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Interleukin-1β Induction of c-fos and Collagenase Expression in Articular Chondrocytes: Involvement of Reactive Oxygen Species

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Abstract Interleukin-1β (IL-1) is implicated in cartilage destruction in arthritis through promotion of matrix metalloproteinase production. Upregulation of collagenase gene expression by IL-1 is known to require the transactivators Fos and Jun. Recently, reactive oxygen species (ROS) have been suggested to act as intracellular signaling molecules mediating the biological effects of cytokines. Here, we demonstrated ROS production by IL-1-stimulated bovine chondrocytes and that neutralizing ROS activity by the potent antioxidant, N-acetylcysteine, or inhibiting endogenous ROS production by diphenyleneiodonium (DPI), significantly attenuated IL-1-induced c-fos and collagenase gene expression. The inhibitory effect of DPI implicates enzymes such as NADPH oxidase in the endogenous production of ROS. Chondrocytes were also found to produce nitric oxide (NO) upon IL-1 stimulation. That NO may mediate part of the inducing effects of IL-1 was supported by the observation that L-N^G-monomethylarginine, a NO synthase inhibitor, partially inhibited IL-1-regulated collagenase expression. Moreover, treatment of chondrocytes with the NO-producing agent, S-nitroso-N-acetylpenicillamine, was sufficient to induce collagenase mRNA levels. In summary, our results suggest that ROS released in response to IL-1 may function as second messengers transducing extracellular stimuli to their targets in the nucleus, leading to augmentation of gene expression. J. Cell. Biochem. 69:19–29, 1998.
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Key words: interleukin-1; reactive oxygen species; nitric oxide; c-fos; collagenase; chondrocytes

Interleukin-1β (IL-1), detected at elevated levels in synovial fluid of patients with osteoarthritis [Loyau and Pujol, 1990], rheumatoid arthritis, and other joint diseases [Westacott et al., 1990], is capable of eliciting a series of responses thought to be involved in the destruction of articular cartilage [Loyau and Pujol, 1990]. Some of these responses include the induction of matrix metalloproteinases such as collagenase and stromelysin [Pelletier et al., 1983], infiltration of inflammatory cells such as lymphocytes and macrophages, and synovial hyperplasia [Dinarello, 1991; Le and Vilcek, 1987].

The cascade of signaling events that follow the occupancy of the IL-1 receptor by its ligand is not well-defined. Depending on the cell types and the cellular responses under study, a myriad of intracellular signals has been suggested to mediate the effects of IL-1. For instances, release of arachidonic acid [Conquer et al., 1992; Chang et al., 1986], activation of protein kinase C and G-proteins [Schutze et al., 1994; O'Neill et al., 1990], hydrolysis of sphingomyelin to ceramide by the action of sphingomyelinase [Schutze et al., 1994; Kolesnick and Golde, 1994] have all been suggested to mediate the effects of IL-1.

In addition, a class of highly diffusible and ubiquitous molecules, termed reactive oxygen species (ROS), may also mediate some aspects of cytokine actions [Lo and Cruz, 1995; Lo et al., 1996; Schreck et al., 1991]. In fact, cytokines have been shown to stimulate the production of ROS in various cell types [Lo and Cruz, 1995; Rathakrishnan et al., 1992; Tawara et al., 1991]. ROS which are highly reactive in nature include species such as hydroxyl radicals, hydro-

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gen peroxide, superoxide, and nitric oxide (NO) [Halliwell and Gutteridge, 1990]. A large body of evidence now supports the idea that ROS can modulate various cellular events from gene expression to cellular proliferation [Devary et al., 1991; Murrell et al., 1990; Lo and Cruz, 1995; Lo et al., 1996; Lander et al., 1996].

Transcriptional regulatory proteins such as c-Fos and c-Jun act as nuclear targets mediating the effects of IL-1 [Lo et al., 1996; Muegge et al., 1989; Lafyatis et al., 1990]. Fos and Jun, which form homo- and hetero-dimers and are referred to as activator protein-1 (AP-1), bind to the AP-1 responsive element in the regulatory domains of several genes including collagenase, stromelysin, and metallothionein [Lafyatis et al., 1990; Zafarullah et al., 1992; Angel et al., 1987]. In the case of the collagenase gene, the AP-1 site and c-fos expression are critical determinants dictating its expression. Treatment with antisense oligonucleotides directed against c-fos was able to completely block the expression of collagenase [Schonthal et al., 1988] and mutation in the AP-1 site of the collagenase promoter also abolished its expression [Angel et al., 1987; Chamberlain et al., 1993]. Interestingly, both c-fos and c-jun gene expression and DNA-binding ability of their protein products can be modulated by exogenous addition of ROS [Devary et al., 1991; Lo and Cruz, 1995; Lo et al., 1996].

