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Roles of Cyclooxygenase (COX)-1 and COX-2 in Prostanoid Production by Human Endothelial Cells: Selective Up-Regulation of Prostacyclin Synthesis by COX-2[1]

Gillian E. Caughey,[2]* Leslie G. Cleland,* Peter S. Penglis,* Jennifer R. Gamble,† and Michael J. James*

The two cyclooxygenase (COX) isoforms, COX-1 and COX-2, both metabolize arachidonic acid to PGH_2, the common substrate for thromboxane A_2 (TXA_2), prostacyclin (PGI_2), and PGE_2 synthesis. We characterized the synthesis of these prostanoids in HUVECs in relation to COX-1 and COX-2 activity. Untreated HUVEC expressed only COX-1, whereas addition of IL-1β caused induction of COX-2. TXA_2 was the predominant COX-1-derived product, and TXA_2 synthesis changed little with up-regulation of COX-2 by IL-1β (2-fold increase). By contrast, COX-2 up-regulation was associated with large increases in the synthesis of PGI_2 and PGE_2 (54- and 84-fold increases, respectively). Addition of the selective COX-2 inhibitor, NS-398, almost completely abolished PGI_2 and PGE_2 synthesis, but had little effect on TXA_2 synthesis. The up-regulation of COX-2 by IL-1β was accompanied by specific up-regulation of PGI synthase and PGE synthase, but not TX synthase. An examination of the substrate concentration dependencies showed that the pathway of TXA_2 synthesis was saturated at a 20-fold lower arachidonic acid concentration than that for PGI_2 and PGE_2 synthesis. In conclusion, endothelial prostanoid synthesis appears to be differentially regulated by the induction of COX-2. The apparent PGI_2 and PGE_2 linkage with COX-2 activity may be explained by a temporal increase in total COX activity, together with selective up-regulation of PGI synthase and PGE synthase, and different kinetic characteristics of the terminal synthases. These findings have particular importance with regard to the potential for cardiovascular consequences of COX-2 inhibition. *The Journal of Immunology,* **2001, 167: 2831–2838.**

The prostanoids prostacyclin (PGI_2)[3] and thromboxane A_2 (TXA_2) play an essential role in the maintenance of vascular homeostasis. PGI_2 is a vasodilator and an inhibitor of platelet aggregation, whereas TXA_2 is a vasoconstrictor and a promoter of platelet aggregation (1). As a consequence of their opposing roles, an imbalance in PGI_2 or TXA_2 production has been implicated in the pathophysiology of many thrombotic and cardiovascular disorders (1–3). Therefore, it is important to understand factors and conditions that might affect the balance of PGI_2/TXA_2 synthesis.

PGI_2 and TXA_2 are products of arachidonic acid (AA) metabolism by cyclooxygenase (COX), followed by metabolism of the COX product, PGH_2, by the terminal synthase enzymes, prostacyclin or TX synthase, respectively. Two isoforms of COX have been identified: COX-1 is expressed constitutively in most cell types, whereas COX-2 is induced by inflammatory stimuli such as bacterial endotoxin and cytokines. Also, several different prostanoid terminal synthases can be present within the one cell, and it is not known what determines the relative rate of production of each individual prostanoid within the same cell. In monocytic cells, it has been observed that the ratio of PGE_2/TXA_2 produced is not fixed, but varies according to which COX isoform is present. For example, in rat peritoneal macrophages, under conditions in which only COX-1 was expressed, TXA_2 was synthesized in excess of PGE_2. However, under conditions of stimulation in which COX-2 was induced, the profile of prostanoid production shifted to favor PGE_2 over TXA_2 production (4, 5).

It is considered that PGI_2 is the main prostanoid synthesized by vascular endothelium and TXA_2 is the main prostanoid produced by platelets. However, the endothelium has been reported to synthesize TXA_2 in addition to PGI_2 (6), and both COX isoforms have been observed, with only COX-1 being detectable in unstimulated cells (6, 7). Endothelial COX-2 can be up-regulated in vitro by inflammatory stimuli (6, 8) and shear stress (7, 9). Because the balance between PGI_2 and TXA_2 production is central in the maintenance of vascular tone and platelet aggregation, determination of the roles of endothelial COX isozymes, particularly with regard to the contribution of COX-2 in the regulation of prostanoid biosynthesis by the endothelium, is important.

In the current study, we examined the synthesis of prostanoids derived from either COX-1 or COX-2 by HUVECs. We observed that TXA_2 is the predominant COX-1 product, whereas up-regulation of COX-2 by IL-1β is associated with a greater increase in the synthesis of PGI_2 and PGE_2 than TXA_2. Both PGI synthase and PGE synthase, but not TX synthase, were up-regulated by IL-1β. Additionally, an examination of the substrate concentration dependencies of PGI_2, PGE_2, and TXA_2 synthesis suggests that different kinetic parameters of the terminal synthases are a major

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[3] Abbreviations used in this paper: PGI_2, prostacyclin; TX, thromboxane; AA, arachidonic acid; ASA, acetylsalicylic acid; COX, cyclooxygenase; MAPK, mitogen-activated protein kinase.

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determinant of the dominance of PGI_2 and PGE_2 production when COX-2 was induced by IL-1β.

