HEPATOCYTE RESISTANCE TO OXIDATIVE STRESS IS DEPENDENT ON PROTEIN KINASE C MEDIATED DOWN-REGULATION OF C-JUN/AP-1*

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Running title: PKC-dependent resistance to death from oxidative stress
SUMMARY

The prevention of injury from reactive oxygen species is critical for cellular resistance to many death stimuli. Resistance to death from the superoxide generator menadione in the hepatocyte cell line RALA255-10G is dependent on down-regulation of the c-Jun N-terminal kinase (JNK)/AP-1 signaling pathway by extracellular signal-regulated kinase 1/2 (ERK1/2). Because protein kinase C (PKC) regulates both oxidant stress and JNK signaling, the ability of PKC to modulate hepatocyte death from menadione through effects on AP-1 was examined. PKC inhibition with Ro-31-8425 or bisindolylmaleimide I sensitized this cell line to death from menadione. Menadione treatment led to activation of PKCµ, or protein kinase D (PKD), but not PKCα/β, PKCζ/λ, or PKCδ/θ. Menadione induced phosphorylation of PKD at Ser744/748, but not Ser916, and translocation of PKD to the nucleus. PKC inhibition blocked menadione-induced phosphorylation of PKD and expression of a constitutively active PKD prevented death from Ro-31-8425/menadione. PKC inhibition led to a sustained overactivation of JNK and c-Jun in response to menadione as determined by in vitro kinase assay and immunoblotting for the phosphorylated forms of both proteins. Cell death from PKC inhibition and menadione treatment resulted from c-Jun activation as death was blocked by adenoviral expression of the c-Jun dominant negative TAM67. PKC and ERK1/2 independently down-regulated JNK/c-Jun as inhibition of either kinase failed to affect activation of the other kinase, and simultaneous inhibition of both pathways caused additive JNK/c-Jun activation and cell death. Resistance to death from superoxide therefore requires both PKC/PKD and ERK1/2 activation in order to down-regulate pro-apoptotic JNK/c-Jun signaling.
INTRODUCTION

The ability of the cell to resist injury from excessive levels of reactive oxygen species (ROS) is a critical survival mechanism in response to a variety of environmental stresses. Until recently oxidative stress was thought to trigger cell death through the adverse effects of biochemical reactions between oxidants and cellular macromolecules. However, it is now known that oxidant-induced death pathways are far more complex with death also resulting from the effects of oxidants on signal transduction pathways (1,2). Central among these signal transducers of oxidant-induced death are the mitogen-activated protein kinases (MAPKs). In the hepatocyte cell line RALA255-10G, resistance to toxicity from the ROS superoxide depends on activation of the MAPK extracellular signal-regulated kinase 1/2 (ERK1/2). Treatment of these cells with the superoxide generator menadione induces ERK1/2 activation (3). Inhibition of ERK1/2 signaling causes sustained activation of the c-Jun N-terminal kinase (JNK)/c-Jun/AP-1 pathway, resulting in cell death from normally nontoxic concentrations of menadione (3). Overactivation of JNK/AP-1 signaling is known to mediate cell death from a number of stimuli in both hepatocytes and non-hepatic cells (4,5). Restricting the duration of this pro-apoptotic AP-1 activation following superoxide-generated cellular stress is required for hepatocyte resistance to oxidative stress.

The critical nature of cellular resistance to oxidant stress suggests the likelihood that redundant or complementary signaling pathways exist in order to protect hepatocytes against oxidant injury. However, upstream inhibitors of AP-1 activation other than ERK1/2 have not been identified after oxidative stress in hepatocytes. In addition to their effects on MAPK signaling, oxidants have been demonstrated to phosphorylate and thereby activate protein kinase C (PKC) isoforms. Multiple PKC isoforms are phosphorylated in response to oxidative stress.
induced by hydrogen peroxide (6,7), including PKCμ or protein kinase D (PKD) (8,9). Although originally described as a PKC family member, PKD has distinct features that make it part of a separate kinase family that also includes PKD2 and PKD3 (10). Both serine and tyrosine phosphorylation of PKD have been reported to result from hydrogen peroxide treatment (8,9). Hydrogen peroxide-induced phosphorylation of Ser744/748 within the PKD activation loop occurs by a PKC-dependent mechanism (11,12). In addition to phosphorylation, PKD activation involves translocation from the cytoplasm to other cellular compartments including the nucleus and mitochondria (13,14). PKD activation has been reported to up-regulate NF-κB signaling, and the protective effects of PKD activation against death from hydrogen peroxide were associated with PKD-dependent NF-κB activation (8). Interestingly, PKD has also been reported to regulate JNK/c-Jun signaling (15-18), suggesting the possibility that PKD activation induced by oxidative stress may also regulate the AP-1 pathway.

The objective of the present study was to examine whether PKC is an upstream regulator of the AP-1 death pathway in a hepatocyte cell line exposed to the superoxide generator menadione. The studies demonstrate that menadione causes a PKC-dependent activation of PKD. Inhibition of PKC/PKD activation leads to increased toxicity from menadione associated with sustained activation of the JNK/AP-1 pathway. Death resulting from PKC/PKD inhibition is blocked by the c-Jun dominant negative TAM67, suggesting that PKD-dependent resistance to menadione toxicity is the result of down-regulation of AP-1 signaling. These data therefore demonstrate for the first time a critical physiologic role for PKC/PKD in the regulation of AP-1 signaling.
EXPERIMENTAL PROCEDURES

Cells and culture conditions

All studies were performed in the adult rat hepatocyte line RALA255-10G (RALA hepatocytes). These cells are conditionally immortalized with a mutant SV40 virus expressing a temperature sensitive T antigen (19). Cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 4% fetal bovine serum (Gemini, Woodland, CA), and antibiotics (Invitrogen) at the permissive temperature of 33°C. For experiments cells were plated and cultured at 33°C for 24 h, and then cultured in DMEM, 2% fetal bovine serum, antibiotics and 1 µM dexamethasone at the restrictive temperature of 37°C, as previously described (20). Under these conditions T antigen expression is suppressed, the cells are nontransformed, and they display a differentiated hepatocyte phenotype (19,21). Cells were then placed in serum-free medium containing dexamethasone for 18 h prior to the start of an experiment.