In the context of arthritis, ROS can have adverse effects on the intactness of cartilage by inhibiting proteoglycan and hyaluronic acid syntheses [Bates et al., 1985a,b] and by destroying its components [Greenwald and Moy, 1979, 1980]. In view of the above, we hypothesized that ROS may also lead to cartilage damage through their roles in the production of cartilage degradative enzymes. Our hypothesis was based on the reasoning that since ROS can stimulate expression of early response genes including c-fos and c-jun, late response gene expression such as that of collagenase might also be activated by these messenger molecules. Therefore, we examined whether IL-1 could induce ROS production in bovine chondrocytes and if such ROS release was involved in the induction of c-fos and collagenase expression.

MATERIALS AND METHODS

Materials

Human recombinant interleukin-1β was generously supplied by Ciba-Geigy (Basel, Switzerland). N-acetylcysteine (NAC) and L-N^G-monomethylarginine (L-NMMA) were from Sigma (St. Louis, MO). Diphenyleneiodonium (DPI) was from Toronto Research Chemicals. S-nitroso-N-acetylpenicillamine (SNAP) was purchased from Biomol Research Laboratories. Radioactive isotopes and X-ray films were from Dupont, New England Nuclear. Sulfanilamide and naphthylethylenediamine, used in assaying nitrite content were from Sigma. Tissue culture dishes were obtained from Becton Dickinson. Antibiotic-antimycotic was purchased from Gibco-BRL.

Chondrocyte Cultures

Primary cultures of bovine chondrocytes were isolated from bovine articular cartilage as described by Cruz et al. [1990]. The cells were plated at 2×10^6 cells/ml in 12 ml of Ham's F-12 medium containing 3% antibiotics and 5% FBS. The cells were allowed to recover for 24 h at 37°C in a humidified atmosphere supplemented with 5% CO_2.

Northern Blot Analysis

Total RNA was isolated by the acidified guanidine isothiocyanate method [Chomczynski and Sacchi, 1987] and subjected to electrophoresis on a denaturing gel. Denatured RNA samples (10–15 µg) were analyzed by gel electrophoresis in a 1% denaturing agarose gel, transferred to a nylon membrane (Bio-Rad), cross-linked with an ultraviolet cross-linker (Stratagene UV Stratalinker 1800), and hybridized with ^{32}P-labelled rat c-fos or human collagenase-1 (MMP-1) cDNA. The blots were subsequently stripped and re-probed with ^{32}P-labelled rat glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA as an internal standard to ensure equal loading of RNA in each lane. When quantitative analysis was performed, the relative levels of mRNA were measured by scanning densitometry of the developed autoradiograms (Image Quant™ Software 3.0, Molecular Dynamics).

Direct Measurement of Intracellular ROS by Fluorescence-Activated Cell Sorting (FACS) Analysis

Intracellular levels of ROS were analyzed as described previously [Lo and Cruz, 1995; Chen et al., 1995]. Briefly, chondrocytes loaded with dihydrorhodamine 123 (DHR; 2 µM; Molecular Probes), a compound irreversibly converted to the green fluorescent compound rhodamine 123

(R123; 500–540 nm) upon reaction with intracellular ROS, were treated with IL-1 (20 ng/ml) for 20 min with or without DPI (5 μM). Both DHR and DPI were dissolved in dimethyl sulfoxide. Cells were then fixed for 20 min in 1.5% paraformaldehyde (Sigma) and the cellular R123 fluorescence intensity of 12,000 chondrocytes was measured by flow cytometry for each sample using an argon laser with the excitation source at 488 nm (Facscalibur 4 color analyzer with CellQuest Software, [Lund-Johansen and Olweus, 1992].

Measurement of NO Production

NO production was measured as the amount of nitrite, the stable end product of NO, released into the culture supernatant. Nitrite concentration was determined in cell-free culture supernatant using the spectrophotometric method based on the Griess reaction [Green et al., 1982]. Briefly, samples were reacted in equal volume with 1% sulfanilamide, 0.1% naphthylethylenediamine, and 5% phosphoric acid at room temperature for 10 min. The nitrite concentration was determined by absorbance at 540 nm in comparison with standard solutions of sodium nitrite prepared in the same medium.

