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Osteopontin is involved in estrogen-mediated protection against diethylnitrosamine-induced liver injury in mice

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ABSTRACT

Diethylnitrosamine (DEN) is a potent hepatotoxin and hepatocarcinogen in animals and possible in humans. Estrogen has been reported to play a protective role against DEN exposure. Osteopontin (OPN), a downstream molecular of estrogen, plays a role in many pathophysiological processes. In this study, we evaluate the role of OPN in estrogen-mediated hepatoprotection in DEN-treated mice. DEN was administrated intraperitoneally to C57BL/6 and *OPN*$^{-/-}$ mice. Compared to male mice, female mice exhibited significantly higher hepatic OPN expression with less liver damage 48 h after DEN treatment. Interestingly, enhanced OPN expression was predominantly detected in hepatocytes after DEN treatment. OPN deficiency enhanced the susceptibility to DEN, which was more apparent in females than males. Estrogen-mediated protection against DEN in males was abrogated by OPN deficiency. The protective activities of estrogen could be mimicked by exogenous OPN. Consistent with liver injury, oxidative stress in liver was enhanced with OPN depletion. OPN reduced DEN-induced oxidative stress likely through inhibition of CYP2A5 expression. In conclusion, we demonstrate that OPN may be involved in estrogen-mediated hepatoprotection in DEN-induced liver injury through enhancement of hepatocyte survival and inhibition of DEN biotransformation. Our findings may provide new insight into gender differences in chemical-induced liver injury and related diseases.

1. Introduction

Diethylnitrosamine (DEN), a nitrosamine compound, is commonly detected in tobacco smoke, food and drinking water (Bartsch and Spiegelhalder, 1996; Boffetta et al., 2008; Verna et al., 1996). DEN can also be generated endogenously from nitrate, nitrite, and secondary amines (Katan, 2009) as well as the metabolism of some therapeutic drugs (Akintonwa, 1985). DEN is a hepatotoxin and hepatocarcinogen in rodents and probably in every species it's been studied. Although no epidemiological studies have specifically evaluated the toxicity of DEN in human, sufficient exposure levels would be expected to cause liver injury and even cancer.

DEN-induced cellular injury is related to enhanced generation of reactive oxygen species (ROS) (Bartsch et al., 1989). The level of 8-hydroxyguanine, which reflects the oxidative damage to DNA, was significantly elevated by DEN in rat liver (Nakae et al., 1997). Free radicals can be detected as early as 1 h after DEN administration in rat liver (Yamada et al., 2006). Moreover, administration of antioxidants has been found to exert protective effects against DEN-induced liver damage (Asamoto et al., 1990; Janani et al., 2009; Shiota et al., 2002). The generation of ROS following DEN exposure is related to its biotransformation by P450 (Bartsch et al., 1989; Gonzalez, 2005). In mouse liver, CYP2A5 and CYP2E1 were found to be the predominant DEN-bioactivating enzymes (Camus et al., 1993). Up to 90% of DEN bioactivation was inhibited by CYP2A5 specific antibody, whereas about 40% of DEN bioactivation was inhibited by antibody against CYP2E1. Robust ROS can induce direct damage to many important cellular components, such

Abbreviations: APAP, acetaminophen; ALT, transaminase; DEN, diethylnitrosamine; DCF-DA, 2′,7′- dichlorodihydrofluorescein diacetate; E2, β-estradiol; ER, estrogen receptor; IHC, immunohistochemistry; GSH, reduced glutathione; LPO, lipid peroxidation; MDA, malondialdehyde; NASH, nonalcoholic steatohepatitis; NKT, Natural killer T cell; OPN, Osteopontin; PPT, propyl-pyrazole-trisphenol; ROS, reactive oxygen species; WT, wild-type.

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as mitochondria, DNA, lipids, and proteins, which result in mitochondria-dependent cell death or activation of cellular signaling cascades that regulate cell proliferation and death (Martindale and Holbrook, 2002; Veal et al., 2007).

