Porcine catabolin stimulates prostaglandin E₂ secretion but does not affect intracellular cyclic AMP production in pig synovial fibroblasts

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SUMMARY
Response in vitro to partially purified porcine leucocyte catabolin were studied in pig synovial fibroblasts. In serum-free cultures catabolin was found to stimulate secretion of prostaglandin E₂ (PGE₂) in a time and concentration-dependent manner. The initial stimulation of PGE₂ secretion occurred only after a latent interval of six hours. In the same cell line catabolin was found to have no effect on the production of cyclic adenosine monophosphate (cAMP) at times ranging from 30 s to 20 min, even at concentrations up to 15 times greater than that required to promote accelerated release of glycosaminoglycans from cultured bovine nasal cartilage. It is therefore concluded that in pig synovial fibroblasts catabolin evokes a delayed secretion of PGE₂ but does not alter cyclic AMP production.

Progressive resorption of articular cartilage is a prominent feature of most of the chronic arthritides and is generally thought to account for much of the joint deformity and loss of function which characterise these diseases.¹ Despite general agreement about the need to prevent this loss of cartilage, limited knowledge of the precise mechanisms responsible for its resorption continues to hamper the development of logical strategies for preventing this degradative process.

Recent research has focused on the possible role of cell messenger proteins which appear to mediate cartilage resorption in vitro.²⁻⁴ These include pig catabolin, an acidic protein (pl 4.9) with a molecular weight of 21 000, produced by pig mononuclear cells when they are cultured with concanavalin A.⁵ Catabolin has been shown to induce resorption of proteoglycans in various types of cartilage, including porcine and human articular cartilage.⁶ ⁷

Recent studies have confirmed that purified porcine mononuclear cell catabolin is a form of interleukin 1.⁸ Since prostaglandin E₂ secretion in vitro is a characteristic connective tissue cell response to interleukin 1,⁹ initial experiments were performed to establish whether porcine catabolin stimulates PGE₂ secretion in synovial fibroblasts.

As it seemed possible that catabolin might activate connective tissue cells by modulating cyclic nucleotide concentrations, experiments were performed to assess the effect of catabolin on cAMP production.

Materials and methods

CELL CULTURE TECHNIQUES
Synovial fibroblasts were isolated from the metacarpophalangeal joint synovium of 4–6-month old pigs by the method of Dayer et al.¹⁰

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PREPARATION OF CATABOLIN

Catabolin was prepared from medium conditioned by concanavalin A stimulated pig leucocytes as described by Saklatvala and Sarsfield.11 Protein fractions from an Ultrogel AcA 54 (LKB) column were assayed for catabolin-like activity in the bovine nasal cartilage system,7 and active fractions (mol. wt 15 000–25 000) were subsequently pooled. A single batch of crude leucocyte catabolin was used for all experiments. The batch was stored in 50 µl aliquots at −20°C. Its catabolic activity was periodically re-examined, and no deterioration in activity was detected over a period of six months.

CYCLIC AMP EXPERIMENTS

The effect of catabolin on cellular cAMP production was investigated in porcine synovial fibroblasts subcultured from the same cell line. Experiments were performed in 35 mm Linbro multiwell plates. When the fibroblasts were confluent culture medium was removed, and the cells were washed twice in Gey’s balanced salt solution and then incubated in air for two hours in DMEM supplemented with 20 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid), 2-4 mM theophylline, and 4 mM glutamine. The fibroblasts were then exposed to identical medium with either no further additives (negative control), prostaglandin E2 0-5 µM (positive control), or catabolin. At appropriate times (see ‘Results’) the cells were fixed in 0-5 ml of 6% trichloroacetic acid (TCA). After one hour the cAMP extracts were transferred to 10×75 mm glass test tubes and the TCA was removed with diethyl ether (four vols×4). Protein precipitates were solubilised in 1 ml of 2 M NaOH and stored at −20°C until assayed.

CYCLIC AMP ASSAY

Cyclic AMP was measured by the radioimmunoassay method of Steiner et al.12 The standards were treated with 6% trichloroacetic acid in an identical manner to the unknown samples. Blanks, standards, and unknown samples were individually succinylated with fresh reagent prepared by dissolving 1 mg of succinic anhydride (Sigma) in 1 ml of dry acetone (AR) and adding this to 5-6 M di-isopropyl triethylamine (Sigma) 100:46, v/v. The assays were performed in duplicate in disposable polypropylene (10×75 mm) tubes. Each tube contained (in order of addition) 100 µl of cAMP antibody (Miles Labs) at a dilution (1:16) sufficient to bind 55–65% of the labelled ligand and 100 µl of tracer (10 µCi/ml (mCi/l) adenosine 3’,5-cyclic phosphoric acid 2’-O-succinyl-3-[125]iodotyrosine methyl ester (Amer- sham International) at a final concentration of approximately 50 pmol/l.

Bound and free 125I ligand were separated with bovine serum albumin (Sigma) and absolute ethanol (AR). Precipitated pellets were counted to within a 1% error using a multichannel gamma spectrometer. A 10 min incubation of five cyclic AMP samples at 30°C with bovine phosphodiesterase (Sigma) and controls caused a reduction in the detectable cyclic AMP of greater than 90% in all of the enzyme treated samples.

