Title of this work
Reduction of endotoxin attenuates liver fibrosis through suppression of hepatic stellate cell activation and remission of intestinal permeability in a rat non-alcoholic steatohepatitis model.

Sub-titles and general headlines
Douhara et al: Reduction of endotoxin attenuates liver fibrosis in NASH model.

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Abbreviations: TLR4, Toll-like receptor 4; NASH, non-alcoholic steatohepatitis; LPS, lipopolysaccharide; CDAA, choline deficiency amino acid; HSC, hepatic stellate cell; LBP, LPS binding protein; CSAA, choline supplemented amino acid; TJP, tight junction protein; NAFLD, non-alcoholic fatty liver disease; HCC, hepatocellular carcinoma; TGF-β, transforming growth factor-β; α-SMA, alpha-smooth muscle actin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Alb, albumin; T-bil, total bilirubin; Glu, glucose; TG, triglyceride; T-cho, total cholesterol.
Abstract

**Background & Aims:** Recent clinical studies showed that endotoxin/Toll-like receptor4 (TLR4) signaling played an important role in the inflammatory pathways associated with non-alcoholic steatohepatitis (NASH). In both human and animal studies, NASH was associated with portal lipopolysaccharide (LPS) and plasma LPS level was thought to have some relations with small intestinal bacterial overgrowth, the change of composition of microbiota, and increased intestinal permeability. The aim of this study is to investigate the role of endogenous endotoxin and TLR4 in the pathogenesis of NASH.

**Methods:** The effect of antibiotics was assessed *in vivo* using a choline deficiency amino acid (CDAA)-induced experimental liver fibrosis model. Antibiotics, polymyxins and neomycins, were orally administered through drinking water.

**Results:** Antibiotics attenuated hepatic stellate cell (HSC) activation, liver fibrosis via controls of TGF-β and collagen in an experimental hepatic fibrosis model. We assessed the mechanism in which antibiotics attenuated LPS-TLR4 signaling and liver fibrosis. Interestingly, TLR4 mRNA level in the liver was elevated in the CDAA group, and the CDAA-induced increase was significantly decreased by antibiotics. But, that in the intestine was not different among all groups. Elevated mRNA level of LPS binding protein (LBP), which was correlated with serum endotoxin levels, was recognized in the CDAA group, and the CDAA-induced increase was significantly reduced by antibiotics. Intestinal permeability of the CDAA group was increased in comparison to the choline supplemented amino acid (CSAA) group. Tight junction protein (TJP) in the intestine determined by immunohistochemical analysis was inversely related to intestinal permeability. Antibiotics improved intestinal permeability and TJP expression.

**Conclusions:** Inhibition of LPS-TLR4 signaling with antibiotics attenuated the liver fibrosis development associated with NASH via inhibition of HSC activation. Our results indicated that reduction of LPS and restoration of intestinal TJP might be a new
therapeutic strategy for treatment of the liver fibrosis development in NASH.
Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in the general population (1). NAFLD includes simple steatosis, non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma (HCC) (2). Although NAFLD is benign, it has been reported that 20% of patients with NAFLD progressed to NASH, cirrhosis and HCC (3, 4). The pathophysiological events and effective therapies for NASH remain unknown.

Recent clinical studies reported that endotoxin/Toll-like receptor4 (TLR4) signaling played an important role in the activation of inflammatory pathways associated with NASH (5). TLR4 is a pattern recognition receptor which recognizes endotoxin and signals through adaptor molecules termed myeloid differentiation primary response gene 88 (MyD88) and Toll/interleukin-1 receptor domain-containing adaptor-inducing interferon-β (TRIF) to activate transcription factors that initiate innate immunity (6). TLR4 is expressed on multiple liver cell types including liver vascular endothelial cells (LEC), Kupffer cells, and hepatic stellate cells (HSC) (7, 8). Indeed, TLR4 on HSC plays a dominant role in fibrosis development through effects on transforming growth factor-β (TGF-β) dependent collagen production (8).

