Retinoic acid modulates lipid accumulation glucose concentration-dependently through inverse regulation of SREBP-1 expression in 3T3L1 adipocytes

Short title: Role of retinoic acid in adipogenesis

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Abstract

It is well known that retinoic acid (RA) suppresses adipogenesis, although there are some contradicting reports. In the present study we examined the effect of extracellular glucose on RA-induced suppression of adipogenesis in 3T3L1 cells culture. When the cells were cultured in normal glucose medium (NG), addition of RA suppressed lipid accumulation. However, when cultured in high glucose medium (HG), addition of RA to the cells enhanced lipid accumulation. These changes were accompanied by parallel alterations in fatty acid synthase (FAS) and sterol regulatory element binding protein (SREBP)-1 gene expression. Transfection of SREBP-1 siRNA abolished RA-induced enhancement of lipid accumulation and FAS expression in the cells cultured with HG. Transfection of the nuclear form of SREBP-1a cDNA into the cells cultured with NG abrogated RA-induced suppression of lipid accumulation and FAS expression. Moreover, RA- and HG-induced SREBP-1a expression occurred at the early phase of adipogenesis, and was dependent on glucocorticoid to induce liver X receptor (LXR) β, peroxisomal proliferator-activated receptor (PPAR) γ and retinoid X receptor (RXR), the key nuclear factors influencing the SREBP-1a gene expression. These results suggest that RA suppresses and enhances lipid accumulation through extracellular glucose concentration-dependent modulation of SREBP-1 expression.

Keywords: 3T3L1; adipogenesis; fatty acid synthase; retinoid; sterol regulatory element binding protein; vitamin A
Introduction

Vitamin A (retinoid) is essential for multiple physiological processes, including vision, immune functions, reproduction, embryonic development, and cellular growth and differentiation (Blomhoff & Blomhoff 2006). Many of these pleiotropic activities of vitamin A are mediated by the activation of specific nuclear receptors, including retinoic acid receptors (RAR) and retinoid X receptors (RXR), which modulate the rates of transcription of numerous target genes upon activation (Shulman & Mangelsdorf 2005; Blomhoff & Blomhoff 2006).

Retinoic acid (RA) suppresses the adipogenic differentiation of mouse 3T3-L1 preadipocytes (Kawada et al. 1990; Schwarz et al. 1997) and primary cultured stromal vascular cells from pig subcutaneous adipose tissues (Brandebourg & Hu 2005). Similar suppressive effects of retinoids on adipogenesis in vivo have been highlighted in previous studies, as outlined below. High vitamin A intake causes reduced adiposity in healthy young adults (Zulet et al. 2008), while the ratio of retinol to retinol binding protein are lower in obese adults, possibly reflecting increased adiposity due to lower retinoid bioavailability (Mills et al. 2008). Dietary vitamin A restriction increases the marbling scores, an index of intramuscular fat, of beef cattle (Gorocica-Buenfil et al. 2007). Moreover, the administration of RA reduces adiposity in mice and is accompanied by remodeling of the white adipose tissue (Mercader et al. 2006). However, RA sometimes displays opposite effects on adipogenesis in vitro and in vivo. 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 mouse periepididymal adipose tissue and also in rat preadipocytes (Safonova et al.1994). Rats given the cafeteria diet supplemented with a high concentration of vitamin A showed increased adiposity compared with rats given the same diet.
containing a low amount of vitamin A (Redonnet et al. 2008). In young lambs, high levels of vitamin A supplementation led to an increase in the total intramuscular lipid level (Arnett et al. 2007).

It has been shown that RA blocks adipogenesis by inhibiting CCAAT/enhancer binding protein (C/EBP) β-mediated transcription, without affecting C/EBPβ protein levels (Schwarz et al. 1997), and a recent report found that RA induces Smad3 expression, resulting in interference with C/EBP β DNA occupancy of the Cebpa promoter (Marchildon et al. 2010). In contrast, other recent findings have shown that RA and carotenoids induce the expression of peroxisomal proliferator-activated receptor (PPAR) γ, a key transcription factor for adipogenesis, in preadipocytes obtained from bovine adipose tissue (Garcia-Rojas et al. 2010). In human HepG2 hepatoma cells, RA induces the expression of the fatty acid synthase (FAS) gene by inducing the transcription and cleavage of sterol regulatory element-binding protein (SREBP)-1c (Roder et al. 2007), a pivotal transcription factor for lipid-metabolizing genes (Eberlé et al. 2004; Raghow et al. 2007; Shimano 2009). Since PPARγ and SREBP-1c act downstream of C/EBP β (Saladin et al 1999; Payne et al. 2010) and PPARγ can be induced by SREBP-1 (Fajas et al. 1999), it is likely that RA induction of SREBP-1 and/or PPARγ accelerates adipogenesis in certain conditions.

