

# Oltipraz ameliorates the progression of steatohepatitis in Nrf2-null mice fed a high-fat diet

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Oltipraz, a synthetic dithiolethione, has chemopreventive effect through nuclear factor erythroid 2-related factor 2 (Nrf2) activation. Nrf2 is known to be involved in the development of experimental steatohepatitis in rodents. In this study, to evaluate the effect of oltipraz on lipid and bile acid metabolism, wild-type and Nrf2-null mice were fed the standard diet (containing 4% soybean oil) with or without oltipraz. Based on these results, we examined the effect of oltipraz on the experimental steatohepatitis in high-fat diet (containing 4% soybean oil and 20% lard) fed Nrf2-null mice. Oltipraz induced hepatic mRNA expression of peroxisome proliferator-activated receptor  $\alpha$ , carnitine palmitoyl transferase 1, and bile salt export pump by Nrf2 independent mechanisms. In Nrf2-null mice fed a high-fat diet for 12 weeks, moderate to severe inflammation and fibrosis were observed. Oral administration of oltipraz suppressed the degree of inflammation and fibrosis in Nrf2-null mouse liver fed a high-fat diet. These histopathological findings approximately corresponded to the data of mRNA expression of tumor necrosis factor  $\alpha$ , monocyte chemoattractant protein-1, Timp-1, and collagen type 1 $\alpha$ 1. These results indicated that oltipraz administration ameliorated liver injury by Nrf2 independent manner in a model of steatohepatitis generated by Nrf2-null mice with high-fat diet.

**Key Words:** nuclear factor erythroid 2-related factor 2, oltipraz, high-fat diet, steatohepatitis, Nrf2-null mice

Oltipraz (OPZ) [4-methyl-5-(2-pyrazinyl)-1,2-dithiol-3-thione], a synthetic dithiolethione, is known as a chemopreventive agent. The chemopreventive effect of OPZ is related to the enhancement of binding activity between nuclear factor erythroid 2-related factor 2 (Nrf2) and antioxidant response element (ARE). At present OPZ is known as a potent Nrf2 activator. The transcription factor Nrf2 is a crucial molecule in the maintenance of redox homeostasis. Nrf2 induces the expression of antioxidant and detoxifying enzymes, glutathione homeostasis, and transport of xenobiotics, including glutathione *S*-transferase A1 (Gsta1), NAD(P)H:quinone oxidoreductase 1 (Nqo1), and multidrug resistance associated proteins (Mrps) by binding to antioxidant responsive elements in the promoters of these genes.<sup>(1,2)</sup> Nrf2 has additional roles in lipid and bile acids metabolism beyond detoxification. With respect to the role of Nrf2 in lipid metabolism, previous studies demonstrated that Nrf2 inhibits lipid accumulation and lipid peroxidation in mouse liver after feeding a high-fat diet (HFD) by interfering with lipid-related pathways. Furthermore, enhanced expression of Nrf2 in Kelch-like ECH-associated protein 1 (Keap1) knockdown mice attenuates fatty liver produced by a methionine- and choline-deficient (MCD) diet.<sup>(3,4)</sup> Concerning the role of Nrf2 in bile acids metabolism, Nrf2

protects against hepatotoxicity induced by an intraperitoneal injection of lithocholic acid.<sup>(5)</sup> Nrf2 modulates bile acid homeostasis in mouse liver, and Nrf2 regulates bile salt export pump (Bsep), multidrug resistance associated protein (Mrp) 4,<sup>(6,7)</sup> and cholesterol 7 $\alpha$ -hydroxylase (Cyp7a1) expression.<sup>(8)</sup> As OPZ is a potent Nrf2 activator, it induces Nqo1 along with other enzymes and bile acid transporters, including Bsep, Mrp2, Mrp3, and Mrp4.<sup>(9,10)</sup>

Nrf2-null mice are known to develop steatohepatitis when fed a MCD diet or a high-fat diet.<sup>(11,12)</sup> The effects of Nrf2 deletion on antioxidant defense mechanisms, bile acid metabolism, and lipid metabolism are involved in the development of experimental steatohepatitis in rodents. Several types of Nrf2 activators have been reported to improve liver injury in the experimental steatohepatitis in rodents.<sup>(13–15)</sup> It has been reported that OPZ protects against alpha-naphthylisothiocyanate (ANIT)-induced hepatotoxicity and choline-deficient, L-amino acid defined diet (CDAA)-induced liver injury.<sup>(16,17)</sup> This hepatoprotective effect of OPZ is thought to be due to Nrf2 activation. However, OPZ also improves ANIT-induced hepatotoxicity in Nrf2-null mice,<sup>(16)</sup> suggesting that OPZ may also have an Nrf2 independent hepatoprotective effect. Indeed, OPZ is also known to activate constitutive androstane receptor (CAR) in addition to Nrf2.<sup>(18)</sup> In the present study, we investigated whether OPZ has a protective effect against HFD-induced liver injury in Nrf2-null mice, and whether OPZ has Nrf2 independent hepatoprotective effect.

