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Molecules and Cells

High Fat Diet Altered the Mechanism of Energy Homeostasis Induced by Nicotine and Withdrawal in C57BL/6 Mice

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Nicotine treatment has known to produce an inverse relationship between body weight and food intake in rodents. Present study determined the effect of repeated treatment with nicotine and withdrawal in control and obese mice, on: (1) body weight, caloric intake and energy expenditure; (2) hypothalamic neuropeptides mRNA expression; and (3) serum leptin. 21-week-old C57BL/6 mice (n = 65) received nicotine (3.0 mg/kg/day; 2 weeks) and saline (1 ml/kg/day; 2 weeks) subcutaneously. Animals were given either a normal-fat (10% kcal from fat, NF) or a high-fat diet (45% kcal from fat, HF) from the 12th week to 25th week. While, nicotine treatment for 14 days induced an increase in hypothalamic agouti-related protein, cocaine- and amphetamine-regulated transcript, pro-opiomelanocortin mRNA expressions, nicotine also produced a reducing effect in body weight gain and leptin concentration in NF mice. High-fat diet induced obese mice showed a blunted hypothalamic and leptin response to nicotine. Remarkable weight loss in obese mice was mediated not just by decreasing caloric intake, but also by increasing total energy expenditure (EE). During nicotine withdrawal period, weight gain occurred in NF and HF groups, which was ascribed to a decrease in EE rather than changes in caloric intake. Hypothalamic AgRP might play a role for maintaining energy balance under the nicotine-induced negative energy status.

INTRODUCTION

Nicotine, a major addictive ingredient of cigarette, and its withdrawal are well known to exert remarkable effects on food intake and body weight (John et al., 2005; Strauss and Mir, 2001). In addition to nicotine induced increase or decrease in caloric intake (Levine et al., 2001), data from clinical trial studies have demonstrated that the thermogenic effect of nicotine cigarettes (Jessen et al., 2003), was overtly exerted in overweight men (Walker and Kane, 2002).

Animal studies have reported that nicotine treatment consistently decreased weight gain in nicotine-treated animals (Bellinger et al., 2003; Nakhate et al., 2009), noted to be prominent in rats fed on a high-fat diet (Wellman et al., 2005). Moreover, the body weight reducing effect of nicotine was reversed during the nicotine withdrawal period (Nakhate et al., 2009). Some studies have also reported that repeated nicotine treatment induced a compensatory increase in food intake, in nicotine-treated animals during the nicotine treatment period (Bellinger et al., 2003; Nakhate et al., 2009).

Acute nicotine treatment is reported to decrease the hypothalamic orexigenic neuropeptide, neuropeptide Y (NPY) mRNA levels while repeated nicotine treatment reversed the acute nicotine treatment induced decrease in the hypothalamic NPY mRNA level (Frankish et al., 1995; Li et al., 2000). Lately, numerous studies have concentrated their efforts in determining the effects of nicotine or its withdrawal in the hypothalamic NPY mediated changes on food intake (Nakhate et al., 2009).

Acute changes in whole-body metabolism are also likely to contribute to the weight modulating effects of nicotine and its withdrawal in female rats (Bishop et al., 2004). In published literature, there is a paucity of data in elaborating the effect of nicotine and its withdrawal on energy balance that would demonstrate the differences in body weight regulation between obese and control groups. Therefore, the present study is intended to investigate the effects of nicotine and its withdrawal on food intake and body weight regulations in control and obese C57BL/6 male mice.

MATERIALS AND METHODS

Animals

The 12-week old C57BL/6 male mice were purchased (Central Lab. Animal Inc., Korea, n = 65), individually housed (18.4 × 29.2 × 12.7 cm) and maintained at a temperature of 23 ± 1.0°C, humidity at 55 ± 5 %, and 12 h dark: light cycle. The mice were fed with normal fat (NF, D12450B: 10.0% Fat, 70.0% Carbohydrate, 20.0% Protein, wt/wt, 3.8 kcal/g) or high-fat (HF, D12451: 44.9% Fat, 35.1% Carbohydrate, 20.0% Protein, wt/wt, 4.7 kcal/g) diet from Research Diets Inc. (Central Lab. Animal Inc., Korea) and water ad libitum. NF or HF diets were supplied from the initiation of the study until the completion of the study at 24 weeks. Body weight and caloric intake of the mice were measured daily using a torsion balance. Daily caloric intake (kcal/

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day) was calculated as (mass of daily caloric intake in g) × (physiological fuel value of the diet in kcal/g). This study was approved by the Korea University's Institutional Animal Care and Use Committee (IACUC).

