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The Liver-Selective Nitric Oxide Donor O^2-Vinyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (V-PYRRO/NO) Protects HepG2 Cells against Cytochrome P450 2E1-Dependent Toxicity

Pengfei Gong, Arthur I. Cederbaum, and Natalia Nieto

Department of Pharmacology and Biological Chemistry, Mount Sinai School of Medicine, New York, New York

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ABSTRACT

HepG2 cells expressing CYP2E1 (E47 cells) are more susceptible to toxicity by arachidonic acid (AA) or after glutathione depletion with an inhibitor of glutamate-cysteine ligase, L-buthionine-(*S*,*R*)-sulfoximine (BSO), compared with control HepG2 cells (C34 cells). The ability of nitric oxide (NO) to protect against CYP2E1-dependent toxicity has not been evaluated. We therefore studied the ability of O^2-vinyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (V-PYRRO/NO), a liver-selective NO donor, to protect against CYP2E1-dependent toxicity and compared this with protection by chemical NO donors. E47 cells incubated with V-PYRRO/NO produced NO, whereas C34 cells did not. Incubation of E47 cells with 50 μM AA or 100 μM BSO for 2 days resulted in a 50% loss of cell viability. V-PYRRO/NO (1 mM) blocked this toxicity of AA and BSO by a mechanism involving NO release via CYP2E1 metabolism of V-PYRRO/NO. NO scavengers hemoglobin and 2-(4-carboxophenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide blocked the protective effects of V-PYRRO/NO. V-PYRRO/NO inhibited CYP2E1 activity and production of reactive oxygen species, whereas hemoglobin prevented these events. AA and BSO induced lipid peroxidation and decreased mitochondrial membrane potential; both of these effects were blocked by V-PYRRO/NO. Unlike V-PYRRO/NO, the chemical donors spermine/NO and (*S*)-nitroso-*N*-acetylpenicillamine release NO directly when added to the medium; however, they could partially protect against the CYP2E1-dependent toxicity. These results suggest that V-PYRRO/NO protects HepG2 cells against CYP2E1-dependent toxicity through inhibition of CYP2E1-derived reactive oxygen species production and lipid peroxidation by the generated NO and that this compound may be valuable in protecting against CYP2E1-dependent toxicity via liver P450-specific generation of NO.

To study the biochemical and toxicological effects of CYP2E1, our laboratory established a HepG2 cell line that constitutively overexpresses CYP2E1 (E47 cells) (Chen and Cederbaum, 1998). Although no toxicity was found with control HepG2 cells that do not express CYP2E1 (C34 cells), the addition of ethanol, iron, or a polyunsaturated fatty acid such as arachidonic acid (AA) to E47 cells decreased cell viability and caused apoptosis (Chen et al., 1997; Cederbaum et al., 2001). These effects were enhanced when cellular glutathione (GSH) levels were lowered by treatment with L-buthionine-(*S*,*R*)-sulfoximine (BSO). Moreover, the treatment of E47 cells to deplete GSH with BSO, an inhibitor of the glutamate-cysteine ligase, resulted in apoptosis and necrosis (Wu and Cederbaum, 2001), whereas no toxicity was found with control C34 or HepG2 cells that expressed CYP3A4 instead of CYP2E1. The antioxidant 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) partially prevented the apoptosis and necrosis after BSO treatment, whereas diallylsulfide, a CYP2E1 inhibitor, was fully protective.

Nitric oxide (NO) is a free radical produced by nitric-oxide synthase (NOS) that has been identified as an important signaling molecule in virtually every tissue in the body. There are three isoforms of NOS found in the liver. Of these isoforms, the inducible NOS and endothelial constitutive NOS are the most important (Clemens, 1999). NO plays important roles in liver homeostasis and disease, and its production is paradoxically implicated in both cytoprotection

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ABBREVIATIONS: E47 cells, HepG2 cell line established after transfection with pCI-neo-CYP2E1; AA, arachidonic acid; GSH, glutathione; BSO: L-buthionine-(*S*,*R*)-sulfoximine; C34 cells, HepG2 cell line established after transfection with pCI-neo; NO, nitric oxide; NOS, nitric-oxide synthase; V-PYRRO/NO, O^2-vinyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate; TNF, tumor necrosis factor; MEM, minimal essential medium; PNP, *p*-nitrophenol; SNAP, *S*-nitroso-*N*-acetylpenicillamine; MTT, 3(4,5-dimethylthiazole-2-yl)2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase; DCF, dichlorofluorescein; ROS, reactive oxygen species; Rh123, rhodamine 123; PI, propidium iodide; L-NMA, N^G-methyl-L-arginine; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; NAP, *N*-acetylpenicillamine; MDA, malondialdehyde.

and cytotoxicity (Taylor et al., 1998; Li and Billiar, 1999). NO seems to be beneficial to hepatocytes, having cytoprotective effects against inflammation and tissue damage and direct cytotoxic effects on invading microorganisms and tumor cells (Suzuki et al., 1995). There are no reports on the ability of NO to affect CYP2E1-dependent toxicity, although NO produced from several NO chemical donors was shown to inhibit CYP2E1 activity in isolated microsomes (Gergel et al., 1997)

V-PYRRO/NO, a stable diazeniumdiolate, is a newly synthesized drug that can circulate freely throughout the body and be metabolized to nitric oxide by enzymes, presumably cytochromes P450 in the liver (Saavedra et al., 1997; Stinson et al., 2002). The liver-selective NO production from V-PYRRO/NO has been demonstrated both in vitro (Saavedra et al., 1997) and in vivo (Stinson et al., 2002; Ricciardi et al., 2001). Because NO has a remarkable array of bioeffector roles in the body (Kerwin et al., 1995), it would be of great benefit to use a liver-selective NO donor such as V-PYRRO/NO for the purpose of liver protection against hepatotoxins, such as TNF-α and galactosamine. V-PYRRO/NO may be a protective NO donor for such hepatoprotection. V-PYRRO/NO has been demonstrated to increase cGMP levels in the liver that promote vasodilation; to reduce in situ hepatic vascular resistance without altering systolic blood pressure (Ricciardi et al., 2001); to protect hepatocytes from TNF-α plus actinomycin D toxicity in vitro (Li and Billiar, 1999); and to protect rats and mice from TNF-α or lipopolysaccharide plus galactosamine hepatotoxicity in vivo (Li and Billiar, 1999; Liu et al., 2002).

The goal of this study was to evaluate whether V-PYRRO/NO can protect against CYP2E1-dependent toxicity, to assess possible mechanisms for this protection, and to compare the protective effects of V-PYRRO/NO with that of typical NO donors that, after metal catalysis, spontaneously release NO.

Materials and Methods

Chemicals. V-PYRRO/NO was a generous gift from Dr. L. Keefer (National Cancer Institute, National Institutes of Health). G418 was purchased from Invitrogen (Carlsbad, CA). The protein DC-20 assay kit was obtained from Bio-Rad (Hercules, CA). Human CYP2E1 polyclonal antibody was kindly provided by Dr. J. M. Lasker (Hackensack Biomedical Research Institute, Hackensack, NJ). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Cell Culture and Treatment. This study was performed using HepG2 cells that constitutively express CYP2E1 (E47 cells) or control HepG2 cells (C34 cells) (Chen and Cederbaum, 1998). Cells were cultured in minimal essential medium (MEM) containing 10% fetal bovine serum and 0.5 mg/ml G418 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine in a humidified atmosphere in 5% CO_2 at 37°C. CYP2E1 expression was routinely monitored by assaying for the oxidation of *p*-nitrophenol (PNP) to *p*-nitrocatechol as described previously (Chen and Cederbaum, 1998). For each experiment, cells were plated and incubated in MEM overnight. The culture medium was replaced with fresh medium, and V-PYRRO/NO, AA, or BSO was added at the different time points selected for each experiment.

