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Early postnatal maternal separation causes alterations in the expression of β3-adrenergic receptor in rat adipose tissue suggesting long-term influence on obesity

Takanori Miki [a,*], Jun-Qian Liu [a], Ken-ichi Ohta [a], Shingo Suzuki [a], Takashi Kusaka [b], Katsuhiko Warita [a], Toshifumi Yokoyama [c], Mostofa Jamal [d], Masaaki Ueki [e], Tomiko Yakura [a], Motoki Tamai [a], Kazunori Sumitani [f], Naohisa Hosomi [g], Yoshiki Takeuchi [a]

[a] Department of Anatomy and Neurobiology, Faculty of Medicine, Kagawa University, Japan
[b] Department of Pediatrics, Faculty of Medicine, Kagawa University, Japan
[c] Department of Bioresource and Agrobiosciences, Graduate School of Science and Technology, Kobe University, Japan
[d] Department of Forensic Medicine, Faculty of Medicine, Kagawa University, Japan
[e] Department of Anesthesia, Nishiwaki Municipal Hospital, Japan
[f] Department of Medical Education, Faculty of Medicine, Kagawa University, Japan
[g] Department of Clinical Neuroscience and Therapeutics, Hiroshima University Graduate School of Biomedical Sciences, Japan

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ABSTRACT

The effects of early postnatal maternal deprivation on the biological characteristics of the adipose tissue later in life were investigated in the present study. Sprague–Dawley rats were classified as either maternal deprivation (MD) or mother-reared control (MRC) groups. MD was achieved by separating the rat pups from their mothers for 3 h each day during the 10–15 postnatal days. mRNA levels of mitochondrial uncoupling protein 1 (UCP-1), β3-adrenergic receptor (β3-AR), and prohibitin (PHB) in the brown and white adipose tissue were determined using real-time RT-PCR analysis. UCP-1, which is mediated through β3-AR, is closely involved in the energy metabolism and expenditure. PHB is highly expressed in the proliferating tissues/cells. At 10 weeks of age, the body weight of the MRC and MD rats was similar. However, the levels of the key molecules in the adipose tissue were substantially altered. There was a significant increase in the expression of PHB mRNA in the white adipose tissue, while the β3-AR mRNA expression decreased significantly, and the UCP-1 mRNA expression remained unchanged in the brown adipose tissue. Given that these molecules influence the mitochondrial metabolism, our study indicates that early postnatal maternal deprivation can influence the fate of adipose tissue proliferation, presumably leading to obesity later in life.

1. Introduction

Early postnatal maternal separation before weaning is acknowledged to be a stressful experience in mammals [1–3]. Numerous reports of preclinical and clinical studies have demonstrated that such adverse experiences in early life profoundly affect normal brain development, leading to functional disruptions in higher brain functions such as learning, memory, cognition, and social interactions [4–6]. In addition to brain disruptions, a growing body of evidence shows that adverse experiences in early life, including maternal separation, influence body-weight gain later in the life [6–10]. These reports investigated the etiological reasons for increased feeding behavior. However, detailed molecular mechanisms underlying this phenomenon are yet to be completely understood. Our primary hypothesis is that the stress induced by early maternal separation potentially affects the biological characteristics of adipose tissue. Therefore, in this study we evaluated the adipose tissue-associated molecules following maternal separation.

Two types of fat, white adipose tissue (WAT) and brown adipose tissue (BAT), exist in mammals including humans and rodents [11]. WAT stores excess calories, and its excessive accumulation causes obesity. BAT dissipates energy to produce heat through non-shivering thermogenesis for protection against cold environments. Therefore, BAT is potentially recognized as a novel target for anti-obesity treatments. BAT functioning is recruited through BAT-specific mitochondrial uncoupling protein 1 (UCP-1). The action of the UCP-1 is known to be mediated through the β3-adrenergic receptor (β3-AR), which is exclusively expressed in both WAT

* Corresponding author. Address: Department of Anatomy and Neurobiology, Faculty of Medicine, Kagawa University, 1750-1 Ikenobe, Miki-cho, Kagawa 761-0793, Japan. Fax: +81 87 891 2088.
E-mail address: mikit@med.kagawa-u.ac.jp (T. Miki).

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and BAT [12]. Prohibitin (PHB) is known to be a ubiquitous and an evolutionarily conserved protein that is localized in the mitochondria [13]. PHB is also known to be specifically required in tissues that undergo extensive cellular proliferation [13,14]. If fact, the depletion of PHB in *Caenorhabditis elegans* results in distinct germ line defects such as diminished oocyte production with smaller blood size [15]. Similarly, deletion of mouse PHB-2, an isoform of PHB, in embryonic fibroblasts results in severely impaired cellular proliferation [14].

The prevalence of diet-induced weight gain, particularly obesity due to high-fat intake in the general population, is one of the serious health problems in the world. Therefore, it is of interest to examine the characteristics of the adipose tissue resulting from high-fat load in animals that were separated from their mothers early in their life. From previous studies that indicate a relationship between maternal separation and body-weight gain [16,17], it is conceivable that a high-fat intake easily affects the characteristics of the adipose tissue. We hypothesized that the maternally separated animals fed with a high-fat diet show increased body mass and adipose tissue proliferation. To test this hypothesis, we used rats to examine whether the stress induced by early MD affects the biological characteristics of the adipose tissue later in life.

