p38 Kinase Regulates Nitric Oxide-induced Apoptosis of Articular Chondrocytes by Accumulating p53 via NFκB-dependent Transcription and Stabilization by Serine 15 Phosphorylation*

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Song-Ja Kim‡, Sang-Gu Hwang, Deug Y. Shin§, Shin-Sung Kang¶, and Jang-Soo Chun||

From the Department of Life Science, Kwangju Institute of Science and Technology, Gwangju 500-712, Korea, ‡Department of Microbiology, Dankook University College of Medicine, Cheonan 330-714, Korea, and §Department of Biology, Kyungpook National University, Daegu 702-701, Korea

Nitric oxide (NO) during primary culture of articular chondrocytes causes apoptosis via p38 mitogen-activated protein kinase in association with elevation of p53 protein level, caspase-3 activation, and differentiation status. In this study, we characterized the molecular mechanism by which p38 kinase induces apoptosis through activation of p53. We report here that NO-induced activation of p38 kinase leads to activation of NFκB, which in turn induces transcription of the p53 gene. Activated p38 kinase also physically associates and phosphorylates the serine 15 residue of p53, which results in accumulation of p53 protein during NO-induced apoptosis. Ectopic expression of wild-type p38 enhanced NO-induced apoptosis, whereas expression of a dominant negative p38 blocked it, indicating that p38 plays an essential role in NO-induced apoptosis of chondrocytes. The increased accumulation of p53 caused expression of Bax, a pro-apoptotic member of the Bcl-2 family that is known to cause apoptosis via release of cytochrome c and caspase activation. These results suggest that NO-activated p38 kinase activates p53 function in two different ways, transcriptional activation by NFκB and direct phosphorylation of p53 protein, leading to apoptosis of articular chondrocytes.

Nitric oxide (NO) production via inducible NO synthase in chondrocytes plays a central role in degenerative diseases of cartilage, for example osteoarthritis and rheumatoid arthritis (1, 2). Although NO-induced cartilage destruction can be caused by various ways, the increased apoptotic cell death of articular chondrocytes appears to be an important contributor (3–6). However, the signaling pathway leading to NO-induced apoptosis of chondrocyte is poorly understood. In other cell types, it has been shown that subtypes of mitogen-activated protein kinase play an important role in NO-induced apoptosis. For example, NO-induced apoptosis of neuronal progenitor cells is mediated by p38 kinase (7), whereas extracellular signal-regulated kinase and c-Jun N-terminal kinase but not p38 kinase are involved in NO-induced apoptosis of cardiomyocytes (8). Our recent study demonstrated that apoptosis of chondrocyte apoptosis caused by direct production of NO with the NO donor sodium nitroprusside (SNP)1 is regulated by opposite functions of two mitogen-activated protein kinase subtypes, extracellular signal-regulated kinase 1/2 and p38 kinase, in association with the elevation of p53 protein level, caspase-3 activation, and differentiation status (9). SNP treatment stimulated activation of both extracellular signal-regulated kinase 1/2 and p38 kinase. The activated extracellular signal-regulated kinase 1/2 plays a role as an inducing signal for dedifferentiation and an inhibitory signal for NO-induced apoptosis, whereas p38 kinase functions as a signal for the maintenance of the differentiated status and as an inducing signal for apoptosis of chondrocytes. NO production is less pro-apoptotic in chondrocytes that are dedifferentiated by a serial monolayer culture that is associated with decreased potential of NO to activate p38 kinase in dedifferentiated cells compared with that in differentiated chondrocytes.

Apoptotic death of NO-treated chondrocytes is due to the ability of p38 kinase to stimulate caspase-3 activity, an inducer of apoptosis, and to stimulate expression and/or accumulation of p53, a known signaling molecule that acts upstream of caspase-3 (9). The signaling pathways leading to caspase activation during apoptosis involves the release of cytochrome c and other apoptogenic factors from injured mitochondria. The release is mediated by the translocation of cytosolic Bax, a pro-apoptotic member of the Bcl-2 family, to mitochondria in response to various apoptotic stimuli (10–13). Several studies indicate that p53 regulates the function of Bax and mitochondrial integrity (14–16). p38 has a short half-life, and the pro-apoptotic function of p53 is achieved by increased expression at the transcriptional level and by post-translational stabilization of the protein by escaping from ubiquitin-dependent degradation (17, 18). Phosphorylation of p53 at multiple sites is the main post-translational modification that is regulated by several different protein kinases depending on types of cells and extracellular stimuli. The protein kinases include ataxia telangiectasia-mutated kinase (19, 20) and the major subtypes of mitogen-activated protein kinase, i.e. extracellular signal-regulated kinases 1 and 2 (21, 22), p38 kinase (22–26), and c-Jun NH2-terminal kinase (27).

