

Cyclic AMP-guanine exchange factor activation inhibits JNK-dependent lipopolysaccharide-induced apoptosis in rat hepatocytes

Kathleen Ponzetti¹
Melissa King¹
Anna Gates¹
M Sawkat Anwer²
Cynthia RL Webster¹

¹Department of Clinical Science,
Tufts Cummings School of Veterinary
Medicine, Grafton MA, USA;

²Department of Biomedical Sciences,
Tufts Cummings School of Veterinary
Medicine, Grafton MA, USA

Abstract: Lipopolysaccharide (LPS) is known to damage hepatocytes by cytokines released from activated Kupffer cells, but the ancillary role of LPS as a direct hepatotoxin is less well characterized. The aim of this study was to determine the direct effect of LPS on hepatocyte viability and the underlying signaling mechanism. Rat hepatocyte cultures treated overnight with LPS (500 ng/mL) induced apoptosis as monitored morphologically (Hoechst 33258) and biochemically (cleavage of caspase 3 and 9 and the appearance of cytochrome C in the cytoplasm). LPS-induced apoptosis was additive to that induced by glycochenodeoxycholate or Fas ligand, was associated with activation of c-Jun N-terminal kinase B (JNK) and p38 mitogen-activated protein kinases (MAPK), and inhibition of protein kinase (AKT). Inhibition of JNK by SP600125, but not of p38 MAPK by SB203580 attenuated LPS-induced apoptosis, indicating JNK dependency. CPT-2-Me-cAMP, an activator of cAMP-GEF, decreased apoptosis due to LPS alone or in combination with glycochenodeoxycholate or Fas ligand. CPT-2-Me-cAMP also prevented LPS-induced activation of JNK and inhibition of AKT. Taken together, these results suggest that LPS can induce hepatocyte apoptosis directly *in vitro* in a JNK-dependent manner and activation of cAMP-GEF protects against the LPS-induced apoptosis most likely by reversing the effect of LPS on JNK and AKT.

Keywords: apoptosis, cAMP-GEF, AKT, exchange protein activated by cAMP (EPAC), lipopolysaccharide, JNK

Introduction

Bacterial cell wall products absorbed from the gastrointestinal tract and delivered to the liver by the portal circulation both induce and aggravate liver injury.¹⁻⁴ Absorption of lipopolysaccharide (LPS), a glycolipid found on the outer membrane of bacteria, from the gastrointestinal tract, occurs with alterations in intestinal permeability, and contributes to the pathology in many liver diseases including; alcoholic and nonalcoholic steatohepatitis, viral and immune hepatitis, and cirrhosis.²⁻⁹ In laboratory rodents, *in vivo* administration of LPS results in hepatic injury and exacerbates damage from known hepatotoxins.^{1,2,10-12} In addition, virally infected, cholestatic, and cirrhotic livers have heightened sensitivity to LPS mediated damage.^{2,13-15} Although the hepatotoxic effect of LPS is considered to be secondary to the release of inflammatory cytokines from LPS activated Kupffer and stellate cells,¹⁶ a direct effect of LPS on hepatocytes has not been ruled out.

Hepatocytes express toll like receptors 4 (TLR4), the plasma membrane receptor for LPS,^{4,17} but the extent to which they respond to LPS by the stimulation of downstream signaling pathways has not been well characterized. When LPS binds to TLR 4

Correspondence: Cynthia RL Webster
Tufts Cummings School of Veterinary
Medicine, 200 Westboro Rd, N. Grafton,
MA 01536, USA
Tel +1 508 887 4711
Fax +1 508 839 8787
Email cynthia.levaille-webster@tufts.edu

in monocytes/macrophages, pro-inflammatory signaling is promoted through activation of the transcription factor, nuclear factor- κ B (NF- κ B), and the stress activated kinases, C-jun-N-terminal kinase (JNK), and p38 mitogen activated kinase (p38 MAPK).^{3,4} Kupffer and stellate cells are triggered by LPS/TLR to activate these signal transduction pathways that promote the synthesis and release of damaging cytokines and inflammatory molecules. Previous studies looking at the direct effect of LPS on hepatocyte function have primarily examined production of acute phase and inflammatory proteins and not hepatocyte signaling mechanisms.^{18–21} A previous study examining the effect of LPS on hepatocyte signaling demonstrated that LPS activates NF- κ B and p42/p44 MAPK in primary mouse hepatocytes.¹⁷ However, the functional consequence of this activation was not studied. A more recent study has shown that hepatocytes are desensitized by LPS in a TLR4 dependent manner.²² Considering the increasing recognition of the importance of LPS/TLR4 signaling in hepatic diseases, the possibility of a direct effect of LPS on hepatocyte toxicity warrants further investigation.

Increasing intracellular cAMP protects hepatocytes from bile acid, Fas ligand and cytokine tumor necrosis factor (TNF- α) mediated cell death.^{23–27} Cyclic AMP transduces intracellular signals through three pathways: a) the serine threonine protein kinase, protein kinase A, b) cyclic nucleotide gated channels, and c) the cAMP binding guanine nucleotide exchange factors (cAMP-GEFs, also known as exchange proteins regulated by cAMP, [EPAC]).²⁸ Cyclic AMP-GEFs catalyze the formation of the active guanosine triphosphate (GTP) bound form of the small GTPase, Rap. Cyclic AMP/cAMP-GEF/Rap signaling pathways exist in hepatocytes and studies show that cAMP-GEF activation is responsible for cAMP's pro-survival effect in hepatocytes.^{23,24,27} Cyclic AMP is known to modulate the liver's response to LPS. *In vivo*, oral administration of phosphodiesterase inhibitors,^{29–31} that increase hepatic cAMP levels, parenteral administration of glucagon³² or cell permeable cAMP analogues³³ protect against LPS-induced liver damage. Although there is some evidence that cAMP's protective effect is at the level of the Kupffer cell,^{34,35} the effect of cAMP on the potential toxic effects of LPS in hepatocytes is unknown, as is information on which arm of cAMP signaling is important in LPS cytoprotection.