DEN administration has been found to induce sex-biased liver injury and tumorigenesis in animal models (Nakatani et al., 2001; Naugler et al., 2007; Verna et al., 1996). The levels of sexual hormone and sexual hormonal receptor have been implicated in this sexual disparity (Kemp et al., 1989; Nakatani et al., 2001; Tejura et al., 1989). This gender difference is mainly due to the protective role of estrogen. Early in 1974, estrogen was found to protect cultured hepatocytes from 7,12-dimethylbenz(a) anthracene- and aflatoxin-induced cytotoxicity (Schwartz, 1974). Administration of estrogen has been shown to protect against DEN and acetaminophen (APAP) induced acute liver damage (Chandrasekaran et al., 2010; Naugler et al., 2007). Nevertheless, the underlying mechanism of estrogen-mediated hepatoprotection against DEN insults is still poorly understood.

Osteopontin (OPN) is a multifunctional protein that is broadly synthesized and identified as a biomarker for a variety of cancers and inflammatory diseases (Denhardt et al., 2001; El-Tanani et al., 2006). Recently, OPN was shown to act as a key stress protein in mechanical stress, oxidative stress and physical stress (Baliga et al., 2011; Ishijima et al., 2007; Wang and Denhardt, 2008). Through interaction with certain integrins and CD44 variants, OPN mediates diverse biological activities, including cell survival, inflammation and proliferation (Rangaswami et al., 2006). In liver, OPN is expressed in different types of liver cells, such as Kupffer cells, hepatic stellate cells and macrophages as well as hepatocytes. The upregulation of OPN expression was observed in various liver injury models, including APAP-induced acute liver injury, ConA-induced hepatitis, nonalcoholic steatohepatitis (NASH), and CCl_4-induced liver injury and fibrosis (Atul Sahai et al., 2004; Diao et al., 2004; Lorena et al., 2006). The distribution of OPN varied with the hepatotoxicant used. In Con A-induced fulminant hepatitis, OPN is mainly synthesized by natural killer T cell (NKT) (Diao et al., 2004). In NASH and CCl_4 mural model, OPN expression was found in hepatocytes. The cell-specific expression of OPN may be closely related to its role in diseases. OPN produced by inflammatory cells was well known as a mediator of hepatic NKT, neutrophil and macrophage recruitment, which was closely involved in inflammatory and toxic hepatic injury (Ramaiah and Rittling, 2008). However, the significance of OPN in hepatocytes is poorly defined.

Here, we use a mouse model to evaluate the role of OPN in estrogen-mediated hepatoprotection in DEN-treated mice. We find that estrogen may enhance OPN production to promote cell survival and eliminate ROS accumulation, which may account for gender difference in DEN-induced liver damage.

2. Materials and methods

2.1. Mice

C57BL/6J mice were purchased from Shanghai Experimental Animal Center of Chinese Academic of Sciences (Shanghai, PR China). *OPN*$^{-/-}$ mice (B6.Cg-Spp1tm1blh/J, cat. No. 004936) were obtained from the Jackson Laboratory. All animals in this study were kept and bred in the Animal Unit of Shanghai Second Military Medical University in environmentally controlled and specific pathogen free conditions.

All animal experimental protocols were approved by and in accordance with the guidelines of the Animal Experiment Committee of the Shanghai Second Military Medical University of the People's Republic of China.

2.2. Animal treatment

Six-week-old mice of both sexes as indicated were injected i.p with DEN (Sigma) (100 mg/kg body weight) and sacrificed at the indicated times. For E2 (Sigma) or propyl-pyrazole-trisphenol (PPT) (Sigma) treatment, the mice received an i.p. injection of 100 μg β-estradiol (E2, Sigma) or indicated doses of PPT diluted in 100 μl corn oil 2 h before DEN administration. Recombinant mouse OPN and mouse mAb 23C3 against human OPN (Fan et al., 2008) were generated in our laboratory. Recombinant mouse OPN was expressed in yeast vector pPICZa and purified for use. Mouse mAb 23C3 showed crossreactivity with mouse OPN (Fan et al., 2008). For OPN treatment, 50 μg of recombinant OPN or PBS was injected i.p for consecutive three days before DEN treatment. For anti-OPN antibody treatment, 200 μg anti-OPN Ab 23C3 or mouse IgG (Sigma) was injected i.p for three times every other day. DEN was coinjected with 23C3 or IgG at the last time. For ovariectomies, six week-old female mice were ovariectomized or sham-operated through a single posterior-approach incision two weeks before DEN treatment (Nakatani et al., 2001).

