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Acrolein cytotoxicity in hepatocytes involves endoplasmic reticulum stress, mitochondrial dysfunction and oxidative stress

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Abstract

Acrolein is a common environmental, food and water pollutant and a major component of cigarette smoke. Also, it is produced endogenously via lipid peroxidation and cellular metabolism of certain amino acids and drugs. Acrolein is cytotoxic to many cell types including hepatocytes; however the mechanisms are not fully understood. We examined the molecular mechanisms underlying acrolein hepatotoxicity in primary human hepatocytes and hepatoma cells. Acrolein, at pathophysiological concentrations, caused a dose-dependent loss of viability of hepatocytes. The death was apoptotic at moderate and necrotic at high concentrations of acrolein. Acrolein exposure rapidly and dramatically decreased intracellular glutathione and overall antioxidant capacity, and activated the stress-signaling MAP-kinases JNK, p42/44 and p38. Our data demonstrate for the first time in human hepatocytes, that acrolein triggered endoplasmic reticulum (ER) stress and activated eIF2 α , ATF-3 and -4, and Gadd153/CHOP, resulting in cell death. Notably, the protective/adaptive component of ER stress was not activated, and acrolein failed to up-regulate the protective ER-chaperones, GRP78 and GRP94. Additionally, exposure to acrolein disrupted mitochondrial integrity/function, and led to the release of pro-apoptotic proteins and ATP depletion. Acrolein-induced cell death was attenuated by N-acetyl cysteine, phenyl-butyric acid, and caspase and JNK inhibitors. Our data demonstrate that exposure to acrolein induces a variety of stress responses in hepatocytes, including GSH depletion, oxidative stress, mitochondrial dysfunction and ER stress (without ER-protective responses) which together contribute to acrolein toxicity. Our study defines basic mechanisms underlying liver injury caused by reactive aldehyde pollutants such as acrolein.

Keywords

liver; apoptosis; mitochondria; ER stress; oxidative stress

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INTRODUCTION

Acrolein, a highly reactive α , β -unsaturated aldehyde, is a common pollutant found in the environment, and in food and water. Acrolein can be formed by combustion of wood, fossil fuels and plastics and is a major component of cigarette smoke (Stevens and Maier, 2008). Acrolein also exists naturally in vegetables, fruits, and herbs (Feron *et al.*, 1991) and is produced during the processing of fat-containing foods and meats (Abraham *et al.*, 2011). Notably, acrolein is also produced endogenously by normal cellular metabolism. Acrolein can be formed in various tissues via lipid peroxidation (Uchida *et al.*, 1998a), metabolism of α -hydroxyamino acids (Esterbauer *et al.*, 1991), polyamines oxidation (Sharmin *et al.*, 2001) and via metabolism of drugs, such as the anticancer drug cyclophosphamide (Kehrer and Biswal, 2000). Acrolein is a strong and highly reactive electrophile, and remains active in the body for several days (Ghilarducci and Tjeerdema, 1995). Humans are exposed to acrolein in industrial, environmental and therapeutic settings, by consumption of certain foods and water, and cigarette smoking. A recent analysis demonstrated that acrolein is a major indoor air pollutant and is one of the most harmful substances in residences across USA (Logue *et al.*, 2012).

Acrolein is primarily metabolized via rapid reaction with sulfhydryl groups of glutathione forming mercapturic acid; this is ultimately eliminated in the urine. Thus, acrolein contributes directly to cellular oxidative stress via loss of glutathione (Kehrer and Biswal, 2000). Acrolein is also a substrate of lung or liver microsomal epoxidase, and liver aldehyde dehydrogenase resulting in oxidation to acrylic acid (Patel *et al.*, 1980). Acrolein can form Michael-type addition adducts with cellular components, particularly proteins and DNA. Increased levels of acrolein adducts have been measured in plasma of patients with renal failure (Lovell *et al.*, 2001; Sakata *et al.*, 2003), Alzheimer's disease (Calingasan *et al.*, 1999) (Lovell and Markesbery, 2001; Lovell *et al.*, 2001), Parkinson's and atherosclerosis (Uchida *et al.*, 1998b) and diabetes (Daimon *et al.*, 2003).

Due to its ubiquitous nature, acrolein and its toxic effects have been extensively studied in various cell types. In hepatocytes, cytotoxicity of acrolein has been reported in vitro (Kaminskas *et al.*, 2005) (Maddox *et al.*, 2004) and in vivo (Arumugam *et al.*, 1999a) (Arumugam *et al.*, 1999b) (Esterbauer *et al.*, 1991). However, the molecular mechanisms and signaling pathways involved in acrolein-induced hepatocellular toxicity are not completely understood. This study examines the cytotoxic mechanisms of acrolein hepatotoxicity in primary hepatocytes and hepatoma cells. Our study demonstrates for the first time that acrolein causes ER stress in hepatocytes leading to cell death. Acrolein also triggers mitochondrial permeability transition and dysfunction, and increases oxidative stress in hepatocytes, thereby invoking multiple cell death mechanisms that together contribute to its hepatotoxic effects.

MATERIALS AND METHODS

Reagents

General chemicals, N-acetyl cysteine (NAC), phenyl butyric acid (PBA), acrolein, and β -actin antibody were purchased from Sigma Aldrich (St. Louis, MO). All other antibodies were purchased from Cell Signaling (Beverly, MA). Cell culture supplies were obtained from Invitrogen (Carlsbad, CA).

Cell culture

HepG2, a human hepatoma cell line obtained from American Type Culture Collection (Rockville, MD) was used as described previously (Joshi-Barve *et al.*, 2003). All treatments were performed on sub-confluent monolayers of cells. Primary human hepatocytes were

obtained from ZenBio (Research Triangle Park, NC) and used in accordance with company instructions. Cells were plated at the following densities: (i) 25,000 cells per well for 96-well plates; (ii) 0.5×10^6 cells per well for 24-well plates; (iii) 1.0×10^6 cells per well for 6-well plates; (iv) 5×10^6 cells per well for 100mm plates.

Cell Viability-MTT assay

Cell survival/cell death was measured in treated cells by the MTT (3, (4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide) assay as described (Joshi-Barve *et al.*, 2003).

DNA fragmentation assay

DNA fragmentation was measured using a commercial ELISA kit (Cell Death Detection ELISA, Roche Applied Sciences, Indianapolis, IN) in accordance with manufacturer instructions.

Cytokeratin 18 Assay

The caspases-3 dependent cleavage of cytokeratin-18 into the M30 fragment was assessed using the M30 CytoDeath™ and the M65 EpiDeath® ELISA kits (ENZO Life Sciences International, Inc., Plymouth Meeting, PA). The ratio of M30/M65 was calculated as described by the manufacturer (Peviva AB, Sweden).

