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Regulation of Human Lung Fibroblast \( \alpha 1(\text{I}) \) Procollagen Gene Expression by Tumor Necrosis Factor \( \alpha \), Interleukin-1\( \beta \), and Prostaglandin E\( _2 \)*

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We investigated the participation of prostaglandin (PG) \( E_2 \) in the regulation of the \( \alpha 1(\text{I}) \) procollagen gene expression by tumor necrosis factor \( \alpha \) (TNF\( \alpha \)), and interleukin-1\( \beta \) (IL-1\( \beta \)) in normal adult human lung fibroblasts. TNF\( \alpha \) (100 units/ml) and IL-1\( \beta \) (100 units/ml) stimulated the production of PGE\( _2 \) and caused a dose-dependent inhibition of up to 54 and 66%, respectively, of the production of type I procollagen. Premature cultures with indomethacin partially reversed the inhibition of procollagen production induced by the cytokines. Cytokine-stimulated endogenous fibroblast PG accounted for 35 and 68% of the inhibition induced by TNF\( \alpha \) and IL-1\( \beta \), respectively. Steady-state mRNA levels for \( \alpha 1(\text{I}) \) procollagen paralleled the changes in collagen production. The transcription rate of the \( \alpha 1(\text{I}) \) procollagen gene was reduced by 58% by TNF\( \alpha \) and by 43% by IL-1\( \beta \). Cytokine-stimulated endogenous PG production accounted for half of these effects. These results indicate that TNF\( \alpha \) and IL-1\( \beta \) inhibit the expression of the \( \alpha 1(\text{I}) \) procollagen gene in human lung fibroblasts at the transcriptional level by a PGE\( _2 \)-independent effect as well as through the effect of endogenous fibroblast PGE\( _2 \) released under the stimulus of the cytokines.

Fibrillar collagens are the most abundant proteins in the lung interstitium and constitute about 15% of the dry weight of the human lung (1). Because of the high turnover of the connective tissue of adult lung (2), the balance between synthesis and degradation must be accurately controlled in order to assure the preservation of normal structure and function. Exaggerated tissue deposition of extracellular matrix proteins is the final outcome of several diseases in which an inflammatory process triggered by various stimuli is the earliest event (3). Tissues undergoing a chronic inflammatory process are often infiltrated by macrophages and lymphocytes. These are the main cell lineages responsible for the production of various cytokines that have been implicated in the initiation, progression, and eventual modulation of a variety of inflammatory and immunologic responses. It has been shown that in addition to their participation in inflammation and the immune response, several cytokines can exert profound effects on fibroblast production of extracellular matrix proteins (4). Thus, it is very likely that they would participate in tissue remodeling and, perhaps, in the development of fibrosis (2).

Tumor necrosis factor \( \alpha \) (TNF\( \alpha \)) and interleukin-1\( \beta \) (IL-1\( \beta \)) are cytokines produced mainly by activated cells of the monocyte/macrophage lineage. These cytokines have many overlapping activities and play a central role in inflammation, T cell activation, and cytokotoxicity (5, 6). It is thought that TNF\( \alpha \) and IL-1\( \beta \) participate in tissue remodeling because of their ability to promote fibroblast growth and angiogenesis and to stimulate the production of collagenase. Multiple studies have examined the effects of TNF\( \alpha \) and/or IL-1 on the production of extracellular matrix proteins by various mesenchymal cells (7-27). In adult and fetal dermal fibroblasts, TNF\( \alpha \) inhibited the production of type I and III procollagens and decreased the levels of their corresponding mRNAs (17-19, 26). However, other reports showed that TNF\( \alpha \) stimulated collagen production in dermal and lung fibroblasts (20, 21, 25). The effects of IL-1 on fibroblast collagen production are also controversial. IL-1 has been shown both to increase and inhibit collagen production. These effects were accompanied by parallel changes in the steady-state levels of the corresponding mRNAs in most studies (20, 22-25), although in other studies an inhibition of collagen production with a paradoxical increase in collagen mRNA levels was found (26, 27).