Nicotine treatment

(-)-Nicotine bitartrate, (Sigma Chemical Co., USA) was dissolved in sterile saline (0.9% NaCl, SAL) and subcutaneously (s.c.) administered at a dose of 1.5 mg/kg of nicotine (prepared as free base, NIC). All mice were subdivided into 4 groups; NF-SAL, NF-NIC, HF-SAL, and HF-NIC groups. For single NIC treatment, 21-week-old mice were killed 2 h after single s.c. NIC solution injection, while for repeated NIC treatment, mice were injected during 14 days with NIC solution twice daily (Rowell and Li, 1997). For NIC withdrawal (NIC-WD), NIC-treated mice were injected with SAL (1 ml/kg, s.c.) twice a day for 14 days. The SAL group also received twice a day (1 ml/kg, s.c.) under the same treatment regimen as the NIC-treated group. According to our preliminary data from this study, 1.5 mg/kg dose of NIC (s.c., once a day) for 2 weeks did not show any drug effects on body weight (g/day) and caloric intake (kcal/day) in the NF ($F_{(13, 169)} = 1.331$, $p = 0.199$ and $F_{(13, 130)} = 0.317$, $p = 0.989$, respectively) and HF ($F_{(13, 169)} = 1.519$, $p = 0.064$ and $F_{(13, 130)} = 0.367$, $p = 0.974$, respectively) group. Previous studies had reported that a dose of NIC lower than 1 mg/kg/day (free base) did not affect weight gain in rats (Bellinger et al., 2003). Thus, 3.0 mg/kg/day dose of NIC (1.5 mg/kg s.c., twice/day) was used in the present study.

Measurement of energy expenditure (EE) and respiratory quotient (RQ)

Dark phase EE and RQ was measured using an open-circuit indirect calorimetric system composed of the LE400-4FL and LE405 gas analyzer (Panlab, S.L., Spain) as per previously described method with suitable modifications (Chen et al., 2002). Before experiment, a gas calibration was performed with a mixture of 5% CO_2, 50% O_2 and 21% O_2 gas standards provided by the manufacturer. In this system, atmospheric air is pulled into the closed acrylic chambers at the rate of 300 ml/min and passed through a separate tube, then onto O_2 and CO_2 sensors for sequential measurement every 5 minutes per channel relative to a reference channel that reads the room air. After SAL or NIC injection at 1800 h, mice were placed in calorimeter chambers and EE and RQ were measured during the dark phase at the 1st and 14th day of NIC treatment and at the 14th day of NIC-WD periods (n = 4-6/group) (Walker et al., 1999). RQ was calculated as the volume of oxygen consumed (vO_2) per carbon dioxide produced (vCO_2) and total EE was calculated as the produce of calorific value of oxygen and vO_2 per kilogram of body weight ($kcal/kg^{0.75}$), according to Weir's equation (Chen et al., 2002; Weir, 1949): EE = 1.44 × vO_2 × (3.815 + 1.232 × RQ) using the Metabolism software v2.0 (Panlab, S.L., Spain). All data were averaged for dark phase.

Measurement of hypothalamic neuropeptide mRNAs

At the 1st and 14th day of NIC treatment and at the 14th day of NIC-WD periods, mice were treated with SAL (1 ml/kg, s.c.) or NIC solution (1.5 mg/kg, s.c.) at 18:00 h and 2 h later (Lage et al., 2007), anesthetized with sodium pentobarbital (100 mg/kg, intraperitoneal injection, i.p.) and then sacrificed. The whole brain was removed from the skull and placed in a brain matrix (Electron Microscopy Sciences, USA). The total hypothalamus was dissected as per the mouse brain stereotaxic coordinates (Paxinos and Franklin, 2001) and was immediately snap-frozen in liquid nitrogen and stored at -80°C until RNA isolation (n = 5-6/group). Total RNA was isolated from individual frozen tissue using 1 ml of Trizol reagent as per the manufacturer's recommendations (Invitrogen Inc., USA). The concentration and purity of total RNA were determined using a NanoDrop spectrophotometer (NanoDrop Technologies Inc., USA) at 260/280 nm and only samples with 260/280 ratio > 1.8 were studied further. The 2 μg of total RNA was mixed on ice with 1 μl of Oligo (dT) primer, 1 μl of 10 mM dNTP mixture and distilled RNase-free water to volume of 12 μl and the mixture was heated at 65°C for 5 min. The following were added: 4 μl of 5× First-Strand Buffer, 2 μl of 0.1 M DTT and 1 μl of superscript II (Invitrogen Inc, USA), and then RNase-free water was added to a total volume of 20 μl. After reacting at 42°C (50 min), the samples were heated at 70°C (15 min). Amplification of 30 μg cDNA of each sample was carried out with 10 mM Tris-HCl (pH 9.0), 30 mM KCl, 1.5 mM MgCl2, 250 μM deoxy-(d)-NTPs, 1 U Taq polymerase, 20 pM of each primer in a total volume of 20 μl (PCR Kit, Bioneer, Korea) using a MyCycler Thermal Cycler System (Bio-Rad Inc., USA).