Nitrite and Nitrate Determination. Cells were grown on 24-well plates (35,000 cells/well) and treated with V-PYRRO/NO (or with SNAP or spermine/NO) in MEM medium without phenol red. After treatment, the culture medium was collected for nitrite or nitrite plus nitrate assay. Nitrite concentration was determined by the Griess reagent (modified) (Sigma-Aldrich) according to the manufacturer's protocol. Nitrite plus nitrate was determined by first reducing the nitrate in the medium to nitrite in the presence of nitrate reductase (Caro et al., 2001) and then determining the concentration of nitrite as described above.

General Methodology. Cell viability was measured by the MTT assay (Perez and Cederbaum, 2001). LDH activity was measured using a commercial lactate dehydrogenase assay kit (Sigma-Aldrich). CYP2E1 activity in intact cells (Perez and Cederbaum, 2001) and microsomes (Chen and Cederbaum, 1998) was measured by studying the oxidation of PNP to *p*-nitrocatechol by methods described previously. The measurement of dichlorofluorescein (DCF) fluorescence was used as a general index of ROS production after incubating the cells with 5 μM DCF-diacetate for 1 h (Perez and Cederbaum, 2002). Lipid peroxidation was determined by measuring the concentration of thiobarbituric acid-reactive species in cell lysates (Perez and Cederbaum, 2002). The mitochondrial membrane potential was examined by monitoring cell fluorescence after double staining with 5 μg/ml rhodamine 123 (Rh123) and 5 μg/ml propidium iodide (PI) as described previously (Bai et al., 1999).

Western Blotting. E47 cells were incubated in the presence or absence of 1 mM V-PYRRO/NO for 48 h. Cells were harvested in phosphate-buffered saline and pelleted by centrifugation at 8000 rpm for 1 min at 4°C. Cells were resuspended in 50 μl of lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.25 mM phenylmethylsulfonyl fluoride, 2 mM EGTA, and 50 mM NaF) and sonicated using a W220 sonicator (Misonix, Inc., Farmingdale, NY). Twenty micrograms of protein were size-fractionated on 12% denaturing polyacrylamide gels and electroblotted onto nitrocellulose membranes. The membrane was incubated with rabbit CYP2E1 polyclonal antibody (1:30,000) as the primary antibody, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000) as the secondary antibody. Detection of the protein bands was performed using the enhanced chemiluminescence immunoblot-detecting reagent ECL (Amersham Biosciences Inc., Piscataway, NJ).

Statistics. Data are presented as mean ± standard deviation with the number of experiments indicated in the figure legends. One-way analysis of variance (with subsequent post hoc comparisons by Sheffé's F test) was performed by version 10.0 (SPSS Inc., Chicago, IL).

Results

V-PYRRO/NO Produces NO via CYP2E1 Metabolism. V-PYRRO/NO is thought to produce NO via P450 metabolism (Saavedra et al., 1997; Stinson et al., 2002). The ability of V-PYRRO/NO to produce NO through CYP2E1 metabolism has not been studied. As previous studies have shown (Chen and Cederbaum, 1998; Wu and Cederbaum, 2001), CYP2E1 content and activity could not be detected in C34 cells but was present in E47 cells. When C34 cells were incubated with 1 mM V-PYRRO/NO, NO production, as measured by nitrite or nitrite plus nitrate, remained unchanged from the basal background level (Fig. 1A). However, incubation with the same amount of V-PYRRO/NO rapidly and significantly increased NO production in E47 cells (Fig. 1A), suggesting that NO production by V-PYRRO/NO in E47 compared with C34 cells is dependent on CYP2E1 activity. Levels of nitrite and nitrite plus nitrate in E47 cells increased rapidly after adding V-PYRRO/NO, reaching a maximum of approximately 5- and 4-fold, respectively, at 8 h, and remaining approximately 2-fold higher after 48 h compared with values in C34 cells ($p < 0.05$ at all time points) (Fig. 1A). Because NO production reached a maximum level at approximately 8 h, we selected this time point for the subsequent NO production experiments. When E47 cells were treated with different doses (0,

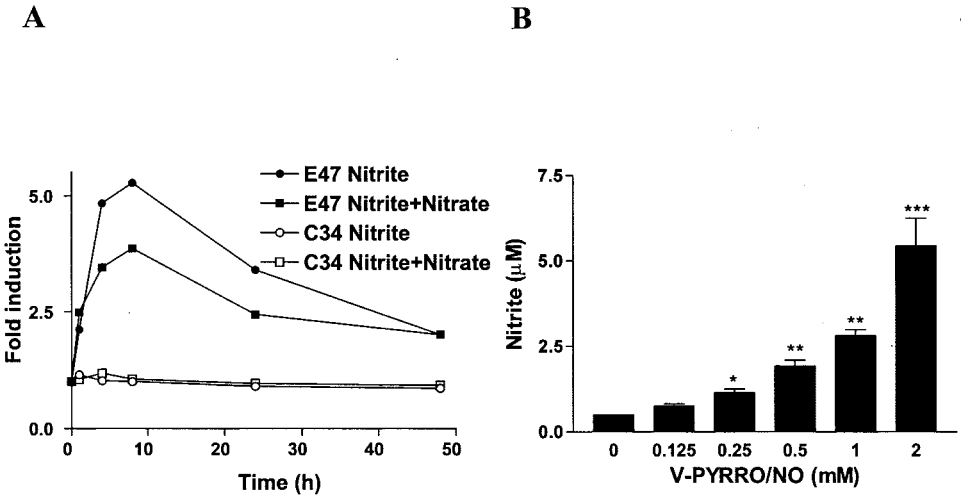

Fig. 1. Nitrite and nitrate production in C34 and E47 cells. A, time course of NO production by V-PYRRO/NO. C34 and E47 cells were incubated with or without 1 mM V-PYRRO/NO for 1, 4, 8, 24, and 48 h. B, dose-dependent NO production by V-PYRRO/NO in E47 cells. E47 cells were treated with 0, 0.125, 0.25, 0.5, 1, and 2 mM V-PYRRO/NO for 8 h. The concentration of nitrite or nitrite plus nitrate in the medium was determined as described under *Materials and Methods*. The amount of nitrite or nitrite plus nitrate produced in the presence of V-PYRRO/NO was expressed as -fold induction compared with that in the absence of V-PYRRO/NO. Results are expressed as mean ± S.D. (n = 3). ⋆, $p < 0.05$; ⋆⋆, $p < 0.01$; ⋆⋆⋆, $p < 0.001$ versus the group without V-PYRRO/NO treatment.

0.125, 0.25, 0.5, 1, and 2 mM) of V-PYRRO/NO for 8 h, nitrite production in E47 cells increased in a V-PYRRO/NO dose-dependent manner (Fig. 1B).

N^G-Methyl-L-arginine (L-NMA) is a specific NOS inhibitor that can inhibit all three isoforms of NOS. To evaluate whether the NO production by V-PYRRO/NO in E47 cells is mediated through stimulation of NOS, the ability of L-NMA to block the V-PYRRO/NO-derived NO production was determined. Figure 2 shows that, in the presence of L-NMA (up to 2 mM), both nitrite plus nitrate (A) and nitrite (B) production by V-PYRRO/NO was not significantly changed ($p > 0.05$ for all doses), suggesting that the NO production by V-PYRRO/NO in E47 cells is not mediated by NOS.

