

# Nitric Oxide Suppresses Apoptosis via Interrupting Caspase Activation and Mitochondrial Dysfunction in Cultured Hepatocytes\*

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Nitric oxide (NO) is a potent inhibitor of apoptosis in many cell types, including hepatocytes. We and others have described NO-dependent decreases in caspase activity in cells undergoing apoptosis. However, previous work has not determined whether NO disrupts the proteolytic processing and thus the activation of pro-caspases. Here we report that NO suppresses proteolytic processing and activation of multiple pro-caspases in intact cells, including caspase-3 and caspase-8. We found that both exogenous NO as well as endogenously produced NO via adenoviral inducible NO synthase gene transfer protected hepatocytes from tumor necrosis factor (TNF)  $\alpha$  plus actinomycin D (TNF $\alpha$ /ActD)-induced apoptosis. Affinity labeling with biotin-VAD-fmk of all active caspase species in TNF $\alpha$ -mediated apoptosis identified four newly labeled spots (activated caspases) present exclusively in TNF $\alpha$ /ActD-treated cells. Both NO and the caspase inhibitor, Ac-DEVD-CHO, prevented the appearance of the four newly labeled spots or active caspases. Immunoanalysis of affinity labeled caspases demonstrated that caspase-3 was the major effector caspase. Western blot analysis also identified the activation of caspase-8 in the TNF $\alpha$ /ActD-treated cells, and the activation was suppressed by NO. Furthermore, NO inhibited several other events associated with caspase activation in cells, including release of cytochrome *c* from mitochondria, decrease in mitochondrial transmembrane potential, and cleavage of poly(ADP-ribose) polymerase in TNF $\alpha$ /ActD-treated cells. These findings indicate the involvement of multiple caspases in TNF $\alpha$ -mediated apoptosis in hepatocytes and establish the capacity of NO to inhibit not only active caspases but also caspase activation.

Apoptosis, or the programmed cell death, is essential to the normal development of multicellular organisms as well as physiologic cell turnover (reviewed in Refs. 1–3). In pathologic states, while a failure to undergo apoptosis may cause abnormal cell overgrowth and malignancy, excessive apoptosis may contribute to organ injury. The signaling pathways leading to apoptosis are

now known to involve the sequential activation of cysteine proteases known as caspases (4), in association with changes in mitochondrial membrane potential and release of cytochrome *c* (5). Caspases are constitutively present in cells as zymogens and require proteolytic cleavage into the catalytic active heterodimer. All activated caspases are comprised of a 17–20-kDa large subunit that contains a redox-sensitive cysteine at the active site and a small subunit of approximately 10 kDa. To date, 14 mammalian caspases have been identified (4, 6, 7). Based on a number of genetic and biochemical studies, the functions of various caspases appear different and these caspases can be tentatively grouped into three categories: caspases that function primarily in cytokine maturation (*e.g.* caspase-1, -4, and -5), effector proteases in the execution phase of apoptosis (caspase-3, -6, and -7), and initiator caspases involved in the early steps of apoptotic signaling (*e.g.* caspase-8, -9, and -10). Several synthetic peptide derivatives that mimic the cleavage sites of natural substrates of caspases have been used to inactivate these caspases. For example, Ac-DEVD-CHO is a caspase-3-like protease inhibitor, Ac-YVAD-CHO a caspase-1-like protease inhibitor, and z-VAD-fmk an irreversible inhibitor for all caspases. Studies using these peptide inhibitors, together with mutational and genetic analysis, unequivocally established the central role of caspases in apoptosis in numerous cell systems (4). While some members of the caspase family are indispensable during mammalian development others seem to be redundant or have limited roles in programmed cell death. However, the specific role of an individual caspase may vary between cell types or apoptotic stimuli (8).

Recent evidence indicates that mitochondria play an essential role in apoptotic cell death induced by various stimuli. Activation of upstream caspases has been shown to lead to the degradation of Bcl-2 family members (9, 10), which promotes the release of cytochrome *c*. This occurs in association with the loss of the inner mitochondrial membrane potential in many situations (11, 12). Released cytochrome *c* subsequently binds to Apaf-1 in the presence of dATP and activates caspase-9 which then activates downstream effector caspases, such as caspase-3 (13).

Although nitric oxide (NO)<sup>1</sup> is cytotoxic in a number of cell types, it suppresses apoptosis in others. Cell types shown to be

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<sup>1</sup> The abbreviations used are: NO, nitric oxide; Ac-, acetyl-; ActD, actinomycin D; CHO, aldehyde; pNA, *p*-nitroanilide; SNAP, *S*-nitroso-*N*-acetylpenicillamine; Oxi-SNAP, oxidized SNAP; NIO, *N*-iminoethyl-L-ornithine; iNOS, inducible nitric oxide synthase; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; PI, propidium iodide; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; PARP, poly(ADP-ribose) polymerase; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing;  $\Delta\Psi_m$ , mitochondrial inner transmembrane potential; AdiNOS, adenoviral iNOS; LacZ,  $\beta$ -galactosidase; AdLacZ, adenoviral LacZ; FADD, Fas-associated death domain protein.

protected from apoptosis by NO include lymphocytes (14, 15), endothelial cells (16), eosinophils (17), splenocytes (18), multiple cell lines (19, 20), certain neurons (21, 22), ovarian follicles (23), and hepatocytes (24, 25). One mechanism for the inhibition of apoptosis by NO is through the suppression of caspase enzymatic activity. NO can directly inhibit caspase activity through *S*-nitrosylation of the active cysteine conserved in all caspases (16, 26). However, it is not clear whether NO interferes only with activated caspases or whether NO blocks pro-caspase processing and activation. In this study we examined this question by determining the number of caspases activated in TNF $\alpha$  plus actinomycin (TNF $\alpha$ /ActD)-treated hepatocytes and assessing the effect of NO on caspase activation. Here we provide evidence that NO prevented the proteolytic activation of all involved caspases, including the most apical caspase, caspase-8. NO also prevented other key events associated with the activation of caspases including the loss of mitochondrial transmembrane potential and release of cytochrome *c*.

#### EXPERIMENTAL PROCEDURES

**Materials**—Williams Medium E, penicillin, streptomycin, L-glutamine, Opti-MEM, and Hepes were purchased from Life Technologies Inc. *N*-Acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-*p*NA), caspase inhibitors *N*-acetyl-DEVD-aldehyde (Ac-DEVD-CHO) and benzyloxy-carbonyl-Val-Ala-Asp fluoromethyl ketone (*z*-VAD-fmk) were from Alexis Co. (San Diego, CA). Stock solutions were prepared at 100 mM in dimethyl sulfoxide. *N*-Acetyl-Ile-Glu-Thr-Asp-*p*-nitroanilide (Ac-IETD-*p*NA), caspase-8 inhibitor *z*-IETD-fmk, and biotin-conjugated VAD-fmk were from Calbiochem (San Diego, CA). *S*-Nitroso-*N*-acetylpenicillamine (SNAP) was synthesized as described (27). Oxidized SNAP (oxi-SNAP) was prepared by incubating SNAP aqueous solution at room temperature for 2 days to completely liberate NO. Mouse recombinant TNF $\alpha$  was obtained from R & D Systems (Minneapolis, MN). Antibodies used in this study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, for caspase-3, iNOS, and Hsp70), Oncogene Research Product (Cambridge, MA, for PARP), and Pharmingen (San Diego, CA, for cytochrome *c*). Caspase-8 monoclonal antibody was kindly provided by Dr. P. H. Kramer (German Cancer Research Center, Germany). Horseradish peroxidase-linked streptavidin and Supersignal<sup>TM</sup> chemiluminescence detection reagents were from Pierce Chemical Co. (Rockford, IL). Unless indicated otherwise, all other chemicals were from Sigma.

