Nitric Oxide Inhibits Apoptosis Downstream of Cytochrome *c* Release by Nitrosylating Caspase 9[1]

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ABSTRACT

Inhibition of the mitochondrial pathway of apoptosis has been implicated as a mechanism contributing to carcinogenesis. Chronic inflammation, which is accompanied by activation of inducible nitric oxide synthase and generation of nitric oxide (NO), is associated with cancer development in a variety of gastrointestinal diseases, including cholangiocarcinoma. Therefore, we examined the effects of NO on the mitochondrial pathway of apoptosis in human cholangiocarcinoma cell lines. Transfection with inducible NO synthase inhibited etoposide-induced apoptosis. *S*-Nitroso-*N*-acetyl-D,L-penicillamine (SNAP), a pharmacological NO donor, did not prevent mitochondrial cytochrome *c* release as assessed by immunoblot analysis or cellular localization of cytochrome *c*-green fluorescent protein. In contrast, SNAP did prevent activation of caspase 9 in etoposide-treated cells. Furthermore, SNAP also blocked caspase 9 activation in a cell-free system and reversibly inhibited catalytic activity of human recombinant caspase 9. As assessed by the Saveille reaction, immunoprecipitated procaspase 9 from SNAP-treated cells released 6-fold more NO than untreated cells, confirming that cellular procaspase 9 is susceptible to nitrosylation. In conclusion, NO inhibits apoptosis downstream of cytochrome *c* release by directly blocking caspase 9 activation.

INTRODUCTION

Cholangiocarcinoma is a highly malignant, generally fatal neoplasm originating from the bile duct epithelial cells or cholangiocytes of the intra- and extrahepatic biliary system (1). Worldwide, cholangiocarcinoma is associated with chronic inflammatory conditions involving the biliary tract including hepatolithiasis, liver fluke infestation, choledochal cysts, and primary sclerosing cholangitis (2). In Western countries, primary sclerosing cholangitis is the main identifiable risk factor for the development of cholangiocarcinoma, and up to 30% of patients with this disease develop cholangiocarcinoma (3). Although it is well known that the above chronic inflammatory conditions involving the bile ducts predispose patients to the development of cholangiocarcinoma, the cellular mechanisms linking chronic inflammation to malignant transformation are unclear.

Although cancers, even of the same cell type such as cholangiocarcinoma, appear to be genetically diverse and biologically heterogeneous, current concepts suggest that only a few "mission critical" events are required for the development of cancers (4). These critical events permit deregulated cell proliferation and suppression of cell death and authorize cell invasion and spread to diverse tissue structures. Of these critical processes, we have been particularly interested in the mechanisms linking chronic inflammation and suppression of apoptosis.

Apoptosis, a form of cell death characterized by stereotypic morphological changes, is responsible for removing genetically altered cells (4). Apoptosis is mediated by activation of caspases, a family of cysteine proteases (5–7). Caspase activation occurs by one of two broad mechanisms, involving death receptors and/or mitochondrial cytochrome *c* release (8, 9). Although either or both pathways can be disrupted in established malignancies, dysregulation of the mitochondrial pathway appears to be very important in cancer development and therapy (10). In this pathway, apoptotic signals are initiated by the release of cytochrome *c* from the mitochondria (9). Cytosolic cytochrome *c* then binds to Apaf-1, a scaffolding protein that facilitates activation of procaspase 9 in the presence of ATP or dATP (11). Once activated, caspase 9 cleaves caspases 3 and 7, resulting in their activation and subsequent stereotypic apoptotic events. Disruption of the mitochondrial apoptotic pathway in melanoma cells with loss of Apaf-1 expression has been demonstrated recently (12). This recent observation highlights the potential importance of inhibiting the mitochondrial apoptotic pathway in carcinogenesis. However, inhibition of the mitochondrial apoptotic pathway by inflammatory mediators is an unexplored potential mechanism linking chronic inflammation to epithelia carcinogenesis.

NO is a candidate inflammatory mediator causing disruption of apoptosis:

(*a*) It is often generated in inflammatory conditions attributable to the induction of NOS[3] in epithelial cells by inflammatory cytokines (13). Because it is induced, this enzyme is referred to as iNOS to distinguish it from endothelial NOS and neuronal NOS (14). iNOS is capable of generating relatively large amounts of NO compared with endothelial NOS and neuronal NOS (15).

