The hepatic bile acid transporters Ntcp and Mrp2 are downregulated in experimental necrotizing enterocolitis

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Necrotizing enterocolitis (NEC) is characterized by an extensive hemorrhagic inflammatory necrosis of the distal ileum and proximal colon and is diagnosed almost exclusively in premature infants (24). While the major risk factors for NEC (prematurity, formula feeding, and bacterial colonization) have previously been identified, the pathophysiology of this disease remains poorly understood.

In addition to pathological effects in the ileum, severe NEC is often accompanied by multisystem organ failure, including the liver (31). We were the first to show that inflammatory mediators in the liver play an important role in the development of experimental NEC (15). Production of hepatic interleukin (IL)-18 and tumor necrosis factor (TNF)-α as well as the number of Kupffer cells were increased in neonatal rats with NEC and correlated with the progression of intestinal damage during disease development. Furthermore, increased levels of TNF-α in the intestinal lumen of rats with NEC was significantly decreased when Kupffer cells were inhibited with gadolinium chloride (15). These results suggest an important role of the liver and the gut-liver axis in NEC pathogenesis.

Bile acids (BAs) are physiological compounds that facilitate emulsification, absorption, and transport of fats and sterols in the intestine and liver. BAs are synthesized in the liver from cholesterol by cholesterol 7α-hydroxylase (CYP7A1) and hepatic mitochondrial 27-hydroxylase (CYP27A1) (22), after which, they are secreted into bile via the bile salt excretory pump (BSEP) and the multidrug resistance-associated protein 2 (MRP2). Most BA reclamation occurs in the distal ileum via the apical sodium-dependent bile acid transporter (ASBT) (10, 38). At the basolateral surface of enterocytes, the heteroergic organic solute transporter (OSTα-OSTβ) removes BAs from the cell and transports them into portal circulation (4). Completion of enterohepatic circulation of BAs is mediated primarily by the sodium-dependent taurocholate-transporting polypeptide (NTCP) (42) as well as members of the organic anion-transporting polypeptide (OATP) family in the liver.

Because the majority of intestinal BA reclamation occurs in the distal ileum, the site of NEC injury, we hypothesized that BAs could play an important role in the pathophysiology of NEC. Using a neonatal rat model, we were the first to show that ileal BA accumulation contributes to ileal damage in NEC (17). Recently, we have expanded our studies to reveal that dysregulation of ASBT in NEC contributes to both the intra-enterocyte accumulation of BAs as well as the incidence and severity of disease in both rat and mouse models of NEC as well as in human patients (19).

Given the integral role of the liver in NEC pathogenesis and the importance of BAs in disease development, we speculated that hepatic BA transporters would also be altered in experimental NEC. Using both rat and mouse models of NEC, we determined that the hepatic BA transporters Bsep, Ntcp, Oatp2, and Oatp4 were downregulated in experimental NEC, whereas removal of IL-18 normalized Mrp2 levels. These data suggest the gut-liver axis should be considered when therapeutic modalities for NEC are developed.
Table 1. Incidence and severity of NEC in experimental models

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Incidence, %</th>
<th>Median Histological Ileal Damage Score</th>
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<tbody>
<tr>
<td>DF (12)</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>NEC (12)</td>
<td>75†</td>
<td>2.5†</td>
</tr>
<tr>
<td>NEC (10)</td>
<td>70</td>
<td>2.5</td>
</tr>
<tr>
<td>NEC + Chol (10)</td>
<td>20†</td>
<td>1.5*</td>
</tr>
<tr>
<td>Asbt+/+ NEC (12)</td>
<td>75</td>
<td>2.5</td>
</tr>
<tr>
<td>Asbt−/− NEC (12)</td>
<td>33†</td>
<td>1.5*</td>
</tr>
<tr>
<td>NEC (10)</td>
<td>70</td>
<td>3.0</td>
</tr>
<tr>
<td>NEC + anti-TNF-α (10)</td>
<td>30*</td>
<td>1.5*</td>
</tr>
<tr>
<td>NEC (9)</td>
<td>67</td>
<td>2.0</td>
</tr>
<tr>
<td>NEC + anti-IL-18 (9)</td>
<td>22*</td>
<td>1.0*</td>
</tr>
<tr>
<td>IL-18+/+ NEC (10)</td>
<td>60</td>
<td>2.5</td>
</tr>
<tr>
<td>IL-18−/− NEC (10)</td>
<td>10*</td>
<td>1.5*</td>
</tr>
</tbody>
</table>

