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Increased oxidative stress and cytotoxicity by hydrogen sulfide in HepG2 cells overexpressing cytochrome P450 2E1

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Abstract

The main objectives of this work were to evaluate the effects of hydrogen sulfide on oxidative stress and cytotoxicity parameters in HepG2 cells and to assess the extent to which cytochrome P450 2E1 (CYP2E1) activity modulates the effects of hydrogen sulfide on oxidative stress and cytotoxicity. Sodium hydrosulfide (NaHS) caused time- and concentration-dependent cytotoxicity in both non-P450-expressing HepG2 cells (C34 cells) and CYP2E1-overexpressing HepG2 cells (E47 cells); however, NaHS-dependent cytotoxicity was higher in E47 than C34 cells. Cytotoxicity by NaHS in C34 and E47 cells was mainly necrotic in nature and associated with an early decrease in mitochondrial membrane potential. NaHS caused increased oxidation of lipophilic (C11-BODIPY^{581/591}) and hydrophilic (DCFH-DA) probes only in E47 cells, at a time point prior to overt cytotoxicity. Trolox, an amphipathic antioxidant, partially inhibited both the cytotoxicity and the increased oxidative stress detected in E47 cells exposed to NaHS. Cell-permeable iron chelators and CYP2E1 inhibitors significantly inhibited the oxidation of C11-BODIPY^{581/591} in E47 cells in the presence of NaHS. NaHS produced lipid peroxidation and cytotoxicity in E47 cells supplemented with a representative polyunsaturated fatty acid (docosahexaenoic acid) but not in C34 cells; these effects were inhibited by α -tocopherol, a lipophilic antioxidant. These data suggest that CYP2E1 enhances H₂S-dependent cytotoxicity in HepG2 cells through the generation of iron-dependent oxidative stress and lipid peroxidation.

Keywords

CYP2E1; HepG2 cells; Hydrogen sulfide; Lipid peroxidation; Oxidative stress; Reactive oxygen species

Introduction

Hydrogen sulfide (H₂S) is not only an environmental contaminant, but it is also generated in mammalian systems exposed to H₂S donors or through enzymatic reactions involved in the metabolism of cysteine. Hydrogen sulfide exerts biological effects ranging from cytotoxic to cytoprotective (Szabo 2007).

Hydrogen sulfide is cytotoxic mainly by reversibly binding to the heme moiety of cytochrome a₃ and inhibiting cytochrome c oxidase activity, followed by depletion of ATP and bioenergetic failure (Thompson et al. 2003). However, experimental evidence suggests that additional mechanisms may contribute to H₂S cytotoxicity. For example, exposure of primary rat hepatocytes to H₂S donors including sodium hydrosulfide (NaHS) and the garlic-derived organic polysulfide diallyl disulfide induced glutathione depletion, dichlorofluorescein oxidation, and cytotoxicity, which were inhibited by antioxidants (Eghbal et al. 2004; Truong et al. 2006; Truong et al. 2009). Free radical-associated DNA damage was detected in Chinese hamster ovary cells exposed to sodium sulfide (Attene-

Ramos et al. 2007). Exposure of a human gingival epithelial cell line to H₂S in a sealed chamber increased the levels of pro-oxidant reactive oxygen species (ROS) in mitochondria (Calenic et al. 2010). These results suggest that an additional mechanism of H₂S-mediated cytotoxicity in cultured cells involves the formation of ROS and the generation of a state of oxidative stress.

In contrast, H₂S decreased oxidative stress in other cultured cells. In rat neurons, NaHS prevented oxidative glutamate toxicity by increasing glutathione levels (Kimura and Kimura 2004). NaHS inhibited peroxynitrite- or hypochlorous-induced protein oxidation and cytotoxicity in human neuroblastoma cells (Whiteman et al. 2004; Whiteman et al. 2005). The administration of NaHS protected mouse brain endothelial cells from oxidative stress and cytotoxicity induced by hyperhomocysteinemia (Tyagi et al. 2009). Exposure to NaHS reduced the levels of ROS in IEC-18 cells (a rat intestinal crypt cell line) (Deplancke and Gaskins 2003). It is evident from these observations that H₂S can promote an antioxidant effect and cytoprotection on the one hand and oxidative stress and cytotoxicity on the other, depending on multiple factors including the cell types under study and administration protocols (donor concentration and duration of exposure).

The liver is a significant target organ for H₂S: H₂S donors have been proposed as therapeutic agents to prevent hepatic ischemia/reperfusion injury (Jha et al. 2008), modulate hepatic microcirculation (Distrutti et al. 2008), and prevent acetaminophen hepatic toxicity (Morsy et al. 2010). H₂S-dependent oxidative stress and cytotoxicity in the liver may be enhanced by cytochrome P450 because nonselective chemical cytochrome P450 inhibitors decreased cell death and formation of ROS in primary rat hepatocytes exposed to NaHS (Eghbal et al. 2004). Cytochrome P450 is a significant source of ROS and oxidative stress in the liver: the NADPH-dependent reduction of O₂ by cytochrome P450 in the presence or absence of substrate to ROS including superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂) is well documented for all forms of the enzyme (Koop 1992). In particular, compared with other isoforms of cytochrome P450, cytochrome P450 2E1 (CYP2E1) shows higher NADPH oxidation rates and capacity to induce ROS and lipid peroxidation (Koop 1992). In addition, ferric chelators decreased cell death and formation of ROS in primary rat hepatocytes exposed to sodium hydrosulfide, suggesting a role for hepatic iron as a catalyst of the formation of ROS in the presence of NaHS (Truong et al. 2006).

Considering the previous information, the objectives of this work were the following: (1) to evaluate the effects of hydrogen sulfide on oxidative stress and cytotoxicity parameters in HepG2 cells (a human hepatoma cell line), (2) to assess the extent to which CYP2E1 activity modulates the effects of hydrogen sulfide on oxidative stress and cytotoxicity in HepG2 cells, and (3) to investigate the possible role of iron in the effects of hydrogen sulfide in HepG2 cells. To accomplish these objectives, HepG2 cells transduced to express human CYP2E1 (E47 cells) were exposed to a hydrogen sulfide donor (NaHS), and the effect on cell viability and oxidative stress parameters were compared with the effect in control HepG2 cells not expressing significantly any cytochrome P450 (C34 cells).

