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Regulation of Lysyl Oxidase and Cyclooxygenase Expression in Human Lung Fibroblasts: Interactions Among TGF-β, IL-1β, and Prostaglandin E

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Abstract Prostaglandin E_2, transforming growth factor-β, and interleukin-1β variably regulate the expression of cyclooxygenase 1, cyclooxygenase 2, and lysyl oxidase in IMR90, human embryo lung fibroblasts. Prostaglandin E_2 at 100 nM upregulates cyclooxygenase 1 mRNA by approximately three-fold while it downregulates lysyl oxidase mRNA levels. Notably, prostaglandin E_2 suppresses the enhancing effect of TGF-β on basal levels of lysyl oxidase mRNA. These changes in steady state mRNA levels reflect transcriptional level control, at least in part. Corresponding changes are seen in the protein levels of lysyl oxidase, cyclooxygenase 1 and cyclooxygenase 2 and in catalytic activities of these enzymes, including net prostaglandin E_2 synthesis. Cyclooxygenase 2 mRNA($t_{1/2}$, 30 min) is considerably less stable than that of cyclooxygenase 1 ($t_{1/2}$, 4 h) while lysyl oxidase mRNA is unusually stable ($t_{1/2} > 14$ h). Taken together with the differing kinetics with which these genes respond to perturbation by these cytokines, the present results suggest a coordinated, autocrine-like mechanism of regulation of cyclooxygenase 1 and cyclooxygenase 2 and further point to the potential of their metabolic product, prostaglandin E_2, to suppress the expression of lysyl oxidase in the inflammatory response to injury.

Key words: lysyl oxidase, cyclooxygenase, transforming growth factor-β, prostaglandin, interleukin-1β

The inflammatory response commonly occurs in the earliest phase of wound healing followed by new connective tissue matrix deposition [Inoue et al., 1995; Bauriedel et al., 1994]. Increases in prostaglandin, interleukin-1, and TGF-β accompany the inflammatory response and appear to act as signals modulating the production of matrix macromolecules by fibrogenic cells at the site of injury [Ridderstad et al., 1991]. We recently reported that the expression of LO, the enzyme which initiates the covalent crosslinking of collagen and elastin, converting these to insoluble fibers, is elevated by TGF-β and reduced by PGE_2 in a lipid-rich population of neonatal rat lung fibroblasts [Boak et al., 1994]. In view of the apparently counteracting effects of PGE_2 and TGF-β on the expression of LO, we further explored the response of both LO and COX-1 and 2 to these effectors as well as to IL-1β in cultured human lung fibroblasts. The two cyclooxygenases are key regulatory enzymes in the conversion of arachidonate and other 20 carbon polyunsaturated fatty acids to endoperoxides and ultimately to prostaglandins. The sequences of the coding regions of the two COX genes are distinctive with COX-1 cDNA hybridizing with COX-2 mRNA only under low stringency [Rosen et al., 1989]. The protein sequences are co-linear except for differences in the length of the amino- and carboxyl-terminal regions. COX-2 is an early gene whose expression is induced rapidly by such cytokines as IL-1 [Ristimaki et al., 1994]. COX-1 appears to function as both a housekeeping and inducible gene. Transcription of this gene is also induced by TGF-β and other cytokines [Jackson et al., 1993].

TGF-β is particularly abundant in the injured lung [Danielpour et al., 1989] and regulates the expression of extracellular matrix proteins, including collagen [Steenfos et al., 1994;

Abbreviations used: Act D, actinomycin D; BAPN, β-aminopropionitrile; LO, lysyl oxidase; COX, cyclooxygenase; DMEM, Dulbecco's Modified Eagle's medium; FBS, fetal bovine serum; IL, interleukin; PGE_2, prostaglandin E_2; RIA, radioimmunoassay; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TGFβ, transforming growth factor β1.

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Bruijn et al., 1994]. This cytokine can be produced by lung fibroblasts [Danielpour et al., 1989; Kelley et al., 1991] and may also derive from inflammatory cells responding to lung injury [Schalch et al., 1991]. Consistent with its stimulation of matrix protein expression, TGF-β enhances wound healing [Pierce et al., 1989]. IL-1β mediates many inflammatory events during the acute phase response, including fever, neutrophilia, and the increased synthesis of acute phase effectors such as prostaglandins. IL-1β stimulates prostaglandin production in numerous cell types while the functional effects of IL-1β are often mediated through prostaglandins [Hughes et al., 1989; Herrmann et al., 1990].

