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OXIDATIVE STRESS MODULATES KLF6$_{Full}$ AND ITS SPLICE VARIANTS

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Abstract

Background & Rationale—Induction of reactive oxygen species (ROS) is a central mechanism in alcohol hepatotoxicity. Krüppel-like factor 6 (KLF6), a transcription factor and a tumor-suppressor gene, is an early responsive gene to injury; however, the impact of ROS and alcohol on KLF6 induction is unknown.

Aim—To investigate the contribution of two sources of ROS, cytochrome P450 2E1 (CYP2E1) and NAD(P)H quinone oxidoreductase (NQO1) and alcohol to the modulation of *KLF6$_{Full}$* expression, splicing to *KLF6_V1* and *KLF6_V2* and the effect on TNFα, a downstream target.

Methods & Results—Endogenous ROS production in CYP2E1-expressing HepG2 cells induced mRNA and protein expression of *KLF6$_{Full}$* and its splice variants compared to control cells. Incubation with pro-oxidants such as arachidonic acid (AA), β-naphtoflavone and H_2O_2, further enhanced *KLF6$_{Full}$* and itssplice variants. The AA effects on *KLF6$_{Full}$* and its splice forms were blocked by vitamin E -which prevents lipid peroxidation- and by diallylsulfide -a CYP2E1 inhibitor-. Menadione and paraquat –two pro-oxidants metabolized via NQO1- induced KLF6$_{Full}$ mRNA in a thiol-dependent manner. Antioxidants and a NQO1 inhibitor suppressed the menadione-dependent increase in *KLF6$_{Full}$* and its splice variants mRNA. Furthermore, primary hepatocytes and livers from chronic alcohol-fed rats, with elevated lipid peroxidation, H_2O_2 and CYP2E1 but with low GSH, showed a ~2-fold increase in *KLF6$_{Full}$* mRNA compared to controls. Inhibition of p38 phosphorylation further up-regulated the CYP2E1 and the AA effects on *KLF6$_{Full}$* mRNA, whereas inhibition JNK and ERK1/2 phosphorylation decreased both. *KLF6_V1* but not *KLF6$_{Full}$* ablation markedly increased TNFα levels in macrophages; thus, TNFα emerges as a downstream target of KLF6_V1.

Conclusion—The novel effect of ROS on modulating *KLF6$_{Full}$* expression and its splice variants could play a relevant role in liver injury and in TNFα regulation.

Keywords

cytochrome P450 2E1; NAD(P)H quinone oxido-reductase; reactive oxygen species; TNFα

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INTRODUCTION

Oxidative stress and the generation of ROS are major pathways mediating ethanol hepatoxicity and liver injury. The major interest on CYP2E1 is due to the ability of this uncoupled enzyme to metabolize ethanol, to generate reactive products from ethanol oxidation, to activate many toxicologically relevant agents such as carbon tetrachloride, acetaminophen and alcohols to more toxic and reactive products, to produce ROS and to be induced by alcohol itself (Koop, 1992; Song, 1996; Tanaka et al., 2000).

Induction of CYP2E1 by ethanol and the subsequent state of oxidative stress is a central mechanism leading to altered gene transcription through activation of transcription factors (Bondy, 1992; Cederbaum, 1989; Cederbaum, 2001; Ishii et al., 1997; Lieber, 2000; Nordmann, 1994; Nordmann et al., 1992; Tsukamoto, 2001). Moreover, antioxidant and/or ROS-responsive elements in the promoter of genes are sensitive to oxidative stress and ROS modify mRNA stability (Sen et al., 2005; Zhao et al., 2004). In contrast to these well-known pathways of transcriptional and post-transcriptional control, nothing is known on whether CYP2E1 and ethanol-induced ROS could modulate mRNA splicing, which is emerging as a critical regulator of tissue repair and differentiation.

Zinc finger proteins have appeared as a major class of eukaryotic transcription factors. The Krüppel-like family of zinc finger transcription factors regulate cell growth, proliferation, apoptosis and angiogenesis (Bieker, 2001; Black et al., 1999; Dang et al., 2000; Kaczynski et al., 2003; Oates et al., 2001; Philipsen and Suske, 1999). All KLF family members possess a highly-conserved C-terminal 81 amino acid Zinc finger DNA-binding domain that can interact with ‘GC-box’ or ‘CACC-box’ DNA motifs in responsive promoters, with each KLF having a distinct N-terminal activation domain (Philipsen and Suske, 1999). While the DNA binding domains of KLFs are identical, their divergent activation domains account for their broad range of biologic activities. KLFs can homo-or heterodimerize with either other KLFs (Merika and Orkin, 1995) or with heterologous transcription factors (Sur et al., 2002; Zhang et al., 2002).

KLF6 is a ubiquitously expressed Krüppel-like transcription factor (Ho and Piquette-Miller, 2007; Sirach et al., 2007a) and a tumor-suppressor gene (Narla et al., 2005; Narla et al., 2001; Narla et al., 2007) whose role *in vivo* has not been fully clarified. Known putative transcriptional targets are placental glycoprotein (Blanchon et al., 2001), *COL1A1* (Ratziu et al., 1998), *TGFβ* (Kim et al., 1998), types I and II *TGFβ* receptors (Kim et al., 1998) and the urokinase type plasminogen activator (Kojima et al., 2000). In humans, KLF6 can undergo splicing to shorter isoforms (*KLF6_V1*, *KLF6_V2* and *KLF6_V3*) (Narla et al., 2005) (Suppl. Fig. 1), which lack all or part of the DNA binding domain. Inducers and repressors of KLF6 as well as the repertoire of downstream targets, particularly targets that may play a key role in liver injury, are not fully characterized to date (Bieker, 2001). Little is known on how $KLF6_{Full}$ and its splice variants may sense ROS and ethanol, whether a shift in their ratios may occur and whether this could affect their down-stream targets, many of which remain unknown. The possible involvement of ROS and ethanol in the expression of $KLF6_{Full}$ and its splice isoforms may help to understand their potential contribution to tissue injury and repair in alcoholic liver disease and other diseased states, where oxidative stress occurs.

MATERIAL AND METHODS

Cell lines and Primary Hepatocytes

Most of our studies were performed with CYP2E1-expressing cells (E47 cells) generated in the laboratory from Arthur I. Cederbaum (Mount Sinai School of Medicine, NY) (Chen and

Cederbaum, 1998; Chen et al., 1997), which are HepG2 cells constitutively expressing human CYP2E1. The control cells (C34 cells) are HepG2 cells transfected with pCI-neo and do not express CYP2E1. The colon cancer HCT116 cells and the murine RAW 246.7 macrophages were obtained from the ATCC (Manassas, VA). Using endotoxin-free collagenase, primary hepatocytes were isolated from rats fed for 8 months, which in humans would be about 20–30 years, with the Lieber-DeCarli diets (Lieber and DeCarli, 1982). The percentage of ethanol-derived calories was 10% for 1 wk, 20% for 1 wk and 35% for 7.5 months. The control group received isocaloric amounts of dextrose (Lieber and DeCarli, 1982). Protocols were approved by the Institutional Animal Care and Use Committee at our Institution. In all cells, CYP2E1 content was measured by Western blot and the catalytic activity by using the fluorescent substrate 7-Methoxy-4-trifluoromethylcoumarin (7-MFC) or by the method of Reinke (Reinke and Moyer, 1985).

Liver Pathology

Blood was collected from the abdominal aorta prior to liver perfusion. Plasma was assayed for the activity of ALT and AST, ethanol concentration and non-esterified fatty acids (NEFA) using kits from Thermo Electron Corporation (Waltham, MA), Sigma (St. Louis, MO) and Wako Chemicals (Richmond, VA), respectively. Liver sections were obtained from the left liver lobe. Samples were fixed overnight in 10% buffered formalin and embedded in paraffin. Sections (5 μm) were stained with H&E and evaluated by a liver pathologist who was blinded from the experimental information.

