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Oxidative stress triggers lipid droplet accumulation in primary cultured hepatocytes by activating fatty acid synthesis

Jaeyong Lee, Takujiro Homma, Toshihiro Kurahashi, Eun Sil Kang, Junichi Fujii*

Department of Biochemistry and Molecular Biology, Graduate School of Medical Sciences, Yamagata University, Japan

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ABSTRACT

Despite the impaired intestinal lipid absorption and low level of visceral fat, the *Sod1*-deficient mouse is susceptible to developing liver steatosis. To gain insights into the mechanism responsible for this abnormal lipid metabolism, we analyzed primary cultured hepatocytes obtained from *Sod1*-deficient and wild-type mice. Lipid droplets began to accumulate in the cultured hepatocytes and was further increased by a *Sod1* deficiency. Levels of enzymes involved in lipogenesis were elevated. It thus appears that lipogenesis is activated by oxidative stress, which is more prominent in the case of *Sod1* deficiency, and appears to participate in liver steatosis.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD), which is defined as the accumulation of fat in the liver exceeding 5–10% by weight, is a common chronic liver disease [1]. Approximately 10%–20% of patients with NAFLD develop nonalcoholic steatohepatitis (NASH) that results in inflammation, fibrosis, and cellular damage in the liver [2]. It is generally thought that the increased inflow of lipids into the liver or elevation in de novo lipid synthesis in the liver, or both, are the cause of NAFLD. While the de novo synthesis of fatty acids accounts for 26% of the hepatic triglycerides in NAFLD patients [3], a high fat-containing diet is often administered to create pathological model animals for NAFLD. However, a high fat diet alone does not spontaneously lead to NASH, and additional factors, such as inflammation, endoplasmic reticulum (ER) stress, and oxidative stress appear to play roles in the pathogenesis of this affliction [1].

Among the deteriorating factors, oxidative stress occurs in various situations, such as insufficient levels of antioxidants and an elevated production of reactive oxygen species (ROS). Superoxide dismutase (SOD) scavenges superoxide radicals, a primary ROS produced in the body, and hence plays a central role in protection against oxidative stress [4]. Body weights of *Sod1*-knockout (KO) mice are lower than wild-type (WT) mice, and the difference between reaches more than 10% at 30 weeks of age [5]. While visceral fat in *Sod1*-KO mice is less than that of WT mice, more lipids accumulate in the livers of *Sod1*-KO mice [6–8]. The impaired secretion of very low density lipoproteins (VLDL), which are rich in triglycerides and contain apolipoprotein B (apoB), from the liver [9] would at least partly explain the lipid accumulation in *Sod1*-KO mice. However, chylomicron secretion from the intestinal epithelia to the blood is also impaired [7], so that the inflow of lipids to the liver is limited in *Sod1*-KO mice.

Both oxidative stress [10] and ER stress [11] have been reported to induce lipid droplet accumulation in cells under culture conditions. Because oxidative stress is a potential cause of ER stress [12], it was hypothesized that ER stress may be involved in the oxidative stress-induced lipogenesis in the *Sod1*-KO mouse liver. In this communication, we report on attempts to validate the role of ROS in the accumulation of lipids using primary cultured hepatocytes.

2. Materials and methods

2.1. Animals

C57BL/6 *Sod1*$^{+/-}$ mice, originally established by Matzuk et al. [13], were purchased from the Jackson Laboratories (Bar Harbor, ME) and backcrossed more than 10 times with C57BL/6 males at our

Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; SOD, superoxide dismutase; KO, knockout; WT, wild type; ER, endoplasmic reticulum; ROS, reactive oxygen species; PBS, phosphate-buffered saline; VLDL, low density lipoproteins; apoB, apolipoprotein B; FAS, fatty acid synthase; ACC, acetyl CoA-carboxylase; SCD1, stearoyl CoA desaturase 1; NAC, N-acetyl cysteine.

