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The gap junction inhibitor 2-aminoethoxy-diphenyl-borate protects against acetaminophen hepatotoxicity by inhibiting cytochrome P450 enzymes and c-jun N-terminal kinase activation

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ABSTRACT

Acetaminophen (APAP) hepatotoxicity is the leading cause of acute liver failure in the US. Although many aspects of the mechanism are known, recent publications suggest that gap junctions composed of connexin32 function as critical intercellular communication channels which transfer cytotoxic mediators into neighboring hepatocytes and aggravate liver injury. However, these studies did not consider off-target effects of reagents used in these experiments, especially the gap junction inhibitor 2-aminoethoxy-diphenyl-borate (2-APB). In order to assess the mechanisms of protection of 2-APB in vivo, male C56Bl/6 mice were treated with 400 mg/kg APAP to cause extensive liver injury. This injury was prevented when animals were co-treated with 20 mg/kg 2-APB and was attenuated when 2-APB was administered 1.5 h after APAP. However, the protection was completely lost when 2-APB was given 4–6 h after APAP. Measurement of protein adducts and c-jun-N-terminal kinase (JNK) activation indicated that 2-APB reduced both protein binding and JNK activation, which correlated with hepatoprotection. Although some of the protection was due to the solvent dimethyl sulfoxide (DMSO), in vitro experiments clearly demonstrated that 2-APB directly inhibits cytochrome P450 activities. In addition, JNK activation induced by phorone and tert-butylhydroperoxide in vivo was inhibited by 2-APB. The effects against APAP toxicity in vivo were reproduced in primary cultured hepatocytes without use of DMSO and in the absence of functional gap junctions. We conclude that the protective effect of 2-APB was caused by inhibition of metabolic activation of APAP and inhibition of the JNK signaling pathway and not by blocking connexin32-based gap junctions.

Introduction

Acetaminophen (APAP) is a widely used analgesic and antipyretic drug, which is safe at therapeutic doses. However, intentional or accidental overdosing can induce severe liver injury and in some patients even acute liver failure (Larson, 2007; McGill et al., 2012). Early animal studies identified reactive metabolite formation, glutathione (GSH) depletion and protein adduct formation as critical events in the toxicity (McGill and Jaeschke, 2013; Mitchell et al., 1973; Nelson, 1990). This mechanistic insight led to the introduction of N-acetylcysteine (NAC) as a clinical antidote against APAP poisoning (Prescott et al., 1977). NAC promotes GSH synthesis and thus protects by enhancing the detoxification capacity for the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) (Corcoran and Wong, 1986; Corcoran et al, 1985). NAC-supported GSH synthesis also replenishes the mitochondrial GSH content, which allows detoxification of reactive oxygen species and peroxynitrite in the mitochondria (Cover et al., 2005; Knight et al., 2002). In addition, surplus NAC not needed to synthesize GSH will be degraded, and the resulting Krebs cycle intermediates support mitochondrial energy metabolism (Saito et al., 2010b). Despite these multiple protective mechanisms of NAC, it is very obvious that NAC is most effective when given as early as possible after the APAP overdose. However, the clinical reality is that many patients only seek medical attention when liver injury is already present (Larson, 2007). Therefore,

Abbreviations: ALT, alanine aminotransferase; APAP, acetaminophen; 2-APB, 2-aminoethoxy-diphenyl-borate; t-BHP, tert-butylhydroperoxide; Cx32, connexin32; DMSO, dimethyl sulfoxide; 7-EFC, 7-ethoxy-4-trifluoromethylcoumarin; GSH, glutathione; HPLC-ECD, high-pressure liquid chromatography with electrochemical detection; JNK, c-jun-N-terminal kinase; p-JNK, phospho-JNK; LDH, lactate dehydrogenase; NAC, N-acetylcysteine; NAPQI, N-acetyl-p-benzoquinone imine; PH, phorone; ROS, reactive oxygen species; TAA, thioacetamide.

