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Original Paper

Mice Deficient in Cyp4a14 Have An Increased Number of Goblet Cells and Attenuated Dextran Sulfate Sodium-Induced Colitis

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Key Words
Colitis • Cyp4a14 • Goblet cells • NADPH oxidase

Abstract
Background/Aims: Cyp4a14 is a member of cytochrome P450 (Cyp450) enzyme superfamily that possesses NADPH monooxygenase activity, which catalyzes omega-hydroxylation of medium-chain fatty acids and arachidonic acid. Study suggests that down-regulation of Cyp4a14 has an anti-inflammatory response in intestine. The present study was to test the function of Cyp4a14 in dextran sulfate sodium (DSS)-induced colitis. ***Methods:*** Female Cyp4a14-knockout (KO) and wild-type (WT) mice were treated with DSS for 6 days to induce colitis. The colon of mice was histologically observed by hematoxylin and eosin (H&E) and periodic acid Schiff (PAS) staining. The serum malondialdehyde (MDA), an endogenous indicator of oxidative stress, was chemically measured. Proinflammatory and NADPH oxidase

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genes were examined by quantitative polymerase chain reaction (qPCR). ***Results:*** Cyp4a14-KO mice had a significantly higher number of goblet cells in the colon and were more resistant to DSS-induced colitis compared with the WT mice. The DSS-treated KO mice had lower levels of MDA. Consistent with the milder inflammatory pathological changes, DSS-treated KO mice had lower levels of IL-1β, IL-6 and TNF-α mRNA in the liver and the colon. Moreover, the colon of DSS-treated Cyp4a14-KO and WT mice had higher mRNA levels of two members of NADPH oxidases, Nox2 and Nox4, suggesting that both Nox2 and Nox4 are inflammatory markers. By contrast, DSS-treated WT and KO mice had drastically decreased epithelium-localized Nox1 and dual oxidase (Duox) 2 mRNA levels, coinciding with the erosion of the mucosa induced by DSS. ***Conclusion:*** These results suggests a hypothesis that the increased goblet cell in the colon of Cyp4a14-KO mice provides protection from mucosal injury and Cyp4a14-increased oxidative stress exacerbates DSS-induced colitis. Therefore, Cyp4a14 may represent a potential target for treating colitis.

Introduction

Inflammatory bowel disease (IBD) is comprised of ulcerative colitis and Crohn's disease. IBD is a refractory condition characterized by chronic inflammation involving ulcers and mucosal erosion of the lower gastrointestinal (GI) tract [1]. Although the exact causes of IBD remain unclear, multifactorial mechanisms, including genetics, immunity, microbiome and environment, contribute to the pathogenesis of IBD [2]. A variety of inflammatory mediators, such as cytokines, growth factors, GI hormones and enzymes producing oxidative stress and lipid peroxidation, are involved in the pathogenesis of IBD [3]. The colon is highly enriched in goblet cells that secrete mucus, primarily mucin-2 (Muc-2), to form a protective barrier against colonization and invasion of luminal microbes [4]. Mice with disrupted Muc-2 gene expression may develop spontaneous colitis and are susceptible to colon cancer development [5, 6]. Decreased goblet cell numbers have been associated with active Crohn's disease and ulcerative colitis in humans [7].

Cytochrome P450s (CYPs) are members of a superfamily of heme proteins, which play a key role in the redox metabolism of endogenous and exogenous molecules [8]. Some CYPs mediate the process of inflammation via converting fatty acids to pro- or anti-inflammatory mediators [9, 10]. The human or rodent CYP4A monooxygenases catalyze the ω-hydroxylation of medium-chain fatty acids and arachidonic acid [11, 12, 13]. CYP4As are involved in the pathogenesis of inflammation, hypertension and cancer [14]. While humans only have one functional CYP4A11 gene in CYP4A family [15], mice have four, namely Cyp4a10, 12a, 12b and 14, which are predominantly expressed in the liver and kidney [16, 17]. Under inflammatory conditions, the expression of CYPs tends to be suppressed [18]. In dextran sulfate sodium (DSS)-induced colitis, the levels of Cyp4a10 and Cyp4a14 mRNA and Cyp4a protein were shown downregulated in the liver [19]. Similarly, in *Citrobacter rodentium*-induced mouse colitis, both Cyp4a10 and Cyp4a14 mRNA and protein levels were downregulated in the liver and kidney [12, 20, 21]. The fact that Cyp4a10- or Cyp4a14-knockout (KO) mice exhibited attenuated colitis induced by *C. rodentium* infection also suggests that down-regulation of Cyp4a gene expression is an anti-inflammatory response. The aim of the present study was to test the function of Cyp4a14 in DSS-induced colitis.