Materials and Methods

Materials

Human rIL-1β was from Genzyme (Cambridge, MA). AA, NS-398 (*N*-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide, rabbit polyclonal Ab against human COX-2, and murine mAb against COX-1 were all purchased from Cayman Chemicals (Ann Arbor, MI). Peroxidase-labeled donkey anti-rabbit and goat anti-mouse Abs, ECL Western blotting system, [^{3}H]PGE_2, [^{3}H]6-keto $PGF_{1\alpha}$, and [^{3}H]TXB_2 were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). PGH_2 and (4-(4-fluorophenyl)-2-(4-methylsulfinyl phenyl)-5-(4-pyridyl)1*H*-imidazole) (SB 203580) were obtained from Calbiochem (San Diego, CA). Abs to the phosphorylated forms of p38 and p44/42 mitogen-activated protein kinases (MAPKs) and the mitogen-activated protein/extracellular signal-related kinase 1 inhibitor PD98059 were purchased from New England Biolabs (Beverly, MA). Rabbit anti-serum against PGE_2 and 6-keto $PGF_{1\alpha}$ and mouse mAb against β-actin were obtained from Sigma (St. Louis, MO).

Cell culture

HUVECs were isolated as described (10). The cells were cultured on gelatin-coated culture flasks in medium M199 with Earle's salts supplemented with 20% FCS, 25 μg/ml endothelial growth supplement (Genome Therapeutics, Waltham, MA), and 25 μg/ml heparin. Cells between passages 2 and 4 were plated in 24-well dishes (1.5×10^5/ml) and allowed to reach confluence (24 h).

Cell stimulation

HUVECs were incubated with RPMI 1640 medium (containing 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml gentamicin), supplemented with 10% heat-inactivated FCS either in the presence or absence of IL-1β (1 ng/ml, 37°C). For short-term stimulation (15 min) with either AA or PGH_2, cells were incubated in serum-free RPMI 1640 medium. To inhibit COX-1 activity, untreated cells were pretreated with aspirin (acetylsalicylic acid (ASA); 10 μg/ml) for 30 min, followed by two washes (11), and then incubated in the appropriate medium with the test agents, according to the specified experiment. Other inhibitors were added 15 min before stimulation. Following the appropriate treatment, cell supernatants were collected and stored at −20°C until analysis for prostanoid measurement by RIA.

Prostanoid measurement

TXB_2, 6-keto $PGF_{1\alpha}$ (the stable hydrolysis products of TXA_2 and PGI_2, respectively), and PGE_2 were measured by RIA using commercially available reagents, except for the TXB_2 antiserum, which was prepared as described previously (12).

Western blotting

Cell lysates were prepared by treating cells with ice-cold lysis buffer (HEPES-buffered HBSS, pH 7.4, 0.5% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin) and sample buffer (0.125 M Trizma base, pH 6.8, 20% glycerol, 4% SDS, 10% 2-ME), followed by 6 min, 95°C before storing at −20°C. Proteins were separated by 9% SDS-PAGE and then transferred onto a Sequi-Blot polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). After blocking the membranes with 5% fat-free dried milk in TBS (25 mM Tris-HCl, 0.2 M NaCl, 0.15% Tween 20, pH 7.6), they were incubated with the appropriate primary Abs, followed by HRP-conjugated donkey anti-rabbit or sheep anti-mouse Ab. Equivalent protein loading and transfer efficiency were verified by staining for β-actin. Bound Abs were revealed with ECL reagent, according to the manufacturer's protocol.

Statistical analysis

Results are expressed as mean ± SEM of triplicate incubations. Statistical significance was examined by Student's *t* test, using $p < 0.05$ as the significance level.

Results

Effect of COX-2 induction on the synthesis of individual prostanoids

COX-2 was not detectable in unstimulated HUVECs (Fig. 1) and remained undetectable in the absence of IL-1β over the 24-h time period examined (data not shown). Treatment of HUVECs with IL-1β (1 ng/ml) for increasing times resulted in expression of COX-2, which was maximal by 16 h (Fig. 1). By comparison, COX-1 was expressed in unstimulated cells, and its expression did not change with IL-1β treatment (Fig. 1). A time course of prostanoid production by HUVECs in response to IL-1β treatment indicated very different synthesis profiles between TXA_2 and PGI_2 or PGE_2. TXA_2 synthesis was evident at the earliest time point of 2 h, when COX-1 was the only isozyme detectable. As COX-2 was induced, there was a modest increase in TXA_2 production by ~2-fold. Even in the absence of IL-1β stimulation, TXA_2 synthesis increased with time (albeit less than IL-1β-stimulated HUVECs) (Fig. 2*A*). By comparison, in the absence of IL-1β stimulation, synthesis of PGI_2 or PGE_2 was not detectable. Treatment of HUVECs with IL-1β resulted in little or no PGI_2 and PGE_2 synthesis up to 4 h, but with the induction of COX-2 by IL-1β, the production of these prostanoids increased by 54- and 84-fold, respectively (production at 2 h compared with that at 24 h) (Fig. 2, *B* and *C*). To determine more specifically the IL-1β-induced changes in synthetic capacity of each prostanoid pathway, HUVECs were treated with IL-1β at time intervals of 0, 8, and 24 h, washed, and then stimulated with AA (10 μM, 10 min, 37°C). This type of examination ensures constant substrate concentration. The results under these conditions were similar to those obtained with measurement of prostanoid accumulation, as described above. TXA_2 was the predominant prostanoid to be synthesized by untreated HUVECs (i.e., time 0), with PGI_2 and PGE_2 being minor products (Table I). At this time, COX-1 was the only isozyme detectable (Fig. 1). With the induction of COX-2 by IL-1β, production of TXA_2 increased by ~2-fold at 20 h, whereas PGI_2 and PGE_2 synthesis increased by 31.6- and 39.3-fold, respectively (Table I). Therefore, it appears that TXA_2 is the major COX-1-derived product, but the induction of COX-2 results in a preferential increase in PGI_2 and PGE_2 synthesis.