In addition to p53, mounting evidence indicates that a nuclear factor κB (NFκB) regulates apoptosis by protecting cells from apoptosis in most cases. However, NFκB also has pro-

1 The abbreviations used are: SNP, sodium nitroprusside; IKK, IκB kinase; NFκB, nuclear factor κB.
apoptotic function, depending on cell type and extracellular stimuli (28). NFκB exists in a latent form in the cytoplasm of unstimulated cells bound to an inhibitory protein, IκB. Upon stimulation of cells, IκB is rapidly phosphorylated at serine residues, leading to ubiquitin-dependent degradation by the 26 S proteasome. The released NFκB dimer from IκB then translocates to the nucleus and activates target genes by binding to the promoter/enhancer region (29). The known target genes of NFκB include both apoptosis-protective genes such as Bcl-2 (30) and pro-apoptotic p53 (31), suggesting that the cell type- and extracellular stimuli-dependent effects of NFκB on apoptosis may be due to its specific effects on the expression of apoptosis-regulating genes.

The functional relationship between NFκB and p53 during the regulation of apoptosis is controversial, especially when NFκB functions as a pro-apoptotic signal. For instance, genotoxic agents stimulate transcription of p53 via activation of NFκB (31), whereas induction of p53 causes activation of NFκB, that correlates with the ability of p53 to induce apoptosis (32). Thus, NFκB appears to function as either an upstream or downstream signaling molecule of p53 during the apoptotic process. Moreover, there is no available evidence indicating the involvement of NFκB and its relationship with p53 in NO-induced apoptosis. We therefore investigated the role and underlying molecular mechanism of NFκB and p53 in NO-induced and p38 kinase-mediated apoptosis of rabbit articular chondrocytes by using SNP as a NO donor. The investigation additionally focused on the characterization of the downstream signal of p53. We report here that NO-induced activation of p38 kinase activates NFκB, which led to increased transcriptional expression of p53. p38 kinase also associated with and phosphorylated the serine 15 residue of p53, which caused accumulation of p53 by stabilization of the protein. The increased expression and accumulation of p53 caused apoptosis by inducing expression and activation of pro-apoptotic Bax.

**EXPERIMENTAL PROCEDURES**

**Culture of Rabbit Articular Chondrocytes and Experimental Condition**—Articular chondrocytes were released from cartilage slices by enzymatic digestion, as previously described (9). To summarize, cartilage slices were dissociated enzymatically for 6 h in 0.2% collagenase type II (381 units/mg solid, Sigma). Individual cells were suspended in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine calf serum, 50 μg/ml streptomycin, and 50 units/ml penicillin, after which they were plated on culture dishes at a density of 5 × 10^6 cells/cm². The cells from day 3 cultures were treated with various concentrations of SNP for the indicated period and reached confluence in 5 days. In some experiments, the cells were pretreated with various inhibitors or activators for 30 min unless otherwise indicated, including 50 μg/ml of SN-50 (Biomed, Plymouth Meeting, PA) to inhibit nuclear translocation of NFκB (33) and SB203580 (Calbiochem) to inhibit p38 kinase (34).

**NO Assay and Determination of Caspase-3 Activity and Apoptosis**—NO production was measured by estimating nitrite using the Griess reagent as previously described (9). Activation of caspase-3 was determined by measuring the absorbance at 405 nm after cleavage of the synthetic substrate acetyl-DEVD-p-nitroanilide as described previously (9). Apoptotic cell death in this study was quantified by a flow cytometric assay based on the number of cells with fragmented DNA (9). Briefly, cells were harvested by centrifugation and fixed in 80% ethanol that had been precooled to −20 °C. The cells were re-suspended in phosphate-buffered saline containing 50 μg/ml propidium iodide, 0.1% Nonidet P-40, and 100 μg/ml RNase A (Sigma) and incubated for 1 h. The number of cells with fragmented DNA was then determined using 1 × 10^6 cells in a FACSort flow cytometer (BD PharMingen).

