Chondroitin sulfate mediates liver responses to injury induced by dual endothelin receptor inhibition

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Chondroitin sulfate mediates liver responses to injury induced by dual endothelin receptor inhibition

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Abstract

Although dual endothelin receptor antagonists (ERAs) show great promise for treating various conditions, their propensity to induce liver injury limits their clinical usage. Inflammation and fibrosis are important processes in liver responses to injury and it has been suggested that they and dual ERA-induced liver injury are mediated by the proteoglycan component chondroitin sulfate (CS), which is synthesized by CHST3 and CHST13. In this study we investigated whether dual ER inhibition in the liver could alter CHST3 and CHST13 expression and thus CS production, and whether liver CS content could prevent inflammatory and fibrosis responses after liver injury. We observed increased CHST3 and CHST13 expression after liver injury in bile duct-ligated mice and histologically confirmed abundant CS deposition in the injured liver. Moreover, treating Hep3B cells with a dual ERA mimic significantly increased CHST3 and CHST13 expression, inflammatory cytokine levels, and glycosaminoglycan deposition. Furthermore, pro-inflammatory and pro-fibrotic markers were observed after dual ERA treatment, while treatment with CS-degrading chondroitinase ABC was able to successfully reverse these phenotypes. These observations suggest that CHST3 and CHST13-induced CS production can mediate liver injury responses caused by dual ER inhibition, thus could be an alternative pathway for treating ERA-induced liver injury.
Keywords: endothelin, endothelin receptor antagonists, liver injury, chondroitin sulfate, inflammation, fibrosis, chondroitinase ABC
INTRODUCTION

The vasoconstrictor, endothelin (ET), exerts a variety of functions in addition to its main role; thus, many studies have investigated ET receptor antagonists (ERAs), as therapeutic agents (Maguire and Davenport 2015; Masaki 2004). Indeed, the blockade of the canonical ET receptor A (ET\textsubscript{A}) and ET receptor B (ET\textsubscript{B}) have been studied in both experimental and clinical trial studies in the hope that ERAs may treat various pathological conditions. Unfortunately, ERAs are associated with too many clinical problems to be administered freely in diverse disease populations; therefore, they are currently only approved to treat select diseases, chiefly pulmonary arterial hypertension (PAH) (Correale et al. 2018; Maguire and Davenport 2015).

One of the main drawbacks of ERAs is their hepatotoxic tendency, particularly when both ET\textsubscript{A} and ET\textsubscript{B} are blocked, as in the case of bosentan usage (Hoeper 2009). Although bosentan is one of the most readily available ERAs worldwide and is widely used as a first-line treatment for PAH, elevated liver enzyme levels are not uncommon after its long-term usage, with around 12 % of bosentan-treated PAH patients displaying such elevations (Humbert et al. 2007). Clinical trials of bosentan in other diseases have reported similar liver enzyme elevation problems; for instance, the ENABLE trial of bosentan in heart failure found that a relatively high number of patients in the bosentan-
treated group experienced liver enzyme elevation (Packer et al. 2017). Thus, an improved understanding of the mechanisms underlying hepatotoxicity in dual ER inhibition is urgently needed to improve the treatment of numerous diseases with ERAs.

Recently, we identified polymorphisms in two genes, CHST3 and CHST13, predicted to be correlated with bosentan-induced liver injury in PAH patients (Yorifuji et al. 2018). CHST3 and CHST13 are members of the carbohydrate sulfotransferase gene family and encode enzymes that catalyze chondroitin sulfation at their respective sites, essentially synthesizing chondroitin sulfate (CS) (Fukuda et al. 2001). CS is a glycosaminoglycan (GAG) composed of alternating sugar chains that is attached to proteoglycans (Avram et al. 2014) and has been implicated in the modulation of several inflammatory processes via various functional capabilities (Jia et al. 2012; Vasiliadis and Tsikopoulos 2017).

There have been conflicting findings regarding whether CS promotes or prevents inflammation in different cell types or under pathogenic conditions; however, several recent studies have suggested that CS plays pro-inflammatory and pro-fibrotic roles in conditions such as heart failure, atherosclerosis, and emphysema (Adhikara et al. 2019; Kai et al. 2015; Zhao et al. 2018).

In this study, we investigated whether dual ER inhibition in the liver could alter CHST3 and CHST13 expression and thus CS production, which mediates liver injury. We also
examined whether altering liver CS content could prevent inflammatory and fibrosis responses after liver injury.

