Mechanisms of Signal Transduction:

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Inhibition of Apoptosis Signal-regulating Kinase 1 by Nitric Oxide through a Thiol Redox Mechanism*

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Nitric oxide is an endogenous thiol-reactive molecule that modulates the functions of many regulatory proteins by a thiol-redox mechanism. NO has now been shown to inhibit the activation of apoptosis signal-regulating kinase 1 (ASK1) in murine fibrosarcoma L929 cells through such a mechanism. Exposure of L929 cells to interferon-γ resulted in the endogenous production of NO and in inhibition of the activation of ASK1 by hydrogen peroxide. The interferon-γ-induced inhibition of ASK1 activity was blocked by N^G-nitro-L-arginine, an inhibitor of NO synthase. Furthermore, the NO donor *S*-nitro-*N*-acetyl-DL-penicillamine (SNAP) inhibited ASK1 activity *in vitro*, and this inhibition was reversed by thiol-reducing agents such as dithiothreitol and β-mercaptoethanol. SNAP did not inhibit the kinase activities of MKK3, MKK6, or p38 *in vitro*. The inhibition of ASK1 by interferon-γ was not changed by 1H- (1,2,4)oxadiazolo[4,3-α]quinoxalin-1-one, an inhibitor of guanylyl cyclase nor was it mimicked by 8-bromo-cyclic GMP. Site-directed mutagenesis revealed that replacement of cysteine 869 of ASK1 by serine rendered this protein resistant to the inhibitory effects both of interferon-γ in intact cells and of SNAP *in vitro*. Co-immunoprecipitation data showed that NO production inhibited a binding of ASK1, but not ASK1(C869S), to MKK3 or MKK6. Moreover, interferon-γ induced the *S*-nitrosylation of endogenous ASK1 in L929 cells. Together, these results suggest that NO mediates the interferon-γ-induced inhibition of ASK1 in L929 cells through a thiol-redox mechanism.

Nitric oxide functions as a signal transducer that contributes to various biological processes in mammals, including vasodilation, the immune response, and synaptic transmission (1–4). This diffusible free radical is produced by nitric-oxide synthase (NOS),[1] the mammalian isoforms of which are categorized as type I or type II (4, 5). The type I enzymes include endothelial NOS and neuronal NOS. These isoforms are expressed constitutively, and their enzymatic activities are regulated by Ca^{2+}-calmodulin (1, 6). The type II enzyme is inducible NOS (iNOS), the expression of which is induced in many cell types, including macrophages, astrocytes, and fibroblasts, in response to cytokines or lipopolysaccaride (7–11). The activity of iNOS is independent of intracellular Ca^{2+} concentration (12).

NO plays an important role in intracellular and intercellular signaling events by regulating the activities of various proteins. It regulates many of these target proteins through *S*-nitrosylation. For example, through direct *S*-nitrosylation, NO activates Ras and the ryanodine receptor (13, 14) as well as inhibits caspase-3, c-Jun NH_2-terminal kinase (JNK), NF-κB, protein kinase C, and *N*-methyl-D-aspartate receptor for glutamate (15–19). *S*-Nitrosylation is thus responsible for both positive and negative regulation of diverse signaling pathways by NO (20).

Apoptosis signal-regulating kinase 1 (ASK1) is a serine/threonine protein kinase that functions as a MAPK kinase kinase in the JNK (also known as stress-activated protein kinase or stress-activated protein kinase) and p38 MAPK signaling pathways (21). ASK1 is activated by exposure of cells to various stimuli, including tumor necrosis factor-α, Fas ligand, hydrogen peroxide, genotoxic agents, microtubule-interfering drugs, and osmotic stress (21, 22). Activation of ASK1 is associated with apoptotic cell death under various conditions (21–23), but it is also implicated in other cellular events such as cell proliferation and differentiation (24, 25). Many lines of evidence suggest that the activity of ASK1 is regulated by physical interaction with proteins including 14-3-3, thioredoxin, glutaminyl-tRNA synthetase, glutathione *S*-transferase Mu, and p21 (also known as Cip1 or WAF1) (26–30).

NO regulates the stress-activated MAPK pathways (31–35). To provide further insight into the mechanism underlying this regulation, we have investigated the possible effect of NO on ASK1 signaling. We now show that the induction of iNOS and the subsequent production of endogenous NO inhibit ASK1 activation though a thiol-redox mechanism. Our observations thus suggest that ASK1 is a physiological target of NO.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Murine fibrosarcoma L929 and HEK293 cells were maintained under an atmosphere of 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For DNA transfection, cells in 100-mm dishes (1×10^6 cells/dish) were transfected with the indicated plasmid vectors either with the use of GenePORTER 2 (Gene Therapy Systems) or by electroporation (Gene Pulser II; Bio-Rad).

Site-directed Mutagenesis—Site-directed mutagenesis of human ASK1 cDNA was performed with a QuikChange kit (Stratagene) and the following mutagenic primers: C835S, 5′-GCTGGCATAAACCCCa-GTACTGAAACTTTTACTGG-3′; C869S, 5′-CTGGTCTCTGGGCaGTA-CAATCATTGAAATGGCC-3′; C918S, 5′-GGCCAAGGCATTCATACTG-

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[1] The abbreviations used are: NOS, nitric-oxide synthase; iNOS, inducible NOS; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; IFN-γ, interferon-γ; HA, hemagglutinin; Diamide, azodicarboxylic acid bis-dimethylamide; SNAP, *S*-nitro-*N*-acetyl-DL-penicillamine; ASK1, apoptosis signal-regulating kinase 1; L-NNA, N^G-nitro-L-arginine; GST, glutathione S-transferase.

