Role of MAPK Phosphatase-1 in Sustained Activation of JNK during Ethanol-induced Apoptosis in Hepatocyte-like VL-17A Cells*

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Ethanol metabolism plays a central role in activating the mitogen-activated protein kinase (MAPK) cascade leading to inflammation and apoptosis. Sustained activation of c-Jun N-terminal kinase (JNK), one of the MAPKs, has been shown to induce apoptosis in hepatocytes. MAPK phosphatase-1 (MKP-1) has been shown to dephosphorylate MAPKs in several cells. The aim of the study is to evaluate the role of MKP-1 in sustained JNK activation as a mechanism to explain ethanol-induced hepatocyte apoptosis. VL-17A cells (HepG2 cells over-expressing alcohol dehydrogenase and cytochrome P450-2E1) were exposed to ethanol for different time periods. Western blots were performed for MKP-1, phospho-JNK, phosphotyrosine, and protein kinase Cζ (PKCζ). Electrophoretic mobility shift assays for AP-1 were performed. Apoptosis was measured using caspase-3 activity assay, TUNEL, and 4′,6-diamidino-2-phenylindole staining. Reactive oxygen species were neutralized by using lentiviral vectors in VL-17A cells. Ethanol incubation markedly decreased the MKP-1 protein levels to 15% of control levels and was associated with sustained phosphorylation of p46 JNK and p54 JNK, as well as increased apoptosis. VL-17A cells over-expressing superoxide dismutase-3 and catalase genes using lentiviral vectors in VL-17A cells. Ethanol incubation markedly decreased the MKP-1 protein levels to 15% of control levels and was associated with sustained phosphorylation of p46 JNK and p54 JNK, as well as increased apoptosis. VL-17A cells over-expressing superoxide dismutase-3 and catalase, treatment with a tyrosine kinase inhibitor, or incubation of the cells with PKCζ small interference RNAs significantly inhibited the ethanol-induced MKP-1 degradation and apoptosis. Ethanol-induced oxidative stress enhanced the tyrosine phosphorylation of PKCζ, which in turn caused the proteasomal degradation of MKP-1, leading to sustained JNK activation and increased apoptosis in VL-17A cells.

Alcoholic liver damage is one of the major causes of cirrhosis. Recent evidence suggests that many cellular responses of ethanol are mediated by the modulation of mitogen activated protein kinases (MAPK) signaling (1, 2). MAPK are involved in various cellular responses, including cell proliferation, differentiation, and apoptosis. Cellular responses mediated by MAPK signaling vary depending upon the intensity and kinetics of MAPKs. One of the members of the MAPK family, JNK, has been shown to be an important mediator of cellular functions. Transient activation of JNK is necessary for liver regeneration (3), whereas sustained activation is involved in apoptosis (4, 5), and ethanol had been shown to induce the sustained activation of both isoforms of JNK (p54 JNK and p46 JNK) in hepatocytes (3, 5–7). Although ethanol was found to be the activator of apoptosis via JNK activation, the mechanism by which ethanol prolongs the JNK activation is not known.

MAPKs are activated by dual phosphorylation on Ser/Thr and Tyr residues, whereas 11 dual-specificity phosphatases or MAPK phosphatases (MKPs) terminate such activation (8). These phosphatases exhibit differential specificity toward MAPK substrates, show distinct subcellular localizations, and have different modes of regulation. Among these phosphatases, MKP-1 was originally identified as an ERK-specific phosphatase (9), and it has also been shown to dephosphorylate and inactivate JNK and p38 MAPK in a cell-specific and context-specific manner (10, 11). Although, MKP-1 expression has been shown to control the phosphorylation of JNK in other cell types, there is no evidence indicating that ethanol modulates JNK phosphorylation via MKP-1 in hepatocytes.

The activation of MAPKs has been shown to be regulated by the activation of tyrosine kinase (12, 13), protein kinase C (PKC) (14), or G-protein (15). Several PKC isoforms have been shown to have important roles in the regulation of cell survival and apoptosis (16, 17), and a series of studies suggests that PKCζ is involved in apoptosis by acting as a proapoptotic signal (18, 19). For example, a selective inhibitor of PKCζ, rottlerin, and a dominant-negative mutant of PKCζ, attenuate apoptosis induced by phorbol ester (20), hydrogen peroxide (H2O2) (21), UV radiation (22), or etoposide (23). These studies demonstrate that PKCζ is one of the prime regulators of apoptosis in several cell types. However, the mechanism by which activation of PKCζ induces cell death remains largely unknown.

