Calcitriol Protects Renovascular Function in Hypertension and Estrogen Deficiency

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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 August 2008 and June 2011. 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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Abstract

Vitamin D modulates ~3% of all gene expression in humans. Recently, vitamin D is reported to be beneficial to the cardiovascular function in addition to its classical effect on calcium homeostasis. However, it remains elusive as to how vitamin D protects renovascular function in pathological states, and whether the protection involves alterations in the protein expression of key biomarkers involved in vascular dysfunction. Using spontaneously hypertensive rats (SHRs) and ovariectomized rats as the animal models and human renal arteries, I investigated whether calcitriol, an active form of vitamin D, could protect against renovascular dysfunction in hypertension and under estrogen deficiency.

In the first part of study, I investigated the anti-oxidative effects of calcitriol in the renovasculature in hypertension. Changes in isometric tension recorded by myographs showed that pronounced endothelium-dependent contractions were observed in the SHR intrarenal arteries. Chronic calcitriol treatment (150 ng/kg/day for 4.5 months) and overnight arterial culture with calcitriol significantly reduced the endothelium-dependent contractions. The contractions were inhibited by losartan [angiotensin II type 1 receptor (AT_1R) antagonist], diphenyleneiodonium [inhibitor of NAD(P)H oxidase] and tempol (ROS scavenger). Western blot analysis showed that *in vivo* and *in vitro* calcitriol treatment reduced the expressions of AT_1R and NAD(P)H oxidase subunits while increasing the expression of superoxide dismutase (SOD)-1 and SOD-2 in the SHR renal arteries. DHE fluorescence and electron paramagnetic resonance revealed a reduction in the ROS level in the calcitriol-treated SHR renal arteries. *In vitro* calcitriol incubation reversed the impaired endothelium-dependent relaxations and reduced the exaggerated endothelium-dependent contractions in Ang II-treated WKY renal arteries. DHE

fluorescence revealed that calcitriol prevented Ang II-induced ROS production in WKY aortic endothelial cells. Noteworthy, *in vitro* calcitriol treatment overnight partially restored the impaired relaxations and normalized the expression of NAD(P)H oxidase subunits in the renal arteries from hypertensive patients. The effect of calcitriol was prevented by a vitamin D receptor (VDR) antagonist, TEI-9647. This part of the study suggests that AT_1R and ROS play critical roles in the pathogenesis of renovascular dysfunction in hypertension. Treatment with calcitriol normalized the elevated expression of AT_1R, NAD(P)H oxidase subunits and ROS production possibly via VDR activation.

In the second part of the study, I examined the anti-inflammatory effects of calcitriol under estrogen deficiency. Incidence of cardiovascular events and osteoporosis increased significantly in women after menopause. While vitamin D is supplemented to postmenopausal women for the treatment and prevention of osteoporosis, it remained unknown whether vitamin-D supplementation could offer protection to the renal arterial function in estrogen deficient states. The present study investigated the changes in the renovascular reactivity in estrogen-deficient ovariectomized rats and whether calcitriol, an active form of vitamin D, could reverse the altered vascular function. Isometric force measurement using myographs showed that acetylcholine-induced endothelium-dependent relaxations were impaired in renal arteries from ovariectomized rats. The impairment was ameliorated by 4.5-month oral treatment with calcitriol (150 ng/kg/day). Acute treatment of non-selective cyclooxygenase (COX) inhibitor (indomethacin), specific inhibitor against COX-2 (celecoxib, NS-398 and DuP-697) or thromboxane-prostanoid (TP) receptor

antagonist (S18886) restored the acetylcholine-induced relaxations, while COX-1 inhibitor (SC-560) was without effect. Western blot analysis showed that calcitriol treatment reduced the elevated expression of COX-2 and TP receptor in renal arteries and aortic endothelial cells from ovariectomized rats. Overnight tissue culture with calcitriol improved relaxations, normalized contractions to a TP receptor agonist (U46619) and downregulated TP receptor in renal artery of ovariectomized rats. Confocal microscopy showed that NO production was attenuated in the aortic endothelial cells cultured from ovariectomized rats. Overnight treatment with calcitriol, celecoxib or S18886 increased the NO production, and calcitriol reduced the expression of endothelial COX-2 and TP receptor. Interestingly, TP receptor expression was also decreased by celecoxib. U46619 impaired the endothelium-dependent relaxations in the renal arteries from sham control, and calcitriol normalized the relaxations and down-regulated the TP receptor expression. The expression of COX-2 and TP receptor in renal arteries from female patients at postmenopausal age was downregulated by calcitriol, of which the effect was prevented by TEI-9647. The results indicated that the impaired endothelium-dependent relaxations under estrogen deficiency are possibly attributed by the release of COX-2-derived prostaglandin(s) that activate the TP receptor and that calcitriol normalizes the renovascular function at least in part through COX-2 and TP receptor down-regulation.

To conclude, I demonstrated a positive role of oxidative stress in the renovascular dysfunction in hypertension and pointed out the dysregulation of

arachidonic acid cascade in the renal arteries under estrogen deficiency. Vitamin D restored the renovascular function via activation of the vitamin D receptor. The present study provides novel evidence of the therapeutic potential of vitamin D in preserving renovascular function under pathological conditions.

论文摘要

维生素 D 可以直接或者间接调节体内大约百分之三的基因表达。除了维持钙磷稳态的作用之外，有报道指出维生素 D 对心血管系统具有保护作用。但是，目前对于维生素 D 是否可以缓解内皮功能障碍，以及作用靶点尚未有明确报道。本实验旨在观察活性维生素 D calitiriol 是否可以延缓自发性高血压大鼠及高血压病人肾血管内皮功能损伤、卵巢切除大鼠及绝经后妇女肾血管内皮功能损伤，及其作用机制。

第一部分实验结果显示 calcitriol 具有潜在的抗氧化功能。具体结果如下：高血压大鼠肾血管的内皮依赖性收缩表现明显增强，以 150ng/Kg/day 的剂量灌胃给予 calcitriol 连续 4.5 个月，或者 calcitriol 过夜孵育高血压大鼠离体肾血管，均可以减弱内皮依赖性收缩。同时，离体血管急性孵育血管紧张素 I 型受体阻断剂 losartan，NADPH 氧化酶的特异性抑制剂 diphenyleneiodonium，以及 ROS 清除剂 tempol 都可以抑制收缩。Western Blot 结果显示高血压大鼠慢性口服 calictriol 后可以将肾血管中表达上调的血管紧张素 I 型受体和 NAD(P)H 氧化酶亚单位，以及表达下降的 SOD-1 和 SOD-2 调节到相对正常的表达水平。高血压大鼠肾血管离体过夜孵育 calcitriol 后，应用 DHE 荧光染色和 EPR 电子捕获技术显示，血管 ROS 含量有所减少，并且能使血管紧张素 I 型受体，NAD(P)H 氧化酶亚单位恢复到正常的表达水平。此外，血管紧张素 II 离体孵育血管正常的雄性 WKY 大鼠肾血管，会诱发内皮依赖性舒张减弱、内皮依赖性收缩明显增强，同时予以 calcitriol 孵育，可以改善内皮依赖性舒张、抑制收缩，还可以将由于 AngII 刺激所诱发的 NAD(P)H 亚单位 NOX-2,NOX-4 的过高表达降低到正常水平。原代培养 WKY 主动脉内皮细胞后 DHE 荧光染色指出 calcitriol 可以降低由血管紧张素 II 刺激导致的 ROS 过度产生。于此相对应的，临床高血压病人肾血管离体应用 calcitriol 后，得到相同的实验结果：内皮依赖性舒张得以改善，同时应用人组织特异性的维生素 D 受体阻断剂后，这种保护作用将会消失。血管紧张素 II 诱发人主动脉内皮

细胞 ROS 过量生成，结果显示 calcitriol 可以逆转这种诱发作用，而在 TEI-9647 存在的时候，calcitriol 这种逆转作用就不再存在。综上结果，血管紧张素 I 型受体和 ROS 在高血压大鼠内皮功能障碍的发病过程当中发挥这关键的作用，长期慢性给予 calcitriol 后，可以通过维生素 D/受体通路，可以下调它们的表达和含量，保护肾血管功能。

第二部分实验结果显示 calcitriol 在卵巢切除大鼠肾血管中具有潜在的抗炎作用。女性心血管疾病和骨质疏松症在绝经后发病率会显著增加，为了预防和治疗骨质疏松，临床会采用维生素 D 疗法，对于维生素 D 治疗骨质疏松的同时是否还可以同样保护血管功能的研究还很少。本实验旨在研究雌性大鼠卵巢切除后是否导致肾血管内皮功能损伤，同时给予 calcitriol 是不是可以肾血管功能损伤及其作用机制。实验结果显示卵巢切除后，肾血管功能受到损伤，内皮依赖性舒张减弱。给予 calcitriol 4.5 月后，肾血管舒张得以改善；在卵巢切除大鼠肾血管急性应用非选择性环氧合酶抑制剂 indomethacin，特异性 2 型环氧合酶抑制剂 celecoxib，NS-398 和 DuP-697，以及血栓素 A_2 受体拮抗剂 S18886 能够改善内皮依赖舒张，I 型环氧合酶抑制剂 SC560 却不能改善舒张。Western Blot 结果显示，卵巢切除后，肾血管内的 COX-2 和 TP 受体表达增加，慢性给予 calcitriol 可以减弱这两种蛋白的表达。离体血管过夜孵育 clacitriol 后，肾血管的舒张有所好转，并且明显抑制由 U46619 诱发的剂量依赖性收缩，此外可以下调 TP 受体的过度表达。共聚焦显微镜技术显示卵巢切除后，主动脉内皮细胞的一氧化氮释放减少，而用 calcitriol, celecoxib 和 S18886 过夜孵育可以增加一氧化氮的释放，另外实验证实卵巢切除后主动脉内皮细胞的 COX-2 表达上调，calcitriol 孵育后使其部分下降;TP 受体表达上调，calcitriol 和 celecoxib 孵育均能将其降至正常水平。使用 U46619 孵育正常雌性大鼠肾动脉后，内皮舒张明显减弱，同时应用 calcitriol, S18886 和 celecoxib 能改善舒张，并且 U466619 孵育过夜能够增强肾血管 TP 受体的表达，同时孵育 calcitriol, S18886 和 celecoxib 能使其恢复到正常表达水平。共聚焦显微镜实验结果表明

U466619 过夜孵育可以导致雌性大鼠内皮细胞一氧化氮释放减少，予以 calcitriol 可以增加一氧化氮的产生。临床实验结果，用 calcitriol 离体孵育年老女性病人肾血管后，血管内 COX-2 和 TP 受体表达下降，同时应用人组织维生素 D 受体拮抗剂剂 TEI-9647 后，calcitriol 的作用被阻断。综上所述，慢性口服给予 calcitriol 能改善卵巢切除导致的肾血管内皮功能障碍，其保护作用有可能是通过维生素 D/ 受体抑制 COX-2/TP 受体这一通路得以实现。

综上所述，我的实验结果显示了活性维生素 D 可以通过其潜在的抗氧化作用缓解高血压肾血管内皮功能损伤、通过其潜在的抗炎作用缓解卵巢切除所致的肾血管内皮功能损伤；以上保护作用都是通过维生素/受体机制得以实现。这些实验结果证明了维生素 D 能够在病理状态下保护肾血管内皮功能，具有潜在的临床治疗功能，为维生素临床治疗使用提供了有力的证据。

Abbreviations

ACh	Acetycholine
Ang II	Angiotension II
AT_1R	Angiotension II type 1 receptor
AT_2R	Angiotension II type 2 receptor
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DHE	Dihydroethidium
DMSO	Dimethyl sulfoxide
EDCF	Endothelium-derived contracting factors
EDRF	Endothelium-derived relaxing factors
OVX	Ovariectomy
PGD_2	Prostaglandin D_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGE_2	Prostaglandin E_2
PGI_2	Prostacyclin I_2
RAS	Renin-angiotensin system
ROS	Reactive oxygen specious
SHR	Spontaneously hypertensive rats
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
TP receptor	Thromboxane-prostanoid receptor
TXA_2	Thromboxane A_2
WKY	Wistar Kyoto rat
VDR	Vitamin D receptor

Publications

Manuscript recently submitted

Dong Jinghui, Wong Siu Ling, Lau Chi Wai, Lee Hung Kay, Ng Chi Fai, Zhang Lihong, Yao Xiaoqiang, Chen Zhen Yu, Vanhoutte Paul M, Huang Yu. Calcitriol protects renovascular function in hypertension by down-regulating angiotensin II type 1 receptors and reducing oxidative stress. (European Heart Journal, 2011, resubmitted)

Manuscripts in preparation

1. Dong Jinghui, Wong Siu Ling, Lau Chi Wai, Yao Xiaoqiang, Chen Zhen Yu, Huang Yu (2011). Calcitriol protects renovascular function of ovariectomized rats through the down-regulation of cyclooxygenase-2 and TP receptor.
2. Dong Jinghui, Wong Siu Ling, Lau Chi Wai, Yao Xiaoqiang, Chen Zhen Yu, Huang Yu (2011). Inhibitory effects of calcitriol on COX-1 and COX-2 expressions in renal vasculature.

Conference abstracts

1. Jinghui Dong, Siu Ling Wong, Chi Wai Lau, Zhen Yu Chen, Xiaoqiang Yao, Yu Huang. Calcitriol protects renovascular function of ovariectomized rats through TP receptor down-regulation. Annual meeting, The Institute of Cardiovascular Science and Meeting, December 2010, Hong Kong. (Poster presentation)
2. Jinghui Dong, Siu Ling Wong, Chi Wai Lau, Zhen Yu Chen, Xiaoqiang Yao, Yu Huang. Cyclooxygenase-2-derived product(s) impairs renovascular function in ovariectomized rats. Scientific Meeting of Asian Society for Vascular Biology.

December 2010, Hong Kong. (Poster presentation)

3. Jinghui Dong, Siu Ling Wong, Chi Wai Lau, Zhen Yu Chen, Xiaoqiang Yao, Yu Huang. Calcitriol protects renovascular function in the spontaneously hypertensive rats. Postgraduate Research Day, School of Biomedical Sciences, the Chinese University of Hong Kong, December 2010, Hong Kong (Chaired poster presentation).
4. Jinghui Dong, Siu Ling Wong, Chi Wai Lau, Zhen Yu Chen, Xiaoqiang Yao, Yu Huang. Calcitriol treatment inhibits endothelium-dependent contractions in the renal arteries of spontaneously hypertensive rats. Annual meeting, Hong Kong Pharmacology Society. December 2010, Hong Kong (Oral presentation).
5. Jinghui Dong, Siu Ling Wong, Xiaoyu Tian, Hung Kay Lee, Wing Yan Lee, Chi Wai Lau, Xiaoqiang Yao, Yu Huang. ROS does not contribute to the acute development of nitroglycerine tolerance in rat aortas. 9th Annual meeting, The Chinese Association of Pathophysiology, November 2010, Nanjing. (Chaired poster presentation)
6. Jinghui Dong, Siu Ling Wong, Xiaoyu Tian, Hung Kay Lee, Wing Yan Lee, Chi Wai Lau, Xiaoqiang Yao, Yu Huang. ROS does not contribute to the acute development of nitroglycerine tolerance in rat aortas. Annual meeting, The Institute of Cardiovascular Science and Meeting, December 2009, Hong Kong. (Poster presentation)

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Chapter I

Introduction

1.1 Introduction of vitamin D

Vitamin D is a fat soluble hormone in our body despite it is categorized as vitamin. Clinically it is used for the treatment of rickets, osteoporosis and hyperparathyroidism.

1.1.1 The discovery of vitamin D

Vitamin D plays a major role in the maintenance of calcium and phosphate homeostasis and bone mobilization (Anderson et al., 2003; Verhave and Siegert, 2010). Research in relation to vitamin D mainly focuses on its effect on bone mineralization and skeletal growth. Its discovery resulted from a non-stop struggling in search for an efficient therapeutic for rickets. Rickets is a bone disease that was very common in the 1600-1900s and is characterized by bone pain, attenuated bone growth, and bone weakness. Epidemiological studies show that the incidence of rickets are significantly higher in people living in densely populated cities compare to those living in the rural area, taking place in cold foggy winter than in sunny summer, implying that sunlight exposure may be associated with rickets. People from different populations are gradually more aware of the importance of sunlight exposure and

outdoor activities as increase exposure to sunlight markedly reduce the incidence of rickets (Roper et al., 2000).

1.1.1.1 Metabolism of vitamin D

Vitamin D can be obtained from the diet and by the action of sunlight on the skin. Indeed, ~80-90% of vitamin D are produced from the skin under UVB stimulation, while the remaining 10-20% is from vitamin-D rich dietary sources such as fish oils, egg yolks, and liver (Judd and Tangpricha, 2009; Nemerovski et al., 2009). Regardless of the initial sources of vitamin D , 25-hydroxylase and 1α-hydroxylase are needed for the its conversion to the active forms, 25-hydroxyvitamin D in the liver and 1,25-dihydroxyvitamin D in the kidney (Holick, 2005b) (Figure 1). This explains why the end vitamin D metabolite (1,25-dihydroxyvitamin D) is clinically prescribed to patients instead of other vitamin D precursors as the liver and kidney function may be impaired in the patients due to disease complications. Indeed, patients with chronic kidney disease are more prone to vitamin D deficiency (Cuppari et al., 2011). Chronic kidney disease is accompanied with a significantly decline in the ability to synthesize the active forms of vitamin D in relation to the reduction of 1α-hydroxylase activity (Bhan et al., 2010). Plasma level of 25-hydroxyvitamin D serves as a clinical biomarker of vitamin D status in the body (Binkley et al., 2010).

Optimal concentration of vitamin D is vital to health, especially in bone mobilization and the homeostasis of calcium and parathyroid hormone levels. Optimal serum vitamin D levels, which is about 75 nmol/L (30 ng/mL), was once evaluated

based on the minimal concentration of 25(OH)D that is needed to synthesize parathyroid hormone. Nowadays, vitamin D levels are defined as (1) severely deficient for ≤25 nmol/L (10 ng/mL), (2) deficient for 25-50 nmol/L (10-20 ng/mL), (3) insufficient for 50-75 nmol/L (20-30 ng/mL), (4) sufficient for ≥75 nmol/L (30 ng/mL) and (5) toxic ≥375 nmol/L (150 ng/mL) (Lee et al., 2008). Vitamin D deficiency is more common in black people and other persons of dark skin, whose cholecalciferol production is limited by the great amount of melanin in their skin. People who live at latitudes far away from the equator lack of sufficient exposure to UVB also resulted in limited production of cholecalciferol. In fact, vitamin D deficiency is a global problem nowadays resulting from decreased outdoors activities (Bener et al., 2008; Puri et al., 2008). Vitamin D deficiency can lead to elevated circulating levels of parathyroid hormone, resulting in negative impacts in bone metabolism and cardiovascular function. In contrast, over-consumption of vitamin D supplements may lead to calcification in which excessive calcium is deposited on vascular wall, kidneys and lungs (Howerth, 1983).

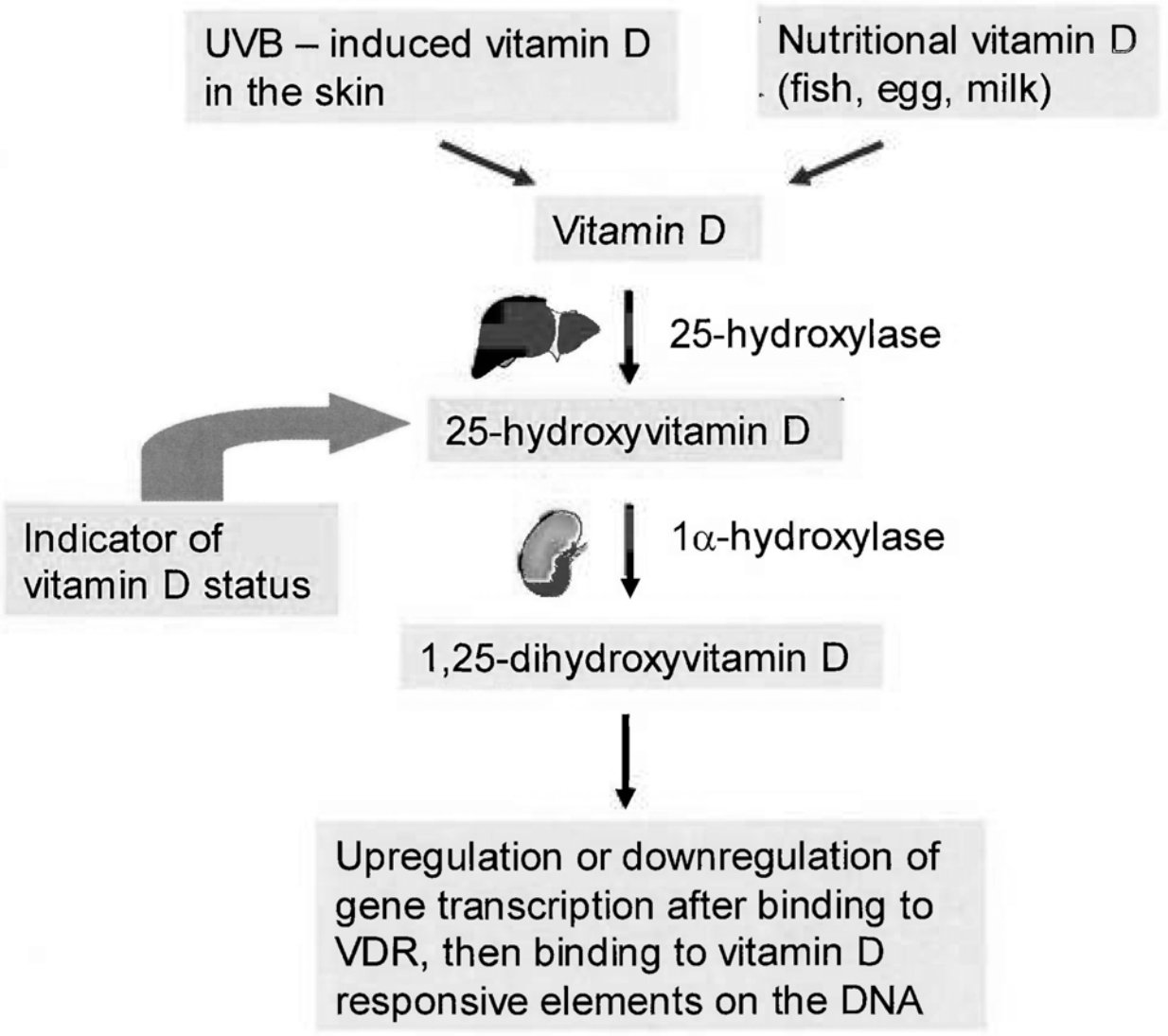

Figure 1.1

The sources, synthesis and metabolism of vitamin D. In the liver and kidney, vitamin D is converted into 25-hydroxyvitamin D, which is used as indicator of vitamin D status and 1.25-dihydroxyvitamin D. Then it can regulate genes transcription by binding to its receptor vitamin D receptor (VDR), which is widely distributed.

1.1.1.2 The clinical application of vitamin D

Vitamin D is generally prescribed for improving bone health, alleviation of rickets and osteoporosis, and treatment of secondary hyperparathyroidism (Chiellini and DeLuca, 2011; Holick, 2005a). For aged women at postmenopausal stage, vitamin D is also medicated to prevent osteoporosis as a result of decreased estrogen production.

1.1.1.3 Vitamin D deficiency

The decreased exposure to sunlight due to outdoors inactivity can lead to a condition called vitamin D deficiency which is becoming a global health problem. Besides bone dysfunction, vitamin D deficiency is associated with impaired cardiovascular function. Exiting evidence suggests an inverse association between the plasma level of vitamin D and dysfunction of the cardiovascular system (Kong et al., 2010; Li, 2003; Vaidya et al., 2011). Epidemiological studies show that the lack of vitamin D is implicated in the development of cancer, hypertension and multiple sclerosis (Giovannucci et al., 2008; Zittermann et al., 2005). A National Health and Nutrition Examination Survey (NHANES) on the general population between 2001 and 2004 shows that about 71% population have their plasma level of 25-hydroxviatmin D above 75 nmol/L, 23% population with 25-75 nmol/L while 6% population with lower than 25 nmol/L of 25-hydroxviatmin D. With regard to patients with chronic kidney disease (CKD), ratio of under 25 nmol/L is become higher and higher with the development of CKD, form CKD stage 3- CKD stage 5.

1.1.1.4 Vitamin D supplementation

The daily consumption of vitamin D is recommended to be 400 international units (IU) for an average adult person. A higher dose of ≤800 IU per day is recommended for pregnant women and children.

1.1.2 Function of vitamin D

The effect of Vitamin D is mediated by its receptor, vitamin D receptor (VDR), which is one of the members in the nuclear receptor superfamily. Upon activation, vitamin D binds to its receptor, VDR, to form a heterodimer complex with the retinoid X receptor (RXR) (Pilz et al., 2009). The VDR-RXR complex can bind to specific DNA sequences termed vitamin D responsive elements, which are sequences located in the promoter regions of various vitamin D-dependent genes. The gene-modulating activity of vitamin D is also conferred through its interaction with transcription factors, such as nuclear factor (NF)-AT and NF-κB which are antagonized by vitamin D/VDR activation (Harant et al., 1997; Takeuchi et al., 1998). Vitamin D can modulate directly or indirectly 3% gene transcription of the whole body including renin, insulin, and cytokines such as interleukin-6 and tumor necrosis factor-α. Owing to the universal expression of VDR, vitamin D can exert its effect on various systems including musculoskeletal, gastrointestinal, prostate, renal, endocrine and cardiovascular system (Holick, 2004; Verhave and Siegert, 2010).

1.1.2.1 Action of Vitamin D in the cardiovascular system

Weishaar et al. was the first group to investigate the relationship between vitamin D and cardiovascular health with the use of a rat model on a diet without vitamin D (Weishaar et al., 1990; Weishaar and Simpson, 1987a, b). In his study, the vitamin D-deficient rats showed significantly elevated blood pressure and greater cardiac and vascular smooth muscle contractile response than the control. His works served as an important basis for subsequent research on the beneficial effect of vitamin D on cardiovascular system.

As cumulating experimental data and clinical evidences pointed out the relationship between vitamin D status and cardiovascular health, scientists began to hypothesize that people with vitamin D deficiency are more susceptible to and have higher risk of cardiovascular disease such as cardiac hypertrophy, hypertension, and myocardial infarction. In fact, a number of clinical studies showed that people living at high latitudes or with dark skin are more prone to have hypertension and higher mortality of cardiovascular disease (Rostand, 1997, 2010; Zittermann, 2006; Zittermann et al., 2006).

Cardiovascular disease is generally associated with hyper-active renin-angiotensin system (RAS). RAS is mainly expressed in the kidney and function to maintain the homeostatic balance between water and electrolyte and thus also plays a role in blood pressure regulation. Vitamin D is an endogenous suppressor of the RAS (Li, 2003; Li et al., 2004b), by blocking the activity of the cyclic AMP response element in the renin gene promoter (Yuan et al., 2007) and thus can inhibit renin

expression directly. The inhibitory effect if vitamin D on renin transcription has been widely demonstrated *in vivo* and *in vitro* (Qiao et al., 2005). VDR knockout and 1α-hydroxylase knockout mice have been employed for investigations (Li et al., 2004b; Xiang et al., 2005b) and cardiac and renal renin expression is found increased compared to the wild type mice. The elevated renin levels results in increased blood pressure. Chronic vitamin D supplementation, along with captopril or losartan, reduces blood pressure in the 1α-hydroxylase knockout mice (Zhou et al., 2008).

1.2.1.1.1 Cardiac protection by vitamin D

In addition to the expression in the kidney, RAS is also present in the heart. Spontaneously hypertensive rats showed elevated expression of RAS in the heart and kidneys (Raizada et al., 2007). Cardiac hypertrophy of SHR is associated with the pronounced expression and activity of the RAS (Georgiopoulou et al., 2010). Vitamin D deficiency or VDR knockout leads to increased heart weight to body weight ratio (O'Connell and Simpson, 1995; Simpson et al., 2007). Co-treatment of paricalcitriol and enalapril ameliorates cardiac oxidative injury in uremic rats (Husain et al., 2009). Hypertrophy of left ventricular cardiomyocytes and the augmented levels of atrial natriuretic peptide are reported in VDR knockout mice, and these abnormalities are reversed by captopril treatment (Xiang et al., 2005a) . Notably, vitamin D is positively co-related to the incidence of congestive heart failure (Zittermann, 2006), indicating a housekeeping role of vitamin D in cardiac health. The expression of VDR in cardiomyocytes implies that the heart is one of the target organ of vitamin D

(Sandgren et al., 1991). Reduced levels of vitamin D increase renin expression and angiotensin II level resulting in cardiomyocyte hypertrophy (Forman et al., 2010; Li et al., 2004a). Low levels of vitamin D also contributes to the pathogenesis of congestive heart failure in which the levels of inflammatory cytokines such as C-reactive protein and interleulin-10 are found elevated (Zittermann et al., 2004). Ayesha Rahman (Rahman et al., 2007) demonstrated that cardiac collagen deposition is increased in VDR knockout mice, accompanied by the augmented activity of matrix metalloprotease (MMP)-2 and MMP-9 and decreased activity of tissue inhibitor of metalloprotease (TIMP). Vitamin D-deficient diet can also elevate collagen deposition in the extracellular space of the myocardium and it is hypothesized that vitamin D can regulate the expression of MMP transcriptionally (Rahman et al., 2007).

In vitro studies using primary cultures of neonatal rat cardiomyocytes showed that vitamin D attenuates ventricular myocyte proliferation and prevent the transcription of gene relating to endothelin-induced myocyte proliferation and hypertrophy such as c-myc, proliferating cell nuclear antigen (PCNA), and atrial natriuretic peptide (O'Connell et al., 1997). Vitamin D may thus modulate the growth of myocytes directly independent on the activity of RAS (Figure 1.2).

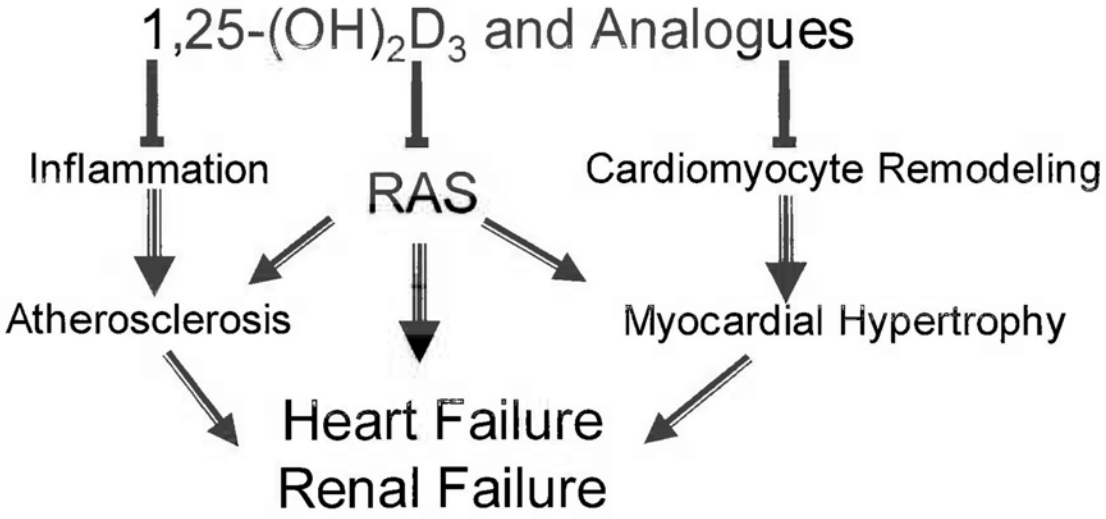

Figure 1.2

1.25-Dihydroxyvitamin D is a potent endogenous suppressor of the renin-angiotensin system (RAS) by directly inhibiting renin gene transcription. 1.25-Dihydroxyvitamin D retards the development of atherosclerosis and myocardial hypertrophy through down-regulating the RAS, which is a key mediator of both heart and renal failure.