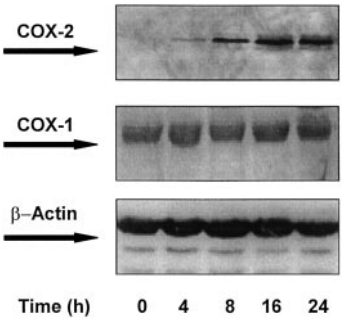

FIGURE 1. Time course of COX-2 protein induction by IL-1β. HUVECs (4.5×10^5) were stimulated with IL-1β (1 ng/ml) at time 0. At the indicated times, cells were processed for Western blot analysis, as described in *Materials and Methods*. Results are representative of at least five separate experiments.

Effect of specific inhibition of COX-1 or COX-2 on HUVEC prostanoid synthesis

To investigate the contributions of the COX isotypes to endogenously derived prostanoid synthesis, selective inhibition of COX-1 and COX-2 activities was required. We have previously documented that transient pretreatment of unstimulated monocytes with aspirin (ASA) results in irreversible inhibition of COX-1 activity, with no effect on activity or induction of COX-2 (11). Inhibition of COX-1 activity by ASA resulted in significant inhibition of TXA_2 synthesis by IL-1β-treated HUVECs (42%), but had

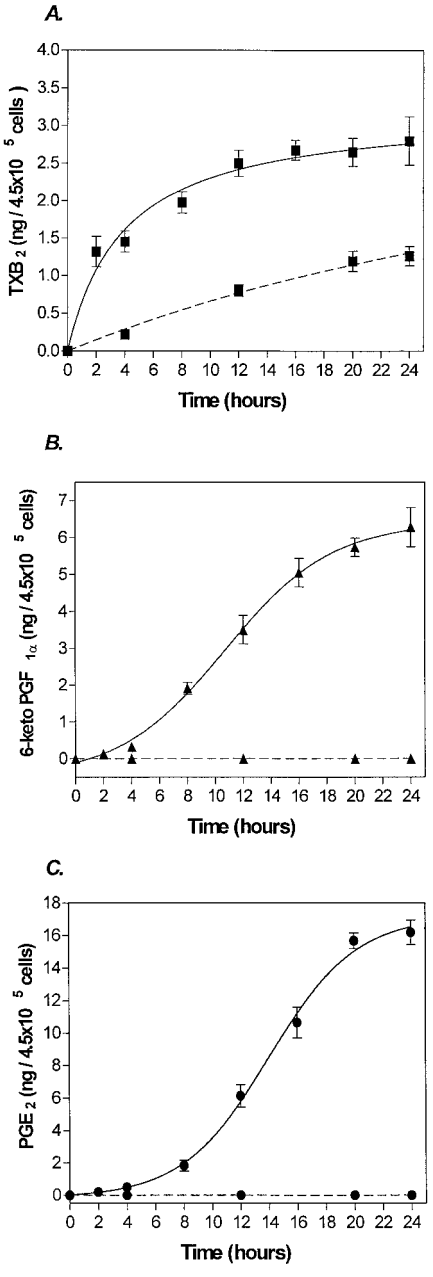

FIGURE 2. Time course of prostanoid synthesis in response to IL-1β treatment. HUVECs (4.5×10^5) were incubated in the presence (solid line) or absence (dashed line) of IL-1β (1 ng/ml) at time 0. At the indicated times, cell supernatants were collected for prostanoid measurement by RIA, as described in *Materials and Methods*. Results are representative of at least five separate experiments.

Table II. *Effect of selective inhibition of COX-1 or COX-2 activity on IL-1β-stimulated prostanoid synthesis*[a]

Treatments	Prostanoid Production (ng/4.5 × 10^5 HUVECs)		
	TXB_2	6-keto $PGF_{1\alpha}$	PGE_2
IL-1β	2.34 ± 0.16	6.28 ± 0.38	8.08 ± 0.82
IL-1β + ASA-wash	1.35 ± 0.28*	5.71 ± 0.84	8.21 ± 0.77
IL-1β + NS-398	1.75 ± 0.36*	0.56 ± 0.35*	0.61 ± 0.26*

[a] HUVECs (4.5×10^5) were preincubated in the presence or absence of aspirin (10 μg/ml) for 30 min followed by two washes (ASA-wash). Cells were then incubated with IL-1β (1 ng/ml, 37°C) with or without NS-398 (0.5 μM) for 20 h. Cell supernatants were collected for prostanoid measurement by RIA; results presented are mean ± SEM of triplicate incubations.

*, $p < 0.05$, by comparison to IL-1β-treatment only. Representative results from at least five separate experiments are shown.

no effect on either PGI_2 or PGE_2 production (Table II). By comparison, treatment with the selective COX-2 inhibitor, NS-398, resulted in almost complete inhibition of IL-1β-induced PGI_2 and PGE_2 synthesis (91% and 92.5%, respectively), but only slightly reduced TXA_2 production (Table II).

