Cyclooxygenase-2–Derived Prostaglandin F$_{2\alpha}$ Mediates Endothelium-Dependent Contractions in the Aortae of Hamsters With Increased Impact During Aging

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Abstract—Hypertension and vascular dysfunction result in the increased release of endothelium-derived contracting factors (EDCFs), whose identity is poorly defined. We tested the hypothesis that endothelial cyclooxygenase (COX)-2 can generate EDCFs and identified the possible EDCF candidate. Changes in isometric tension of aortae from young and aged hamsters were recorded on myograph. Real-time changes in intracellular calcium concentrations ([Ca$^{2+}$]) in native aortic endothelial cells were measured by imaging. Endothelium-dependent contractions were triggered by acetylcholine (ACh) after inhibition of nitric oxide production and they were abolished by COX-2 inhibitors or by thromboxane–prostanoid receptor antagonists. 2-Aminoethoxydiphenyl borate (cation channel blocker) eliminated endothelium-dependent contractions and ACh-stimulated rises in endothelial cell [Ca$^{2+}$], RT-PCR and Western blotting showed COX-2 expression mainly in the endothelium. Enzyme immunoassay and high-performance liquid chromatography-coupled mass spectrometry showed release of prostaglandin (PG)F$_{2\alpha}$ and prostacyclin (PGI$_2$) increased by ACh; only PGF$_{2\alpha}$ caused contraction at relevant concentrations. COX-2 expression, ACh-stimulated contractions, and vascular sensitivity to PGF$_{2\alpha}$ were augmented in aortae from aged hamsters. Human renal arteries also showed thromboxane–prostanoid receptor–mediated ACh- or PGF$_{2\alpha}$-induced contractions and COX-2–dependent release of PGF$_{2\alpha}$. The present study demonstrates that PGF$_{2\alpha}$ derived from COX-2, which is localized primarily in the endothelium, is the most likely EDCF underlying endothelium-dependent, thromboxane–prostanoid receptor–mediated contractions to ACh in hamster aortae. These contractions involved increases in endothelial cell [Ca$^{2+}$]. The results support a critical role of COX-2 in endothelium-dependent contractions in this species with an increased importance during aging and, possibly, a similar relevance in humans. (Circ Res. 2009;104:228-235.)

Key Words: endothelium-derived contracting factors ■ cyclooxygenase-2 ■ thromboxane–prostanoid receptor ■ aging ■ aorta

Besides neuronal and hormonal regulation, vascular tone is modulated locally by a delicate balance between endothelium-derived relaxing (EDRFS) and contracting (EDCFs) factors, with the latter being less well-defined but emerging in hypertension, obesity, hyperlipidemia, diabetes, and aging. A number of molecules have been proposed as possible EDCF candidates under pathophysiological conditions. These include prostaglandin (PG)H$_2$, thromboxane (TX)A$_2$, leukotrienes, endothelin 1, and superoxide anions. The release of these tentative EDCFs can be triggered by acetylcholine (ACh), angiotensins II/III, ADP, and ATP. The contribution of additional cyclooxygenase (COX)-derived metabolites, ie, PGF$_{2\alpha}$, PGD$_2$, and PGF$_{2\alpha}$, has been postulated. The precise nature of these EDCFs varies among species and vascular beds.

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. 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 thromboxane–prostanoid (TP) receptors. In arteries of spontaneously hypertensive or diabetic rats, the expression of COX-1 is upregulated, and the augmented endothelium-dependent contractions are inhibited by COX-1 inhibitors. COX-1–derived prostacyclin, TXA$_2$, or endoperoxides all contribute to endothelium-dependent contractions. However, this generally accepted distinction between “constitutive” and “inducible” isof orm of COX appears to be
an overgeneralization. Indeed, COX-2 can be expressed constitutively in the endothelium of the rat pulmonary and human renal blood vessels and in cultured endothelial cells.\textsuperscript{10,11} A COX-2–specific inhibitor attenuates arachidonic acid–induced vasodilatation of canine coronary arteries,\textsuperscript{12} supporting a physiological role for COX-2 in vascular function. COX-2 is upregulated under pathological conditions including renovascular hypertension,\textsuperscript{13} reflux nephropathy,\textsuperscript{14} and diabetes.\textsuperscript{15} 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,\textsuperscript{16} 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 ACh, probably because of the exaggerated oxidative stress in the vascular wall.\textsuperscript{17}