Cells were pretreated for 1 h with the PKC inhibitors 10 uM Ro-31-8425, 10 uM bisindolylmaleimide I (Bis I), or 10 uM chelerythrine chloride (Calbiochem, San Diego, CA) dissolved in dimethyl sulfoxide (DMSO). Cells were treated with menadione (Sigma, St. Louis, MO) at the concentrations indicated. Some cells were pretreated for 1 h prior to the addition of Ro-31-8425 with 10 uM ebselen (2-phenyl-1,2-benzisoselenazol-3[2H]-one) (Biomol, Plymouth Meeting, PA), 1000 units of catalase polyethylene glycol (Sigma), or 50 uM Val-Ala-Asp-fluoromethylketone (ZVAD; Calbiochem). Ebselen and ZVAD were dissolved in DMSO. In experiments with inhibitors dissolved in DMSO, untreated control cells received equivalent amounts of DMSO.
**MTT assay**

Cell death was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (22). At 24 h after treatment, the cell culture medium was aspirated and an equal volume of a 1 mg/ml MTT solution, pH 7.4 in DMEM was added to the cells. After incubation at 37°C for 1 h, the MTT solution was removed, and 1.5 ml of N-propanol was added to solubilize the formazan product. The absorbance of this compound was measured at 560 nm in a spectrophotometer. The percentage of cell death was calculated by dividing the optical density of a treatment group by the optical density for untreated, control cells, multiplying by 100, and subtracting that number from 100.

**Fluorescence microscopy**

The numbers of apoptotic and necrotic cells were quantified by fluorescence microscopy after costaining with acridine orange and ethidium bromide (23), as previously described (24). Cells with a shrunken cytoplasm, and a condensed or fragmented nucleus as determined by acridine orange staining were considered apoptotic. Necrotic cells were detected by positive staining with ethidium bromide. A minimum of 400 cells per dish were examined, and the numbers of apoptotic and necrotic cells expressed as a percentage of the total number of cells counted.

**Protein isolation, immunoprecipitation and Western blotting**

For the isolation of total cellular protein, cells were harvested in phosphate buffered saline, centrifuged and resuspended in cell lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, pH 7.6, 1% NP 40, 1 ug/ml leupeptin, 2 ug/ml pepstatin, 1 mM...
phenylmethylsulfonyl fluoride, 2 mM β-glycerophosphate, 5 mM sodium pyrophosphate, and 2 mM sodium orthovanadate. Protein concentrations were determined using the BioRad Protein Assay (BioRad, Hercules, CA), according to the manufacturer’s instructions.

To isolate nuclear protein, cells were scraped into hypotonic lysis buffer containing 10 mM Hepes, pH 7.4, 10 mM NaCl, 0.1 mM EDTA, pH 7.6, 0.4% NP 40, 1 ug/ml leupeptin, 2 ug/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 2 mM β-glycerophosphate, 5 mM sodium pyrophosphate, and 2 mM sodium orthovanadate. Lysates were pipetted vigorously, and centrifuged at 800 g, at 4°C for 8 min. Supernatants were transferred into new tubes, and ultracentrifuged at 100,000 g, at 4°C for 60 min. The supernatants were saved as cytosolic fractions. The pellets were lysed in hypertonic lysis buffer (20 mM Hepes, pH 7.4, 400 mM NaCl, 1 mM EDTA, pH 7.6, 1% NP 40, 1 ug/ml leupeptin, 2 ug/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 2 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 2 mM sodium orthovanadate), and centrifuged at 20,000 g, at 4°C for 20 min. The supernatants were used as nuclear fractions. Protein concentrations were determined using the BioRad Protein Assay as above.

For immunoprecipitations, cells were lysed in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, pH 7.6, 1% Triton X-100, 1 ug/ml leupeptin, 2 ug/ml pepstatin and 1 mM phenylmethylsulfonyl fluoride. Protein determination was performed as above and 350 ug of protein were immunoprecipitated by a 1 h incubation with 2 ug of antihemagglutinin (anti-HA) antibody purified from the 12CA5 hybridoma. Samples were then incubated with protein A/G agarose (Sigma) for 30 min. The immune complexes were washed five times with 20 mM Tris, pH 7.5, 500 mM sodium chloride, and resolved on Western blots as described subsequently.
For Western blotting, 50 ug of protein were denaturated at 100°C for 5 min in Laemmli sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 25% glycerol, 0.01% bromophenol blue and 5% β-mercaptoethanol. Samples were applied to 8-10% SDS-polyacrylamide gels and resolved at 100 V over 3 h. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) in transfer buffer containing 25 mM Tris, pH 8.3, 192 mM glycine, 0.01% SDS, and 15% methanol using a Bio-Rad Trans-blot SD semidry transfer cell to which 150 mA were applied for 90 min. Membranes were blocked in 5% non-fat dry milk in 20 mM Tris, pH 7.5, 500 mM sodium chloride, and 0.5% Tween 20 (TBS-T) for 1 h. Membranes were exposed to antibodies against: PKD, PKD phosphorylated at Ser744/748 or Ser916, phosphorylated PKCα/β, phosphorylated PKCδ/θ, phosphorylated PKCζ/λ; phosphorylated and total ERK1/2 (Cell Signaling, Beverly, MA), JNK1 and JNK2, and c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA); protein disulfide isomerase (a kind gift from R.J. Stockert) (25); and Nopp140 (kindly provided by U.T. Meier) (26). Primary antibodies were used at 1:1,000 to 1:6,000 dilutions in 5% non-fat milk or bovine serum albumin in TBS-T for 18 h at 4°C. Goat anti-rabbit and anti-mouse IgG antibodies conjugated with horseradish peroxidase (KPL, Gaithersburg, MD) were used as secondary antibodies at a dilution of 1:10,000 in 5% non-fat milk TBS-T for 1 h. Signals were detected by chemiluminescence (Western Lightning Chemiluminescence Plus, PerkinElmer, Boston, MA) and exposure to x-ray film.

**Transient transfections for PKD overexpression**

RALA hepatocytes were transiently transfected with an expression vector containing the *Escherichia coli* β-galactosidase gene, CMV-βGal, or with PKD.SS744/748EE. PKD.SS744/748EE expresses an HA-tagged mutant PKD in which the activation loop residues
have been replaced with negatively charged Glu resulting in a constitutively active PKD (27). Cells were plated at a lower density and cultured at 37°C for a shorter period of time than in the other experiments to allow for a less confluent culture necessary for optimal transfection efficiency. Transfections were performed with FuGENE 6 (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions. Transfection efficiency was determined by β-galactosidase staining of CMV-βGal-transfected cells using a commercial kit (Invitrogen). Twenty-four hours after transfection, cells were treated with Ro-31-8425 and/or 20 µM menadione as described previously. The percentage cell survival was determined by MTT assay 18 h later.

