Modulation of H⁺ Transport Mechanisms by Interleukin-1 in Isolated Bovine Articular Chondrocytes

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Key Words

Chondrocyte • Cartilage • Na⁺/H⁺ exchange • NHE1 • H⁺-ATPase • Interleukin-1 • Metalloproteinase • Aggrecanase • pH

Abstract

The proinflammatory cytokine interleukin-1 (IL-1) promotes the degradation of articular cartilage by inhibiting matrix synthesis and stimulating degradative enzyme activity. Generation of nitric oxide (NO) in response to IL-1 is implicated in these actions. The catabolic actions of IL-1 can be inhibited by manoeuvres which are predicted to dissipate H⁺ gradients across the chondrocyte plasma membrane. In the present study, the effects of IL-1 on H⁺ extrusion from bovine articular chondrocytes were investigated. pH was measured using the H⁺-sensitive fluorescent dye BCECF. Cells were acidified by ammonium rebound and the contribution of the Na⁺-H⁺ exchanger (NHE) and of the vacuolar H⁺-ATPase to acid extrusion was characterised by ion substitution and inhibitor studies. Overnight (18h) exposure to IL-1 stimulated acid extrusion in a dose-dependent fashion. This effect represented stimulation of both

NHE and the ATPase. Characterisation of the timecourse of this response indicated that, while stimulation of acid extrusion was rapid, effects on the ATPase were only apparent after greater than 8h incubation with the cytokine. In keeping with this observation, the protein synthesis inhibitor cycloheximide abolished the stimulatory effect of IL-1 on ATPase-mediated extrusion. The upregulation of ATPase activity by IL-1 was inhibited by the NOS inhibitor L-NAME and by the NO scavenger PTIO. In cells which had not been exposed to IL-1, treatment with the NO donor SNAP also stimulated acid extrusion by the ATPase. In contrast, NHE activity was not altered by any of these compounds. Taken together, these results imply that IL-1 can stimulate acid extrusion in chondrocytes and that this reflects rapid upregulation of NHE with slower induction of H⁺-ATPase activity which requires elevated levels of NO. While ATPase induction involves protein synthesis, this process may not constitute synthesis of ATPase proteins *per se*, but rather of some associated regulatory process.

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Introduction

Articular cartilage protects the ends of long bones at diarthrodial joints and permits smooth, low-friction movement to occur. The metabolism of chondrocytes, the only cell type normally resident within the tissue, is entirely responsible for determining turnover of the extracellular matrix (ECM), by balancing synthetic and degradative processes. The ECM, which is made up of type II collagen and non-collagenous proteins, as well as large hydrated proteoglycan molecules, confers the tissue with resistance to compressive and tensile stresses and gives cartilage its biomechanical properties. The predominant proteoglycan of articular cartilage is aggrecan which comprises a protein core onto which glycosaminoglycan side chains (keratan sulphate and chondroitin sulphate) are covalently attached. Degenerative articular cartilage disorders (for example, osteoarthritis) are characterised by a reduction in the level of ECM components and are the result not only of impaired matrix synthesis, but also enhanced catabolism of type II collagen and aggrecan [1, 2].

The causes of degenerative cartilage pathology are diverse and manifold. However, it is clear that certain pro-inflammatory cytokines are elevated in degenerative joints and they play a key role in mediating tissue degradation associated with osteoarthritis and rheumatoid arthritis [2, 3]. Of these, interleukin-1 (IL-1) is probably the best studied. IL-1, present at elevated levels in the synovial fluid and extracellular matrix of arthritic tissue [4, 5], inhibits proteoglycan synthesis and stimulates degradative enzyme (matrix metalloproteinases (MMP) and aggrecanase) activity [6-8].

The cellular pathways through which IL-1 exerts its effects on articular cartilage have received considerable attention. Whilst the evidence remains somewhat contradictory, IL-1 seems to exert some of its cellular actions through the generation of NO. Experiments employing NO donors and iNOS inhibitors demonstrate that NO can inhibit collagen and proteoglycan synthesis, and under these conditions proteoglycan catabolism is enhanced [4, 9, 10].

