Ethanolic Extracts of *Brassica campestris* spp. *rapa* Roots Prevent High-Fat Diet-Induced Obesity via β3-Adrenergic Regulation of White Adipocyte Lipolytic Activity

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ABSTRACT The influence of Ethanolic extracts of *Brassica campestris* spp. *rapa* roots (EBR) on obesity was examined in imprinting control region (ICR) mice fed a high-fat diet (HFD) and in 3T3-L1 adipocytes. The ICR mice used were divided into regular diet, HFD, EBR (50 mg/kg/day EBR administered orally), and orlistat (10 mg/kg/day orlistat administered orally) groups. The molecular mechanism of the anti-obesity effect of EBR was investigated in 3T3-L1 adipocytes as well as in HFD-fed ICR mice. In the obese mouse model, both weight gain and epididymal fat accumulation were highly suppressed by the daily oral administration of 50 mg/kg EBR for 8 weeks, whereas the overall amount of food intake was not affected. EBR treatment induced the expression in white adipocytes of lipolysis-related genes, including β3-adrenergic receptor (β3-AR), hormone-sensitive lipase (HSL), adipose triglyceride lipase, and uncoupling protein 2. Furthermore, the activation of cyclic AMP-dependent protein kinase, HSL, and extracellular signal-regulated kinase was induced in EBR-treated 3T3-L1 cells. The lipolytic effect of EBR involved β3-AR modulation as inferred from the inhibition by the β3-AR antagonist propranolol. These results suggest that EBR may have potential as a safe and effective anti-obesity agent via the inhibition of adipocyte lipid accumulation and the stimulation of β3-AR-dependent lipolysis.

KEY WORDS: • β3-adrenergic receptor • anti-obesity effect • *Brassica campestris* spp. *rapa* • lipolysis • white adipose tissue

INTRODUCTION

Obesity is the most common nutritional disorder in the developed world and is a risk factor for the genesis or development of various diseases, including coronary heart disease, hypertension, type 2 diabetes mellitus, cancer, respiratory complications, and osteoarthritis. Attempts to correct the metabolic disparity of obesity include the application of inhibitors of appetite (sibutramine), gastrointestinal lipid uptake (orlistat), and peroxisome proliferator-activated receptor (PPAR)-α (fibrates). However, these drugs can produce adverse side effects. Therapeutically potent and safe anti-obesity reagents such as botanical drugs are urgently required.

The β3-adrenergic receptor (β3-AR) is located on the surface of both white and brown adipocytes. The β3-AR has attracted interest as a potential treatment of obesity because agonists stimulate lipolysis and thermogenesis in rodent and human white adipose tissues (WATs), influencing fuel supply and concomitantly increasing body energy expenditure via stimulation of uncoupling protein (UCP). Furthermore, β3-AR agonists normalize secretion of adipocytokines from adipocytes, which influences insulin sensitivity through the action of adiponectin and tumor necrosis factor-α (TNF-α). Adipocyte lipolysis is stringently regulated by hormones, neurotransmitters, and other effector molecules and by hormone-sensitive lipase (HSL). HSL is an enzyme that hydrolyzes intracellular triacylglycerol and diacylglycerol, and its activity is controlled by phosphorylation of specific serine residues. The binding of agonists to β3-ARs coupled with adenylate cyclase via the stimulatory G-protein leads to an increased production of cyclic AMP and activation of cyclic AMP-dependent protein kinase (PKA). One of the main targets for PKA phosphorylation is HSL.
Collectively, the available data indicate that β3-AR agonists activate not only PKA but also extracellular signal-regulated kinase (ERK), which can stimulate HSL activity.13