MATERIALS AND METHODS

Materials

Lipopolysaccharide (L4391-1MG) and chondroitinase ABC (C3667) were purchased from Sigma-Aldrich (St. Louis, MD, USA). The selective ET\textsubscript{A} blocker BQ-123 was purchased from Abcam (ab141005) and the selective ET\textsubscript{B} blocker BQ-788 was purchased from Phoenix Pharmaceutical (#023-38). The following antibodies were used: anti-STAT3, phospho-Y705 (1:1.000, Abcam, ab76315), anti-STAT3 (1:1.000, Santa Cruz, sc-8019), GAPDH (1:1.000, Cell Signaling Technology, #2118S), CS A (1:200, Cosmo Bio, NU-07-001), and FITC-conjugated α-SMA (1:250, Sigma-Aldrich, F3777-2ML).

Animal study

All animal experiments were approved by the Ethics Review Committee for Animal Experimentation of Kobe Pharmaceutical University, Kobe, Japan. The animals were cared for in accordance with Guide for the Care and Use of laboratory Animals. C57BL6J mice were purchased and maintained with the light-dark cycle of 12:12 h with
access to food and water *ad libitum*. At 12 weeks of age, the mice underwent either a bile duct ligation procedure or a sham procedure, as described previously with slight modifications (Tag et al. 2015). Briefly, mice were anesthetized by 4 % isoflurane inhalation in 100 % oxygen at a flow rate of 3-4 L/min and underwent standard antiseptic procedure prior to the operation. The abdomen was opened and a midline laparotomy was performed with an incision length of approximately 2 cm to expose the peritoneum, which was then cut to expose the peritoneal cavity. After the cavity had been fixed with sutures and a spreader, the bile duct was exposed by lifting and fixing the liver toward the diaphragm and moving the gut caudally. The bile duct was then separated from the adjacent vessels and, for the mice in the ligation group, ligated with 7-0 sutures proximally and distally. After ligation, both abdominal layers were closed using 6-0 sutures. For the mice in the sham group, after the bile duct was isolated the abdominal layers were closed immediately. Post-operation, the mice were allowed to recover in separate lamp-warmed cages until they were fully active, and then moved to normal cages and given food and water *ad libitum*. After 14 days, the mice were sacrificed and their livers harvested for histological sections and quantitative PCR analysis (Abshagen et al. 2015).
Cell culture

For in vitro studies, we utilized Hep3B cells, a liver cancer cell line derived from Hepatocellular Carcinoma cells that arose from differentiated mature hepatocytes. Hep3B cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (Gibco). Cells were cultured until they reached ~90% confluency and then treated with the indicated agents for 24 h before being harvested. In some of the experiments, cells were treated with the selective ET<sub>A</sub> blocker BQ-123 (1 μM/mL) and selective ET<sub>B</sub> blocker BQ-788 (1 μM/mL) to mimic dual ET receptor inhibition, while lipopolysaccharide/LPS (100 ng/mL) was used to induce an inflammatory response. Chondroitinase ABC (ChABC, 5 mU/mL) was used to degrade CS.

Quantitative PCR

Total RNA was extracted from the liver tissue and Hep3B cells using RNAiso plus (TAKARA), purified using a NucleoSpin RNA clean-up kit (Macherey-Nagel), and cDNA synthesis performed (1 μg of total RNA) using a PrimeScript RT reagent kit with gDNA eraser (TAKARA). Quantitative real-time PCR was performed using a LightCycler96 (Roche Applied Science) with FastStart SYBR Green Master Mix (Roche
Applied Science). Target gene mRNA expression levels were normalized to those of 18S ribosomal RNA.

**Immunoblotting**

Immunoblotting was carried out as described previously (Wardhana et al. 2018). Briefly, samples were lysed with RIPA buffer and their protein concentration equalized before boiling in sample buffer. Samples were then separated on SDS-PAGE gels, transferred to 0.45 μm nitrocellulose membranes (BioRad), and probed with primary antibodies overnight and secondary antibodies the following day. Reactive bands were detected by Amersham ECL (GE Healthcare).

**Histopathology**

Liver tissues were fixed with 4 % paraformaldehyde (Wako) for 24 h, dehydrated and embedded in paraffin, and then cut into 5 μm sections. The sections were stained with Alcian blue to detect GAG deposition. For immunofluorescence staining, the deparaffinized sections were treated with antigen unmasking solution (Vector Laboratories) and incubated with anti-chondroitin sulfate A antibodies at 4 °C overnight. The sections were then washed and incubated with Alexa Fluor 488 fluorescence-labeled
rat anti-mouse secondary antibodies (TGI) and mounted with Vectashield mounting medium with DAPI (Vector Laboratories).