To further elucidate the mechanism of NO production in E47 cells by V-PYRRO/NO, the effects of 4-methylpyrazole, an inhibitor of CYP2E1 activity on the production of NO, was examined. Figure 3 shows that 5 mM 4-methylpyrazole inhibited the increase of nitrite production by 1 mM V-PYRRO/NO in E47 cells by approximately 50% at 8 h and completely blocked the increase in nitrite production at 24 and 48 h. Together, these results suggest that V-PYRRO/NO produces NO via CYP2E1 metabolism in E47 cells.

V-PYRRO/NO Protects E47 Cells against AA and BSO Cytotoxicity. CYP2E1-dependent cytotoxicity of AA or BSO on E47 cells has been documented in previous studies (Chen and Cederbaum, 1998; Wu and Cederbaum, 2001). To examine whether V-PYRRO/NO has any protective effects against this cytotoxicity, cell viability, LDH release, and cell morphology were examined. AA or BSO was used at concentrations of 50 and 100 μM, respectively, to produce approximately 50% toxicity after incubation for 2 days (Fig. 4A). At these concentrations, AA and BSO did not cause significant toxicity in C34 cells (data not shown). E47 cells were incubated with 0, 0.125, 0.25, 0.5, 1, and 2 mM V-PYRRO/NO for 48 h. Whereas incubation of E47 cells with 50 μM AA or 100 μM BSO for 48 h resulted in a 46 and 52% loss of cell viability, respectively (Fig. 4A), the addition of V-PYRRO/NO significantly prevented this loss of cell viability in a dose-dependent manner (Fig. 4A). The protection reached a maximum at 1 mM V-PYRRO/NO, restoring cell viability to 90 to 95% of untreated control cells (100% viability). Protection by V-PYRRO/NO was less effective at 2 than at 1 mM concentration. When E47 cells were treated with 50 μM AA or 100 μM BSO in the presence or absence of 1 mM V-PYRRO/NO for 48 h, more than 95% protection by V-PYRRO/NO against AA and BSO cytotoxicity was observed (Fig. 4B). The LDH release assay also showed that V-PYRRO/NO (1 mM) alone was not toxic to E47 cells (Fig. 5). Incubation of E47 cells with 50 μM AA or 100 μM BSO for 48 h resulted in a significant increase of LDH release (from 11.7 to 34.9 and 43.3%, respec-

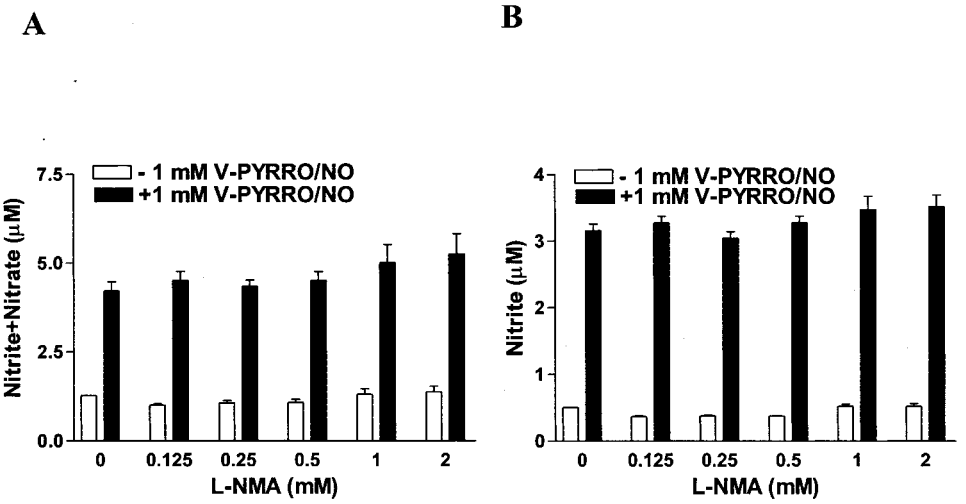

Fig. 2. Effects of L-NMA on nitrite production by V-PYRRO/NO. E47 cells were treated with 1 mM V-PYRRO plus 0, 0.125, 0.25, 0.5, 1, and 2 mM L-NMA for 8 h. Concentrations of nitrite plus nitrate (A) or nitrite (B) in the culture medium were determined as described under *Materials and Methods*. Results are expressed as mean ± S.D. (n = 3).

tively). V-PYRRO/NO at 1 mM reduced the increases of LDH release caused by AA or BSO (from 34.9 to 17.5% and 43.3 to 17.9%, respectively) (Fig. 5). Morphological changes were also observed. For example, E47 cells treated with 50 μM AA or 100 μM BSO for 48 h displayed a round shape and were swollen and lysed (Fig. 6; AA and BSO). These morphological changes were prevented by coincubation with 1 mM V-PYRRO/NO (Fig. 6; AA+V-PYRRO/NO and BSO+V-PYRRO/NO). These results confirmed the V-PYRRO/NO protection in E47 cells against CYP2E1-dependent cytotoxicity induced by AA and BSO.

NO Production Mediates the Protective Effects of V-PYRRO/NO against AA and BSO Toxicity. As shown in Fig. 1, V-PYRRO/NO can significantly increase NO production in E47 cells. Is this increase of NO production responsible for the protection of V-PYRRO/NO against AA and BSO cytotoxicities in E47 cells? Because pyrrolidine, the parental compound of V-PYRRO/NO, does not produce NO (Saavedra et al., 1997), it was used as a control for the effects of V-PYRRO/NO. Whereas addition of 1 mM V-PYRRO/NO significantly reduced the loss of cell viability caused by 50 μM AA or 100 μM BSO (from 52 to 89% viability for AA and 47 to 98% viability for BSO), the same concentration of pyrrolidine showed no protective effects on the cytotoxicity of AA or BSO (Fig. 7A). NO scavengers hemoglobin and carboxy-PTIO blocked the protective effects of V-PYRRO/NO when added at a concentration of 10 and 25 μM, respectively (Fig. 7, A and B). These results suggest that the protection of V-PYRRO/NO against AA and BSO cytotoxicity reflects the effects of NO produced from metabolism of V-PYRRO/NO. To further prove the protective effects of NO and to compare the effectiveness of V-PYRRO/NO with classical chemical NO donors, NO donors such as SNAP and spermine/NO, which spontaneously release NO in the medium, were also tested. SNAP (50 μM) and spermine/NO (5 μM) released NO (Fig. 8A), and similar levels of NO were found when 50 μM SNAP or 5 μM spermine/NO was added to the E47 cells, C34 cells, or just the incubation medium, in contrast with V-PYRRO/NO, which only provided NO in the E47 cells. These compounds can protect E47 cells against AA toxicity (Fig. 8B), whereas the same amount of their parental compounds, NAP and spermine, which did not release NO (Fig. 8A), did not (Fig. 8B). SNAP and spermine/NO restored cell viability from 50 to approximately 75 to 80% of untreated control cells.