2.3. Quantification of liver OPN protein

Livers were removed and snap frozen in liquid nitrogen. Frozen livers were homogenized in ice–cold lysis buffer as described (Malato et al., 2008). Supernatant was pooled for OPN qualification using mouse OPN ELISA kit (R&D Systems). Total protein was quantified by bicinchonic acid (BCA) kit (Pierce).

2.4. RNA isolation and quantitative real-time PCR

Total liver RNA was isolated using the Nucleospin RNA (Macherey–Nagel, Germany). First strand synthesis was performed with random primers and reverse transcription with Quant Reverse Transcriptase (Tiangen Biotech, China). Quantitative real-time PCR was performed using SYBR Green reagent in a Light Cycler (Roche, Germany). Reactions were performed twice in triplicate, and Actin values were used to normalize gene expression. Relative expression of genes was calculated and expressed as $2^{-\Delta\Delta CT}$ as described previously (Livak and Schmittgen, 2001). Gene mRNA relative expression of untreated wt mice was indicated as onefold. Gene fold induction was relative to untreated wild-type mice. The primer sequences and PCR conditions were shown in Table S.

2.5. Biochemical analysis

Serum transaminase (ALT) was measured with commercial kits (Nanjing Jiancheng Biotech, China) according to the manufacturer's instructions. For reduced glutathione (GSH) assay, liver tissue was weighed and homogenized in cold phosphate buffer (20 mM, pH 7.2). Homogenized liver was centrifuged at 10,000g at 4 °C for10 min. Supernatant was used for the quantification of GSH level using commercial kit (Nanjing Jiancheng Biotech, China). Total protein in supernatant was quantified by BCA kit (Pierce). GSH level was expressed at microgram per g protein. For malondialdehyde (MDA) analysis, liver tissue was weighed and homogenized in Tris–HCl buffer (20 mM, pH 7.4). MDA in homogenate was measured with commercial kits (Nanjing Jiancheng Biotech, China). MDA content was expressed at micromole per gram liver.

2.6. Histochemical analysis

Formalin-fixed liver tissue was processed and 5-μm-thick paraffin sections were stained with H&E staining. Necrosis was assessed by analyzing H&E stained sections of liver. Apoptosis was assessed by TUNEL staining of paraffin-embedded slides with the TUNEL Detection Kit (Calbiochemicon). The expression of OPN protein in mouse liver was detected by immunohistochemistry (IHC) assay with anti-OPN mAb 23C3. For antibody control, one set of samples was incubated with non-immune mouse IgG (1:200) instead of primary antibody. To examine the accumulation of superoxide anions, freshly prepared frozen sections were incubated with 5 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA, Sigma) for 30 min at 37 °C, after which they were observed by fluorescent microscopy and photographed (Sakurai et al., 2008). Nuclei were visualized by DAPI staining.

2.7. Statistical analysis

Data expressed are means ± SE. Differences were analyzed by the Student's *t* test, and *P* values <0.05 were considered significant.

3. Results

3.1. OPN expression is enhanced in liver after DEN treatment

To investigate the relationship between OPN expression and liver injury, serum ALT, systemic and hepatic OPN protein were analyzed at different time points after DEN administration. Consistent with previous reports (Naugler et al., 2007), wild-type (WT) male mice exhibited significantly higher serum ALT than females 48 h after DEN treatment (Fig. 1A). Hepatic OPN protein was increased in both sexes after DEN treatment. However, females