1.2.1.1.2 Association between vitamin D and blood pressure.

Accumulating experimental data showed that vitamin D levels are negatively correlated to blood pressure. Hypertension is observed in VDR knockout mice and mice deficient of vitamin D (O'Connell et al., 1997; Xiang et al., 2005a). Supplementation with active vitamin D can restore the elevated blood pressure in nephrectomized and hypertensive rats (Freundlich et al., 2008; Wong et al., 2010a). Hypertensive patients under 3-month ultraviolet B radiation therapy exhibit elevated circulating vitamin D levels with normalized systolic and diastolic blood pressure (Rostand, 1998). These observations are supported by a number of epidemiologic studies (Forman et al., 2007; Martins et al., 2007; Scragg et al., 2007).

In addition to the direct action of vitamin D and its effect on RAS, anti-hypertensive effects of vitamin D are also indirectly conferred by its regulation on calcium and parathyroid hormone (Figure 1.3). Vitamin D deficiency can result in low levels of calcium as vitamin D is needed to enhance intestinal calcium absorption and renal re-absorption of in the kidney. Vitamin D can also stimulate osteoclasts to release calcium. Low levels of calcium can increase the release of parathyroid hormone via a negative feedback mechanism in order to maintain a normal calcium level. Elevated levels of parathyroid hormone can increase blood pressure and infusion of the parathyroid hormone to normotensive subjects is found to induce high blood pressure (Hulter et al., 1986).

A recent clinical study showed there is a high prevalence of hypovitaminosis D in the elder population and that serum 25(OH)-D levels are inversely correlated with

their blood pressure (Almirall et al., 2010). Almirall et al. suggest taking measures to prevent hypovitaminosis D in elder population not only for protecting the skeletal system, but also for promoting cardiovascular health.

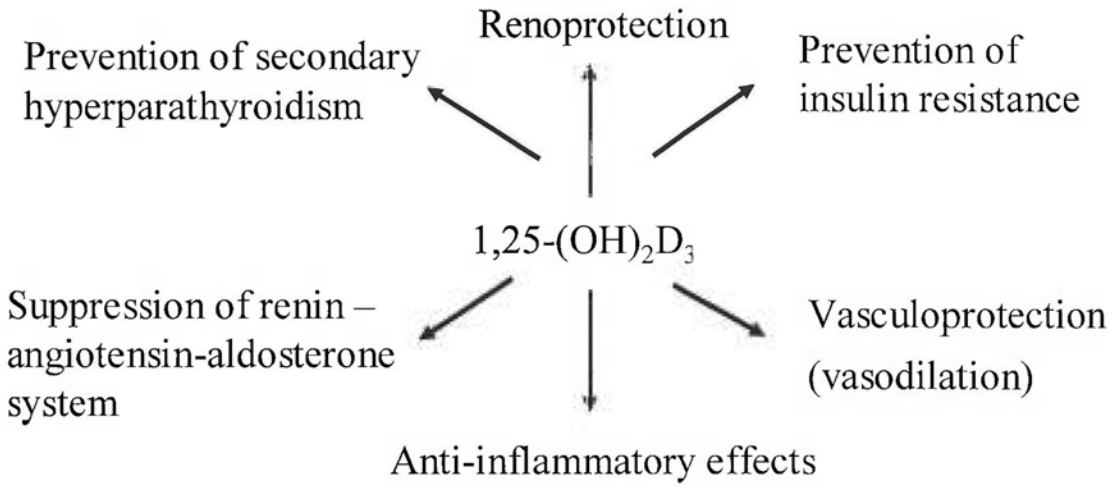

Figure 1.3

The possible mechanisms of the anti-hypertensive effect of 1.25-dihydroxyvitamin D. 1.25-Dihydroxyvitamin D suppresses the renin-angiotensin-aldosterone system, prevents secondary hyperparathyroidism, insulin resistance and inflammation development, benefits the renal function. All these properties are involved in its anti-hypertensive action.

1.2.1.1.3 Vitamin D and endothelial function

Normal endothelial function is attained by the balanced between the production of endothelium-derived relaxing and contracting factors (EDRF and EDCF). When the balance is disturbed such that EDRF production is reduced and the release of EDCF enhanced, endothelial dysfunction occurs.

Although clinically vitamin D supplementation is demonstrated to improve endothelial function in diabetic patients (Sugden et al., 2008), there are limited mechanistic studies regarding how calcitriol improves endothelial function. Vitamin D or its receptor analog is implicated in the regulation of thrombogenic activity (Wu-Wong, 2009). VDR activation can reduce thrombosis and sustain plaque stability in atherosclerotic lesions (Jorde et al., 2010). Whilst plaque vulnerability is closely related to inflammatory states, VDR activation, via its effect to modulate immune response, may attenuate macrophage activation (Wu-Wong, 2009). Wu-Wong et al. found that VDR activation can negatively regulate smooth muscle cell proliferation and differentiation under inflammation during the development of hypertension (Figure 1.4).

Diabetic patients has a high level of advanced glycation end products (AGEs) which cause endothelial dysfunction AGEs decrease endothelial nitric oxide synthase mRNA in human umbilical vein endothelial cells. Calcitriol, being one of vitamin D agonists can prevent the effect of AGE by the reducing the NF-κb-P65 DNA binding activity (Talmor et al., 2008).

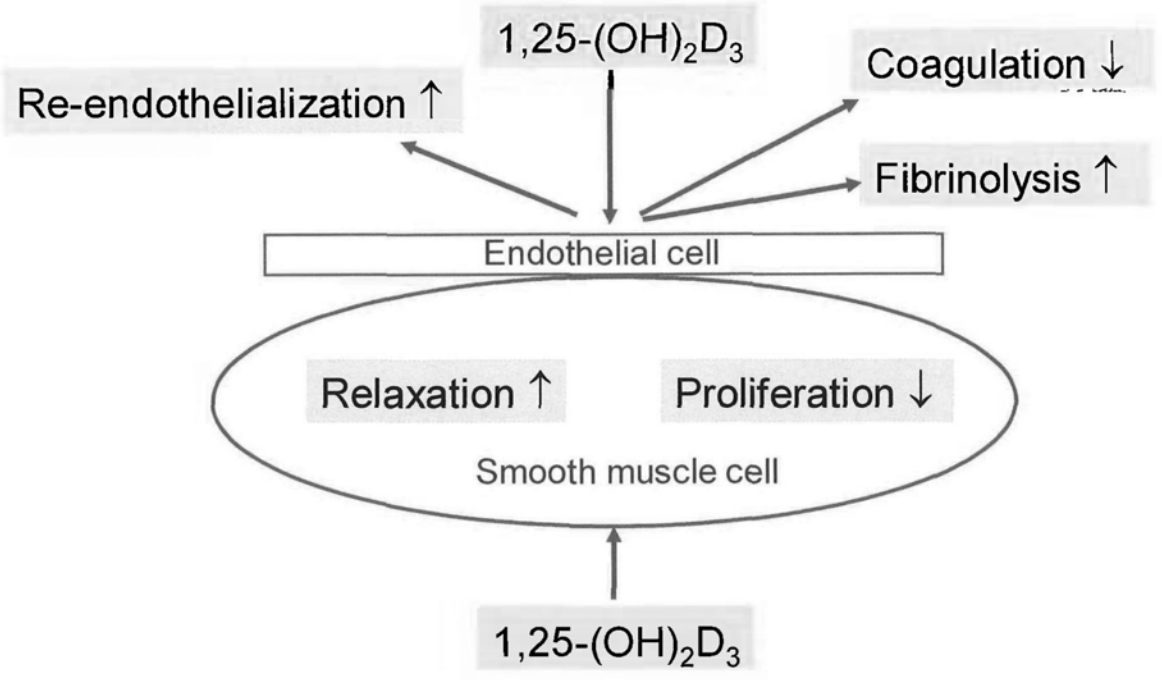

Figure 1.4

The schematic diagram showing the vascular effects of 1.25-dihydroxyvitamin D. 1.25-Dihydroxyvitamin D can act on endothelial cells and smooth muscle cells directly, protecting vascular function by inhibiting coagulation and proliferation, and facilitating re-endothelialization and fibrinolysis.

1.1.2.2 Renal protection by vitamin D

Renal protection of vitamin D is associated with its effects of anti-inflammatory and immunomodulatory properties (Tan et al., 2008; Zhang et al., 2007). Vitamin D is shown to prevent fibrosis in the cultured renal cells and the kidneys in the animal models of renal diseases (Li et al., 2005; Schwarz et al., 1998). It is widely demonstrated that vitamin D is a potent endogenous suppressor of RAS, which play a key role in mediating the pathogensis of kidney function, including kidney fibrosis, glomerular and tubulointerstitial damage, proteinuria, and the deterioration of renal function.(Freundlich et al., 2008; Zhou et al., 2001). Clinical evidence showed that vitamin D can delay the deterioration of kidney function (Doorenbos et al., 2009; Dusso and Tokumoto, 2011; Klaus, 2008; Shroff et al., 2010), and enhance the survival rate of patients with chronic kidney disease by 20-25 % (Teng et al., 2003).

1.1.2.3 Vitamin D and cancer

People living at higher latitudes are associated with higher risk and mortality for Hodgkin's lymphoma, prostate and colon cancers (Feskanich et al., 2004; Gorham et al., 2005; Luscombe et al., 2001).

As to the therapeutic effect of vitamin D in cancer cells, its suppression in the cyclooxygenase-2 (COX-2) pathway is under intense investigation. Calcitriol inhibits COX-2/prostaglandin cascade by reducing COX-2 transcription and increasing the expression of 15-prostaglandin dehydrogenase (15-PGDH), which can inactivate and degrade prostaglandins. Calcitriol can suppress the target receptors of the arachidonic

acid such as FP and EP receptor. In combination with non-steroidal anti-inflammatory drugs that inhibit COX activity, calcitriol treatment represents a potential therapeutic strategy to improve the potency and efficacy of both drugs in the treatment of prostate cancer (Krishnan and Feldman, 2010; Krishnan et al., 2007; Moreno et al., 2005). Vitamin D can also inhibit the COX-2 enzymatic activity directly in cancer cells (Aparna et al., 2008).

Up to date, there are minimal reports whether vitamin D can confer benefical effects to the cardiovascular system by suppressing the arachidonic acid/COX-2 pathway, which contributes significantly to the production of vasoactive constrictors.

In summary, sufficient vitamin D levels are essential for maintaining the normal physiological homeostasis. Of the two main sources of vitamin D, since excessive exposure to sunlight can significantly augment the risk of skin cancer, dietary supplementation with vitamin D is recommended under pathological states.

1.1.2.4 Other noncalcemic effects of vitamin D

In additional, vitamin D can influence pancreatic islet function by enhancing the biosynthetic activity of β-cells and accelerating the conversion of pro-insulin to insulin (Billaudel et al., 1999).

1.2 Endothelial function

Endothelium resides in the innermost layer of the blood vessel and plays a key role in the maintenance of cardiovascular health. By releasing vasoactive factors, it regulates vascular tone, blood coagulation, inflammation and cell growth. Thus endothelium is

not only a quiescent barrier simply delineating the vessel lumen and the interstitial space (Landmesser and Drexler, 2005; Munzel et al., 2008).

1.2.1 Introduction of endothelial function

Endothelial cells regulate the cardiovascular system by synthesizing a number of vasoactive factors. In fact, endothelium not just controls vascular reactivity, healthy endothelium cells also suppress smooth muscle cell proliferation and inhibit leukocyte adhesion (Felmeden and Lip, 2005). Among the vasoactive factors, nitric oxide (NO) is most widely studied and of paramount importance in vascular function. NO dilates blood vessel, prevent platelet aggregation and smooth muscle cell proliferation.

Endothelial dysfunction has been proposed to be one of the initiating events of atherosclerosis. The assessment of endothelial function should be reliable, safe and repeatable for clinical applications, which include forearm blood flow measurement. The golden standard for the assessment of endothelial function is the measurement of coronary blood flow via administration acetylcholine to coronary artery. Assay of nitric oxide and measurement of circulating markers such as plasminogen activator inhibitor-1, C-reactive protein also provide useful information on endothelial health (Felmeden and Lip, 2005). As to animal-based mechanistic studies, endothelial function is usually assessed by the endothelium-dependent relaxations induced by acetylcholine (ACh). ACh can induce at least 80% relaxation in arteries with intact endothelium. In addition to nitric oxide, prostacyclin and endothelium-derived

hyperpolarizing factors are the other two relaxing factors that are derived from the endothelium.

Besides relaxing factors, endothelial cells also synthesize constricting factors. Cardiovascular risk factors, including aging, hypertension, diabetes and menopause, may attenuate the release of EDRFs while augmenting the production of EDCFs, leading to an elevated of the vascular tone and eventually endothelial dysfunction (Forstermann, 2010; Munzel et al., 2008).

1.2.1.1 Endothelial-derived relaxing factors (EDRFs)

1.2.1.1.1 NO

ACh or ligands such as bradykinin, upon binding to their receptors located on the endothelial cells, can induce phosphorylation of endothelial NO synthase at the activation site via calcium influx. NO released from endothelial cells acts on the soluble guanylate cyclase (sGC) on smooth muscle cells, and thereby cause relaxations through the NO-sGC-cGMP cascade. NO is the main vasodilator in conduit arteries.

1.2.1.1.2 Prostacyclin

Prostacyclin is one of the classical vasodilator derived from arachidonic acid. It activates adenylate cyclase and the subsequent cAMP pathway, causing potassium efflux from smooth muscle cell, and eventually leading to relaxation.

1.2.1.1.3 Endothelium-derived hyperpolarizing factor

EDHF is a short-lived cytochrome P450-derived metabolite of arachidonic acid. EDHF from endothelial cells can diffuse across the vascular wall and activates the potassium channels on the vascular smooth muscle cells, thereby resulting in smooth muscle hyperpolarization. EDHF-mediated relaxations are more prominent in smaller vessels including small mesenteric artery and coronary artery, in which their relaxations can be attenuated by inhibitors of cytochrome P450.

1.2.1.2 Endothelium-derived contracting factors (EDCFs)

EDCFs counteract the effect of EDRF. Under the action of cyclooxygenase (COX), PGH_2, the metabolic intermediate of arachidonic acid will be converted into PGE_2, PGD_2, PGI_2, $PGF_{2\alpha}$ and thromboxane A_2 by respective prostaglandin synthases. These factors have their own receptor expressed on smooth muscle cells - EP, DP, IP, FP and TP receptor. Owing to the structural similarity between the prostaglandins, most of them can bind to and activate TP receptor to induce contraction (Figure 1.5).

1.2.2 Endothelial dysfunction

Reduced EDRF production and increased EDCF release can functionally impaired vasodilatation, which is widely observed in vasculatures under pathological states such as hypertension, atherosclerosis, diabetes, aging and menopause.

1.2.2.1 Reactive oxygen specious in endothelial dysfunction

Over-production of reactive oxygen specious is involved in the pathogenesis of endothelial dysfunction. Under physiological condition there is a balance between the oxidant-generating enzymes and oxidant scavenging enzyme. Over-activation of vascular NAD(P)H oxidase, xanthine oxidase and uncoupled eNOS are associated with exaggerated production of reactive oxygen species across the vascular wall in both the endothelial cells and smooth muscle cells.

1.2.2.2 Reduced NO bioavailability in endothelial dysfunction

NO produced in endothelial cell depends on phosphorylation of eNOS, which was found to be attenuated under hypertensive and diabetic conditions. Of note, NO bioavailability can also be reduced by excessive ROS production. ROS not only scavenges NO directly, but also combine with NO to form peroxynitrite, a reactive nitrogen specious that can cause DNA damage and protein nitration.

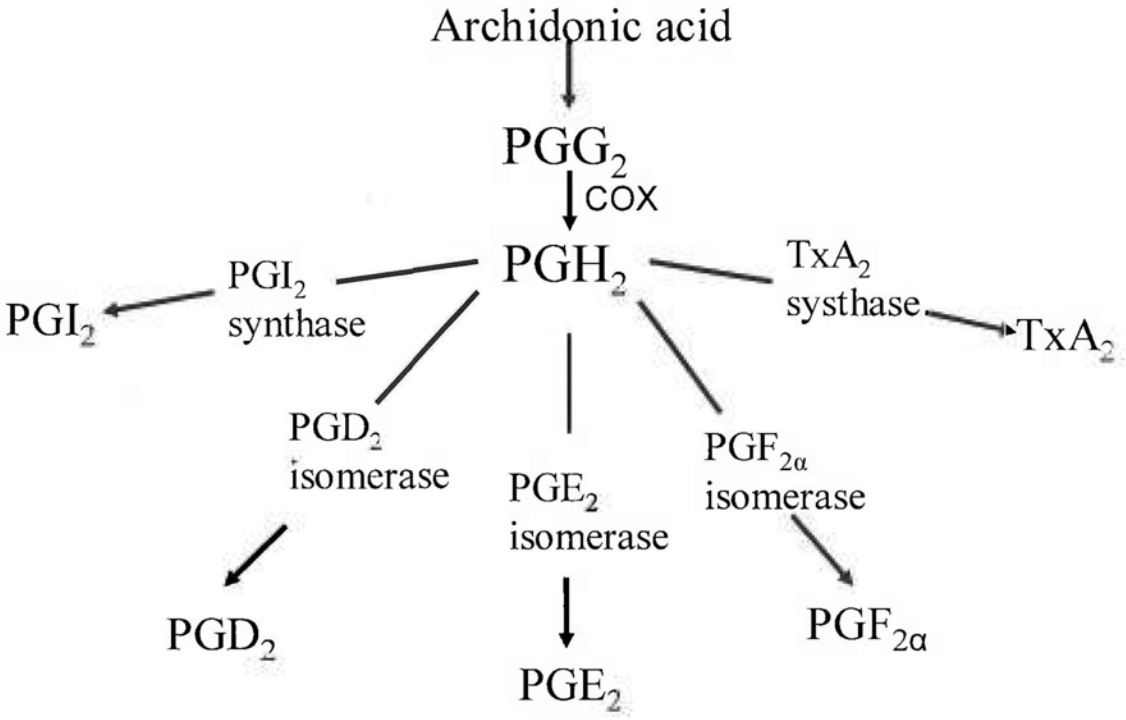

Figure 1.5

The schematic diagram showing the conversion of arachidonic acid into different prostanoids.

1.2.2.3 Pronounced production COX-derived EDCF

Cyclooxygenase is the rate-limiting enzyme in the release of prostanoids. It is present in two isoforms, the constitutively expressed COX-1 and the highly inducible COX-2. Over-expression and/or hyper-activation of these two enzymes leads to over-production of prostanoids, most of being potent vasoactive contractors.

1.2.3 Animal models for the study of endothelial dysfunction in hypertension and estrogen deficiency

Spontaneously hypertensive rats (SHR) and ovariectomized rats are the most common model for the study of endothelial dysfunction in relation to high blood pressure and estrogen deficiency.

1.2.3.1 Endothelial dysfunction in spontaneously hypertensive rats (SHRs)

SHR is one of most commonly adopted hypertensive animal model for cardiovascular research owing to its stability of hypertension development. Vascular dysfunction in SHR is characterized by the steadily elevated blood pressure starting at the age of 3-4 months. SHR aortas and renal arteries exhibited impaired ACh-induced endothelium-dependent relaxations and contractions, which are attributed by the increased ROS generation and over-production of prostanoids resulting from over-expression and/or hyper-activity of COX (Tang and Vanhoutte, 2009; Wind et al., 2010a; Wong et al., 2010b) (Figure 1.6). Since COX is localized in the endothelium and the prostanoids are of endothelial origin, ACh-induced contractions are abolished in arterial rings with endothelium mechanically removed. The impaired relaxations and enhanced contractions are sensitive to COX inhibitors, ROS scavengers and TP receptor antagonist, indicating the involvement of COX-1 and -2, reactive oxygen species and TP receptor in the endothelial dysfunction in hypertension. COX and ROS can be regulating each other – while ROS is found to activate COX-2 in SHR renal arteries (Wong et al., 2010), COX is reported to generate reactive oxygen species in the SHR aortas (Tang et al., 2007).

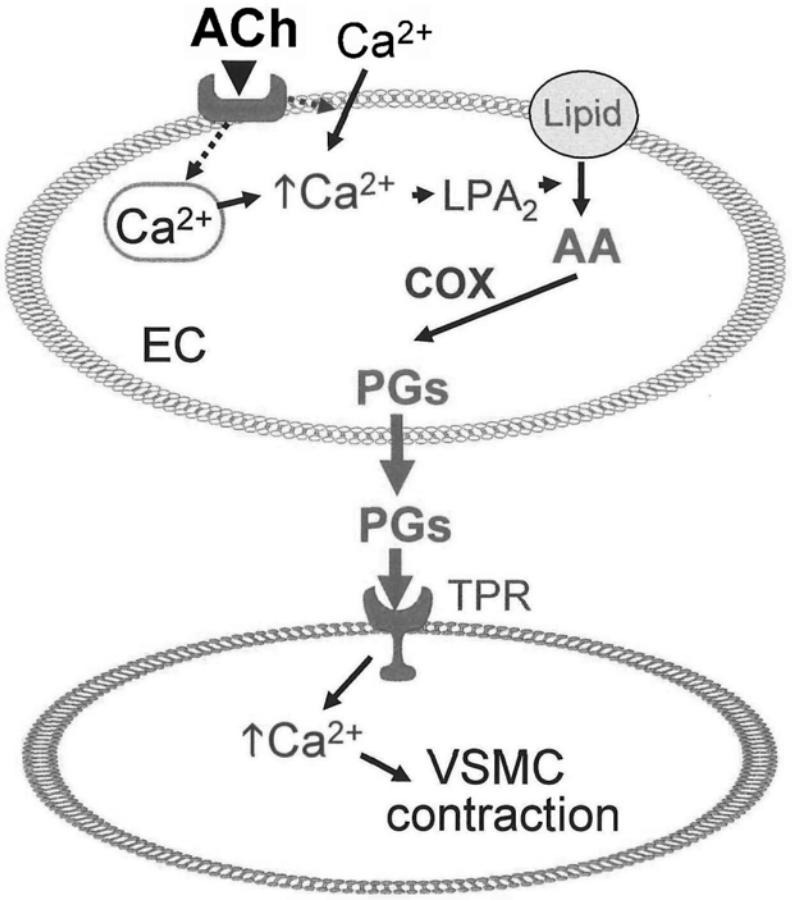

Figure 1.6

The endothelium-dependent contraction in SHR renal arteries. Calcium influx induced by ACh binds to the muscarinic acetylcholine receptor with a rise in intracellular calcium concentration which triggers the production of various prostaglandins from the precursor arachidonic acid (AA) via the activation of cyclooxygense (COX-1 and COX-2). Some constrictive metabolites prostaglandins (PGs) can stimulate the thromboxane-prostanoid receptor (TPR) on smooth muscle cells (VSMC) and cause contraction partly through promoting calcium influx.

1.2.3.1.1 Reactive oxygen specious in hypertension

Animal-based vascular studies on hypertension using animal models such as SHR, angiotensin-II infused rats and diabetic db/db mice provided experimental basis for the possible therapeutic option anti-oxidant treatment in hypertension.

Physiologically, ROS production is under careful control and the ROS level is maintained low, allowing the ROS to exert its function as a signaling mediator but not generating oxidative stress. However, when the relative activity of radical generating and scavenging enzymes are disturbed, over-production of ROS results, leading to a reduction of NO bioavailability, smooth muscle proliferation, and deposition of extracellular matrix proteins.

NAD(P)H oxidase represents the major source of ROS in the vascular wall (Griendling et al., 2000). The NAD(P)H oxidase family is composed of seven members, Nox1-Nox5, Doux1 and Doux2. Among all the subunits NOX-1, NOX-2 and NOX-4 are associated with cardiovascular and widely expressed in cardiovascular system. In rodents, NOX-2 and NOX-4 are mainly expressed in endothelial cell, and NOX-1 and NOX-4 mainly in smooth muscle cells. NAD(P)H oxidase can be activated by cytokines, vasoactive factors and shear stress. Inhibitors against NAD(P)H oxidase and ROS scavengers can restore impaired ACh-induced relaxations and abolish endothelium-dependent contractions (Wind et al., 2010b)Wong et al., 2010). ROS scavengers, SOD mimetics and NAD(P)H oxidase inhibitor can delay the deterioration of kidney function and improve endothelial function and prevent cardiac

hypertrophy (Munzel et al., 2010; Park et al., 2002; Rodriguez-Iturbe et al., 2003; Simonsen et al., 2009; Taddei et al., 2002).

1.2.3.1.2 RAS in hypertension.

RAS serves as the main regulator of water and electrolyte homeostasis (Cheng et al., 2005). Despite being mainly expressed in the kidney, RAS is also present in heart, explaining why RAS is pathologically linked to cardiac dysfunction such as cardiac hypertrophy, and heart failure. Hyper-activated RAS with components renin, angiotensin II, AT_1R is detected in almost all of the animal models for hypertension studies, including SHR, angiotensin II-infused rats, Dahl salt-sensitive hypertensive rats and nephrectomized hypertensive models.

As the precursor for Ang II formation is angiotensinogen, the expression and activity of renin determines the Ang II level. Elevated levels and activity of plasma renin and AT_1R in hypertensive patients and animal models is reported from both clinical studies and animal-based experiments (van Esch et al., 2010). Treatment with renin inhibitor lowers blood pressure in SHR and hypertensive marmosets (Wood et al., 2005). Angiotension receptor blockers (ARBs) are effective drugs for treating patients with essential hypertension. In addition to its effect on lowering blood pressure, ARBs have been reported to possess anti-oxidative properties (Flammer et al., 2007; Yao et al., 2007; Zhu et al., 2007). In fact, as NAD(P)H oxidase is functionally coupled to AT_1R (Endemann and Schiffrin, 2004), blocking the receptor can ameliorate the over-production of ROS. Yao et al. showed that chronic treatment

of losartan for 2 weeks lowers blood pressure and decreases the elevated expression of NAD(P)H oxidase subunits in SHR (Yao et al., 2007).

1.2.3.2 Endothelial dysfunction in ovariectomized rat

Estrogen is reported to be beneficial to the cardiovascular system. Postmenopausal women showed a marked increase in the incidence of cardiovascular disease (Cignarella et al., 2010; Vogelvang et al., 2004). One of the conventional remedy for menopausal symptoms is hormone replacement therapy; however, the outcome of the therapy remained controversial.

1.2.3.2.1 Modulation of NO bioavailability and RAS by estrogen

Estrogen increases NO bioavailability and reduces oxidative stress and the expression and activity of angiotensin converting enzyme (ACE) and AT_1R. Estrogen withdrawal by ovariectomy causes augmented ACE expression and exaggerated ROS production, and eventually resulting in attenuated NO bioavailability. *In vivo* and *in vitro* treatment with ARB improves the attenuated endothelium-dependent relaxations and normalized the elevated expressions of ACE, AT_1R, NAD(P)H oxidase subunits and nitrotyrosine in the aortas from ovariectomized rats (Yung et al., 2011).

1.2.3.2.2 Modulation of COX-1 and COX-2 expressions by estrogen

There are limited findings regarding the effect of estrogen on COX. As estrogen is can exert anti-oxidative effects, deprivation of estrogen by ovariectomy may result in

elevated ROS-mediated COX-2 expression and/or activity. Martorell et al. reported the inhibitory effect of estrogen on the release of prostanoids, yet as to whether estrogen deficiency can modulate the expression and activity of the prostanoid receptor remained unknown.

1.3 Objectives of the present study

Although vitamin D is shown to be beneficial to the cardiovascular system, it remains elusive as to whether and how vitamin D might protect renovascular function in hypertension and under estrogen deficiency. Clear dissection of the effect of vitamin D on the renal vasculature may help to uncover novel therapeutic strategies for vascular complications in hypertensive patients and postmenopausal women. To achieve the objectives, I have investigated:

1. Whether calcitriol, an active form of vitamin D, could prevent endothelial dysfunction in spontaneously hypertensive rats in chronic and *in vitro* treatment, whether similar *in vitro* effects of calcitriol could be observed in the renal arteries from hypertensive patients, and whether the effects of calcitriol are conferred via the alteration of the proteins involved in ROS generation;
2. The underlying mechanism of renovascular dysfunction in estrogen deficiency and whether calcitriol offers renovascular protection using the well-established model of ovariectomized rats.

Chapter II

Methods and Materials

2.1 Animal handling

The study was under approved by the Animal Experimentation Ethics Committee, the Chinese University of Hong Kong (CUHK). Animals were supplied by the CUHK Laboratory Animal Service Center and housed in a temperature-controlled room (22–24 °C) with a 12-h light/dark cycle and supplied with a standard diet and water. Spontaneously hypertensive rats (SHRs), Wistar-Kyoto rats (WKYs) and female Sprague-Dawley rats were used in the study.

2.1.1 Surgical procedure

Adult female Sprague-Dawley rats (weighing 200–220 g) were ovariectomized under anesthesia by ketamine/xylazine 0.2 mL/100g via intraperitoneal injection. The ovaries were removed carefully. For the sham control, sham operation was performed without removing the ovaries.

2.1.2 Chronic vitamin D treatment

chronic vitamin D treatment is started in male SHRs with stably elevated blood pressure at ~180-20 0mmHg at the age of 6 months old and OVX rats 6 months after

ovariectomy. They were received calcitriol at the dose of 150 ng/kg/day via oral gavage for 4.5 months. DMSO was administered as a vehicle control.

2.2 Human artery specimen

The use of human renal arteries for the present study was approved by the Joint Chinese University of Hong Kong–New Territories East Cluster Clinical Research Ethics Committee. The arteries were obtained from nephrectomy specimens from normotensive and hypertensive patients with their consent. Hypertension was defined as having high blood pressure with systolic blood pressure over 140 mmHg and diastolic blood pressure over 90 mmHg. For female patients, menopause age was defined as >55 years. The arteries were incubated overnight in the presence or absence of calcitriol (100 nmol/L) in Dulbecco's Modified Eagle Medium (GIBCO) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (GIBCO) at 37°C. Specific human vitamin D receptor antagonist TEI-9647 (1 μmol/L) was added 30 min before the addition of calcitriol when used.

2.3 Measurement of basic parameters

2.3.1 Blood pressure measurement

Blood pressure was measured by the tail-cuff electrosphygmomanometer system (ADInstruments, Sydney, Australia). Blood pressure was recorded after the rats were stabilized and remained quiescent. An average of 3 readings was taken as the systolic blood pressure of each rat.

2.3.2 Wet weight of uterus

Upon sacrifice of the OVX rats and the sham controls, their uterus were dissected and the weight was recorded. The uterus weight to the body weight ratio regarded as a parameter to determine whether the ovaries were completely removed when the ovariectomy surgery was performed.

2.3.3 Plasma concentration of vitamin D

Serum was collected from OVX rats treated with vehicle and calcitriol upon their sacrifice. Serum level of calcitriol (1,25-dihydroxyvitamin) was measured using a commercially available immunoassay kit (Immunodiagnostic Systems. Fountain Hill, USA) according the manufacturer's instruction.