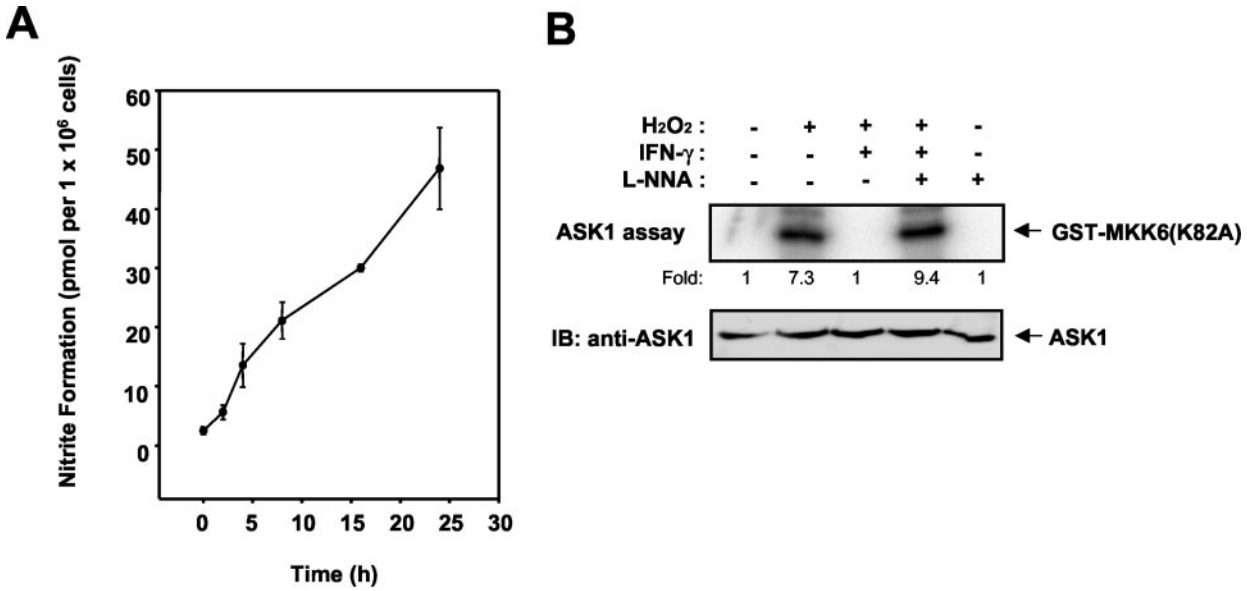

FIG. 1. **IFN-γ-induced NO production inhibits H_2O_2-induced activation of ASK1 in L929 cells.** *A*, L929 cells were incubated for the indicated times with IFN-γ (100 units/ml), after which NO released into the culture medium was determined by the Griess method. *B*, L929 cells were incubated with or without IFN-γ (100 units/ml) for 16 h and then for 30 min in the absence or presence of 2 mM L-NNA. The cells were then left unexposed or exposed to 2 mM H_2O_2 for 20 min. Cell lysates were subjected to immunoprecipitation with anti-ASK1 antibody, and the resulting precipitates were examined for ASK1 activity by immune complex kinase assay with GST-MKK6(K82A) as substrate. The fold activation of ASK1 is indicated *below* each lane. The amount of ASK1 present in cell lysates was revealed by immunoblot analysis (*IB*) with anti-ASK1 antibody.