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2 The abbreviations used are: MAPK, mitogen-activated protein kinase; MKP-1, MAPK phosphatase-1; PKCζ, protein kinase Cζ; eGFP, enhanced green fluores-
The oxidative metabolism of ethanol by hepatocytes elicits a range of responses, including the generation of ROS, one of the important mediators of alcoholic liver injury (24–27). The mechanism by which ethanol induces oxidative stress has been studied by several investigators (28–30). Several reports have shown that reactive oxygen species (ROS), including \( \text{H}_2\text{O}_2 \), superoxide anion \( (\text{O}_2^\cdot) \), and hydroxyl \( (\text{OH}^-) \) ions, are important signaling molecules regulating JNK activity (31, 32). Although it is known that oxidative stress induces the phosphorylation of JNK, the role of ethanol-induced oxidative stress, PKC activation, and the sustained activation of JNK in alcohol-induced injury are not known. In this report we have systematically analyzed the interplay among these molecules, and we demonstrate how ethanol-induced oxidative stress causes the sustained activation of JNK and apoptosis in hepatocytes, via the enhancement of MKP-1 degradation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—For this study, we used a recombinant cell line VL-17A, (derived from HepG2 cells), which constitutively expresses the ethanol-metabolizing enzymes, alcohol dehydrogenase, and cytochrome P450 2E1 (CYP2E1). Cell culture conditions have been followed as described previously (33–35).

**Subcloning of Superoxide Dismutase-3 (SOD3) and Catalase into the Lentiviral Vector**—The fourth generation lentiviral vectors (LVs), containing enhanced green fluorescent protein (eGFP) with the human \( \alpha1-\text{AT} \) promoter, were constructed as described previously (36). The LV-eGFP was used as a control vector, and the human SOD3 construct or catalase was cloned into LV by replacing eGFP. The sequence was verified for its accuracy with the original SOD3 or catalase sequence. All the lentiviral vectors were produced and concentrated as described previously (37). The viral titer was quantitated using the p24 enzyme-linked immunosorbent assay kit (Beckman-Coulter, Fullerton, CA) according to the manufacturer’s instructions and as described previously (38).

**Transduction of Lentiviral Vectors**—Sub-confluent layers of VL-17A cells were transduced with either LV-SOD3 and LV-catalase or LV-eGFP (10\(^6\) transduction units/10\(^6\) cells) in the presence of Polybrene (8 \( \mu \text{g}/\text{ml} \)) for 24 h. The cells were washed with phosphate-buffered saline, and the medium was replenished. Both medium and cells were assayed 5 days after transduction for CAT activity, using an SOD activity kit (EMD Biosciences, San Diego, CA, catalog number 574601) according to the manufacturer’s protocol. The overexpression of SOD3 was also analyzed by real-time PCR as described previously (39). Catalase overexpression was analyzed by both activity assays and Western blots as described by us previously (39).

**Cell Treatments**—VL-17A cells were routinely cultured in the presence of 10% fetal bovine serum. All the ethanol/treatment experiments were performed under serum-free conditions. Prior to treatments, the cells were preincubated with various inhibitors, such as U0126 (ERK inhibitor, 10 \( \mu \text{M} \), Sigma-Aldrich, catalog number U120), SB202190 (p38 MAPK inhibitor, 10 \( \mu \text{M} \), Sigma-Aldrich, catalog number S7067) and SP600125 (JNK inhibitor, 10 \( \mu \text{M} \), Sigma-Aldrich, catalog number S5567), rottlerin (10 \( \mu \text{M} \), EMD Biosciences, catalog number S5573), genistein (30 nm, EMD Biosciences, catalog number S45834), or MG-132 (10 \( \mu \text{M} \), EMD Biosciences, catalog number 474790) for 30–60 min under serum-free conditions. Then ethanol was added, and the mixture was incubated with the inhibitors for various time periods up to 4 h. In all the experiments involving the inhibitors, 0.1% Me\(_2\text{SO} \) was added to control cells. Ethanol was also incubated with cells overexpressing SOD3 and catalase for different time periods up to 4 h. After the treatments, the cells were collected for Western blots, RT-PCR analysis, or apoptosis assays.