General Methodology

Cell viability was monitored using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Intra- and extracellular lipid peroxidation end-products were determined from the production of thiobarbituric acid reactive substances (TBARS) with a standard curve of malonaldehyde bisdimethylacetal (Nieto and Rojkind, 2007). Extracellular H_2O_2 was assessed by the ferrous oxidation-xylenol orange assay (Nourooz-Zadeh et al., 1994). GSH levels were measured in the protein free extract by the recycling method of Tietze (Tietze, 1969). Northern blot analysis for *CYP2E1*, $KLF6_{Full}$ and *GAPDH* mRNA was carried out using cDNA probes developed in our laboratory (Nieto et al., 1999). Western blot analysis for CYP2E1 was performed using an antibody kindly donated by Dr. Jerome Lasker (Puracyp Inc., Carlsbad, CA) (Shimizu et al., 1990). The KLF6 antibody, which recognizes all isoforms, was from Santa Cruz Biotechnology (Santa Cruz, CA). The quantification of the intensity of the signal in Western and Northern blots was expressed under the blots as arbitrary units of densitometry.

Flow-Cytometry Analysis

Intracellular H_2O_2 and $O_2^{\cdot -}$ were assessed using the fluorescent probes 2′, 7′-dichlorofluorescein diacetate (DCFDA) and dihydroethidium (DHE) (Brenner et al., 2003), respectively (Molecular Probes, Eugene, OR). TNFα was measured in cells by flow cytometry analysis using anti-rat TNFα PE-conjugated antibody (BD Pharmingen, San Jose, CA). The intensity of fluorescence was quantified using a FACS Calibur™ Flow Cytometer and analyzed using CellQuest Pro™ software (BD Biosciences, San Jose, CA). Secreted TNFα was measured using an ELISA kit (Invitrogen, Carlsbad, CA).

Quantitative Real-Time PCR

Total RNA was extracted using the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, CA), treated with recombinant DNAse (Roche Diagnostic, IN) and reverse transcribed using the FastStart PCR Master Mix (Roche Diagnostic, Indianapolis, IN). Triplicate qRT-PCR reactions were performed with the LightCycler using the primers

listed in Table 1. Data were analyzed using the LightCyclerR 480 software normalizing the results by GAPDH.

Transfection Experiments and Reporter Assays

RAW 246.7 cells were plated at a density of 5×10^4/well in 12-well plates 1 day prior to transfection. Co-transfection studies were performed using Lipofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The human -982-*TNFα* promoter construct was provided by Dr. Goldfeld (Harvard Medical School, Boston, MA). A total of 1 μg of -982-*TNFα* promoter construct and pGL3 basic and 50 ng of pRenilla-Luc were transfected. Twenty-four hours later scrambled siRNA, siRNA *KLF6*$_{Full}$ or siRNA *KLF6_V1* (20 pmol) were transfected per well. In some experiments, cells were treated with AA for 3 h before harvesting them. Cells were collected 48 h after the transfection with the TNFα reporter construct and both firefly and renilla luciferase activities were measured.

Statistical Analysis

Data were analyzed by a Student's *t* test and by a two-factor ANOVA when applicable. Values are expressed as means ± S.E.M. (N>3).

RESULTS

Overexpression of CYP2E1 -a Source of ROS- Induces KLF6$_{Full}$ and its Splice Forms

To evaluate the contribution of ROS to the expression of *KLF6*$_{Full}$ and its spliced forms, an immortalized HepG2 cell line over-expressing CYP2E1 (Fig. 1A–1C), as a source of endogenous production of ROS (Fig. 1D–1F, white bars) was used and found increased expression of KLF6$_{Full}$ (37–39 kDa) and of its splice variants KLF6_V1 (25 kDa) and KLF6_V2 (30 kDa) when compared with control HepG2 cells (Fig. 2A). Similar results were observed at the mRNA and protein level (Fig. 2B, and not shown). This is likely due to elevated CYP2E1-mediated ROS increase as it does not occur in the control HepG2 cells.

Increased Expression of KLF6$_{Full}$ does not Alter CYP2E1 Levels

Since KLF6 is a transcription factor, to elucidate if there could be a feed-back loop between KLF6$_{Full}$ and CYP2E1, CYP2E1-expressing cells were transfected with the pCI-neo vector containing the cDNA encoding for *KLF6*$_{Full}$ or with the empty vector and 48 h later CYP2E1 expression was analyzed by Northern blot. Similar *CYP2E1* mRNA levels were found in all samples (Fig. 1B). *GCLC* and *GCLM*, two proteins that regulate *de novo* GSH synthesis, which could prevent ROS-induced injury, also remained similar (not shown).

Arachidonic Acid Induces ROS Mostly in CYP2E1-Expressing Cells

To study the role of CYP2E1-derived ROS on *KLF6*$_{Full}$ expression and splicing, we first confirmed the ability of the CYP2E1-expressing cells to generate a state of oxidant stress in the presence of substrates relevant for the development of alcohol-induced liver injury (Nanji, 2004). Both control and CYP2E1-expressing cells were incubated with 0–30 μM AA -a representative polyunsaturated fatty acid (PUFA)- in a time-course experiment up to 9 h (Fig. 1D–1F, grey and back bars). Short time-points were chosen because *KLF6*$_{Full}$ is an early-response gene (Kojima et al., 2000). Cell viability was assessed by the MTT assay and by flow cytometry analysis (not shown) or by monitoring for changes in cell morphology; however, no signs of cytotoxicity were observed at the selected time-points (Fig. 1C). CYP2E1-expressing cells showed higher extra- and intracellular H_2O_2 levels (Fig. 1D–1E, white bars) as well as intracellular O_2^- (Fig. 1F, white bars) than controls. ROS generation was further elevated by treatment with AA (Fig. 1D–1F, gray and black bars). CYP2E1

expression and activity can be modulated by addition of specific inhibitors and the effect mediated by ROS can be prevented by antioxidants. Addition of vitamin E -which prevents lipid peroxidation reactions- (Fig. 1E–1F, light grey bars) and of diallylsulfide -a selective CYP2E1 inhibitor- (Fig. 1E–1F, dark grey bars), prevented the increase in intracellular H_2O_2 and O_2^-, respectively. Levels and activity of CYP2E1 under diallylsulfide treatment are shown in the inset.

Pro-oxidants Increase KLF6$_{Full}$ Mainly in CYP2E1-Expressing Cells

Addition of two pro-oxidants (15 μM menadione or 100 μM paraquat), which undergo two electron reduction through NAD(P)H quinone oxidoreductase, or of a CYP2E1 inducer (100 μM β-naphtoflavone), elevated *KLF6$_{Full}$* expression ~1.9-to-4.8-fold in the CYP2E1-expressing cells as shown by Northern blot analysis (Fig. 3A). A time-course experiment was carried out with menadione to define the time-point of maximal induction (6–12 h, Fig. 3B). Treatment of HCT116 cells -a colon cancer cell line- with menadione caused a similar effect on *KLF6$_{Full}$* (Not shown). A second set of experiments was carried out in the presence of other pro-oxidants such as 30 μM AA, 20 μM H_2O_2, 15 μM menadione and the combination of AA plus H_2O_2 and of AA plus menadione, at doses and times that did not affect cell viability (not shown). There was an induction of *KLF6$_{Full}$* expression under all these treatments, which was more apparent for the CYP2E1-expressing cells than for the control cells (Fig. 3C). Addition of iron -a catalyst of lipid peroxidation-derived reactions- to AA-treated cells further increased *KLF6$_{Full}$* expression (not shown).

Pro-oxidants Also Increase KLF6_V1 and KLF6_V2 mRNA Mainly in CYP2E1-Expressing Cells

To establish a link between ROS, *KLF6$_{Full}$* expression and the possible modulation of the KLF6 splice variants by ROS, control and CYP2E1-expressing cells were incubated with either 30 μM AA or with 15 μM menadione for 6 h and levels of *KLF6$_{Full}$* and its splice forms *KLF6_V1* and *KLF6_V2* were analyzed by qRT-PCR (Fig. 4A–4B). In the absence of treatments, *KLF6$_{Full}$*, *KLF6_V1* and *KLF6_V2* appeared equally induced in the CYP2E1-expressing cells (Fig. 4A–4B, white bars). Addition of AA or menadione further increased mRNA levels of all *KLF6* isoforms mainly in the CYP2E1-expressing cells (Fig. 4A–4B, black bars).

