Basic nutritional investigation

Resveratrol promotes the arcuate nucleus architecture remodeling to produce more anorexigenic neurons in high-fat-diet–fed mice

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ABSTRACT

Objective: Adult hypothalamic neurogenesis has been considered a central regulator of energy balance. Resveratrol (RSV), a natural polyphenol, influences the body fat mass and reduces the amount of adipose tissue. The present study was designed to evaluate the effect of RSV on dynamic of hypothalamic neurons in a diet-induced obesity model of mice.

Methods: Apoptosis, neurogenesis, the expression of the main trophic factors, and the fate of newborn cells were evaluated in the hypothalamus of adult male C57 BL/6 J mice fed a normal diet, a high-fat (HF) diet, or an HF diet supplemented with 400mg/kg RSV (HF + RSV) for 6 wk.

Results: The HF diet caused an increase in neuronal apoptosis in the hypothalamus, which coincided with an increase in the number of newborn cells in the arcuate nucleus, suggesting that compensatory mechanisms developed to overcome deleterious effects of the HF diet. Addition of RSV to the HF diet enhanced the production of newborn cells in all studied regions of the hypothalamus. These changes were paralleled by enhancement of the expression of ciliary neurotrophic factor. Interestingly, a considerable proportion of newborn cells expressed neuropeptide Y in the arcuate nucleus of the HF group, and conversely, most of them differentiated to proopiomelanocortin neurons in HF + RSV mice.

Conclusions: Diets rich in fat changed hypothalamic neuronal balance toward orexigenic versus anorexicogenic neurons. Administration of RSV to the HF diet reversed this balance toward generation of anorexigenic neurons. These data point to the potential for RSV in regulation of body weight, possibly via modulation of hypothalamic neurogenesis.

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in fat could cause neurodegeneration via hypothalamic endoplasmic reticulum stress and induction of apoptosis in hypothalamic neurons [4]. Novel facts have been reported on hypothalamic remodeling and body weight [5,6].

Parallel to recent studies focused on adult neurogenesis in different areas of the mammalian central nervous system, some works have been conducted to evaluate this promising phenomenon in the hypothalamus [7–9]. Consequently, the existence of an active neurogenesis in the hypothalamic area of the adult mammalian brain has been introduced [10,11]. Considering the fact that the hypothalamus is the main central nervous system area for the regulation of food intake and energy expenditure, investigations have been started to evaluate the effects of an HF diet on hypothalamic neurogenesis [12,13]. Despite controversial results, changing the neurogenesis by an HF or calorie-restricted diet is well accepted [14–16]. Because the hypothalamus works as a gate for sensing different nutrients, the little but outstanding previously described data shed a new insight for scientists investigating the effects of other nutritional conditions on neurogenesis in this energy homeostasis circuitry.

Among various natural molecules, resveratrol (RSV) is the most recognized polyphenol; it belongs to the stilbene group and is well known for its phytoestrogenic and antioxidant properties [17]. This phytoalexin compound is present in different plants such as peanuts and the skins of grapes [18,19]. In recent years, rapidly accumulating evidence indicated a remarkable range of biological functions of RSV because this nutrient was revealed to have promising effects in cardioprotection, cancer chemoprevention, and chemotherapy [20,21]. Furthermore, the results of previous in vivo and in vitro studies indicated that RSV can influence body fat mass via decreasing proliferation of preadipocytes, inhibiting differentiation of preadipocytes, promoting lipolysis, and inducing apoptosis of existing adipocytes, thereby reducing the amount of adipose tissue [22,23]. It also stimulates brown fat activation [24,25]. In addition, studies on rodents have found that RSV administration in high doses can increase resistance to obesity during consumption of an HF diet [17,26,27]. Although several studies have consistently reported numerous beneficial antiobesity effects of RSV on peripheral tissues, there is no report about RSV’s potential effects on hypothalamic neurogenesis. The aim of the present study was to determine the effect of dietary RSV on neurogenesis in different areas of the hypothalamus in an HF diet–induced obesity in animal model.

Materials and methods

Animals

Thirty C57 BL/6 male mice (5 wk of age with weight of 15.2 ± 0.2 g) were obtained from Pastor Institute (Karaj, Iran) and kept under standard vivarium conditions on a 12-h light-dark schedule. Mice were housed five per cage with free access to water and subjected to three different dietary regimens as described later. All procedures were conducted in accordance with the guiding principles for the care and use of animals and were approved by the ethics committee of Tehran University of Medical Sciences.

