Rate of Vasoconstrictor Prostanoids Released by Endothelial Cells Depends on Cyclooxygenase-2 Expression and Prostaglandin I Synthase Activity

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Abstract—This study was undertaken to investigate the enzymatic regulation of the biosynthesis of vasoconstrictor prostanoids by resting and interleukin (IL)-1β–stimulated human umbilical vein endothelial cells (HUVECs). Biosynthesis of eicosanoids in response to IL-1β, exogenous labeled arachidonic acid (AA), or histamine, as well as their spontaneous release, was evaluated by means of HPLC and RIA. HUVECs exposed to IL-1β produced prostaglandin (PG) I₂ for no longer than 30 seconds after the substrate was added irrespective of the cyclooxygenase (COX) activity, whereas the time course of PGE₂ and PGD₂ formation was parallel to the COX activity. The ratio of PGE₂ to PGD₂ produced by HUVECs was similar to that obtained by purified COX-1 and COX-2. Production of PGF₂α from exogenous AA was limited and similar in both resting and IL-1β–treated cells. PGF₂α was the main prostanoid released into the medium during exposure to IL-1β, whereas when HUVECs treated with IL-1β were stimulated with histamine or exogenous AA, PGE₂ was released in a higher quantity than PGF₂α. PGF₂α released into the medium during treatment with IL-1β and the biosynthesis of PGE₂ and PGD₂ in response to exogenous AA or histamine increased with COX-2 expression, whereas this did not occur in the case of PGI₂. We observed that PGI synthase (PGIS) mRNA levels were not modified by the exposure to IL-1β, but the enzyme was partially inactivated. When SnCl₂ was added to the incubation medium, the transformation of exogenous AA-derived PGH₂ into PGE₂ and PGD₂ was totally diverted toward PGF₂α. Overall, these results support the conclusions that PGE₂ and PGD₂ (and also probably PGF₂α) were nonenzymatically derived from PGH₂ in HUVECs. The concept that a high ratio of PGH₂ was released by the IL-1β–treated HUVECs and isomerized outside the cell into PGE₂ and PGD₂ was supported by the biosynthesis of thromboxane B₂ by COX-inactivated platelets, indicating the uptake by platelets of HUVEC-derived PGH₂. The IL-1β–induced increase in the release of PGH₂ by HUVECs was suppressed by the COX-2–selective inhibitor SC-58125 and correlated with both COX-2 expression and PGIS inactivation. An approach to the mechanism of inactivation of PGIS by the exposure to IL-1β was performed by using labeled endoperoxides as substrate. The involvement of HO in the PGIS inactivation was supported by the fact that deferoxamine, pyrrolidinedithiocarbamate, DMSO, mannitol, and captopril antagonized the effect of IL-1β on PGIS to different degrees. The NO synthase inhibitor N⁶-monomethyl-L-arginine also antagonized the PGIS inhibitory effect of IL-1β, indicating that NO⁻ was also involved. NO⁻ reacts with O₂⁻ to form peroxynitrite, which has been reported to inactivate PGIS. Homolytic fission of the O-O bond of peroxynitrite yields NO₂⁻ and HO⁻. The fact that 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), which reacts with NO to form NO₂⁻, dramatically potentiated the IL-1β effect suggests that NO⁻ could be a species implicated in the inactivation of PGIS. Cooperation of HO⁻ was supported by the fact that DMSO partially antagonized the effect of carboxy-PTIO. Although our results on the exact mechanism of the inactivation of PGIS caused by IL-1β were not conclusive, they strongly suggest that both NO⁻ and HO⁻ were involved. (Circ Res. 1998;83:353-365.)

Key Words: cyclooxygenase ⋅ prostanoid ⋅ endothelial cell

Endothelium modulates the response of vascular smooth muscle to hormones, neurotransmitters, and platelet products by releasing relaxing factors, such as NO and prostaglandin (PG) I₁ (also termed prostacyclin), and contracting factors, such as endothelin-1, angiotensin II, and vasoconstrictor metabolites of arachidonic acid (AA).¹⁻⁷ PGI₂, PGF₂α, and PGE₂ are the main eicosanoids detected in vitro in the incubates of endothelial cells under different experimental conditions, which include exogenously added AA and several agonists. PGD₂, 12-hydroxyeicosatetraenoic acid (HHT), 15-HETE, and 11-HETE have also been detected in minor amounts.⁸⁻¹¹ All these eicosanoids are derived from

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cyclooxygenase (COX) activity in human umbilical vein endothelial cells (HUVECs).^{12} PGH₂ is the common precursor of the prostanooids whose formation from AA is catalyzed by 2 COX isoenzymes encoded by different genes: COX-1 and COX-2. COX-1 is expressed in a constitutive manner, and COX-2 is the isozyme inducible by mitogens and is overexpressed in inflammatory processes.\(^{13,14}\) Interleukin (IL)-1, together with tumor necrosis factor, plays a pivotal role in the inflammatory response and orchestrates a pleiad of secondary events that orient endothelial cells toward a proinflammatory and prothrombotic function by inducing adhesion molecules, endothelin-1, and coagulation factors.\(^{15}\) A characteristic activity of IL-1 is to induce COX-2 in several cell types, including endothelial cells.\(^{16}–19\)

PGI synthase (PGIS) transforms PGH₂ into what is the most characteristic prostanooid formed by resting endothelial cells, PGF\(_2\)\(_\alpha\).\(^{8,20}\) PGI\(_1\) is the most potent platelet antiaggregatory agent, and it exhibits antiadhesive and smooth muscle–relaxing properties.\(^{2,21}\) PGH₂ also undergoes spontaneous or enzymatic transformation toward PGF\(_2\)\(_\alpha\), PGE\(_2\), and PGD\(_2\).\(^{2,22}\) A growing amount of data support the concept that untransformed PGH₂ is released in vivo by the vascular endothelium under different circumstances.\(^{24–30}\) The predominance of relaxing prostanooids, such as PGI\(_1\), or contracting prostanooids, such as PGH\(_2\) and PGF\(_2\)\(_\alpha\), released by endothelial cells will primarily depend on COX activity but also on the secondary pathways that yield the different prostanooids. PGI\(_1\) release as a consequence of the exposure of endothelial cells to IL-1 has been widely reported.\(^{16,19,21,31–33}\) Nevertheless, when COX activity is overexpressed, PGIS activity could be the limiting step in the biosynthesis of PGI\(_1\), and other prostanooids could be more stimulated by IL-1.\(^{33,36,37}\)

Although release of PGH₂ by endothelium both in vivo and ex vivo has been suggested, its regulation has not been systematically studied, and whether transformation of PGH₂ into PGF\(_2\)\(_\alpha\), PGE\(_2\), and PGD\(_2\) in endothelial cells is spontaneous or enzymatically catalyzed remains unclear. In fact, PGIS is the only enzyme implicated in prostanooid biosynthesis that has been found in endothelial cells in addition to COX. The objective of the present work was to evaluate the hypothesis that the release of vasoconstricting prostanooids is regulated by 2 enzyme activities, COX and PGIS, and that these prostanooids are released predominantly under inflammatory conditions, such as after exposure to IL-1.

