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# Dehydroabietic acid, a phytochemical, acts as ligand for PPARs in macrophages and adipocytes to regulate inflammation

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**Abstract**

Obesity is characterized by an enhanced infiltration of macrophages to adipose tissues, which is closely associated with the low-grade inflammatory state and obesity-related pathologies such as type 2 diabetes and cardiovascular diseases. We showed here that dehydroabietic acid (DAA) is a potent PPARα/γ dual activator. Furthermore, we examined the anti-inflammatory effects of DAA in stimulated macrophages and in the coculture of macrophages and adipocytes. DAA significantly suppressed the production of proinflammatory mediators such as MCP-1, TNF-α, and NO in stimulated RAW 264 macrophages and in the coculture of RAW 264 macrophages and 3T3-L1 adipocytes. These results suggest that DAA is a valuable medicinal and food component for improving inflammatory changes associated with obesity-related diabetes.


*Keywords:* Dehydroabietic acid; Inflammation; MCP-1; TNF-α; NO; PPARα/γ; Macrophage; Coculture; Metabolic syndrome

Obesity is now considered to be epidemic throughout the western world and represents a major risk factor for various life-threatening diseases, such as insulin resistance, cardiovascular diseases, cancer, and type 2 diabetes [1]. In addition, recent studies have indicated that obesity is associated with a low-grade inflammation state, suggesting that inflammation is a potential mechanism by which obesity leads to insulin resistance [2].

The monocyte chemoattractant protein (MCP)-1, a member of the CC chemokine superfamily, plays a pivotal role in monocyte/macrophage trafficking and activation. Yu et al. [3] reported that MCP-1 is crucial in the inflammatory responses of adipose tissues to induce the infiltration and activation of macrophages in these tissues. Macrophages produce various proinflammatory factors including MCP-1, tumor necrosis factor (TNF)-α and nitric oxide (NO). Suganami et al. [4] showed that the macrophage-derived TNF-α establishes a vicious cycle that augments the inflammatory changes and insulin resistance in obese-adipose tissues. Therefore, to prevent obesity-related inflammation, it is important to decrease the production of obese-adipose-tissue-derived proinflammatory factors such as MCP-1 and TNF-α.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear transcription factors. Three subtypes of PPAR have been identified: PPARα, PPARδ, and PPARγ [5]. PPARα is usually involved in the control of lipid metabolism in the liver and skeletal muscle [6]. PPARδ is ubiquitously expressed and is thought to be involved in cell proliferation [7]. PPARγ is strongly expressed in adipocytes and responsible for adipogenesis [8]. PPARγ is also a regulator of insulin resistance in adipose tissues; PPARγ activation promotes adipocyte differentiation and increases the number of small adipocytes with high glucose uptake ability [9]. Recently, many

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PPARα/γ dual agonists have been evaluated for their potency in animal model of type 2 diabetes and shown to have beneficial effects on improving lipid homeostasis, insulin sensitivity, and inflammation in liver, adipose, and vascular tissues [12–14]. However, most studies have investigated the effects of synthetic chemicals and compounds, and little is known about those of natural dual agonists contained in daily foods.

Terpenoids, which are contained in many dietary and herbal plants, have many biological effects [13]. We previously reported that abietic acid (AA), a diterpene, has anti-inflammatory effects on macrophages, which is mediated by PPARγ activation [14]. AA is a major component of the rosin fraction of oleoresin produced by conifer species, such as grand fir (*Abies grandis*) and lodgepo le pine (*Pinus contorta*). These plants also contain many AA derivatives including dehydroabietic acid (DAA). However, the biological efficacies of AA derivatives have not been completely understood.

Here, using a sensitive screening system for PPAR ligands, we showed that DAA is the most potent dual activator of PPARγ and PPARα. Moreover, we demonstrated that DAA suppresses the production of proinflammatory mediators such as MCP-1 and TNF-α in lipopolysaccharide (LPS)-stimulated macrophages and in the coculture of adipocytes and macrophages. These results suggest that DAA exerts its anti-inflammatory properties by activating PPARs. DAA may be a valuable medicinal and food component for improving the inflammatory changes in obese-adipose tissues.

## Materials and methods

*Chemical and cell cultures.* DAA and its derivatives were kindly provided by the Arakawa Chemical Industries, Ltd. (Ibaraki, Japan). All the compounds were diluted with DMSO. DMEM and LPS were from Sigma (MO, USA). Troglitazone was purchased from Sankyo Co., Ltd. (Tokyo, Japan). All the other chemicals were from Sigma or Nacalai Tesuque (Kyoto, Japan) and guaranteed to be of reagent or tissue-culture grade.