Experimental design

After 1 wk of acclimatization, the subjects had free access to a chow diet (control), in-house prepared HF diet (HF), or HF diet + RSV (HF + RSV) for 6 wk. The chow diet was purchased from Behparvar Industries (Tehran, Iran), in which approximately 9% of the total kilocalories were fat (Table 1). In the HF diet, the chow in powder form was mixed by adding soy oil and butter (1:9 volume: weight) up to 60% per total kilocalorie. After homogenization, a dough-like texture was shaped and the obtained blocks were dried and used for feeding. RSV purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan), with purity of more than 99%, was mixed directly with the HF diet at a concentration that provided a 400 mg/kg/d dose. All processes of preparing the diet were performed in dark conditions. The diet containing RSV was stored at −20°C, and food was provided in cages for no more than 2 d. To ensure a 400 mg/kg/d resveratrol dose, at the beginning of the study the food intake was determined using a pair-fed group to calculate the amount of RSV that should be added to the diet. Then, during the study, the amount of RSV added to the chow was adjusted according to changes in body weight and food intake. Because the animals were housed in groups of five mice per cage, each mouse received approximately 400 mg/kg/d resveratrol.

Table 1

<table>
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<tr>
<td>Protein (g/100 g)</td>
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<tr>
<td>Energy (KJ/g)</td>
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</tr>
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* Declared by producer: Behparvar Company, Tehran, Iran.

Food intake and body weight monitoring

To evaluate the food intake throughout the experiment, a preweighed amount of food was provided in standard stainless steel hoppers. After 24 h the amount of food remaining, including any on the bottom of the cages or any that had spilled, was recorded. Intake was calculated as the difference in weight (in grams) between the food provided and the food recovered. Body weight of the animals was measured weekly.

Bromodeoxyuridine labeling

To label proliferating cells in different areas of the hypothalamus, animals of all groups (n = five per each group) received bromodeoxyuridine (BrdU; Sigma, St. Louis, MO, USA) administrated in the morning and evening by intraperitoneal injection at 50 mg/kg of body weight during the first 9 d of the study. To prepare a BrdU solution for injection, BrdU was dissolved in saline and pH was adjusted to 7.35.

Tissue preparation

At the end of the experiments, the animals were deeply anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) and perfused transcardially with 0.9% cold normal saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) with a pH of 7.4. Total intraabdominal white adipose tissues (WAT) were dissected and weighted. After brain and intraabdominal WAT dissection, the tissues were stored in 4% paraformaldehyde for 24 h at 4°C. Subsequently, tissue processing was performed. Then the tissues were embedded in paraffin, and 5-µm-thick coronal sections were prepared using a microtome. Brain sections were collected throughout the caudal hypothalamus (~1.22 to ~2.12 from bregma). The sections were then mounted onto gelatin-subbed slides and used for immunohistochemistry, transferase biotin–deoxyuridine triphosphate nick end labeling (TUNEL) assay, or hematoxylin and eosin (H&E) staining.

H&E staining and analysis of WAT cellularity

For evaluating the fat tissue cellularity, the sections of intraabdominal WAT (three sections per animal) were hydrated with descending grades of alcohol and then stained with H&E. Finally, the tissues were dehydrated in alcohol of ascending concentrations. The photographs captured by a light microscope (Olympus, Japan) were analyzed using Adiposoft, a fully automated software for estimation of WAT cellularity in histologic sections.

Evaluation of apoptosis

Apoptosis in different regions of the hypothalamus was detected employing the TUNEL assay using a commercially available kit (DNA Fragmentation Fluorescence Staining, EMD Millipore, Kankakee, IL, USA) per manufacturer’s instruction. Briefly, brain sections were deparaffinized and incubated with pro-
teinase K (20 mg/mL) for 15 to 30 min at 37°C. Next, sections were equilibrated 10 min in terminal deoxynucleotidyl transferase (TdT) buffer. Sections were then incubated in a TdT–end labeling cocktail in a humid chamber for 60 min at 37°C. The reaction was stopped by a 5 min immersion of the sections in TB buffer at room temperature (RT). Then sections were incubated 20 min at RT with blocking buffer to minimize non-specific staining. Biotin–deoxyuridine triphosphate labeled sections were then incubated with Avidin–flourescin isothiocyanate (FITC) solution for 30 min at RT. Finally, the tissues were counterstained with 4’6-diamidino-2-phenylindole (DAPI, 1 g/mL, Santa Cruz Biotechnology, Heidelberg, Germany) to reveal normal and apoptotic nuclear morphology. In positive controls, tissue slices were pretreated with low concentration of DNase (1 μg/mL during 1 h). TUNEL staining was analyzed using a fluorescent microscope (Olympus, Tokyo, Japan); percentages of TUNEL-positive neurons were determined by dividing the number of TUNEL-positive cells per 100 by the total number of cells per section.

