Regulation of fibroblast procollagen production

Transforming growth factor- β_1 induces prostaglandin E_2 but not procollagen synthesis via a pertussis toxin-sensitive G-protein

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Transforming growth factor- β_1 (TGF β_1) initiates a series of signalling events resulting in diverse cellular responses including stimulation of extracellular matrix protein production. In this study we have investigated the role of pertussis toxin-sensitive G-proteins in mediating the effects of TGF β_1 on fibroblast procollagen metabolism. TGF β_1 stimulated human fetal lung fibroblast procollagen synthesis and production in a dose-dependent manner which was maximal at 0.5 ng/ml. TGF β_1 also decreased the proportion of newly synthesized procollagen degraded intracellularly. Pertussis toxin, a G-protein inhibitor, further stimulated TGF β_1 -induced procollagen synthesis and production, but alone it had no effect on fibroblast procollagen metabolism. Addition of indomethacin also potentiated the TGF β_1 -induced increase in procollagen synthesis and production. The effects of pertussis toxin and indomethacin were not additive. Pertussis toxin and indomethacin did not affect the proportion of newly

synthesized procollagen degraded intracellularly, either alone or in combination, by control cells. The TGF β_1 -induced decrease in intracellular procollagen degradation was maintained but not further affected by pertussis toxin or indomethacin. TGF β_1 increased prostaglandin E_2 (PGE $_2$) compared with PGE $_2$ production by control cells. Addition of pertussis toxin or indomethacin blocked the TGF β_1 -induced increase in PGE $_2$ production. The TGF β_1 -induced increase in PGE $_2$ preceded the increase in procollagen production. These results demonstrate that TGF β_1 -induced procollagen synthesis by lung fibroblasts is modulated by production of PGE $_2$. Pertussis toxin and indomethacin block the production of PGE $_2$ and enhance the effect of TGF β_1 on procollagen synthesis. From these data we conclude that the effects of TGF β_1 on PGE $_2$ production but not procollagen synthesis are mediated via a receptor linked to a pertussis toxin-sensitive G-protein.

INTRODUCTION

Transforming growth factor β_1 (TGF β_1) is one of a family of related polypeptide mediators consisting of five isoforms, three of which have been identified in mammals. The TGF β s are pluripotent mediators, having amongst their many functions the ability to modulate cell growth and stimulate the production and deposition of connective tissue matrix components including collagens, fibronectin and proteoglycans. Altered expression of TGF β s has been implicated in a number of diseases. Increased expression has been observed in fibrotic disorders of the lung [1–3], skin [4], kidney [5] and liver [6]. It has also been suggested that loss of TGF β expression in the skin and skin tumours is associated with hyperproliferation and a high risk of malignancy [7]. It is therefore important to understand the mechanisms by which TGF β s mediate their biological effects.

The effect of TGF β_1 on collagen deposition culminates from a network of interlinked actions. It increases procollagen synthesis via an increase in mRNA [8–10] and decreases the proportion of newly synthesized procollagen degraded intracellularly [11]. TGF β_1 also maximizes its stimulation of collagen deposition by stimulating production of anticollagenases and decreasing the production of collagenase [12–14]. The effect of TGF β_1 on collagen synthesis is known to involve binding of transcription factors to elements in the promoter regions of procollagen genes, resulting in upregulation of gene transcription [15–17]. However, the receptor and signal transduction pathways which lead to these effects are poorly understood.

There are at least seven cell surface receptors/binding proteins

for TGF β_1 (for review see [18]). The type I and II receptors have been cloned and these as well as the type V receptor have been found to contain transmembrane serine/threonine kinase signalling motifs [19–22]. However, the type I receptor also contains regions with sequence similarity for tyrosine kinase and protein kinase C activation [22]. The type III receptor has also been cloned and its product is a membrane-anchored proteoglycan with no obvious signalling motif [23,24]. There is co-operativity between the type I and II and the type II and III receptors, with formation of heteromeric complexes [25–28]. Type I and II receptors have been shown to be involved in all effects of TGF β_1 that have been investigated [25,29–32]. The signalling motifs and functions of the other TGF β_1 receptors/binding proteins have still to be determined and little is known of the intracellular signal transduction pathways.