**RT-PCR**—The cells were collected, total RNA was isolated using Qiagen’s RNA isolation kit, and cDNA was synthesized. The human-specific MKP-1 (catalog number sc35937-PR), PKC\( \delta \) (catalog number sc36253-PR), and glyceraldehyde-3-phosphate dehydrogenase (catalog number sc35448-PR) primers were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). MKP-1 and PKC\( \delta \) were amplified for 25 cycles, and glyceraldehyde-3-phosphate dehydrogenase was amplified for 20 cycles. The PCR conditions were 1 cycle of 94 °C for 5 min, 20–25 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s, followed by 1 cycle of 72 °C for 10 min. The amplified bands were resolved in 1.5% agarose gels.

**Western Blots and Immunoprecipitation**—Cell lysates from different incubations were collected in mammalian cell extraction solution ( Pierce, catalog number 78501), and the protein content was quantified using bicinchoninic acid reagent (Pierce, catalog number 23225). The samples (30–60 \( \mu \text{g}/\text{lane} \)) were separated using 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes and detected with anti-phospho-JNK (Upstate, catalog number 07-175), anti-JNK (Santa Cruz Biotechnologies, catalog number sc571), anti-human MKP-1 (Upstate, catalog number 07-553), PKC\( \delta \) (Santa Cruz Biotechnologies, catalog number sc213), or \( \beta \)-actin (Sigma-Aldrich, catalog number A5441) antibodies as described previously (40). For immunoprecipitation, 500 \( \mu \text{l} \) of protein solution (2 mg/ml) was precleared with protein-A-Sepharose beads for 1 h. This was followed by incubation with anti-PKC\( \delta \) antibodies and protein-A Sepharose beads overnight at 4 °C. Next day, the beads were washed three times using lysis buffer and boiled for 10 min with 20 \( \mu \text{l} \) of 2 \( \times \) sample buffer. The tubes were centrifuged, the supernatant was resolved in polyacrylamide gels, and Western blots were performed using anti-phosphotyrosine antibodies.

**EMSA**—For determining the activation of AP-1 (Promega, Madison, WI, catalog number E3202), electrophoretic mobility shift assays (EMSAs) were performed using a non-radioactive detection method. All the reagents and kits needed for this assay were obtained from Pierce. After different treatments, the cells were washed, and nuclear protein was isolated. AP-1 oligonucleotides were labeled with biotin according to the manufacturer’s instructions. The labeled oligonucleotides were purified using spin columns, and the binding reactions between the labeled oligonucleotides and the nuclear protein were conducted. The bound DNA-protein complex was separated by PAGE electrophoresis, followed by the detection of the shifted bands, as described previously (41, 42).

**ROS Measurements**—VL-17A cells (\( 1 \times 10^6 \) cells/well) were seeded in a 96-well plate and cultured for 24 h. The cells were washed and changed to serum-free media and incubated with...
E100 and 10 μM H₂DCFDA (Invitrogen, catalog number D399). The conversion of H₂DCFDA to fluorescent DCF was measured by spectrofluorometer as described previously (43).

Apoptosis Assays—The cells were administered with different treatments as described above. After 4 h of incubation with ethanol, the cells were washed and analyzed for apoptosis. The TUNEL assay was performed using the ApopTag kit from Sero-logicals Corp. (Billerica, MA, catalog number S7165) as we have done previously (39). In separate wells, DAPI staining was performed using mounting medium containing the DAPI. Caspase-3 activity assay kit was obtained from EMD Biosciences (catalog number 235419), and the assay was performed according to the manufacturer’s instructions.

