Nitric oxide (NO) and tumor necrosis factor-α (TNFα) play important roles in the pathogenesis of liver disease during acute inflammation. The present study was designed to elucidate the effect of NO pre-exposure on TNFα-induced hepatotoxicity. Pretreatment of primary cultures of rat hepatocytes with the NO donor S-nitroso-N-acetylpenicillamine (SNAP) induced the expression of heat shock protein 70 (HSP70) mRNA and protein, which was associated with thermotolerance and cytoprotection from TNFα-induced interference with TNFα-mediated apoptosis correlated with the level of HSP70 expression. SNAP pretreatment inhibited reactive oxygen intermediate generation and lipid peroxidation effects that were reversed by blocking HSP70 expression using an antisense oligonucleotide to HSP70. Finally, endogenous NO formation, induced in hepatocytes stimulated with interferon-γ and interleukin-1β, led to the formation of GSNO and GSSG, induced HSP70, and attenuated TNFα-mediated cytotoxicity. These findings demonstrated that NO can induce resistance to TNFα-induced hepatotoxicity, possibly through the stimulation of HSP70 expression.

Many cell types have the capacity to generate NO from l-arginine. However, the level of NO production and the functional role of NO can vary between cell types. Two constitutive calcium-dependent NO synthases (cNOS)1: neuronal cNOS (nNOS or NOS1) and the endothelial cNOS (eNOS or NOS3), generate small quantities of NO sufficient only for cellular signaling under most circumstances. However, some cells that are exquisitely sensitive to NO, such as neurons, have been shown to exhibit toxicity in response to NOS1 activation (1). A third isoform, NOS2, typically expressed after exposure of cells to inflammatory stimuli (e.g. cytokines and microbial products), originally was referred to as the inducible NOS (iNOS). More recently, it has been recognized that NOS2 is expressed in some resting epithelial cells (2). NO production by NOS2 occurs independent of elevations in basal intracellular calcium concentration (3), and the quantities produced are sufficient to damage or kill susceptible cells (4) or microorganisms (5).

The precise factors that determine cellular sensitivity to NO-mediated toxicity are not clear; however, a number of molecular targets for NO and its reaction products have been identified. It is through the interaction with these targets, typically sulfurhydryl-containing molecules (6, 7) or redox metal-containing proteins (8), that NO affects its biological action. The quantities of NO generated by cNOS isoforms are adequate to activate soluble guanylate cyclase by dislocation of the heme iron within the enzyme (9). Other signaling actions of NO mediated via redox-sensitive sites include inhibition of protein kinase C, activation of tyrosine kinase, inactivation of NF-κB, activation of SoxRS, and activation of G proteins (reviewed in Ref. 10). NO also causes several metabolic alterations by inhibiting the actions of certain thiol- and iron-containing enzymes and thus inhibits mitochondrial respiration, the tricarboxylic acid cycle, DNA synthesis, and antioxidant and DNA repair enzymes. NO and superoxide react together at a diffusion-controlled rate to yield peroxynitrite (ONOO−), which inflicts cellular injury through oxidation of many biological molecules. Furthermore, ONOO− has also been implicated in the inactivation of Mn and Fe superoxide dismutase (11) and aconitase (12, 13). In contrast, NO may protect cells from reactive oxygen intermediate (ROI)-mediated cytotoxicity by scavenging superoxide anions which are implicated in toxicity through the formation of hydrogen peroxide or hydroxyl radical via the Fenton reaction (14). NO also reduces toxic ferryl species to ferrous ion, thereby blocking the hemoprotein-mediated Fenton-like reaction (15). Furthermore, NO has been shown to terminate the propagation of radical-mediated lipid peroxidation (16).

Hepatocytes express NOS2 and produce large amounts of NO in response to the synergistic combination of cytokines such as TNFα, IL-1β, and IFNγ (17) or IL-1β alone in sufficient concentrations (18). This NO synthesis has been associated with DNA condensation, inhibition of protein synthesis, and death.
creased levels of cytochrome P450 and catalase activity in vitro (19). Despite these changes, hepatocytes are rather resistant to NO toxicity. Exposure to NO also protects primary cultured hepatocytes from the cytotoxic effects of higher doses of NO and H$_2$O$_2$ (20). This protective action appears to be mediated by the induction of cytoprotective stress proteins such as heme oxygenase, which, in turn, results from NO-induced alteration in iron homeostasis.

In vivo, large quantities of NO can be generated in the liver in acute inflammation (21, 22). We have suggested that this NO can have protective actions. TNF-α is also produced in the liver in inflammation (23), most likely by the resident macrophages. Studies into the mechanism of hepatic TNF-α toxicity have shown that TNF-α in the presence of RNA or protein synthesis inhibitors induces DNA fragmentation characteristic of apoptosis (24). Furthermore, TNF-α is thought to mediate liver injury in acute inflammation (25) and contribute to fulminant hepatic failure (26, 27). In other cell types, NO has been shown to either promote (28, 29) or inhibit (30, 31) apoptosis. Both NO and TNF-α would be expected to be present in the liver under many inflammatory liver conditions; however, it is unknown if NO exposure potentiates or prevents TNF-α toxicity in hepatocytes. In this study, we examined the consequences of NO exposure on TNF-α-induced apoptosis in cultured rat hepatocytes. We report that NO exposure prevents subsequent TNF-α-induced cell death through the induction of heat shock protein 70 (HSP70).

