Elevated PTEN Levels Account for the Increased Sensitivity of Ethanol-exposed Cells to Tumor Necrosis Factor-induced Cytotoxicity*

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Alcoholic liver disease (ALD) encompasses the spectrum of fatty liver, alcoholic hepatitis, and alcoholic cirrhosis. Fatty liver is a benign and reversible condition that develops in response to short term alcohol abuse. Prolonged alcohol abuse of 80 g of alcohol per day, which is the equivalent of 6–8 drinks daily, predisposes to alcoholic hepatitis that can progress to cirrhosis. Cytokines are critical in initiating and perpetuating the injury that leads to ALD. ALD is associated with an increase in the activity and infiltration of Kupffer cells and neutrophils into the liver parenchyma (1, 2). At this locale, inflammatory cells release a number of proinflammatory cytokines and chemokines. One of the most prevalent and best studied is tumor necrosis factor α (TNF). TNF is a central inciting agent in the genesis of ALD (3–7). This is suggested by the ability of Kupffer cell depletion to prevent the hepatic damage associated with an ethanol intragastric feeding model in rats (8). Additionally, antibodies neutralizing TNF prevented the onset of liver damage (9). In cases of ALD, a correlation was established between TNF levels and measurements of liver damage, such as serum alanine aminotransferase, creatinine, and bilirubin, in addition to a greater likelihood of mortality in patients with higher serum TNF values (10).

Tumor necrosis factor α (TNF) is known to be one of the primary causative cytokines inflicting the characteristic damage to hepatocytes seen in alcoholic liver disease. TNF activates both cell survival and death-inducing signaling pathways. The balance between these two prongs determines the fate of the cell and the onset of disease. Ethanol exposure has been shown to alter mitochondrial function, decreasing their threshold for injury. Importantly, mitochondrial injury is a necessary end point of TNF-induced cell killing. It has been shown that ethanol exposure increases the sensitivity of hepatocytes and HepG2E47 cells to TNF-mediated death. The cumulative and terminal effect of the increased sensitivity to TNF caused by ethanol is an induction of a mitochondrial permeability transition. TNF brings about the mitochondrial permeability transition in ethanol-exposed cells due to amplification in the activity of the p38 stress kinase and a diminution in the activity of the antiapoptotic Akt/PKB kinase. The present report identifies an increase of PTEN expression in ethanol-exposed cells as the main causative factor in altering the balance between prosurvival and prodeath signals initiated by TNF. Suppression of the elevated PTEN levels found in ethanol-exposed HepG2E47 cells through the use of RNA interference reversed the ethanol-induced alterations to TNF signaling, resulting in a preservation of mitochondrial function and cell viability.

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1 The abbreviations used are: ALD, alcoholic liver disease; TNF, tumor necrosis factor; MPT, mitochondrial permeability transition; ASK1, apoptosis signaling kinase 1; TNFR, TNF receptor; PI, phosphatidylinositol; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PBS, phosphate-buffered saline; PMS, phenazine methosulfate; pNA, p-nitroanilide; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; MAPK, mitogen-activated protein kinase; siRNA, small interfering RNA.
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events initiated by TNF to induce the MPT in the compromised mitochondria. Utilizing both primary hepatocytes isolated from control-fed and ethanol-fed rats and the hepatoma cell line HepG2E47, expressing cytochrome P4502E1, we have shown that ethanol exposure alters the intracellular signaling response to TNF (20). Specifically, ethanol-exposed cells exhibit an increase and decrease of p38 and Akt activity, respectively, upon TNF treatment. The increased p38 activity is responsible for mediating the translocation of Bax from the cytosol to the mitochondria. Bax has been shown to bring about mitochondrial damage, including induction of the MPT (21–23). Inhibition of the intensified p38 response prevented Bax activation, mitochondrial dysfunction, and the cell killing induced by TNF in ethanol-exposed cells (20). Conversely, increasing the activity of Akt in ethanol-treated cells by the use of a constitutively active construct of Akt preserved cell viability. Cell surface receptors such as the TNFR activate phosphatidylinositol 3-kinase, which phosphorylates PIP2 to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 is necessary for Akt activation. PTEN (phosphatase and tensin homologue deleted from chromosome 10) is a tyrosine phosphatase with dual protein and lipid phosphatase activity. PTEN recognizes PIP3 as a substrate and removes the D3 phosphate from the inositol ring. Loss of PTEN as seen in certain cancers leads to the accumulation of PIP3, resulting in the constitutive activation of the phosphatidylinositol 3-kinase/Akt pathway. By contrast, an increase of PTEN levels would be expected to decrease the concentration of PIP3 and cause an inhibition of receptor-stimulated Akt activity. The present report identifies an increase of PTEN expression caused by ethanol exposure as being accountable for mediating the array of alterations in TNF signaling seen in ethanol-exposed cells, including the accentuated p38 response, inhibition of Akt and NFkB signaling, and ultimately the mitochondrial damage culminating in cell death.

