Journal of Pharmaceutical Sciences xxx (2016) 1–11

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Pharmacokinetics, Pharmacodynamics and Drug Transport and Metabolism

Localizatoin of Xenobiotic Transporter OCTN1/SLC22A4 in Hepatic Stellate Cells and Its Protective Role in Liver Fibrosis

Yaliang Tang [1], Yusuke Masuo [1], Yoshio Sakai [2], Tomohiko Wakayama [2], Tomoko Sugiura [1], Ryuichi Harada [1], Azusa Futatsugi [1], Takuya Komura [2], Noritaka Nakamichi [1], Hirotaka Sekiguchi [3,4], Keita Sutoh [3,4], Koji Usumi [3], Shoichi Iseki [2], Shuichi Kaneko [2], Yukio Kato [1,*]

[1] *Faculty of Pharmacy, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa 920-1192, Japan*
[2] *School of Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa 920-1192, Japan*
[3] *Life Science Institute Co. Ltd., Chuo-ku, Tokyo 103-0012, Japan*
[4] *Customer Support Division, L·S Corporation Co. Ltd., Chuo-ku, Tokyo 103-0013, Japan*

ARTICLE INFO

Article history:
Received 6 October 2015
Revised 6 December 2015
Accepted 15 December 2015

Keywords:
transporters
membrane transporter
organic cation transporters (OCT)
antioxidants
induction

ABSTRACT

Xenobiotic transporters play key roles in disposition of a certain therapeutic agents although limited information is available on their roles other than pharmacokinetic issues. Here, suppressive effect of multispecific organic cation transporter OCTN1/SLC22A4 on liver fibrosis was proposed in liver injury models. After injection of hepatotoxins such as dimethylnitrosamine (DMN) or concanavalin A, hepatic fibrosis, and oxidative stress, evaluated in terms of Sirius red and 4-hydroxy-2-nonenal staining, respectively, were more severe in liver of *octn1/slc22a4* gene knockout (*octn1*$^{-/-}$) mice than that in wild-type mice. DMN treatment markedly increased α-smooth muscle actin and F4/80, markers of activated stellate and Kupffer cells, respectively, in liver of *octn1*$^{-/-}$, but had less effect in wild-type mice. Thus, *octn1/slc22a4* gene deletion results in more sever hepatic fibrosis, oxidative stress, and inflammation. DMN-treated wild-type mice showed increased Octn1 staining and hepatic concentration of its food-derived antioxidant ergothioneine (ERGO). The upregulated Octn1 was colocalized with α-smooth muscle actin. Functional expression of Octn1 was demonstrated in activated human hepatic stellate cell lines, LI90 and LX-2. Provision of ERGO-rich feed ameliorated DMN-induced liver fibrosis and oxidative stress. Overall, Octn1 is upregulated in activated stellate cells, resulting in increased delivery of its substrate antioxidant ERGO and has protective effect against liver fibrosis.

Introduction

Liver fibrosis is a complex process that includes apoptosis of hepatocytes, infiltration of inflammatory cells, induction of inflammatory cytokines, and proliferation of extracellular matrix (ECM)–producing nonparenchymal cells (NPCs).[1,2] The resultant enhancement of ECM production can disrupt the balance between synthesis and degradation of ECM, progressively worsening the fibrosis. Liver fibrosis can silently progress, leading to liver cirrhosis and hepatocellular carcinoma and is observed in various chronic hepatic diseases that develop after inflammatory stimuli such as viral infection, alcohol, drugs, and nonalcoholic steatohepatitis (NASH).[3,4] Notwithstanding the pivotal role of fibrosis in hepatic diseases, few therapeutic agents that can directly suppress hepatic fibrosis are available.

