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NITRIC OXIDE ACTIVATES METALLOPROTEASE ENZYMES IN ARTICULAR CARTILAGE

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Nitric oxide ($NO^{\cdot}$) is a multifunctional messenger molecule generated by a family of enzymes, collectively termed the nitric oxide synthases. We investigated the role of $NO^{\cdot}$ in the modulation of two metal-dependent proteolytic enzymes (collagenase and stromelysin) which are activated during inflammatory and infective arthritis. The inflammatory mediators interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and the bacterial cell wall fragment endotoxin, induced both nitric oxide synthase activity and stromelysin and collagenase activity in whole cell preparations and in conditioned media from explants of bovine and human cartilage. Both NO_2^- (the stable end-product of $NO^{\cdot}$) and metalloprotease activity were inhibited by competitive inhibitors of nitric oxide synthase. The $NO^{\cdot}$ donor, S-nitroso-N-acetyl-D,L-penicillamine (SNAP) also induced metalloprotease activity in a dose-dependent fashion. These data provide evidence that $NO^{\cdot}$ plays a regulatory role in the activation of metal-dependent proteases in articular chondrocytes and cartilage.

Nitric oxide ($NO^{\cdot}$) is a very small molecule synthesized from the amino acid L-arginine by a family of enzymes, the nitric oxide synthases. Its size (one of the ten smallest molecules) and its unpaired electron (denoted $^{\cdot}$), make it a highly reactive and locally diffusible free radical. Inducible forms of nitric oxide synthase are found in phagocytic cells, hepatocytes, and in cartilage(1-3). Moreover, data from our laboratory suggests that articular chondrocytes are the primary producer of $NO^{\cdot}$ amongst cells normally found in articular joints(1). The inducible forms of nitric oxide synthase release high levels of $NO^{\cdot}$ in response to endotoxin and to inflammatory mediators, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interferon-6(1-3). In the circulatory system the inducible forms of nitric oxide synthase are thought to play a role during host defense and immunological reactions(4). The roles for $NO^{\cdot}$ in cartilage are undetermined.

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Abbreviations: IL-1β, interleukin-1β; L-NAME, Nω-nitro-L-arginine methyl ester; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SNAP, S-nitroso-N-acetyl-D,L-penicillamine; TNF-α, tumor necrosis factor-α.

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The metalloproteases are metal dependent neutral proteases which are synthesized as pro-enzymes and are activated by certain physiological agents after secretion in the extracellular matrix(5). Their activity is greatly enhanced during articular catabolism (eg in septic arthritis, osteoarthritis and rheumatoid arthritis). In articular cartilage, the major metalloproteases include collagenase and stromelysin, a proteoglycanase. Both these enzymes are secreted by chondrocytes and by synovial cells in response to endotoxin, IL1-β and TNF-α(6, 7). Since both nitric oxide synthase and metalloproteases are induced in cartilage by similar stimuli, and since both are elevated in cartilage damage, we hypothesized that $NO^{\cdot}$ may play a regulatory role in the activation of metal-dependent metalloproteases.

METHODS

Materials

Dulbecco's modification of Eagle's medium (DMEM), Ca^{2+} and Mg^{2+} free Dulbecco's phosphate buffered saline (PBS) and Hank's solution, antibiotic-antimycotic solution (#600-5240; 10,000 U/ml penicillin G sodium; 10,000 μg/ml streptomycin sulfate; 25 μg/ml amphotericin B), HEPES solution (238.3 g/l), trypsin solution (0.25% (w/v) trypsin in Hank's solution) and fetal calf serum (FCS) were purchased from Gibco Laboratories Ltd, New York, NY. Tissue culture plates were from Falcon, Becton Dickinson & Co, New York, NY. Picopro scintillation vials and UltimaGold scintillation fluid were purchased from Packard Technologies, Downers Grove, IL. [^{3}H]-type I collagen (1.0 mCi/mg) was purchased from New England Nuclear, Boston, MA. Tumor necrosis factor-α (TNF-α) was a gift from Grace Wong, Genentech Corp, South San Francisco, CA. N^Gmonomethyl-L-arginine monoester was from Calbiochem, LaJolla, CA. S-nitroso-N-acetyl-D,L-penicillamine (SNAP) was from Alexis Corporation, San Diego, CA. All other chemicals and biochemicals, including [^{14}C]-β-casein (15.7 μCi/mg), were purchased from Sigma Chemical Co, St Louis, MO.