Immunohistochemistry

For BrdU incorporation assay or evaluation of the expression of cilary neurotrophic factor (CNTF), immunohistochemistry was performed. Briefly, the brain sections were washed with PBS and then incubated with 2 μM hydrochloric acid for 15 min followed by using 0.1 M sodium tetraborate (Sigma) for 12 min (these steps were done only for BrdU detection). Then the tissues were incubated with 5% normal goat serum and 1% bovine serum albumin in PBS for 60 min. After that, mouse anti-BrdU (1:50; Abcam, Cambridge, MA, USA) or rabbit anti-CNTF (1:20; Santa Cruz Biotechnology) was applied at 4°C overnight. Subsequently, goat antimouse immunoglobulin G (lgG) (Alexa flour 647, 1:600; Invitrogen, Waltham, MA, USA) or goat antirabbit lgG (FITC; 1:700; Abcam) was used for 60 min at RT, and the nuclei were stained with DAPI (1 g/mL, Santa Cruz Biotechnology) or propidium iodide (Molecular Probes, Eugene, OR, USA). Immunolabeled cells were evaluated using a fluorescent microscope (Olympus). The primary antibodies were omitted in control samples in which no immunoreactivity was detected. The percentage of immunopositive cells per area was calculated by the following formula: (number of positive cells × 100) / total number of nuclei.

Total RNA isolation and complementary DNA synthesis

For molecular analysis, animals were deeply anesthetized and decapitated (five mice per group). Then the brain was rapidly removed from the skull, and hypothalamic tissue from the preoptic area to the mammary body was collected. Tissue samples were snap-frozen in liquid nitrogen and stored at −80°C. Hypothalamic total RNA was extracted using Ribo Ex (Hybrid-RTM miRNA, GeneAll, Seoul, Korea) according to the manufacturer’s instructions. The purity and concentration of the RNA extracted from all samples was verified and quantified using a RNA NanoDrop 2000 c Spectrophotometer. RNA samples were then treated with the DNase I kit (Qagen, Hilden, Germany) to remove any contamination with genomic DNA.

For complementary DNA (cDNA) synthesis, 500 ng total RNA from each sample in a total reaction volume of 10 μL was reverse transcribed using the cDNA synthesis kit (Thermo Scientific, Dreieich, Germany) according to the manufacturer’s recommendations. Reactions were incubated initially at 37°C for 15 min and subsequently at 75°C for 10 min. All the cDNA samples were stored at −20°C until quantitative polymerase chain reaction (PCR) analyses.

Quantification of mRNA

Relative mRNA levels of brain-derived neurotrophic factor (BDNF), leukemia inhibitory factor (LIF), sirtuin 1 (Sirt1), α1 catalytic subunit of AMP-activated protein kinase (AMPKα1) and α2 catalytic subunit of AMP-activated protein kinase (AMPKα2), and forkhead box O1 (FoxO1) were quantified using real-time PCR with the CFX 96 Real-Time System (Bio-Rad, Munich, Germany). β-Actin mRNA levels were similarly measured and served as the internal control. The PCR reagent mixture was prepared in a total volume of 20 μL containing 5 μL template, 0.5 μL of each 10 μM primer, 4 μL of 5 × HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia), and 10 μL RNase/DNase-free sterile water (Sigma). The sequences of the target genes’ primers are illustrated in Table 2. Amplification was performed using the following program: an initial denaturation at 95°C for 15 min, 45 cycles of denaturation at 95°C for 30 s, primer annealing at 60°C for 30 s, and elongation at 72°C for 30 s. The procedure was followed by a melting curve analysis to determine product specificity. Meanwhile, wells of no template control and no reverse transcriptase control were prepared as the negative controls to evaluate DNA contamination. All sample mRNA levels were normalized to the values of β-actin, and the results were expressed as fold changes of threshold cycle (Ct) value relative to controls using the 2−ΔΔCt method [28].

Table 2

| Sequences of oligonucleotide primers designed for real-time PCR |
|---------------------------------|-----------------|-----------------|
| Bdnf                           | GTCGCGGAAATACGTTATTGA | CCGGACTTCTTCTAGGACT |
| Lif                            | CCGGAAATACGTTATTGA | CCGGACTTCTTCTAGGACT |
| Sirt1                          | ATATCCCGGACATGTCACAG | TCGAAGAAAGACATGACACT |
| AMPKα1                         | TGATGACATGTTGCAACACTC | ACCACTGTGTCCCTGATA |
| AMPKα2                         | GCCAGGACTATCAGAAGTACA | ATCTGGAGGGGGCTTACAG |
| FoxO1                          | GAATAGGGGGGACAGCAACAG | CCTCCCTCTCCATACCAT |
| Lactin                         | AGGACCTGCTGCTCCCAAAGAG | AGAACCTGCTGCTACCTG |