(*b*) Although controversial, NO can inhibit apoptosis in a variety of cell types including epithelial cells (16). The mechanisms postulated for NO inhibition of apoptosis include disruption of transcription factor-dependent Fas ligand expression (17), inhibition of TNF-α-induced poptosis (18), cGMP-dependent signaling cascades (19), modulation of mitochondrial function (20), and direct nitrosylation of caspases 3 and 8 (21–23). The ability of NO to nitrosylate thiols in the active sites of caspases and inhibit their activity is likely a potent mechanism for NO modulation of apoptosis (24). Despite the potential importance of NO-mediated disruption of apoptosis, fundamental information is lacking regarding the specific effects of NO on the mitochondrial pathway of apoptosis, especially its potential effects on caspase 9 activation. Caspase 9 would be an important potential target of NO because its inactivation has been shown to contribute to cell transformation (25).

On the basis of this information, we formulated the central hypothesis that induction of NOS and subsequent NO production will result in inhibition of the mitochondrial pathway of apoptosis. To begin to test this hypothesis, we addressed the following specific questions: (*a*) Does NO inhibit the mitochondrial pathway of apoptosis? (*b*) Does NO inhibit caspase 9 activation in cells? (*c*) Does NO inhibit mitochondrial cytochrome *c* release? and (*d*) Does NO directly block caspase 9 activation by nitrosylating the proenzyme? We chose human cholangiocarcinoma cell lines for these studies because they respond to inflammatory cytokines with iNOS induction, duplicating

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[3] The abbreviations used are: NOS, nitric oxide synthase; iNOS, inducible NOS; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine; AMC, 7-amino-4-methylcoumarin; GFP, green fluorescent protein; TMRM, tetramethylrhodamine methyl ester; LEHD-AMC, Ac-Leu-Glu-His-Asp 7-amino-4-methylcoumarin; DEVD-AMC, Ac-Asp-Glu-Val-Asp 7-amino-4-methylcoumarin; Apaf-1, apoptotic protease activating factor-1.

the expression of iNOS observed in inflammatory diseases of the human biliary tract (26). Furthermore, the link between chronic inflammation of the biliary tract and the subsequent development of cholangiocarcinoma is clinically established (2). Our data suggest that NO inhibits apoptosis downstream of cytochrome c release by directly blocking caspase 9 activation. NO appears to inhibit caspase 9 directly, likely by nitrosylating the procaspase. These data suggest that inhibition of iNOS induction and/or catalytic activity in inflammatory diseases could be a potential chemopreventive strategy to reduce the risk of cholangiocarcinoma and other human cancers arising from the gastrointestinal mucosa.

MATERIALS AND METHODS

Cell Lines, Cultures, and Treatments. KMBC cells (a kind gift of Dr. Kujiro, Kurume University, Kurume, Japan) and Mz-ChA-1 human cholangiocarcinoma cells (kind gift of Dr. Greg Fitz, Colorado State University, Denver, CO) were cultured in DMEM containing 10% fetal bovine serum. For the studies described here, apoptosis was induced with 10 μM quinidine (to inhibit etoposide efflux by the multidrug resistance protein; Ref. 27) plus 68 μM etoposide for 48 h or 1 μM staurosporine for 4 h. SNAP, 300 μM, was used as a pharmacological NO donor.

Transfection of KMBC Cells with iNOS. KMBC and Mz-ChA-1 cells were transiently transfected using Lipofectamine, according to the instructions of the supplier (Life Technologies, Inc., Gaithersburg, MD). GFP, 1 μg/ml, and 2 μg/ml of the pcDNA3 expression vector containing iNOS cDNA (a kind gift of Dr. T. Billiar, University of Pittsburgh, Pittsburgh, PA) were used for transfection. At 48 h after transfection, 10 μM quinidine and 68 μM etoposide (Sigma Chemical Co., St. Louis, MO) were added. After 48 h of exposure to etoposide, the rate of apoptosis was assessed in GFP-iNOS-transfected and GFP-only-transfected cells (green-labeled cells).

Quantitation of Apoptotic Cells. Apoptosis was quantitated by assessing the nuclear changes of apoptosis using the nuclear binding dye DAPI (5 μg/ml) and fluorescence microscopy.

Immunoblots and Antisera. Whole-cell lysates were prepared as described previously (28) and were diluted in SDS sample buffer to achieve a final concentration of 5 μg/μl. Samples containing 50 μg of protein were heated to 65°C for 5 min and were loaded on a SDS-polyacrylamide gel containing 5–15% acrylamide gradient. At the end of electrophoresis, polypeptides were transferred to nitrocellulose and probed with specific antibodies against caspases 3 and 9. β-Actin served as a control to assure equal protein loading. Bound antibody was visualized using chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL) and exposed to Kodak X-OMAT film.

Caspase Activity Assays. Cytosolic extracts were prepared as described previously (29). Caspase 3/7-like activity in cytosolic extracts was measured using the fluorogenic substrate DEVD-AMC, whereas the activity of human recombinant caspase 9 was determined using LEHD-AMC. Caspase activity was measured, using 100 μM DEVD-AMC or LEHD-AMC in a buffer consisting of 25 mM HEPES, 5 mM MgCl$_2$, 1 mM EGTA, and 1 mM GST. Fluorescence was quantitated in a filter fluorometer (Model 450; Sequoia-Turner, Mountain View, CA) using excitation and emission wavelengths of 360 and 420 nm, respectively. Standard curves were generated with AMC.