Antioxidant Capacity

Cellular antioxidant capacity was measured in total cell extracts using a commercial kit based on the ability of antioxidants in the sample to inhibit the oxidation of ABTS® (2,2'-azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS· + by metmyoglobin (Cayman Chemicals, Ann Arbor, MI).

Western Blot Analysis

Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 4 mM Na₃VO₄, 40 mM NaF, 1% Triton X-100, 1 mM PMSF, 1% protease inhibitor cocktail) and centrifuged at 14,000 g for 10 min. The supernatants were collected and equivalent protein in total cell lysates was resolved by SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane ((Bio-Rad, Hercules, CA). Membranes were blocked for 1 h in blocking buffer (5% nonfat dry milk in 0.1% TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20)) and incubated overnight at 4°C with the primary antibodies diluted in blocking buffer. After washing with 0.1% TBST, the membranes were incubated with appropriate secondary antibodies for 1 h at room temperature. Proteins were visualized using an enhanced chemiluminescence system (ECL, GE Healthcare, Piscataway, NJ) and quantified by densitometry analysis using UNSCANIT (Silk Scientific, Inc, Orem, UT). The density ratio of each band compared to its corresponding GAPDH band was determined. The density ratio was normalized to the untreated value which was set to 1.

Mitochondrial Membrane Potential Assay

Cells were plated in 6-well plates and treated as needed. Cells were stained for 30 min with JC-1 mitochondrial tracker dye (Cayman Chemical Company, Ann Arbor, MI). Fluorescence microscopy using the EVOS– All-in-one fluorescence and phase microscope with monochrome camera (AMG, Advanced Microscopy Group, Bothell, WA) was used to assess mitochondrial membrane permeability.

ATP assay

Cells were plated in 96-well plates and treated as needed. Cellular ATP levels were measured using a commercial CellTiter-Glo® Luminescent ATP Assay kit (Promega Corporation, Madison, WI) in accordance with manufacturer instructions.

RNA isolation and Real Time PCR analysis

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) and subjected to real time PCR using SYBR green I dye reagents with an ABI prism 7500 sequence detection system (Applied Biosystems, Foster City, CA). The specific exon-exon junction primers were designed using Primer-BLAST (NCBI/NIH). The gene expression was analyzed by relative quantification using $2^{-\Delta\Delta C_t}$ method by normalizing with GAPDH or 18s rRNA.

Cellomics

After treatment, hepatocytes were incubated for 1h in growth media containing the dyes (i) Hoechst (for nuclear fluorescence), (ii) TMRM (for mitochondrial membrane potential), (iii) Fluo-4 (for free calcium), and (iv) TOTO-3 (for cell membrane permeability). Cellomics analysis was performed using a Thermo Scientific Array Scan VTI HCS Reader as described by the manufacturer. Cellomics Array Scan 60 software (7.6.2.1-1.00x) was used to determine fluorescence intensities of the four dyes. Well averages, as well as individual cell data were recorded and analyzed.

Statistical Analysis

All data are expressed as mean $\pm$ SD. Data were analyzed by the student's t-test or by unpaired analysis of variance (ANOVA) with Tukey-Kramer post-hoc analysis (0.05), with data from at least three experiments performed in duplicates. Differences were considered statistically significant for $P < 0.05$.

RESULTS

We investigated the mechanisms underlying the cytotoxic effects of acrolein using primary hepatocytes and human hepatoma cells (HepG2 cell line). Most assays were performed in primary hepatocytes; however, certain measurements were done in HepG2 cells. We chose pathophysiologically relevant concentrations of acrolein based on published literature, and estimated levels of acrolein that may be encountered by environmental/accidental exposures and generated within tissues by cellular metabolism and oxidative stress (Calingasan *et al.*, 1999; Lovell *et al.*, 2001) (Sakata *et al.*, 2003) (Kaminskas *et al.*, 2005) (Maddox *et al.*, 2004).

Acrolein induced cell death

Primary hepatocytes were exposed to increasing concentrations of acrolein from 2.5 μ M to 100 μ M, and cell survival after 24h was assayed by the MTT assay. Minimal loss of survival was observed from 2.5 μ M through 25 μ M, and a dose-dependent decrease in survival was seen beyond 25 μ M, with a ~50% loss of viability between 50 μ M and 75 μ M (Figure 1A). To better examine the dose-relationship, we examined acrolein exposure at the added concentration of 60 μ M (at ~50% viability). We then investigated the type of cell death (i.e., apoptosis vs. necrosis) induced by acrolein in primary hepatocytes using two assays of apoptosis. DNA fragmentation, a hallmark of apoptotic cell death, was significantly increased in hepatocytes exposed to acrolein at 50 μ M, 60 μ M and 75 μ M (Figure 1B). Similar results were seen using the M30/M65 CK-18 assay that measures cleavage of cytokeratin-18 by caspase-3, which is activated during apoptosis. Apoptosis was minimal at 25 μ M, and significantly induced at 50 μ M, 60 μ M and 70 μ M acrolein (Figure 1C).

Although the cell death was extensive at 90 μ M and 100 μ M (Figure 1C), we saw no increase in apoptotic markers, suggesting that cell death was likely to be necrotic.

Acrolein induced depletion of cellular antioxidants

The role of oxidative stress in apoptotic cell death is well recognized (Ott *et al.*, 2007). Also, the metabolism of acrolein occurs predominantly by conjugation to GSH (Kehrer and Biswal, 2000), hence, we measured total cellular GSH levels by HPLC in hepatocytes exposed to varying concentrations of acrolein (5 μ M to 75 μ M). We observed a rapid statistically significant depletion of GSH within 3h at all acrolein concentrations, even at those that did not cause significant cell death, i.e., 5 μ M and 10 μ M (Figure 2A).

Additionally, we measured the total antioxidant capacity of hepatocytes exposed to acrolein for 6h and 24h and found that it was substantially diminished at the moderate and high concentrations of acrolein (Figure 2B). Although the antioxidant capacity was significantly reduced at 6h at 10 μ M and 25 μ M acrolein, the levels were restored at 24h, allowing the cells to recover and survive; this did not occur at the higher acrolein concentrations. Thus, cytotoxic acrolein exposure greatly reduced the hepatocyte GSH and the overall capacity to neutralize oxidants.

Acrolein induced activation of cellular stress-signaling kinases

Mitogen-activated protein kinases (MAPKs, or stress-signaling kinases) play important roles in apoptosis and oxidative stress is known to activate MAPKs (Runchel *et al.*, 2011).

Therefore, we investigated the involvement of MAPKs in acrolein-induced hepatocyte death. Activation by phosphorylation of p38, p42/44 and JNK was increased within 15 min in hepatocytes treated with acrolein, particularly at 50 μ M and 75 μ M acrolein (Figure 3).

Activation of all the MAPKs decreased slightly by 60min, but remained above untreated.

