Nitric oxide (NO) has emerged as an important endogenous inhibitor of apoptosis, and here we report that NO prevents hepatocyte apoptosis initiated by the removal of growth factors or exposure to TNFα or anti-Fas antibody. We postulated that the mechanism of the inhibition of apoptosis by NO would include an effect on caspase-3-like protease activity. Caspase-3-like activity increased coincident with apoptosis due to all three stimuli, and treatment with the caspase-3-like protease inhibitor N-acetyl-Asp-Glu-Val-Asp-aldehyde inhibited both proteolytic activity and apoptosis. Endogenous or exogenous sources of NO prevented the increase in caspase-3-like activity in hepatocytes. Exposure of purified recombinant caspase-3 to an NO or NO donor inhibited proteolytic activity. Dithiothreitol (DTT), but not glutathione, reversed the inhibition of recombinant caspase-3 by NO. When lysates from cells stimulated to express inducible NO synthase or cells exposed to NO donors were incubated in DTT, caspase-3-like activity increased to about 55% of cells not exposed to a source of NO. Similarly, administration of an NO donor to rats treated with TNFα and D-galactosamine also prevented the increase in caspase-3-like activity as measured in liver homogenates. The effect of the NO donor was reversed by about 50% if the homogenate was incubated with DTT. TNFα-induced apoptosis and caspase-3-like activity were also reduced in cultured hepatocytes exposed to 8-bromo-cGMP, and both effects were inhibited by the cGMP-dependent kinase inhibitor KT5823. The suppression in caspase-3-like activity in hepatocytes exposed to an NO donor was partially blocked by an inhibitor of soluble guanylyl cyclase, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, (ODQ), while the incubation of these lysates in DTT almost completely restored caspase-3-like activity to the level of TNFα-treated controls. These data indicate that NO prevents apoptosis in hepatocytes by either directly or indirectly inhibiting caspase-3-like activation via a cGMP-dependent mechanism and by direct inhibition of caspase-3-like activity through protein S-nitrosylation.

Apoptosis, or programmed cell death, is distinguished from lytic or necrotic cell death by specific biochemical and structural events. The apoptotic signals trigger specific signaling pathways, including protease activation, which is followed by the appearance of morphologic changes characteristic of cells undergoing apoptosis such as condensation of nuclei and cytoplasm, blebbing of cytoplasmic membranes, and finally fragmentation into apoptotic bodies that are phagocytosed by neighboring cells (reviewed in Ref. 1). Apoptosis is important to many physiologic processes such as cell selection in development and immunologic responses (2), control of organ size in maturation and regeneration (reviewed in Ref. 3), and normal cell turnover throughout the organism (reviewed in Ref. 4). Dysregulated apoptosis may contribute to pathologic states such as autoimmune disease (5) and malignancy (reviewed in Ref. 6).

Our understanding of the biochemical events in apoptosis was significantly advanced by the description of specific cysteine proteases involved in the initiation and amplification of the cell death signaling cascade induced by TNFα (7) and Fas (8). The first identified cysteine protease, caspase-1 (IL-1β-converting enzyme) (9), is a mammalian counterpart of the gene designated as cd3-3, a protease required for programmed cell death in the nematode Caenorhabditis elegans (10). It is now known that caspase-1 is a member of the caspase family of proteases which consists of at least 10 homologs (11). Ectopic expression of any of the caspase family proteases can cause apoptosis; however, not all caspase enzymes have been definitively linked to apoptosis. Caspase-3-like (CPP32-like) proteases that preferentially cleave the amino acid sequence DEVD of biosubstrates, including poly(ADP-ribose) polymerase (PARP) and DNA-dependent kinase (12), are activated in cells following exposure to apoptogenic stimuli (8, 13). Caspase family genes encode proenzyme forms that require proteolytic cleavage for activation. Recent evidence indicates that death signaling involves the mutual activation of several caspase proteases, which in turn cleave several structural and catalytic proteins, resulting in the cleavage of proteins involved in the cellular repair system (12, 14).

The antiapoptotic effect of compounds that inhibit either the activation or activity of caspase-3-like proteases suggests that...
apoptosis can be regulated by modification of the protease signaling cascade. By inhibiting an upstream event in the activation of caspase family proteases, the oncogene product Bcl-2 prevents cell death during physiologic processes (reviewed in Ref. 15). Viruses encode proteins such as CrmA (cowpox virus) (14) and p35 (baculovirus) (16), which protect cell death by blocking caspase family proteases, probably to support viral replication. It is unclear if inhibition of the proteolytic activity of caspase enzymes represents a natural mode of regulation.

The short lived radical nitric oxide (NO) has recently emerged as a novel and potent inhibitor of apoptosis. Endogenous NO synthesis or exposure to low level NO donors was first shown to inhibit apoptosis in human B lymphocytes (17), and similar findings have been reported in splenocytes (18), eosinophils (19), ovarian follicles (20), cardiac myocytes (21), and endothelial cells (22). NO is a diffusible molecule that is produced in low levels by two constitutive NO synthases (eNOS and nNOS) and in much greater levels by the inducible NO synthase (iNOS) (reviewed in Ref. 23). The ubiquitous distribution of the NO isoforms throughout tissues as well as prominent patterns of transient up-regulation in development (24) and several disease processes (25) implicate NO as an important regulator of cell viability. A cGMP-dependent mechanism contributes to the antiapoptotic actions of NO in some cells (18-20), and cGMP has been shown to up-regulate Bcl-2 expression in splenocytes (18). In other cell types, the antiapoptotic mechanism is clearly independent of cGMP (17).