In both human and animal studies, it has been reported that NASH is associated with portal LPS levels through mechanisms involving bacterial translocation (9, 10), and gut microbiota is thought to generate products like lipopolysaccharide (LPS), a cell-wall component of Gram-negative bacteria, which are delivered into liver via portal vein (11, 12). Endotoxin production by gut microbiota could cause an inflammation in patients with obesity, diabetes, metabolic disorder, NAFLD, and NASH (11, 13). Plasma LPS levels are associated with small intestinal bacteria overgrowth, the change of composition of microbiota, and increased intestinal permeability (14).

Polymyxins are antibiotics with a general structure consisting of a cyclic peptide with a long hydrophobic tail and are selectively toxic to Gram-negative bacteria such as E.coli,
*Pseudomonas aeruginosa*, *Enterobacteriaceae*, and *Pneumobacillus* due to their specificity for the LPS molecule that exists within many Gram-negative outer membranes. They are produced by nonribosomal peptide synthetase systems in Gram-positive bacteria such as *Paenibacillus polymyxa* and disrupt the structure of the bacterial cell membrane by interacting with its phospholipids. They are not absorbed through gastrointestinal tract. In clinical settings, they are used for patients with Gram-negative bacterial infections and column of endotoxin apheresis against endotoxemia (15).

Neomycins are aminoglycoside antibiotics, and effective against both Gram-negative and Gram-positive bacteria. They are produced by Gram-positive bacteria such as *Streptomyces fradiae*. They inhibit protein synthesis of bacteria via binding to 30S ribosome. They are also hardly absorbed through gastrointestinal tract, and are useful for Gram-negative bacterial infections in clinical settings.

In this study, we tested the effect of these poorly absorbable antibiotics on intestinal permeability and progression of liver fibrosis. Our results showed that in a rat model of CDAA-induced liver fibrosis, administration of poorly absorbable antibiotics led to less intestinal permeability, as well as decreased liver fibrosis. Consequently, our study elucidated the role of LPS in the pathogenesis of NASH.

Materials and Methods

*Animal Model of Liver Disease*

Six-week-old male Fischer 344 rats (CLEA, Japan) were housed in a room under controlled temperature, and lighting (12/12-h artificial light / dark cycle). Animals were divided into the following three experimental groups and fed for 8 weeks: a) choline deficient amino acid diet (CDAA, n=10), b) choline deficient amino acid diet plus antibiotics (CDAA+AB, n=10), and c) choline supplemented amino acid diet (CSAA, n=5). All rats were sacrificed at the end of week 8. For selective intestinal
decontamination, poorly absorbable antibiotics [1 g/L of polymyxin B sulfate salt (Fluka, Switzerland) and 3 g/L of neomycin trisulfate salt hydrate (Sigma·Aldrich, USA)] were given to the rats of the CDAA+AB group by containing them in drinking water during the experimental period except the first and fifth week. All animal procedures were performed according to standard protocol and in accordance with the standard recommendations for the proper care and use of laboratory animals.

**Histologic Examination**

Conventional histologic examination was performed by Hematoxylin·Eosin and Sirius·Red staining of the excised liver sections, as described previously (16).

**Immunohistochemistry**

For immunostaining of alpha-smooth muscle actin (α-SMA), 5-μm-thick liver sections were stained by indirect immunoperoxidase method with anti-α-SMA antibody (Dako, Japan) as described previously (16). For immunofluorescence examination, frozen liver and intestinal sections were fixed with 4% paraformaldehyde for 10 minutes at 4°C and blocked with 3% bovine serum albumin for an hour at room temperature to eliminate nonspecific background. Tissue sections were then incubated with primary antibodies against ZO-1 (ZO-1; Invitrogen, USA) and Claudin-4 (Claudin-4; Invitrogen; USA) at 4°C overnight. This was followed by incubation with appropriate Alexa Fluor-488 or Alexa Fluor-546 secondary antibodies (Invitrogen, USA) for an hour at room temperature. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) Fluoromount-G (SouthernBiotech, USA). Immunofluorescent staining was visualized with Zeiss Axiovert 40 CEL® (Zeiss, Germany), and images from ZO-1 and Claudin-4 staining were quantified by using AXIO software version 4® (Zeiss, Germany). For quantification, five images were randomly selected for quantification analysis from each sample, and the software program quantified the staining intensity.
of the selected images based on a preselected threshold.

