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PII: S2772-5723(22)00160-1
DOI: https://doi.org/10.1016/j.gastha.2022.09.008
Reference: GASTHA 189

To appear in: Gastro Hep Advances

Received Date: 18 July 2022
Revised Date: 9 September 2022
Accepted Date: 19 September 2022

Please cite this article as: Tong H, Bernardazzi C, Curiel L, Xu H, Ghishan FK, The expression of NHE8 in liver and its role in carbon tetrachloride induced liver injury, Gastro Hep Advances (2022), doi: https://doi.org/10.1016/j.gastha.2022.09.008.

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The expression of NHE8 in liver and its role in carbon tetrachloride induced liver injury

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Conflict of interest: The authors disclose no conflicts of interest.

Authors’ contribution:
Huan Tong: Performed experiments, interpreted data, wrote the manuscript.
Claudio Bernardazzi: Performed experiments, interpreted data.
Leslie Curiel: Performed genotyping.
Hua Xu: Conceived and designed the study, helped interpret data, edited/revised the manuscript.
Fayez K Ghishan: Conceived and designed the study, edited the manuscript.

Fundings: This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant R01-DK-3023890.

Data transparency statement: Data, analytic methods, and study materials will be available on request to interested researchers.

Ethical statement: The corresponding author, on behalf of all authors, jointly and severally, certifies that their institution has approved the protocol for any investigation involving humans or animals and that all experimentation was conducted in conformity with ethical and humane principles of research.

Short title: The role of NHE8 in liver
**Abbreviations:** ALT, alanine aminotransferase; AST, aspartate aminotransferase; BW, body weight; CCl₄, carbon tetrachloride; H&E, hematoxylin-eosin; NHE, sodium-hydrogen exchanger; WT, wild-type; KO, knockout; SE, standard error.
ABSTRACT

Background and Aims Sodium-hydrogen exchanger 8 (NHE8) is expressed in array of tissues and has pleotropic functions beyond simply exchanging sodium and hydrogen across cell membrane. This study investigates the expression pattern of liver NHE8 and its roles in carbon tetrachloride (CCl4)-induced liver injury.

Methods NHE8 expression pattern was investigated in mouse livers of different ages, and in HepG2 cells. CCl4 was given to mice to determine NHE8 expression in CCl4 induced liver injury. TNFα and IL1β were used to treat HepG2 cells to evaluate their effect on NHE8 expression. The CCl4-induced acute and chronic liver injury were also used in NHE8KO mice to determine the role NHE8 deficiency in liver injury.

Results NHE8 was mainly detected in the peripheral area of hepatocytes in mouse liver, and in HepG2 cells. The liver NHE8 expression was 47% ofNHE1, and liver NHE8 expression was the lowest at suckling age and reached plateau at 4weeks of age. Like DSS-colitis reduced intestine NHE8, CCl4-induced acute liver injury also inhibited NHE8 expression. The absence of NHE8 in the liver displayed abnormal hepatocyte morphology and has elevated expression of IL1β and Lgr5. However, unlike NHE8 deficiency enhance DSS-induced colon tissue damage, the absence of NHE8 in the liver did not exacerbate CCl4-induced liver injury. Although both TNFα and IL1β were elevated in CCl4-induced liver injury, they could not inhibit NHE8 expression in hepatocytes, which is in contrast with TNFα-mediated NHE8 inhibition in the intestine.

Conclusion Liver NHE8 has unique roles that are different from the intestine.

Key words: sodium-hydrogen exchanger, hepatocyte, liver injury
Introduction

Sodium-hydrogen exchangers (NHEs) are a super family of various proteins with complex physiological functions. As a vital member of this family, NHE8 is widely expressed in various organs, including heart, lung, skeletal muscle, intestine, kidney, liver, testis and placenta. In line with NHE1, NHE2 and NHE3, the gastrointestinal NHE8 is expressed on the cellular membrane, mainly on the apical surface. NHE8 expression in the gastrointestinal tract is subject to spatiotemporal regulation. Higher NHE8 levels were detected in human stomach, duodenum, and ascending colon; whereas jejenum, ileum and colon have more NHE8 expression in mice. NHE8 expression is also much higher in the stomach and jejunum in young mice than those of adult mice. Recent data suggest that NHE8 has a role in gastric bicarbonate secretion and gastric mucosal protection to reduce the propensity of gastric ulcer, maintenance of mucin production from goblet cells and antimicrobial peptides production from Paneth cells to maintain intestinal mucosal integrity, and suppression of Wnt-β-catenin activation to prevent the development of colitis-associated cancer.

