PPARγ ligands, troglitazone and pioglitazone, up-regulate expression of HMG-CoA synthase and HMG-CoA reductase gene in THP-1 macrophages

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Abstract Recently it has been reported that macrophages express a nuclear receptor, peroxisome proliferator-activated receptor γ (PPARγ). Using a ligand of PPARγ, troglitazone or pioglitazone, we have shown that the expression of two genes involved in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase and HMG-CoA reductase, were increased by activation of PPARγ through a PPAR response element (PPRE) in THP-1 macrophages. In addition, treatment with troglitazone significantly increased the activity of HMG-CoA reductase and the amount of intracellular cholesterol. Thus, we conclude that PPARγ and its agonists increase the cholesterol content of macrophages by the increased expression of genes involved in cholesterol biosynthesis. These findings suggest that PPARγ may play a role in cholesterol metabolism in macrophages. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Peroxisome proliferator-activated receptor γ; Troglitazone; 3-Hydroxy-3-methylglutaryl-CoA synthase; 3-Hydroxy-3-methylglutaryl-CoA reductase; THP-1 cell; Macrophage; Pioglitazone

1. Introduction

Macrophages synthesize various proteins involved in lipid metabolism, such as lipoprotein lipase (LPL) [1] and apolipoprotein E [2]. These findings are observed not only in primary cultured macrophages, but also in macrophages derived from cloned cell-lines such as THP-1 cells [3,4]. However, the mechanism underlying their expression and the physiological role of the gene products have not been fully understood.

Peroxisome proliferator-activated receptors (PPARs) are transcription factors that function as important regulators of cell differentiation and energy homeostasis and act via a PPAR responsive element (PPRE) in the promoter region of target genes. There are several subtypes of PPAR and the subtypes are expressed in a tissue-specific manner. PPARγ is known to be expressed primarily in adipose cells and to be an adipogenic factor that regulates the expression of genes associated with lipid metabolism, such as fatty acid binding protein and LPL [5,6].

It was reported that primary macrophages and THP-1-derived macrophages express PPARγ [7]. Though the role of PPARγ in macrophages remains unclear, effects of PPARγ on the regulation of gene expression in macrophages were reported [8]. Specifically, it was shown that the activation of PPARγ by natural and synthetic agonists induced the expression of genes involved in lipid metabolism [9,10], such as the macrophage-specific receptor CD36, which has been proposed to function as a scavenger receptor for modified low density lipoprotein (LDL). Furthermore, PPARγ was recently reported to be involved in cholesterol efflux in macrophages [11]. These findings suggest a role for PPARγ as a modulator of lipid metabolism in macrophages.

In the current study, using troglitazone or pioglitazone, a synthetic agonist of PPARγ [12], we investigated the expression of two genes involved in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase and HMG-CoA reductase, to assess the effect of PPARγ on cholesterol biosynthesis in THP-1 monocytic cells. We further examined the effect of troglitazone on the enzyme activity and the cholesterol content in THP-1 macrophages.

2. Materials and methods

2.1. Materials

RPMI 1640, fetal calf serum (FCS), and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma (St. Louis, MO, USA). Troglitazone and pravastatin were obtained from Sankyo Pharmaceuticals (Tokyo, Japan), and pioglitazone was from Takeda Pharmaceuticals (Tokyo, Japan). The transfection reagent FuGENE™ 6 was obtained from Roche Diagnostics (Basel, Switzerland).

2.2. Cell culture

The human THP-1 monocytic leukemia cell-line was grown in RPMI 1640 containing 10% FCS. Differentiation of THP-1 cells was induced by resuspending the cells in medium containing PMA (40 ng/ml), which has been shown to induce the conversion of THP-1 cells into mature cells functionally resembling macrophages [13].

2.3. RNA analysis

To generate probes for Northern blot hybridization, the human cDNA probes for HMG-CoA synthase, LPL, and acyl-CoA:choles-

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Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response element; LDL, low density lipoprotein; PMA, phorbol-12-myristate-13-acetate; LPL, lipoprotein lipase; ACAT, acyl-CoA:cholesterol acyltransferase

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terol acyltransferase (ACAT) were constructed by reverse transcription-polymerase chain reaction performed on RNA that had been extracted from differentiated THP-1 cells. A HMG-CoA reductase cDNA was kindly provided by Dr. Shimano (Tsukuba University, Tsukuba, Japan). Total RNA was isolated from cells at the indicated times after induction of differentiation, and 10 μg of total RNA was used for Northern blot hybridization. The results were normalized to those of the 28S rRNA or the elongation factor-1α mRNA in each sample.