## Materials and Methods

**Materials.** Milk casein, corn starch,  $\alpha$ -corn starch, mineral AIN-93G mixture and vitamin AIN-93VX mixture were purchased from Clea Japan (Osaka, Japan). Soybean oil and lard were purchased from Oriental Yeast (Tokyo, Japan) and Yamakei (Osaka, Japan), respectively. OPZ was purchased from LKT Laboratories, Inc. (St. Paul, MN) and all other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan), unless noted.

**Animals.** A colony of wild-type and Nrf2-null mice, generated originally by Itoh *et al.*, were backcrossed with C57BL/6 mice for ten generations (Itoh K *et al.*, 1997). All mice were housed in the same animal care facility controlling for temperature (22  $\pm$  2°C), humidity (55  $\pm$  5%), and light (lights on; 07:00–19:00 h).

**Experiment 1.** To examine the effect of OPZ on lipid and bile acid metabolism, 15-week-old male wild-type and Nrf2-null mice ( $n = 6$ /group) were divided into two groups fed the following

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**Table 1.** Composition of the experimental diets (weight %)

Ingredients	Standard	Standard/OPZ	HFD	HFD/OPZ
Soybean oil	4	4	4	4
Lard	—	—	20	20
Oltipraz	—	0.0067	—	0.0124
Milk casein	14	14	14	14
L-Cystein	0.18	0.18	0.18	0.18
AIN-93G mineral mixture*	3.5	3.5	3.5	3.5
AIN-93VX vitamine mixture*	1	1	1	1
Cellulose powder	5	5	5	5
Corn starch	46.57	46.57	26.57	26.57
$\alpha$ -Corn starch	15.5	15.5	15.5	15.5
Sucrose	10	10	10	10
Choline bitartrate	0.25	0.25	0.25	0.25

\*The amount recommended for use is given by the American Institute of Nutrition. HFD, high fat diet; OPZ, oltipraz.

diets: 1) a standard diet (AIN-93, containing 4% soybean oil) for 4 weeks (Standard group), 2) a standard diet containing OPZ (average intake of OPZ 9.3 mg/kg/day) for 4 weeks (Standard/OPZ group).

**Experiment 2.** Seven-week-old male wild-type and Nrf2-null mice ( $n=6$ /group) were divided into two groups fed the following diets: 1) a high fat diet (HFD) (containing 4% soybean oil and 20% lard) for 12 weeks (HFD group), 2) a HFD for 8 weeks followed by a HFD containing OPZ (average intake of OPZ 8.6 mg/kg/day) for 4 weeks (HFD/OPZ group).

**Diets.** The composition of the experimental diets is shown in Table 1. As OPZ was fine powder, OPZ was thoroughly mixed with other compositions of each diet using a food mixer to obtain the desired concentrations in the feed. The dose and the duration of OPZ administration was chosen with reference to previous reports.<sup>(19,20)</sup> Individual body weights and food intake were recorded once or twice a week, respectively. Based on these data, the amount of OPZ mixed in diets was adjusted each time to administer approximately same total amount of OPZ between groups when diets were prepared. Food was removed two hours prior to collecting tissues and blood samples were collected by heart puncture after anesthesia, and livers were harvested and stored at  $-80^{\circ}\text{C}$  until use. Animals received humane care as outlined in the Guide for the Care and Use of Laboratory Animals (NIH publication 86-23, revised 1985). Studies were approved by Kindai University Faculty of Medicine Animal Care and Use Committee.

**Quantification of serum biochemical markers.** Serum biochemical markers were quantified using biochemistry auto-analyzer Labospect 008 (Hitachi High-Technologies Corporation, Ibaragi, Japan). Serum bile acids (BA) were determined by standard enzymatic-colorimetric assays using bile-acid assay kits in accordance with the manufacturer's protocols (Wako Pure Chemical Industries).

