RESEARCH ARTICLE

Diet-induced obesity regulates the galanin-mediated signaling cascade in the adipose tissue of mice

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Galanin is a neuropeptide that regulates the food intake, neurogenesis, memory, and gut secretion. This study was conducted to evaluate the high-fat diet (HFD)-induced regulation of the galanin receptors (GalRs) and the associated signaling molecules in the adipose tissues of mice. Twenty C57BL/6J mice were given either an HFD or a normal diet for 12 wk. The results of the semiquantitative RT-PCR analyses indicated that the HFD upregulated the expression of GalR1, GalR2, GalR3, resistance to audiogenic seizures, peroxisome proliferator-activated receptorγ2, adipocyte protein 2, and protein kinase Cδ and downregulated the expression of peroxisome proliferative activated receptor γ coactivator 1α and uncoupling protein 1 in the adipose tissues. The immunoblot results showed that the protein levels of peroxisome proliferator-activated receptorγ2 and adipocyte protein 2, and the phosphorylation of c-Raf and extracellular signal-regulated kinase 1/2 were increased, while the phosphorylation of cyclic adenosine monophosphate-responsive element-binding protein, which regulates peroxisome proliferative activated receptor γ coactivator 1α and uncoupling protein 1, was decreased in the epididymal adipose tissues of the HFD-fed mice. These results suggest the possible association of the galanin-mediated signaling pathways in the manifestation of the HFD-induced activation of adipogenesis along with the suppression of thermogenesis in the adipose tissues of mice.

Keywords:
Adipogenesis / Adipose tissue / Galanin / High-fat diet / Obesity

1 Introduction

Galanin, a neuropeptide with 29–30 amino acids, is engaged in the regulation of numerous physiological processes such as food intake, memory, the neuroendocrine function, gut secretion, and motility [1]. It is widely distributed in the central and peripheral nervous system and is expressed abundantly in various regions of the brain [1]. Galanin expression has also been observed in the stomach, visceral adipose tissue, and taste buds, but to a lesser degree than in the brain [1, 2]. Galanin activates three distinct receptor subtypes that have been cloned and characterized pharmacologically: galanin receptor 1 (GalR1), GalR2, and GalR3 [3]. These receptors show distinct distribution patterns depending on the tissue types: GalR1 and GalR2 are abundantly expressed in the brain, muscle, adipose tissue, and stomach [2], whereas GalR2 and GalR3, but not GalR1, are expressed in the lung and kidney [4].

Based on the distribution of galanin and its receptors in the brain regions that regulate ingestive behaviors, Leibowitz and coworkers first tested the effects of centrally administered galanin on feeding and drinking in the rat [5]. Microinjection of nanomole doses of galanin into the

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Abbreviations: AC, adenylyl cyclase; **aP2,** adipocyte protein 2; **CREB,** cyclic AMP-responsive element-binding protein; **ERK,** extracellular signal-regulated kinases 1/2; **GalR,** galanin receptor; **GAPDH,** glyceraldehyde-3-phosphate dehydrogenase; **HFD,** high-fat diet; **HRP,** horseradish peroxidase; **MDI,** differentiation mixture; **ND,** normal diet; **PGC-1α,** peroxisome proliferative activated receptor γ coactivator 1α; **PKC,** protein kinase C; **PPARγ2,** peroxisome proliferator-activated receptorγ2; **PTX,** pertussis-toxin; **Ras,** resistance to audiogenic seizures; **TG,** triglyceride; **UCP1,** uncoupling protein 1

paraventricular nucleus of the hypothalamus of rats induced a dramatic increase in their consumption of standard rodent chow [6]. Chronic administration of galanin into the paraventricular nucleus of the hypothalamus produces complex changes in daily caloric intake, levels of obesity, and regional fat deposition, depending on the fat and carbohydrate content of the diet [7]. An association between galanin synthesis and fatty acid metabolism has been raised from the previous observations that mercaptoacetate-induced blockade of fatty acid metabolism downregulates the galanin expression in the anterior paraventricular nucleus [8] and that the rats fed a high-fat diet (HFD) (but not a high-carbohydrate or high-protein diet) displayed 40% higher hypothalamic galanin levels [9].

GalR activation involves somewhat different signaling pathways depending on the receptor subtypes [3]. GalR1 and GalR3 activate the intracellular effectors through pertussis-toxin (PTX)-sensitive Gi/o proteins [10], which results in the inhibition of adenylyl cyclase (AC) activity and a lower level of cyclic adenosine monophosphate in the cytosol [11]. Although somewhat controversial, additional interactions of GalR2 with Gi-type G-proteins which inhibit AC in a PTX-sensitive manner have been suggested [12]. Therefore, GalR1, GalR2, or GalR3 activation inhibits the phosphorylation of cyclic AMP-responsive element-binding protein (CREB) [10, 13], which leads to decreased expression of peroxisome proliferative activated receptor γ coactivator 1α (PGC-1α) and uncoupling protein 1 (UCP1) (Fig. 1) [14]. Alternatively, GalR1 activation stimulates mitogen-activated protein kinase activity in a protein kinase C (PKC)-independent manner by coupling with a Gi-type G-protein *via* Gβγ subunits [15]. Besides interacting with Gi-type G-proteins, GalR2 also signals through the pathway that involves phospholipase C activation *via* Gq/11-type G-proteins, which catalyzes the cleavage of phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-triphosphate and diacyl glycerol, a PKC activator [16, 17]. Subsequent activation of PKCδ can induce the activation of protein tyrosine kinases that could lead to extracellular signal-regulated kinases 1/2 (ERK) activation [18] which is necessary for the expression of the crucial adipogenic regulators, CCAAT/enhancer-binding protein α, β and δ and peroxisome proliferator-activated receptorγ (PPARγ) (Fig. 1) [19]. Although the galanin-mediated signaling pathways have been characterized in neuronal cells, oral squamous carcinoma cells [20], and rat gastrointestinal tract [3], neither the role of galanin nor the regulation of GalR-mediated signaling

