Hepatocyte growth factor protects hepatocytes against oxidative injury induced by ethanol metabolism

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Original Contribution

Hepatocyte growth factor protects hepatocytes against oxidative injury induced by ethanol metabolism

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ABSTRACT

Hepatocyte growth factor (HGF) is involved in many cellular responses, such as mitogenesis and apoptosis protection; however, its effect against oxidative injury induced by ethanol metabolism is not well understood. The aim of this work was to address the mechanism of HGF-induced protection against ethanol-generated oxidative stress damage in the human cell line VL-17A (cytochrome P450 2E1/alcohol dehydrogenase-transfected HepG2 cells). Cells were pretreated with 50 ng/ml HGF for 12 h and then treated with 100 mM ethanol for 0–48 h. Some parameters of oxidative damage were evaluated. We found that ethanol induced peroxide formation (3.3-fold) and oxidative damage as judged by lipid peroxidation (5.4-fold). Damage was prevented by HGF. To address the mechanisms of HGF-induced protection we investigated the cellular antioxidant system. We found that HGF increased the GSH/GSSG ratio, as well as SOD1, catalase, and γ-glutamylcysteine synthetase expression. To explore the signaling pathways involved in this process, VL-17A cells were pretreated with inhibitors against PI3K, Akt, and NF-κB. We found that all treatments decreased the expression of the antioxidant enzymes, thus abrogating the HGF-induced protection against oxidative stress. Our results demonstrate that HGF protects cells from the oxidative damage induced by ethanol metabolism by a mechanism driven by NF-κB and PI3K/Akt signaling.

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Ethanol (EtOH) abuse remains one of the main factors that cause liver injury. Alcoholic liver disease (ALD) is characterized by several distinct stages of injury ranging from steatosis to cirrhosis [1,2]. These deleterious effects are caused by products of EtOH metabolism such as acetaldehyde and reactive oxygen species (ROS) generated by EtOH metabolism [3]. EtOH is detoxified mainly by alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (Cyp2E1) [3] and both enzymes generate acetaldehyde, a highly toxic metabolite. Additionally, ethanol metabolism by Cyp2E1 generates large amounts of ROS, which can be involved in many harmful processes, such as lipid and protein oxidation, that can promote cellular dysfunction and eventually cell death by apoptosis or necrosis [3–6].

Liver cells possess a number of compensatory mechanisms to deal with ROS and their effects; among these are the induction of antiapoptotic and antioxidant proteins such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GSHPx), and the tripeptide glutathione (GSH), as well as others [5,7]. These mechanisms are induced in response to the actions of some growth factors and cytokines that activate survival signaling pathways, such as **phosphoinositol 3-kinase** (PI3K), Akt, and nuclear factor-κB (NF-κB) [8–10].

Hepatocyte growth factor (HGF), otherwise known as scatter factor, is a multifunctional growth factor that supports diverse cellular process including morphogenesis, motility, proliferation, and apoptosis protection [11]. HGF and its receptor c-Met activate signaling pathways that promote cell survival against apoptotic inducers, including the Fas agonist Jo2. There are several reports that strongly support the antiapoptotic effects of HGF/c-Met [8,12–14], mainly by the induction of antiapoptotic proteins such as Bcl-2, Bcl-X$_L$, or Mcl-1 [14,15]. Although the HGF/c-Met-induced apoptosis protection is well known, the effect on oxidative stress protection remains not fully understood. Some published reports indicate that HGF can protect from oxidative damage [15–17] but the effect of this growth factor on alcoholic liver damage is practically unexplored. Because ROS are thought to be important factors that induce cell injury in ALD, we investigated the capacity of HGF in the protection against ALD, using the recombinant HepG2 cell line VL-17A, which efficiently expresses both ADH and CYP2E1 [18]. We also investigated the mechanisms by which HGF protects cells from the deleterious effects of ROS produced as a result of ethanol metabolism.

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Materials and methods

Cell culture

HepG2 cells that constitutively and stably express ADH (VA-13) or both Cyp2E1 and ADH (VL-17A cells) were produced by Dr. Dahn L. Clemens from the Research Service, The Veterans Affair Medical Center, and the University of Nebraska Medical Center (Omaha, NE, USA). HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). All cell lines were cultured in minimal essential medium containing 10% fetal bovine serum and supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere in 5% CO_2 at 37°C. Cells were plated and incubated in minimal essential medium overnight, the culture medium was replaced with fresh medium, and the various treatments were initiated.

Experimental design

Twenty-four hours after cell plating, the culture media were replaced with a serum-free medium containing or not containing 100 mM EtOH. After incubation for 1, 3, 6, 12, 24, or 48 h, the cells were washed twice with phosphate-buffered saline (PBS), and whole-cell lysates (WCL) were prepared for Western blot analysis. For HGF pretreatments cells were treated for 12 h with 50 ng/ml recombinant human HGF (Peprotech, Rocky Hill, NJ, USA) and then 100 mM EtOH was added to cell culture for 48 h. At the end of treatment the cells were subjected to studies or harvested and prepared for Western blot analysis. To address the participation of some signaling pathways involved in cell survival, hepatocytes were pretreated for 30 min before HGF treatment with 10 mM wortmannin, 10 µM Akt inhibitor II, and 30 µM SN50, a peptide inhibitor of NF-κB.