Despite these advances, the role of NHEs in the liver received less attention. Few studies were published on this topic. NHE1 expression has been studied previously in the liver. Loss of NHE1 could attenuate both de novo lipogenesis and hepatic stellate cell activation, up-regulate farnesoid X receptor, peroxisome proliferator-activated receptor γ and its ligand PGC1α, sestrin 2, and down-regulate liver X receptor α and acetyl CoA carboxylases 1 and 2, thus limiting the stress induced by high-fat diet, and protecting the liver against NAFLD pathogenesis. Furthermore, administration of NHE inhibitor FR183998 in rats prevented fulminant liver failure via inhibiting NF-κB activation. Although NHE8 mRNA was detected in the liver in our earlier study, the expression pattern, and the role of NHE8 in liver injury is still yet to be fully understood. Therefore, the current work aims to investigate these two issues.

Materials and Methods

Animals

The NHE8KO mice were obtained from the offspring of NHE8+/− breeding pairs in a SwissWebster background as previously described. Male NHE8 wild-type (NHE8WT) and NHE8 knockout (NHE8KO)
mice were housed separately in polypropylene cages. Liver tissues were collected for histology assessment, RNA purification, and protein extraction. Sera were collected and stored at -80°C for further analysis. To introduce liver injury, 8-week-old NHE8WT and NHE8KO mice received peritoneal injection(s) of carbon tetrachloride (CCl4, catalog No. 02671, Sigma-Aldrich, St Louis, MO) at the dosage of 0.2μl/g body weight (BW). This dose was tested in our preliminary experiment which could induce liver damage without lethality in the first 24 hours after CCl4 administration. For acute injury, 8-week-old mice were given a single peritoneal injection of CCl4. Sera and liver tissues were collected 24 hours after CCl4 injection. For chronic injury, 8-week-old mice were given a peritoneal injection of CCl4 twice weekly for 4 weeks. Sera and liver tissues were collected 72 hours after the last CCl4 injection. CCl4 was dissolved in olive oil at the volume ratio of 6:94. Control mice were injected with the same volume of olive oil. All the animal works were approved by the University of Arizona Institutional Animal Care and Use Committee.

**Cell culture**

HepG2 cells were obtained from ATCC (Manassas, VA) and cultured in modified Eagle’s minimal essential medium (MEM; catalog No. 11095-080, Thermo Fisher Scientific, Waltham, MA), containing 10% fetal bovine serum, 100U/ml penicillin and 100μg/ml streptomycin, at 37°C in a 5% CO2 atmosphere. Medium was changed every three days. Cells were passaged when 80% of cell density was reached. For cytokine treatment, cells were treated with various concentrations of cytokines for 24 hours and used for RNA and protein preparation.

**RNA purification and quantitative PCR**

Total RNA was isolated from tissue and cells using a Directzol kit (Genesee Scientific, El Cajon, CA). The gene expression was measured using TaqMan probes (Applied Biosystems, Foster City, CA) or SYBR Green probes (Thermo Fisher Scientific, Waltham, MA) on LightCycler96 (Roche Diagnostics, Indianapolis, IN). Liver NHE8, TNFα, IL1β and Lgr5 were measured using the TaqMan reaction system. Collagen 1α (Col 1α) was measured using the SYBR Green system. TATA-binding protein (TBP, for TaqMan probes) and β-actin (for SYBR Green probes) were used as an endogenous reference to normalize gene expression levels.
Western blot