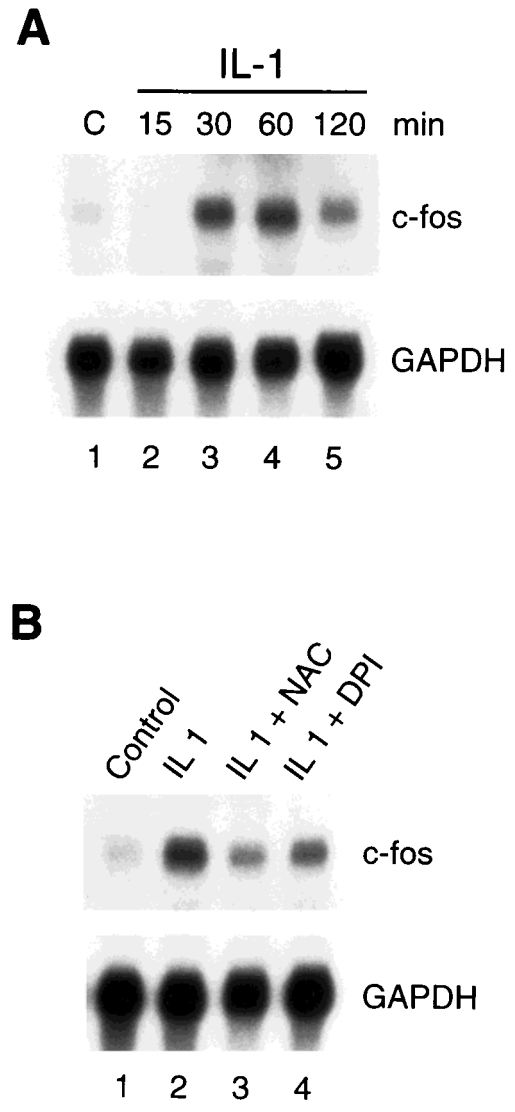
RESULTS

Regulation of c-fos Expression by IL-1 Can Be Inhibited by Blocking the Release or Activity of ROS

Treatment of chondrocyte cultures with IL-1 stimulates c-fos mRNA expression in a time-dependent manner. Induction of c-fos was detectable as early as 30 min after addition of IL-1 and the mRNA levels significantly decreased at around 2 h (Fig. 1A). Since it has been suggested that ROS may act as second messengers in cytokine signaling [Schreck et al., 1991; Lo and Cruz, 1995; Lo et al., 1996], antioxidants which can inactivate ROS would be expected to abolish induction of c-fos by IL-1. We therefore examined whether N-acetylcysteine (NAC), a potent antioxidant, can inhibit IL-1-induced c-fos expression. NAC acts by increasing the intracellular levels of glutathione which in turn neutralizes the activity of ROS [Halliwell and Gutteridge, 1990]. Chondrocytes were pretreated with NAC for 2 h before stimulating with IL-1 for 1 h. Induction of c-fos by IL-1 was significantly attenuated by NAC to 40% of the levels seen with IL-1 stimulation (Fig. 1B and C). Since flavonoid-containing enzymes such as NADPH oxidase are known to produce ROS, we used a specific pharmacological inhibitor of these enzymes, diphenyleneiodonium (DPI) [Stuehr et al., 1991], to determine if they play any role in mediating the effects of IL-1. Similar to NAC, pretreatment of chondrocytes with DPI for 30 min suppressed the induction of c-fos by IL-1 (Fig. 1B and C). NAC or DPI alone did not cause any changes in c-fos gene expression [data not shown, Lo and Cruz, 1995]. These results suggest that ROS released by flavonoid-containing enzymes are important second messengers in IL-1-induced c-fos expression in chondrocytes.

IL-1 Stimulates ROS Production in Bovine Chondrocytes

If ROS play the role of signaling intermediates for IL-1, their production should be increased upon IL-1 exposure. We therefore measured ROS production in bovine articular chondrocytes using dihydrorhodamine 123 (DHR) and fluorescence-activated cell sorting (FACS) analysis [Lund-Johansen and Olweus, 1992]. DHR is oxidized to the membrane-impermeable, fluorescent rhodamine 123 (R123) in the presence of ROS. Incubation of chondrocytes with IL-1 (20 ng/ml) for 20 min resulted in a rightward shift of fluorescence intensity over that of the control, indicating enhanced intracellular ROS production upon cytokine stimulation (Fig. 2). In duplicated experiments, cells treated with IL-1 for 20 min (mean fluorescence intensity = 369.59) had an average of 45% (SD = 3.6%) increase in mean fluorescence intensity compared with control cells (mean fluorescence intensity = 255.8). This amount of ROS increase is similar to that demonstrated for chondrocytes treated with another cytokine tumor necrosis factor α [Lo et al., 1995], and is about twice the amount seen in HeLa cells stimulated with lysophosphatidic acid [Chen et al., 1995]. These findings are also in agreement with other studies which demonstrated that chondrocytes could produce a variety of ROS including hydrogen peroxide and superoxide [Rathakrishnan et al., 1992; Tawara et al., 1991]. Furthermore, pretreatment with DPI inhibited IL-1-induced ROS production (Fig. 2), shifting the fluorescence profile back to the uninduced level. Taken together, this result demonstrated that IL-1 stimulates bovine chondrocytes to produce ROS and that flavonoid-containing enzymes participate in this process.



ROS Production is Involved in IL-1 Stimulation of Collagenase Gene Expression

As c-fos expression is a necessary requirement for the regulation of collagenase mRNA production [Schonthal et al., 1988], we asked if collagenase expression itself is subject to IL-1 stimulation. Enhanced collagenase expression was apparent only after 3 h of IL-1 stimulation. Maximal increase of collagenase mRNA levels was observed by 9 h and remained as high even 18 h after stimulation (Fig. 3A). The slow induction profile of collagenase reflects the requirement of new synthesis of protein factors such as that of c-Fos which is essential for collagenase expression. Hence, the induction pattern of c-fos and collagenase by IL-1 is in keeping with the findings that expression of the latter requires preinduction of the former. Knowing that inhibiting IL-1-induced ROS release suppressed c-fos induction (Figs. 1 and 2), similar analyses using pharmacological inhibitors were performed with respect to collagenase expression. Both DPI and NAC inhibited the IL-1 upregulation of collagenase mRNA levels by approximately 60% (Fig. 3B and C), suggesting that blocking the release and action of ROS is sufficient to repress expression of collagenase. NAC or DPI alone had no effect on collagenase mRNA levels (data not shown). These findings parallel those obtained for c-fos (Fig. 1) and argue that the inhibition seen with collagenase is a direct consequence of blockade of c-fos induction. Taken together, these findings, coupled with