Brassica campestris spp. rapa is a member of the Brassicaceae family, which includes cabbage, broccoli, and turnip. In particular, B. campestris spp. rapa is mainly represented by the Ganghwa turnip, in Korea. It is cultivated commercially in the west coast region of Ganghwa County as the raw material for varieties of kimchi, a Korean traditional fermented vegetable food. The roots of Ganghwa turnip contain several biologically active compounds such as indole alkaloids, sterols, and fatty acid methyl esters.14-16 Ethanolic extracts of B. campestris spp. rapa (EBRs) modulate the deleterious effects of diabetes17 and protect against cisplatin-induced nephrotoxicity.18 Supplementation with EBR (0.26 g/100 g of diet) lowers the weight of epididymal WAT and interscapular brown adipose tissue in type 2 diabetic (C57BL/6J-db/db) mice.17 As part of our screening of ethanolic extracts isolated from various plant sources to detect anti-obesity activities, EBR was found to inhibit adipocyte lipid accumulation and stimulate lipolysis in a dose-dependent manner. In this study, we have more fully investigated the molecular mechanism of the anti-obesity effect of EBR in a 3T3-L1 mouse embryo cell model as well as in a high-fat diet (HFD)-fed imprinting control region (ICR) mouse model.

MATERIALS AND METHODS

Materials

Dulbecco’s modified Eagle’s medium and other materials for cell culture were purchased from Gibco-BRL (Gaithersburg, MD, USA). Adipogenic stimuli including insulin, dexamethasone, and 3-isobutyl-1-methylxanthine were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-phospho-HSL (Ser562) and anti-HSL antibodies (Abs) were purchased from Cell Signaling Technology (Danvers, MA, USA), anti-phospho-PKA (Thr77) and anti-PKA Abs were purchased from Abcam (Cambridge, UK), and all other Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The adipocyte lipolysis assay kit was purchased from Zen-Bio (Raleigh, NC, USA). All other reagents were obtained from Sigma-Aldrich.

Preparation of EBR

B. campestris spp. rapa was obtained from the Ganghwa County Agricultural Technology Center (Incheon, Republic of Korea), and its identity was confirmed by one of the authors (H.-G.C.). A voucher specimen (number 051/57) has been deposited at the Laboratory of Natural Product Chemistry, Kyung Hee University, Suwon, Republic of Korea. The fresh roots were cut and extracted three times with 95% ethanol at room temperature. The combined ethanolic extracts (EBRs) were evaporated in vacuo to obtain 10.6 g per 100 g of fresh root material. The total polyphenol content in the sample, which was determined using the FolinCiocalteu method, was 8.2 ± 0.1 mg of gallic acid equivalents/g of EBR.19 The total flavonoid content, which was determined using a colorimetric method, was 1.4 ± 0.3 mg of quercetin equivalents/g of EBR.19

Animals and diets

Six-week-old male ICR mice were purchased from Central Lab Animal (Seoul, Republic of Korea) and housed in a room having controlled temperature (25 ± 2°C), humidity (50 ± 5%), and lighting (alternating 12-hour periods of light and dark) at the Korean Research Institute of Bioscience and Biotechnology, Daejeon, Republic of Korea. The mice were divided into four groups and fed a standard laboratory regular diet (RD) (AIN-76A; Dyets, Bethlehem, PA, USA) (n = 8), a HFD consisting of 25% beef tallow (Dyets; n = 8) with no supplement, HFD supplemented with EBR (50 mg/kg/day, n = 8), or HFD supplemented with the fat absorption inhibitor orlistat (Xenical® [XEN], Sigma-Aldrich) (10 mg/kg/day, n = 8) as a positive control for 8 weeks. EBR and XEN supplements were dissolved in 0.2 mL of sterilized distilled water containing 0.3% tween-80 and administered orally. Mice were treated in accordance with the Korean Research Institute of Bioscience and Biotechnology Guide for the Care and Use of Laboratory Animals.

Measurement of metabolic parameters

Food intake was monitored daily, and body weight was monitored weekly. Blood was collected from the inferior vena cava into an EDTA-coated tube during feeding periods every other week and at the end of the experiment. There was a 12-hour fast prior to euthanasia. The livers and epididymal fat pads were removed, weighed, and frozen in liquid N2 or stored in RNA later® (Qiagen, Valencia, CA, USA). The plasma concentrations of total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), triglyceride (TG), and glycerol were analyzed using an automatic blood chemical analyzer (CIBA Corning, Medfield, MA, USA). Serum levels of leptin and adiponectin were measured with an enzyme-linked immunosorbent assay kit from R & D Systems (Minneapolis, MN, USA).

