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Topical ER36009, a RARγ-Selective Retinoid, Decreases Abdominal White Adipose Tissue and Elicits Changes in Expression of Genes Related to Adiposity and Thermogenesis

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Chronic topical treatment of rats with a new RARγ-selective retinoid, ER36009, resulted in a significant reduction of epididymal white adipose tissue and a significant increase of interscapular brown adipose tissue without affecting food intake. ER36009 markedly decreased PPARγ, 11β-HSD1, and Bcl-2 mRNA levels, and increased Bax mRNA in white adipose tissue, while it upregulated UCP1 and UCP3 mRNAs in brown adipose tissue and UCP3 mRNA in gastrocnemial muscle. These results suggest that ER36009 has multiple effects on adipose tissue biology and the energy balance. Topically applied ER36009 may have potential for the treatment of obesity.

Key Words: ER36009; retinoid; RARγ; obesity; abdominal white adipose tissue.

Introduction

Obesity, which is a result of an imbalance between energy input (food intake) and energy expenditure, has become a worldwide public health problem. It is generally accepted that abdominal obesity or intraabdominal visceral obesity, caused by overnutrition and physical inactivity, promotes the development of metabolic syndrome, which leads to atherosclerotic disorders, such as cardiovascular disease *(1–7)*. Therefore, there is an urgent need for effective treatments that reduce adiposity, in addition to exercise and dietary control.

Retinoids, natural and synthetic derivatives of vitamin A, exert their effects through two distinct families of nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), both of which are composed of three subtypes, α, β, and γ *(8)*. These nuclear receptors are highly expressed in white and brown adipose tissues *(9,10)*. White adipose tissue (WAT) stores energy in the form of triacylglycerols and its overdevelopment leads to obesity, whereas brown adipose tissue (BAT) dissipates energy as heat by adaptive thermogenesis. In recent years, many studies have shown that retinoids have multiple effects on both brown and white adipose tissues and are involved in the control of adiposity and energy expenditure mechanisms *(10–14)*. Chronic vitamin A supplementation has been reported to cause a slight decrease of body adiposity *(15)*, while animals fed chronically with a vitamin A–deficient diet tended to develop excessive body weight *(16)*. In addition, acute all-*trans* retinoic acid (RA) treatment, whether oral *(17)* or subcutaneous *(16)*, causes a reduction of body weight and adiposity. The RA-induced reduction of adiposity correlated with inhibition of adipocyte differentiation *(18–20)*, with induction of apoptosis in adipocytes *(21,22)*, and with an increase of whole-body thermogenesis *(17,23,24)*.

For many years, RA and related synthetic retinoids have been used to treat various dermatological disorders *(25–30)*. Recently, a new RARγ-selective retinoid, ER36009, has been developed *(31)*. In the previous report, the selectivity of ER36009 for each receptor was indicated as relative 50% inhibitory dose (IC_{50}), obtained by dividing the IC_{50} value of ER36009 for a given receptor by that of RA. Kikuchi et al. showed that ER36009 exhibits a greater affinity for RARγ (relative IC_{50} 3.6) than for RARα (relative IC_{50} 83) or RARβ (relative IC_{50} 21). They also reported that the EC_{30} value (that dose which provides 30% of the maximal transactivation activity in the cotransfection assay) of ER36009 for RARγ was 0.34 relative to that of RA. (The relative EC_{30} values for RARα and RARβ were given as 5.2 and 0.39, respectively.)

This agent has marked comedolytic effects on rhino mouse skin, used as a comedone model *(32)*. Furthermore, we have demonstrated that ER36009 has potent skin-improving effects in a murine model of photoaging compared with RA *(33)*. We also noted a difference in the deposition of abdominal adipose tissue between the control and ER36009-treated groups (unpublished data). In this work, we have addressed this issue in detail, based on our hypothesis that ER36009 may depress adipogenic potential and increase thermogenic potential. We analyzed the effects of topically applied ER36009 and RA on body fat depots and

