Inhibition of the nitrosothiol production of cultured osteoarthritic chondrocytes by rhein, cortisol and diclofenac

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Summary

Objective: Nitric oxide (NO°) is a free molecule produced by NO synthases which acts as a mediator in inflammatory processes. NO° can react with thiol groups of proteins to produce nitrosothiols. Increased concentrations of these bioactive compounds have been found in sera and synovial fluids from patients with osteoarthritis (OA). The aim of this study was to assess the ability of human osteoarthritic chondrocytes to synthesize nitrosothiols and to compare the in vitro effects of rhein, cortisol and diclofenac on nitrosothiol and nitrite production.

Methods: Osteoarthritic chondrocytes were incubated for 24 h with 1 ng/ml of recombinant human interleukin-1β (IL-1β) in the presence or absence of rhein (1.3×10⁻8 M, 6.5×10⁻10 M, or 1.3×10⁻6 M), cortisol (10⁻5 M) or diclofenac (10⁻6 M or 10⁻5 M). Nitrite levels were measured in cell supernatants by the Griess method; nitrosothiol levels were determined in supernatants and cellular lysates by fluorimetry.

Results: At the basal level, nitrosothiols represented 80% of the total of nitrite and nitrosothiol production. After IL-1β stimulation, NO° production was highly increased in the supernatants (45-fold increase in nitrite, 60-fold increase in nitrosothiols) as well as in cell lysates (35-fold increase in nitrosothiols). Rhein caused a dose-dependent decrease in nitrosothiol and nitrite production. In comparison, diclofenac (10⁻6 M) moderately decreased nitrite and nitrosothiol levels in the supernatants but had no effect on lysate nitrosothiol. Cortisol had no significant effect on NO° production.

Conclusions: The IL-1β stimulation increased nitrosothiol production by osteoarthritic chondrocytes. These results demonstrate the need to measure nitrosothiol as well as nitrite production. Rhein inhibited the IL-1β induced NO° production, and may be a suitable treatment for osteoarthritis. © 2001 OsteoArthritis Research Society International

Key words: Nitrosothiols, Nitric oxide, Osteoarthritis, Chondrocyte, Rhein.

Introduction

Nitric oxide (NO°) is a free radical generated enzymatically from L-arginine by NO synthase (NOS) isoenzymes. Several studies have reported that the levels of NO° breakdown products (nitrite and nitrate) are higher in synovial fluids than in serum from patients with rheumatoid arthritis or osteoarthritis (OA), suggesting that NO° may be a local mediator of inflammation in both diseases. NO° contributes to inflammatory and arthritic tissue destruction by inhibition of cartilage macromolecule synthesis, such as collagen type II and proteoglycan, and by enhancing matrix metalloproteases (MMPs). Furthermore, NO° leads to inhibition of DNA replication and protein synthesis and induces apoptosis. Chondrocytes appear to be a significant source of NO° in OA articular sites. In contrast to normal cartilage, OA-affected cartilage can spontaneously release NO° in sufficient quantities to induce cartilage damage in ex-vivo conditions. OA cartilage over-expresses NOS, especially at the cartilage surface, but NOS is not detectable in normal cartilage. In the presence of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-17 or IL-18, chondrocytes over-produce NO°. The inducible OA-affected NOS has similar molecular weight and antibody cross-reactivity as the neuronal NOS isoform.

NO° is a widespread mediator with a short half-life. It can be easily converted into a variety of related molecules such as peroxynitrite and nitrite, or it interacts with sulfhydryl groups of proteins to form nitrosothiols. Nitrosothiols can act as physiological mediators since they have similar biological properties to NO° and they can play a role in the secretion and the storage of NO°. Nitrosothiols were recently identified in certain tissues in micromolar concentrations. Moreover, we have previously reported increased concentrations of nitrosothiols in sera and synovial fluids from patients with rheumatoid arthritis and, to a lesser extent, with OA. These molecules are an important part of bioactive NO° compounds. Measurement of nitrosothiol concentrations could be more important than the measurement of nitrite levels.

Rhein is the active metabolite form of diacerein, a new anti-rheumatic drug. This low molecular weight heterocyclic anthracenic compound has beneficial effects in animal models of OA. Nguyen et al. have shown clinical benefits of rhein in the treatment of OA of the hip. The mechanism of action of this molecule is different from that of non-steroidal anti-inflammatory drugs. Rhein is known to inhibit the effects of IL-1β but has no effect on phospholipase A2 or on cyclooxygenase. Recently, Pelletier et al. have shown an inhibitory effect of diacerein and
rhein on nitrite production by IL-1β stimulated chondrocytes, and a partial inhibition of the expression of the inducible NO synthase. Yaron et al. showed that diacerein and rhein increased IL-1ra levels in cartilage culture media.26 The aim of this study was to assess for the first time the ability of human osteoarthritic chondrocytes to synthesize nitrosothiols and to compare the effects of rhein, diclofenac and cortisol on the production of nitrosothiols.