**Luciferase Assay**—Luciferase activity was assayed using an assay kit purchased from Promega and normalized by β-galactosidase activity.

**Reverse Transcription-PCR**—Total RNA was isolated by a single step guanidinium thiocyanate-phenol chloroform method using RNA STAT-60 (Tel-Test B, Inc., Friendswood, TX) according to the manufacturer’s protocol. Total RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen) for 60 min at 42 °C. Reverse transcription reactions were subjected to PCR with Taq DNA polymerase (Roche Molecular Biochemicals). PCR conditions were 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s for a total of 25 cycles. The PCR primers used were p53 (532-bp product) sense, 5'-CAGCAGCTCTGCACCGAGG-3', and antisense, 5'-ATGCCCTCCCTCAGCAGG-3', and anti-sense, 5'-CCACCAACCTTGTCTGCTA-3'. PCR products were analyzed on a 1.5% agarose gel and visualized by ethidium bromide staining.

**Pulse-Chase Analysis**—Pulse-chase analysis of p53 protein was performed to determine p53 stability. Briefly, chondrocytes, treated with 1 μM SNP for 24 h were preincubated for 20 min in methionine-free medium and then labeled with 50 μCi/ml [35S]methionine for 10 min. The cells were washed twice, and incubated in medium containing excess unlabeled methionine for 0, 1, 2, or 4 h. At each indicated time point, the cells were lysed, and p53 protein was immunoprecipitated as described above. Immuno-precipitated p53 was detected by autoradiography.

**Western Blot Analysis**—Whole cell lysates were prepared by extracting proteins using a buffer containing 50 mm Tris-HCl, pH 7.4, 150 mm...
Levels of IκB—Pro-apoptotic Signal in Articular Chondrocytes

As shown in Fig. 2C, chondrocytes were treated with 1 mM SNP for the indicated period (upper panel) or with different concentrations of SNP for 24 h (lower panel). Levels of IkB were determined by Western blot analysis. The data represent a typical result (A and C) or average values with S.D. (B) (n = 4).

**RESULTS**

**p38 Kinase Stimulates NFκB Activity, Which Functions as a Pro-apoptotic Signal in Articular Chondrocytes**—Treatment of chondrocytes with SNP (1 mM) leads to activation of the NFκB transcription factor as determined by the increased DNA binding activity of NFκB (Fig. 1A), transcripational activation of the NFκB-responsive promoter (Fig. 1B), and degradation of IκB (Fig. 1C). SNP-induced activation of NFκB was blocked by the addition of 50 μg/ml SN-50, an inhibitor peptide for nuclear translocation of the activated NFκB. Because NFκB can either protect or stimulate apoptosis, depending on cell type and extracellular stimuli, we examined whether NFκB activation is essential for NO-induced apoptosis. As shown in Fig. 2A, inhibition of NFκB activity with the SN-50 peptide significantly blocked SNP-induced caspase-3 activation and apoptosis. SNP-induced NFκB activation (Fig. 2B) and apoptosis (Fig. 2C) were also blocked by the transient expression of dominant negative forms of IκB kinases (IκKα and IκKβ) or IκBo. Therefore, the above results clearly indicate that SNP-induced activation of NFκB functions as a pro-apoptotic signal in articular chondrocytes.

The results of a recent study by our group (9) indicate that NO production in chondrocytes activates p38 kinase, which signals the induction of apoptosis. Therefore, in the present study we examined the role of p38 kinase in NFκB activation. Consistent with our previous observation, SNP treatment caused transient activation of p38 kinase in a dose-dependent manner (Fig. 3A). Blocking p38 kinase activity by treatment with SB203580 (an ectopic expression of dominant negative p38 kinase inhibited NO-induced caspase-3 activation and apoptosis (Fig. 3B). Treatment of cells with SN-50 peptide before SNP treatment, a condition that inhibits NFκB activation (Fig. 1A and B) did not affect SNP-induced p38 kinase activation (Fig. 3C). However, inhibition of p38 kinase with SB203580 or expression of dominant negative p38 kinase significantly blocked SNP-induced IκB degradation (Fig. 3C) and NFκB transcriptional activity (Fig. 3D). Therefore, activation of p38 kinase appears to lead to NFκB activation in SNP-treated articular chondrocytes.