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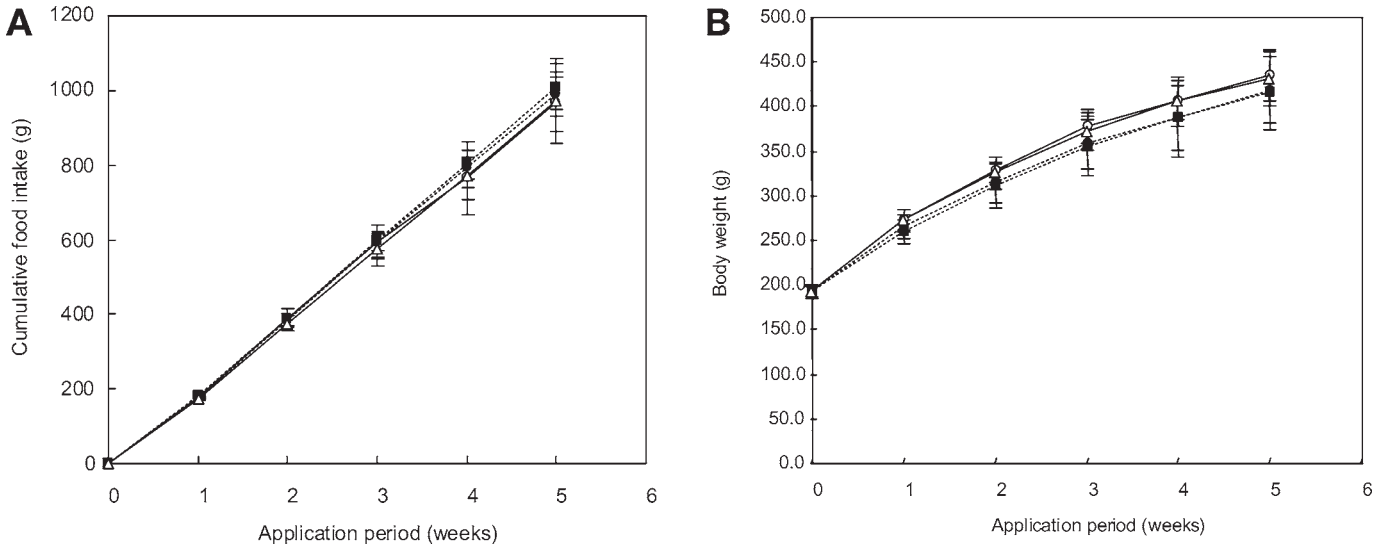


Fig. 1. Cumulative food intake and body weight gain in SD rats treated with ER36009, RA, or vehicle control (acetone). The rats were 7 wk old at the beginning of the study and were divided into four groups. During retinoid treatment, they were fed a commercial diet (CRF-1) *ad libitum* and both body weight and feed intake were checked every week. Each bar represents the mean ± SD ($n = 6$). Open circle: control, solid circle: 0.004 mg/kg ER36009, solid square: 0.008 mg/kg ER36009, open triangle: 0.1 mg/kg RA.