Protein from tissues and cells was extracted in RIPA Buffer containing Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA). BCA protein assay (Thermo Fisher Scientific, Waltham, MA) was used to measure protein concentration. For western blot, total protein (40μg/lane) was used. The primary antibodies used in this study were a rabbit anti-NHE8 antibody (dilution 1:2000), a rabbit anti-caspase-3 antibody (dilution 1:1000; catalog No. 9662S; Cell Signaling Technology Inc., Danvers, MA), and a monoclonal mouse anti-β-actin antibody (dilution 1:6000; catalog No. A5316, Sigma-Aldrich, St Louis, MO). The secondary antibodies were an anti-mouse/rabbit IgG (for NHE 8 detection, dilution 1:12000; Roche Diagnostics, Indianapolis, IN), and a mouse anti-mouse IgG (for β-actin detection, dilution 1:40000; catalog No. A9044; Sigma-Aldrich). Blots were detected with BM Chemiluminescence Western Blotting Substrate kit (catalog No. 11520709001; Roche Diagnostics, Indianapolis, IN) and then exposed on G-BOX Imaging System (Syngene, Frederick, MD). ImageJ software (National Institutes of Health, Bethesda, MD) was used for densitometric analysis.

Immunohistochemical and immunofluorescent staining

Liver tissue sections (4-5μm thick) and cultured human hepatocyte cells (HepG2) were used to detect the expression of NHE8 protein. The NHE8 antibody was used at a dilution of 1:200. For tissue samples, a goat anti-rabbit IgG (dilution 1:500; catalog no. SK-4105; Vector Laboratories, Inc, Burlingame, CA) was used as the secondary antibody, and a DAB detection kit (catalog no. SK-4105; Vector Laboratories, Inc, Burlingame, CA) was used for signal detection. For cell samples, Alexa Fluor 647 goat anti-rabbit IgG (dilution 1:400; catalog No. A27040, Thermo Fisher Scientific, Waltham, MA) was used as the secondary antibody. Stained tissue sections and cells were then observed under microscope (EVOS FL Auto, Thermo Fisher Scientific, Waltham, MA).

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) measurement
The colorimetric activity assay kits for ALT (catalog No. 700260, Cayman Chemical, Ann Arbor, MI) and AST (catalog No 701640, Cayman Chemical) were used to measure the activity of ALT and AST following the manufacturer’s manual. The reactions were recorded at 340nm for 5 minutes using a SpectraMax M3 microplate reader (Molecular Devices, LLC, San Jose, CA). The activity of ALT and AST was calculated according to the method provided in the kits.

Liver necrosis and fibrosis evaluation

For necrosis assay, liver sections (4~5μm-thick) from acute CCl4 injury were stained with hematoxylin-eosin (H&E). H&E staining was done by the University of Arizona Pathology laboratory (Tucson, AZ). The area lacking live cells is considered as necrotic area. For fibrosis assay, liver sections from chronic CCl4 injury were stained with sirius red to evaluate liver fibrosis. The area with sirius red positive stain is considered as fibrotic area. The necrotic foci (without live cells under H&E staining) and fibrotic foci (stained by sirius red) were delineated as region of interest, and the area of region of interest was measured using the Image J program. Three images from each sample were analyzed, and the average of three readings was used as the final value for the sample.

Statistical analysis

Data were presented as Mean ± SE (standard error). One-way ANOVA was used to compare the differences between groups. p value less than 0.05 was considered statistically significant.

Results

NHE8 expression in the liver

NHE8 expression and localization were investigated in both mouse liver tissue and human hepatocyte-origin HepG2 cells. As shown in Figure 1A, a majority of the NHE8 protein was detected in the peripheral area of the hepatocytes in adult mouse liver. In human liver HepG2 cells, NHE8 protein was detected both in the cytosol and at the plasma membrane. Like NHE8 expression in the intestine, liver NHE8 expression is subject to the developmental regulation. In the liver, the expression of NHE8 mRNA
was the lowest at 2 weeks of age and reached a plateau at 4 weeks of age in mice (Figure 1B). The same pattern was also detected at the protein level, where the expression of NHE8 protein was the lowest at 2 weeks of age and plateaued at 4 week of age (Figure 1C).

The detection of membrane expressing NHE isoforms in the liver

To address if NHE isoforms that are known to be expressed at the basal/apical membrane in the intestinal epithelial cells (NHE1, 2, 3, 8) are also expressed in the liver, RNA was extracted from mouse liver and Real-time PCR was applied to detect the expression levels of these NHE isoforms in NHE8WT liver. As shown in Figure 2A, only NHE1, NHE3, and NHE8 but not NHE2 mRNA were detected in mouse livers. The expression level was higher for NHE1 and NHE8 while NHE3 was barely detectable in the liver. Comparing with the expression level of NHE1, the expression levels of NHE8 and NHE3 were ~46.6% and 0.3%, respectively. Furthermore, in the absence of NHE8, the expression of NHE1 and NHE3 was not significantly altered in the liver (Figure 2B).