The products of the cyclooxygenase pathway, such as PGE\( _2 \), also participate in inflammatory and immune responses. TNF\( \alpha \) and IL-1\( \beta \) stimulate PGE\( _2 \) production in several cell lines including macrophages (28), synovial cells, and fibroblasts (29, 30) and increase the steady-state levels of cyclooxygenase mRNA (31). There is evidence that PGE\( _2 \) inhibits collagen production (32) by several mechanisms, including a decreased uptake of proline (33) and an increase in the intracellular degradation of the protein (34). Furthermore, decreased steady-state mRNA levels for \( \alpha 1(\text{I}) \) procollagen have been shown in PGE\( _2 \)-treated fibroblasts, indicating that PGE\( _2 \) also acts at pretranslational levels (35). Several studies have examined the contribution of increased endogenous PG production by TNF\( \alpha \) and IL-1 on the modulation of fibroblast collagen gene expression (18, 19, 22-27). In only two of these studies, it was shown that PGs play a modulatory role (24, 27). Because of our interest on the regulation of fibroblast

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†‡ The abbreviations used are: TNF\( \alpha \), tumor necrosis factor \( \alpha \); IL-1\( \beta \), interleukin-1\( \beta \); PG, prostaglandin; PGE\( _2 \), prostaglandin E\( _2 \); IFN\( \gamma \), \( \gamma \)-interferon.
collagen gene expression by cytokines and cytokine-stimu-
lated endogenous PG, we conducted the studies presented here to clarify the conflicting results described above. We postulated that stimulation of fibroblast PGE2 production by TNFa and IL-1β might play a role in the net effect of these cytokines on type I collagen production, in a manner similar to that shown with TGFβ (36). We present evidence that TNFa and IL-1β inhibit lung fibroblast type I collagen production and decrease the corresponding steady-state mRNA levels and that these effects are mediated by PG-dependent and -independent mechanisms. Furthermore, we found that TNFa, IL-1β, and PGE2 modulation of the steady-state mRNA levels for α1(I) procollagen is largely exerted at the transcriptional level.

**Materials and Methods**

**Human Lung Fibroblast Cell Lines**—Human lung fibroblast cell lines were established from histologically normal lung tissue resected for diagnostic purposes, from the left lingula of a 57-year-old white female (ID), from the right lung of a 52-year-old white female (PM), or from a 20-year-old African female (CCL-210; purchased from ATCC, Rockville, MD).

**Fibroblast Cultures and Labeling Conditions**—Early passage (fifth to eighth passage) fibroblasts were plated at a density of 5 × 10⁴ cells/well in 24-well flat-bottom plates and cultured in Eagle’s minimal essential medium supplemented with 10% fetal calf serum, 1% (v/v) vitamin solution (GHRB), and 2 mM L-glutamine and incubated at 37 °C in a 5% CO₂ atmosphere. The three cell lines reached confluence at approximately 8–9 days. When the cultures reached confluence, the media were removed, and fresh medium containing 5% fetal calf serum, 1% (v/v) vitamin solution, 2 mM L-glutamine, and ascorbic acid (50 µg/ml) was added. After 24-h incubation, fresh medium supplemented with 5% fetal calf serum, 1% (v/v) vitamin solution, 2 mM L-glutamine, and ascorbic acid (50 µg/ml) was added. Samples were then washed in Hanks’ solution and harvested immediately in 4 M guanidinium isothiocyanate. Total RNA was isolated in a CsCl discontinuous gradient as described previously (40). For Northern blot hybridizations, aliquots containing equal amounts of total RNA were denatured in formamide, electrophoresed in 0.8% agarose/glycerol-formaldehyde gel, transferred to nylon membranes (LKB 2016 VacuGene) to nitrocellulose filters (Optiprobe S & U) and UV-cross-linked (UV Stratalinker 2400, Stratagene). The human cDNA clone H677 specific for α1(I) procollagen (41) and a mouse cDNA specific for glyceraldehyde-3-phosphate dehydrogenase (42) were nick-translated with [α-32P]dCTP to specific activities >10⁶ cpm/µg DNA. Filters were washed first in 0.1 M sodium citrate and then sequentially with 2× SSC at 50 °C, 0.2× SSC at 65 °C, and 0.2× SSC at room temperature and then for 15 min in 0.2× SSC at 65 °C and treated with RNase A (10 µg/ml in 2× SSC) for 15 min at 37 °C. The filters were then washed in 2× SSC, 0.1% SDS for 15 min at room temperature and dried. Autoradiographs were obtained and scanned in a laser densitometer. The amount of [3H]32P hybridized to each dot blot was determined by scintillation counting.