The sequences of primers for Neuropeptide Y (NPY), agouti-related peptide (AgRP), cocaine- and amphetamine-regulated transcript (CART), pro-opiomelanocortin (POMC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows : NPY (141 bp), sense, 5′-GCTTGAAGACCCTTCCATT GGTG-3′, antisense, 5′-GGCGGAGTCCAGCCTAGTGG-3′ (60°C/32 cycle); AgRP (251 bp), sense, 5′-GAAGGCCTG ACCAGGCTCTGTTCC-3′, antisense, 5′-AAAGGCATTGAAG AAGCGG CAG-3′ (59°C / 30 cycle); CART (390 bp), sense, 5′-CTGGACATCTACTCTGCCGTGG-3′, antisense, 5′-GTTCCTC GGGGACAGTCACACAGC-3′ (56°C/30 cycle); POMC (301 bp), sense, 5′-TGGTGCCTGGAGAGCAGCCAGTGC-3′, antisense, 5′-TGGAGTAGGAGCGCTTGCCCTCG-3′ (60°C/25 cycle); GAPDH (470 bp), sense, 5′-CCCTTCATTGACCTCAAC TACATGGT-3′, antisense, 5′-GAGGGCCATCCACAGTCTTC TG-3′ (56. 5°C/30 cycle) (Ziotopoulou et al., 2000). The GAPDH gene was used as an internal control gene (Li et al., 2000) because the hypothalamic GAPDH expression has reported to be unaffected by HF diet consumption (45% kcal from fat) for 13 weeks in 16 week-old male mice (Sellayah et al., 2008). After completion of these reactions, the PCR products were electrophoresed on a 1.5% agarose gel containing 0.5% ethidium bromide for UV visualization (ChemiDoc XRS System, Bio-Rad Inc., USA). Gel images were scanned and analyzed using Quantity One 1-D analysis software (v.4.6.1) (BioRad Inc., USA). The gene specific signal normalized to the GAPDH signal were averaged and then used as an expression value for each animal.

Measurement of leptin levels and adipose tissues

Samples for serum leptin, retroperitoneal white adipose tissue (WAT) and interscarpular brown adipose tissue (BAT) were collected on the same day of hypothalamus collection and serum leptin concentration was determined by DuoSet ELISA development kit (R&D System, USA) and adipose tissues were measured using a torsion balance.

Statistics

The Statistical Package for the Social Science (SPSS v12.0) was used to analyze all data. The changes in body weight and caloric intake were analyzed by analysis of variance (ANOVA) with repeated measures and other parameters were analyzed by one-way ANOVA, followed by a *post hoc* Scheffe test. Results are expressed as mean ± SEM.

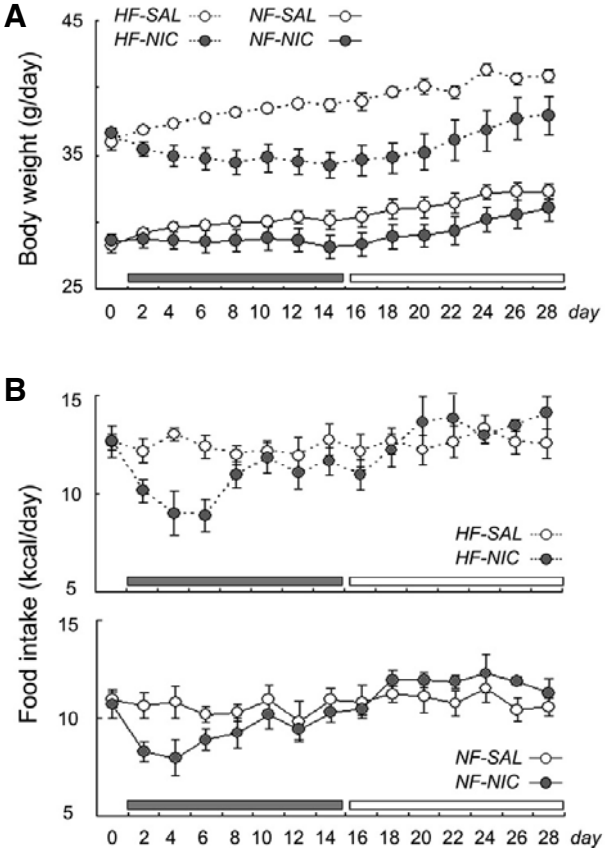

Fig. 1. Changes in body weight (g/day) (A) and food intake (kcal/day) (B) of saline (SAL, 1 ml/kg s.c., twice/day) and nicotine (NIC, 3.0 mg/kg/day s.c., twice/day) exposed mice in the normal-fat (NF, 3.8 kcal/g, solid line) and high-fat (HF, 4.7 kcal/g, dotted line) group during the experimental period (n = 6/group). Results are expressed as mean ± SEM. *, $p < 0.05$ and **, $p < 0.01$ SAL versus NIC; ‡, $p < 0.01$ NF versus HF diet. dark gray bar: NIC treatment (3.0 mg/ kg/day, s.c.) for 2 weeks; open bar: SAL treatment (1 ml/kg, s.c.) for 2 weeks.