2.4. HMG-CoA reductase assay

2×10^6 cells were seeded into culture dishes and maintained in medium containing PMA (40 ng/ml) with or without troglitazone (10 μM) for 24 h, then the cells were scraped into 50 mM TBS, pH 7.4. After centrifugation, each pellet was frozen, then thawed in 0.1 ml buffer containing 50 mM potassium phosphate, pH 7.4, 5 mM dithiothreitol, 5 mM EDTA, 0.2 M KCl, 0.25% Brij 96. After incubation for 10 min at 37°C, the suspension was centrifuged, and the supernatant containing an equal volume of protein (100 μg) was subjected to measurement of HMG-CoA reductase activity as previously described by Brown et al. [14].

2.5. Decoy experiments

The PPRE 'decoy' was selected on the basis of binding of PPARγ to PPREs, as reported previously [15,16]. Decoy (5′-GCG TCG ACA GGG GAC CAG GAC AAA GGT CAC GTT CGG GAG TCG ACG C-3′) or reverse control (5′-GCG TCG ACA GGG GAC CAC TGG ATA CAG GAC GTT CGG CAG TCG ACG C-3′) oligonucleotides were annealed to their respective complementary sequences. THP-1 cells were transfected with either decoy or reversed oligonucleotides in various concentrations using the transfection reagent FuGENE™ 6 for 6 h, according to the manufacturer's protocol. After the 6 h incubation, the cells were resuspended in fresh medium containing PMA in the presence or absence of troglitazone, and gene expression was examined by Northern blot analysis.

2.6. Cellular lipid measurements

THP-1 cells were seeded into six-well culture plates and maintained in medium containing PMA (40 ng/ml) for 2 days, or maintained for a longer period (5 days) in medium containing PMA for the first 2 days and then in medium lacking PMA with or without various agents. Cells were washed twice with phosphate-buffered saline, then incubated in 1 ml of isopropanol:hexane (2:3) for 1 h. The organic layer was collected in a glass tube and dried under nitrogen. Dried extracts were redissolved in the same volume of isopropanol:Triton X (9:1). Total cholesterol (TC) and free cholesterol (FC) masses were measured with a colorimetric enzymatic assay kit (WAKO, Tokyo, Japan). The mass of esterified cholesterol (EC) was determined by subtracting FC from TC.

2.7. Statistical analysis

All experiments were repeated at least twice and similar results were observed in each experiment. The results are expressed as mean ± S.D. Group means were compared using Student's *t*-test. A *P*-value of <0.05 was considered to be statistically significant.

3. Results

3.1. HMG-CoA synthase and HMG-CoA reductase expression in THP-1 cells treated with a PPARγ agonist

Addition of PMA to THP-1 cells for 3 h induced a rapid increase in mRNA levels for HMG-CoA synthase and HMG-CoA reductase; following that, mRNA levels returned to the nearly basal levels observed in undifferentiated THP-1 cells. These results were consistent with the study of Wilkin et al. [17]. In addition, treatment of cells differentiated with PMA with troglitazone resulted in significant increases in mRNA for HMG-CoA synthase and HMG-CoA reductase (Fig. 1). At the peak time (3 h), the levels of both genes were increased about 1.6-fold upon incubation with troglitazone (10 μM), and the decline in mRNA levels for these genes was less in the presence of troglitazone (Fig. 1b). On the other hand,