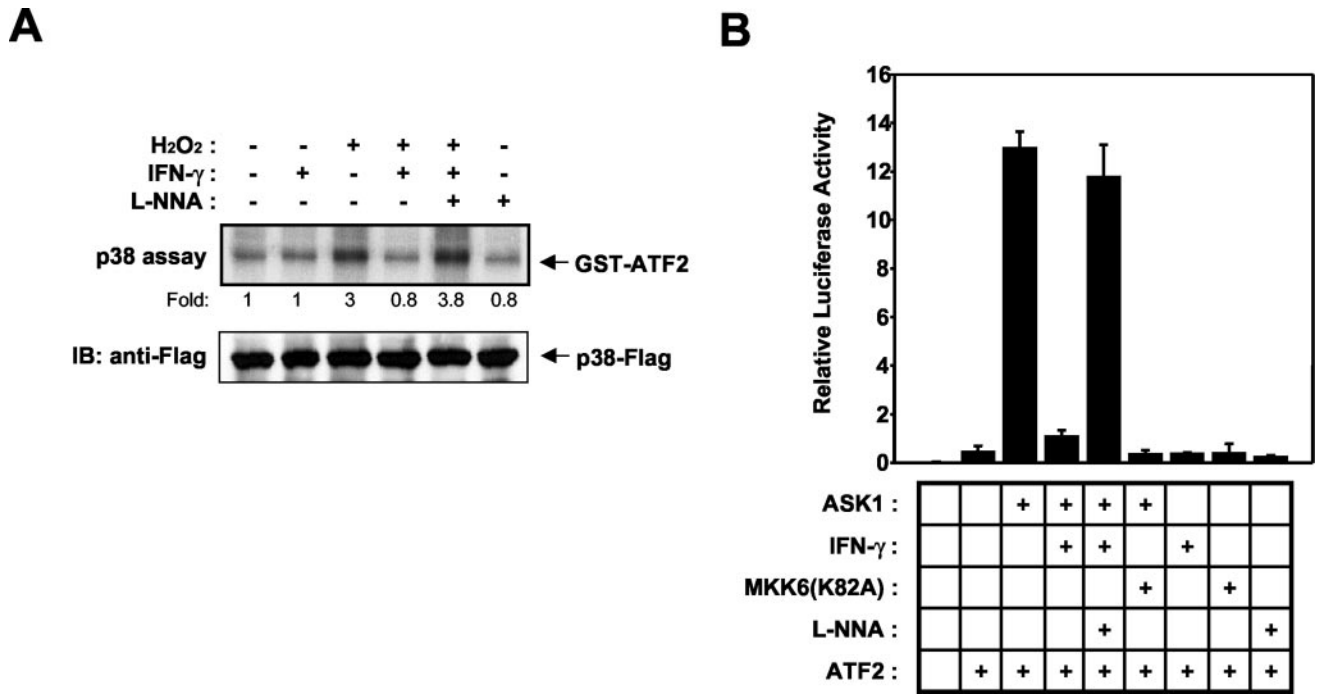

FIG. 2. **Inhibition of the H_2O_2-induced activation of both p38 MAPK and ATF2 by the IFN-γ-induced production of NO in L929 cells.** *A*, L929 cells were transfected for 48 h with an expression vector encoding p38-FLAG, after which they were incubated for 16 h with or without IFN-γ (100 units/ml) and then for 30 min in the absence or presence of 2 mM L-NNA. The cells were then left unexposed or exposed to 2 mM H_2O_2 for 20 min. The cell lysates were subjected to immunoprecipitation with anti-FLAG antibody, and the resulting precipitates were assayed for the kinase activity of p38 with a GST-ATF2 fusion protein as substrate. *B*, L929 cells were transfected for 48 h with pFR-Luc, pFA2-ATF2, and pcDNA3-βgal together with expression vectors encoding ASK1 and MKK6(K82A), as indicated. The transfected cells were incubated for 16 h in the absence or presence of IFN-γ (100 units/ml) and then for 6 h without or with 2 mM L-NNA. The cell lysates were assayed for luciferase and β-galactosidase activities. Luciferase activity was normalized by β-galactosidase activity and then expressed in relative terms. *IB*, immunoblot.

AAAaGTTTTGAACCAGATCC-3′; and C928S, 5′-CCTGACAAGAGAG-CCaGTGCTAACGACTTGC-3′ (mismatches with the ASK1 cDNA template are indicated by lowercase letters). The mutations were verified by automatic DNA sequencing.

Measurement of NO Production—NO production was measured with a kit based on the Griess reaction (Promega) (19).

Immune Complex Kinase Assays—Immune complex kinase assays were performed as previously described (19, 36). In brief, the cells were solubilized with lysis buffer (36), and the lysates were centrifuged at 12,000 × *g* for 10 min at 4 °C. The resulting supernatants were subjected to immunoprecipitation with appropriate antibodies, and the immunoprecipitates were assayed for the enzymatic activities of the indicated protein kinases. Phosphorylated substrates were separated by SDS-polyacrylamide gel electrophoresis, and the extent of their phosphorylation was quantified with a Fuji BAS 2500 PhosphoImager. Protein concentration was determined by the Bradford assay (Bio-Rad).

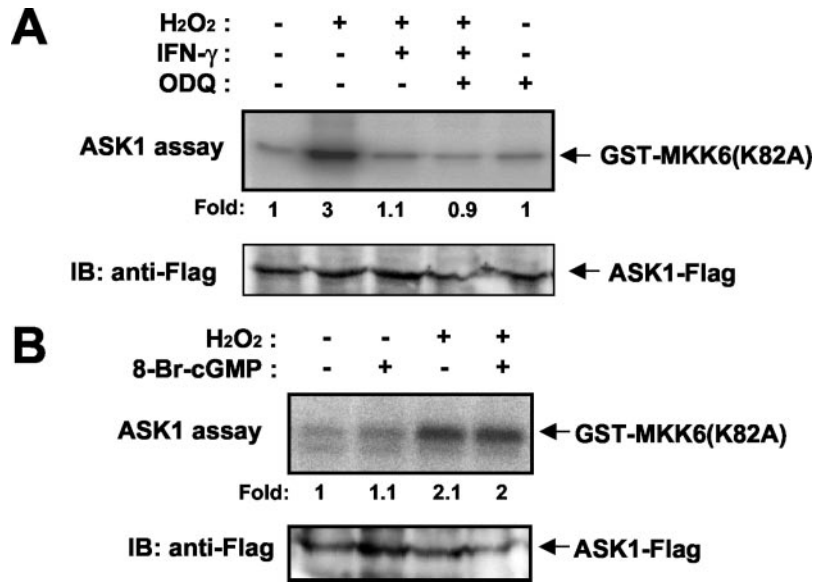

FIG. 3. **Cyclic GMP is not involved in the NO-induced inhibition of ASK1 activity in L929 cells.** *A*, L929 cells were transfected for 48 h with an expression vector encoding ASK1-FLAG. The transfected cells were incubated for 16 h with or without IFN-γ (100 units/ml) and then for 30 min in the absence or presence of 100 μM 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one before exposure to 2 mM H_2O_2 for 20 min. The cell lysates were subjected to immunoprecipitation with anti-FLAG antibody, and the resulting precipitates were assayed for ASK1 activity. *B*, L929 cells were transfected for 48 h with the ASK1-FLAG vector, incubated in the absence or presence of 100 μM 8-bromo-cyclic GMP (*8-Br-cGMP*) for 30 min and then left unexposed or exposed to 2 mM H_2O_2 for 20 min. The cells were then assayed for ASK1 activity as in *A*. *IB*, immunoblot.

Luciferase Reporter Assay for ATF2-dependent Transcription—The transactivation activity of ATF2 was measured with the use of a Path-Detect luciferase reporter kit (Stratagene). Briefly, L929 cells were transfected for 48 h with pFR-Luc, pFA2-ATF2, pcDNA3-β-galactosidase, and the indicated expression vectors. After treatment with the indicated agents, the cells were lysed, and cell lysates were assayed for both luciferase and β-galactosidase activities as described previously (36). The luciferase activity was normalized relative to the β-galactosidase activity of the same cells.

Co-immunoprecipitation Analysis—HEK293 cells were transiently transfected with expression vectors encoding c-Myc epitope-tagged wild-type ASK1 or ASK1(C869S) and HA-tagged MKK3. After 48 h of transfection, the cells were untreated or treated for 1 h with 100 μM *S*-nitro-*N*-acetyl-DL-penicillamine (SNAP). The cell lysates were subjected to immunoprecipitation with mouse monoclonal anti-c-Myc antibody, and the resulting immunoprecipitates were immunoblotted with mouse monoclonal anti-HA antibody (27, 36).

Measurement of S-Nitrosylation—*S*-Nitrosylation of endogenous ASK1 was measured as described previously (19). Briefly, the cells were exposed to interferon-γ (IFN-γ) (100 units/ml) for 16 h at 37 °C and then lysed. The cell lysates were subjected to immunoprecipitation with rabbit preimmune IgG or rabbit polyclonal antibody to ASK1 (Santa Cruz Biotechnology). The resulting precipitates were rinsed five times with lysis buffer and twice with phosphate-buffered saline. The precipitates were then incubated for 30 min at room temperature with 100 μM $HgCl_2$ and 200 μM 2,3-diaminonaphthalene before the addition of 1 M NaOH. A fluorescent triazole generated from the reaction between 2,3-diaminonaphthalene and NO released from *S*-nitrosylated ASK1 was quantified with a spectrofluorometer (PerkinElmer Life Sciences HTS 7000) at excitation and emission wavelengths of 375 and 450 nm, respectively.

