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

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学 位 論 文 の 内 容 要 旨

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

コンドロイチン硫酸はエンドセリン受容体拮抗薬による肝障害を誘導する

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GUSTY RIZKY TEGUH RYANTO

Background and purpose:

Endothelin (ET) is known to exert a variety of functions in addition to its main role as a vasoconstrictor; thus, many studies have investigated ET receptor antagonists (ERAs), blocking the canonical ET receptor A (ET_A) and ET receptor B (ET_B), in both experimental and clinical setting. Unfortunately, ERAs are associated with too many clinical problems to be administered freely in diverse disease populations, which caused ERAs to only be approved to treat select diseases, chiefly pulmonary arterial hypertension (PAH). One of said problems is their hepatotoxic tendency, particularly when both ET_A and ET_B are blocked, as in the case of the widely available and used bosentan, where elevated liver enzyme levels are not uncommon after its long-term usage. Recently, our group identified polymorphisms in two genes, CHST3 and CHST13, predicted to be correlated with bosentan-induced liver injury in PAH patients. 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). CS is a glycosaminoglycan (GAG) composed of alternating sugar chains that is attached to proteoglycans and has been implicated in the modulation of several inflammatory and fibrotic processes via various functional capabilities. In this study, we investigated whether dual ER inhibition in the liver cells could alter CHST3 and CHST13 expression and thus CS production, which in turn could mediate liver injury via its inflammatory and fibrosis response alteration.

Methods:

For *in vivo* study, 12 weeks old C57BL6J mice underwent either a bile duct ligation procedure or a sham procedure. After 14 days, the mice were sacrificed and their livers harvested for histological and quantitative real-time PCR analysis.

In vitro, Hep3B cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum and 1 % antibiotic-antimycotic. Cells were cultured until they reached confluency and then treated with the indicated agents for 24 h before being fixated and immunostained, or harvested for quantitative real-time PCR, immunoblotting analysis. In some of the experiments, cells were treated with the selective ET_A blocker BQ-123 (1 μM/mL) and selective ET_B 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.

Results:

Two-weeks post-ligation, liver CHST3 and CHST13 mRNA expression levels were both significantly higher in the ligated group than in the sham-treated group. 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. Alcian blue staining to identify GAGs deposition in the liver sections confirmed a marked increase in the ligation group compared to the sham group. Furthermore, immunostaining the liver tissue with anti-Chondroitin Sulfate A confirmed increased CS deposition in the liver tissue of the ligated mice.

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-induced inflammatory responses. We found that LPS successfully induced CHST3 and CHST13 upregulation in Hep3B cells, similar to that observed in the *in vivo* BDL-treated liver. To mimic dual ET receptor inhibition, we used BQ-123 and BQ-788 combination treatment in Hep3B cells to block both ET_A and ET_B, respectively, and analyzed its CHST3 and CHST13 mRNA expression, in which we found that the expression both of said genes were drastically higher, even compared to LPS-treated group. 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. In addition, *in vitro* Alcian blue staining in the Hep3B cells confirmed that dual ERA treatment increased CS deposition in hepatocytes similar to the liver model.

We further 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, 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, while immunostaining that ChABC also prevented a substantial increase in α-SMA-positive Hep3B cells compared to the normal vehicle control. Moreover, we found that the activity of STAT3, a known intracellular mediator of inflammation and fibrosis, was increased after dual ERA treatment but was completely abolished after ChABC treatment. As such, our data collectively suggest that chondroitin sulfate mediates the pro-inflammatory and pro-fibrotic responses in the liver cells after dual endothelin receptor inhibition.

Discussion:

Hepatotoxicity is considered a major limitation to using dual ERAs to treat various pathological conditions, limiting their current approved usage to specific diseases such as PAH, and requiring strict liver function monitoring during their administration. Consequently, a deeper understanding of how the liver responds to the blockade of both ET_A and ET_B 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 *in vitro*, 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 studies have reported that CS exerts pro-inflammatory effects in various conditions, such as heart failure and lung fibrosis. 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 phenotype observed in hepatocytes after dual ERA treatment. Although 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, recent developments in ChABC delivery methods have shown promising results for future clinical use, such as local ChABC delivery methods or combination with other agents.

