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Original/*Obesidad*

Time-specific changes in DNA methyltransferases associated with the leptin promoter during the development of obesity

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Abstract

Objective: **The role of epigenetic modifications on leptin expression during the development of obesity has not been clearly determined. This study aimed to investigate changes in the expression of DNA methyltransferases (DNMTs) at the leptin promoter and their effect on gene transcription during the development of obesity.**

Methods: **Using a high-fat diet (HFD)-induced obese (DIO) mouse model, we examined adipose expression of leptin, its promoter associated DNMTs and the methyl CpG-binding domain protein 2 (MBD2) at different time points after HFD feeding.**

Results: **The leptin expression levels in epididymal fat were significantly increased after feeding the mice a HFD for 4, 8, 12 and 18 weeks (w), as opposed to feeding them a standard diet (SD). However, the CpG promoter methylation fractions were significantly reduced at 8 w with a decreased association of MBD2 and DNMT1, and increased at 12 w and 18 w with an increased association of MBD2, DNMT3A and DNMT3B, after HFD feeding. Additionally, the binding of RNA polymerase II was increased at 8 w and decreased at 18 w after HFD feeding compared with SD feeding.**

Conclusions: **These data indicate that time-specific changes in promoter associated DNMTs may be associated with the regulation of leptin expression, indicating that a complex and dynamic epigenetic mechanism underlies aberrant leptin expression during the development of obesity.**

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Key words: *Obesity. Leptin promoter. DNA methyltransferase. Methyl CpG-binding domain protein 2. Mouse.*

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CAMBIOS EN PUNTOS TEMPORALES ESPECÍFICOS EN ADN-METILTRANSFERASAS EN RELACIÓN CON EL PROMOTOR DE LEPTINA DURANTE EL DESARROLLO DE OBESIDAD

Resumen

Objetivo: **El objetivo de las modificaciones epigenéticas sobre la expresión de la leptina durante el desarrollo de obesidad no ha podido ser claramente establecido. Este estudio tiene por objetivo investigar los cambios en la expresión de ADN-metiltransferasas (ADNMTs) en el promotor de leptina y su efecto sobre la trascripción génica durante el desarrollo de obesidad.**

Métodos: **Empleando un modelo de ratones con obesidad inducida por dieta rica en grasa (DRG), examinamos la expresión adiposa de leptina, su promotor asociado ADNMTs y la proteína 2 con dominio de unión a metil-CpG (MBD2) en diferentes momentos tras la alimentación DRG.**

Resultados: **Los niveles de expresión de leptina en grasa epididimal aumentaron significativamente tras la alimentación de los ratones con una dieta DRG durante 4, 8, 12 y 18 semanas (s), contrariamente a la alimentación con dieta estándar (DE). Sin embargo, las fracciones de metilación del promotor CpG se redujeron significativamente en la s8 con una menor asociación de MBD2 y DNMT1, y aumentaron en la s12 y s18 con una mayor asociación de MBD2, DNMT3A y DNMT3B, tras la DRG. Además, la unión de ARN polimerasa II aumentó en la s8 y disminuyó en la s18 tras la DRG en comparación con la alimentación de DE.**

Conclusiones: **Estos datos indican que los cambios en puntos temporales específicos en ADNMTs en relación con un promotor podrían estar relacionados con la regulación de la expresión de leptina, indicando que existe un mecanismo epigenético dinámico y complejo subyacente en la expresión de leptina aberrante durante el desarrollo de obesidad.**

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Palabras clave: *Obesidad. Promotor de leptina. ADN-metiltransferasa. Proteína 2 con dominio de unión a metil-CpG (MBD2). Ratón.*

Introduction

The leptin is primarily expressed in the differentiated adipocytes of white fat tissue and has been shown to play an important role in maintaining the homeostatic control of adipose tissue mass and body weight by regulating food intake and energy expenditure through a variety of neural and endocrine mechanisms[1,2]. In diet-induced obesity (DIO), the expression of the leptin gene and its circulating concentration are elevated without any regulatory effects on body weight, indicating the development of leptin resistance[1,2]. Emerging evidence has shown that leptin transcriptional expression is closely associated with its promoter's methylation. The leptin promoter is highly methylated in pre-adipocytes, and demethylated during their maturation toward terminally differentiated adipocytes, initiating leptin expression[3,4]. In obesity, a hypermethylation of the CpGs in the leptin promoter region has been reported in DIO mice, and this hypermethylation may serve in a feedback role to maintain the leptin concentration within a normal range in DIO mice[5-7]. In humans, women with a better response to dietary intervention showed lower promoter CpG methylation levels of leptin and TNF-alpha compared with the non-responder group, and obese preschool children had reduced CpG methylation levels of the leptin promoter[8,9]. These findings imply that the methylation status of the leptin promoter may vary with age, development and the severity of obesity, affecting gene transcriptional expression.

