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Carvacrol prevents diet-induced obesity by modulating gene expressions involved in adipogenesis and inflammation in mice fed with high-fat diet☆

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Abstract

Carvacrol (2-methyl-5-isopropylphenol) is a monoterpene phenolic constituent of the essential oil produced by numerous aromatic plants and spices. The main objective of this study was to investigate effects of carvacrol in mice fed with a high-fat diet (HFD), which is an important model of obesity, and to study the potential underlying mechanisms focusing on the gene expression involved in adipogenesis, thermogenesis and inflammation. Male C57BL/6N mice were divided in three groups: those who received a normal diet, those fed with HFD and those fed with 0.1% carvacrol-supplemented diet (CSD). Body weight, visceral fat-pads and biochemical parameters were determined. Adipose tissue genes and protein expression levels were also assessed through reverse transcription polymerase chain reaction and Western blot analyses. Mice fed with CSD exhibited significantly reduced body weight gain, visceral fat-pad weights and plasma lipid levels compared with mice fed with HFD. Furthermore, HFD-induced up-regulations of adipose tissue genes and protein associated with the signaling cascades that lead to adipogenesis and inflammation were significantly reversed by dietary carvacrol supplementation. In summary, the major novel finding in our experimental conditions is that carvacrol prevented obesity in HFD-fed mice by decreasing body weight, visceral fat-pad weights and lowering plasma lipid levels. The evidence obtained in this study suggests that carvacrol appears to inhibit visceral adipogenesis probably by suppressing bone morphogenic protein-, fibroblast growth factor 1- and galanin-mediated signaling, and it also attenuates the production of pro-inflammatory cytokines in visceral adipose tissues by inhibiting toll like receptor 2 (TLR2)- and TLR4-mediated signaling.

Keywords: Carvacrol; Anti-obesity; Adipose tissue; Adipogenesis; Inflammation; Mice

1. Introduction

Obesity is one of the independent risk factors for serious health problems associated with chronic diseases such as diabetes [1,2], coronary heart disease [3], hyperlipidemia [4] and cancers [5–7]. High-level consumption of dietary fat is considered a major factor in promoting obesity and associated metabolic diseases in humans as well as in animal models [8]. The acute elevation of plasma free fatty acids (FFAs), which is caused by either increased release from enlarged adipose tissue or elevated intestinal influx from a high-fat diet (HFD), activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pro-inflammatory signaling cascade in the liver and adipose tissues. The result is the increased expression of several pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα), interleukin (IL)-1β and IL-6 observed in these tissues of obese animals [9]. Furthermore, the increased availability of FFAs appears to play a central role in the development of undesirable metabolic changes seen in the adipose tissue, skeletal muscle and liver of insulin-resistant animals and subjects [10].

Carvacrol (2-methyl-5-isopropylphenol) (Fig. 1) is a monoterpene phenolic constituent of essential oil produced by numerous aromatic plants and spices such as dittany of crete (*Origanum dictammus L.*), marjoram (*Origanum majorana L.*), spanish origanum (*Thymus capitatus L.*), summer savory (*Satureja hortensis L.*), thyme (*T. vulgaris L.*), white thyme (*T. serpyllum*), and winter savory (*S. montana L.*) [11–13]. Carvacrol is generally considered safe for consumption and has been included by the Council of Europe in the list of chemical flavorings that may be added to foodstuffs at the 2 ppm level in beverages and 25 ppm in candies. [11]. It has been reported that the median lethal dose of carvacrol in rats is 810 mg/kg of body weight when administered by oral gavages [14].

Oregano oil, whose major components include carvacrol and thymol, has been widely used as a dietary supplement for combating infections and relieving digestive and skin-related problems [15]. *Thymbra spicata var*. extracts, which contain mainly carvacrol, exhibited significant anti-hypercholesterolaemic, anti-oxidant and anti-steatohepatitic activities in mice with HFD-induced obesity [16]. Aside from these reported activities of carvacrol-containing plant

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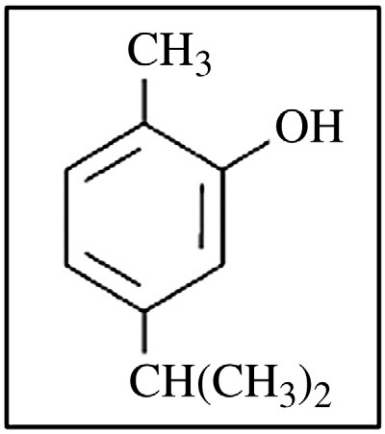


Fig. 1. Structure of carvacrol.

extracts, carvacrol itself has been observed with many diverse physiological actions, such as antibacterial and antifungal [17–19], anti-inflammatory [20], anti-hepatotoxic [21], anti-tumor [22] and anti-carcinogenic activities [23]. Inhibitory effects of carvacrol on 9,10-dimethyl-1,2-benzanthracene-induced tumorigenesis in rats and on the growth of melanomas in vitro were reported [22]. In addition, carvacrol has been reported to be a very potent inhibitor of cell growth in a human non-small cell lung cancer cell line, A549 [23]. More recently, Landa et al. demonstrated in an in vitro study that the possible anti-inflammatory potential of this compound was due to the inhibition of inducible cyclooxygenase-2 [20].

Although various physiological activities of carvacrol have been demonstrated, its link to obesity or metabolic diseases has never been explored. Therefore, the main objective of this study was to investigate anti-obesity effects of carvacrol in HFD-fed mice, which is an important model of obesity, and to explore potential underlying mechanisms, focusing on the expression of adipose tissue genes involved in adipogenesis, inflammation or thermogenesis.

2. Materials and methods

2.1. Chemicals and reagents

Carvacrol (MW 150.22 g/mol, 98% purity) was obtained from Sigma-Aldrich (St. Louis, MO, USA). 3T3-L1 fibroblast cells were purchased from the American Type Culture Collection (Manassas, VA, USA).

2.2. Cell culture and Oil-Red O staining

Mouse embryo 3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium, which was supplemented with 10% bovine calf serum and penicillin/streptomycin at 37°C in 5% CO_2. After the 3T3-L1 cells became confluent, the medium was replaced with differentiation medium containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1 μg/ml insulin, 1 μM dexamethasone (Sigma-Aldrich) and 0.5 mM isobutylmethylxanthine (Sigma-Aldrich) and cultured for 2 days. The cell medium was then replaced with 10% FBS plus 1 μg/ml insulin only, and the cells were fed every 2 days with the same medium. The cells were treated with varying doses of carvacrol (0.1, 1, 10, 50 and 100 μM) for 10 days during differentiation after confluence. On Day 10, cells were twice washed with phosphate-buffered saline (PBS) and fixed with 10% formalin for 1 h at room temperature and then stained with 5% Oil-Red O in isopropanol for 30 min. Cells were then washed with water and images of each dish were taken using Olympus (Tokyo, Japan) microscope. Stained oil droplets were dissolved in isopropanol and quantified by spectrophotometrical analysis at 600 nm.