Acrolein induced mitochondrial dysfunction

The mitochondrial death pathway is involved in many forms of apoptotic cell death. Mitochondrial permeabilization and the loss of mitochondrial membrane potential ($\Delta\Psi$), the subsequent release of proapoptotic proteins such as cytochrome c and AIF from the inter membrane space into the cytosol, and decreased ATP production are hallmarks of apoptosis (Green and Kroemer, 2004). To investigate the involvement of the mitochondria, we examined known parameters of mitochondrial distress in primary hepatocytes treated with acrolein. We tested the effect of acrolein on mitochondrial membrane potential, using the JC-1 cationic mitochondrial dye in HepG2 cells exposed to 25 μ M and 50 μ M acrolein. Since primary hepatocytes exhibited high baseline fluorescence in the absence JC-1 dye, we used HepG2 cells for this experiment. JC-1 accumulates in mitochondria in a membrane potential-dependent fashion forming red fluorescent aggregates. JC-1 outside the mitochondria exists as a green fluorescent monomer. Increased green fluorescence indicating mitochondrial permeability transition (MPT) was observed upon acrolein exposure particularly at 50 μ M (Figure 4A). Moreover, the fluorescent staining pattern changed from punctate to highly diffuse (Figure 4A, 40 μ -higher magnification). Acrolein exposure of hepatocytes also resulted in the release of apoptotic proteins from mitochondria, as seen by an increase in the cytoplasmic levels of cytochrome C and AIF starting as early as 3h, with a parallel drop in the mitochondrial levels of the proteins, particularly cytochrome C (Figure 4B). A consequence of mitochondrial depolarization is decreased production of ATP. The cellular levels of ATP were measured by ELISA following acrolein treatment of hepatocytes for 2h, 6h and 24h (Figure 4C). An early significant drop in ATP was seen only with 100 μ M acrolein, again indicating that the cells were most likely undergoing a rapid necrotic death. By 6h and 24h, a considerable decrease in ATP was seen at all acrolein concentrations except 10 μ M. Interestingly unlike at higher concentrations, the drop in ATP

at 25 μ M acrolein was not progressive; and the cells were able to recover and survive (Figure 4C).

Acrolein induced activation of caspases

Activation of the caspase cascade is an integral part of apoptotic cell death (Orrenius *et al.*, 2011). The release of proapoptotic proteins from the mitochondria (e.g., AIF and cytochrome c) leads to the formation of the apoptosome complex, triggering the caspase cascade by proteolytic activation of procaspase-9 and subsequent activation of procaspase-3. We examined the cleavage and activation of pro-caspases -9 and -3 by immunodetection of their cleavage products (Figure 5). Both caspases were activated by acrolein (60 μ M) in a time dependent manner, with increases in cleavage products observed as early as 3h (Figure 5).

We also assessed the activation of caspase-4, a member of the caspase-1/12 family of caspases. Caspase-4, the human homolog of caspase-12, is localized to the ER and is known to be involved in ER stress-induced apoptotic responses (Bian *et al.*, 2009) (Hitomi *et al.*, 2004). Western blotting analysis showed a the cleavage and activation of pro-caspase-4 (43kDa) into cleaved caspase-4 (10kDa) upon acrolein exposure starting from 1h, suggesting that ER stress may be a component of the hepatotoxic effects of acrolein (Figure 5).

Acrolein induced endoplasmic reticulum stress

The ER stress response is activated by the accumulation of misfolded, malformed or modified proteins in the cell (Xu *et al.*, 2005). Since acrolein is highly reactive and is known to form protein adducts, it is highly likely to cause ER stress. Moreover, the caspase analysis indicated that the ER function may indeed be disrupted by acrolein. Hence, to evaluate the involvement of ER stress in acrolein-induced hepatocyte death, the expression of ER stress markers was examined after treatment of hepatocytes with different concentrations of acrolein (Figure 6A). Phospho-activation of eukaryotic initiation factor 2 α (eIF2 α), an early marker of ER stress, was increased at 50 μ M, 60 μ M and 75 μ M of acrolein. Phosphorylated eIF2 α suppresses overall protein synthesis but selectively allows translation of the transcription factor ATF4, which induces ER proteins that are critical in cell survival/death, such as ATF3 and Gadd153/CHOP; the latter is critical in ER stress-associated apoptosis (Pfaffenbach *et al.*, 2010). Our data demonstrate that acrolein upregulated ATF4, ATF3 and Gadd153/CHOP starting from 50 μ M (Figure 6A). Further, the effects of acrolein on gene expression of ER stress proteins were also investigated at various times after exposure of hepatocytes to 50 μ M acrolein by real time PCR (Figure 6B). An increase in the mRNA levels of GADD153/CHOP (~4.5 fold), ATF3(~3.5 fold) and ATF4 (3.5 fold) was seen within 3h and all mRNAs remained elevated up to 24h after acrolein treatment (Figure 6B).

ER stress involves triggering of both the “alarm” and the “adaptive” phase responses (Xu *et al.*, 2005). The adaptive (protective) phase leads to the upregulation of ER chaperone proteins which assist in the refolding of proteins, relieve ER stress, and reestablish normal ER function. A well-known characteristic of the adaptive/protective response in ER stress is the proteolytic activation of the transcription factor ATF6 (Back *et al.*, 2005), which leads to transcription of the ER chaperones. We examined the effects of acrolein on ATF6 activation and the protein levels of the ER chaperones GRP78 and GRP94 (Figure 6C). Although the proteolytic cleavage of ATF6 (90kDa) into its smaller fragments (50kDa) was apparent, there were no changes in GRP78 and GRP94 at any concentration of acrolein. Moreover, there was no transcriptional upregulation of either GRP78 or GRP94 gene (Figure 6D); rather the level of both mRNAs was decreased by ~ 15–25% at 3h–12h. Thus, acrolein up-

regulated the ER stress genes, but failed to induce the ER protective chaperone genes in hepatocytes.

Analysis of acrolein-induced cell death by Cellomics-HCS

We examined the mechanisms of acrolein induced hepatocyte death using the Cellomics-HCS fluorescent dye imaging, which allows simultaneous assessment of multiple parameters, namely mitochondrial transmembrane potential ($\Delta\psi$) using TMRM, intracellular free calcium accumulation using Fluo-4, and cell permeability using TOTO-3. HepG2 cells were used in Cellomics analyses, since we found that primary hepatocytes exhibited a detectable baseline auto-fluorescence that interfered with the fluorescent dye based Cellomics. The HepG2 cells did not have the same problem and were more amenable to Cellomics. Acrolein exposure caused a dose-related drop in $\Delta\psi$ in HepG2 cells starting at 60 μ M, with a 50% decrease at 75 μ M acrolein, showing that mitochondrial integrity/function was disrupted (Figure 7A, top panel). Interestingly, a slight increase in mitochondrial membrane potential (hyperpolarization) was observed at 40 μ M – 60 μ M acrolein. A dose-associated increase in the levels of free calcium was also seen in acrolein treated cells starting at 50 μ M (Figure 7A, middle panel), showing that ER function is disrupted, causing calcium release from ER. Also, hepatocyte cell death (permeability to TOTO-3) increased with increasing acrolein concentrations (Figure 7A, bottom panel). Similar results were seen upon microscopic examination of hepatocytes treated with increasing acrolein (Figure 7B) with decreased red fluorescence ($\Delta\psi$) and a corresponding increase in green fluorescence (free calcium), showing that acrolein exposure adversely affected both mitochondria and ER.

