0021-972X/95/$03.00/0
Journal of Clinical Endocrinology and Metabolism

Vol. 80, No. 12
Printed in U.S.A.

Prostaglandin E_2 Alters Human Orbital Fibroblast Shape Through a Mechanism Involving the Generation of Cyclic Adenosine Monophosphate*

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ABSTRACT

Orbital fibroblasts from patients with Graves' ophthalmopathy, when treated with prostaglandin E_2 (PGE_2), become stellate and develop prominent cellular processes. In this paper, we describe results of studies designed to characterize the action of PGE_2 on orbital fibroblast shape changes *in vitro*. Orbital and dermal fibroblasts were incubated with PGE_2, one of several prostanoid analogues, 8-br-cAMP or forskolin and were then visualized by phase-contrast microscopy. Other studies involved seeding cells in special chambers equipped with electrodes for cell sensing using electric cell-substrate impedance sensing (ECIS) to detect changes in shape. PGE_2 (10^{-7} mol/L) elicited a rapid and dramatic alteration in the shape of orbital fibroblasts but not those derived from the skin. Cells became stellate and developed prominent cytoplasmic processes that extended out from the central area containing the cell nucleus. The effects were stereoselective in that a number of structurally related compounds, including Sulprostone, PGI_2, $PGF_{2\alpha}$, thromboxane A_2, thromboxane B_2, and 11 deoxy,16,16 dimethyl PGE_2 failed to elicit a similar shape change. Butaprost (10^{-5} mol/L), a specific EP_2 agonist, elicited a similar shape-change as that observed with PGE_2. 16,16-dimethyl PGE_2, a nonselective agonist, could mimic the action of PGE_2. The effect of PGE_2 was apparent at 10^{-8} mol/L, maximal at a concentration of 10^{-7} mol/L and took 4–8 hr to evolve completely. Cycloheximide (10 μg/mL) and actinomycin D (1 μg/mL) failed to block the shape change. The morphologic change could be reproduced by addition of 8-br-cAMP (3 mmol/L) and by forskolin (5 μmol/L). Moreover, PGE_2 and Butaprost treatment elicited in orbital cultures a massive increase in endogenous cAMP production while analogues not affecting cell shape failed to influence cyclic nucleotide generation. Three strains of orbital fibroblasts from patients with Graves' ophthalmopathy and three from normal orbits were tested and all responded to PGE_2 (10^{-7} mol/L). Four strains of dermal fibroblasts failed to respond to PGE_2. The changes in orbital fibroblast morphology were accompanied by a marked decrease in monolayer impedance as assessed by electric cell-substrate impedance sensing. The earliest effects were apparent within 30 min using this sensitive technique. The widely recognized roles of PGE_2 and related compounds in the mediation of the inflammatory response make our current findings in orbital fibroblasts of potential importance to the pathogenesis of Graves' ophthalmopathy. (*J Clin Endocrinol Metab* **80:** 3553–3560, 1995)

PROSTAGLANDIN E_2 (PGE_2) is an important component of the inflammatory cascade and has a diverse set of actions on many target cell types (1, 2). The vast array of physiological roles played by this and related compounds is only now becoming apparent. Prostanoids elicit their biological effects by associating with plasma membrane bound receptors, of which several subtypes have thus far been identified (3–6). Prostanoid receptors have been shown to activate second messenger pathways (7). Human dermal fibroblasts maintained in primary cell culture synthesize and respond to PGE_2 (8). Moreover, the rate of PGE_2 synthesis in fibroblasts is regulated by cytokines and serum through the induction of a cyclooxygenase (9).

Fibroblasts emanating from the human orbit engender phenotypic characteristics *in vitro* that set them apart from those derived from the integument (10–12). These differences exist despite similar morphologies as determined on the light (10) as well as transmission electron microscopic levels (13). In particular, the response domain for interferon gamma differs substantially in orbital and nonorbital fibroblasts (14–16). Even when cultures from distant areas of the skin of individual donors are compared, consistent, distinct patterns of protein synthesis and cytokine response emerge (17–18). We have reported recently that PGE_2 can alter dramatically the morphology of cultured orbital fibroblasts derived from patients with severe Graves' ophthalmopathy (19). These cells were changed from fusiform shapes that resemble most primary human fibroblasts to a stellate morphology. They develop multiple cellular processes that commingle with the bodies and processes of adjacent cells. The changes occurred rapidly, within 3–6 hr of prostanoid addition to the culture medium and were transient in that there was spontaneous reversion toward the native morphology despite the continued presence in the medium of the compound. Readdition of fresh PGE_2 failed to influence this reversion. The shape-changes were preceded and then accompanied by a substantial reduction in culture monolayer impedance, as assessed by electric cell-substrate impedance sensing (ECIS) (20–22). These noninvasive measurements

Received April 1, 1995. Revised May 26, 1995. Re-revised June 8, 1995. Accepted June 13, 1995.

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*This work was supported in part by National Institutes of Health Grant RO1 EY08976 (T.J.S) and by the Research Service of the Department of Veterans Affairs (T.J.S.).

have been demonstrated to correlate well with changes in the morphology of cells adherent to gold electrodes (22).

In this paper, we report the results of studies that characterize the effect of PGE_2 on orbital fibroblast morphology, an action that involves the activation of EP_2 sub-type prostanoid receptors and the generation of 3′,5′ cyclic adenosine monophosphate. These results define a previously unrecognized phenotypic attribute of human orbital fibroblasts.

Materials and Methods

Materials

PGE_2 was from Sigma (St. Louis, MO). Prostacyclin (PGI_2) and prostaglandin $F_{2\alpha}$, 17-phenyl trinor prostaglandin E_2 (17-ph PGE_2), 16,16-dimethyl prostaglandin E_2 (16, 16-dm PGE_2), 11-deoxy-16,16-dimethyl prostaglandin E_2 (11-d-16, 16-dm PGE_2), thromboxanes A_2 and B_2, and Sulprostone were from Cayman, Ann Arbor, MI. Butaprost was from Miles, West Haven CT. Eagle's medium, fetal bovine serum (FBS), and other culture reagents were obtained from Grand Island Biological Company, Grand Island, NY. All other reagents used were of the highest purity commercially available.