MATERIALS AND METHODS

Cell Culture and Treatments—Hepatoma G2E47 (HepG2E47) cells that express cytochrome P-4502E1 (CYP2E1) (kindly provided by Dr. Arthur I. Cederbaum) were maintained in 25-cm² polystyrene culture flasks with 5 ml of minimal essential medium containing 100 units/ml penicillin, 0.4 mg/ml streptomycin, 0.4 mg/ml G418, 1 mM pyruvate, and 10% heat-inactivated fetal bovine serum (complete minimal essential medium). Cells were subcultured 1:5 once a week. The cells were treated with 25 ng/ml TNF-α/H11003/ or into 6-well, 9.3-cm² plates at 1.0 x 10⁶ cells/well, respectively.

Measurements of Cell Viability—Cell viability was determined by trypan blue exclusion and the ability of cells to reduce 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). For the trypan blue exclusion assay, 10 μl of a 0.5% solution of trypan blue was added to the wells. For each data point, we have randomly selected eight microscopic fields and blindly counted a representative group of cells in each well for viability. Each experiment was carried out a minimum of three times. For the MTS assay, the reaction was started by the addition of MTS and phenazine methosulfate (PMS). The absorbance change obtained upon reduction of MTS by viable cells was read 90 min later with a plate reader at 490 nm. 100% cell death was determined by the addition of Triton X-100 to a final concentration of 0.5%, 30 min before MTS and PMS addition. The MTS assay and trypan blue exclusion gave identical results.

Detection of Caspase-3 and Caspase-8 Activity—The assay is based on the ability of the active enzymes to cleave the chromophore pNA from the enzyme substrates DEVD-pNA (caspase-3) or IETD-pNA (caspase-8). Cell extracts were prepared and diluted 1:1 with 2 × reaction buffer (20 mM Tris, pH 7.4, 1 mM dithiothreitol, 2 μM EDTA, 0.1 mM CHAPS, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml leupeptin). DEVD-pNA or IETD-pNA was added to a final concentration of 50 μM, and the reaction was incubated for 1 h at 37 °C. Samples were then transferred to a 96-well plate, and absorbance measurements were made in a 96-well plate reader at 405 nm.

Isolation of Cytosolic and Mitochondrial Fractions—Cells were plated in 25-cm² flasks at a density of 10⁶ cells/flask. After treatments, the cells were harvested by trypsinization followed by centrifugation at 600 x g for 10 min at 4 °C. The cell pellets were washed once in PBS and then resuspended in 3 volumes of isolation buffer (20 mM HEPES, pH 7.4, 10 mM KC1, 1.5 mM MgCl₂, 1 mM sodium-EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1.0 μg/ml leupeptin, 1.0 μg/ml aprotinin in 250 mM sucrose). After being chilled on ice for 3 min, the cells were disrupted by 40 strokes in a glass homogenizer. The homogenate was centrifuged twice at 2,500 x g for 10 min at 4 °C to remove unbroken cells and nuclei. The mitochondria were then pelleted by centrifugation at 12,000 x g for 4 °C for 30 min. The supernatant was removed and filtered through 0.2-μm filters of a glass homogenizer. The pellets were then separated on 12% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. Cytochrome c was detected by a monoclonal antibody (Pharmingen, San Diego, CA) at a dilution of 1:5,000. Secondary goat anti-mouse horseradish peroxidase-labeled antibody (Santa Cruz Biotechnology). Four hundred cells were subjected to the assay and the control was treated with 25 ng/ml TNF-α/H11003/. The cells were discarded, and the samples were incubated for 1 h at 37 °C.

Preparation of Hepatocytes and Ethanol Diet—Hepatocytes were prepared from Charles River Laboratories (Raleigh, NC). Nutritionally adequate liquid diets were formulated according to the method of Seglen (30). Yields of 2–4 x 10⁷ cells/liver with 90–95% viability as assessed by trypan blue exclusion were routinely obtained. Hepatocytes were either plated in 24- or 6-well plates at 1.0 x 10⁶ or 1.0 x 10⁸ cells/well, respectively.