Sample collection and culture conditions

Human cartilage was obtained from a 7 year old female undergoing a knee fusion for proximal femoral focal deficiency. Bovine cartilage was obtained from occipital articular cartilage. Each sterile tissue sample was immediately placed in cold (4°C) tissue culture medium containing 90% (w/v) DMEM, 1% (v/v) antibiotic-antimycotic solution, 0.22% (w/v) $NaHCO_3$ with 10% (v/v) fetal calf serum, pH 7.35. The cartilage was cleaned of non-cartilaginous tissue, washed in sterile normal saline (NS) (x6), 10% (v/v) antibiotic-antimycotic solution in Hank's (x2), and NS (x1). For explant culture, 8 mm diameter cartilage disks were made using an 8 mm skin biopsy punch and were placed with 1 ml media (DMEM with 10% FCS) into each well of a 24-well tissue culture plate and cultured at 37°C. Media were changed every three days throughout the culture period. Chondrocytes were obtained by collagenase digestion(8) of slices of bovine articular occipital cartilage in 0.025% (w/v) collagenase, 1% (v/v) antibiotic-antimycotic solution, 2% (v/v) HEPES solution with gentle agitation at 37°C for 16 hours. The cell suspension was then spun at 30,000 rpm for 15 minutes, the supernatant discarded, the cells washed in 10% (v/v) antibiotic-antimycotic solution and plated in 75 ml culture flasks at 2 x 10^6 cells/flask. When confluent (usually 7-10 days), the cells were trypsinized into 96 well plates for nitrite and metalloprotease assays. All cultured cells were primary or secondary passage.

Nitrite release

Nitrite (NO_2^-), a stable end-product of nitric oxide, was measured in the media of cultured cells and explants utilizing the spectrophotometric method based on the Greiss reaction(9). The absorbance was measured at 550/650 nm with a 340 ATTC microplate photometer (Tecan US Inc, Research Triangle Park, NC).

Cell viability

Cell respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan(10).

Collagenolytic and caseinolytic assay

The assay for radiolabeled collagen degradation (collagenase activity) and for radiolabeled casein degradation (stromelysin activity) of conditioned media of explants of bovine occipital cartilage was that of Williams et al(7). For direct collagenolytic activity and stromelysin (or caseinolytic activity) of cultured chondrocytes, these methods were adapted to a multiwell system. In brief, 10^5 cells/well were seeded in DMEM with 10% (v/v) FCS in 96 well-multiwell tissue culture plates. The following morning, the media was removed and the cells washed with 200 μl PBS (x2). Fresh media (DMEM without FCS) containing 0.256 μCi/ml [^{3}H]-collagen or 0.08 μCi/ml [^{14}C]-casein along with the reagents to be tested were added to each well. The final volume for each well was 250 μl. After incubation for 24 hrs at 37°C, 100 μl of media was transferred for nitrite estimation. 100 μl of media was transferred to 1 ml multiwell tubes for metalloprotease activity, mixed with 100 µl precipitation solution and allowed to precipitate at 4°C overnight. For the collagenase assay the precipitation solution contained 4:1:10 (v/v); 0.2 M EDTA:0.1% (w/v) type I collagen:75% (v/v) saturated $(NH_4)_2SO_4$. For the caseinolytic assay the precipitation solution contained 150 µl ice cold 20% (v/v) trichloracetic acid and 50 µl unlabeled casein (3 mg/ml). The tubes were then centrifuged at 30,000 rpm for 15 min and 100 µl of each supernatant transferred to scintillation vials, mixed with 3 ml of scintillation fluid, and counted in a liquid scintillation spectrophotometer. Maximal substrate degradation was estimated using bacterial collagenase (1,000 ng) or trypsin (0.025% final concentration) and blank reactions were executed with media alone. The inhibitory effects of 1,10-phenanthroline (1 mM) and EDTA (10 mM) on IL-1 (50 ng/ml) induced collagenolytic and caseinolytic activity were quantified by adding these reagents to the reaction mixture.

Statistical analysis

Statistical analysis was performed using two-tailed Student's t tests.

RESULTS

Nitric oxide synthase activity in chondrocytes and cartilage

IL1-β (1-100 ng/ml), TNF-α (1-10 ng/ml) and endotoxin (1-100 µg/ml) induced NO_2^- release in a time and dose-dependent fashion in both human and bovine cartilage explants and in bovine chondrocytes (not shown). Non-stimulated cells did not release measurable NO_2^- (Fig 1a). The release of NO_2^- was inhibited by co-incubation with the nitric oxide synthase inhibitors Nω-nitro-L-arginine methyl ester (L-NAME)(Fig 1a), N^Gmonomethyl-L-arginine monoester or aminoguanidine (not shown), and with the protein synthesis inhibitor, cyclohexamide (Fig 1a). These agents alone did not affect viability (n = 8, data not shown).