siRNA Silencing Studies—PKC8 (catalog number sc36253), the scrambled siRNA oligonucleotides (catalog number sc37007), and the transfection reagent (catalog number sc29528), were obtained from Santa Cruz Biotechnologies. VL-17A cells were seeded at 2 × 10⁵ cells per well in a 6-well culture plate in an antibiotic-free normal VL-17A growth medium with 10% fetal bovine serum. After 24 h of plating, the cells were washed and replaced with 0.8 ml of low serum Opti-MEM medium (Invitrogen). Both siRNA oligonucleotides (50 μM) and 6 μl of transfection reagent were diluted with 100 μl of Opti-MEM medium separately under sterile conditions. Both of the tubes were mixed and incubated at room temperature for 30 min, and this mixture was added to each well. After 6 h of incubation, 1 ml of normal growth media was added, and the mixture was incubated for an additional 18 h. Then the cells were washed and cultured with normal growth media. After 24 h of culture with a change of growth media, the cells were used for the ethanol experiments under serum-free conditions.

Statistical Analysis—All the experiments were performed in triplicates and at least three times. The statistical significance was calculated using one-way analysis of variance. The data were expressed as means ± S.E. and calculated using variance analysis and the Newman-Keuls test for multiple comparisons among groups. p < 0.05 was considered as statistically significant.

RESULTS

VL-17A cells were incubated with 100 mM ethanol (E100) for up to 4 h. The cells were collected, total protein was isolated, and the Western blots for both phospho-JNK and total JNK were performed as described under “Experimental Procedures.” Incubation of VL-17A cells with ethanol caused an increase in the phosphorylation of both p46 JNK and p54 JNK at 30 min that remained increased up to 4 h (Fig. 1A), and this increase was associated with an enhancement in the nuclear translocation of AP-1 (Fig. 1B). There was a 4-fold increase in caspase-3 activity when VL-17A cells were treated for 4 h with E100 (Fig. 1C). Apoptosis was also measured by both the TUNEL assay and DAPI staining. Both apoptotic and total cells were counted in at least 10 high power fields, and the percentage of apoptosis was calculated by the ratio of apoptotic cells to total cells. There was a significant increase in apoptosis with ethanol treatment (Fig. 1C). To evaluate the role of other MAPK cascade in mediating apoptosis, VL-17A cells were preincubated with U0126 (ERK inhibitor, 10 μM), SB202190 (p38 MAPK inhibitor, 10 μM), or SP600125 (JNK inhibitor, 10 μM) for 30 min in serum-free conditions, followed by the addition of E100 for a further period up to 4 h. As a vehicle control, 0.1% Me₃SO was added to the control cells. The cells were washed with phosphate-buffered saline followed by caspase-3 activation assay, or DAPI and TUNEL staining. The results showed that inhibition of either p38 MAPK or JNK caused a significant decrease in the caspase-3 activity (Fig. 1C) as well as DAPI and TUNEL staining (Fig. 1D). Inhibition of ERK did not cause any significant changes in the survival of the cells.
MKP-1 is a dual specificity phosphatase, which is shown to be involved in deactivating JNK in various cells. Hence, we studied the effect of ethanol on MKP-1 expression in VL-17A cells. VL-17A cells were incubated with E100 for 0, 1, and 4 h, and the cells were collected for RNA and protein isolation. RT-PCR was performed for MKP-1 expression, and we found that there was no decrease in the MKP-1 mRNA levels (Fig. 2A). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. Western blot results showed that there was a significant decrease in MKP-1 protein levels in VL-17A cells with ethanol administration (Fig. 2B). The relative gene expression was calculated by the ratio of MKP-1 expression to the housekeeping genes and presented as a densitograph (Fig. 2C).

Ethanol has been shown to induce apoptosis, at least in part, via oxidative stress in hepatocytes. The available data show that inhibition of ethanol-induced oxidative stress decreases cell death. Therefore, we studied whether inhibition of ROS would lead to a recovery of the MKP-1 protein levels by co-expressing both SOD3 and catalase (SOD3/cat) using lentivirus in VL-17A cells. As an experimental control, the cells were transduced with LV-eGFP. Every day, both cells and media were analyzed for SOD3 and catalase expression (data not shown), and maximal expression was observed at 5 days after transduction.