CpG methylation in DNA is catalyzed by enzymes of the DNA methyltransferase (DNMT) family that comprise three active members in mammals sharing a conserved catalytic domain. The interaction of DNMT with gene promoters generally inhibits transcriptional initiation, either by preventing the binding of certain transcription factors or recruiting methylated DNA binding domain (MBD) proteins to generate a repressed chromatin environment[10-13]. However, the three types of DNMTs have been demonstrated to function in a different manner. The founding member, DNMT1, is responsible for maintaining DNA methylation during DNA replication after development. The de novo DNMT3A and DNMT3B enzymes are responsible for the establishment of DNA methylation patterns during development[10,11]. Therefore, based on our previous report demonstrating the dynamic changes in the methylation fraction of CpG sites at the leptin promoter during the development of obesity[7], we investigated the time-specific changes in leptin promoter associated DNMTs in detail and further determined their association with gene transcription during the development of obesity.

Materials and Methods

Diet and animals

Based on the high-fat diet formula (D12492) for DIO mice from Research Diets Inc. (New Brunswick, NJ), a high-fat diet (HFD) (34.9% fat by wt., 60% kcal) was designed using soy and lard oils as the main sources of fat, using a standard diet (SD) (4.3% fat by wt; 10% kcal) as a control. One hundred and twenty male C57BL/6J mice at 3 to 4 weeks of age were obtained from the Laboratory Animal Center at Hospital 304 affiliated with the General Hospital of the People's Liberation Army in China. All of the mice were housed in animal facilities in temperature-, light- and humidity-controlled rooms with a 12-hour (h) light/12-h dark cycle. After one week of recovery from transportation, the mice were classified into two groups (60 mice in each group) and fed the HFD and SD diets, respectively. Their body weights were measured weekly to confirm that the HFD-fed mice gained more weight than the SD-fed ones. At 4-, 8-, 12-, and 18-weeks (w) after feeding, fifteen fasted mice from each of the two groups were anesthetized by an intraperitoneal injection of Avertin (2,2,2-tribromoethanol, T-4840-2, Sigma-Aldrich Chemie GmbH, Steinheim, Germany, 125 mg/kg) to obtain blood samples via heart puncture for the evaluation of blood leptin concentration. The mice were then sacrificed immediately by decapitation. The epididymal fat was dissected from the surrounding tissue, immediately frozen in liquid N_2, and stored at -80 °C for analysis. All of the animal experiments were conducted from 08:00 to 12:00 in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of National Administration Regulations on Laboratory Animals of China. This protocol was approved by the Committee on the Ethics of Animal Experiments of Hospital 304 affiliated with the General Hospital of the People's Liberation Army in China.

Quantitative analysis of mRNA expression

The total RNA was extracted from mouse fat using the TRIzol Reagent (cat. no. 15596-026, Invitrogen, Carlsbad, CA, USA), and the cDNA was prepared from the total RNA using the SuperScript™ III First-Strand Synthesis System for RT-PCR (cat. no. 18080-051, Invitrogen, Carlsbad, CA, USA) according to the procedures provided by the manufacturer. The mRNA levels of leptin and the DNMTs (DNMT1, 3A and 3B) were measured using real-time quantitative RT-PCR with an ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA). The oligonucleotide primers for the targeted genes were used as described in the literature[14,15] and examined using the Primer Express software: leptin (NM_008493.3) (F: TGGCTTTGGTCCTATCTGTC; R: TCCTGGTGACAATGGTCTTG), DNMT1 (NM_010066.4) (F: AAAGTGTGATCCCGAAGATCAAC; R: TGGTACTTCAGGTTAGGGTCGTCTA), DNMT3A (NM_007872.4) (F: CCGCCTCTTCTTTGAGTTCTAC; R: AGATGTCCCTCTTGTCACTAACG), DNMT3B (NM_001003961.4) (F: CCCAAGTTGTACCCAGCAATTC; R: TGCAATTCCATCAAACA-

GAGACA). The PCR conditions were as follows: 95°C for 10 minutes (min); 45 cycles of 95°C for 15 sec, 60°C for 1 min and 72°C for 30 sec; and 7 min at 72°C. The co-amplification of mouse GAPDH mRNA, an invariant internal control, was performed in all of the samples because this house-keeping gene's expression is insignificantly affected by dietary fat. The assays were performed in triplicate, and the results were normalized to the GAPDH mRNA levels using the $2^{-\Delta CT}$ method.