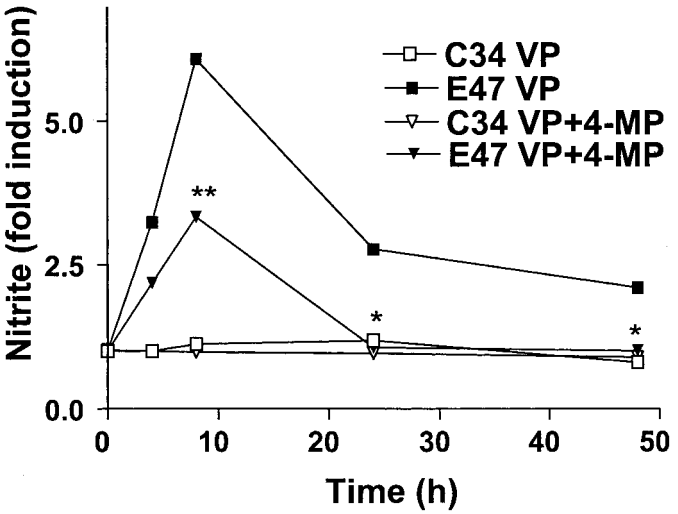

Fig. 3. A CYP2E1 inhibitor, 4-methylpyrazole, inhibits NO production by V-PYRRO/NO. C34 and E47 cells were treated with 1 mM V-PYRRO/NO (C34 VP, E47 VP) or 1 mM V-PYRRO/NO plus 5 mM 4-methylpyrazole (C34 VP + 4-MP, E47 VP + 4-MP) for 0, 4, 8, 24, and 48 h. Concentrations of nitrite in the culture medium were determined as described under *Materials and Methods*. Results are calculated as fold induction and expressed as mean ± S.D. (n = 3). ⋆, $p < 0.05$; ⋆⋆, $p < 0.01$, versus the E47 cell group without 4-methylpyrazole treatment.

V-PYRRO/NO Inhibits CYP2E1 Activity without Affecting CYP2E1 Protein Level. To elucidate the mechanism of the protective effects of V-PYRRO/NO against the CYP2E1-dependent AA and BSO toxicity, we examined the effects of V-PYRRO/NO on CYP2E1 protein content and activity. Western blot analysis showed that the CYP2E1 protein level was unchanged after incubation of 1 mM V-PYRRO/NO for 48 h (Fig. 9B). However, CYP2E1 activity measured by PNP oxidation was dramatically inhibited (Fig. 9A). Incubation of E47 cells with 1 mM V-PYRRO/NO caused time-dependent inhibition of CYP2E1 activity. After 48 h of incubation, CYP2E1 activity was inhibited by 83% (Fig. 9A). To know whether this inhibition is mediated by NO, we examined the effects of Hb on CYP2E1 activity both in microsomes and intact cells treated with 1 mM V-PYRRO/NO. CYP2E1 activity in microsomes isolated from E47 cells (1-h incubation) or in intact E47 cells (24-h incubation) was decreased approximately 80% upon incubation with V-PYRRO/NO (Fig. 9, C and D). Coincubation with 10 μM Hb blocked the inhibition of CYP2E1 activity by V-PYRRO/NO in microsomes and resulted in a 78% recovery of CYP2E1 activity in intact cells (Fig. 9, C and D). These results suggest that protection of V-PYRRO/NO against CYP2E1-dependent

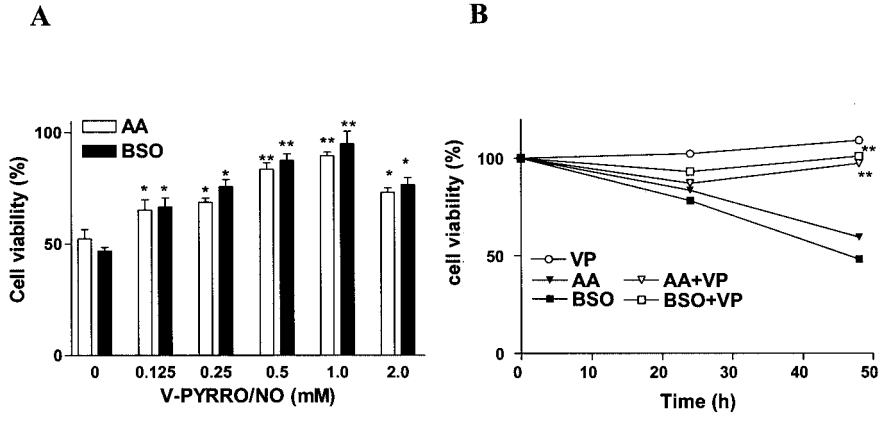

Fig. 4. V-PYRRO/NO protects E47 cells against the loss of viability caused by AA and BSO. A, dose-dependent protection against AA or BSO toxicity by V-PYRRO/NO. E47 cells were treated with 50 μM AA or 100 μM BSO plus 0, 0.125, 0.25, 0.5, and 1 mM V-PYRRO/NO for 48 h. B, time course for V-PYRRO/NO protection of E47 cells from AA and BSO toxicity. E47 cells were treated with 1 mM V-PYRRO/NO (VP), 50 μM AA, 100 μM BSO, 50 μM AA plus 1 mM V-PYRRO/NO (AA+VP), 100 μM BSO plus 1 mM V-PYRRO/NO (BSO+VP), or without treatment for 0, 24, and 48 h. Cell viability was determined by the MTT assay and calculated as percentage of viability compared with that of control E47 cells (100% viability). Results are expressed as mean ± S.D. (n = 3). ⋆, $p < 0.05$; ⋆⋆, $p < 0.01$, compared with the group treated with 50 μM AA or 100 μM BSO alone.

AA and BSO toxicity may be mediated through direct inhibition of CYP2E1 catalytic activity by NO.

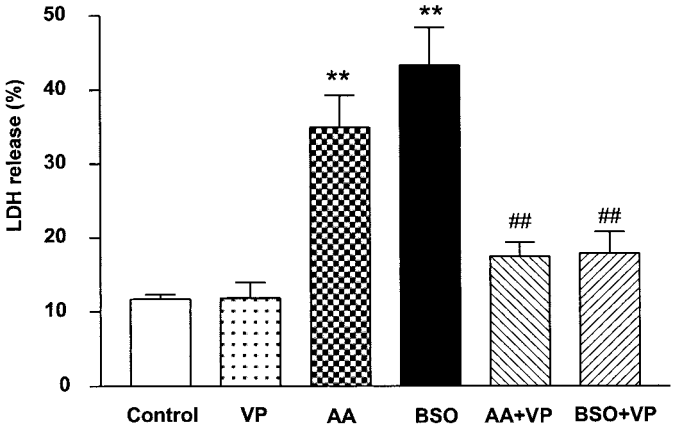

Fig. 5. V-PYRRO/NO prevents LDH release caused by AA and BSO. E47 cells were treated for 48 h with 50 μM AA, 100 μM BSO, 1 mM V-PYRRO/NO (VP), 50 μM AA plus 1 mM V-PYRRO/NO (AA+VP), 100 μM BSO plus 1 mM V-PYRRO/NO (BSO+VP), or with no treatment (Control). LDH activity was determined as described under *Materials and Methods*. LDH release was calculated as $LDH_{out}/(LDH_{out} + LDH_{in}) \times 100\%$, where LDH_{out} is LDH activity in the medium and LDH_{in} is LDH activity in the cell lysates. Results are expressed as mean ± S.D. ($n = 3$). ⋆⋆, $p < 0.01$, compared with control; ##, $p < 0.01$, compared with the corresponding group treated with 50 μM AA or 100 μM BSO alone.