In this study, we sought to determine if NO prevented apoptosis through the modulation of caspase-3-like protease activity. Because NO can modify protein function through nitrosylation of thiol groups (reviewed in Ref. 26), we tested the hypothesis that NO regulates apoptosis through nitrosylation of cysteine at the active site of caspase-3-like proteases. We utilized hepatocytes for these studies because we (27-29) and others (30, 31) have shown that NO is hepatoprotective. In addition, hepatocytes express iNOS under a diverse array of conditions associated with cell death and turnover (reviewed in Ref. 23), including inflammation, infection, and liver transplantation (32). We show here that NO inhibits apoptosis in hepatocytes not only due to an S-nitrosylation-based inhibition of the catalytic activity of caspase-3-like enzymes but also through a cGMP-dependent mechanism that functions either at or upstream of the level of caspase-3-like protease activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Williams medium E, penicillin, streptomycin, l-glutamine, and HEPES were purchased from Life Technologies, Inc. Murine macrophage NOS2 mononuclear antibody was obtained from Transduction Laboratories (Lexington, KY), and anti-PARP monoclonal antibody C2-10 was purchased from PAPR Metabolism Laboratory (Quebec, Canada). N-Acetyl-Asp-Glu-Val-Asp-aldehyde and N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide were purchased from Peptides International Inc. (Louisville, KY) and Quality Controlled Biochemicals, Inc. (Hopkinton, MA), respectively. Red blood cells were prepared from rat blood by centrifugation at 800 g for 10 min. The LDH activity in cell lysate was measured by using an automated procedure on a Technitron RA-500 autoanalyzer (Technitron).

**Assay of Nitrite and Nitrate**—Nitrite plus nitrate (NO$_2$ + NO$_3$) levels in the culture media were measured using an automated high performance liquid chromatography procedure based on the Griess reaction (36) after deproteinization of culture medium with a double volume of 0.5 M NaOH and 10% ZnSO$_4$. In some experiments, NO$_2$ alone was determined by measuring absorbance at 550 nm after mixing 100 μl of sample with an equal volume of the Griess reagent in a microplate reader.

**Treatment of Caspase-3 with NO and Thiol-reactive Agents and Enzyme Activity Assay**—Recombinant human caspase-3 (rh-caspase-3) was fully activated by incubation with 20 mM DTT for 30 min. The DTT was removed by passing the sample through a Sephadex G-25 column equilibrated in buffer A (100 mM HEPES, pH 7.4, 140 mM NaCl, and the protease inhibitors, including 0.5 mM phenylmethylsulfonyl fluoride, 5 μM/ml aprotinin, 5 μM/ml pepstatin, and 10 μg/ml leupeptin). The sample containing 1 μg/ml of rh-caspase-3 was treated with a NO-generating (SNAP or iNOS)-generating (nNOS) donor (29) or carbonyl cyanide 3-chloropropenyl-1,1,1-trifluoroborate, NTB) donor (1 h) or thiol-reactive agents (40 min) on ice. NTB solution was prepared as described previously (37). For some experiments, oxidized SNAP and NTB were prepared by incubation of solutions at room temperature for 48 h to allow for complete release of NO and NO$_3$, as measured by the Griess reaction in the presence of HgCl$_2$. The enzyme was separated from the excess NO donor or thiol-
reductive agents through a Sephadex G-25 column equilibrated with buffer A. For assaying caspase-3-like protease activity in hepatocytes, monolayers of cultured hepatocytes (5 × 10⁶ cells) were harvested from the cultured 100-mm dish by plastic scraper, washed with ice-cold PBS, and resuspended in 140 μl of buffer A. The cell suspension was lysed by three strokes of freezing and thawing. The crude cytosol was obtained as the supernatant from centrifugation at 12,000 g for 20 min at 4 °C. To measure caspase-3-like activity in whole liver, the frozen tissue was homogenized in buffer A, and the crude homogenate was centrifuged at 12,000 g for 20 min at 4 °C. The enzyme reaction mixture contained 0.08 μg of rh-caspase-3 (or 200 μg of cytosolic protein) and 200 μM Ac-EDVD-pNA in 150 μl of buffer B (100 mM HEPES, pH 7.4, 20% glycerol, and protease inhibitors). The enzyme reaction was initiated by adding the substrate to a 96-well plate containing the enzyme solution incubated at 37 °C. The caspase-3-like activity was calculated from the initial velocity by measuring the increased absorbance at 405 nm every 10 min. The reaction mixture without enzyme or substrate was used as a control.

**Northern and Western Blot Analysis**—Isolation of total RNA and Northern blot analysis of iNOS mRNA levels in cultured hepatocytes was carried out using a murine iNOS cDNA probe, as described previously (38). Membranes used for Northern blot analysis were stripped and reprobed for 18 S RNA to assess RNA loading. Western blot for iNOS protein expression was performed as described previously (29). PARP cleavage was analyzed by Western blot analysis using anti-PARP monoclonal antibody C2–10 as described in the manufacturer’s protocol.