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there is clearly a need to develop drugs that are effective after the metabolism phase.

A recent paper by Patel et al. (2012) identified gap junctions containing connexin32 (Cx32) as critical intercellular communication channels responsible for the progression of liver injury after thioacetamide (TAA) and APAP overdose. The authors suggested that gap junctions allow the transfer of a lethal dose of reactive oxygen species (ROS) into neighboring hepatocytes. The most remarkable feature of this study was the identification of a small molecule Cx32-gap junction inhibitor, 2-aminoethoxy-diphenyl-borate (2-APB) (Tao and Harris, 2007), which was not only >99% effective in preventing APAP- or TAA-induced liver injury when given 1 h before drug overdose, but also reduced liver injury by 60% when administered as late as 6 h after the toxicants (Patel et al., 2012). This remarkable protection of 2-APB, especially with delayed administration, garnered significant attention (Fromenty, 2013; Maurel and Rosenbaum, 2012). The editorial commentaries expressed the hope that this might be a novel and promising treatment option for drug-induced liver injury. However, the virtually perfect protection with 2-APB raises some concerns regarding the mechanisms involved. Although Patel et al. (2012) evaluated the formation of TAA metabolites, this was not done with APAP. In addition, 2-APB is only soluble in diluted dimethyl sulfoxide (DMSO), which is known to effectively block APAP toxicity through inhibition of drug metabolism even at very low doses (Jaeschke et al., 2006). Given these serious concerns and the potential benefits of 2-APB treatment, we evaluated the mechanism of protection of 2-APB in a murine model of acetaminophen hepatotoxicity in vivo and in cultured mouse hepatocytes.

Materials and methods

Animals. Male C57Bl/6 mice (8–12 weeks old) purchased from Jackson Laboratories (Bar Harbor, ME) were used in our experiments. The mice were kept in an environmentally controlled room with a 12 h light/dark cycle and free access to food and water. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center and followed the criteria of the National Research Council for the care and use of laboratory animals.

Experiment design. After overnight fasting, mice were treated with 400 mg/kg APAP (Sigma-Aldrich, St. Louis, MO) (i.p.) dissolved in warm saline. A dose of 20 mg/kg of the Cx32 gap junction inhibitor 2-aminoethoxy-diphenyl-borate (2-APB) (Sigma-Aldrich) dissolved in DMSO was administered with APAP, or at 1.5 h, 4.5 h and 6 h after APAP treatment. All vehicle control mice received the same volume of DMSO (0.2 ml/kg) and saline (20 ml/kg). Mice were euthanized at 2 h, 6 h or 24 h after APAP injection and blood and livers were harvested. In addition, some animals were treated with 20 mg/kg 2-APB followed 1 h later by 100 mg/kg phorone (Sigma-Aldrich) dissolved in corn oil and then 1 h later with 1 mmol/kg tert-butylhydroperoxide (tBHP) (Sigma-Aldrich) and euthanized 1 h after tBHP (Xie et al., 2013). Blood was drawn into a heparinized syringe to determine alanine aminotransferase (ALT) activity with a kit from Pointe Scientific (Canton, MI). The liver was removed and pieces were fixed in phosphate-buffered formalin or used for mitochondrial isolation. The rest of the liver was snap-frozen in liquid nitrogen and subsequently stored at −80 °C.

Mouse hepatocyte isolation and cell viability assessment. Primary hepatocytes were isolated from mice by means of a 2-step collagenase perfusion technique as described previously (Bajt et al., 2004). Cell viability was generally more than 90% based on trypan blue exclusion, and cell purity of hepatocytes was more than 95%. The cells were plated in a density of 6×10^5 cells/well in six-well plates (BioCoat collagen I cellware plates; Becton Dickinson, Franklin Lakes, NJ). Cells were grown in Williams E medium (Life Technologies, Grand Island, NY) containing 100 U/ml penicillin/streptomycin, 1×10^{-7} M insulin, and 10% fetal bovine serum. After 3.5 h of attachment to the bottom of the plate, cells were treated with 5 mM APAP and 2-APB at the same time or delayed for 1.5 h at a concentration of 1, 5, 10, or 25 μM. Both APAP and 2-APB were dissolved in 37 °C Williams E medium. Cells were harvested for lactate dehydrogenase (LDH) activity, GSH and APAP-protein adduct determination.