2.3. Animal and diets

Twenty four male C57BL/6N mice (Orient, Gyeonggi-do, Korea) were housed in standard cages and placed in a room where the temperature was maintained at 21±2.0°C, relative humidity at 50±5% and the light at a 12-h light/dark cycle. All the mice consumed a commercial diet and tap water for 1 week before their division into three weight-matched groups (n=8): the normal diet (ND) group, HFD group and 0.1% (w/w) carvacrol-supplemented diet (CSD) group. The ND was a purified diet based on the AIN-76 rodent diet composition. The HFD was identical to the ND except for the addition of that 200 g of fat/kg (170 g of lard plus 30 g of corn oil) and 1% (w/w) cholesterol. The CSD was identical to the HFD and contained 0.1% carvacrol. The diets were given in pellet form for 10 weeks.

The mice's food intake was recorded daily, and their body weights were monitored every week during the feeding period. At the end of the experimental period, the animals were anesthetized with ether, following a 12-h period of fasting. Blood was drawn from the abdominal aorta into an ethylene diamine tetra acetic acid-coated tube, and the plasma was obtained by centrifuging the blood at 2,000×g for 15 min at 4°C. The livers and four different locations of visceral fat-depots (epididymal; mesenteric; perirenal; retroperitoneal) were removed, rinsed with PBS and then weighed. The plasma, liver and visceral fat-pad samples were stored at −70°C until they were analyzed. This study adhered to *the Guide for the Care and Use of Laboratory Animals* developed by the Institute of Laboratory Animal Resources of the National Research Council and was approved by the Institutional Animal Care and Use Committee of Yonsei University in Seoul, South Korea.

2.4. Biochemical analysis

Plasma concentrations of total cholesterol, high-density lipoprotein (HDL) cholesterol, triglyceride, glucose and FFA were enzymatically determined using commercial kits (Bio-clinical System, Gyeonggi-do, Korea). Plasma non-HDL cholesterol concentration was calculated by subtracting the HDL cholesterol concentration from the total cholesterol concentration. Hepatic lipids were extracted using the method developed by Folch et al. [24], and the dried lipid residues were dissolved in 2 ml ethanol. Cholesterol, triglyceride and FFA concentrations in the hepatic lipid extracts were measured using the same enzymatic kits that were applied for the plasma analyses.

2.5. RNA isolation and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from the epididymal and retroperitoneal adipose tissues of each mouse using Trizol (Invitrogen, Carlsbad, CA, USA) and then pooled for the RT-PCR analysis (n=8). Four micrograms of the total RNA was reverse-transcribed using the Superscript II kit (Invitrogen) according to the manufacturer's recommendations. The GenBank accession numbers of the relevant templates and the forward and reverse primer sequences are shown in Table 1. Primers were also designed to amplify the 530-bp cDNA fragment encoding glyceraldehydes 3-phosphate dehydrogenase (GAPDH) as an internal control. The cDNA was served as a template in a 40 μl reaction mixture and was processed using an initial step at 94°C for 5 min, followed by 30–33 amplification cycles (94°C for 30 s; 55–60°C for 45 s; 72°C for 1 min) and 72°C for 10 min. Five microliters of each PCR reaction was mixed with 1 μl of six-fold concentrated loading buffer and loaded onto a 2% agarose gel containing ethidium bromide. RT-PCRs were done in triplicates, and standard errors of means were calculated. Transcription amounts were normalized against the GAPDH transcript.

2.6. Western blot analysis

The epididymal adipose tissue samples obtained from each mouse were homogenized in an extraction buffer containing 100 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM sodium pyrophosphate, 50 mM NaF, 100 mM orthovanadate, 1% Triton X-100, 1 mM phenylmethanesulohonylfluoride, 2 μg/ml aprotinin, 1 μg/ml pepstatin A and 1 μg/ml leupeptin. Homogenates were centrifuged at 1,300×g for 20 min at 4°C. Protein concentrations in homogenates were measured by the Bradford assay (Bio-Rad, Hercules, CA, USA). The protein samples were subjected to 8% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Amersham, Buckinghamshire, UK). The samples were incubated overnight with primary antibodies (diluted 1:1,000) at 4°C. Antibodies to the following proteins were purchased from the indicated sources: extracellular signal-regulated kinase (ERK), phospho-ERK (Thr 202/Tyr 204) and phospho-IRF3 (Ser 396) from Cell Signaling Technology (Beverly, MA, USA); IRF3 from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After the membrane was incubated with the relative secondary antibody, immunoreactive signals were detected using the chemiluminescent detection system (Amersham, Buckinghamshire, UK) and were quantified using the Quantity One analysis software (Bio-Rad).

2.7. Statistical analysis

Results were expressed as mean±S.E.M. of eight mice in each group. The RT-PCR data were presented as means±S.E.M. of the triplicate analyses of the RNA samples pooled from eight mice per group. Statistical significance was calculated using one-way analysis of variance, followed by Duncan's multiple range tests. All the statistical analyses were carried out with the use of SPSS 12.0 software. Differences were considered significant when P<.05.

