The xanthine oxidase inhibitor febuxostat suppresses development of nonalcoholic steatohepatitis in a rodent model

Yusuke Nakatsu,1* Yasuyuki Seno,1* Akifumi Kushiyama,2 Hideyuki Sakoda,3 Midori Fujishiro,4 Aya Katasako,1 Keiichi Mori,1 Yasuka Matsunaga,1 Toshiaki Fukushima,1 Ryuhei Kanaoka,1 Takeshi Yamamotoya,1 Hideaki Kamata,1 and Tomoichiro Asano1

1Department of Medical Science, Graduate School of Medicine, Hiroshima University, Hiroshima, Japan; 2Division of Diabetes and Metabolism, Institute for Adult Disease, Asahi Life Foundation, Tokyo, Japan; 3Division of Neurology, Respiratory, Endocrinology and Metabolism, Department of Internal Medicine, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan; and 4Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Submitted 15 December 2014; accepted in final form 30 April 2015

* Y. Nakatsu and Y. Seno contributed equally to this work.


ELEVATED SERUM CONCENTRATIONS of uric acid (UA), the end product of purine nucleotide degradation, are reportedly associated with the development of cardiovascular and kidney diseases, hypertension, and obesity (5, 9, 17, 18, 26). Although the causative link between hyperuricemia and disease is unclear for gout, many clinical studies have revealed that the prevalence of metabolic syndrome is closely related to graded increases in hyperuricemia, strongly suggesting serum UA levels to be a predictor of metabolic syndrome, including impaired glucose and lipid metabolism (2, 8, 11, 23, 24). Although the endogenous production of UA is mainly from the liver, intestines, and other tissues like muscles, kidneys, and the vascular endothelium, UA is also secreted from adipose tissue, which is reportedly increased in obese subjects (24), supporting the view of UA elevation as a consequence of obesity. On the other hand, UA reportedly reduces nitric oxide (NO) bioavailability and thereby impairs endothelial function (10). Furthermore, several reports have demonstrated favorable effects of xanthine oxidase (XO) inhibitors on the disorders characteristic of metabolic syndrome. For example, administration of allopurinol or febuxostat reportedly prevented fructose-induced hyperinsulinemia, hypertension, and weight gain in rats (19), diabetic renal injury in streptozotocin-treated rats (14), and atherosclerosis development in apolipoprotein E knockout mice (12).

The relationships of elevated serum UA with nonalcoholic fatty liver disease (NAFLD) and steatohepatitis (NASH) have also been demonstrated in several clinical studies (21, 22), but the details of these relationships remain unclear. Serum UA elevation and NASH development may not have a causal relationship, with both instead being consequences of metabolic syndrome. On the other hand, it is also possible that hyperuricemia itself and/or a molecular mechanism(s) associated with it, such as oxidative stress, plays important roles in promoting and/or exacerbating the development of NAFLD, independently of obesity or insulin resistance. If this is the case, therapy aimed at normalization of the serum UA level could be useful for treating NASH, even if the degree of obesity or metabolic factors other than UA show no improvement.

To address this question, we herein examined the effects of an XO inhibitor, febuxostat, on two types of NASH rodent models, with and without elevated serum UA concentrations. We obtained evidence that treatment with this XO inhibitor exerted a strong protective effect against the development of NASH with hyperuricemia induced by a high-fat diet containing trans fatty acid (HFDT), but not on that caused by a methionine choline-deficient (MCD) diet without hyperuricemia. These results suggest the importance of XO in the pathogenesis of NASH development and also raise the possibility of XO inhibitors having therapeutic potential for patients with NASH accompanied by hyperuricemia.
MATERIALS AND METHODS