Bisulfite conversion and sequencing

The examined leptin promoter region includes nucleotides (nts) 29009221-29010220 and spans 16 CpGs within nts -321 to -1 (positions are given relative to the transcription start site [TSS]). The obtained sequence data have been submitted to the GenBank database (http://www.ncbi.nlm.nih.gov) under accession number U18812. The methylation of the leptin promoter was analyzed by bisulfite sequencing. A DNA Purification Kit (cat. no. DN 1008, Biofuture Group Inc., Beijing, China) was used to isolate and purify the DNA from adipose tissue. A bisulfite conversion was performed using the Methylamp™ DNA Modification Kit (cat. no. P-1001, Epigentek Group Inc., Brooklyn, NY). The converted DNA was amplified by nested PCR using Taq DNA Polymerase Master Mix (cat. no. KT201, Tiangen Biotech Inc., Beijing, China) and the following primers were designed using Methprimer: outer F, 5'-GAGTAGTTAGGTTAGGTATGTAAAGAG-3'; inner F, 5'-AGTTTTTTGTAGTTTTTTGTTTTTTG-3'; R, 5'-TAATAACTACCCCAATACCACTTAC-3'. The PCR conditions were as follows: 96°C for 10 minutes (min); 45 cycles of 96°C for 1 min, 51°C for 1 min and 72°C for 1 min; and 10 min at 72°C. The nested PCR was performed using the same conditions as the PCR in the first step. The PCR products were sequenced directly, and the DNA methylation was calculated as described by Lewin et al.[16]

Chromatin immunoprecipitation (ChIP)

Many promoter-associated proteins have been shown to be involved in the epigenetic regulation of gene transcription. We targeted the key histones and enzymes for which specific antibodies were available. The chromatin immunoprecipitation (ChIP) studies were performed using either prepared EpiQuikTM kits (Epigentek Group Inc., NY), i.e., the Tissue Methyl-CpG binding domain protein 2 (MBD2) ChIP Kit (cat. no. P-2018), or the general ChIP kit (Epigentek cat. no. P-2003) with specific antibodies for DNMTs (1, 3a and 3b) (cat. nos. ab13537, ab2850 and ab2851), and RNA Pol II (cat. no. ab5096). Briefly, the procedure was performed as follows. Adipose tissue (20 mg) was cross-linked with 1% formaldehyde for 20 min and then homogenized. The homogenate was sonicated for four pulses of 15 seconds (sec) each at level 2 using the microtip probe of a Branson Digital Sonifier (Model 450, Branson Ultrasonics Corporation, Connecticut, USA), followed by a 40 sec interval on ice between each pulse to generate fragments of genomic DNA ranging from 200 to 1000 bp in length. For the ChIP assays, equivalent amounts of treated chromatin were added to microwells containing immobilized antibodies for the targeted protein or a normal mouse IgG antibody negative control. In addition, a small portion of treated chromatin equivalent to 5% of the extracted genomic DNA was used as the Input DNA to calculate the enrichment of the leptin promoter DNA after immunoprecipitation of the targeted proteins. After incubation for 90 min at 65°C to reverse the cross-links and elute the DNA, Fast-Spin columns were used for DNA purification.

The purified DNA was used for a real-time PCR analysis of the leptin promoter. The sequence data for the leptin gene are available from NCBI (http://www.ncbi.nlm.nih.gov) using the reference sequence NC_000072.6 covering nucleotides (nts) 29060195..29073877. The primers F- 5' TCGAAGCAGGTGCATTCTGT 3' and R- 5' GGGCAACTTGTCTTCCTTTGG 3' were used to generate an 80-bp amplicon (-431 to -510 relative to the TSS). The PCR conditions were as follows: 95°C for 10 min; 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 75°C for 45 sec; and finally 7 min at 75°C and 2 min at 4°C. A melting curve was generated from 70°C to 95°C, and the signal was read every 0.5°C for 5 sec. A co-amplification of mouse GAPDH mRNA was performed in all of the samples, including the ChIP and Input DNA samples. Each sample was normalized to its respective Input PCR product using the $2^{-\Delta CT}$ method. The data were then expressed as the ratio of ChIP to Input, which reflects the quantity of the targeted proteins associated with the leptin promoter.

Statistical analysis

An independent sample t test was performed to compare the means of the DIO and control diet groups. Statistical analyses were performed using SPSS Release 11.5 for Windows with $P<0.05$ considered to be a significant difference.

Results

Changes in body weight, daily food consumption and adipose leptin expression during the development of obesity: Mice were raised from the end of the weaning period on either the HFD or the SD diet for 18 weeks. The HFD mice consumed significantly more calories per day at 2 and 12 weeks (14.76±1.70 and 20.30±1.75 kcal/d/mouse, respectively) than the SD mice (9.45±0.23 and 13.55±1.09 kcal/d/mouse, respectively) (Fig. 1A), resulting in a significant increase in their body weight (BW) starting at 2 weeks (after the HFD feeding,

P<0.05), with the greatest body increase (52.72%) at 18 weeks (Fig. 1B). After feeding the HFD mice for 4, 8, 12, and 18 weeks, the mRNA expression levels and protein concentrations of leptin in epididymal fat were increased compared with the SD fed mice (P<0.05) (Fig. 1C).