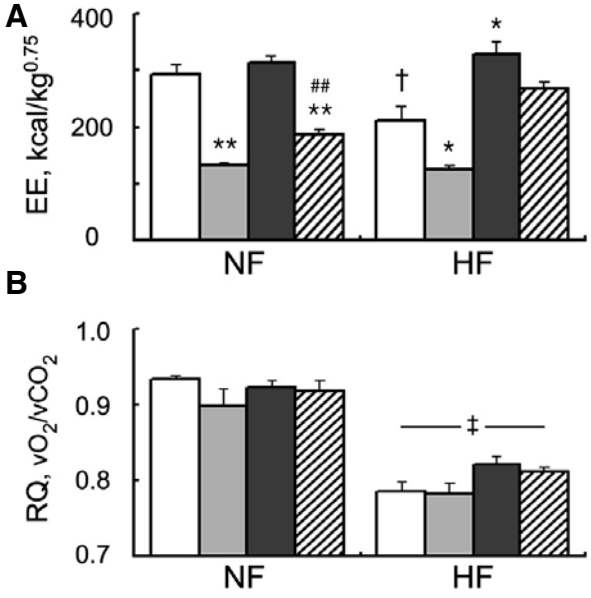

Fig. 2. Effect of nicotine and withdrawal on energy expenditure (EE, kcal/kg$^{0.75}$) and respiratory quotient (RQ, vO_2/vCO_2) for dark phase in SAL (1 ml/kg, open bars), single NIC (1.5 mg/kg, light gray bars), repeated NIC treatment (3.0 mg/kg/day, for 14 days, dark gray bars) and its withdrawal (repeated NIC treatment + SAL for 14 days, stripped bars) in C57BL/6 mice exposed either to a NF and HF diet (n = 4~6/group). Results are expressed as mean ± SEM. *, $p < 0.05$ and **, $p < 0.01$ SAL versus NIC; †, $p < 0.05$ and ‡, $p < 0.01$ NF versus HF diet; ##, $p<0.01$ NIC versus NIC-withdrawal.

RESULTS

Effects of high fat diet on weight changes

Male mice exposed to HF diet showed lower levels of dark phase EE (kcal/kg$^{0.75}$) and RQ (vO_2/vCO_2) (29.4%, $F_{(1, 8)} = 7.9$, $p = 0.029$ and 18.6%, $F_{(1, 8)} = 8.4$, $p = 0.019$, respectively) (Fig. 2) and consumed more calories (18.9%, $F_{(1, 10)} = 40.1$, $p < 0.001$) (Fig. 1B) during experimental period than that of SAL-treated NF group, resulted in a significant increase of body weight (27.1%, $F_{(1, 10)} = 371.6$, $p < 0.001$) (Fig. 1A), WAT and BAT (75.4%, $F_{(1, 20)} = 140.3$, $p < 0.001$ and 60.5%, $F_{(1, 20)} = 34.3$, $p < 0.001$) (Fig. 4B) with a higher level of preprandial serum leptin concentration (17.7%, $F_{(1, 8)} = 6.7$, $p = 0.05$) (Fig. 4A).

Effects of nicotine treatment and withdrawal on body weight and caloric intake

Changes of body weight and caloric intake during the 28 days of NIC treatments and NIC-WD in the NF and HF group are shown in Figs. 1A and 1B. During the NIC treatment for 14 days, the body weight in NIC-treated mice was gradually decreased in the NF (drug*day-$F_{(13, 130)} = 2.3$, $p = 0.01$) and HF (drug*day-$F_{(13, 130)} = 9.4$, $p < 0.001$) group, and the reducing effect of nicotine on body weight was prominent in the HF group (-11.7%, drug -$F_{(1, 10)} = 12.0$, $p = 0.006$) not in the NF group (-6.8%, drug -$F_{(1, 10)} = 1.8$, $p = 0.221$) (Fig. 1A). These results were accompanied by a combined effect of drug × day on caloric intake (kcal/day) in the HF (drug*day-$F_{(13, 130)} = 2.1$, $p = 0.018$) not in the NF (drug*day-$F_{(13, 130)} = 0.9$, $p = 0.482$) group (Fig. 1B). Also, during the 14 days of NIC-WD periods, NIC-WD induced weight gain occurred in the NF (withdrawal*day-$F_{(13, 117)} = 2.9$, $p = 0.001$) and HF (withdrawal*day-$F_{(13, 130)} = 3.2$, $p < 0.001$) group. On the other hand, NIC-WD did not produce an effect in caloric intake (kcal/day) in the NF (withdrawal*day-$F_{(13, 117)} = 1.4$, $p = 0.169$) and HF (withdrawal*day-$F_{(13, 130)}$=1.4, $p = 0.152$) group.

