

Calcium deficiency in the early stages after weaning is associated with the enhancement of a low level of adrenaline-stimulated lipolysis and reduction of adiponectin release in isolated rat mesenteric adipocytes

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Abstract

Dysregulation of visceral adipocytes increases the incidence of metabolic syndrome. Higher production of nonesterified fatty acid and changes in adipocytokine release may trigger insulin resistance. Many studies have suggested that calcium (Ca) deficiency is associated with insulin resistance; however, the mechanisms are poorly understood. We examined the effects of Ca deficiency on adrenaline-induced lipolysis and adipocytokine release in the early stages after weaning using freshly isolated adipocytes from mesenteric fat tissue of 3-week-old male Sprague-Dawley rats fed a normal-Ca (5 g/kg diet) or low-Ca (1 g/kg diet) diet for 4 weeks. The release rate of nonesterified fatty acid in the mesenteric adipocytes after stimulation with a low level of adrenaline (0.2 $\mu\text{g/mL}$) was much higher in the Ca-deficient group than in the control group. In contrast, adiponectin release in the mesenteric fat cells was lower in Ca-deficient rats. Leptin and tumor necrosis factor- α secretion showed a similar tendency without significant intergroup differences, and monocyte chemoattractant protein-1 release was not affected by Ca deficiency. We found that Ca deficiency reduced the average size of fat cells through a large increase in the number of cells slightly smaller than the average size, which may be associated with the changes in the properties of the mesenteric adipose tissue. Our present results suggest that a low intake of Ca in the early stages after weaning is associated with changes in the properties of mesenteric adipocytes, which may be linked to insulin resistance in the future.

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1. Introduction

Metabolic syndrome has become a major social issue both globally as well as in Japan. Obesity is a principal causative factor in the development of metabolic syndrome. The major concerns related to increasing obesity are the associated pathologic signs, including hypertension, dyslipidemia, insulin resistance, and glucose intolerance. The combination of these features dramatically increases the risk of cardiovascular disease [1].

Adipocytes are the primary site of energy storage and the accumulation of triacylglycerol during excess energy intake.

In recent years, it has been accepted that adipocyte dysfunction, particularly in the visceral fat tissue, plays an important role in the development of insulin resistance. Adipocyte synthesizes and secretes biologically active molecules called *adipocytokines* [2,3]. Some of the adipocytokines secreted from hypertrophic adipocytes have been shown to directly or indirectly impair insulin sensitivity through modulation of insulin signaling and glucose and lipid metabolism [4]. The hypertrophic adipocytes produce less adiponectin, which has been reported to lead to the impairment of insulin sensitivity [5]. Higher production of nonesterified fatty acid (NEFA) by adipocytes in the mesenteric fat tissues may also trigger insulin resistance.

Low calcium (Ca) intake is a serious nutritional problem especially in the younger Asian population. Many epidemiologic studies have reported the link between Ca intake and insulin resistance, one of the central abnormalities in obesity or metabolic syndrome [6,7]. The results of several

This study was approved by the Hokkaido University Animal Committee, and the animals were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals.

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observational prospective studies showed that there is a relationship between low/insufficient oral Ca intake and the incidence of type 2 diabetes mellitus [8–10] or metabolic syndrome [11]. Low levels of dietary Ca and dairy products increase the risk of hypertension and insulin resistance syndrome [11–13]. It has been reported that insulin resistance appears to be a consequence of an impairment of intracellular Ca signals with many other cell signaling factors [14,15]. Enhancement of parathyroid hormone (PTH) levels is also known as a factor for impairment of insulin sensitivity [16]. However, these mechanisms are poorly understood.

The aims of this study were to determine the effects of Ca deficiency on the properties of abdominal adipocytes, including lipolytic activity and adipocytokine release, in the early stages after weaning in rats fed a high-sucrose diet, which is a metabolic syndrome-inducible diet. We evaluated adrenaline-induced lipolysis and adipocytokine release using freshly isolated rat mesenteric fat cells after the feeding of a Ca-deficient diet for 4 weeks.

2. Materials and method

2.1. Animals and diets

Male Sprague-Dawley rats (3 weeks old; Japan Clea, Tokyo, Japan) were housed in individual stainless-steel cages with wire-mesh bottoms. The cages were placed in a room with controlled temperature ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$), relative humidity (40%–60%), and lighting (lights on 8:00 AM to 8:00 PM) throughout the study. The rats had free access to deionized water and a semipurified diet based on the AIN93G formulation [17] (normal-Ca diet) for an acclimation period of 7 days. The test diets (normal-Ca diet with 0.5% Ca and Ca-deficient diet with 0.1% Ca) shown in Table 1 were given everyday. This study was approved by the Hokkaido University Animal Committee, and the animals were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals.

