

# Nitric Oxide (NO) Pretreatment Increases Cytokine-induced NO Production in Cultured Rat Hepatocytes by Suppressing GTP Cyclohydrolase I Feedback Inhibitory Protein Level and Promoting Inducible NO Synthase Dimerization\*

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Nitric oxide (NO) regulates the biological activity of many enzymes and other functional proteins as well as gene expression. In this study, we tested whether pretreatment with NO regulates NO production in response to cytokines in cultured rat hepatocytes. Hepatocytes were recovered in fresh medium for 24 h following pretreatment with the NO donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) and stimulated to express the inducible NO synthase (iNOS) with interleukin-1 $\beta$  and interferon- $\gamma$  or transfected with the human *iNOS* gene. NO pretreatment resulted in a significant increase in NO production without changing iNOS expression for both conditions. This effect, which did not occur in macrophages and smooth muscle cells, was inhibited when NO was scavenged using red blood cells. Pretreatment with oxidized SNAP, 8-Br-cGMP, NO<sub>2</sub><sup>-</sup>, or NO<sub>3</sub><sup>-</sup> did not increase the cytokine-induced NO production. SNAP pretreatment increased cytosolic iNOS activity measured only in the absence of exogenous tetrahydrobiopterin (BH<sub>4</sub>). SNAP pretreatment suppressed the level of GTP cyclohydrolase I (GTPCHI) feedback regulatory protein (GFRP) and increased GTPCHI activity without changing GTPCHI protein level. SNAP pretreatment also increased total cellular levels of biopterin and active iNOS dimer. These results suggest that SNAP pretreatment increased NO production from iNOS by elevating cellular BH<sub>4</sub> levels and promoting iNOS subunit dimerization through the suppression of GFRP levels and subsequent activation of GTPCHI.

rotransmission, vasodilation, immune modulation, and regulation of apoptosis (1). It is synthesized from L-arginine by the enzyme nitric-oxide synthase (NOS), which requires the cofactor tetrahydrobiopterin (BH<sub>4</sub>) for maximal activity. NOS catalyzes the oxidation of the terminal guanidinitrogen atom of L-arginine to form L-citrulline and NO. Three isoforms of NOS have been identified and categorized into constitutive and inducible isotypes. Two distinct constitutive NOS (cNOS) isoforms have been cloned from neuronal (2) and endothelial cells (3), which produce nanomolar amounts of NO. A third isoform, inducible NOS (iNOS) is expressed in response to inflammatory cytokines in many cell types including hepatocytes (4), and produces micromolar levels of NO. All of these isotypes are active as homodimers and require NADPH, FAD, FMN, calmodulin, and BH<sub>4</sub> (5). The first four cofactors are usually present in the cells at concentrations that are not limiting for the enzyme activity. The precise role of BH<sub>4</sub> in the iNOS-catalyzed reaction is still equivocal, but it may function as an allosteric cofactor that promotes assembly of iNOS into its active dimeric form (6, 7).

BH<sub>4</sub> is produced *de novo* from GTP by the three consecutive enzymatic reactions: GTP cyclohydrolase I (GTPCHI), 6-pyruvoyl tetrahydrobiopterin synthase, and sepiapterin reductase (reviewed in Ref. 8). BH<sub>4</sub> can also be produced either by a salvage pathway producing dihydrobiopterin via dihydrofolate reductase, or by a recycling pathway that generates 4 $\alpha$ -hydroxytetrahydrobiopterin via 4 $\alpha$ -carbinolamine dehydratase and dihydropteridine reductase (8, 9). However, cellular levels of BH<sub>4</sub> are largely regulated by the activity of GTPCHI, the first and rate-limiting enzyme in *de novo* BH<sub>4</sub> synthesis (8, 10). *GTPCHI* mRNA expression and subsequent BH<sub>4</sub> synthesis can be induced in fibroblasts (11), macrophages (12), smooth muscle cells (13), and hepatocytes (14) by the same proinflammatory stimuli that induce *iNOS* mRNA expression. The catalytic activity of GTPCHI is negatively regulated by the GTPCHI feedback regulatory protein (GFRP) in the presence of BH<sub>4</sub> and GTP (15). In fibroblasts and smooth muscle cells exposed to immune stimuli, NO production is suppressed by inhibition of *de novo* BH<sub>4</sub> synthesis and increased by adding sepiapterin or BH<sub>4</sub> (11, 16). Furthermore, cytokine treatment of endothelial cells enhances NO production by increasing *GTPCHI* expres-

Nitric oxide (NO)<sup>1</sup> is a multifunctional mammalian effector that is involved in numerous biologic processes, such as neu-

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<sup>1</sup> The abbreviations used are: NO, nitric oxide; iNOS, inducible NO synthase; eNOS, endothelial NO synthase; BH<sub>4</sub>, tetrahydrobiopterin; SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine; GTPCHI, GTP cyclohydrolase I; GFRP, GTP cyclohydrolase I feedback regulatory protein; IL-1 $\beta$ , interleukin-1 $\beta$ ; IFN $\gamma$ , interferon- $\gamma$ ; LPS, lipopolysaccharide; RASMC, rat

aortic smooth muscle cells; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; AdiNOS, adenoviral nitric-oxide synthase; 8-Br-cGMP, 8-bromo-cGMP; BisTris, 2-[bis(2-hydroxyethyl)-amino]-2-(hydroxymethyl)propane-1,3-diol.

sion and BH<sub>4</sub> levels without changing endothelial NOS (eNOS) expression (17). These studies indicate that the intracellular level of BH<sub>4</sub> is a rate-limiting factor for NOS activity.

It has been shown that cotreatment with NO donors and cytokines in hepatocytes inhibits NO production by the suppression of NF- $\kappa$ B-dependent iNOS expression (18). However, the effect of NO pretreatment on cytokine stimulation of NO production has not been studied. We investigated the effect of pretreatment with the NO-donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) on the factors influencing NO production following stimulation of hepatocytes with cytokines, IL-1 $\beta$  and IFN $\gamma$ . We found that SNAP pretreatment increased iNOS subunit dimerization and iNOS activity, without changes in expression of iNOS, by augmenting intracellular BH<sub>4</sub> levels through the suppression of GFRP levels and subsequent activation of GTPCHI.