The effect of specific COX inhibition under conditions of constant substrate concentrations was also examined. Following the 20-h incubation in the presence or absence of IL-1β, cells were then washed and incubated with 10 μM AA for 15 min. In untreated cells, inhibition of COX-1 activity resulted in significant inhibition of synthesis of TXA_2 (82%), the predominant COX-1-derived prostanoid synthesized by HUVECs (Fig. 3*A*). Inhibition of COX-1 activity by ASA pretreatment had no significant effect on the synthesis of PGI_2 or PGE_2, but TXA_2 levels were inhibited by 35% (Fig. 3). By contrast, selective inhibition of COX-2 activity by NS-398 (0.5 μM) resulted in almost complete inhibition of PGI_2 and PGE_2 production (Fig. 3, *B* and *C*), but synthesis of TXA_2 was inhibited by only 23% (Fig. 3*A*). Neither ASA nor NS-398 affected the amount of COX-2 protein expressed in response to IL-1β (Fig. 4). These data suggest that while the majority of TXA_2 synthesis is COX-1 dependent, COX-2 can contribute to the synthesis of TXA_2 by IL-1β-treated HUVECs. By comparison, synthesis of both PGI_2 and PGE_2 appears to be predominantly COX-2 derived.

Regulation of terminal synthases by IL-1β

It is possible that the preferential up-regulation of endothelial PGI_2 and PGE_2 synthesis by IL-1β may have resulted from increased amount or activity of PGI synthase and PGE synthase, respectively. To examine this possibility, we studied the effects of IL-1β on the conversion of exogenous PGH_2 to TXA_2, PGI_2, and PGE_2 by the respective terminal synthases. Addition of PGH_2 (10 μM) to untreated HUVECs resulted in production of all prostanoids being examined. Treatment of the cells with IL-1β (1 ng/ml) for

Table I. *Effect of exogenous AA on prostanoid synthesis by HUVECs*[a]

Time (h)	Prostanoid Production (ng/4.5 × 10^5 HUVECs)		
	TXB_2	6-keto $PGF_{1\alpha}$	PGE_2
0	1.68 ± 0.13	0.40 ± 0.03	0.36 ± 0.02
8	2.18 ± 0.23 (1.3)[b]	4.94 ± 0.38 (12.4)	4.13 ± 0.42 (11.5)
20	3.24 ± 0.28 (1.9)	12.65 ± 1.21 (31.6)	14.13 ± 0.47 (39.3)

[a] HUVECs (4.5×10^5) were treated with IL-1β (1 ng/ml, 37°C) for 0, 8, or 20 h, washed, and incubated in serum-free medium, and AA was added (10 μM, 15 min, 37°C). Prostanoids were measured by RIA; results presented are mean ± SEM of triplicate incubations.

[b] In parentheses fold increase over values at 0 time. Representative results from at least three separate experiments are shown.

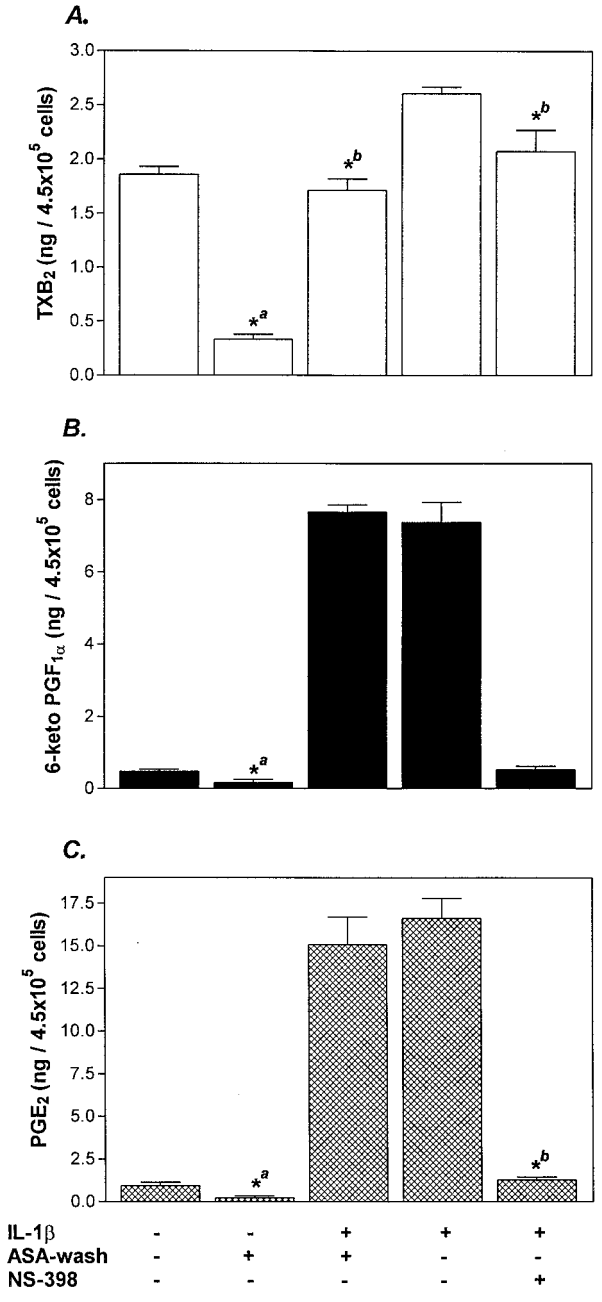

FIGURE 3. Effect of specific inhibition of COX-1 or COX-2 activity on prostanoid synthesis. *A*, TXB_2; *B*, 6-keto $PGF_{1\alpha}$; *C*, PGE_2. HUVECs (4.5×10^5) were preincubated in the presence or absence of aspirin (10 μg/ml) for 30 min, followed by two washes (ASA-wash). Cells were then incubated in either the presence or absence of IL-1β (1 ng/ml) with or without NS-398 (0.5 μM) for 20 h. After this time, cells were washed once and then incubated in serum-free medium with 10 μM AA (15 min, 37°C). When NS-398 was present during the 20-h incubation with IL-1β, it was added also for the 15-min incubation with AA. Cell supernatants were then collected for prostanoid measurement by RIA, as described in *Materials and Methods*. $*^a$, $p < 0.05$, by comparison with untreated cells. $*^b$, $p < 0.05$, by comparison with IL-1β-treated cells. Results are representative of at least five separate experiments.

