Endothelial Cyclooxygenase-2 Mediates Endothelium-dependent Contractions and Angiotensin II-induced Vascular Inflammation

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

The Chinese University of Hong Kong
September 2010

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Declaration

The experiments described in this dissertation were carried out in the School of Biomedical Sciences, the Chinese University of Hong Kong, between August 2006 and June 2010. 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.

Acknowledgements

My graduating friends say that writing up a thesis is a process of recalling the interesting memories throughout the postgraduate study life. To me, not only does it act as a recollection, but also an opportunity to learn to be humble. It is high time to realize and acknowledge my sources of power during these years; without whom, my work simply would not have been possible.

I would like to devote my sincere gratitude to my thesis supervisor Prof Yu Huang for his guidance, trust, encouragements and understandings since my undergraduate days as a final year student in his lab. Prof Huang has enabled me with unlimited opportunities to explore in research and hold scholarly communications at local and international conferences. Recalling the time when I was still an undergraduate student that attached to his laboratory just out of a curiosity in cardiovascular research, he has been so patient in showing me the basics, which has equipped me to become an independent research student later on. I cannot forget how eager he was to rehearse with me for my first open presentation and the lunch discussions with a table of dim sum and inspirational ideas. These are the memorable moments I treasure with Prof Huang in my research life, in addition to the joy when our formulated hypotheses are proven correct with the use of different methodologies that signify we have possibly spotted the correct path to unveil the truth of nature.

Appreciation is also dedicated to Dr Chak Leung Au, Prof Maik Gollasch, Prof Aimin Xu, Prof Paul M Vanhoutte and Prof Xiaoqiang Yao for providing me with constructive comments on my studies. I would like to thank my colleagues in the School of Biomedical Sciences and teammates in the lab. Among them I would like to thank especially Mr Chi Wai Lau, not only in terms of his excellent technical assistance, but also to his everlasting friendship and support.

My family is my source of energy replenishment. Simply words of thanks would not be enough to acknowledge their unconditional support to my personal development. I would like to dedicate this thesis to my two grandfathers, who were keen to see me graduate and had always projected trust and hope in me for continuation of my research careers.

Last but not least, I acknowledge my Lord for planting me with a mind of curiosity, enriching me with courage, leading me on the path and providing me an environment essential for me to advance. Things do not just happen, but with a purpose. Eventually, *those who sow in tears will reap with songs of joy (Psalm 126:5)*.

Abstract

Vascular tone is regulated by the relative contributions of endothelium-derived relaxing (EDRFs) and contracting factors (EDCFs). Augmented release of EDCFs causes an impaired endothelium-dependent relaxation and endothelial dysfunction. Extensive studies suggest a positive role of cyclooxygenase (COX)-1 in the appearance of endothelium-dependent contractions in hypertensive and diabetic animal models, but little is known of the involvement of COX-2, which is over-generalized to be primarily an inducible enzyme during inflammation without a constitutive function. Limited information is available on how COX, in particular COX-2, in the regulation of normal vascular tone.

Aging is regarded as an independent risk factor for the development of cardiovascular diseases. Increased contribution from COX has been demonstrated in hypertensive animal models in which vascular premature aging occurs. However, it remains largely unclear about the role of COX in healthy aging, and which component of the vascular wall contributes to the release of EDCFs. Of importance, COX-2 can be chronically up-regulated by aging and risk factors such as the elevated circulating and tissue level of angiotensin II (Ang II) as reported in hypertensive and/or diabetic patients with vascular complications. In addition to vascular tone regulation, COX-2 is found to be localized in human vascular inflamed tissue indicative of a pro-inflammatory role. It remains obscure whether COX-2 acts directly as a downstream effector in mediating Ang II-induced vascular pathologies. To resolve the missing links of COX-2 in endothelium-dependent contractions and vascular inflammation, the present study was divided into three sub-projects, employing a combination of experimental approaches including functional assessment of vascular reactivity by isometric force measurement, Western blot analysis on relevant protein expression, and knockdown of targets by small interfering RNA. Human renal and mesenteric arteries were used to verify the relevance of the cell- and animal-based studies in human vasculatures.

In the first part of the study, I investigated whether COX-2 participated in the occurrence of endothelium-dependent contractions in the aortae from young (~3 month-old) hamsters and identified the most possible EDCF. Endothelium-dependent

contractions were elicited by acetylcholine and abolished by COX-2 inhibitors (NS-398, DuP-697 and celecoxib) and thromboxane-prostanoid (TP) receptor antagonists (S 18886, L-655,240 and GR 32191), but not by COX-1 inhibitors (valeryl salicylate and sc 560). RT-PCR and Western blot analysis using aortae with and without endothelium revealed that the COX-2 expression was localized mainly in the endothelium. Levels of prostangladin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostacyclin (PGI_2) increased in response to acetylcholine and the release of both prostaglandins was inhibited by COX-2 but not COX-1 inhibitors. Exogenous $PGF_{2\alpha}$ but not PGI_2 caused contractions at a concentration that corresponded to the amount released endogenously. The release of $PGF_{2\alpha}$ was not affected by the presence of nitric oxide (NO). The results of the present study suggest that a novel constitutive role of COX-2 in endothelium-dependent contractions, with its metabolites $PGF_{2\alpha}$ acting as a physiological EDCF in the young hamster aortae.

Based on the results aforementioned, I went on in the second part of the study to examine the impact of aging on EDCF-mediated contractions - the alterations of COX-2-mediated endothelium-dependent contractions and the associated release of prostaglandin(s) in the aortae from aged (>18 month-old) hamsters. Endothelium-dependent contractions in the presence of N^G-nitro-L-arginine methyl ester (L-NAME) were significantly greater in the aortae from aged hamsters and contractions could also be observed without L-NAME, which were sensitive to COX-2 inhibitors and TP receptor antagonists. The levels of COX-2 expression, the release of $PGF_{2\alpha}$ and vascular sensitivity to $PGF_{2\alpha}$ were augmented in aortae of aged hamsters. The present results indicate a positive impact of aging on COX-2-derived $PGF_{2\alpha}$-mediated endothelium-dependent contractions.

In the third part of the study, I investigated the relationship and the intracellular signaling cascades linking two pro-inflammatory factors Ang II and COX-2, and tested whether COX-2 mediated the Ang II-induced vascular pathogenesis. Eight hour-incubation with 100 nmol/L Ang II resulted in maximal COX-2 expression in primary rat endothelial cells and it was inhibited by losartan and RNA synthesis inhibitor (actinomycin-D). Inhibitors of either p38 MAPK or ERK1/2 (respectively SB 202190 and PD 98059) decreased the COX-2 expression, and co-treatment with both inhibitors caused an additive effect, suggesting a joint mediation through both kinases.

Protein kinase C (PKC) inhibitor (GF109203X), and particularly, the specific PKC_{δ} inhibitor (rottlerin), prevented Ang II-induced phosphorylation of ERK1/2 and COX-2 expression, indicating an upstream regulation of ERK1/2 by PKC_{δ}. A pivotal role of PKC_{δ} in Ang II-induced COX-2 expression was further supported by a similar stimulatory effect of PKC activator, signified by the Ang II-stimulated translocation of PKC_{δ} to the membrane and confirmed by its phosphorylation (Tyr^{311}). Small interfering RNA targeting PKC_{δ} ($siPKC_{\delta}$) diminished COX-2 expression, which was abrogated in $siPKC_{\delta}$-treated cells treated with SB 202190, confirming the parallel pathways of PKC_{δ}-ERK1/2 and p38 MAPK. Aortae and renal arteries from Ang II-infused rats exhibited an increased endothelial COX-2 expression and impaired acetylcholine-induced relaxation that was normalized by celecoxib. Human mesenteric arteries incubated with Ang II demonstrated elevated endothelial COX-2 and MCP-1 expressions, of which the former was inhibited by SB 202190 plus rottlerin and the latter prevented by COX-2 inhibitor celecoxib. Renal arteries from hypertensive or diabetic patients revealed an exaggerated expression of COX-2 and MCP-1 in the endothelium. The present novel findings indicate that the activation of PKC_{δ}-ERK1/2 and p38 MAPK is critical in Ang II-induced COX-2 up-regulation in endothelial cells, and identify a COX-2-dependent pro-atherosclerotic cytokine MCP-1.

To conclude, the present study provides novel evidence showing a positive role of COX-2 in mediating endothelium-dependent contractions, how it is up-regulated by Ang II in endothelial cells and the associated inflammatory response. These findings may raise the possibility of curtailing endothelial COX-2 expression and activity as the means of limiting or preventing of vascular inflammation.

論文摘要

血管張力由源於內皮細胞的舒張因子(EDHFs)及收縮因子(EDCFs)所調控。增加 EDCFs 的釋放可引致內皮依賴性舒張作用減弱和內皮功能紊亂。廣泛的研究表明一型環氧化酶(COX-1)在高血壓和糖尿病動物模型中出現的內皮依賴性血管收縮反應中起促進作用，但對於二型環氧化酶(COX-2)的作用則多被忽略，因為 COX-2 一向被過份賦義為一不具恆常作用、主要在炎症下誘發的酶。COX，尤其是 COX-2，在調控正常血管張力方面的研究相當有限。

衰老被認為是心血管疾病發展的獨立危險因子。在高血壓動物血管的過早衰老上 COX 起明顯的作用，但在正常衰老下，COX 的角色和哪一血管壁細胞負責釋放 EDCFs 仍然不清楚。尤其，COX-2 可以被衰老和危險因子（如高血壓和糖尿病血管併發症下，循環與組織血管緊張素 Ang II 的提高）慢性地上調。除了血管張力調控外，COX-2 的表達亦在炎性的血管組織中明顯增加，表明 COX-2 的促炎角色，然而 COX-2 是否直接作為 Ang II 的下游受動器從而介導 Ang II 引發的血管病變卻仍未清楚。為深入闡明 COX-2 在內皮依賴性血管收縮和血管炎性反應中或缺的聯繫，本研究分為三個實驗項目，並利用一系列實驗方法作引證，包括等距張力量度進行血管反應的功能測試、免疫印跡分析相關蛋白表達，以及小干擾 RNA (siRNA)敲低靶點。人類腎動脈和腸繫膜動脈則用作驗證以細胞及動物為基礎的實驗結果在人類血管上的關聯性。

在第一部分實驗，我研究了 COX-2 是否參與年輕(約三個月大)倉鼠主動脈的內皮依賴性收縮和確認了可能的 EDCF。乙酰膽鹼(ACh)引起內皮依賴性收縮可被 COX-2 抑制劑(NS-398、DuP-697 和 celecoxib)及血栓素受體(TP receptor) 拮抗劑 (S 18886、L-655,240 和 GR 32191)所抑制，但不受 COX-1 抑制劑(valeryl salicylate 和 sc 560)影響。RT-PCR 和免疫印跡分析顯示 COX-2 主要分佈在內皮細胞中。前列腺素 $F_{2\alpha}$ ($PGF_{2\alpha}$)和前列環素(PGI_2)水平在 ACh 刺激下明顯增加，它們的釋放均被 COX-2 抑制劑所抑制，但不受 COX-1 抑制劑所影響。外源性的 $PGF_{2\alpha}$ 在檢測的相應內源性濃度下可引起收縮，而 PGI_2 則不能。$PGF_{2\alpha}$ 的釋放不受一氧化氮所影響。這些結果表明了 COX-2 在內皮依賴性收縮上起著一個嶄新的恆常角色，它的代謝產物 $PGF_{2\alpha}$ 在倉鼠主動脈中為正常生理狀態下的 EDCF。

根據以上的結果，我在第二部分實驗中檢測了衰老對 EDCF 介導的內皮依賴性收縮的影響：由 COX-2 介導的內皮依賴性收縮及其與之相關的前列腺素釋放在年老(大於十八個月)倉鼠的主動脈中的改變。年老倉鼠主動脈的內皮依賴性收縮在 N^G-硝基-L-精氨酸甲酯(L-NAME)存在時明顯比年輕的倉鼠大，而且在沒有 L-NAME 的情況下，內皮依賴性

收縮也可被觀察到，這些內皮依賴性收縮對 COX-2 抑制劑和 TP receptor 拮抗劑均敏感。COX-2 的表達、$PGF_{2\alpha}$ 的釋放及血栓素受體的血管敏感度在年老倉鼠主動脈中增加。這些結果表明衰老在 COX-2 衍生、$PGF_{2\alpha}$ 介導的內皮依賴性收縮上起促進作用。

在第三部分實驗中，我研究了兩個促炎因子 Ang II 和 COX-2 之間的關係和細胞內的訊號串聯，並測試了 COX-2 能否介導 Ang II 引起的血管疾病發生。在原始培養的大鼠內皮細胞中，COX-2 的表達在八小時的 Ang II (100 納米克分子濃度) 刺激後達到最高，並能被氯沙坦(losartan，Ang II 一型受體拮抗劑)和 RNA 合成抑制劑(放線菌素 D)抑制。p38 絲裂原活化蛋白激酶(p38 MAPK) 或胞外信號調節激酶 1/2 (ERK1/2)的抑制劑(分別為 SB 202190 和 PD 98059)均可減低 COX-2 的表達，而兩種抑制劑的同步治療更有附加的作用，表明了兩個激酶的共同介導。蛋白激酶 C (PKC) 抑制劑(GF103209X)，尤其是 PKC_{δ} 特異抑制劑(咖馬林粗糠柴苦素，rottlerin)，可抑制 Ang II 引起的 ERK1/2 磷酸化和 COX-2 的表達，提示 PKC_{δ} 是 ERK1/2 的上游調控因子。PKC 激活劑引起的相似刺激作用，Ang II 刺激的 PKC 易位及 PKC 在 Tyr^{311} 的磷酸化進一步驗証 PKC_{δ} 在 Ang II 引起的 COX-2 表達中的關鍵角色。以 PKC_{δ} 作靶點的 siRNA ($siPKC_{\delta}$)減低 COX-2 的表達，而在這些 $siPKC_{\delta}$ 轉染的細胞中，SB 202190 更把餘下的 COX-2 表達完全抑制，確認了 PKC_{δ}-ERK1/2 和 p38 MAPK 的是兩條獨立的細胞內信號通路。Ang II 持續灌流老鼠的主動脈和腎動脈內皮細胞 COX-2 的表達增加以及內皮依賴性舒張減弱。人類腸繫膜動脈在 Ang II 的刺激下顯示內皮 COX-2 及單核細胞趨化蛋白(MCP-1) 表達的提高，前者可被 SB 202190 及 rottlerin 所抑制，而後者的發生則被 celecoxib 所阻止。提升的內皮 COX-2 和 MCP-1 表達亦可見於高血壓或糖尿病病人的腎動脈上。這些新的研究結果表明 PKC_{δ}-ERK1/2 和 p38 MAPK 的激活在 Ang II 引起的 COX-2 表達上調中所起的是關鍵作用，亦確認了促進動脈粥樣硬化的細胞因子 MCP-1 的表達與 COX-2 有關聯。

綜上所述，本研究提供了嶄新的證據顯示 COX-2 在內皮依賴性收縮上的關鍵角色，以及它在內皮細胞內如何被上調和相關炎症反應的作用。這些結果提示了減低內皮 COX-2 表達和活性以達到限制或防止血管發炎的可能性。

Abbreviations

2-APB	2-Aminoethoxydiphenyl borate
ACh	Acetylcholine
Ang II	Angiotensin II
AT_1R	Angiotensin II type 1 receptor
AT_2R	Angiotensin II type 2 receptor
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DETCA	Diethyldithiocarbamate acid
DHE	Dihydroethidium
DMSO	Dimethyl sulfoxide
EDCFs	Endothelium-derived contracting factors
EDHFs	Endothelium-derived hyperpolarizing factors
EDRFs	Endothelium-derived relaxing factors
EIA	Enzyme immunoassay
ERK1/2	Extracellular signal-regulated kinases 1/2
eNOS	Endothelial nitric oxide synthase
EPR	Electron paramagnetic resonance
HPLC-MS	High performance liquid chromatography-coupled mass spectrometry
IL-6	Interleukin-6
L-NAME	N^G-nitro-L-arginine methyl ester
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate oxidase
NO	Nitric oxide
PECAM-1	Platelet endothelial cell adhesion molecule-1
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGI_2	Prostacyclin
PKC	Protein kinase C
ROS	Reactive oxygen species
siRNA	Small interfering RNA
SHR	Spontaneously hypertensive rats
SOD	Superoxide dismutase
SNP	Sodium nitroprusside
TNF-α	Tumor necrosis factor-α
TP receptor	Thromboxane-prostanoid receptor
TxA_2	Thromboxane A_2
VAS	Valeryl salicylate

Awards and Publications

1. Academic Awards and Travel Grants

1. Postgraduate Research Output Award 2009, the Chinese University of Hong Kong.
2. Young Investigator Award (in Oral Presentation) at the 4th International Symposium on Healthy Aging, the Research Centre of Heart, Brain, Hormone and Healthy Aging, Li Ka Shing Faculty of Medicine, the University of Hong Kong (7 March 2009)
3. 1st Prize of Oral Presentation at the 12th Annual Scientific Meeting of the Institute of Cardiovascular Science and Medicine, the University of Hong Kong (14 Dec 2008)
4. 1st Prize of APSAVD Awards for Best Paper Presentations by Young Investigators in 6th Congress of the Asian Pacific Society of Atherosclerosis and Vascular Diseases & 10th Hong Kong Diabetes and Cardiovascular Risk Factors East Meets West Symposium (27 Sept 2008)
5. Joint Winner of EDHF 2008 (5th International EDHF Symposium: Endothelium, Vasoactive Factors and Inflammation), Tampere, Finland (26 Jun 2008).
6. 1st Prize of Poster Presentation at the 11th Annual Scientific Meeting of the Institute of Cardiovascular Science and Medicine, the University of Hong Kong (8 Dec 2007)
7. Outstanding Postgraduate Student Oral Presentation in the 9th Scientific Meeting of Hong Kong Pharmacology Society (16 Dec 2006)
8. Outstanding Poster Presentation at the 10th Annual Scientific Meeting of the Institute of Cardiovascular Science and Medicine, the University of Hong Kong (10 Dec 2006)
9. 3rd Prize of Oral Presentation for Young Investigator Awards, at the 9th Annual Scientific Meeting (Metabolic Syndromes) of the Institute of Cardiovascular Science and Medicine, the University of Hong Kong, incorporating the 5th Scientific Conference on Cardiovascular Sciences across the Strait (4 Dec 2005)

2. Travel Grants

1. Bain Memorial Fund Bursary, British Pharmacological Society (2010)
2. Postgraduate Student Grants for Overseas Academic Activities, The Chinese University of Hong Kong (2008)
3. Postgraduate Student Travel Grant, Hong Kong Pharmacology Society (2008)

3. Scientific Meetings Attended with Oral Presentations

1. WorldPharma 2010, 16th World Congress of Basic and Clinical Pharmacology. Copenhagen, Denmark, June 2010. *(Oral presentation in focused conference Endothelium in Health and Disease - Drug development and novel therapeutic targets and approaches)*
2. Annual Scientific Meeting, Hong Kong Society of Endocrinology, Metabolism and Reproduction. Hong Kong, November 2009. *(Oral Presentation Competition)*
3. Physiology Symposium 2009, Department of Physiology, The University of Hong Kong. Hong Kong, May 2009. *(Oral presentation)*

4. 4th International Symposium on Healthy Aging, Li Ka Shing Faculty of Medicine, the University of Hong Kong. Hong Kong, March 2009. *(Oral presentation Competition)*
5. 12th Annual Scientific Meeting, Institute of Cardiovascular Science and Medicine, The University of Hong Kong. Hong Kong, December 2008. *(Oral Presentation Competition)*
6. 6th Congress of the Asian Pacific Society of Atherosclerosis and Vascular Diseases & 10th Hong Kong Diabetes and Cardiovascular Risk Factors East Meets West Symposium. Hong Kong, September 2008. *(Oral Presentation Competition)*
7. 5th International EDHF Symposium: Endothelium, Vasoactive Factors and Inflammation. Tampere, Finland, June 2008. *(Oral Presentation Competition)*
8. 11th Annual Scientific Meeting, Institute of Cardiovascular Science and Medicine, The University of Hong Kong. Hong Kong, December 2007. *(Poster Presentation Competition)*
9. Physiology Symposium 2007, National Taiwan Normal University. Taiwan, March 2007. *(Oral Presentation)*
10. 9th Scientific Meeting of Hong Kong Pharmacology Society. Hong Kong, December 2006. *(Oral Presentation Competition)*
11. 10th Annual Scientific Meeting, Institute of Cardiovascular Science and Medicine, The University of Hong Kong. Hong Kong, December 2006. *(Poster Presentation Competition)*
12. Faculty Research Day, Faculty of Medicine, the Chinese University of Hong Kong. Hong Kong, August 2006. *(Oral Presentation Competition)*
13. Physiology Symposium 2006, the Chinese University of Hong Kong. Hong Kong, February 2006. *(Oral presentation)*
14. 9th Annual Scientific Meeting, Institute of Cardiovascular Science and Medicine, The University of Hong Kong. Hong Kong, December 2005. *(Oral Presentation Competition)*

3. PUBLICATIONS

Original research articles

1. Liu CQ*, **Wong SL***, Leung FP, Tian XY, Lau CW, Lu L, Yao X, Chen ZY, Yao T, Huang Y. Prostanoid TP receptor-mediated impairment of cyclic AMP-dependent vasorelaxation is reversed by phosphodiesterase inhibitors. Eur J Pharmacol. 2010;632(1-3):45-51. (*co-first authorship)
2. Liu CQ, Leung FP, **Wong SL**, Wong WT, Lau CW, Lu L, Yao X, Yao T, Huang Y. Thromboxane prostanoid receptor activation impairs endothelial nitric oxide-dependent vasorelaxations: the role of Rho kinase. Biochem Pharmacol. 2009;78(4):374-381.
3. **Wong SL**, Leung FP, Lau CW, Au CL, Yung LM, Yao X, Chen ZY, Vanhoutte PM, Gollasch M, Huang Y. Cyclooxygenase-2-derived prostaglandin $F_{2\alpha}$ mediates endothelium-dependent contractions in the aortae of hamsters with increased impact during aging. Circ Res. 2009;104(2):228-235. [Original research article, with Editorial Commentary by Schrör K, Circ Res. 2009;104(2):141-143]

Reviews

1. Wong WT, **Wong SL**, Tian XY, Huang Y. Endothelial dysfunction: the common consequence in diabetes and hypertension. J Cardiovasc Pharmacol. 2010;55(4):300-307.
2. **Wong SL**, Yung LM, Lau CW, Wong WT, Chan YC, Huang Y, Leung FP. Impact of periadventitial adipose tissue on arterial tone. Journal of Internal Medicine Concepts and Practice. 2007;2(3):139-141.

Editorial

1. **Wong SL**, Huang Y. Adventitia as a critical player in the functional integrity of arteries. Circ J. 2010;74(5):854-855.

Book chapter

1. **Wong SL**, Wong WT, Tian XY, Lau CW, Huang Y (2010). Prostaglandins in action: *indispensable roles of cyclooxygenase-1 and 2 in endothelium-dependent* contractions. (recently accepted in Advances in Pharmacology)

Manuscripts recently submitted

1. **Wong SL**, Lau CW, Wong WT, Xu A, Au CL, Ng CF, Ng SSM, Yao X, Huang Y (2010). PKC_{δ}-ERK1/2 and p38 MAPK mediate angiotensin II-induced endothelial cyclooxygenase-2 expression: a link to vascular inflammation and clinical relevance.

Manuscripts in preparation

1. **Wong SL**, Lau CW, Huang Y (2010). Release of endothelium-derived contracting factor is independent of the presence of nitric oxide.

Conference abstracts

1. **SL Wong**, CW Lau, WT Wong, A Xu, CL Au, X Yao, Y Huang. Co-activation of PKC_{δ}/ERK1/2 and p38 MAPK mediates angiotensin II-induced cyclooxygenase-2 up-regulation in endothelial cells and the clinical implications. WorldPharma 2010, 16th World Congress of Basic and Clinical Pharmacology, 17-23 June 2010, Copenhagen, Denmark.
2. **SL Wong**, CW Lau, CL Au, X Yao, Y Huang. Nitric oxide does not affect the release of endothelium-derived contracting factor $PGF_{2\alpha}$ in the hamster aorta. *Journal of the Hong Kong College of Cardiology* 17(2):62 (P18).
3. **SL Wong**, CW Lau, WT Wong, A Xu, X Yao, Y Huang. Joint activation of PKC_{δ}/ERK1/2 and p38 MAPK mediates angiotensin II-induced cyclooxygenase-2 up-regulation in endothelial cells – the clinical relevance. Annual Scientific Meeting, Hong Kong Society of Endocrinology, Metabolism and Reproduction, 22 November 2009.
4. **SL Wong**, CW Lau, X Yao, Y Huang. Release of endothelium-derived contracting factor is unaffected by the presence of nitric oxide in the hamster aorta. The 7th Scientific Conference on Cardiovascular Sciences across the Strait, International Society for Heart Research Chinese Section (ISHRC) and Chinese Association of Pathophysiology, Society for Cardiovascular Sciences, Kunming, China, 14-17 August 2009.
5. **SL Wong**, FP Leung, PM Vanhoutte, X Yao, Y Huang. Aging Exaggerates COX-2-mediated Endothelium-dependent Contractions in Hamster Aortae. Physiology Symposium 2009, Department of Physiology, The University of Hong Kong, 29-30 May 2009.
6. **SL Wong**, FP Leung, CW Lau, XQ Yao, PM Vanhoutte, Y Huang. Aging

Exaggerates COX-2-mediated Endothelium-dependent Vaso-contractions. 4th International Symposium on Healthy Aging, Li Ka Shing Faculty of Medicine, the University of Hong Kong, 7-8 March 2009, OP 27.

7. **SL Wong**, CW Lau, CL Au, X Yao, Y Huang (2008). A Central Role of PKC_{δ} and Activation of p38 MAPK and ERK1/2 Pathways in Angiotensin II-mediated upregulation of COX-2 in Endothelial Cells. *Journal of the Hong Kong College of Cardiology* 16(2): 61 (OC9).
8. **SL Wong**, FP Leung, CW Lau, Y Huang (2008). Ageing enhances COX-2-mediated endothelium-dependent contractions in hamster aortae. *6th Congress of the Asian Pacific Society of Atherosclerosis and Vascular Diseases & 10th Hong Kong Diabetes and Cardiovascular Risk Factors East Meets West Symposium*, 27 September 2008, Ab 19.
9. **SL Wong**, FP Leung, CW Lau, PM Vanhoutte, Y Huang (2008). Endothelium-dependent contractions in hamster aorta: the essential role of COX-2 and prostaglandin $F_{2\alpha}$. *Basic & Clinical Pharmacology & Toxicology* 102(1):15 (S5-6).
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Chapter I

Introduction

1.1 Endothelial function

A monolayer of endothelial cells is located in the innermost compartment of the vessel wall. Instead of serving as an inert barrier between circulating blood and vascular smooth muscle cells, the endothelium is actively contributing to vascular function and homeostasis. Endothelium synthesizes and releases vasoactive factors to regulate the vascular tone in response to physical or chemical stimuli such as shear stress, circulating hormones, cyokines and platelet- or coagulation-derived substances. These vasoactive factors include endothelium-derived relaxing factors (EDRFs) and contracting factors (EDCFs). EDRFs are best characterized to consist of nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factors (EDHFs). As for EDCFs, their identities are less well-defined due to the heterogeneity in vascular beds and among species. Possible candidates include reactive oxygen species (ROS), endothelin-1 and metabolites derived from cyclooxygenase (COX).

In addition to vascular tone regulation, endothelium also confers protection to the vascular wall against inflammatory insults. Healthy endothelium is a non-adhesive and anti-thrombotic surface that minimizes the interaction between endothelium, platelets and immune cells (Nabel, 1991). NO and PGI_2 are by far the most extensively studied molecules participating in the prevention of vascular inflammation and remodeling.

1.1.1 Vascular tone regulation

Vascular tone is the net outcome resulting from the relative contributions of EDRFs and EDCFs.

1.1.1.1 EDRFs

NO, PGI_2 and EDHFs are the main EDRFs that mediate endothelium-dependent relaxations.

NO is generated by the constitutively expressed NO synthase (NOS) in the vascular wall, which mainly resides in the endothelium. NOS converts the substrate L-arginine to NO and L-citrulline (Palmer et al., 1988), with tetrahydrobiopterin as an essential co-factor (Moncada et al., 1991). NO production appears Ca^{2+}-dependent as stimulated by most agonists such as acetylcholine and bradykinin; in contrast, NOS activation by shear stress is dependent on protein tyrosine kinase but not Ca^{2+} (Ayajiki et al., 1996). Upon release, NO freely diffuses to the adjacent vascular smooth muscle layer and activates the soluble guanylate cyclase, leading to a subsequent increase in the cyclic GMP levels (Rapoport and Murad, 1983). The cyclic GMP-dependent protein kinase is then activated, stimulating extrusion of cytosolic Ca^{2+} and membrane hyperpolarization via opening K^+ channels, thereby leading to vasodilatation (Lincoln et al., 1994).

The vasodilatory effect of PGI_2 is dependent on the presence of its natural receptor, the adenylate cyclase-coupled IP receptor (Coleman et al., 1994; Halushka et al., 1989; Kukovetz et al., 1979). Activation of the IP receptor increases the intracellular production of cyclic AMP, which extrudes cytosolic Ca^{2+} (Bukoski et al., 1989) and stimulates membrane hyperpolarization by activating the ATP-sensitive potassium channels (K_{ATP}) (Parkington et al., 2004).

Though NO and PGI_2 can independently mediate vasodilatation, NO potentiates the effect of PGI_2 as NO-induced elevation of cyclic GMP level can sustain the level of cyclic AMP by inhibiting the phosphodiesterase that breaks down cyclic AMP (Delpy et al., 1996; Vanhoutte, 2001).

Contribution of EDHFs in the regulation of vascular tone increases with a reducing size of the arteries (Shimokawa et al., 1996). The exact identity of the EDHFs, however, remains controversial. K^+ (Edwards et al., 1998), cytochrome *P*450 epoxygenase-derived epoxyeicosatrienoic acid (Campbell et al., 1996), hydrogen peroxide (Matoba et al., 2002; Matoba et al., 2000) are proposed to be members of the EDHF family, which can activate K^+ channels including the inwardly rectifying K^+ channels (K_{IR}) (Edwards et al., 1998), the large-conductance Ca^{2+}-activated K^+ channels (K_{Ca}) (Campbell et al., 1996; Gebremedhin et al., 1992; Hu and Kim, 1993; Matoba et al., 2002; Matoba et al., 2000), thus hyperpolarize the plasma membrane of vascular smooth muscle cells, resulting in vasodilatation.

1.1.1.2 EDCFs

Endothelium-dependent contractions are mediated by EDCFs, of which their exact identity is less-well defined. Endothelium-derived ROS such as superoxide anion and hydrogen peroxide are the possible EDCF candidates that mediate vasoconstrictions induced by shear stress (Shimizu et al., 1994) and are involved in Ca^{2+} mobilization in the vascular smooth muscle cells (Suzuki and Ford, 1992). These oxygen radicals also acutely scavenge NO to induce a contractile tone.

To date, a large body of converging evidence suggests that a majority of EDCFs are arachidonic acid metabolites derived by COX. Endoperoxides

prostaglandin (PG) G_2 and PGH_2 (Auch-Schwelk et al., 1989; Ito et al., 1991), thromboxane A_2 (TxA_2) (Shirahase et al., 1988) and PGI_2 (Gluais et al., 2005) are released upon acetylcholine stimulation and they act on TP receptors to mediate endothelium-dependent contractions.

1.1.1.3 Balance between EDRFs and EDCFs

Vasodilatory effect of EDRFs predominates over the constrictions induced by EDCFs in normal vasculature and thus vasodilatation prevails. In pathological states, there is an increase in COX activity and/or reduction in NO bioavailability. The balance of the relative contributions from EDHFs and EDCFs is disturbed, which favors the action of contracting factors, leading to persistent vasoconstrictions (Figure 1.1).

1.1.2 Protection against vascular inflammation

NO not only functions as a potent vasodilator, but also actively protect the endothelium from vascular inflammation. NO inhibits platelet adhesion and aggregation (Radomski et al., 1990), and negatively modulates the expression of pro-inflammatory cytokines and adhesion molecules such as monocyte chemoattractant protein-1 and intracellular adhesion molecule-1 at the transcriptional level (Ikeda et al., 1996; Lee et al., 2002). NO can suppresses the synthesis of endothelin-1 (Alonso and Radomski, 2003), which is a powerful vasoconstrictor and promotes cell proliferation and inflammation. Apart from NO, PGI_2 also attenuate platelet activation and aggregation (Braun et al., 1993; Gimson et al., 1980; Hohlfeld et al., 1992).

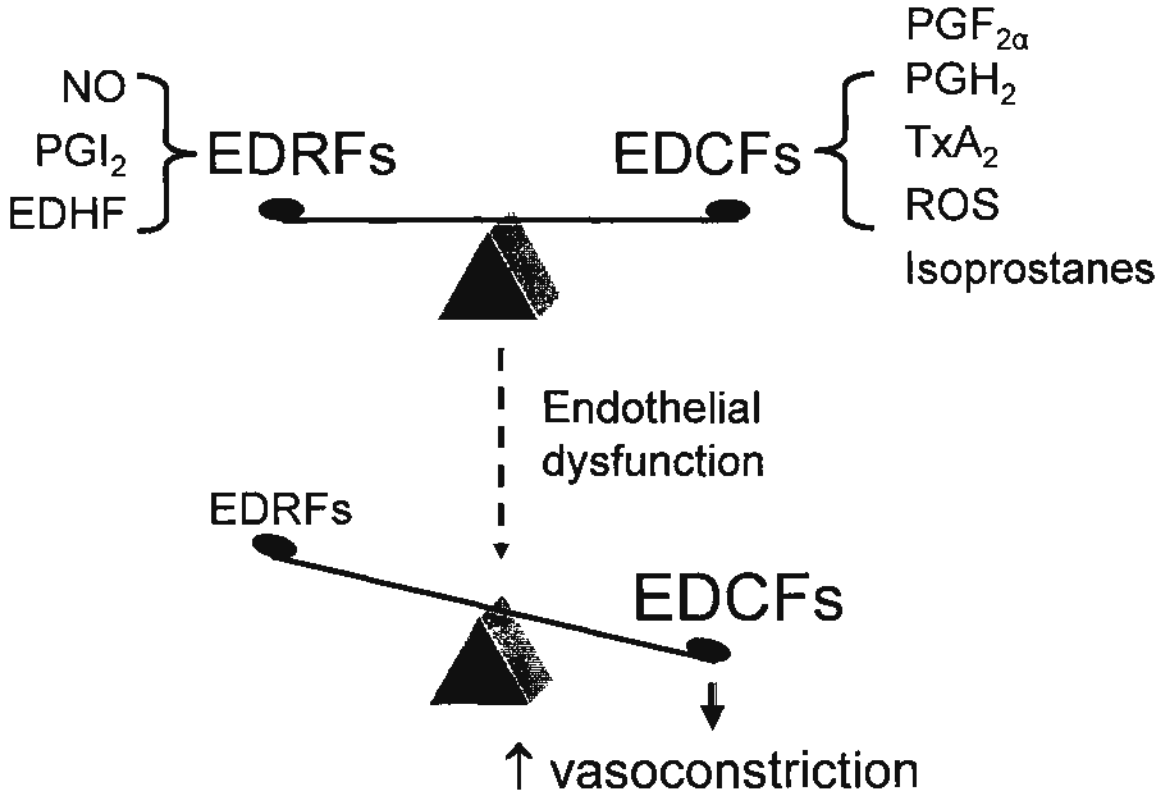

FIGURE 1.1.

Balance of endothelium-derived relaxing factors (EDRFs) and contracting factors (EDCFs) in the regulation of vascular tone. When the production of EDRFs diminishes and/or EDCFs are over-generated, the imbalance favors vasoconstrictions. NO, nitric oxide; PGI_2, prostacyclin; EDHFs, endothelium-derived hyperpolarizing factors; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGH_2, prostaglandin H_2; TxA_2, thromboxane A_2; ROS, reactive oxygen species.

1.2 Endothelial dysfunction

Endothelial dysfunction refers to an abnormal regulation of vascular tone and a diminished protection of the endothelium on the vascular wall which allows the initiation and progression of vascular inflammation. The leading causes of the cardiovascular events associated with endothelial dysfunction include exaggerated oxidative stress, a reduction of NO bioavailability and the activation of protein kinase C (PKC).

1.2.1 Exaggerated oxidative stress

ROS are generated from the reduction-oxidation (redox) reactions involving molecular oxygen in aerobic cells. They are broadly classified into two categories, free radical that contains an unpaired electron and nonradical derivatives. Free radicals can exist independently, but their unpaired electrons make them highly reactive and unstable, thus their half-lives are relatively short. As for nonradicals, they are less reactive and more stable. Examples of free radicals include superoxide anion, hydroxyl radical, lipid peroxide and peroxyl, while hydrogen peroxide and peroxynitrite are nonradicals.

ROS at controlled levels serve as signaling molecules that mediate physiological responses. For instance, hydrogen peroxide acts as an EDHF mediating acetylcholine-induced vasodilatation in murine small mesenteric arteries (Matoba et al., 2000). Intracellular ROS production is tightly regulated by the activity between the radical generating and the anti-oxidative enzymes.

1.2.1.1 Hyperreactive radical generating enzymes

Vascular superoxide anion can be generated by nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) (Rajagopalan et al., 1996;

Ushio-Fukai et al., 1996), xanthine oxidase (Landmesser et al., 2007; White et al., 1996), lipoxygenase (Radogna et al., 2009), COX (Tang et al., 2007), uncoupled endothelial NOS (eNOS) (Vasquez-Vivar et al., 1998) and mitochondrial oxidase (Victor et al., 2009). Elevated expression and activity of NADPH oxidase appears to account for the over-production of superoxide anion in most pathological conditions including diabetes and atherosclerosis. Recently, the role of mitochondrial respiratory chain in free radical generation has caught attention in mediating vascular complications (Dikalova et al., 2010; Jones et al., 2008).

1.2.1.2 Reduced anti-oxidative defense

Anti-oxidative enzymes function to prevent the oxidation of the substrate of radical generating enzymes, thereby preventing ROS production. The major anti-oxidative enzymes are superoxide dismutase (SOD), catalase and glutathione peroxidase (Faraci and Didion, 2004; Johnson and Giulivi, 2005; King et al., 2010; Nishikawa et al., 2009; Valdivia et al., 2009). SOD is presented in three forms, the cytosolic Cu/Zn-SOD (SOD-1), the mitochondrial Mn-SOD (SOD-2) and extracellular SOD (SOD-3) (Faraci and Didion, 2004). SOD dismutates superoxide anion into water and hydrogen peroxide, of which the latter is further converted to water and molecular oxygen by catalase and glutathione peroxidase. Catalase is important when the concentration of cellular hydrogen peroxide is high while glutathione peroxidase predominates when the concentration is low (Nicholis, 1972). Membrane phospholipids are protected from oxidation by glutathione peroxidase which actively donates protons to the phospholipids to maintain it in a reduced state (Li et al., 2009). Deletion of glutathione peroxidase accelerates cardiac hypertrophy and dysfunction

(Ardanaz et al., 2010) while over-expression of anti-oxidantive enzymes can ameliorate ROS-mediated vascular dysfunction (Van Rheen et al., 2010).

1.2.2 Decreased NO bioavailability

NO bioavailability is the key to maintain vascular homeostasis. However, being a radical, it can be easily scavenged by ROS. Treatment with NADPH oxidase inhibitors, apocynin or diphenyliodonium, and SOD mimetic tempol can acutely restore the attenuated endothelium-dependent relaxations in the aortae of diabetic db/db mice (Wong et al., 2010b), indicating that NO is rapidly depleted by ROS upon release and hence renders them non-functional. Reduction in the eNOS expression also impairs NO production. Pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) can directly suppress the eNOS mRNA level in human endothelial cells (Yoshizumi et al., 1993). In the aortae of streptozotocin-induced diabetic rats, ROS downregulate the eNOS expression level, which is normalized by the chronic treatment with apocynin (Olukman et al., 2010). AVE 9488, an eNOS enhancer that increases the expression level of eNOS, protects the heart against ischemia/reperfusion injury and ameliorates portal hypertension (Biecker et al., 2008; Frantz et al., 2009).

1.2.3 PKC activation

Activation of PKC pathways is closely associated with hyperglycemia- or diabetes-induced endothelial dysfunction. PKC directly increases endothelial permeability to albumin (Lynch et al., 1990), possibly mediated by PKC_{α} and $PKC_{\beta 1}$ (Hempel et al., 1997; Nagpala et al., 1996). Hyperglycemia-induced advanced glycation end products, diacylglycerol (DAG) synthesis and oxidative stress can activate PKC, thereby impairing NO-mediated endothelium-

dependent relaxations and promoting the release of endothelin-1 and COX-derived vasoconstrictive prostanoids such as TxA_2 (Cardillo et al., 2002; Cosentino et al., 2003; Hink et al., 2001). Hyperhomocystinemia-induced endothelial dysfunction and reduction in the eNOS activity is reversed by a broad spectrum PKC inhibitor GF 109203X (Jiang et al., 2005). Treatment with a PKC_β inhibitor LY333531 prevents high glucose-induced up-regulation of NADPH oxidase subunits (Quagliaro et al., 2003). Induction of adhesion molecules VCAM-1 is mediated by PKC activation (Deisher et al., 1993). Partmentier et al. (2006) has pointed out a critical role of PKC_ζ in the Ang II-accelerated neointimal growth in the rat carotid after balloon injury.

1.3 COX and endothelium-dependent contractions

COX represents a major source of arachidonic acid-derived vasoconstrictive prostanoids. Chemical agonists such as acetylcholine (receptor-mediated) or Ca^{2+} ionophore (A23187, receptor-independent) can trigger both the NO-mediated vasodilatory pathway and COX-mediated production of vasoconstrictors in response to the increase in intracellular Ca^{2+} levels. Inhibition of the vasodilatory effect from NO allows the unmasking of EDCF-mediated endothelium-dependent contractions.

1.3.1 COX-1 and COX-2

COX exists mainly in two isoforms in endothelial cells, termed as COX-1 and COX-2, localized in the endoplasmic reticulum or nuclear envelope (Smith et al., 2000). COX-1 is expressed constitutively in many cells types, including endothelial cells and vascular smooth muscle cells (Hla and Neilson, 1992), while COX-2 is generally regarded as the inducible isozyme in response to

stimuli such as shear stress (Topper et al., 1996), TNF-α, and lipopolysaccharide (Williams et al., 1999). Recently, the third isoform COX-3 has been identified, which is mainly involved in pain and fever (Schwab et al., 2003).

1.3.2 COX-mediated arachidonic acid cascade

The membrane-bound COX first converts arachidonic acid to PGG_2 and PGH_2, which are then enzymatically transformed by respective synthases or isomerases into conventional prostanoids namely PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TxA_2 (Figure 1.2). These prostanoids are regarded conventional since they act correspondingly to their natural G protein-coupled receptor of DP, EP, FP, IP and TP receptors, the five basic receptors for these prostanoids. COX-1 and COX-2 share similar capacity to generate PGH_2 preceding the chemical conversion into the five conventional prostanoids (Smith et al., 1996). Although COX-2 is a predominant generator of systemic PGI_2, recent studies have demonstrated that local vascular PGI_2 production is mainly derived from endothelial COX-1 but not COX-2, which is not expressed in various human arteries (Flavahan, 2007). The expression of COX isoforms is thus one of the determinants on which of these isozymes is involved in the prostanoid production.

The type(s) of prostanoid produced also depends on the expression and activity of prostaglandin isozymes/synthases. For example, endothelial cells of spontaneously hypertensive rats (SHR) expresses an elevated level of prostacyclin synthase (PGIS) (Tang and Vanhoutte, 2008), thus PGH_2 is more readily converted to PGI_2 under the action of the over-expressed PGIS.

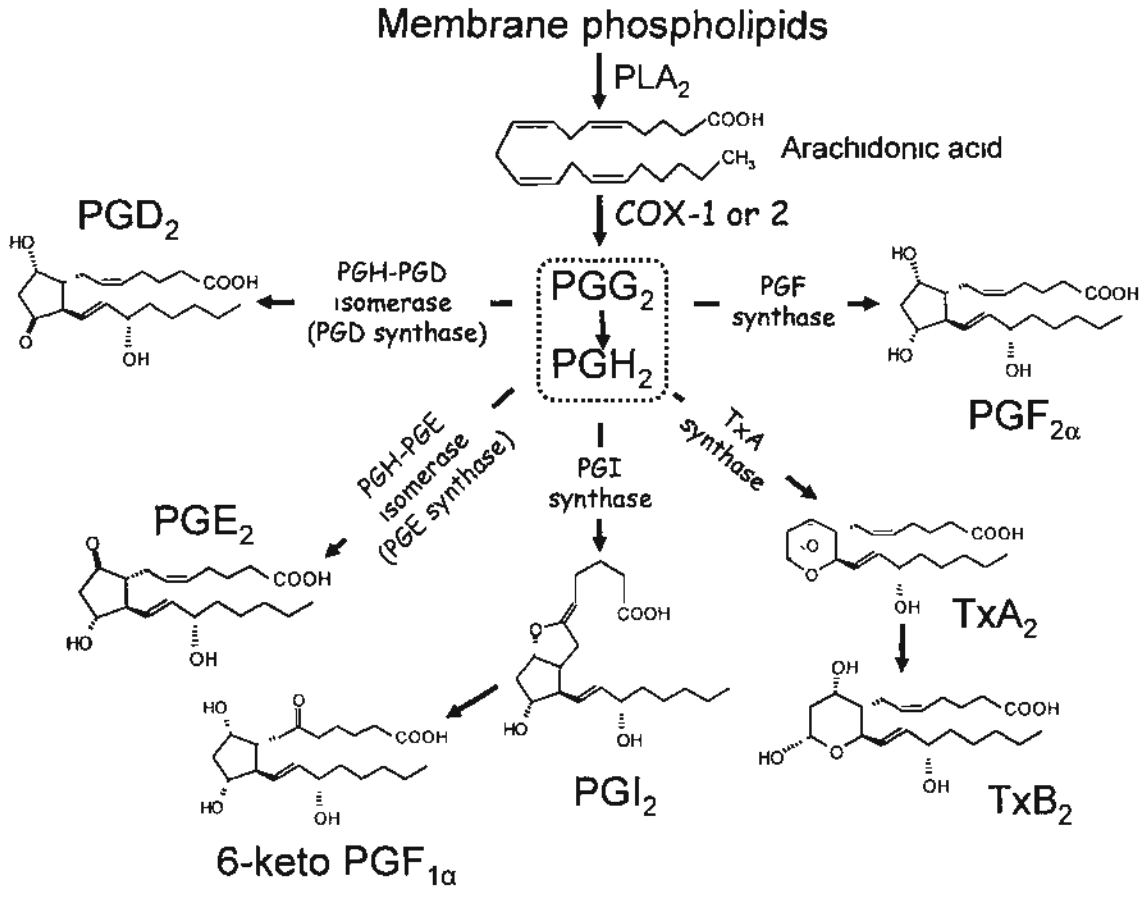

FIGURE 1.2

Arachidonic acid metabolism via cyclooxygenase (COX). Membrane phospholipids are converted to arachidonic acid by phospholipase A_2 (PLA_2). COX-1 or COX-2 then utilizes arachidonic acid as the substrate to generate prostaglandin (PG) G_2 and PGH_2, which is further transformed into various PGs and thromboxane A_2 (TxA_2) under the action of respective synthases. (Modified from Botting, 2004)

1.3.3 COX-independent release of isoprostanes

Utilizing the same substrate as COX, isoprostanes are formed from arachidonic acid, however, via a free radical-catalyzed mechanism independent of COX activity in humans (Davi et al., 1997; Davi et al., 1999). Morrow et al. (1990) is the first to demonstrate the production of isoprotanes in human through peroxidation of arachidonic acid. Indeed, the level of isoprostanes has been taken as a reliable clinical biomarker for cardiovascular diseases with active modulatory effects on vascular tone. Isoprostanes such as 15-F_{2t}-IsoP cause potent TP receptor-mediated contractions in blood vessels *in vitro* and *in vivo*, and the response is enhanced with the removal of endothelium (Cracowski et al., 2001). Besides directly acting on the TP receptor, isoprostanes also stimulate the release of TxA_2 and endothelin-1 from endothelial cells (Daray et al., 2006; Fukunaga et al., 1995; Yura et al., 1999), which in turn might act as EDCFs.

1.3.4 Pre-requisites for endothelium-dependent contractions

Though different prostanoids are proposed to be EDCF candidates, the common pre-requisites for the appearance of endothelium-dependent contractions are the presence of the thromboxane-prostanoid (TP) receptor in the vascular smooth muscle cells and a source of extracellular Ca^{2+}.

1.3.4.1 TP receptor

TP receptor appears to be the common target for the released prostanoids as endothelium-dependent contractions are sensitive to TP receptor antagonism, even though each prostanoid has its own natural receptor. This may be attributed to a non-selective affinity of the TP receptor towards the structurally

similar prostanoids (Figure 1.3). Thus PGI_2, which conventionally acts on its IP receptor to produce a vasodilatory effect, activated the TP receptor resulting in endothelium-dependent contractions in the aortae of SHR (Gluais et al., 2005).

1.3.4.2 A source of extracellular Ca^{2+}

COX-1 and COX-2 are not Ca^{2+}-dependent enzymes, but cytosolic phospholipase A_2 (PLA_2) requires Ca^{2+} ions to convert membrane phospholipids to arachidonic acid as the substrate for COX. This explains why receptor-independent Ca^{2+} ionophore can also trigger prostanoid synthesis and endothelium-dependent contractions (Shi et al., 2008; Shi et al., 2007; Tang et al., 2007). A recent study from Wong et al. (2010a) further substantiates that Ca^{2+} influx is essential to endothelium-dependent contractions and store operated Ca^{2+} channel (SOCC) activated by Ca^{2+}-independent PLA_2 is involved.

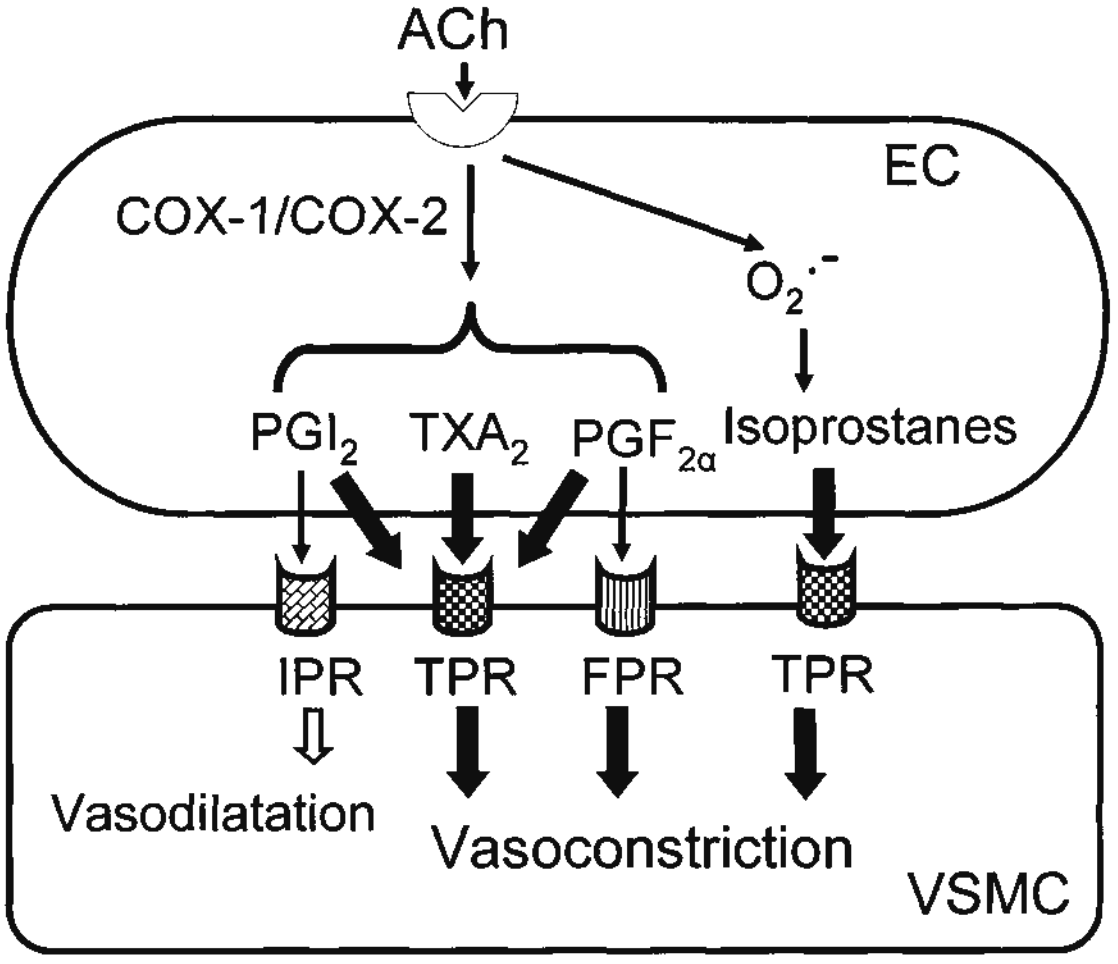

FIGURE 1.3.

Non-selectivity of TP receptor (TPR) to prostanoids. Except its natural agonist thromboxane A_2 (TxA_2), prostacyclin (PGI_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and isoprostanes can also activate TPR. ACh, acetylcholine; COX, cyclooxygenase; $O_2^{\cdot -}$, superoxide anion; IPR, prostacyclin receptor; FPR, FP receptor; EC, endothelial cell; VSMC, vascular smooth muscle cell.

1.3.5 Endothelium-dependent contractions in pathological states

Hypertension, diabetes and aging are the most prominent pathological states in which an increased COX activity is observed. The roles of COX-1 and COX-2 are indispensable in terms of which isoform is involved in which pathological states due to the heterogeneity of vascular beds and species.

1.3.5.1 Hypertension

As first demonstrated in the human brachial artery that non-selective COX inhibitor indomethacin improves vasodilatations in hypertensive patients (Taddei et al., 1997a), it is clear that COX is involved in the pathogenesis of hypertension. In an attempt to elucidate the mechanism of endothelium-dependent contractions in hypertension, several animals models are adopted, among which SHR are most commonly employed. SHR aortae exhibit impaired acetylcholine-induced endothelium-dependent relaxations and pronounced endothelium-dependent contractions as compared with the aortae from age-matched normotensive control Wistar-Kyoto rats (WKY) (Luscher and Vanhoutte, 1986). Indomethacin normalizes the attenuated endothelium-dependent relaxations in SHR aortae and mesenteric arteries (Luscher et al., 1990; Luscher and Vanhoutte, 1986), suggesting that the reduced relaxations to acetylcholine is probably not caused by a reduced production of EDRFs, rather due to an enhanced level of the simultaneously released EDCFs which counteract the vasodilatory effects of relaxing factors on vascular smooth muscle cells. Not only does acetylcholine stimulate greater indomethacin-sensitive endothelium-dependent contractions in SHR aortae, the contractions are also observed in arterial rings stimulated by serotonin (Auch-Schwelk and Vanhoutte, 1991), endothelin (Taddei and Vanhoutte, 1993), adenosine

triphosphate (Mombouli and Vanhoutte, 1993), and Ca^{2+} ionophore (Yang et al., 2004), suggesting that augmented endothelium-dependent contractions may not be related to an increased sensitivity of muscarinic receptors to acetylcholine in arteries from hypertensive animals. More serious investigation into which COX isoform is responsible for the contractions begins with the use of more selective COX inhibitors targeting either isoforms, with which endothelium-dependent contractions in the SHR are preferentially inhibited by the COX-1 inhibitor (Ge et al., 1995).

Oxygen radicals evokes an enhanced endothelium-independent contraction, which is sensitive to COX-1 inhibition and TP receptor antagonism in SHR aortae, while acetylcholine-induced endothelium-dependent contractions are also prevented by superoxide dismutase mimetic (Yang et al., 2002), implying a possible link between ROS and COX-1. ROS may be upstream of COX-1 and activates COX-1 activity, resulting in the release of prostanoids. Since oxygen radical-induced vaso-contractions are suppressed by the COX-1 inhibitor, it is unlikely that ROS or hydroxyl radicals *per se* act as an EDCF. Indeed, oxygen-derived free radicals do not actually trigger a release of prostaglandins (Auch-Schwelk et al., 1990). This may be due to a release of ROS-catalyzed but COX-independent isoprostanes which is yet to be confirmed. This phenomenon is explainable if ROS trigger the production of COX-1-derived ROS, which in turn catalyze the formation of isoprotanes acting on the TP receptor to cause vasoconstrictions. Of note, Tang et al. (2007) has demonstrated that in SHR endothelial cells an ACh-induced COX-mediated production of ROS is inhibited by indomethacin.