As glucose regulates the expression of SREBP-1 in the liver and its target gene in adipocytes (Jones et al. 1998; Shimano et al. 1999; Hasty et al. 2000), in the present study we examined the effect of RA on lipid accumulation in mouse 3T3L1 preadipocytes cultured with different concentrations of glucose and the involvement of SREBP-1 in the process.
Results

Retinoic acid modulates lipid accumulation glucose concentration-dependently through inverse regulation of SREBP-1 expression in 3T3L1 adipocytes

In the absence of all-trans retinoic acid (RA), 3T3L1 cells differentiated in medium containing physiological glucose concentration (5.5 mM) accumulated lipids, comparable to those cultured in high glucose medium (25 mM)(Fig. 1A). Similarly, there was no difference between the cells cultured in normal and high glucose mediums in the mRNA expression of rate limiting enzymes for fatty acid synthesis, fatty acid synthase (FAS) and acetyl-CoA carboxylase-1α (ACC1α) and of an adipogenic differentiation marker, adipose tissue-type fatty acid binding protein (aP2) (Figs. 1B-1D).

In the presence of RA, 3T3-L1 cells cultured in 5.5 mM glucose accumulated lipids less than those cultured in the absence of RA, accompanied with decreases in the mRNA expression of FAS and aP2 (Figs. 1A-1D), indicating that RA suppressed the adipogenic differentiation of mouse 3T3L1 preadipocytes cultured in normal glucose medium as previously reported (Kawada et al. 1990; Schwarz et al. 1997). Surprisingly, the cells cultured in 25 mM glucose in the presence of RA enhanced lipid accumulation more than those cultured in the absence of RA, accompanied with increases in the mRNA expression of FAS, ACC1α and aP2 (Figs. 1A-1D), suggesting that RA accelerated adipogenic differentiation of the cells cultured in high glucose medium.

To determine the mechanism by which RA affects lipid accumulation in a glucose concentration-dependent manner, we next examined the expression of sterol regulatory element binding proteins (SREBP) that is a transcriptional regulator of FAS and ACC gene expression (Shimano 2001; Eberlé et al. 2004; Raghow et al.
2007; Bennett et al. 2008; Im et al. 2009; Shimano 2009). In 3T3L1 cells, SREBP-1c mRNA was not expressed, as reported previously (Shimomura et al. 1997; Inoue et al. 2001), while SREBP-1a and SREBP-2 mRNA was detected (Fig. 1E). Although the presence of RA failed to affect mRNA expression of SREBP-2 irrespective of medium glucose concentrations (Fig. 1G), it suppressed mRNA expression of SREBP-1a mRNA in 5.5 mM glucose medium culture and increased the mRNA in 25 mM glucose medium, respectively (Fig. 1F), concomitant with the changes in lipid accumulation and mRNA expression of FAS (Figs. 1A and 1D).

To verify the involvement of SREBP-1a in the effects of RA on lipid accumulation, we first examined the effect of SREBP-1 siRNA on RA-induced enhancement of lipid accumulation in 3T3L1 cells cultured in the high glucose medium for 3 days. The expression of SREBP-1a mRNA was increased by RA treatment in the cells cultured in high glucose medium (Figs. 1F, 2A and 2B), and this was accompanied by increases in the precursor (125 kDa) and nuclear (68 kDa) forms of SREBP-1 protein (Figs. 2A, 2C, and 2D). Transfection of SREBP-1 siRNA almost completely abrogated the expression of SREBP-1a mRNA and reduced the expression of the precursor and nuclear proteins by 30% and 60%, respectively (Figs. 2A-2D). Importantly, SREBP-1 siRNA abrogated the enhancement of SREBP-1a mRNA and protein expression by RA (Figs. 2A-2D). Similarly, SREBP-1 siRNA decreased FAS mRNA expression by 50% and inhibited its enhancement by RA (Figs. 2E and 2F). Since lipid accumulation was assessed in the cells that had been cultured only for 3 days, the enhancement of lipid accumulation by RA was relatively weak, compared with the lipid accumulation shown in Fig. 1A. However, SREBP-1 siRNA suppressed lipid accumulation and its enhancement by RA (Fig. 2F), supporting the idea that
SREBP-1a plays a role in the enhancement of lipid accumulation by RA in high glucose medium cultures.

Next, we examined the effect of constitutively active SREBP-1a (nuclear form) on the RA-induced suppression of lipid accumulation in normal glucose conditions. Transfection of the nuclear form of human SREBP-1a cDNA for 3 days increased the levels of the nuclear form of SREBP-1a protein (Figs. 3A and 3D). Interestingly, transfection of the human SREBP-1a cDNA increased endogenous SREBP-1a mRNA expression and precursor protein levels (Figs. 3A-3C), suggesting the presence of a self-regulatory mechanism for the gene. RA induced weak but significant suppression of SREBP-1a mRNA and protein expression in 3 day cultures with normal glucose. The transfection of human SREBP-1a cDNA restored the nuclear SREBP-1 protein levels compared to those after empty vector transfection, but failed to prevent the suppressive effect of RA on the expression of endogenous SREBP-1a mRNA and precursor protein (Figs. 3A-3D). Under these conditions, transfection of the SREBP-1a cDNA enhanced the expression of FAS mRNA and lipid accumulation in both the absence and presence of RA (Figs. 3E and 3F), indicating that the restoration of nuclear SREBP-1 protein levels recovered FAS expression and lipid accumulation from RA-induced suppression.