Fig. 1. IL-1 induction of c-fos expression can be inhibited by NAC and DPI. **A:** Effect of IL-1 on c-fos mRNA expression. Chondrocyte cultures were stimulated with IL-1 (20 ng/ml) for various time periods as indicated to determine c-fos mRNA levels. **B:** Effects of DPI and NAC on c-fos induction by IL-1. Chondrocyte cultures were pretreated with DPI (5 μM) for 30 min or NAC (30 mM) for 2 h before the addition of IL-1 (20 ng/ml) for 1 h. Human recombinant IL-1 was dissolved in phosphate buffered saline with 0.1% bovine serum albumin. NAC was first dissolved in Ham's F-12 medium, then neutralized with sodium hydroxide. After treatment of cells with the indicated agents, they were washed in PBS twice before harvest. Total RNA from bovine articular chondrocytes was isolated and the c-fos mRNA levels were determined by Northern blot analysis as described under "Materials and Methods." The blots were subsequently stripped of DNA and re-probed with ^{32}P-labeled rat GAPDH cDNA. The results shown in (A) and (B) are representatives of two and three separate experiments respectively. **C:** Histogram representation of relative c-fos mRNA levels in experiments described in (B). The relative intensity of the bands was determined by densitometric scanning and results were expressed as the mean of three experiments (±S.D.)