Materials and methods

Chemicals

Geneticin sulfate, SITOX® Green and 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a, 4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY^{581/591}) were from Invitrogen (Carlsbad, CA). Fetal bovine serum was from Thermo Scientific Hyclone (Logan, UT). Protein concentration was measured using the Bio-Rad DC protein assay (Hercules, CA). Western blot antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Most of the other chemicals used were from Sigma Chemical Co. (St Louis, MO).

Cell culture

Two human hepatoma HepG2 cell lines described in (Chen and Cederbaum 1998) were used as models in this study: (1) E47 cells, which constitutively express human CYP2E1, and (2) C34 cells, which are HepG2 cells transfected with the empty pCI-neo expression vector. Both cell lines were grown in Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum and 0.5 mg/mL geneticin sulfate supplemented with 100 units/mL penicillin and 100 µg/mL streptomycin in a humidified atmosphere in 5% CO₂ at 37°C. Cells were kept under continuous geneticin selection pressure to ensure maintenance of stably transfected cells expressing the neomycin phosphotransferase gene contained in the pCI-neo expression vector (Chen and Cederbaum, 1998). Cells were subcultured at a 1:10 ratio once a week.

Exposure of cells to H₂S

For the experiments, cells were plated in 25 cm² cell culture flasks at a density of 100,000 cells/mL and incubated for up to 48 h in MEM supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, and 10 mM HEPES at pH 7.4 (MEM_{exps}). Incubations were performed in the presence or absence of NaHS (up to 1.0 mM). The flasks were tightly closed using a plug-seal cap to prevent loss of gaseous hydrogen sulfide. At physiological pH (7.4) and temperature (37°C), the following equilibria are rapidly established:

with pK_a 6.8 and 19 for reactions 1 and 2, respectively (Hughes et al. 2009). Therefore, at physiological pH and temperature, about 25% of the sulfide is present as H₂S, 75% is present as HS⁻, and S²⁻ concentration is negligible. Both H₂S and HS⁻ as major species in solution may contribute to the biological actions of NaHS, and any mention of H₂S refers to H₂S and HS⁻.

Oxidative stress indices

Oxidative stress at the level of the aqueous cellular compartment was evaluated using the hydrophilic probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA permeates across the cell membrane, is de-esterified by cytosolic esterases to 2',7'-dichlorodihydrofluorescein (DCFH) which is oxidized to 2',7'-dichlorofluorescein (DCF) by ROS. Oxidation is associated with an increase in green fluorescence (Caro and Cederbaum 2002a). After the treatment, HepG2 cells were rinsed with MEM, and incubated in MEM supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin with DCFH-DA at a concentration of 5 µM for 30 min. The cells were then washed with MEM, trypsinized, resuspended in MEM, and evaluated by flow cytometry in the F11 channel (green emission at 533±30 nm, with excitation at 488 nm). A minimum of 5,000 events per sample were acquired using an Accuri C6 flow cytometer. For quantification purposes, the percentage of cells displaying high F11 fluorescence (M1%) represents the percentage of cells with high intracellular soluble ROS levels (Caro and Cederbaum 2002a; Seiler et al. 2008).

Oxidative stress at the level of the lipid membrane compartment was evaluated using the lipophilic probe C11-BODIPY^{581/591}. The probe is incorporated into membranes where it is mainly oxidized by chain-propagating species such as peroxy and alkoxyl radicals (Drummen et al. 2002). Oxidation is associated with a spectral fluorescence emission shift from red to green. After the treatment, HepG2 cells were rinsed with MEM and incubated in

MEM supplemented with 100 units/mL penicillin and 100 μ g/mL streptomycin with C11-BODIPY^{581/591} at a concentration of 5 μ M for 30 min. The cells were then washed with MEM, trypsinized, resuspended in MEM, and evaluated by flow cytometry in the FL1 channel (green emission at 533 $\pm$ 30 nm, with excitation at 488 nm). A minimum of 5,000 events per sample were acquired using an Accuri C6 flow cytometer. For quantification purposes, the percentage of cells displaying high FL1 fluorescence (M1%) represents the percentage of cells with high lipid peroxidation levels (Seiler et al. 2008; Aitken et al. 2007).

Cytotoxicity assays

Dead cells were distinguished from live cells by the uptake of propidium iodide (PI) or SITOX® Green by dead cells caused by loss of plasma membrane integrity. The incorporated probes form fluorescent adducts with DNA. After the treatments, cells were trypsinized and resuspended in MEM supplemented with 5 μ g/mL of PI or 1 μ M SITOX® Green. Cellular fluorescence was measured in the FL3 channel of fluorescence for PI (red emission at 670 $\pm$ 30 nm, with excitation at 488 nm) or in the FL1 channel of fluorescence for SITOX® Green (green emission at 533 $\pm$ 30 nm, with excitation at 488 nm). A minimum of 5,000 events per sample were acquired using an Accuri C6 flow cytometer. For quantification purposes, the percentage of cells displaying high fluorescence (M1%) with either probe represents the percentage of nonviable cells (Caro and Cederbaum 2002b).

Apoptotic cells were evaluated by annexin V-FITC labeling. Cells were incubated for 48 h in the presence or absence of 1.0 mM NaHS and after trypsinization, 10⁶ cells were resuspended in 100 μ L buffer (140 mM NaCl, 2.5 mM CaCl₂, 10 mM HEPES at pH 7.4) containing 2 μ L of annexin V-FITC. After a 5-min incubation at room temperature, cellular fluorescence was measured in the FL1 channel of fluorescence for FITC (green emission at 533 $\pm$ 30 nm, with excitation at 488 nm). A minimum of 5,000 events per sample were acquired using an Accuri C6 flow cytometer. For quantification purposes, the percentage of cells displaying high FL1 fluorescence (M1%) represents the percentage of apoptotic cells (Yang et al. 2011).