In a previous study Yao and colleagues reported that LPS can induce apoptosis in cultured rat hepatocytes.³⁶ However, this study used high doses of LPS (5 to 10 μ g/mL) and apoptosis was not confirmed using morphologic criteria. The aim

of the present study was to confirm the direct apoptotic effect of LPS in hepatocytes using both morphologic and biochemical criteria. Additional aims were to characterize the signaling pathway involved in LPS-induced hepatocyte apoptosis and to determine if activation of cAMP-GEF's would protect against LPS mediated damage.

Materials and methods

Cell culture

Hepatocytes were isolated from rat livers by collagenase perfusion as previously described.^{23,24} This isolation procedure has been shown to yield pure hepatocyte cultures free of Kupffer and stellate cell contamination.³⁷ In addition, purity of the cultures was assessed at >99% by light microscopy. These studies were conducted in accordance with Tufts University institutional animal care and use committee and the National Institute of Health guidelines. Cells were plated on to Type I collagen in minimal essential media supplemented with 10% fetal calf serum, 100 ng/mL of insulin, 100 ng/mL of penicillin, and 100 ng/mL of streptomycin. After 1 hour, the media was changed to non-supplemented minimal essential media for 3 hours. LPS (500 ng/mL) from *E. coli* 0127: B8 (Sigma-Aldrich Chemical, St Louis, MO) was added at this time and allowed to incubate overnight. The next day cells were treated with either 100 μ M glycochenodoxycholate (GCDC, Sigma-Aldrich Chemical, St Louis, MO) or 50 ng/mL Fas ligand (Axxora, LLC, San Diego, CA). Some cultures were pretreated for 30 minutes with the p38 MAPK inhibitor, SB 203580 (5 μ M), the JNK inhibitor, SP600125 (15 μ M) (EMD Chemicals, Gibbstown, NJ) or with 20 μ M of the cAMP-GEF activator, 4-(4-chlorophenylthio)-2'-O-methyladenosine-3'5'cyclic monophosphate, (CPT-2-Me-cAMP, Axxora, LLC, San Diego, CA) prior to the addition of LPS.

Immunoblotting

Whole cell lysates were prepared in cell lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 10 μ g/mL aprotinin, 10 μ g/mL leupeptin, 500 nM okadaic acid, and 1 mM orthovanadate, pH 7.5). Proteins were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (PVDF, Millipore, Billerica, MA). Immunoblotting was performed to detect levels of Bim, Bad, Bax (Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-x_L, cleaved caspase 3 or 9, phospho Jun N-terminal Kinase (JNK) (Thr 183 and Tyr 185), phospho protein kinase (AKT) (Ser 473), and

phospho p38 MAPK (Thr 180 and Tyr 182) (Cell Signaling Technology, Danvers MA). Equal protein loading was verified by stripping and immunoblotting with the antibodies to the non-phosphorylated forms of the kinase or with an antibody to actin (EMD Chemicals, Gibbstown, NJ). The blots were developed by chemiluminescence, digitized with Adobe Photoshop®, and quantified using Sigma Gel® software, as we have done previously.^{23,24}

Monitoring of apoptosis

Rat hepatocytes were exposed to LPS overnight or to GCDC for 2 hours or Fas ligand for 4 hours and then monitored morphologically for apoptosis using Hoechst staining.^{23,24} Apoptotic cells were identified by brightly staining condensed chromatin or nuclear fragmentation. A total of 500 cells were counted from random fields on each slide. Apoptosis was expressed as a percentage of the total number of cells counted. Apoptosis was also confirmed biochemically by immunoblotting cell lysates for the 17 kD or 37 kD proteolytic processing fragment associated with activation of caspase 3 or caspase 9, respectively (Cell Signaling Technology, Danvers, MA). Cytochrome C release from mitochondria was determined in cytosolic extracts prepared by a selective digitonin permeabilization technique as previously described.³⁸ Cytosolic proteins were separated by SDS-PAGE transferred to PVDF membrane and immunoblotted for cytochrome C (Santa Cruz Biotechnology). Equal protein loading and purity were assessed with by immunoblotting for actin and anti-cytochrome C oxidase (Molecular Probes), respectively. All immunoblots containing cytosolic protein were negative for the presence of cytochrome C oxidase immunoreactivity confirming lack of mitochondrial contamination.

Statistics

The results represent the mean and standard deviation of at least 3 separate experiments. Comparisons were done with Student's t-test or one way ANOVA and significance was considered at a level of $P < 0.05$.