Enzyme-linked Immunosorbent Assay of Akt, p38 MAPK, and NF-κB Activation—Cells were collected in PBS by centrifugation. The cells were then lysed in cell extraction buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na₃PO₄, 2 mM Na₂VO₃, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture plus 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxy-
cholate). Standards, samples, or controls were added to microtiter wells coated with the appropriate antibody (anti-p38 MAPK, anti-Akt, or anti-p65). The wells were covered and incubated for 2 h at room temperature. The wells were then decanted and thoroughly washed four times. 100 μl of an antibody, either anti-p38 MAPK (pTyr180/182), anti-p38 MAPK, anti-Akt (pS473), anti-Akt, or anti-p65, was added to the wells and incubated for 1 h at room temperature. The solution was decanted, and the wells were washed four times. Afterward, 100 μl of horseradish peroxidase-conjugated anti-rabbit IgG was added to each well. The wells were incubated for an additional 30 min. The wells were decanted and washed four times. 100 μl of stabilized chromogen was added to each well and incubated for 30 min in the dark; 100 μl of stop solution was added to each well. The absorbance of each well was then read at 450 nm on a Synergy HTS microplate reader.

RNA Interference—The Dharmacon SMART selection and SMART pooling technologies are utilized for the RNA interference studies. The SMART selection uses an algorithm composed of 33 criteria and parameters that attempt to limit nonfunctional small interfering RNAs (siRNAs). SMART pooling combines four SMART-selected siRNA duplexes in a single pool to increase the probability of reducing mRNA levels by at least 75%. In addition, the siRNA duplexes comprising the pool were tested individually for their effect on PTEN expression and phenotype of the cells. The siRNAs 1, 2, and 3 all decreased PTEN expression with siRNA 1 giving the most consistent results. Therefore, siRNA 1 was used in subsequent experiments to assess the effects of decreasing PTEN levels on the sensitivity of ethanol-exposed cells to TNF. The siRNA was delivered by a lipid-based method supplied from a commercial vendor (Gene Therapy Systems) at a final siRNA concentration of 100 nM. After formation of the siRNA-liposome complexes, the mix was added to the control or ethanol-exposed HepG2E47 cells in serum-free medium for 4 h. Afterward, the medium was aspirated, and complete medium was added back with or without the addition of 25 mM ethanol for a further 24 h.

RESULTS

Ethanol Exposure Increases PTEN Levels and PTEN Promoter Activity—HepG2E47 cells were exposed to ethanol at a concentration of 25 mM over a 48-h time frame. The level of ethanol in the cell culture medium over a 24-h time course is shown in Fig. 1A. As can be seen, the ethanol level remained relatively constant, reaching a minimum of 22 mM ethanol at 24 h, at which point the cells were washed and fresh medium was added containing 25 mM ethanol, for a total exposure time of 48 h. Afterward, the medium was aspirated, and complete medium was added back with or without the addition of 25 mM ethanol for a further 24 h.
Fig. 2. Suppression of PTEN levels by RNA interference. In all of the panels, HepG2E47 cells were either left untreated or exposed to 25 mM ethanol for 24 h. The cells were then transfected with siRNAs targeting PTEN, luciferase, or lamin A/C. The cells were then cultured for another 24 h in the presence or absence of ethanol. The cells were then harvested, and the levels of PTEN were determined by Western or Northern blotting. A, a Western blot showing the down-regulation of PTEN levels by a pool of four siRNAs targeting PTEN and the lack of effect of siRNAs against luciferase on PTEN levels. B, the down-regulation of PTEN levels by three separate siRNAs constituting the pooled siRNAs against PTEN. In addition, the lack of effect of siRNA targeting lamin A/C on PTEN levels is shown. C, Northern blot analysis demonstrates an increase of PTEN mRNA levels promoted by ethanol exposure and its prevention by siRNA 1 targeting PTEN. D, the specificity of the siRNA targeting PTEN is demonstrated by its lack of effect on lamin A/C levels. Conversely, siRNA targeting lamin A/C results in lamin A/C down-regulation but no effect on PTEN expression. A nontarget control siRNA having no homology to any known gene had no effect on the levels of PTEN or lamin. Results shown are typical of three independent experiments.