RESULTS AND DISCUSSION

NO Mediates IFN-γ-induced Inhibition of ASK1 in L929 Cells—Like many cytokines, IFN-γ induces the expression of iNOS, and thereby increases the intracellular concentration of NO, in macrophages and other cell types (37). IFN-γ thus increased the production of NO in murine fibrosarcoma L929 cells (Fig. 1*A*). We investigated the possible effect of NO on ASK1-mediated signaling in L929 cells. Exposure of these cells to H_2O_2 resulted in the activation of endogenous ASK1, and pretreatment of the cells with IFN-γ blocked this effect of H_2O_2 (Fig. 1*B*). The inhibitory effect of IFN-γ was abolished by N^G-nitro-L-arginine (L-NNA), an inhibitor of NOS. These results thus suggest that IFN-γ inhibits ASK1 activation in L929 cells and that this effect is mediated by NO.

We next examined the effect of IFN-γ on the kinase activity of p38, a downstream MAPK of ASK1, in L929 cells that had been transfected with a plasmid encoding FLAG epitope-tagged p38 (p38-FLAG). IFN-γ inhibited the H_2O_2-induced activation of p38 in the transfected cells, and this inhibitory effect was blocked by L-NNA (Fig. 2*A*). These data suggest that the IFN-γ-induced production of NO was responsible for the inhibition of p38 MAPK activity by this cytokine. The effect of IFN-γ-induced NO production on the transactivation activity of ATF2 was also examined with a luciferase reporter system (Fig. 2*B*). ATF2 is a physiological substrate of p38, and its phosphorylation by this kinase increases its transcription-stimulating activity (38). Overexpression of ASK1 resulted in a marked increase in ATF2-dependent luciferase reporter activity, and this effect was blocked by co-expression of a dominant-negative mutant (MKK6(K82A)) of MKK6, which functions as a MAP2K upstream of p38 (39). These results indicated that the ASK1-induced activation of ATF2 was mediated by the p38 MAPK signaling pathway. IFN-γ also inhibited the ASK1-induced activation of ATF2, and this inhibitory effect of IFN-γ was blocked by L-NNA. The IFN-γ-induced production of NO thus appeared to be responsible for the inhibition of the ASK1-dependent activation of ATF2 by this cytokine in L929 cells.

SNAP Inhibits ASK1 Activity in Vitro—Some of the biological functions of NO are mediated through the activation of guanylyl cyclase, which catalyzes the production of cyclic GMP (37, 40). We therefore examined whether guanylyl cyclase and cyclic GMP mediate the inhibition of ASK1 by NO produced in response to IFN-γ in L929 cells. Pretreatment of the cells with 1H-(1,2,4)oxadiazolo[4,3-a]quinoxalin-1-one, an inhibitor of guanylyl cyclase, did not block the inhibitory effect of IFN-γ on the H_2O_2-induced activation of ASK1 (Fig. 3*A*). Furthermore, pretreatment of the cells with 8-bromo-cyclic GMP, a cell-permeable analog of cyclic GMP, did not inhibit the H_2O_2-induced activation of ASK1 (Fig. 3*B*). These results thus suggested that cyclic GMP was not responsible for the inhibition of ASK1 activation by NO produced in response to IFN-γ in L929 cells.

We next investigated whether NO was able to inhibit ASK1 directly. We examined the effect of the NO donor SNAP on the activities of ASK1, MKK3, MKK6, and p38 *in vitro*. SNAP inhibited the kinase activity of ASK1 that had been isolated from H_2O_2-treated L929 cells by immunoprecipitation (Fig. 4*A*). In contrast, SNAP did not affect the kinase activities of MKK3, MKK6, or p38. SNAP also did not inhibit the activity of MEKK1 *in vitro* (data not shown). The thiol-reducing agents dithiothreitol and β-mercaptoethanol each reversed the inhibitory effect of SNAP on ASK1 activity *in vitro* (Fig. 4*B*). These data thus suggest that ASK1 is a direct target of NO and that

