

Autocrine Function of Inducible Nitric Oxide Synthase and Cyclooxygenase-2 in Proliferation of Human and Rat Pulmonary Artery Smooth-Muscle Cells

Species Variation

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Pulmonary hypertension is characterized by hypertrophy and hyperplasia of vascular smooth muscle occurring via an unknown mechanism. Cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) are expressed under inflammatory conditions and produce mediators that regulate growth in some tissues. We have therefore addressed the question of COX-2 and iNOS involvement in proliferation of human and rat pulmonary artery (PA) smooth-muscle cells (SMC). Interleukin (IL)-1 β suppressed proliferation of both human and rat PA SMC. Moreover, IL-1 β induced COX-2 expression in both cell types. By contrast, IL-1 β stimulated the expression of iNOS protein in rat cells only. COX-2 induced in human cells inhibited proliferation, whereas COX-2 products in rat cells were without affect. However, iNOS activity in rat cells suppressed their proliferation. We conclude that human and rat evolution has diverged such that COX-2 and iNOS, although induced by the same mediator, have different levels of activity and functions in the two species. In humans, induction of COX-2 during pulmonary hypertension may be beneficial for long-term treatment of this disease. **Jourdan, K. B., T. W. Evans, N. J. Lamb, P. Goldstraw, and J. A. Mitchell. 1999. Autocrine function of inducible nitric oxide synthase and cyclooxygenase-2 in proliferation of human and rat pulmonary artery smooth-muscle cells: species variation. *Am. J. Respir. Cell Mol. Biol.* 21:105–110.**

In health, the endothelial layer of blood vessels is the main site of vasoactive hormone release. For example, endothelial cells co-release the vasodilator substances nitric oxide (NO) and prostaglandin (PG) E₂ and prostacyclin (PGI₂). Under physiologic conditions, constitutive forms of NO synthase (cNOS) and cyclooxygenase (COX)-1, both of which are highly localized to the endothelium, catalyze the release of NO and PGs. However, under certain inflammatory conditions in which cytokines such as interleukin (IL)-1 β are elevated, vascular smooth muscle expresses inducible forms of both COX (COX-2) and NOS (iNOS).

Both NOS and COX metabolites are potent modulators of cell growth. In the case of COX metabolites, throm-

boxane (TX) A₂ stimulates proliferation, whereas PGE₂ and PGI₂ inhibit proliferation in a number of smooth-muscle cell types (1, 2). NO is associated most strongly with inhibition of growth (3, 4), although it has been shown to potentiate proliferation of senescent human fibroblasts (5).

Pulmonary hypertension, both primary and secondary to a number of clinical conditions, is now considered to be a chronic inflammatory disease in which circulating levels of cytokines known to induce iNOS and COX-2 (e.g., IL-1 β) are elevated (6), and which is associated with structural alterations to pulmonary vessels typified by hypertrophy and hyperplasia of vascular smooth muscle. However, the pathophysiology of the remodeling process is still poorly understood (7) and treatment remains largely supportive (for review, see [8]). Nevertheless, long-term PGI₂ infusion has been shown recently to cause substantial long-term reductions in pulmonary vascular resistance and possibly to prolong life (9).

We have recently demonstrated in a preliminary report (10, 11) that IL-1 β induces iNOS and COX-2 in pulmonary artery (PA) smooth-muscle cells (SMC). However, the way the expression of these enzymes modulates proliferative responses in PA SMC remains unknown. The purpose of this study was therefore to investigate the effect of

(Received in original form July 27, 1998 and in revised form January 29, 1999)

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Abbreviations: cyclooxygenase, COX; fetal calf serum, FCS; interleukin, IL; inducible NOS, iNOS; N^G nitro L-arginine methyl ester, L-NAME; nitric oxide, NO; NO synthase, NOS; pulmonary artery, PA; prostaglandin, PG; prostacyclin, PGI₂; standard error of the mean, SEM; smooth-muscle cells, SMC; tumor necrosis factor, TNF; thromboxane, TX.

Am. J. Respir. Cell Mol. Biol. Vol. 21, pp. 105–110, 1999
Internet address: www.atsjournals.org

IL-1 β on (1) the release of NO and PGs, (2) the expression of iNOS and COX-2 protein, and (3) smooth-muscle cell proliferation in human and rat PA.