V-PYRRO/NO Decreases ROS Production in E47 Cells. E47 cells show increased production of ROS compared with C34 cells (Chen and Cederbaum, 1998; Mari and Cederbaum, 2000). Incubation with 50 μM AA or 100 μM BSO for 24 h further increased ROS generation in E47 cells by 3.1- and 2.8-fold as measured by the DCF-diacetate fluorescence method (Fig. 10). Incubation with 1 mM V-PYRRO/NO alone decreased the ROS level in E47 cells by 31% ($p < 0.05$). This decrease of the ROS level by V-PYRRO/NO may reflect an antioxidant action of NO or inhibition of ROS production via depression of CYP2E1 activity. The increases of ROS production caused by AA or BSO in E47 cells were almost completely blocked by 1 mM V-PYRRO/NO (Fig. 10).

V-PYRRO/NO Inhibits Lipid Peroxidation Caused by AA and BSO. CYP2E1-dependent toxicity is often associated with elevated lipid peroxidation. Figure 11 shows that lipid peroxidation in E47 cells as measured by the level of the lipid peroxidation end product MDA was significantly increased by AA and BSO. Incubation with 50 μM AA or 100 μM BSO for 48 h increased the MDA level from 0.88 nmol/mg of protein to 2.1 and 1.8 nmol/mg of protein, respectively (Fig. 11). V-PYRRO/NO at a 1-mM concentration slightly but not significantly decreased the MDA level in E47 cells; however, it significantly reduced the increased level of MDA caused by AA (from 2.1 to 0.74 nmol/mg of protein) or BSO (from 1.8 to 0.62 nmol/mg of protein) (Fig. 11).

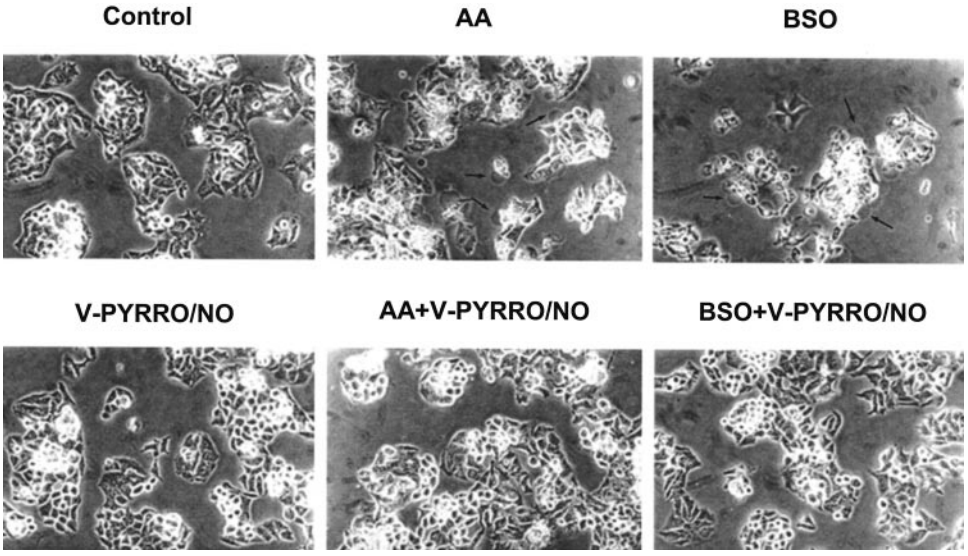

Fig. 6. V-PYRRO/NO protects E47 cells from the morphological changes induced by AA or by BSO. E47 cells were treated for 48 h with 50 μM AA, 100 μM BSO, 1 mM V-PYRRO/NO, 50 μM AA plus 1 mM V-PYRRO/NO (AA+V-PYRRO/NO), 100 μM BSO plus 1 mM V-PYRRO/NO (BSO+V-PYRRO/NO), or with no treatment (Control). Cell morphology was visualized under a light microscope (magnification, 200×).

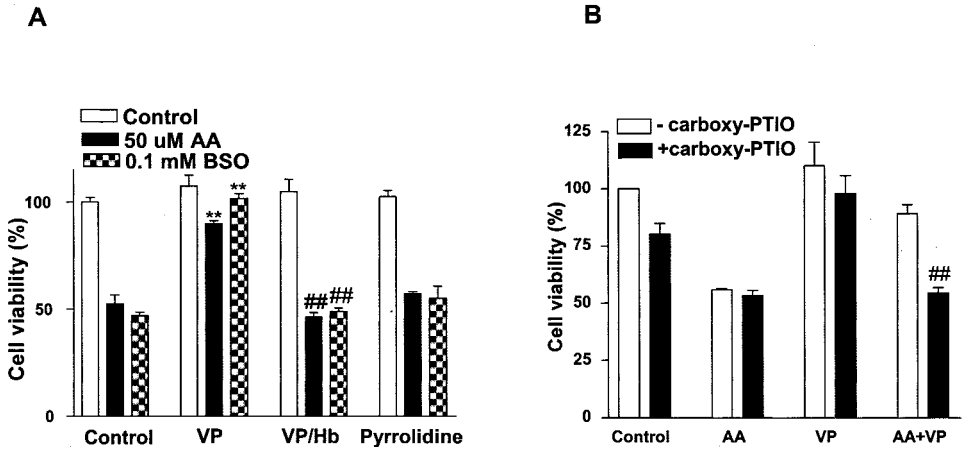

Fig. 7. The protective effects of V-PYRRO/NO are mediated by NO. A, E47 cells were treated with 50 μM AA or 100 μM BSO with or without 1 mM V-PYRRO/NO (VP), 1 mM V-PYRRO/NO plus 10 μM Hb, or 1 mM pyrrolidine for 48 h. B, E47 cells were treated with 50 μM AA or 50 μM AA plus 1 mM V-PYRRO/NO in the presence or absence of 25 μM carboxy-PTIO for 48 h. Cell viability was determined by the MTT assay and expressed as percentage of control E47 cells. Results are expressed as mean ± S.D. ($n = 3$). ⋆⋆, $p < 0.01$, compared with the corresponding group treated with AA or BSO alone. ##, $p < 0.01$, compared with the corresponding group treated with AA plus V-PYRRO/NO or BSO plus V-PYRRO/NO.

V-PYRRO/NO Protects E47 Cells against AA and BSO-induced Changes of Mitochondrial Membrane Potential. Damage to mitochondria is an important feature in the CYP2E1-dependent toxicity (Wu and Cederbaum, 2002). Mitochondrial membrane potential was assayed by flow cytometry after double staining with Rh123 and PI. Rh123 uptake into the mitochondria is proportional to the mitochondrial membrane potential (Lemasters and Nieminen, 1997). PI is imported into the cells and binds to cellular DNA when the integrity of the plasma membrane is lost. As shown in Fig. 12, most of the control E47 cells appear on the low PI and high Rh123 fluorescence field (lower right quadrant), indicative of viable, functional cells. However, treatment of E47 cells with 50 μM AA or 100 μM BSO for 24 h (a time point before strong toxicity) increased the population of cells (from 6.39 to 17.93 and 21.25%, respectively) in the low PI and low Rh123 fluorescence region (lower left quadrant). The population in this specific quadrant refers to cells that are still viable [i.e., PI (−)] but with damaged mitochondria, showing that AA and BSO affect the mitochondria of E47 cells before the onset of overt toxicity. One millimolar V-PYRRO/NO did not change the flow cytometry graph pattern of control E47 cells, confirming its lack of toxicity, but blocked the changes caused by AA or BSO (6.49 and 6.89% of cells in the lower left quadrant after AA or BSO treatment in the presence of V-PYRRO/NO) (Fig. 12), indicating that V-PYRRO/NO prevented the loss of membrane potential observed after AA and BSO treatment.