NFκB-dependent Transcriptional Activation of the p53 Genes during NO-induced Apoptosis—Our previous study showed that p38 kinase-mediated apoptosis of SNP-treated chondrocytes is associated with an increase in p53 protein level (9). To investigate the functional relationship between NFκB activation and p53 protein level, the effects of NFκB inhibition on p53 protein level were first examined. The SNP-induced increase in p53 protein level was abrogated by the inhibition of p38 kinase with SB203580 or expression of dominant negative p38 kinase. Inhibition of NFκB with SN-50 peptide or by forced expression of dominant negative IκKα, IκKβ, or IκBo also abrogated the SNP-induced increase in p53 protein level (Fig. 4A). In contrast, however, ectopic expression of wild-type p53 or dominant negative p53 did not affect SNP-induced IκB degradation (Fig. 4B), indicating that NFκB activity is necessary to increase the p53 protein level in response to NO. We next examined the role of p53 in caspase activation and apoptosis to determine whether the increase in p53 protein level is directly involved in NO-induced apoptosis. Forced expression of a dominant negative mutant form of p53 (p53<sup>ΔN27</sup>) blocked SNP-induced caspase-3 activation and apoptosis of chondrocytes (Fig. 4, C and D). In contrast, ectopic expression of wild-type p53 significantly enhanced SNP-induced caspase activity and apoptosis (Fig. 4, C and D), indicating an essential role for p53 in NO-induced chondrocyte apoptosis.

To elucidate the mechanism leading to the increase in p53 protein level, we first examined whether p53 proteins are accumulated during NO-induced apoptosis by transcriptional activation of the p53 gene. Consistent with the increased protein level, mRNA transcript levels of p53, as determined by reverse transcription-PCR, were also increased in a time-dependent manner in cells treated with SNP (Fig. 5A). The enhanced transcription of p53 was reduced by the inhibition of NFκB (with SN-50 peptide) or p38 kinase (with SB203580 or ectopic expression of a dominant negative p38 kinase) (Fig. 5B), indicating that NFκB-mediated enhanced transcription of p53 leads to accumulation of p53 protein during NO-induced apoptosis.

**p38 Kinase Stabilizes p53 by Direct Phosphorylation at Serine 15**—In addition to transcriptional regulation, p53 activity is more commonly regulated by post-translational stabilization of protein that blocks ubiquitin-dependent degradation by the 26S proteasome (29). Therefore, pulse-chase analyses were used to determine whether NO production causes an alteration in the p53 half-life. The results, shown in Fig. 6A, clearly indicate a dramatically increased half-life of p53 in SNP-treated cells, suggesting that post-translational stabilization of p53 also contributes to the increased p53 protein level upon production of NO.
p38 Kinase-mediated p53 Regulation during Chondrocyte Apoptosis

Because phosphorylation of p53 at multiple sites commonly leads to its stabilization, we determined which site(s) of p53 is phosphorylated in response to SNP treatment by Western blot analysis using phosphorylation site-specific antibodies. Among the phosphorylation sites tested, SNP treatment caused significantly increased phosphorylation at serine 15 (Fig. 6C). The kinetics of dose- and time-dependent phosphorylation on serine 15 (Fig. 6C) is similar to that of the increase in p53 protein level (9). Serine 15 phosphorylation of p53 was reduced to the control level when p38 kinase was blocked with SB203580 or by the expression of dominant negative p38 kinase (Fig. 6D). Inhibition of NFκB activity with SN-50 also abrogated phosphorylation of serine 15, probably because of low available level of p53 protein.

Because the above observation suggests that p38 kinase is responsible for p53 phosphorylation, the role of p38 kinase was further analyzed. As shown in Fig. 7A, immunoprecipitation of p38 kinase or phosphorylated p38 kinase caused co-precipitation of serine 15-phosphorylated p53, indicating a physical association between p38 kinase and p53. To examine whether p38 kinase can directly phosphorylate serine 15 of p33, active p38 kinase was precipitated using phosphorylation-specific antibody, and an in vitro kinase assay was performed using a glutathione S-transferase fusion protein of p53. Western blot analysis indicated that p38 kinase phosphorylates serine 15 of p53 in vitro (Fig. 7B). Thus, activated p38 kinase in SNP-treated cell appears to associate and directly phosphorylate p53.

