0.05 vs *db/db*.

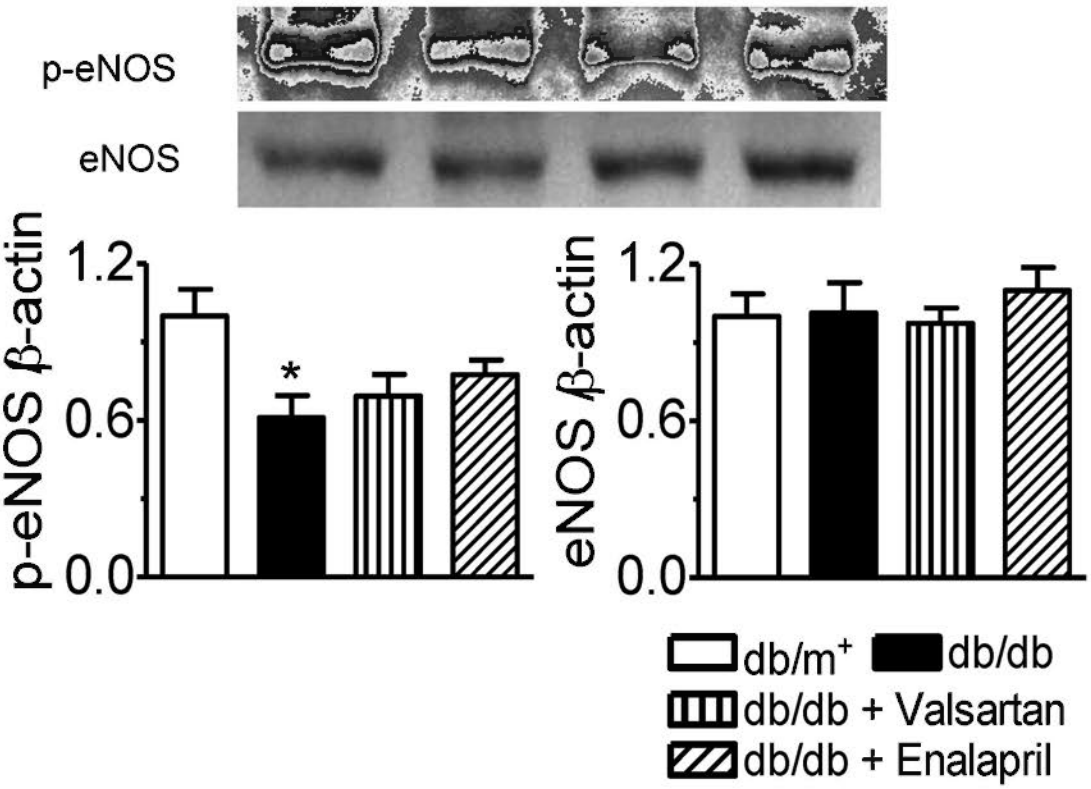


Figure 3.16.
Western blot analysis showing the protein expression levels of eNOS and phosphorylation of eNOS at Ser 1177 following stimulation of ACh in mouse aortas from *db/m+*, *db/db*, *db/db* treated with valsartan and *db/db* treated with enalapril. Results are mean ± SEM of 4 experiments. Statistical significance is indicated by *p<0.05 vs *db/m+* and #p<0.05 vs *db/db*.

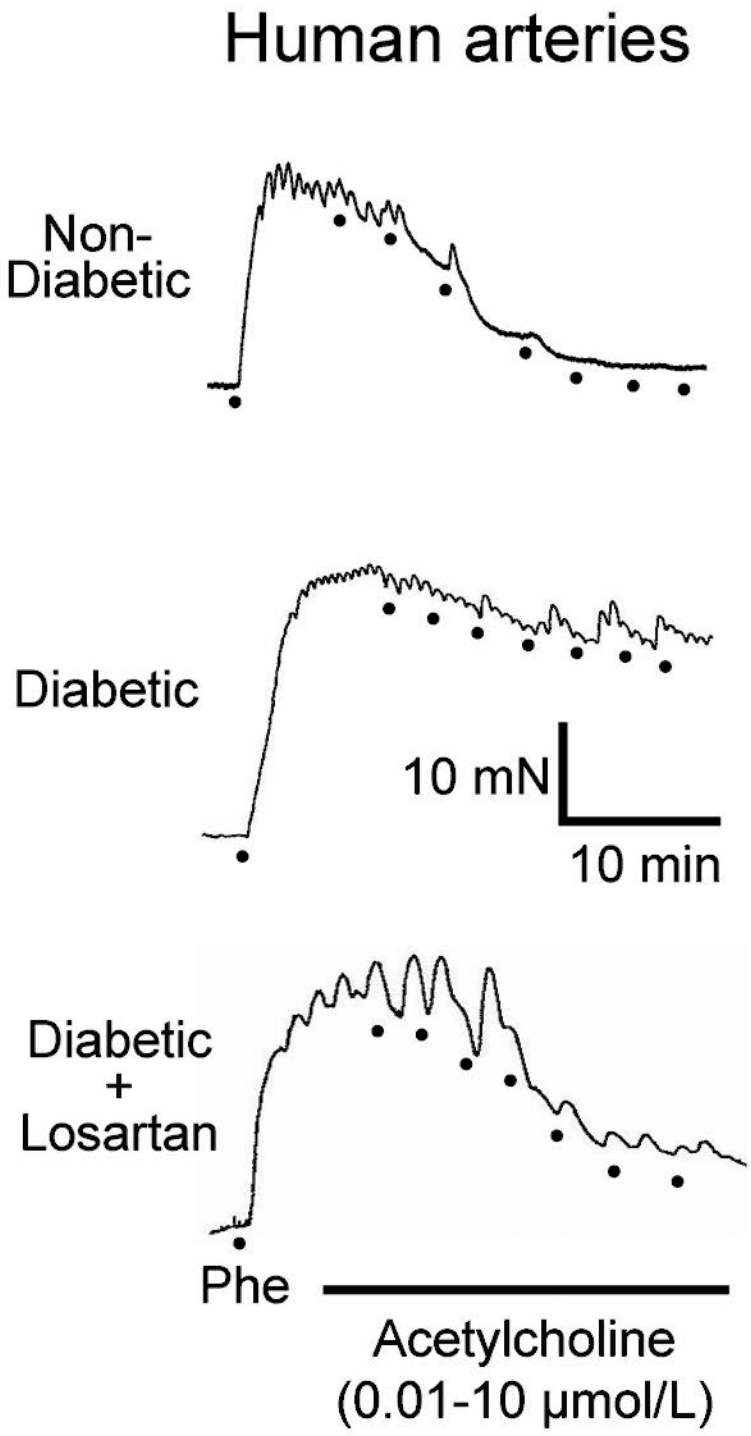


Figure 3.17.
Representative traces showing ACh-induced endothelium-dependent dilatations in renal arteries from non-diabetic **(A)** and diabetic **(B)** patients. **(C)** The effect of 30-min exposure to 3 µmol/L losartan on ACh-induced endothelium-dependent dilatations in diabetic patients.

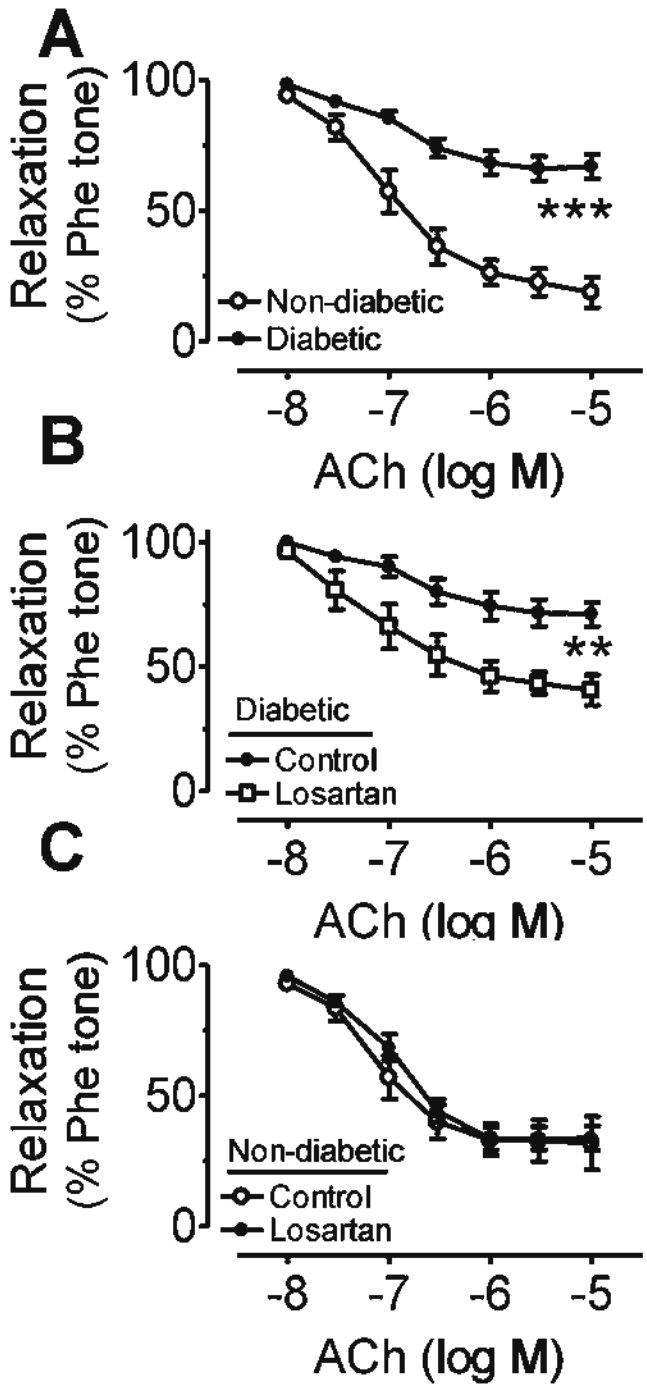


Figure 3.18.
(A) ACh-induced endothelium-dependent dilatations in renal arteries from non-diabetic and diabetic patients; and the effect of 30-min incubation with 3 µmol/L losartan on ACh-induced endothelium-dependent dilatations in diabetic patients **(B)** and non-diabetic patients **(C)**. Results are mean ± SEM. Statistical significance is indicated by ***p<0.001 and **p<0.01.

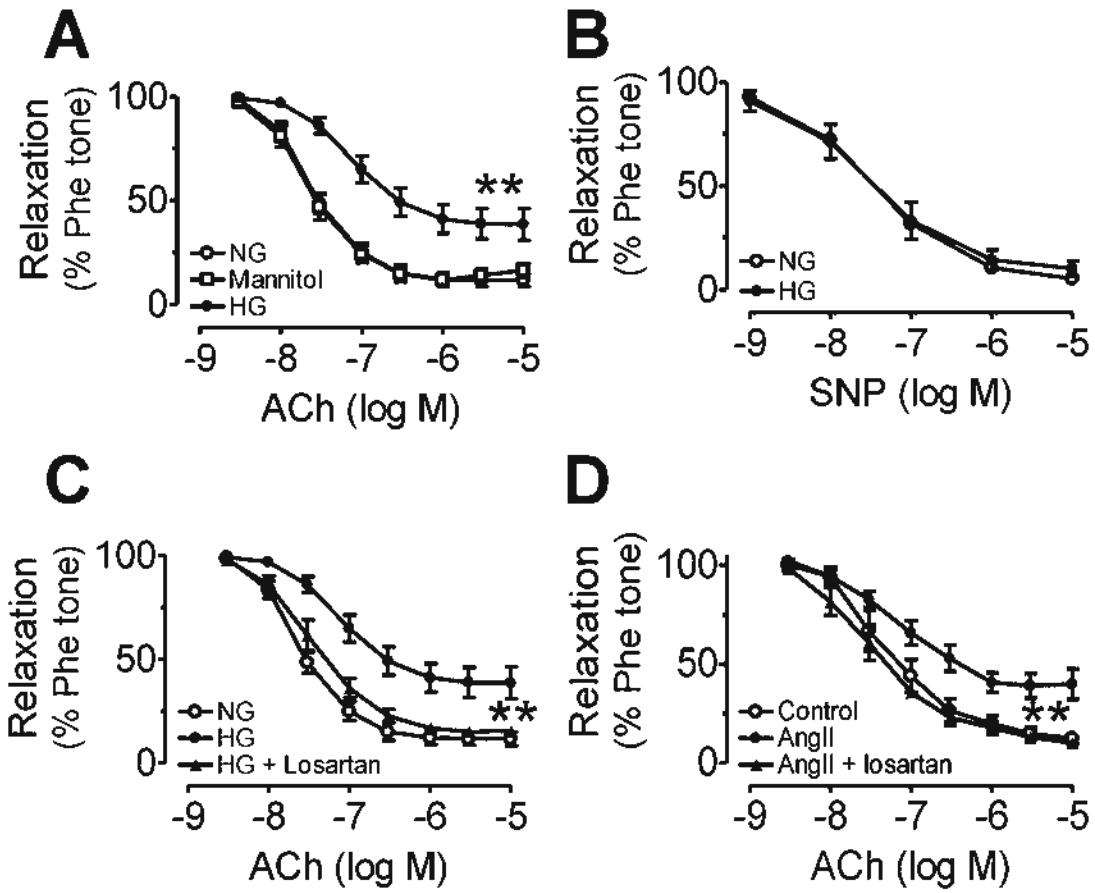


Figure 3.19.
(A) The effects of incubation of c57 mouse aortas in culture medium containing 30 mmol/L glucose (HG), 25 mmol/L mannitol as osmotic control, and 5 mmol/L glucose (NG) for 36 hours. **(B)** SNP-induced endothelium-independent dilatations in HG- and NG-treated mouse aortas. **(C)** Chronic exposure to 3 µmol/L losartan for 36 hours prevented the impairment of ACh-induced endothelium-dependent dilatations in high glucose medium. **(D)** Ang II-impaired ACh-induced vasodilatations was reversed by 3 µmol/L losartan. Results are mean ± SEM of 6-8 experiments. Statistical significance between groups is indicated by **p<0.01.

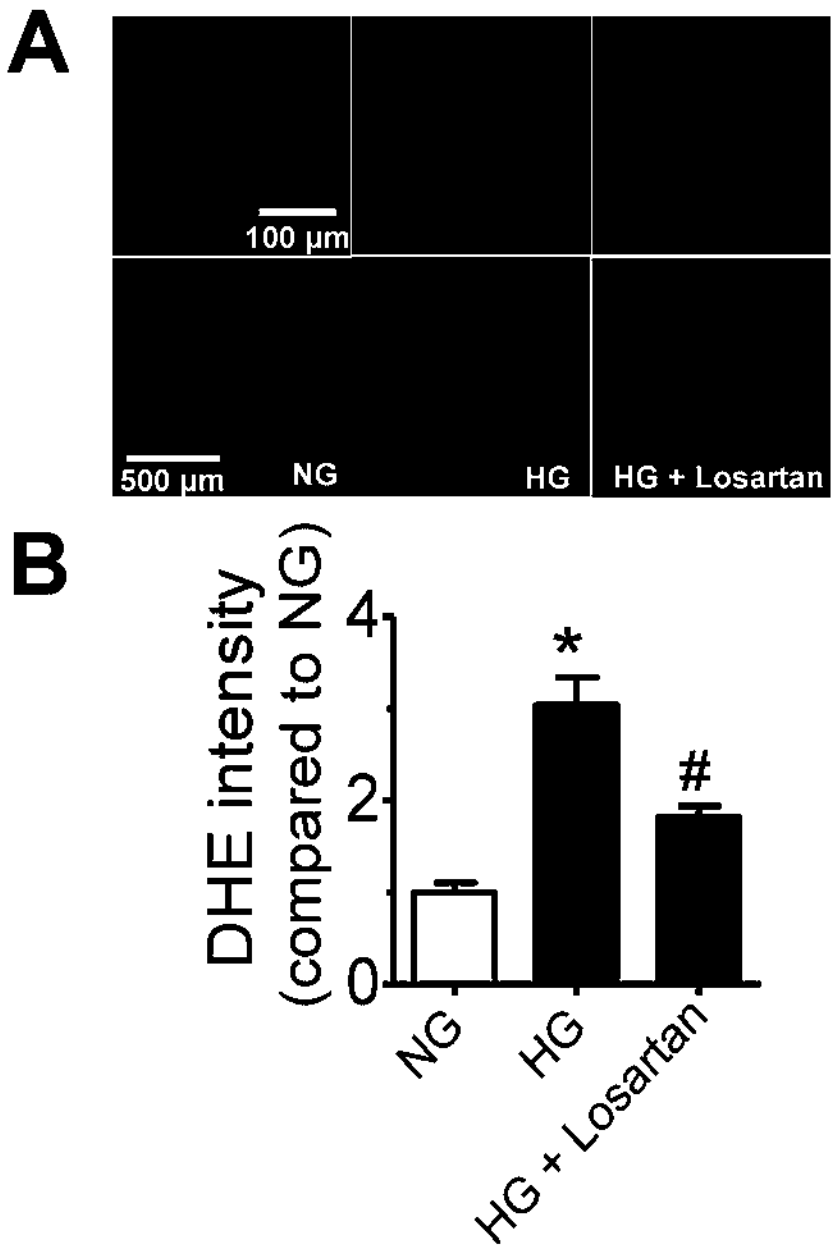


Figure 3.20.
(A) Representative images showing that an increased ROS production in aortic wall under 30 mmol/L glucose medium (HG) was prevented by co-treatment with 3 µmol/L losartan. **(B)** Summarized data for the effects of chronic exposure to 3 µmol/L losartan for 36 hours on the increase in DHE intensity in high glucose medium. Results are mean ± SEM of 6-8 experiments. Statistical significance is indicated by *$p<0.05$ vs NG and #$p<0.05$ vs HG.

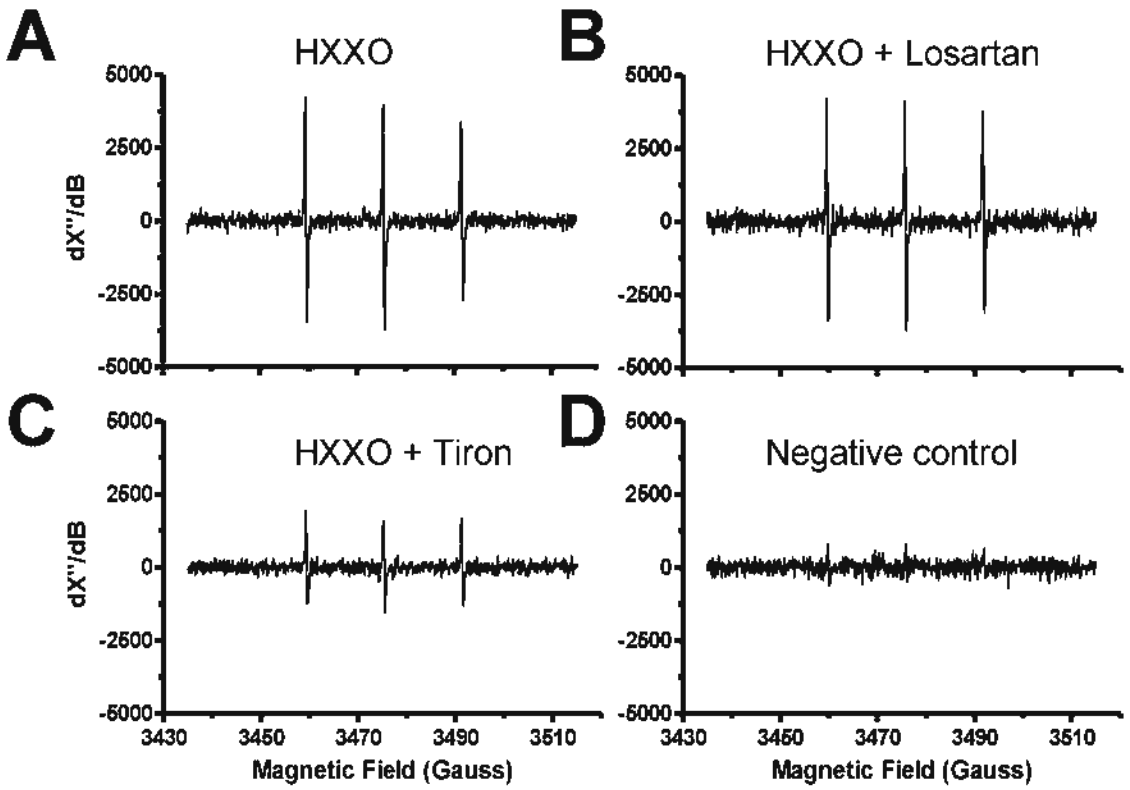


Figure 3.21.
EPR spectra of radical adducts detected by spin trap TEMPONE-H. **(A)** Combination (HXXO) of 100 µmol/L hypoxanthine (HX) and 0.1 U/ml xanthine oxidase (XO) led to a pronounced EPR signal which was inhibited by 1 mmol/L tiron **(C)** but not by 3 µmol/L losartan **(B)**. TEMPONE-H only serves as negative control which showed no EPR signal **(D)**.

3.4 Discussion

The precise mechanisms underlying endothelial dysfunction in type 2 diabetes remain largely unknown. The present study used type 2 diabetic *db/db* mice (C57BL/KSJ) lacking the gene encoding for leptin receptor to investigate the activation of RAS components and associated oxidative stress in endothelial dysfunction. My results clearly showed a key role for AT_1R-mediated ROS over-production in the diminished NO bioavailability which accounts for the impairment of ACh-induced endothelium-dependent dilatations in *db/db* mouse aortas and mesenteric resistance arteries. I provided the first line of experimental evidences demonstrating that losartan as an AT_1R blocker could prevent the impaired endothelial function that was induced by 36-hour exposure of non-diabetic mouse aortas to high glucose (30 mmol/L) in organ culture. The present results implicate that hyperglycemia-induced increase in ROS generation requires AT_1R activation. To substantiate this finding, I treated diabetic *db/db* mice chronically with valsartan (ARB) or enalapril (ACE inhibitor) and showed that drug treatments profoundly ameliorated endothelial dysfunction in *db/db* mouse aortas and mesenteric resistance arteries, which was correlated with marked down-regulation of AT_1R and reduction in ROS production. Further supporting evidences come from my demonstration that acute 30-min exposure to inhibitors of RAS-oxidative stress axis (losartan, apocynin or tempol) improved endothelium-dependent dilatations in *db/db* mouse aortas and resistance arteries. More importantly, I showed for the first time that losartan could also reverse the impaired endothelium-dependent relaxations in renal arteries obtained from patients with diabetes. The results of the present investigation supported and further defined the critical role of AT_1R as the therapeutic target for alleviation of endothelial dysfunction and associated vascular events in diabetes.

The observation of impaired endothelium-dependent dilatations in *db/db* mouse aortas was in accordance with recently reported results (Moien-Afshari et al., 2008; Zhong et al., 2007), whilst endothelium-independent dilatations to NO donor were identical in non-diabetic *db/m*$^+$ and diabetic *db/db* mice and also in mouse aortas with or without high glucose exposure, suggesting that hyperglycemic condition in diabetes does not reduce the relaxing sensitivity of

vascular smooth muscle to NO. I proposed that AT_1R mediates the impaired endothelial function in diabetes based on the following observations. Firstly, acute exposure of diabetic mouse aortas and resistance arteries to the AT_1R blocker losartan significantly enhanced ACh-induced dilatations. Secondly, Ang II stimulation of AT_1R leads to activation and up-regulation of NAD(P)H oxidase which in turn releases ROS. Apocynin was used to inhibit the activity of NAD(P)H oxidases and tempol as a ROS scavenger. Acute treatment with apocynin or tempol enhanced the ACh-induced dilatations to a similar extent. In addition, a combined treatment with losartan and apocynin did not produce additive effects in potentiating vasodilatations, implicating that Ang II signaling involves sequential steps instead of independent actions, initial stimulation of AT_1R followed by activation of NAD(P)H oxidases. Thirdly, in diabetic mouse aortas, AT_1R upregulation was observed, accompanied by over-expression of NAD(P)H oxidases subunits $p22^{phox}$ (membrane bound subunit) and $p47^{phox}$ (cytosolic subunit).

The present study measured the amount of ROS generated intracellularly using DHE fluorescence, in which ethidium binds to DNA when oxidized to emit fluorescence allowing quantification (Robinson et al., 2006). A marked rise in ROS production in the vascular wall of diabetic mouse aortas was indicated by increased DHE fluorescence intensity. ACh stimulated further increase of ROS only in diabetic but not in non-diabetic mouse aortas. The elevated ROS production was blocked by L-NAME, suggesting that this increased portion of ROS is likely driven by eNOS uncoupling as indicated in a previous study showing that eNOS uncoupling is one of the ROS sources in aortas of streptozotocin-induced type 1 diabetes mouse (Oak and Cai, 2007). Of importance, I demonstrated that blockade of RAS and associated oxidative stress by losartan, apocynin, or tempol, greatly reduced the ROS production upon stimulation of ACh. These results indicate that ROS derived from NAD(P)H oxidases may be required for stimulation of eNOS uncoupling to further increase intracellular ROS generation. In addition, I showed that the release of ROS was dependent on the presence of extracellular Ca^{2+} ions which is in line with Guzik et al. who showed Ca^{2+} as an important intracellular activator of NAD(P)H oxidases (Guzik et al., 2008). The present study also

suggested that ACh-stimulated ROS production originates from endothelial cells since ACh failed to increase ROS in rings without endothelium.

Next, I examined the role of AT_1R in the high glucose-induced endothelial dysfunction. We have recently reported that organ culture of mouse aortas with 30 mmol/L glucose for 36 hours resulted in marked reduction in ACh-induced vasodilatations (Wang et al., 2009). The present study showed that losartan can also effectively augment endothelium-dependent dilatations of mouse aortas, which were impaired by high glucose exposure. The improved endothelial function is likely associated with the inhibitory effect of losartan on ROS production through AT_1R blockade. By contrast, the concentration (3 μmol/L) of losartan used in the functional study did not scavenge ROS as indicated by electron paramagnetic resonance spectra, thus discounting the possible ROS scavenging activity of losartan alone. In addition, I provided direct evidence showing that 12-hour exposure of non-diabetic mouse aortas to 100 nmol/L Ang II reduced endothelium-dependent dilatations through an over-production of ROS. The direct reversal effect of losartan on high glucose-induced ROS over-production is novel and important for elucidation of the precise role of AT_1R in hyperglycemia-associated endothelial dysfunction in diabetes.

Chronic oral treatment with valsartan (AT_1R blocker to inhibit Ang II-dependent harmful actions) or enalapril (ACE inhibitor to suppress Ang II formation) markedly improved endothelium-dependent dilatations of *db/db* mouse aortas and resistance arteries. Both RAS-inhibiting agents are commonly used for the treatment of hypertension and diabetes. ACh-induced aortic dilatations in drug-treated diabetic mice were mediated by NO since they were abolished by NOS inhibitor, L-NAME. It is thus postulated that ROS derived from AT_1R-mediated NAD(P)H oxidases lowers the bioavailability of NO by either directly scavenging NO or by reducing the biosynthesis of NO from endothelial nitric oxide synthase (eNOS). The immunoblotting results clearly show a significant upregulation of AT_1R and NAD(P)H oxidases subunits ($p22^{phox}$ and $p47^{phox}$) in *db/db* mouse aortas which could be normalized by chronic treatment with valsartan or enalapril. The over-production of ROS in diabetic mouse aortas, as reflected by increases in nitrotyrosine formation and DHE fluorescence intensity, could also be reversed by

RAS blockade. On the basis of these observations, it is thus logical to conclude that the reduced NO bioavailability is caused by an overproduction of ROS. Interestingly, I also found that ACh-stimulated phosphorylation of eNOS at Ser^{1177} was lower in *db/db* than *db/m*$^+$ mouse aortas, implicating that the maximal capacity of *db/db* mouse aortas to produce NO has been impaired and such impairment can not be reversed by chronic blockade of RAS. This finding further supported the primary role of RAS-dependent oxidative stress in endothelial dysfunction in diabetic mice.

More significantly, I also demonstrated a critical role of AT_1R-mediated ROS in endothelial dysfunction of renal arteries from diabetic patients. Similar to *db/db* mouse aortas, the impaired ACh-induced endothelium-dependent relaxations in human arteries can also be effectively improved by acute treatment with losartan, thus favoring the use of AT_1R blockers for reversing endothelial dysfunction in patients with diabetes. In summary, the present study has provided scientific basis with novel evidence in support of clinical application of selective AT_1R blockers for the prevention and treatment of endothelial dysfunction associated with diabetes.

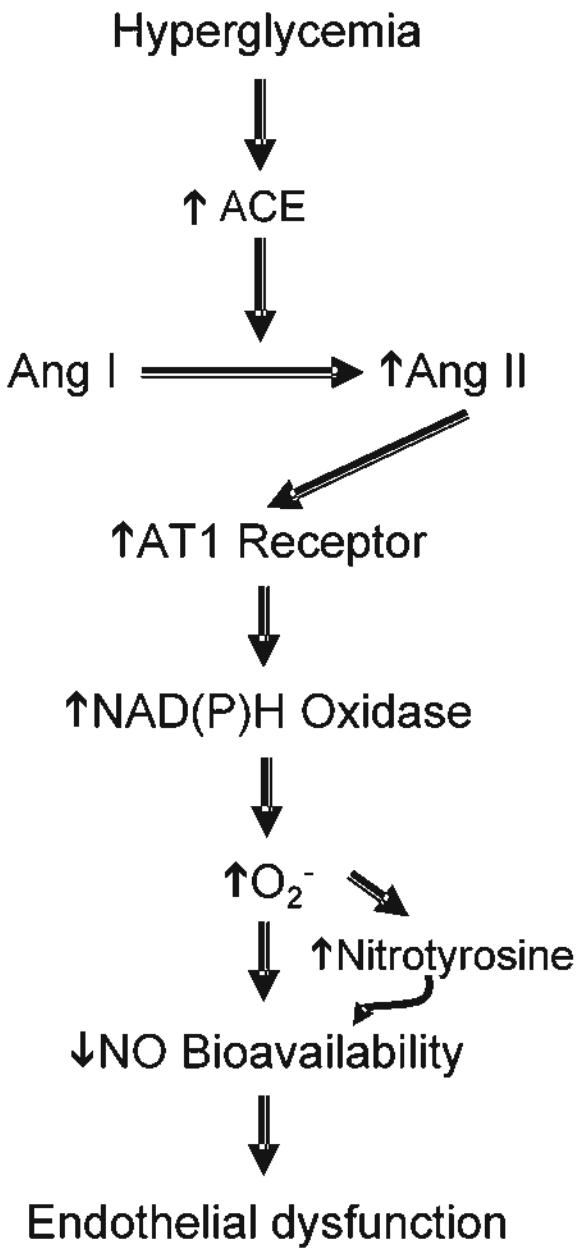


Figure 3.22.
Key roles of the upregulation of expression and activity of the components of the RAS in contributing to endothelial dysfunction through increased oxidative stress in arteries of db/db diabetic mice. Abbreviations: ACE, angiotensin converting enzyme; Ang I, angiotensin I; Ang II, angiotensin II; AT1, angiotensin type 1; NO, nitric oxide; $O2^-$, superoxide anion.

CHAPTER IV

RENIN INHIBITION BY ALISKIREN AMELIORATES ENDOTHELIAL DYSFUNCTION IN SPONTANEOUSLY HYPERTENSIVE RATS

4.1 Introduction

Because renin catalyzes the rate-limiting step in the bio-formation of angiotensin II (Ang II), one of the primary causes of hypertension, a direct renin inhibition has been an important, although until recently elusive, therapeutic strategy. Aliskiren, the first oral renin inhibitor, is approved for once-daily treatment of hypertension (Brown, 2008; Jensen C et al., 2008; Gradman and Kad, 2008). Aliskiren is proven to be effective in long-term reduction of blood pressure with an acceptable safety and tolerability profile in patients with hypertension (Pool 2007; Brown, 2008; Vaidyanathan et al., 2008). In hypertensive patients, aliskiren decreases both systolic and diastolic blood pressure, with a similar efficacy to irbesartan, an angiotensin receptor blocker (ARB) (Gradman et al., 2005). In addition, blood pressure reduction is a critical issue for target organ protection in hypertensive patients. Aliskiren also minimizes the Ang II-dependent cardiac and renal end organ damage (Pilz et al., 2005) and protects the renal function by reducing albuminuria beyond its effect on hypertension in patients with type-2 diabetic nephropathy and hypertension (AVOID trial, Parving et al., 2008). In contrast to other renin-angiotensin system (RAS) blockers such as angiotensin-converting enzyme (ACE) inhibitors and ARBs, renin inhibitors do not increase the circulating level of angiotensin I (Ang I). The pharmacodynamic features of aliskiren are analyzed in human by determination of the plasma renin activity and plasma renin concentration. In normotensive healthy subjects, aliskiren inhibits PRA and increases PRC in a dose-dependent manner, while valsartan (an ARB) increases PRA in normal and mildly sodium-depleted subjects (Azizi et al., 2004). Although suppression induced by ACE inhibitors or ARBs on the RAS and the Ang II-

dependent generation of cytokines and reactive oxygen species (ROS), a high plasma renin activity might also serve as an indicator for cardiovascular risks. High plasma renin activity can turn on other Ang II-generating but ACE-independent pathways (Hollenberg et al., 1998; Alderman et al., 1997). Therefore, aliskiren can become an additional useful drug in uncontrollable hypertensive patients or intolerance to conventional therapies with ACE inhibitors and ARBs.

Limited recent animal studies support a clinical benefit of aliskiren in hypertensive and diabetic patients by exerting an anti-hypertensive effect and target organ protection. In human renin and angiotensinogen double transgenic rat, aliskiren lowers blood pressure with an equal effectiveness as valsartan, prevents cardiac hypertrophy, and protects cardiac function, suggesting that renin inhibition may confer better target organ protection than ARBs (Pilz et al., 2005). In diabetic TG (mRen-2 over-expression) hypertensive rats, aliskiren decreases blood pressure and preserves the renal function (Feldman et al., 2008). In sodium-depleted marmosets, aliskiren produces a rapid and long-lasting blood pressure lowering effect, as in spontaneously hypertensive rats (SHR) in which aliskiren also lowers blood pressure in a dose-dependent manner, and exhibits additive effects administered together with low dosage an ARB or ACE inhibitor (Wood et al., 2005). The ability of aliskiren to stabilize atherosclerotic plaques has been demonstrated in several recent studies. In 2K1C or 1K1C hypertensive $ApoE^{-/-}$ mice, aliskiren and irbesartan (an ARB) prevent atherosclerotic progression and they are more effective than β–adrenoceptor blockers or calcium channel blockers besides the blood pressure-lowering effect and aliskiren therapy shows a less increase in plasma renin concentration and a low level of the plasma renin activity as compared with irbesartan (Nussberger et al., 2008). In low-density lipoprotein receptor-deficient mice fed high fat diet, renin inhibition reduces the formation of aortic atherosclerotic lesions (Lu et al., 2008). In a rabbit model of hyperlipidemic atherosclerosis, aliskiren reduces the plaque area; whereas a combined treatment with aliskiren plus valsartan significantly increases the nitric oxide (NO) bioavailability by restoring eNOS activity, reversing eNOS uncoupling and inhibiting the production of ROS in the vascular wall (Imanishi et al., 2008).

Although aliskiren is shown to exert an anti-hypertensive effect in SHR and renal hypertensive rats, and to augment the NO bioavailability in atherosclerotic rabbits, the impact of renin inhibition on endothelial dysfunction in hypertension has not yet been studied. Endothelial dysfunction is an early and critical predictor of cardiovascular outcomes in hypertensive patients. Therefore, it is necessary and timely to investigate whether renin inhibition can protect endothelial function besides the blood pressure-lowering benefit. Following analysis of recent results published by several groups, I hypothesized that direct renin inhibition could improve endothelial function in hypertension by enhancing the NO bioavailability, reducing the ROS generation and inhibiting the expression of pro-inflammatory factors such as cyclooxygenase-2 (COX-2) or bone morphogenic protein-4 (BMP4) which I have examined in details (see Chapter V and Chapter VI). To test this hypothesis, I employed SHR and normotensive WKY control rats and examined the effect of aliskiren on endothelium-dependent dilatations and contractions in aortas and intralobal renal arteries. I attempted to reveal an inter-connected signaling network among various RAS components, oxidative stress and COX and BMP4 that were likely targeted by aliskiren and provided experimental support for the therapeutic value and potential superiority of renin inhibitors in managing certain subgroups of hypertensive patients uncontrollable by existing conventional drug intervention.

4.2 Methods and Materials

4.2.1 Animals and drug treatment

The experimental protocol was approved by the Ethical Committee for Animal Research, Chinese University of Hong Kong. All rats had free access to an ordinary rat chow diet and tap water and kept on a 12-hour light/dark cycle at room temperature. Six months old SHR and age-matched Wistar Kyoto (WKY) control rats were randomly divided and subjected to oral administration of aliskiren (10 mg/kg/day) or vehicle for 8 weeks. Blood pressure was monitored bi-weekly by a tail-cuff method.

4.2.2 Blood vessel preparation

The rats were killed by cervical dislocation. After the abdominal cavity was opened, the aorta, brain, and both kidneys were removed and placed in ice-cold Krebs solution (in mmol/L): 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11 D-glucose. Both thoracic aorta and intralobal renal arteries were dissected out and cleaned of adhering adipose tissues under a dissection microscope. Each artery was cut into two or more ring segments, 1.5-2 mm (renal arteries), and 3-4mm (aorta) in length. Large-size aortic rings were suspended between two hooks in organ baths. One hook was connected to a Grass force transducer and other one was fixed to the bottom. Changes in isometric tension of aortic rings were continually measured and recorded. A baseline optimal tone of 25 mN was applied to all aortic rings. Each ring segment of the renal artery was mounted in a Multi Myograph System (Danish Myo Technology, Aarhus, Denmark), and changes in vascular reactivity were determined. In brief, two steel wires (each 40 μm in diameter) were passing through the segment's lumen, and each wire was fixed to the built-in jaws of myograph. The organ chamber was filled with 5 mL Krebs solution and oxygenated with a gas mixture containing 95% O_2 plus 5% CO_2. Bathing solution in the chamber was always maintained at 37°C using a built-in heat-exchanger device to give a pH value of ~7.4. Small renal arterial rings were stretched initially to an optimal tension of 2.5 mN respectively, and then allowed to

stabilize under this basal tone for 90 min before the start of each experiment. In some arteries, the endothelium was mechanically removed by rubbing the luminal surface of the arteries with stainless steel wire. Functional disruption of the endothelium was successful if the relaxant effect of 1 µmol/L acetylcholine (ACh) was absent.

4.2.3 Experimental protocol

After an initial equilibration period in organ baths or myograph chambers filled with Krebs solution at 37 °C, each ring was contracted by elevated KCl (60 mmol/L) to ensure a level of contractility that was suitable for a subsequent pharmacological examination. To test endothelium-dependent relaxations in phenylephrine-contracted rings, two consecutive concentration-response curves to ACh (3 nmol/L -30 µmol/L) were constructed in the absence (control) and presence (30-min incubation) of tiron (1 mmol/L, SOD mimetic) plus DETCA (100 µmol/L, ROS scavenger). In some phenylephrine-contracted rings without intact endothelium, sodium nitroprusside (SNP, 3 nmol/L – 10 µmol/L) were applied cumulatively into the bathing solution to test the sensitivity of vascular smooth muscle cells (VSMCs) to NO.

To assess endothelium-dependent contractions, both aorta and renal arteries with an intact endothelium were first incubated with N^G-nitro-L-arginine methyl ester (L-NAME, 100 µmol/L) for 30 min to eliminate the negative influence of endothelium-derived NO on the development of vascular tension prior to cumulative addition of ACh (0.1-100 µmol/L).

4.2.4 Western blotting

Aortas and renal arteries were isolated and were homogenized at 4 °C in RIPA lysis buffer (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, 25 mmol/L sodium pyrophosphate, 1 mmol/L b-glyco-phosphate, 1 mmol/L sodium orthovanadate, 2.1 µmol/L leupeptin, 1 mg/mL aprotinin, 1 mmol/L phenyl-methylsulfonyl fluoride, and 1% Triton X-100) and incubated on ice for 10 min. Samples were then centrifuged at 20000×g for 20 min at 4 °C and the supernatant was collected. Protein concentrations were determined using the Lowry method

(Bio-rad). The protein samples were electrophoresed on a 10% SDS-polyacrylamide gel and transferred onto an immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore). Nonspecific binding sites were blocked by 5% non-fat milk in 0.05% Tween-20 phosphate-buffered saline (PBST), then incubated overnight at 4 °C with primary antibodies including anti-AT_1R (1:1000, Abcam), $p22^{phox}$ (1:500, Santa cruz), $p47^{phox}$ (1:500, Santa cruz), nitrotyrosine (1:1000, Upstate), SOD1 (1:1000, Santa cruz), SOD2 (1:1000, Santa cruz), p-eNOS Ser^{1177}(1:1000, Upstate), eNOS (1:1000, Abcam), BMP4 (1:1000, Sigma), COX-2 (1:500, Cayman) or COX-1 (1:1000) (Cayman). The blots were incubated with secondary antibodies at a dilution of 1:3000 for 1 hour at room temperature, and then washed 3 times in PBST within 10 min. The membranes were developed with an enhanced chemiluminescence detection system (ECL reagents, Amersham Pharmacia), and finally exposed on X-ray films. An equal amount of protein loading was verified with use of a housekeeping anti-GAPDH antibody (Ambion, Inc).

4.2.5 Chemicals and drugs

ACh, L-NAME, phenylephrine, tiron, DETCA (diethyldithiocarbamate acid), SNP were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). All drugs used in this study were dissolved in water.

4.2.6 Statistical analysis

Results are mean ± SEM of n separate experiments. The contractile response was presented as active tension [force (mN) recorded / (2Xlength (mm) of ring)]. The protein expression was normalized to GAPDH and then expressed in comparison with controls. Student's t-test (unpaired two-tailed) was used and concentration-response curves were analyzed by two-way ANOVA followed by Bonferroni post-tests. Levels of probabilities of less than 0.05 were regarded as significantly different.

4.3 Results

4.3.1 Antihypertensive effects of aliskiren in SHR

Eight-week oral treatment of aliskiren at 10 mg/kg/day moderately but significantly reduced systolic blood pressure (SBP) to 155.6 ± 1.87 mmHg of SHR as compared with 165.3 ± 3.2 mmHg in vehicle-treated SHR (n = 5-7, p = 0.034), while chronic aliskiren administration did not affect SBP in WKY rats (123.9 ± 6.1 mmHg in aliskiren and 121.9 ± 4.1 mmHg in vehicle control) **(Figure 4.1)**. In addition, the plasma renin activity remained similar in both WKY and SHR with and without receiving aliskiren treatment **(Table 4.1)**.

4.3.2 Improved endothelium-dependent dilatations in aortas from aliskiren-treated SHR

SHR aortas with endothelium relaxed significantly less in response to different concentrations of ACh than age-matched WKY rat aortas **(Figure 4.2A)** and ACh failed to cause relaxations of aortic rings without endothelium (data not shown). By contrast, endothelium-independent relaxations caused by sodium nitroprusside (SNP) were comparable in aortas from SHR and WKY rats **(Figure 4.2B)**, implying that the relaxing sensitivity or effectiveness of VSMCs to NO is unaffected in SHR. Chronic aliskiren administration clearly improved endothelium-dependent dilatations of aortas from SHR as compared with those from vehicle-treated SHR **(Figure 4.3A).** By contrast, aliskiren treatment did not affect endothelium-dependent dilatations in WKY rats **(Figure 4.3B)**. By contrast, endothelium-independent relaxations to SNP were nearly the same in aortas from the four treatment groups of rats **(Figure 4.4)**, again excluding the possible modulation by aliskiren on VSMC response to exogenous NO.

4.3.3 Effect of ROS scavengers on endothelium-dependent dilatations in aortas of aliskiren- and vehicle-treated rats

ACh-induced dilatations in SHR aortas were enhanced by 30-min pre-exposure to tiron plus DETCA **(Figure 4.5A)**. By contrast, tiron plus DETCA did not affect dilatations in WKY rat aortas **(Figure 4.5B)**. In addition, tiron plus DETCA did not

augment ACh-induced dilatations in aortas from SHR chronically treated with aliskiren **(Figure 4.6A)**. Again, the ACh-induced dilatations were similar with and without acute incubation with tiron plus DETCA in aliskiren-treated WKY rat aortas **(Figure 4.6B)**.

4.3.4 Effect of chronic aliskiren treatment on endothelium-dependent contractions in SHR aortas

In the presence of 100 µmol/L L-NAME, ACh elicited concentration-dependent contractions in SHR aortas with endothelium, but not such contractions were very small in WKY rat aortas **(Figure 4.7A)**. Chronic aliskiren treatment of SHR attenuated ACh-induced contractions with little effect on the maximum contraction **(Figure 4.7B)**. Mechanical removal of the endothelium eliminated endothelium-dependent contractions in both aliskiren- and vehicle-treated SHR **(Figure 4.7C)**. By contrast, contractile responses to phenylephrine were not modified by chronic aliskiren treatment of SHR **(Figure 4.8A)**. Likewise, phenylephrine-induced contractions were comparable in aortas from aliskiren- and vehicle-treated WKY rats **(Figure 4.8B)**.

