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Thioredoxin-Binding Protein-2 Deficiency Enhances Methionine-Choline Deficient Diet-Induced Hepatic Steatosis But Inhibits Steatohepatitis in Mice

Md. Kaimul Ahsan,[1,3] Hiroaki Okuyama,[1] Yuma Hoshino,[1] Shin-Ichi Oka,[2] Hiroshi Masutani,[2] Junji Yodoi,[2] and Hajime Nakamura[1]

Abstract

In nonalcoholic fatty liver disease, oxidative stress is believed to play a crucial role as a second-hit for the progression of simple steatosis to steatohepatitis. Thioredoxin (TRX) is a potent antioxidant molecule that exerts anti-apoptotic and anti-inflammatory functions. TRX-binding protein-2 (TBP-2) is an endogenous negative regulator of TRX. Deficiency of TBP-2 in mice causes hyperlipidemia, hepatic steatosis, hypoglycemia, and bleeding tendency, resembling Reye syndrome in a fasting/glucose-deficient state. The aim of this study was to investigate the role of TBP-2 in the development of nonalcoholic steatohepatitis (NASH). TBP-2-deficient (TBP-$2^{-/-}$) and wild-type (WT) mice were fed either a normal or methionine-choline-deficient (MCD) diet for up to 10 weeks. Compared with WT mice, TBP-$2^{-/-}$ mice showed severe simple steatosis rather than steatohepatitis. However, oxidative stress determined by lipid peroxidation and DNA damage, neutrophil infiltration, and hepatic fibrosis were attenuated in TBP-$2^{-/-}$ mice. PCR analysis showed the expressions of fibrosis-inducing and inflammatory cytokine-related genes were less in TBP-$2^{-/-}$ mice. Moreover, leptin, SREBP1c, PPARγ, and adipogenesis–lipogenesis-related genes were upregulated in TBP-$2^{-/-}$ mice. These results strongly suggested that TBP-2 might be involved in pathogenesis of NASH in WT mice, and inhibitors of TBP-2 could be useful in the prevention or treatment of NASH. *Antioxid. Redox Signal.* 11, 2573–2584.

Introduction

NONALCOHOLIC FATTY LIVER DISEASE (NAFLD) is a chronic and progressive liver disease that causes a wide range of liver damage, from simple steatosis to steatohepatitis (30). Nonalcoholic steatohepatitis (NASH), which has a histopathology similar to that of alcoholic steatohepatitis, appears in individuals who do not have an alcoholic history and is associated with underlying conditions such as obesity, hyperlipidemia, hypercholesterolemia, diabetes, insulin resistance, and the metabolic syndrome (4, 13, 31, 32). A two-hit hypothesis has been advocated in the pathogenesis of NASH (2, 10). In the initial hit, hepatic steatosis is established by the intake of a lipid- or glucose-rich diet, or the presence of one of the above conditions, leading to an increased metabolic rate, peripheral insulin resistance, and accumulation of hepatic triglycerides (TG) (44). The secondary hit includes steatosis-mediated oxidative stress, mitochondrial dysfunction, and increased production of reactive oxygen species (ROS) and pro-inflammatory cytokines, which leads to hepatocellular damage, cellular inflammation, and fibrosis, ultimately development of NASH (9, 16, 17). NASH can progress to liver cirrhosis and finally to end-stage liver disease or hepatocellular carcinoma (21, 33). Therefore, oxidative stress and peroxidative liver damage appear to play a major role in the development of NASH from simple steatosis.

In mammals, two major *in vivo* antioxidant systems are glutathione (GSH)-dependent glutathione peroxidase, and thioredoxin (TRX)-dependent peroxidase peroxiredoxin (Prx) scavenges hydrogen peroxide (H_2O_2). TRX not only acts as an antioxidant through Prx but as an anti-apoptotic, anti-inflammatory, and redox regulatory molecule by its redox-active two-cysteine residues in the active site sequence (23, 36). By use of a two-hybrid screening system, we identified thioredoxin-binding protein-2 (TBP-2) as a negative regulator of TRX (39). TBP-2 is identical to vitamin-D_3-upregulated protein 1 (VDUP1) (7), which is also called thioredoxin-interacting protein (TXNIP) (3). TBP-2 plays an important

[1]Thioredoxin Project, Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, Kyoto, Japan.
[2]Department of Biological Responses, Laboratory of Infection and Prevention, Institute for Virus Research, Kyoto University, Kyoto, Japan.
[3]Department of Surgery, Cardiovascular Research Center, The University of Connecticut Health Center, Farmington, Connecticut.

role in suppression of cellular growth (1, 6, 18, 38), promotion of cellular differentiation (6), induction of apoptosis (6, 24, 28), development of natural-killer (NK) cells (27), and lipid metabolism (3, 40). Overexpression of TBP-2 also involved in an induction of reactive oxygen species (ROS) (45). TBP-2-deficient (TBP-$2^{-/-}$) mice develop hematuria, melena, hypoglycemiam and hypertriglyceridemia in the fasting condition, leading to death due to liver and renal failure, a state resembling Reye syndrome (40). A methionine and choline-deficient (MCD) diet has been widely used to produce an animal model of NASH (20, 34, 48, 49). In this study, we used this dietary model in TBP-$2^{-/-}$ and wild-type (WT) mice in order to elucidate the possible role of TBP-2 in the development of NASH. The results suggest that deficiency of TBP-2 enhances the development of simple hepatic steatosis but inhibits the development of steatohepatitis.

Materials and Methods

Animals and treatments

C57BL/6-strained TBP-2-deficient (TBP-$2^{-/-}$) mice were generated as previously described (40). Six-week-old, wild-type C57BL/6 (WT) mice were purchased from CLEA (Tokyo, Japan) followed by a 2-week acclimatization before beginning the experiments. Eight-week-old TBP-$2^{-/-}$ ($n = 10$) and WT ($n = 10$) mice were divided into four groups (5 mice per group; 3 males and 2 females), housed with 12-h light-dark cycle and fed either a normal diet (Oriental Yeast, Tokyo, Japan) or a MCD diet (Cat no. 960439; ICN, Aurora, OH) for 2–10 weeks. Similar experiments were done twice. Therefore, the total number of animals became 10 in each group ($n = 10$; 6 males and 4 females). The development and progression of liver disease was assessed by means of biochemical, histological, and oxidative-stress tests. All experimental procedures were performed at least three times according to animal guidelines, as permitted by the animal experimentation committee of the Graduate School of Medicine, Kyoto University, Japan.

Serum collection and analysis of biochemical parameters

All groups of mice were anesthetized with intraperitoneal injection of pentobarbital before sampling. Total blood was collected directly from the right ventricle after opening the chest. Serum was separated from total blood by centrifugation in a serum separator tube (Cherry-red capped, Terumo Medical Corp., Somerset, NJ). Serum aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (T-bilirubin), total cholesterol (T-CH), triglycerides (TG), and glucose were measured by the Blood Research Laboratories (Kyoto, Japan).

Histopathological and immunohistochemical analysis

After the animals were sacrificed, their livers were immediately removed. Each liver was divided into three parts: one for RNA extraction, one for protein preparation, and the other for histological analysis. In all experimental groups, $4 \sim 8\,\mu$m thick sections of 10% formalin-fixed and paraffin-embedded liver tissues were stained with hematoxylin and eosin, and examined microscopically. Also, Azan-stained sections and fresh tissue-tech frozen liver tissue sections, stained with Oil-Red-O (Nacalai, Kyoto, Japan), were examined for the degree of hepatic structural change, steatosis, and fibrosis. For immunohistochemical analysis, tissues were first reacted with mouse monoclonal anti-8-hydroxy-2′-deoxyguanosine (8-OHdG; Nikken SEIL Co., Fukuroi, Japan) antibody (1–10 μg/ml in dilution), anti-4-hydroxy-2-nonenal (4-HNE; Nikken SEIL Co.) antibody (10–20 μg/ml in dilution), anti-hexanoyl-lysine adduct (HEL; Nikken SEIL Co.) antibody (2 μg/ml in dilution), or rabbit polyclonal anti-myeloperoxidase (MPO; Neomarkers, Fremont, CA) antibody (1:50 in dilution). Normal rabbit serum (DAKO Corporation, Kyoto, Japan) (1:75 dilution) was used to inhibit nonspecific binding of antibodies. A secondary anti-mouse/rabbit biotinylated antibody (DAKO Corporation) (1:300 dilution) then was applied. Finally, antibody binding was visualized by the use of the avidin-biotin-immuno peroxidase complex method, with the vectastain ABC-AP kit (Vector Laboratories, Burlingame, CA), according to the manufacturer's instructions. Counterstaining was performed with Gill's hematoxylin (Vector Laboratories). Steatosis, lipid peroxidation, DNA damage, neutrophil infiltration, and fibrosis were quantitated in five randomly selected snaps (200 X magnification) derived from at least five individual liver tissues, using Image J software analysis (http://rsb.info.nih.gov/ij/), as previously described (11).