2.4 Isometric force measurement

For endothelium-dependent contractions, the arterial rings from the rats were pre-incubated with N^G-nitro-L-arginine methyl ester (L-NAME, 100 μmol/L) to eliminate the vasodilatory effect of nitric oxide (NO). Cumulative concentrations of acetylcholine (ACh, 0.03–100 μM) were added to trigger contractions. Inhibitors or antagonists, when used, were added along with L-NAME for 30 min prior to the addition of ACh. ACh-induced tension changes in the renal arteries were presented as the tension recorded divided by the tension induced by 60 mmol/L KCl. For human renal arteries, rings were pre-contracted with phenylephrine (3 μmol/L) to attain a stable tension and thereafter ACh was added cumulatively (3 nmol/L to 10 μmol/L) to induce relaxations.

For endothelium-dependent relaxations, after stable contractions were induced by 1 μmol/L phenylephrine, cumulative concentrations of acetylcholine (ACh, 0.003–10 μM) were added to trigger concentration-dependent relaxations.

2.5 Primary culture of rat aortic endothelial cells

Primary endothelial cells were cultured as described. Briefly, aortas were freshly isolated from WKY, SHR, female SD rats and OVX rats, and trimmed of the perivascular adipose tissue in ice-cold phosphate buffered saline. The aortas were then put to enzymatic digestion in 0.2% type 1A collagenase (Sigma) for 15 min at 37 °C. RPMI (GIBCO) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (GIBCO) was added and the cell suspension was then centrifuged at 1500 ×g for 10 min. The supernatant was discarded and the cell pellet was resuspended in the supplemented RPMI medium. Cells were allowed to settle for 1 h and the non-adhering cells were removed by changing the medium. The cells were confirmed to stain positively with the endothelial cell marker, PECAM-1.

2.6 Detection of reactive oxygen species by dihydroethidium (DHE) fluorescence and electron paramagnetic resonance (EPR)

Upon ~80% confluence, WKY aortic endothelial cells were plated on coverslips. Cells were exposed to Ang II (100 nmol/L, 8 h) with or without 30-min pre-treatment of calcitriol or other inhibitors. The cells were then incubated with 5 μmol/L DHE (Molecular Probes) for 15 min at 37°C and imaged using the confocal microscopy system of Olympus Fluoview FV1000 (Olympus America Inc., Melville, NY) with excitation and emission wavelengths of 515 nm and 585 nm respectively. Similar experiments were performed on WKY and SHR renal arteries harvested after

overnight incubation with or without calcitriol and other inhibitors. The arteries were then embedded in OCT compound (Sakura Finetek, the Netherlands), snap frozen in liquid nitrogen and cut into 10 μm-thick cryo-sections. After loading with DHE, the sections were cover-slipped and imaged for the fluorescence. Results are represented as percentages compared to the fluorescence intensity of the control.

ROS production was also confirmed with EPR using 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine hydrochloride (TEMPONE-H, Alexis Biochemical Corp., San Diego, CA, USA) as the spin trapping agent for reactive oxygen species (ROS). Briefly, WKY and SHR renal arteries with or without overnight calcitriol treatment were placed in a chamber containing 2-mL Krebs solution continually oxygenated with 95% O_2 and 5% CO_2 at 37°C. TEMPONE-H (100 μmol/L) and a transition metal chelator diethylenetriaminepentaacetic acid (DTPA, 100 μmol/L, Sigma-Aldrich) were then added and incubated with the tissue for 20 min to trap the ROS produced. The rings were then homogenized in 100-μL bathing solution and placed into glass micropipettes for X-band EPR spectra detection at 21 °C using an EMX EPR spectrometer (Bruker BioSpin GmbH, Siberstreifen, Rheinstetten/Karlsruhe, Germany). Oxidation of TEMPONE-H generates 4-oxo-tempo with a characteristic three-line EPR signal centred at 3474 G. The EPR-settings were as follows: field swept from 3444 G up to 3504 G, microwave power 200 mW, modulation amplitude 2 G, conversion time 655 ms, detector time constant 5245 ms, magnetic field sweep time 671 s.

2.7 Laser confocal fluorescence microscopy for nitric oxide (NO) release

Fluorimetric measurements were performed on primary aortic endothelial cells using an Olympus Fluoview FV1000 laser scanning confocal system mounted on an inverted IX81 Olympus microscope, equipped with a 10× objective (NA 0.5). Intracellular NO production was monitored fluorescent NO indicator 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA). The dye reacts not with NO itself but with NO^+ equivalents, such as nitric anhydride (N_2O_3), which are formed by autoxidation of NO. Cultured rat aortic endothelial cells seeded on glass coverslips were incubated for 15-20 min at room temperature in normal physiological solution or Ca^{2+}-free solution containing 1 μM DAF-FM DA (Invitrogen, USA). The amount of NO was evaluated by measuring the fluorescence intensity excited at 495 nm and emitted at 515 nm. Changes in intracellular NO production were displayed as a ratio of fluorescence relative to the intensity (F_1/F_0). Normal physiological saline solution (NPSS) contained 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 10 mmol/L glucose, and 5 mmol/L HEPES (pH 7.4) .

2.8 Western blot

Renal arteries from the rats and from patients were dissected and homogenized in ice-cold RIPA lysis buffer (1 μg/mL leupetin, 5 μg/mL aprotonin, 100 μg/mL PMSF, 1 mmol/L sodium orthovanadate, 1 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L NaF and 2 mg/mL β-glycerolphosphate). The homogenates were incubated on ice for 20

min and then centrifuged at 20000 ×g for 20 min at 4°C. The supernatant was collected and the protein concentration was determined using the Lowry method (Bio-rad). Equal amount of protein samples were electrophoresed on a 10% SDS-polyacrylamide gel and then transferred onto an immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore). Non-specific binding sites were blocked by 5% non-fat milk or 1% BSA in 0.05% Tween-20 phosphate-buffered saline (PBST), and then incubated overnight at 4°C with primary antibodies against NAD(P)H oxidase subunits (NOX-2, NOX-4 and $p67^{phox}$), nitrotyrosine, angiotensin II type I receptor (AT_1R), AT_2R, cyclooxygenase (COX)-1, COX-2 and thromboxane-prostanoid receptor (TPR) at a dilution of 1:1000, superoxide dismutase (SOD)-1 and SOD-2 at a dilution of 1:2000, GAPDH as house-keeping protein at a dilution of 1:10000. The blots were incubated with appropriate secondary antibodies at a 1:3000 dilution for 1 h at room temperature, and then washed 3 times for 20 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).

2.9 Immunofluorescence microscopy

Changes in the expressions of SOD and NAD(P)H oxidase subunits in the human renal arteries after calcitriol treatment were visualized by immunofluorescence microscopy. Briefly, the arteries were embedded in OCT compound (Sakura Finetek, the Netherlands), snap frozen and cut into 10-μm thick cryostat sections which were then fixed in 4% paraformaldehyde for 30 min and treated with 0.05% Triton X in PBS for 1 min. The sections were blocked with 5% normal donkey serum for 1 h at

room temperature. Primary antibodies against NOX-2, NOX-4, $p67^{phox}$ and SOD-1 were incubated overnight at 4℃. After several washes in PBS, the sections were then incubated with appropriate Alexa Fluor 546 IgG (Invitrogen) for 1 h at room temperature. After cover-slipping, the sections were observed under the confocal microscope Olympus Fluoview FV1000. Images were first acquired with Olympus Fluoview software (version 1.5; FV10-ASW1.5) and then merged using the SPOT advanced software (Version 4.6).

2.10 Drugs, chemicals and other reagents

2.10.1 Chemicals

Chemicals	Description	Solvent	Source
Acetycholine hydrochloride	Muscarinic acetycholine receptor agonist	H_2O	Sigma
Actinomycin D	Transcription inhibitor	DMSO	Sigma
Angiotensin II	Angiotensin II type 1 receptor agonist	H_2O	Tocris
Calcitriol	$1,25(OH)_2VD_3$	DMSO	Tocris
celecoxib	Specific COX-2 inhibitor	DMSO	Pfizer, NY
DPI	NAD(P)H oxidase inhibitor	DMSO	Sigma
Diethylenetriaminepent Aacetic acid (DTPA)	Spin trap reagent	H_2O	Sigma
DuP-697	Specific COX-2 inhibitor	DMSO	Calbiochem
TEI-9647	Human vitamin D receptor antagonist	Ethanol	Teijin Pharma Limited
Hypoxanthine	Substrate for xanthine oxidase	DMSO	Sigma
Indomethacin	Non-selective COX inhibitor	DMSO	Cayman
Losartan	AT_1R block	DMSO	Cayman
L-NAME	Nitric oxide synthase	H_2O	Sigma

	inhibitor		
NS-398	COX-2 inhibitor	DMSO	Tocris
Phenylephrine	α-adrenergic receptor	H_2O	Sigma
SC-560	COX-1 inhibitor	DMSO	Sigma
S18886	TP receptor blocker	DMSO	Sigma
Tempol	SOD mimetic	H_2O	Sigma
TEMPONE-H	Spin trap reagent	H_2O	Sigma
U46619	TP receptor agonist	DMSO	
Xanthine oxidase	Oxidant producing enzyme		Sigma

2.10.2 Compositon of Krebs solution

The Krebs solution is prepared before experiment and pH value is 7.4, with 95% O_2 and 5% CO_2.

Chemicals	Final concentration (mM/L)
NaCl	119
$NaHCO_3$	25
$MgCl_2.6H_2O$	1
KCl	4.7
KH_2PO_4	1.2
$CaCl_2$	2.5
D-glucose	11.1

2.10.3 Reagents for Western blot analysis

2.10.3.1 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

Aprotonin	5 µg/mL
EDTA	1 mmol/L
EGTA	1 mmol/L
β-glycerolphosphate	2 mg/mL
Leupetin	1 µg/mL
Phenylmethysulfonyl fluoride (PMSF)	1 mmol/L
Sodium fluoride	1 mmol/L
Sodium orthovanadate	1 mmol/L

2.10.3.2 Reagents for gel preparation

30% acrylamide		made up to 100mL
	Acrylamide	29.2 g
	Methylene bis-acrylamide	0.8 g
1.5 mol/L Lower tris-base buffer (pH 8.8)		made up to 100mL
	Tris base	18.17g
	10% SDS	4mL
0.5 mol/L Upper tris-base buffer (pH 6.8)		made up to 100mL
	Tris base	6.047
	10% SDS	4 mL
Others		
	Tetramethylethylene diamide (TEMED)	2% in final solution
	Ammonium persulphate (freshly prepared)	0.1% in final solution

2.10.3.3 Buffers for eletrophoresis, transfer and washing

SDS gel loading buffer (2x)		
	Tris (from 1mol/L Tris-HCl,Ph6.8)	125 mmol/L
	SDS	4%
	Glycerol	20%

	Bromophenol blue	0.06%
	B-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
	$KH2PO_4$	0.5 mmol/L
	KCl	1.3 mmol/L
	Tween 20	0.05%

2.10.3.4 Primary antibodies

Primary antibodies for target proteins were diluted in 1% bovine serum albumin (dissolved in PBST) and incubated overnight at 4°C. Corresponging secondary antibodies (DakoCytomation) were used at a dilution of 1:3000 and incubated for 1 hour at room temperature.

Antigen	Host	Type	Application	Company
AT_1R	mouse	monoclonal	WB (1:1000)	Abcam
AT_2R	rabbit	polyclonal	WB (1:1000)	Abcam
COX-1	mouse	monoclonal	WB (1:1000)	Cayman
COX-2	goat	polyclonal	WB (1:1000)	Santa Cruz
GAPDH	mouse	monoclonal	WB (1:10000)	Ambion
Nitrotyrosine	mouse	monoclonal	WB (1:1000)	upstate
NOX-2	rabbit	polyclonal	WB (1:1000)	Abcam

			IHC (1:100)	
NOX-4	rabbit	polyclonal	WB (1:1000) IHC (1:100)	Abcam
P67^{phox}	rabbit	polyclonal	WB (1:1000) IHC (1:100)	Cell signaling
SOD-1	rabbit	polyclonal	WB (1:2000) IHC (1:200)	Santa Cruz
SOD-2	rabbit	polyclonal	WB (1:2000)	Santa Cruz
TP receptor	rabbit	polyclonal	WB (1:1000)	Cayman

2.11 Statistical analysis

Data expressed means ± SEM of five to seven experiments. pD2 is the negative logarithm of the acetylcholine concentration needed to cause 50% of the maximal relaxation and Emax denotes the maximal response produced by the constrictor or dilator. Protein expression analysis were normalized to GAPDH and then presented relative to control. Statistical significance was determined by two-tailed Student's t-test or one-way ANOVA followed by Bonferroni post hoc tests whenever appropriate (GraphPad Software, San Diego, California). *P* values less than 0.05 regarded as statistically significant differences.

Chapter III

Calcitriol protects renovascular function in hypertension by down-regulating angiotensin II type 1 receptors and reducing oxidative stress

3.1 Introduction

Calcitriol (1,25-dihydroxyvitamin D3) is an active form of vitamin D. Besides its classical function of regulating calcium and phosphate homeostasis, calcitriol also acts on the cardiovascular, immune and endocrine systems because of universal distribution of its receptor, vitamin D receptor (VDR), which mediates most of the effects of vitamin D (Holick, 2003; Norman, 2006). Upon binding to its receptor, vitamin D activates the retinoid X receptor and recruits various co-factors to form a transcriptional complex, through which vitamin D modulates ~3% gene transcription of the whole body directly or indirectly.

Vitamin D was first demonstrated as an endogenous suppressor of the renin-angiotensin system (RAS) by direct inhibition on renin transcription, and a majority of research has since focuses on the effect of vitamin D on the cardiovascular system during disease states with hyperactive RAS (Li, 2003; Li et al., 2002; Li et al., 2004; Yuan et al., 2007). Elevated blood pressure is observed in VDR knockout mice (Li et al., 2002) and mice deficient in 1α-hydroxylase (Zhou et al., 2008), an enzyme catalyzing inactive calcidiol to bioactive calcitriol, indicating a housekeeping role for vitamin D in vascular health. Vitamin D also possesses anti-inflammatory, anti-proliferative and anti-hypertrophic properties (Wu-Wong, 2009) which may offer additional cardiovascular benefits. Vitamin D treatment restores cardiac hypertropy in spontaneously hypertensive

rats (SHR) (Mancuso et al., 2008). A recent study reports that vitamin D directly acts on endothelial and smooth muscle cells, thereby increasing re-endothelialization and fibrinolysis while decreasing coagulation (Wu-Wong, 2009). Noteworthy, an inverse correlation is found between vitamin D level and the incidence of heart failure and elevation of blood pressure (Pilz et al., 2009). Patients with chronic kidney disease shows a 70% reduction of cardiovascular mortality (Zittermann et al., 2007) and those receiving 2-year calcitriol therapy exhibit a significantly lower adjusted relative risk of 0.35 for predialysis mortality compared with their untreated counterparts (Kovesdy et al., 2008). In the Health Professionals Follow-up Study on case-control male subjects, those with low circulating 25-hydroxyvitamin D concentrations (≤37.5 nmol/L) have a higher adjusted relative risk of 2.09 for myocardial infarction in contrast to those with levels ≤75 nmol/L (Giovannucci et al., 2008).

Endothelial dysfunction is a predictor of impaired cardiovascular function (Lerman and Zeiher, 2005; Taddei et al., 2002). An altered balance between the release of endothelium-derived relaxing and constricting factors results in endothelial dysfunction. Over-production of constrictors is associated with hypertension (Schiffrin, 2001) and renal insufficiency is common in hypertensive patients (US Renal Data System, 2005). However, little is known as to whether chronic vitamin D supplementation can improve endothelial dysfunction of renal vasculature in hypertension, which is characterized by elevated blood pressure, increased activity of RAS and NAD(P)H oxidase. The present study aimed at investigating whether chronic treatment with calcitriol could protect the renovascular function in renal arteries from SHR and humans, and the mechanisms involved.

3.2 Materials and Methods

The use of human renal arteries was approved by the Joint Chinese University of Hong Kong–New Territories East Cluster Clinical Research Ethics Committee. The study was approved by the CUHK Animal Experimentation Ethics Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

3.2.1 Animals and treatment protocols

Six-month old male SHR and Wistar-Kyoto rats (WKY) were supplied by the Laboratory Animal Services Centre, Chinese University of Hong Kong (CUHK), housed in a temperature-controlled room (22–24 °C) with a 12-h light/dark cycle, and provided with a standard diet and water. The rats were assigned to one of the following three groups, (1) WKY control (2) SHR treated with vehicle DMSO (SHR + vehicle) and (3) SHR treated with calcitriol at 150 ng/kg per day (SHR + calcitriol). Calcitriol treatment was given by oral gavage for 4.5 months. The experimental protocols were approved by the CUHK Animal Experimentation Ethics Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

3.2.2 Blood pressure measurement

Blood pressure was measured by the tail-cuff electrosphygmomanometer system (ADInstruments, Sydney, Australia) after the rats were stabilized and remained quiescent. An average of 3 consecutive readings was taken as the systolic blood pressure of each rat.

3.2.3 Preparation of rat and human arteries

Rats were sacrificed after 4.5-month oral treatment of calcitriol. Intralobal renal arteries were dissected and placed in Krebs solution containing (mol/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 arteries were carefully removed of the surrounding connective tissue and cut into 8–10 ring segments with length ~1.6 mm for functional studies in a Multi Myograph System (610M, Danish Myo Technology A/S, Aarhus N, Denmark) in which changes in isometric tension were recorded. Briefly, two stainless steel wires were passed through the arterial lumen and fixed to jaws of the myograph, which was filled with 5 mL Krebs solution continually oxygenated with 95% O_2 and 5% CO_2 and maintained at 37 °C to give a pH of ~7.4. The rings were stretched to an optimal resting tension of 2.5 mN, and allowed to equilibrate for 60 min before the start of experiments.

The human arteries were obtained from nephrectomy specimens of hypertensive patients with their consent. The mean age of patients was 62 years (range between 55 and 70 years). The arteries were incubated overnight in the presence or absence of calcitriol (100 nmol/L) in Dulbecco's Modified Eagle Medium (GIBCO) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (GIBCO) at 37 °C. Vitamin D receptor antagonist TEI-9647, when used, was added 30 min before the addition of calcitriol.

3.2.4 Experimental protocol of isometric tension recording

The arterial rings were pre-incubated with N^G-nitro-L-arginine methyl ester (L-NAME, 100 μmol/L) to eliminate the vasodilatory effect of nitric oxide (NO). Cumulative concentrations of acetylcholine (ACh, 0.03–100 μM) were added to trigger contractions in rings with endothelium. Each of inhibitors or antagonists, when used, was added

together with L-NAME for 30-min incubation prior to the addition of ACh. ACh-induced contraction was presented as the percentage of 60 mmol/L KCl-induced contraction. For human renal arteries, rings were pre-contracted with phenylephrine (3 μmol/L) to attain a stable tension and thereafter ACh was added cumulatively (3 nmol/L to 10 μmol/L) to induce relaxations.

3.2.5 Primary culture of rat aortic endothelial cells

The detailed information is provided in the methods section in Chapter II.

3.2.6 Western blot analysis

The detailed information is provided in the methods section in Chapter II.

3.2.7 Detection of reactive oxygen species by dihydroethidium (DHE) fluorescence and electron paramagnetic resonance (EPR)

The detailed information is provided in the methods section in Chapter II.

3.2.8 Chemicals

ACh, L-NAME, phenylephrine, losartan, diphenyleneiodonium and tempol were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). Calcitriol and Ang II were from Tocris Bioscience (Avonmouth, UK). (23S)-25-dehydro-1-hydroxyvitamin D3-26,23-lactone (TEI-9647) was a kind gift from Teijin Pharma Limited (Tokyo, Japan). Except ACh, L-NAME, phenylephrine, tempol and Ang II were dissolved in distilled water, and TEI-9647 in ethanol, other drugs were prepared in DMSO.

3.2.9 Statistical Analysis

Data were presented as the means ± SEM of 5-7 experiments. Statistical significance was determined by two-tailed Student's *t*-test or one-way ANOVA followed by Bonferroni post-tests whenever appropriate (GraphPad Software, San Diego, California). $P<0.05$ is considered significantly different.

3.3 Results

3.3.1 *In vivo* calcitriol treatment attenuates augmented endothelium-dependent contractions in SHR renal arteries

There is no difference in animal body weights between vehicle-treated and calcitriol-treated group (402.0 ± 7.3 g in vehicle group and 404.0 ± 12.5 g in calcitriol treatment group, Figure 3.1). The elevated blood pressure in SHR was reduced after 4.5-month treatment with calcitriol (194.6 ± 4.9 mmHg before treatment vs 169.9 ± 4.9 mmHg after treatment, $P<0.05$; Figure 3.2). Pronounced ACh-induced contractions were observed in renal arteries of vehicle-treated SHR (E_{max}: 88.5 ± 5.3 % in SHR + vehicle, n=9) compared to the normotensive WKY control (E_{max}: 13.7 ± 11.0 % in WKY, n=10) (Figure 3.3A and B). The contractions were endothelium-dependent as they were abolished in rings without endothelium (Figure 3.3C). Chronic oral treatment with calcitriol (150 ng/kg/day for 4.5 months) attenuated the endothelium-dependent contractions (E_{max}: 40.9 ± 10.6 % in SHR + calcitriol, n=8; Figure 3.3A and B). Acute (30-min) exposure of renal arteries from SHR + vehicle to calcitriol (100 nM) did not modify the endothelium-dependent contractions (Figure 3.3D). By contrast, the contractions in these arteries were suppressed to the same extent by the acute treatment with losartan (AT_1R antagonist, 3 μmol/L), DPI [NAD(P)H oxidase inhibitor, 100 nmol/L], and tempol (ROS scavenger, 100 μmol/L) (Figure 3.3E).

3.3.2 Calcitriol reduces the AT_1R expression

The AT_1R expression in renal arteries was elevated in SHR (Figure 3.4A) while the AT_2R level remained unchanged (Figure 3.4B). Over-expression of AT_1R was normalized by the oral treatment of calcitriol (Figure 3.4A). Renal arteries from SHR exhibited greater Ang II contractions, which were reversed by an overnight tissue treatment with 100 nmol/L calcitriol (Figure 3.4C).

3.3.3 Calcitriol normalizes the expression of enzymes related to oxidative stress

The level of oxidative stress as reflected by the nitrotyrosine content (Figure 3.5A) and the expressions of NOX-2 (Figure 3.5B) and $p67^{phox}$ (Figure 3.5C), the subunits of NAD(P)H oxidase were augmented in SHR renal arteries, while the levels of superoxide dismutase (SOD)-1 (Figure 3.6A) and SOD-2 (Figure 3.6B) were diminished in these arteries. Chronic treatment with calcitriol corrected the nitrotyrosine level and over-expression of NOX-2 and $p67^{phox}$ (Figure 3.5), and enhanced the expression of both SOD isoforms (Figure 3.6).

Normalization of the altered expressions of NAD(P)H oxidase and SODs resulted in a decreased ROS level. Renal arteries from SHR treated with vehicle exhibited increased ROS accumulation across the vascular wall compared to WKY (Figure 3.7). The elevated ROS level was reduced in arteries from calcitriol-treated SHR (Figure 3.7). By contrast, acute 30-min treatment with calcitriol did not reduce the arterial ROS level, which was acutely inhibited by DPI (100 nmol/L), tempol (100 μmol/L) and losartan (3 μmol/L) (Figure 3.8).

3.3.4 Calcitriol acts directly on renal arteries in tissue culture

The endothelium-dependent contractions in SHR renal arteries were abolished by an

overnight treatment with calcitriol (100 nmol/L), for which the effect was similar to that with losartan (Figure 3.9). Actinomycin-D (2 μmol/L) reversed the inhibitory effect of calcitriol on the contractions (Figure 3.9). Western blot analysis showed that the tissue levels of AT_1R (Figure 3.10A), NOX-2 (Figure 3.10B) and NOX-4 (Figure 3.10C) were diminished in calcitriol-treated SHR renal arteries.

DHE fluorescence showed that the excessive production of ROS in SHR renal arteries was alleviated after 12-h incubation with calcitriol, of which the effect was reversed by actinomycin-D. By contrast, acute calcitriol exposure for 30 min did not reduce ROS levels (Figure 3.11). The results were further confirmed using electron paramagnetic resonance (EPR) on the homogenized renal arteries after treatments. Likewise, SHR renal arteries exhibited a higher ROS level, which was reduced by 8-h calcitriol treatment (Figure 3.12A).

Using a cell-free radical-generating system, hypoxanthine (100 μmol/L)-xanthine oxidase (9 mU/mL) (HXXO), we further demonstrated that the calcitriol-induced ROS reduction was not attributable to acute ROS scavenging effect. HXXO produced a pronounced EPR signal, indicating ROS generation and this increased signal was unaffected by calcitriol but abolished by oxypurinol, the xanthine oxidase inhibitor (Figure 3.12B).

3.3.5 Calcitriol prevents Ang II-induced vascular dysfunction in WKY renal arteries

Overnight incubation of Ang II (100 nmol/L) in renal arteries from normotensive WKY impaired ACh-induced endothelium-dependent relaxations (pD_2: 6.95 ± 0.03, E_{max}: 77.3 ± 1.2 % in control, n=4; pD_2: 6.07 ± 0.36 vs E_{max}: 41.2 ± 7.5 % in Ang II-treated rings, n=5; $P<0.05$; Figure 3.13A and C) and unveiled endothelium-dependent contractions

(E_{max}: 0.4 ± 0.4 % in control, n=4 vs E_{max}: 73.8 ± 4.3 % in Ang II-treated rings, n=6; $P<0.05$; Figure 3.13B and D). Pre-treatment with calcitriol before Ang II exposure prevents attenuation in endothelium-dependent relaxations (pD_2: 7.00 ± 0.08, E_{max}: 69.6 ± 1.8 %, n=5, $P<0.05$ compared with control) and abolished endothelium-dependent contractions (Figure 3.13A-D). Pre-incubation with losartan, DPI and tempol protected renal arteries from Ang II-induced vascular dysfunction (Figure 3.13C-E).

Ang II (100 nmol/L, overnight) augmented the expressions of NOX-2 (Figure 3.14A) and NOX-4 (Figure 3.14B) in WKY renal arteries. NOX-2 and NOX-4 over-expression was reversed by calcitriol and losartan treatment (Figure 3.14). In parallel, the ROS level of primary cultured WKY aortic endothelial cells was elevated by overnight culture with Ang II. Pre-treatment with calcitriol, losartan, tempol and DPI all reversed the ROS increases (Figure 3.15).

3.3.6 Calcitriol enhances dilatations and normalizes ROS production in human renal arteries

The relaxations to the endothelium-dependent dilator acetylcholine were attenuated in renal arteries obtained from hypertensive patients (pD_2: 6.19 ± 0.55, E_{max}: 15.3 ± 6.7%, n=6 in hypertensive patients versus pD_2: 6.96 ± 0.10, E_{max}: 87.1 ± 2.6%, n=4 in normotensive patients; $P<0.05$, Figure 3.16). Overnight *in vitro* treatment with calcitriol (100 nmol/L) enhanced the relaxations (pD_2: 6.34 ± 0.20, E_{max}: 54.1 ± 4.5%, n=4), and this effect of calcitriol was prevented by the human vitamin D receptor (VDR) antagonist, TEI-9647 (1 μmol/L) (Figure 3.16). DHE fluorescence showed that the high ROS level in the vascular wall of renal arteries from hypertensive patients was reduced by calcitriol, an effect also antagonized by TEI-9647 (Figure 3.17).

Immunofluorescence with the reddish orange Alexa Fluor 546 signals from

respective antibodies demonstrated that the expression of AT_1R(Figure 3.18), NOX-2 (Figure 3.19), NOX-4 (Figure 3.20) and $p67^{phox}$ (Figure 3.2l) in renal arteries from hypertensive patients were diminished by overnight exposure to calcitriol, while that of SOD-1 (Figure 3.22) was enhanced. The effects of calcitriol were antagonized by TEI-9647.

Overnight incubation with Ang II (1 μmol/L) increased the ROS level (Figure 3.23A) and up-regulated the expression of NOX-2, NOX-4 and $p67^{phox}$ (Figure 3.23B) in renal arteries from normotensive patients. The increase in the protein expressions and ROS level were prevented by co-incubation with calcitriol, the effect of which was antagonized by TEI-9647 (Figure 3.23). Likewise, human aortic endothelial cells (HAEC) incubated with Ang II overnight exhibited an increased ROS level, which was attenuated by co-incubation with calcitriol or tempol (ROS scavenger, 100 μmol/L). TEI-9647 prevented the effect of calcitriol without modifying that of tempol (Figure 3.24).

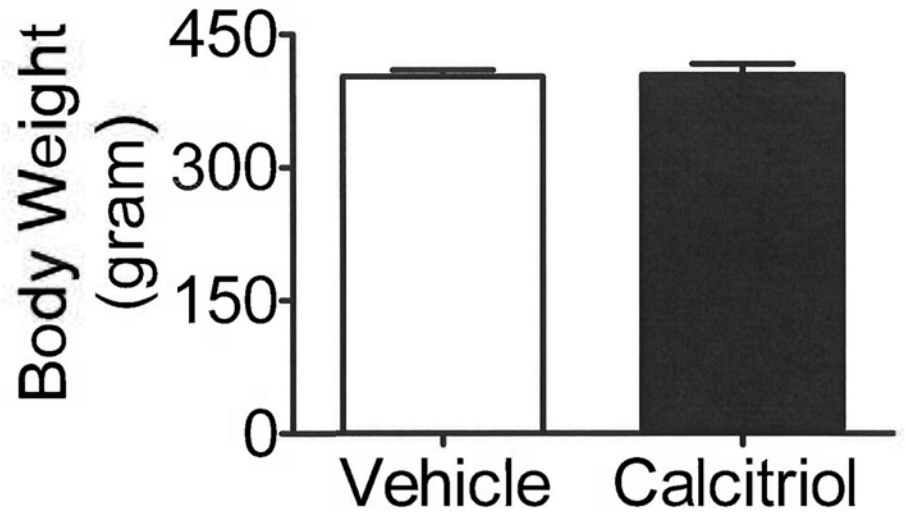

Figure 3.1

Body weight is comparable between vehicle group and calcitriol treatment group. Data are means ± SEM of 5-7 experiments.

