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Macrophage activation by factors released from acetaminophen-injured hepatocytes. Potential role of HMGB1

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Abstract

Toxic doses of acetaminophen (AA) cause hepatocellular necrosis. Evidence suggests that activated macrophages contribute to the pathogenic process; however, the factors that activate these cells are unknown. In these studies, we assessed the role of mediators released from AA-injured hepatocytes in macrophage activation. Treatment of macrophages with conditioned medium (CM) collected 24 hr after treatment of mouse hepatocytes with 5 mM AA (CM-AA) resulted in increased production of reactive oxygen species (ROS). Macrophage expression of heme oxygenase-1 (HO-1) and catalase mRNA was also upregulated by CM-AA, as well as cyclooxygenase (COX)-2 and 12/15-lipoxygenase (LOX). CM-AA also upregulated expression of the proinflammatory chemokines, MIP-1 α and MIP-2. The effects of CM-AA on expression of COX-2, MIP-1 α and MIP-2 were inhibited by blockade of p44/42 MAP kinase, suggesting a biochemical mechanism mediating macrophage activation. Hepatocytes injured by AA were found to release HMGB1, a potent macrophage activator. This was inhibited by pretreatment of hepatocytes with ethyl pyruvate (EP), which blocks HMGB1 release. EP also blocked CM-AA induced ROS production and antioxidant expression, and reduced expression of COX-2, but not MIP-1 α or MIP-2. These findings suggest that HMGB1 released by AA-injured hepatocytes contributes to macrophage activation. This is supported by our observation that expression of the HMGB1 receptor RAGE is upregulated in macrophages in response to CM-AA. These data indicate that AA-injured hepatocytes contribute to the inflammatory environment in the liver through the release of mediators such as HMGB1. Blocking HMGB1/RAGE may be a useful approach to limiting classical macrophage activation and AA-induced hepatotoxicity.

Keywords

liver; macrophages; acetaminophen; high mobility group box-1; reactive oxygen species

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Introduction

Acetaminophen (AA) is a widely used analgesic and antipyretic agent. While safe and effective at therapeutic doses, when ingested in excess, AA causes severe hepatotoxicity, and represents one of the main causes of acute liver failure in the United States (Lee, 2008). Hepatocellular damage and necrosis following AA intoxication result from covalent binding of the reactive intermediate, N-acetyl-para-benzoquinone imine (NAPQI) to critical cellular proteins in centrilobular regions of the liver (Jollow *et al.*, 1973; Mitchell *et al.*, 1973; Dahlin *et al.*, 1984). Accumulating evidence suggests that activated macrophages and inflammatory mediators contribute to AA-induced hepatotoxicity; however, their role in the pathogenic process depends on the timing of their appearance in the liver and mediators they encounter in the hepatic microenvironment, which direct their phenotype and function (reviewed in Laskin, 2009). Thus, whereas early in the pathogenic process, macrophages are classically activated to release cytotoxic and proinflammatory mediators which contribute to AA-induced liver injury, subsequent alternative activation of these cells leads to the release of cytokines and growth factors important in downregulating the inflammatory response and initiating wound repair. Previous studies have shown that hepatocytes injured by AA release factors that stimulate chemotaxis and production of reactive oxygen species (ROS) by Kupffer cells (Laskin *et al.*, 1986; Horbach *et al.*, 1997). These findings suggest that hepatocytes may be a source of mediators that induce early macrophage activation in the liver. However, the identity of these mediators and their effects on other markers of macrophage activation are unknown.

High-mobility group box 1 (HMGB1) is a DNA-binding protein passively released from necrotic cells (Scaffidi *et al.*, 2002). Extracellular HMGB1 functions as a cytokine, inducing classical activation of macrophages and promoting inflammation (Yang, 2005). The biologic actions of HMGB1 are mediated by multiple receptors, including receptor for advanced glycation end-products (RAGE) and Toll-like receptor 4 (TLR4) (Kokkola *et al.*, 2005; Park *et al.*, 2004). Subsequent activation of downstream signaling pathways including p44/42 mitogen-activated protein (MAP) kinase leads to the production of proinflammatory and cytotoxic mediators, such as chemokines, ROS and eicosanoids (Lotfi *et al.*, 2009; Faraco *et al.*, 2007; Pedrazzi *et al.*, 2007). Activation of HMGB1 and downstream signaling pathways are contributing factors in the pathogenesis of sepsis, rheumatoid arthritis, acute lung injury, and hepatic ischemia-reperfusion injury, each of which involves inflammatory macrophages (reviewed in Andersson and Tracey, 2011). We speculate that early macrophage activation in the liver is mediated, in part, by factors such as HMGB1, released from injured hepatocytes and this was investigated in the present studies.

