

Differential Roles of Hydrogen Peroxide and Superoxide in Mediating IL-1-Induced NF- κ B Activation and iNOS Expression in Bovine Articular Chondrocytes

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Abstract Our previous studies showed that reactive oxygen species (ROS) are required for the pro-inflammatory cytokine interleukin-1 β (IL-1) to induce the activity of the Nuclear transcription Factor-kappaB (NF- κ B) and the expression of the inducible isoform of the nitric oxide synthase (iNOS) in bovine articular chondrocytes. This study aimed at elucidating the role of hydrogen peroxide (H₂O₂) and the superoxide radical, two major ROS, in mediating those IL-1-induced responses. The results obtained show that chondrocytes produce both H₂O₂ and superoxide radical in response to IL-1. Treatment of the chondrocyte cultures with H₂O₂ alone did not induce NF- κ B activation or iNOS expression. Addition of H₂O₂ simultaneously with IL-1 did neither enhance nor inhibit NF- κ B activation and iNOS expression, relatively to treatment with IL-1 alone. Accordingly, treatment with catalase did not inhibit those IL-1-induced responses. Treatment with superoxide dismutase, however, effectively prevented IL-1-induced I κ B- α degradation and iNOS expression. Taken together, the results obtained indicate that superoxide mediates IL-1-induced I κ B- α degradation and the consequent NF- κ B activation and iNOS expression in chondrocytes, whereas H₂O₂ does not seem to participate in those IL-1-induced responses. In conclusion, the present study identifies the superoxide radical as the ROS involved in mediating the IL-1-induced signaling pathway that leads to NF- κ B activation and to the expression of NF- κ B-dependent genes in bovine articular chondrocytes. *J. Cell. Biochem.* 88: 783–793, 2003. © 2003 Wiley-Liss, Inc.

Key words: hydrogen peroxide; superoxide radical; interleukin-1 β ; NF- κ B; iNOS expression; chondrocyte

Interleukin-1 β (IL-1), a potent pro-inflammatory cytokine, was shown to induce a series of catabolic responses in chondrocytes that lead to cartilage degradation and inflammation, the major pathogenic events common to all arthritic processes [Pelletier et al., 1993; Weyand, 2000].

The transcription factor, Nuclear Factor kappaB (NF- κ B), seems to play a major role in mediating IL-1-induced cellular responses. NF- κ B regulates the expression of many inflam-

mation-related genes, including cyclooxygenase-2 (COX-2) [Lukiw and Bazan, 1998], inflammatory cytokines [Siebenlist et al., 1994; Yoshida et al., 1999], adhesion molecules [Iademarco et al., 1992; Kaszubska et al., 1993], and the inducible isoform of nitric oxide (NO) synthase (iNOS), which produces large amounts of NO [Xie et al., 1994; Eberhardt et al., 1998]. IL-1 has been shown to activate NF- κ B by inducing the phosphorylation and degradation of I κ B- α , a member of the Inhibitor κ B (I κ B) family of proteins, which allows the translocation to the nucleus of the active NF- κ B dimers [Beg et al., 1993]. Recently, we observed that IL-1 activates NF- κ B in chondrocytes, which is an essential requirement for the expression of iNOS and for the subsequent production of NO [Mendes et al., 2001, 2002a,b,c].

Despite extensive investigation of the responses and signaling pathways utilized by IL-1 in different cells, the mechanisms that link IL-1-binding to its receptor to the activation of

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transcription factors and subsequent gene expression are still incompletely understood. In recent years, reactive oxygen species (ROS) and reactive nitrogen species, namely NO, have been increasingly recognized as signaling intermediates of IL-1-induced responses in many cell types [Blanco and Lotz, 1995; Sen, 1998; Eberhardt et al., 2002]. Changes in the redox state of the cells, resulting from alterations in the rate of ROS generation and/or in the intracellular levels of antioxidants, have been associated with modifications in the pattern of gene expression, which in turn result from changes in the activity of many transcription factors [Allen and Tresini, 2000; Schoonbroodt and Piette, 2000].

Accumulating evidence indicates that NF- κ B is sensitive to redox regulation. Nevertheless, the role of ROS on NF- κ B activation seems to be highly dependent on the cell-type, since the same stimulus, hydrogen peroxide (H_2O_2) for instance, can have opposite effects on the activity of this transcription factor when acting in different cells [Meyer et al., 1993; Wesselborg et al., 1997; Flescher et al., 1998; Lakshminarayanan et al., 1998; Woods et al., 1999].

IL-1 has been shown to induce chondrocytes to produce several types of ROS, including H_2O_2 [Rathakrishnan et al., 1992] and hydroxyl [Tiku et al., 1998] and superoxide [Tawara et al., 1991] radicals. Furthermore, ROS have been shown to be required for IL-1-induced NF- κ B activation and iNOS expression [Mendes et al., 2001], as well as for the expression of c-Fos and collagenase [Lo et al., 1998]. ROS, and H_2O_2 in particular, have also been shown to mediate IL-1-induced expression of c-Jun in articular chondrocytes [Lo et al., 1996]. Moreover, chondrocyte-derived ROS have also been reported to mediate cartilage matrix destruction by inducing aggrecan and collagen degradation [Tiku et al., 1999, 2000].

