Peroxisome Proliferator-Activated Receptor γ (PPARγ)-Independent Specific Cytotoxicity against Immature Adipocytes Induced by PPARγ Antagonist T0070907

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Peroxisome proliferator-activated receptor γ (PPARγ) plays indispensable roles in adipogenesis, which is frequently impaired under pathological conditions such as non-alcoholic steatohepatitis (NASH). Thus, a potent PPARγ antagonist, T0070907 is known as a useful tool for understanding such pathological conditions, while T007097 was also suggested to have PPARγ-independent actions. In the present study, we found that T0070907 inhibited adipogenesis concomitantly with the induction of rapid apoptosis of immature adipocytes within 2 h, whereas another PPARγ antagonist, SR-202 did not show such cytotoxicity. However, T0070907 did not affect the viabilities of pre-adipocytes, mature adipocytes, and NIH-3T3 fibroblasts. The cytotoxic effect of T0070907 was not inhibited by GW1929, a PPARγ agonist, but was inhibited by α-tocopherol, which was previously shown to provide clinical benefit to NASH patients. Interestingly, treatment with high amounts of α-tocopherol alone slightly increased the cellular lipid content in mature adipocytes, but did not affect PPARγ-dependent luciferase reporter expression in COS-7 cells. Moreover, other lipophilic antioxidants, such as tocotrienols, tert-butyldihydroquinone, and butylated hydroxyanisole, also inhibited T0070907-induced apoptosis like α-tocopherol. Consequently, it is suggested that T0070907 efficiently inhibits adipogenesis not only via PPARγ-dependent manner, but also through the induction of apoptosis specifically against immature adipocytes via oxidative stress in a PPARγ-independent manner.

Key words T0070907; immature adipocyte; oxidative stress; peroxisome proliferator-activated receptor γ

Adipogenesis has undergone extensive investigation because of the increasing number of patients with metabolic disorders, including metabolic syndrome and non-alcoholic steatohepatitis (NASH) that are causing serious problems worldwide. Peroxisome proliferator-activated receptor γ (PPARγ) is widely recognized as a critical transcription factor for adipogenesis. Thus, specific modulators of PPARγ have been used in attempts to elucidate the physiological roles of PPARγ in metabolic disorder, and indeed, have contributed to the understanding of the causes and consequences of abnormal adipogenesis, which are frequently observed in patients with metabolic disorders. From this viewpoint, the modulators with greater specificity would provide more precise insights into the biological roles and functional mechanisms of proteins.

T0070907 (IUPAC name: 2-chloro-5-nitro-N-pyridin-4-yl-benzamide) is known as a potent and specific PPARγ antagonist, because it covalently and irreversibly binds to PPARγ.5) Thus, researchers have taken advantage of the properties of T0070907 to investigate the effect of PPARγ inhibition on adipogenesis.5) However, T0070907 also exhibits PPARγ-independent anti-cancer effects including apoptosis induction.2–4) Thus, the PPARγ-independent action of T0070907 might also contribute, at least in part, to its anti-adipogenic effect.

In the present study, we investigated the effect of T0070907 on adipogenesis in 3T3-L1 adipocytes during a maturation stage, and the PPARγ dependency of its effect on adipogenesis. In addition, the mechanism of T0070907-induced PPARγ-independent action was also investigated. Based on these results, we discussed the mechanism regarding anti-adipogenic effect of T0070907.

MATERIALS AND METHODS

Materials The reagents used in the study were purchased as follows: 3,5,3′,4′-tetraiodo-L-thyronine (T3), L-thyroxine (T4), 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX), insulin, α-tocopherol, α-tocotrienol, butylhydroxyanisole, astaxanthin, Hoechst33342, GW1929, T007097, SR-202 and Oil Red O from Sigma Aldrich (St. Louis, MO, U.S.A.), tert-butyldihydroquinone and 10% formalin solution from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), γ-tocotrienol from Santa Cruz Biotechnologies (Santa Cruz, CA, U.S.A.), Dulbecco’s modified Eagle’s medium (DMEM) from Nissui Pharmaceutical (Tokyo, Japan), d-biotin from Nacalai Tesque (Kyoto, Japan), Dulc-Luciferase Reporter Assay System from Promega (Osaka, Japan), Fugene HD from Roche Diagnostics Japan (Tokyo, Japan), 3T3-L1, NIH-3T3 and COS-7 cell lines were obtained from the Japan Health Sciences Foundation (Tokyo, Japan). Antibodies for PPARγ (cat. #2443), and β-actin (cat. #4967) were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.).