Cell lysis and Western blot

WCL were prepared from 1.5×10^6 cells that were collected and washed twice with ice-cold PBS. The cell pellet was resuspended in 150 µl of M-PER (Pierce Chemical, Rockford, IL, USA) lysis buffer supplemented with proteases and phosphatase inhibitors. Lysis was performed on ice for 15 min, and cell debris was removed by centrifugation at 13,000g at 4°C for 10 min. The supernatant was recovered and proteins were separated by electrophoresis using 4–20% gradient Duramide precast gels (Cambrex, Rockland, ME, USA) and transferred to a polyvinylidene difluoride membrane (Invitrogen, Carlsbad, CA, USA). Immunodetection was performed using primary antibody as indicated, anti-SOD1, anti-GSHPx 1/2, anti-actin, γ-glutamylcysteine synthetase (γ-GCS) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-catalase (Sigma, St. Louis MO, USA), followed by horseradish peroxidase-conjugated secondary antibody (Amersham, Piscataway, NJ, USA) and detection by incubation with SuperSignal West Pico chemiluminescent substrate (Pierce) and HyperFilm (Amersham).

Reactive oxygen species measurement

Reactive oxygen species levels were determined using 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma). Cells were treated with 100 mM EtOH for 24 h and then washed with ice-cold PBS and incubated with 3 µM DCFH-DA for 30 min. The cells were trypsinized and suspended in ice-cold PBS in the dark, and ROS generation was measured by flow cytometry using a flow cytometer (Becton–Dickinson). A minimum of 10,000 cell-gate events was acquired and analyzed with CellQuest (Becton–Dickinson) software.

Nuclear extract preparation

Cells were washed twice with ice-cold PBS and scraped off from dishes with a rubber cell lifter. Nuclear extracts were prepared by lysing cells with Igepal CA-630 (0.58%) in 10 mM Hepes, pH 7.9, containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Cells were vigorously mixed and centrifuged at 13,000g at 4°C. Pelleted nuclei were resuspended in 20 mM Hepes, pH 7.9, containing 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF. After being mixed continuously for 15 min at 4°C the samples were centrifuged at 13,000g for 5 min and the supernatants recovered and stored at −80°C.

Electrophoretic mobility shift assay

Activation of NF-κB was examined by electrophoretic mobility shift assay (EMSA) using a consensus oligonucleotide, 5′-AGTTGAGGG-GACTTTCCCAGGC-3′ (Promega, Madison, WI, USA). Probe was labeled by T4 polynucleotide kinase (USB, Cleveland, OH, USA) with [γ-^{32}P] ATP (3000 Ci/mmol; MP Biomedical, Irvine, CA, USA). Labeled probe was purified using Bio-Spin 30 chromatography columns (Bio-Rad, Hercules, CA, USA). Binding reactions were done as described by Roman et al. [19] using 20 µg of protein from nuclear extracts, in 5 µl of incubation buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 5 mM EDTA, 5 mM β-mercaptoethanol, and 20% glycerol) and 1 µg poly(dI–dC). Complexes were separated through nondenaturing 6% polyacrylamide gels and were visualized by autoradiography. In competition experiments 100-fold molar excess of unlabeled oligonucleotide was added to the reaction mix for 5 min before addition of the labeled probe.

Cell viability

Cell viability was assessed by the crystal violet-staining assay according to Nakagawa et al. [20]. Cells seeded at 1×10^5 into 12-well-plates were treated and then washed with PBS, fixed with 3.7% formaldehyde, and stained with 0.2% crystal violet. The absorbance of the 2% sodium dodecyl sulfate extracts was measured at 620 nm. The cell viability was calculated as the percentage relative to untreated cells.

5-Bromo-2′-deoxyuridine (BrdUrd) labeling

Proliferation was analyzed by BrdUrd incorporation using a BrdUrd labeling kit (Roche Diagnostics GmbH, Mannheim, Germany). Cells were pretreated or not with 50 ng/ml HGF for 12 h and then treated with or without 100 mM EtOH for 24, 48, and 72 h and labeled with 10 µM BrdUrd during the last 24 h. Incorporation of BrdUrd was analyzed by luminescence using a multimode detector 880 (Beckman Coulter).

GSH determination by HPLC

GSH and oxidized GSH (GSSG) content was determined by HPLC following a protocol described by Fariss and Reed [21], with some modifications. Briefly, cells were collected in 1 ml of 10% perchloric acid and sonicated on ice for 30 s, and the suspension was centrifuged at 5000g, for 5 min at 4°C. Two hundred fifty milliliters of the supernatant was removed and transferred to a fresh microcentrifuge tube for derivatization. Iodoacetic acid (100 mM) in 0.2 mM m-cresol was added to each sample and to GSH and GSSG solution standards, the pH was adjusted to 9.0 ± 0.2 with a solution of 10 M KOH/3 M $KHCO_3$, and the samples were incubated in the dark at room temperature for 1 h. After that, 1 ml of 1% 1-fluoro-2,4-dinitrobenzene in 100% HPLC-grade ethanol was added to each sample and incubated at room temperature and then at 4°C overnight. The next day, the samples were centrifuged at 13,000g

at room temperature for 1 min and 100 μl N-DNP derivatives was injected into the loop of the HPLC system (Waters, Milford, MA, USA). Separation was achieved on a 3-aminopropyl column (5 μm; 4.6 mm×20 cm; Custom LC, Houston, TX, USA), with initial solvent conditions of 80% solution A (80% grade HPLC methanol), 20% solution B (0.5 M sodium acetate in 64% methanol), which were maintained at 1 ml/min for 10 min. From 10 to 40 min, we a linear gradient was used, to a final ratio of 1% solution A/99% solution B. Eluted N-DNP derivatives were measured by ultraviolet detection at 365 nm.

