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The ROS hydrogen peroxide (H$_2$O$_2$) to the chondrocyte activation of JNK is also observed by direct addition of can act as mediators of JNK activity. Moreover, potent activation of JNK provides strong evidence that ROS reactants species (ROS), which have been suggested to act as second messengers. Here we demonstrate that ROS production by bovine chondrocytes upon cytokine stimulation induces c-jun expression. Since c-jun expression is regulated by its own gene product via phosphorylation by c-j un NH$_2$-terminal kinases (J NKS), we investigated if cytokines and ROS could modulate J NK activity in chondrocyte monolayer cultures. Treatment of bovine chondrocytes with both IL-1 and TNF$\alpha$ leads to rapid induction of J NK activity, stimulating J NK activity 7- and 20-fold, respectively. Importantly, the observation that antioxidant treatment antagonizes IL-1 and TNF$\alpha$ activation of J NK provides strong evidence that ROS can act as mediators of J NK activity. Moreover, potent activation of J NK is also observed by direct addition of the ROS hydrogen peroxide (H$_2$O$_2$) to the chondrocyte cultures. Nitric oxide (NO), a multifunctional ROS, also appears to simulate J NK, albeit to a lesser extent. These findings identify J NK as another molecular target for the actions of NO and H$_2$O$_2$. In addition, the inhibitory effect of diphenyleneiodonium on J NK activation implicates the involvement of flavonoid-containing enzymes in the ROS-mediated signaling process. Overstimulation of J NK activity by excessive production of ROS may, therefore, underlie pathological conditions such as arthritis and cancer.

Interleukin 1 (IL-1) and tumor necrosis factor $\alpha$ (TNF$\alpha$) are known to induce production of reactive oxygen species (ROS), which have been suggested to act as second messengers. Here we demonstrate that ROS production by bovine chondrocytes upon cytokine stimulation induces c-jun expression. Since c-jun expression is regulated by its own gene product via phosphorylation by c-j un NH$_2$-terminal kinases (J NKS), we investigated if cytokines and ROS could modulate J NK activity in chondrocyte monolayer cultures. Treatment of bovine chondrocytes with both IL-1 and TNF$\alpha$ leads to rapid induction of J NK activity, stimulating J NK activity 7- and 20-fold, respectively. Importantly, the observation that antioxidant treatment antagonizes IL-1 and TNF$\alpha$ activation of J NK provides strong evidence that ROS can act as mediators of J NK activity. Moreover, potent activation of J NK is also observed by direct addition of the ROS hydrogen peroxide (H$_2$O$_2$) to the chondrocyte cultures. Nitric oxide (NO), a multifunctional ROS, also appears to simulate J NK, albeit to a lesser extent. These findings identify J NK as another molecular target for the actions of NO and H$_2$O$_2$. In addition, the inhibitory effect of diphenyleneiodonium on J NK activation implicates the involvement of flavonoid-containing enzymes in the ROS-mediated signaling process. Overstimulation of J NK activity by excessive production of ROS may, therefore, underlie pathological conditions such as arthritis and cancer.

Interleukin 1 (IL-1)$^3$ and tumor necrosis factor $\alpha$ (TNF$\alpha$) are multifunctional cytokines involved in inflammation, cell growth, and apoptosis (1–4). The pleiotropic effects of these two cytokines are mediated through distinct cell surface receptors (2, 3, 5). Despite the incomplete understanding of the membrane signaling events following the occupancy of the cytokine receptors, ligand interaction is known to stimulate the release of a wide variety of putative second messengers. Some of the known signal transduction pathways common to both IL-1 and TNF$\alpha$ include coupling to G-proteins (6, 7), activation of phospholipase A$_2$ (8, 9), calcium mobilization (10, 11), and ceramide production (12, 13).