2. Materials and methods

This study was carried out in compliance with the guidelines for experimental use and care of laboratory animals set forth by the Kagawa University Animal Ethics Committee. Six pregnant Sprague–Dawley rats purchased from SLC Japan (Shizuoka, Japan) were used in this study. The day of birth was designated as postnatal day (PND) 0. On PND 2, all pups were placed together and randomly assigned back to the lactating mothers so that each mother received 8 pups (4 male and 4 female). Six sets of litters and their mothers were then randomly assigned to the maternal deprivation (MD) and mother-reared control (MRC) groups (3 sets each group). MD pups were removed from their mother and placed together in a new cage for 3 h per day, between 9 and 12 AM each day from PND 10 to PND 15. After separation, the pups were returned to their mother daily. During this period of separation, the pups were placed in a holding cage under identical conditions as those of the MRC group. The MRC group pups were allowed to remain with their mothers at all times. Animals were weaned on PND 21, and were weighed weekly from PND 21 [3 weeks of age] to 10 weeks of age. After weaning, the animals of both groups were fed with a high-fat diet (18.2% protein, 62.2% fat, and 19.6% carbohydrates) (HFD-60, Oriental Yeast, Tokyo, Japan).

At 10 weeks of age, the female pups of the MD and MRC groups ($n = 9$ each group) were anesthetized with 7% chloral hydrate (0.7 mL/100 g body weight; intraperitoneal injection) and perfused intracardially with medical grade physiological saline. Three female offspring from each set of animals were used. WAT and BAT were immediately collected from the retroperitoneal and interscapular regions, respectively. These fat samples were stored at −80 °C until use.

Homogenization of adipose tissue and extraction of total RNA were performed using QIAshredder and AllPrep DNA/RNA/Protein Mini Kits (Qiagen, Venlo, Netherlands) by following the manufacturer instructions. The concentration and purity of the extracted total RNA were evaluated by optical density measurements at 260 and 280 nm by using NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA). Then, a QuantiTect Reverse Transcription Kit (Qiagen) was used to synthesize the cDNA. The genomic DNA was removed from 0.5 to 1.0 μg of the isolated RNA. To assess an appropriate internal control, co-amplification of a β-actin mRNA was performed for each sample. We used the following forward (F) and reverse (R) primers: for UCP-1 (The national center for biotechnology information (NCBI) reference sequence; NC_005118.3), F: 5′-CTTCTCAGCCGGCGTTTCTG-3′, R: 5′-GGTGATGGTCCCTAAGACACC-3′; for β3-AR (NCBI reference sequence; NC_005115.3), F: 5′-TTCCCAGCGGAGTTTTCATC-3′, R: 5′-AGCGGGTTGAAGGCAGAGT-3′; for PHB (NCBI reference sequence; NC_005109.3) F: 5′-GCGTGGTG AACTCTGCTCTA-3′, R: 5′-TGTACCCAGGGGATGAGGAA-3′; and for β-actin (NCBI reference sequence; NM_031144) F 5′-TTGCTGACAGGATGCAGAA-3′, R 5′-ACCAATCCACACAGAGTACTT-3′. The mRNAs were amplified using the LightCycler system (Roche Diagnostics, Basel, Switzerland). Reactions were performed in a 20-μL volume containing 2 μL of sample cDNA diluted 10-fold with distilled water, 0.5 μM of each forward and reverse primers, and the LightCycler FastStart DNA MasterPLUS SYBR Green I mix (Roche Diagnostics). After the initial denaturation at 95 °C for 20 s, amplification was performed under the following conditions: 40 cycles at 95 °C for 10 s, 58 °C for 5 s, and 72 °C for 20 s. Detection of the fluorescent tracers was carried out at the end of the 72 °C extension period. To confirm the specificity of the amplification, PCR products from each primer pair were subjected to melting-curve analysis after amplification. For melting-curve analysis, the PCR products were denatured by gradually increasing the temperature from 65 °C in 0.2 °C increments. The expression levels of each gene were determined using the ratio of mRNA levels of specific genes relative to those of the housekeeping gene, β-actin. To exclude genomic contamination, electrophoresis of the PCR products, amplified from cDNA using UCP-1, β3-AR, PHB, and β-actin primers, was carried out on a 2% agarose gel, followed by staining with ethidium bromide. A similar electrophoresis of the amplification products without reverse transcription (RT) was also performed as a negative control. The data were analyzed using the LightCycler analysis software. The PCR assay was carried out twice.

mRNA data for the target genes were expressed as the ratio of mRNA levels to those of the housekeeping gene β-actin. Statistical analysis was conducted by two-way repeated measures analysis of variance (ANOVA) for the body weight data and Student's *t*-test for real-time RT-PCR data using the SigmaPlot 12 software (Systat software, Inc., Chicago, IL, USA). The significance level was set at $P < 0.05$.

3. Results

Body weight (g) of MRC and MD rats between 3 and 10 weeks of age is presented in Table 1. Two-way repeated measures ANOVA revealed a significant main effect of age (df 7,112; $F = 868.119$; $P < 0.001$). However, there were no significant differences between the groups (df 1,112; $F = 10587$; $P = 0.226$) and group × age interaction (df 7,112; $F = 0.480$; $P = 0.848$). The post hoc analysis with Tukey–Kramer's test showed that the body weight of MRC and MD animals was statistically similar at all the time-points examined, although age-dependent body-weight gain was seen in animals of both groups.

Fig. 1 illustrates the image of a representative gel electrophoresis of the PCR products showing a single band, which highlights the specificity of amplification. This provides a strong confirmation that the primers we used specifically amplified the target genes UCP-1, β3-AR, PHB, and β-actin, excluding the genomic DNA contamination.

Table 2 shows the mRNA levels, expressed as mean ± standard error of the mean (SEM), of UCP-1, β3-AR, and PHB in WAT and BAT of MRC and MD animals. Statistical analysis using Student's *t*-test revealed that in the WAT there was no significant difference in β3-AR levels between MRC and MD animals, though PHB mRNA levels in MD rats increased significantly. UCP-1 mRNA was not detected in the WAT of either MRC or animals; BAT β3-AR mRNA