**In vitro Alcian blue and immunocytochemical (IHC) staining**

Hep3B cells were seeded into an 8-well chamber at a density of $2 \times 10^4$ cells per well, cultured for 24 h, and treated for a further 24 h. The cells were then fixed using 4 % paraformaldehyde for 15 min, washed, and stained with either Alcian blue to evaluate GAG content or IHC stained for α-SMA. For IHC, the cells were permeabilized with PBS supplemented with 0.2 % Triton-X, blocked with 5 % donkey serum in PBS, and incubated with FITC-conjugated anti-α-SMA primary antibodies at 4 °C overnight. After washing, the cells were mounted with Vectashield mounting medium with DAPI.

**Statistical analysis**

All data are presented as the mean ± standard error of the mean (SEM). Differences between two groups were analyzed using two-tailed Student’s $t$-tests or Mann-Whitney U-tests, as appropriate. Differences between three or more groups were analyzed by one-way ANOVA or Fisher’s exact test. $P$ values of $< 0.05$ were considered statistically significant. All statistical analyses were performed using GraphPad Prism 7.
RESULTS

CHST3 and CHST13 were preferentially upregulated during in vivo liver injury

Previously, we identified CHST3 and CHST13 gene polymorphisms in PAH patients with bosentan-induced liver injury; therefore, we sought to confirm differences in the expression pattern of these genes during liver injury (Yorifuji et al. 2018). To do so, we created an in vivo liver injury model by performing bile duct ligation in 12-week-old C5BL6/J mice alongside appropriate sham-treated control mice. Two-weeks post-ligation, after we confirmed the increased fibrotic deposition in the liver of ligated mice in comparison to sham-treated mice as a proof-of-method (data not shown), liver CHST3 and CHST13 mRNA expression levels were both significantly higher in the ligated group than in the sham-treated group (Fig. 1A).

Hepatic CS deposition increased after liver injury

Since both CHST3 and CHST13 encode enzymes that promote CS synthesis, we investigated whether their upregulation coincided with CS synthesis and deposition in the liver (Fukuda et al. 2001). First, we performed Alcian blue staining in the liver sections, finding a marked increase in the deposition of GAGs, including CS, in the ligation group.
compared to the sham group (Fig. 1B). Furthermore, immunostaining the liver tissue with anti-CS A confirmed increased CS deposition in the liver tissue of the ligated mice (Fig. 1C).

**Dual ERA treatment upregulated CHST3 and CHST13 and increased CS deposition**

Next, we tried to replicate the increased CHST3 and CHST13 expression observed after *in vivo* liver injury *in vitro* by treating Hep3B cells with LPS to mimic the pro-inflammatory phenotype found in the BDL-treated mouse liver. Treating the Hep3B cells with LPS (100 ng/mL) induced CHST3 and CHST13 upregulation similar to that observed in the *in vivo* BDL-treated liver. To mimic dual ET receptor inhibition, we used BQ-123 (1 μM/mL) and BQ-788 (1 μM/mL) combination treatment to block both ET<sub>A</sub> and ET<sub>B</sub>, respectively. We then analyzed whether dual ET receptor blockade could produce similar CHST3 and CHST13 mRNA upregulation to LPS treatment, finding that both CHST3 and CHST13 levels were drastically higher after dual ERA treatment than in the LPS-treated group (Fig. 2A). Moreover, we observed the upregulation of pro-inflammatory and pro-fibrosis genes, such as TNF-α, IL-1β, and collagen 1A1 in both the LPS-treated and dual ERA-treated groups (Fig. 2B). In addition, *in vitro* Alcian blue staining in the Hep3B cells confirmed that dual ERA treatment increased CS deposition.
in hepatocytes, as observed in the in vivo liver injury model (Fig. 2C).

**ChABC abolished the pro-inflammatory and pro-fibrotic phenotype of Hep3B cells after dual ERA treatment**

It has been reported that CS is involved in inflammatory processes in various tissues; therefore, we postulated that CS may mediate pro-inflammatory and pro-fibrotic responses after drug-induced liver injury and that eliminating excess CS could prevent these injury responses. To test this hypothesis, we treated Hep3B cells with dual ERAs and ChABC (5 mU/mL), a CS-degrading enzyme, and observed changes in their inflammatory and fibrosis phenotypes.