4.3.5 Effect of aliskiren treatment on endothelium-dependent dilatations and contractions in intralobal renal arteries of SHR

Endothelium-dependent dilatations to ACh were severely reduced in intralobal renal arteries from SHR as compared with those from age-matched WKY rats **(Figure 4.9A)**. Aliskiren treatment significantly improved ACh-induced dilatation in SHR renal arteries while vehicle treatment had no effect **(Figure 4.9B).** In contrast, aliskiren treatment did not modify endothelium-dependent dilatations in WKY rat renal arteries **(Figure 4.9C)**. In intralobal renal arteries isolated from SHR and treated with 100 µmol/L L-NAME, ACh elicited pronounced contractions but such contractions were significantly less in WKY rat renal arteries **(Figure 4.10A)**. Chronic aliskiren treatment of SHR reduced ACh-induced contractions to similar levels found in untreated WKY rat arteries **(Figure 4.18B)**. Ang II (100 nmol/L)-induced contraction was exaggerated in SHR renal arteries and this contraction

was significantly suppressed by chronic aliskiren treatment **(Figure 4.11A)**. By contrast, the contraction evoked by 60 mmol/L KCl was not modulated by aliskiren in both SHR and WKY rats **(Figure 4.11B)**.

4.3.6 Protein expression for RAS components, SOD, eNOS, COX and BMP4

The up-regulated expression of AT_1R in SHR aortas were normalized by aliskiren treatment **(Figure 4.12A)**, supporting the inhibitory effect of aliskiren on Ang II-evoked contraction **(Figure 4.11A)**. Similarly, the expression level for NAD(P)H oxidases subunits (p47phox and p22phox), and nitrotyrosine were up-regulated in SHR aortas and such up-regulation was inhibited by chronic aliskiren treatment **(Figure 4.13A-C)**. On the other hand, the expression of the antioxidant enzyme, superoxide dismutases (SOD1 and SOD2) was less in SHR aortas and the reduced expression of SOD1 and SOD2 was prevented by aliskiren therapy **(Figure 4.14A-B)**. Aliskiren did not affect the SOD expression in WKY rat aortas **(Figure 4.14)**. In addition, the reduced phosphorylation of eNOS at Ser^{1177} was normalized in SHR aortas by aliskiren treatment whereas the total amount of eNOS remained the same **(Figure 4.15)**. The COX expression was also determined in both aortas and renal arteries. Although the COX-1 expression was unaltered, the COX-2 expression is significantly increased in SHR aortas and renal arteries by aliskiren treatment **(Figure. 4.16 & Figure 4.18)**. The expression of BMP4, which is an upstream activator of vascular inflammation as shown in Chapter VI, was also elevated in SHR aortas and renal arteries and aliskiren treatment greatly inhibited BMP4 up-regulation **(Figure 4.17 & Figure 4.19)**. Again, aliskiren did not affect the BMP4 expression in WKY rat arteries with or without aliskiren treatment **(Figure 4.17 & Figure 4.19)**.

Table 4.1
Effects of chronic aliskiren treatment on systolic blood pressure and plasma renin activity of SHR and WKY rats

	WKY	WKY + Aliskiren	SHR	SHR + Aliskiren
Systolic blood pressure (mmHg)	121.9 ± 4.1	123.9 ± 6.1	165.3 ± 3.2	155.6 ± 1.87*
Plasma renin activity (ng/mL)	3.6 ± 0.33	4.29 ± 0.62	3.5 ± 0.55	3.98 ± 1.09

The results are mean ± SEM of 5 measurements. Statistical difference between SHR and SHR + Aliskiren is indicated by * ($P<0.05$).

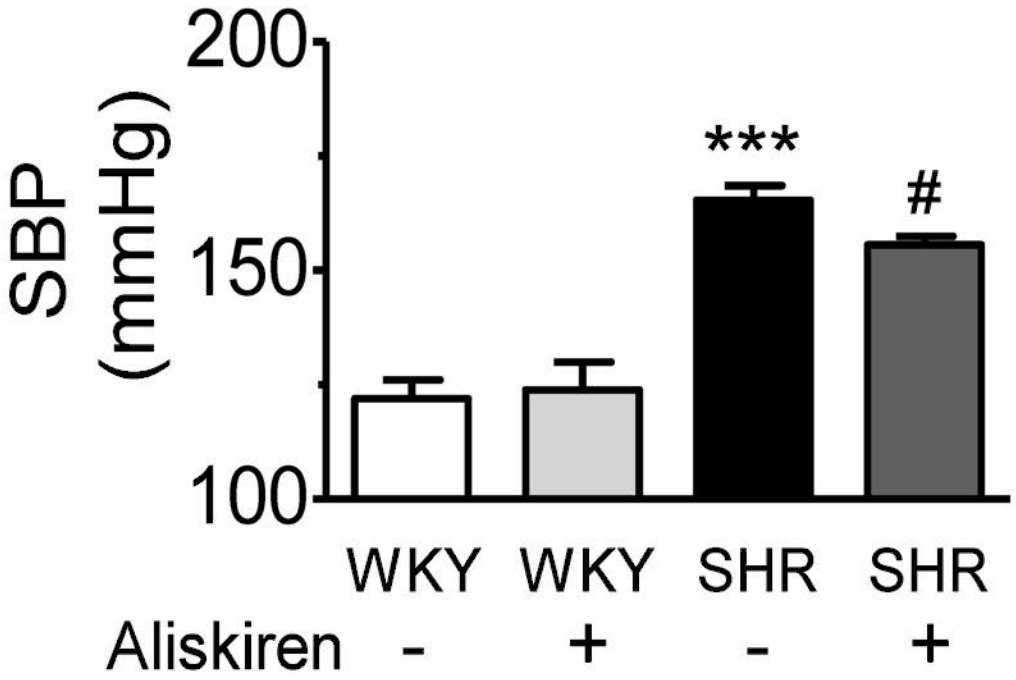


Figure 4.1.
The effect of chronic oral treatment with aliskiren (10 mg/kg/day) for eight weeks on systolic blood pressure (SBP) in Wistar Kyoto (WKY) rats and spontaneously hypertensive rats (SHR). Results are mean ± SEM of 5-7 rats. Statistical significance is indicated by ***$p<0.001$ vs WKY and #$p<0.05$ vs SHR.

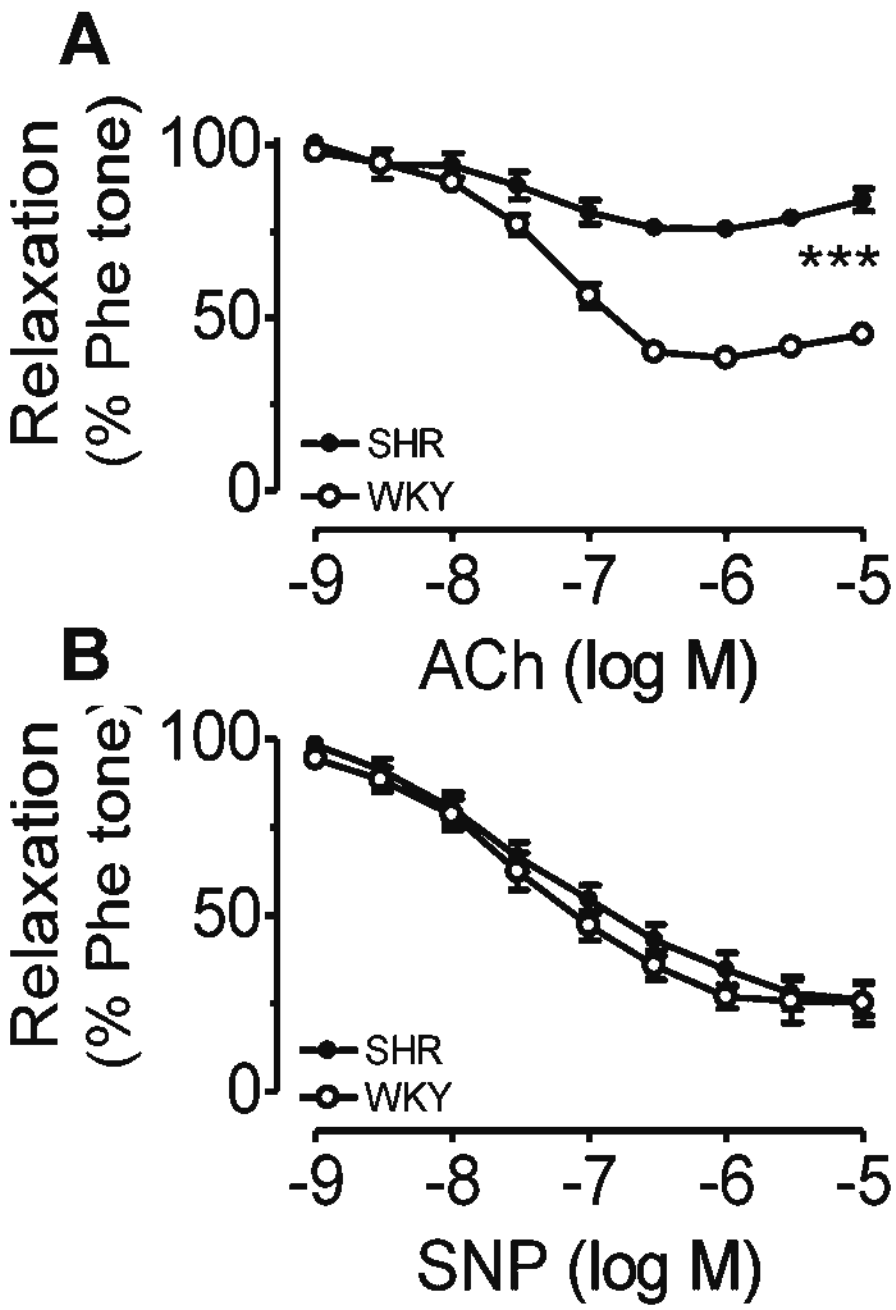


Figure 4.2.
(A) Concentration-response curves showing less acetylcholine (ACh)-induced endothelium-dependent dilatations in aortas from SHR than from WKY. **(B)** Identical endothelium-independent dilatations induced by a NO donor, sodium nitroprusside (SNP) in WKY and SHR aortas. Results are mean ± SEM of 6-8 experiments.

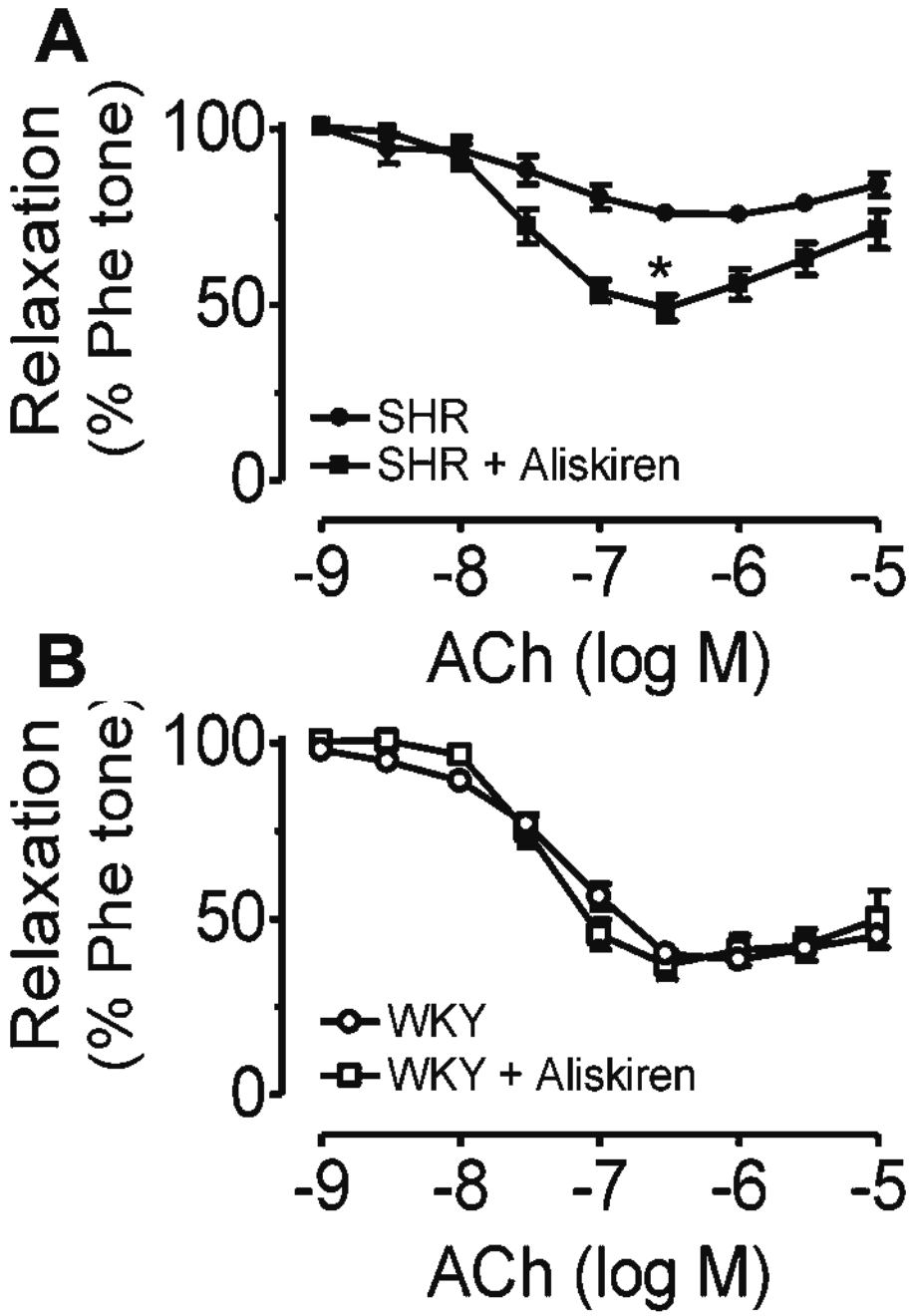


Figure 4.3.
The effect of eight-week treatment with aliskiren (10mg/kg/day) on ACh-induced endothelium-dependent dilatations in SHR **(A)** and WKY **(B)** aortas. Results are mean ± SEM of 6-8 experiments. Statistical significance between groups is indicated by *$p<0.05$.

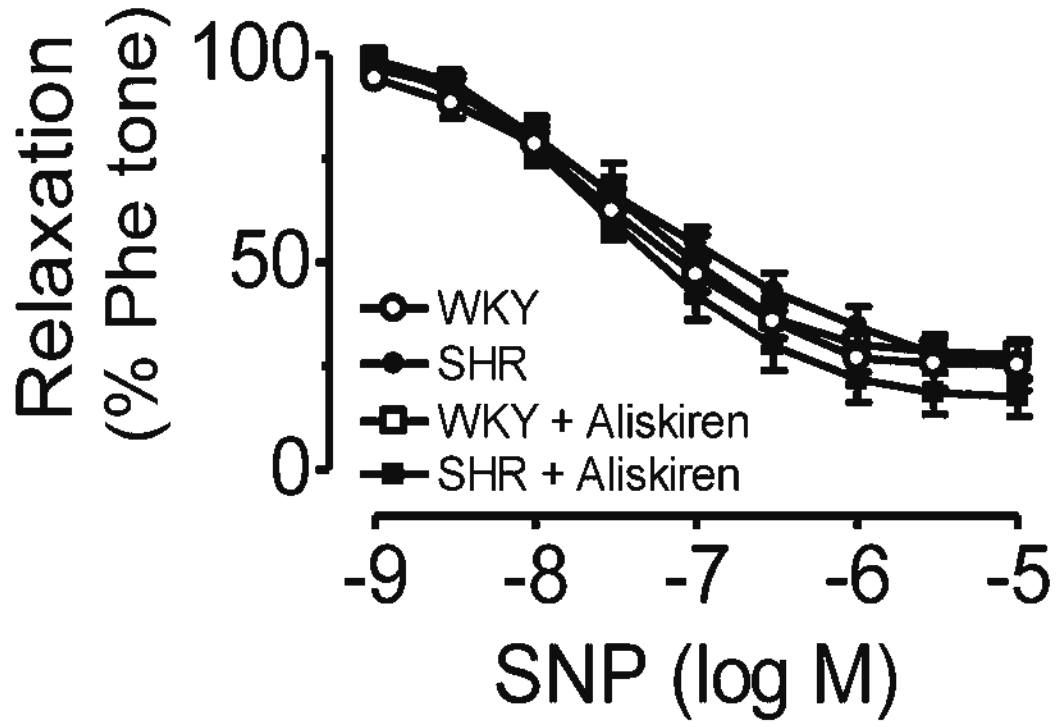


Figure 4.4.
Endothelium-independent dilatations in response to sodium nitroprusside (SNP) in phenylephrine-contracted aortas from aliskiren-treated and vehicle-treated SHR and WKY rats. Results are mean ± SEM of 6-8 experiments.

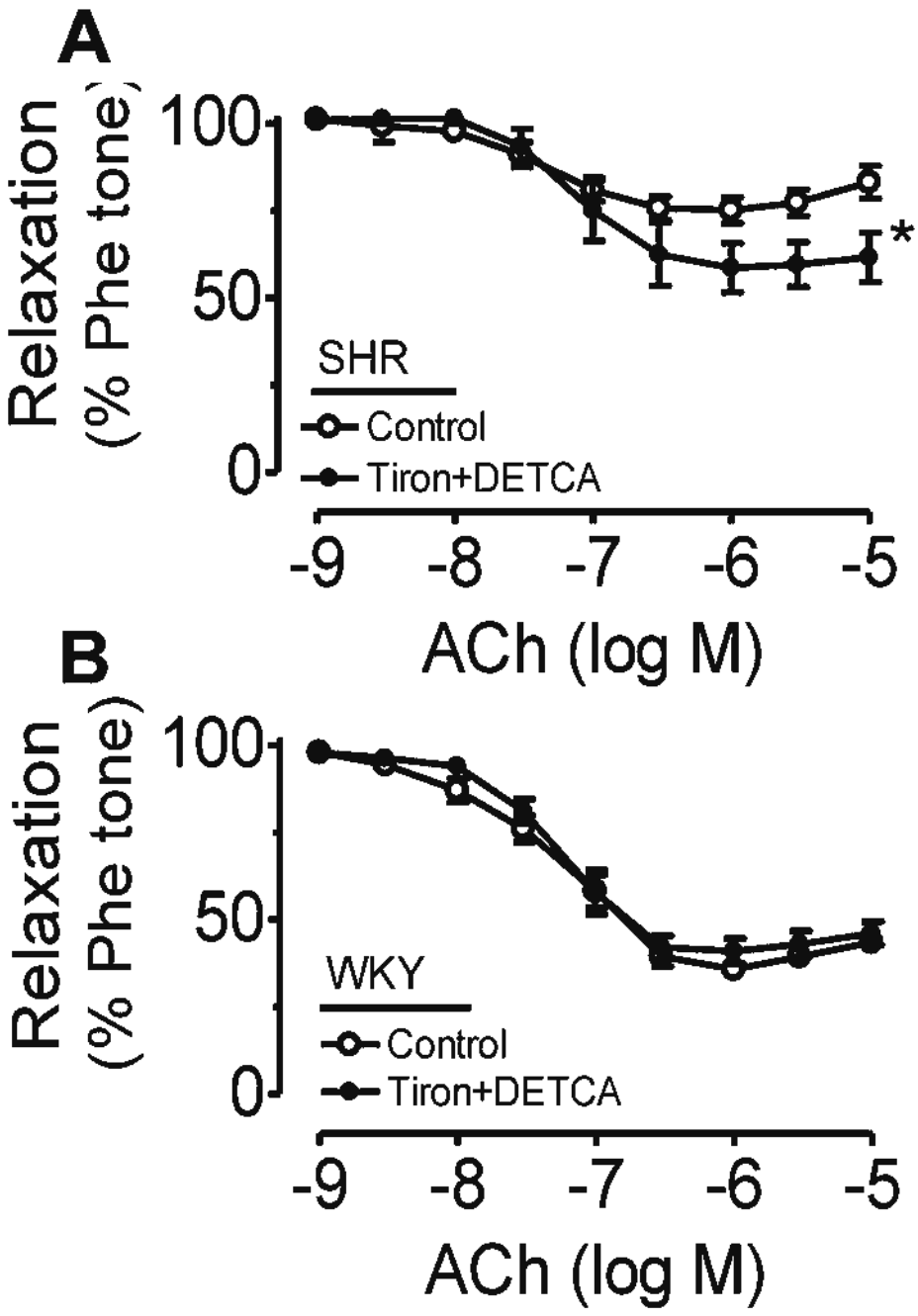


Figure 4.5.
The effect of a combined acute 30-min treatment with 1 mmol/L tiron plus 100 µmol/L DETCA on ACh-induced endothelium-dependent dilatations in aortas from SHR **(A)** and WKY rats **(B)**. Results are mean ± SEM of 5 experiments. Statistical difference between control and treatment group is indicated by *$p<0.05$.

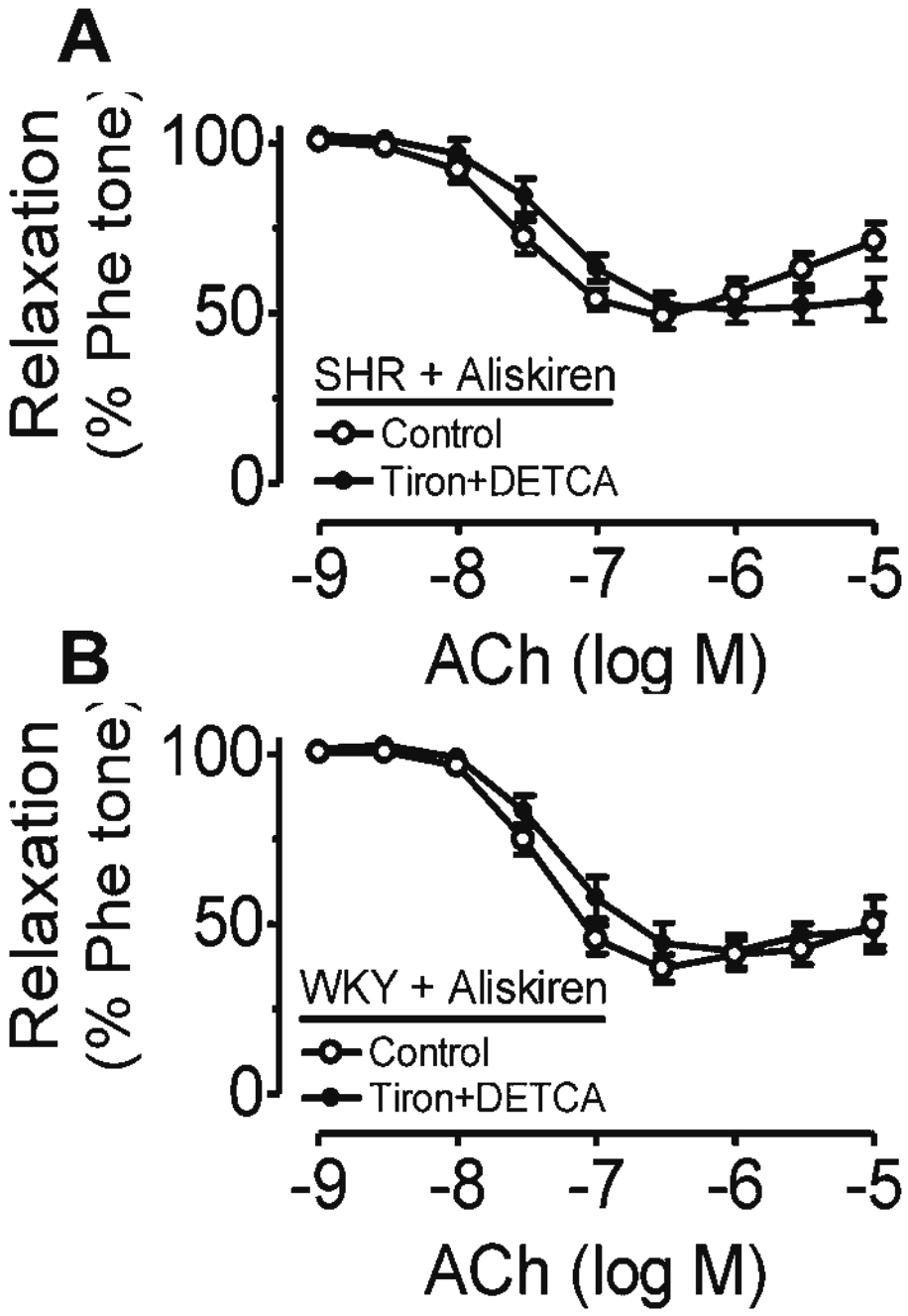


Figure 4.6.
Lack of effect of a combined acute treatment with 1 mmol/L tiron plus 100 µmol/L DETCA on the ACh-induced endothelium-dependent dilatations of aortas from aliskiren-treated SHR **(A)** or aliskiren-treated WKY rats **(B)**. Results are mean ± SEM of 5 experiments.

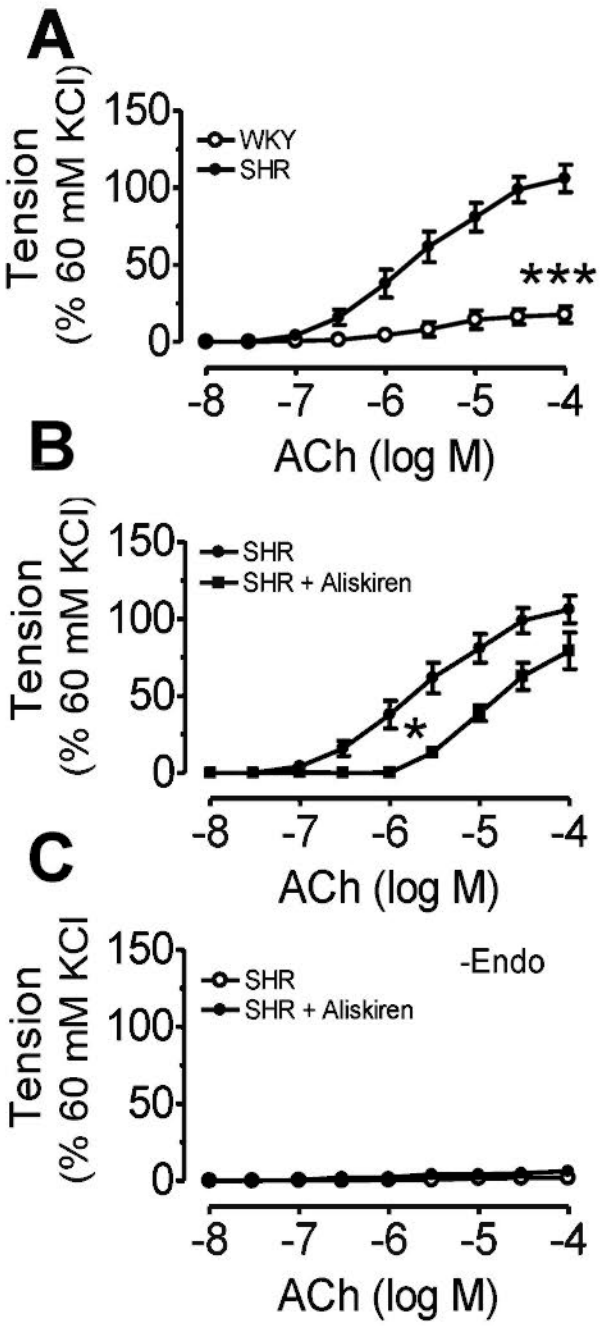


Figure 4.7.

(A) The augmented ACh-induced endothelium-dependent contractions (EDC) in SHR aortas treated by 100 µmol/L L-NAME as compared with aortas from normotensive WKY rats. **(B)** The effect of chronic aliskiren treatment on ACh-induced endothelium-dependent contractions in endothelium-intact SHR aortas. **(C)** The absence of endothelium-dependent contractions in response to ACh in SHR aortas without endothelium. Results are mean ± SEM of 6-8 experiments. Statistical significance between curves is indicated by ***$p<0.001$ and *$p<0.05$.

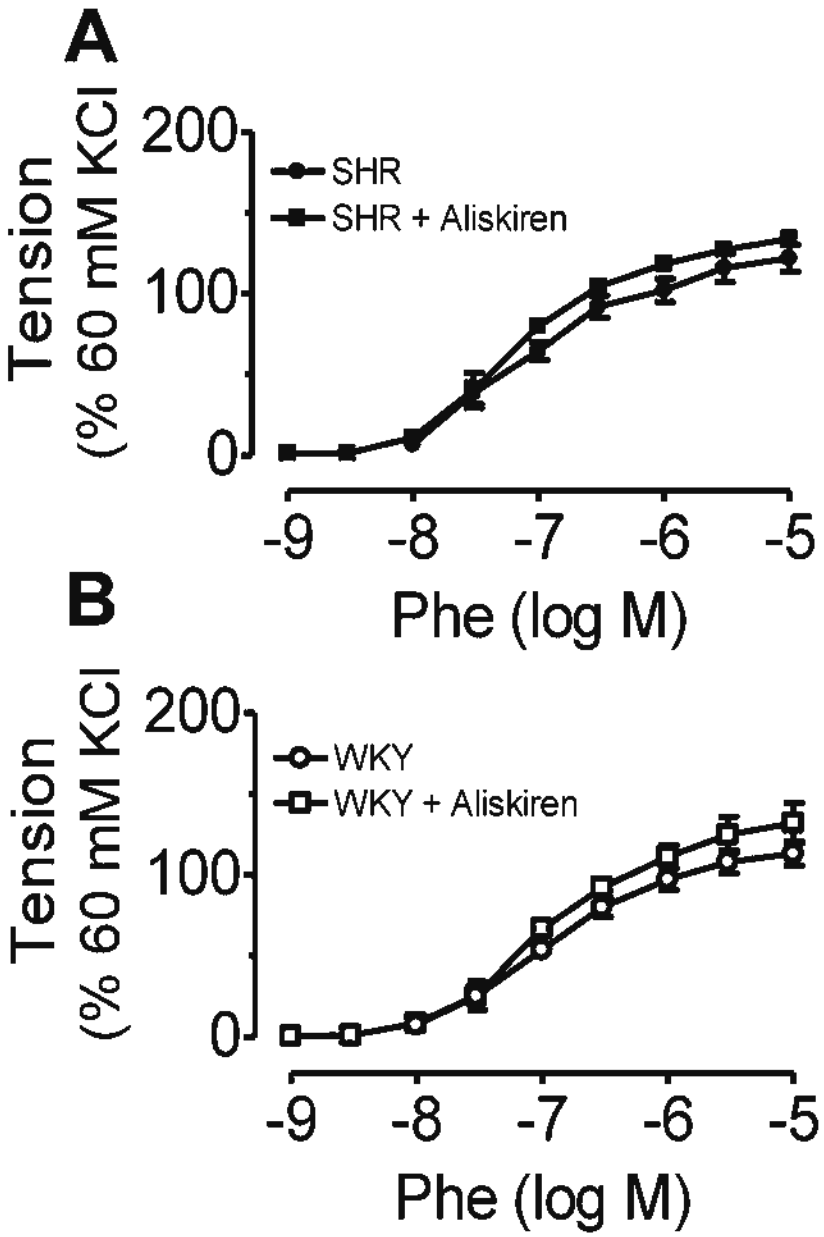


Figure 4.8.
Lack of effect of chronic aliskiren treatment on phenylephrine (Phe)-induced aortic contractions in both SHR **(A)** and WKY **(B)** rats. The contractile response of endothelium-intact aortas was expressed as percentage of a tension induced by 60 mmol/L. Results are mean ± SEM of 4-6 experiments.

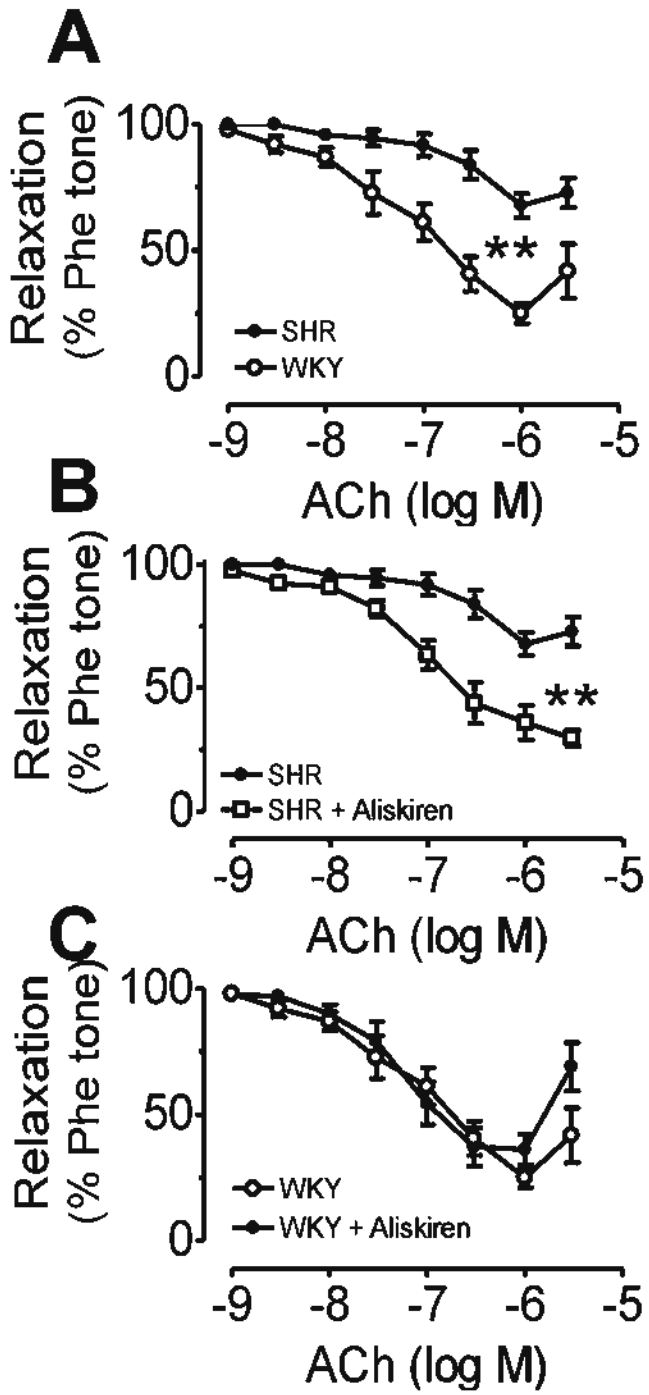


Figure 4.9.
(A) The impaired ACh-induced endothelium-dependent dilatations in SHR intralobal renal arteries as compared with normotensive rat aortas. The effect of chronic aliskiren treatment on ACh-induced dilatations in renal arteries from SHR **(B)** and WKY **(C)** rats. Results are mean ± SEM of 5-7 experiments. Statistical significance between groups is indicated by **p<0.01.

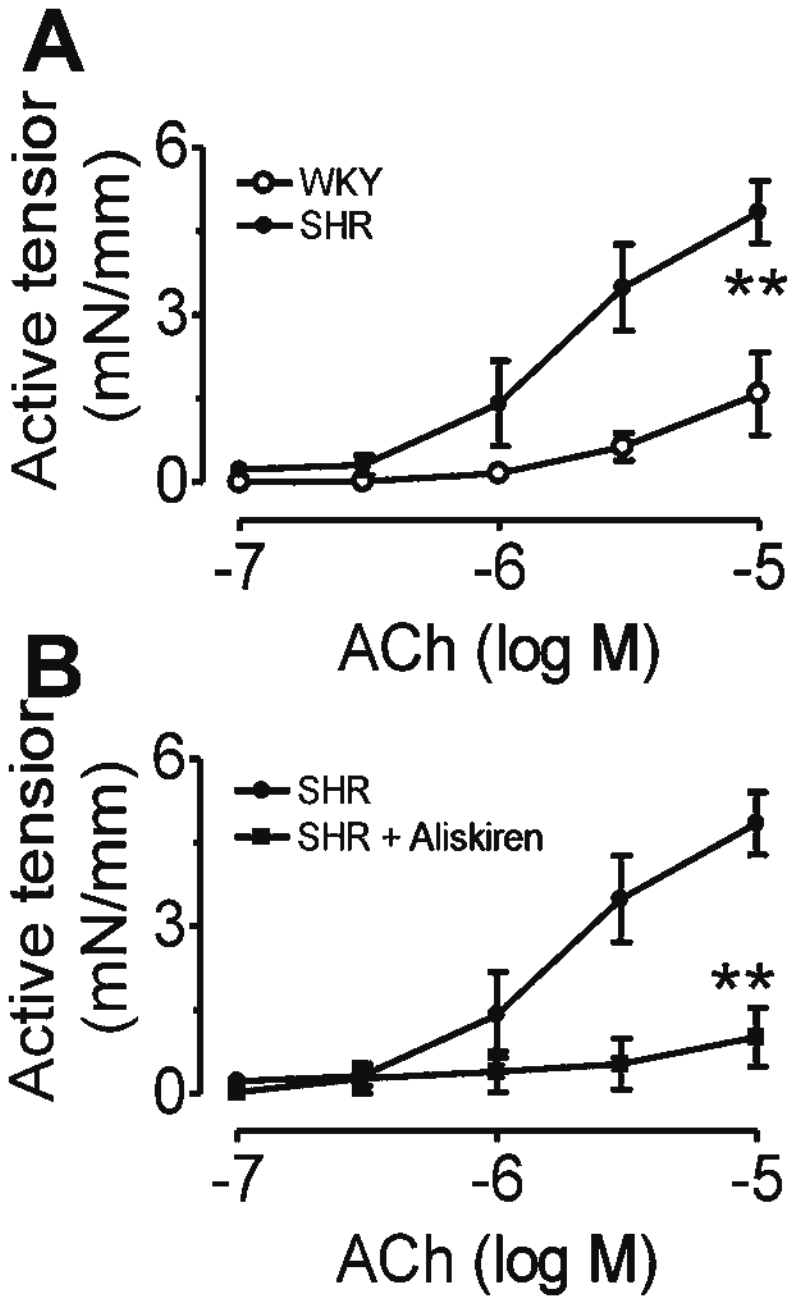


Figure 4.10.
(A) Augmented endothelium-dependent contractions elicited by ACh in L-NAME-treated SHR intralobal renal arteries. **(B)** Effect of chronic aliskiren treatment on the ACh-induced endothelium-dependent contractions in SHR renal arteries. Results are mean ± SEM of 6-8 experiments. Statistical significance between groups is indicated by **$p<0.01$.

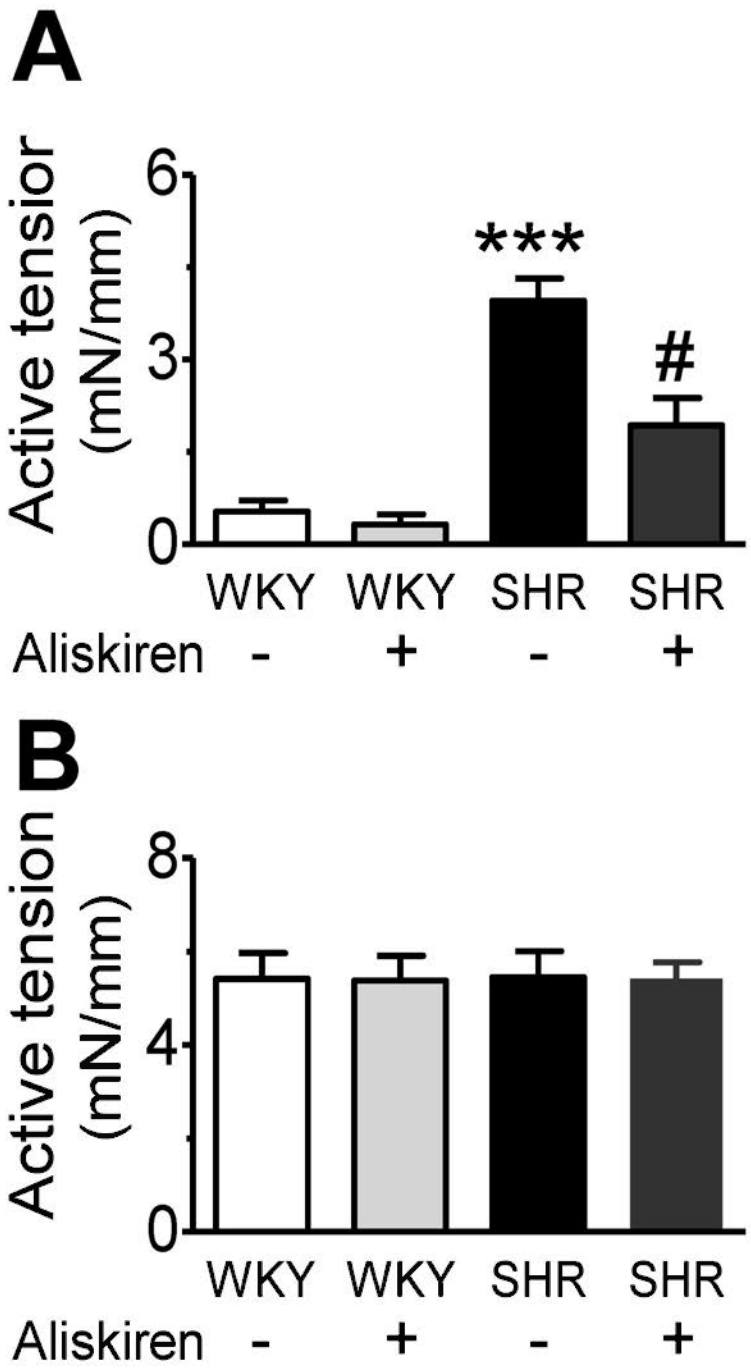


Figure 4.11.
The effect of chronic aliskiren treatment on angiotensin II (100 nmol/L)-induced contraction **(A)** and on contraction induced by 60 mmol/L KCl **(B)** in renal arteries of SHR and WKY rats. Results are mean ± SEM of 5-7 experiments. Statistical significance between groups is indicated by ***p<0.001 vs WKY and #p<0.01 vs SHR.

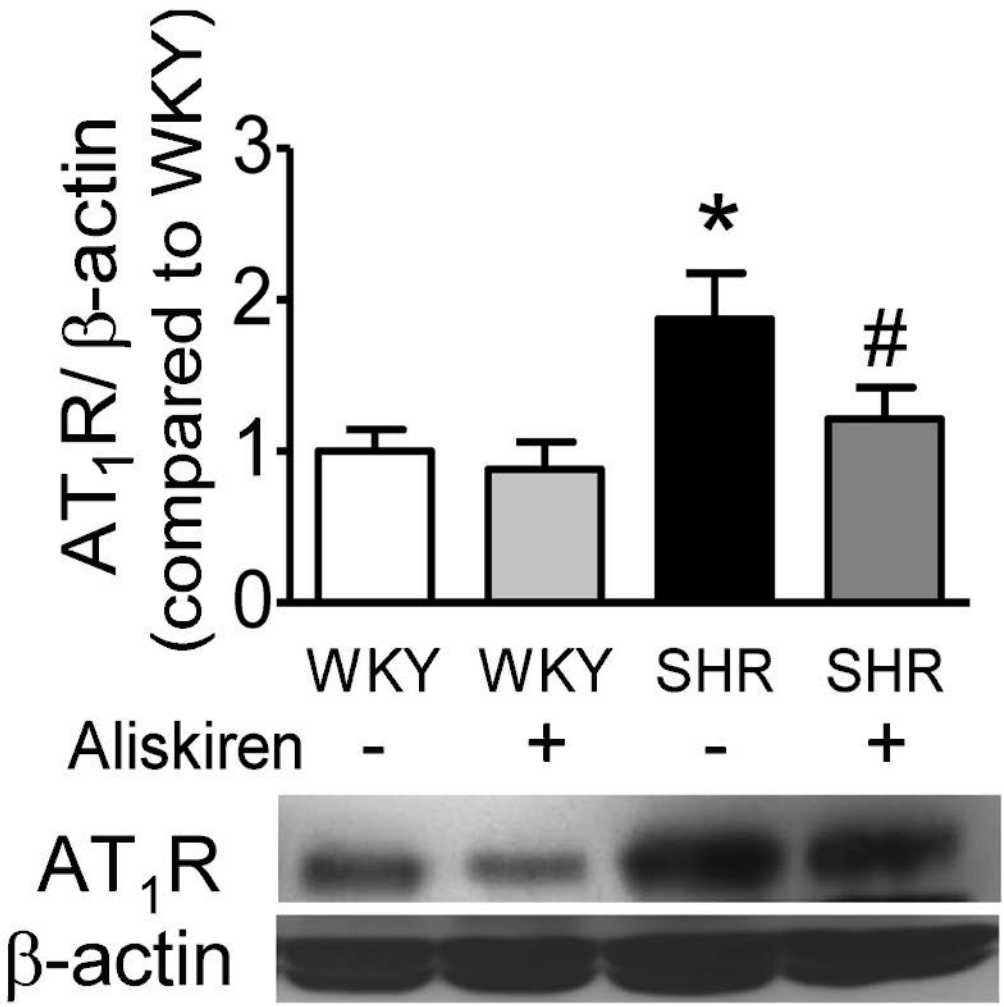


Figure 4.12.
Western blot analysis showing an increase in protein expression of angiotensin II type 1 receptor (AT1R) in SHR aortas and the effect of chronic aliskiren treatment on the level of AT1R. Results are mean ± SEM of 4-6 experiments. Statistical significance between groups is indicated by *$p<0.05$ vs WKY and #$p<0.05$ vs SHR.

