COX-2 Limits Prostanoid Production in Activated HUVECs and Is a Source of PGH$_2$ for Transcellular Metabolism to PGE$_2$ by Tumor Cells

M. Dolores Salvado, Arántzazu Alfranca, Amelia Escolano, Jesper Z. Haeggström, Juan Miguel Redondo

Objective—Inducible expression of cyclooxygenase-2 (COX-2) and terminal prostaglandin synthases (tPGS) has been mainly analyzed in tumor, stromal, and inflammatory cells, and little is known about the regulation of prostanoid biosynthesis by endothelial cells. Here we characterize the profile of prostanoids produced by activated HUVECs and analyze the expression and activities of tPGS.

Methods and Results—Enzyme immunoassays indicated increased endothelial prostanoid production after proangiogenic stimulation, but without parallel upregulation of tPGS. Endothelial prostanoid production instead depended on the induction of COX-2 and was abolished by COX-2 silencing or pharmacological inhibition. COX-2 is functionally coupled to prostacyclin and thromboxane synthases in HUVECs, but these cells show no detectable PGE$_2$ synthase (PGES) activity. Endothelial PGE$_2$ production is partly mediated by nonenzymatic decomposition of COX-2-derived PGH$_2$, but endothelial-produced PGH$_2$ can also be metabolized enzymatically by microsomal PGES-1 in cocultured tumor cells.

Conclusions—Our findings identify a novel transcellular metabolism of PGE$_2$ between the endothelial and tumor compartments. Given the role of PGE$_2$ as a mediator of COX-2 proangiogenic effects, transcellular metabolism of endothelial-derived PGH$_2$ is a potential target for treatment of pathological angiogenesis. (Arterioscler Thromb Vasc Biol. 2009;29:00-00.)

Key Words: ●●●

Numerous studies indicate that enzymes involved in the metabolism of AA and its products might contribute to tumor-induced angiogenesis.$^{1,2}$ Cyclooxygenase-2 (COX-2), which converts AA into the prostanoid precursor PGH$_2$, is induced by the proangiogenic factor VEGF, released by tumor cells. Moreover, proinflammatory cytokines such as IL-1$\beta$ and TNF-$\alpha$, potent COX-2 inducers, also have proangiogenic activity.$^3$ COX-2 expression is elevated in tumors, and genetic inactivation or pharmacological inhibition of COX-2 reduces tumor-induced neovascularization.$^4$ COX-2–associated angiogenesis is thought to be mediated by derived prostanoids, such as PGE$_2$, PGL$_2$, and TXA$_2$. Strong evidence exists for a link between PGE$_2$ and tumor angiogenesis; and of the PGES enzymes (cPGES, mPGES-1, mPGES-2), inducible mPGES-1 appears to be critical for tumor angiogenesis.$^5$ Moreover, in vivo models of TXAS or PGIS overexpression indicate proangiogenic effects for TXA$_2$, and antiangiogenic effects for PGI$_2$.$^6$

Despite the evidence linking prostanoids to tumor angiogenesis, their mechanism of action is poorly understood. In particular, very little is known about the possible contribution made by the regulation of endothelial terminal prostanoid synthases. PGH$_2$ release by endothelium has been shown in vivo and ex vivo$^{7,8}$; however, whether PGH$_2$ is transformed into final prostanoids in endothelial cells spontaneously or enzymatically is unknown.

We investigated the regulation of terminal prostanoid synthases by VEGF and IL-1$\beta$. Our results indicate that although endothelial cells express terminal prostanoid synthases, the key enzyme in the regulation of prostanoid biosynthesis in these cells is COX-2. COX-2–derived PGH$_2$ is released untransformed by endothelial cells, and can serve as a substrate for further metabolism to PGE$_2$ by mPGES-1 expressed in neighboring tumor cells. Transcellular biosynthesis of PGE$_2$ through endothelium-tumor cross-talk suggests a possible role of endothelium-derived PGH$_2$ in tumor angiogenesis.

Methods

Cell Culture and Reagents

HUVECs were cultured as described$^9$ and used within passages 1 to 4. A549 cells were from ATCC (# CCL-185) and cultured as described.$^10$ Cells were grown to confluence and serum-starved overnight before treatments.

VEGF$_{165}$ and IL-1$\beta$ were from Peprotech (UK), and TNF-$\alpha$ from R&D Systems. Primary antibodies were sourced as follows: phospho-Erk-1,2 (Promega); $\alpha$-Tubulin (Sigma); COX-1 and COX-2 (Alexis Biochemicals); mPGES-1, mPGES-2, ePGES, PGI$_2$, and TXAS (Cayman). AA was from Nu-Chek-Prep and PGH$_2$ from...
Larodan Fine Chemicals (Sweden). SC-560 was from Cayman. Etoricoxib was a gift from Dr Godessart (Amirall Prodesfarma).

Sample Preparation, Prostanoid Measurement, and Immunoblot
After treatments, cell supernatants were removed for prostanoid quantification (EIA kits, Cayman), and cell monolayers lysed for immunoblot as described.11

RNA Isolation and RT-PCR
Total RNA was isolated with Tripure Isolation Reagent (Roche Diagnostics), and 2 μg were reverse transcribed to cDNA with MMLV-RT (Invitrogen, CA). Conventional and real-time PCR were conducted as indicated in the supplemental data (available online at http://atvb.ahajournals.org).

Terminal Prostanoid Synthase Activities
Enzyme activities were assessed as previously reported12,13; see the supplemental data for details.

Effect of COX Selective Inhibitors on Prostanoid Formation by Cytokine-Treated ECs
HUVECs were pretreated (1 hour) with 10 μmol/L Etoricoxib (COX-2 inhibitor), 0.01 μmol/L SC-560 (COX-1 inhibitor), or both. After 24-hour stimulation with VEGF (50 ng/mL) or IL-1β (10 ng/mL), samples were processed for prostanoid determination and immunoblot.