**Materials and Methods**

**Cell Culture and Treatment**

Endothelial cells were isolated from human umbilical veins and cultured as described previously.\(^{42}\) When HUVECs cultured with 20% of FBS reached confluence, they were seeded into 6-well plates and maintained without heparin and endothelial cell growth factor for 48 hours before the addition (or not) of 10 U/mL human recombinant IL-1β (50 000 U/μg; purity, >98%; Boehringer Mannheim S.A.) in medium 199 containing 4% FBS and maintained for the indicated period of time until incubation with [\(^{14}\)C]AA or histamine.

Human dermal fibroblasts were isolated and cultured as described previously.\(^{17}\) Cells cultured in 6-well plates, in passages 4 to 6, were maintained with 1% FBS for 48 hours before the addition of 10 nmol/L phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich Química S.A.). After 6 hours, COX-2 inhibition experiments were performed as described below.

The permanent endothelial cell line HUV-EC-C was cultured and treated with 10 nmol/L PMA for 6 hours as described previously\(^{39}\) before COX-2 inhibition experiments. Experiments were performed with the HUV-EC-C line at passages 21 to 27.

Cells of the human erythroleukemia cell (HEL) line were obtained from the American Type Culture Collection (CRL 1730) and cultured in RPMI 1640 medium supplemented with 10% FBS, 1 mmol/L sodium pyruvate, 2 mmol/L l-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO\(_2\) in air and subcultured every 3 to 4 days. To study COX-1 activity, cells were used without further treatment.

**Preparation of Washed Platelet Suspensions**

Peripheral venous blood was drawn from healthy donors who had received no medication in the 2 weeks before extraction. Washed platelet suspensions were prepared as previously described.\(^{40}\) Platelet density was 2×10\(^9\) platelets/mL for the PGH₂ trapping experiments and 1.7×10\(^9\) platelets/mL for the study of COX-1 inhibition. COX-inactivated platelet suspensions were obtained by incubating platelet-rich plasma with 200 μmol/L acetylsalicylic acid (ASA) dissolved in ethanol (final concentration of ethanol, 0.5% [vol/vol]) for 10 minutes at room temperature before the washing procedure.

**Release of Prostanoids From Endogenous AA in Resting and IL-1β–Treated HUVECs**

Cells cultured in 6-well plates were incubated at 37°C in 2.5 mL medium 199 containing 4% FBS and 10 U/mL IL-1β. At the indicated periods of time, prostanooids released into the medium were evaluated after 250-μL aliquots of media were collected and placed in tubes containing indomethacin (final concentration, 10 μmol/L). Samples were kept at −80°C until analysis of PGF\(_2\)\(_\alpha\), PGE\(_2\), and 6-keto-PGF\(_1\)\(_\alpha\) by RIA (PGF\(_2\)\(_\alpha\) was from Amersham Ibérica; PGE\(_2\) and 6-keto-PGF\(_1\)\(_\alpha\) were from Advanced Magnetics Inc).

**Formation of Eicosanoids From Exogenous [\(^{14}\)C]AA and Determination of COX Activity**

After the indicated period of time of exposure to 10 U/mL IL-1β, cells were incubated at 37°C in 0.5 mL medium 199 containing 10 nmol/L HEPES, and 25 μmol/L [\(^{14}\)C]AA (55 to 58 mCi/mmol [1-\(^{14}\)C]AA, Amersham Ibérica) was added in 5 μL of ethanol. At the indicated periods of time, the reactions were stopped by adding 1N HCl to yield pH 3 followed by 1 vol of cold methanol. COX activity was evaluated as the sum of all the eicosanoids formed through the COX pathway.

PGH₂ is an unstable prostaglandin that is converted into PGF\(_2\)\(_\alpha\) by mild reducing agents, such as SnCl\(_2\).\(^{41}\) Therefore, to estimate the PGH₂ released, we calculated the difference between the PGF\(_2\)\(_\alpha\) peak of the samples from cells incubated as previously described and cells incubated in the presence of 200 μg/mL SnCl\(_2\). Samples were kept at −80°C until analysis. HPLC analysis of eicosanoids was performed as previously described.\(^{35,39}\)

**Release of Prostanoids From Endogenous AA in IL-1β–Treated HUVECs in Response to Histamine**

Cells untreated and treated with 10 U/mL IL-1β for the indicated periods of time were incubated at 37°C in the presence of 0.5 mL medium 199 containing 10 nmol/L HEPES and 50 μmol/L histamine. After 10 minutes, supernatants were removed and placed in liquid N\(_2\). Samples were then stored at −80°C until analysis of PGF\(_2\)\(_\alpha\), PGE\(_2\), and 6-keto-PGF\(_1\)\(_\alpha\) by RIA.

**COX-1–and COX-2–Specific mRNA Analysis**

COX-1–and COX-2–specific mRNA levels were determined as previously described.\(^{16,39}\)
PGIS mRNA Analysis

Total RNA was isolated by phenol chloroform extraction according to the protocol described by Chomczynski and Sacchi\(^{12}\) and quantified spectrophotometrically by absorption at 260 and 280 nm. The specific levels of PGIS mRNA were determined by means of a quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Briefly, 1 µg of total RNA was reverse-transcribed into cDNA in the presence of 50 U of reverse transcriptase (Perkin-Elmer) and 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 5 mmol/L MgCl\(_2\), 2.5 µmol/L random hexamers, 20 U of RNAsin (Genex RNA PCR kit, Perkin-Elmer), and 1 mmol/L deoxynucleotide triphosphates (Epiconet Technologies) in a final volume of 20 µL. The reaction was stopped by heating for 5 minutes at 99°C and then 5 minutes at 5°C.