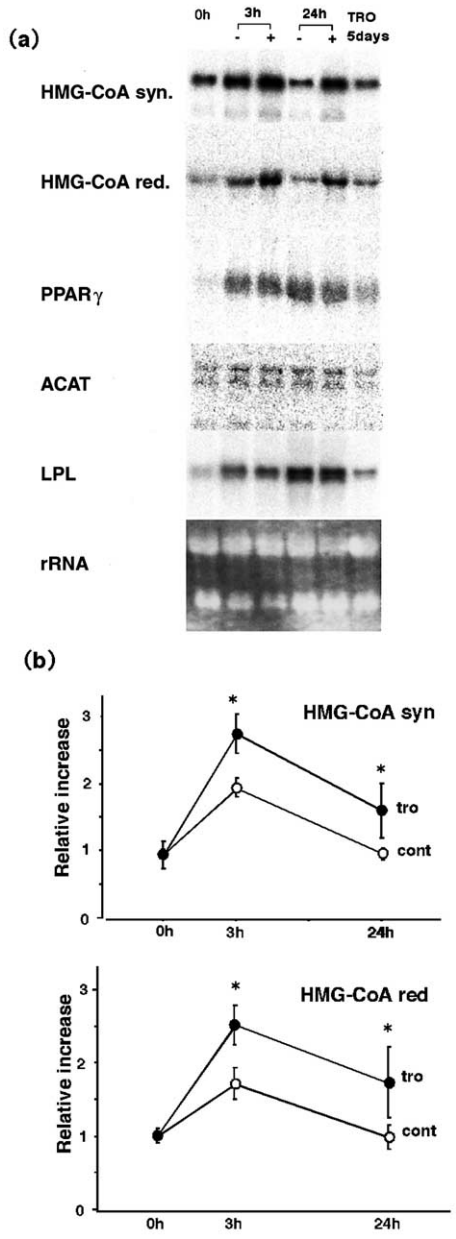

Fig. 1. Induction of differentiation-linked mRNA expression by troglitazone in THP-1 cells. a: THP-1 cells, cultured in media containing PMA (40 ng/ml) to induce differentiation, were treated with (lane; +) or without (lane; −) troglitazone (10 μM). Undifferentiated THP-1 cells were also treated with troglitazone for 5 days and analyzed (lane; TRO 5 days). Abbreviation of each gene is described in the text. b: Changes in expression of HMG-CoA synthase and HMG-CoA reductase in the absence (open circle) or presence (closed circle) of troglitazone for 3 or 24 h are expressed as relative increases. $^*P<0.05$, as compared with time-matched control cells.

treatment of undifferentiated THP-1 cells with troglitazone alone did not result in a significant increase in mRNA levels for these genes. We also examined the mRNA level of PPARγ itself. It increased rapidly along with differentiation of THP-1 cells, however, no significant difference was observed in the level of mRNA in the presence or absence of troglitazone. We further investigated the effect of troglitazone on the expression of two other genes, ACAT and LPL. No significant difference was observed in the levels of these mRNAs regardless of the presence of troglitazone (Fig. 1a).

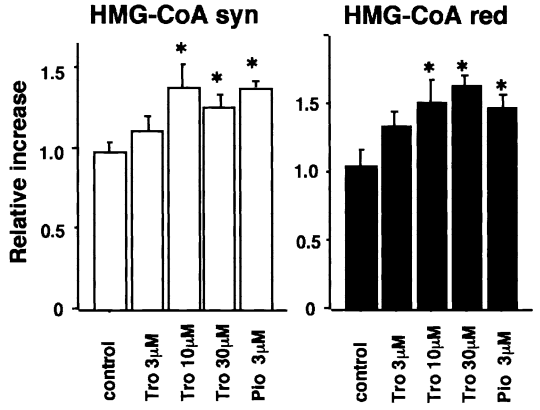

Fig. 2. Induction of HMG-CoA synthase and HMG-CoA reductase expression by troglitazone or pioglitazone in THP-1 cells. THP-1 cells were treated with the indicated concentrations of troglitazone (3–30 μM) (Tro) or pioglitazone (3 μM) (Pio) for 3 h. RNA levels of HMG-CoA synthase and HMG-CoA reductase were normalized by comparison with levels of 28S rRNA and are expressed as relative increases in comparison with the value of control cells, treated with neither troglitazone nor pioglitazone. $*P < 0.05$ versus untreated control.

As shown in Fig. 2, troglitazone increased the expression of mRNA of HMG-CoA synthase and HMG-CoA reductase in a dose-dependent manner. Treatment with pioglitazone, which had been reported to act through a PPARγ [18], also resulted in a significant increase in the mRNA levels.

We also examined the effect of troglitazone on HMG-CoA reductase activity. The results showed that treatment with 10 μM of troglitazone for 24 h along with differentiation increased the enzyme activity of HMG-CoA reductase in THP-1 cells (Table 1). These results confirmed that the increase in enzyme activity observed in cells was comparable to the increase observed in mRNA.