COX-2 can be upregulated by physiological shear stress from pulsatile flow.\textsuperscript{18,19} 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. Because the lipid profile and arachidonic acid metabolism of hamsters resemble that of humans\textsuperscript{20–22} and COX-2 may be important for both the physiological and pathological regulation of vascular reactivity,\textsuperscript{5,18,19} we hypothesized that COX-2 rather than COX-1 is mediating the generation of EDCFs in the aorta of young and healthy hamsters and that COX-2 expression and COX-2–mediated vascular responses increase with aging. We also studied whether this pathway is relevant to humans by studying human renal arteries. The present findings revealed PGF\textsubscript{2\alpha} as a physiological EDCF, which can be generated by COX-2 in the endothelium and is of increasing importance during aging.

Materials and Methods
Most experiments were performed on aortae from young and aged (\textsim{3- or >18-month-old) hamsters. This part of the study was approved by the Animal Ethics Committee, Chinese University of Hong Kong. Human renal arteries were obtained during surgery after informed consent from 4 patients aged 59 to 75 years. An expanded Materials and Methods section can be found in the online data supplement at http://circres.ahajournals.org.

Isometric Force Measurement
Blood vessels were prepared as described in the online data supplement. Briefly, to visualize the endothelium-dependent contractions, aortic rings with endothelium were exposed for 30 minutes to 100 \mu M/L N\textsuperscript{4}-nitro-l-arginine methyl ester (L-NNAME), ACh elicited pronounced contraction of aortic rings with endothelium, with a maximal response of 3.6 ± 0.16 mN/mm (Figure 1A and 1B), corresponding to \approx 70\% of the contractile response (8.55 ± 0.32 mN/mm) induced by 60 nM/L KCl. Removal of the endothelium abolished the contractions to ACh (Figure 1C). The endothelium-dependent contractions were attenuated or eliminated by the nonselective COX inhibitor indomethacin (Figure 2A). Likewise, 3 structurally different selective COX-2 inhibitors (NS-398, DuP-697, and celecoxib) reduced or abolished the endothelium-dependent contractions (Figure 2B through 2D). The specificity of COX-2 inhibition was confirmed by the lack of inhibitory effects of the 3 inhibitors on contractions induced by 60 nM/L KCl and U46619 (supplemental Figure II, A and B). By contrast, neither the COX-1 selective inhibitors (valeryl salicylate [VAS] and sc-560) nor the inhibitor of 5- and 12-lipoxygenase (baicalein) inhibited the response (supplemental Figure I, A through C). The endothelium-dependent contractions were unaffected by treat-

Reverse Transcription–Polymerase Chain Reaction
Expression levels of COX-2 mRNA in rings with and without endothelium were detected by RT-PCR (see the online data supplement).

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') and 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 80°C and 57°C, respectively. PCR products were run on 1.5\% agarose gel in 1\% Tris-acetate-EDTA buffer at 80 V. Ethidium bromide–stained bands were visualized under UV illumination using FluorChem (version 2.00, Alpha Innotech Corp, San Leandro, Calif).

Western Blotting
Expression of COX-1, COX-2, platelet endothelial cell adhesion molecule (PECAM)-1, F-series–prostanoid (FP) receptor, and TP receptor protein in aortic rings was determined by Western blot analysis (see the online data supplement).

Enzyme Immunoassay and High-Performance Liquid Chromatography–Coupled Mass Spectrometry Measurement of Prostaglandins
PGF\textsubscript{2\alpha}, PGE\textsubscript{2}, PGD\textsubscript{2}, 6-keto PGF\textsubscript{1\alpha} (for PG\textsubscript{1\alpha}), TXB\textsubscript{2} (for TXA\textsubscript{2}), and 8-isoprostanes were assayed. Details can be found in the online data supplement.

Detection of Reactive Oxygen Species Formation
Reactive oxygen species (ROS) were detected by electron paramagnetic resonance (EPR). The detailed method can be found in the online data supplement.