**Determination of hepatic lipids and bile acids.** Liver lipid content was extracted according to the method of previous report.<sup>(21)</sup> Hepatic triglycerides and cholesterol levels were determined enzymatically using commercially available kits (Wako Pure Chemical Industries). Hepatic bile acid concentrations were quantified as described previously.<sup>(22)</sup>

**Histopathology.** Liver samples were fixed in 10% formalin prior to routine processing and paraffin embedding. Liver sections (4  $\mu\text{m}$  in thickness) were stained with hematoxylin and eosin (H&E) or van Gieson to evaluate severity of histological changes, including steatosis, inflammation, and fibrosis. Histological findings were scored by using "NAFLD scoring system for rodent model".<sup>(23)</sup>

### RNA isolation and real-time polymerase chain reaction.

Total RNA was isolated using TRIzol reagent according to the manufacturer's protocol (Life Technologies, Tokyo, Japan). The concentration of total RNA in each sample was quantified spectrophotometrically at 260 nm. The mRNA levels were quantified by SYBR real-time polymerase chain reaction (PCR). The primers are listed in Table 2.<sup>(24-26)</sup> The amplification reactions were carried out in an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA). The amount of mRNA was calculated using the comparative CT method which determines the amount of target normalized to an endogenous reference. Each gene was normalized to 18s rRNA.

**Statistical analysis.** The software package, SYSTAT, ver. 11 (Systat Inc., Evanston, IL) was used for statistical analysis. To compare the differences among multiple groups, statistical analysis included analysis of variance, followed by Tukey's multiple range test. Differences were considered statistically significant at  $p<0.05$ . All the experimental data were expressed as mean  $\pm$  SE.

## Results

### Effect of oltipraz on hepatic mRNA expression of lipid and bile acids metabolism related genes in mice with standard diet.

OPZ is known to be Nrf2 activator, and we investigated the extent to which oral administration of OPZ under this experimental condition altered the expression of the gene products related to bile acid metabolism and lipid metabolism (Table 3). With respect to the Nrf2 target gene expression related to bile acid metabolism, Mrp2, Mrp3, small heterodimer partner (Shp), and sterol 12 $\alpha$ -hydroxylase (Cyp8b1) were induced in wild type mice, and not in Nrf2-null mice. OPZ induced hepatic mRNA expression of peroxisome proliferator-activated receptor  $\alpha$  (Ppara) in both wild-type and Nrf2-null mice. Hepatic expression of carnitine palmityl transferase 1 (Cpt1) tended to increase in wild type mice treated with OPZ but not significant, on the other hand, Cpt1 was significantly increased in Nrf2-null mice. With respect to the genes related to bile acid metabolism, hepatic gene expression of Bsep was not significantly increased in wild-type mice treated with OPZ, however, it was significantly increased in Nrf2-null mice by OPZ administration.

### Effect of oltipraz on the experimental steatohepatitis in HFD fed Nrf2-null mice.

**Serum biochemical markers.** Serum total cholesterol, HDL cholesterol, and triglycerides were lower in Nrf2-null mice than in wild-type mice. With respect to liver function test, alkaline phosphatase, alanine aminotransferase, and total bilirubin, tended to be higher in Nrf2-null mice than in wild-type mice. Serum bile

**Table 2.** Real-time PCR primer sequences

Gene symbol	Forward 5'-3'	Reverse 5'-3'
Ppara	TATTCGGCTGAAGCTGGTGATC	CTGGCATTGTCCGGTTCT
Acox	GCCCAACTGTGACTTCCATT	GGCATGTAACCCGTAGCACT
Cpt1	GCACTGCAGCTCGCACATTACAA	CTCAGACAGTACCTCCTTCAGGAAA
Ppar $\gamma$	CAGGCTTGCTGAACGTGAAG	GGAGCACCTTGCGGAACA
Cd36	AATGGCACAGACGCAGCCT	GGTTGTCTGGATTCTGGA
Srebp1c	GTTACTCGAGCCTGCCTTCAGG	CAAGCTTTGGACCTGGGTGTG
Fas	GCTGCGGAAACTTCAGGAAAT	AGAGACGTGTCACTCCTGGACTT
Lxr $\alpha$	TCAGCATCTTCTGCAGACCCGG	TCATTAGCATCCGTGGGAACA
Abcg5	TGGATCCAACACTCTATGCTAAA	GGCAGGTTTTCTCGATGAACTG
Abcg8	TGCCACCTTCCACATGTC	ATGAAGCCGGCAGTAAGGTAGA
Cyp7a1	GCTGTGGTAGTGAGCTGTTGCA	CACAGCCCAGGTATGGAATCA
Cyp7b1	CGAGAAGTGCAGGAGGATATGA	GTGGAGGAAAGAGGGTACAAA
Cyp8b1	GGACTTCCAGTCTATGCAAAAAGA	TGGTACCCAAAACACCTTGAGC
Oatp1a1	CAGATAAATGGATTGCCAG	GTCAACAAATAGTTACAGAG
Shp	CGATCCTCTCAACCCAGATG	AGGGCTCCAAGACTTCACACA
Bsep	GTTTCAGTTCCTCCGTTCAAA	AAGCTGCACTGTCTTTTTCAC
Mrp2	CATAAAACCAGGCCAACTGGTG	TGCCCATGGACATTCTCCAT
Mrp3	CAACTTCTCCGAACTACGCA	TGCTGAGTGTCTTCGAGCAG
Nqo1	AGAGTGGCATCCTGCGTTTCT	TTCCATCCTCCAGGATCTGC
Gclc	GCACGGCATCCTCCAGTTCCT	TCGGATGGTTGGGTTTGTCC
Gclm	GGCTTCGCCTCCGATTGAAGA	TCACACAGCAGGAGGCCAGGT
Tnf $\alpha$	GACCCTCACACTCAGATCATCTTCT	CCTCCACTTGGTGGTTTGTCT
Mcp-1	ACTGAAGCCAGCTCTCTTCTCTC	TTCTTCTTGGGGTTCAGCACAGAC
Timp-1	ACTCGGACCTGGTCATAAGGGC	TTCCGTGGCAGCAAGCAAAGT
Collagen type I $\alpha$ 1	TGTGTTCCCTACTCAGCCGTCT	CATCGGTCATGCTCTCTCCAA
18S rRNA	GCAATTATCCCATGAACA	GGCCTCACTAAACCATCCAA

