Abietic acid activates peroxisome proliferator-activated receptor-γ (PPARγ) in RAW264.7 macrophages and 3T3-L1 adipocytes to regulate gene expression involved in inflammation and lipid metabolism

Nobuyuki Takahashi[a,b], Teruo Kawada[a,b,*], Tsuyoshi Goto[a], Chu-Sook Kim[c], Aki Taimatsu[a], Kahori Egawa[a], Takayuki Yamamoto[a], Mitsuo Jisaka[b,d], Koji Nishimura[b,d], Kazushige Yokota[b,d], Rina Yu[c], Tohru Fushiki[a]

[a] *Laboratory of Nutrition Chemistry, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan*
[b] *Project for Obesity and Lipid Metabolism Regulation, Bio-oriented Research Advancement Institute (BRAIN), Tokyo 105-0001, Japan*
[c] *Department of Food Science and Nutrition, University of Ulsan, Ulsan 680-749, South Korea*
[d] *Department of Life Science and Biotechnology, Faculty of Life and Environmental Science, Shimane University, Shimane 690-8504, Japan*

Received 10 February 2003; revised 16 June 2003; accepted 18 July 2003

First published online 6 August 2003

Edited by Robert Barouki

Abstract **Abietic acid is one of the terpenoids, which are multifunctional natural compounds. It has been reported that abietic acid suppresses effects on inflammation. However, the mechanism underlying the anti-inflammatory effects remains unclear. The present work indicates that abietic acid suppresses the protein expression of tumor necrosis factor-α and cyclooxygenase 2, which are involved in inflammation, in lipopolysaccharide-stimulated macrophages. Moreover, this effect resembles that of thiazolidinedione, a synthetic peroxisome proliferator-activated receptor-γ (PPARγ) ligand. Indeed, abietic acid activates PPARγ in luciferase reporter assays. The activity of abietic acid induces PPARγ target gene expression in RAW264.7 macrophages and 3T3-L1 adipocytes. These data indicate that abietic acid is a PPARγ ligand and that its anti-inflammatory effect is partly due to the activation of PPARγ in stimulated macrophages. The present work suggests a novel possibility that abietic acid, a naturally occurring compound, can be used not only for anti-inflammation but also for regulating lipid metabolism and atherosclerosis.**

Key words: Abietic acid; Anti-inflammatory; Peroxisome proliferator-activated receptor-γ; Adipocyte; Macrophage; Terpenoid; Nutrigenomics

1. Introduction

Terpenoids, which are present in many dietary and herbal plants [1], exhibit many biological effects: for example, antitumor proliferation, antihypercholesterolemia and antidiabetes [1–3]. Therefore, terpenoids have been regarded as valuable medicinal and food components that control many cellular functions and medical conditions in vivo.

Abietic acid, a diterpene, is a major component of the rosin fraction of oleoresin synthesized by conifer species, such as grand fir (*Abies grandis*) and lodgepole pine (*Pinus contorta*) [4]. The chemical structure of abietic acid is shown in Fig. 1A. It was reported that abietic acid has an anti-inflammatory effect [5–7]. In lipopolysaccharide (LPS)-stimulated macrophages, abietic acid suppresses production of prostaglandin E_2 (PGE_2) in vitro and in vivo [7]. However, the mechanism underlying the anti-inflammatory effect of abietic acid has not been clarified.

The anti-inflammatory effect is regulated by many transcriptional factors in vivo. Among the transcriptional factors, peroxisome proliferator-activated receptor-γ (PPARγ), a member of the nuclear receptor superfamily activated by ligands, plays an important role in inflammation [8,9]. The activation of PPARγ signaling enhances macrophage differentiation, inducing the expression of fatty acid translocase (FAT)/CD36, one of the cell's 'scavenger' receptors for the atherogenic low-density lipoprotein (LDL) [10]. Lipoprotein lipase (LPL) also acts as a bridge, which enhances binding to the extracellular matrix and uptake of both native and oxidized LDL by macrophages. These molecules expressed in macrophages may exhibit pro-atherogenic activities. However, thiazolidinedione (TZD), a synthetic ligand for PPARγ, suppresses the production of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in LPS- or phorbol 12-myristate-13-acetate-stimulated macrophages [11,12]. Recent studies showed that, in addition to the anti-inflammatory effect, TZD regulates expression of genes involved in lipid metabolism in macrophages and suppresses their transformation into foam cells [11,13]. Therefore, regulation of PPARγ by TZD in macrophages is indispensable not only for anti-inflammatory events but also for the transformation into foam cells. On the other hand, TZD has been widely used as an antidiabetic drug, which activates PPARγ resulting promotion of adipocyte differentiation ([14], review). TZD not only stimulates glucose uptake into differentiated adipocytes but also induces production of adiponectin (an insulin sensi-

*Corresponding author. Fax: (81)-75-753 6264.
E-mail address: fat@kais.kyoto-u.ac.jp (T. Kawada).

Abbreviations: PPAR, peroxisome proliferator-activated receptor; TZD, thiazolidinedione; BADGE, bisphenol A diglycidyl ether; LPS, lipopolysaccharide; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; aP2, adipocyte fatty acid-binding protein; LPL, lipoprotein lipase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF-α, tumor necrosis factor-α; COX2, cyclooxygenase 2

0014-5793/03/$22.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00859-7

tivity-promoting factor) and suppression of TNF-α (a insulin resistance-inducing factor) through PPARγ activation in adipocytes [15–17]. Thus, ligands for PPARγ such as TZD can show multiple effects including antidiabetes, anti-inflammation, and antiatherosclerosis.

In this study, we show similarity between abietic acid and TZD in terms of suppressive activity for TNF-α and cyclooxygenase 2 (COX2) induction at the protein level in LPS-stimulated macrophages. Moreover, the results demonstrate that abietic acid activates PPARγ in the GAL4 chimera system. Indeed, abietic acid could induce the expression of PPARγ target genes in RAW264.7 macrophages and 3T3-L1 adipocytes. Moreover, the induction of PPARγ target gene expression depended on the expression of PPARγ and a nuclear receptor coactivator, cAMP response element-binding protein (CREB)-binding protein (CBP), in NIH3T3 fibroblasts. These data indicate that abietic acid is an activator of PPARγ and the anti-inflammatory effect of abietic acid is, at least partly, due to its activity as an activator of PPARγ. The results further suggest that abietic acid is a valuable medicinal and food component for use in control functions of macrophages and adipocytes.

2. Materials and methods

2.1. Chemical reagents

T174 TZD, a specific ligand for PPARγ [18], was kindly provided by Tanabe Seiyaku (Osaka, Japan). Abietic acid and bisphenol A diglycidyl ether (BADGE) were from Sigma (St. Louis, MO, USA) or Tokyo Chemical Industry (Tokyo, Japan). These compounds were diluted with dimethyl sulfoxide (DMSO) to prepare the stock solutions (10–100 mM). All other chemicals were from Sigma or Nacalai Tesque (Kyoto, Japan) and of guaranteed reagent grade or tissue culture grade.

2.2. Cell culture

Monkey CV1 kidney cells, mouse 3T3-L1 preadipocytes, mouse NIH3T3 fibroblasts and mouse RAW264.7 macrophage were purchased from American Type Culture Collection. They were cultured in a maintenance medium (10% fetal bovine serum, 200 μM ascorbic acid, 100 units/ml penicillin and 100 μg/ml streptomycin in Dulbecco's modified Eagle's medium) at 37°C in 5% CO_2/95% air under humidified condition. For adipocyte differentiation, 3T3-L1 preadipocytes were incubated in a differentiation medium 2 days after they reached confluence, as described previously [19]. Cell viability was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay as previously described [20].

2.3. Preparation of peritoneal macrophages

BALB/c male mice (Japan SLC, Hamamatsu, Japan) were injected intraperitoneally with 3–4 ml of thioglycollate broth (Difco, Detroit, MI, USA) (3%) 4 days before use. Peritoneal macrophages were prepared as follows: lightly ether-anesthetized mice ($n=4$–6) were injected intraperitoneally with 5 ml of sterilized phosphate-buffered saline. The peritoneal fluid was carefully aspirated to avoid hemorrhage. After centrifugation at $200\times g$ for 10 min, the cell pellet was washed twice with the maintenance medium. Cells were seeded at 1×10^6/ml in the maintenance medium. After incubation for 2 h at 37°C, non-adherent cells were removed by extensive washing with the maintenance medium. All animals received human care, as outlined in the Guide for the Care and Use of Laboratory Animals (Kyoto University Animal Care Committee according to NIH 86-23; revised 1985).

2.4. Measurement of TNF-α and Western blot analysis of COX2

The concentration of TNF-α in culture supernatants was assayed by enzyme-linked immunosorbent assay (ELISA). The assays were conducted utilizing OptEIA™ Mouse TNF-α set (Pharmingen, San Diego, CA, USA). The samples were thawed, diluted properly in assay diluents, and thoroughly mixed. Samples (triplicate) and standards (duplicate) were pipetted into the appropriate wells of the TNF-α antibody-coated ELISA plate. Plates were sealed and incubated for 2 h at room temperature. After rinsing thoroughly, each well was incubated for 1 h with 100 μl of biotinylated mouse TNF-α monoclonal antibody and streptavidin–horseradish peroxidase conjugate. Following another rinse, tetramethylbenzidine substrate solution was placed in each well in the dark for 30 min. The absorbance of each well was read at 450 nm on a microplate reader. Cytokines were quantitated from standard curves using the SOFTmax curve-fitting program (Molecular Devices, Sunnyvale, CA, USA).

For Western blotting to analyze the expression of COX2, mouse monoclonal COX2 antibody (Transduction Labs, Lexington, KY, USA) was used. Western blotting was performed as previously described [20].

2.5. Luciferase assays

Our luciferase assays were performed using an advanced highly sensitive system with coexpression of coactivator CBP developed by modifying the dual luciferase system (Promega, Madison, WI, USA), as previously described [21]. Twenty-four hours after transfection of both reporter and internal control plasmids, the transfected cells were cultured in the medium containing each compound for another 24 h. Luciferase assays were performed using the dual luciferase system according to the manufacturer's protocol.

2.6. RNA preparation and quantification of gene expressions

RNA samples of differentiated 3T3-L1 were prepared using cells cultured on 6-well tissue culture plates 8 days after differentiation induction. The cells were treated with the medium containing PPARγ ligand for 48 h before RNA sample preparation. For analysis of gene expression in RAW264.7 and NIH3T3, pSG5-mPPARγ, the expression vector for PPARγ, was transfected into RAW264.7 cultured on 6-well plates with LipofectAMINE and Plus reagents (Invitrogen, San Diego, CA, USA) as previously described [21]. Twenty-four hours after the transfection, the transfected RAW264.7 and NIH3T3 cells were treated for another 24 h with the experimental medium containing each sample compound. All the total RNA samples were prepared from cells using the SV total RNA isolation system (Promega) according to the manufacturer's protocol.

Aliquots of total RNA were reverse-transcribed using M-MLV reverse transcriptase (Invitrogen) in a thermal cycler (Takara PCR Thermal Cycler SP: Takara Shuzo, Kyoto, Japan) according to the manufacturer's instructions. To quantify mRNA expression, polymerase chain reaction (PCR) was performed using a quantitative real-time RT-PCR system (LightCycler System: Roche Diagnostics, Mannheim, Germany), as described previously [19,21]. All primer sets used in this study were described previously [21]. To compare the mRNA expression levels among samples, the copy numbers of each transcript were divided by that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) showing a constant expression level.

2.7. Statistical analysis

The data are presented as means ± S.E.M. and were statistically analyzed using the unpaired t-test or the Welch t-test when variances were heterogeneous. Differences were considered significant when P was <0.05.

3. Results

3.1. Abietic acid and PPARγ ligand suppress expression of TNF-α and COX2 in LPS-stimulated macrophages

To investigate the anti-inflammatory effect of abietic acid, LPS-stimulated peritoneal macrophages were cultured in the medium containing abietic acid. The addition of 50 μM abietic acid suppressed LPS-induced production of TNF-α (16.3% of the LPS-stimulated vehicle control) as shown in Fig. 1B. This suppressive effect of abietic acid on TNF-α production was dose-dependent. Under the same experimental conditions, 5 μM TZD, which regulates the expression of macrophage differentiation marker genes and inflammation-related genes via PPARγ activation, suppressed approximately 8.9% of the LPS-induced TNF-α production. Moreover, the

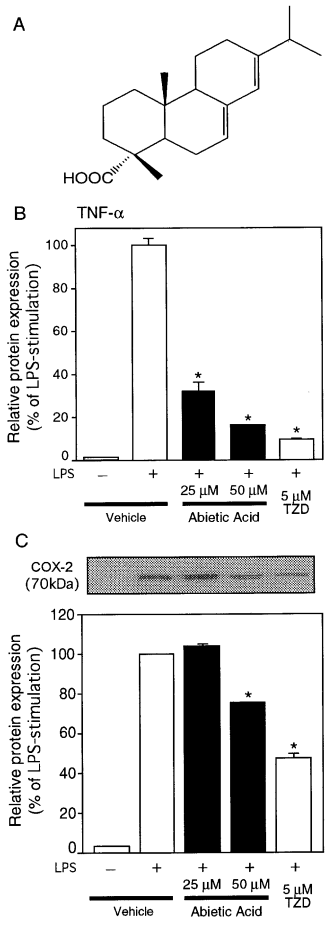

Fig. 1. Abietic acid suppresses protein expression of pro-inflammatory markers in LPS-stimulated macrophages. Structure of abietic acid (A). Peritoneal macrophages were stimulated with 25 ng/ml LPS and incubated for 24 h with abietic acid or TZD. B: Suppressive effects of abietic acid and TZD in LPS-induced TNF-α production. The quantification of TNF-α released from LPS-stimulated macrophages was performed using ELISA as described in Section 2. The production level of LPS-induced TNF-α was set at 100%. The values are means ± S.E.M. of four tests. $*P < 0.05$ compared with LPS-induced TNF-α production. C: Suppressive effects of abietic acid and TZD in LPS-induced COX2 production. Equal amounts of protein (15 μg/lane) were loaded and electrophoresed on a 10% sodium dodecyl sulfate–polyacrylamide gel. After the protein separation, proteins were blotted onto a nitrocellulose membrane, and then COX2 was detected using a chemiluminescent system (ECL). The data are representative of three independent blots. The values are means ± S.E.M. of three tests. $*P < 0.05$ compared with LPS-induced COX2 expression.

protein expression of COX2, which is enhanced in LPS-stimulated macrophages, was suppressed to 75.6% and 45.3% of the LPS-stimulated vehicle control by 50 μM abietic acid and 5 μM TZD, respectively (Fig. 1C), although addition of a lower concentration of abietic acid (25 μM) had no effect. These suppressive effects of abietic acid on the protein expression of TNF-α and COX2 were confirmed at the mRNA level by quantification of both mRNAs in stimulated peritoneal macrophages by using quantitative RT-PCR (the addition of 50 μM abietic acid resulted in 65% and 80% of the vehicle controls for the mRNA expressions of TNF-α and COX2, respectively). These results suggest that abietic acid suppresses the protein expression of both TNF-α and COX2 and that the suppressive effects of abietic acid and TZD are governed by the same mechanism.

3.2. *Abietic acid activates PPARγ as determined by luciferase assays*

We hypothesized that PPARγ is activated by abietic acid as well as TZD, because of the similarity between abietic acid and TZD in terms of their anti-inflammatory effects. Thus, we carried out reporter assays for PPARγ ligands using abietic acid. In the assays, abietic acid induced GAL4/PPARγ chimera transactivation in a dose-dependent manner (Fig. 2A). Using the GAL4/PPARγ chimera system, 30 μM abietic acid induced activation 4.6-fold that of the vehicle control (Fig. 2A). The concentrations of abietic acid used in these assays had no influence on cell viability (data not shown). Moreover, in the GAL4/PPARγ chimera system, BADGE, a PPARγ antagonist [22], suppressed luciferase activity of 10 μM of abietic acid in a dose-dependent manner (Fig. 2B). The activity of abietic acid in the presence of 1 μM BADGE was significantly suppressed at 41% of the activity in the absence of BADGE. It was shown that a binding site of this compound in the ligand-binding domain (LBD) of PPARγ is the same as that of TZD, as determined by competitive assays using ^{3}H-labeled TZD [22]. Therefore, these results indicate that abietic acid activates PPARγ as determined by luciferase assays using the GAL4/PPARγ chimera system and suggest that abietic acid, as well as TZD, binds to the LBD of PPARγ.

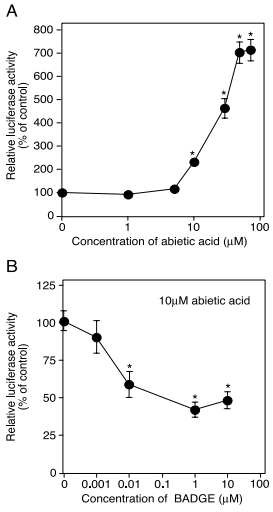

Fig. 2. Abietic acid activates PPARγ as its ligand. A: Luciferase ligand assays using the PPARγ/GAL4 chimera system in CV1 cells. pM-hPPARγ, p4xUASg-tk-luc, pCMX-CBP and pRL-CMV (an internal control) were transfected into CV1 cells and then, 24 h after the transfection, the cells were treated with abietic acid at various concentrations for 24 h. The activity of the vehicle control was set at 100% and the relative luciferase activities are presented as fold induction to that of the vehicle control. The values are means ± S.E.M. of four tests. $*P < 0.05$ compared with the vehicle control. B: Antagonization of activity of abietic acid as a PPARγ ligand by BADGE. The same plasmids as used in ligand assays above were transfected into CV1 cells and 24 h after the transfection 10 μM abietic acid was added into the culture medium with or without BADGE at various concentrations. After another 24 h, luciferase assays were performed. The activity of 10 μM abietic acid without BADGE was set at 100% and the relative luciferase activities are presented as fold induction to that of the positive control. The values are means ± S.E.M. of four tests. $*P < 0.05$ compared with control without BADGE.

3.3. Expression of PPARγ target genes is induced by abietic acid in macrophages and adipocytes

To confirm whether abietic acid activated PPARγ in macrophages, we investigated the mRNA expression of PPARγ target genes in RAW264.7. In transiently PPARγ-expressing RAW264.7 (the transfection efficiencies were approximately 40% through all experiments), the expression of *FAT/CD36* was induced 2.1-fold following the addition of 50 μM abietic acid (closed columns in Fig. 3A), whereas that of *LPL* was induced 4.9-fold (open columns in Fig. 3A). Mock control cells showed no induction after addition of each compound (data not shown). The expressions of *FAT/CD36* and *LPL* are controlled by PPARγ in macrophages [23,24]. Therefore, these results suggest that abietic acid activates PPARγ to regulate expression of PPARγ target genes in RAW264.7 macrophages.

Next, to examine the effects of abietic acid on other cells, we quantified the induction of PPARγ target gene expression in 3T3-L1 adipocytes by abietic acid. In differentiated 3T3-L1 adipocytes, 25 μM abietic acid induced 8.1-fold adipocyte fatty acid-binding protein (aP2) expression 7 days after differentiation induction (closed columns in Fig. 3B). In the case of *LPL*, expression was induced 5.1-fold following the addition of 25 μM abietic acid (open columns in Fig. 3B). Moreover, these effects of abietic acid were dose-dependent, because addition of 50 μM abietic acid resulted in 11.4- and 5.8-fold increases in the expression levels of *aP2* and *LPL*, respectively. This suggests that abietic acid can induce expression of *aP2* and *LPL* via PPARγ activation in adipocytes.

3.4. The PPARγ target gene induction by abietic acid depends on expression of PPARγ and coactivator

To elucidate the details of PPARγ target gene induction by abietic acid, we used transiently PPARγ-expressing NIH3T3 fibroblasts (the efficiency of transient transfection was approximately 55% through all experiments), which differentiate into adipocyte-like cells in a PPARγ-ligand-dependent manner [25]. Addition of 25 μM abietic acid induced the expression of *aP2* 7.2-fold in the transiently PPARγ-expressing NIH3T3 as shown in Fig. 4A. The effect of abietic acid was dose-dependent (50 μM abietic acid induced 32-fold expression). However, induction of *aP2* expression was not observed in the absence of PPARγ expression (Fig. 4A). Similar results were obtained in the induction of *LPL* (data not shown). Mock control cells showed no induction after addition of each compound (data not shown). This strongly suggests that the regulation of PPARγ target gene expression by abietic acid is due to activation of PPARγ. In addition to the dependence on PPARγ expression, the induction of *aP2* by abietic acid depended on the expression of CBP, a coactivator for PPARγ. The coexpression of CBP and PPARγ resulted in a 2.7-fold induction of *aP2* expression in the presence of 25 μM abietic acid in comparison to the expression of only PPARγ, as shown in Fig. 4B. Moreover, the enhanced aP2 induction due to the coexpression of CBP and PPARγ was completely inhibited by adenovirus oncoprotein E1A, which specifically inhibits CBP/p300 functions by its direct binding to the coactivator proteins [26]. This coactivator-dependent activation of abietic acid is the same as that of TZD as previously described [19]. These results suggest that the activation of PPARγ by abietic acid depends on the expression of a coactivator for PPARγ, i.e. CBP.

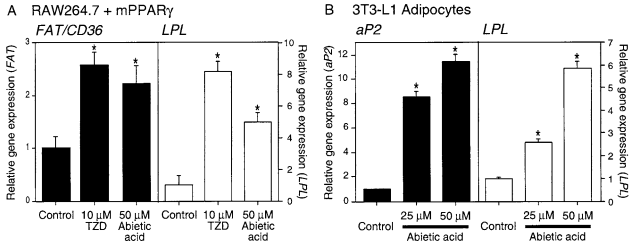

Fig. 3. Abietic acid induces expression of PPARγ target genes in macrophages and differentiated 3T3-L1 adipocytes. A: Transiently PPARγ-expressing RAW264.7 cells were cultured on 6-well plates and 24 h after transfection the stimulated cells were treated with or without abietic acid for another 24 h. B: Differentiated 3T3-L1 adipocytes were treated with vehicle control (DMSO), 10 μM TZD, 25 and 50 μM abietic acid for 48 h 7 days after differentiation induction. In both experiments, expression of mRNAs was estimated using LightCycler, a quantitative real-time RT-PCR (see Section 2). The relative expression levels are presented as fold induction to that of the vehicle control. All values are means ± S.E.M. of six tests. $^{*}P < 0.05$ compared with vehicle controls.

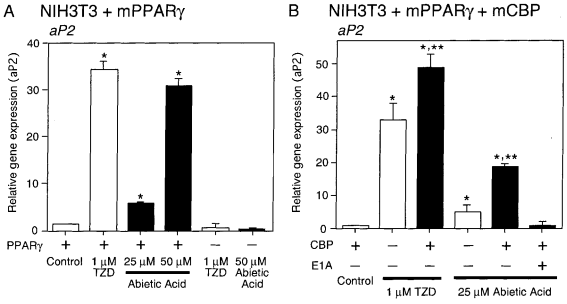

Fig. 4. Abietic acid regulates expression of PPARγ target genes in NIH3T3 cells. A: NIH3T3 cells transfected with or without a PPARγ expression vector (pSG5-PPARγ, 1.0 μg/well) were cultured on 6-well plates and 24 h after transfection TZD or abietic acid was added to the culture medium and further incubated for 48 h. B: CBP expression vector (pCMX-CBP, 1.0 μg/well) with PPARγ expression vector (pSG5-PPARγ, 1.0 μg/well) was transiently introduced into NIH3T3 cells cultured on 6-well plates. Twenty-four hours after the transfection, 25 or 50 μM abietic acid was added into the medium and further incubated for another 24 h. Adenovirus oncoprotein E1A expression vector (1.0 μg/well)-transfected cells were used as the negative control. In both experiments, the expression level of mRNAs was estimated using LightCycler and normalized to the *GAPDH* expression level. The relative expression levels are presented as fold induction to that of the vehicle control. All values are means ± S.E.M. of six tests. $^{*,**}P < 0.05$ compared with vehicle controls and samples without CBP expression, respectively.

4. Discussion

We showed that abietic acid suppresses the protein expression of TNF-α and COX2 in LPS-stimulated macrophages. It was reported that abietic acid has anti-inflammatory effects such as suppression of PGE_2 production and lipoxygenase activity [6,7]. The expression of COX2 is involved in the production of prostaglandins including PGE_2 in macrophages. Therefore, it is suggested that the suppressive effect of abietic acid on PGE_2 production is partly due to the suppression of COX2 expression in macrophages.

Our present study shows that abietic acid activates PPARγ in the GAL4 chimera system. This indicates that abietic acid activates PPARγ as its ligand. The activity of abietic acid as a PPARγ ligand is strongly supported by the competitive effect

of BADGE (an antagonist of PPARγ), which binds to PPARγ LBD, as shown in Fig. 2B. Indeed, abietic acid could regulate the expression of PPAR target genes (*FAT/CD36* and *LPL*) in PPARγ-expressing RAW264.7 macrophages (Fig. 3A). This induction of PPARγ target genes by abietic acid depended on PPARγ expression (Figs. 3A and 4A), suggesting that abietic acid can regulate gene expression via PPARγ activation as its ligand in intact cells. It has been reported that ligands of PPARγ such as TZD and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 suppress the expression and/or the production of several inflammatory cytokines including TNF-α, IL-6, IL-2 and PGE_2 [9]. This suppressive activity of PPARγ is due to inhibition of activities of NF-κB, STAT-1 and AP1 in inflammatory signaling [9]. This is partly because of the competition in binding of CBP/p300 coactivators between PPARγ and other transcriptional factors [27]. CBP/p300 is essential for ligand-dependent activation of PPARγ as a coactivator [19]. The PPARγ activation by abietic acid depended on CBP expression as shown in Fig. 4B. Therefore, it is possible that the anti-inflammatory effects of abietic acid are due to CBP-dependent PPARγ activation in macrophages.

These results indicate that abietic acid is a functional activator of PPARs in intact cells. It has been reported that other terpenoids including phytanic acid and isoprenols can activate PPARs [20,27]. A common characteristic of such compounds is their activity that regulates the expression of adipocyte differentiation marker genes. Phytanic acid and farnesol increase the expression levels of uncoupling protein-1 in primary cultured brown adipocytes [28], and aP2/LPL in 3T3-L1 adipocytes [21], respectively, via PPARγ activation. In our study, abietic acid upregulated the expression of *aP2* and *LPL* in 3T3-L1 adipocytes. Furthermore, in macrophages it was shown that abietic acid increased the expression levels of *FAT/CD36* and *LPL*, which are associated with lipid metabolism and pathological processes such as atherosclerosis. Therefore, there are possibilities that terpenoids including abietic acid might be classified into a novel class of naturally occurring PPAR ligands and that terpenoids might control lipid metabolism in vivo via PPAR activation. In this sense, it is interesting and might be valuable to analyze the relationship between terpenoids in terms of their structures and abilities to induce PPAR activation.

In conclusion, abietic acid suppresses expression of genes involved in inflammation such as TNF-α and COX2 in activated macrophages. This effect is due to the ability of abietic acid to activate PPARγ as its ligand. Abietic acid regulates the expression of PPARγ target genes including *aP2*, *LPL* and *FAT/CD36* in 3T3-L1 adipocytes or RAW264.7 macrophages. In this present work, we propose a possible mechanism of the anti-inflammatory effect of abietic acid and its novel use for transcriptional regulation for management of lipid metabolism in adipocytes or macrophages.

Acknowledgements: We thank Dr. P.A. Grimaldi for the gift of the mouse PPARγ expression vector, Dr. R.H. Goodman for the CBP DNA and Dr. T. Kouzarides for the E1A expression vector. This work was supported in large part by the PROBRAIN Project of the Bio-oriented Technology Research Advancement Institution (BRAIN), Japan and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (13460058).

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