#### EXPERIMENTAL PROCEDURES

**Materials**—William's medium E, penicillin, streptomycin, L-glutamate, and Hepes were purchased from Invitrogen (Gaithersburg, MD). Insulin was obtained from Lilly Inc. (Indianapolis, IN) and calf serum was purchased from Hyclone Laboratories (Logan, UT). Monoclonal iNOS antibody was obtained from Transduction Laboratories (Lexington, KY). SNAP and oxidized SNAP were prepared as described previously (19, 20). Red blood cells were prepared by collecting blood from rats as previously described (21). Antibodies for GTPCHI and GFRP were generated by Dr. Kazuyuki Hatakeyama. All other chemicals and proteins were purchased from Sigma, unless indicated otherwise.

**Cell Culture**—Rat hepatocytes were isolated from male Sprague-Dawley rats (200–250 g, Harlan Sprague-Dawley) by a collagen perfusion method (20). Highly purified hepatocytes (>98%) were obtained by repeated centrifugation at 400  $\times$  *g* followed by further purification over 30% Percoll. Hepatocytes with >95% viability by trypan blue exclusion were suspended in William's medium E supplemented with 1  $\mu$ M insulin, 2 mM glutamine, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and 10% low endotoxin calf serum. Cells were plated in 100-mm Petri dishes (5 ml/dish) and 12-well plates (1 ml/well) at a concentration of 1  $\times$  10<sup>6</sup> and 2  $\times$  10<sup>5</sup> cells/ml, respectively, and precultured in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>, 95% air) at 37 °C for 16 h. Cells were pretreated with SNAP for 8 h, washed three times with fresh medium, and cultured with complete William's medium E containing 5% calf serum for 24 h. Cells were then stimulated with cytokines consisting of 100 units/ml IL-1 $\beta$  (Cistron) and 100 units/ml IFN $\gamma$  (Amgen). Hepatocytes were also transfected with AdiNOS or AdLacZ (10 multiplicity of infections) in serum-free medium for 3 h, washed with fresh medium, and incubated in fresh medium containing 5% serum. Northern and Western blot analyses were performed after stimulation for 10 and 14 h, respectively. NO production was measured after 24 h stimulation. The macrophage cell line RAW264.7 and rat aortic smooth muscle cells (RASMC), isolated from male Sprague-Dawley rats as described previously (22), were plated in 12-well plates at a concentration of 1  $\times$  10<sup>6</sup> cells/ml and treated the same way as described above for hepatocytes, except that RAW264.7 cells were stimulated with 100 ng/ml LPS and 100 units/ml IFN $\gamma$ .

**Western Blot Analysis**—Cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS), and resuspended in 20 mM Tris-HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM phenylmethylsulfonyl fluoride, 5  $\mu$ g/ml aprotinin, 5  $\mu$ g/ml pepstatin A, and 1  $\mu$ g/ml chymostatin). Cells were lysed by three cycles of freeze and thaw, and cytosolic fractions were prepared by centrifugation at 12,000  $\times$  *g* for 10 min at 4 °C. Protein concentration was determined by the BCA method (Pierce). Proteins (40  $\mu$ g) were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific binding was blocked with PBS-Tween (0.01%) (PBS-T) containing 5% nonfat milk for 1 h at room temperature. The membranes were hybridized with antibodies for iNOS, GTPCHI, and GFRP in PBS-T containing 1% nonfat milk for 1 h. Membranes were washed three times with PBS-T and hybridized with horseradish peroxidase-conjugated anti-mouse IgG for 1 h. The membranes were then washed three times with PBS-T, incubated for 2 min with enhanced chemiluminescent solution (Pierce), and exposed to x-ray film (20).

**Northern Blot Analysis**—Total RNA was extracted from hepatocytes by the RNazol method (20). The RNA (20  $\mu$ g) was electrophoresed on 1% agarose gel containing 1% formaldehyde, transferred onto a nylon membrane, and prehybridized with whale sperm DNA at 43 °C over-

night. The probes for iNOS, GTPCHI, and 18 S ribosomal RNA were prepared as described previously (23). The membrane was hybridized with <sup>32</sup>P-labeled probes (~2  $\times$  10<sup>6</sup> cpm/ml) of murine macrophage iNOS or rat GTPCHI, washed three times, and exposed to autoradiography film. To control for mRNA loading, the membranes were rehybridized with <sup>32</sup>P-labeled 18 S ribosomal RNA probe (2  $\times$  10<sup>6</sup> cpm/ml) using the protocol described above.

**Measurement of Nitrite and Nitrate**—Nitrite plus nitrate in the culture medium was determined by an automated HPLC method with the use of a cadmium column after deproteinization (24). Nitrite alone was assayed by the addition of 100  $\mu$ l of Griess reagent to 100  $\mu$ l of culture supernatant in a 96-well plate. Absorbance was measured at 550 nm using a 96-well plate reader, and nitrite concentration was calculated from a standard curve of sodium nitrite.

**Measurement of Cellular Total Biopterin Level**—Total biopterin (biopterin + BH<sub>2</sub> + BH<sub>4</sub>) was measured in hepatocyte lysates after acidic oxidation of reduced forms of biopterin with iodine reagents as previously described (25). In brief, hepatocytes were harvested, washed with ice-cold PBS, and lysed by three cycles of freeze and thaw. Following centrifugation at 12,000  $\times$  *g* for 5 min at 4 °C, the supernatants were treated with the iodine solution (1% I<sub>2</sub> containing 2% KI in 1 N HCl) for 1 h at 37 °C in the dark. The samples were then centrifuged at 12,000  $\times$  *g* for 5 min at 4 °C and the supernatants were treated with 0.1 M ascorbate to remove excess I<sub>2</sub>. Extracts were neutralized with 1 N NaOH followed by 200 mM Tris-HCl (pH 7.8). Biopterin was quantitated by C<sub>18</sub> reverse high performance liquid chromatography using an online fluorescence detector.

**Determination of Monomeric Versus Dimeric iNOS**—Cytosols (100  $\mu$ l, 1 mg protein) were injected onto a Superdex 200 gel filtration column (Amersham Biosciences) at 4 °C and eluted with 40 mM BisTris (pH 7.6), 10% glycerol, 1 mM L-arginine, 4 mM dithiothreitol. Fractions (400  $\mu$ l) were collected and 30- $\mu$ l aliquots were assayed for iNOS activity. The fractions were dialyzed and concentrated to ~50  $\mu$ l in a vacuum centrifuge. iNOS protein was detected by Western blot and the protein band intensity was measured by densitometry.