**Preparation of Primary Hepatocytes and Cell Culture**—Primary rat hepatocytes were isolated and purified from male Sprague-Dawley rats and cultured as described previously (28). Highly purified hepatocytes (>98% purity and >98% viability by trypan blue exclusion) were suspended in Williams medium E supplemented with 10% calf serum, 1  $\mu$ M insulin, 2 mM L-glutamine, 15 mM HEPES (pH 7.4), 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. The cells were plated on collagen-coated tissue culture plates at a density of  $2 \times 10^5$  cells/well in 12-well plates for cell viability analysis or  $5 \times 10^6$  cells/100-mm dish for Western blot and enzyme assays. After 18 h preculture, the cells were washed and further cultured with fresh medium containing 5% calf serum. Apoptosis was induced by incubating the hepatocytes in the culture medium containing 2000 units/ml TNF $\alpha$  and 0.2  $\mu$ g/ml actinomycin D for various time points as specified in the figure legends. Cells were then scraped off the plates and centrifuged, washed twice with cold phosphate-buffered saline and resuspended in a 5-fold volume of hypotonic buffer A (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride, 5  $\mu$ g/ml aprotinin, 5  $\mu$ g/ml pepstatin, and 10  $\mu$ g/ml leupeptin). After three to four cycles of freezing and thawing, cell debris was removed by centrifugation at  $13,000 \times g$  at 4  $^{\circ}$ C for 20 min and the supernatant used as cell lysate. Protein concentration was determined with the BCA assay (Pierce) with bovine serum albumin as standard. Cell viability was determined by the crystal violet method as described previously (27). In brief, cells were stained with 0.5% crystal violet in 30% ethanol and 3% formaldehyde for 10 min at room temperature. Plates were then washed 4 times with tap water. Cells were solubilized with 1% sodium dodecyl sulfate solution and dye uptake was measured at 550 nm using a microplate reader.

**Enzyme Activity Assay**—Caspase activity was evaluated by measuring proteolytic cleavage of chromogenic substrate Ac-DEVD-*p*NA as described previously (26). Ac-DEVD-*p*NA was used as the substrate for caspase-3-like proteases and Ac-IETD-*p*NA as caspase-8 substrate.

Briefly, cell lysate (100  $\mu$ g of protein) was added into buffer A containing 200  $\mu$ M Ac-DEVD-*p*NA or 100  $\mu$ M Ac-IETD-*p*NA in a final volume of 100  $\mu$ l. The reaction mixture was incubated at 37  $^{\circ}$ C for 1 h. The increase in absorbance of enzymatically released *p*NA was measured at 405 nm in a microplate reader.

**Adenoviral iNOS Gene Transfer**—Modified adenoviral vectors carrying the human iNOS or bacterial  $\beta$ -galactosidase cDNA were prepared as described previously (29). After 18 h of preculture, hepatocytes ( $5 \times 10^6$ /10-cm plate) were washed with Hanks' buffered saline and incubated with adenoviral vector containing either the human iNOS or bacterial  $\beta$ -galactosidase (LacZ) cDNA at multiplicity of infection of 1 in a volume of 2 ml of Opti-MEM. Following a 2-h infection, the medium was changed to fresh Williams medium E containing 5% calf serum in the presence or absence of 1 mM of the NOS inhibitor N<sup>G</sup>-monomethyl-L-arginine. The infected hepatocytes were recovered overnight prior to changing to fresh medium and subjecting to induction of apoptosis.

**Affinity Labeling of Active Caspases**—Aliquots of hepatocyte lysate (50  $\mu$ g of protein) were incubated with 1  $\mu$ M biotin-VAD-fmk for 1 h at room temperature in buffer A in a final volume of 50  $\mu$ l. The reaction was stopped by addition of  $\frac{1}{2}$  volume of  $2 \times$  SDS sample buffer followed by heating at 95  $^{\circ}$ C for 5 min. Alternatively, hepatocytes ( $5 \times 10^6$ ) were resuspended in 250  $\mu$ l of buffer A containing 10  $\mu$ g/ml cytochalasin B and 2  $\mu$ M biotin-VAD-fmk, and incubated for 15 min at 37  $^{\circ}$ C. Following 3 cycles of freezing-thawing the cell lysate was prepared as above. Proteins were resolved by 15% SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific binding was blocked with TBS-T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% non-fat milk for 1 h at room temperature. After washing two times with TBS-T, the membrane was probed with horseradish peroxidase-linked streptavidin (1:2000 dilution in TBS-T with 1% bovine serum albumin) for 1 h, and washed for four times with TBS-T. Labeled proteins were then visualized by enhanced chemiluminescence (Supersignal<sup>TM</sup>) according to the manufacturer's instructions.

For two-dimensional electrophoresis of affinity labeled active caspases, after incubation with biotin-VAD-fmk, cell lysates were diluted in dSDS (0.3% SDS, 1%  $\beta$ -mercaptoethanol, 5 mM Tris, pH 8.0) and boiled for 5 min (30). The samples were then snap frozen with liquid nitrogen and vacuum dried. The pellets were solubilized in 40  $\mu$ l of urea buffer (9.0 M urea, 4% (v/v) Nonidet P-40, 2% (v/v)  $\beta$ -mercaptoethanol, 20% Bio-Lyte pH 3–10) and stored at  $-20^{\circ}$ C. Isoelectric focusing electrophoresis (IEF) was performed using a Hoefer Mighty Small II SE 260 tube gel adaptor system (Hoefer Scientific Instruments, CA). Aliquots of samples (25  $\mu$ g of protein) were separated in a 3% acrylamide-IEF (Bio-Rad Ampholyte pH 5–7) according to the instruction from Hoefer (31). For the second dimensional electrophoresis, the tube gels were transferred to the top of 15% SDS-polyacrylamide gel (14  $\times$  16 cm, where each slab gel contained two tube gels) and subjected to SDS-PAGE for 4 h at 60 mA. Proteins were then transferred to nitrocellulose and probed with horseradish peroxidase-linked streptavidin as described above.

**Measurement of  $\Delta\Psi_m$  by Flow Cytometry**—For mitochondrial inner transmembrane potential ( $\Delta\Psi_m$ ) measurements, hepatocytes were incubated with DiOC<sub>6</sub>(3) (80 nM, Molecular Probes Inc.) for 30 min in culture medium at 37  $^{\circ}$ C, 5% CO<sub>2</sub>. DiOC<sub>6</sub>(3) is a fluorescent dye that is incorporated into mitochondria in a  $\Delta\Psi_m$ -dependent manner. As a positive control for  $\Delta\Psi_m$  loss, some hepatocytes were incubated with the uncoupling agent CCCP (50  $\mu$ M), a protonophore that disrupts  $\Delta\Psi_m$ . The cells were then washed twice with ice-cold phosphate-buffered saline and collected by centrifugation at  $200 \times g$ . Incorporation of DiOC<sub>6</sub>(3) by hepatocytes was determined by flow cytometry (Beckton Dickinson) using excitation of a single 488 nm argon laser. Loss of nuclear DNA (hypodiploidy) was determined by propidium iodine staining of ethanol-fixed cells as described previously (32).