20 h, before addition of PGH_2, did not alter the production of TXA_2, but synthesis of PGI_2 and PGE_2 significantly increased by ~10- and 6-fold, respectively (Fig. 5). These results indicate that treatment of HUVECs with IL-1β results in increased COX-2 expression and increased expression of PGI synthase and PGE synthase, but not TX synthase.

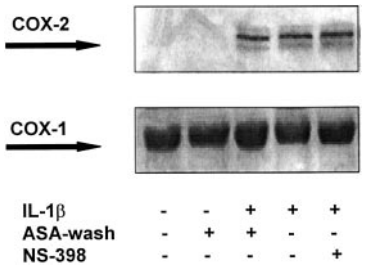

FIGURE 4. Effect of specific inhibition of COX-1 or COX-2 activity on COX-1 and COX-2 protein levels. HUVECs (4.5×10^5) were preincubated in the presence or absence of aspirin (10 μg/ml) for 30 min, followed by two washes (ASA-wash). Cells were then incubated in the presence or absence of IL-1β (1 ng/ml) or NS-398 (0.5 μM) for 20 h. Cells were then processed for Western blot analysis, as described in *Materials and Methods*. Results are representative of four separate experiments.

Kinetic activities of the terminal synthases

We have reported recently that the kinetic properties of the terminal synthases are an important determinant of prostanoid production in human monocytes (13). Therefore, we examined the kinetic properties of TX synthase, PGI synthase, and PGE synthase in HUVECs, in response to increasing substrate (AA) availability. Addition of exogenous AA at doses up to 5 μM to untreated HUVECs, i.e., only COX-1 present, resulted in a dose-dependent increase in TXA_2 production. However, no further increases in TXA_2 production were observed with higher doses of AA (10–100 μM), suggesting saturation of TX synthase or COX-1 at 5 μM AA (Fig. 6*A*). By contrast, both PGI_2 and PGE_2 synthesis increased dose dependently with increasing AA concentrations, up to at least 50 μM. This indicates that COX-1 is not saturated at 5 μM AA (Fig. 6*A*). The concentration of AA required to achieve half-maximal stimulation of TXB_2 synthesis was 1.2 μM compared with PGI_2 and PGE_2, which were 21.9 and 23.6 μM, respectively. Furthermore, at substrate concentrations up to 5 μM, the apparent rate constant of TX synthase was greater than that for PGI or PGE synthase. Similar dose responses to AA were observed in IL-1β-stimulated endothelial cells, in which COX-2 was induced (Fig. 6*B*). TXA_2 production increased dose dependently at doses of AA up to 10 μM, after which no further increases in TXA_2 production were observed. However, synthesis of PGI_2 and PGE_2 increased dose dependently with increasing AA concentrations, up to at least 50 μM. The concentration of AA required to achieve half-maximal

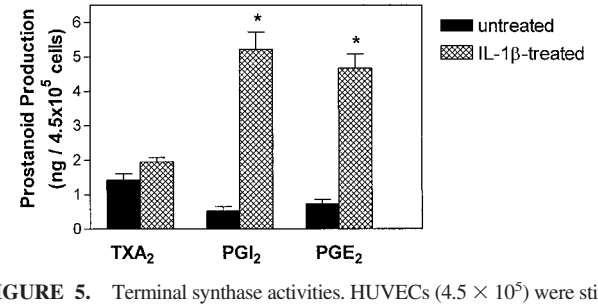

FIGURE 5. Terminal synthase activities. HUVECs (4.5×10^5) were stimulated in the presence or absence of IL-1β (1 ng/ml) for 20 h. Cells were then washed once and incubated with PGH_2 (10 μM) for 15 min, at 37°C in serum-free medium. Cell supernatants were then collected for prostanoid measurement by RIA, as described in *Materials and Methods*. Control incubations were performed in the absence of cells, and no prostanoid production was detectable. Solid bars, untreated HUVECs. Hatched bars, IL-1β-treated HUVECs. *, $p < 0.05$, by comparison with untreated HUVECs. Results presented are representative of three separate experiments.