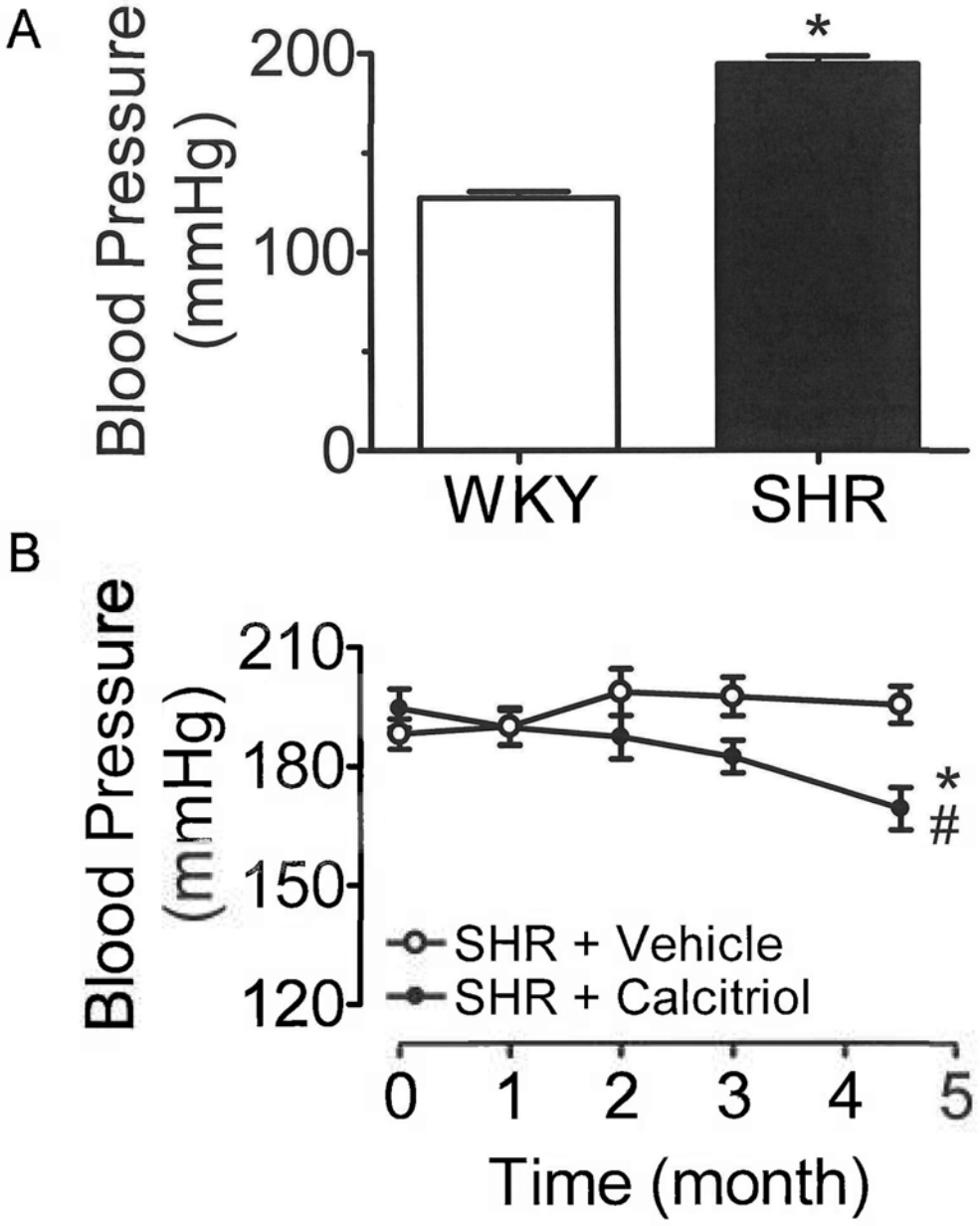

Figure 3.2

(A) Blood pressure was higher in SHR compared to WKY. (B) Chronic treatment of calcitriol decreased blood pressure in SHR. Data are means ± SEM of 8-10 experiments. *$P<0.05$ versus WKY or the calcitriol group of SHR before treatment; $^{\#}P<0.05$ versus the vehicle group of SHR after treatment.

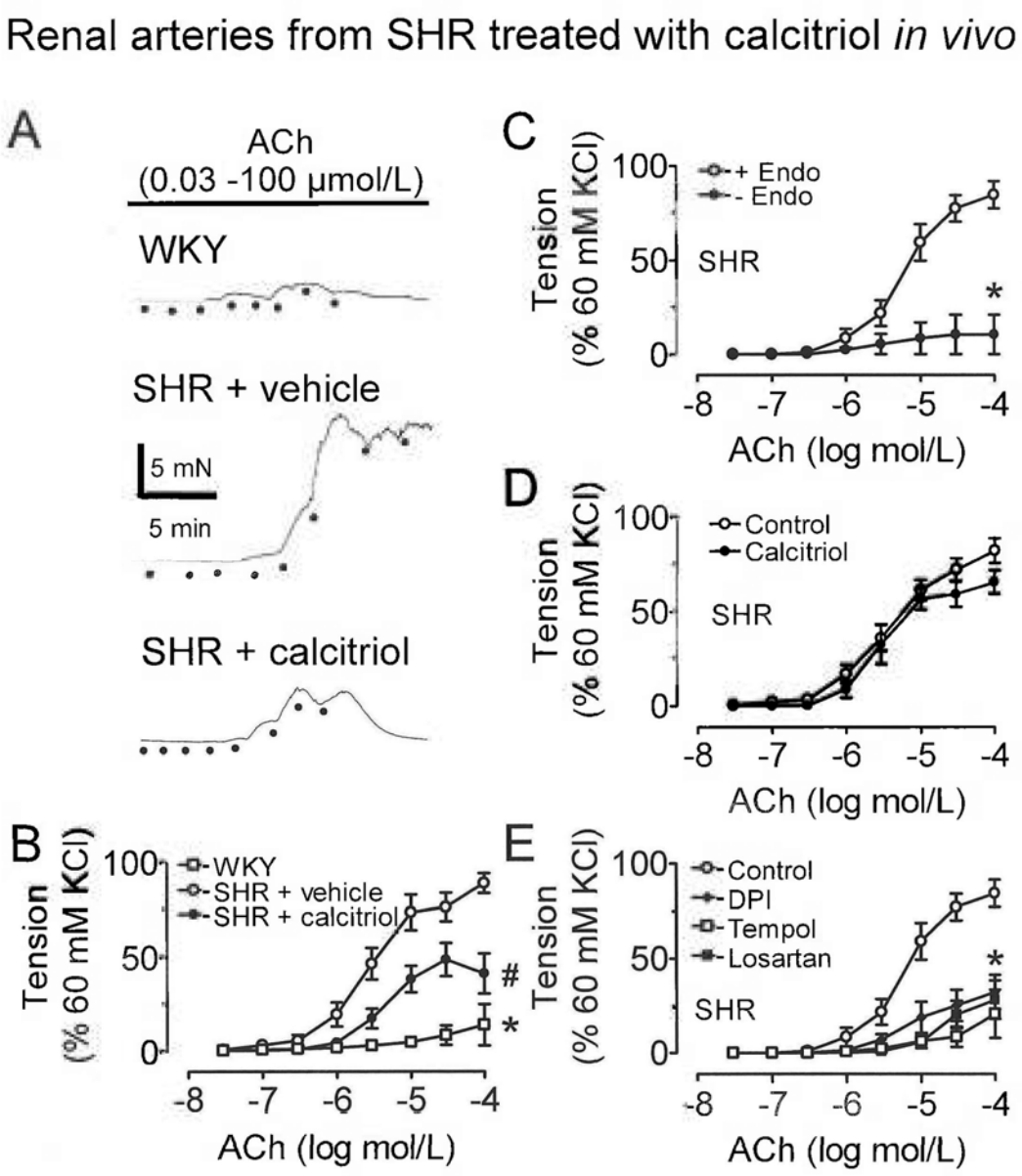

Figure 3.3

(A) Representative traces and (B) concentration-contraction curves showing the inhibitory effect of calcitriol on acetylcholine (ACh)-induced contractions, which were endothelium-dependent as reflected by the absence of contractions in rings without endothelium (-Endo, C). Endothelium-dependent contractions were unaffected by acute exposure (30-min) to calcitriol (100 nmol/L) (D), but attenuated by losartan (3 µmol/L), DPI (100 nmol/L) and tempol (100 µmol/L) (E). Data are means ± SEM of 5 experiments. $^{*}P<0.05$ versus control (SHR + vehicle) or +Endo; $^{\#}P<0.05$ versus vehicle.

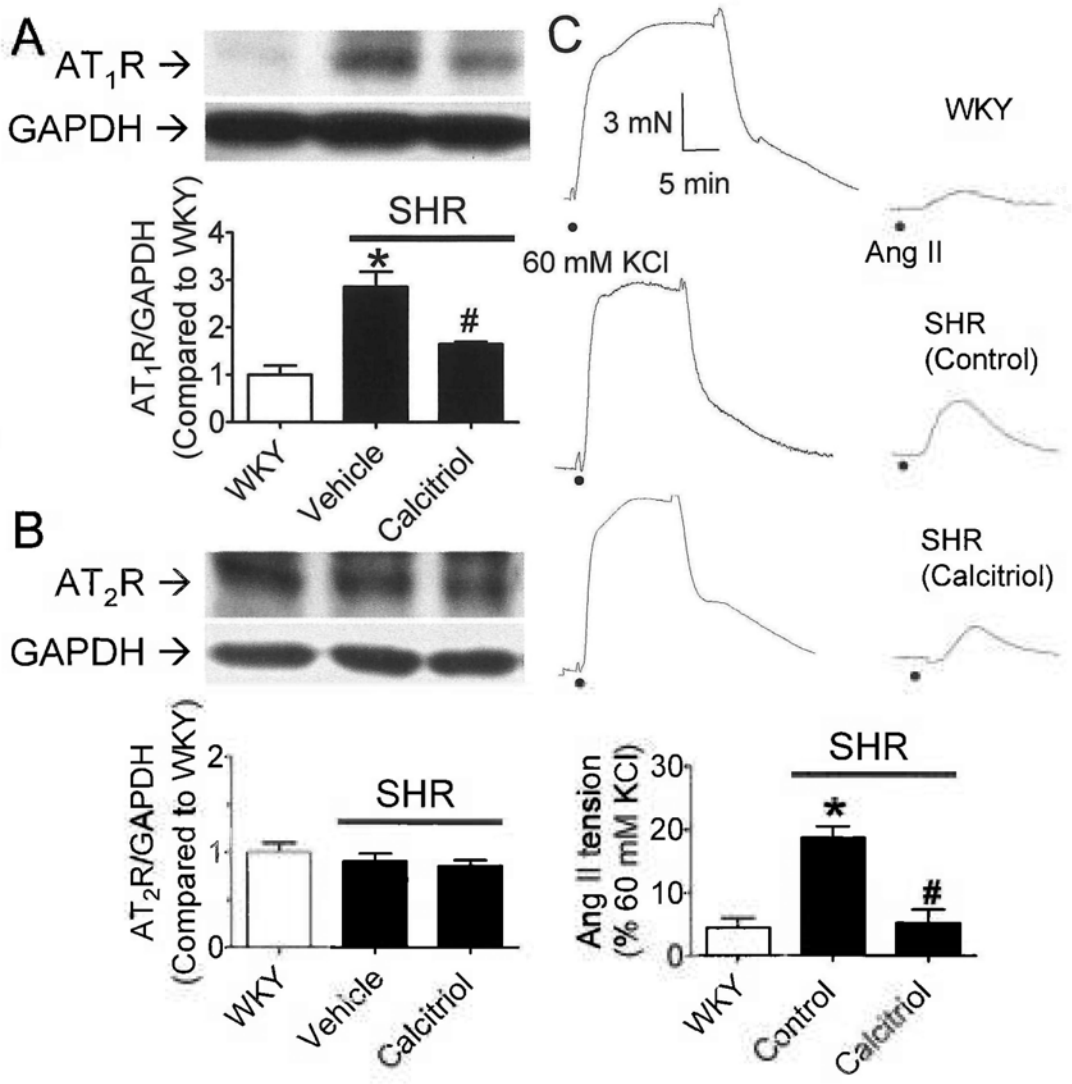

Figure 3.4

(A) Western blot analysis showed that chronic *in vivo* calcitriol treatment inhibited the over-expression of AT_1R in SHR renal arteries. (B) AT_2R expression was similar in renal arteries of WKY, SHR, and calcitriol-treated SHR. Data are means ± SEM of 4 experiments. $^*P<0.05$ compared to WKY; $^{\#}P<0.05$ versus SHR treated with vehicle. (C) Overnight tissue culture of the SHR renal arteries in the presence of calcitriol inhibited Ang II-induced contractions. Data are means ± SEM of 4 experiments. $^*P<0.05$ versus control; $^{\#}P<0.05$ versus calcitriol.

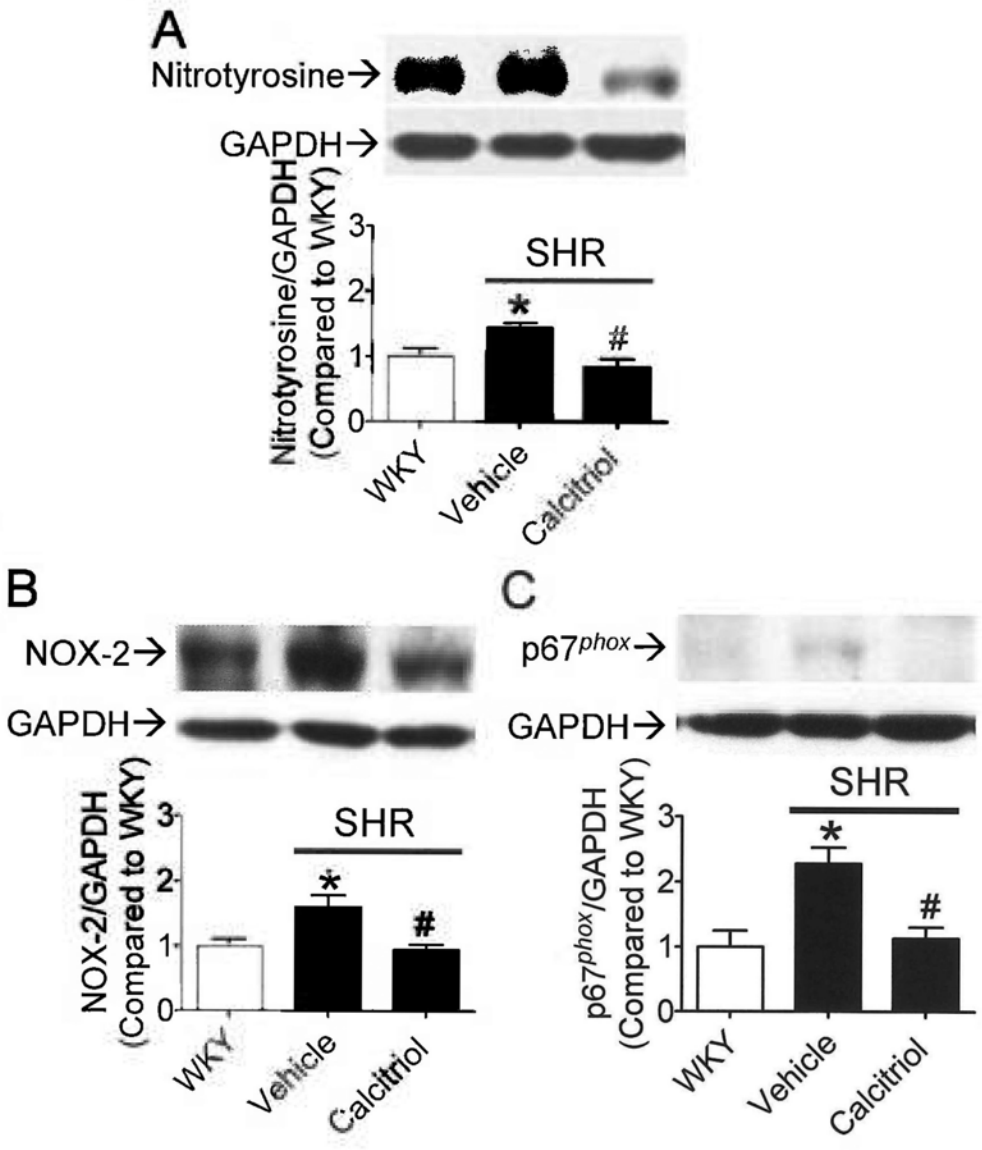

Figure 3.5

Chronic treatment of calcitriol normalized (A) the elevated nitrotyrosine level and down-regulated the over-expression of NAD(P)H oxidase subunits, (B) NOX-2 and (C) $p67^{phox}$ in the SHR renal arteries. Data are means ± SEM of 5 experiments. $^{*}P<0.05$ versus WKY; $^{\#}P<0.05$ versus SHR treated with vehicle.

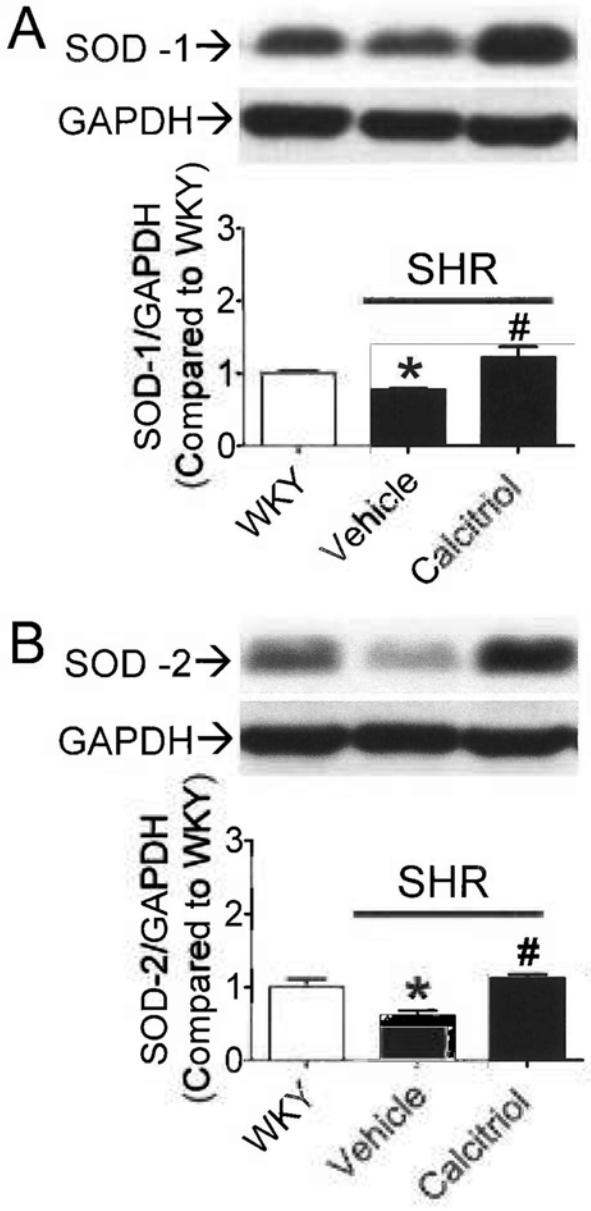

Figure 3.6

Chronic treatment of calcitriol augmented the diminished expression of (A) SOD-1 and (B) SOD-2 in the SHR renal arteries. Data are means ± SEM of 5 experiments. *$P<0.05$ versus WKY; $^{\#}P<0.05$ versus SHR treated with vehicle.

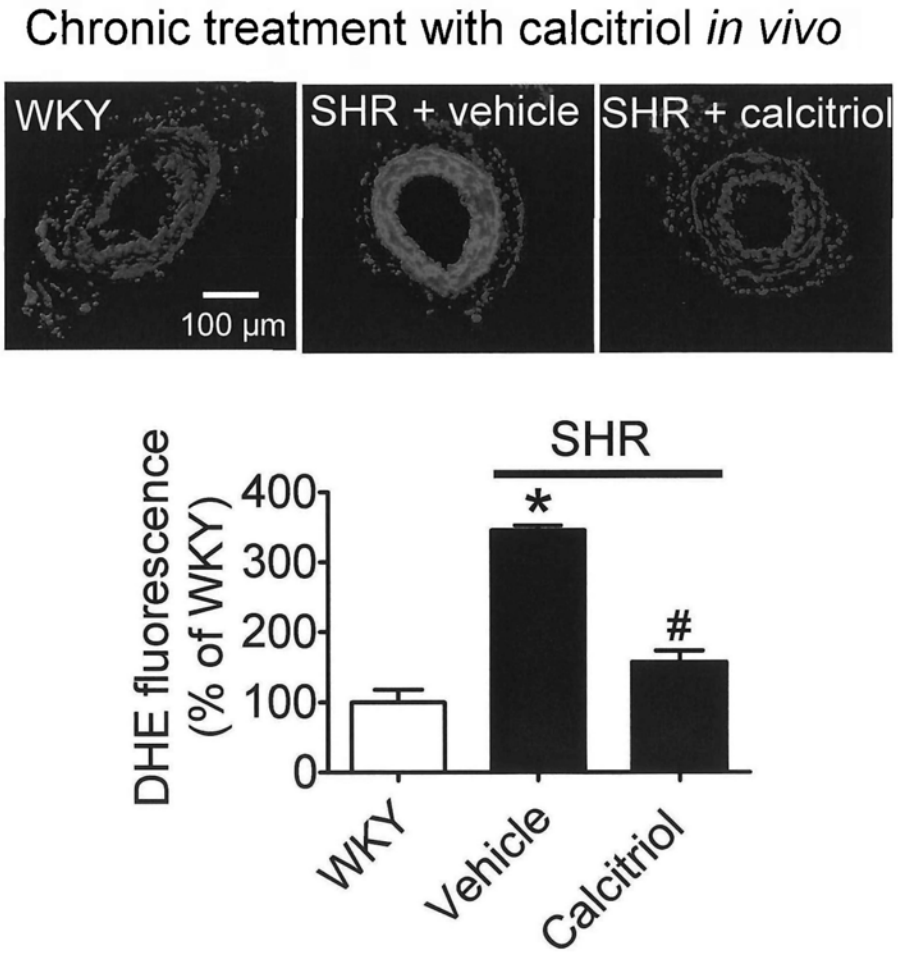

Figure 3.7

Representative images and summarized data showing that the elevated levels of ROS in SHR arteries are reduced by calcitriol. Data are means ± SEM of 4-5 experiments. *$P<0.05$ versus WKY; $^{\#}P<0.05$ versus SHR + vehicle.

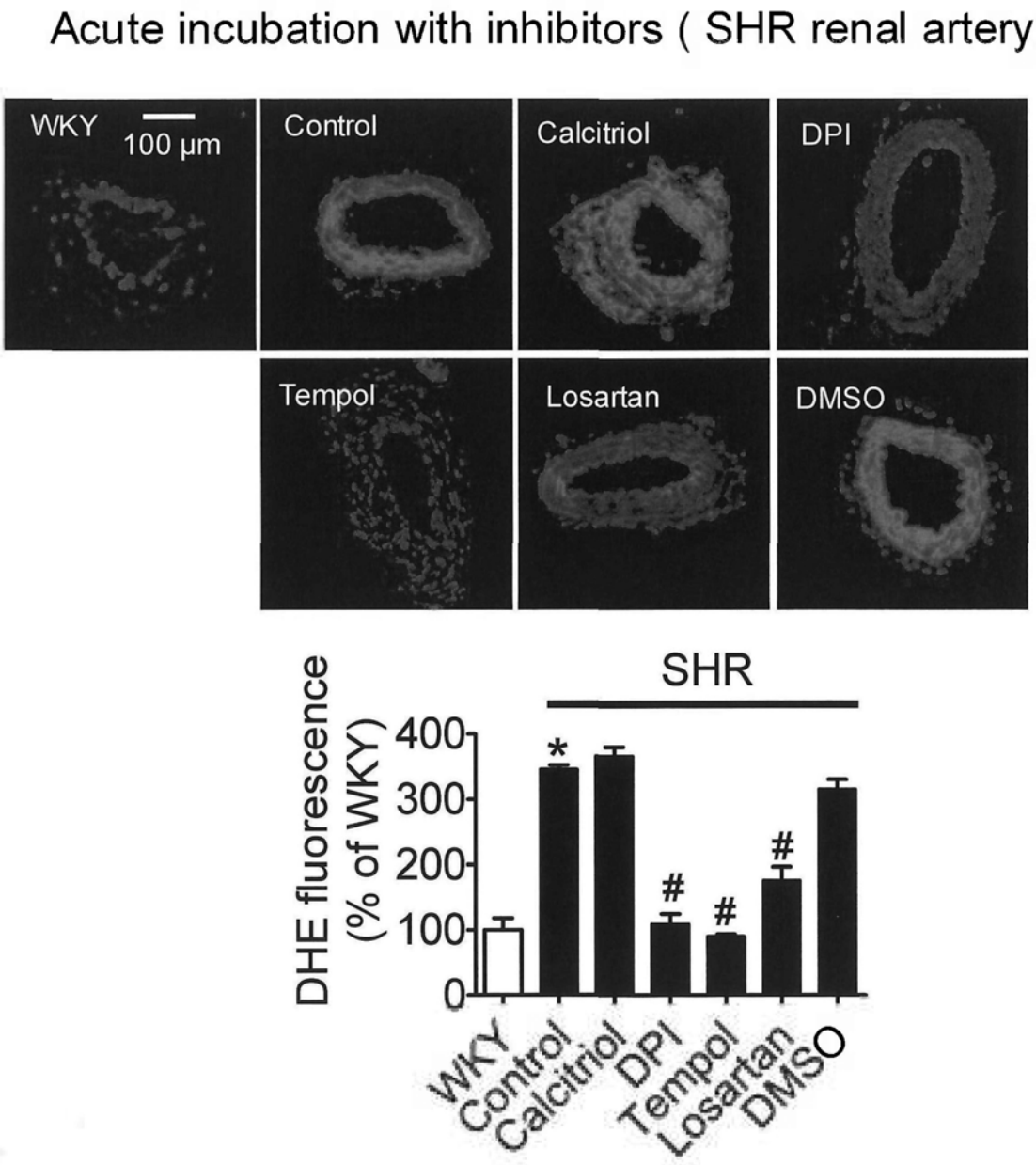

Figure 3.8

Acute exposure (30-min) of SHR renal arteries to DPI (100 nmol/L), tempol (100 µmol/L) and losartan (3 µmol/L) but not calcitriol (100 nmol/L) or DMSO normalized the ROS level. Data are means ± SEM of 4 experiments. *$P<0.05$ versus WKY; $^{\#}P<0.05$ versus SHR control.

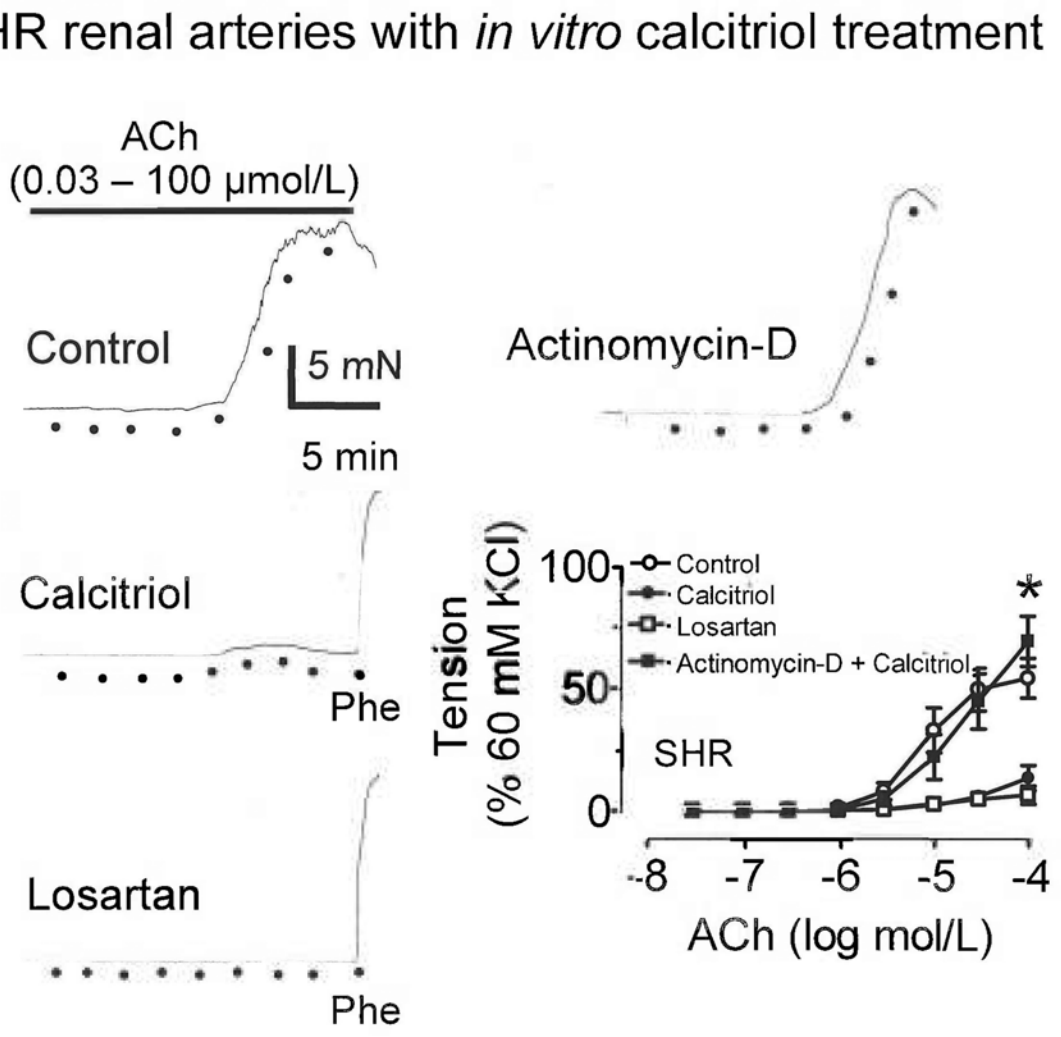

Figure 3.9

Overnight tissue culture with calcitriol (100 nmol/L) or losartan (3 µmol/L) reduced contractions of quiescent SHR renal arteries with endothelium to acetylcholine (ACh). he effect of calcitriol was abolished by combined incubation with actinomycin-D (2 µmol/L). Data are means ± SEM of 4-5 experiments. *P<0.05 compared versus control.

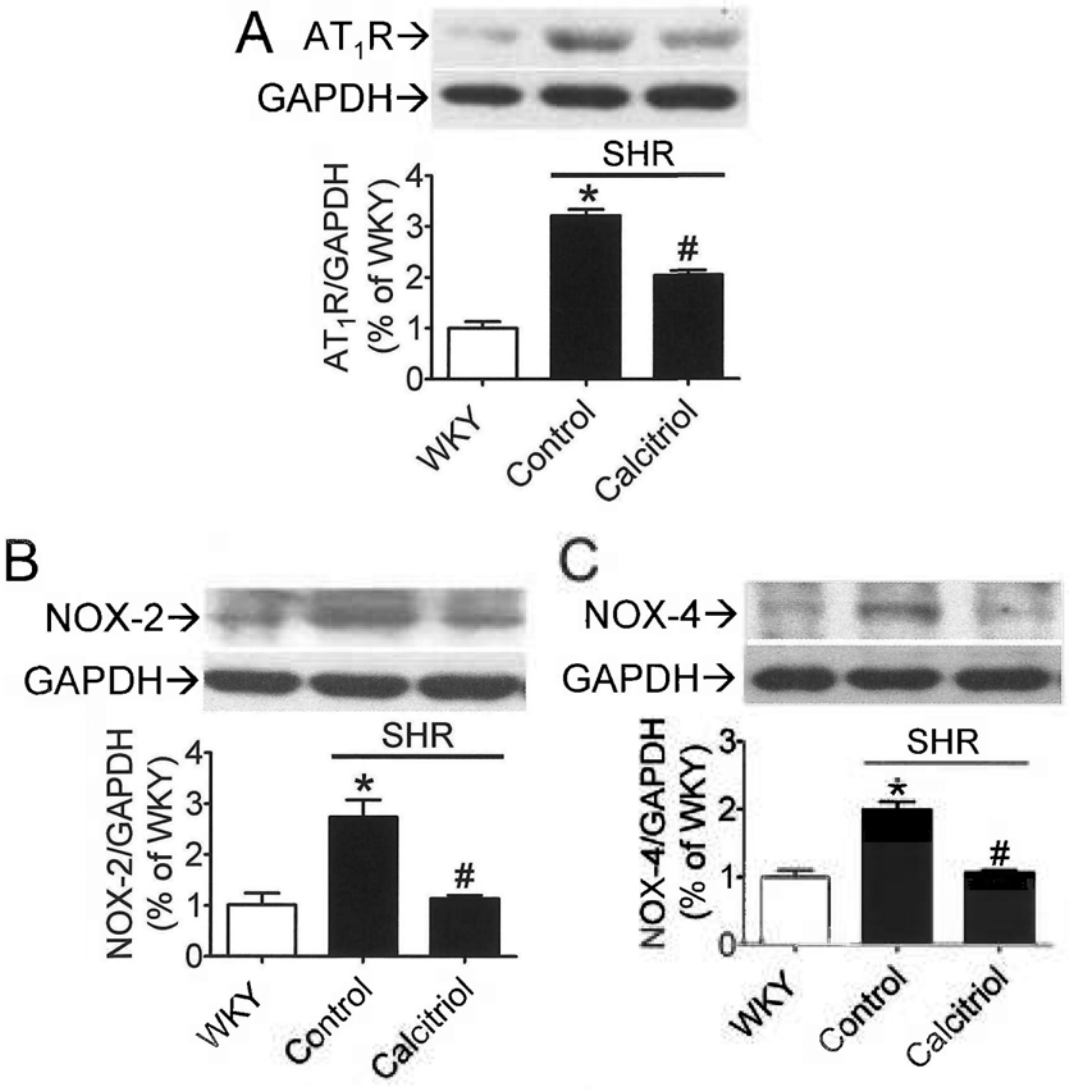

Figure 3.10

Overnight incubation with calcitriol in SHR renal arteries reduced the expressions of (A) AT_1R, (B) NOX-2 and (C) NOX-4. Data are means ± SEM of 4-5 experiments. *$P<0.05$ compared to control. $^{\#}P<0.05$ versus SHR control.