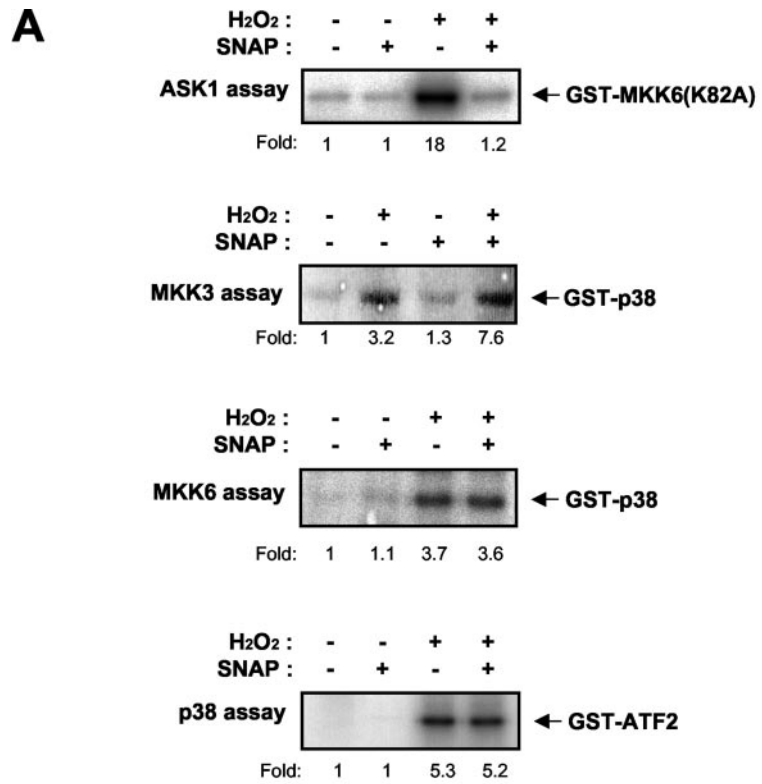

FIG. 4. **SNAP inhibits ASK1 *in vitro*.** *A*, L929 cells were transfected for 48 h with an expression vector encoding ASK1-FLAG, HA-MKK3, HA-MKK6, or p38-FLAG. The cells were then incubated in the absence or presence of 2 mM H_2O_2 for 20 min. The cell lysates were subjected to immunoprecipitation with antibodies to FLAG or to HA. The immunoprecipitates were left unexposed or exposed to 100 μM SNAP for 20 min on ice and then assayed for the indicated protein kinase activities. *B*, ASK1-FLAG immunoprecipitates prepared as in *A* were left unexposed or exposed to 100 μM SNAP for 20 min on ice and then incubated for 20 min on ice without or with 10 mM dithiothreitol (*DTT*) or 10 mM β-mercaptoethanol (β-*ME*). The precipitates were washed three times with 20 mM Hepes (pH 7.4) and then assayed for ASK1 activity.

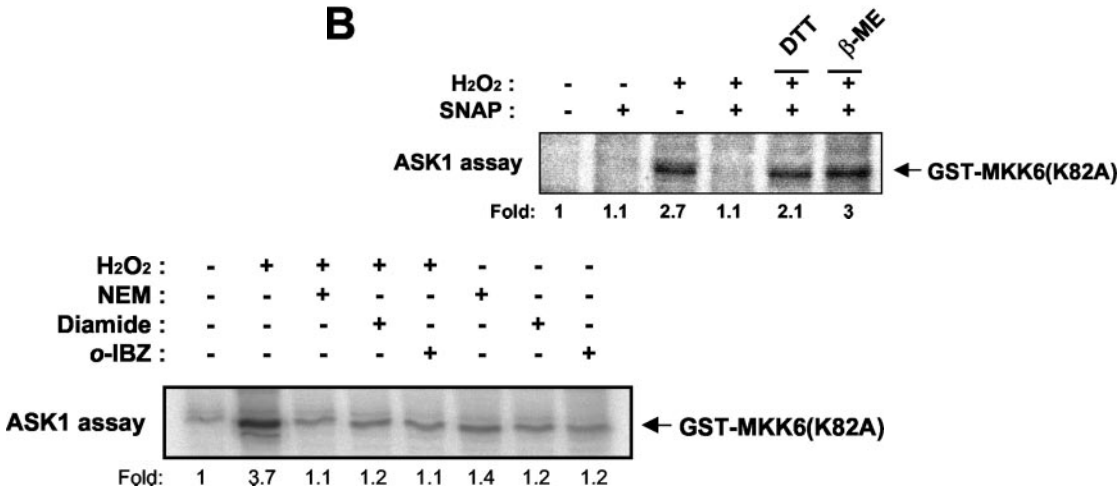

FIG. 5. **Thiol-reactive agents inhibit ASK1 *in vitro*.** L929 cells were transfected for 48 h with an expression vector for ASK1-FLAG and then incubated in the absence or presence of 2 mM H_2O_2 for 20 min. The cell lysates were subjected to immunoprecipitation with antibodies to FLAG. The resulting precipitates were incubated for 20 min on ice in the absence or presence of 500 μM *N*-ethylmaleimide (*NEM*), 500 μM Diamide, or 500 μM *o*-iodosobenzoate (*o-IBZ*) and then examined for ASK1 activity by immune complex kinase assay.

NO inhibits ASK1 activity by a thiol-redox mechanism. We further investigated the thiol-redox regulation of ASK1 by examining the effects of *N*-ethylmaleamide, a thiol-modifying reagent, as well as of azodicarboxylic acid bis-dimethylamide (Diamide) and *o*-iodosobenzoate, each of which oxidizes vicinal thiols (41–43). *N*-Ethylmaleimide, Diamide, and *o*-iodosobenzoate all inhibited ASK1 activity *in vitro* (Fig. 5). Taken together, these data suggest that the kinase activity of ASK1 is dependent on the reduced form of a critical cysteine residue (or residues) present in the protein.

NO Targets the Kinase Domain of ASK1—To characterize further the inhibition of ASK1 by NO, we examined the effects of NO on the kinase activities of two mutants of ASK1, ASK1-ΔN and ASK1-K. ASK1-ΔN is a constitutively active form of ASK1 that encodes amino acids 649–1375 of the protein (27), whereas ASK1-K encodes amino acids 678–936 of ASK1, which include the kinase domain. INF-γ inhibited the kinase activities of HA-tagged ASK1-ΔN and HA-tagged ASK1-K expressed in L929 cells (Fig. 6). SNAP also inhibited the activities of ASK1-ΔN and ASK1-K *in vitro* (Fig. 6). These data suggest that the kinase domain of ASK1 includes the target site (or sites) of NO. The thiol-oxidizing agents *N*-ethylmaleimide, Diamide, and *o*-iodosobenzoate also inhibited ASK1-K activity *in vitro* (data not shown).

Cys^{869} of ASK1 Is Required for Thiol Redox Regulation by NO—The kinase domain of ASK1 contains four cysteine residues: Cys^{835}, Cys^{869}, Cys^{918}, and Cys^{928}. To identify the cysteine residue (or residues) targeted by NO, we constructed mutant proteins in which each of these four residues is individually replaced with serine (C835S, C869S, C918S, and