Semiquantitative RT-PCR

Total RNA was extracted from liver tissue, using Trizol reagent (Invitrogen, Carlsbad, CA). Three micrograms of total RNA was reverse-transcribed, using a SuperScript First-Strand Synthesis System (Invitrogen) with oligo $dT_{12–18,}$ according to the manufacturer's protocol. The cDNA was amplified by PCR, using Ex-Taq DNA-polymerase (TaKaRa Bio Inc., Otsu, Japan) following a standard semiquantitative RT-PCR technique in which the amplified products were not saturated at the number of cycles performed. The primers used for the amplification were enlisted in Table 1. The PCR products were visualized by electrophoresis in 2% agarose gel (Nacalai, Kyoto, Japan) with ethidium bromide (0.5 μg/ml; Nacalai).

Statistical analysis

All data are shown as means $\pm$ SDs. Statistical significance were analyzed with 2-way ANOVA followed by Bonferroni post hoc test using GraphPad Prism software (Version 5.0) and considered significant when the p value is <0.05.

Results

MCD diet causes more rapid development and more severe steatosis in TBP-$2^{-/-}$ mice than in WT mice

Our previous study showed that deficiency of TBP-2 in fasted mice causes impaired fatty acid utilization due to dysregulation of acetyl-CoA and hyperinsulinemia, leading to dyslipidemia, with significantly higher serum triglycerides (TG) levels (40); in normally fed animals, increased triacylglycerol stores in the liver tissue also were present (12). In that work and in the present study, TBP-$2^{-/-}$ mice gained more weight than did WT mice (Fig. 1A) and liver–body weight ratios were also higher in TBP-$2^{-/-}$ mice than obtained in WT mice (Fig. 1B). These results suggest that TBP-2 is involved in fat metabolism and its deficiency might be related

Table 1. Sequences of Primer Used in this Study

Name of genes	*Forward primer (5′- 3′)*	*Reverse primer (5′- 3′)*
TBP-2	ATGGTGATGTTCAAGAAGATCA	TCACTGCACGTTGTTGTTGTTG
TRX	ATGGTGAAGCTGATCGAGA	CAGTAATAGAGGCTTCAAGC
Leptin	AGTGGGAATGAGAAATCACTTAGC	GTGTATTGCTTTCCATCAAGTGTC
Resistin	CTGATGTCGGGGAAGTGAGC	GTGCAGGTGCCTGTAGAGAC
Adiponectin	CGCTTATGTGTATCGCTCAGG	TAGAGTCCATTGTGGTCCCCA
SREBP1c	ATCGGCGCGGAAGCTGTC	CGTTCAAAACCGCTGTGTGTCC
PPAR-γ	ACCCAATGGTTGCTGATTAC	CGGGAAGGACTTTATGTATGAG
aP2	TCCTGTGCTGCAGCCTTTCTCA	CCAGGTTCCCACAAAGGCATCA
Adepsin	TGAACCCTACAAGCGATGGTATG	CCGGTTCATGATTGACACTCTGA
Fas	GTCTGACACTGGCAATCTG	GGTCACACGGGTAGGTAG
Collagen-I	ATGTTCAGCTTTGTGGACCTC	TCCCTCGACTCCTACATCTTC
TGF-β1	TACTGCCGCTTCTGCTCCCACT	TCAGCTGCACTTGCAGGAGCG
NF-κB	ATGGCAGACGATGATCCCTA	CAAGGTCAGAATGCACCAGA
TNF-α	ATGAGCACAGAAAGCATGATCC	TCACAGAGCAATGACTCCAAAG
IL-1β	ATGGCAACTGTTCCTGAACTCA	GGAAGACACAGATTCCATGGTG
INF-γ	ATGAACGCTACACACTGCATC	TCAGCAGCGACTCCTTTTCC
β-Actin	ATGGATGACGATATCGCTGCGCT	TAGAAGCACTTGCGGTGCACGAT

to the development of metabolic diseases. Here we investigated the generation of NAFLD induced by MCD diet feeding for 2–10 weeks in TBP-2$^{-/-}$ and WT mice. Liver tissue from TBP-2$^{-/-}$ mice fed a MCD diet for 1 week began to contain lipid vesicles; however the corresponding tissue from WT mice did not (data not shown). As shown by H&E staining of liver tissues, 2 weeks of MCD-diet feeding resulted in the presence of lipid vesicles that were larger in the TBP-2$^{-/-}$ mice than in WT mice. After 10 weeks of MCD-diet feeding, inflammatory cell infiltration was higher in WT mice than in TBP-2$^{-/-}$ mice (Fig. 1C). Oil-Red-O staining revealed massive steatosis in TBP-2$^{-/-}$ mice but much less in WT mice (Fig. 1D and E). RT-PCR showed that TBP-2 expression was mildly upregulated in MCD-diet fed WT mice (Fig. 1F). No significant difference was observed in TRX expression in MCD diet-fed TBP-2$^{-/-}$ and WT mice, although TRX expression was upregulated in normal diet-fed TBP-2$^{-/-}$ mice compared to that in WT mice. Upregulation of leptin and downregulation of resistin and adiponectin were observed in TBP-2$^{-/-}$ mice compared to WT mice (Fig. 1F). Genes related to adipogenesis and lipogenesis, specifically SREBP1c and PPAR-γ, were upregulated in TBP-2$^{-/-}$ mice compared to WT mice (Fig. 1G).

Serum biochemical analysis revealed significantly more liver injury and lower triglyceride levels in WT mice than in TBP-2$^{-/-}$ mice induced by MCD diet

We next compared serum biochemical markers of hepatic injury induced by MCD diet in the WT and TBP-2$^{-/-}$ mice. Our preliminary data showed that there was no significant difference in the biochemical markers in MCD-fed male and female mice. We also did not find any significant differences in 2 weeks MCD-diet fed WT and TBP-2$^{-/-}$ mice (data not shown). At 10 weeks, AST, ALT, ALP, and total bilirubin values were significantly higher, and T-CH and TG values significantly lower, in WT mice than in TBP-2$^{-/-}$ mice (Table 2). Also, the serum glucose levels were significantly lower in both ND and MCD diet fed TBP-2$^{-/-}$ mice than WT mice. These results are consistent with our previous report (40) that fatty acid utilization in TBP-2$^{-/-}$ mice is impaired and energy is derived only from glucose, leading to hypoglycemia.

MCD diet-induced oxidative stress causes higher lipid peroxidation in liver tissue of WT mice than of TBP-2$^{-/-}$ mice

MCD-diet feeding causes oxidative stress by depletion of GSH and increased generation of H_2O_2 in liver mitochondria (19, 43). Steatosis resulting from oxidative stress seems to be critical for the generation of NASH. Lipid peroxidation, as measured by anti-4-HNE (Fig. 2A) and anti-HEL (Fig. 2B) staining, was enhanced in WT mice compared with TBP-2$^{-/-}$ mice (Fig. 2C). Thus, MCD diet-induced oxidative stress was attenuated by deficiency of TBP-2.

MCD diet-induced oxidative stress causes more DNA damage in liver tissue of WT mice than of TBP-2$^{-/-}$ mice

Oxidative DNA damage, as measured by anti-8-OHdG staining, was significantly higher in WT mice than in TBP-2$^{-/-}$ mice (Fig. 3A). Image J cell counting showed that DNA-damaged cells were 10 times more numerous in WT mice than in TBP-2$^{-/-}$ mice (Fig. 3B).

Neutrophil infiltration is greater in the liver tissue of WT mice than in TBP-2$^{-/-}$ mice induced by MCD diet

Steatosis and oxidative stress-mediated inflammation is thought to be a prerequisite for the development of liver fibrosis in NASH. To address this possibility, we compared hepatic inflammation in 10-week MCD diet-fed WT mice to that in TBP-2$^{-/-}$ mice. Neutrophil infiltration, identified immunohistochemically with anti-MPO staining, was massive in WT mice but minimal in TBP-2$^{-/-}$ mice (Fig. 4A). This observation was confirmed by enumeration of neutrophils through the use of Image J software (http://rsb.info.nih.gov/ij/) (Fig. 4B).