Antioxidants and Inhibitors of NQO1 or CYP2E1 Prevent the Increase in KLF6$_{Full}$ and its Splice Forms by Menadione, Paraquat and AA

Control and CYP2E1 expressing cells were incubated with 0–15 μM menadione or with 0–100 μM paraquat in the absence or presence of antioxidants (an adenovirus over-expressing catalase or SOD, 2–10 mM glutathione ethyl ester (GSH-EE) and 25 μM vitamin E), pro-oxidants (L-buthionine sulfoximine -a GSH depleting agent-) or a NQO1 inhibitor (25 μM dicumarol). Addition of antioxidants and of a NQO1 inhibitor prevented the increase in *KLF6$_{Full}$* by menadione and by paraquat (Fig. 5A–5B). GSH depletion by BSO further elevated the effect of menadione on *KLF6$_{Full}$* expression whereas GSH-EE blunted it (Fig. 5C). In addition, cells were incubated with 0–30 μM AA in the absence or presence of diallylsulfide -a CYP2E1 inhibitor- and of vitamin E -an antioxidant-. AA increased 1.8-and 5.3-fold *KLF6$_{Full}$*, 2.3- and 4.2-fold *KLF6_V1* and 2- and 3.7-fold *KLF6_V2* in control and CYP2E1 expressing cells, respectively (Fig. 5D, black bars *vs.* white bars). This increase was blocked by diallylsulfide (Fig. 5D, light grey bars *vs.* black bars) and by vitamin E (Fig. 5D, dark grey bars *vs.* black bars) or by 0.1 mM sodium diethyldithiocarbamate, another CYP2E1 inhibitor (not shown).

$KLF6_{Full}$ Expression Increases in Primary Hepatocytes from Ethanol-Fed Rats with High CYP2E1

Since alcohol-induced liver injury is mediated in part by ROS, we extended our experiments to primary hepatocytes isolated from rats fed the control or the ethanol Lieber-DeCarli diets, an *in vivo* model known to induce oxidative stress mostly via CYP2E1 induction. The Lieber-DeCarli diets are a very well established protocol for inducing early alcohol-mediated liver injury (Lieber, 1999) and cause moderate steatosis (Fig. 6A, right panel), a 2-fold increase in AST, ALT, plasma ethanol levels of ~120 mg/dL and a 5-fold increase in non-esterified fatty acids (not shown). Oxidative stress was elevated in hepatocytes from ethanol-fed rats as shown by intra-and extracellular TBARS, intracellular H_2O_2 and GSH levels (Fig. 6B–6D) and by CYP2E1 expression (Fig. 6E). qRT-PCR analysis indicated induction of $KLF6_{Full}$ in hepatocytes and in total liver from ethanol-fed rats (Fig. 6F). Similar results were obtained in hepatocytes isolated from pyrazole-injected rats, a chemical known to induce CYP2E1 levels (not shown). The spliced variants were not analyzed as KLF6 splicing has not been described in rat.

Stress-Activated Kinases Participate in the Up-Regulation of $KLF6_{Full}$ Expression

To determine if there was an association between activation of stress-sensitive kinases and $KLF6_{Full}$ expression, we added specific inhibitors of phosphorylation of kinases. SB203580 -an inhibitor of p38 phosphorylation- increased the expression of $KLF6_{Full}$ whereas PD98059 -an inhibitor of MEK1/2 phosphorylation- or SP600125 -an inhibitor of JNK phosphorylation- significantly decreased $KLF6_{Full}$ in CYP2E1 expressing cells compared to controls, establishing a link between ROS, stress-activated kinases and $KLF6_{Full}$ levels (Fig. 7A–7B). Moreover, addition of SB203580 2 h prior to treatment with 30 μM AA, further elevated $KLF6_{Full}$ by 2.5-fold over the AA-mediated increase, while no additive effects were observed by SP600125, Wortmanin or PD98059 (Fig. 7C).

TNFα, a Potential Down-Stream Target of KLF6_V1

To determine whether KLF6 or *KLF6_V1* may affect TNFα, which plays a critical role in alcoholic liver disease and in other modes of liver injury, RAW 264.7 cells, a mouse macrophage cell line, were transfected with the −980-*TNFα* promoter and then cotransfected with control, $KLF6_{Full}$ or *KLF6_V1* siRNAs. In some experiments, cells were treated 48 h after transfections with 30 μM AA for 3 h. Transfection efficiency was evaluated and results were normalized to Renilla-Luc. Transfection with *KLF6_V1* siRNA caused a 2-fold increase and a 6-fold increase in intracellular TNFα in untreated RAW cells and in cell treated with AA, respectively (Fig. 8A) and a slight increase in secreted TNFα was also observed (Fig. 8B); thus, establishing a connection between *KLF6_V1*, oxidant stress and TNFα.

DISCUSSION

Among the mechanisms involved in alcohol toxicity, ROS production is believed to be highly significant and it is currently at the center of considerable research. Generation of ROS, along with a compromised antioxidant defense, can affect gene transcription and the subsequent activation of cellular targets. In addition, a variety of cellular stress signaling pathways can affect the responsiveness to ROS and determine protein function and eventually cell fate (Carreras and Poderoso, 2007). Thus, studies on the effects of ROS on induction of key proteins -such as KLF6- and its potential down-stream targets -such as TNFα- are relevant to better understand the mechanisms of alcohol-induced liver injury and other liver diseases and to develop new strategies for therapy.

To define the impact of ROS on the expression of $KLF6_{Full}$ and its splice variants, we used an immortalized HepG2 cell line over-expressing CYP2E1, as a source of endogenous ROS production, since CYP2E1 is an uncoupled enzyme that metabolizes alcohol and as a result, generates a state of oxidative stress, even in the absence of any added substrate. There was a concurrent increase in the expression of $KLF6_{Full}$ and its splice variants *KLF6_V1* and *KLF6_V2* in CYP2E1-expressing cells when compared with control cells, suggesting a potential link between ROS and $KLF6_{Full}$ expression and splicing. The possibility that KLF6 -as a transcription factor- could modify ROS production *per se* was ruled out as transfection with a plasmid containing the cDNA encoding for $KLF6_{Full}$ neither affected CYP2E1 expression nor nitric oxide synthase 2 (NOS2), GCLC, GCLM or catalase.

We next explored the contribution of two pro-oxidants to the induction of $KLF6_{Full}$ mRNA. Menadione and paraquat are redox cycling compounds, which undergo two-electron reduction through NQO1, generating non-alkylating metabolites that react with O_2 to produce ROS, mostly $O_2^{\cdot -}$, H_2O_2, OH^- and OH. Addition of menadione or paraquat induced $KLF6_{Full}$ mRNA as did β-naphtoflavone -a cytochrome P450 inducer-. Similar results were observed with menadione in the colon cancer cell line HCT116, suggesting that the effects of these pro-oxidants may not be limited to the liver. Moreover, pro-oxidants whose mechanism of action differ such as AA, H_2O_2 and menadione either added alone or in combination, triggered an increase in $KLF6_{Full}$ mRNA mostly in the CYP2E1 expressing cells than in the corresponding controls, suggesting that pro-oxidants either *per se* (e.g. H_2O_2), via CYP2E1-metabolism (e.g. AA, β-naphtoflavone) or through NQO1 metabolism (e.g. menadione and paraquat) enhance $KLF6_{Full}$ mRNA. Furthermore, both AA and menadione induced not only $KLF6_{Full}$ mRNA but also triggered a parallel up-regulation of *KLF6_V1* and *KLF6_V2* mRNA, mostly in CYP2E1-expressing cells, likely reflecting a transcriptional effect rather than increased alternative splicing.

