Cyclic AMP-regulating agents inhibit endotoxin-mediated cartilage degradation

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The influence of cyclic AMP on cartilage degradation was investigated by using phosphodiesterase inhibitors [theophylline and 3-isobutyl-1-methylxanthine (IBMX)], forskolin (which activates the catalytic subunit of adenylate cyclase) and cyclic AMP analogues (dibutyryl and 8-bromo). Breakdown was assessed by quantification of proteoglycans released into the media of 8-day bovine nasal-septum cartilage cultures. Theophylline (1–20 mM), IBMX (0.01–2 mM) and dibutyryl cyclic AMP (0.1–2 mM) had little or no influence on the rate of proteoglycan release from unstimulated (no-endotoxin) cartilages. A small but detectable increase in breakdown was observed with 8-bromo cyclic AMP (0.5–2 mM) and forskolin (50–75 µg/ml). To examine potential inhibitory influences of these agents, the cyclic AMP modulators were added to cultures simultaneously treated with *Salmonella typhosa* endotoxin (12–25 µg/ml), a potent stimulator of cartilage degradation. The 3–4-fold stimulation of breakdown by endotoxin was strikingly inhibited by all three classes of cyclic AMP regulators. Optimal inhibition was found at 10–20 mM-theophylline, 1–2 mM-IBMX, 50–75 µg of forskolin/ml, 2 mM-dibutyryl cyclic AMP and 2 mM-8-bromo cyclic AMP. Inhibition was shown to be reversible, indicating that cartilages were viable after treatment. Sepharose CL-2B chromatography of proteoglycan products released from treated cartilages showed that the endotoxin-stimulated shift to lower average M_r was significantly prevented by cyclic AMP analogues and phosphodiesterase inhibitors. Together, these results show that agents which increase cyclic AMP inhibit both quantitative and qualitative aspects of endotoxin-mediated cartilage degradation.

INTRODUCTION

Cyclic AMP is an important intracellular second messenger and regulator in many biological systems. Several studies suggest that it is important in chondrocyte metabolism. For example, cyclic AMP analogues have been shown to stimulate chondrocyte DNA synthesis [1], RNA synthesis [2], amino acid transport [3,4] and macromolecular synthesis [5–7]. In addition, similar effects have been shown by using agents (such as theophylline and IBMX) which modulate endogenous cyclic AMP concentrations [1,3–5].

Unlike anabolic processes, little is known about the influence of cyclic AMP on cartilage catabolism. Dibutyryl cyclic AMP and 8-bromo cyclic AMP were shown to inhibit the release of matrix proteoglycans synthesized by isolated rat costal cartilage chondrocytes [8]. On the other hand, dibutyryl cyclic AMP stimulated the release of proteoglycans from immature rabbit ear cartilage [9].

We [10–14] and others [15–18] have been studying cartilage degradation using bovine nasal-cartilage discs cultured in the presence of stimulators such as bacterial endotoxin (lipopolysaccharides), rheumatoid synovial membranes or synovium-conditioned media, and interleukin-1 (IL-1; which appears to be similar to or identical with catabolin). Endotoxin, a readily available stimulator, provides an especially useful probe with

which to study the biochemical mechanism of cartilage degradation, because of its striking potency *in vitro*.

In this study, we examined the effects of cyclic AMP modifiers on degradation in unstimulated and endotoxin-stimulated cartilage. Results of experiments using three different methods of altering cartilage cyclic AMP suggest that this versatile molecule acts as a potent inhibitor of endotoxin-mediated cartilage breakdown, while having little influence on unstimulated bovine nasal-cartilage catabolism.

MATERIALS AND METHODS

Materials

All chemicals were reagent grade or the best commercially available. DMEM containing glutamine and glucose (4.5 g/l), penicillin, streptomycin, amphotericin B, Hanks' balanced salt solution and foetal bovine serum (heat-inactivated) were purchased from GIBCO (Chagrin Falls, OH, U.S.A.). 1,9-DimethylMethylene Blue was obtained from Polysciences (Warrington, PA, U.S.A.). $\text{Na}_2^{35}\text{SO}_4$ was obtained from New England Nuclear Corp. (Boston, MA, U.S.A.). The endotoxin used was a phenol-extracted chromatographically purified lipopolysaccharide preparation from *Salmonella typhosa* (Sigma Chemical Co., St. Louis, MO, U.S.A.). Theophylline, IBMX, 8-bromo cyclic AMP, forskolin

Abbreviations used: IBMX, 3-isobutyl-1-methylxanthine; IL-1, interleukin-1; DMEM, Dulbecco's modified Eagle's medium.