**Effects of retinoic acid on expression of nuclear receptors in mouse 3T3-L1 cells cultured in normal and high glucose medium**

Expression of SREBP-1a gene was decreased and increased by the presence of RA in the cells cultured for 6 days in normal and high glucose medium, respectively (Fig. 4A). In contrast, expression of RARα gene was increased and decreased by RA in the cells cultured with normal and high glucose medium, respectively (Fig. 4B). Similarly, expression of RARγ gene was increased by RA in the cells cultured with normal
glucose, but such an enhancement was abolished in the culture with high glucose (Fig. 4C). Expression of RXRα, PPARγ, and liver X receptor (LXR) α genes were decreased by RA in the culture with normal glucose, but these decreases were abrogated in the culture with high glucose (Figs. 4D, 4F, and 4H). Expression of PPARβ/δ gene was decreased and increased by RA with normal and high glucose, respectively (Fig. 4G), whereas expression of RXRβ and LXRβ genes were increased by RA in the culture with high glucose (Figs. 4E and 4I). These results suggest that RA displays extracellular glucose-dependent opposing effects on expression of some nuclear receptor genes like SREBP-1a gene.

Interestingly, glucose concentration-dependent opposing effects of RA on expression of SREBP-1a gene in 3T3-L1 cells were observed when the cells cultured only for 24 h (data not shown). Moreover, gene expression of nuclear receptors, RARα, RARγ, RXRα, RXRβ, PPARγ, PPARβ/δ, LXRα and LXRβ, in the cells cultured for 24 h basically followed the pattern of respective gene expressions in the cells cultured for 6 days (Figs. 4A-4I). Therefore, in the further study, we examined the roles of nuclear receptors in RA- and glucose-regulating SREBP-1a expression in the cells cultured for 1 day.

Role of adipogenic differentiation inducer, dexamethsone, in retinoic acid- and glucose-regulating SREBP-1a and nuclear receptors expression

Confluent 3T3-L1 cells were ordinary treated with a mixture of insulin, isobutylmethylxanthine (IBMX) and dexamethasone (Dex) to induce adipogenic differentiation. To clarify the roles of these components in RA- and glucose-regulating SREBP-1a expression, the cell were treated with each the component either in the presence or absence of RA. In the cells cultured with normal glucose, RA suppressed SREBP-1a expression with and without treatment with a mixture of
adipogenic differentiation inducers (Fig. 5A). Each the component also suppressed SREBP-1a expression irrespective of the presence of RA (Fig. 5A). In the cells cultured with high glucose, RA increased SREBP-1a expression only in the presence of Dex treatment (Fig. 5B), suggesting that glucocorticoid plays a role in modulation of RA responsiveness in high glucose condition.

**Roles of LXRβ, PPARγ and RXR in glucocorticoid- and retinoic acid-dependent induction of SREBP-1a mRNA in the cells cultured in high glucose medium**

To clarify roles of nuclear receptors in glucocorticoid- and retinoic acid-dependent induction of SREBP-1a mRNA in the cells cultured in high glucose medium, effects of nuclear receptor agonists and antagonists were examined. Treatment of the cells with RA (RAR and RXR agonist) and 9-cis-RA (RXR agonist), but not TTNPB (RAR agonist), enhanced expression of SREBP-1a gene in a Dex-treatment dependent manner (Fig. 6, top panel). Treatment with HX531 (RXR antagonist) and T0070907 (PPARγ antagonist) inhibited the Dex- and RA-dependent induction of SREBP-1a mRNA (Fig. 6, second panel). Treatment with troglitazone (PPARγ agonist) and T0901317 (LXR agonist), but not GW0742 (PPARβ/δ agonist), enhanced expression of SREBP-1a gene in a Dex-dependent manner (Fig. 6, bottom panel). Furthermore, inability of PPARβ/δ to affect SREBP-1a gene expression was confirmed by the experiment of PPARβ/δ siRNA transfection (Fig. 7A). These results suggest possible roles of LXR, PPARγ and RXR, but not RAR and PPARβ/δ, in glucocorticoid-, retinoic acid-, and high glucose-dependent induction of SREBP-1a mRNA.

To verify the roles of LXR, the cells were transfected with siRNA for LXRα and/or LXRβ. Transfection with LXRβ siRNA, but not LXRα siRNA, abrogated Dex-dependent enhancement of SREBP-1a expression in the presence of RA and high glucose (Fig. 7A). Transfection with LXRβ siRNA
suppressed LXRβ gene expression (Fig. 7B), and also Dex-dependent enhancement of PPARγ and RXRβ mRNA expression, but not RXRα gene expression (Figs. 7C, 7D and 7E). To verify the roles of PPARγ, the cells were transfected with its siRNA. Transfection with PPARγ siRNA suppressed its expression (Fig. 8A), and Dex-dependent enhancement of SREBP-1a gene expression (Fig. 8B). Although transfection with PPARγ siRNA inhibited the Dex-dependent enhancement of both RXRα and RXRβ genes expression (Figs. 8D and 8E), it failed to suppress the Dex-dependent enhancement of LXRβ gene expression (Fig. 8C). These results indicate pivotal roles of LXRβ and PPARγ in glucocorticoid-, retinoic acid-, and high glucose-dependent induction of SREBP-1a mRNA, and suggest that LXRβ is up-stream regulator for expression of PPARγ.

To confirm the glucocorticoid-dependency of LXRβ, PPARγ, RXRα and RXRβ gene expression though they were induced by RA in the presence of Dex (Fig. 4), these gene expressions were examined in the cells cultured in high glucose medium. As shown in the Figure 9, enhancement of LXRβ, RXRα and RXRβ gene expression required both RA and Dex, while expression of PPARγ gene was increased by Dex alone.