G-proteins act as membrane bound intermediates between cell surface receptors and intracellular effector systems (for review see [33,34]). G-proteins are heterotrimers consisting of α -, β - and γ -subunits. They are cyclically regulated, activation occurs by binding of GTP to the α -subunit which is hydrolysed to GDP and P $_i$ resulting in deactivation followed by dissociation of GDP prior to binding more GTP. The involvement of G-proteins in transduction pathways and identification of their α -subunits is aided by the ability of certain bacterial exotoxins to specifically ADP-ribosylate particular α -subunits and inhibit their activity. Pertussis toxin inhibits α_i -subunits which have a broad cellular distribution and α_o -subunits which appear to predominate in neuronal cells. In contrast, cholera toxin inhibits the ubiquitously distributed α_s -subunits. There is evidence to suggest that some

Abbreviations used: TGF β , transforming growth factor β ; PGE $_2$, prostaglandin E_2 ; NCS, newborn-calf serum.

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actions of $\text{TGF}\beta_1$, including *c-sis* mRNA expression and mitogenesis in fibroblasts as well as inhibition of epithelial cell proliferation and chemotaxis of interleukin-2-activated natural killer cells, are mediated through receptors coupled to G-proteins [35–39]. G_α_1 , G_α_0 and G_α_s have been implicated in some of these actions [38,39]. In this study we have begun to investigate the mechanism by which $\text{TGF}\beta_1$ modulates procollagen production by determining the effects of pertussis toxin on $\text{TGF}\beta_1$ -induced fibroblast procollagen metabolism.

MATERIALS AND METHODS

Cell Culture

Human fetal lung fibroblasts (HFL-1) and fetal rat fibroblasts (Rat 2), both obtained from the American Type Culture Collection, (Rockville, MD, U.S.A.) were cultured in 2.4 cm diameter plates with Dulbecco's modified Eagle's medium + 5% newborn-calf serum (NCS) in a humidified atmosphere of air containing 10% CO_2 at 37°C. When cells appeared confluent they were incubated for a further 24 h. The media was then removed and replaced with 1 ml of pre-incubation medium, which contained 4 mM glutamine, 50 $\mu\text{g}/\text{ml}$ ascorbic acid, 0.2 mM proline and 2% (v/v) NCS and incubated for 24 h. The media were then replaced with 1 ml of fresh preincubation medium containing pertussis toxin (Calbiochem Novabiochem, Nottingham, U.K.) at concentrations between 0 and 500 ng/ml, or indomethacin (Sigma Chemical Co. Ltd., Poole, Dorset, U.K.) at a concentration of 1 $\mu\text{g}/\text{ml}$, and incubated for 3 h. Finally the media were replaced with media containing appropriate concentrations of pertussis toxin, indomethacin and porcine $\text{TGF}\beta_1$ (British Biotechnology, Oxford, U.K.) at concentrations between 0.1 and 10 ng/ml (4–400 pM) and incubated for between 2 and 24 h before harvesting.

Sample preparation

For cultures in which procollagen metabolism was to be assessed, the cell layer was scraped into the medium and aspirated. Each well was washed with 1 ml of PBS and the washings were combined with the initial aspirate. Proteins were precipitated by addition of ethanol to give a final concentration of 67% (v/v) and left at 4°C overnight. The precipitated proteins were extracted by vacuum filtration onto polyvinylidene difluoride filters (Millipore, Durapore 0.45 μm) and the ethanol-soluble material in the filtrate retained. The filtrate was evaporated to dryness using a Dri-Block Sample Concentrator (Technique DB-3, SC-3). The dried filtrates and the proteins adhering to the filters were hydrolysed in 2 ml of 6 M hydrochloric acid at 110°C overnight. Hydrolysates were mixed with approximately 70 mg of charcoal and filtered (Millipore, 0.65 μm) prior to chromatography.

Similar cultures were set up to assess prostaglandin E_2 (PGE_2) production and cell number by measurement of DNA. At the end of incubation the medium was removed and stored at –40°C prior to measurement of PGE_2 . The cell layer was scraped into 1 ml of PBS, aspirated into a microcentrifuge tube and centrifuged at 9000 g for 5 min; the clear supernatant was discarded. Any remaining cells were washed from the well with a further 1 ml of PBS, aspirated and combined with the initial cell pellet. After centrifugation the supernatant was discarded and the pellet was stored at –40°C prior to analysis of DNA. PGE_2 was measured in 10 and 50 μl aliquots of the cell-conditioned medium using a specific enzyme immunoassay (Amersham International, Buckinghamshire, U.K.) and results

were expressed as pg per well. DNA was measured using a modified diphenylamine method as described previously [11].