Histological ileal damage was scored on a scale of 0 (normal) to 4 (necrosis). Animals with an ileal damage score ≥2.0 were considered to have developed necrotizing enterocolitis (NEC). DF, dam fed; Chol, cholestyramine; Asbt, apical sodium-dependent bile acid transporter; TNF-α, tumor necrosis factor-α; IL-18, interleukin-18. *P ≤ 0.05 and †P ≤ 0.01. Incidence of NEC was determined using the χ²-test; median ileal damage score was determined using the Mann-Whitney test.

abdominal distension, respiratory difficulty, etc.) and killed before the end of the study if excessive distress was observed.

**Rat NEC Models**

Sprague-Dawley rats (Charles River Labs, San Diego, CA), delivered via caesarian section one day before scheduled birth from multiple dams, were divided into the following groups: DF (n = 12), pups allowed to feed from a foster mother for 4 days and exposed to asphyxia/cold (A/C) stress (N2 gas for 60 s and 4°C for 10 min) (7, 13, 16), and NEC (n = 12), pups hand-fed with formula for 4 days and exposed to A/C stress.

**Sequestration of luminal BAs.** Neonatal rat pups were hand-fed with formula, exposed to A/C stress, and gavaged with either 120 mg·kg⁻¹·day⁻¹ cholestyramine (Eon Labs, Laurelton, NY) or vehicle (PBS) for 4 days (17). These groups were designated NEC + Chol (n = 10) and NEC (n = 10).

**Neutralization of TNF.** In a separate set of experiments, prematurely delivered pups from multiple litters were placed into one of two experimental groups: NEC + Anti-TNF-α (n = 10), pups hand-fed with formula for 4 days, exposed to A/C stress, and injected once every other day beginning at day 1 with 5 mg/kg monoclonal anti-TNF-α (12) and NEC (n = 10), pups hand-fed with formula for 4 days, exposed to A/C stress, and injected with vehicle alone using the same injection schedule. Neutralization of TNF-α was determined as described (12), based on alterations in disease incidence and severity.

**Neutralization of IL-18.** Prematurely born rat pups were placed into two experimental groups: NEC + Anti-IL-18 (n = 9), pups hand-fed with formula for 4 days, exposed to A/C stress, and injected with 10 mg·kg⁻¹·day⁻¹ goat anti-rat IL-18 (R & D Systems, Minneapolis, MN), and NEC (n = 9), pups hand-fed with formula for 4 days, exposed to A/C stress, and injected with 10 mg·kg⁻¹·day⁻¹ of goat IgG using the same injection schedule. Neutralization of IL-18 was determined based on alterations in disease incidence and severity compared with sham antibody-injected animals.

**Mouse NEC Models**

Neonatal 129S1/SvImJ (ASBT+/+), 129S1/SvImJ-((-/)) (ASBT−/−), B6.129P2-Ile18tm1fsk/J (IL-18−/−), and C57BL/6J (IL-18+/+) mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Homozygous breeder pairs were used, as were newborn pups originating from multiple litters. Pups were subjected to the NEC mouse protocol as follows: mice were collected immediately after birth to prevent suckling of maternal milk. All pups were hand-fed 50 μl formula every 3 h for 3 days using the Hoshiba Nipple: Yajima style (23, 45) developed and manufactured by Meiiji Dairies (Tokyo, Japan) and stressed twice daily with asphyxia (breathing 100% nitrogen gas for 30 s) followed by cold (4°C for 5 min). Groups were designated Asbt−/− NEC (n = 12), Asbt+/+ NEC (n = 12), IL-18+/+ NEC (n = 10), and IL-18−/− NEC (n = 10) (18, 19).