**EXPERIMENTAL PROCEDURES**

**Materials—**Williams medium E, penicillin, streptomycin, l-glutamine, and HEPES were purchased from Life Technologies, Inc. Insulin was purchased from Lilly, and calf serum was obtained from HyClone Laboratories (Logan, UT). Murine macrophage NOS2 monoclones were obtained from Transduction Laboratories (Lexington, KY). TNF-α was purchased from Genzyme (Cambridge, MA); LipofectAMINE was purchased from R&D Systems. Anti-HSP70 monoclonal antibodies were obtained from Sigma or Stress Gen (Victoria, BC, Canada). Di-chlorofluorescein diacetate (DCF-DA) was purchased from Molecular Probes (Eugene, OR), and protein assay reagent was purchased from Pierce. Labeled human TNF-α was purchased from Amersham; NF-κB-specific oligonucleotides and T4 polynucleotide kinase were obtained from Stratagene (La Jolla, CA) and Boehringer Mannheim, respectively. S-Nitroso-N-acetylpenicillamine (SNAP) was synthesized every 2 months as described previously (32), stored frozen as a solid aliquot in the dark, and checked for stoichiometric S-nitrosohydantoin content by the method of Saville (33). N°-Monomethyl-l-arginine (NMA) was purchased from Cyclopes (Salt Lake City, UT). Luciferase assay kits and lysis buffer were purchased from Promega (Madison, WI). HSP70 antisense oligomer (TGGTTCCTTGGCCCAT), HSP70 sense oligomer (ATGGCAGGAAACAA), HSC70 antisense oligomer (AGGTCCTTAGGACAT), and HSC70 sense oligomer (ATGTCCTAGGACCAT) were synthesized from sequences complementary to the initiation codon and four downstream codons of rat HSP70 mRNA (34) and rat HSC70 mRNA (35). All other chemicals and proteins were purchased from Sigma, unless indicated otherwise.

**Hepatocyte Isolation and Culture—**Purified hepatocytes were isolated from male Sprague-Dawley rats (200–300 g, Harlan Sprague-Dawley) by collagenase perfusion using the method of Seglen (36). Hepatocytes were purified to >98% purity by repeated centrifugation at 50 g, followed by further purification over 30% Percoll. Viability at time of plating was consistently 90–95% by trypan blue exclusion. Hepatocytes were cultured in Williams medium E supplemented with 1 μM insulin, 2 μM l-glutamine, 15 μM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% low endotoxin calf serum in 100-mm Petri dishes (5 ml/dish) at a concentration of 1 × 10⁶ cells/ml for 16 h, which represents near-constant culture conditions. Some cells were pre-treated with SNAP (100 μM) for 4 h at 37°C followed by further purification over 30% Percoll. Viability at time of plating was consistently 90–95% by trypan blue exclusion. Hepatocytes were cultured in Williams medium E supplemented with 1 μM insulin, 2 μM l-glutamine, 15 μM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% low endotoxin calf serum in 100-mm Petri dishes (5 ml/dish) at a concentration of 1 × 10⁶ cells/ml for 16 h, which represents near-constant culture conditions. Some cells were pre-treated with SNAP (100 μM) for 4 h at 37°C followed by further purification over 30% Percoll. Viability at time of plating was consistently 90–95% by trypan blue exclusion. Hepatocytes were cultured in Williams medium E supplemented with 1 μM insulin, 2 μM l-glutamine, 15 μM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% low endotoxin calf serum in 100-mm Petri dishes (5 ml/dish) at a concentration of 1 × 10⁶ cells/ml for 16 h, which represents near-constant culture conditions. Some cells were pre-treated with SNAP (100 μM) for 4 h at 37°C followed by further purification over 30% Percoll. Viability at time of plating was consistently 90–95% by trypan blue exclusion.

**Enzyme Activity Assays—**Hepatocytes were collected from Petri dishes, resuspended in PBS containing protease inhibitors, and sonicated (Sonic & Materials, Danbury, CT) with three 15-s bursts while on ice. The solution was centrifuged at maximum speed for 15 min at 4°C in an Eppendorf microfuge. The supernatant (cytosolic fraction) was resuspended in 750 μl lysis buffer (20 mM Tris-HCl, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 1 μg/ml cymostatin) by three freeze-thaw cycles, and the cytosolic fraction was sonicated with 8% SDS-polyacrylamide gel. Cytosolic DNA was precipitated by adding an equal volume of isopropanol. After storing at −20°C overnight, a DNA pellet was obtained by centrifugation at 13,000 × g for 15 min at 4°C and washed twice with 75% ethanol. The pellet was dried and resuspended in 100 μl of 20 mM Tris-HCl, pH 8.0. After digesting RNA with RNase (0.1 mg/ml) at 37°C for 1 h, samples (15 μl) were electrophoresed through a 1.2% agarose gel in 450 mM Tris borate + EDTA (TBE), pH 8.0 buffer. DNA was photographed under visualization with UV light.