Drugs
Chemicals and drugs can be found in the online data supplement.

Data Analysis
Endothelium-dependent contractions were expressed as active tension [force recorded/(2 × ring length)]. Results are means ± 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 2 treatments were compared (GraphPad Software, San Diego, Calif). P < 0.05 was considered significantly different.

Results

Essential Role of COX-2 in Endothelium-Dependent Contractions in Aortae From Young Hamsters
In the presence, but not in the absence of N\textsuperscript{4}-nitro-l-arginine methyl ester (L-NNAME), ACh elicited pronounced contraction of aortic rings with endothelium, with a maximal response of 5.93 ± 0.16 mN/mm (Figure 1A and 1B), corresponding to \approx 70\% of the contractile response (8.55 ± 0.32 mN/mm) induced by 60 nM/L KCl. Removal of the endothelium abolished the contractions to ACh (Figure 1C). The endothelium-dependent contractions were attenuated or eliminated by the nonselective COX inhibitor indomethacin (Figure 2A). Likewise, 3 structurally different selective COX-2 inhibitors (NS-398, DuP-697, and celecoxib) reduced or abolished the endothelium-dependent contractions (Figure 2B through 2D). The specificity of COX-2 inhibition was confirmed by the lack of inhibitory effects of the 3 inhibitors on contractions induced by 60 nM/L KCl and U46619 (supplemental Figure II, A and B). By contrast, neither the COX-1 selective inhibitors (valeryl salicylate [VAS] and sc-560) nor the inhibitor of 5- and 12-lipoxygenase (baicalein) inhibited the response (supplemental Figure I, A through C). The endothelium-dependent contractions were unaffected by treat-

In Situ Endothelial Cell [Ca\textsuperscript{2+}]\textsubscript{i} Imaging
A calcium imaging technique was used to visualize real-time changes in intracellular calcium levels ([Ca\textsuperscript{2+}]\textsubscript{i}) in native endothelial cells of the intact hamster aorta.\textsuperscript{24}
ment with actinomycin-D (10 μmol/L, RNA synthesis inhibitor) or cycloheximide (10 μmol/L, protein synthesis inhibitor) (supplemental Figure I, D).

Endothelium-Dependent Contractions Mediated Through TP Receptors

The endothelium-dependent contractions were attenuated or abolished in aortic rings treated with 3 structurally distinct selective TP receptor antagonists, terutroban (S18886, 3 to 100 nmol/L), L-655,240 (0.1 to 1 μmol/L), or GR 32191 (100 nmol/L) (Figure 3A through 3C). On the contrary, the thromboxane synthase inhibitor ozagrel hydrochloride (10 μmol/L) did not affect the contraction (Figure 3D). 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 (supplemental Figure II, C and D).

Dependency on Extracellular Ca2+

Endothelium-dependent contractions were absent following the removal of extracellular calcium ions. Reintroduction of 2.5 mmol/L CaCl2 to the bathing solution restored contraction to 10 μmol/L ACh (Figure 4A). Exposure of rings to 2-aminoethoxydiphenyl borate (2-APB) (3 to 50 μmol/L, a nonselective cation channel blocker) diminished or abolished the endothelium-dependent contractions (Figure 4B) without affecting the response to 60 mmol/L KCl or U46619 (n=4).

En face fluorescence images from viable individual native endothelial cells of cut-open aortic segments were examined (Figure 4C, a). The fluorescence signal indicative of the [Ca2+]i was absent after mechanical removal of the endothelium. It increased following the addition of ACh in the presence of L-NAME only in arterial tissues with endothelium (Figure 4C, a*). Treatment with 2-APB (50 μmol/L, the concentration that abolished endothelium-dependent contractions), prevented the increases in [Ca2+]i (Figure 4C, b*). By contrast, S18886 (0.1 μmol/L) had no effect on the Ca2+ fluorescence signal (Figure 4C, c*). The ACh-stimulated real-time increase in endothelial cell [Ca2+]i was eliminated by 2-APB but not by S18886 (Figure 4D).