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and *N*⁶,2'-*O*-dibutyryl cyclic AMP were also purchased from Sigma. The dibutyryl cyclic AMP was reported to contain 1–3% of a monobutyryl derivative.

Cartilage degradation assay

Cartilage degradation was assayed by measurement of proteoglycan released during eight days of culture, by a modification of the procedures previously described [10,16,17]. Bovine snouts obtained from a local slaughterhouse were quickly transported to the laboratory, where nasal-septum cartilage was removed, cleaned of adhering tissue and cut into uniform discs by an aseptic technique. Individual cartilage discs (0.5 cm × 0.1 cm or 0.2 cm × 0.1 cm) were placed in culture wells (Falcon 3047 24-well plate, or Falcon 3072 96-well plate respectively) and fed with DMEM containing 10% (v/v) heat-inactivated foetal bovine serum, streptomycin (100 µg/ml), penicillin (100 units/ml) and amphotericin B (5 µg/ml). Various amounts of test substances or control vehicle were added to cartilage cultures, keeping volumes constant (1.5 or 0.25 ml in the larger and smaller wells respectively). Endotoxin, theophylline and dibutyryl cyclic AMP were dissolved in DMEM; forskolin was dissolved in ethanol; IBMX and 8-bromo cyclic AMP were dissolved in dimethyl sulphoxide. Final concentrations of dimethyl sulphoxide and ethanol did not exceed 3% and 1.5% (v/v) respectively. Control cultures containing equivalent amounts of dimethyl sulphoxide or ethanol showed no detectable change in experimental results compared with DMEM controls.

Cultures were incubated at 37 °C in a humidified chamber containing CO₂/air (1:19), were fed by a change of medium after 4 days and were terminated at the end of 8 days. All media were held frozen and later assayed for proteoglycan. Cartilage discs were solubilized by 24 h digestion with papain as described previously [10,12].

Proteoglycan assay

Proteoglycan was assayed by using the metachromatic dye 1,9-dimethylMethylene Blue [19]. A stock solution (16 mg of dye dissolved in 5 ml of ethanol + 95 ml of 0.1 M-formate buffer, pH 3.5) was diluted 1:10 with 0.1 M-formate buffer before use. Dye (5 ml) was added to 10–40 µl of sample (medium or cartilage digest), mixed, and *A*₅₃₅ was measured immediately. Whale chondroitin sulphate (5–40 µg) served as assay standard. Results are expressed as the percentage of proteoglycan (PG) released into culture medium after 8 days incubation:

$$\text{PG release (\%)} = 100 \times \frac{\text{PG in medium}}{\text{PG in medium} + \text{cartilage PG}}$$

Statistical analysis

All experiments were done three or more times in quadruplicate. The Dunnett's [20] analysis was used to determine statistically significant differences between control and experimental groups.

Characterization of [³⁵S]sulphate-labelled products

Radioactively labelled proteoglycan products released from prelabelled cartilage were characterized as previously described [10,13]. Briefly, cartilage discs incubated for 24–48 h in complete DMEM (MgCl₂ substituted for MgSO₄) containing 10–20 µCi of Na₂³⁵SO₄/ml (2 ml/disc) were washed three times in Hanks' solution

and re-incubated for 24 h in non-radioactive (MgSO₄-replete) medium. Using this labelling procedure we have previously shown that nearly all of the incorporated [³⁵S]sulphate is in high-*M*_r proteoglycan complexes, and > 90% was identified as proteoglycan chondroitin sulphate [10,14]. Radioactive discs were then incubated as described above in the 8-day cartilage-degradation assay. Labelled products released into the culture media and residual labelled proteoglycan in the cartilage discs were analysed by gel-filtration chromatography. Cartilage proteoglycan was extracted directly into column elution buffer (see below) by vigorous shaking at 4 °C, with two changes of 2.5 ml each per disc.