Materials and methods

Reagents

5-(and-6)-chloromethyl-2', 7'-dichlorodihydro-fluorescein diacetate acetyl ester (CM-H₂DCFDA) was purchased from Invitrogen (Carlsbad, CA). Liberase 3 Blendzyme was from Roche Applied Sciences (Indianapolis, IN). Rabbit anti-HMGB1 and anti-cyclooxygenase-2 (COX-2) antibodies were from Abcam (Cambridge, MA), rabbit anti-heme oxygenase-1 (HO-1) antibodies from Stressgen (Ann Arbor, MI), goat anti-actin antibody from Santa Cruz (Santa Cruz, CA), and rabbit anti-phospho-p44/42, rabbit anti-p44/42 antibodies, and U0126 from Cell Signaling (Beverly, MA). Non-immune IgG was purchased from ProSci (Poway, CA). Protease inhibitor cocktail consisting of 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, aprotinin, bestatin hydrochloride, N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamine, leupeptin hemisulfate, and pepstatin A, and all other chemicals were from Sigma (St. Louis, MO).

Animals

Male pathogen-free C57Bl/6J mice (8-10 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in microisolator cages and allowed free access to food and water. All animals received humane care in compliance with the institution's guidelines, as outlined in the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health. Mice were fasted overnight prior to hepatocyte isolation.

Hepatocyte isolation and preparation of conditioned medium (CM)

Mice were euthanized with Nembutal (200 mg/kg). Hepatocyte isolation was performed as previously described with some modifications (Gardner *et al.*, 1998). Briefly, the liver was perfused through the portal vein with warm $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's balanced salt solution (pH 7.3) containing 25 mM HEPES and 0.5 mM EGTA, followed by Leibowitz L-15 medium containing HEPES and 0.2 U/ml Liberase 3 Blendzyme. The liver was excised, disaggregated, and the resulting cell suspension filtered through a 220 μm nylon mesh. Hepatocytes were recovered by centrifugation at 50 g, and viability (>90%) assessed by trypan blue dye exclusion. Hepatocytes were plated on type I collagen-coated plates at a density of 7.5×10^6 cells/dish in William's Medium E supplemented with 10% FBS, 1% penicillin-streptomycin, 1 mM sodium pyruvate, 1% insulin-transferrin-selenium, and 2 mM L-glutamine. Non-adherent cells were removed by washing the plates 2-3 h later. After overnight incubation at 37°C, the cells were washed and refed with medium control, or medium containing 5 mM AA. In some experiments, hepatocytes were treated with ethyl pyruvate (30 mM) or with medium control for 1 h, and then with medium containing AA and ethyl pyruvate, or ethyl pyruvate alone. After 24 h, hepatocyte CM was collected in pyrogen-free tubes, centrifuged at 300 g (4°C, 5 min) to remove cell debris, diluted in medium to 30% and assayed immediately.

MTT assay

Hepatocytes were plated onto collagen-coated 96 well dishes at a density of 1.5×10^5 cells/well. Non-adherent cells were removed by washing 2-3 h later. After overnight incubation at 37°C, cells were washed and refed with medium control, or medium containing 1-10 mM AA. At the indicated times, medium was removed and fresh medium containing 0.5 mg/ml MTT added. After an additional 2 h incubation, dimethyl sulfoxide was added, and absorbance measured at 550 nm using a Vmax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). A wavelength of 600 nm was used as reference.

Macrophages

RAW 264.7 murine macrophages (ATCC, Manassas, VA) were cultured in DMEM-GlutaMax with 10% FBS, 0.1% penicillin/streptomycin, and 1 mM sodium pyruvate. Twenty four h prior to initiating the experiments, confluent cells were washed in Hank's balanced salt solution, trypsinized, resuspended in DMEM supplemented with 1% FBS, and plated onto 6-well (1×10^6 cells/well), or 12-well (0.3×10^6 cells/well) dishes. Pyrogen-free reagents, tubes, dishes, pipets, and pipet tips were used in all experiments.

Western blotting

Macrophages were lysed in buffer containing 20 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl_2 , 1 mM diethylene triamine pentaacetic acid (DTPA), 1 mM phenylmethylsulfonylfluoride, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 2 mM sodium orthovanadate, and protease inhibitor cocktail. Protein concentrations were measured using the Bradford Assay (Bio-Rad, Hercules, CA). Proteins were separated on 10.5-14% Tris-glycine polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred onto

nitrocellulose membranes. Non-specific binding was blocked by incubation of the blots for 1 h at room temperature with buffer containing 5% non-fat milk or bovine serum albumin (BSA) for phosphorylated proteins, 10 mM Tris-base, 200 mM sodium chloride, and 0.1% polysorbate 20. Membranes were then incubated overnight at 4 °C with anti-HMGB1 (1:2000), HO-1 (1:1000), COX-2 (1:2000), phospho-p44/42 (1:2000), p44/42 (1:2000) or actin (1:1000) primary antibodies, followed by incubation with isotype-specific HRP-conjugated secondary antibodies (1:10,000) for 1 h at room temperature. Binding was visualized using an ECL Plus chemiluminescence kit (GE Healthcare, Piscataway, NJ).