Materials and Methods

Cell Culture

Human PA (mean diameter, approximately 9 mm) was obtained from healthy sections of lung resected during surgery for lung cancer. Under sterile conditions, vessels were cleaned of connective tissue and the endothelium was gently removed with a rounded scalpel blade. The artery was cut into pieces and placed in a flask with Dulbecco's modified Eagle's medium (DMEM) containing 1 mM sodium pyruvate and phenol red, supplemented with penicillin (100 U/ml), streptomycin (100 μ g/ml), L-glutamine (2 mM), amphotericin B (2.5 μ g/ml), a mixture of nonessential amino acids (L-alanine, L-asparagine, L-aspartate, L-glutamate, glycine, L-proline, and L-serine, at the manufacturer's recommended concentrations; Life Technologies, Paisley, UK), and 16% heat-inactivated fetal calf serum (FCS). The flasks were placed in a cell-culture incubator (37°C, 5% CO₂ and 95% air) and SMC explanted to form a confluent layer in 4 to 8 wk. Rat SMC from the two main branches of the PA taken from male Wistar rats were grown under identical conditions, although they required lower levels of FCS to grow and were therefore cultured in DMEM containing 9% FCS. For experiments measuring the release of mediators, cells were passaged into 96-well plates with 100 μ l medium containing drugs and/or cytokines. The medium was removed from cells after 24 or 48 h; nitrite levels were measured immediately by the Griess reaction (12); and PGE₂, TXB₂ (the breakdown product of TXA₂), or 6-ketoPGF_{1 α} (the breakdown product of PGI₂) was measured by radioimmunoassay either directly or after storage at -20°C (13). For proliferation experiments, cells were passaged into 48-well plates.

Nitrite Measurement

Nitrite was measured in cell supernatants as an index of NO formation after 48 h incubation with or without drugs and cytokines. This period of incubation was required, despite iNOS protein being detected at 24 h, because it is a stable breakdown product that accumulates with time. Aliquots of the cell supernatant were mixed with an equal volume of Griess reagent (0.5% sulfanilamide, 2.5% orthophosphoric acid, and 0.25% naphthylethylenediamine). The absorbency was measured at 540 nm, and nitrite concentration determined using sodium nitrite as a standard.

Proliferation Measurement

Proliferation was measured by the incorporation of tritiated thymidine into the DNA fraction of cells. For these experiments, cells were stimulated for 24 h with FCS and drugs. Cells were then pulsed with 18.5 Bequerels/well of tritiated thymidine for 6 h, after which they were rinsed with 0.01 M phosphate-buffered saline and incubated with 5% trichloroacetic acid for 20 min to precipitate the nucleic acid. The cells were then washed with 95% ethanol and DNA was dissolved in 0.1 M sodium hydroxide and

2% sodium carbonate. Samples were then transferred into vials and liquid scintillant was added. Incorporated thymidine was determined in a liquid scintillation counter (Canberra Packard Tri-Carb 1900CA).

Western Blotting

PA SMC were seeded in 6-well plates and left untreated or treated with IL-1 β (10 ng/ml) for 24 h. The medium was then removed and the cells were lysed with Tris buffer (50 mM; pH 7.4) containing 1% vol/vol Triton X-100, ethylenediaminetetraacetic acid (10 mM), phenylmethylsulfonyl fluoride (1 mM), pepstatin A (0.05 mM), and leupeptin (0.2 mM). Extracts were boiled at a 1:1 ratio with Tris (50 mM; pH 6.8; 4% wt/vol sodium dodecyl sulfate [SDS], 10% vol/vol glycerol, 4% vol/vol 2-mercaptoethanol, and 2 mg/ml bromophenol blue). Samples of equal protein were loaded onto 7.5% Tris-glycine SDS gels, and separated by electrophoresis. After transfer to nitrocellulose, the blots were primed with either a specific antihuman COX-2 antibody (14) (Merck Frosst, Montreal, PQ, Canada) or a specific anti-iNOS antibody (Affiniti Research Products, Exeter, UK), both raised in rabbit. The blots were then incubated with antirabbit immunoglobulin G (raised in goat), conjugated with horseradish peroxidase, and developed by ECL (Amersham International Ltd., Amersham, Kent, UK). Rainbow markers (14 to 200 kD; Amersham) were used for molecular weight determinations.