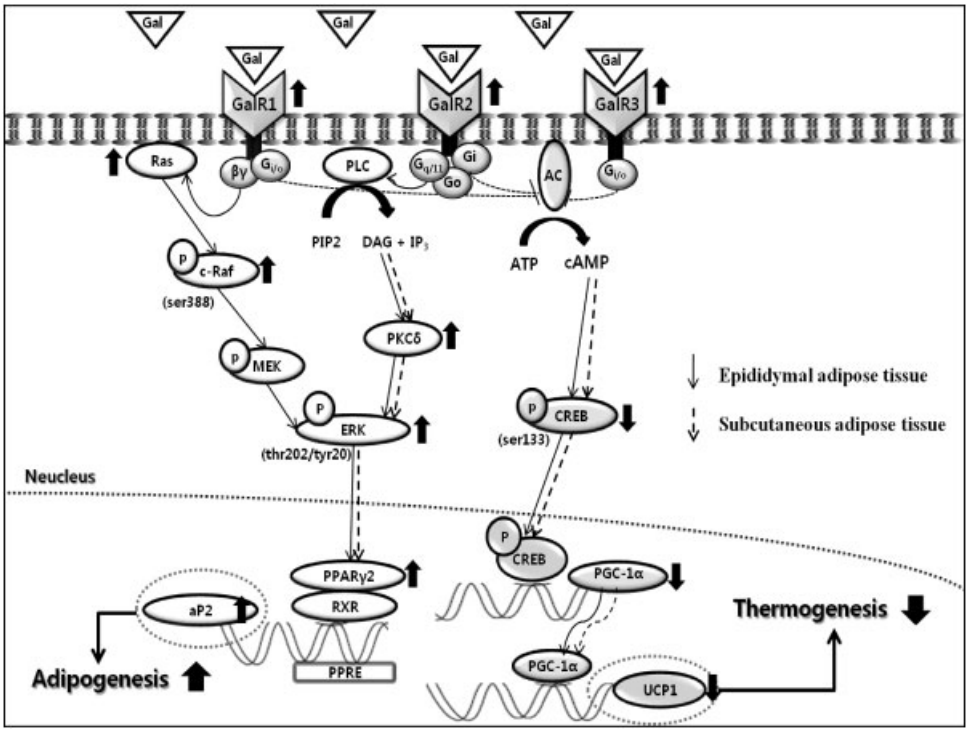

Figure 1. Proposed model for the HFD-induced regulations of the galanin-mediated signaling pathways in the adipose tissues of mice. GalR1, GalR2, and GalR3 genes were overexpressed in the visceral adipose tissues of the HFD-fed mice. The downstream molecules involved in Ras/p-c-Raf/pMEK/pERK or PKCδ/pERK signaling were also upregulated and/or activated by HFD feeding. The upregulation of ERK phosphorylation promoted adipogenesis through the increased expressions of the adipogenic transcription factor PPARγ and its target protein, aP2. Meanwhile, the HFD-induced upregulation of GalR1, GalR 2, and GalR 3 led to the inhibition of AC, resulting in decreased CREB phosphorylation and PGC-1α expression along with a subsequent reduction in UCP1 expression. In the subcutaneous adipose tissues, HFD-induced obesity regulated the PKCδ/pERK and AC/pCREB signaling pathways, leading to increased adipogenesis and suppressed thermogenesis, respectively.

pathways in the adipose tissue of obese mammals has been explored. In this study, the HFD-induced changes in the expression of galanin and its receptors in the visceral and subcutaneous adipose tissues of mice were evaluated. It was also examined whether or not the GalR-mediated signaling pathways are regulated in the adipose tissue of the rodents with diet-induced obesity.

2 Materials and methods

2.1 Animals and diets

Twenty male C57BL/6J mice (7-wk old, 21.5 ± 0.4 g, Jung-Ang Laboratory, Animal, Gyeonggi-do, Korea) were individually housed in standard cages in an environmentally controlled room (21 ± 2.0°C, 50 ± 5% humidity, 12-h light/dark cycle) and given free access to food and water. All the mice consumed a commercial diet (Purina Rodent Chow 5001) and tap water *ad libitum* for 1 wk before their division into two weight-matched groups: the normal diet (ND) group and the HFD group. The ND was formulated based on the AIN-76 rodent diet composition and the HFD contained 200 g of fat *per* kilogram (170 g lard + 30 g corn oil) and 1% cholesterol by weight. The HFD was formulated to provide 40% of the total energy from fat, by replacing carbohydrate energy with lard and corn oil, and had the same amount of vitamins and minerals *per* kilojoule as the ND did. The diets were given in the form of pellets for 12 wk.

The food intake of the mice was recorded every day and their body weights were measured once a week. At the end of the experiment period, the mice were anesthetized with diethyl ether following overnight fasting. Their omental (epididymal, perirenal, retroperitoneal, and mesenteric fat pads) and subcutaneous adipose tissues, and skeletal muscles (gastrocnemius muscles) were removed, rinsed with PBS, and weighed. The samples were immediately frozen with liquid nitrogen and stored at −70°C. All the experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the Yonsei Laboratory Animal Research Center-Institutional Animal Care and Use Committee (YLARC-IACUC), Yonsei University, Korea.