Histological analysis of animal adipocytes

Freshly isolated epididymal WAT sections were fixed in 10% formalin for 24 hours and refixed in Bouin’s solution for 8 hours. Following sufficiently rinsing in flowing water, tissues were processed in a paraffin automatic processor using a programmed cascade. The paraffin-embedded samples were dissected to 8 μm thick and stained with hematoxylin-eosin. After one photograph per sample was obtained for the stained adipose tissue using an optical microscope operating at magnifications of ×200 and ×400, the size of 10 randomly selected adipocytes per photograph was measured using a MetaMorph® computer image analysis program (Molecular Devices, Sunnyvale, CA, USA) to obtain an average value.
Total RNA isolation and quantitative real-time polymerase chain reaction (PCR)

After isolation of total RNA from WAT and 3T3-L1 cells using the RNeasy mini kit (Qiagen) as described by the manufacturer, cDNA was prepared with total RNA (1 µg) using the Superscript cDNA synthesis kit (Qiagen). Real-time PCR was performed using SYBR Green Supermix reagent with the 7500-Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Primer sequences were as follows: β3-AR (NM_013462), forward 5'-AGGCCAACC TGCTGTAAATCA-3' and reverse 5'-TCCACAGTTGC AACGATTT-3'; UCP-2 (NM_011671), forward 5'-GCC TCTACGAACCTGCAA-3' and reverse 5'-CTTCGCA CAGTGGCTCT GTTAT-3'; HSL (NM_010719), forward 5'- TTCAAGGTTGAAGGACT-3' and reverse 5'-ACTTC TGGGTATAGCGCAAT-3'; adipose TG lipase (ATGL) (NM_025802), forward 5'-ACCAACACCAC ACCATCCAG TT-3' and reverse 5'-TTTGCACATTCTCGAGGAA-3'; interleukin (IL)-6 (X54542), forward 5'-GCTACTGGA GTACCATGA AG-3' and reverse 5'-CTCGTACTCCAG TTACTCTG-3' adiponectin (U49915), forward 5'-CATGCC GAGAAGACGTAC-3' and reverse 5'-CGTACACAT AAGGCTCT-3'; and leptin (NM_0008493), forward 5'- TGGCAAGGTGAAGAG-3' and reverse 5'-TCCA GGTCAATGGGCAGG-3'.

Western blot analysis

Cytosolic extracts were prepared with a lysis buffer containing 0.2% Nonidet P-40, 10 mM HEPES, 15 mM KCl, 2 mM MgCl2, 1 mM dithiothreitol, 0.1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride. Cytoplasmic extracts were subjected to immunoblot analysis with Abs to HSL, ATGL, UCP-2, adiponectin, IL-6, TNF-α, phosphoryl-ERK, ERK, phosphoryl-PKA, PKA, phosphoryl-HSL, and HSL. Polyclonal Abs to ERK, phosphoryl-ERK, HSL, phosphoryl-HSL, PKA, and phosphoryl-PKA were purchased from Cell Signaling Technology, and others were purchased from Santa Cruz. Immune complexes were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG Ab (Santa Cruz). The protein levels were determined using an ECL western blot kit (Eliips Biotech, Daejeon) and Image Reader (LAS-3000 Imaging System, Fuji Photo Film, Tokyo, Japan).

3T3-L1 cell culture

Mouse embryo 3T3-L1 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). 3T3-L1 preadipocytes were incubated in a basal medium consisting of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 100 U/mL penicillin-streptomycin at 37°C in a 5% CO2 atmosphere.

Differentiation of adipocytes and Oil red O staining

Two days after confluence, designated day 0, 3T3-L1 preadipocytes were treated with a differentiation medium containing 0.5 mM 3-isobutyl-1-methylxanthine, 1 µM dexamethasone, and 10 µg/mL insulin in basal medium containing 10% fetal bovine serum. On day 3, the medium was changed to Dulbecco’s modified Eagle’s medium containing only 2 µg/mL insulin, and the cells continued to differentiate until day 10 in a basal medium. Cells were incubated with 0-100 µg/mL EBR for the differentiation period. Lipid accumulation in differentiated adipocytes was assessed by Oil Red O staining. Cells were washed twice with phosphate-buffered saline, fixed in 3.7% formaldehyde for 30 minutes, stained for 3 hours with 0.7% (wt/vol) Oil Red O solution in 100% 1,2-propanediol, and washed with 85% and 50% 1,2-propanediol. The stained lipid droplets in the cells were dissolved in isopropanol and quantified by measuring the absorbance at 510 nm. The stained cells were covered with the mounting solution, and photographs were taken.