**Immunoblotting Analysis**—Cytosol fractions were prepared from  $5 \times 10^6$  hepatocytes by homogenization and differential centrifugation in buffer A containing 250 mM sucrose as described previously (33). Cytosolic proteins were used for evaluating cytochrome *c* release. Cell lysates and whole cells were used for immunoblotting analysis of caspases and PARP, respectively. Thirty  $\mu$ g of protein was separated on 14 or 8% SDS-PAGE and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked with TBS-T containing 5% non-fat milk for 1 h at room temperature. Anti-caspase-8 monoclonal antibody C15 was diluted 1:20 in TBS-T containing 1% bovine serum albumin, anti-caspase-3 polyclonal antibody H277 was diluted 1:500, anti-PARP antibody 1:500 and anti-cytochrome *c* antibody 1:1000 in TBS-T containing 1% non-fat milk. After a 1-h incubation at room temperature with agitation, membranes were washed three times with TBS-T. The secondary antibody was incubated at 1:5000 dilution for 1 h. Following 4–5

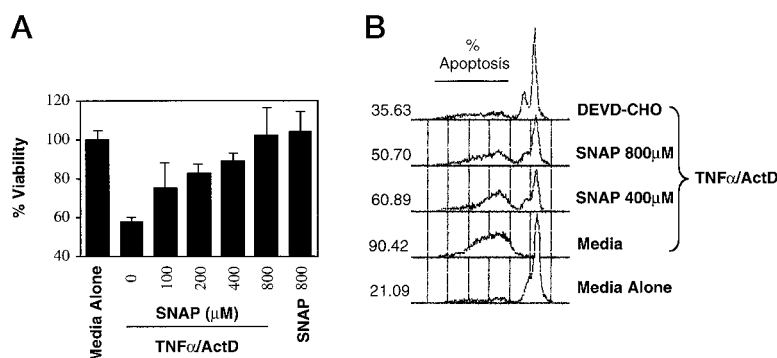


FIG. 1. Nitric oxide inhibits TNF $\alpha$ /ActD-induced apoptosis in cultured primary rat hepatocytes. A, hepatocytes were treated with or without 2000 units/ml TNF $\alpha$  plus 0.2  $\mu$ g of ActD (TNF $\alpha$ /ActD) in the absence or presence of increasing concentrations of SNAP for 12 h. Cell viability was measured by crystal violet staining (mean  $\pm$  S.D.,  $n = 4$ ). B, cells were treated with or without TNF $\alpha$ /ActD as above in the presence of SNAP or 200  $\mu$ M Ac-DEVD-CHO for 10 h. Cells were then labeled with propidium iodide and the percentage of cells exhibiting hypodiploidy was determined in duplicate by flow cytometry as described under "Experimental Procedures." Results are representative of three separated experiments with similar results.

washes with TBS-T, the protein bands were visualized with Supersignal<sup>TM</sup> according to the manufacturer's instructions.

## RESULTS

**NO Inhibits TNF $\alpha$ -induced Apoptosis in Primary Hepatocytes**—Consistent with previous reports (34, 27), TNF $\alpha$  at 2000 units/ml in the presence of actinomycin D (TNF $\alpha$ /ActD) induced massive apoptosis in hepatocytes in a time-dependent manner. The appearance of apoptotic morphology, including cell shrinkage, membrane blebbing, and apoptotic body formation, was seen as early as 6 h, and evident in the majority of cells (>70%) by 8 h (data not shown). Hepatocytes treated with TNF $\alpha$ /ActD for 12 h showed 40–50% decline in cell viability as compared with control cells (Fig. 1A). Consistent with our previous report (24), co-incubation with the NO donor, SNAP, prevented the loss of cell viability in a concentration-dependent manner, whereas SNAP alone had no effect on cell morphology or viability at the concentrations used (Fig. 1A). To confirm that the cell death induced by TNF $\alpha$ /ActD was in fact apoptosis, we analyzed the hypodiploidy of cellular DNA with propidium iodide staining and flow cytometry analysis. In agreement with the cell viability data, SNAP suppressed TNF $\alpha$ /ActD-induced formation of hypodiploidy (Fig. 1B). A caspase-3-like protease inhibitor, Ac-DEVD-CHO, also blocked TNF $\alpha$ /ActD-mediated apoptosis, indicating the involvement of caspase-3-like proteases in TNF $\alpha$ /ActD-induced apoptosis in hepatocytes (Fig. 1B).

**Caspase-3 Is Processed and Activated in TNF $\alpha$ /ActD-induced Apoptosis**—Although we have previously shown that caspase-3-like activity increases in TNF $\alpha$ /ActD-treated hepatocytes, it has not been established that pro-caspase-3 cleavage/activation takes place. Fig. 2 demonstrates that the appearance of the p17 caspase-3 cleavage product coincides with measurable increases in caspase-3-like activity. The earliest increase of caspase-3-like enzyme activity was observed around 3 h while the p17 band was detected at 4 h by immunoblotting analysis (Fig. 2, A and B).

We have shown that NO inhibits increases in caspase-3-like activity in TNF $\alpha$ /ActD-treated hepatocytes (24). To determine if NO blocked pro-caspase-3 processing we exposed TNF $\alpha$ /ActD-treated cells to SNAP and performed Western blotting analysis for the p17 cleavage product. Co-incubation with SNAP prevented the rise of caspase-3-like activity in cells treated with TNF $\alpha$ /ActD (Fig. 2C). As shown in Fig. 2D, SNAP blocked the cleavage/activation of pro-caspase-3, indicating that NO acts either at the level of pro-caspase-3 or disrupts the upstream signal that leads to caspase-3 activation.

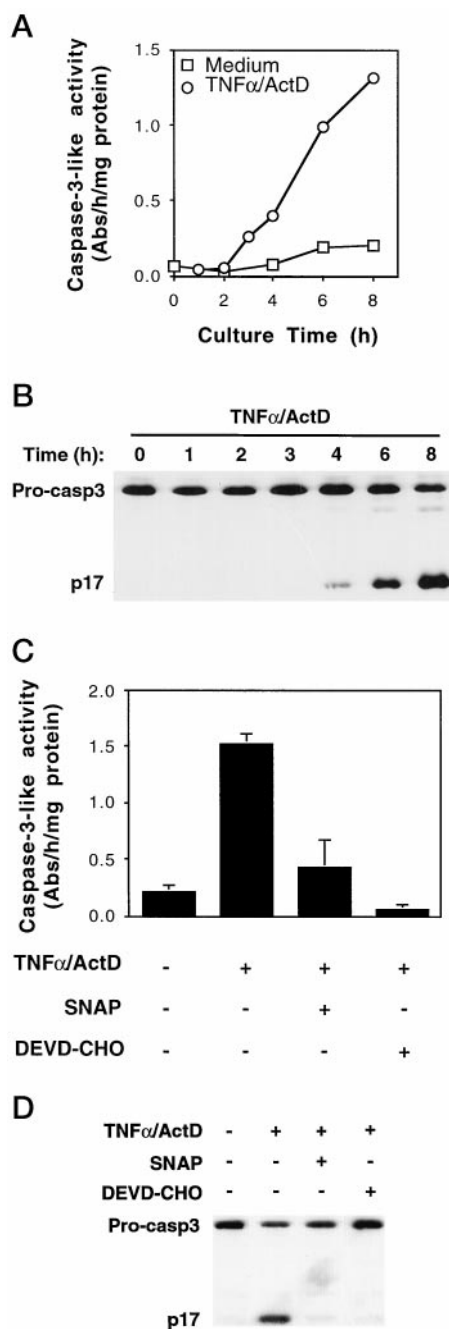
**Endogenously Produced NO Inhibits Apoptosis and Pro-**

**caspase-3 Processing/Activation**—To assure that the effects of the exogenous NO donor also occur when NO was generated by endogenous enzyme, adenoviral iNOS (AdiNOS) and its control partner adenoviral LacZ (AdLacZ) were used to transfect hepatocytes. iNOS expression in AdiNOS-transfected cells was confirmed by immunoblotting analysis and NO production was monitored by measuring NO<sub>2</sub><sup>-</sup> accumulation in the medium (Fig. 3A). NO was produced only in hepatocytes transfected with AdiNOS. The NOS inhibitor, NIO, completely inhibited NO production. It should be noted that in the presence of TNF $\alpha$ /ActD the NO production was only slightly decreased, suggesting that even in the presence of the transcription inhibitor, ActD, enough iNOS protein persists to produce NO throughout the experimental period. This was further substantiated by immunoblotting analysis for iNOS protein (Fig. 3A, inset).