Lipid peroxidation

Endogenous lipid peroxidation was determined by the measurement of thiobarbituric acid reactive substances (TBARS). At the end of the treatment the cells were washed twice with PBS and removed by scraping in PBS plus 0.5 mM 6-hydroxy-2,5,7, 8-tetramethylchroman-2-carboxylic acid (trolox), followed by low-speed centrifugation. The cell pellets were resuspended in PBS plus 0.5 mM trolox (to prevent nonspecific lipid peroxidation during sample preparation). The protein concentration of the cell suspension was determined using a protein assay kit based on the Lowry assay (Bio-Rad DC kit). The cell suspension was mixed with twice its volume of 15% trichloroacetic acid, 0.375% thiobarbituric acid, 0.24 N HCl plus 0.5 mM trolox, and heated for 15 min at 100°C. After centrifugation, the absorbance of the supernatant was measured at 535 nm, and the concentration of TBARS calculated from a standard curve prepared using malonaldehyde bisdimethylacetal (Caro and Cederbaum 2001).

Western blots

After washing the cells with PBS, cells were incubated on ice for 15 min in extraction buffer (250 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1% IGEPAL CA-630), containing 1 mM sodium orthovanadate, 50 μ g/mL aprotinin, and 0.1 mg/mL phenylmethylsulfonyl fluoride. Lysates were centrifuged at 15,000 rpm at 4°C to remove insoluble material, and the protein concentration in whole-cell lysates was measured using the Bio-Rad DC protein assay kit. Protein samples (30 μ g) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and incubated with a primary

antibody anti-CYP2E1 (1:1,000) or anti-beta-actin (1:10,000) and a secondary antibody (Caro and Cederbaum 2006).

CYP2E1 catalytic activity

CYP2E1 activity in HepG2 cells was assessed by the rate of chlorzoxazone hydroxylation in intact cells. Cells were plated in 25 cm² cell culture flasks at a density of 100,000 cells/mL and incubated for 24 h in 5 mL of MEM_{exps} supplemented with 1 mM chlorzoxazone (from a 100-fold concentrated solution in acetonitrile). After the incubation period, the cells were scraped into the medium, and the cell suspension was extracted into 2 mL of ethyl acetate. The organic phase was evaporated under vacuum, and the residue was redissolved in mobile phase (see below). After filtration, 6-hydroxychlorzoxazone was quantified by HPLC as described by Lucas et al. (1996), using an HPLC Waters 510 pump and a Shimadzu SPD-10A UV-vis detector operating at 287 nm. A TSKgel ODS column (15 cm×4.6 mm i.d., Tosoh Corporation) was used, with a mobile phase that consisted of 75% H₂O, 25% acetonitrile, and 0.5% acetic acid delivered at a flow rate of 1.0 mL/min. Under these conditions, the retention times for 6-hydroxychlorzoxazone and chlorzoxazone were 4.7 and 19.0 min, respectively.

Mitochondrial membrane potential

The mitochondrial transmembrane potential was analyzed by the accumulation of rhodamine 123 (R123), a membrane-permeable cationic fluorescent dye. Cells were incubated for 24 h in the presence or absence of 1.0 mM NaHS. At the end of the treatment the cell medium was replaced with MEM_{exps} containing 5 µg/mL R123, and incubated at 37°C for 30 min. The cells were then harvested by trypsinization, washed with PBS, and resuspended in 1 mL of MEM_{exps}. Cellular fluorescence was measured in the FL2 channel of fluorescence for R123 (orange emission at 585±40 nm, with excitation at 488 nm). A minimum of 5,000 events per sample were acquired using an Accuri C6 flow cytometer. For quantification purposes, the percentage of cells displaying low FL2 fluorescence (M1%) represents the percentage of cells with low mitochondrial membrane potential (Caro and Cederbaum 2001).

Total glutathione content

Total glutathione was determined by the enzymatic method of Tietze (Tietze, 1969). Cells were incubated for 24 h in the presence or absence of 1.0 mM NaHS. At the end of the treatment the cells were washed twice with PBS, and detached by trypsinization in PBS, followed by low-speed centrifugation. The cell pellets were resuspended in PBS. The protein concentration of the cell suspension was determined using a protein assay kit based on the Lowry assay (Bio-Rad DC kit). The cell suspension was mixed 1:1 with 10% trichloroacetic acid to extract cellular glutathione. After centrifugation, total glutathione content was assayed by following the increase in absorbance at 412 nm for 1 min in a cuvette containing 0.1 M sodium phosphate buffer (pH 7.5), 5 µM EDTA, 0.6 mM 5,5'-dithiobis(2-nitrobenzoic acid), 0.2-mM NADPH, 1 unit/mL glutathione reductase, and 10 µL of sample in a final volume of 0.2 mL. The increment in absorbance at 412 nm was converted to glutathione concentration using a standard curve (Mari and Cederbaum 2000).

Statistics

Data are expressed as mean±S.E.M. from three to four independent experiments run in duplicate. Oneway analysis of variance with subsequent post hoc comparison by Scheffe was performed using the SigmaStat 2.0 software. A *p*<0.05 was considered as statistically significant.