COX-1 and COX-2 Knockdown
siRNAs (Ambion) are listed in the supplemental data. HUVECs were transiently transfected with Oligofectamine (Invitrogen) (see supplemental data). After 24 hours, cells were stimulated with VEGF or IL-1β, followed by prostanoid determination and immunoblot.

PGE2 Assay in Supernatants of HUVECs Cocultured With A549 Cells
A549 tumor cells were pretreated with IL-1β and COX inhibitors before mixing with HUVECs. Confluent HUVECs were treated with COX inhibitors and IL-1β as required. After 24-hour coculture, supernatants were collected for PGE2 determination. For further details, see the supplemental data.

Statistical Analysis
Data were analyzed by Student t test or by analysis of variance followed by the Newman–Keuls test. Differences were considered statistically significant at P<0.05.

Results
Prostanoid Biosynthesis by Resting and Activated Endothelial Cells
HUVECs were treated with proangiogenic cytokines (VEGF or IL-1β), and PGE2, PGI2 (determined as its stable breakdown product 6-keto PGF1α), and TXA2 (as its stable breakdown product TXB2) were measured in cell supernatants. VEGF and IL-1β increased production of all 3 prostanoids by ~3- to 5-fold (Figure 1). However, whereas VEGF-triggered prostanoid synthesis peaked after 8 hours, the maximal IL-1β–triggered prostanoid response required 24 hours (data not shown).

Endothelial Cell Expression of Terminal Synthases for PGE2, PGI2, and TXA2
To investigate the mechanism underlying the increased prostanoid synthesis, we analyzed the mRNA and protein expression of the terminal prostaglandin synthases for PGE2 (mPGES-1, mPGES-2, and cPGES), PGI2 (PGIS), and TXA2 (TXAS). Analysis by conventional and real-time RT-PCR detected expression of PGIS, TXAS, cPGES, and mPGES-2 in HUVECs; however, exposure to VEGF or IL-1β did not increase expression above the basal levels (supplemental Figure IA and IB). mPGES-1 mRNA was not detected in resting or activated HUVECs; however, expression was induced by IL-1β in A549 cells (supplemental Figure IC), agreeing with previous reports.10,14 Effective activation of HUVECs by the stimuli was confirmed by Erk-1,2 phosphorylation (supplemental Figure IIA). As previously reported,15 VEGF upregulated COX-2 mRNA expression (supplemental Figure IIB). Constitutive expression of cPGES, mPGES-2, PGIS, and TXAS proteins was detected in HUVECs and, as with the mRNA, their expression was unmodified by VEGF (Figure 2A). Constitutive expression of COX-1 was also unaffected by VEGF, whereas COX-2 protein was strongly induced (Figure 2B). IL-1β gave similar results (supplemental Figure III). Agreeing with the mRNA data, mPGES-1 protein expression was not detected in resting or stimulated HUVECs, although mPGES-1 protein was induced by IL-1β in A549 tumor cells (Figure 2C). These results indicate that VEGF- and IL-1β–stimulated increases in HUVEC synthesis of PGE2, PGI2, and TXA2 are not the result of modulated tPGS expression or protein stability.
COX enzymes are generally thought to be functionally coupled to terminal prostanooid synthases. To investigate the coupling of COX isoenzymes to particular TPgs, we measured endothelial prostanooid production in the presence of selective inhibitors of COX-1 (SC-560) and COX-2 (Etoricoxib). The inhibitors diminished PGE2, PG12, and TXA2 biosynthesis in resting and stimulated cells. Although Etoricoxib had a stronger effect on all prostanoids analyzed, SC-560 unexpectedly also partially inhibited prostanooid production, particularly in activated cells (Figure 3A).

The selectivity of COX inhibitors has been questioned; therefore as an alternative we knocked down expression of COX isoenzymes with specific siRNAs. COX-1 or COX-2 protein was effectively and specifically suppressed, and knock-down of one did not alter expression of the other (supplemental Figure VA). COX-2 silencing corroborated the results obtained with Etoricoxib; however, unlike the data obtained with SC-560, suppression of COX-1 expression had no effect on the production of any prostanooid tested, irrespective of treatment (Figure 3B). This lack of effect might have been attributable to an absence of COX-1 enzymatic activity in our experimental system; however, COX-1 activity was detectable in Etoricoxib-treated COX-2-silenced HUVECs, both with and without IL-1β treatment (supplemental Figure VB). These results suggest that even low concentrations of SC-560 affect COX-2 activity and provide evidence of preferential coupling between COX-2 and terminal prostanooid synthases in vascular endothelium.

Enzyme Activities of the Terminal Synthases for PGE2, PG12, and TXA2 in Activated ECs

The functional coupling to upstream COX isoenzymes of terminal prostanooid synthases appears to depend on their kinetic

![Figure 2](http://atvb.ahajournals.org/Downloaded_from_salvadoetal.png)

**Figure 2.** Effects of VEGF on endothelial expression of prostanooid terminal synthases. Immunoblot analysis of the expression of prostanooid terminal synthases (A), or COX-1 and -2 (B) in HUVECs treated with VEGF. C, mPGES-1 expression in HUVECs and A549 treated with IL-1β. Results are representative of 3 independent experiments. Alpha-tubulin was used as loading control.