**Synthesis and Cleavage of 35S-Labeled PARP**—The PARP-containing plasmid (39) was purified with a Qiagen Maxiprep kit and used to prepare 35S-labeled PARP by coupled in vitro transcription/translation with T7 polymerase in a reticulocyte lysate system. Each coupled transcription/translation reaction contained 4 μg of plasmid DNA in a final volume of 200 μl in a methionine-free mixture supplemented with 180 μCi/ml [35S]methionine. The mixture was incubated at 30 °C for 1 h, and the labeled proteins were isolated by passage through a Sephadex G-25 column equilibrated with buffer C (10 mM HEPES, pH 7.4, 1.5 mM MgCl2, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, and protease inhibitors). The labeled substrate (6 μl) was incubated with rh-caspase-3 (8 ng), pretreated with 200 μM SNAP in the presence or absence of red blood cells (400 μg oxyhemoglobin) for 1 h or 200 μM thiol-reactive agents for 40 min on ice. Ac-DEVd-cho was added directly into the enzyme reaction mixture. The final reaction volume of 22 μl was incubated at 37 °C for 1 h and then mixed with an equal volume of 2 × SDS-sample buffer. This mixture was boiled for 3 min and then subjected to electrophoresis on an 8% SDS-polyacrylamide gel. After drying, the gel was exposed to x-ray film at room temperature.

**Preparation of S-100 Fraction from Hepatocytes**—Freshly isolated and purified hepatocytes (2 × 10⁶ cells) were washed twice with ice-cold PBS and resuspended in 5 volumes of ice-cold buffer D (20 mM HEPES, pH 7.4, 10 mM MgCl2, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, and protease inhibitors). After incubaing for 20 min, cells were homogenized on ice by six strokes with a tight pestle in a Dounce homogenizer. The nuclei, intact cells, and cell debris were removed by centrifugation at 1,000 × g for 10 min at 4 °C. The supernatant was further centrifuged at 100,000 × g for 1 h in a Beckman 70 Ti rotor. The supernatant (S-100 fraction) was immediately frozen in liquid nitrogen and stored at −80 °C until used for the in vitro apoptosis assay in the reconstitution system (see below).

**Preparation of Rat Hepatocyte Nuclei**—Freshly isolated and purified rat hepatocytes (1.5 × 10⁶ cells) were washed twice with ice-cold PBS and resuspended in 10 volumes of ice-cold buffer D (20 mM HEPES, pH 7.4, 10 mM MgCl2, 15 mM NaCl, 5 mM EDTA, 0.15 mM spermine, 0.15 mM spermidine, 1 mM phenylmethylsulfonyl fluoride, and 25 mM sucrose). Cells were allowed to swell on ice for 20 min and then were homogenized with a Dounce homogenizer. Greater than 90% lysis was confirmed by microscopy. The homogenate was filtered through four layers of cheesecloth and mixed with an equal volume of buffer E containing 2.3 mM sucrose. The homogenates were layered over 5 ml of buffer E containing 2.3 mM sucrose in a Beckman SW28 centrifuge tube and centrifuged at 22,000 rpm for 90 min at 4 °C. The pellets were washed with buffer E at 800 × g for 10 min. The pellets containing the nuclei were resuspended in buffer E at a concentration of 1 × 10⁶ nuclei/ml and immediately used for an in vitro apoptosis assay.

**Induction of Apoptosis in a Cell-free Reconstitution System**—The reaction mixture contained 40 μl of S-100 (~5 mg/ml), 10 μl of nuclei solution (~1 × 10⁶ nuclei), and 400 ng of pretreated recombinant human caspase-3 in a final volume of 80 μl of buffer F (10 mM HEPES, pH 7.4, 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM MgCl2, 4 mM EGTA, 2 mM ATP, 10 mM creatine phosphate, 50 μg/ml creatine kinase, and 0.2 mg/ml bovine serum albumin). The mixtures were incubated at 37 °C for 140 min and occasionally mixed. The reaction solution was then mixed with 500 μl of buffer G (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% SDS, and 0.2 mg/ml of proteinase K) and incubated at 37 °C for 1 h. The solution was extracted with phenol/chloroform. DNA isolation and electrophoresis were carried out as described previously (29).

**RESULTS**

**Endogenous and Exogenous NO Prevents Spontaneous Hepatocyte Apoptosis**—It has been shown that spontaneous apoptosis occurs in some cell types in vitro in response to nutrient deprivation and to growth factor withdrawal (reviewed in Ref. 4). We characterized the spontaneous decline in viability in primary monolayers of rat hepatocytes cultured on collagen-coated plates. By crystal violet staining, viability dropped precipitously after day 3 (Fig. 1A). Cell death was not associated with LDH release into the medium; instead, most of the LDH activity was retained within nonviable cells, suggesting that spontaneous cell death was not due to necrosis. Apoptotic cell death was confirmed by the detection of fragmented cytoplasmic DNA coincident with the loss of viability starting on day 4 and continuing through day 6 (Fig. 1B). Thus, under these culture conditions, an internally encoded suicide program is spontaneously activated in hepatocytes after 3 days.