Animals, diets, and febuxostat treatment. C57BL/6 mice were purchased from SLC (Hamamatsu, Japan). They were housed in temperature- and light-controlled rooms with free access to food and water. After a 1-wk acclimatization period, the mice were fed normal chow diet (ND) (Oriental Yeast, Tokyo, Japan) or a diet comprising of 50% high fat (40% kcal), 25% high fructose (22% by weight), and 25% high cholesterol (2% by weight), with trans fats serving as the fat source (Primex partially hydrogenated vegetable oil shortening, cat. no. D90100301; Research Diet, New Brunswick, NJ). The HFDT has been deemed to be superior at inducing NASH. Febuxostat (1 mg/kg per day) (Teijin, Tokyo, Japan) was given to the mice by mixing it into their drinking water. After 8 wk, the mice were killed, and their livers and blood were collected. In the other experiment, the mice were randomly divided into two experimental groups and fed the ND (Oriental Yeast) or the MCD diet (Oriental Yeast), with or without febuxostat administration (1 mg/kg per day), for 8 wk. All animals were handled in accordance with the Guidelines for the Care and Use of Experimental Animals published by Hiroshima University, and all protocols were approved by Hiroshima University.

Measurement of serum parameters. Serum was collected after a 10-min centrifugation at 8,000 revolution/min. Alanine transaminase (ALT) and UA were assayed with a Transaminase C-II Test Wako Kit (Wako, Osaka, Japan) and a UA assay kit (Cayman Chemical, Ann Arbor, MI), respectively, according to the manufacturers’ instructions. Homeostasis model of assessment of insulin resistance (HOMA-IR) was calculated as (fasting serum glucose × fasting serum insulin concentration)/405.

Measurement of XO activity. Hepatic XO activity was measured using an XO activity assay kit, according to the manufacturer’s instructions (Sigma, St. Louis, MO).

Fluorescence-activated cell sorting analysis. Livers were homogenized immediately after death of the mice. Liver lysates were washed with RPMI medium four times, and debris was removed through a cell strainer. The cells were stained with brilliant violet-CD3, allophycocyanin-CD45, phycoerythrin-CD8, and 7-aminoactinomycin D (7-AAD) for 30 min and then analyzed with Fortessa (BD Biosciences, San Jose, CA). Macrophages were gated by side scatter and forward scatter (SSC/FLSC), 7-AAD(–), and CD45+ (Cell Signaling, Beverly, MA) overnight at 4°C. For active caspase-3 staining, the slides were reacted with fluorescein isothiocyanate-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. To visualize SMA and 4-HNE, the slides were stained by the dianibenzimidite method. Digital images of lesions were obtained with a multifocal microscope (BZ-9000; KEYENCE, Osaka, Japan).

TUNEL staining was performed, using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI). Briefly, deparaffinized sections were stained according to the manufacturer’s instructions.