**RESULTS**

**Effects of TNFa and IL-1β on Collagen Production in the Presence or Absence of Indomethacin**—Treatment of lung fibroblasts with increasing concentrations of TNFa or IL-1β resulted in a dose-dependent inhibition of type I procollagen production as analyzed by quantitative densitometry of fluorographs from SDS-polyacrylamide gel electrophoresis. Fluorographs of an illustrative experiment with TNFa are shown in Fig. 1A and with IL-1β in Fig. 24. To investigate whether the inhibition of type I procollagen production induced by
TNFα and IL-1β was related to stimulation of endogenous fibroblast PG synthesis by the cytokines, parallel cultures were preincubated with indomethacin (1 µg/ml), a concentration shown previously to produce complete inhibition of cyclooxygenase in these cells. The preincubation of cultures with indomethacin reversed only partially the inhibitory effects of TNFα (Fig. 1B) and IL-1β (Fig. 2B) on type I procollagen production. Densitometric scanning of fluorographs showed a maximal inhibition of newly synthesized type I procollagen of 54% at a concentration of 100 units/ml of TNFα. The concomitant treatment of cells with indomethacin resulted in an inhibition of only 35%. Therefore, endogenous PG accounted for 35% of the total inhibitory effect of TNFα (Fig. 1C).

Similar analysis showed that 100 units/ml of IL-1β caused a maximal inhibition of 66% on type I procollagen production. The concomitant treatment of cells with indomethacin resulted in an inhibition of only 21%. Therefore, endogenous PG accounted for 68% of the total inhibitory effect of IL-1β (Fig. 2C). However, at lower concentrations of IL-1β, indomethacin completely abolished IL-1β inhibition of collagen production, suggesting that at these concentrations, the collagen inhibitory effects of the cytokine are entirely PG-dependent. Next, we examined the effect of the combination of both cytokines on type I procollagen production. Incubation of lung fibroblasts in the presence of constant concentrations of TNFα and increasing concentrations of IL-1β showed that the inhibition of procollagen production was more pronounced (72%) than when the cultures were incubated with a single cytokine (Fig. 3A). Preincubation of cultures with indomethacin showed only a partial reversal of the inhibitory effect of the combination of both cytokines to 55% (Fig. 3B). Thus, the contribution of endogenous PG accounted for only 23% of the total inhibitory effect of that of the combination of both cytokines (Fig. 3C). These results were confirmed by a specific collagenase assay in two separate experiments (Table I).

To exclude the possibility that the observed effects were the result of a global cytotoxic effect of TNFα, cells were labeled with [35S]methionine or with [3H]glucosamine. As shown in Table II, incubation of cells with TNFα (100 units/ml) did not affect the incorporation of [35S]methionine into total proteins. Furthermore, the incorporation of [3H]glucosamine into glycoproteins and glycosaminoglycans was increased in a dose-dependent manner by TNFα, reaching a maximal stimulation of 39% at 100 units/ml. In addition, trypan blue exclusion showed a viability greater than 90% in cells cultured under either control conditions or treated with 50 or 100 units/ml TNFα.

Effects of TNFα and IL-13 on PGE2 Production—As shown in Table III, incubation of lung fibroblasts with TNFα (100 units/ml) caused an increase in PGE2 production from 1.06 to 13.74 ng/ml, and incubation with IL-1β (50 units/ml) caused an increase from 2.16 to 30.56 ng/ml. The incubation with both cytokines resulted in higher PGE2 production than
incubation with either cytokine alone. Preincubation of cells with indomethacin completely abolished PGE_2 production by cells treated with TNFa, IL-1β, or TNFa plus IL-1β (results not shown).