Measurement of hydroxyproline and calculation of procollagen synthesis and the proportion degraded

Hydroxyproline was isolated and measured by reverse-phase HPLC of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)-derivatized hydrolysates as described previously [11,40]. Hydroxyproline content was determined by comparing peak areas of samples from the chromatogram with those generated from standard solutions, derivatized and separated under similar conditions and run on the same day. As the cell monolayer contains a small amount of procollagen and the serum contained hydroxyproline, the amount of hydroxyproline present in the combined culture medium and cell layer at the start of incubation was determined in both the ethanol-soluble and -insoluble fractions. This background level, which represented between 1.95 and 2.62 nmol hydroxyproline per well in the insoluble fraction and between 0.92 and 1.87 nmol hydroxyproline per well in the soluble fraction, was then subtracted from the sample values. This represented between 15 and 22% of the total hydroxyproline measured in each sample after a 24 h incubation. The hydroxyproline present in the ethanol-soluble fraction, containing free amino acids and small peptides, from each culture was taken to represent hydroxyproline derived from the degradation of procollagen during the culture period, whilst the hydroxyproline in the ethanol-insoluble fraction was taken to represent hydroxyproline in intact procollagen and was called procollagen production. The proportion of procollagen degraded can be calculated by dividing the hydroxyproline content of the ethanol-soluble fraction by the sum of the hydroxyproline in the ethanol-soluble and -insoluble fractions. The rate of synthesis was obtained from the combined values for ethanol-soluble and -insoluble fractions, and rates of degradation and production were obtained from values for ethanol-soluble and -insoluble fractions respectively and expressed per μg DNA per hour.

Statistical analysis

Statistical evaluation was performed using an unpaired *t*-test or by one-way analysis of variance for multiple comparisons using the Newman–Keuls procedure. The mean values of various parameters were said to be significantly different when the probability of the differences of that magnitude, assuming the null hypothesis to be correct, fell below 5% (i.e. $P < 0.05$). Where mean values are calculated, standard errors of the mean (S.E.M.) are also given.

RESULTS

Figure 1 shows the effect of $\text{TGF}\beta_1$ on fibroblast procollagen metabolism. Production and synthesis increased in a dose-related manner with maximal stimulation obtained at 0.5 ng/ml which remained constant up to 10 ng/ml. The proportion of procollagen degraded intracellularly decreased from $22.80 \pm 0.69\%$ in control cultures to $19.28 \pm 0.79\%$ in cultures with 1 ng/ml $\text{TGF}\beta_1$ ($P < 0.01$). All further studies were carried out with $\text{TGF}\beta_1$ at a concentration of 1 ng/ml.

The effect of pertussis toxin, at concentrations of 10, 100 and 500 ng/ml, on fibroblast procollagen production is shown in Figure 2. At the two lower doses of pertussis toxin the cells

Figure 1 Effect of TGF β_1 on procollagen synthesis and production by human fetal lung fibroblasts

Procollagen production (solid bars) and synthesis (cross-hatched bars) were measured following incubation of cells for 24 h in the presence of TGF β_1 at the concentrations indicated. Proteins in the combined media and cell layer were precipitated by addition of ethanol to a final concentration of 67% (v/v). Values for procollagen production were obtained from measurements of hydroxyproline in the ethanol-insoluble fraction and values for procollagen synthesis represent the combined measurements of hydroxyproline in the ethanol-soluble and -insoluble fractions. Each value represents the mean $\pm$ S.E.M. for six replicate cultures.