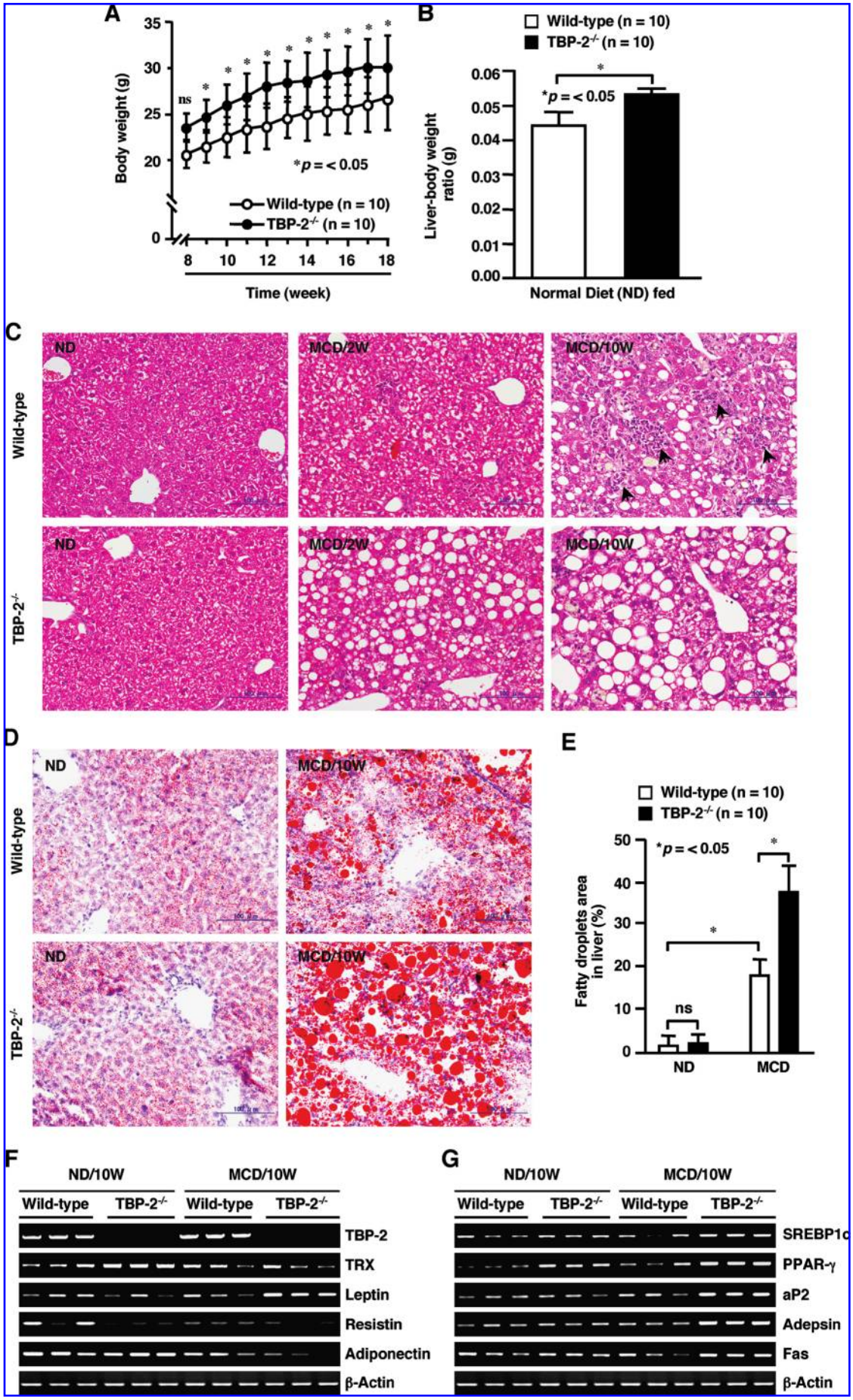
A
Body weight (g)
Time (week)
ns
*p = < 0.05
Wild-type (n = 10)
TBP-2-/- (n = 10)
B
Wild-type (n = 10)
TBP-2-/- (n = 10)
*p = < 0.05
Liver-body weight ratio (g)
Normal Diet (ND) fed
C
Wild-type
TBP-2-/-
ND
MCD/2W
MCD/10W
D
Wild-type
TBP-2-/-
ND
MCD/10W
E
Wild-type (n = 10)
TBP-2-/- (n = 10)
*p = < 0.05
Fatty droplets area in liver (%)
ns
ND
MCD
F
ND/10W
MCD/10W
Wild-type
TBP-2-/-
TBP-2
TRX
Leptin
Resistin
Adiponectin
β-Actin
G
SREBP1c
PPAR-γ
aP2
Adepsin
Fas
β-Actin

Table 2. Serum Biochemical Analysis of Hepatic Injury Induced by MCD Diet Shown in WT and TBP-2$^{-/-}$ Mice

	Normal diet		*MCD diet*	
	WT (n = 10)	*TBP-2$^{-/-}$ (n = 10)*	*WT (n = 10)*	*TBP-2$^{-/-}$ (n = 10)*
AST (IU/L)	55.40 ± 6.07	53.00 ± 8.15	$558.20 \pm 131.12^{\dagger,\S}$	287.60 ± 41.29
ALT (IU/L)	27.00 ± 1.87	20.40 ± 1.34	$623.40 \pm 177.31^{\dagger,\S}$	288.00 ± 56.30
ALP (IU/L)	234.60 ± 40.95	194.20 ± 27.50	$593.00 \pm 81.01^{\dagger,\S}$	268.80 ± 31.07
Total bilirubin (mg/dL)	0.12 ± 0.04	0.10 ± 0.00	$1.14 \pm 0.21^{\dagger,\S}$	0.32 ± 0.13
Total cholesterol (mg/dL)	72.40 ± 12.78	105.80 ± 13.14*	$16.00 \pm 4.74^{\dagger,\S}$	59.80 ± 7.40
Triglycerides (mg/dL)	46.20 ± 16.81	63.00 ± 20.26	$10.40 \pm 2.41^{\dagger,\S}$	31.40 ± 4.93
Glucose (mg/dL)	258.40 ± 29.94	215.20 ± 17.48*	$78.20 \pm 16.04^{\dagger}$	$53.40 \pm 8.35^{\S}$

*, †, and § respectively indicating p value ≤ 0.05, in ND (normal diet) fed WT (C57BL/6) *vs.* TBP-2$^{-/-}$ mice, WT mice ND *vs.* MCD diet, and MCD diet fed WT *vs.* TBP-2$^{-/-}$ mice. Biochemical maker of hepatic injury is significantly higher in WT mice with significantly lower fat parameters. MCD diet fed TBP-2$^{-/-}$ mice are showing hypoglycemia ($p \leq 0.05$).

MCD diet-mediated fibrosis and fibrosis-inducing genes are upregulated in WT mice compared to TBP-2$^{-/-}$ mice

Steatosis, oxidative stress, inflammation, and, finally, fibrosis are considered sequential steps in the development of NASH. Therefore, we analyzed hepatic fibrosis in Azan-stained sections from 10-week MCD diet-fed WT and TBP-2$^{-/-}$ mice. Livers of the WT mice had severe fibrosis in perivenular, periportal, and portocentral spaces, whereas the livers of the TBP-2$^{-/-}$ mice had little fibrosis (Fig. 5A). This observation was confirmed by the use of Image J software analysis (Fig. 5B). Since collagen-I and transforming growth factor beta-1 (TGF-β1) are considered fibrosis-inducing genes (5, 8, 25, 54), we analyzed the expression level of the genes by semiquantitative RT-PCR. As seen in Fig. 5C, the genes were highly expressed in liver tissues of MCD diet-fed WT mice but less expressed in MCD diet-fed TBP-2$^{-/-}$ mice. In the normal diet-fed mice, the expression level of collagen-I was substantially decreased and the level of TGF-β1 moderately decreased in TBP-2$^{-/-}$ mice, compared to corresponding values in WT mice. These results indicate that MCD diet-induced lipid peroxidation (Fig. 2), and DNA damage (Fig. 3) directly stimulates pro-fibrogenic cells to increase synthesis of collagen-I as well as stimulates monocyte or macrophage to upregulate TGF-β1 expression in WT mice. Also, the TBP-2 regulates the expression of collagen-I and TGF-β1. Thus, deficiency of TBP-2 inhibits fibrosis formation by decreasing TBP-2-mediated activation of collagen-I and TGF-β1.

Inflammatory cytokine-related genes, NF-κB, TNF-α, IL-1β, and INF-γ, are also upregulated in WT mice compared to TBP-2$^{-/-}$ mice mediated by MCD diet

We further analyzed the mRNA expression level of cytokines involved in the regulation of inflammation and fibrosis (NF-κB, TNF-α, IL-1β, and INF-γ). In MCD diet-fed WT mice, these genes were highly upregulated, and expression was diminished in TBP-2$^{-/-}$ mice; in animals fed a normal diet, there was less difference in levels of gene expression between TBP-2$^{-/-}$ and WT mice (Fig. 6).These results indicate that deficiency of TBP-2 can reduce the sources of pro-inflammatory cytokines in MCD diet-fed mice, resulting in less hepatic inflammation and fibrosis, the characteristic features of NASH.