![Figure 3](http://atvb.ahajournals.org/Downloaded_from_salvadoetal.png)

**Figure 3.** Contribution of COX enzymes to VEGF- and IL-1β-regulated prostanooid production. A, HUVECs were pre-treated with SC-560 or Etoricoxib and challenged with VEGF or IL-1β. Prostanoids were determined by EIA. B, Prostanoid production in control, COX-1, or COX-2 silenced cells treated with VEGF or IL-1β. Data are means±SD, n=3. **P<0.001, **P<0.01, and *P<0.05 vs untreated.
Transcellular PGE<sub>2</sub> Biosynthesis in Endothelial and Tumor Cocultures

Endothelium is known to release untransformed PGH<sub>2</sub> in certain situations, as homeostatic physiological interactions with platelets. To investigate whether endothelium-derived PGH<sub>2</sub> serves as substrate for mPGES-1 expressed by neighboring tumor cells, we measured PGE<sub>2</sub> production by mixed cultures of HUVECs and A549 cells. A549 cells were activated with IL-1β to ensure optimal induction of mPGES-1, and control and activated cells were treated with COX-inhibitors (A549<sup>COX-INHIB</sup> cells) to limit endogenous prostanoid production (Figure 5A). Coculture of activated A549<sup>COX-INHIB</sup> cells with activated HUVECs yielded significantly greater amounts of PGE<sub>2</sub> than the sum of the productions by the individual cultures (Figure 5B). This increased production was impaired by pretreating HUVECs with COX inhibitors (Figure 5B), suggesting that HUVECs are the source of PGH<sub>2</sub> for transcellular metabolism by A549 cells.

In further experiments, A549 cells were seeded on microporous transwell inserts that allow passage of secreted products but prevent A549-HUVEC contacts. Under these conditions, there was no significant enhancement of PGE<sub>2</sub> synthesis (Figure 5C), suggesting that physical contact between A549 cells and HUVECs is necessary for tumor cell-mediated transcellular PGE<sub>2</sub> synthesis.

To confirm these findings, we examined PGE<sub>2</sub> production by HUVEC-A549 cocultures treated with AA. Exogenous AA induces the release of endothelial-derived PGH<sub>2</sub> to the extracellular medium, where it can be converted to PGF<sub>2α</sub> by addition of the mild reducing agent SnCl<sub>2</sub>. SnCl<sub>2</sub> thus provides a means of selectively inhibiting PGE<sub>2</sub> production from endothelial released PGH<sub>2</sub>. Mixed cultures of activated HUVECs and A549<sup>COX-INHIB</sup> cells produced significantly more PGE<sub>2</sub> in response to exogenous AA than did activated HUVECs alone. This increased PGE<sub>2</sub> production was inhibited by pretreatment with SnCl<sub>2</sub> (Figure 6A), supporting the existence of transcellular metabolism. In HUVECs cultured alone, SnCl<sub>2</sub> reduced PGE<sub>2</sub> production to the levels produced by nonactivated cells (Figure 6B), suggesting that endothelial-derived PGE<sub>2</sub> is produced through nonenzymatic rearrangement of PGH<sub>2</sub>. SnCl<sub>2</sub> did not affect PGE<sub>2</sub> production by single A549 cultures (Figure 6C), indicating that these cells synthesize PGE<sub>2</sub> enzymatically.

### Discussion

COX-2 dependence of tumor angiogenesis has generally been attributed to COX-2 expression in tumor or stromal cells. Our findings, however, support an important contribution by COX-2 expressed in endothelial cells. Moreover, our results provide evidence for a transcellular PGE<sub>2</sub> biosynthetic pathway in which endothelium-derived untransformed PGH<sub>2</sub> serves as substrate for mPGES-1 expressed by tumor cells, suggesting that the tumor prostanoid profile reflects autocrine and paracrine interactions among the different tumor compartments. To our knowledge, this is the first report of PGE<sub>2</sub> production by transcellular metabolism through endothelial-tumor cell crosstalk, and provides insight into the complexity of prostanoid metabolism during angiogenesis.
Given the limited current understanding of how pathways that control prostanoid production and function downstream of COX are regulated, one aim of this study was to characterize the expression and regulation of terminal prostaglandin synthases for PGE2, PGI2, and TXA2 in vascular endothelial cells. Our results show that VEGF and IL-1β, well-documented angiogenesis inducers, induce similar fold increases in the production of PGE2, PGI2, and TXA2 by activated endothelial cells. Although PGI2 is the main product of AA metabolism in vascular endothelium, we detected PGE2 and TXA2 production under basal and activated conditions. The relative expression and characteristic kinetic parameters of tPGS enzymes are thought to be major determinants of the final profile of prostanoids produced by a given cell type, and we therefore hypothesized that increases in the expression or enzymatic activities of these enzymes might contribute to enhanced prostanoid production.

TXA2 and PGI2 can only be biosynthesized enzymatically, and we consistently observed expression and activity of TXAS and PGIS in nonactivated cells (Figures 2A and 4). The lack of any effect of VEGF or IL-1β on these parameters (Figures 2A and 4) suggests that the inducible production of TXA2 and PGI2 depends on upregulation of COX-2 and its functional coupling to TXAS and PGIS. Surprisingly, we did not detect mPGES-1 mRNA or protein in resting or stimulated endothelium. This contrasts with previous reports but agrees with data obtained by Soler et al. We used 2 independent sets of PCR primers and 2 antibodies, tools that effectively detected mPGES-1 mRNA and protein expression in A549 cells. Although such discrepancies have been ascribed to cell passage, we did not detect mPGES-1 in HUVECs from different sources at any passage number.

Figure 5. Coculture with HUVECs increases PGE2 biosynthesis by A549 cells. A, PGE2 released by single cultures of noninhibited (A549) and COX-inhibited A549 (A549COX-INHIB), treated as indicated with IL-1β. B, Control or IL-1β-stimulated HUVECs (ECs) were treated as indicated with COX inhibitors (ECCOX-INHIB) and coincubated with A549COX-INHIB cells (EC/A549COX-INHIB). C, A549COX-INHIB cells were cocultured with HUVECs (ECs) directly or separated by an insert well, and incubated as in B. Data are means±SD, n=3. ***P<0.001, **P<0.01, and *P<0.05 vs untreated; #P<0.01 vs noninhibited A549; ##P<0.001 vs vehicle.