The release of contracting factor(s) can be agonist-specific even in the same vascular bed. While acetylcholine stimulates the release of PGI_2 in SHR

aortae (Gluais et al., 2005), endothelin increases the level of TxA_2 (Taddei and Vanhoutte, 1993) and Ca^{2+} ionophore elevates the level of both prostanoids (Gluais et al., 2006).

Though the aforementioned endothelium-dependent contractions in SHR aortae are mediated primarily by COX-1, evidence also exists that SHR endothelial cells can synthesize and liberate COX-2-derived prostaglandins. For example, endothelial COX-2-derived $PGF_{2\alpha}$ and 8-isoprostane underlie the augmented α-adrenoceptor-induced contractions in SHR arteries (Alvarez et al., 2005), and vasoconstrictions in response to *tert*-butyl hydroperoxide, an oxidative stress from lipid peroxidation, are COX-2-mediated (Garcia-Cohen et al., 2000). In deoxycorticosterone acetate salt-induced hypertensive rats, the COX-2 protein level is elevated and intra-peritoneal administration of a selective COX-2 inhibitor NS-398 attenuates hypertension (Adeagbo et al., 2005).

1.3.5.2 Diabetes

The impaired flow-mediated vasodilatation in the brachial artery is observed in subjects with visceral obesity and diabetes (Hashimoto et al., 1998; Ihlemann et al., 2002), and this has attracted the attention as to whether diabetic vasculopathies are attributed to an enhanced release of contracting factors. Simple experiments using isolated aortae from normal rabbit that are exposed to high glucose have already pinpointed a role of COX and the associated generation of vasoconstricitng prostanoids, TxA_2 and $PGF_{2\alpha}$ under hyperglycemic conditions (Tesfamariam et al., 1990). Early studies show a significant reduction of endothelium-dependent relaxations accompanied by augmented acetylcholine-induced contractions in the aortae of alloxan-induced diabetic rabbits, and that non-selective COX inhibition or TP receptor

antagonism restores the impaired relaxations and abolished the contractions. TxA_2 or its precursor PGH_2 (Tesfamariam et al., 1989), and 15-hydroxyeicosatetraenoic acid (15-HETE) are all proposed as the contracting factors in this preparation while the release of 15-HETE is also elevated in high glucose-treated aortae of normal rabbits (Tesfamariam et al., 1995).

Diabetic vasculopathies are not limited to aortae. Severely attenuated endothelium-dependent relaxations and enhanced acetylcholine-induced contractions are observed in mesenteric arteries of type 2-diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats compared with the age-matched control Long-Evans Tokushima Otsuka (LETO) rats. Acetylcholine-stimulated production of TxA_2 and PGE_2 is increased, accompanied by an elevated expression of COX-1 and COX-2, while the activity of endothelial NO synthase and protein expression of extracellular superoxide dismutase are reduced in the OLETF rats (Matsumoto et al., 2007). Chronic oral treatment with eicosapentaenoic acid to the OLETF rats reverses the imbalance between vasoconstrictions and relaxations, possibly through restoring the NO production and suppressing the COX-2 up-regulation via inhibiting extracellular signal-regulated kinase (ERK) and nuclear factor-κB (NF-κB) (Matsumoto et al., 2009b). Chronic treatment with metformin (an oral anti-diabetic drug), pyrrolidine dithiocarbamate (a thiol antioxidant) or ozagrel (thromboxane synthase inhibitor) improves the NO- and EDHF-mediated relaxations and reduced the EDCF-induced contractions by reducing the production of vasoconstrictive TxA_2, PGE_2, superoxide anion and normalizing NF-κB activity (Matsumoto et al., 2009a; Matsumoto et al., 2008; Matsumoto et al., 2009c). Mesenteric arteries from diabetic db/db mice at age over 12 weeks exhibit a greater transmural pressure-induced myogenic tone, which is sensitive to COX

inhibition and TP receptor antagonism, indicating a positive attribution from vasoconstricting prostanoid(s) (Lagaud et al., 2001).

Streptozotocin induces diabetes in rats by destroying β-cells in the pancreas. Peredo et al. (2006) examined the profile of the released prostanoids upon the induction of diabetic condition with streptozotocin, and found that the prostanoid release in rat aortae remains unaltered in the 1st month after streptozotocin treatment. As diabetes proceeds, PGI_2 production starts to decline from the 4th month onwards with an increased release of vasoconstricting metabolites in both the aortae and mesenteric arteries, indicating that long-term diabetic condition can lead to unfavorable modification in prostanoid production. In the femoral arteries of streptozotocin-treated rats, augmented Ca^{2+} ionophore-induced endothelium-dependent contractions are inhibited by indomethacin, TP receptor antagonist S18886 (terutroban) and thromboxane synthase inhibitor dazoxiben, suggesting a role of TxA_2; when diabetes progresses from 4 to 12 weeks after streptozotocin treatment, COX-1 expression is increased and EP-1 receptor antagonists are needed in addition to terutroban to prevent endothelium-dependent contractions (Shi et al., 2007), implying that PGE_2 may be another prostanoid generated by the dysfunctional endothelium. While the EDCF identity of PGE_2 is yet to be confirmed in streptozotocin-induced diabetic rats, Rutkai et al. (2009) has recently shown that a 4 day-oral administration of EP-1 receptor antagonist AH6809 markedly lowers the elevated systolic blood pressure in db/db mice and that AH6809 reduced the augmented pressure- and angiotensin II-induced tone of pressurized gracilis muscle arterioles from untreated db/db mice. Exogenous PGE_2 or selective EP-1 receptor agonist 17-phenyl-trinor-PGE_2 causes greater contractions in the arterioles and EP-1 expression is higher in the aortae in

db/db mice (Rutkai et al., 2009), which is indicative of a contributory role of PGE_2 in diabetic vascular dysfunction. In alloxan-induced diabetic female Wistar rats, relaxations to acetylcholine in the perfused mesenteric arteriolar bed are reduced, accompanied by an increased production of superoxide anion, and both harmful effects were ameliorated with the COX-2 inhibitor diclofenac (Akamine et al., 2006), suggesting that COX is not only involved in the formation of vasoconstricting prostanoid, $PGF_{2\alpha}$ in this case, but also actively exerting oxidative stress in vasculatures.

Renal pathophysiology is common in diabetic patients (Kamgar et al., 2006; Mogensen and Schmitz, 1988). Renal COX-2 expression and activity are elevated in both streptozotocin-induced type 1 diabetic rats and Zucker type 2 diabetic fatty rats, while the latter also exhibits a reduction in COX-1 expression and an increased urinary excretion of PGE_2 and TxB_2 (Komers et al., 2001; Komers et al., 2005).

1.3.5.3 Aging

The brachial artery NO-dependent vasodilatation is usually taken as an index in evaluating vascular function in humans. Even among normotensive subjects, the dilatation decreases with advancing age. Infusion of indomethacin remarkably potentiates the vasodilatation , indicating an increase release of COX-derived products which counteract the dilatory effect of NO during aging. Hypertension facilitates the early onset of aging in the vascular wall, such that in patients with essential hypertension, impaired vasodilatations that are responsive to COX inhibition are observed at a comparatively younger age (Taddei et al., 1997b). Over-production of COX-derived vasoconstrictors contributes prominently to the development of vascular dysfunction during aging.

Premature aging is also well documented in animal experiments involving spontaneously hypertensive rats (Abeywardena et al., 2002; Arribas et al., 1994; Fujii et al., 1993; Ibarra et al., 2006; Kung and Luscher, 1995).

Since the vasodilatory effect of NO diminishes with age owing to a reduction of its bioavailability, endothelium-dependent contractions are more pronounced and readily observed in aged animals. In the aortae of aged rats, particularly the spontaneously hypertensive rats, endothelium exerts less inhibition on the contractile responses towards 5-HT resulting in greater contractions. While indomethacin does not modify 5-HT-induced contractions in arteries from younger WKY and in endothelium-denuded arteries from young SHR and aged WKY, it prevents the contractions in arteries from young SHR and aged WKY with intact endothelium. Surprisingly, 5-HT-induced contractions in endothelium-denuded arterial rings from aged SHR are also sensitive to indomethcin, indicating that senescent vascular smooth muscles may be another source for COX-derived vasoconstricting factors (Ibarra et al., 2006).

Endothelium-dependent contractions to Ca^{2+} ionophore are significantly greater in the femoral arteries from aged rats when compared with their younger counterparts. While these contractions are eliminated by indomethacin, they can be partially inhibited by specific inhibitors to COX-1 and COX-2, suggesting a joint activation of both COX isoforms during aging. Of note, the protein level of COX-1 and COX-2 are both augmented, whereas the latter was actually undetectable in arteries from younger rats (Shi et al., 2008), which is indicative of an emerging role of COX-2 in aging. Genomic studies on endothelial cells show an increase in the mRNA levels of COX-1, COX-2, thromboxane synthase, PGF synthase, hematopoietic-type PGD synthase, and membrane PGE synthase-2 in aged rats (Tang and Vanhoutte, 2008), indirectly supporting the

exaggerated importance of the arachidonic acid metabolism through COX during aging.

1.4. Vascular inflammation and atherosclerosis

Risk factors such as hypertension, diabetes and hypercholesterolemia are well-known in the initiation of vascular inflammation due to a reduction of protection from the endothelium. Early vascular inflammation can be exaggerated by risk factors and promote the homing of immune cells to the inflamed vascular tissue resulting in atherosclerosis. Atherosclerotic lesions are mainly found in arterial bifurcation such as the aortic arch, carotid, iliofemoral and coronary arteries where the shear stress vectors keep changing (Badimon, 2001). The outer wall of the arterial bifurcations suffers from low shear stress and turbulence, such that the resident time for the interaction between microparticles and immune cells in the circulating blood with the dysfunctional endothelium is longer (Paszkowiak and Dardik, 2003), which may foster leukocyte trafficking and lipid uptake.

The very beginning step of vascular inflammation or atherosclerosis is the recruitment of immune cells to the endothelial layer by pro-inflammatory cytokines, which is followed by the tethering, rolling, flattening and transmigration of the immune cells across the endothelial layer.

1.4.1 Release of inflammatory cytokines

In healthy individuals, endothelium serves as a non-adherent barrier to leukocytes in the presence of the anti-inflammatory endothelium-derived NO and PGI_2. Risk factors such as hyperglycemia can lead to overproduction of ROS, which can activate PKC, mitogen-activated protein kinases (MAPKs) and

NF-κB to upregulate the expression of chemokines and adhesion molecules (Aronson, 2008; Bubici et al., 2006; Gloire et al., 2006; Oliveira-Marques et al., 2009). In addition, the pro-inflammatory cytokines can positively regulate their levels *per se* and induce the expression of others (Goldstein et al., 1996), leading to an acceleration and exaggeration of inflammation.

1.4.1.1 Monocyte chemoattractant protein-1 (MCP-1)

Chemokines (7-14 kDa) are a type of cytokines that is responsible for chemotaxis, a process by which cells migrate along the concentration of a chemotactic factor. They are subdivided into four families, XC, CC, CXC and CX_3C, with the presence of a conserved cysteine residue at the NH_2 terminus and the variable region "X" (Sprague and Khalil, 2009). Well-known examples included fractalkine (CX_3CL1), interleukin-8 (CXCL8) and RANTES (regulated on activation, normal T-expressed and secreted, CCL5). Of note, MCP-1 (CCL2) is by far the most extensively studied CC chemokine on its essential role in the initiation and progression of vascular lesions.

MCP-1 can be released by monocytes, endothelial cells and vascular smooth muscle cells upon pro-inflammatroy insults (Schober et al., 2004). It can promote the accumulation and infiltration of monocytes in the atherosclerotic lesions and even induced migration of smooth muscle cells into the neointima (Libby, 2000; Lo et al., 2005; Weber et al., 2004). These are confirmed by a substantiated increase in both the MCP-1 mRNA and protein levels in the atherosclerotic plaque compared to its minimal expression in normal vasculature (Nelken et al., 1991; Yla-Herttuala et al., 1991).

1.4.1.2 TNF-α

Elevated ROS levels in pathologies like diabetes can activate the NF-κB-mediated transcription of TNF-α (De Martin et al., 2000; Ma et al., 2003), which in turn accentuates the production of superoxide anion from endothelial cells and leukocytes through NADPH oxidase, xanthine oxidase and the uncoupled NOS that acutely depletes NO (Madge and Pober, 2001). As a vicious cycle, the exacerbated oxidative stress further boosts TNF-α production. By activating the IκB kinase-β-dependent NF-κB transcriptional cascade (Bu et al., 2005), TNF-α increases the expression of other pro-atherogenic cytokines such as interleukin-6, MCP-1, adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and E-selectins (Zhang et al., 2005), which are essential for leukocyte adhesion to the vascular wall. TNF-α can also activate matrix metalloproteinases (Hanemaaijer et al., 1993; Toborek and Hennig, 1993) and increase oxidation of low density lipoprotein (Fujiwara et al., 1998). TNF-α release by monocytes was elevated in patients with ischemic heart disease (Tentolouris et al., 2004) and its expression is found in the thrombus of ruptured plaque (Satoh et al., 2008).

1.4.1.3 Interleukin-6 (IL-6)

IL-6 is one of the biomarkers for evaluation of premature atherosclerosis that is closely related to diabetes and acts as an independent predictor of cardiovascular events in hypertensive patients (Kampoli et al., 2009). It mediates the development and destabilization of atherosclerotic plaque by inducing pro-inflammatory cytokines, activating phospholipases in the oxidation of lipoproteins and metalloproteinases in matrix degradation (Schuett et al., 2009; Yudkin et al., 2000), thus accelerating the early stage of atherosclerosis.

IL-6 is found to be expressed in the foam cells and smooth muscle cells in the cap and shoulder of the atherosclerotic lesions (Virani et al., 2008).

1.4.2 Expression of adhesion molecules

After homing to the lesion sites, leukocytes are to anchor on and transmigrate across the endothelium with the help from adhesion molecules.

1.4.2.1 ICAM-1 (CD54) and VCAM-1 (CD102)

Intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are induced in the endothelium in response to pro-inflammatory triggers (Gimbrone, 1999), though the former is also constitutively expressed in the peripheral vascular bed (Miller et al., 1995). ICAM-1 recognizes the leukocytes by β2 integrin and is involved in leukocyte arrest and migration (Rao et al., 2007; Springer, 1990); as for VCAM-1, the counter-receptors are α4β1 and α4β7 and functions in leukocyte arrest and rolling (Cybulsky et al., 2004; Rao et al., 2007). Once the leukocytes adhere to the endothelium, ICAM-1 and VCAM-1 are enriched in clusters and assist in leukocyte extravasation across the endothelium through either junctional or non-junctional transmigration (Barreiro et al., 2002; Carman and Springer, 2004; Shaw et al., 2004).

1.4.2.2 Selectins

Selectins are responsible for leukocyte rolling, which allows them to have close interaction with the activated endothelium to gain access and react with the chemokines presented on the surface of the endothelium (Huo and Xia, 2009). They consist of three subtypes, the P-, E- and L-selectin, and interact with ligands that are all cell-surface glycoproteins, among which the P-selectin

glycoprotein ligand-1 was best characterized (McEver and Cummings, 1997). P-selectin glycoprotein ligand-1 is constitutively present in all leukocytes but at different expression levels, and it is this difference that determines what type(s) of leukocytes are attracted to the vascular lesion (An et al., 2008; Huo and Xia, 2009; Wang et al., 2009).

1.4.3 Degradation of matrix proteins

Smooth muscles switch from quiescent and contractile phenotype to become synthetic and proliferative during vascular remodeling. They can migrate from the medial layer to the neointima by digesting the basement membrane under the action of matrix metalloproteinases (MMPs) (Okada et al., 1997; Pauly et al., 1994; Southgate et al., 1996). The same occurs in the atherosclerotic plaque (Yamada et al., 2008). Intimal thickening happens with the progression of atherosclerosis (Cizek et al., 2007; Nakashima et al., 2007), which is partially mediated by vascular smooth muscle cells migrating from the medial layer by the destruction of matrix proteins. These activated smooth muscle cells, along with the endothelial and infiltrated monocytes can secret MMPs that reduce the stability of the plaque (Galis et al., 1994; Nikkari et al., 1995). Enhanced matrix protein degradation results in an unstable plaque that can finally lead to plaque rupture and atherothrombosis (Hu et al., 2010).

1.4.3.1 Activation of MMPs

MMPs (matrixins), the endopeptidases, can digest extracellular matrix which is consists of matrix proteins like collagen, elastin, gelatins and casein in response to inflammatory stimuli (Page-McCaw et al., 2007). Activation of the metalloproteinases is essential for cell migration and in particular, it contributes

a major role in plaque instability, rupture and eventually atherothrombosis. Prominent expression of a variety of MMPs are demonstrated in the human atherosclerotic region, for example, MMP-1 (collagenase) at the fibrous cap and the carotid plaque shoulder (Back et al., 2010; Galis et al., 1994; Higashikata et al., 2006; Nikkari et al., 1995; Sukhova et al., 1999), MMP-2 and MMP-9 (gelatinases) in the fatty streaks, calcified fibroatheromas and occluded lesions (Back et al., 2010; Kieffer et al., 2001; Li et al., 1996), and MMP-7 (matrilysin) in the foam cells residing on the periphery of the lipid cores (Back et al., 2010; Halpert et al., 1996). Although each subgroup of matrixins has their own substrate, they exhibit a digestion capability on a broad spectrum of matrix components. Noteworthy, certain MMPs not only act on matrix proteins, but also cleave numerous pro-factors into vasoactive molecules, such as MMP-2 and MMP-9 cleaving the pro-TNF-α (Gearing et al., 1994) and pro-IL-1β (Schonbeck et al., 1998) into respective functional cytokines, processing big ET-1 to form the active vasoconstrictor (Fernandez-Patron et al., 1999), and truncating IL-8 to potentiate its potency (Van den Steen et al., 2000). Thus, upregulation of MMP expression and activity not only leads to direct matrix destruction, but also the exaggeration of inflammation.

1.4.3.2 MMP regulation by tissue inhibitor of metalloproteinases

Some MMPs are able to positively regulate the activity of themselves and other MMPs by cleaving the pro- forms into active forms. MMP-2 can activate pro-MMP-1, pro-MMP-2 and pro-MMP-7, MMP-9 cleaves pro-MMP-2, pro-MMP-9 and pro-MMP13 into respective functional matrixins and MMP-3 and -10 convert pro-MMP-1, pro-MMP-7, pro-MMP-8 and pro-MMP-9 into active forms (Back et al., 2010; Lijnen et al., 1998; Nakamura et al., 1998; Rauch et al., 2002).

Along with MMPs, endogenous tissue inhibitors of matrix metalloproteinases (TIMPs) are usually co-expressed, acting as a natural negative modulator on MMP activity. When the pro-MMPs are released, they are bind to the TIMPs until further activation. TIMPs can also lodge the MMPs at the substrate site and thus limit the MMP activity (Raffetto and Khalil, 2008; Shapiro and Senior, 1999). Four TIMPs are identified thus far, and they act in a specific manner to MMPs, such that TIMP-2, TIMP-3 or TIMP-4 acts on pro-MMP-2 and TIMP-1 or TIMP-3 with MMP-9 (Brew and Nagase, 2010).

1.5 Angiotensin II (Ang II)-induced vascular inflammation and remodeling

Ang II is regarded as the most potent end product in the renin-angiotensin system (RAS). It functions more than regulating hemodynamic balance in the sense that it also exerts a direct effect on a variety of vascular cells, including endothelial cells, smooth muscle cells and circulating immune cells. Activation of angiotensin II type 1 receptor (AT_1R) by Ang II not only cause vasoconstriction, but also mediates vascular inflammation and remodeling under pathological states.

1.5.1 RAS and Ang II production

The primary role of RAS is to control blood pressure and electrolyte balance by regulating sodium and water retention (Oparil and Haber, 1974a; b). Starting from the substrate angiotensinogen produced in the liver, the system operates by a stepwise cleavage of this 452 amino acid-long peptide into vasoactive angiotensin peptides. Angiotensinogen is first cleaved by renin released from the kidney into angiotensin (Ang) I (10 amino acids), which is subsequently

cleaved by angiotensin converting enzyme (ACE) and/or chymase (Arakawa and Urata, 2000) into the octapeptide Ang II. Though tissue-specific endopeptidases can further convert Ang I and Ang II to Ang (1-7) (Castro et al., 2005; Santos et al., 2003), the potent Ang II is usually regarded as the terminal effector in the RAS endocrine pathway. In addition to the systemic production, Ang II can also be locally generated with regard to the recent demonstration on the presence of RAS components in vasculature, heart and brain (Li et al., 2008).

1.5.1.1 Ang II and its receptors

Ang II acts on two receptors which exhibit angonistic effects of each other, the angiotensin II type 1 receptor (AT_1R) and type 2 receptor (AT_2R). Activation of AT_1R leads to Ca^{2+}/calmoudulin-mediated vasoconstriction and ROS production from the major oxidant-generating enzyme NADPH oxidase. In contrast, AT_2R appears to be vascular protective as mediated by an AT_2R-NO pathway (Tsutsumi et al., 1999). Activation of AT_2R inhibits Na^+-H^+ exchanges and causes intracellular acidification, which activates the production of bradykinin by kininogenase. Autocrine or paracrine activation of bradykinin B_2 receptor (B_2R) by this bradykinin in the endothelial cells increased eNOS activity and NO production, which mediates vasodilatation. In addition to vascular tone regulation, the ROS and NO released also regulate vascular inflammation in an opposite manner.

1.5.2 Vascular complications mediated by AT_1R activation

AT_1R and AT_2R shared similar affinities for Ang II (Berk, 2003), yet their expression levels are significantly different. AT_1R is the major receptor subtype expressed in adult mammalian cells while AT_2R expresses mostly during the developmental fetal stages and present in a low level for counter-balancing the effect of AT_1R (Berk, 2003; Goodfriend et al., 1996). In view of the predominant expression of AT_1R, physiological, and more importantly pathological, processes are mainly mediated by activation of AT_1R.

1.5.2.1 Endothelial activation and atherosclerosis

Ang II stimulates ROS production, which serve as the most important mediator that in vascular inflammation. Ang II augments the expression of adhesion molecules, chemokines and cytokines (Brasier et al., 2000; Cosentino et al., 2003; Gu et al., 1998; Han et al., 1999; Hernandez-Presa et al., 1997; Pastore et al., 1999; Pueyo et al., 2000; Tummala et al., 1999). Direct evidence by intravital microscopy shows that intraperitoneal injection of Ang II causes significant leukocyte adhesion in both mesenteric arterioles and postcapillary venules, of which the former is dependent on P-selectin and β2 integrins while the latter on P-selectin, β2 and α4 integrins. The expression of adhesion molecules P-selectin, E-selectin, ICAM-1 and VCAM-1 is elevated in both arterioles and venules (Alvarez et al., 2004). While Pueyo et al.(2000) showed that the VCAM-1 expression in rat aortic endothelial cells is mediated through NF-κB activation by Ang II-induced mitochondria-derived ROS, Costanzo et al. (2003) found that redox-sensitive p38 MAPK in addition to NF-κB are involved in the activation of the human umbilical vein endothelial cells (HUVEC). Ang II releases a variety of CC chemokines and cytokines in recruiting leukocytes to

the endothelium, such as MCP-1, RANTES and macrophage inflammatory protein-1α (Mateo et al., 2006). Activation of AT_1R upregulates lectin-like oxidized low density lipoprotein (oxLDL) receptor-1 (LOX-1) in the cultured endothelial cells from human coronary arteries, thus promoting oxLDL uptake, which in turn exaggerates the oxLDL-induced oxidative stress (Li et al., 1999). Ang II up-regulates plasminogen activator inhibitor type 1 (PAI-1) in the endothelial cells (Kerins et al., 1995; Vaughan et al., 1995), which regulates thrombosis and its elevated levels has been reported in myocardial infarction and atherosclerosis. Genetic ablation of AT_1R ($AT_{1a}R^{-/-}$) and pharmacological inhibition of AT_1R by olmesartan markedly attenuate the plaque formation in ApoE deficient mice (Fukuda et al., 2008), suggesting a key role of AT_1R activation in atherogenesis. Pronounced elevation of ACE expression and Ang II is demonstrated in the human atherosclerotic lesions (Diet et al., 1996; Hoshida et al., 2001; Schieffer et al., 2000).

1.5.2.2 Vascular hypertrophy and hyperplasia

Ang II *per se* can act as a growth factor, and induce the expression of other growth factors such as transforming growth factor-β1, insulin-like growth factor and platelet-derived growth factor, stimulating vascular hypertrophy and proliferation (Itoh et al., 1993; Naftilan et al., 1989). Ang II activates MMPs and modify matrix protein composition (Dzau, 2001; Takagishi et al., 1995), thus allowing migration of smooth muscle cells. Ang II-induced cardiac hypertrophy is mediated by an up-regulation of MCP-1, which promotes the differentiation and migration of the bone marrow-derived CD34(+)/CD45(+) fibroblasts (Haudek et al., 2010). Ang II stimulates superoxide anion production by cytochrome *P*450 1B1, which mediates the migration of vascular smooth muscle cells (Yaghini et

al., 2010). In rats exhibiting chronic heart failure after experimental autoimmune myocarditis, angiotensin receptor blocker telmisartan significantly attenuated cardiac fibrosis, hypertrophy and transforming growth factor-β1; elevated levels of IL-6, MCP-1, MMP-2 and MMP-9 are also suppressed by the telmisartan treatment (Sukumaran et al., 2010). Cytoskeleton is actively involved in the adaptive changes during vascular remodeling by conveying mechanical stimulations and signal transduction. Ang II appears to alter the cytoskeletal proteins in pathological remodeling by the activation of Rho/Rho kinase pathway (Wesselman and De Mey, 2002).

1.5.2.3 Plaque instability

Erosion of fibrous cap or plaque rupture is a result from the net destruction of matrix proteins mediated by MMPs, leading to coronary thrombosis and myocardial infarction (Newby, 2005). Ang II upregulates the expression of MMPs (Chen et al., 2004; Galis and Khatri, 2002; Luchtefeld et al., 2005) and plasminogen activator inhibitor-1 (PAI-1) (Vaughan et al., 1995), which disturb the fribrinolytic balance and destabilize the advanced atherosclerotic plaque. Study from Schieffer et al (2000) showed the co-localization of Ang II, AT_1R, ACE and IL-6 in the macrophage accumulated area of the atherosclerotic plaque in human coronary arteries, and that Ang II stimulates the release of IL-6 in cultured smooth muscle cells, indicating a potential contributory role of Ang II in the inflammation and progression of plaque rupture. In an accelerated atherosclerotic model using apoE deficient mice with ligation of the common left carotid artery, Ang II causes the formation of unstable plaques, which exhibit greater intimal and foam cell area, upregulated MMP-2 activity, MCP-1 production and VCAM-1 expression (da Cunha et al., 2006).

1.6 COX-2 and prostanoid-mediated vascular inflammation

Though COX-2 is shown to be constitutively expressed in human pulmonary and renal arteries, its level can be significantly up-regulated due to its highly inducible nature. Conventionally, COX-2 has been regarded as a source of PGI_2 in cardiac tissue and endothelial cells, which confers protection to the cardiovascular system by preventing platelet aggregation and thrombosis. Recent advances, however, have shown an emerging role of COX-2 and the downstream prostaglandin synthases as pro-inflammatory mediates, which not only promotes vasoconstriction by releasing constrictors, its metabolites can also actively participate in vascular inflammation and remodeling.

1.6.1 Induction of COX-2 by various stimuli

COX-2 can be induced in response to both intracellular or extracellular stimulations, including lipopolysaccharides, growth factors (insulin-like growth factor), pro-inflammatory cytokines (TNF-α and IL-1β) and vasoactive peptides (endothelin-1) (Xie et al., 1991), and its level is highly upregulated in cells that are involved in inflammation such as endothelial cells, vascular smooth muscle cells, and immune cells like monocytes and macrophages. Its expression level, on the contrary, is negatively regulated by glucocorticoids and anti-inflammatory IL-10 (Crofford, 1997; Hinz and Brune, 2002; Xie et al., 1991).

The promoter of the immediate-early gene of COX-2 contains a TATA and several enhancer elements including cyclic AMP response element (CRE), AP-1 regulatory element complex and binding sites for transcriptional factors such as NF-κB (Tazawa et al., 1994). Recruitment of which of these transcriptional pathways depends on cells types, the initiating cellular stimuli and the immediate upstream triggers of the transcriptional event, of which mitogen-

activated protein kinase (MAPK), ROS and PKC have been most extensively examined for COX-2 transcription.

1.6.1.1 Role of MAPKs

MAPKs are serine-threonine kinases that are activated to mediate cellular processes like proliferation, differentiation and transformation. Three major mammalian MAPKs are identified - p38 MAPK, the extracellular signal-regulated kinase (ERK) and c-Jun NH_2-terminal kinase (JNK), which is also termed stress-activated protein kinase (SAPK). Each of them consists of several isoforms, p38-α, β, δ and γ, ERK1-8 and JNK1-3. A complete signaling pathway of each of these MAPK axis consist at least two other additional kinase components, an MAPK kinase kinase (MAPKKK) and an MAPK kinase (MAPKK). Upon stimulation, the uppermost MAPKKK is activated, which causes a stepwise phosphorylation of latter protein kinases, the MAPKK and eventually the downstream MAPK (Kim and Choi, 2010).

Each of these pathways can be trigger to mediate inflammatory response, though, recruitment of which depends on what the inflammatory trigger is. Even the triggers are of the same type of stimuli, the signaling pathways involved can be different. Cytokines such as IL-1 or interferon-γ (IFN-γ) induces COX-2 expression, which involves p38 MAPK activation while ERK1/2 plays no role (Caivano and Cohen, 2000; Tsatsanis et al., 2006; Wu et al., 2005). In contrast, TNF-α-induced COX-2 expression is heavily dependent on ERK1/2 and NF-κB (Tsatsanis et al., 2006). As for growth factors, the COX-2 expression induced by insulin-like growth factor is mediated through the activation of PI3K and ERK while p38 MAPK does not participate; on the contrary, transforming growth factor and epidermal growth factor recruit p38 MAPK in addition to ERK1/2 and

PI3K (Chun and Surh, 2004; Tsatsanis et al., 2006). This indicates the complexity of MAPK signaling pathways in regard to even the same inflammatory factor.

1.6.1.2 Role of ROS

ROS are potent triggers to the activation of transcriptional signaling pathways and their levels are found to be elevated in hypertension, hypercholesterolemia and diabetes by a variety of vasoactive peptides, growth factors and cytokines. ROS can indirectly activate serine/threonine kinase by inhibiting the protein serine/threonine phosphatase through the oxidization of the Fe(II)-Zn(II) center of the active site (Rhee et al., 2000). It can also directly stimulate ERK activity by activating G_i and G_o (Nishida et al., 2000). NF-κB is activated by ROS-mediated degradation of IκB (Collins and Cybulsky, 2001). Superoxide anion and hydrogen peroxide can activate AP-1 by inducing the expression of early response gene such as *c-fos* or *c-jun* (Rao et al., 1996). Taken in conjuncture, oxidative stress represents a universal trigger to transcription signaling pathways.

In vascular cells, the COX-2 expression can be highly regulated by intracellular oxidative stress. Human neutrophil peptides released from polymorphonuclear leukocytes induce the expression of COX-2 and endothelin-1 through oxidative stress and activation of p38 MAPK, ERK1/2 and NF-κB (Syeda et al., 2006). TNF-α-induced COX-2 expression in HUVEC is mediated by NADPH oxidase- and mitochondrial-derived hydrogen peroxide through inhibition of serone/threonine phosphatase and activation of p38 MAPK (Eligini et al., 2009). ROS-dependent increase in COX-2 expression prevents macrophage apoptosis (von Knethen et al., 1999) and promotes monocyte

differentiation to macrophage (Barbieri et al., 2003). In addition, RAS activation results in ROS generation, which upregulates cortical COX-2 in the kidney of hypertensive salt-sensitive rats (Jaimes et al., 2008). IL-1, TNF-α and LPS-induced COX-2 expression in meseangial cells is also mediated by ROS (Feng et al., 1995). Levels of ROS and expression of NADPH oxidase subunit $p22^{phox}$ are elevated in atherosclerotic plaque samples from patients undergoing directional coronary atherectomy (Terashima et al., 2007).

1.6.1.3 Role of PKC

PKC consists of a number of isoforms that are subdivided into three main classes, conventional, novel and atypical. Activation of conventional PKCs (α, β1, β2, and γ) requires Ca^{2+}, DAG (endogenous PKC activator) and phospholipid (phosphatidylserine), while novel PKCs (δ, ε, η, and θ) are activated by DAG and phosphatidylserine but not Ca^{2+}. Atypical PKCs (ζ and ι/λ) need neither Ca^{2+} nor DAG (Jaken, 1996).

Activation of PKC has been implicated in diabetic complications. Various studies have pointed to a potential role of PKC in vascular COX-2 expression with the use of pharmacological inhibitors with limited selectivity, and few have exactly defined which isoform(s) is involved and the signaling sequence between PKC and MAPKs. Numerous studies have demonstrated an upregulation of COX-2 expression by a non-selective exogenous PKC activator, phorbol 12-myristate 13-acetate (PMA) in human endothelial cells (Eligini et al., 2005; Miralpeix et al., 1997). PKC inhibitor for a broad spectrum of isoforms (GF109203X) only marginally inhibits Ang II-induced COX-2 expression in cultured human aortic vascular smooth muscle cells (Hu et al., 2002), but completely suppresses the elevated COX-2 mRNA levels evoked by endothelin-

1 in rat aortic endothelial cells (Sugiyama et al., 2004). Another less specific kinase inhibitor of PKC, chelerythrine, also inhibits hyperglycaemia induced COX-2 upregulation, ROS formation and NO reduction in human aortic endothelial cells (Aljofan and Ding, 2010; Costanzo et al., 2003). Acrolein, a toxin in tobacco, induces COX-2 expression and PGE_2 release by p38 MAPK and PKC_δ in HUVEC (Park et al., 2007). Phosphatidylcholine specific phospholipase C and D-induced COX-2 expression in macrophages is also PKC-dependent (Lee et al., 2003).

1.6.2 Localization of COX-2 in inflamed and atherosclerotic tissue

Recent studies have shown that a selective COX-2 inhibitor celecoxib can attenuate the adhesion of colon cancer cells to vascular endothelial cells through suppressing ICAM-1 and VCAM-1 expression, indicating a pro-inflammatory role of COX-2 in endothelial activation, perhaps of a similar relevance to the metastasis of cancer cells (Dianzani et al., 2008). The major component of cigarette smoking, nicotine, induces the expression of COX-2 and ICAM-1 in HUVEC (Zhou et al., 2010). Lipopolysaccharide induces Egr-1/NF-κB-dependent expression of COX-2 and microsomal PGE synthase 1 in macrophages (Diaz-Munoz et al., 2010). Indeed, unlike COX-1, COX-2 expression is minimal in vascular tissue from normal subjects but highly induced in the atherosclerotic plaques co-localized with various inflammatory factors (Burleigh et al., 2005; Cipollone et al., 2001; Schonbeck et al., 1999). COX-2 may play a role in the progression of atherosclerosis by activation of chemotaxis of leukocytes and migration of smooth muscle cells, alteration of vascular permeability, mediation or amplification of cytokine signaling. COX-2 enhances oxLDL- and IL-1-induced monocyte adhesion to activated endothelial cells.

Inflammatory cytokines IL-1, TNF-α or growth factors like transforming growth factor-β can induce COX-2 expression in monocytes (Crofford, 1997). COX-2 expressing macrophages and neutrophils can release PGE_2 (Cipollone et al., 2004), which can activate MMP-2 and MMP-9, causing collagen breakdown, destabilizing the plaque with weakened cap and shoulder, resulting in plaque rupture (Cipollone and Fazia, 2006; Libby et al., 1996).

1.6.3 Controversies of COX-2 inhibitors

Acute infusion of indomethacin augments vasodilatation in hypertensive and aged patients, and a substantial amount of experimental data on animals indicate indispensable roles of COX-1 and COX-2 in improving endothelium-dependent relaxations and inhibiting the exaggerated vasoconstrictions. The concept that chronic COX inhibition may correct the vascular imbalance of endothelium-derived vasoactive factors, however, is antagonized by a number of large randomized clinical trials particularly with the use of specific COX-2 inhibitors, rofecoxib and diclofenac (McGettigan and Henry, 2006; Schror et al., 2005). Instead of conveying beneficial effects on vascular function, COX-2 inhibition causes adverse cardiovascular events, including the increased incidence of thrombosis, myocardial infarction and stroke, leading to the withdrawal of Vioxx from the market in 2004 (Marnett, 2009). Explanation of such detrimental effects of COX-2 inhibition is attributed to the general belief that endothelium-derived COX-2-mediated production of PGI_2 is suppressed while the level of COX-1-mediated TxA_2 in platelets is unaltered, hence favoring platelet activation and aggregation.

Intriguingly, clinical trials do not demonstrate an increased cardiovascular risk in patients treated with another selective COX-2 inhibitor, celecoxib

(Silverstein et al., 2000; White et al., 2007). Retrospective studies from Cho et al. (2003) and meta-analysis from Aw et al. (2005) actually show that patients treated with celecoxib have a lower risk of hypertension development and a slightly decreased systolic blood pressure relative to those treated with rofecoxib. Patients taking celecoxib have a significantly reduced risk for nonfatal myocardial infarction as compared with those not taking any NSAIDs or using rofecoxib (Kimmel et al., 2005). In patients with intermittent claudication associated with peripheral arterial disease, 1-week celecoxib treatment enhances flow-mediated dilatation in the brachial artery and reduced the levels of inflammatory biomarkers such as high-sensitivity C-reactive protein (Florez et al., 2009). These clinical findings are supported and perhaps, accounted by the therapeutic effects of celecoxib against vascular dysfunctions in animal models (Abdelrahman and Al Suleimani, 2008; Cheng et al., 2002; Hermann et al., 2003). It is clear that inhibition of COX-2 activity may not necessarily correlate with the adverse cardiovascular effects; rather, COX-2 inhibitors may be of potential to treat vascular complications in diabetes and hypertension. It is possible that the side effects of rofecoxib are related to its direct inhibition on PGIS (Griffoni et al., 2007). Using the production of PGI_2 (measured in form of its metabolite 6-keto $PGF_{1\alpha}$) as an indicator of the COX-2 activity with PGH_2 supplied exogenously to by-pass the action of COX-2, PGIS activity in the HUVEC has been studied. While non-selective NSAIDs, acetylsalicylic acid and naproxen, and selective COX-2 inhibitor celecoxib poses no significant reduction in the PGIS activity even at a concentration as high as 0.1 mmol/L, rofecoxib suppresses ~30% of the PGIS activity at 0.1 nmol/L, a concentration that does not inhibit COX-2. Rofecoxib inhibition of PGIS activity is even up to ~60% at 10 μmol/L. For a patient taking a single dose of rofecoxib (25 mg), the

plasma level of rofecoxib is around 0.05 to 1 μmol/L, it is thus likely that PGIS activity may be suppressed even within the therapeutic dosage. Inevitably, the use of coxibs should be reviewed extensively with confirmation on whether the inhibitors impose direct suppression of PGI_2 production via PGIS.

1.7 Justification, long term significance and objectives of the present studies

Normal vascular tone is maintained by a delicate balance between EDRFs and EDCFs. When the balance is tilted towards EDCFs due to the over-production of vasoconstrictors and/or diminished production or bioavailability of EDRFs, endothelial dysfunction occurs resulting in exaggerated vasoconstrictions. COX represents a major generator of EDCFs and previous studies have focused on the investigation of the COX isoforms involved in hypertension and diabetes. Limited information is available on how COX, in particular COX-2, in the regulation of normal vascular tone. If EDRFs and EDCFs co-exist, endothelium-dependent contractions should be observable when EDRFs are inhibited even in the healthy vasculature. Using the aortae from young hamsters with constitutive COX-2 expression in the endothelium, the present study aimed to study the role of COX, in particular COX-2, in endothelium-dependent contractions in the healthy state, the signaling pathways and prostaglandin receptor involved and the possible EDCF candidate. Results from the present study should provide novel insights into whether the generally neglected COX-2 isozyme confers a housekeeping function of vascular tone regulation in normal vasculature.

Aging represents an independent risk factor for the development of cardiovascular diseases such as hypertension. A positive role of COX has been

demonstrated in hypertensive animal models in which premature aging in vascular beds occurs. It, however, remains largely unknown on the role of COX in healthy aging, and which component of the vascular wall, endothelium and/or the smooth muscle, contributes to the release of EDCFs. Extended from the study on the aorta of the young hamster, COX-2-mediated endothelium-dependent contractions and the associated release of prostaglandins are investigated in the aged hamsters. The results can help to better define the emerging importance of COX-2 and its metabolites in healthy aging and provides novel information regarding the etiology and potential drug targets in amelioration of vascular dysfunction in the aging vascular bed.

In addition to vascular tone regulation, COX-2, being a highly inducible enzyme by pro-inflammatory factors, also actively participates in vascular inflammation and atherogenesis. RAS activation has been implicated in hypertension and diabetes. However, there is a missing link between Ang II and COX-2 particularly in the endothelial cells, which is the key contributor to vascular dysfunction, and remains to be explored concerning whether COX-2 can acts as an immediate downstream effector upon AT_1R activation and mediates the pathogenic effects of Ang II. Using primary rat endothelial cells which minimally express COX-2 in quiescent states and tissue culture of human renal and mesenteric arteries, the present study investigated the relationship between Ang II stimulation and COX-2 expression, the intracellular signaling pathways that links up these pro-inflammatory factors and the consequence of COX-2 up-regulation in terms of vascular inflammation. In view of the controversies on the clinical use of COX-2 inhibitors in suppressing inflammatory responses due to inhibitor selectivity and the janus biological roles of COX-2 in the vasculature, the present study provides original molecular basis

on how the activity of pro-inflammatory COX-2 and the downstream COX-2-derived inflammatory mediators can be regulated through curtailing the COX-2 expression by selectively targeting the signaling proteins involved in COX-2 upregulation.

To achieve the aforementioned objectives, I have investigated:

1. The novel role of COX-2 and the major physiological COX-2-derived metabolite in endothelium-dependent contractions in the aorta of young hamsters and the relevance of this pathway in human renal arteries;
2. The increasing impact of COX-2 in the aging vasculature;
3. The cellular signaling cascades linking Ang II and COX-2 in the endothelial cells and whether Ang II-induced inflammatory response is mediated by the upregulated COX-2 expression.

Chapter II

Methods and Materials

The studies were approved by the Animal Experimentation Ethics Committee, Chinese University of Hong Kong. This investigation 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). Human renal arteries were obtained during surgery after informed consent from patients.

2.1 Animals

Male Syrian golden hamsters and Sprague-Dawley rats supplied by the Laboratory Animal Services Centre, the Chinese University of Hong Kong were employed in my studies. The animals were housed at constant temperature (21 ± 1 °C) under a 12-hour light/dark cycle and had free access to a standard chow diet and water.

2.1.1 Induction and treatment of Ang II-infused hypertensive rat model

2.1.1.1 Osmotic pump implantation

Sprague-Dawley rats weighing ~250 g were allowed to stabilize one day upon its arrival. The rats were anesthetized with 37.5% ketamine plus 25% xylazine in normal saline (2 ml/kg). Osmotic minipump (ALZET, model 2ML2, Alza Pharmaceutical, Palo Alto, CA, USA) filled with either angiotensin II (Ang II) or the vehicle (normal saline) was implanted subcutaneously in the dorsal region. Ang II was infused at 0.7 mg/kg/day for 9 days.

2.1.1.2 Chronic drug treatment

Four Ang II-infused rats were assigned to chronic treatment with losartan (10 mg/kg/day, dissolved in water) by oral gavage. They were pre-treated one day before osmotic minipump implantation and the treatment continued throughout the infusion period.

2.1.1.3 Blood pressure monitoring

Systolic blood pressure was regularly monitored throughout the infusion period with the tail-cuff electrosphygmomanometer system (PowerLab data acquisition system with LabChart software; ADInstruments, Sydney Area, Australia). The rats remained conscious and quiescent during the measurement. The average of three to four readings was taken as systolic blood pressure of each rat.

2.2 Human artery preparation

Human renal arteries were obtained from patients undergoing surgeries for transitional cell carcinoma or renal cell carcinoma, and small mesenteric arteries from patients with colon cancer. Renal arterial samples were respectively divided into groups of patients with diabetes (fasting plasma glucose level ≥ 7.0 mmol/L), hypertension (systolic and diastolic blood pressure >140 mmHg and >90 mmHg respectively) or neither. For small mesenteric arteries, only those distant from the tumor of the non-hypertensive and non-diabetic patients were used. The patients aged between 40 to 85 years and informed consent for research purposes was obtained.

The arteries were kept in saline and arrived on ice. The arteries were placed in ice-cold Krebs-Henseleit solution (KHS) and dissected free from adventitial adipose tissues. For renal arteries, they were cut into 2-3 mm in

length, either mounted for isometric force measurement in organ baths or preserved in 4% paraformaldehyde for immunohistochemistry. As for the mesenteric arteries, they were dissected in sterile phosphate-buffered saline (PBS) for tissue culture experimemts. The arteries were incubated for 24 h with or without Ang II and pharmacological inhibitors. At the end of the culture experiments, the arteries were preserved in OCT (optimum cutting temperature) compound for cryosectioning and biomarker detection by immunofluorescence.

2.3 Isometric force measurement

Animals were euthanized by carbon dioxide inhalation. Thoracic aortae or renal arteries were excised and dissected in KHS. Following the removal of periadventitial fat, the arteries were cut into segments ~1.0-1.3 mm in length. Each segment was suspended between two stainless steel wires in the chambers of a Multi Myograph System (610M, Danish Myo Technology A/S, Aarhus N, Denmark) for the measurement of isometric force. Each chamber was filled with 5 mL-KHS aerated with 95% O_2 and 5% CO_2 and maintained at 37°C. The rings were stretched to a previously determined optimal resting tension of 10 mN for hamster aorta and 2 mN for rat renal arteries. Thirty minutes after setting up the preparation, they were contracted with 60 mmol/L KCl to test the vessel's contractility, and then washed in normal KHS, finally allowed to equilibrate for 30 min. In some rings, the endothelium was removed mechanically by rolling the luminal surface with a stainless steel wire. Isometric force measurement on human renal arteries was performed in 10 mL-organ baths similar to the settings using myograph. The arteries were allowed to stabilize at 25 mN before commencement of experiments.

2.3.1 Experimental protocols

2.3.1.1 Endothelium-dependent contractions in the hamster aorta

To visualize endothelium-dependent contractions, aortic rings with endothelium were exposed for 30 min to 100 µmol/L N^G-nitro-L-arginine methyl ester (L-NAME) in order to eliminate the relaxant effect of endothelium-derived nitric oxide (NO) prior to the cumulative addition of acetylcholine (ACh, 0.1-10 µmol/L). This contraction was absent in rings without endothelium. Changes in isometric tension to $PGF_{2\alpha}$ (1-30 ng/mL), PGI_2 (3.7-370 ng/mL), cicaprost (10-100 ng/mL) in the presence of L-NAME were also recorded. The effects of various inhibitors and antagonists (e.g., COX-1 and COX-2 inhibitors, TP receptor antagonists) or NO donors (SNP, SIN-1) were tested on ACh- or $PGF_{2\alpha}$-induced contractions following a 30 min-incubation with each drug. Specificity of COX-2 inhibitors and TP receptor antagonists was tested against contraction induced by 60 mmol/L KCl or 50 nmol/L U46619.

2.3.1.2. ACh- or $PGF_{2\alpha}$-induced contractions in human renal arteries

The arteries were pre-incubated with L-NAME (100 µmol/L) for 30 min, upon which cumulative doses of ACh (1-100 µmol/L) were added. To examine the role of TP receptor in the contractions, TP receptor blocker was added when the contractions reached a plateau. Further investigations on the roles of TP and FP receptors in $PGF_{2\alpha}$-induced contractions were performed by pre-contraction of $PGF_{2\alpha}$, followed by the addition of either TP or FP receptor antagonist. Reduction of the $PGF_{2\alpha}$ tone within 30 min was determined. The two receptor antagonists were also pre-treated with L-NAME to observe their inhibitory effects on $PGF_{2\alpha}$-induced contractions.

2.3.1.3. Endothelium-dependent relaxations in rat renal arteries

Endothelium-dependent relaxations were evaluated in arteries from control rats and Ang II-infused rats with or without concomitant oral administration of losartan. The renal arteries were pre-contracted with phenylephrine (0.3 μmol/L) and relaxed with cumulative addition of ACh (0.003-10 μmol/L). Acute effects of celecoxib (3 μmo/L) and sc-560 (0.3 μmol/L) were tested on the relaxations in renal arteries from Ang II-infused rats.

2.4 *In situ* endothelial cell $[Ca^{2+}]_i$ imaging

A calcium imaging technique was employed to visualize real-time changes in intracellular calcium levels, $[Ca^{2+}]_i$ in native endothelial cells of the intact hamster aorta (Leung et al., 2006). Isolated aortic rings with endothelium were labeled for 60 min at 21°C in a solution containing 10 μmol/L Fura-2 AM, 0.025% pluronic F-127, and 1 mmol/L probenacid (to prevent Fura-2 secretion). Thereafter, extracellular Fura-2 AM was removed and each ring was cut open longitudinally and pinned *en face* to a block of silicone elastomer, which was fixed on the base plate of a custom-made perfusion chamber. The chamber was filled with KHS, sealed by a cover glass smeared with high vacuum grease (Dow Corning®, Structure Probe, Inc., West Chester, PA, USA) and then fixed by screws. The chamber was perfused with pre-warmed KHS for 20 min at 1 mL min^{-1} to allow intracellular Fura-2 AM to be cleaved into active Fura-2 by intracellular esterases.

The specimen was illuminated (Polychrome IV light source) on the stage of an IX70 Olympus microscope, fitted with a 20X Olympus water immersion objective. The Fura-2 loaded tissue was excited alternately at 340 and 380 nm, and images of the respective 510-nm emissions were collected at one-second

intervals using the MetaFluor v4.6 software (Universal Imaging Corp., West Chester, PA, USA). The emitted light was transmitted to a collecting device and then to a cooled charge coupled device (CCD) camera. Illumination through the Polychrome IV light source and acquisition by the CCD camera were controlled by MetaFluor software v4.6. Video frames containing images of cell fluorescence were digitized at a resolution of 512 horizontal × 480 vertical pixels. Imaging analysis was performed using a MetaFluor imaging system. After background subtraction, the fluorescence ratio (F340/F380) was obtained by dividing, pixel by pixel, the images at 340 nm and 380 nm. Changes in this ratio reflected changes in $[Ca^{2+}]_i$ and the ratiometric method eliminated potential artifacts caused by variations in cell thickness, intracellular dye distribution or photobleaching.

2.5 Primary culture of rat aortic endothelial cell

Endothelial cells were freshly cultured from rat thoracic aortae as described earlier (Huang et al., 1999). Under aseptic conditions, aorta was excised free and trimmed of perivascular adipose tissue and longitudinally cut open in the ice-cold sterile PBS. The artery was then subjected to digestion in collagenase (Sigma, Type IA) at 37 °C for 15 min. Serum containing medium was added to quench the digestion and the mixture was centrifuged at 800 g for 15 min. The cells were re-suspended in RPMI 1640, supplemented with 10% fetal bovine serum (FBS) plus 1% penicillin/streptomycin (P/S) (GIBCO) and allowed to settle for 1 h upon which the medium was changed. The identity of the endothelial cells was confirmed by the positive immunocytochemical staining for an endothelial cell specific marker, PECAM-1. To avoid the influence of culture condition on endothelial cell phenotype, only cells from passage 1 were used in

the studies.

2.5.1. Experimental protocol

When the cells reached a confluence of ~80-90%, they were serum-deprived for 24 h. Ang II (100 nmol/L) was incubated with for 8 h unless otherwise stated. When used, inhibitors were pre-incubated with the cells for 30 min before the addition of Ang II.

2.6 Reverse-transcription polymerase chain reaction (RT-PCR)

Expression levels of COX-2 mRNA in rings with and without endothelium were detected by RT-PCR. The equipment was cleaned free of RNase either by autoclaving or wiping with RNase Away. The arterial rings were snap frozen in liquid nitrogen and homogenized, and mRNA was extracted using the Aurum total RNA Mini kit (BioRad, Hercules, CA, USA) according to manufacturers' instructions. The extracted RNA was reverse transcribed using the iScript™ cDNA synthesis kit (BioRad), and PCR was performed with *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) with thermal cycles of 5-min 95 °C, 30 cycles of 1-min 95 °C, 1-min T_m (melting temperature), 1-min 72 °C, finally followed by 6-min 72 °C.

Primers for PCR were COX-2 (216-bp) sense (5'-TGA TCC CCA AGG CAC GAA-3') and antisense (5'-ACC TCT CCA CCA ATG ACC TGA-3') (Valeille et al., 2005), GAPDH (171-bp) sense (5'-ACC CAG AAG ACT GTG GAT GG-3') and antisense (5'-CAC ATT GGG GGT AGG AAC AC-3'). Melting temperature for COX-2 and GAPDH primers were 60 °C and 57 °C, respectively. PCR products were run on 1.5% agarose gel in 1X TAE buffer at 80 V. Ethidium bromide-stained bands were visualized under UV illumination using FluorChem

(version 2.00, Alpha Innotech Corp., San Leandro, CA, USA).

2.7 Western blot analysis

2.7.1. Sample preparation

Snap frozen arterial tissues or cells finished with incubation protocols were homogenized or lysed in the ice-cold RIPA buffer with a cocktail of protease inhibitors. The lysates were allowed to incubate on ice for 30 min and were then centrifuged at 20,000 g for 20 min. Supernatants were saved and their protein concentrations were determined by the Lowry method (BioRad).

2.7.2. SDS-PAGE and electroblotting

Equal amount of protein of each sample from the whole cell lysates was aliquoted and kept on ice. After mixing with sample loading buffer containing 5% β-mercaptoethanol, the samples were denatured by boiling for 5 min and then resolved through the 7.5%, 10% or 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) together with the prestained size marker (Biorad). The stacking gel was run at 50 V while the separating at 125 V. The separated proteins were electrophoretically transferred to an Immobilon-P polyvinylidene difluoride (PVDF) membrane (Biorad) by wet transfer at 110 V for 70 min. The membranes were either blocked with 5% non-fat skimmed milk or 1% bovine serum albumin (BSA) dissolved in Tween-20 phosphate-buffered saline (PBST) with gentle shaking for 1 h at room temperature. The membranes were probed overnight at 4 °C with primary antibodies summarized in section 2.17. After 3 washes in PBST each for 5 min, the membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary IgG (Dako, Glostrup, Denmark) at a dilution of

1:3000 for 1 h at room temperature. The membranes were developed with an enhanced chemiluminescence detection system (Amersham ECL reagents; GE Healthcare, Waukesha, WI, USA) and exposed on X-ray films (Fuji). Densitometry was performed using documentation programs (FluorChem, Alpha Innotech Corp., San Leandro, CA, USA or GBOX-CHEM1-HR16, SynGene). GAPDH antibody (Ambion, Inc) was probed as a loading control.

2.8 Cell fractionation and translocation of PKC isoforms

Endothelial cells were incubated with 100 nmol/L Ang II for 1 min and quickly cooled on ice to quench cellular reactions. Inhibitors, when used, were added 30 min prior to the introduction of Ang II. Cytosolic and membranous proteins were extracted with ProteoExtract® Subcellular Proteome Extraction Kit according to manufacturer's instructions (Calbiochem). Protein concentration was determined with the Lowry method and equal amount of proteins were subjected to SDS-PAGE and electroblotting as aforementioned. The membranes were probed with different antibodies specific to particular PKC isoforms. Translocation was determined by comparing the intensity of the membranous fraction in cells under treatment relative to that of control.

2.9 PKC$_\delta$ knockdown with small interfering RNA (siRNA)

When primary rat endothelial cells reached 80% confluence, the cells were transfected with siRNA by electroporation using Amaxa Basic Nucleofector Kit for primary mammalian endothelial cells (Lonza, Germany). Briefly, 2.5 x10^6 cells/mL were trypsinized and washed two times with PBS, and resuspended in 100 µL basic nucleofector solution and transferred to a cuvette containing either 30 pmol scramble siRNA or pre-designed specific siRNA targeting PKC$_\delta$

transcripts (Ambion). The cells were electroporated with the Amaxa Nucleofactor™ apparatus, re-plated in 6-well plates containing pre-warmed complete RPMI medium and left undisturbed for 24 h. The cells were then serum-deprived for 24 h before an 8 h-incubation with Ang II (100 nmol/L). Inhibitors, when use, were incubated for 30 min prior to Ang II addition.

