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Effect of trifluoperazine on toxicity, HIF-1α induction and hepatocyte regeneration in acetaminophen toxicity in mice

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ABSTRACT

Oxidative stress and mitochondrial permeability transition (MPT) are important mechanisms in acetaminophen (APAP) toxicity. The MPT inhibitor trifluoperazine (TFP) reduced MPT, oxidative stress, and toxicity in freshly isolated hepatocytes treated with APAP. Since hypoxia inducible factor-one alpha (HIF-1α) is induced very early in APAP toxicity, a role for oxidative stress in the induction has been postulated. In the present study, the effect of TFP on toxicity and HIF-1α induction in B6C3F1 male mice treated with APAP was examined. Mice received TFP (10 mg/kg, oral gavage) prior to APAP (200 mg/kg IP) and at 7 and 36 h after APAP. Measures of metabolism (hepatic glutathione and APAP protein adducts) were comparable in the two groups of mice. Toxicity was decreased in the APAP/TFP mice at 2, 4, and 8 h, compared to the APAP mice. At 24 and 48 h, there were no significant differences in toxicity between the two groups. TFP lowered HIF-1α induction but also reduced the expression of proliferating cell nuclear antigen, a marker of hepatocyte regeneration. TFP can also inhibit phospholipase A_2, and cytosolic and secretory PLA_2 activity levels were reduced in the APAP/TFP mice compared to the APAP mice. TFP also lowered prostaglandin E_2 expression, a known mechanism of cytoprotection. In summary, the MPT inhibitor TFP delayed the onset of toxicity and lowered HIF-1α induction in APAP treated mice. TFP also reduced PGE_2 expression and hepatocyte regeneration, likely through a mechanism involving PLA_2.

Introduction

Acetaminophen (paracetamol, N-acetyl-p-aminophenol, APAP) toxicity is a very common cause of acute liver failure in the United States (Larson et al., 2005). The role of metabolism in the initiation of APAP toxicity was reported over 40 years ago (Jollow et al., 1973; Mitchell et al., 1973). In addition, oxidative stress has been implicated to be important in the mediation of toxicity (Hinson et al., 1998; Knight et al., 2001; Laskin et al., 1994). The nitration of proteins in APAP toxicity has been recognized, implicating the involvement of the nitrating and oxidizing species peroxynitrite as a mechanism in the development of toxicity (Hinson et al., 1998; Knight et al., 2001).

Mitochondrial permeability transition (MPT) is another mechanism of toxicity in APAP mediated liver injury (Kon et al., 2004; Lemasters et al., 1999; Reid et al., 2005). MPT represents an abrupt increase in the permeability of the inner mitochondrial membrane that results in the loss of ATP and eventual cellular necrosis. The loss of ATP in APAP toxicity was previously demonstrated by Jaeschke (1990). MPT inhibitors, such as cyclosporine A (CYC), have been previously tested using *in vitro* models of APAP toxicity (Kon et al., 2004; Lemasters, 1999; Reid et al., 2005). In addition, MPT inhibitors have been shown to be beneficial in a number of animal models of cellular injury. For example, NIM811, a CYC analog, decreased mitochondrial dysfunction and remnant liver injury in a mouse model of massive partial hepatectomy (Rehman et al., 2011). Few studies have examined the effect of MPT inhibitors on APAP toxicity *in vivo*. We recently reported that the MPT inhibitor CYC decreased toxicity in mice, but CYC also markedly inhibited the metabolism of APAP (Chaudhuri et al., 2010), precluding further study with this compound.

The transcription factor HIF-1α is a master regulator of adaptive responses of cells to hypoxia. The HIF-1 complex is composed of two protein subunits known as HIF-1β, which is constitutively expressed,

Abbreviations: APAP, acetaminophen; ALT, alanine aminotransferase; $cPLA_2$, cytosolic phospholipase A_2; (CYC), cyclosporine A; GSH, glutathione; HIF-1α, hypoxia inducible factor-1α; MPT, mitochondrial permeability transition; PCNA, proliferating cell nuclear antigen; PGE_2, prostaglandin E2; $sPLA_2$, secretory phospholipase A_2; TFP, trifluoperazine; TNFα, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.