In addition to the above messengers, a class of highly diffusible and ubiquitous molecules termed reactive oxygen species (ROS) has recently been recognized to act as signaling intermediates for cytokines, including IL-1 and TNF$\alpha$ (14–16). ROS encompass species such as superoxide, hydrogen peroxide (H$_2$O$_2$), nitric oxide (NO), and hydroxyl radicals (17). These highly reactive molecules are known to regulate many important cellular events, including gene expression (16, 18, 19), transcription factor activation (20), DNA synthesis (21), and cellular proliferation (22).

The signal transduction cascades elicited after exposure to IL-1 and TNF$\alpha$ culminate in a nuclear response characterized by the activation of several key transcriptional regulators, including nuclear factor $\kappa$B (20, 23) and AP-1 (16, 24). Hence, genes with an AP-1 binding site, such as those encoding the metalloproteinases collagenase and stromelysin, are potential targets for the two cytokines (25, 26). AP-1 is composed of the protein products of c-fos and c-jun; their levels of gene expression are also stimulated by cytokines (24). Posttranslational modification of AP-1 transcriptional activity also plays an important role in the control of AP-1-regulated gene expression. Such control is executed in the form of phosphorylation of Fos and J un proteins by members of the mitogen-activated protein kinase family (27–30).

The transcriptional activity of the c-j un protein is greatly enhanced by phosphorylation of two serine residues at positions 63 and 73 in its activation domain (31, 32). Phosphorylation of the NH$_2$ terminus of c-j un is catalyzed by c-j un NH$_2$-terminal kinases (J NKS), a subgroup of the mitogen-activated protein kinase family (28–30). The properties of J NKS that distinguish them from other mitogen-activated protein kinases, such as extracellular signal-related kinases, include their activation by agents such as ultraviolet irradiation, heat shock, protein synthesis inhibitors, and inflammatory cytokines (33, 34). Since many of the J NK activators can be regarded as cellular stress, J NKS have also been termed stress-activated protein kinases (30). Inflammatory cytokines, such as TNF$\alpha$ and IL-1, have been shown to induce J NKS (35) and c-j un expression (2, 24, 26, 36); however, the second messengers responsible for their activation remain unidentified. Our earlier study demonstrated ROS production upon cytokine stimulation in bovine chondrocytes (16); thus, we examined the molecular links between cytokine-induced ROS generation and its downstream signaling events.

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The abbreviations used are: IL-1, interleukin 1; TNF$\alpha$, tumor necrosis factor $\alpha$; ROS, reactive oxygen species; J NK, c-j un NH$_2$-terminal kinase; NAC, N-acetylcysteine; Asc, ascorbic acid; SNAP, S-nitroso-N-acetylpenicillamine; DPI, diphenyleneiodonium; GAPDH, glyceraldehyde phosphate dehydrogenase; WCE, whole cell extract; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; S-P, solid-phase.

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<sup>2</sup> Y. Y. C. Lo, unpublished observations.
Materials and Methods

Reagents—Human recombinant IL-1β was generously supplied by Ciba-Geigy, Basel, Switzerland. Human recombinant TNF-α was from Genzyme Corp. H2O2 was from Fisher Scientific. N-Acetylcyesteine (NAC) and ascorbic acid (Asc) were from Sigma. S-Nitroso-N-acetylpenicillamine (SNAP) was purchased from Biomol Research Laboratories. Diphenylethenone (DPI) was from Toronto Research Chemicals. Radioactive isotopes and x-ray films were from DuPont NEN. Glutathione-Sepharose was obtained from Pharmacia Biotech. Purified anti-human p46 JNK monoclonal antibody was purchased from PharMingen. This antibody recognizes both the M, 46,000 JNK1 and the related M, 55,000 JNK2 proteins.

Cell Culture—Primary cultures of bovine articular chondrocytes were isolated from bovine articular cartilage as described by Cruz et al. (37). Bovine chondrocytes were plated at 2 × 10⁶ cells/ml in 12 ml of Ham's F-12 media containing 3% antibiotics and 5% fetal bovine serum. The cells were allowed to recover for 24 h at 37 °C in a humidified atmosphere supplemented with 5% CO2. Prior to any treatments, chondrocytes were serum-starved overnight.