In the present report, we find that PGE_2, the principal prostanoid produced by the IMR90 [Ricupero et al., 1992], up-regulates the expression of COX-1 but not of COX-2 while it down-regulates the expression of LO in these cells. TGF-β, IL-1β, and PGE_2, in different combinations, variably determine the expression of these three enzymes. The results of these studies suggest that a feedback relationship exists between the prostanoid product of the two COX enzymes and LO which may contribute to the regulation of matrix production following inflammatory tissue injury.

MATERIALS AND METHODS

Cell Culture

IMR90 cells were maintained in culture in DMEM containing 10% FBS, as described previously [Ricupero et al., 1992; Goldstein et al., 1990]. Cultures were rendered quiescent by incubating in DMEM/0.4% FBS for 24 h prior to the addition of cytokines or other perturbants.

Northern Blotting

Total cell RNA was isolated by the method of Chomczynski and Sacchi [1987]. The RNA (15 μg) was fractionated on 1.2% agarose/formaldehyde gels and blotted onto GeneScreen nylon membranes (NEN Dupont). The blots were fixed by UV-crosslinking and hybridized with COX-1, COX-2, and LO DNA probes labeled with ^{32}P by random priming with the Ready to Go kit from Pharmacia. Human COX-1 cDNA was a gift from Dr. T. Hla, Holland Laboratory, American Red Cross. Human COX-2 (a Hind III fragment from a genomic clone containing exons 8–10) and human LO cDNA were prepared in our laboratory. Blots were prehybridized for 20 min at 68°C in Quick Hyb (Stratagene) and hybridized for 2 h in the same solution supplemented with the labeled probe. The blots were then washed at 50°C in 0.2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M NaCitrate, pH 7.0), 0.1% SDS and exposed to Kodak XAR X-ray film at −80°C. The relative levels of mRNA were quantified by scanning densitometry of the developed autoradiograms. Blots were exposed to film for the time needed to obtain an intensity of the bands of interest that was within the linear range of response of the densitometer.

Immunoprecipitation

IMR90 cells were grown to confluence and made quiescent as described. The cells were then cultured in the presence or absence of specific effectors in DMEM containing 0.4% FBS for 20 h. The cells were then incubated for 30 min at 37°C in methionine-free and serum-free DMEM and pulse labeled for 3 h with fresh aliquots of this medium supplemented with 30 μCi/ml of [^{35}S]methionine (673 Ci/mmol, New England Nuclear) in the presence or absence of the specific effectors. Medium and cell layers were harvested and prepared for quantitative immunoprecipitation as described, using rabbit anti bovine lysyl oxidase as the primary antibody [Bedell-Hogan et al., 1993], or rabbit anti COX (Oxford Biomedical, Oxford MI). Identical quantities of radioactivity were immunoprecipitated for each treatment.

Radioimmunoassay (RIA) for Prostaglandins

Polyclonal antibodies to specific prostaglandins were prepared in our laboratory. Cross-reactivity of the antisera against non-targeted prostaglandins was less than 4% [Menconi et al., 1984]. Radioimmunoassays were performed as previously described [Taylor and Polgar, 1980].

Lysyl Oxidase Assays

Lysyl oxidase activity was assayed against a recombinant human tropoelastin substrate labeled with L-[4,5-^3H]lysine as described [Kagan, 1986]. In typical assays, aliquots of conditioned medium were incubated with 375,000 dpm of the tritiated substrate for 3 h at 37°C in 0.15 M NaCl, 0.1 M sodium borate buffer, pH 8.0, in the presence or absence of 0.5 mM BAPN, an active site inhibitor of lysyl oxidase [Tang et al., 1983]. Tritiated water released during the incubation

was isolated by vacuum distillation and counted by liquid scintillation spectrometry. Enzyme activities were corrected for tritium release in BAPN-inhibited controls and normalized against total cell DNA. Purified lysyl oxidase from bovine aorta was used as a positive control in each assay. Total cell DNA was determined by a modified diphenylamine reaction assay [Richards, 1974].