**DNA Isolation and Northern Blot Analysis—**Total RNA was isolated from the cultured hepatocytes as described previously (17). The RNA (20 μg) was electrophoresed on a 1% agarose gel containing 1% formaldehyde, transferred to GeneScreen, hybridized with human HSP70 [32P]cDNA and mouse macrophage NOS2 [32P] cDNA, and exposed to autoradiography film. Relative mRNA levels were quantitated by PhosphorImager.

**Western Blot Analysis—**Western blot analysis was performed using a method modified in our laboratories as described previously (37). Briefly, harvested hepatocytes (5 × 10⁶ cells) were lysed in 100 μl of 20 mM Tris-HCl buffer, pH 7.4, containing protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 1 μg/ml cymostatin) by three freeze-thaw cycles, and the cytosolic fraction was sonicated with 2% SDS-polyacrylamide gel. Membranes were developed with chemiluminescence reagents (DuPont NEN) and exposed to Kodak X-Omat film for 2–10 min.

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**Measurement of Glutathione, Oxidized Glutathione, and S-Nitroso-glutathione—**Hepatocytes (2 × 10⁶ cells) were resuspended in 400 μl of 20 mM Tris-HCl buffer, pH 7.4, containing 50 μg/ml lysophosphatidylcholine and 200 units/ml IL-1β. Cytosolic protein was then extracted and quantitated as described previously (20).

**Measurement of ROI and Lipid Peroxidation—**Intracellular ROI were measured following formation of a fluorescent derivative of DCF-DA (39). Briefly, hepatocytes were incubated with 100 μM DCF-DA (in ethanol) with or without TNF-α+ActD or the equivalent amount of ethanol for 80 min in a 5% CO₂ incubator at 37°C. After incubation, the cells were washed with PBS, harvested, and immediately used to determine the level of fluorescence in a spectrophotofluorimeter (excitation, 488 nm; emission, 520 nm). Cell numbers were determined in parallel, and fluorescent values were normalized to the number of cells (as described in Section 2). Lipid peroxidation was measured by measuring thiobarbituric acid reactive substances (TBARS) at 535 nm (40). Butyraldehyde hydroyzaline (0.04%) was added to the thiobarbituric acid solution to prevent lipid autoxidation during the assay procedure.

**Measurement of Glutathione, Oxidized Glutathione, and S-Nitroso-glutathione—**Hepatocytes (2 × 10⁶ cells) were resuspended in 400 μl of 20 mM Tris-HCl buffer, pH 7.4, containing 50 μg/ml lysophosphatidylcholine and 200 units/ml IL-1β. Cytosolic protein was then extracted and quantitated as described previously (20).
choline and protease inhibitors. The cytosolic fraction was obtained by microcentrifugation at maximum speed at 4°C for 10 min after three cycles of freeze-thaw. Cytosol (150 μl) was mixed with 7.5 μl of 100% trichloroacetic acid and incubated in ice for 5 min. Immediately after the supernatant was obtained by centrifugation, cellular glutathione (GS) and GSH were quantified by the GSH reductase recycling method (41). S-Nitrosothiol glutathione (GSNO) was measured by the method of Clancy et al. (42). Briefly, cytosol was incubated with 0.1 mM NaBH₄ at 37°C for 5 min to break the S-nitroso bond. The solution was acidified to remove excess NaBH₄ and acid-soluble fraction was obtained by centrifugation. Total glutathione was assayed as described above. The concentration of GSNO was calculated as the difference in GSH levels between untreated and NaBH₄-treated cytosolic GSH.

**Electromobility Shift Assay—** Nuclear extracts were prepared by the method of Staal et al. (43) from hepatocytes stimulated with 500 units/ml TNFα for 1 h. The cells were washed with ice-cold PBS, scraped into PBS, and centrifuged at 3,000 rpm in a microcentrifuge at 4°C for 5 min. After discarding the supernatant, the pellet was resuspended in 5 volumes of Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40). The cells were disrupted with 10 hand strokes in a Dounce homogenizer, and nuclei were recovered by centrifugation at 5,000 rpm for 15 min and resuspended in the same volume of Buffer B (Buffer A without Nonidet P-40). After another 15 min centrifugation at 5,000 rpm, nuclear proteins were extracted at 4°C by gently mixing the nuclei in 150 μl of Buffer C (20 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM EDTA, and 0.5 mM dithiothreitol) and adding 50 μl of Buffer D (Buffer C with 400 mM KCl) in a dropwise fashion. Supernatants were collected after 1 h by centrifugation at 13,000 rpm for 30 min. For the electromobility shift assay, NF-κB-specific oligonucleotide was end-labeled with [γ-³²P]ATP using T4 polynucleotide kinase and purified on a 5% nondenaturing polyacrylamide gel in TBE running buffer. Following electrophoresis, gels were dried and subjected to autoradiography.