Table 1
Primer sequences and PCR conditions

Gene description	Primers	Sequences (5′→3)	Annealing temperature (°C)	PCR product (bp)
BMPR	F	CCCAGCTACGCAGGACAATA	55	142
	R	GCCATGGAAATGAGCACAAC		
Shn2	F	GGAGATTCTTCCAGGGTCCA	55	177
	R	CCCTTCCTCTTCCTGCTGAC		
GalR1	F	CCAAGGGGGTATCCCAGTAA	60	134
	R	GGCCAAACACTACCAGCGTA		
GalR2	F	ATAGTGGTGGCATGCTGGAA	60	227
	R	AGGCTGGATCGAGGGTTCTA		
FGFR1	F	CTTGACGTCGTGGAACGATCT	55	344
	R	AGAACGGTCAACCATGCAGAG		
PKCδ	F	CTGAGCGCTGCAAGAAGAAC	60	146
	R	TGGAAACTTTGCCTCCTCCT		
Ctss	F	TCCCGGTCTTTGGCTATTTT	55	149
	R	CCTCCACAGCCTTTATTCCC		
Cyc-D	F	TGGGAAGTTTTGTTGGGTCA	55	144
	R	TCCTTGTCCAGGTAATGCCA		
E2F1	F	CCTCGCAGATCGTCATCATC	55	102
	R	AGAGCAGCACGTCAGAATCG		
SREBP-1c	F	TTGTGGAGCTCAAAGACCTG	55	94
	R	TGCAAGAAGCGGATGTAGTC		
PPARγ2	F	TTCGGAATCAGCTCTGTGGA	55	148
	R	CCATTGGGTCAGCTCTTGTG		
C/EBPα	F	AAGGCCAAGAAGTCGGTGGA	55	189
	R	CCATAGTGGAAGCCTGATGC		
LXR	F	TCCTACACGAGGATCAAGCG	55	119
	R	AGTCGCAATGCAAAGACCTG		
Leptin	F	CTCCAAGGTTGTCCAGGGTT	55	143
	R	AAAACTCCCCACAGAATGGG		
aP2	F	ACATGAAAGTGGGAGTG	55	128
	R	AAGTACTCTCTGACCGGATG		
LPL	F	TGCCGCTGTTTTGTTTTACC	55	172
	R	TCACAGTTTCTGCTCCCAGC		
TLR2	F	GAGCATCCGAATTGCATCAC	55	120
	R	TATGGCCACCAAGATCCAGA		
TLR4	F	TCGAATCCTGAGCAAACAGC	55	199
	R	CCCGGTAAGGTCCATGCTAT		
MyD88	F	AAGAAAGTGAGTCTCCCCTC	55	149
	R	TCCCATGAAACCTCTAACAC		
Tirap	F	GCTTCCAGGGGATCTGATGT	55	183
	R	AAGCAAGCCTACCACGGACT		
TRAF6	F	GCACAAGTGCCCAGTTGACAATGA	55	689
	R	AGTGTCGTGCCAAGTGATTCCTCT		
TRIF	F	ATGGATAACCCAGGGCCTT	55	528
	R	TTCTGGTCACTGCAGGGGAT		
IRF5	F	ACCCGGATCTCAAAGACCAC	55	166
	R	TTATTGCATGCCAACTGGGT		
TNFα	F	TGTCTCAGCCTCTTCTCATT	55	156
	R	AGATGATCTGAGTGTGAGGG		
IFNα	F	GATGCCCAGCAGATCAAGAA	55	105
	R	CCATGCAGCAGATGAGTCCT		
Sirtuin 1	F	AGCTCCTTGGAGACTGCGAT	55	182
	R	ATGAAGAGGTGTTGGTGGCA		
PGC-1α	F	ACTGACAGATGGAGCCGTGA	60	180
	R	GCTGCATGGTTCTGAGTGCT		
RXR	F	TCCATGTCTGCTGTGCTGTG	55	177
	R	TCTGAGTCCCTCCGTGTGAA		
UCP1	F	CTGGGCTTAACGGGTCCTC	60	100
	R	CTGGGCTAGGTAGTGCCAGTG		
UCP3	F	ATGCTGAAGATGGTGGCTCA	55	179
	R	TTGCCTTGTTCAAAACGGAG		
GAPDH	F	AGAACATCATCCCTGCATCC	60	321
	R	TCCACCACCCTGTTGCTGTA		

3. Results

3.1. Effects of carvacrol on adipocyte differentiation

The ability of carvacrol to modulate adipocyte differentiation was screened in the 3T3-L1 cells. Carvacrol exhibited a cell growth inhibitory effect, with IC_{50} values in 273 μM against the 3T3-L1 cells. As shown in Fig. 2A, treating 3T3- L1 cells with carvacrol inhibited adipocyte differentiation at a concentration of 10, 50 and 100 μM. As shown in the Oil-Red O elution of differentiated 3T3-L1 cells (Fig. 2B), carvacrol treatment significantly reduced intracellular fat accumulation in a dose-dependent manner relative to the control cells treated with dimethyl sulfoxide (DMSO). These results indicate that carvacrol may have efficiently blocked adipocyte differentiation in a cultured 3T3-L1 cell line.

3.2. Body and visceral fat-pad weights

Mice fed with CSD exhibited significantly reduced final body weight (24% decrease, $P<.05$) and body weight gain (43% decrease, $P<.05$) compared with the values obtained with HFD-fed mice. As food intake did not differ among experimental groups during the 10-week feeding period, the food efficiency ratio was significantly higher in the HFD mice than in the ND mice and was significantly decreased in the CSD group than in the HFD mice (Table 2). The total visceral fat-pad weight of mice fed with the HFD diet, which was significantly greater than with the ND mice (by 145%, $P<.05$), was reduced when the mice were administered with carvacrol (by 36%, $P<.05$). The epididymal, mesenteric, perirenal and retroperitoneal fat-pad weights of mice given with carvacrol were significantly decreased by 31%, 51%, 55% and 23%, respectively, compared with those for the HFD-fed mice ($P<.05$) (Table 2).

3.3. Plasma and hepatic biochemistries

The HFD-induced hypercholesterolemia was significantly improved by dietary supplementation with carvacrol. Plasma concentrations of total and non-HDL cholesterol were significantly decreased by 29% and 46%, respectively, in mice fed with CSD than in the HFD-fed group. The HFD-induced elevation in the plasma free fatty acid concentration was also significantly reversed when the mice were fed with CSD (80% reduction, $P<.05$). Dietary carvacrol supplementation tended to decrease plasma triglyceride levels compared with that of HFD control mice, without showing a statistical significance (Table 3).

Relative weights of the liver, which were significantly increased in HFD-induced obese mice, were significantly reduced by supplementing the HFD with carvacrol (26% reduction, $P<.05$). Similarly, hepatic cholesterol and triglyceride concentrations, which were elevated prominently in the HFD group than in the ND mice, were found to be significantly decreased by dietary carvacrol supplementation (41%, and 37% reductions in hepatic cholesterol and triglyceride levels, respectively, $P<.05$). The HFD-induced elevation in the hepatic FFA concentration was significantly ameliorated in the liver of CSD mice (58% reduction, $P<.05$) (Table 2).

3.4. Expression of signaling molecules related to adipogenesis

We evaluated the carvacrol-induced changes in the expression of visceral adipose tissue genes associated with adipogenesis through RT-PCR analyses of epididymal adipose tissue of mice. Compared with the ND group, the HFD mice exhibited significantly elevated adipose tissue mRNA levels of cyclin D (Cyc-D), E2F transcription factor 1(E2F1), sterol regulatory element-binding factor 1 (SREBP-1c), peroxisome proliferator-activated receptor gamma 2 (PPARγ2), CCAAT/enhancer binding protein, alpha (C/EBPα), liver X receptor (LXR), leptin, adipocyte protein 2 (aP2), lipoprotein lipase (LPL), bone morphogenetic protein receptor (BMPR), schnurri 2 (Shn2), galanin receptor 1 (GalR1), galanin receptor 2 (GalR2), protein kinase C delta (PKCδ), fibroblast growth factor receptor 1 (FGFR1) and cathepsin S (Ctss). These HFD-induced up-regulations of adipose tissue genes associated with