Hepatocytes can be reconstituted to express iNOS by combinations of IL-1β, TNFα, and IFNγ (38). Consistent with these observations, hepatocytes stimulated with IL-1β and IFNγ (cytokine mixture; CM) expressed both iNOS mRNA and protein, as judged by Northern and Western blot analysis, respectively (Fig. 2A). Hepatocytes exposed to CM on day 0 continued to produce NO for 7 days as determined by the accumulation of...
A experimental conditions described in

DNA fragmentation was detected by 1.2% agarose gel electrophoresis as in A. After collecting hepatocytes, cytosolic DNA was isolated, and antiapoptotic effect of cytokine-stimulated NO production and NO donors. A, induction of iNOS mRNA was detected by Northern blot analysis at 8 h and protein by Western blot analysis at 16 h in rat hepatocytes stimulated with CM (200 units/ml IL-1β plus 100 units/ml IFNγ). Cells were cultured in the presence or absence of the NOS inhibitor NMA (1.5 mM). Cells were cultured with or without NO donors. B, production of NO was measured as accumulation of nitrite plus nitrate in the culture media of hepatocytes (2 × 10⁶ cells/well in 12-well plates) stimulated with the same protocol as in A using the Griess reaction after converting nitrate to nitrite (mean ± S.D., n = 4). C, cell viability was measured under the same experimental conditions described in A by crystal violet staining (mean ± S.D., n = 4). D, cytosolic DNA fragmentation was determined in primary hepatocytes treated under the same conditions as described for A. After collecting hepatocytes, cytosolic DNA was isolated, and DNA fragmentation was detected by 1.2% agarose gel electrophoresis followed by ethidium bromide staining. E, hepatocytes were cultured in 250 μM of NO donor (freshly prepared solution) with or without red blood cells (400 μM of oxyhemoglobin). Cell viability was measured by crystal violet staining (mean ± S.D., n = 4). F, cytosolic DNA fragmentation in hepatocytes treated with 250 μM of either V-PYRRO/NO or SNAP with or without red blood cells for 4 days as in D.

nitrite and nitrate in the culture medium, although the rate of production did decrease with time (Fig. 2B). Neither iNOS mRNA nor protein levels were influenced by the addition of the NOS inhibitor NMA; however, production of nitrite and nitrate was completely inhibited. As shown in Fig. 2, C and D, the spontaneous loss of viability and appearance of apoptosis on day 4 were reversed in an NO-dependent manner in the CM-
treated cells.

To determine whether compounds that release NO could also prevent the spontaneous apoptosis in cultured hepatocytes, SNAP, a compound that releases NO when placed in solution, and V-PYRRO/NO, which must be metabolized to NO within hepatocytes, were added daily to the cultured cells. Both sources of NO substantially reduced and delayed the loss in viability (Fig. 2E) and completely abolished the appearance of fragmented DNA (Fig. 2F). The addition of red blood cells, which scavenge extracellular NO (35), prevented the protective effects of SNAP (Fig. 2, E and F) while the addition of either the parent compound (N-acetyl-DL-penicillamine) of SNAP or oxidized products of SNAP, prepared by allowing SNAP to completely liberate all of its NO, failed to protect (not shown). Thus, the spontaneous cell death due to apoptosis was prevented by both endogenous and exogenous sources of NO.

Both NO and a Caspase-3-like Protease Inhibitor Prevent Spontaneous Hepatocyte Apoptosis—Many of the recently described caspase proteases (11), including caspase-3, -7, and -8 (defined as caspase-3-like proteases), cleave PARP at the site DEVD³⁵⁵-G. Tetrapeptides based on this sequence, susceptible to cleavage by caspase-3-like enzymes, have been used to develop specific assays (Ac-DEVD-pNA) and inhibitors (Ac-DEVD-cho) (8, 16, 40). The capacity of Ac-DEVD-cho to block spontaneous apoptosis in cultured rat hepatocytes was compared with CM treatment (Fig. 3A). Both CM treatment and Ac-DEVD-cho prevented the spontaneous loss of viability (measured on day 4) to a similar degree. The protective effect of CM exposure was lost when NMA was used to block NO synthesis and Ac-DEVD-cho reversed the effect of NMA. The caspase-1 inhibitor Ac-YVAD-cho did not protect the hepatocytes from spontaneous cell death and did not reverse the apoptosis resulting from iNOS inhibition.

To determine whether similar changes in hepatocyte viability occurred in cultured mouse hepatocytes and to establish that iNOS, and not one of the other NOS isoforms, was the source of the NO, we carried out experiments using cultured hepatocytes from iNOS-deficient mice (KO, iNOS−/−) and their wild type counterparts (WT, iNOS+/+) (34). CM treatment resulted in iNOS mRNA and protein expression and production of nitrite and nitrate only from hepatocytes isolated from WT mice (Figs. 3, B and C). In the absence of CM, both WT and KO hepatocytes exhibited a 50% reduction in viability (Fig. 3D). CM partially reversed this only in the WT cells, whereas V-PYRRO/NO and Ac-DEVD-cho prevented loss of viability in both WT and KO hepatocytes. As in the rat cells, Ac-YVAD-cho had no effect on hepatocyte viability. Therefore, in both rat and mouse hepatocytes, an inhibitor (Ac-DEVD-cho) of caspase-3-like proteases inhibits apoptosis similar to NO, and the protective effect of cytokine exposure requires iNOS expression.

NO Inhibits Caspase-3 Activity through S-Nitrosylation—Sequence alignment and inhibitor studies suggest that all caspase family proteases use a cysteine as the catalytic site nucleophile. The reaction products of NO and, in particular, NO⁺ equivalents can interact with sulphhydryl groups on proteins and alter the biological activity of a variety of proteins (reviewed in Ref. 26). To determine if NO or NO⁺ equivalents could directly modify protease activity, purified rh-caspase-3 (16) was incubated with various concentrations of SNAP (NO donor) or NTB (NO⁺ donor) for 1 h. Caspase-3 activity was measured following preincubation of the enzyme with or without 20 mM DTT using a colorimetric assay based on the cleavage of the synthetic peptide Ac-DEVD-pNA. In control experiments, activity loss due to absence of reducing agents was negligible at 30 min and <15% at 60 min (data not shown). Both agents caused a similar concentration-dependent reduc-
tion in caspase-3 activity, with detectable inhibition at 10 µM and nearly complete inhibition at 200 µM or greater (Fig. 4A). Oxidized SNAP or NTB had no effect on caspase-3 activity. When the rate of nitrite release from SNAP was measured, 200–400 µM SNAP released 2–6 µmol of nitrite/liter/h (data not shown), which is similar to the nitrite release rate of CM-treated hepatocytes.