Finally, roles of LXRβ and PPARγ in glucose concentration-dependent expression of SREBP-1a in the presence of RA and Dex were examined. Transfection with LXRβ or PPARγ siRNA, but not LXRα or PPARβ/δ siRNA, abrogated high glucose-dependent enhancement of SREBP-1a expression in the presence of RA and Dex (Fig. 10).
Discussion

In the present study, we have demonstrated for the first time that RA in the pathophysiological concentration range has opposing extracellular glucose concentration-dependent effects on lipid accumulation in cultures of 3T3L1 adipocytes; i.e., RA (1 µM) suppressed lipid accumulation at a normal glucose concentration (5.5 mM), while it enhanced lipid accumulation at a high glucose concentration (25 mM). These opposing effects of RA on lipid accumulation are, at least in part, due to glucose concentration-dependent changes in SREBP-1a expression, which is demonstrated by the following evidence: 1) RA modulated SREBP-1 expression in an extracellular glucose concentration-dependent manner, 2) SREBP-1 has been found to regulate FAS and ACC gene expression (Shimano 2001; Eberlé et al. 2004; Raghow et al. 2007; Bennett et al. 2008; Im et al. 2009; Shimano 2009), 3) suppression of endogenous SREBP-1a expression abolished RA enhancement of FAS expression and lipid accumulation in high glucose medium, and 4) the overexpression of constitutively active SREBP-1a rescued from RA-induced suppression of FAS expression and lipid accumulation in normal glucose medium. Furthermore, the phenomena that glucose-dependent RA suppression and enhancement of lipid accumulation and SREBP expression were reproduced in our preliminary culture of bovine intramuscular adipocytes (M.A. Abd Eldaim and K. Kimura, unpublished data).

RA activates specific nuclear receptors, including RAR and RXR, which modulate the rates of transcription of numerous target genes upon activation (Shulman & Mangelsdorf 2005; Ziouzenkova & Plutzky 2008). Interestingly, similar to SREBP-1a gene expression, expression of these nuclear receptor genes was oppositely regulated by RA during adipogenic differentiation in an extracellular glucose concentration...
dependent manner. For instance, the cells treated with RA in normal glucose medium increased and decreased expression of RARα and RARγ genes and expression of SREBP-1a and RXRα genes, respectively, whereas the cells treated with RA in high glucose medium increased and decreased expression of SREBP-1a and RXRβ genes and expression of RARα gene, respectively. In addition, expression of LXR and PPAR genes, which also dimerize with RXR (Shulman & Mangelsdorf 2005; Ziouzenkova & Plutzky 2008), were inversely regulated by RA in a glucose concentration-dependently. Therefore, it is likely that alteration in the respective amounts of RXR and RXR-binding nuclear receptors such as RAR, LXR and PPAR occurred depending on RA and extracellular glucose, resulting in switching RXR-binding nuclear receptor to modulate SREBP-1a gene expression. It has been reported that activation of RARs in the early stages of adipogenesis inhibits differentiation and C/EBPβ-mediated transcription of PPARγ (Schwarz et al. 1997), a requisite nuclear receptor for adipocyte differentiation (Hamm et al. 1999; Fu et al 2005). In the present study, RA-induced suppression of PPARγ gene expression was confirmed only in the cells cultured in normal glucose medium. Thus, RA-induced suppression of SREBP-1a expression in normal glucose concentration might be attributed to the suppression of PPARγ gene expression.

In high glucose medium, RA-dependent increase of SREBP-1a expression needed a mixture of adipogenic differentiation inducers, insulin, isobutylmethylxanthine (a phosphodiesterase inhibitor) and dexamethasone (a synthetic glucocorticoid). Of these, dexamethasone alone was sufficient for RA-dependent enhancement of the gene expression. In the Dex treated cells, RA also increased expression of PPARγ, PPARβ/δ, RXRα, RXRβ and LXRβ genes, suggesting that glucocorticoid plays a role in modulation of RA responsiveness in high glucose condition. Further, in high
glucose medium with Dex, enhancement of SREBP-1a mRNA was observed in the cells treated with chemical agonists for RXR, PPARγ and LXR, but not for PPARβ/δ and RAR. The enhancement of SREBP-1a mRNA by RA was abrogated by chemical antagonists for RXR and PPARγ, and by siRNA for LXRβ and PPARγ, but not by siRNA for LXRα and PPARβ/δ. These results indicate the central roles of LXRβ, PPARγ and RXR in glucocorticoid-, retinoic acid-, and high glucose-dependent induction of SREBP-1a mRNA.

Both LXRβ and PPARγ were necessary for the RA dependent induction of SREBP-1a expression in response to high glucose medium and LXRβ controlled expression of PPARγ and RXRβ genes. These results suggest that LXRβ is upstream regulator for expression of PPARγ, and may affect the SREBP-1a gene indirectly through induction of PPARγ. In terms of glucose sensitivity, LXRα and LXRβ are targets for glucose-hexosamine-derived O-GlcNAc modification in vivo and in vitro, and upon the modification, LXRα increased expression of SREBP-1c gene (Hasty 2000; Anthonisen 2010). Thus, it is plausibly that LXRβ constitutively expressed and induced by RA and glucocorticoid is modified with O-GlcNAc in high glucose condition, resulting in enhancing expression of PPARγ and RXRβ genes and subsequent of SREBP-1a gene.