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PTEN ablated its expression in both control (lane 3) and ethanol-exposed HepG2E47 cells (lane 4). By contrast, siRNA targeting luciferase had no effect on PTEN levels (lanes 5 and 6). The individual siRNAs constituting the pool were then tested for their ability to decrease expression of PTEN. PTEN levels were repressed by siRNAs 1, 2, and 3 (Fig. 2B). In addition, Fig. 2C demonstrates an increase of PTEN mRNA levels induced by ethanol exposure and its elimination by siRNA 1 targeting PTEN. A siRNA targeting lamin A/C was without effect on PTEN expression but did ablate the expression of lamin A/C (Fig. 2D). Importantly, siRNA 1 against PTEN did not alter the expression of lamin but did down-regulate expression of PTEN (Fig. 2D). Additionally, a nontargeting control siRNA that possesses no homology to any known gene had no effect on the levels of both PTEN and lamin A/C (Fig. 2D). These experiments validate the efficacy and selectivity of the siRNA targeting PTEN to decrease PTEN levels.

Elevated PTEN Levels Inhibit TNF Activation of Akt in Ethanol-exposed Cells—The phosphatidylinositol 3-kinase-Akt signaling pathway is critical in the inhibition of apoptosis. TNF activates Akt, and this is stunted in ethanol-exposed cells. As can be seen in Fig. 3A, TNF treatment of HepG2E47 cells results in a rapid increase in Akt activation, starting as early as 15 min following TNF addition and peaking at 10-fold above basal levels at 60 min. By contrast, HepG2E47 cells exposed to 25 mM ethanol for 48 h exhibited a restrained activation of Akt upon TNF treatment, with a delay in both the onset and extent of Akt activation, with peak levels of only 3-fold above basal levels at 60 min following TNF addition. HepG2E47 cells were subsequently washed and placed in medium without ethanol. Ethanol withdrawal resulted in a sharp decrease of PTEN levels by 16 h, with the PTEN levels returning to near control values by 24 h postexposure and going below them by 48 h. Also shown in Fig. 1C, PTEN levels were found to be elevated in hepatocytes isolated from ethanol-fed rats compared with their control-fed counterparts. This result is consistent with previous studies showing an elevation of PTEN in the livers of ethanol-fed rats (24). The ability of ethanol to increase PTEN levels is partly due to a stimulation of the PTEN promoter. A reporter plasmid was constructed containing 1350 base pairs of the human PTEN promoter (NCBI accession number NM_000314) inserted upstream of the luciferase gene. The plasmid was transfected into HepG2E47 cells, and the luciferase assay was performed at various time points during ethanol exposure. As can be seen in Fig. 1D, there is an ~5-fold increase in PTEN promoter activation after 24 h of ethanol exposure. Conversely, there was a rapid drop in PTEN promoter activity upon ethanol withdrawal, with levels decreasing by half at 24 h after ethanol treatment.

Selective Suppression of PTEN by siRNA—The method of RNA interference was utilized to suppress PTEN levels in ethanol-treated cells. A pool of four siRNA were designed by computer algorithm taking into account 34 separate variables. HepG2E47 cells were either left untreated or exposed to ethanol for 24 h. The cells were then transfected with siRNA targeting PTEN or luciferase. The cells were then incubated for another 24 h in the presence or absence of 25 mM ethanol. As can be seen in Fig. 2A, 48 h post-transfection, the siRNAs targeting PTEN ablated its expression in both control (lane 3) and ethanol-exposed HepG2E47 cells (lane 4). By contrast, siRNA targeting luciferase had no effect on PTEN levels (lanes 5 and 6). The individual siRNAs constituting the pool were then tested for their ability to decrease expression of PTEN. PTEN levels were repressed by siRNAs 1, 2, and 3 (Fig. 2B). In addition, Fig. 2C demonstrates an increase of PTEN mRNA levels induced by ethanol exposure and its elimination by siRNA 1 targeting PTEN. A siRNA targeting lamin A/C was without effect on PTEN expression but did ablate the expression of lamin A/C (Fig. 2D). Importantly, siRNA 1 against PTEN did not alter the expression of lamin but did down-regulate expression of PTEN (Fig. 2D). Additionally, a nontargeting control siRNA that possesses no homology to any known gene had no effect on the levels of both PTEN and lamin A/C (Fig. 2D). These experiments validate the efficacy and selectivity of the siRNA targeting PTEN to decrease PTEN levels.