2.2 Biochemical assays

Plasma concentrations of triglyceride (TG), free fatty acid, total cholesterol, HDL cholesterol, and glucose were enzymatically determined using commercial kits (Bio-Clinical System, Gyeonggi-do, Korea). Hepatic lipids were extracted as described using the method developed by Folch *et al.* [21], and the dried lipid residues were dissolved in 1 mL of ethanol. Cholesterol, TG, and free fatty acid concentrations in the hepatic lipid extracts were measured with the same enzymatic kits that were used for the plasma analyses.

2.3 Cell culture

Mouse embryo 3T3-L1 cells (American Type Culture Collection, Rockville, MD, USA) were cultured in DMEM supplemented with 10% bovine calf serum and penicillin/streptomycin in a humidified atmosphere of 5% CO_2/95% air at 37°C. After the 3T3-L1 cells became confluent, the medium was replaced with a differentiation mixture (MDI) that contained 10% fetal bovine serum, 1% penicillin/streptomycin, 1 μg/mL insulin, 1 μM dexamethasone (Sigma, St. Louis, MO, USA), 0.5 mM isobutylmethylxanthine (Sigma), and was cultured for 2 days. The cell medium was then replaced with 10% fetal bovine serum plus only 1 μg/mL insulin, and the cells were fed every 2 days with the same medium. To investigate the effect of galanin on the GalR expression, postconfluent 3T3-L1 preadipocytes were treated with the MDI (1 μg/mL insulin, 1 μM dexamethasone, and 0.5 mM isobutylmethylxanthine) in the absence or presence of 5 nM galanin (Tocris, Bristol, UK) for 48 and 96 h, and then were harvested and subjected to semiquantitative RT-PCR analyses.

2.4 RNA extraction and semiquantitative RT-PCR

The total RNA was isolated from the epididymal and subcutaneous adipose tissues, and skeletal muscle of each mouse using Trizol (Invitrogen, Carlsbad, CA, USA) and was reverse-transcribed using the Superscript II kit (Invitrogen), according to the manufacturer's recommendations. The primers for the real-time PCR analysis were synthesized by Bioneer (Daejun, Korea). The forward (F) and reverse (R) primer sequences for the genes that were involved in the galanin-mediated signaling pathway are listed in Table 1. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as the internal control for the semiquantitative RT-PCR. The PCR was carried out as follows: 5 min at 94°C, 30 cycles of 94°C for 30 s, 55°C or 60°C for 45 s, and 72°C for 1 min, and 10 min of incubation at 72°C.

2.5 Western blot analysis

The epididymal and subcutaneous fat pads were homogenized at 4°C in an extraction buffer (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 phenylmethanesulphonylfluoride, 2 μg/mL aprotinin, 1 μg/mL pepstatin A, and 1 μg/mL leupeptin). The tissue homogenates were centrifuged (13 000 × *g*, 20 min, 4°C), and the resulting supernatants (whole-tissue extracts) were used for the Western blot analyses. The protein concentrations of each protein extract were quantified using Bradford assay (Bio-Rad, Hercules, CA, USA).

The protein samples (80 μg/lane) were separated with 10% SDS-PAGE and were transferred to a nitrocellulose membrane

Table 1. Primer sequences and RT-PCR conditions

Gene description	Primers	Sequences (5′→3′)	Annealing temperature (°C)	PCR product (bp)
Galanin	F	GAGCCTTGATCCTGCACTGA	60	121
	R	AGTGGCTGACAGGGTCACAA		
GalR1	F	CCAAGGGGGTATCCCAGTAA	56	147
	R	GGCCAAACACTACCAGCGTA		
GalR2	F	ATAGTGGTGGCATGCTGGAA	60	134
	R	AGGCTGGATCGAGGGTTCTA		
GalR3	F	ATCTTCCTGTTGGGCATGGT	60	227
	R	TGTACCGTCTTGCACACGAA		
PPARγ2	F	GGCCAAACACTACCAGCGTA	55	114
	R	TTCTGCCTCTGTTGGATGCC		
aP2	F	AGCATCATAACCCTAGATGG	55	128
	R	TACACCTGGAGCCAGACTTG		
Ras	F	CTGATCCAGAACCACTTTGT	55	124
	R	ACTCTTCTTGACCTGCTGTG		
PKCδ	F	CTGAGCGCTGCAAGAAGAAC	60	146
	R	TGGAAACTTTGCCTCCTCCT		
PGC-1α	F	ACTGACAGATGGAGCCGTGA	60	180
	R	GCTGCATGGTTCTGAGTGCT		
UCP1	F	CTGGGCTTAACGGGTCCTC	60	100
	R	CTGGGCTAGGTAGTGCCAGTG		
GAPDH	F	AGAACATCATCCCTGCATCC	60	367
	R	TCCACCACCCTGTTGCTGTA		

Table 2. Body weight gain, adipose fat pad weight, and plasma and hepatic biochemistries of mouse-fed experimental diets

