Oxidative Stress induced lipid accumulation via SREBP1c activation in HepG2 cells

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Abstract

SREBP1c (sterol regulatory element binding protein 1c) is a metabolic-syndrome–associated transcription factor that controls fatty acid biosynthesis under glucose/insulin stimulation. Oxidative stress increases lipid accumulation, which promotes the generation of reactive oxygen species (ROS). However, we know little about the role of oxidative stress in fatty acid biosynthesis. To clarify the action of oxidative stress in lipid accumulation via SREBP1c, we examined SREBP1c activity in H_2O_2-treated mammalian cells. We introduced a luciferase reporter plasmid carrying the SREBP1c binding site into HepG2 or COS7 cells. With increasing H_2O_2 dose, SREBP1c transcriptional activity increased in HepG2 cells but declined in COS7 cells. RT-PCR analysis revealed that mRNA expression of SREBP1c gene or of SREBP1c-regulated genes rose H_2O_2-dose-dependently in HepG2 cells but dropped in COS7 cells. Lipid accumulation and levels of the nuclear form of SREBP1c increased in H_2O_2-stimulated HepG2 cells. ROS may stimulate lipid accumulation in HepG2 cells via SREBP1c activation.

Keywords: fatty acid biosynthesis, adipogenesis, ROS, transcription factor

Introduction

Lipids play a basic role in energy storage in times of starvation, but in recent years surplus intake of food, leading to obesity and metabolic syndrome, has become a global social issue [1, 2]. Intake of carbohydrates causes a rise in blood glucose level and promotes insulin secretion; it also induces the expression of genes encoding enzymes associated with glycolysis and the biosynthesis of fatty acids. These enzymes include glucokinase (GK), acetyl CoA carboxylase (ACC), fatty acid synthase (FAS), and stearoyl-CoA desaturase-1 (SCD-1) in the liver [3]. One transcription factor that plays a major role in controlling the expression of these lipid synthesis genes in the liver is sterol regulatory element binding protein 1c (SREBP1c) [4].

So far, three isoforms of SREBP have been identified: SREBP1a [5], SREBP1c [6], and SREBP2 [7]. SREBP1c contributes to fatty acid synthesis, SREBP2 works in cholesterol formation, and SREBP1a is involved in both cholesterol formation and biosynthesis of fatty acids. SREBP1a and 1c are generated as splice variants [8]. SREBP protein is generated as a membrane-bound precursor and is bound to granular endoplasmic reticulum (ER) as a complex with SREBP cleavage activating protein (SCAP) [9]. SREBP1c is predominantly produced in the liver and white adipocytes, and it is activated by changes in nutritional status. Generally, SREBP1c–SCAP complex is translocated to the golgi apparatus in response to the action of glucose or insulin; there it is subjected to proteolysis by the binary enzyme combination of site 1 protease (S1P) [10] and site 2 protease (S2P) [11]. This leads to translocation of the N-terminal domain of SREBP1c into the nucleus. Matured SREBP1c in the nucleus then acts on the sterol regulatory element (SRE) (ATCACCCCAC) sequence and E-box (CAXXTG) sequence of the promoter, facilitating the transcription of fatty acid synthesis genes and the gene encoding SREBP1c itself and thus promoting fatty acid biosynthesis [2].

It is now known that excessive caloric intake, obesity, and various obesity-related conditions lead to an increase in oxidative stress. Obesity and oxidative stress are positively correlated [12], and the production of reactive oxygen species (ROS) is enhanced by excess adiposity [13]. Ingestion of large quantities of glucose and free fatty acids provokes acetyl-CoA production and excess nicotinamide adenine dinucleotide (NADH). Accumulation of excess NADH causes a generation of ROS

[14]. Furthermore, adipocytes enlarged through obesity secrete inflammatory cytokines [15]. These adipokines cause systemic arterial sclerosis and insulin resistance; they also trigger an inflammatory response and an increase in oxidative stress in the adipocytes [16]. In contrast, dietary restriction and weight loss cause a decline in ROS generation in the serum [17]. Thus the degree of oxidative stress appears to be closely associated with the progression of obesity-related disorders. However, the etiology has not been fully clarified and the correlations among oxidative stress, obesity, and lipid biosynthesis are not yet well understood.

We therefore focused on the relationship between oxidative stress and the activity of SREBP1c, a major transcription factor in fatty acid biosynthesis.

Materials and Methods

Cell culture. HepG2 cells (RIKEN Institute of Physical and Chemical Research cell bank, Tsukuba, Ibaraki, Japan) and COS7 cells (RIKEN) were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Sanko Junyaku, Chiyoda, Tokyo, Japan).