Figure 6. SnCl2 inhibits PGE2 biosynthesis by AA-stimulated HUVEC/A549 cocultures. A, HUVECs (ECs) were IL-1β-stimulated before coincubation with IL-1β-activated, COX-inhibited A549. AA was added with or without SnCl2, and PGE2 determined. B, Rescaled presentation of data from single HUVEC cultures shown in A. C, PGE2 release from COX-inhibited A549 treated as in A. Data are means±SD, n=3. ***P<0.001 and **P<0.01 vs untreated; ##P<0.001 and #P<0.01 vs IL-1β-treated.
not shown), and therefore the reason for this discrepancy is at present unknown. Moreover, although we detected constitutive expression of cPGES and mpGES-2 in endothelial cells, we did not detect terminal PGE_2 synthase activity; and silencing of these synthases did not affect PGE_2 production in activated HUVECs (Figure 4, supplemental Figure VII). The similar end-product profiles produced after incubation of PGH_2 with HUVECs or cell-free buffer strongly suggests that endothelial-derived PGE_2 forms through nonenzymatic decomposition of PGH_2. This agrees with reports that PGE_2 and PGD_2 can be formed nonenzymatically, and leaves open the question of what role cPGES and mpGES-2 play in endothelium, a subject of recent debate.

It is possible that above a threshold concentration, endothelially-produced PGH_2 cannot be metabolized by the endothelial terminal synthases, particularly TXAS and PGIS, which likely undergo "suicide inactivation" in excess of substrate. Endothelial PGH_2 might then be released, taken up by neighboring cells and subsequently metabolized. Although most cells possess the complete set of enzymes for prostanooid biosynthesis, prostanooid production is often the result of cell–cell interactions that involve transfer of chemically reactive intermediates, a process known as transcellular biosynthesis. It is interesting that transcellular biosynthesis of PGE_2 was notable only when cells were pretreated with IL-1β before coculture. Because IL-1β induces COX-2 and triggers PGE_2 production dependent on COX-2 activity, overexpression and activation of COX-2 appears to be essential for production of PGH_2 sufficient for transcellular metabolism.

Endothelium-derived PGH_2 has been reported to be involved in the transcellular metabolism of TXA_2 by platelets and of PGI_2 by lymphocytes. Many tumor cells express functional mpGES-1 and transcellular metabolism during tumor-endothelium cross-talk could be particularly important in tumor angiogenesis. In this scenario, endothelium would donate PGH_2 to neighboring tumor cells for metabolism by mpGES-1, thus increasing the total yield of PGE_2. Our coculture experiments support this hypothesis. Mixed cultures of HUVECs and A549 produce higher amounts of PGE_2 than single cultures of either type, and the transwell experiments suggest that this effect is dependent on physical interaction. This also indicates that the enhanced PGE_2 production is not attributable to paracrine stimulation.

Additional evidence for the contribution of endothelial-derived PGH_2 to transcellular metabolism comes from experiments with SnCl_2, which substantially reduced PGE_2 production by HUVEC-A549 cocultures (Figure 6A). Furthermore, pretreatment of HUVECs with COX-inhibitors strongly impaired PGH_2 production and reduced PGE_2 biosynthesis in the HUVEC-A549 cocultures (Figure 5).

Angiogenesis is generally believed to be regulated by the profile of downstream COX metabolites (determined by the expression and activities of their respective tPGSs) rather than by the total COX amount expressed in cells. The establishment of COX-2 as the key enzyme regulating endothelial cell prostanooid production may have important implications for understanding of tumor-induced angiogenesis. COX-2 silencing or pharmacological inhibition abrogated basal and VEGF- or IL-1β–induced production of PGE_2, PGI_2, and TXA_2. Surprisingly, the experiments with selective COX inhibitors suggested a partial contribution of COX-1 to prostanooid production in resting and activated endothelial cells (Figure 3A). The fact that this effect was not observed when COX-1 expression was knocked down (Figure 3B) strongly suggests that the COX-1 inhibitor SC-560 affects COX-2 activity. This agrees with reports that, although COX-1 selective under cell-free conditions, SC-560 is not fully COX-1–selective in cells.

COX-1 activity detected in HUVECs (supplemental Figure IVB) is unlikely to reflect a significant contribution of COX-1 to prostanooid biosynthesis, because it was measured in the presence of unphysiologically high concentrations of exogenous arachidonate. In contrast, prostanooid production experiments involve low concentrations of endogenous arachidonate, conditions under which COX-1 might be allosterically inhibited.

Functional coupling of COX-2 to PGIS is consistent with data linking COX-2 and PGI_2, including the recently reported cardiovascular risk derived from COXIB-mediated inhibition of PGI_2 production. Regarding the functional coupling observed between COX-2 and TXAS, although TXA_2 production was originally linked to COX-1 activity, it can vary in heterologous systems expressing both COX isoenzymes. In fact, preferential coupling of TXAS to COX-2 has been reported in macrophages, cancer cells, and even healthy brain and kidney.

A previous study reported a lack of PGE_2 and PGI_2 synthesis in resting HUVECs, associated with the lack of basal COX-2 expression in these cells. Our analysis, in accordance with others, clearly detects basal and cytokine-induced COX-2 expression and prostanooid production. COX-2 expression and PGI_2 production are upregulated under physiological blood flow, which would be consistent with the basal expression of COX-2 we detected.