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Fig. 2. Suppression of PTEN levels by RNA interference. In all of the panels, HepG2E47 cells were either left untreated or exposed to 25 mM ethanol for 24 h. The cells were then transfected with siRNAs targeting PTEN, luciferase, or lamin A/C. The cells were then cultured for another 24 h in the presence or absence of ethanol. The cells were then harvested, and the levels of PTEN were determined by Western or Northern blotting. A, a Western blot showing the down-regulation of PTEN levels by a pool of four siRNAs targeting PTEN and the lack of effect of siRNAs against luciferase on PTEN levels. B, the down-regulation of PTEN levels by three separate siRNAs constituting the pooled siRNAs against PTEN. In addition, the lack of effect of siRNA targeting lamin A/C on PTEN levels is shown. C, Northern blot analysis demonstrates an increase of PTEN mRNA levels promoted by ethanol exposure and its prevention by siRNA 1 targeting PTEN. D, the specificity of the siRNA targeting PTEN is demonstrated by its lack of effect on lamin A/C levels. Conversely, siRNA targeting lamin A/C results in lamin A/C down-regulation but no effect on PTEN expression. A nontarget control siRNA having no homology to any known gene had no effect on the levels of PTEN or lamin. Results shown are typical of three independent experiments.
exposed for 24 h to 25 mM of ethanol followed by transfection with siRNA targeting PTEN or lamin A/C. After a further 24 h of culture in the presence or absence of ethanol, the cells were treated with 10 ng/ml TNF. The cells were harvested at the indicated time points. The level of activated Akt phosphorylated on serine 473 was determined by enzyme-linked immunosorbent assay. B, the level of total Akt was determined in control, HepG2E47 cells exposed to ethanol for 48 h and in ethanol-exposed HepG2E47 cells treated with siRNA targeting PTEN. Results shown are the mean of three independent experiments.

**Fig. 3. Elevated PTEN levels in ethanol-exposed cells blunt activation of Akt by TNF.** A, HepG2E47 cells were either exposed to 25 mM ethanol for 24 h or left untreated. The cells were then transfected with siRNA targeting PTEN or lamin A/C. After a further 24 h of culture in the presence or absence of ethanol, the cells were treated with 10 ng/ml TNF. The cells were harvested at the indicated time points. The level of activated Akt phosphorylated on serine 473 was determined by enzyme-linked immunosorbent assay. B, the level of total Akt was determined in control, HepG2E47 cells exposed to ethanol for 48 h and in ethanol-exposed HepG2E47 cells treated with siRNA targeting PTEN. Results shown are the mean of three independent experiments.

Exposed for 24 h to 25 mM of ethanol followed by transfection with siRNA 1 targeting PTEN or siRNA against lamin A/C, whereupon they were exposed to ethanol for an additional 24 h. The cells were then treated with TNF, and Akt activation was determined. As can be seen in Fig. 3A, suppression of the ethanol-induced elevation of PTEN restored the ability of TNF to activate Akt, with levels of Akt phosphorylation reaching those seen in cells not exposed to ethanol. By contrast, siRNA to lamin A/C did not restore the ability of TNF to activate Akt in cells treated with ethanol. Importantly, as shown in Fig. 3B, the level of Akt protein was the same in control, ethanol-exposed cells and in ethanol-exposed cells treated with siRNA against PTEN, thus showing that the measured decrease in the activation of Akt by TNF in ethanol-treated cells and its restoration by siRNA targeting PTEN cannot be accounted for by a variability of total Akt levels.

**Suppression of PTEN Restores TNF-induced NF-κB Activation in Ethanol-exposed Cells—**The transcription factor NF-κB is a critical mediator in the protective pathways initiated by TNF. Akt has been shown to augment the activation of NF-κB in some contexts (12, 25). As shown in Fig. 4, TNF treatment led to a rapid and robust activation of NF-κB in control HepG2E47 cells over a 2-h time course. By contrast, in cells exposed to ethanol for 48 h, TNF treatment induced only a modest increase of NF-κB activity. However, as with TNF stimulation of Akt, the ability of TNF to activate NF-κB was restored in both rate and extent to that seen in control cells not exposed to ethanol when PTEN levels were ablated by siRNA. By contrast, ethanol-exposed cells transfected with siRNA to lamin A/C displayed no reversal of the inhibition of TNF-induced NF-κB activation induced by ethanol. In order to establish the importance of Akt in mediating TNF activation of NF-κB, stable transfectants expressing a constitutively activated form of Akt (myristoylated Akt) were generated. As can be seen in Fig. 4, ethanol exposure was unable to prevent TNF from activating NF-κB in the cells expressing constitutively active Akt. TNF stimulated NF-κB to the same rate and extent in ethanol-treated cells expressing constitutively activated Akt as in cells not exposed to ethanol. These results indicate that the elevation of PTEN levels evoked by ethanol exposure and the resultant repression of TNF-induced Akt activity are responsible for the decreased stimulation of NF-κB by TNF.