**JNK assay**

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

RALA hepatocytes were cultured as previously described and transiently transfected with reporter genes using Lipofectamine Plus (Invitrogen) 18 h prior to Ro-31-8425/menadione treatments. Cells were transfected with the AP-1-regulated firefly luciferase reporter gene Coll73-Luc (28), and the constitutive *Renilla* luciferase vector pRL-TK (Promega, Madison, WI). Luciferase activities were assayed as previously described (24), and firefly luciferase activity normalized to *Renilla* luciferase activity.

**Adenoviruses**

The adenoviruses Ad5LacZ containing the β-galactosidase gene (29), and Ad5TAM that expresses TAM-67, a dominant negative c-Jun (30), were employed. The adenoviruses were grown in 293 cells, purified by banding twice on CsCl gradients, and titered by plaque assay as previously described (31). RALA hepatocytes were infected at an MOI of 20 as previously described (24).

**Statistical analysis**

All numerical results are expressed as mean ± SE and represent data from three independent experiments with duplicate dishes in each treatment group. Statistical significance was determined by the Student’s *t*-test. Calculations were made with Sigma Plot 2000 (SPSS Science, Chicago, IL).
RESULTS

*PKC inhibitors sensitize RALA hepatocytes to death from menadione*

Menadione is a quinone compound that undergoes redox cycling resulting in the formation of superoxide (32,33). Recent investigations have demonstrated that RALA hepatocyte death from menadione-induced oxidative stress is regulated by both ERK1/2 and JNK MAPKs (3). However, it remains unclear how oxidative stress triggers activation of these MAPKs. To delineate upstream signals that regulate MAPK-dependent oxidant-induced death in RALA hepatocytes, the effect of PKC inhibition on menadione toxicity was examined. Cells were pretreated with vehicle or the PKC inhibitor Ro-31-8425 (34), and non-toxic and toxic concentrations of menadione that have been previously established (3). By 24 h MTT assay, Ro-31-8425 sensitized RALA hepatocytes to 22% and 41% cell death respectively, from the usually non-toxic 20 and 25 μM concentrations of menadione (Fig. 1A). This PKC inhibitor also further increased death from a toxic 30 μM menadione concentration by almost 2-fold (Fig. 1A), indicating that PKC-dependent protective mechanisms were still operative even at toxic levels of oxidative stress. No toxicity occurred from Ro-31-8425 treatment alone (data not shown).

To insure that death was secondary to PKC inhibition, the effect of a second PKC inhibitor, bisindolylmaleimide I (Bis I) (35), on menadione toxicity was investigated. Bis I alone was non-toxic (data not shown), but sensitization of RALA hepatocytes to death from menadione occurred with Bis I pretreatment. Death from Bis I/menadione cotreatment was 28% for 20 μM menadione, and 47% for 25 μM menadione at 24 hours (Fig. 1B), similar to findings for Ro-31-8425/menadione. In contrast, chelerythrine, another purported chemical PKC inhibitor (36), failed to sensitize RALA hepatocytes to death from menadione (data not shown).
Death from Ro-31-8425/menadione cotreatment results from caspase-independent apoptosis

The induction of cell death from combined Ro-31-8425/menadione treatment was additionally confirmed by fluorescence microscopy of acridine orange/ethidium bromide costained cells. At 24 h there was a marked increase in the percentage of apoptotic cells after Ro-31-8425/menadione cotreatment, but only a slight increase in the number of necrotic cells (Fig. 2A). Apoptosis was secondary to oxidative stress as demonstrated by significant inhibition of death by the antioxidants ebselen and catalase (Fig. 2B). Death was not prevented by the caspase inhibitor ZVAD (Fig. 2B). Thus, similar to findings of caspase-independent apoptosis in RALA hepatocytes and non-hepatic cell types from toxic concentrations of menadione (3,37), Ro-31-8425/menadione treatment resulted in an oxidant-induced, caspase-independent apoptosis.

Resistance to menadione toxicity requires early PKC signaling

To delineate the temporal involvement of protective PKC signaling in the menadione death pathway, RALA hepatocytes were examined for menadione-induced cell death after different times of Ro-31-8425 treatment. Conversion of the Ro-31-8425 1 h pretreatment to a 1 h post-treatment still sensitized the cells to significant toxicity from menadione, but reduced death from Ro-31-8425/menadione treatment by 36% (Fig. 3). When Ro-31-8425 treatment was delayed to 2 h after menadione administration, the amount of cell death was not significantly different from that of 25 uM menadione alone (Fig. 3). These data indicate that PKC-dependent signaling mediates an immediate protective response against menadione-induced oxidative stress.
Menadione causes selective PKD Ser744/748 phosphorylation and nuclear translocation

To identify the PKC isoform mediating RALA hepatocyte resistance to menadione toxicity, levels of active, phosphorylated PKC were examined after menadione treatment. Menadione induced an increase in phospho-PKC\(\mu\) or phospho-PKD within 1 h after menadione treatment (Fig. 4A). Menadione-induced phosphorylation was specific for Ser744/748 as no change was detected in the levels of phosphorylation at the Ser916 residue. Levels of total PKD were also unaffected by menadione treatment. Menadione had no effect on the levels of phosphorylated PKC\(\alpha/\beta\), PKC\(\zeta/\lambda\), or PKC\(\delta/\theta\) (Fig. 4A). Selective PKD Ser744/748 phosphorylation was induced by both nontoxic and toxic concentrations of menadione (Fig. 4B).

Once activated, PKD has been reported to translocate from the cytoplasm to cellular organelles including the nucleus and mitochondria (13,14). The levels of active, phosphorylated PKD and PKC were examined in nuclear and cytosolic fractions from RALA hepatocytes after menadione treatment. PKD phosphorylated at Ser744/748 was undetectable in the nuclei of untreated cells, but increased markedly within 1 h after menadione treatment (Fig. 4C). Significant amounts of Ser916 phosphorylated and total PKD were present in the nucleus of untreated cells, but these levels were unchanged by menadione treatment (Fig. 4C). The exclusive presence of the nuclear protein Nopp140 in the nuclear fractions, and of PDI in the cytosolic fractions, demonstrated both the relative purity of the isolates and the equivalence of loading among samples (Fig. 4C). Phosphorylated PKC\(\alpha/\beta\), PKC\(\zeta/\lambda\), and PKC\(\delta/\theta\) were all present in the nuclear fraction of untreated cells, and their levels were unaffected by menadione treatment (data not shown). Menadione treatment failed to lead to mitochondrial translocation of PKD or any PKC isoform (data not shown). Menadione treatment was therefore associated with the translocation of Ser744/748 phosphorylated PKD to the nucleus.
Ro-31-8425 and Bis I inhibit menadione-induced PKD activation

Both Ro-31-8425 and Bis I are general PKC inhibitors (34,35). The specific effects of these two inhibitors on menadione-induced PKD activation were examined. Both compounds blocked the menadione-induced increase in phospho-PKD Ser744/748 without affecting levels of phospho-PKD Ser916 or total PKD (Fig. 5). Chelerythrine, which did not sensitize cells to death from menadione, failed to inhibit PKD Ser744/748 phosphorylation (Fig. 5). Ro-31-8425, Bis I and chelerythrine had no effect on levels of phospho-PKC$\alpha$/$\beta$, phospho-PKC$\zeta$/\$\lambda$, or phospho-PKC$\delta$/\$\theta$ (Fig. 5). Thus, the selective activation of PKD by menadione was specifically inhibited by Ro-31-8425 and Bis I, but not chelerythrine. These data strongly suggested that RALA hepatocyte resistance to menadione-induced death was mediated by protective PKD-dependent signaling.