DTT effectively removes the thiol-bound NO groups from proteins (37, 41). Incubation of SNAP-treated rh-caspase-3 with 20 mM DTT but not glutathione almost completely reversed the inhibition in caspase-3 activity (Fig. 4B), suggesting that NO modified the thiol group in the catalytic site of the protease. To confirm the formation of nitrosylated thiol, we incubated SNAP-pretreated rh-caspase-3 with HgCl₂ to displace the bound NO group and measured nitrite release using a colorimetric method (42). The appearance of a peak in absorbance at 540 nm in the SNAP-pretreated rh-caspase-3-containing solution (Fig. 4C) is consistent with the release of the bound NO group. Based on a molecular extinction coefficient of ε_{540} = 42 mM⁻¹ cm⁻¹ for the diazo product of the Griess reaction, we estimate that 1.5 mol of NO bind to each mole of rh-caspase-3. The addition of HgCl₂ or other thiol-reactive compounds, including N-ethylmaleimide (NEM) and iodoacetamide, directly to rh-caspase-3 all inhibited the activity; however, only the inhibition by HgCl₂ was reversed by 20 mM DTT (Fig. 4D). Taken together, these findings indicate that sulfhydryl modification of caspase-3 leads to the inhibition of protease activity. Furthermore, caspase-3 activity can be inhibited by an NO-dependent thiol redox mechanism that is not reversed by glutathione.

Modification of Caspase-3 by NO Prevents PARP Degradation and DNA Fragmentation—One of the biosubstrates for caspase-3-like protease in cells is PARP (116 kDa), which is cleaved into 85- and 31-kDa fragments (14). The cleavage of
activity of caspase-3 for PARP, the capacity of SNAP and other thiol-modifying agents to block the cleavage of PARP by rh-caspase-3 was tested. rh-caspase-3 was incubated with $^{35}$S-labeled PARP and, after an incubation period of 1 h, PARP cleavage was assessed by SDS-polyacrylamide gel electrophoresis (Fig. 5B). Caspase-3-cleaved PARP, and this was blocked by preincubation of caspase-3 with 200 $\mu$M SNAP, but not if red blood cells were included with the SNAP to scavenge NO. The thiol-modifying agents HgCl$_2$ and NEM also blocked PARP cleavage by caspase-3, as did the specific inhibitor of caspase-3, Ac-DEVD-cho (Fig. 5B). Preincubation of SNAP-treated, but not NEM-treated, rh-caspase-3 with 20 mM DTT restored the capacity of caspase-3 to cleave PARP (Fig. 5C), indicating that the inhibitory effect of SNAP was due to a sulfhydryl modification.

One consequence of caspase-3 activation as apoptosis progresses is intranucleosomal DNA fragmentation, and this function of caspase-3 can be specifically evaluated in a cell-free system (8, 43). No DNA fragmentation was detected by agarose gel electrophoresis when only the cytosolic fraction (S-100) and nuclei from freshly isolated rat hepatocytes were combined (Fig. 5D). The addition of rh-caspase-3 to the reconstitution mixture initiated DNA fragmentation. Preincubation of rh-caspase-3 with 200 $\mu$M SNAP inhibited the capacity of caspase-3 to initiate DNA fragmentation; however, this was not seen if red blood cells were added with SNAP or if NO-depleted oxidized SNAP was used. HgCl$_2$, NEM, and Ac-DEVD-cho, which all inhibit caspase-3 activity (Fig. 4D) and PARP cleavage (Fig. 5B), also blocked caspase-3-dependent DNA fragmentation. When SNAP-treated rh-caspase-3 was preincubated with 20 mM DTT to remove bound nitroso groups, the capacity of caspase-3 to induce DNA fragmentation was restored (Fig. 5E). These data show that NO not only inhibits caspase-3 proteolytic activity but also directly inhibits the capacity of caspase-3 to initiate DNA fragmentation in the reconstitution system.

**NO Inhibits the Caspase-3-like Activity in Cultured Hepatocytes**—We monitored caspase-3-like activity in cultured hepatocytes undergoing spontaneous apoptosis using the same colorimetric assay used for rh-caspase-3. Caspase-3-like activity increased over time in culture with a peak on day 4, the time that apoptosis was first detected (Fig. 6A). Caspase-3-like activity in cytosolic extracts from hepatocytes (day 4) was nearly 90% lower in hepatocytes previously exposed to CM, and this decrease was reversed by NMA (Fig. 6B). Preincubation of the lysates in 20 mM DTT restored the CM-induced inhibition of caspase-3-like activity to about 55% of controls. When hepatocytes were incubated with NO donors (SNAP or V-PYRRO/NO), caspase-3-like activity measured in the cytosolic extract was also inhibited by about 80% (Fig. 6C). The addition of red blood cells to scavenge NO prevented the inhibition of caspase-3-like activity by SNAP. DTT had no effect on caspase-3-like activity when added to extracts from control cells, but again restored about half of the activity in SNAP- or V-PYRRO/NO-treated hepatocytes. Thus, both endogenous and exogenous sources of NO inhibit caspase-3-like activity in cultured hepatocytes, and this inhibition can be partially reversed by the addition of DTT to the cell lysates.