* Corresponding author. Department of Biomolecular Function, Graduate School of Medical Science, Yamagata University, 2-2-2 Iidanishi, Yamagata City, Yamagata 990-9585, Japan.
E-mail address: jfujii@med.id.yamagata-u.ac.jp (J. Fujii).

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institute [5]. A genotypic analysis of the mice was performed using PCR with specific primers, and *Sod1*$^{+/+}$ and *Sod1*$^{-/-}$ mice were used in this study. The animal room was maintained under specific pathogen-free conditions at a constant temperature of 20–22 °C with a 12-hr alternating light–dark cycle. Animal experiments were performed in accordance with the Declaration of Helsinki under the protocol approved by the Animal Research Committee at our institution.

2.2. Isolation of hepatocytes and primary culture

Male WT and *Sod1*-KO mice at 8–10 weeks of age were used for the preparation of hepatocytes. Livers of anaesthetized mice were perfused with Ca^{2+} free Krebs ringer-HEPES buffer, pH 7.6, containing collagenase (Wako, Japan) and a trypsin inhibitor (Sigma, USA) at 37 °C. The resulting cells (1.5×10^6 cells/6 cm dish) were transferred to collagen-coated plastic dishes and cultivated in William's medium (Sigma, USA) supplemented with 10% fetal bovine serum, 100 units penicillin, 0.1 mg/ml streptomycin, 1 mM Glutamax supplement (Life Technologies), and nonessential amino acids under an atmosphere of CO_2 at 37 °C.

2.3. Detection of intracellular lipids by Nile Red staining

Cultured hepatocytes were rinsed with phosphate-buffered saline (PBS) and fixed in 4% formaldehyde solution for 1 h. After 2 washings with PBS, the cells were stained with 0.4 μM Nile Red and 1 μg/ml DAPI for 15 min in the dark. For double staining, cells were permeabilized with 0.5% Tween-20 in PBS for 10 min at room temperature, blocked for 1 h at room temperature in TBST containing 5% skim milk, and incubated overnight at 4 °C with an anti-PLIN2 antibody (Progen, GP40, 1:100) in TBST containing 1% skim milk. After three washes in PBS, the cells were incubated with the Alexa Fluor®-conjugated goat anti-guinea pig antibody (dilution 1:200) for 90 min at room temperature. All images were obtained using a confocal laser-scanning microscope (Zeiss LSM 700, Germany).

2.4. Flow cytometric analysis of intracellular lipids

Hepatocytes were treated with trypsin, collected, and incubated with 0.4 μM Nile Red for 15 min on ice. After washing with PBS, the stained cells were subjected to analysis by FACS (FACSCanto II, BD Biosciences, Tokyo, Japan) at an excitation wavelength of 488 nm and an emission wavelength of 585 nm.

2.5. Immunoblot analysis of proteins

Hepatocytes were rinsed twice and harvested with ice-cold PBS. After centrifugation, the cell pellets were lysed in RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% (w/v) Nonidet P-40, 0.5% (w/v) Deoxycholate, 0.1% SDS) containing 50 mM NaF, 2.5 mM Na-pyrophosphate, 2 mM sodium orthovanadate, 25 mM β-glycerophosphate, 40 μM *p*-aminophenylsulfenyl fluoride, and a protease inhibitor cocktail (Roche) and centrifuged at 17,400× *g* for 10 min at 4 °C. The supernatant was subjected to protein determination using a BCA kit (Pierce) followed by immunoblot analyses as described [7]. The antibodies used were, SOD1 [5] and SOD2 [5], PLIN2, SREBP1 (Santa Cruz Biotechnology, sc-8984), FAS (Santa Cruz Biotechnology, sc-20140), ACC (Abcam, ab45174), SCD1 (Cell Signaling Technology, #2438), β-actin (Santa Cruz Biotechnology, sc-69879) and hyperoxidized PRDX (PRDX-SO_3) (Abcam, ab16830). After incubation with horseradish peroxidase-conjugated 2nd antibodies (Santa Cruz Biotechnology), detection of the immunoreactive bands were performed using Immobilon western chemiluminescent HRP substrate (Millipore). The relative amounts of each protein were quantified using NIH image software.

