Chronic oxidative stress sensitizes hepatocytes to death from 4-hydroxynonenal by JNK/c-Jun overactivation

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Singh R, Wang Y, Schattenberg JM, Xiang Y, Czaja MJ. Chronic oxidative stress sensitizes hepatocytes to death from 4-hydroxynonenal by JNK/c-Jun overactivation. Am J Physiol Gastrointest Liver Physiol 297: G907–G917, 2009. First published September 17, 2009; doi:10.1152/ajpgi.00151.2009.—Sustained activation of the c-Jun NH2-terminal kinase (JNK) signaling pathway mediates the development and progression of experimental diet-induced nonalcoholic fatty liver disease (NAFLD). Delineating the mechanism of JNK overactivation in the setting of a fatty liver is therefore essential to understanding the pathophysiology of NAFLD. Both human and experimental NAFLD are associated with oxidative stress and resultant lipid peroxidation, which have been proposed to mediate the progression of this disease from simple steatosis to steatohepatitis. The ability of oxidents and the lipid peroxidation product 4-hydroxynonenal (HNE) to activate JNK signaling suggested that these two factors may act synergistically to trigger JNK overactivation. The effect of HNE on hepatocyte injury and JNK activation was therefore examined in cells under chronic oxidative stress from overexpression of the prooxidant enzyme cytochrome P450 2E1 (CYP2E1), which occurs in NASH. This CYP2E1-generated oxidative stress sensitized a rat hepatocyte cell line to death from normally nontoxic concentrations of HNE. CYP2E1-overexpressing cells underwent a more profound depletion of glutathione (GSH) in response to HNE secondary to decreased γ-glutamylcysteine synthetase activity. GSH depletion led to overactivation of JNK/c-Jun signaling at the level of mitogen-activated protein kinase kinase 4 that induced cell death. Oxidative stress and the lipid peroxidation product HNE cause synergistic overactivation of the JNK/c-Jun signaling pathway in hepatocytes, demonstrating that HNE may not be just a passive biomarker of hepatic oxidative stress but rather an active mediator of hepatocellular injury through effects on JNK signaling.

nonalcoholic fatty liver disease; glutathione; lipid peroxidation

THE MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) c-Jun-NH2-terminal kinase (JNK) regulates multiple cellular processes that are important in nonalcoholic fatty liver disease (NAFLD) including lipid metabolism, insulin resistance, and cell death. Recent studies have demonstrated that sustained JNK activation occurs in diet-induced models of murine steatohepatitis (33, 39). Both methionine- and choline-deficient and high-fat diet-induced steatohepatitis are associated with increased hepatic levels of phosphorylated JNK and its downstream target c-Jun (33, 39). In diet-treated jnk1 knockout mice, steatosis and liver injury were markedly decreased, demonstrating a critical function for JNK1 in the development of steatohepatitis (33, 39). An antisense oligonucleotide-induced knockdown of JNK1 in established steatohepatitis significantly decreased the degree of steatosis and liver injury, indicating that JNK1 function is also essential for the maintenance/progression of fat accumulation and hepatitis (39). Understanding the mechanism of JNK overactivation in fatty liver disease is critical to NAFLD prevention and treatment. However, the mechanism of hepatocyte JNK overactivation in the setting of steatosis is not known.

Oxidant stress has been implicated as a causal factor in NAFLD development. Both human and experimental NAFLD are associated with chronic oxidative stress and the accumulation of lipid peroxidation products including 4-hydroxynonenal (HNE) (6, 17, 21). The source of oxidative stress is controversial but may result in part from overexpression of the prooxidant enzyme cytochrome P450 2E1 (CYP2E1), which occurs in human and experimental NAFLD (7, 21, 43, 44). Reactive oxygen species (ROS) generated by CYP2E1 or other sources may mediate the progression from steatosis to hepatocyte injury by two mechanisms (11). The first is through impaired cellular function resulting from the direct oxidative modification of cellular macromolecules including lipids, proteins, and DNA. The second mechanism, which has been increasingly implicated in tissue injury, is by direct activation of cell death signaling pathways such as in the induction of hepatocyte apoptosis from menadione-generated superoxide by JNK/c-Jun activation (12). Activation of cell death signaling cascades may occur not only from ROS but also from oxidized byproducts of ROS generation. In particular, the lipid peroxidation product HNE has been shown to activate JNK (5, 31).

As a physiologically relevant model of the involvement of chronic hepatic oxidative stress in hepatocyte injury, the effects of oxidant stress generated by stable CYP2E1 overexpression have been investigated in vitro. Interestingly, CYP2E1 overexpression protected rather than sensitized hepatocytes, hepatoma cells, and NIH3T3 cells to death from acute menadione-generated oxidative stress (15, 19, 28). The explanation of this finding is that cells chronically stimulated by a potentially injurious oxidative stress develop mechanisms of resistance to ROS toxicity. However, it remains possible that injury is promoted by oxidized end products resulting from CYP2E1-dependent oxidant stress. In particular, the fact that CYP2E1 overexpression (22) and HNE (5, 31) are both known stimuli of JNK/c-Jun signaling suggested the hypothesis that ROS and HNE generated from CYP2E1 overexpression and/or other sources of oxidative stress may act in synergy to induce deleterious JNK overactivation.