Cyp4a14 is a major enzyme that induces lipid peroxidation and lipid uptake in the liver [13, 22]; thus, it contributes to hepatic steatosis and fibrosis in mice fed with a high-fat diet or a methionine-and-choline-deficient diet. 20-Hydroxyeicosatetraenoic acid (20-HETE) is one of the primary eicosanoids metabolized by the CYP4A subfamily and can induce the expression of the gp91 and p22phox subunits of Nox2, a member of NADPH oxidase (NOX, Nox in animal) in cardiomyocytes [23, 24]. Since oxidative stress clearly plays a key role in the pathogenesis of IBD, as shown by spontaneous ileocolitis developing in mice deficient in antioxidant enzymes [25, 26], we also evaluated the effect of Cyp4a14 on several members

of Nox in the mouse colon. The NADPH oxidase family has 7 members in humans, namely NOX1-5 and dual oxidases (DUOX)-1 and -2 [27]. Mice express all these members except Nox5 with four members expressed in the colon. NOX1 is highly expressed in the crypt epithelium and plays a role in physiological and inflammatory processes, as well as tissue repair in the colon [28, 29, 30]. DUOXs may play an essential role in host defense against microbes in the GI and respiratory epithelium [31, 32]. NOX2 is present in phagocytes and is crucial for antimicrobial defense [33, 34]; NOX4 is abundant in the kidney and is involved in the pathogenesis of inflammation and colon cancer [35, 36].

In this study, it was confirmed that Cyp4a14 promotes inflammation in DSS-induced colitis. Mice with disrupted Cyp4a14 gene expression had attenuated inflammatory responses to DSS treatment, exhibiting decreased levels of lipid peroxidation in the plasma and a higher number of goblet cells in the colon compared with wild-type (WT) mice. Our results suggest that hepatic Cyp4a14 increases systemic lipid peroxidation and exacerbates DSS-induced colon tissue injury. Therefore, Cyp4a14 may represent a potential target for the treatment of colitis.

Materials and Methods

Animal experimental procedures

WT and *Cyp4a14* KO mice on a 129/SvJ background originally generated by J Capdevila (Vanderbilt University, USA) [37], were kindly provided by YF Zhou (Shenzhen University, China) and housed at the Animal Center of the First Affiliated Hospital of Henan University of Science and Technology. The mice were kept under a 12-hour light/dark cycle and had free access to water and food. The genotype of Cyp4a14-KO mice was confirmed as previously description [38]. Considering the differential gender-dependent expression of Cyp4a14 and its phenotypic sequelae [37], only female mice were studied here since Cyp4a14 is female-predominant in the liver [13]. A total of 40 Cyp4a14-KO and WT mice, aged 6-8 weeks and weighing 18-25 g, were divided into four groups (10 mice per group) as follows: i) Control WT, ii) Control KO, iii) DSS-treated WT and iv) DSS-treated KO. The DSS-treated mice were administered sterilized 3.0% DSS (36-50 kDa molecular weight, MP Biomedicals, USA) in Millipore purified water, while the control mice were only given water. Mouse body weight, stool consistency and presence of rectal bleeding were assessed. The disease activity index (DAI) score, graded on a scale of 0 to 4, was slightly modified from a previously published scoring system [39]. Mice losing ≥20% of their initial body weight were euthanized according to institutional guidelines. At 6 days post-DSS treatment, all mice were euthanized by cervical dislocation under diethyl ether anesthesia. The blood, colons and livers of mice were collected. The length of colons was measured. All experiments were performed according to the protocol approved by the Animal Experimentation Committee of The First Affiliated Hospital of Henan University of Science and Technology.