Nevertheless, it is not clear whether ROS, in general, mediate those IL-1-induced responses, or, instead, a specific type of ROS is responsible for mediating activation of a particular signaling pathway leading to a specific response. Since, under appropriate conditions, H_2O_2 and the superoxide radical can generate other types of ROS, this study first attempted to identify the ability of chondrocytes to produce those two types of ROS in response to IL-1. Then, this study aimed at elucidating the role of H_2O_2 and superoxide in mediating IL-1-induced NF- κ B

activation and iNOS expression in bovine articular chondrocytes.

MATERIALS AND METHODS

Materials

Recombinant human IL-1 β was purchased from R&D Systems (Abingdon, UK). Catalase, superoxide dismutase (SOD), and H_2O_2 were from Sigma Chemical Co. (St. Louis, MO). The anti-iNOS antibody was from Transduction Laboratories (Lexington, KY). The anti-I κ B- α antibody was from New England Biolabs, Inc. (Beverly, MA). The anti-actin antibody was from Boehringer Mannheim (Mannheim, Germany). The oligonucleotide probe containing the consensus binding site for NF- κ B was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The protease inhibitors cocktail, "Complete, Mini" was from Roche Molecular Biochemicals (Mannheim, Germany). The isotopes were from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). The c-DNA probes for iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were a kind gift from Dr. Tony Cruz (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada).

Isolation and Cell Culture

Chondrocytes were isolated from bovine articular cartilage, as described previously [Cruz et al., 1990]. Primary confluent monolayer cultures were set up by plating 2×10^6 cells/ml in Ham's F-12 medium containing 3% antibiotic/antimycotic solution and 5% fetal bovine serum and allowed to recover for 24 h at 37°C in a humidified atmosphere supplemented with 5% CO₂. Prior to any treatments, the cells were serum-starved overnight and, thereafter, maintained in culture medium with 1% antibiotic/antimycotic solution and without serum. In all the experiments using catalase and SOD, the cells, including those left untreated and those treated with IL-1 alone, were permeabilized by addition of digitonin 0.00002% prior to any other treatment.

Measurement of ROS Production

The intracellular production of ROS was measured using 2',7'-dichlorodihydrofluorescein diacetate (DCFH₂-DA) as described previously [Bass et al., 1983; LeBel et al., 1990] with modifications. DCFH₂-DA is a non-fluor-

escent probe that, upon diffusion into the cells, is hydrolyzed by intracellular esterases to 2',7'-dichlorodihydrofluorescein (DCFH₂), which is trapped within the cells. In the presence of ROS, particularly peroxides, DCFH₂ is oxidized to the highly fluorescent compound 2',7'-dichlorofluorescein (DCF).

Chondrocytes (2×10^6) were loaded with 5 μ M DCFH₂-DA in Na⁺ saline solution (140 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 1.0 mM MgCl₂, 1.0 mM NaH₂PO₄, 5.6 mM glucose, 20.0 mM Hepes, pH 7.4) for 20 min, at 37°C. After rinsing, the cells were incubated for 30 min with 80 U/ml of catalase or SOD in Na⁺ saline solution with 0.00002% digitonin. Then, IL-1 (5 ng/ml) was added to the corresponding wells and the cells further incubated for another 30 min. The fluorescence intensity of the cells was measured immediately at 37°C, using a SPEX Fluorolog spectrometer, equipped with a thermostated waterbath, with excitation set at 502 nm and emission set at 550 nm.

Preparation of Cytoplasmic and Nuclear Extracts

The cells were lysed in 400 μ l of buffer 1 (10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P-40, protease inhibitors cocktail at 1:7 dilution, pH 7.5) and incubated on ice for 15 min. The lysates were centrifuged at 2,300g/5min/4°C and the supernatants (cytoplasmic extracts) were collected and stored at -70°C. The pellets were resuspended in 30 μ l of buffer 2 (20 mM HEPES, 5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 300 mM NaCl, 20% Glycerol, protease inhibitors cocktail at 1:7 dilution, pH 7.5). After a 20 min incubation on ice, the pellet lysates were centrifuged at 18,000g/20 min/4°C and the supernatants (nuclear extracts) were collected and stored at -70°C. The protein concentration of the extracts was measured using the bicinchoninic acid/copper (II) sulfate protein assay kit (Sigma Chemical Co., St. Louis, MO).

Western Blot Analysis

The cytoplasmic extracts (25 μ g protein) were diluted in sodium dodecylsulfate (SDS) sample buffer (2.5% SDS, 0.0625 M Tris-HCl, 10% glycerol, 5% 2-mercaptoethanol, 0.05% bromophenol blue, pH 6.8) and boiled for 3 min at 90°C. Proteins were separated by SDS/PAGE [10% (w/v) gel] and transferred onto polyvinylidene difluoride (PVDF) membranes by elec-

troblotting. The membranes were blocked with 5% skim milk in Tris-buffered saline-Tween (TBS-T, 20mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) and then probed with the anti-iNOS or anti-I κ B- α antibodies. After extensive washing with TBS-T, the blots were incubated with a horseradish peroxidase-conjugated anti-rabbit antibody (Dako A/S, Copenhagen, Denmark). The protein-antibody complexes were visualized by chemiluminescence using the ECL Western blotting detection reagents from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). The membranes were subsequently stripped and reprobbed with an anti-actin antibody to ensure an equal protein loading.