Cell Culture and Adipocyte Differentiation of 3T3-L1 Cells 3T3-L1 pre-adipocytes were cultured and induced adipocyte differentiation as shown in Fig. 1A and previously described.5) Briefly, cells were cultured in a basal medium (DMEM supplemented with 10% fetal bovine serum (FBS), 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 0.2% NaHCO₃, 4 mM l-glutamine, 3.5% glucose,
0.2 mM ascorbate, 1 mM T3 and 30 µM T4) at 37°C under a humidified atmosphere with 5% CO₂. In this study, the day when the cells reached confluence was defined as day 0. Adipocyte differentiation was induced by a differentiation medium (the basal medium supplemented with 500 µM IBMX, 1 mM DEX and 1.6 µM insulin) from day 0 to day 2. Hereafter, adipocytes at day 2 were referred to as “immature adipocytes.” Following differentiation, maturation was induced by a maturation medium (the basal medium containing 1.6 µM insulin and 15 µM d-biotin). Following a maturation period during days 2 to 4, the adipocytes at day 5 or later were referred to as “mature adipocytes.” Treatment with α-tocopherol, GW1929 and T0070907 was started from day 2 in the maturation medium with every other day medium changes.

**Oil Red O Staining** Oil Red O staining was performed after incubation for four days (at day 6). Following fixation of mature adipocytes with 10% formalin solution, the cells were incubated with 1.8 mg/mL Oil Red O in 60% isopropanol solution for 10 min at room temperature. Intracellular lipid droplets were visualized by washing the cells with 60% isopropanol solution. Then, Oil Red O dissolved in the lipid droplets was extracted by 100% isopropanol, and its relative concentration was determined by absorbance at 540 nm.

**Detection of Apoptotic Cells** Immature adipocytes at day 2 treated with T0070907 in the maturation medium were simultaneously incubated with Hoechst33342 for 1 h, and then observed by fluorescence microscopy.

**Luciferase Assay** Plasmid DNAs expressing PPARγ2, PPARγ ligand-binding domain (PPARγ LBD) fused with GAL4 DNA-binding domain (GAL4 DBD), firefly luciferase
gene containing PPAR response element (PPRE) and the GAL4-binding sites were kindly provided by Drs. Morikawa and Waku, Osaka University. These plasmid DNAs were co-transfected with renilla luciferase-expressing phRL-TK vector (Promega) into COS-7 cells by Fugene HD, according to the manufacturer's instruction. At 24 h post-transfection, cells were treated with α-tocopherol, GW1929 and T0070907 for further 24 h. Dual luciferase assay was performed according to the manufacturer's instruction. Relative transcriptional activity was calculated by dividing firefly luciferase activity by that of renilla, and was normalized by control values.

RESULTS AND DISCUSSION

The Effects of T0070907 on Adipogenesis of Immature Adipocytes PPARγ protein is present in two isoform, PPARγ1 and PPARγ2, due to different promoter usage and alternative splicing, which add additional amino acids (30 amino acids in mice) to PPARγ2 in its amino terminus.7,8) PPARγ2 is specifically expressed in adipose tissue, in contrast to PPARγ1 with wide tissue distribution. However, despite the structural difference and characteristic expression profile, both isoforms possess the same property to induce adipocyte differentiation.9) Under our experimental conditions, immature 3T3-L1 adipocytes showed a remarkable increase in PPARγ1 expression at day 2, compared to the cells incubated in the basal medium for two days, while PPARγ2 was not observed in immature adipocytes, but was upregulated in mature adipocytes (Fig. 1B). It was demonstrated that PPARγ bound to the PPARγ2 promoter region at 36 h (day 1.5) after induction of adipocyte differentiation, and induced PPARγ2 gene expression.10) Interestingly, in the study, the treatment with T0070907 during the differentiation suppressed the induction of PPARγ2 mRNA expression, while PPARγ1 mRNA was unchanged,10) suggesting that the induction of PPARγ2 by PPARγ has a critical role in adipocyte differentiation.

T0070907 exerts potent inverse agonist activity by irreversibly forming a covalent bond with PPARγ.11) On the basis of these observations, we treated immature 3T3-L1 adipocytes with T0070907. As expected, lipid storage in mature adipocytes at day 6 was significantly decreased by the presence of T0070907 (Fig. 1C).

