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# Effects of retinoic acid and hydrogen peroxide on sterol regulatory element-binding protein-1a activation during adipogenic differentiation of 3T3-L1 cells

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## Abstract

Both retinoic acid (RA) and oxidative stress ( $H_2O_2$ ) increased transcription and cleavage of membrane-bound sterol regulatory element-binding protein (SREBP)-1, leading to enhanced transcription of fatty acid synthase (FAS) in hepatoma cells. On the other hand, RA and  $H_2O_2$  decreased and increased lipogenesis in adipocytes, respectively, although roles of SREBP-1 activation in these effects remain to be elucidated. To elucidate its involvement, we examined the activation of SREBP-1a, expression of FAS genes and lipid accumulation in 3T3-L1 cells in the presence of RA and/or  $H_2O_2$ . RA (1  $\mu$ M) treatment suppressed expression of SREBP-1a and FAS genes and lipid accumulation.  $H_2O_2$  (2  $\mu$ M) treatment induced increased cleavage of SREBP-1a, without affecting amounts of SREBP-1a mRNA and precursor protein, and enhanced expression of FAS gene and lipid accumulation. Increased cleavage of SREBP-1a by  $H_2O_2$  was also observed even in the presence of RA. These results suggest that  $H_2O_2$  enhances a cleavage of SREBP-1a precursor protein, which independently occurs with the RA suppression of SREBP-1a gene expression, and that RA itself has no role in the SREBP-1a activation in adipocytes.

Keywords: adipogenesis, fatty acid synthase,  $H_2O_2$ , SREBP-1, vitamin A

## Introduction

Vitamin A (retinoid) is essential for multiple physiological processes, including vision, immune functions, reproduction, embryonic development, and cellular growth and differentiation<sup>2)</sup>. It

is well documented that retinoic acid (RA) suppresses the adipogenic differentiation of mouse 3T3-L1 preadipocytes<sup>9,15)</sup> and primary cultured stromal vascular cells from bovine perirenal and pig subcutaneous adipose tissues<sup>3,11)</sup>. However, in human HepG2 hepatoma

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cells, RA induces expression of fatty acid synthase (FAS) gene by inducing transcription and cleavage of sterol regulatory element-binding protein (SREBP)-1c<sup>13)</sup>, a pivotal transcription factor for lipid-metabolizing genes<sup>5,12,17)</sup>. Thus, there may be tissue-specific differences in the SREBP activation by RA and subsequent lipogenic gene expression.

On the other hand, oxidative stress is also known to induce lipid accumulation via SREBP-1 activation in HepG2 cells<sup>16)</sup>. Interestingly, such oxidative stress causes lipid accumulation even in adipocytes<sup>10,19)</sup>, although it is unknown whether oxidative stress induces SREBP-1 activation in adipocytes. To clarify the roles of SREBP activation in the RA effects on adipocytes, in the present study we examined the activation of SREBP-1a, the alternative splicing form of SREBP-1 expressed exclusively in 3T3-L1 cells<sup>18)</sup>, expression of SREBP-1a and FAS genes and lipid accumulation after the cells were treated with RA and hydrogen peroxide, an oxidative stressor.

## Materials and Methods

*Cell culture and treatments:* The 3T3-L1 preadipocytes were cultured in Dulbecco's modified Eagles's medium (DMEM, Wako Pure Chemicals Co., Osaka, Japan) containing 10% fetal calf serum (FCS, Trace Scientific Ltd., Melbourne, Australia) in 60 and 100 mm collagen-coated dishes (Iwaki-Asahi Techno Glass, Chiba, Japan) at 37°C and 5% CO<sub>2</sub> under humidified conditions, and the media were changed every 2 days. When the cells had reached confluence (referred to as day 0 hereafter), the cells were further cultured in DMEM containing 10% FCS, 0.5 mM isobutylmethylxanthine (IBMX, Sigma-Aldrich Fine Chemicals, St. Louis, MO, USA), 1 μM dexamethasone (Wako), and 10 μg/ml insulin (Sigma) for the first 2 days, and subsequently cultured in the medium containing 10% FCS and

10 μg/ml insulin until day 6. Where indicated, *all-trans* retinoic acid (RA 1 μM, Wako), DMSO, and/or H<sub>2</sub>O<sub>2</sub> (2–100 μM) were added to the medium from day 0. These treatments had no apparent effect on the cell viability.

*Oil red O staining:* To quantify the lipid accumulation, the cells were fixed with 10% formalin in isotonic phosphate buffer for 1 hr, stained with 0.5% oil red O in 60% isopropyl alcohol for 1 hr, and rinsed extensively with water. After visualization, oil red O stained lipid droplets were extracted with 1 ml of absolute isopropyl alcohol and quantified by measuring absorbance at 540 nm.