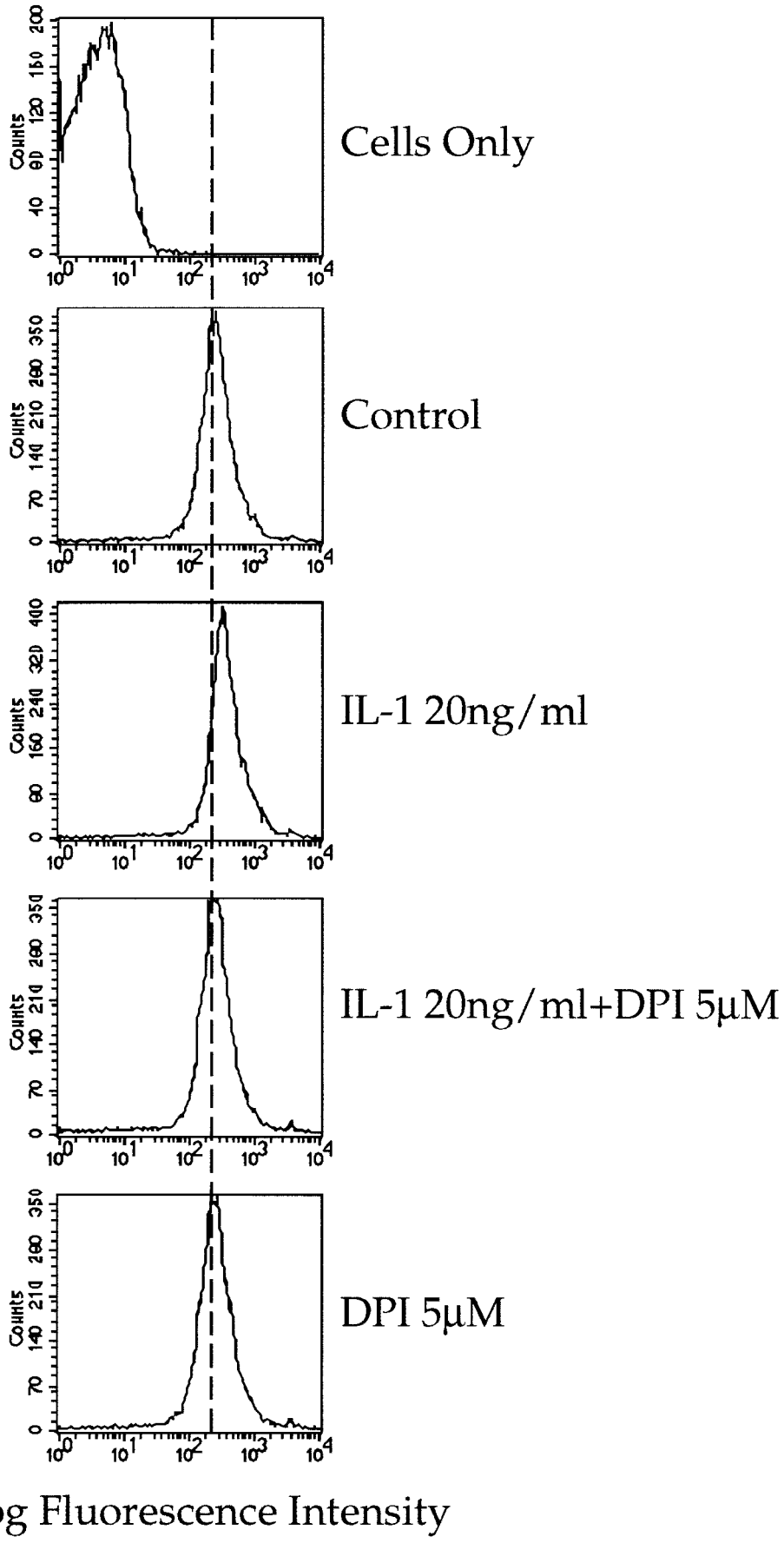


Fig. 2. Direct measurement of ROS production by chondrocytes. Cells were preincubated with DHR for 10 min in the presence or absence of DPI before the addition of IL-1 (20 ng/ml) for another 20 min. The mean fluorescence intensity of the sample treated with DHR alone was used as the control with an average value of 255.89. Average mean fluorescence intensity increased to 369.59 upon IL-1 treatment, representing a 45% (SD = 3.6%) increase over the control. The fluorescence intensities of 12,000 cells were analyzed.

the observation that ROS generation could be detected within 30 min after IL-1 stimulation (Fig. 2) before the first apparent increase in c-fos expression (Fig. 1A), lend further support to the importance of ROS in the IL-1 signaling pathway.

Role of Nitric Oxide in Mediating the Biological Effects of IL-1

IL-1 has been shown to stimulate the generation of another ROS, nitric oxide (NO) [Stadler et al., 1991], which can stimulate the activity of metalloproteinases such as collagenase and stromelysin in articular cartilage [Murrell et al., 1995]. NO has recently gained much recognition as an important physiologic signaling molecules with regulatory roles in the nervous, immune and cardiovascular systems [Bredt and Snyder, 1994]. Figure 4 showed that in our system, IL-1 can also stimulate the release of NO eight-fold. This increase was almost completely blocked by the specific NO synthase (NOS) inhibitor L-N^G-monomethylarginine (L-NMMA, Fig. 4). However, L-NMMA did not affect the constitutive levels of c-fos mRNA nor its induction by IL-1 (Fig. 5A). In agreement with this, we have previously demonstrated that the NO-donating agent, S-nitroso-N-acetylpenicillamine (SNAP), did not stimulate c-fos mRNA expression [Lo and Cruz, 1995]. This finding, however, does not exclude the possibility that NO may still regulate collagenase expression by other mechanisms. Indeed, pretreatment of chondrocytes with L-NMMA for two hours reproducibly caused a partial reduction of IL-1-induced collagenase mRNA levels (Fig. 5B and C). The extent of inhibition by L-NMMA as