3.2. Effects of the PPRE 'decoy' on expression of HMG-CoA synthase and HMG-CoA reductase

It is assumed that the 'decoy' oligonucleotides can compete for binding of activated PPARγ with the promoter region of the gene and thus block PPAR-dependent transcriptional responses [19]. As shown in Fig. 3a,b, the mRNAs for HMG-CoA synthase and HMG-CoA reductase after treatment with PMA for 3 h were significantly suppressed in cells that were transfected with decoy oligonucleotides. The effect of the decoy was dose-dependent and 30 μM decoy suppressed mRNA expression of both the HMG-CoA synthase and HMG-CoA reductase by approximately 60%, as compared to the absence of decoy, whereas transfection of 30 μM of reverse control oligonucleotides caused no significant effect on their expression.

To provide further evidence that a PPAR-dependent pathway was involved in the increased expression of HMG-CoA synthase and HMG-CoA reductase expression by troglitazone, we examined mRNA expression in cells transfected with the indicated oligonucleotides in the presence or absence of troglitazone (10 μM). Treatment with troglitazone caused significant increases in mRNA for both genes in cells trans-

Table 1
Increase of HMG-CoA reductase activity by troglitazone in THP-1-derived macrophages

	HMG-CoA activity (nM/μg protein/min)
Differentiation (−)	0.076 ± 0.022
Differentiation (+)	0.092 ± 0.023
Differentiation (+)+troglitazone	0.121 ± 0.020*

THP-1 cells, cultured in medium containing PMA to induce differentiation, were treated with or without troglitazone (10 μM) for 24 h. The HMG-CoA reductase activity are presented as means ± S.D. $*P < 0.05$ versus undifferentiated THP-1 cells ($n = 4$).

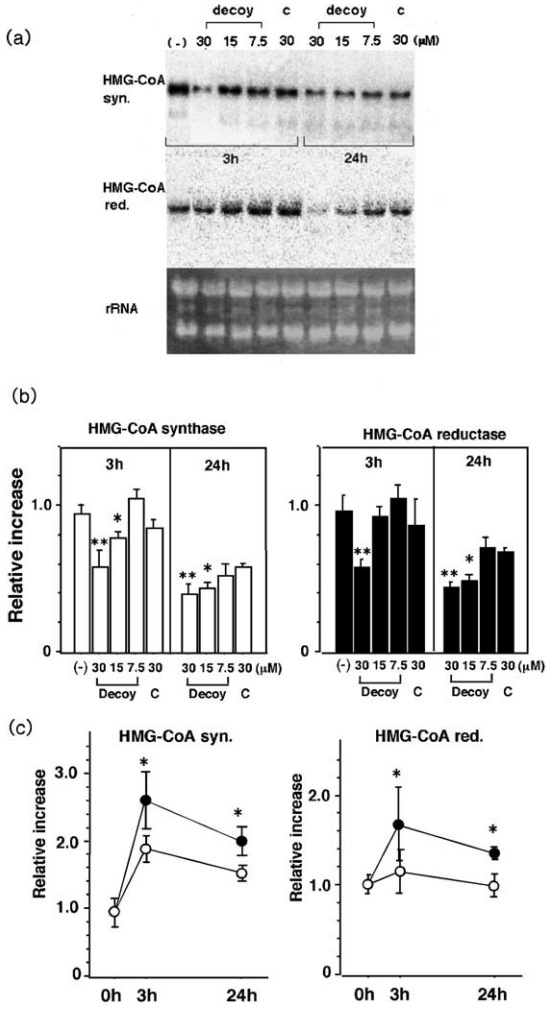

Fig. 3. Effect of the PPRE decoy oligonucleotide on expression of HMG-CoA synthase and HMG-CoA reductase during differentiation of THP-1 cells. a: Undifferentiated THP-1 cells were transfected with various concentrations (7.5–30 μM) of the decoy (decoy) or 30 μM of reverse control (c) oligonucleotide. 6 h after transfection, quantities of the mRNA for HMG-CoA synthase and HMG-CoA reductase were determined at the indicated times of differentiation (3 or 24 h). Cells not transfected with any oligonucleotide (−) were also examined at 3 h. b: RNA levels, normalized by comparison with levels of 28S rRNA, are expressed as relative increases ($n = 3$). $*P < 0.05$, $**P < 0.01$ versus the cells treated with control oligonucleotide. c: THP-1 cells transfected with the PPRE decoy (30 μM) (open circle) or control (30 μM) (closed circle) oligos were differentiated in the presence of troglitazone (10 μM) for 3 or 24 h. The RNA levels are expressed as relative increases in comparison with the value of undifferentiated THP-1 cells. $*P < 0.05$ versus time-matched cells transfected with the PPRE decoy.