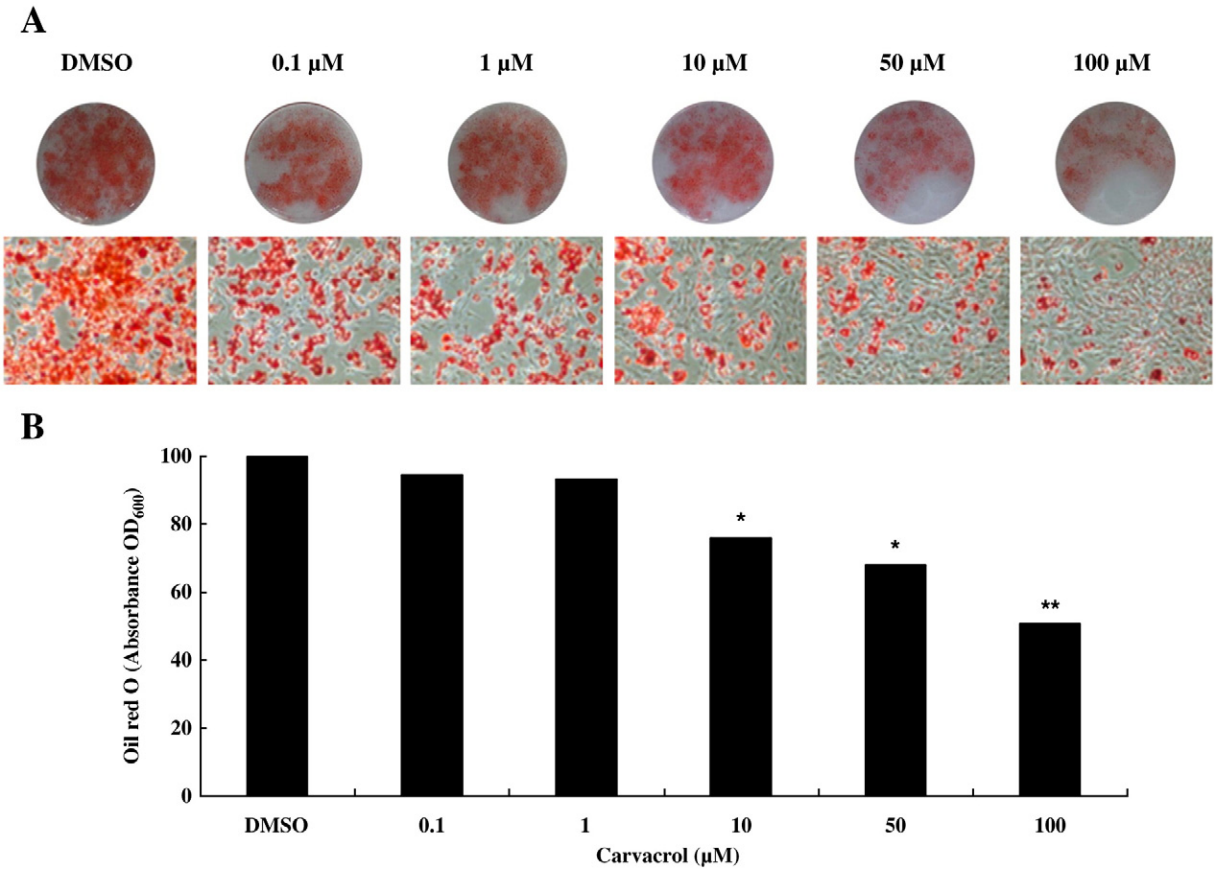


Fig. 2. Inhibition of adipocyte differentiation by carvacrol. 3T3-L1 preadipocytes were grown in the MDI (1-methyl 3-isobutylxanthine, dexamethasone, and insulin) medium for the first 2 days and treated with the indicated concentrations of carvacrol. Ten days after the initiation of differentiation, visualization of triglyceride accumulation by Oil Red O staining and high magnification (×200) of cells was conducted (A). Cells were harvested and the lipid accumulation was measured through a spectrophotometer (B). Each value was expressed as the mean±S.E.M. of five independent experiments. *P <.05 and **P <.01 vs. the control.

the signaling cascades that lead to adipogenesis were significantly reversed by dietary carvacrol supplementation. In the CSD group, the expression was approximately 1.5-fold lower for Shn2, GalR1, GalR2, PKCδ, FGFR1, Cyc-D, E2F1, PPARγ2, LXR, leptin, aP2 and LPL genes and two to three folds lower for BMPR, Ctss, SREBP-1c and C/EBPα genes in their epididymal adipose tissues as compared with the values for the HFD group (Fig. 3A and B).

We also analyzed the protein content of ERK 1/2 and phospho-ERK in the epididymal adipose tissue by the Western blot method. Compared with that of the ND mice, ERK phosphorylation (Thr202/Tyr204) was significantly higher in the visceral adipose tissue of HFD-fed animals and dietary carvacrol supplementation of HFD effectively blocked this HFD-induced up-regulation of ERK phosphorylation (36% reduction in CSD-fed mice *vs.* the HFD group, P<.05) (Fig. 3C).

3.5. Expression of genes related to thermogenesis

Next, we analyzed the mRNA levels of genes involved in thermogenesis in the visceral adipose tissue of mice through semi-

Table 2
Body weight, food intake and visceral fat-pad weight of mice fed experimental diets

Groups	ND	HFD	CSD
Body weight (g)			
Final body weight	30.4±0.50^b	41.5±0.70^a	31.6±1.70^b
Weight gain (g/10 weeks)	11.5±0.86^b	21.5±0.70^a	12.3±1.70^b
Food intake (g/day)	2.75±0.11	2.69±0.00	2.69±0.00
Food efficiency ratio	0.06±0.00^b	0.11±0.00^a	0.06±0.00^b
Visceral fat-pad weight (mg/g body weight)			
Epididymal adipose tissue	22.0±4.80^b	54.4±2.50^a	37.7±6.50^b
Mesenteric adipose tissue	12.0±2.30^b	23.3±2.30^a	11.5±1.90^b
Perirenal adipose tissue	1.73±0.27^b	6.60±0.60^a	3.00±0.70^b
Retroperitoneal adipose tissue	4.58±1.05^c	14.7±0.60^a	11.3±2.20^b
Total	40.3±8.04^b	99.0±4.60^a	63.6±11.0^b

Values are expressed as means±S.E.M. (n=8). Means in the same row not sharing a common superscript are significantly different at P<.05.
FER (food efficiency ratio) = $\frac{\text{Body weight gain for experimental period (g)}}{\text{Food intake for experimental period (g)}}$.

Table 3
Plasma and hepatic biochemistries of mice fed experimental diets

Groups	ND	HFD	CSD
Plasma			
Total cholesterol (mmol/L)	2.02±0.06^c	3.83±0.32^a	2.73±0.30^b
HDL cholesterol (mmol/L)	1.33±0.09^b	1.78±0.07^a	1.68±0.16^{ab}
Non-HDL cholesterol (mmol/L)	0.69±0.15^b	2.05±0.25^a	1.11±0.05^b
Triglyceride (mmol/L)	0.60±0.09^b	1.41±0.12^a	1.18±0.14^a
Free fatty acid (mEq/L)	573±36.4^b	1416±128^a	280±35.2^c
Liver			
Liver weight (g/100 g body weight)	3.60±0.10^c	5.50±0.30^a	4.10±0.10^b
Cholesterol (μmol/g liver)	23.3±2.31^c	76.5±2.51^a	45.4±5.48^b
Triglyceride (mg/g liver)	20.4±1.12^b	35.1±1.22^a	22.0±6.02^b
Free fatty acid (mg/g liver)	8.56±1.13^b	24.3±0.86^a	10.3±0.73^b

Values are expressed as means±S.E.M. (n=8). Means in the same row not sharing a common superscript are significantly different at P<.05.