Electrophoretic Mobility Shift Assay (EMSA)

The NF- κ B oligonucleotide probe was end-labeled with [γ -³²P]ATP by the T4 polynucleotide kinase and purified through Sephadex G-50 spin columns. Nuclear extracts (10 μ g protein) were incubated for 40 min at 4°C in 20 μ l binding reactions containing 20 mM HEPES, 50 mM KCl, 1 mM MgCl₂, 0.5 mM DTT, 4% Ficoll 400, 2 μ g poly(dIdC), 20 μ g BSA, pH 7.9, and 200,000 cpm/reaction of [γ -³²P]-labeled oligonucleotide probe. The DNA-protein complexes were resolved by electrophoresis on 7% native polyacrylamide gels in 0.5 \times TBE for 3 h at 150 V, dried and visualized by autoradiography.

Northern Blot Analysis

Total RNA was extracted using Trizol (Gibco BRL, Life Technologies Inc., Grand Island, NY) and quantitated by spectrophotometry at 260 nm. Denatured RNA samples (20 μ g) were analyzed by gel electrophoresis in a 1% denaturing agarose gel, transferred to a nylon membrane, and cross-linked by heating at 80°C for 30 min. The blots were hybridized with a ³²P-labeled iNOS c-DNA probe, subsequently stripped and reprobbed with a ³²P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) c-DNA as an internal standard to insure a roughly equal loading. The c-DNA probes were labeled with [α -³²P]dCTP by the klenow fragment using the Random Primed DNA Labeling kit from Boehringer Mannheim GmbH (Mannheim, Germany).

Measurement of NO Production

NO production was measured as the amount of nitrite released into the culture supernatant. Nitrite concentration was determined in

cell-free culture supernatants using the spectrophotometric method based on the Griess reaction [Green et al., 1982].

RESULTS

ROS Produced in Response to IL-1 Include H₂O₂ and Superoxide

To measure the intracellular ROS production, we used DCFH₂, a probe that becomes fluorescent upon oxidation and that is particularly sensitive to peroxides, although it can also react with the superoxide radical [Bass et al., 1983; LeBel et al., 1990]. We have previously observed that chondrocytes produce ROS in response to IL-1 [Mendes et al., 2001]. To identify the ROS produced in response to IL-1, we treated the chondrocyte cultures, preloaded with DCFH₂, with catalase or superoxide dismutase (SOD), in the presence of IL-1, as described under Materials and Methods. The results obtained (Table I) show that treatment with IL-1 for 30 min increased the fluorescence intensity of the chondrocytes by approximately 18% relatively to control cells. Pretreatment with catalase reverted the effect of IL-1, so that the fluorescence intensity did not increase above that obtained in control. This indicates that IL-1 induced the production of H₂O₂, which was

hydrolyzed by catalase, thus preventing the oxidation of DCFH₂. On the opposite, when the cells were treated with SOD, the fluorescence intensity was higher than that obtained in cells treated with IL-1 alone. This can be explained by the greater sensitivity of DCFH₂ to oxidation by H₂O₂ than by superoxide, so that, in cells treated with SOD, the superoxide radicals produced were converted to H₂O₂, thus increasing the amount of DCFH₂ oxidized and so the fluorescence intensity of those cells. Taken together, these results indicate that, under the culture conditions used, chondrocytes produced both H₂O₂ and superoxide in response to IL-1.

Superoxide, but not H₂O₂, Mediates IL-1-Induced NF-κB Activation

To study the role of H₂O₂ on NF-κB activation, we determined the levels of IκB-α in the cytoplasmic extracts obtained from cells treated with H₂O₂ in the presence or absence of IL-1. We used the corresponding nuclear extracts to detect the presence of active NF-κB dimers by EMSA analysis with a [γ -³²P]-labeled oligonucleotide probe containing the consensus binding sequence for NF-κB.

The results in Figure 1A show that treatment of chondrocytes with IL-1 (5 ng/ml) for 30 min

TABLE I. Effect of Catalase and Superoxide Dismutase on Interleukin-1β- (IL-1-) Induced Reactive Oxygen Species (ROS) Production

| | Arbitrary units | % Relatively to control |
|--------------------------|--------------------------------------|-------------------------|
| Control | 2.059 ± 0.027 × 10 ⁷ | 100.0 ± 1.3 |
| IL-1, 5 ng/ml | 2.437 ± 0.025 × 10 ⁷ * | 118.4 ± 1.2 |
| Catalase, 80 U/ml + IL-1 | 2.041 ± 0.033 × 10 ⁷ ** | 99.2 ± 1.6 |
| SOD, 80 U/ml + IL-1 | 2.562 ± 0.017 × 10 ⁷ *,** | 124.5 ± 0.8 |

The fluorescence intensity of chondrocyte cultures preloaded with DCFH₂-DA was measured after treatment with interleukin-1β (IL-1), 5 ng/ml, in the presence or absence of the indicated concentration of catalase or superoxide dismutase (SOD), as described in Materials and Methods. Results are expressed in arbitrary units and as a percentage of the fluorescence intensity relatively to that obtained in control cells, which were loaded with DCFH₂-DA and permeabilized with 0.00002% digitonin, but otherwise left untreated. The results presented are the mean ± SD of three independent experiments, each performed in duplicate.