Lipid peroxidation

Lipid peroxidation was assayed by determining the rate of production of thiobarbituric acid-reactive substances (TBARS) by spectrophotometry as described by Buege and Aust [22].

Protein oxidation

Oxidative modification of proteins generates protein carbonyl groups, which represent a hallmark of oxidation status of all proteins. Carbonyl groups were immunodetected using an Oxyblot kit (Chemicon) according to the manufacturer's instructions.

Protein content determination

Protein concentration was determined using a bicinchoninic acid kit (Pierce Chemical), according to the manufacturer's instructions.

Data analysis

Data are reported as means ± SE. The SPSS package version 10 was used to run the analysis. Comparisons among groups were done by means of ANOVA. Tukey's method was used for multiple comparisons. A $p \leq 0.05$ was considered statistically significant.

Results

Ethanol metabolism induces oxidative damage

Hepatocytes metabolize EtOH mainly by the action of Cyp2E1 and ADH. In vitro studies regarding this issue present a challenge because hepatocytes in primary culture rapidly lose the activity of both enzymes. To circumvent this problem we used the VL-17A cell line, which express both Cyp2E1 (Fig. 1A) and ADH, and the VA-13 [23] cell line, which expresses only ADH. It is well known that Cyp2E1 activity generates oxidative stress. Figs. 1B and C show that 100 mM EtOH induced ROS production (Fig. 1B) and lipid peroxidation (Fig. 1C) only in VL-17A cells in comparison with VA-13 and HepG2 cells. As further evidence of oxidative damage we assayed protein oxidation in WCL. Fig. 1D shows that EtOH treatment in VL-17A cells induced significant protein oxidation. These data are in agreement with many reports that Cyp2E1 is responsible for EtOH-induced oxidative damage [3,4]. No differences were observed in lipid peroxidation and ROS production in VA-13 and HepG2 cells (Figs. 1B and C), which confirms that ADH-mediated EtOH metabolism does not produce detectable levels of ROS.

*Cell injury induced by EtOH was prevented by HGF pretreatment*

To address the effects of HGF on EtOH-induced oxidative damage, VL-17A and HepG2 cells were pretreated or not with 50 ng/ml HGF for 12 h and then exposed for 48 h to 100 mM EtOH. HGF-induced protection was explored by examining culture morphology by microscopy. EtOH treatment damaged VL-17A cell culture (Fig. 2F) in comparison with untreated VL-17A (Fig. 2E) and EtOH-treated