Lipolysis assay

The lipolysis assay was performed with 3T3-L1 adipocytes using a lipolysis assay kit (Zin-Bio) as described by the manufacturer. EBR or TNF-α was administered with or without propranolol, a β3-AR antagonist, to the matured adipocytes for 24 hours. Glycerol released into the assay buffer was measured by the absorbance of 540 nm and quantified with the use of a colorimetric assay.

Statistical analysis

Data from WAT, liver, and plasma are presented as mean ± SE values. Significant differences among the groups were determined by one-way analysis of variance using JMP® software (SAS Institute, Cary, NC, USA). Data from 3T3-L1 cells are expressed as mean ± SD values of two independent experiments performed in duplicate. Statistical analysis was done using Student’s t test. Values of P < .05 were considered significant.

RESULTS

We conducted animal studies to assess the effects of EBR on body fat metabolism and its potential role in the prevention of obesity. Consistent with these in vitro bioactivities, EBR was associated with reduced body weight and adipose tissue size in an HFD-induced obese mouse model. After an 8-week feeding period, HFD mice displayed increased body weight compared to mice fed RD (Table 1). Body weight was significantly reduced in the EBR and XEN groups (39.4 ± 1.5 g and 38.9 ± 3.0 g, respectively) compared with the HFD group (43.5 ± 3.7 g) (P < .05). The food intakes were not significantly different between the HFD and EBR groups; however, the food efficiency was significantly lower in the EBR and XEN groups (4.12 ± 0.16 and 4.62 ± 0.04, respectively) compared with the HFD group (6.96 ± 0.12).

To test whether the reduction of body weight was caused by the decrease in adiposity, fat pads of animals were dissected and weighed (Table 1). Abnormalities of epididymal WAT in the HFD group were observed in the necropsy
Table 1. Effects of EBR on Food Intake, Body Weight Gain, Food Efficiency, and Organ Weight

<table>
<thead>
<tr>
<th></th>
<th>RD</th>
<th>HFD</th>
<th>EBR</th>
<th>Orlistat</th>
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<tbody>
<tr>
<td>Food intake (g/day)</td>
<td>3.80 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.75 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.87 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.98 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial (g)</td>
<td>32.4 ± 1.4</td>
<td>31.8 ± 0.9</td>
<td>32.8 ± 0.6</td>
<td>31.7 ± 1.2</td>
</tr>
<tr>
<td>Final (g)</td>
<td>39.2 ± 1.5</td>
<td>43.5 ± 3.7</td>
<td>39.4 ± 1.5</td>
<td>38.9 ± 3.0</td>
</tr>
<tr>
<td>Weight gain (%)</td>
<td>15.86 ± 2.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.15 ± 1.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.66 ± 0.99&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.09 ± 1.38&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Food efficiency (%)</td>
<td>2.86 ± 0.10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.96 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.12 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.62 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Organ weight (g)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Liver</td>
<td>1.70 ± 0.09</td>
<td>2.06 ± 0.13</td>
<td>1.88 ± 0.08</td>
<td>1.81 ± 0.12</td>
</tr>
<tr>
<td>WAT</td>
<td>3.07 ± 0.18</td>
<td>4.31 ± 0.26</td>
<td>2.83 ± 0.29</td>
<td>3.26 ± 0.57</td>
</tr>
</tbody>
</table>

Data are mean ± SE values. Weight gain (%) = (weight gain/final weight) × 100. Food efficiency (%) = (body weight gain [in g/day]/food intake [in g/day]) × 100.
<sup>abc</sup>Means in the same row not sharing a common letter superscript are significantly different (P < .05) between groups.

performed at the end of the experiment. WAT weights were markedly reduced in the EBR group (2.83 ± 0.29 g) in comparison with the HFD group (4.31 ± 0.26 g) and even more than with the XEN group (3.26 ± 0.57 g). In the histological analysis of epididymal WAT, the size of the adipocytes in EBR-treated mice (Fig. 1) was markedly decreased in comparison with that of HFD mice. The liver weight was not significantly different in any group.