Analysis of mouse colon histology by hematoxylin and eosin (H&E) and periodic acid Schiff (PAS) staining

Mouse colon histology was analyzed by H&E and PAS staining. The sections from the distal third of colon were scored for inflammation pathology using an 11-point system modified from our previous studies [40, 41]. These include lymphocyte and neutrophil infiltration (0-3 points), Paneth cell or goblet cell degranulation (0-2 points), epithelium reactivity, such as crypt distortion (0-3 points) and inflammatory foci (0-3 points). The threshold for severe acute inflammation corresponds to a score of 6 points. PAS staining was also conducted on sections from the distal third of the colon using periodic acid solution and Schiff's reagents (cat. no. 395B, Sigma-Aldrich, USA). The number of goblet cells was obtained from a field counting 100 absorptive epithelial cells from the control mice, and five fields were selected from each mouse; the number of goblet cells in DSS-treated mice was only counted in five fields from each mouse due to the destruction of the epithelium and loss of absorptive cells.

Determination of MDA levels in the serum

Serum was separated from the blood by centrifugation for 10 min at 3, 000 rpm at 4°C and stored at -80°C until analysis. The serum MDA levels were assessed using a MDA kit (Beyotime Biotechnology, Shanghai, China) reacting with thiobarbituric acid (TBA). The MDA concentration was calibrated using a standard curve using the MDA provided by the manufacturer.

Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from tissues treated with TRIzol Reagent (Invitrogen, USA), according to the manufacturer's instructions. Total RNA (2 μg) was used for cDNA synthesis. PrimeScript™ RT Master Mix was used in a 40-μl reaction mixture, incubated at 37°C for 15 min, 85°C for 5 sec and 4°C for 10 min to generate cDNA. qPCR was performed using an Applied Biosystems 7500 real-time PCR system (Life Technologies) in a 25-μl qPCR reaction mixture containing 2 μl cDNA mixture, 12.5 μl 2X SYBR Premix Ex TaqTMII (Takara, Japan), 8.5 μl H_2O and 1 μM primers. The PCR primers of Cyp4a14, pro-inflammatory cytokines and Noxs were purchased from Sangon Biotech Co., Ltd (Shanghai, China). The primer sequences are listed in Table 1 were designed by using Primer3.0 website [42]. The cDNA was amplified by incubation at 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec, 58°C for 34 sec and 95°C for 15 sec. As actin mRNA was not changed in Cyp4a14-KO and WT mice in this study (data not shown) therefore actin was used as an internal control. Each sample was analyzed in triplicate.

Statistical analysis

Data are reported as the mean ± standard deviation. A two-way ANOVA test was used when comparing the means of normally distributed parametric data and a Mann-Whitney U test was performed when comparing non-parametric, not normally distributed data. SPSS 20.0 (Chicago, USA) was used for all statistical analyses.

Table 1. Primers sequence for quantitative real-time PCR

mRNA	Gene	Primer sequence	Amplicon (bp)
NM-008361.4	IL-1β	F: 5′-TGC CAC CTT TTG ACA GTG ATG - 3′ R: 5′-AAG GTC CAC GGG AAA GAC AC - 3′	220
NM-031168.1	IL-6	F: 5′-TGG TAC TCC AGA AGA CCA GAG - 3′ R: 5′-AAC GAT GAT GCA CTT GCA GA - 3′	128
NM-013693.3	TNF-α	F: 5′-AGG GTC TGG GCC ATA GAA CT - 3′ R: 5′- CCA CCA CGC TCT TCT GTC TAC - 3′	161
NM-172203.2	Nox1	F: 5′-TTCCTCACTGGCTGGGATAG- 3′ R: 5′-AGTCCGAGGGCCACATAAGA- 3′	189
NM-007807.5	Nox2	F: 5′- TGT TTT CAT TTC CTC ATC AGA AG-3′ R: 5′-CCA ACC ACA CCA GAA TGA CA- 3′	132
NM-015760.5	Nox4	F: 5′-TCT GGA AAA CCT TCC TGC TG- 3′ R: 5′-CCG GCA CAT AGG TAA AAG GA- 3′	143
NM-177610.2	Duox2	F: 5′-TGGTCCCAGCAATTATCTATG- 3′ R: 5′- CCACTGCCCTGATTTGTACTC- 3′	155
NM-007393	β-actin	F: 5′-GGC TGT ATT CCC CTC CAT CG - 3′ R: 5′- CCA GTT GGT AAC AAT GCC ATG - 3′	154