**NF-κB-Luciferase Reporter Construction and Bioassay—** This reporter (NF-κB-pT109) was constructed by inserting three copies of the NF-κB response element (‒GGGGACATTTCGCGGATTCGGCATTCGCTC-3'), Life Technologies, Inc.) into pTK-Luc (44), a plasmid carrying the insertion of the human thymidine kinase promoter (109 to +52) ligated upstream of luciferase. DNA transfections into hepatocytes were carried out in six-well plates using LipofectAMINE, as described (44). The transfected cells were allowed to recover overnight, exposed to 750 μM SNAP for 16 h, and stimulated with TNFα (500 units/ml) for 6 h. After washing twice with PBS, cells were lysed with Reporter lysis buffer. Luciferase activity was assayed using 20 μl of lysate and 100 μl of Luciferase Assay Mixture (Promega, Madison, WI) using a luminometer (LKB 1252 Lumat) at 1000 relative light units (109 to +52) sections were cut using a Leica Ultracut S microtome, mounted on copper grids, counterstained with 2% uranyl acetate (7 min) and 1% lead citrate (2 min), dried, and observed using a JEOl 100CX microscope.

**Statistical Analysis—** Data are presented as mean ± S.D. of at least three separate experiments. Comparisons between two values were performed using paired Student’s t test. Differences were considered significant when the p-value was equal to or less than 0.05.

**RESULTS**

**SNAP Pretreatment Protects Hepatocytes from TNFα Toxicity—** Our laboratory has shown previously that pretreatment of hepatocytes with an NO-donor protects hepatocytes from subsequent H₂O₂ toxicity (20). To determine if NO pretreatment also protects against TNFα-induced toxicity, freshly isolated rat hepatocytes were exposed to 750 μM SNAP as an NO donor, and 18 h later the cells were washed, TNFα was added, and viability was determined by crystal violet staining (Fig. 1). Pretreatment with 750 μM SNAP alone did not reduce hepatocyte viability, while TNFα (28 ng/ml) induced a 12% and 5% cytotoxicity in untreated and SNAP-pretreated hepatocytes, respectively. It has been shown previously that the transcriptional inhibitor ActD markedly increases TNFα toxicity (24). As shown in Fig. 1, viability measured at 14 h after TNFα addition was decreased by 38% when ActD was combined with TNFα. Pretreatment with SNAP for 16 h protected cultured hepatocytes from TNFα + ActD toxicity. If cells were exposed to TNFα + ActD at intervals of 1–6 h after SNAP exposure, no protection was seen (data not shown).

Since TNFα-mediated cytotoxicity has been shown to be associated with increased production of superoxide (46) and blocked by superoxide dismutase (47), we measured intracellular levels of antioxidant enzymes to determine if SNAP pretreatment increased levels of specific intracellular antioxidant(s). As shown in Table I, the cellular levels of the major antioxidants (including catalase, superoxide dismutase, and GSH peroxidase) were not elevated in SNAP-treated hepatocytes, indicating that the SNAP-induced cytoprotective effect was not associated with increases in these cellular antioxidant arms.

**SNAP Pretreatment Protects from TNFα + ActD-induced Apoptosis—** Since TNFα + ActD toxicity in hepatocytes is associated with the induction of apoptosis, we examined the effect of SNAP pretreatment on TNFα + ActD-induced DNA fragmentation. Fig. 2A shows that TNFα + ActD, but not TNFα or ActD alone, induced DNA fragmentation characteristic of apoptosis.
in cultured hepatocytes. Pretreatment with 750 μM SNAP at 4 or 8 h prior to TNFα+ActD addition had no effect; however, if TNFα+ActD were added 12 h after SNAP treatment, the degree of DNA fragmentation was markedly reduced, and by 16 h apoptosis was almost completely inhibited. When concentrations of SNAP ranging from 100 to 750 μM were added to hepatocytes 18 h prior to TNFα+ActD, only SNAP concentrations of 250 μM or greater were found to protect hepatocytes from apoptosis (Fig. 2B).

SNAP Induces HSP70 Expression—The major heat shock protein, HSP70, has been shown to protect various types of cells from TNFα-induced injury (48–50). To determine if SNAP treatment induced hepatocyte HSP70 expression, we exposed hepatocytes to increasing concentrations of SNAP for different time periods and examined HSP70 expression by Northern and Western blot. Untreated hepatocytes expressed a 2.8-kilobase mRNA consistent with the constitutive heat shock protein 70, or HSC70 (Fig. 3A). SNAP treatment resulted in a concentration-dependent increase in the expression of HSC70 as well as the appearance of a 3.1-kilobase mRNA band consistent with inducible HSP70 (51). HSP70 mRNA expression was detected at concentrations of SNAP as low as 100 μM and was maximal at 750 μM. Western blot analysis, using a monoclonal antibody (Stress Gen) that detects both HSC70 and HSP70, demonstrated a dose-dependent increase in expression of HSC70 and appearance of HSP70 protein in hepatocytes treated with SNAP 16 h previously (Fig. 3B). Fig. 3C shows a time course for HSP70 mRNA levels following exposure to 750 μM SNAP and demonstrates that mRNA levels progressively increased until 12 h and then declined. Similarly, the level of HSP70 protein measured using an HSP70-specific monoclonal antibody (Stress Gen) following SNAP pretreatment increased progressively up to 16 h and then decreased (Fig. 3D).