Since this *in vitro* model allows regulating ROS levels, to validate the role of ROS in modulating *KLF6* mRNA, next we incubated cells in the presence of antioxidants and selective inhibitors of the specific source of ROS. Transduction with adenoviruses containing the cDNA encoding either for catalase -to decompose H_2O_2- or for SOD1 -to dismutate $O_2^{\cdot -}$- or treatment with vitamin E -to block lipid peroxidation-derived reactions- partially prevented the increase in $KLF6_{Full}$ mRNA by menadione and by paraquat. Likewise, dicumarol -an inhibitor of NQO1- blunted the effects mediated by menadione and paraquat. Similar results were observed in cells incubated with AA in the presence of vitamin E or diallylsulfide -a CYP2E1 inhibitor- validating the role of CYP2E1 in the transcriptional effects triggered by AA on $KLF6_{Full}$ and its splice variants.

Cellular GSH plays an essential role in preventing damage of oxidative nature to cellular membranes and acts as a hydrogen donor for glutathione peroxidases, which represent a line of defense against lipid peroxidation. Agents that lower GSH, such as L-buthionine sulfoximine, induce cell damage by events related to oxidative stress associated with decrease in protein thiols. Depletion of GSH increased the sensitivity of $KLF6_{Full}$ mRNA to menadione. Conversely, restoration of the GSH pool with GSH-EE blunted the increase in $KLF6_{Full}$ mRNA by menadione, suggesting a direct link between $KLF6_{Full}$ and GSH. It is possible that the menadione effects on *KLF6* are preceded by depletion of intracellular GSH stores due to oxidation to GSSG or to a decrease in protein thiols, likely due to oxidation of –SH groups, although arylation may also occur. The redox transitions of KLF6 –SH groups may affect its activity and cellular function.

As a second approach, we then extended our studies to primary hepatocytes isolated from rats fed the control or the ethanol Lieber-DeCarli diets, a model of early alcohol-induced liver injury. Hepatocytes from ethanol-fed rats displayed increased lipid peroxidation,

elevated H_2O_2 and lower GSH, along with induced CYP2E1. $KLF6_{Full}$ mRNA was analyzed in hepatocytes and in total liver and found to be elevated about two-fold in ethanol-fed rats. Since $KLF6_{Full}$ has not been described to undergo alternative splicing in rats, the splice variants were not analyzed. $KLF6_{Full}$ up-regulation in primary hepatocytes from chronic ethanol-fed rats may reflect an adaptive mechanism to protect against CYP2E1-derived oxidant stress. It could be postulated that in alcohol-induced liver injury, where an oxidative burst occurs due to CYP2E1 up-regulation and alcohol metabolism, modulation of KLF6 expression contributes to cell defense. Splicing of KLF6, at least in humans, may be an early event in alcohol-induced liver injury, leading to changes in KLF6 expression and activation of protein kinases that contribute to liver damage and/or repair. In addition to alcoholic liver disease, KLF6 has been associated with non-alcoholic liver disease (Starkel et al., 2003) and hepatocellular carcinoma (Sirach et al., 2007b; Tarocchi et al., 2011; Zhenzhen et al., 2011).

Since $KLF6_{Full}$ expression appeared regulated by ROS and many protein kinases are highly responsive under oxidant stress conditions, we studied the role of stress-activated kinases to the ROS modulation of $KLF6_{Full}$. Addition of an inhibitor of p38 phosphorylation increased $KLF6_{Full}$ mRNA whereas inhibitors of JNK or of MEK1/2 decreased $KLF6_{Full}$ mRNA. Since AA induced $KLF6_{Full}$ mRNA and SB203580, an inhibitor of p38 phosphorylation has been shown to prevent AA toxicity in the CYP2E1 expressing cells (Wu and Cederbaum, 2003), we next analyzed whether p38 phosphorylation could play a role in modulating $KLF6_{Full}$ mRNA levels. Incubation of cells with SB203580 prior to AA addition further increased $KLF6_{Full}$ mRNA levels, likely contributing to cellular defense; while addition of SP60025 or PD98059, inhibitors of phosphorylation of JNK or MEK1/2 barely lowered $KLF6_{Full}$ mRNA expression.

Because of the essential role of TNFα in alcoholic liver disease, we next investigated whether TNFα could be a target of either $KLF6_{Full}$ or *KLF6_V1*, the main KLF6 isoforms induced by oxidant stress. Knock down of $KLF6_{Full}$ slightly decreased TNFα promoter transactivation and blunted the response to AA. In contrast, *KLF6_V1* ablation increased TNFα promoter transactivation, which was further enhanced by AA. This finding suggests that TNFα may be a direct target of KLF6_V1. These results were also validated in human stellate cells, which also have the ability to generate TNFα upon the onset of liver injury (not shown). In conclusion, these results indicate that several sources of endogenous ROS increase $KLF6_{Full}$ expression and its splice variants; thus, contributing to modulating a critical downstream target such as TNFα.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AA	Arachidonic acid
BSO	L-buthionine sulfoximine
CYP2E1	cytochrome P450 2E1
DAS	diallylsulfide
GSH	glutathione
KLF6	Krüppel-like factor 6
KLF6_V1	KLF6 variant 1
KLF6_V2	KLF6 variant 2
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NEFA	non-esterified fatty acids
NOS2	nitric oxide synthase 2
NQO1	NAD(P)H quinone oxidoreductase
PUFA	polyunsaturated fatty acids
ROS	reactive oxygen species
TBARS	thiobarbituric acid-reactive substances

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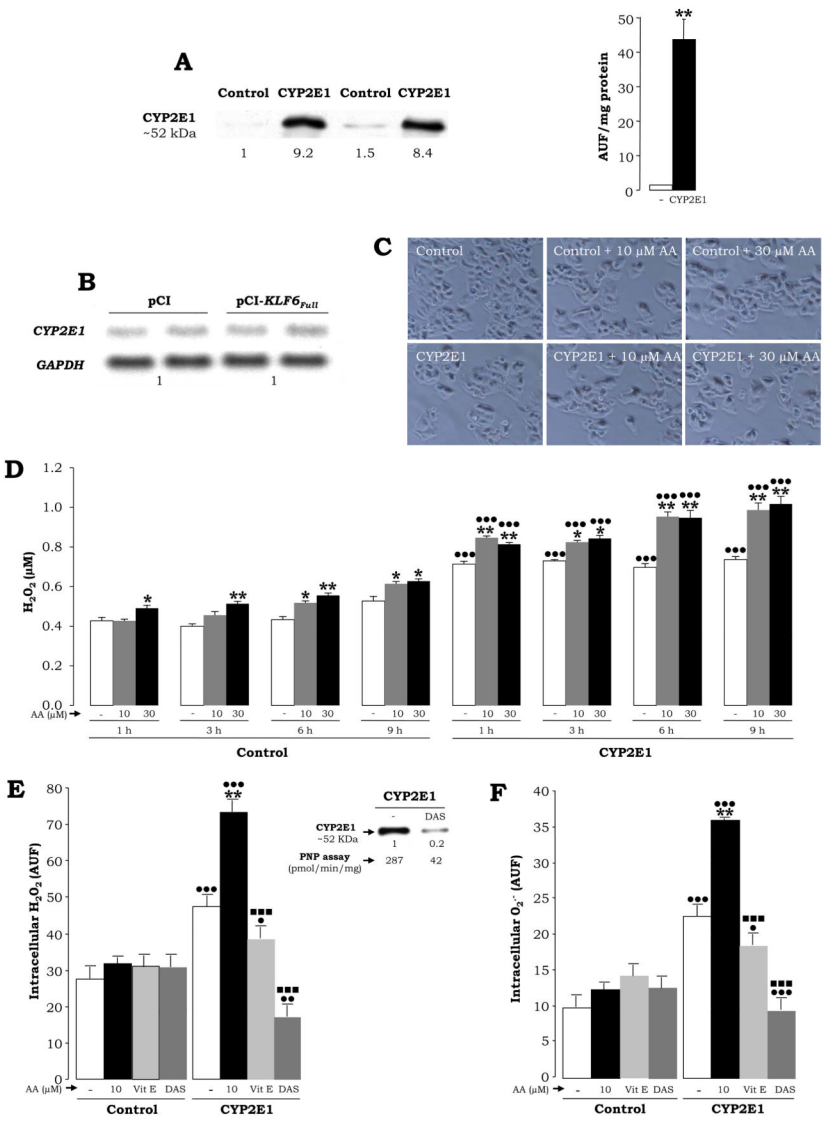