2.10 Immunohistochemistry

Localization of COX-2 or MCP-1 in the aortae and renal arteries from Ang II-infused rats and human renal arteries were determined by immunohistochemistry. The tissues were fixed overnight in 4% paraformaldehyde, processed for embedding in wax and then cut into 5 µm-sections with a microtome (Leica Microsystems, Wetzlar, Germany). Following re-hydration and treatment with 1.4% hydrogen peroxide in absolute methanol for 30 min at room temperature to inhibit endogenous peroxidase activity, antigen retrieval was performed by boiling the sections in 0.01 mol/L sodium citrate buffer (pH 6) for 30-60 s. After rinsing in phosphate-buffered saline (PBS), the sections were blocked with 5% normal donkey serum (Jackson Immunoresearch, West Grove, PA, USA) for 1 h at room temperature. Primary antibodies against COX-2 (Cayman), MCP-1 (Santa Cruz) or Factor VIII (Abcam) diluted to 1:200 in PBS supplemented with 2% BSA were incubated overnight in a humidified chamber at 4 °C. After 3 rinses in PBS each for 5 min, the sections were incubated with corresponding biotin-SP conjugated secondary antibodies (1:500, Jackson Immunoresearch) diluted in PBS for 1 h at room temperature, followed by 1 h incubation with peroxidase-conjugated streptavidin (1:500, Zymed laboratory, San Francisco, CA, USA) after washes. 3,3'-Diamonodenzidine tetrachloride (DAB) chromogen substrate (Vector

Laboratories, California, USA) was used for colour development according to the manufacturer's instruction. Counter-staining of the nucleus was performed with haematoxylin. For rat aortae and renal arteries, counter-staining of cytoplasm was also carried out using eosin to enhance the contrast between cell layers. Negative control was performed in the absence of primary antibodies. Images were viewed and captured under Leica DMRBE microscope coupled to SPOT-RT cooled CCD color digital camera using the objective PL FLUOTAR 20x/0.50 and SPOT Advanced software (Version 3.5.5, Diagnstic Instruments, Sertling Heights, MI, USA).

2.11 Immunofluorescence

Human small mesenteric arteries harvested after a 24 h-incubation protocol were embedded in OCT compound (Sakura Finetek, the Netherlands) in aluminium cryomolds, snap frozen in isopentane pre-cooled in liquid nitrogen and cut into 10 µm thick cryostat sections. The thawed sections were air-dried, post-fixed in 4% paraformaldehyde for 30 min, and then briefly treated with 0.05% Triton X in PBS. The sections were blocked with 5% donkey serum for 1 h at room temperature. Primary antibodies against COX-2 or MCP-1 were incubated overnight at 4 °C. The sections were then labeled with Alexa Fluor 546 donkey anti-goat IgG (Invitrogen, Molecular Probes, California, USA) for 1 h at room temperature. The sections were cover-slipped in anti-fade mounting medium (Vector Laboratories) and viewed under fluorescence microscope (Nikon Eclipse Ti-U) with mercury lamp Nikon Intensilight C-HGFI using the objective Nikon S Fluor 20x/0.75. Images were acquired with SPOT RT3 cooled 2 MP CCD scientific color digital camera and SPOT advanced software (Version 4.6).

2.12. Measurement of prostaglandins

After 30-min incubation in the presence of L-NAME with and without inhibitors in a chamber at 37 °C, aortic rings were transferred to microcentrifuge tubes that contained 200 µL bathing solution and 3 µmol/L ACh. Three minutes later, arterial tissues were removed and solutions were frozen and stored at -80 °C until assay for the determination of the protein content.

2.12.1 Enzyme immunoassay

The levels of arachidonic acid-derived prostanoids were measured by enzyme immunoassay (EIA) kits (Cayman Chemical, Ann Arbor, MI, USA) according to the instruction of the manufacturer. The five prostanoids or their metabolites, $PGF_{2\alpha}$, PGE_2, PGD_2, 6-keto $PGF_{1\alpha}$ (for PGI_2) and TXB_2 (for TXA_2), were assayed. The level of 8-isoprostanes was also determined.

2.12.2 High performance liquid chromatography-coupled mass spectrometry

Release of $PGF_{2\alpha}$ and 6-keto $PGF_{1\alpha}$ was also measured with high performance liquid chromatography-coupled mass spectrometry (HPLC-MS) (Cui et al., 2008). LC-MS experiment was performed on Agilent 1100 series HPLC with binary pump, autosampler and a thermostated column compartment (Agilent Technologies, CA, USA). The separation was performed on a reversed phase column (Alltech Prevail, C8, 2.1 mm × 150 mm, 3 µm) at a flow rate of 0.2 mL/min. The column temperature was maintained at 30°C throughout the analysis. The injection volume was 20 µL. The mobile phases consisted of 0.1 % formic acid in water (A) and in acetonitrile (B). The gradient started at 90 % A, 10 % B for 4 min, followed by a linear increase of solvent B to 55 % at 13 min,

which further increase to 70 % B in 5 min and maintained for 4 min. Finally, solvent B was decreased to 10 % and equilibrated for 15 min before the injection of the next sample. Negative ion electrospray mass spectrometric analysis was carried out using Bruker Daltonics MicrOTOFQ mass spectrometer (Bremen, Germany). The end plate offset and capillary voltage were -550 V and 4000 V respectively. Nitrogen was used as nebulizer gas at 1.4 bar and drying gas at a flow rate of 7.0 L/min at 170 °C. Pure $PGF_{2\alpha}$ and 6-keto $PGF_{1\alpha}$ were dissolved in methanol and serially diluted in KHS to construct standard curves.

2.13 Suspension antibody array-based multiplex immunoassay

Conditioned medium from endothelial cells treated with Ang II (100 nmol/L) for 24 h were harvested and the levels of interleukin-6, tumor necrosis factor-α and monocyte chemoattractant protein-1 were measured with MILLIPLEX MAP rodent Cytokine/Chemokine Panel (Millipore) using Bio-plex Suspension Array System (Bio-Rad), according to manufacturer's instructions.

2.14 Electron paramagnetic resonance spectroscopy

ROS formation was detected with electron paramagnetic resonance (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 trap for superoxide anions and peroxynitrite. Aortic rings were incubated with 2-mL L-NAME (100 μmol/L)-containing Krebs solution, oxygenated with 95% O_2 and 5% CO_2 at 37°C, together with TEMPONE-H (100 μmol/L) and a transition metal chelator diethylenetriaminepentaacetic acid (DTPA, 100 μmol/L, Sigma-Aldrich) to prevent auto-oxidation of hydroxylamine. After incubation, rings were

homogenized in 100-μL bathing solution, placed into glass micropipettes with inner diameter of 1 mm and stored in liquid nitrogen before signal detection. The samples were thawed to room temperature right before the measurement. X-band EPR spectra were measured 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. Interpretations of EPR spectra were done according to hyperfine EPR splitting constants reported by Dikalov *et al.* (1996).

2.15 ROS measurement with dihydroethidium fluorescence

Intracellular production of reactive oxygen species in cells were determined by fluorescence imaging with DHE (Molecular Probes), which binds to DNA upon oxidation to emit fluorescence. Primary rat endothelial cells were seeded on coverslips to ~60-70% confluence. Inhibitors, when used, were pre-incubated for 30 min before Ang II addition. Following a 30 s-treatment of Ang II (100 nmol/L), the cells were rinsed with PBS and incubated in 5 μmol/L DHE for 20 min at 37 °C shielding from light. After a rinse in PBS, fluorescence was observed under a confocal microscope (515-nm excitation; 585-nm long pass filter; Olympus Fluoview). DHE fluorescence intensity was analyzed by Fluoview (version 1.5; FV10-ASW1.5). Data were expressed in fold change compared with untreated control.

2.16 Chemicals and reagents

2.16.1 Chemicals

Stock solutions were kept at -20°C.

Chemical	Description	Solvent	Source
2-Amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT)	iNOS inhibitor	H_2O	Tocris (Bristol, UK)
2-Aminoethoxydiphenyl borate (2-APB)	Non-selective cation channel blocker	DMSO	Calbiochem, EMD Biosciences (La Jolla, CA, USA)
4α-phorbal 12-myristate 13-acetate (4α-PMA)	Negative analog of PKC activator PMA	DMSO	Sigma-Aldrich (St Louis, MO, USA)
Acetylcholine hydrochloride	Muscarinic (M_3) receptor agonist	H_2O	Sigma-Aldrich
Actinomycin-D	RNA synthesis inhibitor	DMSO	Tocris
Angiotensin II	Angiotensin II type 1 receptor (AT_1R) agonist	H_2O	Tocris
Apocynin	Putative NADPH oxidase inhibitor	DMSO	Calbiochem, EMB Biosciences
Baicalein	Lipoxygenase inhibitor	DMSO	Sigma-Aldrich
Celecoxib	COX-2 inhibitor	DMSO	Pfizer (New York, USA)
Cicaprost	IP receptor agonist	H_2O	Cayman (Ann Arbor, MI, USA)
Cycloheximide	Protein synthesis inhibitor	DMSO	Tocris

Diethyldithiocarbamate (DETCA)	Inhibitor of endogenous Cu/Zn-superoxide dismutase (Cu/Zn-SOD)	H_2O	Sigma-Aldrich
Diethylenetriaminepentaacetic acid (DTPA)	Transition metal chelator	H_2O	Sigma-Aldrich
DuP-697	COX-2 inhibitor	DMSO	Tocris
GF109203X	Broad spectrum inhibitor for PKC isoforms	DMSO	Tocris
Go 6976	$PKC_{\alpha/\beta}$ inhibitor	DMSO	Tocris
GR 32191	TP receptor antagonist	H_2O	Tocris
Hydroxylamine	Exogenous NO donor	H_2O	Tocris
Hypoxanthine	Substrate for xanthine oxidase	H_2O	Sigma-Aldrich
Indomethacin	Non-selective COX-inhibitor	DMSO	Sigma-Aldrich
L-655,240	TP receptor antagonist	DMSO	Tocris
L-arginine	Substrate for NOS	H_2O	Sigma-Aldrich
Losartan	AT_1R antagonist	DMSO	Cayman
N^G-nitro-L-arginine methyl ester (L-NAME)	Nitric oxide synthase (NOS) inhibitor	H_2O	Tocris
NS 398	COX-2 inhibitor	DMSO	Tocris
ODQ	Soluble guanylate cylcase inhibitor	DMSO	Tocris
Oxypurinol	Xanthine oxidase inhibitor	NaOH (1 mol/L)	Sigma-Aldrich
PD 123319	AT_2R antagonist	H_2O	Tocris
PD 98059	ERK1/2 inhibitor	DMSO	Tocris
PGE_2	EP receptor agonist	DMSO	Calbiochem, EMD Biosciences
$PGF_{2\alpha}$	FP receptor agonist	DMSO	Cayman

PGI_2	IP receptor agonist	H_2O	Cayman
Phenylephrine	α-adrenergeic receptor agonist	H_2O	Sigma-Aldrich
Phorbal 12-myristate 13-acetate (PMA)	PKC activator	DMSO	Tocris
Rottlerin	PKC_δ inhibior	DMSO	Enzo Life Science (New York, USA)
S18886	TP receptor antagonist	DMSO	Institut de Recherches Servier (Suresnes, France)
SB 202190	p38 MAPK inhibitor	DMSO	Tocris
sc-560	COX-1 inhibitor	DMSO	Sigma-Aldrich
SIN-1	Exogenous NO donor	H_2O	Tocris
Sodium nitroprusside (SNP)	Exogenous NO donor	H_2O	Sigma-Aldrich
SP 600125	JNK inhibitor	DMSO	Tocris
Tempol	SOD mimetic	H_2O	Sigma-Aldrich
TEMPONE-H	Spin trap reagent	H_2O	Alexis (San Diego, CA, USA)
Tiron	SOD mimetic	H_2O	Sigma-Aldrich
U46619	TP receptor agonist	DMSO	Sigma-Aldrich
Valeryl salicylate (VAS)	COX-1 inhibitor	DMSO	Cayman
Xanthine oxidase	Free radical producing enzyme	H_2O	Sigma-Aldrich
εV1-2	PKC_ε inhibior	H_2O	AnaSpec (CA, USA)

2.16.2 Compositions of Krebs-Henseleit solution (KHS)

The solution was freshly prepared before experiments and continuously aerated with 95% O_2 and 5% CO_2 at 37°C to maintain a pH value of 7.4.

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

2.16.3 Reagents for Western blot analysis

2.16.3.1 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 sulphate (SDS)	0.1%
Sodium deoxycholate	0.5%
Cocktail of protease inhibitors	
Aprotinin	5 µg/mL
EDTA	1 mmol/L
EGTA	1 mmol/L
β-glycerolphosphate	2 mmol/L
Leupetin	1 µg/mL
Phenylmethylsulfonyl fluoride (PMSF)	1 mmol/L
Sodium fluoride	1 mmol/L
Sodium orthovanadate	1 mmol/L

2.16.3.2 Reagents for gel casting (stacking and separating)

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

2.16.3.3 Buffers for SDS-PAGE, transfer and washing

Sample loading buffer (2X)	
Tris	125 mmol/L
SDS	4%
Glycerol	20%
Bromophenol blue	0.06%
β-mecaptoethanol (add freshly)	10%
Running buffer for SDS-PAGE (pH 8.3)	
Tris base	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%

Tween-20 Phosphate-buffered saline (PBST) (pH 7.4)	
NaCl	135 mmol/L
NaH_2PO_4	3.2 mmol/L
KH_2PO_4	0.5 mmol/L
KCl	1.3 mmol/L
Tween 20	0.05%

2.17 Primary antibodies

Primary antibodies for target proteins were diluted either in 1% bovine serum albumin (BSA) or 5% non-fat skimmed milk dissolved in PBST for Western blotting (WB), 2% BSA in PBS for immunohistochemistry (IHC) and PBS in immunofluorescence (IF).

Antigen	Host species	Type	Application	Company
COX-1	Mouse	Monoclonal	WB (1:500)	Cayman
COX-2	Rabbit	Polyclonal	WB (1:500)	Cayman
COX-2	Goat	Polyclonal	WB (1:500), IHC (1:200); IF (1:200)	Santa Cruz
FP receptor	Rabbit	Polyclonal	WB (1:500)	Cayman
TP receptor	Rabbit	Polyclonal	WB (1:500)	Cayman
PECAM-1	Goat	Polyclonal	IHC (1:200)	Santa Cruz
ERK1/2	Rabbit	Polyclonal	WB (1:500)	Cell Signaling
Phospho-ERK1/2	Mouse	Monoclonal	WB (1:500)	Cell Signaling
p38 MAPK	Rabbit	Polyclonal	WB (1:500)	Cell Signaling
Phospho-p38 MAPK	Mouse	Monoclonal	WB (1:500)	Cell Signaling
PKC_α	Rabbit	Polyclonal	WB (1:500)	Cell Signaling
PKC_δ	Rabbit	Polyclonal	WB (1:500)	Cell Signaling
PKC_ε	Rabbit	Polyclonal	WB (1:500)	Cell Signaling

PKC_{θ}	Rabbit	Polyclonal	WB (1:500)	Cell Signaling
PKC_{μ}	Rabbit	Polyclonal	WB (1:500)	Cell Signaling
PKC_{ζ}	Rabbit	Polyclonal	WB (1:500)	Cell Signaling
Phospho-PKC_{δ}	Rabbit	Polyclonal	WB (1:500)	Cell Signaling
MCP-1	Goat	Polyclonal	WB (1:500), IHC (1:200); IF (1:200)	Santa Cruz
Factor VIII	Rabbit	Polyclonal	WB (1:500) IHC (1:200)	Abcam
NOX-2	Rabbit	Polyclonal	WB (1:500)	Abcam
GAPDH	Mouse	Monoclonal	WB (1:20000)	Ambion

2.18 Statistical analysis

Endothelium-dependent contractions were expressed as active tension [force recorded/ (2 × ring's length)]. Results are mean ± SEM of n rings from different animals. Protein expression was normalized to GAPDH. For statistical analysis, Student's *t*-test or 2-way ANOVA followed by Bonferroni post-tests were used when more than two treatments were compared (GraphPad Software, San Diego, CA, USA). $P<0.05$ was considered significantly different.

Chapter III

Cyclooxygenase-2-derived $PGF_{2\alpha}$ Mediates Endothelium-dependent Contractions in the Aortae of Young Hamsters

3.1 Introduction

Besides neuronal and hormonal regulation, vascular tone is modulated locally by a delicate balance between endothelium-derived relaxing (EDRFs) and contracting (EDCFs) factors (Furchgott and Vanhoutte, 1989; Luscher and Vanhoutte, 1986), with the latter being less well-defined but emerging as a pathologic marker in hypertension, obesity, hyperlipidemia, diabetes and aging (Vanhoutte et al., 2005).

A number of molecules have been proposed as possible EDCF candidates under pathophysiological conditions. These include prostaglandin H_2 (PGH_2), thromboxane A_2 (TxA_2), leukotrienes, endothelin-1 and superoxide anion. The release of these tentative EDCFs can be triggered by acetylcholine, angiotensins I/II, ADP and ATP (Gollasch, 2002). The contribution of additional cyclooxygenase (COX)-derived metabolites, i.e. PGE_2, PGD_2, and $PGF_{2\alpha}$ has been postulated. The precise nature of these EDCFs varies among species and vascular beds (Luscher and Vanhoutte, 1986; Vanhoutte et al., 2005).

Two isoforms of COX have been identified in blood vessels. COX-1 is constitutively expressed and believed to participate in physiological responses, whereas COX-2 is a highly inducible enzyme (Linton and Fazio, 2004). At least in the rat aorta, EDCFs appear to be COX-1-derived prostanoids generated in the endothelium, which diffuse to contract the underlying vascular smooth muscle by activating TP receptor (Vanhoutte et al., 2005). In arteries of

spontaneously hypertensive or diabetic rats, the expression of COX-1 is up-regulated and the augmented endothelium-dependent contractions are inhibited by COX-1 inhibitors (Gluais et al., 2007; Shi et al., 2007; Yang et al., 2002). COX-1-derived prostacyclin, TxA_2 or endoperoxides all contribute to endothelium-dependent contractions (Gluais et al., 2006; Gluais et al., 2007).

However, this generally accepted distinction between "constitutive" and "inducible" isoform of COX appears to be an over-generalization. Indeed, COX-2 can be expressed constitutively in the endothelium of the rat pulmonary and human renal blood vessels, and in cultured endothelial cells (Baber et al., 2003; Therland et al., 2004). A COX-2 specific inhibitor attenuates arachidonic acid-induced vasodilatation of canine coronary arteries (Hennan et al., 2001), supporting a physiological role for COX-2 in vascular function. COX-2 is up-regulated under pathological conditions including renovascular hypertension (Hartner et al., 2003), reflux nephropathy (Solari et al., 2003), and diabetes (Bagi et al., 2006). For instance, the elevated arteriolar tone and blood pressure in type-2 diabetic mice is associated with the augmented production of COX-2-derived vasoconstrictor prostanoids (Bagi et al., 2005), even though the source of this production is unclear. In deoxycorticosterone acetate salt-induced hypertension, the expression of COX-2 is enhanced and this is related to the increased contraction of the aorta to acetylcholine, probably because of the exaggerated oxidative stress in the vascular wall (Adeagbo et al., 2005).

COX-2 can be up-regulated by physiological shear stress from pulsative flow (Hendrickson et al., 1999; Topper et al., 1996). However, its actual role in the endothelial regulation of the normal vascular tone is uncertain. Identification of COX-2-mediated generation of EDCFs can help to elucidate the cellular mechanisms of endothelial dysfunction and potentially uncover novel

therapeutic targets. Since the lipid profile and arachidonic acid metabolism of hamsters resemble that of humans (Arbeeny et al., 1992; Kris-Etherton and Dietschy, 1997; Nicolosi, 1997) and COX-2 may be important for both the physiological and pathological regulation of vascular reactivity (Hendrickson et al., 1999; Linton and Fazio, 2004; Topper et al., 1996), I hypothesized that COX-2 rather than COX-1 is mediating the generation of EDCFs in the aorta of young and healthy hamsters. The present findings revealed $PGF_{2\alpha}$ as a physiological EDCF, which can be generated by COX-2 in the endothelium.

3.2 Materials and Methods

This study was approved by the Animal Experimentation Ethics Committee, Chinese University of Hong Kong. This investigation 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). Human renal arteries were obtained during surgery after informed consent from four patients aged between 59 and 75 years.

3.2.1 Animals and diet

Experiments were performed on aortae from male Syrian golden hamsters aged ~ 3 month supplied by the Laboratory Animal Services Centre, the Chinese University of Hong Kong. The animals were housed at constant temperature (21 ± 1 °C) under a 12-hour light/dark cycle and had free access to a standard chow diet and water.

3.2.2 Blood vessel preparation

Hamsters were euthanized by CO_2 inhalation. Thoracic aortae were excised and placed in Krebs-Henseleit solution (KHS) containing (mmol/L): NaCl 119, $NaHCO_3$ 25, $MgCl_2$ 1, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, and D-glucose 11.1. Following the removal of periadventitial fat, each artery was cut into segments ~1.0-1.3 mm in length. Each segment was suspended between two stainless steel wires in chambers of a Multi Myograph System (610M, Danish Myo Technology A/S, Aarhus N, Denmark) for the measurement of isometric force. Each chamber was filled with 5 mL-KHS aerated with 95% O_2 and 5% CO_2 and maintained at 37°C. The rings were stretched to a previously determined optimal resting tension of 10 mN. Thirty min after setting up the preparations,

they were contracted with 60 mmol/L KCl to test the vessel's contractility, and washed in normal KHS, and finally allowed to equilibrate for 30 min. In some rings, the endothelium was removed mechanically by rolling the luminal surface with a stainless steel wire.

Human renal arteries were obtained after renal surgery with informed consent from patients. Each artery was cut into 2-3 ring segments, 2-3 mm in length. Rings were suspended in organ bath as described previously (Leung et al., 2006). Each ring was initially stretched to an optimal tension of 25 mN and then allowed to equilibrate for 90 min before the start of the experiments.

3.2.3. Isometric force measurement

Aortic rings with endothelium were exposed for 30 min to 100 µmol/L N^G-nitro-L-arginine methyl ester (L-NAME) prior to the cumulative addition of acetylcholine (0.1-10 µmol/L) for viualization of endothelium-dependent contractions. Changes in isometric tension to $PGF_{2\alpha}$ (1-30 ng/ml), PGI_2 (3.7-370 ng/ml), cicaprost (10-100 ng/ml) in the presence of L-NAME were also recorded. The effects of various inhibitors and antagonists (e.g., COX-1 and COX-2 inhibitors, TP receptor antagonists) or NO donors (SNP, SIN-1) were tested on acetylcholine- or $PGF_{2\alpha}$-induced contractions following a 30-min incubation with each drug. Specificity of COX-2 inhibitors and TP receptor antagonists was tested against contraction induced by 60 mmol/L KCl or 50 nmol/L U46619.

3.2.4 *In situ* endothelial cell $[Ca^{2+}]_i$ imaging

Real-time changes in intracellular calcium levels, $[Ca^{2+}]_i$ in native endothelial cells of the intact hamster aorta were determined by calcium imaging using Fura-2 AM (Leung et al., 2006). Isolated aortic rings with endothelium were

labeled for 60 min at 21 °C in a solution containing 10 μmol/L Fura-2 AM, 0.025% pluronic F-127, and 1 mmol/L probenacid (to prevent Fura-2 secretion). The ring was then cut open and pinned *en face* to a block of silicone elastomer fixed in a perfusion chamber, which was perfused with pre-warmed KHS for 20 min at 1 mL/min.

The specimen was illuminated (Polychrome IV light source) on the stage of an IX70 Olympus microscope, fitted with a 20X Olympus water immersion objective. The Fura-2 loaded tissue was excited alternately at 340 and 380 nm, and images of the respective 510 nm-emissions were collected at one-second intervals using a MetaFluor v4.6 software (Universal Imaging Corp., West Chester, PA, USA). The emitted light was transmitted to a collecting device and then to a cooled charge coupled device (CCD) camera. Illumination through the Polychrome IV light source and acquisition by the CCD camera were controlled by MetaFluor software v4.6. Video frames containing images of cell fluorescence were digitized at a resolution of 512 horizontal × 480 vertical pixels. Imaging analysis was performed using a MetaFluor imaging system. After background subtraction, the fluorescence ratio (F340/F380) was obtained by dividing, pixel by pixel, the images at 340 nm and 380 nm. Changes in this ratio reflected changes in $[Ca^{2+}]_i$.

3.2.5 Reverse-transcription polymerase chain reaction (RT-PCR)

Expression levels of COX-2 mRNA in rings with and without endothelium were detected by RT-PCR. The arterial rings were snap frozen in liquid nitrogen and homogenized, and mRNA was extracted using the Aurum total RNA Mini kit (BioRad, Hercules, CA, USA) according to manufacturers' instructions. The extracted RNA was reverse transcribed using the iScript™ cDNA synthesis kit

(BioRad), and PCR was performed with *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) with thermal cycles of 5 min-95 °C, 30 cycles of 1 min-95 °C, 1 min-T_m (melting temperature), 1 min-72 °C, finally followed by 6 min-72 °C.

Primers for PCR were COX-2 (216 bp) sense (5'-TGA TCC CCA AGG CAC GAA-3') and antisense (5'-ACC TCT CCA CCA ATG ACC TGA-3') (Valeille et al., 2005), GAPDH (171 bp) sense (5'-ACC CAG AAG ACT GTG GAT GG-3') and antisense (5'-CAC ATT GGG GGT AGG AAC AC-3'). Melting temperature for COX-2 and GAPDH primers were 60 °C and 57 °C, respectively. PCR products were run on 1.5% agarose gel in 1X TAE buffer at 80 V. Ethidium bromide-stained bands were visualized under UV illumination using FluorChem (version 2.00, Alpha Innotech Corp., San Leandro, CA, USA).

3.2.6 Western blot analysis

Hamster aortic rings (without or without 3 min-ACh exposure), heart, lung, and human renal arteries were snap frozen in liquid nitrogen and subsequently homogenized in an ice-cold RIPA lysis buffer with a cocktail of protease inhibitors (leupetin, 1 µg/mL; aprotonin, 5 µg/mL; PMSF, 100 µg/mL; sodium orthovanadate, 1 mmol/L; EGTA, 1 mmol/L; EDTA, 1 mmol/L; NaF, 1 mmol/L and β-glycerolphosphate, 2 mg/mL). The lysates were centrifuged at 20,000 xg for 20 min and the supernatants were collected. The protein concentration was determined by the Lowry method (BioRad). Each protein sample (80 µg) was electrophoresed through the 10% SDS-polyacrylamide gels and then transferred to an Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore). The membranes were blocked with 5% non-fat skimmed milk and probed overnight at 4 °C with antibodies against PECAM-1 (Santa Cruz), COX-1 (Santa Cruz), COX-2 (BD Transduction Laboratories), FP or TP receptor

(Cayman Cheimcal). After washes in Tween-20 phosphate buffer saline (PBST), the membranes were incubated with appropriate HRP-conjugated secondary IgG (DakoCytomation) for 60 min at room temperature. The membranes were developed with an enhanced chemiluminescence detection system (ECL reagents; Amersham Pharmacia Biotech, Uppsala, Sweden) and exposed on X-ray films (Fuji). Densitometry was performed using a documentation program (FluorChem, Alpha Innotech Corp., San Leandro, CA, USA). Removal of endothelium was confirmed by probing the membrane with an endothelial cell marker, PECAM-1. GAPDH antibody (Ambion, Inc) was probed as a loading control.

3.2.7 Enzyme immunoassay (EIA) and high performance liquid chromatography-coupled mass spectrometry (HPLC-MS) measurement of prostaglandins

After 30 min-incubation in the presence of L-NAME with or without inhibitors or SNP in a chamber at 37 °C, aortic rings were transferred to microcentrifuge tubes that contained 200 µL bathing solution and 3 µmol/L acetylcholine. Three minutes later, arterial tissues were removed and solutions were frozen and stored at -80 °C until assay for the determination of the protein content.

The levels of arachidonic acid-derived prostanoids were measured by EIA kits (Cayman Chemical, Ann Arbor, MI, USA) according to the instruction of the manufacturer. The five prostanoids or their metabolites, $PGF_{2\alpha}$, PGE_2, PGD_2, 6-keto $PGF_{1\alpha}$ (for PGI_2) and TxB_2 (for TxA_2), were assayed. The level of 8-isoprostanes was also determined.

The release of $PGF_{2\alpha}$ and 6-keto $PGF_{1\alpha}$ was also measured with HPLC-MS (Cui et al., 2008). LC-MS experiment was performed on Agilent 1100 series

HPLC with binary pump, autosampler and a thermostated column compartment (Agilent Technologies, CA, USA). The separation was performed on a reversed phase column (Alltech Prevail, C8, 2.1 mm × 150 mm, 3 μm) at a flow rate of 0.2 mL/min. The column temperature was maintained at 30 °C throughout the analysis. The injection volume was 20 μL. The mobile phases consisted of 0.1 % formic acid in water (A) and in acetonitrile (B). The gradient started at 90 % A, 10 % B for 4 min, followed by a linear increase of solvent B to 55 % at 13 min, which further increase to 70 % B in 5 min and maintained for 4 min. Finally, solvent B was decreased to 10 % and equilibrated for 15 min before the injection of the next sample. Negative ion electrospray mass spectrometric analysis was carried out using Bruker Daltonics MicrOTOFQ mass spectrometer (Bremen, Germany). The end plate offset and capillary voltage were -550 V and 4000 V respectively. Nitrogen was used as nebulizer gas at 1.4 bar and drying gas at a flow rate of 7.0 L/min at 170 °C. Pure $PGF_{2\alpha}$ and 6-keto $PGF_{1\alpha}$ were dissolved in methanol and serially diluted in KHS to construct standard curves.

3.2.8 Detection of reactive oxygen species (ROS) formation

ROS formation was detected with electron paramagnetic resonance (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 trap for superoxide anion and peroxynitrite. Aortic rings were incubated with 2-mL L-NAME (100 μmol/L)-containing Krebs solution, oxygenated with 95% O_2 and 5% CO_2 at 37°C, together with TEMPONE-H (100 μmol/L) and a transition metal chelator diethylenetriaminepentaacetic acid (DTPA, 100 μmol/L, Sigma-Aldrich). After incubation, rings were homogenized in 100-μL bathing solution

and placed into glass micropipettes for signal detection. X-band EPR spectra were measured 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. Interpretations of EPR spectra were done according to hyperfine EPR splitting constants reported by Dikalov et al. (1996).

3.2.9 Drugs and chemicals

Acetylcholine, indomethacin, phenylephrine, baicalein, tiron, tempol, DETCA (diethyldithiocarbamate acid) sodium nitroprusside, L-arginine and U46619 (9,11-dideoxy-11_{α},9_{α}-epoxymethano-prostaglandin $F_{2\alpha}$) were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). DuP-697 (5-bromo-2-(4-fluorophenyl)-3-[4-(methylsulfonyl)phenyl]-thiophene), ODQ, (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), hydroxylamine, SIN-1 (amino-3-morpholinyl-1,2,3-oxadiazolium chloride), L-NAME (N^{G}-nitro-L-arginine methyl ester), actinomycin-D, cycloheximide, GR 32191 ((4*Z*)-7-[(1*R*,2*R*,3*S*,5*S*)-5-([1,1'-biphenyl]-4-ylmethoxy)-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-heptenoic acid hydrochloride), L-655,240 (1-[(4-chlorophenyl)methyl]-5-fluoro-α,α,3-trimethyl-1*H*-indole-2-propanoic acid), NS-398 (N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide) and ozagrel hydrochloride were from Tocris (Avonmouth, UK). 2-APB (2-aminoethoxydiphenyl borate), apocynin and PGE_2 were from Calbiochem, EMD Biosciences (La Jolla, CA, USA). VAS (valeryl salicylate), $PGF_{2\alpha}$, PGI_2, 6-keto $PGF_{1\alpha}$ and cicaprost were from Cayman

Chemical (Ann Arbor, MI, USA). S18886 (3-[(6-amino-(4-chlorobenzensulphonyl)-2-methyl-5,6,7,8-tetrahydronapht]-1-yl) propionic acid) and sc-560 were kind gifts from Institut de Recherches Servier (Suresnes, France). Celecoxib was from Pfizer. Except acetylcholine, GR 32191, L-NAME, phenylephrine, ozagrel hydrochloride, PGI_2, cicaprost, tiron, tempol, DETCA, S18886, sodium nitroprusside, L-arginine, ODQ, hydroxylamine and SIN-1 that were prepared in distilled water, all other drugs were dissolved in DMSO (Sigma-Aldrich).

3.2.10 Data analysis

Endothelium-dependent contractions were expressed as active tension [force recorded/ (2 × ring's length)]. Protein expression was normalized to GAPDH of respective blot. Results are mean ± SEM of n rings from different animals. For statistical analysis, Student's *t*-test or 2-way ANOVA followed by Bonferroni post-tests were used when more than two treatments were compared (GraphPad Software, San Diego, CA, USA). $P<0.05$ was considered significantly different.

3.3 Results

3.3.1 Essential role of COX-2 in endothelium-dependent contractions in aortae from young hamsters

In the presence, but not in the absence of N^G-nitro-L-arginine methyl ester (L-NAME), acetylcholine elicited pronounced contraction of aortic rings with endothelium with a maximal response of 5.93 ± 0.16 mN/mm (Figure 3.1A&B), corresponding to approximately 70% of the contractile response (8.55 ± 0.32 mN/mm) induced by 60 mmol/L KCl. Removal of the endothelium abolished the contractions to acetylcholine (Figure 3.1C). The endothelium-dependent contractions were attenuated or eliminated by the non-selective COX inhibitor indomethacin (Figure 3.2A). Likewise, three structurally different selective COX-2 inhibitors (NS-398, DuP-697 and celecoxib) reduced or abolished the endothelium-dependent contractions (Figure 3.2B-D). The specificity of COX-2 inhibition was confirmed by the lack of inhibitory effects of the three inhibitors on contractions induced by 60 mmol/L KCl and U46619 (Figure 3.3). By contrast, neither the COX-1 selective inhibitors [valeryl salicylate (VAS) and sc-560, Figure 3.4A&B] nor the inhibitor of 5- and 12-lipoxygenase (baicalein) inhibited the response (Figure 3.4C). The endothelium-dependent contractions were unaffected by treatment with actinomycin-D (10 μmol/L, RNA synthesis inhibitor) or cycloheximide (10 μmol/L, protein synthesis inhibitor) (Figure 3.5).

3.3.2 Endothelium-dependent contractions mediated through TP receptors

The endothelium-dependent contractions were attenuated or abolished in aortic rings treated with three structurally distinct selective TP receptor antagonists, terutroban (S18886, 3-100 nmol/L), L-655,240 (0.1-1 μmol/L), or GR 32191 (100 nmol/L) (Figure 3.6A-C). On the contrary, the thromboxane synthase

inhibitor ozagrel hydrochloride (10 µmol/L) did not affect the contraction (Figure 3.6D). The specificity of the TP receptor antagonists was tested against contractions induced by 60 mmol/L KCl and U46619. Treatment with these antagonists inhibited or prevented the U46619-induced contraction without affecting that to 60 mmol/L KCl (Figure 3.7).

3.3.3 Dependency on extracellular Ca^{2+}

Endothelium-dependent contractions were absent following the removal of extracellular calcium ions. Re-introduction of 2.5 mmol/L $CaCl_2$ to the bathing solution restored contraction in the presence of 10 µmol/L acetylcholine (Figure 3.8A). Exposure of rings to 2-APB (3-50 µmol/L, a non-selective cation channel blocker) diminished or abolished the endothelium-dependent contractions (Figure 3.8B) without affecting the response to 60 mmol/L KCl or U46619 (n=4, data not shown).

En face fluorescence images from viable individual native endothelial cells of cut-open aortic segments were examined (Figure 3.9A a). The fluorescence signal indicative of the $[Ca^{2+}]_i$ was absent after mechanical removal of the endothelium. It increased following the addition of acetylcholine in the presence of L-NAME only in arterial tissues with endothelium (Figure 3.9A a*). Treatment with 2-APB (50 µmol/L, the concentration that abolished endothelium-dependent contractions), prevented the increases in $[Ca^{2+}]_i$ (Figure 3.9A b*). By contrast, S18886 (0.1 µmol/L) had no effect on the Ca^{2+} fluorescence signal (Figure 3.9A c*). The acetylcholine-stimulated real-time increase in endothelial cell $[Ca^{2+}]_i$ was eliminated by 2-APB but not by S18886 (Figure 3.9B).

3.3.4 Localization of COX-2

The expression of COX-2 mRNA was significantly higher in aortae with than those without endothelium (Figure 3.10A). The COX-2 protein expression was reduced following the mechanical removal of the endothelium (Figure 3.10B), which I had confirmed by the reduced protein levels of the endothelium-specific marker, PECAM-1 (Figure 3.10D). The protein expression of COX-1 was slightly but insignificantly greater in the aortae with than those without endothelium (Figure 3.10C).

3.3.5 $PGF_{2\alpha}$ as the EDCF

Six possible EDCF candidates, i.e. $PGF_{2\alpha}$, PGE_2, TxA_2, PGD_2, PGI_2 and 8-isoprostanes were assayed chemically. Acetylcholine at 3 μmol/L stimulated a significant rise in the release of $PGF_{2\alpha}$ and PGI_2 (detected as 6-keto $PGF_{1\alpha}$) but not PGE_2, TxA_2 (detected as TxB_2) and PGD_2 from aortic rings with endothelium (Figure 3.11). The release of both $PGF_{2\alpha}$ and 6-keto $PGF_{1\alpha}$ was largely inhibited by removal of the endothelium (Figure 3.12A&B). Among the five assayed prostanoids, only the release of $PGF_{2\alpha}$ (~0.8 ng/mL) and 6-keto $PGF_{1\alpha}$ (~7 ng/mL) evoked by 3 μmol/L acetylcholine was inhibited or abolished by treatment with celecoxib or 2-APB but not by VAS (Figure 3.12A&B). These results are consistent with those obtained by HPLC-MS which showed that the amount of $PGF_{2\alpha}$ (~1.0 ng/mL) was comparable to that (~0.8 ng/mL) assayed by EIA (Figure 3.12C&D).

To further investigate the role of $PGF_{2\alpha}$ and PGI_2 in endothelium-dependent contractions, the effects of $PGF_{2\alpha}$ (1-30 ng/mL), PGI_2 (3.7-370 ng/mL) and its stable analogue cicaprost (10-100 ng/mL) were tested in the presence of 100 μmol/L L-NAME. $PGF_{2\alpha}$ induced contraction of the aortic rings

at the relatively low concentration of 1 ng/mL (Figure 3.13A&B), comparable with the level of acetylcholine-induced release measured by EIA (~0.8 ng/mL). The contraction was reduced by S18886 (0.1 μmol/L). By contrast, neither PGI_2 nor cicaprost produced a contraction (Figure 3.14A-D), even at a concentration fifty-fold higher than that detected in the solution bathing aortae exposed to acetylcholine (~7 ng/mL for 6-keto $PGF_{1\alpha}$). Exogenous PGI_2 did not relax phenylephrine-contracted aortae (Figure 3.14E&F).

PGE_2, whose release was independent of acetylcholine stimulation (Figure 3.15A), produced very small contractions at the assayed concentration (~0.8 ng/mL), and such contractions were insensitive to S18886 (Figure 3.15B).

3.3.6 Non involvement of F-series-prostanoid (FP) receptor

Though $PGF_{2\alpha}$ is a natural agonist for FP receptor, FP receptor did not take a role in $PGF_{2\alpha}$-mediated endothelium-dependent contractions. FP receptor antagonist, AL-8810 (1 μmol/L) did not inhibit acetylcholine- or $PGF_{2\alpha}$-induced contractions (Figure 3.16A&B). The expression of FP receptor is very minimal in the hamster aorta as compared to positive controls of heart and lung (Figure 3.16C).

3.3.7 Non involvement of reactive oxygen species (ROS)

Endothelium-dependent contractions to acetylcholine were unaffected by tiron (1 mmol/L) plus DETCA (100 μmol/L) (membrane-permeable free radical scavengers), tempol (100 μmol/L, superoxide dismutase mimetic) or apocynin (100 μmol/L, NADPH oxidase inhibitor) (Figure 3.17A). The level of 8-isoprostanes was very low (Figure 3.17B) L-NAME-treated aortic rings showed no EPR signal for superoxide anion or peroxynitrite in response to acetylcholine

(Figure 3.18A&B), while the addition of hypoxanthine plus xanthine oxidase (HXXO, a mixture to release superoxide anion) gave rise to three distinct EPR signals (Figure 3.18C).

3.3.8 Human renal arteries

In the presence of 100 µmol/L L-NAME, ACh induced contractions in arteries from patients with hypertension and diabetes mellitus and these contractions were reversed by S18886 (Figure 3.19). $PGF_{2\alpha}$-induced contractions were antagonized by S18886 but not by AL-8810, the FP receptor antagonist (Figure 3.20A&B). Pre-treatment with S18886, but not AL-8810, prevented exogenous $PGF_{2\alpha}$-induced contractions (Figure 3.20C). Western blot analysis revealed little or no expression of FP receptor in human arteries in contrast to well-expressed TP receptor (Figure 3.21A&B). HPLC-MS measurement showed that those arteries released both $PGF_{2\alpha}$ and 6-keto $PGF_{1\alpha}$ in response to 100 µmol/L ACh, but only the release of $PGF_{2\alpha}$ was inhibited by celecoxib (10 µmol/L) while VAS (30 µmol/L) was without effect (Figure 3.21B&C).

3.3.9 Release of $PGF_{2\alpha}$ is unaffected by presence of NO

Acetylcholine did not induced contractions in control arteries without L-NAME exposure. L-NAME (100 µmol/L) unveiled ACh induced contractions with maximal tension of 4.72 ± 0.56 mN/mm at 10 µmol/L. Inhibition of the soluble guanylate cyclase with ODQ also caused similar contractions at 10 µM ACh (4.39 ± 0.47 mN/mm) (Figure 3.22). NO donor, sodium nitroprusside (SNP), at 1 µmol/L inhibited ACh-induced contractions in the presence of L-NAME (Figure 3.23A). In contrast, contractions unmasked by ODQ were not affected by SNP (Figure 3.23B). Another NO donor, SIN-1 (1 µmol/L) and an intermediate for NO

formation, hydroxylamine (30 µmol/L), also suppressed ACh-induced contractions (Figure 3.24A), which were attenuated by L-arginine (1 mmol/L) pre-incubated for 10 min before L-NAME addition (Figure 3.24B).

$PGF_{2\alpha}$ elicited vasoconstrictions in untreated control rings. L-NAME (Figure 3.25A) and removal of endothelium (Figure 3.25B) enhanced the $PGF_{2\alpha}$-induced contractions. SNP at 0.1 µmol/L and 1 µmol/L inhibited $PGF_{2\alpha}$-induced contractions in the presence of L-NAME (Figure 3.25C).

Acetylcholine (3 µmol/L) stimulated the release of $PGF_{2\alpha}$ only in aortic rings with endothelium (72.44 ± 18.10 pg/ml/mm). The release was neither enhanced by 100 µmol/L L-NAME nor prevented by 1 µmol/L SNP (Figure 3.26).

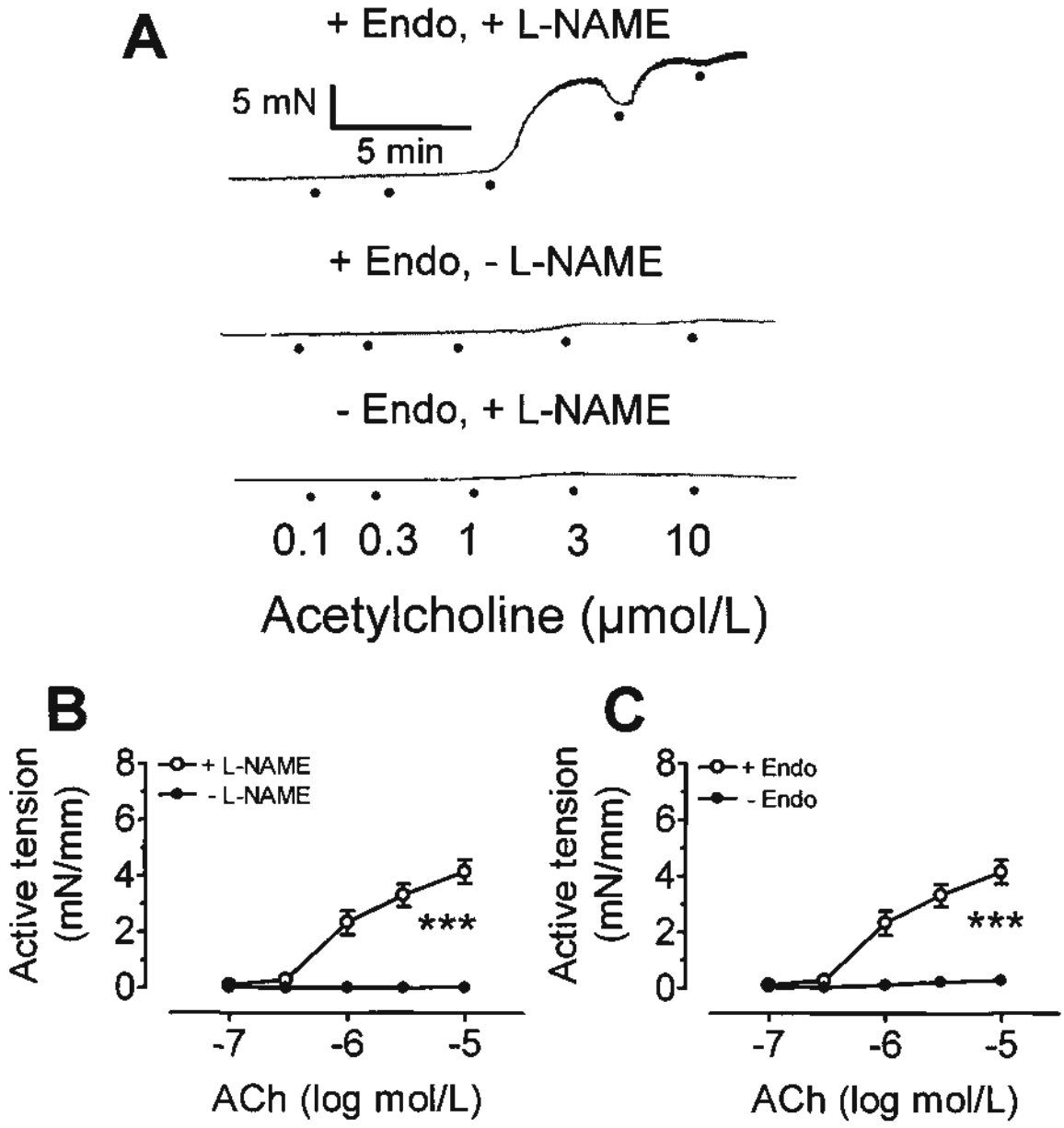

FIGURE 3.1.

(A) Original recordings showing acetylcholine (ACh)-induced contractions present only in rings with endothelium treated with L-NAME (100 µmol/L, 30-min incubation). Concentration-dependent contractions induced by acetylcholine (B) in the presence of L-NAME (+ L-NAME, n=15 vs - L-NAME, n=5) and (C) in rings with endothelium (+ Endo n=15 vs - Endo n=4). Data are mean ± SEM of n experiments. ***P<0.001 compared between +/- L-NAME or +/- Endo.

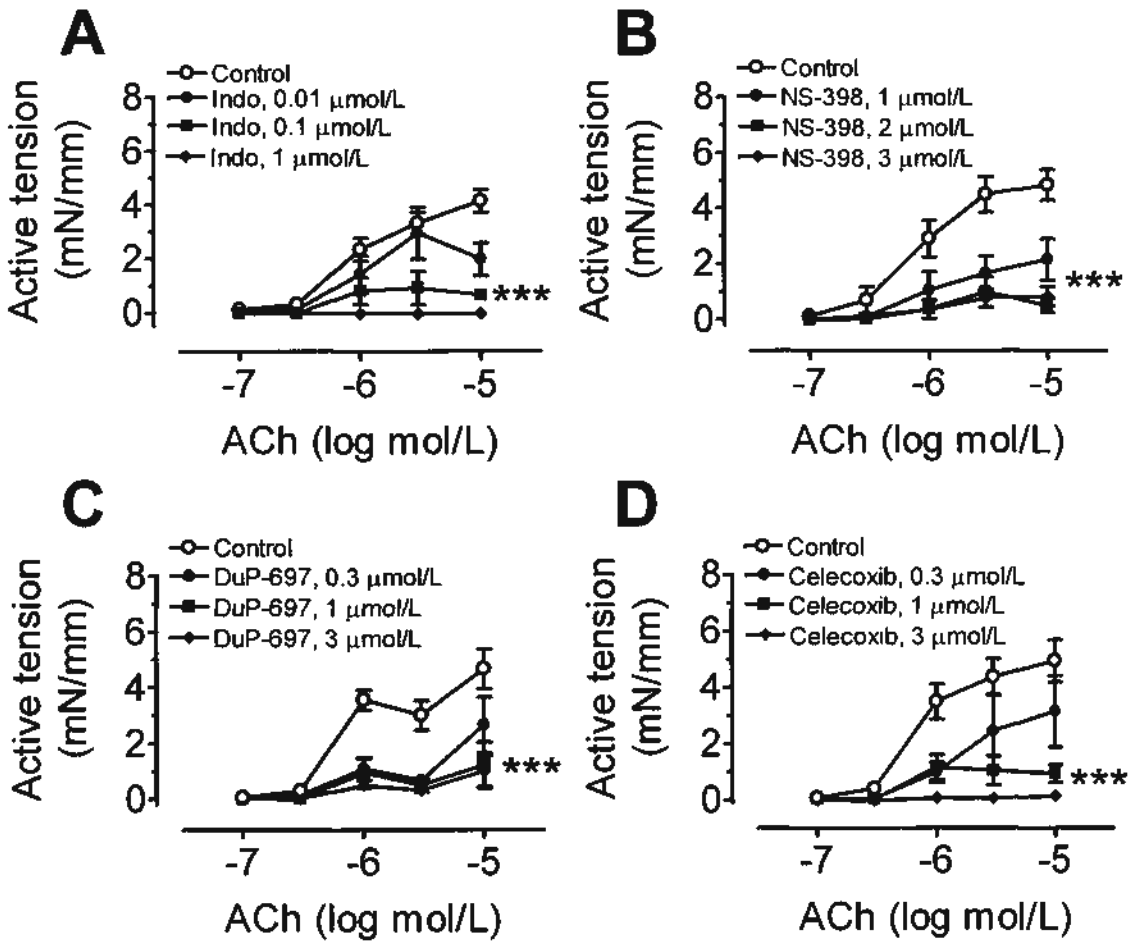

FIGURE 3.2.

Inhibitory effects of a non-selective COX inhibitor indomethacin (Indo) (A, n=6-15) and the selective COX-2 inhibitors, NS-398 (B, n=4-9), DuP-697 (C, n=5-8) or celecoxib (D, n=5-7) on endothelium-dependent contractions. Data are mean ± SEM of n experiments. ***P<0.001 compared with control.

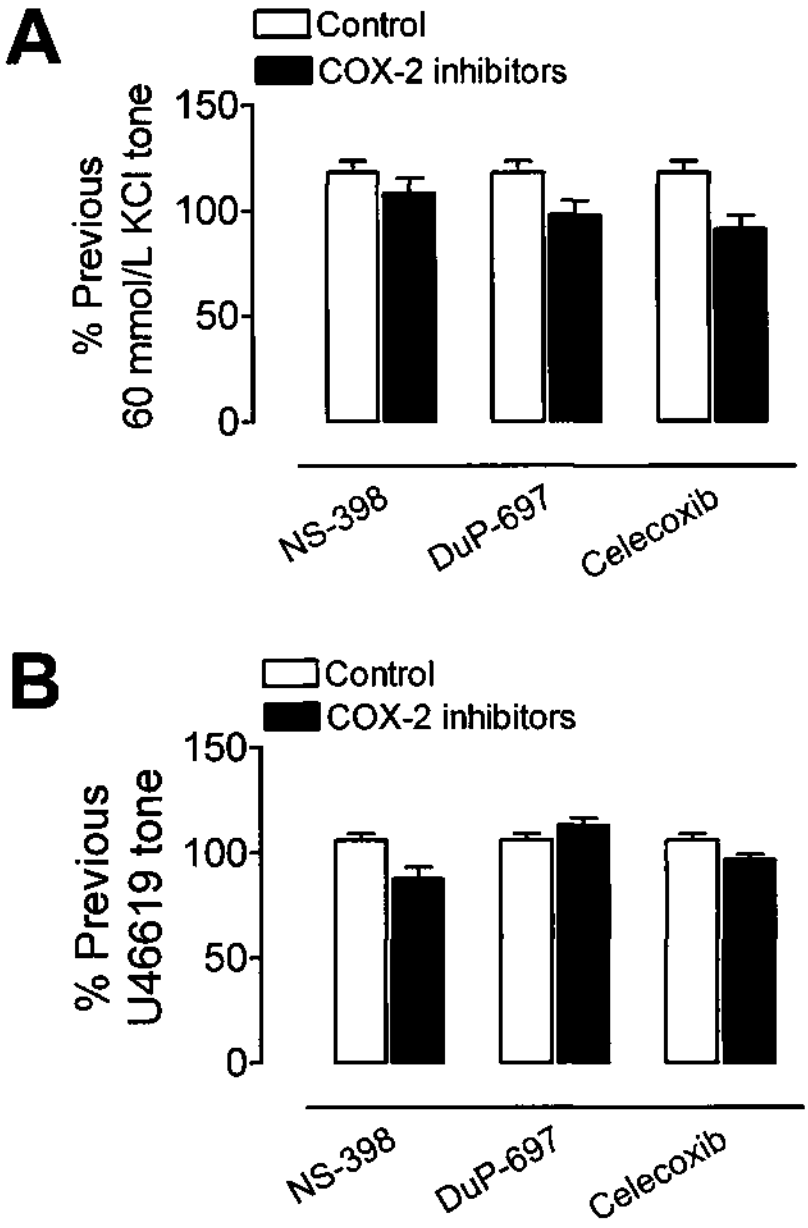

FIGURE 3.3.

Lack of significant effects of COX-2 inhibitors (NS-398, DuP-697 and celecoxib, each at 3 μmol/L) on contractions induced by 60 mmol/L KCl (A) or U46619 (B), confirming the specificity of these inhibitors. Data are mean ± SEM of 4-5 experiments.

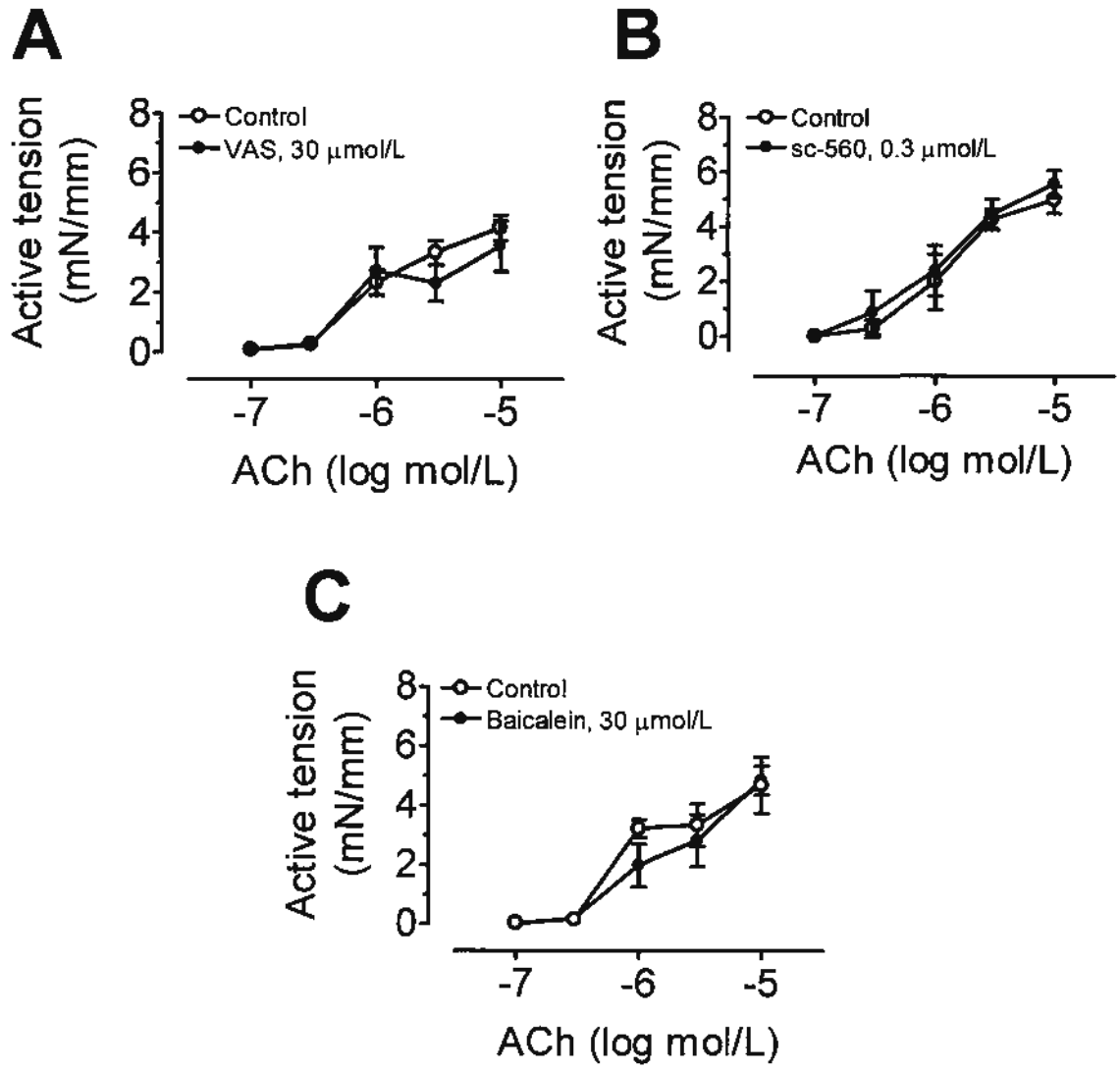

FIGURE 3.4.