	ND	HFD
Body weight gain (g/12 wk)	6.25 ± 0.49	18.5 ± 0.91**
Visceral fat-pad weights (mg/g body weight)		
Epididymal adipose tissue	20.1 ± 1.91	50.2 ± 1.91***
Mesenteric adipose tissue	11.9 ± 0.58	18.9 ± 0.80***
Perirenal adipose tissue	1.89 ± 0.13	5.28 ± 0.34***
Retroperitoneal adipose tissue	5.43 ± 0.38	15.8 ± 0.86***
Total visceral adipose tissue	39.3 ± 3.24	90.2 ± 8.24***
Plasma		
TG (mmol/L)	0.5 ± 0.01	0.89 ± 0.09**
Free fatty acid (μEq/L)	469 ± 0.11	914 ± 73.3**
Total cholesterol (mmol/L)	2.83 ± 0.11	4.46 ± 0.18***
HDL cholesterol (mmol/L)	1.96 ± 0.07	1.16 ± 0.19**
LDL+VLDL cholesterol (mmol/L)	0.87 ± 0.04	3.3 ± 0.18**
Glucose (mmol/L)	3.62 ± 0.41	6.44 ± 0.35***
Liver		
Liver weight (g/100 g body weight)	3.5	5.91 ± 0.19***
TG (μmol/g liver)	28.6 ± 2.33	45.6 ± 1.49**
Cholesterol (μmol/g liver)	10.8 ± 0.64	23.9 ± 1.12***
Free fatty acid (μEq/g liver)	5.88 ± 0.65	16.7 ± 0.43***

**$p < 0.01$, ***$p < 0.001$.
Each value represents the mean ± SEM, $n = 10$

(Amersham, Buckinghamshire, UK). The membranes were blocked for 2 h in 5% bovine serum albumin in Tris-buffered saline/Tween buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20). The membranes were then probed with a 1:1000 dilution of the primary antibody. Antibodies to the following proteins were purchased from the indicated sources: CREB, phospho-CREB (Ser 133), c-Raf, phospho-c-Raf (Ser 259), ERK, and phospho-ERK (Thr 202/Tyr 204) from Cell Signaling Technology (Beverly, MA, USA); PPARγ2, β-actin, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, HRP-conjugated donkey anti-goat IgG, and HRP-conjugated goat anti-rabbit IgG from Santa Cruz Biotechnology (Santa

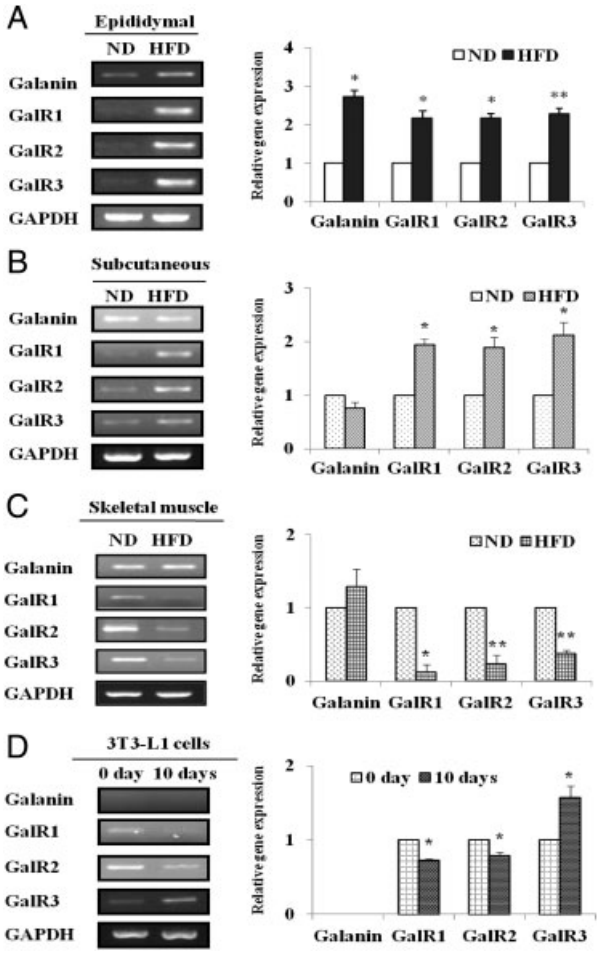

Figure 2. Expression of galanin and its receptors determined *via* the semiquantitative RT-PCR analyses of the epididymal (A) and subcutaneous fat tissues (B) and of the skeletal muscles (C) of the ND- and HFD-fed mice, and in the 3T3-L1 cells, before (0 day) and after (10 day) differentiation (D). The RNA samples that were used for PCR analysis were pooled from ten mice *per* group. The results were normalized to GAPDH mRNA expression. The mRNA levels of the HFD-fed mice were expressed as the fold changes of normal mice. A representative image of the three independent experiments that were conducted is shown in the left part of the figure. The right part of the figure, on the other hand, shows the results of the densitometric analysis of the galanin, mRNA levels of GalR1, GalR2, and GalR3 in the adipose tissues, skeletal muscles, and 3T3-L1 cells. Each bar represents the mean $\pm$ SEM of the three independent experiments ($^{*}p<0.05$, $^{**}p<0.01$).

Cruz, CA, USA); adipocyte protein 2 (aP2) from R&D Systems (Minneapolis, MN, USA); HRP-conjugated goat anti-rat IgG from Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA). After the incubation with the corresponding secondary antibody, signals were detected using the chemiluminescent detection system (Amersham, Uppsala, Sweden) and were quantified using the Quantity One analysis software (Bio-Rad).