The use of alternative promoters generates SREBP-1a and SREBP-1c, which differ only in their first exon (Shimano 2001). In SREBP-1c promoter region, there are the cis-elements for LXR and RXR heterodimer (Repa et al. 2000; Yoshikawa et al. 2001; Shimano 2001; Roder & schweizer 2007) and insulin-mediated activation of the gene are mediated through the combinatorial actions of SREBP, LXR, Sp-1 and NF-Y cis-acting elements (Cagen et al. 2005), although SREBP-1c was not expressed in 3T3-L1 cells as reported previously (Shimomura et al. 1997). In contrast, the
minimal human and mouse SREBP-1a promoters are less than 100 bp upstream of the translation start site of the SREBP-1a gene, but do not contain RAR, LXR, PPAR and RXR binding sites (Zhang et al. 2005; Fernandez-Alvareza et al. 2008). Therefore, the **cis**- elements for these nuclear receptors might be present in the distal region of the SREBP-1a promoter. In addition, it has been reported that RA induction 17β-hydroxysteroid dehydrogenase type 2 expression requires a **cis**-regulatory sequence for SP class of transcription factors and that RARα/RXRα and SP1/SP3 interact with this promoter sequence (Cheng et al. 2008). Therefore, multiple binding sites for SP-1 in the minimal SREBP-1a promoter (Zhang et al. 2005; Fernandez-Alvareza et al. 2008) might be involved in the LXRβ, PPARγ and RXR actions, possibly with SP class of transcription factors. Further works should be taken to reveal the full mechanisms in detail.
Experimental procedures

Materials

The pMESREBP-1a expression vector, which codes for the nuclear form of human SREBP-1a (1-487 amino acids), was donated by Prof. Ryuichiro Sato (University of Tokyo) (Sato et al. 2000). HX531, a RXR antagonist, and troglitazone, a PPARγ agonist, were gifts from Nissan Chemical Industries, Ltd. (Tokyo, Japan) and Daiichi-Sankyo Co. (Tokyo, Japan), respectively.

Dulbecco’s modified Eagle’s medium (DMEM) containing a normal concentration of glucose (5.5 mM), DMEM with a high glucose concentration (25 mM), all-trans retinoic acid (RA), 9-cis-RA, collagenase, and dexamethasone were purchased from Wako Pure Chemicals Co. (Osaka, Japan). Bovine serum albumin (BSA), bovine insulin, isobutylmethylxanthine (IBMX), T0901317, and T0070907 were bought from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). Fetal calf serum (FCS) was obtained from Trace Scientific Ltd. (Melbourne, Australia). The anti-human SREBP-1 antibody (K-10, sc-367) and the control and mouse SREBP-1 siRNA (sc-37007/36558), siRNA for LXRα (sc-38829), LXRβ (sc-45317), PPARβ/δ (sc-36306) and PPARγ (sc-29456) and their transfection reagents were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TTNPB was from Enzo Life Sciences, Inc. (Plymouth, PA, USA), while GW0742 was from Cayman Chemical Co. (Ann Arbor, MI, USA).

Mouse 3T3L1 cell culture

3T3L1 preadipocytes were cultured in DMEM (5.5 mM glucose) containing 10% FCS in collagen-coated dishes, and the media were changed every 2 days. When the cells had reached confluency (referred to as day 0 hereafter), the cells were further cultured in DMEM with a normal glucose concentration (5.5 mM) or DMEM with a
high glucose concentration (25 mM), together with the adipogenic differentiation-inducing cocktail and 10 % FCS for the first 2 days, before subsequently being cultured in either medium containing 10 % FCS and 10 µg/ml insulin until day 6. Where indicated, RA (1 µM) and DMSO were added to the medium from day 0.

The transfection of the nuclear form of SREBP-1a cDNA or the empty vector was performed as follows: 3T3L1 preadipocytes (4x10^5 cells/well) were cultured with DMEM containing 5.5mM glucose, 10 % FCS, and the adipogenic differentiation-inducing cocktail in the presence or absence of RA in 6-well collagen coated plates for 48 h. The cells were then washed with phosphate buffer saline (PBS) and transfected with either cDNA (2 µg/well) with the aid of the Fugene transfection reagent (Roche Diagnostics, Mannheim, Germany) for 24 h. Subsequently, the cells were cultured in DMEM containing 5.5mM glucose, 10 % FCS, 10 µg/ml insulin, and RA (1 µM) (where indicated) for 72 h.

The transfection of SREBP-1 siRNA and the unrelated control siRNA were performed as follows: 3T3L1 preadipocytes (2x10^5 cells/well) were cultured in DMEM containing 5.5mM glucose and 10% FCS in 6-well collagen coated plates for 24 h. The cells were then washed with Opti-MEM (Invitrogen Co. Carlsbad, CA, USA) and transfected with 10 pmol of control or mouse SREBP-1 siRNA together with the siRNA transfection reagent for 7h, before being cultured in DMEM with a high glucose concentration (25 mM) containing 10% FCS and the adipogenic differentiation-inducing cocktail for 48 h. Finally, the cells were cultured in DMEM (25 mM glucose) containing 10 % FCS and 10 µg/ml insulin for 24 h, in the presence or absence of RA.