**Table 3.** Effect of oltipraz on hepatic mRNA expression of lipid and bile acids metabolism related genes in mice with standard diet

	Wild type mice		Nrf2-null mice	
	Standard	Standard/OPZ	Standard	Standard/OPZ
Ppara	1 $\pm$ 0.15	2.28 $\pm$ 0.42*	1.07 $\pm$ 0.18	2.37 $\pm$ 0.33*
Acox	1 $\pm$ 0.17	1.82 $\pm$ 0.41	0.79 $\pm$ 0.14	1.64 $\pm$ 0.23
Cpt1	1 $\pm$ 0.17	2.36 $\pm$ 0.45	1.54 $\pm$ 0.37	5.71 $\pm$ 1.04**
Ppar $\gamma$	1 $\pm$ 0.11	1.35 $\pm$ 0.37	1.76 $\pm$ 0.64	3.11 $\pm$ 0.94
Cd36	1 $\pm$ 0.28	0.61 $\pm$ 0.18	0.79 $\pm$ 0.29	4.12 $\pm$ 2.21
Srebp1c	1 $\pm$ 0.28	1.18 $\pm$ 0.27	1.38 $\pm$ 0.42	1.49 $\pm$ 0.22
Fas	1 $\pm$ 0.46	0.51 $\pm$ 0.13	0.31 $\pm$ 0.08	0.41 $\pm$ 0.07
Lxr $\alpha$	1 $\pm$ 0.12	1.65 $\pm$ 0.36	1.38 $\pm$ 0.16	2.40 $\pm$ 0.19
Abcg5	1 $\pm$ 0.16	1.33 $\pm$ 0.28	1.37 $\pm$ 0.4	1.94 $\pm$ 0.46
Abcg8	1 $\pm$ 0.17	1.81 $\pm$ 0.33	0.97 $\pm$ 0.23	1.52 $\pm$ 0.39
Shp	1 $\pm$ 0.15	1.87 $\pm$ 0.30*	0.76 $\pm$ 0.19	0.70 $\pm$ 0.17
Cyp7a1	1 $\pm$ 0.17	0.92 $\pm$ 0.20	7.73 $\pm$ 4.67	7.15 $\pm$ 2.44
Cyp7b1	1 $\pm$ 0.14	1.41 $\pm$ 0.27	0.58 $\pm$ 0.22	0.57 $\pm$ 0.2
Cyp8b1	1 $\pm$ 0.13	1.99 $\pm$ 0.44*	0.38 $\pm$ 0.09 <sup>#</sup>	0.64 $\pm$ 0.1
Oatp1a1	1 $\pm$ 0.14	1.69 $\pm$ 0.31	0.67 $\pm$ 0.26	0.82 $\pm$ 0.34
Bsep	1 $\pm$ 0.21	1.87 $\pm$ 0.30*	0.81 $\pm$ 0.16	2.12 $\pm$ 0.24*
Mrp2	1 $\pm$ 0.09	1.87 $\pm$ 0.22*	1.21 $\pm$ 0.16	1.99 $\pm$ 0.90
Mrp3	1 $\pm$ 0.13	2.34 $\pm$ 0.33*	0.21 $\pm$ 0.04 <sup>#</sup>	0.49 $\pm$ 0.08

The Data are presented as mean  $\pm$  SE. OPZ, oltipraz. \* $p$ <0.05, \*\* $p$ <0.01, statistically significant differences compared to the control group within each genotype; <sup>#</sup> $p$ <0.05, statistically significant differences compared to wild-type mice within the same diet group.