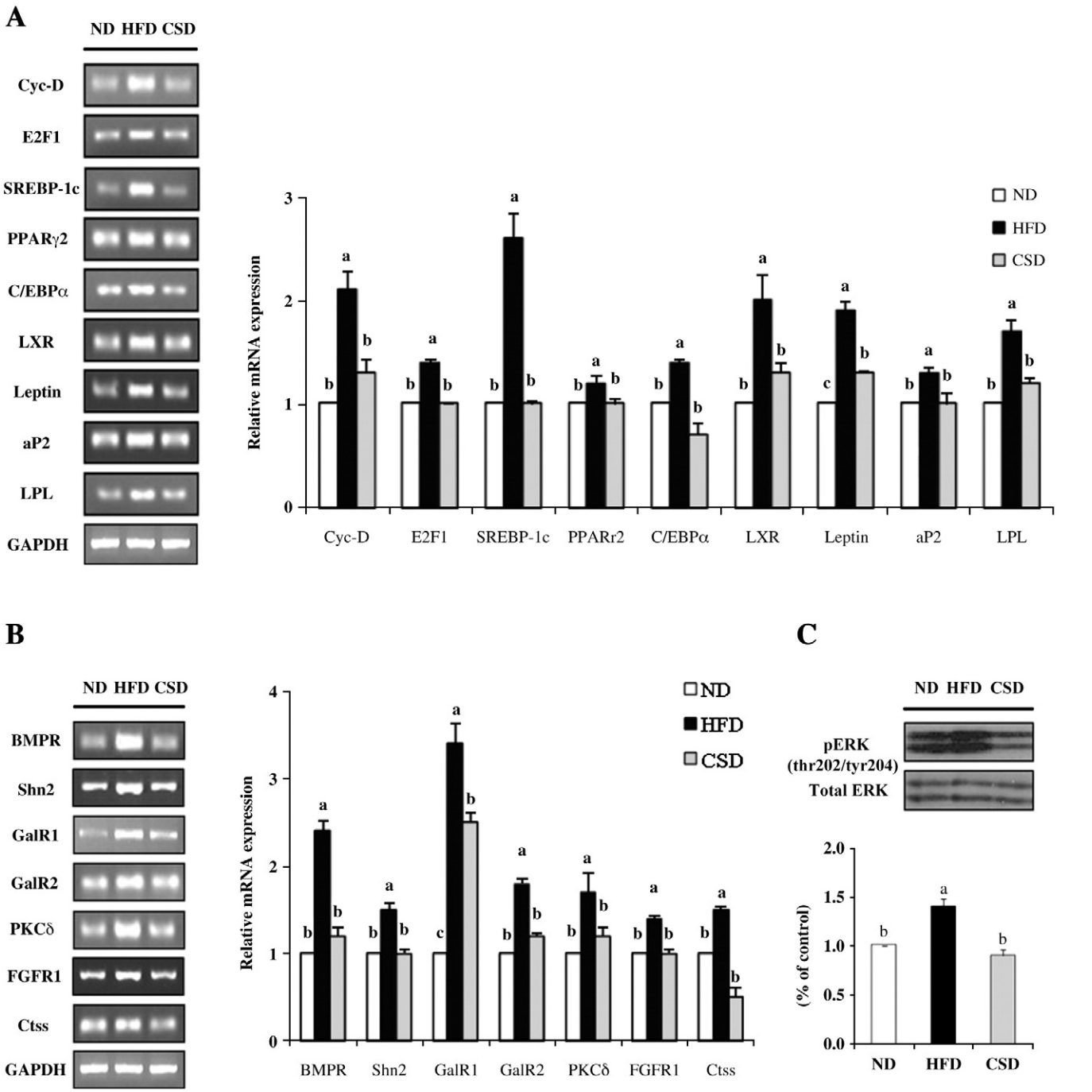


Fig. 3. Effects of dietary carvacrol supplement on mRNA expression of genes related to adipogenesis in the epididymal adipose tissue of mice. Semiquantitative RT-PCR analyses of (A) adipogenic transcription factors and (B) their upstream signaling molecules in epididymal adipose tissue. Bars represent mean±S.E.M., $n=8$. For each gene, means without a common letter differ, $P < 0.05$. The data represent relative density normalized to GAPDH. (C) Protein levels of pERK determined by Western blot.

quantitative RT-PCR analysis (Fig. 4). Compared with the ND group, the HFD mice showed significantly reduced expressions of several adipose tissue genes in relation to thermogenesis, including silent mating type information regulation 2, homolog 1 (SIRT1), peroxisome proliferative activated receptor γ, coactivator 1 alpha (PGC-1α), retinoid X receptor (RXR), uncoupling proteins 1 (UCP1) and uncoupling proteins 3 (UCP3). Carvacrol supplementation failed to recover these HFD-induced down-regulation of thermogenesis-related genes in the visceral adipose tissue of mice and, it further decreased the expression of abovementioned genes below the values observed in the HFD mice (Fig. 4).

3.6. Expression of genes related to inflammation

Feeding mice with HFD for 10 weeks led to significant up-regulations of epididymal adipose tissue genes, such as toll-like receptor 2 (TLR2), toll-like receptor 4 (TLR4), myeloid differentiation primary response gene 88 (MyD88), toll-interleukin 1 receptor domain-containing adaptor protein (Tirap), TNF receptor-associated factor 6 (TRAF6), and TIR-domain-containing adapter-inducing interferon-β (TRIF) as well as their downstream transcription factor, interferon regulatory factor 5 (IRF5), and its target genes such as tumor necrosis factor-alpha (TNFα) and interferon-alpha

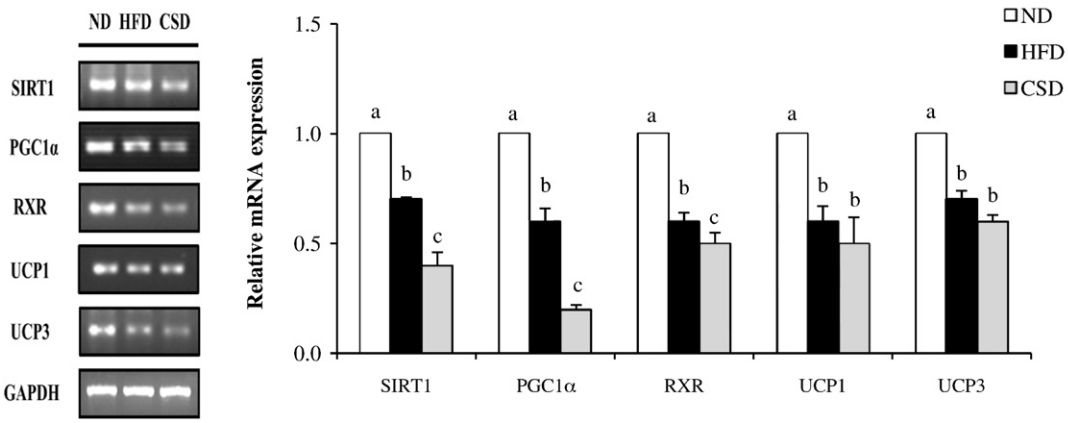


Fig. 4. Effects of dietary carvacrol supplement on mRNA expression of genes related to thermogenesis in retroperitoneal adipose tissue of mice. Semi-quantitative RT-PCR analyses of SIRT1, PGC-1α, RXR, UCP1 and UCP3 in retroperitoneal adipose tissue. Bars represent mean±S.E.M., $n=8$. For each gene, means without a common letter differ, $P<.05$. The data represent relative density normalized to GAPDH.