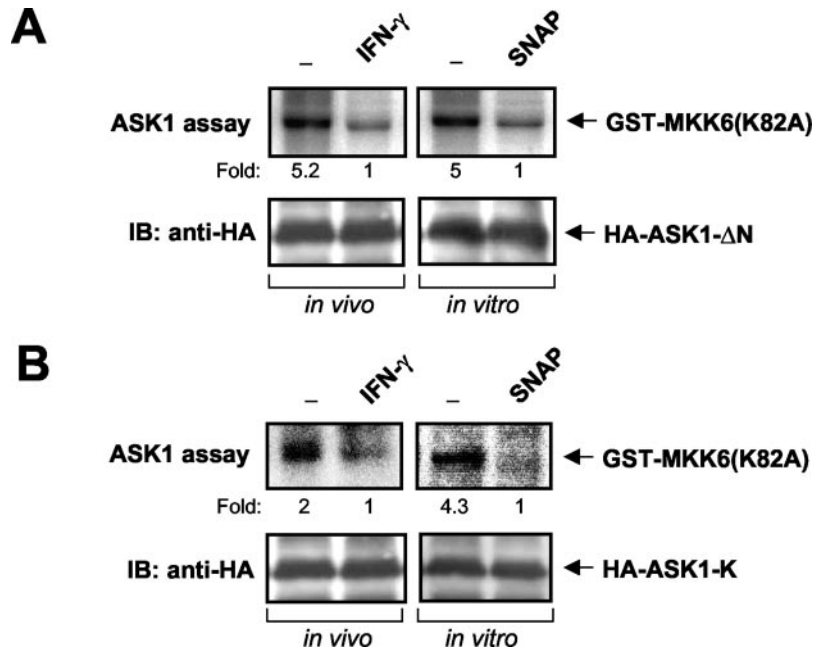

FIG. 6. **NO inhibits ASK1-ΔN and ASK1-K.** L929 cells were transfected for 48 h with an expression vector encoding HA-tagged ASK1-ΔN (*A*) or HA-tagged ASK1-K (*B*). Where indicated, the cells were then incubated in the absence or presence of IFN-γ (100 units/ml) for 16 h. The cell lysates were subjected to immunoprecipitation with anti-HA antibody, and the resulting precipitates were examined for ASK1 activity by immune complex kinase assay. Where indicated, the precipitates were incubated for 20 min on ice in the absence or presence of 100 μM SNAP prior to the immune complex kinase assay for ASK1 activity. *IB*, immunoblot.

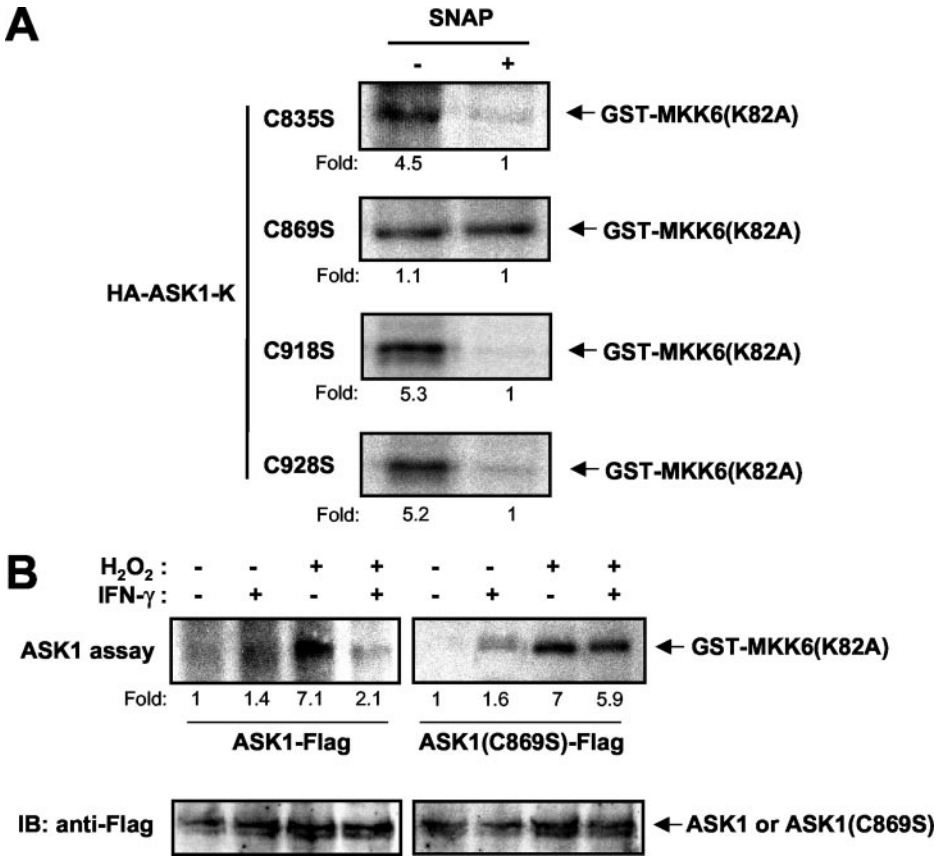

FIG. 7. **Identification of Cys^{869}as a target for NO in the thiol-redox regulation of ASK.** *A*, L929 cells were transfected for 48 h with an expression vector encoding a HA-tagged cysteine mutant of ASK1-K (C835S, C869S, C918S, or C928S). The cell lysates were subjected to immunoprecipitation with anti-HA antibody. The resulting precipitates were incubated for 20 min on ice in the absence or presence of 100 μM SNAP and then assayed for ASK1 activity. *B*, L929 cells were transfected for 48 h with an expression vector encoding ASK1-FLAG or ASK1(C869S)-FLAG, incubated for 16 h in the absence or presence of IFN-γ (100 units/ml), and then left unexposed or exposed to 2 mM H_2O_2 for 20 min. The cell lysates were subjected to immunoprecipitation with anti-FLAG antibody, and the resulting precipitates were examined for ASK1 activity by immune complex kinase assay. The cell lysates were also analyzed by immunoblotting (*IB*) with anti-FLAG antibody.

C928S) and then examined their sensitivity to NO. SNAP inhibited the kinase activities of the C835S, C918S, and C928S mutants of ASK1-K *in vitro* (Fig. 7*A*). In contrast, the C869S mutant was resistant to the inhibition by SNAP. These results suggest that Cys^{869} of ASK1 is the cysteine residue targeted by NO. Next, we transfected L929 cells with an expression vector for FLAG-tagged wild-type ASK1 or ASK1(C869S) and then examined the effect of INF-γ on the H_2O_2-induced activation of the ectopically expressed ASK1 proteins. INF-γ inhibited the H_2O_2-induced increase in the activity of ASK1 but not that in the activity of ASK1(C869S) (Fig. 7*B*). Together, these data suggest that NO produced in response to INF-γ inhibits ASK1 activity through modification of Cys^{869} of the kinase.