2.2. Study design

Acclimated rats were divided into 2 groups of 8 rats and were given one of the abovementioned test diets for 4 weeks.

Table 1
Composition of the experimental diets

Ingredient	g/kg diet	
	0.5% Ca diet	0.1% Ca diet
Casein	250.0	250.0
Corn oil	50.0	50.0
Ca-free mineral mixture ^a	35.0	35.0
Ca carbonate	12.5	2.5
Vitamin mixture ^a	10.0	10.0
Choline chloride	2.5	2.5
Cellulose	50.0	50.0
Sucrose	590.0	600.0

^a The mineral and vitamin mixtures were prepared according to the AIN93G formulation.

Body weight and food intake were measured everyday. Daily food intake in the normal-Ca group was adjusted to that in the Ca-deficient group. Tail blood was collected in heparinized microtubes after 10-hour fasting on the 25th day. On the last day, the rats were killed after the collection of abdominal aortic blood under a pentobarbital anesthesia (Nembutal; sodium pentobarbital, 50 mg/kg body weight; Abbott Laboratories, North Chicago, IL). The liver, kidney, cecum, and abdominal white adipose tissues (mesenteric, retroperitoneal, and epididymal) were immediately excised and weighed. The tail blood plasma and aortic blood serum were collected after centrifugation and stored at -80°C until the subsequent analyses.

The concentrations of Ca, albumin, and total protein; the albumin to globulin (A/G) ratio in the serum; and the concentrations of glucose, NEFA, and triglyceride (TG) in the plasma were measured using enzyme assay kits (Calcium C-test, A/G B-test, Glucose CII-test, and NEFA C-test from Wako Pure Chemical Industries, Osaka, Japan; and TG-EN from Kainos Laboratories, Tokyo, Japan). The serum concentrations of PTH, adiponectin, and leptin were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Rat Whole PTH [1–84 Specific] ELISA kit from Scantibodies Laboratory, Santee, CA; Mouse/Rat Adiponectin ELISA kit from Otsuka Pharmaceutical, Tokyo, Japan; and Rat Leptin ELISA kit from LINCO Research, St Charles, MO).

2.3. Measurement of lipolysis and adipocytokine release in fat cells

Isolated fat cells were obtained from rat mesenteric fat tissues by the method of Rodbell [18]. In brief, mesenteric fat tissues were cut into small pieces with scissors before digestion for 1 hour in isolation medium (Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 118 mmol/L NaCl, 4.7 mmol/L KCl, 2.7 mmol/L CaCl_2 , 1.2 mmol/L KH_2PO_4 , and 24.9 mmol/L NaHCO_3) supplemented with 4% (wt/vol) bovine serum albumin (BSA, fatty acid free; Wako Pure Chemicals), 0.1% (wt/vol) collagenase (Type I; Sigma, St Louis, MO), 0.01% (wt/vol) trypsin inhibitor (TypeII-S, Sigma), and 2.5 mmol/L glucose in a shaking water bath (70 rpm, 37°C). After digestion, the isolated fat cells were filtered from the undigested tissue through 1000- μm nylon mesh and were collected from the isolation medium containing collagenase. The isolated fat cells floated to the surface, and the stromal-vascular fraction (eg, capillary, progenitor cells, fibroblastic cells, and macrophages) was precipitated. The stromal-vascular fractions were removed, and fat cells were washed 2 times with collagenase-free Krebs-Ringer-phosphate buffer containing 3% BSA. The isolated adipocytes were used for studies of the adrenalin-induced lipolysis and adipocytokine release. The isolated mesenteric fat cells (150 μL packed volume) were incubated at 37°C in 750 μL of stimulation medium (Krebs-Ringer-phosphate buffer, pH 7.4, containing 122 mmol/L NaCl, 4.9

mmol/L KCl, and 1.2 mmol/L MgSO₄, 16.7 mmol/L phosphate buffer) supplemented with 3% (wt/vol) BSA (fatty acid free, Wako Pure Chemicals), 5 mmol/L glucose, and 100 μ L of various concentrations of adrenaline (Sigma) solution. The final concentration of adrenaline was 0, 0.1, 0.2, or 0.5 μ g/mL. In preliminary experiment, we examined the NEFA release under varying concentration of adrenaline. We found that 0.5 μ g/mL adrenaline nearly maximized the release of NEFA from fat cells; and in concentration of 0.1 and 0.2 μ g/mL, the NEFA release was definitely raised. Therefore, we used these adrenaline concentrations for the NEFA and adipocytokine release in this study. After incubation for 1 hour, the reaction was stopped on ice. The floating fat cells were removed, and the medium was used for the measurement of NEFA and adipocytokines. The NEFA release was evaluated by subtraction of the NEFA concentration without incubation from the values obtained after incubation.