Results

Mice deficient in Cyp4a14 had milder colitis induced by DSS compared with WT mice

DSS-treated WT mice exhibit suppressed hepatic Cyp4a14 gene expression [12]. To test whether Cyp4a14 monooxygenase exacerbates DSS-induced colitis, we compared the phenotypes between Cyp4a14-KO and WT mice. Both DSS-treated WT and KO mice started to show disease signs at day 3, with lower water and food intake, weight loss, loose stool or diarrhea, and rectal bleeding (Fig. 1A and B). However, the KO mice had a significantly lower disease activity index (DAI) at day 6. In addition, DSS-treated KO mice had a longer colon compared with the treated WT mice (Fig. 1C). As expected, the colons of untreated control Cyp4a14-KO and WT mice had similar lengths, and were both longer compared with those of the DSS-treated mice.

Cyp4a14-KO mouse colons had more goblet cells and milder DSS-induced colitis compared with the WT mice

On histological examination following staining with hematoxylin and eosin (H&E) or periodic acid Schiff (PAS) reagents, the colons of Cyp4a14-KO mice had more goblet cells compared with WT mice in both the control and DSS-treated groups (Fig. 2A and B). The higher number of goblet cells may have protected KO mice from DSS-induced injury. DSS treatment caused extensive exfoliation of crypt epithelial cells, crypt destruction and loss and focal transmural inflammation in the colon (Fig. 2A). As expected, the pathological score in DSS-treated WT mouse colons was significantly higher compared with that in DSS-treated Cyp4a14-KO mice (Fig. 1D).

Cyp4a14-KO mice had lower levels of lipid peroxidation compared with WT mice with or without DSS treatment

Cyp4a14 is a major enzyme inducing hepatic lipid peroxidation and contributing to hepatic steatosis and fibrosis [13, 22]. Cyp4a14 also promotes hypotension, suggesting that