Results

Increased cytotoxicity by NaHS in CYP2E1-overexpressing HepG2 cells

C34 and E47 cells were plated at a density of 100,000 cells/mL, and incubated for up to 48 h in 5 mL MEM_{exp}s supplemented with NaHS (from 0 to 1.0 mM). Cytotoxicity was evaluated by PI staining after trypsinization and expressed as percentage of cells with high FL3 fluorescence (M1%). To confirm that the protocol was competent in detecting plasma membrane permeabilization, a positive control was performed, where C34 cells were pre-incubated with digitonin, which selectively induces cell surface permeabilization (Lorenz et al. 2006). Digitonintreated C34 cells showed a significant increase in the percentage of cells with high FL3 fluorescence, confirming the effectiveness of the protocol (Fig. 1a). At early time points (i.e., before 24 h), no significant effect of 1 mM NaHS on cytotoxicity was observed in either C34 or E47 cells (Fig. 1b). At late time points (i.e., 48 h), NaHS at 1 mM increased cytotoxicity in both C34 and E47 cells with respect to untreated cells, although cytotoxicity was higher in E47 than in C34 cells ($34\pm3\%$ vs. $16\pm3\%$, respectively) (Fig. 1b). Concentration curves for NaHS over the range of 0 to 1.0 mM were performed in C34 and E47 cells incubated for 48 h. In C34 and E47 cells, cytotoxicity by NaHS was significant at concentrations higher than 0.2 mM, and increased with increasing concentrations of NaHS; however, cytotoxicity was higher in E47 cells than in C34 cells at every NaHS concentration tested (Fig. 1c). Cytotoxicity was mainly necrotic in nature, as revealed by the lack of significant annexin V labeling of cells after 48 h of incubation in the presence of 1 mM NaHS (Table 1).

Effect of NaHS on CYP2E1 content and catalytic activity in HepG2 cells

Overexpression of CYP2E1 in E47 cells was confirmed by western blot. The CYP2E1 protein band was detectable in E47 cells, but was almost undetectable in C34 cells, reflecting the lack of significant CYP2E1 expression in HepG2 cells (Fig. 2a). At concentrations of up to 1 mM, NaHS did not significantly affect the expression of CYP2E1 in E47 cells after 24 h of incubation (Fig. 2b).

Overexpression of active CYP2E1 was confirmed by a catalytic assay in situ. Significant conversion of chlorzoxazone to 6-hydroxychlorzoxazone was observed in E47 cells, but not in C34 cells, reflecting the overexpression of active CYP2E1 in E47 cells (Fig. 2c). At 1 mM, NaHS significantly decreased CYP2E1 activity in E47 cells by 27% (Fig. 2d); typical CYP2E1 inhibitors such as 4-methylpyrazole or phenylisothiocyanate (PITC) produced an inhibition of CYP2E1 activity higher than 90% (Fig. 2d). In addition, relevant iron chelators such as deferoxamine mesylate (DFO) and α,α' -dipyridyl (DIP), and antioxidants such as trolox (6-hydroxy-2,5,7, 8-tetramethylchroman-2-carboxylic acid) and α -tocopherol did not significantly affect CYP2E1 activity in E47 cells in situ (Fig. 2d).

Increased oxidative stress indices in CYP2E1-overexpressing HepG2 cells incubated with NaHS

Oxidative stress has been suggested to contribute to the mechanism of H₂S-mediated cytotoxicity in cultured cells (Attene-Ramos et al. 2007; Calenic et al. 2010; Eghbal et al. 2004; Truong et al. 2006; Truong et al. 2009). If oxidative stress causes cell death in HepG2 cells exposed to NaHS, then oxidative stress should occur prior to overt cytotoxicity. Oxidative stress in the lipid or aqueous compartments of HepG2 cells incubated for 24 h with NaHS (0–1.0 mM) was selectively determined by flow cytometry using the lipophilic probe C11-BODIPY^{581/591} or the hydrophilic probe DCFH-DA, respectively (Seiler et al. 2008; Yeum et al. 2003).

In cells incubated in the absence of NaHS, the percentage of cells with high lipid peroxidation was higher in E47 than C34 cells (5.1% vs. 2.1%, respectively) (Fig. 3a–c). These results confirm higher lipid peroxidation in CYP2E1-overexpressing HepG2 cells with respect to non-P450 expressing HepG2 cells (Chen and Cederbaum 1998). Oxidation of C11-BODIPY^{581/591} in C34 cells exposed to NaHS (0.1–1.0 mM) for 24 h did not significantly change with respect to control C34 cells not exposed to NaHS (Fig. 3a, b). Oxidation of C11-BODIPY^{581/591} in E47 cells exposed to NaHS (0.2–1.0 mM) for 24 h significantly increased in a concentration-dependent manner with respect to E47 cells not exposed to NaHS (Fig. 3a, c).

In cells incubated in the absence of NaHS, the percentage of cells with high intracellular soluble ROS levels was higher in E47 than C34 cells (48% vs. 2.0%, respectively) (Fig. 4a–c). These results confirm higher generation of ROS in CYP2E1-overexpressing HepG2 cells when compared with non-P450 expressing HepG2 cells (Mari and Cederbaum 2000). Oxidation of DCFH-DA in C34 cells exposed to NaHS (0.5–1.0 mM) for 24 h did not change significantly with respect to control C34 cells not exposed to NaHS (Fig. 4a, b). Oxidation of DCFHDA in E47 cells exposed to NaHS (0.5–1.0 mM) for 24 h significantly increased in a concentration-dependent manner with respect to E47 cells not exposed to NaHS (Fig. 4a, c).

Recently, production of ROS, depletion of cellular glutathione, and impairment of mitochondrial function have been shown to influence chemical toxicity (Chang et al. 2001). As NaHS increased the production of ROS in CYP2E1-overexpressing cells, we evaluated the possible link between NaHS and depletion of cellular glutathione and disruption of mitochondrial function assessed as mitochondrial membrane potential. NaHS did not produce significant changes in cellular glutathione levels in C34 or E47 cells (Table 1), suggesting that cytotoxicity is not associated with early changes in glutathione levels. NaHS increased the percentage of cells with low mitochondrial membrane potential to the same extent in both C34 and E47 cells (Table 1).