PROSTAGLANDIN EXPERIMENTS

Confluent fibroblasts were washed twice with Gey’s balanced salt solution and then preincubated for two hours in air at 37°C in DMEM supplemented with 20 mM HEPES and 4 mM glutamine. The cells were then incubated in medium containing identical supplements with either catabolin or no further additives (controls). At various time intervals (see ‘Results’) the medium was collected and immediately frozen in liquid nitrogen. Samples were then stored at −20°C until required for assay.

Purification of thawed medium was carried out with (Sep-pak, Waters) octadecylsilica columns by a method modified from that of Powell.13 100 µl aliquots of medium were diluted with 0-2 M citric acid (pH 3) and loaded onto columns pre-equilibrated with diluted (approx 0-1 mM) HCl (pH 3). The columns were then washed with 10 ml of 2% ethanol/water to remove polar material, and 10 ml of petroleum ether (AR, b.p. 40–60°C) to remove any non-polar lipids and fatty acids. The prostaglandins were then eluted in 5 ml of methyl formate (Aldrich). Preliminary experiments gave recoveries of 87-3±7-7% (SEM) (n=6), and subsequent assay values were corrected accordingly. The methyl formate fractions were collected in plastic vials and evaporated to dryness under reduced pressure at room temperature. Prostaglandin fractions were redissolved in 2 ml of assay buffer (0-01 M sodium phosphate pH 7-3, 0-1 M Na3N, 0-1% bovine gammaglobulin), and 100 µl samples were assayed in duplicate by the Seragen radioimmunoassay procedure as described in the manufacturer’s instructions. The rabbit PGE2 antiserum used in the assay had the following cross-reactivity profile at 50% maximal binding; PGE1 100%, PGA2 6%, PGA1 3%, 6-keto PGE1 1%, PGF2a 1-5%, and PGF1α, PGB1, and PGB2 all <1%. Although the antiserum is not specific for PGE2, all prostaglandin detected was assumed to be PGE2, since previous studies have shown that this is the major prostanoid produced by synovial cells under similar culture conditions.14

PROTEIN ASSAYS

Solubilised protein samples were neutralised with an
equal volume of 2 M hydrochloric acid. Protein was then assayed in duplicate samples by the Coomassie Blue dye method with bovine serum albumin as standard.¹⁵

**STATISTICAL METHODS**

Student's two-tailed t test was used to determine the significance of differences between the means of two groups.

**Results**

**CATABOLIN ACTIVITY**

Concentration-response studies indicated that the stock preparation of catabolin was active at 60 nl/ml (µl/l) of culture medium (Fig. 1). Maximal resorption of proteoglycans (measured by release of glycosaminoglycans into the medium of cultured bovine nasal cartilage discs) occurred at 100 nl/ml (µl/l) of culture medium. (Catabolin concentrations are subsequently expressed in units per ml. One unit is here defined as that quantity of catabolin which produces a 100% increase in release of glycosaminoglycans over that observed in controls.)

**Fig. 1** Percentage release of glycosaminoglycans (GAG) (chondroitin sulphate) from discs of bovine nasal cartilage cultured in the presence of serum and catabolin for eight days. Values are mean±SEM (n=5). *p<0.001. (µl/ml=µl/l).

**Fig. 2** PGE₂ concentrations in media from pig synovial fibroblasts cultured in the presence (○) and absence (△) of catabolin. Values are mean±SEM (n=9). *p<0.001. (ng/ml=µg/l).

**PROSTAGLANDIN E₂ SECRETION**

Prostaglandin (PGE₂) secretion was evident in both control and catabolin-stimulated cells. The PGE₂ released was similar in both groups at three and six hours. However, at nine hours PGE₂ secretion was increased substantially in the cells exposed to 15 units of catabolin (Fig. 2). Concentration-response
Table 1  PGE$_2$ concentrations* in pig synovial fibroblast media after the cells were exposed to partially purified catabolin for nine hours

<table>
<thead>
<tr>
<th></th>
<th>PGE$_2$ (ng/ml)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>486±229</td>
<td></td>
</tr>
<tr>
<td>Catabolin (units/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-15</td>
<td>512±487</td>
<td>NS</td>
</tr>
<tr>
<td>0-90</td>
<td>1018±203</td>
<td>p&lt;0-01</td>
</tr>
<tr>
<td>4-50</td>
<td>1205±64</td>
<td>p&lt;0-01</td>
</tr>
<tr>
<td>15-00</td>
<td>1396±233</td>
<td>p&lt;0-01</td>
</tr>
</tbody>
</table>

*Values are mean±standard deviation (n=4).
†ng/ml=µg/l.
‡NS=not significant.

studies performed at nine hours indicated that the increment in secretion was concentration dependent (Table 1).