Liver changes in the absence of NHE8

Tissue histology was evaluated to detect the effect of NHE8 on liver morphology. Although loss of NHE8 did not significantly change the liver weight (1.59±0.08g in WT vs 1.67±0.07g in KO) or the liver/BW ratio (5.61±0.91% in WT vs 4.47±0.03% in KO), it altered the morphology of hepatocytes. As shown in Figure 2C, a significantly higher number of mice with hypochromatic hepatocytes were observed in NHE8KO mice compared with NHE8WT mice (84.6% in KO mice vs 44.4% in WT mice; n=9-13 mice in each group, p=0.047). Since the hypochromatic appearance was similar to hepatocyte swelling change, the expression of TNFα and IL1β was analyzed to assess liver inflammation. Lgr5 expression in the liver is considered as a marker following liver damage 10, and Lgr5+ hepatocytes have been shown to contribute to liver homeostasis 11. Therefore, Lgr5 expression was also analyzed to assess liver injury. As shown in Figure 2D, the expression of IL1β was significantly induced in NHE8KO livers (2.41±0.55 in KO vs 1.13±0.20 in WT, n=8 mice in each group, p=0.029) although the expression of TNFα was insignificantly increased in NHE8KO liver. The expression of Lgr5 was also increased in NHE8KO liver (1.32±0.12 in
KO vs 1.02±0.10 in WT, n=6 mice in each group, p=0.042). The expression of both Caspase-3 mRNA (1.13±0.09 in KO vs 1.04±0.10 in WT, n=10 mice in each group, p=0.261) and Caspase-3 protein (1.69±0.19 in KO vs 1.20±0.26 in WT, n=3 mice in each group, p=0.110) were not altered between NHE8WT and NHE8KO livers (Figure 2E). Furthermore, the expression levels of TNFα and IL1β in older NHE8KO livers (12- to 20-weeks old) did not increase further compared with that of in 8-weeks-old NHE8KO livers (data not shown).

*Expressions of hepatic NHE8 under CCl4 treatment*

NHE8 expression has been shown to be inhibited during intestinal inflammation in mice, it was interesting to know if the expression of NHE8 in the liver is also affected by liver injury. Since CCl4 is a commonly used chemical reagent in liver injury studies, we treated normal mice with CCl4 for 24 hours to introduce acute liver injury. Liver NHE8 mRNA and protein were measured in CCl4-injected mice as well as oil-injected mice. As shown in Figure 3, CCl4 injection decreased not only NHE8 mRNA by approximately 50% (0.52±0.04 in CCl4 group vs 1.02±0.06 in oil group, n=6 in each, p<0.001), but also NHE8 protein level by 76.9% (0.13±0.05 in CCl4 group vs 0.54±0.08 in oil group, n=6 in each, p=0.003).

*Effect of NHE8 in CCl4-induced acute liver injury*

As liver NHE8 expression was down-regulated in normal mice after expose to CCl4, the next question was whether NHE8 deficiency alters the response in mice with CCl4-induced liver injury. NHE8KO mice and NHE8WT mice were thereafter given a single dose of CCl4 injection. Liver tissues and blood samples were collected 24 hours after CCl4 injection. Although the liver weight was similar between NHE8WT and NHE8KO mice at 24 hours after CCl4 injection, the liver/BW ratio was significantly higher in NHE8KO mice compared with NHE8WT mice (5.08±0.07 % in KO vs 4.56±0.16% in WT, n=6 mice in each group, p=0.01). The levels of ALT and AST were also elevated in CCl4-treated NHE8WT and NHE8KO mice (Table 1). Tissue histology assessment revealed that both NHE8WT mice and NHE8KO mice displayed liver damage after CCl4 injection, but NHE8KO mice displayed less necrotic areas compared with NHE8WT mice (48.4% in WT vs. 40.8% in KO, n=6 mice in
each group, p=0.027) (Figure 4A). Comparing with oil-injected NHE8WT livers, the gene expression of TNFα and IL1β was significantly increased in CCl4-injected NHE8WT livers, CCl4-injected NHE8KO livers and oil-injected NHE8KO livers. Interestingly, no further increase in the expression of TNFα and IL1β was observed in CCl4-injected NHE8KO livers compared with oil-injected NHE8KO livers (Figure 4B).

