PPARγ Activation and Adipocyte Differentiation Induced by AS-6, a Prenyl-phenol Antidiabetic Antibiotic

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The prenyl-phenol antibiotics ascochlorin-related compounds, are known to reduce serum cholesterol and triglyceride, suppress hypertension, and ameliorate types-I and II diabetes. However, little is known about the molecular mechanism for these physiological effects. Here we report that the ascochlorin derivative, 4-*O*-carboxymethyl ascochlorin (AS-6) acts as a potent activator of the nuclear hormone receptor, PPARγ, although it does not activate the related receptors, PPARα, PPARδ or RARα. AS-6 interacts directly with the PPARγ molecule *in vitro*, and induces differentiation of the mouse preadipocyte cell line 3T3-L1. Our results suggest that AS-6 is a partial agonist for PPARγ with a novel chemical structure.

Adipocytes play a central role in lipid homeostasis and the maintenance of energy balance in vertebrate organisms, storing energy in the form of triglycerides during periods of nutritional abundance and releasing it in the form of free fatty acids at times of nutritional deprivation. An imbalance in lipid homeostasis can lead to obesity, which is associated with other serious medical conditions: type-II diabetes and cardiovascular disease[1,2]. The ability to isolate and characterize cell lines that progress from undifferentiated progenitor cells to mature adipocytes following appropriate stimulation has made it possible to identify the factors involved in adipocyte development[3]. Among these is the nuclear hormone receptor peroxisome proliferator activated receptor (PPAR)γ[3~5].

PPARγ is activated by the thiazolidinediones (TZDs), compounds originally synthesized as drug candidates to treat hyperlipidemia and insulin resistance[6,7]. These compounds bind to PPARγ with high affinity ($Kd=50\sim700$ nM) and enhance differentiation of preadipocyte cell lines into mature adipocytes. Three reagents of this class, pioglitazone, rosiglitazone and troglitazone, were approved by the United States Food and Drug Administration, and have been used largely in the treatment of type II diabetes. However, troglitazone was withdrawn from the market due to its hepatotoxicity. Several endogenous ligands including 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and linoleic acid were identified although their affinities are well below those of most other nuclear hormone receptors[4]. Because drugs targeted to PPARs have been demonstrated to be effective for hyperlipidemia and insulin resistance, there is an increasing interest in developing new compounds which activate or antagonize these receptors with high specificity and high affinity, as well as in searching for endogenous high-affinity ligands for these receptors.

Although ascochlorin-related compounds isolated from the fungus, *Ascochyta viciae*, were originally found as anti-

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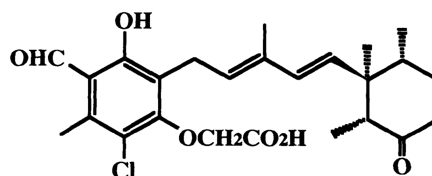
viral antibiotics[8], they can also modulate energy and lipid metabolism in animals. Ascofuranone significantly reduces serum lipid levels when orally administered to rats fed a cholesterol-rich diet[9]. This compound also markedly reduces hepatic and cardiac cholesterol content without affecting body weight[10]. 4-*O*-Methylascochlorin (MAC) effectively lowers plasma total cholesterol by i) enhancing the output of biliary cholesterol; ii) inhibiting intestinal cholesterol reabsorption followed by an increment of fecal neutral sterol loss; and iii) modulating cholesterol partition in the plasma[11~13]. MAC and ascofuranone attenuate hyperlipidemia and hypertension in deoxycorticosterone acetate-loaded uninephrectomized rats[14].

Another ascochlorin derivative, AS-6 (Fig. 1), potentiates insulin action both in normal and streptozotocin-induced diabetic rats[15,16]. It ameliorates polydipsia, polyuria and glycosuria; prevents pancreatic islet degeneration; and reduces insulin resistance in db/db genetically obese diabetic mice. AS-6 changes fatty acid composition[17] and significantly restores functional abnormalities and the calcium binding ability of the adipocyte plasma membrane to levels comparable to lean littermates[18,19]. These results suggest functional similarity of AS-6 to the thiazolidinediones in their therapeutic activities against diabetes. In this paper, we tested AS-6 for activation of PPARs, its interaction with PPARs *in vitro*, and its effect on adipocyte differentiation.

Fig. 1. Structure of AS-6.