MTT assay. HepG2 and COS-7 cells (1.0×10^4) cultured in a 96-well plate in DMEM medium were treated with H_2O_2 (0, 100, 250, 500, 1000 μM) for 3 hrs. After medium was exchanged, HepG2 cells were cultured for 48 hrs (24 hrs for COS7 cells) and treated by 5 mg / ml MTT (Thiazolyl Blue Tetrazolium Bromide) solution (Sigma) for 3 hrs. After cells were dissolved in 0.04 N HCl (in isopropanol), formazane level was analyzed by measuring OD_{570} nm (against OD_{630} nm).

Transfection of DNA. HepG2 and COS-7 cells (1.0×10^6) were cultured in a 35-mm dish in DMEM culture medium for 24 hrs. Cells were treated with plasmids (pGL2-basic SREBP1c (-2.6 kb) promoter Luc (a gift from Dr. Shimano, University of Tsukuba), pCMV-β-gal plasmid) and Lipofectamine™ 2000 (Invitrogen, CA, USA) for 45 hrs. Then, cells were treated by H_2O_2 (0, 100, 250, 500, 1000 μM) for 3 hrs, and were lysed by Reporter Lysis Buffer (Promega, Madison, WI, USA) for luciferase assay.

Luciferase assay. After brief centrifugation (12,000 rpm, 2 min, MX-100, Tomy, Tokyo, Japan) of the cell lysate, supernatant was collected and mixed with Luciferase Assay Reagent (Promega). Luciferase activity was measured with a Luminometer

MicroLumat LB96p (Berthold Technology, Germany). A β-gal assay was performed to normalize plasmid induction efficiency.

RT−PCR. The cDNA was synthesized using M-MLV Reverse Transcriptase (Takara Bio, Otsu, Shiga, Japan), and PCR (95 °C for 5 min; 23–38 cycles of 95 °C for 30 s, 52 °C for 30 sec, 72 °C for 1 .5 min; 72 °C for 7 min) was performed using the specific primers (5'-GGAGGGGTAGGGCCAACG-3' and 5'-AGGGGTGGAGCTCAACTG-3') for SREBP1c, (5'-GAGGGAAGGGAATTAGAA-3' and 5'-ATCACCCCAGGGAGATAC-3') for ACC1 (acetyl CoA carboxylase 1), (5'-AGGCTGTGAAGCCATTCG-3' and 5'-CGCACCTCCTTGGCAAAC-3') for FAS (fatty acid synthase), (5'-TGGAAGACGACATTCGCG-3' and 5'-TGAAGCACATCAGCAGCA-3') for SCD1 (stearoyl-CoA desaturase 1), (5'-CACGTGCACTTCAAGGAG-3' and 5'-GGTCTGCTAGCTCCATCC-3') for SCAP (SREBP cleavage activating protein), (5'-TCATCACCAAGGCCATCG-3' and 5'-CCACCACCCTGTTGCTGT-3') for GAPDH (glyceraldehyde 3-phosphate dehydrogenase).

Western blotting. Cells were treated with H_2O_2 (0, 100, 250, 500, 1000 μM) for 6 hrs, and protein fraction was extracted. SDS-PAGE was performed with 30 μg of protein, and the separated proteins were transferred to a PVDF membrane (Schleicher & Schuell BioScience, Keene, NH, USA). The blotted membrane was soaked in 5% skim milk and incubated with primary antibody for 1 hour. The filter was incubated with second antibody for 1 hour, and detected by lumiGLO reagent (Cell Signaling, Danvers, MA, USA). Finally, each protein band was detected by chemiluminescence (Las1000, FujiFILM, Minato, Tokyo, Japan), and analyzed with Image Gauge Software (FUJI-FILM). anti-SREBP-1 (H-160) rabbit polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and anti-β-actin (Cell Signaling Technology) were used as primary antibodies.

Oil red O Staining. HepG2 and COS-7 cells (5.0×10^4) were cultured in a 24-well (gelatine coated) dish in DMEM culture medium for 24 hrs, and treated with H_2O_2 (0, 500 μM) for 3 hrs. After forty-eight hrs incubation, cells were fixed with 4% paraformaldehyde for 1 hour, and it was dyed using 3 mg/ml Oil red O (in 60% isopropanol) for 10 min. After dyeing, cell was washed with sterile water, and observed under a microscope (DMIRBE M2FLIII (Leica Microsystems Inc., Bannockburn, IL, USA)).