The amount of NEFA released from the fat cells was measured using an enzyme assay kit (NEFA C test from Wako Pure Chemicals), and lipolysis was expressed as micromoles of NEFA released per milliliter packed fat cells per hour. Tumor necrosis factor- α (TNF- α), monocyte chemoattractant protein-1 (MCP-1), adiponectin, and leptin released by the fat cells were quantified with ELISA kits (Rat TNF- α ELISA kit from Bender Medsystems, Vienna, Austria; MCP-1 Rat ELISA system from Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom; Mouse/Rat Adiponectin ELISA kit from Otsuka Pharmaceutical; and Rat Leptin ELISA kit from LINCO Research).

2.4. Measurement of fat cell size

Isolated fat cells from mesenteric fat tissues were stained with oil red O. Fat cell size was evaluated as average cell area for more than 100 cells per rat. Cell areas were measured

Table 2

Initial and final body weight and food intake in rats fed the test diets for 4 weeks

	0.5% Ca diet	0.1% Ca diet	P
Initial body weight (g)	78.2 \pm 1.5	78.3 \pm 1.1	.968
Final body weight (g)	281.9 \pm 2.5	286.3 \pm 4.6	.420
Food intake (g/d)	18.5 \pm 0.2	18.1 \pm 0.2	.286
	Wet g/100 g body weight		
Liver weight	5.23 \pm 0.16 ^b	5.73 \pm 0.15 ^a	.035
Kidney weight	0.82 \pm 0.02 ^b	0.92 \pm 0.02 ^a	.003
Cecum weight	0.98 \pm 0.08	1.01 \pm 0.05	.757
Mesenteric fat weight	0.98 \pm 0.07 ^a	0.81 \pm 0.05 ^b	.046
Retroperitoneal fat weight	1.49 \pm 0.13 ^a	1.03 \pm 0.11 ^b	.019
Epididymal fat weight	1.33 \pm 0.05 ^a	1.00 \pm 0.02 ^b	<.001

Values are means \pm SEM (n = 8). Values in a row with a different letter are significantly different by Student *t* test (*P* < .05).

Table 3

Serum concentrations of Ca, PTH, adiponectin, leptin, albumin, and protein, and the A/G ratio in the abdominal aortic blood after 4 weeks and plasma concentrations of triacylglycerol, NEFA, and glucose in the tail blood after 10 hours of fasting on the 25th day of rats fed the 0.5% Ca or 0.1% Ca diet

	0.5% Ca diet	0.1% Ca diet	P
Aortic blood serum			
Ca (m mol/L)	2.86 \pm 0.06 ^a	2.59 \pm 0.04 ^b	.003
PTH (pg/mL)	11.37 \pm 0.94 ^b	38.74 \pm 3.30 ^a	<.001
Adiponectin (μ g/mL)	5.87 \pm 0.94 ^a	2.72 \pm 0.22 ^b	.009
Leptin (ng/mL)	6.01 \pm 0.87 ^a	3.14 \pm 0.45 ^b	.011
Albumin (g/dL)	3.92 \pm 0.05	3.96 \pm 0.12	.778
Protein (g/dL)	5.71 \pm 0.07	5.69 \pm 0.10	.867
A/G ratio	2.20 \pm 0.04	2.32 \pm 0.13	.402
Tail blood plasma after 10-h fasting			
Triacylglycerol (mmol/L)	1.58 \pm 0.33	1.11 \pm 0.17	.228
NEFA (mmol/L)	0.64 \pm 0.05	0.59 \pm 0.03	.463
Glucose (mmol/L)	6.88 \pm 0.22	6.90 \pm 0.13	.935

Values are means \pm SEM (n = 7–8). Values in a row with a different letter are significantly different by Student *t* test (*P* < .05).

using Image J version 1.41 (National Institutes of Health, Bethesda, MD) software.