SNAP Pretreatment Induces GSH Oxidation and GSNO Formation Responsible for HSP70 Induction—Because NO reacts with intracellular thiols such as GSH (10), we examined intracellular GSH and its derivatives. As shown in Fig. 4A, intracellular GSH levels in hepatocytes exposed to 750 μM SNAP decreased by about 50% at 4–6 h following exposure to 750 μM SNAP and then slowly recovered to control level (15 h). Intracellular GSNO levels increased to a maximum of 28% of total GSH at 4 h, while GSSG levels became maximal at 34% of total GSH at 6 h. Since it is known that conjugation, depletion, or oxidation of GSH increases the levels of cytoprotective heat shock proteins, including HSP70 (52, 53), in some cell types we studied the level of HSP70 mRNA in hepatocytes with three thiol-modulating agents, each with a different mode of action. HSP70 mRNA was induced by exposure to diamide (a GSH-oxidizing agent) and N-ethylmaleimide (a GSH-conjugating agent), but not by buthionine sulfoximine, which blocks GSH synthesis (Fig. 4B).

SNAP Pretreatment Induces Resistance to Heat Shock—Since our data showed that SNAP exposure induced HSP70 expression, we next examined whether pretreatment of hepatocytes with SNAP could result in the expected resistance to subsequent heat challenge. Hepatocytes were pretreated with concentrations of SNAP ranging from 0 to 1 mM for 16 h and then exposed to a heat challenge of 43.5 °C for 4 h. Viability was assayed after culturing the cells with fresh medium for another 12 h at 37 °C. Fig. 5 reveals that hepatocytes developed resistance to heat-induced killing following exposure to SNAP. Increases in viability were seen following exposure to SNAP concentrations of 250 μM, and maximum resistance was ob-

### Table I

| Enzymes                   | Activity * | Control | SNAP (16 h) |
|---------------------------|------------|---------|-------------|
| Catalase                  | 824.4 ± 98.7 | 728.0 ± 84.6 |
| Superoxide dismutase      | 77.1 ± 6.7 | 69.8 ± 7.0 |
| GSH peroxidase            | 383.8 ± 61.6 | 421.5 ± 81.2 |

* One unit of enzyme activity: catalase, the decomposition of 1.0 μmol of H$_2$O$_2$/min/mg of protein; superoxide dismutase, the change of 0.025 absorbance/min/mg of protein; GSH peroxidase, the oxidation of nmol NADPH/min/mg of protein.

* Values represent mean ± S.D. for three separate experiments.

### Fig. 2. SNAP pretreatment protects hepatocytes from TNFα-mediated apoptosis in a time-dependent (A) and dose-dependent (B) manner. Hepatocytes were pretreated with 750 μM SNAP (A) or different concentrations (B), for various time periods (A) or 16 h (B), washed twice with fresh medium, and incubated with TNFα+ActD for 9 h. Cells were washed twice with ice-cold PBS, harvested, and lysed with 20 mM Tris buffer, pH 8.0, containing 10 mM EDTA and 0.5% Triton X-100. Cytoplasmic DNA was isolated as described under “Experimental Procedures” and electrophoresed on an agarose gel after normalizing DNA amount by protein concentration. DNA was visualized with UV light and photographed.

### Fig. 3. SNAP pretreatment induces hepatocyte HSP70 expression. A) Northern blot analysis of HSP70 protein in hepatocytes treated with SNAP 16 h and then decreased (Fig. 3B). Similarly, the level of HSP70 protein measured using an HSP70-specific monoclonal antibody (Stress Gen) following SNAP pretreatment increased progressively up to 16 h and then decreased (Fig. 3D).
The capacity for SNAP pretreatment to protect against TNFα-ActD-mediated apoptosis correlated well with the stimulation of inducible HSP70 expression, suggesting that HSP70 prevented the apoptosis. Therefore, we hypothesized that induction of HSP70 by heat should also prevent apoptosis by TNFα-ActD. Hepatocytes were exposed to heat (43.5°C) for 0–1.5 h and HSP70 protein expression determined 16 h later by Western blot analysis (Fig. 6A). A time-dependent increase in inducible HSP70 protein expression was noted, and the degree of HSP70 expression correlated well with protection from TNFα-ActD-induced DNA fragmentation (Fig. 6B). No significant injury (>90% viability as judged by crystal violet staining) was observed served at 750 μM SNAP pretreatment.