Effects of nicotine treatment and withdrawal on EE and RQ

Effects of NIC treatment and NIC-WD on dark phase EE (kcal/kg$^{0.75}$) and RQ (vO_2/vCO_2) in the NF and HF group are shown in Fig. 2. An ANOVA test indicated a significant drug effect on dark phase EE in the NF ($F_{(3, 14)} = 35.2$, $p < 0.001$) and HF ($F_{(3, 14)} = 16.9$, $p < 0.001$) group, but there was no drug effect on RQ in the NF ($F_{(3, 15)} = 0.9$, $p = 0.447$) and HF ($F_{(3, 14)} = 0.9$, $p = 0.218$) group. Post hoc comparisons indicated that single NIC treatment produced a significant decrease in EE in the NF (-56.3%, $p < 0.001$) and HF (-41.5%, $p = 0.025$) group. However, while NIC treatment for 14 days did not produce a significant drug effect in the NF group (-2.6%, $p = 0.98$) compared with those of SAL-treated NF group, 14 days of NIC treatment produced a significant increase in EE in the HF group (54.5%, $p = 0.022$). On the other hand, NIC-induced EE was decreased by NIC-WD in the NF group (-38.5%, $p < 0.001$), but was slightly decreased without significant difference in the HF group (-18.5%, $p = 0.308$).

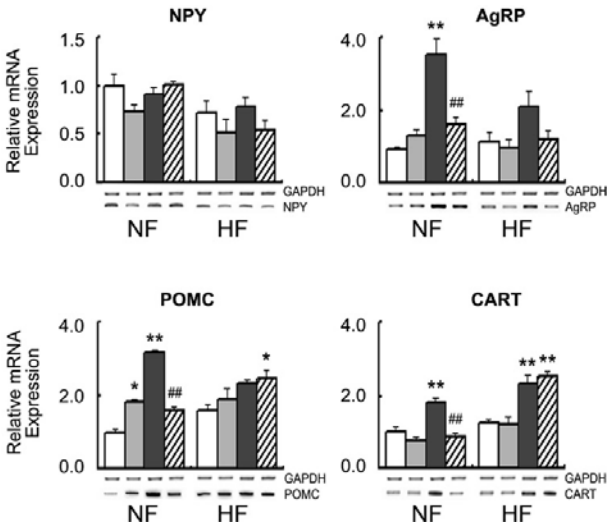

Fig. 3. Effect of nicotine and withdrawal on the hypothalamic NPY, AgRP, POMC and CART mRNA expressions in SAL (1 ml/kg, open bars), single NIC (1.5 mg/kg, light gray bars), repeated NIC treatment (3.0 mg/kg/day for 14 days, dark gray bars) and its withdrawal (repeated NIC treatment + SAL for 14 days, stripped bars) in C57BL/6 mice exposed either to a NF and HF diet (n = 5~6/group). Results are expressed as mean ± SEM. *, $p < 0.05$ and **, $p < 0.01$ SAL versus NIC; ##, $p < 0.01$ repeated NIC versus NIC-withdrawal.

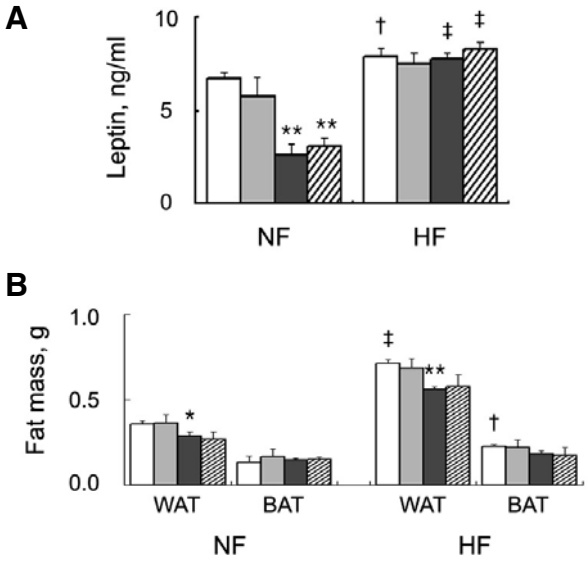

Fig. 4. Effect of nicotine and withdrawal on (A) preprandial leptin levels (ng/ml), (B) retroperitoneal adipose tissue (WAT) and interscarpular brown adipose tissue (BAT) (g) in saline (1 ml/kg, open bars), single NIC (1.5 mg/kg, light gray bars), repeated NIC treatment (3.0 mg/kg/day for 14 days, dark gray bars) and its withdrawal (repeated NIC treatment + SAL for 14 days, stripped bars) in C57BL/6 mice exposed either to a NF and HF diet (n = 4~7/group). Results are expressed as mean ± SEM. *, $p < 0.05$ and **, $p < 0.01$ saline versus nicotine; †, $p < 0.05$ and ‡, $p < 0.01$ NF versus HF diet.