Hence, the transduced cells were used 5 days after LV transduction in all experiments. The cells were analyzed for SOD3 expression by real-time RT-PCR, and we found a significant increase in SOD3 mRNA levels (Fig. 3A). This correlated with an increase in both intracellular and secreted SOD activity (Fig. 3B). The catalase expression was studied by both Western blots and catalase activity, and there was increased protein expression (Fig. 3C) and activity (Fig. 3D) in these cells. The percentage of in vitro transduction efficiency of LV was determined as described previously (38). The eGFP-positive cells were used as
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A transduction control in determining the effects of LV in these cells. The results showed that LV did not cause any significant changes in MKP-1 protein expression. SOD3/cat-overexpressing cells were used in ROS neutralization experiments.

Both the transduced cells and untransduced cells were incubated with E100 for 1 h, the ROS release was measured by the conversion of H2DCFDA to fluorescent DCF, and the mean fluorescence intensity was calculated and corrected with the protein content. Ethanol caused an increase in the ROS release in VL-17A cells, and overexpression of SOD3/cat caused a significant inhibition of ROS production (Fig. 4A). Incubation of SOD3/cat-overexpressing cells with E100 did not cause any change in MKP-1 mRNA levels (data not shown) but caused a significant recovery in ethanol-induced MKP-1 protein degradation, as determined by Western blot (Fig. 4B). β-Actin was used as a loading control in these Western blots.

The mechanism by which oxidative stress induced the MKP-1 degradation was analyzed. It had been shown in other systems that tyrosine phosphorylation of PKCδ can induce proteasomal activation. Hence, we determined whether ethanol-induced oxidative stress caused the tyrosine phosphorylation of PKCδ. Either inhibition of tyrosine kinase by a specific tyrosine kinase inhibitor, genistein (30 nM), or use of the SOD3/cat-overexpressing cells, decreased the ethanol-induced PKCδ tyrosine phosphorylation (Fig. 5A), whereas treatment of cells with either the proteasomal inhibitor (MG-132) or PKCδ inhibitor (rottlerin, 10 μM) did not decrease the tyrosine phosphorylation of PKCδ. Next, the effect of tyrosine phosphorylation of PKCδ on MKP-1 degradation was studied. The cells were preincubated for 1 h with either rottlerin, genistein, or the proteasomal inhibitor MG-132 (10 μM) followed by co-incubation with E100 for an additional 4 h. In parallel experiments, SOD3/cat-overexpressing cells were also incubated with E100 for 4 h. Incubation of the cells with either of these inhibitors abolished ethanol’s effects on the MKP-1 protein degradation (Fig. 5B). Next, we studied whether the recovery of MKP-1 would result in the transient activation of JNK in these cells. Hence, MG-132 was preincubated with the cells for 1 h in serum-free conditions, which was followed by the incubation of E100 for 0 min, 30 min, 1 h, and 4 h. In parallel experiments, SOD3/cat-overexpressing cells were also incubated with E100 at different time points. Incubation with ethanol caused a transient activation of JNK in these cells (Fig. 5C). To confirm that PKCδ is involved in ethanol-induced MKP-1 degradation, we designed siRNA-mediated PKCδ-silencing experiments. VL-17A cells were transfected with scrambled siRNAs or with PKCδ siRNA oligonucleotides as described under “Experimental Procedures.” The siRNA transfection
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FIGURE 6. Effect of PKCδ siRNA oligonucleotides on MKP-1 and apoptosis in VL-17A cells. VL-17A cells were transfected with scrambled or PKCδ siRNA oligonucleotides as described under “Experimental Procedures.” Expression of PKCδ was analyzed by both RT-PCR (A) and Western blots (B). A, total RNA was isolated, and cDNA was synthesized followed by amplification of PKCδ or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using 25 or 20 cycles, respectively. An agarose (2%) gel was run for visualizing the amplicons. B, the total protein was isolated, and Western blots were run for PKCδ (30 μg/lane) or β-actin (30 μg/lane). C, the siRNA-transfected cells were treated with E100 for 4 h, the protein was isolated, and Western blots for MKP-1 (60 μg/lane) were performed. Lane 1, untransfected cells, no ethanol treatment; lane 2, scrambled siRNA-transfected cells with E100; lane 3, PKCδ siRNA-transfected cells with E100. D, VL-17A cells were transfected with either scrambled siRNA or PKCδ siRNAs as described under “Experimental Procedures.” The transfected cells were incubated with E100 for 0–4 h. Western blots were performed for the phosphorylation of JNK using anti-phospho JNK antibodies. Panel i shows the scrambled siRNA-transfected cells that were incubated with ethanol for different time points. Panel ii shows the PKCδ siRNA-transfected cells that were incubated with ethanol for different time periods. Total JNK (panel iii) was detected to show the expression of JNK in these cells. E100-treated cells for 0 min (lane 1); 30 min (lane 2); 1 h (lane 3); and 4 h (lane 4). This is representative of three experiments. E, VL-17A cells were transfected with either scrambled siRNA or PKCδ siRNA as described under “Experimental Procedures.” These cells were treated with E100 for 4 h, and the nuclear protein was analyzed by anti-AP-1 translocation by EMSA. To confirm the translocation is due to AP-1 binding, excess unlabeled AP-1 oligonucleotides were mixed in the reaction tube containing samples from E100-treated VL-17A cells. This is representative of three experiments.