To test this hypothesis, the ability of chronic oxidant stress from CYP2E1 expression to sensitize hepatocytes to death from HNE was examined. CYP2E1 overexpression sensitized cells to death from usually nontoxic concentrations of HNE. Death was mediated by sustained JNK/c-Jun activation resulting from cellular depletion of the antioxidant glutathione (GSH). Thus the increased levels of ROS and HNE that occur in the setting of a steatotic liver may synergistically activate JNK/c-Jun signaling that promotes liver injury.

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MATERIALS AND METHODS

Cells and culture conditions. Experiments were performed in the rat hepatocyte line RALA255-10G (RALA) cultured as previously described (20). This hepatocyte cell line is conditionally immortalized rat hepatocyte line RALA255-10G (RALA) cultured as previously discarded, and 1.5 ml of volume of a 1 mg/ml MTT solution, pH 7.4, in DMEM was added to after treatment, the cell culture medium was aspirated, and an equal subtracting that number from 100.

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ROS assay. ROS levels were measured by means of the 2',7'- dichlorodihydrofluorescin diacetate (DCFH-DA) assay. Cells were treated with DCFH-DA (6.25 μg/ml) alone, or in combination with 85 μM HNE or 3.5 mM H2O2 (Sigma). After 2 h, the cells were washed and lysed in 0.1% Triton X-100, and the fluorescent intensity of the lysate was determined at excitation and emission wavelengths of 492 and 520 nm, respectively. Background fluorescence was determined from vehicle-treated cells and subtracted from the sample values. Values were expressed as relative fluorescent intensity compared with untreated VEC cells.

HNE-adduct ELISA. Levels of HNE-histidine protein adducts were measured by ELISA using a commercial kit (Cell Biolaboratories, San Diego, CA).

GST activity. GST S-transferase (GST) activity was determined as previously described (30). Cell protein was isolated in 1% Triton X-100, and protein concentration was determined using the Bio-Rad (Hercules, CA) protein assay according to the manufacturer’s instructions. The reaction mixture contained 50 μg of protein, 1 mM GSH, and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB). The change in absorbance at 340 nm, indicating formation of the glutathione-CDNB (GS-CDNB) conjugate, was monitored spectrophotometrically. GST activity was expressed as nanomoles of GS-CDNB formed per minute per milligram of protein (ε = 9,600 M⁻¹ cm⁻¹).

GSH assay. Cellular total GSH content was determined by the 5,5'-dithiobis(2-nitrobenzoic acid)-GSH disulfide recycling assay (1), as previously described (46). Protein concentrations were determined on the same lysates, and GSH levels were calculated as nanomoles per milligram of protein.

GCS activity. γ-Glutamylcysteine synthetase (GCS) activity was determined by a spectrophotometric assay, as previously described (37). The assay employed a reaction mixture of Tris·HCl (100 mM, pH 7.5, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, and protease and phosphatase inhibitors, as described previously (42). The cells were then sonicated, and the lysate was used for Western blotting after determination of the protein concentration.

Western blotting was performed by denaturing 50 μg of protein at 100°C for 5 min in Laemmli sample buffer containing 62.5 mM Tris, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, and 5% β-mercaptoethanol. Samples were applied to 12% SDS-polyacrylamide gels and resolved at 100 V over 3 h. Proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell Bioscience, Keene, NH) in transfer buffer containing 25 mM Tris, pH 8.3, 192 mM glycine, 0.01% SDS, and 15% methanol using a Bio-Rad Transblot SD semidry transfer cell to which 150 mA were applied for 90 min. Membranes were blocked in 5% nonfat dry milk, 20 mM Tris, pH 7.5, 500 mM sodium chloride, and 0.5% Tween 20 (TBS-T) for 1 h. Membranes were exposed to antibodies that recognized phosphorylated and total JNK1 and JNK2, phosphorylated and total c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated and total extracellular signal-regulated kinase (ERK) 1/2, phosphorylated MAPK kinase 4 (MKK4) (Cell Signaling, Beverly, MA), and protein disulfide isomerase (kind gift of Richard Stochkert, Albert Einstein College of Medicine, Bronx, NY). These primary antibodies were used at 1:1,000 to 1:2,000 dilutions in 5% bovine serum albumin or nonfat milk for 18 h at 4°C. Membranes were exposed to anti-rabbit and anti-mouse secondary antibodies conjugated with horseradish peroxidase (KPL, Gaithersburg, MD) at a dilution of 1:10,000 in 5% nonfat milk TBS-T for 1 h at room temperature. Signals were detected with a chemiluminescence detection system (Western Lightning Chemiluminescence Plus, PerkinElmer Life Sciences, Boston, MA) and exposure to X-ray film.