It seems probable that the proteolytic degradation of extracellular matrix is predominantly the result of activity of two enzymes: collagenase 3, MMP13, and the aggrecanase ADAMTS4. MMP13 activity is regulated both at the transcriptional level and by cell-surface processing of a pro-form by MT1-MMP. In contrast, ADAMTS4 activity is controlled largely by post-transcriptional activation involving furin and MT4-MMP.

There is reason to speculate that H^+ homeostasis by chondrocytes may be altered by processes promoting collagenolysis and aggrecanolysis. Bafilomycin, which inhibits H^+ -ATPase activity, and chloroquine, which can dissipate H^+ gradients, both prevent the proteoglycan degradation initiated by IL-1 [11, 12]. Moreover, bafilomycin can inhibit ATPase-dependent degradation of MT1-MMP in intracellular vesicles.

As for all cells, chondrocyte intracellular pH (pH_i) is determined by the balance of constitutive acid-loading processes (cellular metabolism, inward leak of H^+) and efflux of H^+ across the cell membrane, and the uptake and release of H^+ from intracellular inclusions. In chondrocytes from a number of different species, both Na^+ - H^+ exchange (isoform 1, NHE1) and a NBD-Cl (chloro-7-nitrobenz-2-oxa-1,3-diazole)/bafilomycin sensitive H^+ -ATPase have been shown to operate to varying degrees [13-16]. In mammalian chondrocytes, HCO_3^- -dependent membrane transport processes do not operate to a great extent, probably because the expression and activity of carbonic anhydrase is low [17]. While NHE1 has been shown to be expressed in the cell membrane, the location of the H^+ -ATPase is not fully resolved: studies on other cell types suggest that it can be located either in the membrane of intracellular organelles or in the cell membrane itself (for example, [18]).

In the present study, the effects of IL-1 treatment on H^+ transport in articular chondrocytes have been determined using fluorescence measurements of pH_i . The findings show that IL-1 modifies acid extrusion from chondrocytes, and raise the possibility that altered distribution of H^+ ions in cartilage may be associated with cartilage degradation.

Materials and Methods

HOE694 (3-methylsulfonyl-4-piperidinobenzoyl)-guanidine methanesulfonate was the kind gift of AventisPharma, Frankfurt, Germany. All other chemicals were purchased from Sigma-Aldrich, Poole, UK.

All experiments were performed in HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) buffered solutions at 37°C. HEPES-buffered saline (HBS) comprised (in mM): NaCl (145), KCl (5), glucose (10), HEPES (15) and pH was corrected to 7.40 using concentrated NaOH. Na^+ -free solution was identical to HBS except N-methyl-D-glucamine chloride (NMDG-Cl) replaced NaCl and pH was corrected to 7.4 using concentrated HCl. To calibrate the BCECF (2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein) fluorescence signal, high K^+ solution was used with composition (mM): KCl (150), glucose (10), HEPES (15) and pH was adjusted to the desired

level using concentrated KOH. Stock solutions of inhibitors were prepared at concentrations such that the solvent never accounted for more than 1% (v:v) of the experimental volume.

Stock solutions of interleukin-1 α (IL-1) were prepared in sterile filtered HBS supplemented with 1% bovine serum albumin, at a concentration 100 times greater than that at which it was finally used.