Ro-31-8425-induced sensitization to death from menadione is prevented by PKD expression

To mechanistically link inhibition of PKD activation with cell death from menadione, the effect of PKD expression on death from Ro-31-8425/menadione was determined. RALA hepatocytes were transiently transfected with a $\beta$-galactosidase expressing control vector or with the vector PKD.SS744/748EE that expresses a constitutively active PKD (27). Similar to primary hepatocytes, RALA hepatocytes are difficult to transfect, and the transfection efficiency was only 30% as assessed by $\beta$-galactosidase staining of control vector transfected cells. Transfection with PKD.SS744/748EE resulted in PKD expression as demonstrated by immunoprecipitations with an anti-HA antibody followed by Western blotting for PKD (Fig. 6A). Expression of the constitutively active PKD decreased cell death from Ro-31-8425/menadione by 40% (Fig. 6B). While the inhibition of death was incomplete, this
percentage of inhibition was commensurate with the transfection efficiency. The relative amount
of cell death from Ro-31-8425/menadione was higher than in the previous experiments as less
confluent cultures were employed in order to maximize transfection efficiency, and cell death
from menadione is proportional to cell density. These data directly link PKC-dependent PKD
activation to hepatocellular resistance to menadione-induced cell death.

**Ro-31-8425 pretreatment results in overactivation of ERK1/2 and JNK MAPKs in response to
menadione**

The early temporal involvement of PKC/PKD signaling in the regulation of RALA
hepatocyte death from menadione suggested that their activation may affect other cell signals
that ultimately mediate resistance to toxic oxidative stress from menadione. Previous studies
identified ERK1/2 activation as critical for RALA hepatocyte resistance to menadione toxicity
(3), and PKD overexpression in non-hepatic cell types has been shown to induce ERK1/2
activation (16). In light of these findings, the possibility that PKC/PKD inhibition sensitized
RALA hepatocytes to death from menadione by blocking ERK1/2 activation was examined by
Western blotting for phospho-ERK1/2. Surprisingly, PKC inhibition by Ro-31-8425 led to
activation rather than inhibition of ERK1/2 at a low dose of menadione that by itself failed to
significantly affect ERK1/2 phosphorylation (Fig. 7A). ERK1/2 activation that occurred with
toxic concentrations of menadione was further increased by Ro-31-8425 cotreatment (Fig. 7B).
Sensitization to menadione toxicity by PKC inhibition therefore could not be explained by a
block in ERK1/2 MAPK signaling.

The mechanism of ERK1/2-mediated resistance to menadione toxicity is through the
down-regulation of pro-apoptotic JNK/c-Jun/AP-1 signaling (3). Despite high levels of ERK1/2
activation, Ro-31-8425/menadione cotreatment led to sustained JNK1/2 and c-Jun activation as reflected by increased levels of these phospho-proteins on immunoblots (Fig. 7C). Levels of total JNK1/2 and c-Jun were unaffected by Ro-31-8425 or menadione treatment. JNK activation as measured by an in vitro kinase assay was markedly increased in both Ro-31-8425/menadione- and Bis I/menadione-treated cells as compared to cells treated with menadione alone (Fig. 7D and 7E). JNK activity was unaffected by administration of Ro-31-8425 at times later than 1 h after menadione treatment (Fig. 7F), corresponding to the inability of delayed administration of this inhibitor to sensitize cells to death from menadione (Fig. 3). Thus, PKC/PKD inhibition converted the RALA hepatocyte response to menadione to one of sustained JNK/c-Jun overactivation despite increased activation of ERK1/2.

Death from Ro-31-8425/menadione is mediated by c-Jun/AP-1 overactivation

Increased phosphorylation of c-Jun leads to its transcriptional activation as a subunit of the transcription factor AP-1. To assess levels of AP-1 activity, RALA hepatocytes were transiently transfected with an AP-1 driven luciferase reporter gene Coll73-Luc. Treatment with 25 uM menadione had no effect on AP-1 dependent luciferase activity (Fig. 8A). Ro-31-8425 treatment alone led to a modest increase in activity, while cotreatment with menadione led to a 2.5-fold increase in AP-1-dependent gene expression (Fig. 8A).

To determine whether increased AP-1 activity resulting from PKC/PKD inhibition mediated cell death from menadione, the effect of blocking c-Jun function was examined by adenoviral expression of the c-Jun dominant negative TAM67. TAM67 expression has been previously demonstrated to effectively inhibit AP-1 transcriptional activity in RALA hepatocytes (38). Cells were infected with the adenovirus Ad5LacZ as a control for the nonspecific effects of
viral infection. Ad5LacZ-infected cells were sensitized to toxicity from menadione by Ro-31-8425 similar to uninfected cells (Fig. 8B). Infection with the TAM67-expressing adenovirus Ad5TAM completely blocked death from PKC inhibition and menadione treatment at the 25 μM concentration, and inhibited death at 30 μM menadione by 50% (Fig. 8B). PKC/PKD inhibition therefore sensitized RALA hepatocytes to death from menadione through overactivation of the c-Jun/AP-1 pathway.