causation >70% inhibition of PKCδ mRNA (Fig. 6A) and protein (Fig. 6B) expression. As expected, transfection of the cells with the PKCδ siRNA did not decrease the ethanol-induced ROS release (data not shown); however, the ethanol-induced degradation of MKP-1 protein was restored in VL-17A cells transfected with PKCδ siRNA oligonucleotides but not in cells transfected with scrambled siRNAs (Fig. 6C). This MKP-1 restoration was associated with a decrease in the ethanol-induced sustained phosphorylation of p46 JNK and P54 JNK in PKCδ-transfected VL-17A cells (Fig. 6D, panel ii), whereas the scrambled siRNA-transfected VL-17A cells did not show a decrease in JNK activity with ethanol (Fig. 6D, panel i). To assess whether the inhibition of sustained JNK activation could decrease the nuclear translocation of AP-1, one of the down-stream targets of JNK, we conducted EMSA experiments. Incubation of VL-17A cells with E100 caused an increase in AP-1 nuclear translocation in scrambled siRNA transfected cells. On the other hand, ethanol-induced AP-1 nuclear translocation was inhibited in PKCδ siRNA-transfected cells (Fig. 6E).

VL-17A cells were transfected with PKCδ or scrambled siRNAs followed by incubation with E100 for 4 h. In parallel experiments, the cells were preincubated with rottlerin, genestein, or MG-132, followed by incubation with E100 for 4 h. SOD3/cat-overexpressing cells were also incubated with E100 for 4 h. We determined that E100 treatment increased apoptosis in cells transfected with scrambled siRNA, whereas this increase was significantly inhibited in the PKCδ siRNA-transfected cells, cells pretreated with inhibitors, or SOD3/cat-overexpressing cells, as measured by the caspase-3 activity assay (Fig. 7A) and DAPI and TUNEL staining (Fig. 7, B and C).

DISCUSSION

In the present study we investigated the molecular mechanism by which ethanol induces a sustained activation of p46 JNK and p54 JNK and causes apoptosis in VL-17A cells. Several studies have demonstrated that the duration and magnitude of JNK activation are important for determining whether cells undergo cell proliferation or apoptosis (3, 5–7). Transient activation of JNK is required for liver regeneration (3), whereas prolonged and robust JNK activation promotes cell death (4, 5), and the addition of ethanol to primary rat hepatocytes has been shown to induce a rapid and robust induction of JNK (44). However, the mechanism of this JNK induction by ethanol has not been determined previously. In our studies, we have used VL-17A cells, which are HepG2 cells overexpressing both alcohol dehydrogenase and CYP2E1, to study the molecular regulation of the sustained activation of JNK. These cells are well characterized and possess primary hepatocyte-like characters (33–35). In agreement with other findings, VL-17A cells showed a sustained increase in JNK phosphorylation and increased apoptosis with ethanol administration. We also tested the activation of ERK1/2 and p38 MAPK. In our study we found that the induction of ERK1/2 by ethanol was slow and increased to a maximum at 4 h (data not shown). However, the addition of ERK inhibitor did not cause any significant changes in the cell survival at 4 h. Ethanol also induced the activation of p38 MAPK (data not shown), and its inhibition caused a decrease in apoptosis in VL-17A cells. Although previous reports have shown the mechanisms by which oxidative stress induces p38 MAPK (45, 46), it is possible that MKP-1 degradation is responsible for the sustained activation of p38 MAPK. Further studies are needed to establish this mechanism.