**Effects of TNFa and IL-1β on α(1) Procollagen mRNA Levels**—In order to investigate the mechanisms of the inhibition of fibroblast type I procollagen production by TNFa and IL-1β, the steady-state mRNA levels for α(1) procollagen were examined by Northern blot hybridizations with a specific human cDNA. TNFa produced a dose- and length of incubation-dependent reduction of the steady-state mRNA levels for α(1) procollagen with maximal reduction of 76% at a concentration of 100 units/ml (Fig. 4A) and of 67% after 16 h of incubation with the cytokine (Fig. 4B).

Northern hybridization analysis of total RNA from lung fibroblasts treated with increasing concentrations of IL-1β showed a dose-dependent reduction of α(1) procollagen steady-state mRNA levels that reached a 68% at 100 units/ml (Fig. 5A). IL-1β induced a length of incubation-dependent reduction of α(1) procollagen steady-state mRNA levels that reached 91% at 24 h (Fig. 5B).

**Participation of Endogenous PG on the Reduction of α(1) Procollagen mRNA Levels by TNFa and IL-1β**—To investigate the participation of PG on the effects of TNFa on the α(1) procollagen steady-state mRNA levels, control and TNFa-treated cells were incubated with or without indomethacin or exogenous PGE_2 and total RNA was analyzed by Northern hybridization (Fig. 6). In agreement with a previous report (35), the treatment of control cultures with PGE_2 (100 ng/ml) resulted in a marked decrease (up to 60%) of the α(1) procollagen steady-state mRNA levels. Treatment of cultures with TNFa (100 units/ml) reduced the α(1) procollagen steady-state mRNA levels by 38%. This decrease was partially reversed by preincubation with indomethacin, as only a 22% diminution was observed in cultures treated with TNFa plus indomethacin and PGE_2 and total RNA was analyzed by Northern hybridization (Fig. 6). In agreement with a previous report (35), the treatment of control cultures with PGE_2 (100 ng/ml) resulted in a marked decrease (up to 60%) of the α(1) procollagen steady-state mRNA levels. Treatment of cultures with TNFa (100 units/ml) reduced the α(1) procollagen steady-state mRNA levels by 38%. This decrease was partially reversed by preincubation with indomethacin, as only a 22% diminution was observed in cultures treated with TNFa plus indomethacin and PGE_2 and total RNA was analyzed by Northern hybridization (Fig. 6).
Table III

Effect of TNFα and IL-1β on PGE₂ production by cultured human lung fibroblasts

Confluent human lung fibroblasts (cell line PM) were incubated for various intervals with TNFα (100 units/ml) or IL-1β (50 units/ml). At the end of the incubations PGE₂ was determined in the media by radioimmunoassay, and total RNA was extracted from cell layers for Northern hybridizations shown in Figs. 4 and 5.

<table>
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<tr>
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<th>TNFα</th>
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<tr>
<td>h</td>
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<tr>
<td>0</td>
<td>1.06 ± 0.32</td>
<td>2.16 ± 0.28</td>
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<tr>
<td>0.5</td>
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<td>4</td>
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<td>10.12 ± 0.3</td>
</tr>
<tr>
<td>8</td>
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<tr>
<td>16</td>
<td>10.68 ± 0.86</td>
<td>20.12 ± 6.3</td>
</tr>
<tr>
<td>24</td>
<td>13.74 ± 2.84</td>
<td>30.56 ± 0.96</td>
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Fig. 4. Time- and dose-dependent reduction of α(1) procollagen steady-state mRNA levels by TNFα in cultured human lung fibroblasts. Confluent human lung fibroblasts (cell line PM) were incubated for 18 h with various concentrations of TNFα (A) or with TNFα (200 units/ml) for various intervals (B). Total RNA was extracted as described under "Material and Methods." Samples containing 10 μg of total RNA were denatured, electrophoresed on a 0.8% agarose gel, and transferred to a nitrocellulose filter. The filter was hybridized to a radiolabeled human cDNA specific for α(1) procollagen and processed by autoradiography.