**Heat Exposure Mimics SNAP Treatment**—The capacity for SNAP pretreatment to protect against TNFα-ActD-mediated apoptosis correlated well with the stimulation of inducible HSP70 expression, suggesting that HSP70 prevented the apoptosis. Therefore, we hypothesized that induction of HSP70 by heat should also prevent apoptosis by TNFα-ActD. Hepatocytes were exposed to heat (43.5°C) for 0–1.5 h and HSP70 protein expression determined 16 h later by Western blot analysis (Fig. 6A). A time-dependent increase in inducible HSP70 protein expression was noted, and the degree of HSP70 expression correlated well with protection from TNFα-ActD-induced DNA fragmentation (Fig. 6B). No significant injury (>90% viability as judged by crystal violet staining) was observed served at 750 μM SNAP pretreatment.

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Antisense Oligomer to HSP70 Blocks HSP70 Expression and Protection against TNFα1 ActD-induced Apoptosis—

To determine if HSP70 was directly responsible for the inhibition of apoptosis induced by SNAP exposure, hepatocytes were incubated with HSP70 antisense oligonucleotide (10 μM) during a 750 μM SNAP pretreatment, followed by TNFα1 ActD exposure. Antisense oligomers blocked induction of HSP70 expression (Fig. 7A) and inhibited SNAP-induced protection from TNFα1 ActD-mediated apoptosis (Fig. 7B). Sense oligomers had no effect on induction of HSP70 protein expression and did not inhibit the SNAP-induced cytoprotection. In contrast, HSC70 antisense oligomers (10 μM), which blocked HSC70 expression, had no effect on SNAP-stimulated HSP70 protein expression or protection from TNFα cytotoxicity following SNAP exposure (data not shown).

Microscopic Examination of Apoptosis—We further confirmed the protective effect of SNAP pretreatment on TNFα1 ActD-induced apoptosis by light and electron microscopy. Chromatin condensation and peripheral and nuclear blebbing, typical characteristics of apoptotic morphology, were apparent on electron microscopic examination of TNFα1 ActD-treated hepatocytes (Fig. 8, A and B), but not control cells (Fig. 8C). Similarly, the percentage of apoptotic hepatocytes following TNFα1 ActD treatment was significantly increased from 7% to 53% (Fig. 8D). Pretreatment with SNAP or heat shock reduced TNFα1 ActD-induced apoptosis to 17% and 15%, respectively. Antisense oligomers to HSP70 inhibited the apoptotic effect of SNAP pretreatment.

TNFα Binding and NF-kB Activation Are Not Reduced by SNAP Pretreatment—Since TNFα cytotoxicity can be reduced by inhibiting TNFα binding to its receptors (54) and blocking its signal transduction (55), we examined whether NO modified TNFα surface binding or TNFα signal transduction. SNAP pretreatment did not change the specific binding of TNFα to cell surface receptors (Fig. 9A). Similarly, TNFα-mediated NF-kB activation as determined by electromobility shift assay was unchanged by SNAP pretreatment (Fig. 9B). The lack of an effect of SNAP treatment on TNFα-induced NF-kB activation was further confirmed in cultured hepatocytes transfected with an NF-kB-reporter (luciferase) construct. Induction of luciferase activity in response to TNFα was not significantly different between SNAP-treated and control hepatocytes (Fig. 9C), indicating no change in the NF-kB activation in this assay which tests NF-kB function.

SNAP Pretreatment or Heat Inhibits ROI Formation and Lipid Peroxidation—TNFα cytotoxicity is associated with overproduction of activated oxygens (O2· and H2O2) from the mitochondrial respiratory chain (46). Therefore, we next examined whether SNAP pretreatment inhibited TNFα-induced ROI formation as measured by the oxidation of the cell-permeable fluoregenic marker DCF-DA. TNFα exposure enhanced ROI-induced oxidation of DCF-DA in untreated hepatocytes, whereas pretreatment with SNAP or heat shock significantly reduced the TNFα-induced oxidation of DCF-DA (Fig. 10A). Antisense oligomers of HSP70 prevented the effect of SNAP on
whether endogenous NO could protect hepatocytes from TNFα cytotoxicity, we examined TNFα cytotoxicity in hepatocytes induced to produce NO by cytokine exposure. When incubated with IFNγ and IL-1β, rat hepatocytes expressed NOS2 protein as confirmed by Western blot analysis (Fig. 11A) and produced NO as judged by the accumulation of stable end products of NO, nitrite and nitrate (Fig. 11B). NO synthesis was nearly completely inhibited by the NOS inhibitor NMA. Although total GSH levels (GSH+GSSG+GSNO) were unchanged in IFNγ+IL-1β-treated cells, intracellular GSNO and GSSG levels were elevated and GSH significantly reduced. This change was also reversed by NMA (Fig. 11C). Under these conditions, HSP70 protein levels were increased by IFNγ+IL-1β exposure and this increase was attenuated by NMA (Fig. 12A). When hepatocytes were subsequently exposed to TNFα+ActD, IFNγ- and IL-1β-stimulated hepatocytes were protected from both TNFα-induced DNA fragmentation (Fig. 12B) and cytotoxicity (Fig. 12C). The protective effects were inhibited by NMA.

