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S-Nitrosation Regulates the Activation of Endogenous Procaspase-9 in HT-29 Human Colon Carcinoma Cells*

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Nitric oxide-mediated signals have been suggested to regulate the activity of caspases negatively, yet literature has provided little direct evidence. We show in this paper that cytokines and nitric-oxide synthase (NOS) inhibitors regulate *S*-nitrosation of an initiator caspase, procaspase-9, in a human colon adenocarcinoma cell line, HT-29. A NOS inhibitor, N^G-methyl-L-arginine, enhanced the tumor necrosis factor-α (TNF-α)-induced cleavage of procaspase-9, procaspase-3, and poly-(ADP-ribose) polymerase, as well as the level of apoptosis. N^G-Methyl-L-arginine, however, did not affect the cleavage of procaspase-8. These results suggest that nitric oxide regulates the cleavage of procaspase-9 and its downstream proteins and, subsequently, apoptosis in HT-29 cells. Labeling *S*-nitrosated cysteines with a biotin tag enabled us to reveal *S*-nitrosation of endogenous procaspase-9 that was immunoprecipitated from the HT-29 cell extracts. Furthermore, the treatment with TNF-α, as well as NOS inhibitors, decreased interferon-γ-induced *S*-nitrosation in procaspase-9. Our results show that *S*-nitrosation of endogenous procaspase-9 occurs in the HT-29 cells under normal conditions and that denitrosation of procaspase-9 enhances its cleavage and consequent apoptosis. We, therefore, suggest that *S*-nitrosation regulates activation of endogenous procaspase-9 in HT-29 cells.

A family of caspases (cysteine-containing aspartate-specific proteases) is a key operator in the apoptotic process (1, 2). Based on molecular ordering, apoptotic caspases are generally categorized into initiator (*e.g.* caspase-8, -9) and executioner (*e.g.* caspase-3, -6, -7) caspases (3, 4). Inactive procaspases, existing as latent zymogens under normal conditions, become cleaved into their active forms composed of two large subunits and two small subunits either autocatalytically or via other activated caspases during apoptotic signaling pathways (4). In a death receptor-mediated apoptotic pathway, binding of tumor necrosis factor-α (TNF-α)[1] to its cognate receptor triggers a cascade of protein-protein interactions, forming a death-inducing signaling complex (5). Procaspase-8 becomes recruited to the death-inducing signaling complex (6–9) and undergoes autocleavage because of its increased local concentration, which is explained by a proximity-induced model (10). In type I cells, a large amount of activated caspase-8 directly cleaves executioner caspases such as caspase-3 (3, 11, 12). On the other hand, a lower level of caspase-8 formed in type II cells leads to further downstream events that mediate the release of cytochrome *c* from mitochondria into cytosol (13–17), which, in turn, activates another initiator caspase, procaspase-9 (18, 19). In the presence of cytochrome *c* and dATP (20, 21), apoptotic protease-activating factor-1 (Apaf-1) binds to procaspase-9 via a caspase activation recruitment domain (22), forming a complex called the apoptosome (20, 21, 23–27). In the apoptosome, caspase-9 is activated to process other downstream caspases including caspase-3 (19, 28–30). Active executioner caspase-3 can further cleave downstream substrates involved in apoptotic changes (31–37), such as poly-(ADP-ribose) polymerase (PARP) (38). Likewise, the cleavage of procaspases, an irreversible post-translational modification, has been used as an indicator of apoptosis. On the other hand, reversible modifications, such as phosphorylation (39, 40) or *S*-nitrosation (41–47), have been implied to inactivate procaspases, although only a few reports on direct identification of these modifications in endogenous procaspases are available. Considering that caspases are key mediators of the apoptotic process, identifying any regulatory modifications of these proteases is crucial to elucidate mechanisms of cellular balancing between survival and death.

The role of nitric oxide in apoptosis has been controversial and multi-faceted. Thus, depending on not only cell types but also concentration and duration of nitric oxide produced, nitric oxide can function as either a pro- or anti-apoptotic factor (48–50). The general consensus is that normal levels of nitric oxide protect cells whereas abnormal production of nitric oxide results in cell death. Also, nitric oxide-induced *S*-nitrosation of proteins, including receptors (51–53), kinases (54), G-proteins (55–57), redox regulatory proteins (58), transcription factors (59–61), and extracellular matrix proteins (62), has been reported as a regulatory modification in cell signaling pathways (63, 64) including the apoptotic process. *S*-Nitrosation of caspases has also been suggested to decrease their activity or cleavage in diverse cell types treated with nitric oxide donors or nitric-oxide synthase inhibitors, although these studies did not directly demonstrate *S*-nitrosation (41–47, 65, 66). On the other hand, *S*-nitrosation of recombinant active caspase-3 treated with a nitric oxide donor, *S*-nitroso-*N*-acetyl-penicillamine (SNAP), was identified by electrospray ionization mass spectrometry (67). This report, however, raises the issue of relevance to endogenous caspases in the cells and did not characterize the modification site. Nitrosation of the active-site cysteine in endogenous procaspase-3 and its denitrosation