JNK assay. JNK activity was measured in cell lysates using a stress-activated protein kinase/JNK assay kit (Cell Signaling), according to the manufacturer’s instructions. An NH2-terminal c-Jun-(1-89) fusion protein bound to GSH-Sepharose beads was used to immobilize JNK from cell lysates that contained 250 μg of total protein. After washing, the kinase reaction was performed in the presence of cold ATP using the c-Jun fusion protein as a substrate. Samples were resolved on 10% SDS-polyacrylamide gels, and the amount of phosphorylated c-Jun was detected with an antibody specific for c-Jun phosphorylated at serine 63. As a control for the loading of equivalent
amounts of protein among samples, total c-Jun levels were analyzed by immunoblotting with a phosphorylation-independent c-Jun antibody (Santa Cruz Biotechnology). Proteins were visualized using a secondary antibody and chemiluminescence substrate as described above.

**Luciferase assay.** RALA hepatocytes were cultured as previously described and transiently transfected with reporter genes using Lipofectamine Plus (Invitrogen) 18 h before HNE treatments. Cells were transfected with the activator protein-1 (AP-1)-driven firefly luciferase reporter gene 2XtTRELuc (14) and the constitutive Renilla luciferase vector pRL-TK (Promega, Madison, WI). Luciferase activities were assayed as previously described (24), and firefly luciferase activity was normalized to Renilla luciferase activity.

**Adenovirus preparation and infection.** To inhibit c-Jun function, cells were infected with the adenovirus Ad5TAM that expresses TAM-67, a dominant negative c-Jun (4). Ad5LacZ, which expresses the *Escherichia coli* β-galactosidase gene (16), served as a control for the nonspecific effects of adenoviral infection. Viruses were amplified in 293 cells, purified by banding twice on CsCl gradients as previously described (45), and titered by plaque assay. Infections were performed as previously described (45), at an multiplicity of infection of 20.

**Dephosphorylation assay.** The rate of JNK and c-Jun dephosphorylation following exposure of hepatocytes to heat shock was examined by a method adapted from Yaglom et al. (47). To activate JNK/c-Jun, heat shock was performed by putting the cells in 42°C medium and a 42°C incubator for 15 min. After heat shock, cells were treated with 10 mM 2-deoxyglucose and 5 μM rotenone to inhibit further protein phosphorylation. At different times, the cells were harvested on ice, washed twice with cold phosphate-buffered saline, supplemented with 1 mM sodium orthovanadate, and lysed in buffer containing 1% Triton X-100, 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 2 mM sodium vanadate, 1 μg/ml leupeptin, 2 μg/ml pepstatin, and 1 mM PMSF. The lysates were subjected to Western blotting as described above and immunoblotted with JNK and c-Jun antibodies.

**Statistical analysis.** All numerical results are reported as mean ± SEM and represent data from a minimum of three independent experiments. Groups were compared by the Student’s t-test. Statistical significance was defined as *P* < 0.05. Calculations were made with Sigma Plot (Jandel Scientific, San Rafael, CA).

**RESULTS**

**CYP2E1 overexpression sensitizes to death from HNE.** To determine whether chronic oxidative stress may synergize with lipid peroxides to induce hepatic injury, sensitivity to HNE-induced cell death was examined in a hepatocellular model of chronic oxidative stress induced by overexpression of the enzyme CYP2E1. Studies were performed in a polyclonal RALA hepatocyte cell line stably transfected with empty vector (VEC) cells that lacks CYP2E1 activity and in a clone with physiological overexpression of CYP2E1 following stable transfection with a CYP2E1 expression vector (S-CYP15 cells) (19, 34). VEC cells were resistant to HNE toxicity as reflected in their low levels of cell death (<10%) at HNE concentrations up to 100 μM as determined by 24-h MTT assay (Fig. 1A). In contrast, CYP2E1-overexpressing S-CYP15 cells were sensitized to death from HNE with levels of concentration-dependent cell death ranging from 34–100% with treatment with 75–100 μM HNE (Fig. 1A).

S-CYP15 cell sensitization to HNE toxicity was confirmed by fluorescence microscopic examination of acridine orange/ethidium bromide costained cells for the steady-state levels of apoptosis and necrosis. Untreated S-CYP15 cells had slight increases in the numbers of apoptotic and necrotic cells compared with VEC cells, as previously reported (19). At 8 and 12 h after treatment with HNE, steady-state levels of apoptotic and necrotic cells were increased significantly in S-CYP15 cells compared with VEC cells (Fig. 1B). The amount of necrosis was greater than the degree of apoptosis, but the numbers of necrotic cells may have been inflated by the inclusion of apoptotic cells undergoing secondary necrosis.

To insure that the sensitivity of S-CYP15 cells to HNE-mediated cell death was secondary to CYP2E1 overexpression and not nonspecific clonal variation, the extent of death following HNE treatment was examined in two additional S-CYP clones. The clone S-CYP29 has increased CYP2E1 protein levels and enzymatic activity similar to S-CYP15 cells, whereas S-CYP43 cells do not overexpress CYP2E1 despite...
transfection with the CYP2E1 expression vector and antibiotic selection (35). Similar to the findings in S-CYP15 cells, S-CYP29 cells were sensitized to death from HNE as reflected in their nearly 100% cell death from 80 and 85 μM HNE concentrations (Fig. 1C). In contrast, the S-CYP43 clone, which fails to overexpress CYP2E1, was resistant to HNE toxicity similar to the findings in VEC cells (Fig. 1C). Thus sensitization to HNE toxicity was the result of cellular effects of CYP2E1 overexpression.

