The Differential Effect of Prostaglandin \( \text{E}_2 \) on Transforming Growth Factor-\( \beta \) and Insulin-induced Collagen Formation in Lung Fibroblasts*

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We examined the effect of prostaglandin (PG) \( \text{E}_2 \) on transforming growth factor-\( \beta \) (TGF-\( \beta \)) and insulin-stimulated collagen formation in lung fibroblast cultures. TGF-\( \beta \) increased type I collagen production 2-3-fold as determined by the densitometric analysis of autoradiograms from polyacrylamide gels and by measuring the amount of nondialyzable hydroxyproline. This was associated with a 5-6-fold increase in \( \alpha(1) \) mRNA levels. \( \text{PGE}_2 \) at \( 10^{-7} \text{ M} \) strongly inhibited type I collagen formation in TGF-\( \beta \)-stimulated cultures by 60-70%. \( \text{PGE}_2 \) blocked collagen formation in TGF-\( \beta \)-stimulated cultures by decreasing levels of \( \alpha(1) \) gene expression. \( \text{PGE}_2 \) inhibited \( \alpha(1) \) gene expression in part through inhibition of transcription. In contrast, insulin increased type I collagen production 2-fold and was associated with a 30-40% increase in \( \alpha(1) \) mRNA. Although \( \text{PGE}_2 \) also decreased \( \alpha(1) \) mRNA levels in insulin-treated cultures, \( \text{PGE}_2 \) had no effect on collagen formation. Taken together, these results demonstrate that collagen formation may proceed through two distinct pathways. These two pathways are distinguishable by their sensitivity to \( \text{PGE}_2 \) treatment and their relationship to increases in collagen mRNAs.

In fibroblast cultures, prostaglandin \( \text{E}_2 \) (\( \text{PGE}_2 \)) inhibits amino acid uptake (1), cell proliferation (2), and collagen production (3). We found previously that \( \text{PGE}_2 \) exerts a relatively specific effect on collagen synthesis, inhibiting collagen production more dramatically than overall protein synthesis (3). The mechanism whereby \( \text{PGE}_2 \) inhibits collagen production may involve increased intracellular degradation of newly synthesized collagen molecules (4, 5) and possibly decreased rates of collagen gene transcription (6). In contrast to the inhibitory effect of \( \text{PGE}_2 \) effector molecules such as insulin-like peptides and transforming growth factor \( \beta \) (TGF-\( \beta \)) are potent stimulators of collagen formation (7, 8). Administration of these effector molecules individually to fibroblast cultures does not stimulate cell proliferation (7, 8).

Insulin and insulin-like growth factor I both activate collagen formation in lung fibroblasts through interactions with insulin-like growth factor I receptor (7). TGF-\( \beta \) activates collagen formation through interactions with its own specific cell surface receptor (8-11). Insulin has been shown to phosphorylate the 56 ribosomal protein whereas TGF-\( \beta \) does not phosphorylate this protein (12-15). Phosphorylation of this protein may be involved in regulating ribosomal function (12-16). Taken together, these data indicate that TGF-\( \beta \) and the insulin-like peptides may activate collagen synthesis through separate cellular pathways.

In this paper, we investigated the mechanism of \( \text{PGE}_2 \)-mediated inhibition of collagen production by examining the differential effect of \( \text{PGE}_2 \) on insulin- and TGF-\( \beta \)-induced collagen formation. We found that \( \text{PGE}_2 \) inhibited TGF-\( \beta \)-induced collagen production. In contrast, \( \text{PGE}_2 \) did not block insulin-induced collagen production.

**MATERIALS AND METHODS**

Human embryonic lung fibroblasts (IMR-90, Institute for Medical Research, Camden, NJ) were grown in Dulbecco's modified Eagle's medium with 0.37 g of sodium bicarbonate/100 ml, 10% fetal bovine serum, 100 units of penicillin/ml, 10 \( \mu \)g of streptomycin/ml, and 0.1 mM nonessential amino acids. The cells were maintained in a humidified 5% CO\(_2\)-95% air incubator at 37°C. The cell cultures were grown to confluence in 150-(Lux), 100-, or 35-mm dishes (Falcon). After confluence was reached, the cells were plated into the quiescent state by reducing the serum content to 0.4% and then treated with insulin (Collaborative Research) or TGF-\( \beta \) (Research Diagnostic Systems) in the presence or absence of \( \text{PGE}_2 \).