Cell Culture

Orbital fibroblasts were obtained from individuals undergoing surgery for the treatment of severe Graves' ophthalmopathy or for a condition that did not involve inflammation of the orbit. As far as we know, the donors were not receiving glucocorticoids at the time of surgery. Dermal fibroblasts were obtained from punch biopsies of apparently normal skin of the abdominal wall or pretibium. None of the donors had clinically apparent dermopathy. Some of the tissue samples used were kindly provided by Dr. Michael Kazim, College of Physicians and Surgeons of Columbia University, New York. In the authors' opinion, methods for securing human tissue were humane, included proper consent and approval, and complied with the Declaration of Helsinki. Institutional Review Board approval at the Albany Medical College has been obtained to participate in these studies. Biopsies were placed on plastic substratum and covered with Eagle's medium supplemented with Earle's salts, 10% FBS, glutamine, and antibiotics as described previously (23, 24). Culture strains were maintained in a humidified, 37 C, 5% CO_2 incubator and passaged serially following disruption of monolayers with trypsin/EDTA treatment and stored long-term in liquid N_2. Stock cultures were subdivided to inoculate the ECIS wells and sterile coverslips for microscopy. Cultures were not used beyond the 12th passage from initiation. These cultures fail to express either smooth muscle-specific α-actin or Factor VIII, documenting that they are not contaminated with either vascular smooth muscle or endothelial cells (25).

Phase contrast microscopy

Fibroblasts were inoculated on sterile 22 × 22 mm glass coverslips at a seeding density of about 10^4 cells/coverslip. The cells were allowed to attach for one day before the initiation of experiments. At the times indicated, cultures were shifted to medium supplemented with 1% FBS without or with the test compound. Following incubation, cells were fixed in 2% para-formaldehyde in phosphate buffered saline (PBS, pH 8.0). The coverslips were washed twice and mounted on glass slides with glycerol/PBS (1:1). Microscopy was performed with a Nikon Labophot (Nikon Corp., Melville, NY) equipped with a 35 mm camera. Photographs were taken on Kodak Tri-Max 400 film (Eastman Kodak, Rochester, NY). The final magnification was 400 ×.

Quantitation of the number of cells undergoing shape change was accomplished by viewing at least four random fields and performing manual cell counts, examining at least 100 cells in each culture. All comparisons of morphology were conducted with diluent-treated (control) cultures incubated under identical experimental conditions included in each study. Criteria for judging a cell as responding to the test compound included evidence of elevation of the central, perinuclear area above the plane of the substratum, presence of a circumferential halo and cytoplasmic retraction, and the formation of cellular processes as described previously (19). All observations reported were conducted by a single observer (H.-S. Wang) and were verified independently by two other individuals. Interassay variation for the primary observer was 5% or less.

Impedance measurements with ECIS

The electrical measurement of cultured fibroblast morphology was based on the technique described previously (20, 21). Details concerning the instrumentation have appeared recently (22). In brief, the cells were inoculated onto a plastic surface which had gold electrodes attached to it. After at least 1 day, monolayers were shifted to medium containing 1% FBS and the test compound. Control cultures received diluent alone. The fibroblasts adhered directly to the electrodes, and the culture medium served as an electrolyte. An approximately constant current source applied an ac signal of 1 μA between a small active electrode and a larger counter electrode, while voltage was monitored with a lock-in amplifier. Voltage and phase data were stored and processed with a personal computer. The same computer controledthe output of the amplifier and switched the measurement to different electrodes in the course of an experiment.

When cells attached and spread on the gold electrodes, the insulating plasma membranes blocked the current path, and the impedance increased several-fold, depending on the cell type. To measure this change in impedance it was important that the active electrode was small in area (10^{-3} cm^2), otherwise the solution resistance would have dominated the measurement and would have masked the effect caused by the cells on the electrode. The impedance continued to fluctuate after the cells reached confluence because of the small spontaneous changes in cell morphology.

ECIS electrode arrays consisted of a row of 5 tissue culture wells mounted on a 1 × 3 in plastic slide. The following fabrication steps were employed: a 50 nm gold layer was first sputtered on 20 mil polycarbonate films. The gold layer was patterned using standard photolithography procedures to form contact pads, a large counter electrode, and an area that would finally include the active ECIS electrode. A 6 μm thick layer of photoresist was spread over the patterned gold surface via spin coating. Using standard procedures, the photoresist was selectively removed from areas of the slide including the 250 μm diameter active electrodes. The photoresist was hard baked and finally cleaned and sterilized using an oxygen plasma etching procedure. To complete the arrays, sterile 10 mm × 10 mm glass cloning cylinders were mounted to the plastic slide using a tissue compatible silicone adhesive. The completed array mounted in a holder that made electrical contact through the gold contact pads and was connected to the ECIS instrumentation.

Assay for cyclic adenosine monophosphate production

Confluent fibroblast monolayers were cultivated in 24-well cluster plates and shifted to medium containing 1% FBS and supplemented with the phosphodiesterase inhibitor, RO-20–1724 (0.25 mM, RBI Research Biochemicals, Natick, MA) for 48 hr. Cultures were treated with diluent or the test compounds for the durations indicated in the results section. Medium was removed and cAMP quantitated with an RIA assay system (Amersham, Arlington Heights, IL) according to instruction from the manufacturer.