Real-time PCR

Total RNA was isolated from macrophages using an RNeasy kit (Qiagen, Valencia, CA). RNA purity and concentration were measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). RNA was converted into cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to manufacturer's directions. Standard curves were generated using serial dilutions from pooled randomly selected cDNA samples. Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on a 7900HT thermocycler (Applied Biosystems, Foster City, CA). All PCR primer pairs were generated using Primer Express 2.0 (Applied Biosystems), and synthesized by Integrated DNA Technologies (Coralville, IA). For each sample, gene expression changes were normalized relative to 18s RNA. Data are expressed as fold change relative to control. Primer sequences were: catalase, CCAGGGCATCAAAACTTG and GCCCTGAAGCTTTTGTGTCAG; HO-1, CCTCACTGGCAGGAATCATC and CCTCGTGGAGACGCTTTACATA; 12/15-lipoxygenase (12/15-LOX), ACCAGCAAGGACGACGTGAC and ATCAGGTAGCGACCCCATCA; COX-2, GCTTCGGGAGCACAACAGA and GCTCATCACCCCACTCAGGAT; RAGE, TCCCGATGGCAAAGAAACA and GAGTCCCGTCTCAGGGTGTCT; macrophage inflammatory protein-1 α (MIP-1 α), TCTTCTCAGGCCATATGGA and TCCGGCTGTAGGAGAAGCA; MIP-1 β , CAGCACCAATGGGCTCTGA and GCCGGAGGTGTAAGAGAAAC; MIP-2, ACTGACCTGGAAAGGAGGAGC and TGGTTCTTCCGTTGAGGGAC; TLR4, AGCCATTGCTGCCAACATC and ACCTTCCGGCTCTTGTGGA; tumor necrosis factor- α (TNF- α), AAATTTCGAGTGACAAGCCGTA and CCCTTGAAGAGAACCTGGGAGTAG; interleukin-1 (IL-1), CCAAAAGATGAAGGGCTGCT and TCATCTGGACAGCCCAGGTC; 18sRNA, CGGCTACCACATCCAAGGAA and GCTGGAATTACCGCGGCT.

Measurement of ROS production

5-(and-6)-chloromethyl-2', 7'-dichlorodihydro-fluorescein diacetate acetyl ester (CM-H₂DCFDA) was used to assess ROS production by macrophages (Gomes *et al.*, 2005). Macrophages were incubated in polypropylene tubes with 1 μ M CM-H₂DCFDA for 15 min at 37°C in a shaking water bath and then plated onto 96-well dishes (5×10^4 cells/well). Cells were treated with control or AA (1.5 mM), or with CM from control or AA-treated hepatocytes, and then analyzed for fluorescence using a SpectraMax M5 fluorescent spectrophotometer (Molecular Devices, Sunnyvale, CA) every 2 min for 30 min. The excitation and emission wavelengths were 495 nm and 520 nm, respectively.

Statistical analysis

All experiments were repeated at least three times. Data were analyzed using the Student's *t* test or one-way ANOVA followed by Tukey's post hoc analysis. A *p* value of 0.05 was considered statistically significant.

Results

Effects of hepatocyte-derived mediators on macrophages

Initially, we determined if AA-induced cytotoxicity in primary cultures of mouse hepatocytes was associated with the release of mediators that activate macrophages for cytotoxic/proinflammatory activity. For these studies, we used conditioned medium (CM) collected from hepatocytes 24 h after treatment with 5 mM AA, a dose which caused approximately 50% reduction in viability, and morphological evidence of cytotoxicity, including cell shrinkage and partial detachment from the culture dishes (Fig. 1). CM from AA-treated hepatocytes (CM-AA) caused a time-dependent increase in ROS production by macrophages (Fig. 2, upper panel). This activity was not observed in cells incubated with CM from hepatocytes treated with control (CM-CTL). A similar lack of biological activity was noted after treatment of macrophages with AA by itself (not shown). Treatment of macrophages with CM-AA also resulted in a 2.5 fold increase in mRNA expression of the antioxidant catalase, which was evident within 6 h, and persisted for at least 24 h (Fig. 3, upper panel). HO-1 mRNA expression was also upregulated in macrophages following treatment with CM-AA; however, this response was transient, reaching a maximum after 6 h. Upregulation of HO-1 mRNA in response to CM-AA was correlated with increases in HO-1 protein at both 6 h and 24 h post-treatment (Fig. 3, lower panel).

We next analyzed the effects of hepatocyte CM on enzymes mediating eicosanoid biosynthesis. Incubation of macrophages with CM from AA-treated, but not from control hepatocytes or AA alone, upregulated COX-2 protein and mRNA expression (Figs. 3 and 4). CM-AA also increased 12/15-LOX mRNA expression. In contrast, CM-AA caused a small, but reproducible decrease in expression of 5-LOX (Fig. 4).

We also assessed macrophage expression of proinflammatory mediators including CC and CXC chemokines in response to CM from AA-treated hepatocytes. Expression of MIP-1 α (CCL3) and MIP-2 (CXCL2) mRNA was upregulated in macrophages by CM-AA (Fig. 5). Interestingly, AA by itself was also found to upregulate MIP-2 expression, although not as effectively as CM-AA. In contrast, basal levels of MCP-1 (CCL2) mRNA expression were downregulated by CM-AA, while no changes were noted in MIP-1 β (CCL4) expression. CM-AA also had no significant effects on macrophage expression of the proinflammatory cytokines TNF- α and IL-1 (not shown).

