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Pterostilbene inhibits dimethylnitrosamine-induced liver fibrosis in rats

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ABSTRACT

Pterostilbene, found in grapes and berries, exhibits pleiotropic effects, including anti-inflammatory, antioxidant, and anti-proliferative activities. This study was conducted to investigate the effect of pterostilbene on liver fibrosis and the potential underlying mechanism for such effect. Sprague–Dawley rats were intraperitoneally given dimethyl n-nitrosamine (DMN) (10 mg/kg) 3 days per week for 4 weeks. Pterostilbene (10 or 20 mg/kg) was administered by oral gavage daily. Liver function, morphology, histochemistry, and fibrotic parameters were examined. Pterostilbene supplementation alleviated the DMN-induced changes in the serum levels of alanine transaminase and aspartate transaminase ($p < 0.05$). Fibrotic status and the activation of hepatic stellate cells were improved upon pterostilbene supplementation as evidenced by histopathological examination as well as the expression of α-smooth muscle actin (α-SMA), transforming growth factor-β1 (TGF-β1), and matrix metalloproteinase 2 (MMP2). These data demonstrated that pterostilbene exhibited hepatoprotective effects on experimental fibrosis, potentially by inhibiting the TGF-β1/Smad signaling.

1. Introduction

Stilbene phytoalexins, such as resveratrol (*trans*-3,4′,5-trihydroxystilbene) and its natural dimethylether analogue, pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene), are plant's defense means in response to environmental stresses, including microbial infection and ultraviolet radiation (Adrian, Jeandet, Douillet-Breuil, Tesson, & Bessis, 2000; Douillet-Breuil, Jeandet, Adrian, & Bessis, 1999). We and others have shown that pterostilbene, mainly from blueberries and grape vines (Szajdek & Borowska, 2008), exhibits pleiotropic pharmacological effects including anti-inflammatory, antioxidant, anti-proliferative, anti-cancer, and pain-relieving activities in cell culture and animal studies (Chiou et al., 2010; Pan et al., 2009; Remsberg et al., 2008). The potential chemopreventive and therapeutic effects of resveratrol in hepatocarcinogenesis has been reviewed (Mann et al., 2009); however, the effect of pterostilbene on earlier stages of liver injury such as fibrosis and cirrhosis has not been studied extensively.

When suffering from chronic injury, liver undergoes wound-healing processes, accumulate extracellular matrix proteins, and consequently, fibrosis or scarring ensues. Multiple etiologies, including viral hepatitis, alcohol intoxication, drug abuse, metabolic disorders due to mineral overload, and autoimmune diseases, attribute to liver fibrosis (Friedman, 2003; Kisseleva & Brenner, 2006), which may further evolve into cirrhosis. Liver cirrhosis often results in high mortality (Friedman, 2003) and is also a risk factor in the development of hepatocellular carcinoma (HCC) (Bataller & Brenner, 2005), which ranks the fifth of the cancer incidence worldwide (Caldwell & Park, 2009).

Hepatic stellate cells (HSC; also known as ito cells or lipocytes) are involved in the fibrogenesis. During liver injury, hepatic stellate cells switch from quiescent, vitamin A-storing epithelial features to activated, vitamin A-losing and α-smooth muscle actin (α-SMA)-expressing myofibroblastic phenotypes (Friedman, 2008b; Gressner, Weiskirchen, & Gressner, 2007). Alpha-smooth muscle actin has been shown as a fairly reliable marker of the activation of the HSC in both experimental and clinical settings (Moreira, 2007). In addition to the morphological changes, HSC also produce excessive extracellular matrix proteins, including, and in particular, collagen type I, (Friedman, 2008a). A wide range of cell types, including hepatocytes and Kupffer cells, in liver, and cytokines such as platelet-derived growth factor (PDGF) and transforming

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growth factor-beta (TGF-β), play important roles during the process of liver injury (Kisseleva & Brenner, 2006; Moreira, 2007). Previously, liver fibrosis and cirrhosis were presumed irreversible responses whereas current thoughts hold otherwise, if the underlying etiology is eradicated (Rossi, Adams, Bulsara, & Jeffrey, 2007; Zhou & Lu, 2009). Therefore, establishing effective anti-fibrotic strategies could ultimately manage liver fibrosis and cirrhosis and provide favourable prognosis for chronic liver diseases (Friedman, 2003).

Dimetyl n-nitrosamine (DMN) is a potent hepatotoxin, which is metabolised by microsomal cytochrome p450IIE1 in liver (Yang, Tu, Koop, & Coon, 1985; Yoo, Guengerich, & Yang, 1988). Administration of DMN can induce liver damage in rats, which mimics the progression of liver fibrosis and cirrhosis in humans (George, Rao, Stern, & Chandrakasan, 2001). Therefore, DMN-treated animal models are widely used to study the biochemical and pathological manifestations of liver injury (Ala-Kokko, Pihlajaniemi, Myers, Kivirikko, & Savolainen, 1987; George & Chandrakasan, 1996; George et al., 2001).