Fig. 1. CYP2E1-Expressing Cells Generate ROS in the Absence or Presence of AA

Control and CYP2E1-expressing cells were analyzed for CYP2E1 protein by Western blot. The quantification of the signal was referred to the first control, which was assigned a value of 1. CYP2E1 activity was monitored by fluorimetry using 7-MFC as substrate. Results refer to mean ± SEM (N=3, **P<0.01) **(A)**. CYP2E1-expressing cells were transfected with either pCi-Neo or pCi-Neo-$KLF6_{Full}$ vectors and *CYP2E1* and *GAPDH* expression were analyzed by Northern blot **(B)**. Control and CYP2E1-expressing cells were treated with 0–30 μM AA for 6 h (100% viability) and light micrographs were taken **(C)**. Time-course study (1–9 h) of extracellular H_2O_2 in the presence of 0–30 μM AA **(D)**. Intracellular H_2O_2 and $O_2^{\cdot -}$ at 6 h after 30 μM AA-treatment in the presence of either 25 μM vitamin E or 5 mM diallylsulfide (DAS) using the fluorescent probes DCFDA **(E)** and DHE **(F)**. Results in (D) to (F) are means ± SEM (N=6; *P<0.05 and **P<0.01 for AA-treated *vs.* untreated, •P<0.05, ••P<0.01 and •••P<0.001 for CYP2E1-expressing cells *vs.* control, ■■■P<0.001 for co-treated *vs.* AA-treated).

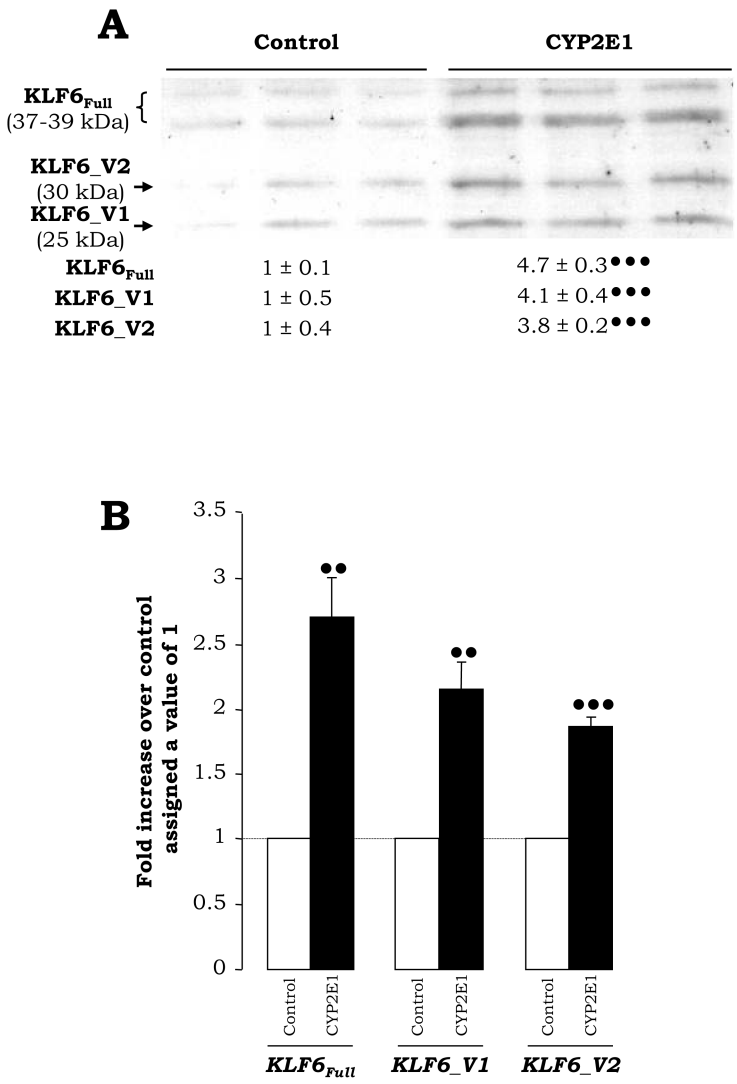

Fig. 2. Expression of KLF6$_{Full}$ and its Splice Variants in CYP2E1-Expressing Cells

The expression of *KLF6$_{Full}$*, *KLF6_V1* and *KLF6_V2* protein was analyzed by Western blot. The quantification of the signal was referred to the average signal in the controls, which was assigned a value of 1 **(A)**. qRT-PCR for *KLF6$_{Full}$*, *KLF6_V1* and *KLF6_V2* in control and CYP2E1-expressing cells **(B)**. In both panels, results are expressed as fold increase over the control which was assigned a value of 1 (dashed line in (B)) and refer to means ± SEM (N=3, ••P<0.01 and •••P<0.001 for CYP2E1-expressing cells *vs.* control).

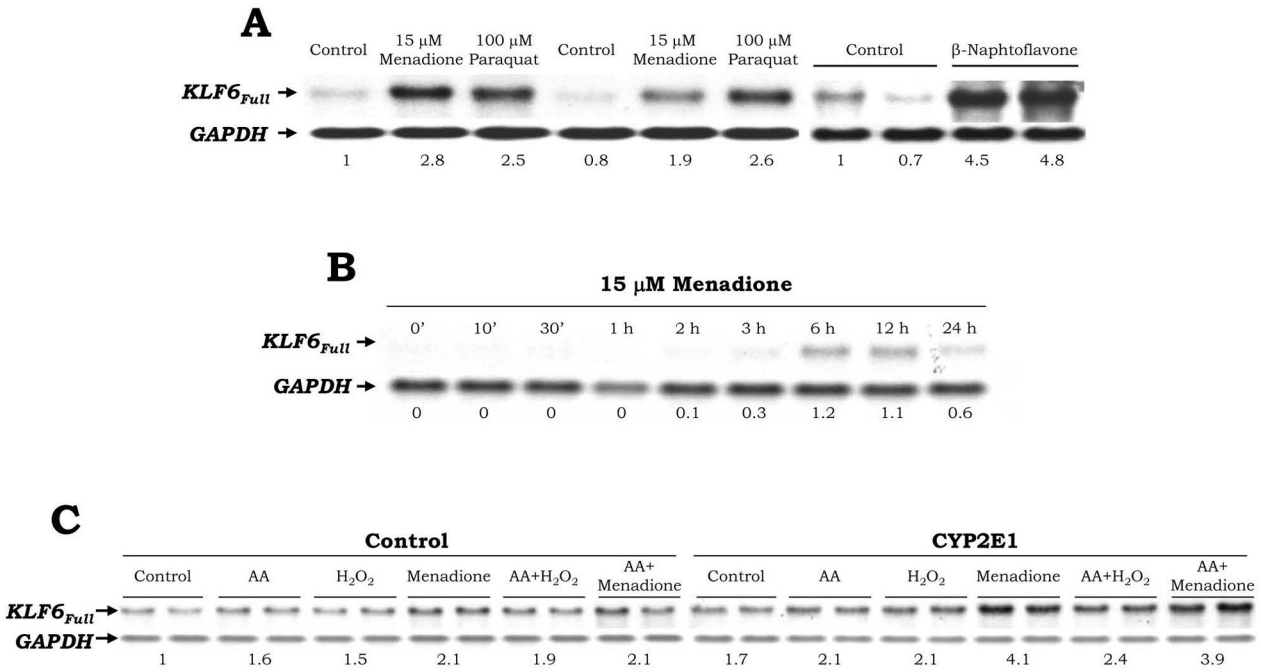

Fig. 3. Pro-oxidants Increase KLF6$_{Full}$

CYP2E1-expressing cells were treated in duplicate with 15 μM menadione, 100 μM paraquat or 100 μM β-naphtoflavone for 6 h **(A)** or in a time-course study up to 24 h with 15 μM menadione **(B)** and *KLF6$_{Full}$* expression was evaluated by Northern blot analysis. A value of 1 was given to the untreated cells in (A) and to the first time-point to show any signal in (B). Control and CYP2E1-expressing cells were treated with 30 μM AA, 20 μM H_2O_2, 15 μM menadione or the combination of AA plus H_2O_2 and AA plus menadione for 6 h and *KLF6$_{Full}$* expression was assessed by Northern blot analysis. The quantification of the signal indicated under the blots is the average of *N*=2. The signal for the untreated controls was assigned a value of 1 **(C)**.