HepG2 cells (Fig. 2B), which do not metabolize EtOH. HGF pretreatment abrogated the deleterious effects of EtOH metabolism, preventing cell damage (Fig. 2F). To confirm these observations we decided to determine protein oxidation and lipid peroxidation; results show that EtOH increased the content of carbonyl groups in WCL (Fig. 2I) and lipid peroxidation products (TBARS) 4.8-fold compared to untreated cells (Fig. 2J), which indicates an oxidative damage process. HGF pretreatment prevented the EtOH metabolism-induced oxidative damage to control values. These data are in agreement with the cell viability experiment determined by crystal violet-staining assay. EtOH decreased cell viability 35% compared with untreated cells and HGF pretreatment protected cells against EtOH toxicity (Fig. 2K).
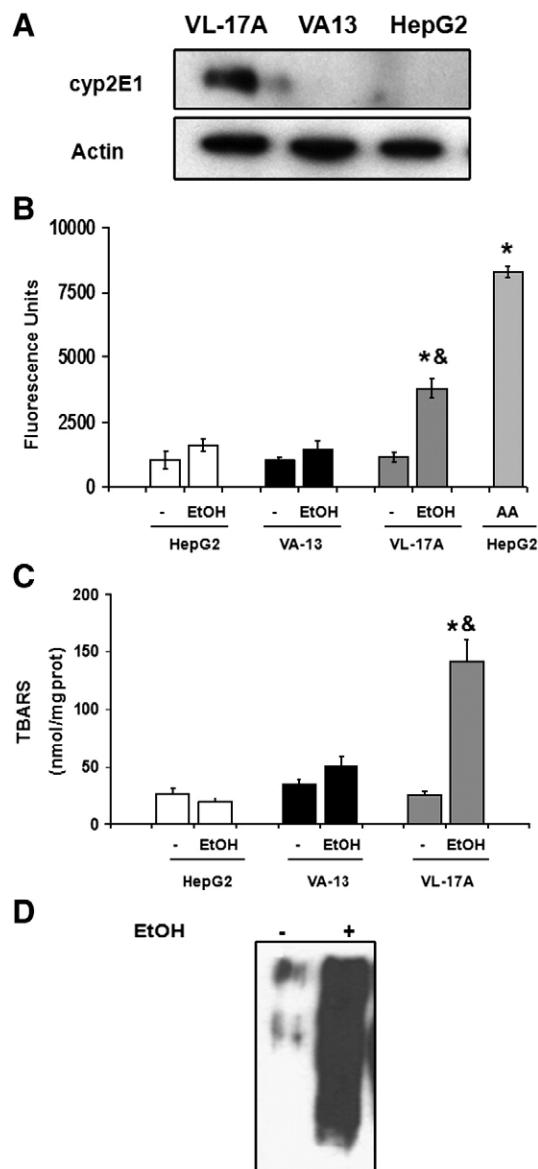
Fig. 1. Ethanol metabolism induces oxidative stress. VL-17A, VA-13, and HepG2 cells were treated or not with 100 mM EtOH for 48 h. (A) Whole-cell lysates prepared from serum-staved (16 h), untreated cells were immunoblotted with anti-cytochrome P450 2E1 to determine the expression of the enzyme. (B) ROS determination. After EtOH treatment cells were incubated with the oxidative-sensitive probe DCFH-DA (3 μM) for 30 min and fluorescence was recorded by flow cytometry as indicated under Materials and methods. Antimycin A (AA) was used as a positive control. (C) Lipid peroxidation determined by the content of thiobarbituric acid-reactive substances (TBARS) as indicated under Materials and methods. (D) Protein oxidation determined by protein carbonyl group content. Each column represents the mean ± SEM of at least three independent experiments. *$p < 0.05$ against HepG2 treated with EtOH. ᵃ$p < 0.05$ against untreated VL-17A cells.
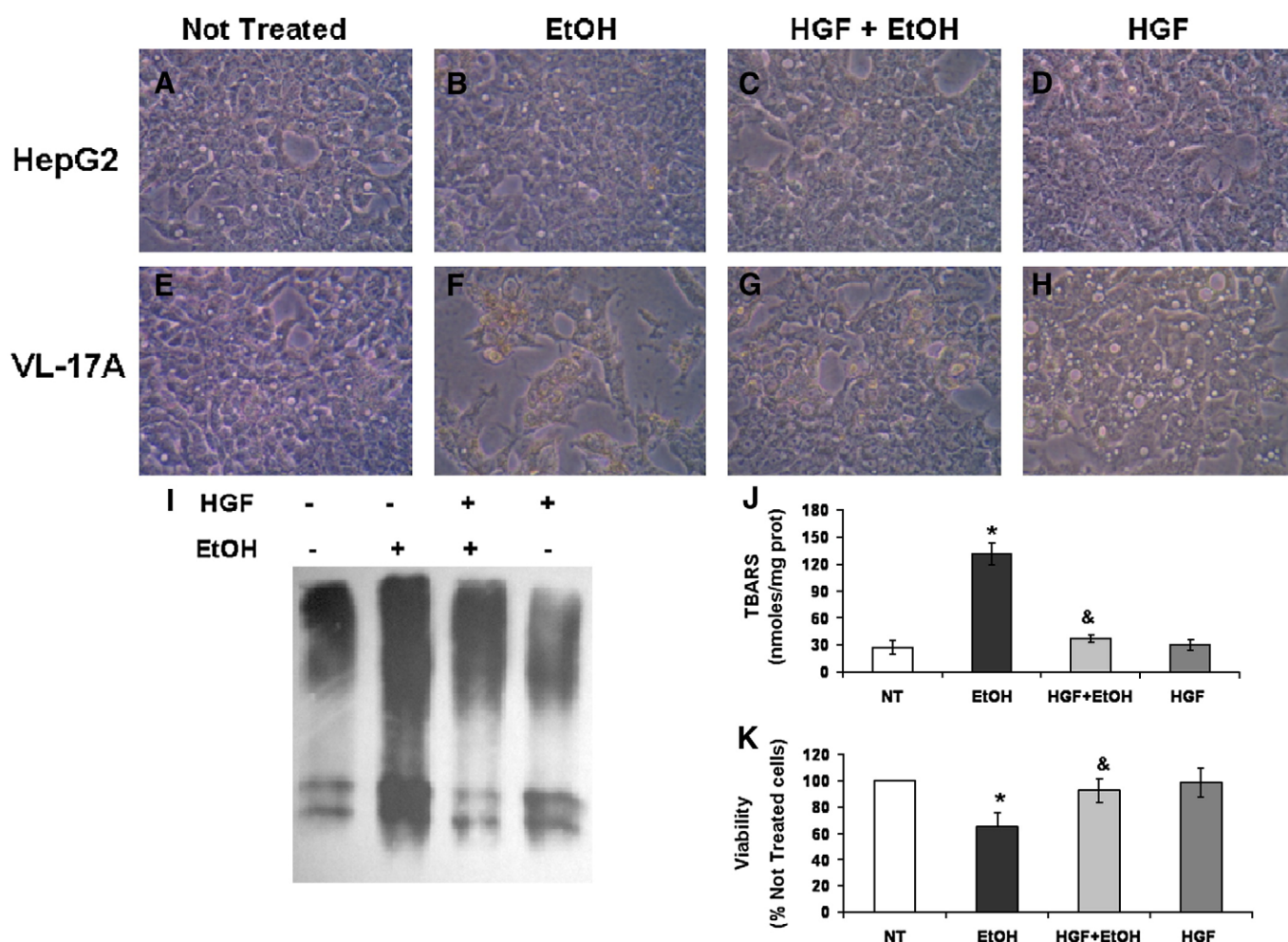
Fig. 2. HGF protects against ethanol metabolism-induced oxidative damage. HepG2 or VL-17A cells were pretreated or not with 50 ng/ml HGF for 12 h and then treated or not with 100 mM EtOH for 48 h. (A–H) Images show cell morphology under all experimental conditions in HepG2 and VL-17A cells. Original magnification 200×. (I) Protein oxidation determined by protein carbonyl group content. (J) Lipid peroxidation determined by the content of TBARS as indicated under Materials and methods. (K) Viability of VL-17A cells as determined by crystal violet assay. Each column represents the mean ± SEM of at least three independent experiments. *$p < 0.05$ against not-treated (NT) cells. [&]$p < 0.05$ against EtOH-treated VL-17A cells.