Triglyceride assay. Cells were lysed in lysis buffer (20 mM HEPES [pH 7.6], 420 mM NaCl, 1% triton X-100, 0.1% SDS) and total fat was extracted by Bligh & Dyer method [18]. The cell extract (600 ml) was incubated with 2 ml methanol and 1 ml chloroform for 1 hour, and then 1 ml chloroform and 1 ml of sterile water were added, centrifuged briefly to collect chloroform phase. This extract was dried for overnight, and was dissolved in 10% triton-isopropanol solution. According to a manual of triglyceride E-test Wako (Wako), the quantity of Triglyceride was measured. The quantity of triglycerides (μg/μl) was normalized by each protein content (μg/μl).

Results

Effect of H_2O_2 on transcriptional activity of SREBP1c

To analyze the effects of ROS on SREBP1c, we treated cells of the human hepatic cell line HepG2 and the monkey kidney cell line COS7 with H_2O_2 (0, 100, 250, 500, 1000 μM) for 3 h. We performed an MTT assay to analyze the viability of the cells under H_2O_2 stimulation. No cell toxicity was detected under H_2O_2 exposure at the lesser dose than 500 μM (Fig. 1A).

Cells were transfected by a reporter plasmid carrying the SREBP1c promoter and exposed to H_2O_2. Transcriptional activity of SREBP1c increased dose-dependently up to a dose of 500 μM and then declined in HepG2 cells (Fig. 1B). With 500 μM H_2O_2 treatment, SREBP1c transcriptional activity was more than double that without treatment. When cells were treated with 1.0 mM H_2O_2 transcriptional activity decreased slightly. Because the cell toxicity was high with 1.0 mM H_2O_2, we considered that the β-gal assay had not normalized the plasmid transfection efficiency.

In contrast, luciferase activity was reduced in COS7 cells in response to increasing dose of H_2O_2; activity after treatment with H_2O_2 at 500 μM was less than 50% of that without H_2O_2 (Fig. 1B). These results indicate that endogenous SREBP1c activity was regulated by H_2O_2 stimulation.

Effect of H_2O_2 on expression of mRNAs of SREBP1c target genes and the SREBP1c

gene itself

We used RT-PCR to analyze the effects of H_2O_2 on endogenous expression of the mRNA of the SREBP1c gene itself; of SREBP1c target genes such as the fatty acid metabolism genes encoding ACC, FAS, and SCD-1; and of the non-target gene encoding SCAP, which is essential for the regulation of SREBP1c-binding to the ER membrane and SREBP1c translocation to the golgi body. Expression of mRNA of the genes encoding ACC, FAS, SCD-1, and SREBP1c itself increased with increasing H_2O_2 dose in HepG2 cells (Fig. 2A). Additionally, expression of the non-target gene encoding SCAP was upregulated under H_2O_2 stimulation. These changes were particularly marked under treatment with 500 μM or more H_2O_2. In contrast, expression of all of these genes in COS7 cells was dose-dependently downregulated under H_2O_2 stimulation (Fig. 2A).

These results suggested that H_2O_2 induces fatty acid synthesis via activation of SREBP1c in HepG2 cells, whereas synthesis is reduced in COS7 cells. Although PCR products were efficiently detected in HepG2 cells with 25 to 32 amplification cycles, more amplification (with as many as 35 to 38 cycles) was required for COS7 cells, suggesting that these genes are expressed at much higher levels in HepG2 cells.

Effect of H_2O_2 on production of matured SREBP1c protein

We then analyzed the production of mature-form SREBP1c protein (68 kDa) in cells treated with H_2O_2. Cells were exposed to H_2O_2 (0, 100, 250, 500, 1000 μM) for 6 h. Western blot analyses revealed that the level of this protein increased in HepG2 cells with increasing H_2O_2 dose (Fig. 2B). Because SREBP1c is translocated into the nucleus after processing of the 125-kDa immature form into the 68-kDa mature form, the above result suggests that H_2O_2 stimulated some of the steps of SREBP1c translation, processing, or nuclear translocation in HepG2 cells. In contrast, the mature form of SREBP1c protein, which was detected as double bands, did not change under H_2O_2 stimulation in COS7 cells (Fig. 2B). Because the antibody for SREBP1 (H-160, Santa Cruz) that we used recognizes both SREBP1c and SREBP1a, the second band may have represented SREBP1a.

