27-Hydroxycholesterol suppresses lipid accumulation by down-regulating lipogenic and adipogenic gene expression in 3T3-L1 cells

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Abstract Cholesterol oxidation products (oxysterols) are produced from cholesterol by automatic and/or enzymatic oxidation of the steroidal backbone and side-chain. Oxysterols are present in plasma and serum, suggesting that oxysterols are related to the development and progression of various diseases. However, limited information is available about the absolute amounts of oxysterols in organs and tissues, and the physiological significance of oxysterols in the body. In the present study, we quantified the levels of 13 oxysterols in white adipose tissue (WAT) of mice and then evaluated correlations between each oxysterol level and WAT weight. The sum of the levels of 13 oxysterols in WAT (white adipose tissue) was 15.9 ± 3.4 $\mu\text{g/g}$ of WAT weight and approximately 1 % of cholesterol level. Among oxysterols, the levels of 27-hydroxycholesterol (27-OH), an endogenous oxysterol produced by enzymatic oxidation, and the relative WAT weights were significantly negatively correlated. Next, we evaluated the effects of 27-OH on lipogenesis and adipogenesis in 3T3-L1 cells. TO901317 (TO), a potent and selective agonist

for LXR α , significantly increased intracellular TAG contents, while 27-OH significantly reduced the contents to half when compared with control (DMSO) and completely abolished the effect of TO. In addition, 27-OH significantly reduced the mRNA levels of lipogenic (LXR α and FAS) and adipogenic genes (PPAR γ and aP2) during adipocyte maturation of 3T3-L1 cells. In conclusion, our results indicate that 27-OH suppresses lipid accumulation by down-regulating lipogenic and adipogenic gene expression in 3T3-L1 cells.

Keywords 27-Hydroxycholesterol · LXR α · PPAR γ · Lipid accumulation · 3T3-L1 adipocytes

Introduction

Adipocytes are well recognized as endocrine cells that are controlled by energy intake and expenditure, and the endocrine function of adipocytes depends on adipocyte size. Large adipocytes [i.e. intracellular triacylglycerol (TAG) accumulation in adipocytes] have been shown to play crucial roles in the development of metabolic syndrome through secretion of fatty acids and adipocytokines such as tumor necrosis factor- α (Matsuzawa 2006). Thus, reduction or suppression of intracellular TAG accumulation in adipocytes is a target to prevent and/or alleviate obesity and metabolic syndrome.

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The nuclear receptor liver X receptor alpha (LXR α) is predominantly expressed in central organs of lipid metabolism such as the liver and adipose tissue (Willy et al. 1995). Growing evidence indicates that LXR α has important regulatory roles in adipocytes (Laurençikienė and Rydén 2012). Most nuclear receptors are activated by endogenous lipophilic ligands, and LXR α is activated by oxysterols (Janowski et al. 1996; Schroeffer 2000). Oxysterols are produced from cholesterol by automatic and/or enzymatic oxidation of the steroidal backbone and side-chain. Oxysterols are present in plasma and serum, suggesting that oxysterols are related to the development and progression of various diseases such as cardiovascular disease and Alzheimer's disease (Björkhem and Diczfalussy 2002; Sottero et al. 2009). However, limited information is available about the absolute amounts of oxysterols in adipose tissue and the physiological significance of oxysterols in adipose tissue and adipocytes.

In the present study, we quantified the levels of 13 oxysterols in white adipose tissue (WAT) of mice and then evaluated correlations between each oxysterol level and WAT weight. In addition, we evaluated the function of an oxysterol found a significant correlation on intracellular TAG accumulation in adipocytes.