Measurement of LDH and GSH. The measurement of LDH activities was performed as described in detail (Bajt et al., 2004). In short, after removing the medium, cells were scraped off and lysed in cell lysis buffer (25 mM HEPES, 5 mM EDTA, 0.1% CHAPS, and 1 mg/ml each of pepstatin, leupeptin, and aprotinin). The lysates were sonicated and centrifuged for 10 min at 20,000 *g* at 4 °C. Both the resulting supernatant and previous cell medium were incubated with potassium phosphate buffer containing pyruvate and NADH, and LDH activities were determined by the decline of absorbance at 340 nm. GSH levels in primary mouse hepatocyte were determined using a modified Tietze assay (Yan et al., 2010). In brief, primary cells were lysed and homogenized in 3% sulfosalicylic acid and centrifuged to remove precipitated proteins. The supernatant was further diluted with 0.1 M potassium phosphate buffer, pH 7.4. The samples were then assayed with dithionitrobenzoic acid as described (Jaeschke and Mitchell, 1990). A 10% sodium dodecyl sulfate solution was applied to the pellet to solubilize the protein. Protein concentrations were determined using the bicinchoninic acid kit (Pierce, Rockford, IL). The GSH levels were expressed per mg protein.

APAP protein adducts and cytochrome P450 activity. APAP-protein adducts in liver tissues were measured by high-pressure liquid chromatography with electrochemical detection (HPLC-ECD) according to the method of Muldrew et al. (2002) with some modifications (Ni et al., 2012). APAP-protein adduct concentration in primary mouse hepatocytes was determined as described (McGill et al., 2011). The cytochrome P450 activity was determined using the 14,000 *g* supernatant from a total liver homogenate in the 7-ethoxy-4-trifluoromethylcoumarin (7EFC) deethylase assay (Buters et al., 1993), as described in detail (Ramachandran et al., 2011). The substrate 7EFC is known to be metabolized by Cyp1A2 and 2E1 (Buters et al., 1993).

Histology and immunoprobing. Formalin-fixed tissue samples were embedded in paraffin and 5 μm thickness sections were cut and stained with hematoxylin and eosin (H&E) for blinded evaluation of tissue necrosis as described (Gujral et al., 2002). Western blotting was performed as described in detail previously (Bajt et al., 2000). Briefly, a 20% liver homogenate was prepared for each liver sample in homogenizing buffer (25 mM HEPES, 5 mM EDTA, 2 mM DTT, 0.1% CHAPS supplemented with 1 μg/ml leupeptin, aprotinin and pepstatin) with 10 stroke of a tight fitting Teflon pestle in a mortar. The homogenate was centrifuged at 14,000 *g* for 20 min and the supernatant was collected. After determination of protein concentration using the bicinchoninic acid kit (Pierce, Rockford, IL), 30 μg of protein was loaded per lane. Western blotting for JNK was performed using rabbit anti-JNK and anti-phospho-JNK antibodies (Cell Signaling Technology, Danvers, MA) with horseradish peroxidase-coupled donkey anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) (Bajt et al., 2000). Proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech., Inc., Piscataway, NJ).

Statistics. All data were expressed as mean ± SEM. For normally distributed data, statistical significance was evaluated by one-way analysis of variance (ANOVA), followed by Student Newman–Keul's test for multiple comparisons. For non-normally distributed data, ANOVA was performed on ranks, followed by Dunn's multiple comparisons. $P < 0.05$ was considered significant.

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