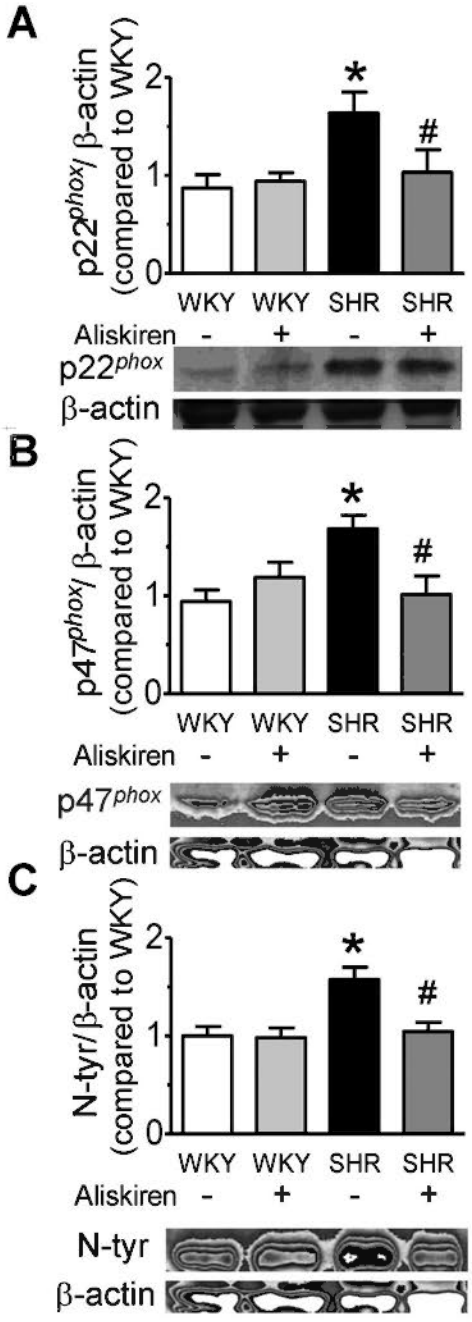


Figure 4.13.
The effect of chronic aliskiren treatment on the protein expression level of NAD(P)H oxidase subunit p22*phox* **(A)**, p47*phox* **(B)**; and nitrotyrosine **(C)** in endothelium-intact aortas from SHR and WKY rats. Results are mean ± SEM of 4 experiments. Statistical significance between groups is indicated by *p<0.05 vs WKY and #p<0.05 vs SHR.

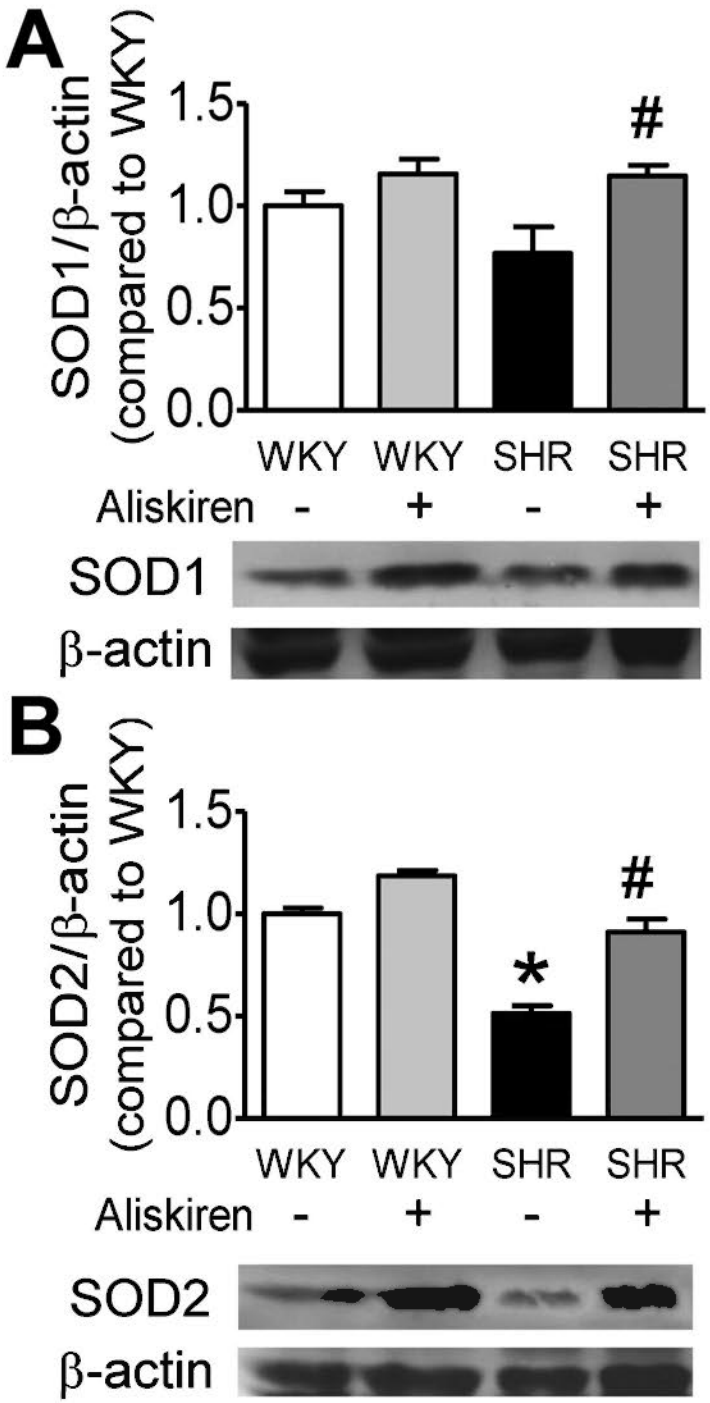


Figure 4.14.
The effect of chronic aliskiren treatment on the protein expression level of SOD1 **(A)** and SOD2 **(B)** in aortas from SHR and WKY rats. Results are mean ± SEM of 4 experiments. Statistical significance between groups is indicated by *p<0.05 vs WKY and #p<0.05 vs SHR.

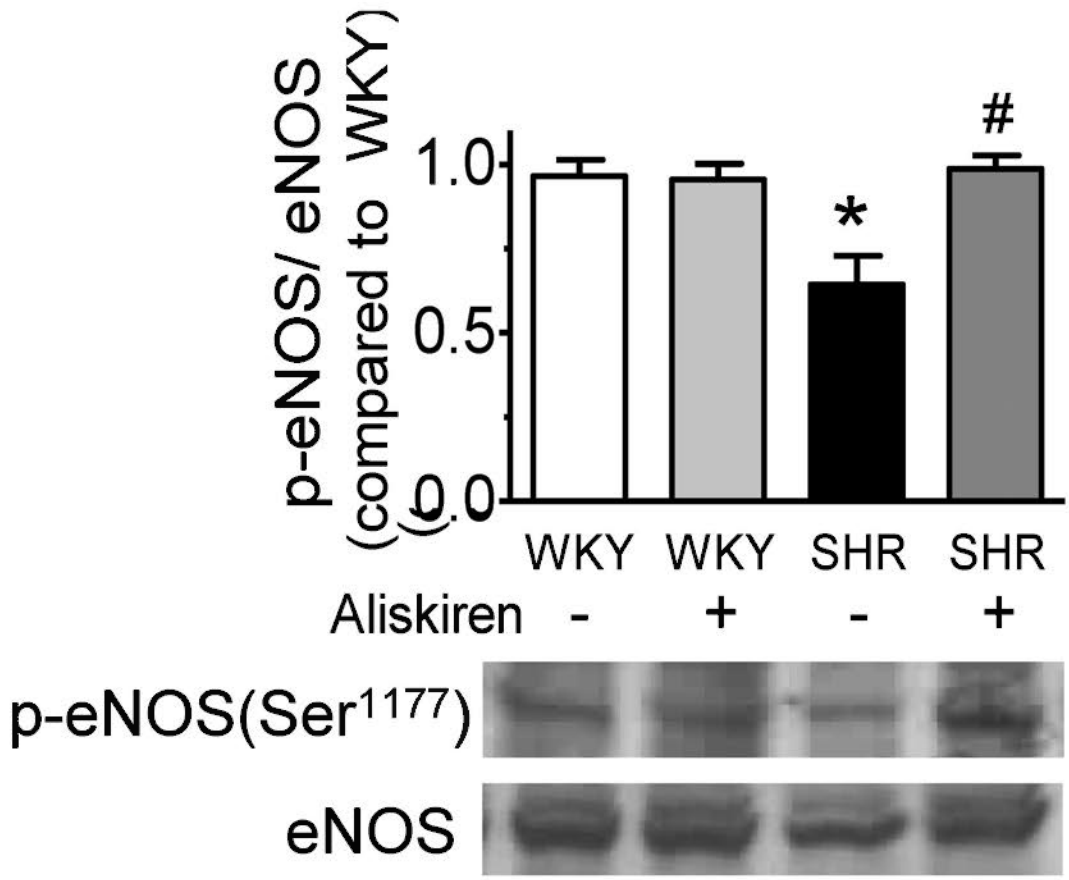


Figure 4.15.
The effect of chronic aliskiren treatment on the protein expression level of endothelial nitric oxide synthase (eNOS) and phosphorylation of eNOS at Ser1177 (p-eNOS) in aortas from SHR and WKY rats. Results are mean ± SEM of 4 experiments. Statistical significance between groups is indicated by *p<0.05 vs WKY and #p<0.05 vs SHR.

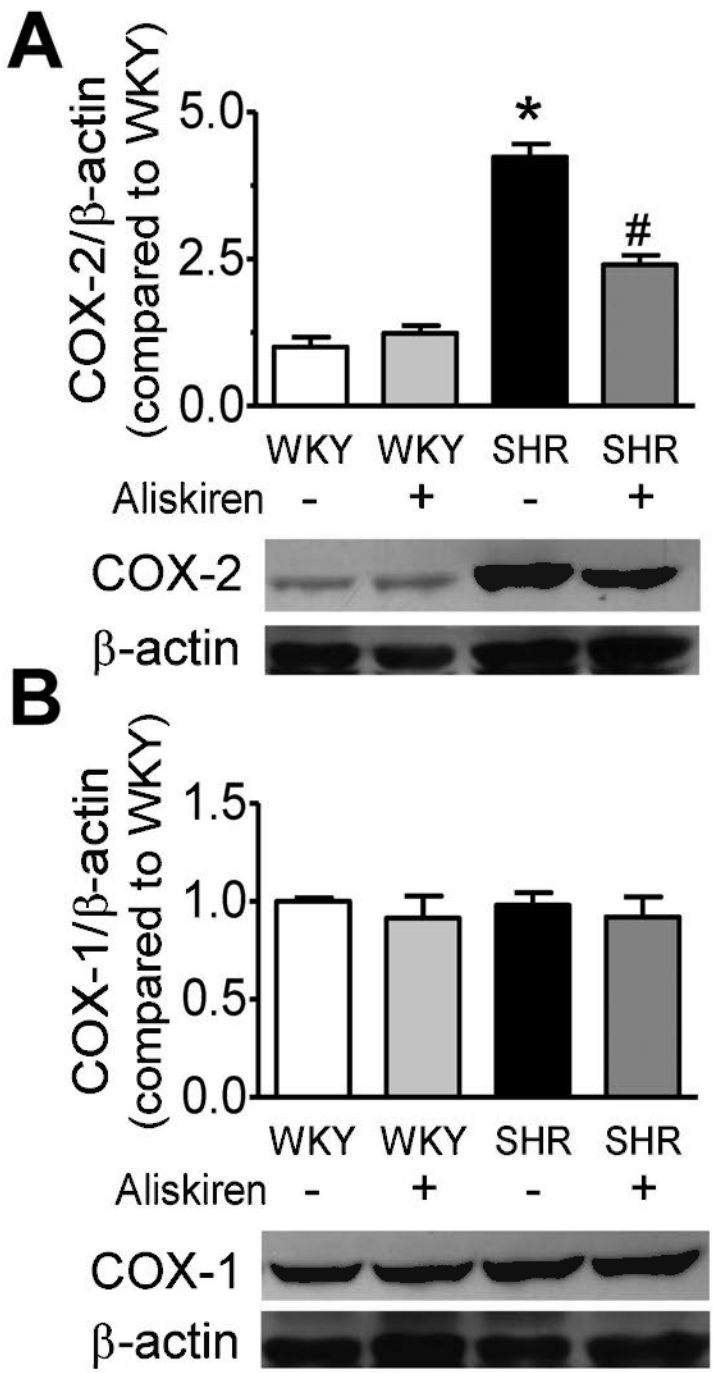


Figure 4.16.
The effect of chronic aliskiren treatment on the protein expression level of COX-2 **(A)** and COX-1 **(B)** in aortas from SHR and WKY rats. Results are mean ± SEM of 4 experiments. Statistical significance between groups is indicated by *p<0.05 vs WKY and #p<0.05 vs SHR.

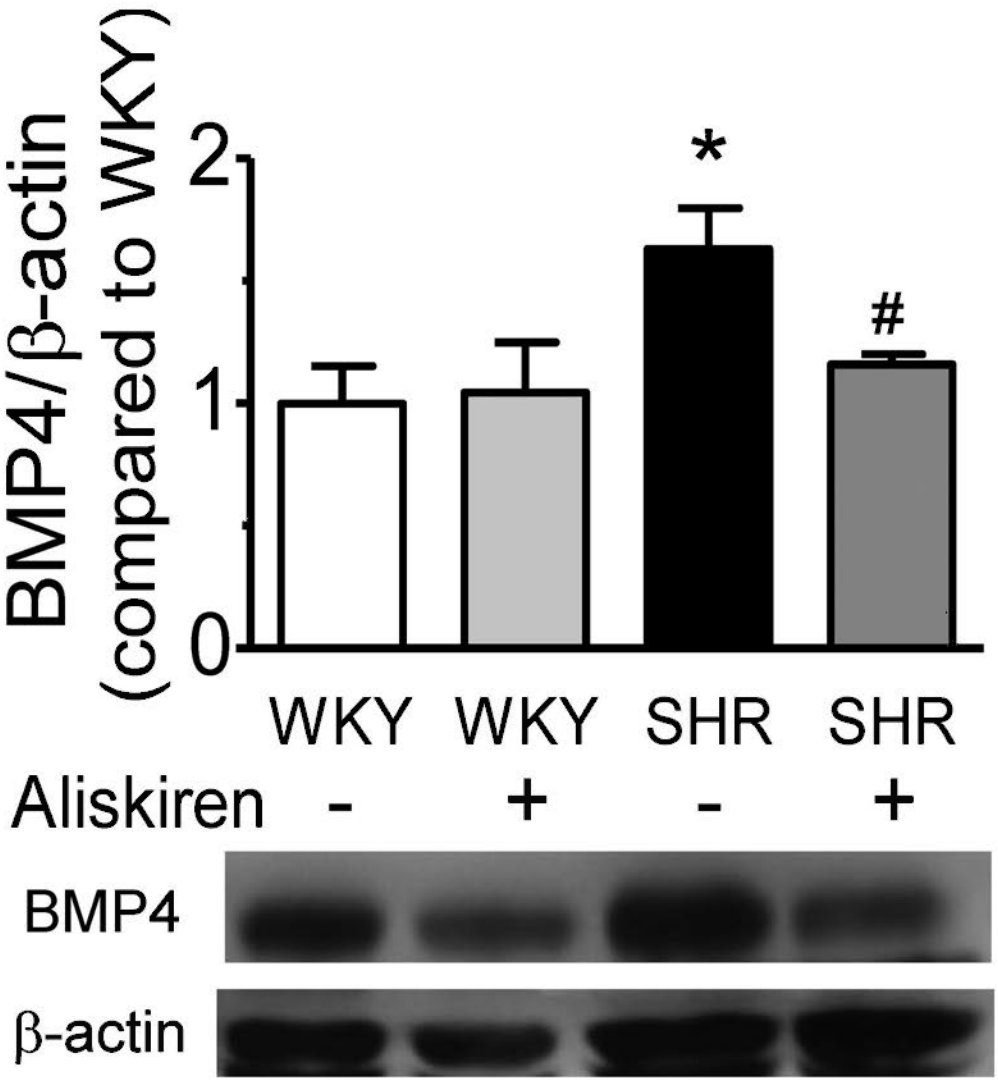


Figure 4.17.
The effect of chronic aliskiren treatment on the protein expression level of bone morphogenic protein-4 (BMP4) in aortas from SHR and WKY rats. Results are mean ± SEM of 4 experiments. Statistical significance between groups is indicated by *p<0.05 vs WKY and #p<0.05 vs SHR.

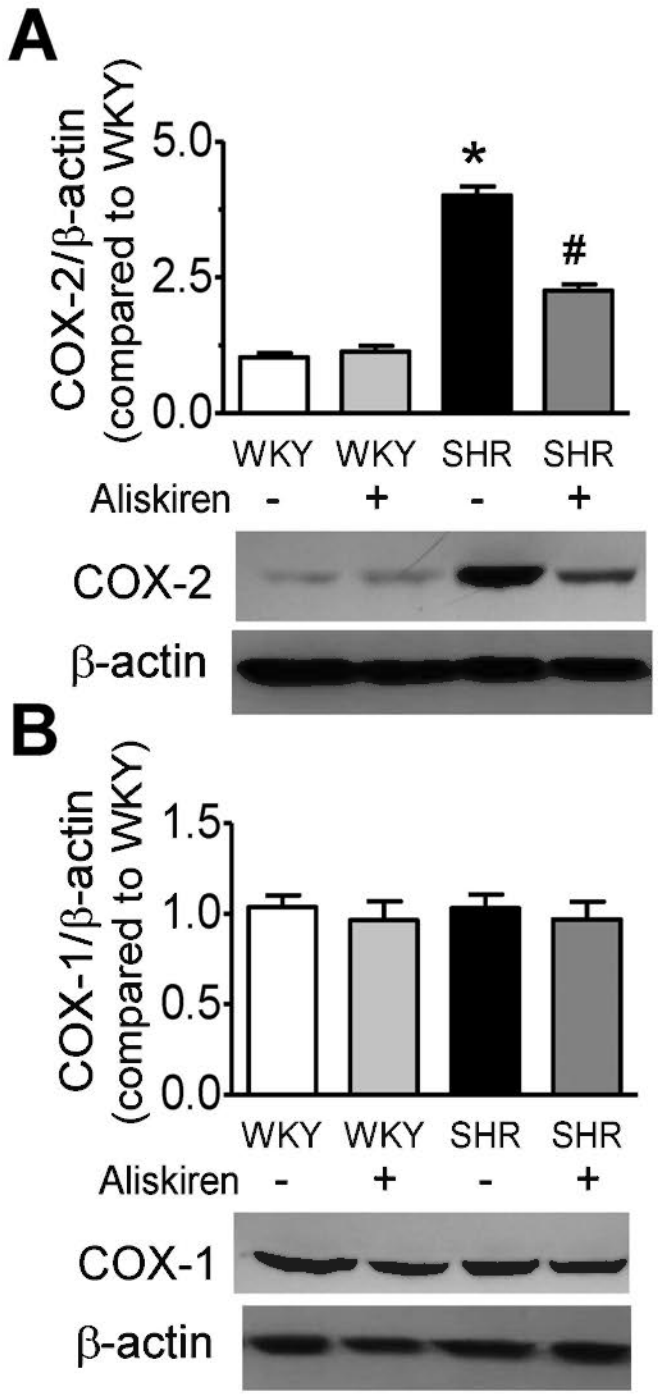


Figure 4.18.
Western blot analysis showing chronic aliskiren treatment on protein expression levels of COX-2 **(A)** and COX-1 **(B)** in renal arteries from SHR and WKY rats. Results are mean ± SEM of 4 experiments. Statistical significance is indicated by *$p<0.05$ vs WKY and #$p<0.05$ vs SHR.

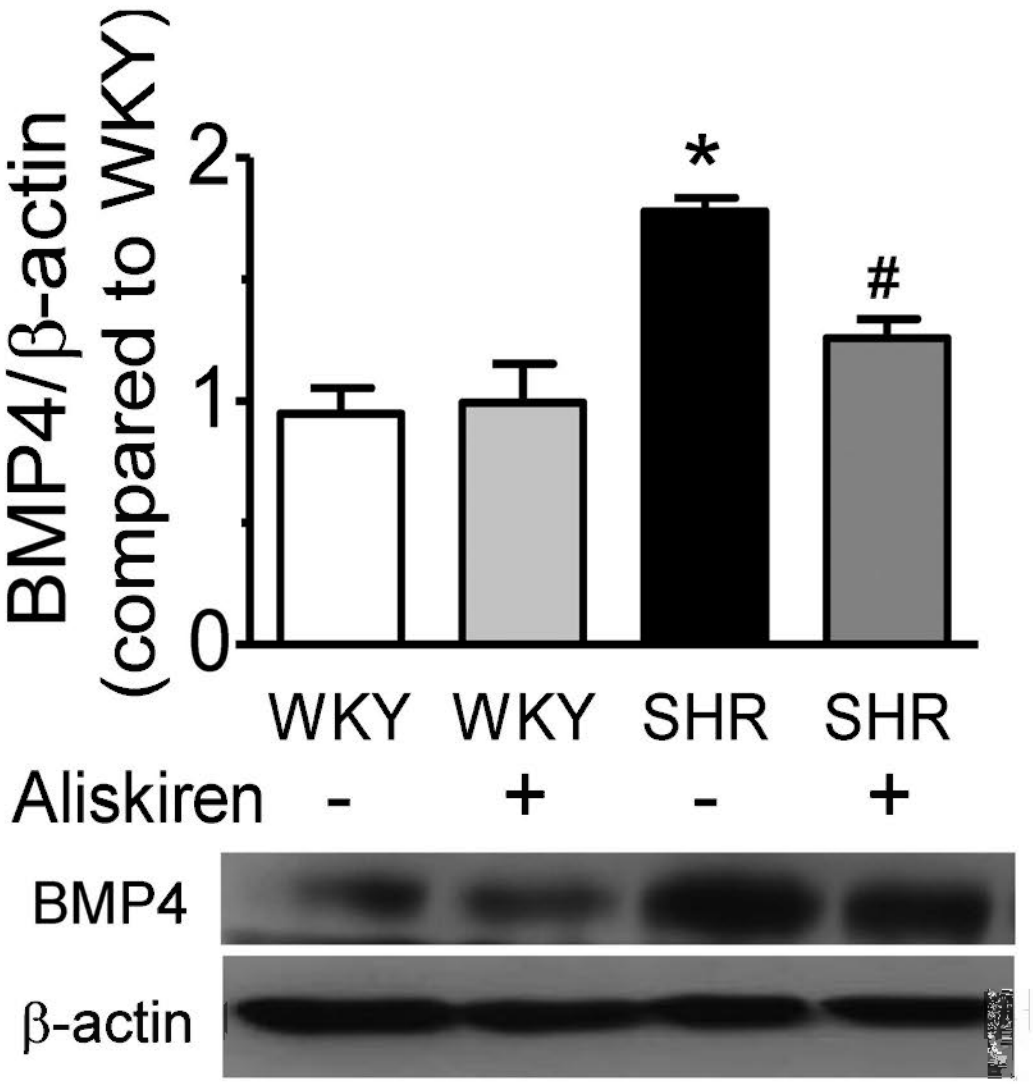


Figure 4.19.
Western blot analysis showing chronic aliskiren treatment on protein expression levels of BMP4 in renal arteries from SHR and WKY rats. Results are mean ± SEM of 4 experiments. Statistical significance is indicated by *p<0.05 vs WKY and #p<0.05 vs SHR.

4.4 Discussion

The present study examined the effect of direct renin inhibition by aliskiren on endothelial dysfunction in spontaneously hypertensive rats (SHR). The new findings of this study support the vascular benefit of aliskiren as an anti-hypertensive drug. Firstly, chronic daily oral administration of low-dose aliskiren to SHR improved endothelium-dependent dilatations and attenuated endothelium-dependent contractions in both aortas and renal arteries. Secondly, the vascular benefit of aliskiren was related to favourable modulation of the RAS through suppression of the up-regulated expression of RAS components and RAS-dependent oxidative stress. Thirdly, chronic aliskiren therapy increased the expression of the anti-oxidant enzyme SOD, and restored the reduced phosphorylation of eNOS at serine-1177. Finally, aliskiren reduced the over-expression of BMP4 and COX-2; the two important markers of vascular inflammation (also see Chapter VI for details). The results obtained in this study may enhance the clinical prospective of aliskiren and other more specific renin inhibitors in the prevention and treatment of hypertension-related vascular dysfunction.

In line with results from previous studies (Wood et al., 2005; Nussberger et al., 2008), I also found a moderate reduction of systolic blood pressure by low-dose aliskiren treatment in eight-month old SHR with established hypertension (≥ 165 mmHg). Aliskiren treatment improved endothelium-dependent dilatations in SHR aortas and intralobal renal arteries. The improvement was more prominent in renal arteries than in aortas, possibly due to varied degrees of contribution of the RAS and oxidative stress to endothelial dysfunction in different vascular beds. The beneficial effect in renal arteries could be more significant in consideration of the importance of control of renovascular tone in the kidney function in hypertension while impaired dilatations in conduit arteries are more related to occurrence of atherosclerosis and also altered ventricular function. The present study indicates a positive role of overproduced ROS in endothelial dysfunction in SHR since acute removal of ROS augmented ACh-induced dilatations in SHR aortas. Interestingly, such improvement in aortas was lost in SHR receiving eight-week oral

administration of aliskiren, suggesting that aliskiren therapy might reduce oxidative stress in the vascular wall, a common condition in hypertension, and thus contributes to better endothelial function. Likewise, I also showed that in renovascular hypertensive rats, ROS scavenging improve endothelial function in renal arteries (see Chapter V for details), suggesting that oxidative stress is likely to be a common cause of endothelial dysfunction in hypertension. In a recent study using pressurized myograph, the impaired endothelium-dependent vasodilatations in mesenteric resistance arteries of SHR can be rescued by the NAD(P)H oxidase inhibitor apocynin (Virdis et al., 2009), which is L-NAME sensitive, again indicating that ROS reduces the NO bioavailability and thus actively participates in endothelial dysfunction. In consistence with this notion, the data I obtained in db/db mouse mesenteric resistance arteries also show that the augmented dilatation to ACh by acute exposure to tiron plus DETCA can be fully inhibited by L-NAME (see Chapter III for details). However, the present results do not permit me to distinguish whether improved endothelial function contributes to a moderate blood pressure-lowering effect of aliskiren or vice versa. This concern can be addressed in organ culture of blood vessels I have developed, which allows me to determine a possible direct action of aliskiren on arteries in the future extended study.

The results from functional examinations indicate that the impaired endothelial function in SHR is associated with increased oxidative stress as shown previously by many other groups. The expression of various components along the RAS-oxidative stress axis was significantly enhanced in SHR arteries and they include the AT_1 receptor, $p22^{phox}$, $p47^{phox}$, and nitrotyrosine in aortic tissue of SHR. Each of up-regulations was suppressed by chronic aliskiren treatment. It is known that NADPH oxidase is one of the most important oxidant enzymes catalyzing the generation and release of ROS in the onset of hypertension. In SHR and salt-induced hypertensive rats, $p22^{phox}$ is up-regulated (Ulker et al., 2003; Shah and Singh, 2007), and apocynin protects vascular function in hypertensive rats (Jimenez et al, 2007). In 28-week-old SHR, tiron can partially improve ACh-induced vasodilatation, while SOD was devoid of such benefit (Ulker et al., 2003). In order to exclude the impact of endogenous SOD on the effect of aliskiren, I used

a combination of tiron plus DETCA. Therefore, the improved endothelial function following such treatment is likely to cause mainly by scavenging of ROS derived from the up-regulated NAD(P)H oxidase. Moreover, the up-regulation of BMP4, a novel and upstream activator of vascular inflammation, which was reported before to activate NAD(P)H oxidase and then mediate the ROS production (Miriyala et al., 2006), was also inhibited by aliskiren therapy. A recent study shows that palmitate induced acute vascular inflammation or high fat feeding lead to the up-regulation of BMP4 and NOX4 in mouse aortas (Maloney et al., 2009). I have also investigated the pathological role of BMP4 in hypertension and its association with over-expression of COX-2, another pro-inflammatory factor (see Chapter VI for details). Taken together, these findings support active roles of BMP4 and NAD(P)H oxidase in the induction of endothelial dysfunction and aliskiren is very effective in inhibiting both of them.

As discussed elsewhere in this thesis, a balance between anti-oxidant and oxidant enzymes is crucial to determine whether ROS is physiological regulators of vascular function or serve as pathological initiators for vascular dysfunction. In the present study, I found that the up-regulated expression of SOD1 and SOD2, major forms of the anti-oxidant enzyme were inhibited by aliskiren and this effect shall be independent of direct renin inhibition if renin alone does not trigger the ROS production in the vascular wall. It is probable that reduced expression/activity of NAD(P)H oxidase and increased expression/activity of SOD account for inhibition of oxidative stress stemming from aliskiren treatment.

Moreover, the lost level of phosphorylation of eNOS at serine-1177 in SHR aortas is rescued following chronic aliskiren treatment, suggesting such benefit also contributes to the increased NO bioavailability and endothelial function. This benefit might be unique since chronic treatment with valsartan (ARB) or enalapril (ACE inhibitor) did not increase eNOS phosphorylation in aortas from diabetic db/db mice (see Chapter III for details). My results in SHR are consistent with those obtained in rabbits in which aliskiren protects endothelial function and inhibits atherosclerotic changes by augmenting the NO bioavailability via an increased phosphorylation of eNOS and Akt (Imanishi et al., 2008). Some discrepancies exist

regarding the level for the eNOS expression in different animal models of hypertension. For instance, the eNOS mRNA level is decreased in salt-induced hypertension (Shah and Singh, 2007; Zhou et al., 2004), while the expression of both eNOS mRNA and protein is increased in SHR arteries (Ulker et al., 2003). However, my study shows the total amount of eNOS is unaffected in SHR aortas. Taken together, aliskiren in comparison with other RAS inhibitors may offer better vascular protection by preserving eNOS activity.

The present study also provides the first piece of evidence that renin inhibition by aliskiren almost prevented endothelium-dependent contractions in SHR aortas and renal arteries. Endothelial dysfunction in hypertension is not only caused by blunted endothelium-dependent dilatations but also by a concomitant release of endothelium-derived contracting prostanoids (Vanhoutte, 2009). The augmented endothelium-dependent contractions are apparent in SHR aortas due to the increased release of COX-dependent prostanoids (Luscher et al., 1990). Similarly, in SHR renal arteries, EDCF-mediated contraction is more pronounced with NOS inhibition and is caused by stimulation of the TP receptor by COX-dependent prostanoids (Michel et al., 2008). Taking my new findings presented in Chapter V and Chapter VI, my results suggest that aliskiren-induced suppression of the EDCF response in SHR arteries may be partly mediated by its inhibition of COX activities. To support this claim, I did show that aliskiren can actually reduce the already-overexpressed COX-2 in SHR aortas and renal arteries. By contrast, the expression of COX-1 is unchanged in SHR and WKY rat arteries and aliskiren treatment produced no effect although COX-1 is implicated to mediate EDCF action in SHR arteries in earlier studies (Yang et al., 2003).

In summary, the present study clearly demonstrates that renin inhibition by aliskiren protects endothelial function in SHR by increasing the NO bioavailability via two major mechanisms: preserved eNOS activity and reduced oxidative stress and also by inhibiting endothelium-dependent contractions probably by inhibition of up-regulated expression and/or activity of COX-2 and BMP4 in the vascular wall. Although more detailed examination is needed, the present data do support the clinical efficacy of aliskiren in the treatment of hypertensive patients.

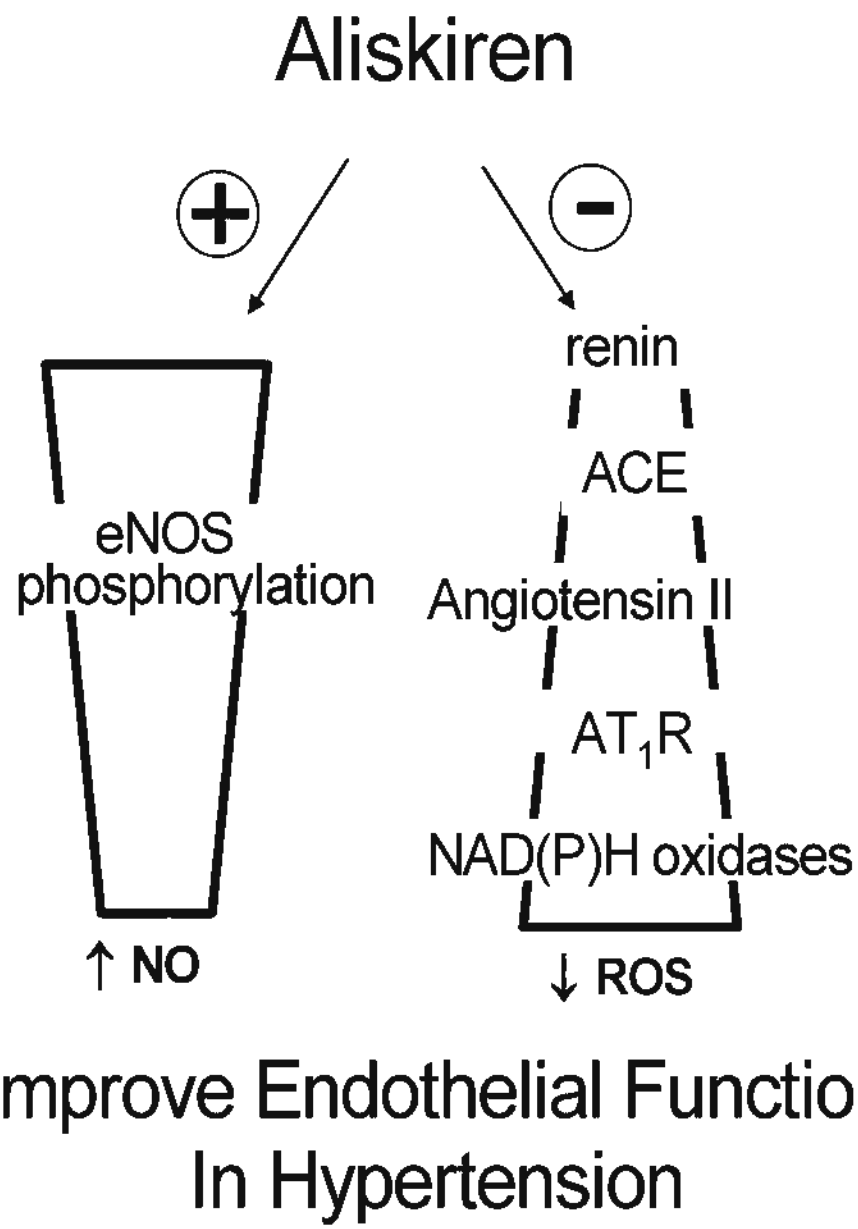


Figure 4.20.
Schematic figure summarizing the possible mechanisms undelying the vasoprotective effects of renin inhibitor, aliskiren, in improving endothelial function in hypertension. Abbreviations: ACE, angiotensin converting enzyme; AT_1R, angiotensin type 1 receptor; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; ROS, reactive oxygen species.

CHAPTER V

CYCLOOXYGENASE-2 DERIVED PROSTAGLANDIN $F_{2\alpha}$ MEDIATES ENDOTHELIUM-DEPENDENT CONTRACTIONS IN INTRALOBAL RENAL ARTERIES OF RENOVASCULAR HYPERTENSIVE RATS

5.1 Introduction

Endothelial cells modulate the tone of the underlying vascular smooth muscle by releasing endothelium-derived relaxing (EDRF) and endothelium-derived contracting factors (EDCF). EDCF, a characteristic of endothelial dysfunction, was reported to be the cyclooxygenase (COX)-mediated products of arachidonic acid and EDCF activates thromboxane prostanoid receptors in the vascular smooth muscle cells to cause contraction (Auch-Schwelk et al., 1990; Yang et al., 2002; Tang et al., 2005; Vanhoutte et al., 2005; Gluais et al., 2005; Koga et al., 1989; Gluais et al., 2005). Endothelium-dependent contractions are augmented by hypertension (Yang et al., 2002; Tang et al., 2005; Koga et al.,1989; Auch-Schwelk et al., 1989), diabetes (Shi et al., 2007; Shi et al., 2007), and ageing (Wong et al., 2009). Although EDCF-mediated contractions can be observed in arteries from aging, or diabetic rats, and in humans with essential hypertension, acute estrogen deficiency and heart failure (Taddei et al., 2002), much less is known about the chemical identify of EDCFs and endothelium-derived factor(s) that may modify the ability of endothelial cells to liberate EDCFs in blood arteries from rats with experimental induced renovascular hypertension, a widely used model for the study of nephropathy in hypertension and the cellular and molecular mechanisms involved in the induction of endothelium-dependent contractions are only partially understood.

Arachidonic acid-derived metabolites, namely prostaglandin I_2 (PGI_2), prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$),

isoprostane, and thromboxane A_2 (TXA_2), are proposed to be among the EDCF candidates (Lüscher and Vanhoutte, 1986; Vanhoutte et al., 2005). The chemical conversion of arachidonic acid into prostaglandin H_2 (PGH_2) by COX is an important enzymatic step in the regulation of prostaglandin biosynthesis. Several earlier studies concerning the degradation of arachidonic acid to EDCF candidate(s) showed that selective COX-1 inhibitors could prevent acetylcholine (ACh)-induced endothelium-dependent contractions in the aortas of spontaneously hypertensive rats (SHR) (Ge et al., 1995; Yang et al., 2002). Expression of COX-1 was found to be markedly up-regulated in SHR aortas as compared with those of normotensive Wistar-Kyoto (WKY) rats (Ge et al., 1995). It was thus suggested that endothelium-dependent contractions are mediated by COX-1, the constitutively expressed isoform of COX. On the other hand, COX-2 can be induced by the presence of the elevated level of reactive oxidative species (ROS), which leads to endothelial dysfunction with aging (Heymes et al., 2000; Ge et al., 1995; de Sotomayor et al., 2005; Kang et al., 2007). A positive role of COX-2 in the appearance of endothelium-dependent contractions has been clearly demonstrated in healthy hamster aortas (Wong et al., 2009).

ROS not only attenuate endothelium-dependent vasodilatations by scavenging nitric oxide (Rubanyi et al., 1986), but also play a role in endothelium-dependent contractions (Yang et al., 2002; Shi et al., 2007; Katusic et al., 1989; Tang et al., 2007). Superoxide anions had been proposed as an EDCF in dog basilar arteries (Katusic et al., 1989; Tang et al., 2007), whereas hydroxyl radicals in aortas of spontaneously hypertensive rats (SHRs) (Yang et al., 2002) and femoral arteries of streptozotocin-induced type I diabetes (Shi et al., 2007). Besides, oxygen-derived free radicals can facilitate endothelium-dependent contractions in the aortas of SHRs and rabbits (Yang et al., 2002; Auch-Schwelk et al., 1989; Tesfamariam et al 1992) and the femoral arteries of experimentally induced diabetic rats (Shi et al., 2007).

Nevertheless, it is basically unknown whether arachidonic acid derivatives and ROS act independently of each other as EDCFs or they can actually interact and control the expression and activity of the downstream enzymes such as COXs

to release most probable EDCF(s) in hypertension. The present study aimed to identify whether endothelium dependent contractions could be induced in the intrarenal arteries of the 2-kidney 2-clip rat model of renovascular hypertension. I have delineated a sequence of events leading to the release of $PGF_{2\alpha}$ as the major EDCF and elucidated a critical role of COX-2 in the induction of endothelium-dependent contractions and impaired endothelium-dependent dilatations in rat renal arteries.

5.2 Methods and Materials

5.2.1 Animals and surgical procedure

The experimental protocol was approved by the Ethical Committee for Animal Research, Chinese University of Hong Kong. Renovascular hypertension rats (RHR) were induced as described previously (Zeng et al., 1998). In brief, male Sprague-Dawley rats (80-100 g) were anaesthetized with a mixture of 35 mg/kg ketamine and 7 mg/kg xylazine and a midline laparotomy was performed. Two silver clips with an internal diameter of 0.3 mm were then placed around the root of left and right renal arteries. The sham-operated normotensive rats were subjected to laparotomy and renal artery separation only. All rats had free access to an ordinary rat chow diet and tap water and kept on a 12-hour light/dark cycle at room temperature (~22-24 °C).

5.2.2 Blood pressure measurement

Systolic blood pressure was measured weekly for consecutive 10 weeks by a tail-cuff method in conscious rats before and after renal artery constriction.

5.2.3 Chronic treatment with celecoxib or valsartan

Five weeks after renal artery stenosis, RHR with blood pressure over 180 mmHg were randomly divided into three groups and subjected to oral administration of celecoxib (10 mg/kg/day; specific COX-2 inhibitor), valsartan (10 mg/kg/day; angiotensin type-1 receptor blocker); or vehicle solvent control for 5 weeks. The sham-operated rats were also treated with celecoxib at the same duration of time.

5.2.4 Wet weight of the heart and kidney

After the opening of abdominal and chest cavity, the heart and kidneys were taken out. The wet weights of heart, left and right kidneys in different groups of rats were measured.

5.2.5 Blood vessel preparation

The rats were killed by cervical dislocation. After the abdominal cavity was cut open, the kidney from both sides were removed and placed in ice-cold Krebs solution (in mmol/L): 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11 D-glucose. The intralobal renal arteries (mean diameter of ~250 µm) were dissected out, cleaned of adhering fatty tissues, and cut into two ring segments, each ~2 mm in length. Each segment was mounted in a Multi Myograph System (Danish Myo Technology, Aarhus, Denmark), and changes in isometric tension of the artery were recorded. Briefly, two steel wires (each 40 µm in diameter) were inserted through the segment's lumen and each wire was fixed to the jaws of a myograph. The organ chamber was filled with 5-mL Krebs solution and oxygenated with a mixture of 95% O_2-5% CO_2. Krebs solution in the chamber was maintained at 37°C using a built-in heat-exchanger device to give a pH value of ~7.4. Each ring was stretched initially to 3 mN, an optimal tension based on the length-tension relationship, and then allowed to stabilize at this baseline tension for 90 min before the start of each experiment. In some arteries, the endothelium was mechanically removed by rubbing the luminal surface of an artery with a small stainless steel wire. Functional removal of the endothelium was verified if the artery did not relax in response to 10 µmol/L acetylcholine.

5.2.6 Vascular reactivity

5.2.6.1 Endothelium-dependent dilatations

The first series of experiments tested the alteration of endothelium-dependent dilatations (EDR) in intralobal renal arteries of RHR. After an initial period of a 30-min equilibration in myograph chambers filled with Krebs solution at 37 °C, each ring was contracted by KCl (60 mmol/L). Two consecutive concentration-response curves to ACh (3 nmol/L - 30 µmol/L) were then constructed in the absence (control) and presence (30-min incubation) of a drug. The drugs under testing included SC560 (0.3 µmol/L; specific COX-1 inhibitor), celecoxib (3 µmol/L; specific COX-2 inhibitor), tiron (1 mmol/L; SOD mimetic) plus DETCA (100 µmol/L; ROS scavenger), or S18886 (0.1 µmol/L; TP-receptor antagonist). In some rings without

endothelium that were contracted by phenylephrine (1 µmol/L), sodium nitroprusside (SNP, 1 nmol/L - 10 µmol/L) were applied cumulatively to relax the arteries in order to test the sensitivity of vascular smooth muscle cells to NO.

5.2.6.2 Endothelium-dependent contractions (EDC)

5.2.6.2.1 Role of endothelium and NO

Renal arteries with an intact endothelium were incubated with N^G-nitro-L-arginine methyl ester (L-NAME, 100 µmol/L) for 30 min to eliminate the negative influence of NO on the vascular tone prior to cumulative applications of acetylcholine (ACh, 0.1-100 µmol/L). Endothelium-dependent nature of ACh-induced contractions was confirmed by the lack of contractile responses to ACh in intralobal renal arteries without endothelium.

5.2.6.2.2 Involvement of non-selective cation channels and extracellular calcium ions

Renal arteries with an intact endothelium were incubated with 2-aminoethoxydiphenyl borate (2-APB; 50 µmol/L), a non-selective cation channel blocker in the presence of 100 µmol/L L-NAME for 30 min prior to cumulative addition of ACh. To examine the dependency on the presence of external calcium ions, the same experiments were repeated in the L-NAME-treated endothelium-intact rings bathed in a in a Ca^{2+}-free Krebs solution.

5.2.6.2.3 Involvement of COX and thromboxane-prostanoid (TP) receptor

In the presence of L-NAME, arteries with an intact endothelium were first exposed for 30 min to indomethacin (1 µmol/L), a non-selective COX inhibitor, ACh was then added cumulatively to bathing solution. In order to distinguish which COX isoform was likely involved, both selective COX-2 inhibitors (NS-398, DuP-697 and celecoxib, 3 µmol/L each), and COX-1 inhibitors (valeryl salicyclate at 0.3 mmol/L; VAS and sc-560 at 0.3 µmol/L) were used. Cycloheximide (10 µmol/L), a protein synthesis inhibitor was employed to determine whether COX isoform-related endothelium-dependent contractions were inducible. Finally, in the presence of L-

NAME, arterial rings with an intact endothelium were treated for 30 min with the specific TP-receptor antagonist, S18886 (0.1 µmol/L) prior to cumulative addition of ACh.

5.2.6.2.4 Role of ROS

Renal arterial rings with an intact endothelium were subjected to 30-min treatment with tempol (SOD mimetic, 100 µmol/L), tiron (superoxide anion scavenger, 1 mmol/L) plus DETCA (100 µmol/L) in the presence of L-NAME before cumulative addition of ACh.

5.2.6.2.5 Specificity of inhibitors/antagonists

The specificity of individual pharmacological inhibitors tested on endothelium-dependent contractions were evaluated by examining their effects on contractions induced by the following agonists utilizing different cellular mechanisms to trigger VSMC contraction: elevated KCl (60 mmol/L, contraction by membrane depolarization), phenylephrine (1 µmol/L, contraction by activation of α1-adrenoceptor), or U46619 (100 nmol/L, contraction by activation of TP receptors). Two consecutive contractile responses were evoked by each agonist in control and in the presence of different inhibitor aforementioned. Data were expressed as a ratio of the amplitude of the second contraction over that of the first contraction.

5.2.6.3 Responses to other vasoconstrictors

Intralobal renal arteries from RHR and sham-operated control rats were examined for their responsiveness to three commonly used vasoconstrictors, phenylephrine (0.01-10 µmol/L), U46619 (1-100 nmol/L) or endothelin-1 (1-50 nmol/L) to determine whether contractive responses could be altered in hypertension. Several prostanoids including $PGF_{2\alpha}$, PGI_2 and 8-isoprostane were also tested for their potential impact on the vascular tone and for the possible altered contracting sensitivity of renal arteries to constrictive prostanoids.