**Elevated PTEN Levels Potentiate TNF Activation of p38 in Ethanol-exposed Cells—**We have shown that ethanol exposure results in a magnified response of the stress kinase, p38, to TNF treatment. These results were reproduced and are shown in Fig. 5. TNF stimulates an initial moderate activation of p38 in control cells that peaks at 15 min and rapidly decays after 1 h. TNF stimulated p38 to the same rate and extent in ethanol-exposed cells as in nonexposed controls during the initial 45 min of treatment. However, there was a second and much greater increase of p38 activation induced by TNF in the ethanol-exposed cells, with p38 activation reaching 6-fold above basal levels at 2 h and being sustained for up to 4 h. By contrast, in ethanol-exposed cells in which PTEN expression was suppressed by siRNA, there was a restrained stimulation of p38 by TNF treatment, with the cells exhibiting only the early onset and modest p38 activation seen in control cells and
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FIG. 5. Potentiation of TNF-induced p38 activity in ethanol-exposed cells is dependent on an elevation of PTEN. HepG2E47 cells were either left untreated or exposed to 25 mM ethanol for 24 h. The cells were then either left alone or transfected with siRNA against lamin A/C. After a further 24 h of incubation in 25 mM ethanol, the cells were treated with 10 ng/ml TNF. Alternatively, HepG2E47 cells expressing constitutively active myristoylated Akt (Myr-Akt) were either left untreated or exposed to 25 mM ethanol for 48 h. The cells were washed, and the lysates were assayed using an enzyme-linked immunosorbent assay for active p38 phosphorylated on tyrosine 180 and 182. Results shown are the mean of three independent experiments.

FIG. 6. Ethanol exposure prevents ASK-1 phosphorylation by Akt in TNF-treated cells, resulting in a potentiation of p38 activity. A, HepG2E47 cells were either left untreated (lane 1) or exposed to 25 mM ethanol for 24 h. The cells were then transfected with a pool of siRNAs targeting PTEN (lane 2), siRNA targeting lamin A/C (lane 3), or lamin A/C (lane 4). B, HepG2E47 cells were left untreated or exposed to 25 mM ethanol for 24 h. The cells were then transfected with siRNA targeting ASK-1 (lane 5) or lamin A/C (lane 6). C, HepG2E47 cells were left untreated or exposed to 25 mM ethanol for 24 h. The cells were transfected with siRNA targeting ASK-1 (lane 7) or lamin A/C (lane 8). The elevation of PTEN levels in ethanol-exposed cells results in a potentiation of p38 activity.

PTEN Prevents Inactivation of ASK-1 by TNF in Ethanol-treated Cells—Apoptosis signaling kinase-1 (ASK-1) is a MAPK kinase kinase that is capable of activating the p38 stress kinase pathway. It is regulated by both oxidative stress and phosphorylation. Akt negatively regulates ASK-1. Akt phosphorylates ASK-1 on serine 83, preventing it from activating p38. By contrast, ASK-1 phosphorylation on serine 83 is determined by Western blotting utilizing a phosphospecific antibody. B, HepG2E47 cells were either left untreated or transfected with siRNA targeting ASK-1 or PTEN. The levels of total ASK-1 were determined by Western blotting. C, HepG2E47 cells were either left untreated or exposed to 25 mM ethanol for 24 h. The cells were then either left alone or transfected with siRNA targeting ASK-1. For all conditions, at the indicated time points, the cells were harvested, and the lysates were assayed using an enzyme-linked immunosorbent assay for active p38 phosphorylated on tyrosine 180 and 182. Results shown are the mean of three independent experiments.

The levels of the activated form of NF-κB in the nuclear extracts were measured by an ability to bind the NF-κB consensus site coated in a 96-well plate. After binding and washing, the p65 subunit was detected by primary and secondary antibodies, providing a colorimetric readout. Results shown are the mean of three independent experiments.

