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Apocynin reduced doxycycline-induced acute liver injury in ovariectomized mice

Satoru Mitazaki [a], Midori Hashimoto [a], Yui Matsuhashi [a], Shigeyoshi Honma [b], Miwako Suto [c], Naho Kato [c], Kouichi Hiraiwa [c], Makoto Yoshida [b], Sumiko Abe [a,*]

[a] Laboratory of Forensic Toxicology, Faculty of Pharmacy, Takasaki University of Health and Welfare, 60 Nakaorui-machi, Takasaki 370-0033, Japan
[b] Laboratory of Pathophysiology, Faculty of Pharmacy, Takasaki University of Health and Welfare, 60 Nakaorui-machi, Takasaki 370-0033, Japan
[c] Department of Legal Medicine, Fukushima Medical University, 1 Hikarigaoka, Fukushima 960-1295, Japan

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ABSTRACT

To determine the physiological role of estrogen in the development of liver injury, we examined the sensitivities of sham and ovariectomy (ovx) mice against doxycycline (DOXY)-induced acute liver injury. Ovx or sham operation was performed in C57BL/6J wild-type female mice of eight weeks of age. Sham mice and ovx mice were treated with DOXY (240 mg/kg ip) 8 weeks after the operation, 30 min after apocynin (5 mg/kg) or saline administration. Blood and liver samples were obtained at 3 and 6 h after DOXY administration. Liver dysfunction occurred soon after DOXY administration and became more severe in ovx mice than in sham mice. At early phase after DOXY injection, TNF-α and iNOS inductions upregulated almost the same levels in sham and ovx mice. On the other hand, expression levels of IL-6, IL-10, c-fos, cox-2 and HO-1, downstream genes of TNF-α, were significantly increased in ovx mice compared to those in sham mice, correlated with liver dysfunction. In addition, apocynin, a NADPH oxidase (Nox) inhibitor, totally improved DOXY-induced liver injury in both sham and ovx mice, indicating that reactive oxygen species generated through Nox activation by DOXY are responsible for development of acute liver injury.

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1. Introduction

Liver tissue has great potential for inducing local and/or systemic inflammatory responses [2] and appears to be a preferred source for and target of cytokine signaling. In fact, hepatic failure might represent the collapse of hepatic homeostasis as a result of an imbalance between damaging and protective signals that are very tightly regulated under physiological conditions [16,30]. Estrogen and its derivatives are powerful endogenous antioxidant agents that are able to reduce lipid peroxide levels in the liver and blood [22]. Most of the evidence of estrogen's role in liver metabolism and inflammation has been obtained by measurements of enzyme expression in ovariectomized (ovx) or aromatase-deficient animals. Ovx mice have been used as estrogen-deficient models that reflect pathologic changes in peri- or post-menopausal women [20,23]. Doxycycline (DOXY), one of the tetracycline-derived compounds, is known to induce acute liver failure (ALF) [25,14]. Thus, we established a DOXY-induced ALF model in sham- and ovx-operated mice to evaluate the role of estrogen during the development of liver injury.

Interleukin-6 (IL-6) is a cytokine that provokes a broad range of cellular and physiological responses, including immune response, inflammation, hematopoiesis, and oncogenesis, by regulating cell growth, gene activation, proliferation, survival, and differentiation. IL-6 activates the phosphorylation of JAK kinases, signal transducers and activators of transcription-3 (STAT3) and Src homology-2 [9,10]. We previously reported that IL-6 induction was observed during the development of cisplatin-induced acute renal failure (ARF) in wild-type (WT) mice and that predominant progression of renal dysfunction in IL-6 knockout (IL-6$^{-/-}$) mice was also observed at the early stage, indicating that IL-6 plays a protective role in the progress of ARF [17–19]. We also reported that IL-6 deficiency results in an increase of oxidative stress caused by a decrease of superoxide dismutase (SOD; an anti-oxidative enzyme) activity and over-expressed 4-hydroxy-2-nonenal protein (an

Abbreviations: Ovx, ovariectomy; DOXY, doxycycline; ALF, acute liver failure; ARF, acute renal failure; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; STAT3, signal transducers and activators of transcription-3; SOD, superoxide dismutase; Nox, NADPH oxidase; ROS, reactive oxygen species; ALT, alanine aminotransferase; iNOS, inducible nitric oxide synthase; cox-2, cyclooxygenase-2; HO-1, heme oxygenase-1.

* Corresponding author. Fax: +81 27 352 1118.
E-mail address: sabe@takasaki-u.ac.jp (S. Abe).

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oxidative stress marker) in proximal tubular epithelial cells during cisplatin-induced ARF, indicating that IL-6 modulates the generation of oxidative stress and plays a protective role against oxidative stress evoked by cisplatin administration [18].

TNF-α is a potent proinflammatory cytokine that is primarily secreted from monocytes and macrophases in response to inflammation, infection, and other environmental stresses.