indomethacin. Therefore, endogenous PG accounted for 43% of the effect of TNFα. The addition of exogenous PGE₂ (100 ng/ml) to cultures treated with TNFα (100 units/ml) plus indomethacin (1 μg/ml) resulted in an additive decrease of 70% on the α(1) procollagen steady-state mRNA levels. Incubation of lung fibroblasts with IL-1β decreased the α(1) procollagen steady-state mRNA levels by 61%. However, when IL-1β-treated cells were preincubated with indomethacin the α(1) procollagen mRNA steady-state levels decreased by only 35% (Fig 7). Incubation of cultures with TNFα plus IL-1β caused a 72% reduction of the steady-state mRNA levels for α(1) procollagen. This inhibition was not reversed by preincubation of the cultures with indomethacin, indicating that when the cells were exposed to a combination of TNFα plus IL-1β at these concentrations, the main mechanisms affecting the α(1) procollagen steady-state mRNA levels were PG-independent.

Effect of TNFα, IL-1β, and PGE₂ on the Transcription Rate of the α(1) Procollagen Gene—In order to investigate whether the decrease on the α(1) procollagen steady-state mRNA levels induced by TNFα is mediated by transcriptional mechanisms, lung fibroblasts were incubated for 24 h with TNFα with or without indomethacin or with PGE₂ plus indomethacin, and the transcription rates of the α(1) procollagen gene were measured by an in vitro nuclear transcription assay. Control cultures were incubated with indomethacin to eliminate any influence of endogenous PG and allow maximal expression of the α(1) procollagen gene. Treatment of cells with indomethacin plus PGE₂ (100 ng/ml) resulted in a 37% inhibition of the transcription rate of the α(1) procollagen gene (Fig. 8). Treatment of cells with TNFα (100 units/ml) resulted in greater inhibition (68%) of the transcription rate of the gene. This effect was partially reversed by the preincubation of cells with indomethacin (inhibition of only 25%). Therefore, endogenous PG accounted for 57% of the inhibitory effect of TNFα. In a separate experiment lung fibroblasts were incubated with vehicle alone (EtOH), indomethacin alone, IL-1β alone, or IL-1β plus indomethacin (Fig. 8). Cells treated with vehicle alone showed a modest inhibition of the transcription rate of the α(1) procollagen gene (20%) as compared with indomethacin-treated cells. This level of inhibition, therefore, reflects the level of inhibition induced by endogenous PG under basal conditions. Treatment with IL-1β alone caused a 43% inhibition of the transcription rate of the α(1) procollagen gene, and preincubation of IL-1β-treated cultures with indomethacin partially reversed the inhibitory effect of IL-1β to only 18%. Therefore, endogenous PG accounted for 58% of the inhibitory effect of IL-1β. These observations indicate that the decrease in the transcription rate of the gene by TNFα and IL-1β results from a combination of direct inhibitory effects of the cytokines plus the inhibitory effects of endogenous PGE₂.

Discussion

Here we present evidence that TNFα and IL-1β down-regulate the production of type I procollagen in normal human lung fibroblasts by modulating the steady-state mRNA levels...
for the protein and that these effects occur largely through inhibition of transcription. Blockade of endogenous fibroblast PG production reversed only partially these effects. Addition of exogenous PGE₂ reduced the steady-state mRNA levels and the transcription rate of the α1(I) procollagen gene. These results demonstrate that the inhibitory effect of TNFα and IL-1β on lung fibroblast collagen production is partially due to the effects of newly synthesized fibroblast PG in response to TNFα and IL-1β stimulation. The inhibitory effects of TNFα were selective for collagen and were not related to toxicity as demonstrated by the absence of changes in the incorporation of [³⁵S]methionine, the increased synthesis of glycosaminoglycans and glycoproteins (20, 45), and the high level of cell viability as measured by trypan blue exclusion. Although the results presented have clearly demonstrated an effect of IL-1 and TNFα on collagen synthesis, an additional mechanism for the inhibition on the production of collagen by TNFα and IL-1β must be that of intracellular and extracellular degradation induced by the increased production of PG and increased production of collagenase, respectively (6).

We found that the inhibitory effects of the cytokines on the steady-state mRNA levels were variable within the same cell line (60–90% for IL-1β in PM) or for the same cytokine in different cell lines (TNFα, ~70% in PM and 40% in ID).