RESULTS

IMR90 cells incubated with TGF-β IL-1β exhibit increased steady state levels of COX-1, COX-2, and LO mRNA. The maximum response to cytokines occurs in COX-1 mRNA at 18 h, in COX-2 mRNA at 4 h, and in LO mRNA at 8 h (data not shown). As shown (Fig. 1), PGE_2 (35 ng/ml) triples the COX-1 mRNA level while it decreases LO mRNA below the basal level but does not alter the levels of COX-2 mRNA. TGF-β (4 ng/ml) and IL-1β (50 pg/ml) increase the mRNA for all three enzymes when present individually. TGF-β and IL-1β in combination more than triple COX-2 mRNA levels, well above the effect of IL-1β and TGF-β alone. The inclusion of PGE_2 at 35 ng/ml with either TGF-β or IL-1β largely suppresses the stimulating effect of both cytokines on steady-state LO mRNA levels.

The corresponding effect of these agents on LO activity secreted into the medium during 24 h incubations is illustrated in Fig. 2. The total enzyme activity is increased approximately three-fold by TGF-β and approximately two-fold by IL-1β. When present alone, PGE_2 reduces the accumulated levels of enzyme activity slightly, whereas, when present together with IL-1β or TGF-β or with a combination of these two cytokines, PGE_2 completely suppresses the increases in LO activity induced by either of these two agents or by their combination.

COX and LO proteins, pulse-labeled with [^{35}S]methionine in cultures incubated in the presence or absence of the cytokines, were immunoprecipitated from the cell layer and medium. Autoradiography of the bands resolved by SDS-PAGE revealed that COX protein content increases in response to treatment with TGF-β and to a greater degree in response to PGE_2 (Fig. 3b). In contrast, PGE_2 (Fig. 3a) decreases the LO protein level and, when present together with TGF-β, prevents the marked increase in LO protein seen in response to TGF-β, alone. The bands seen at 46 and 50 kDa in the cell layer extract correspond to prolysyl oxidase and its N-glycosylated derivative, respectively, while that seen in the conditioned medium and cell layer extract at 30 kDa corresponds to the mature, proteolytically processed catalyst [Trackman et al., 1992]. The higher molecular weight bands predominantly seen in the conditioned