Cell-Free System for Caspase 3 and 9 Activation. HL-60 cell cytosol was prepared as described previously (29). Cell-free caspase activation reactions (40-μl final volumes) were assembled on ice in 96-well microtiter plates by adding a final concentration of 1 mM ATP and 0.5 μg/μl cytochrome c to cytosolic extracts (50 μg protein). Reactions were initiated by placing the microtiter plate in a humidified 30°C incubator. After incubation for the selected time periods, reaction mixtures were harvested for immunoblotting to assess caspase 3 and caspase 9 activation.

Detection of Cytochrome c-GFP Release from Mitochondria. Release of cytochrome c from mitochondria was assessed using both morphological and biochemical approaches.

The expression vector pEGFP-cytochrome c (a gift of Dr. Anna-Liisa Nieminen, Case Western Reserve University, Cleveland, OH) encodes a polypeptide that contains GFP fused to the COOH-terminal of cytochrome c. Mz-ChA-1 and KMBC cells (80% subconfluent) were transiently transfected using Lipofectamine (Life Technologies, Inc.). One ml of a mixture containing 10 μl of Lipofectamine and 2 μg of pEGFP-cytochrome c plasmid was used for the transfection. At 48 h after transfection, cells were treated with either 300 μM SNAP for 3 h and then with staurosporine (1 μM) for 4 h or with staurosporine only. Mitochondria were identified using the fluorescent cationic dye TMRM. The cells were also stained with the nuclear binding dye DAPI (5 μg/ml). Confocal microscopy was performed using a laser scanning confocal microscope (LSM 510; Carl Zeiss, Inc., Thornwood, NY). The excitation and emission wavelengths for GFP were 488 and 505 nm, for DAPI 360 and 385 nm, and for TMRM 543 and 580 nm, respectively.

To detect cytosolic cytochrome c biochemically, immunoblots were prepared using cytosolic fractions of cells (29). Samples containing 40 μg of protein were loaded after heat denaturation onto a 15% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane, and cytochrome c was detected using a specific antibody. β-Actin served as a control to assure equal protein loading.

Immunoprecipitation of Caspase 9. Mz-ChA-1 cells were harvested and coeluted by centrifugation at 800 rpm for 5 min. After washing the pellet twice with cold PBS, cells were lysed by incubation for 20 min in lysis buffer (150 mM NaCl, 50 mM HEPES, 10% glycerol, 5 mM MgSO$_4$, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml trasylol, 1% thiodiglycol, and 1% Triton X-100). After centrifugation at 12,000 rpm for 15 min, the protein concentration of the supernatant was measured, and 5 mg of cytosolic protein were immunoprecipitated using procaspase 9-conjugated Sepharose beads (a kind gift of Y. Lazebnik, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY). After overnight incubation on an end-over-end shaker at 4°C, the beads were washed four times with cold radioimmunoprecipitation assay buffer [1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate (pH 7.2), and 2 mM EDTA]. Polypeptides were released from beads by adding 50 μl of SDS sample buffer to the beads and incubating them at 65°C for 20 min. Beads were sedimented at 12,000 rpm for 2 min, and supernatant was recovered for SDS-PAGE analysis.

Saville Reaction. Quantification of the NO group, displaced from the thiol by mercuric ion, forms the basis of this sensitive method (30, 31). Briefly, after immunoprecipitation, procaspase 9 was released from the beads by adding 50 μl of buffer containing 0.06 M Tris and 2% SDS and incubation at 65°C for 20 min. The samples then were treated with HgCl$_2$ (0.2%, dissolved in a buffer containing 1% sulfanilamide in 0.5 M HCl). Samples were incubated for 5 min to allow the formation of the diazonium salt. A 0.02% solution of N-(1-naphtyl)ethylenediamine dihydrochloride dissolved in 0.5 M HCl was then added, and the azo dye formation was detected by spectrophotometry. Background nitrate concentration was deducted by performing a parallel experiment, where HgCl$_2$ is omitted from the sulfanilamide solution.

Statistical Analysis. All data represent at least three experiments using cells, or extract from a minimum of three separate isolations, and are expressed as the mean $\pm$ SE unless otherwise indicated. Differences between groups were compared using an ANOVA for repeated measures and a *post hoc* Bonferroni test to correct for multiple comparisons. All statistical analyses were performed with the statistical software package InStat from GraphPAD (San Diego, CA).

Reagents. The pharmacological NO donor, SNAP, and L-N-monomethyl arginine citrate were from the Caymen Chemicals (Ann Arbor, MI). Staurosporine, etoposide, quinidine, DAPI, and cytochrome c were from Sigma Chemical Co. (St. Louis, MO). TMRM was from Molecular Probes (Eugene, OR). The fluorogenic substrates DEVD-AMC and LEHD-AMC were from Peptides International (Osaka, Japan). Rabbit antisera recognizing the neo-epitopes of activated caspases 3 and 9, generated using synthetic peptides (CIETD and CPEPD, respectively), coupled to keyhole limpet hemocyanin, were obtained from Dr. Scott Kaufmann (Mayo Clinic, Rochester, MN). Antibodies against procaspases 3 and 9 and cytochrome c were derived from Transduction Laboratories (Lexington, KY). Peroxidase-coupled, affinity-purified secondary antibodies were from Kirkegaard & Perry (Gaithersburg, MD).

RESULTS

NO Inhibits the Mitochondrial Pathway of Apoptosis. KMBC and Mz-ChA-1 cells were transfected with iNOS for 24 h before
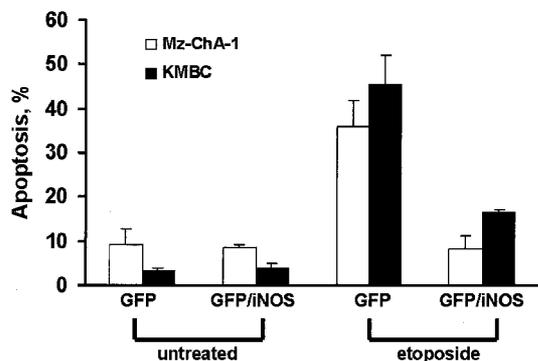
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Fig. 1. iNOS inhibits etoposide-induced apoptosis. Mz-ChA-1 and KMBC cells were transiently transfected with 1 µg/ml GFP and 2 µg/ml of the pcDNA3 expression vector containing iNOS cDNA, or GFP only, using the Lipofectamine method. At 24 h after transfection, 68 µM etoposide and 10 µM quinidine were added. After 48 h of exposure to etoposide, the rate of apoptosis was assessed in transfected (GFP-positive) cells, using the nuclear binding dye DAPI, and fluorescent microscopy.

treating with etoposide [known to induce apoptosis by the mitochondrial pathway (32)] and quinidine for an additional 48 h (quinidine was used to block efflux of etoposide via P-glycoprotein; Refs. 27, 33). After 48 h of treatment with etoposide/quinidine, $36 \pm 6\%$ and $45 \pm 7\%$ of Mz-ChA-1 and KMBC cells, respectively, transfected with GFP displayed the morphological features of apoptosis (Fig. 1). In contrast, the rate of apoptosis was reduced by >65% in the iNOS and GFP cotransfected cells ($36 \pm 6\%$ *versus* $8 \pm 3\%$ for the Mz-ChA-1 cells and $45 \pm 7\%$ *versus* $16 \pm 1\%$ for the KMBC cells; $P < 0.01$). These composite data implicate NO as a potential inhibitor of the mitochondrial apoptotic pathway.

NO Inhibits Caspase 9 Activation in Cells. We next measured caspase catalytic activity in Mz-ChA-1 cells treated with the pharmacological NO donor SNAP (26), obtaining cytosolic extracts and performing caspase catalytic assay using the fluorogenic substrate DEVD-AMC. During treatment of these cells with staurosporine or etoposide/quinidine (both agents induce the mitochondrial apoptotic pathway), caspase activity increased during apoptosis but was reduced during treatment with SNAP (Fig. 2A). To more directly examine the polypeptides responsible for the caspase activity, we performed im-

munoblot analysis for active caspases 3 and 9 (Fig. 2B). Active subunits for caspases 3 and 9 were readily detected in etoposide/quinidine-treated cells. However, treatment with SNAP blocked etoposide activation of caspases 3 and 4. Because caspase 9 is upstream of caspase 3 in the apoptotic pathway, NO appears to inhibit apoptosis by blocking caspase 9 activation.

NO Does Not Block Cytochrome *c* Release from Mitochondria. In the mitochondrial pathway of apoptosis, caspase 9 is activated by cytosolic cytochrome *c*. Therefore, the effect of NO on cellular cytochrome *c* compartmentation was examined after treatment with etoposide/quinidine in the absence and presence of SNAP (Fig. 3A). As assessed by immunoblot analysis, cytosolic cytochrome *c* was observed during treatment with etoposide/quinidine despite treatment with SNAP. To exclude potential loss of mitochondrial integrity during fractionation, we also assessed the cellular compartmentation of cytochrome *c* in cytochrome *c*-GFP transfected KMBC and Mz-ChA-1 cells (Fig. 3B). In control cells, cytochrome *c*-GFP fluorescence colocalized with the mitochondrial dye TMRM (data not shown). We used staurosporine as a proapoptotic agent in these experiments because it induces apoptosis already after 4 h of treatment, whereas induction by etoposide takes 48 h. After treatment with staurosporine (34, 35), with or without SNAP, there was a redistribution of cytochrome *c*-GFP fluorescence from a punctate to a diffuse pattern in both cell lines, consistent with its release from mitochondria into the cytosol. On the basis of these observations, NO does not appear to prevent caspase 9 activation by inhibiting cytochrome *c* release from mitochondria.

NO Inhibits Caspase 9 Activation in Cell-Free Systems. The above results suggested that NO inhibits caspase 9 activation downstream of cytochrome *c* release. To more directly test this interpretation, a cell-free system was used to ascertain whether NO inhibits activation of caspase 9 after addition of cytochrome *c*. Previous findings have demonstrated that incubation of cytosolic extracts with ATP and cytochrome *c* results in caspase 9 and 3 activation in a time-dependent manner (29). If the cytosol was preincubated with SNAP, however, no activation of caspases 9 and 3 was observed (Fig. 4A). These observations suggested that NO might be inhibiting caspase 9 activation directly. Indeed, SNAP also directly inhibited
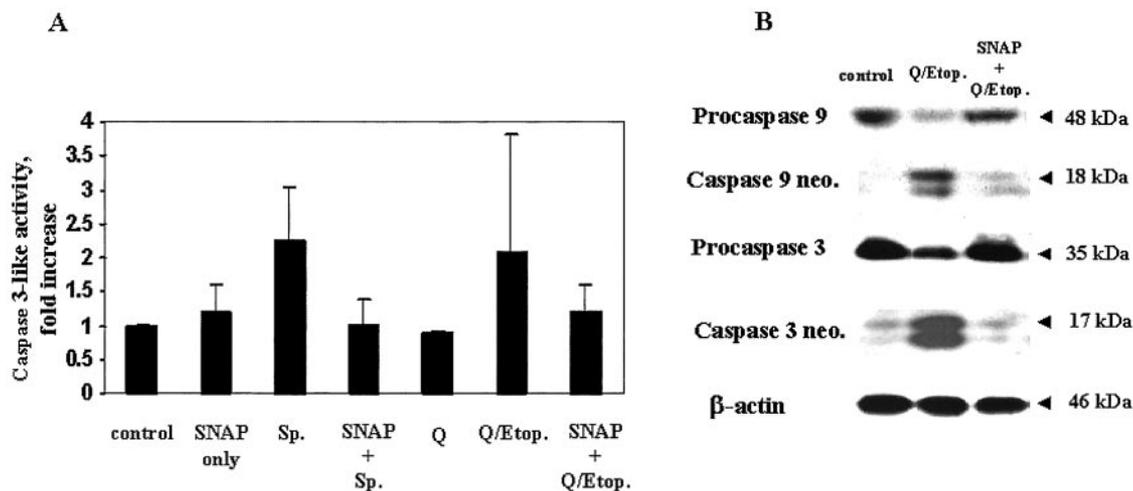

Fig. 2. NO inhibits caspase 9 activation in cells as assessed by caspase catalytic activity (*A*) and immunoblot analysis (*B*). *A,* cytosolic extracts were prepared from Mz-ChA-1 cells as described previously (29). Caspase activity was measured using DEVD-AMC as fluorogenic substrate. Enzyme activity in control (untreated) cells was set arbitrarily as 1. Treatment of these cells with staurosporine or etoposide/quinidine (both agents induce the mitochondrial apoptotic pathway), caspase activity increased during apoptosis but was reduced during treatment with SNAP. Only quinidine did not induce significant apoptosis. *Bars,* SD. *B,* whole-cell lysates were prepared from each experimental condition as described previously (28). Samples were loaded on 5–15% density gradient polyacrylamide SDS gels. At the end of electrophoresis, transfer of proteins and immunoblotting with specific antibodies detecting the pro-form and the active large subunit of caspases 3 and 9 (*Caspase 3 neo.* and *Caspase 9 neo.,* respectively) were performed. Immunoblotting for β-actin assured equal loading of the lanes. Note that in the presence of SNAP, the major form of caspase 9 is the inactive pro-form. *Sp,* staurosporine; *Etop,* etoposide; *Q,* quinidine.
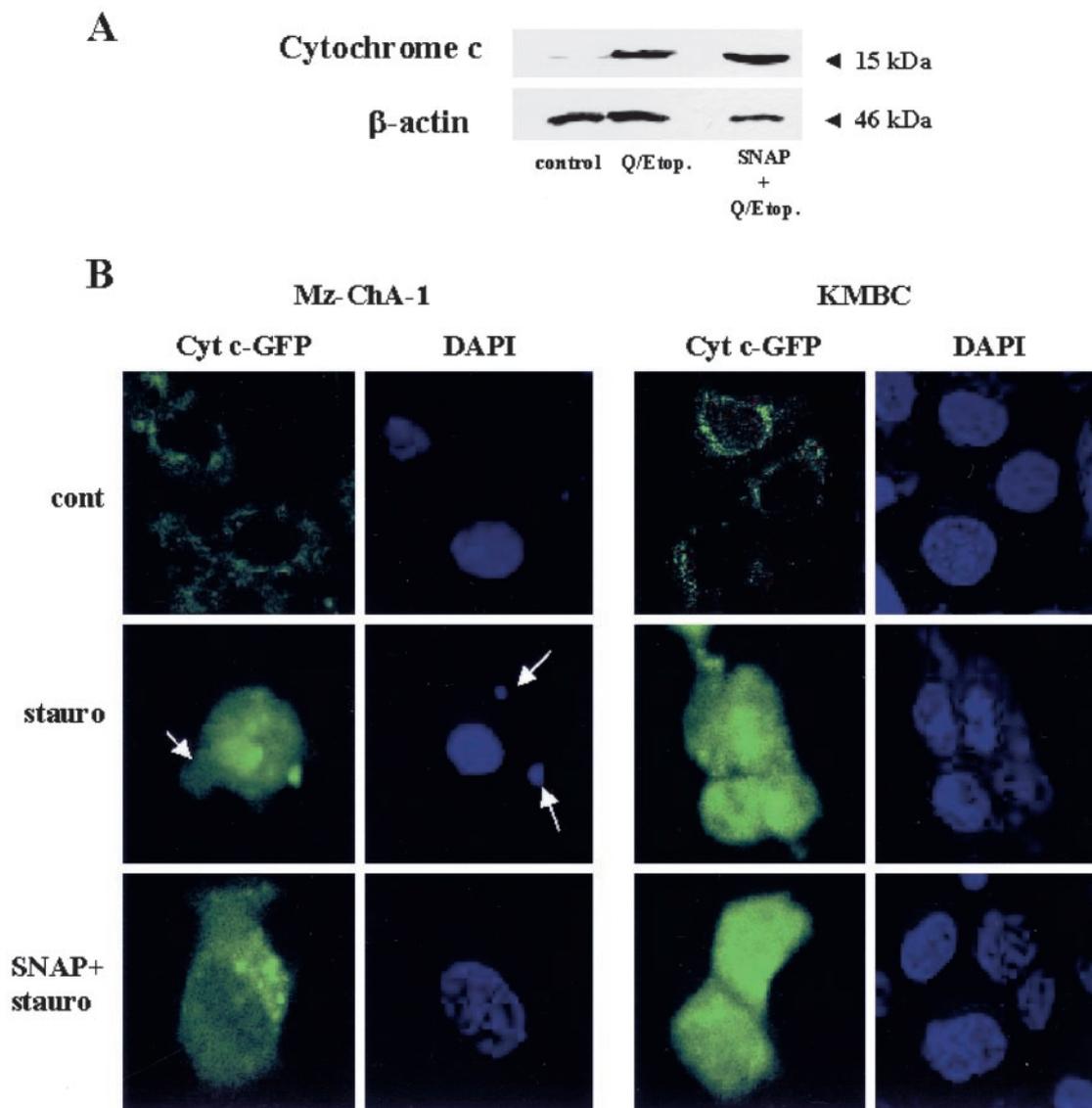
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Fig. 3. Cytochrome *c* release from mitochondria is not inhibited by NO. *A*, Western blot analysis was performed on cytosolic extracts of Mz-ChA-1 cells, after treating cells with 68 μM etoposide or SNAP plus etoposide. Note that NO does not inhibit cytochrome *c* release. Equal loading of the lanes was assured by blotting for β-actin. *Etop,* etoposide; *Q*, quinidine. *B*, Mz-ChA-1 and KMBC cells were transfected with cytochrome *c*-GFP. Forty-eight h after transfection, cells were incubated with 1 μM staurosporine, with or without previous incubation in 300 μM SNAP. Cells were stained with DAPI and viewed under confocal microscopy. Apoptosis (*arrows*, apoptotic nuclei) and cytochrome *c* release were assessed, and these data demonstrate that NO prevents nuclear fragmentation but not cytochrome *c* release.

human recombinant caspase 9 catalytic activity in the absence of cellular constituents (Fig. 4*B*). This inhibition was reversed by DTT, which is known to reduce S-NO bonds and restore activity of nitrosylated enzymes. This result suggests that NO is capable of nitrosylating caspase 9 and inhibiting its activity.