Results

LPS induces apoptosis in hepatocytes and enhances fas ligand-and bile acid-induced apoptosis

Treatment of hepatocytes with LPS (500 ng/mL) overnight resulted in the morphologic appearance of apoptosis in 6.5 ± 0.56 % of the hepatocytes (Figures 1A and 1B) representing an almost 3 fold increase in the small amount of

basal apoptosis seen in untreated hepatocytes. Biochemically, LPS treatment was associated with an increase in the generation of the 17 kD and 37 kD cleavage fragment of caspase 3 and caspase 9, respectively (Figures 1C and 1D). Analysis of cytosolic extracts revealed the appearance of cytochrome C in LPS treated hepatocytes (Figure 1E), indicating activation of the mitochondrial pathway. These results indicate that LPS, in a clinically relevant dose, induces hepatocyte apoptosis directly in the absence of signals from Kupffer or stellate cells, and that this apoptosis involves a mitochondrial mediated pathway.

Hepatocytes exposed to GCDC (2 hours at 100 μ M) or Fas ligand (4 hours at 50 ng/mL) undergo apoptosis with an average of 2.5 and 5.2 fold increase in cell death, respectively, compared to untreated cultures (Figures 2A and 2B). When LPS sensitized hepatocytes were exposed to GCDC or Fas ligand, apoptosis increased approximately 60% over that

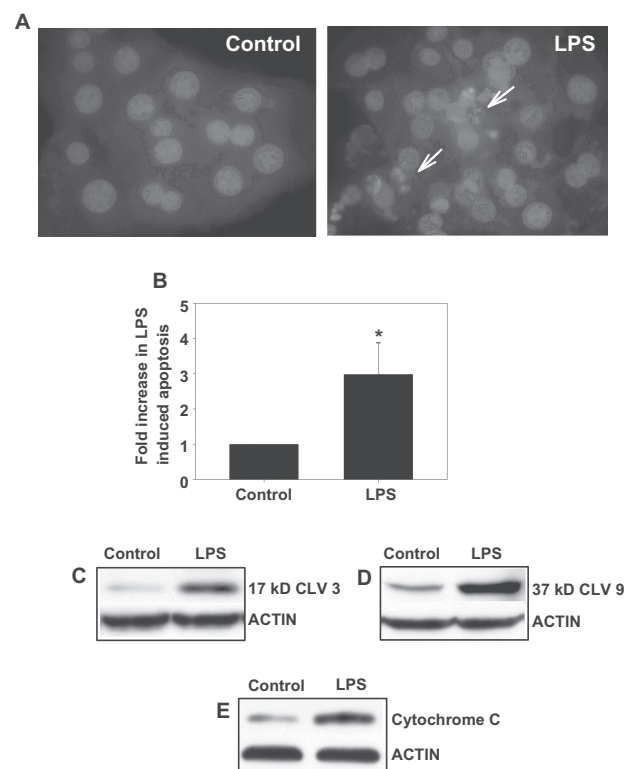


Figure 1 LPS induces apoptosis in hepatocytes. Hepatocytes were treated overnight with 500 ng/mL LPS and cells were evaluated morphologically by Hoechst staining for the presence of apoptosis. Representative photomicrographs (100 \times) are shown; the arrows depict apoptotic hepatocytes (**A**). The amount of apoptosis was quantified by counting the number of hepatocytes with apoptotic morphology and is expressed as a percentage of the amount of apoptosis seen in the control and represents mean \pm SD [$n = 3-5$] (**B**). Biochemical evidence of apoptosis was also obtained by immunoblotting for the 17 kD or 37 kD cleavage product of caspase 3 or caspase 9, respectively (**C** and **D**). Cytosolic preparations were immunoblotted for the appearance of mitochondrial cytochrome C (**E**). *Significantly different from control, $P < 0.05$.

Abbreviations: LPS, lipopolysaccharide; SD, standard deviation.