HGF induces cell survival by a mechanism mediated by NF-κB

To study whether HGF-induced protection is a process mediated by the well-known survival factor NF-κB, we explored the activation of this transcription factor. HGF treatment in VL-17A cells exerted a time-dependent increase in DNA binding of NF-κB as early as 30 min, which peaked at 120 min (Fig. 3A). To confirm this effect, we analyzed the degradation of the natural inhibitor of NF-κB (IκB-α) by Western blot. IκB-α content decreased in a time-dependent manner and was correlated with NF-κB activation (data not shown). To clarify that ethanol by itself does not induce NF-κB activation, we performed an EMSA with nuclear protein from hepatocytes treated with 100 mM EtOH from 30 to 360 min (Fig. 3B), and no changes in the activation of NF-κB were observed. Furthermore we analyzed whether pretreatment with HGF for 2 h followed by treatment with EtOH for an additional 2 h could induce the activation of the transcription factor. Fig. 3C demonstrates that HGF + EtOH did not induce any change. To support the participation of NF-κB in HGF-induced cell protection, we determined cell viability by crystal violet-staining assay in cells pretreated for 30 min with SN50, a peptide inhibitor of NF-κB, and after that, we proceeded with the HGF and EtOH treatments, for 12 and 48 h, respectively. As Fig. 3D shows, SN50 inhibitor abrogated the protective effect of HGF, decreasing viability by 61% compared to HGF + EtOH treatment. This result confirms the participation of NF-κB in the HGF-induced cell

protection against oxidative stress generated by EtOH metabolism. The effect of SN50 on toxicity of EtOH alone was a slight decrease in viability in comparison with EtOH (data not shown) confirming that abrogation of basal NF-κB activity sensitizes cells to die.

HGF-induced NF-κB activation is mediated by the PI3K/Akt pathway

To address the signaling events underlying HGF-induced NF-κB activation, we decided to explore the inhibition of the main upstream steps that lead NF-κB activation. PI3K/Akt is the main signaling pathway that is activated by HGF and other growth factors [8,10,24]. VL-17A cells were pretreated for 30 min with wortmannin, Akt inhibitor, and SN50 inhibitor as a positive control and then treated with 50 ng/ml HGF for 120 min, and then EMSA was performed. As Fig. 4 shows, both wortmannin and Akt inhibitor abrogated HGF-induced NF-κB activation, suggesting that PI3K and Akt are involved in NF-κB activation.