As expected, treating the Hep3B cells with dual ERAs enhanced the expression of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, as well as the fibrosis marker collagen 1A1. Interestingly, concurrent ChABC treatment was able to prevent all of the changes in the expression of these genes (Fig. 3A), while immunostaining that ChABC also prevented a substantial increase in α-SMA-positive Hep3B cells compared to the normal vehicle control (Fig. 3B). Moreover, we found that the activity of STAT3, a known intracellular mediator of inflammation and fibrosis, increased after dual ERA treatment but was completely abolished after ChABC treatment (Fig 3C).
DISCUSSION

Hepatotoxicity is considered a major limitation to using dual ERAs to treat various pathological conditions, in part limiting their current approved usage to specific diseases such as PAH, and requiring strict liver function monitoring during their administration (Hoeper 2009; Humbert et al. 2007). Consequently, a deeper understanding of how the liver responds to the blockade of both ET<sub>A</sub> and ET<sub>B</sub> receptors and the underlying mechanisms is crucial to develop solutions for ERA hepatotoxicity.

In this study, we showed that the expression of the CS production-promoting CHST3 and CHST13 genes is upregulated in the injured livers of bile duct-ligated mice alongside increased CS deposition. Moreover, dual ER inhibition with BQ-123 and BQ-788 induced similar upregulation of both genes and caused a pro-inflammatory and pro-fibrotic response in hepatocytes <i>in vitro</i>, a phenomenon that did not appear when hepatocytes were separately treated with either BQ-123 or BQ-788, which was abolished by ChABC treatment.

The role of CS in inflammation and fibrosis remains poorly understood due to the conflicting evidence surrounding its exact role. Although traditionally known to be anti-inflammatory and widely used as supplemental treatment for osteoarthritis, several recent
papers have reported that CS exerts pro-inflammatory effects (Dyck et al. 2018; Jia et al. 2012; Vasiliadis and Tsikopoulos 2017; Zhao et al. 2018). Zhao et al. reported CS deposition in failing remodeled hearts and demonstrated that CS can directly bind to TNF-α and contribute toward the remodeling process (Zhao et al. 2018). In addition, Kai et al. showed that silencing CHST3 gene expression could ameliorate the pro-inflammatory and pro-fibrotic phenotype of lungs from in vivo emphysema and lung fibrosis models (Kai et al. 2017; Kai et al. 2015). Our results suggest that CS exerts similar pro-inflammatory effects in the liver, specifically hepatocytes; thus, CS may act differently in different cell types and/or in response to different stimuli.

We also showed that depleting abundant CS accumulation using ChABC could be a potential treatment for the pro-inflammatory and pro-fibrotic phenotype observed in hepatocytes after dual ERA treatment. Until recently, the clinical applications of ChABC have remained elusive, partly due to the difficulty of effectively delivering and maintaining ChABC concentrations over a long period of time as a result of its temperature sensitivity (Avram et al. 2014; Cheng et al. 2015). ChABC activity has been shown to drastically decrease 3-5 days after administration under 37 °C conditions (normal human body temperature), thus is likely unable to withstand the continuous upregulation of CS-synthesizing genes as demonstrated in this study (Cheng et al. 2015;
Mahajan 2018). Consequently, a strategy is required that maintains essential ChABC activity to continually combat CS accumulation, and recent developments in this particular aspect have shown promising results for future clinical use. Although no human studies have yet been conducted, several studies in the field of neuroinflammation have suggested various alternatives that deliver and maintain ChABC activity for longer, such as local ChABC delivery methods or combination with other agents (Cheng et al. 2015; Hu et al. 2018; Mahajan 2018).

Interestingly, this study highlighted how blocking ET receptors in the liver can induce inflammatory responses, contrary to previous studies that have reported the potential anti-inflammatory and anti-fibrotic effects of ERAs in other cell types under conditions such as arthritis, sepsis, and atherosclerosis. Indeed, some studies have specifically linked these effects to ERAs inhibiting the production of pro-inflammatory cytokines, such as TNF-α and IL-1β, by ERAs (Imhof et al. 2011; Kowalczyk et al. 2015; Watson et al. 2010). Nonetheless, our results suggest that hepatocytes may respond differently to the blockade of both ETₐ and ET₇ to produce pro-inflammatory cytokines. Moreover, due to the hepatic elimination and accumulation of ERAs, hepatocyte-specific mechanisms may allow them to induce rather than inhibit inflammation by activating pathways that have not yet been linked to ET receptor blockade (Lepist et al. 2014).
Notably, we demonstrated that STAT3 pathway activation is increased after dual ERA treatment in hepatocytes, indicating that total blockade of the canonical ET pathway could activate hepatocyte inflammation and fibrosis injury responses in a similar way to other stimuli, such as LPS in this study, or in other models of injury, such as CCl4 treatment (Kasembeli et al. 2018; Wang et al. 2011). STAT3 has recently been reported to be a major regulator of pro-fibrotic tissue responses to injury, while it has previously been shown that STAT3 is an intracellular signaling mediator of inflammatory cytokine-related signaling pathways (e.g. IL-6/glycoprotein130 pathway) and activates the acute phase response in liver injury and inflammation (Bode et al. 2012; Chakraborty et al. 2017; Kasembeli et al. 2018). Thus, we believe that STAT3 could explain the inflammatory and fibrotic activity of hepatocytes. To our knowledge, this is the first study to directly link ET receptor blockade to the STAT3-mediated pathway in the liver. Indeed, little evidence has yet linked ET to the STAT3 pathway and it is highly likely that other complex mechanisms link these two pathways. The resume of our findings and our proposed working model are illustrated in figure 4.