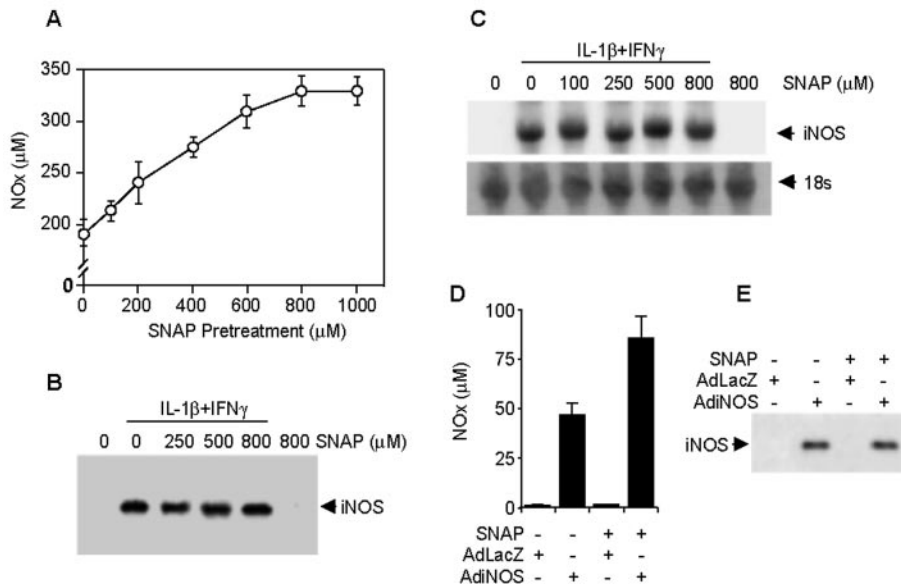
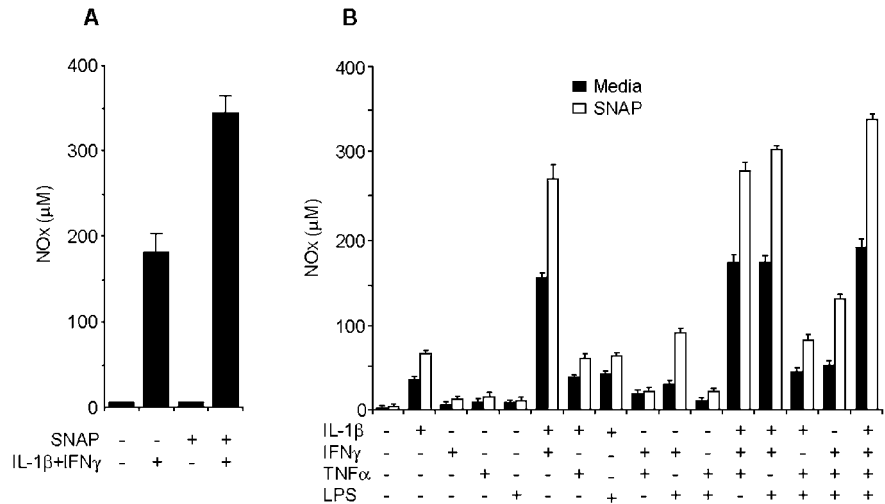
**Enzyme Assays**—Hepatocytes were harvested, washed with ice-cold PBS, and homogenized with an Ultra-Turrax homogenizer in 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.5  $\mu$ g/ml leupeptin, 0.35  $\mu$ g/ml pepstatin, and 1 mM dithiothreitol. The lysates were centrifuged at 12,000  $\times$  *g* for 5 min at 4 °C and the supernatants were used for enzyme assays. GTPCHI activity was measured by the modified method of Duch *et al.* (26) and Geller *et al.* (23) as follows. The enzyme reaction was initiated by the addition of the enzyme solution to the reaction mixture (500  $\mu$ l), containing 0.1 M Tris-HCl (pH 7.8), 0.3 M KCl, 2.5 mM EDTA, 10% glycerol, and 1 mM GTP. The reaction mixture was incubated in the dark at 37 °C for 1 h and then the reaction was stopped by the addition of 50  $\mu$ l of the iodine solution. After 1 h at room temperature, excess iodine was removed by the addition of 50  $\mu$ l of 2% ascorbate. The mixture was supplemented with 50  $\mu$ l of 1 N NaOH and then incubated with 3 units (100  $\mu$ l) of alkaline phosphatase at 37 °C for 1 h. The reaction was terminated by adding 100  $\mu$ l of 1 N acetic acid. After centrifugation at 12,000  $\times$  *g* for 5 min, the supernatant was applied to a Whatman Partisil 10 ODS column (4.6  $\times$  250 mm) connected to a Cosmosil 10 C<sub>18</sub> column (4.6  $\times$  50 mm). Neopterin was eluted with a solvent of 50 mM sodium acetate buffer (pH 5.0) containing 0.1 mM EDTA and 5% methanol at a flow rate of 0.8 ml/min. The column temperature was maintained at 25 °C. The elute was monitored with a fluorimeter (excitation, 350 nm; emission, 440 nm). iNOS activity was determined as previously described (27). In brief, cytosols or column fractions (30  $\mu$ l) were pre-incubated with 100  $\mu$ M BH<sub>4</sub> for 40 min at 37 °C prior to assay of NO synthesis activity in the presence of 1 mM NADPH, 20  $\mu$ M FAD, 20  $\mu$ M FMN, 4 mM L-arginine, and 5 mM glutathione, in a final volume of 200  $\mu$ l of Tris-HCl (pH 7.7). The reaction was terminated at various times by addition of 400  $\mu$ l of 0.5 mM NaOH and 400  $\mu$ l of 10% ZnSO<sub>4</sub>. After centrifugation at 12,000  $\times$  *g* for 10 min at 4 °C, nitrite plus nitrate were measured in the supernatant.

**Data Analysis and Statistics**—The data are presented as mean  $\pm$  S.D. from more than three independent experiments. Statistical comparisons between groups were performed using the Student's *t* test.