2.5. Statistics

Student *t* test was used for comparisons between the 2 groups (Tables 2 and 3, Fig. 3 for each cell size). The effects of diet and adrenaline were analyzed by 2-way repeated-measures analysis of variance (ANOVA), and the differences among treatment groups were determined using Tukey-Kramer test (Figs. 1 and 2) in case that interaction in 2-way

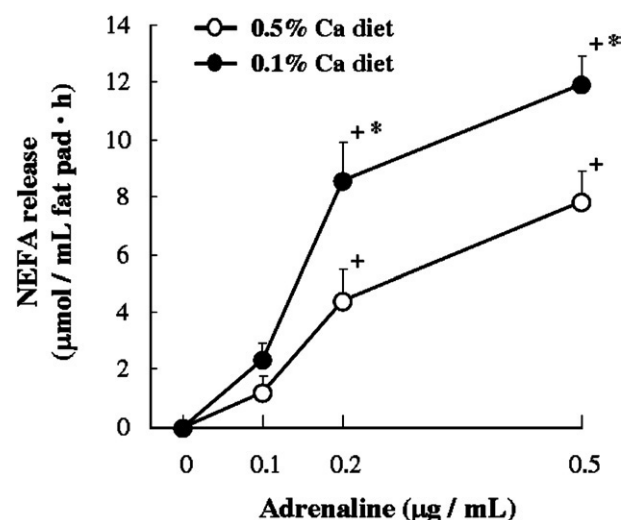


Fig. 1. Release rates of NEFA by stimulation with adrenaline in isolated mesenteric fat cells from rats fed the 0.1% or 0.5% Ca diet. The rates of NEFA release were evaluated by subtraction of the values without incubation from those obtained after incubation. Values are means \pm SEM (n = 8). *P* values by 2-way ANOVA were < .001 for diet and adrenaline, and .032 for diet \times adrenaline. +Significant difference from adrenaline 0 μ g/mL in the same diet group. *Significant difference between 2 diet groups in the same adrenaline dose, *P* < .05.