**P* < 0.05 relatively to control.

***P* < 0.05 relatively to IL-1.

Fig. 1. Effect of H₂O₂ on nuclear transcription factor-kappaB (NF-κB) activation. **A:** Effect of H₂O₂ on IκB-α degradation, in the presence or absence of interleukin-1β (IL-1); **(B)** effect of H₂O₂ on NF-κB-DNA binding activity, in the presence or absence of IL-1. Chondrocyte cultures were treated with IL-1 (5 ng/ml) and/or the indicated concentrations of H₂O₂ for 30 min. The cytoplasmic extracts obtained from those cells were used to detect IκB-α protein levels by Western blot (panel A) and the corresponding nuclear extracts were used to detect NF-κB binding to a specific

oligonucleotide probe by EMSA (panel B), as described under Materials and Methods. The results shown are the mean ± SD of the intensity, in arbitrary units, of the bands obtained, for each condition, in six different experiments using six distinct sets of cell cultures. **P* < 0.01 relatively to control cells; ♦*P* < 0.01 relatively to IL-1. Statistical significance was assessed by the unpaired Student's *t*-test. The autoradiographies shown are representative of six independent experiments.

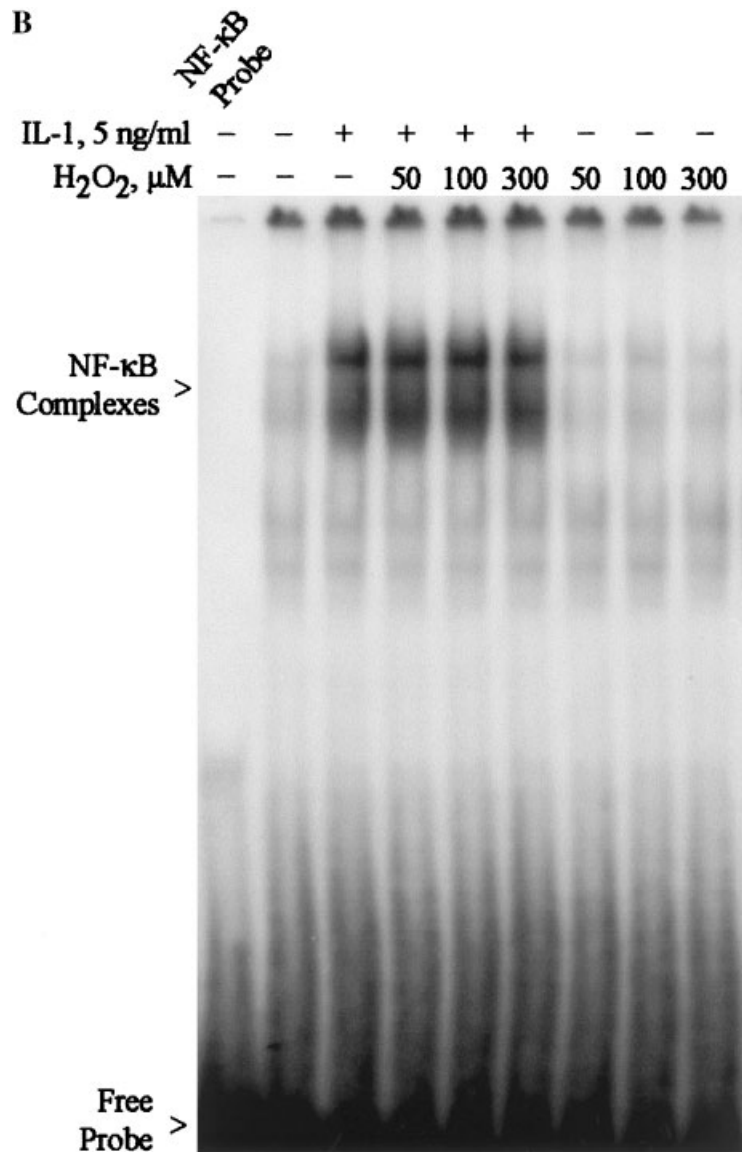
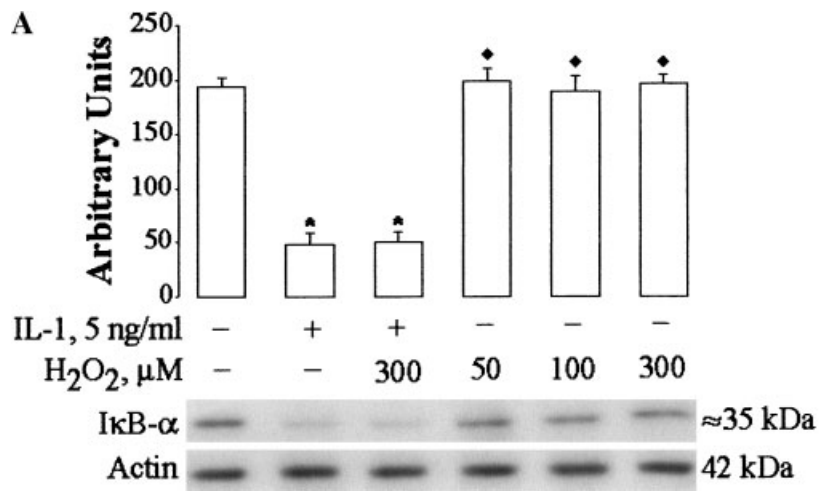