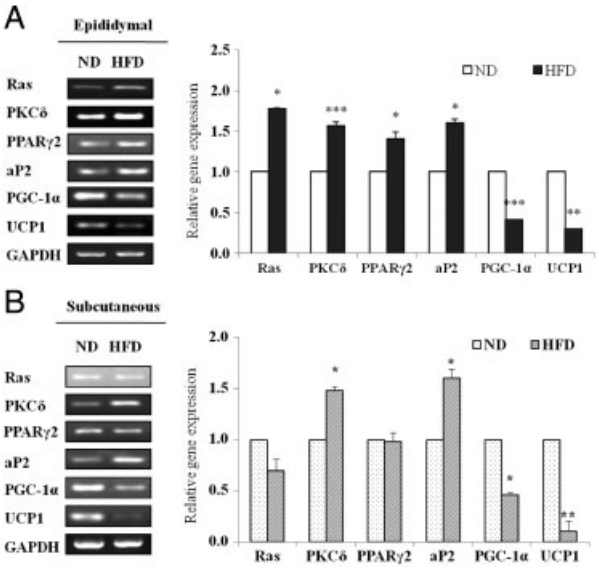

Figure 3. Expression of the galanin-mediated signaling molecules determined *via* the semiquantitative RT-PCR analyses of the epididymal (A) and subcutaneous fat tissues (B) from the ND- and HFD-fed mice. The RNA samples that were used for PCR analysis were pooled from ten mice *per* group. The results were normalized to GAPDH mRNA expression. The mRNA levels of the HFD-fed mice were expressed as the fold changes of normal mice. A representative image of the three independent experiments that were conducted is shown in the left part of the figure. The right part of the figure, on the other hand, shows the results of the densitometric analysis of the Ras, PKCδ, PPARγ2, aP2, PGC-1α, and UCP1 mRNA expression in the epididymal (A) and subcutaneous (B) adipose tissues. Each bar represents the mean $\pm$ SEM of the three independent experiments ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

2.6 Statistical analyses

The data on the body weight gain, visceral fat pad weights, liver weights, and blood and hepatic biochemistries are presented as means $\pm$ SEM of ten mice. The RT-PCR and Western blot data are presented as means $\pm$ SEM of the triplicate analyses of the RNA and protein samples, respectively, which were pooled from ten mice *per* group. Student's *t*-test using the SPSS software was used to evaluate the differences between the values for the HFD and ND groups, and a *p*-value of <0.05, <0.01, or <0.001 was considered significant.

3 Results

3.1 Body weight gain, tissue weights, and biochemistries

After 12 wk, the body weight gain in the mice that were given the HFD was 18.5 ± 0.91 g, *versus* 6.25 ± 0.49 g in the controls ($p<0.01$). The relative weights of the epididymal,

mesenteric, perirenal, and retroperitoneal fat pads were 150, 59, 179, and 191% greater, respectively, in the mice that were fed the HFD than in the ND-fed mice ($p<0.001$). The relative weight of the liver was also significantly greater in the HFD group than in the ND group ($p<0.001$) (Table 2).

The plasma TG (78% higher, $p<0.01$), free fatty acid (95% higher, $p<0.01$), total cholesterol (58% higher, $p<0.001$), LDL+VLDL cholesterol (279% higher, $p<0.01$), and glucose (78% higher, $p<0.001$) concentrations of the HFD group were significantly higher than those of the control mice. Conversely, the concentration of plasma HDL cholesterol was significantly lower in the HFD group than in the ND group ($p<0.01$). The HFD-fed mice exhibited a significantly elevated level of plasma glucose (78% increase) compared with the ND-fed mice. The hepatic TG (59% increase, $p<0.01$), cholesterol (121% increase, $p<0.001$), and free fatty acid (184% increase, $p<0.001$) levels of the mice that were fed the HFD were also higher than those observed in the ND-fed animals (Table 2).

3.2 Expression of the galanin and GalR genes

The galanin and GalR expressions were investigated through semiquantitative RT-PCR analyses of the adipose tissues, and skeletal muscles of the ND- and HFD-fed mice as well as in pre- and fully differentiated 3T3-L1 adipocytes (Fig. 2). The mRNA levels of galanin were significantly elevated in the epididymal adipose tissues, but were not affected in the subcutaneous adipose tissues and skeletal muscles of the HFD-fed mice. Giving the mice an HFD significantly upregulated the expression of the GalR1, GalR2, and GalR3 genes in both their epididymal (from 2.17- to 2.29-fold) and subcutaneous adipose tissues (from 1.89- to 2.12-fold), whereas it significantly downregulated the expression of the GalR1, GalR2, and GalR3 genes in their skeletal muscles (from 0.12- to 0.38-fold) (Figs. 2A–C).

Next, it was determined whether the galanin and GalR expressions are regulated in the 3T3-L1 cells during terminal differentiation. The expressions of the GalR1 and GalR2 genes were significantly downregulated (0.73- and 0.79-fold, respectively) in the fully differentiated 3T3-L1 cells (at day

