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

5

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23 guidelines for the care and use of laboratory animals.

24

ABSTRACT

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

19

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 pathological 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 non-esterified 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 epidemiological 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 observational prospective studies showed that there is a relationship between low/insufficient oral Ca intake and the incidence of Type 2 diabetes [8-10] or metabolic syndrome [11]. Low levels of dietary calcium and dairy products increase the risk of hypertension and insulin resistance syndrome (IRS) [11-13]. It has been reported that

1 insulin resistance appears to be a consequence of an impairment of intracellular Ca signals
2 with many other cell signaling factors [14, 15]. Enhancement of PTH levels is also known as
3 a factor for impairment of insulin sensitivity [16]. However, these mechanisms are poorly
4 understood.

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

10

11

MATERIALS AND METHOD

12 **Animals and diets**

13 Male Sprague-Dawley rats (3 weeks old; Japan Clea, Tokyo, Japan) were housed in
14 individual stainless-steel cages with wire-mesh bottoms. The cages were placed in a room
15 with controlled temperature ($22 \pm 2^\circ\text{C}$), relative humidity (40-60%) and lighting (lights on
16 08:00-20:00 h) throughout the study. The rats had free access to deionized water and a
17 semipurified diet based on the AIN93G formulation [17] (Normal Ca diet) for an acclimation
18 period of 7 days. The test diets (Normal Ca diet with 0.5% Ca and Ca-deficient diet with 0.1%
19 Ca) shown in Table 1 were given everyday. This study was approved by the Hokkaido
20 University Animal Committee and the animals were maintained in accordance with the
21 Hokkaido University guidelines for the care and use of laboratory animals.

22 **Study design**

23 Acclimated rats were divided into two groups of eight rats, and were given one of the

1 abovementioned test diets for 4 weeks. Body weight and food intake were measured everyday.
2 Daily food intake in the normal Ca group was adjusted to that in the Ca-deficient group. Tail
3 blood was collected in heparinized micro tubes after 10h fasting on the 25th day. On the last
4 day, the rats were killed after the collection of abdominal aortic blood under a pentobarbital
5 anesthesia (Nembutal: sodium pentobarbital, 50 mg/kg body weight, Abbott Laboratories,
6 North Chicago, IL, USA). The liver, kidney, cecum, and abdominal white adipose tissue
7 (mesenteric, retroperitoneal, and epididymal) were immediately excised and weighed. The tail
8 blood plasma and aortic blood serum were collected after centrifugation, and stored at -80°C
9 until the subsequent analyses.

10 The concentrations of Ca, albumin, and total protein, and the Albumin/Globulin ratio
11 (A/G ratio) in the serum, and the concentrations of glucose (Glc), non-esterified fatty acid
12 (NEFA) and triglyceride (TG) in the plasma were measured using enzyme assay kits
13 (Calcium C-test, A/G B-test, Glucose CII-test and NEFA C-test from Wako Pure Chemical
14 Industries, Ltd., Osaka, Japan; and TG-EN from Kainos Laboratories, Tokyo, Japan). The
15 serum concentrations of parathyroid hormone (PTH), adiponectin and leptin were measured
16 using an ELISA kit (Rat Whole PTH (1-84 Specific) ELISA kit from Scantibodies Laboratory,
17 Inc., Santee, Canada; Mouse/Rat Adiponectin ELISA kit from Otsuka Pharmaceutical, Tokyo,
18 Japan; and Rat Leptin ELISA kit from LINCO Research, Missouri, USA).