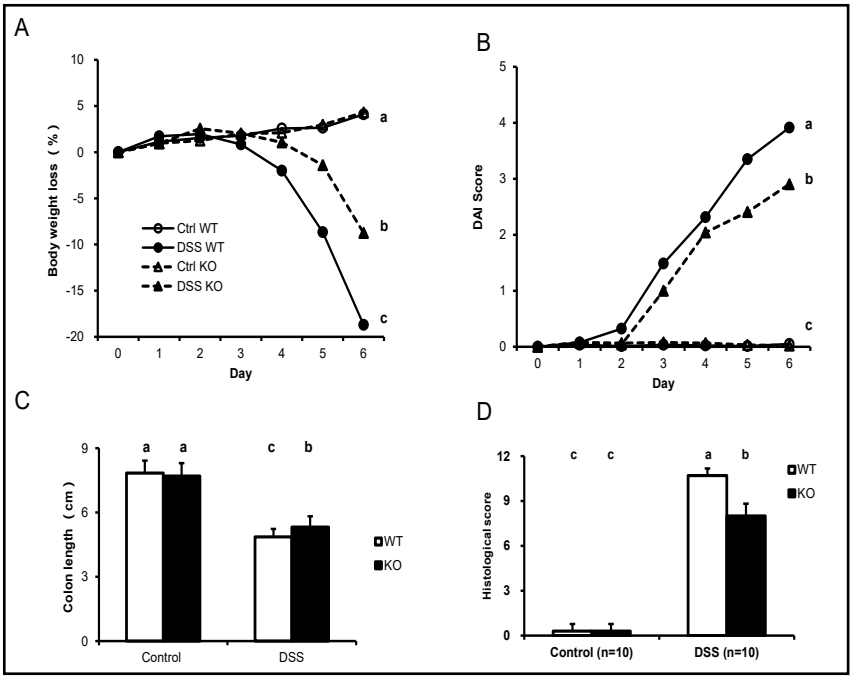

Fig. 1. Comparison of DSS effect on Cyp4a14-KO with WT mice. (A) Body weight changes presented as the percentage of initial weight during the 6 day treatment. (B) Disease activity index (DAI) scored during the course of treatment. (C) Colon length measured at the end of 6 day treatment. (D) Histological score determined on the H&E-stained colon sections. The statistical comparisons were done on Day 6. The groups with different letters indicate that they are significantly different. The group with the letter a has a higher mean than the group with the letter b, which has a higher mean than the group with the letter c, i.e., a>b>c, P<0.05.