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[1] The abbreviations used are: TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; NOS, nitric-oxide synthase; NMA, N^G-methyl-L-arginine; GSNO, *S*-nitrosoglutathione; SNAP, *S*-nitroso-*N*-acetyl-penicillamine; Apaf-1, apoptotic protease-activating factor-1; PARP, poly-(ADP-ribose) polymerase; biotin-HPDP, *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide; iNOS, inducible NOS.

through Fas signaling were observed in various immune cells by using photolysis chemiluminescence (68). Also, the relation of *S*-nitrosation to cellular localization of procaspase-3 was addressed (69). These reports applied more direct methods to endogenous procaspase-3 compared with previous literature. One concern is that they used the whole immunoprecipitates of procaspases, and, therefore, it is possible that *S*-nitrosation could be detected also from other proteins precipitated with procaspase-3.

Combining the separation of the components precipitated with procaspase-9 by molecular weight and a labeling method for *S*-nitrosated cysteine, we were able to visualize *S*-nitrosation of endogenous procaspase-9. Furthermore, nitric-oxide synthase inhibitors and a death signal decreased *S*-nitrosation of procaspase-9. These results suggest that denitrosation of endogenous procaspase-9 enhances its cleavage and consequently apoptosis.

EXPERIMENTAL PROCEDURES

Cell Culture and Chemical Treatment—Human colon epithelial adenocarcinoma cell line, HT-29 (generously provided from the Peter Sorger laboratory in the Department of Biology, MIT), was maintained in McCoy's 5A medium (Invitrogen) supplemented with 10% fetal bovine serum and L-glutamine (Invitrogen) at 37 °C under 5% CO_2. Cells were seeded onto culture plates at a density of $5 \times 10^4/cm^2$ and grown for 24 h. Then, 200 units/ml of interferon-γ (IFN-γ) (Roche Applied Science) was applied for 24 h to sensitize the cell line to death signals (70, 71), followed by treatment with 50 ng/ml of TNF-α (Peprotech, Rocky Hill, NJ) for the indicated hours. Cells were co-treated with TNF-α and nitric oxide inhibitors, N^G-methyl-L-arginine (NMA) (Sigma), 1400w, L-N^5-(1-iminoethyl)-ornithine, and L-thiocitrulline (Calbiochem), whereas *S*-nitrosoglutathione (Sigma) was applied for 2 h before TNF-α treatment. In the Biotin Switch Method, cells were treated with NMA or 1400w for 4 h following 24 h of IFN-γ treatment.

Cell Death Assay—Apoptosis was measured with cell death detection enzyme-linked immunosorbent assay plus (Roche Applied Science) according to the manufacturer's instruction. Briefly, cell lysates equivalent to 10^3 cells were incubated with both anti-histone antibody labeled with biotin and anti-DNA antibody conjugated with peroxidase in streptavidin-coated microplates for 2 h. Microplate wells were washed and incubated with substrates for colorimetric measurement at wavelength 405 nm with reference at 490 nm.

Western Blotting—Cells lysates were prepared in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.5% Igepal, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 1 μg/ml leupeptin, 1 μg/ml bestatin, 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 14,000 × *g* for 30 min. Total protein concentration in the supernatant was measured by the BCA protein assay (Pierce). Equal amounts of proteins were then separated in 15% Tris-HCl gel (Bio-Rad) except 10% gel for PARP by using a Bio-Rad mini-SDS-PAGE system and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked in TBS buffer (20 mM Tris-HCl, 150 mM NaCl) with 0.1% Tween 20 and 5% non-fat milk and then incubated with a primary antibody at 4 °C overnight. Anti-mouse caspase-8 antibody, anti-rabbit cleaved caspase-9 antibody, anti-rabbit cleaved caspase-3 antibody (Cell Signaling Technology, Beverly, MA), anti-mouse PARP antibody, anti-mouse caspase-9 antibody, and anti-mouse Apaf-1 antibody (BD Biosciences) were used as primary antibodies. After washing with TBS buffer with 0.1% Tween 20, the membranes were incubated in the blocking buffer with secondary anti-IgG antibody conjugated with horseradish peroxidase (Pierce) for 1 h. The membranes were then developed with supersignal West Femto substrate (Pierce).

Immunoprecipitation—50 μl of polyclonal anti-rabbit procaspase-9 antibody (BD Biosciences) was immobilized onto 150 μl of Aminolink plus coupling gel beads (Pierce) according to the manufacturer's instruction and stored in the same volume of phosphate-buffered saline. For control beads, normal rabbit serum was immobilized under the same conditions. Cell lysates containing 5 mg of total protein were incubated with 20 μl of the immobilized antibody at 4 °C overnight. After centrifugation, the supernatant was removed, and the beads were washed with TBS buffer containing 0.1% Igepal and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Sigma) extensively. Bound proteins were eluted by 0.1 M of glycine (pH 2.9) and immediately neutralized with ammonium hydroxide.