Not the later and more robust p38 activation stimulated by TNF in cells exposed to ethanol. By contrast, siRNA to lamin A/C had no effect on the augmented activation of p38 by TNF in ethanol-treated cells. Furthermore, as shown in Fig. 5, expression of constitutively active Akt (Myr-Akt) was also able to suppress the augmented stimulation of p38 brought about by TNF in ethanol-exposed cells. Such results indicate that the elevated PTEN levels characteristic of ethanol exposure inhibit the TNF-initiated Akt activity that is necessary to dampen the response of p38 to TNF treatment.

The levels of total ASK-1 were determined by Western blotting. C, HepG2E47 cells were either left untreated or exposed to 25 mM ethanol for 24 h. The cells were then either left alone or transfected with siRNA targeting ASK-1. For all conditions, at the indicated time points, the cells were harvested, and the lysates were assayed using an enzyme-linked immunosorbent assay for active p38 phosphorylated on tyrosine 180 and 182. Results shown are the mean of three independent experiments.

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Fig. 7. Suppression of elevated PTEN levels in ethanol-exposed cells prevents the TNF-induced activation of Bax and caspase-3 and the release of cytochrome c into the cytosol. HepG2/E47 cells were either left untreated or exposed to 25 mM ethanol for 24 h. The cells were then transfected with siRNA targeting PTEN and exposed to ethanol for a further 24 h. A, the cells were harvested at 2 h post-TNF treatment, the cell lysates were assayed for caspase-8 and caspase-3 activity as described under “Materials and Methods.” In B and C, the cell lysates were assayed for caspase-8 and caspase-3 activity as described under “Materials and Methods.” The levels of cytochrome c (Cyt. C) or Bax were assessed by Western blotting. The results shown are typical of three independent experiments.

Suppression of PTEN Prevents TNF from Promoting Bax-induced Mitochondrial Injury and Cell Killing in Ethanol-exposed Cells—We have shown that cell death in ethanol-exposed cells treated with TNF is independent of caspase-8 but is accompanied by caspase-3 stimulation. As can be seen in Fig. 7A, suppression of PTEN did not decrease substantially the activation of caspase-8 by TNF treatment in ethanol-exposed cells but did prevent the elevation of caspase-3 activity provoked by TNF. The increase of caspase-3 activity in ethanol-exposed cells treated with TNF is independent on mitochondrial injury. As can be seen in Fig. 7B, in ethanol-treated cells there is a small increase of the proapoptotic protein Bax in the mitochondria (Fig. 7B, lane 2). However, treatment of ethanol-exposed cells with TNF induces a rapid and robust translocation of Bax from the cytosol to the mitochondria in ethanol-exposed cells (Fig. 7B, lane 3), a translocation prevented by suppressing PTEN levels (Fig. 7B, lane 4). Concomitantly, lowering PTEN levels prevented the mitochondrial damage promoted by TNF in ethanol-exposed cells. As shown in Fig. 7C, ethanol exposure resulted in a TNF-induced release of the intermembrane space protein, cytochrome c, from the mitochondria and its accumulation in the cytosol. Repressing PTEN levels in ethanol-exposed cells treated with TNF prevented the loss of cytochrome c from mitochondria and its localization to the cytosol (Fig. 7C). Similarly, PTEN repression prevented mitochondrial membrane depolarization and cell death induced by TNF in ethanol-exposed cells. TNF treatment of ethanol-exposed cells caused a rapid drop in mitochondrial membrane potential that was complete by 3 h (Fig. 8A, circles). The siRNA against PTEN prevented mitochondrial depolarization in ethanol-exposed cells treated with TNF (Fig. 8A, squares). TNF also induced cell killing in ethanol-exposed cells over a 6-h time course, with 80% of the cells losing viability by 6 h (Fig. 8B, circles). Suppression of PTEN levels substantially inhibited TNF-induced cell killing, with less than 20% of the ethanol-exposed cells losing viability at 6 h post-TNF treatment (Fig. 8B, squares).