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and HIF-1α, which is not present in normal cells but is induced under hypoxic conditions. The HIF-1α subunit is continuously synthesized and degraded by the prolyl hydroxylase system under normoxic conditions, while it accumulates rapidly following exposure to low oxygen tensions. HIF-1α may also be induced by oxidative stress. HIF-1α is induced in the early stages of APAP toxicity in the mouse and in freshly isolated hepatocytes incubated under a stream of oxygen (James et al., 2006). Moreover, HIF-1α induction occurs at sub-toxic doses of APAP, suggesting the presence of low levels of oxidative stress (Chaudhuri et al., 2010) without overt toxicity (e.g., ALT elevation). Treatment of mice with low dose CYC reduced HIF-1α, supporting the hypothesis that HIF-1α induction in APAP toxicity is secondary to oxidative stress. However, high dose CYC inhibited the metabolism of APAP, preventing further studies with this compound. To further examine the role of MPT in APAP toxicity, the effect of the MPT inhibitor trifluoperazine (TFP) was studied in APAP treated mice. Previous studies have shown TFP to be protective in APAP toxicity but mechanisms of the protection were not well defined (Dimova et al., 1995; Yamamoto, 1990). We hypothesized that TFP would reduce toxicity in mice treated with high doses of APAP and that treatment with TFP would reduce HIF-1α induction in the liver. Since TFP is also a phospholipase A_2 inhibitor, the effects of TFP on the cyclooxygenase pathway were examined, in addition to later events in APAP toxicity.

Materials and methods

Drugs and reagents. APAP was obtained from Sigma Chemical Co. (St. Louis, MO). Trifluoperazine was obtained from Sigma-Aldrich Co. (St. Louis, MO) Coomassie Plus Protein Assay Reagent was purchased from Pierce Chemical Co. (Rockford, IL). DTT (dithiothreitol; Cleland's reagent) was obtained from Bio-Rad Laboratories (Hercules, CA). Gills Hematoxylin II and Permount were acquired from Fisher Scientific, Inc. (Pittsburgh, PA). Anti-HIF-1α monoclonal antibody was purchased from Novus Biologicals (Littleton, CO) and diluted 1:1000 immediately before use.

Experimental animals. Six-week old male B6C3F1 mice (mean weight, 25.1 g) were obtained from Harlan Sprague Dawley (Indianapolis, IN). All animal experimentations were in accordance with the criteria of the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences. Protocols for animal experimentation were approved by the University of Arkansas for Medical Sciences Animal Care and Use Committee. Mice were acclimatized one week prior to the planned experiments and fed *ad libitum*. Animals were housed 3 per cage and maintained on a 12 h light/dark cycle. On the day prior to experiments, mice were fasted overnight and dosing studies began at 0800 the following morning. Food was returned to the mice 4 h after APAP. In a preliminary dose response study, TFP was administered by oral gavage (5, 7.5 or 10 mg/kg; 3 mice/dose group) 1 h before administration of APAP (200 mg/kg) IP. Other mice received APAP (200 mg/kg). Control mice received saline IP. Mice were euthanized at 1 or 2 h after APAP. In the time course study, the APAP/TFP mice received additional doses of TFP by oral gavage (10 mg/kg) at 7 and 36 h after APAP (Dimova et al., 1995; Yamamoto, 1990). The mice were euthanized at 1, 2, 4, 8, 24, or 48 h after APAP (6–7 mice/dose group). Animals were anesthetized with CO_2 for blood sampling. Blood was removed from the retro-orbital plexus, allowed to coagulate at room temperature, centrifuged, and the serum was used for measurement of alanine aminotransferase (ALT). Mice were then euthanized in a CO_2 atmosphere followed by cervical dislocation and removal of the livers. The livers were weighed and a portion was preserved in formalin for histological sections. The remaining livers were snap frozen in liquid nitrogen and stored at −80 °C for additional analyses.