**ERK1/2 and PKC/PKD are independent signals for resistance to menadione toxicity**

The present data together with previous studies (3), indicate that ERK1/2 MAPK and PKC/PKD signaling are both critical for RALA hepatocyte resistance to menadione toxicity. These two signals may act sequentially or in parallel. ERK1/2 signaling was not downstream of PKC activation as inhibition of PKC increased rather than decreased ERK1/2 activation in response to menadione (Fig. 7A and 7B). Inhibition of ERK1/2 signaling by U0126 similarly failed to affect menadione-induced PKD activation (Fig. 9A). These data suggested that the two signaling pathways acted independently to protect RALA hepatocytes from menadione toxicity. To examine this possibility, the effect of co-inhibition of ERK1/2 and PKC on cell death from menadione was determined. At two concentrations of menadione, co-administration of Ro-31-8425 and U0126 led to a significantly increased amount of cell death over that from either inhibitor alone (Fig. 9B). In addition, cotreatment with both inhibitors led to a greater increase in phospho-JNK1/2 and phospho-c-Jun levels in response to menadione than did either inhibitor by itself (Fig. 9C). These data indicate that ERK1/2 and PKC/PKD are independent signals that down-regulate JNK/c-Jun after menadione treatment.
DISCUSSION

The present study demonstrates that resistance to superoxide toxicity in a hepatocyte cell line is mediated through a PKC-dependent serine phosphorylation and activation of PKD that temporally restricts pro-apoptotic AP-1 signaling. The critical findings that support this conclusion are: (1) the ability of the PKC inhibitors Ro-31-8425 and Bis I but not chelerythrine to sensitize RALA hepatocytes to death from normally nontoxic concentrations of menadione; (2) the specific phosphorylation of PKD at Ser744/748 by menadione, and prevention of this activation by Ro-31-8425 and Bis I but not chelerythrine; (3) that expression of a constitutively active PKD prevents death from PKC inhibition and menadione; (4) the demonstration of overactivation of all three elements of the JNK/c-Jun/AP-1 pathway with the inhibition of PKC/PKD activation; and (5) that expression of the c-Jun dominant negative TAM67 blocked the sensitization of RALA hepatocytes to death from PKC inhibition and menadione. Consistent with the involvement of PKC/PKD signaling in the inherent resistance of RALA hepatocytes to death from superoxide was that death from PKC inhibition and low dose menadione occurred through oxidant-dependent, caspase-independent apoptosis identical to death from toxic concentrations of menadione (3). The failure of chelerythrine to alter resistance to menadione toxicity was undoubtedly secondary to its inability to inhibit PKC or PKD activation. Despite its widespread use as a PKC inhibitor, other studies have demonstrated that this compound in fact does not inhibit PKC activity (39).

Menadione specifically induced PKD phosphorylation at Ser744/748 but not at Ser916. PKD activation by phosphorylation of the Ser744/748 site has been previously demonstrated to occur by a PKC-dependent mechanism in non-hepatic cells (11,12). In contrast, phosphorylation at Ser916 occurs by PKC-independent autophosphorylation (40). It is also known that the PKC
inhibitors employed in this study do not inhibit PKD directly (41). Thus, both the selective phosphorylation of Ser744/748 but not Ser916, and the inhibition of this phosphorylation by Ro-31-8425 and Bis I, are consistent with cellular resistance to menadione being a PKC-dependent process mediated through PKD. The present studies do not identify the PKC isoform responsible for PKD phosphorylation. The lack of activation by menadione, or inhibition by Ro-31-8425 and Bis I, of the phosphorylated forms of PKCα/β, PKCζ/λ, and PKCδ/θ, exclude these isoforms as the mediators of PKD phosphorylation. The PKC isoforms likely responsible for menadione-induced PKD phosphorylation are PKCε or PKCη based on their established role in Ser744/748 phosphorylation of PKD in other cell types (12,16). However, we were unable to examine this possibility experimentally because of the unavailability of rat-reactive antibodies for these activated PKC isoforms.

Hydrogen peroxide has been previously demonstrated to induce phosphorylation of both serine and tyrosine sites on PKD (8,11,27). Both serine and tyrosine phosphorylation activate PKD (11,42), and together they lead to synergistic activation (27). In contrast to the PKC dependence of Ser744/748 phosphorylation, tyrosine phosphorylation of PKD occurs through Src-Abl signaling (8,42). We were unable to examine for changes in tyrosine phosphorylation in our cells because of the lack of cross-reactivity between the phospho-tyrosine specific PKD antibody (42), and rat cells. However, while we cannot exclude a role for tyrosine phosphorylation of PKD in RALA hepatocyte resistance to superoxide toxicity, the PKC-dependent nature of our findings strongly suggests that PKD Ser744/748 phosphorylation mediates PKD activation in our model.

In addition to its regulation by phosphorylation, PKD activity is a function of its translocation to different cellular compartments in response to stimuli. PKD predominantly
resides in the cytoplasm although smaller amounts have been reported in Golgi and mitochondria in some cell types (10,13,14). In response to an activating stimulus, PKD moves briefly to the plasma membrane, returns to the cytoplasm, and then translocates to the nucleus (43). While PKD was predominantly located in the cytosol of RALA hepatocytes, PKD was detectable by immunoblotting in the nuclei of untreated cells. Interestingly, while the Ser744/748 phosphorylated form of PKD was not found in the nucleus, significant levels of phospho-PKD Ser916 were present. In response to menadione, no significant change could be detected in nuclear levels of total PKD or phospho-PKD Ser916. Yet significant levels of phospho-PKD Ser744/748 were found in the nucleus in response to menadione treatment. Phospho-PKD Ser744/748 was also present in the cytoplasm only after menadione treatment. These findings are consistent with the demonstration of shuttling of activated PKD between cytoplasm to nucleus (43). PKD activation in response to oxidative stress therefore involves both phosphorylation and translocation. However, the present studies do not exclude the possibility of PKD phosphorylation in the nucleus, especially in light of the failure to detect an increase in total nuclear PKD following menadione treatment.

PKD’s survival effect in HeLa cells and NIH 3T3 fibroblasts was previously demonstrated to occur through activation of NF-κB (8). Inhibition of PKD function blocked NF-κB activation and sensitized cells to death from hydrogen peroxide (8). Although the present studies demonstrate a similar survival effect of PKD in RALA hepatocytes treated with superoxide, this protective effect is clearly not mediated by NF-κB. Menadione alone or cotreatment with Ro-31-8425 and menadione led to equivalent levels of NF-κB activation in RALA hepatocytes as determined by luciferase reporter assay (data not shown). We have also previously demonstrated that although NF-κB activation is protective from tumor necrosis factor...
toxicity (20), NF-κB promotes RALA hepatocyte death from menadione or hydrogen peroxide (44). These data effectively exclude the possibility that NF-κB mediates PKC-dependent resistance to menadione in our cells.