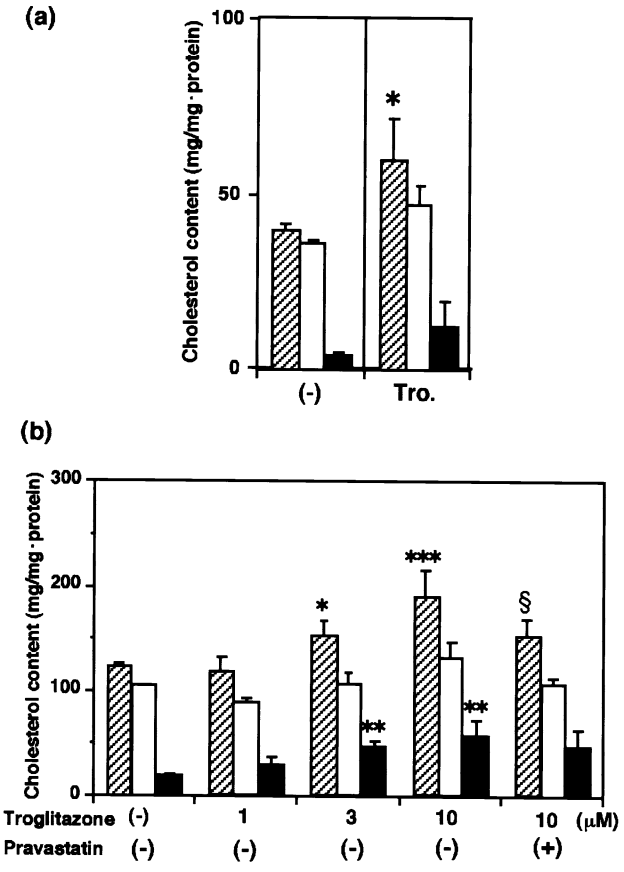

Fig. 4. The role of troglitazone on accumulation of cholesterol in THP-1-derived macrophages. THP-1 cells were incubated for the first 2 days in the presence of PMA (a), and for three additional days in the absence of PMA (b) with various concentrations of troglitazone (1–10 μM) or with troglitazone (10 μM) and pravastatin (30 μM). The amount of each type of cholesterol (TC, striped bar; FC, open bar; EC, solid bar) was normalized by the value of total cell protein ($n=4$). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$ versus untreated control. $^{\S}<0.02$ versus cells treated with troglitazone (10 μM) only.

fected with the reverse control oligonucleotides, while the decoy inhibited the increase in expression (Fig. 3c).

3.3. *Effect of a PPARγ agonist on accumulation of cholesterol in THP-1 cells*

To determine whether the increase in mRNA for the enzymes lead to a corresponding increase in intracellular cholesterol, we measured TC and FC in THP-1 cells. After incubation in the presence of PMA for 2 days, THP-1 cells loaded with troglitazone contained approximately 1.3-fold more TC than cells not treated with troglitazone. However, no significant difference was seen in FC, suggesting that the increased cholesterol in cells treated with troglitazone was due to an increase in EC (Fig. 4a). With prolonged incubation (for the first 2 days in the presence of PMA, and for three additional days in the absence of PMA), a much greater accumulation of cholesterol was observed in cells treated with troglitazone, despite deprivation of PMA; approximately a 1.8-fold increase in TC and a six-fold increase in EC were observed as compared with untreated cells. These effects of troglitazone on cholesterol mass were dose-dependent, and pravastatin, a specific inhibitor of HMG-CoA reductase, inhibited the increases in TC and EC caused by treatment with troglitazone (Fig. 4b).

4. Discussion

Recent studies have demonstrated that PPARγ is expressed in primary macrophages in vitro and in vivo [7,20] and is upregulated during differentiation of monocyte into macrophages [21]. In addition, both natural and synthetic PPARγ ligands are reported to regulate the expression of not only genes associated with inflammation, such as iNOS and TNF-α [7,22], but also genes associated with phagocytic activity, such as CD36 [8,23] in macrophages. These observations suggest that PPARγ plays an essential role in modulating the specific functions of macrophages.

In THP-1 cells, not only the expression of HMG-CoA synthase and HMG-CoA reductase but also the expression of other genes involved in lipid homeostasis such as LPL [3,24] and apolipoprotein E [3] are induced during the differentiation of this monocyte cell-line to macrophage-like cells by phorbol esters. Thus, it had been thought that the expression of these genes was mainly regulated through a PKC-dependent mechanism [17,25].

Recently, Tontonoz et al. reported that PPARγ ligands upregulate expression of the macrophage-specific scavenger receptor, CD36, and promote uptake of modified LDL via CD36 in THP-1 cells [8], suggesting a novel role of PPARγ in gene regulation, differentiation and lipid metabolism in macrophages. Their studies showed that treatment of THP-1 cells with troglitazone alone resulted in a small increase in CD36 mRNA, and the effect of a combination of troglitazone and phorbol ester was much greater than that of troglitazone alone. In the present study, we obtained similar results; troglitazone increased the effect of PMA on the expression of HMG-CoA synthase and HMG-CoA reductase. As the increased expression of these genes by troglitazone was inhibited by a PPRE decoy, we suggest that the transcriptional response was dependent on the interaction of PPARγ with PPRE. We suggest that the expression of HMG-CoA synthase and HMG-CoA reductase during the differentiation of THP-1 cells are regulated as follows: first, PPARγ is induced by phorbol ester through a PKC-dependent pathway, and then troglitazone interacts with PPARγ and regulates the transcription of mRNA, directly or indirectly. Interestingly, in our experiment, levels of the mRNA for LPL were not increased by troglitazone treatment despite this gene having been reported to have an element recognized by PPARγ in its promoter [26]. The reason why LPL mRNA was unaffected is unclear, but it might be due to a tissue-specific transcriptional regulation of this gene by distinct types of PPAR [6]. In fact, PPARα and its agonists were reported to increase LPL gene expression in macrophages [27]. It might also be the result of different PPARγ subtypes. THP-1-derived macrophages reportedly express only PPARγ1 and PPARγ3 [28], whereas LPL promoter is activated by PPARγ2 in other types of culture cells [26]. It is possible that LPL mRNA is mainly regulated by PPARγ2.

In these experiments, we also demonstrated that activation of PPARγ resulted in an increase of intracellular cholesterol even in the absence of modified LDL, and that pravastatin inhibited these increases in cholesterol caused by treatment with troglitazone. This suggests that the increased amount

of cholesterol by troglitazone observed in the present study was the result of increased de novo synthesis, rather than the uptake of modified LDL, or than its esterification by the action of ACAT, because ACAT expression was not increased. What is the meaning of increased cholesterol in macrophages? Recently it has been reported that cholesterol is essential for entry of mycobacteria into macrophages [29]. In this report, the authors demonstrate that the entry of mycobacteria is inhibited in macrophage depleting its cholesterol. Therefore, increase of the mRNAs for cholesterogenic genes by activated PPARγ may play an important role in the acquisition of some macrophage-specific abilities, such as phagocytosis. Moreover, it has been recently reported that the expression of PPARγ is observed in atherosclerotic legions colocalized with macrophages [20,28], and that PPARγ ligand, troglitazone, significantly inhibits the development of atherosclerotic foam cell lesions [30–32]. However, troglitazone exerted apparently an opposite effect in the current experiments. The anti-atherogenic effects of PPARγ ligand appear to be mediated by favorable changes in the extracellular environment such as the suppression of hyperinsulinemic hyperglycemia, inflammation and oxidation [22,33]. To understand the complex roles of PPARγ in the process of atherosclerosis, further studies on cholesterol metabolism in macrophages are needed.

In conclusion, we demonstrated for the first time that the expression of two genes involved in cholesterol metabolism, HMG-CoA synthase and HMG-CoA reductase, is increased by a ligand of PPARγ, troglitazone, in macrophages. Our results also showed that the activity of HMG-CoA reductase, which is important in the regulation of intracellular cholesterol mass, and the cholesterol content were concomitantly increased by troglitazone. An increase in intracellular cholesterol may contribute to regulation of macrophage-specific functions or regulation of atherogenesis.

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