Solute carrier SLC22A4 was first identified as carnitine/organic cation transporter 1 (OCTN1) in human fetal liver but is expressed ubiquitously in organs.[5,6] OCTN1/SLC22A4 is multispecific transporter which accepts various types of therapeutic agents as substrates.[5,6] Metabolome analysis using cell lines transfected with *SLC22A4* gene *in vitro* and *octn1/slc22a4* gene knockout (*octn1*$^{-/-}$)

Abbreviations used: SLC, solute carrier; ECM, extracellular matrix; NPC, nonparenchymal cells; NASH, nonalcoholic steatohepatitis; HSCs, hepatic stellate cells; OCTN1, carnitine/organic cation transporter 1; ERGO, ergothioneine; DMN, dimethylnitrosamine; ConA, concanavalin A; α-SMA, alpha-smooth muscle actin; 4HNE, 4-hydroxy-2-nonenal; [^{3}H]ERGO, [^{3}H]Ergothioneine; ERGO-d9, Deuterium-labeled L-ergothioneine; HFD, high-fat diet; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NOX, NADPH oxidase; siControl, control siRNA; FBS, fetal bovine serum; TNF, tumor necrosis factor 1; TGF, transforming growth factor.
Conflicts of interest: The authors report no conflict of interest.
This article contains supplementary material available from the authors by request or via the Internet at http://dx.doi.org/10.1016/j.xphs.2016.02.023.

* *Correspondence to:* Yukio Kato (Telephone/Fax: +81-76-234-4465).
E-mail address: ykato@p.kanazawa-u.ac.jp (Y. Kato).

http://dx.doi.org/10.1016/j.xphs.2016.02.023
0022-3549/

mice *in vivo* have demonstrated that OCTN1 most efficiently transports a naturally occurring food-derived antioxidant, ergothioneine (ERGO).[7,8] ERGO is highly hydrophilic, and its uptake through plasma membranes is primarily mediated by OCTN1, as evidenced by the fact that food-derived ERGO is detected at μM to sub-mM level in almost all organs in wild-type mice but minimally detected in *octn1*$^{-/-}$ mice.[8] After oral ingestion, ERGO is efficiently absorbed in the gastrointestinal tract by OCTN1, then almost completely taken up by the liver and apparently not metabolized in the body.[8,9] Thus, ERGO is mainly delivered to the liver after oral ingestion. Interestingly, ERGO is minimally taken up into liver parenchymal cells but selectively taken up into NPCs.[9] This is consistent with the minimal immunoreactivity of hepatocytes with OCTN1 antibody.[9] Thus, OCTN1 could be unique drug transporter expressed in NPCs in the liver.

Hepatic NPCs contribute to production and secretion of multiple cytokines and inflammation-related substances and are constantly exposed to oxidative stress.[10,11] In particular, hepatic stellate cells (HSCs) are activated by oxidative stress to collagen-producing myofibroblasts, which promote liver fibrosis by producing ECM.[12,13] Oxidative stress may also injure parenchymal cells, leading to further progression of liver injury. It was recently reported that antioxidant and radical-scavenging enzymes can ameliorate hepatic fibrosis and injuries.[14,15] Thus, proper control of the oxidative stress-antioxidant balance may inhibit the onset and/or progression of hepatic fibrosis.

Because ERGO is a stable antioxidant that can directly scavenge reactive oxygen species,[16,17] it may have a protective role against oxidative stress after its uptake into NPCs. Nevertheless, pathophysiological role of ERGO and its transporter OCTN1 is minimally understood. Information on localization and function of the drug transporter in disease-related cells such as NPCs may give an insight in novel drug delivery and pharmacotherapeutics. The purpose of the present study is to test the hypothesis that OCTN1 plays a role in suppression of oxidative stress and hepatic fibrosis by mediating delivery of its food-derived antioxidant substrate ERGO into hepatic NPCs. To examine this hypothesis, hepatic fibrosis models were constructed by 2 different hepatotoxins, dimethylnitrosamine (DMN), and concanavalin A (ConA) in wild-type and *octn1*$^{-/-}$ mice. DMN is widely used to produce model of hepatic fibrosis because the model can reproduce most of the features of human hepatic fibrosis.[18-20] On the other hand, immunological responses are involved in pathogenesis of hepatic fibrosis in human, and administration of ConA leads to activation of immune system, resulting in hepatic fibrosis, which reproduces one of major pathology in human.[21,22] NASH model was also constructed to evaluate OCTN1 expression during chronic liver injury.

