Hydrogen peroxide mediates interleukin-1β-induced AP-1 activation in articular chondrocytes: Implications for the regulation of iNOS expression

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Abstract

The pro-inflammatory cytokine interleukin-1β (IL-1) induces articular chondrocytes to produce reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), which mediate some IL-1-induced responses. This study aimed at elucidating the role of ROS, particularly H_2O_2, in mediating IL-1-induced activation of the transcription factor activator protein-1 (AP-1) in primary cultures of articular chondrocytes. AP-1 may function either as an inducer or as a repressor of the inducible nitric oxide synthase (iNOS) gene promoter. Since we observed that AP-1 is not required for iNOS expression in chondrocytes, we also investigated whether it is a repressor of this gene. The results of electrophoretic mobility shift assays showed that both IL-1 and H_2O_2 activated AP-1 and that inhibition of IL-1-induced ROS production abrogated AP-1 activation. The AP-1 complexes, induced by either IL-1 or H_2O_2, contained c-Fos/c-Jun and c-Fos/JunD heterodimers, but IL-1 activated AP-1 with a kinetics slower than that observed with H_2O_2. Pre-activation of AP-1, before stimulation of the cells with IL-1, did not inhibit iNOS mRNA and protein synthesis, relative to cells treated with IL-1 alone. These results indicate that H_2O_2 is a major mediator of IL-1-induced AP-1 activation in articular chondrocytes and that inhibition of ROS production is an effective strategy to block this IL-1-induced response. This study also identifies c-Fos/c-Jun and c-Fos/JunD heterodimers as the AP-1 transcription factors induced by IL-1, which, although not involved in the transcriptional regulation of the iNOS gene, may be important for the regulation of other genes also relevant in arthritic diseases, namely the collagenase-1 and IL-8 genes.

Abbreviations: AP-1, activator protein-1; DPI, diphenyleneiodonium chloride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H_2O_2, hydrogen peroxide; IL-1, interleukin-1β; IL-8, interleukin-8; iNOS, inducible nitric oxide synthase; MMP, matrix metalloproteinase; NF-κB, nuclear factor-kappaB; NO, nitric oxide; ROS, reactive oxygen species

Introduction

Arthritic diseases have in common clinical features of inflammation and cartilage degradation, which, at least in part, result from the augmented synthesis and release of pro-inflammatory cytokines, among which interleukin-1β (IL-1) has a prominent role (Pelletier et al., 1993; Weyand, 2000). IL-1 induces articular chondrocytes to produce inflammatory media-

tors, namely prostaglandins (Knott et al., 1994) and nitric oxide (NO) (Palmer et al., 1993), and cartilage matrix degrading enzymes, such as the matrix metalloproteinases-1, -3 and -13 (MMPs-1, -3 and -13 or collagenase-1, stromelysin-1 and collagenase-3, respectively) (Catterall et al., 2001; Lo et al., 1998; Mengshol et al., 2001), whereas the synthesis of cartilage matrix proteins is inhibited (Goldring et al., 1994; Taskiran et al., 1994). NO and the MMPs are thought to play major roles in the pathophysiology of arthritic diseases, contributing to the irreparable degradation of the cartilage matrix (Lotz et al., 1995). Therefore, understanding the cellular mechanisms that control the production of these mediators is of great importance for the development of new therapeutic strategies that may help revert the degradation of the cartilage matrix that occurs in arthritic diseases. Despite extensive investigation, the signal transduction pathways utilized by IL-1 to induce those responses in chondrocytes are still poorly understood.

Increasing evidence indicates that reactive oxygen species (ROS) are important mediators of IL-1-induced cellular responses (Eberhardt et al., 2002; Kuo et al., 1997). In chondrocytes, IL-1 has been shown to induce the production of various types of ROS, including hydrogen peroxide (H_2O_2) (Rathakrishnan et al., 1992; Mendes et al., 2003), singlet oxygen (Rathakrishnan et al., 1992) and hydroxyl (Tiku et al., 1998) and superoxide anion (Mendes et al., 2003; Tawara et al., 1991) radicals, as well as the reactive nitrogen species NO (Palmer et al., 1993). We have recently observed that ROS, particularly the superoxide anion radical, are required for activation of the nuclear transcription factor-kappaB (NF-κB) and for the expression of the inducible NO synthase (iNOS) gene induced by IL-1 in bovine articular chondrocytes (Mendes et al., 2001, 2003). Other ROS-mediated IL-1-induced cellular responses in chondrocytes include expression

of the *c-jun* (Lo et al., 1996), *c-fos* and *collagenase* (Lo et al., 1998) genes and activation of the c-Jun NH2-terminal kinase (Lo et al., 1996), which phosphorylates c-Jun increasing its transcriptional activity (Kyriakis et al., 1994). Furthermore, chondrocyte-derived ROS have also been shown to mediate cartilage matrix destruction by inducing aggrecan and collagen degradation (Tiku et al., 1999; Tiku et al., 2000).

The protein products of the *c-fos* and *c-jun* genes can heterodimerize to form an Activator Protein-1 (AP-1) transcription factor. AP-1 plays an essential role in mediating some effects of IL-1, namely the expression of the *collagenase-1* gene (Lafyatis et al., 1990). Furthermore, AP-1 has recently been implicated in the transcriptional induction of the collagenases-1 and -3 in response to IL-1 and other stimuli, both in chondrocytes and synoviocytes (Catterall et al., 2001; Mengshol et al., 2001; Sun et al., 2002). Nevertheless, the role of AP-1 on the expression of other genes whose promoter regions also contain binding sites for this transcription factor is not always so clear. For instance, the promoter region of the iNOS gene from different species contains binding sites for AP-1 (Lowenstein et al., 1993; Chu et al., 1998), but, in different cells, the role of this transcription factor on iNOS expression has been shown to range from induction (Marks-Konczalik et al., 1998; Kristof et al., 2001; Giri et al., 2002) to repression (Kleinert et al., 1998; Pance et al., 2002).

Accumulating evidence indicates that AP-1 is sensitive to redox regulation. However, the role of reactive oxygen and nitrogen species on AP-1 activation seems to be highly dependent on the cell type, since the same oxidative stimulus, such as H_2O_2 (Devary et al., 1991; Meyer et al., 1993; Flescher et al., 1998; Lakshminarayanan et al., 1998) and NO (Rossi et al., 2000; Zouki et al., 2001), can either induce or repress the activity of this transcription factor when acting in different cells.

In chondrocytes, we observed previously that NO activates AP-1 DNA-binding, mimicking the effect of IL-1 (Mendes et al., 2002a). Moreover, the requirement of ROS for IL-1 induction of both c-fos and c-jun expression (Lo et al., 1996, 1998), as mentioned above, suggests that ROS may mediate IL-1-induced AP-1 activation. Nevertheless, this question has never been addressed directly in chondrocytes and, although H_2O_2 has been shown to mimic IL-1-induced c-jun expression in these cells (Lo et al., 1996), its ability to modulate AP-1 activity is unknown. Therefore, the present study aimed at elucidating the role of ROS, particularly that of H_2O_2, in mediating IL-1-induced AP-1 activation in articular chondrocytes.

On the other hand, we have also observed that AP-1 activation is not required for IL-1-induced iNOS expression in chondrocytes (Mendes et al., 2002b). Therefore, we investigated whether AP-1 is not involved or functions as a negative regulator of the *iNOS* gene in IL-1-stimulated chondrocytes.

Materials and methods

Reagents

Recombinant human IL-1β was purchased from R&D Systems (Abingdon, UK), H_2O_2 from Sigma Chemical Co. (St. Louis, MO, USA), diphenyleneiodonium chloride (DPI) from Calbiochem (San Diego, CA, USA), the anti-iNOS antibody was from Transduction Laboratories (Lexington, KY, USA), the anti-actin antibody was from Boehringer Mannheim (Mannheim, Germany). The antibodies against the protein subunits of the Fos and Jun families and the oligonucleotide probes were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The protease inhibitors cocktail, "Complete, Mini", was from Roche Molecular Biochemicals (Mannheim, Germany).

The isotopes were from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, USA). 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% CO_2. Prior to any treatments, the cells were serum-starved overnight and, thereafter, maintained in culture medium with 1% antibiotic/antimycotic solution and without serum.

Preparation of cytoplasmic and nuclear extracts

The cells were lysed in 400 μl of buffer 1 (10 mmol/L Tris-HCl, 10 mmol/L NaCl, 3 mmol/L $MgCl_2$, 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 2300g/5 min/4°C and the supernatants (cytoplasmic extracts) were collected and stored at –70°C. The pellets were resuspended in 30 μl of buffer 2 (20 mmol/L HEPES, 5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 1 mmol/L DTT, 300 mmol/L 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 18000g/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).

Electrophoretic mobility shift assay (EMSA)

The AP-1 oligonucleotide probe was end-labeled with [γ-^{32}P]ATP by the T4 polynucleotide kinase and purified through Sephadex G-50 spin columns. Nuclear extracts (10 μg protein) were incubated on ice, for 40 min, in 20 μl binding reactions containing 20 mmol/L HEPES, 50 mmol/L KCl, 1 mmol/L MgCl$_2$, 0.5 mmol/L DTT, 4% Ficoll 400, 2 μg poly (dIdC), 20 μg BSA, pH 7.9 and 150 000 cpm/reaction of [γ-^{32}P]-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. For supershift analysis, nuclear extracts from IL-1- or H$_2$O$_2$-treated cells were incubated on ice for 2 h with 2 μg/reaction of each antibody, before the addition of the radiolabeled oligonucleotide. Competition assays were performed by simultaneous incubation of the IL-1-treated nuclear extracts with the labeled AP-1 probe and with a 100-fold excess of the corresponding unlabeled probe or with a 100-fold excess of unlabeled octamer-1 (Oct-1) probe.

Western blot analysis

The cytoplasmic extracts (25 μg protein) were diluted in sodium dodecylsulfate (SDS) sample buffer (2.5% SDS, 0.0625 mol/L 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 electroblotting. The membranes were blocked with 5% skim milk in Tris-buffered saline-Tween (TBS-T, 20 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% Tween-20) and then probed with the anti-iNOS antibody. 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. The membranes were subsequently stripped and reprobed with an anti-actin antibody to ensure an equal protein loading.

Northern blot analysis

Total RNA was extracted using Trizol (Gibco Brl, Life Technologies Inc., Grand Island, NY, USA) and quantitated by spectrophotometry at 260 nm. Denatured RNA samples (20 μg) 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 [^{32}P]-labeled iNOS c-DNA probe, subsequently stripped and reprobed with a [^{32}P]-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-^{32}P]dCTP by the klenow fragment using the Random Primed DNA Labeling kit from Boehringer Mannheim GmbH.

Results

Inhibition of IL-1-induced ROS production prevents AP-1 activation

To detect AP-1 activation, we evaluated, by EMSA analysis, the ability of the proteins present in the nuclear extracts from cells treated with IL-1, in the presence or absence of DPI, to form complexes with a [γ-^{32}P]-labeled oligonucleotide probe containing the consensus binding sequence for that transcription factor.

The results in Figure 1A show that nuclear extracts from IL-1-treated cells elicit the formation of an intense slower migrating band
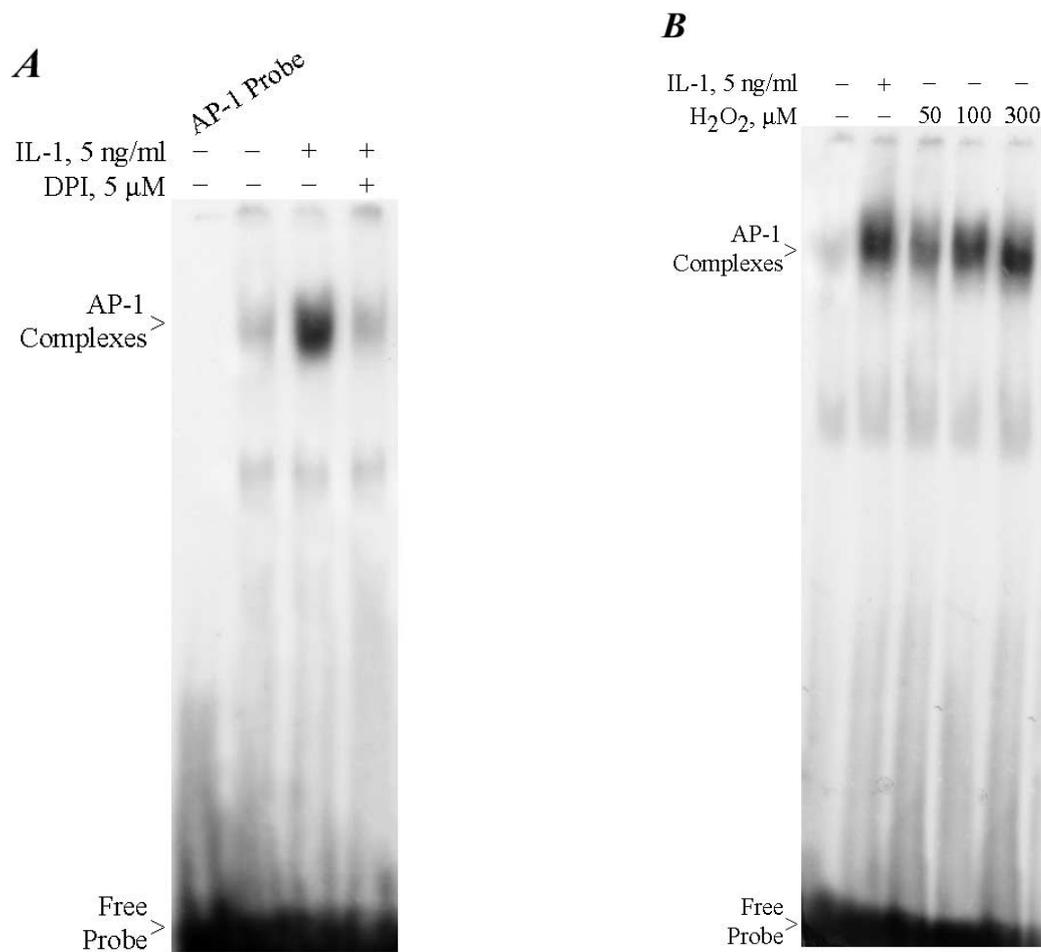
Figure 1. Reactive oxygen species, namely H_2O_2, mediate IL-1-induced AP-1 activation. A: Inhibition of ROS production prevents IL-1-induced AP-1 activation. Chondrocyte cultures were pretreated with DPI (5 μmol/L) for 2 h, before the addition of IL-1 (5 ng/ml) for 3 h. B: Dose-dependent activation of AP-1 by H_2O_2. Chondrocyte cultures were treated with the indicated concentrations of H_2O_2 for 3 h. The nuclear extracts, prepared as described under "Materials and methods", were used to detect AP-1 binding to a specific oligonucleotide probe by EMSA. The autoradiography shown in panel A is representative of three distinct experiments and that shown in panel B is representative of four distinct experiments.

corresponding to the binding of AP-1 proteins to the [γ-^{32}P]-labeled oligonucleotide probe. Competition assays, using either a 100-fold excess of unlabeled AP-1 probe or a 100-fold excess of unlabeled Oct-1 probe (Figure 3A), indicated that the retarded band detected on the autoradiography represents the formation of specific complexes between AP-1 proteins and the AP-1-specific oligonucleotide probe.

Diphenyleneiodonium chloride (DPI), which completely inhibits IL-1-induced ROS production (Lo et al., 1998; Mendes et al., 2001), was effective in preventing IL-1-induced AP-1 activation, as indicated by the absence of the corresponding band in the electrophoretic mobility shift assay shown in Figure 1A. This inhibition cannot be accounted for by toxic effects of DPI, since we observed previously that, in concentrations up to 10 μmol/L, this compound does not affect the viability of

articular chondrocytes, cultured under conditions identical to those used in this study (Mendes et al., 2001).

H_2O_2 is sufficient to induce AP-1 activation

The results in Figure 1B show that H_2O_2 induced the formation of complexes migrating more slowly than the unbound, free oligonucleotide probe. As shown for IL-1, competition assays indicated that the retarded band detected on the autoradiography obtained with nuclear extracts from H_2O_2-treated cells represents the formation of specific complexes between AP-1 proteins and the AP-1-specific oligonucleotide probe (Figure 3B). Furthermore, the intensity of the bands shown in Figure 1B increased with increasing concentrations of H_2O_2, thus showing a dose-dependent activation of AP-1 by H_2O_2. Taken together, these results and those in Figure 1A show that H_2O_2 is sufficient to induce AP-1 activation and that ROS, namely H_2O_2, mediate AP-1 activation in response to IL-1.

To further evaluate the relevance of AP-1 activation by H_2O_2, we studied the pattern of AP-1 activation as a function of the incubation time with IL-1 and with H_2O_2. The results in Figure 2 show that after 30 min incubation with H_2O_2, the AP-1 complexes were already detectable, reaching maximum intensity at 3 h and decreasing thereafter. In contrast, 1 h incubation with IL-1 was required for the AP-1 complexes to be detectable, increasing thereafter in a manner identical to what happened with H_2O_2 treatment. These results further suggest that H_2O_2 mediates IL-1-induced AP-1 activation. Indeed, if H_2O_2 has to be produced, in response to IL-1, for AP-1 activation to occur, it is not surprising that direct addition of H_2O_2 to the cell cultures activated AP-1 more rapidly than treatment with IL-1.
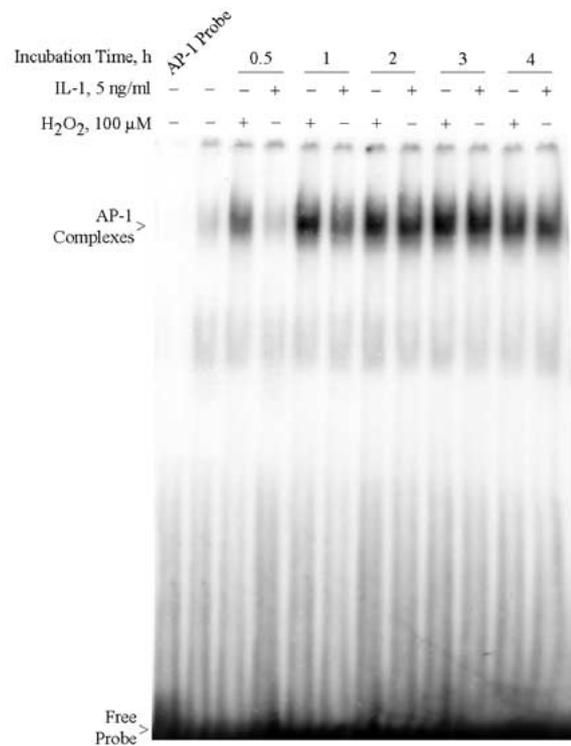

Figure 2. Time-dependent activation of AP-1 by IL-1 and H_2O_2. Chondrocyte cultures were treated with IL-1 (5 ng/ml) or with H_2O_2 (100 µmol/L) for the indicated time periods (0.5–4 h). The nuclear extracts, prepared as described under "Materials and methods", were used to detect AP-1 binding to a specific oligonucleotide probe by EMSA. The autoradiography shown is representative of three distinct experiments.

The AP-1 complexes, induced by IL-1 and by H_2O_2, contain c-Fos/c-Jun and c-Fos/JunD heterodimers

Supershift analysis with antibodies against the protein subunits of the Fos and Jun families was used to characterize and compare the protein composition of the AP-1 complexes induced by IL-1 and by H_2O_2. The results obtained show that incubation of the nuclear extracts from either IL-1-treated cells (Figure 3A) or H_2O_2-treated cells (Figure 3B) with antibodies against the proteins c-Fos and JunD, prior to the addition of the [γ-^{32}P]-
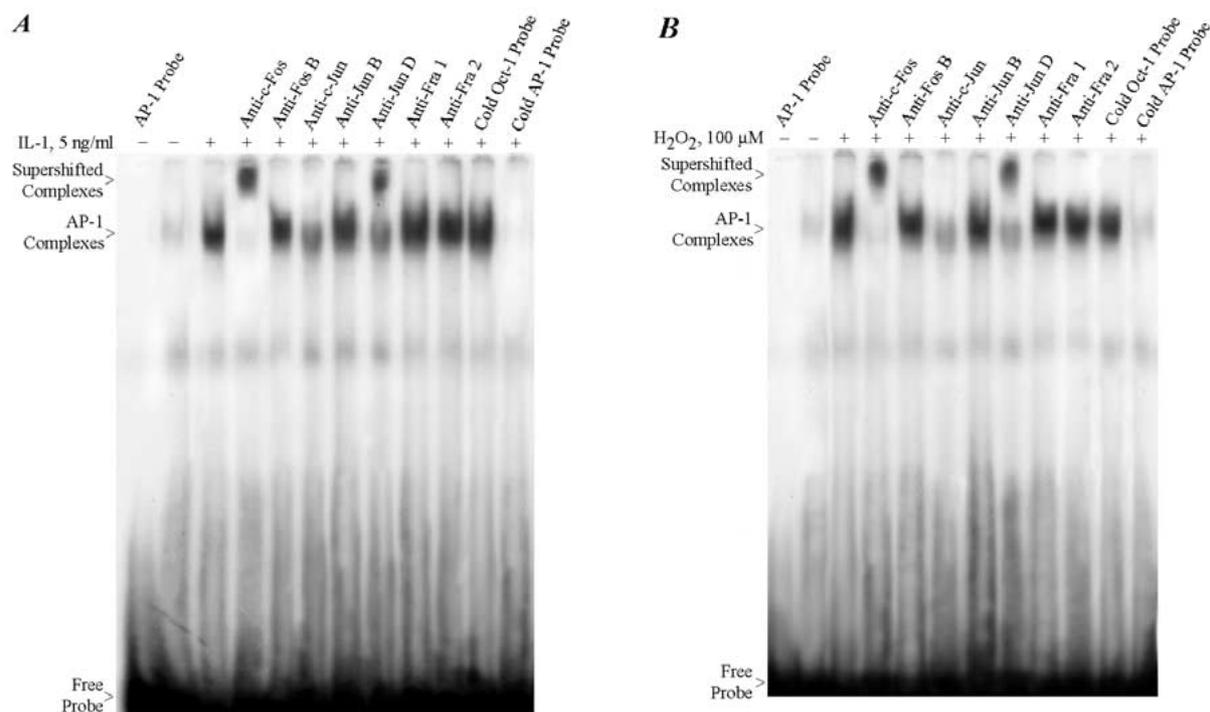
Figure 3. Characterization of the AP-1 complexes induced by IL-1 and by H$_2$O$_2$. A: characterization of the AP-1 complexes induced by IL-1; B: characterization of the AP-1 complexes induced by H$_2$O$_2$. Chondrocyte cultures were treated with IL-1 (5 ng/ml) or with H$_2$O$_2$ (100 μmol/L) for 3 h. Competition and supershift assays were performed as described under "Materials and methods". The autoradiographies shown are representative of three distinct experiments.

labeled AP-1 probe, elicited the formation of slower migrating complexes that appear in the autoradiography closer to the origin of the gel. This indicates that the supershifted bands contain the proteins c-Fos and JunD. In identical assays using an antibody against the c-Jun protein, no supershifted bands were detected, but the intensity of the AP-1–oligonucleotide probe complexes was greatly reduced (Figures 3A and 3B). This indicates that binding of the antibody to AP-1 dimers containing the protein c-Jun prevented the binding of those dimers to the oligonucleotide probe. Antibodies against the other members of the Fos and Jun families, namely anti-FosB, anti-JunB, anti-Fra1 and anti-Fra2, had no appreciable effect on the intensity or on the electrophoretic mobility of the AP-1 oligonucleotide probe complexes (Figures 3A and 3B). Therefore, it seems that the AP-1 dimers, induced by either IL-1 or H$_2$O$_2$ in articular chondrocytes, are mainly composed of the proteins c-Fos, c-Jun and JunD. The Jun proteins are probably arranged in heterodimers with c-Fos, since the Fos proteins cannot form homodimers (Angel and Karin, 1991) and the anti-c-Fos antibody completely supershifted the AP-1 complexes, whereas the anti-c-Jun and the anti-JunD antibodies only partially abrogated or supershifted the AP-1 complexes.

AP-1 activation does not repress IL-1-induced iNOS expression

To investigate the role of AP-1 on the transcriptional regulation of iNOS expression in articular chondrocytes, we took advantage of the observed ability of H_2O_2 to induce AP-1 activity (Figures 1B, 2 and 3) without inducing or even being required for iNOS expression (Mendes et al., 2003). Thus, AP-1 activity was induced by treating the chondrocyte cultures with H_2O_2 (50–300 µmol/L) for 2 h before the addition of IL-1. The cells were then incubated with IL-1 (5 ng/ml) for 5 or 16 h and the iNOS mRNA and protein levels, respectively, were determined. The results in Figure 4 show that pre-treatment with H_2O_2 had no effect on the iNOS mRNA and protein levels induced by IL-1. Therefore, these results show that AP-1 activation does not repress IL-1-induced iNOS expression in articular chondrocytes.

Discussion

In agreement with other reports (Tawara et al., 1991; Rathakrishnan et al., 1992; Tiku et al., 1998), our previous studies showed that chondrocytes produce ROS in response to IL-1 (Mendes et al., 2001, 2003) and that H_2O_2 constitutes a large fraction of the total ROS produced (Mendes et al., 2003). We have also observed that DPI, a selective inhibitor of flavonoid-containing enzymes, such as NADPH oxidase, NADH oxidase, quinone oxidoreductase and cytochrome P450 reductase, prevents IL-1-induced ROS production in a concentration-dependent manner (Mendes et al., 2001). In the present study, we also found that DPI effectively prevented IL-1-induced AP-1 activation (Figure 1A), thus indicating that ROS are required for and mediate this response to IL-1.

H_2O_2 has been extensively used as an oxidative stimulus to study the role of ROS on many cellular processes, namely on AP-1 activation, in a variety of cells. The ability of H_2O_2 to activate AP-1 in different cells is well documented (Devary et al., 1991; Lakshminarayanan et al., 1998; Zhou et al., 2001). However, the opposite effect, that is, inhibition of AP-1 activation by pro-oxidant stimuli or induction by antioxidants, has also been reported (Meyer et al., 1993; Wesselborg et al., 1997; Flescher et al., 1998). It has been suggested that these discrepancies may reflect cell type-specific mechanisms of AP-1 regulation (Karin et al., 1997; Shaulian and Karin, 2002). The results presented here show that, in articular chondrocytes, H_2O_2 activates AP-1 in a dose-dependent manner (Figure 1B), mimicking the response induced by IL-1 (Figure 1A). The protein composition (Figure 3) and the temporal pattern (Figure 2) of AP-1 activation in response to H_2O_2 and IL-1 were identical. Nevertheless, AP-1 dimers, capable of binding to an AP-1-specific oligonucleotide probe, were induced more rapidly by H_2O_2 than by IL-1 (Figure 2). This indicates that H_2O_2 has to be produced in response to IL-1, before AP-1 activation can occur. Taken together, the results presented show that H_2O_2 is a major ROS involved in mediating IL-1-induced AP-1 activation in articular chondrocytes.

The promoter region of the iNOS gene from different species contains AP-1-binding sites (Lowenstein et al., 1993; Chu et al., 1998), but the role of this transcription factor in the expression of iNOS seems to vary considerably in different cells, even within the same species. For instance, in human lung epithelial cells (Marks-Konczalik et al., 1998; Kristof et al., 2001) and in C6 glioma cells (Giri et al., 2002), AP-1 has been shown to mediate induction of iNOS expression, whereas in human colon tumor epithelial cell lines it was shown to have a repressor effect (Kleinert et al., 1998; Pance et al., 2002). We have previously observed that AP-1 is not required for IL-1-induced iNOS expression in articular chondrocytes, indicat-
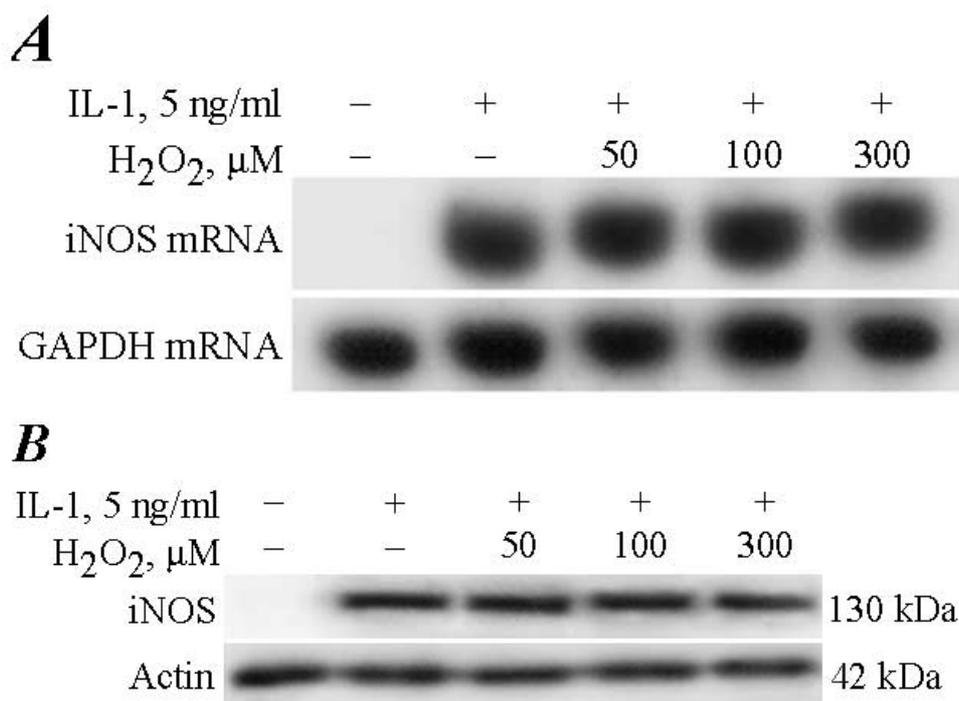
A

IL-1, 5 ng/ml	$-$	$+$	$+$	$+$	$+$
H_2O_2, μM	$-$	$-$	50	100	300

iNOS mRNA

GAPDH mRNA

B

IL-1, 5 ng/ml	$-$	$+$	$+$	$+$	$+$
H_2O_2, μM	$-$	$-$	50	100	300

iNOS   130 kDa

Actin   42 kDa

Figure 4. H_2O_2 pre-treatment does not affect IL-1-induced iNOS expression. A: effect of H_2O_2 pre-treatment on IL-1-induced iNOS mRNA levels; B: effect of H_2O_2 pre-treatment on IL-1-induced iNOS protein levels. Chondrocyte cultures were treated with the indicated concentrations of H_2O_2 for 2 h before the addition of IL-1 (5 ng/ml) and then further incubated for 5 h (A) or 16 h (B). The iNOS mRNA and protein levels were determined by Northern and Western blot analysis, respectively, as described under "Materials and methods". The autoradiographies shown are representative of three distinct experiments.

ing that this transcription factor is not an inducer of the iNOS promoter (Mendes et al., 2002b). However, this finding does not exclude the possibility that AP-1 can behave as a negative regulator or repressor of the iNOS promoter in articular chondrocytes.

To elucidate this question, we took advantage of the ability of H_2O_2 to activate this transcription factor (Figures 1B, 2 and 3B), without interfering with iNOS expression (Mendes et al., 2003). In fact, we found recently that, although ROS, particularly the superoxide anion, are required for IL-1-induced iNOS expression mediated by NF-κB (Mendes et al., 2001, 2003), H_2O_2 does neither induce by itself nor does it mediate, enhance or inhibit IL-1-induced NF-κB activation and the subsequent expression of iNOS in articular chondrocytes (Mendes et al., 2003). Hence, we used H_2O_2 to specifically activate AP-1, before stimulation of the cells with IL-1, so that the role of this transcription factor on iNOS expression could be evaluated. The results presented here show that pre-activation of AP-1, by treatment of the cells with H_2O_2, did not inhibit IL-1-induced iNOS expression (Figure 4). Therefore, we can conclude that AP-1 is not a repressor of the iNOS promoter and, therefore, does not participate in the transcriptional regulation of the *iNOS* gene in bovine articular chondrocytes.

Recent reviews, concerning the activation modes and functions of AP-1, suggest that different AP-1 dimers may have distinct roles

in the regulation of gene transcription (Karin et al., 1997; Shaulian and Karin, 2002). The heterodimer c-Fos/c-Jun has the highest affinity for the AP-1 site and the highest transactivation activity (Karin et al., 1997; Shaulian and Karin, 2002). However, recent studies indicate that other AP-1 dimers, distinct from the classical c-Fos/c-Jun, can participate on the regulation of gene expression. Indeed, various AP-1 complexes containing either Fra2/JunD (Marks-Konczalik et al., 1998; Kristof et al., 2001), JunB, c-Jun and JunD (Cho et al., 2002) or Fra1 (Giri et al., 2002), were reported to be involved in inducing the transcription of the *iNOS* gene in different cells. Hence, at least, some of the discrepancies concerning the role of AP-1 on the transcriptional regulation of the *iNOS* gene may result from the induction of different AP-1 dimers in response to a given stimulus in different cells, or in response to distinct stimuli in the same cell-type. Nevertheless, the presence of active AP-1 dimers in the cultured chondrocytes, at the time of IL-1 treatment, did not alter iNOS expression relatively to cells treated with IL-1 alone (Figure 4). Therefore, these results exclude a role for AP-1, regardless of its composition, on the transcriptional regulation of the *iNOS* gene in articular chondrocytes.

Although not required for the expression of iNOS, the AP-1 complexes induced by IL-1 and H_2O_2 in chondrocytes may be relevant for the induction of other genes whose protein products also play significant roles in the pathophysiology of arthritic diseases. One of those is interleukin-8 (IL-8), a cytokine that has been implicated in a variety of inflammatory diseases, including arthritis (Luster, 1998). Various stimuli, namely IL-1, have been shown to induce IL-8 expression in articular chondrocytes (Recklies and Golds, 1992; Pulsatelli et al., 1999). On the other hand, the c-Fos/JunD complex has been reported to mediate induction of the IL-8 gene in response to either tumor necrosis factor-α or H_2O_2 in A549

epithelial cells (Lakshminarayanan et al., 1998). Interestingly, we found that, besides c-Fos and c-Jun, the AP-1 complexes, induced by either IL-1 or H_2O_2, also contained JunD, probably arranged in heterodimers with c-Fos (Figure 3). Therefore, it will be interesting to study the role of this particular AP-1 transcription factor on IL-1-induced IL-8 expression in articular chondrocytes. Moreover, the c-Fos/JunD complex has also been shown to bind to AP-1 sites in the promoter region of the *collagenase-1* gene and to be essential for its transcriptional induction (White and Brinckerhoff, 1995). Thus, our results suggest that this particular AP-1 transcription factor may also play an important role on H_2O_2-mediated IL-1-induced collagenase-1 expression in articular chondrocytes.

Conclusion

The results presented here indicate that H_2O_2 is a major ROS mediating IL-1-induced AP-1 activation, in articular chondrocytes, by inducing the formation of c-Fos/c-Jun and c-Fos/JunD dimers. Furthermore, this study also shows that inhibition of ROS production, namely suppression of H_2O_2 synthesis, is an effective strategy to prevent IL-1-induced AP-1 activation in articular chondrocytes. On the other hand, this study shows that, in articular chondrocytes, AP-1 is not a repressor of the iNOS gene promoter. Nevertheless, given the importance of IL-1-induced gene expression in arthritic diseases, the roles of the distinct AP-1 complexes in mediating the various IL-1-induced responses, as well as the mechanisms that regulate the activity and specificity of those complexes, deserve to be further investigated.

Acknowledgments

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