Exposure to TNF $\alpha$ /ActD resulted in apoptosis in control cells and AdLacZ-transfected cells (Fig. 3B). In contrast, cells transfected with AdiNOS were protected significantly from apoptosis and this protection was completely reversed by the NOS inhibitor NIO (Fig. 3B). Likewise, caspase-3-like activity was inhibited only in AdiNOS-transfected cells but not in the AdLacZ cells upon TNF $\alpha$ /ActD exposure. When NO synthesis was inhibited by NIO, the caspase-3-like activity was brought back to the level similar to that detected in the AdLacZ group (Fig. 3C). Furthermore, processing and activation of pro-caspase-3 was nearly completely blocked by NO produced by iNOS (Fig. 3D).

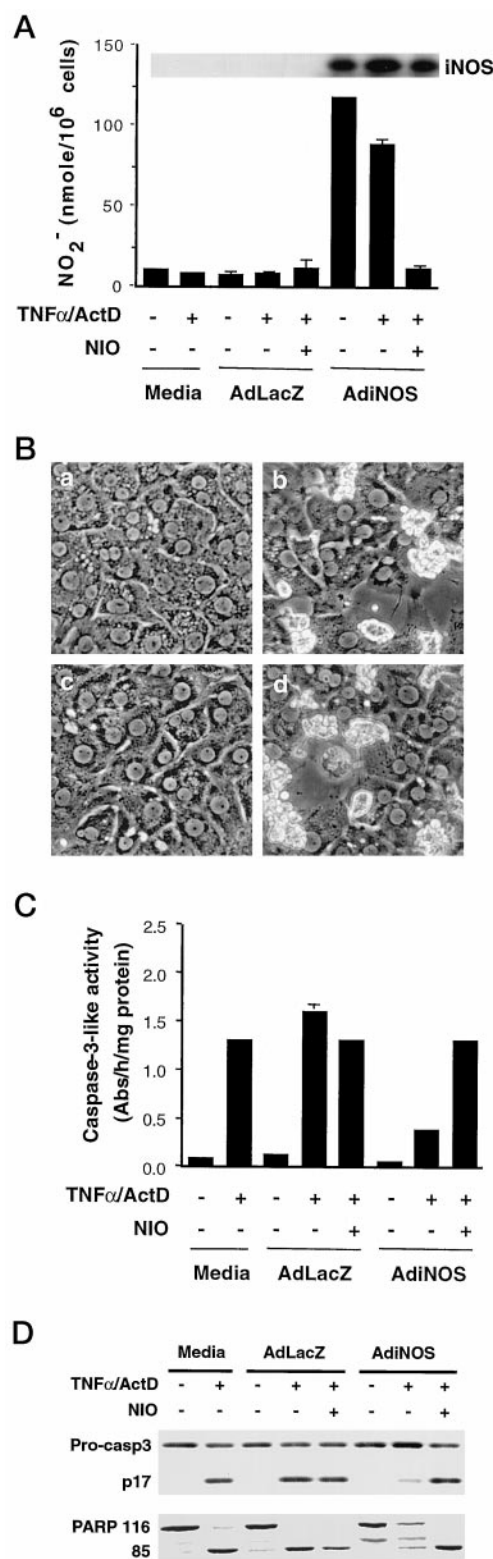
In many cell systems apoptosis is accompanied by the proteolytic cleavage of poly(ADP-ribose) polymerase (PARP) from a 116-kDa polypeptide to a 85-kDa fragment. In primary hepatocytes, PARP, an intracellular substrate of caspase-3 and -3-like proteases, was cleaved during TNF $\alpha$ /ActD-mediated apoptosis to its characteristic 85-kDa fragment (Fig. 3D). In agreement with the decreased caspase-3-like activity and suppressed pro-caspase-3 activation in AdiNOS transfected cells upon TNF $\alpha$ /ActD treatment, PARP cleavage was suppressed by NO (Fig. 3D).

**NO Inhibits Processing/Activation of Multiple Caspases in Primary Hepatocytes Induced to Undergo Apoptosis**—Numerous caspases may be activated in the apoptosis cascade. To determine whether multiple caspases are activated in hepatocytes treated with TNF $\alpha$ /ActD, we used the biotin-linked caspase inhibitor, biotin-VAD-fmk, to label all the active caspase species present in hepatocytes undergoing apoptosis. Z-VAD-fmk is an irreversible caspase inhibitor that covalently binds to the active cavity of a wide spectrum of active caspases.