Caspase 9 Can Be Nitrosylated in Cells. To provide further evidence that caspase 9 is nitrosylated in cells, we treated cells with SNAP, lysed the cells, and determined whether the immunoprecipitated caspase 9 protein contained NO. This analysis indicated that immunoprecipitated procaspase 9 from SNAP-treated cells released 6-fold more NO (0.064 μmol/mg protein) than untreated cells (0.011 μmol/mg protein; Fig. 5), confirming that procaspase 9 is susceptible to nitrosylation *in situ*.

DISCUSSION

In the present study, we have demonstrated that: (*a*) NO inhibits the mitochondrial pathway of apoptosis; (*b*) NO inhibits apoptosis down-stream of cytochrome *c* release; and (*c*) NO inhibits caspase 9 activation in cells, in a cell-free system, and in an *in vitro* assay using recombinant caspase 9. These data indicate that NO may inhibit the mitochondrial pathway of apoptosis by nitrosylating caspase 9. These results not only have implications for the mechanisms of carcinogenesis arising in the background of chronic inflammation but also suggest prevention strategies for human cancer. Each of these findings is discussed in greater detail below.

The role of NO in modulating apoptosis has been controversial. Many investigators have found that NO potentiates apoptosis (36), whereas others have observed NO inhibition (17, 22) of apoptosis. These differences have been attributed to the concentrations of NO used, the redox status of the cell, the cell type, and the context of the cell. Recently, Kim *et al.* (37) have suggested that cellular non-heme iron content is a determinant of the effects of NO on cells. Cells with low levels of non-heme iron (*e.g.,* macrophage cell lines) appear susceptible to NO-mediated cytotoxicity, whereas cells with increased

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concentrations of non-heme iron (*e.g.,* hepatocytes) are resistant to NO-mediated cell death and other forms of cell death by apoptosis. This was attributed to the formation of intracellular dinitrosyl-iron complexes upon NO exposure, which suppressed caspase activity. Our current studies using multiple models of apoptosis in human cholangiocarcinoma cell lines suggest that these cells, such as hepatocytes, the other epithelial cell type in the liver, are protected from cell death by NO.

NO has been postulated to inhibit apoptosis by a variety of mechanisms including inhibition of transcription factor-dependent Fas ligand expression (17), cGMP-dependent signaling cascades (19), and direct nitrosylation of caspases 3 and 8 (21–23). It was also shown that in melanoma cells endogenously produced NO has antiapoptotic effects (38). The models of apoptosis used in the present study are not Fas dependent, and NO exerted its inhibitory effect on apoptosis even when soluble guanylyl cyclase (ODQ) and PKG (KT5823) were blocked (data not shown). Therefore, we pursued the possibility that NO inhibits apoptosis by nitrosylating caspase 9. Our studies using the cell-free system and recombinant enzyme caspase 9 suggest that caspase 9 can be directly nitrosylated. Evidence for *in vivo* caspase 9 nitrosylation was provided by demonstration of the release of NO by $HgCl_2$ from immunoprecipitated caspase 9. Collectively, these data suggest that NO may also inhibit apoptosis by nitrosylating caspase 9.

The current study demonstrated NO inhibition of apoptosis downstream of cytochrome *c* release. The critical commitment step in the
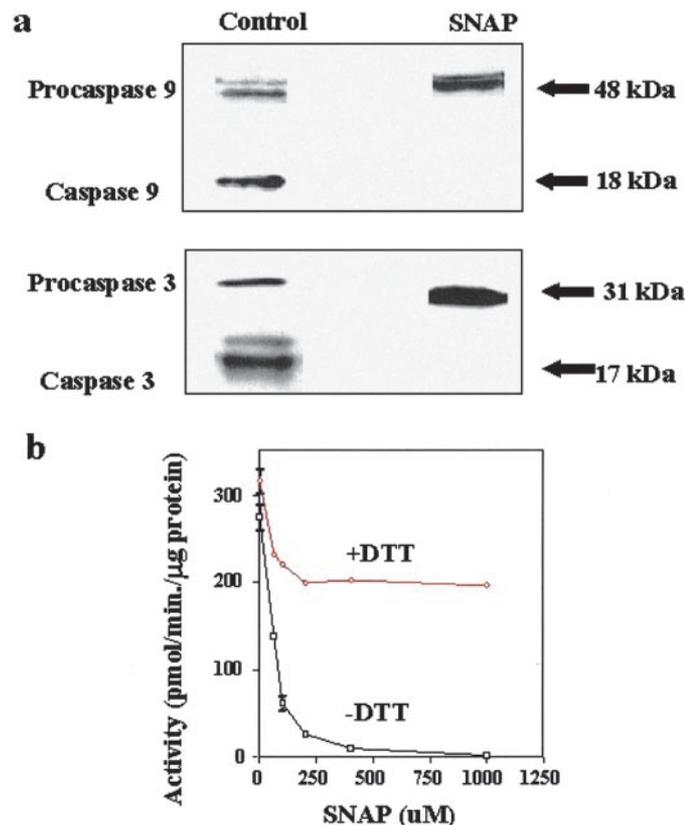