Localization of COX-2

The expression of COX-2 mRNA was significantly higher in aortae with endothelium than those without (Figure 5A). The COX-2 protein expression was reduced following the mechanical removal of the endothelium (Figure 5B), which we had confirmed by the reduced protein levels of the endothelium-specific marker PECAM-1 (Figure 5C). The protein expression of COX-1 was slightly but insignificantly greater in the aortae with endothelium than those without (supplemental Figure I, E).
Role of ROS

Endothelium-dependent contractions to ACh were unaffected by tiron (1 mmol/L) plus diethylthiocarbamate acid (100 μmol/L) (membrane-permeable free radical scavengers), tempol (100 μmol/L, superoxide dismutase mimetic), or apocynin (100 μmol/L, NADPH oxidase inhibitor) (supplemental Figure III, A). L-NAME–treated aortic rings showed no EPR signal for superoxide anions or peroxynitrite in response to ACh (supplemental Figure III, B and C), whereas the addition of hypoxanthine plus xanthine oxidase (HXXO, a mixture to release superoxide anions) gave rise to 3 distinct EPR signals (supplemental Figure III, D).

PGF<sub>2α</sub> As the EDCF

Six possible EDCF candidates, ie, PGF<sub>2α</sub>, PGE<sub>2</sub>, TXA<sub>2</sub>, PGD<sub>2</sub>, PGI<sub>2</sub>, and 8-isoprostanes were assayed chemically. ACh at 3 μmol/L stimulated a significant rise in the release of PGF<sub>2α</sub> and PGL<sub>2</sub> (detected as 6-keto PGF<sub>1α</sub>) but not PGE<sub>2</sub>, TXA<sub>2</sub> (detected as TXB<sub>2</sub>), and PGD<sub>2</sub> from aortic rings with endothelium (Figure 6A and 6B). The release of both PGF<sub>2α</sub> and 6-keto PGF<sub>1α</sub> was largely inhibited by removal of the endothelium (Figure 6C and 6D). The level of 8-isoprostanes was very low (supplemental Figure III, E). Among the 5 assayed prostanooids, only the release of PGF<sub>2α</sub> (~0.8 ng/mL) and 6-keto PGF<sub>1α</sub> (~7 ng/mL) evoked by 3 μmol/L ACh was inhibited or abolished by treatment with celecoxib or 2-APB but not by VAS (Figure 6C and 6D). These results are consistent with those obtained by high-performance liquid chromatography-coupled mass spectrometry (HPLC-MS), which showed that the amount of PGF<sub>2α</sub> (~1.0 ng/mL) was comparable to that (~0.8 ng/mL) assayed by enzyme immunoassay (EIA) (supplemental Figure IV).

To further investigate the role of PGF<sub>2α</sub> and PGI<sub>2</sub> in endothelium-dependent contractions, the effects of PGF<sub>2α</sub> (1 to 30 ng/mL), PGI<sub>2</sub> (3.7 to 370 ng/mL), and its stable analog cicaprost (10 to 100 ng/mL) were tested in the presence of 100 μmol/L L-NAME. PGF<sub>2α</sub> induced contraction of the aortic rings at the relatively low concentration of 1 ng/mL (Figure 6E), comparable with the level of ACh-induced release measured by EIA (~0.8 ng/mL). The contraction was reduced by S18886 (0.1 μmol/L). By contrast, neither PGI<sub>2</sub> nor cicaprost produced a contraction (Figure 6F and 6G), even at a concentration 50-fold higher than that detected in the solution bathing aortae exposed to ACh (~7 ng/mL for 6-keto PGF<sub>1α</sub>). Exogenous PGI<sub>2</sub> did not relax phenylephrine-contracted aortae (supplemental Figure V).

PGF<sub>2α</sub>, whose release was independent of ACh stimulation (Figure 6A), produced very small contractions at the assayed concentration (~0.8 ng/mL), and such contractions were insensitive to S18886 (supplemental Figure V).