*Real time PCR:* To quantify the levels of SREBP-1a and FAS mRNA expression, real-time PCR was performed as follow. The RNA (2 μg) was treated at 70°C for 10 min and reverse transcribed using 100 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA), 50 pmol of poly (dT) primer, and 20 nmol of dNTP in a total volume of 20 μl at 37°C for 1 hr. After heating at 94°C for 5 min, PCR amplification was performed with 2.5 units Taq polymerase (Ampliqon, Herlev, Denmark), 3 mM MgCl<sub>2</sub>, and 50 pmol of forward and reverse primers specific to the respective genes in a total volume of 25 μl. The primers and annealing PCR conditions for mouse SREBP-1a, mouse FAS, and mouse β-actin are summarized in Table 1. Denaturation of PCR was performed at 94°C for 30 sec, while extension was performed at 72°C for 60 sec. The PCR products were subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). The nucleotide sequence of each cDNA was confirmed, and the cDNA were used as standards for real-time PCR. The real-time PCR was performed with a fluorescence thermal cycler (Light Cycler System, Roche Diagnostics, Basel, Switzerland) using 0.5 μM of each primer as shown in Table 1. The fluorescence of SYBR Green (Qiagen, Hilden, Germany) at 530 nm was recorded at the end of

**Table 1. Primer list**

Gene (Accession No)	Forward primer sequence Reverse primer sequence (PCR product size) [Annealing conditions]
mSREBP-1a (BC056922.1)	5 -ATGGACGAGCTGGCCTTCGGTGAGGCGGC-3 5 -CAGGAAGGCTTCCAGAGAGGA-3 (+ 44 to + 296, 255 bp) [60°C, 60 s, 30 cycles]
mFAS (AF 127033)	5 -GGCATCATTGGGCACTCCTT-3 5 -GCTGCAAGCACAGCCTCTCT-3 (+ 1769 to + 1851, 82 bp) [60°C, 60 s, 30 cycles]
m $\beta$ -actin (NM 007393)	5 -TCGTACCACAGGCATTGTGAT-3 5 -TGCTCGAAGTCTAGAGCAAC-3 (+ 517 to + 753, 237 bp) [58°C, 30 s, 30 cycles]

the extension phase and analyzed using the Light Cycler Software (Version 3). The level of  $\beta$ -actin mRNA was also determined as an internal control.

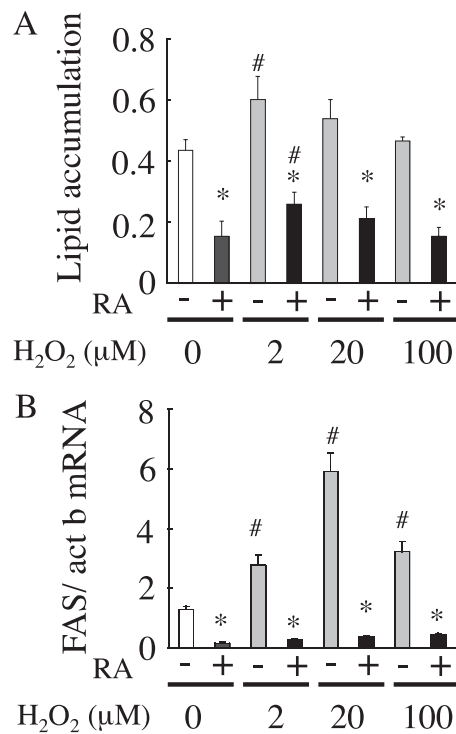
**Western blot:** To analyze SREBP-1 protein and its activation, Western blot was performed as follow. The cells were washed twice with ice-cold PBS and lysed with a Dounce homogenizer in 400  $\mu$ l of lysis buffer (10 mM Tris/HCl, 10 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.5% Nonidet 40, and protease inhibitor cocktail (Complete, Boehringer Mannheim, Mannheim, Germany). After removing cell debris by centrifugation (12,000 x g) at 4°C for 10 min, the supernatant was stored at -80°C. An aliquot of the supernatant (20  $\mu$ g protein) was resolved by SDS-PAGE (8% gel) under reducing condition, and the proteins were then electroblotted onto a PVDF membrane (Immobilon, Millipore, Bedford, MA, USA). The membrane was then blocked for 1 hr at room temperature in 5% (w/v) skimmed milk in 20 mM Tris/HCl (pH 7.5), 0.15 M NaCl, and 0.01% Tween 20, before being incubated with anti-SREBP-1 antibody (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The membrane was then washed 5 times with 20 mM Tris/HCl (pH 7.5), 0.15 M NaCl, and 0.01% Tween 20 and incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit IgG antibody (1:5000, Zymed laboratories, Inc.,

South San Francisco, CA, USA) for 1 h at room temperature. Visualization was performed using an enhanced chemiluminescence detection system (Millipore) according to the manufacturer's instructions. The intensity of chemiluminescence for the corresponding protein was analyzed by NIH Image, a public-domain image processing and analysis program.