**Disease Evaluation**

Pathological changes in intestinal architecture were evaluated using our previously published NEC scoring system (7, 17, 18). Histological changes were scored by a blinded evaluator and graded as follows: 0 (normal), no damage; 1 (mild), slight submucosal and/or lamina propria separation; 2 (moderate), moderate separation of submucosa and/or lamina propria and/or edema in submucosal and muscular layers; 3 (severe), severe separation of submucosa and/or lamina propria and/or severe edema in submucosal and muscular layers, region villous sloughing; 4 (necrosis), loss of villi and necrosis. Intermediate scores of 0.5, 1.5, 2.5, and 3.5 were also used to more accurately assess levels of ileal damage when necessary (6, 7, 13, 15, 16, 18). To determine the incidence of NEC, only animals with histological scores of two or greater were considered to have developed experimental NEC (6, 7, 13, 15, 16, 18).

**BA Levels**

Total ileal luminal BA levels were determined by flushing a section of distal ileum with cold PBS. After being flushed, the ileal segment was weighed. Total intraenterocyte were determined from homogenates of a weighed piece of liver as described for the intraenterocyte procedure. BA-containing supernatants were frozen at −70°C until assayed, and BA levels were determined using the Total Bile Acids Assay Kit (Diazyme, San Diego, CA) according to the manufacturer’s protocol.

**Branched DNA assay for detection of RNA.** Specific oligonucleotide probes for Cyp7a1, Cyp27a1, Bsp, Mrp2, Mrp3, Ntcp, Oat1, and Oatp4 were diluted in lysis buffer supplied by the Quantigene HV Signal Amplification Kit (Genospectra, Fremont, CA). Substrate solution, lysis buffer, capture hybridization buffer, amplifier, and label probe buffer used in the analysis were all obtained from the Quantigene HV Signal Amplification Kit.

**Table 2. Total luminal and intraenterocyte BAs in experimental NEC models**

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Luminal BAs</th>
<th>Intraenterocyte BAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>DF (12)</td>
<td>0.5 ± 0.4</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>NEC (12)</td>
<td>1.7 ± 0.7*</td>
<td>1.1 ± 0.4*</td>
</tr>
<tr>
<td>NEC (10)</td>
<td>1.9 ± 0.4</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>NEC + Chol (10)</td>
<td>0.3 ± 0.1†</td>
<td>0.2 ± 0.2*</td>
</tr>
<tr>
<td>Asbt+/+ NEC (12)</td>
<td>2.0 ± 0.2</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>Asbt−/− NEC (12)</td>
<td>2.9 ± 0.4*</td>
<td>0.3 ± 0.1*</td>
</tr>
<tr>
<td>NEC (10)</td>
<td>2.1 ± 0.6</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>NEC + anti-TNFα (10)</td>
<td>0.5 ± 0.2†</td>
<td>0.4 ± 0.1*</td>
</tr>
<tr>
<td>NEC (9)</td>
<td>1.9 ± 0.4</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>NEC + anti-IL-18 (9)</td>
<td>0.7 ± 0.3*</td>
<td>0.6 ± 0.3*</td>
</tr>
<tr>
<td>IL-18+/+ NEC (10)</td>
<td>2.9 ± 1.1</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>IL-18−/− NEC (10)</td>
<td>0.9 ± 0.3*</td>
<td>0.5 ± 0.1*</td>
</tr>
</tbody>
</table>

Data are expressed as mean μg·μl total bile acids (BAs)⁻¹·mg ileum⁻¹± SD. *P ≤ 0.05 and †P ≤ 0.01 by ANOVA.
Gene Discovery Kit (Genospectra). The assay was performed in 96-well format with 5 µg total RNA added to the capture hybridization buffer and 50 µl of the diluted probe set. The total RNA was then allowed to hybridize to the probe set overnight at 53°C. Hybridization steps were performed per the manufacturer's protocol the following day. Luminescence of the samples was measured with a Quantiplex 320 bDNA luminometer interfaced with Quantiplex Data Management Software Version 5.02.

**Protein Preparation**

Individual frozen liver samples were homogenized in a 5× volume of ice-cold homogenization buffer (50 mM Tris·HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 0.1% SDS; 1% sodium deoxycholic acid; 1% Triton X-100; 50 mM diithiothreitol; 50 µg/ml aprotinin; 50 µg/ml leupeptin; and 5 mM phenylmethylsulfonyl fluoride). The homogenates were centrifuged at 10,000 rpm for 5 min at 4°C, and the supernatant was collected. Total protein concentration was quantified using the Bradford protein assay.