**Table 4.** Serum biochemical markers, hepatic lipid profiles and hepatic bile acids

	Wild type mice		Nrf2-null mice	
	HFD	HFD/OPZ	HFD	HFD/OPZ
<b>Serum</b>				
Total cholesterol (mg/dl)	169 ± 11	134 ± 21	76 ± 11 <sup>#</sup>	106 ± 17
Triglycerides (mg/dl)	24 ± 11	19 ± 4	11 ± 2 <sup>*</sup>	18 ± 3
HDL cholesterol (mg/dl)	90 ± 2	74 ± 11	45 ± 7 <sup>#</sup>	61 ± 8
ALT (IU/L)	33 ± 5	39 ± 7	64 ± 20	56 ± 14
ALP (IU/L)	173 ± 7	193 ± 27	372 ± 73	224 ± 28
Bilirubin (mg/dl)	0.02 ± 0.02	0.05 ± 0.03	0.18 ± 0.05	0.13 ± 0.03
Bile acids (µmol/L)	3.96 ± 1.51	8.16 ± 1.09	45.85 ± 16.51 <sup>#</sup>	13.93 ± 5.32
<b>Liver</b>				
Cholesterol (mg/g liver)	5.94 ± 0.29	7.34 ± 0.51	7.48 ± 0.75	5.87 ± 0.17
Triglycerides (mg/g liver)	152.5 ± 33.3	147 ± 25.6	197.4 ± 54.7	117.4 ± 28.7
Bile acids (µmol/g liver)	0.11 ± 0.03	0.1 ± 0.04	0.11 ± 0.01	0.07 ± 0.01 <sup>*</sup>

The Data are presented as mean ± SE. HFD, high fat diet; OPZ, oltipraz. <sup>\*</sup>*p*<0.05, statistically significant differences compared to the control group within each genotype; <sup>#</sup>*p*<0.05, statistically significant differences compared to wild-type mice within the same diet group.

**Table 5.** Effect of oltipraz on hepatic mRNA expression of lipid and bile acids metabolism related genes in mice with high fat diet

	Wild type mice		Nrf2-null mice	
	HFD	HFD/OPZ	HFD	HFD/OPZ
Ppara	1 ± 0.12	1.93 ± 0.39	1.34 ± 0.16	2.53 ± 0.39 <sup>*</sup>
Acox	1 ± 0.21	1.27 ± 0.35	1.21 ± 0.21	2.04 ± 0.29 <sup>*</sup>
Cpt1	1 ± 0.12	1.71 ± 0.28	1.45 ± 0.1	2.52 ± 0.43 <sup>*</sup>
Pparγ	1 ± 0.22	1.62 ± 0.44	1.30 ± 0.39	1.30 ± 0.17
Cd36	1 ± 0.49	0.94 ± 0.37	0.80 ± 0.19	0.99 ± 0.17
Srebp1c	1 ± 0.21	1.84 ± 0.68	1.08 ± 0.22	1.07 ± 0.14
Fas	1 ± 0.22	1.23 ± 0.37	1.32 ± 0.30	1.49 ± 0.21
Lxrα	1 ± 0.10	1.81 ± 0.24	1.79 ± 0.22	2.37 ± 0.29
Abcg5	1 ± 0.31	1.04 ± 0.18	0.91 ± 0.28	1.27 ± 0.24
Abcg8	1 ± 0.23	1.25 ± 0.26	0.97 ± 0.32	1.42 ± 0.10
Cyp7a1	1 ± 0.22	1.73 ± 0.38	4.31 ± 1.73	4.27 ± 1.30
Cyp7b1	1 ± 0.27	0.82 ± 0.17	0.73 ± 0.29	1.19 ± 0.21
Cyp8b1	1 ± 0.18	1.06 ± 0.18	0.45 ± 0.10	0.77 ± 0.12
Oatp1a1	1 ± 0.15	1.54 ± 0.23	1.53 ± 0.34	2.35 ± 0.27
Shp	1 ± 0.11	1.13 ± 0.29	0.71 ± 0.12	1.21 ± 0.20
Bsep	1 ± 0.16	1.79 ± 0.2	1.52 ± 0.25	1.94 ± 0.21
Mrp2	1 ± 0.11	1.35 ± 0.12	1.18 ± 0.12	1.56 ± 0.18
Mrp3	1 ± 0.11	1.36 ± 0.17	0.26 ± 0.04 <sup>#</sup>	0.33 ± 0.06
Nqo1	1 ± 0.26	1.34 ± 0.21	0.29 ± 0.07 <sup>#</sup>	0.36 ± 0.04 <sup>#</sup>
Gclc	1 ± 0.12	1.38 ± 0.11	0.74 ± 0.10	0.96 ± 0.12
Gclm	1 ± 0.15	1.40 ± 0.14	0.94 ± 0.19	1.32 ± 0.16