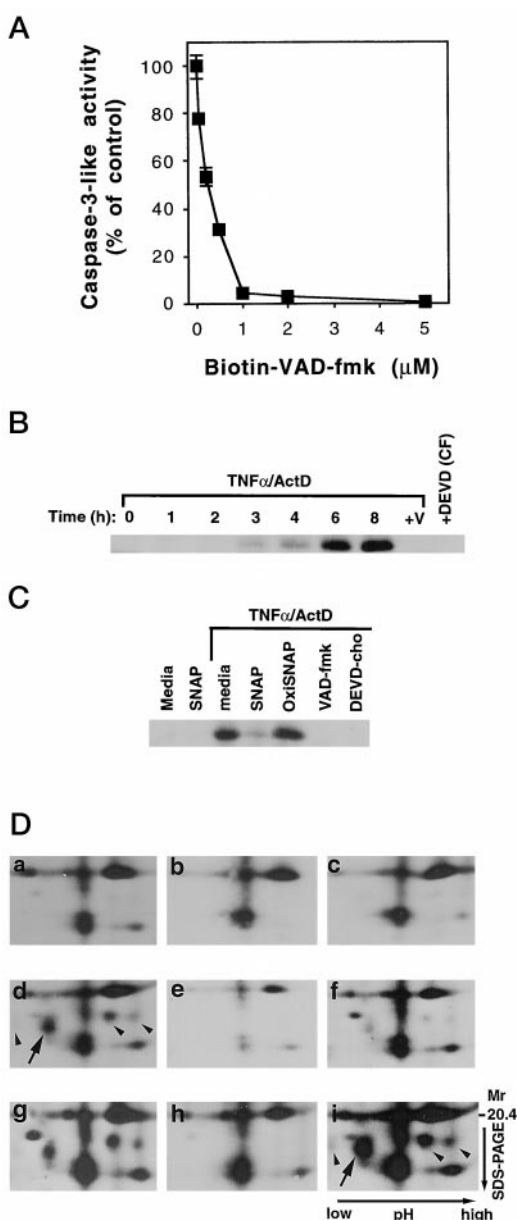


**FIG. 2. Activation of caspase-3 in TNF $\alpha$ /ActD-induced apoptosis and its inhibition by NO and caspase inhibitor Ac-DEVD-CHO.** *A*, time-dependent activation of caspase-3-like activity in apoptotic hepatocytes. Cells were treated with or without TNF $\alpha$ /ActD (2000 units/ml and 0.2  $\mu$ g/ml, respectively). At indicated time points, cells were collected, washed with ice-cold phosphate-buffered saline, and cell lysate was prepared. Caspase-3-like activity was determined using a colorimetric assay with Ac-DEVD-pNA. *B*, immunoblotting analysis of caspase-3 activation. Thirty  $\mu$ g of cell lysates obtained as above was loaded into each lane, separated on 14% SDS-PAGE, transferred onto a nitrocellulose membrane, and visualized as described under "Experimental Procedures." *C* and *D*, hepatocytes were treated with TNF $\alpha$ /ActD in the presence of 800  $\mu$ M SNAP or 200  $\mu$ M Ac-DEVD-CHO for 8 h. Caspase-3-like activity (mean  $\pm$  S.D.,  $n = 3$ ) (*C*) and caspase-3 Western blot (*D*) was carried out as above. Results are representative of three separate experiments with similar results.

Z-VAD-fmk (100  $\mu$ M) effectively prevented TNF $\alpha$ /ActD-induced apoptosis in cultured primary hepatocytes (data not shown). When incubated with cell lysates prepared from TNF $\alpha$ /ActD-treated hepatocytes, biotin-VAD-fmk inhibited caspase-3-like activity in a dose-dependent manner (Fig. 4A). Thus, biotin-



**FIG. 3. AdiNOS gene transfer suppresses TNF $\alpha$ /ActD-induced apoptosis.** Primary hepatocytes were infected with AdLacZ or AdiNOS and then cultured with or without TNF $\alpha$ /ActD in the absence or presence of 1 mM NIO for 7 h. *A*, NO<sub>2</sub><sup>-</sup> levels were measured in the culture media by Greiss reaction (mean  $\pm$  S.D.,  $n = 3$ ). *Inset* is a Western blot for iNOS for the same groups. *B*, phase-contrast photomicrographs showing cellular morphology of control untreated cells (*a*), AdLacZ transfected, TNF $\alpha$ /ActD-treated cells (*b*), AdiNOS transfected, TNF $\alpha$ /ActD-treated cells (*c*), and AdiNOS transfected, TNF $\alpha$ /ActD plus NIO-treated cells (*d*). Photomicrographs were taken at magnification of  $\times 20$ . *C*, caspase-3-like activity; and *D*, Western blots of caspase-3 and PARP of the same groups. Results are representative of at least three independent experiments with similar results.



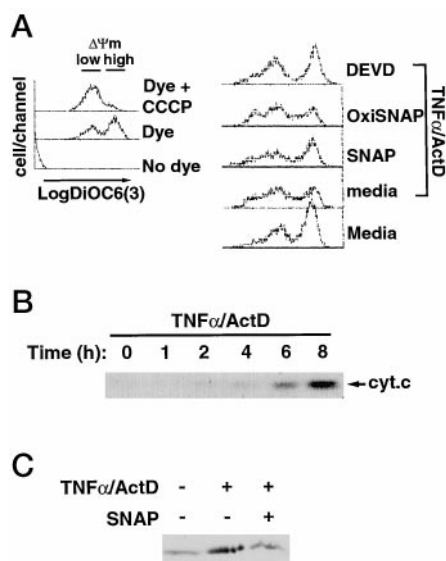
**FIG. 4. NO suppresses the activation of multiple caspases.** *A*, aliquots (50  $\mu\text{g}$  of protein) of cell lysate from hepatocytes treated with  $\text{TNF}\alpha/\text{ActD}$  for 7 h were incubated with increasing concentrations of biotin-VAD-fmk for 1 h at room temperature. Caspase-3-like activity in the aliquots was then determined by the colorimetric assay. *B*, affinity labeling blot of active caspases. Hepatocytes were induced to undergo apoptosis with  $\text{TNF}\alpha/\text{ActD}$  in the absence and presence of 100  $\mu\text{M}$  z-VAD-fmk (+V) for indicated times or for 7 h. Cell lysates were prepared and active caspases were labeled with 1  $\mu\text{M}$  biotin-VAD-fmk. The labeled caspases were resolved on SDS-PAGE, transferred to nitrocellulose membrane, and visualized as described under "Experimental Procedures." To determine the specificity of the labeling, cell lysate (50  $\mu\text{g}$  of protein) from cells treated with  $\text{TNF}\alpha/\text{ActD}$  for 7 h was incubated with biotin-VAD-fmk as above in the presence of 10-fold excess of Ac-DEVD-CHO in the cell-free system (*last lane*). *C*, biotin-VAD-fmk affinity labeling of cell lysate prepared from hepatocytes treated with indicated reagents for 7 h. *D*, two-dimensional electrophoresis analysis of biotin-VAD-fmk labeling of active caspases. Hepatocytes were treated under specified conditions for 7 h. Cell lysate was labeled with biotin-VAD-fmk and resolved by two-dimensional electrophoresis as described under "Experimental Procedures." *a*, control cells without treatment; *b*,  $\text{TNF}\alpha$  alone; *c*, ActD alone; *d*,  $\text{TNF}\alpha/\text{ActD}$  (arrow and arrowheads indicate new spots, black arrow points the p17 subunit of caspase-3); *e*, 800  $\mu\text{M}$  SNAP alone; *f*,  $\text{TNF}\alpha/\text{ActD}$  plus 800  $\mu\text{M}$  SNAP; *g*,  $\text{TNF}\alpha/\text{ActD}$  plus 800  $\mu\text{M}$  Oxidized SANP (OxiSNAP); *h*,  $\text{TNF}\alpha/\text{ActD}$  plus 200  $\mu\text{M}$  Ac-DEVD-CHO, and *i*, overexposed image of blot *d* to better demonstrate the faint new spots. Results are representative of two to four similar experiments.

VAD-fmk interacts directly with activated caspases, establishing the basis for affinity labeling of all the active caspases.

Affinity labeling of active caspases with biotin-VAD-fmk was carried out under conditions where more than 95% of caspase-3-like activity was inactivated in lysates from  $\text{TNF}\alpha/\text{ActD}$ -treated cells. Fig. 4*B* shows labeled active caspases in hepatocytes treated with  $\text{TNF}\alpha/\text{ActD}$  for various periods of time. No active caspases were detected when cell lysate from control cells was incubated with biotin-VAD-fmk. In contrast, 3 h after treatment with  $\text{TNF}\alpha/\text{ActD}$  a double band representing active caspases was detected with the biotin probe. The intensity of the labeling increased with time in a manner similar to caspase-3-like activity (see above Fig. 2*A*). The presence of a 10-fold excess of Ac-DEVD-CHO abolished the ability of biotin-VAD-fmk to label caspases (*last lane* in Fig. 4*B*), demonstrating the labeling specificity for active caspases. Furthermore, there was no labeling of active caspases if cells were previously exposed to  $\text{TNF}\alpha/\text{ActD}$  in the presence of z-VAD-fmk, providing further support for the conclusion that these new bands represent active caspases. As shown in Fig. 4*C*, cell treatment with SNAP effectively prevented the formation of active caspases whereas oxidized SNAP had no effect.