S-CYP cells have constitutively increased ROS production but normal HNE levels. CYP2E1 overexpression generates ROS, which could result in the formation of lipid peroxidation products such as HNE if ROS levels exceed the antioxidant capacity of the hepatocyte. The increased HNE toxicity in S-CYP cells may result therefore from a greater concentration of HNE in these cells that is the sum of both endogenous and exogenous sources. As expected, untreated S-CYP15 cells had increased ROS levels by the DCFH-DA assay compared with VEC cells (Fig. 2A). HNE treatment increased ROS production in both cell types, but levels remained significantly greater in S-CYP cells (Fig. 2A). In contrast, the amount of ROS generated by treatment with the oxidant H2O2 was equivalent in the two cell types. Despite the constitutive increase in ROS in S-CYP cells, VEC and S-CYP15 cells had equivalent low levels of HNE as determined by the quantity of HNE-protein adducts (Fig. 2B). In response to HNE treatment, no increase in adducts occurred in VEC cells, but levels were markedly increased in S-CYP15 cells (Fig. 2B), suggesting a defect in HNE metabolism in these cells. Thus sensitization of S-CYP cells to death from HNE did not result simply from higher concentrations of HNE occurring from the combination of CYP2E1-generated endogenous production and exogenous supplementation.

S-CYP cell sensitivity to HNE toxicity is not secondary to decreased GST activity. A possible mechanism of S-CYP cell sensitization to HNE toxicity could be the failure of S-CYP cells to metabolize and thereby eliminate this toxic hydroxyaldehyde. The relative increase in levels of HNE-protein adducts in S-CYP cells with HNE treatment was also suggestive of this possibility. In hepatocytes, the major pathway of HNE metabolism is through GST-mediated conjugation to GSH to form an HNE-GSH conjugate that then effluxes from the cell (13). To determine whether S-CYP cells had impaired GST function, GST activity was measured in untreated and HNE-treated VEC and S-CYP15 cells. Levels of GST activity were equivalent in the two cell types untreated and at various times after HNE treatment (Fig. 3A), demonstrating that S-CYP cell sensitization to HNE toxicity was not the result of decreased GST activity.

HNE toxicity results from GSH depletion in CYP2E1-overexpressing RALA hepatocytes. Previous studies have shown that, in contrast to the ability of wild-type or VEC hepatocytes to survive a profound decrease in levels of the cellular antioxidant GSH, S-CYP cells undergo cell death in response to chemical GSH depletion by DEM (19). Upon exposure to the death receptor ligand TNF, only hepatocytes with CYP2E1 overexpression undergo GSH depletion and resultant cell death (22). Thus CYP2E1 overexpression makes hepatocytes susceptible to GSH depletion and resultant cell death, suggesting that the mechanism of HNE toxicity in S-CYP cells may be from GSH depletion resulting from GSH consumption for HNE conjugation. To examine for this possibility, GSH content was measured in VEC and S-CYP15 cells at different times after 80 μM HNE treatment. As previously reported (19), basal GSH levels were equivalent in the two cell types (Fig. 3B). GSH content decreased significantly within 15 min of HNE treatment in both VEC and S-CYP15 cells, consistent with the known rapid conjugation of HNE with GSH (13). However, at 0.5 h after HNE treatment, GSH levels were decreased 86% in S-CYP15 cells compared with only 57% in VEC cells (Fig. 3B). GSH levels reached their nadir in both cell types at 1 h after HNE treatment at which point levels in S-CYP15 cells were fivefold lower than in VEC cells (Fig. 3B). In addition, S-CYP cells experienced a lag in the recovery of their GSH content back to normal levels. GSH levels normalized in VEC cells within 4 h, whereas levels in S-CYP15 cells were still markedly depressed at 8 h (Fig. 3B). GSH levels did return to normal in S-CYP15 cells surviving at 24 h (Fig. 3B).

These data suggested that CYP2E1 overexpression may sensitize hepatocytes to death from HNE by promoting a fatal depletion of GSH. To determine the functional significance of GSH depletion in S-CYP cell death from HNE, the effect of GSH levels on cell death was examined. Chemical GSH depletion of VEC cells by DEM, which was nontoxic by itself, sensitized VEC cells to HNE toxicity (Fig. 3C). Conversely, pretreatment with 2 mM GSH ethyl ester, a membrane-permeable form of GSH (1), completely inhibited S-CYP15 cell death.