**NO Prevents Apoptosis in Response to TNF$\alpha$ or Anti-Fas Antibody**—Experiments were performed to establish whether NO also prevents TNF$\alpha$- or anti-Fas antibody-induced apoptosis through inhibition of caspase-3-like protease activity. Rat hepatocytes treated with TNF$\alpha$ and mouse hepatocytes exposed to anti-Fas antibody both exhibited a 50% reduction in viability in the presence of the transcriptional inhibitor actinomycin D (ActD) (Fig. 7A). This loss of viability was associated
with a significant increase in caspase-3-like activity (Fig. 7, B and C) and could be inhibited by SNAP, V-PYRRO/NO, or Ac-DEVD-cho but not Ac-YVAD-cho (Fig. 7A). SNAP or V-PYRRO/NO also suppressed caspase-3-like activity in cultured hepatocytes exposed to TNFα or anti-Fas antibody, while incubation of the cell lysates in 20 mM DTT restored, in part, the caspase-3-like activity (Fig. 7, B and C). These data show that both TNFα- and anti-Fas antibody-induced apoptosis include increases in caspase-3-like activity, which is subject to NO inhibition by thiol modification.

![Graph](image)

**Fig. 6.** NO inhibits caspase-3-like protease activity in cultured rat hepatocytes. **A,** time course of caspase-3-like activity. Cytosolic extract was prepared from hepatocytes cultured with or without CM (200 units/ml IL-1β plus 100 units/ml IFNγ) in the presence or absence of 1.5 mM NMA. Caspase-3-like protease activity was measured with Ac-DEVD-pNA using a colorimetric assay (mean ± S.D., n = 3). B, effect of DTT on cytosolic caspase-3-like activity. Crude extract from hepatocytes treated with CM for 4 days was incubated with or without 20 mM DTT on ice for 30 min, and caspase-3-like activity was measured with the Ac-DEVD-pNA colorimetric assay (mean ± S.D., n = 3). C, cytosolic caspase-3-like activity in hepatocytes treated with NO donors. Cytosolic extract isolated from hepatocytes treated with 250 μM SNAP or V-PYRRO/NO daily for 4 days was preincubated with or without 20 mM DTT on ice for 30 min, and caspase-3-like enzyme activity was measured with the Ac-DEVD-pNA colorimetric assay (mean ± S.D., n = 3).

**Evidence for S-Nitrosylation of Caspase-3-like Proteases in Vivo**—We have previously shown that V-PYRRO/NO selectively releases NO in the liver in vivo and that this compound blocks the massive liver damage due to apoptosis in TNFα plus D-galactosamine-treated rats (33). To establish whether NO inhibited caspase-3-like activity in vivo, rats received a continuous intravenous infusion of either saline or V-PYRRO/NO via an Alzet osmotic minipump and were treated with an intraperitoneal injection of TNFα plus D-galactosamine. As shown in Fig. 8, at 8 h following injection of TNFα plus D-galactosamine, caspase-3-like activity was markedly elevated in homogenates from whole liver. Preincubation of the homogenate in 20 mM DTT did not alter the already elevated caspase-3-like activity. Infusion of V-PYRRO/NO alone had no effect on caspase-3-like activity, but when infused following TNFα plus D-galactosamine, caspase-3-like activity was only 20% that of animals receiving TNFα plus D-galactosamine after infusion with saline. Preincubation of this homogenate with DTT restored approximately 50% of the caspase-3-like activity. These data suggest that the reduction in caspase-3-like activity in vivo by NO is due to S-nitrosylation.

**NO Inhibits Increases in Caspase-3-like Activity via a cGMP-dependent Pathway**—The above findings indicate that NO is a potent inhibitor of caspase-3-like activity; however, failure to completely restore enzyme activity with DTT suggested that mechanisms in addition to S-nitrosylation contributed to the NO-induced suppression of caspase-3-like activity. In other cell types, NO has been shown to prevent apoptosis via a cGMP-dependent mechanism (18–20). We have recently shown that the cGMP analog 8-bromo-cGMP prevents TNFα-induced cell death in hepatocytes in a concentration-dependent manner between 1 and 1,000 μM (33). Consistent with these previous observations, 8-bromo-cGMP mimicked the capacity of SNAP to inhibit cell death as measured by crystal violet and the appearance of DNA fragmentation in response to TNFα plus ActD (Fig. 9, A and B). When caspase-3-like activity was measured in cell lysates, both SNAP and 8-bromo-cGMP inhibited the TNFα plus ActD-induced increase in activity; however, preincubation of the cell lysates with DTT partially restored activity only in the SNAP-treated cells (Fig. 9C). The addition of the soluble guanylyl cyclase inhibitor ODQ with SNAP prevented approximately 50% of the SNAP-induced drop in caspase-3-like activity. The remainder of the activity was restored if the lysate was preincubated in DTT; ODQ alone had no effect. Thus, NO-stimulated cGMP synthesis prevents either caspase-3 activation or signaling events upstream of caspase-3-like protease activation, and this accounts for the non-DTT-reversible inhibition of caspase-3-like activity.