#### RESULTS

**SNAP Pretreatment Increases Cytokine-induced NO Production**—To determine whether NO pretreatment regulates IL-1 $\beta$  and IFN $\gamma$ -induced NO production in rat primary cultured hepatocytes, cells were treated with or without the NO donor SNAP (800  $\mu$ M) for 8 h, recovered in fresh medium for 24 h, and

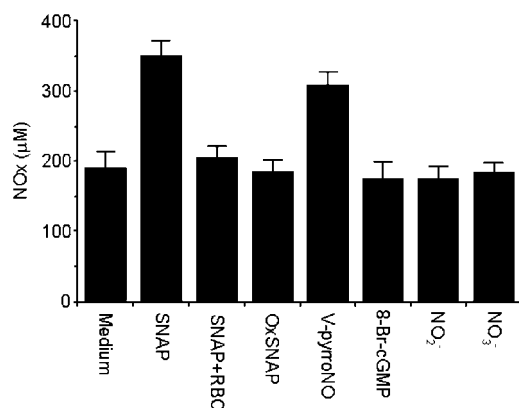
**FIG. 1. SNAP pretreatment increases cytokine-induced NO production in hepatocytes.** Hepatocytes were pretreated with or without 800  $\mu\text{M}$  SNAP for 8 h, washed three times with fresh medium, cultured in medium containing 5% calf serum for 24 h, and then stimulated with IL-1 $\beta$  plus IFN $\gamma$  (A) or different combinations of IL-1 $\beta$ , IFN $\gamma$ , tumor necrosis factor- $\alpha$  (*TNF- $\alpha$* ), and LPS (B). After 24 h, NO $_x$  was determined in the culture medium. Data represent the mean  $\pm$  S.D. of four independent experiments.



**FIG. 2. Pretreatment with SNAP increases cytokine-induced NO production in a dose-dependent manner without changing iNOS expression levels.** Hepatocytes were treated with different concentrations of SNAP for 8 h, washed three times with fresh medium, cultured in medium containing 5% calf serum for 24 h, and then stimulated with IL-1 $\beta$  and IFN $\gamma$ . A, after 24 h, the effect of SNAP concentration on NO production was determined by measuring accumulation of NO $_x$  in the culture medium. Data represent the mean  $\pm$  S.D. of four independent experiments. B, after 14 h, cell lysates were prepared and levels of iNOS protein were measured by Western blot analysis. C, after 8 h, levels of iNOS mRNA were measured by Northern blot analysis. D and E, hepatocytes were treated with or without 800  $\mu\text{M}$  SNAP for 8 h, washed three times with fresh medium, cultured in medium containing 5% calf serum for 24 h, and then transfected with AdiNOS or AdLacZ (10 multiplicity of infections) in serum-free medium. After 3 h, cells were incubated in fresh medium containing 5% serum. After an additional 24 h, NO $_x$  (D) and iNOS protein levels (E) were determined by HPLC and Western blot, respectively.

stimulated with cytokines for 24 h. Hepatocyte viability remained >95% throughout the experiment. Hepatocytes treated with 800  $\mu\text{M}$  SNAP for 8 h generated  $25 \pm 4 \mu\text{M}$  NO $_x$ /h in the culture medium (data not shown), which is similar to the levels of NO produced by cytokine-stimulated hepatocytes (24) and LPS-injected rats (28). Cytokine treatment induced a large rise in NO production in hepatocytes, and the NO production was further increased by SNAP pretreatment (Fig. 1A). Control or SNAP-pretreated cells without cytokine stimulation did not accumulate NO $_x$  in the culture medium. We also examined the effects of individual cytokines (IL-1 $\beta$ , IFN $\gamma$ , or tumor necrosis factor- $\alpha$ ), known to regulate iNOS expression (4), on NO production in hepatocytes with or without SNAP pretreatment (Fig. 1B). When hepatocytes were stimulated with a single cytokine, NO production was detected in hepatocytes stimulated with IL-1 $\beta$  only, but not with any other single cytokine. As we have previously shown (28), mixtures containing both IL-1 $\beta$  and IFN $\gamma$  were most effective in NO production. However, NO production was significantly higher in SNAP-pretreated hepatocytes than in control cells, for all of the cytokine treatment conditions (Fig. 1B).

**SNAP Pretreatment Increases NO Production in Hepatocytes Stimulated with Cytokines or Transfected with the iNOS Gene without Changing iNOS Expression Level**—To investigate the effect of SNAP concentration on cytokine-induced NO production, hepatocytes were pretreated with various SNAP concentrations for 8 h prior to a 24-h recovery and stimulated with IL-1 $\beta$  and IFN $\gamma$ . NO production was significantly increased in a manner that correlated with the concentration of SNAP and appeared to be maximally produced at 800  $\mu\text{M}$  SNAP (Fig. 2A). We have reported previously that cytokines produce NO from human and rat hepatocytes through the transcriptional expression of the *iNOS* gene (4, 20, 27). Whereas cytokine exposure revealed iNOS protein expression, SNAP pretreatment caused no further increase in iNOS protein levels measured by Western blot (Fig. 2B). In addition, Northern blot analysis revealed that SNAP pretreatment did not affect the levels of *iNOS* mRNA expression above that seen with cytokines alone (Fig. 2C). Moreover, hepatocytes transfected with *iNOS* using an adenoviral vector (AdiNOS) significantly increased NO production and this NO production was also increased by SNAP pretreatment (Fig. 2D). SNAP pretreatment did not increase iNOS protein levels compared with those of



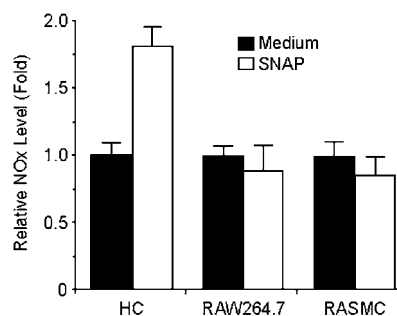
**FIG. 3. Effects of NO donors, red blood cells, oxidized NO products, and 8-Br-cGMP on cytokine-induced NO production in hepatocytes.** Hepatocytes were treated with SNAP (800 µM), red blood cells (800 µM hemoglobin), oxidized SNAP (OxSNAP, 800 µM), V-PyrroNO (800 µM), 8-Br-cGMP (800 µM), nitrite (800 µM), or nitrate (800 µM) in 10-cm plates for 8 h, washed three times with fresh medium, recovered in medium containing 5% calf serum for 24 h, and stimulated with IL-1β and IFNγ. After 24 h incubation, nitrite plus nitrate (NOx) was determined in the cultured medium. Data represent the mean ± S.D. of three independent experiments.