Metalloprotease activity in chondrocytes and cartilage

IL1-β (50 ng/ml) stimulated a 3-fold increase in collagenolytic activity of cultured bovine chondrocytes. This activity was completely inhibited with 1 mg/ml of the nitric oxide synthase inhibitor, L-NAME or with 20 µg/ml of the protein synthesis inhibitor, cyclohexamide (Fig 1b). Endotoxin (10 µg/ml) and IL-1β (100 ng/ml) also induced stromelysin activity in the media of cultured bovine cartilage by 50-100% (Fig 2b & 2d). In each case, the increase in metalloprotease

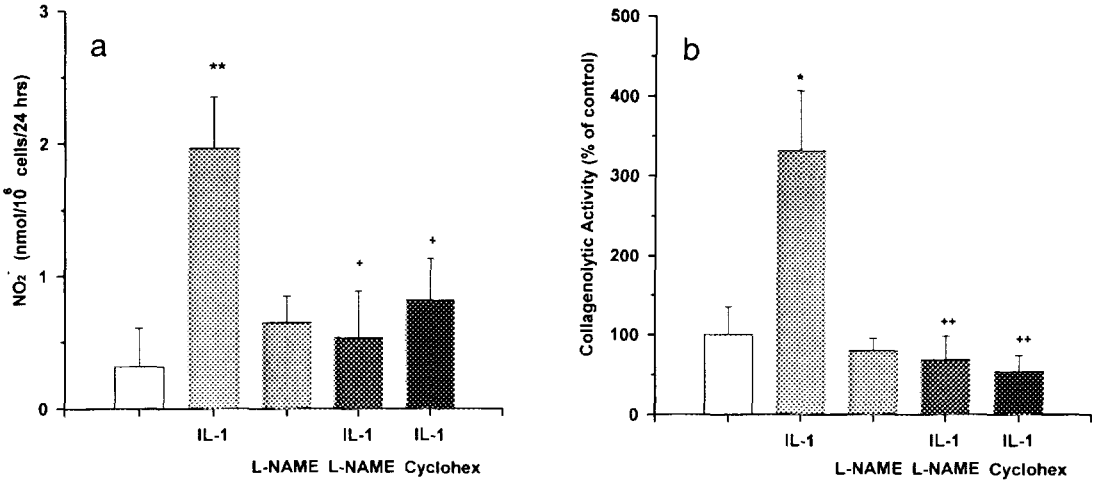

Figure 1. Nitric oxide synthase and collagenolytic activity in cultured bovine chondrocytes. (a) Nitrite (NO_2^-) release, (b) collagenolytic activity in cell/media over 24 hrs. Note that NO_2^- production and collagenolytic activity was stimulated by IL-1 (50 ng/ml interleukin-1β) and this stimulation was inhibited by 1 mg/ml of the nitric oxide synthase inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME) or 20 μg/ml of the protein synthesis inhibitor, cyclohexamide (cyclohex). N = 6 for each group; mean ± SEM. Mean collagenolytic activity in control group = 1.2 μg collagen/10^6 cells/24 hrs. *** = $p < 0.001$ as compared with control, ** = $p < 0.01$ and * = $p < 0.05$, as compared with IL-1 alone using un-paired two-way Student's t test. No treatment inhibited mitochondrial-dependent reduction of MTT to formizan.

activity was accompanied by an increase in nitric oxide synthase activity and was inhibited by the nitric oxide synthase inhibitor, L-NAME (Figs 1a,1b,2a-d). The caseinolytic activity of IL-1β treated medium from bovine cartilage was inhibited by the metal chelators 1,10-phenanthroline and EDTA (by 87% and 83% respectively; $p < 0.003$). The collagenolytic activity of IL-1β treated medium from bovine cartilage was inhibited by EDTA (by 73%; $p < 0.001$) but not 1,10-phenanthroline (NS).

Metalloprotease activity versus nitric oxide synthase activity

Unstimulated cartilage explants released basal amounts of stromelysin activity. When stimulated by endotoxin, this activity increased in a near linear fashion. There was an excellent correlation between metalloprotease activity and NO_2^- levels in the media of explants of articular cartilage was established ($r = 0.8$, $p < 0.001$; Fig 3).

Effects of exogenous NO· on metalloprotease activity

NO· exogenously generated with the NO·-donor SNAP increased caseinolytic activity of cultured bovine chondrocytes in a dose-dependent fashion (Fig 4).