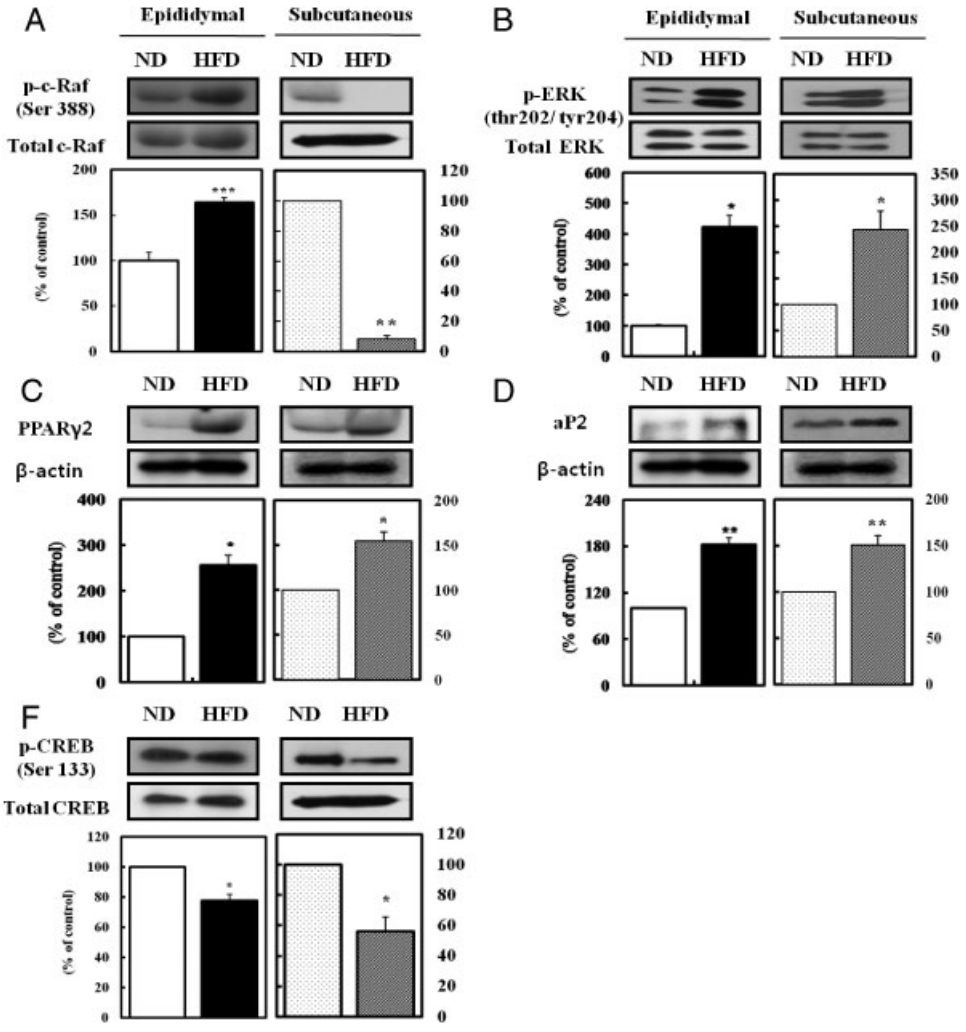

Figure 4. Immunoblots of the downstream molecules of the galanin-mediated signaling pathway in the epididymal and subcutaneous fat tissues from the ND- and HFD-fed mice. The protein in the whole tissue extract (80 μg/lane) from the epididymal adipose tissue was probed using the following antibodies: (A) anti-phospho-c-Raf (Ser 259) and anti-c-Raf; (B) anti-phospho-ERK (Thr 202/Tyr 204) and anti-ERK; (C) anti-PPARγ2; (D) anti-aP2; and (E) anti-phospho-CREB (Ser 133) and anti-CREB. A representative image of the ND and HFD groups for each protein is shown in the upper part of each figure. The lower part of each figure shows the results of the densitometric analysis. Each bar represents the mean ± SEM of the three independent experiments ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

10) compared with the preadipocytes (at day 0), whereas a significant upregulation of the GalR3 mRNA (1.57-fold) was observed in the differentiated 3T3-L1 cells compared with the preadipocytes. At the same time, neither the preadipocytes nor the differentiated 3T3-L1 adipocytes expressed galanin (Fig. 2D).

3.3 mRNA levels of the galanin-mediated signaling molecules

Since HFD-induced obesity is associated with a rise in the expression of galanin and its receptors in the adipose tissue, the *in vivo* regulation of the galanin-mediated signaling molecules in a mouse model of obesity was checked. The HFD-fed mice exhibited significantly higher levels of the resistance to audiogenic seizures (Ras) (77% increase, $p<0.05$), PKCδ(56% increase, $p<0.05$), PPARγ (40% increase, $p<0.05$), and aP2 (60% increase, $p<0.05$) gene expressions, along with significantly lower expression levels of the PGC-1α (59% decrease, $p<0.05$) and UCP1 (70% decrease, $p<0.05$) genes in their epididymal adipose tissues compared with the ND-fed animals (Fig. 3A). Similarly, in the subcutaneous adipose tissues, the expressions of the PKCδ (48% increase, $p<0.05$) and aP2 (50% increase, $p<0.05$) genes were significantly upregulated, whereas the PGC-1α (54% decrease, $p<0.05$) and UCP1 (90% decrease, $p<0.05$) gene expressions were significantly downregulated in the HFD-fed mice compared with the ND-fed mice. HFD did not alter the mRNA levels of Ras and PPARγ2 in the subcutaneous adipose tissues of the mice (Fig. 3B).