Cell isolation

Chondrocytes were isolated using a standard enzyme digestion protocol modified from one which has previously been described [19]. Briefly, cartilage was dissected under sterile conditions from the articular surfaces of metacarpophalangeal joints of 18 to 36-month-old steers obtained within 2h of slaughter. Cartilage shavings were placed in Dulbecco's modified Eagle's medium (DME), supplemented with 1% (v:v) penicillin (1250 U ml⁻¹) - streptomycin (5mg ml⁻¹) solution. Cartilage was then digested overnight (18h, 37°C, 5% CO₂) with 0.1% collagenase (w:v) in the presence or absence of IL-1 (concentration dependent on experiment). In some cases, cells were isolated in DME supplemented with the nitric oxide synthase inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME, 1mM), or the nitric oxide scavenger 2-phenyl-4,4,5,5-tetramethyl-imidazole-1-oxyl 3-oxide (PTIO, 50 μ M) or the protein synthesis inhibitor cycloheximide (1mM). In other experiments the nitric oxide donor S-nitroso-N-acetylpenicillamine (SNAP, 200 μ M) was added to the cartilage digestion medium, in the absence of IL-1, 6h before the end of the isolation. At the end of the isolation the cell suspension was filtered, first through a coarse strainer (pore size approximately 500 μ m), then through a 20 μ m nylon filter mesh. The final filtered solution was centrifuged and washed twice (10min, 3000 $\times$ g) before the cell pellet was resuspended at a final concentration of approximately 1 $\times$ 10⁶ cells ml⁻¹ in HBS. Cell viability and number were assessed using the Trypan blue exclusion technique with cells visualised in an improved Neubauer haemocytometer under light microscopy [20]. In all cases, cell preparations were only used when viability was greater than 95%. For certain experiments, cells were prepared as described above in the absence of IL-1, and then incubated at 37°C for a further period in the presence of IL-1 (0.5ng ml⁻¹), with aliquots of cells removed for experimental use at given time points (1-18h).

pH recording

pH_i was measured using the H⁺-sensitive fluoroprobe BCECF in conjunction with standard cuvette fluorimetric techniques [21]. Cells suspended in HBS at 37°C (1 $\times$ 10⁶ cells ml⁻¹) were incubated with the membrane permeant ester, BCECF-AM (10 μ M), for at least 20min and for not more than 60min. Cells were then microcentrifuged (10,000 $\times$ g) and subjected to acidification by ammonium prepulse as required (below) before resuspension in the appropriate experimental solution. pH_i was calculated from the BCECF fluorescence emission measured using a Hitachi F2000 spectrofluorimeter (excitation wavelengths 490nm and 439nm; emission wavelength 535nm). The 490nm/439nm ratio was converted to pH using a calibration curve constructed from cells treated with the ionophore

nigericin (2 μ M) and suspended in a high-K⁺ solution at different pH values (pH 6-8) [21]. Separate calibrations were performed in the presence of NBD-Cl to discount effects of the inhibitor on the fluorescent signal.

Acid equivalent extrusion pathways were characterised for cells pre-acidified by ammonium prepulse (20mM NH₄Cl in HBS, 15min). Given that the activity of many acid extruding processes is steeply dependent on pH_i, care was taken to ensure that the acidification imposed was of consistent magnitude.

Acidified cells were resuspended in Na⁺-free solution or in HBS supplemented with NHE inhibitors HOE694 (10 μ M, specific for NHE1) or amiloride (1mM, a non-specific dose) or with the H⁺-ATPase inhibitors NBD-Cl (chloro-7-nitrobenz-2-oxa-1,3-diazole; 100 μ M) or bafilomycin A₁ (200nM). Acid equivalent fluxes (J_{H⁺}, mM min⁻¹) were calculated for the first 180s of recovery using the equation J_{H⁺} = Δ pH_i/ Δ t $\times$ buffering power (β _i), where, for this cell type, β _i = -43.3pH_i + 377 for pH_i values between 6.4 and 7.2 [22].

Statistics

Unless otherwise stated, results are presented as mean $\pm$ standard error of the mean (SEM), where *n* is the number of batches of cells liberated by digestion of cartilage from different joints on which experiments were performed. For each individual batch, each experiment was performed in triplicate. Significant differences were determined by Student's unpaired *t*-test.

Results

For all of the experiments described here, isolated chondrocytes were acidified by ammonium prepulse and the subsequent recovery of pH_i was monitored using BCECF fluorescence. The mean pH of chondrocytes following acidification was 6.50 $\pm$ 0.01 (*n* = 92).