Materials and methods

SOURCES

Ham’s F12 medium and phosphate-buffered saline (PBS) were obtained from Gibco (Grand Island, U.S.A.), and fetal calf serum (FCS) was obtained from BioWhittaker (Verviers, Belgium). Clostridia type I collagenase, porcine trypsin, sodium nitrite, sulfanilamide, N-1-naphthylethylenediamide dihydrochloride, 2,3-diaminonaphthalene (DAN), glutathione, NH4-sulfamate, HgCl2, cortisol, and diclofenac were obtained from Sigma (St Louis, MO, U.S.A.). Human recombinant interleukin-1β (IL-1β) was a generous gift of Negma laboratories. Rhein was a generous gift of Negma laboratories. Human cartilage specimens were isolated from surgical samples obtained during hip surgery on patients with OA. Ten patients (nine F/one M, aged 78±10 years, mean±S.D.) were selected for this study. Diagnosis was made according to the American College of Rheumatology criteria.27

CARTILAGE DISSECTION AND CHONDROCYTE CULTURE

Chondrocytes were obtained as previously described.28 Briefly, cartilages were carefully dissected from the underlying bone. Small fragments of cartilage were enzymatically digested using trypsin and collagenase. Cells were suspended in Ham’s F12 medium containing 10% FCS, netilmicin, ceftazidin, vancomycin and amphotericin B, and were plated at 1.5×10⁶ cells in 75 cm² culture flasks. Cells were cultured until confluence in an atmosphere containing 5% CO2. The medium was changed every 3 days. When confluence was attained, a passage was carried out, using trypsin. Cells (7×10⁶) were suspended in 1 ml of Ham’s F12 with 10% FCS and placed in 4 cm² 12-well plates at 37°C (5% CO2). All experiments were performed on confluent cultures at first passage.

CHONDROCYTE TREATMENT

The growth medium of the chondrocyte cultures was replaced by fresh medium without FCS. To determine the NO⁺ production over time, cells were incubated for 6, 18 or 24 h either with or without IL-1β (1 ng/ml). To determine the effect of rhein, diclofenac and cortisol, cells were co-incubated for 24 h with (1) no additives, (2) IL-1β (1 ng/ml) and rhein (1.3×10⁻⁵ M, 6.5×10⁻⁶ M or 1.3×10⁻⁶ M), (3) IL-1β (1 ng/ml) and diclofenac (10⁻⁵ M), (4) IL-1β (1 ng/ml) and cortisol (10⁻⁵ M). In other experiments cells were first stimulated by IL-1β (1 ng/ml) for 6 h then drugs were introduced into the medium for the last 18 h.

NITROSOTHIOL MEASUREMENTS

Nitrosothiol levels were determined by a fluorimetric method as described by Marzinzig et al.29 For intracellular nitrosothiol determination, cells were lysed by incubation with 20 μl 1 M NaOH for 10 min. Basic pH did not influence the NO liberation from nitrosothiols. Lysates were neutralized with 1 M HCl. The supernatant or lysate (50 μl) was diluted in 50 μl distilled water. NH4-sulfamate (50 μl of 0.1 mM) was added to trap nitrite. The mixture was incubated for 10 min, 50 μl of reaction mixture (one part 1.1 mM HgCl₂ and four parts 0.05 mg/ml DAN in 0.62 M HCl) was added, and the complete mixture was incubated for 10 min at room temperature in the dark. Then, 2.8 M NaOH (20 μl) was added. Acid pH liberated NO from nitrosothiols. The fluorescence intensity was measured in a 96-well microtiter plate (Pharmacia) at excitation/emission wavelengths of 360 nm and 450 nm, respectively. Nitrosothiol stocks for generating standard curves were prepared as follows: 50 μl of glutathione at various concentrations (0.15 to 10.0 μM in 1 m HCl) was incubated with 50 μl NaNO2 (10 μmol/l) at room temperature in the dark for 2 h. The reaction mixture was incubated with 50 μl NH4-sulfamate (100 μmol/l). Total cell protein was assayed according to Lowry et al.30 with bovine serum albumin as a standard. Results are expressed as nmol/mg of cellular protein.