Augmented Endothelium-Dependent Contractions in Aortae From Aged Hamsters

ACh-induced endothelium-dependent contractions were significantly higher in aortae from aged (>18-month-old) than young (~3-month-old) hamsters (Figure 7A), and ACh was able to trigger contractions even in the absence of L-NAME in aortae from the aged animals (supplemental Figure VI, H), which were inhibited or abolished by celecoxib, DuP-697, and S18886 (Figure 7B and 7C) and by 2-APB but not by VAS or sc-560 (supplemental Figure VI). Aged aortae ex-
pressed a significantly higher level of COX-2, which was again mainly localized to the endothelium (Figure 7D), whereas the expression of COX-1 was comparable in the 2 age groups (supplemental Figure VI, J). Finally, ACh tended to stimulate more release of PGF2α and 6-keto PGF1α in aortae from aged than young hamsters, and the release of PGF2α was inhibited significantly by celecoxib (Figure 7E and supplemental Figure VI, K).

PGF2α produced larger contractions in L-NAME–treated aortae from aged than young hamsters (Figure 7F), whereas contractions to KCl (30 to 50 mmol/L) or phenylephrine (0.1 to 1 μmol/L) were comparable (data not shown). The protein expression of TP receptors was similar in aortae from both age groups (supplemental Figure VI, L). By contrast, in the aged hamster aortae, PG12 produced neither a relaxation nor a contraction, and PGE2-induced contractions were again insensitive to S18886 (supplemental Figure V).

**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 (supplemental Figure VII, A). PGF2α-induced contractions were antagonized by S18886 but not by AL-8810, the FP receptor antagonist (supplemental Figure VII, B and C). Pretreatment with S18886, but not AL-8810, prevented exogenous PGF2α-induced contractions (supplemental Figure VII, D). Western blot analysis revealed little or no expression of FP receptor in human arteries in contrast to well-expressed TP receptor (supplemental Figure VII, E). HPLC-MS measurement showed that those arteries released both PGF2α and 6-keto PGF1α in response to 100 μmol/L ACh, but only the release of PGF2α was inhibited by celecoxib (10 μmol/L), whereas VAS (30 μmol/L) was without effect (supplemental Figure VII, F).

**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, 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 PGF2α, 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.

Similar to previous observations in other blood vessels, 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. Because the contraction is not observed in the absence of the inhibitor of NO synthase, endothelium-dependent relaxations would have predominated over contractions in the aorta of young healthy hamsters.

Arachidonic acid, released from cell membranes by phospholipases, can be metabolized via different pathways to generate vasoactive substances. Lipooxygenases convert arachidonic acid to HETEs (hydroperoxyeicosatetraenoic acids) and then to either HETEs (hydroxyeicosatetraenoic acids) or leukotrienes. Cyclooxygenases oxygenate arachidonic acid to form PGG2 and PGH2, which are further converted to various prostanoids including PGD 2, PGE2, TXA2, and PGI2 via their respective synthases. In the present study, baicalein was used to inhibit the lipooxygenase pathway, yet this caused no suppression of the ACh-induced endothelium-dependent contractions. By contrast, incubation with a relatively low concentration of indo- mecain 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, whereas COX-2 is highly inducible by proinflammatory cytokines, tumor promoters, and mitogens. Recent studies suggest that COX-2 is also constitutively expressed in...
the kidney, brain, and arteries. In the cardiovascular system, endothelial cells express COX-2 in response to shear stress under normal physiological condition. When COX-2 is present, it contributes to PGI₂ synthesis and can activate silent reservoirs of PGI₂ synthase in most tissues. In the present study, the endothelium-dependent contractions of the aorta from healthy hamsters were mediated by COX-2, whereas the constitutively expressed COX-1 did not play a major role, as evidenced by the pronounced attenuation of the response by NS-398, DuP-697, and celecoxib but not by sc-560 and VAS. 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 ACh. The molecular biological comparison of aortae with and without endothelium permitted the conclusion that COX-2 mRNA and protein expressions, demonstrated by 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. After 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 receptor 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 ACh. In contrast to the well-expressed TP receptor, the FP receptor is minimally expressed in hamster aortae. Besides, ACh- or PGF₂α-induced contractions were not reduced by the FP receptor antagonist AL-8810 (supplemental Figure VIII), thus discounting a significant role of the FP receptor in the endothelium-dependent contractions, although its natural agonist PGF₂α is proposed to be the EDCF in hamster aortae.