The rate of pH_i recovery in HEPES-buffered solution for cells isolated in the presence of IL-1 was markedly greater than that for cells isolated in its absence. Using an estimate of intrinsic (non-HCO₃⁻) buffering capacity (β _i) derived previously for this cell type [22] the rate of pH_i recovery was converted to acid equivalent efflux (J_{H⁺}, mM min⁻¹; Figure 1).

To determine the dose dependence of the effect of IL-1 on H⁺ efflux from acidified articular chondrocytes, the 18h isolation protocol was performed in the presence of variable concentrations of the cytokine. The magnitude of acid equivalent efflux, measured over the first 180s of recovery from acidification increased with rising doses of IL-1 (0.05 - 2ng ml⁻¹; Figure 2).

For chondrocytes isolated in the absence of IL-1, H⁺ efflux during the first 180s following acidification was almost entirely dependent on the presence of extracellular

Fig. 1. Effect of IL-1 on acid extrusion from bovine articular chondrocytes. Cells were isolated over 18h in DME lacking or supplemented with IL-1 (0.5 ng ml⁻¹). Acid equivalent efflux (J_H) was calculated from the recovery of pH in the 180s following ammonium prepulse. $n = 3$, * $p < 0.05$.

Fig. 2. The effect of increasing doses of IL-1 on acid extrusion from articular chondrocytes. Cells were isolated over 18h in DME supplemented with IL-1 (0-2 ng ml⁻¹). Acid equivalent efflux (J_H) was calculated from the recovery of pH in the 180s following ammonium induced acidification. $n = 3$, * $p < 0.05$.

Na^+ ions (2.64 ± 0.09 mM min⁻¹ in the presence, 0.53 ± 0.09 mM min⁻¹ in the absence of Na^+ ions, $P < 0.01$). The addition of NBD-Cl (100 μ M) had no significant effect on acid extrusion ($J_H = 2.66 \pm 0.25$ mM min⁻¹).

Fig. 3. Na^+ -dependent and NBD-Cl-sensitive components of acid extrusion in IL-1 treated bovine articular chondrocytes. (A) Representative recordings of recovery of pH_i in IL-1 treated bovine articular chondrocytes following ammonium prepulse. Cells were isolated over 18h in DME supplemented with IL-1 (0.5 ng ml⁻¹). Recovery was recorded for cells suspended in HBS, in Na^+ -free solution, and in HBS containing NBD-Cl (100 μ M). Data are representative of at least nine identical recordings. (B) Mean acid equivalent efflux (J_H) values calculated from multiple experiments performed in this way. $n = 3$, * $p < 0.05$.

In contrast, for chondrocytes isolated in the presence of IL-1 (0.5ng ml⁻¹, 18h), the pH_i recovery was less sensitive to Na^+ substitution, and inhibited to a greater extent (approximately 40%) by NBD-Cl (Figure 3). There was no statistically significant difference in the degree of inhibition of H^+ extrusion by removal of Na^+ ions, amiloride (1mM) or HOE694 (60 μ M) ($J_H = 0.83 \pm 0.09$,

Fig. 4. The timecourse of IL-1 induced up-regulation of acid extrusion in bovine articular chondrocytes.

(A) Representative recordings of recovery of pH_i in bovine articular chondrocytes following ammonium prepulse. Experiment performed using cells isolated in the absence of IL-1 or cells exposed to IL-1 (0.5 ng ml^{-1}).

(B) Change in acid extrusion with time of exposure to IL-1. Cells were isolated over 18h in the absence of IL-1, then incubated for a further period of 18h in fresh DME supplemented with IL-1 (0.5 ng ml^{-1}). Aliquots of cells were removed at various timepoints during incubation with IL-1, and the Na^+ -dependent and NBD-Cl-inhibited components of acid extrusion from cells which had been acidified using ammonium prepulse were measured. Acid equivalent efflux (J_H) was calculated from the recovery of pH in the 180s following acidification. Data are presented as the difference from the flux measured at $t = 0\text{h}$. $n = 3$, * $p < 0.05$.