Discussion

Nonalcoholic steatohepatitis (NASH) is an advanced and progressive chronic liver disease. In this study, we investigated the pathogenesis of NASH in our TBP-2$^{-/-}$ mice model with MCD diet feeding. The MCD diet is widely used to produce an animal model of NASH (34, 53). We found that 10 weeks of MCD-diet feeding promoted the development of NASH-like pathology, including a high grade of oxidative stress, neutrophil infiltration, and fibrosis in the liver tissue of WT mice (Figs. 2–5). The mechanism of MCD diet-mediated generation of NASH is known to include the development of steatosis through the

FIG. 1. Deficiency of TBP-2 in mice enhanced MCD diet-induced hepatic simple steatosis. (**A**) Body weights and (**B**) liver–body weight ratios of wild-type ($n = 10$) and TBP-2$^{-/-}$ ($n = 10$) mice fed a normal diet. Values are in gram (g)/mice, means $\pm$ SDs (*$p \leq 0.05$). (**C**) Hematoxylin and eosin (H&E) staining, and (**D**) *Oil-Red-O* staining of representative liver tissue sections from WT mice (*upper rows*) or TBP-2$^{-/-}$ mice (*lower rows*) fed a normal diet (ND) or methionine- and choline-deficient (MCD) diet for 2 weeks (2W) to 10 weeks (10W). The original magnification was 200$\times$ and *arrows* mark indicating cellular infiltrations. (**E**) The degree of simple steatosis was measured on the basis of percentage of fatty droplets in area analysis by Image-J software in Oil-Red-O-stained liver tissue sections from WT (*white bars*, $n = 10$) or TBP-2$^{-/-}$ (*black bars*, $n = 10$) mice fed a MCD diet or normal diet for 10 weeks. Data are shown as means $\pm$ SDs (*$p \leq 0.05$ and ns, indicating no significance). (**F**) Semiquantitative RT-PCR analysis of mRNA expression of TBP-2, TRX, leptin, resistin, and adiponectin (with β-actin as a housekeeping gene) in the liver tissues of 10-week ND or MCD diet-fed WT or TBP-2$^{-/-}$ mice. Experiments were performed in triplicate. Similar results were obtained from three separate experiments. (**G**) Semiquantitative RT-PCR analysis of mRNA expression of the adipogenic and lipogenic specific genes SREBP1c, PPAR-γ, aP2, adepsin, and Fas (with β-actin as a housekeeping gene) in the liver tissues of 10-week ND- or MCD diet-fed WT or TBP-2$^{-/-}$ mice. Experiments were performed in triplicate. Similar results were obtained from three separate experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

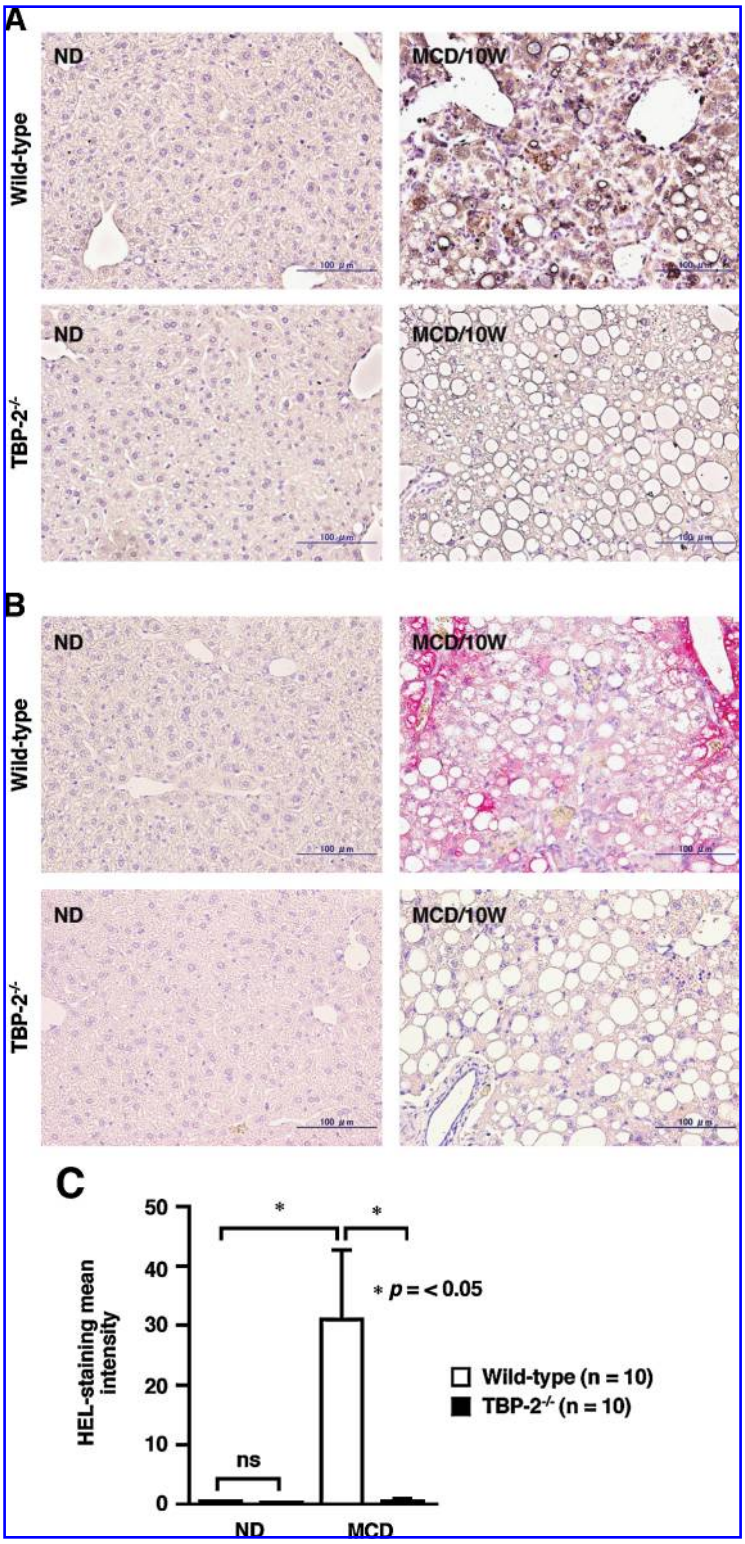

FIG. 2. Deficiency of TBP-2 in mice inhibited MCD diet-induced lipid peroxidation. (**A**) 4-HNE staining and (**B**) HEL staining of representative liver tissue sections from 10-week normal diet (ND) or methionine- and choline-deficient (MCD) diet-fed WT mice (*upper rows*) or TBP-$2^{-/-}$ mice (*lower rows*). The original magnification was 200×. (**C**) The degree of lipid peroxidation was measured on the basis of HEL-stained intensity recorded by Image-J software in 10-week ND- or MCD diet-fed WT (*white bars*, $n = 10$) or TBP-$2^{-/-}$ (*black bars*, $n = 10$) mice. Data are shown as means ± SDs (*$p \leq 0.05$ and ns, indicating no significance). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

inhibition of phosphatidylcholine synthesis, leading to decreased hepatic export of VLDL and increased TG accumulation in liver (52, 55, 56). In addition, MCD-diet feeding causes mitochondrial dysfunction, decreases ATP synthesis in the liver cells, and increases mitochondrial production of H_2O_2 and ROS, with, ultimately, increased oxidative stress (19, 47). Furthermore, methionine is an essential component for biosynthesis of the endogenous antioxidant molecule GSH (42, 43). MCD feeding decreases the synthesis of GSH, thus enhancing oxidative stress. Oxidative stress can result in increased lipid peroxidation, stellate cell activation, increased release of pro-inflammatory cytokines and inflammatory cell infiltration (14). NASH and hepatic fibrosis then develop.

In the current study, MCD diet increased leptin and decreased resistin and adiponectin in liver tissue. Development of severe steatosis with upregulation of adipogenic and lipo-

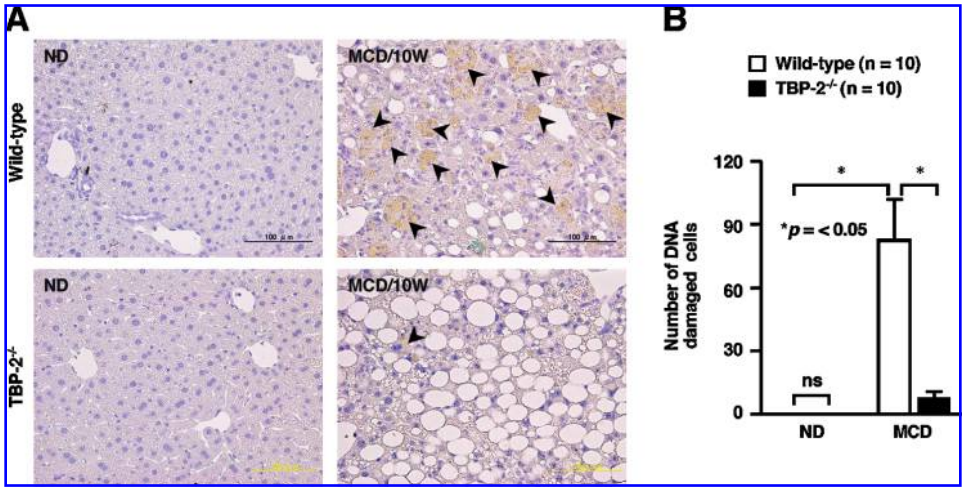

FIG. 3. Deficiency of TBP-2 in mice inhibited MCD diet-induced DNA damage. (**A**) 8-OHdG staining of representative liver tissue sections from 10-week normal diet (ND)-fed or methionine- and choline-deficient (MCD) diet-fed WT mice (*upper row*) or TBP-2$^{-/-}$ mice (*lower row*). The original magnification was 200×. (**B**) DNA damage was quantified by DNA-damaged cell counting with Image-J software, using 8-OHdG-stained liver tissue sections from 10-week ND- or MCD diet-fed WT (*white bars*, $n = 10$) or TBP-2$^{-/-}$ (*black bars*, $n = 10$) mice. Data are shown as means ± SDs (*$p \leq 0.05$ and ns, indicating no significance). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