Table 1. Designed primers

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Sequence</th>
<th>Gene ID</th>
<th>AmpliCon Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC1</td>
<td>F: gag agg ggt caa ctt cc 14433 R: ctt ggg atc cac act ctc ca 16176</td>
<td>107476</td>
<td>200 bp</td>
</tr>
<tr>
<td>FASN</td>
<td>F: tgg ctt cca cta cag att gc 11430 R: aac agg ctc aga ggc aca at 16176</td>
<td>14104</td>
<td>192 bp</td>
</tr>
<tr>
<td>PPARx</td>
<td>F: atg cca gta ctc cgg tt 21858 R: ggc ccc gtt ctt gtt 13177</td>
<td>19013</td>
<td>220 bp</td>
</tr>
<tr>
<td>CYP4A10</td>
<td>F: cca gca gtt ccc acc atc cc 21858 R: tga tgg ccc cag aat ctc tt 13117</td>
<td>13117</td>
<td>190 bp</td>
</tr>
<tr>
<td>CYP4A14</td>
<td>F: ggt ttc aat ggg ctc ct 21858 R: tct gct gga gct ctt gc 13119</td>
<td>13119</td>
<td>165 bp</td>
</tr>
<tr>
<td>ACOX</td>
<td>F: ttc aag aca gag ccc tga aa 21858 R: cag agg caa ggg tca cat cc 13117</td>
<td>11430</td>
<td>222 bp</td>
</tr>
<tr>
<td>Collagen1a1</td>
<td>F: ccg tgc ttc tca gaa ca 21858 R: gag cag cca tgg act agg ac 12842</td>
<td>12842</td>
<td>158 bp</td>
</tr>
<tr>
<td>Collagen1a2</td>
<td>F: ccc tgc ttc tca gaa ca 21858 R: gag cag cca tgg act agg ac 12843</td>
<td>12843</td>
<td>168 bp</td>
</tr>
<tr>
<td>SMA</td>
<td>F: acc aac tgg gac gac agt gaa 21858 R: tga ctc gct ggg cgg atc 11475</td>
<td>11475</td>
<td>90 bp</td>
</tr>
<tr>
<td>TIMP1</td>
<td>F: att cca ggc tgg gaa aa 19013 R: ctc cta gta cgc cag gga ac 21857</td>
<td>21857</td>
<td>183 bp</td>
</tr>
<tr>
<td>MCP1</td>
<td>R: gcg tct gct gga gct cct 12842</td>
<td>21858</td>
<td>155 bp</td>
</tr>
<tr>
<td>IL-1B</td>
<td>F: cgg gcc ttc aag gaa ga 21858 R: tct gga ccc act ctt tc 12029</td>
<td>20296</td>
<td>249 bp</td>
</tr>
<tr>
<td>IL-6</td>
<td>F: cgg gcc ttc aag gaa ga 19013 R: tct gga ccc act ctt tc 16176</td>
<td>16176</td>
<td>86 bp</td>
</tr>
<tr>
<td>IL-12</td>
<td>F: tgc agg cca ctc acc act ca 21858 R: rct ctc cag cta tgg gg 16193</td>
<td>16193</td>
<td>165 bp</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: tga tgg ctc cag ac 21858 R: cct gtc ggc taa ggt cct 14433</td>
<td>14433</td>
<td>178 bp</td>
</tr>
</tbody>
</table>

F.; Fas; R.; reverse. ACC1, acetyl-CoA carboxylase 1; FASN, fatty acid synthase; PPAR, peroxisome proliferator-activated receptor; ACOX, acyl coenzyme A oxidase; SMA, smooth muscle actin; TIMP, tissue inhibitor of metalloproteinase; MCP1, monocyte chemotactic protein 1.
sections were treated with Pro K. After being washed, the sections were reacted with terminal deoxynucleotidyl transferase and fluorescein-12-dUTP. After being washed, the slides were mounted employing DAPI.

Thiobarbituric acid reactive substances assay. Thiobarbituric acid reactive substances (TBARS) measurement was performed using a TBARS assay kit (Cayman Chemical), according to the manufacturer’s instructions. Briefly, malondialdehyde (MDA) in lysates was reacted with thiobarbituric acid at 100°C to produce the MDA-TBA adduct, and the concentration produced was measured at 535 nm.

Statistical analysis. Results are expressed as means ± SE. Statistical significance was assessed using ANOVA followed by Tukey’s honestly significant difference test. Variables not normally distributed were log transformed before the analysis. P < 0.05 was taken to indicate a statistically significant difference.

RESULTS

Febuxostat reduced the elevated serum UA concentration and increased liver weight induced by HFDT feeding. Mice were divided into four groups based on the combinations of HFDT and ND with or without febuxostat in the drinking water. After the 8-wk treatment, body and liver weights were greater in the HFDT-fed mice than in the ND-fed mice (Fig. IA). Febuxostat administration slightly but significantly reduced liver weight without affecting whole-body weights. Whereas febuxostat administration did not alter blood glucose concentrations or HOMA-IR scores (Fig. 1B), serum UA concentrations and hepatic XO activity raised by HFDT feeding were completely normalized by febuxostat (Fig. 1, C and D), with no change in the amount of hepatic XO protein (Fig. 1E). Serum ALT levels were markedly elevated by HFDT feeding for 8 wk, but the degree of these increases was significantly suppressed by febuxostat administration (Fig. 1F).