Materials and methods

Materials

5-cholesten-3 β ,19-diol (19-hydroxycholesterol, 19-OH), 5-cholesten-3 β ,4 β -diol (4 β -hydroxycholesterol, 4 β -OH), 5-cholesten-3 β ,7 α -diol (7 α -hydroxycholesterol, 7 α -OH), 5-cholesten-3 β ,7 β -diol (7 β -hydroxycholesterol, 7 β -OH), 5-cholesten-3 β ,22(R)-diol [22(R)-hydroxycholesterol, 22(R)-OH], 5-cholesten-3 β ,24(S)-diol [22(S)-hydroxycholesterol, 22(S)-OH], 5-cholesten-3 β ,25-diol (25-hydroxycholesterol, 25-OH), 5 α ,25R-cholesten-3 β ,26-diol (27-hydroxycholesterol, 27-OH), 5 α -cholestan-3 β -ol-6-one (6-ketocholestanol, 6-keto), 5-cholesten-3 β -ol-7-one (7-ketocholesterol, 7-keto), cholestan-5 α ,6 α -epoxy-3 β -ol (5 α ,6 α -epoxycholesterol, 5,6 α -epoxy), cholestan-5 β ,6 β -epoxy-3 β -ol (5 β ,6 β -epoxycholesterol, 5,6 β -epoxy), cholestan-3 β ,5 α ,6 α -triol (α -cholestantriol, α -triol), and cholestan-3 β ,5 α ,6 β -triol (β -cholestantriol, β -triol)

were purchased from Steraloids Inc. (Newport, RI, USA). 5 α -Cholestane was purchased from SIGMA-ALDRICH Japan K.K. (Tokyo, Japan). TO901317 (TO) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco-BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Life Technologies (Carlsbad, CA, USA). Ascorbic acid, dexamethasone, and 3-isobutyl-1-methylxanthine were purchased from Nacalai Tesque (Kyoto, Japan). Penicillin and streptomycin were purchased from Meiji Seika Pharma Co., Ltd. (Tokyo, Japan). Dimethyl sulfoxide (DMSO) and insulin were purchased from Wako Pure Chemicals (Tokyo, Japan).

Animal experiments

Six-week-old male C57BL/6 J mice ($n = 8$) were obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The mice were housed individually in plastic cages in a temperature-controlled room (21–23 °C) under a 12-h light/dark cycle. Experimental diet was prepared according to the AIN-76 formula (American Institute of Nutrition 1977) with some modifications and contained (g/kg): 200 casein, 150 corn starch, 100 olive oil, 50 cellulose, 35 mineral mixture (AIN-76), 10 vitamin mixture (AIN-76), 3 DL-methionine, 2 choline bitartrate, 0.5 cholesterol, and sucrose to 1000 g. The mice were initially fed a commercial diet (NMF, Oriental Yeast Co., Tokyo, Japan) for 3 days and then fed the experimental diet for 9 weeks. All the mice were allowed free access to water. The body weight for each mouse was measured every week and consumption of the feed for each mouse was measured every day. At the end of the feeding period, the mice were subjected to 6-h fasting, anesthetized with Sonmopentyl[®] (Kyoritsu Seiyaku Corporation, Tokyo, Japan) and then sacrificed by aortic exsanguination. Perirenal WAT was immediately excised and weighed. The samples were kept at –30 °C until analysis.

This experiment was conducted according to the Guidelines for Animal Experiments of Kyushu University (Fukuoka, Japan) and the law (no. 105) and notification (no. 6) of the government of Japan. The animal protocol was approved by the review committee of Kyushu University (authorization no. A22-159-3).

Analysis of cholesterol and oxysterols in perirenal WAT of mice

Total lipids in perirenal WAT were extracted with chloroform:methanol (2:1, v/v) containing 0.01 % (w/v) butylated hydroxytoluene (BHT) (Nacalai Tesque, Kyoto, Japan). The levels of cholesterol in perirenal WAT were analyzed by gas chromatography (GC) using 5 α -cholestane as an internal standard, as described previously (Matsuoka et al. 2014). The levels of oxysterols in perirenal WAT were analyzed according to the previous report (Ando et al. 2002) with a few modifications. After total lipids were saponified overnight, unsaponified lipids were extracted with hexane. The extracted lipids were applied to a Sep-pak Vac silica cartridge (Nihon Waters, Tokyo) to separate the oxysterols from cholesterol. The cartridge equilibrated with hexane was sequentially eluted with a solvent mixture composed of hexane and 2-propanol (1:200, v/v), and one composed of hexane and 2-propanol (3:7, v/v), which allowed for sequential elution of cholesterol, and 19-OH plus oxysterols, respectively. After removal of the oxysterol fraction-containing solvent under N₂, the dried residues were converted to trimethylsilyl ethers for 30 min at room temperature. Determination of oxysterol was performed using GC–MS (Shimadzu GC-17A version 3 coupled with a SPB-1 fused silica capillary column, 60 m $\times$ 0.25 mm, 0.25 μ m thickness, Spelco Inc., Bellefonte, PA, USA) connected to a QP5050A series mass-selective detector (Shimadzu). The following temperature program was applied with helium (high-purity 99.9999 %) as a carrier gas at a flow rate of 1.5 ml/min: 180 °C for 1 min; from 180 to 250 °C at 20 °C/min; from 250 to 290 °C at 5 °C/min; 290 °C for 32.5 min. The total run time was 45 min. The injector was operated in the split ratio of 1:5 and was kept at 300 °C, and the detector transfer line was kept at 300 °C. The individual oxysterol concentrations were measured with 19-OH as an internal standard. The mass spectrometer was operated in the electron impact (EI) mode (70 eV). A quantitative analysis was performed by the internal standard method for mass spectrometry in the selected ion monitoring mode. The monitored ion during the chromatographic run was varied as a function of time

and the characteristic ion for each oxysterol was recorded. To quantify each oxysterol, calibration curves measuring the peak area of each oxysterol versus that of the internal standard were used. Peak identification was confirmed by the relative retention time and a mass spectral comparison with authentic standards.

Cell culture and treatment

3T3-L1 preadipocytes (JCRB9014) were maintained in the maintenance medium (DMEM supplemented with 10 % FBS, 200 μ M ascorbic acid, 100 units/ml penicillin, and 100 μ g/ml streptomycin) at 37 °C in 5 % CO₂. After 3T3-L1 preadipocytes reached confluence, the cells were incubated for an additional 48 h. Then, the cells were differentiated in the differentiation medium (the maintenance medium supplemented with 0.25 μ M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 10 μ g/ml insulin) for 48 h. The medium was then replaced with the maturation medium (the maintenance medium supplemented with 5 μ g/ml insulin), and changed to fresh medium every 2 days. 27-OH and TO were added to the maturation medium as DMSO solution. The final concentration of DMSO was adjusted to 1 % so as not to affect cell growth.

Measurement of intracellular triacylglycerol contents in 3T3-L1 adipocytes

Ten days after induction of differentiation, the cells were washed twice with ice-cold PBS buffer containing 0.02 % EDTA, harvested in ice-cold RIPA buffer, and lysed by sonication. Intracellular TAG contents in the lysates were measured using a commercial enzyme assay kit (Triglyceride E-Test from Wako Pure Chemicals). Protein contents in the lysates were measured as described previously (Lowry et al. 1951). Intracellular TAG contents were expressed as a relative value after normalization to protein contents.

Oil red O staining

Ten days after induction of differentiation, the cells were washed twice with PBS buffer, fixed with 10 %

formalin for 1 h, washed twice with distilled water, and then stained with Oil Red O dye (Lipid assay kit from Cosmo Bio Co., Ltd., Tokyo, Japan) for 15 min. Stained cells were washed three times with distilled water, and then photographed.

Analysis of mRNA expression

Total RNA from 3T3-L1 adipocytes was isolated using a guanidinium thiocyanate/cesium chloride ultracentrifugation method (Chirgwin et al. 1979) and was reverse transcribed with a Transcriptor First Strand cDNA Synthesis kit (Roche, Berlin, Germany) to obtain cDNA. Quantitative real-time RT-PCR analysis was performed with a SYBR Premix EX Taq II kit (Takara, Shiga, Japan) and a real-time PCR system (Mx3000P qPCR system; Agilent Technologies, Santa Clara, CA, USA). Primer sequences used were as follows: liver X receptor alpha (LXR α) (forward primer, 5'-CCGACAGAGCTTCGTCCACA-3'; reverse primer, 5'-CCCACAGACACTGCACAG-3'), peroxisome proliferator-activated receptor gamma (PPAR γ) (forward primer, 5'-GCCCTTTGGTGACTTTATGG-3'; reverse primer, 5'-CAGCAGGTTGTCTTGGATGT-3'), fatty acid synthase (FAS) (forward primer, 5'-TTGCTGGCACTACAG AATGC-3'; reverse primer, 5'-AACAGCCTCAG AGCGACAAT-3'), adipocyte fatty acid-binding protein (aP2) (forward primer, 5'-AAGACAGCTCCTCCTCG AAGGTT-3'; reverse primer, 5'-TGACCAAATCCCC ATTTACGC-3'), β -actin (forward primer, 5'-AGCCA TGTACGTAGCCATCC-3'; reverse primer, 5'-TCCCT CTCAGCTGTGGTGGTGAA-3'). Results were expressed as a relative value after normalization to β -actin expression.

Statistical analysis

All values are expressed as the mean $\pm$ standard error of mean (SEM). Linear regression analysis was used to assess the relation between oxysterol levels in WAT and relative WAT weights (Table 1). Comparisons among groups were performed by using one-way ANOVA followed by the Dunnett's multiple comparison post hoc test (Fig. 2) or Tukey–Kramer multiple comparison post hoc test (Figs. 3, 4). Differences were considered significant at $P < 0.05$. Statistical analysis was performed using Excel 2011 (Microsoft, USA) with the add-in software Statcel 3 (Yanai 2011).

Results and discussion

27-Hydroxycholesterol levels in WAT are negatively correlated with relative WAT weights

In the present study, after a 9 week feeding period, final body weights, perirenal WAT weights, relative perirenal WAT weights, and cholesterol levels in WAT of C57BL/6 J mice were 27.8 ± 0.6 g, 0.290 ± 0.009 g, 1.04 ± 0.02 g/100 g of body weight, and 1.42 ± 0.02 mg/g of WAT weight (mean $\pm$ SEM), respectively. Knowledge of the levels of oxysterols in tissues including adipose tissue is very limited. We thereby measured the levels of 13 oxysterols in WAT of mice. As shown in Table 1, oxysterol levels (μ g/g tissue) were as follows (in order of decreasing mean values): 5,6 α -epoxy (2.27), 7-keto (2.22), 5,6 β -epoxy (2.15), β -triol (1.93), 4 β -OH (1.48), 6-keto (1.24), 27-OH (1.15), 7 α -OH (1.14), 7 β -OH (0.909), 25-OH (0.513), α -triol (0.419), 24(S)-OH (0.403), 22(R)-OH (0.100). Total oxysterol level in WAT was 15.9 ± 3.4 μ g/g of WAT weight and approximately 1 % of the cholesterol level. To understand physiological significance of oxysterols in WAT, we assessed correlations between each oxysterol level and relative WAT weight (Table 1). The levels of 27-OH, an endogenous oxysterol produced from cholesterol by the mitochondrial enzyme sterol 27-hydroxylase (CYP27A1) (Fig. 1), and the relative WAT weights were significantly negatively correlated ($r = -0.727$, $P < 0.05$), suggesting that 27-OH levels in WAT regulates adipose tissue mass and intracellular TAG contents. On the other hand, cholesterol levels in WAT did not correlate with the relative WAT weights (data not shown).

27-Hydroxycholesterol suppresses intracellular TAG accumulation during adipocyte maturation of 3T3-L1 cells

CYP27A1 is widely expressed throughout different tissues with highest levels not only in liver and muscle but also in kidney, intestine, lung, and so on (Bikle 2001). Although 27-OH is generated by CYP27A1, limited information is available about the physiological significance of 27-OH in the body. Based on the results of Table 1, we evaluated the effect of 27-OH on lipid accumulation in 3T3-L1 cells. The results

Table 1 Oxysterol levels in white adipose tissue of mice and correlation coefficients between each oxysterol level and white adipose tissue weight

	Oxysterol levels	Correlation coefficient	
	(μg/g tissue)	<i>r</i>	<i>P</i> value
Autoxidation			
7β-OH	0.909 ± 0.404	0.560	N.S.
5,6β-epoxy	2.15 ± 0.22	−0.405	N.S.
5,6α-epoxy	2.27 ± 0.26	0.218	N.S.
β-triol	1.93 ± 0.63	0.578	N.S.
6-keto	1.24 ± 0.53	0.612	N.S.
α-triol	0.419 ± 0.217	0.536	N.S.
7-keto	2.22 ± 0.29	0.466	N.S.
Auto- and/or Enzymatic-oxidation			
7α-OH	1.14 ± 0.58	0.541	N.S.
25-OH	0.513 ± 0.262	0.541	N.S.
Enzymatic oxidation			
4β-OH	1.48 ± 0.41	0.392	N.S.
22(R)-OH	0.100 ± 0.049	0.430	N.S.
24(S)-OH	0.403 ± 0.120	0.454	N.S.
27-OH	1.15 ± 0.07	−0.727	0.041
Total oxysterols	15.9 ± 3.4	0.525	N.S.

The data are expressed as the mean $\pm$ SEM ($n = 8$)

4 β -OH, 4 β -hydroxycholesterol; 7 α -OH, 7 α -hydroxycholesterol; 7 β -OH, 7 β -hydroxycholesterol; 22(R)-OH, 22(R)-hydroxycholesterol; 22(S)-OH, 22(S)-hydroxycholesterol; 25-OH, 25-hydroxycholesterol; 27-OH, 27-hydroxycholesterol; 6-keto, 6-ketocholesterol; 7-keto, 7-ketocholesterol; 5,6 α -epoxy, 5 α ,6 α -epoxycholesterol; 5,6 β -epoxy, 5 β ,6 β -epoxycholesterol; α -triol, α -cholestantriol; β -triol, β -cholestantriol; N.S. not significant

Fig. 1 The chemical structures of cholesterol and 27-hydroxycholesterol

showed that 27-OH dose-dependently suppressed intracellular TAG accumulation during adipocyte maturation of the cells (Fig. 2). Endogenous oxysterols act as ligands for LXR α (Janowski et al. 1996; Schroepfer 2000). LXR α is highly expressed in the liver and adipose tissue (Willy et al. 1995) and regulates expression of lipogenic genes (Kim and Spiegelman 1996; Joseph et al. 2002). Thus, we suggest that the suppression of TAG accumulation in WAT by 27-OH may contribute to its antagonistic action on LXR α .

Intracellular TAG accumulation by the induction of TO901317, a potent and selective agonist for LXR α , is cancelled by the addition of 27-hydroxycholesterol

It is well recognized that TO is a potent and selective agonist for LXR α (Schultz et al. 2000). LXR α activation accelerates lipid accumulation in adipocytes (Juvet et al. 2003). To examine further the effect of 27-OH on lipid accumulation in 3T3-L1 cells, we investigated whether 27-OH suppresses intracellular TAG accumulation induced by TO treatment. TO treatment significantly increased intracellular TAG contents by twofold, while 27-OH treatment significantly reduced the contents to half when compared

with control and completely abolished the effect of TO (Fig. 3).

27-Hydroxycholesterol suppresses lipogenesis and adipogenesis in 3T3-L1 cells

To gain insight into the effect of 27-OH on lipogenesis and adipogenesis during adipocyte maturation of 3T3-L1 cells, we analyzed the expression levels of mRNA related to them. TO significantly increased LXR α mRNA levels, whereas 27-OH significantly reduced the mRNA levels when compared with control and completely abolished the effect of TO (Fig. 4A). LXR α also directly regulates FAS expression through interaction with its promoter (Joseph et al. 2002). Consistent with the observed dynamics of LXR α mRNA, 27-OH markedly reduced FAS mRNA levels and completely abolished the effect of TO (Fig. 4C).

Several stimuli that interfere with adipogenesis, such as trans10, cis12-conjugated linoleic acid (Granlund et al. 2005) and ultraviolet light A (Lee et al.

Fig. 2 27-OH suppresses intracellular TAG accumulation during adipocyte maturation of 3T3-L1 cells. The cells were treated with DMSO (the vehicle control), 0.5, 1, 7, and 10 μ M 27-OH for 10 days after induction of differentiation. TAG level of the vehicle control was arbitrarily set at 1. Data are presented as the mean $\pm$ SEM ($n = 5$). Columns marked with asterisk(s) are significantly different from the control (** $P < 0.01$). DMSO, dimethyl sulfoxide; 27-OH, 27-hydroxycholesterol; TAG, triacylglycerol

Fig. 3 Intracellular TAG accumulation by the induction of TO is cancelled by the addition of 27-OH. The cells were treated with DMSO (the vehicle control), 1 μ M TO, 7 μ M 27-OH, and 1 μ M TO + 7 μ M 27-OH for 10 days after induction of differentiation. The cells were stained with Oil Red O dye. Intracellular TAG contents were measured. TAG level of the vehicle control was arbitrarily set at 1. Data are presented as the mean $\pm$ SEM ($n = 5$). Columns marked with different letters are significantly different at $P < 0.05$. DMSO, dimethyl sulfoxide; 27-OH, 27-hydroxycholesterol; TAG, triacylglycerol; TO, TO901317

Fig. 4 27-OH suppresses lipogenesis and adipogenesis of 3T3-L1 cells. The cells were treated with DMSO (the vehicle control), 1 μ M TO, 7 μ M 27-OH, and 1 μ M TO + 7 μ M 27-OH for 10 days after induction of differentiation. Each gene expression level (LXR α , PPAR γ , FAS, and aP2) was normalized to the β -actin level. Each gene expression of the vehicle control was arbitrarily set at 1. Data are presented as the

mean $\pm$ SEM ($n = 5$). Columns marked with *different letters* are significantly different at $P < 0.05$. aP2, adipocyte fatty acid-binding protein; DMSO, dimethyl sulfoxide; FAS, fatty acid synthase; LXR α , liver X receptor alpha; 27-OH, 27-hydroxy-cholesterol; PPAR γ , peroxisome proliferator-activated receptor gamma; TAG, triacylglycerol; TO, TO901317

2010) reduce not only the expression of LXR α but also simultaneously downregulate the expression of PPAR γ , a master regulator of adipogenesis. As shown in Fig. 4B, PPAR γ mRNA levels were also significantly reduced by 27-OH treatment, the results that is in agreement with previous studies. In addition, 27-OH significantly reduced the mRNA levels of aP2, a PPAR γ target gene and also called fatty acid binding protein 4 (FABP4) (Fig. 4D). PPAR γ and CCAAT/enhancer-binding protein alpha (C/EBP α) cooperatively regulate adipogenesis (Lefterova et al. 2008). Recently, it has been reported that 27-OH treatment exhibited anti-adipogenic activity through the down-regulation of C/EBP α and FABP4 mRNA

levels in 3T3-L1 cells (Li et al. 2014). Our results provide supportive evidence for the previous study (Li et al. 2014). Further studies are necessary to clarify the mechanisms of down-regulating gene expression related to lipogenesis and adipogenesis by 27-OH.

In conclusion, the present study indicates that 27-hydroxycholesterol suppresses intracellular TAG accumulation by down-regulating lipogenic and adipogenic gene expression during adipocyte maturation of 3T3-L1 cells. Whether increased circulating level of 27-OH and/or increased synthesis of 27-OH in WAT reduce lipid accumulation in WAT and then prevent obesity and metabolic syndrome, would be of great interest for future study.

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Authors' contributions B.S. wrote the manuscript. B.S., K.K., Y.H., Y.N., Y.F. and L.T.T. participated in the experimental work and collected and analyzed data. B.S. and M.S. contributed to the study design, supervised the study, and commented on the manuscript. All authors have read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest None.

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