Role of p44/42 MAP kinase in CM-AA-induced macrophage activation

In our next series of studies, we analyzed the role of p44/42 MAP kinase in regulating macrophage responses to factors released from AA-injured hepatocytes. Treatment of macrophages with CM-AA resulted in a rapid increase in expression of activated p44/42 MAP kinase, which was evident within 30 min. In contrast, no significant changes were noted in expression of total p44/42 protein (Fig. 6, upper panel), or in expression of total or phospho-p38 MAP kinase or JNK (not shown). Pretreatment of macrophages with the MEK1/2 inhibitor U0126, completely blocked CM-induced expression of phospho-p44/42, with no effect on total p44/42. U0126 also blocked expression of COX-2 protein induced by CM-AA with no effect on HO-1 (Fig. 4, lower panel). Expression of MIP-1 α and MIP-2 mRNA was also significantly reduced by the MEK1/2 inhibitor (Table 1). In contrast, U0126 had no effect on CM-AA-induced expression of catalase or 12/15-LOX mRNA.

Potential role of hepatocyte-derived HMGB1 in macrophage activation

HMGB1 is a proinflammatory protein released by necrotic cells implicated in macrophage activation and tissue injury (Yang *et al.*, 2005). In further experiments, we determined if AA-injured hepatocytes release HMGB1 and if this protein is involved in macrophage

activation. Western blot analysis of hepatocyte culture supernatants showed that treatment with 5 and 10 mM AA, but not control or 2.5 mM AA, was associated with the release of HMGB1 (Fig. 1, middle panel). HMGB1 was not detectable in CM-AA after immunoprecipitation with anti-HMGB1 antibody. Additionally, pretreatment of hepatocytes with ethyl pyruvate, which has been reported to block HMGB1 release (Tang *et al.*, 2010), prior to AA, resulted in a loss of detectable HMGB1 in CM-AA (Fig. 1, lower panel). Ethyl pyruvate by itself had no effect on control hepatocytes, and did not alter the survival of hepatocytes treated with AA (Fig. 1 and not shown).

To assess the potential role of HMGB1 in macrophage activation induced by hepatocyte-derived mediators, we pretreated hepatocytes with ethyl pyruvate and then with AA or CTL. The effects of hepatocyte CM on macrophages were then analyzed. Whereas ethyl pyruvate pretreatment had no effect on the activity of CM-CTL, the ability of CM-AA to stimulate macrophage ROS production was completely suppressed (Fig. 2). A similar loss of ROS-generating activity was observed when CM-AA was subjected to immunoprecipitation with anti-HMGB1 antibodies. We also found that CM-AA-induced expression of catalase and HO-1 mRNA, as well as HO-1 and COX-2 protein in macrophages was blunted when hepatocytes were pretreated with ethyl pyruvate (Table 2 and Fig. 3). Similar decreases in mRNA expression of HO-1 were also observed following pretreatment of CM-AA with anti-HMGB1 antibody (not shown). In contrast, CM-AA induced expression of MIP-1 α and MIP-2 in macrophages was unaltered by ethyl pyruvate pretreatment of hepatocytes (not shown).

RAGE is a macrophage receptor for HMGB1 (Kokkola *et al.*, 2005). Treatment of macrophages with CM from AA-injured hepatocytes resulted in a marked upregulation of RAGE mRNA expression (Fig. 5). Conversely, no major effects were observed on expression of TLR4, which has also been identified as an HMGB1 receptor (Park *et al.*, 2004).

Discussion

Macrophages have been implicated as key cellular effectors in the pathogenesis of AA-induced hepatotoxicity. These cells, which consist of resident and infiltrating macrophages, are rapidly activated following AA intoxication to release cytotoxic and proinflammatory mediators which contribute to liver damage (Gardner and Laskin, 2007; Laskin, 2009; Laskin *et al.*, 2011). Previous studies have suggested that macrophage activation is mediated in part, by factors released from AA-injured hepatocytes (Laskin *et al.*, 1986). Characterization of these factors and their biological effects may provide a better mechanistic understanding of pathways leading to hepatotoxicity.

The present studies demonstrate that AA-induced cytotoxicity in primary mouse hepatocytes was associated with the release of macrophage activating factors. Thus, in response to CM from AA-injured hepatocytes, macrophage production of ROS and expression of COX-2, 12/15-LOX, MIP-1 α , MIP-2 and RAGE were increased, indicating macrophage activation to a proinflammatory phenotype. Surprisingly, CM-AA had no effect on expression of TNF α or IL-1, two proinflammatory mediators generated by classically activated cytotoxic/proinflammatory macrophages (Mosser and Edwards, 2008). These findings suggest that there are multiple pathways regulating macrophage activation in the liver during the early stages of AA-induced hepatotoxicity.