Figure 2 Effect of pertussis toxin on normal and TGF β_1 -induced procollagen production

Procollagen production by human fetal lung fibroblasts was measured in cultures with and without TGF β_1 (1 ng/ml) at the concentrations of pertussis toxin indicated. Each value represents the mean $\pm$ S.E.M. for six replicate cultures. Where no error bars are shown they were within the point.

appeared normal microscopically, and procollagen production was not affected, but at 500 ng/ml some cells lost their characteristic spindle shape and became rounded, a small decrease in procollagen production was also observed. In the presence of TGF β_1 and pertussis toxin, procollagen production was increased. Maximal stimulation of about 22% ($P < 0.001$) compared with the increase induced by TGF β_1 alone was obtained with pertussis toxin at 100 ng/ml. With 500 ng/ml pertussis toxin the procollagen production was reduced to values similar to those obtained with TGF β_1 alone. Pertussis toxin did not affect the proportion of newly synthesized procollagen degraded intracellularly at any concentration tested (control values in this experiment were $28.0 \pm 1.1\%$). The TGF β_1 -induced decrease in intracellular procollagen degradation was maintained in the presence of pertussis toxin. Values at 10 ng/ml pertussis

Figure 3 Effect of pertussis toxin and indomethacin on normal and TGF β_1 -induced procollagen production

Each bar shows the procollagen production of human fetal lung fibroblasts in the presence of the indicated combinations of pertussis toxin (100 ng/ml), indomethacin (1 μ g/ml) and TGF β_1 (1 ng/ml). Each value represents the mean $\pm$ S.E.M. for six replicate cultures.

toxin were $29.5 \pm 0.64\%$ for pertussis toxin alone and $24.83 \pm 1.43\%$ in the presence of pertussis toxin plus TGF β_1 . At 100 ng/ml pertussis toxin values were $28.64 \pm 0.76\%$ for pertussis toxin alone and $25.35 \pm 0.48\%$ in the presence of pertussis toxin and TGF β_1 ($P < 0.05$ and $P < 0.005$ respectively).

Similar results were obtained with another fibroblast line (Rat 2). Control rates of procollagen synthesis were 5.24 ± 0.29 pmol hydroxyproline per μ g DNA per hour. This was increased by 64% to 8.61 ± 0.33 pmol hydroxyproline per μ g DNA per hour ($P < 0.001$) in the presence of TGF β_1 (5 ng/ml) with a further increase compared with that of TGF β_1 alone of 27% to 10.91 ± 0.51 pmol hydroxyproline per μ g DNA per hour in the presence of TGF β_1 and 100 ng/ml pertussis toxin ($P < 0.01$).

Figure 3 shows the effects of pertussis toxin (100 ng/ml) and the cyclooxygenase (prostaglandin endoperoxide synthase) inhibitor, indomethacin, on fibroblast procollagen production. Indomethacin (1 μ g/ml) increased procollagen production by 20% from 34.11 ± 1.72 pmol hydroxyproline per μ g DNA per hour to 40.97 ± 0.81 pmol hydroxyproline per μ g DNA per hour ($P < 0.01$). In the presence of TGF β_1 , indomethacin further stimulated procollagen production from 44.04 ± 1.21 pmol hydroxyproline per μ g DNA per hour to 50.32 ± 1.07 pmol hydroxyproline per μ g DNA per hour ($P < 0.01$). The effects of pertussis toxin and indomethacin were not additive. For cells incubated with pertussis toxin and indomethacin values were 38.74 ± 0.76 pmol hydroxyproline per μ g DNA per hour which was similar to the values obtained for each agent alone. In the presence of TGF β_1 , as well as pertussis toxin and indomethacin, values were 49.24 ± 0.70 pmol hydroxyproline per μ g DNA per hour which was greater than the value for TGF β_1 alone ($P < 0.05$) but was not significantly different to the values obtained for TGF β_1 in the presence of pertussis toxin or indomethacin. At the dose of indomethacin used, there were no changes in intracellular procollagen degradation. In this experiment, control values were $22.80 \pm 0.69\%$, which decreased to $19.28 \pm 0.79\%$ in the presence of TGF β_1 ($P < 0.01$). Values for indomethacin and indomethacin plus TGF β_1 were $22.41 \pm 0.62\%$ and $18.53 \pm 0.53\%$ respectively. The effects of indomethacin and pertussis toxin on TGF β_1 -induced procollagen synthesis showed similar changes to those for production (Table 1).

Table 1 Effects of pertussis toxin and indomethacin on TGF β_1 -induced stimulation of procollagen synthesis by lung fibroblasts

Human fetal lung fibroblasts were incubated in the presence of the indicated combinations of pertussis toxin (100 ng/ml) and indomethacin (1 μ g/ml), with or without TGF β_1 (1 ng/ml). Each value represents the mean $\pm$ S.E.M. for six replicate cultures. *Significantly different compared with its respective control; †significantly different compared with the value for TGF β_1 alone.