DISCUSSION

This study is the first to show that induction of chondrocyte metalloprotease activity is dependent upon and mediated via nitric oxide. The induction of nitric oxide synthase activity in chondrocytes

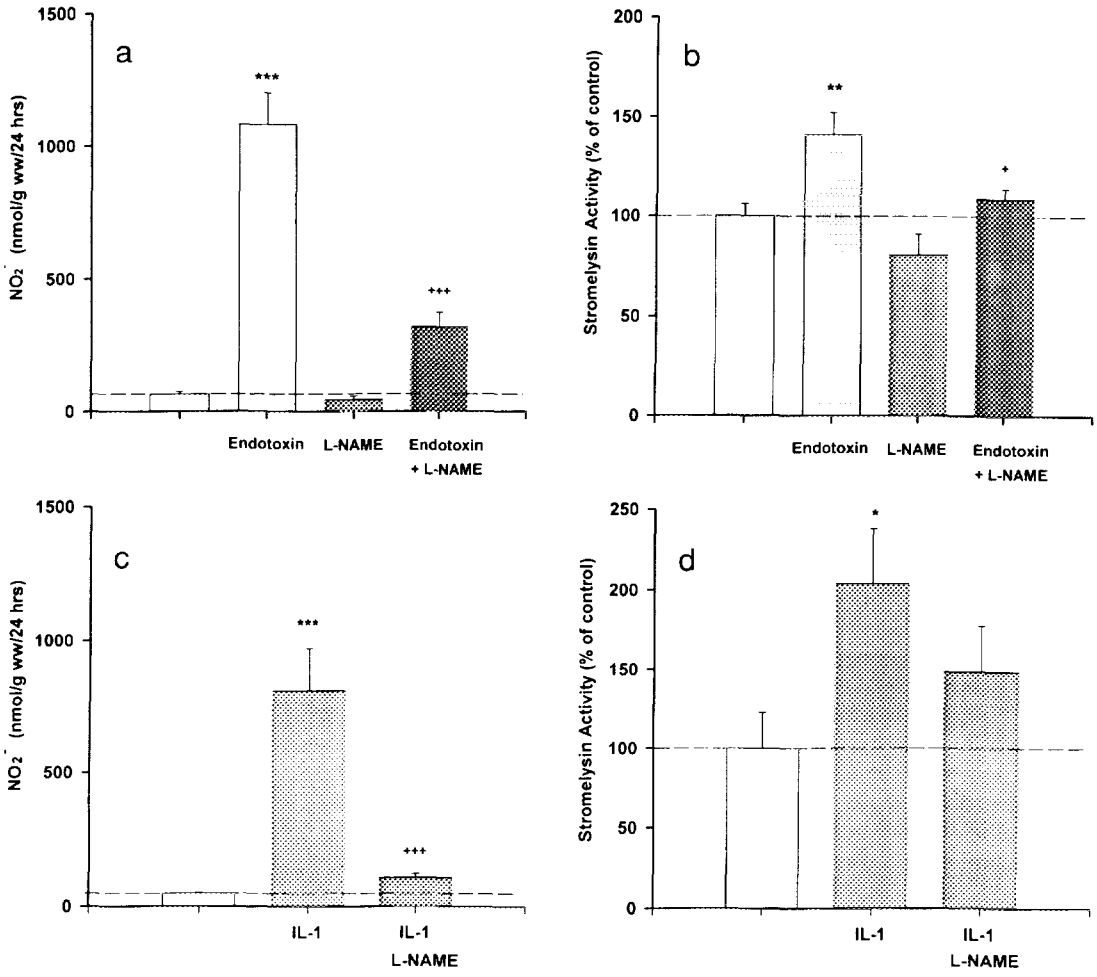

Figure 2. Nitric oxide and stromelysin activity in cultured bovine cartilage explants. (a) Nitrite (NO_2^-) release and (b) caseinolytic (stromelysin) activity of the media of explants removed after 24 hrs incubation with 10 μg/ml endotoxin and 1 mg/ml of the nitric oxide synthase inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME). (c) Nitrite (NO_2^-) release and (d) stromelysin activity of the media of explants removed after 72 hrs incubation with 100 ng/ml interleukin-1β (IL-1) and L-NAME. Note that NO_2^- production and stromelysin activity was stimulated by endotoxin and IL-1 and this stimulation was inhibited by the nitric oxide synthase inhibitor, L-NAME. N = 6 for each group; mean $\pm$ SEM. Mean stromelysin activity in control group in (b) = 8.6 μg casein/g w/w cartilage/24 hrs, in (d) = 25 μg casein/g w/w cartilage/24 hrs. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ as compared with control, + = $p < 0.05$, +++ = $p < 0.001$ as compared with endotoxin alone using un-paired two-way Student's t test.

was a time-dependent process that involved protein synthesis. The induction of metalloprotease activity also involved protein synthesis. Once induced, nitric oxide synthase activity correlated with increased metalloprotease activity in chondrocyte culture and in conditioned media from cartilage explants. When nitric oxide synthase activity was inhibited, metalloprotease activity was also inhibited. Exogenous $NO^{\cdot}$ also induced metalloprotease activity.