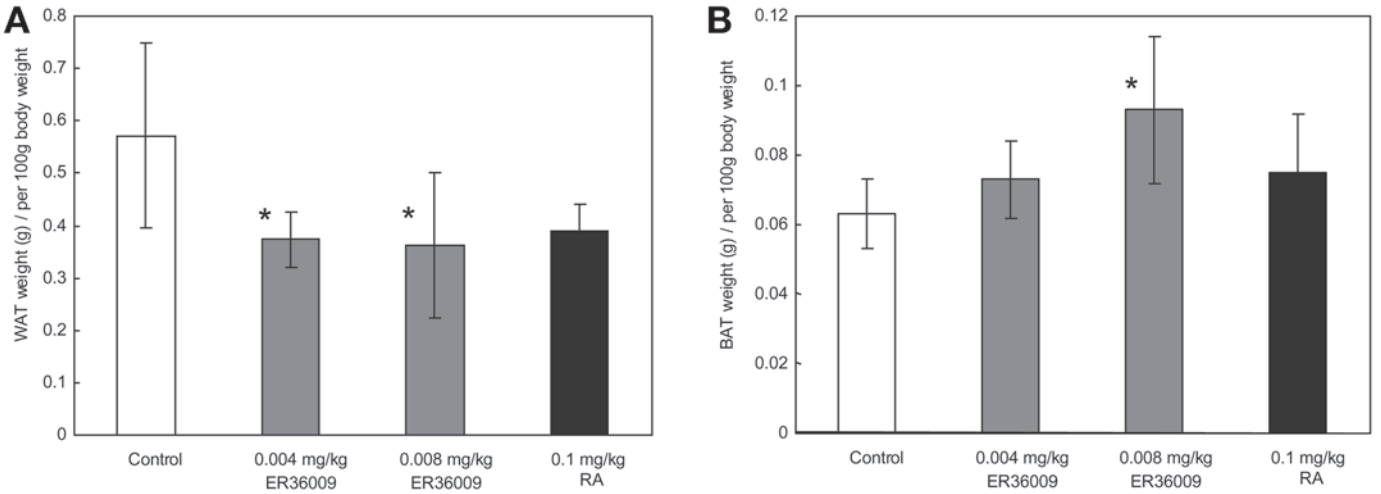


Fig. 2. Effect of retinoid treatment on the adipose tissues. (**A**) Epididymal white adipose tissue mass to body weight ratio. (**B**) Interscapular brown adipose tissue mass to body weight ratio. After the 5-wk retinoid treament, each adipose tissue was excised and weighed. Each bar represents the mean ± SD ($n = 5$–6). *$p < 0.05$ versus control.

on the expression of various genes related to adiposity and thermogenesis.

Results

Impact of Topical ER36009 on Body Weight Gain and Food Intake

Mild scaling and erythema were observed in all retinoid groups in the early period. Within 2 wk, these changes faded completely and the application area became smooth. We checked the food intake every week in all groups. There were no significant differences among any of the groups throughout the experiment (Fig. 1A). Although topical administration of ER36009 or RA caused subtle scaling at the application area, there was no effect on food intake, and weight gain was not suppressed (Fig. 1B).

ER36009 Affected Deposition of Adipose Tissue

Previous studies have demonstrated that body fat distribution is closely related to the occurrence of metabolic disorders; an excess of abdominal fat is a pivotal component of the metabolic syndrome. ER36009 significantly inhibited the accumulation of epididymal WAT in a dose-dependent manner (Fig.2A).

On the other hand, ER36009 dose-dependently increased interscapular BAT. The effect was statistically significant at 0.008 mg/kg ER36009 (Fig. 2B). RA administration had no significant effect on BAT or WAT.

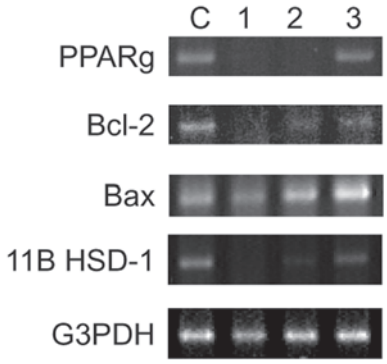


Fig. 3. Effect of retinoid treatment on gene expression of epididymal fat. Total RNA isolated from epididymal white adipose tissue was reverse-transcribed and amplified using specific primers. PPARγ, Bcl-2, Bax, 11β-HSD-1 and G3PDH mRNA were amplified for 32, 35, 35, 35, and 27 cycles, respectively. C: control (acetone), 1: 0.004 mg/kg ER36009, 2: 0.008 mg/kg ER36009; 3: 0.1 mg/kg RA.