Hepatosteatosis induced by HFDT was reversed by febuxostat administration. Hematoxylin-eosin staining showed that the livers of mice fed HFDT developed marked macrovesicular steatosis (Fig. 2A). In contrast, the mice fed HFDT with febuxostat showed much less hepatic fat accumulation than HFDT-fed mice not given febuxostat (Fig. 2, A and B). To reveal the molecular mechanism underlying febuxostat-induced suppression of hepatosteatosis, the mRNA levels of the genes regulating lipid metabolism were investigated. Febuxostat had no significant effect on mRNA levels of acetyl-CoA carboxylase and fatty acid synthase, which are both involved in lipid synthesis (Fig. 2C). Neither the peroxisome proliferator-activated receptor-α nor the CYP4A10 mRNA

Fig. 1. Febuxostat reduced serum uric acid (UA) elevations and liver weight increases induced by high-fat diet containing trans fatty acid (HFDT) feeding. Mice were divided into 4 groups based on the combination of HFDT and normal chow diet (ND) with or without febuxostat in drinking water (n = 8 per group). After the 8-wk treatment, the indicated parameters were measured. A: whole-body and liver weights. B: blood glucose concentrations in the fed and fasted states and homeostasis model of assessment of insulin resistance (HOMA-IR). C: serum UA concentration. D: xanthine oxidase (XO) activity in livers. E: immunoblotting (IB) of hepatic XO. Cont, control; Feb, febuxostat. F: serum alanine transaminase (ALT) level. All data are shown as means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001; n.s., not significant.
level was affected by the difference in diets or by febuxostat administration (Fig. 2D). On the other hand, although the expressions of fatty acid oxidation-related genes such as acyl coenzyme A oxidase (ACOX) and CYP4A14 were reduced by HFDT feeding, febuxostat normalized these reductions (Fig. 2E).

**Febuxostat inhibits liver fibrosis attributable to HFDT feeding.** To elucidate the impact of febuxostat on liver fibrosis caused by HFDT feeding, collagen deposition in the liver was estimated by Sirius red staining. The mice fed HFDT showed marked liver fibrosis, whereas almost no collagen deposition was observed in the livers of HFDT-fed mice administered febuxostat (Fig. 3, A and B). Hydroxyproline accumulation in the liver was also caused by HFDT feeding, and febuxostat suppressed this effect (Fig. 3C). Consistent with the results of Azan staining, whereas a marked increase was observed in HFDT-fed mice, mRNA expressions of collagen-1a1/1a2 in the febuxostat-treated mice were not increased by HFDT feeding and were significantly lower than those in the untreated mice fed HFDT (Fig. 3D). In addition, hepatic collagen proteins were up-regulated by HFDT, and febuxostat administration reduced the resulting accumulation of collagen (Fig. 3E).

Because activation of hepatic stellate cells reportedly plays a critical role in the development of hepatic fibrosis, immunofluorescence staining was carried out using anti-SMA antibody, as a marker of stellate cell activation. Numerous SMA-positive cells were observed in the livers of HFDT-fed mice, and this finding was normalized by febuxostat administration (Fig. 4A). These immunochemical staining data were supported by the analysis of mRNA expressions of SMA and tissue inhibitors of metalloproteinases (TIMPs) (Fig. 4B).

**Febuxostat attenuates oxidative stress and inflammatory cytokine releases.** Oxidative stress and inflammatory cytokines are reportedly involved in the activation of hepatic stellate cells. Thus the accumulation of 4-HNE as a marker of oxidative stress was examined (Fig. 5A). Although the amount of 4-HNE was markedly increased in the livers of HFDT-fed mice, febuxostat administration was found to suppress the accumulation of 4-HNE (Fig. 5A). In addition, the amount of
MDA, a product of lipid peroxidation, was also elevated by HFDT feeding but was completely normalized by febuxostat administration (Fig. 5B).

We also investigated the expressions of cytokines such as monocyte chemoattractant protein 1 (MCP-1), IL-1β, IL-6, and IL-12. In our NASH model, MCP-1 expressions were markedly elevated by HFDT, but the expressions of IL-1β, IL-6, and IL-12 were unchanged or only slightly elevated. In contrast, febuxostat administration markedly reduced the expressions of MCP-1 and IL-6, while only tending to reduce the expressions of IL-1β and IL-12 without statistical significance (Fig. 5C). The populations of immune cells were not affected by either HFDT or febuxostat (Fig. 5D).