The primers used for the amplification of the PGIS-specific cDNA were purchased from Oxford Biomedical Research, Inc. The cDNA for GAPDH was amplified and used as the internal control, and the sense and antisense primers for GAPDH were 5'-CACCCATGTGCAAAATTTCCATGGCA-3' and 5'-TCTAGAAGCAGGTCGCTGACC-3', respectively.\(^{38,39}\) The PCR was carried out in a DNA Thermal Cycler 480 (Perkin-Elmer) with a reaction mixture (100 µL) containing 100 µmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2.5 mmol/L MgCl\(_2\), 0.2 µmol/L deoxynucleotide triphosphates, 0.25 µmol/L for PGIS and 1 µmol/L for GAPDH sense or anti-sense primers, 2.5 U Taq polymerase (Perkin-Elmer), and 4 µCi of \[^3H\]deoxycytidine triphosphate (48 to 71 Ci/mmol, Amersham International). Aliquots were removed after 27, 30, and 35 cycles in order to test the linearity of the amplification. The profile of the amplification cycles was 94°C for 1 minute, 55°C for 2 minutes, and 72°C for 90 seconds. Amplification products were separated in a 1.5% low-melting-point agarose gel (GIBCO BRL) for 1 hour, and 72°C for 90 seconds. Amplification products were separated in a 1.5% low-melting-point agarose gel (GIBCO BRL) containing ethidium bromide (0.5 µg/mL) and visualized under UV light and cut with a circular template. Radioactivity associated to the specific band was determined as described previously\(^{38,39}\) and normalized with respect to GAPDH.

Western Blotting of COX-1 and COX-2

Lysates of control cells and HUVECs treated with IL-1β for the indicated period of time were prepared as previously described.\(^{19}\) Total protein equivalents of each sample were submitted to SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore). After blocking nonspecific binding of antibody, membranes were incubated with a specific polyclonal antibody, membranes were incubated with a specific polyclonal antibody for COX-1 and COX-2 (Oxford Biomedical Research). Positive controls were performed by using isolated COX-1 from ram seminal vesicles (Oxford Biomedical Research) or COX-2 purified from sheep placenta (Cayman Chemical). Detection was performed with a peroxidase-conjugated second antibody, and the membrane was developed with an ECL System (Amersham) according to the manufacturer’s instructions. To estimate the relative levels of each COX protein, the films were scanned in order to obtain digital images. The digital images of the bands were quantified, with the use of PC-Image analysis software (Foster Findlay Associates Ltd), by determining the sum of the gray density value of all pixels inside a rectangular area was obtained for each band. Results were then expressed as arbitrary units or normalized to the darkest band.

Trapping of HUVEC-Derived PGH\(_3\) by Platelets

HUVECs in 6-well plates, untreated and cultured with 10 U/mL IL-1β for 24 hours, were incubated at 37°C in 0.450 mL medium 199 containing 10 mmol/L HEPES and 25 µmol/L [\(^{14}\)C]AA. After 0.5 minutes, 10\(^6\) COX-inactivated platelets in 50 µL were added to the wells, and the reactions were stopped after another 9.5 minutes, as described previously. Inactivation of COX in the platelet suspensions was monitored previously by incubating 0.5-mL aliquots of the washed platelet suspensions with 25 µmol/L [\(^{14}\)C]AA at 37°C for 5 minutes.

Trapping of PGH\(_3\) from endogenous AA derived from resting and IL-1β-treated HUVECs by platelets was achieved by adding 50 µL of a platelet suspension containing 10\(^6\) COX-inactivated platelets to HUVECs 0.5 minutes after cell challenge with 50 µmol/L histamine. After 10 minutes of the addition of histamine, supernatants were removed and placed in liquid N\(_2\). Samples were then kept at −80°C until the analysis of 6-keto-PGF\(_{1α}\) and thromboxane (Tx) B\(_2\) by RIA (TxB\(_2\), Advanced Magnetics Inc).

Incubation With Isolated COX-1 and COX-2 and Obtaining of \[^14\]C-Labeled Endoperoxides

COX-1 (5 U/0.5 mL) isolated from ram seminal vesicles (Oxford Biomedical Research) or COX-2 purified from sheep placenta (Cayman Chemical) was incubated in 100 mmol/L Tris-HCl (pH 8.5) containing 2 mmol/L phenol and 5 µmol/L [\(^{14}\)C]AA. Samples were incubated at 37°C for 5 minutes, after which the reaction was stopped by adding 10 U/mL indomethacin by 1 mol cold methanol. The products were subjected to HPLC analysis.

For the experiments concerning PGIS inactivation, an incubation of 180 U/mL of isolated COX-1 with 25 µmol/L [\(^{14}\)C]AA at 30°C for 1 minute was used as a source of \[^14\]C-labeled endoperoxides. The reaction was stopped by adding an excess of indomethacin (final concentration, 10 µmol/L). In these conditions, the remaining untransformed [\(^{14}\)C]AA was <10%, and the percentage of nondenatured endoperoxides, evaluated by the difference of PGF\(_{2α}\) after addition of 1 mol methanol or 1 mol methanolic solution of 400 µg/mL SnCl\(_2\), was always >80%. Hence, the incubation mixture was immediately used as a source of labeled endoperoxides without further manipulation.

Effect of COX-2–Selective Inhibition on the Release of PGH\(_3\)

IL-1β-treated HUVECs expressed both COX-1 and COX-2 in all experimental conditions. Therefore, in order to determine the concentration of the selective COX-2 inhibitor SC-58125\(^{39,44}\) (Laboratorio Almirall-Prodesfarma) to be used with HUVECs, cultures of PMA-treated human dermal fibroblasts and the HUV-EC-C line were used for COX-2 tests, and cultures of the HEL line and fresh human platelets were used for COX-1 tests. Fibroblasts and HUV-EC-C attached in 6-well plates, a 0.5-mL aliquot of HEL suspension (5×10\(^5\) cells/mL), or a 0.15-mL platelet suspension were incubated at 37°C in the presence of the indicated concentration of SC-58125 for 5 minutes. [\(^{14}\)C]AA (25 µmol/L) was later added, and cells were incubated for another 5 minutes. Reactions were stopped, and COX-derived eicosanoids were analyzed as described above. COX activity was evaluated as the sum of all the chromatographic peaks corresponding to COX-derived eicosanoids.