Materials

Tritiated PGE₂, TXB₂, and 6-keto PGF_{1 α} were obtained from Amersham International. IL-1 β and tumor necrosis factor (TNF)- α were purchased from Boehringer Mannheim (Lewes, East Sussex, UK). Amphotericin B and nonessential amino acids were purchased from Life Technologies. L-745,337 was a kind gift from Merck Pharmaceuticals (Montreal, PQ, Canada). All other materials were purchased from Sigma Chemical Co. (Poole, Dorset, UK).

Statistical Analysis

All data are reported as means \pm standard error of the mean (SEM) for *n* experiments. Data were compared either by one-way analysis of variance or by one sample *t* test where appropriate, with statistical significance at *P* < 0.05.

Results

Comparison of COX Activity in Rat and Human PA SMC

Both human and rat PA SMC released low but detectable amounts of PGE₂, TXB₂, or 6-ketoPGF_{1 α} , under control culture conditions (Figure 1A). Stimulation of cells with IL-1 β (10 ng/ml) resulted in an increased release of PGE₂ primarily from both cell types (Figure 1A), with lower levels of 6-keto PGF_{1 α} (10 \pm 3 ng/ml for rat cells and 5 \pm 1 ng/ml for human cells) being released. TXB₂ remained undetectable in either human or rat cells after stimulation with IL-1 β (data not shown). Under control culture conditions there was no COX-2 protein, as detected by Western blot analysis, in human or rat cells. However, after 24 h stimu-

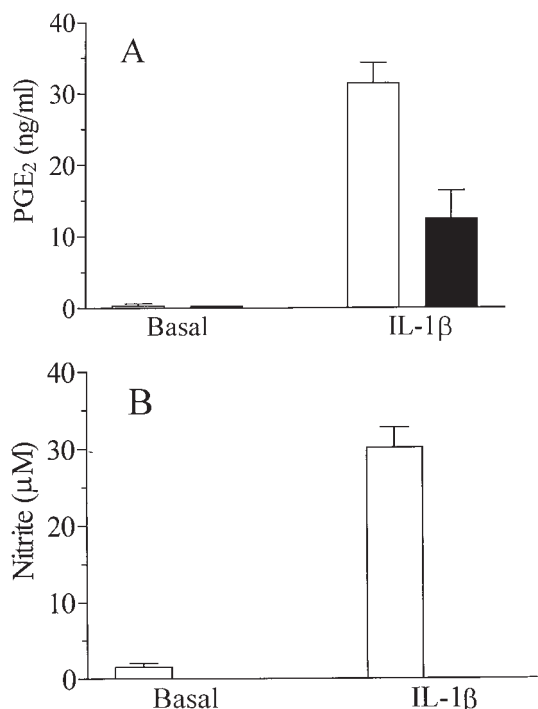


Figure 1. Effect of IL-1β (10 ng/ml) on (A) PGE₂ and (B) nitrite production by isolated PA SMC. Data shown in (A) were obtained after 24 h incubation and in (B) after 48 h incubation with IL-1β. Open columns, rat cells; filled columns, human cells. Data are means ± SEM of n = 8–10 experiments.

lation with IL-1β, both species of cells contained COX-2 protein (Figure 2). Indomethacin, an inhibitor of both COX-1 and COX-2, and of L-745,337, a selective COX-2 inhibitor, concentration-dependently inhibited IL-1β-induced release of PGE₂ from human and rat PA SMC. L-745,337 was less potent than indomethacin as an inhibitor of PGE₂ release by human (122-fold) and rat (9-fold) cells (Table 1). In separate experiments, TNF-α (10 ng/ml) did not stimulate the release of significant amounts of

TABLE 1
Concentrations that produce 50% inhibition of effect (IC₅₀) for indomethacin, L-745,337, L-NAME, and aminoguanidine on the release of NOS and COX products, nitrite and PGE₂, respectively, from human and rat PA SMC

	Nitrite		PGE ₂	
	Human	Rat	Human	Rat
Indomethacin	—	No effect	8.2 nM ± 5.7	3.6 nM ± 0.4
L-745,337	—	No effect	1.0 μM ± 0.9	31.7 nM ± 20
L-NAME	—	100 μM ± 23.6	—	487 μM ± 212
Aminoguanidine	—	140 μM ± 26.3	—	No effect

Data are means ± SEM of n = 2–4 separate experiments, each carried out in triplicate.
IC₅₀ values calculated by GraphPad Prism.

