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Effect of bradykinin, TGF-β1, IL-1β, and hypoxia on COX-2 expression in pulmonary artery smooth muscle cells

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Bradbury, D. A., R. Newton, Y.-M. Zhu, J. Stocks, L. Corbett, E. D. Holland, L. H. Pang, and A. J. Knox. Effect of bradykinin, TGF-β1, IL-1β, and hypoxia on COX-2 expression in pulmonary artery smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 283: L717–L725, 2002. First published May 10, 2002; 10.1152/ajplung.00070.2002.—Prostanoids are major regulators of smooth muscle function that are generated by cyclooxygenase (COX). Here we hypothesized that cytokines and mediators that regulate the pulmonary circulation would alter COX expression and prostanoid generation in pulmonary artery smooth muscle cells. Bradykinin, transforming growth factor-β1 (TGF-β1), and interleukin-1β (IL-1β) increased inducible COX-2 expression and prostaglandin E_2 (PGE_2) release. Transfection studies using a COX-2 promoter construct demonstrated that all three agents acted transcriptionally. Constitutive COX-1 protein expression was unchanged. The COX inhibitor indomethacin, the COX-2 inhibitor NS-398, the protein synthesis inhibitor cycloheximide, and the glucocorticoid dexamethasone abrogated the increased PGE_2 levels. Dexamethasone and cycloheximide prevented COX-2 induction. Hypoxia (3% O_2-5% CO_2-92% N_2) for 24 h selectively augmented TGF-β1-stimulated PGE_2 production and COX-2 induction but had no effect alone. Prolonged hypoxic culture alone for 48 and 72 h enhanced COX-2 induction and increased PGE_2. These studies show that a number of stimuli are capable of inducing COX-2 in pulmonary artery smooth muscle cells. The interaction between hypoxia and TGF-β1 may be particularly relevant to pulmonary hypertension.

prostaglandin E_2; human; cell culture

PULMONARY HYPERTENSION OCCURS as a result of narrowing of the lumen of the pulmonary arteries due to vasoconstriction or hyperplasia of pulmonary vascular smooth muscle cells. Primary pulmonary hypertension (PPH) is a condition of unknown etiology (14), whereas secondary pulmonary hypertension can accompany a number of chronic hypoxic lung disorders (44). Vascular smooth muscle cells and endothelial cells play important roles in the development of pulmonary hypertension (43). Increased plasma levels of several factors responsible for the regulation of pulmonary vascular tone and smooth muscle cell proliferation have been associated with pulmonary hypertension, including interleukin (IL)-1, IL-6, endothelin-1, and prostanoids (18, 24, 41). The production of prostanoids may act as a negative-feedback mechanism, inasmuch as prostaglandin (PG) E_2 and PGI_2 are potent vasodilators and inhibitors of vascular remodeling (3, 46).

Prostanoids are arachidonic acid metabolites produced by cyclooxygenase (COX), which converts arachidonic acid to PGH_2. PGH_2 is then converted to prostanoids by specific terminal synthases. COX exists as two isoforms, COX-1 and COX-2. COX-1 is constitutive and produces physiological levels of prostaglandins and thromboxanes. In contrast, COX-2 is induced in inflammatory conditions and is responsible for the increased prostanoid production seen in pathological states (26). In many biological systems, COX-2 induction is mediated by inflammatory cytokines and peptides, such as IL-1β and bradykinin (BK). COX-2 products can regulate a number of cellular processes involved in inflammation and remodeling, including chemokine, matrix metalloproteinase, and angiogenic factor production and adenylyl cyclase function (3, 32–35). Although prostanoids are known to be potent regulators of human pulmonary artery smooth muscle cell (PASMC) function, surprisingly little is known about the mechanisms involved in their endogenous generation in pulmonary vascular smooth muscle cells, although IL-1β was shown to induce COX-2 in one study associated with large increases in PGE_2 production (20, 21).

We have studied the effects of three candidate stimuli for COX-2 induction in cultured human PASMC [transforming growth factor (TGF)-β, BK, and hypoxia] and used IL-1β, the only known inducer of COX-2 in these cells, as a positive control. The effects of hypoxia on COX-2 expression and PGE_2 production in PASMC treated with BK, TGF-β1, and IL-1β were also studied. We measured PGE_2 as a representative prostanoid, inasmuch as this is the main prostaglandin produced by PASMC (46).

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TGF-β is a polypeptide cytokine that exists in three isoforms: TGF-β1, TGF-β2, and TGF-β3. TGF-β isoforms, particularly TGF-β1, can regulate smooth muscle cell proliferation and vascular remodeling (5). BK, a kinin and potent vasodilator, is an important regulator of cardiovascular function that acts in part via prostanoid generation in several tissues, including vascular smooth muscle cells and endothelial cells (28). Hypoxia is recognized as a key stimulus for pulmonary blood vessel remodeling and development of pulmonary hypertension. Inasmuch as hypoxia transcriptionally regulates COX-2 in human umbilical vein endothelial cells (HUVEC) (40) and induces COX-2 in rat lung (7), we were interested to determine whether the same were true in PASMC.