Double immunohistochemistry

For finding the fate of newborn cells, a double immunohistochemistry technique was performed. Briefly, the brain sections were washed with PBS. Next, the slides were incubated with 2 M HCl for 15 min. Then the sections were incubated in 0.1 M sodium tetraborate (Sigma) for 12 min. Next, 5% normal goat serum and 1% bovine serum albumin in PBS were used for 60 min. After that, mouse anti-BrdU (1:50; Abcam) in combination with rabbit antiproteinase (POMC; 1:200; Abcam) or rabbit antineuropeptide Y (NPY; 1:1000; Abcam) was applied at 4°C overnight. Subsequently, goat antimouse IgG (FITC; 1:700; Abcam) and goat antirabbit IgG (Alexa Fluor 647, 1:600; Invitrogen) were used for 60 min at RT. The nuclei were stained with DAPI (1 g/mL, Santa Cruz Biotechnology). By using a fluorescent microscope (Olympus, Japan), immunopositive cells were analyzed. The percentage of BrdU-positive cells, which expressed a defined marker (POMC or NPY) per area, was calculated by the following formula: (number of BrdU-defined marker-positive cells × 100) / total number of BrdU-positive cells.

Statistical analyses

Results are presented as mean ± standard error of the mean. Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) software (Version 23; IBM Corp., Armonk, NY, USA). One-way analysis of variance was used for the analyses in which change over groups was assessed. Pair-wise comparison of means was accomplished by least significant difference post hoc test. In all analyses, P < 0.05 was regarded as statistically significant.

Results

Body weight and food intake

A significant increase in body mass was identified in all mice groups compared with the control group at the fourth and fifth weeks of the experiment (P < 0.05, Fig. 1A). The body weight of the mice in the HF + RSV group was significantly less than that of the HF group at the sixth week of the experiment (20.2 ± 0.3 g versus 21.4 ± 0.5 g, respectively, P < 0.05). Despite starting a decreasing trend by the HF + RSV group at the end of the present study, there was still a significant difference between the weight of the HF + RSV group and that of the control group (20.2 ± 0.3 g versus 17.8 ± 0.3 g, P < 0.001). As shown in Figure 1B, there was no difference in food intake among the three groups.

Adipogenesis

The weight of intraabdominal WAT in the HF group (151.4 ± 6.3 percentage of control) was significantly higher than that of the HF + RSV group (92.6 ± 8.4 percentage of control, P < 0.01, Fig. 2). In addition, histologic evaluation of WAT in H&E-stained sections indicated that there was no significant difference in adipocyte area among the three groups. However, this index tended to decrease in the HF + RSV group. Another index of distribution of adipocytes, the number of adipocytes per evaluated area, was considerably higher in the HF group (57.5 ± 9.9) than that of the control (37.1 ± 4.4) and HF + RSV (34.2 ± 5.3) groups (P < 0.05). As
indicated in Figure 2G, fat pads of mice on the HF diet had a greater frequency of small adipocytes compared with the other groups.

Briefly, adding RSV to the HF diet decreased the amount of intraabdominal fat pad. In this context, reduction of the number of adipocytes was remarkable.

Hypothalamic nerve cell damage

To evaluate the amount of cell damage in the hypothalamus, the distribution of apoptotic cells was analyzed by means of the TUNEL method (Fig. 3). In the arcuate nucleus (ARC), the percentage of TUNEL-positive cells in the HF group (31.1 ± 4) was significantly higher than that of the control and HF + RSV groups (11.4 ± 4.3 and 16.3 ± 3.2, respectively, P < 0.05). In the medial hypothalamus (MH), although the HF diet tended to increase the percentage of apoptotic cells (19.8 ± 2.6) compared with that of the control and HF + RSV groups (11.3 ± 4.8 and 10.4 ± 2.2, respectively), the difference was not significant (P = 0.21). In the lateral hypothalamus (LH), the percentage of TUNEL-immunoreactive cells in the HF group (28.3 ± 7) was significantly higher than that of the control and HF + RSV groups (7.5 ± 3.5 and 9.1 ± 1.2, respectively, P < 0.05).

Collectively, the HF diet induced cell damage in all studied areas of the hypothalamus, especially in the ARC and LH. However, addition of RSV to the HF diet reduced this phenomenon in all studied regions of the hypothalamus.

Hypothalamic generation of newly born cells

BrdU cell proliferation assay was done to evaluate production of newly born cells in different areas of the hypothalamus. As shown in Figure 4, the percentage of BrdU-positive cells in the ARC of the HF (28.9 ± 1.4) and HF + RSV (34.7 ± 5.6) groups increased remarkably compared with the control group (16.5 ± 3, P < 0.05). In the MH, this index didn’t change in the HF group (12.1 ± 1.4) compared with the control group (11.2 ± 1.4), whereas the percentage of BrdU-positive cells in the MH of the HF + RSV group (23.4 ± 3.8) increased significantly compared with two other groups (P < 0.05). Similarly, in the LH, the percentage of BrdU-positive cells of the HF (9.6 ± 2.4) and control (10.4 ± 0.8) groups didn’t differ significantly, but this index increased remarkably in the HF + RSV group (17.4 ± 2.3, P < 0.05).

