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Postweaning low-calcium diet promotes later-life obesity induced by a high-fat diet

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Abstract

The aim of this study was to investigate the effects of a postweaning low-calcium diet on later obesity and explore the underlying mechanisms. Ninety-six male rats were weaned at 3 weeks of age, fed standard (STD: 0.50% calcium, n=48) and low-calcium (LC: 0.15% calcium, n=48) diets for 3 weeks, and then fed the standard diet for a 3-week washout period successively. Finally, the STD rats were divided into STD control and high-fat diet (HFD) groups, and the LC ones into LC control and LC+HFD (LCHF) groups. The STD and LC rats were fed the standard diet, while the HFD control and LCFD ones were fed a high-fat diet for 6 weeks to induce obesity. During the three feeding periods, adenosine-monophosphate-activated protein kinase (AMPK) and its responsive proteins phospho-acetyl-coA carboxylase, carnitine palmitoyltransferase 1 and uncoupling protein 3 were persistently down-regulated in the LC group (decreased by 18%, 24%, 18% and 20%, respectively) versus the STD group, and these effects were significantly more pronounced in the LCHFD group (decreased by 21%, 30%, 23% and 25%, respectively) than the HFD group by a later high-fat stimuli, causing more fat and body weight in adulthood. However, lipolysis enzymes, serum leptin, insulin and lipids were not significantly affected until the body weight and fat content changed at 15 weeks of age. The results suggest that the low-calcium diet after weaning promotes rat adult-onset obesity induced by high-fat diet, which might be achieved by programming expressions of genes involved in AMPK pathway.

Keywords: Postweaning; Low-calcium diet; Adult obesity; AMPK; Metabolism programming

1. Introduction

Obesity is a major public health problem, which increases the risk of mortality and morbidity from hypertension, dyslipidemia, type 2 diabetes, stroke, coronary heart disease, etc. [1]. It is a multifactorial disease that develops from the interaction between genetic, environmental and psychosocial factors [2]. Nutrition is a critical influential factor of adult obesity. In recent decades, the effect of early nutrition on later obesity has been focused [3]. It has been reported that early nutrition may have potentially important long-term effects on blood lipids, plasma insulin, obesity, etc. [4]. Animal models supported the hypothesis that prenatal and early postnatal nutrition permanently affects later obesity [5]. Human investigation also revealed that breastfeeding might help prevent childhood overweight [6].

Calcium plays an essential and varied role in the body and is vital for health [7]. However, many studies have shown that, in most countries, calcium intake is much lower than the international recommendations [8–10]. Low calcium intake is considered to be a risk factor for some disorders, such as osteoporosis [11], hypertension [12], cancer [13] and insulin resistance [14]. Recently, low calcium intake has been identified as a potential contributing factor to obesity [15,16]. Epidemiologic data suggest that people with low calcium intake have a higher prevalence of overweight or obesity [16]. Some studies have demonstrated that calcium modulated adipocyte metabolism by influencing intracellular calcium levels and fatty acid absorption from the gastrointestinal tract [17,18]. However, most of these studies focused on the effect of calcium intake in adults on lipid metabolism. Very few studies have investigated the role of calcium deficiency in the development of obesity, especially in early life after weaning when mammals exhibit rapid growth and development.

We previously demonstrated that postweaning isocaloric hypersoybean oil versus a hypercarbohydrate diet reduced obesity in adult rats induced by a high-fat diet [19]. Later, we further verified that high dietary intake of medium-chain fatty acids during pregnancy in rats prevented later-life obesity in their offspring [20]. Since the status of early nutrition plays an important role in later health, as one of the most important mineral nutrients, whether calcium deficiency in the early stage of life affects later obesity is not clear. Therefore, in the present study, we investigated the effect of a postweaning low-calcium diet on later obesity and the gene expressions related to obesity to explore the underlying mechanisms.

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2. Materials and methods

2.1. Experimental design and animal care

Timed pregnant Wistar rats (SLACCAS Laboratory Animal Co., Ltd., Shanghai, China) were housed in individual plastic shoebox cages with free access to both food and water and were maintained at a constant-temperature (22°C±3°C) animal room with a 12-h light–dark cycle. The number of male offspring per litter was standardized to seven males within 24 h of birth to minimize variation among litters. Male offspring were weaned on day 21 and housed in wire-mesh hanging cages at an ambient temperature (22°C±3°C) with a 12-h light–dark cycle and free access to water. These offspring were allocated to standard (STD: 0.50% calcium, w/w) (n=48) and low-calcium diet (LC: 0.15% calcium, w/w) (n=48) groups based on purified AIN-93G diet [21] for 3 weeks. The vitamin mix and mineral mix, except for $CaCO_3$, were based on AIN-93M-MX [21]. The rats were then fed a standard AIN-93G diet for a 3-week washout period. At 9 weeks of age, rats from the STD group were divided randomly into two groups, STD and high-fat diet (HFD), and those from LC group were divided into two groups, LC and LC+high-fat diet (LCHFD). The STD and LC groups were fed the standard diet, while the HFD and LCFD groups were fed a high-fat diet for 6 weeks to induce obesity. The ingredients of diets are given in Table 1. The flowchart of the procedure is shown in Fig. 1. Food intake was measured daily, and body weight was measured weekly. The weight-adjusted energy intake was calculated as:

$$\text{kilocalorie intake} \times 100 / \text{body weight}$$

To determine whether postweaning low-calcium diet induced changes in the later life of rats, we chose dynamic observation points 6 (low-calcium diet feeding for 3 weeks after weanling), 9 (near adulthood) and 15 (after 6 weeks of obesity inducing) weeks of age. At each time point, 12 rats from each group were fasted for 12 and then anesthetized using pentobarbital (15–20 mg/kg, intraperitoneal) and sacrificed by exsanguination from the abdominal aorta. The blood samples were centrifuged at 2500 rpm for 15 min to extract serum. Liver, skeletal muscle, and perirenal and epididymal fat pads were dissected from each animal according to defined anatomical landmarks. Tissues were weighed immediately after dissection to avoid evaporative weight loss and then frozen at −80°C for subsequent analysis. Body fat content was calculated as:

$$100\ (\text{perirenal} + \text{epididymal fat pads}) / \text{body weight}$$

Animal care and experimental procedures were approved by the Animal Experimental Committee of Harbin Medical University.

2.2. Measurements of blood lipids, glucose, serum insulin and leptin

Serum contents of triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were assayed by standard enzymatic colorimetric methods with commercial kits (Biosino Biotechnology, Beijing, China) and with an autoanalyzer (Autolab, PM 4000, AMS, Rome, Italy). Blood glucose was measured with blood glucose meter (Johnson & Johnson Company, USA). Serum insulin was detected using a rat insulin radioimmunoassay (RIA) kit (Beijing North Institute of Biological Technology, Beijing, China), and leptin was measured using a commercial rat leptin RIA kit (Linco Research, St. Charles, MO, USA) according to the manufacturer's instructions.

Table 1
Ingredients of diets used for feeding the rats

Ingredients	Amount (g/100 g diet)		
	STD	LC	HFD
Casein	20	20	20
L-Cystine	0.3	0.3	0.3
L-Methionine	0.16	0.16	0.16
Carbohydrates	66.84	64.87	55.84
Fat	7.00[a]	7.00[a]	18.00[b]
Cellulose	1	1	1
Calcium carbonate	1.25	0.375	0.375
Vitamin mix, AIN-93G	1	1	1
Mineral mix, AIN-93G	3.5	3.5	3.5
Choline bitartrate (50% choline)	0.2	0.2	0.2
Sources of energy (%)			
Protein	20	20	17
Carbohydrates	65	65	48
Fat	15	15	35

n=12 per group.
[a] Soybean oil.
[b] Lard (14.0g) plus soybean oil (4.0g).

2.3. Western blot analysis

The protein expressions of hormone sensitive lipase (HSL), adipose triglyceride lipase (ATGL) in white adipose tissue, adenosine-monophosphate-activated protein kinase (AMPK), phospho-AMPKα^{Thr172} (P-AMPK), carnitine palmitoyltransferase 1 (CPT-1), uncoupling protein 3 (UCP3), acetyl-coA carboxylase (ACC) and phospho-ACC^{Ser79} (p-ACC) in skeletal muscle were determined by Western blot. The white adipose tissue or skeletal muscle was washed twice with ice-cold phosphate-buffered solution, crushed in a mortar with liquid nitrogen and then lysed in cold lysis butter [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1 μg/ml leupeptin, 50 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride] for 30 min. The lysates were centrifuged at 12 000 rpm for 20 min at 4°C. Protein concentrations were measured, and equal amounts of protein were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and electrotransferred to polyvinylidene difluoride membranes. The membranes were blocked with 1% bovine serum albumin and probed with primary antibodies against HSL, ATGL (Abcam, Cambridge, UK), AMPKα, P-AMPKα^{Thr172}, ACC, p-ACC^{Ser79} (Cell Signaling Technology, Beverly, MA, USA), CPT-1, UCP3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin (Boster Biotechnology, Wuhan, China) overnight. The membranes were washed three times with TBS-T buffer [150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.4), 0.05% Tween-20] for 10 min, incubated with rabbit IgG antibody for 1 h at 37°C and then washed three times with TBS-T buffer. The blots were detected with alkaline phosphatase. Data were presented as the ratios of target protein to β-actin. Experiments were replicated at least three times, and representative blots were shown.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of fatty acid synthase (FAS) in liver

The messenger RNA (mRNA) levels of FAS in liver were determined by a qRT-PCR method. Total RNA was extracted from frozen liver using a commercially available Trizol reagent (Invitrogen Life technologies, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized using a cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The amplification procedure was as follows. Samples were predenatured at 95°C for 10 min and then subjected to 40 cycles of amplification that consisted of 15 s at 95°C, 30 s at 55°C and 30 s at 72°C. β-Actin was used as the internal control. The primer sequences of the FAS and β-actin were described previously [20]. The reaction was followed by melt curve analysis to verify specificity. The levels of each mRNA were determined with the ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems) using a SYBR Green PCR Master Mix (Applied Biosystems). Samples were analyzed by the $2^{-[\text{delta}][\text{delta}]\text{Ct}}$ method according to the methods of Livak et al. [22].

2.5. Statistical analysis

Statistical analyses were performed using SPSS 13.0 statistical program (version 13.01S; Beijing Stats Data Mining Co. Ltd.). Data were expressed as mean±S.D. One-way analysis of variance was used to analyze continuous variables, and P values less than .05 were considered statistically significant.

3. Results

3.1. Body weight, fat content and adjusted energy intake

There was an increasing tendency for the fat content in the LC group than the STD control at 6, 9 and 15 weeks of age. When the rats were fed a high-fat diet, the body weight and fat content were increased significantly in the high-fat diet (HFD and LCHFD) groups compared with the STD group at 15 weeks of age. In particular, the body weight and fat mass were significantly in the LCHFD group higher than the HFD group at 15 weeks of age. Body weight and body fat content are given in Table 2. There were no significant differences in adjusted energy intake between the LC and STD groups (Fig. 2A, B), or the LCHFD and HFD groups (Fig. 2B).

3.2. Levels of blood lipids, glucose, serum insulin and leptin

There were no significant differences in levels of blood TG, TC, HDL-C, LDL-C, insulin, glucose or leptin between the LC and STD groups at 6, 9 and 15 weeks of age. At 15 weeks of age, the levels of TG, TC, LDL-C and leptin were significantly higher but HDL-C was lower in the high-fat diet (HFD and LCHFD) groups than the STD group. Compared with the HFD group, the LCHFD group had higher levels of

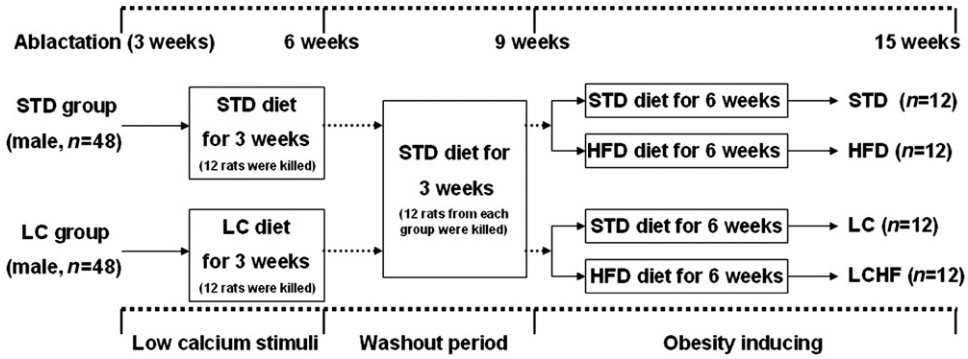

Fig. 1. Flowchart of the study design.

serum TG, TC, LDL-C and leptin but lower level of HDL-C. The blood parameters are given in Table 2.

3.3. HSL and ATGL protein levels in white adipose tissue

HSL and ATGL are two key enzymes of lipolysis in rat white adipose tissue, which play key roles in the development of obesity. In the present study, both expressions of HSL and ATGL tended to be decreased in the LC group than in the STD group at 6, 9 and 15 weeks of age. After 6 weeks of high-fat diet feeding, the expressions of HSL and ATGL were significantly increased in the HFD group than the STD group. Compared with the HFD group, rats in the LCHFD group had significantly lower expressions of HSL and ATGL at 15 weeks of age (Fig. 3A, B).

Table 2
Average body weight, body fat content, blood parameters

Parameter	STD	LC	HFD	LCFA
Body weight, g				
3 weeks	50.98±3.67	51.07±4.59		
6 weeks	144.40±3.11	148.10±9.33		
9 weeks	197.58±34.58	204.83±30.93		
15 weeks	323.16±18.59	326.77±26.38	336.06±15.81*	349.85±16.55*,**
Body fat content, 100 (perirenal+epididymal fat pads) g/(body weight) g				
6 weeks	1.10±0.23	1.27±0.27		
9 weeks	2.20±0.37	2.36±0.34		
15 weeks	4.39±0.28	4.46±0.40	4.64±0.26*	5.85±0.38*,**
Serum TG , mmol/L				
6 weeks	0.66±0.12	0.62±0.32		
9 weeks	0.64±0.16	0.59±0.27		
15 weeks	0.72±0.17	0.68±0.18	1.21±0.27*	1.42±0.22*,**
TC, mmol/L				
6 weeks	1.28±0.18	1.31±0.56		
9 weeks	1.33±0.19	1.39±0.21		
15 weeks	1.44±0.28	1.47±0.36	2.51±0.29*	2.98±0.22*,**
Serum HDL-C, mmol/L				
6 weeks	0.77±0.34	0.73±0.43		
9 weeks	0.84±0.22	0.86±0.23		
15 weeks	0.86±0.26	0.82±0.22	0.75±0.15*	0.62±0.11*,**
Serum LDL-C, mmol/L				
6 weeks	0.41±0.16	0.48±0.18		
9 weeks	0.39±0.16	0.42±0.15		
15 weeks	0.58±0.19	0.60±0.16	0.82±0.12*	0.98±0.17*,**
Fasting blood glucose, mmol/L				
6 weeks	3.53±0.64	3.85±0.42		
9 weeks	4.26±0.52	4.33±0.58		
15 weeks	4.12±0.22	3.98±0.24	4.20±0.19	4.24±0.24
Fasting serum insulin, μIU/ml				
6 weeks	13.62±7.09	15.59±6.14		
9 weeks	6.22±1.56	5.54±1.91		
15 weeks	8.15±3.97	9.77±4.09	7.33±3.43	7.89±3.92
Fasting serum leptin, ng/ml				
6 weeks	0.24±0.18	0.25±0.16		
9 weeks	0.53±0.15	0.64±0.39		
15 weeks	4.54±1.75	4.86±1.37	6.36±1.22*	7.87±1.27*,**

n=12 per group; data are mean±S.D.
* P<.05 vs. STD group.
** P<.05 vs. HFD group.

3.4. FAS mRNA level in liver

FAS is the key enzyme in *de novo* lipogenesis, catalyzing the reactions for the synthesis of long-chain fatty acids [23], which is related to serum TG level and obesity closely. Our data showed that FAS mRNA levels in the LC group had an increasing tendency but were not significant at 6, 9 and 15 weeks of age. Compared with the STD group, rats in the high-fat diet (HFD and LCHFD) groups had higher

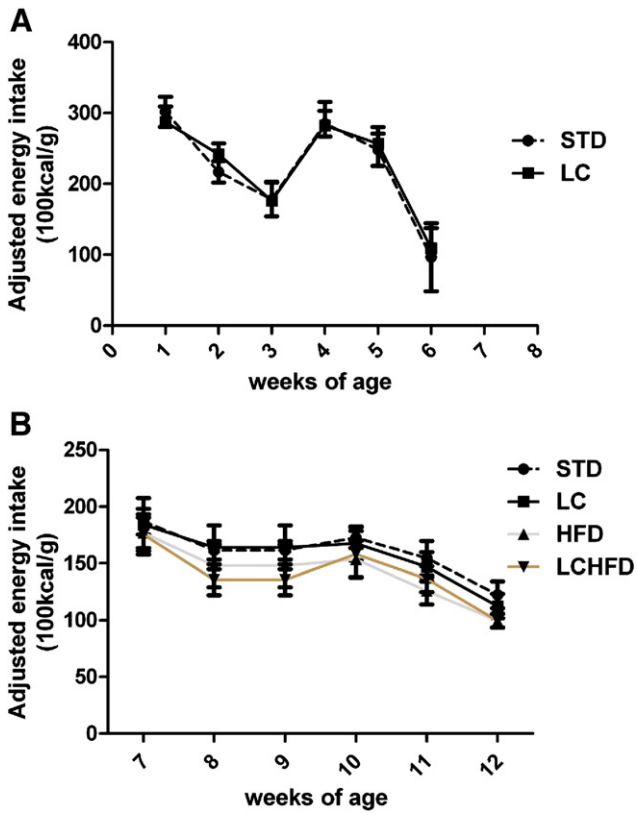

Fig. 2. The adjusted energy intake of postweaning low-calcium diet-fed rats. (A) Adjusted energy intake of rats fed with a low-calcium diet for 3 weeks and with successive standard diet for another 3 weeks. (B) Adjusted energy intake of rats fed with high-fat diet for 6 weeks. Values are means±S.D. No significant difference in adjusted energy intake was observed between the STD and LC groups, or between the LCHFD and HFD groups.

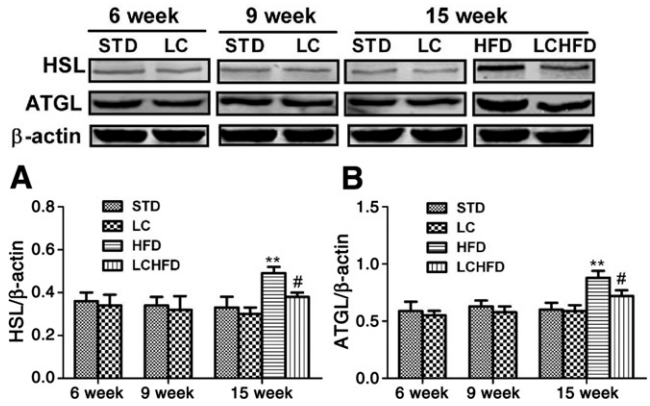

Fig. 3. Effects of postweaning low-calcium diet on expressions of HSL and ATGL in white adipose tissue. Values are means±S.D., n=12. The experiment was performed in triplicate. **P<.01 vs. the STD group. $^{\#}P$<.01 vs. the HFD group at 15 weeks of age.

FAS mRNA level at 15 weeks of age. However, there were no significant differences in FAS mRNA levels between the LCHFD and HFD groups at 15 weeks of age (Fig. 4.).

3.5. ACC, CPT1 and UCP3 expressions in skeletal muscle

ACC plays an important role in energy balance by controlling malonyl-CoA synthesis. ACC activity was inactivated by phosphorylation of the protein. Our data showed that the expressions of p-ACC in the LC group were significantly decreased by 18%, 24% and 20% at 6, 9 and 15 weeks of age compared with the STD group, respectively. The expression of p-ACC in LCHFD group was significantly decreased by 30% than that in the HFD group at 15 weeks of age (Fig. 5). In skeletal muscle, ACC is thought to be a physiological regulator of mitochondrial CPT1, the rate-limiting enzyme in FFA oxidation [24]. Therefore, we further detected the expression of CPT1 in skeletal muscle. UCP3 is another protein that affects skeletal muscle capacity for fatty acid transport and oxidation. In the present study, CPT1 expressions in the LC group were observed to be persistently decreased significantly by 18%, 15% and 14% than those in the STD group at 6, 9 and 15 weeks of age, respectively. Likely, the low-calcium diet caused 20%, 14% and 19% increases in UCP3 expressions than the STD group at 6, 9 and 15 weeks of age, respectively. When exposed to the high-fat diet, rats in the LCHFD group showed significantly lower expressions of CPT1 and UCP3 by 23% and 25%, respectively, compared with the HFD group at 15 weeks of age (Fig. 6A, B).

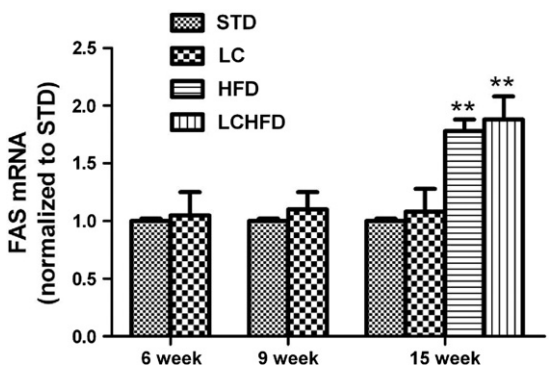

Fig. 4. Effects of postweaning low-calcium diet on mRNA level of FAS in liver. Values are means±S.D., n=12. The experiment was performed in triplicate. **P<.01 vs. the STD group.

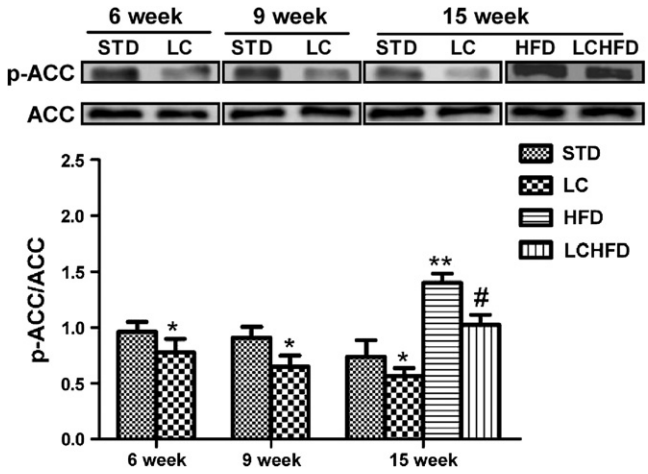

Fig. 5. Effects of postweaning low-calcium diet on expressions of p-ACC and ACC in skeletal muscle. Values are means±S.D., n=12. The experiment was performed in triplicate. *P<.05 vs. the STD group. **P<.01 vs. the STD group. $^{\#}P$<.01 vs. the HFD group at 15 weeks of age.

3.6. Expression and activity of AMPK in skeletal muscle

AMPK is a metabolic gauge regulating whole-body energy homeostasis. In skeletal muscle, AMPK phosphorylates and thus inactivates ACC, and then regulates CPT1. The data showed that the postweaning low-calcium diet persistently down-regulated the expression of AMPK in the LC group by 14%, 18% and 15% compared with the STD group at 6, 9 and 15 weeks of age, respectively. The effect was decreased by 21% in the LCHFD group versus the HFD group at 15 weeks of age. More importantly, this down-regulated expression of AMPK was accompanied by significantly decreased AMPK activity (the quotient of P-AMPK/AMPK) (Fig. 7).

4. Discussion

The notion that nutrition in early life can alter organ function, and thereby predispose or program individuals to adult disease, has been of great interest to researchers. The majority of the studies focused on the effect of sugar or protein restriction in early life on later life obesity. It has been reported that calcium intake can lead to a reduction of body weight and body fat in both animal and human studies [16]. On the contrary, low calcium intake has been considered

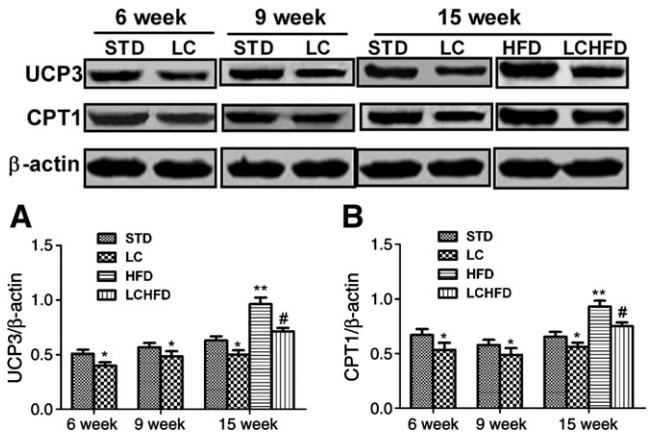

Fig. 6. Effects of postweaning low-calcium diet on expressions of UCP3 and CPT1 in skeletal muscle. Values are means±S.D., n=12. The experiment was performed in triplicate. *P<.05 vs. the STD group. **P<.01 vs. the STD group. $^{\#}P$<.01 vs. the HFD group at 15 weeks of age.

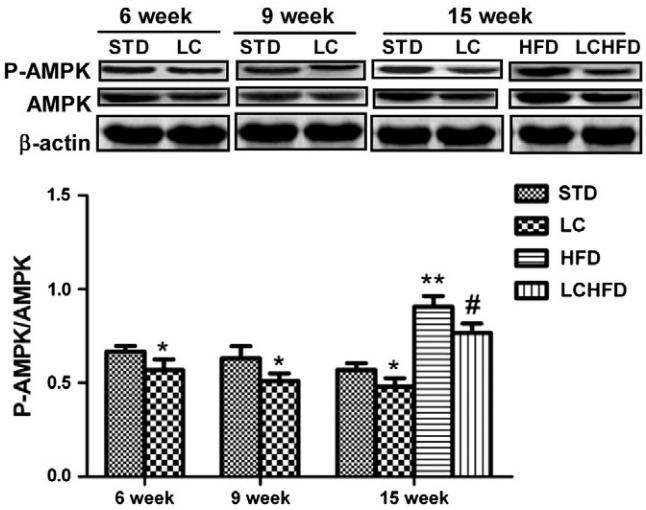

Fig. 7. Effects of postweaning low-calcium diet on expressions of p-AMPK and AMPK in skeletal muscle. Values are means±S.D., $n=12$. The experiment was performed in triplicate. *$P<.05$ vs. the STD group. **$P<.01$ vs. the STD group. #$P<.01$ vs. the HFD group at 15 weeks of age.

a risk factor for development of obesity. There are increasing studies on effect of maternal nutrient intake on later health in animals [5]. However, very few researches have paid attention to the effect of low-calcium diet during early life, especially after weaning when animals experience a transitional stage from breastfeeding to formula and exhibit a rapid growth and development.

In the present study, we found that an early-life low-calcium diet caused increasing tendencies for fat mass and body weight and became significant when rats were exposed to a high-fat diet in their later life. Excess energy intake is a main factor contributing to fat stores or obesity [2]. To avoid the interference of the confounding factor, energy intake was adjusted with the body weight of postweaning rats. During the whole feeding period, there were no significant differences in adjusted energy intake among groups. So the differences in fat store and body weight in adulthood can be explained by the difference of calcium intake in early life. Correspondingly, the levels of serum lipids were also not significantly different between the HFD and LCHFD groups until the rats were given the high-fat stimuli in adulthood. This result was inconsistent with previous studies that showed that calcium intake caused beneficial effect on lipid and lipoprotein profiles [18,25], but consistent with recent study failing to find a relationship between calcium intake and serum lipid level [26]. In a word, the rats that had ever undergone a postweaning low-calcium diet were prone to suffering a disturbance of lipid metabolism and exhibiting obesity when exposed to a high-fat stimuli in adulthood, which reminded us that it was likely that poor calcium intake in early life installed a switch which could be turned on by later-life stimulus.

To further explore the underlying mechanism by which low calcium intake in early life affected adult obesity, we determined the regulating factors responsible for the obesity occurrence. The lipolytic reaction in adipose tissue is one of the most important reactions in the management of bodily energy reserves. A reduced lipolytic activity may lead to the accumulation of adipose tissue stores and may contribute to the symptoms of obesity. ATGL and HSL are two major enzymes in adipose tissue TG catabolism. ATGL catalyzes the initial step in TG lipolysis [27], while HSL degrades diglyceride to monoacylglycerol and free fatty acid (FFA) [28]. It has been shown that the protein expressions of lipolytic enzymes are major determinants of lipolytic capacity in fat cells [29]. Hence, we firstly detected expressions of these two enzymes. The results showed that the postweaning low-calcium diet did not significantly change the expressions of HSL and ATGL in adipose tissue. However, these two enzymes were up-regulated by a later high-fat intervention. Most importantly, we observed that the rats who ever suffered a postweaning low-calcium diet showed significantly lower expressions of HSL and ATGL compared with those fed a normal-calcium diet in early life. These findings suggested that the low calcium intake in early life made rats susceptible to obesity by high-fat induction in adulthood, resulting into significantly changes in body weight, fat mass and lipid profiles.

Liver is a major organ for *de novo* lipogenesis [30], and FAS in liver is responsible for saturated fatty acid synthesis. FAS is regulated primarily at the level of transcription and is sensitive to nutritional and hormonal regulation [23]. Down-regulation of FAS leads to the prevention of obesity and decreased level of TG in plasma [31,32]. Therefore, we wanted to know whether FAS in liver was affected by the postweaning low calcium like HSL and ATGL described above. The data showed that the early-life low-calcium diet intake did not significantly change the gene expression FAS in liver before exposure to the high-fat diet. Though the high-fat diet up-regulated mRNA level of FAS, there were not yet significant differences in FAS mRNA levels between the HFD and LCHFD groups at 15 weeks of age, suggesting that the postweaning low-calcium diet caused changes in body weight and fat accumulation, which might not be related to the fatty acid anabolism in liver.

It is well known that obesity is associated with leptin that acts on receptors in the central nervous system to inhibit food intake and promote energy expenditure [33]. Obesity in human and rodents is almost always associated with leptin deficiency or resistance [34]. We observed significantly increased body weight and fat mass accompanied by higher leptin level in the LCHFD group than the HFD group. The findings were in agreement with those studies in which a strong correlation between serum leptin level and body weight was found [35–37]. However, the serum leptin levels were not affected significantly by low calcium in early life until the rat body weight and fat mass changed in adulthood. We speculated that this change in leptin might be a reflection of body weight and that leptin might not be involved in the programming by low calcium intake in early life.

Insulin is another hormone that is closely related to lipid metabolism. Insulin inhibits lipolysis by activation of phosphodiesterase3B pathway resulting in the reduction of cAMP levels and thus protein kinase A activity [38]. That fetal hyperinsulinemia may promote the development of excess adipose tissue mass has been reported [39]. In the present study, the insulin levels were not significantly changed during the whole experimental period. So it can be speculated that the changes in fat accumulation and lipid metabolism were not related to insulin regulation in later life.

Obesity is an energy balance problem and occurs when energy intake exceeds energy expenditure. As described above, the adjusted energy intake among groups were similar before and after feeding the rats the high-fat diet. Thus, we speculated that alterations in energy expenditure would be responsible for the fat store and disturbance of lipid profile. CPT1 is considered a key regulatory enzyme in FFA oxidation that produces energy in skeletal muscle. It catalyzes the formation of long-chain acylcarnitine, thus committing FFAs to β-oxidation in the mitochondria. Increased CPT1 expression can decrease the intracellular concentration of TG, fat content and body weight [40]. UCP3 expressed mainly in skeletal muscle is another protein that regulates fuel metabolism. It has been reported that UCP3 was a mediator of adaptive thermogenesis in humans [41] and correlated negatively with body mass index [42]. Hence, we next evaluated whether the postweaning low-calcium diet influenced the expressions of CPT1 and UCP3 in skeletal muscle. As a result, our data showed that these two protein expressions were persistently decreased by feeding the rats a postweaning low-calcium diet.

These changes were still displayed when the low-calcium intervention disappeared and were significantly more pronounced after high-fat diet exposure for 6 weeks.

In skeletal muscle, AMPK is phosphorylated and then inactivates ACC [43], which catalyzes the carboxylation of cytosolic acetyl-CoA to form malonyl-CoA. The inactivation of ACC causes a reduction of malonyl-CoA, which is an allosteric inhibitor of carnitine CPT1, the rate-limiting enzyme of FFA oxidation [24]. Activation of AMPK achieved by phosphorylation also up-regulates the expressions of both CPT1 and UCP3 [44,45]. Hence, we determined the activities and expressions of AMPK and ACC in the skeletal muscle. Results from this study showed that the postweaning low-calcium diet significantly down-regulated the expressions of p-AMPK and p-ACC, which still persisted even in the absence of low-calcium stimuli. These changes were exaggerated after exposure to the high-fat diet. AMPK regulates metabolism in response to energy demand and supply. AMPK can be activated by calcium-mediated signaling and is responsible for phosphorylating a wide variety of substrates [46]. Based on the above findings, it seemed that it was the high-fat diet that triggered the "energy switch" placed by low calcium intake in early life, subsequently affecting the downstream functional proteins involved in energy expenditure. Therefore, we speculated that the postweaning low-calcium diet promoted the occurrence of obesity possibly through programming the expression and activity of AMPK and then the AMPK-responsive proteins of ACC, CPT1 and UCP3.

From the above results, we recognized that one thing that genes involved in energy expenditure including AMPK, ACC, CPT1 and UCP3 had in common is that their expressions were persistently repressed by the postweaning low calcium in early life even when the low-calcium stimuli was withdrawn. Moreover, the magnitude of gene repression was amplified upon being exposed to the high-fat diet in later life. A similar phenomenon was often observed in DNA methylation, which is susceptible to occurrence in response to environmental stimuli such as diet or toxins [47] and can lead to stable long-term repression of gene expression. Transient nutritional stimuli at specific ontogenic stages may have long-lasting influences on gene expression by influencing DNA methylation patterns, even in the absence of initial stimuli [47]. Thus, we conjecture that the genes involved in AMPK pathway might be methylated under a low-calcium condition. Of course, further studies are needed to validate this hypothesis.

In the present study, the postweaning low-calcium diet caused persistent down-regulation of enzymes involved in AMPK pathway in rats. These rats were prone to obesity and were more likely to suffer a disturbance of lipid metabolism when exposed to a high-fat diet in later life. A possible explanation is that postweaning low calcium might alter epigenetic regulation of those genes which were programmed to be triggered when rats were exposed to the high-fat diet.

In conclusion, our findings revealed that the low-calcium diet in postweaning rats promoted later life obesity, which might be achieved by programming the gene expressions of AMPK pathways involved in fatty acid metabolism.

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