1.19 ± 0.41 , $1.42 \pm 0.37 \text{ mM min}^{-1}$; respectively, $P > 0.05$). Bafilomycin A_1 (200 nM) reduced acid extrusion in IL-1-treated cells to levels comparable to those recorded in the presence of NBD-Cl ($J_H = 3.69 \pm 0.2$ vs $3.32 \pm 0.17 \text{ mM min}^{-1}$, respectively).

The timecourse of the effect of IL-1 on amiloride-sensitive and NBD-Cl-sensitive components of H^+ extrusion from acidified chondrocytes was determined. For cells isolated in the absence of IL-1, stimulation of acid efflux was apparent within 15min following exposure to IL-1 (0.5 ng ml^{-1} ; Figure 4a). In further experiments, cells were isolated in the absence of IL-1, and then incubated at 37°C for a further period in the presence of IL-1 (0.5 ng ml^{-1}), with aliquots of cells removed for experimental use at given time points (1-18h). The results from these experiments are summarised in Figure 4b.

With increasing duration of exposure to IL-1, a progressive increase in both the Na^+ -dependent and the NBD-Cl inhibited components of efflux was apparent, albeit with different timecourses. The Na^+ -dependent component reached a plateau by 8h; in contrast, an increase in the component which could be inhibited by NBD-Cl was not apparent until after 10h of exposure to IL-1 and continued to rise until 18h. For cells subjected to this procedure, the contribution of the NBD-Cl inhibited flux after 18h was greater than 50% of the total IL-1 induced flux.

To test for involvement of protein synthesis in the IL-1 effects on H^+ extrusion, chondrocytes were co-incubated with cycloheximide ($100 \mu\text{M}$) during overnight isolation in IL-1-supplemented media. In cells exposed to cycloheximide, while the amiloride sensitive component

Fig. 5. The effect of modifiers of NO levels on NBD-Cl-sensitive acid extrusion from bovine articular chondrocytes. Cells were isolated over 18h in DME lacking or supplemented with IL-1 (0.5 ng ml⁻¹). For cells exposed to IL-1, some batches were also exposed to either the NOS inhibitor L-NAME (1mM) or to the NO scavenger PTIO (50μM) during isolation. For cells not isolated in the presence of IL-1, some batches were exposed to the NO donor SNAP (200μM) for the last 6h of digestion. Acid equivalent efflux (J_H) was calculated from the recovery of pH in the 180s following ammonium induced acidification. n = 3, * p < 0.05.

of recovery was still apparent (2.33 ± 0.15 mM min⁻¹), the NBD-Cl-sensitive flux was abolished (0.00 ± 0.21 mM min⁻¹).

Figure 5 summarises the effects of three treatments designed to modify NO levels in chondrocytes. H⁺ efflux was significantly reduced when cells were isolated in IL-1 supplemented media containing either the nitric oxide synthase inhibitor L-NAME (1mM) or with the nitric oxide scavenger PTIO (50μM). This effect represented a reduction in the NBD-Cl sensitive component of efflux; the amiloride-sensitive component was unaltered.

Moreover, H⁺ efflux was stimulated in IL-1 untreated cells exposed to the nitric oxide donor SNAP (200μM) for the last 6h of their isolation. This was accounted for by a significant increase in the NBD-Cl inhibited component of efflux, which was of comparable magnitude to that induced by IL-1.

Discussion

The pro-inflammatory cytokine IL-1 inhibits proteoglycan synthesis and stimulates degradative enzyme activity in articular cartilage. Bafilomycin and chloroquine, two compounds which interfere with H⁺ homeostasis in vertebrate cells, have been found to protect against proteoglycan degradation initiated by IL-1. In the

present study, the effects of IL-1 on H⁺ transporters have been characterised for the first time in bovine articular chondrocytes.