**Western Blotting**

For protein analysis, samples were run on a 10–20% gradient polyacrylamide gel and were transferred to Immuno-Blot PVDF membranes (Bio-Rad). Membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20. Membranes were incubated with anti-Ntcp (kindly provided by Dr. Meenakshisundaram Ananthanarayanan, Yale School of Medicine, New Haven, CT) or anti-Mrp2 (Kamiya Biomedical, Seattle, WA) antibody overnight at 4°C. After being washed, the membranes were incubated at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody followed by enhanced chemiluminescence detection. The density of the bands was measured using ImageJ software.

**Fig. 1.** Hepatic bile acid (BA) levels and hepatic mitochondrial 27-hydroxylase (Cyp27a1) in experimental necrotizing enterocolitis (NEC) models. A: dam-fed (DF) (n = 4), NEC (n = 7) and NEC + cholestyramine (Chol) (n = 5), Asbt⁻/⁻ DF (n = 4), Asbt⁻/⁻ NEC (n = 7), Asbt⁻/⁻ DF (n = 4), and Asbt⁻/⁻ NEC (n = 8); DF (n = 4), NEC (n = 6), and NEC + anti-tumor necrosis factor (TNF)-α (n = 6); and DF (n = 4), NEC (n = 7), and anti-interleukin (IL)-18 NEC (n = 5) were evaluated for total hepatic BA levels. Asbt, apical sodium-dependent bile acid transporter. *P ≤ 0.05 vs. DF and NEC + Chol. **P ≤ 0.05 vs. DF, RLU, relative light units. B: the same groups were evaluated for Cyp27a1 RNA. #P ≤ 0.01 vs. DF. *P ≤ 0.05 vs. Asbt⁻/⁻ DF. **P ≤ 0.05 vs. Asbt⁻/⁻ DF. †P ≤ 0.05 vs. DF. 

**Fig. 2.** RNA levels of hepatic BA transporters in NEC. Livers from DF (n = 10) and NEC (n = 10) were evaluated for RNA levels of hepatic BA transporters bile salt excretory pump (Bsep), multidrug resistance-associated protein (Mrp) 2, Mrp3, sodium-dependent taurocholate-transporting polypeptide (Ntcp), organic anion-transporting polypeptide (Oatp) 2, and Oatp4. #P ≤ 0.01 by ANOVA.
conjugated secondary antibody. Proteins were visualized with a chemiluminescent system (Pierce, Rockford, IL) and exposed to X-ray film. Membranes were stripped and probed with anti-pan cadherin (Abcam, Cambridge, MA) to determine equal loading of protein. Densitometry was performed to compare protein expression between groups using Bio-Rad QuantityOne software, and data were normalized based on densitometry readings for pan-cadherin.

Statistics

Statistical analyses between groups were performed using ANOVA followed by Fisher protected least-significant difference test. The χ²-test was used to determine statistical differences in disease incidence, and the Mann-Whitney test was used to determine statistical significance of median ileal damage scores. All numerical data are expressed as means ± SD unless otherwise noted. The number of animals studied in each group was determined based on calculating sample size using a 95% confidence level.

RESULTS

Hepatic BAs and Cyp27a1 are Elevated During Development of NEC

As expected from previous publications, both the incidence and severity of NEC were significantly increased in pups subjected to the NEC protocol compared with dam-fed (DF) animals (Table 1). In addition, ileal luminal and intraenterocyte BA levels were significantly elevated in the NEC vs. DF group (Table 2). When pups were given cholestyramine to sequester luminal BAs (NEC + Chol), incidence and severity of NEC were significantly reduced, with corresponding decreases in ileal BAs (Tables 1 and 2). Hepatic BA levels were also significantly increased in the NEC group compared with DF littermates and decreased in the NEC + Chol group (Fig. 1A). Because BAs are synthesized in the liver from cholesterol via Cyp7a1 and Cyp27a1, expression of these genes was examined. Cyp27a1 was significantly increased in NEC and NEC + Chol compared with DF (Fig. 1B); Cyp7a1 levels were similar between groups (data not shown).