We next examined whether the EBR-associated alterations in adiposity correlated with changes in blood levels of lipids and adipokines. An 8-week administration of EBR decreased the levels of TC and TG and increased the ratio of HDL-C to TC in the HFD group (Table 2), but the values were not significantly different. On the other hand, the TG and glycerol levels of the XEN group were significantly decreased by 57% and 78% of those in the HFD group, respectively. The adipokines leptin and adiponectin correlated positively and negatively, respectively, with obesity. Consistent with this, EBR administration produced a 62% decrease in leptin level (P < .01) and a 13% increase in adiponectin level (P < .05) compared with the HFD group. These observations are consistent with the suggestion that EBR might suppress the development of obesity by regulating adiposity formation independent of the plasma level of lipids and might prevent metabolic disease by improving the adipokine profile.

![Image](image1.png)

**FIG. 1.** EBR treatment reduces the size of white adipocytes in HFD-fed ICR mice. Epididymal WAT freshly isolated from each mouse group was fixed in 10% formalin and embedded in paraffin. Sections 8 mm thick were stained with hematoxylin and eosin, and cell morphology and size were analyzed. <sup>abc</sup>Means not sharing a common letter are significantly different between groups (P < .05).
Table 2. Effects of EBR on Plasma Level of Lipid Parameters and Adipokines

<table>
<thead>
<tr>
<th>Lipid parameters</th>
<th>RD</th>
<th>HFD</th>
<th>EBR</th>
<th>XEN</th>
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<tr>
<td>TC (mg/dL)</td>
<td>188.0±10.3</td>
<td>245.1±22.4</td>
<td>218.0±10.4</td>
<td>208.3±18.7</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>116.3±5.3</td>
<td>168.3±13.0</td>
<td>159.5±6.0</td>
<td>162.0±9.8</td>
</tr>
<tr>
<td>HDL-C/TC</td>
<td>0.621±0.017</td>
<td>0.693±0.016</td>
<td>0.735±0.014</td>
<td>0.633±0.091</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>152.5±15.6b</td>
<td>206.9±8.1a</td>
<td>171.0±17.8a</td>
<td>118.5±11.1b</td>
</tr>
<tr>
<td>Glycerol (mg/mL)</td>
<td>65.8±4.4a</td>
<td>67.4±3.2a</td>
<td>63.2±2.4b</td>
<td>52.5±2.6b</td>
</tr>
<tr>
<td>Adipokines</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>10.6±1.0b</td>
<td>28.0±3.9a</td>
<td>10.6±2.1b</td>
<td>13.0±3.7b</td>
</tr>
<tr>
<td>Adiponectin (×10^7 ng/mL)</td>
<td>33.7±1.8a</td>
<td>30.5±0.9b</td>
<td>34.6±1.1b</td>
<td>28.4±1.8b</td>
</tr>
</tbody>
</table>

Data are mean ± SE values.

abMeans in the same row not sharing a common letter superscript are significantly different (P<.05) between groups.

