Basic Science

S100A16 mediation of weight gain attenuation induced by dietary calcium

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ABSTRACT

Dietary calcium influences the regulation of energy metabolism, and weight gain is attenuated by a high-calcium diet. S100A16 is a novel calcium-binding signaling protein of the EF-hand superfamily that promotes adipogenesis. This study aimed to investigate the effect of S100A16 on weight gain attenuation with a calcium-rich diet. An obese rat model was produced after feeding with a high-fat diet. Animals were randomly divided into 4 groups according to the diet provided over 8 weeks: normal diet group; high-fat, normal-calcium diet group; high-fat, high-calcium diet (HH) group; and high-fat, low-calcium diet group. Serum biochemistry was analyzed, and body weight and visceral fat pads were measured. Expression of S100A16 was assayed by Western blotting. Adipogenesis was detected by oil red O staining. Increases in body weight and visceral fat weight were attenuated in the HH group. High-calcium diets decreased the concentrations of serum total cholesterol and triglyceride. Expression of S100A16 decreased in the HH group. Using the 3T3-L1 preadipocyte model, it was observed that elevation of intracellular Ca2+ via calcium ionophores led to the exclusion of S100A16 from the nucleus. Overexpression of S100A16 in 3T3-L1 preadipocytes enhanced adipogenesis, although a significant reduction in Akt phosphorylation was also detected. High-calcium diets were associated with a significant reduction in body weight gain. High-calcium diets may lead to nuclear exclusion of S100A16, which results in the inhibition of adipogenesis and enhanced insulin sensitivity.

1. Introduction

S100 proteins are small acidic proteins (molecular mass, 10-13 kd) characterized by distinctive homo- or heterodimeric architecture and 2 highly conserved Ca2+-binding domains: a classic C-terminal EF-hand with a canonical Ca2+-binding loop and an S100-specific N-terminal EF-hand. Owing to their elevated expression in a broad range of tumor tissues, S100 proteins are considered to be biomarkers for cancer. Furthermore, altered expression levels of S100 proteins have been...
S100A16, a novel member of the S100 family, is ubiquitously expressed, although the tissue-specific physiological functions of S100A16 are still largely undefined [3,4].

Dietary calcium plays a pivotal role in the regulation of energy metabolism. High-calcium diets attenuate adipocyte lipid accretion and weight gain during periods of overconsumption of an energy-dense diet and increase lipolysis, thereby accelerating weight loss. However, the mechanism by which this occurs is unclear [5]. It has been reported that calcium attenuates the direct effects of vitamin D on adipocyte lipid metabolism, which accounts for at least part of the "antiobesity" effects of dietary calcium [6,7]. Alterations in metabolic efficiency and increased core temperature and uncoupling protein-2 expression, in addition to decreased efficiency of energy utilization, have also been attributed to the effects of calcium. This study focused on the expression and functional roles of S100A16 in an obese rat model fed with a high-fat, high-calcium diet.

2. Materials and methods

2.1. Animals and experimental protocol

Forty healthy male specific pathogen-free Sprague-Dawley rats, aged 8 weeks, were purchased from Nanjing Qingzilan Technology (Nanjing, China). The diet was also supplied by this company. Animals were acclimatized for 1 week before the experiment. Animals were housed at 23°C ± 1°C with 12-hour/12-hour light/dark cycles and 45% ± 5% humidity and allowed free access to normal chow diet and water. The rats were cared for in accordance with the institution guidelines. Animals were then randomly divided into 4 groups (n = 10) according to the experimental diet that was fed for 8 weeks as follows: normal diet (N); high-fat, normal-calcium diet (HN); high-fat, high-calcium diet (HH); and high-fat, low-calcium diet (HL). The compositions of the diets are shown in Table 1.

The experimental protocol has been previously described [8]. Body weight was measured weekly for 8 weeks, after which the animals were killed by CO2 overdose. Blood was collected from the abdominal vena cava and centrifuged at 1000g for 15 minutes at 4°C. Serum biochemistry (high-density lipoprotein [HDL], triglyceride [TG], glucose [GLU], total cholesterol [TC]) was analyzed using an automatic chemistry analyzer (Olympus Japan). The weight of the visceral fat pads was recorded. All procedures performed were approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University.

2.2. Cell culture and treatments

3T3-L1 preadipocytes were cultured in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37°C (5% CO2). Adipocyte differentiation was induced as previously described [9]. Briefly, 3T3-L1 cells were seeded and allowed to grow to confluence over 2 days (designated as d0) before being cultured in DMEM containing 10% FBS, 0.5 mmol/L 3-isobutyl-1-methylxanthine, 1 μg/mL porcine insulin, and 1 mmol/L dexamethasone (Sigma, St Louis, MO). After incubation for 48 hours (d 2), the medium was replaced with DMEM containing 10% FBS and 1 μg/mL insulin. On d 4, the culture
medium was replaced with DMEM containing 10% FBS; and the incubation was continued for 4 days with 2 media changes.

### 2.3. Construction of S100A16 overexpression plasmids and stable clone selection

Overexpression plasmid pcDNA3.1A(-)-myc-S100A16 was constructed. Full-length mouse S100A16 cDNA was amplified by reverse transcriptase polymerase chain reaction from mRNA isolated from mouse white adipose tissue using the following primers: F: 5′-GGTGAAT-TCATGGCTGAGTCTATACAG-3′; R: 5′-GCTGTCGACCGGATCCGTAGCTGCTGCCCTGCT-3′. The polymerase chain reaction products were then subcloned into the pcDNA3.1A(-)-myc expression vector. The constructs were transfected into 3T3-L1 preadipocytes using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA) according to the instructions of the manufacturer. Selection medium containing 400 to 800 μg/mL G418 (Sigma) was added after 48 hours and replaced every 2 days. Cultures were maintained for 1 week in medium containing 800 μg/mL G418. Resistant colonies were maintained for further analysis in culture medium supplemented with 200 μg/mL G418.

### 2.4. Oil red O staining

Oil red O staining was carried out according to a previously published protocol [10]. Cells were washed 3 times with phosphate-buffered saline (PBS) and fixed with 10% formalin for 5 minutes at room temperature. After fixation, cells were washed twice with PBS and stained with filtered oil red O solution (stock solution, 3 mg/mL in isopropanol; working solution, 60% of the stock solution and 40% distilled water) for 20 minutes at room temperature. The cells were then washed with distilled water to remove unbound dye, visualized, and photographed using light microscopy (×200 magnification).

### 2.5. Protein extraction and Western blotting analysis

The adipose tissue was removed from around the testes or ovaries, washed twice with ice-cold PBS, and treated with lysis buffer (50 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, 1% [vol/vol] Nonidet-P40, 1 mmol/L EDTA, 1 mmol/L NaF, 10 μg/mL aprotinin, 10 μmol/L leupeptin, and 1 mmol/L phenyl-methanesulfonyl fluoride). Each tissue sample was then homogenized and centrifuged at 4°C, and the protein content was then supernatant.

Equal amounts of protein (60 μg) were electrophoresed on 12% sodium dodecyl sulfate polyacrylamide electrophoresis gels and electrophoretically transferred to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were probed overnight at 4°C with antibodies to S100A16 (1:1,000, #11456-AP; Proteintech Group), p-AKT (Ser473; #9271), total-AKT (Cell Signaling Technology), and monoclonal antitubulin (1:5,000) in TBST containing 1% bovine serum albumin (wt/vol). The blots were then incubated for 2 hours with antirabbit or antimouse secondary antibodies. The immune complexes were detected using an ECL plus detection kit (Pierce, Rockford, IL) and analyzed using a scanning densitometer with molecular analysis software (Bio-Rad, Hercules, CA).

### 2.6. Statistical analysis

The data were analyzed by 2-tailed Student t test for single comparisons and by one-way analyses of variance with a correction for multiple comparisons. P < .05 was considered to be statistically significant.

### 3. Results

#### 3.1. High-calcium diets attenuated weight gain and visceral adipocyte lipid accretion

The body weights of experimental animals were recorded at weekly intervals during the course of the experiment (Fig. 1A). Animals were also weighed at 16 weeks of age (8 weeks after the study period). The N group weighed 423.15 ± 31.34 g, the HN group weighed 543.27 ± 32.29 g, and the HH group weighed 426.17 ± 35.18 g, and the HL group weighed 625.12 ± 29.48 g (P < .05). During the 8-week study period, the following body weight gains were recorded: N group, 358 g; HN group, 474 g; HH group, 410 g; and HL group, 559 g. At the end of the study, animals in the HL group weighed 36% more than those in the HH group. The weight of the visceral fat pads in the HL group consistently exceeded that of the HH group (Fig. 1B, P < .05).

#### 3.2. High-calcium diets decreased the concentration of serum TC and TG

No differences were recorded in the mean concentrations of GLU and HDL among the 4 groups (N, HN, HH, and HL) at the end of the study (Fig. 2A, B). However, the serum TC and TG levels in the HH group were significantly lower than those of the HL group (1.85 ± 0.37 vs 2.83 ± 0.36 μmol/L [TC], 0.63 ± 0.06 vs 1.04 ± 0.09 μmol/L [TG], P < .05) after 8 weeks (Fig. 2C, D). The mean serum TC and TG levels of the HN and HL groups were all higher than those of the N group. Furthermore, the levels in the HN group increased those in the other groups after 8 weeks.

#### 3.3. High-calcium diets decreased S100A16 expression in fat tissues, and overexpression of S100A16 in 3T3-L1 preadipocytes enhanced adipogenesis

Western blot analysis showed that S100A16 expression was downregulated in the HH group compared with the other 3 groups (Fig. 3A, B). To investigate the effect of S100A16 on adipogenesis, S100A16 overexpression plasmids were constructed; and a stably transfected clone of 3T3-L1 cell was selected (Fig. 3C). Differentiation was induced in the presence of methylisobutylxanthine. Following oil red O staining, a greater density of lipid droplets was observed in S100A16 overexpressing 3T3-L1 cells compared with control cells (Fig. 3C).

#### 3.4. Intracellular Ca2+ led to S100A16 nuclear exclusion, and S100A16 negatively influenced insulin activity by downregulation of Akt phosphorylation

The effect of Ca2+ on the location of the Ca2+-binding protein S100A16 was investigated to determine the role of S100A16
in adipogenesis. 3T3-L1 cells were treated with ionomycin (a Ca\(^{2+}\) ionophore supplemented with extracellular Ca\(^{2+}\)) or the Ca\(^{2+}\) antagonist, ethylene glycol tetraacetic acid (EGTA), for different periods of time. Western blot analysis of nuclear extracts of 3T3-L1 cells suggested that a high intracellular Ca\(^{2+}\) level induced S100A16 nucleolar exit, whereas lowering intracellular Ca\(^{2+}\) concentration led to S100A16 nuclear translocation (Fig. 4A).

To further confirm the cell signaling pathway, insulin-stimulated signaling, particularly Akt phosphorylation, was

![Graph showing body weight and visceral fat weight changes](image1)

**Fig. 1** – Body weight and visceral fat weight changes in experimental groups. Sprague-Dawley rats (aged 8 weeks) were fed with N, HN, HH, or HL for 8 weeks. The body weight (A) and the visceral fat pad weight (B) were measured. Results are expressed as the mean ± standard deviation (*P ≤ .05).

![Graph showing GLU, HDL, TC, and TG concentrations](image2)

**Fig. 2** – High-calcium diets decreased the concentrations of serum TC and TG. The serum concentrations of GLU (A), HDL (B), TC (C), and TG (D) were assayed at the end of the study using an automatic chemistry analyzer (Olympus Japan). 1, N; 2, HN; 3, HH; 4, HL. Results are expressed as the mean ± SD (n = 10, *P ≤ .05).
evaluated. Treatment of 3T3-L1 adipocytes with insulin induced a dose-dependent increase in Akt phosphorylation. However, this increase was significantly attenuated in cells that overexpressed S100A16 (Fig. 4B, C). In combination, these data suggest that insulin activity in 3T3-L1 adipocytes is negatively influenced by increased expression of S100A16.

4. Discussion

Dietary calcium plays an important role in the maintenance of skeletal integrity, blood pressure regulation, and modulation of chronic disease risk [11,12]. Reports have indicated that dairy-rich diets reduce the risk of hypertension and cardiovascular disease and, furthermore, are implicated in the prevention and treatment of obesity. It has been reported that increasing dietary calcium from approximately 400 to 1000 mg/d for 1 year resulted in a 4.9 kg reduction in body fat [5]. This observation is in accordance with the results of this study, which suggest that dietary calcium plays a pivotal role in the regulation of energy metabolism. Specifically, a low-calcium diet was associated with accelerated weight gain and fat accretion, whereas high-calcium diets result in the distinct suppression of both fat accretion and weight gain in animals maintained with identical caloric intakes (Figs. 1 and 2). However, the molecular mechanisms responsible for these phenomena are unknown. Small calcium binding mitochondrial carriers [13], uncoupling protein [14], and reactive oxygen species [15] have been reported to regulate the dietary calcium–induced inhibition of adipocyte lipid accretion.

Previous studies have reported that S100A16 is highly expressed in adipocytes. S100A16, a member of the S100 protein family, is considered as to be a marker for cancer [16]. It is ubiquitously expressed and highly conserved in mammals [17]. In this study, it was observed that S100A16 was downregulated in the adipose tissue of animals fed with an HH (Fig. 3A). To further study the effect of S100A16 on adipogenesis, S10016A was overexpressed in the mouse adipocyte model and 3T3-L1 cells. Cell differentiation into adipocytes was induced in the presence of methylisobutylxanthine. Enhanced adipogenesis and differentiation were clearly observed in 3T3-L1 preadipocytes that overexpressed S100A16 (Fig. 3D). However, the mechanism of this effect remains unclear.

The relocation of S100 proteins within cells has been reported in response to [Ca^{2+}] oscillation [3]. Therefore, Ca^{2+}-
dependent translocation of S100A16 was investigated in 3T3-L1 cells exposed to either ionomycin, a Ca\(^{2+}\) ionophore supplemented with extracellular Ca\(^{2+}\), or EGTA, a Ca\(^{2+}\) antagonist. It was observed that S100A16 was excluded from the nucleus following elevation of intracellular Ca\(^{2+}\). Furthermore, overexpression of S100A16 in 3T3-L1 adipocytes was associated with significant reduction in insulin signaling (Fig. 4B). Three members of the S100 protein family, S100B, S100A2, and S100A4, are known to interact directly with a number of cellular proteins, particularly the tumor suppressor protein p53 [18-21], which is also known to be a negative regulator of adipogenesis and a positive regulator of insulin sensitivity [22-24]. Preliminary evidence revealed interaction of S100A16 with p53 in immunoprecipitation assays [25].

In this study, we considered that vitamin D is also important for efficient calcium absorption [26] and adjusted the diet to contain an equivalent amount of vitamin D. Opening the B ring of vitamin D precursors would increase the fluidity of the membrane and so make the membrane more permeable to calcium, thus providing a signaling mechanism for the action of S100A16 [27]. However, the relationship between vitamin D and S100A16 requires further investigation.

In conclusion, this study has demonstrated that high-calcium diets are associated with exclusion of S100A16 from the nucleus, resulting in decreased interaction of S100A16 with p53. Furthermore, high-calcium diets were associated with a significant reduction in body weight gain. Therefore, the results of this study suggest that high-calcium diets lead to inhibition of adipogenesis and enhanced insulin sensitivity via a signaling mechanism mediated by S100A16.

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**Conflicts of Interest**

The authors have nothing to declare.

**References**