Recent studies have shown that levels of oxidative stress due to reactive oxygen species (ROS) were increased and that levels of enzymatic antioxidants were decreased in ovx mice. NADPH oxidase (Nox) is an enzyme catalyzing the univalent reduction of oxygen to produce the superoxide anion radical, which in turn can be converted into other ROS [4]. Apocynin (4-hydroxy-3-methoxy-acetophenone) is an inhibitor of the intracellular translocation of two critical cytosolic components of the Nox complex present in the cell membrane, which results in the inhibition of peroxynitrite ($ONOO^-$) formation. Apocynin has also been reported to interrupt activation of redox-sensitive transcription factors [12]. Therefore, we hypothesized that apocynin attenuates DOXY-induced ALF.

The aim of the present study was to elucidate the role of estrogen in and the effect of apocynin on the development of DOXY-induced ALF by determining various physiological parameters and expression of genes related to inflammation and oxidative stress in sham and ovx mice.

2. Materials and methods

2.1. Animals and reagents

Eight-week-old female wild-type C57BL/6J mice (CLEA Japan, Inc., Japan) weighing 15–20 g were used for all of the mouse studies. All animal experiments in this study were approved by the Ethics Committee of Animal Experiments in accordance with the Guidelines for Animal Experiments of Takasaki University of Health and Welfare and the Japanese Government Animal Protection and Management Law. Efforts were made to minimize suffering and to reduce the number of animals used. Mice were maintained on a standard diet and water was freely available. Mice were housed 2–5 per cage under a 12-h light and dark schedule for at least 1 week before surgery. Doxycycline (DOXY, MP Biomedicals, LLC, USA) was freshly prepared in sterile saline adjusted to pH 6 at a concentration of 24 mg/ml. Apocynin (Sigma–Aldrich, Japan) was also freshly prepared in sterile saline at a concentration of 0.5 mg/ml. An ovariectomy (ovx) or sham operation was performed in C57BL/6J female mice at 8 weeks of age and maintained on a standard diet. Eight weeks after surgery, mice were given either apocynin (5 mg/kg) or saline ip 30 min before DOXY (240 mg/kg) or saline ip administration. This single dose of DOXY produced severe liver injury and different survival rates in sham and ovx mice. Under deep anesthesia with Nembutal (Dainippon Sumitomo Pharma, Japan), blood samples were collected via cardiac puncture and kidneys were removed 3 and 6 h after DOXY administration (n = 4–17 per time point).

2.2. Determination of blood alanine aminotransferase and IL-6

Liver function was assessed by determination of blood alanine aminotransferase (ALT) using ALT Test Wako (Wako Pure Chemical Industries, Ltd., Japan). Blood IL-6 levels were determined using an ELISA kit (Invitrogen Corp., USA) according to the manufacturer's instructions. All samples were measured as triplicates and the mean was calculated for data analysis. A calibration curve and a negative control (a blank well without plasma) were run for each test plate.

2.3. Histology

Liver samples were fixed in 10% phosphate-buffered saline and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin for histopathological analysis. Histopathological evaluation was performed with a quantitative method. Assessment of tissue alterations in 5 different fields for each section examined under a Lyca DFC280 light microscope by Leica Q Win and Image Analysis System (Leica Micros Imaging Solutions Ltd., Cambridge, U.K.). The area consist of normal hepatocytes were calculated in each samples. Representative images from 2–4 mice/group were selected.

2.4. Real-time RT-PCR

Total RNA was obtained from kidney homogenate using TRIZOL Reagent (Invitrogen Corp., USA). RNA extract was treated with DNase using a DNase treatment kit (Takara Bio, Japan) prior to RT-PCR amplification. The expression levels of selected genes were quantified by real-time RT-PCR using an MX-3000P (Agilent Inc., USA). Briefly, total RNA was reverse-transcribed with SuperScript III reverse transcriptase and oligo-dT primers (Invitrogen Corp., USA). SYBR Premix Ex Taq II (Takara Bio. Japan) was used for real-time PCR analysis. The forward and reverse primer sequences for selected genes previously reported [17–19] are listed in Table 1. The relative quantity (RQ) value ($2^{-\Delta\Delta Ct}$, where Ct is the threshold cycle) normalized to GAPDH amplified was calculated for each sample. RQ values were calculated as fold change in gene expression relative to the control group.

2.5. Statistical analyses

Survival data were compared using the logrank test. Comparisons between sham and ovx groups were performed using analysis of variance (ANOVA) followed by the Dunn-Bonferroni test. Significance was defined as $p < 0.05$.

Table 1
Primer sequences used in real-time PCR.

Gene	Forward	Reverse	References
TNFα	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC	[21]
iNOS	GGCAGCCTGTGAGACCTTTG	GCATTGGAAGTGAAGCGTTTC	NM0109027
c-fos	CAACGCCGACTACGAGGCGTCAT	GTGGAGATGGCTGTCACCG	[7]
IL-6	TCCAGTTGCCTTCTTGGGAC	GTGTAATTAAGCCTCCGACTTG	[11]
IL-10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT	NM0105482
Cox-2	TTTGTTGAGTCATTCACCAGACAGAT	AGGATGTGTAAGGTTTCAGGGAGAAG	[8]
SOD1	CCTGGGCAATGTGACTGCTG	CAATCACTCCACAGGCCAAG	[5]
SOD2	AACTCAGGTCGCTCTTCAGC	GAACCTTGGACTCCCACAGA	[15]
HO-1	CCTCACTGGCAGGAAATCATC	CCTCGTGGAGACGCTTTACATA	[19]
GAPDH	TTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA	[8]

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