Ntcp and Mrp2 are Decreased in NEC

Initial studies also assessed mRNA levels of the canalicular BA transporters Bsep and Mrp2 and the basolateral transporters Ntcp, Oatp2, Oatp4, and Mrp3 in both DF and NEC groups. These transporters were chosen because they are expressed at birth. Ntcp and Mrp2 mRNA levels were significantly reduced; there were no significant changes in any of the other transporters examined (Fig. 2). We confirmed that Ntcp and Mrp2 protein levels were decreased in experimental NEC (Fig. 3). Based on these results, we concentrated the rest of the studies on Cyp27a1, Ntcp, and Mrp2.

Fig. 3. Ntcp and Mrp2 protein levels are significantly reduced in NEC. A: representative Western blots from DF and NEC groups. B: densitometry from DF (n = 12) and NEC (n = 12) groups. After normalization with pan-cadherin, mean optical density (OD) for the DF group was assigned a value of 1.0. The mean OD for the NEC group was determined relative to this number. *P < 0.05 by ANOVA.

Fig. 4. Ntcp and Mrp2 protein levels are unchanged when BAs are sequestered during development of NEC. Newborn rats were subjected to the NEC protocol and given cholestyramine (Chol) to sequester luminal BAs or given vehicle alone (NEC). A: representative Western blots from NEC and NEC + Chol groups. B: densitometry from NEC (n = 10) and NEC + Chol (n = 10) groups. After normalization with pan-cadherin, mean OD for the NEC group was assigned a value of 1.0. The mean OD for the NEC + Chol group was determined relative to this number. Statistical analysis was performed using ANOVA.
Elevated Ileal BA Levels are not Responsible for Reduced Hepatic Ntcp and Mrp2 in NEC

To determine if increased ileal BAs play a role in the reduction of Ntcp and Mrp2 in experimental NEC, neonatal rats were subjected to the NEC protocol with or without cholestyramine. When ileal BAs were removed, there was no statistically significant difference in either Ntcp or Mrp2 protein (Fig. 4). Because cholestyramine can sequester more than just BAs, we also developed NEC in mice in which the Asbt gene was knocked out (Asbt/−/− NEC). Because these mice lack the transporter that is responsible for the majority of BAs transferred into the enterocyte, enterohepatic circulation is disrupted. The incidence and severity of NEC was significantly reduced in the Asbt/−/− NEC group compared with wild-type (Asbt+/+/−) NEC (12). To determine if elevated TNF-α plays a role in the decreased Ntcp and Mrp2 in NEC, anti-TNF-α antibody was injected in neonatal rats subjected to the NEC protocol. Administration of anti-TNF-α in the rat NEC model significantly reduced the incidence and severity of disease (Table 1), as well as ileal luminal and intraenterocyte BA levels (Table 2). Ntcp protein levels were significantly increased in the group given anti-TNF-α (Fig. 6). However, there was no change in levels of Mrp2. These data suggest that a factor other than TNF-α is responsible for the downregulation of Mrp2 in experimental NEC. Hepatic BAs were significantly increased in the NEC group compared with DF pups, but there was no decrease in animals in which TNF-α was neutralized. Cyp27a1 was increased in both groups subjected to the NEC protocol (Fig. 1).

Neutralization of TNF-α Normalizes Ntcp, but not Mrp2

Because disruption of BA recirculation did not alter the levels of Ntcp and Mrp2, we examined other factors overproduced in experimental NEC that could be responsible for the reductions of these transporters. Hepatic TNF-α is elevated in NEC (15), and TNF-α has been shown to downregulate both Ntcp (11) and Mrp2 (2, 21). We have also shown that neutralization of TNF-α markedly reduces the severity and incidence of NEC (12). To determine if elevated TNF-α plays a role in the decreased Ntcp and Mrp2 in NEC, anti-TNF-α antibody was injected in neonatal rats subjected to the NEC protocol. The mean OD for the Asbt/−/− NEC group was assigned a value of 1.0. The mean OD for the Asbt/−/− NEC group was determined relative to this number. Statistical analysis was performed using ANOVA.
Elimination of IL-18 Results in Upregulation of Mrp2