**Effect of NHE8 in CCl4-induced chronic liver injury**

To study the role of NHE8 in chronic CCl4 liver injury, mice were given CCl4 twice a week for 4 weeks. During the 4-week-long experiment, lethality was observed in both CCl4-treated NHE8WT and NHE8KO mice. The survival rate was 41.7% and 63.6% for NHE8WT and NHE8KO, respectively. Although no difference was observed in the liver weight or the liver/BW ratio between NHE8WT and NHE8KO mice in control group, the liver weight in NHE8KO mice was heavier than that of in NHE8WT mice in the chronic CCl4-treated group (2.29±0.16g in KO vs 1.91±0.02g in WT, n=5-7 mice in each group, p=0.036). The similar observation was also seen in liver/BW ratio in NHE8KO mice (6.35±0.25% in KO vs 5.31±0.01% in WT, n=5-7 mice in each group, p=0.008). The levels of ALT were significantly elevated in both CCl4 treated NHE8WT and NHE8KO mice, but the levels of AST were not elevated in CCl4-treated NHE8WT or NHE8KO mice (Table 1). Since chronic CCl4 liver injury may induce fibrosis, we analyzed fibrosis markers in these mice. As shown in Figure 5A, a mild fibrosis was observed in liver sections in both CCl4-treated NHE8WT and NHE8KO mice. The calculated fibrosis area was similar in CCl4-treated NHE8WT and NHE8KO mice (2.6±0.2% in WT vs 2.6±0.4% in KO). A similar change on the expression of TNFα and IL1β was also detected in CCl4-treated NHE8WT mice and CCl4-treated NHE8KO mice (Figure 5B). Moreover, the expression of collagen 1α, a marker for fibrosis, was dramatically increased in both CCl4-treated NHE8WT and NHE8KO mice (Figure 5C).

**Effect of cytokines on NHE8 expression in HepG2 cells**

NHE8 expression has been shown to be downregulated by TNFα in the intestinal epithelial cells and goblet cells. Therefore, it was interesting to know if the expression of NHE8 in the liver is also
subject to the regulation of inflammatory cytokines. Since TNFα and IL1β expression were elevated in the liver after CCl4 treatment, and previous studies showed that HepG2 responded to TNFα and IL1β challenges \(^{14-17}\), we decided to treat HepG2 cells with these two cytokines to determine the effect of cytokine on NHE8 expression. Interestingly, the cytokine treatment did not change the expression levels of NHE8 mRNA and NHE8 protein compared with non-treated cells. The expression of NHE8 mRNA was similar in TNFα-treated HepG2 cells compared with in non-treated HepG2 cells. The NHE8 protein abundancy was also similar between TNFα-treated HepG2 cells and non-treated HepG2 cells (Figure 6A). The same result was also observed in IL1β treated cells. The NHE8 mRNA expression and the NHE8 protein abundancy remained the same regardless of whether HepG2 cells were exposed to IL1β or not (Figure 6B).

**Discussion**

Four NHE family members (NHE1, 2, 3, 8) are expressed at the apical or basolateral membrane in the gastrointestinal epithelia. These NHEs play important physiological roles in the digestive system, ranging from sodium absorption, cell volume regulation, and intracellular pH regulation, to mucosal protection \(^1\). So far, only NHE1 and NHE3 are the most studied NHEs in the liver. NHE1 is expressed in the nuclear membrane and nucleoplasm in the liver \(^{18}\). Loss of NHE1 has been shown to protect liver against NAFLD pathogenesis mediated by high-fat diet \(^8\). NHE3 is located on the apical membrane of cholangiocytes in the liver and plays a role in fluid absorption from the lumens \(^{19}\). Although our earlier work has detected NHE8 mRNA expression in the liver, the localization of this protein and its role in the liver are unknown.