Discussion

V-PYRRO/NO has been suggested to be a liver-selective NO donor, presumably producing NO through metabolism by cytochrome P450 in the liver (Saavedra et al., 1997; Stinson et al., 2002). Our study demonstrated that HepG2 cells containing CYP2E1 can metabolize V-PYRRO/NO and produce NO in a dose-dependent manner. V-PYRRO/NO did not produce NO in C34 cells (HepG2 cells transfected with an empty vector). A specific NOS inhibitor, L-NMA, did not affect the CYP2E1-dependent NO production, whereas an effective CYP2E1 inhibitor, 4-methylpyrazole, inhibited NO production, indicating that V-PYRRO/NO produces NO not through

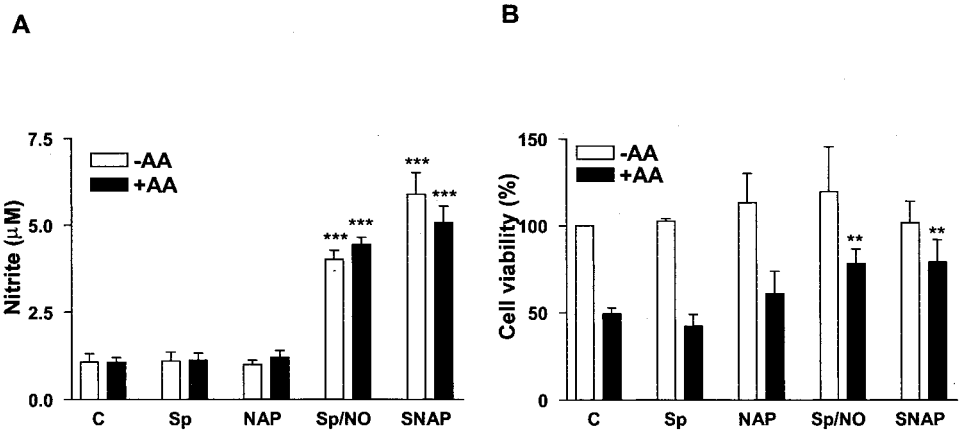

Fig. 8. SNAP and Spermine/NO protect E47 cells against AA toxicity. E47 cells were treated with 5 μM spermine (Sp) or spermine/NO or 50 μM NAP or SNAP in the absence or presence of 50 μM AA. At 8 h after treatment, the culture medium was collected and the nitrite in the medium was determined (A). Cell viability of E47 cells treated for 48 h with or without 50 μM AA was determined by MTT assay (B). Results are expressed as mean ± S.D. (n = 3). ⋆⋆, $p < 0.01$l; ⋆⋆⋆, $p < 0.001$, compared with the control E47 cells.

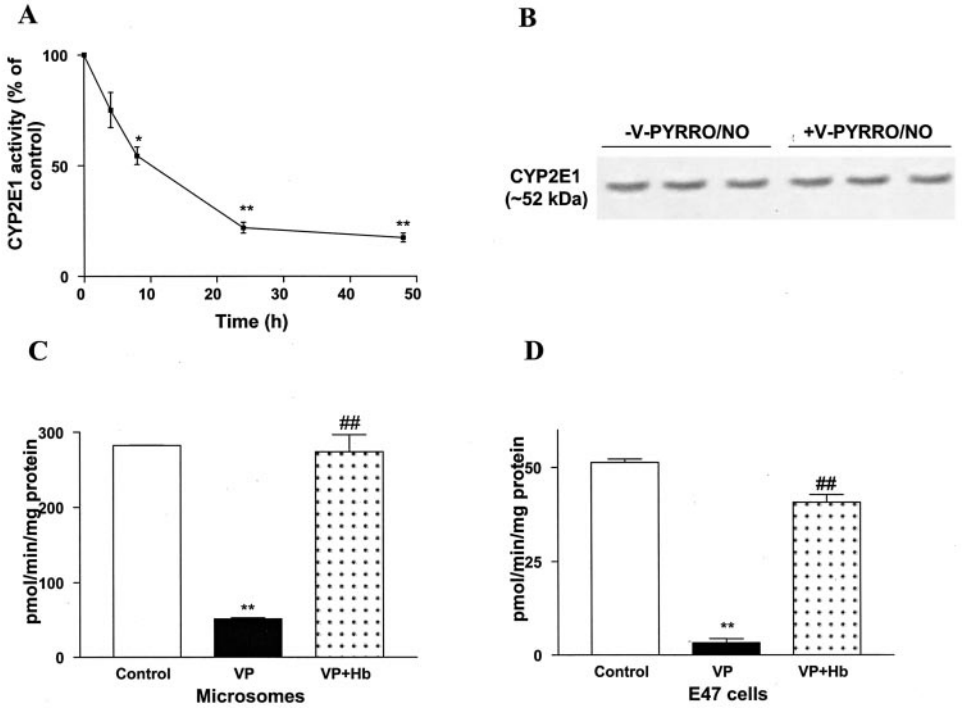

Fig. 9. V-PYRRO/NO inhibits CYP2E1 activity but does not change the CYP2E1 protein level. A, time-dependent inhibition of CYP2E1 activity by V-PYRRO/NO. E47 cells were treated with or without 1 mM V-PYRRO/NO for 0, 4, 8, 24, and 48 h in the presence of 0.4 mM PNP. CYP2E1 activity of E47 cells treated with V-PYRRO/NO was expressed as percentage of untreated cells. B, E47 cells were treated with or without 1 mM V-PYRRO/NO for 48 h. CYP2E1 protein content was detected by Western blot analysis. Microsomes prepared from the E47 cells (C) or intact E47 cells (D) were incubated with buffer (Control) or treated with 1 mM V-PYRRO/NO (VP) or 1 mM V-PYRRO/NO plus 10 μM Hb (VP + Hb) in the presence of 0.4 mM PNP for 1 h (microsomes) or 24 h (E47 cells). CYP2E1 activity was determined from the rate of oxidation of PNP (picomoles per minute per milligram of protein). Results are expressed as mean ± S.D. (n = 3). ⋆, $p < 0.05$; ⋆⋆, $p < 0.01$, compared with the control group. ##, $p < 0.01$, versus the group treated with V-PYRRO/NO alone.

stimulation of endogenous NOS, but through CYP2E1 metabolism. These results do not imply that CYP2E1 is the only or major P450 for the metabolism of V-PYRRO/NO to NO. Indeed, in collaborative studies with Dr. Larry Keefer, we found that microsomes from phenobarbital-treated rats with a high level of CYP2B1 were even more reactive than microsomes from pyrazole-treated rats with high levels of CYP2E1 in generating NO from V-PYRRO/NO. Nevertheless, CYP2E1 is a P450 that can effectively produce NO from V-PYRRO/NO. The specific mechanism at the molecular level by which CYP2E1 oxidizes V-PYRRO/NO to release NO was not investigated in this study. Scheme 2 of Saavedra et al. (1997) speculated on epoxide formation from the vinyl moiety of V-PYRRO/NO by P450 epoxidation followed by hydrolysis of the two-carbon epoxide to glycoaldehyde to produce PYRRO/NO; the latter, similar to SNAP or spermine/NO, rapidly releases NO at room temperature and at neutral pH (Saavedra et al., 1997). The results with V-PYRRO/NO differ from the results with chemical NO donors such as spermine/NO or SNAP, which generate NO, even in the absence of cells.