Evidence suggests that ROS including superoxide anion, hydrogen peroxide and hydroxyl radicals derived from inflammatory phagocytes contribute to hepatic injury induced by diverse xenobiotics (reviewed in Laskin *et al.*, 2011). ROS can induce membrane, protein

and DNA damage leading to cytotoxicity and this may be important in the pathogenesis of AA-induced hepatotoxicity (Jaeschke *et al.*, 2003). ROS generation is a characteristic feature of classically activated macrophages (Mosser and Edwards, 2008). We found that CM from AA-injured mouse hepatocytes stimulated macrophage ROS production. These results are in accord with previous reports that AA-treated rat hepatocytes release factors that induce a respiratory burst in Kupffer cells (Laskin *et al.*, 1986). These data support the idea that hepatocytes contribute to the proinflammatory environment in the liver following AA intoxication. CM from AA-injured hepatocytes was also found to upregulate catalase and HO-1 expression in macrophages. These antioxidants play an important role in protecting against AA-induced hepatotoxicity (Chen *et al.*, 2002; Ferret *et al.*, 2001; Chiu *et al.*, 2002). Increased expression of antioxidants by macrophages may be a compensatory response to oxidative stress induced by hepatocyte-derived proinflammatory mediators.

COX-2 is the major enzyme mediating macrophage biosynthesis of proinflammatory prostaglandins, including PGE₂. It is induced in macrophages by inflammatory stimuli and activation is associated with tissue injury (Stables and Gilroy, 2011). The present studies show that CM from AA-injured hepatocytes upregulated COX-2 expression in macrophages, which is consistent with a cytotoxic/proinflammatory phenotype of these cells (Mosser and Edwards, 2008). Treatment of animals with AA results in increased expression of COX-2 in the liver (Reilly *et al.*, 2001; Oz and Chen, 2008; Gardner *et al.*, 2010). Surprisingly, loss of COX-2 is associated with increased susceptibility of mice to AA-induced liver injury (Reilly *et al.*, 2001). This may be due to reduced generation of the anti-inflammatory prostanoids PGD₂ and 15d-PGJ₂ by COX-2. Recent studies suggest that activation of COX-2 and the generation of PGE₂ during the onset phase of inflammation may also indirectly elicit pro-resolution effects by inducing the transcription of enzymes involved in the generation of anti-inflammatory lipoxins (Serhan *et al.*, 2008). It is possible that early activation of COX-2 is important in converting macrophages into anti-inflammatory/wound repair cells and initiating the resolution of the inflammatory response to AA.

Macrophages activated by inflammatory stimuli are also known to synthesize leukotrienes via a family of LOX enzymes including 5-LOX, 12-LOX, and 15-LOX. Whereas 5-LOX induces the generation of proinflammatory leukotrienes such as leukotriene B₄, and is thought to be a pathogenic factor in acute liver injury, 12-LOX and 15-LOX are mainly involved in the production of anti-inflammatory lipoxins which serve a protective function, initiating the resolution of inflammation (Stables and Gilroy, 2011). Interestingly, CM from AA-treated hepatocytes was found to upregulate expression of 12/15-LOX, but to downregulate expression of 5-LOX. These data suggest a potential mechanistic pathway, in addition to COX-2, involved in the transition of classically activated proinflammatory macrophages into alternatively activated immunosuppressive cells, an important step in wound repair.

Expression of the proinflammatory chemokines MIP-1 α and MIP-2 is increased in the liver after AA administration to rodents (Lawson *et al.*, 2000; Liu *et al.*, 2004; Bourdi *et al.*, 2007). These chemokines are important in macrophage and neutrophil trafficking into inflamed tissues and have been implicated in hepatotoxicity (Hogaboam *et al.*, 1999). Moreover, increased expression of chemokines, including MIP-1 α and MIP-2, is a feature of classically activated macrophages (Mosser and Edwards, 2008; Mantovani *et al.*, 2004). We found that hepatocytes injured by AA release factors that upregulate macrophage expression of both MIP-1 α and MIP-2. These data are in accord with reports that hepatocytes treated with cytotoxic doses of AA or with ethanol release chemotactic factors for rat Kupffer cells, as well as neutrophils, and provide additional support for the idea that injured hepatocytes aggravate hepatotoxicity via activation of phagocytic leukocytes (Laskin *et al.*, 1986; Takada *et al.*, 1995; Horbach *et al.*, 1997; Perez *et al.*, 1984; Shiratori *et al.*, 1993).

Interestingly, AA by itself upregulated expression of MIP-2 in macrophages. These results were unexpected since previous studies showed that AA has no effect on macrophage functional responses (Laskin *et al.*, 1986). It may be that gene expression is a more sensitive marker of macrophage activation than functional analyses. In contrast to its effects on MIP-1 α and MIP-2, CM from AA-injured hepatocytes had no effect on expression of MIP-1 β . These data suggest the presence of multiple factors in hepatocyte CM that differentially modulate macrophage accumulation in the liver. We also found that mRNA expression of MCP-1 was downregulated by CM-AA in macrophages. MCP-1 expression is increased in the liver after AA administration and it has been shown to be required for emigration of anti-inflammatory/repair macrophages into the tissue (Dambach *et al.*, 2002). The fact that CM-AA suppresses MCP-1 supports the idea that hepatocytes damaged by AA release factors that predominantly induce proinflammatory macrophage accumulation and activation.

In further studies we assessed potential biochemical mechanisms mediating the effects of hepatocyte-derived activating factors on macrophages. MAP kinases comprise a family of protein-serine/threonine kinases that transduce extracellular signals into a variety of cellular activities including proliferation, differentiation, survival and inflammation (Dong *et al.*, 2002). Following treatment of macrophages with CM from AA-injured hepatocytes, activation of the p44/42 MAP kinase pathway was noted within 30 min. Additionally, CM-AA induced expression of COX-2, MIP-1 α , and MIP-2 was dependent on p44/42. Thus, pretreatment of macrophages with the MEK1/2 inhibitor U0126, blocked the effects of CM-AA on expression of these genes. Evidence suggests that the biological actions of a number of classical macrophage activators, including lipopolysaccharide (LPS) and HMGB1, involve activation of the p44/42 MAP kinase signaling pathway (Kokkola *et al.*, 2005; Pedrazzi *et al.*, 2007). These data suggest a common biochemical mechanism mediating macrophage proinflammatory activity.

The precise identity of the hepatocyte-derived factors that activate macrophages for proinflammatory activity in the liver during the pathogenesis of AA-induced toxicity is unknown. The present studies suggest that HMGB1 may be responsible, at least in part, for this activity. This is based on our findings that AA-induced hepatocyte cytotoxicity was associated with the release of HMGB1, a response that was blocked by pretreatment of hepatocytes with ethyl pyruvate, which prevents HMGB1 release (Tang *et al.*, 2010). Additionally, both CM-AA and purified HMGB1 upregulate expression of COX-2, MIP-1 α , MIP-2, RAGE, and phospho-p44/42 (Pedrazzi *et al.*, 2007; Li *et al.* 1998; Kokkola *et al.* 2005). Recent studies have shown that AA treatment of immortalized hepatocytes leads to HMGB1 release (Martin-Murphy *et al.*, 2010). However, higher doses of AA were required to induce this response, which may reflect the reduced sensitivity of transformed cells, relative to primary cells, to the cytotoxic effects of AA, potentially as a result of diminished metabolic capacity.

Ethyl pyruvate pretreatment of hepatocytes also blocked CM-AA-induced macrophage ROS production, a response that was also suppressed by immunoprecipitation of CM-AA with anti-HMGB1 antibody; these data suggest that the ROS-inducing activity in CM-AA is due to HMGB1. These findings are in accord with previous reports that HMGB1 released from necrotic cells induces a respiratory burst in eosinophils, and that purified HMGB1 stimulates neutrophil ROS production (Lotfi *et al.*, 2009; Fan *et al.*, 2007). Pretreatment of hepatocytes with ethyl pyruvate was also found to suppress CM-AA-induced expression of COX-2, as well as HO-1. These results are consistent with reports that HO-1 deficiency is associated with enhanced release of HMGB1 and decreased survival during endotoxemia. The fact that inhibition of HO-1 and COX-2 was not complete suggests that CM-AA contains factors, in addition to HMGB1, that contribute to macrophage activation. This is supported by our

findings that ethyl pyruvate pretreatment of hepatocytes had no effect on CM-AA-induced macrophage chemokine expression. The mechanisms underlying the ability of ethyl pyruvate to block HMGB1 release have not been established. In lung epithelial cells, ethyl pyruvate-mediated inhibition of HMGB1 release appears to be due to a switch from necrotic to apoptotic cell death (Lim *et al.*, 2007). It remains to be determined if a similar mechanism is involved in the inhibitory actions of ethyl pyruvate on AA-injured hepatocytes.

A major macrophage receptor for HMGB1 is RAGE, which is reported to be activated during inflammation and in response to oxidative stress (Sims *et al.*, 2010). RAGE activation is detrimental in various models of inflammatory injury including sepsis, LPS-induced lung injury, arthritis, partial hepatectomy, and liver ischemia-reperfusion injury (Herold *et al.*, 2007; Zhang *et al.*, 2008; Zeng *et al.*, 2004). Blockade of RAGE has also been reported to protect against lethal doses of AA (Ekong *et al.*, 2006). We found that macrophage expression of RAGE is upregulated in response to CM-AA. Consequences of RAGE activation include the generation of ROS, and increased expression of COX-2, HO-1 and chemokines, such as MIP-2 (Lotfi *et al.*, 2009; Wautier *et al.*, 2001; Shanmugam *et al.*, 2003; Zeng *et al.*, 2009). Our findings that CM-AA induced similar responses in macrophages, and that this correlated with increased RAGE expression, provide additional support for a role of HMGB1 in inducing these activities.