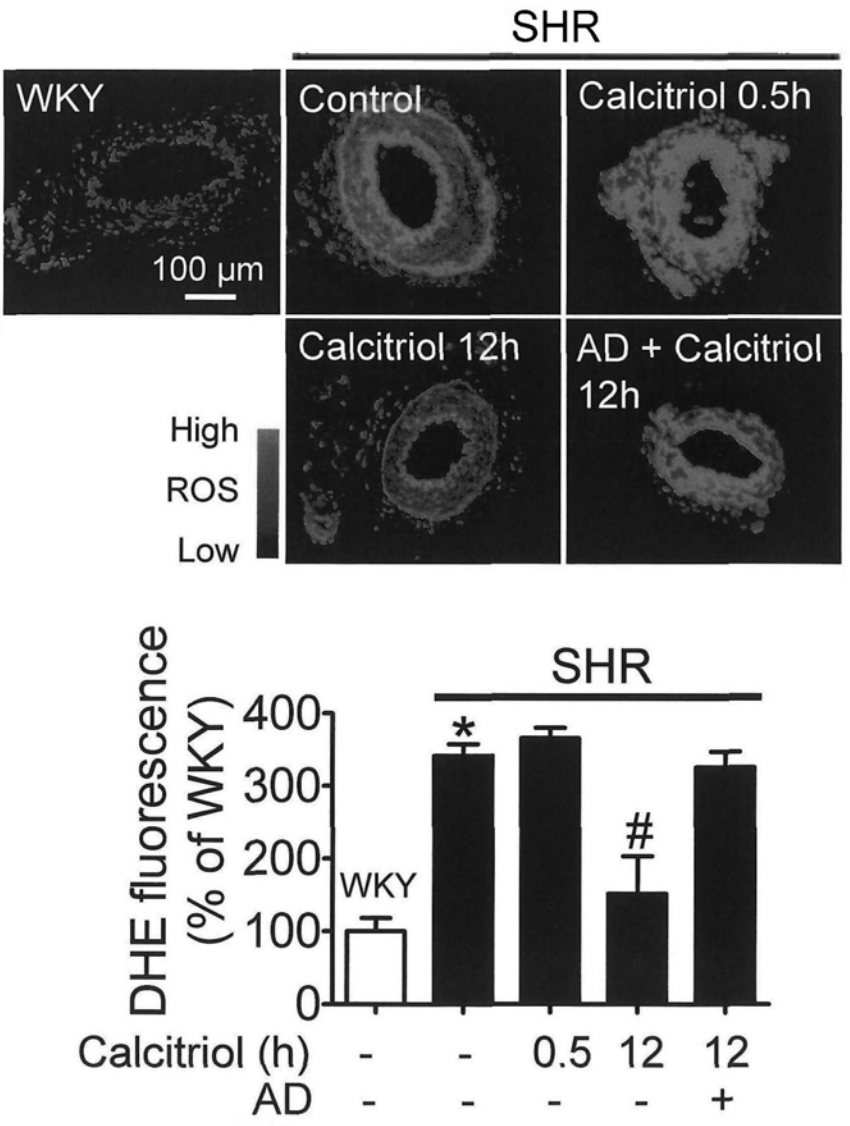

Figure 3.11

DHE fluorescence showed that SHR renal arteries exhibited elevated ROS level compared to WKY arteries. Twelve-hour calcitriol treatment reduced the ROS level and this effect was abolished by actinomycin-D (AD; 2 µmol/L). In contrast, acute exposure (30 min) of SHR renal arteries to calcitriol (100 nmol/L) did not reduce ROS level. Data are means ± SEM of 4-5 experiments. $^{*}P<0.05$ compared to control. $^{\#}P<0.05$ versus SHR control.

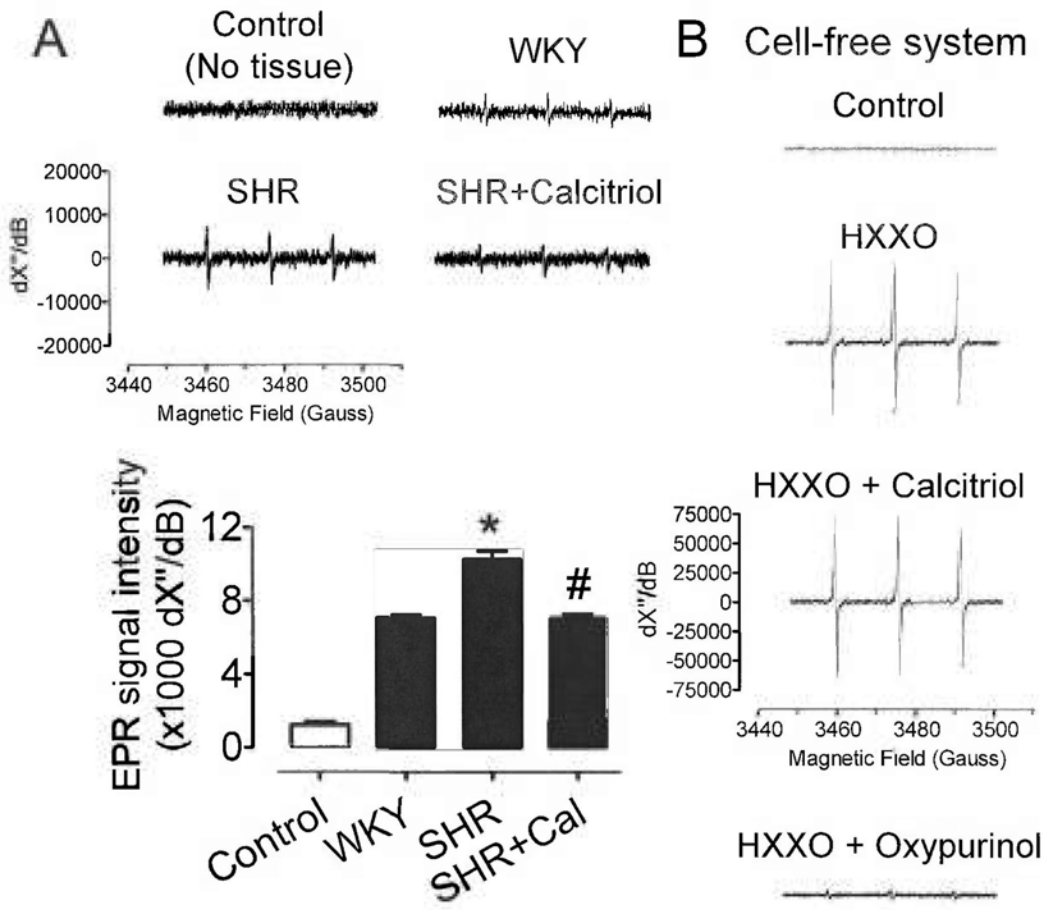

Figure 3.12

(A) The ROS level measured by electron paramagnetic resonance (EPR) was greater in renal arteries from SHR compared to those from WKY and calcitriol reduced the elevated ROS level in SHR renal arteries. Data are means ± SEM of 4 experiments. $^{*}P<0.05$ versus WKY; $^{\#}P<0.05$ versus SHR without treatments. (B) Data from EPR showed that hypoxanthine (HX, 100 μmol/L) and xanthine oxidase (XO, 9 mU/mL) generated reactive oxygen species in a cell-free system compared to the control. The HXXO-induced ROS production was not altered by calcitriol (100 nmol/L) but abolished by oxypurinol (100 μmol/L), an xanthine oxidase inhibitor.

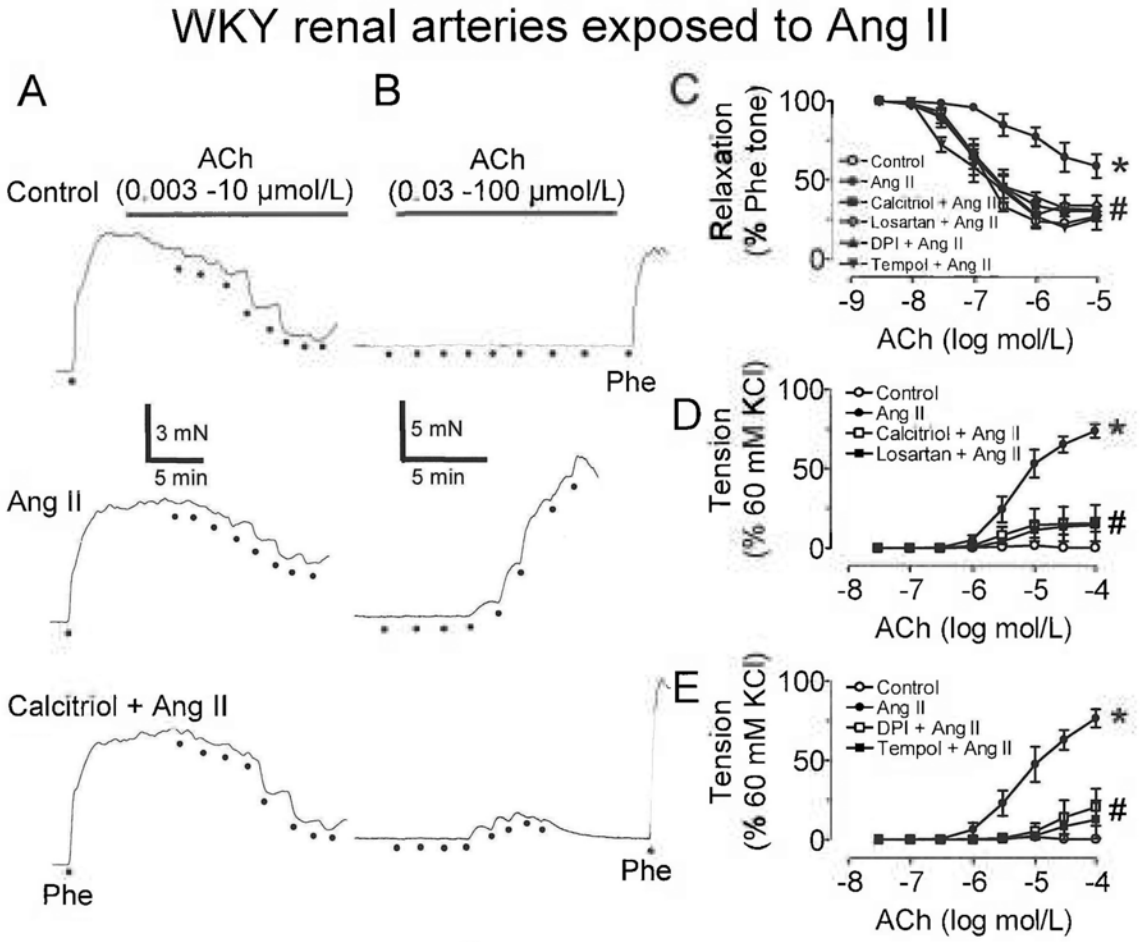

Figure 3.13

Overnight exposure to Ang II (100 nmol/L) (A) impaired relaxations to acetylcholine (ACh) and (B) unmasked contractions to the muscarinic agonist in renal arteries with endothelium from normotensive WKY. (A-D) Combined exposure to calcitriol (100 nmol/L) enhanced the relaxations and reduced the contractions. (C-E) Losartan (3 µmol/L), DPI (100 nmol/L) and tempol (100 µmol/L) reversed endothelial dysfunction in Ang II-treated WKY renal arteries. Data are means ± SEM of 4-5 experiments. $^{*}P<0.05$ versus control; $^{\#}P<0.05$ versus Ang II.

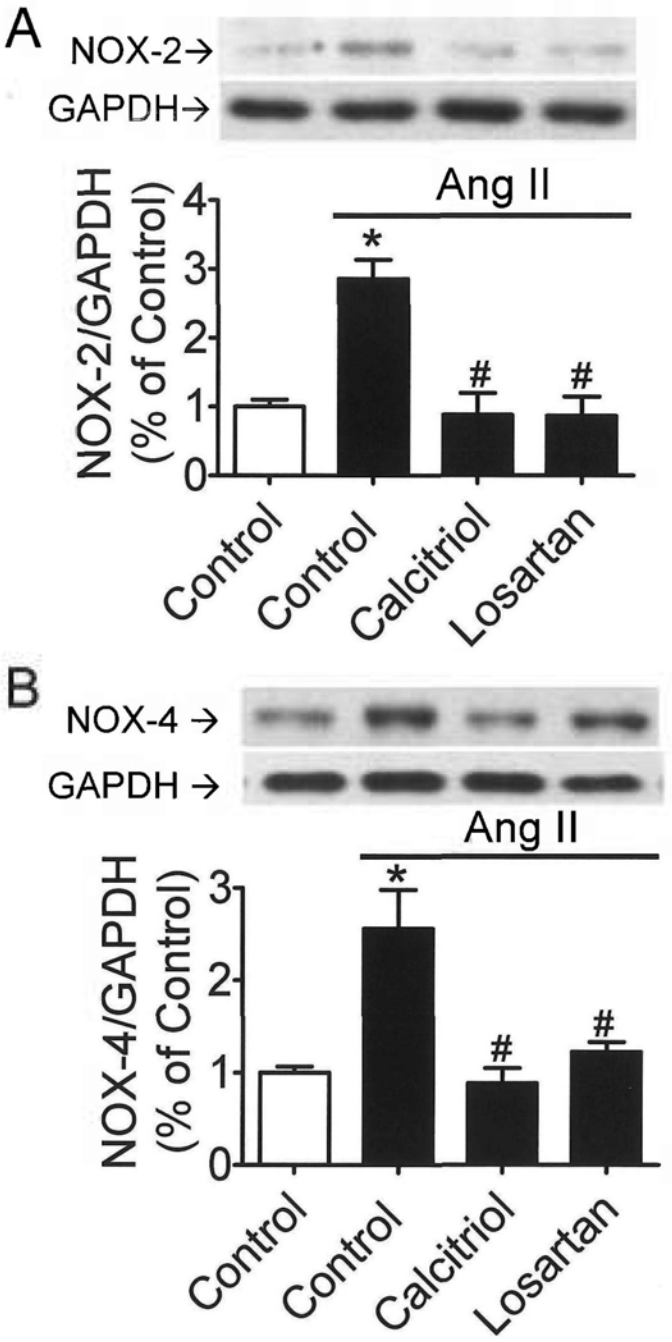

Figure 3.14

Overnight Ang II (100 nmol/L) exposure elevated the expression of (A) NOX-2 and (B) NOX-4 in renal arteries of WKY. The NOX-2 and NOX-4 expression was normalized by the co-incubation with calcitriol (100 nmol/L) or losartan (3 μmol/L). Data are means ± SEM of 4 experiments. *P<0.05 versus control; $^{\#}P$<0.05 versus Ang II.

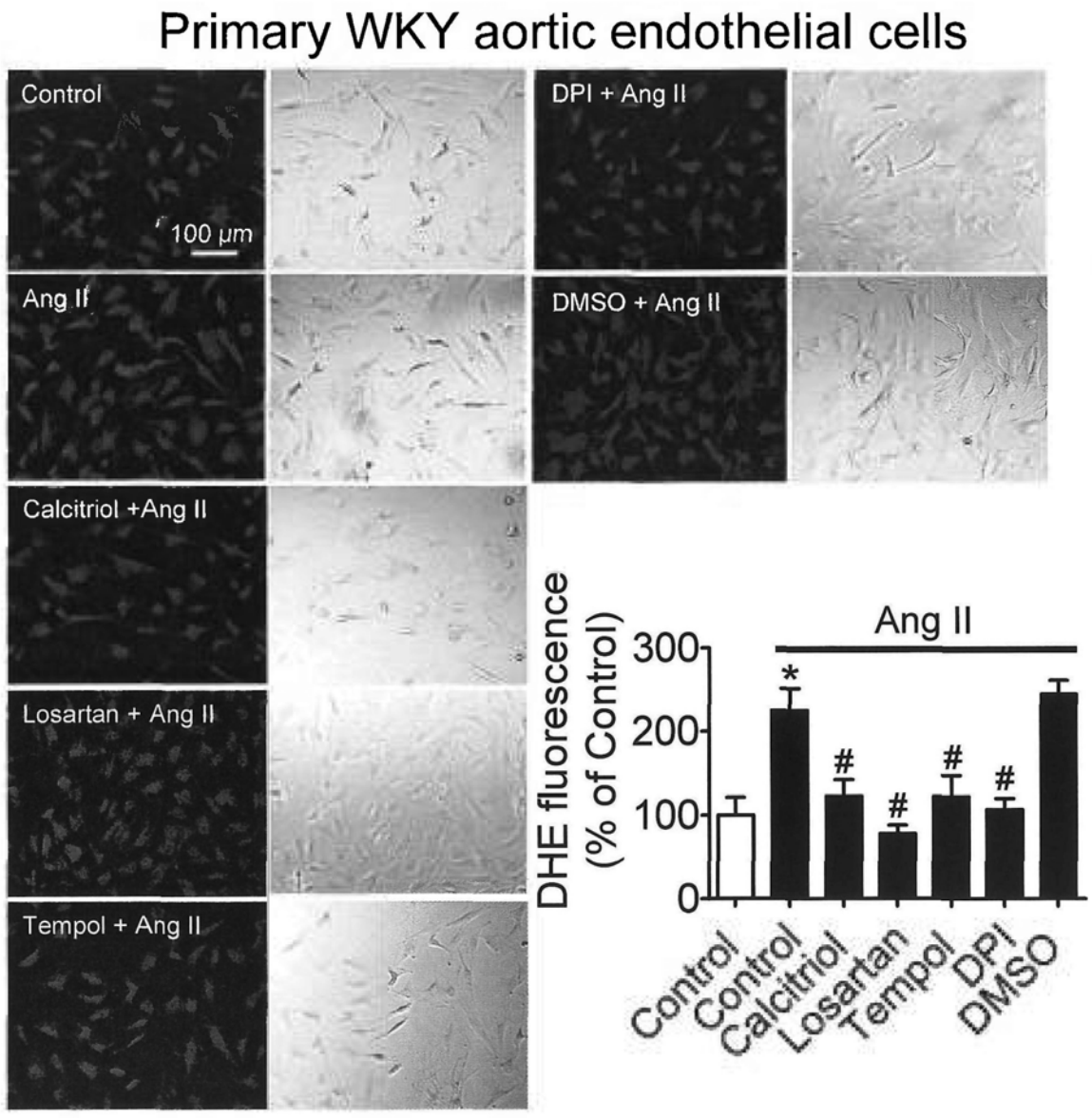

Figure 3.15

Ang II (100 nmol/L, overnight) increased the ROS level in primary WKY endothelial cells. The ROS level was reduced by treatment with calcitriol (100 nmol/L), losartan (3 μmol/L), tempol (100 μmol/L) or DPI (100 nmol/L). DMSO as the solvent control did not alter the ROS level. Data are means ± SEM of 5 experiments. $^{*}P<0.05$ versus control; $^{\#}P<0.05$ versus Ang II.

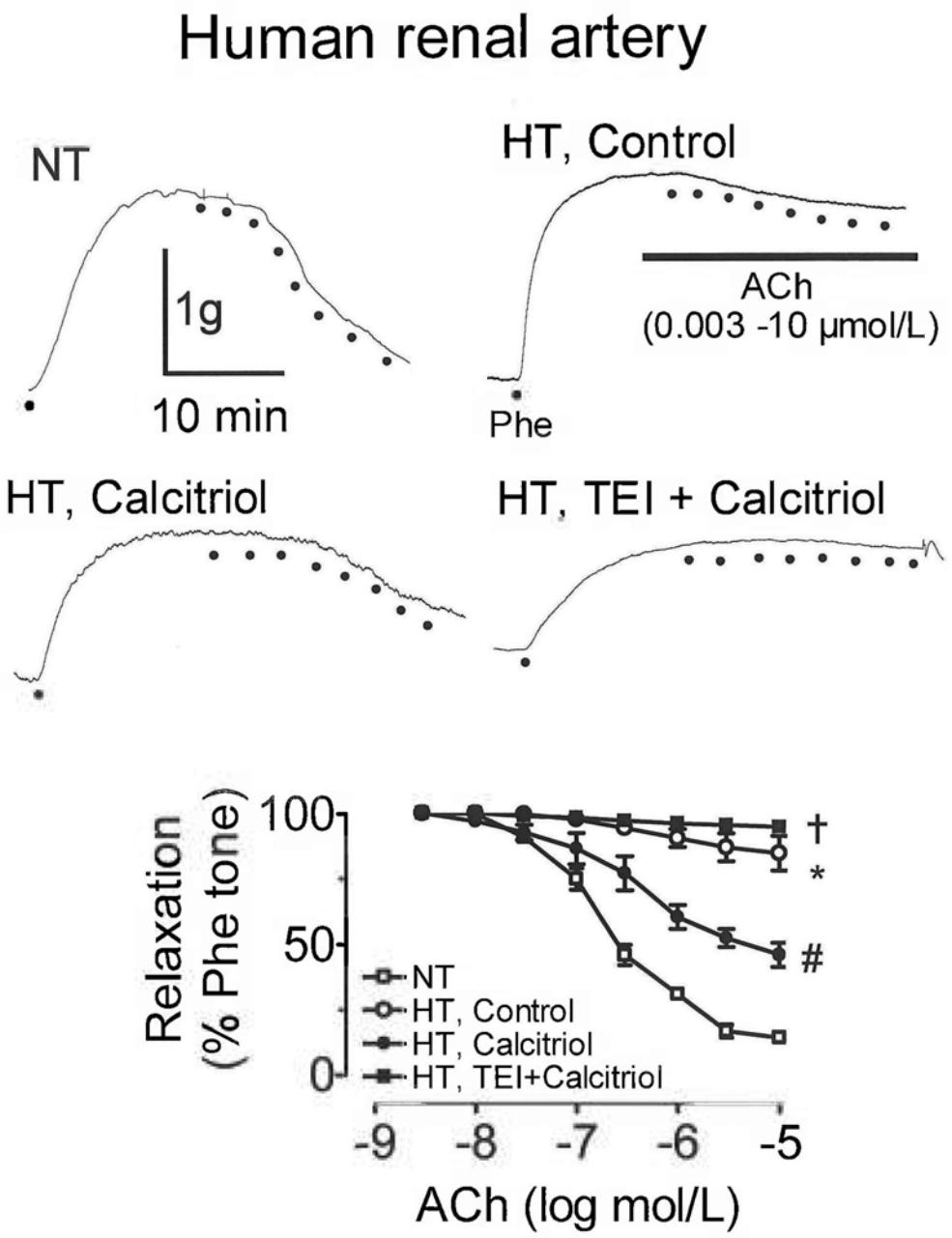

Figure 3.16

ACh-induced relaxations were enhanced by calcitriol (100 nmol/L) treatment; this effect was prevented by a specific antagonist against human VDR, TEI-9647 (TEI, 1 µmol/L). The graph shows means ± SEM of 3-6 experiments on samples from different patients, with relaxations expressed as percentage of the stable contraction to phenylephrine (Phe). $^{*}P<0.05$ versus normotensive patients (NT); $^{\#}P<0.05$ versus control from hypertensive patients (HT, Control) and $^{\dagger}P<0.05$ versus HT.

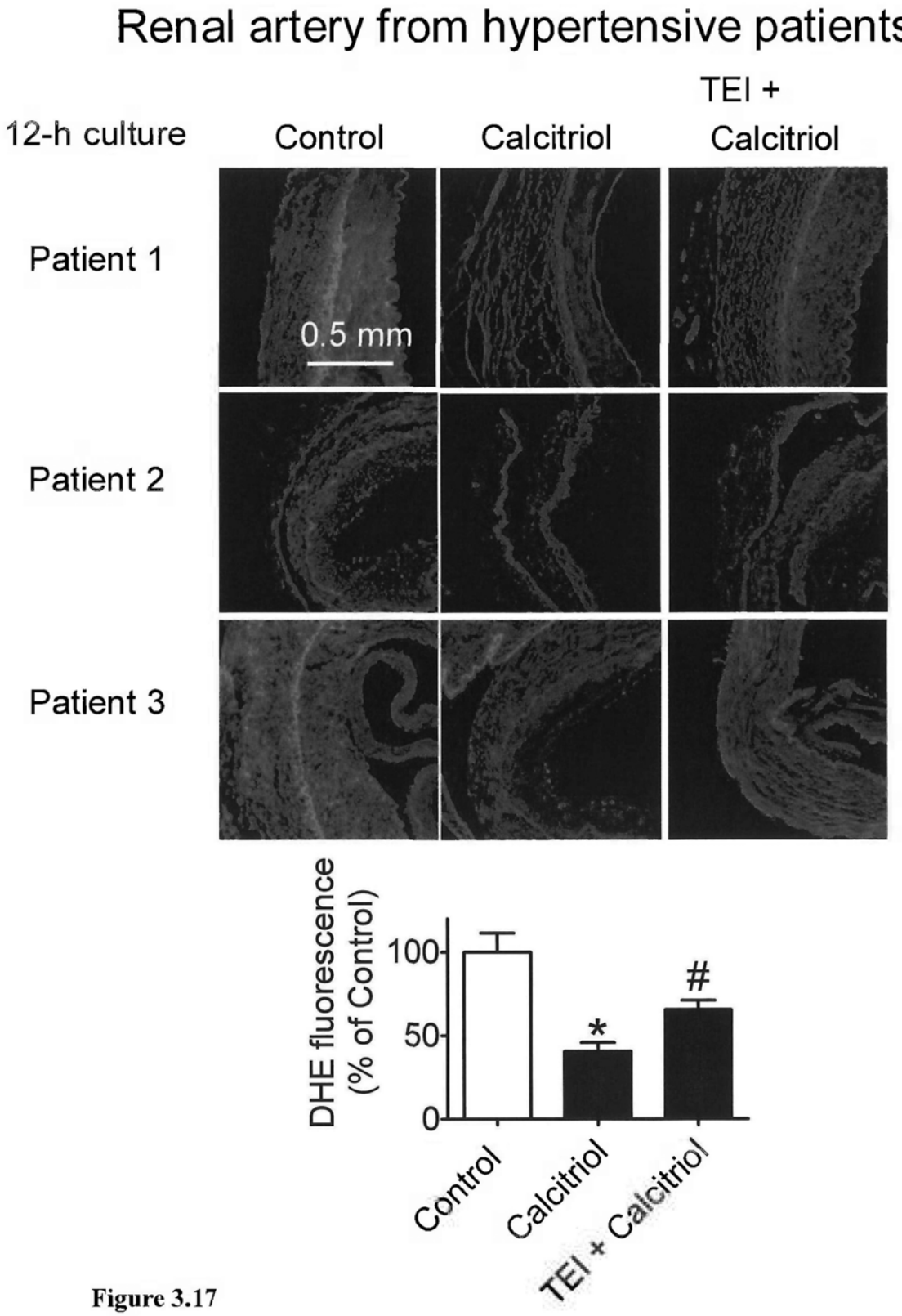

Figure 3.17

In renal artery of three hypertensive patients, DHE fluorescence of ROS showed that calcitriol reduced ROS level, an effect was antagonized by TEI-9647. $^{*}P<0.05$ versus Control; $^{\#}P<0.05$ versus Calcitriol.

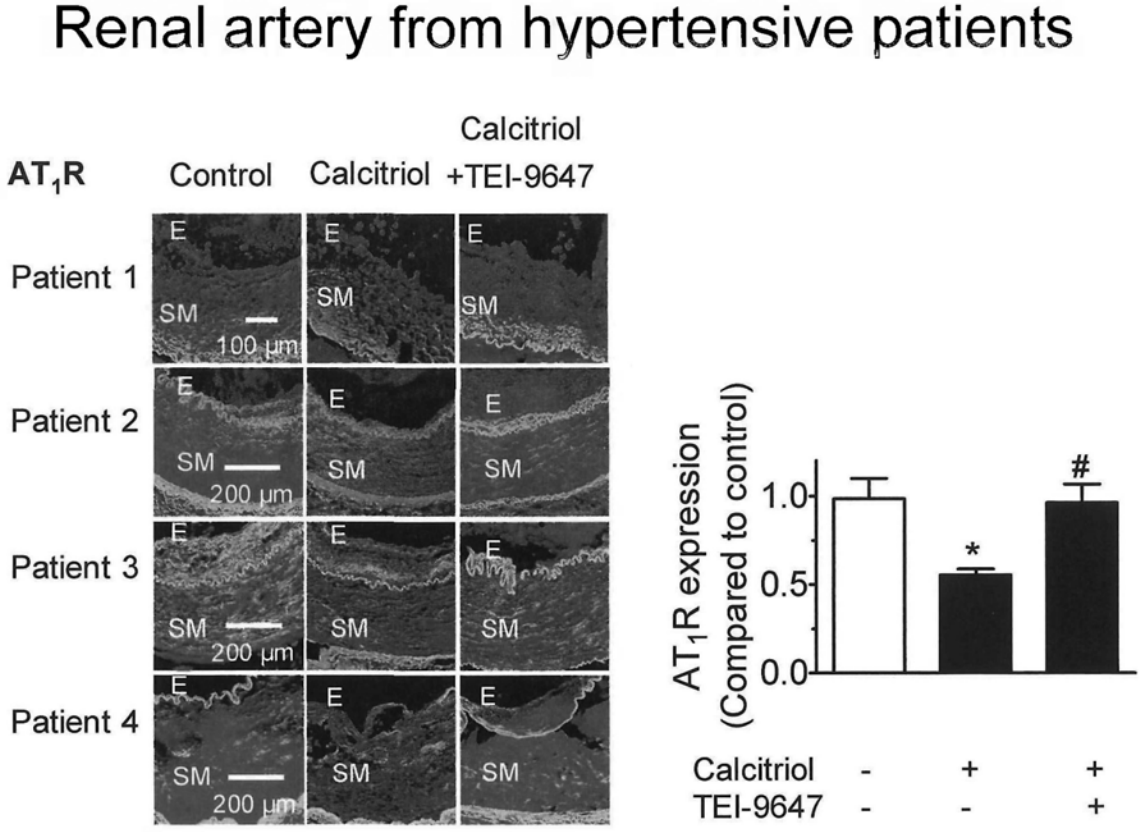

Figure 3.18

Renal arteries from four hypertensive patients exhibit altered expression levels of the oxidative stress-related proteins which are all normalized by calcitriol incubation as detected by immunofluorescence microscopy. Yellowish-green autofluorescence indicated the elastin of the internal and external elastic laminae, of which the former delineated the vessel wall into the luminal endothelium and the medial smooth muscle layer while the latter separated the smooth muscle layer from the adventitia. Signals from Alexa Fluor 546-conjugated secondary antibodies attached to primary antibodies. High levels of arterial AT_1R signified by the intense reddish orange color were reduced by calcitriol. The calcitriol-induced modulation was prevented by TEI-9647. $^{*}P<0.05$ versus Control; $^{\#}P<0.05$ versus Calcitriol.