Northern Blot Analysis—Total RNA was isolated by the acidified guanine isothiocyanate method (38) and quantitated by spectrophotometry at 260 nm. Denatured RNA samples (10–15 μg) were analyzed by gel electrophoresis in a 1% denaturing agarose gel, transferred to a nylon membrane (Bio-Rad), and cross-linked with an ultraviolet cross-linker (Stratagene UV Stratalinker 1800). The blots were hybridized with 32P-labeled human c-jun cDNA, subsequently stripped, and re-probed with 32P-labeled rat glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA as internal control.

Whole Cell Extract (WCE) Kinase Assay—Following treatment of chondrocytes with various agents, cells were washed twice with phosphate-buffered saline and harvested according to published procedures by Hirota et al. (29). Briefly, the cells were suspended in WCE buffer and rotated at 4 °C for 30 min. The supernatant was collected as WCE after centrifugation at 10,000 g for 30 min. The supernatant was then centrifuged at 100,000 g for 30 min. The resulting supernatant was diluted according to the procedure described by Hirota et al. (29). Extract containing 100–300 μg of protein was added to 10 μl of glutathione-Sepharose suspension prebound with 20 μg of GST-c-Jun or GST. After being centrifuged at 10,000 × g for 10 min, the pellet eluted in Laemmli buffer. Following the addition of protein to an equal volume of Laemmli buffer and boiling for 5 min, the proteins were then subjected to SDS-PAGE, followed by drying, autoradiography, and quantitation by phosphorimaging analysis.

Solid-phase (S-P) Kinase Assay—WCE was diluted according to the procedures described by Hirota et al. (29). Extract containing 100–300 μg of protein was added to 10 μl of glutathione-Sepharose suspension prebound with 20 μg of GST-c-Jun or GST. The mixture was rotated at 4 °C for 3 h and centrifuged at 10,000 × g for 20 s. The pellet eluted in Laemmli buffer was washed with buffer (20 μl HEPES, pH 7.7, 5.0 mM NaCl, 2.5 mM MgCl2, 0.1 mM EDTA, 0.05% Triton X-100, 0.5 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride) and then resuspended in 30 μl of kinase buffer (20 μl HEPES, pH 7.6, 20 mM MgCl2, 2 mM dithiothreitol, 25 mM β-glycerophosphate, and 0.1 mM Na2VO4) containing 20 μM ATP and 5 μCi of [γ-32P]ATP. After being incubated at 30 °C for 20 min, the reaction was terminated by boiling in Laemmli buffer. Samples containing phosphorylated proteins were first resolved by SDS-PAGE, followed by drying, autoradiography, and quantitation by phosphorimaging analysis.

Results and Discussion

In our previous studies, we have implicated the involvement of ROS in TNF-α- and IL-1-mediated nuclear response, such as the induction of c-fos gene expression (16). Not only were antioxidants shown to inhibit cytokine induction of c-fos, we and others have also demonstrated enhanced expression of c-fos expression by exogenous addition of H2O2 in bovine chondrocytes, Hela cells, and osteoblasts (16, 18, 19). In view of this, we decided to examine the effects of IL-1, TNF-α, and ROS on another early response gene c-jun, of which the protein product heterodimerizes with Fos to form the AP-1 transcription factor (24). Time course analyses showed that IL-1 and TNF-α caused a transient increase in the steady-state c-jun mRNA levels with optimal expression at around 30 min (Fig. 1A). To assess the relevance of ROS as putative second messengers in the induction of c-jun by IL-1 and TNF-α, we tested if endogenous ROS were required in cytokine-mediated c-jun regulation. To do this, chondrocytes were first treated with the antioxidants, NAC and Asc, before the addition of cytokines. Both NAC and Asc are effective free radical scavengers; the former is known to increase intracellular glutathione levels, which in turn modulate the concentration of ROS via glutathione peroxidase (17). On the other hand, Asc itself is highly reactive toward radicals and has proven to be a versatile scavenger (39). NAC and Asc, which neutralize the activities of ROS, reduced both IL-1- and TNF-α-induced c-jun mRNA levels (Fig. 1B), implicating ROS in the signaling cascade leading to c-jun induction.

