Research Article

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Deleterious effect of oltipraz on extrahepatic cholestasis in bile duct-ligated mice

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Background & Aims: Oltipraz (4-methyl-5(pyrazinyl-2)-1-2-dithiole-3-thione), a promising cancer preventive agent, has an antioxidative activity and ability to enhance glutathione biosynthesis, phase II detoxification enzymes and multidrug resistance-associated protein-mediated efflux transporters. Oltipraz can protect against hepatotoxicity caused by carbon tetrachloride, acetaminophen and alpha-naphthylisothiocyanate. Whether oltipraz has hepato-protective effects on obstructive cholestasis is unknown.

Methods: We administered oltipraz to mice for 5 days prior to bile duct ligation (BDL) for 3 days. Liver histology, liver function markers, bile flow rates and hepatic expression of profibrogenic genes were evaluated.

Results: Mice pretreated with oltipraz prior to BDL demonstrated higher levels of serum aminotransferases and more severe liver damage than in control mice. Higher bile flow and glutathione secretion rates were observed in unoperated mice treated with oltipraz than in control mice, suggesting that liver necrosis in oltipraz-treated BDL mice may be related partially to increased bile-acid independent flow and biliary pressure. Oltipraz treatment in BDL mice enhanced α-smooth muscle actin expression, consistent with activation of hepatic stellate cells and portal fibroblasts. Matrix metalloproteinases (Mmp) 9 and 13 and tissue inhibitors of metalloproteinases (Timp) 1 and 2 levels were increased in the oltipraz-treated BDL group, suggesting that the secondary phase of liver injury induced by oltipraz might be due to excessive Mmp and Timp secretions, which induce remodeling of the extracellular matrix.

Conclusions: Oltipraz treatment exacerbates the severity of liver injury following BDL and should be avoided as therapy for extrahepatic cholestatic disorders due to bile duct obstruction.

Keywords: Oltipraz; Nrf2; Obstructive cholestasis; Bile secretion; Hepatic stellate cells.

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Abbreviations: BDL, bile duct ligation; Mmp, matrix metalloproteinases; Timp, tissue inhibitors of metalloproteinase; NqoI, NAD(P)H quinone oxidoreductase; UDCA, ursodeoxycholic acid; PBC, primary biliary cirrhosis; GSH, glutathione; Mrp, multidrug resistance-associated protein; BSEP, bile salt efflux pump; LD50, lethal dosage; CMC, carboxymethylcellulose; EDTA, ethylenediaminetetraacetic acid; CK19, cytokeratin 19; ALT, alanine aminotransferase; FXR, farnesoid X receptor; ECM, extracellular matrix; IL-1β, interleukin-1 beta; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha; TGF-β1, transforming growth factor-beta 1; α-SMA, alpha smooth muscle actin; HSC, hepatic stellate cells; PDGFβ, platelet-derived growth factor-beta; BADF, bile acid-dependent bile flow; BAIF, bile acid-independent bile flow; PF, portal fibroblasts; AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; ANIT, alpha-naphthylisothiocyanate; Keap1, kelch-like ECH-associated protein 1; OPZ, oltipraz.

Introduction

Cholestasis is associated with reductions in bile flow, hepatic accumulation of bile acids, and hepatocellular injury and fibrosis. Exposure of hepatocytes to these increasing levels of toxic bile acids can result in production of reactive oxidative species, leading to oxidative stress and progressive liver damage [1]. Increased levels of the by-products of oxidative stress have been detected in obstructive cholestasis in rodents and humans [2,3]. In an attempt to restore redox balance between radical-generating and radical-scavenging capacity, specific pathways can be activated in order to prevent further oxidative injury in the liver [1]. This adaptive stress response involves enhancing the expressions of antioxidant genes, including NAD(P)H quinone oxidoreductase (NqoI), heme oxygenase-1, glutathione-*S*-transferase and UDP-glucuronosyltransferase1A6 [4].

Ursodeoxycholic acid (UDCA), the only drug approved by the FDA specifically for the treatment of primary biliary cirrhosis (PBC), has both direct and indirect antioxidant properties [5]. It acts directly by scavenging hydroxyl radicals and indirectly through the induction of endogenous antioxidant defenses, including increasing the expression of γ-glutamylcysteine synthetase regulatory subunit and increasing the rate of glutathione (GSH) synthesis. Recently, a novel therapeutic mechanism of

UDCA action via Nrf2 activation has been suggested and may represent another drug target in cholestatic liver diseases [5,6]. Nonetheless, the effectiveness of UDCA is limited to the early stages of PBC [7]. Thus, alternative therapeutic interventions are clearly needed for patients with cholestatic liver diseases.

Oltipraz is a known antioxidant and a promising chemo-preventive agent [8]. To date, oltipraz has proved effective as anticarcinogen in experimental models for breast, bladder, liver, forestomach, colon, tracheal, lung, and skin cancer [9]. Oltipraz is undergoing clinical trial evaluation in Qidong, China, as a possible chemoprotective agent against aflatoxin B1 in humans. The antitumorigenic effects of oltipraz in rodents include inhibition of certain cytochrome P450 (CYP1A2) and induction of phase II detoxifying enzymes, including microsomal epoxide hydrolase, UDP-glucuronyltransferase and glutathione-*S*-transferase. Moreover, oltipraz has the ability to modulate liver regeneration [10], and to inhibit hepatitis B virus [11] and human immunodeficiency virus replication [12]. Furthermore, oltipraz can protect against hepatotoxicity caused by carbon tetrachloride [13], acetaminophen [14] and α-naphthylisothiocyanate, a cause of intrahepatic cholestasis in an animal disease model [15]. In addition, oltipraz can up-regulate the gene expression of canalicular efflux transporters, including the multidrug resistance-associated protein (Mrp) 2 [16] and bile salt efflux pump (BSEP) [17], as well as the alternative basolateral efflux transporters, Mrp3 and Mrp4 having an essential role in the adaptive response to obstructive cholestatic liver injury [18,19]. Considering its antioxidant and many favorable pharmacological effects on the liver, oltipraz may be an attractive candidate drug for the treatment of cholestatic liver diseases.