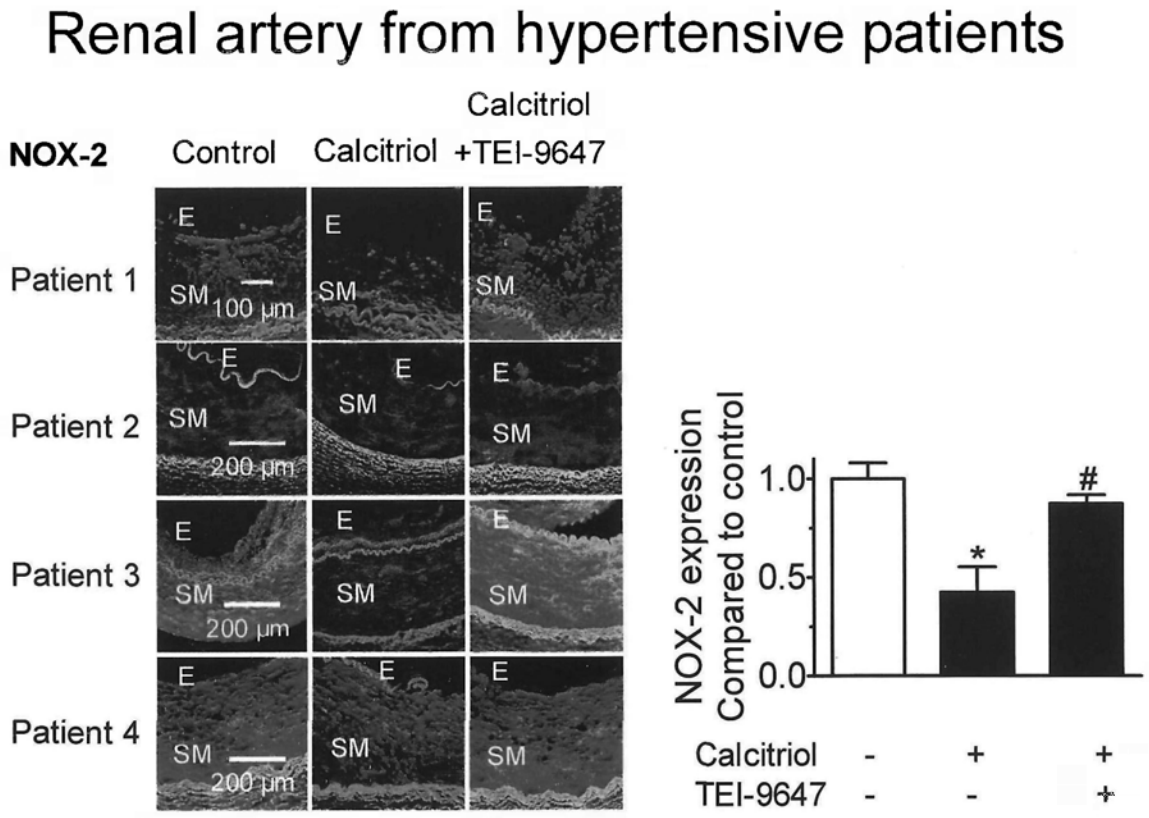

Figure 3.19

Renal arteries from four hypertensive patients exhibit altered expression levels of the oxidative stress-related proteins which are all normalized by calcitriol incubation as detected by immunofluorescence microscopy. Yellowish-green autofluorescence indicated the elastin of the internal and external elastic laminae, of which the former delineated the vessel wall into the luminal endothelium and the medial smooth muscle layer while the latter separated the smooth muscle layer from the adventitia. Signals from Alexa Fluor 546-conjugated secondary antibodies attached to primary antibodies. High levels of arterial NOX-2 signified by the intense reddish orange color were reduced by calcitriol. The calcitriol-induced modulation was prevented by TEI-9647. $^{*}P<0.05$ versus Control; $^{\#}P<0.05$ versus Calcitriol.

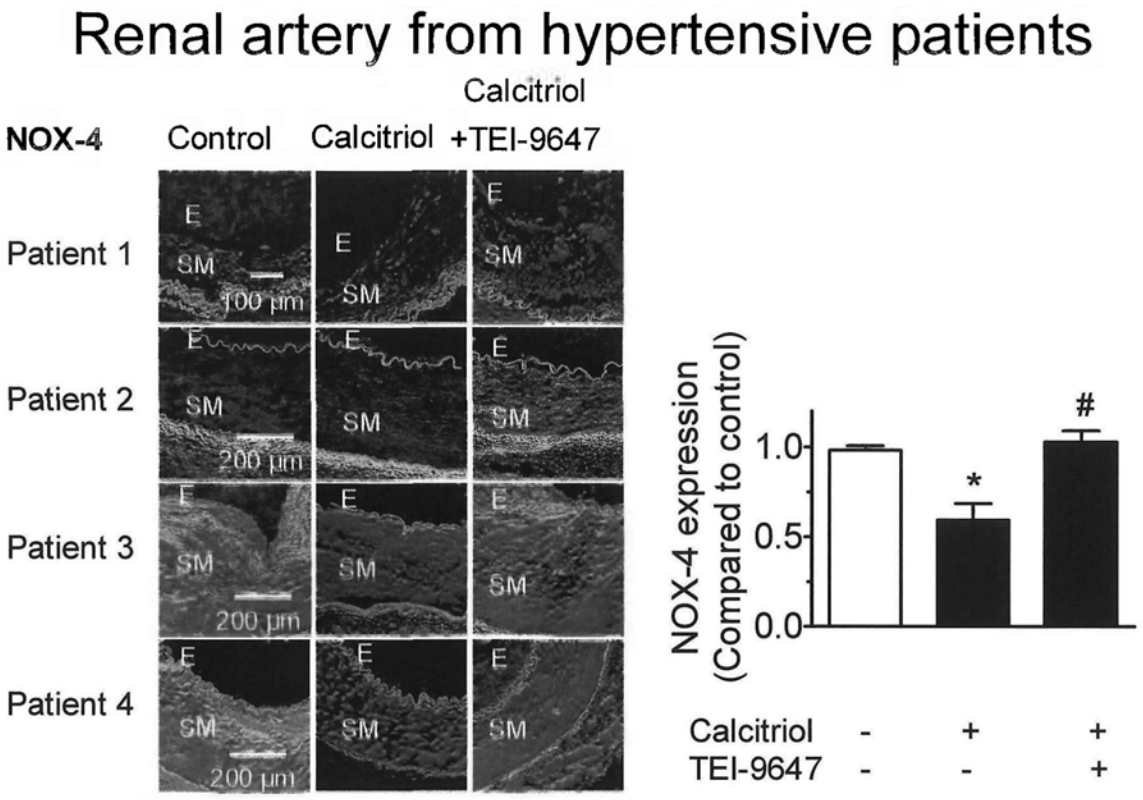

Figure 3.20

Renal arteries from four hypertensive patients exhibit altered expression levels of the oxidative stress-related proteins which are all normalized by calcitriol incubation as detected by immunofluorescence microscopy. Yellowish-green autofluorescence indicated the elastin of the internal and external elastic laminae, of which the former delineated the vessel wall into the luminal endothelium and the medial smooth muscle layer while the latter separated the smooth muscle layer from the adventitia. Signals from Alexa Fluor 546-conjugated secondary antibodies attached to primary antibodies. High levels of arterial NOX-4 signified by the intense reddish orange color were reduced by calcitriol. The calcitriol-induced modulation was prevented by TEI-9647. $^{*}P<0.05$ versus Control; $^{\#}P<0.05$ versus Calcitriol.

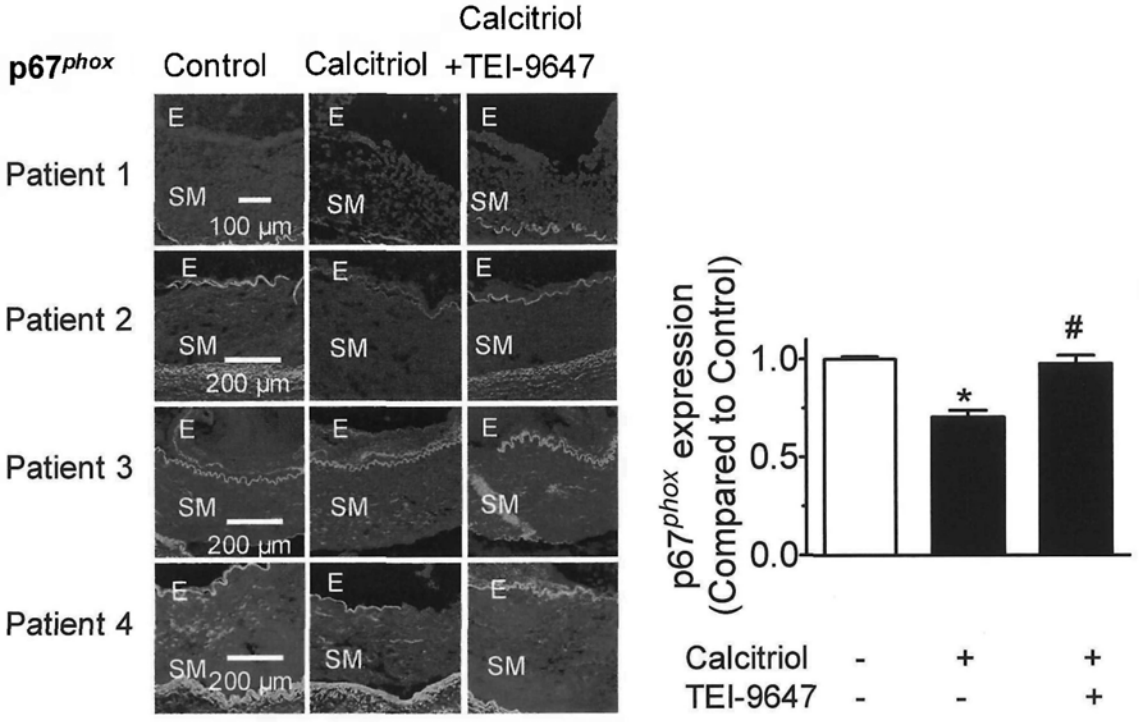

Figure 3.21

Renal arteries from four hypertensive patients exhibit altered expression levels of the oxidative stress-related proteins which are all normalized by calcitriol incubation as detected by immunofluorescence microscopy. Yellowish-green autofluorescence indicated the elastin of the internal and external elastic laminae, of which the former delineated the vessel wall into the luminal endothelium and the medial smooth muscle layer while the latter separated the smooth muscle layer from the adventitia. Signals from Alexa Fluor 546-conjugated secondary antibodies attached to primary antibodies. High levels of arterial $p67^{phox}$ signified by the intense reddish orange color were reduced by calcitriol. The calcitriol-induced modulation was prevented by TEI-9647. $^{*}P<0.05$ versus Control; $^{\#}P<0.05$ versus Calcitriol.

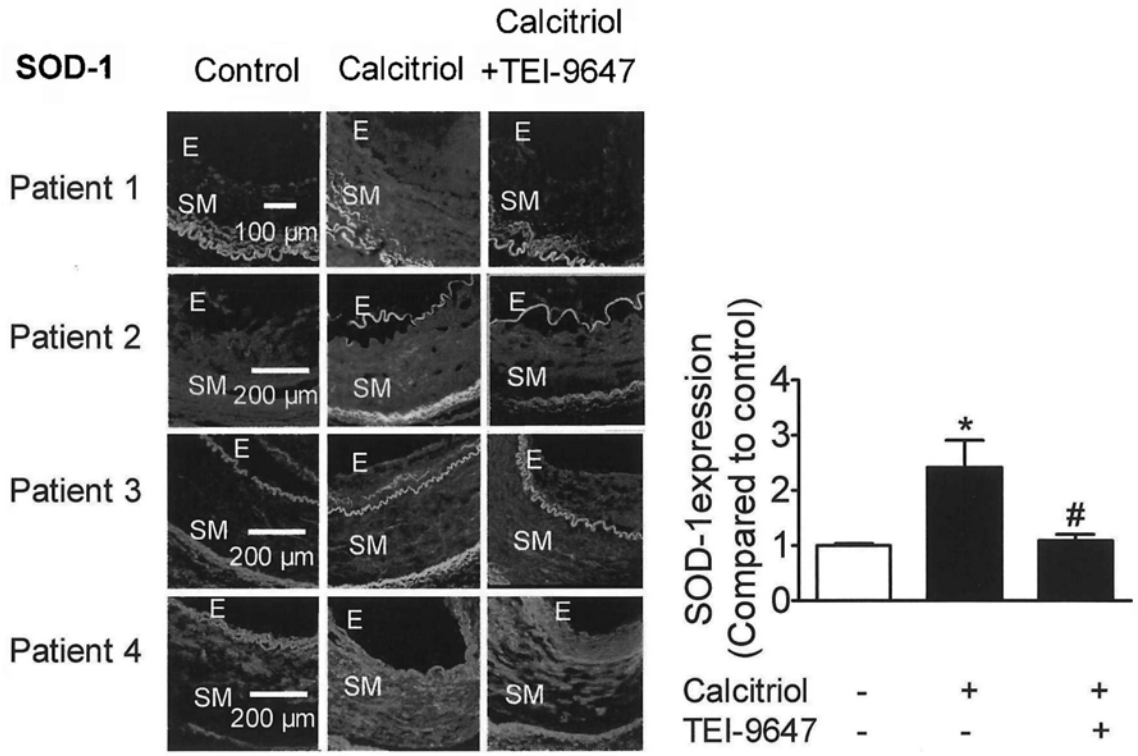

Figure 3.22

Renal arteries from four hypertensive patients exhibit altered expression levels of the oxidative stress-related proteins which are all normalized by calcitriol incubation as detected by immunofluorescence microscopy. Yellowish-green autofluorescence indicated the elastin of the internal and external elastic laminae, of which the former delineated the vessel wall into the luminal endothelium and the medial smooth muscle layer while the latter separated the smooth muscle layer from the adventitia. Signals from Alexa Fluor 546-conjugated secondary antibodies attached to primary antibodies. Low level of arterial SOD-1 signified by the intense reddish orange color were increased by calcitriol. The calcitriol-induced modulation was prevented by TEI-9647. $^{*}P<0.05$ versus Control; $^{\#}P<0.05$ versus Calcitriol.

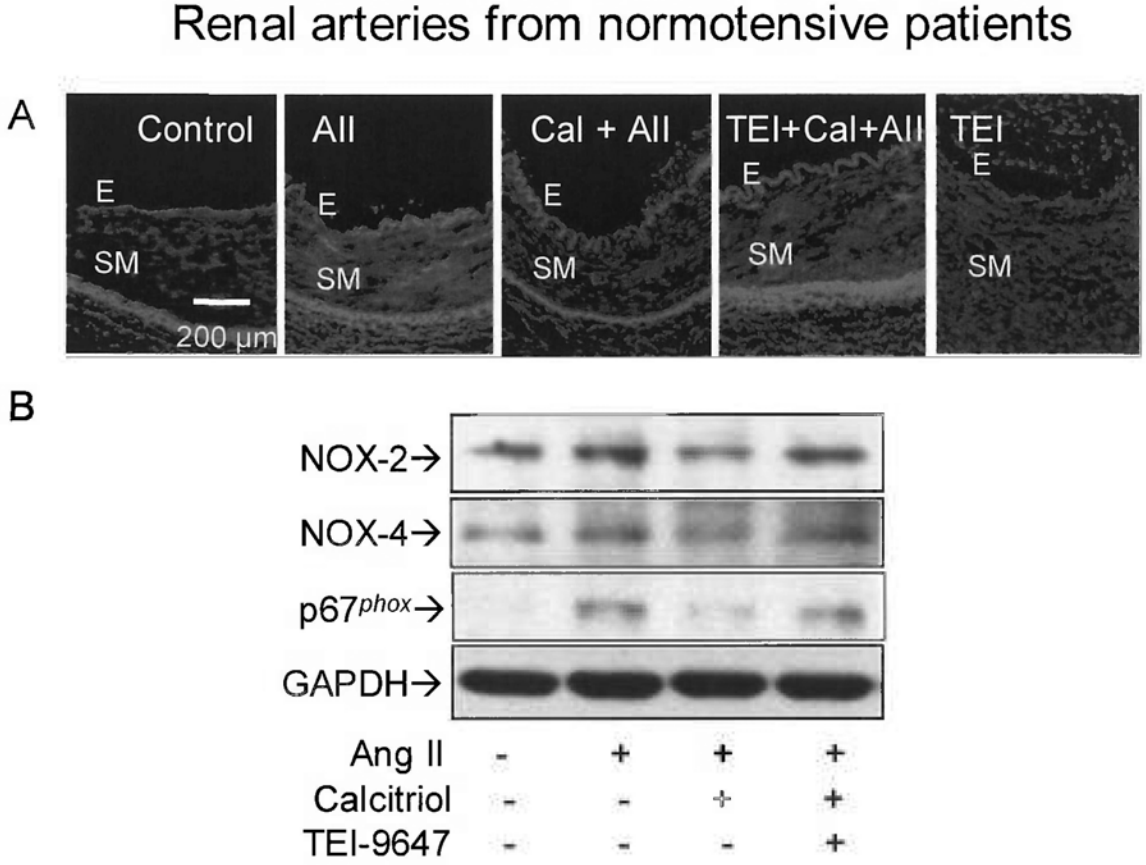

Figure 3.23

DHE fluorescence and Western blotting, respectively, showed that Ang II (1 µmol/L, overnight) increased (A) the arterial ROS level and (B) the expression of NOX-2, NOX-4 and $p67^{phox}$, all of which were reduced by calcitriol treatment. (A and B) TEI-9647 abolished the effects of calcitriol. Photomicrographs and blots are representative images from experiments performed on samples from three different patients.

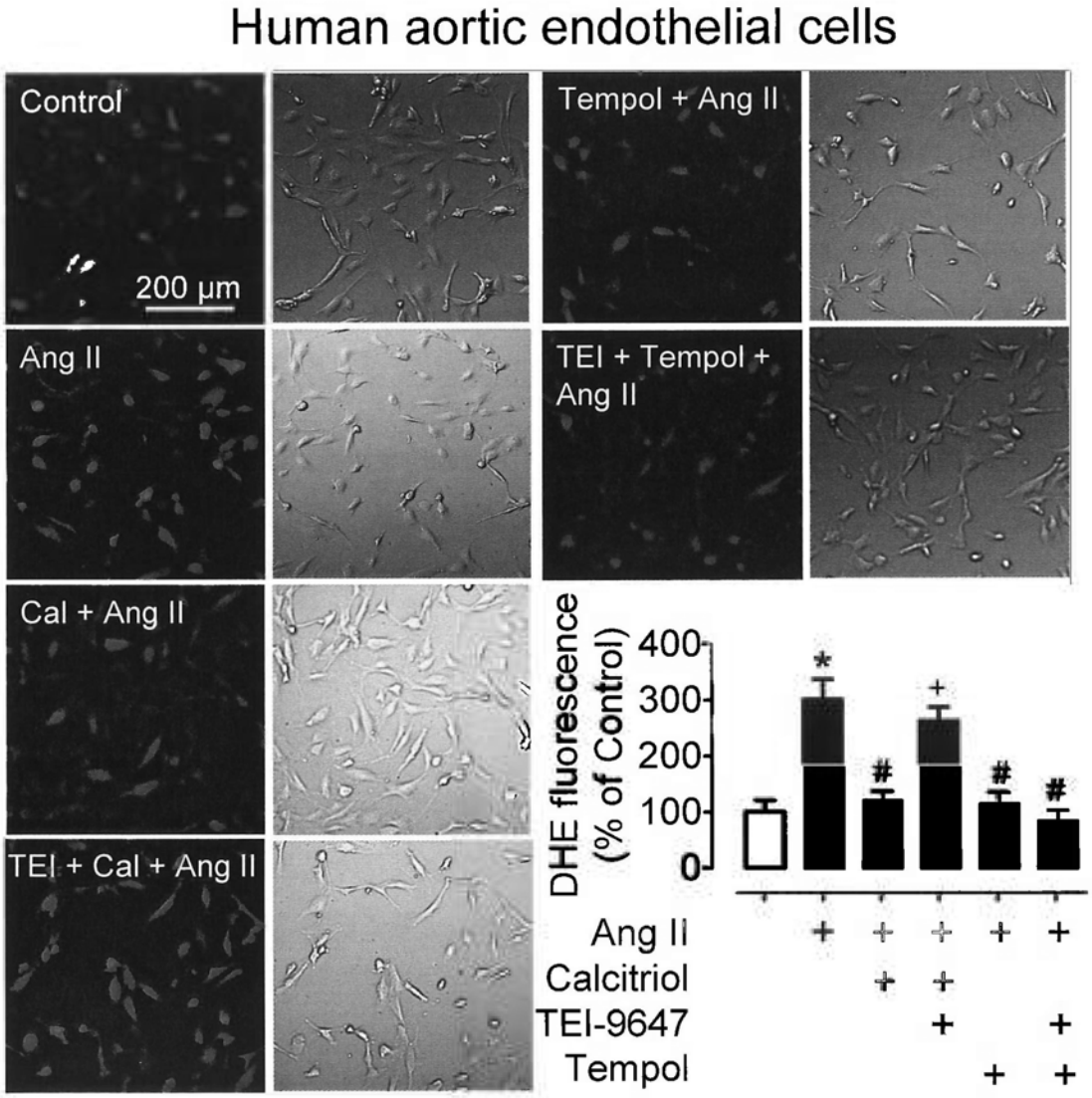

Figure 3.24

DHE fluorescence showed that the ROS level was reduced by calcitriol (Cal) and tempol, but only the effect of calcitriol was antagonized by TEI-9647 (TEI; 1 µmol/L). The bar graph represents means ± SEM of 4 experiments. *$P<0.05$ compared to control; $^{\#}P<0.05$ versus Ang II; $^{\dagger}P<0.05$ versus combined treatment with Ang II plus calcitriol.

3.4 Discussion

The present study provides evidence of the protective effect of calcitriol, an active form of vitamin D, on renovascular function in hypertension. The major novel findings include, (1) calcitriol, by either chronic oral administration or overnight tissue culture, diminishes the exaggerated endothelium-dependent contractions in SHR renal arteries via a down-regulation of AT_1R and NAD(P)H subunits, NOX-2 and $p67^{phox}$, and a up-regulation of SOD-1 and SOD-2; (2) calcitriol *per se* does not possess radical scavenging activity, instead, it reduces oxidative stress via the transcriptional regulation of the radical generating and scavenging enzymes; (3) Ang II, representing a major circulating cytokine in hypertensive patients, impairs endothelium-dependent relaxations and unveils endothelium-dependent contractions in renal arteries of normotensive rats, and the impairment was prevented by co-treatment with calcitriol; and (4) more importantly, we have demonstrated for the first time that impaired endothelium-dependent relaxations in renal arteries from hypertensive patients can be partially restored by calcitriol, of which the effect is antagonized by a specific human VDR antagonist, TEI-9647, pinpointing the positive role of VDR in the calcitriol-induced protective effects.

ROS are involved in the impairment of vascular function and development of hypertension (Touyz, 2004; Wind et al., 2010; Xu and Touyz, 2006), both of which are improved by treatment with anti-oxidants or ROS scavengers (Park et al., 2002; Rodriguez-Iturbe et al., 2003; Simonsen et al., 2009). Increased ROS production is implicated in hypertensive patients (Delles et al., 2008) and in animal models such as SHR, Ang II- or norepinephrine-infused hypertensive rats, or renin over-expression in transgenic mice (Munzel et al., 2008). ROS decrease the bioavailability of NO and its excessive production impairs endothelium-dependent relaxations and unmask

endothelium-dependent contractions (Urso and Caimi, 2011; Vanhoutte, 2011; Vanhoutte et al., 2005; Versari et al., 2009; Wong et al., 2010b). ROS are generated from various enzymes, of which NAD(P)H oxidase represents the major source. ROS scavengers and inhibition of the hyper-active NAD(P)H oxidase can prevent the development of endothelial dysfunction in the SHR (Knight et al., 2010; Wong et al., 2010b). In addition to ROS, the increased RAS activity is reported in vascular tissue of hypertensive patients and thus AT_1R blockers represent a major class of anti-hypertensive drugs. *In vitro* and *in vivo* studies show that vitamin D is a potent endogenous suppressor of *RAS* by inhibiting renin transcription (Qiao et al., 2005). Chronic treatment with calcitriol or other active forms of vitamin D prevents the development of cardiac hypertrophy in SHR by suppressing the cardiac RAS (Kong et al., 2010). However, how vitamin D improves endothelial dysfunction, particularly in the renal vasculature which exhibits elevated ROS and RAS in hypertension, remain elusive.

The present findings support the regulatory role of vitamin D on the RAS and ROS production in hypertension. First, we demonstrate functionally that chronic oral administration or overnight treatment with calcitriol reverse the exaggerated endothelium-dependent contractions and the impaired endothelium-dependent relaxations in renal arteries from SHR; both alterations are sensitive to the AT_1R antagonist, inhibitor of NAD(P)H oxidase and ROS scavengers, indicative of a pathogenic involvement of AT_1R and NAD(P)H oxidase. Second, Western blot analysis shows an up-regulation of AT_1R and NAD(P)H oxidase subunits, NOX-2 and $p67^{phox}$, and a down-regulation of SOD-1 and SOD-2 in SHR arteries compared with those of WKY, which are all normalized by calcitriol. The reduction in the AT_1R level is reflected by a diminished contraction in response to Ang II. Third, ROS detection on

SHR renal arteries and aortic endothelial cells by DHE fluorescence and EPR confirms ROS over-production, which is reduced by calcitriol. Mimicking hypertension in which the circulating level of Ang II is elevated, renal arteries from normotentive rats exposed to Ang II overnight impairs endothelium-dependent relaxations, unveils endothelium-dependent contractions, and augments the expression of NOX-2 and NOX-4. The Ang II-induced endothelial dysfunction is again normalized by co-treatment with calcitriol. Similar findings were obtained in renal arteries from hypertensive patients and in human aortic endothelial cells, supporting a clinical relevance and therapeutic role of vitamin D by correcting the elevated RAS activity and ROS over-production in hypertension.

The present study, in demonstrating the inhibitory effect of a specific antagonist against human VDR TEI-9647,(Diaz et al., 2009) establishes a crucial role of VDR in the calcitriol-induced restoration of renovascular function in hypertension. Indeed, the impaired endothelium-dependent relaxations of renal arteries from hypertensive patients were partially rescued by overnight incubation with calcitriol, and this was antagonized by the VDR antagonist, strongly suggesting that calcitriol exerts its beneficial effects via VDR activation. Consistently, overnight incubation with Ang II increases the ROS level in human aortic endothelial cells, and this is prevented by both calcitriol and the anti-oxidant tempol. However, only the effect of calcitriol, but not that of tempol, is antagonized by TEI-9647, demonstrating that the protective effect of calcitriol can indeed be attributed to VDR activation.

The vaso-protective effect of calcitriol observed in the present study cannot be explained by direct scavenging of ROS as demonstrated by the lack of direct effect of calcitriol on generation of free radicals by the cell-free hypoxanthine-xanthine oxidase reaction. It is not immediate, in contrast to the inhibition of endothelium-dependent

contractions reported in the SHR aortae, which is non-genomic in nature and has been attributed to inhibition of endothelial calcium influx Wong et al (Wong et al., 2008). in a non-genomic manner. Instead, calcitriol exerts its effect through genomic regulation. This conclusion is prompted by the present measurements of the protein expression of radical generating and scavenging enzymes, with calcitriol reducing the expression of the former [NAD(P)H oxidase and its subunits] but augmenting that of the latter (SOD-1 and SOD-2). The genomic impact of calcitriol is confirmed by the experiments with the mRNA synthesis inhibitor, actinomycin-D, which abolishes this protective effect against endothelium-dependent contractions and increases in ROS levels. A genomic action is consistent with the conclusion that the effects of calcitriol reported in the present study are due to VDR activation. Indeed, upon binding of and activation by vitamin D, VDR forms a heterodimer complex with the retinoid X receptors (RXR). The VDR-RXR complex can bind to specific DNA sequences, termed vitamin D responsive elements, located in the promoter regions of various vitamin-D-dependent genes (Carlberg and Seuter, 2009; Dusso et al., 2005; Sanchez-Martinez et al., 2008).

The present findings substantiate the capability of chronic treatment with vitamin D to reduce ROS level in rat arteries (Wong et al., (2010a) but provide novel insights into the possible mechanisms underlying by which it regulates radical homeostasis. Calcitriol is a potent suppressor of the RAS (Qiao et al., 2005). Since the AT_1R expression is decreased following calcitriol treatment, the modification of the NAD(P)H and SOD expressions and the reduction of ROS level observed upon chronic treatment with the vitamin may be due in part to a lesser presence of AT_1R, which is a key mediator of the pathogenesis of hypertension and the activation of which serves as a primary trigger for ROS production by NAD(P)H oxidase (Lassegue and Clempus, 2003). Cyclooxygenases (COX) are the major source of endothelium-derived

contracting factors and ROS facilitate their production/action(Feletou et al., 2011; Vanhoutte, 2011; Vanhoutte et al., 2005). Chronic administration of calcitriol reduces the expression of COX-1 in the rat aortae (Wong et al., 2010a). Paricalcitriol, another active form of vitamin D, reduces excessive ROS production by down-regulating pro-inflammatory factors such as inducible nitric oxide synthase, tumor necrosis factor-α and COX-2 (Husain et al., 2010). Endothelium-dependent contractions of the SHR renal arteries are prevented by inhibitors of cyclooxygenase (Michel et al., 2008).Thus, it is likely that the reduction in ROS production demonstrated in the present study leads to decreased expression/presence of endothelial cyclooxygenase(s) which then explains the attenuation of endothelium-dependent contractions caused by the exposure to calcitriol.

The present study confirms that chronic oral administration of calcitriol reduces the elevated blood pressure of the SHR (Wong et al., 2010a) Whether or not there is a relationship between low plasma levels of vitamin D and elevated arterial blood pressure remains controversial (Pilz et al., 2009). Nevertheless, the present findings, together with those from limited clinical and animal-based studies (Freundlich et al., 2008; Krause et al., 1998; Wong et al., 2010a) support the view that vitamin D supplement can lower arterial blood pressure, which may have indirect effects not due necessarily to a direct genomic action on the blood vessel wall. However the protective effect of calcitriol on renal arteries is not likely to be secondary to the blood pressure reduction. Indeed, in the present study, the inhibition by calcitriol on the exaggerated endothelium-dependent contractions of isolated SHR renal arteries was similar after an overnight *in vitro* incubation after chronic *in vivo* treatment, strongly suggesting that calcitriol directly improves endothelial function.

In summary, chronic treatment with the active form of vitamin D, calcitriol, protects renovascular function in hypertension. The calcitriol-induced protection is

likely mediated through VDR by down-regulation of the expressions of AT_1R, NAD(P)H subunits and up-regulation of SOD-1 and -2, resulting in a reduction in ROS over-production. Both *in vitro* and *in vivo* animal-based results are supported by the findings from human renal arteries and human endothelial cells, the present study suggests calcitriol/VDR activation as a novel therapeutic strategy to ameliorate hypertension-associated vascular dysfunction.