Detection of S-Nitrosation by the Biotin Switch Method—The procedure was performed according to the protocol by Jaffrey and Snyder (72, 73). Briefly, eluates of procaspase-9 immunoprecipitation and rabbit muscle creatine phosphokinase (Sigma) as controls were, respectively, incubated with 20 mM methyl methanethiosulfonate (Sigma) followed by acetone precipitation. Precipitates were centrifuged and resuspended in HENS buffer (25 mM Hepes-NaOH, 0.1 mM EDTA, 0.01 mM neocuproine, and 1% SDS) and then incubated with 1 mM ascorbic acid and 4 mM *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (biotin-HPDP) (Pierce) for 1 h. Because biotin-HPDP is cleavable under the reducing conditions, prepared samples were loaded onto SDS-PAGE gels without dithiothreitol. Biotinylated samples were then detected by blotting as described above except that 3% bovine serum albumin was used in the blocking buffer instead of non-fat milk, incubation with primary antibody was omitted because of the biotin labeling, and Neutravidin conjugated with horseradish peroxidase (Pierce) was used instead of secondary antibody. If necessary, each membrane was reprobed with anti-mouse caspase-9 antibody by Western blotting.

RESULTS

N^G-Methyl-L-Arginine Enhanced the TNF-α-induced Apoptosis of HT-29 Cells—Treatment with the nitric-oxide synthase (NOS) inhibitor, NMA, enhanced the level of apoptosis induced by TNF-α at the 24-h point (Fig. 1*A*). Also, treatment with NMA alone induced apoptosis of IFN-γ-sensitized cells. The cell death assay detects only the end point of apoptosis, so we did not observe significant increases of cell death at earlier time points. Our results demonstrated that blocking the production of nitric oxide enhanced the effect of a death signal, suggesting, in turn, that endogenously produced nitric oxide can protect cells. Next, we examined the effects of a nitric oxide donor, *S*-nitrosoglutathione (GSNO), on apoptosis. We used GSNO, because it is a more likely endogenous nitric oxide donor in the cells than other agents. Treatment with GSNO decreased the induction of apoptosis by TNF-α and/or NMA but did not show statistical significance (Fig. 1*B*). The partial inhibitory effect by GSNO could result from its poor permeability into the cells. Another NOS inhibitor, 1400w, also enhanced the TNF-α-induced apoptosis (Fig. 1*C*).

Nitric-oxide Synthase Inhibitors Enhanced the TNF-α-induced Cleavage of Caspases—Fig. 2 shows that co-treatment with NMA and TNF-α enhanced the cleavage of procaspase-9, -3, and PARP compared with the treatment with TNF-α alone at both the 8- and 24-h time points. However, co-treatment with NMA did not affect the cleavage of procaspase-8. Procaspase-3 and PARP are well known downstream substrates of caspase-9 and -3, respectively, meaning that their cleavage can represent the activity of upstream enzymes. Therefore, NMA treatment up-regulated both the cleavage and activity of caspase-9 and -3. Treatment with NMA alone induced the cleavage of procaspase-9 in IFN-γ-sensitized cells (data not shown). These observations suggest that nitric oxide-mediated signals act on the downstream events of caspase-8, which include the activation of procaspase-9. Also, other NOS inhibitors, *i.e.* 1400w, L-N^5-(1-iminoethyl)-ornithine, and L-thiocitrulline, enhanced the cleavage of procaspase-9 by TNF-α (data not shown). In addition, pretreatment with GSNO as a nitric oxide donor decreased the cleavage of procaspase-9, -3, and PARP by TNF-α, again not affecting procaspase-8, and reversed the effect of NMA to enhance the cleavage of procaspase-9, -3, and PARP by TNF-α (data not shown).

The Biotin Switch Method Visualized S-Nitrosation of Procaspase-9—The results mentioned above indicated that altering the level of cellular nitric oxide by using nitric oxide-related chemicals affected the cleavage of caspases, as well as the level of apoptosis. Nitric oxide-mediated signals thus are apparently involved in regulating the cleavage of procaspase-9 and its downstream proteins and consequent apoptosis. Previous reports have implicated *S*-nitrosation of caspases as a negative