Overall, the HF diet increased mitotically, dividing cells only in the ARC. Adding RSV to the HF diet enhanced the number of new cells in all three studied areas of the hypothalamus.

Hypothalamic CNTF-positive neurons distribution

Using immunohistochemistry, the distribution of CNTF in different areas of the hypothalamus was investigated (Fig. 5). Although the percentage of CNTF-positive cells in the ARC of the HF group (31 ± 1) didn’t significantly differ from that of the control group (25.4 ± 3), this index increased significantly in the HF + RSV group (39.5 ± 2.5) compared with the other groups (P < 0.05). In the MH, the percentage of CNTF-positive cells significantly increased in both the HF (33.1 ± 2.3) and HF + RSV (34.3 ± 1.3) groups compared with the control group (24.9 ± 1.8, P < 0.05). In the LH, there was no significant difference in the percentage of CNTF-positive cells among the control, HF, and HF + RSV groups (15.6 ± 2.9, 22.4 ± 3.8 and 26.2 ± 3.9, respectively; P < 0.05).

In summary, adding RSV to an HF diet increased the percentage of CNTF-positive cells in the MH as well as ARC and had a tendency to raise it in the LH, although the HF diet enhanced this index only in the MH.

Hypothalamic BDNF and LIF expression

Real-time PCR studies were used to investigate the levels of trophic factors BDNF and LIF in the hypothalamus. As shown in Figure 6, the expression of BDNF in the HF group (1.8 ± 0.5) did
not differ in the control group (1.07 ± 0.19). In addition, there was no remarkable changes in the expression of BDNF in the HF + RSV group (1.6 ± 0.3) compared with the other groups. Although it seems the HF diet tended to raise BDNF level in the hypothalamus, adding RSV to this diet couldn’t have a significant effect on the expression of this growth factor.

The mRNA level of LIF significantly increased in the HF group (1.9 ± 0.3) compared with the control group (1 ± 0.1, \( P < 0.05 \)). On the other hand, there was no outstanding change in the expression of LIF in the HF + RSV group (1.4 ± 0.3) compared with the other groups (Fig. 6). Thus, RSV couldn’t affect the hypothalamic LIF levels enhanced by the HF diet.

Expression of RSV downstream targets in hypothalamus

As indicated in Figure 6, the expression of Sirt1 tended to increase in the HF + RSV group (1.37 ± 0.18) compared with the control (1.01 ± 0.13) and HF groups (1.01 ± 0.14), but this change was not significant (\( P = 0.07 \)). There was no remarkable difference in expression of AMPKα1, AMPKα2, and FoxO1 among the groups. The results indicated that mRNA expression of the mentioned targets of RSV did not change in the hypothalamus after adding RSV to the HF diet.

Hypothalamic remodeling in ARC

To evaluate whether RSV modulated the generation of new neurons in mice treated with an HF diet, the distribution of POMC-BrdU-immunoreactive neurons were investigated in the ARC in different mice groups. Double immunofluorescence analysis was carried out to detect POMC- and BrdU-coexpressing cells. As shown in Figure 7, a significantly higher percentage of BrdU-positive cells in the ARC of the HF + RSV group (62.4 ± 6.3) was identified as POMC-expressing neurons compared with that of the HF group (30.5 ± 6.5, \( P < 0.05 \)). There was no significant difference in the percentage of POMC- and BrdU-coexpressing cells between the control and HF groups, but this factor tended to be lower in the HF group compared with the control group (44.17 ± 5.71, \( P = 0.15 \)).

To evaluate the production of new orexigenic neurons, NPY-BrdU-positive cells were quantified in the ARC of all groups (Fig. 8). By double immunofluorescence analysis, a higher percentage of BrdU-positive cells were found to express NPY in the ARC of the HF group (75.3 ± 2.7) compared with the HF + RSV group (47.7 ± 5.3) and control group (48.3 ± 7; \( P < 0.05 \)).

Taken together, by the HF diet, orexigenic neuron production from new cells increased, and adding RSV to the HF diet...
changed the fate of new cells to anorexigenic ones and consequently remodeled the ARC architecture.