In addition to PPARγ agonists, efforts have been made to develop novel medications with improved efficacy for the treatment of metabolic disorders. Among them, vitamin E was shown to have clinical efficacy for NASH in the Pioglitazone vs. Vitamin E vs. Placebo for Treatment of Non-diabetic Patients with Nonalcoholic Steatohepatitis (PIVENS) trial,11) in which a greater improvement of liver histology in NASH patients was found in response to vitamin E than to a PPARγ agonist, pioglitazone. Vitamin E is a naturally abundant lipophilic antioxidant and is composed of four tocopherols and four tocotrienols. In particular, the main vitamin E isoform, α-tocopherol, which was used in the PIVENS trial,11) was also found to induce the expression and activation of PPARγ in 3T3-L1 adipocytes.12,13) These observations suggest that α-tocopherol may affect adipogenesis through PPARγ in NASH patients. Thus, the effect of α-tocopherol on T0070907-induced decrease in lipid content of immature adipocytes was examined. The T0070907-induced inhibition of adipogenesis was almost completely blocked by α-tocopherol, although the amount of α-tocopherol was higher than usual in vitro experimental condition (Fig. 1C). Moreover, although the sizes of cellular lipid droplets decreased in cells treated with T0070907 and α-tocopherol compared to those in untreated cells, the numbers remained almost the same (Fig. 1D).

To address whether this result was due to the induction of adipogenesis by α-tocopherol through PPARγ activation, we treated immature adipocytes with α-tocopherol alone. However, unexpectedly, α-tocopherol resulted in only a slight increase in intracellular lipid storage in mature adipocytes (Fig. 1E). The amount of lipid in the cells was saturated when treated with 800 µM α-tocopherol. This increase in lipid storage was abolished when mature adipocytes were treated with 800 µM of α-tocopherol from days 5 to 9 (data not shown). Thus, it was suggested that the increased lipid storage was unlikely to be a consequence of the intracellular accumulation of α-tocopherol, and that the concentration of α-tocopherol tested in the present study was adequate. These results indicate that α-tocopherol positively regulates adipogenesis in the presence of T0070907. However, α-tocopherol alone showed only a minimal effect on the adipogenesis of immature adipocytes, compared to that expected from its inhibitory activity against T0070907 (Figs. 1C, D). Thus, α-tocopherol may also involve a PPARγ-independent mechanism in the regulation of adipogenesis during the maturation phase, at least in our experimental conditions, whereas significant activation of PPARγ by α-tocopherol was previously reported in mature adipocytes.1,15)

The Cytotoxicity of T0070907 against Immature Adipocytes As shown in Fig. 2A, we observed that there were a number of spots where mature adipocytes became detached within 2 h after incubation with T0070907. In order to evaluate the effect of T0070907 treatment against viabil-

Fig. 2. T0070907 Induced Apoptosis in Early-Differentiated Immature 3T3-L1 Adipocytes

(A) Representative microscopic images of differentiated 3T3-L1 adipocytes on day 2 after treatment for 2 h with 0 (non-treatment) or 10 μM T0070907 alone, or in combination with 800 µM α-tocopherol, or 10 nM GW1929. (B) Representative microscopic images of Hoechst33342 staining of differentiated 3T3-L1 adipocytes on day 2 after treatment for 1 h with 0 (non-treatment) or 10 µM T0070907.
ity status, the cells were stained with nuclear stain reagent Hoechst33342. Hoechst33342 staining revealed that cells treated with T0070907 exhibited condensed nuclei, which are a characteristic hallmark of apoptosis (Fig. 2B). However, we failed to quantitatively evaluate the apoptosis induction, due to the insufficient number of apoptotic cells. Because, besides the inhibitory activity against PPARγ, it was reported that T0070907 also possessed potent anti-tumor activity,2-4 it was suggested that T0070907 treatment induced apoptosis in the immature 3T3-L1 adipocytes. However, the spots were refilled with survived 3T3-L1 cells even in the presence of T0070907. These cells reached confluence again, but without lipid droplet formation (Fig. 1D). Although the nature of the survived cells is currently unknown, the cytotoxic effect of T0070907 was not observed in mature lipid-laden adipocytes (Fig. 3A), in which both PPARγ1 and PPARγ2 were expressed (Fig. 1B). Moreover, confluent 3T3-L1 pre-adipocytes in the basal medium, but not in the differentiation medium, in which PPARγ1 was predominantly expressed (Fig. 1B), remained undamaged by T0070907 (Fig. 3B). These results suggest that the cytotoxic effect of T0070907 is specific to immature adipocytes. Moreover, this cytotoxic effect of T0070907 in immature adipocytes was prevented by the addition of α-tocopherol, but not by GW1929 (Fig. 2A), implying an independent action of T0070907 from PPARγ. This result indicates that the blockade of cytotoxicity by α-tocopherol is possibly related to the amelioration of T0070907-induced adipogenesis inhibition.