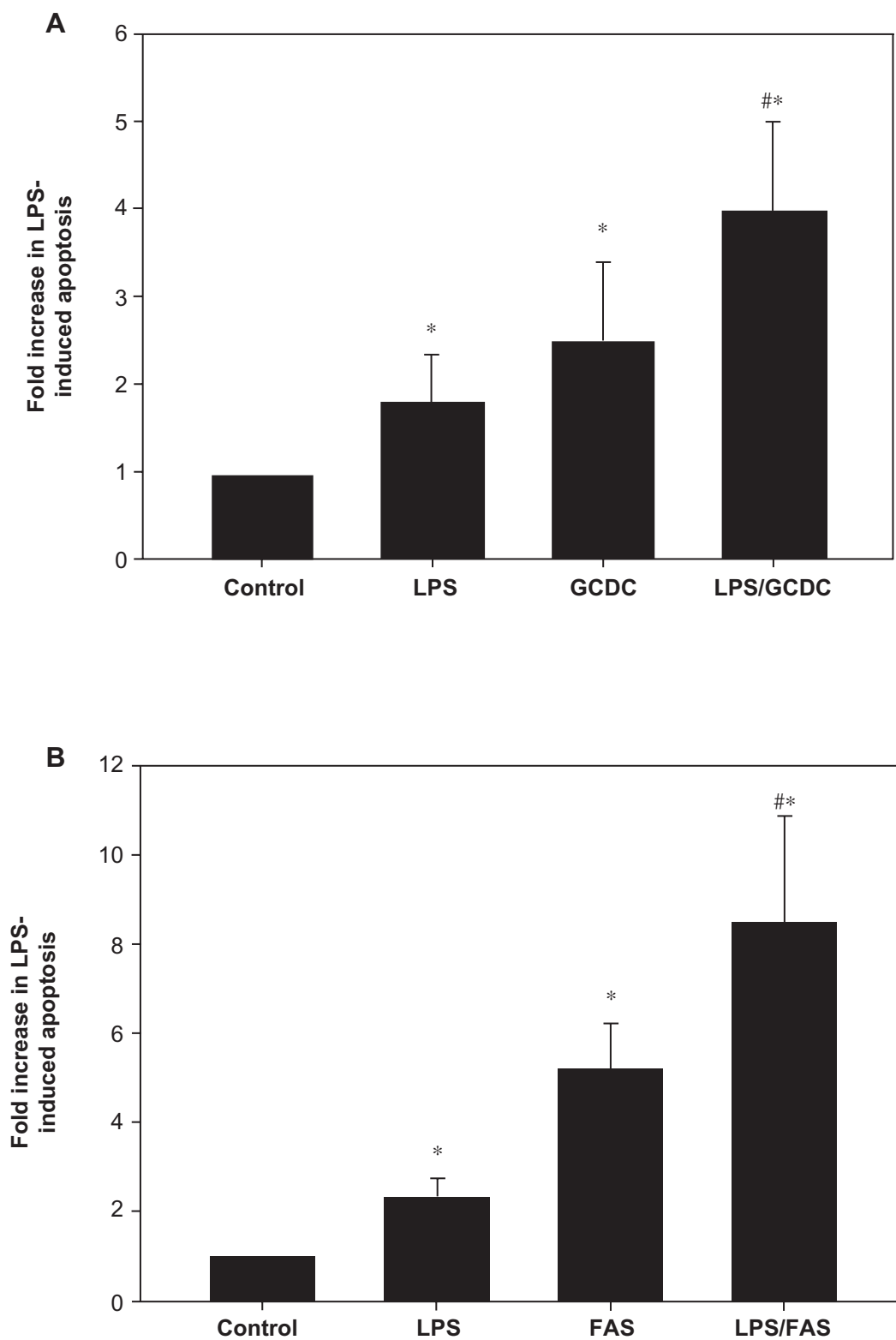


Figure 2 LPS-mediated apoptosis is additive to that induced by bile acids or Fas ligand. Hepatocytes were treated with 500 ng/mL LPS overnight followed by treatment with 100 μ M GCDC for 2 hours (A) or 50 ng/mL Fas L for 4 hours (B). Cells were evaluated morphologically by Hoechst staining for the presence of apoptosis. Results are expressed as a percentage of the amount of apoptosis seen in the control and represent mean \pm SD (n = 3–5). *Significantly different from control, #Significantly different from LPS alone, $P < 0.05$.

Abbreviations: LPS, lipopolysaccharide; GCDC, glycochenodoxicholate; SD, standard deviation; FAS, Fas ligand.

seen in hepatocytes without pre-treatment with LPS. The increase in apoptosis seen in the presence of GCDC or Fas ligand appeared to be additive to that seen in the presence of LPS alone.

LPS activates stress-activated kinases in hepatocytes

The stress-activated protein kinases, JNK³⁹⁻⁴³ and p38 MAPK^{44,45} have been implicated in facilitating hepatocyte apoptosis, while AKT has cytoprotective properties.^{23,24,46-48} Thus we investigated the temporal effect of LPS on the phosphorylation state (and hence activation) of these 3 kinases. LPS induced a 1.5 to 2 fold increase in JNK phosphorylation at 2 hours that persisted for up to 24 hours (Figures 3A and 3D). At the same time LPS induced an early (by 30 minutes) and sustained (up to 24 hours) increase (1.4 to 2 fold) in p38 phosphorylation (Figures 3B and 3D).

LPS decreased AKT phosphorylation, but only after overnight incubation (Figures 3C and 3D). In support of a proapoptotic role for decreased AKT phosphorylation, overnight treatment with 20 μ M LY 294002, a PI3K/AKT inhibitor, resulted in $12.5\% \pm 1.2\%$ apoptosis. LPS had no effect on the expression of total p38, JNK or AKT proteins for up to 24 hours (data not shown). It may be noted that LPS did not affect extracellular receptor kinase ERK phosphorylation (data not shown).

In order to determine whether LPS induced the activation of JNK or p38 MAPK was necessary for LPS induced apoptosis, hepatocytes were pre-treated with a specific inhibitor of JNK (SP600125) or p38 MAPK (SB203580) for 30 minutes prior to the application of LPS. The results of these experiments show that the inhibition of JNK, but not p38 MAPK attenuated LPS induced apoptosis (Figure 3E). Treatments with the kinase inhibitors alone had no effect on apoptosis