CYCLIC AMP
In both short (0–180 seconds) and long (3–20 minutes) time course experiments a high concentration of catabolin (15 units/ml) did not significantly alter intracellular concentrations of cyclic AMP (Figs 3 and 4). In contrast, 0·5 µM PGE$_2$ stimulated a marked increase in cAMP in agreement with previous results. Likewise no significant alteration in cyclic AMP production was observed at 0·5 or 3 minutes in fibroblasts exposed to concentrations of catabolin ranging from 1·5 to 75 units/ml (Table 2).

Fig. 3  Intracellular cAMP in cultured pig synovial fibroblasts exposed to PGE$_2$ 0·5 µM (▲), catabolin 15 units/ml (●), and control medium (○). Values are mean±SEM (n=5).

Fig. 4  Intracellular cAMP in cultured pig synovial fibroblasts exposed to PGE$_2$ 0·5 µM (▲), catabolin 15 units/ml (●), and control medium (○). Values are mean±standard deviation (n=3).

Table 2  Intracellular cAMP in pig synovial fibroblasts exposed to partially purified catabolin for 0·5 and 3 minutes

<table>
<thead>
<tr>
<th></th>
<th>0·5 min</th>
<th>3·0 min</th>
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<tbody>
<tr>
<td></td>
<td>(n=5)</td>
<td>(n=5)</td>
</tr>
<tr>
<td>Control</td>
<td>14±3*</td>
<td>14±7</td>
</tr>
<tr>
<td>Catabolin (units/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1·5</td>
<td>18±8 NS†</td>
<td>17±6 NS</td>
</tr>
<tr>
<td>15</td>
<td>15±4 NS</td>
<td>33±19 NS</td>
</tr>
<tr>
<td>75</td>
<td>—</td>
<td>12±6 NS</td>
</tr>
<tr>
<td>PGE$_2$ (0·5 µM)</td>
<td>79±15</td>
<td>p&lt;0·001 175±68 p&lt;0·001</td>
</tr>
</tbody>
</table>

*Values are mean±standard deviation.
†NS=not significant (p>0·05).

Discussion
Catabolin has been previously shown to affect connective tissue cell functions in a variety of tissues, including nasal and articular cartilage. More recently, purified catabolin has been shown to
have comitogenic activity in the mouse thymocyte assay, indicating that it is a form of interleukin 1.8

Precisely how it affects target cells and in particular whether there are intracellular mediators of activation is unknown. Cyclic AMP was considered in this regard because it is commonly involved in the transduction of peptide hormone signals. Synovial fibroblasts, which are reported to secrete prostaglandin E in response to interleukin 1,9 10 were used to examine the effects of catabolin on cyclic AMP production.9 10 The ability of these cells to secrete PGE2 in response to catabolin was established, but irrespective of the time after exposure or concentration of catabolin no significant increment or decrement in cAMP production was observed. The results are therefore inconsistent with the idea that catabolin activates connective tissue cells via a cAMP dependent mechanism. They are, however, in agreement with the findings of Dayer and others who found that within the first hour after exposure to human mononuclear cell factor, rheumatoid synovial cells did not produce more cAMP.17

Whether cAMP is involved in thymocyte mitogenesis or chondrocyte activation is a matter for further investigation.

Partially purified catabolin was found to be a potent stimulus for PGE2 secretion. This response was concentration dependent, and the concentration-response profile coincided with that for the percentage of glycosaminoglycans released from cultured discs of bovine nasal cartilage. There was a notable delay of at least six hours before catabolin stimulated the secretion of PGE2. A latency of this order suggests that secretion may be contingent upon some as yet unknown intracellular activation process and not simply confined to a direct effect on the release of cell membrane phospholipids. In this respect it would be of interest to determine whether the secretion of PGE2 depends on the integrity of transcriptional or translational mechanisms.

The findings are consistent with those of Saklatvala and others who have recently shown that PGE2 secretion is markedly increased when pig synovial fibroblasts and other connective tissue cells are cultured in the presence of purified catabolin for four days.8

Whether catabolin or related molecules stimulate prostaglandin secretion in vivo is still unknown. Several indirect lines of evidence suggest that such stimulation may occur in inflammatory joint disease. Raised levels of PGE2 have been detected in synovial fluid18 and recently Wood et al. reported the presence of an interleukin 1 like factor in synovial fluid.19 The probability that these two findings are more than coincidentally related is strengthened by in-vitro evidence that cells derived from rheumatoid synovium secrete PGE2 when exposed to interleukin 1.8 20

The role of prostaglandins in proteoglycan resorption is less conjectural. In experiments with prostaglandin synthetase inhibitors Sheppeard and his associates have shown that the proteoglycan resorption induced by synovial catabolin does not depend on prostaglandin production.21 Likewise Decker, Henney, and Dingle have found that inhibitors of prostaglandin synthesis do not alter the catabolic activity of porcine heart valve catabolin (unpublished data). In bone the prostaglandins have been implicated in matrix resorption.22 However, Gowen and associates, who have shown that partially purified human interleukin 1 stimulates bone resorption, found this to be independent of prostaglandin production.23

Thus there is evidence in vitro that catabolin and related molecules stimulate prostaglandin secretion by connective tissue cells, but this does not account for the many activities ascribed to these cytokines, and it remains for further research to clarify their precise mechanism of action.

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References

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