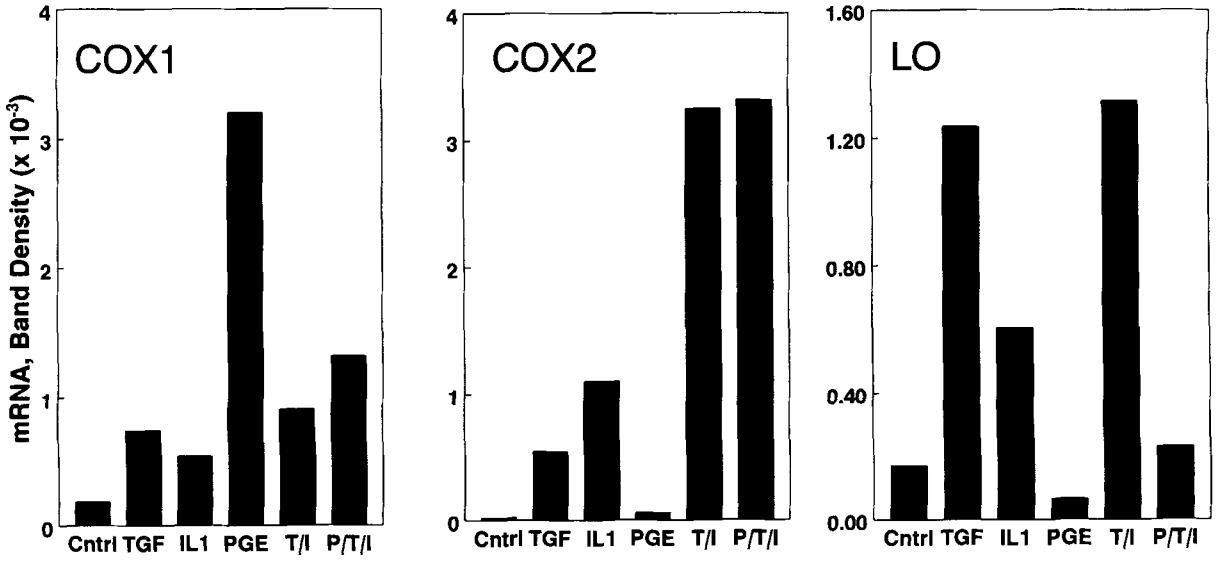

Fig. 1. Effect of TGF-β, IL-1β, and PGE_2 on COX-1, COX-2, and LO mRNA levels in IMR90 cells. Confluent, quiescent IMR90 cells were treated with 4 ng/ml TGF-β, 50 pg/ml of IL-1β, and 35 ng/ml of PGE_2 for 4 h (COX-2) and 8 h (LO) and 18 h (COX-1). RNA was extracted and a Northern blot prepared and probed as described in the Material and Methods section. The film was scanned with a Molecular Dynamics Computing Densitometer. Abbreviations: T or TGF = TGFβ, I or IL1 = IL-1β, PGE or P = PGE_2.

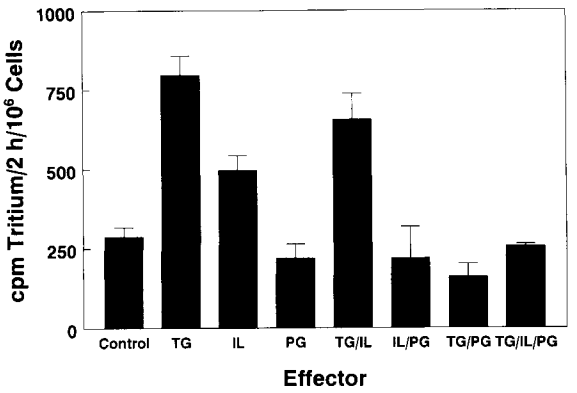

Fig. 2. Lysyl oxidase activity in IMR90 incubated in the presence or absence of TGF-β, IL-1β, or PGE_2. Confluent, quiescent IMR90 cells were incubated for 24 h in presence or absence of TGF-β (4 ng/ml), IL-1β (50 pg/ml), or PGE_2 (35 ng/ml). The culture medium was removed and assayed for enzyme activity as described. Each bar height is an average of values obtained from three separate cultures. The heights of the vertical lines represent two standard deviations. Abbreviations are as shown in Figure 1.

medium of the TGF-β-treated cultures are assumed to be proteins co-precipitating with the enzyme, consistent with the affinity of LO for matrix macromolecules [Kagan, 1986].

Incubation of IMR90 cultures with actinomycin D (1 μg/ml) suppresses the accumulation of detectable quantities of COX-2 mRNA in cells cultured in the presence or absence of IL-1β, TGF-β or in the combined presence of these effectors. Actinomycin D also suppresses the increase in COX-1 mRNA seen in response to TGF-β or PGE_2. This inhibitor of transcription also prevents the increase in LO mRNA levels induced both by IL-1β and TGF-β (Fig. 4).

The effect of the cytokines on PGE_2 synthesis by the IMR90 cells was assessed in the presence and absence of Act D. The action of phospholipase A_2 was bypassed by supplying the cells with excess (10 μM) free arachidonate. As illustrated in Fig. 5, TGF-β and IL-1β, when present individually, increase net synthesis of PGE_2. Treatment of the cells with a combination of these two cytokines increased synthesis well above the effects of the individual cytokines. Act D blocks the action of both effectors alone or in combination.

The relative stabilities of the transcripts for LO, COX-1, and COX-2 were determined by measuring mRNA levels for each at selected times after the addition of Act D to the IMR90 cultures. This procedure was performed on cultures previously stimulated with a mixture of TGF-β and IL-1β to generate levels of these transcripts sufficient for this kinetic analysis. As shown (Fig. 6), COX-2 mRNA decreases rapidly after Act D addition with a $t_{1/2}$ of approximately 30 min. COX-1 is more stable with a $t_{1/2}$ of approximately 4 h. Lysyl oxidase mRNA appears to be quite stable, with no apparent change seen throughout the 14 h after Act D addition.

DISCUSSION

The current data reveal that mRNA species coding for COX-1 and COX-2, enzymes critically involved in the production of prostaglandin, are markedly elevated in response to cytokines which are commonly elevated in inflammatory processes. Moreover, PGE_2, a specific downstream metabolic product of the prostaglandin biosynthetic pathway initiated by the action of these enzymes, potently stimulates the accumulation of the mRNA species for COX-1, a catalytic progenitor of PGE_2.