Lack of effects of COX-1 inhibitors, valeryl salicylate (VAS, 30 μmol/L, A) and sc-560 (0.3 μmol/L, B), 12-lipoxygenase inhibitor, baicalein (30 μmol/L, C) on acetylcholine-induced endothelium-dependent contractions. Data are mean ± SEM of 5-6 experiments. NS, no statistical significance.

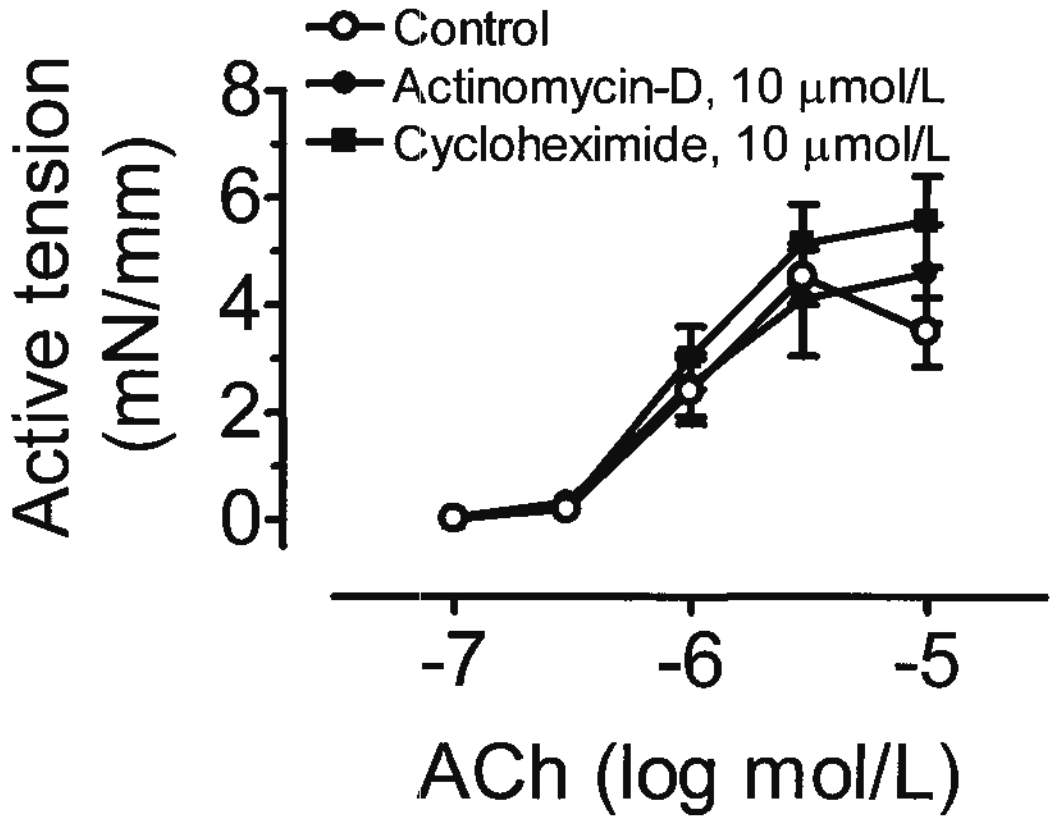

FIGURE 3.5.

Acetylcholine-induced endothelium-dependent contractions were unaffected by transcription inhibitors, the RNA synthesis inhibitor, actinomycin-D (10 μmol/L) or protein synthesis inhibitor, cycloheximide (10 μmol/L). Data are mean ± SEM of 6 experiments.

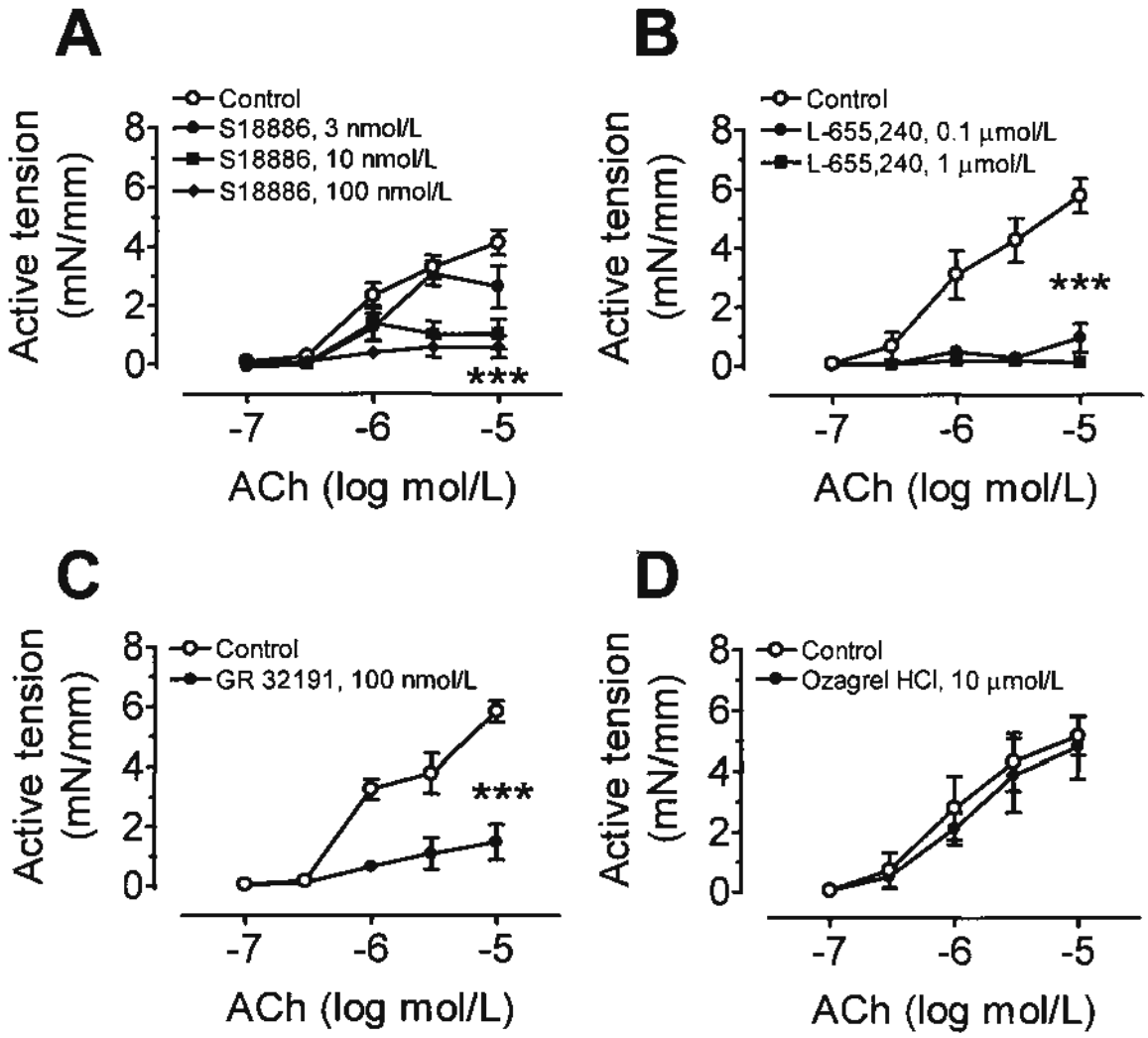

FIGURE 3.6.

Inhibitory effects of the TP receptor antagonists, S18886 (A, n=5-15), L-655,240 (B, n=4-6), and GR 32191 (C, n=5-6) and lack of the effect of the thromboxane synthase inhibitor ozagrel (D, n=5) on endothelium-dependent contractions. Data are mean ± SEM of n experiments. ***P<0.001 compared with control.

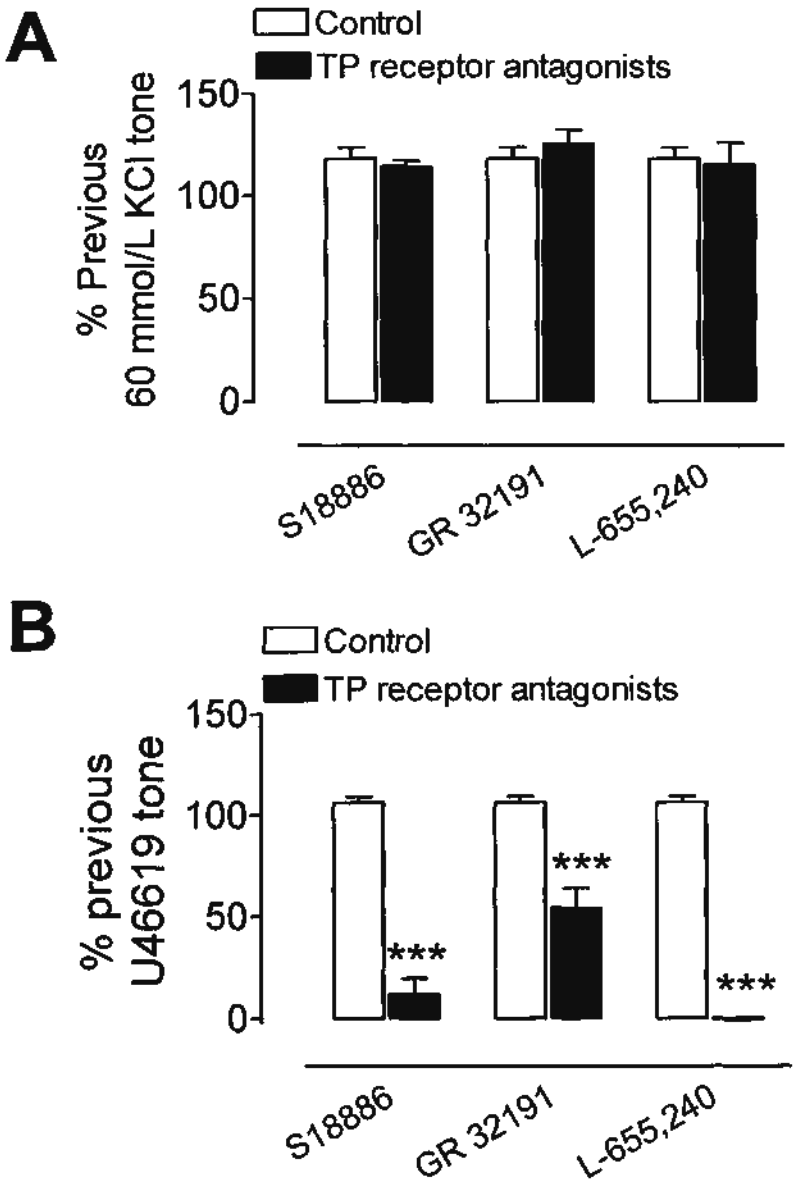

FIGURE 3.7.

(A) Lack of effect of selective TP receptor antagonists (S18886, 100 nmol/L; GR 32191, 100 nmol/L; L-655,240, 1 µmol/L) on contractions induced by 60 mmol/L KCl. (B) These TP receptor antagonists significantly inhibited U46619-induced contractions. Data are mean ± SEM of 4-5 experiments. ***P<0.001 between control and antagonist groups.

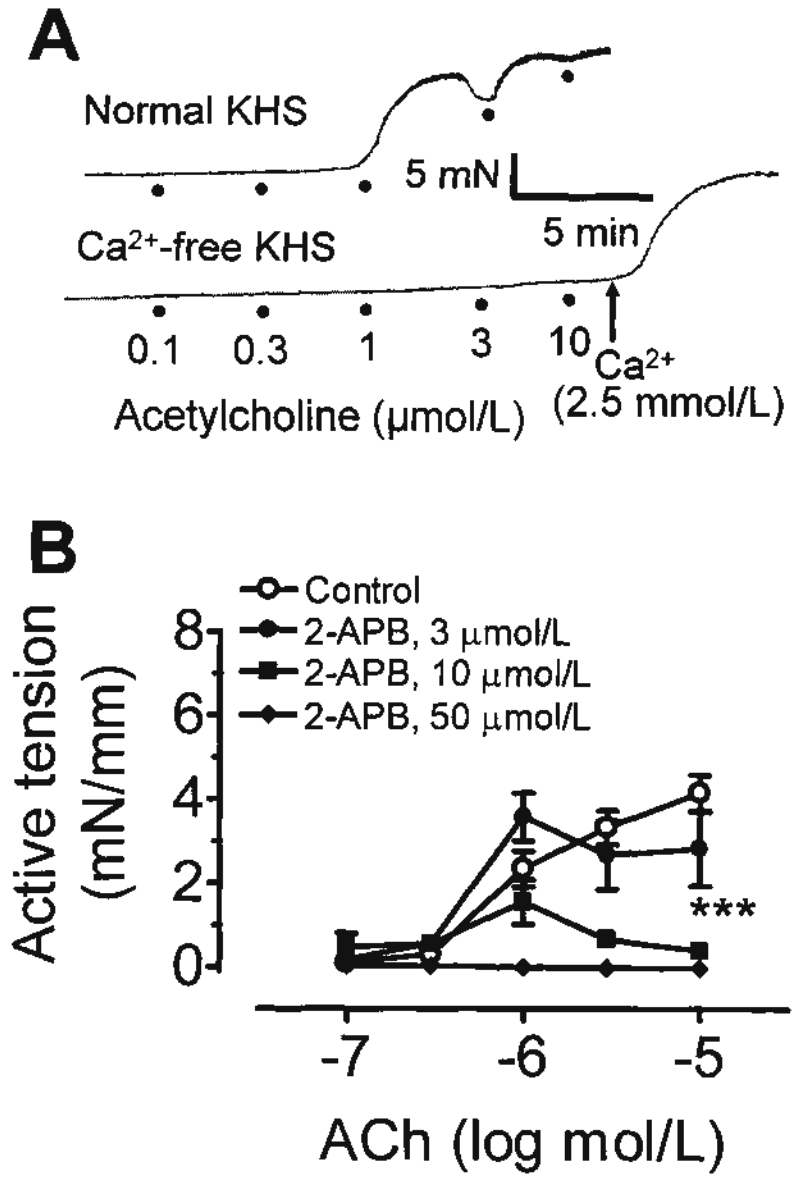

FIGURE 3.8.

(A) Dependency of endothelium-dependent contractions on the presence of extracellular calcium ions. (B) Inhibitory effect of 2-APB on endothelium-dependent contractions. The scale bar applies to both traces. Data are mean ± SEM of 5-15 experiments. ***P<0.001 compared with control.

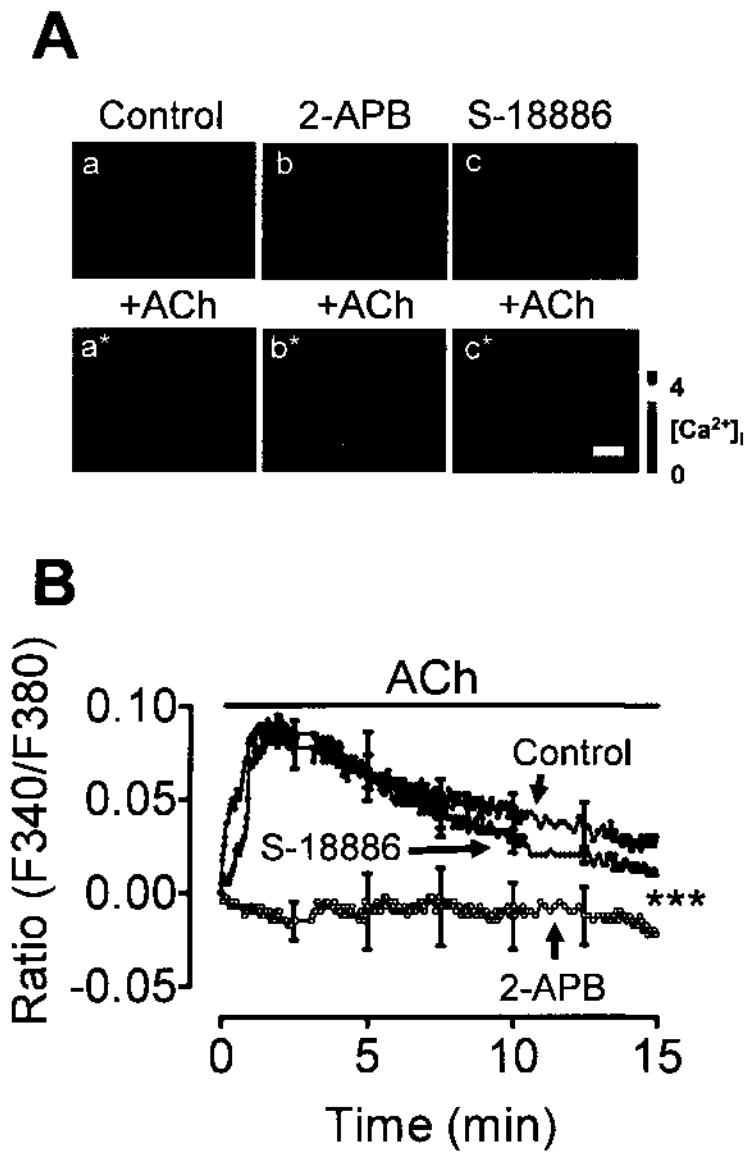

FIGURE 3.9.

(A) Calcium levels in *en face* preparations of native endothelial cells of the aorta measured by ratiometric fluorescence imaging. Images showing increases in $[Ca^{2+}]_i$ *in situ* induced by acetylcholine (a*) in the presence of L-NAME, which was abolished by 50 µmol/L 2-APB (b*), but unaffected by 0.1 µmol/L S18886 (c*). The scale bar represents 50 µm. (B) Time course of the effect of acetylcholine on $[Ca^{2+}]_i$ of endothelial cells under different treatments. Data are mean ± SEM of 4 experiments. ***P<0.001 compared with control.

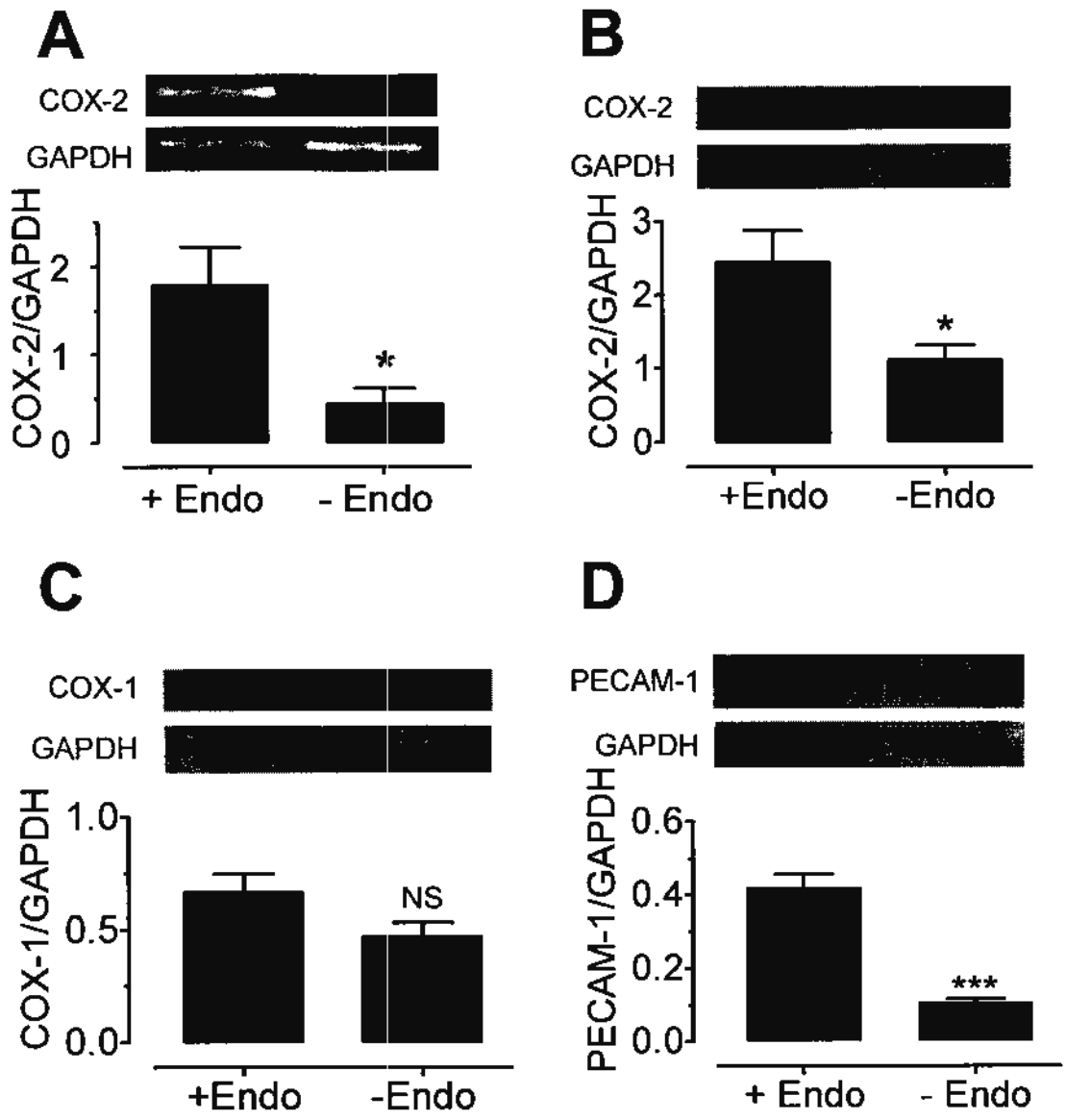

FIGURE 3.10.

Reduced levels of COX-2 mRNA and protein in preparations without, compared with those with endothelium, as revealed by RT-PCR (A, n=5) and Western blot analysis (B, n=8). COX-1 expression was similar in hamster aorta with or without endothelium (C, n=6). Western blotting for an endothelial cell marker, PECAM-1 confirmed the removal of endothelium (D, n=7). Data are mean ± SEM of n experiments. *P<0.05 and ***P<0.001 between rings with and without endothelium.

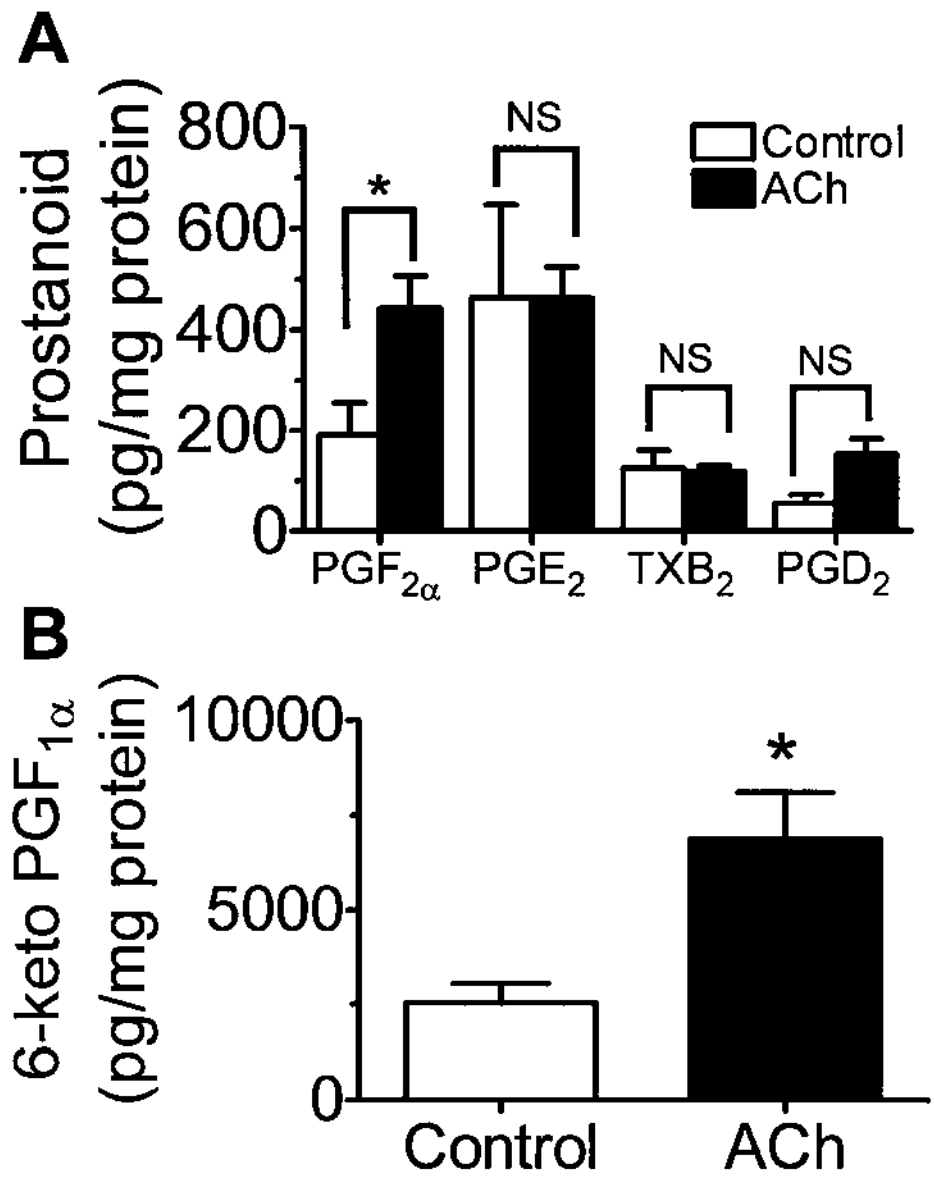

FIGURE 3.11.

EIA measurement of the putative arachidonic acid metabolites, $PGF_{2\alpha}$, PGE_2, TxB_2, PGD_2 (A) and 6-keto $PGF_{1\alpha}$ (B) in the bathing solution of L-NAME-treated aortae with and without exposure to acetylcholine (ACh vs Control). Among the five prostanoids, only the release of $PGF_{2\alpha}$ and 6-keto $PGF_{1\alpha}$ was augmented significantly by acetylcholine. Data are mean ± SEM of 3-5 experiments. *P<0.05 compared with control. NS, no statistical significance.

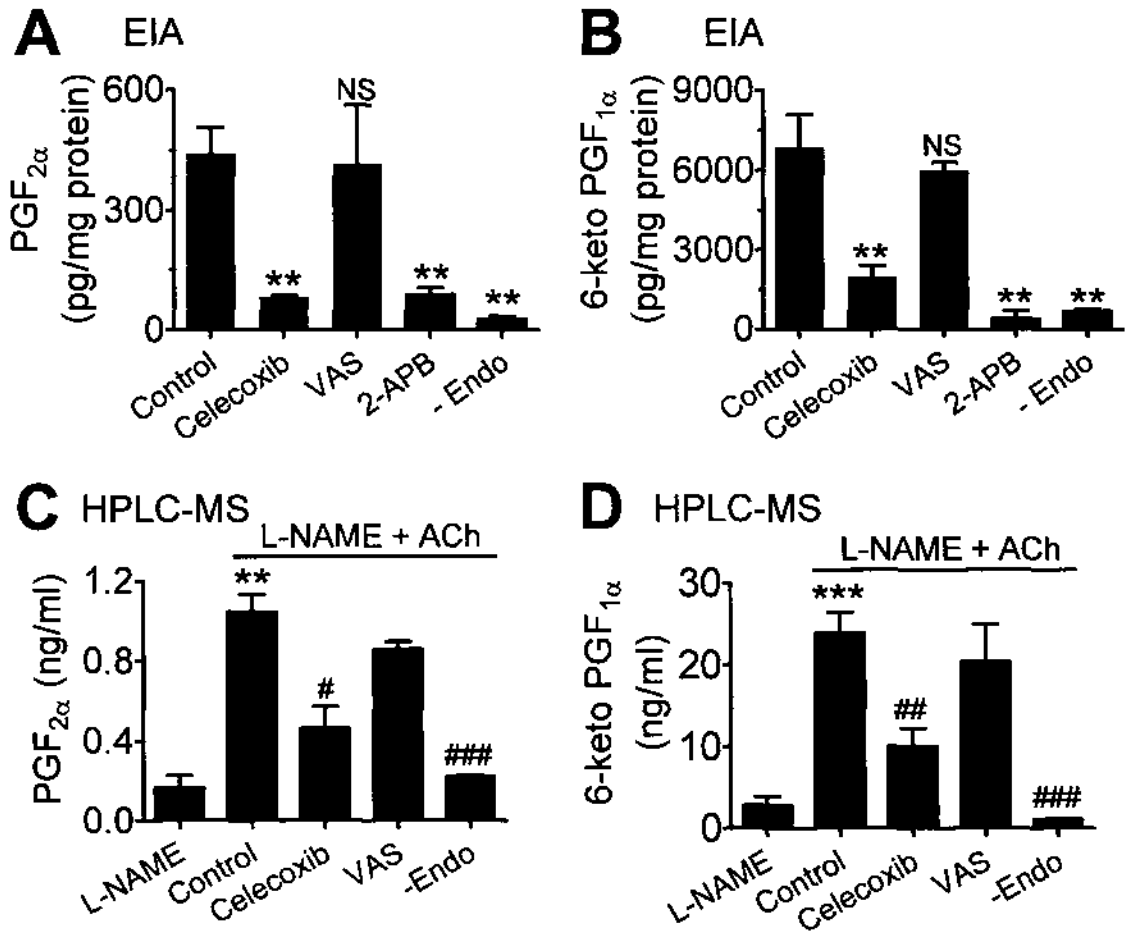

FIGURE 3.12.

Release of $PGF_{2\alpha}$ and 6-keto $PGF_{1\alpha}$ measured by EIA (A&B) and HPLC-MS (C&D). The ACh-stimulated release of $PGF_{2\alpha}$ (A&C) and 6-keto $PGF_{1\alpha}$ (B&D) depended on the presence of endothelium and was inhibited by celecoxib or 2-APB but unaffected by VAS. Data are mean ± SEM of 3-5 experiments. $^{*}P<0.05$ and $^{**}P<0.01$ compared with control. NS, no statistical significance.

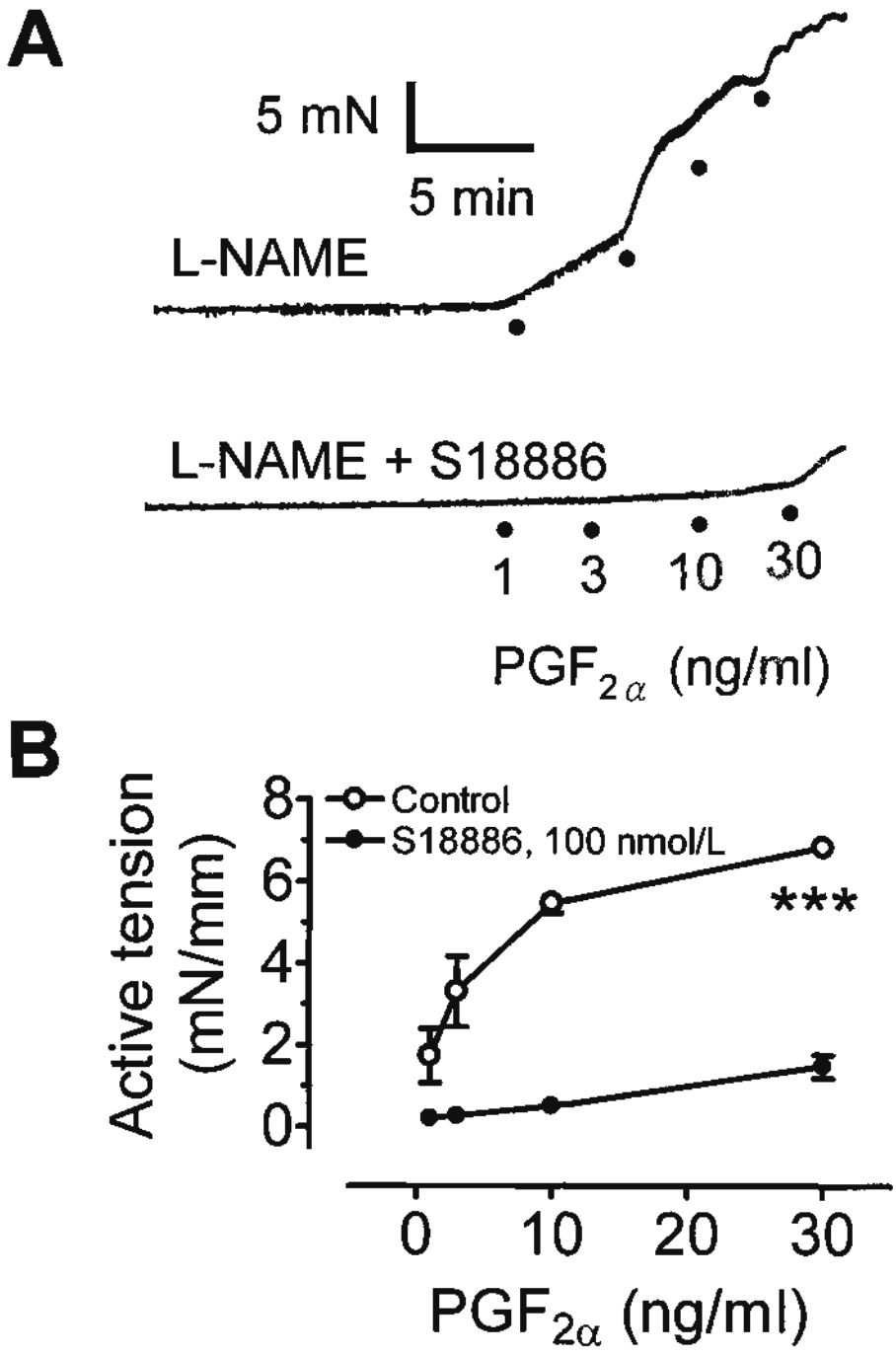

FIGURE 3.13.

Trace (A) and summarized results (B) showing the responses of L-NAME-treated rings with endothelium to $PGF_{2\alpha}$. Inhibitory effect of 0.1 µmol/L S18886 on PGF2α-induced contractions (A&B). Data are mean ± SEM of 5 experiments. ***P<0.001 compared with control.

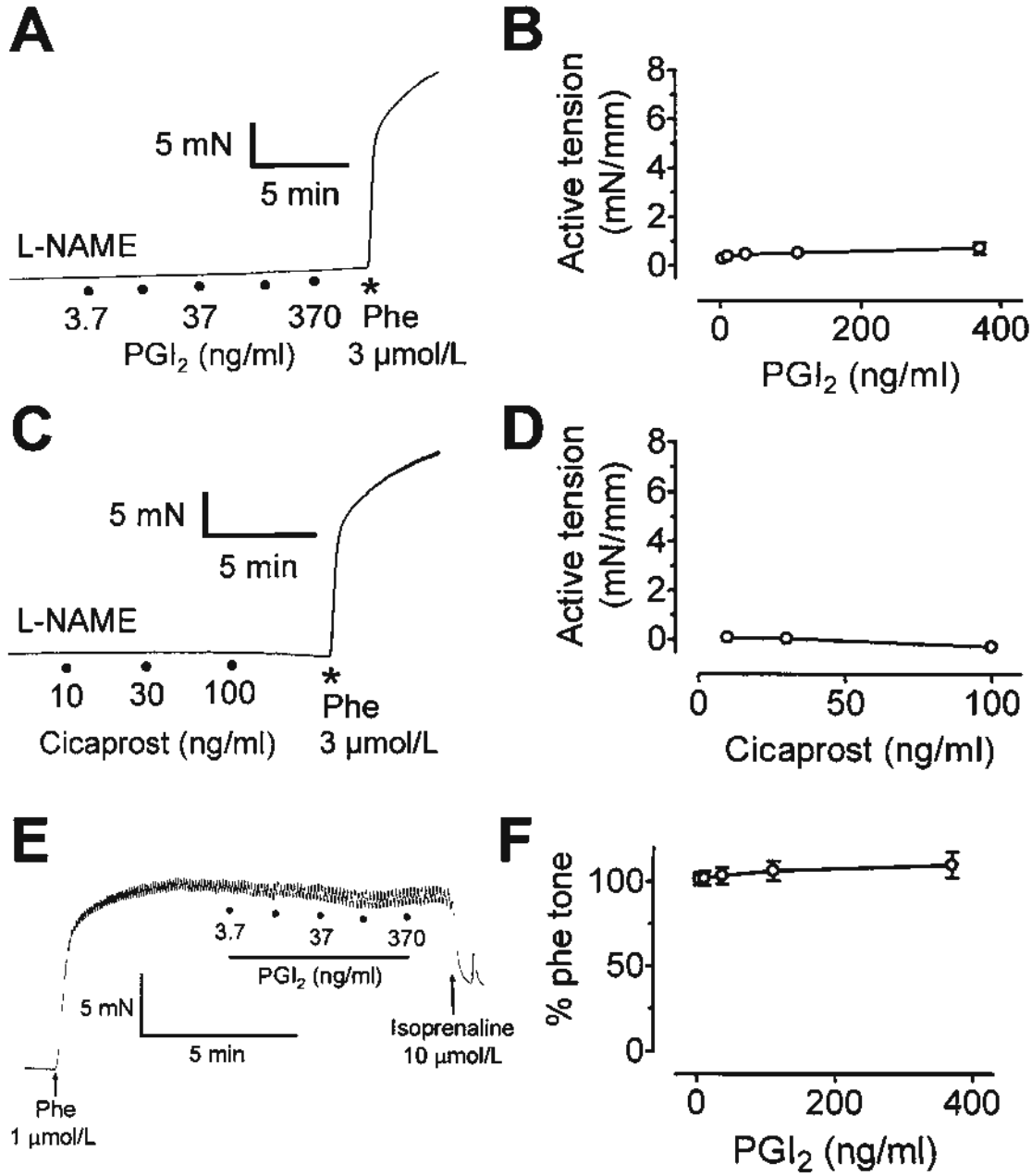

FIGURE 3.14.

Trace and summarized results showing the responses of L-NAME-treated rings with endothelium to PGI_2 (A&B) and cicaprost (C&D). (E&F) PGI_2 did not relax pre-contracted aortae. Data are mean ± SEM of 5 experiments. ***P<0.001 compared with control.

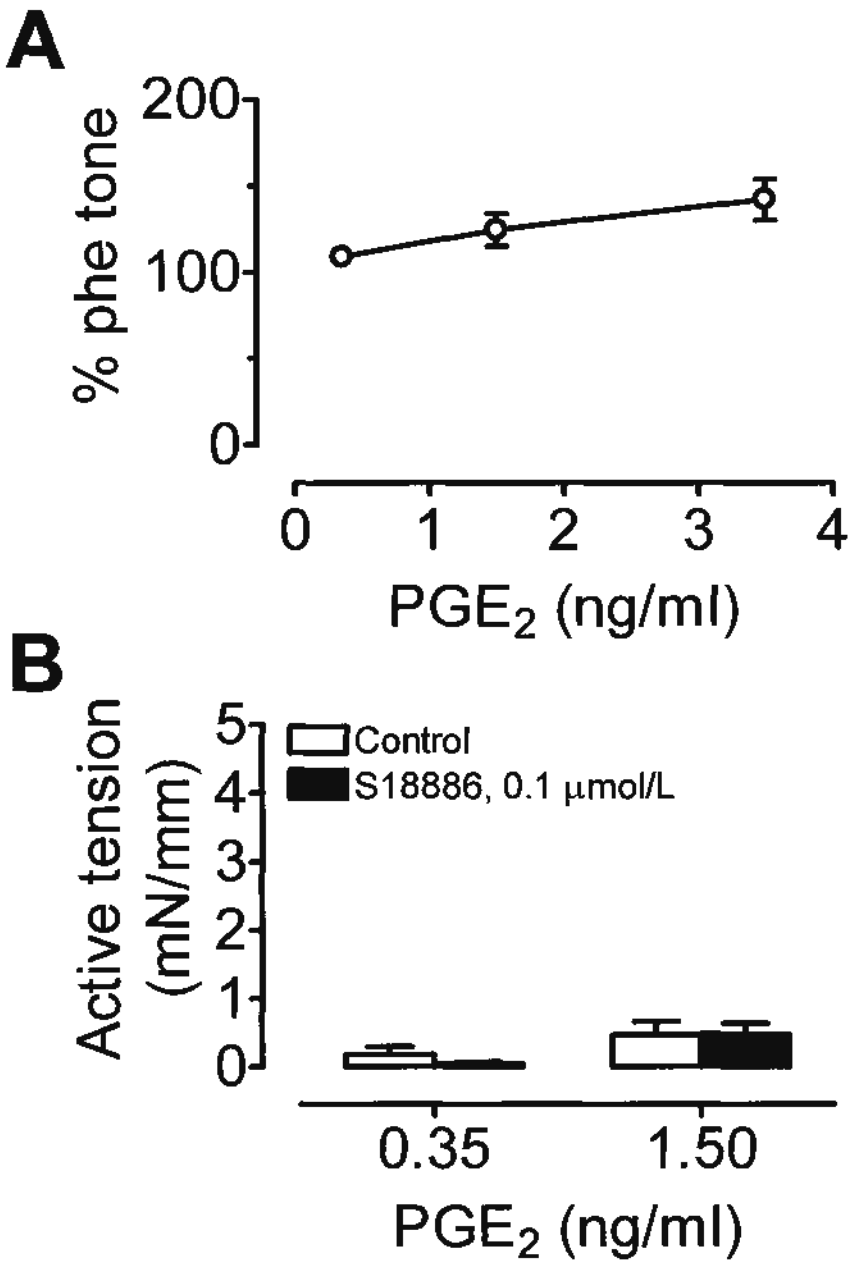

FIGURE 3.15.

(A) PGE_2 did not relax but caused contractions in the aortae. These contractions were not inhibited by TP receptor antagonist, S18886 (0.1-0.3 µmol/L, B). Data are mean ± SEM of 4 experiments.

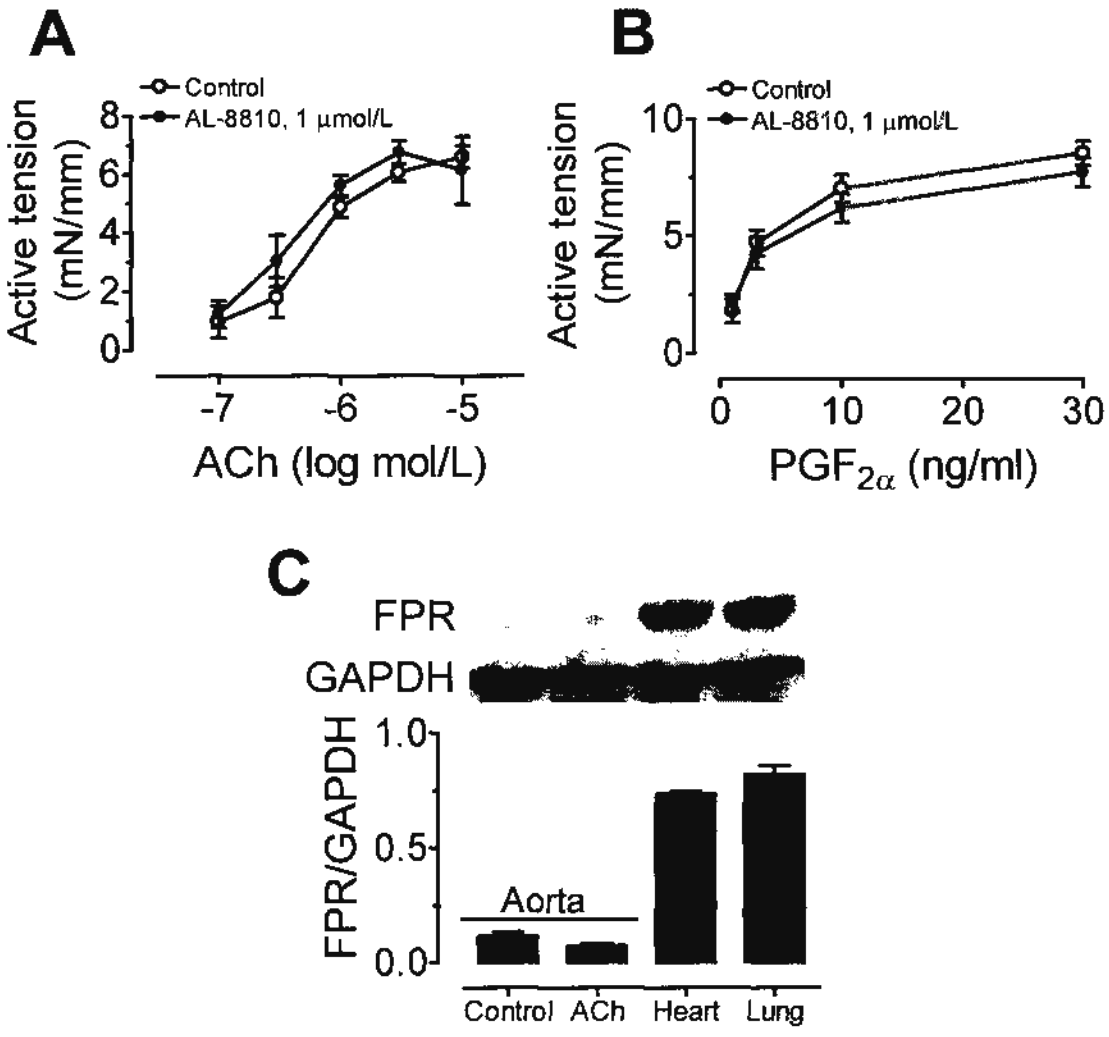

FIGURE 3.16.

Lack of effect of the FP receptor antagonist AL-8810 (1 µmol/L) on contractions induced by acetylcholine (A) and $PGF_{2\alpha}$ (B) in the L-NAME-treated aortae. (C) The protein expression level of FP receptor (FPR) in hamster aortae, heart and lung. Data are mean ± SEM of 5 experiments.

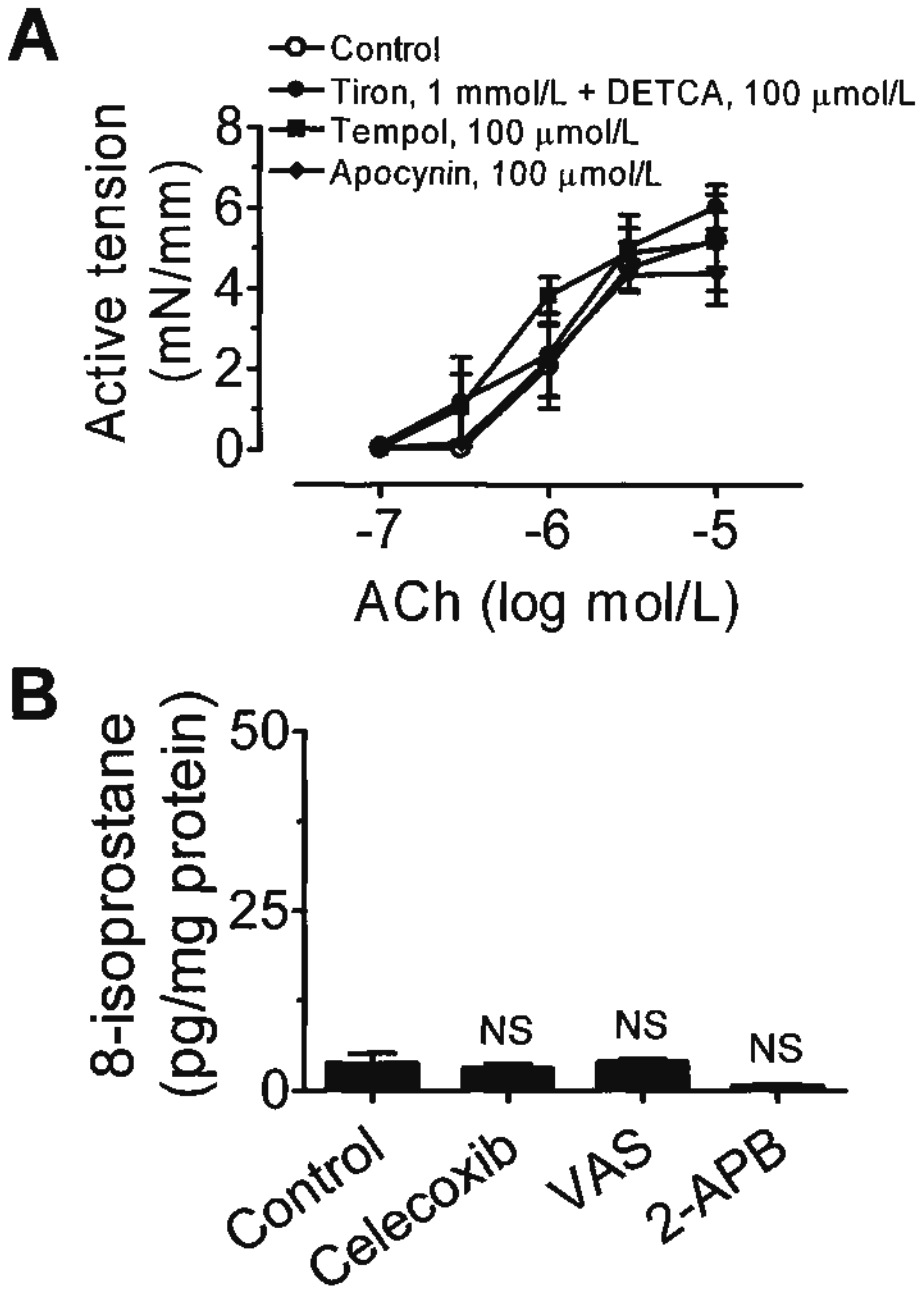

FIGURE 3.17.

(A) Lack of effects of free radical scavengers (1 mmol/L tiron plus 100 μmol/L DETCA or 100 μmol/L tempol) or an NADPH oxidase inhibitor, apocynin (100 μmol/L) on endothelium-dependent contractions. (B) EIA measurement of 8-isoprostane in the bathing solution of L-NAME-treated aortic rings exposed to acetylcholine; the level of 8-isoprostane was not affected by celecoxib, VAS or 2-APB. Data are mean ± SEM of 4 experiments.

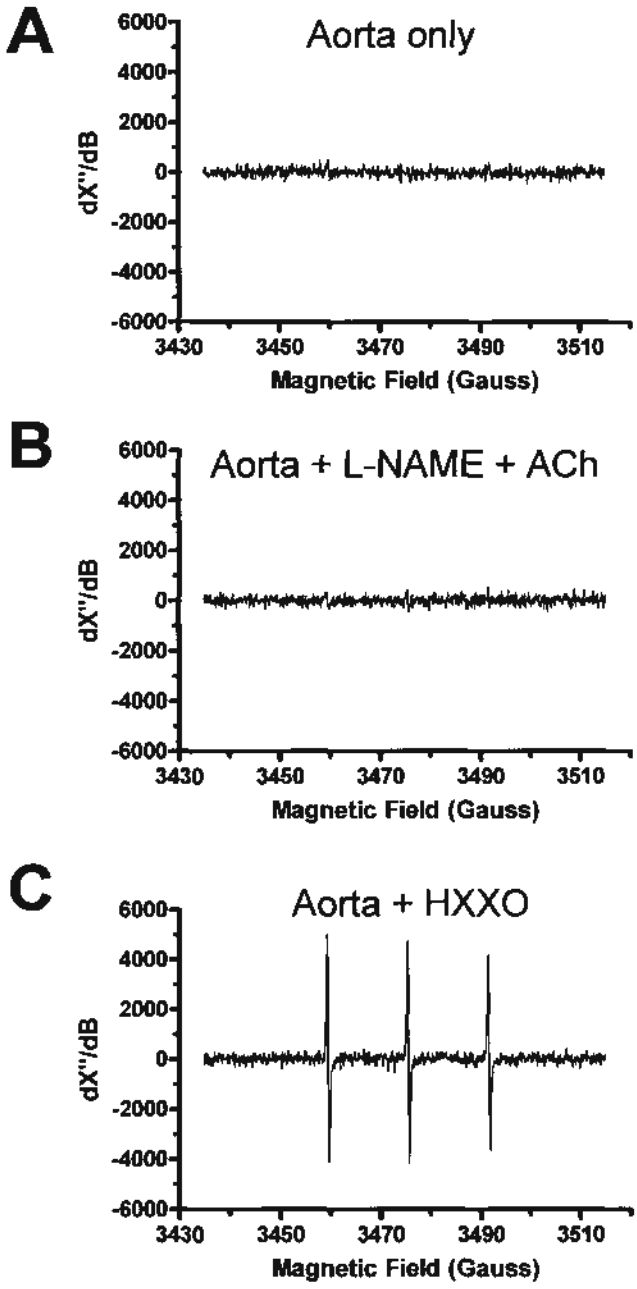

FIGURE 3.18.

Recordings with the electron paramagnetic resonance technique showing the absence of ROS signal in a control aorta (A) and in an L-NAME-treated aorta exposed to acetylcholine (B). (C) ROS signal as positive control in an aorta after addition of hypoxanthine plus xanthine oxidase. Data are mean ± SEM of 3 experiments.

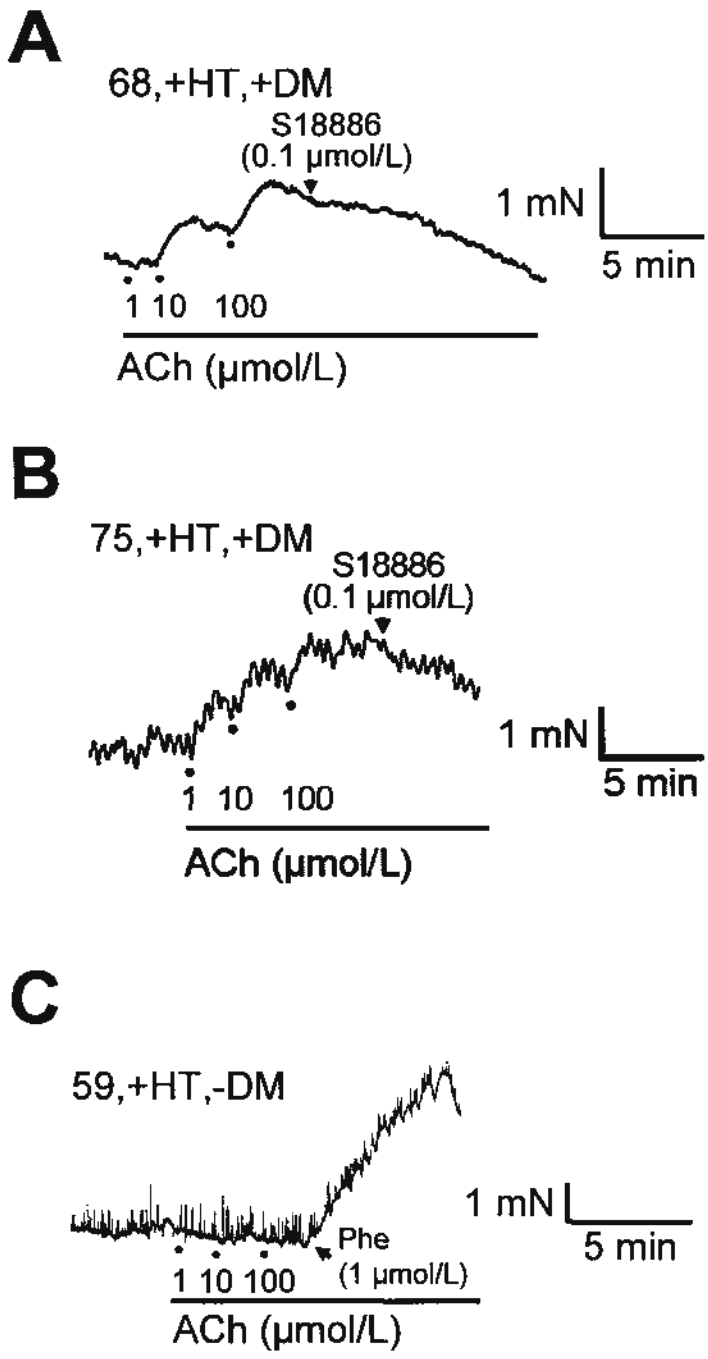

FIGURE 3.19.

ACh-induced contractions in human renal arteries from patients with hypertension and diabetes at age of 68 (A) and 75 (B) and from patient with hypertension only at age of 59 (C). The number indicates the age of the patient; HT, hypertension; DM, diabetes.