2.6. Statistical analysis

Statistical analyses of the data were performed using the Student's *t*-test. *; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$.

3. Results

3.1. Lipid droplet formation in the hepatocytes under cultural conditions

In order to elucidate the molecular mechanism responsible for the accelerated development of hepatic steatosis in the *Sod1*-deficient mouse [6,9], we isolated hepatocytes from WT and *Sod1*-deficent mice at 8–10 weeks of age when the liver was not yet affected and performed a primary culture of them under conventional conditions. Although cells express SOD2 in the mitochondrial matrix in addition to SOD1, no difference was observed in the levels of the SOD2 protein between WT and *Sod1*-KO hepatocytes (Fig. 1). The *Sod1*-deficient hepatocytes were flat compared to the cobblestone appearance of the WT hepatocytes. We also noted that substantial numbers of globular bodies had accumulated in the *Sod1*-deficient hepatocytes, and the numbers increased in only 24 h. The amounts of the globular bodies continued to increase with increasing culture time. When the cells were treated with a lipophilic fluorescent probe Nile Red at 24 h after isolation, the globular bodies were stained red. Immunostaining of the cells with an antibody against PLIN2, which is the peripheral protein component essential for lipid droplet formation [14], showed a green fluorescence at the surface of the globular bodies. Thus, we concluded that the globular bodies were lipid droplets covered with PLIN2 and that the droplets grew relatively rapidly in the *Sod1*-deficient hepatocytes under the culture conditions used.

3.2. Accelerated lipid droplet formation under Sod1 deficiency

We then performed a quantitative analysis of the Nile Red-stained hepatocytes by flow cytometry and observed a small difference in the lipid-derived fluorescence between *Sod1*-KO and WT hepatocytes at 2 h (Fig. 2). At 24 h after isolation, the fluorescent intensity increased in both the WT and *Sod1*-KO hepatocytes, but was increased more in the *Sod1*-KO hepatocytes. Thus the primary cultured hepatocytes gained more lipids in only a one day-culture period. In the following experiments, we performed analyses of the hepatocytes mainly at 2 h and 24 h after isolation because prolonged incubation affected other cellular characteristics, including the morphology of the cells.

3.3. Elevation of expression of genes involved in the lipid synthesis

Because a direct inflow of lipids into the hepatocytes would not account for the formation of the lipid droplets in the *Sod1*-KO cells under the culture conditions, we focused on the gene products responsible for lipogenesis (Fig. 3). We confirmed the elevation of PLIN2, which supports lipid droplet formation by covering the surface of the droplets. It has also been reported that the sterol-regulatory element binding protein 1 (SREBP1) is elevated in the *Sod1*-KO mouse liver [8]. We also confirmed elevated levels of the precursor form of SREBP1 and BiP, an ER stress marker, in the mouse liver in vivo (Supplemental Fig. 1), but did not detect the activated form of SREBP1 probably due to its short lifetime. In the case of primary cultured hepatocytes, their levels were decreased somewhat at 24 h, which suggested that, the expression of the

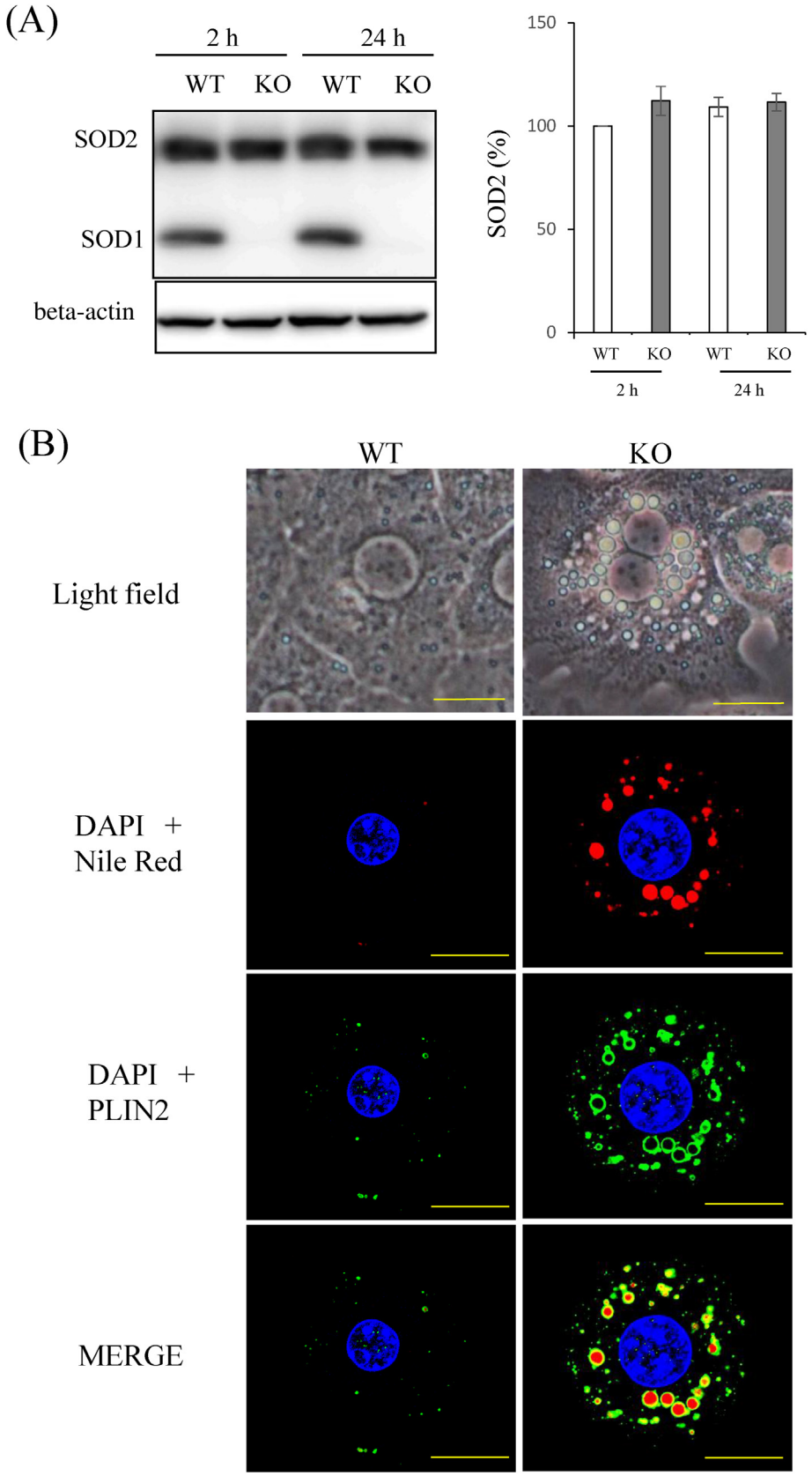

Fig. 1. Accumulation of lipid droplets in primary cultured hepatocytes. (A) Proteins extracted from the hepatocytes at 2 and 24 h after isolation were subjected to immunoblot analysis. SOD2 immune reactive band was semi-quantified by scanning the blot membranes. Columns and bars represent the mean ± SEM ($n = 3$). (B) Lipid droplet accumulation in primary cultured hepatocytes from the mice. Hepatocytes isolated from each genotype were cultivated for 24 h under conventional conditions. After fixing in formaldehyde, cells were stained with DAPI (blue), Nile Red (red), and anti-PLIN2 antibody (green) and subjected to analysis by a confocal laser scanning microscopy. The typical appearance of WT and *Sod1*-KO heaptocytes are shown. Bars indicate 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

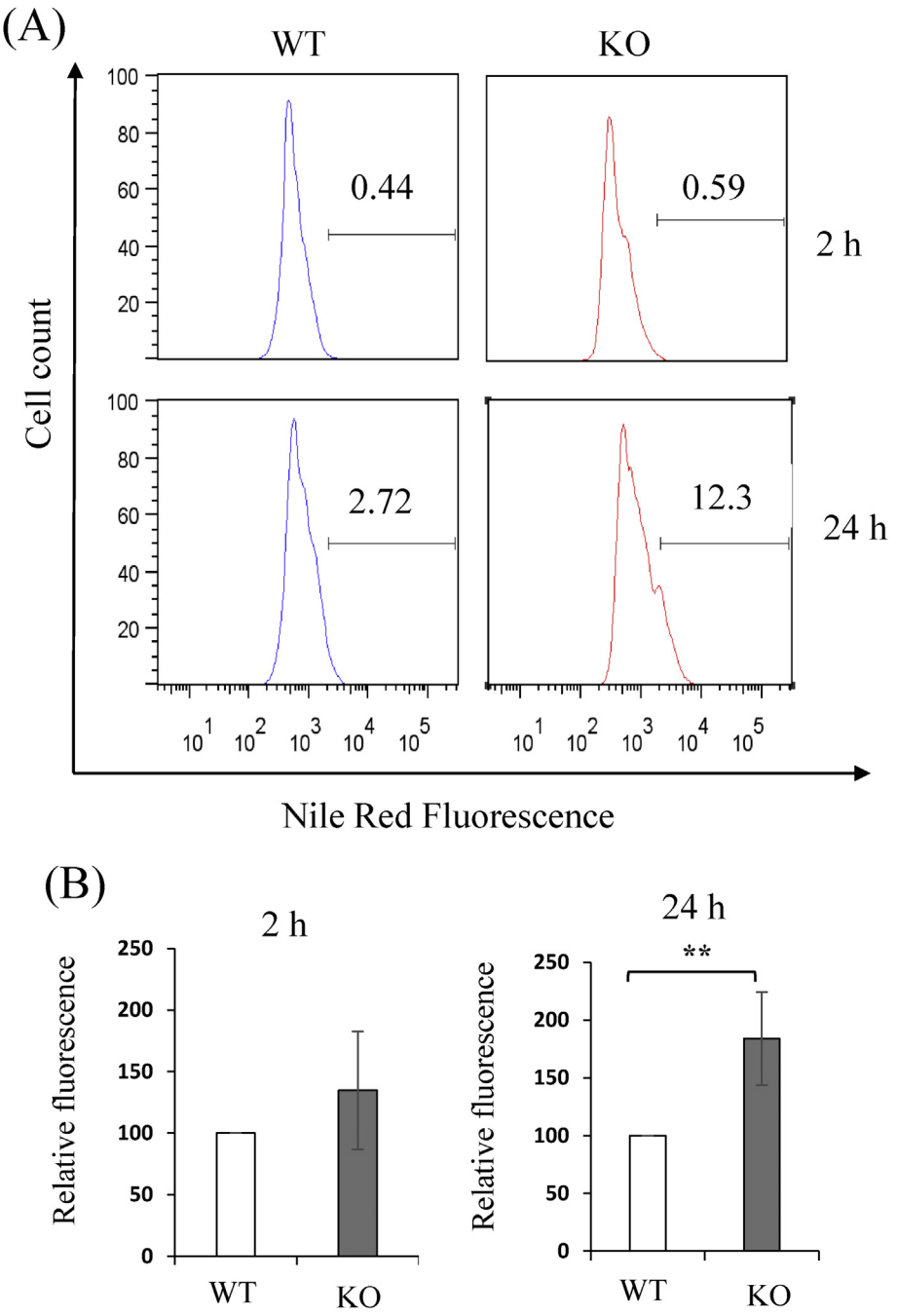

Fig. 2. FACS analyses of Nile Red-stained hepatocytes. (A) Hepatocytes cultured for 2 h and 24 h were reacted with Nile Red and subjected to flow cytometric analysis. (B) Values for Nile Red fluorescence in *Sod1*-KO hepatocytes relative to those of WT hepatocytes are shown. (n = 4). $^{**}p < 0.01$.

SREBP1 precursor was downregulated or that it had undergone proteolytic activation under hyperoxic conditions (about 20% oxygen) compared to in vivo situation (about 5% oxygen).

In order to further elucidate the mechanism responsible for this, we examined the issue of whether the expression of the downstream genes had changed under the culture conditions used. We chose three genes, fatty acid synthase (FAS), acetyl CoA-carboxylase (ACC), and stearoyl CoA desaturase 1 (SCD1) that are key lipogenic genes upregulated by SREBP1 [15]. The levels of all three proteins in the *Sod1*-KO cells were originally high at 2 h and the high levels were maintained up to 24 h. Thus, we concluded that SREBP1 was activated by proteolytic cleavage at Golgi bodies, which resulted in the stimulation of lipogenesis by upregulating target genes. A *Sod1* deficiency is accompanied by an increase in ROS levels, which, in turn, causes cellular components to be oxidatively modified. In fact we confirmed the existence of elevated oxidative stress in the *Sod1*-KO cells by detecting hyperoxidized Prx ($Prx\text{-}SO_3$), which is elevated in cells with excessive hydrogen peroxide and, hence, constitutes a hallmark of oxidative stress.

3.4. Antioxidants partially suppress lipid droplet accumulation

Finally we examined the issue of whether an antioxidant could suppress lipid droplet accumulation by decreasing ROS levels