Here we report for the first time that TGF-β and BK can induce COX-2 in PASMC and that COX-2 induction was inhibited by the protein synthesis inhibitor cycloheximide and the glucocorticoid dexamethasone. PGE_2 release was abrogated by indomethacin, a nonselective COX inhibitor, and NS-398, a COX-2-selective inhibitor. Furthermore, short-term hypoxia selectively potentiated the effect of TGF-β1. Prolonged hypoxic incubation increased COX-2 induction and PGE_2 release on its own.

METHODS

Cell culture. Proximal, *Mycoplasma*-negative PASMC from a 22-yr-old Caucasian man were purchased at *passage 3* from Clonetics (BioWhittaker, Wokingham, Berkshire, UK). The PASMC were cultured to *passage 6* in smooth muscle cell growth medium-2 BulletKit (Clonetics BioWhittaker).

All experiments were set up in triplicate or quadruplicate using *passage 6* cells cultured in 6- or 24-well plates at 37°C in a 5% CO_2 humidified incubator (Leec, Colwick, Nottingham, UK). The confluent cells were growth arrested by serum withdrawal for 24 h. The medium was replaced with fresh serum-free medium containing the cytokines or peptides under investigation. In the inhibition experiments, the cells were preincubated for 1 h with the inhibitors. This time was chosen on the basis of our previous studies in cultured cell systems (34). At the end of the incubation time, the culture supernatants were harvested and the protein was extracted from the cells. In experiments where hypoxic culture conditions were used, a 37°C humidified 5% CO_2 incubator connected to a nitrogen generator to give 3% O_2 was used (Napco, Winchester, VA). Inasmuch as previous studies have shown that COX-2 is induced in response to hypoxia in HUVEC in vitro, these cells were used as a positive control in the hypoxia experiments (19, 40).

Assessment of cell viability. One well of the 24-well plate in each condition was detached using 0.025% trypsin and 0.01% EDTA. The cell number and percent viability were assessed using a hemocytometer and 0.4% trypan blue (Sigma, Poole, UK). The cell viability was also measured using a FACSCalibur flow cytometer (Becton Dickinson, Cowley, UK) and propidium iodide (Sigma).

PGE_2 assay. PGE_2 levels were assessed by radioimmunoassay as described previously (2, 10). The bound [^{3}H]PGE_2 was measured using the Tri-Carb 2100TR liquid scintillation analyzer (Packard Bioscience, Pangbourne, UK). The PGE_2 levels were calculated with Riasmart software (Packard Bioscience).

Western blot analysis. Western blotting for COX-1 and COX-2 was performed as described previously (34). Staining intensity of the bands was measured using a densitometer (Syngene, Braintree, UK) together with Genesnap and Genetools software (Syngene). The COX-2 densitometry values shown in Fig. 6*C* were normalized for COX-1 expression.

Transfections. The PASMC were transiently cotransfected for 2 h with ~1 kb of intact COX-2 promoter (C2.1) ligated with a luciferase reporter plasmid (pGL3-Basic, Promega, Southampton, UK) and the internal control plasmid pRL-SV40 (Promega) containing the *Renilla* luciferase gene using

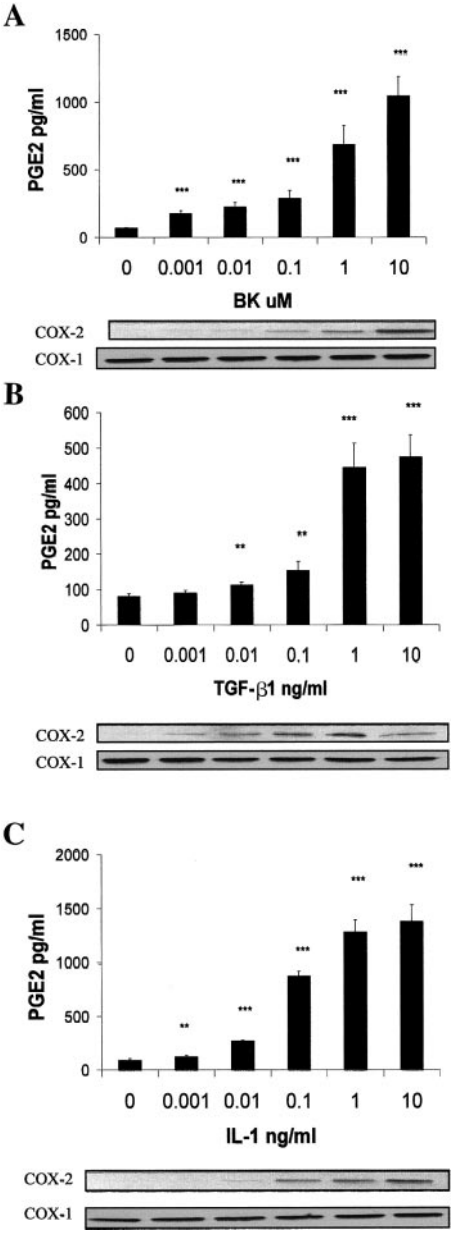
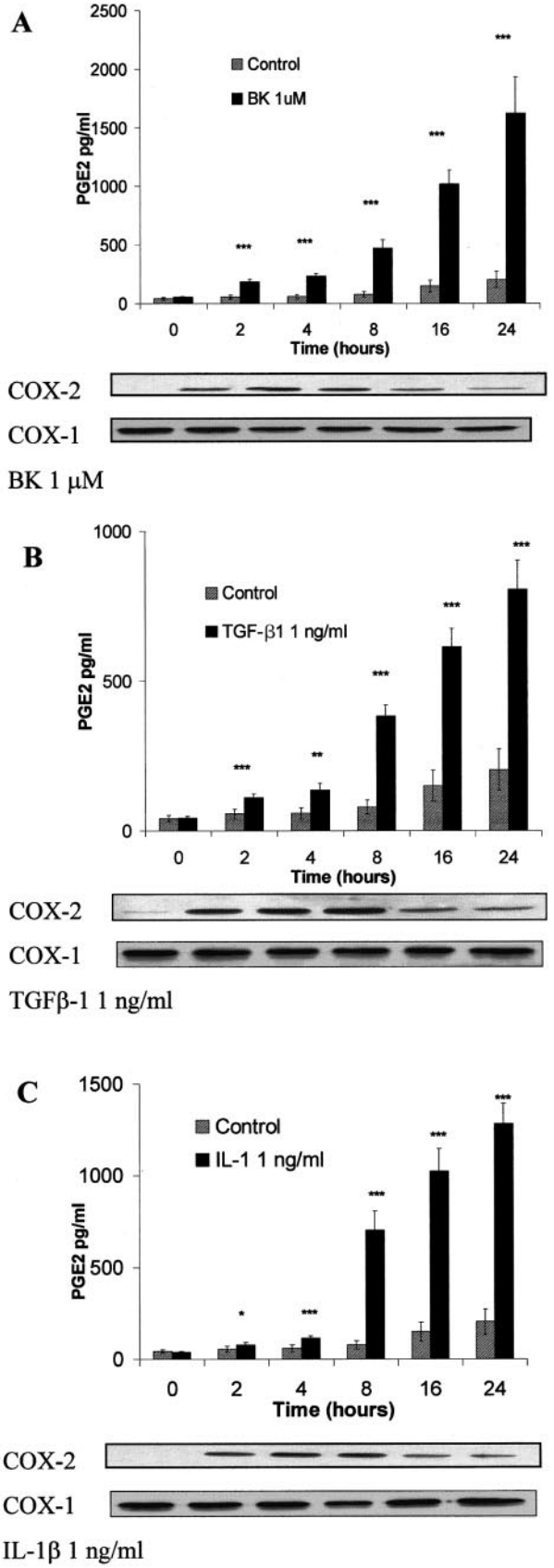