A question arises on potential differences in responsiveness of RAW 264.7 macrophages and primary liver macrophages to CM-AA. RAW 264.7 macrophages exhibit many functional characteristics of primary mouse macrophages, including phagocytosis and pinocytosis, antibody-dependent killing, responsiveness to bacterial products, and high secretory profile [Raschke *et al.*, 1978]. Moreover, in previous studies we demonstrated that primary cultures of Kupffer cells respond to hepatocyte CM with increased chemotaxis and oxidative metabolism [Laskin *et al.*, 1986]. These findings suggest that RAW 264.7 macrophages responses are indeed reflective of Kupffer cells.

In conclusion, the present studies suggest that factors released from AA-injured hepatocytes, including HMGB1, activate macrophages to produce cytotoxic and proinflammatory mediators known to be involved in hepatotoxicity (Laskin, 2009; Laskin *et al.*, 2011). Inhibition of HMGB1 release or neutralization of its biological activity may represent a potential approach to downregulating activated macrophages, a key step in mitigating AA-induced hepatotoxicity.

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Abbreviations

AA	acetaminophen
CM-H₂DCFDA	5-(and-6)-chloromethyl-2', 7' -dichlorodihydro-fluorescein diacetate acetyl ester
COX	cyclooxygenase
DTPA	diethylene triamine pentaacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N',N' -tetraacetic acid
EP	ethyl pyruvate
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HMGB1	high-mobility group box 1
HO-1	heme oxygenase-1
LOX	lipoxigenase
LPS	lipopolysaccharide
MAP kinase	mitogen activated protein kinase

MIP	macrophage inflammatory protein
MTT	methylthiazolyldiphenyl-tetrazolium bromide
NAPQI	N-acetyl-para-benzoquinone imine
RAGE	receptor for advanced glycation end-products
ROS	reactive oxygen species
TLR	Toll-like receptor

Article Highlights

> These studies analyze macrophage activation by mediators released from acetaminophen-damaged hepatocytes. > Factors released from acetaminophen-injured hepatocytes induce macrophage ROS production and expression of COX-2, chemokines, and RAGE. > Hepatocyte-mediated macrophage activation involves p44/42 MAP kinase signaling. > HMGB1 is released from acetaminophen-injured hepatocytes and contributes to macrophage activation.

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Fig. 1. AA-induced cytotoxicity in hepatocytes and the release of HMGB1. *Upper panel.* Hepatocytes were treated with 1-10 mM acetaminophen (AA) or medium control (CTL). After 24 h, cells were washed and refed with medium containing 0.5 mg/ml MTT. DMSO was added 2 h later and absorbance measured at 550 nm. Each bar represents the mean $\pm$ SE ($n = 8-12$). ^aSignificantly different ($p < 0.05$) from CTL. *Middle Panel.* Hepatocytes were treated with 1-10 mM AA or CTL. Equal volumes of culture supernatants were collected 24 h later, concentrated using Microcon centrifugal filter devices (Millipore, Billerica, MA), and analyzed for HMGB1 by western blotting. One representative blot from three independent experiments is shown. *Lower Left Panel.* Hepatocytes were treated with medium or with 30 mM ethyl pyruvate (EP) for 1 h, followed by AA (5 mM) or CTL. Culture supernatants were collected 24 h later, concentrated, and analyzed for HMGB1 by western blotting. One representative blot from three independent experiments is shown. *Lower Right Panel.* Culture supernatants from hepatocytes treated with AA (5 mM) or CTL for 24 h were incubated overnight with anti-HMGB1 antibody followed by immobilized protein G. Samples were then centrifuged and supernatants analyzed for HMGB1 by western blotting.

Fig. 2. Effects of hepatocyte conditioned medium (CM) on macrophage ROS production. Macrophages were preincubated with CM-H₂DCFDA for 15 min, and then with 30% CM from control (CTL) hepatocytes (CM-CTL), acetaminophen (AA)-treated hepatocytes (CM-AA), CM-AA immunoprecipitated with anti-HMGB1 antibody, or with CM from hepatocytes pretreated with ethyl pyruvate (EP) for 1 h, and then with AA for 24 h. Fluorescence was measured spectrophotometrically every 2 min for 30 min. Each point represents the mean $\pm$ SE (n = 3-5). Note that at all times greater than 6 min, CM-AA was significantly different ($p < 0.05$) from CM-CTL, CM-AA + anti-HMGB1, and CM-AA + EP.

Fig. 3. Effects of hepatocyte conditioned medium (CM) on macrophage expression of catalase, HO-1, and COX-2. *Upper panel.* Macrophages were treated with medium control (CTL), 1.5 mM acetaminophen (AA), or 30% CM from CTL or AA-treated hepatocytes. After 6 h or 24 h, macrophage mRNA expression for catalase and HO-1 was analyzed by RT-PCR. Data were normalized to 18s RNA and expressed as fold change relative to time-matched CTL. Each bar represents the mean $\pm$ SE (n = 3). ^aSignificantly different (p < 0.05) from CTL. ^bSignificantly different (p < 0.05) from CM-CTL. ^cSignificantly different (p < 0.05) from AA. *Lower panel.* Macrophages were treated with CTL, AA (1.5 mM), or 30% CM from CTL or AA-treated hepatocytes, or with CM from hepatocytes pretreated for 1 h with medium (MED) or with ethyl pyruvate (EP) and then with AA or CTL. Macrophage COX-2 and HO-1 protein expression was analyzed by western blotting 6 h and/or 24 h later. One representative blot from three independent experiments is shown.