	Procollagen synthesis (pmol hydroxyproline per μ g DNA per hour)	
	Control	TGF β_1
	44.27 $\pm$ 2.44	54.59 $\pm$ 1.65*
Pertussis toxin	44.76 $\pm$ 1.28	67.23 $\pm$ 0.72*†
Indomethacin	52.83 $\pm$ 1.19*	61.75 $\pm$ 1.03*†
Pertussis toxin + indomethacin	48.48 $\pm$ 1.08	61.09 $\pm$ 0.60*†

Figure 4 Effect of TGF β_1 , pertussis toxin and indomethacin on human fetal lung fibroblast PGE₂ production

PGE₂ was measured in the culture medium 24 h after addition of TGF β_1 (1 ng/ml), pertussis toxin (100 ng/ml) and indomethacin (1 μ g/ml) in the combinations indicated. Each value represents the mean $\pm$ S.E.M. for five replicate cultures.

The effect of TGF β_1 on PGE₂ production by human fetal lung fibroblasts is shown in Figure 4. Control cultures contained 239 $\pm$ 90 pg of PGE₂ per well. In the presence of TGF β_1 the PGE₂ production increased about 5-fold to 1175 $\pm$ 291 pg per well ($P < 0.01$). Pertussis toxin and indomethacin alone showed a tendency to decrease basal PGE₂ production but this was not significant. However, pertussis toxin and indomethacin completely blocked the TGF β_1 -induced increase in PGE₂ ($P < 0.01$ in both cases).

Figure 5 shows the changes in PGE₂ (Figure 5a) and procollagen production (Figure 5b) at various times up to 24 h. In control cultures PGE₂ levels remained constant over the 24 h incubation period. In the presence of TGF β_1 , PGE₂ levels were not significantly different compared with control values at 2 h. However, by 4 h PGE₂ had increased almost 4-fold [control, 107 $\pm$ 10 pg of PGE₂ per well; TGF β_1 , 403 $\pm$ 25 pg of PGE₂ per

Figure 5 Kinetics of TGF β_1 -induced PGE₂ and procollagen production

PGE₂ (a) and procollagen (b) production by human fetal lung fibroblasts with and without TGF β_1 (1 ng/ml) were measured at various times up to 24 h. Each value represents the mean $\pm$ S.E.M. for six replicate cultures. Where no error bars are shown they were within the point.

well ($P < 0.001$)] and continued to increase up to 24 h when values were approximately seven times the control values [control, 116 $\pm$ 16 pg of PGE₂ per well; TGF β_1 , 826 $\pm$ 94 pg of PGE₂ per well ($P < 0.001$)]. In contrast there were no consistent differences in procollagen production between control and TGF β_1 -treated cells up to 8 h. At 16 and 24 h procollagen production was increased in TGF β_1 -treated cells by about 20 and 45% respectively ($P < 0.001$ in both cases).

DISCUSSION

In these studies we have shown that TGF β_1 stimulates the synthesis and production of procollagen by fetal fibroblasts and that intracellular degradation of newly synthesized procollagen is decreased. Pertussis toxin did not inhibit basal or TGF β_1 -induced procollagen metabolism. These findings suggest that TGF β_1 -induced procollagen metabolism is not mediated by receptor-coupling to pertussis toxin-sensitive G-proteins. These conclusions are consistent with those of a recent study of steady-state mRNA levels for type I procollagen in bovine adrenal capillary endothelial cells [41]. In contrast to this previous study, in our experiments pertussis toxin reproducibly further stimulated the TGF β_1 -induced increase in procollagen synthesis and production.

One interpretation of the pertussis toxin enhancement of TGF β_1 's stimulation of collagen synthesis and production is that pertussis toxin may be blocking a TGF β_1 -induced inhibitory pathway of procollagen metabolism. One such pathway might involve prostaglandins. Prostaglandins of the E type are potent inhibitors of procollagen metabolism [42–46]. TGF β_1 has been reported to stimulate cyclooxygenase 1 but not cyclooxygenase 2 gene expression and production of PGE₂ by lung fibroblasts [46,47]. Furthermore, this increase in PGE₂ limits the TGF β_1 -induced stimulation of collagen production [46]. To investigate the potential role of PGE₂ in modulating the TGF β_1 -induced

procollagen synthesis and its potentiation by pertussis toxin, experiments were performed in the presence of indomethacin, and fibroblast PGE₂ production was measured. Indomethacin alone stimulated procollagen synthesis, indicating that the cells synthesized prostaglandins under basal culture conditions. In the presence of TGF β_1 , indomethacin also potentiated the increase in procollagen synthesis and production to a similar extent to that induced by pertussis toxin. The effects of pertussis toxin and indomethacin on TGF β_1 -induced procollagen production and synthesis were not additive, suggesting that both agents were acting on the same pathway. TGF β_1 increased PGE₂ production and this increase was blocked by pertussis toxin and indomethacin. These results suggest that the potentiation of TGF β_1 -stimulated procollagen synthesis and production by pertussis toxin is due to inhibition of TGF β_1 -induced PGE₂ synthesis and further suggests that TGF β_1 -induced fibroblast production of prostaglandins is mediated either directly or indirectly through a pertussis toxin-sensitive G-protein. PGE₂ is also a potent inhibitor of fibroblast proliferation [48] and may be at least partly responsible for TGF β -induced inhibition of cell proliferation observed in many cells.

PGEs have previously been shown to inhibit procollagen synthesis and increase the proportion of newly synthesized procollagen degraded intracellularly by human lung fibroblasts [42,44–46]. In the present studies TGF β_1 induced PGE₂ production, which had an inhibitory effect on procollagen synthesis but did not affect the proportion of procollagen degraded intracellularly. This difference may be explained by the amount of PGE₂ produced by the fibroblasts which resulted in concentrations of about 1–2 ng/ml in the media of cells incubated with TGF β_1 . Previous studies have shown effects of exogenously applied PGE₂ on procollagen synthesis at concentrations in the range of 10–250 ng/ml [44–46], whereas effects on degradation appear to require concentrations of at least 2 μ g/ml [42,44].

In this study we have also shown that the effects of TGF β_1 on PGE₂ production precede the increase in procollagen production. PGE₂ production was increased within 4 h of addition of TGF β_1 but stimulation of procollagen production was not apparent until 16 h. To our knowledge this is the first report of the time-course of TGF β_1 -induced procollagen production. However, procollagen mRNA levels have been reported to be increased in as little as 2 h following treatment of rat kidney fibroblasts with TGF β_1 [9]. This apparent discrepancy in the time-course of changes in mRNA and protein synthesis could be explained either by differences in cell type or by the increase in PGE₂ production, which may negate the effect of increased procollagen mRNA on procollagen synthesis at the early times.

The receptor(s) mediating these effects of TGF β_1 is(are) uncertain, although it is likely that receptors I and II are involved. TGF β_1 -mediated effects on cell proliferation have previously been attributed to the type I receptor [49] and these effects were shown to be coupled through pertussis toxin-sensitive G-proteins [37]. However, studies demonstrating the cooperative nature of the TGF β type I and II receptors [25] suggest that the antiproliferative effects of TGF β_1 are mediated through the type II receptor in conjunction with the type I receptor [30]. Both type I and type II receptors have been shown to contain cytoplasmic serine/threonine kinase signalling motifs and the type II receptor has been shown to require its kinase activity to enable signalling of growth inhibition [25]. However, it is known that receptors may be coupled to multiple signalling motifs which enable a diverse range of responses to receptor activation, and it has been reported that a consensus G-protein activating sequence has been found in the type II receptor (Arg³⁵⁶-Lys³⁸¹) but this has yet to be confirmed [39]. Alternatively, it has recently been proposed

that the multifunctional nature of responses to TGF β_1 may be due to different isoforms of receptor I [29,31,32]. Whether TGF β_1 -induced prostaglandin and procollagen synthesis are mediated through one of these receptors or another of the less well characterized TGF β receptors/binding proteins remains to be determined.

These results demonstrate that the effects of TGF β_1 on fibroblast procollagen metabolism are modulated by production of PGE₂ which is inhibited by pertussis toxin or indomethacin with a concomitant increase in procollagen synthesis. From these data we conclude that the effects of TGF β_1 on PGE₂ but not procollagen synthesis are mediated via a receptor linked, directly or indirectly, to a pertussis toxin-sensitive G-protein.

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