5.2.6.4 Hydrogen peroxide or HX-XO-induced vasoconstrictions

Hydrogen peroxide (H_2O_2, 100 μmol/L) or a combination of hypoxanthine (HX; 100 μmol/L) and xanthine oxidase (XO; 0.01 unit/mL) used to generate superoxide anions ($O_2^{\cdot-}$) or hydroxyl free radicals ($OH^{\cdot}$) were tested on their positive effect in developing the vascular tension of intralobal renal arteries in the presence of 100 μmol/L L-NAME. In order to determine whether ROS could stimulate the activity of up-regulated COX to release constrictive prostanoids and thus causes endothelium-dependent contractions, the intralobal renal arteries with or without endothelium were incubated individually with SC560 (0.3 μmol/L), celecoxib (3 μmol/L) or S18886 (0.1 μmol/L) prior to the challenge of H_2O_2 or HX-XO. ROS-elicited vascular tension was compared among different treatment groups.

5.2.7 Western blot

Renal arteries were dissected out and were homogenized at 4°C in RIPA lysis buffer (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, 25 mmol/L sodium pyrophosphate, 1 mmol/L b-glyco-phosphate, 1 mmol/L sodium orthovanadate, 2.1 μmol/L leupeptin, 1 mg/mL aprotinin, 1 mmol/L phenyl-methylsulfonyl fluoride, and 1% Triton X-100) and incubated on ice for 10 min. The samples were then centrifuged at 20000×g for 20 min at 4°C and the supernatant was collected. Protein concentrations were determined using the Lowry method (Bio-rad). The protein samples were electrophoresed on a 10% SDS-poly-acrylamide gel and transferred onto an immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore). Nonspecific binding sites were blocked by 5% non-fat milk in 0.05% Tween-20 phosphate-buffered saline (PBST), and then incubated overnight at 4°C with primary antibodies including anti-COX-2 (1:1000) or anti-COX-1 (1:1000) (Cayman). The blots were incubated with secondary antibodies at 1:3000 dilutions for 1 h at room temperature, and then washed 3 times for 10 min in PBST. The membranes were then developed with an enhanced chemiluminescence detection system (ECL reagents, Amersham Pharmacia), and finally exposed to X-ray films. Equal protein loading was verified with use of a housekeeping anti-GAPDH antibody (Ambion, Inc).

5.2.8 Electron paramagnetic resonance (EPR) spectroscopy

The formation of ROS was measured by a reaction with the spin trap TEMPONE-H using EPR spectroscopy, using the method adopted from Dikalov et al., 1998. DTPA was used to decrease the self-oxidation of hydroxylamine catalyzed by traces of transition metal ions. Renal arteries were dissected from RHR or sham-operated rats, and incubated in DTPA (100 µmol/L) and TEMPONE-H (80 µmol/L) at 37°C in oxygenated Krebs solution for 30 min. Acetylcholine was added for 5 min to trigger the release of ROS in the presence of L-NAME. Tissues together with 200 µL incubation medium were collected, homogenized, and stored in glass Pasteur pipettes (tip inner diameter: 1 mm) at -80°C. The samples were thawed in room temperature before the start of the measurement. EPR spectra were recorded at room temperature using a Bruker EMX EPR spectrometer (Bruker, Karlsruhe, Germany). The EPR settings were as follows: field center, 3475 G; field sweep, 60 G; microwave frequency, 9.72 GHz; microwave power, 20 mW; magnetic field modulation, 100 kHz; modulation amplitude, 2.0 G; conversion time, 655 msec; detector time constant, 1024 msec; and acquisition of ten scans. The generation of ROS by chemical interaction between xanthine oxidase (XO, 0.01 U/mL) and hypoxanthine (HX, 100 µmol/L) (XO-HX) was taken as the positive control. A mixture of Krebs solution containing DTPA and TEMPONE-H served as the negative control. TEMPONE-H were dissolved in oxygen-free (Argon bubbled) double-distilled water.

5.2.9 Measurement of prostanoids by enzyme immunoassay

The levels of arachidonic acid-derived prostanoids were measured by EIA kits (Cayman Chemical, Ann Arbor, MI, USA) according to the instruction of the manufacturer. Rat renal arteries were exposed to 100 µmol/L ACh for 5 min in a 500-µL bathing Krebs solution with and without acute drug treatment and the solution was then collected and kept at -80°C until later processing; The six prostanoids or their metabolites, $PGF_{2\alpha}$, PGE_2, PGD_2, 6-keto $PGF_{1\alpha}$ (for PGI_2) and TXB_2 (for TXA_2) and 8-isoprostane, were assayed.

5.2.10 Chemicals and drugs

Acetylcholine, indomethacin, L-arginine, N^G-nitro-L-arginine methyl ester (L-NAME), phenylephrine, tiron, tempol, diethyldithiocarbamate acid (DETCA), sodium nitroprusside (SNP) and U46619 (9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$) were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). Cycloheximide, 2-aminoethoxydiphenyl borate (2-APB) was purchased from Calbiochem, EMD Biosciences (La Jolla, CA, USA). Valeryl salicylate (VAS), $PGF_{2\alpha}$ and PGI_2 were from Cayman Chemical (Ann Arbor, MI, USA). Endothelin-1, 5-bromo-2-(4-fluorophenyl)-3-[4-(methylsulfonyl)phenyl]-thiophene (DuP-697) and N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS-398) and 8-isoprostane were from Tocris (Avonmouth, UK). sc-560 and 3-[(6-amino- (4-chlorobenzensulphonyl)-2-methyl- 5,6,7,8- tetrahydronapht] -1 -yl) propionic acid (S18886) were kind gifts from Institut de Recherches Servier (Suresnes, France). Except acetylcholine, L-NAME, phenylephrine, endothelin-1, SNP, PGI_2, tiron, tempol, DETCA, and S18886 that were prepared in water, all other drugs were dissolved in DMSO (Sigma-Aldrich).

5.2.11 Statistical analysis

Results are mean ± standard error of the mean (SEM) of n experiments. Tontractions were expressed as active tension [force recorded/(2×length of ring)]. Specificity of inhibitors/antagonists was determined by the percentage of the second contraction (in the presence of the inhibitor/antagonist) with respect to the first contraction induced by elevated extracellular KCl, phenylephrine or U46619. The protein expression was normalized to the level of GAPDH and then expressed in comparison with the control value. Student's t-test (unpaired two-tailed) was used and concentration-response curves were analyzed by two-way ANOVA followed by Bonferroni post-tests. Levels of probabilities of less than 0.05 were regarded as statistically significant between groups.

5.3 Results

5.3.1 Impaired endothelium-dependent dilatations in RHR

Representative traces in **Figure 5.1A** show that acetylcholine (ACh)-induced endothelium-dependent dilatations were significantly blunted in intralobal renal arteries of RHR when compared with those in the sham-operated control rats. The renal arteries of RHR rats exhibit a biphasic response to ACh, initial relaxations by ACh at concentrations lower than 1 μmol/L followed by contractions by ACh at concentrations greater than 1 μmol/L **(Figure 5.1A&B)**. By contrast, the level of endothelium-independent dilatations to sodium nitroprusside (SNP) was comparable between the two groups of rats **(Figure 5.1C)**, suggesting that the relaxing sensitivity of VSMCs to exogenous NO is unaffected following the induction of renovascular hypertension. Thirty-minute pre-exposure of RHR renal arteries to tiron plus DETCA **(Figure 5.2A)**, S18886 **(Figure 5.2B)**, or celecoxib **(Figure 5.2C)** profoundly improved the ACh-induced endothelium-dependent dilatations. But the selective inhibition of COX-1 by SC560 did not restore the impaired endothelial function in RHR renal arteries **(Figure 5.2D)**.

5.3.2 Augmented endothelium-dependent contractions (EDC) in RHR

Representative traces in **Figure 5.3A** clearly show that ACh elicited marked increases in the tension of RHR intralobal renal arteries in the presence of L-NAME but failed to cause any contraction in renal arteries from sham-operated control rats. The amplitude of ACh-induced contraction in RHR renal arteries approximated the contraction produced by 60 mmol/L KCl (data not shown). The level of the augmented contractions to ACh depended on the time after introduction of renal artery stenosis; vascular contractions were much greater at 10-week RHR than 5-week RHR **(Figure 5.3B)**. The characteristic features of the augmented ACh-elicited contractions in RHR renal arteries were described in the following sub-sections:

5.3.2.1 Endothelium-dependent contractions require extracellular Ca^{2+}

ACh-elicited contractions were recorded in RHR intralobal renal arteries with an intact endothelium only in the presence of L-NAME, with a maximum contraction of 2.13 ± 0.38 mN/mm in response to 100 µmol/L ACh **(Figure 5.4A)**, indicating that endothelium-derived NO had masked the visualization of endothelium-dependent contractions even under a hypertensive condition. The mechanical removal of the endothelium prevented the ACh-induced contractions **(Figure 5.4B)**, thus confirming the dependency of ACh-induced contractions on the presence of a functional endothelium. The appearance of endothelium-dependent contractions in response to ACh relied entirely on the presence of extracellular calcium ions as the evoked contractions were absent in RHR renal arteries bathed in a calcium-free Krebs solution **(Figure 5.4C)**. Acute 30-min treatment with 2-APB abolished ACh-induced endothelium-dependent contractions of RHR renal arteries bathed in normal calcium-containing Krebs solution **(Figure 5.4D)**. By contrast, 2-APB at 50 µmol/L did not affect contraction induced by 60 mmol/L KCL, phenylephrine, or U46619 **(Figure 5.6B)**.

5.3.2.2 COX-2 mediated endothelium-dependent contraction through acting on the TP receptor

Endothelium-dependent contractions were eliminated by acute exposure to the non-selective COX inhibitor, indomethacin **(Figure 5.5A)**, supporting a positive role of COX. Further examination showed that three structurally distinct and specific inhibitors of COX-2, NS-398, DuP-697 and celecoxib, abolished ACh-induced endothelium-dependent contractions in RHR renal arteries **(Figure 5.5B)**. The specificity of indomethacin and COX-2 inhibitors were confirmed by the lack of inhibitory responses on contractions evoked by 60 mmol/L KCl **(Fig 5.6B-E)**, phenylephrine or U46619. In contrast, COX-1 selective inhibitors (VAS or sc-560) failed to inhibit the contraction responses **(Figure 5.5C&D)**. The acetylcholine-elicited endothelium-dependent contractions were also unaffected by treatment with cycloheximide, the protein synthesis inhibitor **(Figure 5.5E)**. The TP receptor antagonist S18886 abolished the ACh-induced endothelium-dependent

contractions **(Figure 5.5F)** without affecting contractions induced by 60 mmol/L KCl or phenylephrine **(Figure 5.6F)**.

5.3.2.3 ROS-mediated endothelium-dependent contractions

In RHR intralobal renal arteries, endothelium-dependent contractions were attenuated by 30-min incubation with tempol **(Figure 5.7A)** or with a combination of tiron and DETCA **(Figure 5.7B)**. Results obtained from EPR spectroscopy confirmed an increased generation of superoxide anions during the time course of endothelium-dependent contractions in RHR renal arteries **(Figure 5.7C&D)**. Exogenously applied H_2O_2 and HX-XO induced a greater contraction in RHR intralobal renal arteries (3.43±0.56 mN/mm for H_2O_2 and 2.87±0.66 mN/mm for HX-XO) when compared to sham-operated control rats (1.09±0.13 mN/mm for H_2O_2 and 0.11±0.03 mN/mm for HX-XO) **(Figure 5.8A&B)**, which was comparable to the endothelium-dependent contraction of 2.99±0.24 mN/mm induced by 100 µmol/L ACh **(Figure 5.3B)**. Celecoxib and S18886 prevented ROS-induced contractions in RHR intralobal renal arteries while sc-560 had no effect **(Figure 5.8A&B)**. In addition, endothelium denuation markedly reduced the level of contraction elicited by H_2O_2 and HX-XO.

5.3.2.4 Agonist-induced vasoconstriction

Despite both ACh-induced endothelium-dependent contractions and ROS-mediated contractions were markedly augmented in RHR intralobal renal arteries, the contractile responses to other vasoconstrictors, such as phenylephrine, U46619 and endothelin-1 in the renal arteries were similar between hypertensive and normotensive rats **(Figure 5.9A-C)**.

5.3.3 Chronic treatment of celecoxib improves endothelial function in RHR renal arteries

5.3.3.1 Improvement of endothelium-dependent dilatations in celecoxib- treated RHR

Representative traces in **Figure 5.10A** show clearly that the impaired ACh-induced endothelium-dependent dilatations in RHR intralobal renal arteries were prevented

by the chronic five-week treatment with either celecoxib or valsartan. The impairment of endothelium-dependent dilatations appeared to be time-dependent as ACh-induced vasodilatations were deteriorated progressively from 5-week to 10-week after induction of renovascular hypertension **(Figure 5.10B**). Chronic celecoxib treatment not only improved the ACh-induced dilatations but also eliminated contractions in response to ACh at concentrations greater than 1 μmol/L **(Figure 5.10A&C)**. Likewise, valsartan, serving as a positive control, produced almost the same protective effect as celecoxib in preserving endothelium-dependent dilatations in RHR intralobal renal arteries **(Figure 5.10D)**.

5.3.3.2 Abolition of endothelium-dependent contractions in celecoxib-treated RHR

Endothelium-dependent contractions were also examined in celecoxib- or valsartan-treated rats. ACh-induced endothelium-dependent contractions were significantly greater in intralobal renal arteries in RHR as compared with their time-matched sham-operated control rats **(Figure 5.11A)**. Chronic oral administration of celecoxib to both RHR **(Figure 5.11C)** and sham control rats **(Figure 5.11B)** resulted in absence of endothelium-dependent contractions in response to ACh in L-NAME-treated renal arteries. Similarly, chronic valsartan treatment also prevented the endothelium-dependent contractions in RHR intralobal renal arteries **(Figure 5.11D)**.

5.3.4 Increased COX-2, but not COX-1 expression in RHR renal arteries

The protein level of COX-2 in RHR renal arteries with endothelium was significantly higher than those of normotensive control rats **(Figure 5.12A&B)**. Chronic oral treatment of celecoxib to RHR did not alter the enhanced expression of COX-2 expression **(Figure 5.12A&B)**, but chronic valsartan treatment significantly inhibited the COX-2 up-regulation **(Figure 5.12A&B)**. By contrast, the protein levels of COX-1 were comparable in renal arteries between RHR and control rats

and chronic therapy with either celecoxib or valsartan treatment did not alter the COX-1 expression in RHR renal arteries **(Figure 5.12C)**.

5.3.5 Increased release of prostaglandins in RHR renal arteries

The level of six arachidonic acid metabolites, $PGF_{2\alpha}$, PGE_2, PGD_2, PGI_2, TXA_2, and 8-isoprostane that were released by renal arteries into the bathing solution, was assayed by ELISA kits. ACh at 100 μmol/L caused a significantly greater release of prostanoids, namely $PGF_{2\alpha}$, PGE_2, PGD_2, PGI_2 (detected as 6-keto $PGF_{1\alpha}$), TXA_2 (detected as TXB_2), and 8-isoprostane, in RHR renal arteries compared to normotensive controls **(Figure 5.13 & Figure 5.14)**. The order for the released amount of each prostanoid followed a descending order as: PGI_2 > $PGF_{2\alpha}$ > PGE_2 > PGD_2 > TXB_2 > 8-isoprostane. Chronic celecoxib treatment significantly reduced the release of prostanoid $PGF_{2\alpha}$, PGE_2, PGD_2, and 8-isoprostane **(Figure 5.14B,C,D&F)** but not 6-keto $PGF_{1\alpha}$ or TXB_2 in renal arteries. Valsartan treatment, however, significantly reduced all the prostanoids measured in RHR renal arteries. Acute 30-min exposure of RHR renal arteries to the combination of tiron and DETCA, or to celecoxib also prevented the acetylcholine-stimulated rise of several prostanoids in RHR renal arteries **(Figure 5.14 A-F)**. On the contrary, sc-560 was only able to reduce the release of PGE_2, PGD_2 and TXB_2 **(Figure 5.14C-E)** but had no effect on 6-keto $PGF_{1\alpha}$, $PGF_{2\alpha}$ or 8-isoprostane **(Figure 5.14A,B&F)**.

5.3.6 $PGF_{2\alpha}$ serves as an EDCF

Since ACh-elicited endothelium-dependent contractions in RHR intralobal renal arteries were only inhibited by COX-2 inhibitors but not COX-1 inhibitors **(Figure 5.5)**; and the prostanoid ELISA assay demonstrated that celecoxib but not sc-560 reduced the release of 6-keto $PGF_{1\alpha}$, $PGF_{2\alpha}$ or 8-isoprostane **(Figure 5.14A,B&F)**, suggesting they might serve as possible candidates of EDCF. Importantly, hydrogen peroxide also stimulated the release of $PGF_{2\alpha}$ in RHR renal arteries which was inhibited by celecoxib (COX-2 inhibitor) but not by sc-560 (COX-1 inhibitor). To confirm this, exogenous PGI_2, $PGF_{2\alpha}$ or 8-isoprostane were applied

to see whether they were able to evoke contractions in L-NAME-treated intralobal renal arteries and whether there was a difference in the contracting sensitivity to these prostanoids. Indeed, PGI_2, $PGF_{2\alpha}$ or 8-isoprostane produced concentration-dependent contractions in both RHR and control intralobal renal arteries. It is clear that PGI_2 produced an identical constrictive effect in renal arteries from both groups of rats but $PGF_{2\alpha}$ or 8-isoprostane were more effective in contracting RHR than control renal arteries **(Figure 5.17B&C)**. $PGF_{2\alpha}$ at ~38.1 nmol/L the equivalent amount released by RHR renal arteries as measured by ELISA, contracted RHR renal arteries to a magnitude of force ~2.1 mN/mm **(Figure 5.17B)**, which was similar to the endothelium-dependent contraction produced by 100 µmol/L ACh. By contrast, PGI_2 and 8-isoprostane at respective amount as determined by ELISA, i.e. ~0.38 µmol/L and ~2.12 nmol/L, did not cause obvious contractions in RHR intralobal renal arteries. In addition, PGI_2 and PGE_2 did not relax intralobal renal arteries that were precontracted by phenylephrine in either RHR or sham-operated control rats (data not shown). Finally PGE_2 did not evoke a contraction in intralobal renal arteries. Like the inhibitor effect of TP-receptor antagonist S18886 on ACh-induced endothelium-dependent contractions, S18886 prevented contractions induced by these prostanoids (PGI_2, $PGF_{2\alpha}$, or 8-isoprostane) (data not shown). To further elucidate the role of $PGF_{2\alpha}$ on endothelial function, acetylcholine-induced endothelium-dependent relaxations in renal arteries were impaired by 7 nmol/L $PGF_{2\alpha}$ which were reversed by S18886 (TP receptor antagonist) **(Figure 5.16A)**; while unaffecting the SNP-induced relaxations **(Figure 5.16B)**.

5.3.7 Basic parameters and blood pressure alteration following drug treatments

RHR had the similar averaged body weight as sham-operated control rats. Chronic treatment with either celecoxib or valsartan did not modify the body weight of RHR **(Figure 5.18A)**. The ratio of heart weight over body weight (expressed in % HW/BW) was significantly higher in RHR than in sham control and this value was significantly reduced by celecoxib or valsartan treatment in RHR **(Figure 5.18B)**.

On the other hand, the weight of the left and right kidney did not change among all four groups of rats when expressed as percentage of the body weight **(Figure 5.18C&D)**. Systolic blood pressure increased gradually immediately after surgical stenosis of renal arteries and reached a plateau (~200 mmHg) at week five, the time at which drug treatment was initiated. As shown in **Figure 5.19**, treatment with celecoxib or valsartan exerted a rapid blood pressure–lowering effect one week after drug therapy and such effect maintained during the remaining duration of drug treatment. It is clear that valsartan was more effective than celecoxib.

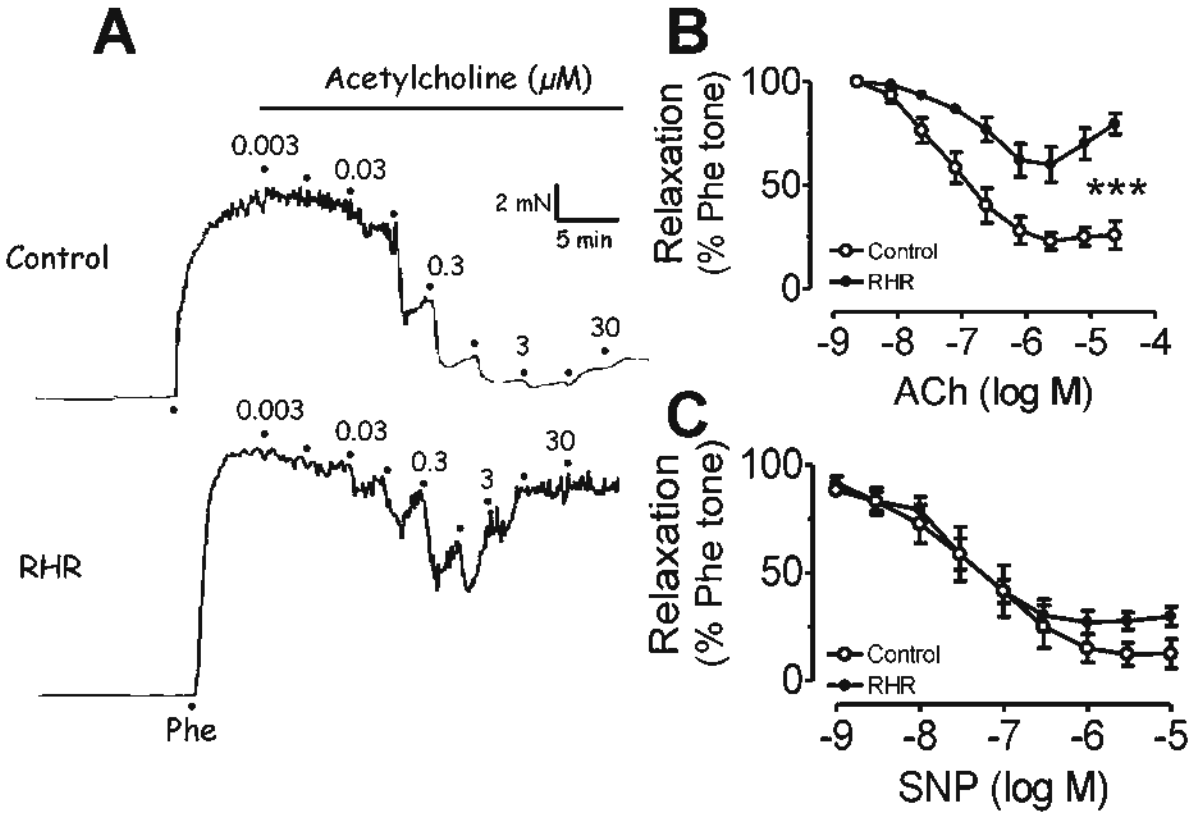


Figure 5.1.
(A) Representative traces showing blunted acetylcholine (ACh)-induced endothelium-dependent dilatations of intralobal renal arteries of (renovascular hypertensive) RHR compared to those of sham-operated control rats. Concentration-response curves for dilatations induced by ACh (**B**) or sodium nitroprusside (SNP) (**C**) in RHR and control rat intralobal renal arteries. **(C)**. Results are mean ± SEM of 8-12 experiments. Statistical significance between control and RHR groups is indicated by ***p<0.001.

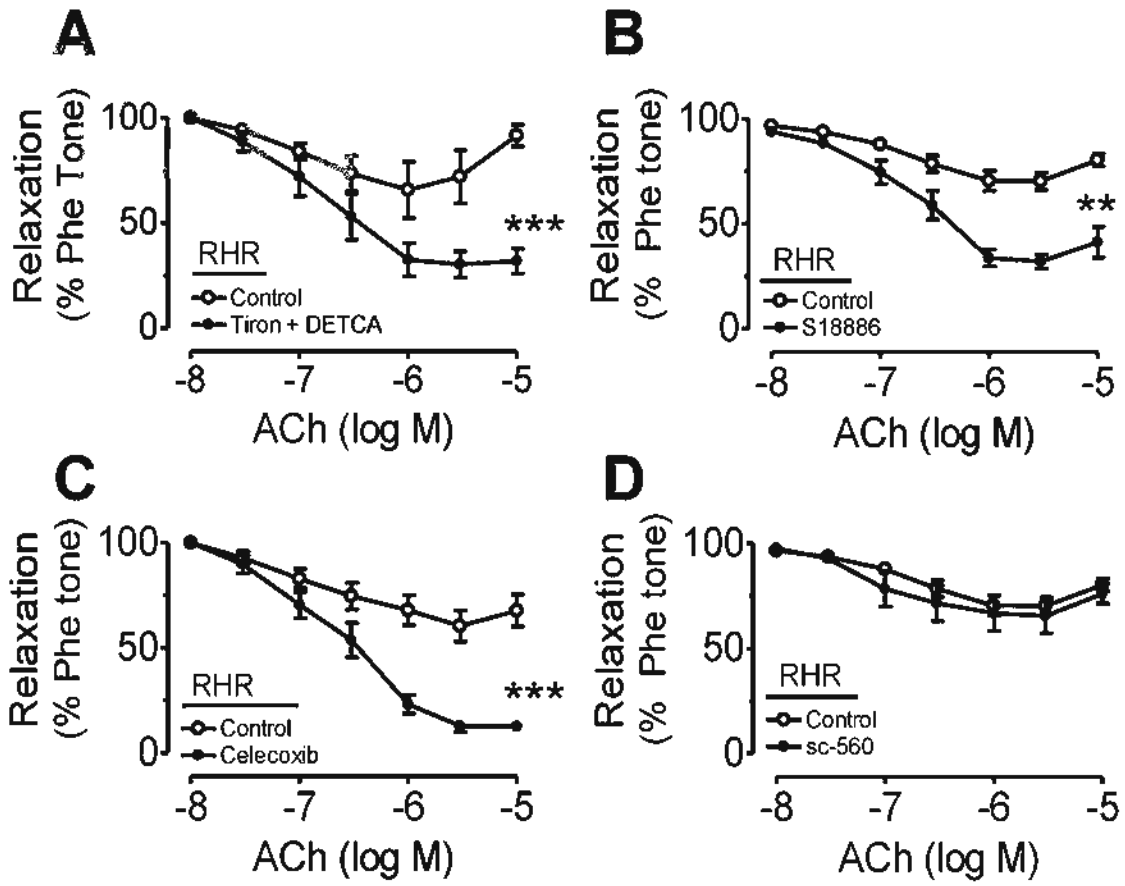


Figure 5.2.
Effects of acute 30-min exposure to (**A**) 1 mmol/L Tiron plus 100 µmol/L DETCA (Tiron + DETCA); (**B**) 0.1 µmol/L S18886; (**C**) 3 µmol/L celecoxib; and **(D)** 0.3 µmol/L sc-560 on ACh-induced endothelium-dependent dilatations in RHR intralobal renal arteries. Results are mean ± SEM of 6-8 experiments. Statistical significances between control and treatment groups are indicated by *** $p<0.001$ or ** $p<0.01$.

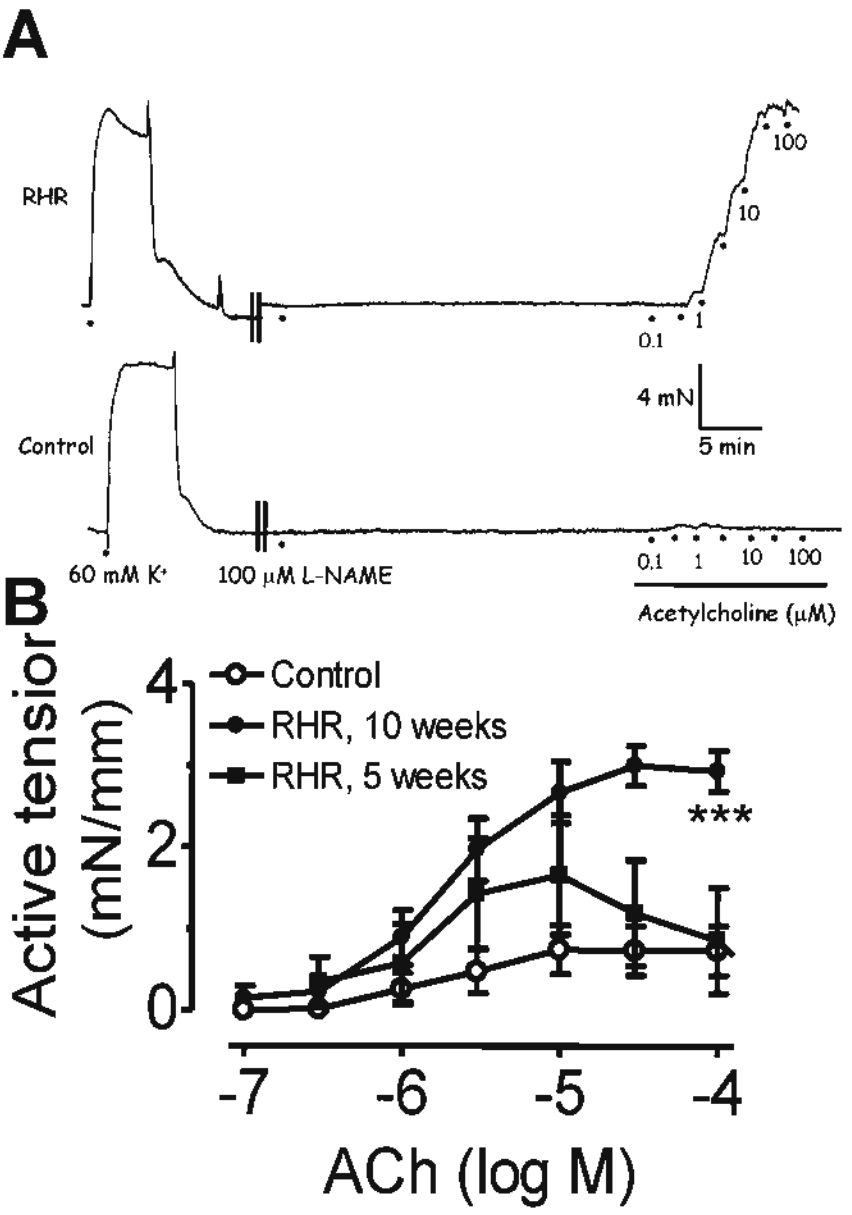


Figure 5.3.
(A) Representative traces showing augmented ACh-induced endothelium-dependent contractions in RHR intralobal renal arteries compared with those from sham-operated control rats. **(B)** Concentration-response curves for ACh-induced contractions in the presence of 100 µmol/L in intralobal renal arteries from 5-week RHR, 10-week RHR and time-matched (10-week) sham-operated control rats. Results are mean ± SEM of 8-12 experiments. Statistical significance between groups is indicated by *** $p<0.001$.

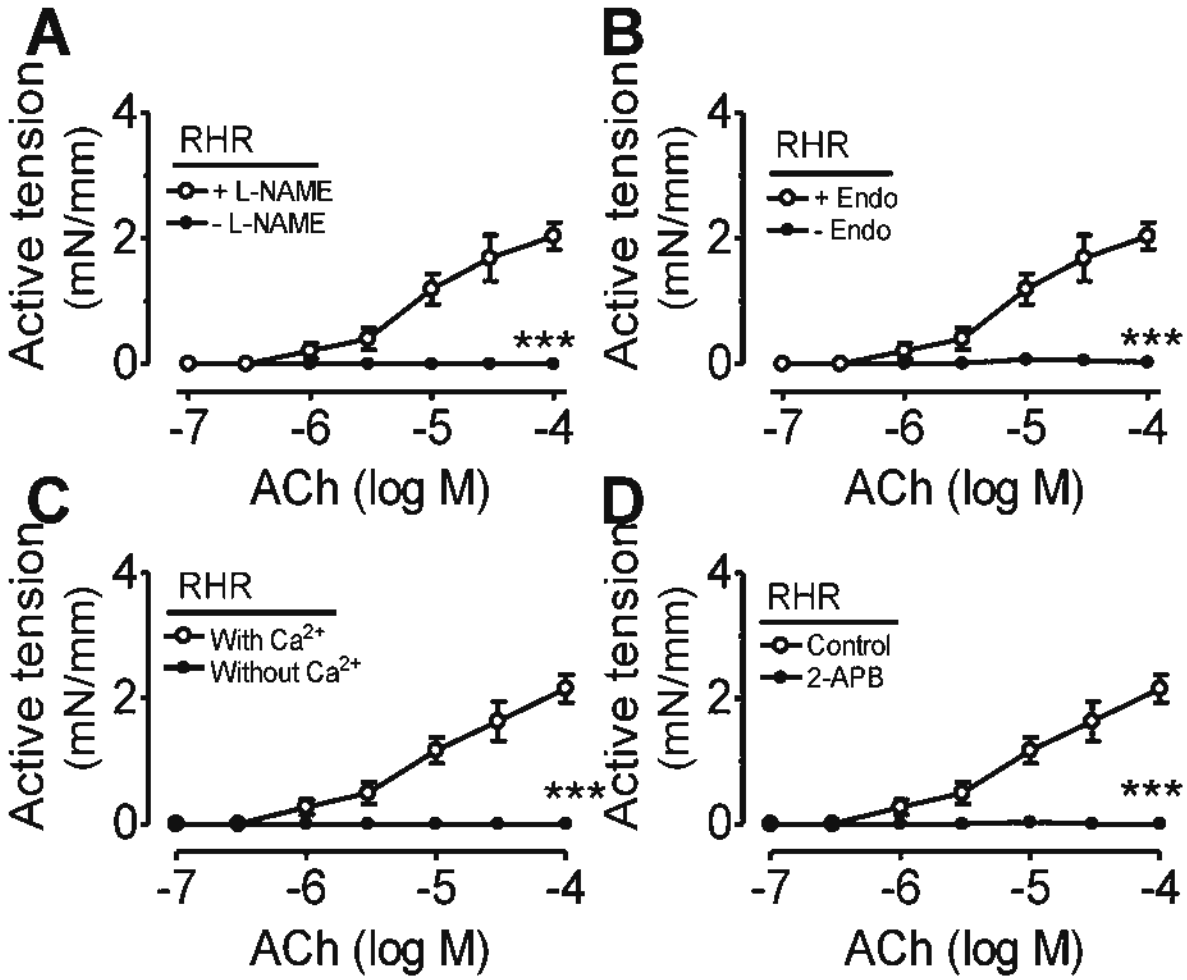


Figure 5.4.
Concentration-dependent contractions evoked by ACh in RHR intralobal renal arteries occurring only in the presence of 100 µmol/L L-NAME **(A)**; with endothelium **(B)**; with the presence of extracellular calcium ions **(C)**; and eliminated by 50 µmol/L 2-APB (a non-selective cation channel blocker) **(D)**. Results are mean ± SEM of 4-6 experiments. Statistical significance between control and treatment groups is indicated by *** $p<0.001$.

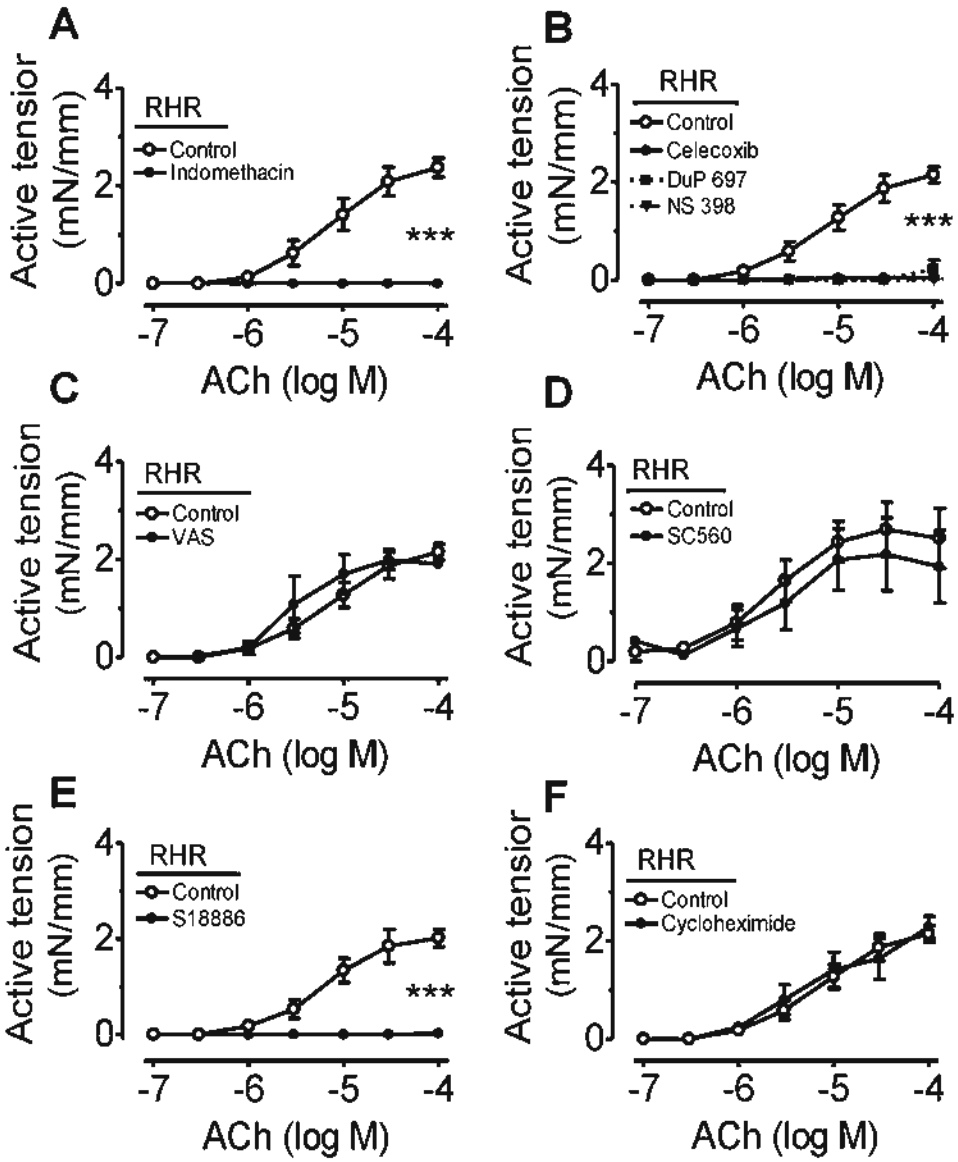


Figure 5.5.
Effects of acute 30-min exposure to **(A)** non-selective COX inhibitor, 1 μmol/L indomethacin; **(B)** three structurally different COX-2 inhibitors (celecoxib, DuP697 and NS398, each at 3 μmol/L); selective COX-1 inhibitors **(C)** 0.3 mmol/L valeryl salicyclate and **(D)** 0.3 μmol/L sc-560; **(E)** TP-receptor antagonist, 0.1 μmol/L S18886; and **(F)** protein synthesis inhibitor, 10 μmol/L cycloheximide on ACh-elicited endothelium-dependent contractions in RHR intralobal renal arteries in the presence of L-NAME. Results are mean ± SEM of 6-8 experiments. Statistical significance between control and treatment groups is indicated by *** $p<0.001$.

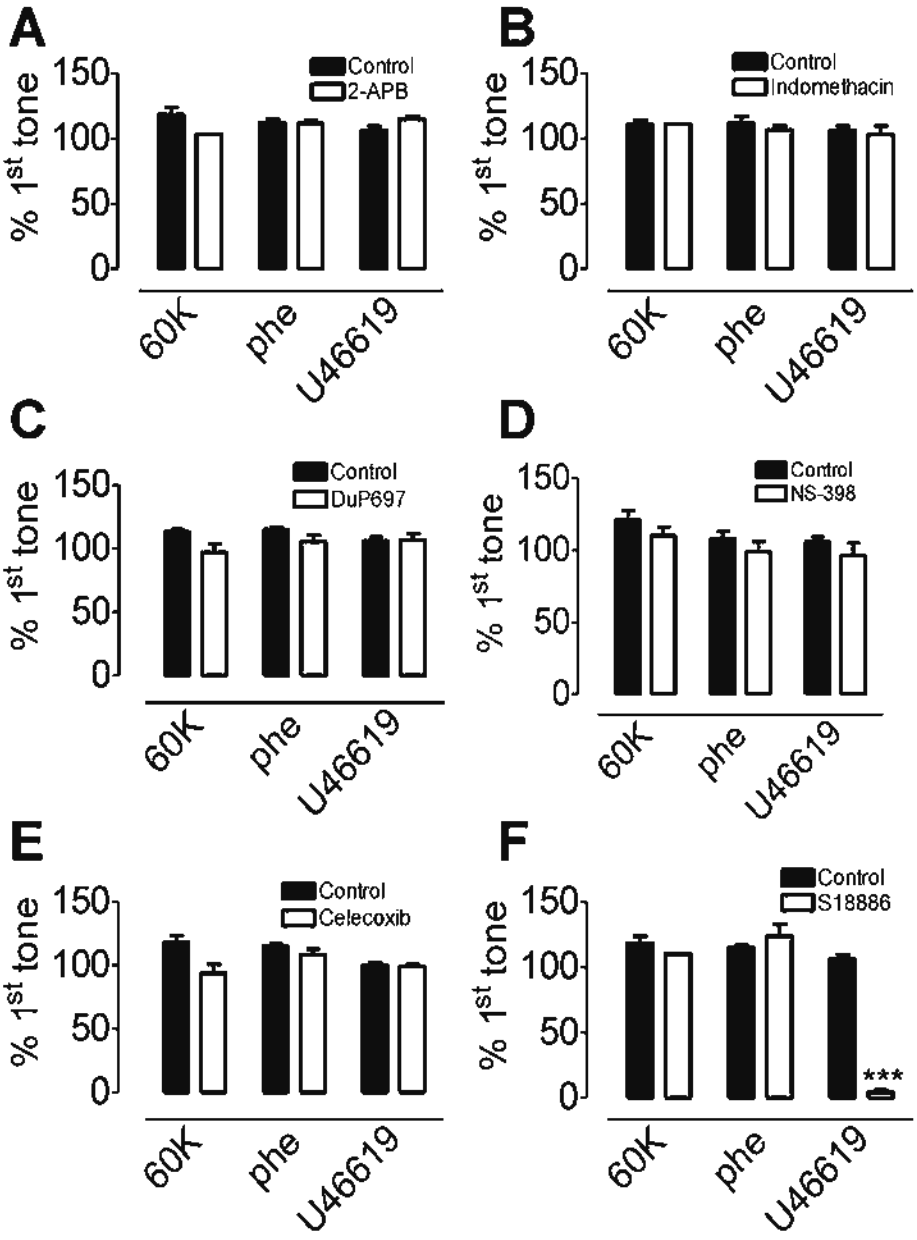


Figure 5.6.
Specificity test for effects of 2-APB **(A)**, 1 µmol/L indomethacin **(B)**, 3 µmol/L DuP697 **(C)**, 3 µmol/L NS398 **(D)**, 3 µmol/L celecoxib **(E)**, and 0.1 µmol/L S18886 **(F)** on contraction elicited by 60 mmol/L KCl, phenylephrine (Phe)-, and U46619 in control intralobal renal arteries. Results are mean ± SEM of 4-6 experiments. Statistical significance between control and treatment groups is indicated by *** $p<0.001$.

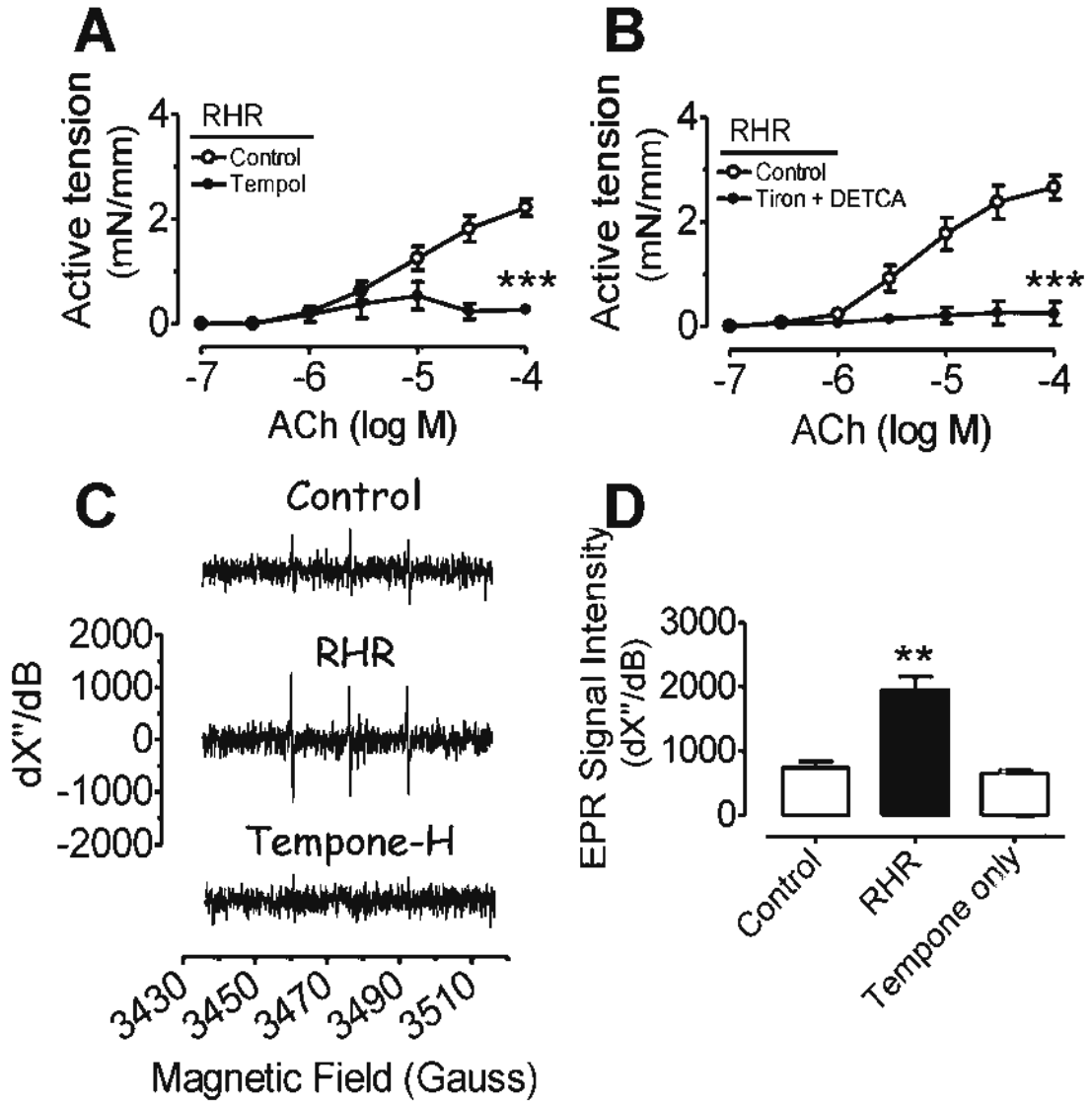


Figure 5.7.
The inhibitory effects of **(A)** SOD mimetic, 100 µmol/L tempol; and **(B)** a combined treatment with 1 mmol/L tiron plus 100 µmol/L DETCA on the ACh-induced endothelium-dependent contractions in RHR intralobal renal arteries. **(C)** Representative EPR spectroscopy recordings showing a markedly increased amplitude of ROS signal in response to 100 µmol/L ACh in RHR renal arteries and the summarized values **(D)**. Results are mean ± SEM of 4-6 experiments. Statistical significance between control and treatment groups is indicated by *** $p<0.001$ and ** $p<0.01$.