We next investigated a possible mechanism by which the targeting of NO on Cys^{869} of ASK1 resulted in the inhibition of the kinase activity. We examined whether NO production could block the interaction between ASK1 and its substrate MKK3 after HEK293 cells were transfected with plasmid vectors encoding HA-tagged MKK3 and c-Myc epitope-tagged ASK1 or ASK1(C869S). Immunoblot analysis with anti-HA antibody of

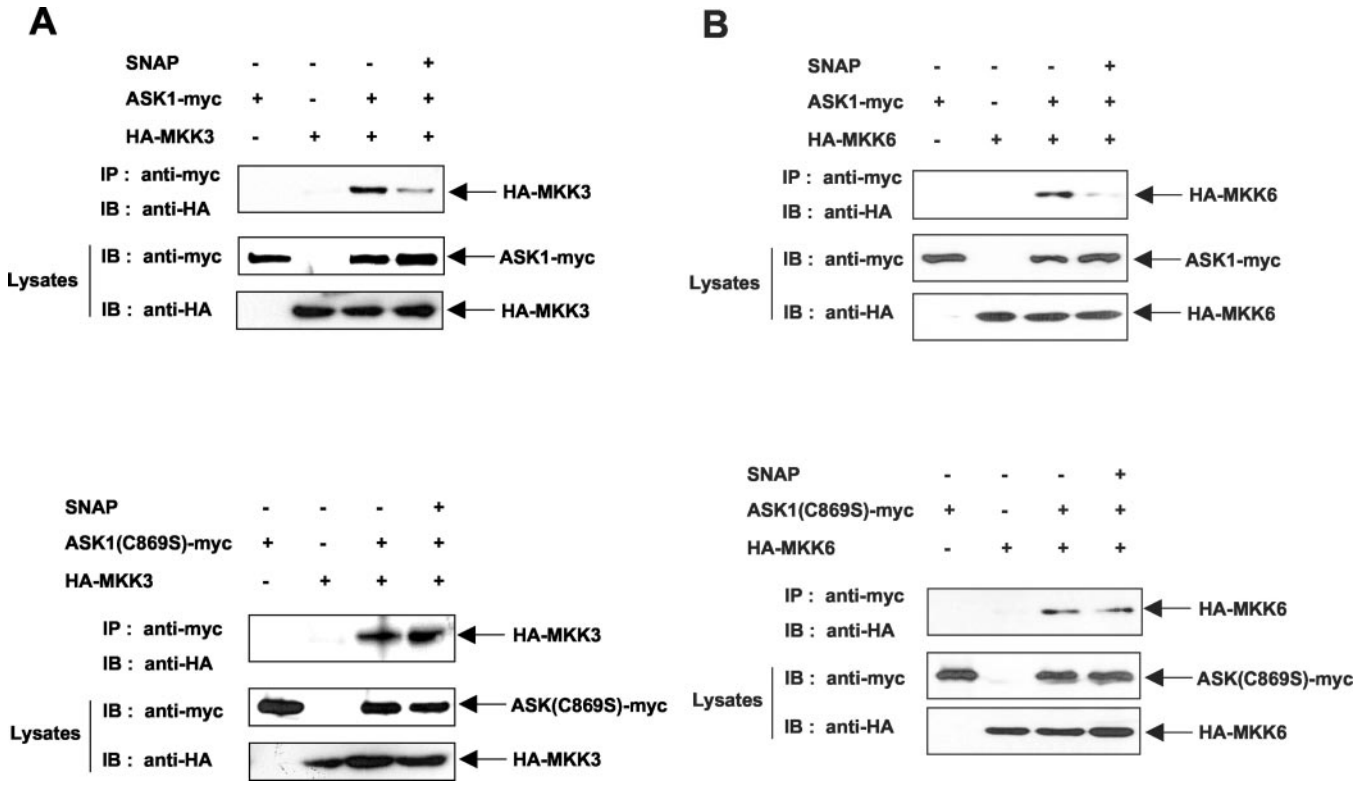

FIG. 8. **The effect of SNAP on the binding of ASK1 or ASK1(C869S) to MKK3 or MKK6.** HEK293 cells were transfected for 48 h with plasmid vectors encoding c-Myc epitope-tagged ASK1 (*ASK1-myc*) or ASK1(C869S)-Myc along with HA-MKK3 (*A*) or HA-MKK6 (*B*). The transfected cells were untreated or treated for 1 h with 100 μM SNAP, and the cell lysates were subjected to immunoprecipitation (*IP*) with mouse monoclonal anti-c-Myc antibody. The resulting immunoprecipitates were subjected to immunoblot analysis with mouse monoclonal anti-HA antibody. The cell lysates were also directly subjected to immunoblot (*IB*) analysis with an antibody to c-Myc or HA.