genic genes in TBP-2$^{-/-}$ mice was also observed (Fig. 1F and G). The higher expression of PPARγ associated with higher expression levels of adipogenic–lipogenic genes aP2 and Fas in MCD diet fed liver tissue, indicating adipogenic–lipogenic transformation of hepatocytes (26, 46). Decreased adiponectin expression may enhance triglyceride deposition in the liver tissue to produce simple severe steatosis (51). The deficiency of TBP-2 seemed to lead to loss of control of activation of PPAR-γ and SREBP-1c, including activation of other adipogenic and lipogenic genes, such as aP2 and adepsin, as well as Fas. Moreover, impaired acetyl-CoA utilization (40) might help to induce the steatosis in TBP-2$^{-/-}$ mice. Despite less severe steatosis, the cellular infiltration (Fig. 1C) and liver injury were greater in WT mice than in TBP-2$^{-/-}$ mice (Table 2). The serum TG and total cholesterol levels were also increased in TBP-2$^{-/-}$ mice. It was previously reported that the TGs might help in protection against the MCD diet-induced liver damage in TBP-2$^{-/-}$ mice (50). Also another possibility is that the increased levels of serum TG and total cholesterol in TBP-2$^{-/-}$ mice might be simply the hallmark of a more deranged and pro-lipogenic metabolic state rather than a protective scenario.

Since TBP-2 is a negative regulator of TRX (39) and overexpression of TBP-2 mediates oxidative stress via suppressing

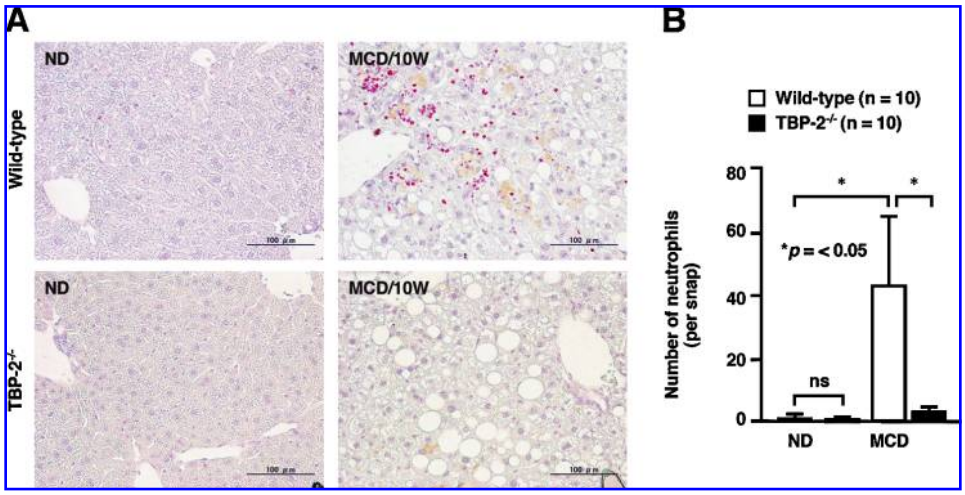

FIG. 4. Deficiency of TBP-2 in mice inhibited MCD diet-induced neutrophil infiltration. (**A**) MPO staining of representative liver tissue sections from 10-week normal diet (ND)- or methionine- and choline-deficient (MCD) diet-fed WT mice (*upper row*) or TBP-2$^{-/-}$ mice (*lower row*). The original magnification was 200×. (**B**) Neutrophil infiltration was quantified by a cell counting with Image-J software in MPO-stained liver tissue sections from 10-week ND- or MCD diet-fed WT (*white bars*, $n = 10$) or TBP-2$^{-/-}$ (*black bars*, $n = 10$) mice. Data are shown as means ± SDs (*$p \leq 0.05$ and ns, indicating no significance). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

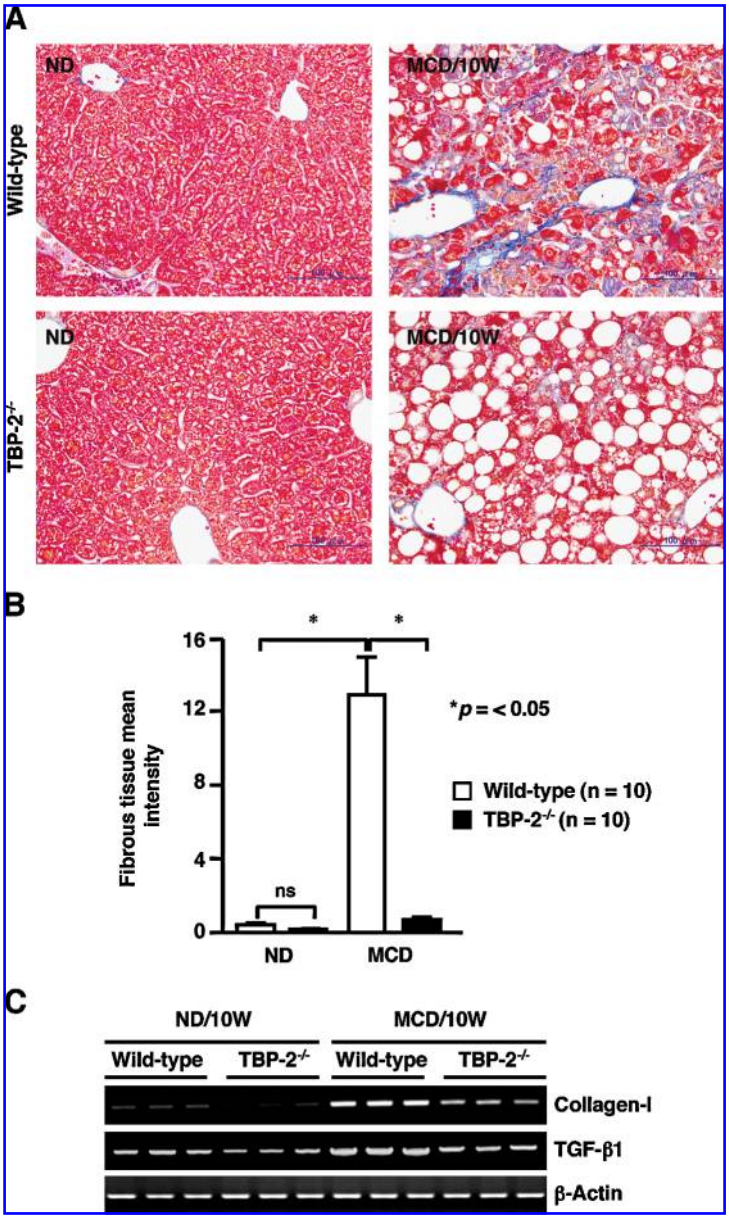

FIG. 5. Deficiency of TBP-2 in mice inhibited MCD diet-induced induction of fibrosis and fibrosis-inducing genes. (**A**) Azan staining of representative liver tissue sections from 10-week normal diet (ND)- or methionine- and choline-deficient (MCD) diet-fed WT mice (*upper row*) or TBP-$2^{-/-}$ mice (*lower row*). The original magnification was 200×. (**B**) The degree of fibrosis was quantified from HEL-stained intensity by Image-J software in the 10-week ND- or MCD diet-fed WT (*white bars*, $n = 10$) or TBP-$2^{-/-}$ (*black bars*, $n = 10$) mice. Data are shown as means ± SDs (*$p \leq 0.05$ and ns, indicating no significance). (**C**) Semiquantitative RT-PCR analysis of mRNA expression of the fibrosis-inducing genes collagen-I and TGF-β1 (with β-actin as housekeeping gene) from the liver tissues of 10-week ND- or MCD diet-fed WT or TBP-$2^{-/-}$ mice. Experiments were performed in triplicate. Similar results were obtained from three separate experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