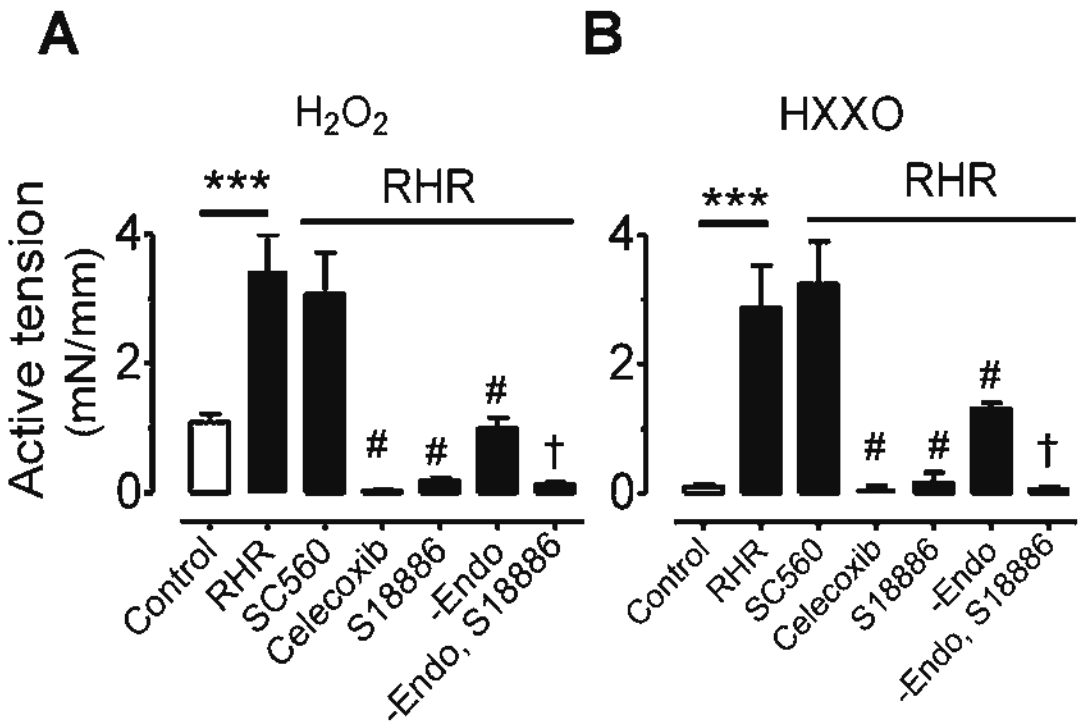


Figure 5.8. (A) H_2O_2- and **(B)** HX-XO-generated contraction in intralobal renal arteries from RHR and control rats. Effects of 0.3 µmol/L sc-560, 3 µmol/L celecoxib, 0.1 µmol/L S18886 and removal of the endothelium on contractions evoked by **(A)** 100 µmol/L H2O2 and **(B)** HX-XO (100 µmol/L hypoxanthine plus 0.01 units/ml xanthine oxidase). Results are mean ± SEM of 5-8 experiments. Statistical significance between groups is indicated by *** $p<0.001$; # $p<0.001$ vs RHR; † $p<0.01$ vs RHR (-Endo). – Endo denotes arteries without endothelium.

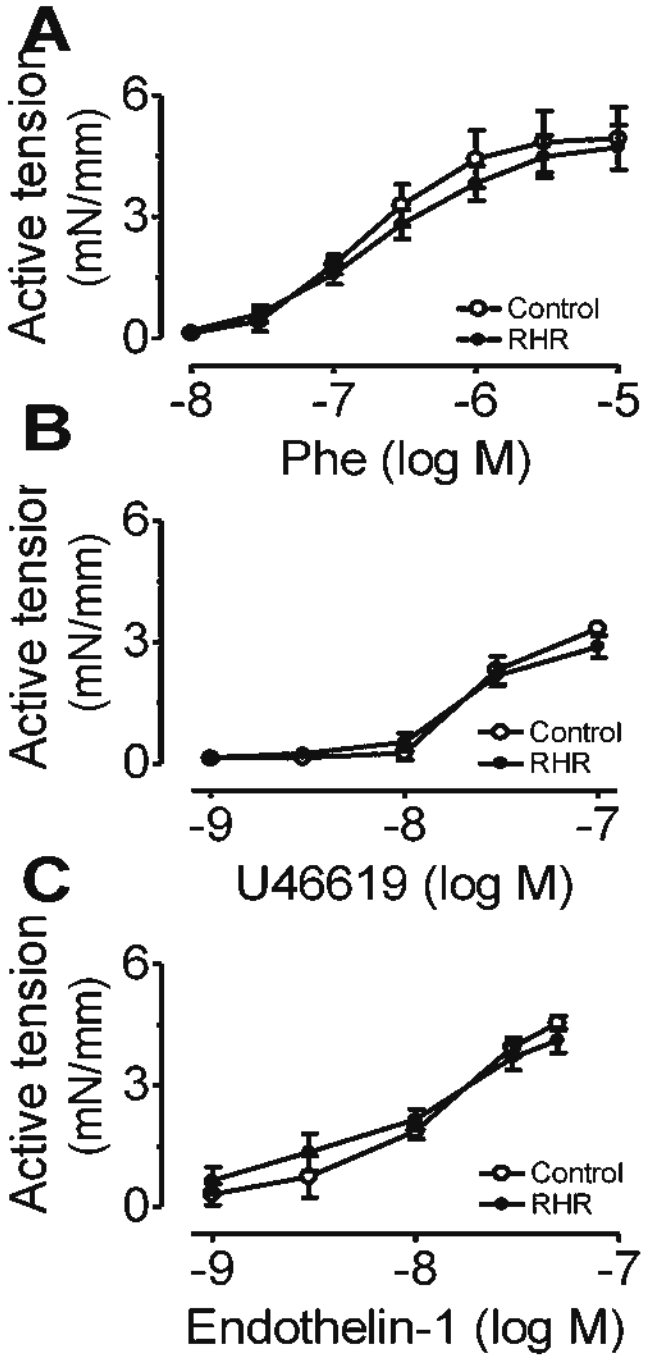


Figure 5.9.
(A) phenylephrine (Phe), **(B)** U46619 and **(C)** endothelin-1 elicited comparable levels of contractions in intralobal renal arteries between RHR and sham control rats. Results are mean ± SEM of 6-8 experiments.

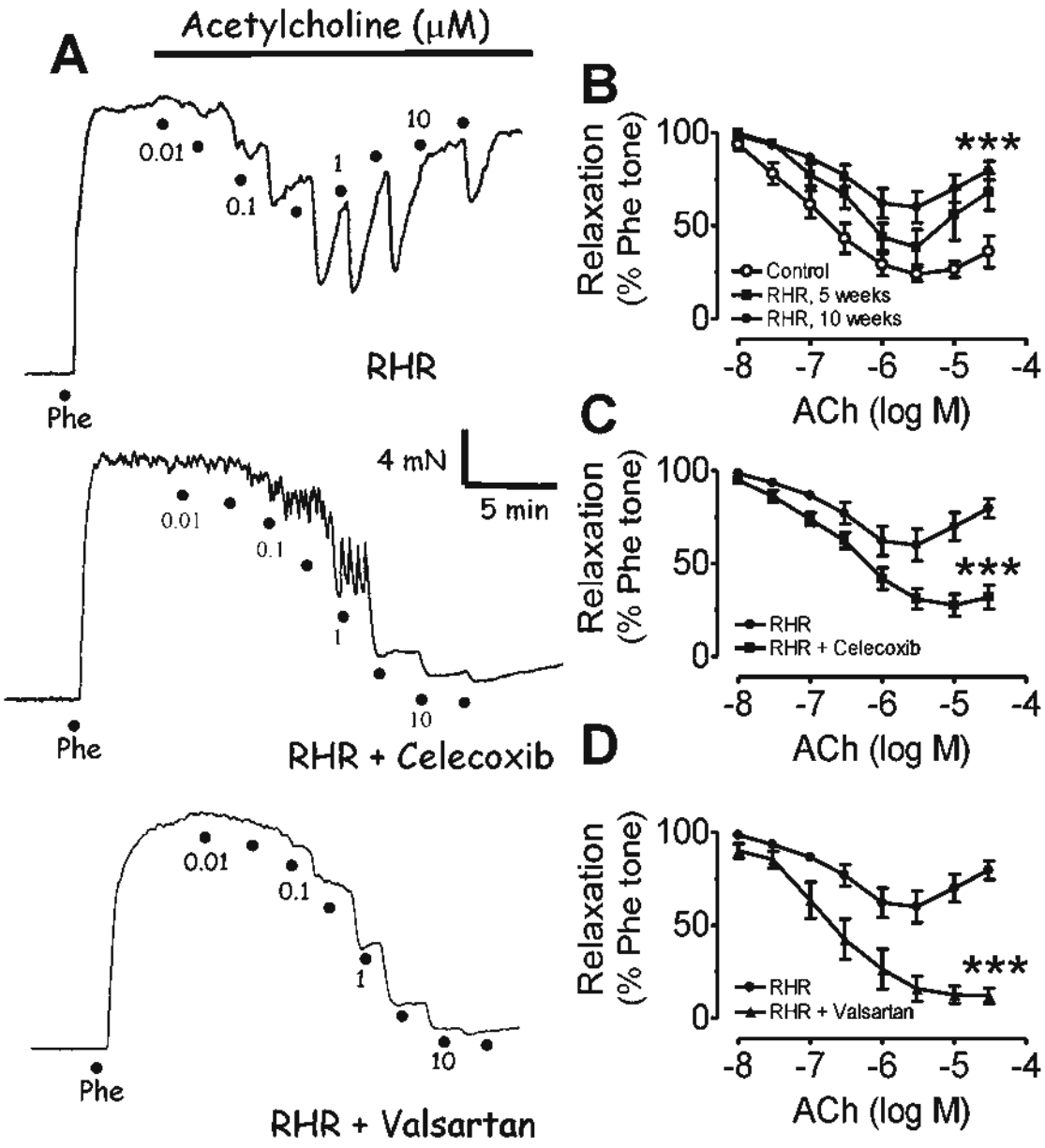


Figure 5.10.
(A) Representative traces showing the impaired ACh-induced endothelium-dependent dilatations were prevented by chronic five-week treatment with celecoxib or valsartan. Concentration-response curves for ACh-induced dilatations in RHR treated by **(C)** celecoxib and **(D)** valsartan. **(B)** Time-dependent impairment of ACh-induced vasodilatations in 5-week and 10-week RHR. Results are mean ± SEM of 6-8 experiments. Statistical significance between control and treatment groups is indicated by *** $p<0.001$.

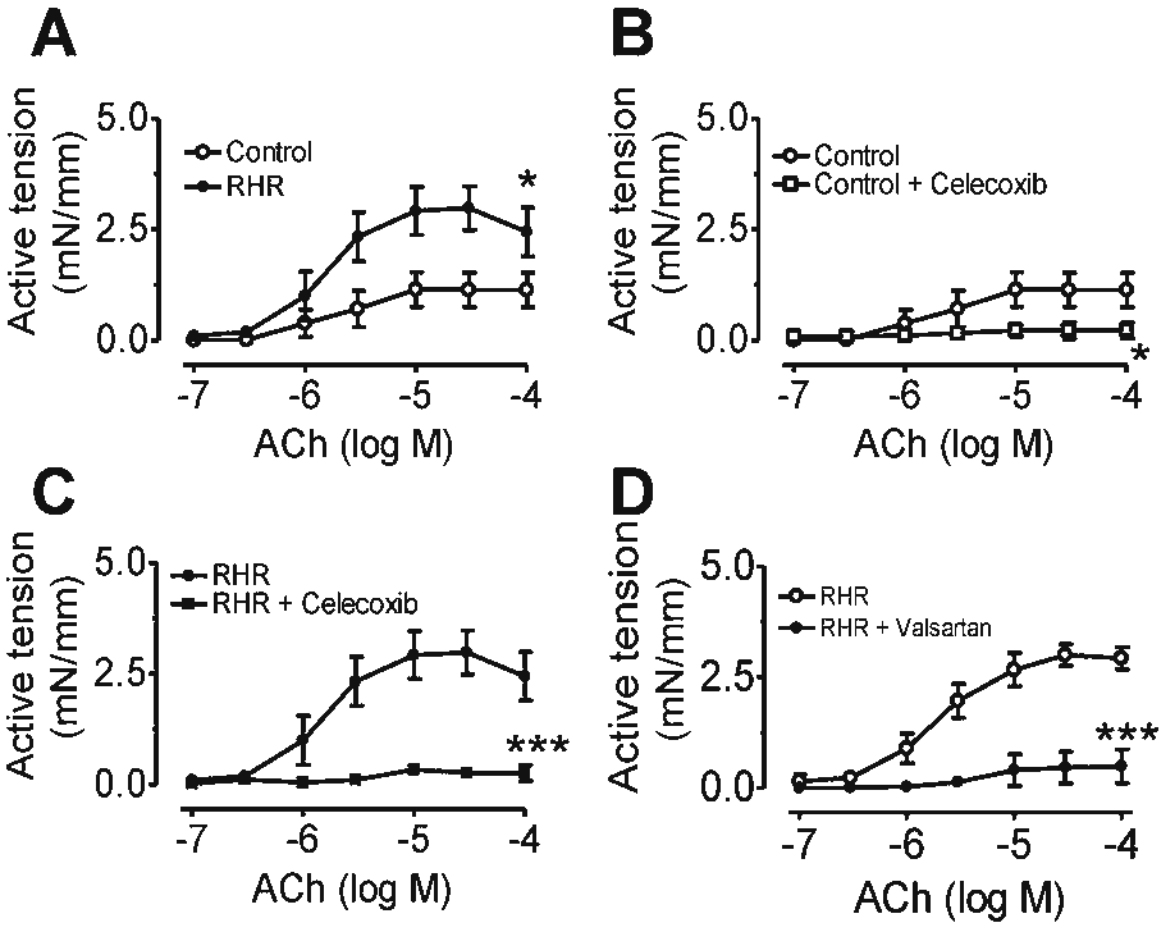


Figure 5.11.
(A) Augmented endothelium-dependent contractions in response to ACh in RHR intralobal renal arteries. Effects of chronic five-week treatment with celecoxib on endothelium-dependent contractions in intralobal renal arteries from **(B)** sham control rats and **(C)** RHR. **(D)** Abolition of ACh-induced endothelium-dependent contractions by five-week valsartan treatment. Results are mean ± SEM of 6-8 experiments. Statistical significances between groups are indicated by *** $p<0.001$ and * $p<0.05$.

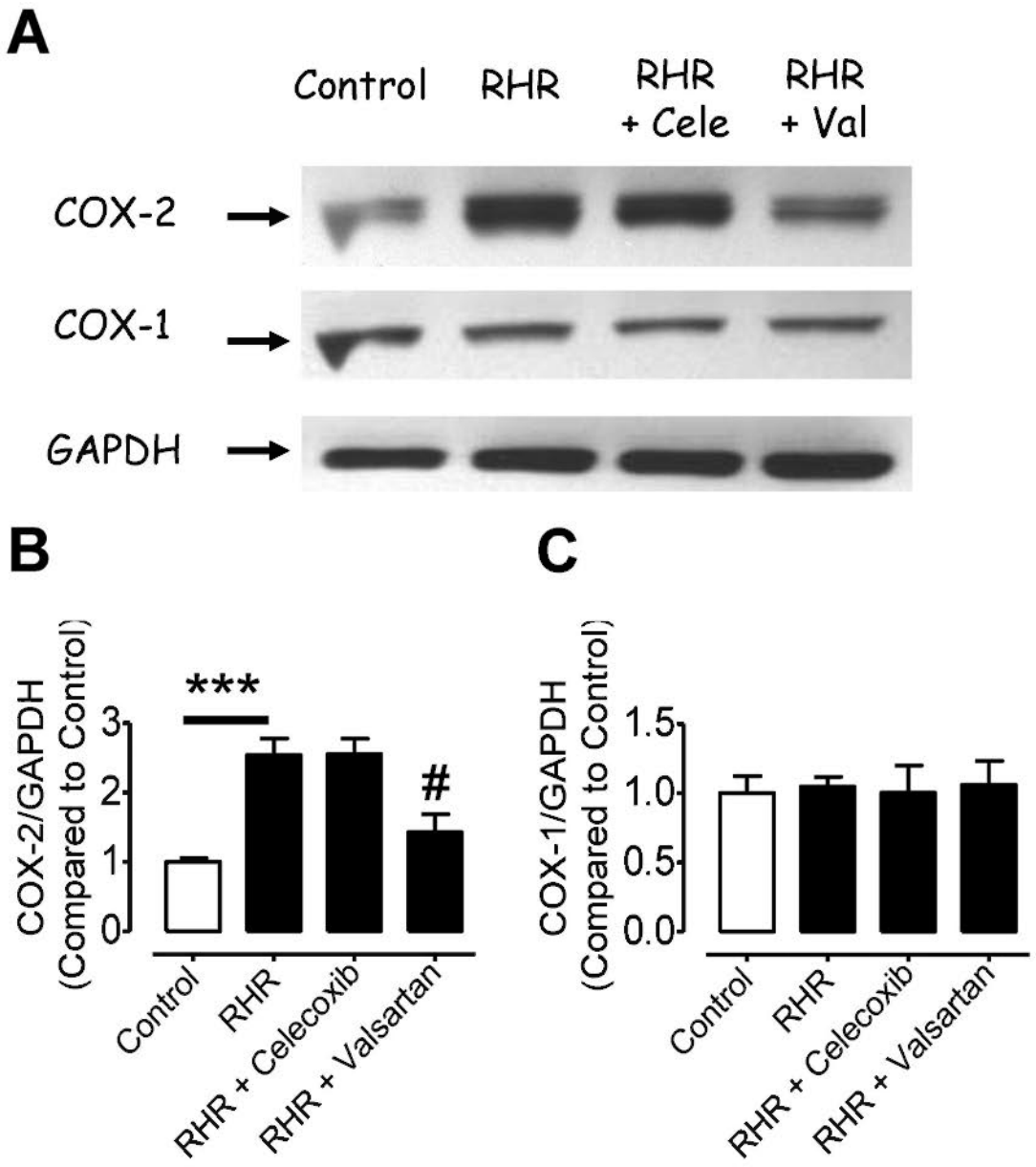


Figure 5.12.
(A) Western blot analysis showing the expression of protein levels for COX-2 (72kDa) and COX-1 (72 kDa) in renal arteries from control rats, RHR, RHR treated with celecoxib, and RHR treated with valsartan **(A).** Summarized values for the expression of **(B)** COX-2 and **(C)** COX-1. Results are mean ± SEM of 4 experiments. Statistical significance between control and treatment groups is indicated by *** $p<0.001$; # $p<0.001$ vs RHR group.

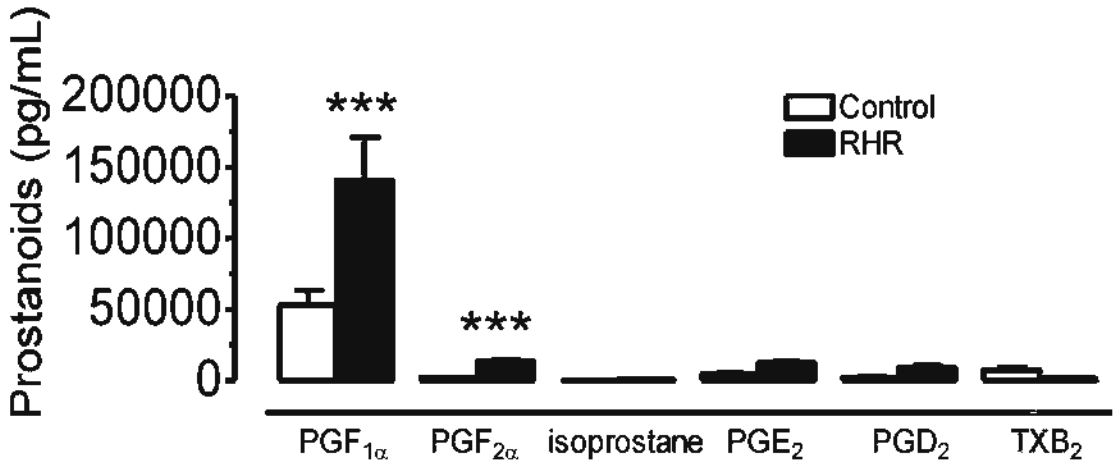


Figure 5.13.
The amount of six prostanoids that were released in response to 100 µmol/L ACh stimulation in solution bathing the renal arteries from RHR and sham operated control rats. The level of 6-ketoprostaglandin 1α (metabolites of PGI2) was the most abundant. Results are mean ± SEM of 4 experiments. Statistical significance between control and RHR groups is indicated by *** $p<0.001$.

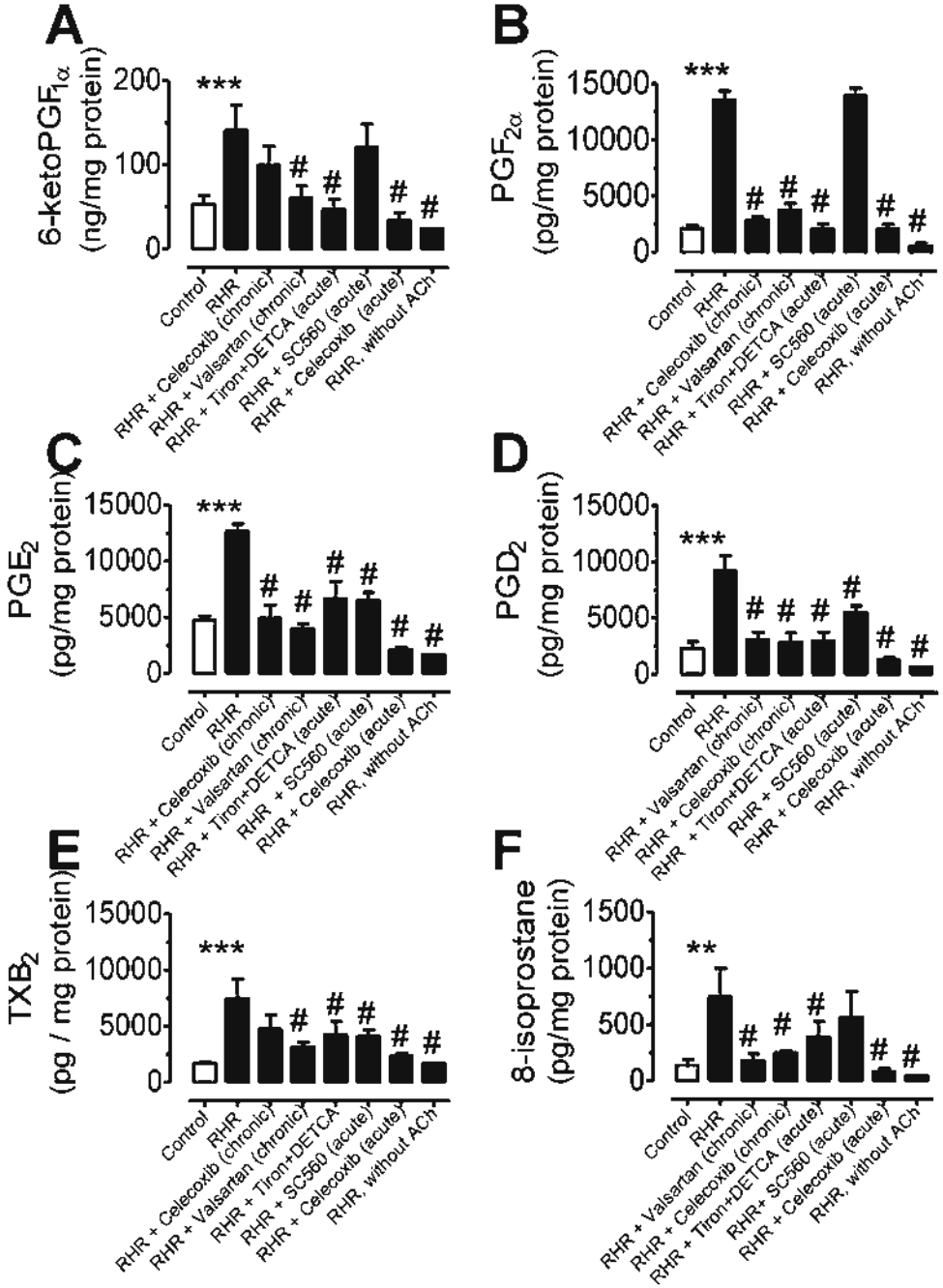


Figure 5.14.
Effects of acute exposure to 0.3 µmol/L sc-560, 3 µmol/L celecoxib or 1 mmol/L tiron plus 100 µmol/L DETCA on the amount of individual prostanoid that was released in renal arteries in response to ACh in solutions bathing renal arteries from control rats, RHR, RHR treated with celecoxib or with valsartan **(A-F)**. Results are mean ± SEM of 4 experiments. Statistical significance between control and RHR groups is indicated by *** $p<0.001$; # $p<0.01$ vs RHR group.

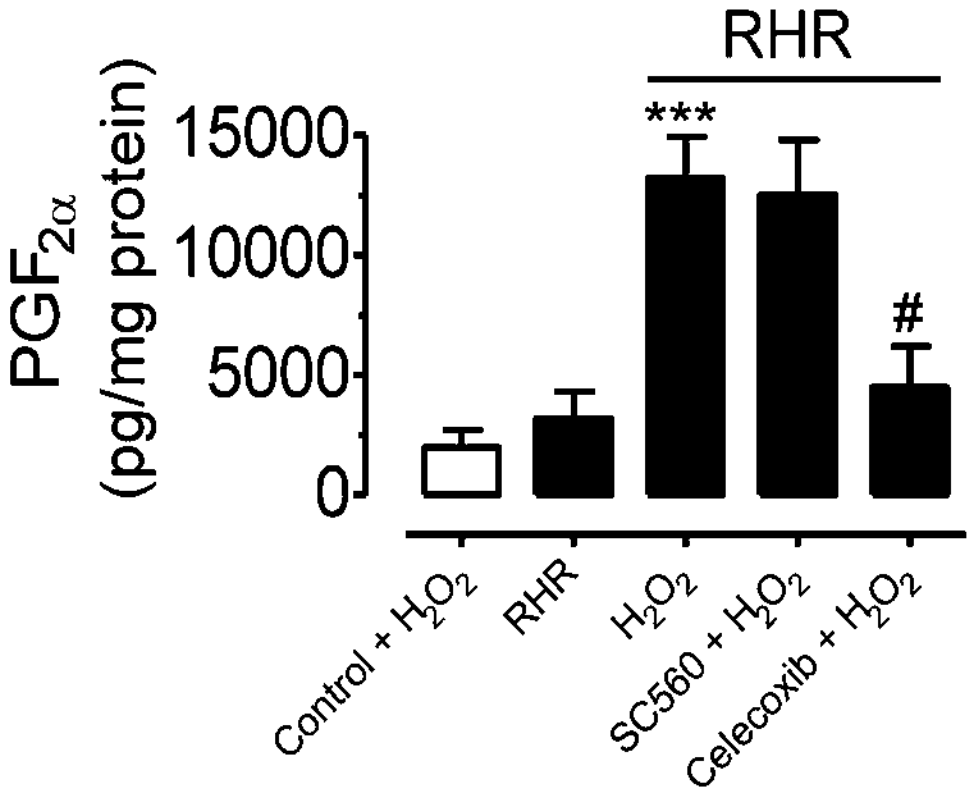


Figure 5.15.
Effects of H_2O_2 on the release of $PGF_{2\alpha}$ in renal arteries of sham-operated and RHR. Results are mean ± SEM of 4 experiments. Statistical significance between control and RHR groups is indicated by *** $p<0.001$; # $p<0.01$ vs RHR group.

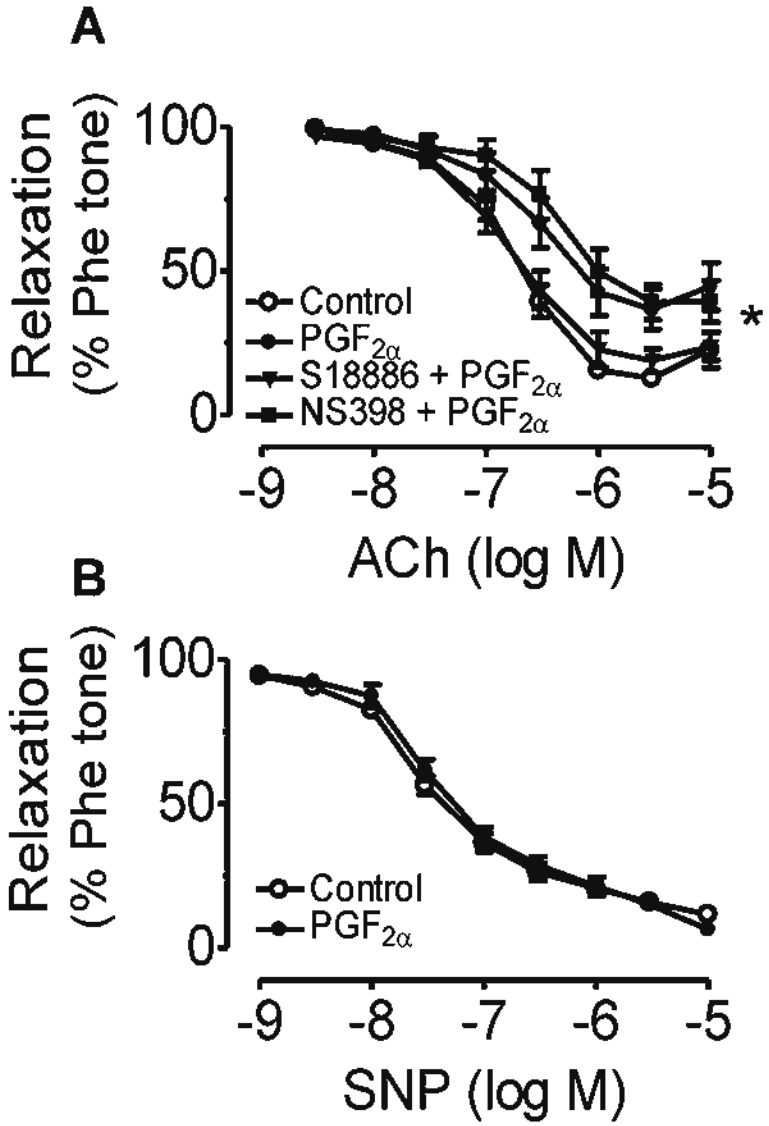


Figure 5.16.
Effects of acute exposure to 7 nmol/L PGF2α on **(A)** ACh-induced endothelium-dependent relaxations and **(B)** SNP-induced endothelium-independent relaxations in rat intralobal renal arteries. **(A-F)**. Results are mean ± SEM of 6 experiments. Statistical significance between control and RHR groups is indicated by * $p<0.05$.

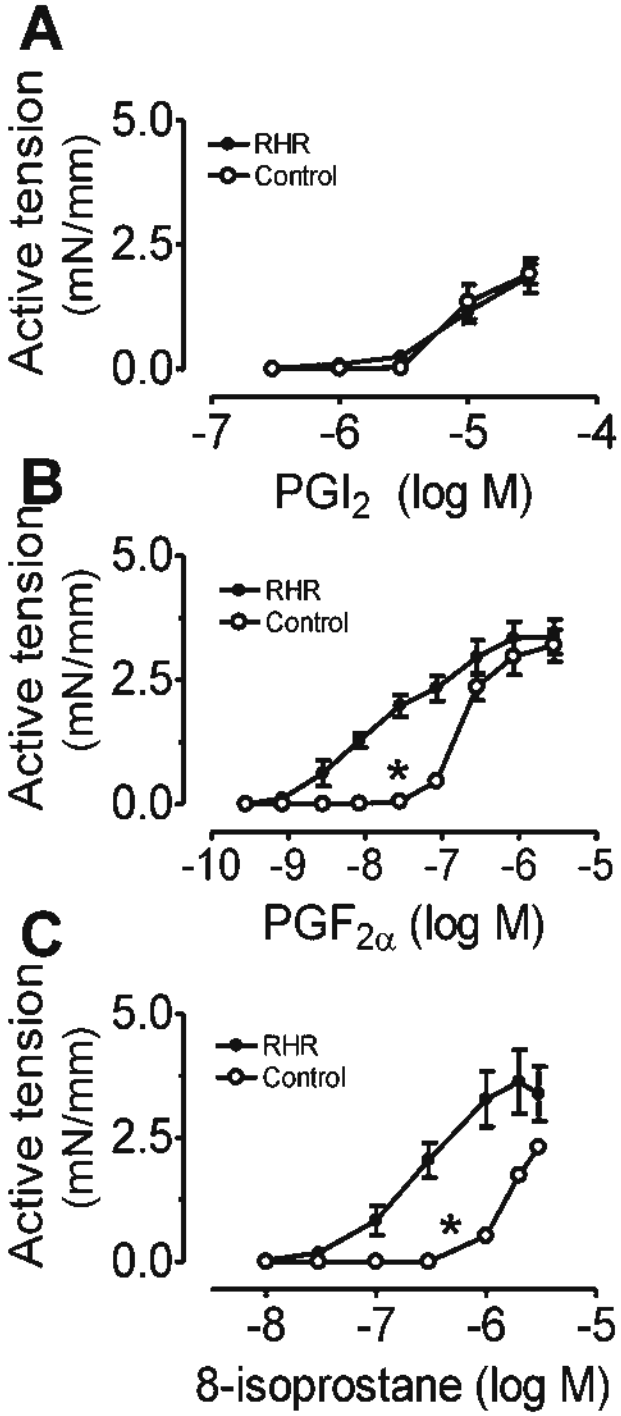


Figure 5.17.
Concentration-response curves for contractions induced **(A)** prostacyclin (PGI2); **(B)** prostaglandin F2α (PGF2α); and **(C)** 8-isoprostane. Results are mean ± SEM of 5-6 experiments. Statistical significance between control and RHR groups is indicated by *** $p<0.001$.

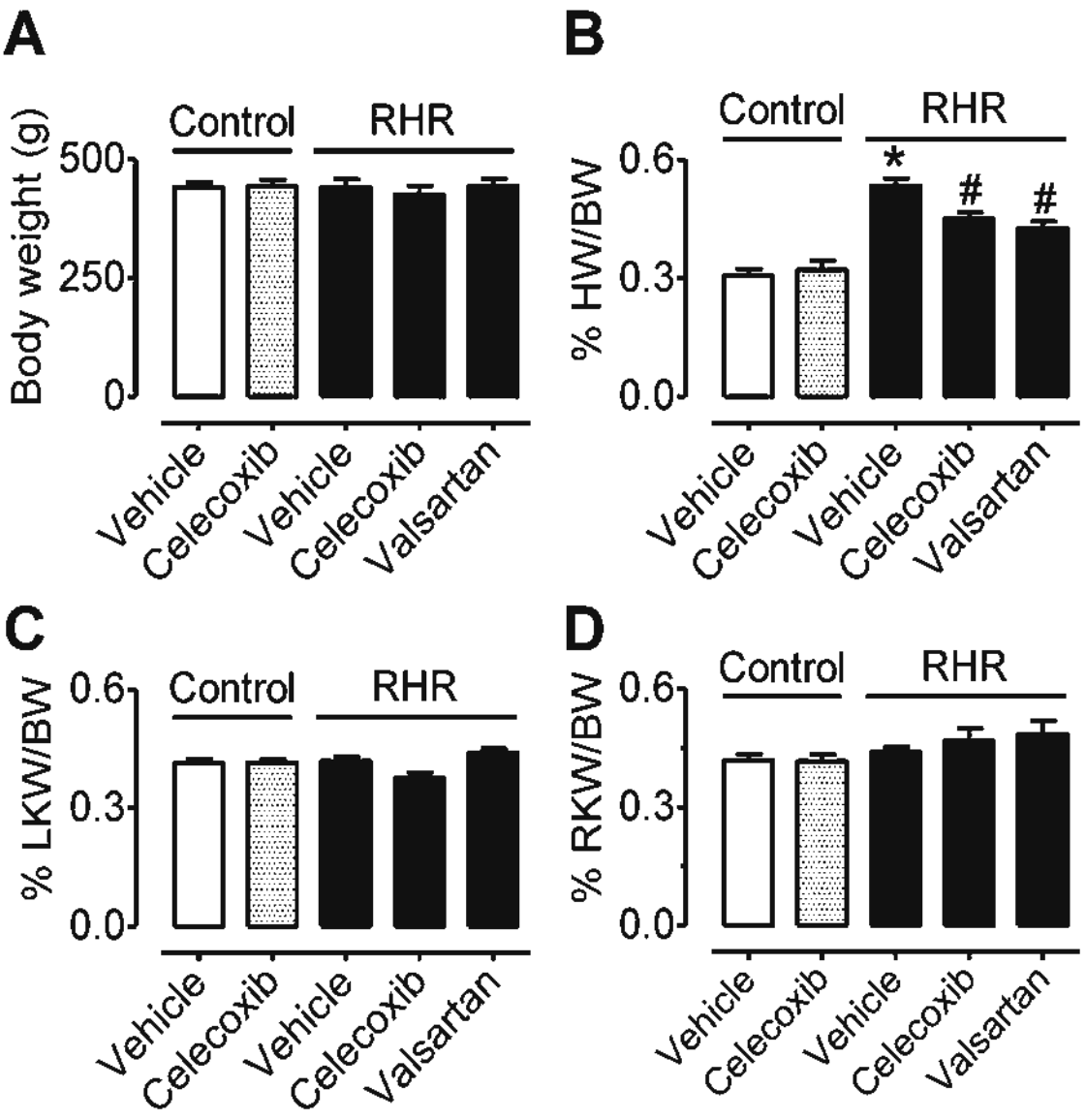


Figure 5.18.
Basic parameters including **(A)** body weight, **(B)** heart weight to body weight (HW/BW), **(C)** left kidney to body weight (LKW/BW), and **(D)** right kidney to body weight (RKW/BW). Results are mean ± SEM of 8-10 rats. Statistical significance between control and RHR groups is indicated by * $p<0.01$; and # $p<0.05$ vs RHR vehicle group.

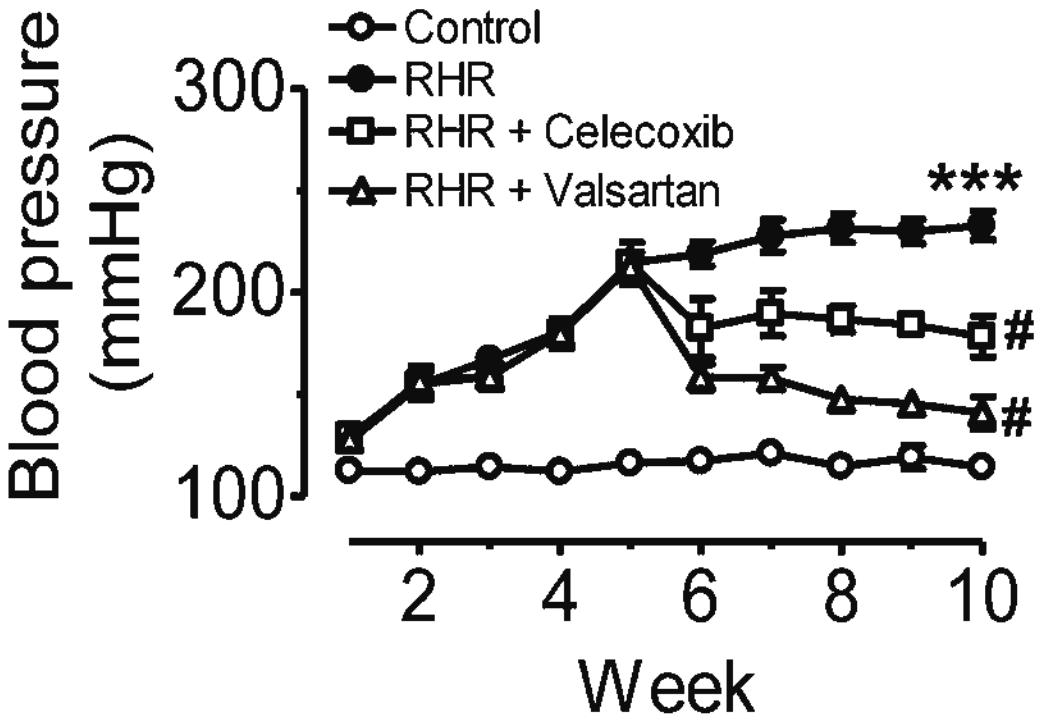


Figure 5.19.
Systolic blood pressures measured weekly following surgical induction of renal artery stenosis and subsequent oral treatment with celecoxib and valsartan to RHR at week 5. Results are mean ± SEM of 6-8 rats. Statistical significance between control and RHR groups is indicated by *** $p<0.001$; and # $p<0.05$ vs RHR vehicle group.

5.4. Discussion

From the present study, I have obtained novel findings regarding a significant role of COX-2-derived $PGF_{2\alpha}$ in endothelial dysfunction of intralobal renal arteries from renovascular hypertensive rats (RHR). Firstly, I confirm the severely impaired ACh-induced endothelium-dependent dilatations in RHR renal arteries, and found that ROS scavengers, selective COX-2 inhibitors, and TP receptor antagonist largely or fully restored endothelium-dependent dilatations and inhibited contractions induced by higher concentrations (> 1 μmol/L) of ACh. Secondly, I observe the augmented endothelium-dependent contractions in the presence of L-NAME and characterize the sequence of intracellular signaling events leading to the generation of EDCFs which then interact with the TP receptor on VSMCs to trigger contractile responses in RHR renal arteries. To summarize, I have revealed a critical cascade of cellular steps beginning from the ACh-stimulated ROS production to the trigger of the release of a most likely EDCF, $PGF_{2\alpha}$, from the up-regulated COX-2, leading to the appearance of endothelium-dependent contractions as well as an impaired endothelial function in renal arteries from a rat model of renovascular hypertension.

Renovascular hypertension associated with renal artery stenosis accounts for a small portion of overall hypertension, but it is considered to be a potentially curable form of secondary hypertension in humans (Dunnick and Sfakianakis, 1991; Bosmans and De Broe ME, 2004). Renovascular hypertension is related to the activation of the RAS which in turn causes an increased oxidative stress (Higashi et al., 2002). In the present study, I employ the two-kidney two-clip (2K2C) renovascular hypertensive rats as an experimental model to examine the renovascular dysfunction in isolated intralobal renal arteries, which allowed me to dissect out more precise and detailed mechanisms responsible for endothelial dysfunction in an *ex vivo* experimental setting, and to exclude the possible confounding factors from any systemic changes in the cardiovascular function. The 2K2C model is an alternative to two-kidney one-clip (2K1C) and one-kidney one clip (1K1C) Goldblatt hypertensive models. All models pathophysiologically resemble human renovascular hypertension.

Endothelial dysfunction has been observed in different types of RHR animal models. For example, the impaired agonist-induced endothelium-dependent dilatations are reported in aortas (Castro et al., 2008) and in small mesenteric arterioles (Fortes et al., 1992) from 2K1C hypertensive rats. In addition, endothelium-dependent dilatations triggered by electric field stimulation are blunted in penile small arteries (Martinez 2006) and in superior mesenteric arteries from 1K1C hypertensive rats (Stankevicius, 2002). ACh-induced endothelium-dependent relaxations were reduced in 2K2C rat aortas (de la Riva et al., 2000). However, there is no report in literature about pathological alterations of vascular function in renal arteries during the development of renovascular hypertension in view of a significant role of unfavorably altered renal artery reactivity in renovascular hypertension. Against this background, I used intralobal renal arteries of internal diameter of ~250 µm to investigate mechanisms involved in endothelial dysfunction in renovascular hypertension. Firstly, I have demonstrated that ACh-induced endothelium-dependent dilatations were greatly reduced in RHR renal arteries and I observe that the dilatation was converted into contractile responses when the concentration of ACh was raised above 1 µmol/L. By contrast, endothelium-independent dilatations to a NO donor, SNP were almost identical in renal arteries between normotensive control rats and RHR, confirming that the relaxing sensitivity of VSMCs to NO remains unchanged and that impaired ACh-induced dilatations are primarily caused by the dysfunction of endothelial cells. Secondly, ROS scavengers or SOD mimetic acutely restore the impaired ACh-induced dilatations, which is in accordance to the previous study showing that blunted ACh-induced endothelium-dependent relaxations of aortas in 2K1C-induced hypertensive mice were prevented by deletion of NAD(P)H oxidase subunit $gp91^{phox}$ (Jung et al., 2004), suggesting a significant role of ROS production in contributing to the development of endothelial dysfunction in RHR.