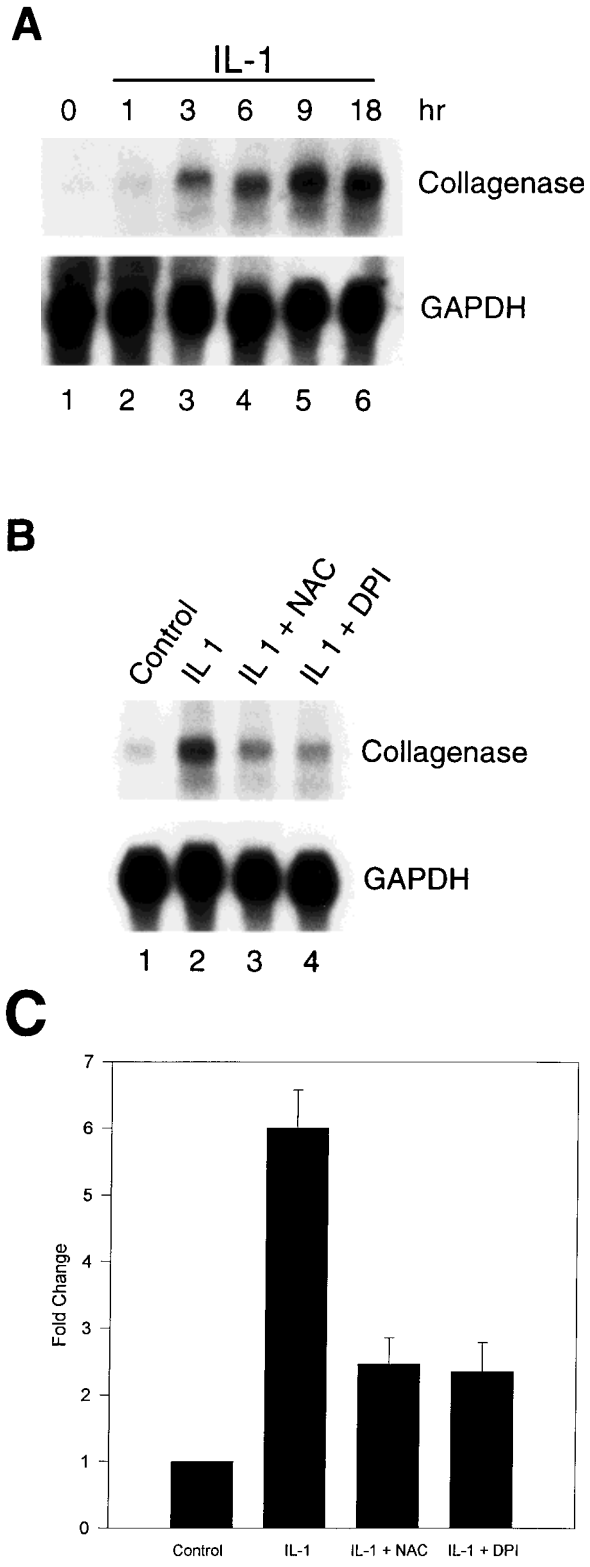


Fig. 3. Diphenyleneiodonium and N-acetylcysteine inhibit the induction of collagenase expression by IL-1. **A:** Time course of IL-1 induction of collagenase gene expression. Chondrocytes were treated with IL-1 (20 ng/ml) for the various time periods up to 18 h. RNA was isolated and Northern blot analysis was performed to examine collagenase mRNA expression. Measurement of GAPDH mRNA levels was as described under Figure 1. Similar results were obtained in two separate experiments. **B:** DPI and NAC inhibit IL-1-induced collagenase mRNA levels. After preincubation with DPI (5 μM; 30 min) or NAC (30 mM; 2 h), chondrocytes were then treated with IL-1 for 18 h. Collagenase and GAPDH mRNA levels were determined. The results are representatives of three independent experiments. **C:** Relative collagenase mRNA levels in experiments described in (B). Quantitation of densitometric scans is shown as means ± S.D., n = 3.

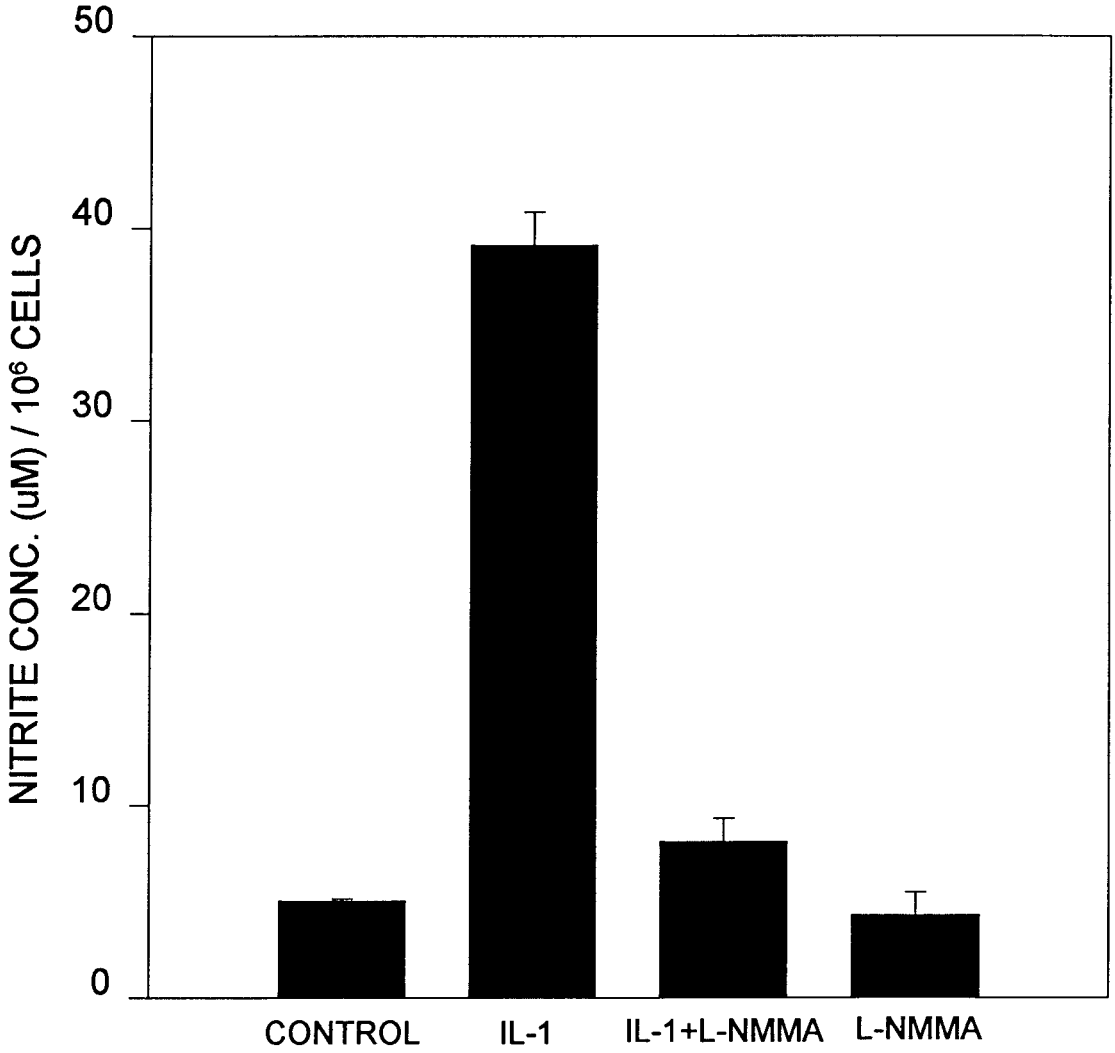


Fig. 4. Effect of L-NMMA on IL-1-induced nitric oxide production. Chondrocytes were first treated with 250 μM L-NMMA for 2 h prior to the addition of IL-1 (20 ng/ml). After 72 h of incubation, nitrite content was determined as described in "Materials and Methods." Values shown are means ± S.D. of a typical experiment done in triplicate.