**Statistic analysis:** The results are expressed as means  $\pm$  S.E.M. Statistical analysis was performed using two-way ANOVA and Fischer's post hoc test, with  $p < 0.05$  being considered statistically significant.

## Results

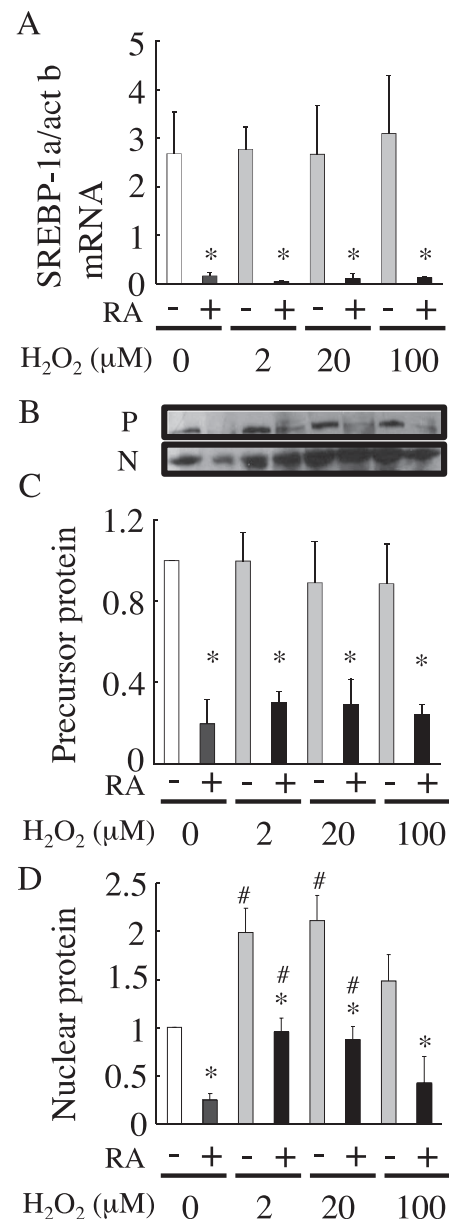
The 3T3-L1 cells accumulated lipid on the day 6 of culture. Treatment of the cells with RA during the culture suppressed lipid accumulation, while treatment with H<sub>2</sub>O<sub>2</sub> at the lowest concentration (2  $\mu$ M) significantly increased lipid accumulation (Fig. 1A). Treatment with RA also suppressed FAS gene expression, while treatments with H<sub>2</sub>O<sub>2</sub> (2-100  $\mu$ M) increased it (Fig. 1B). Treatment with RA even in the presence of H<sub>2</sub>O<sub>2</sub> inhibited lipid accumulation, though H<sub>2</sub>O<sub>2</sub> treatment somehow counteracted RA inhibition (Fig. 1A). On the other hand, treatment with RA abolished H<sub>2</sub>O<sub>2</sub>-induced FAS



**Fig. 1. Effects of retinoic acid and hydrogen peroxide on lipid accumulation (A) and expression of fatty acid synthase gene (B).** 3T3-L1 cells were cultured to confluence with DMEM and 10% FCS. These cells were then cultured with DMEM and 10% FCS containing retinoic acid (RA, 1 μM; +, presence; -, absence) and/or H<sub>2</sub>O<sub>2</sub> (2-100 μM). The cells were also treated with an adipogenic differentiation cocktail for the first two experimental days, and each medium was subsequently changed every 2 days. **A:** On day 6 of culture, the cells were stained with oil red O, and the dye remaining in the cells, which reflected the amount of accumulated lipids, was measured using the absorbance at 540 nm after extracting the cells with isopropanol. **B:** Total RNA was extracted from the cells, and FAS gene expression was determined by real-time PCR, and the levels of the gene expression are shown after being normalized to the level of β-actin (*act b*) mRNA expression.\* and # indicate statistical significance between the controls and RA treatment or H<sub>2</sub>O<sub>2</sub> treatment, respectively ( $p < 0.05$ ).

gene expression with statistical significance between the two factors (Fig. 1B). That is, the effect of RA on the lipid accumulation was opposite to the effect of H<sub>2</sub>O<sub>2</sub>, and these effects were possibly, at least in part, explained by their influences on FAS gene expression.