Instead the protective effect of PKD in our model occurred through inhibition of AP-1 signaling. This conclusion is derived from findings of overactivation of AP-1 with PKD inhibition, and the ability of a c-Jun dominant negative to prevent death resulting from PKC inhibition and menadione treatment. AP-1 is a critical regulator of multiple cell processes including proliferation, and cell death (4). This is particularly true in the liver as c-Jun deficient mice undergo lethal hepatic degeneration from apoptosis (45). However, the role of c-Jun/AP-1 and the upstream kinase JNK in cell death is controversial, as evidence exists in a variety of cell systems for both pro- and anti-apoptotic functions of these molecules (4,5). These diverse findings may result from distinct effects of these signals depending on the cell type, stimulus and the physiologic context. They may also reflect the different biological effects of the two forms of JNK, JNK1 and JNK2, and c-Jun, as well as interactions with other transcription factors (46). However, it is clear that a critical factor that regulates the cellular effect of JNK/AP-1 signaling is the length of time of this activation. In both RALA hepatocytes and primary hepatocytes, inhibition of NF-κB sensitizes to death from tumor necrosis factor-α by inducing a prolonged activation of JNK/AP-1 (47,48). A typical transient activation of JNK/AP-1 may therefore promote cell proliferation or survival, whereas sustained activation triggers cell death (4,5). The findings in the present study are consistent with this concept as death from menadione occurred when prolonged AP-1 activation occurred in the absence of PKD signaling.

The effect of PKC/PKD signaling on the AP-1 pathway was distinct from that of ERK1/2. Findings supporting this conclusion include: (1) the fact that inhibitors of either
ERK1/2 or PKC sensitized cells to death from menadione in the absence of any effect on the other signaling molecule; (2) combined inhibition of both pathways led to a modest increase in JNK/c-Jun activation over that achieved by either inhibitor alone; and (3) simultaneous inhibition of both pathways led to increased sensitization to death from menadione. The mechanism by which ERK1/2 activation down-regulates AP-1 signaling is unknown but likely secondary to the induction of phosphatase expression (3). In contrast, PKC-dependent PKD signaling is known to inhibit JNK and c-Jun activation through direct interactions of PKD with both JNK and c-Jun (15-18). The present study is the first identification of a physiologic process modulated by PKC/PKD-dependent inhibition of JNK/c-Jun/AP-1 signaling. The cell requires ERK1/2- and PKC/PKD-dependent signaling mechanisms to sufficiently down-regulate AP-1 in order to resist death from superoxide. The existence of these multiple, independent pathways may reflect the importance to the organism of eliminating cells significantly damaged by oxidative stress. Impairment of either pathway in disease states may sensitize the hepatocyte to death from oxidative stress, and efforts to up-regulate these signal transduction pathways may be a means to promote cellular survival.
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FOOTNOTES

1The abbreviations used are: Bis I, bisindolylmaleimide I; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; PKC, protein kinase C; PKD, protein kinase D; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PDI, protein disulfide isomerase; ROS, reactive oxygen species; ZVAD, Val-Ala-Asp-fluoromethylketone.
REFERENCES

1. Czaja, M. J. (2002) Antioxid. Redox. Signal. 4, 759-767

2. Gabbita, S. P., Robinson, K. A., Stewart, C. A., Floyd, R. A., and Hensley, K. (2000) Arch. Biochem. Biophys. 376, 1-13

3. Czaja, M. J., Liu, H., and Wang, Y. (2003) Hepatology 37, 1405-1413

4. Shaulian, E., and Karin, M. (2002) Nat. Cell Biol. 4, E131-E136

5. Czaja, M. J. (2003) Am. J. Physiol Gastrointest. Liver Physiol 284, G875-G879

6. Konishi, H., Tanaka, M., Takemura, Y., Matsuzaki, H., Ono, Y., Kikkawa, U., and Nishizuka, Y. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11233-11237

7. Konishi, H., Yamauchi, E., Taniguchi, H., Yamamoto, T., Matsuzaki, H., Takemura, Y., Ohmae, K., Kikkawa, U., and Nishizuka, Y. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6587-6592

8. Storz, P., and Toker, A. (2003) EMBO J. 22, 109-120

9. Waldron, R. T., and Rozengurt, E. (2000) J. Biol. Chem. 275, 17114-17121

10. Van Lint, J., Rykx, A., Maeda, Y., Vantus, T., Sturany, S., Malhotra, V., Vandenheede, J. R., and Seufferlein, T. (2002) Trends Cell Biol. 12, 193-200

11. Waldron, R. T., Rey, O., Iglesias, T., Tugal, T., Cantrell, D., and Rozengurt, E. (2001) J. Biol. Chem. 276, 32606-32615

12. Waldron, R. T., and Rozengurt, E. (2003) J. Biol. Chem. 278, 154-163

13. Rey, O., Sinnett-Smith, J., Zhukova, E., and Rozengurt, E. (2001) J. Biol. Chem. 276, 49228-49235

14. Storz, P., Hausser, A., Link, G., Dedio, J., Ghebrehiwet, B., Pfizenmaier, K., and Johannes, F. J. (2000) J. Biol. Chem. 275, 24601-24607

15. Brandlin, I., Eiseler, T., Salowski, R., and Johannes, F. J. (2002) J. Biol. Chem. 277, 45451-45457

16. Brandlin, I., Hubner, S., Eiseler, T., Martinez-Moya, M., Horschinek, A., Hausser, A., Link, G., Rupp, S., Storz, P., Pfizenmaier, K., and Johannes, F. J. (2002) J. Biol. Chem. 277, 6490-6496

17. Hurd, C., and Rozengurt, E. (2001) Biochem. Biophys. Res. Commun. 282, 404-408

18. Hurd, C., Waldron, R. T., and Rozengurt, E. (2002) Oncogene 21, 2154-2160
19. Chou, J. Y. (1983) *Mol. Cell Biol.* 3, 1013-1020

20. Jones, B. E., Lo, C. R., Liu, H., Srinivasan, A., Streetz, K., Valentino, K. L., and Czaja, M. J. (2000) *J. Biol. Chem.* 275, 705-712

21. Chou, J. Y., and Yeoh, G. C. (1987) *Cancer Res.* 47, 5415-5420

22. Mosmann, T. (1983) *J. Immunol. Methods* 65, 55-63

23. Duke R.C. and Cohen J.J. (1992) In Coligan J.E., Kruisbeek A.M., Marguiles D.H, Shevack E.M., and Strober W., ed. *Current Protocols in Immunology*, Wiley & Sons, New York

24. Liu, H., Lo, C. R., Jones, B. E., Pradhan, Z., Srinivasan, A., Valentino, K. L., Stockert, R. J., and Czaja, M. J. (2000) *J. Biol. Chem.* 275, 40155-40162