measured by densitometric analysis was around 45%. Also, the NO-donating agent SNAP alone could stimulate collagenase expression in a time-dependent manner similar to that when IL-1 is used (Fig. 5D). Collectively, these results indicate that NO may play a role in the induction of collagenase expression by IL-1, independent of signaling pathways affecting c-fos mRNA levels.

DISCUSSION

In this study, we describe a potential signaling pathway by which IL-1 may stimulate c-fos and collagenase expression in chondrocytes. Our data strongly suggested that ROS form part of the signaling systems mediating the biological effects of IL-1 in these cells. The notion that ROS may act as signaling molecules has also gained attention recently because of the demonstration of their importance in mediating the effects of growth factors [Sundaresan et al., 1995; Lo and Cruz, 1995]. Moreover, oncogenic but not wild type versions of the oncogene Ras appear to stimulate constitutive production of ROS in cancer cells [Irani et al., 1997]. More importantly, inhibiting ROS production by NAC in these cells dampened their ability to exhibit uncontrolled proliferation [Irani et al., 1997].

The importance of ROS in IL-1 signaling does not imply it is the only pathway mediating the

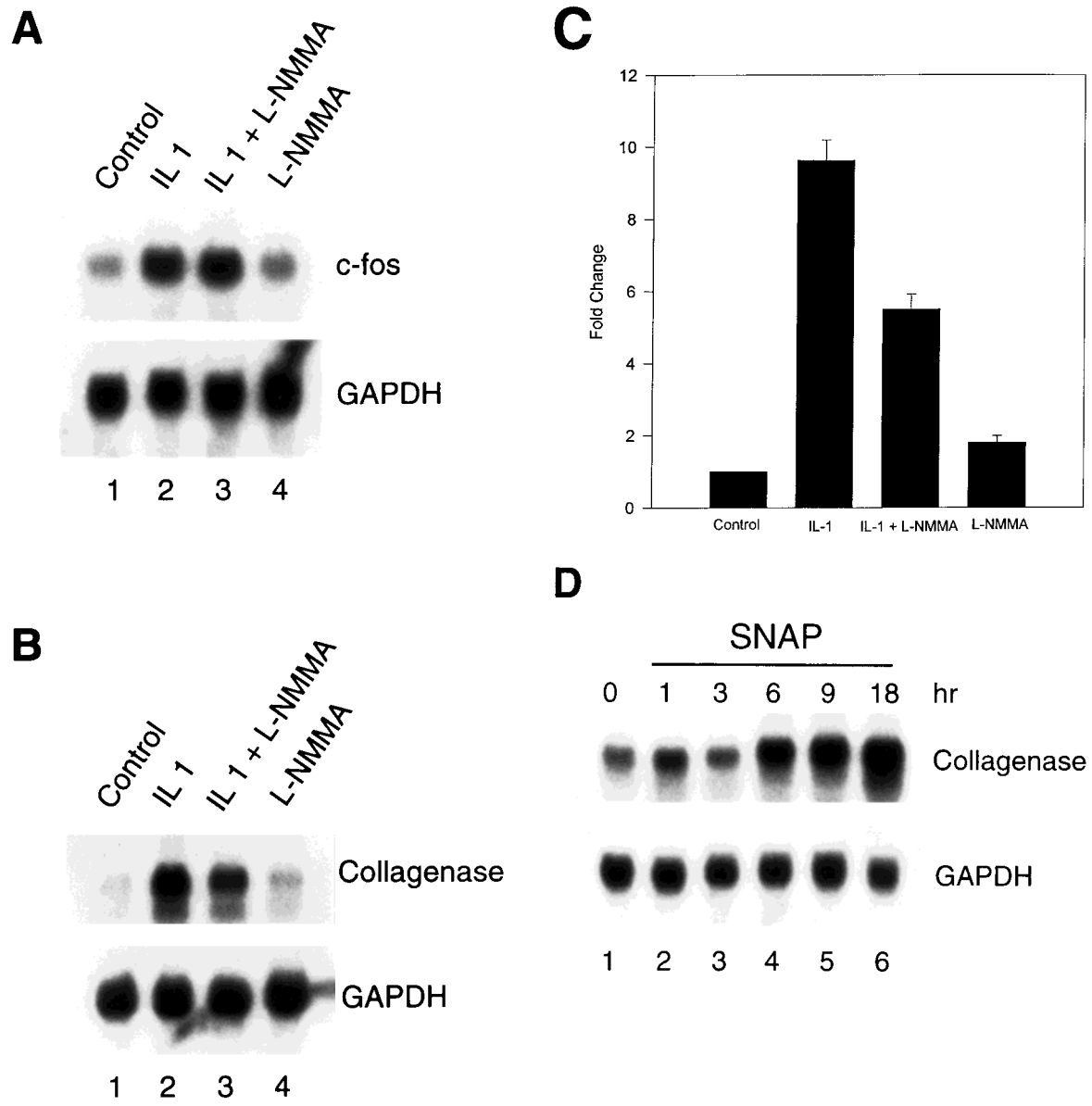


Fig. 5. Role of NO in the regulation of c-fos and collagenase expression. **A:** L-NMMA has no effect on IL-1-induced c-fos mRNA levels. Chondrocyte cultures were first incubated with L-NMMA (250 μM) for 2 h before adding IL-1 (20 ng/ml). After 1 h, total RNA was isolated and c-fos mRNA levels were determined as described in Figure 1. **B:** Effect of L-NMMA on collagenase induction by IL-1. Pretreatment of chondrocytes with L-NMMA (250 μM) for 2 h caused a partial reduction of IL-1-induced collagenase (18 h) mRNA levels. **C:** Quantitative analysis of collagenase expression in experiments described in (B). Data are expressed as means ± S.D., n = 3. **D:** Time course of SNAP induction of collagenase gene expression. Chondrocytes were stimulated with SNAP (100 μM) for various time periods as indicated to determine collagenase mRNA levels. The experiments in (A) and (D) were performed twice with similar results.

wide range of effects of the cytokine. Indeed, several signal transducers which can interact with the IL-1 receptor have recently been identified [Cao et al., 1996a; Malinin et al., 1997]. An IL-1-dependent protein kinase has also been cloned [Cao et al., 1996b]. Thus, many signaling systems might exist that couple IL-1 receptor to downstream targets depending on the ligands and cell types being studied.

Our studies implicated flavonoid-containing enzymes in the production of ROS. However, the mechanisms by which IL-1 stimulates flavonoid-containing enzymes and ROS production are not known. In previous studies, IL-1 was