Effect of H_2O_2 on lipid accumulation

As indicated above, H_2O_2 stimulation of HepG2 cells increased expression of both the SREBP1c gene itself and the SREBP1c target genes involved in fatty acid biosynthesis. We therefore analyzed whether intracellular adiposity would increase in response to H_2O_2 stimulation. Cells were first treated with H_2O_2 (0, 500 μM) for 3 h and then cultured for a further 48 h after removal of the H_2O_2; this was followed by MTT assay to analyze cell viability. Because this treatment hardly had an influence on cell survival rate (Fig. 3A), these conditions were used for further experiments. Cells were stimulated with H_2O_2 and then observed after staining with Oil Red O, which can detect intracellular lipids. Many red-stained lipid droplets were detected in HepG2 cells after H_2O_2 stimulation (Fig. 3B). Although the lipid droplets in untreated HepG2 cells were stained to some extent, more of them were stained, and stained more strongly, after cell stimulation with 500 μM H_2O_2. Therefore, H_2O_2 activated fatty-acid-biosynthesis genes, including SREBP1c, and accelerated intracellular lipid accumulation. In addition, many HepG2 cells stimulated with H_2O_2 at 500 μM developed a slim, extended, fiber-like shape (Fig. 3B). These changes in cell shape led to the formation of many gaps among the cells.

In contrast, kidney cells, including COS7 cells, do not accumulate fat; in these cells the expression and function of fatty-acid–biosynthesis genes are very weak. This was supported by the results of our physiological analysis, which revealed that H_2O_2 caused negligible lipid accumulation in COS7 cells (Fig. 3B).

Effect of H_2O_2 on triglyceride accumulation

Furthermore, we analyzed triglyceride accumulation in cells treated with H_2O_2. Cells were stimulated with H_2O_2 (0, 250, 500 μM) for 3 h and then cultured for a further 48 h in fresh medium without H_2O_2 for triglyceride assay. In HepG2 cells the triglyceride level rose with increasing H_2O_2 dose (Fig. 4). This result was in accordance with that of our Oil Red O staining, indicating that H_2O_2 stimulated lipid accumulation in the HepG2 cells. The rise in intracellular triglyceride level was very small, but this may have been because some triglycerides were drained from the culture medium as lipoproteins. In contrast, the triglyceride assay revealed no effect

of H_2O_2 on COS7 cells (Fig. 4), a result similar to that seen with Oil Red O staining (Fig. 3B).

Discussion

Our results clearly revealed that H_2O_2 stimulation increased SREBP1c transcriptional activity (Fig. 1B) in HepG2 cells and induced lipid accumulation in these cells (Figs. 3B, 4) through upregulation of the genes encoding SREBP1c and other genes involved in fatty acid metabolism (Fig. 2A). In contrast, in COS7 cells H_2O_2 suppressed SREBP1c transcriptional activity (Fig. 1B) and thereby reduced the expression of the genes encoding SREBP1c and other genes involved in fatty acid synthesis (Fig. 2A) Because the expression and function of fatty acid synthetic genes are very low in COS7 cells, lipid accumulation was almost undetectable in these cells (Figs. 2A, 3B, 4). These results indicate that ROS stimulation is critically involved in lipid accumulation through regulation of the expression and function of SREBP1c in HepG2 cells.

It is conceivable that oxidative stress can itself activate the insulin signal cascade and SREBP activity in HepG2 cells. H_2O_2 has an insulin-like action and thereby acts as a second messenger in the insulin signal cascade [19]. Because SREBP1c is activated under the regulation of insulin signaling, H_2O_2 may thus activate SREBP1c via activation of the insulin-signaling cascade. As evidence to support this interpretation, generation of ROS through infection with hepatitis C virus causes activation of phosphoinositide 3-kinase/ protein kinase B and inactivation of phosphatase and tensin homolog resulting in the cleavage and activation of SREBP [20]. As a second possible mechanism for the activation of SREBP activity, oxidative stress may evoke ER stress and thus activate SREBP. ER stress evoked by the accumulation of misfolding proteins under the stimulation of ROS and other stressors activates c-Jun N-terminal kinase [21] and SREBP1, 2 [22, 23]. Therefore, H_2O_2 may have activated SREBP1c by the evocation of ER stress.

The difference in action of H_2O_2 between HepG2 and COS7 cells may be due to a difference in nutrient metabolic function. The liver is a major organ for nutrient metabolism. Various signaling pathways, such as those for insulin signaling, glycolysis, and fatty acid biosynthesis, play important roles in the liver that are

influenced by insulin and glucagon. In contrast, the kidney has poor insulin sensitivity, and its ability to biosynthesize fatty acids in response to insulin signaling is weak. Such differences in nutrient metabolic function between the two tissues may cause differences in SREBP1c activity in response to changes in nutritional status.

This is the first report to indicate that oxidative stress has a strong influence on SREBP1c activity. This is an important clue to the causal relationship between oxidative stress and obesity and obesity-related disorders.