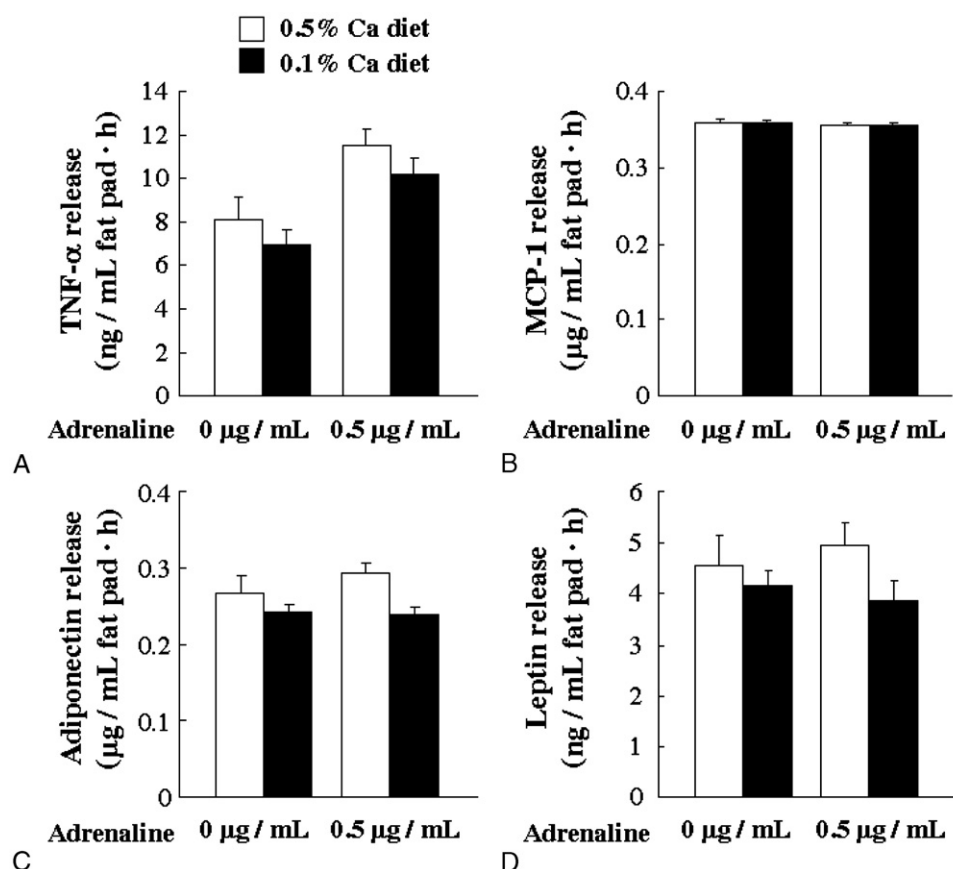


Fig. 2. Release rates of TNF- α (A), MCP-1 (B), adiponectin (C), and leptin (D) in adrenaline-nonstimulated and -stimulated isolated mesenteric fat cells from rats fed the 0.1% or 0.5% Ca diet. Values are means \pm SEM ($n = 8$). P values by 2-way ANOVA were .144 for diet, <.001 for adrenaline, and .920 for diet \times adrenaline (TNF- α); .960 for diet, .507 for adrenaline, and .996 for diet \times adrenaline (MCP-1); .008 for diet, .454 for adrenaline, and .301 for diet \times adrenaline (adiponectin); and .114 for diet, .953 for adrenaline, and .456 for diet \times adrenaline (leptin). $P < .05$ was regarded as a significant difference.

analysis of variance is significant. A difference with $P < .05$ was considered significant. Pearson correlation coefficients were calculated (Fig. 4).

3. Results

Final body weight and food intake, which were in the reference range for 8-week-old rats, were similar between the 0.5% Ca and 0.1% Ca diet groups, whereas the relative weights of the mesenteric, retroperitoneal, and epididymal fat tissue were 17%, 30%, and 25% lower in the 0.1% Ca diet group than in the 0.5% Ca diet group, respectively (Table 2). Relative wet weights of the liver and kidney were greater in the 0.1% Ca diet group than in the 0.5% Ca diet group; however, there were no differences in the weights of the cecum together with its contents between the 2 groups.

In the aortic blood serum, Ca concentration was significantly lower and PTH level was much higher in the 0.1% Ca diet group than in the 0.5% Ca diet group (Table 3). The serum adiponectin and leptin concentrations were lower in the 0.1% Ca diet group compared with the 0.5% Ca diet group. Feeding with the Ca-deficient diet had no effects on

the serum levels of albumin and total protein or on the A/G ratio. Furthermore, the ingestion of 0.5% or 0.1% Ca diet did not cause any changes in TG, NEFA, and glucose concentrations in the tail blood plasma of fasted rats. However, TG levels of the rats in this study were much higher than the reference range (51.5 ± 15.0 mg/dL, 10 weeks old) officially provided by the breeder (Japan Clea). Glucose levels were within reference range (163.7 ± 28.4 mg/dL, 10 weeks old).

Adrenaline-induced lipolysis in isolated mesenteric fat cells was increased in both groups in a dose-dependent manner (Fig. 1). Release of NEFA by stimulation with adrenaline was higher in the Ca-deficient group than in the normal-Ca group according to the result of 2-way ANOVA ($P < .001$, Fig. 1 legends). At 0.2 and 0.5 μ g/mL adrenaline, NEFA release was 2- and 1.5-fold higher in the 0.1% Ca diet group than that in the 0.5% Ca diet group, respectively. Nonstimulated levels of NEFA release were very low, and no intergroup differences were observed between diet groups (data not shown).

Adipocytokine release by both adrenaline-stimulated and -nonstimulated isolated mesenteric fat cells is shown in Fig. 2. Although diet had no significant effect on TNF- α