Effect of signaling pathway inhibitors on acrolein-induced hepatocyte death

Thus far, our data show that exposure to acrolein results in the activation of several stress/injury pathways in hepatocytes. To assess the contribution of each of these processes to acrolein-induced hepatotoxicity, we used inhibitors to block specific death-signaling pathways and determined the effects on acrolein-induced hepatocyte cell death. We investigated the potential protective effects of the following compounds (i) JNK inhibitor SP600025 (25 μ M); (ii) pan caspase inhibitor Z-VAD-FMK (25 μ M); (iii) antioxidant GSH-prodrug N-acetyl cysteine, NAC (1mM); and (iv) chemical chaperone ER stress inhibitor phenyl butyric acid, PBA (10 μ M). Hepatocytes were treated with three acrolein concentrations that caused considerable apoptosis (50 μ M, 60 μ M, and 75 μ M), without or with a pretreatment with each of the four inhibitors, and cell survival was measured by MTT assay (Figure 8). At the concentrations used, none of the inhibitors had any inherent toxicity on hepatocytes. Although a significant protective effect was conferred by all the inhibitors, the effect was only partial in each case. NAC appeared to be the most effective in preventing cell death, suggesting that oxidative stress and loss of GSH were critical components of acrolein-induced hepatotoxicity. Further studies with more specific inhibitors may be necessary for a detailed understanding of the contribution of various pathways.

DISCUSSION AND CONCLUSIONS

The adverse effects of acrolein on human health are relevant since acrolein is a ubiquitous pollutant present in the environmental, food and water, and human exposures are common. Being an endogenous toxin, acrolein is especially insidious. Moreover, acrolein can easily move across cell membranes and tissues due to its solubility in water and alcohol, and hence, high concentrations of acrolein produced by lipid peroxidation can spread from the dying cell of origin to damage/kill adjacent cells. However, because of its high reactivity at the sites of exposure/generation, acrolein is thought to have limited dispersal in the body. Nevertheless, elevated levels of acrolein and acrolein-adducts have been found in

unanticipated sites in the body, such as plasma of patients with renal failure (Sakata *et al.*, 2003), livers of mice fed alcohol or a high fat diet (Joshi-Barve, unpublished observation), and brains and spinal cords of people with neurologic disorders (Weinhold, 2011).

Reported or calculated levels of acrolein exposure and/or generation vary widely. Human serum levels of acrolein are estimated to normally range as high as 50 $\mu\text{mol/L}$ (Satoh *et al.*, 1999). Acrolein concentrations are reported to reach 80 $\mu\text{mol/L}$ in fluids lining the respiratory tract of smokers (Eiserich *et al.*, 1995). Patients treated with the anticancer drug cyclophosphamide had serum acrolein levels that reached up to 10.2 μM (Ren *et al.*, 1999), and elevated urinary acrolein that caused urotoxicity (Kaijser *et al.*, 1993). A blood level of 129 $\mu\text{mol/L}$ acrolein was reported in a patient who died of acrolein-induced acute cardiotoxicity after oral ingestion of the herbicide allyl alcohol which is metabolized to acrolein in the liver (Toennes *et al.*, 2002). Additionally, acrolein-FDP-lysine adducts have been detected in many body fluids including serum, plasma, bronchial lavage, urine, and cerebrospinal fluid (Calingasan *et al.*, 1999; Lovell *et al.*, 2001). The acrolein-lysine adduct levels vary widely, reaching up to 50.15 $\mu\text{mol/L}$ in CSF from patients with bacterial meningitis (Tsukahara *et al.*, 2002), and 180 μM in the plasma of renal failure patients (Sakata *et al.*, 2003). Despite such studies, it is difficult to extrapolate the acrolein concentrations that may be relevant in hepatocytes *in vivo*. Since acrolein exposures can come about externally and by endogenous generation, the tissue-specific and localized levels of acrolein at sites of generation and/or exposure are likely to be high. Notably, the liver is a major metabolic and detoxification organ with high mitochondrial activity, and can experience greatly elevated levels of lipid peroxidation and oxidative stress, particularly in disease states. Hence, high localized hepatic levels of acrolein may be reached in pathophysiological disease conditions. The higher acrolein concentrations used in our *in vitro* study are greater than expected in normal conditions, but are in the range anticipated in the liver under pathological conditions.

Acrolein is known to have diverse effects in various cell types. Acrolein can decrease the proliferation of cells (Rudra and Krokan, 1999), and can induce apoptosis (Pugazhenthir *et al.*, 2006) (Tanel and Averill-Bates, 2005), as well as necrosis (Luo *et al.*, 2005) (Liu-Snyder *et al.*, 2006). Interestingly, acrolein inhibits cell death of neutrophils (Finkelstein *et al.*, 2005) and can activate endothelial cells via ER stress without cell death (Haberzettl *et al.*, 2009). Additionally, hepatotoxic effects in cigarette smokers may be ascribed to acrolein, since acrolein is the major toxic component in cigarette smoke. Clinical studies have linked cigarette smoking to hepatotoxicity, where smoking was associated with increased liver fibrosis (Pessione *et al.*, 2001), cirrhosis (Yu *et al.*, 1997), risk of hepatocellular carcinoma (Mori *et al.*, 2000) and higher 5-year mortality in alcoholics (Pessione *et al.*, 2003).