To test whether the reduction of fat mass in EBR-treated mice was accompanied by changes in the expression of genes involved in lipolysis, total RNA and protein lysates were prepared from epidydymal WAT followed by quantitative real-time PCR and western blot analysis, respectively (Fig. 2). The mRNA level of HSL, a major rate-limiting enzyme of lipolysis, was markedly induced about threefold, with mRNA levels of β3-AR and ATGL also being elevated about two- and 1.5-fold, respectively. On the other hand, the quantity of mRNA of lipoprotein lipase, the enzyme responsible for the catabolism of TG-rich lipoproteins, did not differ significantly in any group (Fig. 2A). It is interesting to note that the pattern of expression of the adipokines adiponectin and leptin and the cytokines IL-6 and TNF-α according to diet-induced obesity in the HFD group was restored by oral administration of EBR (Fig. 2B). The western blot analysis of these transcriptional factors (Figs. 2C and D) showed that the expression of HSL, ATGL,
FIG. 3. Effects of EBR on regulation of genes and activation of various kinases involved in lipolysis in 3T3-L1 adipocytes. (A) 3T3-L1 adipocyte differentiation was induced, 2 days after confluence, by 0.5 mM 3-isobutyl-1-methylxanthine, 1 mM dexamethasone, and 10 μg/mL insulin in basal medium containing 10% fetal bovine serum. After 72 hours, the induction medium was changed to a maturation medium containing only 2 μg/mL insulin and 10% fetal bovine serum. The maturation medium was changed 2 days later, and the cells were maintained in basal medium that was replenished every other day for 6 days until harvested. Cells were incubated with 0–100 μg/mL EBR for the differentiation period. The levels of mRNA were measured by quantitative real-time PCR and normalized to glyceraldehyde 3-phosphate dehydrogenase. *P < .05 versus preadipocytes. **P < .05 versus adipocytes. (B) Cells were treated with 50 μM and 100 μg/mL EBR for 2 hours (HSL and PKA) or 12 hours (ERK) and lysed. Total or phosphorylated (p-) PKA, HSL, and ERK were detected by western blot using their specific antibodies. The images are representative of two independent experiments. Intensity of the detected bands was compared using a model GS800 calibrated densitometer (Bio-Rad, Hercules, CA, USA) with Quantity One software (version 4.4.0). The total protein of kinase was used as the internal control.

UCP-2, and adiponectin was significantly increased and that expression of IL-6 and TNF-α was decreased in the EBR group compared to the HFD group.

The influence of EBR treatment on the regulation of gene expression was also confirmed at the cell level using 3T3-L1 adipocytes. Treatment with 100 μg/mL EBR markedly induced the gene expression of β3-AR about sevenfold as well as the gene expression of HSL and ATGL about twofold each in 3T3-L1 cells (Fig. 3A). β3-AR agonists stimulate adrenergic receptors, followed by the increase of intracellular cyclic AMP and activation of PKA, which, in turn, phosphorylates HSL, the rate-limiting enzyme catalyzing lipolysis. Moreover, β3-AR agonists not only activate PKA, but also activate the mitogen-activated protein kinase pathway and ERK. Phosphorylation of HSL is associated with an increase in hydrolytic activity of the enzyme and the translocation of HSL from the cytosol to the lipid droplet in some physiological settings. We next investigated whether the kinase activation for HSL as well as PKA and ERK, a downstream target of β3-AR, was also induced by EBR treatment. As shown in Figure 3B, significant increases in PKA phosphorylation were detectable within 2 hours, whereas the total PKA amounts were almost identical at each time point, suggesting their involvement in the lipolytic process. EBR treatment also induced HSL phosphorylation in a manner similar to that observed for PKA phosphorylation. Moreover, EBR treatment for up to 12 hours induced ERK phosphorylation, which regulates HSL activation, suggesting that the HSL activation due to treatment with EBR occurs via several kinase signaling pathways.

To more specifically evaluate the influence on obesity of EBR, assays of adipogenesis and lipolysis were performed in 3T3-L1 cells. Oil Red O staining showed that the treatment of EBR during adipocyte differentiation inhibited the lipid accumulation in a dose-dependent manner (Fig. 4A and B), with accumulation being nearly abrogated by 100 μM EBR. Otherwise, as a nonselective β-blocker, propranolol can be used in the study for β3-AR agonists. The glycerol release from the mature adipocytes was increased with EBR treatment in a dose-dependent manner (Fig. 4C). However, when the mature adipocytes were treated with propranolol (10 μM) and the β3-AR was blocked, EBR did not increase the release of glycerol from the mature adipocytes (Fig. 4A and C). As gathered from all the results above, it is clear that the anti-obesity effect of EBR is due to the lipolytic potency via β3-AR modulation with inhibition of the lipid accumulation in adipocytes.