**Polyacrylamide Gel Electrophoresis (PAGE)**—Confluent quiescent fibroblast cultures were labeled in serum-free medium containing \( ^{3} \text{H} \)proline (2 \( \mu \)Cl/ml) and ascorbate (90 \( \mu \)g/ml) in the presence or absence of \( \text{PGE}_2 \). During labeling of proteins, cells received no further additions or were incubated with either insulin or TGF-\( \beta \). Previously we have found that greater than 90-95% of collagen derived from IMR-90 cells is type I collagen and that nearly 90-95% of total collagen is in a soluble form in the medium (8). Therefore, only the medium was processed for PAGE. After the pulse period, the medium from individual culture plates received a solution of protease inhibitors yielding a final concentration of 10\(^{-4} \) M phenylmethylsulfonyl fluoride, 10\(^{-3} \) M hydroxymercurobenzoate, and 2 \( \times \) 10\(^{-3} \) M EDTA. The medium was then dialyzed against H\(_2\)O at 4°C and lyophilized. Medium fractions were digested with pepsin prior to PAGE on 6% gels (17). Autoradiography was performed according to the method of Bonner and Laskey (18).

**Collagen Production and Total Protein Production**—Collagen production was assessed by determining the total nanomoles of nondialyzable hydroxyproline. Confluent quiescent cells were stimulated as described above but without \( ^{3} \text{H} \)proline in the medium. The medium received a solution of protease inhibitors and was dialyzed against H\(_2\)O at 4°C and then lyophilized. The samples were subsequently hydrolyzed in 6 \( \text{N} \) HCl for 20 h at 106°C. To determine nanomoles of hydroxyproline in the hydrolysates, the amino acids were reacted with ninhydrin and assayed colorimetrically. Total protein production was assessed by determining acid-precipitable counts in cultures radiolabeled with \( ^{3} \text{H} \)proline (19).

**RNA Isolation and Northern Isolation**—Total cellular RNA was isolated by lysing cells with a solution containing 5 \( \text{M} \) guanidine monothiocyanate, precipitated with LiCl, and purified following extractions with phenol-chloroform. After extraction, saturated ammonium acetate was added to the RNA which was then precipitated with 100% ethanol, collected by centrifugation, lyophilized, and redissolved in H\(_2\)O. RNA was quantitated by absorbance at 260 nm. Purity was assayed by absorbance at 280 and 310 nm. RNA (10 \( \mu \)g) was electrophoresed on a 1% agarose-6% formaldehyde gel and trans-
ferred to a nitrocellulose filter. Equal loading of RNA was confirmed by size determination of ribosomal bands fractionated on agarose-formaldehyde gels stained with ethidium bromide. Hybridization was performed using 0.5-1.0 \times 10^6 \text{ cpm}/lane of labeled probe (specific activity, 4-10 \times 10^6 \text{ cpm}/\mu g). The filter was washed according to methods described by Thomas (20) and exposed to x-ray film for autoradiography at several different times to ensure that the bands could be quantified by densitometry within the linear range. The probes utilized in these experiments were pAlR1 and pRAH3.2, which, respectively, are a rat cDNA \text{a1(1)} clone (21) and a \text{SalI-PvuI}I fragment of the mouse histone gene (22). These probes specifically identify corresponding human mRNAs.

Nuclear Run-off Assay—Medium was removed from 150-mm dishes, the cells washed twice with Puck’s saline, and scraped into a nonident P-40 lysis buffer (23). Following two low speed spins, the pellet was reconstituted in a glycerol buffer (24). In vitro labeling of nascent RNA transcripts, purification of RNA, and hybridization of labeled RNA with DNA probes immobilized on nitrocellulose filters were performed according to methods outlined by Greenberg and Ziff (23) and Groudine et al. (24).