the TRX function (22), deficiency of TBP-2 in mice may enhance the antioxidant and anti-inflammatory functions of TRX (23, 35). In support of this evidence, we observed the higher TRX expression in ND-fed TBP-$2^{-/-}$ mice (Fig. 1F). The enhanced activities of TRX might account for the decreased development of steatohepatitis in the TBP-$2^{-/-}$ mice. TBP-2 also has unique functions independent to TRX, such as extensive involvement in fat metabolism, cell-growth suppression, induction of apoptosis, and NK-cell development (1, 6, 18, 24, 27). Therefore, deficiency of TBP-2 also might account for the attenuation of MCD diet-induced apoptosis in liver parenchyma cells and ultimately decreased development of steatohepatitis in the TBP-$2^{-/-}$ mice. Although we have no data in the present study, further studies will show the direct evidence that TBP-2 deficiency may inhibit the pro-apoptotic effect of TBP-2. Deficiency of TBP-2 in TBP-$2^{-/-}$ mice results in impaired acetyl-CoA utilization and increased triacylglycerol stores in liver by enhancing lipogenesis and fatty acid esterification (12). A Reye-like syndrome has been reported in fasted TBP-$2^{-/-}$ mice, characterized by hypertriglyceridemia, hepatic steatosis, renal failure, severe coagulopathy, and hypoglycemia (40). Enforced expression of TBP-2 increased ROS production in endothelial cells (45). In contrast, TBP-2 transgenic mice had higher ROS production (41). Therefore, deficiency of TBP-2 in TBP-$2^{-/-}$ mice may interfere with the production of ROS in MCD diet-fed animals, resulting in less production of pro-inflammatory cytokines and less inflammation. Indeed, here we showed that MCD diet-mediated oxidative stress-induced lipid peroxidation and DNA damage were less in TBP-$2^{-/-}$ mice than in WT mice (Figs. 2 and 3). The increased lipid peroxidation and DNA damage in WT mice fed a MCD diet likely induces a high level of oxidative stress, which induces the production of fibrosis by increasing the synthesis of collagen and TGF-β (14, 17, 25, 33, 37). In support of this possibility, it was our finding that MCD diet-induced induction of collagen-I and TGF-β1

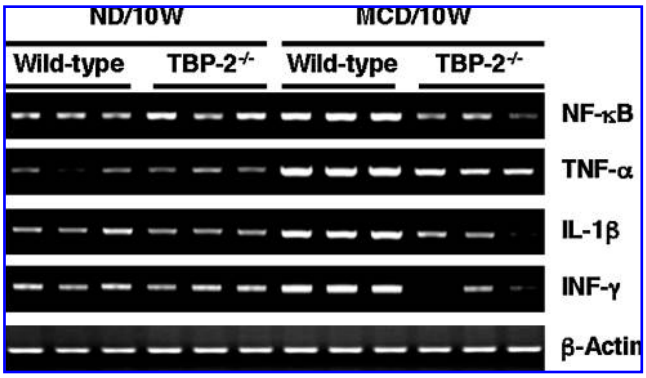

FIG. 6. Semiquantitative RT-PCR for representative cytokines involved in the induction of inflammation. The mRNA level of genes for NF-κB, TNF-α, IL-1β, and INF-γ (with β-actin as housekeeping gene) from the liver tissues of 10-week normal (ND)- or methionine- choline-deficient (MCD)-diet fed WT or TBP-2$^{-/-}$ mice. Experiments were performed in triplicate. Similar results were obtained from three separate experiments.

expression was less in TBP-2$^{-/-}$ mice than in WT mice (Fig. 5). The collagen-I expression in TBP-2$^{-/-}$ mice was down-regulated only by deficiency of TBP-2. This finding coincides with the results of previous reports that the overexpression of TBP-2 causes upregulation of collagen synthesis, and addition of anti-TGF-β antibody significantly suppressed collagen-I synthesis as well as TBP-2 (24, 25). Moreover, it was also reported that TBP-2 is upregulated by TGF-β1 (18). Therefore, TBP-2 might have important regulatory effect on collagen-I as well as on TGF-β1. However, further investigation should be done to clarify the regulatory mechanism. In the present study, MCD diet feeding promoted more oxidative stress and the recruitment of more hepatic inflammatory cells (Fig. 4), via activation of NF-κB (29) and other cytokines (TNF-α, IL-1β, and INF-γ) (Fig. 6) in WT mice than in TBP-2$^{-/-}$ mice. The induction of TNF-α in WT mice may cause mitochondrial dysfunction, leading to more ROS production and exaggerated pathogenesis of NASH (15). MCD diet-induced inflammation was less in TBP-2$^{-/-}$ mice than in WT mice. This result might be explained by the presence of fewer NKT cells, leading to less production of NKT cell related cytokines in the TBP-2$^{-/-}$ mice (41), as it was already known that TBP-2 is required for the development of NKT cells (27). However, further investigation will be needed to clarify the exact mechanism. Our proposed molecular mechanism by which TBP-2 deficiency can inhibit MCD diet-induced development of NASH is illustrated in Fig. 7.

In summary, in the presence of TBP-2 in WT mice, MCD diet feeding enhanced the development of NASH by inducing oxidative stress, increasing the release of pro-inflammatory cytokines and resultant inflammatory cellular infiltrate, and activating fibrosis. In contrast, lack of TBP-2 in TBP-2$^{-/-}$ mice inhibited the development of NASH by lowering oxidative stress, blocking pro-inflammatory cytokine release, inhibiting cellular infiltration and fibrosis, and may be attenuating apoptosis of liver parenchyma cells. Thus, it appears that TBP-2 plays a key role in the pathogenesis of NASH, at least in MCD diet-induced animal model. Various compounds have been reported to induce TBP-2 expression (6, 7, 18, 24), but no TBP-2 inhibitor has thus far been described. We suggest the possibility that TBP-2 inhibitor substances could be useful in the prevention or treatment of NASH.

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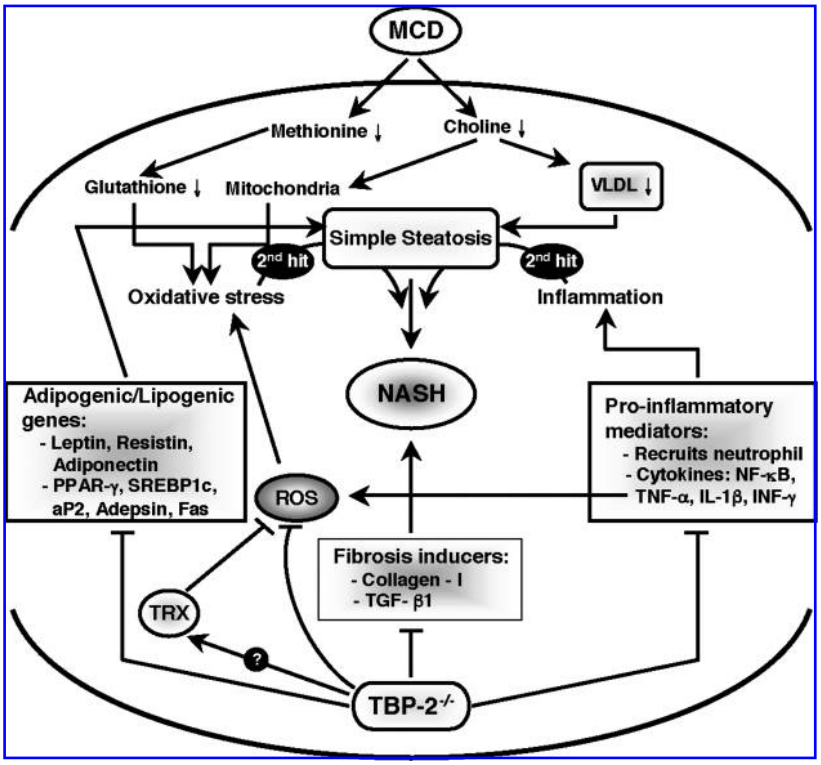

FIG. 7. Schematic representation of the proposed role of TBP-2 in the progression of simple steatosis to NASH in MCD diet-induced NAFLD. Deficiency of TBP-2 enhances simple severe steatosis by upregulating genes controlling adipogenesis and lipogenesis. At the same time, deficiency of TBP-2 suppresses ROS production, either directly or indirectly, via activation of the antioxidative gene TRX, resulting in less oxidative stress. Deficiency of TBP-2 also suppresses the production of pro-inflammatory mediators and fibrosis-inducing genes, resulting in less inflammation and fibrosis in the liver. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

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Author Disclosure Statement

No competing financial interests exist.