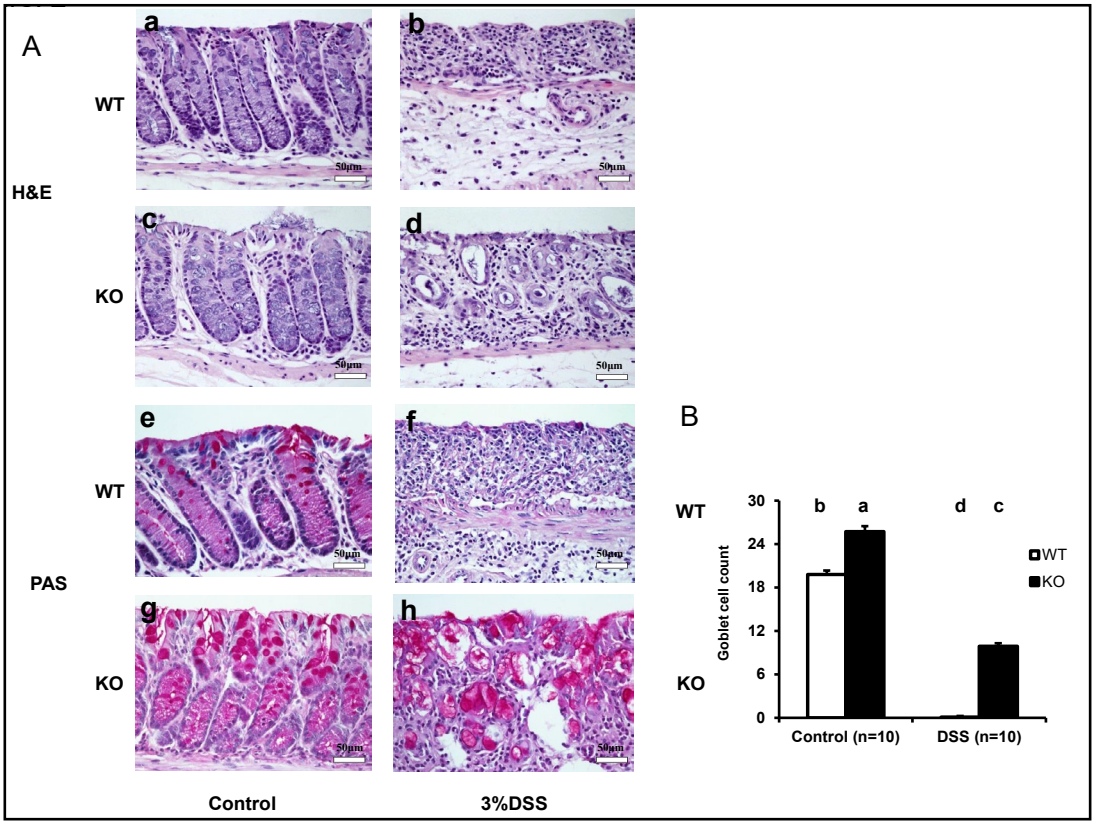

Fig. 2. Histology of mouse colon stained with H&E and periodic acid Schiff (PAS) reagents. (A) Panels a-d are H&E stained colon sections from WT control (a), WT DSS-treated (b), Cyp4a14 KO control (c) and KO DSS-treated (d) mice. Analysis is shown in Fig. 1D. Panels e-h are PAS-stained colon sections from WT control (e), WT DSS-treated (f), KO control (g) and KO DSS-treated (h) mice. PAS stains carbohydrate as pink color. Magnification, x400. (B) Goblet cell number analyzed on the PAS-stained sections. The groups with different letters indicate that the means are different, where a>b>c>d, P<0.05.

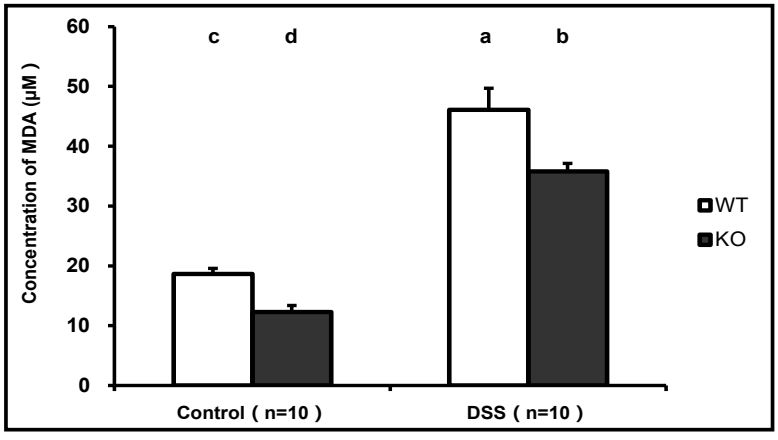

Fig. 3. Comparison of MDA levels between Cyp4a14-KO and WT mice with and without DSS treatment. MDA levels were increased following DSS treatment. Cyp4a14-KO mice had lower MDA levels compared to WT mice with or without DSS treatment. The groups with different letters indicate that the means are different, where a>b>c>d, P<0.05.

hepatic and renal monooxygenases have systemic effects [37]. We quantified systemic lipid peroxidation by analyzing the level of MDA in the serum. In both the control and DSS-treated groups, the sera of Cyp4a14-KO mice had a significantly lower level of MDA compared with WT mice (Fig. 3). The fact that DSS treatment drastically increased serum MDA levels in both Cyp4a14-KO and WT mice supports the hypothesis that colitis is associated with increased lipid peroxidation.

Table 2. Cytokine mRNA levels in mouse colon and liver

	IL-1β		IL-6		TNF-α	
	Colon	Liver	Colon	Liver	Colon	Liver
WT Ctrl	1.0±0.1 [b]	1.0±0.1 [c]	1.0±0.0 [c]	1.0±0.1 [c]	1.0±0.1 [c]	1.0±0.1 [b]
KO Ctrl	1.9±0.0 [b]	1.1±0.1 [c]	0.8±0.0 [c]	1.1±0.1 [c]	0.9±0.1 [c]	0.9±0.1 [b]
WT DSS	286.0±144.7 [a]	8.2±1.7 [a]	103923.9±67037.9 [a]	84.6±16.2 [a]	6.2±0.6 [a]	2.4±0.2 [a]
KO DSS	211.4±49.1 [a]	3.1±0.2 [b]	7458.1±3053.5 [b]	4.7±0.3 [b]	2.7±0.2 [b]	3.7±0.2 [a]