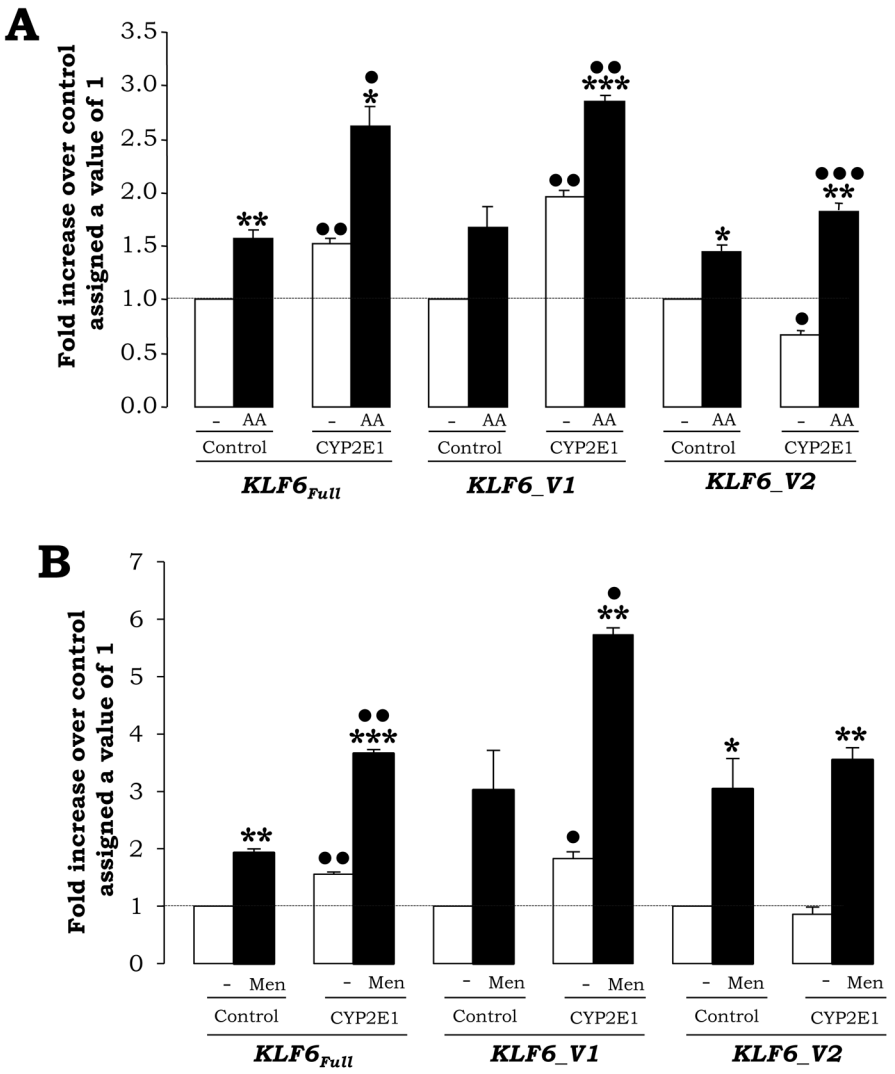

Fig. 4. Pro-oxidants Increase KLF6$_{Full}$ and its Splice Forms More in CYP2E1-Expressing Cells than in Controls

Control and CYP2E1-expressing cells were treated with 30 μM AA **(A)** or with 15 μM menadione **(B)** for 6 h and the expression of *KLF6$_{Full}$* and its splice forms *KLF6_V1* and *KLF6_V2* was assessed by qRT-PCR. Results are expressed as fold increase over the untreated controls, which were assigned a value of 1 (dashed line) and are means ± SEM (N=3; *P<0.05, **P<0.01 and ***P<0.001 for treated *vs.* control and •P<0.05, ••P<0.01 and •••P<0.001 for CYP2E1-expressing cells *vs.* control).

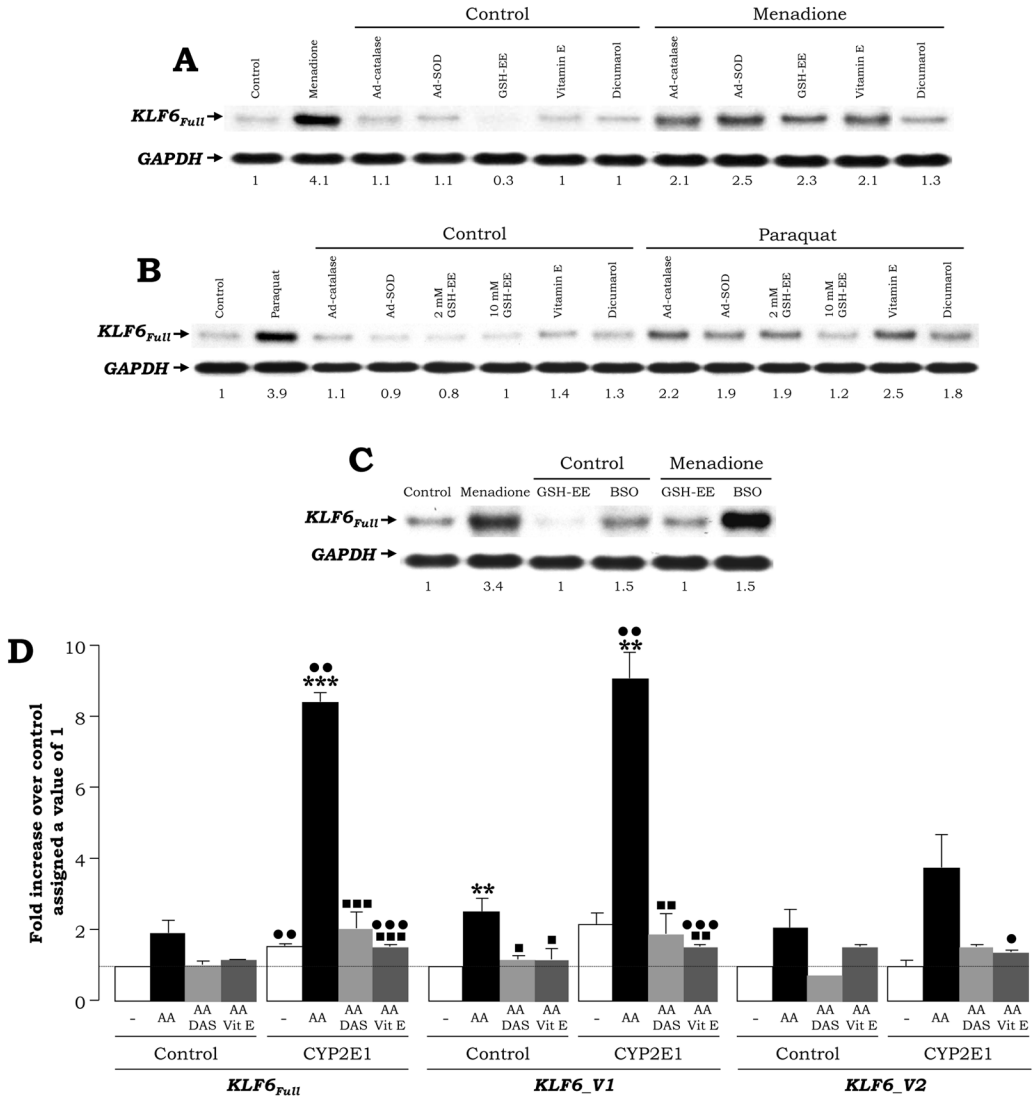

Fig. 5. Antioxidants and NQO1 and CYP2E1 Inhibitors Prevent the Increase in $KLF6_{Full}$ and its Splice Forms