References

1. Ahsan MK, Masutani H, Yamaguchi Y, Kim YC, Nosaka K, Matsuoka M, Nishinaka Y, Maeda M, and Yodoi J. Loss of interleukin-2-dependency in HTLV-I-infected T cells on gene silencing of thioredoxin-binding protein-2. *Oncogene* 25: 2181–2191, 2006.
2. Angulo P and Lindor KD. Non-alcoholic fatty liver disease. *J Gastroenterol Hepatol* 17 Suppl: S186–190, 2002.
3. Bodnar JS, Chatterjee A, Castellani LW, Ross DA, Ohmen J, Cavalcoli J, Wu C, Dains KM, Catanese J, Chu M, Sheth SS, Charugundla K, Demant P, West DB, de Jong P, and Lusis AJ. Positional cloning of the combined hyperlipidemia gene Hyplip1. *Nat Genet* 30: 110–116, 2002.
4. Bogdanova K, Poczatkova H, Uherkova L, Riegrova D, Rypka M, Feher J, Marchesini G, and Vesely J. Non-alcoholic fatty liver disease (NAFLD)–a novel common aspect of the metabolic syndrome. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* 150: 101–104, 2006.
5. Border WA and Noble NA. Interactions of transforming growth factor-beta and angiotensin II in renal fibrosis. *Hypertension* 31: 181–188, 1998.
6. Butler LM, Zhou X, Xu WS, Scher HI, Rifkind RA, Marks PA, and Richon VM. The histone deacetylase inhibitor SAHA arrests cancer cell growth, up-regulates thioredoxin-binding protein-2, and down-regulates thioredoxin. *Proc Natl Acad Sci USA* 99: 11700–11705, 2002.
7. Chen KS and DeLuca HF. Isolation and characterization of a novel cDNA from HL-60 cells treated with 1,25-dihydroxyvitamin D-3. *Biochim Biophys Acta* 1219: 26–32, 1994.
8. Comporti M, Signorini C, Arezzini B, Vecchio D, Monaco B, and Gardi C. Isoprostanes and hepatic fibrosis. *Mol Aspects Med* 29: 43–49, 2008.
9. Cortez–Pinto H, de Moura MC, and Day CP. Non-alcoholic steatohepatitis: From cell biology to clinical practice. *J Hepatol* 44: 197–208, 2006.
10. Day CP and James OF. Steatohepatitis: A tale of two "hits"? *Gastroenterology* 114: 842–845, 1998.
11. De S, Swanson P, Sinanan M, Rosen J, Dagan A, Hannaford B. Assessment of tissue damage due to mechanical stresses. *Intl Conf Biomed Robotics Biomech (IEEE/RAS-EMBS)* (First ed.), Pisa, Tuscany, Italy, 2006, p. 823–828.
12. Donnelly KL, Margosian MR, Sheth SS, Lusis AJ, and Parks EJ. Increased lipogenesis and fatty acid reesterification contribute to hepatic triacylglycerol stores in hyperlipidemic Txnip−/− mice. *J Nutr* 134: 1475–1480, 2004.
13. Festi D, Colecchia A, Sacco T, Bondi M, Roda E, and Marchesini G. Hepatic steatosis in obese patients: Clinical aspects and prognostic significance. *Obes Rev* 5: 27–42, 2004.
14. George J, Pera N, Phung N, Leclercq I, Yun Hou J, and Farrell G. Lipid peroxidation, stellate cell activation and hepatic fibrogenesis in a rat model of chronic steatohepatitis. *J Hepatol* 39: 756–764, 2003.
15. Goossens V, Grooten J, De Vos K, and Fiers W. Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. *Proc Natl Acad Sci USA* 92: 8115–8119, 1995.
16. Grant LM and Lisker–Melman M. Nonalcoholic fatty liver disease. *Ann Hepatol* 3: 93–99, 2004.
17. Green RM. NASH—Hepatic metabolism and not simply the metabolic syndrome. *Hepatology* 38: 14–17, 2003.
18. Han SH, Jeon JH, Ju HR, Jung U, Kim KY, Yoo HS, Lee YH, Song KS, Hwang HM, Na YS, Yang Y, Lee KN, and Choi I. VDUP1 upregulated by TGF-beta1 and 1,25-dihydroxyvitamin D3 inhibits tumor cell growth by blocking cell-cycle progression. *Oncogene* 22: 4035–4046, 2003.
19. Hensley K, Kotake Y, Sang H, Pye QN, Wallis GL, Kolker LM, Tabatabaie T, Stewart CA, Konishi Y, Nakae D, and Floyd RA. Dietary choline restriction causes complex I dysfunction and increased H(2)O(2) generation in liver mitochondria. *Carcinogenesis* 21: 983–989, 2000.
20. Ip E, Farrell GC, Robertson G, Hall P, Kirsch R, and Leclercq I. Central role of PPARalpha-dependent hepatic lipid turnover in dietary steatohepatitis in mice. *Hepatology* 38: 123–132, 2003.
21. Jansen PL. Nonalcoholic steatohepatitis. *Neth J Med* 62: 217–224, 2004.
22. Junn E, Han SH, Im JY, Yang Y, Cho EW, Um HD, Kim DK, Lee KW, Han PL, Rhee SG, and Choi I. Vitamin D3 up-regulated protein 1 mediates oxidative stress via suppressing the thioredoxin function. *J Immunol* 164: 6287–6295, 2000.
23. Kaimul Ahsan M, Nakamura H, Tanito M, Yamada K, Utsumi H, and Yodoi J. Thioredoxin-1 suppresses lung injury and apoptosis induced by diesel exhaust particles (DEP) by scavenging reactive oxygen species and by inhibiting DEP-induced downregulation of Akt. *Free Radic Biol Med* 39: 1549–1559, 2005.
24. Kaimul AM, Nakamura H, Masutani H, and Yodoi J. Thioredoxin and thioredoxin-binding protein-2 in cancer and metabolic syndrome. *Free Radic Biol Med* 43: 861–868, 2007.
25. Kobayashi T, Uehara S, Ikeda T, Itadani H, and Kotani H. Vitamin D3 up-regulated protein-1 regulates collagen expression in mesangial cells. *Kidney Int* 64: 1632–1642, 2003.
26. Larter CZ, Yeh MM, Williams J, Bell–Anderson KS, and Farrell GC. MCD-induced steatohepatitis is associated with hepatic adiponectin resistance and adipogenic transformation of hepatocytes. *J Hepatol* 49: 407–416, 2008.
27. Lee KN, Kang HS, Jeon JH, Kim EM, Yoon SR, Song H, Lyu CY, Piao ZH, Kim SU, Han YH, Song SS, Lee YH, Song KS, Kim YM, Yu DY, and Choi I. VDUP1 is required for the development of natural killer cells. *Immunity* 22: 195–208, 2005.
28. Liyanage NP, Fernando MR, and Lou MF. Regulation of the bioavailability of thioredoxin in the lens by a specific thioredoxin-binding protein (TBP-2). *Exp Eye Res* 85: 270–279, 2007.
29. Lu Y and Wahl LM. Oxidative stress augments the production of matrix metalloproteinase-1, cyclooxygenase-2, and prostaglandin E2 through enhancement of NF-kappa B activity in lipopolysaccharide-activated human primary monocytes. *J Immunol* 175: 5423–5429, 2005.
30. Ludwig J, Viggiano TR, McGill DB, and Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc* 55: 434–438, 1980.
31. Marchesini G, Brizi M, Morselli–Labate AM, Bianchi G, Bugianesi E, McCullough AJ, Forlani G, and Melchionda N.