Fig. 1

induced the degradation of I κ B- α , which was not altered by simultaneous incubation with H₂O₂ (300 μ M). On the other hand, the levels of I κ B- α in the cytoplasmic extracts from cells treated for 30 min with H₂O₂ alone, in concentrations ranging from 50 to 300 μ M, did not differ from those found in control cells, thus indicating that H₂O₂ alone does not induce I κ B- α degradation (Fig. 1A).

EMSA of nuclear extracts from IL-1-treated cells detected the binding of proteins to the [γ -³²P]-labeled oligonucleotide probe containing the consensus sequence for NF- κ B (Fig. 1B), thus reflecting NF- κ B activation. When H₂O₂ (50–300 μ M) was added to the chondrocytes simultaneously with IL-1, no changes in the intensity of the NF- κ B–oligonucleotide probe complexes were detected relatively to those induced by IL-1 alone. In the absence of IL-1, nuclear extracts from chondrocyte cultures treated with H₂O₂ (50–300 μ M) for 30 min did not show the formation of specific complexes between nuclear proteins and the NF- κ B oligonucleotide probe, as compared with extracts from control cells (Fig. 1B). These results are in agreement with those obtained for the cytoplasmic levels of I κ B- α and show that H₂O₂ alone is not sufficient to induce NF- κ B activation in chondrocytes. Furthermore, these results also

show that H₂O₂ does neither inhibit nor enhance IL-1-induced NF- κ B activation.

Nevertheless, these results do not exclude the possibility that H₂O₂ may be required, although not sufficient, for IL-1-induced NF- κ B activation. To investigate this question, we treated the chondrocytes cultures with catalase or SOD in the presence of IL-1 and determined the cytoplasmic levels of I κ B- α . Figure 2 shows that pretreatment with SOD prevented IL-1-induced I κ B- α degradation, whereas pretreatment with catalase had no effect on the cytoplasmic levels of I κ B- α .

Since treatment with catalase was effective in preventing IL-1-induced DCFH₂ oxidation and the consequent increase in the fluorescence intensity, the lack of inhibition of I κ B- α degradation by catalase indicates that H₂O₂, although being produced, is not required for this IL-1-induced response. Furthermore, since neither H₂O₂ nor catalase had any effect on I κ B- α degradation, increased levels of H₂O₂ resulting from superoxide dismutation, cannot account for the inhibitory effect observed with SOD treatment. Instead the inhibitory effect observed with SOD treatment is more likely the result of decreased superoxide levels. Taken together, these results indicate that superoxide mediates IL-1-induced I κ B- α degradation and

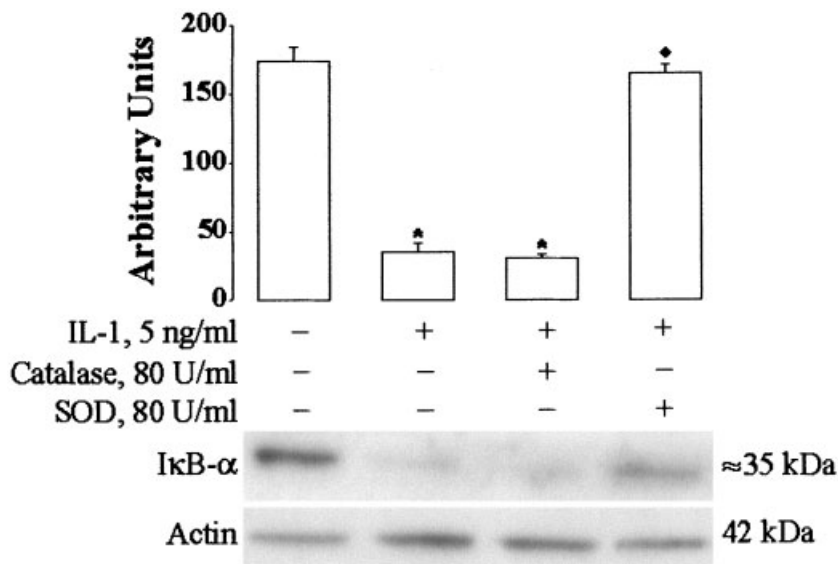


Fig. 2. Effect of catalase and superoxide dismutase (SOD) on interleukin-1 β - (IL-1-) induced I κ B- α degradation. Chondrocyte cultures were treated with catalase (80 U/ml) or SOD (80 U/ml) for 30 min before the addition of IL-1 (5 ng/ml) and then further incubated for another 30 min. The cytoplasmic extracts obtained from those cells were used to detect I κ B- α protein levels by Western blot, as described under Materials and Methods. The

results shown are the mean \pm SD of the intensity, in arbitrary units, of the bands obtained, for each condition, in three different experiments using three distinct sets of cell cultures. * P < 0.01 relatively to control cells; ♦ P < 0.01 relatively to IL-1. Statistical significance was assessed by the unpaired Student's t -test. The autoradiography shown is representative of three independent experiments.

the consequent NF- κ B activation in chondrocytes, whereas H₂O₂ does not seem to participate in this IL-1-induced response.