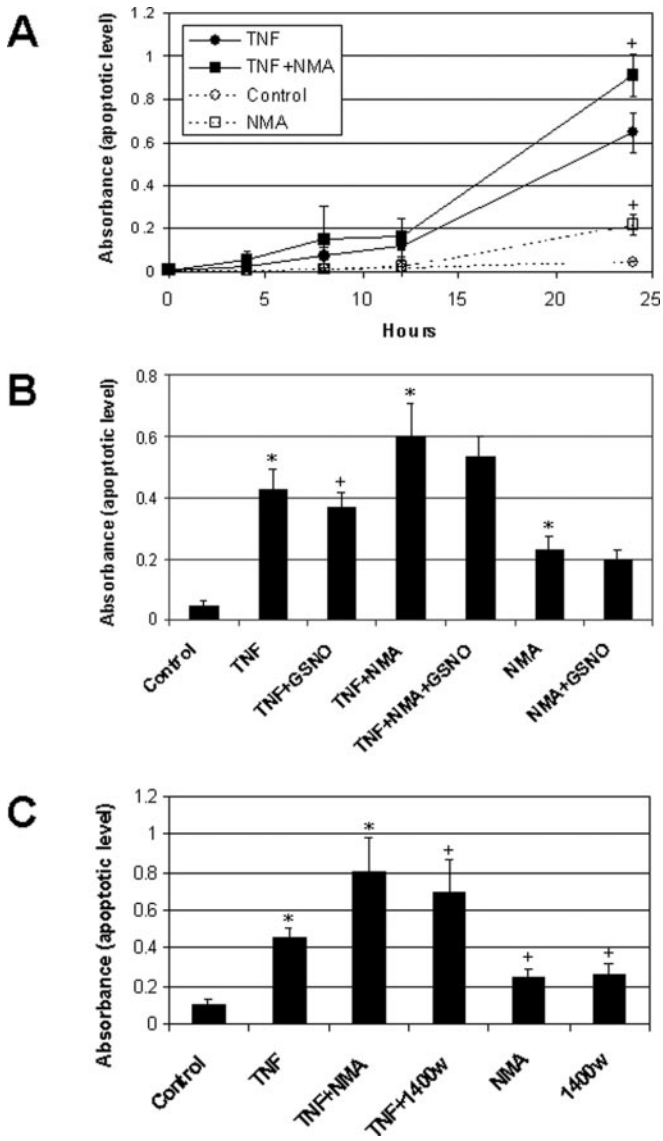

FIG. 1. **The effects of nitric-oxide synthase inhibitors on the level of apoptosis induced by TNF-α.** The apoptotic level was measured by enzyme-linked immunosorbent assay for cytosolic histone-associated DNA fragment. *Panel A*, cells were treated with 50 ng/ml of TNF-α and/or 5 mM of NMA for the indicated hours following treatment with 200 units/ml of IFN-γ for 24 h. *Panel B*, cells were treated with 50 ng/ml of TNF-α and/or 5 mM of NMA for 24 h and, in some cases, pretreated with 100 μM of GSNO for 2 h following treatment with 200 units/ml of IFN-γ for 24 h. *Panel C*, cells were treated with 50 ng/ml of TNF-α and/or 5 mM of NMA or 20 μM of 1400w for 24 h following treatment with 200 units/ml of IFN-γ for 24 h. In all the graphs, * represents that the value is statistically significant at $p < 0.01$ level. + represents that the value is statistically significant at $p < 0.05$ level. Statistical significance was tested with paired Student's t test with $n = 8$.

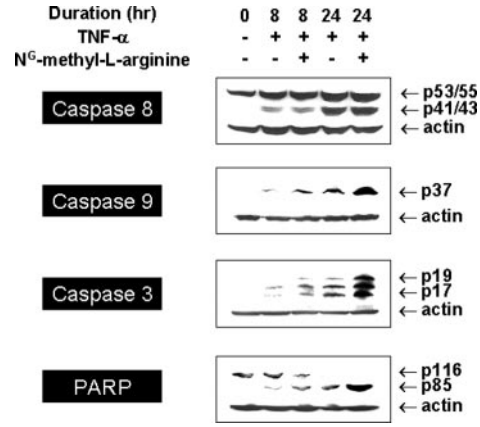

FIG. 2. **The effects of nitric-oxide synthase inhibitors on the cleavage of caspases by TNF-α.** The intact and/or cleaved form of each protein was detected by Western blotting from the cells incubated with 50 ng/ml of TNF-α and 5 mM of NMA for 8 or 24 h following treatment with 200 units/ml of IFN-γ for 24 h. *p53/55* of caspase-8 and *p116* of PARP are their intact forms. *p41/43* of caspase-8, *p37* of caspase-9, *p17/19* of caspase-3, and *p85* of PARP represent their cleaved forms. The figures represent three similar experimental results.