Many mechanisms have emerged that contribute to toxicity and cell death (Orrenius *et al.*, 2011). The mode of cell death induced by acrolein appears to be dose- and cell type-dependent. Our study reveals the molecular mechanisms and signaling pathways that contribute to acrolein toxicity in hepatocytes, and shows that multiple mechanisms of oxidative stress, mitochondrial dysfunction and ER stress are activated (Figure 9). Acrolein-induced cell death process may be initiated in multiple different intracellular compartments, with cross talk between these compartments that together contribute to cytotoxicity. The novel findings are that acrolein triggers ER stress in hepatocytes, concurrent with activation of stress signaling MAPKs. To our knowledge, this is the first report of acrolein-induced ER stress leading to upregulation of apoptosis inducing protein GADD153/CHOP and causing cell death in hepatocytes. Acrolein also caused mitochondrial dysfunction by altering mitochondrial membrane potential, leading to the release of cytochrome c and AIF, and depletion of cellular ATP. Interestingly, we observed mitochondrial membrane hyperpolarization at intermediate concentrations of acrolein (40 μM – 60 μM). This

mitochondrial hyperpolarization may be an adaptive response to the toxic stimulus or, on the other hand, may be a harbinger of cell death as shown in T-cells (Banki *et al.*, 1999). Recent reports demonstrate that ER stress and activation of the stress kinases JNK and p38MAPK are major contributors to hepatic injury in fatty liver disease (Tarantino and Caputi, 2011) and palmitate-mediated cell death (Pfaffenbach *et al.*, 2010). Moreover, the sustained activation of the stress-kinase JNK is believed to mediate hepatocyte apoptosis, leading to enhanced liver damage (Singh *et al.*, 2009; Verma and Datta, 2012). These studies emphasize the relevance of our findings in acrolein induced hepatocyte injury.

Interestingly, the adaptive/protective phase of ER stress was not activated by acrolein in hepatocytes. Adaptive responses allow cells to function normally in the face of an adverse stimulus; however, if the adaptive response does not occur or is overwhelmed, the cells are eliminated by apoptosis. It is likely that the higher concentrations of acrolein are particularly cytotoxic because they prevent adaptive responses. The specific mechanisms as to why the ER adaptive response is not functional despite a robust activation of ER stress by acrolein remain unclear. Our data show that although ATF6 was activated, up regulation of ER chaperones did not occur. The activation of the ER stress-induced bZIP transcription factor XBP-1 by alternate splicing is known to result in ER chaperone gene transcription (Back *et al.*, 2005). We are currently investigating several upstream events involved in the ER adaptive response (including XBP-1 splicing) to determine how acrolein exposure selectively impairs the ER protective mechanisms ultimately leading to apoptosis.

The depletion of cellular glutathione by acrolein has been documented (Lam *et al.*, 1985) (Horton *et al.*, 1997) (Horton *et al.*, 1999). Similar to these studies, we found that acrolein causes a rapid and strong reduction in GSH. Additionally, we found that acrolein reduced the overall antioxidant capacity of hepatocytes. Thus, acrolein elicits cellular oxidative stress and decreases the antioxidant capacity of hepatocytes; this may be a trigger for cell death, may render the cells more susceptible to further injury, and may contribute to pathological processes in the liver. In our study, hepatocytes exposed to the low levels of acrolein were able to recover and replete their cellular antioxidant stores by 24h; this did not occur at the higher concentrations of acrolein at which cell death was observed. The causal relationship between GSH and cell death/apoptosis is not entirely clear; both the extent and the duration of GSH depletion may be critical determinants. Our data indicate at 10 μ M acrolein, cellular GSH and antioxidant capacity was considerably depleted early on and was restored by 24h, suggesting that extensive depletion alone is insufficient for cell death and that the duration of depletion may be more important. Notably, GSH was shown to be indispensable for effective protein folding and maturation at the ER (Chakravarthi and Bulleid, 2004). This is in keeping with our data showing that acrolein exposed hepatocytes with low GSH have an activated ER stress response that ultimately leads to cell death.

Based on our study, we find that the use of GSH pro-drugs (NAC) and inhibitors (of JNK, caspases and ER stress) may be beneficial for the prevention and treatment of pathological conditions associated with excessive acrolein generation and/or accumulation. NAC is already approved for clinical use and is routinely used to treat overdose of the hepatotoxic drug acetaminophen (Perry and Shannon, 1998). Caspase inhibitors are under consideration by USFDA for human use, while chemical chaperones are already approved (Ozcan *et al.*, 2006). Our results showed that each inhibitor was only partial effective in preventing acrolein-induced hepatocyte death, emphasizing that acrolein was associated with multiple modes of cell death. Based on this partial attenuation of acrolein cytotoxicity with inhibitors, it is likely that combinations of the inhibitors may provide greater protection and hence, combinatorial therapies may be a novel modality against acrolein hepatotoxicity.

In summary, our study demonstrates that exposure to acrolein induces a variety of stress responses in hepatocytes, of which GSH depletion, oxidative stress, mitochondrial dysfunction and the novel ER stress (in the absence of ER adaptive/protective responses) are critical components. Moreover, our data also suggest that the use of antioxidants and inhibitors in combination may be attractive therapeutic options for preventing acrolein hepatotoxicity. The findings in this study are relevant in multiple settings, including direct hepatotoxicity via environmental and accidental exposures to acrolein; in the use/side effects of the anticancer drugs (e.g. cyclophosphamide); during the regulation of proliferation/tumor growth by polyamines, and in the case of various alcoholic and non-alcoholic liver diseases where acrolein generation/accumulation may be elevated. Further detailed studies on the toxic mechanisms of acrolein are necessary to determine the temporal sequence of events; whether one death pathway triggers another or whether they are all coordinately/concurrently activated; the relative contribution of MAPKs; the comparative susceptibility of mitochondria and ER; and the inter-dependence or cross-talk between cell death mechanisms.

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Abbreviations

MAPK	Mitogen activated protein kinase
JNK	Jun-activated kinase
ER	endoplasmic reticulum
eIF2α	eukaryotic initiation factor 2-alpha
Gadd153/CHOP	growth arrest- and DNA damage-inducible gene 153 or C/EBP homologous protein (CHOP)
ATF	activating transcription factor
GRP	glucose regulated protein
DALY	disability adjusted life year
GSH	glutathione
AIF	apoptosis inducing factor
ELISA	enzyme linked immunosorbent assay
PCR	polymerase chain reaction
NAC	N-acetyl cysteine
bZIP	Basic Leucine Zipper
XBP	X-box binding protein-1
cyt C	cytochrome C
MPT	mitochondrial permeability transition
IRE	inositol-requiring enzyme