Medium (0.5 ml) from control or experimental groups was mixed with an equal volume of elution buffer and applied to a 1.5 cm × 29 cm Sepharose CL-2B column. Cartilage extracts were applied directly. Column elution was at 12 ml/h with a dissociative buffer composed of 4 M-guanidinium chloride, 0.15 M-sodium acetate buffer (pH 7.0) and proteinase inhibitors (phenylmethanesulphonyl fluoride, benzamidinium hydrochloride and iodoacetamide, all 1 mM). Fractions (1 ml) were collected and counted for radioactivity in a Packard 3320 liquid-scintillation counter. Calibration markers (and their peak elution positions) included Na₂³⁵SO₄ (*V*_s, fraction no. 45) and Blue Dextran 2000 (*V*₀, fraction no. 15). The *M*_r distribution of products was based on their elution volume (*V*_e).

RESULTS

Effects of the phosphodiesterase inhibitors theophylline (1–20 mM) and IBMX (0.01–2 mM) on unstimulated and endotoxin-enhanced cartilage degradation are shown in Fig. 1. Neither agent, at any of the concentrations used, caused a significant change in basal release of cartilage proteoglycan. In contrast, endotoxin-stimulated breakdown (approx. 2-fold) was inhibited in a dose-dependent fashion, detectable at about 5 mM-theophylline and 0.01 mM-IBMX. At maximal inhibitory doses (10–20 mM-theophylline and 1–2 mM-IBMX), breakdown was decreased nearly to control values.

Like the phosphodiesterase inhibitors, forskolin (5–75 µg/ml) greatly decreased endotoxin-stimulated catabolism in a dose-dependent manner (Fig. 2). Detectable inhibition was found at 5 µg/ml, with optimal effect at approx. 75 µg/ml. Samples treated with forskolin alone showed a minor increase in basal proteoglycan release.

As a third independent mechanism to increase cartilage cyclic AMP, dibutyryl cyclic AMP and 8-bromo cyclic AMP were added to unstimulated and endotoxin-treated cartilages (Fig. 3). Both of these analogues depressed endotoxin-mediated breakdown in a concentration-dependent manner. Dibutyryl cyclic AMP (2 mM) decreased proteoglycan release from endotoxin-treated samples by 75%, whereas 2 mM-8-bromo cyclic AMP depressed breakdown to control values. When added without endotoxin, 8-bromo cyclic AMP appeared to enhance breakdown to a small degree at 0.5–2 mM (Fig. 3b), whereas dibutyryl cyclic AMP had no detectable effect (Fig. 3a).

Inhibition by the cyclic-AMP-modulating agents was found to be reversible. Thus, although endotoxin-mediated breakdown was largely inhibited during an initial 4-day culture period in the presence of theo-

Fig. 1. Dose-response of phosphodiesterase inhibitors on cartilage degradation

Cartilage discs were cultured for 8 days in the absence (○) or presence (●) of 12.5 μ g of endotoxin/ml, at the indicated concentrations of theophylline and IBMX. Values are means $\pm$ S.E.M. ($n = 4-12$).

Fig. 2. Dose-response of forskolin on cartilage degradation

Cartilage discs were cultured for 8 days in the absence (○) or presence (●) of 12.5 μ g of endotoxin/ml at the indicated concentrations of forskolin. Values are means $\pm$ S.E.M. ($n = 8$).

Fig. 3. Dose-response of cyclic AMP analogues on cartilage degradation

Cartilage discs were cultured for 8 days in the absence (○) or presence (●) of 12.5 μ g of endotoxin/ml at the indicated concentrations of dibutyryl cyclic AMP and 8-bromo cyclic AMP. Values are means $\pm$ S.E.M. ($n = 5-9$).

phylline, forskolin or dibutyryl cyclic AMP, degradation was completely restored when the agents were removed on day 4 and endotoxin alone was added. Indeed, the rates of proteoglycan depletion during days 4-8 in previously inhibited cultures were comparable with the initial rates (days 0-4) induced by endotoxin in the absence of each inhibitor. By day 8 of culture, proteoglycan depletion was extensive (approx. 90%) in cartilages treated with endotoxin alone for the last 4 days of culture. This was true after relief of inhibition, whether the initial (day 0-4) cultures were made in the presence or absence of endotoxin.