The present data show that Ca²⁺ 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 Ca²⁺-free solution showed no contraction until Ca²⁺ was reintroduced into the bathing solution. Ca²⁺ ions possibly enter endothelial cells via nonspecific cation channels, as evidenced by the effect of 2-APB in attenuating endothelium-dependent contractions and abolishing the ACh-stimulated elevation of [Ca²⁺]i in situ imaging of endothelial cells. In addition, 2-APB inhibited the ACh-induced release of COX-2-derived prostanoids in the aorta with endothelium, whereas 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, PGF₂α appears to be the most likely EDCF candidate. Indeed, PGF₂α was released...
endogenously from the aortic endothelium in physiological amounts that correspond to its potent effect in eliciting contraction of the smooth muscle. Although PGi2 was also released in considerable amounts, it failed to evoke any contraction or relaxation per se, even at a concentration 50 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. The present study can discount the possible involvement of PGi2 because its release was not stimulated by ACh and the small contractions induced by exogenous PGE2 were insensitive to the TP receptor antagonism.

Because TP receptors are involved in the response, it is logical to speculate that TXA2 may contribute to endothelium-dependent contractions in the hamster aorta. However, this possibility is made unlikely by 2 observations. First, in the presence of ozagrel, a thromboxane synthase inhibitor, endothelium-dependent contractions to ACh remained unaltered. Second, ACh did not increase the release of TXA2. Thus TXA2 is not a major EDCF candidate in the hamster aorta. By contrast, TXA2 contributes to the endothelium-dependent contractions of the canine basilar artery and the SHR aorta.7,33

COX is involved in the generation of ROS in vascular tissues, which are normally neutralized by NO. It appears necessary to test whether ROS play a role in the production and the action of EDCF, because 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 ACh. The EPR study showed that basal levels of superoxide anions and peroxynitrite formed by the combination of superoxide and NO were undetectable in the hamster aorta and remained so even in the presence of L-NAME and ACh. The EIA study demonstrated that 8-isoprostanones, which are generated in vivo by the free radical-catalyzed, nonenzymatic peroxidation of arachidonic acid, were released in considerable amounts in the hamster aorta of the healthy hamster. The present data show that COX-2 catalyzes the formation of PGF2α, which allows the influx of extracellular Ca2+ to raise the [Ca2+]i, resulting in activation of phospholipase A2 and the subsequent activation of the constitutively expressed COX-2, thus leading to the generation of prostanoids from arachidonic acid. Among the prostanoids released, PGF2α is the most likely candidate of EDCF and diffuses toward adjacent vascular smooth muscle cells (VSMCs), where it activates the TP receptors and causes contraction of VSMCs. AA indicates arachidonic acid.

Increased vascular sensitivity to PGF2α with unaltered expression of the TP receptor in aortae from aged hamsters.

The preliminary results obtained in human renal arteries imply that a COX-2 metabolite, possibly PGF2α, produces TP receptor–dependent vasoconstrictions consistent with the observations on hamster aortae. In addition, we provide preliminary evidence that the release of PGF2α but not that of PGI2, 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.

To conclude, the present study demonstrates a role of endothelial COX-2 in the regulation of vascular tone in the aorta of the healthy hamster. The present data show that endothelial COX-2 catalyzes the formation of PGF2α, which represents a physiological EDCF at least in this preparation. Through binding to TP receptors, PGF2α produces endothelium-dependent contractions to ACh (Figure 8). The present preliminary data in human arteries could have clinical relevance in humans because the same or a similar pathway also exists in the human renal arteries being tested. Our data also demonstrate that this pathway is of increasing importance during aging in hamsters.

Sources of Funding
This study was supported by the Hong Kong General Research Fund (Chinese University of Hong Kong) (4362/04M and 465308), the Chinese University of Hong Kong Li Ka Shing Institute of Health Sciences, the Chinese University of Hong Kong Focused Investment Scheme, and the Germany–Hong Kong Joint Research Scheme/Deutsche Forschungsgemeinschaft. S.L.W. is a postgraduate student and F.P.L. is a postdoctoral fellow.

Disclosures
None.
References


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Circ Res. 2009;104:228-235; originally published online December 18, 2008; doi: 10.1161/CIRCRESAHA.108.179770

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Supplement Material

Methods

Animals and diet

Experiments were performed on aortae from young (∼3-month old) and aged (>18-month old) male Syrian golden hamsters 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.