Materials and Methods

Materials

DMN and ConA were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Sigma-Aldrich, Inc (St. Louis, MO), respectively. N-morpholinosydnonimine hydrochloride was purchased from Dojindo Laboratories (Kumamoto, Japan). Mouse monoclonal antibodies against human α-smooth muscle actin (α-SMA) and 4-hydroxy-2-nonenal (4HNE) and rat monoclonal antibodies against F4/80 were obtained from Dako North America Inc (Carpinteria, CA), Wako Pure Chemical Industries and Abcam Inc (Cambridge, MA), respectively. [^{3}H]Ergothioneine ([^{3}H]ERGO; 293 Ci/mol) was purchased from Moravek Biochemicals (Brea, CA), diluted with transport buffer and stored at −30°C. Its quality was verified by checking the difference in the uptake between HEK293 cells stably transfected with *SLC22A4* gene and vector alone. Deuterium-labeled L-ergothioneine (ERGO-d9) was obtained from tetrahedron (Vincennes, France). All other chemicals were commercial products of analytical grade.

Animals

Mice were used for all experiments at 6-8 weeks of age. The *octn1*$^{-/-}$ mice[8] and littermates were of a mixed genetic background (C57BL/6J and 129Sv/Ev), produced by intercrossing *octn1*$^{+/-}$ mice. They had free access to food (Sankyo-labo, Toyama, Japan), which contained approximately 0.2 μg ERGO/g chaw and water, except during the oral administration study of ERGO-rich feed. The present study was carried out in accordance with the guide for the care and use of laboratory animals in Kanazawa University.

Models of Liver Fibrosis

Liver fibrosis was induced in female mice by intraperitoneal administration of DMN at 10 μg/g body weight (1% wt/vol dissolved in saline solution) for the first 3 consecutive days of the week under light ether anesthesia as described previously.[23] The ConA-induced hepatitis model was constructed in male mice by injection of ConA (20 μg/g body weight) dissolved in PBS via the jugular vein once a week for 2 weeks.[24] Control mice received an equal volume of vehicle only. To develop NASH model, male mice were started at 7 weeks of age on an atherogenic high-fat diet (HFD) composed of cocoa butter, cholesterol, cholate, and corticotropin-releasing factor-1 (Oriental Yeast Co, Tokyo, Japan) for 14, 24, or 36 weeks.

Oral Administration of ERGO-Rich Feed

Powdered extract of golden oyster mushroom containing 1% wt/wt ERGO (aminothioneine®, L·S Corporation, Tokyo, Japan) and ERGO-free feed (basal diet®, TestDiet, St. Louis, MO) which contains less than 0.01 μg ERGO/g chaw according to our liquid chromatography-tandem mass spectrometry (LC-MS/MS) determination. These were mixed in the ratio of 95:5 to prepare ERGO-rich diet containing 0.05% wt/wt of ERGO. C57BL/6J female mice were fed with ERGO-free or ERGO-rich diet for 8 weeks, and DMN treatment was performed in the final week. Mice were then sacrificed, and liver was excised.

Histology and Immunohistochemistry

The liver samples were harvested from mice and immersion-fixed overnight in PBS containing 4% paraformaldehyde. The fixed liver was sequentially cryoprotected in 10%, 20%, and 30% sucrose in PBS, embedded in optimal cutting temperature compound and frozen at −30°C. Free-floating sections were cut at 10 μm using a Leica cryostat. Tissue sections were stained with picro-Sirius Red solution (0.1% Direct Red 80 dissolved in saturated picric acid) to visualize collagen deposition. The staining signals were visualized using a BIOREVO BZ-9000 microscope (Keyence, Osaka, Japan) and quantified using dynamic cell count BZ-H1C software (Keyence). For quantification, the number of pixels showing the red color of stained collagen fibers was extracted after establishing a color threshold, and the percentage of those fibers in the liver was calculated by dividing the total red-colored area by the total area of the liver. Frozen sections were also heated at 92°C for 15 min in 20-mM Tris buffer (pH 9.0) for antigen activation. After successive pretreatments with 0.3% Tween 20/PBS for 20 min, 0.3% H_2O_2/PBS for 30 min and blocking solution (1% bovine serum albumin, 5% skim milk and 1.5% goat serum in PBS) for 30 min, the sections were incubated overnight with anti-OCTN1, anti-α-SMA, anti-F4/80, or 4HNE antibody at 4°C, followed by washing with PBS and further