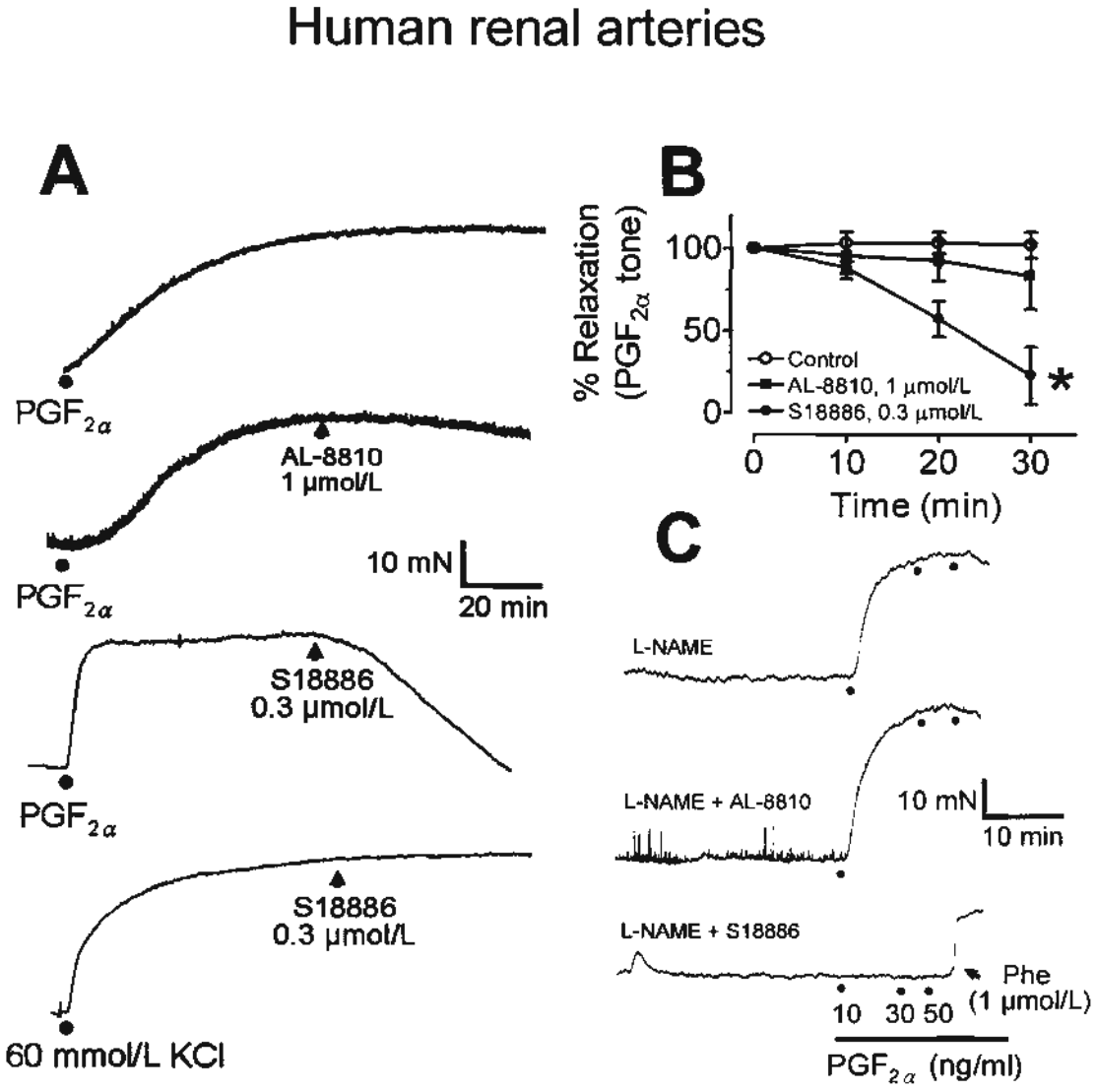

FIGURE 3.20.

(A&B) TP receptor antagonist (S18886) but not FP receptor antagonist (AL-8810) reversed $PGF_{2\alpha}$-induced contractions within 30 minutes after addition of the antagonists (n=4). (C) Pre-treatment with S18886, but not AL-8810, prevented $PGF_{2\alpha}$-induced contractions. Data are mean ± SEM of 3-4 experiments. *P<0.05 compared with control.

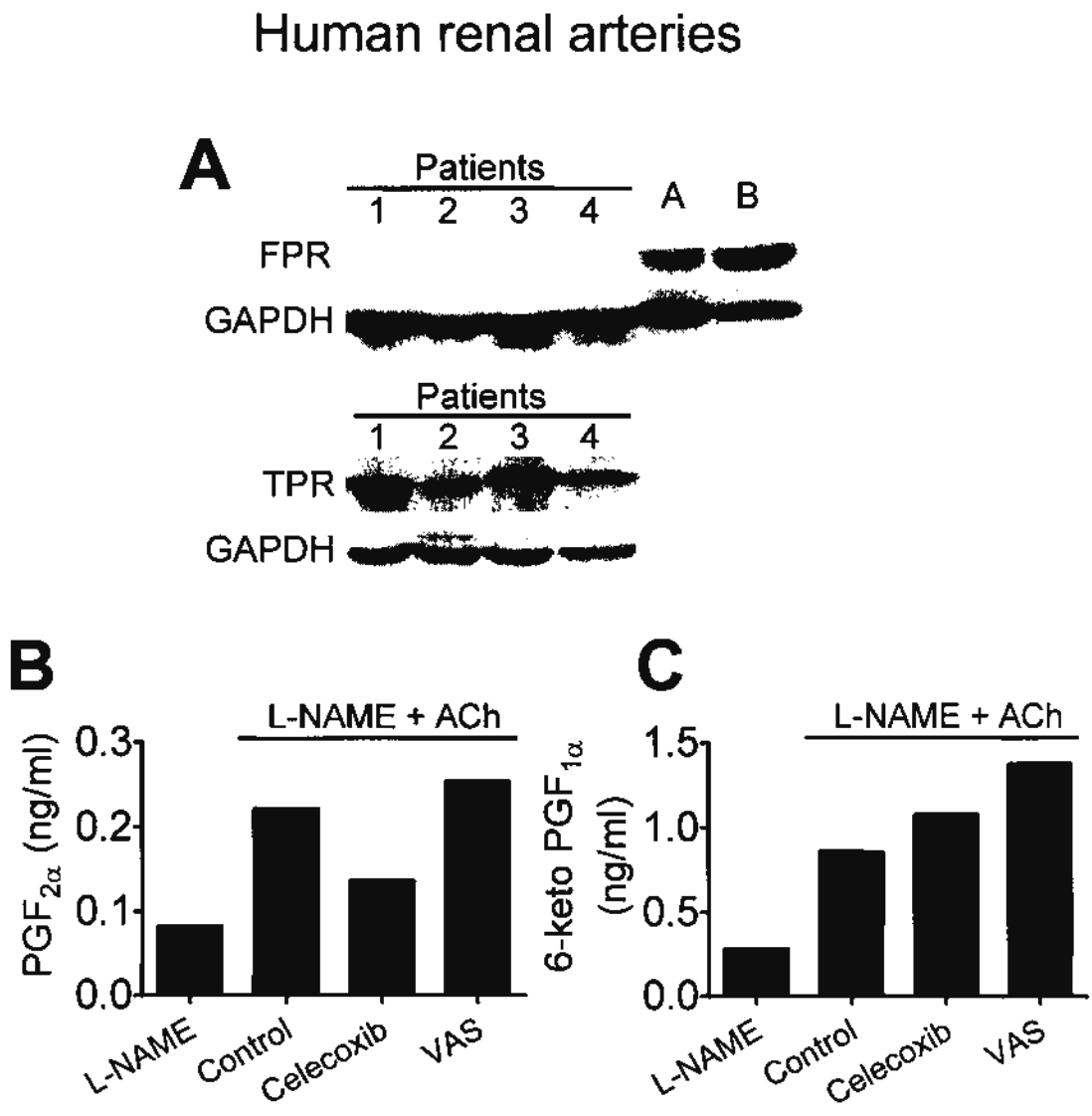

FIGURE 3.21.

(A) Western blotting analysis showed that human renal arteries expressed the TP receptor (TPR) but not the FP receptor (FPR). Lanes A (hamster heart) & B (hamster lung) served as positive controls. Increased release of (B) $PGF_{2\alpha}$, but not (C) PGI_2 (assayed in form of 6-keto $PGF_{1\alpha}$) was inhibited by celecoxib and not by VAS in human renal arteries from two hypertensive and diabetic patients (data are average of two patients).

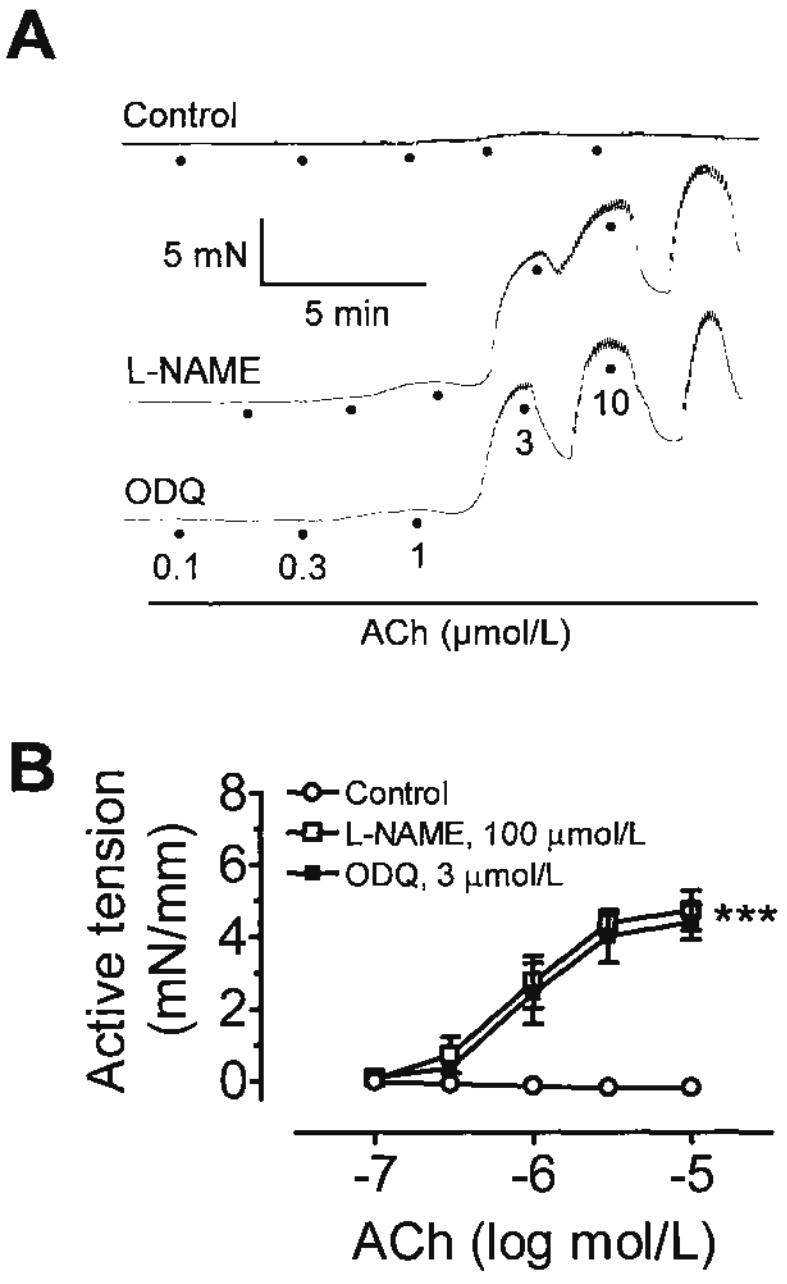

FIGURE 3.22.

(A) Representative traces of ACh-induced contractions in the presence of L-NAME and ODQ. (B) L-NAME (100 μmol/L) and ODQ (3 μmol/L) unveiled similar ACh-induced contractions. Data are presented as mean ± SEM of 5-6 experiments from different rats. ***P<0.001 compared with control.

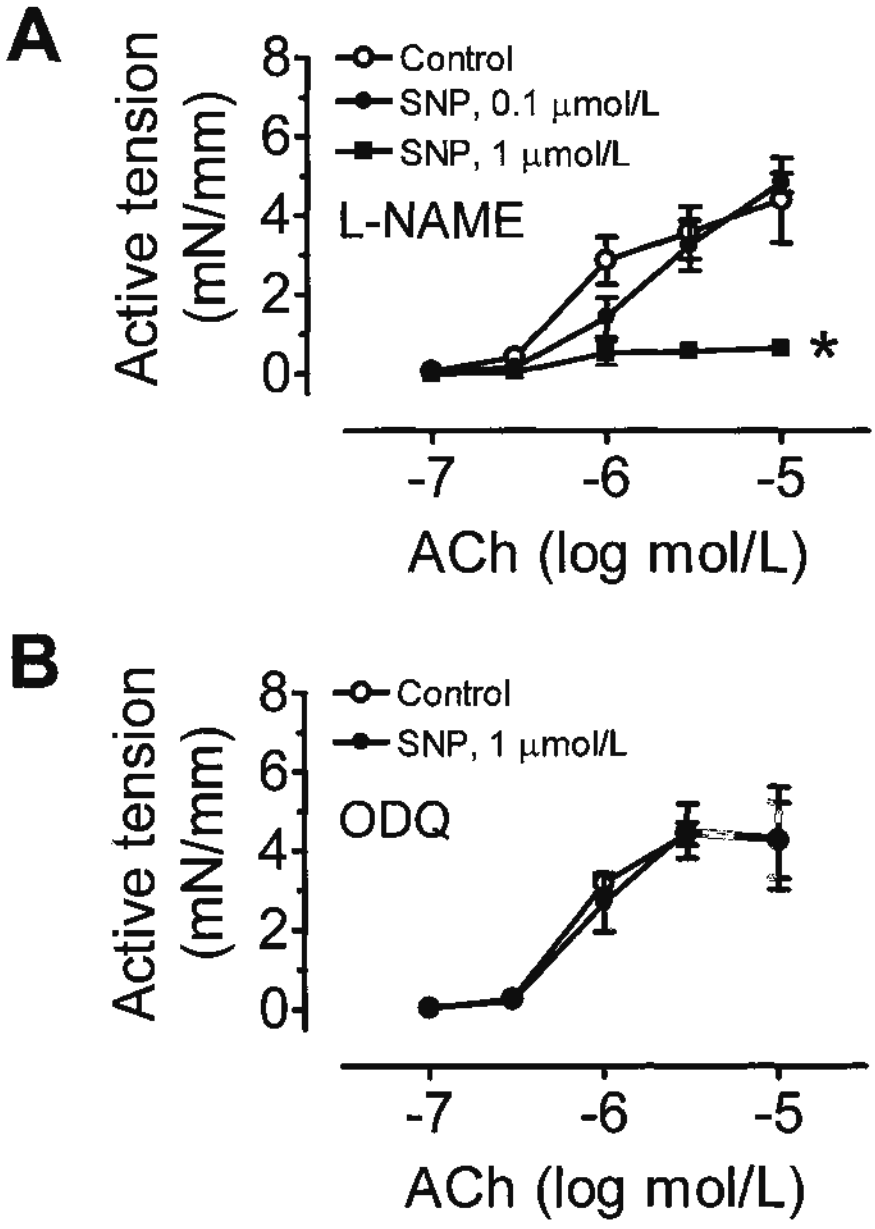

FIGURE 3.23.

SNP at 1 μmol/L prevented ACh-induced contractions unmasked by (A) L-NAME but not those by (B) ODQ. Data are presented as mean ± SEM of 4 experiments from different rats. *$P<0.05$ compared with control.

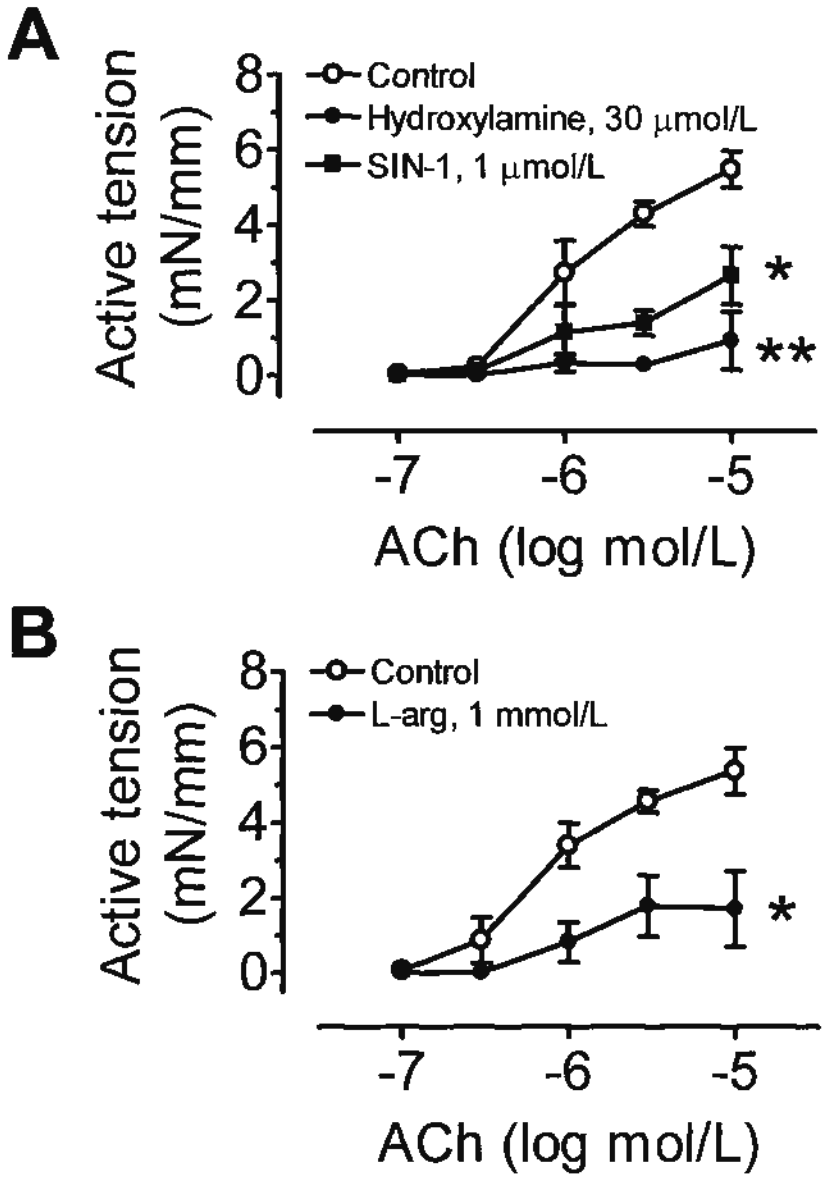

FIGURE 3.24.

(A) Inhibitory effects of hydroxylamine (30 μmol/L), SIN-1 (1 μmol/L) and (B) L-arginine (L-arg, 1 mmol/L) on ACh-induced contractions in the presence of L-NAME. Data are presented as mean ± SEM of 4-5 experiments from different rats. *P<0.05 and **P<0.01 compared with control.

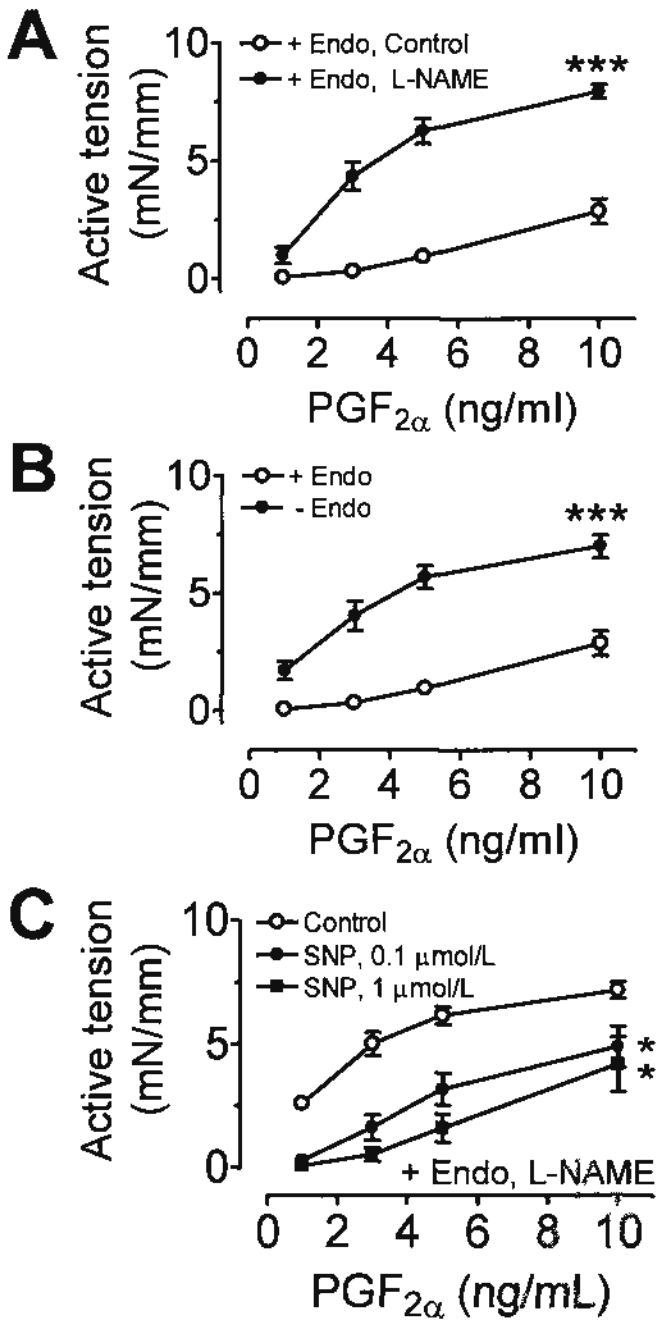

FIGURE 3.25.

$PGF_{2\alpha}$-induced vasoconstrictions were potentiated by (A) L-NAME or (B) removal of endothelium. (C) SNP at 0.1 μmol/L and 1 μmol/L suppressed $PGF_{2\alpha}$-induced contractions in the presence of L-NAME. Data are presented as mean ± SEM of 4-6 experiments from different rats. **P*<0.05 and ****P*<0.001 compared with control or + Endo.

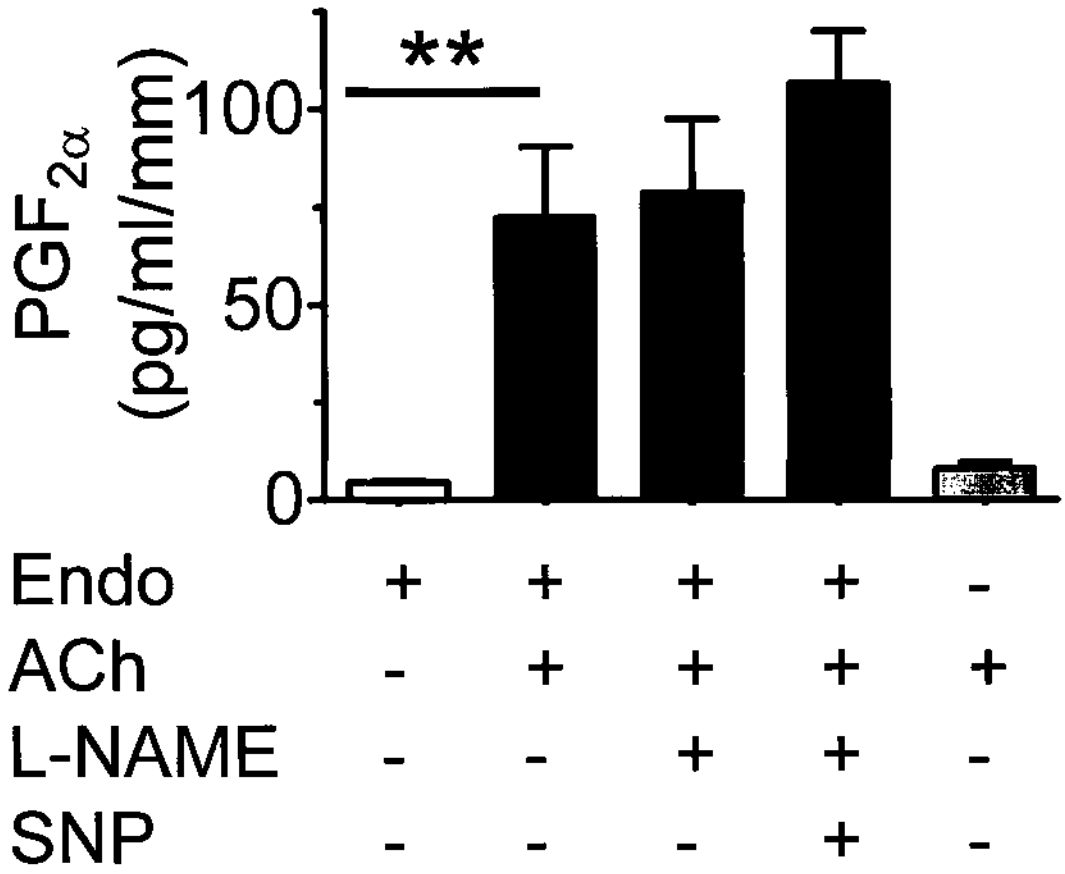

FIGURE 3.26.

ACh induced endothelium-dependent release of $PGF_{2\alpha}$, which was unaffected by the presence of L-NAME and SNP. Data are presented as mean ± SEM of 4 samples from different rats. $^{**}P<0.01$ compared with control.

3.4 Discussion

Endothelium-dependent contractions are observed generally in arteries of aged or diseased animals, including high fat diet-induced obese mice, spontaneously hypertensive rats and diabetic rats (Shi et al., 2007; Traupe et al., 2002; Vanhoutte et al., 2005), in which endothelial function is already impaired. The present study demonstrates that in the aorta of young and healthy hamsters endothelium-dependent contractions can be evoked via COX-2-mediated production of $PGF_{2\alpha}$ which acts on the TP receptor in vascular smooth muscle cells. Our studies on human renal arteries revealed that this pathway could be of relevance also in humans.

Arachidonic acid, released from cell membranes by phospholipases, can be metabolized via different pathways to generate vasoactive substances. Lipoxygenases convert arachidonic acid to HPETEs (hydroperoxyeicosatetraenoic acids) and then to either HETEs (hydroxyeicosatetraenoic acids) or leukotrienes. Cyclooxygenases oxygenate arachidonic acid to form PGG_2 and PGH_2, which are further converted to various prostanoids including PGD_2, PGE_2, $PGF_{2\alpha}$, TxA_2 and PGI_2 via their respective synthases (Simmons et al., 2004). In the present study, baicalein was used to inhibit the lipoxygenase pathway, yet this caused no suppression of the acetylcholine-induced endothelium-dependent contractions. By contrast, incubation with a relatively low concentration of indomethacin abolished the response. These observations permit the conclusion that arachidonic acid metabolites formed under the catalytic action of cyclooxygenases are the most likely EDCF candidate(s) mediating the endothelium-dependent contractions in the aorta of healthy hamsters.

COX-1 is known to be expressed constitutively in most tissues while

COX-2 is highly inducible by pro-inflammatory cytokines, tumor promoters and mitogens (Fries and Grosser, 2005). Recent studies suggest that COX-2 is also constitutively expressed in the kidney, brain and arteries (Baber et al., 2003; FitzGerald and Patrono, 2001; Therland et al., 2004). In the cardiovascular system, endothelial cells express COX-2 in response to shear stress under normal physiological condition (Topper et al., 1996). When COX-2 is present, it contributes to PGI_2 synthesis (Bolego et al., 2006; McAdam et al., 1999), and can activate silent reservoirs of PGI_2 synthase in most tissues (Klumpp et al., 2005). In the present study, the endothelium-dependent contractions of the aorta from healthy hamsters were mediated by COX-2 while the constitutively expressed COX-1 did not play a significant role, as evidenced by the pronounced attenuation of the response by NS-398, DuP-697 and celecoxib (COX-2 inhibitors), but not by sc-560 and VAS (COX-1 inhibitors). Inhibition of RNA synthesis and protein synthesis by actinomycin-D and cycloheximide, respectively, did not alter the endothelium-dependent contractions, indicating that COX-2 was expressed constitutively in the aorta and that its presence was not induced acutely by acetylcholine. The molecular biological comparison of aortae with and without endothelium permitted the conclusion that COX-2 mRNA and protein expressions, demonstrated using RT-PCR and Western blot analysis, respectively, are localized mainly in the endothelium. Thus, endothelial COX-2 appears to represent the major enzyme responsible for the generation of EDCF(s) in the aorta of healthy hamsters. Upon the production of EDCF(s) by COX-2 in endothelial cells, it diffuses to the vascular smooth muscle cells where it acts on the TP receptor to cause contraction. The involvement of TP receptors was demonstrated in the present study by the use of specific antagonists (S18886, GR 32191 and L-655,240), which markedly

decreased or abolished the endothelium-dependent contractions evoked by acetylcholine. In contrast to the well-expressed TP receptor, the FP receptor is minimally expressed in hamster aortae. Besides, ACh- or $PGF_{2\alpha}$-induced contractions were not reduced by the FP receptor antagonist, AL-8810, thus discounting a significant role of the FP receptor in the endothelium-dependent contractions although its natural agonist $PGF_{2\alpha}$ is proposed to be the EDCF in hamster aortae.

The present data show that Ca^{2+} influx into endothelial cells is crucial for the occurrence of the endothelium-dependent contractions. This conclusion is based on the observation that preparations incubated in Ca^{2+}-free solution showed no contraction until Ca^{2+} was re-introduced into the bathing solution. Ca^{2+} ions possibly enter endothelial cells via non-selective cation channels, as evidenced by the effect of 2-APB in attenuating endothelium-dependent contractions and abolishing the acetylcholine-stimulated elevation of $[Ca^{2+}]_i$ in *in situ* imaging of endothelial cells. In addition, 2-APB inhibited the acetylcholine-induced release of COX-2-derived prostanoids in the aorta with endothelium, while it did not affect U46619- or KCl-induced contraction of aortic rings, illustrating that it acts on the endothelial cells.

By comparing the results from EIA and HPLC-MS and the subsequent functional studies performed using the myograph with exogenously added prostanoids, $PGF_{2\alpha}$ appears to be the most likely EDCF candidate. Indeed, $PGF_{2\alpha}$ was released endogenously from the aortic endothelium in physiological amounts which correspond to its potent effect in eliciting contraction of the smooth muscle. Although PGI_2 was also released in considerable amounts, it failed to evoke any contraction or relaxation *per se*, even at a concentration fifty times higher than the one detected, suggesting that it may not contribute to

endothelium-dependent contractions as it does in the aorta of spontaneously hypertensive rats (Gluais et al., 2006; Gluais et al., 2007). The present study can discount the possible involvement of PGE_2 because its release was not stimulated by acetylcholine and the small contractions induced by exogenous PGE_2 were insensitive to the TP receptor antagonism.

Since TP receptors are involved in the response, it is logical to speculate that TxA_2 may contribute to endothelium-dependent contractions in the hamster aorta. However, this possibility is made unlikely by two observations. First, in the presence of ozagrel, a thromboxane synthase inhibitor (Buccellati et al., 2002), endothelium-dependent contractions to acetylcholine remained unaltered. Second, acetylcholine did not increase the release of TxA_2. Thus TxA_2 is not a major EDCF candidate in the hamster aorta. By contrast, TxA_2 contributes to the endothelium-dependent contractions of the canine basilar artery and the SHR aorta (Gluais et al., 2006; Katusic et al., 1988).

COX is involved in the generation of ROS in vascular tissues (Tang et al., 2007), which are normally neutralized by NO. It appears necessary to test whether ROS play a role in the production and the action of EDCF, since L-NAME inhibits NO production and unmasks the endothelium-dependent contractions. The present evidence from functional, EPR and EIA studies points against such a possibility. In the functional studies, neither free radical scavengers nor an NADPH oxidase inhibitor attenuated the endothelium-dependent contractions to acetylcholine. The EPR study showed that basal levels of superoxide anion and peroxynitrite formed by the combination of superoxide and NO (Szabo et al., 2007), were undetectable in the hamster aorta, and remained so even in the presence of L-NAME and acetylcholine. The

EIA study demonstrated that 8-isoprostanes, which are generated *in vivo* by the free radical-catalyzed, non-enzymatic peroxidation of arachidonic acid (Morrow, 2006), were released in very small amounts compared with other prostaglandins. Thus, in the healthy blood vessels examined in the present studies ROS play no role in catalyzing the production and release of EDCF, unlike in the aorta of the SHR (Yang et al., 2002).

The preliminary results obtained in human renal arteries imply that a COX-2 metabolite, possibly $PGF_{2\alpha}$, produces TP receptor-dependent vasoconstrictions consistent with the observations on hamster aortae. In addition, we provide preliminary evidence that the release of $PGF_{2\alpha}$ but not that of PGI_2 in response to ACh was inhibited by celecoxib, thus indirectly indicating a crucial role of COX-2 in ACh-induced contractions of human renal arteries.

Agreeing with previous observations in other blood vessels (Shi et al., 2007; Traupe et al., 2002; Vanhoutte et al., 2005; Yang et al., 2002), the occurrence of endothelium-dependent contractions in the aorta of the young hamster is unmasked by the presence of L-NAME, which eliminates the production of endothelium-derived NO. This raises a question of whether NO inhibits the release of $PGF_{2\alpha}$, which subsequently prevents $PGF_{2\alpha}$-mediated endothelium-dependent contractions or it is the predominance of vasodilatory effect of NO that has counteracted the contractions in the vascular smooth muscle cells. The first possibility, however, was made unlikely based on two observations. First, ODQ, an inhibitor of soluble guanylate cyclase, also unmasked similar endothelium-dependent contractions as L-NAME did, indicating $PGF_{2\alpha}$ co-exists with NO and that inhibition to the downstream effector signaling of NO is equally effective as inhibiting NO production for the appearance of endothelium-dependent contractions. Second, exogenous NO

donors did not affect the $PGF_{2\alpha}$ release, but inhibits ACh- or exogenous $PGF_{2\alpha}$-induced contractions. Taken together, it is likely that NO exerts a downstream vasodilatory effect on vascular smooth muscle cells that appear predominant over endothelium-dependent contractions, instead of a direct inhibition on the COX-2 activity and subsequent $PGF_{2\alpha}$ production and release.

To conclude, the present study demonstrates a positive role of endothelial COX-2 in the regulation of vascular tone in the aorta of the healthy hamster. The present results show that endothelial COX-2 catalyzes the formation of $PGF_{2\alpha}$, which represents a physiological EDCF at least in this preparation. Through binding to TP receptors, $PGF_{2\alpha}$ produces endothelium-dependent contractions in response to acetylcholine (Figure 3.27). The masking effect of NO on endothelium-dependent contractions is possibly due to its predominant vasodilatory action in the vascular smooth muscle cells, rather than direct inhibition on COX-2 activity and the release of $PGF_{2\alpha}$ in the endothelial cells (Figure 3.28). The present preliminary data in human arteries could have clinical relevance in humans since the same or similar pathway also exists in the human renal arteries being tested.

Limitation of the study on human arteries

A limitation of the present study on human arteries was the limited amount and supply of arterial samples, which made it difficult to repeat the complete set of experiments performed on hamster aortae and to study the effects of age dependency. Nevertheless, the results would imply that my findings on hamsters could be similarly observed in the human situations. In order to validate the functional integrity of the human samples, the human artery was first tested for ACh-induced contractions. This made pre-incubation with various

inhibitors, particularly the COX-2 inhibitors, difficult to perform, as I could not differentiate whether the lack of ACh-induced contractions was due to COX-2 inhibition or the loss of functional integrity of the artery *per se*. Therefore, the TP receptor antagonist, S18886, was added only after ACh-induced contractions occurred.

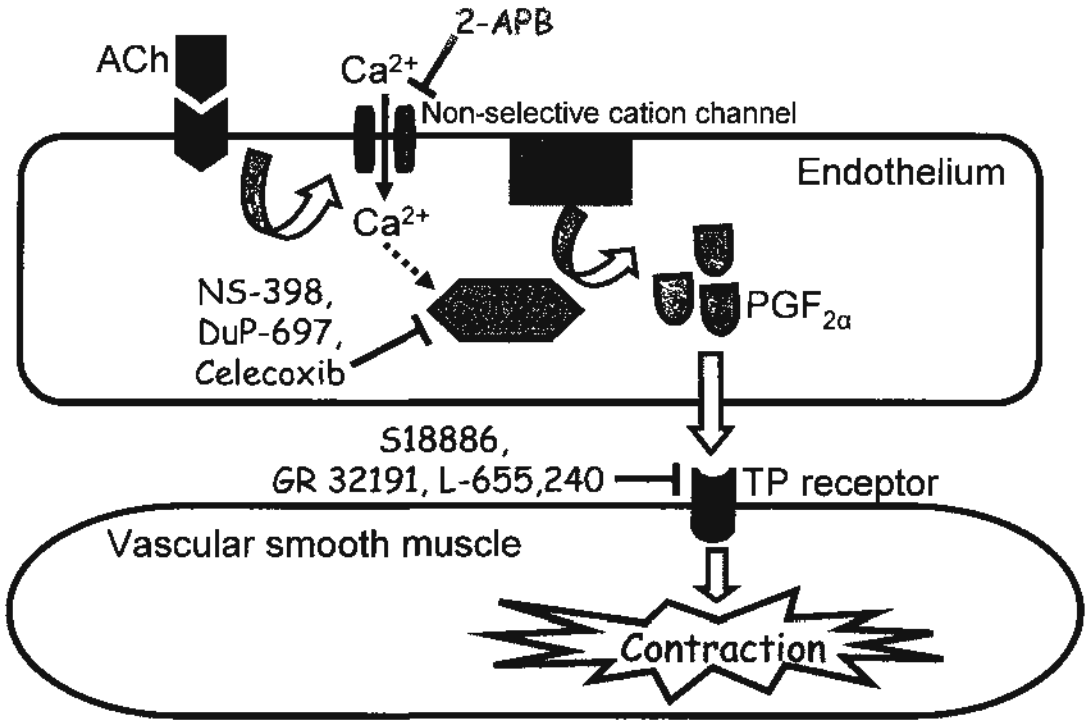

FIGURE 3.27.

Postulated cellular mechanisms mediating endothelium-dependent contractions in the aorta of healthy hamsters. Stimulation by acetylcholine of the muscarinic receptors on the endothelium triggers the opening of 2-APB-sensitive non-selective cation channels, which allow the influx of extracellular Ca^{2+} to raise the $[Ca^{2+}]_i$, resulting in activation of phospholipase A_2 and the subsequent activation of the constitutively expressed COX-2, and thus leading to the generation of prostanoids from arachidonic acid. Among the prostanoids released, $PGF_{2\alpha}$ is the most likely candidate of EDCF, and diffuses towards adjacent vascular smooth muscle cells (VSMCs) where it activates the TP receptors and causes contraction of VSMCs. ACh, acetylcholine; 2-APB, 2-aminoethoxydiphenyl borate; AA, arachidonic acid; COX-2, cyclooxygenase-2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; TP receptor, thromboxane-prostanoid receptor.

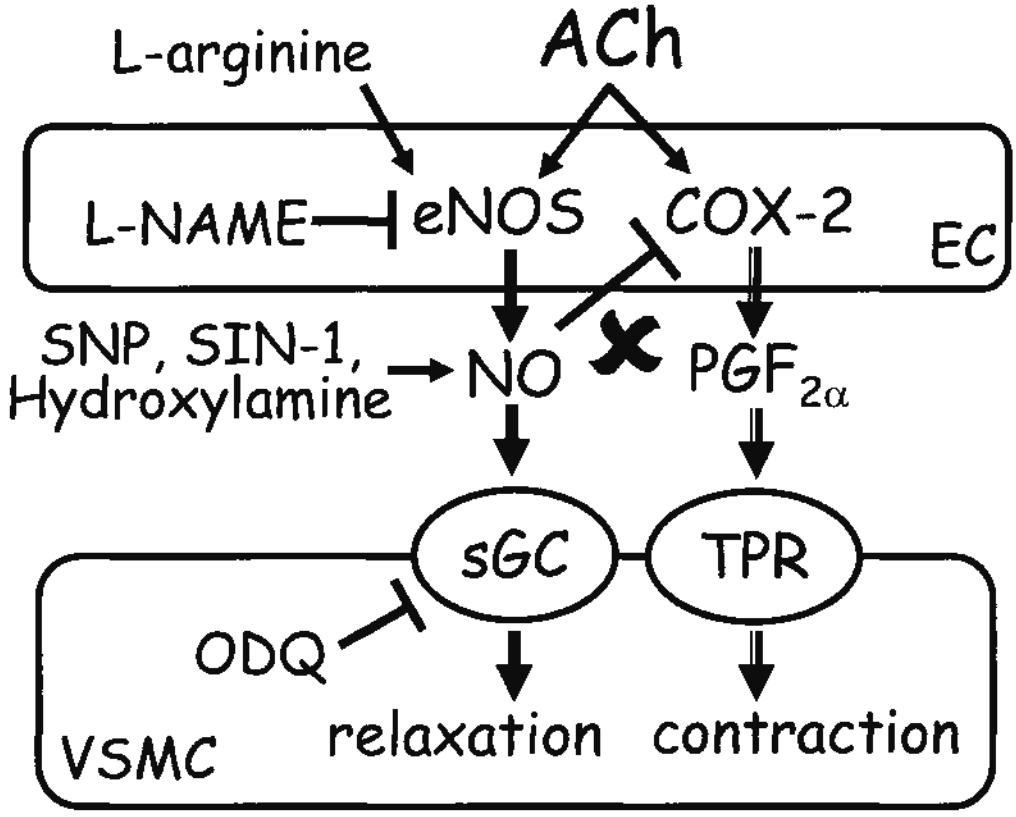

FIGURE 3.28.

Schematic diagram showing the interaction between NO and $PGF_{2\alpha}$. ACh can simultaneously trigger the release of NO and $PGF_{2\alpha}$ respectively from eNOS and COX-2, of which the former leads to sGC-mediated relaxations and the latter causes contraction by TP receptor activation. Inhibition on either NO production or NO signaling allows the appearance of ACh-induced contractions. The masking effect of NO, however, is unlikely due to an inhibition on COX-2 activity, nor to a chemical inactivation of $PGF_{2\alpha}$.

Chapter IV

Enhanced impact of Cyclooxygenase-2 and $PGF_{2\alpha}$ in endothelium-dependent contractions during aging

4.1 Introduction

Epidemiological studies suggest that aging represents an independent risk factor for the development of cardiovascular diseases such as hypertension and atherosclerosis (Csiszar et al., 2007). Vascular dysfunction in aging is characterized by severely attenuated endothelium-dependent vasodilatations, possibly attributed by the detrimental imbalance between relaxing and contracting factors (Mombouli and Vanhoutte, 1999). Progressive reduction in nitric oxide (NO) production and augmentation of vasoconstrictor release such as endothelin-1 have favored the persistent contractions of vascular beds (Donato et al., 2009; Ferrari et al., 2003; Matz and Andriantsitohaina, 2003). Brachial artery NO-dependent vasodilatation is usually taken as an index in evaluating vascular function in humans. Even among normotensive subjects, the dilatations decrease with advancing age. Infusion of a non-selective cyclooxygenase (COX) inhibitor, indomethacin, remarkably potentiates the vasodilatations (Taddei et al., 1997), indicating an emerging role of COX products which counteracts the dilatory effect of NO during aging.

Though there are discrepancies in the conclusions from different studies on whether the expression of nitric oxide synthase (eNOS) is increased or decreased during aging (Briones et al., 2005; Cernadas et al., 1998; Matz et al., 2000; Soucy et al., 2006; van der Loo et al., 2000), a consensus was reached that in general eNOS activity was reduced in aged animals (Smith et al., 2006),

attributed by a decrease of Akt-mediated phosphorylation of eNOS at the activation site Ser^{1177} (Soucy et al., 2006). Of note, aging is usually accompanied by other risk factors like the elevation of reactive oxygen species (ROS) (Hamilton et al., 2001), which is possibly due to the impairment in the endogenous cellular anti-oxidative defense mechanism mediated by Cu/Zn superoxide dismutase (SOD), Mn-SOD and extracellular SOD (Brown et al., 2006; Chen and Chen, 2006; Didion et al., 2006; Xu et al., 2006), and the overproduction of oxidants by the dysfunctional and hyperreactive free radical-generating enzymes such as NADPH oxidase and xanthine oxidase (Brandes et al., 2005), resulting in uncontrollable ROS accumulation. The exaggerated oxidative stress in the vascular wall acutely scavenges and depletes NO, and the subsequent formation of peroxynitrite can in turn further inactivates the MnSOD and deplete tetrahydrobiopterin (Laursen et al., 2001), a co-factor essential for eNOS function. Soluble guanylate cyclase expression is downregulated in aging (Cernadas et al., 1998; Kloss et al., 2000; Moritoki et al., 1992; Tschudi et al., 1996). Taken together, the participation of NO in the regulation of vascular function is severely diminished during aging.

The improvement of attenuated endothelium-dependent relaxations by acute treatment with indomethacin or thromboxane-prostanoid (TP) receptor antagonist SQ-29548 in the aorta of the aged spontaneously hypertensive rats (SHR) pinpoints the contributions of COX metabolites in aging-related vascular dysfunction (Graham and Rush, 2009). Endothelium-dependent contractions to Ca^{2+} ionophore are significantly greater in the femoral arteries from aged rats when compared with their younger counterparts (Shi et al., 2008). While these contractions are abolished by indomethacin, they can be partially inhibited by specific inhibitors of COX-1 and COX-2. Genomic studies on endothelial cells

show an increase in the mRNA levels of COX-1, COX-2, thromboxane synthase, PGF synthase, hematopoietic-type PGD synthase, and membrane PGE synthase-2 in aged rats (Tang and Vanhoutte, 2008), indirectly supporting the exaggerated importance of the arachidonic acid metabolism through COX during aging.

Based on my findings on a constitutive role of COX-2 in endothelium-dependent contractions in the aorta of young hamster detailed in Chapter III (Wong et al., 2009), I further investigated the role of COX in healthy aging in an attempt to address (1) whether there was a switch of functions of the COX isoforms in aged animals, (2) the prostaglandin(s) that mediated the endothelium-dependent contractions in aging and (3) the vascular component that contributed to the augmentation of COX-derived metabolites.

4.2 Materials and Methods

Experiments were performed on aortae from aged (> 18 months old) male Syrian golden hamsters. This part of the study was approved by the Animal Ethics Committee, Chinese University of Hong Kong.

4.2.1 Animals and diet

The aged hamsters were supplied by the Laboratory Animal Services Centre, the Chinese University of Hong Kong. The animals were housed at constant temperature (21 ± 1 °C) under a 12-hour light/dark cycle and had free access to chow diet and water.

4.2.2 Blood vessel preparation

Hamsters were euthanized by CO_2 inhalation. Thoracic aortae were excised and placed in Krebs-Henseleit solution (KHS) containing (mmol/L): NaCl 119, $NaHCO_3$ 25, $MgCl_2$ 1, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, and D-glucose 11.1. Following the removal of periadventitial fat, each artery was cut into segments ~1.0-1.3 mm in length. Each segment was suspended between two stainless steel wires in chambers of a Multi Myograph System (610M, Danish Myo Technology A/S, Aarhus N, Denmark) for the measurement of isometric force. The rings were stretched to a previously determined optimal resting tension of 10 mN. Thirty min after setting up the preparation, they were contracted with 60 mmol/L KCl to test the vessel's contractility, and washed in normal KHS, and finally allowed to equilibrate for 30 min. In some rings, the endothelium was removed mechanically by rolling the luminal surface with a stainless steel wire.

4.2.3. Isometric force measurement

Aortic rings were exposed to 100 µmol/L N^G-nitro-L-arginine methyl ester (L-NAME) for 30 min followed by the cumulative application of acetylcholine (ACh, 0.1-10 µmol/L). The effects of various inhibitors and antagonists (e.g., COX-1 and COX-2 inhibitors, TP receptor antagonists) were tested on ACh-induced endothelium-dependent contractions following a 30 min-incubation with each drug. Contractions to $PGF_{2\alpha}$, PGI_2, PGE_2, KCl and phenylephrine were examined in L-NAME treated aortae from both young and aged hamsters.

4.2.4 Western blot analysis

Aortic rings were snap frozen in liquid nitrogen and subsequently homogenized in an ice-cold RIPA lysis buffer with a cocktail of protease inhibitors (leupetin, 1 µg/mL; aprotonin, 5 µg/mL; PMSF, 100 µg/mL; sodium orthovanadate, 1 mmol/L; EGTA, 1 mmol/L; EDTA, 1 mmol/L; NaF, 1 mmol/L and β-glycerolphosphate, 2 mg/mL). The lysates were centrifuged at 20,000 xg for 20 min and the supernatants were collected. The protein concentration was determined by the Lowry method (BioRad). Each protein sample (80 µg) was electrophoresed through the 10% SDS-polyacrylamide gels and then transferred to an Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore). The membranes were blocked with 5% non-fat skimmed milk and probed overnight at 4 °C with antibodies against COX-1 (Santa Cruz) and COX-2 (BD Transduction Laboratories). After washes in Tween-20 phosphate buffer saline (PBST), the membranes were incubated with appropriate HRP-conjugated secondary IgG (DakoCytomation) for 60 min at room temperature. The membranes were developed with an enhanced chemiluminescence detection system (ECL reagents; Amersham Pharmacia Biotech, Uppsala, Sweden) and

exposed on X-ray films (Fuji). Densitometry was performed using a documentation program (FluorChem, Alpha Innotech Corp., San Leandro, CA, USA). GAPDH antibody (Ambion, Inc) was probed as a loading control.

4.2.5 Enzyme immunoassay (EIA) and high performance liquid chromatography-coupled mass spectrometry (HPLC-MS) measurement of prostaglandins

After 30-min incubation in the presence of L-NAME with and without inhibitors in a chamber at 37 °C, aortic rings were transferred to microcentrifuge tubes that contained 200 μL bathing solution and 3 μmol/L ACh. Three minutes later, arterial tissues were removed and solutions were frozen and stored at -80 °C until assay for the determination of the protein content.

The levels of $PGF_{2\alpha}$ and 6-keto $PGF_{1\alpha}$ (for PGI_2) were measured by EIA kits (Cayman Chemical, Ann Arbor, MI, USA) according to the instruction of the manufacturer.

Release of $PGF_{2\alpha}$ and 6-keto $PGF_{1\alpha}$ was also measured with HPLC-MS (Cui et al., 2008). LC-MS experiment was performed on Agilent 1100 series HPLC with binary pump, autosampler and a thermostated column compartment (Agilent Technologies, CA, USA). The separation was performed on a reversed phase column (Alltech Prevail, C8, 2.1 mm × 150 mm, 3 μm) at a flow rate of 0.2 mL/min. The column temperature was maintained at 30°C throughout the analysis. The injection volume was 20 μL. The mobile phases consisted of 0.1 % formic acid in water (A) and in acetonitrile (B). The gradient started at 90 % A, 10 % B for 4 min, followed by a linear increase of solvent B to 55 % at 13 min, which further increase to 70 % B in 5 min and maintained for 4 min. Finally, solvent B was decreased to 10 % and equilibrated for 15 min before the

injection of the next sample. Negative ion electrospray mass spectrometric analysis was carried out using Bruker Daltonics MicrOTOFQ mass spectrometer (Bremen, Germany). The end plate offset and capillary voltage were -550 V and 4000 V respectively. Nitrogen was used as nebulizer gas at 1.4 bar and drying gas at a flow rate of 7.0 L/min at 170 °C. Pure $PGF_{2\alpha}$ and 6-keto $PGF_{1\alpha}$ were dissolved in methanol and serially diluted in KHS to construct standard curves.

4.2.6 Drugs and chemicals

ACh, phenylephrine and U46619 (9,11-dideoxy-11_{α},9_{α}-epoxymethano-prostaglandin $F_{2\alpha}$) were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). N^{G}-nitro-L-arginine methyl ester (L-NAME), DuP-697 (5-bromo-2-(4-fluorophenyl)-3-[4-(methylsulfonyl)phenyl]-thiophene) were from Tocris (Avonmouth, UK). 2-APB (2-aminoethoxydiphenyl borate) and PGE_2 were from Calbiochem, EMD Biosciences (La Jolla, CA, USA). VAS (valeryl salicylate), $PGF_{2\alpha}$ and PGI_2 were from Cayman Chemical (Ann Arbor, MI, USA). S18886 (3-[(6-amino-(4-chlorobenzensulphonyl)-2-methyl-5,6,7,8-tetrahydronapht]-1-yl) propionic acid) and sc-560 were kind gifts from Institut de Recherches Servier (Suresnes, France). Celecoxib was from Pfizer. Except ACh, L-NAME, phenylephrine, PGI_2 and S18886 that were prepared in distilled water; all other drugs were dissolved in DMSO (Sigma-Aldrich).

4.2.7 Data analysis

Endothelium-dependent contractions were expressed as active tension [force recorded/ (2 × ring's length)]. Results are mean ± SEM of n rings from different animals. Protein expressions determined by Western blotting were relative to

GAPDH of the respective blot. For statistical analysis, Student's *t*-test or 2-way ANOVA followed by Bonferroni post-tests were used when more than two treatments were compared (GraphPad Software, San Diego, CA, USA). $P<0.05$ was considered significantly different.

4.3. Results

4.3.1 Augmentation of endothelium-dependent contractions

In the presence of L-NAME (100 μmol/L), ACh caused greater contractions in aortae from aged hamsters than those from the younger counterparts (Figure 4.1A). These contractions were not observed in rings without endothelium (Figure 4.1B) and were abolished by 2-APB (50 μmol/L) (Figure 4.1C).

Selective COX-2 inhibitors DuP-697 (Figure 4.2A) and celecoxib (Figure 4.2B) (both at 3 and 10 μmol/L), and TP receptor antagonist S18886 (0.1 and 0.3 μmol/L, Figure 4.2C) significantly reduced the endothelium-dependent contractions. These inhibitors when used at the concentrations that completely abolished the maximal ACh (10 μmol/L)-induced endothelium-dependent contractions in the aortae from young hamsters (DuP-697, celecoxib, 3 μmol/L; S18886, 0.1 μmol/L), could only partially inhibit the contractions in the aortae from aged hamsters (Figure 4.2D). Further attenuation to the endothelium-dependent contractions was achieved by higher concentrations of these inhibitors (Figure 4.2A-C). In contrast, selective COX-1 inhibitors valeryl salicylate (VAS, 30 and 300 μmol/L, Figure 4.3A) and sc-560 (0.3 and 1 μmol/L, Figure 4.3B) did not affect the endothelium-dependent contractions even at much higher concentrations (VAS, 300 μmol/L; sc-560, 1 μmol/L) compared to those used in the aortae from young hamsters (VAS, 30 μmol/L; sc-560, 1 μmol/L). SOD mimetic tempol (100 μmol/L, Figure 4.4A) or NADPH oxidase inhibitor apocycnin (100 μmol/L, Figure 4.4B) did not inhibit the ACh-induced contractions.

4.3.2 Appearance of endothelium-dependent contractions in the absence of L-NAME

ACh-induced endothelium-dependent relaxations were remarkably impaired in the aortae from aged hamsters. At high concentrations of ACh (1-100 μmol/L), pronounced contractions were observed (Figure 4.5). ACh-induced contractions were observed in the aortic rings from aged hamsters in the absence of L-NAME, although smaller than those in rings treated with L-NAME (Figure 4.6A). These contractions were abolished by the removal of endothelium (Figure 4.6B), and abrogated by DuP-697 (10 μmol/L), S18886 (0.3 μmol/L) (Figure 4.6C) and 2-APB (50 μmol/L) (Figure 4.6D), but remained unaffected by VAS (30 μmol/L) (Figure 4.6C).

4.3.3 Exaggerated COX-2 expression in the aorta from aged hamsters

Protein expression of COX-2 was markedly augmented in the aortic rings of aged hamsters. Removal of endothelium resulted in a diminished level of COX-2 protein detected (Figure 4.7A). In contrast, COX-1 expression was comparable in rings with or without endothelium from both young and aged hamsters (Figure 4.7B).

4.3.4 Enhanced release and responsiveness to $PGF_{2\alpha}$

Release of ACh-induced $PGF_{2\alpha}$ was augmented, though not significantly, in the L-NAME-treated aortae from aged hamsters as determined by EIA and HPLC-MS (Figure 4.8A&B). Release of PGI_2 was also increased by aging (Figure 4.8C&D). The release of $PGF_{2\alpha}$ and PGI_2 was abrogated by celecoxib but remained unaffected in the presence of VAS. The release was inhibited by endothelium removal (Figure 4.8E&F).

$PGF_{2\alpha}$ caused contractions in the aortae from aged hamsters. At the same concentration of $PGF_{2\alpha}$ (1 or 3 ng/ml), the L-NAME-treated aortae from aged hamsters contracted more than those from their younger counterparts (Figure 4.9A). On the contrary, contractions to KCl (30 – 50 mmol/L) or phenylephrine (0.1 – 1 µmol/L) were similar in both aged and young hamsters (Figure 4.9B). Augmentation of $PGF_{2\alpha}$-evoked contractions, however, was not attributed by an increase in TP receptor expression, which appeared comparable in the aortae from both ages (Figure 4.9C).