Experiments using KT5823, a cGMP-dependent kinase inhibitor, were performed to determine if the effects of cGMP on cell viability and caspase-3 activation were mediated via cGMP-dependent kinase. The addition of KT5823 had no effect on TNFα plus ActD-induced cell death or caspase-3 activation (Fig. 10, A and B); however, KT5823 partially reversed the effects of SNAP and almost completely prevented the effects of 8-bromo-cGMP on cell viability and caspase-3-like activity. When cytosolic extracts were incubated with 20 mM DTT, caspase-3-like activity was restored only in SNAP-treated hepatocytes (data not shown). Therefore, cGMP signaling for the inhibition of apoptosis occurs through cGMP-dependent kinase.

**DISCUSSION**

The main conclusion of this study is that NO or its reaction products acts as an endogenous inhibitor of apoptosis by two distinct mechanisms. Working through a cGMP-dependent mechanism, NO acts either at the level of caspase-3-like protease activation or upstream of this event to prevent the activation of the protease. NO also directly inhibits the activity of caspase-3-like protease by S-nitrosylation of the enzyme. The end result is a suppression of caspase-3-like activity, which rescues the cell from a suicidal death. The key role of caspase-3-like proteases in apoptotic signal transduction, coupled with the established widespread expression patterns of the three NO synthases in both normal and disease states, predicts an important role for NO as an endogenous regulator of apoptosis.

It is now known that NO prevents apoptosis in a diverse array of cell types, and previous studies have provided some mechanistic insights. Low level iNOS expression in Epstein-Barr virus-transformed B lymphocytes protected cells from apoptotic cell death (17). These authors postulated that the effect of NO was mediated through the down-regulation of the expression of the immediate early Epstein-Barr virus transactivator Zta. This NO-dependent process was independent of cGMP and was thought to be regulated by changes in sulfhydryl redox states. Others have reported that B lymphocytes...
Fig. 7. NO inhibits apoptosis and caspase-3-like activity in liver cells exposed to TNFα or anti-Fas antibody. A, rat hepatocytes were treated with 32 ng/ml rmTNFα plus 0.2 μg/ml ActD, mouse hepatocytes were exposed to 0.5 μg/ml anti-Fas antibody plus 0.2 μg/ml ActD, and viability was determined by crystal violet staining 12 h later (mean ± S.D., n = 4). B and C, crude extract was obtained from hepatocytes 8 h after treatment with TNFα plus ActD (B) or anti-Fas antibody plus ActD (C), as described for A. Caspase-3-like activity was measured in cytosolic extract with Ac-DEVD-pNA using a colorimetric assay (mean ± S.D., n = 3).

Fig. 8. NO prevents increases in caspase-3-like activity in vivo. Rats were anesthetized with pentobarbital and Alzet osmotic mini-pumps containing either saline or the liver-selective NO donor V-PYRRO/NO were implanted with catheters positioned in the jugular vein. At the same time, rats were injected intraperitoneally with 10 μg/kg TNFα and 700 mg/kg d-galactosamine (GalN). 8 h later, rat livers were isolated after perfusion with PBS, and caspase-3-like activity was measured in the liver homogenates with or without preincubation with 20 mM DTT on ice for 30 min (n = 6 rats per group, mean ± S.D.).

Fig. 9. 8-Bromo-cGMP inhibits TNFα-induced apoptosis and suppresses caspase-3-like protease activation in hepatocytes. A and B, rat hepatocytes were treated with 32 ng/ml rmTNFα plus 0.2 μg/ml ActD in the presence or absence of 400 μM SNAP or 800 μM 8-bromo-cGMP for 12 h. Viability was measured by crystal violet staining (A), and apoptosis was assessed by DNA fragmentation (B). C, caspase-3-like protease activity was measured in the cytosolic extract with Ac-DEVD-pNA using a colorimetric assay. Hepatocytes were exposed to TNFα plus ActD as above with 400 μM SNAP, 100 μM ODQ, or 800 μM 8-bromo-cGMP as indicated for 8 h. Half of the extract was incubated with (open bar) or without (closed bar) 20 mM DTT on ice for 30 min before the caspase-3-like activity was measured (mean ± S.D., n = 3).
phages by a p35-dependent mechanism (44). NO reacts rapidly with superoxide to form the highly toxic peroxynitrite, which can induce apoptosis (52). Therefore, the interaction of NO with superoxide would not only lead to the formation of more toxic radicals, it would also remove NO from the system. Thus, whether NO protects from apoptosis or induces cell death most likely depends on the rate of NO formation and the prominence of caspase-3-like protease activation in the apoptotic process as well as other factors such as the presence of other radicals and antioxidants.

Whether a cell undergoes apoptosis appears to be due to a shift in the balance between anti- and proapoptotic factors in favor of the “pro” factors. Our results indicate that physiologically relevant levels of NO can contribute to this balance by modulating the level of caspase-3-like protease activity through both direct and indirect mechanisms. Both exogenous (53) and endogenous (54) sources of NO increase cGMP levels in hepatocytes. We show that NO-stimulated cGMP synthesis either directly or indirectly reduces TNFα-induced caspase-3-like protease activation through cGMP-dependent kinase. These studies were performed in the presence of ActD, indicating that the cGMP effects did not require new gene expression. Although the possibility of a direct effect on caspase-3-like protease is not excluded, an interaction of cGMP-dependent kinase with the apoptotic signaling pathway upstream of caspase-3-like protease activation seems more likely. One potential pathway for caspase-3 activation involves the upstream activation of caspase-1 with proteolytic cleavage of procaspase-3 to the active enzyme. We found that the caspase-1 inhibitor had no effect on spontaneous or TNFα-induced cell death, suggesting that caspase-1 is not involved as caspase-3-like protease activation in hepatocytes. Activation of caspase-3 proteases has been shown to be associated with phosphorylation/dephosphorylation upstream signaling (55). Thus, cGMP-dependent kinase could regulate upstream signaling by directly or indirectly modulating protein phosphorylation events. cGMP analogs also prevented spontaneous apoptosis in cultured hepatocytes (data not shown), demonstrating that neither ActD nor TNFα was required to reveal the antiapoptotic potential of cGMP. The failure of cGMP to completely suppress caspase-3 activation may indicate that this is an inefficient system for the down-regulation of apoptotic signaling. Alternatively, a separate signaling pathway not susceptible to cGMP-dependent down-regulation of apoptotic signaling may also be involved in caspase-3-like protease activation.