Fig. 5. NO directly binds to the thiol groups of procaspase 9. Immunoprecipitated procaspase 9 from control and SNAP (300 μM)-exposed Mz-ChA-1 cells was subjected to $HgCl_2$ treatment in a sulfanilamide solution. Background nitrate concentration was deducted by performing a parallel experiment, where $HgCl_2$ was omitted from the sulfanilamide solution. Procaspase 9 immunoprecipitated from SNAP-treated cells released more NO than control cells (0.064 μmol/mg immunoprecipitated protein *versus* 0.011 μmol/mg immunoprecipitated protein ($P < 0.05$). The *inset* shows that equal amounts of procaspase 9 were tested in the SNAP-treated and control experiments. *Bars,* SE.

mitochondrial pathway of apoptosis has not been firmly established. Although mitochondrial dysfunction with cytochrome *c* release has been suggested as the commitment step for apoptosis (9), there are several recent findings suggesting that caspase 9 activation by Apaf-1 and ATP (dATP) represents the linchpin step in this cascade. For example, microinjection of cytochrome *c* into cells is not universally associated with apoptosis (39), and similarly, cytochrome *c* release from the mitochondria does not uniformly induce apoptosis (40, 41). Heat-shock proteins 27 and 70 have also been shown to inhibit apoptosis after cytochrome *c* release (18) by interacting with cytosolic cytochrome *c* or preventing recruitment of procaspase 9 to the apoptosome complex. Caspase 9-deleted embryonic stem cells are also resistant to apoptosis despite translocation of cytochrome *c* to the cytosol (42). These observations suggest that caspase 9 activation is essential for, and likely represents, the commitment step for the mitochondrial pathway of apoptosis. Nitrosylation of caspase 9 by iNOS-generated NO would appear to be another mechanism negatively regulating this pathway of apoptosis.

Dysregulation of apoptosis has been postulated to contribute to carcinogenesis (10, 43, 44). Because many growth-promoting oncogenes induce apoptosis (oncogene-dependent apoptosis), apoptosis must be inhibited for some oncogenes to transform cells. Oncogene-dependent apoptosis is mediated by caspase 9 (45), and deletion of caspase 9 will substitute for p53 loss in promoting the oncogenic transformation of Myc-expressing cells (25). Our current studies suggest that the inflammatory mediator NO can disrupt caspase 9 function and in this respect would potentially dysregulate apoptosis and promote carcinogenesis.

Although it is tempting to speculate that the reactive inflammatory mediator NO mechanistically links chronic inflammation to carcinogenesis, additional studies are required to evaluate its true physiological importance. Additional studies using various models of carcinogenesis induced by chronic inflammation in wild-type and iNOS knockout mice should not only provide a more complete picture of the role of iNOS in this process but also evaluate iNOS inhibitors as potential chemopreventive drugs for reducing the occurrence of chronic inflammation-associated cancers.

ACKNOWLEDGMENTS

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Fig. 4. NO inhibits caspase 3 and 9 activation in cytosol under cell-free conditions. *a,* cytosol was prepared as described previously (29). Cell-free caspase activation reactions (40-μl final volumes) were assembled on ice in 96-well microtiter plates by adding 1 mM ATP, 0.5 μg/μl cytochrome *c* (at final concentration) to the cytosolic extracts (50 μg of protein). After incubation for the selected time periods, reaction mixtures were harvested for immunoblots to assess caspase 3 and 9 activation. *b,* SNAP reversibly inhibits caspase 9 activation; 1 unit of human recombinant caspase 9 was incubated in the presence of increasing concentrations of SNAP for 1 h at room temperature. Caspase activity was measured using the fluorogenic substrate LEHD-AMC (caspase 9). In parallel experiments, 5 mM DTT was added to the incubation buffer after SNAP.
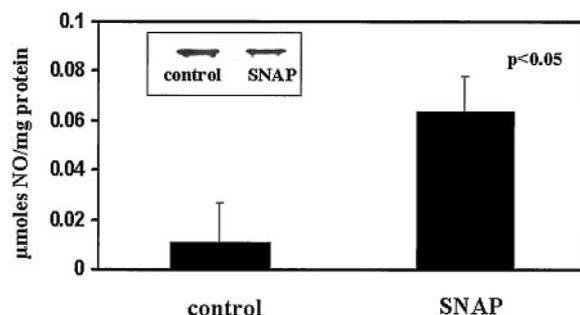
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Nitric Oxide Inhibits Apoptosis Downstream of Cytochrome *c* Release by Nitrosylating Caspase 9

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