CYP2E1 expressing cells were incubated with 15 μM menadione **(A)** and with 100 μM paraquat **(B)** in the absence or presence of adenoviruses expressing catalase or SOD, 2–10 mM GSH-EE, 25 μM vitamin E and 25 μM dicumarol. CYP2E1 expressing cells were also incubated with 15 μM menadione in the presence of 0–0.2 mM BSO **(C)** and Northern blot analysis were carried out to evaluate for $KLF6_{Full}$ expression. A value of 1 was given to the untreated cells. Control and CYP2E1-expressing cells were incubated with 0–30 μM AA in the presence of 25 μM vitamin E or of 5 mM diallylsulfide (DAS) for 6 h and the expression of $KLF6_{Full}$ and its splice forms *KLF6_V1* and *KLF6_V2* was assessed by qRT-PCR **(D)**. Results are expressed as fold increase over the untreated controls which were assigned a value of 1 (dashed line) and are means ± SEM (N=3; **P<0.01 and ***P<0.001 for AA-treated *vs.* control, •P<0.05, ••P<0.01 and •••P<0.001 for CYP2E1-expressing cells *vs.* control and ■P<0.05, ■■P<0.01 and ■■■P<0.001 for co-treated *vs.* AA-treated).

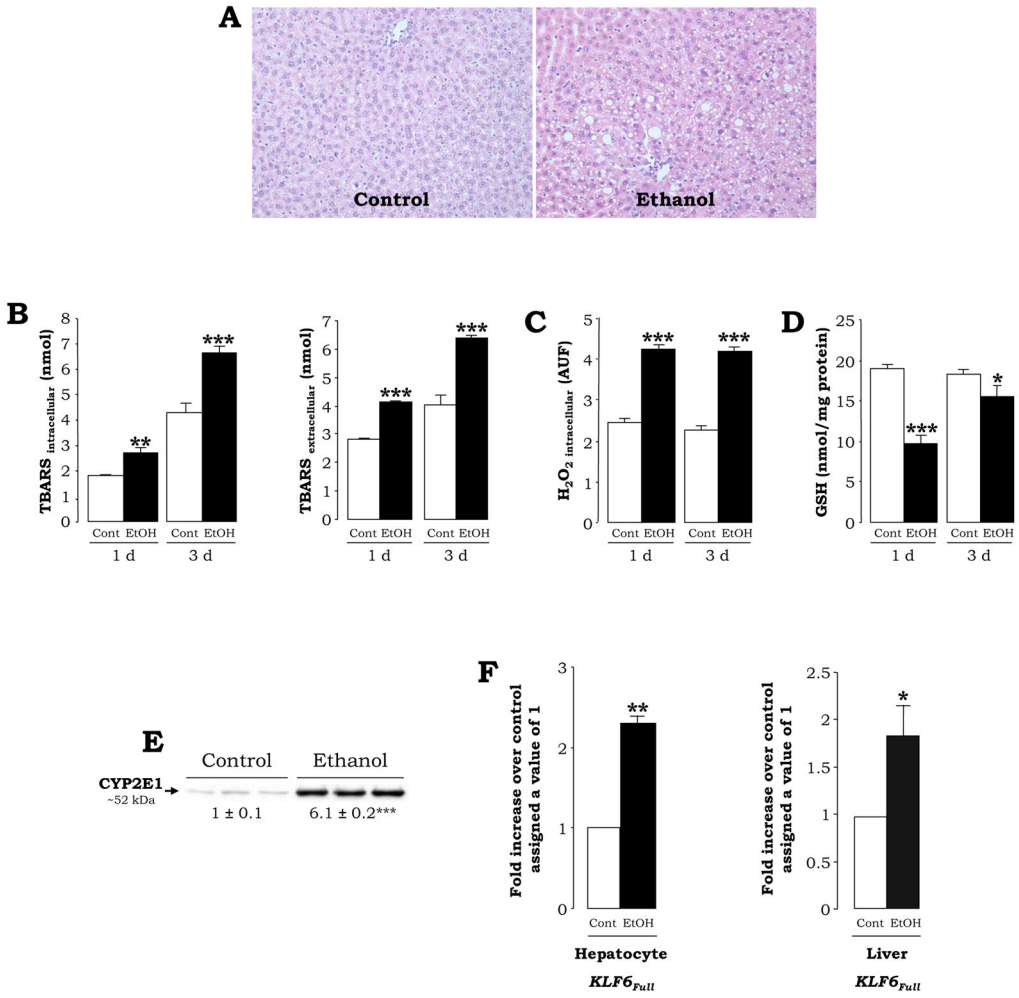

Fig. 6. Primary Hepatocytes and Livers from Ethanol-Fed Rats show Increased expression of $KLF6_{Full}$

H&E staining **(A)** in rats fed the control Lieber-DeCarli diet showed minimal steatosis (left), while rats fed the ethanol Lieber-DeCarli diet showed periportal and pericentral micro- and macro-vesicular steatosis (right) (original magnification=200x). Levels of intra- and extracellular lipid peroxidation (TBARS) **(B)**, intracellular H_2O_2 **(C)** and GSH **(D)**. Western blot analysis for CYP2E1 expression in hepatocytes from control and ethanol-fed rats **(E)**. Induction of $KLF6_{Full}$ expression in hepatocytes and in total liver **(F)** from ethanol-fed rats was determined by qRT-PCR. Results are expressed as fold-increase over the untreated controls, which were assigned a value of 1. In all panels results are means ± SEM (N=6–10, *P<0.05, **P<0.01 and ***P<0.001 for ethanol *vs.* control).