Consistent with this observation, p53 phosphorylation was not affected by the addition of wortmannin (Fig. 7C), which inhibits ataxia telangiectasia-mutated kinase (41), a well known upstream kinase of p53. The increased accumulation of p53 and potentiation of apoptosis and caspase activity in cells transfected with wild-type p53 was reduced by the co-transfection of a dominant negative p38 kinase (Fig. 4, B-D) or inhibition of p38 kinase with SB203580 (data not shown), indicating the importance of p38 phosphorylation of p53 kinase for stabilization of p53 protein. Finally, ectopic expression of p53 mutant in which serine 6, 9, 15, 20, 33, and 37 and threonine 18 are mutated to alanine (39) reduced NO-induced apoptosis.

p53 Induces Transcriptional Expression of Pro-apoptotic Bax—p53 functions as a transcription factor to regulate target genes involved in various processes including apoptosis. To determine the transcriptional activity of accumulated p53, the expression of p21, that is a well known target gene of p53, was examined. As shown in Fig. 8A, expression level of p21 was increased in SNP-treated cells, and the increase in p21 was blocked by the inhibition of p38 kinase with SB203580 or NFκB with SN-50 or expression of dominant negative forms IKKα, IKKβ, or IκBα, indicating that p53 induces p21 during NO-induced apoptosis.
To elucidate the pro-apoptotic role of p53 in SNP-treated cells, we next investigated whether p53 regulates expression and/or activity of Bax, a pro-apoptotic member of the Bcl-2 family. Bax level was low in control cells and was dramatically increased by NO production. The kinetics of Bax expression were similar to those of the expression/accumulation and severe 15 phosphorylation of p53 (Fig. 8B, upper panel). The increase in Bax expression was blocked by the inhibition of p38 kinase with SB203580 or NFκB inhibitor IκB (Fig. 8B). Our results are consistent with other reports, which indicate the potential role of p38 kinase in NFκB activation in other experimental systems (44–46). Blocking apoptosis by the inhibition of NFκB activation (Fig. 2B) indicates that the activated NFκB functions as a pro-apoptotic signal in SNP-treated chondrocytes. Although NFκB is known to protect cells from apoptosis in most cases, it is also known to contribute to apoptosis depending on cell types and extracellular stimuli (28). The pro-apoptotic effects of activated NFκB may be due to transcriptional stimulation of pro-apoptotic genes such as p53 (31). We confirmed in this study that activated NFκB in SNP-treated articular chondrocytes regulates expression of pro-apoptotic p53, as evidenced by the observation that inhibition of NFκB activation abrogated an increase in p53 (Fig. 4A) and mRNA transcript (Fig. 5B). Because ectopic expression of p53 did not affect IκB degradation (Fig. 4B), NFκB appears to regulate p53 gene expression in articular chondrocytes (Fig. 9), although the possibility that SNP treatment increases stability of p53 mRNA transcript could not be eliminated.

FIG. 4. NFκB regulates expression and/or accumulation of p53. A, chondrocytes were untreated (Control) or treated with 1 mM SNP for 24 h that was pretreated for 30 min with vehicle alone, 50 μg/ml SN-50, or 20 μM SB203580 (upper panel). Cells were transfected with the dominant negative form of IKKα, IKKβ, or IκBα. After incubation of cells in complete medium for 24 h, the cells were untreated or treated with 1 mM SNP for 24 h (lower panel). Total p53 level was determined by Western blot analysis. B–D, cells were transfected with empty vector or vector containing wild-type p53, dominant negative mutant p53 (p53m), or both wild-type p53 and dominant negative p38 kinase (p53 + Δp38). After incubation of cells in complete medium for 24 h, the cells were untreated or treated with 1 mM SNP for 24 h. Levels of IκB and p53 were determined by Western blot analysis (B). Apoptotic cells (C) and caspase-3 activity (D) was determined as described under “Experimental Procedures.” The data represent a typical result or average values with S.D. (n = 4).

FIG. 5. NFκB regulates transcriptional expression of p53. A, chondrocytes were treated with 1 mM SNP for the indicated period. B, chondrocytes were pretreated for 30 min with vehicle alone or 50 μg/ml SN50. Alternatively, the cells were transfected with dominant negative p38, cultured for 24 h in complete medium, and untreated or treated for 30 min with SN50. The cells were then incubated with 1 mM SNP for 24 h. After isolation of total RNA, reverse transcription-PCR was performed to detect p53 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts. The data represent results of a typical experiment performed at least four times. MW, molecular weight.