Blood vessel preparation

Hamsters were euthanized by CO₂ inhalation. Thoracic aortae were excised and placed in Krebs-Henseleit solution (KHS) containing (mmol/L): NaCl 119, NaHCO₃ 25, MgCl₂ 1, KCl 4.7, KH₂PO₄ 1.2, CaCl₂ 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 tungsten wires in chambers of a Multi Myograph System (610M, Danish Myotechnology A/S, Aarhus N, Denmark) for the measurement of isometric force. Each chamber was filled with 5 mL-KHS aerated with 95% O₂ and 5% CO₂ and maintained at 37°C. The rings were stretched to a previously determined optimal resting tension of 10 mN. Thirty minutes after setting up the preparation, they were contracted with 60 mM 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 tungsten 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 previously¹. 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.
In situ endothelial cell \([\text{Ca}^{2+}]_i\) imaging

A calcium imaging technique was employed to visualize real-time changes in intracellular calcium levels, \([\text{Ca}^{2+}]_i\), in native endothelial cells of the hamster aorta. Isolated aortic rings with endothelium were labeled for 60 min at 21°C in a solution containing 10 \(\mu\)M 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 (luminal side up) 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 a 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 X 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 \([\text{Ca}^{2+}]_i\) and the ratiometric method eliminated potential artifacts caused by variations in cell thickness, intracellular dye distribution or photobleaching.
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.

Western blotting

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 PECAM-1, COX-1 (Santa Cruz), COX-2 (BD Transduction Laboratories), FP or TP receptor (Cayman Chemcal). 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 antibody (Santa Cruz). GAPDH antibody (Ambion, Inc) was probed as a loading control.

Measurement of prostaglandins by enzyme immunoassay (EIA) and high performance liquid chromatography-coupled mass spectrometry (HPLC-MS)

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 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.

Release of PGF$_2\alpha$ and 6-keto PGF$_{1\alpha}$ was also measured with HPLC-MS. 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\textsubscript{2α} and 6-keto PGF\textsubscript{1α} were dissolved in methanol and serially diluted in KHS to construct standard curves.

**Detection of reactive oxygen species (ROS) formation**

To test whether ROS were involved in endothelium-dependent contractions, 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. Briefly, aortic rings were incubated with 2-mL L-NAME (100 µmol/L)-containing Krebs solution, oxygenated with 95% O\textsubscript{2} and 5% CO\textsubscript{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) and Janzen and Hare (1990).\textsuperscript{2,3}

**Chemicals**

Acetylcholine, indomethacin, L-NAME (N\textsuperscript{G}-nitro-L-arginine methyl ester), phenylephrine, baicalein, tiron, tempol, DETCA (diethyldithiocarbamate acid) and U46619 (9,11-dideoxy-11\textsubscript{a},9\textsubscript{a}-epoxymethano-prostaglandin F\textsubscript{2α}) were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). DuP-697 (5-bromo-2-(4-fluorophenyl)-3-[4-(methylsulfonyl)phenyl]-thiophene), actinomycin-D, cycloheximide, GR 32191 ((4Z)-7-[(1R,2R,3S,5S)-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-1H-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 PGE2 were from Calbiochem, EMD Biosciences (La Jolla, CA, USA). VAS (valeryl salicylate), PGF2α, PGI2, 6-keto PGF1α 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, PGI2, cicaprost, tiron, tempol, DETCA and S18886 that were prepared in distilled water; all other drugs were dissolved in DMSO (Sigma-Aldrich).