Fig. 2. S-CYP cells have constitutively increased reactive oxygen species production but normal levels of HNE. A: relative fluorescent intensity in untreated VEC and S-CYP15 (S-CYP) control cells (Con) and cells treated for 2 h with 85 μM HNE or 3.5 mM H2O2 (*P < 0.02; #P < 0.0001 compared with VEC cells with the same treatment). B: levels of HNE-histidine adducts in VEC and S-CYP cells untreated or treated for the indicated number of hours with 85 μM HNE (*P < 0.02 compared with VEC cells with the same treatment). Results are means ± SE from 3 independent experiments each with duplicate data points.
pressed cells were that consumption of GSH was greater in S-CYP cells or that S-CYP cells had a decreased capacity for GSH synthesis that delayed normalization of their GSH content. To distinguish between these two possibilities, the recovery of GSH levels after chemically induced depletion from DEM was compared in VEC and S-CYP15 cells. DEM induced an equally profound depletion of GSH in VEC and S-CYP15 cells within 2 h (Fig. 4A). However, GSH content normalized at different rates in the two cell types. Within 12 h of DEM administration VEC cell GSH content had almost returned to baseline, whereas levels were still markedly depressed in S-CYP15 cells (Fig. 4A). These data suggested that S-CYP cells have an inherent problem in their GSH synthetic capacity that limits their ability to normalize GSH content after chemical- or oxidant-induced depletion.

To examine whether S-CYP cells have an insufficiency in GSH synthesis, activity levels of GCS, the rate-limiting enzyme in GSH synthesis (26), were assayed in the two cell types. HNE has been reported to upregulate GSH synthesis in nonhepatic cells by inducing GCS expression (25). GCS activity was unchanged in VEC cells following HNE treatment (Fig. 4B). Activity was modestly but significantly reduced in untreated S-CYP15 cells compared with VEC cells (Fig. 4B). S-CYP15 cell GCS activity further decreased with HNE treat-

Fig. 3. S-CYP cells undergo marked glutathione (GSH) depletion with HNE treatment. GSH S-transferase activity (A) and cellular GSH content (B) were measured as described in MATERIALS AND METHODS in VEC and S-CYP15 (S-CYP) cells untreated and treated with 80 μM HNE for the indicated times shown. Data are from 3 independent experiments performed in duplicate (*P < 0.04; #P < 0.0001 compared with VEC cells with the same treatment). C: percentage cell death in VEC cells treated with 80 μM HNE, diethyl maleate (DEM), or a combination of the two and in S-CYP15 cells treated with 80 μM HNE, 2 mM GSH ethyl ester, or both agents together. Results are means ± SE from 3 independent experiments (*P < 0.03; #P < 0.0002 compared with cells treated with HNE alone).

Fig. 4. S-CYP cells undergo increased GSH depletion from DEM and have decreased γ-glutamylcysteine synthetase (GCS) activity. A: GSH levels were determined in VEC and S-CYP15 (S-CYP) cells untreated and treated with DEM for the number of hours shown. Results are means ± SE from 3 independent experiments (*P < 0.01 compared with VEC cells at the same time point). B: GCS activity in VEC and S-CYP15 cells untreated and treated for the indicated number of hours with 80 μM HNE. The data are means ± SE from 4 independent experiments (*P < 0.003; #P < 0.001 compared with VEC cells at the same time point).
ment to levels that were only 50% of those in VEC cells at 2 and 4 h (Fig. 4B).

**HNE-induced death in S-CYP-overexpressing cells is associated with JNK/c-Jun overactivation.** A possible mechanism for S-CYP cell death from HNE-induced GSH depletion could be that, in the absence of sufficient levels of the antioxidant GSH, CYP2E1-generated ROS have direct, injurious biochemical effects on cellular macromolecules. Alternatively, ROS or HNE itself may affect cell signaling pathways that regulate hepatocyte death and survival. Previous studies have demonstrated that CYP2E1 overexpression promotes a proapoptotic overactivation of the MAPK JNK in response to TNF (22). Activation of the JNK/c-Jun pathway in VEC and S-CYP15 cells by HNE treatment was therefore assessed by immunoblotting for levels of phosphorylated JNK and its downstream substrate c-Jun. In VEC cells treated with 80 μM HNE, JNK activation was barely detectable, and a transient and low-level activation of c-Jun occurred at 1–2 h as reflected in increased levels of the phosphorylated forms of JNK and c-Jun (Fig. 5A). In contrast, marked increases in phospho-JNK and phospho-c-Jun occurred in HNE-treated S-CYP15 cells at 1 h and persisted for greater than 12 h (Fig. 5A and data not shown). The effect of HNE on a second MAPK, ERK1/2, was also examined. HNE treatment induced a sustained overactivation of ERK1/2 in S-CYP15 but not VEC cells (Fig. 5A). No changes were observed in the levels of total JNK, c-Jun, or ERK1/2 in the two cell types with HNE treatment (Fig. 5A). HNE treatment in the setting of CYP2E1 overexpression therefore led to sustained activation of the JNK and ERK1/2 MAPK signaling pathways.