3.4 Immunoblot analysis of the galanin-mediated signaling molecules

To determine whether HFD altered the protein levels of the signaling molecules involved in the galanin-mediated signaling pathways, Western blot analyses were performed using the proteins extracted from the epididymal and subcutaneous adipose tissues of the ND- and HFD-fed mice. The immunoblot results of the epididymal adipose tissues showed that in the HFD-fed mice, the phosphorylations of c-Raf at Ser388 (64% increase, $p<0.001$) were elevated compared with those for the ND-fed mice. In contrast to the results observed in the epididymal adipose tissues, c-Raf phosphorylation was dramatically reduced in the subcutaneous adipose tissue by feeding the HFD (91% decrease, $p<0.01$). The HFD-fed mice showed 323 and 143% increases in the expression of ERK phosphorylation in their epididymal and subcutaneous adipose tissues, respectively ($p<0.05$) (Figs. 4A and B). The protein levels of PPARγ2 ($p<0.05$) and its target protein, aP2 ($p<0.01$) were also significantly elevated in the epididymal and subcutaneous adipose tissues of the HFD-fed mice (Figs. 4C and D). The phosphorylations of CREB at Ser133 in the epididymal (22% reduction, $p<0.05$) and subcutaneous adipose tissues (43% decrease, $p<0.05$) were significantly reduced in the HFD-fed mice than in the ND group (Fig. 4E).

3.5 Galanin-induced changes in the GalRs expression of the 3T3-L1 cell

Next, we determined the mRNA levels of GalR1, GalR2 and GalR3 in the 3T3-L1 cells differentiated for 46 and 96 h in the absence or presence of 5 nM galanin. The GalR1 and GalR2 gene expressions were not altered in the 3T3-L1 cells at 48 and 96 h following MDI treatment, whereas the GalR3

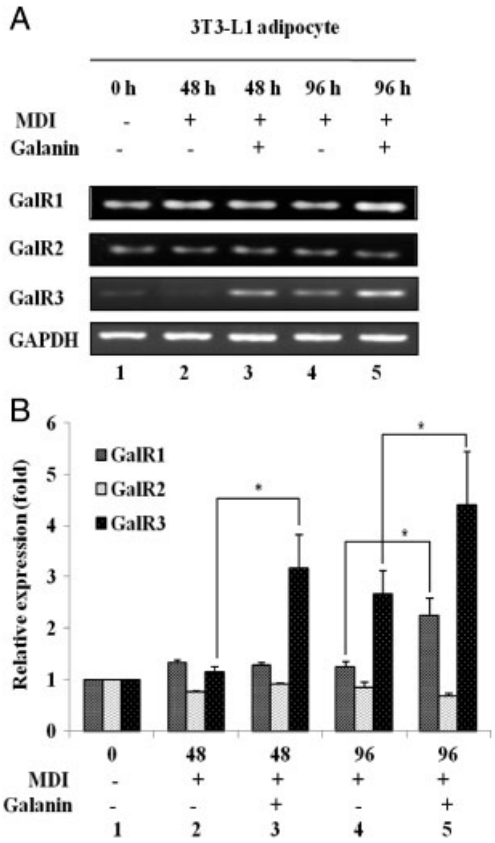

Figure 5. Effect of the galanin treatment on the expression of the GalRs in the 3T3-L1 adipocytes. The total RNA from the 3T3-L1 cells incubated in the presence or absence of 5 nM galanin along with MDI was isolated at the indicated time point, and semi-quantitative RT-PCR analysis was performed. The expression levels before the galanin treatment were set as onefold. (A) A representative image of the three independent experiments is shown in the left part of the figure. (B) The right part of the figure shows the results of the densitometric analysis of the mRNA expressions of GalR1, GalR2, and GalR3 in the 3T3-L1 cells. Each bar represents the mean±SEM of the three independent experiments (*$p<0.05$). Lane 1, 0 h MDI (–) galanin (–); lane 2, 48 h MDI (+) galanin (–); lane 3, 48 h MDI (+) galanin (+); lane 4, 96 h MDI (+) galanin (–); and lane 5, 96 h MDI (+) galanin (+).

expression was significantly upregulated at 96 h following MDI treatment as compared with undifferentiated cells (at 0 h). GalR1 gene expression was significantly upregulated at 96 h following galanin treatment, while GalR3 gene expression was significantly upregulated at 48 and 96 h, in a time-dependent manner, in 3T3-L1 cells undergoing differentiation (Fig. 5).

4 Discussion

The HFD that was used in the current study successfully induced the accumulation of the omental and subcutaneous adipose tissues, along with other obesity-related complications including dyslipidemia, insulin resistance, hyperinsulinemia, hyperglycemia, and fatty liver in mice (Table 1). Galanin is a highly inducible neuropeptide, showing a distinct upregulation after pathological disturbance within the nervous system [22]. The expression of galanin at both the mRNA and the peptide levels are elevated following estrogen administration, neuronal activation, and/or nerve injury, as well as during the development of the nervous system [23, 24]. In this study, galanin was expressed at a significantly higher level in the epididymal adipose tissues of the mice with HFD-induced obesity compared with the ND-fed mice, which could lead to the increased release of galanin from the visceral adipose tissues into the circulation. Although the blood levels of galanin were not determined in the HFD- or ND-fed mice in the present study due to the commercial unavailability of the mouse galanin antibody, an observation that significantly higher levels of plasma galanin were detected in obese women than in lean women [25] supports the hypothesis of this study. Therefore, it is plausible that, in diet-induced obesity, the elevated blood level of galanin will upregulate the expression of the GalRs located in the plasma membrane of the visceral adipose tissue, which may, in turn, stimulate the galanin-mediated signaling pathways in the adipose tissue. It was observed that the mRNA levels of GalR1, GalR2, and GalR3 were significantly upregulated in the enlarged epididymal and subcutaneous adipose tissues of the mice with HFD-induced obesity *versus* ND-fed mice, whereas in the skeletal muscles, the HFD-induced changes in the expression of the GalR1, GalR2, and GalR3 genes were reversed (Fig. 1). These results suggest the possibility that the galanin-mediated signaling pathways are among the important signaling cascades related to adipogenesis and adipocyte differentiation along with several known signaling pathways.