Next we asked whether exogenous addition of ROS could mimic the effects of IL-1 and TNF-α with respect to induction of c-jun. Hydrogen peroxide, a membrane-permeable reagent physiologically produced in large amounts by cells such as granulocytes (17), has been widely used to assess the effects of ROS. Hydrogen peroxide was found to activate c-jun gene expression with induction first apparent at 30 min (Fig. 1C), whereas the expression of the housekeeping gene GAPDH was not affected. The induction of c-jun mRNA expression by H2O2 was transient and is similar to the c-jun induction profiles obtained with TNF-α and IL-1. These data provide further evidence that ROS can indeed act as second messengers in signaling c-jun expression in chondrocyte cultures.

The findings that ROS are involved in inducing expression of c-jun prompted us to investigate the underlying mechanisms. Interestingly, the c-jun promoter contains two AP-1-like sequences termed JUN1 and JUN2 (18, 40, 41). Each of these sites is bound by a heterodimer of c-jun in association with another transcription factor, ATF2. Hence, the c-jun promoter is autoregulated by its own gene product (40, 41). Activation of the c-jun promoter involves phosphorylation of the prebound c-jun-ATF2 heterodimers by JNK/stress-activated protein kinases, which are members of the mitogen-activated protein kinase family (28–30). Phosphorylation of serine-63 and serine-73 in the activation domain of c-jun increases its activation potential (31, 32). These findings, coupled with our analyses of c-jun induction described above, led us to hypothesize that JNKs in chondrocytes might also be regulated by cytokines with ROS as activating intermediates. WCE and S-P assays were used to examine JNK activity. Following the treatment of chondrocytes with various agents, a whole cell lysate was prepared by lysis with detergent. This extract could be assayed directly with a GST-c-jun fusion protein as substrate in the WCE assay. Alternatively, in the S-P assay, the extract was first incubated with the GST-c-jun fusion protein to allow...
Activity. Treatment with IL-1 and TNFα reaction. We first looked at the effects of cytokines on JNK c-Jun-specific kinases now acted as a substrate in the kinase radioisotopes. The GST-c-Jun fusion protein used to bind the washed off, and kinase activity was analyzed in the presence of binding of endogenous JNKs. Then, unbound proteins were probed with 32P-labeled rat Northern blot analysis. The blots were subsequently stripped and regular chondrocytes, and the c-Jun mRNA levels. Chondrocyte cultures were preincubated with NAC (30 mM) or Asc (100 mM) for 2 h before the addition of IL-1 (35 ng/ml) or TNFα (30 ng/ml) for 30 min. Human recombinant TNFα and IL-1 were dissolved in phosphate-buffered saline with 0.1% bovine serum albumin. C, hydrogen peroxide stimulates c-Jun gene expression in chondrocytes. Primary chondrocyte cultures were treated with 300 μM H2O2 at various time points as indicated. Cells were washed in phosphate-buffered saline twice. Total RNA was then isolated from bovine articular chondrocytes, and the c-Jun mRNA levels were determined by Northern blot analysis. The blots were subsequently stripped and reprobed with 32P-labeled rat GAPDH cDNA. The housekeeping gene GAPDH was used as an internal control to show similar loading of RNA. The results shown are representative of three independent experiments, with the exception of the time course experiment with cytokine treatments, which was performed twice.