The coculture experiments presented here establish the principle that endothelial prostanooids can be metabolized by neighboring tumor cells. However, further work will be needed to confirm the existence of transcellular prostanooid metabolism in vivo and to determine whether addition of proangiogenic cytokines to static cell cultures accurately models the tumor/vasculature interface. Transcellular metabolism of endothelial-derived PGH_2 illustrates the complex interactions between endothelium and other cell compartments. The use of endothelial prostanooid precursors to sustain pathological angiogenic signals provides a vivid demonstration of the ability of tumor cells to coopt an organism’s pathophysiological responses and redirect them toward tumorigenic outcomes. Greater understanding of the complexity of prostanooid metabolism during angiogenesis, and of how complex autocrine and paracrine interactions determine tumor growth, is likely to provide new avenues for the development of treatments for pathological angiogenesis.

Acknowledgments
We thank M. Hamberg for invaluable help with GCMS, N. Godessart for providing Etoricoxib, and L. Vila for helpful discussion. S. Bartlett provided editorial assistance.

Sources of Funding
The Centro Nacional de Investigaciones Cardiovasculares (CNIC, Spain) is supported by the Spanish Ministry of Science and Innov-
References


COX-2 Limits Prostanoid Production in Activated HUVECs and Is a Source of PGH₂ for Transcellular Metabolism to PGE₂ by Tumor Cells

M. Dolores Salvador, Arántzazu Alfranca, Amelia Escofano, Jesper Z. Haeggström and Juan Miguel Redondo

Arterioscler Thromb Vasc Biol. published online May 7, 2009;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2009/05/07/ATVBAHA.109.188540.citation

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2009/05/07/ATVBAHA.109.188540.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
SUPPLEMENTAL DATA

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

**RNA isolation and RT-PCR.** 2 μg of total RNA were reverse transcribed (RT) by random primer extension with MMLV-reverse transcriptase (Invitrogen, Carlsbad, CA). Primers and cycle numbers for amplification of cDNAs were as follows: mPGES-1 (30 cycles), sense 5’-CCT CCC GGC CTT CCT GCT CT-3’, antisense 5’-TGG GGG CCT CCG TGT CTC AG-3’; cPGES (30 cycles), sense 5’-CAA TGC AGC CTG CTT CTG CAA AG-3’, antisense 5’-CTT TAC TCC AGA TCT GGC-3’; mPGES-2 (30 cycles), sense 5’-GAG ATG AAG TGG CGG CAG TGG-3’, antisense 5’-CCC ACA GCA GCC ACC CAC TT-3’; PGIS (30 cycles), sense 5’-GGA GCA AAT GGC TGG AGA GTT ACC-3’, antisense 5’-GAT CCG TCA GGG TTC AGG AAT CG-3’; TXAS (35 cycles), sense 5’-CCT TCT CCT GGC TCA TTT A-3’, antisense 5’-TCG TCT CGG TTC TTA TTG G-3’; β-Actin (30-35 cycles), sense 5’-TGA CGG GGT CAT CCA CAC TGT GCC CAT CTA-3’, antisense 5’-CTA GAA GCA TTT TCG GTG GAC GAT GGA GGG-3’.

After denaturation at 95°C for 2 min, PCR amplification cycles were as follows: mPGES-1, 30s at 95°C, 45s at 65°C, and 1 min at 72°C; cPGES, 30s at 95°C, 45s at 60°C, and 1min at 72°C; mPGES-2, 30s at 95°C, 45s at 64°C, and 30s at 72°C; PGIS, 30s at 95°C, 45s at 62°C, and 1min at 72°C; TXAS, 30s at 95°C, 45s at 54°C, and 1min at 72°C. All products were elongated for 10 min at 72°C. PCR products were resolved by electrophoresis on 1.5% (w/v) agarose gels.

Quantitative analysis of mRNA expression by real-time PCR was performed in an ABI Prism 7900 thermal cycler using predesigned validated assays for the selected genes.
Supplemental Material

(TaqMan Gene Expression Assays; Applied Biosystems, Foster City, CA). 1 µL of the reverse transcription reaction was amplified using TaqMan Universal PCR Master Mix and universal thermal cycling parameters. Relative mRNA expression was quantified by the Comparative Ct method (User bulletin 2, Applied Biosystems) and normalized to 18S as an endogenous control. Untreated cells were used as calibrator.

**Assay of terminal prostanoid synthase activities.** HUVEC (2\(\times\)10^6 cells per condition) were rinsed twice with phosphate-buffered saline (PBS), and incubated (10 min, 37 °C) with 18 µM PGH\(_2\) in 1M potassium phosphate buffer (pH 7.4) containing GSH (5mM) and DTT (0.5mM). The reaction was terminated by adding stop solution (25 mM FeCl\(_2\), 50 mM citric acid). 1µg each of deuterated PGE\(_2\), 6-keto-PGF\(_{1\alpha}\) and TXB\(_2\) (Cayman) were added to supernatants as internal standards and solid phase extraction was performed immediately using Sep-Pak C18 cartridges (Water Associates, Milford, MA). Prostanoids, eluted in 80% methanol, were dried under an N\(_2\) stream and derivatized to trimethylsilyl ethers as previously described\(^{42}\). The products were analyzed by CG/MS. Non-cell samples, to assay for non-enzymatic degradation of substrate, were run in parallel.

**Knock-down experiments.** siRNAs (Ambion, Inc., USA) included predesigned annealed siRNAs targeted to COX-1 (siRNA ID # 2391), cPGES (siRNA ID # 140054) and mPGES-2 (siRNA ID # 130829), a Silencer custom COX-2 siRNA (# AM16104) targeted to bases 1774–1792 of NCBI ID:M90100 (5\(\acute{\text{e}}\)-gggctgtccctttacttca-3\(\acute{\text{e}}\)), and a scrambled negative control (# 4365). HUVEC were transiently transfected with 20 nmol/L (COX-1, COX-2) or 200 nmol/L (cPGES, mPGES-2) of the required siRNA in 16 µL Oligofectamine transfection reagent (20µl for cPGES, mPGES-2) (Invitrogen).
Twenty-four h after transfection (32 h for cPGES and mPGES-2), cells were serum starved and stimulated with VEGF or IL-1β. Samples were processed for prostanoid quantification and western blot as described above or for assessment of COX-1 enzymatic activity as described below.