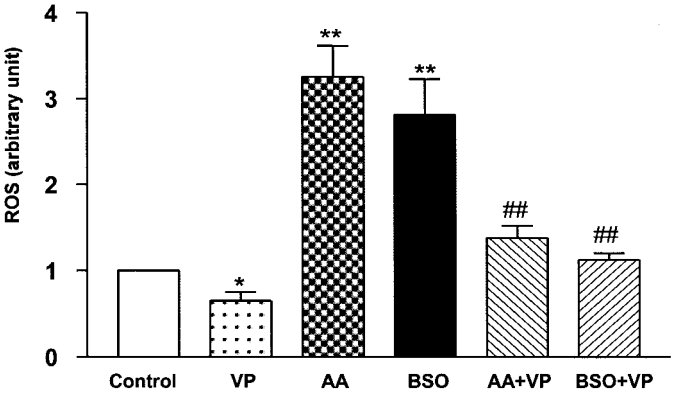

Fig. 10. V-PYRRO/NO decreases ROS production in E47 cells. E47 cells were treated with 50 μM AA, 100 μM BSO, 1 mM V-PYRRO/NO (VP), 50 μM AA plus 1 mM V-PYRRO/NO (AA+VP), 100 μM BSO plus 1 mM V-PYRRO/NO (BSO+VP), or with no treatment (Control) for 24 h. ROS levels were determined by the DCF-diacetate method and were calculated as -fold increase in fluorescence compared with that of control E47 cells. ROS levels in the control group were arbitrarily assigned a fluorescence value of 1. Results are expressed as mean ± S.D. ($n = 3$); ⋆, $p < 0.05$; ⋆⋆, $p < 0.01$, compared with the control group; ##, $p < 0.01$, compared with the corresponding group without 1 mM V-PYRRO/NO.

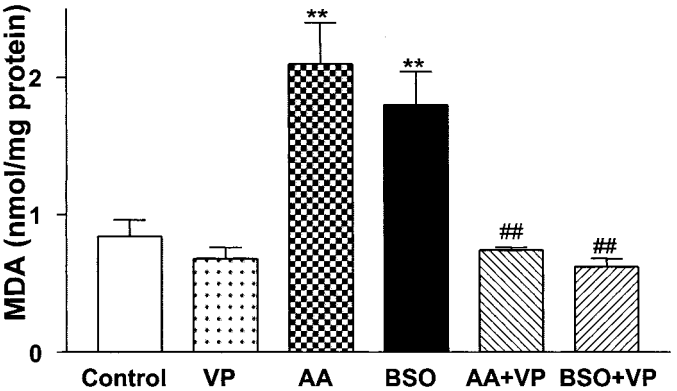

Fig. 11. V-PYRRO/NO inhibits lipid peroxidation induced by AA and BSO in E47 cells. E47 cells were treated for 48 h with 50 μM AA, 100 μM BSO, 1 mM V-PYRRO/NO (VP), 50 μM AA plus 1 mM V-PYRRO/NO (AA + VP), 100 μM BSO plus 1 mM V-PYRRO/NO (BSO + VP), or with no treatment (Control). MDA content was determined by the thiobarbituric acid reactive species method as described under *Materials and Methods*. Results are expressed as mean ± S.D. ($n = 3$). ⋆⋆, $p < 0.05$, compared with the control group; ##, $p < 0.05$, compared with the corresponding group without 1 mM V-PYRRO/NO.

V-PYRRO/NO rapidly increased NO production in E47 cells, reaching a maximum at approximately 8 h and then gradually decreasing to 2-fold of basal level at 24 h. This change may be caused by subsequent inhibition of CYP2E1 catalytic activity from NO or decreased concentration of the V-PYRRO/NO. NO produced from V-PYRRO/NO metabolism by CYP2E1 can, in turn, inhibit CYP2E1 activity. Although it seems to be a later and incomplete process, this inhibition may affect subsequent NO production and probably requires a build-up of effective levels of NO, as NO continued to be produced even after 48 h of incubation with V-PYRRO/NO. CYP2E1 activity in intact E47 cells was gradually inhibited after the addition of V-PYRRO/NO. After 24 h, CYP2E1 activity was inhibited approximately 80 to 90%, whereas the NO level in E47 cells treated with V-PYRRO/NO was still 2-fold higher than untreated control cells. This persisting elevation of NO may be important for the inhibition of CYP2E1 activity and protection from CYP2E1-depedent toxicity.

AA and BSO induced a variety of CYP2E1-dependent effects in E47 cells, such as an increase in cell cytotoxity, elevated ROS production and lipid peroxidation, and a decrease in mitochondrial membrane potential, as documented previously (Chen and Cederbaum, 1998; Chen et al., 1997; Perez and Cederbaum, 2001). V-PYRRO/NO can protect the CYP2E1-expressing cells against these toxicities, increasing the cell survival rate, decreasing the LDH release, preventing the toxic morphological changes, decreasing the level of ROS and lipid peroxidation, and preventing the decrease of mitochondrial membrane potential caused by AA and BSO. These protective effects of V-PYRRO/NO are mediated by NO produced through CYP2E1 metabolism because pyrrolidine, the parental compound of V-PYRRO/NO, which cannot release NO, showed no protection. Importantly, two NO scavengers (hemoglobin and carboxy-PTIO) decreased the protective effects of V-PYRRO/NO on the cytotoxicity caused by AA and BSO. Although NO production in E47 cells showed a V-PYRRO/NO dose-dependent increase, the protection of V-PYRRO/NO against AA and BSO-induced cytotoxicity was somewhat less effective at concentrations higher than 1 mM. The explanation for this is unclear, but it may relate to the increased production of NO at 2 mM V-PYRRO/NO, which could suggest that delivering an appropriate amount of NO is important for the NO-mediated protection. These considerations may reflect the antioxidant versus pro-oxidant actions of NO. For example, an appropriate amount of NO protects hepatocytes from oxidative toxicity; however, excess NO enhanced rather than protected the oxidative toxicity (Joshi et al., 1999).

It has been demonstrated that, when reduced by NADPH-cytochrome P450 reductase, CYP2E1 is a loosely coupled enzyme that displays high NADPH oxidase activity and is very reactive in catalysis of lipid peroxidation and production of ROS relative to other P450 isoforms (Ekstrom and Ingelman-Sundberg, 1989). Increased CYP2E1 activity is usually accompanied by increased ROS production and lipid peroxidation. CYP2E1 is also found in the mitochondria (Neve and Ingelman-Sundberg, 1999; Robin et al., 2001). Increased production of ROS and lipid peroxidation by

CYP2E1 can further damage the mitochondrial membrane (Wu and Cederbaum, 2002). Such damage can result in the release of cytochrome *c* and a decrease in ATP generation and lead to apoptosis or necrosis of cells (Liu et al., 1996; Yang et al., 1997; Kowaltowski et al., 1999). A major mechanism by which V-PYRRO/NO protects E47 cells against AA- and BSO-induced CYP2E1-dependent toxicity is probably through inhibition of CYP2E1 activity, because V-PYRRO/NO dramatically inhibited CYP2E1 activity in either the in vitro-isolated microsome preparation or the intact E47 cells. Inhibition of CYP2E1 activity can be achieved by reducing the amount of the enzyme or direct inhibition of the catalytic activity of the enzyme. Because V-PYRRO/NO did not change the protein level of CYP2E1 as shown by Western blotting, it may act through direct inhibition of the catalytic activity of CYP2E1. This inhibitory effect is very likely to be mediated by NO because the NO scavenger hemoglobin allows the recovery of the CYP2E1 activity inhibited by V-PYRRO/NO. We assume that hemoglobin traps NO diffusing out of the cells and that in its presence the NO gradient is outwardly directed, thereby lowering intracellular NO. NO has been shown to inhibit the activity of several P450s (Khatsenko et al., 1993; Wink et al., 1993; Stadler et al., 1994; Kim et al., 1995a). Gergel et al. (1997) showed that NO can inhibit CYP2E1 catalytic activity by directly binding to the heme component of the enzyme and forming a stable heme-NO complex. A decrease in ROS production and lipid peroxidation was associated with the inhibition of enzyme activity (Gergel et al., 1997).