Indomethacin, acting as a non-selective COX inhibitor, was shown before to normalize the blunted relaxations in 2K1C small mesenteric arterioles (Fortes et al., 1992). The present study elucidates that COX-2, rather than COX-1, plays a key role in inducing the production and release of vasoconstricting prostanoids that

harm endothelium-dependent dilatations in RHR renal arteries based on the following four pieces of findings in RHR renal arteries: (1) acute selective COX-2 inhibition by celecoxib results in complete restoration of ACh-induced dilatations; (2) acute COX-1 inhibition by sc-560 does not exert such beneficial effect; (3) three structurally distinct inhibitors of COX-2 (celecoxib, NS-398 and DuP697) abolish endothelium-dependent contractions while COX-1 inhibitors (VAS and SC-560) had no effect (also see below); and (4) neither celecoxib nor sc-560 modifies contractions induced by phenylephrine, 60 mmol/L KCl and U46619.

More significantly, I observe for the first time that COX-2-derived prostanoids, by acting on the TP receptor to impair endothelial function as antagonism of the TP receptor by S18886 entirely restores the impaired ACh-induced dilatations of RHR renal arteries. Further support comes from my recent demonstration that $PGF_{2\alpha}$ is able to inhibit ACh-induced dilatations in normotensive SD rat renal arteries and this inhibition is prevented by S18886.

Endothelium-dependent contractions can be readily visualized in conduit arteries from spontaneously hypertensive rats and diabetic rats (Vanhoutte et al., 2005; Shi et al., 2007), which is regarded as an important pathophysiological index of vascular dysfunction. However, endothelium-dependent contractions in renovascular hypertension were yet to be demonstrated. In the present study, I characterize the cellular and molecular mechanisms participating in endothelium-dependent contractions in intralobal renal arteries from 2K2C RHR and again demonstrate a critical role of COX-2. The ACh-induced contractions occur only in arteries with intact endothelium and such a vascular response has been defined as endothelium-dependent contractions in many previous studies (Vanhoutte et al., 2005; Wong et al., 2009). Visualization of endothelium-dependent contractions requires the presence of L-NAME. This strongly indicates that endogenous NO whose production can be elevated by ACh, a ligand usually used to trigger endothelium-dependent dilatations, may have masked the contractile response mediated by EDCFs in arteries in which the NO production were not eliminated. But a few circumstances such as aging may uncover endothelium-dependent contractions in the absence of L-NAME (Wong et al., 2009) as the eNOS activity or

NO-generating capacity may have been severely inhibited. Secondly, my results indicate that calcium influx into endothelial cells could be an initiative step in the induction of endothelium-dependent contractions in RHR renal arteries on the basic of the two observations: (1) renal arteries bathed in a Ca^{2+}-free solution can not contract in response to ACh, but contract upon re-addition of 2.5 mmol/L $CaCl_2$, suggesting contractions require extracellular calcium ions but it can not be distinguished whether the release of an EDCF in endothelial cells requires calcium ions or calcium ions are needed for VSMCs to develop a tension; and (2) a nonselective cation channel blocker, 2-APB inhibited ACh-induced contractions without affecting contractions evoked by elevated KCl, phenylephrine or U46619, suggesting that Ca^{2+} entry occurs in the endothelial cells rather than in VSMCs to trigger endothelium-dependent contractions although types of the calcium channel involved are unclear. Likewise, a recent study clearly shows that 2-APB prevents ACh-stimulated rises in endothelial cell $[Ca^{2+}]_i$ *in situ* of endothelium-intact hamster aortas and the same concentration of 2-APB abolished endothelium-dependent contractions (Wong et al., 2009). In addition, phenylephrine-, U46619- and endothelin 1-induced contractions were similar in renal arteries between normotensive rats and RHR, implicating that renovascular hypertension does not alter the contractile activity of VSMCs.

The impaired endothelium-dependent dilatations can be effectively rescued by ROS removal, suggesting a contributory role of ROS to endothelial dysfunction in renovascular hypertension. It was unknown whether ROS scavengers could attenuate endothelium-dependent contractions in RHR. Earlier studies by other research groups suggested that ROS may act as one of candidates for EDCF, for example, in canine basilar arteries (Katusic et al., 1993) and in rat femoral arteries of streptozotocin-induced diabetes (Shi et al., 2007) and also showed that or ROS enhance the EDCF response in aortas of spontaneously hypertensive rats (Yang et al., 2003). On the contrary, ROS plays no role in the induction of endothelium-dependent contractions and ROS removal does not affect endothelium-dependent relaxations in hamster aortas (Wong et al., 2009), suggesting a potential impact of species on the role of ROS in the induction of endothelial dysfunction.

The increased level of H_2O_2 is implicated in endothelium-dependent contractions in femoral arteries of streptozotocin-induced diabetic rats (Shi et al., 2008). Likewise, the ROS over-generation contributes to an enhanced contractile response to phenylephrine in mesenteric resistance arteries of db/db diabetic mice (Pannirselvam et al., 2005). Indeed, H_2O_2 can cause a direct contraction in normotensive rat renal arteries (Gao et al., 2005). In the present study, I demonstrate a critical involvement of ROS in the occurrence of endothelium-dependent contractions in renovascular hypertension because ACh-induced contractions of RHR renal arteries can be prevented by tempol (SOD mimetic) or tiron (SOD mimetic) plus DETCA (HO^- scavenger). To confirm such role for ROS, I measured the tissue level of ROS in control and in RHR renal arteries and found that RHR arteries contain significantly higher levels of ROS as determined by EPR spectroscopy. ROS generated in RHR renal arteries are unlikely to cause a direct contraction because COX-2 inhibitor celecoxib or TP receptor antagonist S18886 eliminate ACh-induced contractions. It is likely that ROS may stimulate COX-2 to release prostanoids that activate the TP receptor in VSMCs. If this were a case, exogenous ROS should be able to trigger contractions and either celecoxib or S18886 should block such contractions. Indeed, O_2^- generated through a chemical reaction between hypoxanthine and xanthine oxidase (HX-XO) or H_2O_2 produced significantly greater contractile responses in RHR renal arteries than those of control rats. As expected, H_2O_2- or HX-XO-induced contraction is abolished by celecoxib and S18886, while COX-1 inhibitor sc-560 was without effect. Intriguingly, ROS-induced contractions were significantly reduced in renal arteries without endothelium. It is probable that ROS is capable of releasing constrictive prostanoids from both endothelial cells and VSMCs as the COX-2 expression is up-regulated in both cell types. This have been verified by chemical assay of COX-2-dependent prostanoids in RHR renal arteries exposed to H_2O_2 or HX-XO. It is now clear that ACh-stimulated increases in various prostanoids as measured by EIA method were reduced by ROS scavengers, tiron plus DETCA in RHR renal arteries, further supporting a triggering role of ROS in the ACh-stimulated release of EDCFs in renovascular hypertension.

There exist two main isoforms of COX. COX-1 is expressed constitutively in most of mammalian cells including endothelial cells and VSMCs, whereas COX-2 is generally considered as an inducible isoform in most of tissues and cells and its expression is elevated upon inflammatory stimulation (Parente and Perretti, 2003). A few studies do show a constitutive presence of COX-2 in blood vessels, kidney and brain (Baber et al., 2003; FitzGerald and Patrono, 2001; Therland et al., 2004; Wong et al., 2009). In the present study, I provide solid results in favor of a critical role of COX-2 but not COX-1 in mediating endothelial dysfunction and induction of endothelium-dependent contractions in RHR renal arteries. The main supporting findings are (1) that ACh-induced contractions of RHR renal arteries are abolished by acute exposure to three structurally different selective COX-2 inhibitors, NS-398, DuP-697, and celecoxib, but not by COX-1 inhibitors, sc-560 and VAS; (2) acute COX-2 inhibition but not COX-1 inhibition improved ACh-induced dilatations in RHR renal arteries; (3) chronic treatment with celecoxib restores the blunted endothelial function and abolishes endothelium-dependent contractions in RHR renal arteries; (4) that the COX-2 expression is markedly increased while the COX-1 expression is unchanged in RHR renal arteries; and (5) that celecoxib but not sc-560 inhibits the release of $PGF_{2\alpha}$, the most likely EDCF found in this study (see below). I have probably obtained the first line of evidence for a positive role of COX-2 in endothelial dysfunction and EDCF responses in renal arteries.

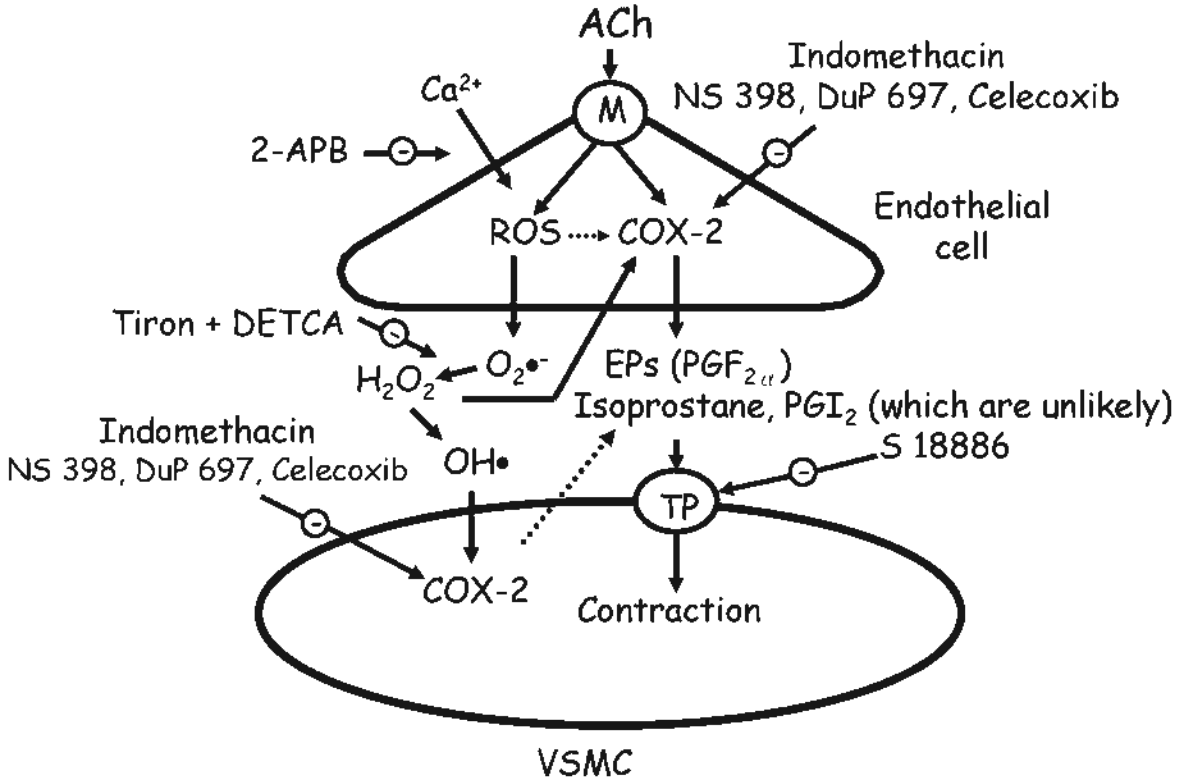
The results from functional studies point to a primary contribution of COX-2-derived arachidonic acid products to trigger endothelium-dependent contractions. From the EIA results and subsequent examination of the effect of exogenously applied prostanoids on the vascular tone, $PGF_{2\alpha}$ seems to be the most likely EDCF in response to ACh in RHR renal arteries. The level of all six prostanoids (6-keto $PGF_{1\alpha}$ as the end product of PGI_2, $PGF_{2\alpha}$, PGE_2, PGD_2, TXB_2 as the end product of TXA_2, and 8-isoprostane) in ACh-stimulated renal arteries is significantly higher in RHR than control rats. Among these prostanoids, only $PGF_{2\alpha}$ causes contraction at the amount equivalent to that measured by the EIA assay in RHR renal arteries bathed in Krebs solution and challenged by ACh. Although PGI_2 can also cause contractions, the concentration required is much higher than that released by RHR

renal arteries. The amount of the released 8-isoprostane can be neglected because such amount is far below a threshold level needed for a contraction. Of importance, the release of $PGF_{2\alpha}$, is inhibited only by COX-2 inhibitor but not by COX-1 inhibitor, strongly supporting that the EDCF is derived from COX-2 in RHR renal arteries. Although celecoxib inhibits the level of all six arachidonic acid metabolites, the involvement of PGD_2, PGE_2 and TXA_2 can be discounted because the release of the former two are inhibited by COX-1 inhibitor sc-560 which does not affect endothelium-dependent contractions and because sc-560 but not celecoxib inhibits the level of TXA_2. Nevertheless, it is still unclear whether the activity and expression of $PGF_{2\alpha}$ synthase would be altered by the induction of renovascular hypertension.

The present study shows clearly that blood pressure increased steadily after surgical procedure of renal artery stenosis and the rise leveled off at five week. Daily oral administration of celecoxib lowered blood pressure at the first week of treatment to a relative stable level for the next four weeks. Valsartan serving as a positive control drug produced a greater blood pressure-lowering effect. However, there are contradictory data in literature concerning the blood pressure-reducing action of COX-2 inhibition. COX-2 inhibitors reduce mean arterial blood pressure in 2K1C hypertensive rats (Okumura et al., 2002; Wang et al., 1999); however, another study reported no reduction of blood pressure in the same model (Hartner et al., 2003). The blood-pressure lowering effects as observed in the present study were accompanied by a reduction in the HW/BW ratio, suggesting the beneficial effects of COX-2 inhibition on hypertension and cardiac hypertrophy. It is yet to be determined whether blood pressure reduction inhibits cardiac hypertrophy or COX-2 inhibitors exert two separate benefits.

This study is probably the first functional characterization of the important role of COX-2 in endothelial dysfunction and of $PGF_{2\alpha}$ as the most likely EDCF in RHR renal arteries. Induction of renovascular hypertension leads to ROS overproduction and COX-2 up-regulation in renal arteries. In response to ACh, increased $[Ca^{2+}]_i$ in endothelial cells may trigger release of ROS which in turn stimulate the activity of the up-regulated COX-2; the latter catalyzes the chemical

conversion of arachidonic acid/ PGH_2 into $PGF_{2\alpha}$ and $PGF_{2\alpha}$ triggers ACh-induced endothelium-dependent contractions through binding to the TP receptor in VSMCs (**Figure 5.18**). Since COX-2 is expressed in various human vascular beds, the present data might be highly relevant for cardiovascular regulation in humans. Indeed, the present data in RHR renal arteries aorta supports an important role of COX-2 in the impaired endothelial function in hypertension. Furthermore, since COX-2 is a well-established inflammatory enzyme, the present results also suggest that the endothelium-dependent contractions of RHR renal arteries be a useful and reliable model for pharmacological investigations in COX-2-dependent inflammatory responses as occur in hypertension.



Constitutive expression of COX-2 and COX-2 up-regulation in hypertension

Figure 5.18.
A schematic diagram illustrating a cascade of events leading to ACh-elicited endothelium-dependent contractions in intralobal renal arteries in a rat model of renovascular hypertension. ACh stimulates influx of calcium ions in endothelial cells and elevated $[Ca^{2+}]_i$ stimulates the production of ROS which in turn activate the already up-regulated COX-2 to catalyze formation of several prostanoids from arachidonic acid in both endothelial cells and VSMCs. $PGF_{2\alpha}$ is the most likely EDCF to activate the TP receptor in VSMCs to trigger contractions. Inhibition of calcium influx by 2-APB, ROS removal by various scavengers, COX-2 inhibition and TP receptor antagonism all abolish ACh-induced endothelium-dependent contractions. Arrows in red indicate inhibition.

CHAPTER VI

COX-2 UP-REGULATION AND PROSTAGLANDIN $F_{2\alpha}$ MEDIATE BONE MORPHOGENIC PROTEIN 4-INDUCED ENDOTHELIAL DYSFUNCTION IN HYPERTENSION

6.1 Introduction

Bone morphogenic protein 4 (BMP4) belongs to the BMP family, six of which (BMP2 to BMP7) fall into the transforming growth factor-β (TGF-β) superfamily. BMP4 was originally discovered to participate in embryonic development, bone and cartilage formation (Hogan, 1996; Li and Wozney, 2001; Massague, 2000). Recent studies indicate that BMP4 is involved in inflammation, a critical initiator of atherogenic event (Dhore et al., 2001; Vendrov et al., 2006).

BMP4 is a mechano-sensitive and pro-inflammatory gene; and disturbed flow increases BMP4 production in cultured endothelial cells. BMP4 promotes the expression of intracellular adhesion molecules and monocyte adhesion via a reactive oxygen species (ROS)-dependent manner (Chang et al., 2007; Sorescu et al., 2004; Sorescu et al., 2003). BMP4 expressed in vascular smooth muscle enhances pulmonary vascular remodeling in pulmonary hypertension (Frank et al., 2005) and BMP4 infusion induces hypertension in mice partly through stimulating the expression and activity of vascular NADPH oxidases and subsequent overproduction of ROS disturbs endothelial function (Miriyala et al., 2006).

However, the exact underlying mechanisms remain largely unclear with regard to the BMP4 cascade culminating in endothelial dysfunction. Other signaling pathways may be also involved and cyclooxygenase-2 (COX-2) could be one of possible mediators. COX-2 up-regulation was detected in atherosclerotic lesions (Schonbeck et al., 1999) and COX-2 catalyzes production of a majority of vascular prostanoids in human atherosclerotic areas (Belton et al., 2000). COX-2 inhibition improves endothelial function in patients with hypertension and coronary heart disease (Chenevard et al., 2003; Widlansky et al., 2003). On the other hand, COX-2 is also expressed constitutively in vascular

endothelial cells (Baber et al., 2003; Therland et al., 2004) and its expression is up-regulated with ageing (Wong et al., 2009), suggesting that COX-2 plays an important role in both physiological and pathological regulation of vascular function depending on the level of the expression and activity.

Against this background, I therefore hypothesized that the up-regulated COX-2 in endothelial cells played a critical role in BMP4-induced endothelial dysfunction. In the present study, I used mouse, rat and human arteries to investigate whether BMP4 could impair endothelium-dependent dilatations (EDR), and trigger endothelium-dependent contractions (EDC), and to elucidate the linker between BMP4 stimulation and downstream COX-2 up-regulation, and finally to verify the clinical relevance of this BMP4/COX-2 pathway in hypertension.

6.2 Methods and Materials

This study was approved by the Animal Ethics Committee at Chinese University of Hong Kong (CUHK) and the Joint CUHK-New Territories East Cluster Clinical Research Ethics Committee. C57BL/6J mice, spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats were supplied by CUHK Laboratory Animal Center while COX-$1^{-/-}$ or COX-$2^{-/-}$ mice were supplied by University of Hong Kong.

6.2.1 Blood vessel preparation

Adult male mice and rats were sacrificed by CO_2 suffocation and mouse aorta or rat intralobal renal artery were removed and placed in ice-cold Krebs solution (mmol/L): 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11 D-glucose. Arteries were cleaned of adhering adipose tissue and cut into ring segments of 2 mm in length. As recently described (Wang et al., 2009), mouse aortic rings were incubated for 12 h in Dulbeco's Modified Eagle's Media (DMEM, Gibco) culture media with 10% fetal bovine serum (FBS, Gibco), 100 IU penicillin and 100 µg/ml streptomycin, and placed in a CO_2 incubator with 95 % O_2 plus 5 % CO_2 with and without BMP4. After 12-h incubation, rings were mounted in a Multi Myograph System (Danish Myo Technology, Denmark) for recording of changes in vascular reactivity. Briefly, two steel wires (40-µm in diameter) were inserted through vessel's lumen, and each wire was fixed to the jaws built in the myograph. The organ chamber was filled with 5 ml Krebs solution and constantly gassed by 95% O_2-5% CO_2 at 37°C (pH ~7.4). Each ring was stretched initially to 3 mN, an optimal tension, and then allowed to stabilize for 90 min before the start of each experiment.

6.2.2 Functional studies

Some arterial rings were exposed to BMP4 in control and in the presence of each of the following inhibitors: noggin (BMP4 antagonist, 100 ng/mL), apocynin (NADPH oxidase inhibitor, 100 µmol/L), celecoxib and NS398 (COX-2 inhibitors, 3 µmol/L), SB202190 (p38 MAPK inhibitor, 10 µmol/L) or cycloheximide (protein synthesis inhibitor, 10 µmol/L). After incubation with BMP4 for 12 h rings were mounted in myograph and then subjected to acute 30-min exposure to tempol

(SOD mimetic, 100 μmol/L), tiron (superoxide anion scavenger, 1 mmol/L), DETCA (ROS scavenger, 100 μmol/L), S18886 (TP receptor antagonist, 100 nmol/L), or celecoxib (3 μmol/L).

The first series of experiments tested the alteration of EDR. Rings were contracted by phenylephrine (1 μmol/L) to establish a stable tension and then acetylcholine (ACh) was added cumulatively (1 nmol/L – 10 μmol/L) to produce concentration-dependent dilatations. ACh-induced dilatations were abolished by 100 μmol/L N^G-nitro-L-arginine methyl ester (L-NAME, NOS inhibitor), or by endothelium removal. The second set of experiments examined endothelium-dependent contractions (EDC). Aortic rings were first treated for 30 min with 100 μmol/L L-NAME to eliminate the interference of endothelium-derived nitric oxide (NO), a procedure commonly adopted to visualize EDC, and then contractions were elicited by cumulative addition of ACh (0.1 - 30 μmol/L).

Intralobal renal arteries were dissected from SHR and WKY rats and subjected to 12-h organ culture in DMEM in control and in the presence of 100 ng/mL noggin or 3 μmol/L celecoxib. Both EDR and EDC were studied and compared in different treatment groups.

Human renal arteries were harvested from nephrectomy specimen in patients, with or without history of hypertension after informed consent obtained. The mean age of our patients was 62.5 (range 44-70) years old. The indications of surgery include tumor (renal cell carcinoma, transitional cell carcinoma, and renal leiomyosarcoma) and non-functioning kidney. History of hypertension was defined as having persistent elevated blood pressure, systolic pressure over 140 mmHg or diastolic pressure over 90 mmHg, and required medical therapy. Human arteries were treated using the same protocol as for rat renal arteries. Due to the limited availability of human arteries, we only studied EDR.

6.2.3 ROS detection by EPR spin trapping and H_2O_2-sensitive fluorescent dye

To measure the amount of ROS released from arterial tissues, electron paramagnetic resonance (EPR) was performed with 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine hydrochloride (TEMPONE-H, Alexis) and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO, Alexis) as spin trap agents.

All EPR samples were placed in 100 µL glass tubes and suspended in Krebs solution. In order to inhibit reactions catalyzed by transition metals, DTPA (0.2 mmol/L) was added. X-band EPR spectra were measured at room temperature using an EMX EPR spectrometer (Bruker). The EPR settings were as follows: magnetic field, 3480 G; sweep width, 100 G, microwave frequency, 9.746 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 0.3 G; conversion time, 10.24 msec; time constant, 0.64 msec.

6.2.4 Intracellular ROS measurement

The method was modified from Matoba *et al.* (2000). Mouse thoracic aortas were dissected out and cut into several ring segments. After different treatments, the aortic rings were fluorescently labeled with 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Molecular Probes) in Krebs solution for 30 minutes. Extracellular CM-H_2DCFDA was washed away by rinsing arterial tissues for three times in Krebs solution. Each ring was cut open longitudinally. The vascular strips were placed on a rectangular slide glass (24 mm X 50 mm) with the endothelium facing downwards and covered with another square cover glass (22 mm X 22 mm). The fluorescently labeled vascular strips were examined using an Olympus Fluoview FV1000 laser scanning confocal system (Olympus America Inc., Melville, NY) mounted on an inverted IX81 Olympus microscope, equipped with a 20X water-immersion objective (NA 0.5) under a laser scanning confocal microscope (Olympus), with an excitation wavelength of 480 nm and an emission wavelength at 520 nm at room temperature. Fluorescence images of the endothelium were obtained and the fluorescence intensity was determined using Olympus confocal analytical software.

6.2.5 Western blotting

Isolated arteries were subjected to similar procedures as in functional studies, frozen in liquid nitrogen, and homogenized in an ice-cold RIPA lysis buffer that contained 1 µg/mL leupeptin, 5 µg/mL aprotonin, 100 µg/mL PMSF, 1 mmol/L sodium orthovanadate, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L sodium fluoride, and 2 µg/mL β-glycerolphosphate. The tissue lysates were centrifuged at 20,000 ×g for 20 min. The supernatants were collected and protein

concentrations were analyzed using Lowry method (Bio-rad). The protein samples were electrophoresed on a 10% SDS-poly-acrylamide gel and transferred onto an immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore). Nonspecific binding sites were blocked by 5% non-fat milk in 0.05% Tween-20 phosphate-buffered saline (PBST), then incubated overnight at 4°C with primary antibodies including anti-COX-2 or anti-COX-1 (Cayman), eNOS, phosphor-eNOS at Ser^{1177}, p38 MAPK (Cell Signaling Technology), or BMP4 (Sigma-Aldrich), followed by a HRP-conjugated swine anti-rabbit or anti-mouse IgG (DakoCytomation), developed with an enhanced chemiluminescence detection system (ECL reagents, Amersham Pharmacia), and finally exposed to X-ray films. Equal protein loading was verified with use of a housekeeping anti-GAPDH antibody (Ambion, Inc).

6.2.6 Measurement of prostanoids by enzyme immunoassay

The levels of arachidonic acid-derived prostanoids were measured by EIA kits (Cayman Chemical, Ann Arbor, MI, USA) according to the instruction of the manufacturer. The five prostanoids or their metabolites, $PGF_{2\alpha}$, PGE_2, PGD_2, 6-keto $PGF_{1\alpha}$ (for PGI_2) and TXB_2 (for TXA_2), were assayed.

6.2.7 Constructs, lentivirus production and transduction

I have designed two shRNAs (short hairpin RNA) targeting mouse BMP receptor 1a : shRNA1 (5'- GCT GTT AAA TTC AAC AGT GAC ACA AAT G -3') and shRNA2 (5'- TCT CTC TAT GAC TTC CTG AAA TGT GCC A -3'); and one shRNA targeting firefly luciferase: 5'-TGC GCT GCT GGT GCC AAC CCT ATT CT-3' as a control. DNA fragments containing shRNAs sequence were synthesized and cloned into lentiviral RNAi (RNA interference) vector pLUNIG after annealing as previously described (Chen et al., 2003; Chen et al., 2008).

The VSV-G-pseudotyped lentiviruses were produced by co-transfecting 293T cells with the transfer vector and three packaging vectors: pMDLg/pRRE, pRSV-REV, and pCMV-VSVG as previously described. Subsequent purification was performed using ultracentrifugation. Mouse blood vessels were cultured in 24-well plates and were transduced with lentivirus and 8 μg/ml polybrene (Sigma).

6.2.8 Quantitative reverse transcription PCR (q-RT-PCR)

The QRT-PCR was carried out using SYBR green QRT-PCR Master Mix, 2-Step kit from ABI. Briefly, total RNA was extracted using Trizol reagent as described by the manufacturer (Invitrogen). The cDNA was generated using oligo dT primer mix and total RNA. The cDNA was PCR amplified using primers specific for mouse Bmpr1a and GAPDH. The PCR amplication was carried out in an ABI 7500 machine. The Ct (threshold cycle) value of BMP receptor 1a amplification was normalized to that of GAPDH control. The product size of Bmpr1a is 470 bp. The primers for QRT-PCR were Bmpr1a forward: 5- GTA CAG GAC GCG TGC GAA TCA GAC -3; Bmpr1a reverse: 5- TGC CAT CAA AGA ACG GAC CTA TAA C -3; GAPDH forward: 5-AGG TGA CCG CAT CTT CTT GT-3; GAPDH reverse: 5-CTT GCC GTG GGT AGA GTC AT-3.

6.2.9 Drugs

BMP4, acetylcholine, L-NAME, phenylephrine, noggin, tiron, tempol, DETCA (diethyldithiocarbamate acid) and sodium nitroprusside (SNP) were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). Cycloheximide, NS-398 (N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide), SB202190, PD98059 and SP600125 were from Tocris (Avonmouth, UK). $PGF_{2\alpha}$ and PGI_2 were from Cayman Chemical (Ann Arbor, MI, USA). Apocynin and PGE_2 were from Calbiochem, EMD Biosciences (La Jolla, CA, USA). S18886 (3-[(6-amino-(4-chlorobenzensulphonyl) -2-methyl-5,6,7,8- tetrahydronapht] -1-yl) propionic acid) and SC-560 were kind gifts from Institut de Recherches Servier (Suresnes, France). Celecoxib was from Pfizer. Acetylcholine, L-NAME, phenylephrine, PGI_2, tiron, tempol, DETCA and S18886 were prepared in distilled water and others in DMSO (Sigma-Aldrich).

6.2.10 Statistical analysis

Results were mean ± SEM from different individuals. Concentration-response curves were analyzed by non-linear regression curve fitting using GraphPad Prism software (Version 4.0, San Diego, CA) to estimate E_{max} as the maximal response and pD_2 as the negative logarithm of the drug concentration that produced 50% of E_{max}. The protein expression was quantified by densitometer

(FluorChem, Alpha Innotech, San Leandro, CA) and normalized to GAPDH and then compared with control. Statistical significance was determined by two-tailed Student's t-test or one-way ANOVA followed by Bonferroni post-tests when more than two treatments were compared. $P < 0.05$ was regarded as significantly different.

6.3 Results

6.3.1 BMP4 impairs endothelium-dependent dilatations

ACh-induced endothelium-dependent dilatations (EDR) in mouse aortas were progressively impaired by 12-h exposure to the increasing concentrations of BMP4 **(Figure 6.1A)** and prolonged BMP4 exposure caused further impairment **(Figure 6.1B, Table 1)** and aortas started to contract in response to ACh over 1 μmol/L **(Figure 6.2A&B)**. By contrast, endothelium-independent relaxations to sodium nitroprusside (SNP) were unaffected by 20 ng/ml BMP4 **(Figure 6.1C)**.

6.3.2 BMP-4-induced endothelial dysfunction and endothelium-dependent contractions are mediated by the up-regulated ROS and COX-2

BMP4 antagonist noggin (100 ng/mL) prevented BMP4-induced impairment of EDR **(Figure 6.3A)**. Co-treatment (12-h) with apocynin (100 μmol/L, **Figure 6.4A)** or acute incubation with tempol (100 μmol/L, **Figure 6.4C)** ameliorated BMP4-induced endothelial dysfunction. In the presence of L-NAME, endothelium-dependent contractions (EDC) of BMP4-treated aortas were eliminated by noggin **(Figure 6.3B)**, apocynin **(Figure 6.4B)** and tempol **(Figure 6.4D)**. Tiron (1 mmol/L) plus DETCA (100 μmol/L) also acutely restored the endothelial function in BMP4-treated aortas **(Figure 6.5A)**, thus collectively supporting an essential role of ROS in BMP4-induced endothelial dysfunction.

ACh-stimulated phosphorylation of eNOS at Ser^{1177} was unaltered by BMP4 or by noggin **(Figure 6.6)**, implying that the elevated ROS lowers the NO bioavailability.

Knocking down BMP receptor Ia by RNAi **(Figure 6.7C)** eliminated the ability of BMP4 to trigger EDC in mouse aortas **(Figure 6.7A&B)**, suggesting that the vascular action is mediated through BMP receptors.

Acute (30 min) or chronic (12 h) treatment with celecoxib (3 μmol/L) prevented the impaired EDR **(Figure 6.8A)** and EDC **(Figure 6.8B)** induced by BMP4. But COX-1 inhibitor SC560 showed no effect **(Figure 6.5B)**. In BMP4-treated aortas, EDR was improved while EDC was abolished by selective TP receptor antagonist S18886 (100 nmol/L, **Figure 6.8C&D)**.

In contrast to the harmful effect on endothelial function in wild-type mice

(Figure 6.9A&B), BMP4 did not affect EDR **(Figure 6.9C)** nor trigger EDC **(Figure 6.9D)** in COX-$2^{-/-}$ mouse aortas, thus indicating that increased COX-2 expression or activity mediates BMP4-induced endothelial dysfunction.

6.3.3 BMP4 induces ROS overproduction detected by EPR spectroscopy and fluorescence imaging

ROS scavengers effectively improved endothelial function and prevented EDC, indicating that BMP4 is likely to cause oxidative stress in endothelial cells. I also used the H_2O_2-sensitive fluorescent probe CM-H_2DCFDA to measure intracellular ROS. Exposure (12-h) to BMP4 also increased the basal intracellular ROS levels as measured *in situ* on endothelial cell layer in endothelium-intact aortas and ROS scavengers suppressed the ROS production **(Figure 6.10A&B)**.

In addition, 12-h incubation in 20 ng/ml BMP4 significantly elevated ROS production in ACh-stimulated mouse aortas as measured by EPR spectroscopy. This increase was abolished by noggin, apocynin, tempol **(Figure 6.11A)**, while COX-1 inhibitor (SC560) and COX-2 inhibitors (NS398 and celecoxib) had no effect **(Figure 6.11B and Figure 6.12A)**. Likewise, the increased ROS was primarily derived from endothelial cells as ACh failed to stimulate a rise of ROS in aortas without endothelium **(Figure 6.12B)**. By contrast, ROS increase remained unchanged in aortas from wild-type, COX-$1^{-/-}$ and COX-$2^{-/-}$ mice **(Figure 6.13A&B)**.

6.3.4 BMP4 induces COX-2 up-regulation in mouse aortas

BMP4 exposure led to 2-fold increase of COX-2 expression **(Figure 6.14A)** in mouse aortas and the COX-2 level is significantly less in aortas without endothelium **(Figure 6.14B)**, in concert with my functional results that show an enhanced COX-2 activity, COX-2 over-expression induced by BMP4 was also attenuated by noggin, apocynin and tempol **(Figure 6.3 & Figure 6.4)**. In cultured mouse aortic endothelial cells, 12-hour incubation with BMP4 also up-regulated the COX-2 expression and this effect was inhibited by noggin **(Figure 6.14C)**. The COX-2 up-regulation appears to be induced by BMP4 as co-treatment (12 hr) with cycloheximide prevented BMP-induced blunted ACh-induced dilatations **(Figure 6.15A)**, and both cycloheximide and

actinomycin D, each at 10 µmol/L abolished the COX-2 expression **(Figure 6.15B)**.

Moreover, treatment with p38 MAPK inhibitor (SB202190, 10 µmol/L) but not inhibitors of ERK (PD98059, 10 µmol/L) and JNK (SP600125, 10 µmol/L) reduced COX-2 over-expression **(Figure 6.16A)**, improved EDR **(Figure 6.16B)** and abolished EDC of BMP4-treated aortas **(Figure 6.16C)**, suggesting that p38 MAPK participates in the over-expression and/or activity of COX-2.

In contrast, BMP4 (20 ng/mL) did not change the COX-1 expression in mouse aortas with and without co-treatment with noggin, tempol, apocynin or SB202190 **(Figure 6.17)**.

6.3.5 COX-2-derived $PGF_{2\alpha}$ mediates endothelium-dependent contractions

Treatment with either COX-2 inhibitor celecoxib or TP receptor antagonist S18886, abolished EDC in BMP4-treated aortas **(Figure 6.8)**, it is therefore probable that COX-2-dependent arachidonic acid metabolites act as endothelium-derived contracting factors (EDCF) in BMP4-induced EDC. Under the same working condition as in functional studies, ACh (10 µmol/L) stimulated a significant increase in the level of released $PGF_{2\alpha}$ (~1.5 ng/L, **Figure 6.18A)**, but not in PGE_2, PGD_2 (in its stable metabolite of PGD-MOX), PGI_2 (in its stable metabolite of $PGF_{1\alpha}$) and TXA_2 (in its stable metabolite of TXB_2) **(Figure 6.18A)**. The ACh-stimulated release of $PGF_{2\alpha}$ was prevented by 3 µmol/L celecoxib **(Figure 6.18B)**. To further elucidate the role of $PGF_{2\alpha}$ in EDC, the effect of $PGF_{2\alpha}$ (1-100 ng/L) was tested in control and in BMP4-treated aortas with endothelium in the presence of L-NAME. $PGF_{2\alpha}$ induced contraction of aortic rings at a relatively low concentration **(Figure 6.19)**. $PGF_{2\alpha}$ at 1.4-1.5 ng/L (an amount equivalent to that of $PGF_{2\alpha}$ measured by EIA), elicited a tension of ~0.85 mN **(Figure 6.19)** which is comparable with ~0.98 mN in EDC **(Figure 6.3B)**. $PGF_{2\alpha}$-induced contractions were abolished by S18886 (100 nmol/L) **(Figure 6.19)**. By contrast, PGI_2 and PGE_2 did not cause contractions in mouse aortas (data not shown).

6.3.6 Association between BMP4 and COX-2 in hypertensive rats and human subjects

To further elucidate the pathophysiological significance of BMP4 in endothelial dysfunction, I examine the vascular protective effect of BMP4 antagonist noggin in spontaneously hypertensive rats. Normotensive WKY renal arteries relaxed better in response to ACh than SHR arteries **(Figure 6.20A)**. ACh produced endothelium-dependent contractions only in SHR arteries (**Figure 6.20B**). Treatment (12-h) with noggin (100 ng/mL) or celecoxib (3 μmol/L) restored the endothelial function **(Figure 6.20C)** and abolished EDC **(Figure 6.20D)**. Noggin also prevented the enhanced expression of both BMP4 and COX-2 in SHR renal arteries **(Figure 6.21)**.

Similar observation can be made in renal arteries from hypertensive patients. As shown in **Figure 6.22**, noggin or celecoxib treatment significantly enhanced ACh-induced dilatations **(Figure 6.22A&B)**. Again, the expression levels of both BMP4 and COX-2 were higher in hypertensive than normotensive human arteries **(Figure 6.22C)**, and largely inhibited by noggin **(Figure 6.22D)**. Finally, BMP4 (20 ng/mL) caused up-regulation of COX-2 in normotensive human arteries **(Figure 6.23A)** and reduced ACh-induced relaxations **(Figure 6.23B)**.

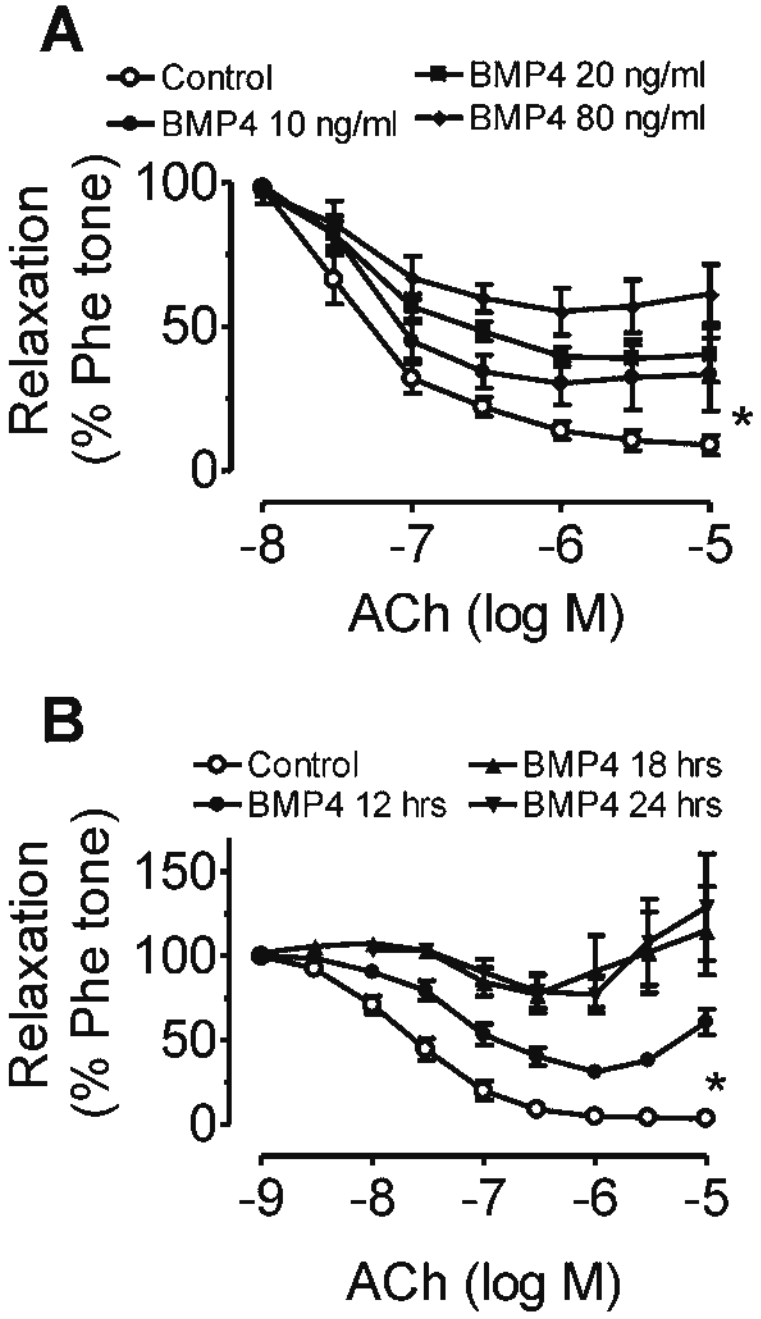


Figure 6.1.
(A) BMP4 (12-h incubation) impaired ACh-induced dilatations in C57BL/6J mouse aortas in a concentration-dependent manner (0-80 ng/mL). **(B)** BMP4 impaired ACh-induced dilatations in a time-dependent manner (12-24 h). Data are mean ± SEM of 5 mice. *P<0.05 versus control.

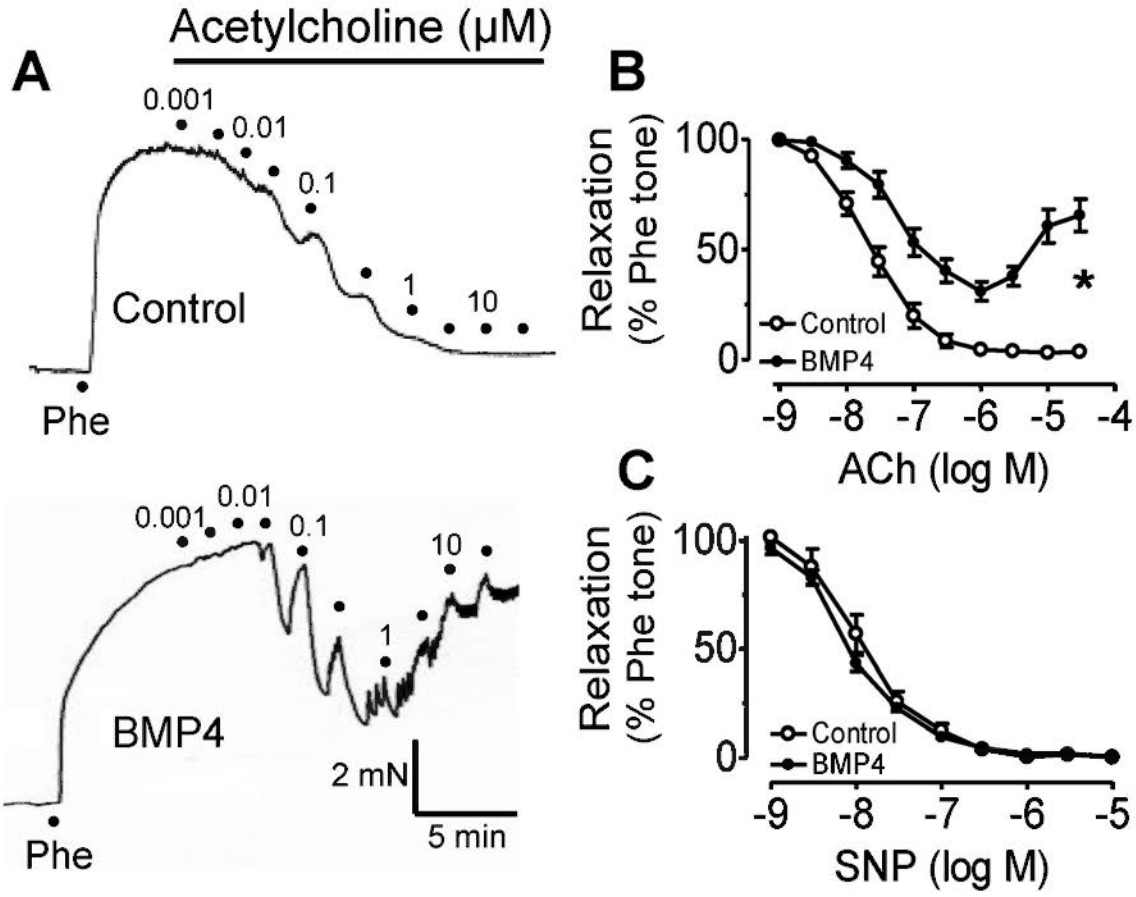


Figure 6.2.