Chapter IV

Calcitriol Restores Renovascular Function in Estrogen-deficient Rats through the Down-regulation of Cyclooxygenase-2 and TP Receptor

4.1 Introduction

Estrogen is reported to be beneficial to cardiovascular function (Gragasin et al., 2003; Mendelsohn, 2000; Strehlow et al., 2003; Wagner et al., 2001) and is essential for women to maintain bone mineral density (Adriana S. Dussoa, 2009; Riggs et al., 1995) Postmenopausal women exhibit a significantly increase in cardiovascular morbidity (Rosano et al., 2009; Strehlow et al., 2003) and osteoporosis (Kaunitz et al., 2009; Ribot and Tremollieres, 2006) due to the reduced level of estrogen. Calcitriol (1,25-dihydroxyvitamin D3), being an active form of vitamin D that modulate calcium absorption, is a recommended medication for postmenopausal women to treat or prevent osteoporosis. In fact, besides its classical function in bone mineralization, vitamin D also exerts its effect in the cardiovascular, immune and endocrine system owing to the universal expression of the vitamin D receptor (VDR) (Holick, 2003; Norman, 2006), which can regulate gene expression after activation (Pilz et al., 2009).

Recent studies pointed out an anti-inflammatory role of vitamin D in the arachidonic acid cascade via the suppression of cyclooxygenase (COX)-2 in prostate cancer cells (Moreno et al., 2005; Moreno et al., 2006). Cyclooxygenase converts the substrate arachidonic acid to prostaglandin (PG) G_2 and H_2, which are further metabolized to various prostanoids by tissue-specific prostaglandin synthases. The

vasoactive prostanoids exert their effects by binding to their respective receptor subtypes such that $PGF_{2\alpha}$ acts on FP receptor, PGE_2 on EP receptor, PGD_2 on DP receptor, PGI_2 on IP receptor and thromboxane A_2 (TxA_2) on the TP receptor. Although prostanoids exhibit greater affinity to their own receptors, most prostanoids are able to bind to and activate TP receptor owing to the structural similarity between the prostanoids. Prostanoids such as thromboxane A_2 are involved in the proinflammatory and oxidative events in cardiovascular diseases in both humans and experimental models (Remuzzi et al., 1992; Sebekova et al., 2007). TP receptor agonists, including TxA2 and other vasoconstrictive prostanoids, play a key role in vascular dysfunction such as proliferation of smooth muscle cells and platelet aggregation (Belhassen et al., 2003; Versari et al., 2009). TP receptor is thus regarded as a therapeutic target for prostanoid-mediated inflammation of emerging importance.

Kidneys and the renal vasculature are very sensitive to risk factors; however, the mechanism of renovascular dysfunction in estrogen deficiency remained largely unknown. Clinical data (Agarwal et al., 2005; Alborzi et al., 2008; Francois et al., 2011) and animals-based evidences from spontaneously hypertensive rats (SHR), diabetic rodent models and nephrectomized rats (de Zeeuw et al., 2010; Doorenbos et al., 2009; Koleganova et al., 2009) suggest a protective effect of vitamin D on the kidney function in hypertension and diabetes. The present study characterized the changes in renovascular reactivity in the well-established rodent model of estrogen deficiency, the ovariectomized rats, and opted to investigate whether and how vitamin D exerts a therapeutic effect on the vascular function.

4.2 Materials and Methods

The use of human renal arteries was approved by the Joint Chinese University of Hong

Kong–New Territories East Cluster Clinical Research Ethics Committee. The study was approved by the CUHK Animal Experimentation Ethics Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

4.2.1 Animals and reagents

Adult female Sprague-Dawley rats weighing 200-220 g were supplied by the Laboratory Animal Services Centre, the Chinese University of Hong Kong (CUHK) and were housed in a temperature-controlled room (22–24 °C) with a 12-h light/dark cycle. The animals have free access to a standard diet and water. Ovariectomy was performed with anesthesia via an intraperitoneal injection of ketamine plus xylazine (0.2 ml per 100g body weight). Rats were randomly assigned to one of the following three groups six months after surgery: (1) Sham control, (2) ovariectomized (OVX) with treatment of vehicle DMSO (OVX + vehicle) and (3) OVX with calcitriol treatment at 150 ng/kg per day (OVX + calcitriol). Calcitriol treatment was given by oral gavage for 4.5 months. Ovariectomy was validated by the marked reduction in the weight of uterus.

4.2.2 Blood pressure measurement

Blood pressure was measured by the tail-cuff electrosphygmomanometer system (ADInstruments, Sydney, Australia) after the rats were stabilized and remained quiescent. An average of 3 consecutive readings was taken as the systolic blood pressure of each rat.

4.2.3 Measurement of serum calcitriol level

Serum was collected from OVX rats treated with vehicle and calcitriol upon their

sacrifice. Serum level of calcitriol (1,25-dihydroxyvitamin) was measured using a commercially available immunoassay kit (Immunodiagnostic Systems. Fountain Hill, USA) according the manufacturer's instruction.

4.2.4 Preparation of rat arteries

Rats were sacrificed after 4.5-month oral treatment of calcitriol. Intralobal renal arteries were dissected and placed in Krebs solution containing (mol/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 arteries were carefully removed of the surrounding connective tissue and cut into 8–10 ring segments with length ~1.6 mm for functional studies in a Multi Myograph System (610M, Danish Myo Technology A/S, Aarhus N, Denmark) in which changes in isometric tension were recorded. Briefly, two stainless steel wires were passed through the arterial lumen and fixed to jaws of the myograph, which was filled with 5 mL Krebs solution continually oxygenated with 95% O_2 and 5% CO_2 and maintained at 37 °C to give a pH of ~7.4. The rings were stretched to an optimal resting tension of 2.5 mN, and allowed to equilibrate for 60 min before the start of experiments.

4.2.5 Isometric tension measurement

The arteries were allowed to equilibrate for 60 min after suspended in the myograph chamber. After a stable contraction to 1 μmol/L phenylephrine was obtained, cumulative relaxations to acetylcholine (0.003-10 μmol/L) were recorded. Inhibitors or antagonists, when used, were pre-incubated for 30 min before addition of acetylcholine. Endothelium-dependent relaxations were expressed as percent of maximal relaxation induced by different dose acetylcholine by dividing the peak contraction (mN) of phenylephrine tension. Contractions were expressed as a percentage to the tension

induced by 60 mmol/L KCl.

4.2.6 Human renal artery preparation

Human arteries were collected from female patients > 55 years old after informed consent. The rings incubated overnight in DMEM (GIBCO, Grand Island, NY) culture media supplemented with 10% fetal bovine serum (GIBCO) and 1% penicillin/streptomycin in the presence or absence of calcitriol (100 nmol/L). Vitamin D receptor antagonist, TEI-9647 (1 μmol/L) was added 30-min before the addition of calcitriol. The rings were snap frozen in liquid nitrogen and processed for Western blotting.

4.2.7 Primary cell culture of rat aortic endothelial cells

The detailed information is provided in the methods section in Chapter II.

4.2.8 Western blotting

The detailed information is provided in the methods section in Chapter II.

4.2.9 Confocal microscopy on the real-time nitric oxide (NO) production in the endothelial cells

The detailed information is provided in the methods section in Chapter II.

4.2.10 Chemicals

ACh, L-NAME, phenylephrine, indomethacin, SC-560, celecoxib, DuP-697 and NS-398 were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). Calcitriol were from Tocris Bioscience (Avonmouth, UK). TEI-9647 was a kind gift from Teijin

Pharma Limited (Tokyo, Japan). Except ACh, L-NAME, phenylephrine, were dissolved in distilled water, and TEI-9647 in ethanol, other drugs were prepared in DMSO.

4.2.11 Statistical Analysis

Data were expressed as the means ± SEM of 5-7 experiments. Statistical significance was determined by two-tailed Student's *t*-test or one-way ANOVA followed by Bonferroni post-tests whenever appropriate (GraphPad Software, San Diego, California). $P<0.05$ is considered significantly different.

4.3 Results

4.3.1 Basic physical and biochemical parameters

Successful ovariectomy was validated by a significant reduction in the weight if uterus (Figure 4.1A). Oral gavage of calcitirol to OVX rats resulted in an increased in serum calcitriol level (Figure 4.1B). Blood pressure remained similar among the sham control, OVX + vehicle and OVX + calcitriol (Figure 4.1C), while body weight was increased in the OVX rats compared to the sham control, but was not affected by calcitriol treatment (Figure 4.1D).

4.3.2 *In vivo* calcitriol treatment enhances endothelium-dependent relaxations in renal arteries from OVX rats

Impaired ACh-induced relaxations were observed in renal arteries of vehicle-treated OVX rats compared to the sham control (pD_2: 6.53 ± 0.10, E_{max}: 39.6 ± 4.9 % in OVX + vehicle, n=5 vs pD_2: 6.80 ± 0.17, E_{max}: 64.6 ± 10.5 % in sham, n=4). Relaxations were enhanced in the renal arteries from OVX + calcitriol (pD_2: 6.30 ± 0.09, E_{max}: 68.6 ± 3.9 %) (Figure 4.2A and B). In contrast, the SNP-induced relaxations were not different

among sham control, OVX + vehicle and OVX + calcitriol (Figure 4.2C).

4.3.3 COX-2 inhibitors and TP receptor antagonist acutely improve endothelium-dependent relaxations in renal arteries from OVX rats

Thirty-minute incubation with indomethacin (3 μmol/L), a non-specific COX inhibitor, restored the impaired ACh-induced relaxations in renal arteries from OVX rats (Figure 4.3A). Similar results were obtained with 30-min exposure to celecoxib, DuP-697 and NS-398 (all at 3 μmol/L) while COX-1 inhibitor SC-560 (10 nmol/L) was without effect. TP receptor antagonist, S18886 (0.3 μmol/L) also enhanced relaxations (Figure 4.3D).

4.3.4 Calcitriol normalizes the expressions of COX-2 and TP receptor

The expression of COX-2 and TP receptor were augmented in the renal arteries from OVX rats (Figure 4.4A and B). Chronic treatment with calcitriol attenuated the COX-2 and TP receptor expression. In contrast, COX-1 expression remained unchanged in the renal arteries from the sham control, OVX + vehicle and OVX + calcitriol (Figure 4.4C).

4.3.5 *In vitro* calcitriol exposure improves relaxations and inhibits U46619-induced contractions

Endothelium-dependent relaxations were attenuated in renal arteries from OVX rats and were restored by an overnight treatment with calcitriol (100 nmol/L) (Figure 4.5A and B). In contrast, acute exposure (30 min-incubation) of the arteries to calcitriol did not modify the relaxations (Figure 4.5A and C). Renal arteries exhibit greater S18886 sensitive U46619-induced contractions, which were normalized by *in vitro* calcitriol incubation overnight (Figure 4.6A). Western blot analysis showed that overnight incubation with calcitriol or celecoxib decreased TP receptor expression in renal artery

(Figure 4.6B).

4.3.6 Calcitriol prevents U46619-induced vascular dysfunction in renal arteries from sham control

Overnight incubation of U46619 (10 nmol/L) in renal arteries from sham control impaired ACh-induced endothelium-dependent relaxations (pD_2: 6.95 ± 0.08, E_{max}: 84.3 ± 3.7 % in control, n=5 in sham; pD_2: 5.98 ± 0.15 vs E_{max}: 49.8 ± 6.2 % in U46619-treated rings, n=5; $P<0.05$; Figure 4.7). Pre-treatment with calcitriol prevents attenuation in endothelium-dependent relaxations (pD_2: 6.68 ± 0.08, E_{max}: 86.0 ± 2.6 %, n=4, $P<0.05$ compared with U4-treated rings) (Figure 4.7). U46619 (10 nmol/L) overnight exposure elevated TP receptor expression in renal arteries from sham control (Figure 4.8) and the over-expression was reversed by calcitriol (100 nmol/L), S18886 (0.3 μmol/L) and celecoxib (3 μmol/L).

4.3.7 *In vitro* calcitriol incubation enhances NO production in aortic endothelial cells cultured from OVX rats

NO production in aortic endothelial cells cultured from OVX rats was markedly attenuated compared to those cultured from sham control in response to ACh. Overnight treatment with calcitriol, S18886 or celecoxib partially restored the ACh-stimulated NO production (Figure 4.9). In parallel, Western blot results showed that cultured endothelial cells from OVX rats have an elevated endothelial expression of COX-2 and TP receptor, which were markedly reduced by calcitriol treatment (Figure 4.10A and B). TP receptor expression was also reduced by celecoxib (Figure 4.10B). COX-1 expression remained unchanged in endothelial cells cultured from sham control or OVX rats and was not affected by calcitriol (Figure 4.10C).

Incubation of U46619 (100 nmol/L) markedly decreased the NO production in the endothelial cells cultured from sham control. The ACh-stimulated NO production was augmented with overnight co-treatment with calcitriol (Figure 4.11)

4.3.8 Calcitriol downregulates COX-2 and TP receptor expression in human renal arteries

Western blot results revealed that overnight calcitriol exposure decreased the expression of COX-2 and TP receptor in renal arteries from female patients in the age of menopause (age > 55 years). Pre-treatment with human vitamin D receptor antagonist, TEI-9647 (1 μmol/L) prevented the calcitriol-induced effects (Figure 4.12).

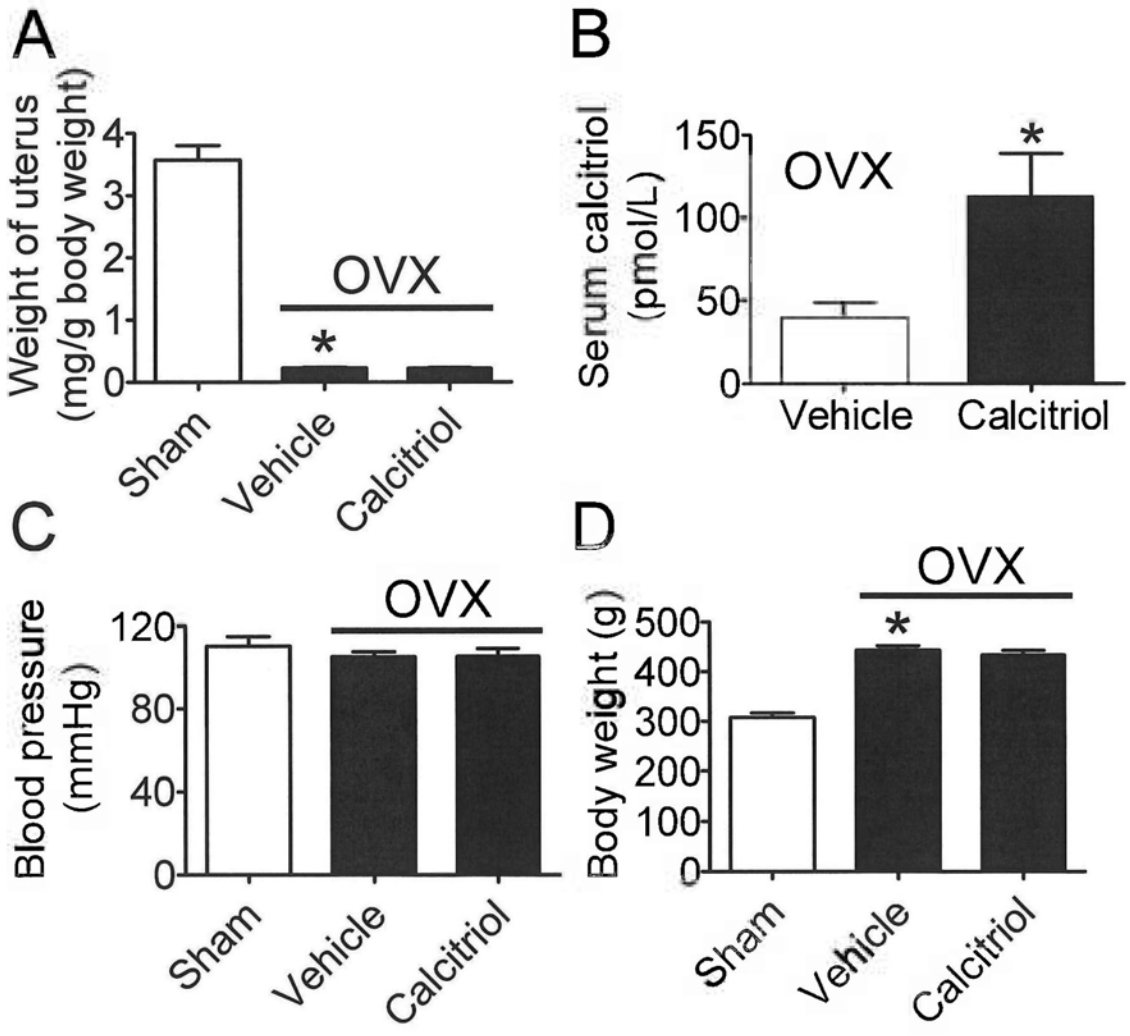

Figure 4.1

(A) Weight of uterus decreased significantly in OVX rats. (B) Serum calcitriol in OVX increased with oral treatment of calcitriol. (C) Blood pressure remained similar among the sham control, OVX rats with or without calcitriol treatment. (D) OVX rats had increased body weight, which was not modified by calcitriol treatment. Data are means ± SEM of 4-6 experiments. *P<0.05 versus sham control.

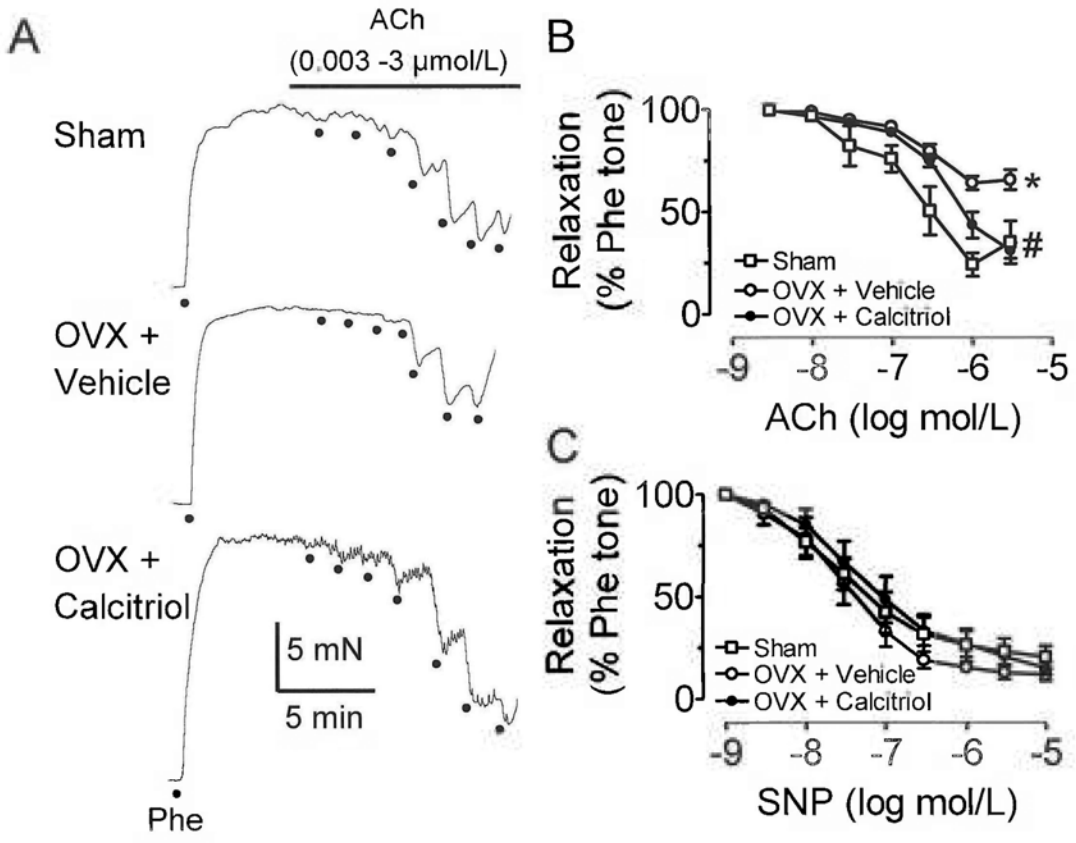

Figure 4.2

(A and B) Renal arteries from OVX rats exhibited impaired ACh-induced endothelium-dependent relaxations, which were restored in OVX rats orally treated with calcitriol. (C) Endothelium-independent sodium nitroprusside (SNP)-induced relaxations were similar in renal arteries from sham control and OVX rats with or without calcitriol treatment. Data are means ± SEM of 5-8 experiments. $^{*}P<0.05$ versus sham control; $^{\#}P<0.05$ versus OVX + vehicle.

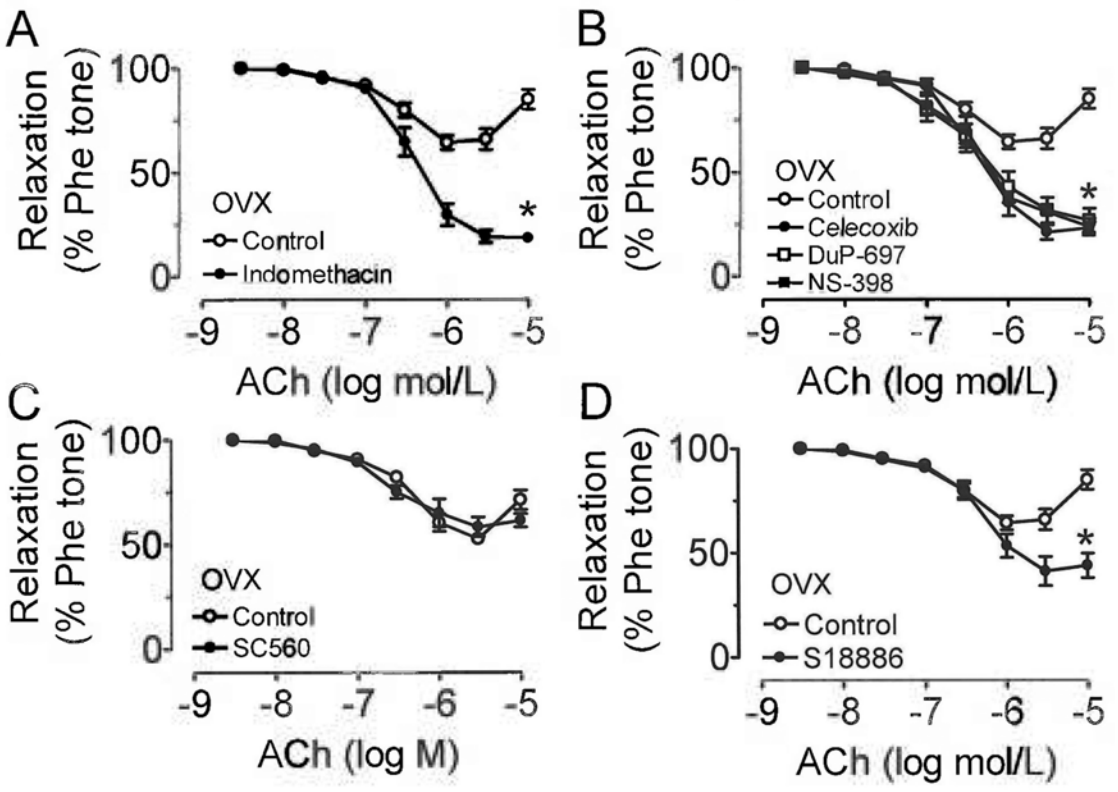

Figure 4.3

ACh-induced relaxations in the renal arteries from OVX rats were restored by non-selective COX inhibitor, indomethacin (A, 3 μmol/L), COX-2 inhibitors, celecoxib, DuP-697 and NS-398 (B, all at 3 μmol/L) and TP receptor antagonist, S18886 (D, 0.3 μmol/L), but remained unaffected by COX-1 inhibitor SC-560 (C, 10 nmol/L). Data are means ± SEM of 3-6 experiments. *P<0.05 versus sham control.

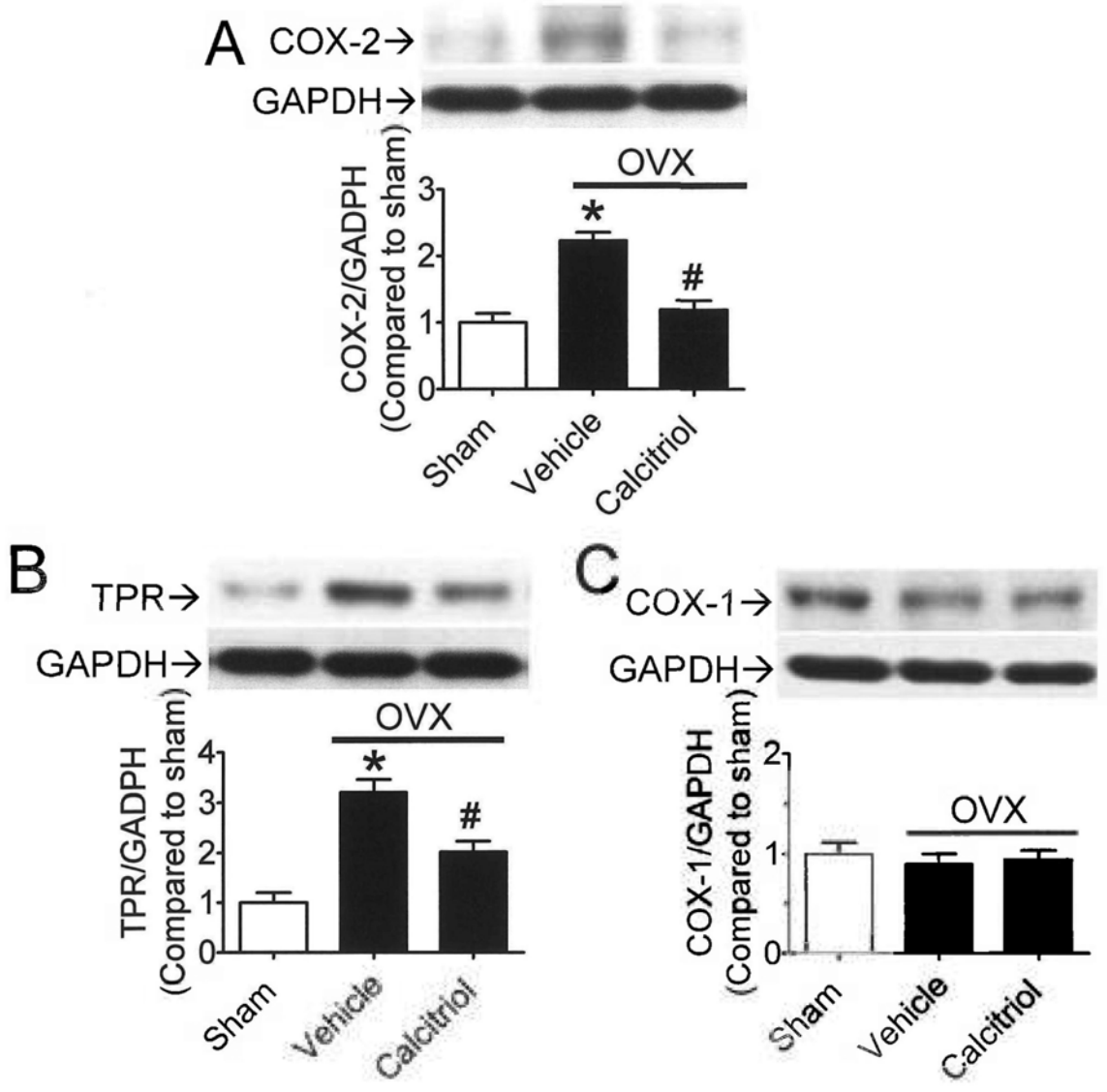

Figure 4.4

Western blotting showed that COX-2 (A) and TP receptor (B, TPR) expression was augmented in renal arteries from OVX rats and the expression was normalized in OVX rats treated with calcitriol. In contrast, COX-1 expression was not changed in renal arteries from OVX rats compared to the sham control. Data are means ± SEM of 3-5 experiments. *P<0.05 versus sham control; $^{\#}P$<0.05 versus OVX + vehicle.

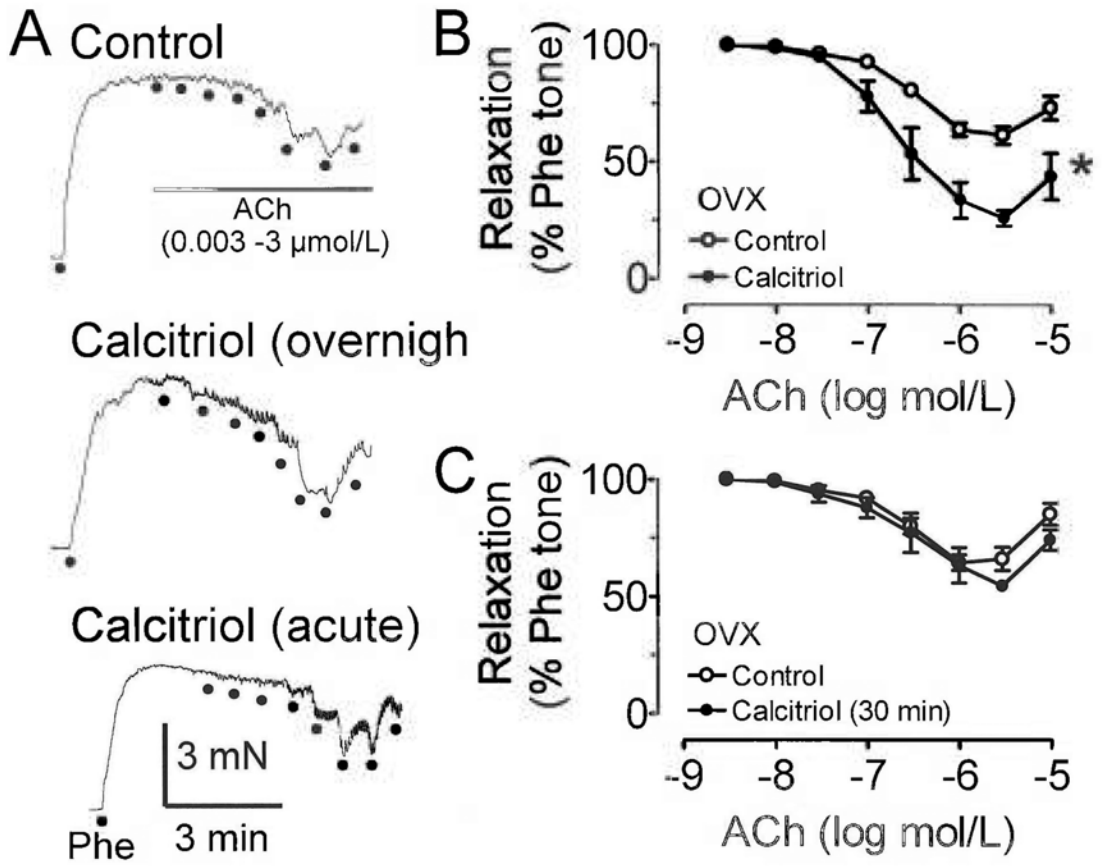

Figure 4.5

Overnight tissue culture of renal arteries from OVX rats with calcitriol (100 nmol/L) improved the ACh-induced endothelium-dependent relaxations (A and B), while acute exposure of the arteries to calcitriol was without effect (A and C). Data are means ± SEM of 4-6 experiments. *P<0.05 versus control.