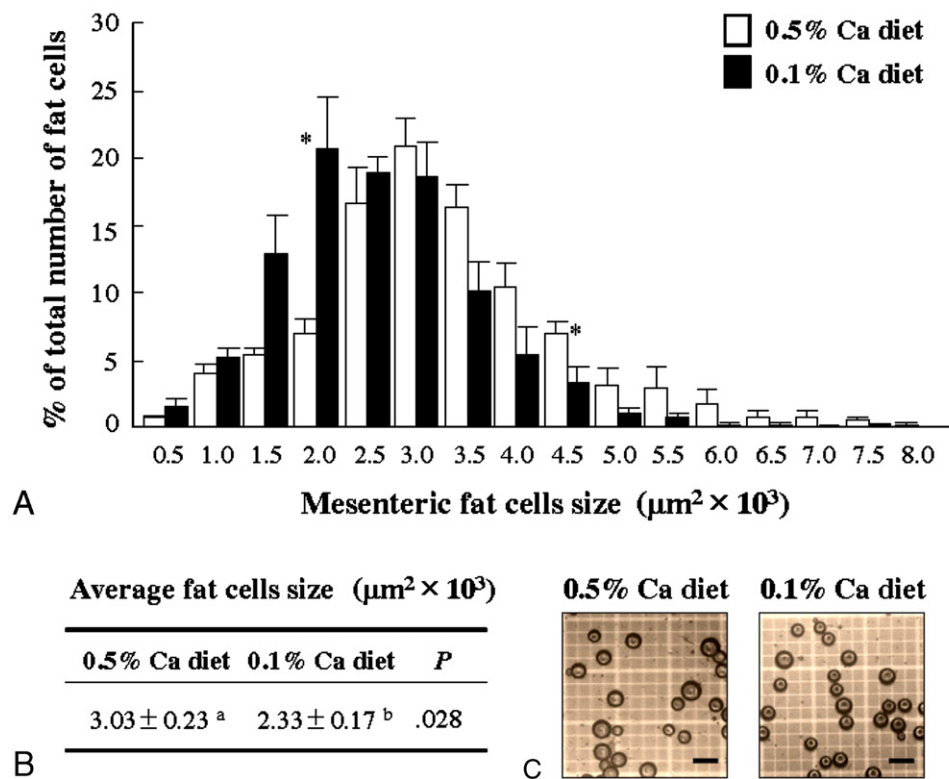


Fig. 3. Size distribution (A) and average size (B) of the isolated mesenteric fat cells in rats fed the 0.1% or 0.5% Ca diet. Values are means \pm SEM ($n = 8$). *Mean value was significantly different between the 0.5% Ca diet and the 0.1% Ca diet, $P < .05$ (A). Means not sharing a common letter differ significantly, $P < .05$ (B). Oil red O–stained isolated fat cells (scale bar: 100 μm) from the 0.1% or 0.5% Ca diet group (C).

release, adrenaline (0.5 $\mu\text{g}/\text{mL}$) stimulated clearly the adipocytokine release. The results of 2-way ANOVA show that adrenaline and diet had no effect on MCP-1, but diet did affect adiponectin release, which was significantly lower in the 0.1% Ca diet group compared with that in the 0.5% Ca diet group. Change in leptin release showed a similar tendency to that of adiponectin without any significant intergroup differences.

The Ca-deficient diet affected average adipocyte size and distribution in the mesenteric fat tissues (Fig. 3). Feeding with the 0.1% Ca diet was associated with a reduction in average adipocyte size in mesenteric fat cells compared with that observed after feeding with the 0.5% Ca diet. The number of smaller-sized fat cells, $1.5 \mu\text{m}^2 \times 10^3$, tended to be greater ($P < .051$) and that of $2.0 \mu\text{m}^2 \times 10^3$ was significantly greater in the 0.1% Ca diet group than in the 0.5% Ca diet group, respectively, whereas that of large-sized fat cells, $4.5 \mu\text{m}^2 \times 10^3$, was lower in the Ca-deficient group.

The cell size inversely correlated with adrenaline-induced lipolysis ($R = -0.756$, $P = .001$, $n = 16$) and positively correlated with those in release of adiponectin ($R = 0.706$, $P = .002$, $n = 16$) and TNF- α ($R = 0.503$, $P = .047$, $n = 16$), but not in leptin release ($R = 0.445$, $P = .084$, $n = 16$). Furthermore, fat cell size positively correlated to adiponectin release in Ca-deficient group ($R = 0.913$, $P = .002$, $n = 8$, Fig. 4); however, there was

no correlation in 0.5% Ca group ($R = 0.369$, $P = .369$, $n = 8$, Fig. 4). Blood PTH levels also correlated inversely with adiponectin ($R = -0.698$, $P = .004$, $n = 15$). There was no correlation with TNF- α ($R = -0.229$, $P = .413$, $n = 15$) and leptin ($R = 0.290$, $P = .294$, $n = 15$). The correlation coefficient between PTH levels and cell size was -0.696