Fig. 4.

Effects of hepatocyte conditioned medium (CM) on expression of macrophage eicosanoid biosynthetic enzymes. Macrophages were treated with medium control (CTL), 1.5 mM acetaminophen (AA), or 30% CM from CTL or AA-treated hepatocytes. After 6 h, macrophage mRNA expression of eicosanoid biosynthetic enzymes was analyzed by RT-PCR. Data were normalized to 18s RNA and expressed as fold change relative to time-matched CTL. Each bar represents the mean $\pm$ SE (n = 3). ^aSignificantly different (p < 0.05) from CTL. ^bSignificantly different (p < 0.05) from CM-CTL. ^cSignificantly different (p < 0.05) from AA.

Fig. 5. Effects of hepatocyte conditioned medium (CM) on macrophage expression of chemokines and HMGB1 receptors. Macrophages were treated with medium control (CTL), 1.5 mM acetaminophen (AA), or 30% CM from CTL or AA-treated hepatocytes. After 6 h, macrophage mRNA expression for chemokines and HMGB1 receptors was analyzed by RT-PCR. Data were normalized to 18s RNA and expressed as fold change relative to CTL. Each bar represents the mean $\pm$ SE (n = 3). ^aSignificantly different (p < 0.05) from CTL. ^bSignificantly different (p < 0.05) from CM CTL. ^cSignificantly different (p < 0.05) from AA.

Fig. 6. Role of p44/42 MAP kinase in hepatocyte CM-induced COX-2 and HO-1 expression. *Upper Panel.* Macrophages were pretreated for 2 h with 10 μ M U0126 or control, and then with medium control (CTL), 1.5 mM acetaminophen (AA), or 30% CM from CTL or AA-treated hepatocytes for 30 min. Expression of phosphorylated and total MAP kinase p44/42 was analyzed by western blotting. *Lower Panel.* Macrophages were pretreated for 2 h with 10 μ M U0126 or DMSO, and then with medium CTL or AA, or with CM from control (CTL) or AA-treated hepatocytes for 6 h. COX-2 and HO-1 protein expression were analyzed by western blotting. One representative blot from three independent experiments is shown.

Table 1
Effects of p44/42 MAP Kinase Inhibition on Macrophage Gene Expression

	CM-CTL		CM-AA	
	DMSO	U0126	DMSO	U0126
Catalase	0.7 ± 0.2	0.9 ± 0.1	2.3 ± 0.7 ^{a,b}	2.5 ± 0.1
12/15-LOX	1.0 ± 0.4	1.1 ± 0.2	3.2 ± 0.2 ^{a,b}	2.0 ± 0.2
MIP-1α	1.1 ± 0.1	1.2 ± 0.3	2.3 ± 0.1 ^{a,b}	0.7 ± 0.1 ^c
MIP-2	1.1 ± 0.1	1.2 ± 0.3	8.0 ± 0.3 ^{a,b}	4.3 ± 0.4 ^c

Macrophages were treated with U0126 or DMSO for 2 h, followed by conditioned medium (CM) from control (CTL) or acetaminophen (AA)-treated hepatocytes. Six h later, mRNA expression was analyzed by RT-PCR. Data are expressed as fold change relative to CTL (mean ± SE, n=3).

^aSignificantly different (p = 0.05) from CTL;

^bSignificantly different (p = 0.05) from CM-CTL;

^cSignificantly different (p = 0.05) from DMSO.

Table 2
Effects of Ethyl Pyruvate Pretreatment of Hepatocytes on Macrophage Gene Expression

	CM-CTL		CM-AA	
	Med	EP	Med	EP
Catalase				
6 h	0.7 ± 0.1	1.4 ± 0.1	3.7 ± 0.6 ^a	1.0 ± 0.1 ^b
24 h	0.9 ± 0.2	2.1 ± 0.3	4.4 ± 0.4 ^a	0.9 ± 0.1 ^b
HO-1				
6 h	0.7 ± 0.1	4.2 ± 0.3	64.3 ± 6.5 ^a	2.7 ± 0.2 ^b
24 h	0.8 ± 0.1	3.4 ± 0.2	39.6 ± 2.0 ^a	5.4 ± 0.2 ^b

Macrophages were treated with conditioned medium (CM) from hepatocytes pretreated for 1 h with medium control (Med) or ethyl pyruvate (EP), and then with acetaminophen (AA) or control (CTL). mRNA expression was analyzed 6 and 24 h later by RT-PCR. Data are expressed as fold change relative to CTL (mean ± SE, n = 3).

^aSignificantly different (p = 0.05) from CM-CTL.

^bSignificantly different (p = 0.05) from CM-AA without pretreatment.