Fig. 1. Increase in prostaglandin E_2 (PGE_2) release and cyclooxygenase-2 (COX-2) expression after 24 h of incubation with increasing concentrations of bradykinin (BK, *A*), transforming growth factor (TGF)-β1 (*B*), and interleukin-1β (IL-1β, *C*). Values are means ± SE of 4 experiments, each performed in quadruplicate. COX-1 protein levels remained unchanged. Western blotting shown is representative of 4 blots. Data were analyzed by ANOVA: $^{**}P < 0.01$; $^{***}P < 0.001$.



a liposomal transfection system (Lipofectamine LF2000, GIBCO Life Technologies, Paisley, UK). After 2 h of incubation with 10 ng/ml IL-1β, 10 μM BK, or 10 ng/ml TGF-β1, the cells were harvested and the firefly and *Renilla* luciferase activities were measured using the dual-luciferase assay system kit (Promega) and tube luminometer (Berthold Technologies, Bad Wildbag, Germany). Lipofectamine has a transfection efficiency of 10–20% in our hands using human PASMC and green fluorescent protein. In preliminary experiments, this was similar to Fugene 6 (Hoffmann-La Roche, Basel, Switzerland) and more efficient than electroporation (data not shown).

Flow cytometric assessment of TGF-β receptor II expression. PASMC were detached using a sterile solution of 0.02% EDTA in PBS (Sigma), washed, and fixed in Facslyse (Becton Dickinson) following the manufacturer's instructions. The cells were stained with FITC-conjugated TGF-β receptor II antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or FITC-conjugated negative control antibody (Dako, Ely, UK). Using the FACSCalibur flow cytometer (Becton Dickinson) and logarithmic amplification of the green fluorescence channel (FL-1), 10,000 events were acquired and analyzed with CellQuest software (Becton Dickinson).

TGF-β1 ELISA. TGF-β1 was measured with a specific ELISA (DuoSet, R & D Systems, Abingdon, UK).

Materials. Recombinant human IL-1β and TGF-β1 were purchased from R & D Systems. BK, indomethacin, dexamethasone, and cycloheximide were purchased from Sigma. The selective COX-2 inhibitor *N*-(2-cyclohexyloxy-4-nitrophenyl)-methanesulphonamide (NS-398) was purchased from Cayman Chemical (Alexis, Bingham, UK).

Statistical analysis. The results of the PGE_2 levels were expressed as the mean of the triplicate or quadruplicate wells for that experiment. The experiments were repeated at least three times, and the results represent the means ± SE. Analysis of variance or Wilcoxon signed rank test was used to determine significant differences. Flow cytometric data were compared using Kolmogorov-Smirnov statistics, where $D > 0.1$ was considered significantly different (48).

RESULTS

BK, TGF-β1, and IL-1β induce COX-2 protein expression and increase PGE_2 production in a concentration-dependent manner in cultured human PASMC. PGE_2 release over 24 h in control cells was 203 ± 69 pg/ml ($n = 7$ experiments). Incubation for 24 h with 1 nM–10 μM BK induced an increase in PGE_2 release into the culture supernatant in the human PASMC in a concentration-dependent manner (Fig. 1*A*). The same pattern of PGE_2 release was seen when PASMC were incubated with 1 pg/ml–10 ng/ml TGF-β1 (Fig. 1*B*) and 1 pg/ml–10 ng/ml IL-1β (Fig. 1*C*). This PGE_2 production was associated with the induction of COX-2 protein. Western blotting showed a dose-dependent induction of the COX-2 protein in response to BK, TGF-β1, and IL-1β (Fig. 1). There was a significant increase in COX-2 expression and PGE_2 production compared with unstimulated control values when cells were incubated

Fig. 2. Time-dependent induction of COX-2 and increase of PGE_2 with 1 μM BK (*A*), 1 ng/ml TGF-β1 (*B*), and 1 ng/ml IL-1β (*C*). Western blotting for COX-1 shows that COX-1 protein was not changed. Western blotting is representative of 4 blots. Data were analyzed by ANOVA: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

with 1 μM BK, 1 ng/ml TGF-β1, or 1 ng/ml IL-1β. Western blotting for COX-1 showed that COX-1 expression was constitutive and did not change with any concentration of BK, TGF-β1, or IL-1β (Fig. 1). Inasmuch as BK has been shown to increase release of IL-1β in fibroblasts (31), we considered whether release of IL-1β might mediate the effect of BK. However, PASMC cultured in the presence of BK and neutralizing antibodies to IL-1β showed no reduction in COX-2 expression or PGE_2 levels, suggesting that BK-mediated COX-2 induction was not secondary to IL-1β induction (data not shown).

Kinetics of PGE_2 production and COX-2 induction in human PASMC in response to culture with 1 μM BK, 1 ng/ml TGF-β1, or 1 ng/ml IL-1β. When the cells were cultured with 1 μM BK, 1 ng/ml TGF-β1, or 1 ng/ml IL-1β for 2, 4, 8, 16, and 24 h, there was a significant increase in PGE_2 production compared with *time 0* levels and the unstimulated control cells at the same time points (Fig. 2). The time course for COX-2 induction showed expression of the enzyme at 2, 4, 8, 16, and 24 h for PASMC incubated with 1 μM BK, 1 ng/ml TGF-β1, and 1 ng/ml IL-1β. The strongest COX-2 bands were present at 4 and 8 h (Fig. 2). There were no detectable COX-2 bands when the PASMC were cultured for the same time periods in the absence of BK, TGF-β1, and IL-1 β (see Fig. 5*B*). Western blotting for COX-1 showed that COX-1 was expressed constitutively, and the intensity of the protein did not change with incubation times (Fig. 2).

Inhibition of BK, TGF-β1, and IL-1β induced PGE_2 production by indomethacin, NS-398, dexamethasone, and cycloheximide in PASMC. Concentration-response experiments were performed to determine the optimum concentrations of the respective inhibitors that would significantly reduce PGE_2 production in response to 24 h of incubation with BK, TGF-β1, or IL-1β. PASMC were preincubated for 1 h with indomethacin, NS-398, dexamethasone, or cycloheximide at 100 nM–100 μM. DMSO (0.2%) in culture medium was used as a vehicle control. BK, TGF-β1, or IL-1β was then added to give final concentrations of 1 μM BK, 1 ng/ml TGF-β1, or 1 ng/ml IL-1β, and the cells were incubated for a further 24 h. The optimum concentrations of inhibitor that gave a significant reduction in PGE_2 induced by BK, TGF-β1 or IL-1β without affecting the cell viability were 1 μM indomethacin, 1 μM NS-398, 0.1 μM dexamethasone, and 10 μM cycloheximide (Fig. 3). The COX-2 protein induction after 4 h of incubation with 1 μM BK, 1 ng/ml TGF-β1, or 1 ng/ml IL-1β was inhibited by preincubation of the cells with 0.1 μM dexamethasone and 10 μM cycloheximide. However, there was no reduction in COX-2 expression by preincubation with 1 μM indomethacin or 1 μM NS-398 (Fig. 3).

Transcriptional regulation of COX-2 by BK, TGF-β1, and IL-1β. PASMC that had been transfected with ~1 kb of the COX-2 promoter (C2.1) ligated to a firefly luciferase reporter gene showed increased luciferase activity in cells subsequently treated with BK, TGF-β1, or IL-1β compared with unstimulated control cells (Fig. 4). This suggests that all three agents were acting transcriptionally. There were no significant differences between control and stimulated cells that were transfected with empty vector only (pGL3-Basic).