The kinetics of the increases induced by TGF-β and IL-1β in the mRNA levels for COX-1 and 2 suggest a coordinated response to these cytokines. COX-2 mRNA increases relatively quickly with a maximum effect noted at 4 h after addition of TGF-β and IL-1β to the IMR90 cultures. COX-1 mRNA, in contrast, becomes noticeably elevated only at a later time with a maximum level accumulating at 18 h after introduction of the cytokines. The product of the acute, early response of COX-2, PGE_2, could reasonably be expected to induce a subsequent increase in COX-1 expression, thus maintaining elevated levels of this prostaglandin in the affected tissue environment. The differing half-lives of the two COX mRNAs appear to accommodate this scenario well, since the COX-2 mRNA is considerably less stable than that of COX-1. Indeed, we previously observed that the COX-2 mRNA is essentially extinguished by 16 h after treatment of IMR90 cells with these cytokines [Jackson et al., 1993]. These effects appear to be consistent with an early, possibly acute phase role for COX-2 in prostaglandin production and a subsequent, more sustained role for COX-1 in maintaining prostaglandin levels in injured tissues.

The stimulation of COX-1 and 2 and LO mRNA levels by TGF-β and IL-1β reflects events occurring at least at the transcriptional level as indicated by the suppression of these effects by Act D. TGF-β has also been shown to activate the promoter for α1(I) collagen in IMR90 cells [Ritzenthaler et al., 1993] while both TGF-β and IL-1β are known to effect gene transcription in other cell types [Armendariz-Borunda et al.,