The *in vitro* findings that the mRNA levels of GalR1 and GalR2 were decreased in the fully differentiated 3T3-L1 cells but not in the preadipocytes partially conflicts with the *in vivo* results (Figs. 1A, B and D). This discrepancy might have been caused in part by the fact that the adipose tissues were affected by the upregulated plasma galanin level of obese animals [25], whereas the 3T3-L1 cells were not subjected to this influence (galanin stimulation) in the medium. Therefore, the effect of galanin treatment on the GalRs expression in the 3T3-L1 preadipocytes was further investigated. As shown in Fig. 5, the galanin treatment induced a significant increase in the expression of the mRNA levels of GalR1 (at 96 h) and GalR3 (at 48 and 96 h) compared with the untreated control cells. These results suggest the possibility that galanin upregulation increases the endogenous galanin-mediated signaling pathway *in vitro*, as well as *in vivo via* GalR1 and GalR3. Increased GalR expression has been reported in the amygdala of the early stage Alzheimer's disease [26]. In the small-cell lung cancer cells, the activation of GalR2 was found to initiate multiple signaling pathways, including the mitogen activated protein kinase pathway, resulting in the stimulation of clonal growth [27, 28]. In contrast, the GalR2 activation led to the inhibition of cell proliferation and the induction of apoptosis in the human SH-SY5Y neuroblastoma cells transfected with human GalR2 [29].

There are many pathways that influence adipocyte differentiation, such as insulin-, insulin-like growth factor 1- [30], Wnt10b- [31], transforming-growth factor β- [32], fibroblast growth factor- [33], and bone morphogenetic proteins- [34] mediated signaling. To determine whether the galanin-mediated signaling pathways are regulated in the adipose tissues of the mice with diet-induced obesity, the effects of HFD feeding on the expression and/or activation of the downstream signaling molecules and their target proteins in the epididymal and subcutaneous adipose tissues of the mice were further explored. 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 [12, 35]. It was found in the present study that the prolonged consumption of HFD led to increases in the expression of the galanin-mediated signaling molecules, such as Ras and PKCδ at the mRNA level, along with the enhanced phosphorylation of the c-Raf and ERK proteins in the epididymal adipose tissues of mice (Figs. 2 and 3). Besides, HFD strongly induced the PPARγ2 and aP2 protein expressions in the epididymal adipose tissue (Figs. 2 and 3). The activation of ERK signaling promotes adipogenesis by enhancing the PPARγ2 and CCAAT/enhancer-binding proteinα gene expressions during the differentiation of 3T3-L1 preadipocytes [19]. Therefore, the activation of the galanin-mediated signaling pathways appears to play a role, at least in part, in the development of HFD-induced adipogenesis in the epididymal adipose tissues of mice (Fig. 1).

Here, the fat depot-specific effect (epididymal *versus* subcutaneous) of an HFD on the regulation of the galanin-mediated signaling pathway in mice was observed. Unlike those observed in the visceral adipose tissue (epididymal fat), in the subcutaneous fat tissue, the mRNA levels of Ras in the two groups of mice were not different, and phosphorylated c-Raf was significantly decreased by HFD feeding (Fig. 4). Collectively, these results suggest that the HFD-induced activation of galanin-mediated adipogenesis signaling occurs

through the GalR2/PKCδ/ERK pathway in the subcutaneous adipose tissue. This depot-specific biology may underlie the differences in the response of visceral *versus* subcutaneous fat to an HFD. The fact that adipose tissues from various anatomic locations contribute differently to endocrine-metabolic regulation is a well-accepted phenomenon supported by a large body of experimental evidences [36]. The venous drainage of intra-abdominal fat is different from that of subcutaneous fat may partially underlie such phenomenon [36]. In addition, the adipose tissue consists of a mixed population of cells, and its composition differs from one fat depot to another and may change dynamically in response to nutritional and genetic factors.

A suggested consequence of the increased GalRs expression in the enlarged adipose tissue is the inhibition of the thermogenesis-signaling cascade. The stimulation of the GalRs expressed in the GalR2-transfected cell lines inhibits forskolin-stimulated cyclic adenosine monophosphate production in a PTX-sensitive manner, which leads to reduced CREB activation [12]. Downregulated phospho-CREB decreases the expression of PGC-1α and UCP1 in the brown adipose tissues and liver [37]. Findings in the epididymal and subcutaneous adipose tissues of mice demonstrated that the HFD-induced overexpressions of galanin and its receptors were accompanied by the downregulation of the phosphorylated CREB protein and the subsequent reductions in the PGC-1α and UCP1 mRNA levels.

In conclusion, 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, was observed in this study. Moreover, HFD feeding stimulated the expression of the key adipogenic transcription factors, PPARγ2 and its target protein, aP2, in their visceral and subcutaneous adipose tissues of mice. Meanwhile, HFD was found to inhibit the activation and expression of phospho-CREB, PGC-1α, and UCP1, which are the downstream molecules and target proteins that are related to thermogenesis. These results suggest the possible association of the galanin-mediated signaling pathways in the manifestation of the HFD-induced activation of adipogenesis along with suppression of thermogenesis in the adipose tissues of mice. From a therapeutic point of view, the results of this study suggest that GalR antagonists or inhibitors may find clinical application in the control of diet-induced obesity and fat deposition.

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5 References

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