regulatory modification (41–47). Because caspase-9 is an upstream caspase, and its cleavage was enhanced by the treatment with NOS inhibitors, we focused on visualizing *S*-nitrosation of endogenous procaspase-9 by using a more direct tool, the Biotin Switch Method (72, 73). Because of the process of biotin labeling and lack of reducing agents in the sample loading buffer for electrophoresis, bands of blotting after the Biotin Switch Method tend to show more streaking and bending during electrophoresis than usual Western blotting bands. We applied this method to the procaspase-9 immunoprecipitates from the cell extracts of HT-29 cell line pretreated with IFN-γ. IFN-γ is known to up-regulate apoptosis-related genes in our cell line (74), so we used this cytokine to increase the level of low-abundance apoptotic proteins such as procaspase-9. Protein bands representing *S*-nitrosation appeared at the size of procaspase-9 (47 kDa) and higher molecular mass (Fig. 3*A*, *left*), which was shown to be a complex of procaspase-9 formed during the immunoprecipitation process, most likely via disulfide bonds (data not shown). Reprobing the membrane in the *left column* of Fig. 3*A* with caspase-9 antibody showed that bands of *S*-nitrosation corresponded to those of procaspase-9 (Fig. 3*A*, *right*). Cross reactivity of the antibody was not detected in Western blotting of procaspase-9 following its immunoprecipitation (Fig. 3*B*). Also, pretreatment of the procaspase-9 immunoprecipitates with ascorbic acid before the Biotin Switch Method, to reduce *S*-nitrosated cysteines (72, 73), resulted in weaker or no *S*-nitrosation than the un-pretreated sample (Fig. 4*A*). In addition, no *S*-nitrosation was visible without the biotin tag (Fig. 4*B*). These results indicated that combining immunoprecipitation and the Biotin Switch Method enabled us to visualize *S*-nitrosation of procaspase-9. There was a possibility that procaspase-9 formed complexes with other proteins, and *S*-nitrosation in higher molecular weight could result also from those proteins. Accordingly, we detected Apaf-1, the only protein known to bind to the proform of caspase-9, forming the apoptosome during the apoptotic process (20, 21, 23–27). Dithiothreitol, which was not present in the sample loading buffer to maintain the cleavable biotin tag intact, was used in *lanes 2*, *4*, and *6* to reduce disulfide bonds that may have occurred during the immunoprecipitation step. Apaf-1 was detected in the original cell extracts. However, as expected, it did not co-immunoprecipitate with procaspase-9, because the cells were not stimulated with apoptotic agents (Fig. 4*C*). To avoid the possibility of artificial *S*-nitrosation by acidified nitrite during elution from the antibody, immunoprecipitates were extensively washed to remove possible nitrite from cell extracts. Also, a control experiment with creatine phosphokinase under the same conditions as that with procaspase-9 showed that elution condition did not affect the level of *S*-nitrosation. In summary, we could demonstrate *S*-nitrosation of endogenous procaspase-9 by using immunoprecipitation and the Biotin Switch Method.

Nitric-oxide Synthase Inhibitors and an Apoptotic Agent Decreased S-Nitrosation of Procaspase-9, Which Was Enhanced by IFN-γ Treatment—Because NOS inhibitors enhanced the TNF-α-induced cleavage of procaspase-9, we separated the effects of NOS inhibitors and TNF-α. Because the Biotin Switch Method

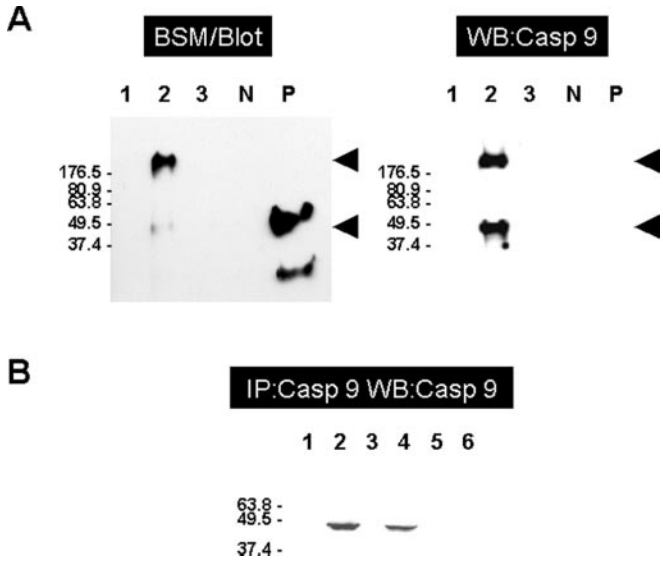

FIG. 3. **The Biotin Switch Method visualizes *S*-nitrosation of procaspase-9.** *Panel A*, *left*, the Biotin Switch Method (*BSM*) was applied to the procaspase-9 immunoprecipitates. *S*-Nitrosated proteins labeled with biotin tag were visualized by blotting with Neutravidin conjugated with horseradish peroxidase. *Lane 1*, cell extracts precipitated with control beads; *lane 2*, cell extracts precipitated with antibody-immobilized beads; *lane 3*, no cell extracts precipitated with antibody-immobilized beads; *lane N*, negative control, creatine phosphokinase treated with vehicle and prepared by the Biotin Switch Method; *lane P*, positive control, creatine phosphokinase treated with 1 mM SNAP and prepared by the Biotin Switch Method. *Panel A*, *right*, the *left column* was reprobed with caspase-9 antibody. *Panel B*, immunoprecipitation (*IP*) of procaspase-9 followed by Western blotting (*WB*) with caspase-9 (*Casp 9*) antibody. *Lane 1*: cell extracts precipitated with control beads; *lane 2*, cell extracts precipitated with antibody-immobilized beads; *lane 3*, no cell extracts precipitated with antibody-immobilized beads; *lane 4*, supernatant of *lane 1*; *lane 5*, supernatant of *lane 2*; *lane 6*, supernatant of *lane 3*.