**Febuxostat reduced cell apoptosis induced by HFDT feeding.** Finally, the effect of febuxostat on the apoptosis of hepatocytes was investigated. HFDT feeding markedly increased the number of cells positive for TUNEL staining, and this was almost completely reversed by febuxostat administration (Fig. 6, A and B). In addition, active caspase-3-positive cells were significantly increased in the livers of HFDT-fed mice, a finding that was normalized by the administration of febuxostat (Fig. 6, C and D).

Febuxostat had no effect on the NASH development induced by MCD diet feeding. Although mice fed the MCD diet had slightly but not significantly elevated serum UA concentrations compared with those fed the ND, febuxostat administration did not affect the serum UA concentration in the MCD diet-fed mice (Fig. 7A). In addition, febuxostat did not normalize the serum ALT elevation caused by MCD diet feeding (Fig. 7B). Hematoxylin-eosin staining revealed marked increases in fat droplets, increased inflammatory cell infiltration, and balloon-like structures in the livers of the MCD diet-fed mice, and febuxostat intervention did not improve these abnormalities (Fig. 7C). In addition, the results of Sirius red staining indicated that febuxostat had no effect on collagen deposition induced by the MCD diet (Fig. 7C). Similarly, febuxostat administration showed no significant normalizing effects on the elevations of collagen-1a1, collagen-1a2, TIMP1, and TIMP2 mRNA caused by MCD diet feeding (Fig. 7D). Taken together, these results allow us to conclude that febuxostat does
DISCUSSION

NASH is highly related to metabolic syndrome and obesity, and its incidence has been increasing in many, particularly advanced, nations. However, there are currently no treatment options specifically for NASH, and this disease is usually managed with lifestyle changes such as diet, exercise, and weight reduction. Although several recent lines of evidence have demonstrated a relationship between hyperuricemia and NASH (21, 22), the present study showed clearly that febuxostat exerts a strong protective effect against NASH development induced by HFDT feeding, based on our observations of the inductions of fibrosis genes, steatohepatitis, and inflammatory cytokine expressions. On the other hand, interestingly, febuxostat had no protective effect against the development of MCD diet-induced NASH that is not accompanied by hyperuricemia.

Regarding the pathogenesis of NASH, the “two-hit theory” has been proposed as a mechanism underlying the development of NASH (15). The first hit involves simple steatosis, whereas the second hit, factors such as oxidative stress and inflammatory cytokines, exacerbates NASH once it has developed. These two hits are both present in the NASH rodent models created by HFDT and MCD diet feeding, with phenotypic features such as obesity, insulin resistance, glucose intolerance, and hyperuricemia all being present in the HFDT- but not the MCD diet-induced NASH models. Whereas febuxostat did not affect body weight gain, insulin resistance, or glucose intolerance induced by HFDT feeding, serum UA elevation, observed in the HFDT- but not the MCD diet-fed mice, was completely normalized. With the aforementioned issues in consideration, it may be reasonable to speculate that febuxostat protects against HFDT-induced NASH development via normalization of the elevated UA concentration itself and/or accompanying molecular mechanism(s). Recent reports have shown increased XO activity in HFDT-fed mice and genetically obese mice (24, 25). In good agreement with these reports, XO activity in the livers of HFDT-fed mice was shown to be increased (Fig. 1D). Unexpectedly, XO activity was revealed to also be upregulated in the MCD diet-fed mice (MCD, 197 ± 11006 vs. ND, 115 ± 77926 mol/mg protein per min), despite there being no serum UA elevation. However, with consideration that MCD-fed mice are malnourished with atrophic adipose tissue, UA production might be suppressed by a deficiency of source materials in the liver and adipocytes, the two major tissues producing UA. On the other hand, HFDT-fed mice with excessive nutrition and hypertrophic adipocytes would presumably release more UA than the ND-fed mice.