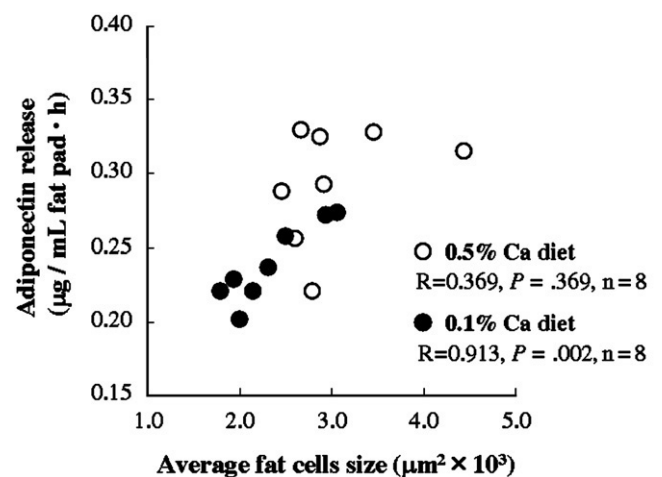


Fig. 4. Correlations between fat cell size and adiponectin release in adrenaline-stimulated isolated mesenteric fat cells from rats fed the 0.1% or 0.5% Ca diet. $P < .05$ was regarded as a significant correlation.

($P = .004$, $n = 15$), which was very similar to the value between PTH and adiponectin as shown above.

4. Discussion

The present study shows that adrenaline-induced lipolysis was increased and adiponectin release was decreased in isolated mesenteric fat cells by Ca deficiency. There have been many studies to examine the effects of food components in cultured fat cells such 3T3-L1 cells. We found alterations in the mesenteric adipocytes in isolated fat cells from rats adapted to a low-Ca diet for 4 weeks in the early stages after weaning. This is the first report indicating that Ca deficiency changes lipolytic activity and adipocytokine release in abdominal fat cells. Epidemiologic studies have suggested that Ca deficiency is associated with insulin resistance as described in “Introduction.” Food habits, for example, low Ca intakes, often become unbalanced during an early stage of life. Our findings may have implication for prevention of metabolic syndrome in human.

As mentioned above, lipolysis in the mesenteric adipocytes was enhanced after stimulation with adrenaline in Ca-deficient rats. The relative difference in the NEFA release rate between the diet groups was larger at a low dose of adrenaline ($0.2 \mu\text{g/mL}$) than at a high dose ($0.5 \mu\text{g/mL}$). The results of 2-way ANOVA showed that a Ca-deficient diet clearly affects adrenaline-induced lipolysis and that the interaction between diet and adrenaline is also significant. These results suggest that adrenaline sensitivity, rather than the lipolytic activity of the fat cells, is enhanced by 4 weeks of feeding with a low-Ca diet. It has been reported that higher levels of NEFA impair insulin sensitivity and present a risk factor for metabolic syndrome [19,20]. Stimulation of NEFA release from abdominal fat cells with low levels of adrenaline possibly induces insulin insensitivity. Low Ca intakes have been reported to be associated with glucose intolerance and higher risks for diabetes in previous epidemiologic studies [8–10]. This study showed that the liver weight was increased by Ca deficiency. The higher release of NEFA from mesenteric fat cells possibly induces fat accumulation and increases weight of the liver. The kidney weight was also increased by Ca deficiency without increase in water content. Cause for the increase in the kidney weight is still unknown.

There are few studies on Ca and adipocyte lipid metabolism. Previous studies have shown that a high-Ca diet stimulates lipolysis and inhibits *de novo* lipogenesis via decreasing intracellular Ca concentrations and suggested that 1α , 25-dihydroxyvitamin D₃ (calcitriol) and PTH levels are involved in these changes in adipocytes [21–23]. However, these results seem to disagree with our present findings in that we demonstrated that the Ca-deficient diet caused marked increases in lipolysis in isolated mesenteric fat cells. It is possible that Ca deficiency regulates lipolysis by a mechanism other than that working in the presence of excess amounts of Ca. Several studies indicate a relationship

between PTH level and insulin resistance or metabolic syndrome [10,24–26]. An increase in PTH level seems to be associated with metabolic syndrome in older men [27,28]. We showed that the concentration of serum PTH was markedly increased in the Ca-deficient diet group. It is possible that the changes in lipolysis and adiponectin release induced by Ca deficiency might be directly associated with the increased levels of PTH in the blood. Further research on the underlying mechanism is required.

