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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 eIF2α, 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.

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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

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.

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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, 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 100 mm 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 $2^{-\Delta\Delta Ct}$ method by normalizing with GAPDH or 18s rRNA.

Cellomics. After treatment, hepatocytes were incubated for 1 h 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 (7.6.2.1-1.00×) 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 ± 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; Kaminskas et al., 2005; Lovell et al., 2001; Maddox et al., 2004; Sakata et al., 2003).

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