4.4.5. Non-involvement of PGE_2 and PGI_2 in endothelium-dependent contractions

In contrast to $PGF_{2\alpha}$, PGI_2 produced neither a relaxation (Figure 4.10) nor a contraction (Figure 4.11) in the aortae from aged hamsters as it did not in those from young hamsters. PGE_2, another prostaglandin that can regulate vascular tone, did not cause relaxations either (Figure 4.12A). PGE_2-evoked contractions were not inhibited by S18886 (Figure 4.12B).

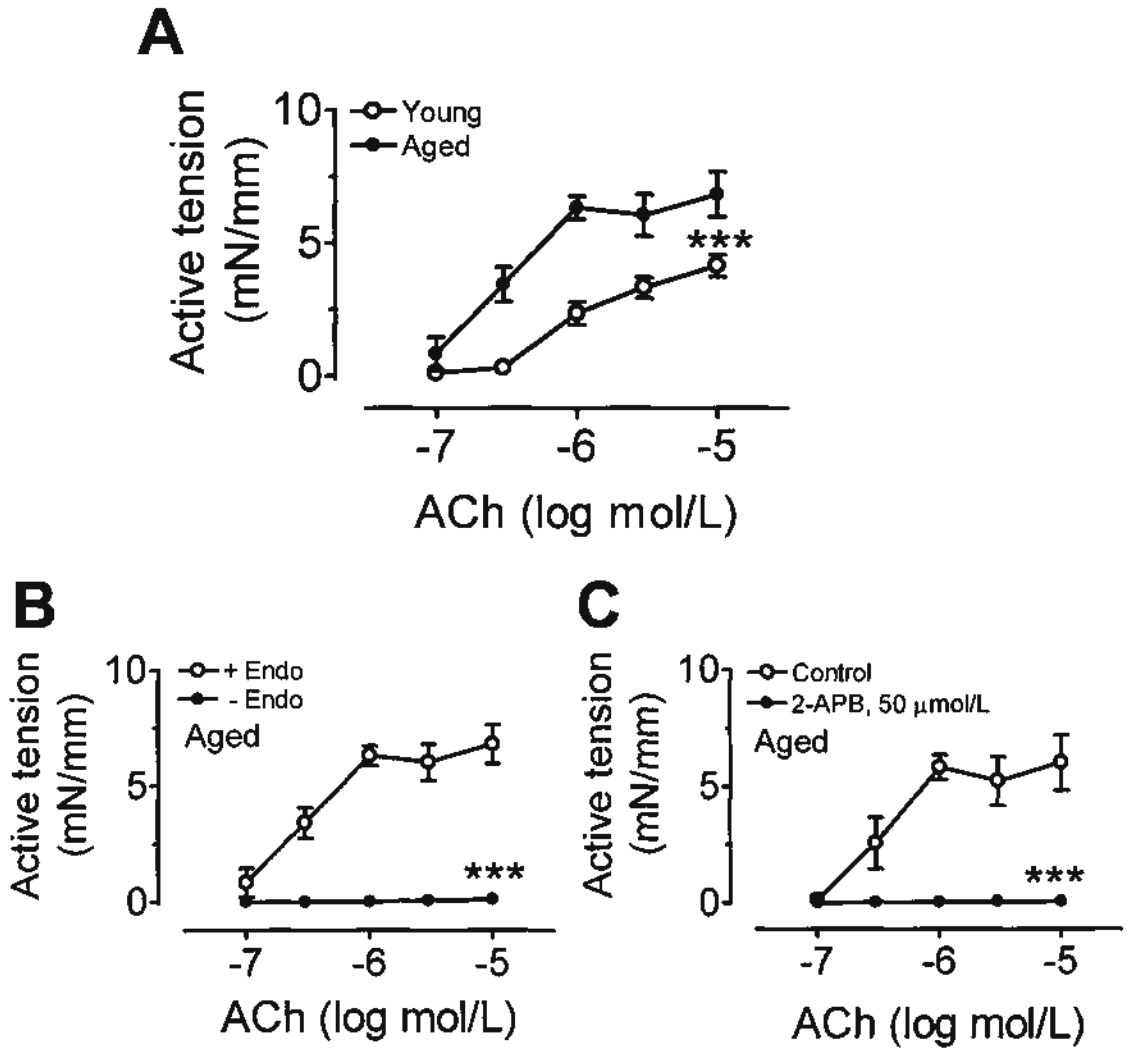

FIGURE 4.1.

Acetylcholine (ACh)-induced contractions were greater in aortae from aged hamsters (A, n=8-15). These contractions were only observed in rings with endothelium (B, n=5), which were abolished by 50 µmol/L 2-APB (C, n=5). Data are mean ± SEM of n experiments. ***$P<0.001$ compared between young and aged group, between + Endo and - Endo or with control.

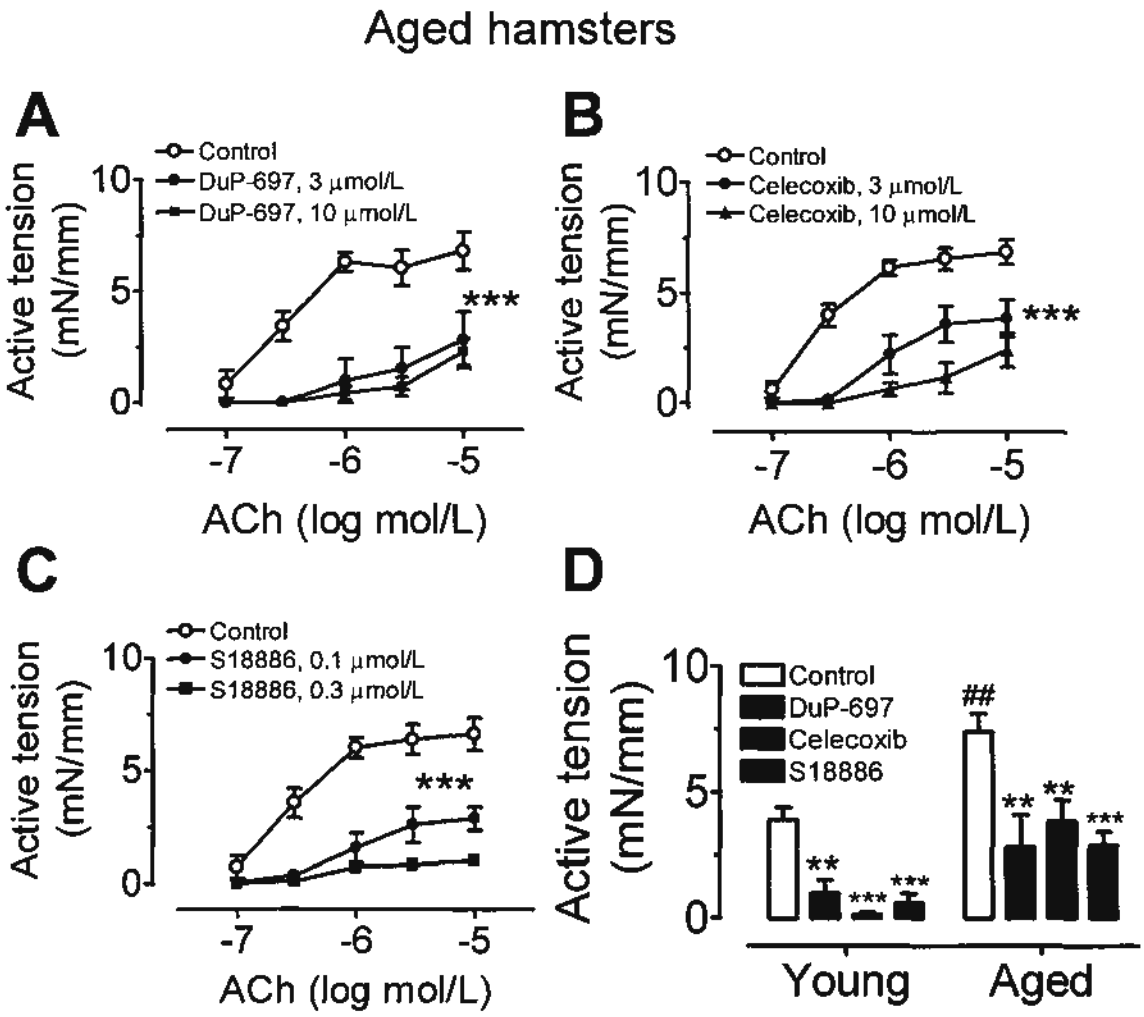

FIGURE 4.2.

Inhibitory effects of 3-10 μmol/L DuP-697 (A), 3-10 μmol/L celecoxib (B), or 0.1-0.3 μmol/L S18886 (C) on endothelium-dependent contractions in the ring treated with L-NAME. (D) Maximal ACh (10 μmol/L)-induced contractions in the presence of 3 μmol/L DuP-697, 3 μmol/L celecoxib, and 0.1 μmol/L S18886 in aortic rings from young and aged aortae. Data are mean ± SEM of 4-8 experiments. $^{**}P<0.01$ and $^{***}P<0.001$ compared with control in the same age group; $^{\#\#}P<0.01$ between the control groups of young and aged hamsters in rings with endothelium.

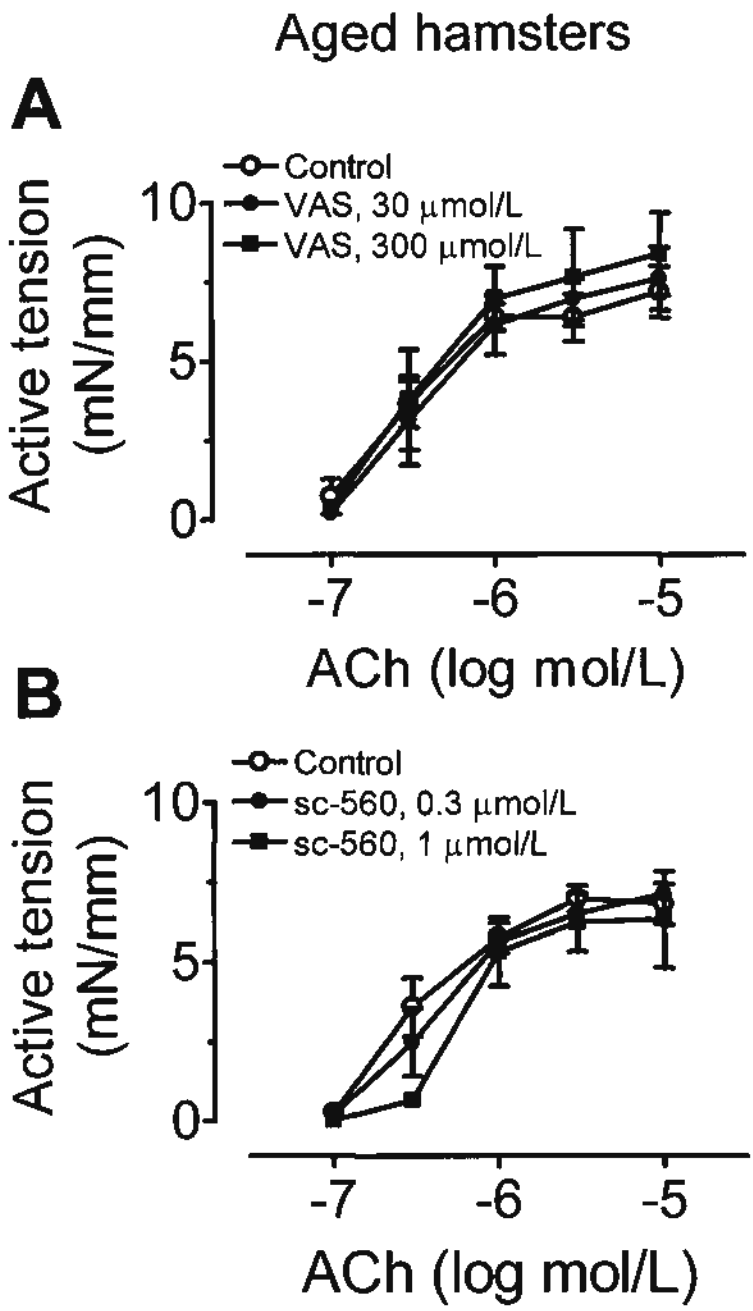

FIGURE 4.3.

Lack of effect of COX-1 inhibitors (A) VAS (30-300 μmol/L) and (B) sc-560 (0.3-1 μmol/L) on ACh-induced contractions in aortae from aged hamsters. Data are mean ± SEM of 4-5 experiments.

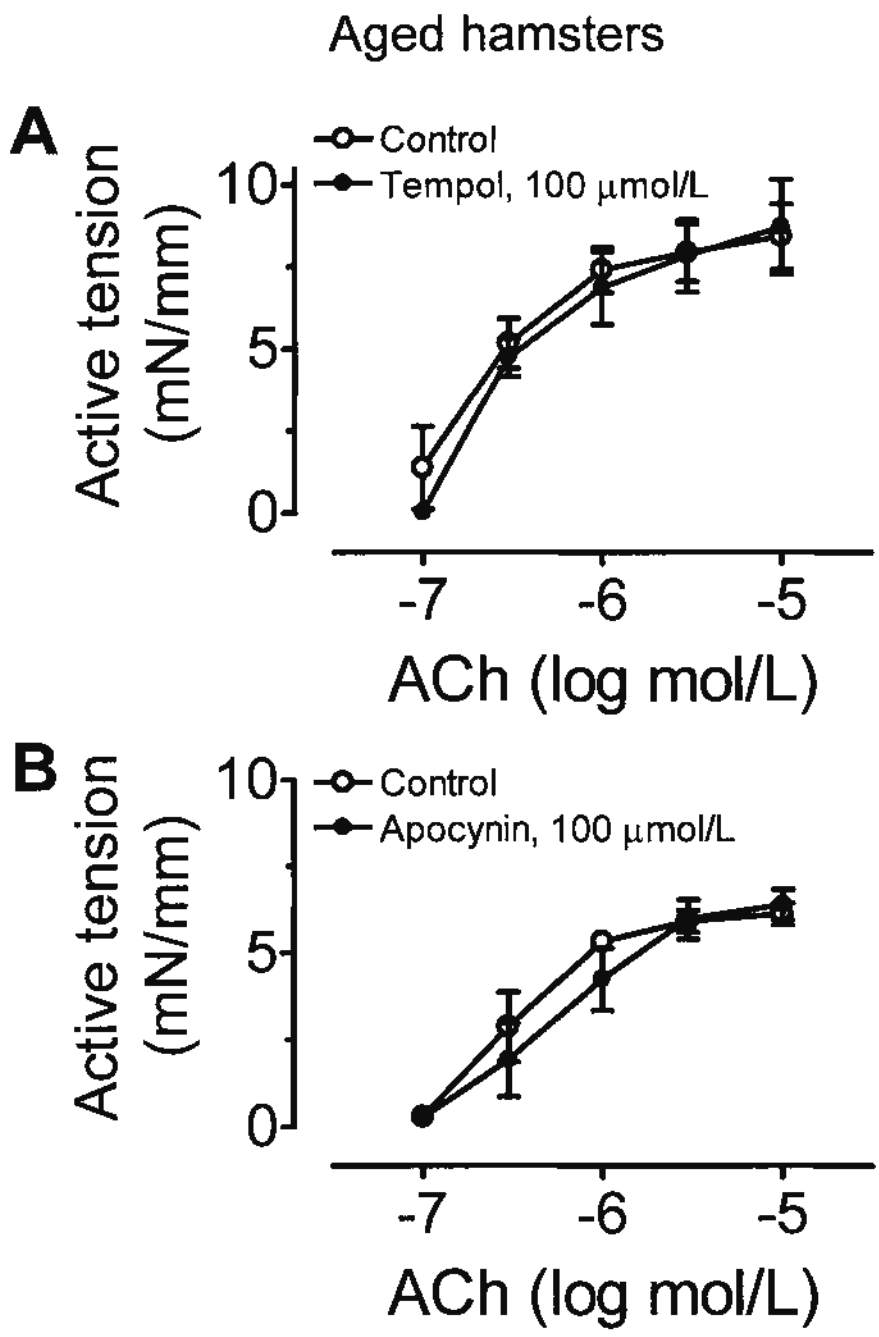

FIGURE 4.4.

Lack of effect of (A) SOD mimetic, tempol (100 μmol/L) and (B) NADPH oxidase inhibitor, apocynin (100 μmol/L) on ACh-induced contractions in aortae from aged hamsters. Data are mean ± SEM of 3 experiments.

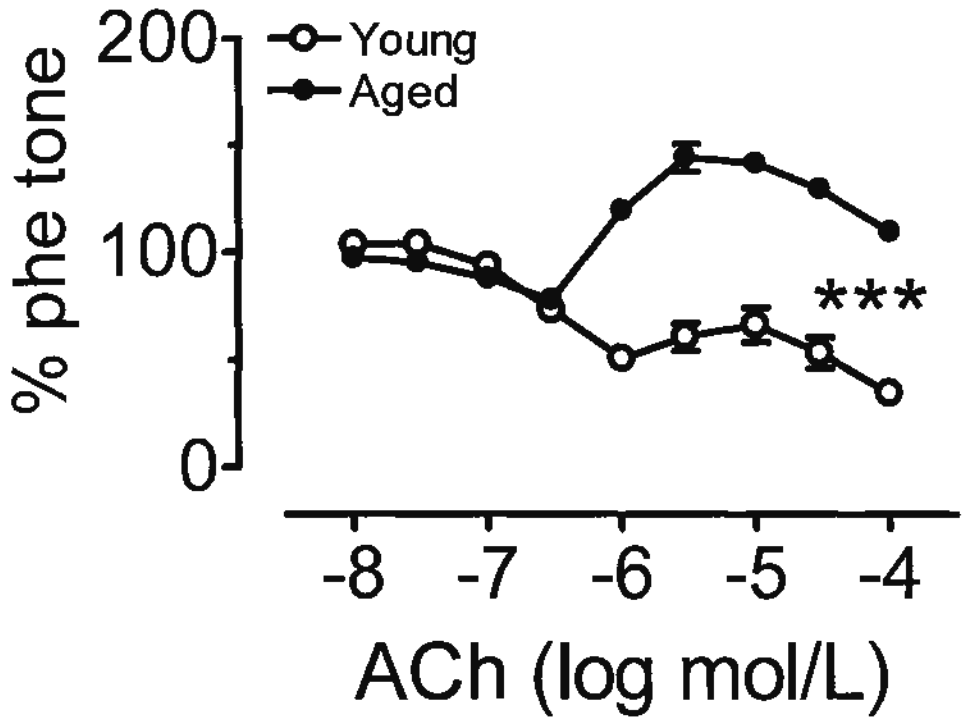

FIGURE 4.5.

Significantly impaired ACh-induced endothelium-dependent relaxations in the aortae from aged hamsters. Data are mean ± SEM of 9-10 experiments. ***P<0.001 compared between the young and aged hamsters.

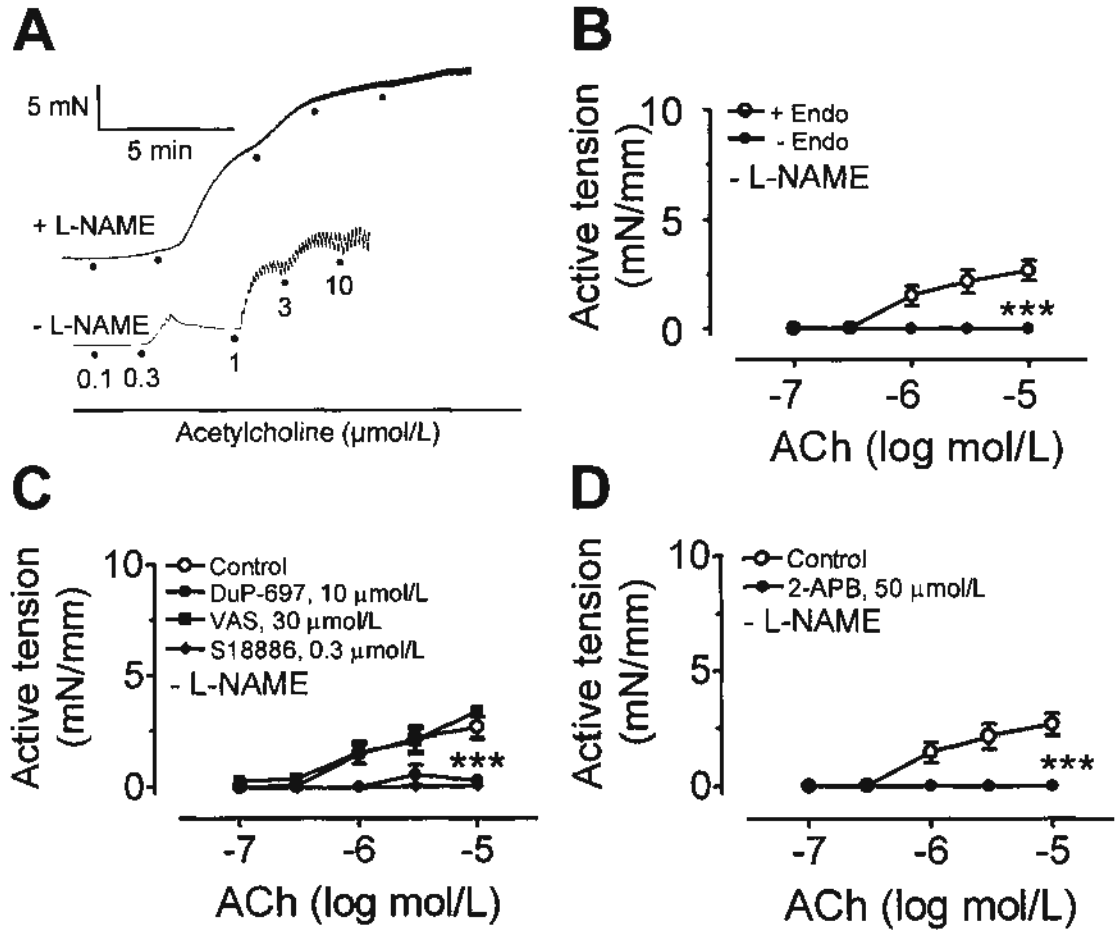

FIGURE 4.6.

(A) Representative traces showing ACh-induced contractions in aortae from aged hamsters in the presence or absence of L-NAME. The contractions were endothelium-dependent (B), and were abolished by DuP-697, S18886 (C) or 2-APB (D), but not VAS (C). Data are mean ± SEM of 5-10 experiments. ***P<0.001 compared with control or between + Endo and - Endo.

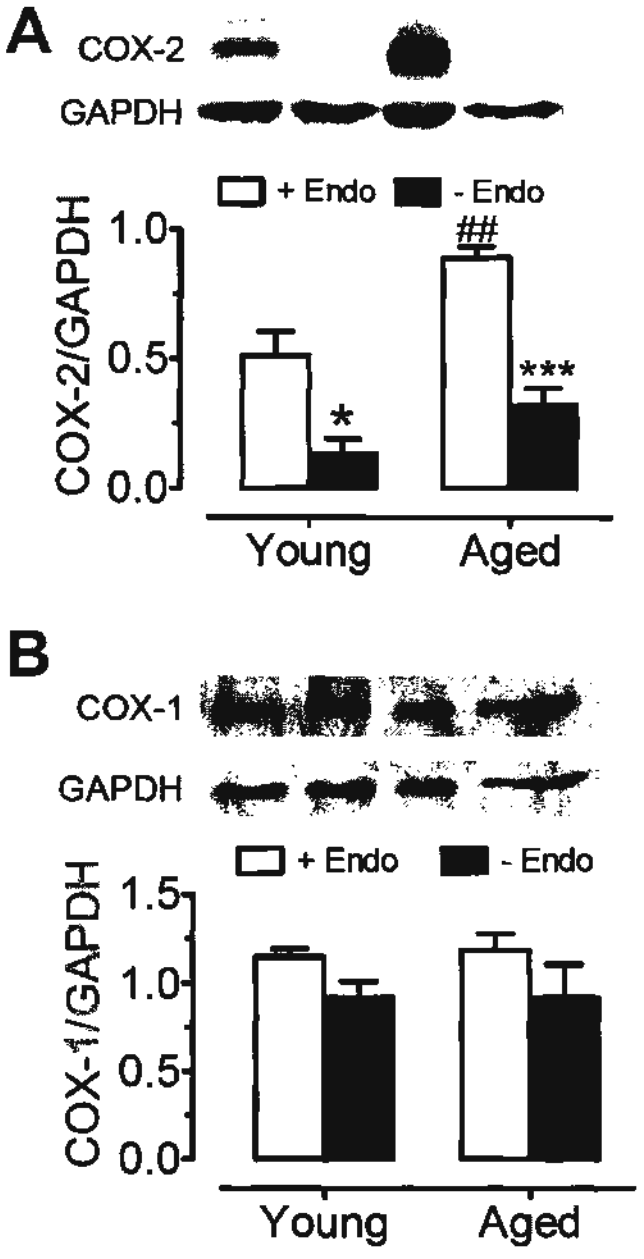

FIGURE 4.7.

(A) COX-2 expression was augmented in the aortae from aged hamsters and it was mainly localized in the endothelium. (B) COX-1 expression was similar in the aortae from both young and aged hamsters with or without endothelium. Data are mean ± SEM of 4 experiments. ***P<0.001 compared between + Endo and - Endo. ##P<0.01 between the control groups of young and aged hamsters in rings with endothelium.

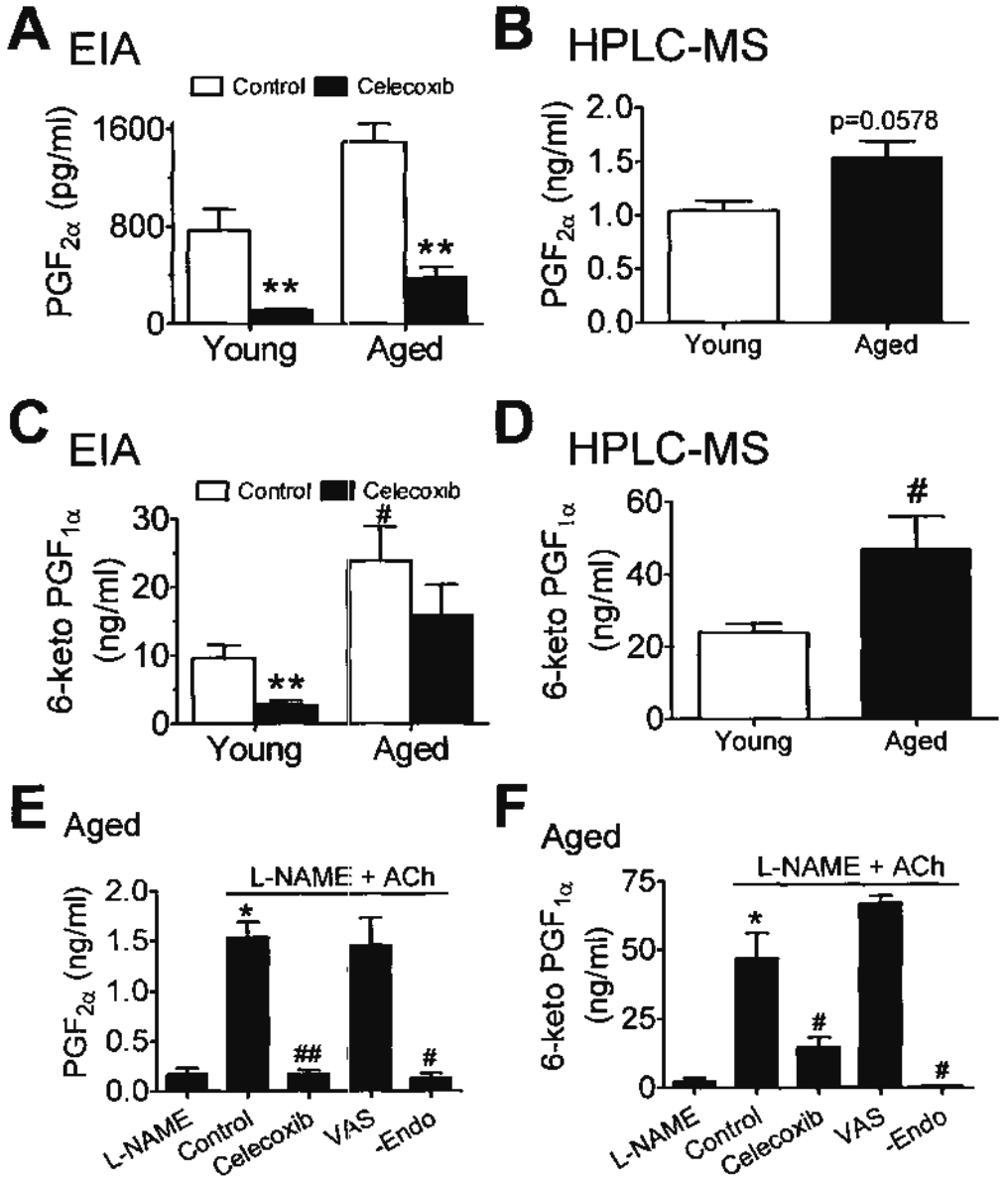

FIGURE 4.8.

ACh-stimulated release of $PGF_{2\alpha}$ (A&C) and PGI_2 (assayed in form of 6-keto $PGF_{1\alpha}$) (B&D) in the bathing solution of L-NAME-treated aortae determined by enzyme immunoassay (EIA, A&B) and high performance liquid chromatography-coupled mass spectrometry (HPLC-MS, C&D). $^{**}P<0.01$ compared with control in the same age group; $\#P<0.05$ between aged and young aortae with endothelium in control. Release of $PGF_{2\alpha}$ (E) and PGI_2 (F) were endothelium- and COX-2 dependent. $^{*}P<0.005$ compared with L-NAME only; $\#P<0.05$ and $\#\ \#P<0.01$ among the L-NAME + ACh treated groups. Data are mean ± SEM of 3-4 experiments.

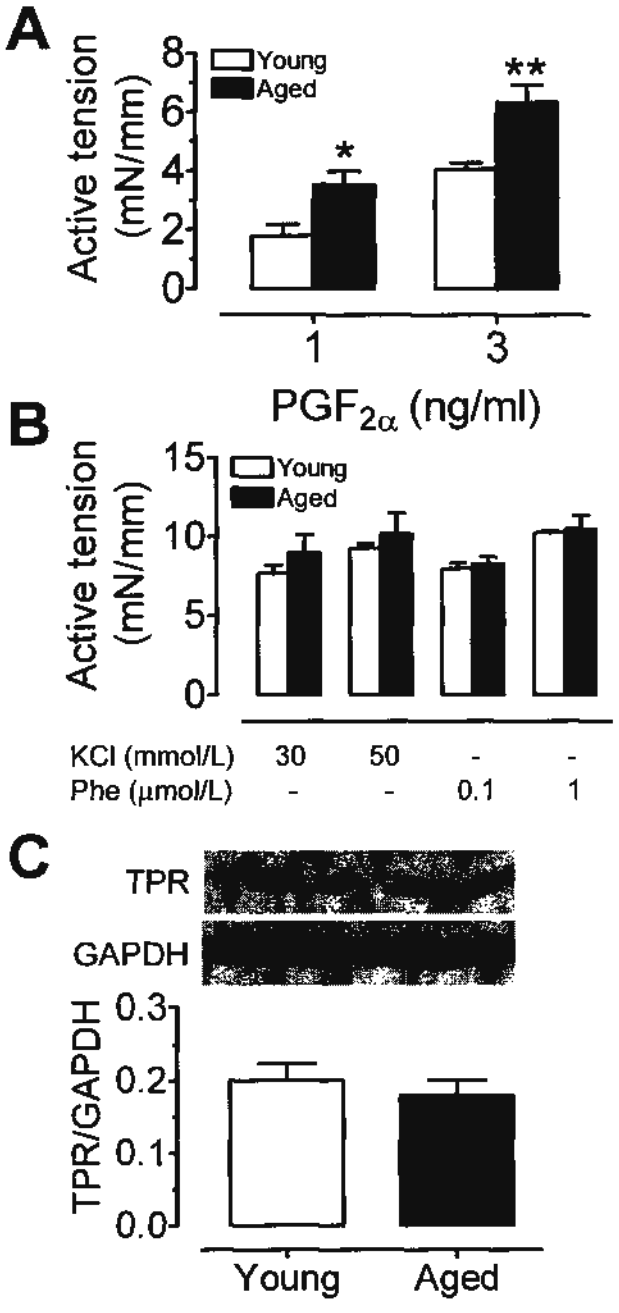

FIGURE 4.9.

(A) $PGF_{2\alpha}$-induced contractions in L-NAME-treated aortae from young and aged hamsters (n=5). *P<0.05 and **P<0.01 compared between the young and aged group. (B) Contractions to KCl and phenylehprine (Phe) was similar in the aortae from both age groups (n=3-4). (C) Protein expression of TP receptor (TPR) were comparable in aortae from young and aged hamsters (n=4). Data are mean ± SEM of n experiments.

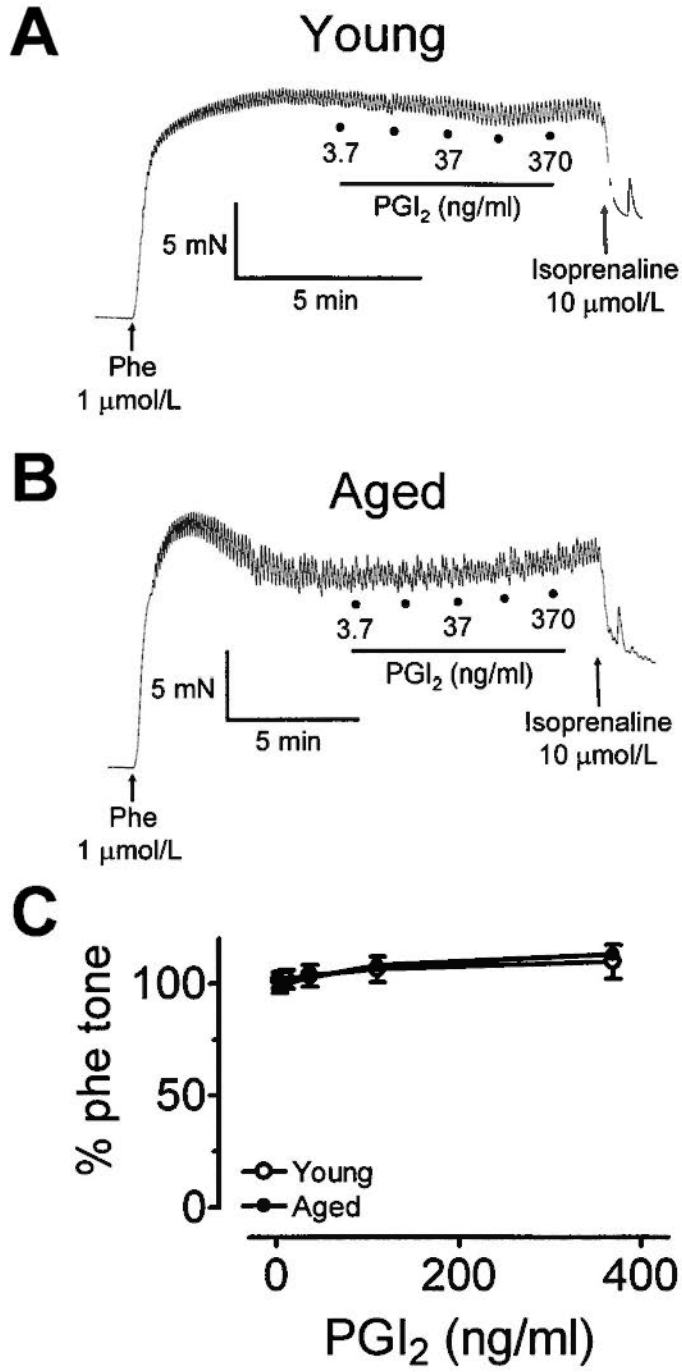

FIGURE 4.10.

Representative trace (A&B) and summarized data (C) showing PGI_2 did not relax aortae from both young and aged hamsters, while isoprenaline at 10 μmol/L caused relaxation (A&B). Data are mean ± SEM of 4 experiments.

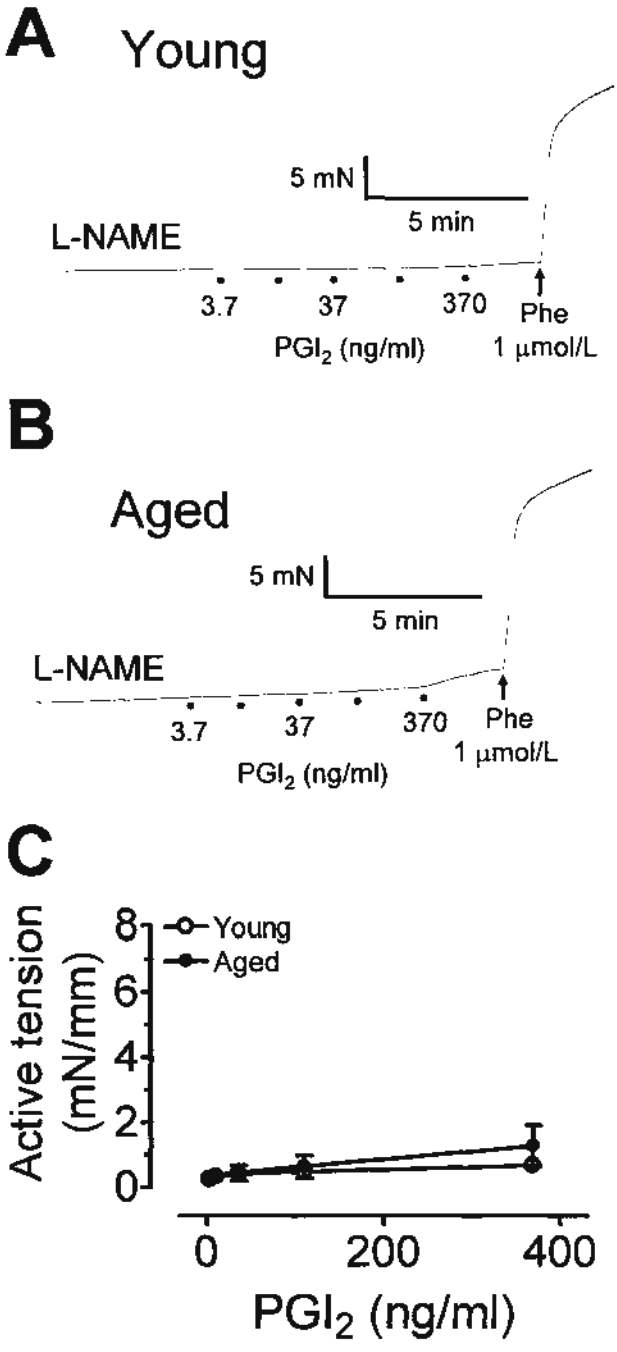

FIGURE 4.11.

Representative trace (A&B) and summarized data (C) showing PGI_2 did not contract aortae from both young and aged hamsters, while phenylephrine at 1 µmol/L evoked pronounced contractions (A&B). Data are mean ± SEM of 4 experiments.

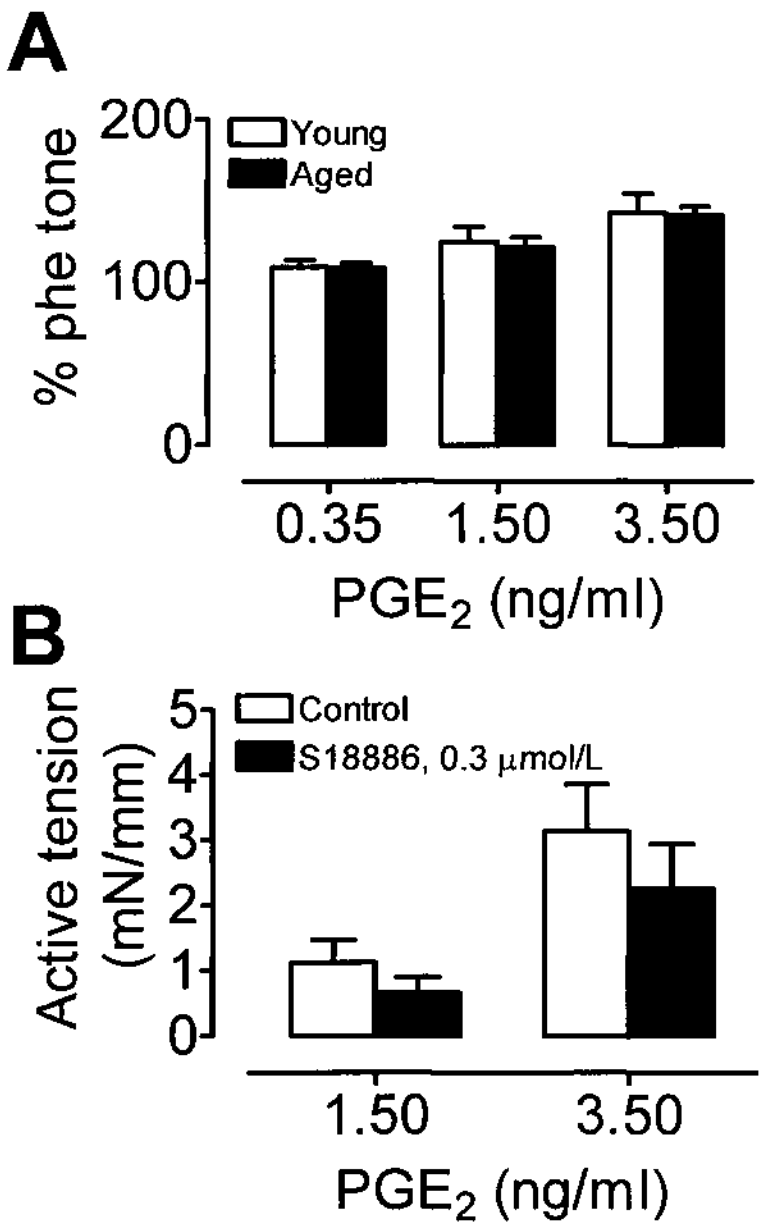

FIGURE 4.12.

PGE_2 caused contractions in aortae from aged hamsters as it did those from the young hasmters (A). These contractions were not inhibited by TP receptor antagonist, S18886 (0.3 μmol/L, B). Data are mean ± SEM of 4 experiments.

4.4 Discussion

Limited studies have cast their focus on the role of COX-2 in the vascular bed during healthy aging. In the present study, I have made novel observations in (1) an emerging role of COX-2 in endothelium-dependent contractions in the aorta of aged hamster without a contribution from COX-1; (2) a more physiological appearance of endothelium-dependent contractions without the need of pharmacological inhibition of NOS by L-NAME, and (3) the enhanced release and vascular sensitivity of $PGF_{2\alpha}$, which is solely endothelium-derived.

Aortae of aged hamsters exhibited greater endothelium-dependent contractions than those from the younger counterparts. These contractions were exclusively contributed by COX-2 with little contribution from COX-1, as evidenced by the pronounced attenuation caused by two structurally distinct COX-2 inhibitors in contrast to the lack of effect of COX-1 inhibitors even at higher concentrations. This is different from the endothelium-dependent contractions in the femoral arteries of aged rats, in which both COX-1 and COX-2 are involved (Shi et al., 2008). The present study was, however, in agreement with Shi et al.'s that COX-2 was elevated in the arteries from aged animals. Indeed, COX-2 was not expressed in the femoral arteries of younger rats, and was induced during aging. The nature of COX-2 expression augmentation may be similar in both species though the COX-2 in hamsters was constitutively expressed at a young age.

ACh-induced relaxations were impaired in the aortae from aged hamsters, suggesting that there may be a reduction in NO bioavailability or impairment in NO signaling (Cernadas et al., 1998; Kloss et al., 2000; Moritoki et al., 1992; Smith et al., 2006; Tschudi et al., 1996). It is possible that this reduced contribution of NO in vascular tone regulation allows the appearance of ACh-

induced endothelium-dependent contractions, which may represent physiological interaction between NO signaling and the action of endothelium-derive contracting factors (EDCF) *in vivo*. These contractions were again prevented by COX-2 inhibitor and TP receptor antagonist, but not by COX-1 inhibitor. Endothelium-dependent contractions unveiled with or without L-NAME were also extracellular Ca^{2+}-dependent, as suggested by its abrogation by 2-APB.

I also determined the prostaglandin(s) release from the aortae of aged hamsters upon ACh stimulation. Both $PGF_{2\alpha}$ and PGI_2 tended to be increased in the aortae from aged hamsters. The measurements agreed well between EIA and HPLC-MS. Release of both prostaglandins was sensitive to inhibition of COX-2 but not COX-1, which matched well with functional observations. Though PGI_2 is conventionally regarded as a vasodilator, recent studies from Gluais et al. (2005; 2006) suggest that excessive PGI_2 production may activate TP receptor to cause vasoconstrictions. To test whether $PGF_{2\alpha}$ and PGI_2 could act as the EDCF in the aorta of aged hamsters, vascular reactivity of exogenous $PGF_{2\alpha}$ and PGI_2 were examined. $PGF_{2\alpha}$ caused contractions; at the same concentrations of $PGF_{2\alpha}$, the L-NAME-treated aortae from aged hamsters significantly contracted more than those from the young hamsters. By contrast, contractions to KCl or phenylephrine remained unaltered in L-NAME-treated rings from both ages. The increased response to $PGF_{2\alpha}$ is unlikely to be attributed by an increased TP receptor expression, which was comparable in the aortae from both young and aged hamsters. Though PGI_2 shared a similar release profile as $PGF_{2\alpha}$, it can neither relax nor contract the arteries, thus ruling out the possibility for PGI_2 acting as the EDCF. PGE_2, another prostaglandin that can contract blood vessels, was found to contract the aortae

from aged hamsters. These contractions, however, were not sensitive to TP receptor antagonism. Taken in conjuncture, $PGF_{2\alpha}$, but not PGI_2 nor PGE_2, represents a novel prostaglandin that mediates endothelium-dependent contractions in aging.

Noteworthy, COX-2 expression was significantly reduced in endothelium denuded rings, suggesting COX-2 was mainly localized in the endothelium, without much expression in the vascular smooth muscle cells. Since the release of $PGF_{2\alpha}$ was COX-2 derived and removal of endothelium abrogated the release, these observations permit the conclusion that endothelium is the only source of the enhanced EDCF production, in contrast to previous studies by Ibarra et al. (2006) showing that senescent vascular smooth muscle cells can also generate COX-derived vasoconstrictors second to the endothelium during aging in SHR.

ROS have been well-identified as the culprit in endothelial dysfunction during aging. In the present study, the COX-2-dependent endothelium-dependent contractions were neither attenuated by a putative NADPH oxidase or SOD mimetic, indicating that ROS are unlikely to acutely mediate the contractions. Nevertheless, the participation of ROS in the chronic modification of the arachidonic acid cascade cannot be excluded, especially in COX-2 upregulation. Superoxide-derived hydroxyl radical can positively modulate COX-2 expression (Jaimes et al., 2008). In addition, release and plasma concentration of pro-inflammatory cytokines such as interleukin-1β and tumor necrosis factor-α are augmented in both aged animals and humans (Belmin et al., 1995; Bruunsgaard et al., 2000; Csiszar et al., 2003; 2004; Saito et al., 2003; Straub et al., 2001; Wei et al., 1992). COX-2, being highly inducible by redox challenges and cytokine stimulation (Rimarachin et al., 1994; Vane et al., 1998),

may have been chronically upregulated with advancing age, especially when the protection from NO is diminished.

To conclude, the present study provided first-line evidence for a positive impact of aging on COX-2-derived $PGF_{2\alpha}$-mediated endothelium-dependent contractions. Aging is likely to reduce NO bioavailability, ultimately leading to the occurrence of endothelium-dependent contractions without pharmacological inhibition of NO synthesis. The enhanced endothelium-dependent contraction is most likely related to an increased expression and activity of COX-2, a slightly augmented ACh-stimulated release of $PGF_{2\alpha}$, and most importantly, the increased vascular smooth muscle contractility to $PGF_{2\alpha}$ with unaltered expression of the TP receptor in aortae from aged hamsters (Figure 4.13).

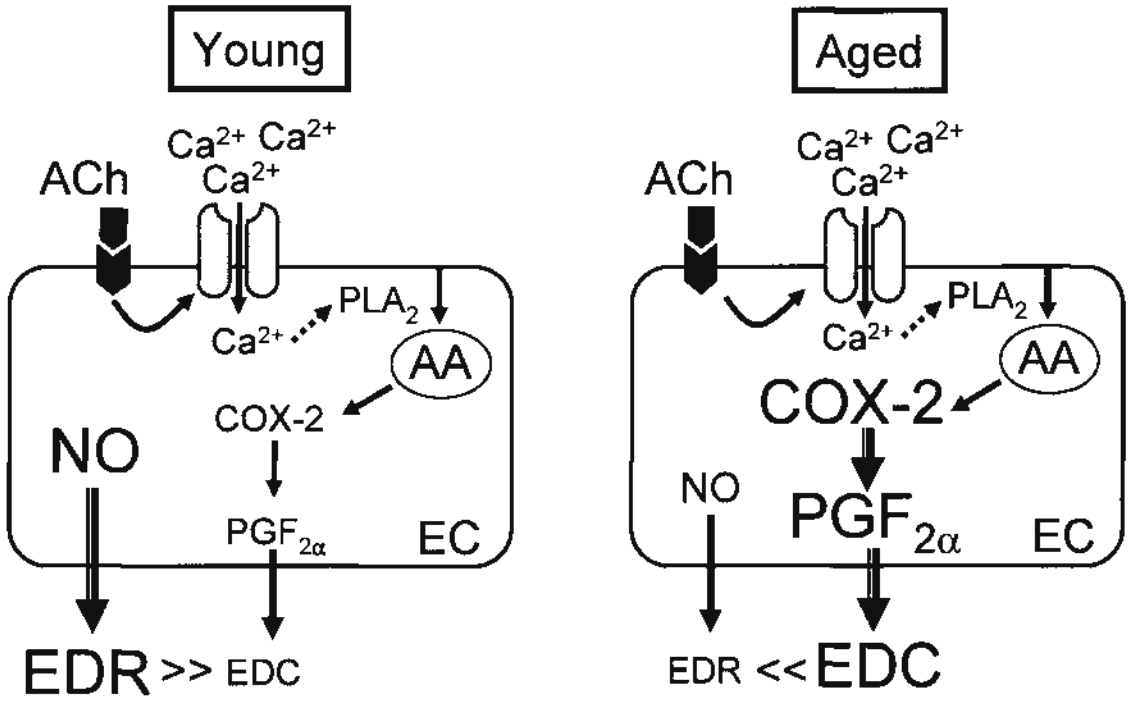

FIGURE 4.13.

In the aorta from young hamsters, acetylcholine (ACh) triggers an influx of extracellular calcium (Ca^{2+}) into the endothelial cells (EC), and COX-2 is activated to produce prostaglandin (PG) $F_{2\alpha}$. During aging, COX-2 expression is augmented which leads to increased release of $PGF_{2\alpha}$, resulting in greater endothelium-dependent contractions (EDC). Since nitric oxide (NO) production is diminished, EDC overrides endothelium-dependent relaxations (EDR), such that EDC are observable without pre-treatment of NO synthase inhibitor, which is required in the aortic rings from the young hamsters. PLA_2, phospholipase A_2; AA, arachidonic acid.

Chapter V

PKC$_\delta$-ERK1/2 and p38 MAPK Mediate Angiotensin II-induced Endothelial Cyclooxygenase-2 Expression

A Link to Vascular Inflammation and Clinical Relevance

5.1 Introduction

Angiotensin II (Ang II), the most prominent vasoactive peptide in the renin-angiotensin system, causes vascular dysfunction through an exaggerated production of reactive oxygen species (ROS) and vascular hyper-contractility through stimulation of Ang II type 1 receptor (AT_1R) (Choi et al., 2008; Landmesser et al., 2007; Mollnau et al., 2002; Savoia and Schiffrin, 2007). Ang II is also a potent inducer of the expression of pro-inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, monocyte chemoattractant protein-1 (MCP-1) and adhesion molecules like ICAM-1 (intercellular adhesion molecule-1), VCAM-1 (vascular cell adhesion molecule-1) or selectins (Ando et al., 2004; Cheng et al., 2005; Costanzo et al., 2003; Pastore et al., 1999; Pueyo et al., 2000). These factors are prerequisite for the initiation of atherosclerosis. In addition, Ang II activates matrix metalloproteinases (MMP), thereby promoting cell migration and adverse vascular remodeling (Parmentier et al., 2006; Savoia and Schiffrin, 2007; Yaghini et al., 2005). Ang II is thus closely associated with diseases accompanied by vascular inflammation.

Cyclooxygenase-2 (COX-2) is minimally expressed in healthy vascular tissues, but it is highly inducible upon stimulation by growth factors, pro-inflammatory cytokines and bacterial toxins (Bawolak et al., 2008; Eligini et al., 2005; Hu et al., 2002; Rikitake et al., 2001). A marked up-regulation of COX-2 is

reported in inflamed vascular tissues (Yogi et al., 2009), in vascular remodeling of wire-injured mouse femoral arteries (Ogawa et al., 2009), and in human atherosclerotic plaques (Barbieri and Weksler, 2007; Cipollone et al., 2004; Cipollone et al., 2001; Gomez-Hernandez et al., 2006). The expression of COX-2, prostanoid synthases, and prostaglandin receptors are up-regulated in blood mononuclear cells and plaques of patients with carotid atherosclerosis (Gomez-Hernandez et al., 2006). The plasma level of prostaglandin E_2, known to activate MMP, is augmented in patients with atherosclerosis (Cipollone et al., 2004; Cipollone et al., 2001; Gomez-Hernandez et al., 2006). Recent studies show that non-steroidal anti-inflammatory drugs (NSAID) can reduce vascular inflammation (Costanzo et al., 2003) and COX-2 inhibition is beneficial in decreasing adhesion molecule expression in cancer cell lines (Dianzani et al., 2008).

Both Ang II and COX-2 are associated with vascular inflammation and remodeling. However, it remains to be explored whether COX-2 could play a direct role as a downstream effector in mediating Ang II-induced vascular pathogenesis. The altered endothelial cell function is the key contributor to vascular inflammation. I hypothesized that Ang II could induce COX-2 expression in endothelial cells, which in turn is related to the generation of pro-inflammatory cytokines. The present study aimed at investigating the relationship between Ang II stimulation and COX-2 expression, the intracellular signaling pathways linking the two pro-inflammatory inducers, and the clinical relevance of COX-2 up-regulation in the human vasculature.

Using primary culture of rat endothelial cells, *in vivo* animal model of Ang II-infused hypertensive rats, and tissue culture of human arteries, I demonstrated here for the first time, to the best of my knowledge, the up-

regulation of endothelial COX-2 by Ang II. The present findings featured a novel role of protein kinase C_δ (PKC_δ) activated at Tyr^{311} and the key mediations via extracellular signal-regulated kinases 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (p38 MAPK) in the Ang II-induced endothelial cell COX-2 expression. Results with renal arteries from patients with hypertension or diabetes point to a clinical relevance of the animal-based findings.

5.2 Materials and Methods

This study was approved by the Animal Experimentation Ethics Committee, Chinese University of Hong Kong. Male Sprague Dawley rats (260-280 g) were supplied by Laboratory Animal Service Centre, Chinese University of Hong Kong. This investigation 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). Human small mesenteric and renal arteries were obtained during surgery after getting informed consent from patients.

5.2.1 Cell culture

Endothelial cells were freshly cultured from rat thoracic aortae as described earlier (Huang et al., 1999). Briefly, the aorta was excised free, cleaned of perivascular adventitial adipose tissue and subjected to digestion in collagenase (Sigma, Type IA) at 37 °C for 15 min. Serum containing medium was added to quench the digestion and the mixture was centrifuged at 800 g for 15 min. The cells were re-suspended in RPMI 1640, supplemented with 10% fetal bovine serum (FBS) plus 1% penicillin/streptomycin (P/S) (GIBCO) and allowed to settle for 1 h upon which the medium was changed. The identity of the endothelial cells was confirmed by immunocytochemical staining for an endothelial cell specific marker, PECAM-1 (Figure 5.1). To avoid the influence of culture condition on endothelial cell phenotype, only cells from passage 1 were used in the present study.

5.2.2 SDS-PAGE and Western blot analysis

Cells were serum-deprived for 24 h and incubated with Ang II (100 nmol/L) for 8 h unless otherwise stated. When used, inhibitors were added to with the cells for 30 min before the addition of Ang II. After the incubation, cells were lysed in an ice-cold RIPA buffer with a cocktail of protease inhibitors (leupetin, 1 µg/ml; aprotonin, 5 µg/ml; PMSF, 100 µg/ml; sodium orthovanadate, 1 mmol/L; EGTA, 1 mmol/L; EDTA, 1 mmol/L; NaF, 1 mmol/L and β-glycerolphosphate, 2 mg/ml). The lysates were centrifuged at 20,000 g for 20 min and protein concentration was determined by the Lowry method (BioRad). Equal amount of proteins from the whole cell lysates were separated by SDS-PAGE as described (Wong et al., 2009) and the membranes were probed with antibodies against COX-2 (Cayman), COX-1 (Cayman), p38 MARK, ERK1/2 and PKC isoforms (Cell Signaling).