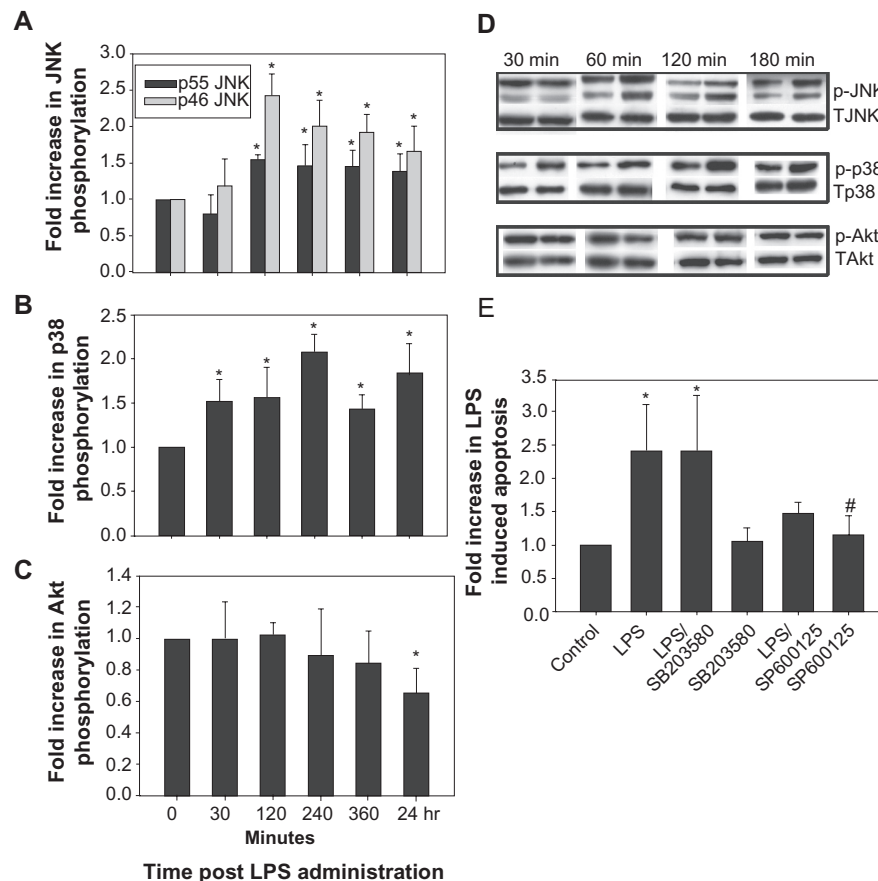


Figure 3 LPS activates stress activated kinases in hepatocytes. Cultured rat hepatocytes were treated with 500 ng/mL LPS for the time points indicated and cell lysates were prepared. The lysates were immunoblotted for phospho JNK (**A** and **D**), phospho p38 MAPK (**B** and **D**), and phospho AKT (**C** and **D**). The blots were developed by chemiluminescence, digitized and quantified. Results of quantification of at least 3 separate experiments are shown in A, B and C and representative gels in D. Hepatocytes were pretreated for 30 min with either a JNK (SP 600125, 15 μ M) or p38 MAPK inhibitor (SB 203580, 5 μ M) and then overnight with 500 ng/mL of LPS (**E**). Cells were evaluated morphologically by Hoechst staining for the presence of apoptosis. Results are expressed as the percentage increase in apoptosis compared to control and represent mean \pm SD ($n = 3$). *Significantly different from control, #Significantly different from LPS, $P < 0.05$.

Abbreviations: LPS, lipopolysaccharide; JNK, c-Jun N-terminal kinase; pJNK, phospho JNK; MAPK, p38 mitogen-activated protein kinases; AKT, protein kinase; SD, standard deviation.

(Figure 3E). Previous studies by others and us demonstrated inhibition of JNK and p38 MAPK by these inhibitors under similar experimental conditions.^{49,50} These results suggest that LPS-induced apoptosis is JNK-dependent.

Activation of cAMP-GEF attenuates LPS-induced apoptosis and opposes LPS-induced modulation of JNK and AKT phosphorylation

Increases in cAMP protect hepatocytes from a variety of apoptotic stimuli.^{23,24,27} The cAMP cytoprotective pathway in rat hepatocytes involves protein kinase A independent activation of a cAMP-GEF/phosphoinositol-3-kinase/AKT pathway.^{23,24} We used a cAMP analogue (CPT-2-Me-cAMP) that selectively activates cAMP-GEF's, but not protein

kinase A in hepatocytes^{23,27} to investigate the role of this pathway in LPS induced apoptosis. Pretreatment with 20 μ M CPT-2-Me-cAMP inhibited morphologic evidence of LPS-induced apoptosis by 60% (Figure 4A) and prevented caspase 3 cleavage (Figure 4B). Since we have already shown that CPT-2-Me-cAMP protects against GCDC and Fas ligand mediated apoptosis in hepatocytes,²³ we investigated whether this cytoprotective effect persisted in the presence of LPS. cAMP-GEF activation was capable of inhibiting Fas ligand and GCDC induced apoptosis in the presence of LPS (Figures 4C and 4D).

In order to determine if the cytoprotective effect of CPT-2-Me-cAMP was associated with modulation of LPS induced changes in JNK or AKT phosphorylation, we treated hepatocytes sequentially with CPT-2-Me-cAMP and LPS and then determined the effect on JNK phosphorylation