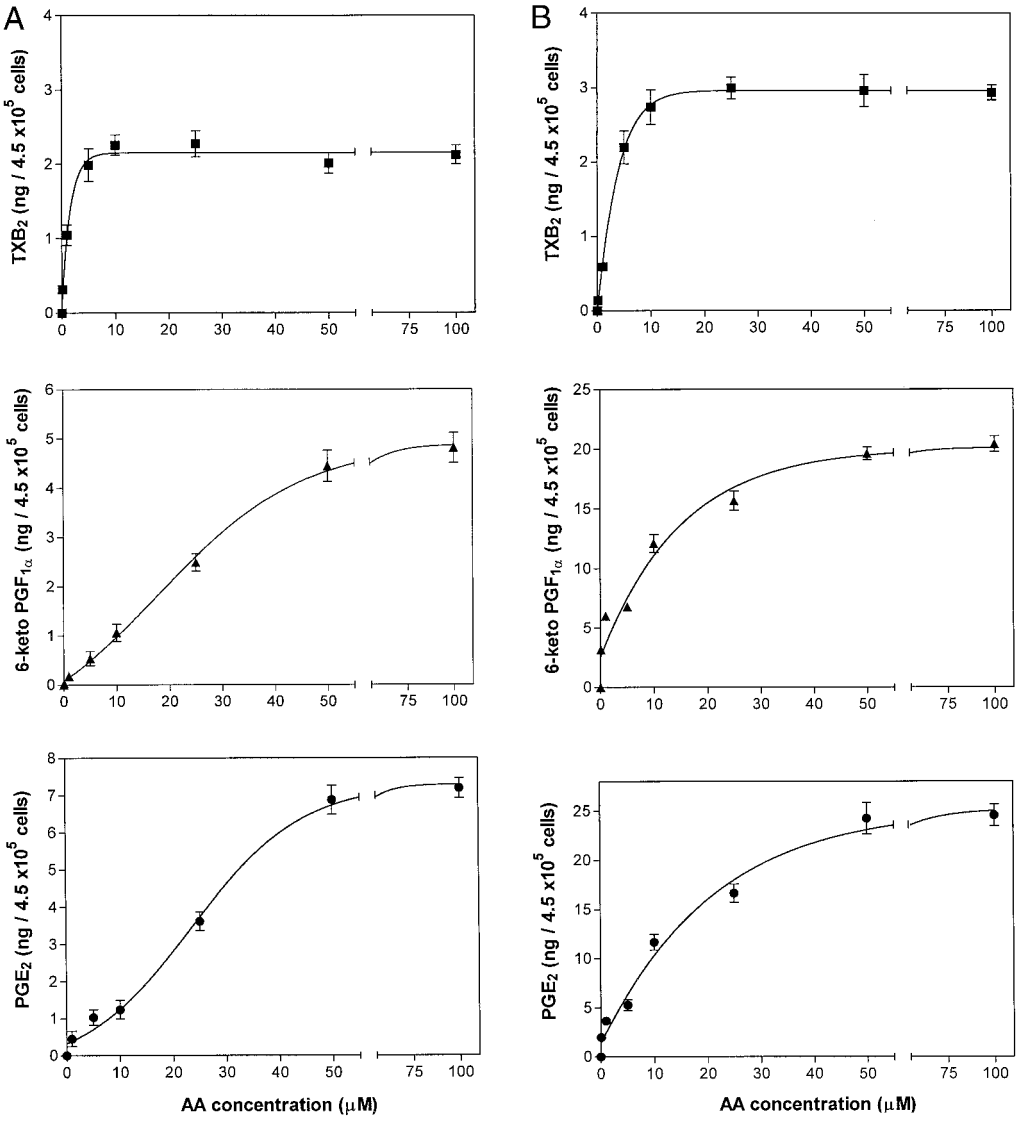

FIGURE 6. Dose response with exogenous AA. Untreated HUVECs (4.5×10^5) (*A*) or IL-1β-treated HUVECs (4.5×10^5) (*B*) were cultured in serum-free medium in the presence of increasing concentrations of AA (0–100 μM) for 15 min, 37°C. Cell supernatants were then collected for prostanoid measurement by RIA, as described in *Materials and Methods*.

TXA_2 synthesis was 2.6 μM, which was considerably less than that required for half-maximal production of PGI_2 (8.5 μM) and PGE_2 (13.2 μM).

Regulation of COX-2 induction by p38 and p44/42 MAPKs

The MAPK cascade is one of the major signaling pathways leading from cellular activation to gene transcription. Induction of COX-2 has been reported to be mediated by both the p38 and p44/42 MAPK pathways in various cell types, in response to either LPS or cytokine stimulation (14–16). Therefore, we examined the effect of IL-1β addition (1 ng/ml) on p38 and p44/42 activation in HUVECs. As shown in Fig. 7, IL-1β induced phosphorylation of both p38 and p44/42 MAPK in a time-dependent manner. Activation of p38 MAPK peaked at 15 min after exposure to IL-1β, and maximal activation of p44/42 MAPK was observed 30 min post-IL-1β treatment. In the absence of IL-1β, there was no detectable phosphorylation of either p38 or p44/42 MAPK (data not shown).

Although it appears that IL-1β can activate both the p38 and p44/42 MAPK pathways in HUVECs, we wanted to establish the potential roles of these MAPKs in the induction of COX-2 expression by HUVECs in response to IL-1β. Addition of SB 203580 (p38 MAPK inhibitor) or PD 98059 (inhibitor of mitogen-activated protein/extracellular signal-related kinase 1 activation) had no effect on prostanoid synthesis by untreated HUVEC (data not shown). However, addition of either SB 203580 or PD 98059 to IL-1β-treated HUVECs resulted in significant inhibition of both PGI_2 and PGE_2 synthesis (Table III). By comparison, addition of

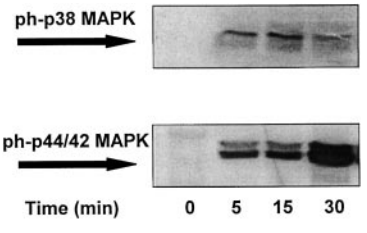

FIGURE 7. Time courses of p38 and p44/42 MAPK activation by IL-1β. HUVECs (4.5×10^5) were incubated with IL-1β (1 ng/ml) for 0, 5, 15, and 30 min. Cells were then processed for Western blot analysis, as described in *Materials and Methods*. Results are representative of three separate experiments.