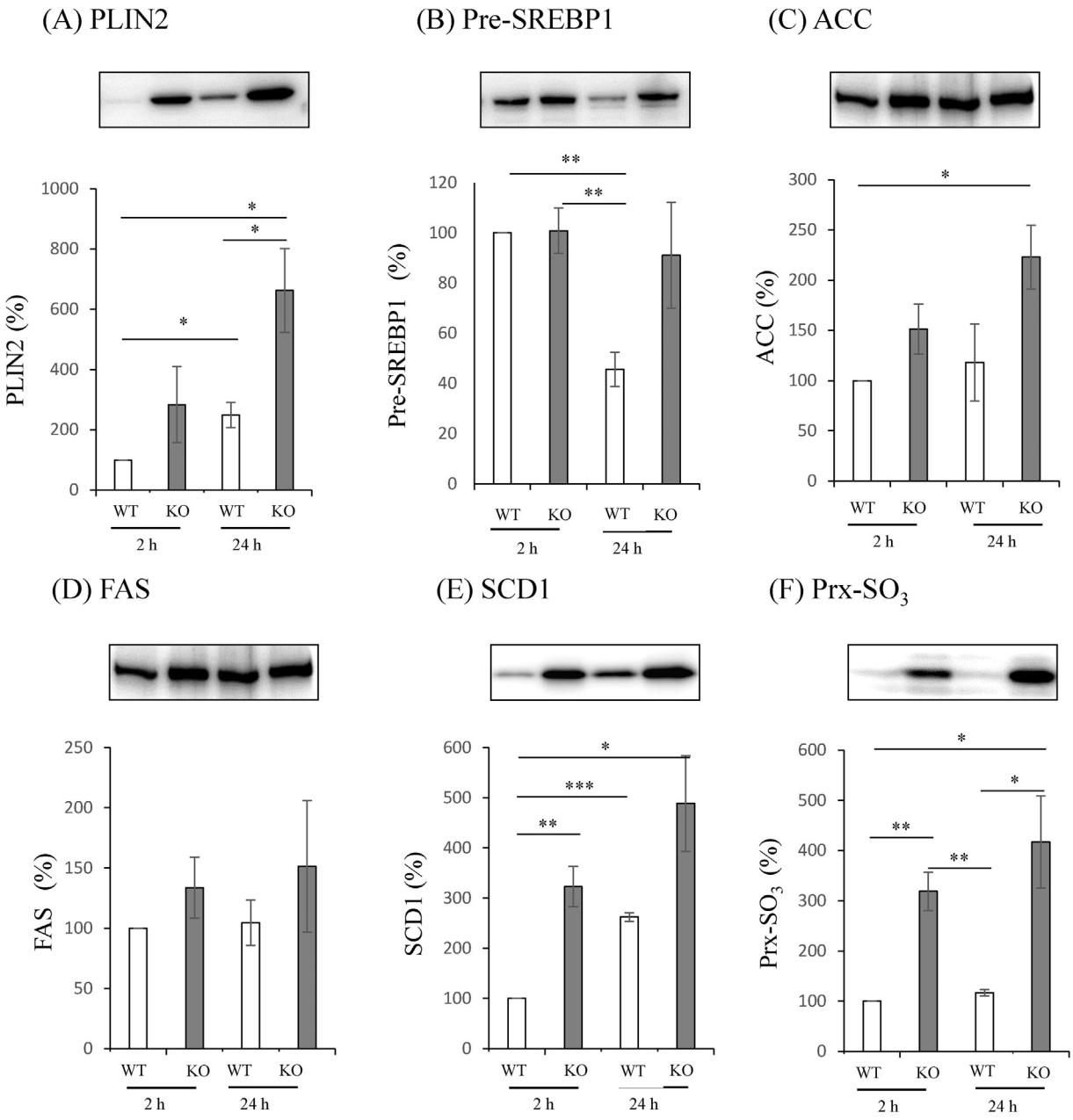

Fig. 3. Levels of proteins responsible for hepatic lipid metabolism. Extracted proteins were subjected to immunoblot analysis using antibodies against PLIN2 (A), SREBP1 (B), ACC (C), FAS (D), SCD1 (E), and Prx-SO_3 (F). Each immune reactive band was semi-quantified by scanning the blot membranes and normalized to the corresponding β-actin. Columns and bars represent the mean ± SEM ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

(Fig. 4). We examined several antioxidative compounds such as Trolox and Tiron, and found them mostly either toxic or non-effective except for N-acetyl cysteine (NAC) (data not shown). NAC at 2 mM was found to be effective in the WT cells but not in the *Sod1*-KO cells. Insufficient protection of NAC against an *Sod1* deficiency can be attributed to the low antioxidant capacity of the compound. Thus, the results would at least partly support the hypothetical role of oxidative stress in lipid droplet accumulation under cultural conditions.

4. Discussion

The findings reported herein show that lipid droplet formation was triggered in hepatocytes after isolation and was enhanced by a *Sod1* deficiency under conventional culture conditions (Figs. 1 and 2). Because the absence of PLIN2 prevents hepatic steatosis caused by alcohol administration [14], PLIN2 is elevated as a compensatory action to enhance lipogenesis and support lipid droplet accumulation.

SREBPs are localized at the ER membrane in their inactive precursor forms. In the classical pathway, the depletion of sterols cause SREBPs to be translocated to the Golgi apparatus and proteolytic cleavage, leading to activating genes that are involved in synthesis and uptake of cholesterol and fatty acids [16]. The membrane-bound SREBP1 precursor is more abundant in the liver of the *Sod1*-KO mouse than that of the WT mouse [8, and Supplemental Fig. 1], but the levels were relatively less in the case of cultured hepatocytes (Fig. 3). However, because SREBP1-regulated genes as

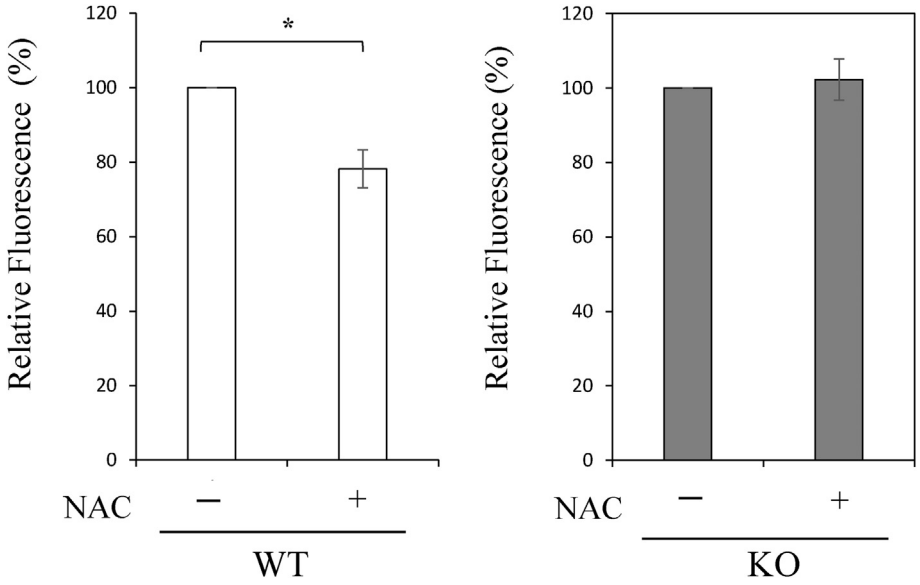

Fig. 4. FACS analyses of NAC-treated, Nile Red-stained hepatocytes. At 2 h after isolation of hepatocytes, NAC (2 mM) was added to the culture media. After incubation for 22 h, the hepatocytes were reacted with Nile Red and subjected to flow cytometric analysis. Values for Nile Red fluorescence in hepatocytes relative to those of hepatocytes without NAC are shown. (n = 3). $^{*}p < 0.05$.

well as lipid droplets were elevated in both types of hepatocytes, SREBP1 appears to be spontaneously activated under the culture conditions used in this study. Two Golgi-associated proteases, site-1 protease (S1P) and site-2 protease (S2P) are involved in the proteolytic cleavage of SREBPs [16]. For processing SREBP1, S2P appears to be more important than S1P because an inhibitor of S2P but not of S1P affected the processing [17]. Other than sterols, stress on the cells is known to activate SREBPs and hence lipogenesis [10,11].

The primary cultured hepatocytes suffer from severe oxidative stress compared to the in vivo situation because the culture is exposed to atmospheric oxygen, the level of which is several folds higher than that in peripheral tissues. Tormos et al. [18] showed that the isolation of hepatocytes per se causes the simultaneous oxidation of glutathione, which is consistent with elevated oxidative stress under culture conditions, and cell cycle entry. Although we attempted to culture the cells under low oxygen conditions (5%), which can allow *Sod1*-deficient mouse embryonic fibroblasts to survive by suppressing oxidative stress [19], the viability of the hepatocytes was markedly decreased (data not shown). We then examined several antioxidants to determine whether they suppressed lipid droplet accumulation. Among the antioxidants examined, including Trolox and Tiron, NAC suppressed the lipogenesis in hepatocytes without damaging the cells (Fig 4). NAC can be metabolized to cysteine that consequently increases cellular glutathione levels.

Superoxide plays a role in the degradation of apoB by polyunsaturated fatty acid-mediated apoB degradation via the peroxidation of the lipids [20]. ROS accelerate disulfide bridge formation between nearby sulfhydryls, which increases the rate of occurrence of misfolded proteins in the ER and hence leads to ER stress [21,22]. The ApoB protein possesses several cysteine residues at the amino-terminal 5% region [23], some of which are essential for VLDL assembly and secretion [24]. Aberrant oxidation due to elevated ROS by a *Sod1* deficiency would cause the inappropriate folding of apoB and would impair VLDL secretion. Thus, the oxidative stress-induced ER stress could play roles in the defect in the secretion of VLDL and chylomicrons from the *Sod1*-deficient liver and the intestinal epithelium, respectively [7,9]. In short, our data showed that oxidative stress caused under cultural conditions resulted in activation of lipogenesis in WT hepatocytes and at extreme level in *Sod1*-deficient hepatocytes.

Conflicts of interest

We declare no conflict of interest between the authors or with any institution in relation to the contents of this article.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.121.

Transparency document

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