Several studies have shown that MKP-1-deficient macrophage cells exhibit prolonged JNK activation as well as enhanced production of tumor necrosis factor-α and interleukin-6 compared with wild-type cells (47–49). In neuronal cells MKP-1 degradation is associated with increased glutamate toxicity (50). These reports suggested to us that MKP-1 degradation might play a role in ethanol-induced sustained JNK activation and subsequent apoptosis. Therefore, we incubated VL-17A cells with ethanol and studied the expression of MKP-1. Ethanol incubation caused a significant decrease in the
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expression of MKP-1. Although we found a decrease in the protein level of MKP-1, RT-PCR data showed that there was no decrease in MKP-1 mRNA levels, suggesting that the regulation might be at a post-transcriptional level. Recently it was shown that ROS could promote a sustained JNK activation causing cell death induced by tumor necrosis factor-α (32). In hepatocytes, the oxidative metabolism of ethanol elicits the generation of ROS, and is involved in inducing apoptosis (1, 5, 24, 25, 27). Increased oxidative stress has been shown to activate the MAPKs, but the magnitude of the activation depends on the dose and potency of the stimulus. Hence, we co-expressed both SOD3 and catalase, and our data showed that ethanol-induced ROS release was significantly inhibited by SOD3/cat-overexpressing cells. These results showed that these enzymes were biologically active in the in vitro system. In our studies the lentiviral-mediated overexpression of antioxidant genes restored the ethanol-induced degradation of MKP-1.

To explore the molecular mechanism by which MKP-1 degradation is enhanced by ethanol-induced oxidative stress, we conducted further experiments. Previous studies have shown that the tyrosine phosphorylation of PKCδ could lead to MKP-1 degradation in neuronal cells (50). Also, other reports have shown that oxidative stress can induce the tyrosine phosphorylation of PKCδ in several cells (18, 19, 21). Hence, we hypothesized that ethanol-induced oxidative stress could induce the tyrosine phosphorylation of PKCδ, which in turn would activate the proteasomal degradation of MKP-1 in hepatic cells. To test this hypothesis, first we studied whether ethanol incubation increased the tyrosine phosphorylation of PKCδ and that inhibition of ROS could inhibit this process. Our data indicated that either overexpression of antioxidant genes or genistein, a specific tyrosine kinase inhibitor, caused a significant decrease in PKCδ tyrosine phosphorylation in VL-17A cells. But, incubation of MG-132 with ethanol did not decrease the PKCδ tyrosine phosphorylation, which suggested that proteasomes act downstream of PKCδ. Our data also confirmed that inhibition of either PKCδ or its tyrosine phosphorylation, or proteasomal activation reversed ethanol-induced MKP-1 degradation. Previous data have shown that chronic administration of ethanol either in vivo (51, 52) or in vitro (53) caused an inhibition of proteasomal activation. In our study, we show that acute administration of ethanol induces the activation of proteasomes. These data confirm that the effects of ethanol on signaling pathways differ depending on its concentration, means of administration, and its metabolism (1). Our data further indicate that ethanol-induced oxidative stress enhanced the tyrosine phosphorylation of PKCδ, followed by proteasomal activation and MKP-1 degradation. To further attest to this mechanism, we determined whether the recovery of MKP-1 levels could normalize the JNK activation. When we incubated the cells with MG-132 prior to ethanol exposure or treated SOD3/cat-overexpressing cells with ethanol, there was a recovery of MKP-1 leading to transient rather than sustained activation of JNK, and a resulting decrease in apoptosis.

Previously it was shown that the incubation of a PKC inhibitor (GF109203X) or a tyrosine kinase inhibitor (genistein) with primary rat hepatocytes for 1 h did not cause a decrease in the 200 mM ethanol-induced JNK activation (44). In contrast to their findings, our data showed that inhibition of PKCδ using siRNA oligonucleotides caused a significant inhibition of etha-
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