AdiNOS alone (Fig. 2E). These results indicate that increases in NO production following SNAP pretreatment are not likely because of an increase in iNOS expression at the protein or mRNA levels.

**NO<sub>2</sub><sup>-</sup>, NO<sub>3</sub><sup>-</sup>, or cGMP Does Not Mimic the Effect of NO on Cytokine-induced NO Production in Hepatocytes**—NO activates soluble guanylyl cyclase to produce cGMP from GTP, resulting in a variety of biological phenomena such as cellular signaling and protection against apoptosis (1, 24). NO also interacts with oxygen molecules and various metal-containing biomolecules, which results in production of the stable end products nitrite and nitrate (29). To investigate whether pretreatment with NO donors, oxidized NO products, or cGMP would increase cytokine-induced NO production, hepatocytes were pretreated with these molecules, recovered for 24 h, and stimulated with IL-1β and IFNγ. Twenty-four hours later, NOx production was measured in the culture medium (Fig. 3). Pretreatment with SNAP (a general NO donor) and V-PyrroNO (a hepatocyte-specific NO donor that mimics endogenous NO sources) (30) significantly increased cytokine-induced NO production compared with that of control cells, whereas the control oxidized SNAP (not liberating NO) did not affect NO production. SNAP pretreatment in the presence of red blood cells, the scavenger of NO, inhibited the effect of SNAP on cytokine-induced NO production. The membrane-permeable cGMP analog 8-Br-cGMP, NO<sub>2</sub><sup>-</sup> or NO<sub>3</sub><sup>-</sup> did not increase cytokine-induced hepatocyte NO production compared with control cells.

**NO Pretreatment Does Not Augment Cytokine-induced NO Production in Smooth Muscle Cells or Macrophages**—To examine whether SNAP pretreatment increases NO production in other cells known to express iNOS, the macrophage cell line RAW264.7 and RASMC were pretreated with SNAP, recovered in fresh medium for 24 h, and stimulated with LPS + IFNγ and IL-1β + IFNγ, respectively (Fig. 4). In contrast to SNAP-pretreated hepatocytes, cytokine-induced NO production did not increase in either RAW264.7 macrophages or RASMC.

**SNAP Pretreatment Increases iNOS Activity in Intact Cells and Cytosols**—To determine whether SNAP pretreatment increases iNOS activity within cells stimulated with cytokines, hepatocytes were stimulated with IL-1β and IFNγ for 12 h after a 24-h recovery period following SNAP pretreatment. Cells were washed and cultured in fresh medium containing



**FIG. 4. Comparative effect of SNAP pretreatment on cytokine-induced NO production in hepatocytes, macrophages, and smooth muscle cells.** Hepatocytes (HC), macrophage cell line RAW264.7 cells, and RASMC were treated with SNAP in 12-well plates for 8 h, washed three times with fresh medium, recovered in medium containing 5% calf serum for 24 h, and then stimulated with IL-1β + IFNγ (hepatocytes and RASMC) or LPS + IFNγ (RAW264.7 cells). After 24 h incubation, NOx was determined in the culture medium. Data represent the mean ± S.D. of three independent experiments.

5% serum for 4 h. NO production was then measured in the culture medium (Fig. 5A). SNAP-pretreated hepatocytes significantly increased cytokine-induced NO production compared with control cells, whereas unstimulated hepatocytes following SNAP pretreatment did not produce NO (Fig. 5A). To examine whether the effect of SNAP pretreatment on cytokine-induced NO production was because of increased iNOS activity, cytosolic fractions were prepared from hepatocytes of the same time point and the enzyme activity was measured *in vitro*. It has been shown that BH<sub>4</sub> functions as an allosteric cofactor for *in vitro* and *in vivo* assembly of iNOS proteins into its active dimeric form (6, 7). When measured in the absence of exogenous BH<sub>4</sub>, iNOS activity of the cytosol from SNAP-pretreated hepatocytes was significantly increased compared with that from cells without SNAP pretreatment (Fig. 5B). When the cytosols were preincubated with 100 µM BH<sub>4</sub> for 30 min, cytosolic iNOS activities were not different between control and SNAP-pretreated hepatocytes (Fig. 5C). The same results were observed in iNOS gene-transfected hepatocytes (data not shown). The suppression of iNOS activity without preincubation of the cytosols with BH<sub>4</sub> suggests that GTPCHI activity may be elevated by NO pretreatment because BH<sub>4</sub> is known to act as an allosteric inhibitor of further BH<sub>4</sub> production (31).

**SNAP Pretreatment Increases Cellular Biopterin Level and GTPCHI Activity**—To determine whether SNAP pretreatment increases intracellular BH<sub>4</sub> levels, total cellular biopterin levels (BH<sub>4</sub> and more oxidized states) were measured in the cytosolic fractions from hepatocytes pretreated with or without SNAP. SNAP pretreatment increased the total cellular biopterin level from a basal concentration of 18 to 28 nmol/mg of protein. These levels were further increased to 29 and 40 nmol/mg of protein following stimulation with IL-1β and IFNγ (Fig. 6A). Because GTPCHI is known to be a rate-limiting enzyme in the *de novo* synthesis pathway of BH<sub>4</sub> (8, 10), the effect of SNAP pretreatment on GTPCHI activity was measured. SNAP pretreatment increased GTPCHI activity from the control level of 17 to 33 nmol/mg of protein/h (Fig. 6B). These enzyme activities were further increased to 37 and 56 nmol/mg of protein/h by stimulation with cytokines. These results indicate that elevation of the intracellular BH<sub>4</sub> level in SNAP-pretreated hepatocytes is because of an increase in GTPCHI activity.

**SNAP Pretreatment Suppresses GFRP Level But Did Not Alter GTPCHI Expression**—To determine whether SNAP pretreatment increases GTPCHI expression, GTPCHI mRNA and protein were detected by Northern and Western blot analyses.