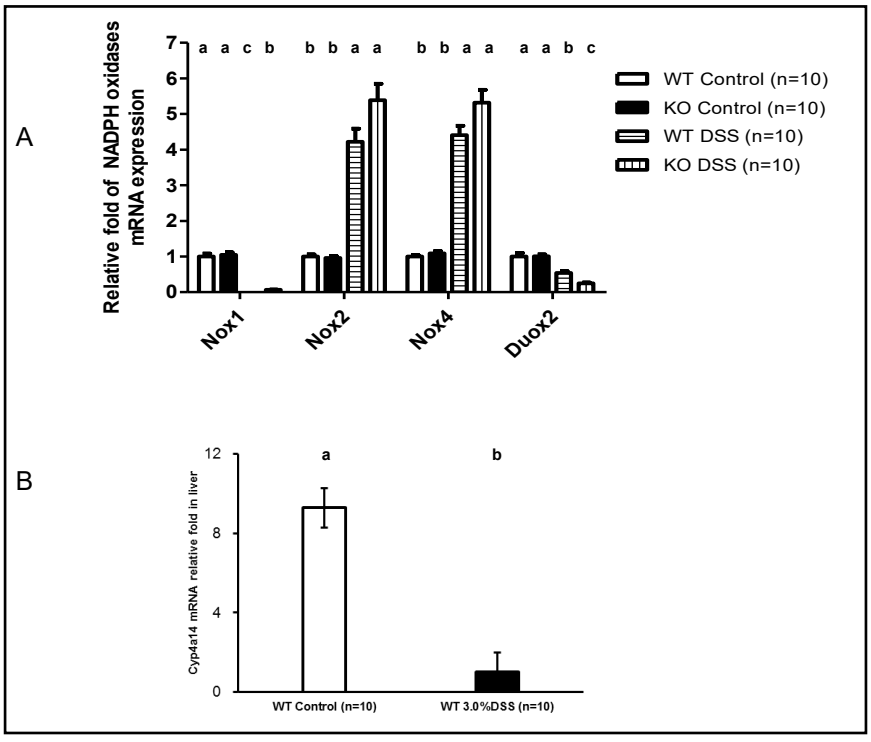

Fig. 4. Comparison of the mRNA levels of NADPH oxidase Nox1, Nox2, Nox4 and Duox2 in the colon and Cyp4a14 in the liver with or without DSS treatment. (A) Comparison of the levels of Nox1, Nox2, Nox4 and Duox2 mRNA in the colon of Cyp4a14-KO and WT mice with or without DSS treatment. Comparison of the mean was made for each gene individually. (B) Comparison of the levels of Cyp4a14 mRNA in the liver of WT mice with and without DSS treatment. The groups with different letters have different means, where a>b, P<0.05.

Cyp4a14-KO mice had lower levels of pro-inflammatory cytokine mRNAs in the colon and the liver after DSS treatment compared with WT mice

DSS treatment increased the mRNA levels of IL-1β, IL-6 and TNF-α in the liver and colon of Cyp4a14-KO and -WT mice (Table 2). The WT mice had higher levels of these pro-inflammatory cytokines in either the colon (TNF-α), the liver (IL-1β), or both (Il-6), compared with KO mice after DSS treatment. There was no difference in the liver and colon mRNA levels of these cytokines between untreated WT and KO mice. This result supports the fact that DSS-induced colitis is milder in Cyp4a14-KO compared with that in Cyp4a14-WT mice.

CYP4A14 had a minimal effect on NADPH oxidase gene expression in the colon

Cyp4a hydrolyzes arachidonic acid into 20-HETE, which induces the expression of the gp91 and p22phox subunits of Nox2in cardiomyocytes [23, 24]. Thus, the mRNA levels of Nox1, Nox2, Nox4 and Duox2 were also analyzed in the colons of Cyp4a14-KO and -WT mice. DSS treatment markedly decreased the mRNA levels of Nox1 and Duox2, two epithelium-specific NADPH oxidases (Fig. 4A). This drastic decrease in epithelium-expressed Nox1 and Duox2 mRNA levels is likely due to the loss of colonic epithelium induced by DSS treatment.

The mRNA levels of Nox2 and Nox4 were found to be the same in the colon compared between Cyp4a14-KO and WT mice with or without DSS treatment, suggesting that Cyp4a14 does not affect Nox2 or Nox4 gene expression in the colon (Fig. 4A). However, both Nox2 and Nox4 mRNAs were markedly elevated in the inflamed colon after DSS treatment. This finding suggests that both Nox2 and Nox4 are inflammatory markers expressed by non-epithelial cells.