DISCUSSION

Differentiated chondrocyte, which is the only cell type found in normal mature cartilage, is responsible for the maintenance of homeostasis and integrity of cartilage. The homeostasis is destroyed in degenerative diseases such as osteoarthritis and rheumatoid arthritis (42). The generation of NO in chondrocytes is primarily caused by inflammatory cytokines and is one of the leading causes of the destruction of cartilage homeostasis, including apoptotic death of chondrocytes (43). Our recent study (9) provides evidence that NO-induced activation of p38 kinase functions as an inducing signal for apoptosis of chon-
protein kinase (22, 23, 25), increases p53 protein half-life due to the interference of Mdm2 function, resulting in inhibition of ubiquitination and degradation of p53 (48). Phosphorylation sites of interest include serines 15, 20, and 37 and threonine 18, which are located within or close to the Mdm2 binding site of p53. Among these sites, serine 15 has been studied more closely because it is the site phosphorylated by ataxia telangiectasia-

FIG. 6. NO-induced phosphorylation of p53 at serine 15. A, chondrocytes were untreated as a control or treated with 1 mM SNP for 24 h. The cells were pulsed with [35S]methionine for 30 min and then chased for the indicated time period. After immunoprecipitation, radiolabeled p53 protein was detected by autoradiography. B, chondrocytes were untreated as a control (CON) or treated with 1 mM SNP for 24 h, and phosphorylation of p53 was determined by Western blot analysis using phosphorylation site-specific antibodies. C, chondrocytes were treated with 1 mM SNP for the indicated period (upper panel) or with the indicated concentrations of SNP for 24 h (lower panel). Serine 15 phosphorylation of p53 was determined by Western blot analysis. D, chondrocytes were pretreated for 30 min with vehicle alone, 50 μg/ml SN50, or 20 μM SB203580 and untreated (Control) or treated with 1 mM SNP for 24 h. Alternatively, the cells were transfected with dominant negative p38 and cultured for 24 h in complete medium and treated with 1 mM SNP for 24 h. The data represent results of a typical experiment performed at least three times with similar results.

FIG. 7. p38 kinase associates and directly phosphorylates serine 15 of p53. A, chondrocytes were untreated or treated with 1 mM SNP for 24 h. p38 kinase or phosphorylated active p38 kinase (pp38) was immunoprecipitated (IP). p53, serine 15 phosphorylated p53, and p38 kinase were detected by Western blot analysis from the immune complex. B, chondrocytes were untreated (−) or treated (+) with 1 mM SNP for 24 h. After immunoprecipitation of phosphorylated and active p38 kinase (pp38), in vitro kinase assay was performed using the glutathione S-transferase fusion protein of p53 as a substrate. Serine 15-phosphorylated p53 was detected by Western blot analysis (WB). C, chondrocytes were treated for 30 min with the indicated concentrations of wortmannin (WT) followed by incubation with vehicle alone (−) or 1 mM SNP for 24 h. Serine 15 phosphorylation of p53 was determined by Western blot analysis. D and E, chondrocytes were transfected with empty vector or mutant p53 in which serine 6, 9, 15, 20, 33, 37 and threonine 18 were mutated to alanine. After incubation in complete medium for 24 h, the cells were either left untreated or treated with 1 mM SNP for 24 h. Apoptotic cell death (D) and p53 protein levels (E) were determined. The data represent results of a typical experiment performed at least four times.

FIG. 8. p53 induces Bax expression. A, cells were untreated (Control) or treated with 1 mM SNP for 24 h that was pretreated for 30 min with 50 μg/ml SN-50 or 20 μM SB203580 or untreated (upper panel). Cells were transfected with dominant negative form of IKKα, IKKβ, or IκBα. After incubation of cells in complete medium for 24 h, the cells were treated with 1 mM SNP for 24 h (lower panel). Total p21 level was determined by Western blot analysis. B, cells were treated with 1 mM SNP for the indicated period, and expression of Bax was determined by Western blot analysis (upper panel). Cells were untreated (Control) or treated with 1 mM SNP for 24 h that was pretreated for 30 min with 50 μg/ml SN-50 or 20 μM SB203580 or untreated (middle panel). Cells were transfected with the dominant negative form of IKKα, IKKβ, or IκBα. After incubation of cells in complete medium for 24 h, the cells were treated with 1 mM SNP for 24 h (lower panel). Expressions of Bax were determined by Western blot analysis.