Discussion

The present study examined the effects of the HF diet and RSV on weight and energy intake, as well as the hypothalamic neurogenesis and remodeling in male mice. Mice on an HF diet became obese, whereas RSV treatment protected mice from diet-induced obesity. This antiobesity effect of RSV was accompanied by a decrease in fat as reflected in the mass and morphologic changes of the intraabdominal white fat pad. On the other hand, an HF diet and RSV consumption were accompanied by several changes in the hypothalamus, the control center of body weight and appetite, as the pattern of neurogenesis in the hypothalamic areas was altered by these factors. Both the HF diet and the HF diet containing RSV increased the number of new cells and neurogenesis in the hypothalamus of mice. In addition, the HF diet–induced neurogenesis was associated with an increase in programmed cell death in different regions of the hypothalamus, the phenomenon that decreased in the HF + RSV group. In this study, we provide evidence that HF diet consumption leads to higher conversion of the newly generated cells to NPY-expressing neurons, and conversely, treatment with RSV decreases this differentiation. Instead, RSV causes a high percentage of the newly generated cells to take on POMC neuro-
nal cell fate. To our knowledge, our study was the first to evaluate the effects of an HF diet on the production of new neurons of ARC. Moreover, there has been no study about RSV effects on hypothalamic neurogenesis and remodeling so far.

Animal models are important for studying the molecular aspects of obesity and its pathophysiological effects. One of these models gaining increased attention is the diet-induced obesity model in mice [29,30]. In the present study we used C57BL/6 mice as an outstanding choice for creating a reliable model of obesity and fed them with a diet containing common sources of fatty acids, butter, and soy oil [2]. Based on our results, the frequency of adipocytes increased in the HF group, which could be due to impaired adipogenesis/fat storage in intraabdominal white adipose tissue. According to previous studies, both size and number of adipocytes can be increased after HF diet consumption. This phenomenon is dependent on the strain of animals, type of fat depot, and contents of diet. For instance, Jo et al. [30] reported that compared with the FVB/N strain (obesity-resistant), the C57 BL/6 strain (obesity-prone) has greater recruitment of small adipose cells. On the other hand, Fang et al. [31] revealed that subcutaneous fat had a greater capacity for storage than visceral adipose tissue. Although, most researchers reported that large adipocytes are pathologic cells, few investigations indicated that small ones are responsible for the pathology of obesity. Fang et al. [31] reported an increase in the fraction of small adipocytes in obese diabetic patients. McLaughlin et al. [32]

Fig. 5. Pattern of CNTF expression in hypothalamus after RSV treatment. To evaluate the distribution of hypothalamic CNTF (A), the immunohistochemistry was performed followed by counting the CNTF-immunoreactive cells (green cells) in (B) ARC, (C) MH, and (D) LH of the control (n = 5), HF (n = 4), and HF + RSV (n = 5) groups. A magnified representation of CNTF-positive cells is inside the yellow circle in E. The nuclei were stained with propidium iodide and are seen in red. (F) Quantification of CNTF-positive cells. *P < 0.05 versus control and HF groups. †P < 0.05 versus control group. 3V, third ventricle; ARC, arcuate nucleus; CNTF, ciliary neurotrophic factor; HF, high-fat diet; LH, lateral hypothalamus; MH, medial hypothalamus; RSV, resveratrol.

Fig. 6. Expression of probable RSV target genes in hypothalamus after RSV treatment. To analyze the expression of BDNF, LIF, AMPKα1, AMPKα2, and FoxO1, real-time PCR was done in the control (n = 5), HF (n = 5), and HF + RSV (n = 5) groups. *P < 0.05 versus control group. HF, high-fat diet; PCR, polymerase chain reaction; RSV, resveratrol.
reported that in insulin-resistant patients, the ratio of small to large adipocytes increased. Because C57 BL/6 mice are a strain that becomes obese, hyperglycemic, and insulin resistant when fed an HF diet [33], it is not surprising that in our study, fat accumulation in C57 BL/6 mice occurred by producing of many small adipocytes. In addition, McLaughlin et al. [34] reported that with expanding body fat mass, impairment in the ability to generate mature “large” triacylglycerol-storing adipose leads to accumulation of small adipose cells. This impairment in adipogenesis and abnormal remodeling of adipose cells

Fig. 7. Distribution of POMC-BrdU–positive cells in ARC after RSV treatment. To assess relative abundance of newborn POMC expressing neurons, the double immunohistochemistry method was performed by counting the POMC-BrdU–immunoreactive cells in ARC of the control (n = 5), HF (n = 4), and HF + RSV (n = 5) groups. (A) BrdU-positive cells (green); (B) POMC-positive cells (red); (C) nuclei (blue); (D) merged picture. (E) Magnified representation of the yellow box in D in which an example of BrdU-POMC–positive neuron is shown in the yellow circle. *P < 0.05 versus HF + RSV. 3V, third ventricle; ARC, arcuate nucleus; BrdU, bromodeoxyuridine; HF, high-fat diet; POMC, antiproopiomelanocortin; RSV, resveratrol.