5.2.3 Cell fractionation and translocation study of PKC isoforms

Endothelial cells were incubated with 100 nmol/L Ang II for 1 min and quickly cooled on ice to quench cellular reactions. Inhibitors, when used, were added 30 min prior to the introduction of Ang II. Cytosolic and membranous proteins were extracted with ProteoExtract® Subcellular Proteome Extraction Kit according to manufacturer's instructions (Calbiochem). Protein concentration was determined with the Lowry method and equal amount of proteins were subjected to SDS-PAGE as aforementioned.

5.2.4 PKC_{δ} knockdown with small interfering RNA (siRNA)

When primary rat endothelial cells reached 80% confluence, the cells were transfected with siRNA by electroporation using Amaxa Basic Nucleofector Kit

for primary mammalian endothelial cells (Lonza, Germany). Briefly, 2.5 $x10^6$ cells/mL were trypsinized and washed two times with PBS, and resuspended in 100 µl basic nucleofector solution and transferred to a cuvette containing either 30 pmol scramble siRNA or pre-designed specific siRNA targeting PKC_δ transcripts (Ambion). The cells were electroporated with the Amaxa Nucleofactor™ apparatus, re-plated in 6-well plates containing pre-warmed complete RPMI medium and left undisturbed for 24 h. The cells were then serum-deprived for 24 h before an 8 h-incubation with Ang II (100 nmol/L). Inhibitors, when use, were incubated for 30 min prior to Ang II addition.

5.2.5 ROS detection DHE fluorescence

Intracellular superoxide anion production was measured with DHE (Molecular Probes), which binds to DNA upon oxidation to emit fluorescence. Following a 30 s-treatment of Ang II, primary rat endothelial cells seeded on cover-slips were incubated in 5 µmol/L DHE for 20 min at 37 °C. After a rinse in PBS, fluorescence was observed under a confocal microscope (515-nm excitation; 585-nm long pass filter; Olympus Fluoview). DHE fluorescence intensity was analyzed by Fluoview (version 1.5; FV10-ASW1.5). Data were expressed in fold change compared with untreated control.

5.2.6 Suspension antibody array-based multiplex immunoassay

Conditioned medium from endothelial cells treated with Ang II (100 nmol/L) for 24 h were harvested and the levels of IL-6, TNF-α and MCP-1 were measured with MILLIPLEX MAP rodent Cytokine/Chemokine Panel (Millipore) using Bio-plex Suspension Array System (Bio-Rad), according to manufacturer's instructions.

5.2.7 Ang II infusion in rats

Sprague-Dawley rats weighing ~250 g were infused with Ang II at 0.7 mg/kg/day or vehicle (normal saline) for 9 days using an osmotic pump (ALZET, model 2ML2, Alza Pharmaceutical, Palo Alto, CA, USA) implanted subcutaneously in the dorsal region. Blood pressure was regularly monitored throughout the infusion period with a tail-cuff electrosphygmomanometer system, after which aortae and renal arteries were excised, dissected free from perivascular connective tissue and preserved in 4% paraformaldehyde for immunohistchemical staining. Interlobal renal arteries were used for functional evaluation of endothelium-dependent relaxations.

5.2.8 Tissue culture of human small mesenteric arteries

Human small mesenteric arteries were treated with Ang II (1 μmol/L) for 24 h in DMEM supplemented with 10% FBS and 1% P/S at 37 °C. Inhibitors were added 30 min prior to the addition of Ang II. Arteries were preserved for cryo-sectioning and immunofluorescence localization of COX-2 and MCP-1.

5.2.9 Immunohistochemical staining

Localization of COX-2 or MCP-1 in the aortae and renal arteries from Ang II-infused rats and human renal arteries were determined by immunohistochemistry. The tissues were fixed overnight in 4% paraformaldehyde, processed for embedding in wax and then cut into 5 μm-sections. Following re-hydration and treatment with 1.4% hydrogen peroxide in absolute methanol for 30 min at room temperature to inhibit endogenous peroxidase activity, antigen retrieval was performed by boiling the sections in 0.01 mol/L sodium citrate buffer (pH 6) for 30-60 s. After rinsing in PBS, the

sections were blocked with 5% donkey serum and incubated overnight with primary antibodies against COX-2 (Cayman), MCP-1 (Santa Cruz) or Factor VIII (Abcam) diluted in PBS supplemented with 2% BSA in a humidified chamber at 4 °C. The sections were then incubated with corresponding biotinylated secondary antibodies for 1 h at room temperature, followed by 1 h incubation with peroxidase-conjugated streptavidin. DAB (Vector Laboratories, California, USA) was used for color development according to the manufacturer's instruction. Counter-staining of the nucleus was performed with haematoxylin. For rat aortae and renal arteries, counter-staining of cytoplasm was also carried out using eosin to enhance the contrast between cell layers. Negative control was performed in the absence of primary antibodies.

5.2.10 Immunofluorescence localization

Human small mesenteric arteries harvested after a 24 h-incubation protocol were embedded in OCT compound (Sakura Finetek, the Netherlands) in aluminium cryomolds, snap frozen in isopentane pre-cooled in liquid nitrogen and cut into 10 µm thick cryostat sections. The thawed sections were air-dried, post-fixed in 4% paraformaldehyde for 30 min, and then briefly treated with 0.05% Triton X in PBS. The sections were blocked with 5% donkey serum for 1 h at room temperature. Primary antibodies against COX-2 or MCP-1 were incubated overnight at 4 °C. The sections were then labeled with Alexa Fluor 546 donkey anti-goat IgG (Invitrogen, Molecular Probes, California, USA) for 1 h at room temperature. The sections were cover-slipped in anti-fade mounting medium (Vector Laboratories) and views under immunoflourescence microscope (Nikon Eclipse Ti-U).

5.2.11 Functional examination with myography

Endothelium-dependent relaxations of the interlobal renal arteries were determined in myographs. After subjecting the rats to a 9 day-infusion of Ang II, interlobal renal arteries were dissected and suspended in the myograph (Danish Myo Technology, Aarhus, Denmark) with stainless steel wires. Each chamber was filled with 5 mL Krebs-Henseleit solution containing (mmol/L): NaCl 119, $NaHCO_3$ 25, $MgCl_2$ 1, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, and D-glucose 11.1, and aerated with 95% O_2-5% CO_2 at 37 °C to give a pH of ~7.4. The arterial rings were stretched to a pre-determined optimal resting tension of 2 mN and allowed to stabilize at this basal tone for 60 min before experiments began. Acetylcholine (ACh)-induced endothelium-dependent relaxations were evaluated in arteries from control rats and Ang II-infused rats with or without concomitant oral administration of losartan (10 mg/kg/day). The acute effects of celecoxib (3 μmo/L) and sc-560 (0.3 μmol/L) were tested on the relaxations in renal arteries from Ang II-infused rats.

5.2.12 Chemicals

Angiotensin II, losartan, PD 123319, actinomycin-D, SB 202190, PD 98059, SP 600125, L-NAME, AMT, GF109203X, Go 6976, NS 398 and phorbol 12-myristate 13-acetate were purchased from Tocris (Avonmouth, UK). Rottlerin was from Enzo Life Sciences (New York, USA) and εV1-2 from AnaSpec (California, USA). Oxypurinol, tiron, tempol, DETCA, 4α-phorbol 12-myristate 13-acetate and sc-560 were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). Apocynin were from Calbiochem, EMD Biosciences (La Jolla, CA, USA). Celecoxib was from Pfizer. Angiotensin II, PD 123319, N^G-nitro-L-arginine methyl ester, AMT, tiron, tempol, DETCA and εV1-2 were dissolved in distilled

water and the others in dimethyl sulphoxide (DMSO, Sigma). DMSO served as solvent control.

5.2.13 Data Analysis

Protein expression was normalized to GAPDH and relative to the controls unless otherwise stated. Results represent means ± SEM of n separate experiments. Statistical significance was determined by two-tailed Student's *t*-test or one-way ANOVA followed by Bonferroni post-tests when more than two treatments were compared (GraphPad Software, San Diego, California). A *P* value of less than 0.05 was regarded as statistically different.

5.3 Results

5.3.1 Ang II induces COX-2 expression in endothelial cells

COX-2 expression was undetectable in the untreated quiescent endothelial cells and it was not induced by culturing in serum-deprived medium. Ang II at 100 nmol/L increased COX-2 expression, reaching a maximum after an 8 h-incubation and this effect was concentration-dependent (3-100 nmol/L) (Figure 5.2A & 5.3A). Treatment with actinomycin-D (10 μmol/L), an inhibitor of RNA synthesis, prevented Ang II-induced COX-2 expression (Figure 5.4A) without affecting COX-1 expression (Figure 5.4B), indicating the inducible nature of COX-2 in endothelial cells in response to Ang II. By contrast, COX-1 was constitutively expressed in endothelial cells and its level was unaffected by Ang II (Figure 5.2B & 5.3B).

5.3.2 Ang II up-regulates COX-2 expression via AT_1R

Treatment with losartan (3 μmol/L), an AT_1R blocker, abolished Ang II-induced COX-2 expression, while PD 123319 (1 μmol/L), an AT_2R blocker, was without effect (Figure 5.4A).

5.3.3 p38 MAPK and ERK1/2 jointly mediate Ang II-induced COX-2 expression

Of the three well-known kinase pathways, only p38 MAPK inhibitor (SB 202190, 10 μmol/L) and ERK1/2 inhibitor (PD 98059, 20 μmol/L) reduced Ang II-induced COX-2 expression, each by ~50%, while SP 600125 (10 μmol/L), a c-Jun-N-terminal kinase (JNK) inhibitor, had no effect. Co-treatment with SB 202190 and PD 98059 produced additive inhibition (Figure 5.5A), suggesting a parallel involvement of p38 MAPK and ERK1/2 kinases in mediating Ang II-induced

COX-2 expression. By contrast, these inhibitors did not alter the expression of COX-1 (Figure 5.5B). When the time course of ERK1/2 and p38 MAPK activation by Ang II was examined, peak phosphorylation of both kinases was noted ~ 5 min after the addition of 100 nmol/L Ang II (Figure 5.5C&D).

5.3.4 PKC activation is upstream of ERK1/2

Treatment with GF 109203X (GFX, 2 μmol/L), an inhibitor for a broad spectrum of PKC isoforms, markedly reduced Ang II-induced COX-2 expression. Rottlerin (PKC_{δ} inhibitor, 10 μmol/L) but not Go 6976 ($PKC_{\alpha/\beta}$ inhibitor, 1 μmol/L) inhibited COX-2 expression, indicating that PKC_{δ} is the mediator of Ang II-induced COX-2 expression (Figure 5.6A). Again, these inhibitors did not affect COX-1 expression (Figure 5.6B). Next, I determined the sequence of events involving PKC, ERK1/2 and p38 MAPK upon Ang II stimulation. PKC inhibition would be expected to prevent the activation of ERK1/2 and p38 MAPK if PKC is upstream regulator of the two latter pathways. ERK1/2 phosphorylation was inhibited by GFX and rottlerin but not Go 6976, with PD 98059 serving as a positive control for ERK1/2 inhibition (Figure 5.6C). By contrast, p38 MAPK phosphorylation was prevented by losartan, but unaltered by GFX, Go 6976 and rottlerin (Figure 5.6D), indicating that unlike ERK1/2, activation of p38 MAPK was independent of PKC_{δ} regulation.

5.3.5 PKC_{δ} plays a key role with little involvement of other PKC isoforms

Studies with the pharmacological inhibitor of PKC_{δ}, rottlerin, implied the participation of PKC_{δ} in Ang II-induced COX-2 expression. To confirm the involvement of PKC_{δ}, a time-dependent phosphorylation of PKC_{δ} at the activation site Tyr^{311} was determined. PKC_{δ} was activated in less than 1 min

after the addition of Ang II (Figure 5.7).

Cytoplasm to membrane translocation of various PKC isoforms was examined by fractionating the cytosolic and membranous portion of total protein from endothelial cells. I validated this method by a clear detection of large amount of cytosolic GAPDH in the cytosol but minimal in the membranous fraction, and vice versa for membrane-bound NOX-2 subunit of NADPH oxidase (Figure 5.8A&B). The results showed that PKC_{δ} and PKC_{ε} were activated upon 1-min stimulation with Ang II, which was signified by their increased levels in membranous fractions. By contrast, α, θ, and μ isoforms did not increase in the membranous portions of Ang II-stimulated cells compared with that of control. PKC_{ζ} was minimally expressed in endothelial cells (Figure 5.8C & 5.9). PKC_{δ} translocation was abolished by losartan (Figure 5.10A). SB 202190 and PD 98059 failed to inhibit AngII-induced PKC_{δ} translocation (Figure 5.10B), again confirming the notion that ERK1/2 can only be downstream target of PKC_{δ}, but not vice versa, while p38 MAPK and PKC_{δ} are independent of each other. Co-treatment with SB 202190 and rottlerin produced additive effects in suppressing the Ang II-induced COX-2 expression, without affecting COX-1 expression (Figure 5.11A&B).

Transfection with small interfering RNA targeting PKC_{δ} ($siPKC_{\delta}$) further supports the key role of PKC_{δ}. Western blot analysis confirmed that $siPKC_{\delta}$ successfully knock-downed PKC_{δ} without affecting PKC_{ε} (Figure 5.12A,D&E). In $siPKC_{\delta}$-transfected cells, Ang II-induced COX-2 expression was reduced by ~50% and the remaining portion could almost be abolished by co-treatment with SB 202190, again indicating the operation of dual signal transduction pathways involving PKC_{δ} and p38 MAPK. By contrast, scramble siRNA had no effect on Ang II-induced COX-2 expression, when compared with the non-transfected

endothelial cells (Figure 5.12A-C).

As shown in the study of PKC translocation, PKC_{ε} was also activated by Ang II (Figure 5.8C & 5.9C). However, PKC_{ε} inhibitor peptide, εV1-2, did not prevent Ang II-induced COX-2 expression (Figure 5.11C). $siPKC_{\delta}$ did not suppress PKC_{ε} levels (Figure 5.12A&E) but abolished COX-2 expression in combination with p38 MAPK inhibitor, SB 202190 (Figure 5.12A&C), thus suggesting that PKC_{ε} is unlikely to be one of the mediators of COX-2 expression.

The role of PKC_{δ} in COX-2 expression was also pinpointed using an exogenous PKC activator, phorbol 12-myristate 13-acetate (PMA). PMA at 1 μmol/L time-dependently increased COX-2 expression while its negative analog, 4α-PMA had no effect (Figure 5.13A&B). Such increase in COX-2 expression was again sensitive to inhibition by GFX and rottlerin, but not Go 6976 (Figure 5.13A&C).

5.3.6 Ang II-induced release of MCP-1 is COX-2 dependent

The level of MCP-1 in the conditioned medium from endothelial cells treated with 100 nmol/L Ang II for 24 h increased (Figure 5.14), while the release of IL-6 and TNF-α fell below detectable levels (data not shown). The MCP-1 release was inhibited by losartan and two structurally different COX-2 inhibitors, celecoxib and NS 398 (both at 3 μmol/L) (Figure 5.14).

5.3.7 Renovascular dysfunction and elevated endothelial COX-2 expression in Ang II-infused rats

Systolic blood pressure of rats rose up from 104.3 ± 4.6 mmHg to 174.0 ± 9.6 mmHg during a 9-day period of Ang II infusion, and this increase was prevented

by the concomitant oral treatment of losartan (Figure 5.15A). Endothelium-dependent relaxations were impaired in interlobal renal arteries from the Ang II-infused rats and this impairment was abolished by losartan treatment (Figure 5.15B). The attenuated relaxations were restored by acute treatment with COX-2 inhibitor celecoxib (3 μmol/L) (Figure 5.15C), but not by COX-1 inhibitor sc-560 (data not shown). Immunohistochemical staining revealed that endothelial COX-2 expression (indicated by red arrows) was augmented in both aortae (Figure 5.16A) and renal arteries (Figure 5.16B) of Ang II-infused rats and the increased COX-2 staining was attenuated by losartan.

5.3.8 Ang II-induced COX-2 expression and COX-2-dependent MCP-1 expression in human mesenteric arteries

The Ang II-induced expression of pro-inflammatory MCP-1 was also demonstrated in the endothelial layer of human mesenteric arteries. The first column of Figure 5.17A and B demonstrated the green autofluorescence from elastin of the internal elastic lamina which delineated the artery into endothelial and smooth muscle layers. The second column featured the fluorescence emitted from Alexa fluor 546-conjugated secondary antibodies which were tagged to primary antibodies against either COX-2 (Figure 5.17A) or MCP-1 (Figure 5.17B), appearing in reddish orange color together with the noise from autofluorescence. In the overlay images on the third column, the autofluorescence and noise appeared yellowish green, and the remaining reddish orange signified the signals from COX-2 or MCP-1. Referring to the third column of Figure 5.17A, endothelial COX-2 expression was remarkably augmented after 24 h-exposure to Ang II (1 μmol/L), which was prevented by co-treatment with SB 202190 and rottlerin, but remained unaffected in solvent

control exposed to DMSO. Noteworthy, this COX-2 expression may contribute to the MCP-1 expression, as evidenced by an increase of endothelial MCP-1 expression in the same arteries treated with Ang II, which was inhibited by pre-incubation with celecoxib but not the solvent DMSO (Figure 5.17B).

5.3.9 Co-expression of COX-2 and MCP-1 in renal arteries from patients with hypertension or diabetes

Nine renal arterial samples were collected from patients undergoing renal surgery for transitional cell carcinoma or renal cell carcinoma, among them three were identified non-diabetic and non-hypertensive, three were diagnosed diabetic (fasting plasma glucose level ≥ 7.0 mmol/L) and three were hypertensive (systolic and diastolic blood pressure >140 mmHg and >90 mmHg respectively). Two patients from the hypertensive group were female, and all other patients were male. The average age of patients in each group is ~60 years old, ranging from ~ 40 to ~ 85 years old. Expressions of COX-2 (indicated by red arrows) and MCP-1 (indicated by green arrows) were higher in renal arteries from patients with hypertension or diabetes as demonstrated by the dark brown DAB staining compared with those from non-diabetic and non-hypertensive subjects (Figure 5.18). Factor VIII served as a specific marker for endothelium which was stained dark brown.

5.3.10. Reactive oxygen species (ROS) do not mediate COX-2 up-regulation

Based on the fact that Ang II is well-documented to increase oxidative stress in vascular cells and the signaling molecules p38 MAPK and ERK1/2 are redox-sensitive (Costanzo et al., 2003; Granger et al., 2004; Hu et al., 2002), I tested

whether ROS play a role in Ang II-induced COX-2 expression.

Dihydroethidium (DHE) fluorescence in endothelial cells showed that the level of reactive oxygen species was raised in ~30 s after Ang II addition, which was prevented by losartan, NADPH oxidase inhibitor apocynin (100 μmol/L) and ROS scavengers, tiron (1 mmol/L) plus DETCA (100 μmol/L) (Figure 5.19). Inhibitors of ROS-producing enzymes including apocynin (100 μmol/L), oxypurinol (xanthine oxidase inhibitor, 100 μmol/L), 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT, iNOS inhibitor, 30 μmol/L), N^G-nitro-L-arginine methyl ester (L-NAME, non-specific NOS inhibitor, 100 μmol/L), ROS scavengers tempol, and tiron plus DETCA, however, did not decrease the Ang II-induced COX-2 expression (Figure 5.20A), thus ruling out the participation from ROS. Indeed, exogenous H_2O_2 only induced a small increase in COX-2 expression compared with the marked stimulation by Ang II (Figure 5.21A).

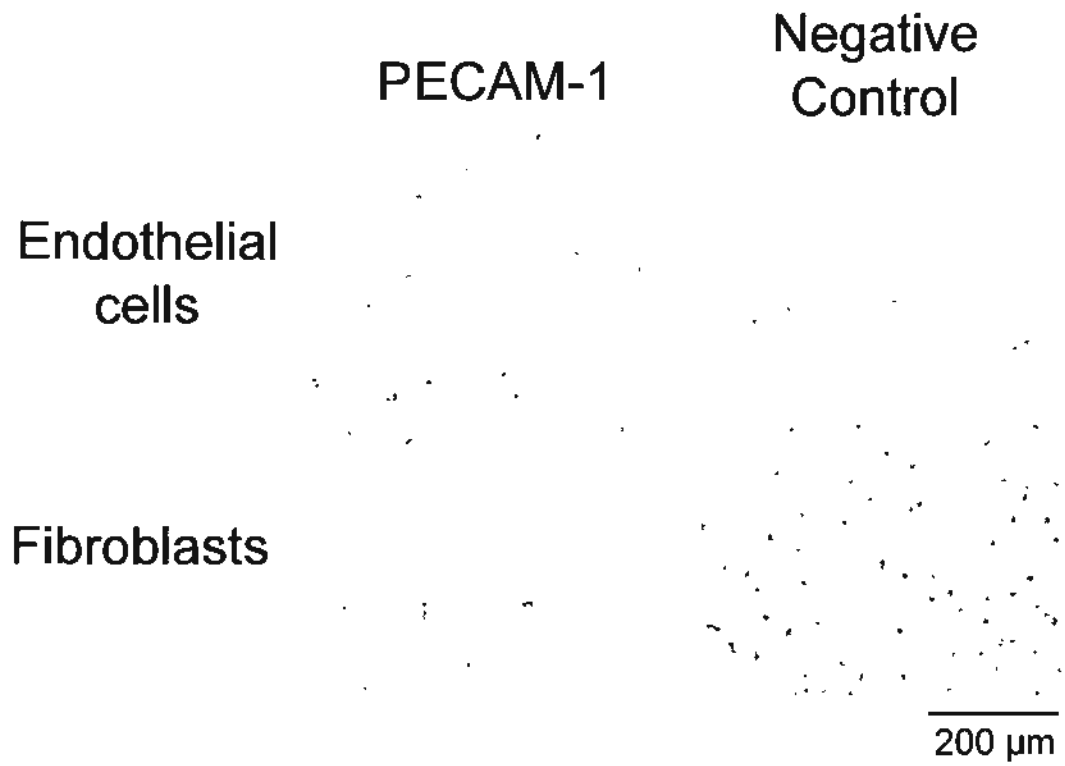

FIGURE 5.1.

Primary culture of cells from rat thoracic aortae were confirmed to be endothelial cells, which were positively stained with an endothelial cell-specific maker, PECAM-1. Fibroblasts served as a negative control to the staining.

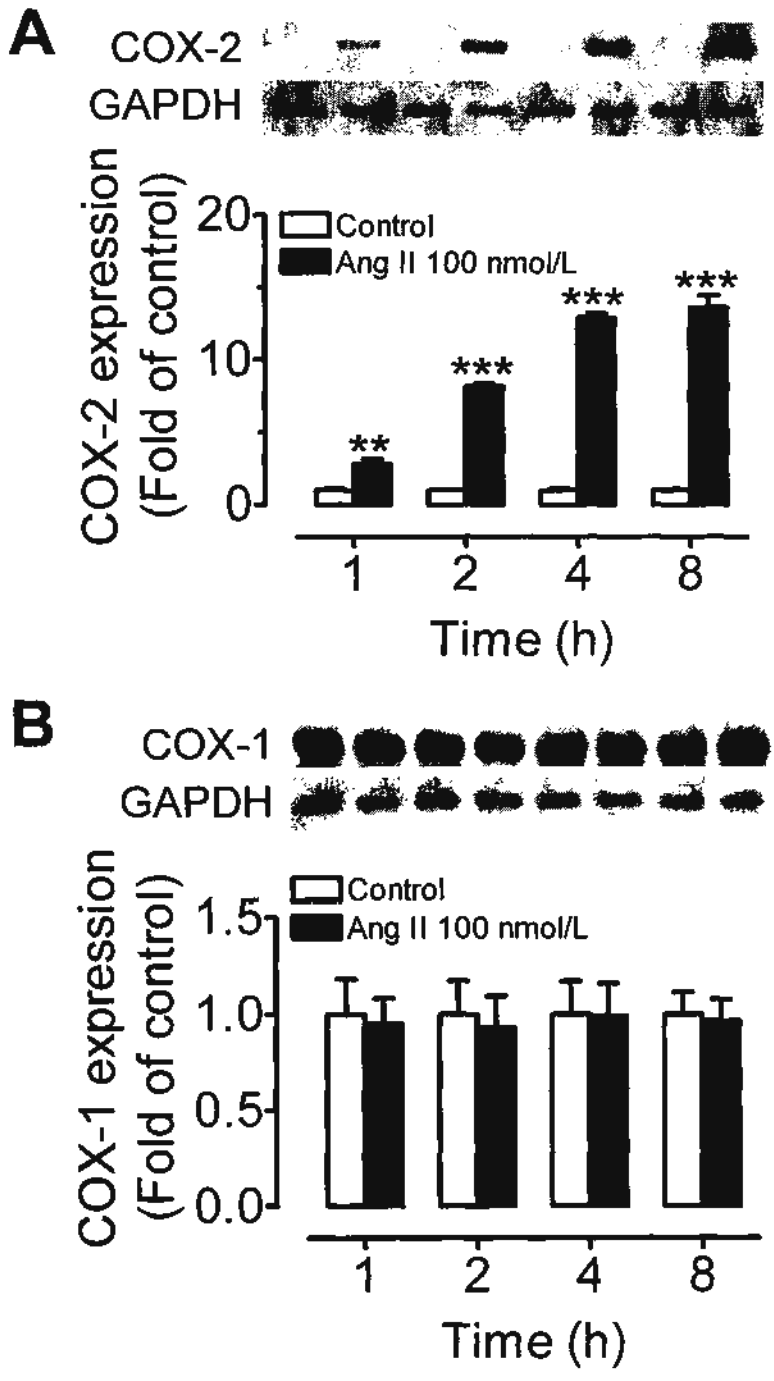

FIGURE 5.2.

(A) COX-2 expression in primary rat endothelial cells increased in response to 100 nmol/L Ang II in a time-dependent (1-8 h) manner, while (B) COX-1 expression was not affected by Ang II incubation across time. Data are mean ± SEM of 5-6 experiments. $^{**}P<0.01$, $^{***}P<0.001$ versus control.

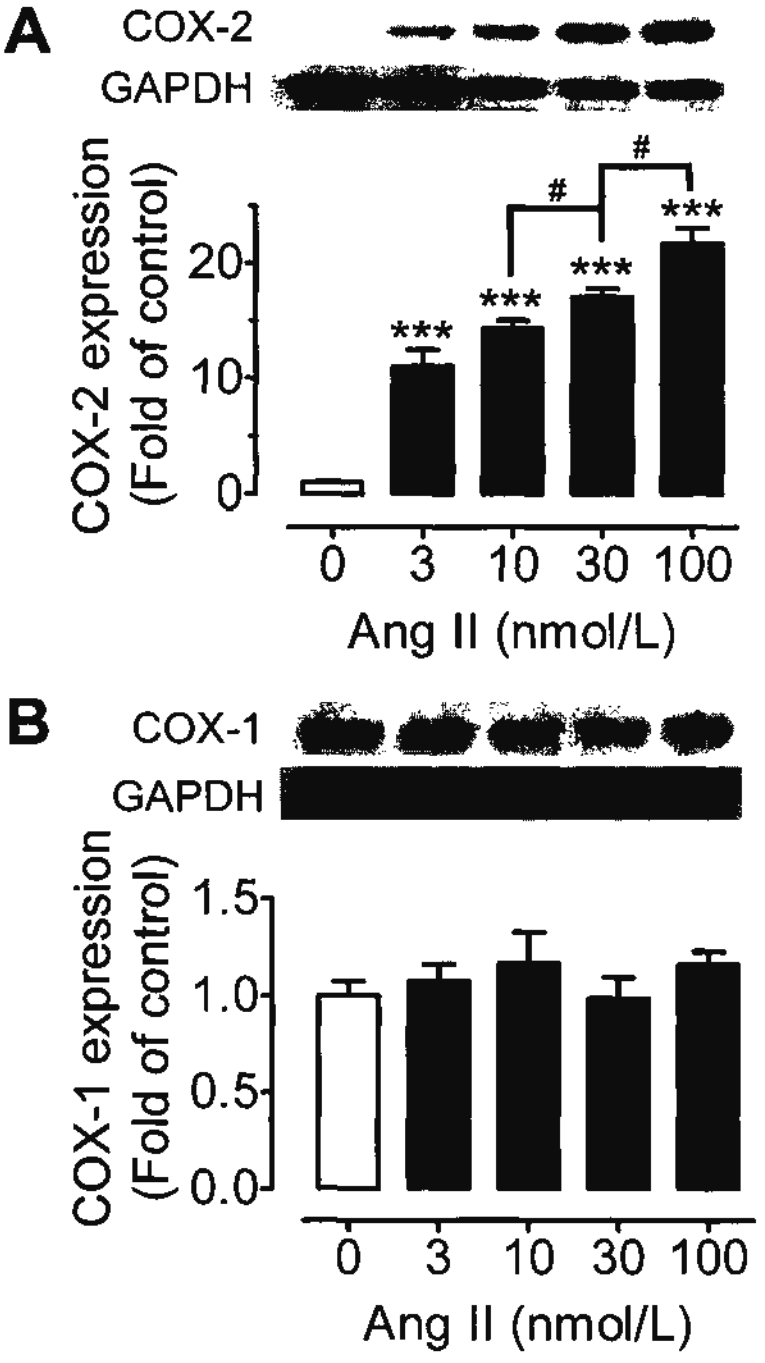

FIGURE 5.3.

(A) Ang II induced a concentration (0-100 nmol/L)-dependent expression of COX-2 in 8 h-incubation. (B) COX-1 expression remained unaffected by Ang II incubation at different concentrations. Data are mean ± SEM of 5-6 experiments. ***P<0.001 versus control; $^{\#}P$<0.05 between groups treated with Ang II.

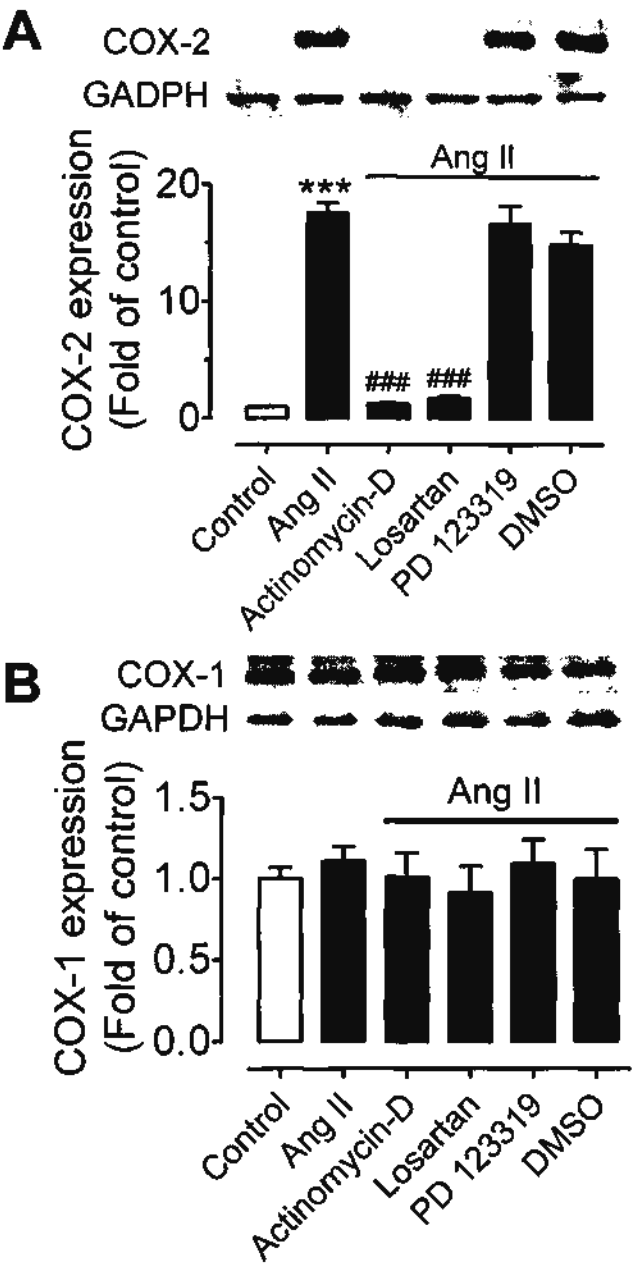

FIGURE 5.4.

(A) Ang II (100 nmol/L)-induced COX-2 expression was prevented by actinomycin-D (10 µmol/L) and losartan (3 µmol/L), but unaffected by the presence of PD 123319 (1 µmol/L) and the solvent DMSO. (B) COX-1 expression was not affected by these inhibitors. Data are mean ± SEM of 5 experiments. ***P<0.001 versus control; ###P<0.001 between Ang II and other groups co-treated with Ang II.

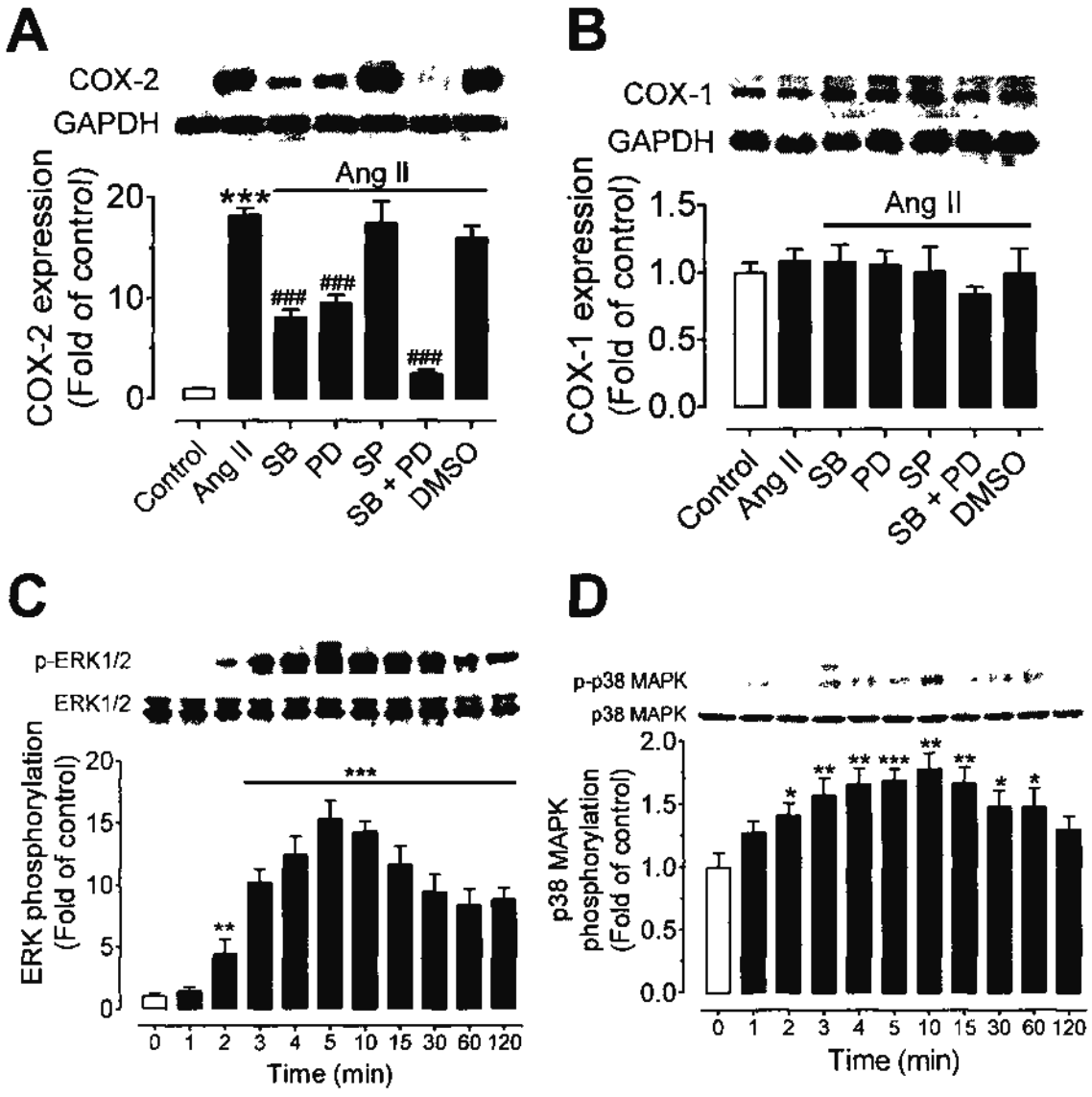

FIGURE 5.5.

(A) COX-2 expression was inhibited by SB 202190 (SB, 10 μmol/L) or PD 98059 (PD, 20 μmol/L), but not by SP 600125 (SP, 10 μmol/L). Co-treatment of SB and PD further reduced the COX-2 expression. (B) COX-1 expression was not affected by these inhibitors. (C&D) Time course for phosphorylation of ERK1/2 and p38 MAPK in response to Ang II (100 nmol/L). Data are mean ± SEM of 5 experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus control; $^{\#\#\#}P<0.001$ between Ang II and other groups co-treated with Ang II.

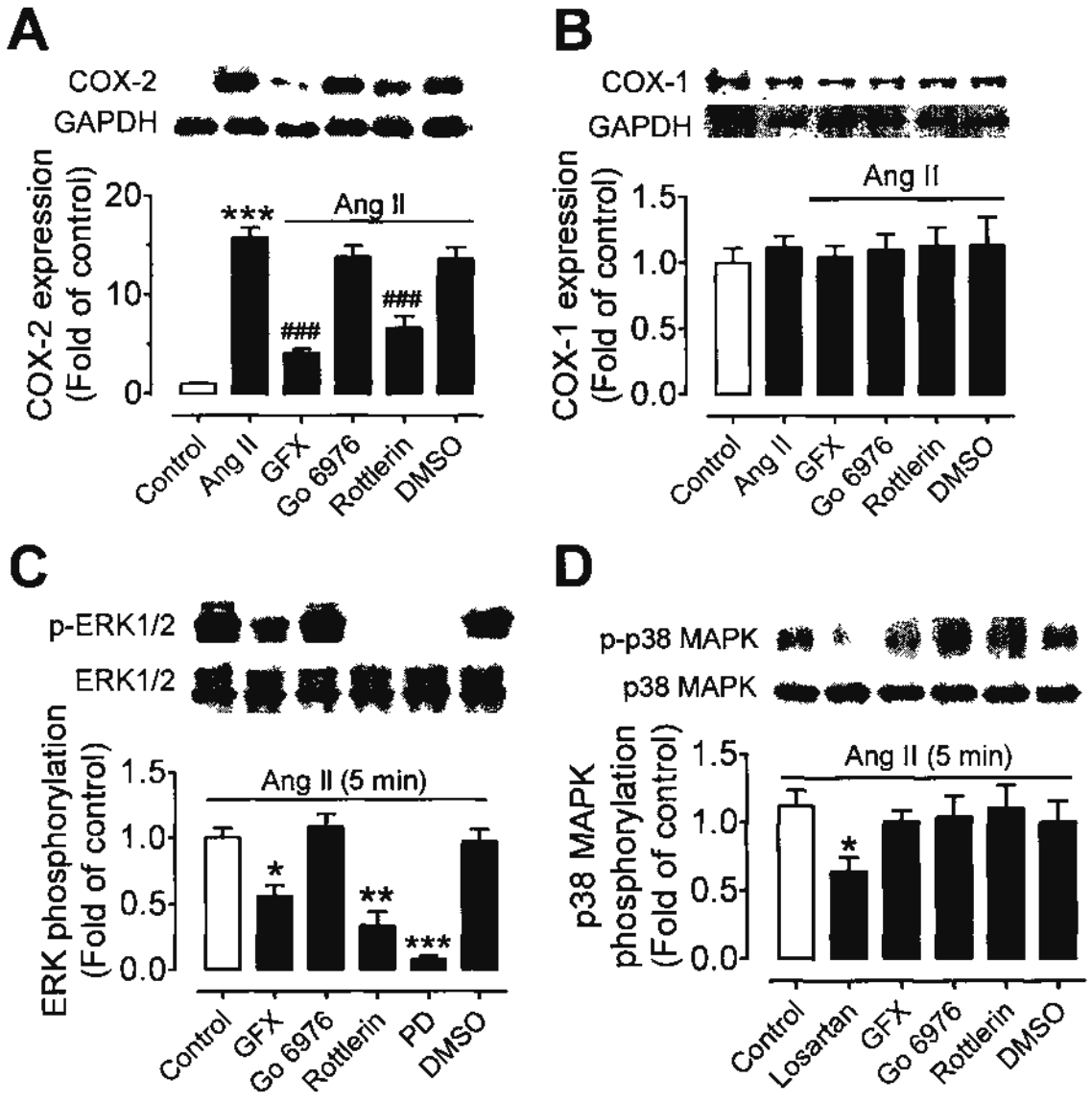

FIGURE 5.6.

(A) COX-2 expression was inhibited by GF 109203X (GFX, 2 μmol/L) or rottlerin (10 μmol/L), but not Go 6976 (1 μmol/L). (B) COX-1 expression remained unchanged with these inhibitors. (C) ERK1/2 phosphoryation was inhibited by GF 109203X (GFX, 2 μmol/L), rottlerin (10 μmol/L) or PD 98059 (PD), but not Go 6976. (D) Phosphorylation of p38 MAPK was inhibited by losartan (3 μmol/L) but not PKC inhibitors. **P*<0.05, ***P*<0.01, ****P*<0.001 versus control; ###*P*<0.001 between Ang II and other groups co-treated with Ang II.

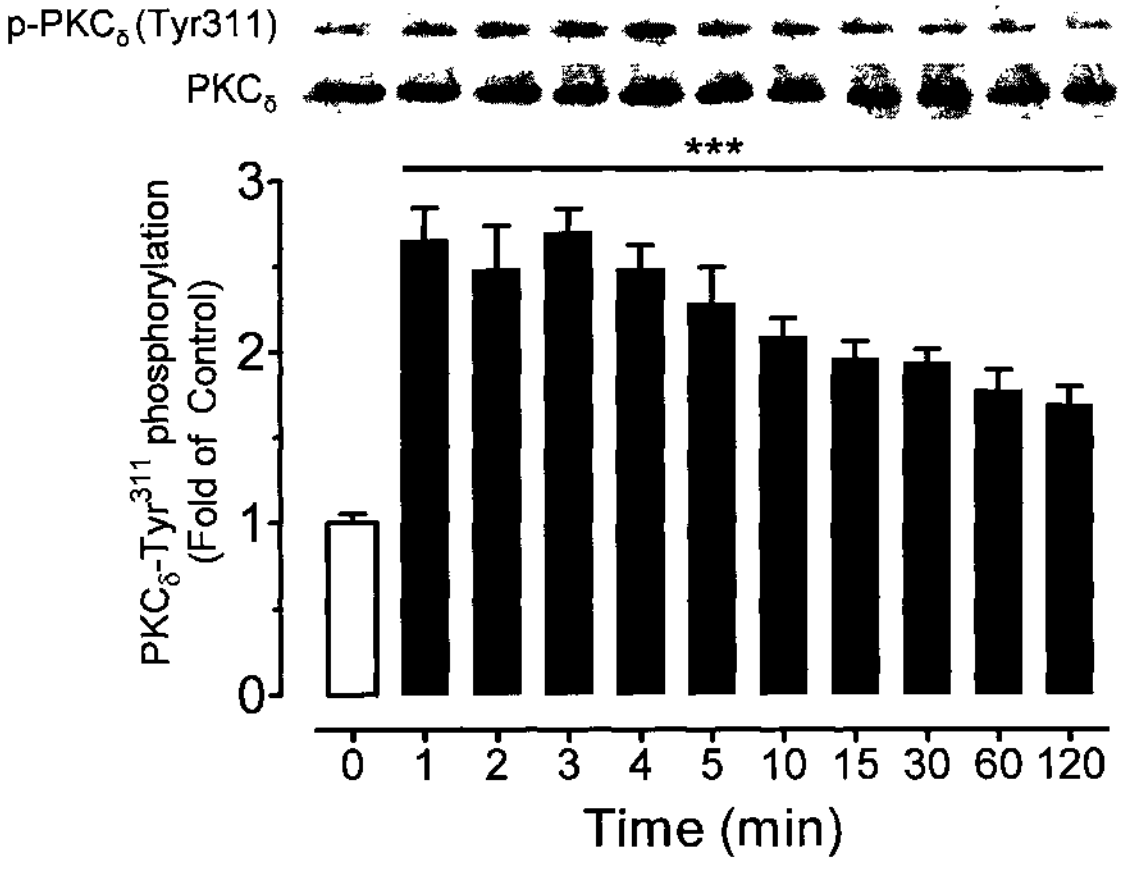

FIGURE 5.7.

PKC_δ was phosphorylated at Tyr^{311} within 1 min of Ang II addition. Data are mean ± SEM of 5 experiments. ***P<0.001 versus control.

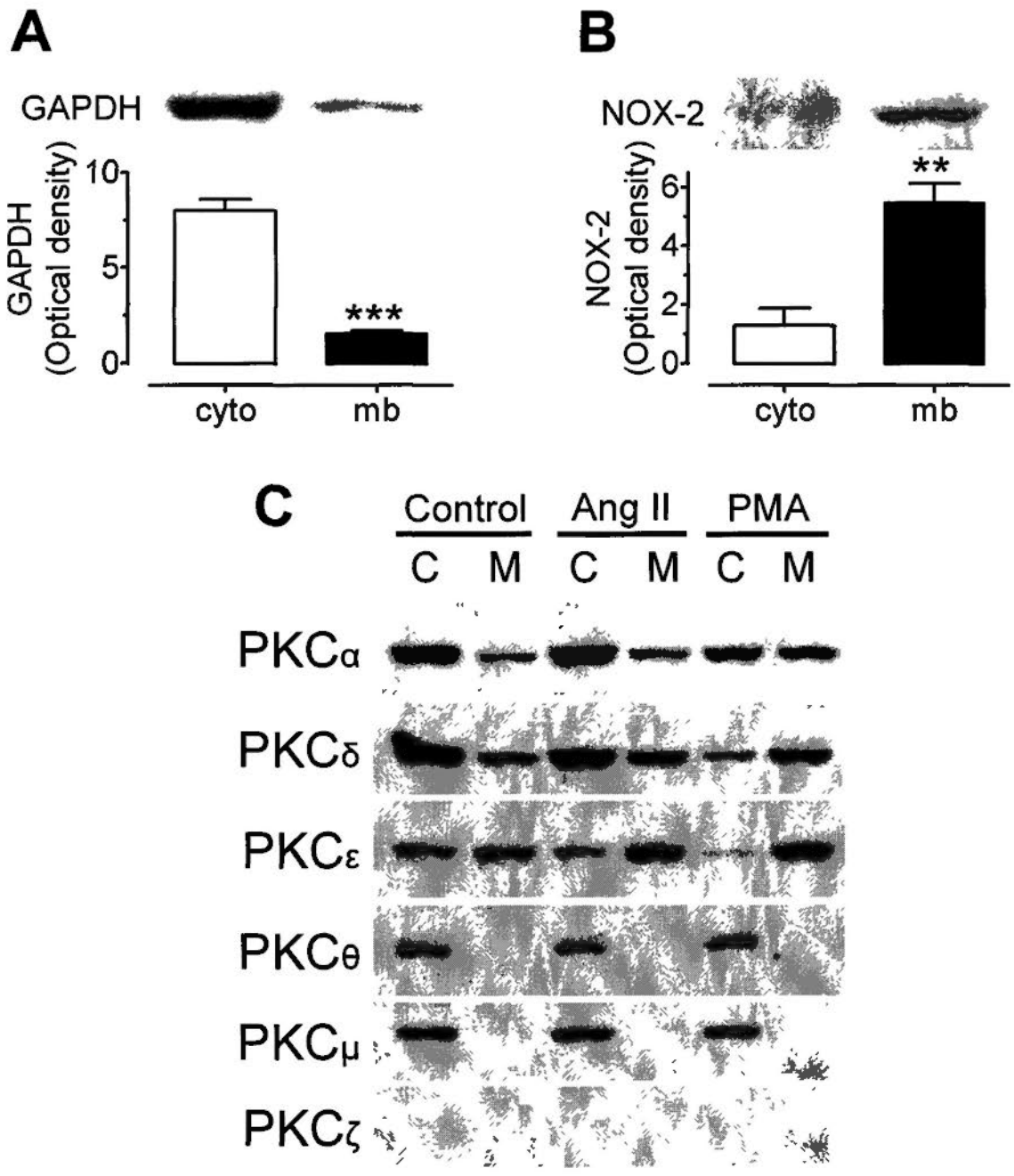

FIGURE 5.8.

(A&B) Validation on the extraction method of cytosolic (cyto) and membranous (mb) protein by detecting cytosolic GAPDH and membrane-bound NOX-2. Data are mean ± SEM of 6 experiments. $^{**}P<0.01$, $^{***}P<0.001$ versus cyto. (B) Representative immunoblots of translocation of various PKC isoforms upon Ang II (100 nmol/L) and PMA (1 μmol/L) stimulation. C, cytosol; M, membrane.

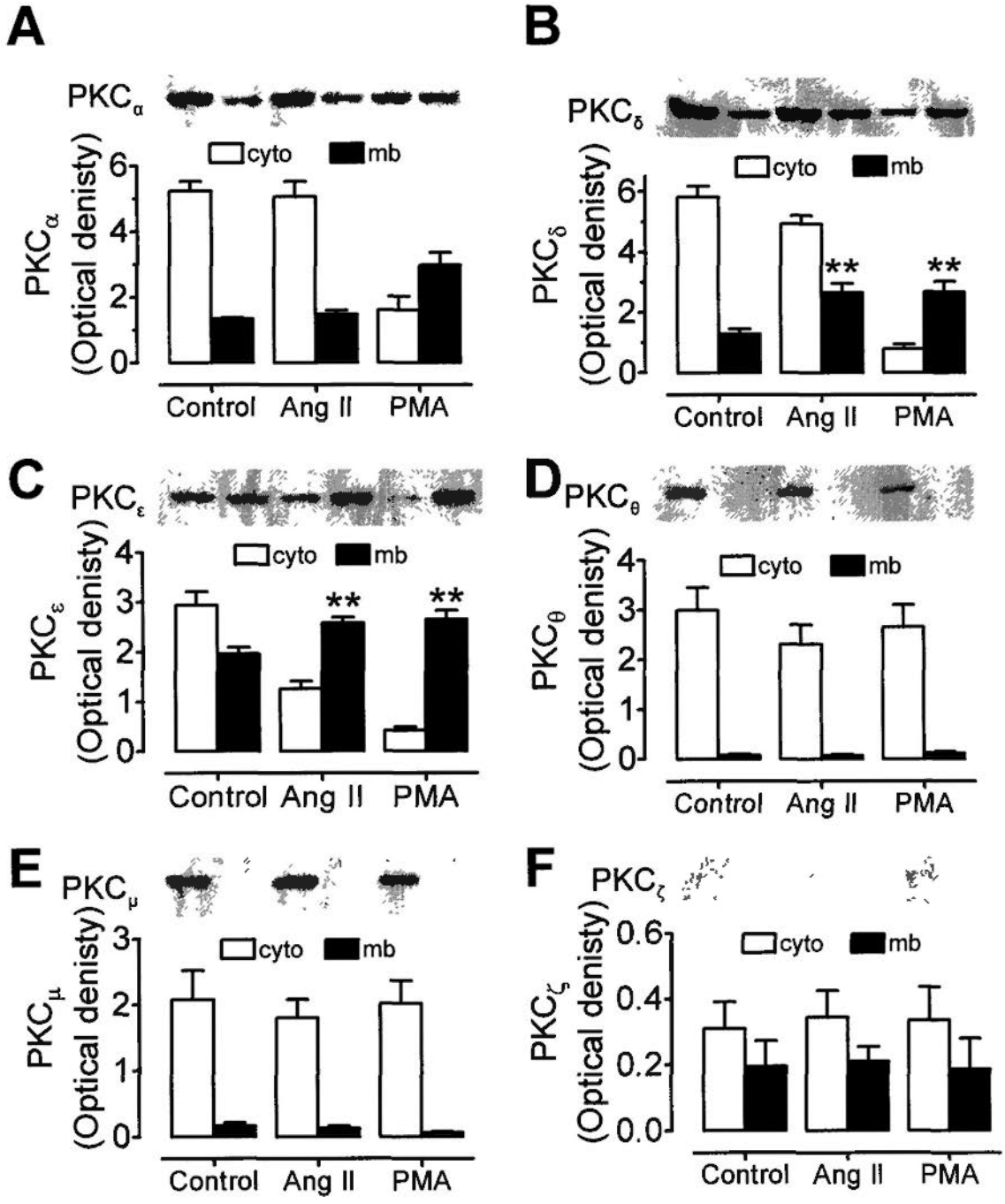

FIGURE 5.9.

Summarized data on the translocation of PKC isoforms (A) α, (B) δ, (C) ε, (D) θ, (E) μ and (F) ζ upon stimulation by Ang II (100 nmol/L) and PMA (1 μmol/L). Data are mean ± SEM of 4-5 experiments. $^{**}P<0.01$ versus mb of the control. Cyto, cytosol; mb, membrane.

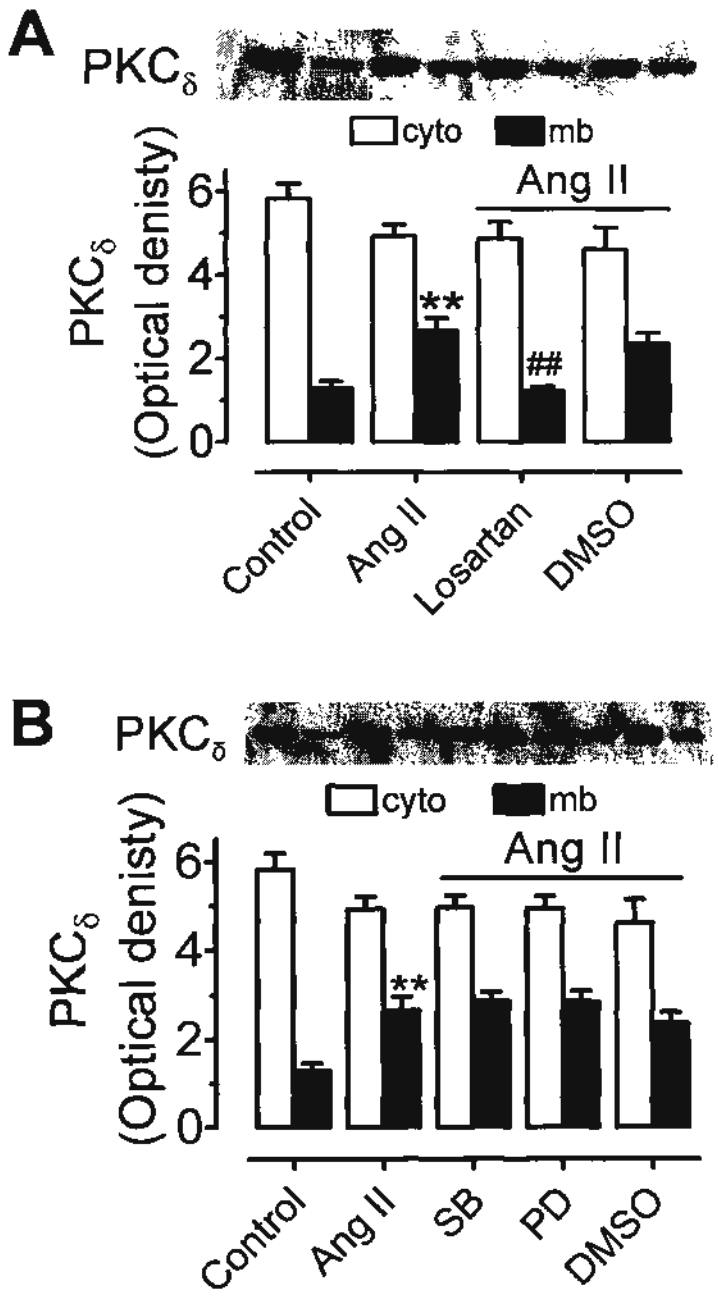

FIGURE 5.10.

Ang II stimulated PKC_δ to translocate to the membrane, which was (A) prevented by losartan (3 μmol/L), but (B) not by SB 202190 (SB, 10 μmol/L) and PD 98059 (PD, 20 μmol/L). Data are mean ± SEM of 4-5 experiments. $^{**}P<0.01$ versus mb of the control, $^{\#\#}P<0.01$ versus mb of Ang II. Cyto, cytosol; mb, membrane.