FIG. 9. Schematic summary of p38 kinase regulated apoptosis of articular chondrocytes. NO-induced activation p38 kinase activates NFκB that leads to increased transcriptional expression p53. p38 kinase also associates and phosphorylates serine 15 residue of p53, which causes accumulation of p53 by stabilization of protein. The increased expression and accumulation of p53 causes expression and activation of pro-apoptotic Bax, which ultimately activates caspase-3 to cause apoptosis.
mutated kinase (19, 20) and also by extracellular signal-regulated kinase (22) and p38 kinase (22, 26). In addition to serine 15, p38 kinase has been shown to phosphorylate serine 33 (25) and 37 and threonine 18 are mutated to alanine reduced NO-induced apoptosis and accumulation of p53 protein (Fig. 7), which further supports the importance of serine 15 phosphorylation in p53 protein stabilization. Although stabilization of p53 is important for transcriptional activation, our current results also suggest that transcriptional expression of p53 is also essential for p53 accumulation. This conclusion is based on the observation that inhibition of p53 expression after the inhibition of NF-κB activity blocked accumulation of p53 (Fig. 6D). Under this condition, serine 15 phosphorylation of p53 is also weak; this may be due to the level of p53 available for phosphorylation by p38 kinase.

There is little evidence available that indicates a pro-apoptotic role of p53 in chondrocytes. For example, a study by Yatsugi et al. (5) suggests the possible involvement of p53 in chondrocyte apoptosis and cartilage destruction. Immunohistochemical analyses of p53 in the articular cartilage of patients with rheumatoid arthritis and osteoarthritis showed increased staining of the tumor suppressor protein in chondrocytes with apoptotic morphology. Although activation of p53 is believed to play an important role in NO-induced apoptosis of various cell types (50), some recent studies using p53−/− cells indicate that p53 is not required for NO-induced apoptosis in certain cell types including alveolar macrophages (51) and vascular smooth muscle cells (52). However, our recent observation that the potentiation of SNP-induced apoptosis and caspase activation in p53-transfected cells and that inhibition of apoptosis by the ectopic expression of mutant p53 (Fig. 4) strongly suggests that increased p53 protein levels contribute to NO-induced apoptosis of articular chondrocytes.

To date, the mechanism of p53 regulation of apoptosis has not been clearly elucidated. However, the best understood function of p53 is as a transcription factor, an activity that contributes to apoptotic function (53). We confirmed in this study the transcriptional activity of p53 by examining the expression of p21\textsuperscript{Waf1/Cip1}, a well known target gene of p53. Few genes are known to regulate p53-mediated apoptotic function, including Bax, although a large number of cellular genes that are transcriptionally regulated by p53 have been described. Among the genes, the function of Bax is best studied, and it clearly contributes to apoptosis (14). In response to apoptotic signals, Bax translocates to the mitochondria from the cytosol, where it causes a decline in mitochondrial membrane potential followed by cytochrome c release and caspase activation (10, 12, 13, 15). Although the promoter sequence of Bax contains a putative NF-κB binding site, NF-κB does not directly regulate Bax expression (54). Our current study suggests that p53 directly regulates Bax expression. In addition, it is also likely that NF-κB-dependent transcriptional expression of Bax is mediated by p53. This idea is consistent with many other observations that indicate direct regulation of Bax expression by p53 (14, 55). Although it is possible that the response of p53 is dependent on the coordinate expression of several gene products (56), it is likely that p53-mediated expression and redistribution of Bax causes SNP-induced apoptosis via release of cytochrome c and caspase activation as depicted in Fig. 9.

In summary, we demonstrate that NO-induced activation of p38 kinase stimulates NF-κB, which leads to the increased transcriptional expression of p53. p38 kinase also associates with and phosphorylates the serine 15 residue of p53, resulting in accumulation of p53 by stabilization of the protein. The increased expression and accumulation of p53 causes Bax expression by inducing expression and activation of pro-apoptotic factors.

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