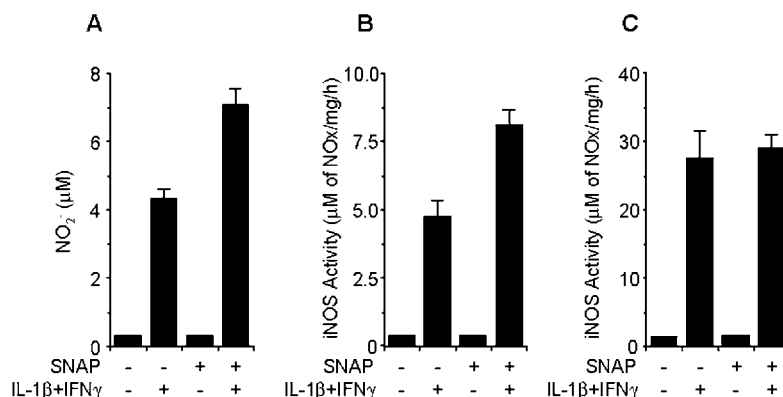


FIG. 5. **Effect of SNAP pretreatment on iNOS activity.** Hepatocytes were treated with or without SNAP (800  $\mu\text{M}$ ) for 8 h, washed twice with fresh medium, recovered in 5% serum-containing medium for 24 h, and stimulated with or without IL-1 $\beta$  and IFN $\gamma$  for 12 h. A, after 12 h, cells were washed three times with fresh medium and incubated in 5% serum-containing medium. After 4 h, nitrite was measured in the culture medium using Griess reagent. B, cells were harvested, washed with ice-cold PBS, and lysed by three cycles of freeze and thaw. Cytosolic fractions were prepared by centrifugation at  $12,000 \times g$  for 10 min. Cytosolic iNOS activity was measured in the presence of 1 mM NADPH, 20  $\mu\text{M}$  FAD, 20  $\mu\text{M}$  FMN, 4 mM L-arginine, and 5 mM glutathione without exogenous BH<sub>4</sub>. C, cytosolic iNOS activity was measured as shown in B following preincubation with 100  $\mu\text{M}$  BH<sub>4</sub> at 37  $^{\circ}\text{C}$  for 30 min. Data represent the mean  $\pm$  S.D. of more than three independent experiments.

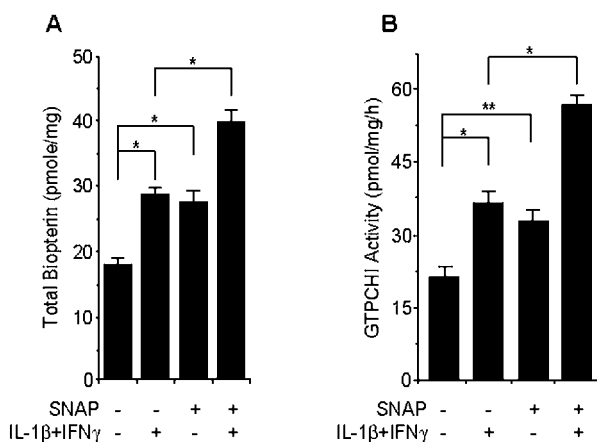


FIG. 6. **Effect of SNAP pretreatment on cellular total bioppterin levels.** Hepatocytes were treated with or without SNAP (800  $\mu\text{M}$ ), washed twice with fresh medium, recovered in 5% serum-containing medium for 24 h, and stimulated with or without IL-1 $\beta$  and IFN $\gamma$  for 16 h. A, cells were harvested, washed with ice-cold PBS, and lysed by three cycles of freeze and thaw in the presence of protease inhibitors. Cellular total bioppterin levels (A) and GTPCHI activities (B) were measured by HPLC method as described under "Experimental Procedures." Data represent the mean  $\pm$  S.D. of three independent experiments. \*,  $p < 0.01$ ; \*\*,  $p < 0.05$ .

GTPCHI mRNA and protein were constitutively expressed and the levels were increased by cytokines. Neither the basal nor stimulated levels were altered by SNAP pretreatment (Fig. 7, A and B). Thus, the level of GTPCHI expression did not correlate with the cellular bioppterin level and GTPCHI activity (Fig. 6). GTPCHI activity can be post-translationally inhibited by BH<sub>4</sub> in the presence of GFRP, and this inhibition is reversed by phenylalanine (31). We next measured the effect of exogenous BH<sub>4</sub> and phenylalanine on GTPCHI activity in the cytosols from control and SNAP-pretreated hepatocytes. As shown in Fig. 7C, GTPCHI activity was significantly higher in SNAP-pretreated hepatocyte cytosols than control cytosols. When BH<sub>4</sub> (100  $\mu\text{M}$ ) was added to the enzyme assay mixture, GTPCHI activity was markedly inhibited in the control cytosols, and this inhibition was much less in the SNAP-pretreated hepatocyte cytosols. The inhibitory effect of BH<sub>4</sub> was reversed only in the controls by the addition of phenylalanine, but not tyrosine and tryptophan. These effects of BH<sub>4</sub> and phenylalanine suggest that NO may suppress GFRP level in hepatocytes. To better