binding of endogenous JNKs. Then, unbound proteins were washed off, and kinase activity was analyzed in the presence of radiolabels. The GST-c-jun fusion protein used to bind the c-jun-specific kinases now acted as a substrate in the kinase reaction. We first looked at the effects of cytokines on JNK activity. Treatment with IL-1 and TNFα potently activated JNK activity, as measured by phosphorylation of the substrate GST-c-jun in WCE assay (Fig. 2A). Phosphorylation was observed as early as 15 min and gradually decreased over the course of 2 h. Phosphorimaging analysis showed that maximum fold induction of JNK activities by IL-1 and TNFα were 7- and 20-fold, respectively. The S-P assay in which c-jun-specific kinases were first bound to the substrate gave essentially the same results. This indicated that the phosphorylation signals seen in the "whole cell extract" assay were indeed from c-jun-specific kinases. As a control, phosphorylation was not observed with the GST moiety alone as substrate (data not shown). We again exploited the abilities of antioxidants to perturb intracellular ROS levels, to assess the role of endogenous ROS in regulating the c-jun un kinases. Antioxidant treatment antagonized the stimulating effects of IL-1 and TNFα on c-jun kinase activity (Fig. 2B), indicating that in vivo ROS production does play a role in modulating JNK activity. Next, we tested the effects of two ROS, H2O2 and NO generated by SNAP, on the activity of JNK. The ROS NO has recently gained substantial recognition as a signaling molecule with the property of both neurotransmitter and hormone (42). NO production by many cell types, including chondrocytes, is also significantly enhanced in the presence of cytokines (16, 43). Fig. 2C clearly shows that the direct addition of H2O2 dramatically activated JNK. Interestingly, NO also stimulated the kinase activity, albeit to a much lesser extent. Both NO and H2O2 stimulated JNK after 15 min treatment (Fig. 2C). Signal quantitation indicated that the maximum fold-activation of JNK activity by H2O2 was around 8-fold. These data provided the first direct evidence that ROS can modulate the activity of JNK.

FIG. 1. Effects of cytokines, antioxidants and H2O2, on c-jun mRNA expression. A, time courses of IL-1 and TNFα stimulation of c-jun gene expression. Bovine chondrocyte cultures were treated with either TNFα (30 ng/ml) or IL-1 (35 ng/ml) for various time periods as indicated. B, effects of antioxidants and IL-1- and TNFα-induced c-jun mRNA levels. Chondrocyte cultures were preincubated with NAC (30 mM) or Asc (100 μM) for 2 h before the addition of IL-1 (35 ng/ml) or TNFα (30 ng/ml) for 30 min. Human recombinant TNFα and IL-1 were dissolved in phosphate-buffered saline with 0.1% bovine serum albumin. C, hydrogen peroxide stimulates c-jun gene expression in chondrocytes. Primary chondrocyte cultures were treated with 300 μM H2O2 at various time points as indicated. Cells were washed in phosphate-buffered saline twice. Total RNA was then isolated from bovine articular chondrocytes, and the c-jun mRNA levels were determined by Northern blot analysis. The blots were subsequently stripped and reprobed with 32P-labeled rat GAPDH cDNA. The housekeeping gene GAPDH was used as an internal control to show similar loading of RNA. The results shown are representative of three independent experiments, with the exception of the time course experiment with cytokine treatments, which was performed twice.

FIG. 2. Regulation of JNK activity by cytokines with ROS as signaling intermediates. A, effects of the cytokines IL-1 and TNFα on JNK activity. Chondrocytes were treated with IL-1 (35 ng/ml) or TNFα (30 ng/ml) for various time periods as shown. Similar results were obtained in two separate experiments. B, the antioxidants, NAC and Asc, attenuate IL-1 and TNFα stimulation of JNK activity. Pretreatment of chondrocyte cultures with NAC (30 mM) or Asc (100 μM) for 2 h was followed by the addition of IL-1 (35 ng/ml) or TNFα (30 ng/ml) for 30 min. The experiment was repeated three times. C, time courses of H2O2 and NO activation of JNK. Chondrocytes were incubated with either H2O2 (300 μM) or SNAP (100 μM) for different time points, and whole cell lysates were isolated for JNK activity determination. SNAP, which evokes NO upon dissolving, was prepared in ethanol immediately before use and was directly added to culture medium. Extracts were used to determine JNK activity by both the WCE assay and the S-P assay as described under "Materials and Methods." The data shown are representative of three independent experiments.