Monkey CV1 kidney cells were purchased from American Type Culture Collection and murine macrophages were obtained from RIKEN BioResource Center (Ibaragi, Japan). They were cultured in DMEM containing 10% FBS (JRH Bioscience, KS, USA) and 100 U/ml penicillin/100 μg/ml streptomycin (Gibco BRL, NY, USA) at 37 °C in 5% $CO_2$/95% air under humidified condition. RAW 264 cells were seeded in 6-well plates ($2 \times 10^6$ cells/ml/well) and treated with 100 ng/ml LPS and various concentrations of DAA in serum-free medium for 18 h. Cell viability was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [15].

Adipocytes and macrophages were cocultured in a contact system as previously described [4]. Briefly, RAW 264 cells ($1 \times 10^5$ cells/ml) were plated onto dishes with serum-starved and hypertrophied 3T3-L1 cells. The coculture was incubated in serum-free DMEM for 24 h. RAW 264 and 3T3-L1 cells were separately cultured under the same conditions as those for the control cultures. DAA was added to the coculture at various concentrations. Twenty-four hours after the treatment, culture supernatants were collected and stored at −80 °C until measurements.

*Luciferase assay.* PPAR luciferase assay was performed using a GAL4/PPAR chimera system as previously described [16]. CV1 cells were cultured on 24-well plates. Transfection was performed using LipofectAMINE (Invitrogen, CA, USA) according to the manufacturer's protocol. Five hours after the transfection, the cells were treated with each compound for an additional 24 h, and then luciferase assay was performed according to the manufacturer's protocol (Dual-Luciferase Reporter Assay System; Promega, WI, USA).

*Measurement of MCP-1, TNF-α and NO production.* The concentrations of MCP-1 and TNF-α in each culture supernatant were determined by ELISA. The assays were conducted utilizing a READY-SET-GO! mouse MCP-1 and TNF-α (eBiosciense, CA, USA) according to the manufacturer's protocol. The amount of nitrite in each culture supernatant was measured using Griess reagent. Briefly, 100 μl of supernatant was mixed with an equal volume of Griess reagent (1:1 (v/v) composed of 0.1% *N*-1-naphthyl-ethylenediamine in distilled water and 1% sulfanilamide in 5% phosphoric acid) on a 96-well flat-bottom plate. The absorbance at 550 nm was measured after 10 min (using the Microplate Reader; Bio-Rad Laboratories, CA, USA). The amount of NO was calculated from a standard curve plotted using known concentrations of $NaNO_2$.

*Western blotting.* RAW 264 cells were carefully washed twice with ice-cold PBS and placed immediately in lysis buffer containing 20 mM Tris–HCl (pH 7.5), 15 mM NaCl, 1% Triton X-100, and a protease inhibitor cocktail (Nacalai Tesuque). The resulting lysate was centrifuged at 15,000 rpm for 5 min, and the supernatant was stored for subsequent analysis. The protein concentration of the cell lysate was determined using DC protein assay reagents (BioRad Laboratories). Fifteen micrograms of protein was subjected to 8% SDS–PAGE, and the separated products were transferred to an Immobilon-P membrane (Millipore, MA, USA). After blocking with 3% skim milk in PBS/0.1% Tween 20, the membrane was incubated with primary antibodies overnight and then with a secondary antibody conjugated to horseradish peroxidase for 1 h. The secondary antibody was visualized using chemiluminescence with an ECL Western blotting detection reagent (Amersham Biosciences, NJ, USA). The primary and secondary antibodies used were as follows: anti-iNOS antibody (BD Transduction Laboratories, CA, USA) and anti-mouse IgG (Upstate, NY, USA).

*Statistical analysis.* The data were presented as means ± SEM. and statistically analyzed using one-way ANOVA when their variances were heterogeneous and unpaired *t*-test. Differences were considered significant at $p < 0.05$.

## Results

### *DAA stimulates PPARα and PPARγ activities*

We screened for novel PPAR ligands in AA derivatives (50 μM) using a luciferase reporter assay system (Fig. 1). DAA (the structure is shown in Fig. 2A) the most strongly activated GAL4/PPARα and GAL4/PPARγ by approximately 7.5- and 10.4-fold, respectively, of the 10 compounds examined. However, none of the compounds had any effect on GAL4/PPARδ transactivation. The activity of DAA was in a dose-dependent manner in both PPARα (Fig. 2B) and PPARγ (Fig. 2C) transactivation levels. The concentration of DAA used in these assays had no effect on cell viability, as determined by MTT assays (data not shown). These results indicate that DAA is a strong agonist for both PPARα and PPARγ.

### *DAA inhibits secretion of MCP-1, TNF-α, and NO in LPS-stimulated macrophages*

Some synthetic ligands for PPARγ are reported to have an anti-inflammatory effect on macrophages and adipocytes. Thus, we focused on the PPARγ agonist activation of DAA and investigated whether DAA inhibits the pro-