Effects of nicotine treatment and withdrawal on hypothalamic neuropeptide mRNAs expression

Effects of NIC treatments and NIC-WD on the hypothalamic neuropeptides mRNA expression in the NF and HF group are shown in Fig. 3. An ANOVA indicated a significant effect of NIC on the expression levels of hypothalamic AgRP ($F_{(3, 15)} = 15.6$, $p < 0.001$), POMC ($F_{(3, 15)} = 118.9$, $p < 0.001$) and CART ($F_{(3, 15)} = 16.1$, $p < 0.001$) mRNAs in the NF group; however, the effect of NIC was detected in the POMC ($F_{(3, 15)} = 6.2$, $p = 0.001$) and CART ($F_{(3, 18)} = 85.8$, $p < 0.001$) mRNA expression, not in the NPY ($F_{(3, 15)} = 0.8$, $p = 0.538$) and AgRP ($F_{(3, 15)} = 0.664$, $p = 0.59$) mRNA expression in the HF group. Post hoc analysis also showed that single NIC treatment increased the POMC mRNA expression (81.8%, $p = 0.010$) in the NF group and 14 days of NIC treatment increased the expression levels of hypothalamic POMC, AgRP and CART mRNA in the NF group (218.3%, $p < 0.001$; 276.8%, $p < 0.001$; 72.3%, $p = 0.005$, respectively). Unlike in the NF group, NIC treatment for 14 days increased the CART (85.4%, $p < 0.001$) mRNA expression, but the others were not affected by NIC treatment in the HF group. After NIC-WD period, the increased hypothalamic AgRP, POMC and CART mRNA levels were decreased in the NF group (-55.1%, $p = 0.003$; -50.7%, $p < 0.001$ and -51.4%, $p < 0.001$, respectively) compared to the NIC-treated NF group, but there was no significant difference between NIC-treated and NIC-WD HF group and POMC and CART mRNA expressions in the HF group were sustained higher than that of the SAL group (87.2%, $p = 0.022$ and 106.5%, $p < 0.001$, respectively).

Effects of nicotine treatment and withdrawal on leptin levels and adipose tissues

The effect of NIC treatments and NIC-WD on the leptin levels (ng/ml) and fat mass (g) in the NF and HF group are shown in Fig. 4. An ANOVA test indicated a significant NIC effect in the preprandial leptin levels in the NF group ($F_{(3, 16)} = 10.6$, $p < 0.001$) not in the HF group ($F_{(3, 16)} = 0.7$, $p = 0.578$) (Fig. 4A). Post hoc comparisons also indicated that NIC treatment for 14 days produced a significant decrease in the serum leptin levels in the NF group (-61.0%, $p = 0.003$) compared with that of SAL-treated NF group, which was unaffected by 14 days of NIC-WD compared with NIC-treated NF group (18.9%, $p = 0.971$). In the HF group, however, while the preprandial leptin levels in the NIC treated-HF group were unaffected by NIC treatment or NIC-WD compared with those of SAL-treated HF group (-1.6%, $p = 0.927$ and 4.8%, $p = 0.616$), the leptin levels in the NIC- and WD-treated HF group were higher compared with those of NIC-treated and NIC-WD NF groups (196.9%, $p < 0.001$ and 165.9%, $p < 0.001$, respectively).

An ANOVA test indicated a significant NIC effect in the weight of WAT in the HF group ($F_{(3, 22)} = 4.5$, $p = 0.013$) but not in the NF group ($F_{(3, 27)} = 2.4$, $p = 0.086$) (Fig. 4B). However, *post hoc* comparisons also indicated that NIC treatment for 14 days produced a significant decrease in the WAT of NF (-19.5%, $p = 0.044$) and HF (-21.1%, $p = 0.005$) group, whereas 14 days of NIC-WD did not affect to weight of WAT (g) compared with NIC-treated group in the NF (-6.7%, $p = 0.576$) and HF (2.2%, $p = 0.830$) group. On the other hand, there was no significant effect of NIC in the weight of BAT (g) in the NF ($F_{(3, 27)} = 1.3$, $p = 0.300$) and HF ($F_{(3, 22)} = 2.1$, $p = 0.130$) group.

DISCUSSION

In this study, mice exposed to HF diet for 13 weeks showed a lower EE during dark phase (Hu et al., 2004) (Fig. 2) and a higher caloric intake compared with that of NF group, which leads to a significant increase in adipose tissues (Fig. 4B) and body weight in the HF group (Figs. 1A and 1B). As seen in

previous studies (Bellinger et al., 2003; Wellman et al., 2005), NIC treatment for 14 days lead to a reduction of body weight in both groups, particularly in the HF group and also produced an inverse relationship between body weight change and caloric intake after NIC treatment period (Figs. 1A and 1B). These results seem to be caused at least in part by NIC-induced increase of AgRP mRNA expressions in the NF group, which may be mediated by NIC-induced decreased leptin levels (Figs. 3 and 4), subsequently, a combination of these influences may lead to this compensated caloric intake.