is not quantitative, we normalized the density of the *S*-nitrosation band to that of the procaspase-9 band reprobed with caspase-9 antibody to compare the effects of different chemicals. Compared with untreated cells, treatment with IFN-γ enhanced *S*-nitrosation of procaspase-9, which was decreased by both NMA and 1400w (Fig. 5). These results suggest that *S*-nitrosation of procaspase-9 may be regulated by a NOS induced by IFN-γ. The other interesting observation is that we could visualize *S*-nitrosation of procaspase-9 in the untreated cells, suggesting that a constitutive level of *S*-nitrosation may protect cells. In addition, *S*-nitrosation of procaspase-9 was decreased at the 12-hour point of the incubation with TNF-α (Fig. 6, *BSM/Blot*). At the same time, we also observed the cleaved forms of caspase-9, -3, and PARP, whereas the level of Apaf-1, the activator of caspase-9, was not changed (Fig. 6). Western blotting of procaspase-9 did not reveal a significant change, implying that only a small fraction of procaspase-9 becomes cleaved by TNF-α (data not shown). These results suggest that TNF-α leads to denitrosation of procaspase-9, promoting its cleavage.

DISCUSSION

TNF-α induces apoptosis through its receptor-mediated signaling pathway, in which upstream caspases, caspase-8 and -9, and a downstream executioner, caspase-3, become activated via cleavage. Considering that these caspases are key mediators of apoptosis, endogenous regulatory mechanisms, such as post-translational modifications, for their activation must be crucial in maintaining the cellular balance. An anti-apoptotic role of nitric oxide via a post-translational modification, *S*-nitrosation, of proteins including caspases has been suggested. Pro-

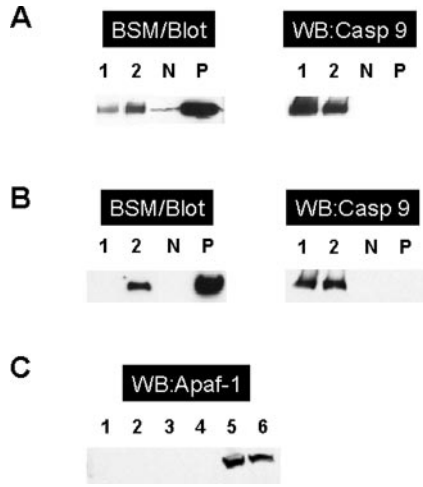

FIG. 4. **The confirmation of *S*-nitrosation in procaspase-9.** *Panel A*, the effect of pretreatment with ascorbic acid before the Biotin Switch Method (*BSM*). *Left*, *lane 1*, immunoprecipitates of procaspase-9 pretreated with ascorbic acid before the Biotin Switch Method; *lane 2*, immunoprecipitates of procaspase-9 followed by the Biotin Switch Method; *lane N*, negative control, creatine phosphokinase treated with vehicle and prepared by the Biotin Switch Method; *lane P*, positive control, creatine phosphokinase treated with 1 mM SNAP and prepared by the Biotin Switch Method. *Panel A*, *right*, the *left column* was reprobed with caspase-9 (*Casp 9*) antibody. *Panel B*, the detection of biotin labeling is specific. *Left*, *lane 1*, immunoprecipitates of procaspase-9 followed by the Biotin Switch Method without biotin-HPDP; *lane 2*, immunoprecipitates of procaspase-9 followed by the Biotin Switch Method with biotin-HPDP; *lane N*, negative control, creatine phosphokinase treated with vehicle and prepared by the Biotin Switch Method; *lane P*, positive control, creatine phosphokinase treated with 1 mM SNAP and prepared by the Biotin Switch Method. *Panel B*, *right*, the *left column* was reprobed with caspase-9 antibody. *Panel C*, *Apaf-1* was not precipitated with procaspase-9. Apaf-1 was detected by Western blotting (*WB*). *Lane 1*, immunoprecipitates of procaspase-9 followed by the Biotin Switch Method and loaded onto electrophoresis gel without dithiothreitol; *lane 2*, immunoprecipitates of procaspase-9 followed by the Biotin Switch Method and loaded onto electrophoresis gel with dithiothreitol; *lane 3*, immunoprecipitates of procaspase-9 loaded onto electrophoresis gel without dithiothreitol; *lane 4*, immunoprecipitates of procaspase-9 loaded onto electrophoresis gel with dithiothreitol; *lane 5*, cell extracts without dithiothreitol; *lane 6*, cell extracts with dithiothreitol.