We have previously shown that, in addition to TNF-α, increases in the proinflammatory cytokine IL-18 play a crucial role in the development of NEC (15, 16, 18). To evaluate if IL-18 could affect the levels of Mrp2 in NEC, we injected NEC-induced rats with or without anti-IL-18 to neutralize this cytokine. When subjected to the NEC protocol, rats injected with IL-18 (NEC + anti-IL-18) had significant decreases in incidence and severity of disease (Table 1) and luminal and intraenterocyte BAs (Table 2) compared with rats given sham antibody (NEC). In addition, rats given anti-IL-18 had similar levels of Ntcp protein but significantly increased Mrp2 compared with those injected with sham antibody (Fig. 7). Again, pups subjected to the NEC protocol had increased levels of hepatic BAs compared with DF, with Cyp27a1 also elevated in both NEC and NEC + anti-IL-18 groups (Fig. 1). To confirm that IL-18 can reduce expression of Mrp2 in NEC, we utilized IL-18 knockout mice. When subjected to the NEC protocol, Mrp2 levels in mice lacking functional IL-18 (IL-18−/− NEC) were significantly higher than wild-type mice (IL-18+/+ NEC), results similar to those observed in the rat NEC model when IL-18 was neutralized (Fig. 8).

DISCUSSION

Elevated ileal BAs have been shown to play a critical role in the development of NEC, and ileal BA transporters are dysregulated in both experimental and human disease (14, 17, 30). In the data presented herein, we show that, while the hepatic BA transporters Ntcp and Mrp2 are downregulated in experimental NEC, neither elevated ileal nor hepatic BAs appear to be responsible for these decreases. The data do suggest that elevated TNF-α and IL-18 play a significant role in the diminished levels of Ntcp and Mrp2, respectively. In addition, with the lone exception of Asbt−/− mice, hepatic BA levels were significantly elevated in animals subjected to the NEC protocol; this increased expression was significantly lowered only in the NEC + Chol group. Cyp27a1 levels were also significantly increased in all groups subjected to the NEC protocol. In experimental groups with reduced incidence and severity of disease (NEC + Chol, Asbt−/− NEC, NEC + anti-TNF, and NEC + anti-IL-18), Cyp27a1 levels remained elevated.

Transport of ileal BAs has profound effects on hepatic BA transport and production. We have shown that while BAs accumulate in the ileum and liver during NEC, decreased ileal BA levels in groups with less disease continue to have elevated hepatic BA levels. Thus, hepatic BA accumulation alone does not seem to influence disease progression. Here, we show that, during development of experimental NEC, expression of the hepatic BA efflux transporters Bsep and Mrp2 is not increased.

Fig. 7. Mrp2 protein, but not Ntcp, is significantly increased after injection of anti-IL-18 during development of NEC. Newborn rats were subjected to the NEC protocol and injected with either sham antibody (NEC) or anti-IL-18 (NEC + anti-IL-18). A: representative Western blots from NEC and NEC + anti-IL-18 groups. Ab, antibody. B: densitometry from NEC (n = 9) and NEC + anti-IL-18 (n = 9) groups. After normalization with pan-cadherin, mean OD for the NEC group was assigned a value of 1.0. The mean OD for the NEC + anti-IL-18 group was determined relative to this number. *P ≤ 0.05 by ANOVA.

Fig. 8. Mrp2 protein, but not Ntcp, is significantly increased in IL-18 knockout mice during NEC. Newborn IL-18+/+ and IL-18−/− mice were subjected to the NEC protocol. A: representative Western blots from IL-18+/+ NEC and IL-18−/− NEC groups. B: densitometry from IL-18+/+ NEC (n = 10) and IL-18−/− NEC (n = 10) groups. After normalization with pan-cadherin, mean OD for the IL-18+/+ NEC group was assigned a value of 1.0. The mean OD for the IL-18−/− NEC group was determined relative to this number. *P ≤ 0.05 by ANOVA.
which could provide an explanation for the lack of increased BAs in the jejunum we have previously reported (16). For all experimental models tested, with the exception of hepatic BAs in the Asbt−/− mice, there were significantly higher levels of hepatic BA and Cyp27a1 in DF compared with NEC groups. The lack of a significant change in hepatic BA levels in the Asbt−/− mice may reflect the changes in enterohepatic circulation that occur with a complete knock out of the main transporter responsible for active transport of BAs across the apical membrane of enterocytes. Furthermore, the only group in which the incidence and severity of NEC was decreased that also had a decrease in hepatic BA levels was the group treated with cholesteramine. It is interesting to note that this group was, similar to the Asbt−/− mice, the only one with which enterohepatic circulation was specifically interfered, rather than alterations in proinflammatory mediators.