25. Terada, K., Manchikalapudi, P., Noiva, R., Jauregui, H. O., Stockert, R. J., and Schilsky, M. L. (1995) *J. Biol. Chem.* 270, 20410-20416

26. Meier, U. T. (1996) *J. Biol. Chem.* 271, 19376-19384

27. Storz, P., Doppler, H., and Toker, A. (2004) *Mol. Cell Biol.* 24, 2614-2626

28. Webb, P., Lopez, G. N., Uht, R. M., and Kushner, P. J. (1995) *Mol. Endocrinol.* 9, 443-456

29. Iimuro, Y., Nishiura, T., Hellerbrand, C., Behrens, K. E., Schoonhoven, R., Grisham, J. W., and Brenner, D. A. (1998) *J. Clin. Invest* 101, 802-811

30. Bradham, C. A., Hatano, E., and Brenner, D. A. (2001) *Am. J. Physiol Gastrointest. Liver Physiol* 281, G1279-G1289

31. Xu, Y., Bialik, S., Jones, B. E., Iimuro, Y., Kitsis, R. N., Srinivasan, A., Brenner, D. A., and Czaja, M. J. (1998) *Am. J. Physiol* 275, C1058-C1066

32. Monks, T. J., Hanzlik, R. P., Cohen, G. M., Ross, D., and Graham, D. G. (1992) *Toxicol. Appl. Pharmacol.* 112, 2-16

33. Thor, H., Smith, M. T., Hartzell, P., Bellomo, G., Jewell, S. A., and Orrenius, S. (1982) *J. Biol. Chem.* 257, 12419-12425

34. Merritt, J. E., Sullivan, J. A., Tse, J., Wilkinson, S., and Nixon, J. S. (1997) *Cell Signal.* 9, 53-57

35. Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., and Loriolle, F. (1991) *J. Biol. Chem.* 266, 15771-15781

36. Herbert, J. M., Augereau, J. M., Gleye, J., and Maffrand, J. P. (1990) *Biochem. Biophys. Res. Commun.* 172, 993-999
37. Joza, N., Susin, S. A., Daugas, E., Stanford, W. L., Cho, S. K., Li, C. Y., Sasaki, T., Elia, A. J., Cheng, H. Y., Ravagnan, L., Ferri, K. F., Zamzami, N., Wakeham, A., Hakem, R., Yoshida, H., Kong, Y. Y., Mak, T. W., Zuniga-Pflucker, J. C., Kroemer, G., and Penninger, J. M. (2001) *Nature* **410**, 549-554

38. Liu, H., Jones, B. E., Bradham, C., and Czaja, M. J. (2002) *Am. J. Physiol Gastrointest. Liver Physiol* **282**, G257-G266

39. Lee, S. K., Qing, W. G., Mar, W., Luyengi, L., Mehta, R. G., Kawanishi, K., Fong, H. H., Beecher, C. W., Kinghorn, A. D., and Pezzuto, J. M. (1998) *J. Biol. Chem.* **273**, 19829-19833

40. Matthews, S. A., Rozengurt, E., and Cantrell, D. (1999) *J. Biol. Chem.* **274**, 26543-26549

41. Zugaza, J. L., Sinnett-Smith, J., Van Lint, J., and Rozengurt, E. (1996) *EMBO J.* **15**, 6220-6230

42. Storz, P., Doppler, H., Johannes, F. J., and Toker, A. (2003) *J. Biol. Chem.* **278**, 17969-17976

43. Rey, O., Zhukova, E., Sinnett-Smith, J., and Rozengurt, E. (2003) *J. Cell Physiol* **196**, 483-492

44. Jones, B. E., Lo, C. R., Liu, H., Pradhan, Z., Garcia, L., Srinivasan, A., Valentino, K. L., and Czaja, M. J. (2000) *Am. J. Physiol Gastrointest. Liver Physiol* **278**, G693-G699

45. Hilberg, F., Aguzzi, A., Howells, N., and Wagner, E. F. (1993) *Nature* **365**, 179-181

46. Qiao, L., Han, S. I., Fang, Y., Park, J. S., Gupta, S., Gilfor, D., Amorino, G., Valerie, K., Sealy, L., Engelhardt, J. F., Grant, S., Hylemon, P. B., and Dent, P. (2003) *Mol. Cell Biol.* **23**, 3052-3066

47. Liu, H., Lo, C. R., and Czaja, M. J. (2002) *Hepatology* **35**, 772-778

48. Schwabe, R. F., Uchinami, H., Qian, T., Bennett, B. L., Lemasters, J. J., and Brenner, D. A. (2004) *FASEB J.* **18**, 720-722
**FIGURE LEGENDS**

**Fig. 1.** PKC inhibition sensitizes RALA hepatocytes to death from menadione. (A) RALA hepatocytes were cultured as described in Experimental Procedures and pretreated for 1 h with DMSO or Ro-31-8425 (Ro) prior to treatment with the indicated uM concentrations of menadione (Men). The percentage of cell death was determined by MTT assay 24 h after menadione treatment. (B) Percentage of cell death 24 h after treatment with DMSO or Bis I and 20 or 25 uM menadione. Results are from duplicate samples from each of three independent experiments.

**Fig. 2.** PKC inhibition leads to caspase-independent apoptosis from menadione. (A) The numbers of apoptotic and necrotic cells were determined in acridine orange and ethidium bromide costained cells by fluorescence microscopy as described in Experimental Procedures. Cells were untreated (Con), or treated with 25 uM menadione (Men), Ro-31-8425 (Ro) or a combination of the two (Ro/Men). The percentages of cells that were apoptotic or necrotic were determined at 12 h after menadione treatment. (B) Percentage of cell death at 24 h in RALA hepatocytes treated with Ro-31-8425 and 25 uM menadione alone (∅), or together with pretreatment with ebselen (Eb), catalase (Cat), or Val-Ala-Asp-fluoromethylketone (ZVAD). The data are from three independent experiments performed in duplicate (*P<0.0001).

**Fig. 3.** Early inhibition of PKC signaling is required to sensitize cells to death from menadione. RALA hepatocytes were treated with 25 uM menadione alone (Men) or with Ro-31-8425 as a 1 h pretreatment (-1h), or as a treatment 1 h, 2 h, or 4 h after the menadione. The percentage cell
death was determined at 24 h by MTT assay. The results represent data from three independent experiments performed in duplicate.