Materials and Methods

Cell Culture

U2OS, a human osteosarcoma, was provided by C.-L. Wu (MGH Cancer Center, Charlestown, MA, USA). 3T3-L1[20], the preadipocyte cell lines, obtained from the Institute of Fermentation (Osaka, Japan). These cell lines were cultured in D-MEM supplemented with 5% fetal bovine serum (FBS) at 37°C in a humidified incubator under 10% CO_2 atmosphere.

Plasmids

Expression vectors of intact human nuclear receptors, corresponding Gal4-fusion nuclear receptors, and reporter plasmids for PPAR, RAR, and Gal4-fusion nuclear receptors[21~24] were kind gifts from S. KATO (University of Tokyo, Tokyo, Japan). pCMV-β-galactosidase was provided by N. H. HEINTZ (University of Vermont, Burlington, VT, USA). GST-tagged PPARγ was constructed by introducing the intact human PPARγ gene into pGEX plasmid (Promega).

Transient Transfection and Enzyme Assays

U2OS cells (1×10^6 cells in a 10 cm-diameter dish) were transfected with pCMV-β-galactosidase (8 μg) plasmid as an internal control, a reporter plasmid (8 μg) and a corresponding expression vector of the nuclear receptor (2 μg), and a carrier (DNA from salmon sperm, 6 μg) through the calcium phosphate precipitation method as described previously[25]. Cells were cultured for 12 hours and washed three times with PBS. After 4 hours culture in fresh medium, cells were trypsinized, resuspended in phenol red-free D-MEM containing 5% charcoal-stripped FBS and further cultured with drugs for 20 hours. Chloramphenicol acetyltransferase (CAT) expression as well as enzymatic activities of luciferase (Boehringer) and β-galactosidase activities (Clontech) were determined by commercial kits. CAT activation and luciferase activities were normalized by the β-galactosidase activity.

Adipocyte Differentiation

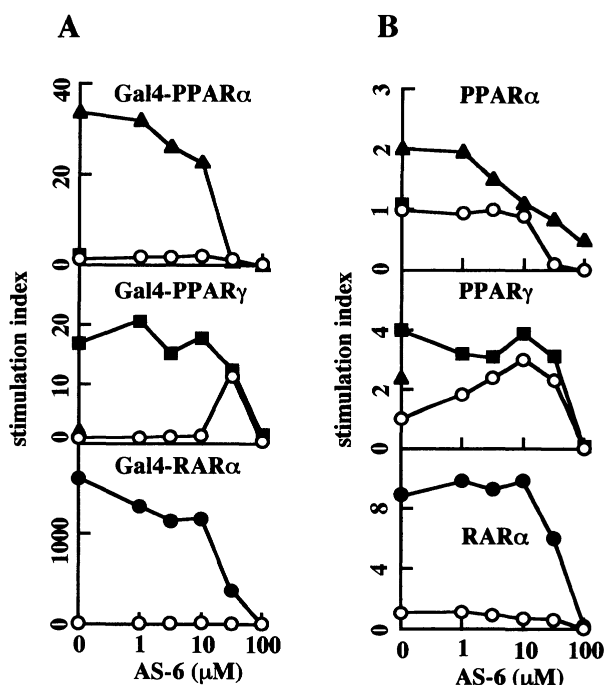
3T3-L1 (6×10^4 cells/well in a 24 well plate) was grown for 48 hours until confluence, treated with 1 μM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) for 48 hours, and then treated with 1 μg/ml insulin and drugs for 5 days. At the termination of the culture, differentiation was determined with triglyceride accumulation and glycerol phosphate dehydrogenase (GPDH) activity[26] in the cell lysate. The amount of triglyceride was determined with a commercial assay kit (Determiner TG, Kyowa Co., Tokyo, Japan).

In Vitro Interaction of AS-6 with PPAR-γ

The Biacore-biosensor system (Pharmacia) was used to determine the interaction between the drugs and the receptor PPARγ. Anti-GST antibody was immobilized on the sensor tip according to the supplier's instructions. GST-PPARγ expressed in bacteria was purified with glutathione-beads. The purified protein (10 μg/ml) followed by the test drug in running buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P-20) were run over the

sensor tip for 4 minutes with a flow rate of 10 μl/minute, and the tip was then washed with running buffer. The interaction was determined by relative resonance unit.

Fig. 2. AS-6 specifically activates PPARγ.