In the neutral pathway, Cyp7a1 is the rate-limiting factor in conversion of cholesterol to BAs. The acidic pathway utilizes Cyp27a1 and plays a more significant role in BA production in neonates (29, 37). This may explain why, in these studies, Cyp27a1, but not Cyp7a1, was altered in experimental NEC. Previously, we have shown that BA levels in the jejunum of DF and NEC rats are similar (17). Coupled with the current findings of increased hepatic BAs in NEC, these data suggest that the downregulation of Mrp2, and, perhaps, the static levels of Bsep, contribute to an accumulation of BAs in the liver. Examination of temporal changes in these parameters at earlier time points may elucidate when the increased hepatic BAs occur.

While proinflammatory mediators have many effects on the expression of BA transporters (2, 33, 39) and Cyp7a1 (28), stimulation of cytokines with LPS does not upregulate Cyp27a1 (5). The data presented herein show that neutralization of TNF-α and IL-18, cytokines induced by LPS, has no effect on expression of Cyp27a1. This may partially explain why Cyp27a1 reductions coincided with reductions in hepatic BA levels only in animals in which luminal BAs were removed (NEC + Chol). Interestingly, dexamethasone increases Cyp27a1 expression in neonatal rats (29), the circulating BA pool is increased in neonates whose mothers were treated with dexamethasone while pregnant (44), and Pietz et al. observed that steroids contribute to an increased risk of developing NEC (36). We have previously shown that corticosterone levels are significantly elevated in neonatal rats subjected to the NEC protocol (3). Thus, increased Cyp27a1 seen in experimental NEC models may be influenced by increased glucocorticoids rather than cytokines or BAs.

Most work that examines hepatic BA transporters has been accomplished in adult systems. For example, in adult rats fed cholic acid, Ntcp was decreased while Mrp2 was increased (8), and cholesteramine treatment diminished the cholic acid-induced increase in Mrp2 (25). While little is known about how the neonatal liver responds to BAs during inflammatory processes, it has been shown that bile formation is limited at birth (41), and hepatic BA transport mechanisms are developmentally regulated (20, 32, 34, 40, 43). In our current work, when ileal luminal and intraenterocyte BA levels are significantly elevated in NEC, both Ntcp and Mrp2 levels are decreased, and cholesteramine treatment had no significant effect on either transporter. Our data show that TNF-α and IL-18, not BAs, contribute to decreased Ntcp and Mrp2, respectively, in models of experimental NEC. TNF-α and IL-18 are important in NEC