Similar to other reports, the hepatic Cyp4a14 mRNA levels were decreased by more than 9-fold after DSS-treatment in the WT mice (Fig. 4B). As expected, Cyp4a14 mRNA expression was not detected in the colon (data not shown), as this gene is mainly expressed in the liver and kidney [16].

Discussion

In this study, it was demonstrated that mice deficient in Cyp4a14 developed milder DSS-induced colitis compared with WT mice. Cyp4a14 is predominantly expressed in the liver and kidney, and is a major enzyme involved in lipid peroxidation. Unlike other Cyp4a isozymes, Cyp4a14 does not catalyze arachidonic acid directly to produce 20-HETE, an inflammation promoter [43, 44, 45, 46]. However, Cyp4a14 appears to induce Cyp4a10 and Cyp4a12 gene expression, which produce lipid peroxidation, since Cyp4a14-KO mouse liver exhibits significantly reduced mRNA levels of other Cyp4as [13]. The lipid peroxidation generated in the liver and kidney apparently has a systemic impact, affecting the colon, as well as other tissues, such as the cardiovascular and pulmonary systems [46, 47]. A systemic effect of lipid peroxidation induced by Cyp4a14 was also detected in the present study, as Cyp4a14-KO mice exhibited lower levels of plasma MDA, a stable product of lipid peroxidation, compared with WT mice with and without DSS treatment.

Mice deficient in Cyp4a14 also had more goblet cells in the colon compared with WT mice, which may also contribute to their resistance to DSS-induced colitis. To the best of our knowledge, this is the first time description of this phenomena. The increased goblet cells in the colon of these mice could be a consequence of a developmental effect of Cyp4a14 deficiency since from our study and others there is no direct evidence that Cyp4a14 has promotion function of proliferation of colonic goblet cell. Cyp4a14 not only plays an important role in lipid metabolism, but also indirectly leads to oxidative stress [48]. Cyp4a14-induced oxidative stress affects the redox-sensitive cell differentiation pathways in the intestinal epithelium. Other studies have demonstrated that decreased oxidative stress in Nox1-KO mice (since Nox1 produces superoxide in the crypt epithelium) resulted in increased levels of goblet cells, presumably by modulating the Wnt/β-catenin and Notch1 signaling pathways, which regulate differentiation of epithelial cells into either absorptive enterocytes or secretory cells (such as goblet cells) [49]. Our results suggest a hypothesis that Cyp4a14-generated oxidative stress affects colonic epithelial cell differentiation.

We also analyzed the effect of Cyp4a14 on the four members of the NADPH oxidase family in the colon. Evidently, Cyp4a14 does not affect Nox gene expression, since the mRNA levels of all four members, Nox1, Nox2, Nox4 and Duox2, were the same in the colon of both KO and WT mice, with and without DSS treatment. However, DSS markedly decreased the mRNA levels of Nox1 and Doux2, two epithelium-specific enzymes that are involved in the pathogenesis of IBD [26, 50]. The downregulation of Nox1 and Duox2 gene expression is most likely due to the loss of epithelial cells. By contrast, Nox2 and Nox4 gene expression levels were markedly increased in inflamed colons. Since Nox2 is highly expressed in the inflammatory cells (neutrophils and monocytes), elevation of Nox2 mRNA level may simply reflect the increase in infiltrating inflammatory cells. The fact that Nox4 expression was also highly induced by DSS treatment suggests that it is also expressed in inflammatory or immune cells.

In conclusion, the present study suggests that hepatic or renal Cyp4a14 increased systemic lipid peroxidation, which exacerbated DSS-induced colitis. The Cyp4a14-KO mice

were found to have more goblet cells in the colon, which provides additional protection from DSS-induced mucosal injury. The mechanism of consequent dual effects of lipid peroxidation and colon epithelial cell differentiation in Cpy4a14-deficient mice were worth to further study and might make Cyp4a14 an attractive therapeutic target for the treatment of colitis.

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Disclosure Statement

The authors declare no conflict of interest.

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