The Transcriptional Function of PPARγ Is Not Affected by α-Tocopherol On the basis of the aforementioned observations, we investigated the effect of α-tocopherol on the transcriptional activity of PPARγ. Luciferase reporter assays were performed to address this issue. First, a luciferase reporter plasmid containing GAL4-binding sites was co-transfected into COS-7 cells with the expression vector PPARγ LBD fused with GAL4 DBD.6 Although α-tocopherol slightly activated PPARγ LBD-mediated transcriptional activation, the effect was not observed in the presence of GW1929, which induced a greater level of activation than that observed with α-tocopherol (Fig. 4A). T0070907 showed inverse agonist activity, and completely suppressed the transcriptional activation by GW1929 and by α-tocopherol (Fig. 4A). This result was consistent with a previous study demonstrating that α-tocopherol did not affect the function of PPARγ LBD in HeLa cells.12 Thus, PPARγ LBD is unlikely to be involved in the mechanism by which α-tocopherol restored the anti-adipogenic effect of T0070907.

Next, we investigated the effect of α-tocopherol on the transcriptional activity of full-length PPARγ2, which is consist

Fig. 3. T0070907 Induces Cytotoxicity in an Immature Adipocyte-Specific Manner

(A) Representative microscopic images of terminally-differentiated mature 3T3-L1 adipocytes treated with the indicated concentrations of T0070907 (T), α-tocopherol, or GW1929 for 24h. (B) Representative microscopic images of confluent 3T3-L1 pre-adipocytes treated with the indicated concentrations of T0070907 (T), α-tocopherol, or GW1929 in the basal medium for 24h.

Fig. 4. α-Tocopherol Did Not Influence PPARγ Transcriptional Activity

(A, B) The expression vectors of the PPARγ ligand-binding domain fused with GAL4 DNA-binding domain (A) or full-length PPARγ2 (B) were co-transfected into COS-7 cells with firefly luciferase reporter plasmid by Fugene HD. At 24h post-transfection, cells were treated with 800 µM α-tocopherol (αT), and/or 10 nM GW1929 (GW) in the presence of 0 (blank columns) or 10 (filled columns) µM T0070907 for a further 24h. The data were normalized to the non-treatment control. Data are shown as means±S.D. NS, not significant; *, p<0.05; **, p<0.01; ***, p<0.001 (Student t-test).
of the same amino acid sequence as PPARγ, except for additional amino acids at the amino terminus. Post-translational modifications are a fundamental mechanism of regulating the diverse biological functions of proteins. The transcriptional activity of PPARγ is regulated by phosphorylation and ubiquitination at Ser112 and small ubiquitin-like modifier–ylation at Lys107. These modifications occur outside of PPARγ LBD (317–505 amino acids), indicating that α-tocopherol may also activate PPARγ by modifying other PPARγ domains. Thus, another luciferase reporter plasmid containing PPRE was co-transfected with the full-length PPARγ2 expression vector. However, as observed by PPARγ LBD, α-tocopherol did not reverse the inverse agonist activity of T0070907 (Fig. 4B). Moreover, α-tocopherol neither directly activated PPARγ2 nor enhanced GW1929-induced PPARγ2 activation (Fig. 4B). Landrier et al. demonstrated in COS-1 cells that α-tocopherol enhanced the full-length PPARγ-dependent activation of the adiponectin promoter, which contains the functional PPRE. They also found that α-tocopherol enhanced the production of an endogenous PPARγ ligand, 15-deoxy-D12,14-prostaglandin J2 (PGJ2) in 3T3-L1 cells. Although the mechanism of action remains unclear, the production of PGJ2 in COS-7 cells might be absent or low. Moreover, no significant difference was observed in the expression levels of PPARγ mRNA in immature 3T3-L1 adipocytes treated with T0070907 in the presence or absence of α-tocopherol for two days (data not shown). Our results suggest that α-tocopherol does not directly activate the transcriptional activity of PPARγ as a ligand, and imply that a PPARγ-independent mechanism might be, at least in part, involved in T0070907-induced apoptosis in immature adipocytes.