COX products by either rat or human cells (n = 6; data not shown).

Comparison of NOS Activity in Rat and Human PA SMC

No nitrite was detected in the supernatant of human cells cultured for 24 or 48 h with or without IL-1β (10 ng/ml) stimulation. Similarly, rat cells released undetectable levels of nitrite after 24 h stimulation with IL-1β. However, rat cells released detectable levels of nitrite after 48 h treatment with IL-1β (Figure 1B). Correspondingly, only rat cells showed induction of iNOS protein after treatment with IL-1β (Figure 3). Both N^G nitro L-arginine methyl ester (L-NAME), the nonselective NOS inhibitor, and aminoguanidine, a selective iNOS inhibitor (in some *in vitro* systems [15]), concentration-dependently inhibited the release of nitrite from rat PA SMC treated with IL-1β. L-NAME and aminoguanidine inhibited nitrite release with equal potencies (Table 1).

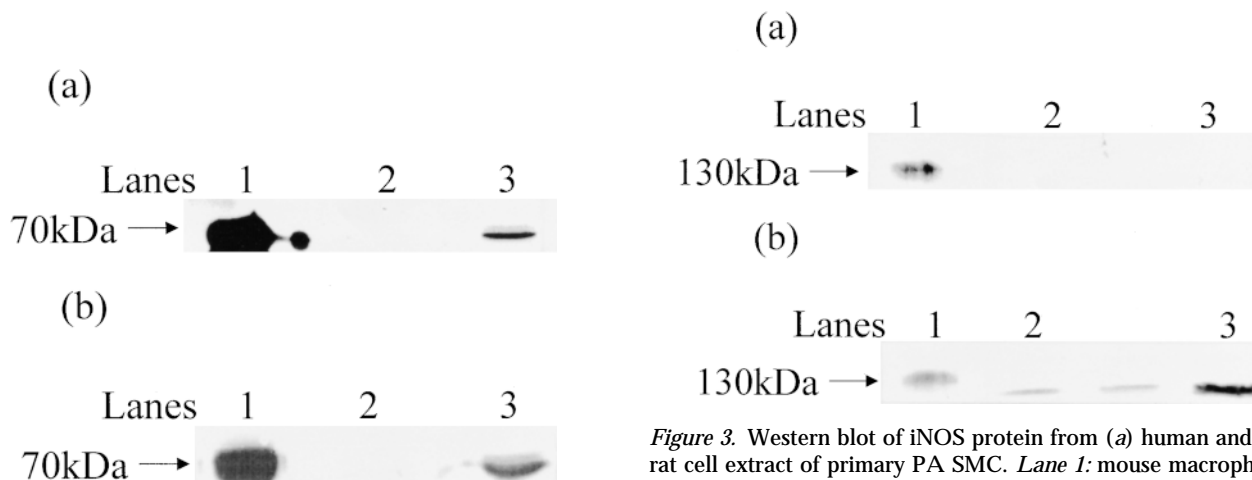


Figure 2. Western blot of COX-2 protein from (a) human and (b) rat cell extract of primary PA SMC. Lane 1: COX-2 protein standard; lane 2: untreated cells; lane 3: cells treated with IL-1β for 24 h.