NITRITE MEASUREMENTS

Nitrite was measured in cultured cell supernatants by diazotization reaction of Griess with a microplate adaptation.31 Briefly, 100 μl of culture supernatant was incubated with 100 μl of 1% sulfanilamide, 0.1% N-1-naphthylethylenediamine dichloride in 25% H3PO4 at room temperature for 10 min. Optical densities were measured at 570 nm using a Dynatech MR 5000 microplate reader (Dynex Technology S.A., France). Background levels of nitrite were determined in cell-free Ham’s F12 medium with or without additives and were subtracted from the total amount of nitrite formed. The nitrite concentration was calculated from a NaNO2 standard curve. The detection limit for nitrite was 0.1 μmol/l. Results are expressed as nmol/mg of cellular protein.

CELL VIABILITY

Cell viability was calculated from the release of lactate dehydrogenase (LDH) into supernatants. For this determination, culture supernatants were removed at the end of incubation and the cells were lysed with 200 μl of 1% Triton X-100. The LDH activities of supernatants and lysed cells were calculated using the following formula: LDH release (%) = 100×supernatant LDH/(supernatant LDH+cellular LDH). Cellular viability was calculated as follows: 100−LDH release (%).

STATISTICAL ANALYSIS

Results are expressed as mean±S.E.M. of triplicate measurements. Comparisons were made using the Mann–Whitney U-test. Differences with P-values of less than 0.05 were considered significant.
Results

NITROSOThIOL AND NITRITE PRODUCTION BY OA CHONDROCYTES

Table I shows the levels of nitrosothiols and nitrite before and after 24 h of stimulation by IL-1β (1 ng/ml). At the basal level, nitrosothiols represented 80% of the total nitrite and nitrosothiol production by OA chondrocytes. After 24 h of stimulation, NO° production was highly increased relative to the untreated cells in the supernatants and in the lysates:

<table>
<thead>
<tr>
<th></th>
<th>Before IL-1β stimulation (nmol/mg of protein)</th>
<th>After IL-1β stimulation (nmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrite (supernatant)</td>
<td>1.56±2.42</td>
<td>92.34±80.1</td>
</tr>
<tr>
<td>Nitrosothiol (supernatant)</td>
<td>3.67±0.85</td>
<td>168±74.9</td>
</tr>
<tr>
<td>Nitrosothiol (lysate)</td>
<td>2.96±0.93</td>
<td>101.3±85.2</td>
</tr>
</tbody>
</table>

Results are expressed as mean±1 s.d. of five patients.

Fig. 1. NO° production by OA chondrocytes over time. Cells were stimulated by IL-1β (1 ng/ml). Nitrite levels were determined in cellular supernatants and nitrosothiol levels were measured in supernatants and cell lysates, as described in Materials and methods, at 0, 6, 18 and 24 h. Results are expressed as mean±1 s.d.; results are representative of three independent experiments.

Fig. 2. Effects of co-incubation of OA chondrocytes with IL-1 and rhein, diclofenac or cortisol on production of (A) nitrite in supernatants, (B) nitrosothiol in supernatants, (C) nitrosothiol in cellular lysates. Cells were incubated with IL-1β (1 ng/ml) for 24 h with or without rhein, diclofenac or cortisol at the indicated final concentration. Results are expressed as mean percentage±1 s.e.m. *P<0.05, **P<0.01, ***P<0.001 vs IL-1β treated cells.
about 45 times for nitrosothiols in the supernatants, about 60 times for nitrite in the supernatants and about 35 times for nitrosothiols in the lysates. Time-comparison experiments revealed an increasing production of nitrosothiols in both basal and stimulated cells (Fig. 1).

EFFECTS OF DRUGS ON NO\(^\circ\) PRODUCTION AFTER 24 H

The effects of rhein, diclofenac and cortisol on nitrosothiol production were studied on OA chondrocytes (N=5) treated for 24 h with a combination of IL-1\(\beta\) (1 ng/ml) and one of these drugs. As shown in Fig. 2, rhein induced a dose-dependent decrease in nitrosothiol and nitrite production. The maximal effect was obtained at the concentration of \(1.3 \times 10^{-4}\) mol/l. At this concentration, there was a 78% inhibition of nitrosothiols in the lysates and nitrite in the supernatants, and a 68% inhibition of nitrosothiols in the supernatants. A cytopathic effect was observed when the concentration was higher (\(1.3 \times 10^{-4}\) M) (Table II). In comparison, diclofenac (\(10^{-5}\) M) moderately decreased the production of nitrite and nitrosothiols in the supernatants (about 20%) and had no significant effect on nitrosothiols in the lysates. Cortisol (\(10^{-5}\) M) had no significant effect on NO\(^\circ\) production.