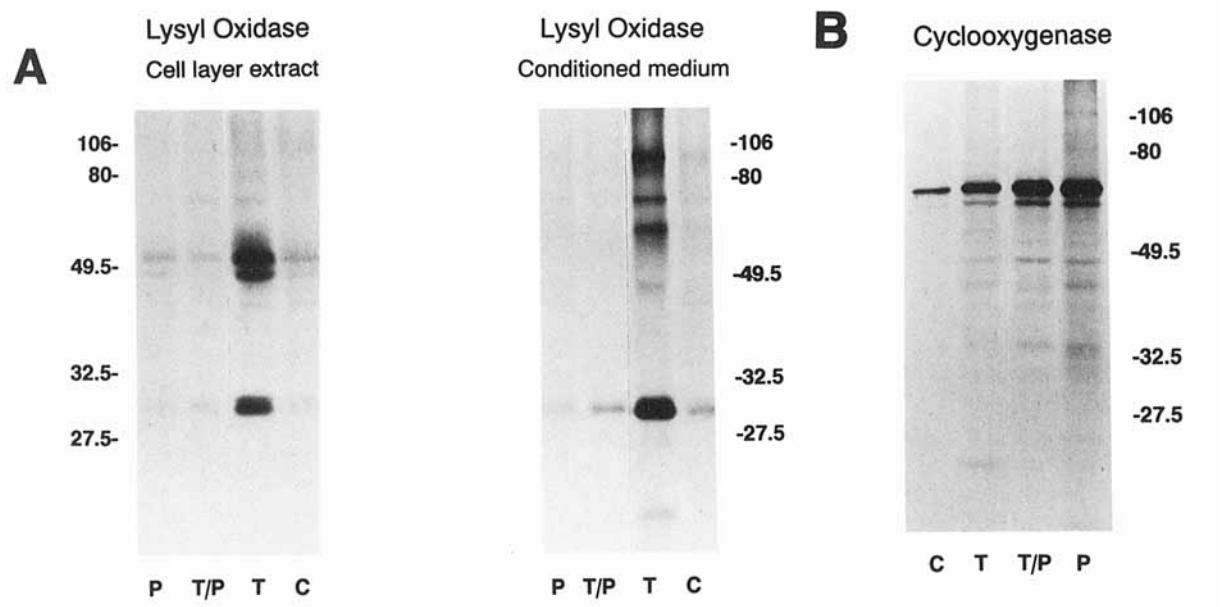

Fig. 3. SDS-PAGE of [^{35}S] methionine-labeled proteins immunoprecipitated from IMR90 cells. Confluent, quiescent cells were treated with TGF-β, 4 ng/ml; PGE_2 (35 ng/ml) or their combinations. (**A**) Immunoprecipitation with anti-lysyl oxidase of cell extracts (*Lanes 1–4*) or medium. The prominent bands at 50 and 46 kDa in the cell layer extract represent the N-glycosylated and non glycosylated forms of the LO proenzyme. The bands at 30 kDa in the cell layer and medium reflect the mature enzyme forms [Trackman et al., 1992]. (**B**) Immunoprecipitation with anti-cyclooxygenase of cell extracts. Abbreviations are as shown in Figure 1.

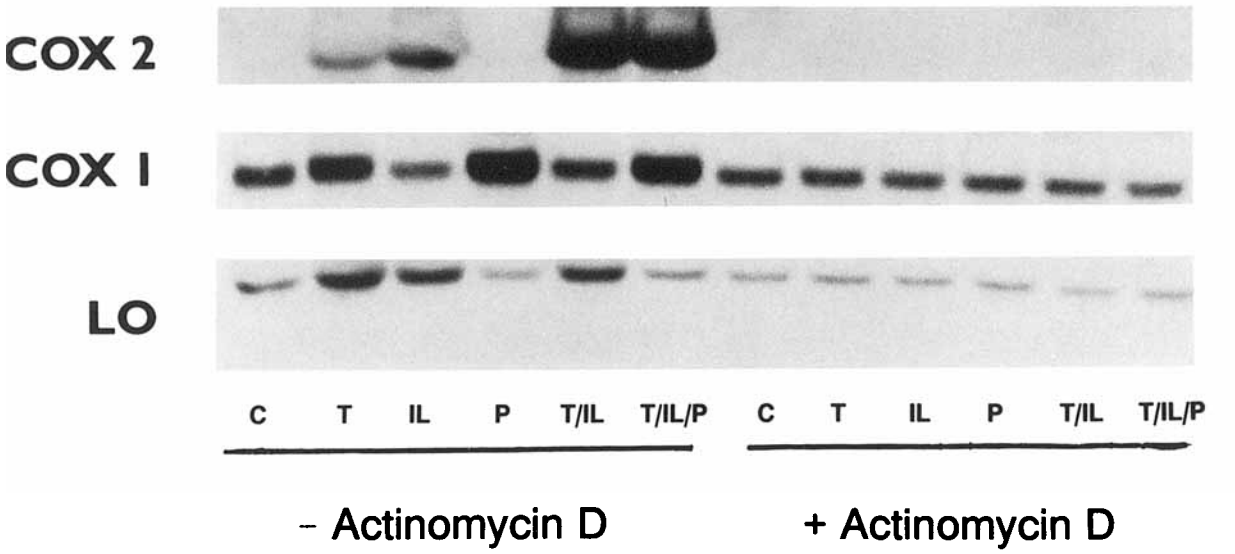

Fig. 4. Effect of Act D on mRNA Levels. Confluent, quiescent IMR90 cells were incubated for 7 hours with TGF-β (4 ng/ml), IL-1β (50 pg/ml), PGE_2 (35 ng/ml), or combination as indicated. A parallel set of cultures was incubated with the same effectors in the presence of an effector and Act D (1 μg/ml). RNA was extracted and analyzed by Northern blotting as described. Abbreviations are as shown in Figure 1.

1992, 1994]. The effects of these cytokines on the activity of the three gene products explored here may also involve post transcriptional regulation of gene expression. For example, TGF-β has been reported to increase the efficiency of translation of the α2(I) collagen transcript in the IMR90 [Fine et al., 1995] while TGF-β down regulates cdk4 expression [Ewen et al., 1995] and tumor necrosis factor expression [Bogdan et al., 1992] by inhibiting their translation.