Although UA is reportedly capable of acting as a prooxidant like other reducing substances such as ascorbic acid, the reaction catalyzed by XO converting xanthine to UA requires oxygen (1). XO is abundantly expressed in the liver and generates hydroxyperoxide when catalyzing substrates. Thus serum UA elevation is considered to accompany increased accumulation of oxidative stress generated by hepatic XO. In fact, 4-HNE or MDA produced by lipid peroxidation was found to be higher in mice given HFDT, but this elevation was normalized by febuxostat administration. In contrast, the expression levels of apoptosis-related genes such as FADD and Bim mRNAs did not significantly differ between the HFDT and ND groups (data not shown). It is considered that oxidative stress affects the membrane potential of mitochondria and

Fig. 4. Febuxostat inhibits HFDT-induced activation of hepatic stellate cells. A: sections prepared from the livers of ND- or HFDT-fed mice with or without febuxostat administration were stained with anti-smooth muscle actin (SMA) antibody, as a marker of stellate cell activation. Representative photographs of each group are shown. B: mRNA levels of SMA, tissue inhibitor of metalloproteinase 1 (TIMP1), and TIMP2. All data are shown as means ± SE. *P < 0.05.
triggers the release of cytochrome c into the cytosol, inducing apoptosis (6). Therefore, we speculate the involvement of oxidative stress on the induction of hepatocyte apoptosis probably via the impaired mitochondrial function. Furthermore, accumulated oxidative stress reportedly leads to increased expressions of inflammatory cytokines, such as MCP-1 and IL-6, findings also observed in mice given HFDT and normalized by febuxostat. These findings may represent phenomena similar to those reported in the previous study showing the suppressive effect of febuxostat on lipopolysaccharide (LPS)-induced MCP-1 release from macrophages (20), but further study is necessary to clarify this issue.

The accumulation of oxidative stress and inflammatory cytokine expressions are also likely to be involved in, not only the inflammation and fibrosis occurring as a result of the “second hit,” but also in lipid accumulation because continuous injection of LPS into rats reportedly results in fatty liver development (7). This study revealed that expressions of fatty acid oxidation-related genes such as ACOX and CYP4A14 are reduced by HFDT feeding and that these abnormalities were normalized by febuxostat administration. Because MCD diet feeding decreases the expressions of catalase and superoxide dismutase, oxidative stress also accumulates in the livers of mice given the MCD diet (4, 13, 16) although its generation mechanism is not related to either the XO activity or the UA concentration. Thus it is reasonable to speculate that this difference in the process of generating oxidative stress explains why febuxostat exerts no effect on MCD diet-induced NASH development. Finally, it should be noted that, besides the mechanism involving increased oxidative stress, an elevated UA concentration may itself also contribute to hepatic lipid accumulation because a previous report showed UA to promote triglyceride accumulation in hepatocytes by inhibiting AMP kinase (3).

In conclusion, to our knowledge, this is the first study clearly demonstrating that an XO inhibitor exerts a strong protective effect against NASH development, independently of the normalization of impaired glucose metabolism or
obesity. Because the results obtained for the HFDT-fed NASH model are more clinically relevant than those of the MCD diet-fed model, our results raise the possibility of an XO inhibitor such as febuxostat as effective for the treatment of at least some human patients with NASH, perhaps those with borderline or high serum UA concentrations. Clinical trials will be required to test the therapeutic potential of XO inhibitors.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


Fig. 6. Febuxostat reduced cellular apoptosis induced by HFDT feeding. Sections prepared from the livers of ND- or HFDT-fed mice with or without febuxostat administration were subjected to TUNEL staining (A and B) or active caspase-3 staining (C and D). TUNEL-positive cells or active caspase-3 cells were counted in 3 randomly selected areas for each mouse (magnification fields ×600). **P < 0.01.
Fig. 7. Febuxostat had no effect on the nonalcoholic steatohepatitis development induced by methionine choline-deficient (MCD) diet feeding. A: serum UA concentration. B: serum ALT level. C: paraffin-embedded sections were stained with hematoxylin and eosin (HE) or with Sirius red. Representative photographs of each group are shown. D: mRNA levels of collagen 1α1, collagen 1α2, TIMP1, and TIMP2. All data are shown as means ± SE. *P < 0.05.