In our current study, we found that NHE8 is highly expressed in the liver. Unlike the intestinal NHE8 where it is located on the apical membrane of the enterocytes, the NHE8 is mainly detected inside the hepatocytes in mouse liver and in human hepatocyte cells. During developmental maturation, liver NHE8 expression was at the lowest level at suckling age, and peaked after weaning, following the similar pattern seen in the mouse colon \(^{20}\). Among the four membrane-expressing NHE isoforms (NHE1, 2, 3 and 8), NHE8 expression is relatively abundant in the liver while the expression of NHE3 is very low and the
expression of NHE2 is absent. In the intestine, NHE2 and NHE3 could compensate for the loss of NHE8, but no such compensation was observed in the liver. Furthermore, NHE1 does not compensate for the loss of NHE8 in the liver. These observations suggest that NHE8 might bear a unique physiological function in the liver.

To understand the possible role of NHE8 in the liver, we analyzed liver morphology in NHE8KO mice. Although liver weight and liver/BW ratio were similar between NHE8WT and NHE8KO mice, abnormal morphology of hepatocytes was observed in NHE8KO mice. The enlarged hepatocytes were seen in the absence of NHE8 in liver along with the elevated proinflammatory cytokine expression. These observations suggest that loss of NHE8 might introduce hepatocyte damage in the liver, but this damage was not potent enough to induce apoptosis in the liver as Caspase-3 did not increase significantly in the absence of NHE8. We have reported that NHE8 deficiency resulted in increased Lgr5 expression in the intestine. Here, we observed a similar pattern in the liver. An approximately 1.3-fold increase in Lgr5 mRNA expression was detected in NHE8KO liver. This result suggests that injured liver cells in the absence of NHE8 might stimulate a liver repair process since Lgr5 proliferative stem cells are absent in the homeostatic liver but are activated by liver injury. Altogether, our observations suggest that loss of NHE8 in liver induces mild inflammation which may contribute to the altered liver homeostasis.

CCl4 is a potent liver toxin and is widely used to induce liver damage. The commonly used CCl4 dose in liver injury studies ranges from 0.6µl/g BW to 1µl/g BW CCl4. In our study, we found that 0.2µl/g BW CCl4 still induces liver damage but does not have fatal effect in our experimental mice in the first 24 hours after CCl4 injection. Using this lower CCl4 dose in our acute liver injury study, we observed a dramatic reduction of NHE8 mRNA expression and NHE8 protein abundancy. Therefore, NHE8 expression is susceptible to CCl4-induced liver injury. We have previously reported that loss of NHE8 in the intestine increases the severity of DSS colitis in mice, therefore, we wanted to test if NHE8 deficiency will further deteriorate the liver injury induced by acute CCl4 treatment. Our data showed that NHE8KO mice displayed liver injury that was not worse than NHE8WT mice after exposure to one dose of CCl4 injection. The activities of ALT and AST in NHE8KO mice were elevated but slightly lower than that of NHE8WT mice. The expression of liver TNFα and IL1β in CCl4-treated NHE8KO mice was also
increased to the level observed in CCl4-treated NHE8WT livers. These observations imply that loss of NHE8 function does not exacerbate CCl4-induced acute liver injury. It is interesting to notice that oil injection significantly increased the expression of TNFα and IL1β in NHE8KO liver, suggesting a possible role of NHE8 in lipid metabolism in the liver; although more studies will be conducted to address the role of NHE8 in lipid metabolism.

Liver fibrosis has been linked to persistent injury and recovery. The development of liver fibrosis involves the accumulation of extracellular matrix secreted by myofibroblasts derived from epithelial cells, mesenchymal stromal cells, fibrocytes, hepatic stellate cells and/or portal fibroblasts upon liver chronic injury. To address whether NHE8 plays any role in liver fibrosis development induced by chronic CCl4 treatment, we gave mice CCl4 injections twice a week for 4 weeks. At the end of the experiment, Sirius red staining detected a very small fibrosis area (< 3%) in the liver in CCl4-treated NHE8WT and CCl4-treated NHE8KO mice. The lack of noticeable fibrosis area in our study is most likely due to the low CCl4 dose and the short duration used in our study, because fibrosis formation requires higher dosage CCl4 injection and longer treatment duration. Although no obvious fibrosis area was seen in chronic CCl4-injected NHE8KO and NHE8WT mice, a significant increase in the expression of collagen 1α was detected in these mice. When comparing cytokine gene expression, we noticed that chronic CCl4 treatment increased the expression of TNFα but not IL1β in both NHE8WT and NHE8KO livers. This may be attributed to the role of TNFα in mitigating liver injury. These observations suggest that loss of NHE8 in liver does not contribute to the severity of chronic CCl4 liver injury in mice.