**Real-time PCR**

Total RNA was extracted from the liver and intestinal tissue samples using acid guanidinium thiocyanate-phenol-chloroform extraction. The mRNA levels of collagen Iα, TGF-β, TLR4, and LPS-binding protein (LBP) in the liver and TLR4 in the intestine were measured by real-time PCR using the Applied Biosystems Step One Plus real-time PCR® (Applied Biosystems, USA), as described previously (17). Primer sequences were as follows: B-actin-forward 5'-GGA GAT TAC TGC CCT GGC TCC TA-3' and reverse 5'-GAC TCA TCG TAC TCC TGC TTG CTG-3'; TLR4-forward 5'-CCG CTC TGG CAT CAT CTT CA-3' and reverse 5'-CCC ACT CGA GGT AGG TGT TTC TG-3'; LBP forward 5'-AAC ATC CGG CTG AAC ACC AAG-3' and reverse 5'-CAA GGA CAG ATT CCC AGG ACT GA-3'; TGF-β forward 5'-CGG CAG CTG TAC ATT GAC TT-3' and reverse 5'-AGC GCA CGA TCA TGT TGG AC-3'; Collagen Iα forward 5'-AGC TCC TGG GCC TAT CTG ATG A-3' and reverse 5'-AAT GGT GCT CTG AAA CCC TGA TG-3'.

**Protein Expression Analysis**

Hepatic tissue was homogenized in lysis buffer (Tissue protein extraction reagent, Thermo Scientific, Japan) containing a mixture of protease and phosphatase inhibitors (Roche, Switzerland). Total collagen volume in the liver was measured by Sircol collagen assay kit® (Biocolor, UK). TGF-β levels in the liver were measured by ELISA (R&D Systems, USA).

**Determination of Rat Intestinal Permeability**

FITC-dextran 40 kDa (Sigma-Aldrich, USA), 25 mg each, was orally administered on the day of sacrifice. Four hours after FITC-dextran gavage, each rat was anesthetized and blood was drawn from its portal vein. Each plasma was analyzed by fluorescence
measurement at the excitation wavelength of 490 nm and the emission wavelength of 520 nm.

**Statistical Analysis**

Results, expressed as mean ± SD, were analyzed by using Student’s t-test for unpaired data (IBM SPSS Statistics version 22, USA). A P value of <0.05 was regarded as statistically significant.

Result

**General findings**

The general findings of each experimental group at the time of sacrifice are shown in Table 1. The relative weights of the liver in the CDAA group and the CDAA+AB group were more than that of the CSAA group, whereas no significant differences were observed between two previous groups. Regarding the serological data between the CDAA group and the CDAA+AB group, no significant differences were observed in the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin (Alb), total bilirubin (T-bil), glucose (Glu), triglyceride (TG), total cholesterol (T-cho), and high density lipoprotein cholesterol (HDL-cho).

**Effect of poorly absorbable antibiotics on liver fibrosis development**

We initially examined the effects of poorly absorbable antibiotics on liver fibrosis, induced by CDAA intake. As shown in Fig. 1A, although quite severe fibrosis was observed in the CDAA group, no fibrosis could be seen in the CSAA control group, and poorly absorbable antibiotics attenuated the CDDA-induced fibrosis. Because it is generally known that activated HSC played a key role on fibrogenesis, we next carried out an immunohistochemical analysis of α-SMA to examine the effects of poorly absorbable antibiotics on HSC activation during the liver fibrosis development.
Distinctly decreased level of α-SMA expression was observed in the CDAA+AB group (Fig. 2). Semi-quantitative analysis performed by Image J software (NIH, USA) showed significant decrease of α-SMA in the CDAA+AB group in comparison with the CDAA group (Fig. 2A). Additionally, markedly suppressed levels of hepatic TGF-β and total collagen were revealed in the CDAA+AB group, compared with the CDAA group (Fig. 1C, 2C). RT-PCR also showed that these inhibitory effects closely correlated with the changes of mRNA expression levels of TGF-β and collagen-Iα (Fig. 1B, 2B). According to these data above, we considered that poorly absorbable antibiotics attenuated hepatic stellate cell activation and liver fibrosis via controls of TGF-β and collagen in this experimental hepatic fibrosis model.