Proteoglycan products released into the culture medium and those remaining in cartilage at the end of the experiment were analysed by gel-filtration chromatography (Fig. 4). In unstimulated cultures, [35 S]proteoglycans extracted from cartilage after an 8-day period were eluted as a single broad proteoglycan-monomer peak largely excluded from the gel (Fig. 4c). This was comparable with the elution profile of monomers from uncultured cartilage. Products released into the culture medium showed a minor decrease in peak radioactivity, a slight shift to the right, as well as the

Fig. 4. Gel-filtration chromatography of proteoglycan products

Cartilage discs prelabelled with [^{35}S]sulphate as described in the Materials and methods section were cultured for 8 days in the absence (○) or presence (●) of $12.5\text{ }\mu\text{g}$ of endotoxin/ml. Cultures were maintained either in the absence of cyclic AMP regulators (a, b, c) or in the presence of 1 mM-dibutyl cyclic AMP (d, e, f) or 1 mM-IBMX (g, h, i). Media harvested on day 4 (a, d, g) and day 8 (b, e, h) and residual day-8 cartilage extracts (c, f, i) were applied to a column (1.5 cm $\times$ 29 cm) of Sepharose CL-2B and eluted under dissociative conditions. ^{35}S radioactivity profiles, shown for one of duplicate cultures in each condition, are expressed as percentages of total applied radioactivity (c.p.m.). The vertical bar in each panel indicates the elution peak position of proteoglycan monomers extracted from labelled cartilage at the outset of culture.

appearance of a small oligosaccharide peak (Figs. 4a and 4b). These changes are consistent with limited monomer degradation in control cultures, as we [11] and others [21] have previously reported. In contrast, endotoxin treatment produced a major shift to lower average M_r values during both culture periods (Figs. 4a and 4b), which was largely prevented by simultaneous treatment with either dibutyl cyclic AMP (Figs. 4d and 4e) or IBMX (Figs. 4g and 4h). Residual proteoglycan extracted from 8-day endotoxin-treated cartilage, representing approx. 5% of total proteoglycan, was devoid of larger- M_r species, and demonstrated a polydisperse distribution, with K_{av} approx. 0.6 (Fig. 4c). Simultaneous treatment with either class of inhibitor restored the cartilage proteoglycan profiles toward control levels, with retention of a high- M_r exclusion peak, and limited contribution from smaller species (Figs. 4f and 4i).

DISCUSSION

Endotoxin, a potent stimulator of proteoglycan release from cartilage *in vitro*, is a useful probe with which to study the biochemical events associated with cartilage degradation. Although the mechanism of its

action is not clearly understood, it seems likely that endotoxin directly promotes chondrocyte degradative activity. Indeed, isolated bovine articular chondrocytes in confluent culture demonstrate a similar endotoxin response, with extensive breakdown of soluble proteoglycan monomers [22] and release of large amounts of proteoglycan-degrading neutral proteinase (J. J. Steinberg, unpublished work). On the other hand, the bovine nasal cartilage used in the present experiments, even though from the relatively avascular portion of the septum, may have small vascular channels which will contain endothelial and blood cells. Hence, the tissue may be a source of IL-1, a potent stimulator of cartilage degradation [21,23–25]; the possibility that endotoxin stimulates breakdown via endogenous IL-1 production cannot be entirely excluded. Moreover, the present findings that enhanced cyclic AMP concentrations inhibit breakdown could be consistent with that possibility, in view of the report that cyclic AMP inhibits IL-1 production in U937 leukaemic cells [26].