(A) Representative traces showing BMP4-induced endothelial dysfunction. Concentration-response curves for dilatations induced by ACh **(B)** and by sodium nitroprusside SNP (**C**). Data are mean ± SEM of 5-7 mice. *P<0.05 versus control.

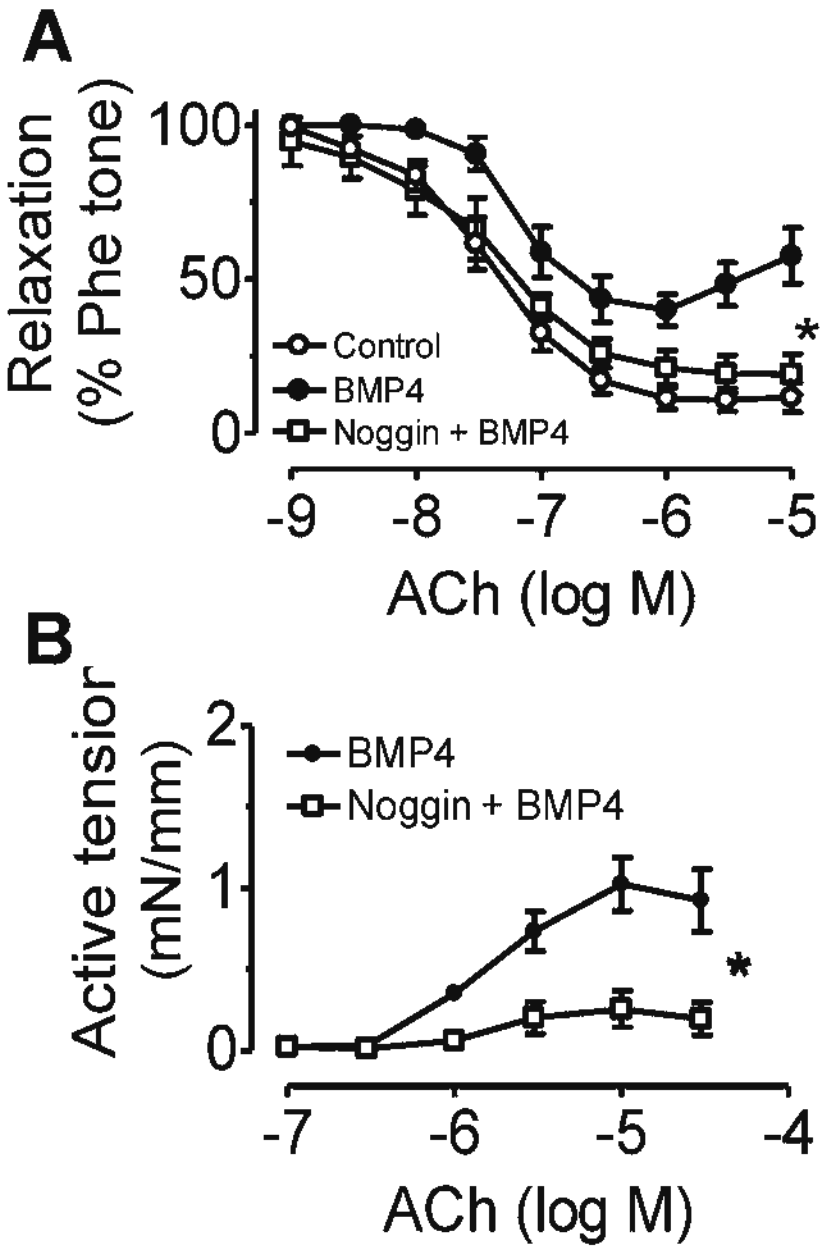


Figure 6.3.
(A) BMP4 (20 ng/mL)-induced endothelial dysfunction was inhibited or prevented by concomitant incubation with 100 ng/mL noggin. **(B)** BMP4 (20 ng/mL) treatment unmasked ACh-induced endothelium-dependent contractions and noggin (100 ng/mL) blocked this effect. All experiments were performed on C57BL/6J mouse aortas. Data are mean ± SEM of 6-8 mice. *P<0.05 between BMP4 and drug treatment.

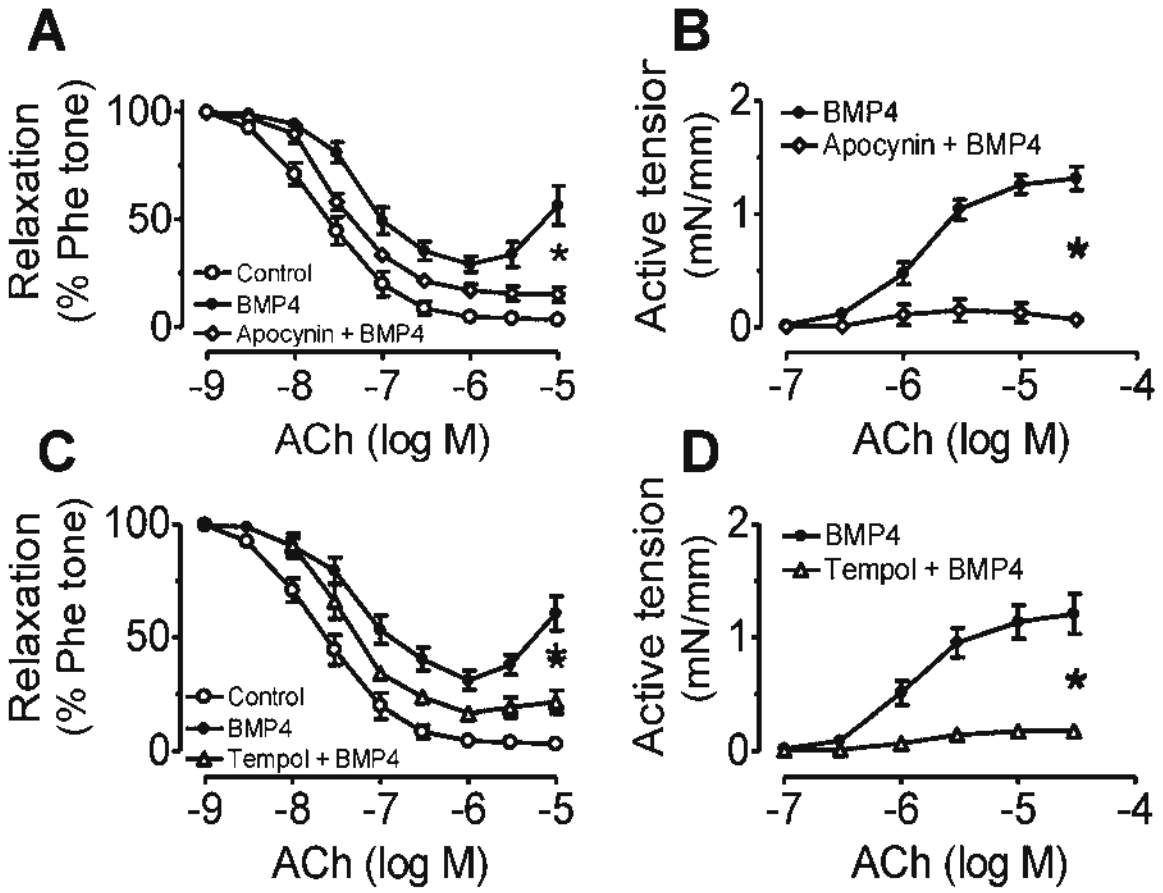


Figure 6.4.
Effects of 100 µmol/L apocynin **(A)** and acute 30-min treatment with 100 µmol/L tempol **(C)** on BMP4-induced impairment of ACh-induced endothelium-dependent aortic dilatations. The BMP4-exaggerated endothelium-dependent contractions in the presence of L-NAME (100 µmol/L) were abolished by concomitant incubation with apocynin **(B)** and 30-min treatment with tempol **(D)**. All experiments were performed on C57BL/6J mouse aortas. Data are mean ± SEM of 6-8 mice. *P<0.05 between BMP4 and drug treatment.

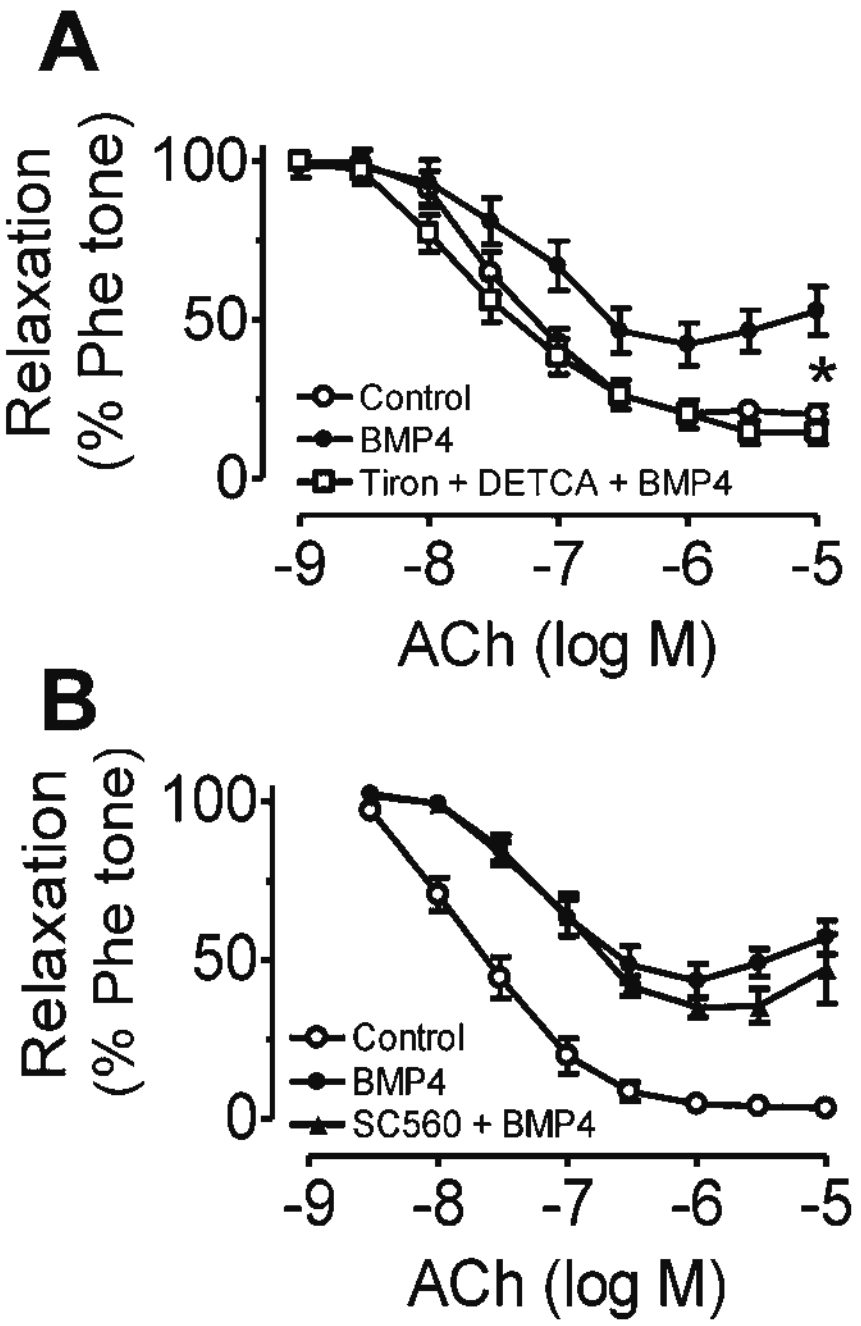


Figure 6.5.
(A) Effect of acute treatment with 100 µmol/L tiron plus 1 mmol/L DECTA on BMP4 (20 ng/mL)-induced impairment of ACh-induced endothelium-dependent dilatations. **(B)** Lack of an effect of SC560 on BMP4-indude endothelial dysfunction. All experiments were performed on C57BL/6J mouse aortas. Data are mean ± SEM of 6 mice. *P<0.05 between BMP4 and drug treatment.

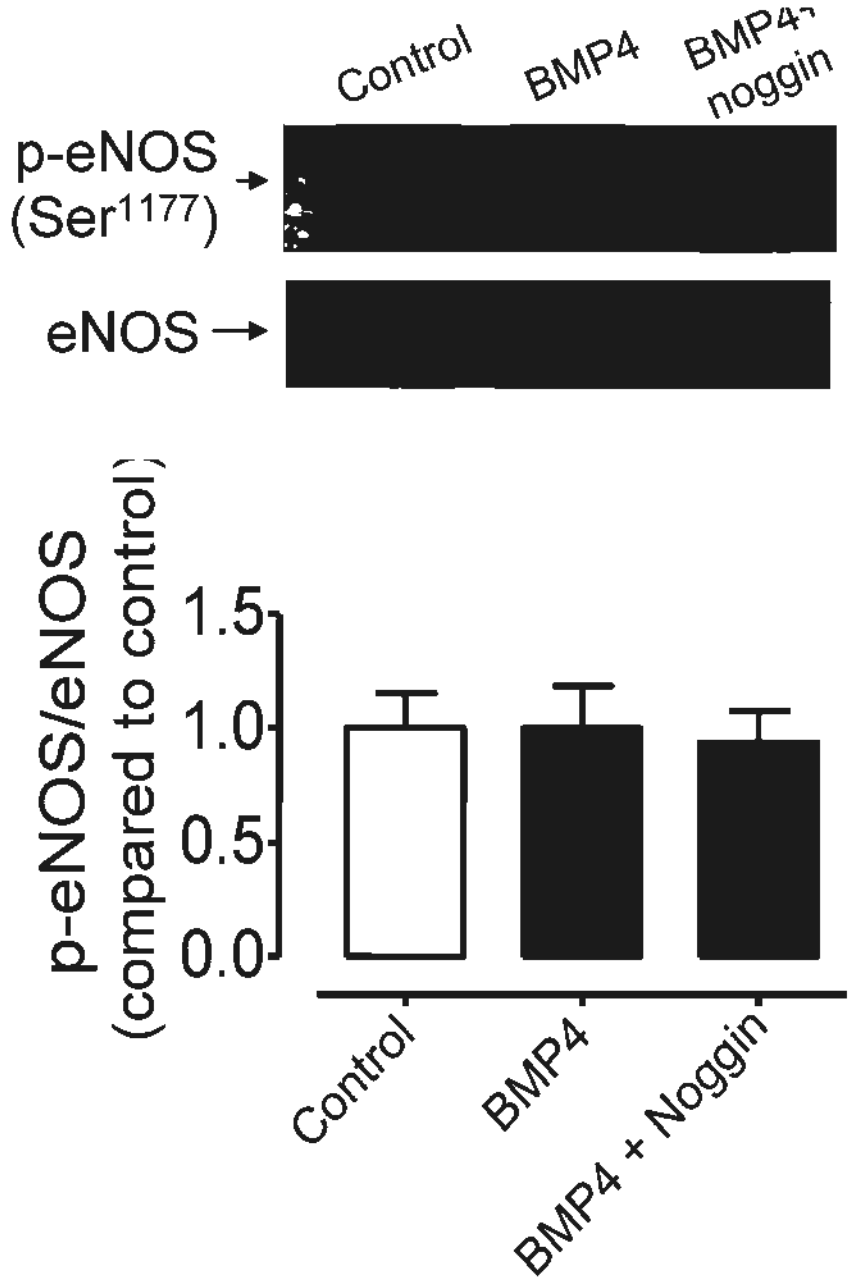


Figure 6.6.
The level of phosphorylation of eNOS (p-eNOS) at Ser^{1177} in response to 1 µmol/L ACh in C57BL/6J mouse aortic rings with and without BMP4 (20 ng/mL) treatment. Data are mean ± SEM of 4 mice.

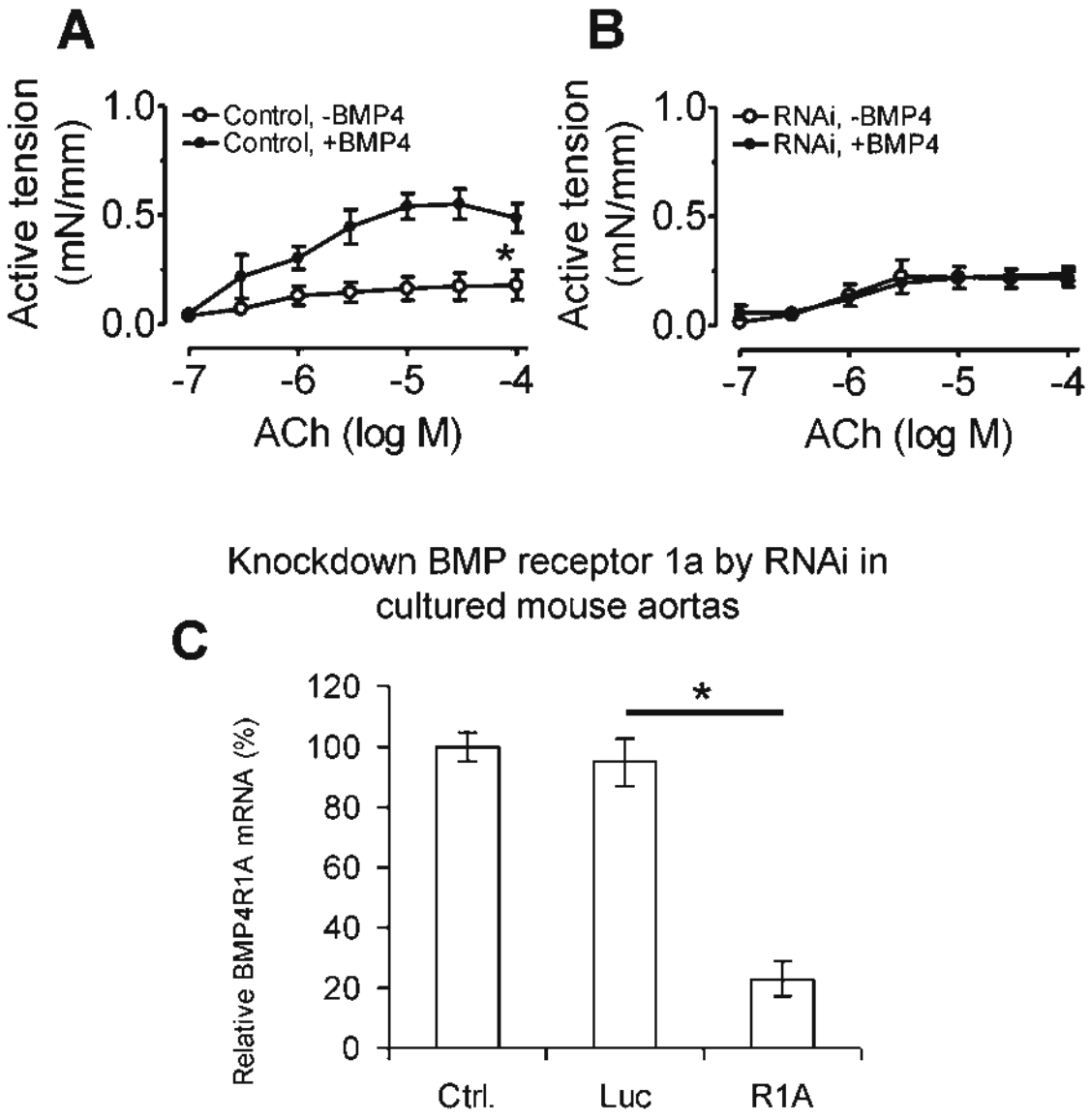


Figure 6.7.
(A&B) RNAi knocking down of BMP receptor 1a abolished endothelium-dependent contractions in BMP4-treated mouse aortas. **(C)** The mRNA level for BMP4 receptor 1a with and without its knockdown. All experiments were performed on C57BL/6J mouse aortas. Results are mean ± SEM of 3 mice. *P<0.05 with and without BMP4 treatment or between Luc and R1A.

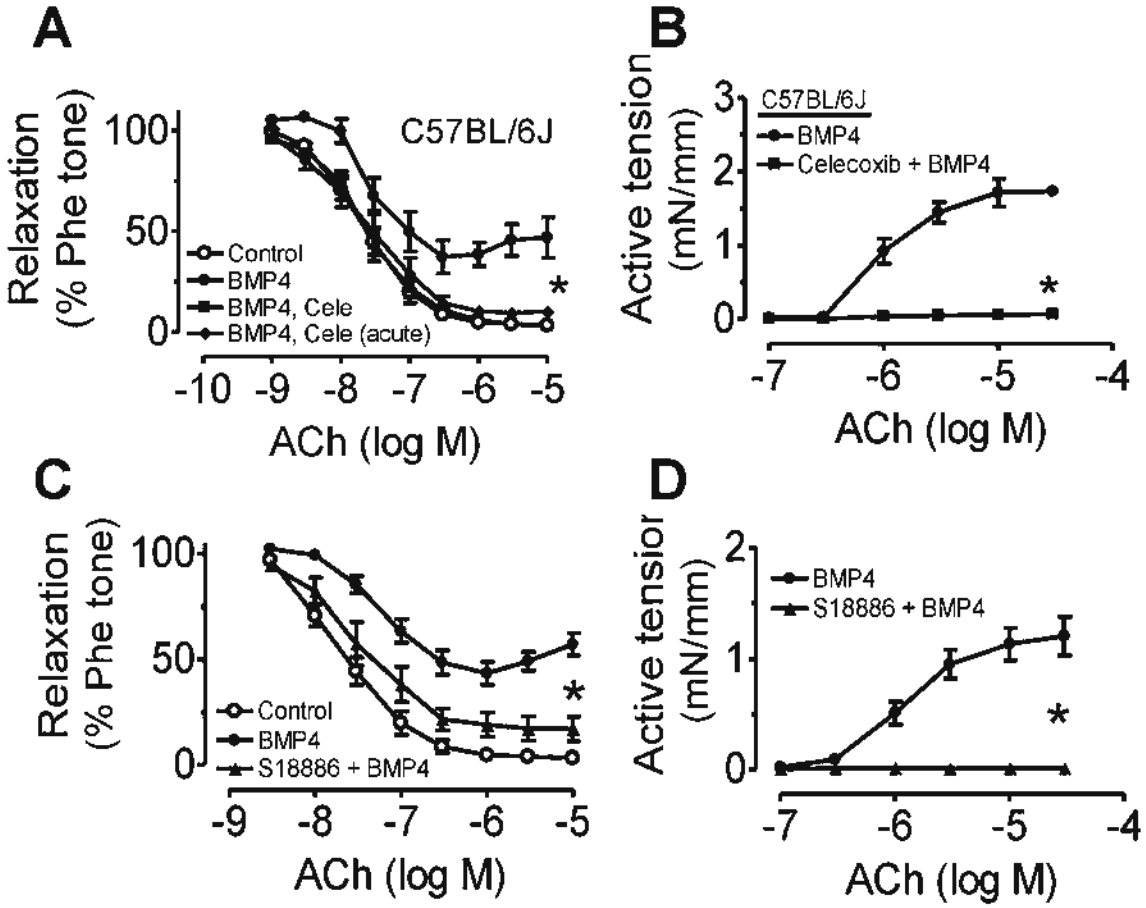


Figure 6.8.

Treatment (12-h or acute, 30-min) with 3 µmol/L celecoxib restored the impaired ACh dilatations **(**EDR, **A)** and prevented endothelium-dependent contractions (EDC) in the presence of L-NAME **(B)** in C57BL/6J mouse aortas. S18886 (100 nmol/L) exerted the same effect as celecoxib on EDR **(C)** and EDC **(D)**. Data are mean ± SEM of 6-8 mice. **P*<0.05 between BMP4 and drug treatment.

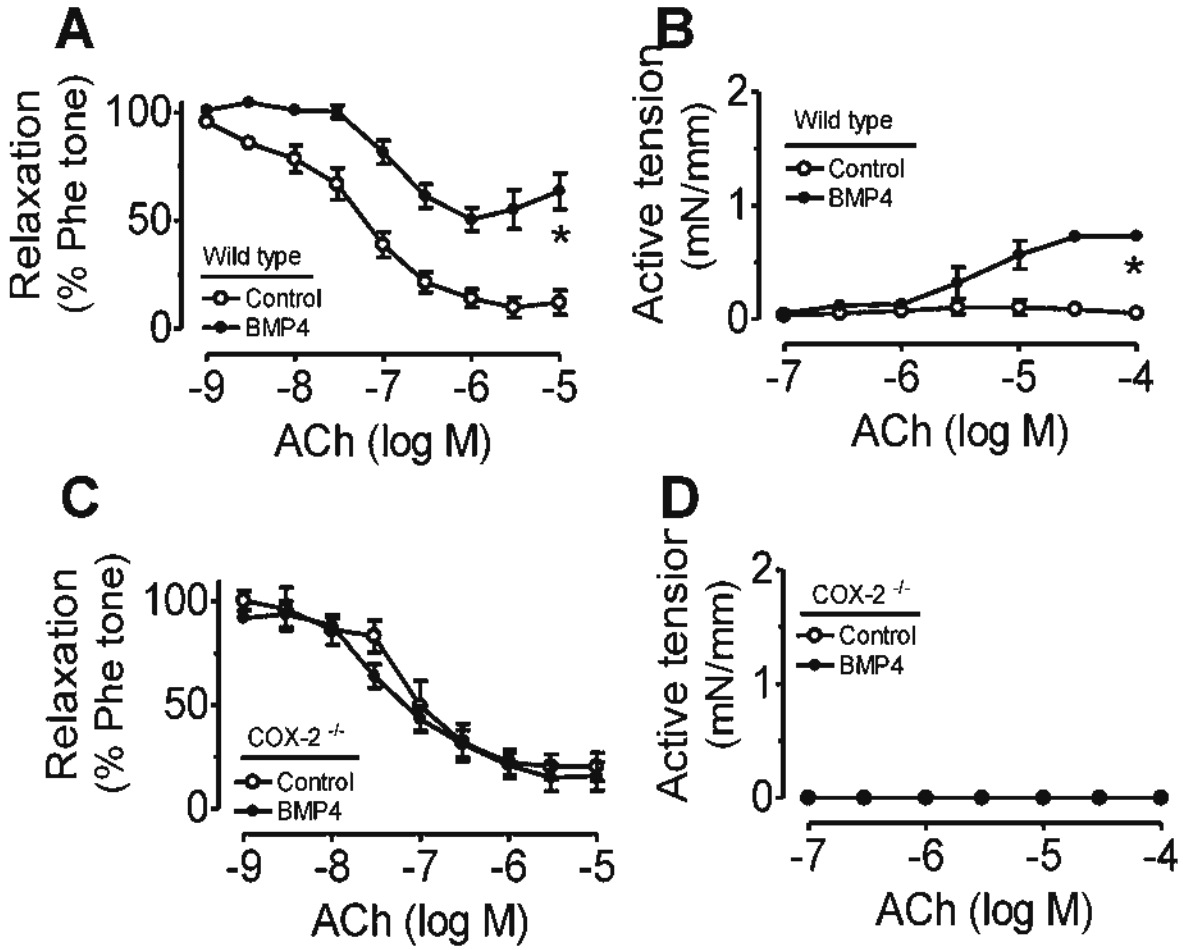


Figure 6.9.
BMP4 (20 ng/mL) impaired endothelial function **(A)** and elicited endothelium-dependent contractions **(B)** in wild-type mouse aortas. BMP4-induced effects on both endothelium-dependent dilatations **(C)** and endothelium-dependent contractions **(D)** were eliminated in COX-2$^{-/-}$ mice. Data are mean ± SEM of 5-7 mice. *P<0.05 versus control.

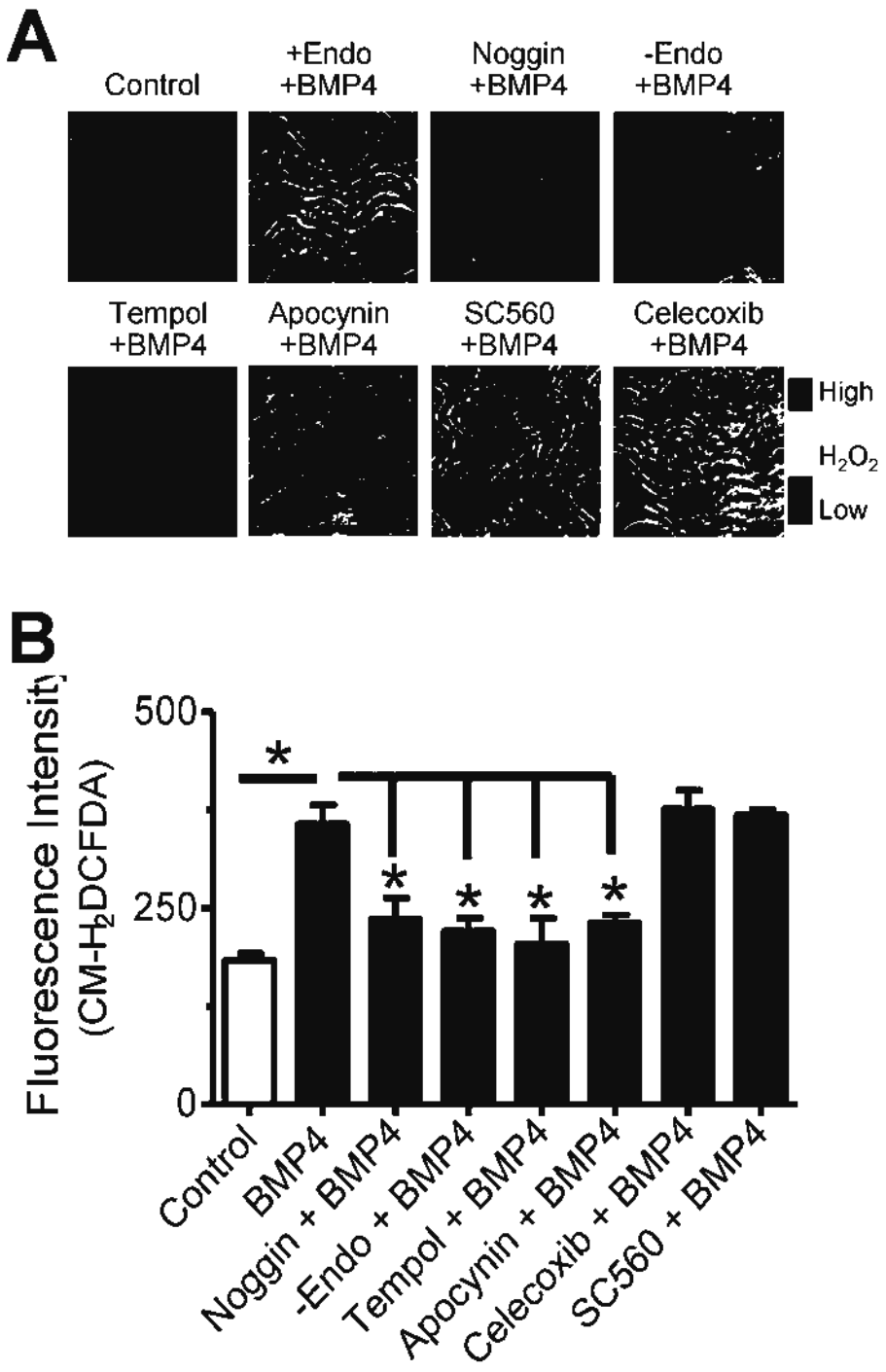


Figure 6.10.
(A) The stimulatory effect of BMP4 (20 ng/mL) on the intracellular level of ROS in endothelial cell *in situ* in cut-open freshly isolated mouse aortas as visualized by CM-H2DFDA fluorescent dye under various pharmacological treatments. **(B)** Summarized value for each fluorescence intensity. Data are mean ± SEM of 3-4 experiments from different mice.

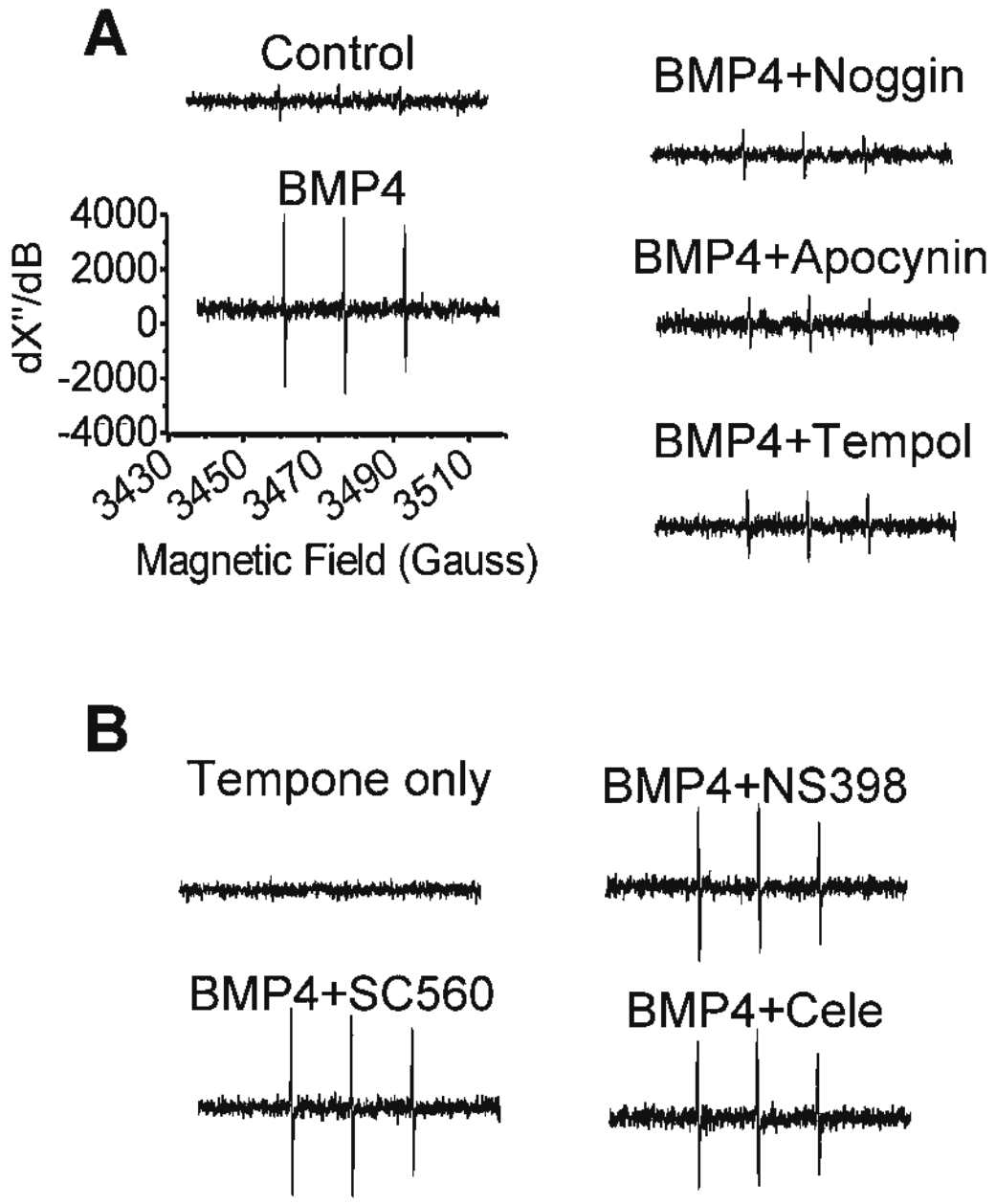


Figure 6.11.
Representative EPR spectra showing a markedly increased amplitude of ROS signal in BMP4 (20 ng/mL)-treated mouse aortas in response to ACh was inhibited by noggin, apocynin, tempol **(A)** but unaffected by SC560, NS398 and celecoxib **(B)**. Data are mean ± SEM of 4-6 mice. *P<0.05 between BMP4 and control or drug treatment.

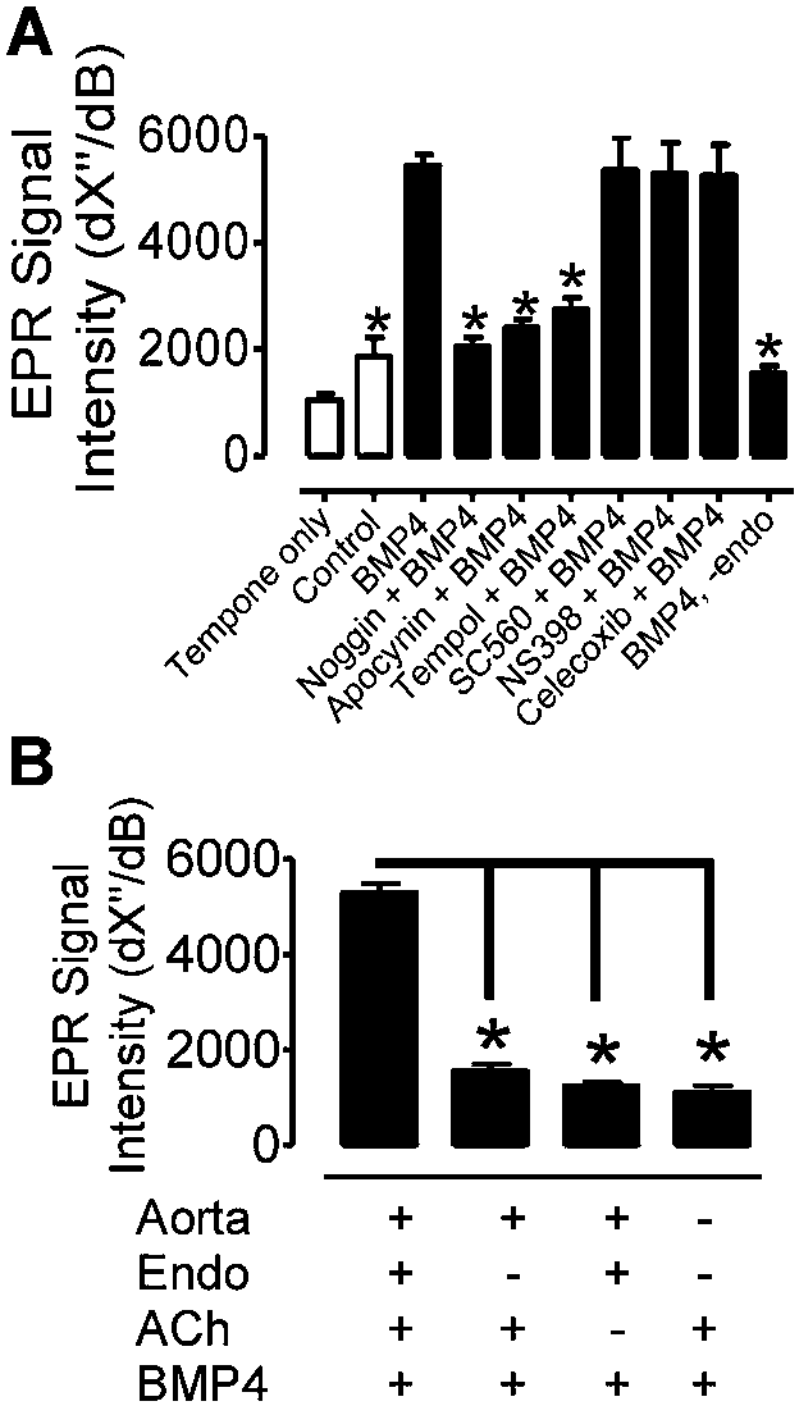


Figure 6.12.
(A) Summarized data for EPR spectra intensity under various pharmacological treatments of BMP4 (20 ng/mL)-exposed mouse aortas shown in Figure 6.11. **(B)** ACh-stimulated increase in EPR spectra intensity required the presence of endothelium. Data are mean ± SEM of 4-6 mice. *P<0.05 between BMP4 and control or drug treatment.

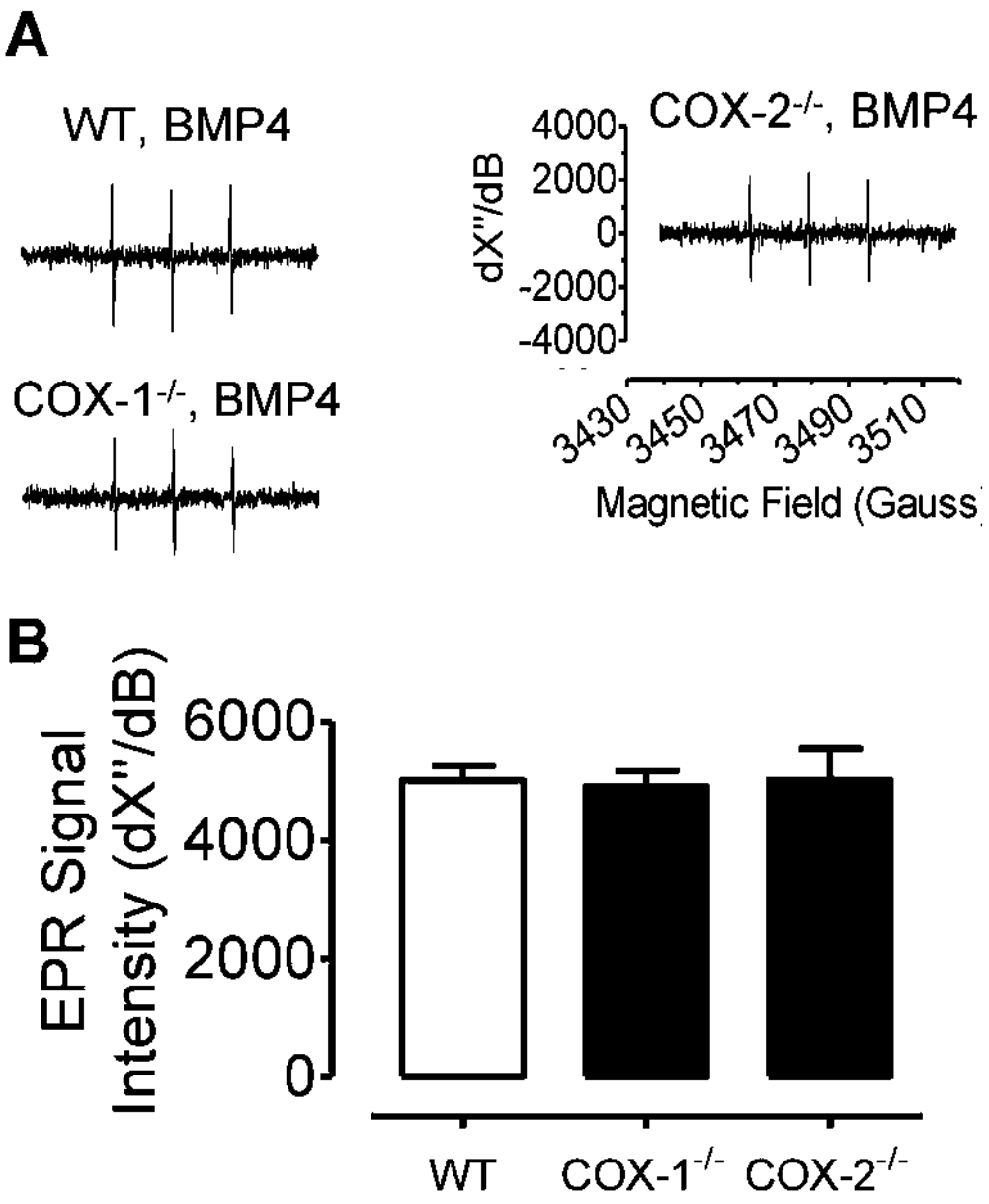


Figure 6.13.
(A&B) Representative EPR spectra showing the markedly increased amplitude of ROS signals was comparable in BMP4 (20 ng/mL)-treated mouse aortas of wild-type COX-$1^{-/-}$ and COX-$2^{-/-}$ mice. Data are mean ± SEM of 4 mice. *P<0.05 between BMP4 and control or drug treatment.

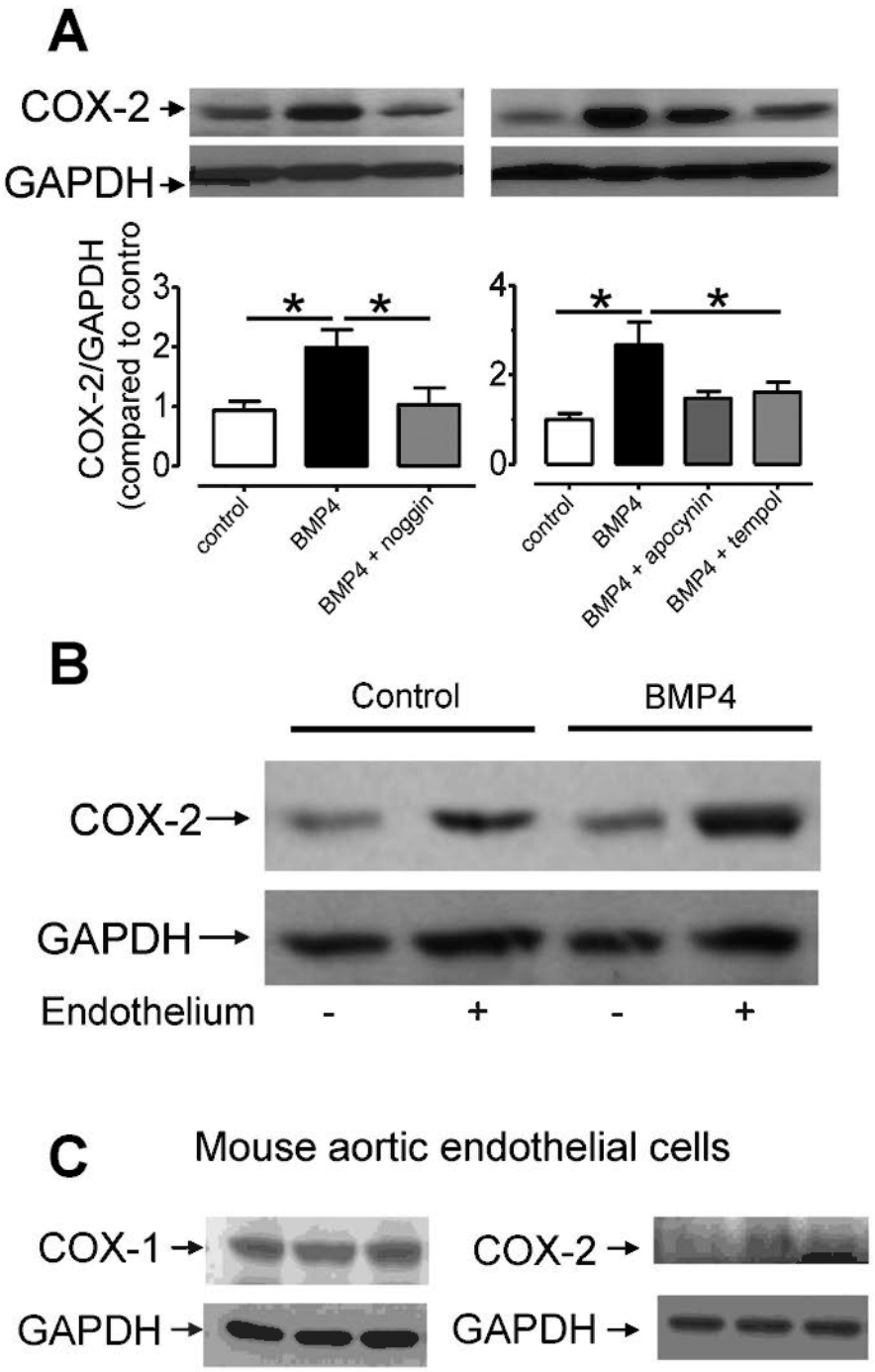


Figure 6.14.
Western blot analysis showing that BMP4 (20 ng/mL) up-regulated the expression of COX-2, which was prevented by co-treatment with noggin, apocynin or tempol **(A)**. The level of COX-2 expression was less in aortas without endothelium **(B)** and in cultured mouse aortic endothelial cells in response to BMP4 and noggin **(C)**. Data are mean ± SEM of 4-6 mice. *P<0.05 between curves.