Next, effects of RA on SREBP-1 activation were examined by detecting its nuclear form. Treatment with RA decreased SREBP-1a gene



**Fig. 2. Effects of retinoic acid and hydrogen peroxide on expression of SREBP-1a gene (A) and proteins (B-D).** 3T3-L1 cells were cultured as described in the legend for Fig. 1. RNA and proteins were extracted from the cells, and the expression levels of SREBP-1a mRNA and the precursor and nuclear forms of SREBP-1 were examined by real time PCR (A) and Western blot analysis (B-D), respectively. **A:** The expression levels of SREBP-1a genes are shown after being normalized to the level of β-actin (*act b*) mRNA expression. **B:** Shown are representative results for the SREBP-1a precursor (P) and nuclear form (N). **C and D:** Densitometric analysis was performed for the precursor (C) and nuclear (D) forms of SREBP-1. \* and # indicate statistical significance between the controls and RA treatment or H<sub>2</sub>O<sub>2</sub> treatment, respectively ( $p < 0.05$ ).

expression, whereas treatments with  $H_2O_2$  (2–100  $\mu M$ ) had no effect on the gene expression (Fig. 2A). Similarly, RA decreased the amount of SREBP-1a precursor protein while  $H_2O_2$  was without effect on it, possibly reflecting the gene expression (Fig. 2A–C). On the other hand, treatment with  $H_2O_2$  increased the amount of nuclear form of SREBP-1a, while RA either in the presence or absence of  $H_2O_2$  decreased it (Fig. 2D). However, treatments with  $H_2O_2$  (2–20  $\mu M$ ) partly recovered the amount of nuclear form of SREBP-1a from RA suppression (Fig. 2D). These results suggest that  $H_2O_2$  treatment enhances the cleavage of SREBP-1a precursor protein, which independently occurs with the RA suppression of SREBP-1a gene expression, and that RA itself has no role in the SREBP-1a activation in adipocytes.

## Discussion

In the present study, we have demonstrated that RA strictly suppresses expression of SREBP-1a and FAS genes and lipid accumulation, without affecting SREBP-1a activation. As SREBP-1 has been found to regulate lipid-metabolizing genes including FAS genes<sup>5,12,13,17</sup>, the suppression of SREBP-1a at transcription levels, but not at post-translational levels, is likely to be a key step for RA effects on adipocytes. The phenomena also suggest that the regulatory mechanisms of SREBP-1 expression are different from the hepatoma cells<sup>13</sup>. However, RA sometimes displays enhancement of adipogenesis *in vitro* and *in vivo*. For example, RA at a concentration lower than 10 nM enhances the activity of glycerol-3-phosphate dehydrogenase, a marker of adipogenesis, in Ob17 cells established from the periepididymal adipose tissue of the genetically obese C57/BL6J mouse and in rat preadipocytes<sup>14</sup>. In young lambs, high levels of vitamin A supplementation lead to an increase in the total intramuscular lipid level<sup>1</sup>. Since

vitamin A supplementation and restriction influence meat quality<sup>1,7,8</sup>, elucidation of the regulatory mechanisms of SREBP-1 transcription by RA in adipocyte might be important for controlling adiposity of animals, especially beef cattle, in future.

We have also demonstrated that  $H_2O_2$  treatment induces SREBP-1a activation, without affecting amounts of SREBP-1a mRNA and precursor protein. The latter indicates  $H_2O_2$  has no roles in SREBP-1a expression at transcription and translation. This is in contrast with the fact that  $H_2O_2$  at much higher concentrations (100–1000  $\mu M$ ) affects SREBP-1c expression at transcription in HepG2 and COS7 cells<sup>16</sup>. It has been shown that  $H_2O_2$  mimics insulin action<sup>10,19</sup>, and that insulin stimulates a plasma membrane NADPH oxidase system present in adipocytes and transiently generates  $H_2O_2$ <sup>6</sup>. The role of insulin-generating  $H_2O_2$  in insulin action is proposed to be an inhibitor of cyclic AMP-activated PKA and subsequent lipolysis, and of protein tyrosine phosphatases, leading to sustained insulin signaling<sup>4,6</sup>. As  $H_2O_2$  at 2  $\mu M$  increased lipid accumulation accompanied with enhancement of FAS gene expression and SREBP-1a activation in adipocytes, SREBP-1 may be a novel target of insulin-generating  $H_2O_2$ .

In summary, RA and  $H_2O_2$  inhibited and activated SREBP-1a-dependent expression of FAS and lipid accumulation, respectively. The former occurred at the transcription levels of SREBP-1a, while the latter did at the levels of post-translational cleavage of SREBP-1a.

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