REFERENCES

- Abraham K, Andres S, Palavinkas R, Berg K, Appel KE, Lampen A. Toxicology and risk assessment of acrolein in food. *Mol Nutr Food Res*. 2011; 55:1277–1290. [PubMed: 21898908]
- Arumugam N, Sivakumar V, Thanislass J, Pillai KS, Devaraj SN, Devaraj H. Acute pulmonary toxicity of acrolein in rats--underlying mechanism. *Toxicol Lett*. 1999a; 104:189–194. [PubMed: 10079053]
- Arumugam N, Thanislass J, Ragunath K, Niranjali Devaraj S, Devaraj H. Acrolein-induced toxicity--defective mitochondrial function as a possible mechanism. *Arch Environ Contam Toxicol*. 1999b; 36:373–376. [PubMed: 10227855]
- Back SH, Schroder M, Lee K, Zhang K, Kaufman RJ. ER stress signaling by regulated splicing: IRE1/HAC1/XBP1. *Methods*. 2005; 35:395–416. [PubMed: 15804613]
- Banki K, Hutter E, Gonchoroff NJ, Perl A. Elevation of mitochondrial transmembrane potential and reactive oxygen intermediate levels are early events and occur independently from activation of caspases in Fas signaling. *J Immunol*. 1999; 162:1466–1479. [PubMed: 9973403]
- Bian ZM, Elnor SG, Elnor VM. Dual involvement of caspase-4 in inflammatory and ER stress-induced apoptotic responses in human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci*. 2009; 50:6006–6014. [PubMed: 19643964]
- Calingasan NY, Uchida K, Gibson GE. Protein-bound acrolein: a novel marker of oxidative stress in Alzheimer's disease. *J Neurochem*. 1999; 72:751–756. [PubMed: 9930749]
- Chakravarthi S, Bulleid NJ. Glutathione is required to regulate the formation of native disulfide bonds within proteins entering the secretory pathway. *J Biol Chem*. 2004; 279:39872–39879. [PubMed: 15254031]
- Daimon M, Sugiyama K, Kameda W, Saitoh T, Oizumi T, Hirata A, Yamaguchi H, Ohnuma H, Igarashi M, Kato T. Increased urinary levels of pentosidine, pyrraline and acrolein adduct in type 2 diabetes. *Endocr J*. 2003; 50:61–67. [PubMed: 12733710]
- Eiserich JP, van der Vliet A, Handelman GJ, Halliwell B, Cross CE. Dietary antioxidants and cigarette smoke-induced biomolecular damage: a complex interaction. *The American journal of clinical nutrition*. 1995; 62:1490S–1500S. [PubMed: 7495250]
- Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med*. 1991; 11:81–128. [PubMed: 1937131]
- Feron VJ, Til HP, de Vrijer F, Woutersen RA, Cassee FR, van Bladeren PJ. Aldehydes: occurrence, carcinogenic potential, mechanism of action and risk assessment. *Mutat Res*. 1991; 259:363–385. [PubMed: 2017217]
- Finkelstein EI, Ruben J, Koot CW, Hristova M, van der Vliet A. Regulation of constitutive neutrophil apoptosis by the alpha,beta-unsaturated aldehydes acrolein and 4-hydroxynonenal. *Am J Physiol Lung Cell Mol Physiol*. 2005; 289:L1019–1028. [PubMed: 16040627]
- Ghilarducci DP, Tjeerdema RS. Fate and effects of acrolein. *Rev Environ Contam Toxicol*. 1995; 144:95–146. [PubMed: 8599034]
- Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. *Science*. 2004; 305:626–629. [PubMed: 15286356]
- Haberzettl P, Vladyskovskaya E, Srivastava S, Bhatnagar A. Role of endoplasmic reticulum stress in acrolein-induced endothelial activation. *Toxicol Appl Pharmacol*. 2009; 234:14–24. [PubMed: 18951912]
- Hitomi J, Katayama T, Eguchi Y, Kudo T, Taniguchi M, Koyama Y, Manabe T, Yamagishi S, Bando Y, Imaizumi K, Tsujimoto Y, Tohyama M. Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Aβeta-induced cell death. *J Cell Biol*. 2004; 165:347–356. [PubMed: 15123740]
- Horton ND, Biswal SS, Corrigan LL, Bratta J, Kehrer JP. Acrolein causes inhibitor kappaB-independent decreases in nuclear factor kappaB activation in human lung adenocarcinoma (A549) cells. *J Biol Chem*. 1999; 274:9200–9206. [PubMed: 10092592]

- Horton ND, Mamiya BM, Kehrer JP. Relationships between cell density, glutathione and proliferation of A549 human lung adenocarcinoma cells treated with acrolein. *Toxicology*. 1997; 122:111–122. [PubMed: 9274807]
- Joshi-Barve S, Barve SS, Butt W, Klein J, McClain CJ. Inhibition of proteasome function leads to NF-kappaB-independent IL-8 expression in human hepatocytes. *Hepatology*. 2003; 38:1178–1187. [PubMed: 14578856]
- Kaijser GP, Korst A, Beijnen JH, Bult A, Underberg WJ. The analysis of ifosfamide and its metabolites (review). *Anticancer research*. 1993; 13:1311–1324. [PubMed: 8239501]
- Kaminskas LM, Pyke SM, Burcham PC. Differences in lysine adduction by acrolein and methyl vinyl ketone: implications for cytotoxicity in cultured hepatocytes. *Chem Res Toxicol*. 2005; 18:1627–1633. [PubMed: 16300370]
- Kehrer JP, Biswal SS. The molecular effects of acrolein. *Toxicol Sci*. 2000; 57:6–15. [PubMed: 10966506]
- Lam CW, Casanova M, Heck HD. Depletion of nasal mucosal glutathione by acrolein and enhancement of formaldehyde-induced DNA-protein cross-linking by simultaneous exposure to acrolein. *Arch Toxicol*. 1985; 58:67–71. [PubMed: 4091658]
- Liu-Snyder P, McNally H, Shi R, Borgens RB. Acrolein-mediated mechanisms of neuronal death. *J Neurosci Res*. 2006; 84:209–218. [PubMed: 16619238]
- Logue JM, Price PN, Sherman MH, Singer BC. A method to estimate the chronic health impact of air pollutants in u.s. Residences. *Environ Health Perspect*. 2012; 120:216–222. [PubMed: 22094717]
- Lovell MA, Markesbery WR. Ratio of 8-hydroxyguanine in intact DNA to free 8-hydroxyguanine is increased in Alzheimer disease ventricular cerebrospinal fluid. *Arch Neurol*. 2001; 58:392–396. [PubMed: 11255442]
- Lovell MA, Xie C, Markesbery WR. Acrolein is increased in Alzheimer's disease brain and is toxic to primary hippocampal cultures. *Neurobiol Aging*. 2001; 22:187–194. [PubMed: 11182468]
- Luo J, Robinson JP, Shi R. Acrolein-induced cell death in PC12 cells: role of mitochondria-mediated oxidative stress. *Neurochem Int*. 2005; 47:449–457. [PubMed: 16140421]
- Maddox JF, Domzalski AC, Roth RA, Ganey PE. 15-deoxy prostaglandin J2 enhances allyl alcohol-induced toxicity in rat hepatocytes. *Toxicol Sci*. 2004; 77:290–298. [PubMed: 14657518]
- Mori M, Hara M, Wada I, Hara T, Yamamoto K, Honda M, Naramoto J. Prospective study of hepatitis B and C viral infections, cigarette smoking, alcohol consumption, and other factors associated with hepatocellular carcinoma risk in Japan. *Am J Epidemiol*. 2000; 151:131–139. [PubMed: 10645815]
- Orrenius S, Nicotera P, Zhivotovsky B. Cell death mechanisms and their implications in toxicology. *Toxicol Sci*. 2011; 119:3–19. [PubMed: 20829425]
- Ott M, Gogvadze V, Orrenius S, Zhivotovsky B. Mitochondria, oxidative stress and cell death. *Apoptosis*. 2007; 12:913–922. [PubMed: 17453160]
- Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO, Gorgun CZ, Hotamisligil GS. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science*. 2006; 313:1137–1140. [PubMed: 16931765]
- Patel JM, Wood JC, Leibman KC. The biotransformation of allyl alcohol and acrolein in rat liver and lung preparations. *Drug metabolism and disposition: the biological fate of chemicals*. 1980; 8:305–308. [PubMed: 6107226]
- Perry HE, Shannon MW. Efficacy of oral versus intravenous N-acetylcysteine in acetaminophen overdose: results of an open-label, clinical trial. *J Pediatr*. 1998; 132:149–152. [PubMed: 9470017]
- Pessione F, Ramond MJ, Njapoum C, Duchatelle V, Degott C, Erlinger S, Rueff B, Valla DC, Degos F. Cigarette smoking and hepatic lesions in patients with chronic hepatitis C. *Hepatology*. 2001; 34:121–125. [PubMed: 11431742]
- Pessione F, Ramond MJ, Peters L, Pham BN, Batel P, Rueff B, Valla DC. Five-year survival predictive factors in patients with excessive alcohol intake and cirrhosis. Effect of alcoholic hepatitis, smoking and abstinence. *Liver Int*. 2003; 23:45–53. [PubMed: 12640727]
- Pfaffenbach KT, Gentile CL, Nivala AM, Wang D, Wei Y, Pagliassotti MJ. Linking endoplasmic reticulum stress to cell death in hepatocytes: roles of C/EBP homologous protein and chemical