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, modulated partly by inhibition of pro-inflammatory cytokines production (e.g. TNF-α, IL-1β) in other cell types under various conditions. Nonetheless, our results suggest that hepatocytes may respond differently to the blockade of both ET_A and ET_B 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.

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. 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 and activates the acute phase response in liver injury and inflammation. 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 and it is likely that other complex mechanisms link these two pathways.

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.

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.

神戸大学大学院医学(系)研究科（博士課程）

論文審査の結果の要旨			
受付番号	甲 第 2988 号	氏 名	GUSTY RIZKY TEGUH RYANTO
論文題目 Title of Dissertation	コンドロイチン硫酸はエンドセリン受容体拮抗薬による肝障害を誘導する Chondroitin sulfate mediates liver responseto injury induced by dual endothelin receptor inhibition		
審査委員 Examiner	主 査 Chief Examiner 児玉裕三 副 査 Vice-examiner 古屋敷智之 副 査 Vice-examiner 永川 祥		

（要旨は１，０００字～２，０００字程度）

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Methods

For *in vivo* study, 12 weeks old C57BL6J mice underwent either a bile duct ligation procedure or a sham procedure. After 14 days, the mice were sacrificed and their livers harvested for histological and quantitative real-time PCR analysis.

In vitro, Hep3B cells were treated with the agents for 24 h before being fixated and immunostained, or harvested for quantitative real-time PCR, immunoblotting analysis. In some of the experiments, cells were treated with the selective ET_A blocker BQ-123 (1 μM/mL) and selective ET_B blocker BQ-788 (1 μM/mL) to mimic dual ET receptor inhibition.

Results

Two-weeks post-ligation, liver CHST3 and CHST13 mRNA expression levels were both significantly higher in the ligated group than in the sham-treated group. Alcian blue staining to identify glycosaminoglycan (GAG) deposition in the liver sections confirmed a marked increase in the ligation group compared to the sham group. Immunostaining confirmed increased CS deposition in the liver tissue of the ligated mice.

The authors found that LPS induced CHST3 and CHST13 upregulation in Hep3B cells. The authors used BQ-123 and BQ-788 combination treatment in Hep3B cells to block both ET_A and ET_B, respectively, and found that the expression both CHST3 and CHST13 mRNA were drastically higher, even compared to LPS-treated group. The authors observed the upregulation of pro-inflammatory and pro-fibrosis genes in both the LPS-treated and dual ERA-treated groups. *in vitro* Alcian blue staining in the Hep3B cells confirmed that dual ERA treatment increased CS deposition in hepatocytes similar to the liver model.

The authors treated Hep3B cells with dual ERAs and ChABC, 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. concurrent ChABC treatment was able to prevent all of the changes in the expression of these genes, while immunostaining that ChABC also

prevented a substantial increase in α-SMA-positive Hep3B cells compared to the normal vehicle control. Moreover, the authors found that the activity of STAT3, a known intracellular mediator of inflammation and fibrosis, was increased after dual ERA treatment but was completely abolished after ChABC treatment.

Discussion

A deeper understanding of how the liver responds to the blockade of both ET_A and ET_B receptors and the underlying mechanisms is crucial to develop solutions for ERA hepatotoxicity. The author's 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. The authors also showed that depleting abundant CS accumulation using ChABC could be a potential treatment for the phenotype observed in hepatocytes after dual ERA treatment.

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, modulated partly by inhibition of pro-inflammatory cytokines production (e.g. TNF-α, IL-1β) in other cell types under various conditions. Nonetheless, the author's results suggest that hepatocytes may respond differently to the blockade of both ET_A and ET_B 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.

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In conclusion, the author's 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.

The candidate, having completed studies on the mechanism by which endothelin receptor inhibition induces liver injury, with a specialty in CHST3 and CHST13-induced chondroitin sulfate production, is hereby recognized as having qualified for the degree of Ph.D. (Medicine) .