Our data would suggest that caspase-3 activation that is not effectively blocked by cGMP is then subject to direct inhibition by NO through S-nitrosylation. This could be an effective backup mechanism to assure that any caspase-3-like protease that becomes activated, even in the presence of elevated cGMP, is inhibited. NO modulation of the function of many proteins occurs through interaction with redox regulatory thiols (reviewed in Ref. 26). Thiol nitrosylation by NO or its reaction products has been reported for glyceraldehyde-3-phosphate dehydrogenase (37), hemoglobin (56), albumin (57), tissue-type plasminogen activator (58), cysteine protease cathepsin B (57), and the nonprotein thiol glutathione (59). Functional modification by S-nitrosylation of the bacterial transcriptional factor OxyR is the mechanism for the activation of genes encoding protective proteins (60), while S-nitrosylation of the N-methyl-d-aspartate type glutamate receptor protects neurons from N-methyl-d-aspartate-mediated toxicity (61). Caspase family proteases are plausible targets for S-nitrosylation, since their catalytic mechanisms require a cysteine residue at an active site. Of the eight cysteine residues in caspase-3, cysteine 163 resides in the catalytic site and is required for protease activity (14). The inhibition of caspase-3 activity with thiol-reactive compounds as well as NO and NO+ equivalents indicates that thiol modification results in enzyme inhibition, while the detection of 1.5 mol of S-nitrosothiol/mol of caspase-3 exposed to a NO donor argues that the single cysteine in the catalytic site is most susceptible to NO-dependent redox modification. The reversal of NO-mediated inhibition of caspase-3-like activity by DTT, but not GSH, is consistent with S-nitrosylation as shown for glyceraldehyde-3-phosphate dehydrogenase (37, 41).

Because the NO radical does not effectively nitrosylate thiol groups, a NO reaction product is implicated in the S-nitrosylation and inhibition of caspase-3-like protease. NO+ equivalent nitrosylates thiols (61) and mimics the effect of NO donors when added to cells. Of note, NO+ release by NTB has a much shorter half-life than NO release by SNAP; however, both inhibited caspase-3 in an identical concentration-dependent manner, suggesting that NO+ was a much more efficient inhibitor of the protease. Reactive nitrogen oxide species, including NO+ and its equivalents, can be generated by the reaction of NO with O2 (62), iron-sulfur clusters (63), or heme iron (64). Hepatocytes are rich in heme and iron-sulfur cluster-containing proteins, and NO production in hepatocytes leads to the formation of iron-nitroso complexes, as detected by electron paramagnetic resonance spectroscopy (35). These complexes have been shown to carry out transnitrosative reactions (63). In our in vitro experiments, the cells were grown under aerobic conditions, and the interaction of NO with O2 could form N2O3, which has been shown to cause S-nitrosylation via the formation of NO+ equivalents in activated macrophages (65). We also present evidence that efficient S-nitrosylation of caspase-3-like protease can occur in vivo. Intravenous infusion of V-PYRRO/NO, an NO donor that selectively releases NO in the liver (33), reduced TNFα-induced caspase-3-like activity in a manner that could be partially reversed by DTT. The partial reversal by DTT also indicates that other mechanisms are involved, and we have shown that hepatic cGMP levels are elevated during V-PYRRO/NO infusion (33).

The capacity of relatively low concentrations of NO to modulate elevations in caspase-3-like protease activity is contrasted by the capacity of higher concentrations of NO to inhibit TNFα-induced apoptosis in hepatocytes through the induction of heat shock protein 70 (29). We found that induction of heat shock following NO exposure required several hours, while direct inhibition of caspase-3 activity was immediate, indicating distinct mechanisms for protection. Others
have recently shown in endothelial cells that NO prevents TNFα-induced apoptosis through a mechanism involving suppression of caspase-3-like protease activity (22). This indicates that the quantities of NO produced by endothelial NO synthase are adequate to suppress caspase-3-like proteases in endothelial cells. Whether NO also suppresses protease activation in endothelial cells was not studied.

Our studies further associate the expression of NO and especially iNOS with the regulation of apoptosis. Initially, iNOS was thought to be expressed only after exposure of cells to stimuli such as cytokines, microbes, or microbial products. We now know that iNOS is also expressed constitutively in respiratory (66) and intestinal epithelium (67) as well as tumor (68) and especially iNOS with the regulation of apoptosis. Initially, we have recently shown in endothelial cells that NO prevents TNFα-induced apoptosis through a mechanism involving suppression of caspase-3-like protease activity (22). This indicates that the quantities of NO produced by endothelial NO synthase are adequate to suppress caspase-3-like proteases in endothelial cells. Whether NO also suppresses protease activation in endothelial cells was not studied.

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