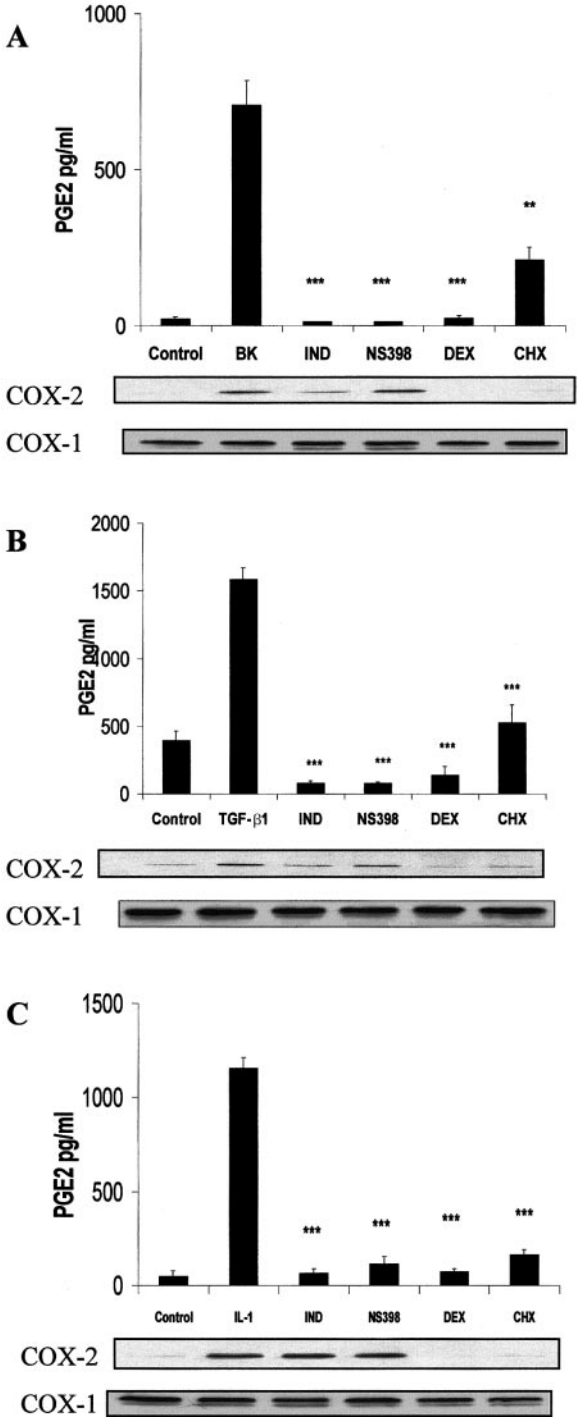


Fig. 3. Inhibition of BK (*A*)-, TGF-β1 (*B*)-, and IL-1β (*C*)-induced PGE_2 production by 1 μM indomethacin (IND), 1 μM NS-398, 0.1 μM dexamethasone (DEX), and 10 μM cycloheximide (CHX). Western blotting showed that DEX and CHX inhibited COX-2 induction and that COX-1 protein expression was not changed. Data were analyzed by ANOVA: $^{**}P < 0.01$; $^{***}P < 0.001$.

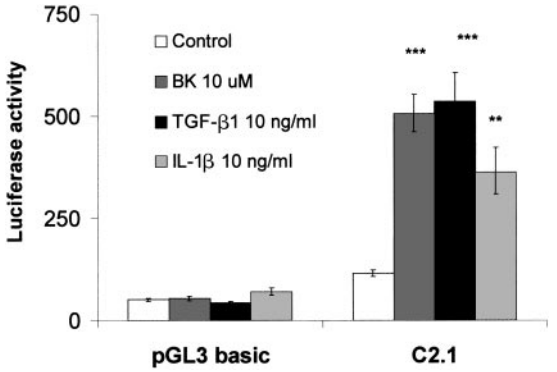


Fig. 4. Luciferase activity in pulmonary artery smooth muscle cells (PASMC) transfected with ~1 kb of the COX-2 promoter (C2.1) or control vector (pGL3-Basic) ligated to a luciferase reporter gene. Luciferase expression was significantly increased in cells transfected with COX-2 promoter and treated with 10 μM BK, 10 ng/ml TGF-β1, or 10 ng/ml IL-1β compared with unstimulated control cells when analyzed using ANOVA: $^{**}P < 0.01$; $^{***}P < 0.001$.

Effect of short-term hypoxia on COX-2 protein expression and PGE_2 production in unstimulated and BK-, TGF-β1-, and IL-1β-treated human PASMC. Comparison between unstimulated PASMC cultured for 2, 4, 8, 16, and 24 h (short-term culture) under normoxic (20% O_2) and hypoxic (3% O_2) conditions showed no differences in induction of COX-2 protein or PGE_2 production (Fig. 5, *A* and *B*) or in autocrine TGF-β1 levels (Fig. 5*C*). There were no significant differences between PGE_2 release and COX-2 expression in PASMC cultured under hypoxic compared with normoxic conditions and in the presence of 1 μM BK or 1 ng/ml IL-1β at these time points (data not shown). The amount of COX-1 protein was also unchanged. HUVEC were used as a positive control in the hypoxia experiments, inasmuch as previous studies showed that COX-2 is induced by hypoxic culture in these cells (Fig. 5*B*). However, when PASMC were treated with 1 ng/ml TGF-β1 and the hypoxic and normoxic culture conditions were compared, there was a significant increase in PGE_2 levels with hypoxia at 4, 8, and 16 h (Fig. 6*A*). Densitometry of the COX-2 Western blotting showed stronger bands with hypoxic than with normoxic culture (Wilcoxon signed rank test, $P = 0.02$; Fig. 6, *B* and *C*). Again, COX-1 expression was unchanged.