**Effect of poorly absorbable antibiotics on LPS-TLR4 signaling**

TLR4 enhances hepatic inflammation and fibrogenesis (8, 18). This finding led us to hypothesize that poorly absorbable antibiotics might attenuate LPS-TLR4 signaling and liver fibrosis would also be ameliorated as a result. TLR4 mRNA expression in the liver and intestine were examined as a next step. Interestingly, TLR4 mRNA level in the liver was elevated in the CDAA group, and the CDAA-induced increase was significantly decreased by antibiotics (Fig. 3A). However, TLR4 mRNA levels in the intestine were not different in all groups (Fig. 3B). These data suggested that TLR4 related signaling in the intestine was not important for liver fibrosis, whereas TLR4 in the liver was essential. Then, we measured mRNA levels of LBP, which was indispensable for LPS to bind TLR4 and was correlated with serum endotoxin levels (24). Significantly, elevated mRNA level of LBP was recognized in the CDAA group and this increase was reduced in the CDAA+AB group (Fig. 3C).

**Effect of poorly absorbable antibiotics on intestinal permeability and tight junction protein**
Elevated mRNA level of LBP suggested that serum LPS level was increased in the CDAA group. Serum LPS level was thought to be involved in gut permeability. So, we examined gut permeability by analyzing the fluorescent levels of portal vein after oral gavage loading with FITC-dextran. The fluorescent levels of portal vein in the CDAA group were increased when compared to the CSAA group. The increase of intestinal permeability of the CDAA group was improved by the addition of poorly absorbable antibiotics (Fig. 4). Because gut permeability was controlled by tight junction protein, including ZO-1 and Claudin-4 (14, 20), we examined immunohistochemical analyses of ZO-1 and Claudin-4 in the intestinal sections. As shown below, immunohistochemical analyses showed that strong expressions of ZO-1 and Claudin-4 were predominant in the intestinal sections of CSAA control group (Fig. 5A, B). On the other hand, the delocalization and substantial decrease in the intestinal sections of CDAA group were dramatically improved by poorly absorbable antibiotics administration.

Discussion

In this study, we examined the effect of poorly absorbable antibiotics, polymyxin and neomycin, on the development of hepatic fibrosis and intestinal permeability. We demonstrated that the antibiotics not only reduced CDAA-induced hepatic fibrosis and HSC activation but also improved intestinal permeability.

Liver is the main target of intestinally-derived bacterial products, and the rate of bacterial translocation increases in various models of hepatic disease, rendering LPS a likely candidate mediator of TLR4-dependent profibrogenic effects. Accordingly, we found increased LBP mRNA expression in the CDAA group, indicating that LPS should be increased. Moreover, LBP mRNA expression and fibrogenesis were reduced in rats treated with poorly absorbable antibiotics, suggesting that the intestinal flora would be the main source of LPS and that intestinally-derived LPS would drive fibrogenesis.

Translocated LPS derived from the gut microflora mediates TLR4 activation in the
liver. But, this translocation might be independent of intestinal TLR4 (21). We tested the mRNA expression of TLR4 in the liver and intestine. The mRNA expression levels in the liver of CDAA-induced NASH model were increased. In contrast, those in the intestine of CDAA-induced NASH model were not increased. However, Guo et al. reported that LPS caused an increase in intestinal permeability via an intracellular mechanism involving TLR4-dependent up-regulation of CD14 membrane expression (22). The relationship between LPS and TLR4 on intestinal permeability has been still controversial.