Statistics—Statistical evaluation of the data was performed using a Student’s \(t\) test for means of equal and unequal size (25). Probability values <0.05 were considered significant.

**RESULTS**

We determined the effect of PGE\(_2\) treatment (10\(^{-7}\) M) on the amount of nondialyzable hydroxyproline in the medium (nanomoles/ml) in unstimulated cultures and in cultures following stimulation with TGF-\(\beta\) (2 ng/ml) or insulin (2 \mu g/ml). PGE\(_2\) decreased the amount of nondialyzable hydroxyproline in unstimulated cultures and in TGF-\(\beta\)-stimulated cultures (Table I). In contrast, PGE\(_2\) had no significant effect on the amount of nondialyzable hydroxyproline in the medium of insulin-stimulated cultures. To determine the inhibitory effect of PGE\(_2\) on the formation of intact collagen peptide chains induced by TGF-\(\beta\) or insulin, we performed PAGE of pepsin digests of medium proteins radiolabeled with \[^{[H]}\]proline (Fig. 1). Densitometric analysis demonstrated that TGF-\(\beta\) increased types I and III collagen production approximately 3-fold and that insulin increased type I collagen production 2-fold. PGE\(_2\) strongly inhibited types I and III collagen formation stimulated by TGF-\(\beta\) but did not inhibit types I or III collagen formation stimulated by insulin.

To determine the effect of PGE\(_2\) on total protein synthesis in TGF-\(\beta\)- and insulin-stimulated cultures, we measured acid-precipitable counts in cultures labeled with \[^{[H]}\]proline. TGF-\(\beta\) (10 ng/ml) increased total protein production by approximately 2–3-fold (Fig. 2) while insulin (2 \mu g/ml) increased total protein production by approximately 2-fold. PGE\(_2\) significantly decreased total protein synthesis in cultures stimulated with TGF-\(\beta\). In contrast, PGE\(_2\) did not significantly decrease protein synthesis in cultures stimulated with insulin.

**TABLE I**

*The effect of PGE\(_2\) on TGF-\(\beta\) and insulin-induced collagen formation*

| Hydroxyproline | Control | PGE\(_2\) | Inhibition % |
|---------------|---------|-----------|-------------|
| Unstimulated  | 8.8 ± 0.8 | 5.9 ± 1.1  |
| TGF-\(\beta\)  | 17.5 ± 1.5 | 6.6 ± 1.4  |
| Insulin       | 14.9 ± 1.5 | 13.7 ± 1.3 |

*Denotes \(p < 0.001\) for corresponding control.

**FIG. 1.** The effect of PGE\(_2\) on collagen production in unstimulated, TGF-\(\beta\)-stimulated, and insulin-stimulated cultures. Confluent quiescent cultures containing \[^{[H]}\]proline (2 \mu Ci/ml) and ascorbate (50 \mu g/ml) in the medium were unstimulated (lanes 1 and 4), TGF-\(\beta\)-stimulated at 2 ng/ml (lanes 2 and 5), or insulin-stimulated at 2 \mu g/ml (lanes 3 and 6) for 24 h. The medium was processed and pepsin-treated before equal aliquots from individual dishes were separated on 6% PAGE for analysis by autoradiography. Lanes 4–6 received medium from dishes which were also treated with PGE\(_2\) during the 24-h incubation.

**FIG. 2.** The effect of PGE\(_2\) (10\(^{-7}\) M) on total protein production in unstimulated, TGF-\(\beta\) (10 ng/ml)-stimulated, and insulin (2 \mu g/ml)-stimulated cultures. Medium proteins were radiolabeled with \[^{[H]}\]proline in confluent quiescent cultures stimulated for 24 h with effector molecules in the absence (open bars) or presence (cross-hatched bars) of PGE\(_2\). Total protein production was assessed by determining acid-precipitable counts (19). Data is expressed as mean ± S.E. for three or four determinations. Asterisk denotes \(p < 0.001\).

