Research Article

Inflammatory regulation of steroid sulfatase: A novel mechanism to control estrogen homeostasis and inflammation in chronic liver disease

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Background & Aims: Chronic inflammatory liver diseases are associated with estrogen excess and feminization in men, which is thought to be due to compromised liver function to break down estrogens. The goal of this study is to determine whether the inflammatory induction of steroid sulfatase (STS), which converts inactive estrogen sulfates to active estrogens, may have contributed to the estrogen excess in chronic liver disease.

Methods: We performed bioinformatic analysis, real-time PCR, immunohistochemistry, and UPLC/MS-MS to analyze hepatic STS expression and serum estrogen levels in patients with chronic liver diseases. The crosstalk between NF-κB pathway and STS-regulated estrogen signaling was investigated by electrophoretic mobility shift assay, chromatin immunoprecipitation, luciferase assay and gene knockdown experiments in human hepatocytes.

Results: Hepatic STS was induced in patients with chronic inflammatory liver diseases, which was accompanied by increased circulating estrogen levels. The human *STS* gene, but not the mouse *Sts* gene, was induced by inflammatory stimuli in hepatic cells. Mechanistically, STS was established as a novel NF-κB target gene, whose induction facilitated the conversion of inactive estrogen sulfates to active estrogens, and consequently attenuated the inflammatory response. In contrast, genetic or pharmacological inhibition of STS or a direct blockade of estrogen signaling sensitized liver cells to the transcriptional activation of NF-κB and inflammatory response, possibly through the inhibition of IκB kinase activation.

Conclusions: Our results suggest a negative feedback loop in chronic inflammatory liver diseases, in which the inflammatory activation of NF-κB induces *STS* gene expression. The induced STS facilitates the conversion of inactive estrogen sulfates to active estrogens, which in return attenuates the NF-κB-mediated inflammation.

Keywords: Steroid sulfatase; Estrogens; Estrogen metabolism; Inflammation; Liver disease.

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Abbreviations: ChIP, chromatin immunoprecipitation; CRP, C-reactive protein; CYP, cytochrome P450; E_2, estradiol; E_2S, estradiol sulfate; EMSA, electrophoretic mobility shift assay; ER, estrogen receptor; EST, estrogen sulfotransferase; FBS, fetal bovine serum; HPH, human primary hepatocytes; ICI, ICI 182,780; IHC, immunohistochemistry; IL-8, interleukin-8; IKK, IκB kinase; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; PDTC, pyrrolidine dithiocarbamate; PMA, phorbol 12-myristate 13-acetate; STS, steroid sulfatase; STX, STX64; TFF1, trefoil factor 1; TNFα, tumor necrosis factor α; VCAM, vascular cell adhesion molecule; VEH, vehicle; WT, wild-type; XBP1, X-box binding protein 1.

Introduction

Abnormal estrogen metabolism in liver disease has been long recognized in the clinic. Concomitants of liver diseases are clinical signs and symptoms like palmar erythema, spider nevus, gynecomastia, and infertility due to disturbed homeostasis of steroid hormones, especially the estrogens. Studies have reported increased estrogen levels and signs of endocrine disturbance in patients with chronic liver diseases [1]. The hormone levels are positively correlated to the severity of the liver disease [2], whereas treating patients towards improved liver function resulted in regression of endocrine disturbance [3]. The liver is the primary site of estrogen metabolism through phase I oxidation reactions, which are mainly catalyzed by CYP1A2 and

CYP3A4 [4], and phase II conjugation reactions mediated by the estrogen sulfotransferase (EST) [5]. It is thought that damage to the liver impairs its capacity to metabolize and inactivate estrogens, resulting in increased estrogen levels in the circulation [6]. However, there have been reports that changes in steroid hormone levels may occur before the liver functions are compromised [7], suggesting additional mechanisms by which liver disease causes estrogen excess. Although estrogens are known to be the anti-inflammatory hormones, it is unclear whether the estrogen excess can affect the clinical outcome of the underlying liver diseases.

Estrogen sulfation and desulfation represent an important and unique mechanism to control estrogen homeostasis by a reversible metabolic process of conjugation and deconjugation, rather than the destruction of estrogens [8]. Estrogens can be sulfated and deactivated by several sulfotransferases such as EST, SULT1A1 and SULT2A1, with EST being the primary estrogen sulfotransferase at the physiological concentrations [5]. Unlike estrogens, estrogen sulfates cannot bind to the estrogen receptor (ER) and thus are biologically inactive; but they have higher concentrations and prolonged half-life in the circulation, acting as a reservoir for regenerating active estrogens through the STS-mediated desulfation reaction [9]. STS is believed to be the only enzyme responsible for the desulfation of estrogen sulfates.

Consistent with the role of STS in hormonal homeostasis, *STS* gene deletion or mutation is associated with reproductive manifestations, such as cryptorchidism in males and failed labor progression in females due to disrupted steroid hormone homeostasis [10]. High expression of STS is detected in malignant breast tissues and predicts poor prognosis [11], suggesting an important function of STS in enhancing local estrogen signaling and promoting the development of hormone-dependent breast cancer. Cytokines have been suggested to regulate the expression and activity of STS, but the results have been contradicting. Interleukin (IL)-1 decreased the expression and activity of STS in endometrial stromal cells [12]. However, IL-6 and tumor necrosis factor α (TNFα) were reported to increase STS activity in breast cancer cells, probably through post-translational mechanisms [13]. It is possible that the effects of cytokines on the expression of STS depend on the cellular context. Nevertheless, little is known about the transcriptional regulation of STS, especially in the liver, the major estrogen-metabolizing organ.