Effect of trolox on oxidative stress and cell toxicity by NaHS in HepG2 cell overexpressing CYP2E1

If oxidative stress increases cell death in CYP2E1-overexpressing cells exposed to NaHS, then antioxidants should decrease both oxidative stress and cytotoxicity. Trolox (an analogue of vitamin E) was used as antioxidant in this model because, owing to its amphipathic properties, it is active both in the hydrophilic and lipophilic compartments (Pulido et al. 2003). Trolox partially decreased lipid peroxidation (assessed as percentage of cells displaying high C11-BODIPY^{581/591} F11 fluorescence) in E47 cells exposed to 1.0 mM NaHS (from 35% to 21%; Fig. 5a). In contrast, the effect of trolox on the oxidation of C11-BODIPY^{581/591} in C34 cells treated with NaHS was nonsignificant (Fig. 5a). Trolox partially decreased the levels of intracellular ROS (assessed as percentage of cells displaying high DCFH-DA F11 fluorescence) in E47 cells exposed to 1.0 mM NaHS (from 64% to 42%; Fig. 5b). In contrast, the effect of trolox on the oxidation of DCFH-DA in C34 cells treated with NaHS was nonsignificant (Fig. 5b). Trolox partially decreased cytotoxicity (assessed as percentage of cells with high PI F13 fluorescence) in E47 cells exposed to 1.0 mM NaHS (from 30% to 17%; Fig. 5c). In contrast, the effect of trolox on cytotoxicity in C34 cells treated with NaHS was nonsignificant (Fig. 5c).

Increased lipid peroxidation in CYP2E1 overexpressing HepG2 cells incubated with NaHS

If NaHS increases oxidative stress in the lipophilic compartment of HepG2 cells, then NaHS should increase the oxidation of cellular polyunsaturated fatty acids, which are endogenous substrates for lipid oxidation. C34 and E47 cells were preloaded with a representative

polyunsaturated fatty acid, docosahexaenoic acid (DHA). DHA was selected because it is a dietary component and it is very susceptible to lipid peroxidation, containing six double bonds in its structure (Kikugawa et al. 2003). DHA significantly increased lipid peroxidation evaluated as TBARS in E47 cells exposed to NaHS (Table 2). Importantly, DHA did not increase lipid peroxidation in C34 cells exposed to NaHS. Lipid peroxidation induced by DHA and NaHS in E47 cells was partially inhibited by a lipid-soluble antioxidant, α -tocopherol (Table 2). DHA and NaHS induced cytotoxicity (evaluated by the uptake of SYTOX® Green) only in E47 cells, an effect that was partially blocked by α -tocopherol (Table 2).

Effect of iron chelators and CYP2E1 inhibitors on lipid oxidation by NaHS in HepG2 cells overexpressing CYP2E1

If the increased oxidative stress in the lipid compartment of E47 cells exposed to NaHS depends on redox-active free iron, then oxidation of C11-BODIPY^{581/591} should be inhibited by iron chelators that form stable redoxinactive complexes. DFO and DIP were selected as iron chelators because they are cell permeable and have been shown to inhibit iron-dependent oxidative stress in several models (Hermes-Lima et al. 1998; Huang et al. 2002). The iron chelators tested significantly decreased the oxidation of C11-BODIPY^{581/591} in E47 cells in the presence of NaHS (Table 3). The effect of iron chelators on lipid peroxidation of C34 cells in the presence of NaHS was nonsignificant (Table 3). The effect of iron chelators on CYP2E1- and H₂S-dependent cytotoxicity could not be evaluated due to significant cytotoxicity of the iron chelators themselves after 48 h of incubation (data not shown).

If the increased oxidative stress in the lipid compartment of E47 cells exposed to NaHS depends on CYP2E1 activity coupled to ROS generation, then oxidation of C11-BODIPY^{581/591} should decrease in the presence of CYP2E1 inhibitors. 4-Methylpyrazole and PITC were selected as CYP2E1 inhibitors because they have been shown to inhibit CYP2E1 activity in hepatocytes (Gong et al. 2003; Nieto et al. 2002). The CYP2E1 inhibitors tested significantly decreased the oxidation of C11-BODIPY^{581/591} in E47 cells in the presence of NaHS (Table 3). The effect of CYP2E1 inhibitors on lipid peroxidation of C34 cells in the presence of NaHS was nonsignificant (Table 3).

Discussion

The data presented in this work suggest that CYP2E1 overexpression enhances H₂S-dependent cytotoxicity in HepG2 cells through the generation of a state of oxidative stress. The evidence to support this conclusion is the following: (1) NaHS caused time- and concentration-dependent cytotoxicity in both C34 (non-cytochrome P450-expressing) and E47 (CYP2E1-overexpressing) cells; however, NaHS-dependent cytotoxicity was higher in E47 than C34 cells; (2) NaHS caused increased oxidation of lipophilic and hydrophilic probes in CYP2E1-overexpressing HepG2 cells at a time point prior to overt cytotoxicity; no significant increase in probe oxidation was observed in C34 cells exposed to NaHS; (3) trolox, an amphipathic antioxidant, partially inhibited both the cytotoxicity and the increased oxidative stress detected in CYP2E1-overexpressing HepG2 cells exposed to NaHS; no significant effect of trolox was observed in C34 cells exposed to NaHS. Cytotoxicity by NaHS in C34 cells was not associated with increased oxidation of hydrophilic or lipophilic probes, and was not significantly inhibited by trolox, suggesting that it was mediated by non-oxidative mechanisms. Cytotoxicity in C34 and E47 cells was associated with early impairment of mitochondrial function (assessed as mitochondrial membrane potential), suggesting a role for early mitochondrial dysfunction in NaHS-mediated cytotoxicity in HepG2 cells.

In this work, NaHS significantly increased cytotoxicity and oxidative stress in CYP2E1-overexpressing HepG2 cells at 0.2 mM and higher concentrations up to 1.0 mM. This concentration range is comparable to the one recently reported by other investigators to alter cellular functions in vitro. For example, NaHS increased calcium-activated potassium channel activity of rat pituitary tumor cells with a median effective concentration (EC_{50}) of 0.167 mM (Sitdikova et al. 2010). Also, NaHS induced the relaxation of mouse mesenteric artery segments with an EC_{50} of 0.12 mM (Yang et al. 2008). Recent studies suggest that whole tissue free H_2S concentrations in vivo are in the nanomolar range (Furne et al. 2008). However, exogenously applied H_2S can be stored in vivo as bound sulfur that could be released under physiological conditions (Ishigami et al. 2009). Therefore, the overall amount of hydrogen sulfide could be adequate in vivo to modulate pathophysiological reactions following exposure to H_2S donors (Muellner et al. 2009).