IL-1 was found to increase H⁺ extrusion from acid-loaded chondrocytes in a dose-dependent fashion, with stimulation being maximal at around 0.5ng ml⁻¹. Previous studies have shown that two systems - Na⁺-H⁺ exchange (NHE) and a V-type H⁺-ATPase - operate in chondrocytes to remove H⁺ from the cytoplasm, with the exchanger playing the dominant role. The observation that both amiloride and NBD-Cl could attenuate the enhancement induced by IL-1 indicates that the activity of both NHE and the ATPase is increased. While NBD-Cl is known best for its actions as an ATPase inhibitor, it is not truly specific and has been reported to inhibit other membrane transport pathways, including a H⁺ channel [23]. Nevertheless, our finding that NBD-Cl inhibited acid extrusion to the same extent as another ATPase inhibitor, bafilomycin, is strong evidence that the NBD-Cl sensitive flux which is augmented by IL-1 represents the activity of the H⁺-ATPase.

There is limited information concerning the actions of IL-1 on acid extruders in other cell types. Nevertheless, in agreement with our findings, the activity of NHE has been reported to be upregulated in rat peritoneal fibroblasts and rat astrocytes by IL-1 at doses comparable to those employed here [24, 25]. Similarly, a V-type H⁺-ATPase

has been found to be stimulated by IL-1 in mouse peritoneal macrophages [26]. While the effects of IL-1 on NHE in these other studies was apparent within minutes, the increase in ATPase activity was observed only after a lag period of several hours and required *de novo* protein synthesis.

In the present study, a similar differential response was observed: the timecourse of the response to IL-1 demonstrated two distinct phases. Although acid extrusion was stimulated within 15min of exposure to IL-1, this effect was entirely the result of augmented NHE activity which was maximal after 8h; it was only at timepoints greater than 10h that increases in ATPase activity became apparent. The NHE response to growth factors and cytokines commonly reflects the activation of this protein by direct phosphorylation, Ca²⁺-calmodulin complex binding or altered interactions with regulatory proteins, and the rapidity of our response suggests similar events are occurring here. In chondrocytes, growth factors up-regulate the activity of the NHE1 isoform, as well as inducing expression of NHE3 [13]. Here, however, the observation that NHE-1 specific HOE694, amiloride and removal of Na⁺ ions all inhibited acid extrusion to the same degree indicates that the enhanced NHE activity is solely mediated by NHE1. The slower onset of ATPase activation and its inhibition by the protein synthesis inhibitor cycloheximide is similar to that observed in macrophages and suggests that this response reflects the appearance of new ATPase protein or of another protein which determines ATPase activity.

At least some of the effects of IL-1 can be attributed to the induction of NO synthesis by this cytokine. While the involvement of NO in inhibiting synthetic processes is relatively clear-cut, there is also some evidence for a role in initiating degradative events [9]. In the present study, three interventions were employed to assess the role of NO in the IL-1 response we observed. Both

inhibition of NOS using L-NAME and reduction of NO levels using the scavenger PTIO markedly reduced the stimulation of acid extrusion by IL-1. In both cases the effect represented ablation of ATPase-mediated acid extrusion, with no effect on NHE apparent. Similarly, when NO levels were elevated using the NO donor SNAP, upregulation of ATPase-mediated acid extrusion occurred while NHE activity was unchanged. These results indicate that NO signalling cascades underlie the alteration in ATPase activity which we observe. It seems unlikely that the fundamental event underlying increased acid extrusion in response to IL-1 (and NO) can be stimulation of the H⁺-ATPase since, in other studies, NO has been found to inhibit this transporter through cGMP-mediated events [27]. Our data may indicate therefore that some other process, permissive for ATPase activity, is the target for IL-1 actions.

The existence of parallel anion 'shunt' pathways must, to some extent dictate the activity of the electrogenic ATPase [28]. In other cells, NO has been shown to stimulate Cl⁻ currents [29]: it is therefore possible that it is a Cl⁻ conductance rather than the H⁺-ATPase *per se* which is stimulated in response to IL-1.

While this question remains to be addressed, the present findings shed further light on the diverse range of events by which IL-1 promotes cartilage destruction. They may suggest a role for the establishment of acidic compartments in cartilage in response to IL-1 which might facilitate the degradation of the extracellular matrix by IL-1.

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