Superoxide, but not H₂O₂, Is Required for IL-1-Induced iNOS Expression

The results presented in Figure 3 show that H₂O₂ alone did not induce iNOS mRNA or protein synthesis, at any of the concentrations tested (50 – 300 μ M), and, when added simultaneously with IL-1 to the chondrocyte cultures, it did not alter the effect of the cytokine. Accordingly, treatment of the chondrocyte cultures with catalase, prior to the addition of IL-1 also had no effect on IL-1-induced iNOS mRNA (Fig. 4A) and protein (Fig. 4B) levels, as well as on its enzymatic activity (Fig. 4C) which was assessed as the amount of NO produced. In contrast, treatment with SOD decreased both the expression (Figs. 4A,B) and the enzymatic activity (Fig. 4C) of iNOS induced by IL-1. Therefore, superoxide, but not H₂O₂, seems to be required for the transcriptional induction of iNOS in response to IL-1, which is in agreement with the results obtained relatively to NF- κ B activation.

DISCUSSION

In the present study, we first evaluated the ability of chondrocytes to produce H₂O₂ and

superoxide radical in response to IL-1. The results obtained showed that chondrocytes produced both types of ROS in response to this cytokine (Table I), which is in agreement with previous studies [Tawara et al., 1991; Rathakrishnan et al., 1992].

In most cell types, treatment with H₂O₂ [Meyer et al., 1993; Wesselborg et al., 1997; Manna et al., 1998], or with enzymes that increase its intracellular level, such as superoxide dismutase [Schmidt et al., 1995], results in the activation of NF- κ B. Accordingly, treatment with antioxidants, such as *N*-acetylcysteine [Meyer et al., 1993] and pyrrolidine dithiocarbamate [Schreck et al., 1992], or with antioxidant enzymes, like catalase [Schmidt et al., 1995] and thioredoxin [Jin et al., 1997], effectively blocks induction by various stimuli. Since inhibition of IL-1-induced ROS production prevented NF- κ B activation and the subsequent iNOS expression in chondrocytes [Mendes et al., 2001], here we investigated whether H₂O₂ could be the mediator of that response. However, the results show that H₂O₂, either alone or in combination with IL-1, had no effect on NF- κ B activation (Fig. 1) and iNOS expression (Fig. 3) and that catalase was unable to block induction by IL-1 (Figs. 2 and 4). These results suggest that H₂O₂ is not required for NF- κ B activation and iNOS expression in bovine articular chondrocytes.

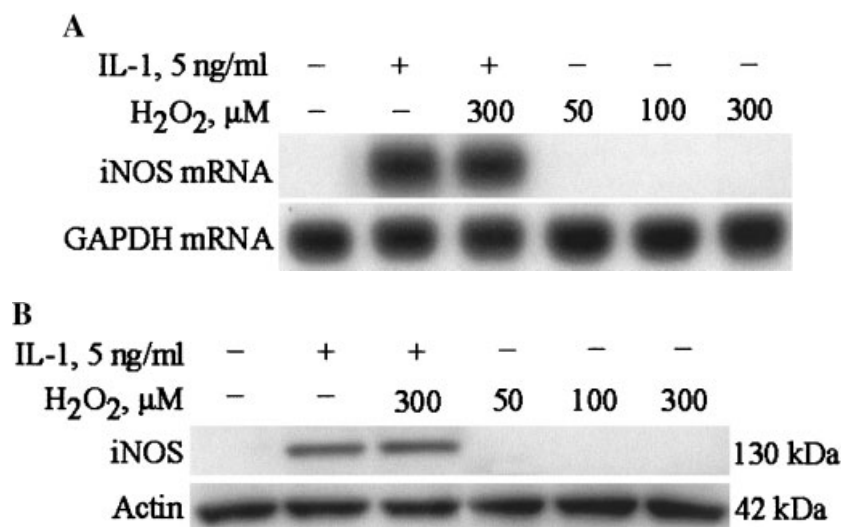


Fig. 3. Effect of H₂O₂ on the expression of the inducible isoform of the nitric oxide synthase (iNOS). **A:** Effect of H₂O₂ on iNOS mRNA levels, in the presence or absence of interleukin-1 β (IL-1); **(B)** effect of H₂O₂ on iNOS protein levels, in the presence or absence of IL-1. Chondrocyte cultures were treated simultaneously with H₂O₂ (300 μ M) and IL-1 (5 ng/ml), or with the indicated concentrations of H₂O₂ alone (from 50 to 300 μ M), for

6 (panel A) or 16 h (panel B). Northern blot and Western blot analysis of iNOS mRNA and protein levels, respectively, were performed as described under Materials and Methods. The autoradiographies shown in panels A and B are representative of six independent experiments, which were performed using six distinct sets of cell cultures.