(IFNα). These HFD-induced elevations in TLRs mediated pro-inflammatory signaling molecules were almost completely abolished by dietary carvacrol supplementation. In the CSD group, the visceral adipose tissue expression was approximately 1.5-fold lower for TLR2, TLR4, Tirap, TRAF6, IRF5, TNFα and IFNα genes and two to three folds lower for MyD88 and TRIF genes than in the HFD group (Fig. 5A and B).

The protein levels of interferon regulatory factor 3 (IRF3) and phospho-IRF3 in the epididymal adipose tissue of mice were determined by the Western blot method. Ser396 phosphorylation of IRF3 in the adipose tissue was 240% higher in the HFD mice than in the ND mice and 41% lower in the CSD-fed mice than in the HFD group ($P<.05$) (Fig. 5C).

4. Discussion

Carvacrol-treated 3T3-L1 adipocytes during differentiation inhibited lipid accumulation in a dose-dependent manner at concentrations of 10, 50 and 100 μM by 24%, 32% and 49% ($P<.05$), respectively. The maximum reductive effects on lipid accumulation of carvacrol observed in this present study are lower than the values for resveratrol (94.3% reduction at 50 μM [25]), genistein (94% reduction

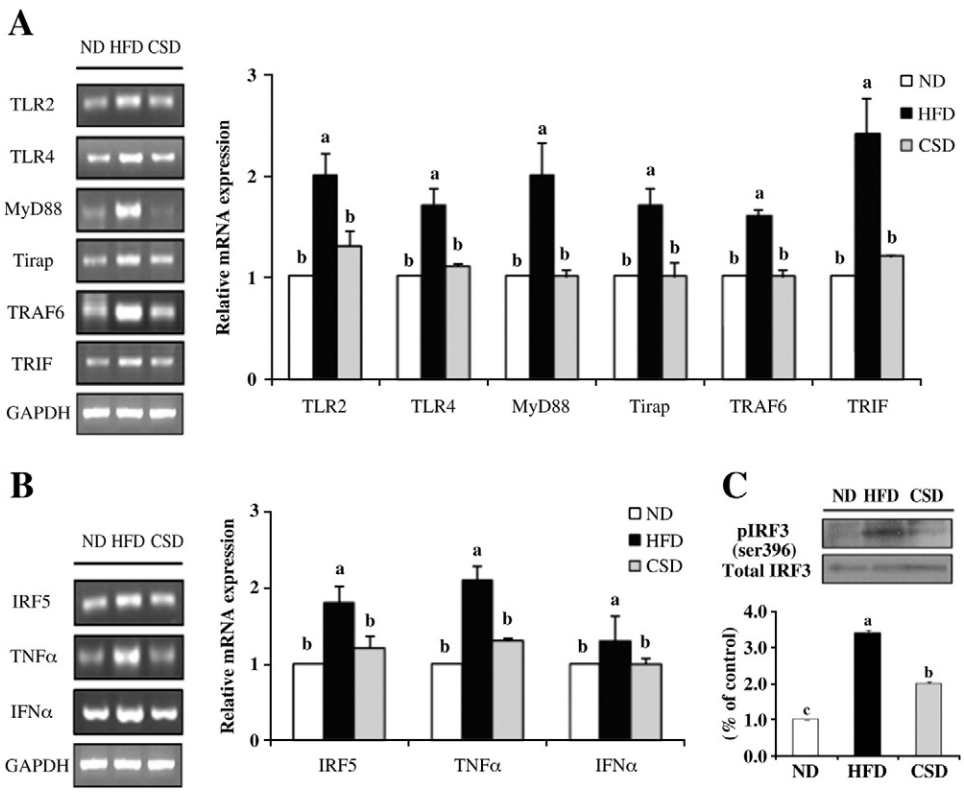
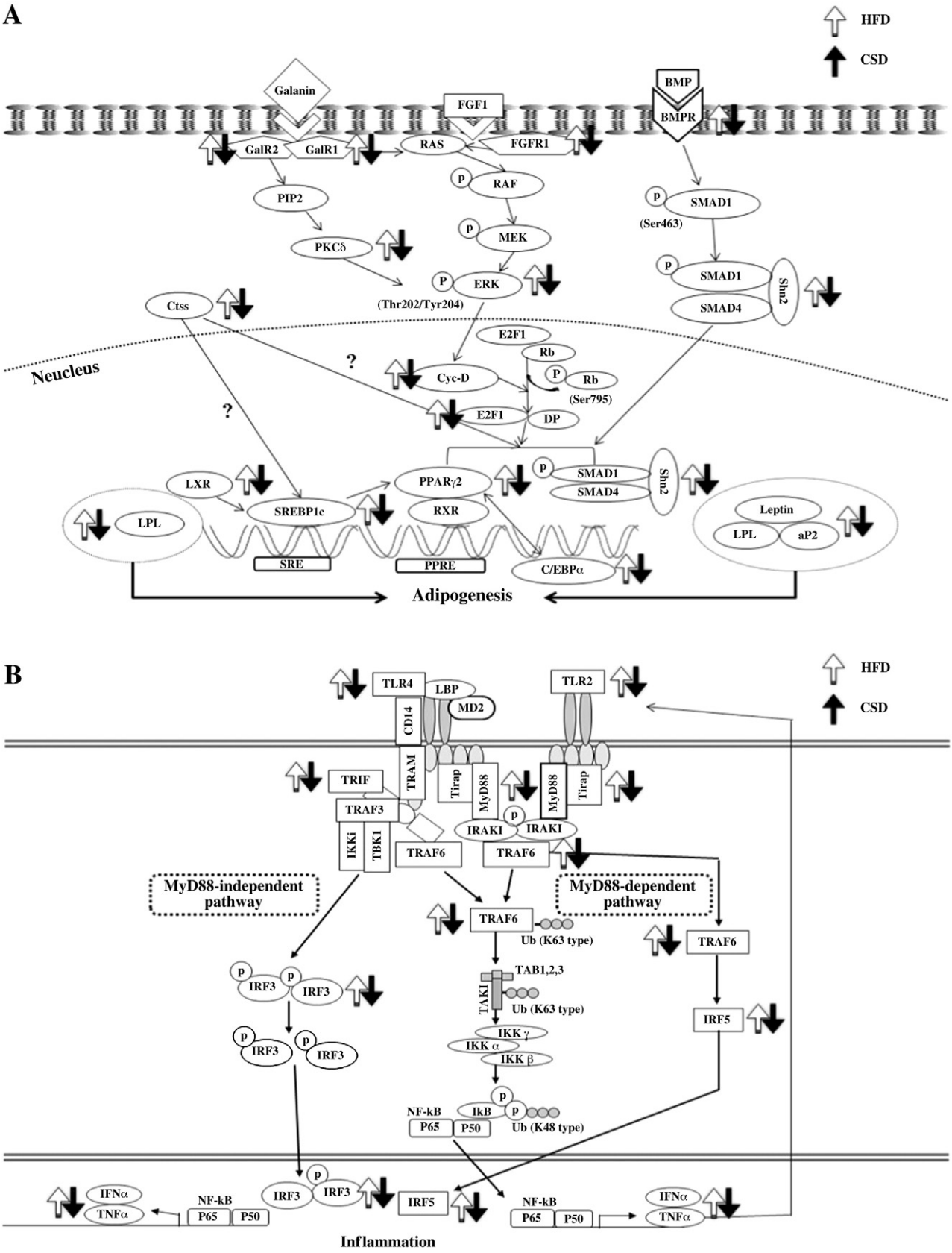