Importantly, while PGE_2 increases COX-1 mRNA levels and, thus, potentially increases its

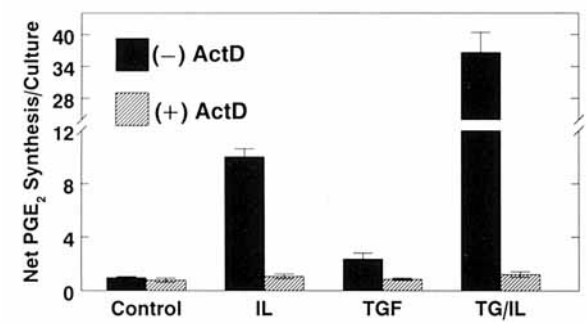

Fig. 5. Synthesis of prostaglandin E in response to cytokines in presence and absence of Act D. IMR90 cells were exposed to either IL-1β (50 pg/ml) or TGF-β (4 ng/ml) for 14 hours in the presence or absence of Act D (1 μg/ml). The cells were then washed free of serum and incubated with 10 μM arachidonate for 20 min. PGE_2 in the medium was determined by RIA. The data are presented as the mean of values obtained from quadruplicate cultures ± SEM.

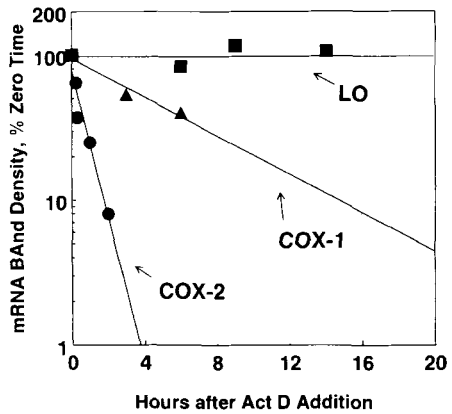

Fig. 6. Stability of COX-1, COX-2, and LO mRNA. Confluent, quiescent IMR90 cells were incubated with a mixture of TGF-β (4 ng/ml) and IL-1β (50 pg/ml) for different periods (COX-1, 18 h; COX-2, 4 h; LO 8 h) as required to generate the maximum increase in mRNA levels for each transcript. The cultures were then refed with fresh medium supplemented with Act D (1 μg/ml) in the absence of the cytokines. mRNA levels were determined by Northern blotting, as described.

own enzymatic production, this prostanoid essentially completely suppresses the marked increases in LO expression induced by TGF-β and/or IL-1β. Since PGE_2 lowers basal LO mRNA level only marginally, this prostanoid appears to have little effect on the intrinsic level of the LO mRNA, which as shown in these studies, is highly stable in these cells. The present results contribute to a growing body of evidence which suggests the participation of prostanoids, particularly PGE_2, in the regulation of extracellular matrix synthesis. For example, PGE_2 has been reported to inhibit the induction of the gene for collagen [Raisz et al., 1993]. This inhibition apparently occurs at the transcriptional level [Pilbeam et al., 1993]. PGE_2 has also been reported to regulate the expression of the collagenase gene [Pentland et al., 1995].

The analysis of proteins synthesized de novo and of PGE_2 synthesis confirm that cytokine and PGE_2 induced changes in steady state mRNA levels are accompanied by corresponding changes in the levels of protein and the catalytic action of the COX enzymes as well as LO. Furthermore, the corresponding changes seen in the mature (30 kDa) and proenzyme (50 and 46 kDa) forms of LO [Trackman et al., 1992] in response to these effectors indicate that the changes in mature enzyme levels do not stem from differing degrees of proteolytic processing of the proenzyme to the mature catalyst.

In summary, there appears to be a coordinated induction of the COX1 and COX2 genes resulting in the increased production of prostaglandin in response to cytokines. Further production of prostaglandin can occur through feedback induction of COX1 mRNA by accumulated PGE_2. In turn, these events may limit the production of connective tissue matrices in inflammatory responses to injury driven by such cytokines as TGF-β and IL-1β. Cyclooxygenase is the primary chemotherapeutic target of a number of non-steroidal anti-inflammatory agents which act by inhibiting prostaglandin production. These agents may have secondary, fibrotic effects by preventing the production of PGE_2 which would normally inhibit lysyl oxidase production. It is also important to take note of the recent evidence that lysyl oxidase exhibits potent chemotactic activity for monocytes and lymphocytes in vitro [Lazarus et al., 1995]. Thus, the prevention of the increased expression of LO by prostaglandin, the production of which is itself stimulated by inflammatory processes, may be important as a biological mechanism for limiting the degree of inflammation and of the degree of fibrosis accompanying the inflammatory response.

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