Changes in CpG methylation of the leptin promoter during the development of obesity: A total of 16 CpG sites on the leptin promoter were determined and each site was assigned a number. The methylation fractions of sites 1, 5, 7-9 and 11-13 were reduced at 8 w, whereas those of sites 1-10 were increased at 12 w and 18 w after the HFD feeding, compared with the SD feeding (P<0.05), leading to a reduction in the average total methylation fraction at 8 w and an increase in the average total methylation fraction at 12 w and 18 w after HFD feeding (P<0.05). No changes were found in the CpG methylation at 2 w and 4 w after the HFD feeding (P>0.05) (Table I).

Changes in MBD2 and RNA Pol II binding at the leptin promoter during the development of obesity: Compared with SD feeding, HFD feeding affected the binding of MBD2 to the leptin promoter, with the promoter methylation changes shown in Table I, indicating that less MBD2 was associated with the promoter after HFD feeding at 8 w but more MBD2 was associated with the promoter after HFD feeding at 12 w and 18 w, with no changes at 4 w after HFD feeding (Fig. 2A). This is consistent with the general rule that the methylation of CpG dinucleotides, parti-

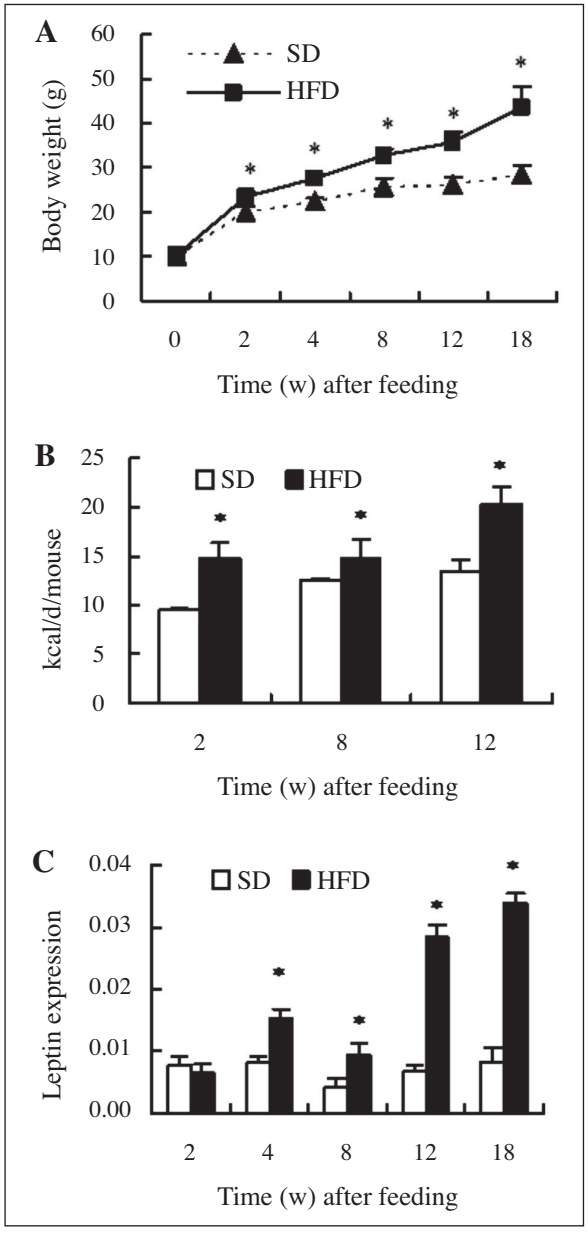

*Fig. 1.—**Changes in body weight, daily caloric intake and leptin mRNA expression after HFD feeding:** Three to four-week-old C57BL/6J male mice were fed a HFD for 2 to 18 w, with SD mice used as a control group. At 2-, 4-, 8-, 12-, and 18-weeks after feeding, the leptin mRNA expression in epididymal fat was determined by real-time quantitative RT-PCR. A: body weight; B: caloric intake; C: leptin mRNA expression. The values are expressed as the mean ± SEM; n=15 in each group at each time point and are compared with the SD feeding group, *P<0.05.*