**Assay of COX-1 enzymatic activity.** HUVEC (1x10⁶ cells per condition) were transiently transfected with 20 nmol/L of COX-2 siRNA or a control siRNA as described above. Twenty-four h after transfection, cells were serum starved and stimulated with IL-1β for 24h. Cells were harvested by scraping in 0.2 mL Tris-HCL pH 7.5 and homogenized by sonication. Protein content was quantified and 1.5 mg of total cell lysate was used for reactions, which were performed in a final volume of 60 µL containing 10 mM CaCl₂, 200 nM 12(S)-HpETE (Cayman) and where indicated Etoricoxib 10 µM or DMSO (Vehicle). The samples were incubated with 25 µM AA (Cayman) for 30 min at 37 °C. PGE₂ contents were determined by EIA (Cayman).

**PGE₂ assay in supernatants of HUVEC co-cultured with A549 cells.** A549 tumor cells (2 x 10⁵ per condition) were activated (48 h) with IL-1β (10 ng/mL) or IL-1β mixed with TNF-α (10 ng/mL). Before mixing with HUVEC, A549 cells were pretreated (16 h) with COX selective inhibitors (1µM SC-560 and 15 µM Etoricoxib), rinsed with PBS, trypsinized, and resuspended in medium 199, 0.5% FBS. Confluent HUVEC, cultured in gelatin-coated 24-well plates (2 x 10⁵ cells per well), were serum-starved and subsequently treated (16 h) with COX inhibitors and IL-1β (10 ng/mL) as required. After rinsing twice with PBS, HUVEC were incubated with serum-starvation medium (control EC) or with resting or activated COX-inhibited A549 cells (24 h, 37 °C) in a final volume of 0.7 mL. Cell supernatants were collected for determination of PGE₂.
To test whether PGE$_2$ production in HUVEC-A549 co-cultures involves direct contact, A549 cells were placed in a transwell insert (0.4µm-Millipore). The final volumes of the luminal and abluminal compartments were 0.6 and 0.1 mL, respectively.

To assess transcellular biosynthesis of PGE$_2$ from exogenously added AA, untreated and IL-1β-treated HUVEC were co-cultured with A549 cells as above. After 7h, 25µM AA (in ethanol) was added, and co-incubations continued for 30 min at 37 °C. PGH$_2$ is unstable and is converted into PGF$_{2\alpha}$ by mild reducing agents such as SnCl$_2$ 2, 38,28. Therefore, in some experiments, to evaluate the release of PGH$_2$ in the co-cultures, cells were pretreated with SnCl$_2$ (200 µg/mL) for 10 min before addition of AA.

**Assay of cPLA$_2$ expression and enzymatic activity in endothelial cells.** After treatments, cell monolayers were immediately washed twice in ice-cold phosphate-buffered saline (PBS) and lysed for western blot analysis as described 11. Total protein equivalents of each sample were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Milford, MA). Membranes were probed with cPLA$_2$ Antibody (#2832; Cell Signaling Technology Inc.), Phospho-cPLA$_2$ (Ser505) Antibody (#2831; Cell Signaling Technology Inc.), COX-2 antibody (ALX-804-112; Alexis Biochemicals,Carlsbad, CA) or α-Tubulin (T9026; Sigma, Poole, UK) as required. Immunoreactive bands were detected by enhanced chemiluminescence (GE HealthCare, Amersham, UK).

To assess cPLA2 enzymatic activity, HUVEC monolayers (2x10$^6$ cells per condition) were harvested by scraping in 0.2 mL of Tris-HCL pH 7.5 containing 1mM EDTA, and homogenized by sonication. After centrifugation (10,000 x g, 15 min, 4°C), cell supernatants were assayed with a cPLA$_2$ Assay kit (Cayman) according to the manufacturer’s instructions.
SUPPLEMENTAL REFERENCES


DETAILED LEGENDS TO MANUSCRIPT FIGURES

Figure 1.-Profile of prostanoids released from HUVEC activated with VEGF and IL-1β. Endothelial cells (HUVEC) were challenged with VEGF (50 ng/mL) or IL-1β (10 ng/mL) as indicated. PGE₂, PGI₂ (as 6-keto-PGF₁α) and TXA₂ (as TXB₂) were measured in culture supernatants by enzyme immunoassay (EIA). Results are expressed as pg per 4 x 10⁵ cells, and data are means ± SD of three independent experiments performed in duplicate. *** p<0.001 vs. untreated; ** p<0.01 vs. untreated (Student’s t test).

Figure 2.-Effects of VEGF and IL-1β on endothelial expression of prostanoid terminal synthases. A., Immunoblot analysis of the expression of prostanoid biosynthetic enzymes. HUVEC were treated with VEGF (50 ng/mL, 8h). B., For detection of mPGES-1, HUVEC and A549 cells were treated with IL-1β for 48h and analyzed in parallel. Results shown are representative of at least three independent experiments. α-tubulin was used as a loading control.

Figure 3.-Contribution of COX enzymes to VEGF- and IL-1β-regulated endothelial prostanoid production. A., Effect of selective COX-1 and COX-2 inhibitors. HUVEC were pretreated (1 h) with 0.01 µM SC-560 or 10 µM Etoricoxib or both and challenged with VEGF (50 ng/mL) or IL-1β (10 ng/mL) for 24-h. Prostanoid concentrations were measured in culture supernatants by EIA. B., siRNA silencing. Prostanoids were determined in culture supernatants of controls (siControl) and COX-1 (siCOX-1) or COX-2 (siCOX-2) silenced cells treated with VEGF or IL-1β as in A. Results are expressed as pg per 4 x 10⁵ cells, and data are means ± SD of three independent
experiments performed in duplicate. ***, p<0.001; **, p<0.01; *, p<0.05 vs. untreated. (ANOVA).