On the other hand, with an exception of CART mRNA expression, NIC-induced responsiveness of hypothalamic neuropeptides mRNA expression was less sensitive in the HF group, when compared with those of NIC-treated NF group. This result may be due to sustained higher levels in leptin concentration in the NIC-treated HF group compared with that of NIC-treated NF group (Fig. 4), suggesting that obese mice may regulate their caloric intake through HF diet-induced altered hypothalamic and peripheral mechanisms.

It is well established that HF diet consumption can produce an increase in body weight, thus inducing an animal model of obesity in rodents (Mitchell et al., 2009; Ziotopoulou et al., 2000) with a higher plasma leptin concentrations (Shi et al., 2009; Shiraev et al., 2009). Leptin, a potent mediator in energy homeostasis (Ahima et al., 2000), has been reported to act in the hypothalamus (Enriori et al., 2006; Hewson et al., 2002; Morris and Rui, 2009) to regulate food intake (Henry and Clarke, 2008) and body weight (Oswal and Yeo, 2009).

Circulating leptin, an important peripheral hormonal signal in the regulation of energy homeostasis (Anubhuti and Arora, 2008; Fruhbeck et al., 1997), also showed rapid changes as a sensor of energy balance in conditions of negative or positive energy balance, i.e. fasting or refeeding (Anubhuti and Arora, 2008; Hardie et al., 1996). On the other hand, leptin concentration also displays patterns of circadian rhythms, wherein the maximum was noticed in the nocturnal phase (Cano et al., 2008; Perello et al., 2006). On the contrary, leptin in HF diet-induced obese animal model showed the peak level during the diurnal phase and the minimum level was seen during nocturnal phase (Cano et al., 2009). Although we measured the leptin levels at the initial time of dark phase, when the difference of circulating leptin levels between the NF and HF group has begun to disappeared from its peak for diurnal phase, still we observed a significant difference in the preprandial leptin levels (Fig. 4A), thus, suggesting an altered dietary regulation of circulating leptin levels in the HF group.

Additionally, in accordance with previous observation (Sanigorski et al., 2002), we found that NIC treatment for 14 days reduced body weight with a decreased preprandial leptin levels only in NIC-treated NF group but not in obese NIC-treated group (Fig. 4A). Although, there are no substantial experimental evidence to indicate that physiological mechanisms are responsible for this blunted leptin response to nicotine in obese mice, while, it is well documented that leptin plays a role in the regulation of energy balance directly coupled with functional leptin receptors (Lollmann et al., 1997; Wang et al., 1999) or leptin-mediated energy utilization through beta-adrenoceptors (Collins et al., 1996; Commins et al., 2000) in adipose tissues. It is also hypothesized that sympathetic modulation of leptin expression from adipose tissues is implicated in energy balance (Mark et al., 2003; Scarpace and Matheny, 1998; Scarpace et al., 2000). There are evidences suggesting that stimulation of beta-adrenergic mechanisms leads to suppression of leptin mRNA expression and secretion from adipose tissues (Ricci et al., 2005) as well as circulating leptin levels (Li et al., 1997; Sakane et al., 1998; Zhang et al., 2002).

On the other hand, animal studies have also reported that high fat diet-induced obese mice showed a decreased beta-adrenergic regulation in WAT as well as a higher plasma leptin level compared to the control group (An et al., 2010; Collins et al., 1999; Shi et al., 2009) and a defective adrenergic receptor signaling related with energy utilization in adipose tissue (Gaidhu et al., 2010). Human studies have also shown blunted leptin-mediated sympathetic nerve activity in obesity (Masuo et al., 2006; 2008). Moreover, diet induced-obese mice have been reported to show a blunted response in the hypothalamus (Mitchell et al., 2009; Sahu et al., 2002) and circulating leptin concentration (Lindqvist et al., 2005; O'Doherty and Nguyen, 2004) under fasting-induced negative energy status.

Animal and clinical studies have co-relating evidence that nicotine increase thermogenesis by involving catecholaminergic activation in adipose tissues both in animals (Brees et al., 2008; Lupien and Bray, 1988; Yoshida et al., 1990) and human (Andersson and Arner, 2001), whereas NIC also induced a decrease of circulating leptin levels in animals exposed to standard chow diet (Li and Kane, 2003; Sanigorski et al., 2002) and human (Reseland et al., 2005). Moreover, in humans, cigarette smoking-induced decrease of plasma leptin levels has shown to be mediated by smoking-induced increased plasma catecholamine levels in smoking men (Reseland et al., 2005). Thus, it is possible to speculate that although we did not directly measure the sympathetic activation or relevant indicators, the leptin levels in NIC-treated obese mice may be mediated by indirect action of nicotine on circulating leptin concentration through HF diet-induced diminished sympathetic nerve activity.