**Fig. 4.** Menadione induces selective activation of PKD Ser744/748. (A) Protein was isolated from untreated RALA hepatocytes, and cells treated with 25 μM menadione for the indicated number of hours. Aliquots of protein were immunoblotted with antibodies against PKD phosphorylated at Ser744/748 (P-PKD(Ser744/8)) or Ser916 (P-PKD(Ser916)), total PKD, and phosphorylated forms of PKCα/β (P-PKCα/β), PKCζ/λ (P-PKCζ/λ), and PKCδ/θ (P-PKCδ/θ). (B) Immunoblots performed with the same antibodies on cells untreated, or treated with the indicated μM concentrations of menadione for 2 h. (C) RALA hepatocytes were untreated or treated with 25 μM menadione for the indicated number of minutes. Nuclear and cytosolic protein fractions were obtained as detailed in Experimental Procedures. Protein aliquots were immunoblotted with the PKD antibodies plus antibodies to Nopp140 and protein disulfide isomerase (PDI). The results are representative of three independent experiments. Numerical results under the PKD Westerns represent the relative signal intensity among samples from densitometry scanning of the three experiments.

**Fig. 5.** Ro-31-8425 and Bis I selectively block PKD Ser744/748 phosphorylation in response to menadione. RALA hepatocytes were untreated, or treated with 25 or 30 μM menadione for 2 h as indicated. Some of the menadione-treated cells were also pretreated with the PKC inhibitors Ro-31-8425 (Ro), bisindolylmaleimide I (Bis I) or chelerythrine chloride (Chel). Total protein was isolated and aliquots immunoblotted with antibodies against PKD phosphorylated at Ser744/748 (P-PKD(Ser744/8)) or Ser916 (P-PKD(Ser916)), total PKD, and phosphorylated
forms of PKCα/β (P-PKCα/β), PKCζ/λ (P-PKCζ/λ) and PKCδ/θ (P-PKCδ/θ). All results are typical of three independent experiments.

**Fig. 6.** Constitutive PKD expression blocks death from Ro-31-8425/menadione. (A) RALA hepatocytes were transiently transfected with a β-galactosidase expressing vector (Vec) as a control, or with PKD.SS744/748EE (PKD). Cell lysates were immunoprecipitated with an anti-HA antibody (HA), and immunoblotted with an anti-PKD antibody to demonstrate overexpression of constitutively active PKD. (B) Percentage cell death as determined by MTT assay in Vec and PKD transfected cells 18 h after treatment with Ro-31-8425 and 20 μM menadione. Results are from three independent experiments performed in duplicate (* P<0.001).

**Fig. 7.** PKC inhibition causes sustained ERK1/2 and JNK MAPK activation in response to menadione. (A-B) RALA hepatocytes were pretreated with DMSO or Ro-31-8425 (Ro) followed by 25 μM (A) or 30 μM (B) menadione as indicated. Total protein was isolated at the indicated number of hours after menadione treatment, and immunoblots performed with antibodies against phosphorylated ERK1/2 (P-ERK1/2) and total ERK1/2. (C) Protein was isolated from cells treated with 25 μM menadione and DMSO or Ro-31-8425 for the indicated number of hours and immunoblotted with antibodies for phospho-JNK1 and JNK2 (P-JNK1/2), total JNK1 and JNK2 (JNK1/2), phosphorylated c-Jun (P-c-Jun), and total c-Jun. (D) Identically treated cells were assayed for JNK activity by an in vitro kinase assay. JNK activity is indicated by levels of phosphorylated c-Jun (P-c-Jun) on immunoblots, while levels of total c-Jun serve as a control for protein loading. (E) Equivalent studies of JNK activity when cells were pretreated with the inhibitor Bis I. (F) In vitro kinase assay with cells treated with Ro-31-
8425 1 h prior to menadione administration (-1h), or 1 h (+1h) or 2 h (+2h) after menadione treatment. Results shown are representative of three independent experiments.

**Fig. 8.** PKC inhibition sensitizes RALA hepatocytes to death from AP-1 overactivation. (A) Cells were transiently transfected with an AP-1 regulated luciferase reporter gene and relative luciferase activity determined as described in Experimental Procedures. Cells were untreated (Con), treated with 25 uM menadione (Men) or Ro-31-8425 (Ro) alone, or a combination of the two (Ro/Men). Luciferase activity was determined 6 h after menadione treatment. (B) Percentages of cell death in Ad5LacZ- or Ad5TAM-infected cells 24 h after treatment with Ro-31-8425 and 25 or 30 uM menadione as indicated. Data are from duplicate samples from three independent experiments (* P<0.01; # P<0.001).

**Fig. 9.** PKC and ERK1/2 independently regulate menadione-induced JNK/AP-1 activation. (A) Total protein was isolated from cells pretreated with DMSO, Ro-31-8425 (Ro) or U0126 followed by 1 or 2 h of 25 uM menadione as indicated. Aliquots of protein were immunoblotted with antibodies against PKD phosphorylated at Ser744/748 (P-PKD(Ser744/8)) or Ser916 (P-PKD(Ser916)) and total PKD. (B) The percentage cell death was determined by 24 h MTT assay after treatment with 20 or 25 uM menadione alone, or combined with Ro-31-8425 and/or U0126. At the two concentrations of menadione, the amount of cell death resulting from cotreatment with both inhibitors was significantly increased over that from either inhibitor by itself. (C) RALA hepatocytes were pretreated with Ro-31-8425 and/or U0126 and then 20 uM menadione as indicated. After 2 h of menadione treatment, total protein was isolated and immunoblotted with antibodies for phospho-JNK1 and JNK2 (P-JNK1/2), total JNK1 and JNK2 (JNK1/2),
phosphorylated c-Jun (P-c-Jun), and total c-Jun. Results are from or representative of three independent experiments (* P<0.01; # P< 0.03).
Fig. 1A
Fig. 1B
Fig. 2A
Fig. 2B
Fig. 3
Fig. 4A
Fig. 4B
Fig. 4C
Fig. 5
Fig. 6A
Fig. 6B

The graph shows the percentage of cell death (% of Cell Death) for Men and Ro/Men groups under Vector and PKD conditions. The Ro/Men group shows a significant increase in cell death compared to Men and other conditions, marked with an asterisk (*).
Fig. 7A
Fig. 7B
Fig. 7C
Fig. 7D
Fig. 7E
Fig. 7F
Fig. 8A

Relative Luciferase Activity

|    | Con | Men | Ro   | Ro/Men |
|----|-----|-----|------|--------|
|    | ![Bars] | ![Bars] | ![Bars] | ![Bars] |

- *: Indicates statistical significance compared to baseline.
- #: Indicates a different comparison.
Fig. 8B
Fig. 9A
Fig. 9B
Fig. 9C
Hepatocyte resistance to oxidative stress is dependent on protein kinase C mediated down-regulation of c-Jun/AP-1
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