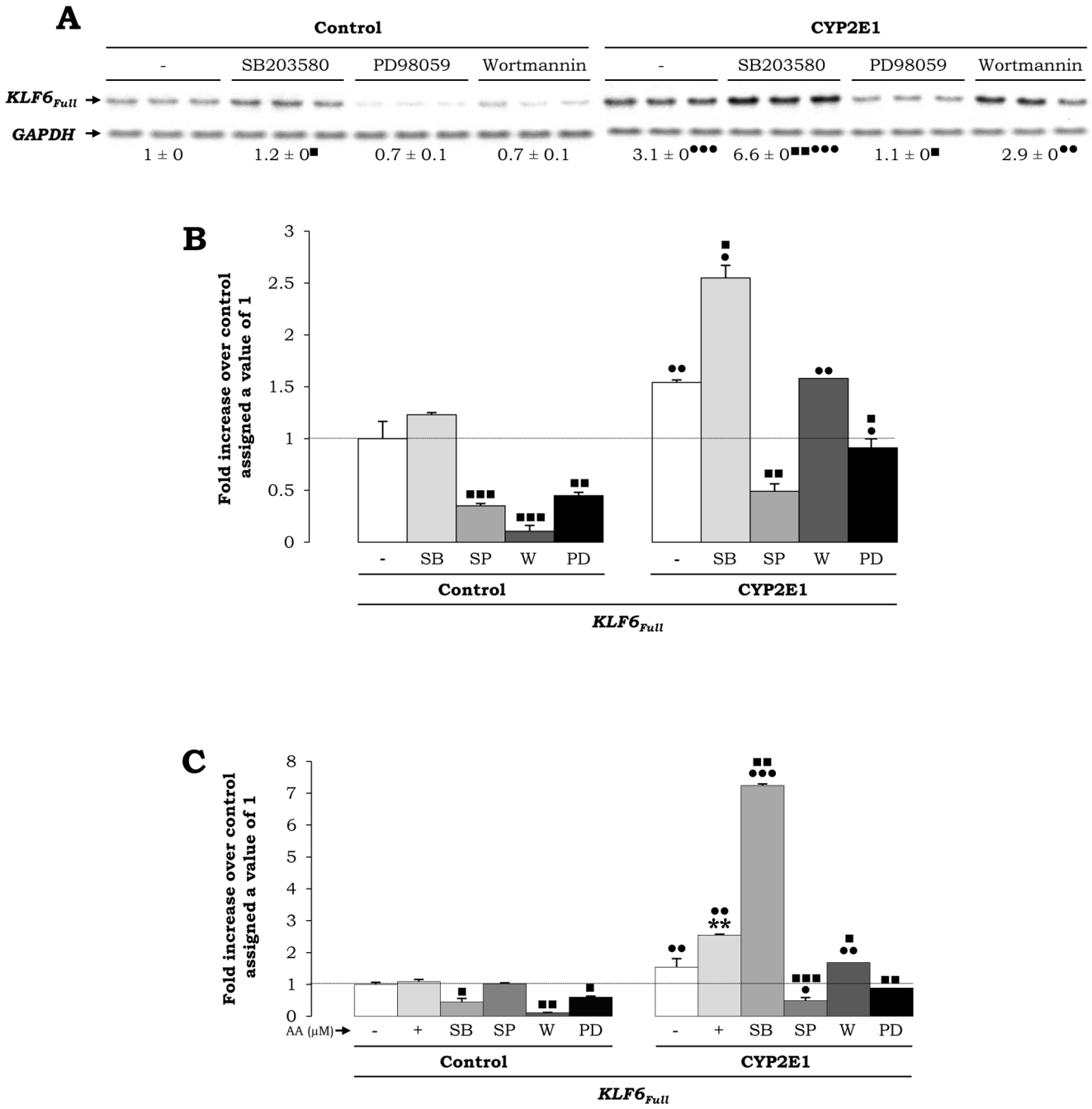

Fig. 7. KLF6$_{Full}$ Expression is Regulated by Stress-Activated Kinases

Control and CYP2E1-expressing cells were incubated in the presence of inhibitors of phosphorylation of p38 (SB203580), MEK1/2 (PD95059) and PI3K (Wortmannin) and the expression of *KLF6$_{Full}$* was assessed by Northern blot analysis. Untreated controls were assigned a value of 1 **(A)**. qRT-PCR for *KLF6$_{Full}$* in control and CYP2E1-expressing cells incubated with the above indicated inhibitors and with the inhibitor of JNK phosphorylation SP60025 added in the absence **(B)** or in presence of 30 μM AA **(C)**. In all panels, results are expressed as fold increase over the untreated control cells assigned a value of 1 and refer to means ± SEM. (*N*=3; ***P*<0.01 for AA-treated *vs.* control, •*P*<0.05, ••*P*<0.01 and

•••$P<0.001$ for CYP2E1-expressing cells *vs.* control and ■$P<0.05$, ■■$P<0.01$ and ■■■$P<0.001$ for co-treated *vs.* AA-treated).

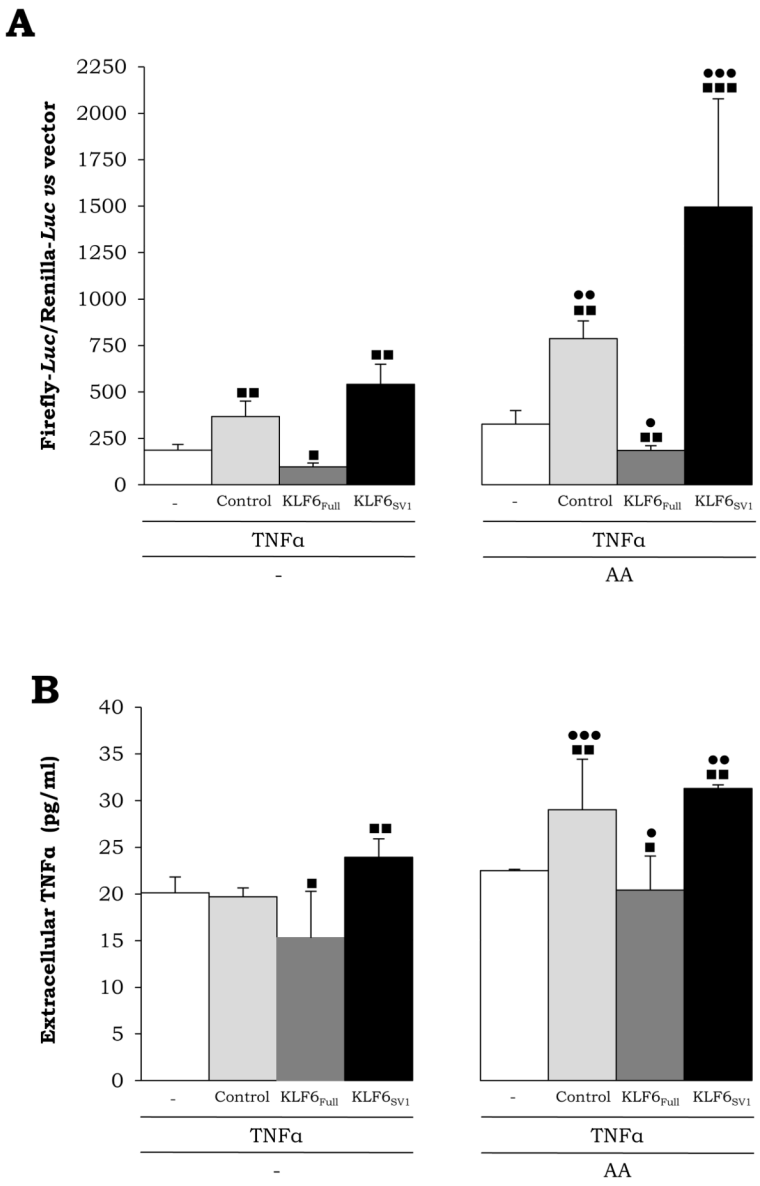

Fig. 8. KLF6_V1 Ablation Up-Regulates TNFα in the presence of AA

RAW 246.7 macrophages were transfected with the -982-*TNFα* promoter. Then, a co-transfection was carried out with control, $KLF6_{Full}$ or *KLF6_V1* siRNAs. In some experiments, cells were treated with 30 μM AA for 3 h. Luc activity after 48 hours of transfection was corrected by transfection efficiency and normalized with Renilla-Luc **(A)**. Secreted TNFα was measured in the respective supernatants by ELISA **(B)**. Results are expressed as mean ± SEM (N=6; ■P<0.05, ■■P<0.01 and ■■■P<0.001 for any siRNA *vs.* control and •P<0.05, ••P<0.01 and •••P< 0.0 01 f or A A-treated *vs.* control).

Table 1

Primers

mRNA	Forward primer	Reverse primer	Amplicon size
KLF6Fu ll	5′-**CGGACGCACACAG**GAGAAAA-3′ **Exon 2**-Exon 3 (nt 763–782)	5′-CGGTGTGCTTTCGGAAGTG-3′ Exon 3	103 bp
KLF6_V 1	5′-**CCTCGCCAGGGAAG**GAGAA-3′ **Exon 2-Exon 3** (nt 509–780)	5′-CGGTGTGCTTTCGGAAGTG-3′ Exon 3	104 bp
KLF6_V 2	5′-**GTCGGGGAAGCCA**GAGAA-3′ **Exon 2**-Exon 3 (nt 607–780)	5′-CGGTGTGCTTTCGGAAGTG-3′ Exon 3	103 bp
GAPDH	5′-CAATGACCCCTTCATTGACC-3′	5′-GATCTCGCTCCTGGAAGATG-3′	113 bp