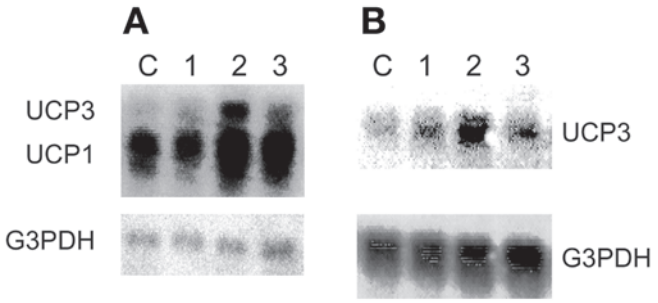


Fig. 4. Effect of retinoid treatment on the expression of UCP genes of brown adipose tissue and gastrocnemius muscle. Poly (A)+ RNAs (5 ng/lane) were prepared from interscapular brown adipose tissue or gastrocnemius muscle and Northern blot analysis was performed. (**A**) Interscapular brown adipose tissue, (**B**) gastrocnemius muscle. C: control (acetone), 1: 0.004 mg/kg ER36009, 2: 0.008 mg/kg ER36009; 3: 0.1 mg/kg RA.

Effects of ER36009 on Expression of PPARγ, Bcl-2, Bax, and 11β-HSD-1 Genes in Epididymal White Adipose Tissue

Adipose tissue secretes various bioactive substances, known as adipocytokines, and their dysregulation in abdominal or visceral obesity may participate in the development of the metabolic syndrome. To determine whether ER36009 affected gene expression in WAT, we examined the expression levels of several genes related to obesity or adiposity after the 5 wk administration of ER36009 (Fig. 3). ER36009 treatment markedly decreased the expression of the adipogenic transcription factor PPARγ in the epididymal WAT. It also dramatically reduced the expression of the antiapoptotic factor Bcl-2 and increased the expression of the pro-apoptotic protein Bax. The expression of 11β-HSD1 that was reported to be associated with the development of visceral obesity was also greatly decreased by administration of ER36009. On the other hand, RA also exerted similar effects on the expression of these genes, but except in the case of Bax, the effects of RA were weaker than those of ER36009.

ER36009 Treatment Upregulated Expression of UCPs in Brown Adipose Tissue and in Muscle

Because ER36009 dose-dependently increased interscapular BAT, we examined changes in the expression of UCP mRNAs in this tissue during chronic administration of retinoids. ER36009 (0.008 mg/kg) and RA upregulated UCP1 and -3 expression (Fig. 4A). However, the effect of ER36009 was superior to that of RA. In contrast, no marked change was seen in the low-dose ER36009 treatment group.

In addition, the UCP3 mRNA level in gastrocnemius muscle was markedly increased in 0.008 mg/kg ER36009-treated rats (Fig. 4B). Low-dose ER36009 and RA slightly upregulated the expression of the UCP-3 gene compared with the control group.

Discussion

Here we have shown that chronic treatment of mice with the new RARγ-selective retinoid ER36009 resulted in a significant reduction of epididymal WAT, as well as changes in the expression of various genes related to adiposity and thermogenesis in epididymal WAT, interscapular BAT, and gastrocnemial muscle. To our knowledge, this is the first report showing that a topically applied retinoid can improve abdominal obesity. It will be interesting to examine whether or not topical ER36009 also affects the subcutaneous WAT from an esthetic perspective, although we focused here on abdominal WAT because of the possible implications for treatment or prevention of metabolic syndrome.

In this study, ER36009 significantly inhibited accumulation of epididymal WAT, while having no effect on body weight gain. Felipe et al. reported that chronic dietary vitamin A supplementation retarded the increase of body weight only when animals were fed a high fat diet *(24)*. Thus, ER36009 may have had no effect on body weight because the animals were given a normal fat diet. Indeed, we confirmed that ER36009 and RA at higher doses both reduced body weight gain in animals on the normal fat diet (data not shown).

UCP1 is expressed predominately in brown adipose tissue *(34,35)* and is a key molecule for thermogenesis in this tissue, which is an important organ for cold- and diet-induced thermogenesis in rodents *(35,36)*. In contrast, the precise role of UCP3 in mitochondria is still controversial. Several transgenic animal studies suggest that UCP3 can increase energy expenditure and regulate body weight *(37–39)*. Other groups have suggested that the primary physiological role of UCP3 may be the mitochondrial handling of fatty acids, rather than the regulation of energy expenditure through thermogenesis *(40,41)*. If UCP3 protects mitochondria against fatty acid accumulation, it may help to maintain muscular fat oxidative capacity. Thus, in either

case, UCP3 is a candidate target for the prevention and/or treatment of obesity and diabetes. Our results show that topically applied ER36009 promoted the expression of both UCP1 mRNA and UCP3 mRNA in BAT, and also upregulated UCP3 expression in muscle. At least some of the effects of ER36009 may be exerted through these molecules.