Since the large subunits of all known caspases have similar molecular weights, one-dimensional SDS-gel electrophoresis may not adequately distinguish various active caspases. Therefore, we used two-dimensional isoelectric focusing/SDS-gel electrophoresis to better resolve the biotin-VAD-fmk-labeled active caspases. The two-dimensional isoelectric focusing/SDS-gel electrophoresis analysis at the 7-h time point revealed at least four newly labeled spots present in  $\text{TNF}\alpha/\text{ActD}$ -treated cells that were absent in the control cells and in cells treated with  $\text{TNF}\alpha/\text{ActD}$  in the presence of Ac-DEVD-CHO. Spots for two of the active caspases were of much higher intensity (Fig. 4*D*). The major spot (black arrow, Fig. 4*D*) was identified as the large subunit of caspase-3 by immunoblotting analysis of the same blot (data not shown). In agreement with our above one-dimensional electrophoresis results, NO nearly completely blocked the activation of all the caspases including caspase-3 when cells were treated with  $\text{TNF}\alpha/\text{ActD}$  and SNAP (Fig. 4*D*). In contrast, oxidized SNAP had no effect on the processing of these caspases. No active caspases were detected in cells treated with ActD or  $\text{TNF}\alpha$  alone.

**Involvement of Mitochondria in  $\text{TNF}\alpha/\text{ActD}$ -mediated Apoptosis and the Effect of NO**—The above data indicate that NO prevents the activation of multiple caspases. Caspase activation is thought to contribute to changes in mitochondrial function with release of cytochrome *c*. These mitochondrial events can also lead to activation of downstream caspases. To determine the involvement of mitochondria in hepatocyte apoptosis, we first examined the changes of mitochondrial transmembrane potential upon induction of apoptosis with  $\text{TNF}\alpha/\text{ActD}$ . Mitochondrial inner transmembrane potential ( $\Delta\Psi\text{m}$ ) was monitored by incorporated fluorescence of the cationic lipophilic dye DiOC<sub>6</sub>(3), a potential-sensitive dye. The pattern of the fluorescence of DiOC<sub>6</sub>(3) taken up by control cultured hepatocytes revealed two cell populations, the major one with higher fluorescence representing cells with intact high  $\Delta\Psi\text{m}$  and a second smaller population with low  $\Delta\Psi\text{m}$  (Fig. 5*A*). The presence of a population with low  $\Delta\Psi\text{m}$  was not surprising since there was a basal level of apoptosis in these cultures of primary cells as revealed by the propidium iodine staining of DNA (Fig. 1*B*) and by Annexin V labeling analysis (data not shown). When cells were labeled with DiOC<sub>6</sub>(3) in the presence of 50  $\mu\text{M}$  CCCP, a protonophore that disrupts  $\Delta\Psi\text{m}$ , the population of cells with higher  $\Delta\Psi\text{m}$  was converted to the low  $\Delta\Psi\text{m}$  cells (Fig. 5*A*), confirming that the dye was sensitive to mito-



**FIG. 5. NO inhibits mitochondrial membrane potential changes and cytochrome *c* release.** *A*: *left panel*, hepatocytes were loaded with 80 nM DiOC<sub>6</sub>(3) in the absence and presence of 50 μM CCCP for 30 min at 37 °C. Incorporation of the dye was evaluated with flow cytometry. *Right panel*, hepatocytes were cultured in the absence and presence of 2000 units/ml TNFα plus 0.2 μg/ml ActD supplemented with or without 800 μM SNAP, 800 μM Oxi-SNAP, or 200 μM Ac-DEVD-CHO as indicated for 7.5 h prior to addition of DiOC<sub>6</sub>(3). Cells were labeled for 30 min and mitochondrial membrane potential was then determined by flow cytometry. *B* and *C*, immunoblots for cytosolic cytochrome *c*. Cells were cultured with TNFα (2000 units/ml) plus ActD (0.2 μg/ml) in the absence and presence of 800 μM SNAP for the indicated times (*B*) or for 8 h (*C*). Cytosolic extract was obtained by centrifugation at 100,000 × *g* for 1 h at 4 °C after homogenization of cells in buffer A containing 250 mM sucrose. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and detected with anti-cytochrome *c* antibody as detailed under "Experimental Procedures." Results are representative of two similar experiments.

chondrial transmembrane depolarization. The amount of DiOC<sub>6</sub>(3) dye taken up by cells remained unaltered for at least 6 h after TNFα/ActD treatment (data not shown). However, a reduction in ΔΨ<sub>m</sub> was detected 8 h after exposure to TNFα/ActD. Coincubation of SNAP with TNFα/ActD suppressed the disruption of ΔΨ<sub>m</sub>, whereas oxidized SNAP had no effect on the alteration of ΔΨ<sub>m</sub> in response to TNFα/ActD-induced apoptosis. The caspase-3-like protease inhibitor Ac-DEVD-CHO also abolished the mitochondrial depolarization, indicating that the activation of the Ac-DEVD-CHO-inhibitable proteases occurred prior to mitochondrial depolarization in primary hepatocytes.

We next investigated whether translocation of cytochrome *c* to cytoplasm occurs in TNFα/ActD-treated hepatocytes. Release of cytochrome *c* to cytoplasm in apoptotic cells was detected by immunoanalysis. As shown in Fig. 5*B*, cytosol from untreated cells contained negligible cytochrome *c* whereas cytochrome *c* started to accumulate in the cytosol as early as 4 h in cells exposed to TNFα/ActD with additional increases through 8 h. The level of cytochrome *c* in cytosol of hepatocytes treated with TNFα/ActD for 8 h was brought back down to the control levels by co-treatment of cells with SNAP, demonstrating that NO acts at or above the level of cytochrome *c* release (Fig. 5*C*).

**NO Inhibits Caspase-8 Activation**—It is well described that the most proximal caspase activated following interaction of TNFα with its receptor is caspase-8 (35). Activation of caspase-8 can lead to direct activation of other caspases or to the release of cytochrome *c* from mitochondria (9, 10, 36). The observation that NO inhibits the proteolytic activation of all caspases as well as apoptosis-associated mitochondrial changes