Previous studies have showed that NHE8 expression is regulated by inflammation in the intestine. Intestinal inflammation reduced NHE8 expression at both mRNA and protein levels. Mechanistic study showed that TNFα inhibits NHE8 expression through reducing NHE8 basal promoter activation both in the intestinal epithelial cells and in goblet cells. Here, we observed that the expression of NHE8 was reduced in CCl4-induced liver injury. But mechanistic study showed that TNFα and IL1β treatment does not alter the expression of NHE8 at both gene expression and protein expression levels in hepatocytes. As TNFα and IL1β treatment has no effect on NHE8 expression in hepatocytes, the CCl4-induced NHE8 reduction in livers is most likely due to CCl4-induced necrosis in hepatocytes.
Together with previous studies on NHE8 expression during the intestinal inflammation, these observations suggest that cytokine-mediated NHE8 expression regulation is tissue specific.

In summary, our current study identified the expression of NHE8 in the liver and that NHE8 expression is inhibited in CCl4-induced acute liver injury. Like NHE8 deficient in the intestine, loss of NHE8 expression results in mild liver inflammation and possible altered liver homeostasis. Unlike NHE8 deficiency in the intestine, NHE8 deficiency in the liver did not change the outcome of CCl4-induced acute or chronic liver injury, while NHE8 deficiency in the intestine increased the susceptibility of DSS-colitis. Furthermore, liver NHE8 expression was not regulated by inflammatory cytokines while the intestinal NHE8 expression was downregulated by inflammatory cytokine. Therefore, the liver NHE8 has unique roles that are different with the intestinal NHE8.
References


Figure legends

Figure 1. NHE8 expression and localization in the liver

(A) NHE8 protein localization in mouse liver (top) and human hepatocytes (bottom). Liver sections from 8-week-old mice were stained with NHE8 antibody and detected by DAB method (brown color indicates NHE8 protein). HepG2 cells were reacted with NHE8 antibody and detected with fluorescence-conjugated anti rabbit IgG (Red color indicates NHE8 protein, blue color indicates nuclei). Sections were observed under EVOS FL Auto microscope.

(B) NHE8 mRNA expression in mouse liver at different ages. Liver tissues were collected from 2-, 4-, 8-, and 12-week-old mice. RNAs were isolated from tissues and used for Real-time PCR to quantitate the expression level of NHE8. Data are represented as means ± SE. * P ≤ 0.05 2wk vs other age groups.

(C) NHE8 protein expression in mouse liver at different ages. Liver tissues were collected from 2-, 4-, 8-, and 12-week-old mice. Total protein was extracted and used for western blot detection. Data are represented as mean±SE. * p ≤ 0.05 2wk vs other age groups.

Figure 2. The expression of NHE isoforms in liver and effect of NHE8 deficiency in liver

(A) NHE1, 3 and 8 mRNA expression in normal liver. Liver tissues were collected from 8-week-old mice. RNAs were isolated from tissues and used for Real-time PCR to quantitate the expression level of targeted NHE isoforms. Data are represented as mean±SE. * p ≤ 0.05 NHE8 and NHE3 vs NHE1; # p ≤ 0.05 NHE3 vs NHE8 and NHE1.

(B) NHE1, 3 and 8 mRNA expressions in NHE8WT and NHE8KO livers. Liver tissues were collected from 8-week-old NHE8WT and NHE8KO mice. RNAs were isolated from tissues and used for Real-time PCR to quantitate the expression level of targeted NHE isoforms. Data are represented as mean±SE.* p < 0.05 WT vs KO.