**Figure 4.-Enzyme activities of terminal prostanoid synthases in endothelial cells.**
HUVEC (2 x 10^6 per condition) were stimulated with VEGF (50 ng/mL, 8 h) or IL-1β (10 ng/mL, 24 h). Cells were then washed twice and incubated with PGH₂ (18 µM) for 10 min at 37°C. Activities of tPGES, TXAS and PGIS were indexed by the cell supernatant contents of PGE₂, TXA₂ (as TXB₂) and PGI₂ (as 6-keto-PGF₁α) measured by GCMS. Control PGH₂ incubations were performed without cells (hatched bars). Results are expressed as ng per 2 x 10^6 cells and data are the means ± SD of three independent experiments. (Student’s t test).

**Figure 5.-Co-culture with HUVEC increases PGE₂ biosynthesis by A549 cells. A.,**
PGE₂ concentrations released by single cultures of non-inhibited (A549) and COX-inhibited A549 (A549COX-INHIB) cells. A549 cells were treated with IL-1β (10 ng/mL) for 48h to ensure optimal induction of mPGES-1.Where indicated, COX selective inhibitors (1µM SC-560 and 15 µM Etoricoxib) were added during the last 16h to reduce endogenous PGH₂ production. Cells were replated and cultured (24 h) in serum deprived medium, and culture supernatant PGE₂ determined by EIA. B. Confluent HUVEC (EC) were serum-deprived and stimulated (16h) with IL-1β (10 ng/mL). Where indicated, EC were simultaneously pretreated with COX inhibitors (1µM SC-560, 10 µM Etoricoxib: EC\(^{COX-INHIB}\)). After washing, HUVEC monolayers were incubated either with cell-free serum-starvation medium (EC) or with a suspension of A549\(^{COX-INHIB}\) cells obtained as in A (EC/A549\(^{COX-INHIB}\)). PGE₂ was determined after 24h. PGE₂ production by single A549\(^{COX-INHIB}\) cultures is shown for comparison. Untreated and IL-1β refer to pretreatments of both cell types. C., To test whether PGE₂ production in HUVEC-A549 co-cultures involves direct contact, A549\(^{COX-INHIB}\) cells
were co-cultured with HUVEC (EC) directly (cell-to-cell contact) or separated by means of an insert well, and incubated as in B. Results are expressed as pg/mL and data are the means ± SD of three independent experiments. ***, p<0.001 vs. untreated; **, p<0.01 vs. untreated; *, p<0.05 vs. untreated; #, p<0.01 vs. non-inhibited A549 cells; ##, p<0.001 vs. vehicle (ANOVA).

Figure 6.- SnCl₂ inhibits PGE₂ biosynthesis by HUVEC/A549 co-cultures treated with AA. A., HUVEC (EC) were serum-deprived and stimulated with IL-1β (10 ng/mL, 16h) before co-incubation with IL-1β-activated COX-inhibited A549 tumor cells (see legend to Figure 5). Untreated and IL-1β refer to pretreatments of both cell types. After 7h coculture, AA (25µM) was added for 30 min with or without SnCl₂ (200 µg/mL). PGE₂ was determined in cell supernatants by EIA. B., Re-scaled presentation of data in A showing PGE₂ release from single cultures of HUVEC. C., PGE₂ release from single cultures of COX-inhibited A549 cells incubated with AA and SnCl₂ as in A. Results are expressed as pg/mL and data are the means ± SD of three independent experiments. ***, p<0.001 vs. untreated; **, p<0.01 vs. untreated; ##, p<0.001 vs. IL-1β-treated; #, p<0.01 vs. IL-1β-treated.
SUPPLEMENTAL FIGURE LEGENDS

**Figure I.-Effects of VEGF and IL-1β on endothelial expression of prostanoid terminal synthases.** A,B., HUVEC were treated with 50 ng/mL VEGF (A) or 10 ng/mL IL-1β (B), and mRNA expression of prostanoid synthases was assessed by conventional PCR (upper panels) or real-time quantitative PCR (q-PCR; lower panels). C., HUVEC and A549 cells were treated with IL-1β (24 h) and mPGES-1 mRNA expression was analyzed by conventional PCR (upper panel) or q-PCR (lower panels). For conventional PCR (A,B,C, upper panels), a representative experiment of four performed is shown. Equal loading was confirmed by analysis of β-Actin expression. For q-PCR (A,B,C, lower panels), mRNA expression is expressed relative to that of untreated cells at time 0, after normalization to 18 S rRNA. Data are the means of two independent experiments in which each q-PCR was assessed at least twice in duplicate. ***, p<0.001 vs. untreated. (ANOVA).

**Figure II.- Positive control of HUVEC stimulation by VEGF, IL-1β, EGF or bFGF**

A., Western blot showing phospho-ERK-1/2 induction in whole cell lysates of HUVEC treated with VEGF (50 ng/mL, 10 min) or IL-1β (10 ng/mL, 30 min). Vertical lines indicate grouping of regions of the same gel from which irrelevant lanes have been deleted. A representative of four independent experiments is shown. Equal loading was confirmed by western blot for the cytosolic protein α-tubulin. B., Endothelial cells were challenged with VEGF (50 ng/mL, 2h) and COX-2 mRNA expression was evaluated by real-time quantitative PCR (q-PCR). For q-PCR, the levels of mRNA are expressed relative to that of untreated cells at time 0, after normalization to 18 S rRNA. Data are
the means of two independent experiments in which each q-PCR was assessed at least twice in duplicate. **, p<0.01 vs. untreated. (ANOVA).