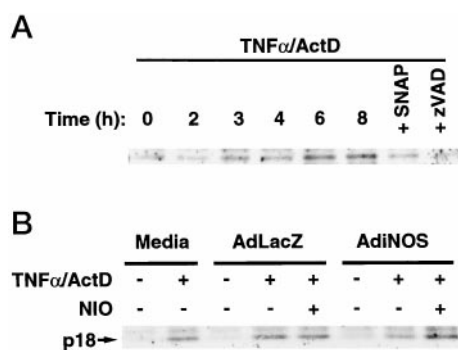
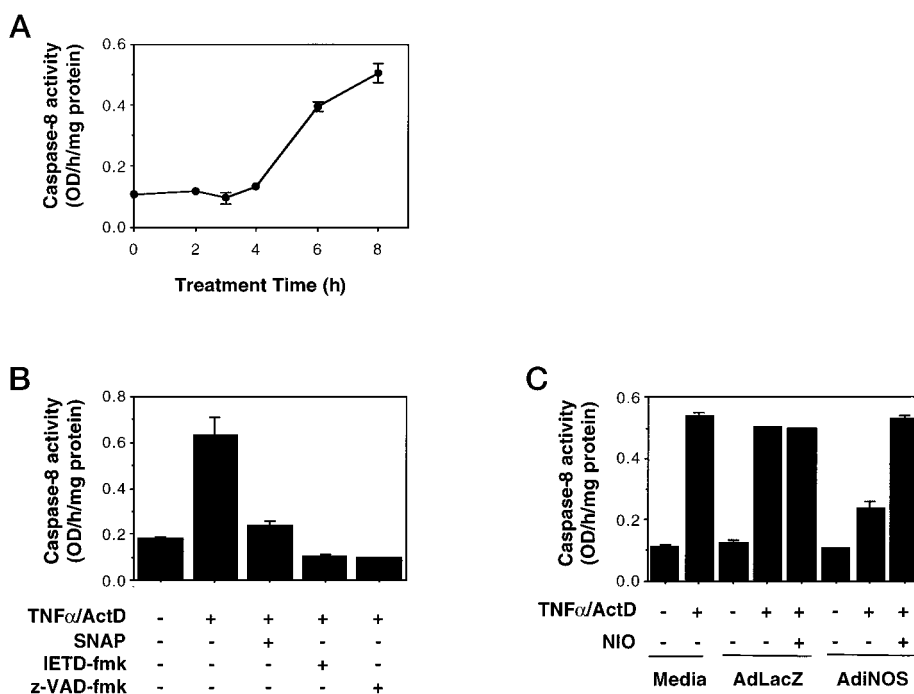
suggests that activation of caspase-8 was inhibited by NO. To further determine whether activation of caspase-8 occurs and whether it is in fact inhibited by NO, we first examined the time course for caspase-8 activity in cytosolic extracts from TNFα/ActD-treated hepatocytes using the caspase-8-specific substrate IETD-*p*NA. IETD-*p*NA is preferentially cleaved by caspase-8, with a  $k_{cat}/K_m$  value around 60-fold higher when compared with that of caspase-3 (37). Caspase-8 activity was increased in TNFα/ActD-treated cells in a time-dependent manner (Fig. 6*A*) and was significantly inhibited by SNAP (Fig. 6*B*). Like z-VAD-fmk, the caspase-8 inhibitor z-IETD-fmk (50 μM, Ref. 38) inhibited the caspase-8 activity and rescued the cells from TNFα/ActD-induced cell death, providing further supports for the involvement of caspase-8 and the role for NO-dependent caspase-8 inhibition. Furthermore, caspase-8 activity was suppressed in cells transfected with iNOS and subsequently exposed to TNFα/ActD, a suppression completely reversed by the iNOS inhibitor NIO (Fig. 6*C*). Finally, we performed immunoblotting analysis specifically for the large subunit of caspase-8. Using a monoclonal antibody that recognizes the large subunit of caspase-8, we found that a ≈18-kDa polypeptide was detectable as early as 3 h after cell exposure to TNFα/ActD, and that its intensity increased in a time-dependent manner (Fig. 7*A*). The catalytically active subunit of caspase-8 was seen only in apoptotic cells, but not in the control cells. When cells were treated with TNFα/ActD in the presence of SNAP or z-VAD-fmk, processing of caspase-8 was inhibited (Fig. 7*A*). Likewise, AdiNOS transfection significantly suppressed the appearance of this proteolytic fragment whereas addition of NIO reversed this suppression (Fig. 7*B*), demonstrating that in TNFα/ActD-treated hepatocytes caspase-8 was indeed activated and its proteolytic processing was markedly inhibited by NO.

## DISCUSSION

This study was undertaken to determine if NO inhibited caspase activation and the associated mitochondrial changes in TNFα/ActD-induced apoptosis. We first identified the number of caspases activated in hepatocytes. We show that at least four caspases were activated including caspase-3 and caspase-8. Furthermore, caspase activation occurred in association with loss of mitochondrial membrane potential and accumulation of cytochrome *c* in the cytosol. Our results demonstrate that exposure to NO, either via a NO donor or through expression of iNOS, markedly suppresses activation of all of the caspases and prevents loss of mitochondrial membrane potential and release of cytochrome *c*. Thus, NO effectively inhibits not just the activity of active caspases but also the proteolytic cleavage/activation of pro-caspases in cells.

Participation of caspases has been demonstrated in hepatocyte apoptosis induced by TNFα (24, 25), staurosporine (39), transforming growth factor-β (39), aging in culture and Fas (40). We (24, 26) and others (39–41) have previously shown that caspase-3-like protease activity increases in hepatocytes undergoing spontaneous apoptosis or apoptosis in response to transforming growth factor-β, TNFα or Fas, while caspase-1-like activity does not. The caspase-3-like family of proteases is defined by a specificity for the DEVD substrate sequence and includes caspases-3 and -7 as well as caspase-8 due to the overlap in substrate specificity (26, 42, 43). The identity of specific caspases activated under these conditions has not been well established. Here two-dimensional electrophoresis analysis of affinity labeled activated caspases in TNFα/ActD-treated cells revealed at least four different active caspase species. Immunoblotting analysis of the two-dimensional gel electrophoresis identified one major spot as caspase-3. The identity of the other active caspases detected by this approach is uncertain

**FIG. 6. NO inhibits caspase-8 activation.** *A*, time-dependent activation of caspase-8 in apoptotic hepatocytes. Cellular proteins isolated from hepatocytes exposed to TNF $\alpha$ /ActD for indicated times were analyzed for Ac-IETD-pNA cleavage activity. *B*, cells were treated with or without TNF $\alpha$ /ActD (2000 units/ml and 0.2  $\mu$ g/ml, respectively) in the presence of SNAP (800  $\mu$ M), z-IETD-fmk (50  $\mu$ M), or z-VAD-fmk (50  $\mu$ M) for 8 h and cell lysate were prepared. Caspase-8 activity was determined using a colorimetric assay with Ac-IETD-pNA (100  $\mu$ M). *C*, hepatocytes were infected with AdLacZ or AdiNOS and then cultured with or without TNF $\alpha$ /ActD in the absence or presence of 1 mM NIO for 6 h. Caspase-8 activity in the cell lysates was measured as above. Results are one representative of two independent experiments measured in duplicates.



**FIG. 7. NO suppresses caspase-8 activation.** *A*, time-dependent activation of caspase-8 in apoptotic hepatocytes. Cellular proteins isolated from hepatocytes treated with TNF $\alpha$ /ActD for indicated times or treated in the presence of 800  $\mu$ M SNAP or 100  $\mu$ M z-VAD-fmk for 8 h were separated on SDS-PAGE and transferred onto a nitrocellulose membrane. The catalytic subunit p18 of caspase-8 was visualized by immunoblotting analysis. *B*, NO-dependent suppression of caspase-8 activation. Hepatocytes were infected with AdLacZ or AdiNOS as described in the legend to Fig. 3 and under "Experimental Procedures." The large subunit of caspase-8 was visualized by immunoblotting analysis.

and currently under investigation in our laboratory. Based on the fact that TNF $\alpha$ /ActD-induced hepatocyte apoptosis requires the death receptor TNFR1 and current knowledge on death receptor-mediated signaling pathway, it is tempting to speculate that at least one of the active caspase species labeled by biotin-VAD-fmk is caspase-8, the most apical caspase in the cascade. This is in line with our immunoblotting analysis with a monoclonal antibody that recognizes the large subunit of caspase-8 (Fig. 7), where pro-caspase-8 was processed/activated to p18 in TNF $\alpha$ /ActD-treated hepatocytes but not in control cells. Two-dimensional electrophoresis analysis of biotin-VAD-fmk-labeled recombinant human caspase-8 revealed three active species with the same sizes but slightly different charges. Two of the three caspase-8 species closely co-migrated with two of the four labeled active caspases in the TNF $\alpha$ /ActD-treated rat hepatocytes (same IEF positions but migrated slightly slower in SDS-PAGE, probably due to species difference) (data not shown), indicative of the identity of two labeled

spots as caspase-8.