Generally, adipose tissue mass is regulated by both the number and volume of adipocytes. Several lines of evidence suggest that in vivo, PPARγ is critical for adipocyte differentiation *(42–44)*. Its role in the control of body weight in animal models has been demonstrated *(45)*, and, more recently, a similar role has been suggested in humans *(46)*. In this study we found that ER36009 treatment led to a marked decrease in the expression of the PPARγ in the epididymal WAT. This result suggests that ER36009 prevents abdominal WAT deposition, at least in part, by attenuating the expression of PPARγ.

The proteins Bcl-2 and Bax are involved in apoptosis, and the Bcl-2/Bax ratio appears to determine the susceptibility of adipocytes to apoptosis *(47,48)*. We found that ER36009 markedly increased expression of the Bax gene and decreased that of the Bcl-2 gene in the epididymal WAT, resulting in a decrease of the Bcl-2/Bax ratio. In our previous study, we had shown that ER36009 administration restored normal apoptosis at the granular layer of rhino mouse skin, which is a mutant strain of hairless mouse with horn-filled utricles reminiscent of human microcomedos. Thus, ER36009 may induce apoptosis via altered expression of Bcl-2/Bax.

Transgenic mice overexpressing 11β-HSD-l, which converts inactive 11-dehydrocorticosterone [cortisone in humans] into active corticosterone (cortisol) selectively in adipose tissue, were recently shown to develop a full metabolic syndrome with visceral obesity, diabetes, and dyslipidemia *(49)*, whereas 11β-HSD-1 deficiency or inhibition has beneficial metabolic effects and results in reduced visceral fat accumulation upon high-fat feeding *(50–52)*. We found that ER36009 markedly inhibited 11β-HSD-l mRNA expression in abdominal WAT. Thus, ER36009 could have a beneficial effect on abdominal obesity by downregulation of 11β-HSD-l. PPARγ ligands inhibit adipocyte 11β-HSD1 expression and activity *(53)*. However, in this study, the expression of PPARγ was downregulated by administration of ER36009. Thus, the inhibitory effect of ER36009 on the expression of 11β-HSD-l appears to be independent of PPARγ.

In accordance with our previous findings, the new RARγ agonist ER36009 exerted potent effects at lower doses than the pan-RAR agonist RA. In WAT depots, RARα, RARγ, and RXRα are highly expressed, whereas RARβ and RXRγ are poorly expressed *(9,54)*. Indeed, the expression of RARγ in adult rodents is restricted to skin, lung *(55)*, and adipose tissue *(54)*. These results support the notion that RARγ may play a key role in the effects of retinoids on obesity. Our preliminary study confirmed that the synthetic RARα agonist ER34617 *(31)* had no effect on the deposition of abdominal fat in the same model, while the synthetic RXR pan-agonist ER35794 *(56)* increased intraabdominal obesity (data not shown). However, we cannot completely rule out the possibility that our present findings are specific to ER36009. Thus, it will necessary to confirm the findings with another RARγ-specific retinoid.

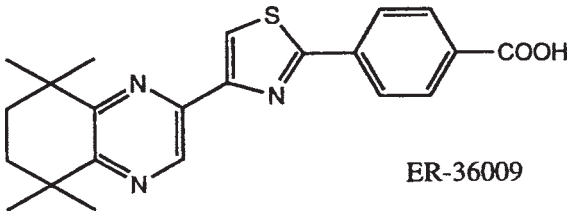


Fig. 5. Structure of ER36009.