In this study, we investigated the effect of pterostilbene on a well-characterised animal model of DMN-induced liver fibrosis. We found that pterostilbene improved serum parameters of liver function, inhibited the activation of HSC, reduced the expression of α-SMA and collagen I, and alleviated the progression of liver injury, potentially by inhibiting the TGF-β1/Smad-mediated signaling.

2. Materials and methods

2.1. Reagents and chemicals

Pterostilbene was synthesised as described previously (Pan et al., 2009). All reagents and chemicals were purchased from Sigma, Inc. (St. Louis, MO) unless specified otherwise. *N*-Nitrosodimethylamine (dimethyl n-nitrosamine; DMN) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Alpha-SMA and matrix metalloprotease 2 (MMP2) antibodies were obtained from Epitomics, Inc. (Burlingame, CA). Beta-actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). TGF-β1, p-Smad2, pSmad3, and Smad2/3 antibodies were purchased from Transduction Laboratories (BD Biosciences, Lexington, KY).

2.2. Animals and treatment

Thirty-two male Sprague–Dawley rats, weighing 200–250 g, were purchased from the National Laboratory Animal Center (Taipei, Taiwan). All animal experimental protocols used in this study were approved by Institutional Animal Care and Use Committee of the National Kaohsiung Marine University (IACUC, NKMU). The animals were housed in a humidity-controlled room at 25 ± 1 °C with a 12-h dark/light cycle with free access to Laboratory rodent diet 5001 (PMI Nutritional International, Brentwood, MO) and water. After 2-week of acclimation, the animals were randomly assigned into three DMN-treated groups and one control group with eight rats in each group. The DMN-treated animals were administered DMN (10 mg per kg body weight) via intraperitoneal (i.p.) injection on Monday, Wed, and Friday for four consecutive weeks. Control, untreated animals were given an equal volume of normal saline. Two DMN groups were also administered 10 mg and 20 mg pterostilbene per kg body weight, respectively, by oral gavage daily.

At the end of the study period, all animals were killed under CO_2 anesthesia. Blood was collected by cardiac puncture and serum was harvested and stored at −80 °C until analysis. After rinsing with normal saline, the weights of livers, spleens, and kidneys were recorded. The liver samples were either immediately frozen in liquid nitrogen and kept at −80 °C for further analysis or fixed with 10% buffered neutral formalin and embedded in paraffin for histological examination. Liver tissue sections (5 μm thickness) were subjected to Sirius red and immunohistochemistry staining methods for collagen distribution and α-SMA expression, respectively.

2.3. Biochemical analysis of liver function

Liver function was assessed by the serum levels of aspartate transaminase (AST), alanine transaminase (ALT), triacylglycerol (TG), and total cholesterol (T-chol). Briefly, serum was spotted onto respective Fujifilm Dri-Chem slides (Fujifilm, Kanagawa, Japan) and each biochemical indicator was determined using a blood biochemistry analyzer (Fujifilm Dri-Chem 3500s; Fujifilm, Kanagawa, Japan) according to the manufacturer's instructions.

2.4. Tissue protein extraction, Western blot analysis, and detection

Liver tissues were homogenised and total proteins were extracted using gold lysis buffer (20 mM Tris–HCl, pH 7.4; 1 mM NaF; 150 mM NaCl; 1 mM ethylene glycol tetraacetic acid (EGTA); 1 mM phenylmethanesulphonyl fluoride; 1% NP-40; and 10 μg/mL leupeptin). Protein concentrations were measured by Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). Fifty micrograms of protein were mixed with 5× sample buffer (0.3 M Tris–HCl (pH 6.8), 25% 2-mercaptoethanol, 12% sodium dodecyl sulphate (SDS), 25 mM EDTA, 20% glycerol, and 0.1% bromophenol blue), subjected to 10% SDS–polyacrylamide gel electrophoresis, and electrotransferred onto immobile membranes (PVDF; Millipore Corp., Bedford, MA). The membranes were immunoblotted with primary antibodies including α-SMA, MMP2, TGF-β1, p-Smad2, pSmad3, and Smad2/3, and β-actin (Transduction Laboratories, BD Biosciences, Lexington, KY) at room temperature for 1 h. Detection was achieved by measuring the chemiluminescence of blotting agent (ECL, Amersham Corp., Arlington Heights, IL) and analyzed by densitometric scanning (Alliance 4.7, UVItec, Cambridge, UK).

2.5. Statistical analysis

All data were expressed as mean ± SD. All statistical analyses were performed by *Student's* t test using Sigma Plot 10.0. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of pterostilbene on the relative organ weights of the DMN-treated rats

The average body weight of the three DMN-treated groups was significantly lower than that of the control group at the end of the study, whereas no significant difference was observed among the DMN-treated animals with or without pterostilbene supplementation (Fig. 1). In the DMN-alone group, the weight of liver was significantly lower than that of the control group, whereas the weights of kidney and spleen were significantly higher than those of the control group (Table 1). Pterostilbene supplementation (20 mg/kg body weight) significantly attenuated the changes in weights of liver, kidney, and spleen caused by DMN treatment (Table 1).