EFFECTS OF TIME ON THE ASSOCIATION OF IL-1\(\beta\) AND THE DRUGS

To determine the influence of time on the association of IL-1\(\beta\) with the drugs, cells were first treated with IL-1\(\beta\) alone, then drugs were introduced in the growth medium for the last 18 h. As indicated in Fig. 3, cortisol (\(10^{-5}\) M) and diclofenac (\(10^{-5}\) M) had no significant effect on the NO\(^\circ\) production. Only the highest concentrations of rhein decreased nitrosothiol production in the supernatants (about 50% for \(10^{-5}\) M rhein and about 40% for \(6.5 \times 10^{-6}\) M rhein). \(10^{-5}\) M rhein induced a 40% reduction in nitrite production in the cell supernatants, compared with a reduction of 30% at a dose of \(6.5 \times 10^{-6}\) M. Rhein had only a moderate effect on the level of nitrosothiols in the cell lysates (about 15% reduction for \(10^{-5}\) M rhein).
Discussion

This study is the first to assess the capacity of human cells, especially chondrocytes, to produce nitrosothiols, a bioactive metabolite of NO. At the basal level, nitrosothiols make up a significant fraction of total NO metabolites (more than double the level of nitrite in the supernatant). Nitrosothiols are increased after IL-1β induction to the same extent as nitrite. Therefore, a measure of nitrosothiols instead of nitrite in the supernatants and cell lysates separately could be important when studying NOS activity. In articular sites under IL-1 stimulation, chondrocytes are probably at least partially the source of nitrosothiols found in synovial fluids from OA patients. It would be expected that some part of NO generated from cells can form adducts with cellular SH-compounds, such as glutathione, to form nitrosothiols. This relatively stable complex may diffuse to remote sites and release bioactive NO. Moreover, nitrosothiols themselves have biological properties similar to NO and constitute a real and terminal mobilizable pool. Nitrosothiols have been implicated in intracellular signaling processes and are responsible for the nitrosylation and the activation of p21. Nitrosothiols may also be toxic to cells by disrupting protein structure or interfering with the catalytic activity of enzymes.

OA treatment is based currently on analgesics and non-steroidal anti-inflammatory drugs (NSAIDs). However, these drugs do not slow down the progression of OA. Recent findings have shown a beneficial effect of inhibiting NO production in vivo in experimental OA, suggesting a complementary approach with drugs that inhibit iNOS may be useful. Our study confirms an inhibitory effect of rhein on the NO production induced by IL-1β. This effect occurs in a dose-dependent manner and at the therapeutic concentration for OA patients. Rhein has a similar effect on nitrite (the end-metabolite of NO) and nitrosothiol production. When rhein is introduced 18 h after IL-1β at a therapeutic concentration, it inhibits about 50% of nitrosothiol production, suggesting that this molecule could have a strong effect on damages produced by acute, subacute or chronic inflammatory states. Our results are in accordance with those of Pelletier et al., who showed on a similar cellular model that diacerein and its active metabolite rhein partially inhibited the production of iNOS mRNA induced by IL-1β. Few therapeutic drugs have the ability to inhibit NO production. An inhibitory effect has been reported for tetracyclines, aspirin, and partially for corticoids but most NSAIDs do not inhibit the NO production induced by IL-1β in cultured chondrocytes. Our results confirm these observations since we did not observe any effect of diclofenac on nitrosothiol or nitrite production. We did find an effect of cortisol. However, effects of corticoids on the inducible NO synthase are still not well known. Our results are similar to those reported by Grabowski et al., 25 who showed on a similar cellular model that diacerein and its active metabolite rhein partially inhibited the production of iNOS mRNA induced by IL-1β. Few therapeutic drugs have the ability to inhibit NO production. An inhibitory effect has been reported for tetracyclines, aspirin, and partially for corticoids but most NSAIDs do not inhibit the NO production induced by IL-1β in cultured chondrocytes. Our results confirm these observations since we did not observe any effect of diclofenac on nitrosothiol or nitrite production. We did find an effect of cortisol. However, effects of corticoids on the inducible NO synthase are still not well known. Our results are similar to those reported by Grabowski et al., 25 who showed on a similar cellular model that diacerein and its active metabolite rhein partially inhibited the production of iNOS mRNA induced by IL-1β. Few therapeutic drugs have the ability to inhibit NO production. An inhibitory effect has been reported for tetracyclines, aspirin, and partially for corticoids but most NSAIDs do not inhibit the NO production induced by IL-1β in cultured chondrocytes. In conclusion, this study demonstrates that human OA chondrocytes, after IL-1β stimulation, are able to produce high quantities of nitrosothiols, a bioactive form of NO.

These results illustrate the need to measure nitrosodiol as well as nitrite production, since pharmacological molecules can modify the cellular redox balance and liberate NO from nitrosothiols or divert the NO pathway. Rhein had a significant inhibitory effect on NO production by OA chondrocytes. In view of pharmacological properties, these results add some more information to the body of evidence obtained previously with diacerein and rhein on in vitro and animal models. Further studies on joint structure modifications are proceeding.

References

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