Fig. 9. Effects of SNAP on specific TNFα binding (A), NF-κB activation (B), and NF-κB promoter activity (C). A, hepatocytes were incubated with recombinant human 125I-TNFα in the presence or absence of excess unlabeled TNFα at 4 °C for 3 h after 18 h of treatment with (●) or without (○) 750 μM SNAP. Specific binding of TNFα was calculated as the difference between the binding in the presence and absence of excess unlabeled TNFα. B, hepatocytes were pretreated with different concentrations of SNAP for 16 h and stimulated with TNFα (500 units/ml) for 1 h. Nuclear extract preparation and electromobility shift assay were performed as described under “Experimental Procedures.” In competition experiments, excess unlabeled NF-κB oligomer (cold probe, CP) was added. C, for NF-κB promoter activity, hepatocytes were transfected with NF-κB luciferase reporter plasmid, treated with or without 750 μM SNAP, and then stimulated with TNFα (500 units/ml) for 6 h. Cell lysates were isolated and assayed for luciferase activity assay.

DISCUSSION

In the present study, we have shown that pretreatment with the NO-generating compound SNAP protects cultured rat hepatocytes from TNFα+ActD-induced cytotoxicity and apoptosis. Both time-course and dose-response studies revealed that induction of HSP70 mRNA and protein occurred in parallel to protection from TNFα+ActD-induced apoptosis. Antisense oligomers to HSP70, but not HSF70 sense nor HSC70 antisense oligomers, inhibited NO-induced HSP70 expression and rendered hepatocytes again susceptible to TNFα+ActD-induced apoptosis. Expression of HSP70 induced by hyperthermia also protected hepatocytes from TNFα+ActD cytotoxicity. SNAP pretreatment did not increase antioxidant enzyme activities, strongly suggesting that the protective effect of SNAP pretreatment was not due to enhanced cellular antioxidant capacity through these enzyme systems. Furthermore, the decomposition products of SNAP (1 mM), N-acetylpenicillamine (1 mM, the parent compound of SNAP), and 8-bromo-cGMP (500 μM) had no effect on HSP induction or the cytoprotection (data not shown), suggesting that NO liberated from SNAP was the mediator through a cGMP-independent mechanism. TNFα receptor binding and TNFα-mediated signal transduction in hepatocytes were unchanged, as analyzed by the specific binding of iodinated TNFα and NF-κB activation. Finally, endogenous NO formation following IFNγ+IL-1β exposure caused increased HSP70 expression and protection from TNFα+ActD-induced cytotoxicity. Thus, we conclude that one mechanism by which NO protects hepatocytes from TNFα-induced cytotoxicity is through the induction of HSP70.
The cytotoxic action of TNFα has been associated with the activation of phospholipase A₂, the cytosolic release of ceramide, and the formation of ROI. Overproduction of ROI has been identified as a key component of apoptotic pathways involving activation of endogenous endonucleases (57) and direct DNA fragmentation (58). The potential importance of ROI in TNFα-induced cytotoxicity and apoptosis has been demonstrated in studies where overexpression of Mn-superoxide dismutase, catalase, and GSH peroxidase, were not changed in hepatocytes following SNAP pretreatment. Furthermore, even though intracellular GSH levels were transiently suppressed by SNAP exposure, the levels had returned to base line by the time of TNFα exposure. Instead, SNAP pretreatment induced the expression of HSP70, which has been shown to have anti-apoptotic or cytoprotective effects against TNFα toxicity. Thus, it is likely that the preinduction of HSP70 by SNAP protected the cells from TNFα+ActD-mediated apoptosis.

NO can act as an antioxidant by scavenging O₂⁻ directly. Therefore, another possible explanation of our findings is that NO neutralized O₂⁻. This seems unlikely because the NO donor was added 16 h before the TNFα+ActD. Furthermore, no protective effect from DNA fragmentation was seen when hepatocytes were treated simultaneously with 750 μM SNAP and TNFα (data not shown). In fact, the protection from TNFα+ActD-induced apoptosis was not detected until 12 h following SNAP exposure. Because SNAP has a half-life of only about 4.5 h under our experimental conditions (data not shown), no SNAP-derived NO should remain at the time of TNFα treatment. We also could not detect the presence of possible delayed NO-generating sources in hepatocytes, such as GSNO (Fig. 4) or iron-nitrosyl complexes (data not shown) 16 h following SNAP pretreatment. In contrast to our findings, others have presented evidence that simultaneous NO production protects from apoptosis (30, 31). Thus, it is likely that the mechanism of NO-mediated protection varies depending on the cell type and the quantity, as well as the timing and duration, of NO exposure.