A human osteosarcoma, U2OS, was transfected with expression vectors for Gal4-fused nuclear receptors and a luciferase reporter plasmid bearing a Gal4 binding motif (A), or expression plasmids for full length nuclear receptors and corresponding CAT reporter plasmids (B) together with pCMV-β-galactosidase. The transfectants were further incubated for 20 hours in the presence of indicated concentrations of AS-6 without (open circle) or with 100 μM Wy14,643 (solid triangle), 10 μM pioglitazone (solid square) or 1 μM all-*trans*-retinoic acid (solid circle). Enzyme expression in the cytoplasm was determined. The stimulation index is the enhancement of enzyme expression normalized by β-galactosidase activity.

Results

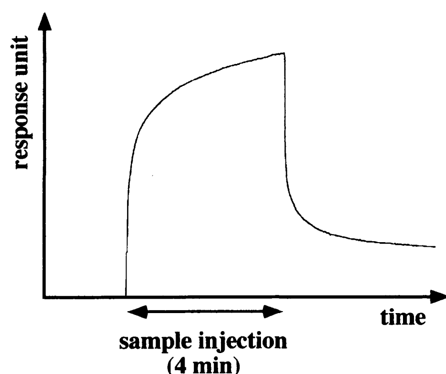
It was reported recently that PPARγ promotes adipocyte differentiation and plays a critical role in the control of insulin sensitivity and development of diabetes and that its expression occurs in a highly adipose-selective manner[4,5,27]. To study the effect of AS-6 on PPARs, we carried out reporter assays based on fusion proteins made up of the PPAR ligand binding domain and the Gal4 DNA binding domain. Because the Gal4 fusion nuclear receptor binds Gal4 binding elements on the reporter DNA, subtype specific transactivation activity through an ectopically expressed fusion receptor can be evaluated in a manner independent of endogenous PPARs. U2OS, a human osteosarcoma, was transfected with the expression vector of the fusion nuclear receptors and the reporter plasmid containing the Gal4 binding element upstream of the thymidine kinase promoter and luciferase gene; the induction of luciferase was determined 24 hours after the addition of AS-6 (Fig. 2A).

We found that AS-6 specifically activates the expression of PPARγ, having its maximum at 30 μM. This level of activation is 49% lower than the degree of activation produced by 10 μM pioglitazone. AS-6 does not activate either PPARα or RARα, while Wy14,643 increases transactivation activity 89-fold through Gal4-PPARα, and all-trans-retinoic acid increases transactivation 69-fold through Gal4-RARα. AS-6 does not activate Gal4-PPARδ (data not shown). Although AS-6 inhibits the PPARγ activity induced by pioglitazone at 30 and 100 μM, the inhibition is not specific, since the activities of Gal4-RARα induced by all trans-retinoic acid and Gal4-PPARα induced by Wy14,643 are also decreased by these concentrations of AS-6.

We also examined the effects of AS-6 on reporter experiments by transfecting U2OS cells with the expression vector of full-length human PPARα, PPARγ or RARα, and a corresponding reporter plasmid containing specific elements for each nuclear receptor upstream of the thymidine kinase promoter and the CAT gene (Fig. 2B). Similar to the results obtained with Gal4 fused nuclear receptors, AS-6 specifically activated PPARγ but not other nuclear receptors. However, the stimulation index was less than that obtained with Gal4-fusion nuclear receptors because of the higher background signal. Activation of these nuclear receptors by specific ligands was suppressed nonspecifically at higher concentrations of AS-6. These results suggest that AS-6 is a specific agonist for PPARγ.

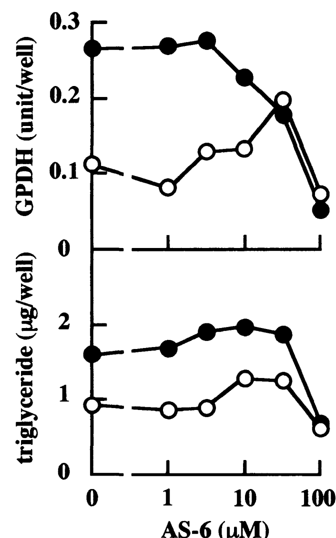
To confirm the direct interaction of AS-6 with PPARγ, GST-fused full-length PPARγ produced in bacteria was purified, and the interaction was assessed by Biacore, which detects the interaction between ligands and receptors fixed on the sensor tip as change of surface plasmon resonance. Anti-GST antibody was fixed on the sensor tip and the purified GST-PPARγ protein was bound. A dramatic increase in response was detected during injection of AS-6 (Fig. 3); the signal was partially returned to the basal level by washing with running buffer. This result demonstrates that AS-6 interacts with PPARγ directly and reversibly.