As a result of these experiments, SC-58125 concentrations of 1 and 10 µmol/L were chosen to study the effect of selective inhibition of COX-2 on the release of PGH\(_3\) by HUVECs. Resting HUVECs and HUVECs treated with 10 U/mL IL-1β overnight were incubated with 1 and 10 µmol/L of SC-58125 for 5 minutes. HUVECs were then incubated in the presence of 25 µmol/L [\(^{14}\)C]AA alone or in the presence of 200 µg/mL SnCl\(_2\) for another 5 minutes as previously mentioned. The untransformed PGH\(_3\) was then estimated as described above.

Effect of IL-1β on the Inactivation of PGIS

To determine the PGIS activity after treatment of HUVECs with IL-1β, cells cultured in 6-well plates were treated with 10 U/mL IL-1β for 6 hours, without any additive or in the presence of final concentrations of the following: 1 mmol/L deferoxamine (Sigma), 1% (vol/vol) DMSO (Sigma), 1 mmol/L captopril (Laboratorios Cidfa), 200 µmol/L pyrrolidine-dithiocarbamate (PDTC, Sigma), 1 mmol/L 1,2,3-oxadiazolium, 5-amino-3-(4-morpholin)-chloride (Sin-1, Cayman), 100 µmol/L 2-(4-carboxyphenyl)-4,4,5,5-tetramethyldihydroazoline-1-oxyl-3-oxide (carboxy-PDTC, Cayman), 50 µmol/L superoxide dismutase (Sigma), 500 U/mL catalase (Sigma), 1 mmol/L N\(^\circ\) monomethyl-L-arginine (L-NMMA, Cayman), 5 mmol/L mannitol (Merck), or 100 µmol/L tyrosine (Sigma) (all dissolved in culture medium) or 0.5 mmol/L phenylbutazone, 10 µmol/L indomethacin, or 50 µg/mL vitamin E (all from Sigma) dissolved in ethanol (final ethanol concentration, 0.25% [vol/vol]). Parallel incubations of HUVECs with the scavengers and inhibitors in the absence of IL-1β were performed as controls. The culture
medium was then removed, and HUVECs were treated with 100 μmol/L ASA in medium 199 for 10 minutes at 37°C. The ASA-containing medium was removed, and 200 μL of the solution containing the 14C-labeled endoperoxides was added. HUVECs were allowed to stand at 37°C for another 5 minutes. The final concentration of 14C-labeled endoperoxides was ~18 μmol/L. 14C-labeled 6-keto-PGF1α was evaluated by HPLC as described. Resting (control) and IL-1β-treated HUVECs for each condition were always incubated with the same batch solution of 14C-labeled endoperoxides. Negative controls were performed using cells boiled for 15 minutes. Synthesis of NO in untreated and in IL-1β-treated cells was determined by assay of the culture supernatants for NO2− (nitrite) using the Griess reagent, which contains 1 vol of 10 g/L sulfanilamide in 50 mL/L H3PO4, and 1 vol of 1 g/L N-(1-naphthyl)ethylenediamine dihydrochloride in water (all from Sigma). An aliquot of 500 μL of the culture medium was reacted with an equal volume of the Griess reagent for 10 minutes at room temperature in the dark. The optical densities of the assay samples were measured at 550 nm. Concentrations of NO2− were determined from a standard curve prepared using NaNO2 (Sigma) diluted in culture medium.

Statistics
Sigma-Stat software was used for statistical analysis. Statistical significance between groups was assessed using the Student t test for 2 groups. One-way ANOVA and the Student-Newman-Keuls test were used to compare >2 groups. Correlations were evaluated by linear regression. A value of P<0.05 was considered significant.

Results
AA Metabolism in HUVECs
As expected, resting and IL-1β–treated HUVECs incubated with exogenous [14C]AA produced 6-keto-PGF1α, PGE2, and PGF2α as major eicosanoids, and PGD2, HHT, 15-HETE, and 11-HETE were minor compounds.

COX-1 and COX-2 Expression
The time course of mRNA and protein expression of COX-1 and COX-2 was measured by incubating HUVECs with or without 10 U/mL of IL-1β during various time intervals. Specific mRNAs encoding for both COX-1 and COX-2 were induced by IL-1β in a time-dependent manner (Figure 1), although only the increase of COX-2 was statistically significant (P<0.05). The levels of COX-1 mRNA were slightly enhanced, whereas for COX-2 they were enhanced 12.5-fold by IL-1β. The maximum levels of COX-2 mRNA were observed between 3 and 6 hours of exposure to IL-1β.

The Western blot analysis of the COX isoforms present in HUVECs is shown in Figure 2. In the absence of IL-1β, the antibody against COX-1 recognized a band in HUVEC samples of ~70 kDa corresponding to the migration of purified COX-1 from ram seminal vesicles. The antibody against COX-2 did not detect the purified COX-1 from ram seminal vesicles and had a slight reaction with a protein band of the unstimulated cells. In contrast, after 1 hour of IL-1β treatment, the antibody against COX-2 peptide recognized a protein band in HUVEC samples of ~70 kDa corresponding to the migration of purified COX-2 from sheep placenta. The densitometric analysis of the bands showed that after 3 hours, IL-1β significantly increased the expression of COX-2 in a time-dependent manner, whereas modifications in the protein band corresponding to COX-1 over time were minimal. COX-2 expression reached a maximum level between 6 and 9 hours of IL-1β exposure, followed by a slight decrease at 24 hours. Figure 2 shows that the total COX activity also increased in a time-dependent manner with the IL-1β treatment, although it was only statistically significant after 6 hours of exposure to the cytokine. It was notable that whereas COX-2 protein levels decreased after 24 hours of exposure to IL-1β, the total activity continuously increased until 24 hours.

Evaluation of Release of Prostanoids by HUVECs in Response to IL-1β
Release of prostanoids by resting and 10 U/mL IL-1β–treated cells was determined by monitoring the most significant
prostanoids over 24 hours: PGE\textsubscript{2}, PGF\textsubscript{2\alpha}, and 6-keto-PGF\textsubscript{1\alpha}.

Results in Figure 3 show that after 24 hours, treatment with IL-1\beta caused a higher increment in the quantity of PGF\textsubscript{2\alpha} (\approx 20-fold) and PGE\textsubscript{2} (\approx 16.5-fold) than of 6-keto-PGF\textsubscript{1\alpha} (\approx 3.5-fold) accumulated in the medium compared with no treatment. Levels of 6-keto-PGF\textsubscript{1\alpha} in the medium were abruptly enhanced 1 hour after exposure of cells to IL-1\beta, whereas PGE\textsubscript{2} and PGF\textsubscript{2\alpha} were released into the medium more slowly, following the pattern of COX-2 expression. We should point out that 6-keto-PGF\textsubscript{1\alpha} was the only compound whose accumulated quantity in the culture medium was significantly enhanced (\approx 7-fold) 1 hour after exposure to IL-1\beta, when COX-2 protein and activity was not enhanced (Figure 2). Release of PGE\textsubscript{2}, PGF\textsubscript{2\alpha}, and 6-keto-PGF\textsubscript{1\alpha} by control cells was quantitatively slight, and the 3 progression curves had similar patterns.

### Biosynthesis of Eicosanoids From Exogenous AA

To observe the differences concerning synthesis of the different eicosanoids between those cells that overexpress COX-2 and resting cells, progression curves after the addition of labeled substrate were evaluated, and results are shown in Figure 4. The maximum levels of 6-keto-PGF\textsubscript{1\alpha} and PGF\textsubscript{2\alpha} were similar in both resting and IL-1\beta–stimulated HUVECs (10 U/mL IL-1\beta for 24 hours). Nevertheless, the amount of PGI\textsubscript{2} increased to a maximum 10 minutes after substrate addition in resting HUVECs, whereas the synthesis of PGI\textsubscript{2} practically ceased 1 minute after addition in the cells treated with IL-1\beta. Furthermore, the amount of 6-keto-PGF\textsubscript{1\alpha} produced after 10 minutes by IL-1\beta–treated cells was significantly lower than that produced by control cells. HHT was produced in a greater amount in IL-1\beta–treated than in resting HUVECs, whereas the synthesis of PGI\textsubscript{2} practically ceased 1 minute after addition in the cells treated with IL-1\beta. Furthermore, the amount of 6-keto-PGF\textsubscript{1\alpha} produced after 10 minutes by IL-1\beta–treated cells was significantly lower than that produced by control cells. HHT was produced in a greater amount in IL-1\beta–treated than in resting HUVECs. However, production stopped 1 minute after incubation, and the progression curve showed a pattern similar to that of 6-keto-PGF\textsubscript{1\alpha}. In contrast, an increasingly higher production of PGE\textsubscript{2}, PGD\textsubscript{2}, and HETEs was observed in IL-1\beta–treated cells compared with control cells (Figure 4).
Effect of IL-1β on the Expression of PGIS

Results presented in Figure 4 suggest that IL-1β does not exert any effect on PGIS expression. To confirm this, PGIS mRNA levels were determined by RT-PCR, and results are shown in Figure 5. Results from 4 experiments indicated that PGIS mRNA was not increased in HUVECs exposed overnight to IL-1β compared with control cells.

Characterization of the Biosynthetic Pathway of PGE_2 and PGD_2 in HUVECs

The slow time course of PGE_2, PGD_2, and HETE formation and the fact that they were the only eicosanoids that increased as a consequence of the induction of the COX-2 expression when an excess of exogenous substrate was supplied suggested that all of these eicosanoids could be formed in HUVECs by the exclusive intervention of COX. To verify that transformation of PGH_2 into PGE_2 and PGD_2 was not enzymatic, 2 different experimental approaches were carried out.

Figure 6 shows representative chromatograms corresponding to samples of HUVECs and isolated COX incubated with exogenous [1^4C]AA. Isolated COX-1 and COX-2 yield a ratio of PGE_2 to PGD_2 of 4.2±0.8 and 4.4±1.2 (mean±SD, n=5), respectively, similar to the ratio obtained in samples from HUVECs (3.9±0.99) (n=10). No 6-keto-PGF_1α was detected in the isolated COX incubations. In addition, the presence of SnCl_2 in the incubations of HUVECs with [1^4C]AA caused a complete diversion of the transformation of HUVEC-derived PGH_2 toward PGF_2α instead of toward PGE_2 plus PGD_2 (Figure 6).

Comparison Between the Metabolism of Exogenous and Endogenous AA as a Function of Time of Incubation With IL-1β

After treatment of HUVECs with 10 U/mL IL-1β for different periods of time, cells were incubated in the presence of
25 μmol/L [14C]AA or 50 μmol/L histamine. Histamine-induced release of endogenous AA and the formation of the major PGs were evaluated by RIA. Results in Figure 7 show some differences between the effect of IL-1β on the metabolism of exogenous and endogenous AA. When substrate was supplied exogenously, we observed a slight reduction in the ability of HUVECs to form PGI₂ (evaluated as 6-keto-PGF₁α) as a function of time of incubation with IL-1β. In contrast, the ability to form PGE₂ and PGD₂ increased with the time of exposure to the cytokine and correlated with COX-2 expression. Whereas formation of PGI₂ and PGF₂α from endogenous substrate increased suddenly after 1 hour of incubation with IL-1β and a sustained ability to form these PGs was then observed, statistically significant correlations were observed only between the increment in COX-2 protein expression and the increment in PGE₂ and PGD₂ formation from exogenous substrate and between the increment in COX-2 protein expression and the increment in PGE₂ formation in response to histamine (formation of PGD₂ from endogenous substrate was not determined; Figure 7).

**Effect of IL-1β on the Release of Untransformed PGH₂ by HUVECs From Exogenous and Endogenous Substrate**

The amount of untransformed PGH₂ released by HUVECs from exogenous [14C]AA was estimated as the difference in the labeled PGF₂α between the samples incubated in the presence and absence of SnCl₂. After any period of time exposure to IL-1β, the formation of 6-keto-PGF₁α and HETEs from exogenous substrate was only minimally modified by the presence of SnCl₂ in the medium. In contrast, PGE₂ and PGD₂ completely disappeared in the samples containing SnCl₂, and the levels of PGF₂α increased concomitantly. HHT levels were also substantially reduced in the presence of SnCl₂ but were not totally suppressed (not shown). Figure 8 shows the estimated release of PGH₂ and PGI₂ (evaluated as 6-keto-PGF₁α) relative to the resting cells as a function of time of incubation with IL-1β. The increment in the release of PGH₂ into the medium was statistically correlated with the increase in COX-2 protein and with the decrement in PGI₂ production (Figure 8). The ratio of PGH₂ to PGI₂ increased ~4-fold as a consequence of treatment with IL-1β.

PGE₂, PGF₂α and 6-keto-PGF₁α released from endogenous sources in HUVECs stimulated with histamine were also evaluated, after several periods of time of exposure to IL-1β, both in the absence and presence of SnCl₂. Formation of 6-keto-PGF₁α was lower in the presence of SnCl₂ than in its absence (Figure 9), in contrast with results from the experiments adding exogenous [14C]AA. As expected, PGE₂ levels were substantially lower in the presence of SnCl₂ but were not totally suppressed. In addition, the production of PGF₂α was similar both in the absence and in the presence of SnCl₂ in particular when the activity of COX was maximum. Data from exogenous substrate experiments indicated that SnCl₂ did not modify COX and PGIS activities. Hence, the lower levels of prostanoids observed in the presence of SnCl₂ should be due to a less
effective release of AA. This led to a decreased formation of PGH\textsubscript{2} and therefore a decreased production of PGI\textsubscript{2}. The less effective mobilization of endogenous AA would also explain why PGF\textsubscript{2}\textalpha did not increase as much as expected as a consequence of the diversion of the PGH\textsubscript{2} toward formation of PGF\textsubscript{2}\textalpha instead of PGE\textsubscript{2} and PGD\textsubscript{2}. Hence, although the results indicated that untransformed PGH\textsubscript{2} was also released from endogenous AA, the estimation of the amount of PGH\textsubscript{2} released was not possible when this indirect method was used.

To evaluate the untransformed PGH\textsubscript{2} released by HUVECs from endogenous sources, PGH\textsubscript{2} was trapped by ASA-treated platelets, and its transformation by Tx synthase was evaluated. First, platelets treated with 200 \textmu mol/L ASA were added to the wells of HUVECs and incubated in the presence of [\textsuperscript{14}C]AA. ASA-treated platelets did not produce any eicosanoid derived from COX activity. Preliminary experiments served to choose the appropriate time to add the platelet suspensions to the HUVECs. COX-inactivated platelets were added to the HUVECs treated with IL-1\beta at increasing periods of time after the addition of 25 \textmu mol/L [\textsuperscript{14}C]AA to the HUVECs. The maximum conversion of labeled PGH\textsubscript{2} into labeled TxB\textsubscript{2} and HHT was observed when 10\textsuperscript{8} platelets were added to HUVECs 0.5 minutes later than the addition of [\textsuperscript{14}C]AA (not shown). Therefore, a delay of 0.5 minutes between the addition of [\textsuperscript{14}C]AA and platelet suspensions was chosen for the following experiments.

Results in the Table show that the presence of platelets in the incubation mixture did not modify the amount of PGI\textsubscript{2} produced by HUVECs from endogenous or exogenous AA. ASA-treated platelets trapped HUVEC-derived PGH\textsubscript{2} from both exogenous and endogenous AA. HHT in platelets is formed by the action of Tx synthase. The ratio of HHT to TxB\textsubscript{2} formed in our conditions was evaluated by incubating suspensions of platelets untreated with ASA with 25 \textmu mol/L [\textsuperscript{14}C]AA and determining both compounds by HPLC. This ratio was 1.47±0.18 (mean±SD, n=5), which allowed us to estimate the amount of PGH\textsubscript{2} trapped by ASA-treated platelets in the experiments using histamine. The estimated amounts of PGH\textsubscript{2} trapped by platelets from HUVECs incubated with [\textsuperscript{14}C]AA and those incubated with 50 \textmu mol/L histamine were comparable, indicating a substantial release of PGH\textsubscript{2} from endogenous AA.
Effect of the Selective Inhibition of COX-2 on the Release of PGH$_2$

To select the most suitable concentration of the selective inhibitor in our experimental system, concentration-dependent inhibition curves on both COX-1 and COX-2 whole-cell systems were obtained by using human platelets and HEL line suspensions for COX-1 and the HUV-EC-C line and human dermal fibroblasts treated with PMA for COX-2. The $[^{14}C]$AA concentration used was the same as that in HUVEC experiments. The COX activity in these systems was measured as the sum of all COX-derived compounds. As a result of these experiments, we chose 1 and 10 μmol/L SC-58125, since at these concentrations, inhibition of COX-2 was high and COX-1 was not substantially inhibited (see Figure 10). As expected, the inhibition of the release of PGH$_2$ evaluated using the SnCl$_2$ indirect method was consistent with a major COX-2 origin.

Inactivation of PGIS During Incubation of HUVECs With IL-1β

After treatment of HUVECs with 10 U/mL IL-1β for 6 hours, cells were incubated with $[^{14}C]$AA for 10 minutes. Non-detectable levels of 6-keto-PGF$_1$α in the unstimulated control cells were increased to detectable levels in the stimulated cells (Figure 11). These results clearly indicate that PGIS was partially inactivated during the treatment with IL-1β.

To approach the mechanism of inactivation of PGIS in response to IL-1β, cells were incubated with several radical scavengers and inhibitors during exposure to IL-1β. Vitamin E and extracellular addition of catalase and superoxide dismutase did not exert any effect. In contrast, the Fe$^{3+}$-chelating agent deferoxamine and the free radical scavenger carboxy-PTIO dramatically increased the PGIS inhibitory effect of IL-1β. The effect of carboxy-PTIO was significantly prevented by the NO synthase L-NMMA but not by L-tyrosine. This was consistent with a significant 1.5-fold increase of the NO$_2^-$ levels in the culture medium of IL-1β–treated cells compared with control cells ($492.3 \pm 117$ and $320.5 \pm 63.3$ pmol/10$^6$ cells for 6 hours, respectively; mean±SD, n=6, P<0.05). In contrast, the presence the NO scavenger carboxy-PTIO dramatically increased the PGIS inhibitory effect of IL-1β.
In 1978, Marcus et al. suggested that PGE₂ and PGD₂ could be formed nonenzymatically in HUVECs. Their suggestion was based on the observation that PGH₁ incubated in the absence of cells yielded a mixture of all the prostanoids except PGI₁. Nevertheless, COX-2 had not yet been discovered at that time. We have now extended this research in order to verify the hypothesis that endothelial cells express only 2 enzymes that are involved in prostanoid biosynthesis, COX and PGIS, and that when COX-2 is overexpressed, accumulated PGH₁ is effectively released outside the cell.

Our results indicate that HUVECs do not transform PGH₃ into PGE₂ and PGD₂ enzymatically. This concept is supported by the following: (1) Progression curves indicate that the formation of PGE₂ and PGD₂ is substantially slower than that of the other prostanoids and overlap those of 15-HETE and 11-HETE, whose formation does not require later enzymatic steps. (2) The ratio of PGE₂ to PGD₂ formed by HUVECs from exogenous substrate was almost identical to that yielded by isolated COX. (3) The presence of SnCl₂ totally diverted exogenous AA-derived PGH₁ toward PGF₂α, instead of PGE₂ and PGD₂.

In agreement with other reports, we observed that release of PGI₁ by HUVECs was increased in cells exposed to IL-1β compared with resting cells. However, the release of PGF₂α and PGE₂ was substantially more induced by IL-1β than was the release of PGI₁. The mobilization of AA from cell lipids induced by IL-1β could account for the fact that the cytokine-induced release of PGI₁ occurred (Figure 3) before an appreciable increase in the COX expression and activity (Figure 2). In contrast, a slow PGF₂α and PGD₂ accumulation in the medium was observed as COX-2 increased. This indicated that a COX-2–dependent excess of PGH₁, which was not transformed into PGH₂, was formed during the incubation with IL-1β. On the other hand, the ability of cells to form PGE₂ and PGD₂ from exogenous AA was greatly enhanced with the expression of COX-2 (Figure 7). The same phenomenon was observed when mobilization of endogenous AA was induced by histamine; the ability to synthesize PGE₂ (in this case PGD₂ was not measured) also correlated with COX-2 expression (Figure 7). This was consistent with the data reported by Bull et al., who found that PGF₂α was the most enhanced eicosanoid in response to histamine after pretreatment of dermal microvascular endothelial cells with IL-1β. The fact that the increased ability to form PGI₁ in response to histamine was already observed after 1 hour of IL-1β-exposure, when COX activity was not appreciably induced, suggests that IL-1β exerted priming in the histamine-induced AA mobilization.

Since biosynthesis of PGE₂ and PGD₂ are nonenzymatic in HUVECs, PGH₁ could be transformed into a mixture of these position isomers outside the cell. The ratio of untransformed [¹⁴C]AA-derived PGH₁ to PGI₁ released by HUVECs as a function of time of exposure to IL-1β overlapped with the increase in the COX activity and COX-2 expression. This indicates that the presence of COX-2 causes a dramatic increase in the ratio of PGH₁ to PGI₁ released by HUVECs when exogenous substrate is supplied. The fact that the COX-2–selective inhibitor SC-58125 inhibited, in a concentration-dependent manner, the release of PGH₁ at concentrations that did not appreciably inhibit COX-1 also supports this conclusion (Figure 10). A substantial release of PGH₁ formed from endogenous AA could be demonstrated by trapping with ASA-treated platelets. In contrast to that which occurred in the exogenous substrate experiments, SnCl₂ was unable to completely suppress PGE₂ formation when IL-1β–treated cells were stimulated with histamine. This may be due to the endogenous and exogenous AA having access to the COX located in different intracellular compartments. PGH₁ generated from endogenous sources could be partially converted nonenzymatically into PGE₂ inside the cell.

The formation of PGF₂α by 2-electron reduction of PGH₁ could occur nonenzymatically inside or partially outside the cells. Our results are not conclusive with respect to these points. Nevertheless, unlike 6-keto-PGF₁α, formation of PGF₂α showed a slow progression curve consistent with a nonenzymatic reduction of PGH₁. In contrast with that which
occurred with PGE₂ and PGD₂. PGF₂α did not increase with the COX activity when an excess of exogenous substrate was supplied. This was consistent with an enzymatic and/or a electron donor–dependent synthesis. These facts taken together strongly suggest that formation of PGF₂α in endothelial cells is limited by the presence of some cell-derived electron donor rather than by an enzymatic activity posterior to COX. The cellular nature of the electron donor is suggested by the fact that the relative quantity of PGF₂α formed in the incubations with isolated COX was negligible. We can speculate that a slow turnover of such an electron donor(s) could account for the predominant formation of PGF₂α when the slow release of prostanooids caused by the presence of IL-1β was measured (Figure 3). In contrast, when an excess of exogenous AA was supplied, or a high quantity of AA was suddenly released by the action of histamine, a great amount of PGH₂ was rapidly formed. As a consequence, this factor(s) could be rapidly depleted and finally exhausted, thus limiting the transformation of PGH₂ into PGF₂α. Under such conditions, PGE₂ and PGD₂ would be the predominant nonenzymatic formations of PGH₂ (Figures 4 and 7).

These considerations lead to the idea that COX and PGIS are the only enzymes involved in the biosynthesis of prostanooids in endothelial cells. Moreover, progression curves indicate that PGE₂, PGD₂, and PGF₂α were formed in detectable amounts, mainly when PGIS became inactive, which occurred faster in the IL-1β–treated cells than in the resting HUVECs (Figure 4). The faster inactivation of PGIS under COX-stimulated conditions yielding an excess of PGH₂ is consistent with the “suicide” behavior of PGIS.48 The fact that the levels of mRNA for PGIS were not induced by IL-1β indicates that the only enzyme activity regulated at the transcriptional level was COX, particularly COX-2. This was consistent with the fact that the PGI₂ that formed when an excess of exogenous substrate was supplied did not increase according to the increment of COX-2 protein and activity. Moreover, PGI₂ formation ability was reduced as a function according to the increment of COX-2 protein and activity.

Moreover, PGI2 formation ability was reduced as a function of COX-2 expression but also with PGIS inactivation (Figure 4 and 7). These authors proposed an O-O homolytic cleavage in the peroxynitrite molecule caused by the heme iron of the PGIS. This could finally yield NO₂⁻· nitrosating species, which may attack tyrosine or tyrosinate.60 Nevertheless, nitration of tyrosyl residues by NO₂⁻ is possible through the formation of tyrosyl radicals,61,62 which could be favored by HO·. Our results from carboxy-PTIO experiments are consistent with an inactivation of the PGIS mediated by NO₂⁻. The fact that the presence of DMSO in addition of carboxy-PTIO decreased the ratio of inactivated PGIS, when compared with carboxy-PTIO alone, suggests that HO· was also involved. Consistently, another mechanism by which deferoxamine could prevent the effect of IL-1β on PGIS inactivation may be the direct reaction with peroxynitrite.59 We also observed that SIN-1, which releases both NO· and O₂⁻ as a source of peroxynitrite, inactivated PGIS in both resting and IL-1β–treated HUVECs. In summary, our results about the exact mechanism of the partial inactivation of PGIS caused by the exposure of HUVECs to IL-1β are not conclusive, but they strongly suggest that both NO· (which could generate peroxynitrite that undergoes homolytic cleavage of the O-O bond to yield NO₂⁻) and HO· were involved.

Evidence that the HO· radical was involved in the inactivation of PGIS was provided by the following: (1) The use of DMSO, PDTC, mannitol, and captopril, powerful HO· scavengers,59-64 partially or totally antagonized the effect of IL-1β. (2) Iron-dependent HO· formation from O₂⁻·-generating systems such as Fenton or Haber-Weiss reactions was inhibited by deferoxamine, a powerful Fe³⁺ chelator,69 which also blocked the inactivation of PGIS induced by IL-1β. (3) α-Tocopherol did not antagonize the IL-1β–induced inactivation of PGIS, since α-tocopherol did not react efficiently with HO·. Catalase and superoxide dismutase added to the extracellular phase did not exert any effect on the inactivation of PGIS, probably because of the fast intracellular generation of HO· from H₂O₂ generated by dismutation of O₂· or from homolytic fission of ONOOH, with the extracellular presence of these enzymes being not effective.

Peroxidase activity as a source of free radicals has been reported to be involved in the inactivation of PGIS.64 Deferoxamine and PDTC are also inhibitors of the peroxidase activity of COX.55 Since most of the COX inhibitors do not exert any effect on peroxidase activity,55 the fact that indomethacin and phenylbutazone partially (but without statistical significance) inhibited the inactivation of PGIS by IL-1β would indicate that PGG₁ contributed to the production of the inactivating oxygen-derived free radicals.

Another explanation has been given by Zou and Ullrich,56 who found that peroxynitrite irreversibly inhibits PGIS. Our results from L-NMMA experiments, which inhibited inactivation of PGIS by IL-1β, are consistent with those of these authors, who suggested that NO· formation is the limiting step in the peroxynitrite production in endothelial cells and may be induced by the inflammatory cytokines. Actually, exposure to IL-1β caused a significant 1.5-fold increase in the NO·-released by HUVECs in terms of NO₂⁻, although we did not evaluate the expression of the inducible NO synthase. The fact that the NO· scavenger carboxy-PTIO57 significantly increased the inhibitory effect of IL-1β in the present study was in apparent contradiction with this. Nevertheless, carboxy-PTIO equimolarly reacts with NO· to yield 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl plus NO₂⁻·. Homolytic fission of peroxynitrous acid, formed by protonation of peroxynitrite, yields NO₂⁻ and HO·.58,59 Zou et al60 recently reported that nitration of an active site–related tyrosine could account for the PGIS inactivation produced by peroxynitrite. These authors proposed an O-O homolitic cleavage in the peroxynitrite molecule caused by the heme iron of the PGIS. This could finally yield NO₂⁻·-nitrating species, which may attack tyrosine or tyrosinate.60 Nevertheless, nitration of tyrosyl residues by NO₂⁻ is also possible through the formation of tyrosyl radicals,61,62 which could be favored by HO·. Our results from carboxy-PTIO experiments are consistent with an inactivation of the PGIS mediated by NO₂⁻. The fact that the presence of DMSO in addition of carboxy-PTIO decreased the ratio of inactivated PGIS, when compared with carboxy-PTIO alone, suggests that HO· was also involved.
Present results provide a biochemical explanation for the production and release of the constrictive prostanoids, PGH₂ and PGF₂α, by endothelial cells. Predominance of relaxing or constricting prostanoids will depend on both COX and PGIS activities. An impairment of endothelial cell function occurs under inflammatory conditions, and COX activity is increased by means of the induction of COX-2 and by partial inactivation of PGIS. Under such conditions, endothelial cells could produce an excess of PGH₂ that could exert proinflammatory and prothrombotic activities before its conversion to other prostaglandins inside and outside the endothelium. PGH₂ from endothelial cells enriched in COX-2 could also be converted into TxA₂ by platelets and macrophages. MicrovesSEL expression of COX-2 could contribute to the platelet activation observed in the disseminated intravascular coagulation, usually associated with systemic inflammatory responses. By use of an antagonist of the PGH₁/TxA₂ receptor, it has been shown that endogenous PGH₁ decreases the thrombolytic activity of tissue plasminogen activator. 61 PGH₂ and TxA₂ stimulate proliferation of vascular smooth muscle cells in vitro. 62–66 Expression of COX-2 could also contribute to the myointimal hyperplasia observed in atherosclerosis by mediating the formation of PGH₂ (and transcellular TxA₂) and hydroxy linoleic acids, 19 which elicit smooth muscle cell mitogenesis. 67

In summary, our results show that resting HUVECs and HUVECs exposed to IL-1β produce only PGH₂ and PGI₂ enzymatically. Production of PGF₂α could apparently be linked to the presence of a limiting intracellular electron donor. Overexpression of COX-2 turns the endothelial cell toward a phenotype characterized by an increased production of PGH₁, which cannot be totally transformed into PGI₂ partly because of its inactivation. Despite the fact that caution and more research is required to assess the biological relevance of our findings in vivo, the present data increase our knowledge about the metabolism of AA by endothelial cells and about the possible biological role of the induction of COX-2 in the vascular endothelium.

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