Acknowledgments

We wish to thank Dr. Hitoshi Shimano (University of Tsukuba) for providing plasmid of pGL2-basic SREBP1c (-2.6 kb) promoter Luc. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan.

Figure Legends

Fig.1 Effect to transcriptional activity of SREBP1c by H_2O_2. (A) Viability of the cell treated with H_2O_2 was indicated. HepG2 (◆) and COS-7 (●) cells cultured in a 96-well plate were treated with H_2O_2 (0, 100, 250, 500, 1000 μM) for 3 hrs, and analyzed for MTT assay. (B) Luciferase activity in the cell treated with H_2O_2 was indicated. Cells were transfected with plasmids pGL2-basic SREBP1c (-2.6 kb) promoter Luc (0.5 μg/dish) and pCMV-β-gal (0.5 μg/dish). After 45 hrs incubation, cells were treated with H_2O_2 (0, 100, 250, 500, 1000 μM) for 3 hrs, and analyzed for luciferase assay. Luciferase activity was corrected by β-galactosidase activity. An error bar shows standard deviation of the mean, $n = 6$.

Fig.2 Effect to expression of SREBP1c-controlled genes by H_2O_2. (A) The mRNA level of each genes was analyzed by RT-PCR. Cells cultured in a 60 mm plate were treated with H_2O_2 (0, 100, 250, 500, 1000 μM) for 3 hrs, and RNA was extracted for RT-PCR assay. GAPDH was used as an internal control of RNA. (B) The protein level of matured SREBP1c (68 kDa) was analyzed by western blot assay. Cells were treated with H_2O_2 (0, 100, 250, 500, 1000 μM) for 6 hrs, and nuclear protein was

prepared for western blot analysis using anti-SREBP1c antibody. β-actin was used as a loading control of protein.

Fig.3 Effect to accumulation of adipose by H_2O_2. (A) Cell viability after the treatment with H_2O_2 was indicated. HepG2 (◆) and COS-7 (●) cells were treated with H_2O_2 (0, 100, 250, 500, 1000 μM) for 3 hrs, and after 24 hrs or 48 hrs culture, cells were analyzed for MTT assay. (B) Accumulation of adipose after the treatment of H_2O_2. Red-stained region indicates adipose droplet. Arrow indicates fiberization of the cell. Cells were cultured on 24-well plates and treated with H_2O_2 (0, 500 μM) for 3 hrs, and after 48 hrs incubation, cells were fixed for Oil red O staining.

Fig.4 Effect to accumulation of Triglyceride by H_2O_2. Triglyceride level was analyzed in the H_2O_2-treated HepG2 and COS7 cells. Cells were treated with H_2O_2 (0, 250, 500 μM) for 3 hrs, and after 48 hrs culture, lipid was extracted for triglyceride assay using Bligh and Dyer methods [18]. Cells were analyzed for MTT assay. Triglyceride level was corrected by total protein.

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Fig.1

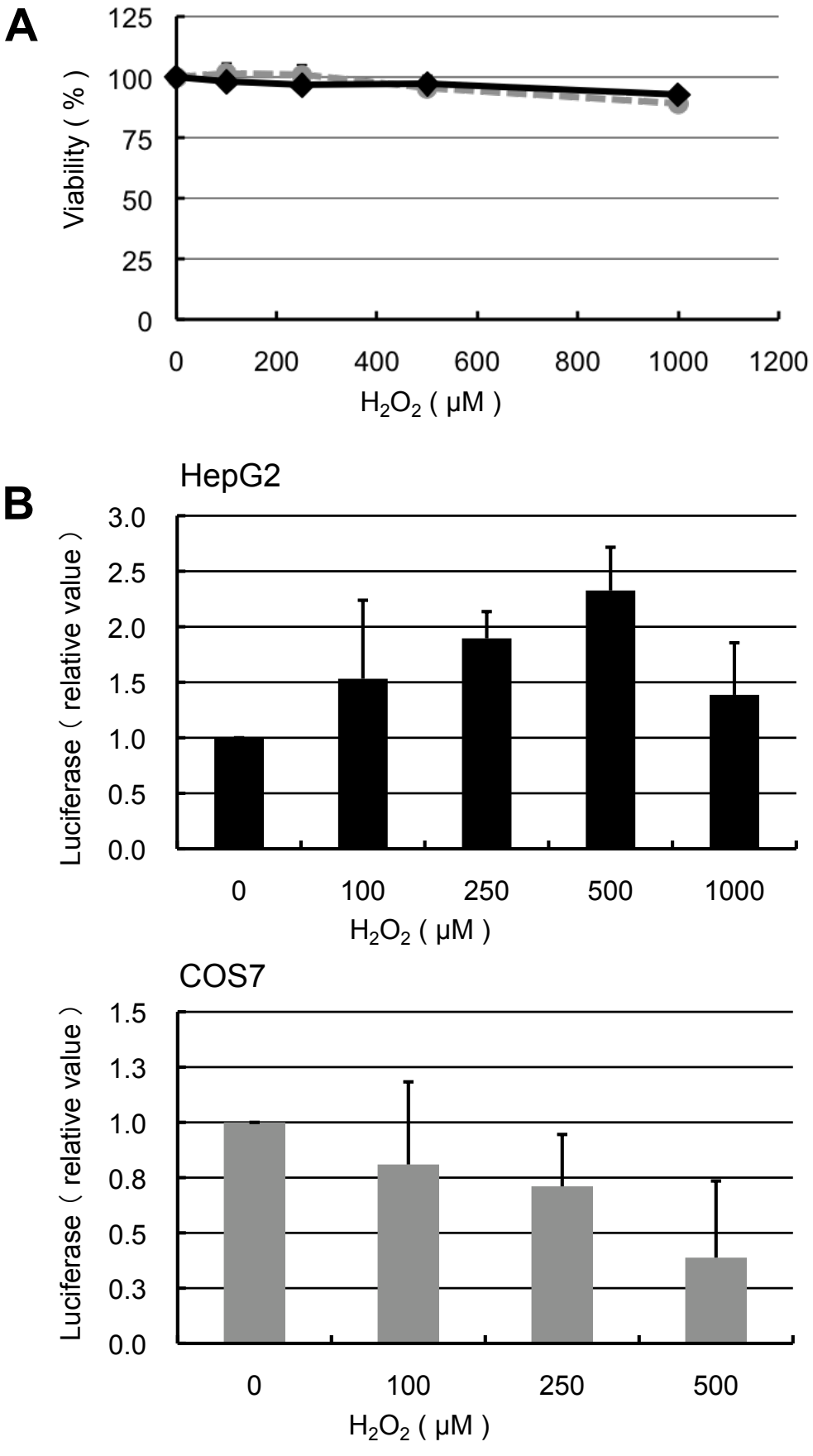
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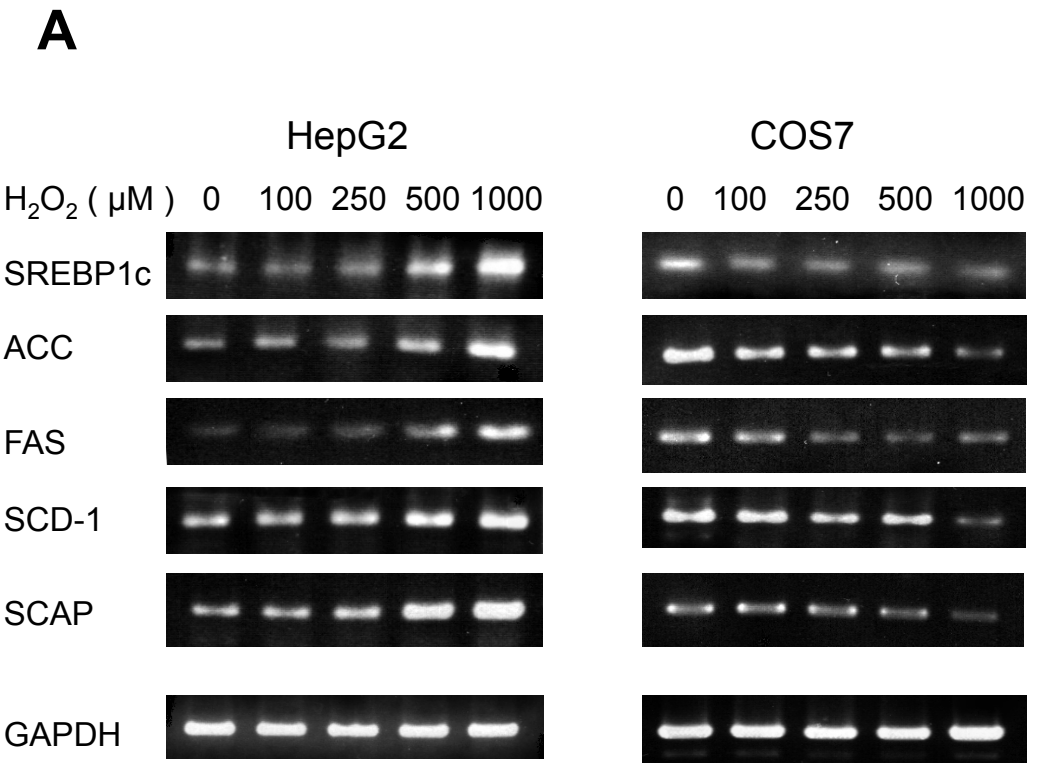
Fig.2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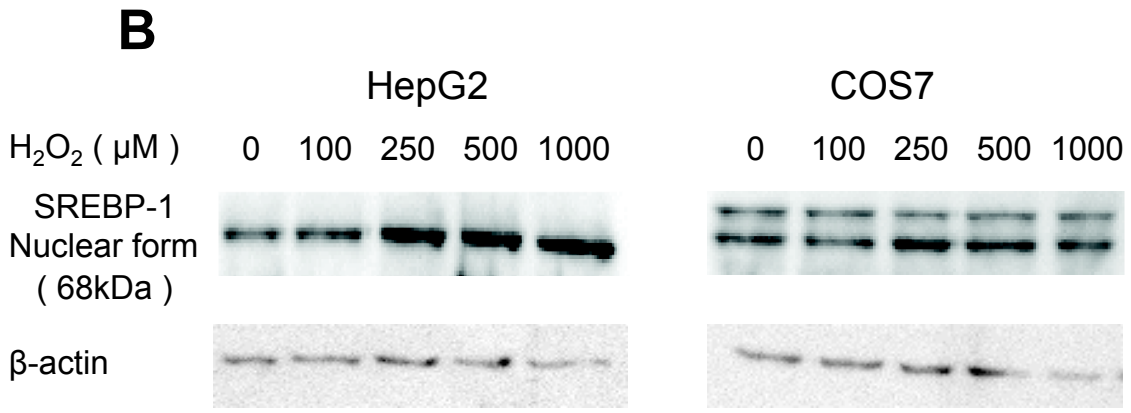
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SREBP-1
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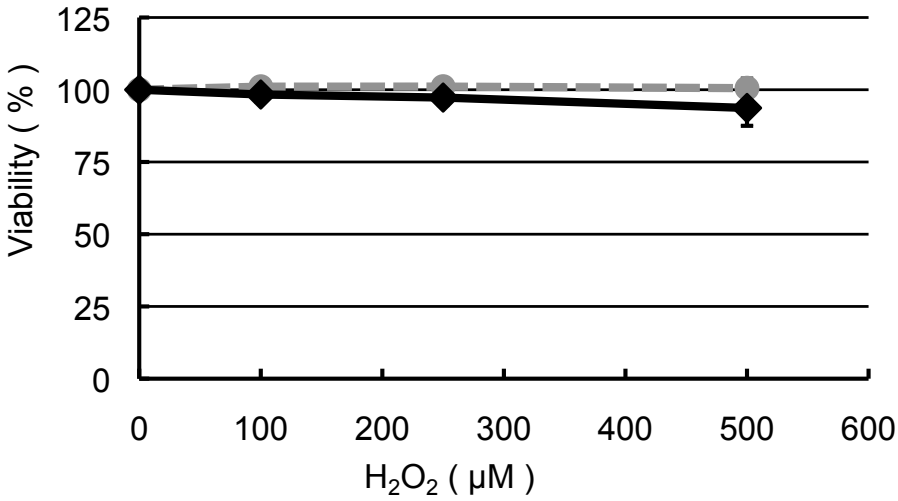
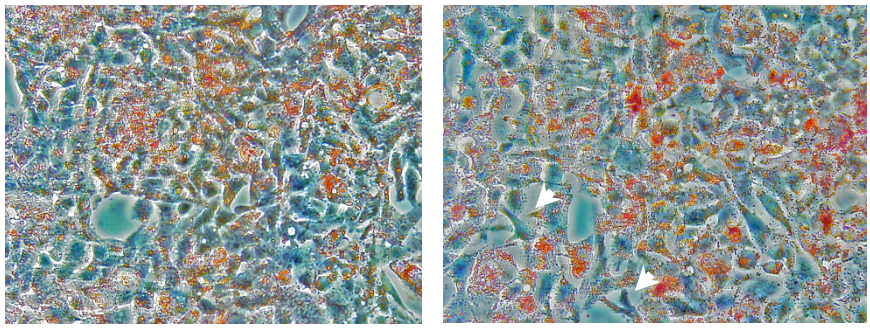
Fig.3

A



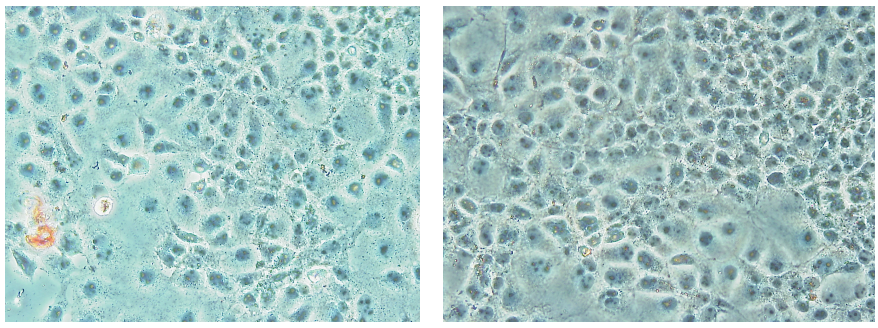
B

HepG2



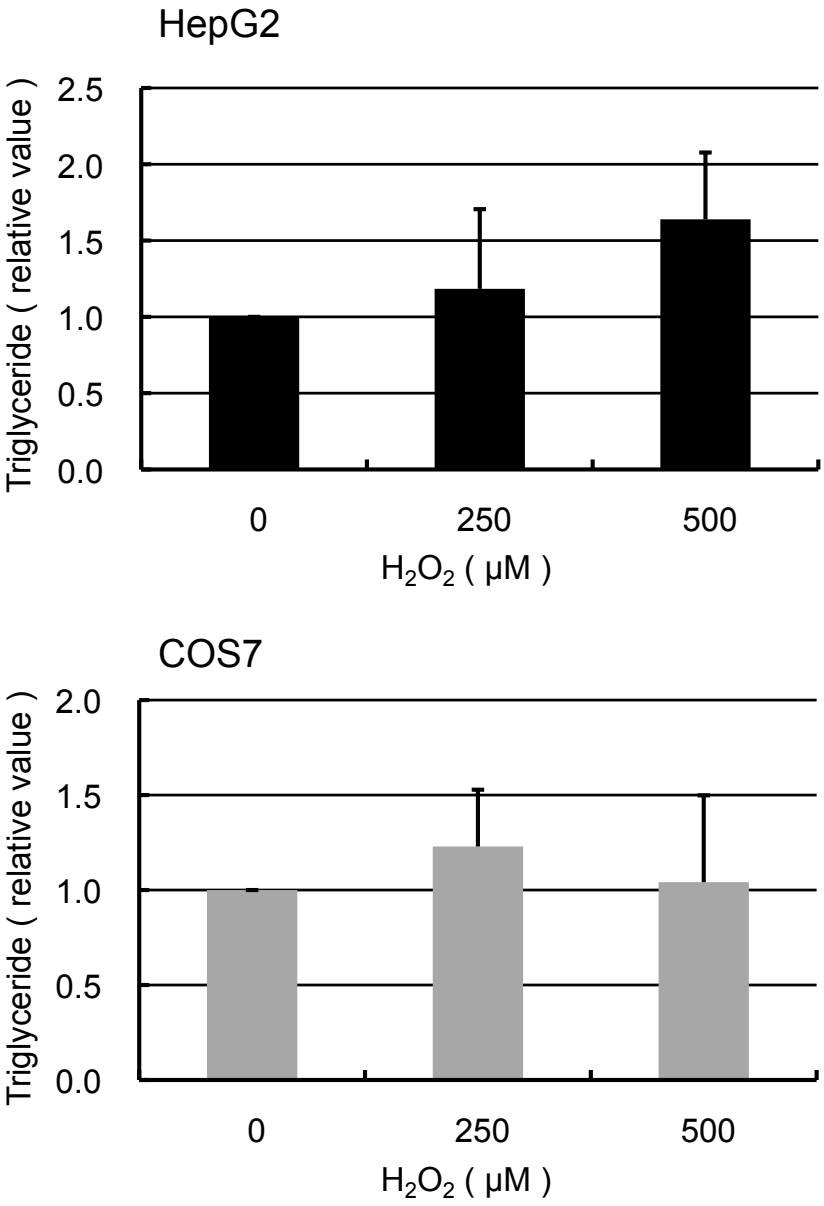
H_2O_2 (μM) 0 500

COS7



H_2O_2 (μM) 0 500

Fig.4


HepG2
Triglyceride (relative value)
2.5
2.0
1.5
1.0
0.5
0.0
0
250
500
H2O2 (μM)
COS7
Triglyceride (relative value)
2.0
1.5
1.0
0.5
0.0
0
250
500
H2O2 (μM)