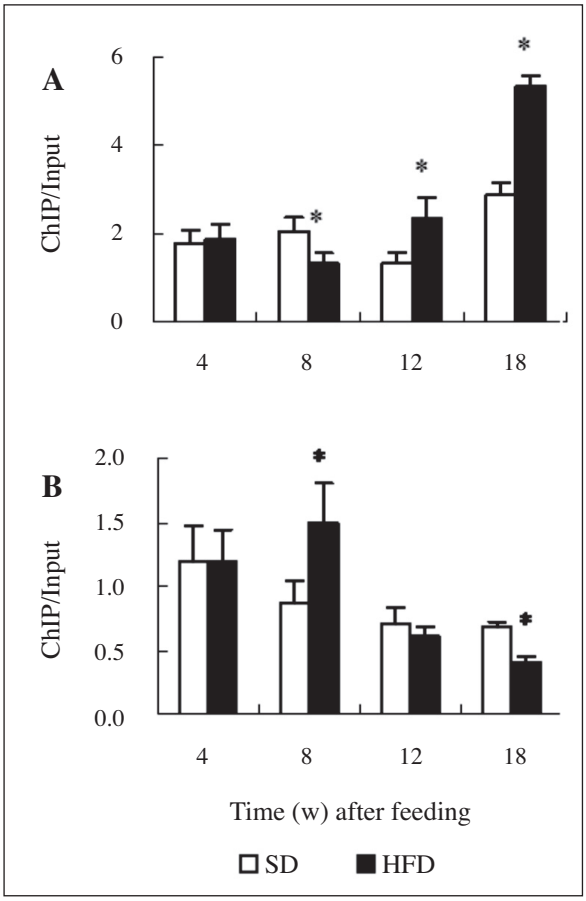

*Fig. 2.—**Changes in MBD and RNA Pol II binding at the leptin promoter during the development of obesity:** Mice were fed a HFD using mice fed a SD diet as controls, and their epididymal fat was dissected 2-, 4-, 8-, 12-, and 18-weeks after feeding. Binding of MBD2 (A) and RNA Pol II (B) to the leptin promoter was determined by ChIP-PCR. The ratio of the PCR signal from the protein ChIP DNA to the PCR signal from the total genomic DNA (Input) is plotted as an estimation of the protein levels. The values are expressed as the mean ± SEM (n=15 for each group) compared with the SD feeding group, *P<0.05.*

Table I

Quantitative methylation analysis of the leptin promoter

Time	*Diet*	*CpG sites*																
		a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*	*k*	*l*	*m*	*n*	*o*	*p*	*average*
2 w	DIO	0.61 ± 0.05	0.75 ± 0.04	0.61 ± 0.06	0.68 ± 0.05	0.76 ± 0.06	0.67 ± 0.05	0.81 ± 0.03	0.80 ± 0.04	0.69 ± 0.05	0.79 ± 0.04	0.81 ± 0.05	0.86 ± 0.04	0.88 ± 0.03	0.86 ± 0.02	0.90 ± 0.03	0.96 ± 0.03	0.75 ± 0.02
	Control	0.60 ± 0.07	0.72 ± 0.05	0.57 ± 0.04	0.68 ± 0.05	0.75 ± 0.05	0.66 ± 0.03	0.79 ± 0.04	0.81 ± 0.04	0.66 ± 0.04	0.77 ± 0.04	0.78 ± 0.04	0.86 ± 0.04	0.84 ± 0.03	0.82 ± 0.03	0.88 ± 0.04	0.95 ± 0.02	0.76 ± 0.02
4 w	DIO	0.64 ± 0.06	0.76 ± 0.05	0.59 ± 0.04	0.68 ± 0.05	0.73 ± 0.05	0.66 ± 0.05	0.77 ± 0.04	0.79 ± 0.03	0.64 ± 0.03	0.76 ± 0.04	0.75 ± 0.05	0.81 ± 0.03	0.82 ± 0.02	0.81 ± 0.03	0.85 ± 0.02	0.93 ± 0.01	0.75 ± 0.02
	Control	0.64 ± 0.07	0.74 ± 0.06	0.57 ± 0.04	0.66 ± 0.06	0.76 ± 0.04	0.66 ± 0.04	0.76 ± 0.04	0.78 ± 0.03	0.65 ± 0.03	0.74 ± 0.04	0.77 ± 0.06	0.61 ± 0.03	0.81 ± 0.02	0.81 ± 0.03	0.85 ± 0.01	0.93 ± 0.01	0.75 ± 0.03
8 w	DIO	0.54 ± 0.02*	0.68 ± 0.02	0.56 ± 0.03	0.62 ± 0.03	0.71 ± 0.03*	0.64 ± 0.02	0.74 ± 0.02*	0.77 ± 0.02*	0.63 ± 0.02*	0.75 ± 0.03	0.77 ± 0.02*	0.83 ± 0.03*	0.84 ± 0.04*	0.84 ± 0.02	0.89 ± 0.04	0.94 ± 0.02	0.73 ± 0.02*
	Control	0.57 ± 0.03	0.69 ± 0.04*	0.56 ± 0.02	0.64 ± 0.04*	0.73 ± 0.03*	0.65 ± 0.03*	0.76 ± 0.03	0.80 ± 0.02*	0.66 ± 0.03	0.76 ± 0.03*	0.81 ± 0.03	0.66 ± 0.02*	0.87 ± 0.02*	0.85 ± 0.03*	0.89 ± 0.02	0.94 ± 0.01	0.75 ± 0.02
12 w	DIO	0.57 ± 0.06*	0.72 ± 0.04*	0.60 ± 0.05*	0.66 ± 0.05*	0.77 ± 0.03*	0.67 ± 0.04*	0.80 ± 0.02*	0.82 ± 0.02*	0.67 ± 0.03*	0.77 ± 0.06*	0.78 ± 0.03	0.84 ± 0.03	0.85 ± 0.04	0.83 ± 0.04	0.87 ± 0.04	0.94 ± 0.03	0.76 ± 0.03*
	Control	0.52 ± 0.05	0.66 ± 0.05*	0.53 ± 0.05	0.61 ± 0.05*	0.71 ± 0.03*	0.63 ± 0.04*	0.74 ± 0.03	0.77 ± 0.03*	0.64 ± 0.05	0.74 ± 0.04*	0.78 ± 0.04	0.83 ± 0.03*	0.84 ± 0.03*	0.83 ± 0.03*	0.88 ± 0.03	0.94 ± 0.01	0.73 ± 0.03
18 w	DIO	0.78 ± 0.06*	0.87 ± 0.02*	0.71 ± 0.04*	0.80 ± 0.04*	0.86 ± 0.06*	0.75 ± 0.04*	0.88 ± 0.02*	0.89 ± 0.02*	0.70 ± 0.04	0.85 ± 0.02*	0.83 ± 0.02	0.88 ± 0.02	0.87 ± 0.03	0.84 ± 0.02	0.86 ± 0.03	0.94 ± 0.01	0.83 ± 0.02*
	Control	0.61 ± 0.04	0.76 ± 0.04	0.63 ± 0.04	0.71 ± 0.04	0.76 ± 0.04	0.70 ± 0.04	0.79 ± 0.03	0.82 ± 0.03	0.68 ± 0.03	0.79 ± 0.04	0.81 ± 0.03	0.87 ± 0.04	0.85 ± 0.04	0.84 ± 0.03	0.88 ± 0.02	0.94 ± 0.03	0.78 ± 0.03