Results

Orbital fibroblasts, whether emanating from individuals without inflammatory disease or from patients with Graves' ophthalmopathy, morphologically resemble other types of human fibroblasts when maintained in primary culture. Only subtle differences are present on the light microscopic level (Fig. 1a,c,e) (10) or when the cells are examined with transmission electron microscopy (13). When fibroblasts from patients with Graves' ophthalmopathy are treated with PGE_2, their fusiform shapes are altered dramatically as

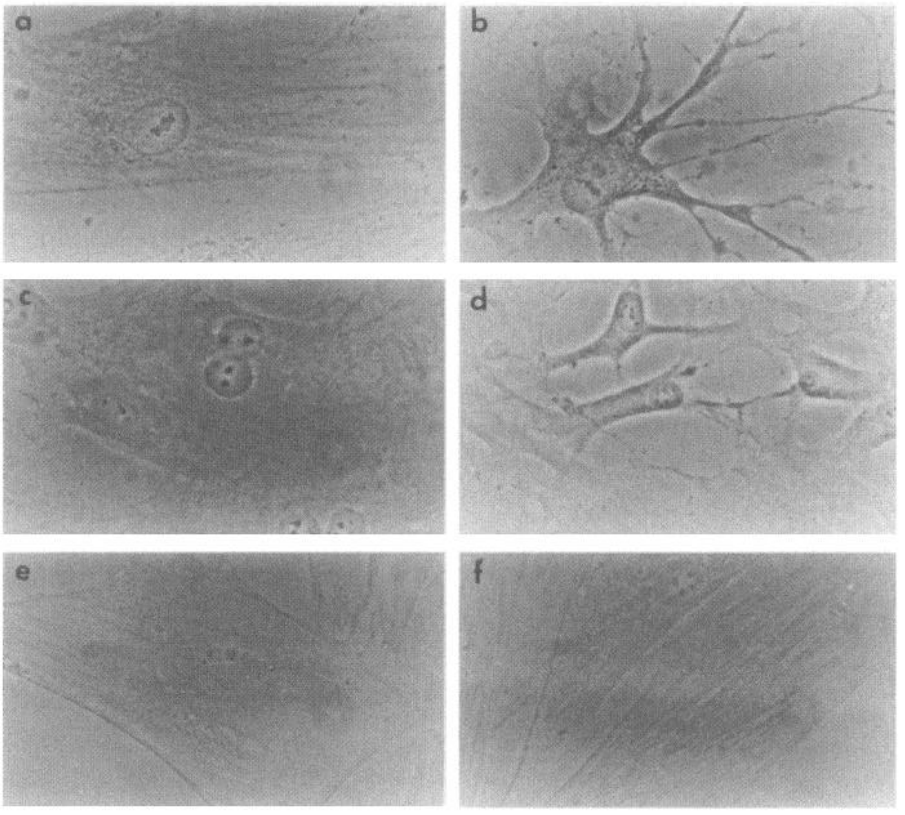


FIG. 1. Phase-contrast microscopy demonstrating the effect of PGE_2 on the morphology of orbital fibroblasts emanating from a patient with Graves' ophthalmopathy, a normal orbit and a dermal fibroblast strain from normal tissue. Cultures were initiated on glass coverslips and allowed to attach to the substratum and then were treated with PGE_2, (10^{-7} mol/L, final concentration) in medium supplemented with 1% FBS. Cultures were exposed to the compound for 4 hr, and then monolayers were fixed and photographed. Panel a, Graves' untreated orbital; Panel b, Graves' PGE_2-treated orbital; Panel c, normal untreated orbital; Panel d, normal PGE_2-treated orbital; Panel e, normal untreated dermal; Panel f, normal PGE_2-treated dermal (Final magnification × 1600)

prominent processes develop around the central, nucleus containing part of the cells. As Fig. 1b suggests, the PGE_2-treated (10^{-7} mol/L) Graves' orbital fibroblasts are stellate and appear to extend higher above the plane of the substratum than do untreated cells (Fig. 1a). These cultures received the prostanoid 4 hr before they were photographed. There exists considerable variability among individual cells with regard to the morphologies they assume following PGE_2 treatment. Some of the cells have 20 or more processes while others have changed minimally. In general, the cytoplasm of cells undergoing shape change appears more granular than untreated cells. The processes have irregular outlines, with evidence of some fine projections emanating from them.

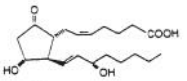
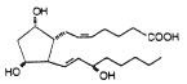
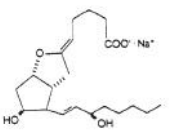
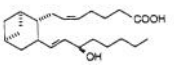
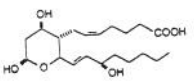
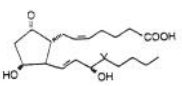
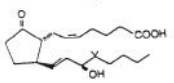
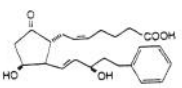
Fig. 1 also contains micrographs of control (Fig. 1c) and PGE_2-treated orbital fibroblasts (Fig. 1d) from normal orbital tissue. The changes elicited by the prostanoid are qualitatively similar to those seen in cultures from a patient with Graves' ophthalmopathy. In contrast, PGE_2 failed to alter the morphology of dermal fibroblasts under identical experimental conditions (Figs. 1e and 1f). The duration of time in culture or passage number failed to alter the cellular response to PGE_2. Moreover, PGE_2, in the concentrations used in these studies, failed to alter the viability of either the responding or the nonresponding cells.

Table 1 contains data concerning the responses of orbital fibroblasts from a patient with Graves' ophthalmopathy to PGE_2 and several structurally-related compounds including $PGF_{2\alpha}$, PGI_2, 16,16 dimethyl PGE_2, 17-phenyl trinor PGE_2, 11 deoxy, 16,16 dimethyl PGE_2, thromboxane A_2 and thromboxane B_2 present at a concentration of 1×10^{-7} mol/L. Of these compounds, 16,16 dimethyl PGE_2, an agonist which has been shown to be nonselective, exhibited morphogenic actions similar to those of PGE_2. By contrast, 11 deoxy, 16,16 dimethyl PGE_2 was completely inactive. In a study conducted at a higher prostanoid concentration (10^{-5} mol/L), Butaprost, a selective agonist that interacts with the EP_2 receptor subtype was very active (Table 2). By contrast, even at this high concentration, Sulprostone, a selective EP_3 agonist and 17-phenyl trinor PGE_2, an EP_1 agonist, failed to alter the shape of most cells examined.

The time-course of PGE_2-dependent changes in cell morphology is shown in Fig. 2. As the serial micrographs of a single field of cells attest, when orbital fibroblasts are treated with the prostanoid (10^{-7} mol/L), the cells begin to change within the first 2 hr of exposure (note the absence of change at 20 min, Fig 2b). At 2 hr, a number of cells have begun to round up (Fig. 2c) and by 4 hr, cell processes become prominent (Fig. 2d). The effect is pronounced in the 8 hr view (Fig. 2e), when some cells possess many prominent processes and are maximally refractile. The 21 hr view (Fig. 2f) shows most of the cells that had undergone shape change are now flattened out, and the processes are far less prominent. As Fig. 2 suggests, the changes in shape elicited by PGE_2 occur at different times in the population of cells in a given culture.

Morphological changes associated with the addition of PGE_2 to the culture medium of orbital fibroblasts are accompanied by a substantial decrease in the impedance of the culture monolayer. The effects of PGE_2 on monolayer impedance are dose-dependent (Fig. 3). The threshold of the effect was around 10 nmol/L and was maximal at 100 nmol/L. Higher concentrations of the prostanoid consistently yielded smaller changes in impedance. Within 2 hr of

TABLE 1. Effect of PGE_2 and several related compounds on the morphology of orbital fibroblasts from a patient with Graves' ophthalmopathy

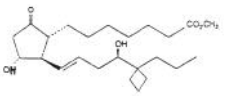
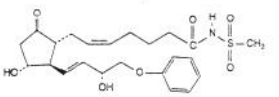
Treatment	Molecular structure	Percent cell change
Control		1
PGE_2		50
$PGF_{2\alpha}$		4
PGI_2		2
Thromboxane A_2		2
Thromboxane B_2		3
16,16-dimethyl PGE_2		30
11-deoxy-16,16-dimethyl PGE_2		4
17-phenyl trinor PGE_2		4

Sterilized glass coverslips were inoculated with cells, which were allowed to attach for 24 hr in medium containing 10% FBS. Cultures were then shifted to medium supplemented with 1% serum and the test compounds indicated (all at a final concentration of 0.1 μM), incubated at 37 C for 4 hr, fixed and subjected to manual cell counts. At least 100 cells per treatment group were inspected.

PGE_2 addition, normalized resistance reached its nadir of 20% below control values. Impedance at this concentration remained low over the next 5.5 hr. The rapid changes in impedance present in the individual data curves are caused by cellular micromotion (26). In contrast to the effects seen with PGE_2, the prostanoids found not to alter cell morphology failed to elicit changes in monolayer impedance (data not shown).

A number of the actions of PGE_2 are mediated through the activation of adenylate cyclase and generation of cAMP. We tested the ability of exogenous cAMP to elicit the changes in orbital fibroblast morphology by adding to the culture medium the cell membrane permeable analogue 8-br-cAMP (3 mmol/L). As the micrographs in Fig. 4 suggest, PGE_2 (100 nmol/L) (Panel B) and 8-br-cAMP (Panel D) alter cell morphology identically within 4 hr of addition to the medium. The adenylate cyclase activating compound, forskolin (5 μmol/L) (Panel C) also changed orbital fibroblast morphology. Unlike the cellular response to PGE_2, which was transient, the shape of cells treated with 8-br-cAMP remain altered for as long as the cyclic nucleotide is present in the culture medium (tested for up to 96 hr).

TABLE 2. Effects of PGE_2 and its analogues on the morphology of orbital fibroblasts from a patient with Graves' ophthalmopathy

Treatment	Molecular structure	Percent cell change
Control		2
PGE_2		25
Butaprost		33
Sulprostone		2
17-phenyl trinor PGE_2		9
Thromboxane A_2		3
Thromboxane B_2		5

The study was conducted in a similar manner to that in Table 1, except that the prostanoid concentration was 10 μM.

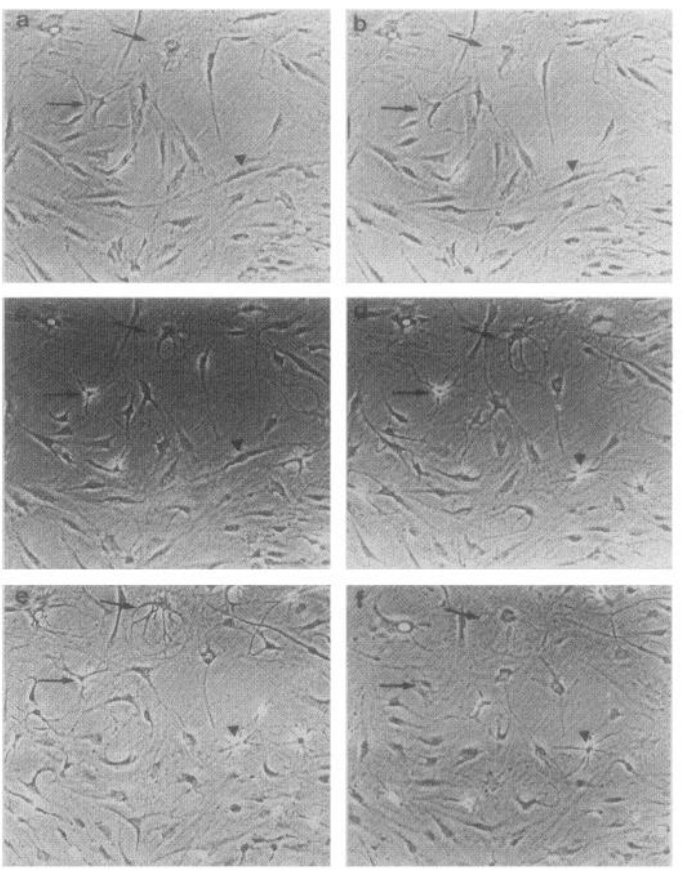


FIG. 2. Time-dependence of the effects of PGE_2 on cell morphology. A representative field of cells in a culture derived from the orbit of a patient with Graves' ophthalmopathy was photographed serially at different intervals following the addition of PGE_2 (10^{-7} mol/L) to the medium. Panel a, control; Panel b, 20 min; Panel c, 2 hr; Panel d, 4 hr; Panel e, 8 hr; Panel f, 21 hr. The two arrows point to cells that begin to change within 2 hr of prostanoid addition, while the arrowhead defines a cell undergoing change later. (Final magnification ×400.)

We next determined whether PGE_2 might be acting through a pathway involving the endogenous generation of cAMP in its effects on cell morphology. As the data in Fig. 5

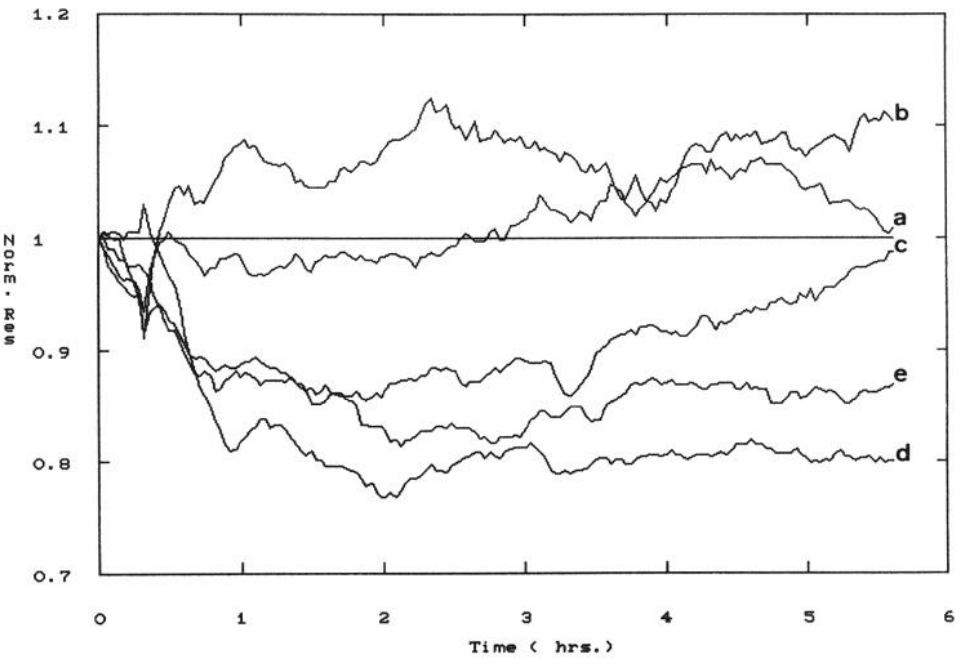


FIG. 3. Effect of increasing concentrations of PGE_2 on the normalized resistance of orbital fibroblast monolayers (the electrode-cell system is treated as a series RC circuit for convenience). Cells from a patient with severe Graves' ophthalmopathy were seeded in electrode chambers as described in *Materials and Methods*. Fibroblasts were allowed to attach for 24 hr and were then shifted to medium supplemented with 1% FBS and the concentration of prostanoid indicated. The concentrations of PGE_2 in the cultures were as follows: a; control; b, 10^{-9} mol/L; c, 10^{-8} mol/L; d, 10^{-7} mol/L; e, 10^{-6} mol/L.

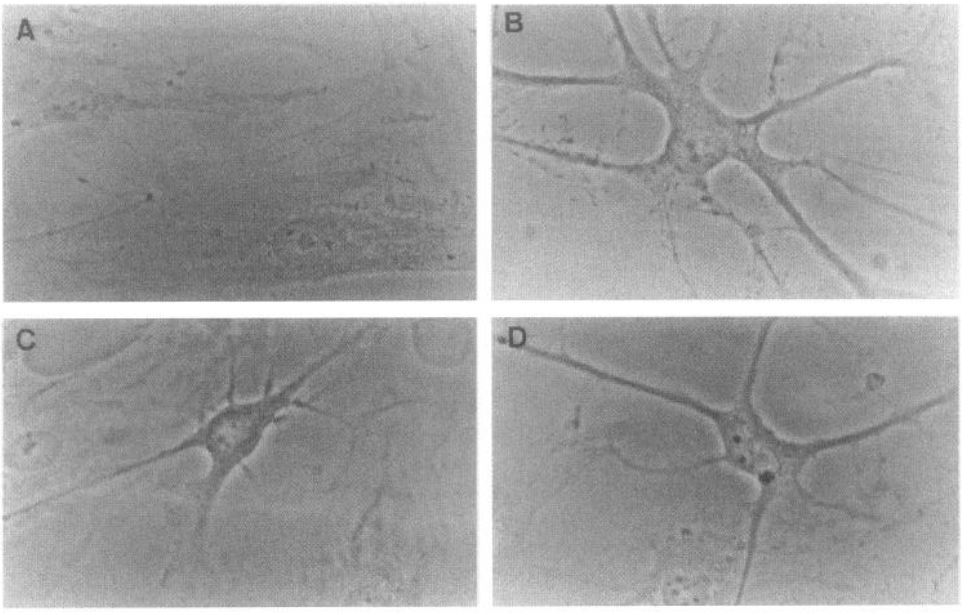


FIG. 4. Phase-contrast microscopy demonstrating the effects of PGE_2 (100 nmol/L, Panel B), forskolin (5 μmol/L, Panel C), and 8-br-cAMP (3 mmol/L, Panel D) on the morphology of orbital fibroblasts. Cultures were treated with nothing (control, Panel A) or the test compound for 4 hr (Final magnification $\times$ 1400.)

clearly demonstrate, PGE_2 (100 nmol/L) and Butaprost (10 μmol/L) both increase cAMP generation in orbital fibroblasts massively, several hundred-fold, above the levels seen in untreated control cultures. By contrast, Sulprostone, which failed to elicit any change in cell shape, did not increase cAMP levels in these fibroblasts.

To determine whether ongoing gene transcription or protein translation are necessary for the mediation of the morphogenic effects of PGE_2 in orbital fibroblasts, actinomycin D (1 μg/mL) or cycloheximide (10 μg/mL) was added to culture medium in addition to the prostanoid. Neither inhibitor affected the morphogenic action of PGE_2. These inhibitor concentrations are associated with greater than 90% inhibition of RNA and protein synthesis, respectively, in human fibroblasts (27).

In our early studies, we were impressed by susceptibility to PGE_2-mediated shape change of orbital fibroblasts from patients with ophthalmopathy. However, the question of whether this effect was a function of the orbital disease or reflected a property common to cells from individuals without orbital pathology was not confronted. We therefore examined several strains of orbital fibroblasts from normal donors and as well as those from patients with severe Graves' ophthalmopathy. As the data in Table 3 suggest, fibroblast strains from normal orbital connective tissue manifest shape changes following exposure to PGE_2 (10^{-7} mol/L) for 4 hr.

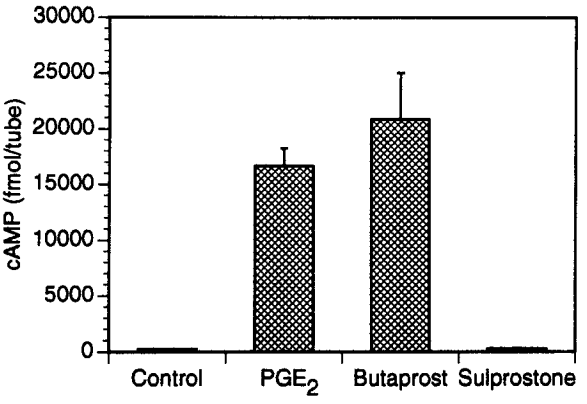


FIG. 5. The effect of PGE_2 (100 nmol/L), Butaprost (10 μmol/L), and Sulprostone (10 μmol/L) on the generation of cAMP in orbital fibroblasts. Confluent monolayers were cultivated in 24-well clusters and were treated as descibed in the *Materials and Methods* section. The data are expressed as the mean ±SEM (n = 3).

TABLE 3. Effect of PGE_2 on the morphology of several strains of human fibroblasts in primary cell culture

Strain type	Percent cell change
Graves' orbit-strain 1	
control	0
PGE_2	45
Graves' orbit-strain 2	
control	1
PGE_2	44
Graves' orbit-strain 3	
control	1
PGE_2	50
Normal orbit-strain 1	
control	1
PGE_2	23
Normal orbit-strain 2	
control	2
PGE_2	29
Normal orbit-strain 3	
control	2
PGE_2	22
Abdominal dermal-strain 1	
control	1
PGE_2	1
Abdominal dermal-strain 2	
control	1
PGE_2	2
Pretibial dermal-strain 1	
control	0
PGE_2	1
Pretibial dermal-strain 2	
control	0
PGE_2	1

Each culture was initiated on sterilized glass coverslips 24 hr before experimental manipulations. Then culture medium containing 10% FBS was removed, and fresh medium containing 1% serum without (control) or with PGE_2 (10^{-7} mol/L) was added. Cultures were incubated at 37 C for 4 hrs, fixed, and manual cell counts conducted. Each strain was examined in four different fields per coverslip, and at least 100 cells per culture were inspected.

The number of cells undergoing changes appears somewhat fewer than in cultures derived from patients with Graves' ophthalmopathy. This difference does not relate to a higher threshold in the normal cultures because the addition of higher concentrations of PGE_2 (up to 10^{-5} mol/L) failed to increase the response rate in these cells. In fact, fewer cells responded at these higher concentrations of PGE_2 (see Table 2). Moreover, studies carried out for greater than 24 hr demonstrated the differences between normal and Graves' derived fibroblasts. As the data in Table 3 also indicate, none of the four dermal fibroblast strains tested responded to the prostanoid, regardless of their anatomic region of origin or whether the tissue donor had Graves' disease. Graves' orbital strain 1, abdominal dermal strain 2, and pretibial dermal strain 2 were all derived from a single donor with severe ophthalmopathy but without clinically apparent dermopathy.

Discussion

Human orbital fibroblasts in culture engender intrinsic characteristics that set them apart from dermal and other extraorbital fibroblasts. These differences are apparent despite long-term maintenance in cell culture. PGE_2 addition to the medium causes a marked alteration in the morphology of orbital fibroblast cultures that is transient and involves the development of cellular processes. We do not yet understand the functional implications of the shape changes to orbital tissue *in vivo*. Moreover, the function of the cellular processes is undefined. Time-lapse microscopy suggests that these processes participate in considerable dynamic interplay with similar terminations on adjacent cells, suggesting a potential role in cell-to-cell communication (Wang, H-S., M.G.Hogg, T.J.Smith, unpublished observations).

The PGE_2-treated fibroblasts undergoing shape-change resemble a number of cell types previously described including the IL-1 producing stellate cells that emanate from the synovial membranes involved in rheumatoid arthritis (28). Dendritic cells and Langerhans cells are antigen presenting lymphoid-derived components of the immune system found in the circulation and in many nonlymphoid tissues (29). These cells are also stellate in shape; however, we have no evidence that orbital fibroblasts undergoing shape-change possess immunologic functions distinct from those of other fibroblast types. PGE_2 has been shown to regulate the shape of corneal endothelial cells, an action mediated by the EP_2 class prostanoid receptors through the generation of cAMP (30, 31). Moreover, epidermal growth factor apparently elicits its influence on HeLa cell and rat-1 fibroblast morphology through an induction of cyclooxygenase, the resultant metabolites from which mediate the breakdown of actin stress fibers (32). In the current study, we present data suggesting a similar mechanism with regard to prostanoid actions in orbital fibroblasts. PGE_2 appears to be eliciting its effects on cell shape through the activation of EP_2 receptors and the generation of extremely high levels of cAMP.

The shape changes observed in these studies occurred rapidly. Changes in cell monolayer impedance were first detectable within 30–60 min. of PGE_2 addition to the cultures. This was substantially earlier than any alterations seen at the light microscopic level. Thus the ECIS method used here provides insight into the proximate cellular events associated with prostanoid action. It is possible, with this technique, to quantitate previously unrecognized, subtle changes in cell shape that might be associated with cell-small mole-

cule interactions. The noninvasive nature of these measurements allows dynamic assessment of the same population of cells over considerable periods of time. The rapidity of the PGE_2 effects coupled with their insensitivity to treatment with cycloheximide and actinomycin D strongly support PGE_2 acting through a post-translational mechanism. We have found that cells undergoing shape-changes exhibit substantial evidence of cytoskeletal reorganization (Wang, H-S., T. J. Smith; manuscript in preparation). Whether these ultrastructural changes represent primary actions of PGE_2 or are a consequence of the perturbation of other cellular processes is uncertain. The transient nature of the PGE_2 effects is consistent with prostanoid receptor desensitization. By contrast, the ability of 8-br-cAMP to sustain the shape change lends support for this possibility.

While orbital fibroblasts from donors with severe ophthalmopathy exhibit a marked susceptibility to the morphogenic effects of PGE_2, cells from normal orbits are somewhat less responsive. The molecular basis for this apparent difference is not yet understood. Differences in the proportion of responsive cells in Graves' and non-Graves' derived fibroblasts could result from the methods used to initiate cultures, where a considerable bias might be introduced into the make-up of the cell population. It is also possible that responsive cells are over-represented in connective tissue involved with Graves' ophthalmopathy and therefore in the biopsies from diseased orbits. Quantitation of relative expression of relevant prostanoid receptors in Graves' and non-Graves' derived cultures may prove enlightening. Clearly, examination of several additional strains derived from both Graves' diseased and normal orbital tissue will be necessary before any conclusions concerning differential responses to PGE_2 can be drawn. The possibility that these cultures of orbital fibroblasts are contaminated with endothelial or vascular smooth muscle cells has been addressed by our finding that none of the cells expresses Factor VIII or smooth muscle-specific α-actin (25).

The effects of PGE_2 on orbital cell morphology exhibit considerable stereo-selectivity in that several structurally related compounds are unable to mimic the action of that compound. Because four human prostanoid receptors have been identified to date, each with characteristic ligand-binding specificities and signal transduction coupling properties (33–36), this finding of stringent stereochemical requirements for activity implies that a discrete receptor type(s) is involved in the mediation of the PGE_2-dependent shape change.

The current set of observations suggests another major difference in the phenotype of orbital fibroblasts as compared to cells of dermal origin. Orbital fibroblasts express a profile of gangliosides distinct from that found in dermal fibroblasts (37). We have demonstrated previously that orbital fibroblasts are considerably more susceptible to certain actions of interferon gamma (14–18) and the newly described T cell product, leukoregulin (38, 39), than are other types of fibroblasts. These or related orbital fibroblast responses to lymphocyte-derived factors may account for orbital tissue manifestations of a systemic immunologic disease. Presumably the orbital fibroblast is a major source of the hyaluronan accumulating in the orbital tissue of patients with Graves' disease (40). While the mechanisms involved in the shape change are as yet incompletely defined, it would appear that the cytoskeleton of orbital fibroblasts is considerably less stabile than that of dermal fibroblasts. This previously unrecognized property of orbital fibroblasts in culture may be relevant to the unique circumstances *in situ* confronting the connective tissue from which it is derived. Further inquiry into possible functional roles of cells with altered morphology is warranted. Whether the differences between orbital and dermal fibroblasts relate to the neural crest origin of the former (41) is not known. In any event, the central pathogenic role of an inflammatory response in active Graves' ophthalmopathy makes the observation that orbital fibroblasts are differentially sensitive to prostaglandin action potentially important.

Acknowledgments

The authors acknowledge the generous provision of Butaprost by Dr. Harold Kluender, Miles, West Haven, CT.

References

1. **Samuelsson B, Goldyne M, Granström E, Hamberg M, Hammarström S Malmsten C.** 1978 Prostaglandins and thromboxanes. Annu Rev Biochem. 47:997–1029.
2. **Moncada S, Flower RJ, Vane JR.** 1985 Prostaglandins, prostacyclin, thromboxane A_2 and leukotrienes. In: Gilman AG, Goodman LS, Rall TW, Murad F, eds. The pharmacological basis of therapeutics. 7th ed. New York: Macmillan Publishing; 660–673.
3. **Coleman RA, Kennedy I, Humphrey PPA, Bunce K, Lumley P.** 1990 Prostanoids and their receptors. In: Hansch C, Sammes PG, Taylor JB, eds. Comprehensive medicinal chemistry, Vol. 3, Oxford: Pergamon Press; 643–714.
4. **Coleman RA, Kennedy I, Sheldrick RLG, Tolowinska IY.** 1987 Further evidence for the existence of three subtypes of PGE_2-sensitive (EP-) receptors. Br J Pharmacol. 91:407P.
5. **Negishi M, Sugimoto Y, Irie A, Narumiya S, Ichikawa A.** 1993 Two isoforms of prostaglandin E receptor EP_3 subtype. J Biol Chem. 268:9517–9521.
6. **Namba T, Oida H, Sugimoto Y, et al.** 1994 cDNA cloning of a mouse prostacyclin receptor. J Biol Chem. 269:9986–9992.
7. **Negishi M, Namba T, Sugimoto Y, et al.** 1993 Opposite coupling of prostaglandin E receptor EP_{3C} with G_S and G_0. J Biol Chem. 268: 26067–26070.
8. **Mauviel A, Rédini F, Hartmann DJ, Pujol J-P, Evans CH.** 1991 Modulation of human dermal fibroblast extracellular matrix metabolism by the lymphokine leukoregulin. J Cell Biol. 1455–1462.
9. **O'Banion MK, Winn VD, Young DA.** 1992 cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. Proc Natl Acad Sci USA. 89:4888–4892.
10. **Smith TJ, Bahn RS, Gorman CA.** 1989 Hormonal regulation of hyaluronate synthesis in cultured human fibroblasts: evidence for differences between retroocular and dermal fibroblasts. J Clin Endocrinol Metab. 69:1019–1023.
11. **Smith TJ, Kottke RJ, Lum H, Andersen TT.** 1993 Human orbital fibroblasts in culture bind and respond to endothelin. Am J Physiol. 265:C138-C142.
12. **Smith TJ.** 1984 Dexamethasone regulation of glycosaminoglycan synthesis in cultured human skin fibroblasts: similar effects of glucocorticoid and thyroid hormones. J Clin Invest. 74:2157–2163.
13. **Henrikson RC, Smith TJ.** 1995 Ultrastructure of cultured human orbital fibroblasts. Cell Tissue Res. 278:629–631.
14. **Smith TJ, Bahn RS, Gorman CA, Cheavens M.** 1991 Stimulation of glycosaminoglycan accumulation by interferon gamma in cultured human retroocular fibroblasts. J Clin Endocrinol Metab. 72:1169–1171.
15. **Smith TJ, Ahmed A, Hogg MG, Higgins PJ.** 1992 Interferon-γ is an