**T0070907 Induces Cytotoxicity in an Adipocyte-Specific and PPARγ-Independent Manner** To further explore the cell specificity of T0070907, we treated NIH-3T3 mouse fibroblasts with T0070907. The cells were viable after treatment with 10–100 µM T0070907 for 2h and with 10–100 µM for 24h (Fig. 5A). Although severely damaged cells appeared after 24h in the presence of 100 µM T0070907, NIH-3T3 cells remained viable even at 80 µM and adhered to the culture dish after 48h (Fig. 5A, data not shown). NIH-3T3 cells undergo adipogenesis by the ectopic expression of PPARγ and appropriate ligand stimulation. Moreover, we observed the apparent expression of PPARγ in immature adipocytes both in the present and previous studies (Fig. 1B). Thus, the PPARγ2-expressing

![Fig. 5. Adipocyte-Specific and PPARγ-Independent Cytotoxicity of T0070907](image)
vector was transfected into NIH-3T3 cells. However, the transfected cells were also refractory to 10 \( \mu M \) T0070907 (Fig. 5B). We further examined the effect of SR-202, IUPAC name: [(4-chlorophenyl)-dimethoxypyrophosphorylmethyl]dimethyl phosphate, another chemically-unrelated PPAR\(\gamma\)-specific antagonist, on immature adipocytes. However, in contrast to T0070907, SR-202 did not induce cell death (Fig. 5C). These results suggest that the T0070907-induced cytotoxicity involves a 3T3-L1 adipocyte-specific and, at least in part, PPAR\(\gamma\)-independent mechanism. Further studies are required to identify the signaling pathway involved in the mechanism.

**T0070907 Increases Oxidative Stress in Immature Adipocytes**

Because \( \alpha \)-tocopherol is a potent lipophilic antioxidant, we addressed whether its antioxidant activity plays a role in the amelioration of T0070907-induced cytotoxicity. In addition to \( \alpha \)-tocopherol, various lipophilic antioxidants including \( \alpha \)-tocotrienol, \( \gamma \)-tocotrienol, tert-butylhydroquinone, and butylhydroxyanisole completely reversed the T0070907-induced cytotoxicity (Fig. 6). Astaxanthin is also a potent antioxidant, but did not exhibit the same effect. A clinical trial demonstrated that astaxanthin reduces serum triglyceride levels and increases serum adiponectin levels. Thus, our observation implies that astaxanthin has another mechanism of action involved in the regulation of adipogenesis, in addition to antioxidant activity. Another possible explanation is that this result may be caused by insufficient solubility, because astaxanthin is a highly lipophilic compound. In this case, solubility could be improved by pharmaceutical intervention, such as liposome formation.

In the present study, we found that T0070907 could have a PPAR\(\gamma\)-independent action in the inhibition of adipogenesis. Although direct evidence was not obtained in immature adipocytes, the following findings, in particular, would strongly support the notion: (1) \( \alpha \)-Tocopherol did not affect the suppressive action of T0070907 on the transcriptional activity of PPAR\(\gamma\) in COS-7 cells (Fig. 4), in marked contrast to the observation in immature 3T3-L1 adipocytes; and (2) T0070907 did not exert any effects even in mature adipocytes that express both PPAR\(\gamma1\) and PPAR\(\gamma2\) (Figs. 1B, 3A), and PPAR\(\gamma\)-overexpressing NIH-3T3 cells (Fig. 5B).

The present findings strongly suggest that researchers should carefully interpret the results obtained by using T0070907 in 3T3-L1 adipocytes. Although the precise mechanisms by which T0070907 increases oxidative stress and induces apoptosis remain to be clarified, \( \alpha \)-tocopherol plays a role as an antioxidant in the suppression of T0070907-induced anti-adipogenic effects. The present study also provided molecular insights into \( \alpha \)-tocopherol-mediated clinical efficacy against metabolic syndrome and NASH. It is of note that NIH-3T3 fibroblasts were damaged by T0070907 at a much higher concentration than 3T3-L1 adipocytes. This observation suggests that adipocytes were more sensitive to oxidative stress. Insulin resistance is a fundamental pathology of NASH, diabetes mellitus, and metabolic syndrome, and is caused in part by oxidative stress. It was reported that obesity and a high-fat diet aggravate oxidative stress in adipose tissue, and impair the insulin sensitivity of adipocytes. Thus, medication with the potential to protect adipocytes from oxidative stress may be important and hold promise in the treatment of insulin resistance and its-related diseases.

In conclusion, from the results in this study, it is suggested that T0070907 efficiently inhibits adipogenesis, not only via PPAR\(\gamma\)-dependent manner, but also through the induction of apoptosis specifically against immature adipocytes via oxidative stress in a PPAR\(\gamma\)-independent manner. T0070907 has contributed to the understanding of the causes and consequences of abnormal adipogenesis, on one hand. However, on the other hand, our study demonstrated that T0070907 is also a useful tool to investigate oxidative stress-related pathologies in adipose tissue.

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