The Data are presented as mean ± SE. HFD, high fat diet; OPZ, oltipraz. <sup>\*</sup>*p*<0.05, statistically significant differences compared to the control group within each genotype; <sup>#</sup>*p*<0.05, statistically significant differences compared to wild-type mice within the same diet group.

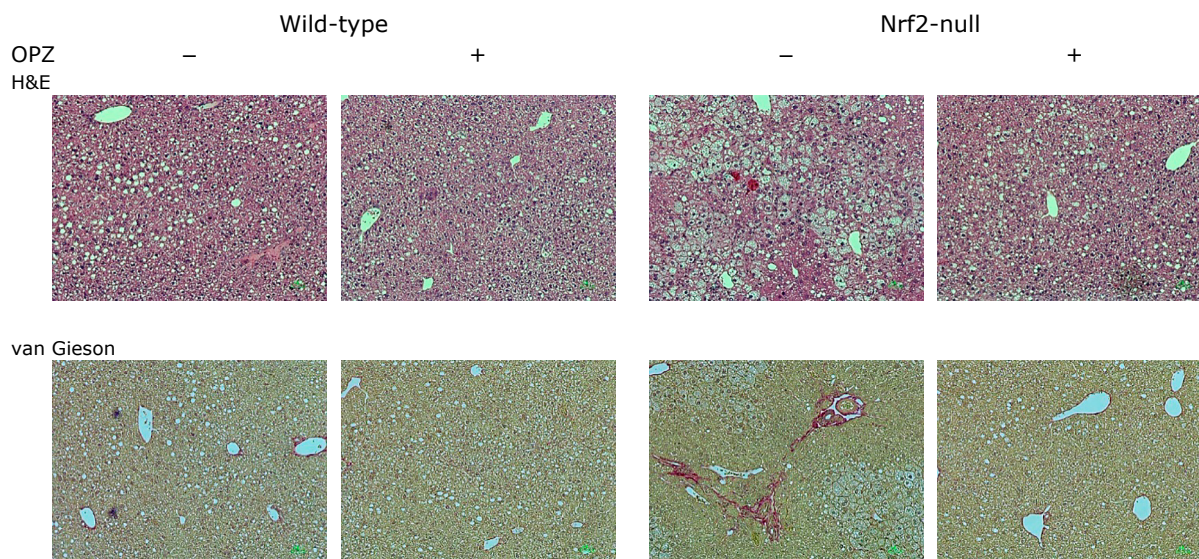
acid was significantly higher in Nrf2-null mice than in wild-type mice. Serum bile acid concentration was tended to decrease in Nrf2-null mice compared with wild-type mice, but not significant (Table 4).

**Hepatic lipid profiles and bile acids.** There was no difference in hepatic concentration of cholesterol and triglyceride among the four groups. In Nrf2-null mice, OPZ reduced hepatic bile acid concentrations (Table 4).

**Hepatic gene expression of lipid, bile acids metabolism and detoxification related genes.** OPZ induced the gene expression of Ppara and its target genes, Acox and Cpt1 in Nrf2-null mice, but not in wild-type mice. With respect to the Nrf2 target gene expression related to bile acid metabolism, Mrp3, Shp, and Cyp8b1 were not induced in either wild-type or Nrf2-null mice. OPZ did not induce the gene expression of Nqo1, Gclc and Gclm both in either wild-type or Nrf2-null mice (Table 5).

**Table 6.** Degree of steatosis, inflammation, and fibrosis of liver in wild type and Nrf2-null mice with high fat diet

		Wild type mice		Nrf2-null mice	
		HFD	HFD/OPZ	HFD	HFD/OPZ
(Score)		Mean of the score			
Steatosis	(0–9)	2.5	2.5	4.2	2.5
Inflammation	(0–3)	0	0.2	1.7	0.8
Fibrosis	(Degree)	(n = 6)	(n = 6)	(n = 6)	(n = 6)
	No	6	5	0	3
	Mild	0	1	5	3
	Moderate	0	0	1	0



**Fig. 1.** H&E and van Gieson staining in wild-type and Nrf2-null mouse liver. Representative photomicrographs of H&E-stained and van Gieson-stained liver sections from wild-type and Nrf2-null mice after 12 weeks of each diet. Representative images are shown.