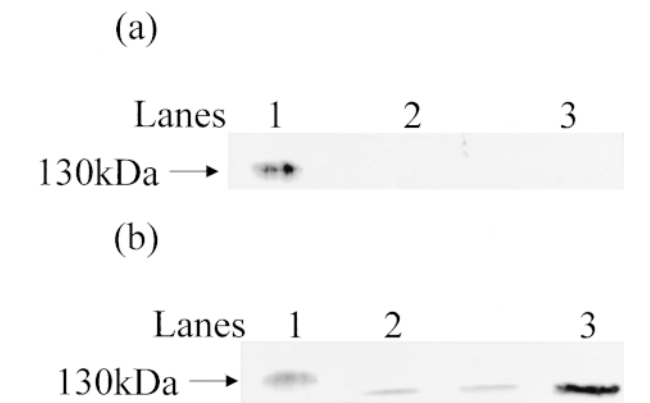


Figure 3. Western blot of iNOS protein from (a) human and (b) rat cell extract of primary PA SMC. Lane 1: mouse macrophage protein standard; lane 2: untreated cells; lane 3: cells treated with IL-1β for 24 h. The sample present in the unmarked lane between lanes 2 and 3 was extracted from cells treated with TNF-α (10 ng/ml for 24 h).

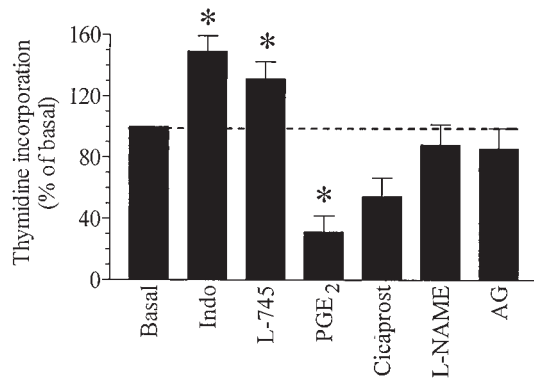


Figure 4. Effects of indomethacin, L-745,337, PGE₂, and cicaprost, all 10 μM; and L-NAME and aminoguanidine, both 1 mM, on control levels of serum-induced proliferation (10% FCS) of human PA SMC determined by the uptake of tritiated thymidine. Data are means ± SEM of *n* = 5–10 experiments. **P* < 0.05 by one sample *t* test compared with Basal.

NOS and COX Cross Talk in Rat PA SMC

Neither indomethacin nor L-745,337 (up to 100 μM) affected the release of nitrite from rat PA SMC stimulated with IL-1β (*n* = 3; data not shown). Moreover, aminoguanidine had no effect on the release of PGE₂ from rat PA SMC (up to 1 mM; *n* = 9). By contrast, L-NAME at concentrations in excess of those required to block iNOS inhibited the release of PGE₂ from rat PA cells stimulated with IL-1β (Table 1).

Serum-Induced Proliferation of Human and Rat PA SMC

FCS stimulated the uptake of tritiated thymidine into both rat and human PA SMC. Rat cells proliferated at a higher rate than did human cells, and required less serum to do so. Indeed, the optimal concentration of FCS required to cause proliferation was 5% for rat and 10% for human. Rat PA SMC cultured without serum incorporated 10,393 ±

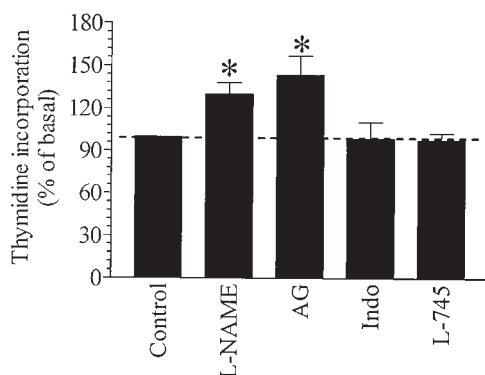


Figure 5. Effects of indomethacin and L-745,337, both 10 μM; and L-NAME and aminoguanidine, both 1 mM, on control levels of serum-induced proliferation (5% FCS) of rat PA SMC determined by the uptake of tritiated thymidine. Data are means ± SEM of *n* = 5–10 experiments. **P* < 0.05 by one sample *t* test compared with Control.