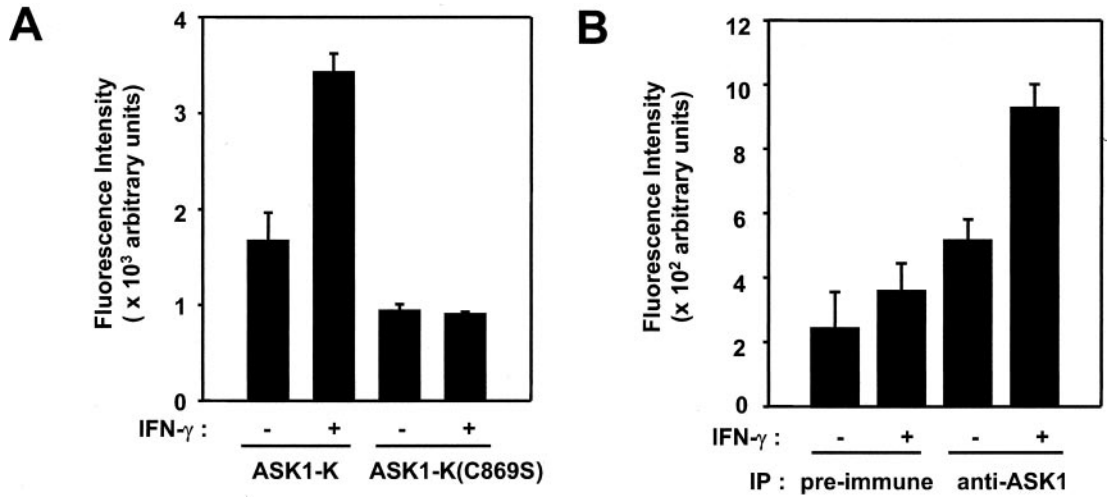

FIG. 9. **IFN-γenhances *S*-nitrosylation of ASK1 in L929 cells.** *A*, L929 cells were transfected with an expression vector encoding HA-tagged ASK1-K or ASK1-K(C869S). After 48 h of transfection, the cells were incubated for 16 h in the absence or presence of IFN-γ (100 units/ml). The cell lysates were precleared with the use of mouse preimmune IgG and then subjected to immunoprecipitation (*IP*) with mouse monoclonal anti-HA antibody. The resulting immunoprecipitates were incubated with 200 μM $HgCl_2$ and 200 μM 2,3-diaminonaphthalene for 30 min at room temperature and then added with 1 M NaOH. NO released from *S*-nitrosylated ASK1 was quantified by fluorometry. *B*, L929 cells were incubated with IFN-γ (100 units/ml) and analyzed as in *A*, with the exception that immunoprecipitation was performed with either preimmune rabbit IgG or rabbit polyclonal antibodies to ASK1, and both of the resulting precipitates were assayed for *S*-nitrosylation. These data represent the results from three independent experiments.

the c-Myc immunoprecipitates indicated that treatment of the cells with SNAP inhibited the interaction between ASK1 and MKK3. In contrast, SNAP did not affect the interaction between ASK1(C869S) and MKK3 (Fig. 8*A*). SNAP also inhibited the interaction between ASK1 and its another substrate MKK6, whereas it did not affect the interaction between ASK1(C869S) and MKK6 (Fig. 8*B*). Our results thus suggest that the suppression of the interaction between ASK1 and its substrate kinases is involved in the mechanism underlying the inhibition of ASK1 by NO.

IFN-γ Enhances the S-Nitrosylation of Endogenous ASK1 in L929 Cells—We next investigated whether the INF-γ-induced production of NO results in the *S*-nitrosylation of ASK1 in L929 cells. The cells were transfected with an expression vector for

HA-tagged ASK1-K or ASK1-K(C869S) and then treated with INF-γ, after which immunoprecipitates of the recombinant proteins analyzed for *S*-nitrosylation by fluorometry with 2,3-diaminonaphthalene (19). INF-γ increased the extent of *S*-nitrosylation of ASK1-K but not that of ASK1-K(C869S) (Fig. 9*A*). These results suggest that INF-γ treatment enhances the *S*-nitrosylation of Cys^{869} of ASK1 in intact cells. Finally, we examined the effect of INF-γ on the *S*-nitrosylation of endogenous ASK1 in L929 cells. INF-γ also increased the extent of *S*-nitrosylation of the endogenous protein (Fig. 9*B*).

NO, an endogenous thiol-reactive agent, modifies many regulatory proteins by a thiol-redox mechanism and thereby modulates their biological functions (13–19). We have now shown that ASK1 is a target protein of NO and that NO acts at a site in the kinase domain of ASK1. Although NO exerts some of its biological effects by activating guanylyl cyclase and thereby inducing the accumulation of cyclic GMP, guanylyl cyclase and cyclic GMP do not appear to contribute to the negative regulation of ASK1 by NO. NO inhibits the activity of ASK1 both *in vitro* and in intact cells by a thiol-redox mechanism. Furthermore, site-directed mutagenesis revealed that Cys^{869} is the redox-sensitive cysteine residue of ASK1. IFN-γ, by inducing the expression of iNOS and the consequent production of NO, induces *S*-nitrosylation of endogenous ASK1 and thereby inhibits ASK1 activation in L929 cells. Collectively, our results indicate that NO functions as a physiological inhibitor of ASK1. It is noteworthy that H_2O_2 does not inhibit ASK1 activity *in vitro* (data not shown). Furthermore, replacement of Cys^{869} of ASK1 by serine has no inhibitory effect on the kinase activity of the protein. Our findings thus strongly suggest that the inhibition of ASK1 by *S*-nitrosylation results from the presence of a nitrosyl group not from the absence or a simple oxidation of a sulfhydryl group, at Cys^{869}. Cys^{869} is located in the catalytic domain of ASK1, where Asp^{803} functions as the catalytic aspartate and the activation loop spans the region between Asp^{822} and Glu^{849} (44, 45). The activation loop includes Thr^{838} and Thr^{842}, the phosphorylation sites of ASK1 (45). Autophosphorylation of Thr^{838}, in particular, is required for ASK1 activation (45). Phosphorylation within the activation loop of a protein kinase induces a conformational change that may allow better access of a substrate to the catalytic site (44, 46). One intriguing question is whether *S*-nitrosylation at Cys^{869} impedes autophosphorylation of Thr^{838}. Further studies are needed to investigate a possibility that the inhibition of Thr^{838} autophosphorylation by *S*-nitrosylation at Cys^{869} might be the mechanism by which NO suppresses a physical association between ASK1 and its substrates.

NO regulates stress-activated MAPK signaling pathways under various conditions (31–35). Given that ASK1 is a proximal component of such signaling pathways that result in the activation of JNK or p38, the thiol-redox regulation of ASK1 by NO may be an efficient means by which to modulate the activities of these pathways. We also recently showed that NO inhibits JNK through *S*-nitrosylation of a specific cysteine residue in this protein (19). Our previous and present findings together thus suggest that NO regulates stress-activated signaling by *S*-nitrosylation of two target proteins: ASK1 and JNK. Our present results also suggest that the negative regulation of ASK1 by IFN-γ might contribute to some of the biological effects of this cytokine in cells that express iNOS.

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