HGF enhances the antioxidant system to deal with EtOH toxicity

Because GSH is the main hepatoprotector molecule in the liver, we decided to explore the GSH/GSSG ratio and the expression of γ-GCS, the key-step enzyme in GSH synthesis. Fig. 5A shows that HGF increased the GSH/GSSG ratio by 3-fold in comparison with untreated cells. These data are in agreement with γ-GCS content determined by
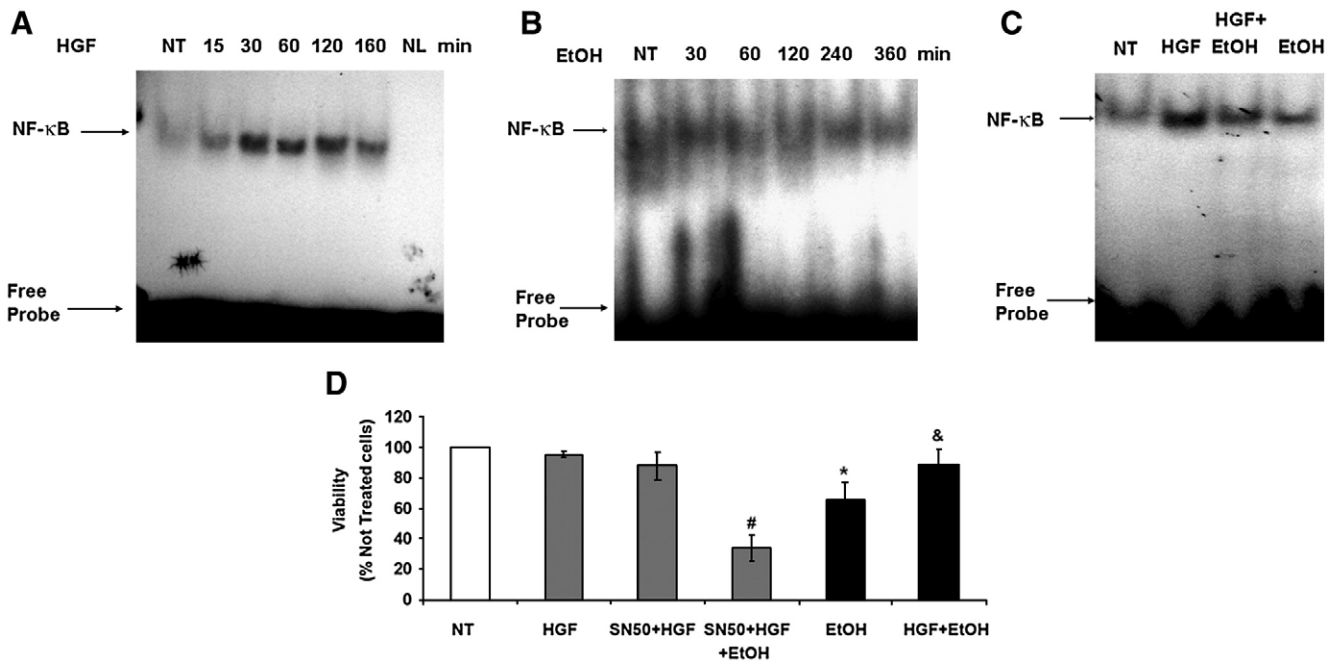
Fig. 3. HGF induces NF-κB activation-dependent cell survival. (A) Electromobility shift assay of NF-κB DNA-binding activity in serum-starved (16 h) VL-17A cells treated with (A) HGF (50 ng/ml) for 15, 30, 60, and 120 min; (B) ethanol (100 mM) for 30, 60, 120, 240, and 360 min; and (C) HGF (50 ng/ml) for 120 min and/or ethanol (100 mM) for 120 min. (D) Viability of VL-17A cells as determined by crystal violet assay. Each column represents the mean ± SEM of at least three independent experiments. *$p < 0.05$ against not-treated (NT) cells. &$p < 0.05$ against HGF + EtOH-treated cells. #$p < 0.05$ against EtOH-treated VL-17A cells.

Western blot (Fig. 5B), by which HGF treatment induced the enzyme 2.8-fold compared to untreated cells. EtOH treatment decreased the GSH/GSSG ratio, but the pretreatment with HGF in the presence of EtOH restored the ratio to control levels (Fig. 5C).

In addition to the GSH system we investigated the expression of the antioxidant proteins SOD1, catalase, and GSHPx 1/2 by Western blot. HGF induced the expression of SOD1 and catalase (Fig. 6A), but failed to induce GSHPx 1/2 (data not shown). The expression of SOD1 and catalase was time-dependent and peaked at 12 h of HGF exposure (2.2- and 2.5-fold, respectively) and was associated with the cytoprotective effect observed in Figs. 2H, I, J, and K. To address the participation of PI3K/Akt/NF-κB signaling in the regulation of

antioxidant enzymes we pretreated cells with 10 mM wortmannin, 10 μM Akt inhibitor II, and 30 μM SN50. The three inhibitors abrogated the HGF-induced catalase, SOD1, and γ-GCS (Fig. 6B). These data suggest that HGF transduces its antioxidant response via the PI3K/Akt/NF-κB signaling pathway.

HGF improves the EtOH-mediated impairment of cell proliferation

Finally, to correlate the molecular findings with a physiological process we analyzed cell proliferation, because it is well known that EtOH impairs cell proliferation [25]. As Fig. 7 shows, EtOH decreased proliferation at 48 h of treatment compared to untreated cells, whereas HGF pretreatment moderated this effect significantly.

Discussion

Alcohol-induced damage is a multifactorial process that involves genetic and environmental factors. Some of these factors are ROS derived from Cyp2E1-mediated EtOH metabolism in addition to those generated by mitochondrial dysfunction [26]. Cyp2E1 activity is increased by 10- to 20-fold in the livers of alcohol-treated rodents [27]. In humans an increase has been observed, not only in alcohol abusers, but also in moderate alcohol consumers [28]. This overactivity of Cyp2E1 in ALD is tightly linked to ROS production and cell damage [3,4,29,30], as we confirmed in this work. The recombinant HepG2 cells VL-17A, which express Cyp2E1 and alcohol dehydrogenase, presented oxidative damage when they were treated with 100 mM EtOH for 48 h (Figs. 1 and 2F), in comparison with untreated VL-17A cells or HepG2 cells that lack Cyp2E1 activity. Venugopal et al. [30] reported that VL-17A cells treated with 100 mM EtOH generate ROS that activate PKCδ and proteasome-mediated degradation of mitogen-activated protein kinase (MAPK) phosphatase-1, a protein phosphatase involved in c-Jun N-terminal kinase dephosphorylation, leading a sustained activation of this kinase and apoptosis progression.