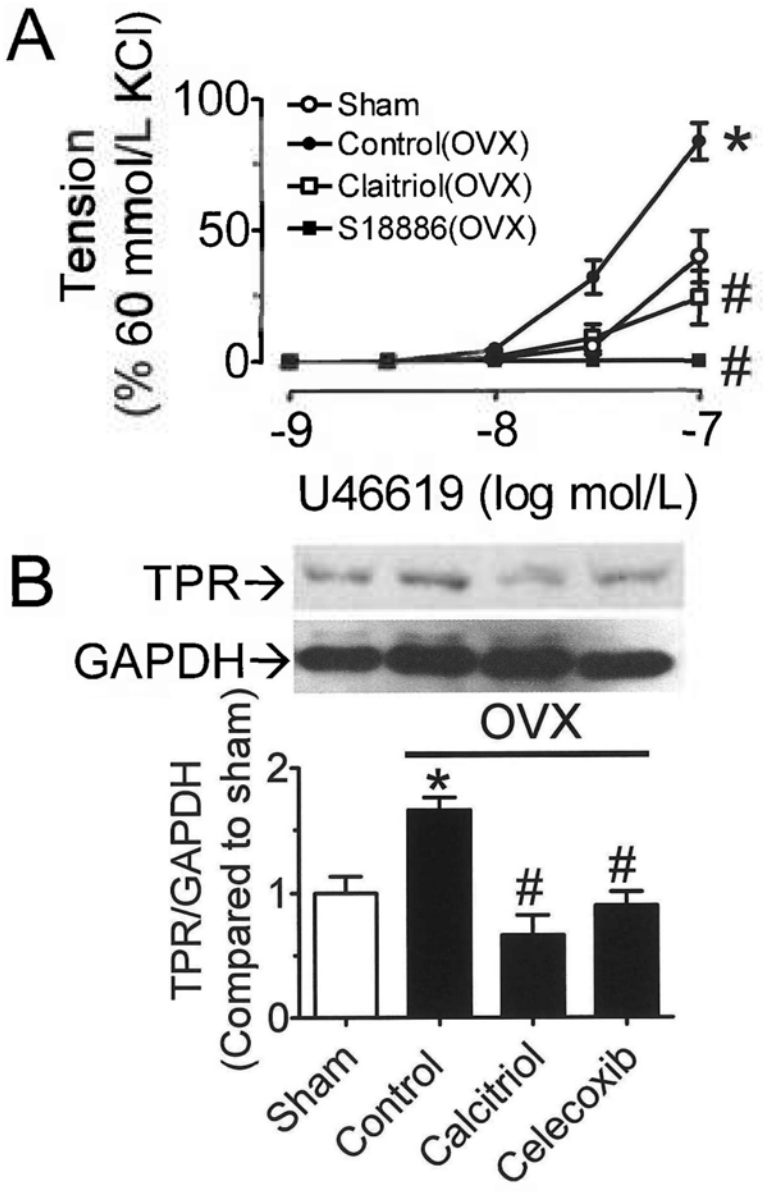

Figure 4.6

Renal arteries from OVX rats exhibited enhanced U46619-induced contractions, which were normalized by the overnight treatment of calcitirol and S18886. Western blot showed that protein expression of TP receptor (TPR) was elevated in renal arteries from OVX rats, and the over-expression was reversed by overnight exposure to calcitriol and celecoxib. Data are means ± SEM of 4-6 experiments. $^{*}P<0.05$ versus sham control; $^{\#}P<0.05$ versus OVX control.

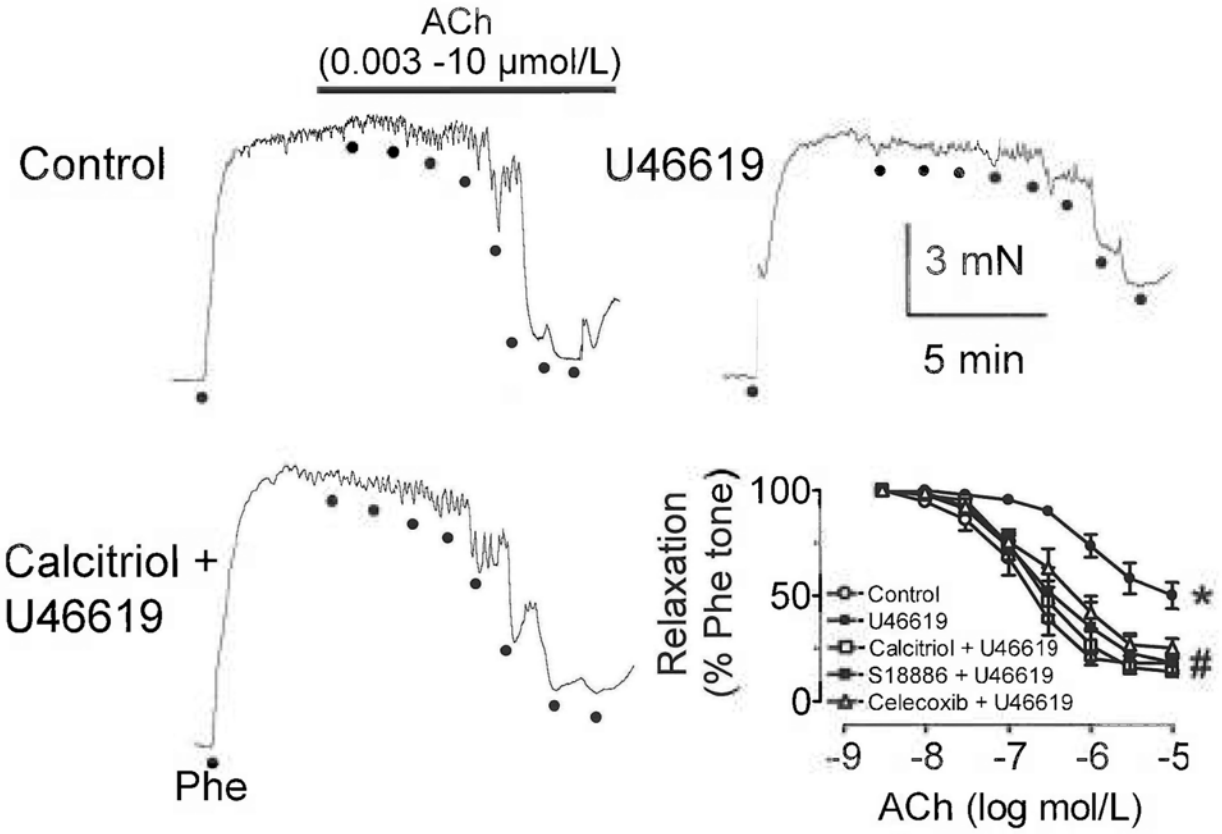

Figure 4.7

Overnight incubation of renal arteries from sham control with U46619 (10 nmol/L) resulted in impaired ACh-induced relaxations, which were prevented by co-incubation with calcitriol, S18886 and celecoxib. Data are means ± SEM of 4-6 experiments. $^{*}P<0.05$ versus control; $^{\#}P<0.05$ versus U46619.

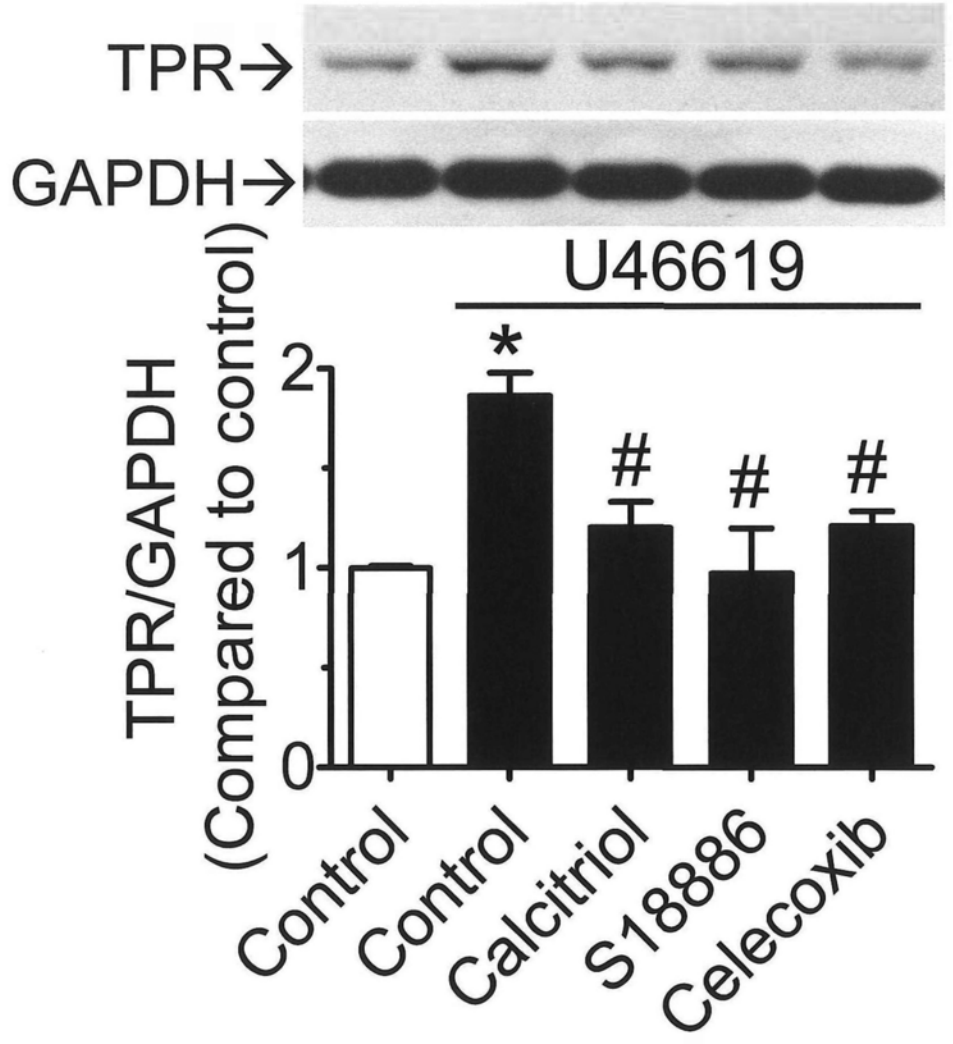

Figure 4.8

Western blot analysis showed an elevated expression of the TP receptor (TPR) in renal arteries from sham control after exposure to 10 nmol/L U46619. The U46619-induced TPR upregulation was prevented by co-exposure of the arteries to calcitriol (100 nmol/L), S18886 (0.3 μmol/L) and celecoxib (3 μmol/L). Data are means ± SEM of 3-4 experiments. *P<0.05 versus control (without U46619); $^{\#}P$<0.05 versus U46619 control.

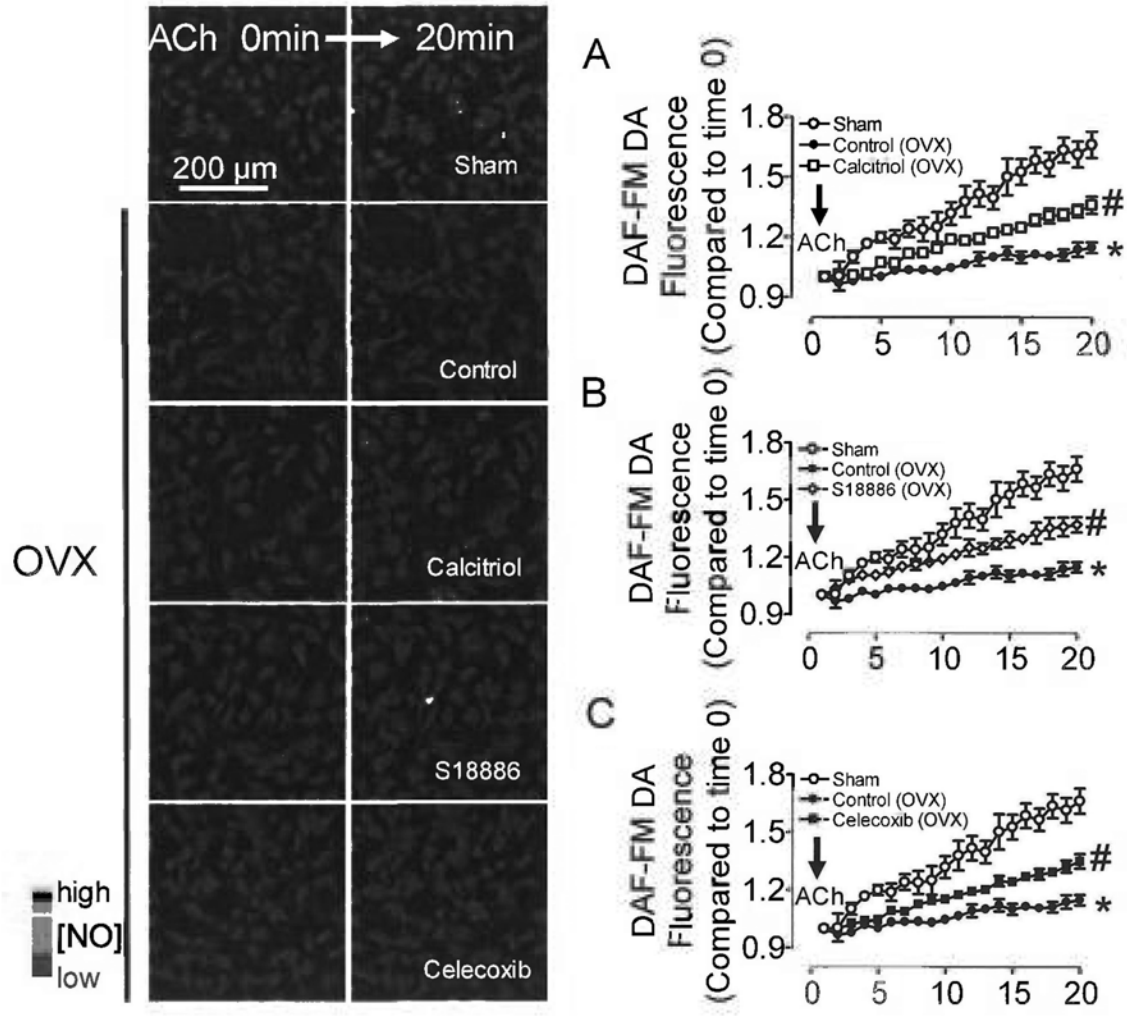

Figure 4.9

Confocal microscopy showed that ACh-stimulated NO production was reduced in endothelial cells cultured from the aortas of OVX rats compared to the sham control. Overnight treatment with calcitriol(A), S18886(B) and celecoxib(C) enhanced the NO production. Data are means ± SEM of 4-5 experiments. $^{*}P<0.05$ versus sham; $^{\#}P<0.05$ versus OVX control.

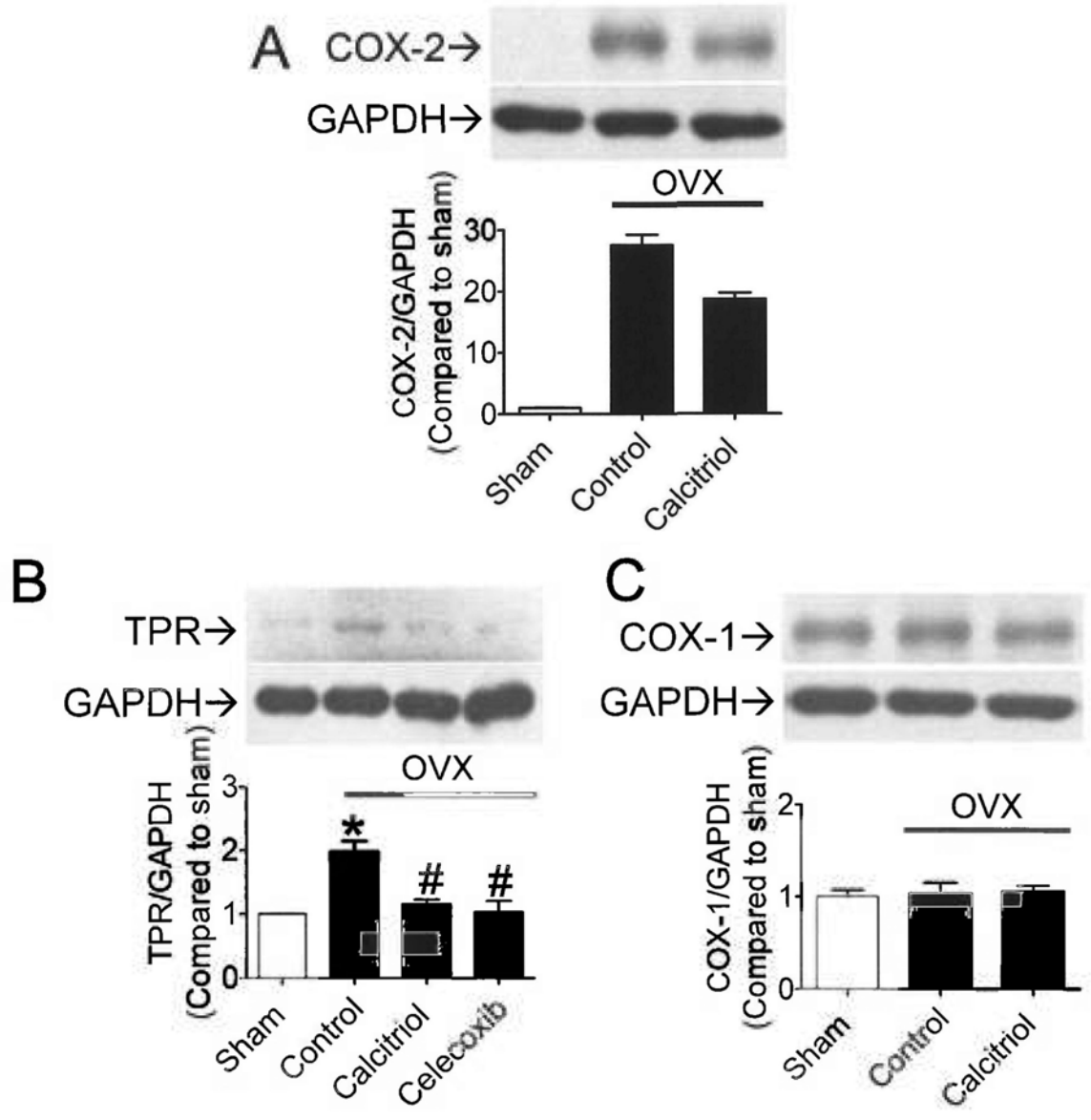

Figure 4.10

COX-2 and TP receptor (TPR) expression was increased in the endothelial cells cultured from the aortas of the OVX rats compared to those from the sham control. The elevated COX-2 and TPR expression was normalized by the overnight *in vitro* treatment of calcitriol (100 nmol/L) and the latter also by celecoxib (3 μmol/L). COX-1 expression remained unchanged among the sham control, and OVX rats with or without calcitriol treatment. Data are means ± SEM of 3-5 experiments. $^{*}P<0.05$ versus control; $^{\#}P<0.05$ versus U46619.

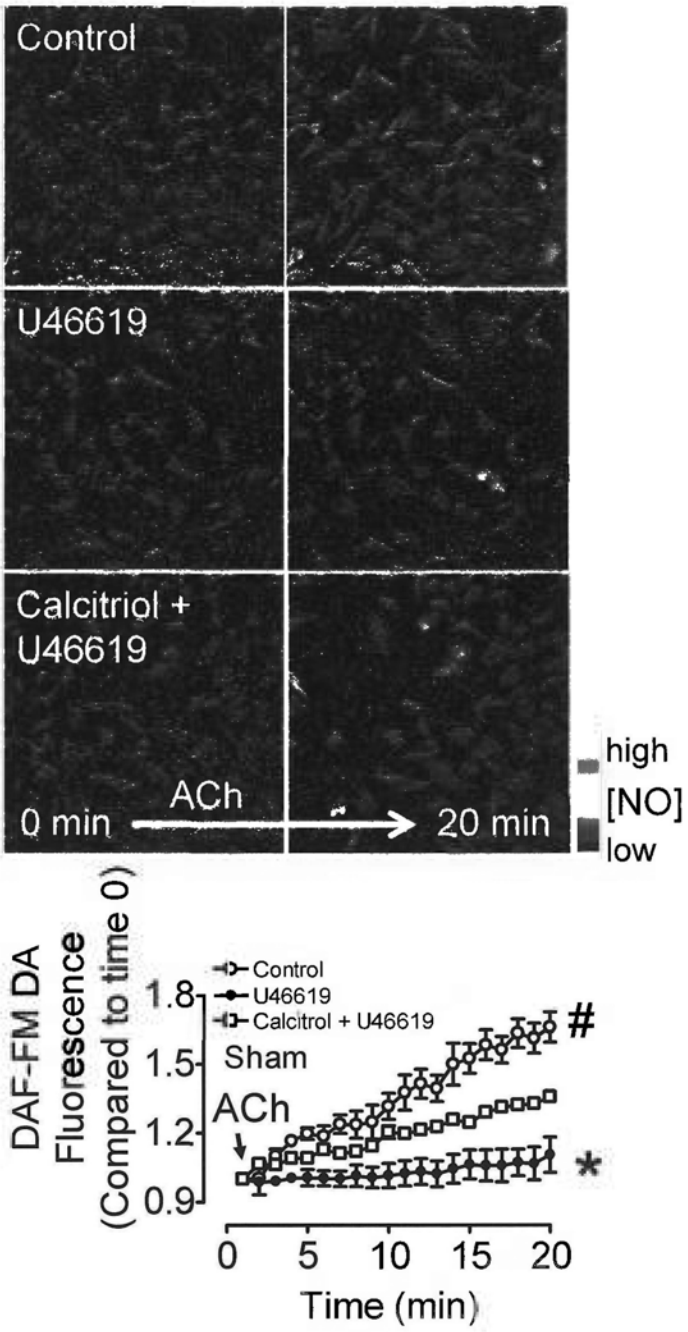

Figure 4.11

Overnight treatment of the aortic endothelial cells from sham control with U46619 (10 nmol/L) resulted in blunted response in NO production. Co-treatment with calcitriol (100 nmol/L) enhanced the NO production in the U46619-treated endothelial cells. Data are means ± SEM of 3-5 experiments. $^{*}P<0.05$ versus control; $^{\#}P<0.05$ versus U46619.

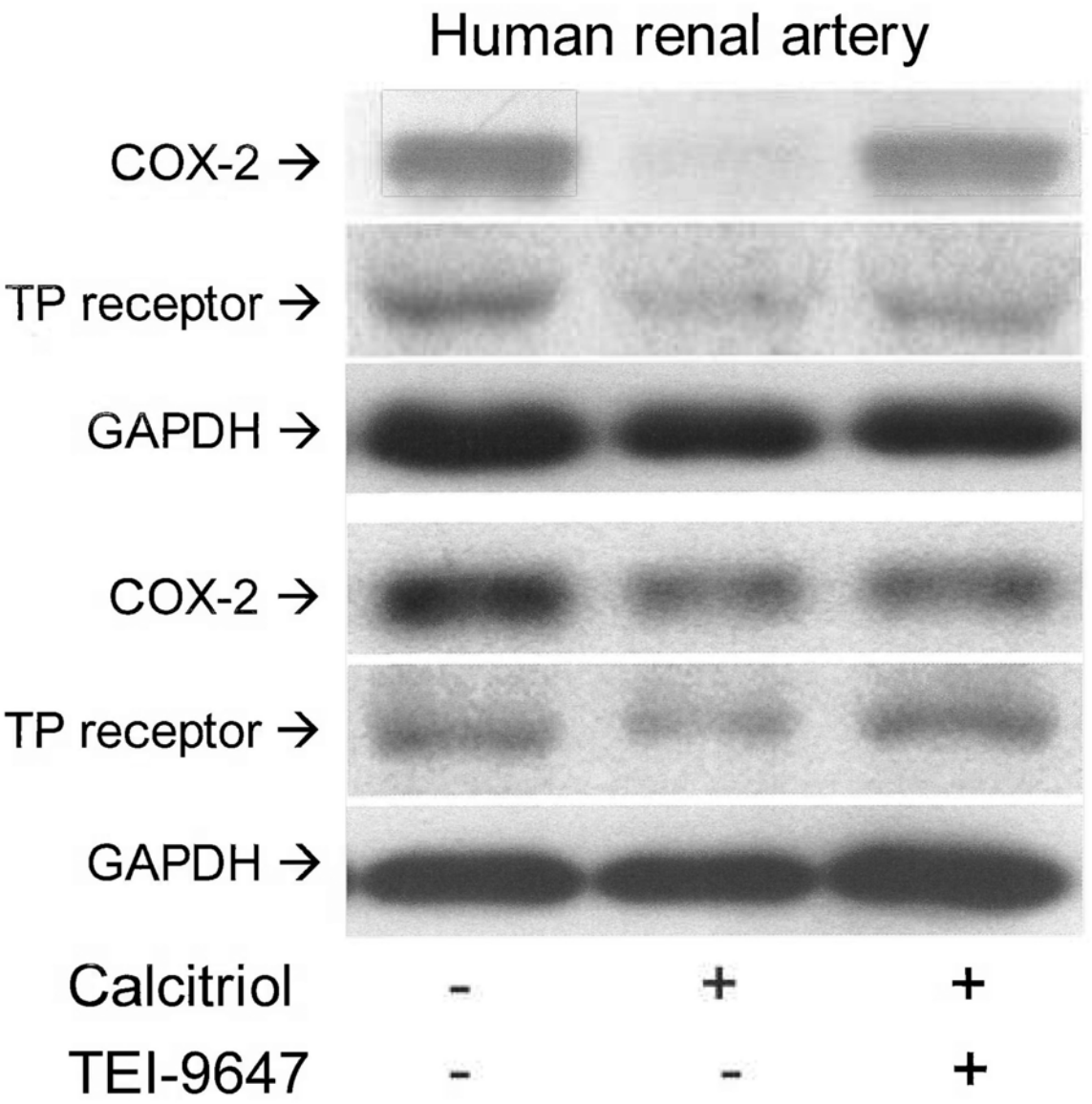

Figure 4.12

Representative Western blot showed that COX-2 and TP receptor (TPR) expression levels were reduced by the overnight treatment with calcitriol (100 nmol/L), of which the effect was prevented by the human vitamin D receptor antagonist TEI-9647 (1 μmol/L).

4.4 Discussion

The present study demonstrated that calcitriol, an active form of vitamin D, protects the renovascular function under estrogen deficiency by down-regulating the COX-2/TP receptor pathway. The major novel findings include: (1) *in vivo* and *in vitro* calcitriol treatment improves endothelium-dependent relaxations and normalizes the elevated expressions of COX-2 and TP receptor in renal arteries of the OVX rats. The attenuated relaxations can be rescued by an acute exposure to a non-selective COX inhibitor (indomethacin), selective COX-2 inhibitors (celecoxib, NS-398 and DuP-697) and TP receptor antagonist (S18886), but not by specific COX-1 inhibitor (SC-560), suggesting the positive involvement of COX-2 and TP receptor. (2) Acute calcitriol incubation did not enhance endothelium-dependent relaxations, implying that the protective effects of calcitriol may likely pass through the transcriptional and/or translational events. (3) Western blot analysis from endothelial cells isolated from OVX rat aortas revealed a marked increase in COX-2 and TP receptor expression under estrogen deficiency and the over-expression of COX-2 and TP receptor is downregulated by the *in vitro* incubation of calcitriol. (4) U46619, a potent activator of TP receptor which is involved in the development of kidney dysfunction (Sebekova et al., 2007), impairs endothelium-dependent relaxations and augments TP receptor expression in renal arteries form sham control. Co-incubation of calcitriol, S18886 or celecoxib with U46619 prevents the development of U46619-induced vascular dysfunction. (5) ACh-stimulated NO production was blunted in endothelial cells cultured from the OVX rats and was improved by overnight exposure to calcitriol, S18886 and celecoxib. Importantly, Western blot analysis showed that calcitriol down-regulated the COX-2 and TP receptor expression in the renal arteries from postmenopausal women and the effect of calcitriol was antagonized by human vitamin D receptor antagonist (TEI-9647), indicating that

the action of calcitriol is likely mediated through the vitamin D receptor.

Recent findings report that vitamin D not only inhibits the RAS, but also down-regulates the COX-2 pathway in the cancer cells. Since vitamin D is prescribed to postmenopausal women to treat or prevent osteoporosis, I tested the hypothesis on whether vitamin D offers extra benefits on the cardiovascular system during estrogen deficiency by down-regulating the pro-inflammatory proteins such as COX-2, of which its critical involvement in endothelial dysfunction is recently established (Wong et al., 2010) (Wong et al., 2011; Wong et al., 2009) .

Results from the present study revealed an up-regulation of COX-2 and TP receptor in the renal arteries during estrogen deficiency, and the impaired endothelium-dependent relaxations of the arteries were rescued by the acute treatment of the COX-2 inhibitor and TP receptor antagonist, suggesting a key role of the arachidonic acid cascade in renovsacular dysfunction in estrogen-deficient rats. COX-2 over-expression may result in the over-production of constrictive protanoids that may activate the TP receptor, which on the smooth muscle cells causes vasoconstriction while on the endothelial cells inhibits the NO production (Liu et al., 2009). Indeed, I demonstrate that overnight tissue culture with U46619 impaired endothelium-dependent relaxations, increased the TP receptor levels and blunted ACh-stimulated NO production. In fact, TP receptor is involved in the development of cardiovascular disease (Cayatte et al., 2000; Michel et al., 2008) and its receptor antagonist S18886 (terutroban) was shown to prevent the development of cardiovascular diseases (Belhassen et al., 2003; Lesault et al., 2011) and introduced into clinical trials (Osende et al., 2004). Since celecoxib normalized the TP receptor expression in renal arteries from OVX rats and those treated with U46619, it is likely that endothelial COX-2-derived metabolites may act in an autocrine and paracrine manner that positively regulates the TP receptor expression. In

contrast, the involvement of COX-1 in the renovascular dysfunction during estrogen deficiency is less likely since the COX-1 inhibitors did not modify the endothelium-dependent relaxations and the expression of COX-1 remained unchanged with ovariectomy.

Vitamin D is involved in the regulation of gene transcription including that of many cytokines such as tumor necrosis factor-α (Nagpal et al., 2005). However, the findings on how vitamin D regulates the expression of pro-inflammatory proteins such as COX-2 are limited, and a majority was reported in prostate cancer cells (Aparna et al., 2008; Thill et al., 2010). The present study provides novel evidence on the regulation of the COX-2 and TP receptor expression by both *in vivo* and *in vitro* calcitriol treatment in renovascular dysfunctions. Calcitriol restored celecoxib- and S18886-sensitive ACh-induced relaxations in renal arteries from OVX rats and the U46619-impared relaxations in the renal arteries from sham control. Molecularly, calcitriol normalized the expression of COX-2 and TP receptor in renal arteries from OVX rats and U46619-treated renal arteries from sham control. Western blot analysis demonstrated the endothelial expression of COX-2 and TP receptor in the OVX rats, which were again down-regulated with overnight treatment of calcitriol. Since the blood pressure of the OVX rats was not modified by the chronic oral treatment of calcitriol and well-matched functional and molecular results are obtained from both *in vivo* and *in vitro* treatment with calcitriol, calcitriol is likely to act on the vasculature directly. Notably, similar results were obtained in the human renal arteries obtained from postmenopausal female patients, in which the expression of COX-2 and TP receptor were markedly reduced by calcitriol. The effect of calcitirol was prevented by co-treatment with TEI-9647, indicating the involvement of vitamin D receptor in the renovascular protective effects. The specificity of TEI-9647 in inhibiting vitamin D or other vitamin D receptor agonist

has been confirmed by study of Ishizuka et al (Ishizuka et al., 2004). Since celecoxib can down-regulate TP receptors, we cannot exclude the possibility of calcitriol functions to suppress COX-2 expression, which thereafter leads to a reduction in the prostaglandin synthesis and results in a down-regulation of TP receptor expression. Whether and how calcitriol regulates COX-2 and TP receptor expression remain to be investigated.

In summary, chronic supplementation with the active form of vitamin D, calcitriol, protects renovascular function under estrogen deficiency at least in the OVX model. The calcitriol-induced protection is likely mediated through vitamin D receptor by down-regulation of the COX-2 and TP receptor expression. Both *in vitro* and *in vivo* animal-based results are supported by the findings from human renal arteries. The present study suggests calcitriol/VDR activation as a novel therapeutic strategy to ameliorate renovascular complications under estrogen deficiency in addition to its classical application in bone mineralization for treatment or prevention of osteoporosis in postmenopasal women.

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