Other mechanisms besides inhibition of CYP2E1 activity may also contribute to the protection by V-PYRRO/NO against CYP2E1-dependent toxicity. For example, NO has been reported to block apoptosis by inhibiting caspases in hepatocytes (Li and Billiar, 1999). NO can also prevent the propagation reactions of the lipid peroxidation cascade (d'Ischia et al., 2000), suppress the opening of the mitochondria permeability transition pore by depolarizing the mitochondria membrane and inhibiting Ca^{2+} fluxes (Brookes et al., 2000), and induce certain antioxidant genes such as the 70-kDa heat shock protein and hemoxygenase-1 in hepatocytes, which may protect these cells from death (Kim et al., 1995b, 1997). Because AA- or BSO-induced CYP2E1-dependent toxicity partially involves apoptosis and mitochondrial damage, these other possible mechanisms of V-PYRRO/NO protection require further study. Studies conducted by Liu et al. (2003) have shown that V-PYRRO/NO is effective in both protecting against acetaminophen-induced liver injury in vivo in mice and ameliorating the aberrant gene expression seen with hepatotoxicity. This protective effect did not seem to be caused by decreased acetaminophen bioactivation (which was indirectly assayed by measuring cellular GSH levels); rather, it seemed to involve inhibition of oxidative stress, blocking of apoptosis, and possibly the maintenance of hepatic vasculature to prevent congestion and progression of critical toxic insults.

Is the protection afforded by NO released from V-PYRRO/NO unique or more effective than protection by NO produced from chemical donors such as SNAP or spermine/NO? If the protection by NO is largely caused by inhibition of CYP2E1, it is tempting to speculate that generation of NO by CYP2E1 may indeed be more effective in inhibiting CYP2E1 in the same environment than NO generated in the medium, which would have to diffuse to the endoplasmic reticulum to react with CYP2E1, with a high probability of reacting with other enzymes or with GSH. However, spermine/NO or SNAP could also partially protect the E47 cells against AA toxicity, whereas the parent compounds spermine and NAP did not. Although in general the protection against AA toxicity by V-PYRRO/NO seems to be somewhat "better" than the protection by spermine/NO or SNAP, restoring cell viability from approximately 50% in the absence of any NO donors to approximately 90 to 95% viability (Figs. 4 and 7) (cell viability in the presence of AA plus spermine/NO of approximately 78%, cell viability in the presence of AA plus SNAP of approximately 79%), these "differences" are not sufficiently robust to claim any unique effects by the NO released from V-PYRRO/NO, and more work would be necessary to examine this. Neverthless, these results show that NO can protect against CYP2E1-dependent toxicity and suggest that a major advantage of V-PYRRO/NO is that it avoids many of the limitations encountered by previous NO donors

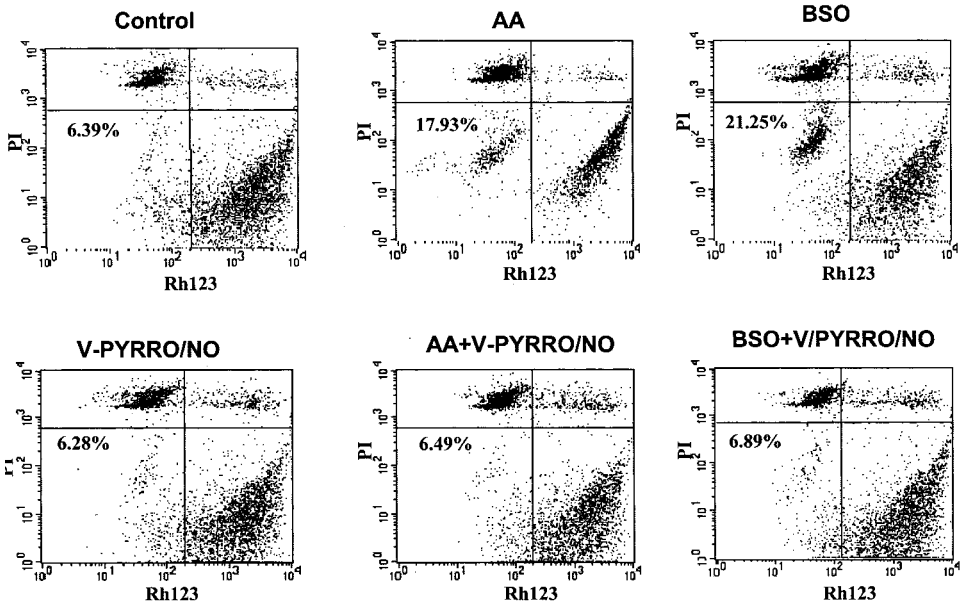

Fig. 12. Flow cytometry assay of the mitochondrial membrane potential. E47 cells were treated with 50 μM AA or 100 μM BSO with or without 1 mM V-PYRRO/NO for 24 h. Mitochondrial membrane potential was determined with Rh123 as described under *Materials and Methods*. The data presented here correspond to one representative image of each group. Control, E47 cells with no treatment; AA, E47 cells treated with 50 μM AA; BSO, E47 cells treated with 100 μM BSO; V-PYRRO/NO, E47 cells treated with 1 mM V-PYRRO/NO; V-PYRRO/NO+AA, 1 mM V-PYRRO/NO plus 50 μM AA; and V-PYRRO/NO+BSO, 1 mM V-PYRRO/NO plus 100 μM BSO.

(SNAP, diethylamine/NO, 3-morpholinosydnonimine, etc.), such as nonselectivity with respect to tissue generation of NO, spontaneous production of NO, often at very high initial rates that can cause toxicity, requiring metals for their eventual decomposition to NO, and the extracellular rather than intracellular generation of NO.

CYP2E1 metabolizes and activates many toxicologically important substrates such as ethanol, carbon tetrachloride, acetaminophen, and *N*-nitrosodimethylamine to more toxic products (Guengerich et al., 1991). Induced CYP2E1 activity and oxidative stress by ethanol may contribute to the pathogenesis of alcoholic liver disease. NO delivered by V-PYRRO/NO can inhibit CYP2E1 activity and CYP2E1-dependent toxicity and protect CYP2E1-expressing cells against toxicity by a polyunsaturated fatty acid or by GSH depletion. The latter two are believed to be important for the development of alcoholic liver injury. Interestingly, increased generation of NO via arginine infusion prevented alcoholic liver injury (Nanji et al., 2001). Liver-selective delivery of NO by V-PYRRO/NO could be a valuable approach in protecting against alcohol- and drug-induced liver damage.

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Address correspondence to: Dr. Arthur Cederbaum, Department of Biochemistry and Molecular Biology, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1020, New York, NY 10029. E-mail: arthur.cederbaum@mssm.edu