- chaperones in palmitate-mediated cell death. *Am J Physiol Endocrinol Metab.* 2010; 298:E1027–1035. [PubMed: 20159858]
- Pugazhenth S, Phansalkar K, Audesirk G, West A, Cabell L. Differential regulation of c-jun and CREB by acrolein and 4-hydroxynonenal. *Free Radic Biol Med.* 2006; 40:21–34. [PubMed: 16337876]
- Ren S, Kalhorn TF, Slattery JT. Inhibition of human aldehyde dehydrogenase 1 by the 4-hydroxycyclophosphamide degradation product acrolein. *Drug metabolism and disposition: the biological fate of chemicals.* 1999; 27:133–137. [PubMed: 9884322]
- Rudra PK, Krokan HE. Acrolein cytotoxicity and glutathione depletion in n-3 fatty acid sensitive- and resistant human tumor cells. *Anticancer research.* 1999; 19:461–469. [PubMed: 10226583]
- Runchel C, Matsuzawa A, Ichijo H. Mitogen-activated protein kinases in mammalian oxidative stress responses. *Antioxid Redox Signal.* 2011; 15:205–218. [PubMed: 21050144]
- Sakata K, Kashiwagi K, Sharmin S, Ueda S, Irie Y, Murotani N, Igarashi K. Increase in putrescine, amine oxidase, and acrolein in plasma of renal failure patients. *Biochem Biophys Res Commun.* 2003; 305:143–149. [PubMed: 12732208]
- Satoh K, Yamada S, Koike Y, Igarashi Y, Toyokuni S, Kumano T, Takahata T, Hayakari M, Tsuchida S, Uchida K. A 1-hour enzyme-linked immunosorbent assay for quantitation of acrolein- and hydroxynonenal-modified proteins by epitope-bound casein matrix method. *Analytical biochemistry.* 1999; 270:323–328. [PubMed: 10334850]
- Sharmin S, Sakata K, Kashiwagi K, Ueda S, Iwasaki S, Shirahata A, Igarashi K. Polyamine cytotoxicity in the presence of bovine serum amine oxidase. *Biochem Biophys Res Commun.* 2001; 282:228–235. [PubMed: 11263996]
- Singh R, Wang Y, Schattenberg JM, Xiang Y, Czaja MJ. Chronic oxidative stress sensitizes hepatocytes to death from 4-hydroxynonenal by JNK/c-Jun overactivation. *Am J Physiol Gastrointest Liver Physiol.* 2009; 297:G907–917. [PubMed: 20501438]
- Stevens JF, Maier CS. Acrolein: sources, metabolism, and biomolecular interactions relevant to human health and disease. *Mol Nutr Food Res.* 2008; 52:7–25. [PubMed: 18203133]
- Tanel A, Averill-Bates DA. The aldehyde acrolein induces apoptosis via activation of the mitochondrial pathway. *Biochim Biophys Acta.* 2005; 1743:255–267. [PubMed: 15843039]
- Tarantino G, Caputi A. JNKs, insulin resistance and inflammation: A possible link between NAFLD and coronary artery disease. *World J Gastroenterol.* 2011; 17:3785–3794. [PubMed: 21987620]
- Toennes SW, Schmidt K, Fandino AS, Kauert GF. A fatal human intoxication with the herbicide allyl alcohol (2-propen-1-ol). *Journal of analytical toxicology.* 2002; 26:55–57. [PubMed: 11888018]
- Tsukahara H, Haruta T, Todoroki Y, Hiraoka M, Noiri E, Maeda M, Mayumi M. Oxidant and antioxidant activities in childhood meningitis. *Life sciences.* 2002; 71:2797–2806. [PubMed: 12383885]
- Uchida K, Kanematsu M, Morimitsu Y, Osawa T, Noguchi N, Niki E. Acrolein is a product of lipid peroxidation reaction. Formation of free acrolein and its conjugate with lysine residues in oxidized low density lipoproteins. *J Biol Chem.* 1998a; 273:16058–16066. [PubMed: 9632657]
- Uchida K, Kanematsu M, Sakai K, Matsuda T, Hattori N, Mizuno Y, Suzuki D, Miyata T, Noguchi N, Niki E, Osawa T. Protein-bound acrolein: potential markers for oxidative stress. *Proc Natl Acad Sci U S A.* 1998b; 95:4882–4887. [PubMed: 9560197]
- Verma G, Datta M. The critical role of JNK in the ER-mitochondrial crosstalk during apoptotic cell death. *J Cell Physiol.* 2012; 227:1791–1795. [PubMed: 21732347]
- Weinhold B. Acrolein and neuro disorders. *Environ Health Perspect.* 2011; 119:A68. [PubMed: 21285012]
- Xu C, Bailly-Maitre B, Reed JC. Endoplasmic reticulum stress: cell life and death decisions. *J Clin Invest.* 2005; 115:2656–2664. [PubMed: 16200199]
- Yu MW, Chiang YC, Lien JP, Chen CJ. Plasma antioxidant vitamins, chronic hepatitis B virus infection and urinary aflatoxin B1-DNA adducts in healthy males. *Carcinogenesis.* 1997; 18:1189–1194. [PubMed: 9214602]

HIGHLIGHTS

- Human primary hepatocytes and cultured cell lines are used.
- Multiple cell death signaling pathways are activated by acrolein.
- Novel finding of acrolein-induced ER stress
- Acrolein fails to activate ER stress-induced protective responses.
- Combinatorial therapies may be needed for preventing acrolein hepatotoxicity.