Fig. 9. Model for the gut-liver axis in experimental NEC. In response to risk factors for NEC (prematurity, formula feeding, hypoxia, etc.), Cyp27a1 is elevated (1), and BAs produced in the liver are transported into the intestine via Bsep and Mrp2 (2). In the ileum, Asbt is significantly elevated, leading to accumulation of BAs within ileal enterocytes. This accumulation promotes the initiation of ileal damage and production of inflammatory mediators, particularly IL-18 (3). Ileal-derived IL-18 and some BAs travel back to the liver via the portal circulation; these BAs are transported into the liver via Ntcp (4). Intestinally derived IL-18 activates Kupffer cells (KC) in the liver (5), which produce additional IL-18 as well as TNF-α. IL-18 in the liver downregulates Mrp2 (6), which, when coupled with elevated Cyp27a1, may contribute to BA accumulation in the liver. Hepatic-derived TNF-α and IL-18 enter the intestine (7), enhancing ileal inflammation and damage. Ntcp is downregulated by hepatic TNF-α, either directly from activated Kupffer cells or indirectly through the enterohepatic circulation (8), allowing even less BAs to be recirculated. The accumulated hepatic BAs contribute to increased hepatic inflammation, and, along with elevated proinflammatory mediators, lead to the increased markers of liver injury seen in experimental NEC. Ost, organic solute transporter.
pathogenesis (12, 15), and TNF-α has been shown to downregulate Ntcp, Oatp4, and Mrp2 mRNA levels (2, 9, 11, 21). Previous work has shown that the proinflammatory cytokine IL-1β decreases NTCP and MR2 in human hepatocytes (27), Mrp2 in mice (21), and Ntcp in rats (11). In addition, anti-IL-1β treatment prevents downregulation of Mrp2 in rats with endotoxemia (9). Our current studies suggest that Mrp2 can be downregulated by IL-18 in NEC. To our knowledge, this is the first report of IL-18 influencing levels of hepatic BA transporters. Interestingly, both IL-18 and IL-1β are synthesized as a 23-kDa inactive precursor that is cleaved by caspase-1 to produce the active form. Because our experiments were conducted only in neonatal animals, it remains unclear if IL-18 can downregulate Mrp2 in older animals. Additional work using nonneonatal models of inflammation and/or primary hepatocyte cultures from adult and neonatal sources will help to clarify this distinction. It is intriguing that, while our studies show that IL-18 and TNF-α have different effects on Mrp2 and Ntcp, both cytokines utilize NF-κB signaling pathways. Thus, it seems likely that, during NEC development, there are additional signaling pathways that control these differential effects of these cytokines on Mrp2 and Ntcp. Further elucidation of the mechanisms by which these transporters are downregulated in our experimental models is currently ongoing.

Several studies have shown a link between inflammation/oxidative stress and the regulation of membrane transporters at distal tissue sites. In a rat model of intestinal ischemia-reperfusion injury, the expression of Mrp2 was found to be reduced both in the intestine as well as the liver (35). The decrease in Mrp2 expression coincides with elevated serum levels of the proinflammatory cytokine IL-6, suggesting a possible role for this cytokine in the regulation of Mrp2 expression. Additionally, in the rat model of trinitrobenzene sulfonic acid-induced colitis, intestinal and hepatic Mrp2 expression was also diminished with increased oxidative stress being a possible mechanism (26). Further characterization of the role of oxidative stress in the regulation of hepatic Mrp2 in NEC is needed, however.

We propose the following working hypothesis for the interaction between the gut-liver axis in experimental NEC (Fig. 9): In response to risk factors for NEC (prematurity, formula feeding, hypoxia, etc.), Cyp27a1 is elevated and BAs produced in the liver are transported into the intestine via Bsep and Mrp2. In the ileum, Asbt is significantly elevated, leading to accumulation of BAs within ileal enterocytes (17, 19). This accumulation promotes the initiation of ileal damage and production of inflammatory mediators, particularly IL-18 (16, 18). Ileal-derived IL-18 and some BAs travel back to the liver via portal circulation (15, 17); these BAs are transported into the liver via Ntcp. Intestinally derived IL-18 activates Kupffer cells in the liver, which produce additional IL-18 as well as TNF-α (15). Heptic IL-18 downregulates Mrp2, which, when coupled with elevated Cyp27a1, may contribute to BA accumulation in the liver. Hepatic-derived TNF-α and IL-18 enter the intestine, enhancing ileal inflammation and damage (15). Ntcp is downregulated by hepatic TNF-α, either directly from activated Kupffer cells or indirectly through enterohepatic circulation, allowing even less BAs to be recirculated. The accumulated hepatic BAs contribute to increased hepatic inflammation (1), and, along with elevated proinflammatory mediators, lead to the increased markers of liver injury seen in experimental NEC, such as Nos2, nitrotrosyne, and serum transaminases (15). Additional accumulation of ideal BAs further exacerbates intestinal damage and inflammation at the site of NEC injury. Thus, both ileal and hepatic BA transporters contribute to development of experimental NEC.

In conclusion, we showed that the hepatic transporters, Ntcp and Mrp2, are downregulated, whereas Cyp27a1 is upregulated in rodent models of NEC. Our results demonstrate that increased levels of the proinflammatory cytokines TNF-α and IL-18 in experimental NEC may play a role in the regulation of Ntcp and Mrp2, respectively. These data also suggest the importance of the gut-liver axis when therapeutic modalities for NEC are developed.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