To deal with oxidative stress, hepatocytes have available a wide range of antioxidants and survival factors that are induced by changes in the redox status as an adaptation response and by growth factors as
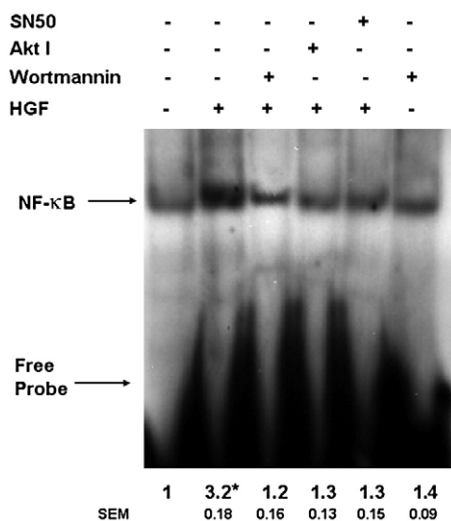
Fig. 4. HGF-induced NF-κB activation is mediated by PI3K and Akt. Electromobility shift assay of NF-κB DNA binding activity in serum-starved (16 h) cells. VL-17A cells were pretreated with 100 nM wortmannin, 10 μM Akt inhibitor II, or 30 μM SN50 for 30 min and then treated with HGF (50 ng/ml) for 2 h. Image is representative of at least three independent experiments. *$p < 0.05$ against untreated cells.
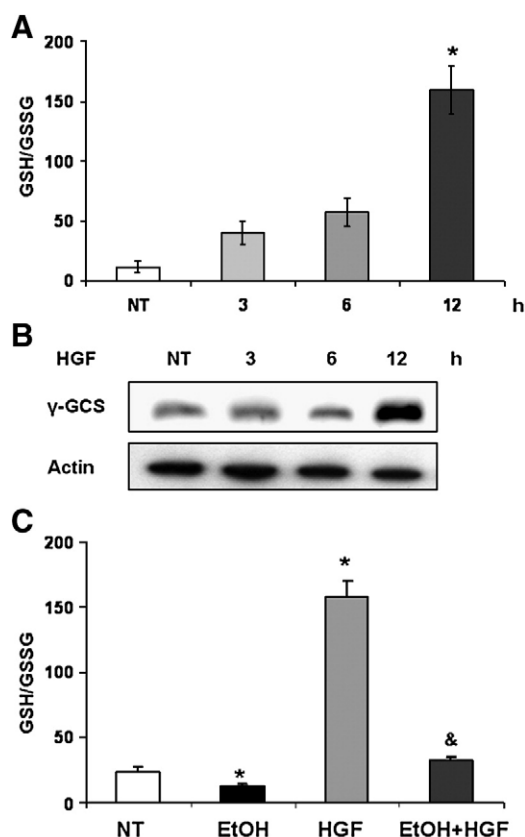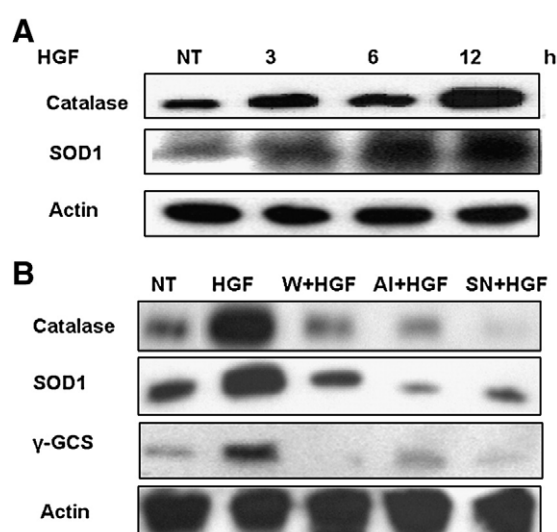
Fig. 5. HGF increases GSH synthesis. (A) The ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) determined by HPLC in serum-starved cells that were treated with HGF (50 ng/ml) for 0–12 h. Each column represents the mean ± SEM of three independent experiments. *$p < 0.05$ against untreated cells. (B) Expression of γ-glutamylcysteine synthetase (γ-GCS). Whole-cell lysates prepared from VL-17A cells treated with HGF (50 ng/ml) at 0, 3, and 12 h were immunoblotted with anti-γ-GCS. A representative Western blot of three experiments is shown. (C) The ratio of GSH/GSSG in hepatocytes treated or not with HGF (50 ng/ml) for 12 h, or ethanol (100 mM) for 48 h, or the combination of both. Each column represents the mean ± SEM of three independent experiments. *$p < 0.05$ against untreated cells. &$p < 0.05$ against EtOH-treated cells.