The development of many chronic inflammatory liver diseases is more common in men than in women. The prognosis of hepatocellular carcinoma is also worse for male than for female patients [14]. These gender differences may be accounted for by sex hormones. Although it is not a classic target organ of sex steroid hormones, the liver has been shown to express functional ER and respond to estrogen stimulation. Since estrogens are known for their anti-inflammatory activities [15], they may provide benefit in inhibiting the progression of chronic inflammatory liver diseases.

In this study, we showed that STS is a novel NF-κB target gene that is induced in the liver of patients with hepatitis and cirrhosis. Our data strongly suggest that the inflammatory induction of STS may have contributed to the estrogen excess in chronic liver disease. Our results also suggested an STS-mediated negative feedback loop to inhibit inflammation, in which the NF-κB responsive activation of STS increases active estrogen levels, which in turn attenuates the NF-κB-mediated inflammation.

Materials and methods

Microarray and bioinformatic analysis

We previously performed microarray analyses [16] on liver samples from human subjects with normal (<10 mg/L) or elevated (>10 mg/L) C-reactive protein (CRP) levels. The data have been deposited in the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) database (GSE32504). The microarray results were verified by TaqMan real-time PCR using the following predesigned TaqMan assays obtained from Life Technologies (Carlsbad, CA): Hs00996676_m1 (STS); Hs00960941_m1 (SULT1E1), and Hs00174103_m1 (IL8). A standard curve was generated for each assay using pooled cDNA from the liver sample collections. With the help of the standard curve, a quantitative measure was calculated which was normalized on the housekeeping gene *RPLP0*. The Gene Expression Omnibus database was also mined for *STS* expression in subjects with chronic inflammatory liver disease. The GSE28619 dataset, which contained 15 liver samples from alcoholic hepatitis patients and 9 control liver samples, was analyzed on the Affymetrix Human Genome U133 Plus 2.0 microarrays.

Immunohistochemistry (IHC), real-time PCR analysis, and Western blot

For IHC analysis, we used a commercial tissue microarray and archived paraffin sections from West China Hospital, Sichuan University, China. The tissue microarray slides of human liver diseases (Cat #LV1201) were purchased from US Biomax (Rockville, MD). The slides were provided in a single core per case with clinical information of sex, age, and pathological diagnosis. Samples contained on the tissue microarray were from autopsy and surgical resection, according to the information provided by the vendor (http://www.biomax.us/faq.php). The tissue microarray contains 66 liver cases, including 22 cases of hepatitis, 30 cases of cirrhosis and 14 cases of controls. One of the cirrhosis cases was excluded because of the lack of hepatocytes. Additional liver tissue sections from 20 patients, including 10 cases of control liver samples and 10 cases of chronic hepatitis liver samples, were collected from surgical resection at West China Hospital, Sichuan University, China. Informed written consent was obtained from each patient, and the Ethics Committee of the West China Hospital approved the study. The IHC staining was performed using monoclonal anti-STS antibody (dilution 1:50) purchased from Abcam (Cambridge, MA) following the heat-induced antigen-retrieval procedures. The stained slides were evaluated by a surgical pathologist in a blinded fashion and scored according to the staining intensity.

Real-time PCR analyses were performed on the GSE32504 samples, as well as samples derived from the primary human hepatocytes and the human hepatoma HuH7 cells. For real-time PCR analysis, cDNA was synthesized from total RNA by reverse transcription with random hexamer primers and Superscript RT III enzyme from Invitrogen. SYBR Green-based real-time PCR was performed with the ABI 7300 real-time PCR system. Data were normalized against the *GAPDH*.

All Western blot analyses were performed on primary human hepatocytes or HuH7 cells. For Western blotting, the anti-STS antibody was used at 1:200 dilution. The anti-IKKα (3G12), anti-IKKβ (D30C6), anti-Phospho-IKKα (Ser176)/IKKβ (Ser177) (C84E11) and anti-p65 (D14E12) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Quantification of the Western blots was performed using the NIH Image J software.

Human serum samples and estrogen measurement

Patients with alcoholic cirrhosis were eligible for recruitment from University of Louisville Hospitals between January 2013 and November 2014. Blood was drawn from the cubital vein of subjects at fasting state. The serum samples were collected at the time of blood collection and stored at −80 °C before analysis. A control group of serum samples were collected concurrently from healthy patients undergoing screening colonoscopy that corresponded to patients by gender and age. We collected six alcoholic cirrhosis samples and three control samples from male subjects. Informed consent was obtained from all participants, and the study was approved by the Institutional Review Boards at the University of Louisville. To measure the level of serum estrogen and estrogen metabolite, UPLC/MS-MS were carried out with a Waters Acquity UPLC system connected with the Xevo TQ triple quadrupole mass spectrometer as we have previously described [17].

Cell culture and drug treatment

Human primary hepatocytes were obtained through the Liver Tissue Procurement and Distribution System (Pittsburgh, PA). The HuH7 and HEK 293 cell lines were obtained from the American Type Culture Collection (Manassas, VA). In