**Table 7.** Hepatic gene expression of proinflammatory mediators and fibrogenesis markers in wild-type and Nrf2-null mice

	Wild type mice		Nrf2-null mice	
	HFD	HFD/OPZ	HFD	HFD/OPZ
Tnfa	1 ± 0.27	1.78 ± 0.45	5.04 ± 1.11 <sup>##</sup>	3.34 ± 1.48
Mcp-1	1 ± 0.12	2.92 ± 0.78	18.46 ± 3.50 <sup>##</sup>	7.83 ± 1.61 <sup>**</sup>
Timp-1	1 ± 0.28	1.96 ± 0.6	30.3 ± 9.47 <sup>#</sup>	17.7 ± 8.31
Collagen type 1α1	1 ± 0.19	1.06 ± 0.36	5.10 ± 2.23	2.75 ± 0.69

The Data are presented as mean ± SE. HFD, high fat diet; OPZ, oltipraz. <sup>\*\*</sup> $p < 0.01$ , statistically significant differences compared to the control group within each genotype; <sup>#</sup> $p < 0.05$ , <sup>##</sup> $p < 0.01$ , statistically significant differences compared to wild-type mice within the same diet group.

**Histology findings of the liver.** In wild-type mice, both HFD and HFD/OPZ groups demonstrated moderate to severe hepatic steatosis, however there were no inflammation or fibrosis. On the other hand, in Nrf2-null mice, moderate to severe inflammation and fibrosis were observed in the HFD group. In Nrf2-null mice, the degree of inflammation and fibrosis was milder in the HFD/OPZ group compared with the HFD group (Table 6 and Fig. 1).

**Hepatic gene expression of proinflammatory mediators and fibrogenesis markers.** Hepatic expression of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), monocyte chemoattractant protein-1 (Mcp-1),

tissue inhibitor of metalloproteinase-1 (Timp-1), and collagen type I $\alpha$ 1, reflecting the degree of liver inflammation and fibrosis, was not changed by OPZ administration in wild-type mice. The expression of Tnfa, Mcp-1, and Timp-1 were higher in Nrf2-null mice without OPZ than in wild-type mice without OPZ. In Nrf2-null mice, the gene expression of Mcp-1 was significantly decreased by OPZ administration. Hepatic expression of Tnfa, Timp-1, and collagen type I $\alpha$ 1 tended to be attenuated by OPZ in Nrf2-null mice, but these changes were not statistically significant (Table 7).

## Discussion

Because the administration of Nrf2 activators ameliorates hepatic lipid accumulation and hepatic fibrosis,<sup>(12–15)</sup> and because Nrf2 deficiency develops steatohepatitis,<sup>(11,12)</sup> it is thought that Nrf2 is one of the key molecules for the development of steatohepatitis in rodents. This phenomenon is thought to be related to the expression of several detoxifying and antioxidant enzymes that are regulated by Nrf2,<sup>(27,28)</sup> as well as the regulatory effect of Nrf2 on lipid and bile acid metabolism.<sup>(3–7,16,29)</sup> OPZ has been reported to attenuate hepatic fibrosis in experimental rodent models of steatohepatitis.<sup>(17)</sup> Therefore, the pharmacological effect of OPZ on this experimental steatohepatitis was thought to be Nrf2-dependent. On the other hand, OPZ has also been reported to have Nrf2-independent pharmacological effect.<sup>(16,18)</sup> In this study, we demonstrated that OPZ had the Nrf2 independent effect on lipid and bile acid metabolism, and that OPZ ameliorated the experimental steatohepatitis caused by 12 weeks of HFD treatment in Nrf2-null mice. This finding suggests that the pharmacological action of OPZ for this experimental steatohepatitis is through Nrf2 independent manner.

Under condition fed a standard diet containing 4% soybean oil, hepatic expression of Nrf2 target genes such as Mrp2, Mrp3, Shp, and Cyp8b1 was increased by OPZ in wild-type mice, but not in Nrf2-null mice. Therefore, the oral administration dose and the duration of OPZ in this study was thought to be an appropriate level as Nrf2 activator. On a standard diet, hepatic expression of fatty acid oxidation related gene, including Ppara, and Cpt1 and bile acid transporters, including Bsep was induced by OPZ in Nrf2-null mice. These results suggest that OPZ has the effects on regulation of the gene product related to lipid and bile acid metabolism not only through Nrf2 dependent pathway but also through Nrf2 independent pathway. On the other hand, under condition fed a high fat diet, there were little induction of Nrf2 target gene by OPZ. These results suggest that OPZ is no longer able to further induce the Nrf2 pathway in HFD condition.