The transfection of siRNA for LXR α, LXR β, PPAR β/δ, PPAR γ and the unrelated control were performed essentially as described above. Transfected 3T3L1
preadipocytes were cultured in DMEM with a high glucose concentration (25 mM) containing 10% FCS and the adipogenic differentiation-inducing cocktail for 24 h.

**Oil red O staining**

The cells that had undergone adipogenic differentiation were fixed with 10% formalin in isotonic phosphate buffer for 1 h, stained with 0.5% oil red O in 60% isopropyl alcohol for 1 h, 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 their absorbance at 540 nm (Sagara et al. 2013).

**Conventional and real-time RT-PCR**

Total cellular RNA was isolated from the cultured cells by the guanidine-isothiocyanate method using RNAiso reagent (Takara Bio, Shiga, Japan).

The RNA (2 µg) was treated at 76 °C for 10 min and reverse transcribed using 100 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen), 50 pmol of poly (dT) primer, and 20 nmol of dNTP in a total volume of 20 µl at 37 °C for 1 h. After heating at 94 °C for 5 min, PCR amplification was performed with 2.5 units Taq polymerase (Ampliqon, Herlev, Denmark), 3 mM MgCl₂, 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, SREBP-1c, and SREBP-2; human SREBP-1a; bovine SREBP-1; mouse fatty acid synthase (FAS); mouse acetyl-CoA carboxylase-1α (ACC1α); mouse adipose tissue-type fatty acid binding protein (aP2); and mouse β-actin (act b) are summarized in Table 1. Denaturation was performed at 94 °C for 30 sec, while extension was performed at 72 °C for 60 sec. The PCR products were analyzed by electrophoresis in 1.5% agarose gel and stained with ethidium bromide.
To confirm the amplification of the genes, the PCR products were subcloned into the pGEM-T Easy vector (Promega; Madison, WI, U.S.A). Then, the nucleotide sequence of each cDNA was confirmed, and the cDNA were used as standards for real-time PCR.

To quantify gene expression levels, real-time PCR was performed with a fluorescence thermal cycler (Light Cycler System, Roche Diagnostics, Basel, Switzerland) using 0.5 µM of each primer (Table 1 and 2). The fluorescence of SYBR Green (Qiagen, Hilden, Germany) at 530 nm was recorded at the end of the extension phase and analyzed using the Light Cycler Software (Version 3). The levels of mouse β-actin mRNA were also determined as an internal control.

**Western blot**

The cells were washed twice with ice-cold PBS and lysed with a Dounce homogenizer in 400µl of lysis buffer (10 mM Tris/HCl, 10 mM NaCl, 3 mM MgCl₂, 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.

Aliquots of the resultant cell lysate (20 µg protein) were resolved by SDS-PAGE (8% gel) under reducing conditions, and the proteins were electroblotted onto a PVDF membrane (Immobilon; Millipore, Bedford, MA, USA). The membrane was then blocked for 1 h at room temperature in 5% (w/v) skimmed milk in 20mM Tris/HCl (pH 7.5), 0.15 M NaCl, and 0.01% Tween 20, before being incubated with anti-SREBP-1 antibody (1:2000) 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 of the corresponding proteins was analyzed by NIH Image, a public-domain image processing and analysis program.

Statistical Analysis

The results are expressed as means ± S.E.M. Statistical analysis was performed using ANOVA and Fischer’s post hoc test, with $p < 0.05$ being considered statistically significant.
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<th>Gene</th>
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<th>Reverse primer sequence</th>
<th>(PCR product size)</th>
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Figure legends

Fig. 1  Retinoic acid suppressed and enhanced lipogenic gene expression in 3T3-L1 cell cultured in normal and high glucose medium, respectively

3T3-L1 cells were cultured to confluence with DMEM containing 5.5mM glucose. The cells were then cultured with DMEM containing 5.5mM glucose and DMEM containing 25 mM glucose, either in the presence (+) or absence (-) of retinoic acid (RA, 1 μ M). These 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, 3T3-L1 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-G: RNA was also prepared, and the expression levels of the aP2 (B), ACC-1 α (C), FAS (D), SREBP-1a (F), and SREBP-2 (G) genes were quantified by real time PCR. The expression levels of these genes are shown after being normalized to the level of β-actin (act b) mRNA expression. Statistically significant differences (p < 0.05) are indicated by * (control vs. RA treatment) and # (5.5 mM glucose vs. 25 mM glucose). Representative conventional RT-PCR results for the expression of SREBP-1a, -1c, and -2, and act b genes in 3T3-L1 cells (E) are shown, where W and L indicate RNA from mouse white adipose tissue and liver tissue, respectively.

Fig. 2  Suppression of SREBP-1 by its siRNA abrogated the retinoic acid-induced enhancement of lipid accumulation and FAS mRNA expression in 3T3-L1 cells cultured with a high concentration of glucose.