In conclusion, chronic topical treatment of rats with a new RARγ-selective retinoid, ER36009, reduced abdominal obesity and elicited changes in the expression of various genes related to adiposity and thermogenesis in epididymal WAT, interscapular BAT, and gastrocnemial muscle. Therefore, topically applied ER36009 not only improves such skin disorders as acne and photoaging, but may also provide a new therapeutic strategy to treat obesity. Further work is required to elucidate in detail the molecular mechanisms of ER36009 action.

Materials and Methods

Chemicals

All-*trans*-retinoic acid (RA) was purchased from BASF (Wyandotte, MI). The synthetic retinoid ER36009 (Fig. 5) was kindly provided by Eisai Tsukuba Research Laboratories.

Animals

Twenty-four male Sprague–Dawley (SD) rats were obtained from Charles River Japan, Inc. (Yokohama, Japan). The rats were 7 wk old at the beginning of the study and were randomly divided into four groups, so that the mean body weights of the groups were identical. The groups were an acetone (control) group, a 0.004 mg/kg ER36009 group, a 0.008 mg/kg ER36009 group, and a 0.1 mg/kg RA group. They were fed a commercial diet (CRF-1, 0riental Yeast Co., Ltd., Tokyo, Japan) *ad libitum* and feed intake was checked three times a week to obtain the cumulative intake, which was recorded. This study was approved by the Shiseido Animal Care Committee.

Treatment

RA and ER36009 were each dissolved in acetone. Each stock solution was prepared in subdued light to a concentration of 0.1%, and diluted as required just before application. Between applications, stock solutions were stored under argon at 4°C. Aliquots of 100 μL of test materials were applied with a pipet on the dorsal skin of the animal, once

Table 1
Primer Sequences for Rat PPARγ, Bcl-2, Bax, 11β-HSD-1 and G3PDH

	Primer		Position
Rat PPARγ	5'-GGG AAT TCG GTG AAA CTC TGG GA-3'	[Sense]	53–435 *(57)*
	5'-ATG GAT CCT CTT CAT GTG GCC TG-3'	[Antisense]	
Rat Bcl-2	5'-CGG AAT TCA TAA CCG GGA GAT CGT-3'	[Sense]	263–569 *(58)*
	5'-TAG GAT TCA TCT CTG CAA AGT CGC-3'	[Antisense]	
Rat Bax	5'-GCG AAT TCA CAA CAT GGA GCT GC-3'	[Sense]	109–322 *(59)*
	5'-ATG GAT CCA AGT CCA GTG TCC AG-3'	[Antisense]	
Rat 11bHSD-1	5'-CGG AAT TCT TTG CTC TGG ATG GG-3'	[Sense]	889–1277 *(60)*
	5'-TTG GAT CCA TTT GTG GGT AGG GC-3'	[Antisense]	
Rat G3PDH	5'-GCG AAT CC AGA ACA TCA TCC CT-3'	[Sense]	633–813 *(61)*
	5'-TAG GAT CCT TCA CCA CCT TCT TG-3'	[Antisense]	

daily for 5 consecutive days per week (excluding Saturday and Sunday) during 5 wk. The control group was treated with acetone alone. All sites of solution application were shaved routinely with electric clippers.

Tissue Collection

Following anesthesia, interscapular BAT and epididymal WAT were excised, weighed, and rapidly frozen in liquid nitrogen. Gastrocnemius muscle was also sampled and frozen in liquid nitrogen. Organ weights were divided by the body weight of the rat and are given as relative values.

Isolation of Total RNA from Rat Tissues

To minimize RNA degradation, tissues were frozen in liquid nitrogen immediately after sampling and homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) with a SPEX CertiPrep 6700 cryogenic mill (SPEX CertiPrep, Metuchen, NJ). Total RNA was isolated with TRIzol reagent according to the manufacturer's protocol. The RNA pellet was dissolved in RNase-free water, and the amount of RNA was determined by measuring the absorbance at 260 nm with a spectrophotometer.

Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Complementary DNA (cDNA) was synthesized from 5 μg of total RNA using random primer in a 20 μL reaction volume containing reverse transcription reaction buffer (pH 8.3), 0.01 *M* dithiothreitol, 0.5 m*M* deoxynucleotide phosphate, and 200 U of Superscript II (Invitrogen). The mixture was incubated at 42°C for 50 min and then at 70°C for 15 min.