Association of nonalcoholic fatty liver disease with insulin resistance. *Am J Med* 107: 450–455, 1999.
32. Marchesini G, Bugianesi E, Forlani G, Cerrelli F, Lenzi M, Manini R, Natale S, Vanni E, Villanova N, Melchionda N, and Rizzetto M. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. *Hepatology* 37: 917–923, 2003.
33. Matteoni CA, Younossi ZM, Gramlich T, Boparai N, Liu YC, and McCullough AJ. Nonalcoholic fatty liver disease: A spectrum of clinical and pathological severity. *Gastroenterology* 116: 1413–1419, 1999.
34. Nagasawa T, Inada Y, Nakano S, Tamura T, Takahashi T, Maruyama K, Yamazaki Y, Kuroda J, and Shibata N. Effects of bezafibrate, PPAR pan-agonist, and GW501516, PPAR-delta agonist, on development of steatohepatitis in mice fed a methionine- and choline-deficient diet. *Eur J Pharmacol* 536: 182–191, 2006.
35. Nakamura H, De Rosa SC, Yodoi J, Holmgren A, Ghezzi P, and Herzenberg LA. Chronic elevation of plasma thioredoxin: inhibition of chemotaxis and curtailment of life expectancy in AIDS. *Proc Natl Acad Sci USA* 98: 2688–2693, 2001.
36. Nakamura H, Masutani H, and Yodoi J. Extracellular thioredoxin and thioredoxin-binding protein 2 in control of cancer. *Semin Cancer Biol* 16: 444–451, 2006.
37. Nieto N, Friedman SL, Greenwel P, and Cederbaum AI. CYP2E1-mediated oxidative stress induces collagen type I expression in rat hepatic stellate cells. *Hepatology* 30: 987–996, 1999.
38. Nishinaka Y, Nishiyama A, Masutani H, Oka S, Ahsan KM, Nakayama Y, Ishii Y, Nakamura H, Maeda M, and Yodoi J. Loss of thioredoxin-binding protein-2/vitamin D3 up-regulated protein 1 in human T-cell leukemia virus type I-dependent T-cell transformation: implications for adult T-cell leukemia leukemogenesis. *Cancer Res* 64: 1287–1292, 2004.
39. Nishiyama A, Matsui M, Iwata S, Hirota K, Masutani H, Nakamura H, Takagi Y, Sono H, Gon Y, and Yodoi J. Identification of thioredoxin-binding protein-2/vitamin D(3) up-regulated protein 1 as a negative regulator of thioredoxin function and expression. *J Biol Chem* 274: 21645–21650, 1999.
40. Oka S, Liu W, Masutani H, Hirata H, Shinkai Y, Yamada S, Yoshida T, Nakamura H, and Yodoi J. Impaired fatty acid utilization in thioredoxin binding protein-2 (TBP-2)-deficient mice: A unique animal model of Reye syndrome. *FASEB J* 20: 121–123, 2006.
41. Okuyama H, Yoshida T, Son A, Oka S, Wang D, Nakayama R, Masutani H, Nakamura H, Nabeshima Y, and Yodoi J. Thioredoxin-binding protein 2 modulates lipid metabolism and natural killer T cell-dependent innate immunity in the liver. *Antioxid Redox Signal* 11: 000–000, 2009.
42. Oz HS, Chen TS, and Neuman M. Methionine deficiency and hepatic injury in a dietary steatohepatitis model. *Dig Dis Sci* 53: 767–776, 2008.
43. Oz HS, Im HJ, Chen TS, de Villiers WJ, and McClain CJ. Glutathione-enhancing agents protect against steatohepatitis in a dietary model. *J Biochem Mol Toxicol* 20: 39–47, 2006.
44. Pagano G, Pacini G, Musso G, Gambino R, Mecca F, Depetris N, Cassader M, David E, Cavallo–Perin P, and Rizzetto M. Nonalcoholic steatohepatitis, insulin resistance, and metabolic syndrome: Further evidence for an etiologic association. *Hepatology* 35: 367–372, 2002.
45. Piao ZH, Yoon SR, Kim MS, Jeon JH, Lee SH, Kim TD, Lee HG, Bae KH, Min JK, Chung SJ, Kim M, Cho YS, Oh DB, Park SY, Chung JW, and Choi I. VDUP1 potentiates Ras-mediated angiogenesis via ROS production in endothelial cells. *Cell Mol Biol (Noisy-le-grand)* 55 Suppl: OL1096–1103, 2009.
46. Rahman SM, Schroeder–Gloeckler JM, Janssen RC, Jiang H, Qadri I, Maclean KN, and Friedman JE. CCAAT/enhancing binding protein beta deletion in mice attenuates inflammation, endoplasmic reticulum stress, and lipid accumulation in diet-induced nonalcoholic steatohepatitis. *Hepatology* 45: 1108–1117, 2007.
47. Serviddio G, Sastre J, Bellanti F, Vina J, Vendemiale G, and Altomare E. Mitochondrial involvement in non-alcoholic steatohepatitis. *Mol Aspects Med* 29: 22–35, 2008.
48. Weltman MD, Farrell GC, Hall P, Ingelman–Sundberg M, and Liddle C. Hepatic cytochrome P450 2E1 is increased in patients with nonalcoholic steatohepatitis. *Hepatology* 27: 128–133, 1998.
49. Weltman MD, Farrell GC, and Liddle C. Increased hepatocyte CYP2E1 expression in a rat nutritional model of hepatic steatosis with inflammation. *Gastroenterology* 111: 1645–1653, 1996.
50. Yamaguchi K, Yang L, McCall S, Huang J, Yu XX, Pandey SK, Bhanot S, Monia BP, Li YX, and Diehl AM. Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with nonalcoholic steatohepatitis. *Hepatology* 45: 1366–1374, 2007.
51. Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama–Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P, and Kadowaki T. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med* 7: 941–946, 2001.
52. Yao ZM and Vance DE. The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. *J Biol Chem* 263: 2998–3004, 1988.
53. Yu J, Ip E, Dela Pena A, Hou JY, Sesha J, Pera N, Hall P, Kirsch R, Leclercq I, and Farrell GC. COX-2 induction in mice with experimental nutritional steatohepatitis: Role as pro-inflammatory mediator. *Hepatology* 43: 826–836, 2006.
54. Yu L, Border WA, Huang Y, and Noble NA. TGF-beta isoforms in renal fibrogenesis. *Kidney Int* 64: 844–856, 2003.
55. Zeisel SH. Choline: Critical role during fetal development and dietary requirements in adults. *Annu Rev Nutr* 26: 229–250, 2006.
56. Zeisel SH and Blusztajn JK. Choline and human nutrition. *Annu Rev Nutr* 14: 269–296, 1994.

Address correspondence to:
Hajime Nakamura, M.D., Ph.D.
Department of Preventive Medicine
The Tazuke Kofukai Medical Research Institute Kitano Hospital
Department of Preventive Medicine
2-4-20 Oogimachi, Kitaku
Osaka, Japan 530-8480

E-mail: hj-nakamura@kitano-hp.or.jp

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Abbreviations Used

ALP = alkaline phosphatase
ALT = alanine aminotransferase
ANOVA = analysis of variance
aP2 = adaptin protein complex 2
AST = aspartate aminotransferase
Fas = fatty acid synthetase
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
GSH = glutathione
HEL = hexanoyl-lysine adduct
4-HNE = 4-hydroxy-2-nonenal
H_2O_2 = hydrogen peroxide
IL-1β = interleukin-1β
INF-γ = interferon-γ
MCD = methionine and choline deficient
MPO = myeloperoxidase
mRNA = messenger RNA
NAFLD = nonalcoholic fatty liver diseases
NASH = nonalcoholic steatohepatitis
NK = natural-killer
NKT = natural-killer T
O_2^- = superoxide
8-OHdG = 8-hydroxy-2′-deoxyguanosine
PCR = polymerase chain reaction
PPAR-γ = peroxisome proliferators-activated receptor-γ
Prx = peroxiredoxin
ROS = reactive oxygen species
SDs = standard deviation of the mean
SOD = superoxide dismutate
SREBP1c = sterol regulatory element binding protein 1c
T-bilirubin = total bilirubin
TBP-2 = thioredoxin-binding protein-2
TBP-$2^{-/-}$ = thioredoxin-binding protein-2 deficient
T-CH = total cholesterol
TG = triglycerides
TGF-β1 = transforming growth factor-β1
TNF-α = tumor necrosis factor-α
TRX = thioredoxin
TXNIP = thioredoxin-interacting protein
VDUP1 = vitamin-D_3 upregulated protein 1
WT = wild-type

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2. Yuxing Zhang , Yanzhi Du , Weidong Le , Kankan Wang , Nelly Kieffer , Ji Zhang . 2011. Redox Control of the Survival of Healthy and Diseased Cells. *Antioxidants & Redox Signaling* **15**:11, 2867-2908. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
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4. Jacquelyn J. Maher . 2011. New Insights from Rodent Models of Fatty Liver Disease. *Antioxidants & Redox Signaling* **15**:2, 535-550. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
5. Maurizio Parola , Fabio Marra . 2011. Adipokines and Redox Signaling: Impact on Fatty Liver Disease. *Antioxidants & Redox Signaling* **15**:2, 461-483. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
6. Ying Xiong , Joachim D. Uys , Kenneth D. Tew , Danyelle M. Townsend . 2011. S-Glutathionylation: From Molecular Mechanisms to Health Outcomes. *Antioxidants & Redox Signaling* **15**:1, 233-270. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
7. Marika Lindahl , Alejandro Mata-Cabana , Thomas Kieselbach . 2011. The Disulfide Proteome and Other Reactive Cysteine Proteomes: Analysis and Functional Significance. *Antioxidants & Redox Signaling* **14**:12, 2581-2642. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
8. Hyo-Jung Kwon, Young-Suk Won, Yeo-Dae Yoon, Won-Kee Yoon, Ki-Hoan Nam, In-Pyo Choi, Dae-Yong Kim, Hyoung-Chin Kim. 2011. Vitamin D3 up-regulated protein 1 deficiency accelerates liver regeneration after partial hepatectomy in mice. *Journal of Hepatology* **54**:6, 1168-1176. [CrossRef]
9. Susan J. Fairweather-Tait , Yongping Bao , Martin R. Broadley , Rachel Collings , Dianne Ford , John E. Hesketh , Rachel Hurst . 2011. Selenium in Human Health and Disease. *Antioxidants & Redox Signaling* **14**:7, 1337-1383. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
10. Kiyokazu Koga , Agnes Kenessey , Saul R. Powell , Cristina P. Sison , Edmund J. Miller , Kaie Ojamaa . 2011. Macrophage Migration Inhibitory Factor Provides Cardioprotection During Ischemia/Reperfusion by Reducing Oxidative Stress. *Antioxidants & Redox Signaling* **14**:7, 1191-1202. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
11. Chiara Nediani , Laura Raimondi , Elisabetta Borchi , Elisabetta Cerbai . 2011. Nitric Oxide/Reactive Oxygen Species Generation and Nitroso/Redox Imbalance in Heart Failure: From Molecular Mechanisms to Therapeutic Implications. *Antioxidants & Redox Signaling* **14**:2, 289-331. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
12. Paola Chiarugi . 2009. Survival or Death: The Redox Paradox. *Antioxidants & Redox Signaling* **11**:11, 2651-2654. [Citation] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]