Discussion

The present study demonstrates that the alterations to TNF signaling pathways induced by ethanol exposure are largely attributable to an increase of PTEN levels. Suppression of the elevated PTEN levels induced by ethanol through the use of RNA interference resulted in a reversal in the responses of ethanol-exposed cells to TNF to those characteristic of cells not exposed to ethanol, including a restoration of TNF-induced Akt and NF-κB activation, a decrease in the exaggerated p38 re-
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The adenoviral early region 1A protein mediates sensitization to different cell death-inducing stimuli including TNF (43). E1A expression was shown to result in a down-regulation of Akt activity and increase of p38 activity, inhibition of which prevents sensitization by E1A to TNF. Similarly, in the present study, the restoration of TNF-initiated Akt activation by repression of PTEN levels in ethanol-exposed cells diminished the p38 response to TNF, resulting in an inhibition of the translocation of Bax to the mitochondria and its attendant damage. In addition, constitutively active Akt that is unresponsive to PTEN inhibition prevented the magnified p38 activation in ethanol-exposed cells treated with TNF, thereby also preserving mitochondrial integrity and cell viability. Interestingly, the only parameter unaltered by the expression of PTEN was the activation of caspase-8. This observation suggests that the activation of caspase-8 by TNF in and of itself does not cause cell death in ethanol-exposed hepatocytes.

The increased p38 activity seen in ethanol-exposed cells is associated with a decreased phosphorylation of ASK-1 by Akt. ASK-1 is a MAPK kinase kinase that can activate the c-Jun N-terminal kinase and p38 MAPK cascades (44). By phosphorylating ASK-1 on serine 83, Akt negatively regulates ASK-1 activity (45). In control cells not exposed to ethanol, TNF treatment induced an increased phosphorylation of ASK-1 on serine 83. By contrast, TNF was unable to induce ASK-1 phosphorylation in ethanol-exposed cells, a feature reversed by suppression of PTEN levels and expression of constitutively active Akt.

These results suggest that the elevated PTEN level found in ethanol-treated cells is responsible for preventing the negative regulation of ASK-1 by Akt. The importance of ASK-1 in instigating the augmented p38 response to TNF during ethanol treatment is shown by the ability of siRNA targeting ASK-1 to reduce p38 activity in ethanol-exposed cells treated with TNF.

The response of p38 to TNF treatment of ethanol-exposed cells is biphasic. In the first phase, there is a modest increase of p38 activity, starting at 15 min and regressing by 45 min. By contrast, the second phase of the p38 response to TNF is seen only in ethanol-exposed cells, starting at 60 min and being maintained at levels 2-fold above control through 4 h post-treatment. It is this second phase of p38 activation that appears to be critical in mediating increased cell sensitivity to TNF, since the first phase is also observed in control cells not exposed to ethanol and resistant to TNF-induced cytotoxicity.

We have shown previously that the first phase of p38 activation is dependent on the formation of reactive oxygen species but that the second is not (20). Importantly, antioxidants delayed but did not prevent the increased cell killing seen in ethanol-exposed cells treated with TNF. In that study, such results were seen in both HepG2E47 and primary hepatocytes isolated from ethanol-fed rats.

Interestingly, elimination of ASK-1 expression did not prevent the first phase of p38 activity but did prevent the second phase seen only in ethanol-exposed cells. The increased p38 activity evoked by TNF treatment can be partly explained by the inability of Akt to phosphorylate and inhibit ASK-1 due to the ethanol-induced elevation of PTEN. However, this does not account for how ASK-1 is stimulated to begin with and whether ethanol exposure has any influence on this aspect of ASK-1 control. ASK-1 binds to thioredoxin and is regulated by reactive oxygen species (46). However, ASK-1 can also be controlled by reactive oxygen species-independent mechanisms such as through the formation of ceramide that is triggered by TNF (47). Additionally, the question remains as to how ethanol exposure activates the PTEN promoter as was shown in the present study (Fig. 1D). Unfortunately, little is known about the transcriptional elements in the PTEN promoter. However,
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it has recently been shown that the transcription factors p53 and Erg-1 (early growth response gene) can up-regulate PTEN expression (48–50). Both of these transcription factors possess growth-suppressing activities and are triggered by cellular stresses. It is possible that ethanol can impose a stress on the cell that can lead to activation of such or similar transcription factors through as yet uncharacterized processes. It must also be acknowledged that the elevated PTEN levels induced by ethanol exposure may be due to a diminution of degradation of the PTEN mRNA. The resulting up-regulation of PTEN levels can lead to activation of such or similar transcription factors through as yet uncharacterized processes. It must also be acknowledged that the elevated PTEN levels induced by ethanol exposure may be due to a diminution of degradation of the PTEN mRNA. The resulting up-regulation of PTEN levels can lead to activation of such or similar transcription factors through as yet uncharacterized processes.

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