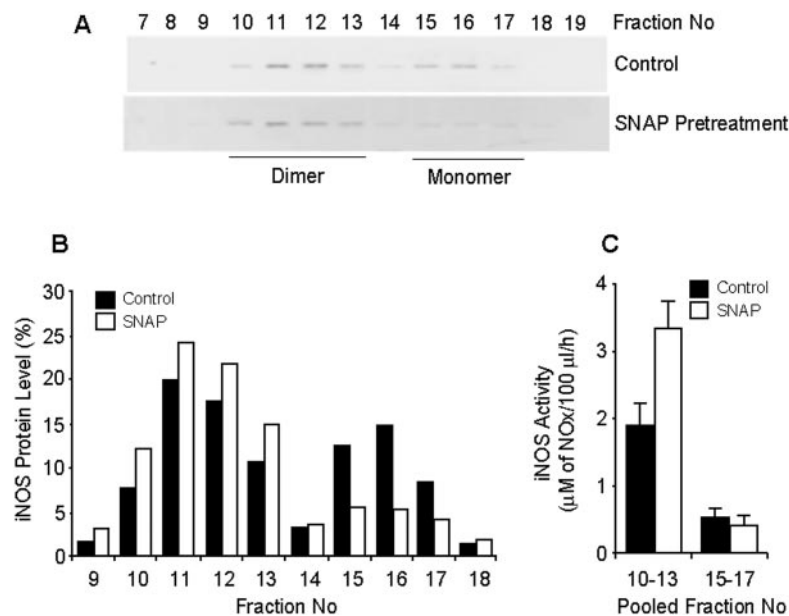
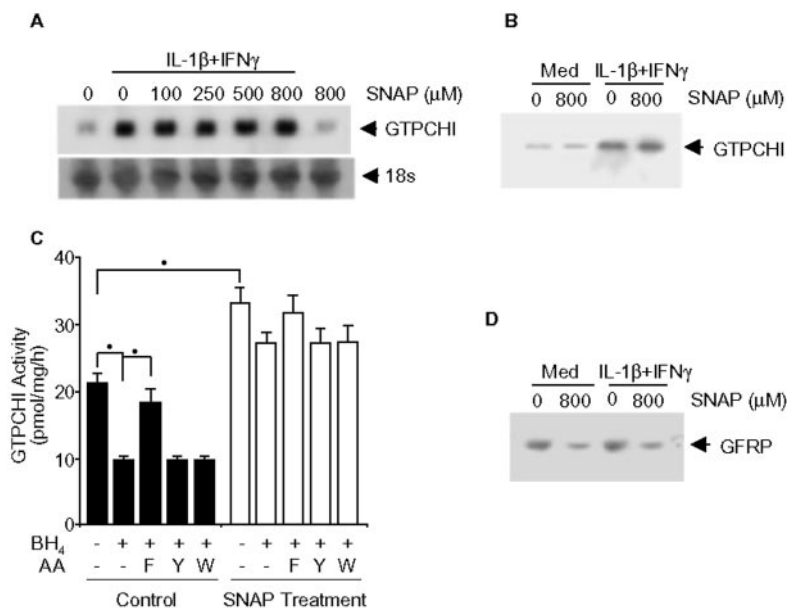
characterize the nature of increased GTPCHI activity in the hepatocytes following SNAP pretreatment, cellular GFRP levels were assessed by Western blot. GFRP was constitutively expressed in control hepatocytes, and this level was suppressed by SNAP pretreatment (Fig. 7D). Cytokine stimulation did not alter the GFRP levels in hepatocytes with or without SNAP pretreatment. These results suggest that SNAP pretreatment increases GTPCHI activity through the suppression of GFRP level.

**SNAP Pretreatment Increases iNOS Subunit Dimerization**—It has been shown that subunit dimerization is required for iNOS to synthesize NO *in vitro* and *in vivo* (6, 7). We next examined whether SNAP pretreatment increased dimerization in hepatocytes. Cytosols were prepared from hepatocytes stimulated with IL-1 $\beta$  and IFN $\gamma$  following SNAP pretreatment, equal amounts of protein (1 mg) were fractionated by gel filtration chromatography, and the iNOS protein level and activity of each fraction were determined. Western blot analysis showed that there was more iNOS protein in the dimeric form after SNAP pretreatment compared with controls (Fig. 8A). Analyses of the protein intensity revealed an estimated dimeric iNOS to monomeric ratio of 2:1 in the control cytosols but the ratio was 3.5:1 in the cytosols from SNAP-pretreated cells (Fig. 8B). SNAP pretreatment increased NO synthesis activity in the pooled fractions containing dimeric iNOS (fraction numbers 10–13), but not in the pooled iNOS monomeric fractions 15–17 (Fig. 8C). These results suggest that SNAP pretreatment increases iNOS activity in hepatocytes by promoting subunit dimerization.

## DISCUSSION

It has been reported that cotreatment with the NO donor SNAP and cytokines (including LPS) suppresses iNOS expression and NO production in hepatocytes (18), macrophages (32, 33), and epithelial cells (34). However, in this study we have shown that SNAP pretreatment had the opposite effect in hepatocytes through a novel mechanism including BH<sub>4</sub> synthesis. When hepatocytes were treated with SNAP and then stimulated with the cytokines IL-1 $\beta$  and IFN $\gamma$ , or transfected with AdiNOS, NO production was significantly increased by enhancing iNOS activity without changing the levels of iNOS mRNA and protein. Only NO, but not cGMP, nitrite, or nitrate, pretreatment enhanced cytokine-induced NO production. This response appeared to be cell type-specific to hepatocytes and was not observed in smooth muscle cells or macrophages. Finally,

**FIG. 7. Effect of SNAP pretreatment on GTPCHI expression, its activity, and GFRP level.** Hepatocytes were treated with or without various concentrations of SNAP, washed twice with fresh medium, recovered in 5% serum-containing medium for 24 h, and stimulated with or without IL-1 $\beta$  and IFN $\gamma$ . *A*, after 8 h stimulation, *GTPCHI* mRNA was determined by Northern blot analysis. *B*, after 12 h stimulation, the levels of GTPCHI protein were determined by Western blot analysis. *C*, cells were harvested and lysed by three cycles of freeze and thaw in the presence of protease inhibitors. Cytosolic GTPCHI activity was measured in the presence or absence of exogenous BH $_4$  (100  $\mu$ M), L-phenylalanine (F, 2 mM), L-tyrosine (Y, 2 mM), or L-tryptophan (W, 2 mM). Data represent the mean  $\pm$  S.D. of three independent experiments. *D*, the levels of GFRP expression were determined by Western blot analysis. \*,  $p < 0.01$ .



**FIG. 8. Effect of SNAP pretreatment on iNOS dimerization.** Hepatocytes were treated with or without SNAP (800  $\mu$ M), washed twice with fresh medium, recovered in 5% serum-containing medium for 24 h, and stimulated with or without cytokine for 16 h. Cytosols were prepared and 1-mg protein samples were fractionated by gel filtration chromatography. *A*, each fraction was separated on SDS-PAGE, and iNOS protein levels were determined by Western blot using an iNOS-specific antibody. *B*, the intensities of iNOS protein bands were measured by densitometry. Data present average value from two independent experiments. *C*, dimeric (fraction numbers 10–13) and monomeric iNOS fractions (fraction numbers 15–17) were pooled. iNOS activities of each pool were measured as described in the legend to Fig. 5. Data present the mean  $\pm$  S.D. of two independent experiments.

the mechanism appeared to occur through the enhancement of BH $_4$  levels by suppressing GFRP expression levels. These results demonstrate a novel regulatory effect of NO on GFRP expression and a potentially important mechanism for NO to amplify subsequent iNOS activity.