Fig. 8. Distribution of NPY-BrdU–positive cells in ARC after RSV treatment. To assess relative abundance of newborn NPY expressing neurons, the double immunohistochemistry method was performed by counting the NPY-BrdU–immunoreactive cells in ARC of the control (n = 5), HF (n = 4), and HF + RSV (n = 5) groups. (A) BrdU-positive cells (green); (B) NPY-positive cells (red); (C) nuclei (blue); (D) merged picture. (E) Magnified representation of the yellow box in D in which an example of BrdU-NPY–positive neuron is demonstrated in the yellow circle. (F) The quantification of NPY-BrdU–positive cells. *P < 0.05 versus control and HF + RSV groups. 3V, third ventricle; ARC, arcuate nucleus; BrdU, bromodeoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; HF, high-fat diet; NPY, antineuropeptide Y; RSV, resveratrol.
has been suggested to have a significant association with inflammation.

In the next step, we evaluated the central effects of RSV as an antiobesogenic compound in an HF diet. RSV dose was selected based on the study of Lagouge et al. [17], which found that mice treated with 400 mg/kg of RSV administered in an HF diet tended to gain significantly less body weight compared with the controls. Our results indicated a significant difference in the weight of mice that received RSV and mice on an HF diet presented in the last week of study (the sixth week). It seems that RSV required a lag phase to overcome the obesogenic effect of the HF diet. Previous studies using the same dose of RSV (400 mg/kg body weight) indicated a similar pattern of weight loss [17,35]. Furthermore, in this study, similar to previous studies, the weight of intraabdominal white adipose tissue was significantly lower in HF diet mice treated with RSV compared with that in HF diet mice [17,36]. It is worth pointing out that these protective effects of RSV were not caused by decreased food intake, because the amount of food consumed per mouse over a 24-h period was unchanged compared with that of the HF group. This antiobesity effect of RSV could be due to the increase of energy expenditure [25]. The present study indicated the neuroprotective effect of RSV on HF-induced hypothalamic nuclei damage via an antiapoptosis mechanism. Based on the results of previous investigations, an HF diet induces lipotoxicity not only in peripheral tissues, including the liver, muscle, and pancreas, but also in the central nervous system, specifically the hypothalamus [4,37]. This pathologic accumulation of lipids leads to activation of immune responses and apoptosis in hypothalamic nuclei [38]. Tissue-specific pro- and antiapoptotic effects of RSV have been reported. The proapoptotic effect of RSV is beneficial for activating programmed cell death in cancer cells [39,40]. In addition, it induces apoptosis in adipocytes [41,42]. On the other hand, RSV has an antiapoptotic effect on cortical neurons and hippocampal cells via regulation of the expressions of Bcl-2, Bax, and caspase-3 proteins in mitochondria and suppression of the mitochondrial death pathway [43]. Moreover, our results illustrated that this antiapoptotic role of RSV was more prominent in the ARC and LH. Together, this dual role of RSV (peripherally proapoptotic role on adipocytes versus centrally antiapoptotic role on hypothalamic nuclei) may lead to weight loss.

Recently, hypothalamic subependymal niche has been introduced as a novel site of the adult neurogenesis [44]. Subsequently, study of the probable relationship between hypothalamic neurogenesis and energy homeostasis has been growing [10,45]. In our study, evaluation of BrdU-positive cells’ distribution in different areas of the hypothalamus represented that the number of newly born cells increased, especially in ARC after HF diet consumption. The study conducted by Bless et al. [46] supports the present results. They reported that an HF diet increased the number of BrdU-positive cells in the hypothalamus of adult female mice. In addition, Gouazé et al. [47] found that cell renewal in the hypothalamus of adult mice transiently increased after HF diet consumption. Interestingly, blocking the hypothalamic cell proliferation led to weight gain. Contrarily, Li et al. [48] found a decreasing trend of newborn production after HF diet consumption. Additionally, McNay et al. [6] revealed that an HF diet generated fewer new hypothalamic cells. However, they concluded that diet-induced obesity leads to an expansion of the pool of hypothalamic neural stemlike cells while leading to a depletion of the pool of highly proliferative progenitor-like cells. These discrepancies can be explained by differences in design of the studies. In both of the latest investigations, animals were exposed to an HF diet for a period longer than that of our study (8 or 16 wk versus 6 wk). In addition, contrary to both studies, BrdU was administered in the beginning of our investigation. Based on our results and the report by Pierce and Xu [44], the increase in hypothalamic new cell generation may be a compensatory mechanism after HF diet–induced hypothalamic damage. In the present study, this compensatory mechanism was highlighted in ARC, which may be due to the proximity of this area to the hypothalamic neurogenic niche located in the ventral hypothalamic ventricular zone [12]. The closer to the niche, the more powerful the damaged signal to the niche and the more migration of the new cells to the neighbor area (ARC).