Effect of hypoxia on TGF-β receptor II expression. These experiments were performed to determine whether the hypoxic enhancement of TGF-β1-mediated COX-2 induction was due to upregulation of the TGF-β type II receptor. PASMC were subjected to hypoxic or normoxic culture for 2, 4, 24, and 48 h and then stained with FITC-conjugated polyclonal TGF-β type II receptor antibody or FITC-conjugated negative control antibody. The number of positive cells together with the median peak fluorescence, which is a measure of the receptor density, was assessed using flow cytometry. The histograms were analyzed using Kolmogorov-Smirnov statistics; $D > 0.1$ was considered significantly different. Overall, there were no significant differences in percent positive cells or TGF-β type II receptor expression between hypoxic and normoxic culture at these times (data not shown).

Extended culture of PASMC results in induction of COX-2. Figure 7*A* shows the PGE_2 release in hypoxic and normoxic culture in unstimulated PASMC with incubation times >24 h. There was a significant increase in hypoxic PGE_2 production at 48 and 72 h with a corresponding increase in COX-2 protein expression (Fig. 7*B*).

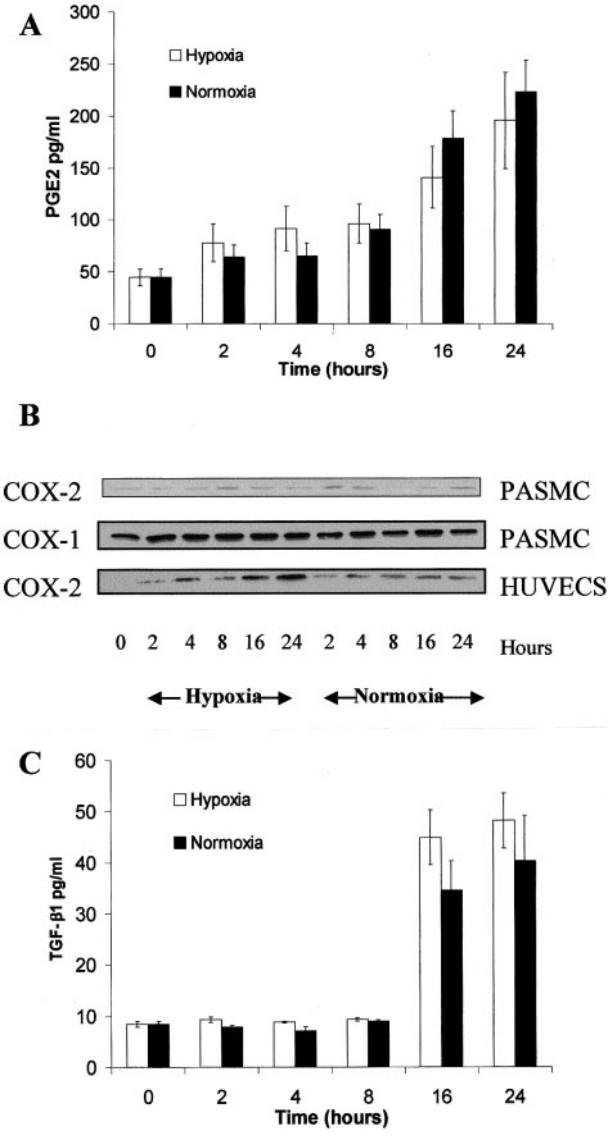


Fig. 5. *A*: PGE_2 release in unstimulated PASMC cultured under hypoxic and normoxic culture conditions at time points up to 24 h. Values are means ± SE of 4 experiments, each done in quadruplicate. There were no significant differences in PGE_2 production between hypoxia and normoxia at any of the time points. *B*: Western blotting for COX-1 and COX-2 expression under conditions described in *A;* blot is representative of 4 blots. Induction of COX-2 protein by hypoxic culture in human umbilical vein epithelial cells (HUVECs), which were used as a positive control, is also shown. There were no significant differences in the levels of autocrine TGF-β1 release between hypoxic and normoxic culture at these times (*C*).