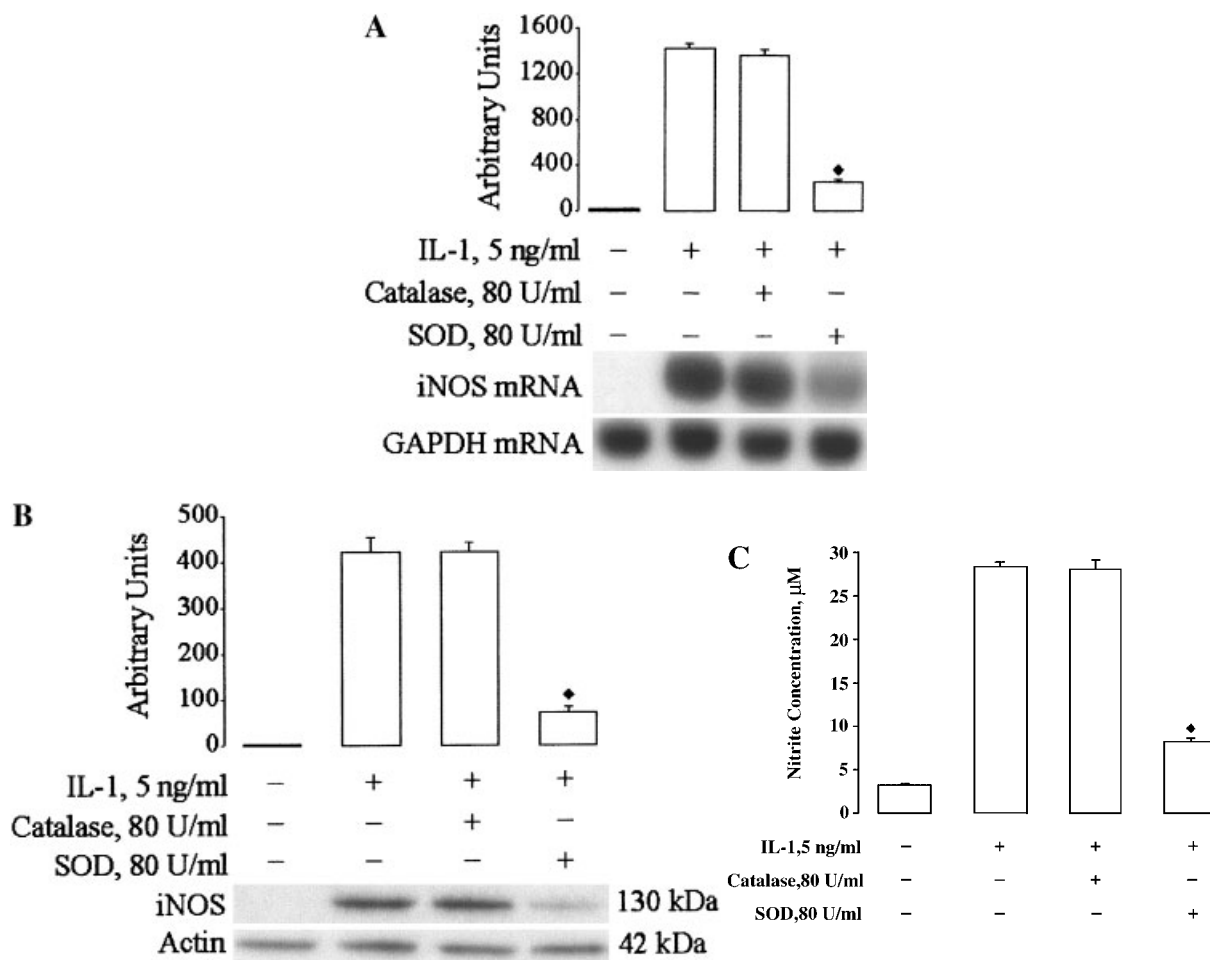


Fig. 4. Effect of catalase and superoxide dismutase (SOD) on interleukin-1 β - (IL-1-) induced expression and activity of the inducible isoform of the nitric oxide synthase (iNOS). **A:** Effect of catalase and SOD on IL-1-induced iNOS mRNA levels; **(B)** effect of catalase and SOD on IL-1-induced iNOS protein levels. **C:** Effect of catalase and SOD on IL-1-induced iNOS activity, assessed as the amount of nitrite accumulated in the culture supernatants. Chondrocyte cultures were treated with catalase (80 U/ml) or SOD (80 U/ml) for 30 min before the addition of IL-1 (5 ng/ml) and then further incubated for 6 (panel A) or 16 h (panels B and C). Northern blot and Western blot analysis of iNOS mRNA and protein levels, respectively, and measurement of nitrite

concentration were performed as described under Materials and Methods. In panels A and B, the results shown are the mean \pm SD of the intensity, in arbitrary units, of the bands obtained, for each condition, in three different experiments which were performed using three distinct sets of cell cultures. The autoradiographies shown in panels A and B are representative of three independent experiments. In panel C, the results shown are the mean \pm SD of the nitrite concentration determined in three independent experiments, each performed in triplicate. $\blacklozenge P < 0.01$ relatively to IL-1. Statistical significance was assessed by the unpaired Student's *t*-test.