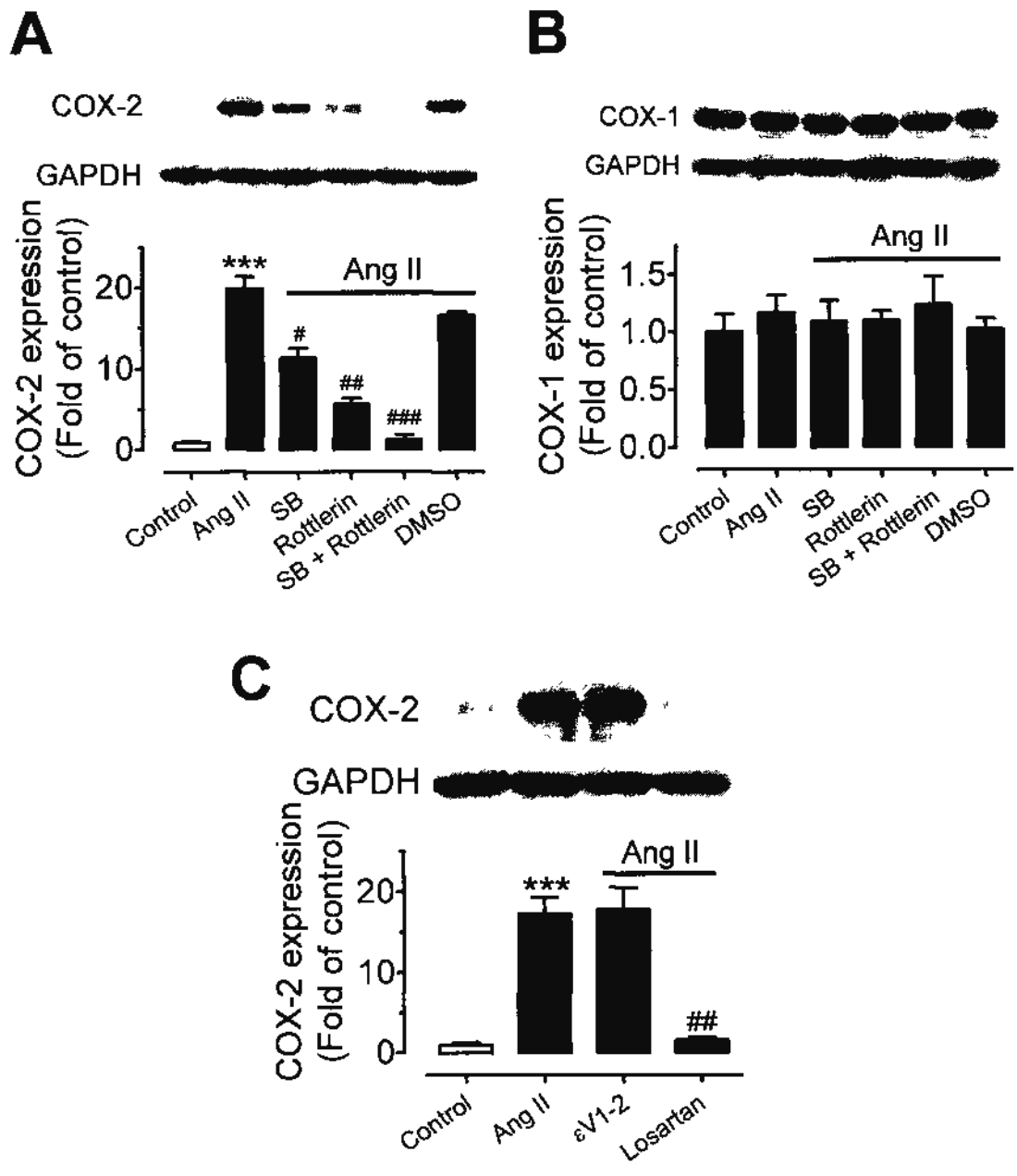

FIGURE 5.11.

(A) Co-treatment of SB 202190 (SB, 10μmol/L) and rottlerin (10 μmol/L) abolished Ang II-induced COX-2 expression, (B) while these inhibitors did not affect COX-1 expression. (C) εV1-2 (10 μmol/L) did not reduce COX-2 expression. Data are mean ± SEM of 4-6 experiments. ***P<0.001 versus control, #P<0.05, ##P<0.01 and ###P<0.001 versus Ang II.

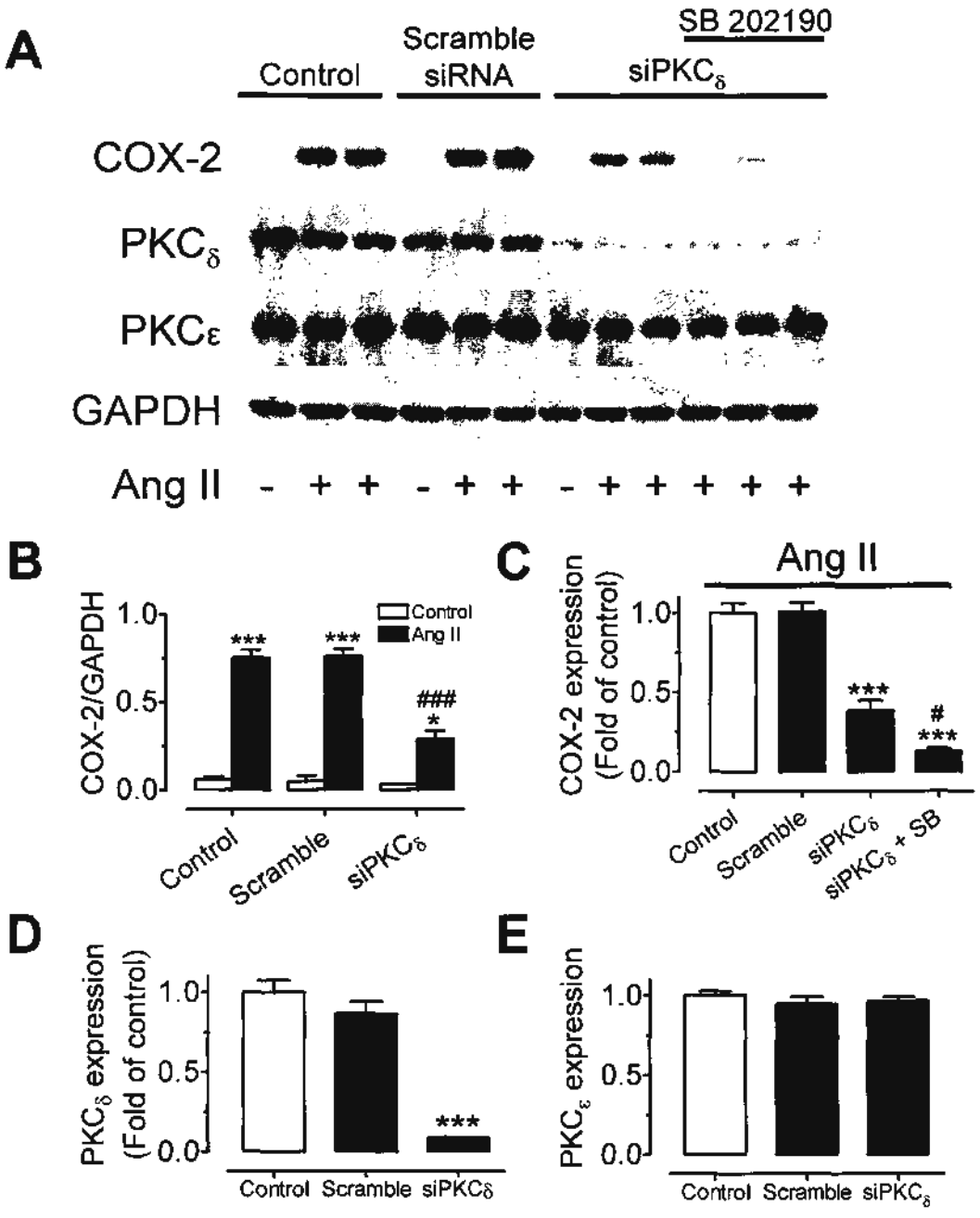

FIGURE 5.12.

(A) Representative blot and (B&C) summarized data showing small interfering RNA (siRNA) targeting PKC_δ ($siPKC_\delta$) significantly reduced Ang II-induced COX-2 expression, which was further abolished by SB 202190. (D&E) $siPKC_\delta$ abolished expression of PKC_δ but not PKC_ε. Data are mean ± SEM of 4 experiments. $^{***}P<0.001$ versus respective controls; $^{\#}P<0.05$ and $^{\#\#\#}P<0.001$ versus scramble siRNA-transfected group treated with Ang II.

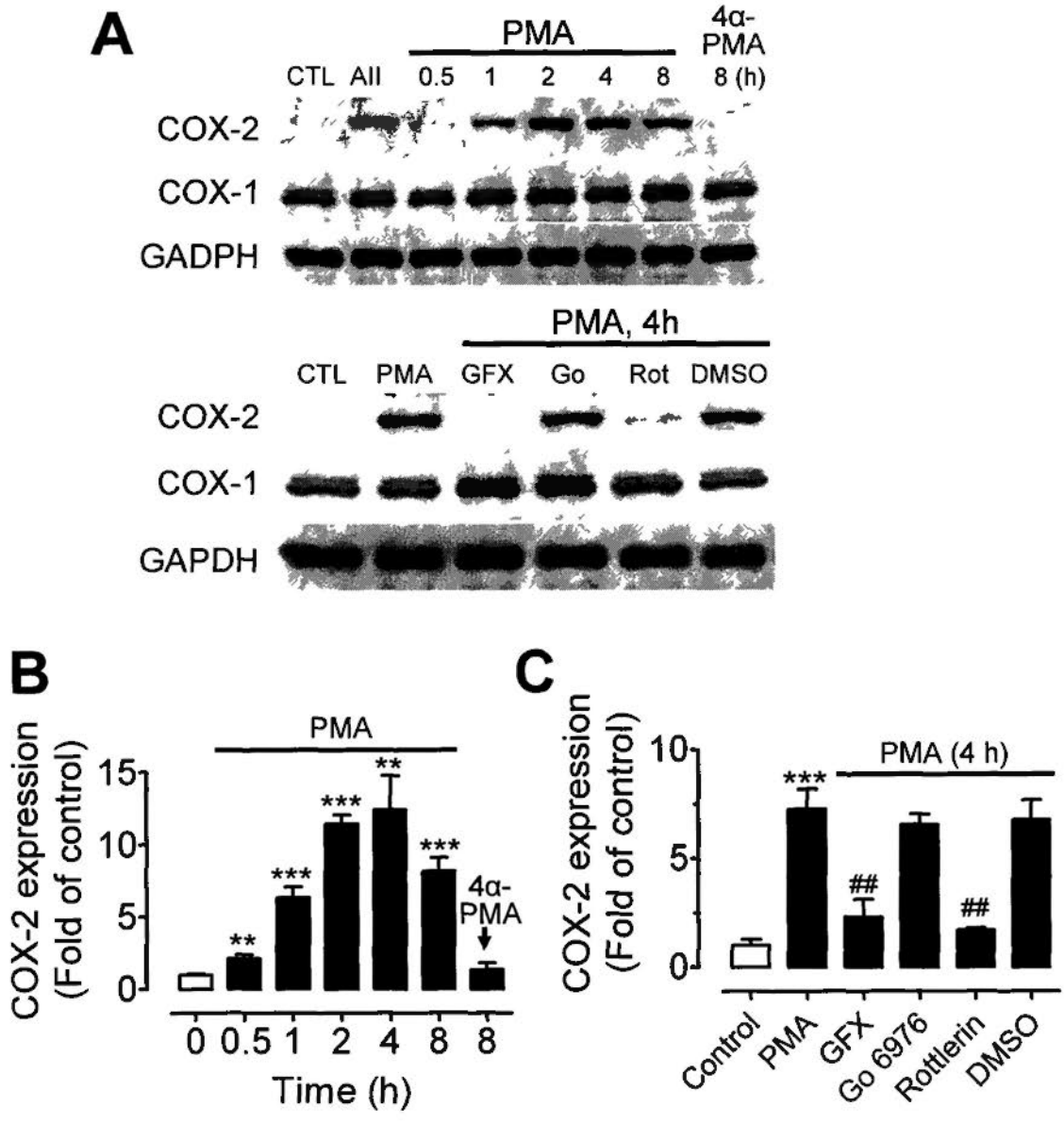

FIGURE 5.13.

(A) Representative blot and (B) summarized data showing PMA (1 μmol/L) induced COX-2 expression but not its negative analogue 4α-PMA (1 μmol/L). (A&C) The COX-2 expression was inhibited by GF 109203X (GFX, 2 μmol/L) and rottlerin (10 μmol/L), but not Go 6976 (1 μmol/L). Data are mean ± SEM of 4 experiments. $^{**}P<0.05$ and $^{***}P<0.001$ versus control; $^{\#\#}P<0.01$ versus PMA-treated group.

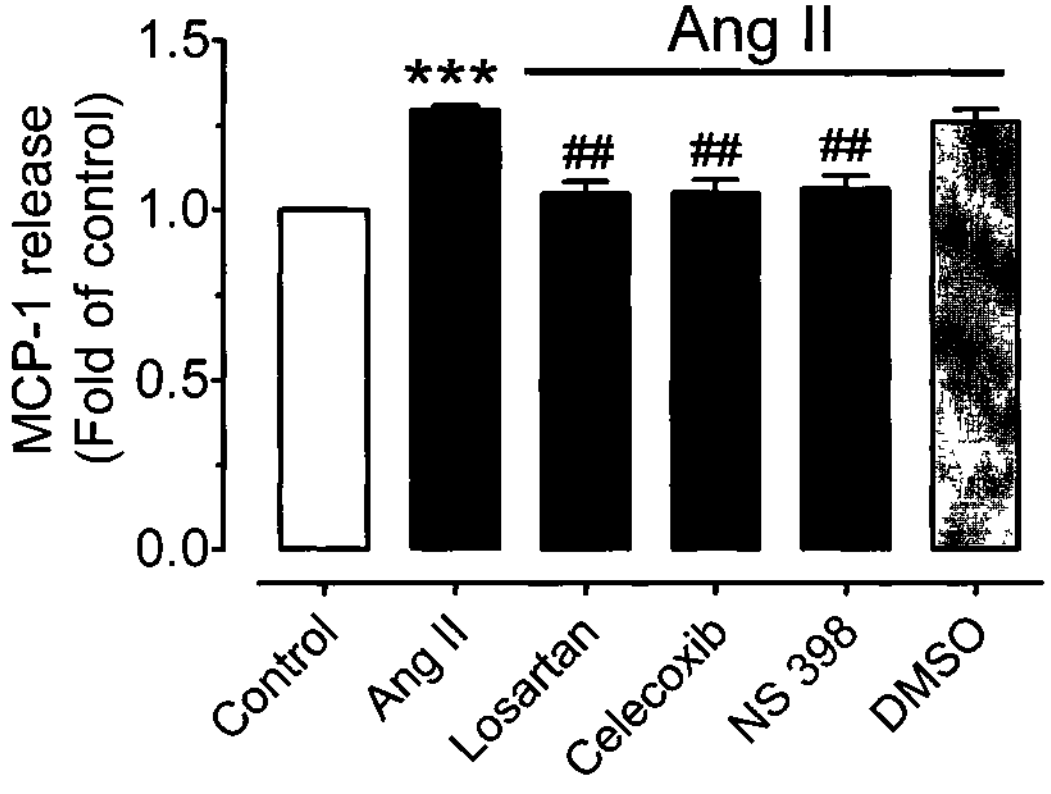

FIGURE 5.14.

MCP-1 release increased after 24 h-incubation with Ang II (100 nmol/L), which was prevented by losartan (3 µmol/L) and COX-2 inhibitors, celecoxib and NS 398 (each 3 µmol/L). Data are mean ± SEM of 4 experiments. ***$P<0.001$ versus control; ##$P<0.01$ versus Ang II-treated group.

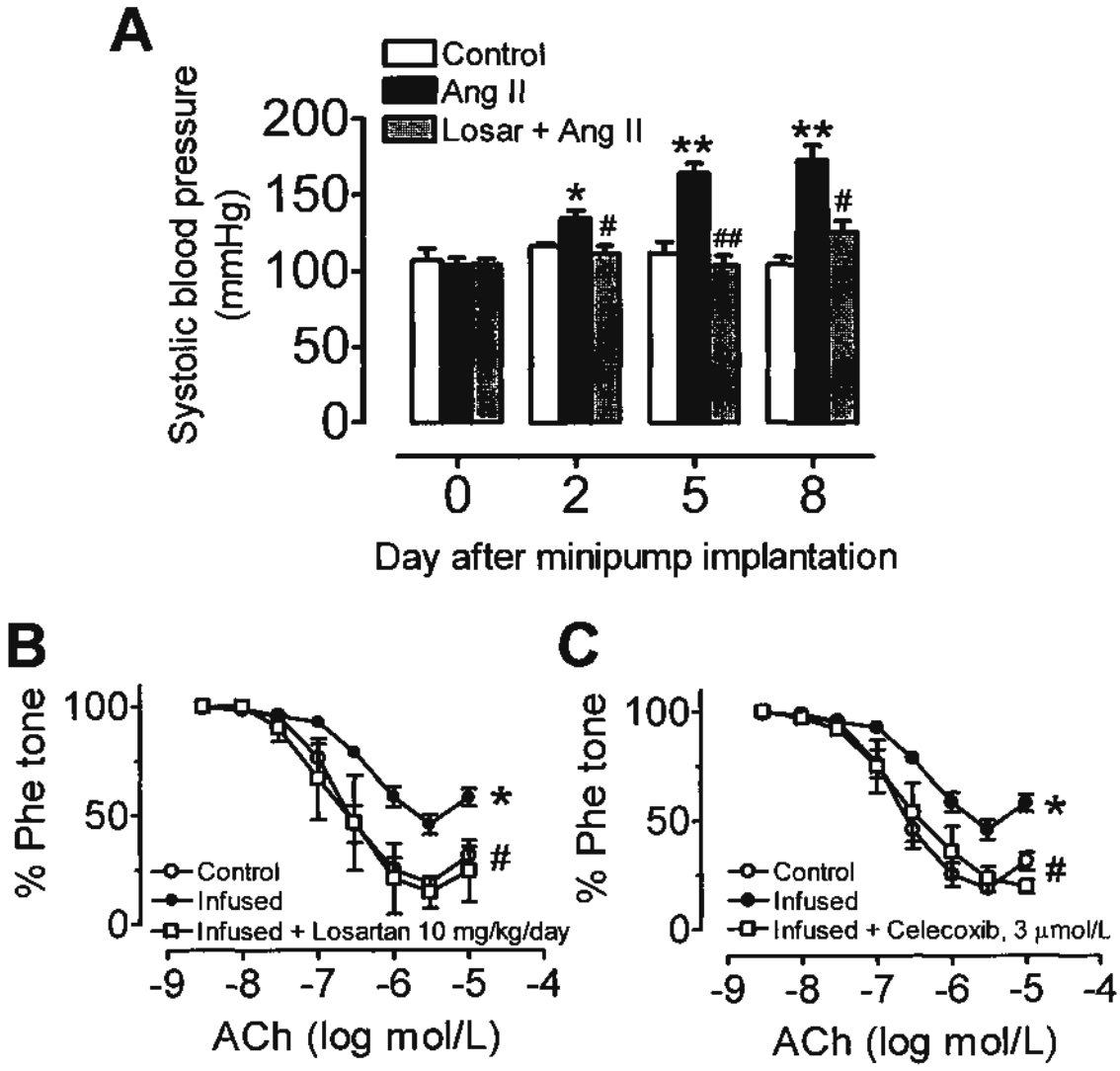

FIGURE 5.15.

(A) Systolic blood pressure increased markedly in Ang II (0.7 mg/kg/day)-infused rats, which was prevented by oral administration of losartan (10 mg/kg/day). (B) ACh-induced endothelium-dependent relaxations were attenuated in the interlobal renal arteries from Ang II-infused rats but not in those orally administered with losartan. (C) Celecoxib (3 µmol/L) acutely restored the impaired endothelium-dependent relaxations. Data are mean ± SEM of 4 experiments. **P*<0.05 and ***P*<0.01 versus control; #P<0.05 and ##P<0.01 versus Ang II-infused group.

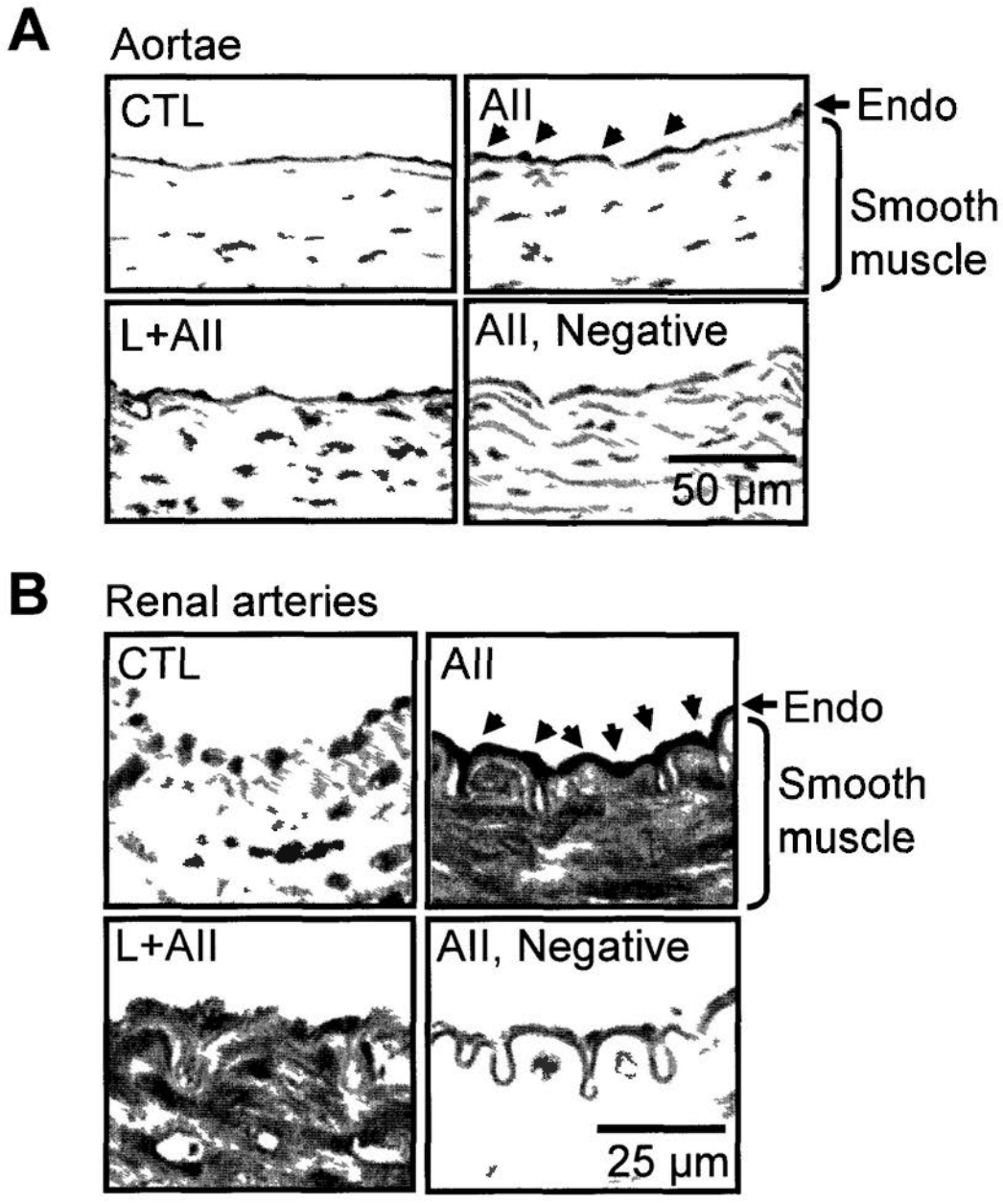

FIGURE 5.16.

Endothelial COX-2 expression (indicated by red arrows) was augmented in the (A) aortae and (B) renal arteries of Ang II-infused rats (AII). The expression was reduced in those from Ang II-infused rats orally-treated with losartan (L+AII). Similar observations for each group were made in arteries from 4 rats. CTL, control; Endo, endothelium.

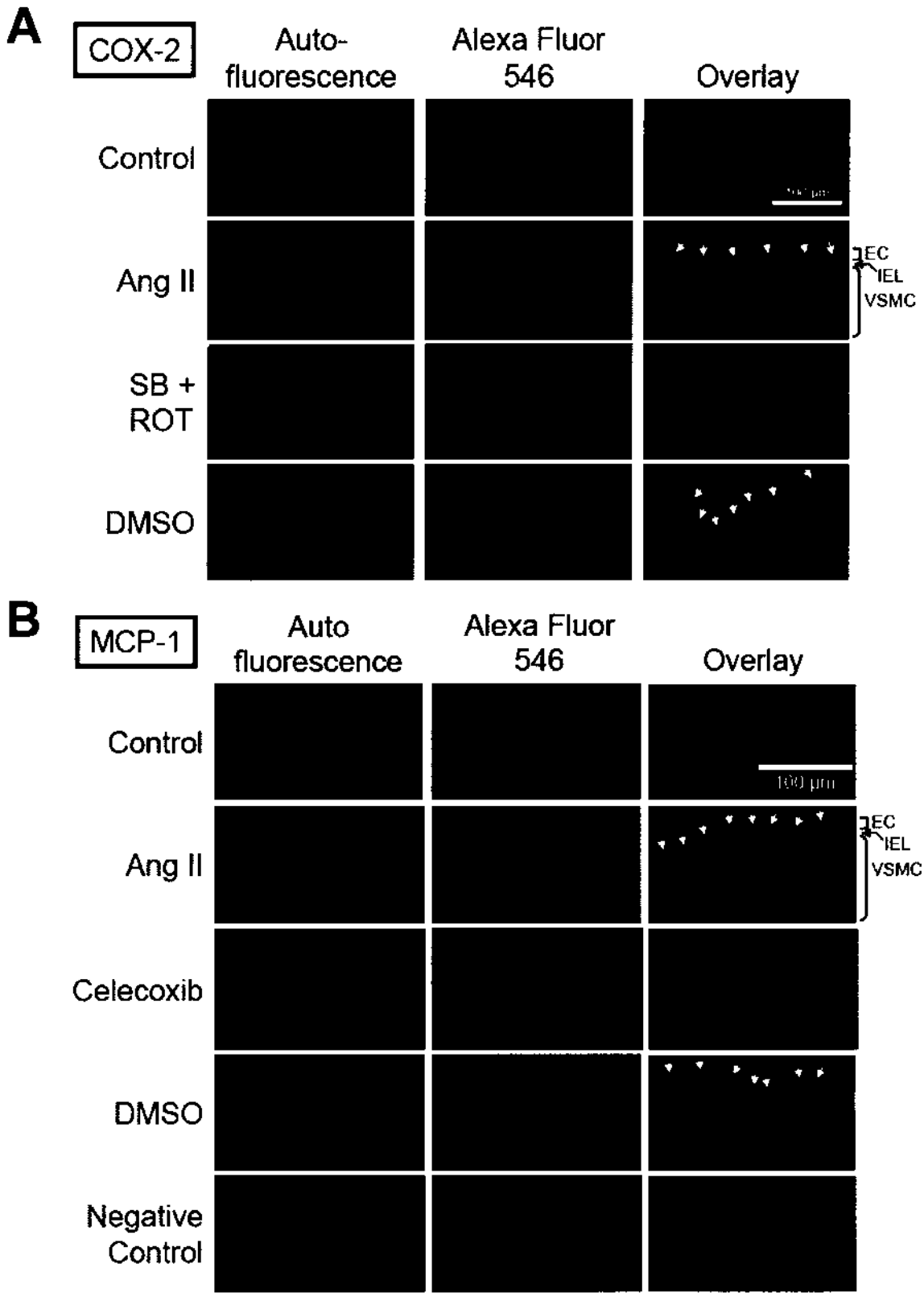
A
COX-2
Auto-fluorescence
Alexa Fluor 546
Overlay
Control
Ang II
SB + ROT
DMSO
EC
IEL
VSMC
B
MCP-1
Auto fluorescence
Alexa Fluor 546
Overlay
Control
100 µm
Ang II
Celecoxib
DMSO
Negative Control
EC
IEL
VSMC

FIGURE 5.17.

FIGURE 5.17.

Autofluorescence (first column in green) signified the elastin from internal elastic lamina (IEL) in the vessel wall. It delineated the layers of endothelial cells (EC) and vascular smooth muscle cells (VSMC). Detection for Alexa Fluor 546 (second column in reddish orange) gave the signals from both the elastin and antibody-fluorophore complex. Third column features the overlay images from the first and second column, with the elastin appearing yellow. The reddish orange color in the third column demonstrates the pure signal from antibodies. (A) Ang II (1 μmol/L) induced endothelial COX-2 expression (indicated by white arrows), which was sensitive to SB 202190 (SB, 10 μmol/L) and rottlerin (ROT, 10 μmol/L). (B) Ang II elevated endothelial MCP-1 expression, which was inhibited by celecoxib (3 μmol/L). DMSO did not affect the expressions of both Ang II-induced COX-2 and MCP-1. When primary antibody was neglected (i.e. negative control), the signals were purely from the elastin.

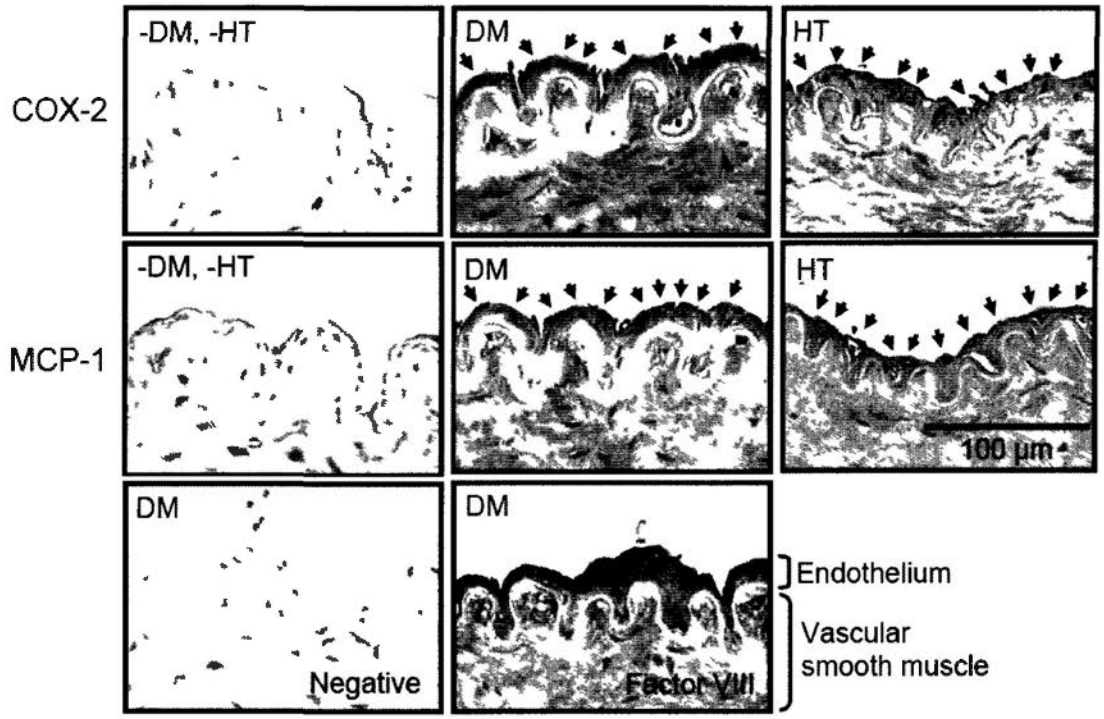

FIGURE 5.18.

COX-2 (stained in brown, indicated by red arrows) and MCP-1 (stained in brown, indicated by green arrows) were elevated in renal arteries from patients with diabetes (DM) or hypertension (HT), compared with subjects without diabetes and hypertension (-DM, -HT). Heavy brown color in the lowest right panel indicates factor VIII staining in the endothelium.

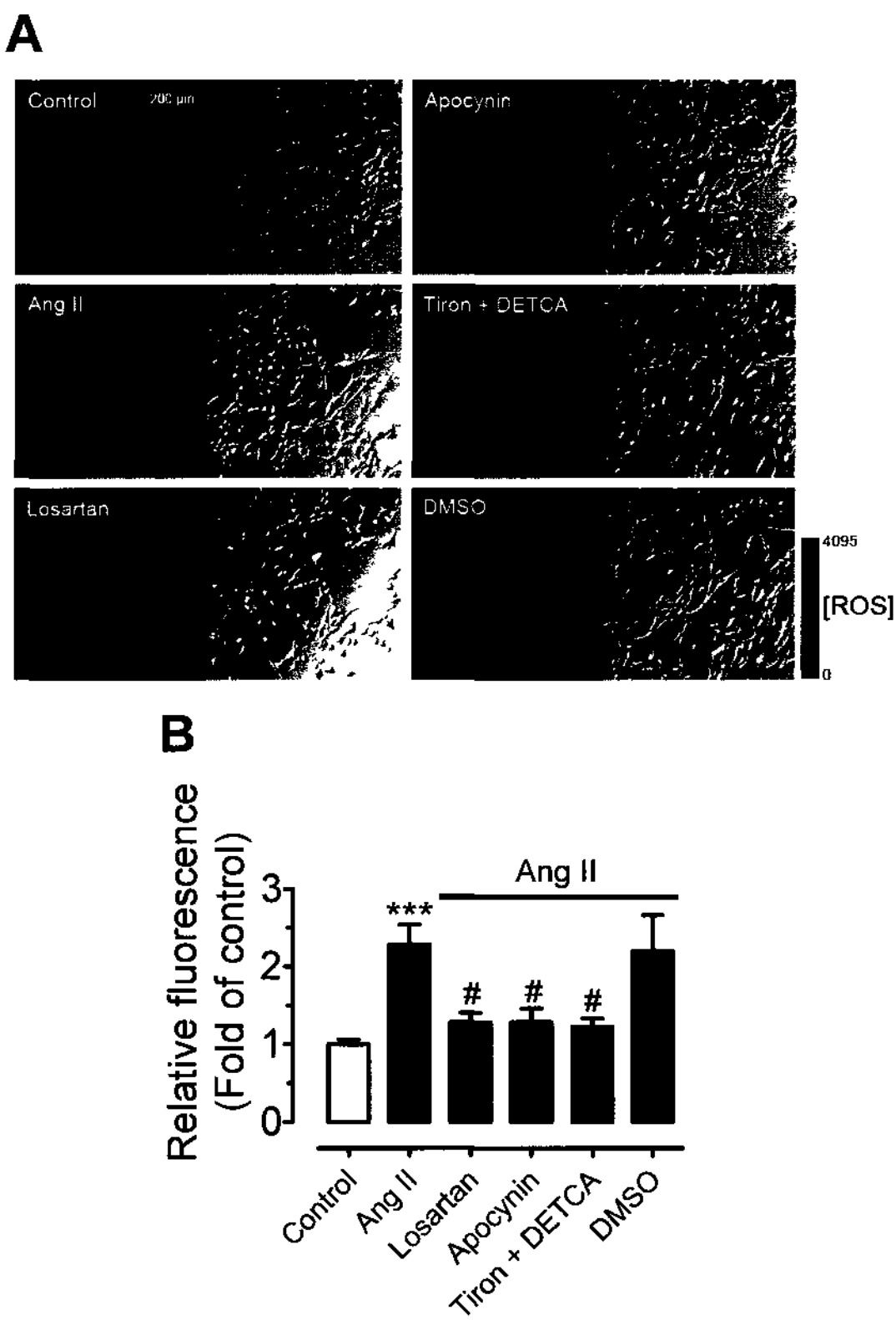
A
Control
Apocynin
Ang II
Tiron + DETCA
Losartan
DMSO
4095
[ROS]
0
B
Relative fluorescence
(Fold of control)
Ang II
***
#
#
#
3
2
1
0
Control
Ang II
Losartan
Apocynin
Tiron + DETCA
DMSO

FIGURE 5.19.

FIGURE 5.19.

(A) Representative images of DHE fluorescence. The left panel of each pair of images showed the DHE fluorescence signal (in red) on the production of reactive oxygen species like superoxide anion, while the right panel showed the brightfield images of the endothelial cells. (B) Summarized data showed that there was about 2-fold increase of oxidant production 30 s after Ang II (100 nmol/L) addition, which were inhibited by losartan (3 μmol/L), apocynin (100 μmol/L) and tiron (1 mmol/L) plus DETCA (100 μmol/L). Data are mean ± SEM of 4 experiments. ***$P<0.001$ versus control; $^{\#}P<0.05$ versus Ang II-treated group.

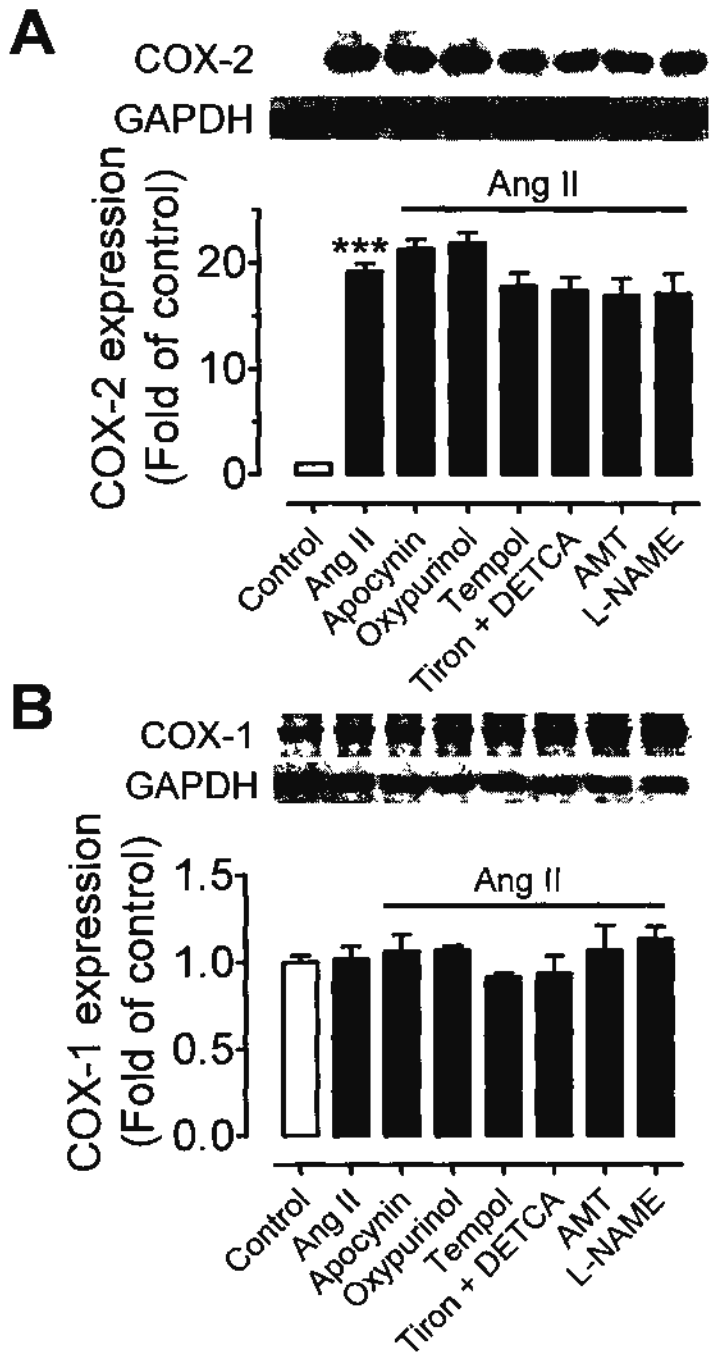

FIGURE 5.20.

Expression of neither Ang II-induced (A) COX-2 nor (B) constitutive COX-1 was affected in the presence of inhibitors against reactive oxygen species (apocynin, oxypurinol, tempol, DETCA and L-NAME, each at 100μmol/L; Tiron, 1 mmol/L; AMT, 30 μmol/L). Data are mean ± SEM of 3-4 experiments.

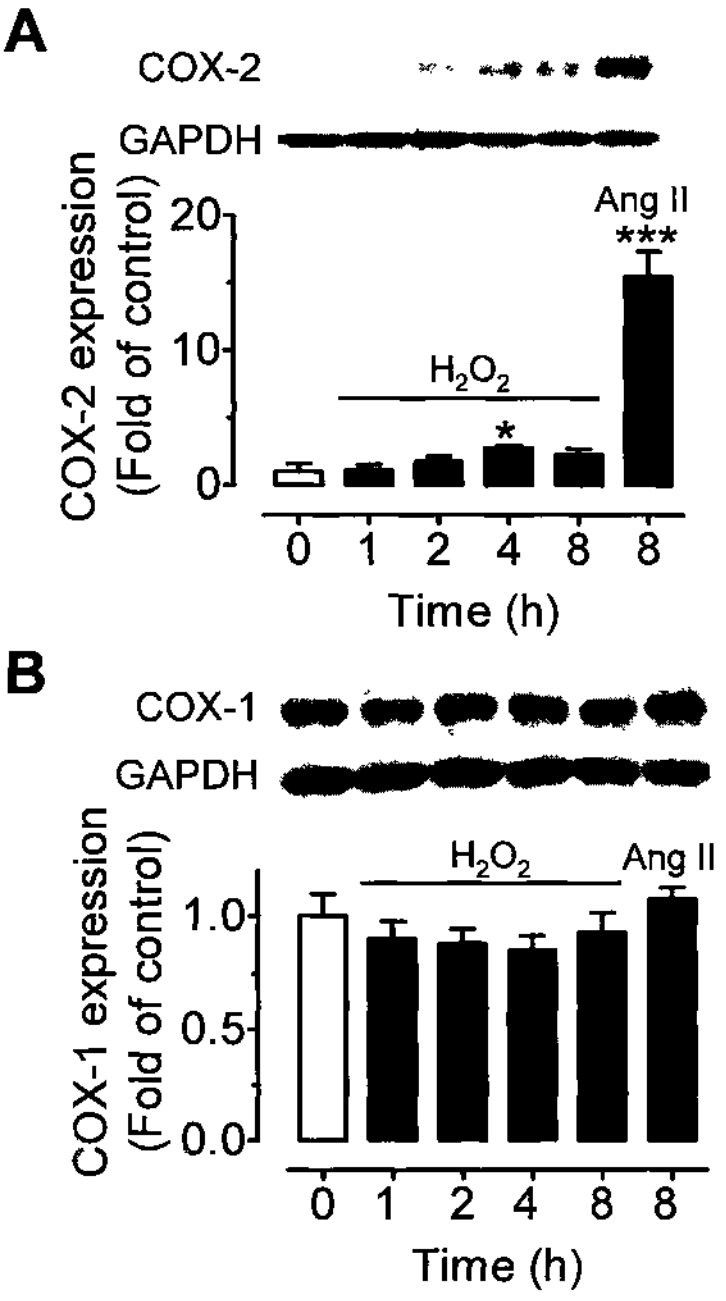

FIGURE 5.21.

(A) H_2O_2 (100 µmol/L) only induced a very low level of COX-2 expression compared to Ang II, (B) and COX-1 expression was not altered with either H_2O_2 or Ang II treatment. Data are mean ± SEM of 4 experiments. *P<0.05 and ***P<0.001 versus control.

5.4 Discussion

The present study shows for the first time, to my best knowledge, the up-regulation of endothelial COX-2 expression by Ang II and elucidates the underlying sequence of intracellular events pinpointing a novel role of PKC_{δ} activated at Tyr^{311} and joint contributions of ERK1/2 and p38 MAPK. I also defined the Ang II-induced release or expression of a COX-2 dependent pro-atherosclerotic cytokine MCP-1. The present results were obtained not only from primary culture of rat endothelial cells, but could also be confirmed in *in vivo* animal model of Ang II-infused hypertensive rats and extended to tissue culture of human arteries. Results from renal arteries of patients with hypertension or diabetes further point to the clinical relevance of the present experimental findings.

Ang II, a potent vasoconstrictive peptide plays a central role in vascular dysfunction associated with hypertension and diabetes, and AT_1R blockers represent a major class of anti-hypertensive drugs that interrupt Ang II-initiated intracellular signaling (Schmieder, 2005). While Ang II serves as a powerful ligand to trigger transcriptions of pro-atherosclerotic cytokines (Ando et al., 2004; Cheng et al., 2005; Costanzo et al., 2003; Pastore et al., 1999; Pueyo et al., 2000), the role of the highly inducible pro-inflammatory enzyme COX-2 in vascular diseases has recently gained attention as its over-expression is also detected in human atherosclerotic plaques (Gomez-Hernandez et al., 2006). However, the relationship between Ang II and COX-2 remains elusive as to whether COX-2 acts as a direct downstream effector of Ang II to mediate vascular inflammation, especially in endothelial cells which represent the first-line initiation point of atherosclerosis. The present study first demonstrated that Ang II could markedly up-regulate COX-2 expression in endothelial cells upon

activation of AT_1R. The COX-2 induction was jointly mediated by p38 MAPK- and ERK1/2-dependent signaling pathways, as the inhibitor of either kinases, SB 202190 or PD 98059, could only partially suppress the COX-2 expression while their co-treatment resulted in additive inhibition that almost abolished COX-2 expression. It appeared that JNK was unlikely to be involved as its inhibitor did not alter Ang II-induced COX-2 expression.

Since p38 MAPK and ERK1/2 are sensitive to redox triggers (Hsu et al., 2004; Jaulmes et al., 2009; Matesanz et al., 2007), I initially suspected that Ang II-induced oxidative stress might contribute to the COX-2 expression. This possibility, however, was excluded by two observations. First, Ang II-stimulated superoxide anion production was prevented by NADPH oxidase inhibitor, apocynin and ROS scavengers, tiron plus DETCA; however Ang II-induced COX-2 expression was unaltered by these inhibitors and by inhibitors against xanthine oxidase, inducible NOS and NOS uncoupling. Second, H_2O_2, which was reported to increase COX-2 expression in HUVEC (Eligini et al., 2009), induced a very small increase in COX-2 expression. The present results disagree with a previous report showing that in human saphenous vein endothelial cells, IL-1α-induced COX-2 expression involves both NADPH oxidase-derived superoxide anion and H_2O_2 (Massaro et al., 2006).

PKC appears to be another possible upstream target since PKC is associated with the G-protein-coupled AT_1R. Herein I defined a novel role of PKC_δ, activated at Tyr^{311}, to mediate COX-2 expression in Ang II-treated rat aortic endothelial cells. Pharmacologically, Ang II- and PMA (PKC activator)-induced COX-2 expression were both sensitive to inhibition by GF 109203X (a broad spectrum of PKC inhibitor) and rottlerin (PKC_δ inhibitor). Of note, GF 109203X and rottlerin also inhibited Ang II-induced ERK1/2 phosphorylation,

indicating that PKC$_\delta$ is likely the upstream activator of ERK1/2. Activation of p38 MAPK was likely independent of PKC regulation because its phosphorylation was unaffected by PKC inhibitors.

To address the concern over the specificity of pharmacological inhibitors used, I studied the treatment-induced translocation of PKC$_\delta$ from the cytoplasm to the plasma membrane and excluded the participation of other PKC isoforms. Translocation of soluble cytosolic PKC to the particulated membranous form is a key feature that signifies PKC activation (Salamanca and Khalil, 2005). Comparing the portion of membranous PKC after Ang II stimulation with that of control, only PKC$_\delta$ and PKC$_\varepsilon$ were found to be responsive to Ang II. While PKC$_\alpha$ was activated by PMA but not Ang II, PKC$_\theta$ and PKC$_\mu$ remained unresponsive to either treatments and PKC$_\zeta$ was not expressed in endothelial cells. Indeed, though PKC$_\alpha$ was activated by PMA, treatments with PKC$_{\alpha/\beta}$ inhibitor Go 6976 failed to suppress the PMA-induced COX-2 expression, thus contrasting a complete abrogation by rottlerin. Likewise, Ang II activated both PKC$_\delta$ and PKC$_\varepsilon$, yet the role of PKC$_\varepsilon$ in mediating COX-2 expression appears to be negligible as indicated by the following two observations. First, PKC$_\varepsilon$ inhibitor εV1-2 did not affect Ang II-induced COX-2 expression. Second, siPKC$_\delta$ markedly suppressed COX-2 expression without affecting PKC$_\varepsilon$ expression. If PKC$_\varepsilon$ were to be significantly involved in mediating Ang II action, its non-suppressed levels in siPKC$_\delta$-transfected cells with or without concomitant exposure to p38 MAPK inhibitor - SB2020190, would have maintained the inducible response of COX-2 expression as in the control or scramble siRNA-transfected cells. Taken together, these data indicate that even a number of PKC isoforms might be activated upon Ang II stimulation, PKC$_\delta$ is probably the main isoform that is essentially coupled with endothelial COX-2

expression. Noteworthy, inhibition of p38 MAPK by SB 202190 in combination with PKC_{δ} knockdown cells prevented the induction of COX-2 expression, thus proving a parallel involvement of the PKC_{δ}-ERK1/2 and p38 MAPK pathways. Since PKC_{δ} can posttranslationally modify the AU-rich element binding protein HuR (Doller et al., 2008), it is not impossible that PKC_{δ} also serve to stabilize COX-2 mRNA and thereby increasing the COX-2 expression level. Such findings contrast to Ang II-induced COX-2 up-regulation in vascular smooth muscle cells which mainly depends on the ERK1/2 activity without the participation of PKC or p38 MAPK (Hu et al., 2002). Pre-treatment by inhibitor of either ERK1/2 or p38 MAPK did not affect Ang II-induced PKC_{δ} translocation, again confirming that PKC_{δ} is upstream of ERK1/2 activation and independent of p38 MAPK regulation.

Up-regulation of endothelial COX-2 by Ang II could be demonstrated not only in cultured cells. Indeed, the present study clearly showed an augmentation of endothelial COX-2 protein expression in the aorta and renal arteries of Ang II-infused rats, which was inhibited by concomitant oral treatment of losartan. Impairment of endothelium-dependent relaxations was acutely restored by the COX-2 inhibitor celecoxib, indicating COX-2-derived vasoconstrictive prostanoids may contribute to vascular dysfunctions, as documented in diabetic, hypertensive and aging animal models (Adeagbo et al., 2005; Akamine et al., 2006; Wong et al., 2009).

Tissue culture experiments on human mesenteric arteries substantiate the findings in cultured rat endothelial cells. Ang II-induced endothelial COX-2 expression was prevented by co-treatment of SB 202190 and rottlerin. More importantly, increased COX-2 expression was accompanied by a concomitant rise in MCP-1 expression with both proteins being immunolocalized to the

endothelium. This MCP-1 increase was sensitive to COX-2 inhibition by celecoxib, clearly suggesting a causal relationship between these two pro-inflammatory and pro-atherosclerotic cytokines. Indeed, Ang II-induced MCP-1 release into the conditioned medium of cultured rat endothelial cells was also prevented by the two specific COX-2 inhibitors, celecoxib and NS 398. Taken together, endothelial COX-2-dependent release of MCP-1 may represent a new concept in our understanding of the Ang II-induced vascular inflammation and atherosclerotic plaque formation involving macrophages.

Co-localization of COX-2 and MCP-1 does not solely appear in current experimental settings. In renal arteries from patients with hypertension or diabetes, expressions of both COX-2 and MCP-1 were clearly detected in the endothelium, which was in sharp contrast to the non-existence of both proteins in arteries from non-diabetic and non-hypertensive subjects.

Assembling the time course of maximal activation of signaling proteins, and studies using pharmacological inhibitors and siRNA, I propose the following cellular pathways of Ang II-induced endothelial COX-2 expression (Figure 8). Ang II stimulates AT_1R to activate PKC_δ in <1 min and subsequently ERK1/2 in ~5 min, along with maximal PKC-independent p38 MAPK activation in ~5 min. COX-2 expression was detected at 1 h and reaches the maximum at 8 h. MCP-1 release detected at 24 h is COX-2 dependent.

Significance of the findings

Clinical use of COX-2 inhibitors in suppressing inflammatory responses remains a hot topic in recent years due to their reported adverse cardiovascular effects. While COX-2 inhibition was shown to increase blood pressure and thrombotic events (Bresalier et al., 2005; Capone et al., 2008), it also produces benefits in

patients with hypertension and coronary artery disease (Chenevard et al., 2003; Widlansky et al., 2003). These seemingly contradictory findings between animal and human studies may suggest that COX-2 may take up a janus role in producing different types of metabolites probably depending on the nature of triggers under physiological and pathological states. COX-2 can release either vasoprotective prostacyclin or deleterious vasoconstrictors. While the exact role of COX-2 remains unresolved and preferentially targeting COX-2 in particular systems is a challenging task, the present study provides a novel insight into how COX-2 action can be modulated by regulating its expression. In this study, I demonstrate an important role of PKC_{δ} in mediating Ang II-induced COX-2 expression. Intriguingly, a recent study has shown that adiponectin, a cardioprotective adipokine, up-regulates endothelial COX-2 expression by activating calreticulin/CD91-PI3K/Akt and promotes revascularization after ischemia (Ohashi et al., 2009). It is noteworthy that Ang II-induced COX-2 expression does not involve PI3K pathways (data not shown), implying that COX-2 expression can be mediated by different agonist-dependent pathways. If we selectively target PKC_{δ} isoform in a strategic manner to suppress the up-regulation of pro-inflammatory COX-2, the expression of protective COX-2 can remain undisturbed and thus preventing the occurrence of the scenario in which non-selective inhibition towards these functionally different COX-2 abrogates the beneficial effects from the protective ones. Indeed, PKC_{β} and PKC_{δ} are preferentially activated in response to hyperglycemia and diabetes (Meier and King, 2000) and PKC inhibitors such as ruboxistaurin targeting the β isoform has been introduced in clinical trials (Geraldes and King, 2010). While there is no available antagonist or neutralizer for MCP-1 over-production, inhibiting COX-2 and subsequent down-regulation of MCP-1 release may offer an

alternative to retard the initiation of atherosclerosis. With the advancement in the development of phosphopeptide mimetics, specific peptide inhibitors of PKC_{δ} may uncover novel therapeutic potential to combat vascular inflammatory disease.

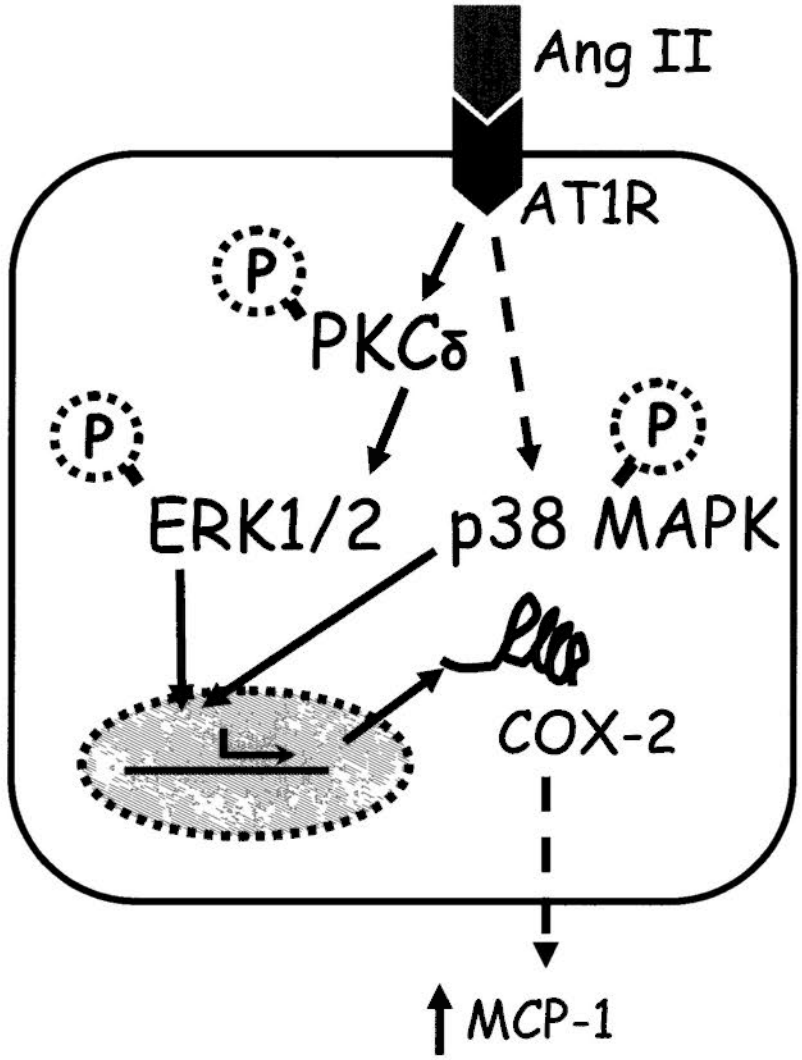

FIGURE 5.22.

Postulated cellular mechanisms on Ang II-induced COX-2 expression in endothelial cells and time course of events. Ang II acts on angiotensin II type 1 receptor (AT_1R), which then activates PKC_δ in < 1 min. Activations of PKC_δ-dependent ERK1/2 and -independent p38 MAPK occur at ~ 5 min. COX-2 begins to be expressed after ~1 h and reaches a maximal expression at ~ 8 h. COX-2-dependent MCP-1 release was detected in 24 h.

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