Fig. 5. Effects of dietary carvacrol supplement on mRNA expression of genes related to inflammation in the epididymal adipose tissue of mice. Semi-quantitative RT-PCR analyses of (A) TLR2- and 4-mediated signaling molecules and (B) pro-inflammatory cytokines in epididymal adipose tissue. Bars represent mean±S.E.M., $n=8$. For each gene, means without a common letter differ, $P<.05$. The data represent relative density normalized to GAPDH. (C) Protein levels of pIRF3 determined by Western blot.


A
HFD
CSD
Galanin
FGF1
BMP
BMPR
GalR2
GalR1
RAS
FGFR1
PIP2
RAF
SMAD1
(Ser463)
PKCδ
MEK
SMAD1
SMAD4
Shn2
ERK
(Thr202/Tyr204)
Ctss
E2F1
Rb
Neucleus
Cyc-D
Rb
(Ser795)
E2F1
DP
LXR
PPARγ2
SMAD1
SMAD4
Shn2
Leptin
LPL
SREBP1c
RXR
LPL
aP2
SRE
PPRE
C/EBPα
Adipogenesis
B
HFD
CSD
TLR4
LBP
MD2
CD14
TLR2
TRAM
TRIF
Tirap
MyD88
MyD88
Tirap
TRAF3
IRAK1
IRAK1
IKKi
TBK1
TRAF6
TRAF6
MyD88-independent pathway
MyD88-dependent pathway
TRAF6
Ub (K63 type)
TRAF6
IRF3
IRF3
TAB1,2,3
TAK1
Ub (K63 type)
IRF5
IRF3
IRF3
IKK γ
IKK α
IKK β
NF-kB
IkB
Ub (K48 type)
P65
P50
IRF3
IRF3
IRF5
IFNα
TNFα
NF-kB
P65
P50
NF-kB
P65
P50
IFNα
TNFα
Inflammation

at 50 μM [26]) and capsaicin (84% reduction at 100 μM [27]) but higher than the values for berberine (20% reduction at 100 μM [28]), epigallocatechin gallate (EGCG) (19% reduction at 10 μM [29]) and quercetin (15.9% reduction at 25 μM [30]) as determined through Oil-Red O staining. We conducted an animal feeding study on HFD-fed mice to investigate the anti-obesity effect of carvacrol, having found this compound to inhibit adipocyte differentiation in mouse pre-adipocytes. In our preliminary study, carvacrol supplemented to the HFD at 0.01%, 0.05% and 0.1% levels for 28 days exhibited a dose-dependent reduction in the body weight of mice (data not shown). On the basis of these results, animals were fed 0.1% carvacrol for a longer period in the present study. Considering that the LD_{50} value for a single intragastric administration of carvacrol to rat was 810 mg/kg body weight in an acute toxicity study [31], the 0.1% carvacrol supplemented in the diet (equivalent to 100 mg/kg body weight) appears to have no harmful effect. After 10 weeks of feeding, the body weight gain and visceral fat-pad weights of the CSD group were significantly lower than that of HFD mice. It is noteworthy that daily food intake during the entire feeding period did not differ among groups, indicating that body weight loss induced by carvacrol was not attributable to food intake inhibition. Thus, we hypothesized that dietary carvacrol reduces the body weight of mice through modulation of adipogenesis and thermogenesis in visceral adipose tissues. To test this hypothesis, we investigated the effect of carvacrol supplementation on the expression of adipose tissue genes that are involved in adipogenesis and thermogenesis.

PPARγ, a nuclear receptor critical for adipogenesis, results in the recruitment of coactivator C/EBPα, assembly of a transcriptional complex, and regulates the transcription of its target genes such as aP2, LPL, leptin and FAS. Although the biologically relevant endogenous PPARγ ligand is unknown, exogenous ligands for PPARγ include FFAs, prostanoids and the synthetic high-affinity antidiabetic agents, thiazolidinediones [32]. Plasma FFA, elevated in obesity, can activate PPARγ through direct interaction with the ligand-binding domain of this receptor [33]. The inhibitory effects of carvacrol on adipogenesis and on the expressions of transcription factors PPARγ and C/EBPα and its target genes such as aP2, LPL and leptin observed in the present study might be associated to its ability to lower FFA levels in blood and tissues. LXR is a key regulator of lipid expression of a lipogenic transcription factor SREBP-1c, which induces PPARγ expression and activates the expression of mRNAs for LPL and FAS [34]. Ctss, which has been known as a novel marker of adiposity [35], also increases the gene expression of adipocyte markers (PPARγ, SREBP-1c, aP2, and CD36) [36].

FGF-1 has been recently known as a key regulator of human adipogenesis as FGF-1-treated human preadipocytes exhibited PPARγ expression and FGFR inhibitor treatment decreased FGF-1-induced phosphorylation of ERK1/2 in preadipocytes [37]. The activation of ERK signaling promotes adipogenesis by enhancing PPARγ and C/EBPα gene expression during the differentiation of 3T3-L1 pre-adipocytes [37]. Furthermore, current literature indicates that the cyc-D/pRb/E2F pathway lies downstream of the mitogenically activated Ras/Raf/MEK/ERK cascade [38]. We observed the up-regulations of FGFR1, pERK, cyc-D, pRb and E2F1 genes, compared to their normal weight controls, in the adipose tissues of *ob*/*ob* and DIO mice (unpublished data). Galanin is one of the most inducible neuropeptides that is known to stimulate the intake of a fat-rich diet [39]. The conventional galanin-mediated signaling involves the activation of the Ras/c-Raf/MEK/ERK (*via* GalR1) and PKCδ/ERK (*via* GalR2) pathways, which have been reported in the nerve systems and tumor cells [40,41]. On the other hand, the overexpression of galanin and its receptors in the epididymal adipose tissues of HFD-fed mice, along with the increased expression and/or activation of the downstream molecules related to adipogenesis such as the Ras/pRaf/pERK and PKCδ/pERK signaling molecules, were observed previously [42]. BMP also induces transcriptional activation of PPARγ. It has been reported that in mouse embryonic fibroblast, upon BMP stimulation, Shn-2 is recruited to the PPARγ2 promoter via an interaction with SMAD1/4 during adipocyte differentiation [43]. To our knowledge, this study provides the first evidence showing that BMP-mediated signaling might be associated with enhanced adipogenesis in the visceral adipose tissue of mice with HFD-induced obesity.