Table III. *Effect of inhibition of p38 and p44/42 MAPK pathways on prostanoid production*[a]

Treatments	Prostanoid Production (ng/4.5 × 10^5 HUVECs)		
	TXB_2	6-keto $PGF_{1\alpha}$	PGE_2
Untreated	2.74 ± 0.14*	0.87 ± 0.20*	0.45 ± 0.18*
IL-1β	3.81 ± 0.34	9.06 ± 1.63	12.2 ± 1.41
IL-1β, SB 203580 (1 μM)	3.24 ± 0.16	0.41 ± 0.16*	0.49 ± 0.27*
IL-1β, PD 98059 (5 μM)	3.66 ± 0.25	1.75 ± 0.19*	2.19 ± 0.18*

[a] HUVECs (4.5 × 10^5) were treated with IL-1β (1 ng/ml, 20 h, 37°C) in the presence or absence of the p38 (SB 203580; 1 μM) or p44/42 (PD 98059; 5 μM) MAPK inhibitors. Cells were then washed and incubated in serum-free medium, and AA was added (10 μM, 15 min, 37°C). Prostanoids were measured by RIA; results presented are mean ± SEM of triplicate incubations.

*, $p < 0.05$, by comparison to IL-1β treatment only. Representative results from five separate experiments are shown.

either of these inhibitors had no effect on TXA_2 production (Table III). Recently, doubts have been raised concerning the selectivity of the MAPK inhibitors SB 203580 and PD 98059. In particular, they have been reported to interfere directly with AA metabolism via the COX pathway in platelets (17). However, we did not observe inhibition of COX-1-derived prostanoids, namely TXA_2, indicating that at the concentrations used, neither compound inhibits COX-1 or TX synthase activities. Western blot analysis demonstrated that both SB 203580 and PD 98059 inhibited IL-1β-induced COX-2 induction (Fig. 8). Thus, IL-1β appears to up-regulate COX-2 expression in endothelial cells through a mechanism involving both the p38 MAPK and the p44/42 MAPK pathways. The p38 MAPK pathway has been reported to be involved in the regulation of COX-2 mRNA stability (16, 18), while the p44/42 MAPK has been shown to regulate COX-2 at the transcriptional level (16). Whether this specificity of COX-2 regulation by these MAPKs occurs in our cell system is yet to be elucidated.

Discussion

Up-regulation of COX-2 in a cell contributes to the total cellular COX activity. It would be anticipated that this would result in a general and uniform increase in prostanoid synthesis. However, in the present study, we demonstrated that the profile of prostanoid synthesis in HUVECs is dependent on the specific isotype of COX that was present. In unstimulated HUVECs, which express COX-1, but no detectable COX-2, TXA_2 was the predominant prostanoid synthesized, with both PGI_2 and PGE_2 being relatively minor products. When COX-2 was induced by IL-1β, the synthesis of PGI_2 and PGE_2 increased substantially, whereas only a modest increase in TXA_2 production was observed. These differential changes in prostanoid synthesis were observed when production arising from either endogenous or exogenous AA was measured.

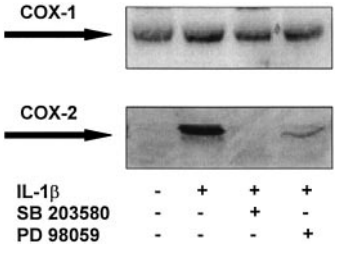

FIGURE 8. Effect of p38 or p44/42 MAPK inhibition on IL-1β-stimulated COX-2 induction. HUVECs (4.5 × 10^5) were preincubated for 15 min with either the p38 MAPK inhibitor (SB 203580; 1 μM) or the p44/42 MAPK inhibitor (PD 98059; 5 μM), followed by addition of IL-1β (1 ng/ml) and further incubation for 20 h at 37°C. Cells were then processed for Western blot analysis as described in *Materials and Methods*. Results are representative of five separate experiments.

Selective inhibition of either COX-1 or COX-2 supported the apparent dependencies of TXA_2 synthesis of COX-1 and PGI_2 and PGE_2 synthesis on COX-2 in endothelial cells.

Similar associations have been observed in rat peritoneal macrophages. COX-1 was linked with TXA_2 production, whereas the induction of COX-2 by LPS shifted prostanoid synthesis to favor PGE_2 (5, 19) and PGI_2 synthesis (5). Differences in the subcellular distributions of COX-1 and COX-2 were proposed as an explanation for the different prostanoid synthesis profiles associated with the different COX isozymes (5). However, this is unlikely, as COX-1 and COX-2 are reported to be located within the same subcellular locations (20).