We again exploited the abilities of antioxidants to perturb intracellular ROS levels, to assess the role of endogenous ROS in regulating the c-jun un kinases. Antioxidant treatment antagonized the stimulating effects of IL-1 and TNFα on c-jun kinase activity (Fig. 2B), indicating that in vivo ROS production does play a role in modulating JNK activity. Next we tested the effects of two ROS, H2O2 and NO generated by SNAP, on the activity of JNK. The ROS NO has recently gained substantial recognition as a signaling molecule with the property of both neurotransmitter and hormone (42). NO production by many cell types, including chondrocytes, is also significantly enhanced in the presence of cytokines (16, 43). Fig. 2C clearly shows that the direct addition of H2O2 dramatically activated JNK. Interestingly, NO also stimulated the kinase activity, albeit to a much lesser extent. Both NO and H2O2 stimulated JNK after 15 min treatment (Fig. 2C). Signal quantitation indicated that the maximum fold-activation of JNK activity by H2O2 was around 8-fold. These data provided the first direct evidence that ROS can modulate the activity of JNK.

One class of enzymes that are known to give rise to various
Chondrocyte cultures were preincubated with DPI (5 μM) for 30 min before the addition of IL-1 (35 ng/ml) or TNFα (30 ng/ml) for 30 min. Whole cell lysates were prepared by lysis with detergent. Extracts were used to determine JNK activity by both the WCE assay and S-P assay as described under "Materials and Methods." Three separate experiments were performed with similar results.

Types of ROS is the flavonoid-containing enzymes. Therefore, we examined the effect of DPI, a potent inhibitor of flavonoid-containing enzymes, such as NADPH oxidase and nitric oxide synthase (44), on IL-1 and TNFα induction of JNK activity. As with antioxidants, the addition of DPI also attenuated cytokine stimulation of JNK activity (Fig. 3). In bovine chondrocytes, DPI has previously been shown to inhibit IL-1 and TNFα stimulation of intracellular ROS production (16). Hence, the above findings are in keeping with a role for ROS production by flavonoid-containing enzymes in the cytokine stimulation of JNK activity.

Since JNK phosphorylation of c-Jun is critical for its transcriptional activity (28-32) and c-Jun autoregulates its own promoter (40, 41), our observation of enhanced JNK activity upon exposure to cytokines is consistent with the cytokine induction of c-Jun mRNA levels in chondrocytes. The exact pathway from ROS release to JNK activation has yet to be identified. Our finding that DPI inhibits cytokine stimulation of JNK implicates the involvement of flavonoid-containing enzymes such as NADPH oxidase. This is particularly noteworthy considering that one of the critical components of NADPH oxidase, Rac1, has been shown recently to be an upstream regulator of JNK (45-47). Perhaps the enhanced activity of the enzyme NADPH oxidase in the presence of Rac1 gives rise to increased ROS levels, which then initiates a kinase cascade culminating in the activation of JNK. The cellular mechanism of ROS sensing and the identity of the direct sensor molecules await further characterization. JNKs themselves do not appear to be the direct targets of ROS because both H2O2 and SNAP could not activate JNKs immobilized onto GST-c-Jun glutathione beads (data not shown). Nonetheless, intracellular ROS sensing may occur through the redox regulation of sensor molecules. Such form of regulation has been demonstrated for the transcription factors Fos and Jun in which oxidation-reduction of a key cysteine residue modulates DNA binding activity (48). Furthermore, similar redox modification may also underlie direct modulation of p21waf1 activity by ROS such as H2O2 and NO (49, 50).