Despite this variability, the effects of TNFα and IL-1 on the two cell lines described here and in an additional cell line (CCL-210; not shown) were consistently inhibitory on the α1(I) procollagen gene expression, at the three levels examined, i.e. rates of gene transcription, steady-state mRNA levels, and protein production. Furthermore the participation of endogenous prostaglandins on this inhibitory effect was found also at the three levels of protein biosynthetic pathway examined. This consistency makes it very unlikely that a clonal selection of a particular cell could be responsible for the results we obtained (46). The discrepancies with previous reports that examined the influence of PG on TNFα and IL-1β effects on collagen production could be due to intrinsic differences in the ability of different cell types to produce or to respond to endogenous PG. It is also possible that under particular experimental conditions such as serum-free or low serum conditions, the endogenous PG production could be too low to cause detectable effects on collagen production. On the other hand, very high concentrations of TNFα or IL-1β or their combination could inhibit the expression of the procollagen gene by PG-independent mechanisms that cannot be reversed by inhibition of endogenous PG production. The observations described here when cultures were exposed to a
of cell to cell and cytokine interactions is required for the increased deposition of extracellular matrix in lung fibrosis. For example, TNFα in combination with IL-1 and interferon-γ (IFNγ) increases the adherence of T-lymphocytes to human lung fibroblasts (51), presumably through the induction of the intercellular adhesion molecule-1 (ICAM-1). TNFα in conjunction with IFNγ induces or amplifies the expression of HLA class II antigens in monocytes (52) and T cells (53), providing an additional mechanism for cell adherence, and more importantly, enhancing their antigen presenting capability. TNFα and IL-1β also increase the expression of high affinity IL-2 receptors in T cells (53), enhancing their proliferative response. The macrophage and lymphocyte activation caused by TNFα and IL-1β would stimulate the production of powerful fibrogenic factor(s) such as TGFβ by these cells. These cytokine interactions turn more complex if their interdependence with the products of arachidonic acid metabolism are considered. In conclusion, human lung fibroblasts TNFα and IL-1β inhibit the production of α1(I) procollagen largely at the transcriptional level by PGE2-dependent and independent mechanisms. Although these cytokines appear to be important mediators in the early inflammatory stages of lung fibrosis, the chronic deposition of extracellular matrix proteins leading to fibrosis must be the result of more complex cellular responses.

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**FIG. 8. Effects of TNFα and IL-1β and PGE2 on the transcription rate of the α1(I) procollagen gene in cultured human lung fibroblasts.** In two separate experiments (Experiment 1, lanes 1–4; Experiment 2, lanes 5–8) confluent human lung fibroblasts (Experiment 1, cell line ID; Experiment 2, cell line PM) were incubated for 24 h under the following conditions. Lane 1, indomethacin (1 μg/ml); lane 2, indomethacin (1 μg/ml) + PGE2 (10 ng/ml); lane 3, TNFα (100 units/ml); lane 4, TNFα (100 units/ml) + indomethacin (1 μg/ml); lane 5, vehicle (10 μl/ml 10% EtOH); lane 6, indomethacin (1 μg/ml); lane 7, IL-1β (50 units/ml); lane 8, IL-1β (50 units/ml) + indomethacin (1 μg/ml). Nuclei were isolated and in vitro transcription assays performed as described under "Materials and Methods." Labeled transcripts from each sample (3 × 106 cpm in Experiment 1 and 1 × 106 cpm in Experiment 2) were hybridized to the filter-bound cDNAs for α1(I) procollagen and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and to the plasmid pBR322. After washing and digestion with RNase A, the filters were processed by autoradiography (A); autoradiograms were scanned in a laser densitometer, the pBR322 background was subtracted, and the integrated areas corresponding to hybridized α1(I) procollagen transcripts were normalized with the areas corresponding to hybridized glyceraldehyde-3-phosphate dehydrogenase transcripts. The resulting values are expressed in arbitrary densitometric units (AU) as a percentage relative to values from samples incubated with indomethacin alone (B).
Collagen Regulation by TNFα, IL-1β, and PGE₂