NO can have adverse effects on host survival, ranging from direct cellular cytotoxicity (28, 29) to the damage of cellular components leading to the mutagenesis (60). Therefore, it is not surprising that a high level of NO exposure induces protective stress responses. Although our observation that NO stimulates HSP70 expression in hepatocytes has not been shown before, Kim et al. (19) have reported previously that NO induces heme oxygenase, or HSP32, in rat hepatocytes. The increased expression of HSP32 protects the cells from subsequent toxic concentrations of NO (20). However, the inhibitor of HSP32, Sn-protoporphyrin, did not reverse the induction of HSP70 or the SNAP-induced protection from TNFα toxicity (data not shown). Thus, it is likely that HSP70 and HSP32 are both induced by...
NO exposure, but while HSP32 protects from oxidative injury, HSP70 protects against TNFα-induced apoptosis. Although the protection in both instances may involve antioxidant properties, it is likely that HSP32 and HSP70 protect through distinct mechanisms.

HSP70 is induced by several environmental stimuli such as free radicals, heat, heavy metals (61), serum-free culture media (62), and agents that modulate intracellular ratio of GSSG to GSH in hepatocytes (52, 53, 63). Van Remmen et al. (62) recently reported that the type of serum-free culture medium profoundly influenced the spontaneous induction of HSP70 and HSC70 in cultured rat hepatocytes. Incubation in L15 medium resulted in marked spontaneous induction associated with a decrease in the GSH/GSSG ratio, whereas incubation in Williams medium E had little effect on HSP expression. Here we used Williams medium E and serum and, in agreement with this previous study, found no spontaneous induction of HSP70.

The mechanism by which NO stimulates the expression of HSP70 may involve the interaction of NO with thiol-containing molecules. Ample evidence exists to support the view that NO readily oxidizes low molecular weight thiols, forming S-nitrosothiols and disulfide. Of cellular low molecular weight thiols, glutathione is the most abundant as well as being one of the intracellular targets of NO. Here, pretreatment of hepatocytes with NO was shown to alter the redox state accompanied by oxidation of GSH and formation of GSNO (Fig. 4A). A GSH oxidizing agent (diamide) and a GSH alkyllating agent (N-ethylmaleimide) both induced HSP70 mRNA, but a GSH synthesis inhibitor (buthionine sulfoximine) did not (Fig. 4B). In addition, we show here that hepatocytes stimulated to produce NO by cytokine exposure expressed HSP70 associated with an intracellular GSH redox change. This cellular effect was attenuated by the NOS inhibitor NMA, strongly indicating that, like SNAP, cytokine-induced endogenous NO is also capable of inducing HSP70 expression in vitro. It is worth noting that even in the presence of NMA there was still a significant increase in HSP70 induction, which may indicate cytokine-dependent, but NO-independent, up-regulation (64). Taken together, these results suggest that it is possible that induction of HSP70 could be regulated by GSH-dependent cellular redox changes in response to NO.

Although heat exposure can cause apoptosis, it has been shown that heat shock also induces resistance to a subsequent challenge of other apoptotic agents, including ROI, NO, and glucocorticoid in mouse thyocytes (65, 66), as well as serum withdrawal in neuroblastoma ND7 cells (67). HSPs may protect cells by acting as molecular chaperons, guiding the folding and trafficking of damaged proteins (68). Induction of HSP protects cells not only from damage due to heat but also from damage due to oxidative injury and cytokine-mediated cytotoxicity. We show here that both ROI production and lipid peroxidation are inhibited by SNAP-induced HSP70 expression. Jäättelä et al. (48) demonstrated that pretreatment of mouse fibrosarcoma cells (WEHI) with heat-protected cells from TNF-induced cytolyis, and that the protective effect roughly correlated with the kinetics of HSP induction. Furthermore, only cells overexpressing HSP70 were found to be protected from both ROI- and TNFα-induced cytotoxicity, whereas overexpression of HSP27 protected only from exogenous ROI exposure but not TNFα cytotoxicity (49, 50). Recent data have shown that HSP may protect from TNFα toxicity by inhibiting the action of ROI on mitochondrial membrane potential (69). Inhibition of TNFα toxicity by SNAP pretreatment could occur through the inhibition of ROI production in mitochondria, preventing ROI-mediated alterations in mitochondrial membrane potential. This could prevent cytochrome c release, which is involved in apoptosis through activation of caspase proteases such as CPP32/Yama (70); however, since HSP70 is not a mitochondrial protein, it is unlikely that HSP70 acts directly as a mitochondrial antioxidant. HSP70 may instead block signal transduction to the mitochondria, resulting in the inhibition of mitochondrial ROI production by inhibiting either second lipid messenger(s) to mitochondria (61) or by preventing the interaction between the death domain of TNFα receptor and signal molecule(s) (71). Alternatively, it is also possible that HSP70 may enhance the chaperon-mediated import of precursor proteins into mitochondria which control mitochondrial function (72–74) leading to decreased ROI formation. Further experiments will be required to establish whether any of these mechanisms account for the protection from TNFα-mediated apoptosis.

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