Primer sequences for rat PPARγ, Bcl-2, Bax, 11β HSD-1, and G3PDH are shown in Table 1 *(57–61)*. PCR was conducted in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) in a 50 μL reaction volume using AmpliTaq Gold (Applied Biosystems).

Reaction conditions were as follows; initial denaturation at 95°C for 10 min, 95°C for 30 s, 51°C for 30 s, 72°C for 30 s for 32 cycles (PPARγ); 95°C for 30 s, 51°C for 30 s, 72°C for 30 s for 35 cycles (Bcl-2); 95°C for 30 s, 51°C for 30 s, 72°C for 30 s for 35 cycles (Bax); and 95°C for 30 s, 51°C for 30 s, 72°C for 30 s for 35 cycles (11βHSD-1); 95°C for 30 s, 51°C for 30 s, 72°C for 30 s for 27 cycles (G3PDH).

The PCR products were analyzed by electrophoresis on 2% agarose gels and stained with ethidium bromide. All bands were excised from the gel, cloned into pBluescript II KS vector (Stratagene, La Jolla, CA), and sequenced.

RNA Probes for Northern Blot Analysis

The cDNAs for rat UCP1, UCP3, and G3PDH (rUCP1, rUCP3, and rG3PDH) were generated using RT-PCR. The following primers were designed for RT-PCR: sense primer, 5'-CGGAATTCAAGATGGTGAGTTCGAC-3' and antisense primer, 5'-TAGGATCCGTATCGTAGAG GCCAAT-3', for rUCP1 *(62)* ; sense primer, 5'-GCGAAT TCACTGTGGAAAGGGACT-3', and antisense primer, 5'-TAGGATCCATCCCAGACGCAGAAA-3', for rUCP3 *(63)*; sense primer, 5'-GCGAATTCCAGAACATCATCC CT -3', and antisense primer, 5'- TAGGATCCTTCACCAC CTTCTTG-3', for rG3PDH *(61)*. The underlined sequences correspond to the restriction sites for *EcoR*I and *BamH*I. The expected fragments correspond to the nucleotide sequences 177–472 for rUCP1, 519–841 for rUCP3, and 633–813 for G3PDH.

Total RNA from rat embryo (Ambion K.K., Tokyo) for rG3PDH, rat muscle (OriGene Technologies, Inc., Rockville, MD) for rUCP3, and rat BAT for rUCP1 were used for RT-PCR.

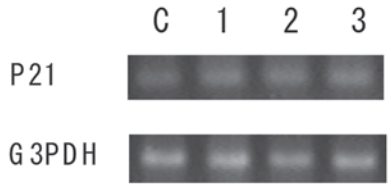
PCR parameters included initial denaturation at 95°C for 10 min, then 95°C for 30 s, 51°C for 30 s, 72°C for 30 s for 35 cycles. The isolated cDNA was digested with *EcoR*I/*BamH*I and then ligated into the *EcoR*I/*BamH*l site of pBluescript II KS vector (Stratagene, La Jolla, CA) , and sequenced. Digoxigenin (DIG)-labeled RNA probes for northern blot analysis were prepared with a Northern Starter Kit (Roche Diagnostics Japan, Tokyo) according to the manufacturer's protocol.

Northern Blot Analysis

Poly (A) + RNA were prepared by Oligotex-dT30 (Takara Bio Inc., Shiga). RNA samples were separated by electrophoresis on 1% formaldehyde/agarose gel and transferred onto a Hybond N+ membranes (Amersham Biosciences KK., Tokyo), then cross-linked by UV irradiation. DIG-labeled rUCP1, rUCP3, and rG3PDH antisense RNA probes were prepared as above. Hybridization was performed according to the DIG Northern Starter Kit's protocol. The hybridized probe was detected with an alkaline-phosphatase-conjugated anti-DIG antibody based on a chemiluminescence reaction. Detection of alkaline phosphatase activity with CDP-Star results in light emission that can be recorded by a Molecular Image System GS-250 (Bio-Rad, Hercules, CA).