GalR1, GalR2, FGFR1 and BMPR mRNA expressions were reduced in the visceral adipose tissues of the CSD-fed mice compared with those for the HFD mice. GalR1-, GalR2- and FGFR1-mediated ERK down-regulation is associated with the inhibition of Cyc-D and E2F1. The inhibition of these upstream molecules results in decreased expressions of the key adipogenic transcription factors PPARγ and C/EBPα and their target genes such as leptin, LPL and aP2. Therefore, the decreases in visceral fat-pad weights observed in the CSD-fed mice than in the HFD mice might be manifested through suppressed BMP-, FGF-1- and galanin-mediated signaling cascades that would eventually lead to decreased adipogenesis in the visceral adipose tissue. Moreover, carvacrol supplemented to a HFD also reversed the HFD-induced increases in the LXR, SREBP-1c and Ctss expressions at their mRNA levels in the visceral adipose tissue of mice (Fig. 6A).

Adaptive thermogenesis, which is directly linked to oxidative metabolism and controlled by mitochondria, is an important component of energy homeostasis and a metabolic defense against obesity [44]. UCP1 and UCP3 were down-regulated in white adipose tissues of diet-induced obesity and *ob*/*ob* animals, leading to decreased thermogenesis and energy expenditure that induce fat accumulation [45,46]. SIRT1 activates PGC1α deacetylation and is involved in mitochondrial biogenesis [47]. PGC-1α coactivates other transcription factors (including RAR, RXR, PPARα/β/γ2, C/EBPα/β) and induces UCP1 in human adipocytes [48,49]. In this study, SIRT1, PGC-1α, RXR, UCP1 and UCP3 mRNA expression, which were down-regulated by the HFD, were not affected by carvacrol supplementation. Accordingly, the body weight-lowering effect of carvacrol observed in mice appears to be unrelated to thermogenesis regulation.

The local application of carvacrol-based dental gel from *Alecrim pimenta* has an anti-inflammatory effect in experimental periodontitis [50]. Furthermore, carvacrol from *Lavandula multifida* ethanol extract was able to reduce the Croton oil-induced ear edema in mice [51]. Nevertheless, the beneficial effects of carvacrol on metabolically triggered inflammation have not yet been investigated. Obesity is associated with a chronic inflammatory responses characterized by

Fig. 6. (A) Schematic representation of the genes regulated by carvacrol in BMP-, FGF-1- and galanin-mediated signaling pathways. GalR1, GalR2 and FGFR1 mRNA expressions were reduced in the visceral adipose tissues of the CSD-fed mice compared with those for the HFD mice. GalR1-, GalR2- and FGFR1-mediated ERK down-regulation is associated with the inhibition of Cyc-D and E2F1. Moreover, carvacrol supplemented to an HFD significantly suppressed the expression of BMPR and Shn2. The inhibition of these upstream molecules results in decreased expressions of the key adipogenic transcription factors PPARγ and C/EBPα and their target genes such as leptin, LPL and aP2. Ctss and LXR induce SREBP-1c expression, leading to the expression of PPARγ. Carvacrol also reversed the HFD-induced increases in the expressions of these molecules. (B) Schematic representation of the genes regulated by carvacrol in TLR2- and 4-mediated signaling pathway. TLR4 uses MyD88-dependent and MyD88-independent pathways, whereas TLR2 signals only in the MyD88-dependent manner. The MyD88-dependent pathway uses TRAF6 and IRF5, leading to its nuclear translocation and co-operation with NF-κB. The MyD88-independent pathway uses TRIF in activating NF-κB in either a TRAF6-dependent or a TRAF6-independent mechanism. TRIF associates with TBK1 and IKKi, which in turn leads to pIRF3. The mRNA and protein levels of TLR2, TLR4, downstream molecules (MyD88, Tirap, TRIF, TRAF6, IRF5 and IRF3) and pro-inflammatory cytokines (TNFα, IFNα) were reduced in the visceral adipose tissues of the CSD-fed mice compared with those for the HFD mice.

abnormal pro-inflammatory cytokines production and activation of inflammatory signaling pathways [52,53]. TLRs, which are essential for the development of innate immunity to pathogens, trigger the production of pro-inflammatory cytokines [54]. TLR2 and 4 bind lipid-based structures via their long-chain saturated FFA moieties [55] and FFA alone are capable of activating TLR2 and TLR4 [55,56]. TLR2 and TLR4 stimulated with agonist recruit adaptor molecules (MyD88, Tirap, TRIF and TRAM [TRIF-related adaptor molecule]) to activate downstream signaling pathways [57]. TLR4 uses MyD88-dependent and MyD88-independent pathways, whereas TLR2 signals only in the MyD88-dependent manner. The MyD88-dependent pathway uses TRAF6 and IRF5, leading to its nuclear translocation and co-operation with NF-κB. The MyD88-independent pathway uses TRIF in activating NF-κB in either a TRAF6-dependent or a TRAF6-independent mechanism. TRIF associates with TBK1 and IKKi, which in turn leads to pIRF3. The mRNA and protein levels of TLR2, TLR4, downstream molecules (MyD88, Tirap, TRIF, TRAF6, IRF5 and IRF3) and pro-inflammatory cytokines (TNFα, IFNα) were reduced in the visceral adipose tissues of the CSD-fed mice compared with those for the HFD mice (Fig. 6B). Moreover, the plasma FFA concentration, which was prominently elevated in HFD-fed mice, was found to be significantly decreased by carvacrol. Accordingly, these results suggest that carvacrol may improve metaflammation by both down-regulating TLR2 and TLR4 expressions and lowering plasma FFA levels.In summary, the major novel finding in our experimental conditions is that carvacrol prevented obesity in the HFD-fed mice by decreasing body weight and visceral fat-pad weights and lowering plasma lipid levels. Several phytochemicals, such as quercetin, EGCG, capsaicin and genistein, were reported to efficiently induce apoptosis through the activation of mitogen-activated protein kinase pathway to enhance fatty acid oxidation through AMP-activated protein kinase activation and/or to inhibit adipogenesis through the down-regulation of adipogenic transcription factors (PPARγ and C/EBPα) in 3T3-L1 preadipocytes or in adipose tissues of obese animals [30,58,59]. The evidence obtained in the present study suggests that carvacrol appears to inhibit visceral adipogenesis possibly by suppressing BMP-, FGF-1- and galanin-mediated signaling cascades and to attenuate the production of pro-inflammatory cytokines in the visceral adipose tissues by inhibiting TLR2- and TLR4-mediated signaling pathways. These modes of action on modulating genes associated with adipogenesis and inflammation by carvacrol are considered relatively novel compared with the mechanisms reported for other anti-obesity phytochemicals.

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