It has been shown that NO production in both human and murine hepatocytes depends on the expression of *iNOS* in response to LPS and/or inflammatory cytokines (20, 27, 33). The expression of *iNOS* can be regulated at the level of transcription, and NF- $\kappa$ B activation is essential for its expression (35). In this study, NO production was higher in SNAP-pretreated cells, even though the levels of *iNOS* mRNA and protein (Fig. 2) as well as NF- $\kappa$ B activation (data not shown) were similar to those of non-pretreated control cells. The observation that SNAP pretreatment increased NO production in hepatocytes transfected with *AdiNOS* indicates that typical transcription pathways for *iNOS* expression are not the sites of action.

The biologically active form of iNOS is a homodimer and requires NADPH, FAD, FMN, and BH $_4$  as cofactors for maximal enzyme activity *in vitro* (5). BH $_4$  functions as an allosteric

cofactor for promoting assembly of the iNOS monomer into its active dimeric form *in vitro* and in cells (6, 7). Inhibitors of *de novo* synthesis of BH $_4$  have been shown to inhibit LPS and IFN- $\gamma$ -induced NO production (11, 16), probably through the inhibition of iNOS subunit dimerization (7, 36). It has been also demonstrated that NIH3T3 cells, deficient in *de novo* BH $_4$  biosynthesis, express mostly monomeric forms of iNOS and generate a minimal amount of NO following transfection with the human *iNOS* gene (7). However, NIH3T3 cells grown with supplemental BH $_4$  generated NO by increasing the percentage of dimeric iNOS. LPS and cytokines stimulate the *de novo* synthesis of BH $_4$  by increasing *GTPCHI* expression in various types of cells (11, 23), and the increased BH $_4$  production supplies an essential cofactor to increase synthesis of NO by iNOS (17). Furthermore, cytokine treatment of endothelial cells enhances NO production by increasing cellular BH $_4$  levels without changing *eNOS* expression (17). Thus, the intracellular level of BH $_4$  appears to be a rate-limiting factor for NOS activity. We showed that SNAP pretreatment increased total cellular biopterin concentrations (BH $_4$  and more oxidized states)

and promoted iNOS subunit dimerization. We also found that BH<sub>4</sub> supplementation elevated NO synthesis activity in cytosols from hepatocytes stimulated with cytokine alone and that SNAP pretreatment reduced the cellular level of nonfunctional iNOS monomers by 50% (30 versus 14%). These results indicate that SNAP pretreatment increases cytokine-induced hepatocyte NO production by elevating the intracellular total biopterin levels and promoting iNOS subunit assembly into an active dimer.

Intracellular BH<sub>4</sub> levels are regulated by GTPCHI, which is the first and rate-limiting enzyme in *de novo* synthesis of BH<sub>4</sub>. We determined the levels of GTPCHI mRNA and protein in SNAP-pretreated hepatocytes by Northern and Western blotting analyses. SNAP pretreatment did not change the expression levels of GTPCHI. However, we found that SNAP increased GTPCHI activity. To explore further the mechanism by which SNAP pretreatment increases GTPCHI activity, we tested the possibility that SNAP pretreatment may regulate cellular GFRP level. It has been shown that GTPCHI interacts with GFRP in the presence of BH<sub>4</sub> and GTP, resulting in the formation of an inhibitory protein complex, which suppresses *de novo* synthesis of BH<sub>4</sub> (15, 31). This inhibition is reversed by phenylalanine (31). We examined the effects of exogenous BH<sub>4</sub> and phenylalanine on GTPCHI activity in hepatocyte cytosols. Exogenous BH<sub>4</sub> inhibited markedly GTPCHI activity in control cytosols, but only partially in SNAP-pretreated hepatocyte cytosols. We also found that phenylalanine overcame feedback inhibition of GTPCHI activity by BH<sub>4</sub> in controls, but not by SNAP-pretreated hepatocyte cytosols. These results suggest that NO may suppress the cellular level or biological activity of GFRP. Indeed, Western blot analysis showed that SNAP pretreatment suppressed the GFRP level. This suppression may increase GTPCHI activity and subsequently intracellular total biopterin content, resulting in increased iNOS subunit assembly and NO synthesis activity. How NO regulates GFRP expression is unclear, but it is known that NO suppressed gene expression and protein synthesis in hepatocytes (37) and other cell types (33). Further studies are required to define the molecular mechanism.

Pretreatment of hepatocytes with the hepatocyte-specific NO donor V-PyrroNO also increased cytokine-induced NO production following a 24-h recovery. SNAP and V-PyrroNO produce NO in the culture media and within hepatocytes, respectively (24, 30). This indicates that NO-generating sites whether autocrine or paracrine can increase cytokine-induced NO production. Although NO is oxidized to nitrite and nitrate and activates guanylyl cyclase in hepatocytes to generate cGMP (38) (a key mediator of NO-dependent cellular signaling) pretreatment with 8-Br-cGMP, nitrite or nitrate did not increase cytokine-induced NO production. NO, but not NO<sub>2</sub><sup>-</sup>, NO<sub>3</sub><sup>-</sup>, or cGMP, induces nitrosative stress by interacting with cellular thiols and iron complexes (39), which then can regulate enzyme activity (28, 40) and transcriptional gene expression (29). The nitrosative stress occurs effectively in hepatocytes but not in macrophages and smooth muscle cells because of the relatively high iron content of hepatocytes (39). This stress is probably associated with NO-mediated suppression of GFRP expression, which leads to increases in cellular BH<sub>4</sub> level and subsequent elevation of NO production.

Although under certain conditions, NO can induce hepatotoxicity, in general, NO is a protective molecule in cultured hepatocytes and liver *in vivo* (20, 24, 30, 39, 41). We have previously shown that NO production protects hepatocytes from tumor necrosis factor- $\alpha$ -induced apoptotic cell death by

nitrosative S-nitrosylation of caspase family proteases, which involve the apoptotic signaling cascade (24). In this study, we demonstrated that SNAP pretreatment increased iNOS subunit dimerization and NO production in cultured hepatocytes by augmenting the cellular BH<sub>4</sub> level through the suppression of GFRP levels and activation of GTPCHI. This suggests that the suppression of GFRP expression by NO is a mechanism to amplify NO production and protect hepatocytes from injury during acute inflammatory states.

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