Three to four-week-old C57BL/6J male mice were fed a HFD using mice fed a SD diet as controls. At 2-, 4-, 8-, 12-, and 18-weeks after feeding, the genomic DNA isolated from epididymal fat was analyzed for the methylation of 16 CpG sites (*a, b, … p*) at the indicated positions spanning nucleotides -321 to -1 relative to the TSS in the leptin gene promoter. The methylation fraction for each CpG site is represented as the mean ± the SD determined from 15 mice in each group *compared with the control group, P<0.05.

-321agccttctgtagcctcttgctccctgcggtgctggaagcaccatcccaagggacccgtccttaaactaccgctgctcagtagctgctggccggacctcgaggattaccggctcataccaagcgcccccaa

a ***b*** ***c*** ***d*** ***e***

acttgcactcgagggcgcggctgaagttctccctcgaggcgcctagaatggagcactaggttgctgctgccactgttgctggcccgctgggtgggggcgggagttggcgctcgcagggactggggctggccg

f ***g*** ***h*** ***I*** ***j*** ***k*** ***l*** **Sp1** ***m*** ***n*** **Lp1** ***o***

gacagttgcgcaagtggcactggggcagttataagaggggcaggcaggcatggagccccg -1 (TSS)

p **C/EBP** **TATA-box**

cularly at gene promoters and regulatory regions, has been shown to recruit MBD regulating gene transcription[17,18]. The binding of RNA Pol II was shown to be increased at 8 w and decreased at 18 w after HFD feeding compared with SD feeding (P<0.05) (Fig. 2B).

Changes in DNMTs associated with the leptin promoter during the development of obesity: DNMTs associated with the leptin promoter underwent alterations during the development of diet-induced obesity. HFD feeding reduced the binding of DNMT1 to the leptin promoter at 4 and 8 w and then increased the binding of DNMT1 to the leptin promoter at 18 w. The binding of DNMT3a and DNMT3b to the leptin promoter was increased at 8, 12 and 18 w after HFD feeding (Fig. 3).

Changes in the expression of DNMTs in adipose tissue during the development of obesity: As shown in figure 4, the changes in mRNA expression of the three types of DNMTs were similar, illustrating significantly reduced expression levels at 4 w and 8 w but increased expression levels at 12 w and 18 w after HFD feeding.