Figure 1. Effect of acrolein on cell survival and apoptosis

Primary human hepatocytes were untreated (UT) or treated with acrolein at varying concentrations (as indicated) for 24h. (A) Cell viability/survival was determined by MTT assay. The data were normalized to untreated values (set to 100%), and expressed as mean ±

SD (n=3, independent experiments). **(B)** DNA fragmentation was measured by ELISA. The data were normalized to untreated values (set to 1), and expressed as mean $\pm$ SD (n=3, independent experiments). **(C)** Caspase-dependent cleavage of cytokeratin 18 was assessed by ELISA and expressed as a ratio of cleaved to intact protein (M30/M65 ratio, right panel). The data were normalized to untreated values (set to 1) and expressed as mean $\pm$ SD (n=3, independent experiments).). *P < 0.05 as compared with untreated for **(A)**, **(B)** and **(C)**.

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Figure 2A. Effect of acrolein on cellular GSH

Hepatocytes were treated with acrolein (5, 10, 25, 50, 75 μM) for 6 hours, and total cellular GSH levels was analyzed by HPLC. The data were normalized to cell count and expressed as mean ± SD (n=3, replicates). *P < 0.05 as compared with untreated.

Figure 2B. Effect of acrolein on cellular antioxidant capacity

Hepatocytes were treated with acrolein (5, 10, 25, 50, 75, 100μM) for 6 or 24hours, and total cellular antioxidant capacity was measured by ELISA. The data were normalized to untreated values (set to 100%), and expressed as mean ± SD (n=3, independent experiments). *P < 0.05 as compared with untreated.

Figure 3. Effect of acrolein on stress-signaling MAPKs

Hepatocytes were untreated (UT) or treated with acrolein (25, 50, 75 μ M) for 15 or 60 min, and phosphorylation of p42/44-, p38, and JNK MAP-kinases was analyzed by Western blotting, with GAPDH as loading control. Results are representative of three independent experiments. The density of each band was compared to its corresponding GAPDH, and represented as a density ratio. The density ratio was normalized to the untreated value which was set to 1.

Figure 4. Effects of acrolein on mitochondrial function

(A) Mitochondrial membrane potential: HepG2 cells were untreated (UT) or treated with 25 or 50 μ M acrolein for 6h. Loss of mitochondrial membrane potential was monitored using the JC-1 mitochondrial dye by fluorescence microscopy at low (10 $\times$) and high (40 $\times$) magnification. Results shown are representative of at least two experiments performed in duplicate.

(B) Release of pro-apoptotic mitochondrial proteins: Hepatocytes were treated with 50 μ M acrolein for 0, 3, 6, 12 and 24h. Levels of cytochrome c and AIF in the cytoplasm and mitochondria were examined by Western blot analysis. Results shown are representative of three experiments.

(C) Cellular ATP levels: Hepatocytes were untreated (UT) or treated (in triplicate) with acrolein (10, 25, 50, 75 and 100 μ M) for 2, 6, or 24h, and the total cellular levels of ATP were analyzed as described in Methods. The data represent relative fluorescence units (RLU) and are expressed as mean $\pm$ SD (n=3, replicates). *P < 0.05 compared to untreated.

Figure 5. Effect of acrolein on caspases

Hepatocytes were treated with 50 μ M acrolein for 1, 3, 6, 12 and 24h. Levels of cleaved caspase-3, 4 and 9 were examined by Western blotting with GAPDH as loading control. Results shown are representative of three experiments. The density of each band was compared to its corresponding GAPDH, and represented as a density ratio. The density ratio was normalized to the untreated value which was set to 1.

Figure 6. Effect of acrolein ER stress

(A) and (C): Hepatocytes were untreated (UT) or treated with 25, 50, 60, 75 and 100 μ M acrolein for 24h. Protein levels of ER stress proteins phospho-eIF2 α , ATF4, ATF3 and GADD153/CHOP in (A), and ATF6, ER chaperones GRP78 and GRP94 in (C) were analyzed by Western blotting with GAPDH as loading control. Results shown are representative of three experiments. The density of each band was compared to its corresponding GAPDH, and represented as a density ratio. The density ratio was normalized to the untreated value which was set to 1. (B) and (D): Hepatocytes were untreated (UT) or treated with 50 μ M acrolein for 1, 3, 6, 12, or 24h. The mRNA levels of GADD153/CHOP, ATF3 and ATF4 in (B) and GRP78 and GRP94 in (D) were analyzed by real time qPCR analysis. Fold induction of mRNA was calculated as described in Methods and expressed as mean $\pm$ SD (n=3). *P < 0.05 compared to untreated.

Figure 7. Effect of acrolein on hepatocyte mitochondrial potential, calcium release and cell permeability by Cellomics-HCS analysis

Hepatocytes were exposed to acrolein (5, 10, 20, 30, 40, 50, 60, 70, 80 or 100μM) for 24hours. The cells were loaded for 1h with the four fluorescent dyes (Hoechst, TMRM, Fluo-4 and TOTO-3), and analyzed by Cellomics-HCS to measure mitochondrial membrane potential (top); release of free calcium (middle); and cell death/permeability (bottom). **(A):** The data were analyzed by averages of treated cells and expressed in arbitrary fluorescence units (AU) as mean $\pm$ SD from two experiments in triplicate. **(B):** The cells were examined microscopically and representative images from cells exposed to 20, 40, 60, 80 or 100μM

acrolein are shown (Magnification 20×). The red fluorescence shows mitochondrial membrane potential and release of free calcium is represented by green fluorescence.

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Figure 8. Effect of inhibitors on acrolein induced hepatocyte death

Hepatocytes were untreated (UT) or treated for 24h with acrolein (50, 60 or 75μM), without or with a pretreatment with (i) 25μM JNK inhibitor SP600025 (JNK); (ii) 25μM pan caspase inhibitor Z-VAD-FMK (Casp); (iii) 1mM antioxidant GSH-prodrug N-acetyl cysteine (NAC); and (iv) 10μM chemical chaperone and ER stress inhibitor, phenyl butyric acid (PBA). Cell survival/viability was determined by MTT assay. The data were normalized to untreated values (set to 100%), and expressed as mean $\pm$ SD (n=3). *P < 0.05 compared to untreated. #P<0.05 compared with the corresponding acrolein treatment.

Figure 9.
Schematic of acrolein cytotoxicity in hepatocytes.