Liver histology. Hematoxylin and eosin (H&E) staining was performed for histological examination of the liver samples. A reviewer blinded to treatment group examined the liver sections and scored them for hepatocellular necrosis and hemorrhage using a 0 (no lesion) to 4 (severe change) scoring system that included lobular localization as per previous publications (Chaudhuri et al., 2010).

Metabolism and toxicity assays. Serum ALT levels were measured using an Alera chemistry analyzer (Alfa Wassermann, West Caldwell, New Jersey). APAP covalently bound to protein in liver was measured by initial protease treatment of liver homogenates, followed by high performance liquid chromatography-electrochemical (HPLC-EC) analysis for APAP-cysteine as previously described (Muldrew et al., 2002). Glutathione (GSH) was measured using Ellman's reagent as previously described (Rehman et al., 2011; Yamamoto, 1990).

HIF-1α nuclear extraction. HIF-1α expression was measured by Western blot as previously described (James et al., 2006). Liver tissue for HIF-1α was prepared using Active Motif's (Carlsbad, CA) Nuclear Extract kit. Briefly, 30 μg of protein was separated by SDS-PAGE and blocked for 1 h at room temperature and then incubated in the primary monoclonal antibody (Novus Biologicals, Littleton, CO) was used at a dilution of 1:1000 overnight at 4 °C. A peroxidase-conjugated goat anti-mouse IgG secondary antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) was used (1:2000 dilution) for 1 h at room temperature. Band detection was performed using ECL Plus detection (Amersham, Piscataway, NJ).

Expression of proliferating cell nuclear antigen (PCNA). Immunoblots of mouse liver for PCNA expression were performed using a monoclonal antibody (Carpentaria, CA) at 1:500 as per our previous publications (Donahower et al., 2006). In addition, immunohistochemical assays for PCNA in liver sections were performed as per our previous publication (Donahower et al., 2006), using a mouse monoclonal PCNA antibody (Dako, Carpinteria, CA) (1:75) and Gills Hematoxylin II as the counterstain. Quantification of PCNA staining of hepatocyte nuclei was performed using Aperio imaging. Quantitative pathological analysis hardware and software, Aperio Scanscope T2 and ImageScope software (Aperio, Vista, CA), were used to quantify the staining in the proliferating hepatocyte nuclei in each tissue section.

Growth factor and cytokine assays. Supernatants of homogenized liver were assayed for vascular endothelial growth factor (VEGF) using an ELISA kit available from R & D (Minneapolis, MN) as per our previous publications (Donahower et al., 2006). Serum samples were analyzed for tumor necrosis factor alpha (TNFα) using an ELISA kit available from Enzo Life Sciences (Plymouth Meeting, PA).

PLA_2 activity and PGE_2 levels in liver. PLA_2 activity in liver was measured using a PLA_2 activity kit (Cayman Chemicals, Ann Arbor, MI) as per the manufacturer's instructions and following published methods (Reyes et al., 2006). Liver samples were homogenized and centrifuged at 14,000 for 40 min using a cellulose membrane filter with a cut-off of 30 kDa (Spin-X 500 UF Concentrators, 30 K MWCO, Corning Scientific, Wilkes Barre, PA) to separate the PLA_2 isoforms. The higher molecular weight fraction was used to measure cPLA2 activity and the lower molecular weight fraction was used to measure sPLA2 activity. To avoid the measurement of $iPLA_2$ in the sample, bromoenol lactone was used. Results are expressed as nmol/mg/mL. PGE_2 was measured in liver homogenates using the Luminex Prostaglandin E_2 kit from Cayman Chemicals (Ann Arbor, MI) as per the manufacturer's instructions.

Statistical analysis. Results are expressed as means ± SE. A p value of 0.05 was considered significant for all analyses. Comparisons between multiple groups were performed by one-way analysis of variance