**Figure III.-** Effects of IL-1β on endothelial expression of prostanoid terminal synthases. Immunoblot analysis of the expression of prostanoid biosynthetic enzymes in HUVEC treated with IL-1β (10ng/mL, 24h). α-tubulin was used as loading control.

**Figure IV.-** Effects of VEGF and IL-1β on cPLA₂ expression and enzymatic activity in endothelial cells. A., Western blot showing phospho-cPLA₂ (p-cPLA₂) induction in HUVEC treated for different times with IL-1β (10 ng/mL) (upper panels) or VEGF (50 ng/ml) (lower panels). Total cPLA₂ (cPLA₂) expression did not change with either treatment. Equal loading was confirmed by western blot for the cytosolic protein α-tubulin (TUB). B., Graphic shows cPLA₂ activity in lysates of endothelial cells disrupted after the indicated treatments. cPLA₂ activity is expressed in nmol/min/mL. C., Western blot showing total cPLA₂ (cPLA₂) expression in HUVEC treated for different times with IL-1β (10 ng/mL) or VEGF (50 ng/ml). COX-2 western blot was used as positive control for VEGF and IL-1β HUVEC stimulation (asterisk indicates specific band). Equal loading was confirmed by western blot for α-tubulin (TUB).

**Figure V.-** Specific knock-down of COX-1 and COX-2 protein expression and assessment of COX-1 activity in endothelial cells. HUVEC were transfected with siRNA (20nM) specific for COX-1 or COX-2. Control cells were transfected with scrambled siRNA. A., Western blots show appropriate suppression of COX-1 and COX-2 protein expression in cells treated with or without IL-1β (10 ng/mL) for 24h; a representative experiment of 4 is shown. The vertical lines indicate grouping of regions of the same gel from which irrelevant lanes have been deleted. Equal loading was
confirmed by western blot for α-tubulin. **B.**, Endothelial COX-1 activity was indexed by the PGE₂ produced from AA-treated lysates of COX-2-silenced endothelial cells pre-treated with or without IL-1β (10 ng/mL) for 24h, and Etoricoxib (10 μM) where indicated. A representative experiment out of 3 performed is shown. Results are expressed as pg per 1 x 10⁶ cells.

**Figure VI.- Comparison of tPGES enzymatic activities of HUVEC and A549 tumor cells.** **A.**, PGE₂ production from PGH₂ incubations in culture medium without cells or in the presence of HUVEC or A549 tumor cells treated with IL-1β for 48h. Results are expressed as ng per 6 x 10⁶ cells and data are the means ± SD of three independent experiments. ***, p<0.01 vs. No Cells. (Student’s t test). **B.**, Representative GC chromatograms showing products formed in PGH₂ incubations.

**Figure VII.- Specific knock-down of cPGES and mPGES-2 protein expression in endothelial cells by siRNA.** HUVEC were transfected with siRNA (200nM) specific for cPGES (sicPGES) or mPGES-2 (simPGES-2). Control cells were transfected with scrambled siRNA (siControl). **A.**, Western blots show appropriate suppression of cPGES (upper panel) and mPGES-2 (lower panel) protein expression in cells treated with or without IL-1β (10 ng/mL) for 24h. Loading control (LC) refers to α-tubulin (upper panel) or α-cPGES (lower panel). **B.**, PGE₂ levels were measured in culture supernatants of controls (siControl) and cPGES (sicPGES) or mPGES-2 (simPGES-2) silenced cells treated with IL-1β as indicated. Results are expressed as pg per 4 x 10⁵ cells.
A. VEGF (50 ng/mL)  
- cPGES  
- mPGES-2  
- PGiS  
- TXAS  
- β-Actin  

0 0.5 1 2 4 8 24 hours  

B. IL-1β (10 ng/mL)  
- cPGES  
- mPGES-2  
- PGiS  
- TXAS  
- β-Actin  

0 0.5 1 2 4 8 24 hours  

C.  
- HUVEC  
- A549  
- mPGES-1  
- β-Actin  

Relative mRNA expression  

HUVEC + IL-1β 24h  

mPGES-1  

Relative mRNA expression:  
- untreated  
- IL-1β 24h

***
Fig. II

A. 
untreated  VEGF 10' IL-1β 30'

B. 

<table>
<thead>
<tr>
<th></th>
<th>COX-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>untreated</td>
<td><img src="bar_graph_un-treated" alt="" /></td>
</tr>
<tr>
<td>VEGF 2h</td>
<td>![bar_graph_VEGF_2h]</td>
</tr>
</tbody>
</table>

Relative mRNA expression

- ![](bar_graph_un-treated)
- ![bar_graph_VEGF_2h]
Fig. III  IL-1β (10 ng/mL)

<table>
<thead>
<tr>
<th>Protein</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COX-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cPGES</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mPGES-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGIS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TXAS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Tubulin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. IV
Fig. V

A.  

Control siRNA  COX-1 siRNA  
IL 1β 24h  -  +  -  +  
COX-1  
COX-2  
α-Tubulin  

Control siRNA  COX-2 siRNA  
IL 1β 24h  -  +  -  +  
COX-2  
COX-1  
α-Tubulin  

B.  

PGE₂ (ng/10⁶ cells)  

siControl  siCOX-2+Etoricoxib  

untreated  IL-1β
Fig. VI
Fig. VII

A.

<table>
<thead>
<tr>
<th></th>
<th>Control siRNA</th>
<th>cPGES siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL 1β 24h</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>cPGES</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>LC</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

B.

Graph A: 
- siControl
- siPGES
- simPGES

Graph B: 
- IL 1β 24h

Bars: 
- PGE2 (pg/4x10^6 Cells)