In this study we determined if oltipraz has hepato-protective effects in a murine model of obstructive cholestasis. Unexpectedly, our findings demonstrate that oltipraz treatment significantly exacerbates the severity of liver injury following bile duct ligation (BDL). We conclude that prospective clinical studies with oltipraz require caution and that oltipraz should be avoided as therapy for cholestatic disorders related to bile duct obstruction.

Materials and methods

Materials

All reagents were obtained from Sigma-Aldrich (St Louis, MO). Oltipraz was purchased from Axxora (San Diego, CA). Polyclonal antibody to Mrp2 was a gift from Dr. Bruno Stieger (University of Zurich). Mrp4 and Bsep antibodies were purchased from Everest Biotech (Oxfordshire, UK), and Kamiya Biomedical (Seattle, WA), respectively. Mrp3 antibody was developed in our laboratory [20]. SH-PTP1 antibody was obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). DAB peroxidase substrate kit was supplied from Vector Laboratories (Burlingame, CA).

Animals and surgical procedures

Male C57BL/6J mice (8–9 weeks old) were obtained from the Jackson Laboratory, Bar Harbor, ME. Mice were housed in the Yale animal facility, and were kept in controlled light (12-h light, 12-h dark cycle) and temperature (22 °C), and provided with food and water *ad libitum*. The study protocol was approved by the Yale Animal Care and Use Committee, and was in accordance with National Institutes of Health guidelines (protocol no. 2009-07458). Mice were randomized and pretreated orally with oltipraz at a dose of 150 mg/kg body weight that is known to be the safety dose (LD50 >5000 mg/kg) or the solvent control (carboxymethylcellulose, CMC) for 5 days prior to bile duct ligation [21] for 3 days. Total duration for oltipraz treatment was 8 days. Animals were fasted overnight prior to sacrifice. Plasma, bile, urine and liver tissue were collected and stored at −80 °C or fixed in 4% neutral buffered formaldehyde for blinded histological evaluation by J.L.B.

Immunohistochemical analysis

Immunohistochemistry for cytokeratin 19 was performed on paraffin sections to quantify bile duct proliferation. Antigen retrieval using 1 mM EDTA, pH 8.0, in a steamer was done prior to overnight incubation with the monoclonal rat anti-CK19 (Troma III) as previously described [22].

Hepatocellular function

Plasma ALT and total bilirubin levels were measured as indicators of hepatic injury using standard diagnostic kits (Thermo Scientific, Middletown, VA). Bile acid concentrations in the plasma, gallbladder, and liver were determined using a commercial total bile acid kit (Diazyme Laboratories, Poway, CA) as previously described [22].

Quantitative real-time polymerase chain reaction

RNA was isolated from frozen liver samples using Trizol reagent according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA). Complementary DNA (cDNA) synthesis and subsequent quantitative real-time PCR using TaqMan technology were performed as described previously [17]. The gene-specific primers used were provided by Applied Biosystems and are listed in Supplementary Table 1. *Gapdh*, a house keeping gene, was run for each sample to normalize expression.

Western blot analysis for bile acid transporter expression

Membrane enriched liver proteins were isolated as previously described [23]. Western blot analysis was performed as previously described with some modifications [17]. Briefly, samples were loaded onto 4–12% NuPAGE Bis-Tris gradient gels and were electrophoresed with MOPS running buffer (Invitrogen). Proteins were transferred onto nitrocellulose membrane and incubated with the antibodies listed above. IRDye680 or IRDye800 conjugated to rabbit or goat IgG were used as secondary antibodies (Li-Cor, Lincoln NE). The relative quantities of protein expression were analyzed using the Odyssey infrared image system (Li-Cor).

Measurement of bile flow

Bile flow was determined in a separate group of mice, which were treated with either CMC or oltipraz for 8 days. Bile was collected every 5 min for 60 min in pre-weighed eppendorf tubes containing water or 6% sulfosalicylic acid for bile acid and glutathione determination, respectively, as previously described [24].

Glutathione content determination

Glutathione content in the liver homogenate or in bile samples was determined using a glutathione assay kit (Sigma).

Statistical analysis

All data are expressed as the mean ± standard deviation (S.D.). Statistical analyses were performed using analysis of variance with Bonferroni post-testing (SigmaStat program, Jandel Scientific). Differences were considered statistical significant at $p < 0.05$.

Results

Oltipraz exacerbates BDL-induced liver damage

Oltipraz treatment had no effect on body weight either in normal animals or when given prior to BDL (Supplementary Fig. 1A). However, oltipraz treatment significantly increased the liver weight in BDL animals compared to CMC-BDL mice (Supplementary Fig. 1B). Liver histology was normal in unoperated mice