To further confirm the presence of JNK/c-Jun overactivation in S-CYP15 cells, the effects of HNE treatment on JNK kinase activity and levels of c-Jun-dependent, AP-1-driven transcription were examined. JNK activity was measured by an in vitro kinase assay with c-Jun as substrate. HNE treatment induced a twofold increase in JNK activity in VEC cells at 1 h, but activity returned to baseline within 2 h (Fig. 5B). In CYP2E1-overexpressing S-CYP15 cells, HNE treatment led to a sixfold increase in JNK activity at 1 h that persisted for greater than 4 h (Fig. 5B). JNK phosphorylation of c-Jun increases its ability to transcriptionally activate the AP-1 promoter. Effects of HNE on AP-1 activity were determined in VEC and S-CYP15 cells transiently transfected with the AP-1-driven reporter 2XTRILuc. S-CYP15 cells had levels of AP-1 transcriptional activity that were increased significantly threefold over VEC cells after HNE treatment (Fig. 5C). In contrast, consistent with the minimal JNK/c-Jun activation that occurred in VEC cells, AP-1 activity was unaffected by HNE treatment in these cells (Fig. 5C). Therefore, CYP2E1 overexpression led to elevated AP-1 transcriptional activation that was further amplified by HNE treatment.

To confirm that JNK overactivation resulted from CYP2E1 overexpression and not nonspecific clonal variation, immunoblot analysis for phosphorylated JNK, c-Jun, and ERK1/2 was performed in two additional HNE-treated S-CYP clones. HNE induced JNK, c-Jun, and ERK1/2 overactivation in the CYP2E1-overexpressing clone S-CYP29 similar to S-CYP15 cells (Fig. 5D). As expected, JNK, c-Jun, and ERK1/2 overactivation did not occur in the S-CYP43 clone, which, despite transfection, fails to overexpress CYP2E1 (Fig. 5D). No changes were observed among cell types in the levels of total JNK, c-Jun, or ERK1/2.
Inhibition of JNK/c-Jun signaling blocks HNE-mediated death in CYP2E1-overexpressing cells. Prolonged activation of the JNK/c-Jun signaling pathway triggers cell death in hepatocytes (12, 23, 27, 36). To determine whether JNK/c-Jun function sensitizes S-CYP cells to death from HNE, the ability of JNK/c-Jun inhibition to block HNE-induced cell death was examined. JNK/c-Jun function was blocked by infecting cells with the adenovirus AdSTAM that expresses TAM67, a dominant negative form of c-Jun lacking the transactivation domain (4). AdSTAM effectively blocks c-Jun function in RALA hepatocytes as assessed previously by the effects of AdSTAM on AP-1 transcriptional activity (23). S-CYP cells infected with the control adenovirus Ad5LacZ, that expresses the β-galactosidase gene or Ad5STAM were treated with 80 or 85 μM HNE, and cell death was determined by MTT assay. Inhibition of c-Jun function decreased S-CYP15 cell death from 80 and 85 μM HNE by 75% and 64%, respectively (Fig. 6A). In contrast, cell death was unaffected by pharmacological inhibition of ERK1/2 function with PD98059 (data not shown).

JNK/c-Jun overactivation results from GSH depletion. The mechanistic involvement of both GSH depletion and prolonged JNK/c-Jun signaling in S-CYP cell death from HNE suggested that GSH depletion might be the upstream event mediating JNK/c-Jun overactivation. Consistent with this possibility was the fact that the greater depletion of GSH in S-CYP cells after DEM treatment (Fig. 4A) was associated with increased MAPK activation as reflected in increased levels of phosphorylated JNK, c-Jun, and ERK1/2 (Fig. 6B). To specifically examine the role of GSH depletion in HNE-induced JNK/c-Jun activation, the effects of GSH ethyl ester supplementation on levels of HNE-induced JNK and c-Jun phosphorylation were determined. HNE-induced phosphorylation of JNK/c-Jun was completely blocked by GSH supplementation (Fig. 6C), demonstrating that JNK/c-Jun overactivation resulted from GSH depletion. ERK1/2 MAPK activation was similarly inhibited by GSH (Fig. 6C). To confirm that JNK/c-Jun activation was downstream of GSH depletion, the effect of c-Jun inhibition on HNE-induced GSH depletion was determined. Inhibition of c-Jun signaling by AdSTAM infection failed to block HNE-induced GSH depletion (Fig. 6D). These data demonstrate that JNK/c-Jun overactivation is the downstream effect of GSH depletion, as GSH supplementation blocked JNK/c-Jun activation and death, whereas AdSTAM inhibited cell death without affecting GSH levels.

Oxidative stress mediates S-CYP cell death from HNE. To demonstrate that oxidant stress resulting from CYP2E1 overexpression was the mechanism of cell sensitivity to death from HNE, the effect of the antioxidant catalase on death from HNE was examined. Catalase treatment significantly decreased the degree of GSH depletion in HNE-treated S-CYP cells (Fig. 7A). This inhibition of GSH depletion resulted in decreased HNE-induced JNK activation as determined by levels of phosphorylated JNK and c-Jun (Fig. 7B). As a result, catalase treatment blocked cell death from HNE (Fig. 7C). The increased produc-
tion of ROS by CYP2E1 overexpression therefore mediated cell death from HNE.