(C) Morphological changes of liver tissue in NHE8WT and NHE8KO mice. Liver tissues were collected from 8-week-old mice and tissue sections were subjected H&E staining. Sections were observed under EVOS FL Auto microscope.
(D) TNFα, IL1β, and Lgr5 mRNA expressions in NHE8WT and NHE8KO livers. Liver tissues were collected from 8-week-old NHE8WT and NHE8KO mice. RNAs were isolated from tissues and used for Real-time PCR to quantitate the expression level of target genes. Data are represented as mean±SE. * p<0.05 WT vs KO for IL1β, # p<0.05 WT vs KO for Lgr5.

(E) Caspase-3 expression in NHE8WT and NHE8KO livers. Liver tissues were collected from 8-week-old NHE8WT and NHE8KO mice. RNAs were isolated from tissues and used for Real-time PCR to quantitate the expression level of Caspase-3 gene. Total protein was extracted and used for western blot detection. Data are represented as mean±SE.

Figure 3. NHE8 expression in NHE8WT mice with CCl4-induced acute liver injury

(A) NHE8 mRNA expressions in NHE8WT liver. Liver tissues were collected from 8-week-old NHE8WT mice at 24 hours either received oil or CCl4 injection. RNAs were isolated from tissues and used for Real-time PCR to quantitate the expression level of target genes. Data are represented as mean±SE. * p<0.05 CCl4 group vs oil group.

(B) Liver NHE8 protein expressions. Total protein lysates were prepared from liver tissues collected from 8-week-old NHE8WT mice at 24 hours either received oil or CCl4, and used for western blot analysis. Data are represented as mean±SE. * p<0.05 CCl4 group vs oil group.

Figure 4. Effect of NHE8 deficiency in CCl4-induced acute liver injury

(A) Hematoxylin-eosin staining and liver necrosis area of liver. Liver tissues were collected from CCl4-treated mice and tissue sections were subjected H&E staining. Sections were observed under EVOS FL Auto microscope. Black arrows indicate some necrosis foci.

(B) TNFα and IL1β expressions in acute liver injury livers. Liver tissues were collected from CCl4-treated and oil-treated mice. RNA was isolated and used for Real-time PCR to compare the fold induction of TNFα and IL1β expressions in CCl4 group over oil group. Data are represented as mean±SE. * p<0.05 NHE8WT CCl4 group vs NHE8WT oil group, † p<0.05 NHE8WT oil group vs NHE8KO oil group.
Figure 5. Effect of NHE8 deficiency in CCl4-induced chronic liver injury.

(A) Hematoxylin-eosin staining and Sirius red staining of liver. Liver sections (4~5μm-thick) from chronic CCl4 injury were stained with hematoxylin-eosin (H&E) for morphological observation. Sirius red staining was used to evaluate liver fibrosis. Tissue sections were observed under EVOS FL Auto microscope. The area of fibrotic area was analyzed using Image J program.

(B) The expression of TNFα and IL1β in the liver. RNA was extracted from tissues and used for Real time PCR to compare the fold induction of target gene in CCl4 group over oil group. Data are represented as mean±SE. * p<0.05 NHE8WT CCl4 group vs NHE8WT oil group, # p<0.05 NHE8KO CCl4 group vs NHE8KO oil group.

(C) The expression of Col1α and αSMA in the liver. RNA was extracted from tissues and used for Real time PCR to compare the fold induction of target gene in CCl4 group over oil group. Data are represented as mean±SE. * p<0.05 NHE8WT CCl4 group vs NHE8WT oil group, # p<0.05 NHE8KO CCl4 group vs NHE8KO oil group.

Figure 6. Effect of cytokine on NHE8 expression in HepG2 cells

HepG2 cells were treated with various concentrations of TNFα and IL1β for 24 hours. Cells were harvested for RNA extraction and protein purification. Real-time PCR was used to detect the expression of NHE8 mRNA. Western blot was used to detect NHE8 protein abundancy. Data are represented as mean±SE.

(A) The effect of TNFα on NHE8 expression

(B) The effect of IL1β on NHE8 expression
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<th>Table 1. Changes in liver injury</th>
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*a p<0.05 WT Oil vs WT CCl4, b p<0.05 KO Oil vs KO CCl4, c p<0.05 WT CCl4 vs KO CCl4.