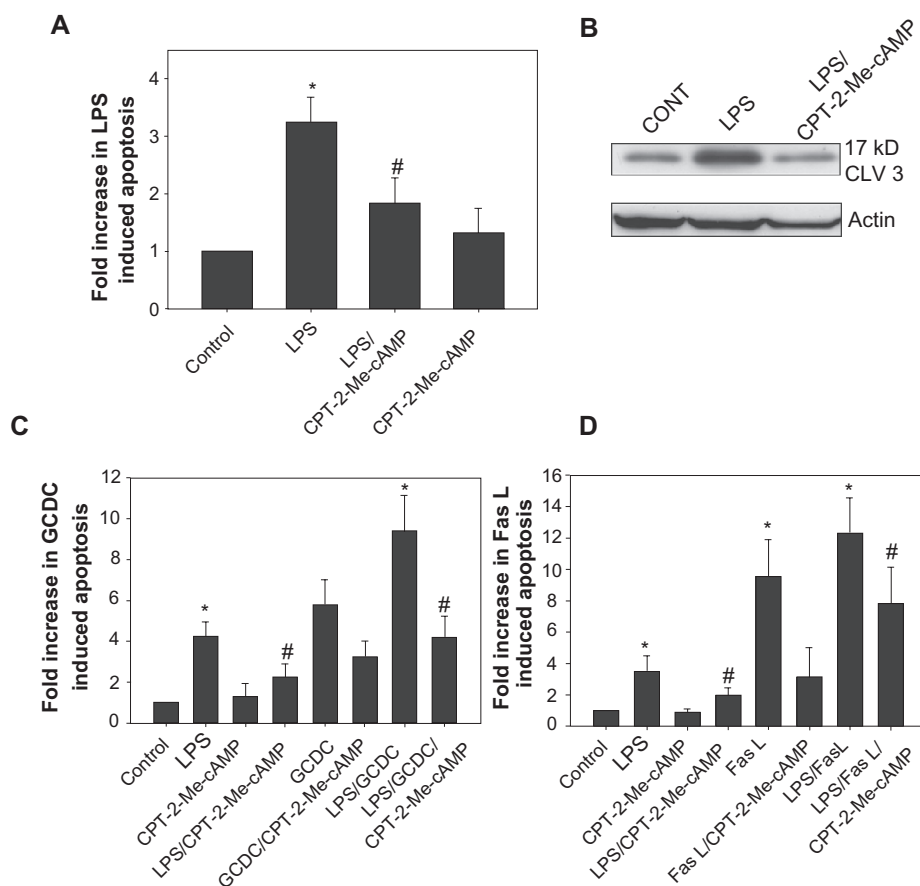


Figure 4 Activation of cAMP-GEF's attenuates LPS induced hepatocyte apoptosis. Hepatocytes were pretreated with 20 μ M CPT-2-Me-cAMP or vehicle for 30 minutes followed by treatment with vehicle or 500 ng/mL LPS overnight. Cells were evaluated morphologically by Hoechst staining for the presence of apoptosis (A). Results are expressed as a percentage of the amount of apoptosis seen in the control and represent mean \pm SD (n = 4–6). Whole cell lysates were immunoblotted for the 17 kD cleaved fragment of caspase 3 and equal protein loading was verified by immunoblotting for actin (B). Hepatocytes were pretreated with 20 μ M CPT-2-Me-cAMP for 30 minutes and then treated overnight with 500 ng/mL LPS \pm pretreatment before exposing to 100 μ M GCDC for 2 hours (C) or 50 ng/mL of Fas L for 4 hours (D). *Significantly different from control. #Significantly different from LPS, $P < 0.05$.

Abbreviations: LPS, lipopolysaccharide; cAMP, cyclic adenosine monophosphate; CPT-2-Me-cAMP, 4-(4-chlorophenylthio)-2'-O-methyladenosine-3'5' cyclic monophosphate; GCDC, glycochenodoxycholate; SD, standard deviation.

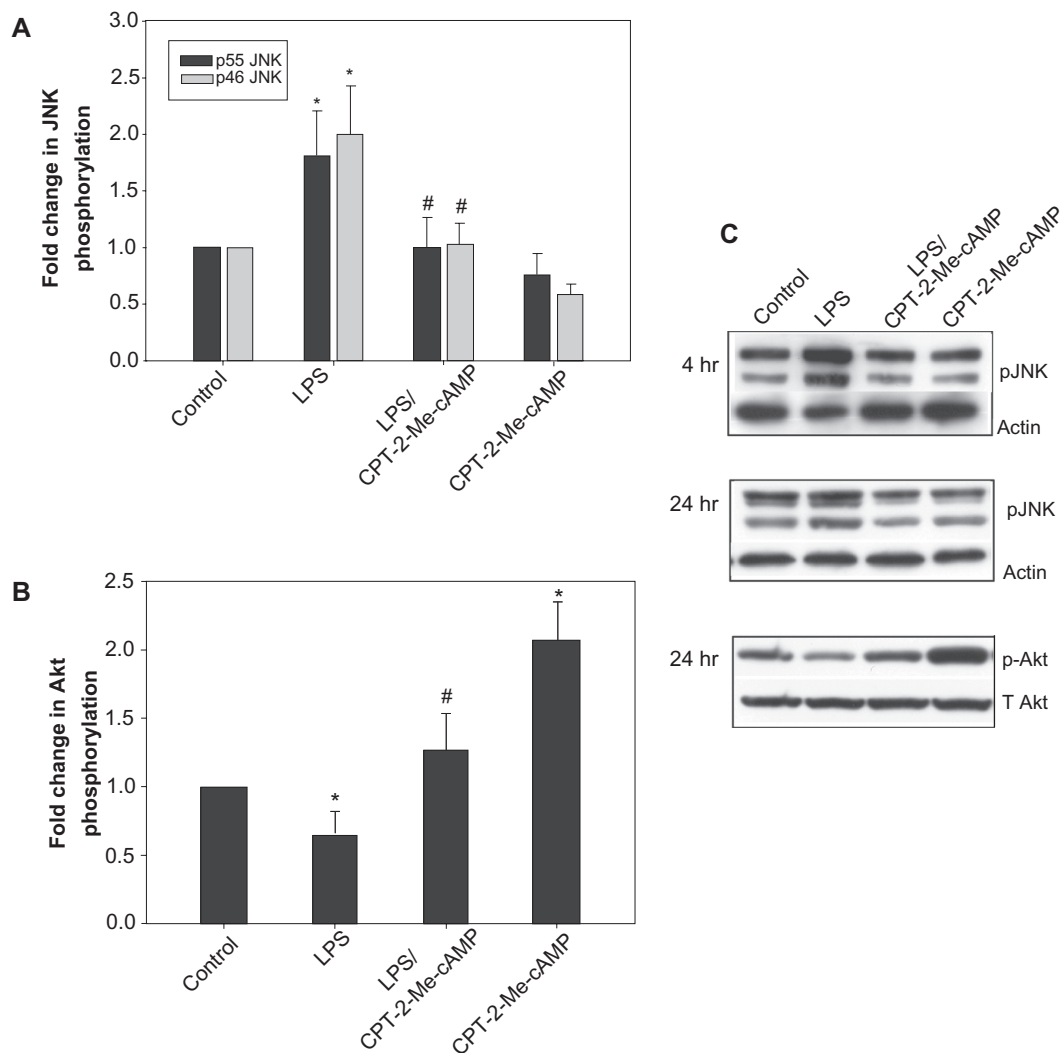


Figure 5 Activation of cAMP-GEF inhibits LPS induced phosphorylation of JNK. Hepatocytes were treated with 500 ng/mL of LPS +/- pretreatment with 20 μ M CPT-2-Me-cAMP for 4 or 24 hrs. Whole cell lysates were prepared. The lysates were immunoblotted for phospho JNK (A and C) and phospho AKT (B and C) and developed by chemiluminescence, digitized and quantified. Representative gels are shown in C and the results of quantification of at least 3 separate experiments are shown in A and B. *Significantly different from control, $P < 0.05$. #Significantly different from LPS, $P < 0.05$.

Abbreviations: LPS, lipopolysaccharide; cAMP, cyclic adenosine monophosphate; CPT-2-Me-cAMP, 4-(4-chlorophenylthio)-2'-O-methyladenosine-3'5'cyclic monophosphate; JNK, c-Jun N-terminal kinase; AKT, protein kinase; SD, standard deviation.

at 4 and 24 hours and AKT phosphorylation at 24 hours. CPT-2-Me-cAMP opposed the LPS induced increase in JNK and decrease in AKT phosphorylation (Figure 5). CPT-2-Me-cAMP alone had no effect on JNK phosphorylation, and as we have previously reported, stimulated AKT phosphorylation.^{23,24} These results suggest that cAMP-GEF activation may prevent LPS-induced apoptosis by modulation of AKT and/or JNK.

LPS has no effect on the expression of Bcl-2 proteins

Hepatocytes are Type II cells that rely on the mitochondrial pathway to execute apoptosis. The B-cell lymphoma-2 (Bcl-2) family proteins are important modulators of the

mitochondrial response to toxic injury. Previous studies of LPS-induced injury in nonhepatic cells have suggested that LPS can modulate the expression of Bcl-2 proteins.^{51,52} Thus, we investigated the effect of overnight treatment with LPS on the expression of the proapoptotic proteins, Bcl-2-associated X protein (Bax), Bcl-2 antagonist of cell death protein (Bad), and BH3-only protein (Bim), and antiapoptotic protein, Bcl-x_L. LPS had no significant effect on the total expression of these Bcl-2 members (data not shown).

Discussion

The present study demonstrates that treatment of rat hepatocytes with LPS induces hepatocyte apoptosis. LPS treatment

of hepatocytes is accompanied by sustained phosphorylation of JNK and p38 MAPK and decreases in AKT phosphorylation. Inhibition of LPS-induced phosphorylation of JNK with chemical inhibitors or by activation of cAMP-GEF's attenuates LPS-induced apoptosis.

Our study shows that LPS-induced rat hepatocyte apoptosis can result from a direct effect of LPS on hepatocytes. Our findings with a more clinically relevant dose of LPS and morphological and biochemical confirmation of apoptosis establish that LPS can cause hepatocyte apoptosis independent of its ability to activate Kupffer or stellate cells. Thus, it would appear that although LPS-induced hepatocytes apoptosis *in vivo* results primarily from the release of cytokines from activated Kupffer and stellate cells, LPS can also have a direct toxic effect on hepatocytes.

The present study provides further insight into the signaling mechanism involved in LPS-induced hepatocyte apoptosis. The finding of sustained activation of p38 MAPK and JNK by LPS raises the possibility that these kinases may be involved in LPS-induced apoptosis since both JNK and p38 MAPK have been implicated in hepatocyte apoptosis.³⁹⁻⁴⁵ Studies with inhibitors of p38 MAPK and JNK however showed that LPS-induced apoptosis may be mediated via JNK and not p38 MAPK. Sustained JNK activation is associated with several models of hepatocyte apoptosis/cell death including bile acid, fatty acid and TNF- α mediated apoptosis, ischemia-reperfusion injury and acetaminophen toxicity,^{39-44,53} although a recent *in vivo* study suggested that it was likely nonparenchymal cell JNK activation that was most important in TNF- α mediated apoptosis.⁵⁴ The mechanisms whereby JNK controls hepatocyte cell death has not been fully elucidated, but has been associated with modulation of Bim and TNF related apoptosis inducing ligand (TRAIL) death receptors, trafficking of Fas death receptors to plasma membrane and accelerated turnover of the antiapoptotic protein, cFLIP.^{39,42,44,52,55}