However, for several reasons we consider that alterations in endogenous IL-1 production, if they do occur, are unlikely to account for the present findings. Firstly, articular cartilage shows a similar, but slower,

degradative response to endotoxin ([27]; J. J. Steinberg, unpublished work). Such cartilage is not penetrated by blood vessels and therefore is unlikely to show significant IL-1 production, although an unconfirmed report suggests that chondrocytes may themselves be a source of IL-1 [28]. Secondly, endotoxin-stimulated breakdown is largely unaffected by simultaneous treatment with anti-(human IL-1) serum (Genzyme, Boston, MA, U.S.A.) at concentrations that effectively block (by 60% or more) the cartilage-degradative response to IL-1 (M. Yodlowski, J. R. Hubbard & J. J. Steinberg, unpublished work). Although high-affinity membrane receptors for human IL-1 have been demonstrated in bovine cells [29], a difference in species-specific antibody responsiveness cannot be excluded. Thirdly, the degradation of proteoglycan monomers in response to endotoxin (Fig. 4) is far more extensive than that generally produced by IL-1 [21,24,25]. Lastly, differential responses to inhibitors further distinguish the two agents: cortisol inhibits endotoxin-stimulated breakdown in a dose-dependent manner [30], but has little or no effect at concentrations as high as $10\text{ }\mu\text{M}$ on IL-1-induced breakdown [24]; polymyxin B treatment has been shown to abolish the cartilage matrix-degrading activity of endotoxin, but not of blood mononuclear-cell factors containing IL-1 [15].

In this investigation we have shown that five different modulators of cellular cyclic AMP, acting via three independent mechanisms [(i) blockade of cyclic AMP degradation through phosphodiesterase inhibition, (ii) activation of the catalytic subunit of adenylate cyclase through forskolin action, and (iii) directly elevating cyclic AMP concentrations by the use of membrane-permeable cyclic AMP analogues] cause both quantitative and qualitative inhibition of endotoxin-mediated breakdown of bovine nasal cartilage *in vitro*. These observations strongly suggest that increasing intracellular cyclic AMP blocks the cartilage-destructive potential of endotoxin. Since little is known about the mechanism of endotoxin action on cartilage or about cartilage catabolism in general, the present findings may lead to a clearer understanding of how cyclic AMP is related to these processes. Whether cyclic AMP acts to prevent the production of a second regulator or directly blocks a catabolic cascade is yet to be determined. Studies on the specificity of this cyclic AMP response and its potential clinical value are also of future interest.

In previous studies, endotoxin has been shown to cause proteoglycan release from living, but not from dead, cartilage [15,31]. Hence the restoration of a potent degradative response to endotoxin after relief from the inhibitory effects of cyclic AMP modulators indicates that these agents were not merely exhibiting a toxic effect on the tissue.

The results of this study are consistent with our observation that at high concentrations indomethacin blocks the destructive action of endotoxin on cartilage [32]. At similar concentrations, Newcombe *et al.* [33] showed that indomethacin acts as a potent inhibitor of articular-cartilage cyclic nucleotide phosphodiesterase.

Our findings are also consistent with those of Miller & Erickson-Lucas [8], who found suppression of proteoglycan release by 8-bromo cyclic AMP and by dibutyryl cyclic AMP in a foetal-rat chondrocyte system. However, stimulation of breakdown by theophylline and dibutyryl cyclic AMP was noted by Shinmei *et al.* [9], using immature rabbit ear cartilage. Such conflicting reports

may reflect the differences in the experimental conditions or sources of cartilage. Studies are required to determine if the observations discussed in the present paper are similar for different types of cartilage and cartilage from different species.

Endotoxin-stimulated breakdown resulted in the release of most of the cartilage proteoglycan during an 8-day culture period. The released products, although much decreased in hydrodynamic size, were broadly distributed over a range of relatively high M_r values. We have previously shown [10] that this pattern is indicative of extensive core-protein proteolysis. The current findings suggest that enhancement of cyclic-AMP-dependent mechanisms inhibits the generation of endotoxin-induced breakdown products by a decrease in core proteolysis.

We thank Elayne A. Bornslaeger of the Department of Biology, University of Pennsylvania, for her advice on the use of phosphodiesterase inhibitors and cyclic AMP analogues. We also thank Danette Mattmueller and Pat Mark for their excellent technical assistance and Terri Sayles for preparation of the manuscript. This work was aided by a grant from the Orthopaedic Research and Education Foundation to J.R.H. and by N.I.H. grants AM-35906 and AM-36983. F.A.B. is a Visiting Medical Student from Albany Medical College, Albany, NY, U.S.A.

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Received 11 August 1986/7 January 1987; accepted 26 January 1987