Interestingly, other studies also suggest that, in some cells, H₂O₂ does not have a prominent role on NF- κ B activation and, in a few cases, H₂O₂ has been found to regulate this transcription factor negatively. In COS-1 cells, for instance, overexpression of catalase had no effect on NF- κ B activation induced by TNF or phorbol esters [Suzuki et al., 1995], whereas, in stimulated T-lymphocytes, acute or chronic exposure to H₂O₂ blocked NF- κ B activation [Flescher et al., 1998]. On the other hand, overexpression of manganese-SOD completely abolished TNF-

induced NF- κ B activation in a breast cancer cell line [Manna et al., 1998]. However, this study also reported that overexpression of SOD potentiated H₂O₂-induced NF- κ B activation, rendering it difficult to ascertain which type of ROS was involved in NF- κ B activation in those cells.

On the other hand, treatment with SOD prevented IL-1-induced I κ B- α degradation (Fig. 2) and iNOS expression (Fig. 4). These inhibitory effects can only be accounted for by the action of this enzyme, that is, either by the reduced levels of superoxide radical or by the presence of

increased amounts of H₂O₂. As to the last possibility, increased amounts of H₂O₂ could either inhibit or enhance the effects of IL-1. These possibilities were ruled out by the use of catalase and by the direct addition of H₂O₂ to chondrocyte cultures treated or not with IL-1. The absence of positive or negative effects in cells treated either with catalase and IL-1 (Figs. 2 and 4) or with H₂O₂, alone or in the presence of IL-1 (Figs. 1 and 3) shows that H₂O₂ is not only unable to induce NF- κ B activation and iNOS expression in chondrocytes, but more importantly, that it does neither mediate nor inhibit those responses induced by IL-1. Moreover, Tiku et al. [1998] showed that the formation of the hydroxyl radical in chondrocytes requires the addition of exogenous iron and the presence of H₂O₂. Therefore, our results also exclude the hydroxyl radical, or any other ROS produced from H₂O₂, as being the mediator of IL-1-induced NF- κ B activation and iNOS expression in chondrocytes. Hence, the inhibitory effects observed by treatment with SOD most probably result from the decreased availability of the superoxide radical. Taken together, the results presented indicate that the superoxide radical is the ROS involved in mediating IL-1-induced I κ B- α degradation and the consequent NF- κ B activation and iNOS expression in chondrocytes.

Our findings are in agreement with recent reports indicating that superoxide, rather than H₂O₂, is the ROS involved in mediating some IL-1-induced responses. Superoxide has been shown to act as a costimulatory factor amplifying matrix metalloproteinase-9 expression in glomerular mesangial cells by enhancing IL-1-induced activation of the mitogen-activated protein kinases, p38 and extracellular signal-regulated kinase (ERK), and of the transcription factors NF- κ B and activator protein-1 [Eberhardt et al., 2000]. In agreement with this report, IL-1-induced ERK activation and matrix metalloproteinase-9 expression, in vascular smooth muscle cells, were shown to be mediated by superoxide [Gurjar et al., 2001]. Interestingly, Beck et al. [1998] and Kuo et al. [2000] reported independently that addition of superoxide to rat mesangial cells or to hepatocyte cultures, respectively, enhanced IL-1-induced iNOS expression, which, although not showing that superoxide mediates this IL-1-induced response, is in agreement with our results presented here.

On the other hand, peroxynitrite, which results from the reaction of superoxide with NO, is another ROS that has been recently recognized as an important mediator of some IL-1-induced responses in various cells. In bovine articular chondrocytes, it was reported to mediate IL-1-induced inhibition of proteoglycan synthesis [Oh et al., 1998]. However, it is unlikely that the inhibition of NF- κ B activation and iNOS expression that occurred in SOD-treated cells, as shown in Figures 2 and 4, resulted from the unavailability of peroxynitrite. In fact, we have previously observed that NO exerts an inhibitory effect on NF- κ B activation and iNOS expression [Mendes et al., 2002b], which argues against a role for peroxynitrite in those IL-1-induced responses. Furthermore, we also observed that upon IL-1-treatment, the constitutive isoform of NOS (or NOS I) is rapidly degraded, whereas the inducible isoform takes a longer time to be expressed [Mendes et al., 2002b]. Therefore, the amount of NO produced by chondrocytes immediately after IL-1 treatment is probably very low, thus making the production of significant amounts of peroxynitrite improbable. Taken together, these results suggest that peroxynitrite is not the ROS required for IL-1 induction of NF- κ B activation and iNOS expression in bovine articular chondrocytes.

In conclusion, the results presented here confirm our previous observations that ROS are required for IL-1-induced NF- κ B activation and iNOS expression [Mendes et al., 2001]. More significantly, the present study identifies the superoxide radical as the ROS involved in mediating the IL-1-induced signaling pathway that leads to NF- κ B activation and to the expression of NF- κ B-dependent genes in bovine articular chondrocytes.

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