**Increased phospho-JNK/c-Jun levels in HNE-treated S-CYP cells are secondary to increased MKK4 activation.** Steady-state levels of phosphorylated JNK and c-Jun induced by any stimulus including HNE reflect the balance between the rates of phosphorylation by upstream kinases and dephosphorylation by phosphatases. In addition, HNE has been demonstrated to directly bind and activate JNK in hepatic stellate cells (31). Immunoprecipitation of HNE-treated S-CYP15 cells with an anti-JNK or anti-HNE antibody followed by immunoblotting with the other antibody failed to detect JNK-bound HNE (data not shown). This finding suggested that JNK/c-Jun activation was the result of altered kinase and/or phosphatase activity and not a direct effect of HNE on JNK.

HNE-treated S-CYP15 cells were then examined for a differential upregulation of upstream kinase MKK4 of JNK. HNE treatment of VEC cells failed to cause any significant increase in levels of active, phosphorylated MKK4 as detected by immunoblotting (Fig. 5A). In contrast, a sustained increase in MKK4 activation occurred in HNE-treated S-CYP15 cells (Fig. 5A). This increase in MKK4 activation was the result of GSH depletion, as MKK4 phosphorylation was blocked by GSH supplementation (Fig. 6C).

To assess whether an inherent decrease in JNK phosphatase activity also contributed to JNK overactivation in S-CYP cells, rates of JNK dephosphorylation were compared in the two cell types. The marked difference in levels of JNK phosphorylation in VEC and S-CYP cells in response to HNE made this stimulus unsuitable for comparative studies of dephosphorylation rates; therefore, heat shock was used to activate JNK. The amount of JNK phosphorylation induced by heat shock was equivalent in the two cell types (Fig. 8A). The rate of JNK dephosphorylation after heat shock was slightly greater in S-CYP15 cells than VEC cells, but the differences were not significant (Fig. 8, A and B). Overactivation of JNK in response to HNE therefore did not result from HNE binding to JNK or a deficiency in phosphatase activity but rather from increased activation of the upstream kinase MKK4.

**S-CYP cells are not sensitized to death from a lipid peroxidation product that fails to activate JNK/c-Jun.** Finally, to determine the specificity of HNE toxicity in CYP2E1-overexpressing cells, the resistance of these cells to a second lipid peroxidation product, MDA, was investigated. In contrast to the findings with HNE, VEC and S-CYP15 cells were both resistant to high concentrations of MDA. At low levels of MDA, S-CYP cells were in fact more resistant to toxicity, and
at higher concentrations, low levels of cell death were equivalent inVEC and S-CYP cells (Fig. 9A). Consistent with its relative lack of toxicity, MDAtreatment failed to induce JNK or c-Jun activation in either VEC or S-CYP15 cells (Fig. 9B). Thus CYP2E1-overexpressing cells were specifically sensitized to death from the lipid peroxide HNE.

**DISCUSSION**

Overactivation of the MAPK JNK has been identified as a central mechanism in the development and progression of murine steatohepatitis (33, 39). However, the factors that trigger sustained JNK activation in these models are unknown. Both experimental and human NAFLD are associated with oxidant stress and increased levels of HNE generated by ROS-induced lipid peroxidation (6, 17, 21). The lipid peroxidation products produced in this disease are considered passive markers of the effects of oxidative stress. However, similar to ROS, lipid peroxidation products may affect cell signaling pathways (5, 31) and thereby actively alter cellular responses to injury. This study specifically examined whether HNE promoted hepatocyte death pathway signaling in the setting of chronic oxidative stress. Overexpression of CYP2E1 was employed as a model of chronic oxidative stress because expression of this enzyme is increased in both nonalcoholic and alcoholic fatty liver disease (43, 44). Findings of increased CYP2E1 expression in concert with oxidant stress have suggested a prooxidant etiological role for this enzyme in the development of steatohepatitis; however, the function of CYP2E1 in these diseases remains controversial. Nonetheless, CYP2E1 expression serves as a physiological model for the effects of chronic oxidant stress on hepatocytes. Although CYP2E1 overexpression is protective against an acute exogenous oxidant stress (15, 19, 28), this chronic oxidative stress sensitizes hepatocytes to death from other factors (22, 34). The known induction of the proapoptotic JNK/c-Jun pathway by oxidants and HNE suggested that the synergistic effects of these two factors may be a mechanism of JNK/c-Jun overactivation and hepatocyte death.

RALA hepatocytes transfected with vector alone were resistant to high concentrations of HNE, consistent with prior findings in primary hepatocytes (38). CYP2E1 overexpression sensitized these cells to toxicity from HNE concentrations of 75 μM or greater. Death did not occur from an increased baseline concentration of HNE from CYP2E1 overexpression, as untreated S-CYP15 cells had normal HNE levels despite elevated ROS production. Mean steady-state whole liver HNE levels in response to oxidative stress are ~10 μM (32, 41), and localized cellular concentrations may reach as high as 5 mM (2, 18). Our in vitro model employed a single treatment with HNE, which was metabolized rapidly by the hepatocyte within a few minutes (13, 38). Therefore, RALA hepatocyte HNE exposure in our experiments was relatively limited compared with the sustained elevations that hepatocytes are exposed to in vivo in liver disease. These facts suggest that the sensitization of CYP2E1-overexpressing hepatocytes to death occurred at a physiologically relevant HNE concentration.