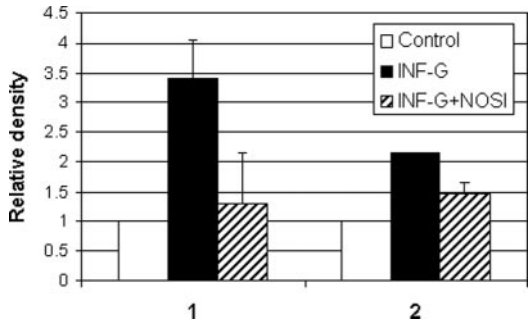

FIG. 5. **The effects of nitric-oxide synthase inhibitors on *S*-nitrosation of procaspase-9.** The Biotin Switch Method was applied to immunoprecipitates of procaspase-9 followed by blotting of biotin-labeled proteins. The *graph* shows the relative level of *S*-nitrosation in procaspase-9. The density of *S*-nitrosation measured by using Scion Image software (Scion Corp., Frederick, MD) was normalized to that of the procaspase-9 band from Western blotting. *Control*, no treatment; *INF-G*, 200 units/ml of IFN-γ; *IFN-G+NOSI*, 200 units/ml of IFN-γ with 5 mM of NMA (*1*, *left side* of the *graph*) or 20 μM of 1400w (*2*, *right side* of the *graph*).

caspase-3 has been the major target for the detection of endogenous *S*-nitrosation (68). Also, *S*-nitrosation was detected in recombinant procaspase-8 added to hepatocyte lysates (43), whereas our results show that the application of a NOS inhibitor, NMA, did not affect the cleavage of endogenous pro-

caspase-8 in HT-29 cells. On the other hand, nitric-oxide synthase inhibitors enhanced the TNF-α-induced cleavage of an upstream initiator, procaspase-9, which led us to focus on visualizing its *S*-nitrosation. We were able to detect *S*-nitrosation of endogenous procaspase-9 from HT-29 cell extracts by combining immunoprecipitation and labeling endogenously nitrosated cysteine(s). Our data also demonstrated that NOS inhibitors not only enhanced the TNF-α-induced cleavage of procaspase-9, but also decreased *S*-nitrosation of procaspase-9 based on the Biotin Switch Method. Furthermore, treatment with TNF-α decreased *S*-nitrosation of procaspase-9, whereas it induced the cleavage of procaspase-9 and apoptosis. These results demonstrate that TNF-α triggers the cleavage of procaspase-9 via its denitrosation and imply that denitrosation is part of the regulatory mechanism during the apoptotic process (Fig. 7). Also, procaspase-9 was *S*-nitrosated in untreated cells, which suggests that nitric oxide-mediated signals may constitutively protect HT-29 carcinoma cells via *S*-nitrosation of procaspase-9.

Because treatment with IFN-γ enhanced *S*-nitrosation, an important question is which NOS regulated by IFN-γ is involved in increasing *S*-nitrosation of procaspase-9. IFN-γ-dependent expression of inducible nitric-oxide synthase (iNOS) has been observed in diverse cell types (75, 76). Particularly, co-treatment with interleukin-1α and IFN-γ increased the gene expression of iNOS in HT-29 cells (77). We thus examined the level of iNOS protein, as well as the alteration of *S*-nitrosation of procaspase-9 by these cytokines. iNOS was detected by Western blotting in the cells treated with both interleukin-1α and IFN-γ for 24 h but not with each cytokine alone (data not shown). Also, we could detect neither neuronal nitric-oxide synthase nor endothelial nitric-oxide synthase, although potential inhibitors of neuronal nitric-oxide synthase and endothelial nitric-oxide synthase enhanced the cleavage of procaspase-9 by TNF-α (data not shown). Our inability to detect an NOS by Western blotting suggests that the source of nitric oxide may come from a very low level of an NOS compartmentalized with procaspase-9. Such compartmentalization could also explain why *S*-nitrosoglutathione only partially reversed the effects of NMA on apoptosis (Fig. 1*B*), possibly because of the limited accessibility of GSNO to a location of procaspase-9 and its instability because of the reactivity inside of the cells. Recent research has attempted to identify a new NOS isoform in mitochondria (77–82). Whether a NOS exists in mitochondria of HT-29 cells is not known, but it could be a candidate for *S*-nitrosation of procaspase-9 in our cell line. The problem of identifying a regulatory NOS isoform is thus also related to the cellular localization of both the proform and cleaved form of caspase-9. Mannick *et al.* have attempted to answer this question (69), concluding that *S*-nitrosation of procaspase-3 did not affect its localization, although a larger fraction of caspase-3 and -9 was *S*-nitrosated in mitochondria than in cytosol. This conclusion implies that *S*-nitrosation of procaspase-3 and -9 occurs in the mitochondria. They also reported heme-nitrosation of cytochrome *c* (83), further supporting a role of a mitochondrial NOS in the regulation of apoptosis. Therefore, the identification of an NOS in mitochondria and its relation to *S*-nitrosation of proteins will provide important information on the regulation of apoptosis. The present limitation is lack of a specific antibody to detect mitochondrial NOS. Antibodies to three pre-existing NOS isoforms have been used to detect mi-