Hydrogen sulfide can reduce metal ions including iron according to (Tapley et al. 1999):

Both products of reaction (reduced iron and sulphhydryl radical) might interact with CYP2E1-derived reactive ROS (superoxide anion and hydrogen peroxide) to produce hydroxyl radical ($^\bullet OH$) which can initiate lipid peroxidation, according to (Tapley et al. 1999):

According with this proposed mechanism, iron chelators that promote the formation of redox-inactive complexes should inhibit the generation of hydroxyl radical and lipid peroxidation in the presence of CYP2E1-derived ROS and H_2S . The inhibition of lipid peroxidation by iron chelators and CYP2E1 inhibitors in E47 cells exposed to NaHS (but not in C34 cells) suggests that active iron redox cycling and CYP2E1 activity are required to induce lipid peroxidation in HepG2 cells exposed to H_2S , and supports the mechanism proposed. The mechanism proposed is also supported by the observation that H_2S -dependent oxidative stress and cytotoxicity in primary hepatocytes was inhibited by nonspecific cytochrome P450 inhibitors (Eghbal et al. 2004) and deferoxamine (Truong et al. 2006).

Additional mechanisms that might contribute to the pro-oxidant effect of NaHS in CYP2E1-overexpressing HepG2 cells include: (1) hydrogen sulfide inhibits catalase activity through heme binding (Nicholls 1961); lower catalase activity might exacerbate oxidative stress in CYP2E1-overexpressing cells; (2) hydrogen sulfide might increase the generation of mitochondrial ROS by blocking cytochrome *c* oxidase and keeping the respiratory chain components in its reduced state (Eghbal et al. 2004); this extra oxidative challenge might exacerbate damage in CYP2E1-overexpressing cells already under oxidative stress; (3) autoxidation of H_2S catalyzed by a metal containing enzyme such as cytochrome P450 could contribute to the generation of ROS in cytochrome P450 expressing cells (Eghbal et al. 2004). Interestingly, NaHS at 1 mM produced a low, but significant inhibition of CYP2E1 activity in E47 cells. NaHS has been shown to bind to the heme iron and inhibit the activity of heme proteins such as cytochrome *c* oxidase and catalase (Nicholls 1961; Thompson et al. 2003). ROS formation in primary hepatocytes exposed to NaHS has been associated with possible redox interactions of H_2S with the heme iron in cytochrome P450 (Eghbal et al. 2004). Therefore, partial binding of H_2S to the heme iron of CYP2E1 might contribute both

to the partial CYP2E1 inhibition and the increased generation of ROS observed in E47 cells. The combination of mechanisms by which H₂S and CYP2E1 interact to promote oxidative stress in HepG2 cells is currently under evaluation.

Oxidation of C11-BODIPY^{581/591} or DCFH-DA was used to evaluate the relative participation of lipid peroxidation or accumulation of intracellular soluble ROS, respectively, as early events or mediators of cytotoxicity (Seiler et al. 2008). H₂S significantly increased both C11-BODIPY^{581/591} and DCFH-DA oxidation in CYP2E1-overexpressing cells. However, the increase in the percentage of E47 cells with high lipid peroxidation after incubation with NaHS (from 5% in the absence of NaHS to 28% in the presence of 1 mM NaHS) was higher than the increase in the percentage of E47 cells with high intracellular soluble ROS levels (from 48% in the absence of NaHS to 62% in the presence of 1 mM NaHS). These results suggest that although both lipid peroxidation and soluble ROS increased in H₂S-treated E47 cells, lipid peroxidation might play a more prominent role in cytotoxicity caused by the combination of H₂S and CYP2E1. Endogenous lipid peroxidation was confirmed in NaHS-treated CYP2E1-overexpressing cells supplemented with a polyunsaturated fatty acid (Table 2). A lipophilic antioxidant such as α -tocopherol decreased both endogenous lipid peroxidation and cytotoxicity, further supporting the conclusion that lipid peroxidation is a critical step in NaHS- and CYP2E1-dependent cytotoxicity.

In other cellular models, H₂S has been identified as an antioxidant, decreasing the oxidation of probes or endogenous biomolecules, and promoting cytoprotection (Jha et al. 2008). The antioxidant component of H₂S has been associated with direct scavenging of reactive species (such as O₂⁻, H₂O₂, ONOO⁻, HClO⁻, lipid hydroperoxides or 4-hydroxy 2-nonenal) (Geng et al. 2004; Schreier et al. 2010; Jeney et al. 2009; Whiteman et al. 2004; Whiteman et al. 2005) and/or increased production of antioxidant defenses under chronic conditions (such as glutathione by increasing the activity of γ -glutamylcysteine synthetase) (Kimura and Kimura 2004). However, other factors might affect the antioxidant capacity of hydrogen sulfide. For example, the antioxidant activity of NaHS in vitro was substantially lowered by competing reactions of NaHS with molecular oxygen (Stasko et al. 2009). In addition, the antioxidant potential of H₂S is limited by its relatively low redox potential ($\text{HS}^- \rightarrow \text{S}^\circ + \text{H}^+ + 2\text{e}^-$, $E^\circ = 0.17$ V at pH 7.0) and lower concentration with respect to other intracellular thiols such as glutathione and cysteine (Kabil and Banerjee 2010). Therefore, the antioxidant/pro-oxidant balance of H₂S is affected by many factors that interplay in cells, including cell type, concentration, administration protocol, oxygen and iron levels, and expression of oxidative enzymes such as CYP2E1.

The results presented suggest that hydrogen sulfide donors or pathophysiological conditions where H₂S is elevated (such as sepsis (Zhang et al. 2007), acute pancreatitis (Bhatia et al. 2005), periodontal disease (Chen et al. 2010), ulcerative colitis, and colorectal cancer (Attene-Ramos et al. 2010) could impose an oxidative challenge in tissues with elevated CYP2E1. In these conditions, amphipathic antioxidants such as trolox might prove protective.