DISCUSSION

Adipose tissue is the major site for storage of TGs. Mobilization of free fatty acids and glycerol via lipolysis provides a rapid source of fuel for other organs in response to fasting, infection, and inflammation. A decreased lipolytic effect by β3-AR-dependent stimulation in adipose tissue has repeatedly been demonstrated in obesity and may be a cause of excess accumulation of body fat. The maximum lipolytic capacity of fat cells was significantly decreased in obesity when measured using a nonselective β-receptor agonist (isoproterenol). Likewise, enzyme activity, protein expression, and mRNA of HSL are significantly decreased in adipocytes of obese subjects. In the current study, EBR-treated mice had a significant reduction in epididymal WAT contents as well as weight gain. Microscopic analysis ascertained that the reduction was due to the restricted adipocyte size. So, it is clear that EBR can affect adipocyte formation in ICR mice fed HFD.

The role of UCP-2 in the energy balance is not clear, although a role of this protein in energy balance and thermogenesis has been suggested. In this study, the expression of UCP-2 was down-regulated in the epididymal adipose tissue of HFD-induced ICR mice. These results are
in accordance with the previous observation that UCP-2 and UCP-3 mRNA levels decrease in WAT of HFD-induced rats. The expression of UCP-2 was significantly increased in the EBR group compared to the HFD group. This result suggests that the decrease of WAT adiposity in the EBR group is through an increase in the uncoupled respiration in WAT. On the other hand, UCP-2 may be important for the regulation of insulin secretion and the protection of the organism against free oxygen radicals.

Considering that HSL functions as the rate-limiting enzyme catalyzing the lipolysis of triglycerol and diacylglycerol, we attempted to identify the primary signaling pathways involved in EBR-induced lipolysis and determined that a variety of kinases, particularly PKA and ERK, were concomitantly involved in induction of lipolysis, probably via HSL activation. Under complex hormonal control, HSL is the target of both lipolytic and antilipolytic hormones. The traditional epinephrine-mediated lipolysis pathway involves the PKA cascade. Activated PKA phosphorylates HSL, which is subsequently translocated to the surfaces of lipid droplets, catalyzing lipid hydrolysis. HSL can also be activated via ERK. ERK pathway activation appears to be able to regulate adipocyte lipolysis via the phosphorylation of HSL and increases in the activity of HSL. In the present study, the lipolytic effect of EBR was found to be associated with stimulated β₃-AR activity and coincident induction of cyclic AMP-dependent PKA activation and phosphorylation of HSL (Figs. 2 and 3).
Notably, EBR induced lipolytic activation through the expression of genes involved in lipolysis in epididymal WAT and 3T3-L1 cells. HSL expression was also induced by two-thirds of white adipocytes by EBR treatment. Although the precise mechanism of the transcriptional regulation of the HSL gene remains to be elucidated, it has been demonstrated that there is a functional peroxisome proliferator responsive element in the mouse HSL promoter and that both PPAR and retinoid X receptor interact with this region. These results indicate that the HSL gene is transcriptionally regulated by a PPAR/retinoid X receptor heterodimer. Recently, it was reported that the PPARγ agonist rosiglitazone increases the level of HSL mRNA in rat primary brown adipocytes and rat WAT explants. Similar to other nuclear receptors, PPARs are phosphoproteins, and their transcriptional activity is affected by cross-talk with kinases and phosphatases. Phosphorylation by ERK and PKA affects their activity in a ligand-dependent or -independent manner. Considering that kinases were shown in the present study to be activated by EBR treatment, we suggest that cross-talk between ERK and PPAR is linked with the induction of HSL expression.

CONCLUSIONS

Our results implicate EBR as having potential as a safe and effective botanically derived compound in the treatment of obesity via the stimulation of β3-AR-dependent lipolysis in 3T3-L1 cells and epididymal adipocytes of HFD-induced obese mice, although the precise active component of EBR remains to be elucidated. EBR treatment induces the coincident activation of PKA, HSL, and ERK and the expression of the lipolysis-related genes β3-AR, HSL, ATGL, and UCP-2 in white adipocytes. The expression pattern of adipokines, adiponectin, and leptin according to diet-induced obesity in HFD mice can be restored to that in mice fed RD by oral administration of EBR. The relative biochemical complexity of EBR may pleiotropically influence several lipolysis targets simultaneously.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES