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Modulation of collagen production by fibroblasts

Effects of chronic exposure to agonists that increase intracellular cyclic AMP

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Cultured human lung fibroblasts were evaluated for their responsiveness to isoprenaline (isoproterenol) or prostaglandin E_2 before and after chronic incubation with the agonist. Cells incubated for 6h with either agonist were suppressed in terms of collagen production and exhibited increased intracellular cyclic AMP. Cells incubated for 72h with the agonist and then re-challenged for 6h with the same agonist did not demonstrate suppressed collagen production or increased cyclic AMP. Cells incubated for 72h with isoprenaline still responded to prostaglandin E_2 when challenged for 6h; however, when the order of agonist exposure was reversed, cells incubated with prostaglandin E_2 did not respond to a challenge by isoprenaline. If cells were allowed to recover for 48h without the agonist after a 72h chronic incubation, they recovered their responsiveness to the agonist. The results indicate that, although cultured fibroblasts may become desensitized to one agonist, they may retain their sensitivity to a second agonist and that chronic suppression of collagen production may be achieved by alternate exposure to isoprenaline and prostaglandin E_2.

Current concepts of collagen metabolism suggest that fibroblasts tightly control the amount and type of collagen that they produce (Hance & Crystal, 1977; Breul *et al.*, 1980). There is evidence, however, that such cells are capable of modulating collagen production in response to endogenous factors such as aging or viral transformation, or to extracellular influences such as hormones, pharmacological agents, proteinases, or serum factors (Manner *et al.*, 1974; Hassel *et al.*, 1976; Narayanan & Page, 1977). In this regard, studies by Baum *et al.* (1978) and Berg *et al.* (1981) have shown that agents that cause cells to increase their cyclic AMP, such as isoprenaline (isoproterenol), prostaglandins of the E series and cholera toxin, rapidly cause soft-tissue fibroblasts to suppress the amount of collagen that they produce.

The modulation of collagen production by agents that influence intracellular cyclic AMP are of interest for several reasons. First, physiological concentrations of these hormones may play an important role in modulating the amount of collagen produced in various tissues. Second, since a variety of pharmacological agents will increase cyclic AMP within collagen-producing cells, it is reasonable to hypothesize that such agents might be useful in suppressing collagen production in those conditions in which there may be increased collagen deposited in tissues, such as in fibrotic disorders.

It is important to realize, however, that association of increased intracellular cyclic AMP with suppression of collagen production has only been demonstrated in short-term experiments where cultured fibroblasts have been incubated with the hormone for 6h or less. Since chronic exposure of fibroblasts to hormones that increase cyclic AMP often leads to 'desensitization' to that hormone, with a return of cyclic AMP towards the baseline concentration, it is possible that the effects of such hormones on suppressing collagen production might be short-lived. Thus chronic exposure of soft-tissue fibroblasts to β-agonists or prostaglandin E_2 might lead to an initial suppression of collagen production, but, after chronic exposure to these agents, the fibroblast may soon return to producing larger amounts of these macromolecules.

In this context, the present study was designed to evaluate the effects of a β-agonist (isoprenaline) and prostaglandin E_2 on collagen production by diploid lung fibroblasts under a variety of conditions: acute

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exposure (6h), chronic exposure (3 days) and intermittent exposure (chronic exposure, followed by a recovery period, followed by acute exposure). In addition, desensitization to these agents was evaluated by chronically exposing fibroblasts to each agent and then exposing the fibroblasts to the other agent. The results suggest that chronic exposure of fibroblasts to a β-agonist or prostaglandin E_2 does cause fibroblasts to become desensitized to each of these agents. However, intermittent exposure with a given hormone or alternating exposure with one hormone followed by the second hormone does, in some instances, result in continuous suppression of collagen production, suggesting that such agents may have a role in the therapy of the fibrotic disorders.

Experimental

Materials

Hanks balanced salt solution and Dulbecco's modified Eagle's medium were obtained from both the N.I.H. Media Unit and Grant Island Biological Co., Grand Island, NY, U.S.A. Foetal-calf serum was from Colorado Serum Co., Denver, CO, U.S.A.; penicillin–streptomycin solution was from Grand Island Biological Co.; β-aminopropionitrile fumarate, ascorbate, glutamine and *N*-ethylmaleimide were from Sigma Chemical Co., St. Louis, MO, U.S.A.; prostaglandin E_2 was a gift from J. Pike, Upjohn, Kalamazoo, MI, U.S.A.; DL-isoprenaline was from Sigma; bacterial collagenase (form III) was from Advanced Biofactures Co., Lynbrook, NY, U.S.A.; [^{14}C]proline (265mCi/mmol) was from either Schwarz/Mann, Orangeburg, NY, U.S.A., or Amersham Corp., Arlington Heights, IL, U.S.A. All other reagents were analytical grade. Cells were disrupted with a Sonifier Cell Disrupter, from Heat Systems–Ultrasonics Inc., Plainview, NY, U.S.A.

Cell cultures

All studies were done with a diploid fibroblast line designated HFL-1 (American Tissue Culture Collection no. CCL 153), derived from a foetal human lung of 16 weeks gestation. These fibroblasts have a doubling time of 24h, have a normal karyotype, exhibit contact inhibition, and contain no mycoplasma or viral contaminants. All studies were carried out with cells in subcultivations 10–20. The fibroblasts were cultured in 10ml of growth medium (Dulbecco's modified Eagle's medium supplemented with 10% foetal-calf serum, 100 units of penicillin/ml, 100μg of streptomycin/ml and 0.06% glutamine). Fibroblasts were plated at 5×10^5cells/100mm culture dish in 10ml of growth medium.

Quantification of collagen production

To measure collagen production, each plate of fibroblasts was incubated at 37°C for 6h in labelling medium {10ml of Hanks' balanced salts solution supplemented with 50μg of β-aminopropionitrile fumarate/ml, 50μg of ascorbate/ml and 16μCi of [^{14}C]proline, with or without an agonist (see below)}. After the labelling period, the medium and cell layer were harvested together, heated at 100°C for 15min, sonicated (with a microtip at 40W for 30s), and dialysed against deionized water at 4°C until less than 100c.p.m. of [^{14}C]proline appeared in the diffusate. The percentage of [^{14}C]proline in newly synthesized collagen relative to total protein was determined by using bacterial collagenase as previously described (Baum *et al.*, 1978; Breul *et al.*, 1980).

Quantification of cyclic AMP content

To measure intracellular cyclic AMP content, plates of fibroblasts parallel to those used to quantify collagen production were incubated at 37°C for 6h in labelling medium in which 2mM-[^{12}C]proline was substituted for [^{14}C]proline. After incubation, the plates were placed on ice, the media removed by aspiration, and the cells harvested with 2ml of 5% (w/v) trichloroacetic acid. Intracellular concentration of cyclic AMP was determined with a radioimmunoassay kit (New England Nuclear, Boston, MA, U.S.A.). Protein was determined by the method of Lowry *et al.* (1951), and intracellular cyclic AMP was expressed as pmol/mg of cellular protein.

Exposure of fibroblasts to agents that mediate intracellular cyclic AMP concentrations

To determine the effectiveness of collagen suppression by exposure to agonists which increase intracellular cyclic AMP, HFL-1 fibroblasts were plated at 5×10^5cells/100mm culture dish in growth medium. At 3 days after subcultivation, the medium was aspirated and replaced by fresh medium containing either DL-isoprenaline (2μM), prostaglandin E_2 (0.3μg/ml), or no agonist. Six types of incubation were utilized: (1) 'control' = 6h incubation without added agonist; (2) 'acute exposure' = 6h incubation with isoprenaline or prostaglandin E_2; (3) 'chronic exposure' = cells incubated with isoprenaline or prostaglandin E_2 for 72h; (4) 're-challenged' = cells incubated with isoprenaline or prostaglandin E_2 for 72h, the media removed, and the cells re-exposed to isoprenaline or prostaglandin E_2 for 6h; (5) 'chronic exposure with recovery' = cells incubated with isoprenaline or prostaglandin E_2 for 72h, the media removed, and the cells incubated for 48h in fresh media without either agonist; and (6) 'chronic exposure with recovery followed by re-challenge' = cells incubated with isoprenaline or prostaglandin E_2 for 72h, the

media removed, the cells incubated for 48 h in fresh media without agonists and then the cells re-challenged with media containing isoprenaline or prostaglandin E_2. For all incubation conditions, collagen production and intracellular cyclic AMP content were determined as described above.

Statistical evaluation

All data are presented as means ± S.D., each mean representing an average of three culture plates. Comparisons were made by using the Wilcoxon Mann–Whitney rank sum test for non-parametric analysis (Kraft & Van Eeden, 1968).

Results

Acute and chronic effects of agonists on collagen production

When fibroblasts were exposed to isoprenaline or prostaglandin E_2 for 6 h ('acute exposure'), intracellular cyclic AMP was increased and collagen production suppressed ($P < 0.5$, both comparisons) (Fig. 1). In contrast, when exposure to either agonist was continued for 3 days ('chronic exposure'), the cyclic AMP concentrations returned to normal ($P > 0.4$ for isoprenaline; $P > 0.2$ for prostaglandin E_2) and the suppression of collagen production was lost ($P > 0.2$ for isoprenaline; $P > 0.3$ for prostaglandin E_2) (Fig. 1). Thus isoprenaline and prostaglandin E_2 are capable of suppressing collagen production in diploid human fibroblasts in the short term, but are not capable of maintaining this suppressive affect for 72 h.

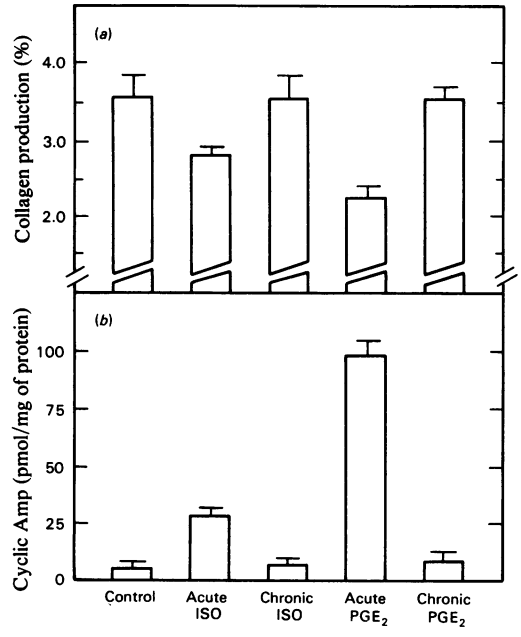

Fig. 1. *Effect of acute and chronic exposure to agonists on (a) collagen production and (b) intracellular cyclic AMP in normal human fibroblasts*

HFL-1 fibroblasts were incubated for 3 days (chronic exposure) or 6 h (acute exposure) in the presence of isoprenaline (ISO) or prostaglandin E_2 (PGE_2) as described in the text. The effect on collagen production is represented as the percentage of [^{14}C]proline in newly synthesized collagen relative to total protein; the effect on intracellular content of cyclic AMP is given as pmol/mg of cell protein. Data are presented as means ± S.D., each mean representing the average of three culture plates.

Agonist re-challenge of chronically treated fibroblasts

The loss of the acute suppression of collagen production observed when fibroblasts were chronically treated with agonists was not due to a loss or inactivation of the agonists in the media. For example, when fibroblasts were chronically treated with isoprenaline and then 're-challenged' acutely with media including fresh isoprenaline, cyclic AMP concentrations remained low ($P > 0.1$) and collagen production was not suppressed ($P > 0.2$) (Fig. 2). Similar observations were made when fibroblasts were chronically exposed to prostaglandin E_2 and then 're-challenged' acutely with fresh prostaglandin E_2 ($P > 0.1$ for cyclic AMP content; $P > 0.5$ for collagen production) (Fig. 3).

In contrast, cells chronically treated with isoprenaline were responsive to acute exposure to prostaglandin E_2, even though the cells had become 'desensitized' to the β-agonist isoprenaline i.e. cyclic AMP increased ($P < 0.5$) and collagen production was suppressed ($P < 0.01$) (Fig. 2). This was not true, however, when the order of the agonist exposure was reversed. When fibroblasts treated with prostaglandin E_2 for 72 h and then acutely challenged with isoprenaline, no suppression of collagen production ($P > 0.2$) or increase in cyclic AMP was observed ($P > 0.3$) (Fig. 3). Thus, in terms of collagen production, when cells become desensitized to prostaglandin E_2, there is an apparent crossed desensitization to a β-agonist.

Recovery of responsiveness to hormones

Although the acute suppression of collagen production induced by isoprenaline or prostaglandin E_2 was lost on chronic exposure, the responsiveness could be recovered by allowing the cells a period of time in which they were no longer exposed to either agonist. When fibroblasts were exposed to isoprenaline for 72 h and then allowed to recover by incubation for an additional 48 h without hormone, collagen production was not suppressed

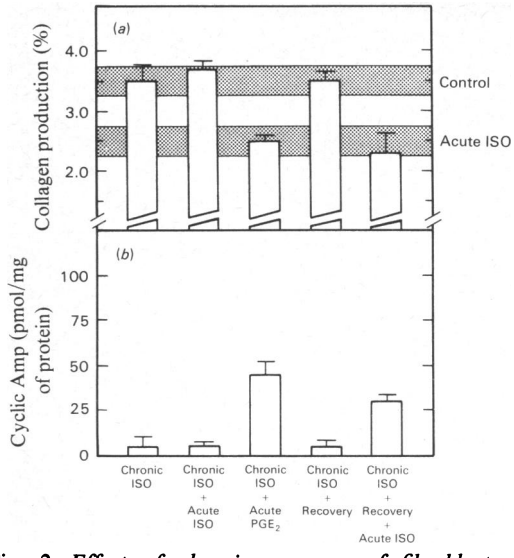

Fig. 2. *Effect of chronic exposure of fibroblasts to isoprenaline on the subsequent response of the cells to acute exposure to isoprenaline or prostaglandin E_2*
HFL-1 fibroblasts were incubated with isoprenaline for 3 days ('Chronic ISO'). Parallel plates of cells were similarly treated and then: (1) re-challenged with isoprenaline ('Chronic ISO + Acute ISO'); (2) re-challenged with prostaglandin E_2 ('Chronic ISO + Acute PGE_2'); (3) isoprenaline was removed and the cells were allowed to recover for 48 h ('Chronic ISO + Recovery'); or (4) isoprenaline was removed, the cells were allowed to recover for 48 h, and then re-challenged with isoprenaline ('Chronic ISO + Recovery + Acute ISO'). (*a*) Effect on collagen production is represented as the percentage of [^{14}C]proline in newly synthesized collagen relative to total protein; (*b*) effect on intracellular content of cyclic AMP is given as pmol/mg of cell protein. Data are presented as means ± S.D., each mean representing the average of three culture plates. Shaded areas represent the percentage of collagen produced in untreated HFL-1 cells ('Control') and in cells exposed to isoprenaline for 6 h ('Acute ISO').

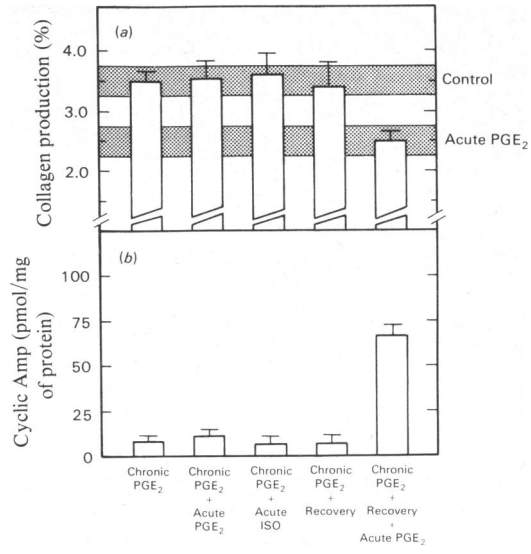

Fig. 3. *Effect of chronic exposure of fibroblasts to prostaglandin E_2 on the subsequent response of the cells to acute exposure with isoprenaline or prostaglandin E_2*
HFL-1 cells were incubated with prostaglandin E_2 for 3 days ('Chronic PGE_2'). Parallel plates of cells were similarly treated and then: (1) re-challenged with prostaglandin E_2 ('Chronic PGE_2 + Acute PGE_2'); (2) re-challenged with isoprenaline ('Chronic PGE_2 + Acute ISO'); (3) prostaglandin E_2 was removed and the cells were allowed to recover for 48 h ('Chronic PGE_2 + Recovery'); or (4) prostaglandin E_2 was removed, the cells allowed to recover for 48 h, and then re-challenged with prostaglandin E_2 ('Chronic PGE_2 + Recovery + Acute PGE_2'). (*a*) Effect on collagen production is represented as the percentage of [^{14}C]proline in newly synthesized collagen relative to total protein; (*b*) effect on intracellular content of cyclic AMP is given as pmol/mg of cell protein. Data are presented as means ± S.D., each mean representing the average of three culture plates. Shaded areas represent the percentage of collagen production in untreated HFL-1 cells ('Control') and cells exposed to prostaglandin E_2 for 6 h ('Acute PGE_2').

($P > 0.3$) and cyclic AMP remained at its baseline concentration ($P > 0.4$) (Fig. 2). When the 'recovered' cells were then re-challenged with isoprenaline, collagen production was again suppressed and intracellular cyclic AMP increased ($P < 0.05$, both comparisons) (Fig. 2). A similar effect was seen with prostaglandin E_2; cells chronically exposed and then allowed to recover were again responsive to this agent ($P < 0.05$, both comparisons) (Fig. 3).

Discussion

The mechanism through which diploid human fibroblasts regulate collagen production appears to be closely correlated with the concentration of intracellular cyclic AMP (Baum *et al.*, 1978, 1980; Berg *et al.*, 1981). When intracellular concentrations of cyclic AMP are elevated above basal value during a 6 h (acute) exposure to isoprenaline or prostaglandin E_2, HFL-1 fibroblasts produce significantly less collagen relative to total protein. However, when fibroblasts are chronically exposed to either agonist, intracellular cyclic AMP does not remain increased and collagen production returns to its value in unstimulated cells. When these fibroblasts are allowed an adequate period of time with which to recover from long-term exposure to either

agonist and then re-challenged with the same agonist, cyclic AMP is again increased and collagen production is again suppressed. This desensitization to isoprenaline is specific for isoprenaline, since fibroblasts chronically treated with isoprenaline are still responsive to acute challenge by prostaglandin E_2 even though the fibroblasts are desensitized to further challenges by isoprenaline. However, when the order of agonist exposure is reversed, cells desensitized to prostaglandin E_2 do not respond to a challenge by isoprenaline.

Although the mechanism underlying the response of collagen production to chronic exposure to agents that increase intracellular cyclic AMP in HFL-1 fibroblasts is not known, it is likely to involve a desensitization mechanism similar to that described for the response to hormones in other cell lines (Remold-O'Donnell, 1974; Chlapowski *et al.*, 1975; Mickey *et al.*, 1975; Newcombe *et al.*, 1975; Shear *et al.*, 1976; Su *et al.*, 1976). In these cells, the adenylate cyclase system consists of unique receptors that couple with specific hormones, a regulatory protein that binds GTP, and a catalytic unit responsible for converting ATP into cyclic AMP. The regulatory protein appears to be, in part, responsible for the affinity of each agonist to its receptor, as well as the coupling of the receptor to the catalytic unit of adenylate cyclase (Levitzki, 1978; Levitzki & Helmreich, 1979). Crossed desensitization has also been described in a variety of cell lines in which desensitization induced by one hormone results in the loss of responsiveness to another hormone (Su *et al.*, 1976; Tuck *et al.*, 1980). In the present study, chronic exposure to prostaglandin E_2 resulted in crossed desensitization to the β-agonist isoprenaline, whereas chronic exposure of HFL-1 fibroblasts to isoprenaline did not result in desensitization to prostaglandin E_2. By analogy with other systems that have been studied, this suggests that in HFL-1 fibroblasts desensitization to isoprenaline may occur at the level of the receptor, whereas desensitization to prostaglandin E_2 may occur subsequent to the receptor and is non-specific.

Although the desensitization–resensitization mechanism found in the present system may not be apparent in all cell lines, it appears to be an important aspect of the adenylate cyclase system in HFL-1 fibroblasts. Since short-term exposure to cyclic AMP derivatives and agents that increase cyclic AMP alters the rate of collagen production relative to total protein, it is obvious that intracellular cyclic AMP concentrations may be one method by which the fibroblasts regulate collagen production (Hsie *et al.*, 1971; Peterkofsky & Prather, 1975, 1979; Baum *et al.*, 1978, 1980; Berg *et al.*, 1981).

The fact that collagen, a secretory protein, is subject to a host of external factors which alter its production by fibroblasts may have important clinical consequences, since current concepts suggest that, under constant physiological conditions, collagen production by HFL-1 fibroblasts remains unchanged (Breul *et al.*, 1980). In several clinical conditions there exists an imbalance between the rate of collagen synthesis and the rate of collagen degradation, such that excessive amounts of collagen accumulate in the extracellular space (Uitto & Lichenstein, 1976). Since the present study indicates that chronic suppression of collagen production can be obtained through interrupted or alternate exposure to either isoprenaline or prostaglandin E_2, such an approach may serve as a model for regulating collagen production in clinical situations involving collagen overproduction.

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