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Abbreviations

C11-BODIPY^{581/591}	4,4-Difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid
C34	HepG2 cell line derived after transfection with pCI-neo vector
CYP2E1	Cytochrome P450 2E1
DCFH-DA	2',7'-Dichlorodihydrofluorescein diacetate
DFO	Deferoxamine mesylate
DIP	α,α' -Dipyridyl
E47	HepG2 cell line derived after transfection with pCI-neo vector containing the human CYP2E1 cDNA
MEM	Minimal essential medium
MEM_{exps}	MEM supplemented 100 units/mL of penicillin, 100 μ g/mL of streptomycin, and 10 mM HEPES at pH 7.4
PITC	Phenylisothiocyanate
PI	Propidium iodide
Rhodamine 123	R123
ROS	Reactive oxygen species
TBARS	Thiobarbituric acid reactive substances
Trolox	6-Hydroxy-2,5,7, 8-tetramethylchroman-2-carboxylic acid

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Fig. 1.

Effect of NaHS on viability in HepG2 cells. **a** Positive control of plasma membrane permeabilization. A suspension of C34 cells in MEM with propidium iodide was left untreated (–digitonin), or was exposed for 2 min to 50 μ g/mL digitonin (+ digitonin), and then analyzed by flow cytometry. A representative histogram (cell count vs. FL3 fluorescence at 488–670 nm excitation–emission) is shown. M1% represents the percentage of cells with high FL3 fluorescence (i.e., nonviable cells). **b** Time course of plasma membrane permeabilization. C34 (circles) or E47 (squares) cells were incubated for up to 48 h in the absence (0 mM NaHS, white symbols) or presence (1 mM NaHS, black symbols) of 1 mM NaHS, followed by PI staining as described in “Materials and methods.” In every condition, the percentage of cells with high FL3 fluorescence (M1%) is reported. **c** Concentration curve of plasma membrane permeabilization. C34 cells (black circles) or E47 cells (black squares) were incubated for 48 h with increasing concentrations of NaHS (from 0 to 1.0 mM), followed by PI staining as described in “Materials and methods.” In every condition, the percentage of cells with high FL3 fluorescence (M1%) is reported. * $p < 0.05$, significantly different with respect to the same cell type incubated in the absence of NaHS; # $p < 0.05$, significantly different with respect to C34 cells incubated in the same conditions

Fig. 2.

Effect of NaHS on CYP2E1 expression and activity in HepG2 cells. **a** C34 or E47 cells were incubated for 24 h in MEM_{exp}. Protein extracts were collected, resolved by SDS polyacrylamide gel electrophoresis, and immunoblotted with an antibody against CYP2E1 or β actin as a loading control. **b** E47 cells were incubated for 24 h in the presence of increasing concentrations of NaHS up to 1.0 mM in MEM_{exp}. Protein extracts were collected, resolved by SDS polyacrylamide gel electrophoresis, and immunoblotted with an antibody against CYP2E1 or β actin as a loading control. **c** C34 or E47 cells were incubated for 24 h in MEM_{exp} supplemented with 1 mM chlorzoxazone. The product of CYP2E1 enzymatic catalysis, 6-hydroxychlorzoxazone, was identified according to the retention time of an authentic standard (indicated with an *arrow*). **d** E47 cells were incubated for 24 h in MEM_{exp} supplemented with 1 mM chlorzoxazone and various additions as shown in the figure. The product of CYP2E1 enzymatic catalysis, 6-hydroxychlorzoxazone, was quantified by HPLC with respect to an authentic standard; 100% enzyme activity represents a velocity of 72 μ M 6-hydroxychlorzoxazone/24 h/10⁶ cells. **p*<0.05, significantly different with respect to E47 cells incubated with no additions

Fig. 3.

Effect of NaHS on C11-BODIPY^{581/591} oxidation in HepG2 cells. **a** Concentration curve of C11-BODIPY^{581/591} oxidation. C34 (black circles) or E47 (black squares) cells were incubated for 24 h in the absence or presence of NaHS (from 0.1 to 1.0 mM), followed by C11-BODIPY^{581/591} incorporation as described in “Materials and methods.” The percentage of cells with high green FL1 fluorescence (i.e., high lipid peroxidation) is expressed as M1 (%). **b** Representative histogram (cell count vs. FL1 fluorescence at 488–533 nm excitation–emission) of C34 cells incubated in the absence (C34 0 mM NaHS, red line) or presence (C34 1 mM NaHS, black line) of 1.0 mM NaHS for 24 h, followed by C11-BODIPY^{581/591} incorporation. The percentage of cells with high green FL1 fluorescence (i.e., high lipid peroxidation) is expressed as M1 (%). **c** Representative histogram (cell count vs. FL1 fluorescence at 488–533 nm excitation–emission) of E47 cells incubated in the absence (E47 0 mM NaHS, black line) or presence (E47 1 mM NaHS, red line) of 1.0 mM NaHS for 24 h, followed by C11-BODIPY^{581/591} incorporation. The percentage of cells with high green FL1 fluorescence (i.e., high lipid peroxidation) is expressed as M1 (%). * $p < 0.05$, significantly different with respect to the same cell type incubated in the absence of NaHS; # $p < 0.05$, significantly different with respect to C34 cells incubated in the same conditions

Fig. 4.