3T3L1 cells were cultured in antibiotic free DMEM containing 5.5mM glucose in 6-well plates at a density of 2x10^5 cells/ well for 24h, before being incubated in serum
and antibiotic free Opti-MEM medium and transfected with either the control or SREBP-1 siRNA (10 pmol each) for 7 h. The cells were then cultured further with DMEM containing 25mM glucose and an adipogenic differentiation cocktail in the presence (+) or absence (-) of retinoic acid (RA, 1 μM) for 72h. A-E: RNA and proteins were extracted from the cells, and the expression levels of SRBEP-1a mRNA and the precursor and nuclear forms of SREBP-1 protein were examined by conventional RT-PCR and Western blot analysis, respectively (A). The expression levels of the SREBP-1a (B) and FAS (E) genes were quantified by real time PCR, and these are expressed after being normalized to the level of β-actin (act b) mRNA expression. Densitometric analysis was performed for the precursor (C) and nuclear (D) forms of SREBP-1. F: Intracellular lipid was stained with oil red O and quantified after isopropanol extraction. Statistically significant differences (p < 0.05) are indicated by * (control vs. RA treatment) and # (control siRNA vs. SREBP-1 siRNA).

Fig. 3  Constitutively active SREBP-1a abrogated the retinoic acid-induced suppression of lipid accumulation and FAS mRNA expression in 3T3-L1 cells cultured with a normal glucose concentration.

3T3L1 cells were cultured with DMEM containing 5.5mM glucose in 6-well plates at a density of 4x10⁵ cells/well for 24 h and treated with an adipogenic differentiation cocktail for 48 h. The cells were then transfected with a human SREBP-1a (1-487 amino acid) expression vector or an empty vector (2 μg/well), before being further cultured in the presence (+) or absence (-) of retinoic acid (RA, 1 μM) for 72h. A-E: RNA and proteins were extracted from the cells, and conventional RT-PCR was performed to check the expression of human and mouse (endogenous) SRBEP-1a, whereas Western blot analysis was performed to measure those of the precursor and
nuclear forms of SREBP-1 (A). The expression levels of the mouse SREBP-1a (B) and FAS (E) genes were quantified by real time PCR and normalized to the level of β-actin (act b) mRNA expression. Densitometric analysis was performed for the precursor (C) and nuclear (D) forms of SREBP-1. F: Intracellular lipid was stained with oil red O and quantified after isopropanol extraction. Statistically significant differences (p < 0.05) are indicated by * (control vs. RA treatment) and # (empty vector transfection vs. SREBP-1 cDNA transfection).

Figure 4. Effects of retinoic acid on expression of nuclear receptor genes in 3T3-L1 cells cultured in normal and high glucose medium.

3T3L1 cells were cultured for 6 days as described in the legend for Fig. 1 in the presence (+) or absence (-) of retinoic acid (RA, 1 µM). The expression of nuclear receptors, RARα (B), RARγ (C), RXRα (D), RXRβ (E), PPARγ (F), PPARβ/δ (G), LXRα (H) and LXRβ (I), and of SREBP-1a (A) as a reference were quantified by real time PCR, and normalized to the level of β-actin (act b) mRNA expression. Statistical significant differences (p < 0.05) are indicated by * (control vs. RA treatment) and # (5.5 mM glucose vs. 25 mM glucose).

Figure 5. Roles of adipogenic differentiation inducers in retinoic acid-dependent SREBP-1a expression in 3T3-L1 cells cultured for 1 day in normal and high glucose medium.

3T3-L1 cells were cultured to confluence and then cultured with DMEM containing 5.5mM glucose (A) or 25 mM glucose (B). They were also treated with a mixture of adipogenic differentiation cocktail (Mix) and each component (Ins, insulin 10 µg/ml; IBMX, isobutylmethylxanthine 0.5 mM; Dex, dexamethasone 1µM) for 24 h.
Expression of SREBP-1a was quantified by real time PCR, and normalized to the level of β-actin (act b) mRNA expression. Statistical significant differences ($p < 0.05$) are indicated by * (control vs. RA treatment) and # (control vs. a Ins/IBMX/Dex treatment).

Figure 6. Effect of nuclear receptor agonists and antagonists on dexamethasone-dependent SREBP-1a expression in 3T3-L1 cells cultured in high glucose medium.

3T3-L1 cells were cultured in DMEM containing 25 mM glucose either in the presence (+) or absence (−) of dexamethasone (Dex, 1 μM) for 24 h. In the top panel, the cells were also treated with DMSO as a vehicle control (C), retinoic acid (RA, 1 μM), TTNPB (RAR agonist, 1 μM) and 9-cis-RA (RXR agonist, 1 μM). In the second panel, the cells were treated with DMSO (C), HX531 (HX, RXR antagonist, 1 μM) and T0070907 (T007, PPARγ antagonist, 1 μM) in the presence of RA (1 μM). In the bottom panel, the cells were treated with DMSO (C), GW0742 (GW, PPARβ/δ agonist, 1 μM), Troglitazone (Trog, PPARγ agonist, 10 μM) and T0901317 (T090, LXR agonist, 1 μM) in the absence of RA. Expression of SREBP-1a were quantified by real time PCR, and normalized to the level of β-actin (act b) mRNA expression. Statistical significant differences ($p < 0.05$) are indicated by * (control vs. Dex treatment) and # (control vs. an agonist/antagonist treatment).