To examine whether PGE\(_2\) inhibited collagen formation by affecting collagen gene expression, we determined the effect of PGE\(_2\) on TGF-\(\beta\)- and insulin-induced \(\text{a1(1)}\) mRNA levels. Total RNA was isolated from cells after 16 h in insulin-stimulated cells and after 24 h in TGF-\(\beta\)-stimulated cells. We have previously found that nearly maximal levels of \(\text{a1(1)}\) mRNA are induced at these time points following stimulation.
with each of these molecules (7). Densitometry indicated that TGF-β increased α1(I) mRNA 5–6-fold (Fig. 3A). PGE₂ treatment reduced α1(I) mRNA levels by 90% in TGF-β-stimulated cultures (Fig. 3A). In contrast, insulin increased α1(I) mRNA levels by only 30–40% (Fig. 3B). PGE₂ treatment reduced α1(I) mRNA levels by approximately 80% in insulin-treated cultures.

To determine whether PGE₂ reduced α1(I) mRNA levels by inhibiting gene transcription, we performed nuclear run-off assays on nuclei isolated from cultures that were stimulated with TGF-β in the presence or absence of PGE₂. We found that TGF-β increased the rate of transcription of the α1(I) gene 2-fold above unstimulated cultures (Fig. 4). PGE₂ reduced transcriptional rates in untreated cultures and in TGF-β-treated cultures. TGF-β had no effect on histone gene transcription (Fig. 4) whereas stimulation with medium containing 10% serum increased histone gene transcription (data not shown). The effect of PGE₂ on collagen gene transcription was relatively specific because treatment with PGE₂ had no effect on histone gene transcription. Hybridization of labeled nascent transcripts to immobilized plasmids (pUC18) without inserts yielded no nonspecific binding.

**DISCUSSION**

The findings in this paper suggest that collagen formation may proceed through two distinct pathways. These two pathways are distinguishable by both their sensitivity to PGE₂ and by their dependence on increases in collagen mRNA levels. Our results demonstrate that insulin-induced increases in collagen formation were associated with only small increases in collagen mRNAs. In contrast, TGF-β-induced collagen formation was accompanied by larger increases in mRNA levels. Though PGE₂ reduced collagen gene expression in both TGF-β- and insulin-treated cultures, PGE₂ inhibited collagen production in cultures stimulated with TGF-β but not in cultures stimulated with insulin.

Other investigators have shown that TGF-β enhances collagen gene expression by affecting both transcriptional and post-transcriptional mechanisms (26–28). Rossi et al. (28) found that TGF-β stimulates transcription of the mouse α2(I) gene by activating the interaction of a nucleoprotein with the α2(I) gene promoter. Raghow et al. (26) found that TGF-β increased type I collagen mRNAs in human dermal fibroblasts by stabilization of mRNAs. Our data is in agreement with both these observations since we found that TGF-β increased α1(I) mRNA by 5–6-fold whereas α1(I) gene transcription was increased 2-fold.

The small increases in α1(I) gene expression in insulin-treated cultures suggest that insulin activates collagen production primarily through effects at the translational level. Insulin induces phosphorylation of the S6 ribosomal protein (12–15). Increases in the initiation rate and movement of mRNAs into polysomes are temporally correlated with phosphorylation of S6 (14,15). The in vitro translation of globin mRNA was dramatically enhanced when ribosomal subunits were phosphorylated (16). Whether the effect of insulin on collagen production results from alterations at the ribosomal complex is unclear at this time.

In this paper, we found that PGE₂ inhibited collagen synthesis in fibroblasts stimulated with TGF-β but did not inhibit collagen synthesis in cultures stimulated with insulin. This inhibitory effect was mediated in part by the effect of PGE₂ on α1(I) gene transcription. Whether PGE₂ exerts a selective effect on the collagen α1(I) gene promoter has not been clarified. During tissue injury, the effectiveness of PGE₂ may depend on whether collagen production is directed by mech-
anisms which require increases in collagen gene expression. Inflammatory exudates containing effector molecules which activate collagen synthesis through cellular pathways not susceptible to PGE₂ may eventuate a more extensive fibrotic response.

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