To the best of our knowledge, our results provide the first evidence that adding RSV to an HF diet increased the number of newly born cells in the ARC, MH, and LH. Previous studies focused on other areas of the mammalian brain, especially the hippocampus, to evaluate the effect of RSV on neurogenesis. Overall, a growing pool of evidence supports the view that RSV increases hippocampal neurogenesis. Kumar et al. [49] reported that low concentrations (10 μM) of RSV stimulated proliferation of cultured neural progenitor cells, whereas high concentrations (>20 μM) exhibited inhibitory effects. Then they administered RSV to adult rats and found that the number of newly generated cells in the hippocampus increased with upregulation of p-CREB and SIRT1 proteins. Madhyastha et al. [50] reported that RSV enhanced hippocampal neurogenesis in the prenatally stressed animals. Harada et al. [51] indicated that RSV increased production of insulin–like growth factor-1 and neurogenesis in the hippocampus of adult mice. In addition, Moriya et al. [52] indicated that RSV improved neurogenesis and expression of BDNF mRNA in the hippocampus. Based on these results, we hypothesized that generation of newborn cells in the hypothalamus after RSV administration can also be influenced by the main trophic factors.

The results of this study indicated that the expression of LIF mRNA but not BDNF mRNA was remarkably enhanced by an HF diet. Moreover, the number of CNTF-positive cells were significantly high only in MH area. According to the anorexigenic role of these factors, it can be concluded that elevation of the level of these factors could be a compensatory mechanism to reduce undesirable effects of diets high in fat [53]. Based on our food intake data, it seems that the probable compensatory effects of the endogenous type of CNTF on weight and calorie intake of these obesity-prone mice cannot occur. On the other hand, adding RSV to an HF diet didn’t have a significant effect on mRNA levels of BDNF and LIF but considerably enhanced CNTF in ARC and MH. Previous studies indicated that centrally administered CNTF-induced cell proliferation in feeding centers of the hypothalamus and blocking this cell proliferation inhibited the antiobesity effect of CNTF [10]. Together, our results revealed that RSV increased endogenous CNTF levels, which are able to act as both proliferative and antiobesogenic agents.

To evaluate the direct effects of RSV on the hypothalamus, the expression of Sirt1 as one of the main targets of RSV, Foxo1 as a probable target of sirtuin1, and AMPKα1 and AMPKα2 as probable downstream transcripts of RSV were measured in the hypothalamus. Adding RSV to an HF diet tended to increase the expression of Sirt1, but this change was not statistically significant. Further investigations are required to evaluate downstream targets of RSV in post-translational levels and in defined hypothalamic areas specifically in POMC neurons.

To better understanding the fate of the new proliferating cells identified in our study, we evaluated remodeling of ARC by study of POMC and NPY neurons production. Interestingly, the pattern of cell differentiation was opposite in HF and HF + RSV groups,
so that an HF diet promoted NPY neuron production, but adding RSV to the HF diet changed the fate of new cells to POMC neurons. Considering the fact that NPY neurons are orexigenic and POMC ones are anorexigenic, we can hypothesize that cell differentiation induced by an HF diet remodeled the ARC for more weight gain but administration of RSV changed the architecture of this main feeding center for weight loss. One of the recent studies examined the fate of ARC neurons in an animal model of diet-induced obesity [6]. The results of the study indicated that the number of both POMC + BrdU–positive neurons and NPY + BrdU–positive cells were increased. In this study, BrdU labeling was performed during embryogenesis and then animals were exposed to an HF diet during 6 to 16th weeks of experiments. Thus, the study indeed evaluated the retention of embryonic proliferating cells, not differentiation of adult hypothalamic neurogenesis. In contrast to our results, another study revealed that an HF diet enhanced the percentage of POMC + BrdU–positive cells in ARC [47]. This study evaluated the fate of new neurons after the 21st day post–HF diet consumption; we examined this index at 42nd day of the HF diet. It is possible that before day 42 the brain was in unsuccessful compensatory phase, which led to failure as HF diet intake was continued.

In conclusion, the quantity of new proliferating cells in the hypothalamus increased after HF diet consumption, probably because of tissue damage, and adding RSV enhanced this index possibly via an increase in trophic factors. The fate of these new cells was different in various contexts so that the orexigenic versus anorexigenic balance in the HF regimen differed from the HF + RSV diet. We need to bear in mind that neurogenesis has several steps, including cell proliferation, migration, differentiation, maturation, and integration [54]. During each step, neural stem/progenitor cells are influenced by various signals of microenvironment [55]. Thus, further studies focusing on these steps are needed to evaluate the effect of RSV on hypothalamic neurogenesis.

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References