Fig. 3. Direct interaction of AS-6 and PPARγ.



GST-PPARγ produced by bacteria was bound to a sensor tip, and the interaction with AS-6 (100 μM) was detected by monitoring surface plasmon resonance.

Fig. 4. Induction of adipocyte differentiation by AS-6.



Confluent 3T3-L1 cells were treated with 1 μM dexamethasone and 0.5 mM IBMX for 48 hours, followed by 1 μg/ml insulin and 10 μM AS-6 in the presence (solid circle) or absence (open circle) of 10 μM pioglitazone for 5 days. Adipocyte differentiation was determined by triglyceride content and glycerol phosphate dehydrogenase (GPDH) activity in the cytoplasm.

PPARγ is known to be strongly expressed in adipocytes, and its ectopic expression in fibroblasts has been shown to result in adipocyte differentiation[5,27,28]. The treatment of preadipocyte and stem cell lines with high-affinity PPARγ ligands induces their conversion into mature adipocytes[28~30]. Confluent 3T3-L1 cells were incubated with dexamethasone and isobutyl-3-methylxanthine (IBMX), an inducer of cAMP, for 2 days, followed by incubation with AS-6 or pioglitazone in the presence of insulin for 5 days. Pioglitazone at 10 μM efficiently converted the cells into adipocytes as judged by triglyceride accumulation and GPDH, a marker enzyme of mature adipocytes (Fig. 4). AS-6 at 10 μM also induced triglyceride accumulation and GPDH activity, although the extent of differentiation were less than those observed with pioglitazone.

Discussion

Ascochlorin-related compounds modulate energy and lipid metabolism of animals and have therapeutic effects on disease models including diabetes and hyperlipidemia. However, the molecular mechanism for these physiological effects is unclear. In this report we have found that AS-6, a synthetic derivative of ascochlorin, like pioglitazone, activates PPARγ. AS-6 activates PPARγ specifically without activating other PPAR isotypes or RARα. We observed a direct interaction between AS-6 and PPARγ, as demonstrated by the Biacore sensor system. These results suggest that AS-6 is a specific agonist for PPARγ with a novel chemical structure and that the therapeutic effects of AS-6 on metabolic diseases are attributable to the agonistic activity for PPARγ.

Recent studies have shown that TZD family compounds can act as full agonists, partial agonists or antagonists depending on target cell types and the manner binding to PPARγ. For instance, troglitazone was shown to antagonize rosiglitazone-stimulated PPARγ transcriptional activity and at the same time act as a full agonist of PPARγ in 3T3-L1 cells[31]. MCC-555 has been shown to be a more potent activator of PPARγ than any other known TZD, yet its binding affinity to this receptor is less than 1/10 that of rosiglitazone[32]. The *N*-terminus of PPARγ is not required for activation by MCC-555, and MCC-555 does not stimulate corepressor recruitment to PPARγ. This selectivity of MCC-555 may contribute to its enhanced hypoglycemic potency *in vivo* despite its reduced affinity for PPARγ relative to other TZDs. These differences could

be caused by variations in the number of specific cofactors, differences in PPAR response elements, or the presence of different isoforms of PPARγ.

In another study, it was suggested that TZD-induced activation of PPARγ does not correlate directly with antidiabetic activity. Another new TZD, NC-2100, which has been shown to be a weak PPARγ activator and a weak inducer of adipogenesis of 3T3-L1 cells, efficiently lowered plasma glucose levels in KKAy obese mice in low doses[33]. It even caused the smallest body weight increase among the TZDs tested, which seems to be explained by the finding that NC-2100 significantly induces uncoupling proteins (UCPs) -1 and -2 in white adipose tissue.

In these respects, AS-6 seems to act as a partial agonist of PPARγ, although we failed to detect specific antagonism with a full agonist, pioglitazone, because of the toxicity of AS-6. It is possible that AS-6, which has a structure entirely different from TZD, activates PPAR-γ through a mechanism distinct from that of TZD. Further studies aiming at determining how AS-6 interacts with and activates PPARγ will reveal the mechanisms of the unique physiological activities of ascochlorin-related compounds.

AS-6, as a member of a new class of drugs with a unique pharmacological profile, may offer benefit for the treatment of human metabolic diseases. The chemical structure of ascochlorin is promising for the design of new compounds as modulators of nuclear receptors.

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