Death from HNE in CYP2E1-overexpressing cells was associated with overactivation of JNK/c-Jun signaling as demonstrated by increased levels of 1) phospho-JNK and phosphoc-Jun by Western blotting, 2) JNK activity by in vitro kinase assay, and 3) AP-1-driven transcriptional activity. JNK/c-Jun signaling mediated cell death from HNE as expression of the c-Jun-dominant negative TAM67 significantly increased cell survival. HNE has been identified as a JNK activator in other cell types (5, 31), but we believe this to be the first report to identify HNE as an inducer of JNK signaling in hepatocytes. A mechanism of JNK activation by HNE in some cells is through HNE binding to JNK (31). Direct HNE-JNK interaction was not the mechanism of JNK activation in HNE-treated S-CYP cells, as HNE and JNK did not communoprecipitate and activation of the upstream JNK kinase MKK4 occurred. The mechanism of JNK/c-Jun overactivation resulting from CYP2E1 overexpression and HNE treatment was a prolonged reduction in levels of the principal nonenzymatic antioxidant GSH. GSH depletion, JNK overactivation, and cell death were mediated by CYP2E1-induced oxidant stress, as the antioxidant catalase inhibited all of these events. MAPK activity is known to be redox sensitive, and the fact that ROS can inactivate MAPK phosphatases (40) suggested that prolonged JNK activation may be secondary to a failure to dephosphorylate this protein. However, the rate of JNK dephosphorylation in S-CYP cells was equivalent to that in control VEC cells. Instead HNE-treated S-CYP cells had marked overactivation of MKK4, demonstrating that the effect of GSH depletion occurred upstream of JNK. The mechanism by which HNE activates MKK4 remains to be determined.

The susceptibility of S-CYP cells to GSH depletion was not specific for HNE, as these cells also underwent a more profound GSH reduction in response to the chemical DEM. The
initial GSH depletion was marked with both treatments, equiva-

tent in the two cell types after DEM treatment, but signifi-

cantly greater in HNE-treated S-CYP cells than in VEC cells.

Both treatments led to a more sustained period of GSH deple-
tion in S-CYP cells. Rat liver GSH has a rapid 2–3-h turnover,

and GCS is the rate-limiting enzyme in GSH synthesis (26).

GCS activity was reduced in S-CYP cells especially after HNE
treatment. Levels of GCS activity can regulate hepatocyte
injury as evidenced by the ability of GCS overexpression to
ameliorate acetaminophen-induced liver injury resulting from
GSH depletion (3). The more extensive GSH depletion in S-
CYP cells therefore had two mechanisms. One was the greater
degree of GSH depletion that occurred from the com-

bined effects of CYP2E1-generated oxidative stress and HNE

conjugation. Second, GSH levels in these cells could not
recover as quickly because of reduced GCS activity and GSH
synthetic capacity. These findings suggest the presence of a
 novel mechanism of decreased GCS activity in S-CYP cells

that remains to be defined. It is somewhat surprising that
S-CYP cells would downregulate an antioxidant pathway

that might be expected to be upregulated as a compensatory
protective response to their chronic oxidative stress. However,
the effect on GCS might be the result of the upregulation of a

critical compensatory signaling response that had a secondary
deleterious effect of decreasing GCS activity. Consistent with
this speculation is that we previously demonstrated that S-CYP

cells have increased ERK1/2 signaling that protects them from
oxidant stress (19) but also acts to sensitize them to death from
fatty acids (34).

The results of this study differ from those previously re-
ported in HepG2 hepatoma cells in which stable CYP2E1
overexpression increased their resistance to HNE toxicity (28).
This discrepancy is likely secondary to differences between the
nontransformed RALA hepatocytes employed in the present
studies and hepatoma cells. Transformed cell lines have
marked differences in their antioxidant levels compared with
normal cells. HepG2 cells have GSH levels that are threefold
greater than those in primary hepatocytes or RALA cells (8).
In contrast, HepG2 cells have a 10-fold decrease in their GST
activity compared with normal liver (28). Therefore, the reg-
ulation of GST expression and resultant HNE toxicity obvi-
ously differs in these cells compared with normal liver and not
surprisingly from RALA hepatocytes as well.

Despite an up-

regulation of GSH synthesis in CYP2E1-overexpressing HepG2
cells, consistent with our findings in RALA hepatocytes is
that CYP2E1-overexpressing HepG2 cells undergo cell
death from chemical GSH depletion (8), suggesting that these
cells also have an impaired ability to replete GSH.

The present findings are a novel demonstration of the ability
of ROS and a lipid peroxidation product to have synergistic
effects on cellular JNK MAPK signaling and provide a phys-

iologically relevant mechanism for the overactivation of JNK/
c-Jun signaling in the steatotic liver. Although the hepatocyte
can adapt to the oxidant stress, the cell is still vulnerable to
injury from HNE because of cumulative effects on JNK sig-

naling. Thus the lipid peroxide HNE generated in NAFLD may

not be merely a passive marker of oxidative stress in the liver
but an actual mediator of liver injury. This finding supplies a
new potential mechanism by which oxidant stress may promote
the development of this disease.

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