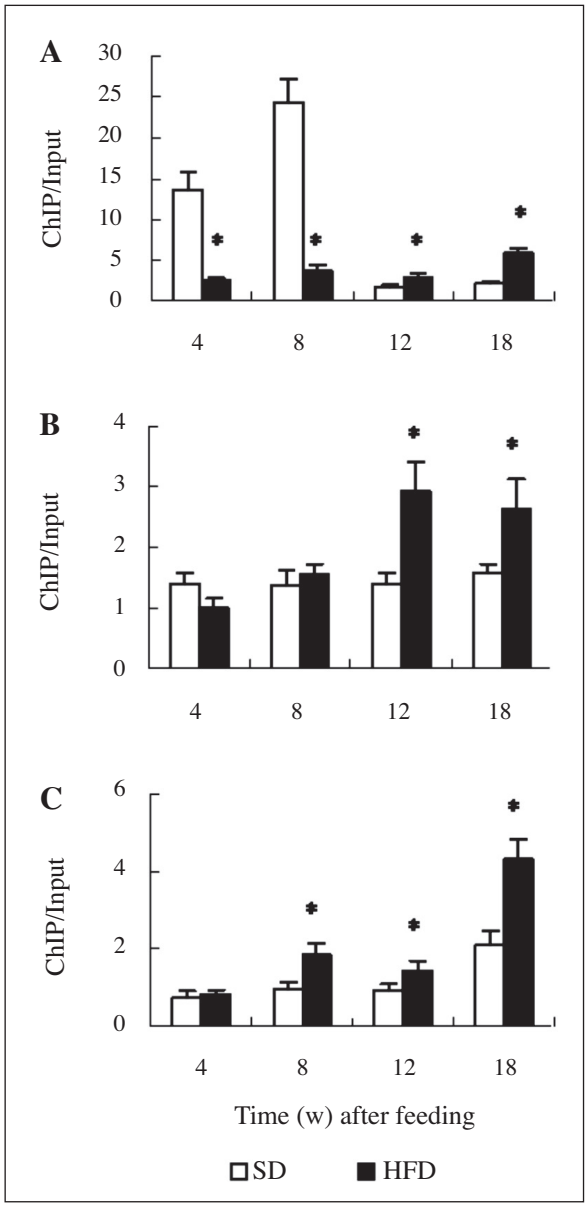

*Fig. 3.—**Changes in expression of the DNMTs binding at the leptin promoter during the development of obesity:** Mice were fed a HFD using mice fed a SD diet as controls, and their epididymal fat was dissected at 2-, 4-, 8-, 12-, and 18-weeks after feeding. Binding of DNMT1 (A), DNMT3a (B) and DNMT3b (C) to the leptin promoter was determined by ChIP-PCR. The ratio of the PCR signal from the protein ChIP DNA to the PCR signal from the total genomic DNA (Input) is plotted as an estimation of the protein levels. The values are expressed as the mean ± SEM (n=15 for each group) *compared with the SD feeding group, P<0.05.*

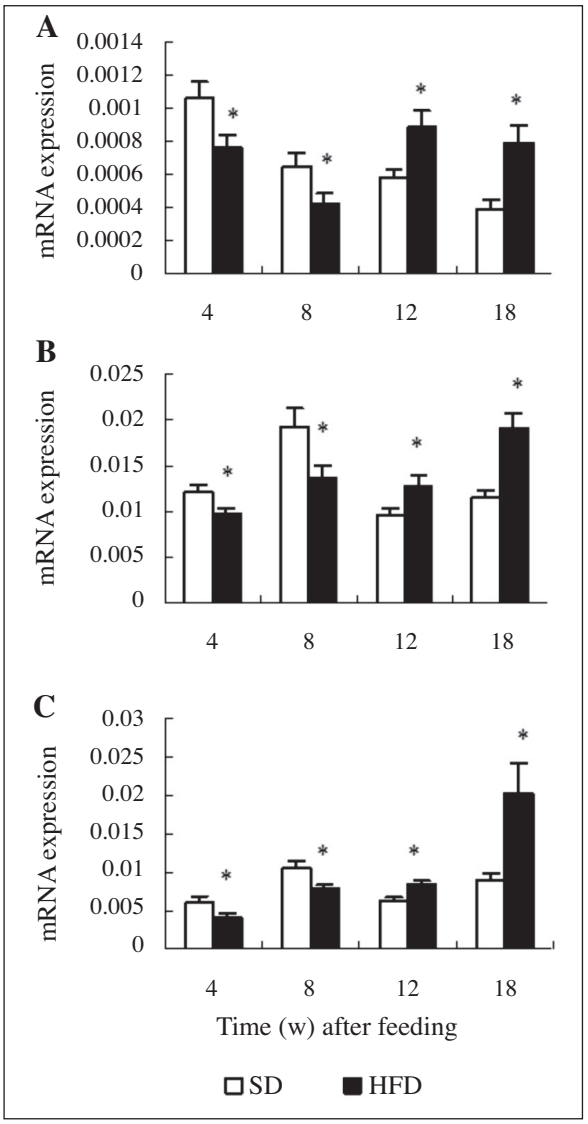

*Fig. 4.—**Changes in the expression of DNMTs in adipose tissue during the development of obesity:** Mice were fed a HFD using mice fed a SD as controls, and their epididymal fat was dissected 2-, 4-, 8-, 12-, and 18-weeks after feeding. The mRNA expression of DNMT1 (A), DNMT3a (B) and DNMT3b (C) was determined by real-time quantitative RT-PCR. The values are expressed as the mean ± SEM; n=15 in each group *compared with the SD feeding group, P<0.05.*