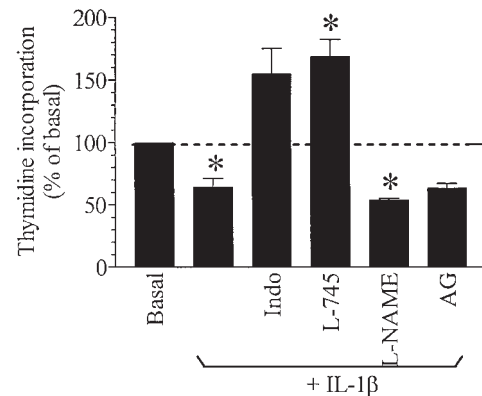


Figure 6. Effects of IL-1β (10 ng/ml) without and with indomethacin, L-745,337, L-NAME, and aminoguanidine on serum-induced proliferation (10% FCS) of human PA SMC determined by tritiated thymidine uptake. Data are means ± SEM of *n* = 5–10 experiments. **P* < 0.05 by one sample *t* test compared with Basal.

5,896 cpm of thymidine, which was increased to 64,318 ± 6,540 cpm with 5% FCS. Human cells under control culture conditions incorporated 549 ± 152 cpm without serum, which was increased to 12,065 ± 1,353 cpm after treatment with 10% FCS.

COX-2 and iNOS Involvement in PA Smooth-Muscle Cell Proliferation: a Comparison between Rats and Humans

When cells were stimulated with serum, indomethacin and L-745,337 (10 μM for each) increased proliferation of human PA SMC was seen (Figure 4). By contrast, PGE₂ and cicaprost, a prostacyclin analogue, significantly inhibited serum-induced proliferation of human PA SMC (Figure 4). However, serum-induced proliferation of rat PA SMC was unaffected by either indomethacin or L-745,337 (Figure 5) or PGE₂ (data not shown). Further, L-NAME and aminoguanidine (1 mM for each) had no effect on the pro-

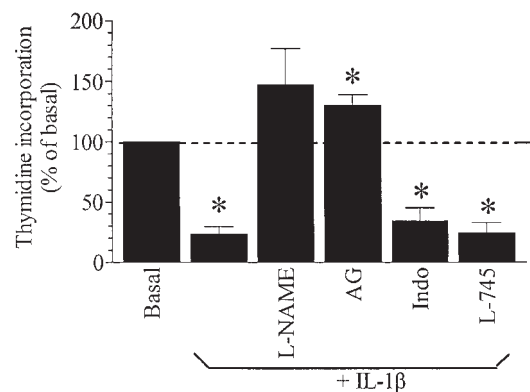


Figure 7. Effects of IL-1β (10 ng/ml) without and with L-NAME, aminoguanidine, indomethacin, and L-745,337 on serum-induced proliferation (5% FCS) of rat PA SMC determined by tritiated thymidine uptake. Data are means ± SEM of *n* = 5–10 experiments. **P* < 0.05 by one sample *t* test compared with Basal.

liferation of human SMC but significantly increased tritiated thymidine uptake in rat cells.

Inhibition of Serum-Induced Proliferation by IL-1 β

IL-1 β (10 ng/ml) significantly inhibited serum-induced proliferation of human and rat SMC by 40 and 77%, respectively (Figures 6 and 7). The inhibition of proliferation of human PA SMC by IL-1 β was reversed by both indomethacin and L-745,337 (both 10 μ M). By contrast, the nonselective iNOS inhibitor and iNOS inhibitors L-NAME and aminoguanidine had no effect on IL-1 β -induced inhibition of proliferation in human PA SMC (Figure 6). However, in rodent cells, neither indomethacin nor L-745,337 affected IL-1 β -induced inhibition of proliferation, whereas L-NAME and aminoguanidine (both 1 mM) reversed it (Figure 7).

Discussion

We have shown that rat PA SMC co-express iNOS and COX-2 after stimulation with IL-1 β . By contrast, under the same conditions, human PA SMC express COX-2 without iNOS. Further, these inducible enzymes clearly modulated cellular proliferation of both rat and human PA cells. However, rat cells appeared to be exclusively regulated by iNOS and human cells by COX-2.

A number of studies have established that rat vascular SMC from systemic vessels express iNOS and release large amounts of NO after stimulation with IL-1 β (16, 17). The levels of iNOS expressed and nitrite released by PA cells in our study are in line with those reported previously for other vascular smooth-muscle types (18, 19). Because rat PA SMC release very high levels of NO, iNOS may well have autocrine functions in these cells. This is clearly the case for the mechanical responsiveness of PA, which becomes hyporesponsive to contractile agents after iNOS induction (20). Proliferation of vascular SMC is inhibited by NO or NO containing compounds (4, 21, 22) and may therefore represent a function modulated by iNOS expression. Indeed, we found that when rat cells were treated with IL-1 β to induce iNOS, proliferation was greatly reduced.