The mechanism by which LPS activates JNK and p38 MAPK in hepatocytes is unclear at this time. Studies in non-hepatic cells suggest that LPS binding to toll-like receptor 4 protein (TLR4) results in the activation of the transcription factor, NF- κ B and the stress activated kinases, JNK and p38 MAPK.^{3,4} Since hepatocytes express TLRs, it is likely that the activation of JNK and p38 MAPK observed after LPS exposure in the present study is mediated by TLR activation. However, a recent study with mouse hepatocytes showed that LPS failed to activate JNK and p38 MAPK, although LPS-induced TLR4-dependent phosphorylation of p42/p44 mitogen-activated kinase

and activation of NF- κ B¹⁷ This discrepancy may be due to the time frames examined (not mentioned in the mouse study) or represent differences due to culture conditions or variation within species. It is, however, also possible that LPS-induced activation of JNK and p38 MAPK may not be mediated via TLR4 in hepatocytes. Experiments to determine if LPS activates these stress activated kinases in hepatocytes isolated from TLR 4^{-/-} mice would help to answer this question.

Our studies show that LPS mediated apoptosis in hepatocyte proceeds through a mitochondrial dependent pathway as evidenced by cytochrome C release and caspase 9 cleavage. Activation of JNK can mediate mitochondrial dysfunction. In acetaminophen and ischemia-reperfusion injury in hepatocytes, JNK translocates to the mitochondria and promotes cell death by induction of the mitochondrial membrane permeability transition (MPT).^{40,43} Since our studies concur with previous studies which support a role for mitochondrial dysfunction in LPS-mediated toxicity in the liver^{56,57} the role of JNK in LPS-mediated mitochondrial damage deserves further clarification. Another mechanism by which JNK modulates mitochondrial integrity is by controlling the balance of pro- and antiapoptotic Bcl-2 proteins. Free fatty acid-induced JNK activation is associated with an increase in expression of the pro-apoptotic proteins Bax and Bim expression.^{41,58} We were unable to show any changes in the total expression of Bax or Bim with LPS treatment. However, since these proteins are also controlled by post-translational events such as phosphorylation and translocation to the mitochondria we cannot rule out a role for LPS in the modulation of their action. Indeed, acetaminophen induced JNK activation results in mitochondrial translocation of Bax without changes in total Bax expression.⁴⁰

We and others have shown that increases in intracellular cAMP can protect hepatocytes from a variety of toxic injuries including bile acid, Fas ligand- and TNF- α -mediated apoptosis.²³⁻²⁷ We extend these observations to show that cAMP acting through cAMP-GEF's, also protects hepatocytes from LPS-mediated damage. This cytoprotective effect of cAMP-GEF against a wide variety of apoptotic stimuli in hepatocytes may suggest that cAMP-GEF exerts its cytoprotective effect at the level of mitochondria. In Type II cells, such as hepatocytes, death receptor-mediated apoptosis is believed to require a mitochondrial amplification loop and cAMP-GEF has been shown to produce a cytoprotective effect by inhibiting the mitochondrial pathway.⁵⁰

The cytoprotective effect is accompanied by attenuation of LPS-mediated activation of JNK- and LPS-induced dephosphorylation of AKT, both of which would oppose hepatocyte apoptosis.^{23,24,27,38,39,41} In addition, cAMP-GEF activates the antiapoptotic AKT signaling pathway. Thus, cAMP appears to reverse LPS-induced hepatocyte apoptosis by inhibiting LPS-induced pro-apoptotic stimuli as well as activating antiapoptotic signals. Hepatocytes express several receptors, including those for glucagon and epinephrine, which increase cAMP synthesis. It is tempting to speculate that increases in intracellular cAMP which occur in response to these stress hormones may serve as a protective surveillance mechanism against hepatocyte damage from intestinally derived LPS. A recent study showing that lack of the glucagon receptor in mice predisposes them to toxic injury is consistent with this hypothesis.²⁷

Our study also showed that LPS-induced apoptosis is additive to that induced by GCDG or Fas ligand in hepatocytes. This observation is consistent with *in vivo* studies in which LPS enhances hepatic injury from a variety of insults including viral, cholestatic and toxic events.^{2,10,13–15} In some cases, this enhanced sensitivity to injury is associated with upregulation of TLR4 in the liver.^{59–61} Our results raise the possibility that this heightened sensitivity may, in part, be due to the direct effect of LPS on hepatocytes could be even more profound, if the LPS effect is mediated via TLR, in the damaged liver with increased TLR4 expression.

Conclusion

This is the first study to show that LPS induces JNK-dependent hepatocyte apoptosis *in vitro* in the virtual absence of Kupffer and stellate cells, and that LPS's effect is additive to the apoptosis caused by bile acids or Fas ligand. Our study in isolated hepatocytes demonstrates that LPS not only causes liver injury independently, but may also augment injury when other pro-apoptotic stimuli are present. Since these results are similar to what has been observed *in vivo*, it is likely that the direct apoptotic effect of LPS on hepatocytes plays a role in liver disease. Selective strategies to prevent LPS-mediated toxic injury, such as augmenting intracellular cAMP levels, may have therapeutic application in the myriad of hepatic diseases in which LPS has been implicated to play a pathologic role.

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Disclosures

The authors report no conflicts of interest in this work.

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