Both PGI synthase and PGE synthase have been shown to be inducible enzymes. Expression of PGI synthase was increased by shear stress in HUVECs (7), and inflammatory stimuli have been reported to up-regulate PGE synthase activity in rat peritoneal macrophages and A549 cell line (19, 21, 22). With regard to PGE synthase, a cytosolic constitutive and a membrane-associated inducible form have recently been identified (21, 23). Based on coexpression studies in transfected HEK293 cells, cytosolic constitutive PGE synthase and membrane-associated inducible PGE synthase are reported to be functionally linked with COX-1 or COX-2, respectively (21, 23). In the present study, up-regulation of endothelial COX-2 was accompanied by specific up-regulation of the terminal synthases, PGI synthase and PGE synthase, but not TX synthase. Although this may explain in part the selective increase in PGI_2 and PGE_2 production with cell stimulation, other factors appear to be involved. Synthesis of PGI_2 and PGE_2 increased by 50- to 80-fold with endogenous AA, or 31- to 39-fold with exogenous AA, whereas PGI and PGE synthase activities increased only by ~6- to 10-fold. To account for the magnitude of the increased ratios of PGI_2/TXA_2 and PGE_2/TXA_2 with COX-2 induction, we propose that different kinetic characteristics of the terminal synthases may be involved also. Examination of increasing concentrations of substrate in either untreated or IL-1β-treated cells demonstrated that at the lower doses of AA ($\leq$10 μM), TXA_2 synthesis exceeded that of PGI_2 and PGE_2, suggesting that TX synthase has a higher rate constant than that for PGI and PGE synthase. However, at doses of AA >10 μM, synthesis of TXA_2 did not increase, whereas synthesis of both PGI_2 and PGE_2 increased. Because production of PGI_2 and PGE_2 was responsive to doses of AA >10 μM and up to 50 μM, this demonstrates that COX was not saturated. Therefore, the lack of responsiveness of TXA_2 synthesis indicates saturation of TX synthase at AA concentrations <10 μM. These results are in accordance with the differences in K_m values reported for TX and PGE synthase in human monocytes, which were 1 and 17 μM, respectively (13). Consideration of the kinetic characteristics of the terminal synthases allows an explanation of findings without invoking linkage

of COX isotypes with terminal synthases in different subcellular locations. Thus, it is proposed that under conditions of low total COX activity, as observed when COX-1 only is present, TXA_2 production predominates due to a higher rate constant of TX synthase. Upon cell stimulation, total COX activity increases due to COX-2 induction, TX synthase becomes rapidly saturated with PGH_2, whereas PGI and PGE synthases respond to the increased COX activity with increased synthesis of PGI_2 and PGE_2. In this explanation, the linkages between COX-1 and TXA_2 synthesis and between COX-2 and PGI_2/PGE_2 are apparent linkages only. Thus, a major determinant of increased PGI_2 and PGE_2 production over TXA_2 by IL-1β treatment is increased total COX activity in combination with different kinetic characteristics of the terminal synthases. The selective increase in PGI synthase and PGE synthase activity further augments the increases in PGI_2 and PGE_2 synthesis over that of TXA_2.

This study indicates that the role of endothelial COX-2 induction in vascular homeostasis is important due to its action of altering the ratio of prostanoids from a prothrombotic (high TXA_2/PGI_2) to an antithrombotic (high PGI_2/TXA_2) mixture. Although the induction of COX-2 appears to be important in many physiological processes, the induction of COX-2 has generally been associated with production of deleterious prostanoids due to the involvement of COX-2 in inflammatory disorders such as rheumatoid arthritis and osteoarthritis (24). Consequently, there has been rapid development of selective COX-2 inhibitors. These have been shown to suppress unwanted inflammation in patients with rheumatoid arthritis and osteoarthritis, with decreased upper gastrointestinal side effects compared with conventional agents. The selective COX-2 inhibitors are now in clinical use (24, 25). However, little is known regarding the physiological role of COX-2 in the vasculature, and there is evidence to suggest that it may be cardioprotective (26). COX-2 knockout mice are reported to develop cardiac fibrosis (27), and administration of COX-2 inhibitors abolished the cardioprotective effect of ischemic preconditioning in a model of myocardial infarction. In this model, up-regulation of myocardial COX-2 by ischemia was associated with PGI_2 and PGE_2 synthesis (28). Our study indicates that induction of COX-2 in the endothelium would result in increased synthesis of PGI_2, but not TXA_2, favoring an antithrombotic state. Therefore, up-regulation of COX-2 by these cells may represent an important protective mechanism against vascular injury or insult. This could result in an exacerbation of the potential for thrombotic complications with the use of selective COX-2 inhibitors. Additionally, COX-2 inhibitors do not suppress COX-1-derived TXA_2 production by platelets, unlike conventional nonsteroidal anti-inflammatory drugs, and this would further alter the TXA_2/PGI_2 balance toward a prothrombotic state. Two recent studies examining the effects of selective COX-2 inhibition in healthy volunteers have demonstrated inhibition of systemic PGI_2 production without inhibition of platelet-derived TXA_2 production synthesis (29, 30). Systemic PGI_2 synthesis is measured by urinary excretion of 2,3 dinor-6 keto $PGF_{1\alpha}$, and is believed to reflect mainly blood vessel-derived PGI_2 synthesis. COX-2 may be up-regulated in large straight tracts of vasculature that are exposed to the biomechanical stimulus of uniform laminar flow (9, 31), and this may account for the COX-2-derived PGI_2 production. However, the situation in vivo remains unclear, because COX-2 was not detected in healthy arterial and venous tissues, but was highly expressed in atherosclerotic lesions (32–35). The cardiovascular consequences of COX-2 inhibition are further emphasized by a recent large clinical trial in rheumatoid arthritis that reported a 4-fold increase in myocardial infarction in patients using a selective COX-2 inhibitor (36).

In summary, the results of this study indicate a mechanism through which the initial prothrombotic vascular response to injury by endothelial cells becomes self-limiting, through the induction of COX-2 and the increased production of PGI_2. The results further suggest that the changing total cellular COX activity in conjunction with the kinetic properties of the terminal prostanoid synthases and the selective induction of PGI synthase, but not TX synthase, mediate this response. Furthermore, preliminary results from specific COX-2 inhibition in healthy volunteers and clinical arthritis trials support the contention that vascular COX-2 is an important protein for maintaining vascular homeostasis.

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