Two JNK isoforms (M, 46,000 JNK1 and M, 55,000 JNK2) have been identified in HeLa cells (29); both of them are capable of phosphorylating the JNK activation domain. To distinguish the different isoforms in bovine chondrocytes, we used a S-P in-gel kinase assay to determine the sizes of JNKs being activated upon cytokine stimulation. After the chondrocytes were treated with IL-1 or TNFα in the presence or absence of the antioxidant NAC, whole cell extracts were isolated and first incubated with GST-c-Jun un-glutathione beads to selectively bind JNK un-specific kinases. Then protein kinase activities were examined following SDS-PAGE on gels that were polymerized with either GST or GST-c-Jun un. After electrophoresis, the proteins were renatured in the gel and incubated in kinase buffer in the presence of [γ-32P]ATP, as indicated under "Materials and Methods." The results are representative of three independent experiments.

Western blot analysis was also used to definitively identify the c-Jun-associating kinases in primary chondrocytes. We used a purified anti-human JNK1 monoclonal antibody that also cross-reacts with human JNK2, as seen in Fig. 5, lane 1, with HeLa whole cell extract. The anti-human JNK1 antibody reacted with two proteins having identical sizes as human JNK1 and JNK2 in chondrocyte extract (Fig. 5, lanes 1 and 2). Although the JNK2 band was less prominent than the JNK1 band in the chondrocyte extract, lane 2 indicates only that both JNK1 and JNK2 are present in chondrocytes and does not necessarily reflect the relative amounts of each c-Jun un kinase. Interestingly, the JNK1 protein band appears as a doublet in extracts stimulated with either IL-1 or TNFα (Fig. 5, lanes 3-5). The upper band of the doublet probably corresponds to phosphorylated, and thus activated, form of JNK1 (51). It is interesting to note that the JNK2 band was less prominent than the JNK1 band in the chondrocyte extract, lane 2 indicates only that both JNK1 and JNK2 are present in chondrocytes and does not necessarily reflect the relative amounts of each c-Jun un kinase.
known that unactivated JNKs can associate with c-Jun; however, activation leads to enhanced binding (51). In this study, JNK2 was barely detectable by this antibody, probably due to poor cross-reactivity of the antibody toward the bovine counterpart of human JNK2 or lower amount of bound JNK2, as suggested by the S-P in-gel kinase experiment, or both (Fig. 4). The relatively weak interaction of JNK2 with c-Jun seen in our chondrocyte system is also consistent with similar findings in human cells (29). Taken together, this study demonstrates that ROS is a link between cytokine-receptor interaction and activation of the JNK cascade. Any hyperproduction of ROS may become more important in pathological conditions in which an abnormally high concentration of cytokines leads to increased NO production (42, 54). Overstimulation of JNK activity by cytokine-stimulated ROS production (e.g. NO and H2O2) may cause deregulation of c-Jun expression. The c-Jun protein product, as a key component of AP-1, may then induce inappropriate transcriptional responses, leading to overexpression of metalloproteinases and uncontrolled cellular proliferation. Thus, abnormal activation of the JNK cascade may underlie disease conditions such as arthritis and cancer, which often are characterized by overproduction of ROS and metalloproteinases (54–57).

Fig. 5. Western blot analysis of JNK polypeptides associated with GST-c-Jun. Chondrocyte cultures were treated with IL-1 (35 ng/ml) or TNFα (30 ng/ml) for 30 min. Whole cell extracts were used either directly or first incubated with GST-c-Jun prior to SDS-PAGE. Lanes 1 and 2, untreated HeLa and chondrocyte whole cell extracts, respectively. Samples from lanes 3–5 represent proteins previously bound to GST-c-Jun and eluted by boiling in Laemmli buffer. Lane 3, unstimulated chondrocyte extract; lane 4, IL-1-treated chondrocyte extract; lane 5, TNFα-treated chondrocyte extract. After SDS-PAGE, the resolved proteins were transferred to Immobilon-P membrane, blotted with anti-JNK1 polyclonal antibodies, and detected by enhanced chemiluminescence. The experiment was performed twice with essentially identical results.
Reactive Oxygen Species Mediate Cytokine Activation of c-Jun NH$_2$-terminal Kinases
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