Discussion

We found that leptin mRNA expression levels in epididymal fat were consistently increased at different time points after HFD feeding, reconfirming the high levels of leptin associated with obesity[1,2]. It has been previously shown that epigenetic modifications are responsible for chromatin structure and stability and the modulation of tissue-specific gene expression and that DNA methylation is the only known modification targeting the DNA itself and is usually associated with gene silencing[17,19]. We observed changes in the methylation of the leptin promoter in adipose tissue, indicating that the methylation fraction of the CpG sites was reduced at 8 w, but increased at 12 w and 18 w in obese mice after HFD feeding. CpG methylation at gene promoters has been demonstrated to silence transcription not only by blocking transcription factor binding but also by the recruitment of MBD, which recruits histone deacetylases and histone methyltransferase complexes to DNA causing histone deacetylation and the methylation of specific lysine residues[17,18]. In response, MBD2 promoter binding varied with changes in promoter CpG methylation, showing a reduction at 8 w and an increase at 12 w and 18 w after HFD feeding. The expression of RNA pol II, a key enzyme for initiating gene transcription, was also affected after HFD feeding, showing an increase at 8 w and a decrease at 18 w. These data suggest that in the early stage of obesity, epigenetic changes in the leptin promoter may result in increased leptin mRNA expression to counteract increasing body weight[1,2] and that with the further development of obesity, epigenetic modifications of the promoter may reduce leptin levels by inhibiting leptin transcription. Paradoxically, we found that leptin mRNA expression in adipose tissue was increased, rather than reduced, in DIO mice after 12 w of HFD feeding. To the best of our knowledge, the changes in epigenetic modifications at the leptin promoter may result from feedback due to increased gene expression. These epigenetic modifications are not sufficient to return gene expression to normal levels, and suggest a mechanism to counteract leptin over-expression[7]. The paradox between epigenetic modification and transcription of the leptin gene warrants further exploration.

The enzymes responsible for DNA methylation at CpG nucleotides are members of the DNMT protein family, including DNMT1, DNMT3A and DNMT3B. These enzymes methylate the cytosine residue in the presence of the cofactor SAM (S-Adenosyl methionine), which donates the –CH3 group and is converted to SAH (S-Adenosyl homocysteine)[3,4]. In the current study, after 12 w of HFD feeding to induce obesity, the DNMTs associated with the leptin promoter varied in expression, with a large decrease of DNMT1 initially (4 w and 8 w after HFD feeding) followed by an increase of DNMT3A and DNMT3B at later stages (12 w and 18 w after HFD feeding). This may lead to early hypomethylation and later hypermethylation of promoter CpG sites during the development of HFD induced obesity, and ultimately results in the above mentioned changes in gene transcription.

Regarding global genomic methylation, DNMTs are closely associated with cell proliferation and differentiation during body development. DNMT1 is responsible for maintaining DNA methylation during DNA replication after development due to its high affinity for hemimethylated DNA templates[20]. The de novo DNMT3A and DNMT3B enzymes are responsible for the establishment of DNA methylation patterns during development by affecting the methylation status of normally unmethylated CpG sites[21,22]. DNMT3B is more prevalent in early embryonic stages and is the main enzyme responsible for DNA methylation during implantation, whereas DNMT3A is expressed in later embryonic stages and differentiated cells[3,4]. However, several reports indicate that DNMT1, DNMT3A and DNMT3B can have both "de novo" and maintenance functions "in vivo", cooperating with each other to establish global DNA methylation patterns[23,24]. In this study, the total expression of DNMT1 in epididymal fat gradually decreased with aging, whereas the total expression of DNMT3A and DNMT3B steadily increased, with a peak at 12 w of age (8 w after feeding) after which their expression decreased and remained stable. This may keep pace with adipose tissue development, indicating that subcutaneous adipocytes are committed and differentiated much earlier during embryogenesis than gonadal adipocytes, and the number of adipocytes remains very stable postnatally, whereas gonadal adipocytes undergo differentiation gradually over a relatively long period of time after birth[25]. In vitro, DNA methyltransferase 1 (DNMT1) levels in adipocytes are controlled in part by the enzyme ATP-citrate lyase (ACL) and the silencing of DNMT1 can accelerate adipocyte differentiation[26]. Furthermore, the expression of DNMTs was affected by HFD feeding, showing an early reduction in expression (4 w and 8 w) and an increase in expression after 12 w. These changes corresponded with changes in adipocytes induced by the HFD, showing that a significant number of new adipocytes formed after 8 w and 12 w of HFD feeding[25,27].

In summary, the DNA methylation patterns of the leptin promoter were affected by the development of obesity. The time-specific changes in promoter associated DNMTs observed may be associated with the regulation of leptin expression, suggesting a complex and dynamic underlying epigenetic mechanism for aberrant leptin expression in obesity. The cause of this over-expression of leptin needs to be elucidated in the future.

Conflict of interest

The authors have no conflicts of interest.

Acknowledgments

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