Statistical Analysis

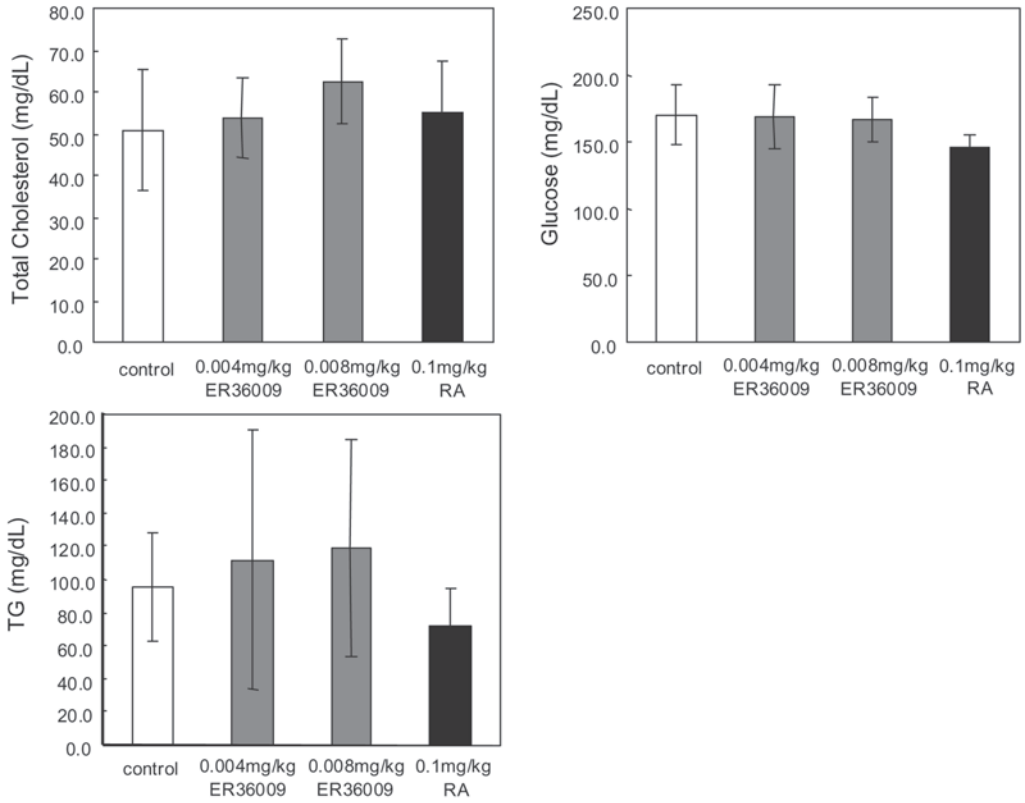
The significance of intergroup differences of white and brown adipose tissue mass were assessed by using Tukey's multiple comparison tests.



Appendix 1: Effect of Retinoid Treatment on the P21 Gene Expression

Total RNA isolated from epididymal white adipose tissue was reverse-transcribed and amplified using specific primers. P21 and G3PDH mRNA were amplified for 35 and 25 cycles, respectively. C: control (acetone), 1: 0.004 mg/kg ER36009, 2: 0.008 mg/kg ER36009, 3: 0.1 mg/kg RA.

The following primers were designed for RT-PCR: sense primer, 5'-AGGCAAGAGTGCCTTGACGA-3' and antisense primer, 5'-TCCTCTTGACCTGCTGTGTCG-3', for rat P21; sense primer, 5'-TGATTCTACCCACGGCAAGTT-3', and antisense primer, 5'-TGATGGGTTTCCCATTGATGA-3', for rat G3PDH.



Appendix 2: Effect of Retinoid Treatment on the Serum Total Cholesterol, Glucose and Triglyceride

Cholesterol E-test WAKO, Glucose CII-test WAKO, and Triglyceride E-test WAKO (WAKO, Tokyo) were used to assay. Each bar represents the mean ± SD (n = 6).

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