Although we revealed a connection between CS, dual ERAs, and injury-induced inflammation and fibrosis in this study, further studies are required to confirm our results and elucidate the underlying mechanisms that could link these three major aspects.
Moreover, the exact mechanism via which dual ER inhibition could upregulate CS-synthesizing genes and how CS chains could specifically induce the injury response in the liver remain unclear. Furthermore, the limitations of our current study is that we did not perform dual ET blockade in *in vivo* setting, and performing this study in the future could further confirm the link between CS and dual ET blockade. In conclusion, our study suggests that CHST3 and CHST13-induced CS production could mediate liver injury responses due to dual ER inhibition and that interfering with this pathway could be a promising alternative for treating ERA-induced liver injury.

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Packer, M., McMurray, J.J.V., Krum, H., Kiowski, W., Massie, B.M., Caspi, A., Pratt,


FIGURE CAPTIONS

Figure 1. CHST3 and CHST13 upregulation and chondroitin sulfate deposition in the injured liver. (A) Relative CHST3 and CHST13 mRNA expression in the livers of bile duct-ligated and sham-treated mice (n = 6-7 per group). (B-C) Representative histological images of Alcian blue staining for GAG content (B) and immunostaining for chondroitin sulfate (C) in the liver sections of the same mice. Scale bars, 100 μm. ***P < 0.001, **P < 0.01, *P < 0.05, error bars represent the mean ± SEM.

Figure 2. Dual ERA treatment caused CS overproduction and an inflammatory phenotype in Hep3B cells. (A) Relative CHST3 and CHST13 mRNA expression in Hep3B cells after treatment with lipopolysaccharide (100 ng/mL), BQ-123 (1 μM/mL) + BQ-788 (1 μM/mL), or a vehicle for 24 h (n = 4-6 per group). (B) Relative TNF-α, IL-1β, and collagen 1A1 mRNA expression in the same cells. Scale bars, 100 μm. (C) Alcian blue staining in Hep3B cells treated with BQ-123+BQ-788 or a control vehicle for 24 h. Scale bars, 100 μm. ***P < 0.001, **P < 0.01, *P < 0.05, error bars represent the mean ± SEM.

Figure 3. Chondroitinase ABC treatment ameliorated dual ERA-induced...
**hepatocyte inflammation and fibrosis.** (A) Relative TNF-α, IL-1β, IL-6, and collagen 1A1 mRNA expression in Hep3B cells after treatment with chondroitinase ABC (5 mU/mL), BQ-123 (1 μM/mL) + BQ-788 (1 μM/mL), or all three agents for 24 h with a control vehicle group (n = 6 per group). (B) Representative immunocytochemistry images for α-SMA in Hep3B cells after the same treatment. Scale bars, 100 μm. (C) Immunoblots and relative densitometry quantification analysis for phospho-STAT3 relative to total STAT3 with GAPDH as a loading control in Hep3B cells after the same treatment (n = 3 per group). ***P < 0.001, **P < 0.01, *P < 0.05, error bars represent the mean ± SEM.

Figure 4. Schematic figure of the proposed mechanism of CS-mediated liver injury after dual ER inhibition.
Figure 1

A

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B

Sham

Ligated

C

Chondroitin Sulfate A  
DAPI  
Merged

Sham

Ligated

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Figure 2

(A) Relative mRNA expression of CHST3 and CHST13.

(B) Relative mRNA expression of TNFα, IL-1β, and Collagen 1A1.

(C) Images of Vehicle and BQ123+BQ78 treated samples.
Figure 3

A

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B

C

Phospho-STAT3

Total STAT3

GAPDH

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Figure 4

Endothelin Receptor Antagonists

Hepatocytes

Liver Injury

Inflammation & Fibrosis

CHST3

CHST13

Chondroitinase ABC

STAT3 activity

Chondroitin Sulfate

Inflammatory/Fibrotic Cytokines

ETA

ETB

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