It is reported that adiponectin expression is significantly higher in visceral fat than in subcutaneous adipose tissue in lean animals [29]. In our present study, we revealed a significant reduction in adiponectin and a tendency to decrease in leptin release from isolated mesenteric fat cells in rats fed a low-Ca diet. We also found large decreases in the levels of aortic blood adiponectin and leptin by the Ca deficiency, which agree with the results of isolated mesenteric fat cells. Serum adiponectin was strongly correlated with adiponectin released from isolated fat cells ($R = 0.745$, $P = .001$, $n = 16$), but there was just a tendency for correlation in the case between serum leptin and fat cell release ($R = 0.493$, $P = .052$, $n = 16$). This finding suggests that the adiponectin released from mesenteric fat tissues may largely contribute to changes in blood level of this adipocytokines. Therefore, fat cell property may be altered by Ca deficiency. Adiponectin is exclusively produced by adipocytes; and recent research has demonstrated that this adipocytokine acts as an antidiabetic, antiatherogenic, and anti-inflammatory hormone [30,31]. Plasma adiponectin concentrations are found to be lowered in obese and insulin-resistant states [30–32]. We also found that release of TNF- α from mesenteric fat cells was increased by adrenaline stimulation in both the diet groups. This finding has not been reported previously and possibly demonstrates a mechanism for adrenaline-induced glucose intolerance because TNF- α impairs insulin sensitivity [33,34]. Our present results suggest that low intakes of Ca in the early stages after weaning may induce insulin resistance and increase the risk of metabolic syndrome through changes in properties of the mesenteric adipocytes.

The size of fat cells is an important determinant of their functions. In general, larger fat cells have a higher capacity to release NEFA [35,36] and a lower responsiveness to insulin [37,38]. We found that Ca deficiency reduced average adipocyte size, which was accompanied by large increases in number of cells of a slightly smaller than average size, 1.5 to $2.0 \mu\text{m}^2 \times 10^3$. The conventional method for measuring fat cell size is the histologic method using fixed sections of fat tissue. However, it is difficult to evaluate the exact fat cell size using this method because of differences in the shape of each cell. Our method using isolated fat cells is useful to accurately measure cell size because all fat cells are round in shape. The increase in the number of fat cells just below the average size may be associated with the reduction in adipocytokine release and is responsible for a loss of adipose tissue weight in rats in the Ca-deficient diet group.

Adiponectin has the ability to induce differentiation of preadipocytes into mature adipocytes, stimulating a transcriptional cascade. The reduction in adiponectin associated with Ca deficiency is possibly involved in the size reduction in the mesenteric adipocytes through autocrine function. Recent research has demonstrated that intracellular Ca^{2+} also plays a complex role in the differentiation of committed preadipocytes into mature, fat-laden adipocytes [39].

We analyzed the correlation between fat cell size and lipolysis or adipocytokine release with 0.5 $\mu\text{g/mL}$ adrenaline. The cell size inversely correlated with adrenaline-induced lipolysis and positively correlated with those in release of adiponectin. Interestingly, fat cell size positively correlated to adiponectin release in Ca-deficient group with very high correlation coefficient ($R = 0.913$); however, there was no correlation in 0.5% Ca group ($R = 0.369$) as shown in Fig. 4. These results of correlation between cell size and adiponectin release within each diet group suggest that cell size is not sole factor, but change in fat cell properties with Ca deficiency is responsible for lower adiponectin release because average cell size in individual rats was overlapped in a considerable part between 0.1% and 0.5% Ca groups.

We found that blood PTH levels also correlated inversely with adiponectin and that fat cell size also negatively correlated with PTH with very similar correlation coefficients (-0.698 and -0.696). These results suggest that enhanced PTH level equally affects fat cell size and adiponectin release. The enlarged adipocyte usually reduces adiponectin release, which is an important factor for insulin resistance. However, our present results reveal that the reduction of fat cell size does not increase, but decreases, adiponectin release in Ca-deficient group. In multiple regression analysis, fat cell size was most associated with adiponectin release ($R = 0.953$, $P = .01$, $n = 8$) in 0.1% Ca group. This result suggests that low levels of adiponectin might decrease the fat cell size and also suggests that the reduction of adiponectin is caused by changes in mesenteric adipocyte properties induced with hyperparathyroidism. There are some reports showing that elevated levels of PTH impair insulin sensitivity [16,40–42]. The present study showed that largely increased blood PTH levels possibly induce insulin resistance, suppress lipogenesis in fat cells, and reduce the cell size in Ca-deficient rats.

In conclusion, low Ca intake leads to a reduction in fat cell size and stimulates low-level adrenaline-induced lipolysis while suppressing adiponectin release in mesenteric fat cells. Consequently, Ca deficiency might be a factor for the development of metabolic syndrome.

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