Substantial evidence is emerging that mitochondria participate in both the initiation and the execution phases of apoptosis. Release of cytochrome *c* or apoptosis inducing factor from mitochondria to cytoplasm induces activation of effector caspases and nuclear apoptosis in a cell-free system (13, 44). Microinjection of cytochrome *c* into cells has been shown to initiate the apoptotic cascade (45). The mechanism by which cytochrome *c* is translocated from the mitochondrial intermembrane space to the cytoplasm remains elusive. Mitochondrial permeability transition has been implicated in cytochrome *c* release, probably through the rupture of the mitochondrial outer membrane. Onset of permeability transition results in membrane depolarization, uncoupling of oxidative phosphorylation, and mitochondrial swelling. Disruption of mitochondrial transmembrane potential ( $\Delta\Psi_m$ ) has been reported to precede caspase-3 activation and the final execution stage of apoptosis in many situations, but not all (46). In TNF $\alpha$ /ActD-induced hepatocyte apoptosis, changes in mitochondrial  $\Delta\Psi_m$  were not evident until the late phase of apoptosis (>8 h following apoptosis induction) when caspase-3 was fully activated (Fig. 5A). The TNF $\alpha$ /ActD-induced disruption of  $\Delta\Psi_m$  was blocked by the caspase inhibitor Ac-DEVD-CHO, suggesting that mitochondrial depolarization requires caspase activation. In contrast, mitochondrial cytochrome *c* was released by 4 h into cytoplasm in TNF $\alpha$ /ActD-treated hepatocytes and paralleled caspase-3-like protease activation (Fig. 5B). Most recently Fas-mediated activation of caspase-8 has been shown to cleave Bid, a Bcl-2 interacting protein, resulting in the translocation of cleaved Bid to the mitochondria where it triggers cytochrome *c* release (9, 10). The effect of caspase-8 may be amplified by cytochrome *c*-dependent downstream caspase activation, leading to an amplification loop between caspases and mitochondria (36, 47). Loss of cytochrome *c* could uncouple oxidative phosphorylation and thus result in disruption in  $\Delta\Psi_m$ . The capacity of NO to suppress all of these events could be explained by the inhibition of caspase activation by NO.

Inhibition of iNOS during endotoxemia results in increased hepatic apoptosis and this effect is inhibited by the simultaneous infusion of a NO generator (48, 49). Likewise, infusion of a NO donor blocks TNF $\alpha$  + *N*-galactosamine-induced hepatic

injury and apoptosis *in vivo* (48). Thus, NO is a potent endogenous inhibitor of apoptosis in the liver. Previous mechanistic studies have shown that NO directly inactivates active caspases via S-nitrosylation of the catalytic cysteine residue (26). In the present study, we demonstrate that NO prevents the proteolytic activation of multiple pro-caspases in intact cells. This includes the inhibition of the most proximal protease, caspase-8 as well as the executioner caspase-3. Two non-exclusive mechanisms have been implicated for the propagation of the apoptotic signaling cascade from caspase-8 to caspase-3 in TNF $\alpha$ -mediated cell death. These include the direct activation of caspase-3 by caspase-8 and the cytochrome *c*-dependent activation of caspase-9. According to current understanding, death receptors (TNFR1 and Fas) induce the recruitment of FADD (Fas-associated death domain protein) and pro-caspase-8, thus forming a membrane anchored death-inducing signal complex (35). Protein-protein interaction between the death effector domain of pro-caspase-8 and FADD results in the cleavage/activation of pro-caspase-8. It has been shown in a cell-free system that at higher concentrations active caspase-8 can directly activate downstream caspases such as caspase-3 and thereby promotes the apoptotic cascade. However, at lower concentrations of caspase-8, the mitochondrial release of cytochrome *c* is necessary for death signal amplification and a complete engagement of the apoptotic machinery (36). It is also possible that direct activation of caspase-3 by an upstream caspase can lead to mitochondrial changes and release of cytochrome *c* through the capacity of caspase-3-like proteases to cleave the Bcl-2 family members (50, 51). This inhibition of cytochrome *c* release and loss of mitochondrial  $\Delta\Psi_m$  by NO found in this study could have been due to the inhibition of caspase-8 activation, thus preventing the downstream events. Furthermore, NO does not appear to interrupt the death signal transduced from Fas receptor to FADD/pro-caspase-8, since NO was still able to suppress apoptosis induced by overexpression of FADD or caspase-8 in Jurkat cells (20). It is not clear at present how NO inhibits the processing of pro-caspase-8. In the previous studies showing inactivation of caspase enzymes via S-nitrosylation, including caspase-8 (20, 26), only processed and activated caspases were used. It is likely that in intact cells such as the system used in this study, a limited amount of caspase-8 was activated upon the formation of the death-inducing signal complex but subsequently inactivated by NO through S-nitrosylation. As a result, further activation of caspase-8 and other effector caspases was diminished. This possibility is supported by our previous work (24) showing that the reducing agent dithiothreitol partially reversed the NO-mediated suppression of caspase activity in cells. Alternatively, NO may S-nitrosylate the essential cysteine residue of the pro-caspase-8 and thus prevent the zymogen from undergoing proteolytic activation. Despite evidence for NO-dependent S-nitrosylation of activated caspases, it remains unclear whether NO can effectively S-nitrosylate pro-caspases and whether this S-nitrosylation has any effect on pro-caspase processing.

The anti-apoptotic function of NO is not unique to hepatocytes, neither is it restricted only to death receptor- or growth factor withdrawal-mediated apoptosis. Endogenous NO synthesis or exposure to low level NO donors has been shown previously to prevent apoptosis in human B lymphocytes (15), splenocytes (18), eosinophil (17), ovarian follicles (24), and endothelial cells (16). Various mechanisms for the anti-apoptotic effect of NO have been proposed in addition to S-nitrosylation of caspases. They include, NO-induced up-regulation of protective proteins such as heat shock protein 70 (Hsp70) (27) and Bcl-2 (18), NO-dependent disruption of JNK activation (17),

and increases in cGMP levels (22, 24). Results in the present study revealed that NO inhibited TNF $\alpha$ /ActD-induced apoptosis even when transcription was inhibited, demonstrating an interaction of NO or products of NO with the death cascade. Furthermore, levels of Hsp70 and Bcl-2 were not altered in TNF $\alpha$ /ActD-treated hepatocytes upon simultaneous exposure to NO donor or to NO produced from iNOS in the cells.<sup>2</sup> In addition, we have previously shown that the NO-mediated protection in hepatocytes is only partially due to increases in cGMP levels (24). Even though much of the protection by NO is cGMP-independent, it is interesting to note that high concentrations of cGMP analogues effectively inhibit apoptosis in hepatocytes (24). Finally, NO has no effect on the activation of JNK kinase induced by TNF $\alpha$ /ActD.<sup>2</sup> Taken together, our data demonstrate that the anti-apoptotic effect of NO is a result of the capacity of NO to prevent caspase cleavage/activation into catalytic active form and consequently the apoptosis resulting from caspase activation. Considering the central roles of caspases in apoptosis and the wide distribution of NO synthase in tissues, one might speculate that the regulation of apoptosis by NO by modulating caspase activation could be of great significance.

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