inducer of plasminogen activator inhibitor type-1 in human orbital fibroblasts. Am J Physiol. 263:C24-C29.
16. **Higgins PJ, Smith TJ.** 1993 Pleotrophic action of interferon gamma in human orbital fibroblasts. Biochim Biophys Acta. 1181:23–30.
17. **Smith TJ, Higgins PJ.** 1993 Interferon gamma regulation of *de novo* protein synthesis in human dermal fibroblasts in culture is anatomic site dependent. J Invest Dermatol. 100:288–292.
18. **Smith TJ, Higgins PJ.** 1993 Bidimensional gel electrophoretic analysis of protein synthesis and response to interferon-γ in cultured human dermal fibroblasts. Biochim Biophys Acta. 1181:300–306.
19. **Smith TJ, Wang H-S, Hogg MG, Henrikson RC, Keese CR, Giaever I.** 1994 Prostaglandin E_2 elicits a morphological change in cultured orbital fibroblasts from patients with Graves' ophthalmopathy. Proc Natl Acad Sci USA. 91:5094–5098.
20. **Lo C-M, Keese CR, Giaever I.** 1991 Monitoring motion of confluent cells in tissue culture. Exp Cell Res. 204:102–109.
21. **Tiruppathi C, Malik AB, Del Vecchio PJ, Keese CR, Giaever I.** 1992 Electrical method for detection of endothelial cell shape change in real time: assessment of endothelial barrier function. Proc Natl Acad Sci USA. 89:7919–7923.
22. **Giaever I, Keese CR.** 1993 A morphological biosensor for mammalian cells. Nature. 366:591–592.
23. **Smith TJ, Murata Y, Horwitz AL, Philipson L, Refetoff S.** 1982 Regulation of glycosaminoglycan synthesis by thyroid hormone *in vitro*. J Clin Invest. 70:1066–1073.
24. **Smith TJ.** 1987 n-Butyrate inhibition of hyaluronate synthesis in cultured human fibroblasts. J Clin Invest. 79:1493–1497.
25. **Smith TJ, Sempowski GD, Wang H-S, Del Vecchio PJ, Lippe SD, Phipps RP.** 1995 Evidence for cellular heterogeneity in primary cultures of human orbital fibroblasts. J Clin Endocrinol Metab. 80: 2620–2625.
26. **Giaever I, Keese CR.** 1991 Micromotion of mammalian cells measured electrically. Proc Natl Acad Sci USA. 88:7896–7900.
27. **Smith TJ.** 1988 Glucocorticoid regulation of glycosaminoglycan synthesis in cultured human skin fibroblasts: evidence for a receptor mediated mechanism involving effects on specific *de novo* protein synthesis. Metabolism. 37:179–184.
28. **Goto M, Sasano M, Yamanaka H, et al.** 1987 Spontaneous production of an interleukin 1-like factor by cloned rheumatoid synovial cells in long-term culture. J Clin Invest. 80:786–796.
29. **Steinman RM.** 1991 The dendritic cell system and its role in immunogenicity. Annu Rev Immunol. 9:271–296.
30. **Jumblatt MM, Matkin ED, Neufeld AH.** 1988 Pharmacological regulation of morphology and mitosis in cultured rabbit corneal endothelium. Invest Ophthalmol Vis Sci. 29:586–593.
31. **Jumblatt MM, Paterson CA.** 1991 Prostaglandin E_2 effects on corneal endothelial cyclic adenosine monophosphate synthesis and cell shape are mediated by a receptor of the EP_2 subtype. Invest Ophthalmol Vis Sci. 32:360–365.
32. **Peppelenbosch MP, Tertoolen LGJ, Hage WJ, de Laat SW.** 1993 Epidermal growth factor-induced actin remodeling is regulated by 5-lipoxygenase and cyclooxygenase products. Cell. 74:565–575.
33. **Funk CD, Furci L, FitzGerald GA, et al.** 1993 Cloning and expression of a cDNA for the human prostaglandin E receptor EP_1 subtype. J Biol Chem. 268:26767–26772.
34. **Bastien L, Sawyer N, Grygorczyk R, Metters KM, Adam M.** 1994 Cloning, functional expression, and characterization of the human prostaglandin E_2 receptor EP_2 subtype. J Biol Chem. 269:11873–11877.
35. **Kunapuli SP, Fen Mao G, Bastepe M, et al.** 1994 Cloning and expression of a prostaglandin E receptor EP_3 subtype from human erythroleukaemia cells. Biochem J. 298:263–267.
36. **Regan JW, Bailey TJ, Pepperl DJ, et al.** 1994 Cloning of a novel human prostaglandin receptor with characteristics of the pharmacologically defined EP_2 subtype. Mol Pharmacol. 46:213–220.
37. **Berenson CS, Smith TJ.** 1995 Human orbital fibroblasts in culture express ganglioside profiles distinct from those in dermal fibroblasts. J Clin Endocrinol Metab. 80:2668–2674.
38. **Smith TJ, Wang H-S, Evans CH.** 1995 Leukoregulin is a potent inducer of hyaluronan synthesis in cultured human orbital fibroblasts. Am J Physiol. 268:C382-C388.
39. **Hogg MG, Evans CH, Smith TJ.** 1995 Leukoregulin induces plasminogen activator inhibitor type-1 in human orbital fibroblasts. Am J Physiol. 269:C359–C366.
40. **Smith TJ, Bahn RS, Gorman CA.** 1989 Connective tissue, glycosaminoglycans and diseases of the thyroid. Endocr Rev. 10:366–391.
41. **Noden DM.** 1982 Periocular mesenchyme: neural crest and mesodermal interactions. In: Jakobiec FA, ed. Ocular anatomy, embryology, and teratology. Philadelphia: Harper and Row; 97–119.