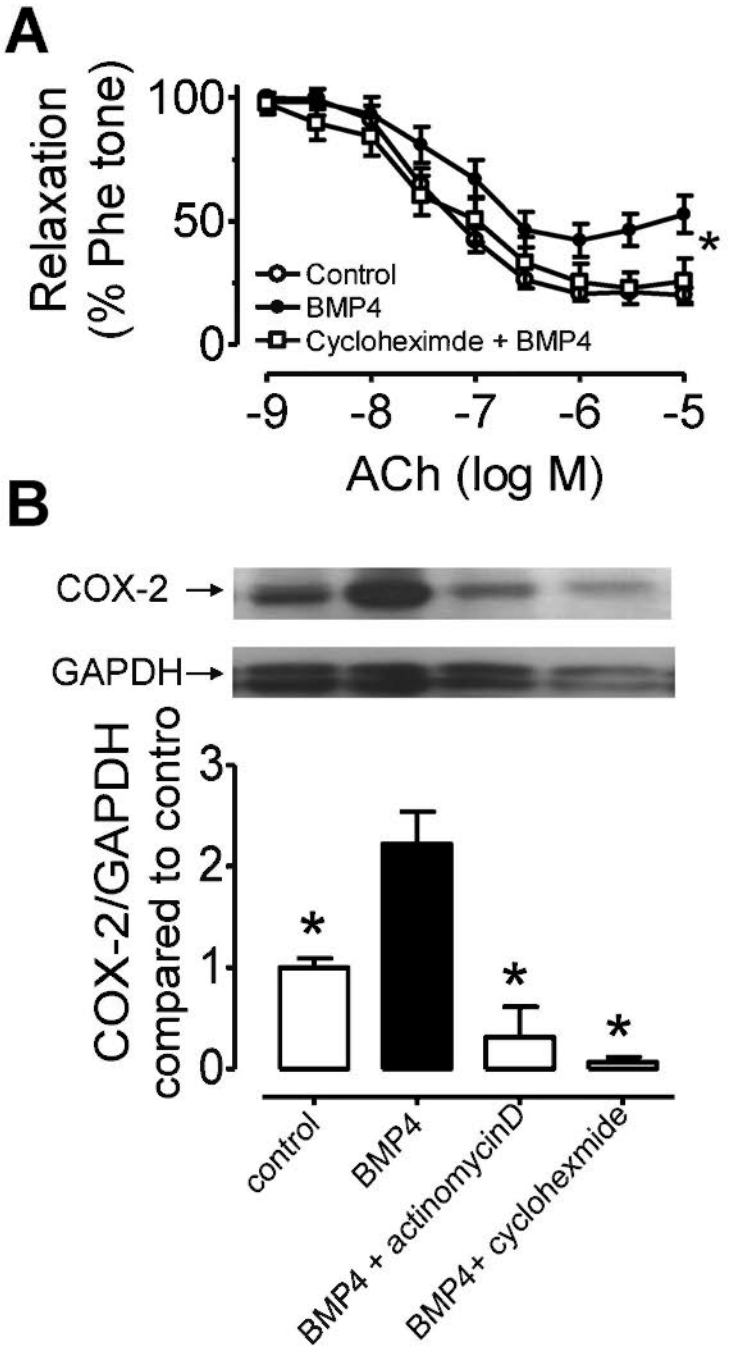


Figure 6.15.
(A) Prevention of BMP4 (20 ng/mL)-impaired endothelium-dependent dilatations by co-treatment (12 hr) with 10 µmol/L cycloheximide in mouse aortas. **(B)** Co-treatment with cycloheximide or 10 µmol/L actinomycin D inhibited the BMP4-induced up-regulation of COX2. Data are mean ± SEM of 4 mice. *P<0.05 versus BMP4.

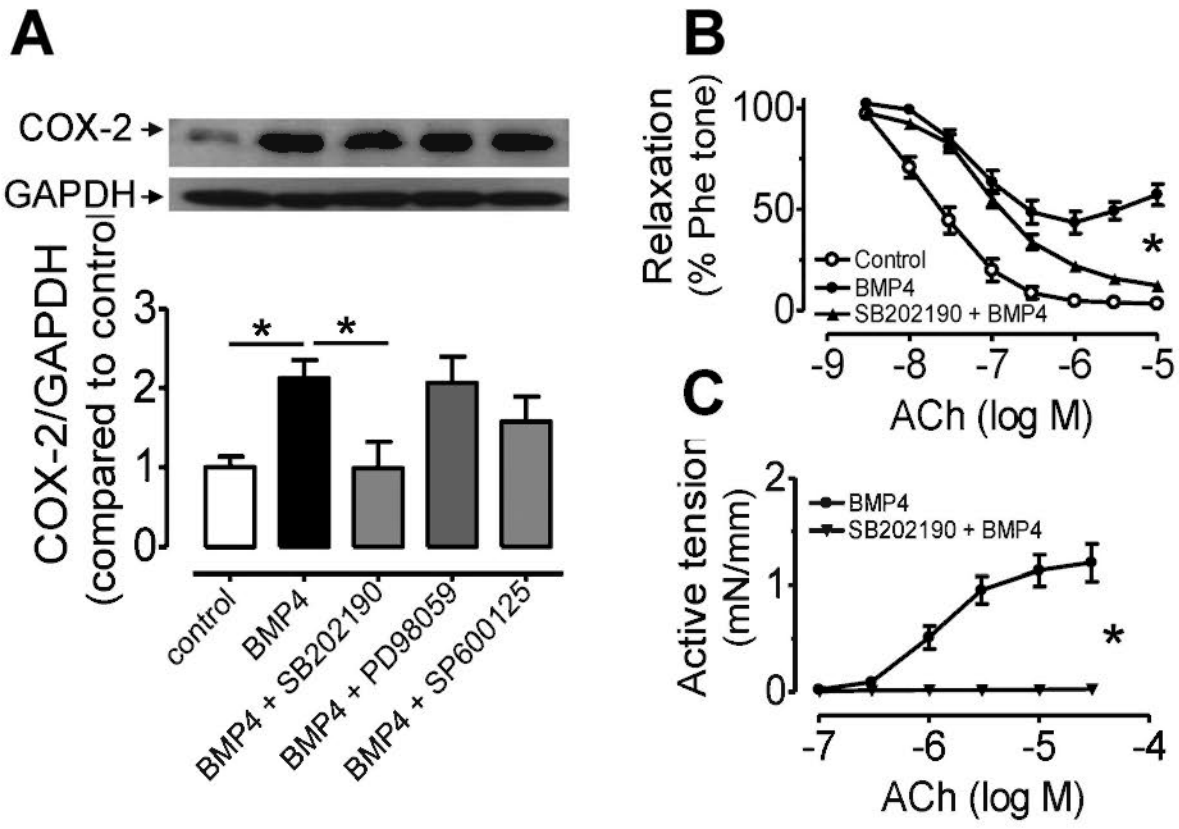
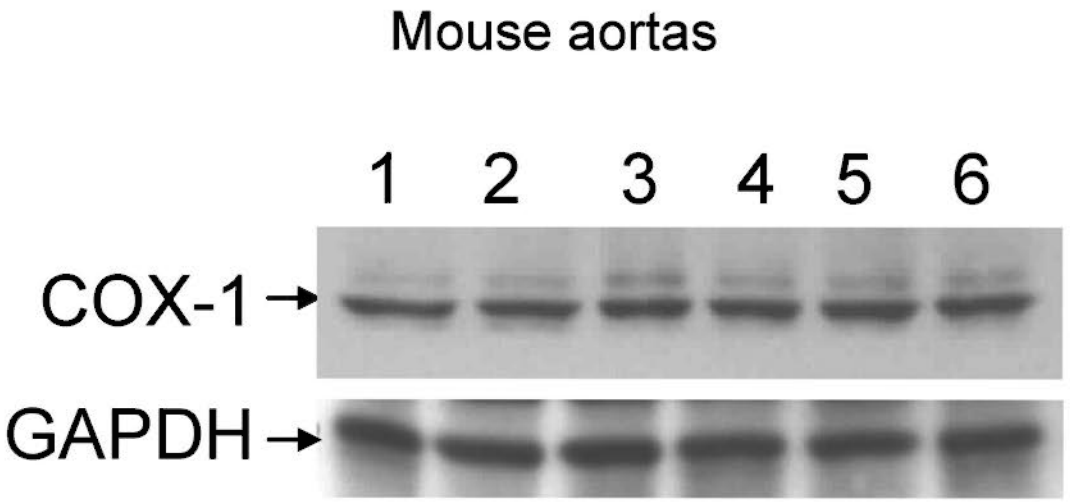


Figure 6.16.

(A) Inhibition of p38 MAPK but not ERK or JNK prevented BMP4 (20 ng/mL)-induced COX-2 over-expression. Co-treatment with SB202190 (p38 MAPK inhibitor, 10 µmol/L) ameliorated BMP4-induced endothelial dysfunction **(B)** and prevented endothelium-dependent contractions **(C)**. Data are mean ± SEM of 4-6 mice. **P*<0.05 between curves.



1. Control
2. BMP4
3. Noggin + BMP4
4. Tempol + BMP4
5. Apocynin + BMP4
6. SB202190 + BMP4

Figure 6.17
The expression of COX-1 was unchanged by treatment with BMP4 alone or in combination with noggin (100 ng/mL), tempol (1 mmol/L), apocynin (100 µmol/L) or SB202190 (10 µmol/L). Data are representative from three separate experiments.

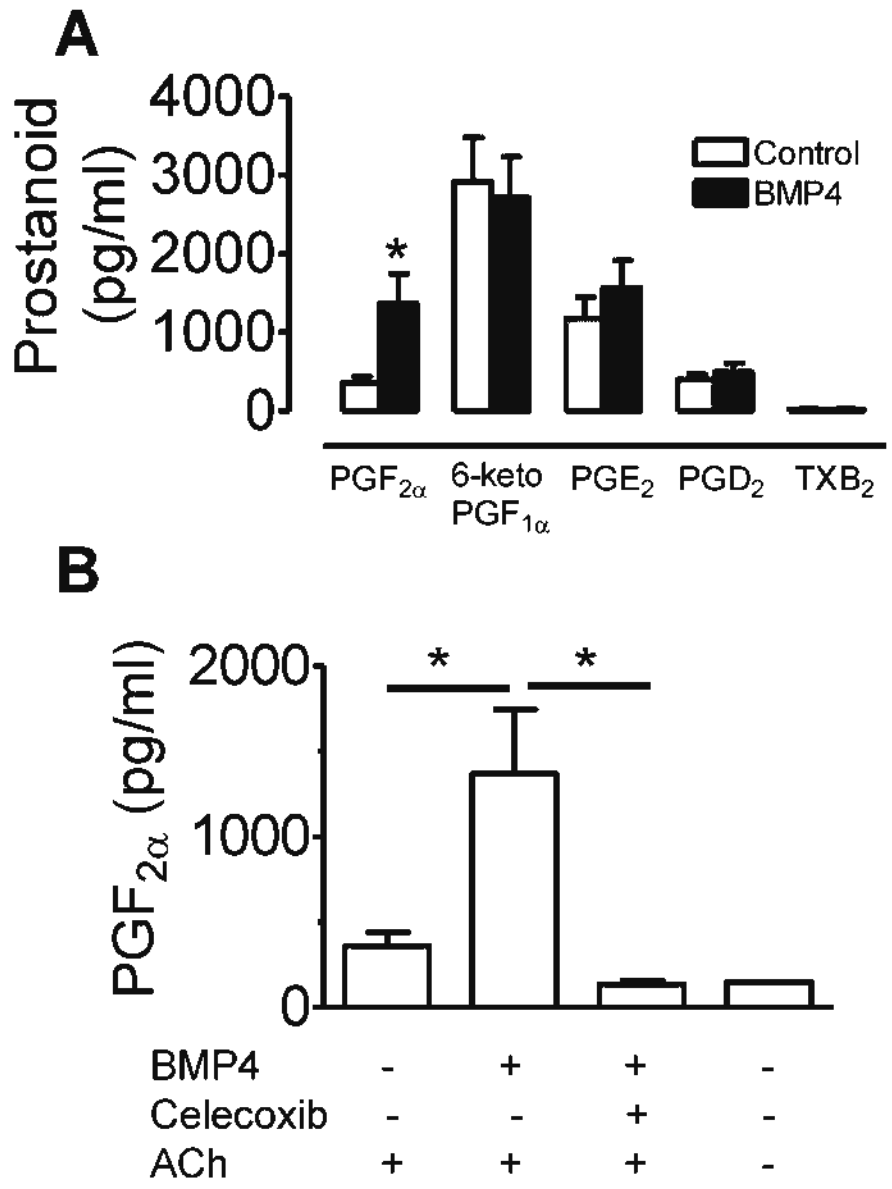


Figure 6.18.
(A) EIA measurement of the putative arachidonic acid metabolites, $PGF_{2\alpha}$, PGE_2, PGD_2, 6-keto $PGF_{1\alpha}$ and TXB_2 in the bathing solution of L-NAME-treated aorta in response to 10 µmol/L ACh. Among the five prostanoids, only the release of $PGF_{2\alpha}$ was augmented significantly by ACh. **(B)** ACh-stimulated $PGF_{2\alpha}$ increase was abolished by 3 µmol/L celecoxib. Mouse aortas were incubated with BMP4 (20 ng/mL) for 12 hours. Data are mean ± SEM of 3-5 experiments from different mice. *P<0.05 versus control.

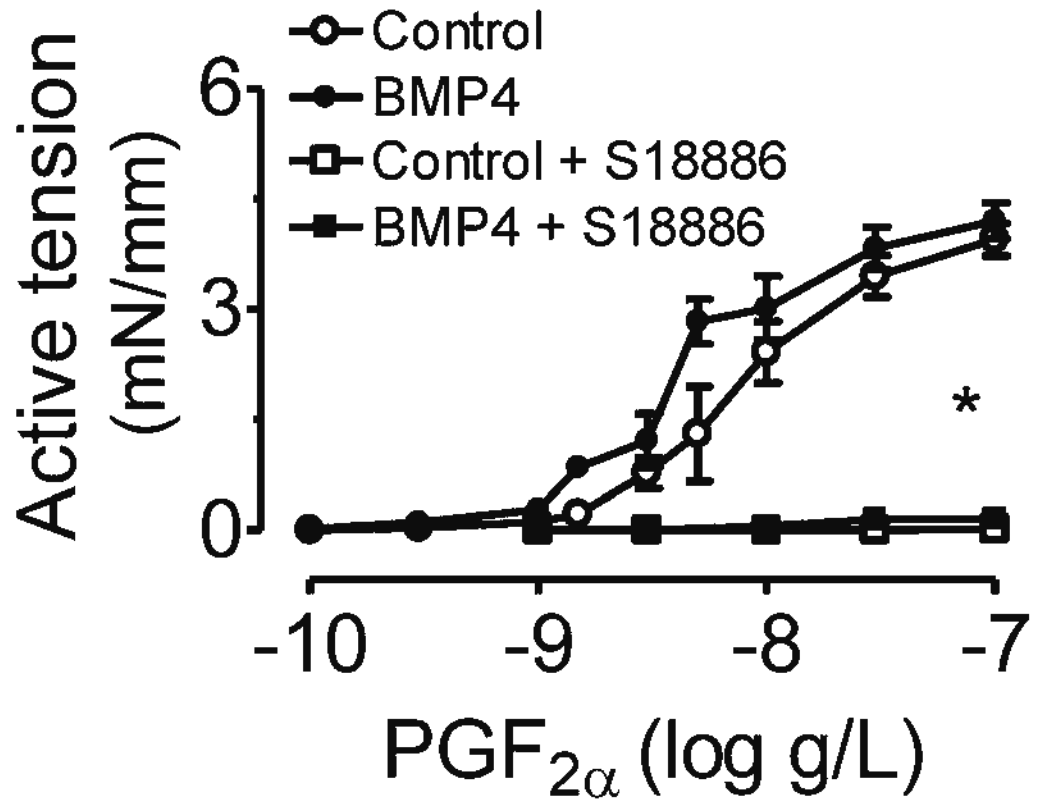


Figure 6.19.
Exogenous $PGF_{2\alpha}$ produced concentration-dependent contractions in aortic rings with and without BMP4 treatment and its abolition by 100 nmol/L S18886, the TP receptor antagonist. Data are mean ± SEM of 3-5 experiments from different mice. *P<0.05 between curves.

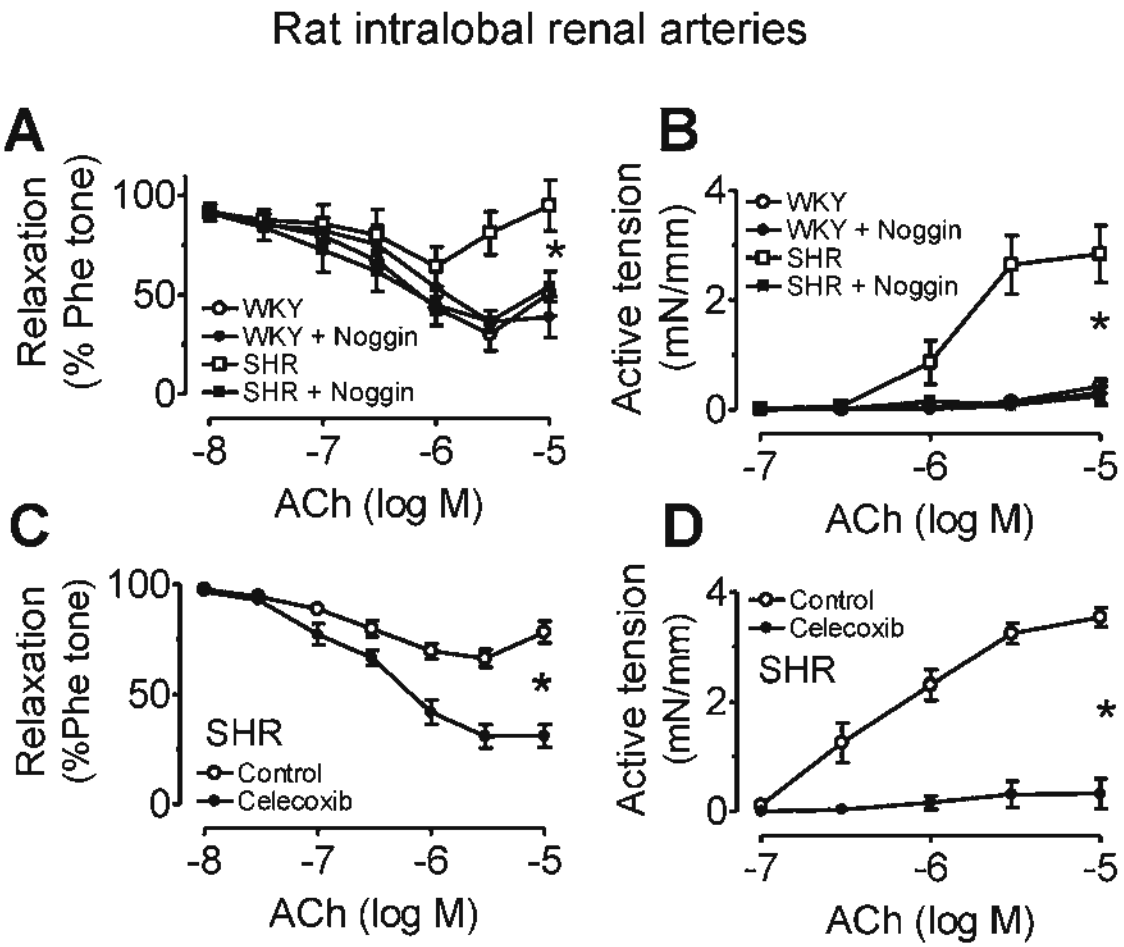


Figure 6.20.
Treatment (12-h) with 100 ng/mL noggin improved ACh-induced dilatations **(A)** and abolished endothelium-dependent contractions **(B)** in intralobal renal arteries of spontaneously hypertensive rats (SHR) but not of normotensive (WKY) rats. Celecoxib improved endothelial function **(C)** and prevented endothelium-dependent contractions **(D)**. Data are mean ± SEM of 6-8 rats. *P<0.05 between curves.

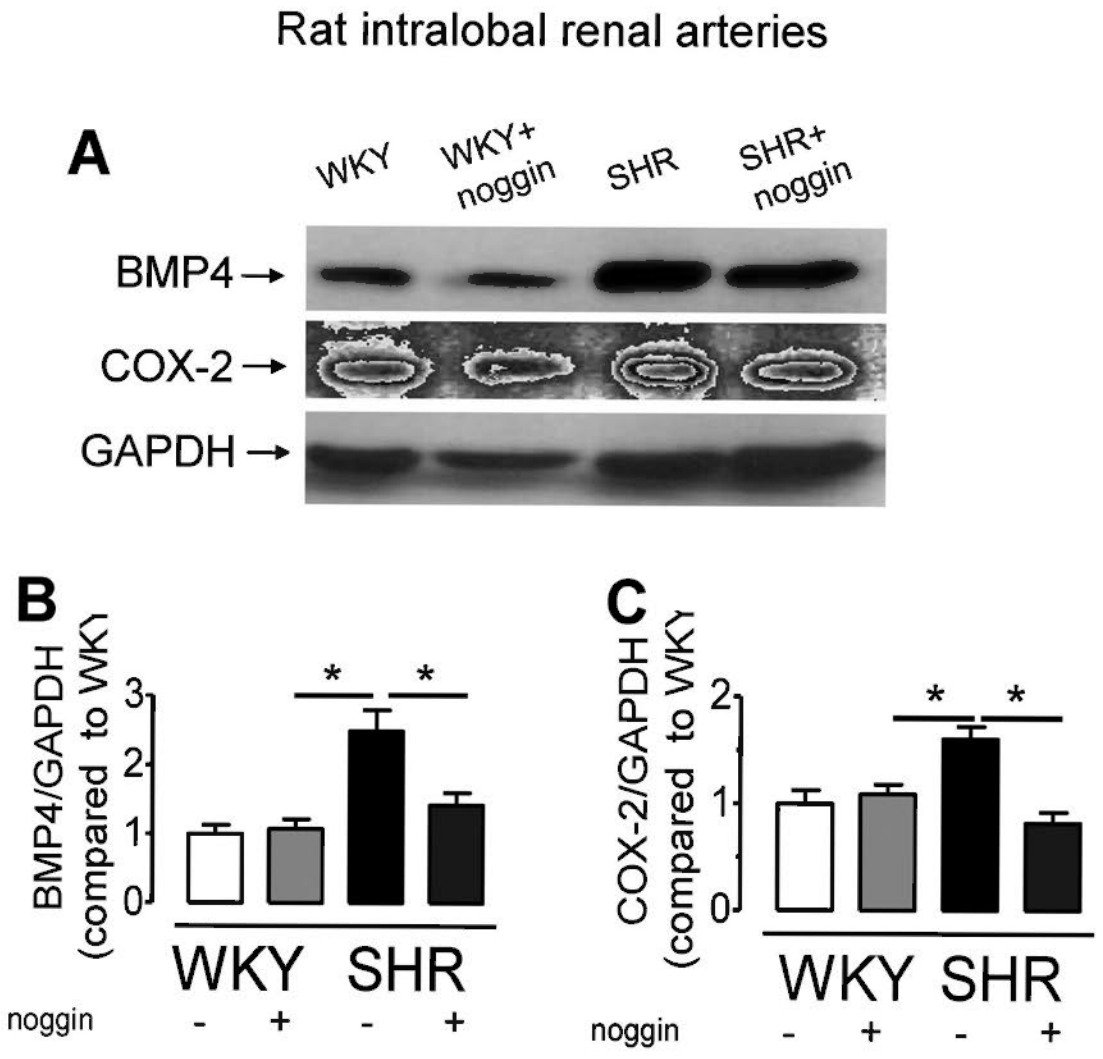


Figure 6.21.

(A) The increased expression of BMP4 and COX-2 was detected only in SHR renal arteries. Noggin treatment lowered the expression of BMP4 **(B)** and COX-2 **(C)**. Data are mean ± SEM of 6-8 rats. **P*<0.05 between curves.

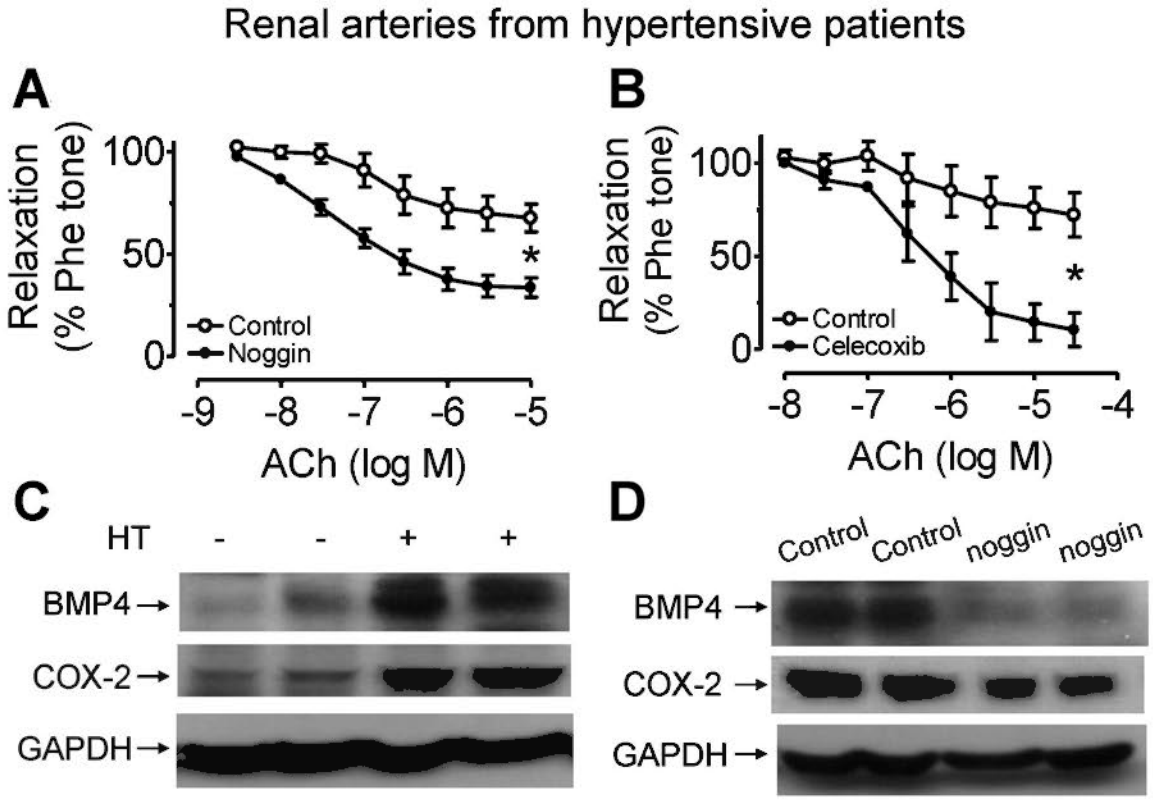


Figure 6.22.

Noggin **(A**, 100 ng/mL**)** or celecoxib **(B**, 3 µmol/L**)** treatment (12-h) improved ACh-induced relaxations in renal arteries from hypertensive patients. Increased expression of BMP4 and COX-2 in hypertensive patients compared with normotensive patients **(C)** and noggin reduced BMP4 and COX-2 expression in renal arteries from hypertensive patients **(D)**. Data are mean ± SEM of 3-5 subjects. **P*<0.05 between curves. HT, hypertension

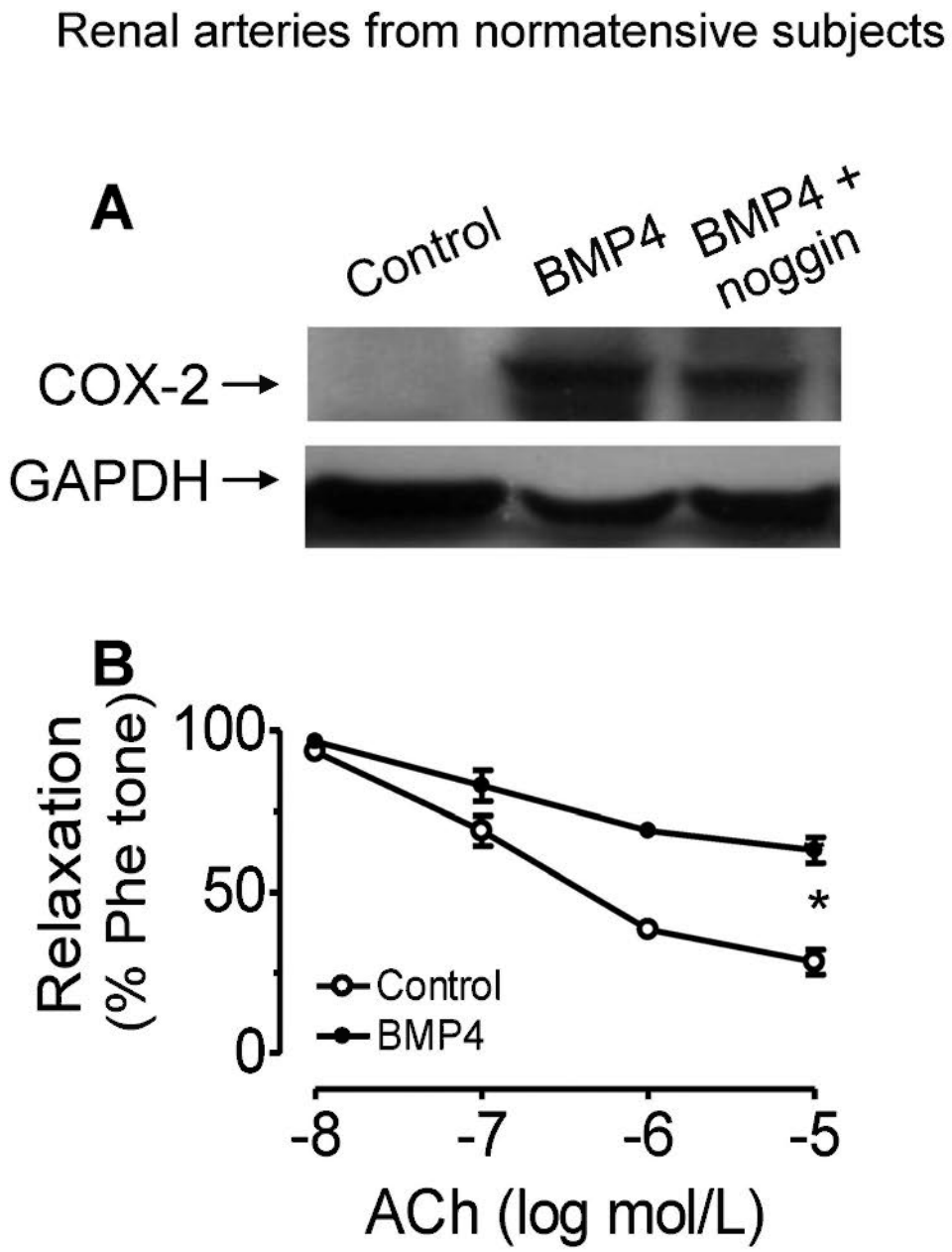


Figure 6.23.
BMP4 stimulated over-expression of COX-2 and noggin prevented such effect **(A)** and BMP4 impaired ACh-induced relaxations **(B)** in renal arteries from normotensive subjects. Data are mean ± SEM of 3-5 subjects. *P<0.05 between curves.

6.4 Discussion

The present study elucidates for the first time that up-regulated expression and activity of COX-2 plays an essential role in BMP4-induced endothelial dysfunction. Firstly, I demonstrate that BMP4 impairs endothelium-dependent dilatations and elicited endothelium-dependent contractions in mouse aortas, which can be abolished by BMP4 antagonist, BMP receptor Ia RNAi, COX-2 inhibitors, ROS scavengers, or in COX-2 deficient mice. Secondly, BMP4 exposure leads to increased expression and activity of constitutive COX-2 situated primarily in endothelial cells which release $PGF_{2\alpha}$ as an EDCF. Thirdly, COX-2 up-regulation is stimulated by oxidative stress and activation of p38 MAPK. Fourthly, BMP4 increases ROS production in mouse aortas which is sensitive to ROS scavengers and NAD(P)H oxidase inhibitor, but unaffected by COX-2 inhibition. Lastly, I provide novel evidence for a significant contribution of BMP4 and COX-2 to endothelial dysfunction in spontaneously hypertensive rats and its relevance to human hypertension. Collectively, the present findings clearly support that BMP4 as an upstream activator triggers the increased expression of COX-2 in endothelial cells as an important downstream target enzyme responsible for initiation and maintenance of endothelial dysfunction. A pathophysiological significance of BMP4 in hypertension is thus revealed.

Earlier work by others has demonstrated the involvement of BMP4 in atherosclerosis and hypertension, which was first described to mediate inflammatory responses of endothelial cells induced by shear stress via a ROS-dependent mechanism (Sorescu et al., 2003; 2004). BMP4 infusion causes hypertension and impairs vasorelaxations in mice through stimulation of NADPH oxidase (Miriyala et al., 2006). This study suggests that BMP4 could serve as a potential novel predictor of vascular dysfunction. Indeed, my results clearly show that BMP4 directly impaired endothelium-dependent dilatations in different vasculatures from three species. BMP4 blunted EDR in human arteries as well as in mice and rats. My data is in line with the recently reported results (Csiszar et al., 2006; Csiszar et al., 2008). The harmful effect of BMP4 on vasculature or endothelial cells can be inhibited by noggin or BMP4 siRNA (Sorescu et al., 2003; Chang et al., 2007). In addition, the present results show that the detrimental vascular action of BMP4 is mediated through BMP receptor Ia. BMP4 binds to BMP receptor with high affinity, which is involved in

endothelial development and survival (Kaneko et al., 2008).

I show for the first time that BMP4 can trigger endothelium-dependent contractions (EDC) which is a unique pathophysiological response seen in hypertension. Similar to the previous demonstration of a pivotal role of COX-2 in the appearance of EDC in hamster aortas (Wong et al., 2009), BMP4-induced EDC is abolished by COX-2 inhibitor. Of importance, $PGF_{2\alpha}$, released upon ACh stimulation acts as the major EDCF in BMP4-treated aortas. By contrast, ACh did not cause rises of other prostanoids, such as PGE_2, PGI_2, PGD_2 or TXA_2 in bathing solution of aortic rings. By comparing the results from EIA and subsequent functional studies on aortas with exogenously applied prostanoids, I found that exogenous $PGF_{2\alpha}$ at an equivalent amount detected by EIA produced a comparable level of contraction to EDC. This conclusion is consistent with the previous findings in hamster and human arteries that COX-2-derived $PGF_{2\alpha}$ mediates TP receptor-dependent EDC (Wong et al., 2009). By contrast, other prostanoids (PGE_2, PGI_2 and PGD_2) did not trigger contractions in mouse aortas, while TXA_2 existed only in very small amount as detected by EIA.

I next investigated whether the vascular effect of BMP4 depends on the activation of COX-2 which could be the target enzyme in response to BMP4-induced oxidative stress. The critical role of COX-2 in BMP4-induced endothelial dysfunction is well supported by the following observations. Co-treatment (12 h) with or acute exposure (30 min) to celecoxib prevented (1) BMP4-induced impairment of ACh dilatations, (2) endothelium-dependent contractions, and (3) BMP4-stimulated release of $PGF_{2\alpha}$. More importantly, BMP4 lost its ability to reduce endothelial function in COX-$2^{-/-}$ mice. ROS produced by NAD(P)H oxidase is reported to be the major downstream target of BMP4 to mediate inflammatory vascular responses. Two recent studies show that BMP4 induced ROS production in endothelial cells and in isolated rat arteries (Sorescu et al., 2004; Csiszar et al., 2006). With use of H_2O_2-sensitive florescent dye and EPR spectroscopy spin trap to assess the ROS production in mouse aortas *in situ*, I were able for the first time to confirm the endothelial origin of ROS production induced by BMP4 with and without ACh stimulation. I also found that the ROS production was not reduced by chemical inhibition of COX-2 nor in COX-$2^{-/-}$ mice. However, ROS removal annulled BMP4-induced COX-2 over-expression despite an earlier report that COX may be involved in the

generation of ROS in the vascular wall (Tang et al., 2007). The discrepancy in a causal relationship between ROS and COX-2 may also depend upon species, systems and methods used in different studies. Taken together, ROS is the upstream activator of BMP4-mediated COX-2 up-regulation. COX-2 is constitutively expressed in mouse endothelial cells. Likewise, the constitutive presence of COX-2 was also reported in hamster, rat and human arteries, and in cultured endothelial cells (Baber et al., 2003; Therland et al., 2004; Wong et al., 2009). Moreover, BMP4-induced COX-2 up-regulation may occur at the transcription level as both cycloheximide and actinomycin D prevented COX-2 up-regulation. By contrast, specific COX-1 inhibitor did not influence the vascular effect of BMP4. Finally, BMP4 did not alter expression of COX-1 in mouse aortas. Collectively, I provide mechanistic evidences showing that COX-2 is most likely to serve as the missing link between BMP4 and endothelial dysfunction.

The present study also indicates that p38 MAPK activation participates in the regulation of the COX-2 expression since the MAPK inhibitor attenuated COX-2 up-regulation, improved endothelial function, and prevented endothelium-dependent contractions in BMP4-treated mouse aortas. ROS was shown before to enhance the activity of p38 MAPK in the vascular tissues (Goettsch et al., 2009; Usatyuk et al., 2003; Wang et al., 2004). BMP4 can activate MAPK (p38 and p44/42) in endothelial cells and myocytes (Csiszar et al., 2006, Yang et al., 2007). I have hence provided the first line of evidence that MAPK activation is functionally coupled to COX-2-dependent EDC and impairs vasodilatation in response to BMP4.

The role of BMP4 and COX-2 in altered vascular reactivity is also revealed in spontaneously hypertensive rats (SHR). Treatment of SHR renal arteries with either noggin or celecoxib normalized dilatations to the level in WKY arteries and abolished endothelium-dependent contractions. BMP4 infusion causes hypertension in mice (Miriyala et al., 2006) and promotes vascular remodeling in pulmonary hypertension (Frank et al., 2005). However, whether BMP4 is linked to essential hypertension is unclear. The present study shows a marked increase of BMP4 and COX-2 in hypertensive animals and both were attenuated by noggin, thus suggesting an association between BMP4 and COX-2 in hypertension. I also revealed such association in renal arteries from hypertensive patients.

To conclude, the present study demonstrates a critical role of endothelial COX-2 in BMP4-induced endothelial dysfunction **(Figure 6.24)**. BMP4-induced oxidative stress lowers NO bioavailability and up-regulates COX-2 in endothelial cells. Endothelial COX-2 catalyzes the formation of $PGF_{2\alpha}$, which represents a pathological EDCF in mouse aortas challenged by BMP4. Besides, BMP4 antagonist noggin protects endothelial function in hypertension. Since both BMP4 and COX-2 are expressed in hypertensive human renal arteries, the present data might be highly relevant for cardiovascular disease. Indeed, the present findings in mouse, rat and human arteries supports an increasingly important role of BMP4-dependent COX-2 in endothelial dysfunction in hypertension. Although the cardiovascular adverse effects are associated with the long-term use of COX-2 inhibitors (Hinz et al., 2007), these drugs continue to be instrumental in better understanding of how the COX-2 signaling pathway is disrupted in vascular disorders. As COX-2 is a well-established pro-inflammatory enzyme and BMP4 is a novel upstream activator of vascular inflammation, the present results also suggest that the BMP4 signaling cascade could be potentially useful targets for pharmacological intervention of COX-2-dependent vascular events, particularly in view of recently publicized cardiovascular toxicity of COX-2 inhibitors.

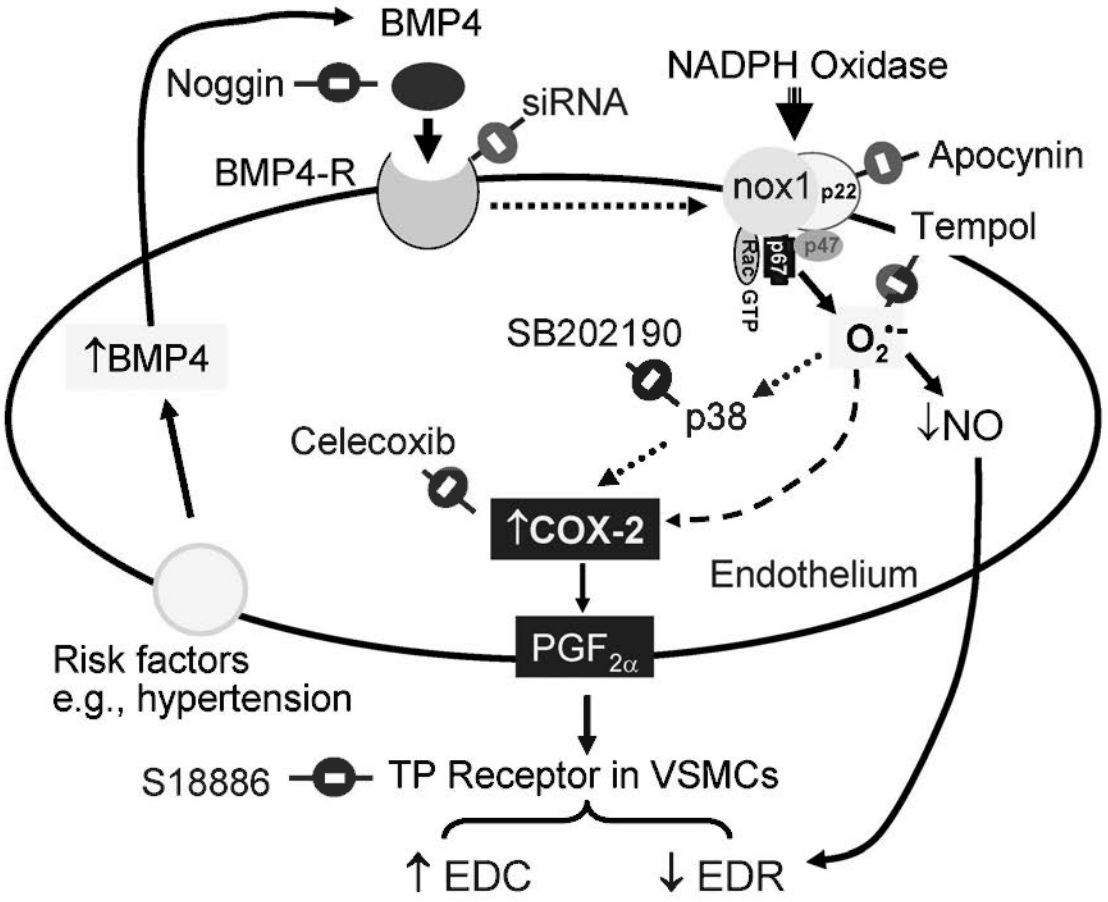


Figure 6.24.

Proposed cellular signaling cascade mediating BMP4-induced endothelial dysfunction and endothelium-dependent contractions. BMP4 by acting on BMP receptor 1a in the endothelial cells, causes an increased production of NAD(P)H oxidase-dependent superoxide anions, resulting in MAPK activation that leads to over-expression and activity of COX-2. COX-2-dependent generation of prostanoids from arachidonic acid impair endothelium-dependent relaxations and trigger endothelium-dependent contractions. $PGF_{2\alpha}$ is the most likely candidate of endothelium-derived contracting factor (EDCF) which diffuses towards adjacent vascular smooth muscle cells (VSMCs) and activates TP receptors on VSMCs to trigger contraction. Risk factors such as hypertension increase the expression of BMP4 in the endothelium which may act as autocrine regulator for COX-2 over-expression to impair endothelial function. COX-2, cyclooxygenase-2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; TP receptor, thromboxane-prostanoid receptor. EDR, endothelium-dependent relaxations; EDC, endothelium-dependent contractions.

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