In contrast to rat cells, few studies have been published showing induction of iNOS in human vascular SMC, although iNOS messenger RNA (mRNA) has been detected in human aorta after treatment with inflammatory cytokines and lipopolysaccharide (23). Nevertheless, the relative ability of human and rat cells to release NO after iNOS expression has not been addressed. In this study we were unable to detect either iNOS protein or NO production from human cells. Moreover, unlike the observations made using rat cells, we were unable to show any "functional" role for iNOS in the proliferative responses of human cells. It may, however, be the case that iNOS is expressed in very low amounts in human PA cells but is still able to modulate some other aspect of vascular responses.

In contrast to iNOS expression, COX-2 was induced in both human and rat cells after exposure to IL-1 β . Interestingly, COX-2 did not affect proliferation in rat cells but reduced tritiated thymidine uptake by human PA cells. Further, exogenous PGE₂ or the PGI₂ analog cicaprost

mimicked the actions of COX-2 in human PA cells. These observations suggest that COX-2, through the release of PGI₂ and/or PGE₂, inhibits proliferation of these cells via EP or IP receptor (2) activation. Similar observations have recently been reported for human systemic arterial SMC stimulated to express COX-2 with platelet-derived growth factor (24). Thus, under these *in vitro* conditions, COX-2 has an anti-inflammatory, antiangiogenic role in human PA cells through its ability to inhibit the proliferation of SMC. This anti-inflammatory role for COX-2 is not seen in rat cells, despite the facts that COX-2 protein is induced in these cells and that they release PGE₂ after treatment with IL-1 β . These observations suggest that rat and human evolution have diverged such that NOS and COX, although induced by the same stimulus, have different levels of activity and function in the two species.

Under some conditions but not others, the coinduction of iNOS and COX-2 results in "cross talk" between the enzyme pathways (25–31). However, we found no effect of indomethacin or L-745,337 on the production of nitrite. This is in contrast to inhibition by aspirin and ibuprofen of transcription of human iNOS gene transfected into rat vascular SMC (32) or enhanced levels of mRNA and nitrite production stimulated by indomethacin in rat mesangial cells (33), but is in agreement with the lack of effect of these drugs on activated macrophages (29, 30) and whole blood vessels in culture (34). Similarly, inhibition of the production of NO by aminoguanidine was without effect on PGE₂, suggesting that iNOS has no role in COX-2 activity in these cells. We have made similar observations using whole human PA in culture (35). Paradoxically we found that the mixed cNOS/iNOS inhibitor L-NAME significantly reduced the release of PGE₂. However, these effects of L-NAME occurred at higher concentrations than were required to inhibit nitrite release, and are therefore probably due to another property of the drug.

The observations made in this study may have relevance to the disease processes underlying pulmonary hypertension, in which inappropriate proliferation of PA SMC occurs. IL-1 β is elevated in the lungs of patients with pulmonary hypertension and may lead to the induction of COX-2. It is tempting to speculate that COX-2 is expressed in the pulmonary vessels of pulmonary hypertensive patients. If this is the case, we suggest that COX-2 products limit the already overactive proliferation of SMC that typifies this disease. Alternatively, the pulmonary vessels of patients who succumb to diseases such as pulmonary hypertension may have a dysfunction in the COX-2 pathway. Indeed, Wilborn and coworkers (36) have shown that fibroblasts isolated from patients with idiopathic pulmonary fibrosis have a diminished capacity to synthesize PGs and to express COX-2. Clinical data showing that infusion of PGI₂ analogs limit the progression of pulmonary hypertension (9) support this hypothesis. Many side effects are associated with the continuous delivery of this PG, such as pump reliability and line infection. Upregulation of endogenous COX-2 may represent an alternative therapeutic strategy.

Acknowledgments: One author (K.B.J.) is a MRC research assistant. Another author (J.A.M.) is a Wellcome Trust career development fellow. The authors thank Cathy Ratcliffe for her help with collection of the human PA.

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