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 our 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 we 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.
References


Online Figure I

**Online Figure I.** Lack of effects of the COX-1 inhibitors, valeryl salicylate (VAS) (A, n=6) or sc-560 (B, n=4), a 5- and the 12-lipoxygenase inhibitor, baicalein (C, n=5), or actinomycin and cycloheximide (D, n=6) on endothelium-dependent contractions. The protein expression of COX-1 in the aorta with and without endothelium (E, n=6). Data are means±SEM of n experiments.
Online Figure II

Online Figure II. Lack of significant effects of COX-2 inhibitors on contractions induced by 60 mmol/L KCl (A) or U46619 (B), confirming the specificity of these inhibitors. (C) Lack of effects of selective TP receptor antagonists on contraction induced by 60 mmol/L KCl. (D) Inhibitory effects of the TP receptor antagonists on U46619-induced contraction. Data are means±SEM of 4-5 experiments. ***P<0.001 compared with control.
**Online Figure III.** Lack of effects of free radical scavengers (tiron plus DETCA and tempol) or an NADPH oxidase inhibitor, apocynin on endothelium-dependent contractions (A, n=4). Recordings with the electron paramagnetic resonance technique showing absence of ROS signal in a control aorta (B) and in an L-NAME-treated aorta exposed to acetylcholine (C). (D) ROS signal as positive control in an aorta after addition of hypoxanthine plus xanthine oxidase. DETCA, diethyldithiocarbamate acid. (E) EIA measurement of 8-isoprostane in the bathing solution of L-NAME-treated aortic rings exposed to acetylcholine. Data are means±SEM of three experiments. NS, not significant.
Online Figure IV

**Online Figure IV.** Release of PGF$_{2\alpha}$ and PGI$_2$ (assayed in form of 6-keto PGF$_{1\alpha}$) in aortae from young and aged hamsters determined by high performance liquid chromatography-coupled mass spectrometry (HPLC-MS) (A-D, n=3-4). *P<0.05, **P<0.01 and ***P<0.001 compared between the groups of L-NAME and L-NAME + ACh. #P<0.05, ##P<0.01 and ###P<0.001 within the group of L-NAME + ACh. Comparison of the prostanoid release in response to ACh in aortae from young and aged hamsters (E&F, n=3-4). Data are means±SEM of n experiments.
Online Figure V. PGI₂ did not relax (A) nor contract (B) aortae from both young and aged hamsters (n=4). PGE₂ caused contractions in aortae from young and aged hamsters, and these contractions were not inhibited by TP receptor antagonist, S18886 (C, n=4).
Online Figure VI
Online Figure VI

Online Figure VI. Acetylcholine-elicited contractions were endothelium-dependent in L-NAME-treated aortae from aged hamsters (A, n=3-8), and the contractions were inhibited or abolished by 2-APB (B, a non-selective cation channel blocker, n=3), DuP-697 and celecoxib (C&D, specific COX-2 inhibitors, n=4-12), S18886 (E, a selective TP receptor antagonist, n=6-10), but not by VAS and sc-560 (F&G, specific COX-1 inhibitors, n=3-9). Endothelium-dependent contractions in the absence of L-NAME in the aortae from aged hamsters (H, n=4), which were abolished by 2-APB (I, n=3). Protein expression of COX-1 in aortae with (+ Endo) and without (- Endo) endothelium from young and aged hamsters (J, n=4). Release of 6-keto PGF$_{1\alpha}$ in response to ACh in the bathing solution of L-NAME-treated aortae from young and aged hamsters (K, n=3-4). The protein expression of the TP receptor in aortae from young and aged hamsters (L, n=7-8). Data are means±SEM of n experiments. **P<0.01 and ***P<0.001 compared with control within the same age group; #P<0.05 between the young and aged groups. Data are means±SEM of n experiments.
Online Figure VII
Online Figure VII

**Online Figure VII.** ACh-induced contractions in human renal arteries (A). The number indicates the age of the patient; HT, hypertension; DM, diabetes. 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 (B&C, n=4). Pre-treatment with S18886, but not AL-8810, prevented PGF$_{2\alpha}$-induced contractions (D). Western blotting analysis showed that human renal arteries expressed the TP receptor (TPR) but not the FP receptor (FPR) (E). Lanes A (hamster heart) & B (hamster lung) served as positive controls. Increased release of PGF$_{2\alpha}$, but not 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 (F, data are average of two patients).
**Online Figure VIII.** The FP receptor (FPR) was minimally expressed in the hamster aortae with reference to the positive expression in the heart and lung from hamsters (A, n=4). The FP receptor antagonist, AL-8810 did not attenuate ACh- or PGF$_{2\alpha}$-induced contractions (B&C, n=4).