In addition to increase of AgRP mRNA expression, 3.0 mg/kg/day of NIC treatment for 14 days also produced an increase in the hypothalamic POMC and CART mRNA expression, simultaneously (Fig. 3). While previous studies have reported that intermittent NIC administration (1.4 mg/kg/day, i.p., 4 times a day) for 9 days did not influence the hypothalamic AgRP, NPY, POMC mRNA levels, but reduced the CART mRNA levels in the dorsomedial nucleus of hypothalamus in rats compared to that of SAL-treated group (Kramer et al., 2007), the exact mechanisms by which NIC or its withdrawal modulates hypothalamic neuronal circuitries is known only to a limited extent (Fornari et al., 2007; Frankish et al., 1995; Li et al., 2000; Nakhate et al., 2009). The inconsistency of these results may be due to changes in food intake as the animals in these experiments are either maintained or suppressed food intake over the course of the experiment, which may be affected by various dosing regimens (Kirshenbaum et al., 2008).

Hypothalamic AgRP is also well known to exert a role in maintaining energy balance that regulate feeding and energy expenditure (Ilnytska and Argyropoulos, 2008; Stutz et al., 2005). For instance, the hypothalamic AgRP mRNA expression was significantly increased with decreased preprandial leptin levels, without any changes of hypothalamic POMC and CART mRNA expressions under negative energy status in rats (Palou et al., 2009; Vogler et al., 2008). Thus, our result of decreased leptin levels in the NIC-treated NF mice may induce hypothalamic AgRP mRNA expression changes that resist further weight loss by confusing the animal towards a compensated food intake during 14 days of NIC treatment period in this experimental regime (Figs. 1B and 3), thus, showing a reciprocal mechanism between central and peripheral regulation to balance energy homeostasis under negative energy status.

Meanwhile, anorectic effects of nicotine in feeding has been reported to be mediated by endogenous cholinergic systems in

the lateral hypothalamus (Jo et al., 2002), our results of increased POMC and CART mRNA expression may be ascribed to direct effects of NIC on neuropeptides regulation *via* nicotinic acetylcholine receptors in the hypothalamus. Additionally, although obese mice exposed to HF diet showed an altered leptin-dependent hypothalamic regulation that modulate food intake, anorectic effects of nicotine on food intake (kcal/day) during early part of the NIC treatment were more prominent than NIC-treated NF group. Therefore, further research is needed to determine the early development of compensatory food intake in response to long term NIC treatments.

Interestingly, there are very few studies on the mechanisms involved in NIC-induced body weight regulation by which NIC affects metabolic rate in obese mice. Although animal studies, including data from this study showed a conflicting effect of NIC on metabolic rate (Bishop et al., 2004; Wager-Srdar et al., 1984); however, recent study also reported that the inhibitory effect of nicotine on weight gain was caused by a decrease in food intake but not an increase in metabolic rate in rats fed with a diet that consists of 4% fat, 55.3% carbohydrate, and 20% protein (Bellinger et al., 2010). And we found that 3.0 mg/kg of NIC treatment and withdrawal did not affect respiratory quotient; however, NIC treatment significantly increased dark phase EE in obese mice. Thus, reducing effect of NIC treatment on body weight in obese mice resulted from a combined effect between increased EE and decreased caloric intake (Figs. 1B and 2).

Our results also showed that NIC-WD induced weight gain in both groups may be due to a decrease in dark phase EE (Fig. 2), as there were no significant increase in caloric intake (Fig. 1) during the NIC-WD period. Additionally, the circulating leptin levels observed in the NIC-WD group was not affected by the 14 days of NIC-WD period compared to that of NIC-treated group, while it showed a positive relationship between the circulating leptin levels and the weight of WAT (Figs. 4A and 4B) (Considine and Caro, 1997; Handjieva-Darlenska and Boyadjieva, 2009) even after the 14 days of NIC-WD period. Thus, the circulating leptin levels in the NIC-WD group may be ascribed to the changes in WAT observed in the NIC-WD group (Fig. 4B). These results may be due to the 14 days of NIC-WD period, which was not sufficient to recover the weight of WAT. Considering a physiological role of leptin in the body weight regulation (Lindqvist et al., 2005; O'Doherty and Nguyen, 2004; Shi et al., 2009), our results may suggest that the changes observed in this study with regard to weight gain induced by NIC-WD would probably be more dependent on factors such as peripheral energy utilization rather than on energy consumption through hypothalamic regulation.

It seems to be more effective for obese mice to control weight gain during nicotine treatment, also NIC-WD-induced weight gain occurred because of decreased dark phase EE rather than leptin-dependent hypothalamic appetite regulation in mice. The current study has provided new information for understanding of the basis of mechanism involved in nicotine-induced energy balance in mice.

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