found to stimulate phospholipase A_2, promoting release of arachidonic acid [Conquer et al., 1992; Chang et al., 1986]. Since arachidonic acid was found to activate NADPH oxidase to produce superoxide [Rubinek and Levy, 1993], it is possible that this fatty acid may serve as an intermediate in the activation of enzymes leading to the production of ROS.

Although NO did not upregulate c-fos expression in chondrocytes [Lo and Cruz, 1995], inhibition of its production using a specific NOS inhibitor partially reduced the IL-1 induction of collagenase expression. Also, treatment of chondrocytes with a NO-donating agent is able to stimulate collagenase gene expression. One possible explanation is that NO may directly modulate AP-1 activity through post-translational modification of AP-1 proteins, without affecting their mRNA expression. Interestingly, Fos and Jun proteins are indeed subject to a novel form of regulation termed redox regulation in which oxidation-reduction of a key cysteine residue modulates DNA binding activity [Abate et al., 1990]. Similar redox regulation has also been demonstrated with Ras [Lander et al., 1995]. In this case, modulation of Ras activity by NO occurs through direct nitrosylation of a critical cysteine residue [Lander et al., 1996]. Whether NO can affect AP-1 activity through similar mechanism awaits further experimentation.

Our findings indicate that blocking ROS production or decreasing ROS levels using DPI and NAC respectively, can inhibit IL-1-induced c-fos and collagenase expression in chondrocytes. Not only do these data further strengthen our hypothesis that ROS are essential second messengers in IL-1 induction of c-fos and collagenase expression, but they are also in keeping with the fact that antioxidants such as vitamin C, E, β-carotene, and selenium have proved useful in the treatment of arthritis [Deucher, 1992]. Although it has been shown that ROS can directly destroy the biochemical integrity of proteoglycan, hyaluronic acid and collagen [Greenwald and Moy, 1979; Greenwald and Moy, 1980], this study suggests that another potential means of cartilage destruction could be the secondary consequence of ROS-induced production of collagenase. Finally, the inhibitory effect of L-NMMA on collagenase expression suggests that the nitric oxide synthase pathway may provide another therapeutic target for the treatment of arthritis and other joint diseases. Indeed, employing rats with induced arthritis, two studies showed that inhibition of NO synthesis profoundly reduced the severity of the disease or even conferred a protective effect [Stefanovic-Racic et al., 1994; McCartney-Francis et al., 1993]. In particular, Stefanovic-Racic et al. showed that even a modest decrease in NOS activity can have a large protective effect on the joint [Stefanovic-Racic et al., 1994]. In view of this study, the success of blocking NOS in treating arthritis may be due partly to the reduction of collagenase expression in addition to obliterating the cytotoxic effects mediated by NO. Taken together, specific NOS inhibitors and antioxidants may have a place in battling the chronic debilitating disease arthritis.

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