Several previous studies have shown that targeted deletion of Nrf2 leads to enhanced susceptibility to hepatic injury, and rapid progression of steatohepatitis in Nrf2-null mice with high-fat diet.<sup>(12)</sup> In the present study, after 12 weeks of HFD feeding, wild-type mice showed fatty deposits, but no inflammation or fibrosis in the liver. On the other hand, in Nrf2-null mice fed a HFD for 12 weeks, hepatic fat deposits as well as inflammation and fibrosis were observed in the liver as previously reported.<sup>(12)</sup> Increase in pro-inflammatory cytokines such as TNF $\alpha$  as well as dysregulation of antioxidative system is thought to be related to the susceptibility of Nrf2-null mice to steatohepatitis in HFD.<sup>(12)</sup> In addition to these mechanisms, the hepatic damages are thought to be caused by dysregulation of fatty acid and bile acid metabolism in the absence of Nrf2. However, in this study, there was no difference in hepatic lipid concentrations and hepatic expression of fatty acid metabolism related gene product between HFD-fed wild-type and Nrf2-null mice. Regarding bile acid metabolism, Cyp7a1 expression tended to be higher and Mrp3 expression was lower in Nrf2-null mice, but there was no significant difference in hepatic bile acid concentration between wild-type and Nrf2-null mice. Because of these results, we could not

conclude that the steatohepatitis in Nrf2-null mice fed a HFD is due to disturbance of fatty acid or bile acid metabolism caused by deficiency of Nrf2.

Number of studies demonstrated that Nrf2 activators have been shown to inhibit liver fibrosis in rodent models of steatohepatitis.<sup>(13–15,17)</sup> These findings suggest that Nrf2 activation may be a target for the treatment of steatohepatitis. In the present study, OPZ reduced fat deposition and the expression of genes associated with inflammation and fibrosis, including Tnf $\alpha$ , Mcp-1, Timp-1 and collagen type 1a1 in Nrf2-null mouse liver with HFD feeding. These findings suggest that OPZ may inhibit fat deposit, inflammation, and fibrosis by Nrf2 independent mechanisms. Although the mechanism underlying the Nrf2-independent pharmacological effect of OPZ observed in this study is not clear, we demonstrated that OPZ induced the expression of Ppara and its target gene Cpt1 in Nrf2-null mice. Ppara has a key role in regulation of fatty acid  $\beta$ -oxidation and it has been reported that HFD induced  $\beta$ -oxidation by Nrf2 independent mechanisms.<sup>(12)</sup> These findings suggest that OPZ may further activate fatty acid  $\beta$ -oxidation even in the presence of HFD-induced activation of PPAR $\alpha$  pathway in Nrf2 null mice. In addition to the role in fatty acid  $\beta$  oxidation, Ppara suppresses inflammatory cytokines by competitively inhibiting nuclear factor-kappa B (NF-kappa B), a master regulator of inflammation.<sup>(30)</sup> In fact, Ppara is known to protect the liver from HFD-induced steatosis and inflammation.<sup>(31,32)</sup> Furthermore, it has been reported that pemafibrate, a selective PPAR $\alpha$  modulator, improves hepatic steatosis, inflammation, and fibrosis in rodent experimental steatohepatitis model.<sup>(33)</sup> PPAR $\alpha$  is considered to have a protective role against the pathogenesis of steatohepatitis. Therefore, the activation of Ppara is one of the candidate mechanisms for Nrf2-independent hepatoprotective effect of OPZ against steatohepatitis in Nrf2-null mice fed a HFD. OPZ is also known as a CAR activator.<sup>(18)</sup> With respect to the relation between PPAR $\alpha$  and CAR, it has been reported that PPAR $\alpha$  agonist induced the drive of CAR into hepatocyte nuclei.<sup>(34)</sup> Furthermore, PPAR $\alpha$ -null mice showed enhanced hepatocyte proliferation in response to CAR agonist.<sup>(35)</sup> These findings suggest that there may be some functional relation between PPAR $\alpha$  and CAR.

In conclusion, this study showed that OPZ administration ameliorated steatohepatitis developed in Nrf2-null mice when placed on a HFD in an Nrf2-independent manner. Therefore, OPZ has hepatoprotective effects via Nrf2 dependent and independent mechanisms.

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## Conflict of Interest

No potential conflicts of interest were disclosed.

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