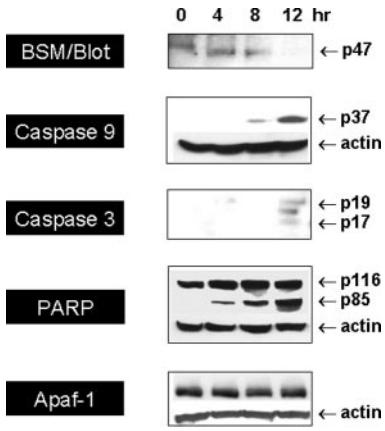

FIG. 6. **The effect of TNF-α on *S*-nitrosation of procaspase-9.** *BSM/Blot*, the Biotin Switch Method was applied to immunoprecipitates of procaspase-9 from HT-29 cells treated with TNF-α for the indicated hours following the treatment with 200 units/ml of IFN-γ. The rest of *blots*, the intact and/or cleaved forms of each protein were detected by Western blotting from the cells incubated with 50 ng/ml of TNF-α for the indicated hours following the treatment with 200 units/ml of IFN-γ.

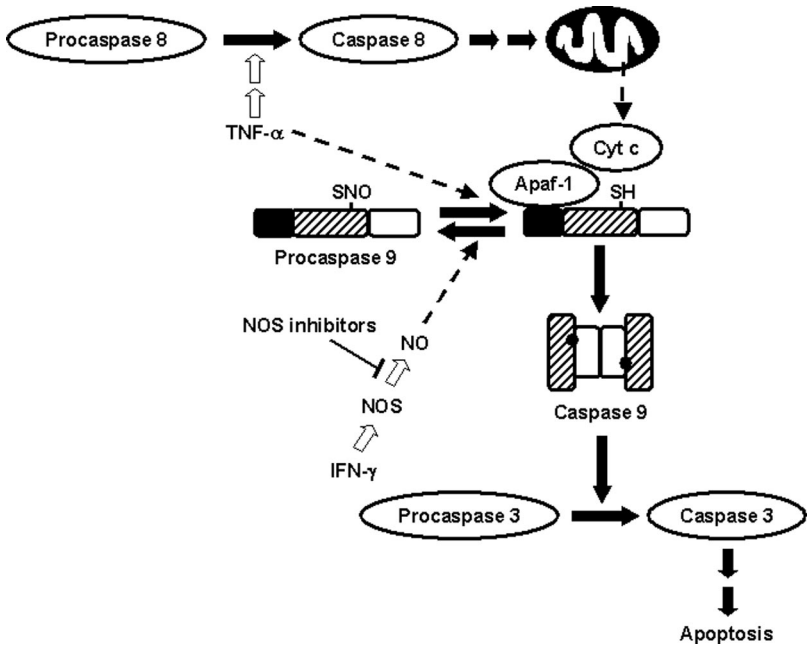

FIG. 7. **A scheme of the regulation of procaspase-9 by cytokines.** TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; Cyt c, cytochrome C; Apaf-1, apoptotic protease-activating factor-1; NOS, nitric-oxide synthase; NO, nitric oxide; SNO, *S*-nitroso; SH, sulfhydryl.

tochondrial NOS (78, 80, 81), but other investigators have failed to confirm these results (82).

Characterization of intracellular nitrosating agents, as well as the mechanism of protein denitrosation, remains as important questions. We used NOS inhibitors to block the sources of cellular nitrosating agents. N_2O_3 and nitrosated thiols originating from nitric oxide have been suggested as nitrosating agents (63, 64, 84), but the actual mechanism is not fully understood. Our results show that TNF-α triggers a signaling pathway that leads to denitrosation. Similarly, another death signal, Fas ligand, reduces *S*-nitrosation of procaspase-3 measured by photolysis chemiluminescence (68). Therefore, death signals seem to cause denitrosation, but the molecules or steps involved in the mechanism of denitrosation remain to be characterized. The level of Apaf-1, an activator of procaspase-9, was not affected by TNF-α treatment, whereas procaspase-9 became cleaved. Denitrosation may promote the interaction of procaspase-9 with Apaf-1 via conformational change.

Because of limitations in techniques, direct identification of *S*-nitrosation in proteins has been difficult. Currently, the Biotin Switch Method (72, 73) is the best technique to visualize the modification, although this method does not provide quantitative information. *S*-Nitrosation sites in proteins have also been deduced from mutations of cysteine moieties, particularly active site cysteines. In these types of experiments, protein activity or interactions were measured after the mutations, but the modification sites have not been identified directly. We have been focusing on the direct identification of *S*-nitrosation sites in endogenous procaspase-9 using mass spectrometry with the challenge of detecting a potential modification site of a peptide in a low-abundance protein.

Using a biotin labeling method combined with immunoprecipitation, we were able to visualize *S*-nitrosation of endogenous procaspase-9 in the HT-29 cell line. We suggest that nitric oxide-mediated signals induced by IFN-γ protect cells from apoptosis, via *S*-nitrosation of procaspase-9, which then is removed during the apoptotic process induced by TNF-α as outlined in Fig. 7. *S*-Nitrosation could be a major negative regulatory mechanism to explain the role of nitric oxide in protecting cells from apoptosis. Also, denitrosation could be one of apoptotic events induced by TNF-α to speed up the cleavage of procaspase-9.

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