NAFLD is associated with increased intestinal permeability and small intestinal bacteria overgrowth (21, 23). These findings have been thought to be associated with the severity of hepatic steatosis. The increased intestinal permeability might be the condition for the hypothesis of the contribution of gut-liver axis to development of NAFLD (14). The intestinal barrier defect might be caused by disruption, imbalance of proliferation and apoptosis, intestinal mucosal atrophy and edema associated with portal hypertension or absence of bile acids, and systemic increases in inflammatory cytokines, and oxidative stress produced from the liver (24, 25, 26). LPS causes an increase in intestinal permeability via an intracellular mechanism involving TLR4-dependent up-regulation of CD14 membrane expression (22).

Caco-2 cells grown in zinc-deficient media have reduced transepithelial electrical resistance (TEER) and altered expression of ZO-1 and Occludin, which are one of the intestinal TJP, compared with Caco-2 cells grown in zinc-replete media (27). In clinical practice, zinc deficiency is likely to occur in patients with liver cirrhosis (28, 29). Zinc deficiency in patients with liver cirrhosis may reduce TJP in the intestine and increase the permeability. In our NASH model, CDAA-induced hepatic fibrosis, endogenous LPS and systemic increases in inflammatory cytokines might disrupt intestinal tight junction proteins. From this point of view, the recruitment of tight junction proteins by using probiotics and zinc preparation, for example, would be a new strategy for NASH
Intestinal microflora is involved in liver fibrosis. In this in vivo model, dietary habits through increasing the percentage of intestinal endotoxin producers such as Gram-negative bacteria might accelerate liver fibrogenesis, introducing dysbiosis as a co-factor contributing to chronic liver injury in NAFLD (30). Endo et al. also showed butyrate-producing probiotics reduced NAFLD progression in rats (9). These data indicated that intestinal microflora could be a new target for NASH treatment.

In conclusion, inhibition of LPS-TLR4 signaling with poorly absorbable antibiotics attenuated the liver fibrosis development of NASH via inhibition of HSC activation. Our results indicated that reduction of LPS and restoration of the intestinal tight junction protein might be a new therapeutic strategy for treatment of the liver fibrosis development in NASH.

Conflict of interest
The authors declare that they have no conflicts of interest.

Figure legends
Fig.1 Antibiotics ameliorated liver fibrosis induced by CDAA diet. (A) Collagen deposition was evaluated by Sirius-Red staining. Extensive fibrosis was observed in the CDAA group. Treatment with antibiotics showed significant inhibitory effect against liver fibrosis. No fibrosis was observed in the CSAA group. Semi-quantitative analysis confirmed histological findings. (B) Collagen-Iα mRNA expression in the liver was significantly increased in the CDAA group when compared with the CSAA group. Treatment with antibiotics markedly suppressed the expression of Collagen-Iα. (C) Compared with the CSAA group, total collagen level of the CDAA group was increased,
and this increase was significantly suppressed by antibiotics. Data are reported as mean ± SD. *P<0.05, **P<0.01.

Fig. 2 Activated HSCs were reduced by administration of antibiotics. (A) Compared with the CDAA group, a significantly decreased number of α-SMA immunopositive cells was recognized after the treatment of antibiotics. No α-SMA immunopositive cells were observed in the CSAA group. Semi-quantitative analysis confirmed that α-SMA immunopositive cells were decreased in the antibiotics treated group in parallel with the reduction of liver fibrosis. (B) Compared with the CSAA group, significantly increased TGF-β mRNA expression in the liver of the CDAA group was demonstrated. Treatment with antibiotics suppressed the expression of TGF-β in comparison with CDAA group. (C) Increased TGF-β protein level in the CDAA group was significantly suppressed by antibiotics. The degree of TGF-β suppression by antibiotics was at similar magnitude of the inhibition of α-SMA positive cells. Data are reported as mean ± SD. *P<0.05, **P<0.01.