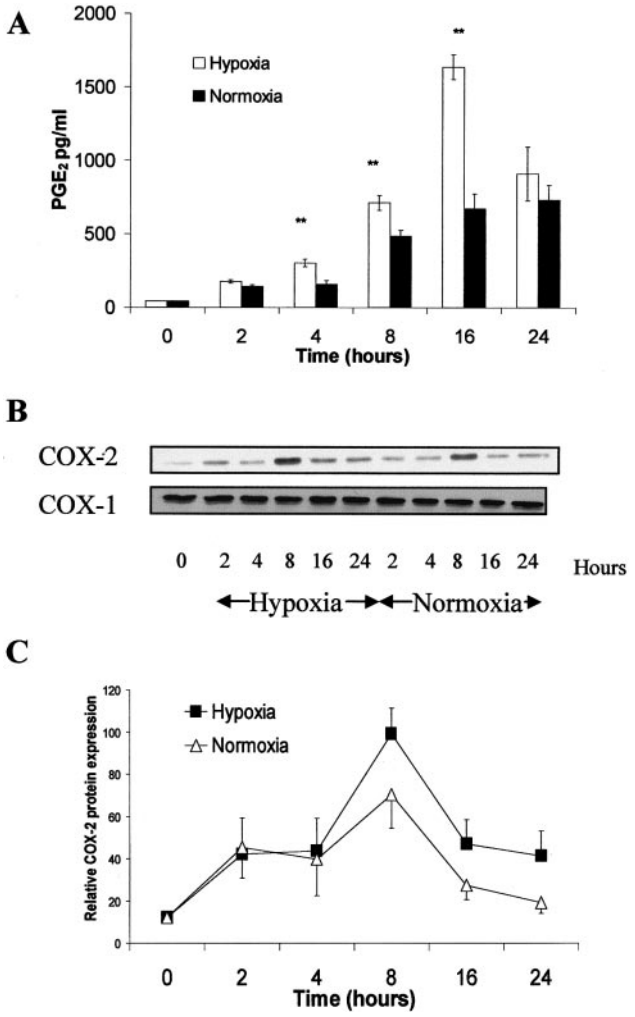


Fig. 6. *A*: PGE_2 release (means ± SE, $n = 3$) in cells treated with 1 ng/ml TGF-β1 cultured under hypoxic and normoxic conditions at time points up to 24 h. TGF-β1 caused a greater increase in PGE_2 production under hypoxic than under normoxic conditions. $^{**}P < 0.01$ (by ANOVA). *B*: consistent increase in COX-2 with TGF-β1 under hypoxic compared with normoxic conditions. Western blot is representative of 3 blots. COX-1 protein was not changed in these experiments. *C*: densitometry results for COX-2 Western blotting ($n = 3$). COX-2 protein expression was increased in TGF-β1-treated cells under hypoxic compared with normoxic culture.

DISCUSSION

There are several novel findings in this study. It is the first to show that BK or TGF-β1 can induce COX-2 in human PASMC and the first to study the effects of hypoxia. Hypoxia alone did not induce COX-2 over short-term incubation (24 h) but selectively potentiated TGF-β1-mediated COX-2 induction and PGE_2 production. More prolonged hypoxic incubation enhanced COX-2 and PGE_2 production on its own. The cytokines and peptides that were used in these experiments induced COX-2 over a wide range of concentrations: 100 nM–10 μM BK, 100 pg/ml–10 ng/ml IL-1β, and 1 pg/ml–10 ng/ml TGF-β1. These concentrations were within the ranges measured in vivo in a number of pathological conditions, including pulmonary hypertension (1, 18, 23, 24, 29, 30, 36, 38). We found that IL-1β produced changes in COX-2 expression and PGE_2 production similar to those reported previously in human PASMC (3, 4, 20, 21). We found that BK also induced COX-2 and PGE_2 production in cultured human PASMC in a concentration- and time-dependent manner. Although no previous studies have looked at the effect of BK on COX-2 expression in human PASMC, BK also enhanced COX-2 expression and prostanoid release in airway smooth muscle cells and in the lung vasculature of lipopolysaccharide-treated rats (17, 35). BK increases PGE_2 release in cells in a two-step process: an initial increased arachidonic acid release and a delayed induction of COX-2 (35). Inasmuch as others have shown that BK can activate cytoplasmic phospholipase A_2 to release arachidonic acid in human PASMC (45), we did not measure arachidonic acid mobilization.

TGF-β1 stimulates collagen production in vascular smooth muscle cells, which is important in vascular fibrosis and tissue remodeling and has been implicated in the pathophysiology of pulmonary hypertension (6). No previous studies have looked at the effect of TGF-β on COX-2 in PASMC, although our group has shown that TGF-β1 induced COX-2 and PGE_2 release in cultured human airway smooth muscle cells (13). In the present study, we found that TGF-β1 induced COX-2 and increased PGE_2 production in human PASMC in a concentration- and time-dependent manner. The effect of TGF-β is likely to be mediated via the TGF-β response element on the COX-2 promoter gene (47). Our findings with TGF-β may be of relevance to recent studies looking at the genetics of pulmonary hypertension. The effects of TGF-β in vascular cells are inhibi-

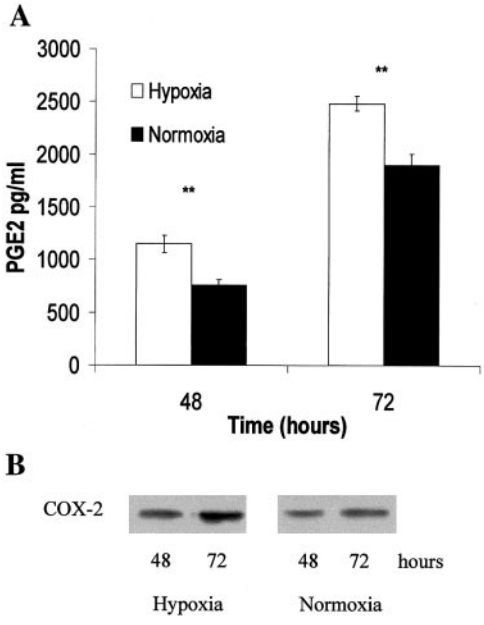


Fig. 7. PGE_2 release (*A*) and COX-2 expression (*B*) in unstimulated PASMC under hypoxic and normoxic conditions for extended culture (48 and 72 h). There was a significant increase in PGE_2 production and COX-2 protein at both time points under hypoxic compared with normoxic conditions. PGE_2 data were analyzed by ANOVA: $^{**}P < 0.01$.

tion of cell growth, induction of differentiation, and stimulation of collagen synthesis. Somatic mutations in these growth-inhibitory pathways involving TGF-β receptor I and TGF-β receptor II lead to uncontrolled tumor cell proliferation (5). Inasmuch as PGE_2 is a potent vasodilator and inhibitor of cell proliferation and collagen production, impaired TGF-β-mediated COX-2 induction and PGE_2 generation might contribute to pulmonary vasoconstriction and remodeling in PPH. Mutations in the bone morphogenic protein type II receptor (BMPR-II) gene (BMPR2) results in familial PPH, and germ-line mutations of BMPR2 have been reported in >26% of sporadic cases of PPH (11, 42). BMPR2 encodes a type II receptor member, BMPR-II, of the TGF-β superfamily of cell-signaling molecules (25). Further studies are required to determine whether bone morphogenic proteins and BMPR-II are also involved in COX-2 regulation. It would also be interesting to compare responses in different regions of the pulmonary vasculature.