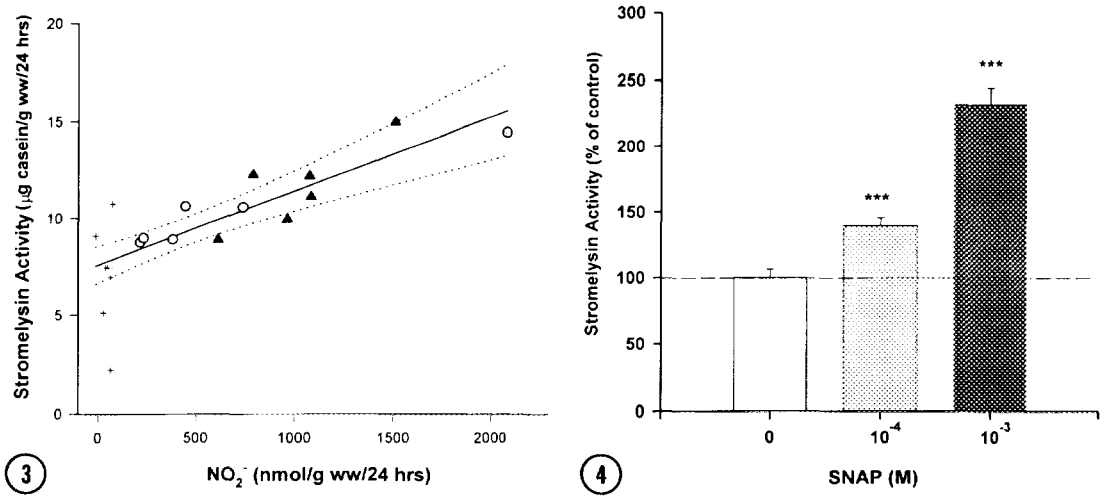

Figure 3. Correlation between stromelysin activity and nitrite of the media of cultured bovine cartilage explants. The explants were incubated for 24 hrs in media alone (○), 1 mg/ml Nω-nitro-L-arginine methyl ester, L-NAME (+), 10 μg/ml endotoxin (▲), or endotoxin and L-NAME (◍). Correlation coefficient (r) = 0.8, $p < 0.001$.

Figure 4. Effects of exogenously generated nitric oxide ($NO^{\cdot}$) on stromelysin activity of cultured chondrocytes. $NO^{\cdot}$ was generated by the $NO^{\cdot}$ donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP). Mean stromelysin activity of the control group = 2.4 μg casein/10^6 cells/24 hrs. *** = $p < 0.001$ as compared with control using un-paired two-way Student's t test. No treatment inhibited mitochondrial-dependent reduction of MTT to formizan.

The proteolytic activities in these assays were noted at a neutral pH and were inhibited by either EDTA or 1,10-phenanthroline, both metal chelators, and corresponded to type I collagenase and stromelysin(7).

The mechanism whereby $NO^{\cdot}$ modulated metalloprotease activity is undetermined. A direct catabolic effect of $NO^{\cdot}$ was unlikely as media that had been exposed to the cartilage and $NO^{\cdot}$, but which had time for all the active free radicals to deplete had very similar effects of collagen and casein degradation. $NO^{\cdot}$ can directly interact with other metal containing enzymes to either stimulate (ie soluble guanylate cyclase(11) and cyclo-oxygenase(12)) or inhibit (eg aconitase(13)) the enzymatic activity. Thus it is conceivable that $NO^{\cdot}$ directly interacted with the metal containing active site of collagenase and stromelysin to alter the enzyme's configuration and activate the metalloproteases from their proenzyme state.

In summary, we have demonstrated in whole cell and whole tissue systems that chondrocytes when stimulated by inflammatory mediators and endotoxin have the ability to produce large amounts of $NO^{\cdot}$ via induction of nitric oxide synthase, and that this $NO^{\cdot}$ activates metalloproteases. $NO^{\cdot}$, therefore, represents a key molecule in inflammatory-induced cartilage catabolism. The L-arginine-nitric oxide synthase pathway may be a novel therapeutic target for the prevention and management of arthritis.

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