Fig. 3 Antibiotics attenuated LPS-TLR4 signaling.
(A) TLR4 mRNA expression in the liver of the CDAA group was significantly increased when compared to the CSAA group. Treatment with antibiotics suppressed the expression of TLR4 in the liver. (B) However, TLR4 mRNA level in the small intestine was not different among all groups. (C) LBP mRNA level was elevated in the CDAA group when compared to the CSAA group and this increase was significantly decreased by antibiotics. Data are reported as mean ± SD. * P <0.05, ** P <0.01. n.s., not significant.

Fig. 4 Antibiotics improved intestinal permeability.
The portal fluorescent level in the CDAA group was significantly increased in
comparison to the CSAA group. The increase of intestinal permeability in the CDAA group was improved by poorly absorbable antibiotics. Data are reported as mean ± SD. *$P<0.05$, **$P<0.01$. n.s., not significant.

Fig.5 Antibiotics improved tight junction protein expression in the small intestine. (A, B) Immunohistochemical analyses showed adequate expressions of ZO-1 and Claudin-4 in the intestinal sections of the CSAA control group. It should be noted that antibiotics dramatically improved the delocalization and substantial decrease in the intestinal sections of the CDAA rats. Semi-quantitative analysis confirmed immunohistochemical findings. Data are reported as mean ± SD. *$P<0.05$, **$P<0.01$.

References


Table 1

Table 1. Characteristic features of the experimental groups.

<table>
<thead>
<tr>
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<th>CSAA (n = 5)</th>
<th>CDAA (n = 10)</th>
<th>CDAA+AB (n = 10)</th>
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<tr>
<td>Body weight (g)</td>
<td>304.0 ± 11.6</td>
<td>291.3 ± 18.5</td>
<td>250.6 ± 12.4*</td>
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<td>Liver weight (g)</td>
<td>10.4 ± 0.7</td>
<td>18.6 ± 1.4*</td>
<td>15.6 ± 1.5*</td>
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<td>Liver weight (% body)</td>
<td>3.4 ± 0.2</td>
<td>6.4 ± 0.2*</td>
<td>6.2 ± 0.4*</td>
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<td>AST (IU/l)</td>
<td>57.6 ± 6.0</td>
<td>361.2 ± 39.0*</td>
<td>384.5 ± 46.3*</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>25.4 ± 8.6</td>
<td>244.8 ± 55.2*</td>
<td>259.4 ± 53.0*</td>
</tr>
<tr>
<td>T-bil (mg/dl)</td>
<td>0.03 ± 0.01</td>
<td>0.13 ± 0.02*</td>
<td>0.13 ± 0.01*</td>
</tr>
<tr>
<td>ALB (g/dl)</td>
<td>3.0 ± 0.2</td>
<td>3.3 ± 0.3</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>T-cho (mg/dl)</td>
<td>42.6 ± 5.9</td>
<td>26.1 ± 3.3*</td>
<td>24.6 ± 2.1*</td>
</tr>
<tr>
<td>HDL-cho (mg/dl)</td>
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<td>14.3 ± 2.5</td>
<td>14.9 ± 1.6</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>116.6 ± 18.9</td>
<td>10.6 ± 7.6*</td>
<td>6.4 ± 1.6*</td>
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<td>Glucose (mg/dl)</td>
<td>135.0 ± 28.8</td>
<td>101.9 ± 11.3</td>
<td>105.3 ± 29.6</td>
</tr>
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</table>

Data are reported as mean ± SD.

* Statistically significant as compared with CSAA, respectively (P < 0.01).
Figure 1

A

CSAA

CDAA

CDAA+AB

B

C

Collagen Jr/b Actin: mRNA

Collagen µg/mg protein
Figure 2

A

CSAA

CDAA

CDAA+AB

B

C

TGF-β / b. Actin
mRNA

TGF-β pg/mg protein

CSAA  CDA  CDAA+AB

CSAA  CDA  CDAA+AB
Figure 3

A

TLR4/βACT mRNA
Liver

CSAA  CDAA  CDAA+AB

B

TLR4/βACT mRNA
Intestine

CSAA  CDAA  CDAA+AB

C

LBP/βactin mRNA

CSAA  CDAA  CDAA+AB

**  *