The increased PGE_2 production in response to all three agents, BK, TGF-β1, and IL-1β, was inhibited by the nonselective COX inhibitor indomethacin and the COX-2 selective inhibitor NS-398, consistent with a COX-2-mediated increase. Furthermore, preincubation with dexamethasone or cycloheximide inhibited BK-, TGF-β1-, and IL-1β-induced PGE_2 production and COX-2 protein expression. COX-1 protein, which was expressed constitutively in the PASMC, remained constant and was not diminished by either inhibitor, consistent with its housekeeping role. There is evidence to suggest that a posttranscriptional destabilization of IL-1-induced COX-2 mRNA by dexamethasone occurs in synovial fibroblasts (37). Dexamethasone is also known to inhibit gene transcription by repressing glucocorticoid receptor translocation to the nucleus and preventing nuclear factor-κB transactivation (9).

To determine whether BK, TGF-β1, and IL-1β were acting transcriptionally, we transfected cells with a COX-2 promoter construct linked to a luciferase/*Renilla* reporter system. These studies demonstrated that BK-, TGF-β1-, and IL-1β-mediated COX-2 induction was at least in part transcriptionally mediated. However, the fact that IL-1β was a less potent activator of the reporter construct suggests that posttranscriptional mechanisms may also be important with IL-1β.

Hypoxia is the major contributor to secondary pulmonary hypertension. Inasmuch as hypoxia has been shown to induce COX-2 in HUVEC and rat lung cells (7, 40), we were interested to determine whether there was a direct effect of hypoxia on COX-2 in human PASMC. It has previously been shown that prostaglandin metabolism was altered in cultured pulmonary artery endothelial cells exposed to 3% O_2 (12). We therefore studied the effect of hypoxic culture (3% O_2) directly on human PASMC by comparing the induction of COX-2 protein and PGE_2 release in unstimulated cells and in BK-, TGF-β1-, and IL-1β-treated cells. We found that short-term hypoxic exposure (<24 h) did not induce COX-2 expression or increase PGE_2 release compared with normoxic culture in unstimulated cells. We also studied the effect of short-term hypoxic exposure on the effects of TGF-β1, BK, and IL-1β. We found that hypoxic exposure did not enhance COX-2 induction or PGE_2 generation in response to BK or IL-1β. However, the induction of COX-2 and PGE_2 release by TGF-β1 was enhanced by hypoxia in PASMC. We considered several explanations for this effect. One possibility is that upregulation of the TGF-β receptor occurs in response to hypoxia. In rat hepatocytes, hypoxia restored the growth-inhibitory effects of TGF-β1 via an increase in mRNA for TGF-β receptor types I, II, and III (22). However, we found no differences in receptor number or density under hypoxic conditions compared with normoxic conditions in our study. We then investigated whether hypoxic culture induced an increase in autocrine TGF-β1 production. This might explain the synergistic effect between hypoxia and TGF-β1 by increasing the local concentration of TGF-β1. However, at the 4-, 8-, and 16-h time points, where hypoxia augmented the exogenous TGF-β1 induction of COX-2, there were no detectable differences in endogenous TGF-β1 levels between hypoxic and normoxic culture. An alternative explanation for the enhancement of TGF-β1-mediated COX-2 induction by hypoxia is an interaction between hypoxia inducible factor and Smad proteins, as has been shown in the regulation of the vascular endothelial growth factor gene (39), but this requires further study. Prolonged culture of unstimulated PASMC for 48 and 72 h was associated with COX-2 induction and PGE_2 release in hypoxic and normoxic conditions, with higher expression in the hypoxia-treated cells. The cells were growth arrested by serum depletion, and this may account for the presence of COX-2 in long-term normoxic culture as the cells become quiescent in the G_0 phase. Studies in human fibroblasts have shown that COX-2 induction by phorbol 12-myristate 13-acetate and IL-1β is more pronounced in the G_0 phase than in cells in cycle (16).

Induction of COX-2 may potentially be advantageous in pulmonary hypertension, inasmuch as it results in high levels of PGI_2 and PGE_2 (46), both of which have vasodilatory and antiproliferative properties. This hypothesis would be consistent with studies showing that long-term PGI_2 therapy has been reported to be beneficial in PPH (27), that PGI_2 analogs are potent inhibitors of PASMC proliferation (8), and that overexpression of prostacyclin synthase protects against the development of hypoxic pulmonary hypertension in transgenic mice (15).

In conclusion, we have shown that, in human PASMC, BK, TGF-β1, and IL-1β induce COX-2 through transcriptional mechanisms. With short-term culture, hypoxia alone did not induce COX-2 but selectively increased the effect of TGF-β1. Long-term hypoxia enhanced the induction of COX-2 in unstimulated human PASMC and significantly increased PGE_2 release.

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