Effect of NaHS on DCFH-DA oxidation in HepG2 cells. **a** Concentration curve of DCFH-DA oxidation. C34 (black circles) or E47 (black squares) cells were incubated for 24 h in the absence or presence of NaHS (from 0.5 to 1.0 mM), followed by DCFHDA incorporation as described in “Materials and methods.” The percentage of cells with high green FL1 fluorescence (i.e., high intracellular soluble ROS) is expressed as M1 (%). **b** Representative histogram (cell count vs. FL1 fluorescence at 488–533 nm excitation–emission) of C34 cells incubated in the absence (C34 0 mM NaHS, black line) or presence (C34 1 mM NaHS, red line) of 1.0 mM NaHS for 24 h, followed by DCFH-DA incorporation. The percentage of cells with high green FL1 fluorescence (i.e., high intracellular soluble ROS) is expressed as M1 (%). **c** Representative histogram (cell count vs. FL1 fluorescence at 488–533 nm excitation–emission) of E47 cells incubated in the absence (E47 0 mM NaHS, black line) or presence (E47 1 mM NaHS, red line) of 1.0 mM NaHS for 24 h, followed by DCFH-DA incorporation. The percentage of cells with high green FL1 fluorescence (i.e., high intracellular soluble ROS) is expressed as M1 (%). * $p < 0.05$, significantly different with respect to the same cell type incubated in the absence of NaHS. # $p < 0.05$, significantly different with respect to C34 cells incubated in the same conditions

Fig. 5.

Effect of trolox on oxidative stress and viability parameters in NaHS-treated HepG2 cells. **a** Effect of trolox on C11-BODIPY^{581/591} oxidation. C34 or E47 cells were pre-incubated for 1 h in the absence or presence of 0.2 mM trolox, followed by incubation with NaHS (0 or 1.0 mM) for 24 h. C11-BODIPY^{581/591} oxidation is expressed as the percentage of cells with high green FL1 fluorescence (*BODIPY M1 (%)*). **b** Effect of trolox on DCFH-DA oxidation. C34 or E47 cells were pre-incubated for 1 h in the absence or presence of 0.2 mM trolox, followed by incubation with NaHS (0 or 1.0 mM) for 24 h. DCFH-DA oxidation is expressed as the percentage of cells with high green FL1 fluorescence (*DCFH-DA M1 (%)*). **c** Effect of trolox on cytotoxicity evaluated as PI incorporation. C34 or E47 cells were pre-incubated for 1 h in the absence or presence of 0.2 mM trolox, followed by incubation with NaHS (0 or 1.0 mM) for 48 h. PI incorporation is expressed as the percentage of cells with high red FL3 fluorescence (*PI M1 (%)*). * $p < 0.05$, significantly different with respect to the same cell type incubated in the same conditions but in the absence of trolox

Table 1

Characterization of NaHS-dependent cytotoxicity in HepG2 cells

Cell type	NaHS (mM)	Annexin V M1 (%)	Total GSH (nmol/mg prot)	R123 M1 (%)
C34	0	4±2	78±17	5±1
C34	1	7±3	85±21	22±5 *
E47	0	7±4	97±30	7±1
E47	1	6±2	78±22	28±3 *

C34 or E47 cells were incubated in the absence or presence of 1.0 mM NaHS in MEM_{exps}, followed by the determination of apoptosis (expressed as the percentage of cells with high annexin V fluorescence (annexin V M1)), total glutathione (expressed as nmol/mg prot), and mitochondrial membrane potential (expressed as the percentage of cells with low R123 fluorescence or R123 M1), as described under "Materials and methods"

* $p < 0.05$, significantly different with respect to the same cell type incubated in the absence of NaHS

Table 2

Effect of NaHS on lipid peroxidation in HepG2 cells

Cell type	DHA (μ M)	α -Tocopherol (mM)	NaHS (mM)	TBARS (nmol/mg prot)	SYTOX Green MI (%)
C34	50	0	0	0.054 $\pm$ 0.004	3.2 $\pm$ 1.2
C34	50	0	1	0.042 $\pm$ 0.005	3.9 $\pm$ 0.9
E47	50	0	0	0.077 $\pm$ 0.011	5.1 $\pm$ 1.1
E47	50	0	1	0.175 $\pm$ 0.022 *	37 $\pm$ 3 *
E47	50	0.2	0	0.043 $\pm$ 0.010	4.2 $\pm$ 1.5
E47	50	0.2	1	0.081 $\pm$ 0.009 *#	12 $\pm$ 2 *#

C34 or E47 cells were pre-incubated for 24 h in the presence of 50 μ M docosahexaenoic acid (DHA) with or without 0.2 mM α -tocopherol, washed with PBS, and incubated with NaHS (0 or 1.0 mM) in MEMexps for 24 h. Lipid peroxidation was evaluated by the concentration of thiobarbituric reactive substances (TBARS) in whole cells, and cytotoxicity was evaluated by the incorporation of SYTOX® Green as described in "Materials and methods"

* $p < 0.05$, significantly different with respect to the same cell type incubated in the same conditions but in the absence of NaHS

$p < 0.05$, significantly different with respect to the same cell type incubated in the same conditions but in the absence of α -tocopherol

Table 3
Effect of iron chelators and CYP2E1 inhibitors on H₂S- and CYP2E1-dependent lipid peroxidation

Cell type	NaHS (mM)	Addition	BODIPY M1 (%)
C34	0	None	1.1±0.1
C34	1	None	1.2±0.1
C34	1	0.1 mM DFO	1.1±0.1
C34	1	0.1 mM DIP	0.9±0.1
C34	1	5 mM 4-methylpyrazole	1.2±0.1
C34	1	10 μM PITC	1.4±0.1
E47	0	None	2.4±0.2
E47	1	None	24±2
E47	1	0.1 mM DFO	9.5±1.3*
E47	1	0.1 mM DIP	10.1±1.2*
E47	1	5 mM 4-methylpyrazole	9.0±1.0*
E47	1	10 μM PITC	11.2±0.9*

C34 or E47 cells were pre-incubated for 1 h in the absence or presence of iron chelators (0.1 mM DFO or 0.1 mM DIP) or CYP2E1 inhibitors (5 mM 4-methylpyrazole or 10 μM PITC), followed by incubation with NaHS (0 or 1.0 mM) for 24 h. C11-BODIPY^{581/591} oxidation is expressed as the percentage of cells with high green FL1 fluorescence (BODIPY M1 (%))

* $p < 0.05$, significantly different with respect to the same cell type incubated in the same conditions but in the absence of iron chelator or CYP2E1 inhibitor