Figure 7. Roles of LXR and PPARβ/δ in retinoic acid- and dexamethasone-dependent SREBP-1a expression in 3T3-L1 cells cultured in high glucose medium.
3T3L1 cells were transfected with either siRNA for LXRα, LXRβ, LXRα plus LXRβ, PPARβ/δ or control (10 pmol each) as described in the legend of Fig. 2. The cells were cultured further in DMEM containing 25 mM glucose and retinoic acid (RA, 1 μM) either in the presence (+) or absence (-) of dexamethasone (Dex, 1 μM) for 24 h. Expression of SREBP-1a (A), LXRβ (B), PPARγ (C), RXRα (D) and RXRβ (E) were quantified by real time PCR, and normalized to the level of β-actin (act b) mRNA expression. Statistical significant differences (p < 0.05) are indicated by * (control vs. Dex treatment) and # (control siRNA vs. the other siRNA treatment).

Figure 8. Role of PPARγ in retinoic acid- and dexamethasone-dependent SREBP-1a expression in 3T3-L1 cells cultured in high glucose medium.
3T3L1 cells were transfected with either siRNA for PPARγ or control (10 pmol each) as described in the legend of Fig. 2. The cells were cultured further in DMEM containing 25 mM glucose and retinoic acid (RA, 1 μM) either in the presence (+) or absence (-) of dexamethasone (Dex, 1 μM) for 24 h. Expression of PPARγ (A), SREBP-1a (B), LXRβ (C), RXRα (D) and RXRβ (E) were quantified by real time PCR, and normalized to the level of β-actin (act b) mRNA expression. Statistical significant differences (p < 0.05) are indicated by * (control vs. Dex treatment) and # (control siRNA vs. PPARγ siRNA).

Figure 9. Effect of retinoic acid and dexamethasone on LXRβ, PPARγ, RXRα and RXRβ expression in 3T3-L1 cells cultured in high glucose medium.
3T3-L1 cells were cultured in DMEM containing 25 mM glucose in the presence (+) or absence (-) of dexamethasone (Dex, 1 μM) and retinoic acid (RA, 1 μM) for 24 h. Expression of LXRβ (A), PPARγ (B), RXRα (C) and RXRβ (D) were quantified by
real time PCR, and normalized to the level of β-actin (act b) mRNA expression. Statistical significant differences ($p < 0.05$) are indicated by * (control vs. RA treatment) and # (control vs. Dex treatment).

**Figure 10. Roles of LXR and PPAR in retinoic acid- and dexamethasone-dependent SREBP-1a expression in 3T3-L1 cells cultured in normal and high glucose medium.**

3T3L1 cells were transfected with either siRNA for LXRα, LXRβ, LXRα plus LXRβ, PPARβ/δ, PPARγ or control (10 pmol each) as described in the legend of Fig. 2. The cells were cultured further in DMEM containing 5.5 mM (N) or 25 mM (H) glucose in the presence of retinoic acid (RA, 1 μM) and dexamethasone (Dex, 1 μM) for 24 h. Expression of SREBP-1a were quantified by real time PCR, and normalized to the level of β-actin (act b) mRNA expression. Statistical significant differences ($p < 0.05$) are indicated by * (N vs. H glucose) and # (control siRNA vs. the other siRNA treatment).
Fig. 1

A. Lipid accumulation

B. aP2/act b mRNA

C. ACC1a/act b mRNA

D. FAS/act b mRNA

E. 3T3-L1

F. SREBP-1a/act b mRNA

G. SREBP-2/act b mRNA

RA Glucose 5.5mM 25mM
Fig. 2

A
SREBP-1a (m)
Act b
precursor nuclear
RA - + - + control siRNA

B
SREBP-1a /act b mRNA
RA - + - + control siRNA

C
Precursor protein
RA - + - + control siRNA

D
Nuclear protein
RA - + - + control siRNA

E
FAS /act b mRNA
RA - + - + control siRNA

F
Lipid accumulation
RA - + - + control siRNA
**Fig. 3**

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**Fig. 4**

A. SREBP-1α/act b mRNA

B. RARα/act b mRNA

C. RARγ/act b mRNA

D. RXRα/act b mRNA

E. RXRβ/act b mRNA

F. PPARγ/act b mRNA

G. PPARβ/δ/act b mRNA

H. LXRα/act b mRNA

I. LXRβ/act b mRNA
Fig. 5
Fig. 6

Graph showing the effect of various treatments on SREBP-1a/act on b mRNA expression under Glucose 25mM conditions. The treatments include Control, Dex, GW 0742 (-), Gw0742 (Dex), Trog (-), Trog (Dex), T0901317 RA (-), T0901317 (Dex), HX-531 (RA), HX-531 (RA & Dex), T0070907 (RA), T0070907 (RA & Dex).
Fig. 7

**Glucose 25mM+RA**

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Glucose 25mM+RA

A

B

C

D

E

Fig. 8
Fig. 9

A

LXRγ/act b mRNA

RA
- - + +
Dex
- + - +

B

PPARγ/act b mRNA

RA
- - + +
Dex
- + - +

C

RXRα/act b mRNA

RA
- - + +
Dex
- + - +

D

RXRβ/act b mRNA

RA
- - + +
Dex
- + - +

Glucose 25mM
Fig. 10

SREBP-1a/act b mRNA

+RA +Dex

Glucose

N H N H N H N H N H N H N H

C α β α+β β/δ γ

siRNA

LXR PPAR

0 1 2 3 4 5

SREBP-1a/act b mRNA

+RA +Dex

Glucose

N H N H N H N H N H N H

C α β α+β β/δ γ

siRNA

LXR PPAR