Fig. 6. HGF enhances the main antioxidant enzymes. (A) Expression of catalase and SOD1. Whole-cell lysates prepared from VL-17A cells treated with HGF (50 ng/ml) for 0, 3, 6, and 12 h were immunoblotted with anti-catalase and anti-SOD1. A representative Western blot of three experiments is shown. (B) VL-17A cells were pretreated with the PI3K inhibitor wortmannin (W; 100 nM), Akt inhibitor II (AI; 10 μM), or NF-κB inhibitor SN50 (SN; 30 μM) for 30 min and then treated with HGF (50 ng/ml) for 12 h. Antioxidant enzymes were immunoblotted with anti-catalase and anti-SOD1. A representative Western blot of three experiments is shown.

survival responses. Recently it has been proposed that HGF and its receptor c-Met can regulate the expression of many genes involved in oxidative stress regulation [31] and even in the regulation of oxidative stress-related carcinogenesis [17]; however, the mechanism is not fully characterized. We observed that pretreatment with HGF protects hepatocytes against oxidative damage induced by EtOH (Fig. 2), which is in agreement with the effect observed in other cell types [7,15,16].

It is well known that HGF can activate NF-κB, a key downstream regulatory factor of cell survival [32,33], so we decided to explore the activation of this transcription factor in VL-17A cells treated with HGF. Our data revealed that HGF induced NF-κB activation as rapidly as 30 min after treatment; this result is in agreement with those reported by Fan et al. [32]. These results suggest that HGF exerts a rapid response of cell survival, mediated by NF-κB, preparing the cells to deal with insults such as apoptosis inducers [12], hypoxia/reoxygenation-induced oxidative stress [24], or alcohol metabolism-mediated damage as we presented in this work.

Our data suggest that this response was mediated by PI3K and Akt activation. It is well documented that this pathway modulates many functions related to cell survival, proliferation, and liver regeneration [34] by activating downstream mediators and transcription factor such as MAPK, the signal transducers and activator of transcription proteins, and NF-κB [35]. All of these downstream kinases are able to regulate antiapoptotic response, proliferation, and regeneration, but in terms of antioxidant defense are not fully characterized.

GSH is the main antioxidant protector against oxidative stress in the liver and its relevance in ALD [26] and other pathologies has been well documented. GSH is synthesized in the cytosol as a response to many signals, including oxidative stress and growth factors [6]. Tsuboi [36] reported that HGF treatment increased GSH synthesis in rat hepatocytes via induction of γ-GCS. Our data are in agreement with these observations; HGF treatment increased the GSH/GSSG ratio in VL-17A cells as well as γ-GCS expression and these effects were correlated with cell survival (Fig. 2K). Furthermore, recently Takami et al. [17] found that c-Met knockout mice present a disruption in redox homeostasis, which conditioned them to N-nitrosodiethylamine-induced hepatocarcinogenesis. This effect was abrogated with oral administration of N-acetyl-L-cysteine (NAC), a well-known GSH precursor and antioxidant. The same result was observed in c-Met knockout hepatocytes that present an enhanced sensitization to Fas-induced apoptosis, which was abrogated by NAC treatment [37]. On the other hand, it has been reported that HGF can induce gene
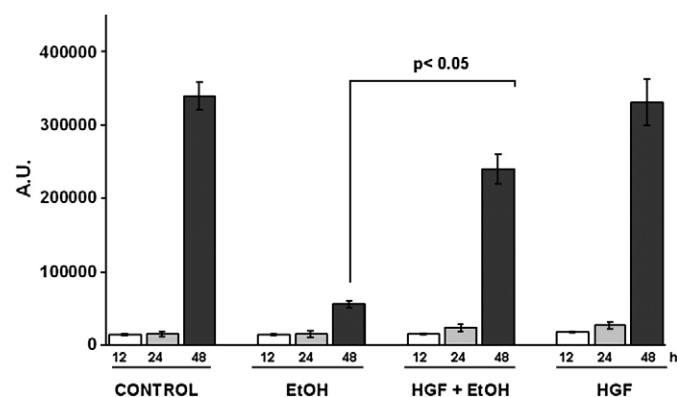
Fig. 7. HGF improves the EtOH-mediated impairment of cell proliferation. Proliferation was analyzed by BrdUrd incorporation using a BrdUrd labeling kit. VL-17A cells were pretreated or not with 50 ng/ml HGF for 12 h and then treated with or without 100 mM EtOH for 24, 48, and 72 h and labeled with 10 μM BrdUrd during the last 24 h. Incorporation of BrdUrd was analyzed by luminescence. Each column represents the mean ± SEM of six independent experiments.

expression of γ-GCS in bile duct epithelial cells [7] and an increased GSH synthesis in rat hepatocytes and H4IIE cells [33,36]. It is clear that GSH homeostasis is one of the main survival systems that are modulated by HGF in response to oxidative stress.

In addition to GSH synthesis and γ-GCS expression promoted by HGF, our data revealed that some antioxidant proteins are induced by the action of this growth factor. HGF stimulated the expression of the main antioxidant proteins such as SOD1 and catalase, but failed for GSHPx. Recently it has been published that in bile duct epithelial cells, HGF induced the expression of γ-GCS and GSH reductase, whereas no effect was observed for SOD1, catalase, and GSHPx [7]. This differential gene expression suggests that HGF activities are cell-type-dependent, even in liver cell populations. Hepatocytes are the main cell type that detoxifies EtOH, and it is in these cells that more antioxidant protection is needed in addition to the GSH system.

In conclusion, this work sustains the predominant role of HGF in the antioxidant protection against EtOH-induced oxidative stress, by a mechanism dependent on the activation of the PI3K/Akt/NF-κB signaling pathway.

Acknowledgments

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