19 **Measurement of lipolysis and adipocytokine release in fat cells**

20 Isolated fat cells were obtained from rat mesenteric fat tissues by the method of
21 Rodbell [18]. In briefly, mesenteric fat tissues were cut into small pieces with scissors before
22 digestion for 1 hour in isolation medium (Krebs-Ringer-bicarbonate buffer, pH 7.4,
23 containing 118 mmol/L NaCl, 4.7 mmol/L KCl, 2.7 mmol/L CaCl₂, 1.2 mmol/L KH₂PO₄ and

1 24.9 mmol/L NaHCO₃) supplemented with 4% (w/v) bovine serum albumin (BSA, fatty acid
2 free, Wako Pure Chemicals, Osaka, Japan), 0.1% (w/v) collagenase (Type I, Sigma, St Louis,
3 MO, USA), 0.01% (w/v) trypsin inhibitor (TypeII-S, Sigma, St Louis, MO, USA) and 2.5
4 mmol/L glucose in a shaking water bath (70 rpm, 37°C). After digestion, the isolated fat cells
5 were filtered from the undigested tissue through 1000 µm nylon mesh, and were collected
6 from the isolation medium containing collagenase. The isolated fat cells floated to the surface,
7 and the stromal-vascular fraction (for example; capillary, progenitor cells, fibroblastic cells,
8 and macrophages) were precipitated. The stromal-vascular fractions were removed, and fat
9 cells were washed 2 times with collagenase-free Krebs-Ringer-phosphate buffer containing
10 3% BSA. The isolated adipocytes were used for studies of the adrenalin-induced lipolysis and
11 adipocytokine release. The isolated mesenteric fat cells (150 µL packed volume) were
12 incubated at 37°C in 750 µL of stimulation medium (Krebs-Ringer-phosphate buffer, pH 7.4,
13 containing 122 mmol/L NaCl, 4.9 mmol/L KCl, and 1.2 mmol/L MgSO₄, 16.7 mmol/L
14 phosphate buffer) supplemented with 3% (w/v) bovine serum albumin (BSA, fatty acid free,
15 Wako Pure Chemicals, Osaka, Japan), 5 mmol/L glucose and 100 µL of various
16 concentrations of adrenaline (Sigma, St Louis, MO, USA) solution. The final concentration of
17 adrenaline was 0, 0.1, 0.2, or 0.5 µg/mL. In preliminary experiment, we examined the NEFA
18 release under varying concentration of adrenaline. We found that 0.5 µg/mL adrenaline nearly
19 maximized the release of NEFA from fat cells, and in concentration of 0.1 and 0.2 µg/mL the
20 NEFA release definitely raised. Therefore, we used these adrenaline concentrations for the
21 NEFA and adipocytokine release in this study. After incubation for 1 hour, the reaction was
22 stopped on ice. The floating fat cells were removed, and the medium was used for the
23 measurement of NEFA and adipocytokines. NEFA release was evaluated by subtraction of

1 the NEFA concentration without incubation from the values obtained after incubation.

2 The amount of NEFA released from the fat cells was measured using an enzyme
3 assay kit (NEFA C test from Wako Pure Chemicals, Osaka, Japan) and lipolysis was
4 expressed as micromoles of NEFA released per ml packed fat cells per h. Tumor necrosis
5 factor α (TNF- α), monocyte chemoattractant protein-1 (MCP-1), adiponectin and leptin
6 released by the fat cells were quantified with ELISA kits (Rat TNF- α ELISA kit from Bender
7 Medsystems, Vienna, Austria; MCP-1 Rat ELISA system from Amersham Pharmacia Biotech,
8 Buckinghamshire, UK; Mouse/Rat Adiponectin ELISA kit from Otsuka Pharmaceutical,
9 Tokyo, Japan; and Rat Leptin ELISA kit from LINCO Research, Missouri, USA).

10 **Measurement of fat cell size**

11 Isolated fat cells from mesenteric fat tissues were stained with oil red O. Fat cell size
12 was evaluated as average cell area for more than 100 cells per rat. Cell areas were measured
13 using Image J version 1.41 (National Institutes of Health, Bethesda, MD, USA) software.

14 **Statistics**

15 Student's *t*-test was used for comparisons between the two groups (Table 2 and 3,
16 Fig. 3 for each cell size). The effects of diet and adrenaline were analyzed by two-way
17 repeated measures ANOVA, and the differences among treatment groups were determined
18 using Tukey-Kramer's test (Fig. 1 and 2) in a case that interaction in two-way ANOVA is
19 significant. A difference with $P < 0.05$ was considered significant. Pearson's correlation
20 coefficients were calculated (Fig. 4).

21

RESULTS

Final body weight and food intake, which normal range for 8 weeks 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 two 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. Further, 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 normal range (51.5 ± 15.0 mg/dL, 10 weeks old) officially provided by the breeder (Japan Clea, Tokyo, Japan). Glucose levels were within normal 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 two-way ANOVA ($P < 0.001$, Fig. 1 legends). At $0.2 \mu\text{g/mL}$ and $0.5 \mu\text{g/mL}$ adrenaline, NEFA release was 2-fold and 1.5-fold higher in the 0.1% Ca diet group than that in the 0.5%

1 Ca diet group, respectively. Non-stimulated levels of NEFA release were very low and no
2 inter-group differences were observed between diet groups (Data not shown).

3 Adipocytokine release by both adrenaline stimulated and non-stimulated isolated
4 mesenteric fat cells is shown in Fig.2. Although diet had no significant effect on TNF- α
5 release, adrenaline (0.5 $\mu\text{g}/\text{mL}$) stimulated clearly the adipocytokine release. The results of
6 two-way ANOVA show that adrenaline and diet had no effect on MCP-1, but diet did affect
7 adiponectin release, which was significantly lower in the 0.1% Ca diet group compared with
8 that in the 0.5% Ca diet group. Change in leptin release showed a similar tendency to that of
9 adiponectin without any significant inter-group differences.

10 The Ca-deficient diet affected average adipocyte size and distribution in the
11 mesenteric fat tissues (Fig. 3). Feeding with the 0.1% Ca diet was associated with a reduction
12 in average adipocyte size in mesenteric fat cells compared with that observed after feeding
13 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 ($P <$
14 0.051), and $2.0 \mu\text{m}^2 \times 10^3$ was significantly greater in the 0.1% Ca diet group than in the 0.5%
15 Ca diet group, respectively, whereas, that of large-sized fat cells, $4.5 \mu\text{m}^2 \times 10^3$, was lower in
16 the Ca-deficient group.

17 The cell size inversely correlated with adrenaline-induced lipolysis ($R = -0.756$, $P =$
18 0.001 , $n = 16$), and positively correlated with those in release of adiponectin ($R = 0.706$, $P =$
19 0.002 , $n = 16$) and TNF α ($R = 0.503$, $P = 0.047$, $n = 16$), but not in leptin release ($R = 0.445$, $P =$
20 0.084 , $n = 16$). Further, fat cell size positively correlated to adiponectin release in Ca-deficient
21 group ($R = 0.913$, $P = 0.002$, $n = 8$, Fig. 4), however, there was no correlation in 0.5% Ca group
22 ($R = 0.369$, $P = 0.369$, $n = 8$, Fig. 4). Blood PTH levels also correlated inversely with
23 adiponectin ($R = -0.698$, $P = 0.004$, $n = 15$). There were no correlation with TNF α ($R = -0.229$,

1 $P=0.413$, $n=15$) and leptin ($R= - 0.290$, $P= 0.294$, $n=15$). The correlation coefficient between
2 PTH levels and cell size was $- 0.696$ ($P= 0.004$, $n=15$), which was very similar to value
3 between PTH and adiponectin as shown above.
4

5 **DISCUSSION**

6 The present study shows that adrenaline-induced lipolysis was increased and
7 adiponectin release was decreased in isolated mesenteric fat cells by Ca deficiency. There
8 have been many studies to examine the affects of food components in cultured fat cells such
9 3T3-L1 cells. We found alterations in the mesenteric adipocytes in isolated fat cells from rats
10 adapted to a low Ca diet for 4 weeks in the early stages after weaning. This is the first report
11 indicating that Ca deficiency changes lipolytic activity and adipocytokine release in
12 abdominal fat cells. Epidemiological studies have suggested that Ca deficiency is associated
13 with insulin resistance as describe in introduction. Food habits, for example low Ca intakes,
14 often become unbalanced during an early stage of life. Our findings may have implication for
15 prevention of metabolic syndrome in human.

16 As mentioned above, lipolysis in the mesenteric adipocytes was enhanced after
17 stimulation with adrenaline in Ca-deficient rats. The relative difference in the NEFA release
18 rate between the diet groups was larger at a low dose of adrenaline ($0.2 \mu\text{g/mL}$) than at a high
19 dose ($0.5 \mu\text{g/mL}$). The results of two-way ANOVA showed that a Ca-deficient diet clearly
20 affects adrenaline-induced lipolysis, and that the interaction between diet and adrenaline is
21 also significant. These results suggest that adrenaline sensitivity, rather than the lipolytic
22 activity of the fat cells, is enhanced by 4 weeks feeding with a low Ca diet. It has been
23 reported that higher levels of NEFA impair insulin sensitivity and present a risk factor for

1 metabolic syndrome [19, 20]. Stimulation of NEFA release from abdominal fat cells with low
2 levels of adrenaline possibly induces insulin insensitivity. Low Ca intakes have been reported
3 to be associated with glucose intolerance and higher risks for diabetes in previous
4 epidemiological studies [8-10]. This study showed that the liver weight was increased by Ca
5 deficiency. The higher release of NEFA from mesenteric fat cells possibly induces fat
6 accumulation and increases weight of the liver. The kidney weight was also increased by Ca
7 deficiency without increase in water content. Cause for the increase in the kidney weight is
8 still unknown.

9 There are few studies on Ca and adipocyte lipid metabolism. Previous studies have
10 shown that a high Ca diet stimulates lipolysis and inhibits de novo lipogenesis via decreasing
11 intracellular Ca concentrations, and suggested that $1\alpha, 25$ -dihydroxyvitamin D₃ (calcitriol)
12 and PTH levels are involved in these changes in adipocytes [21-23]. However, these results
13 seems to disagree with our present findings in that we demonstrated that the Ca-deficient diet
14 caused marked increases in lipolysis in isolated mesenteric fat cells. It is possible that Ca
15 deficiency regulates lipolysis by a mechanism other than that working in the presence of
16 excess amounts of Ca. Several studies indicate a relationship between PTH level and insulin
17 resistance or metabolic syndrome [10, 24-26]. An increase in PTH level seems to be
18 associated with metabolic syndrome in older men [27, 28]. We showed that the concentration
19 of serum PTH was markedly increased in the Ca-deficient diet group. It is possible that the
20 changes in lipolysis and adiponectin release induced by Ca deficiency might be directly
21 associated with the increased levels of PTH in the blood. Further research on the underlying
22 mechanism is required.

23 It is reported that adiponectin expression is significantly higher in visceral fat than in

1 subcutaneous adipose tissue in lean animals [29]. In our present study, we revealed a
2 significant reduction in adiponectin and a tendency to decrease in leptin release from isolated
3 mesenteric fat cells in rats fed a low Ca diet. We also found large decreases in the levels of
4 aortic blood adiponectin and leptin by the Ca deficiency, which agrees with the results of
5 isolated mesenteric fat cells. Serum adiponectin was strongly correlated with adiponectin
6 released from isolated fat cells ($R=0.745$, $P=0.001$, $n=16$), but just tendency in the case
7 between serum leptin and fat cell release ($R=0.493$, $P=0.052$, $n=16$). These finding suggests
8 that the adiponectin released from mesenteric fat tissues may largely contribute changes in
9 blood level of this adipocytokines. Therefore, fat cell property may be altered by Ca
10 deficiency. Adiponectin is exclusively produced by adipocytes, and recent research has
11 demonstrated that this adipocytokine acts as an anti-diabetic, anti-atherogenic and
12 anti-inflammatory hormone [30, 31]. Plasma adiponectin concentrations are found to be
13 lowered in obese and insulin-resistant states [30-32]. We also found that release of TNF- α
14 from mesenteric fat cells was increased by adrenaline stimulation in both the diet groups. This
15 finding has not been reported previously, and possibly demonstrates a mechanism for
16 adrenaline-induced glucose intolerance because TNF- α impairs insulin sensitivity [33, 34].
17 Our present results suggest that low intakes of Ca in the early stages after weaning may
18 induce insulin resistance and increase the risk of metabolic syndrome through changes in
19 properties of the mesenteric adipocytes.

20 The size of fat cells is an important determinant of their functions. In general, larger
21 fat cells have a higher capacity to release NEFA [35, 36] and a lower responsiveness to
22 insulin [37, 38]. We found that Ca deficiency reduced average adipocyte size, which was
23 accompanied by large increases in number of cells of a slightly smaller than average size, 1.5

1 - $2.0 \mu\text{m}^2 \times 10^3$. The conventional method for measuring fat cell size is the histological
2 method using fixed sections of fat tissue. However, it is difficult to evaluate the exact fat cell
3 size using this method due to differences in the shape of each cell. Our method using isolated
4 fat cells is useful to accurately measure cell size because all fat cells are round in shape. The
5 increase in the number of fat cells just below average size may be associated with the
6 reduction in adipocytokine release, and is responsible for a loss of adipose tissue weight in the
7 Ca-deficient diet group rats. Adiponectin has the ability to induce differentiation of
8 pre-adipocytes into mature adipocytes stimulating a transcriptional cascade. The reduction in
9 adiponectin associated with Ca deficiency is possibly involved in the size reduction in the
10 mesenteric adipocytes through autocrine function. Recent research has demonstrated that
11 intracellular Ca^{2+} also plays a complex role in the differentiation of committed pre-adipocytes
12 into mature, fat laden adipocytes [39].

13 We analyzed the correlation between fat cell size and lipolysis or adipocytokine
14 release with $0.5 \mu\text{g/mL}$ adrenaline. The cell size inversely correlated with adrenaline-induced
15 lipolysis, and positively correlated with those in release of adiponectin. Interestingly, fat cell
16 size positively correlated to adiponectin release in Ca-deficient group with very high
17 correlation coefficient ($R= 0.913$), however, there was no correlation in 0.5% Ca group ($R=$
18 0.369) as shown in Fig.4. These results of correlation between cell size and adiponectin
19 release within each diet group suggest that cell size is not sole factor, but change in fat cell
20 properties with Ca deficiency is responsible for lower adiponectin release because average
21 cell size in individual rats were overlapped in a considerable part between 0.1% and 0.5% Ca
22 groups.

23 We found that blood PTH levels also correlated inversely with adiponectin and fat cell

1 size also negatively correlated with PTH with very similar correlation coefficients (- 0.698
2 and - 0.696). These results suggest that enhanced PTH level equally affects fat cell size and
3 adiponectin release. The enlarged adipocyte usually reduces adiponectin release, which is an
4 important factor for insulin resistance. However, our present results reveals that the reduction
5 of fat cell size do not increased, but decreased adiponectin release in Ca-deficient group. In
6 multiple regression analysis, fat cell size was most associated with adiponectin release
7 ($R=0.953$, $P=0.01$, $n=8$) in 0.1% Ca group. This result suggests that low levels of adiponectin
8 might decrease the fat cell size and also suggest that the reduction of adiponectin is caused by
9 changes in mesenteric adipocyte properties induced with hyperparathyroidism. There are
10 some reports showing that elevated levels of PTH impair insulin sensitivity [16, 40-42]. The
11 present study showed largely increased blood PTH level possibly induce insulin resistance,
12 suppress lipogenesis in fat cells, and reduce the cell size in Ca-deficient rats.

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

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FIGURE LEGENDS

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Figure 1. Release rates of non-esterified fatty acid (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 two-way ANOVA were < 0.001 for diet and adrenaline, and 0.032 for diet \times adrenaline. +; Significant difference from adrenaline 0 μ g/mL in the same diet group. *; Significant difference between two diet groups in the same adrenaline dose, *P* < 0.05.

Figure 2. Release rates of TNF- α (A), MCP-1 (B), Adiponectin (C) and Leptin (D) in adrenaline non-stimulated 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 two-way ANOVA were 0.144 for diet, < 0.001 for adrenaline and 0.920 for diet \times adrenaline (TNF- α); 0.960 for diet, 0.507 for adrenaline and 0.996 for diet \times adrenaline (MCP-1); 0.008 for diet, 0.454 for adrenaline and 0.301 for diet \times adrenaline (Adiponectin) and 0.114 for diet, 0.953 for adrenaline and 0.456 for diet \times adrenaline (Leptin). *P* < 0.05 was regarded as a significant difference.

Figure 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* < 0.05 (A). Means not sharing a common letter differ significantly, *P* < 0.05 (B). Oil red O stained isolated fat cells (scale bar: 100 μ m) from the 0.1% or 0.5% Ca diet group (C).

1 Figure 4. Correlations between fat cell size and adiponectin release in adrenaline stimulated
2 isolated mesenteric fat cells from rats fed the 0.1% or 0.5% Ca diet. $P < 0.05$ was regarded as
3 a significant correlation.
4

Table 1. Composition of the experimental diets

Ingredient	0.5% Ca diet	0.1% Ca diet
	g / kg diet	
Casein	250.0	250.0
Corn oil	50.0	50.0
Calcium-free Mineral mixture *	35.0	35.0
Calcium carbonate	12.5	2.5
Vitamin mixture *	10.0	10.0
Choline Chloride	2.5	2.5
Cellulose	50.0	50.0
Sucrose	590.0	600.0

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

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	<i>P</i>
Initial body weight (g)	78.2 ± 1.5	78.3 ± 1.1	0.968
Final body weight (g)	281.9 ± 2.5	286.3 ± 4.6	0.420
Food intake (g / day)	18.5 ± 0.2	18.1 ± 0.2	0.286
	wet g / 100 g body weight		
Liver weight	5.23 ± 0.16 ^b	5.73 ± 0.15 ^a	0.035
Kidney weight	0.82 ± 0.02 ^b	0.92 ± 0.02 ^a	0.003
Cecum weight	0.98 ± 0.08	1.01 ± 0.05	0.757
Mesenteric fat weight	0.98 ± 0.07 ^a	0.81 ± 0.05 ^b	0.046
Retroperitoneal fat weight	1.49 ± 0.13 ^a	1.03 ± 0.11 ^b	0.019
Epididymal fat weight	1.33 ± 0.05 ^a	1.00 ± 0.02 ^b	<0.001

Values are means ± SEM (n = 8). Values in a row with a different letter are significantly different by Student's *t*-test (*P* < 0.05).

Table 3. Serum concentrations of Calcium, Parathyroid hormone, Adiponectin, Leptin, Albumin, and Protein, and the Albumin/Globulin ratio in the abdominal aortic blood after 4 weeks and Plasma concentrations of Triacylglycerol, Non-esterified fatty acid and Glucose in the tail blood after 10h 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	<i>P</i>
Aortic blood serum			
Calcium (m mol / L)	2.86 ± 0.06 ^a	2.59 ± 0.04 ^b	0.003
Parathyroid hormone (pg / mL)	11.37 ± 0.94 ^b	38.74 ± 3.30 ^a	<0.001
Adiponectin (µg / mL)	5.87 ± 0.94 ^a	2.72 ± 0.22 ^b	0.009
Leptin (ng / mL)	6.01 ± 0.87 ^a	3.14 ± 0.45 ^b	0.011
Albumin (g / dL)	3.92 ± 0.05	3.96 ± 0.12	0.778
Protein (g / dL)	5.71 ± 0.07	5.69 ± 0.10	0.867
Albumin / Globulin ratio	2.20 ± 0.04	2.32 ± 0.13	0.402
Tail blood plasma after 10h fasting			
Triacylglycerol (m mol / L)	1.58 ± 0.33	1.11 ± 0.17	0.228
Non-esterified fatty acid (m mol / L)	0.64 ± 0.05	0.59 ± 0.03	0.463
Glucose (m mol / L)	6.88 ± 0.22	6.90 ± 0.13	0.935

Values are means ± SEM (n=7-8). Values in a row with a different letter are significantly different by Student's *t*-test (*P* < 0.05).

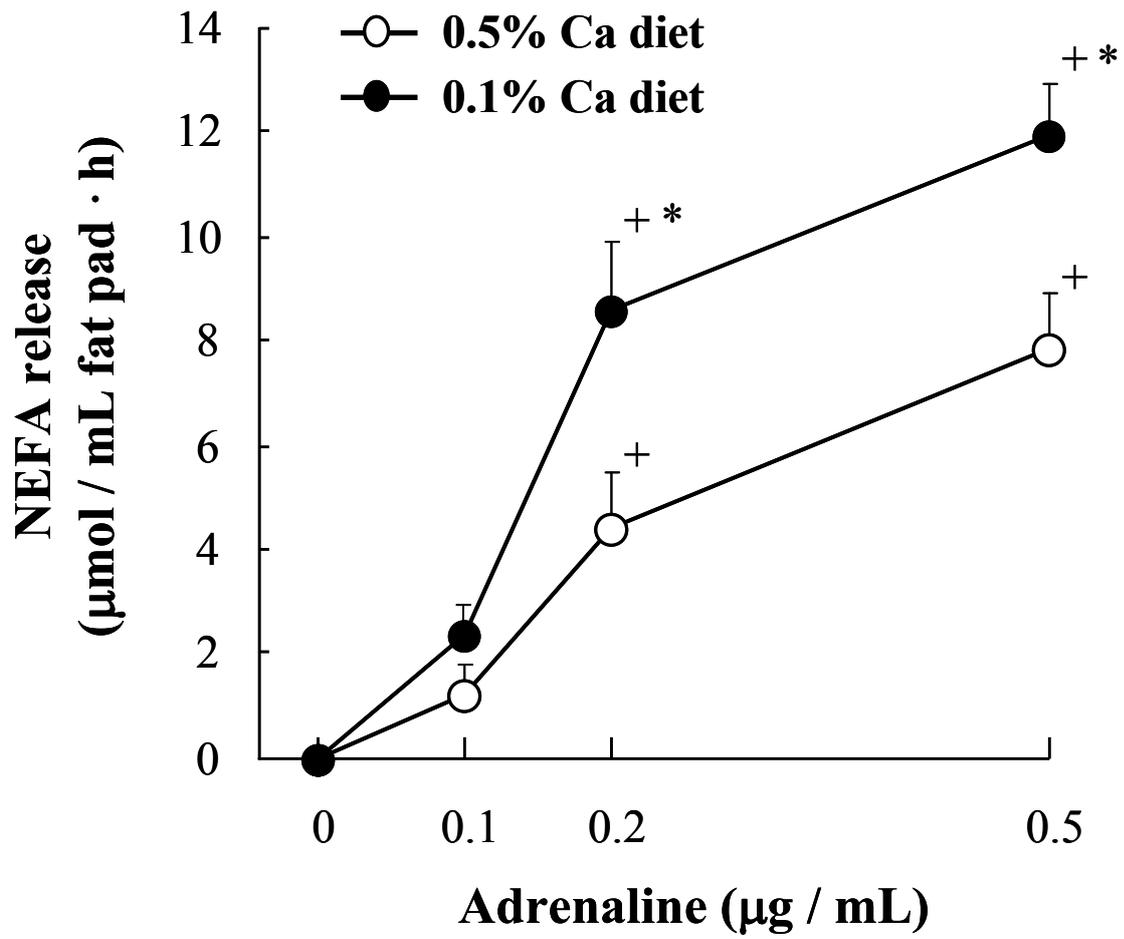


Figure 1

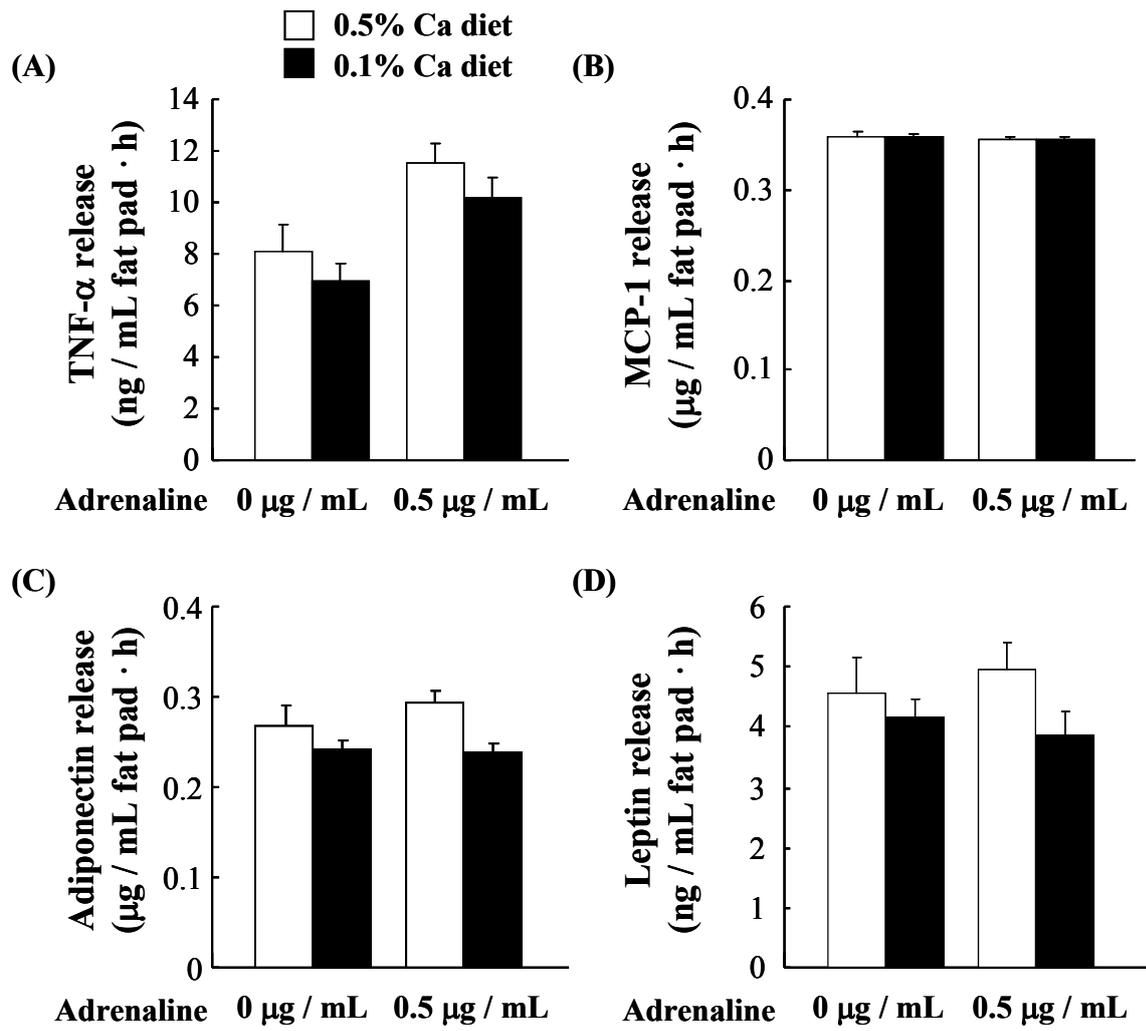


Figure 2

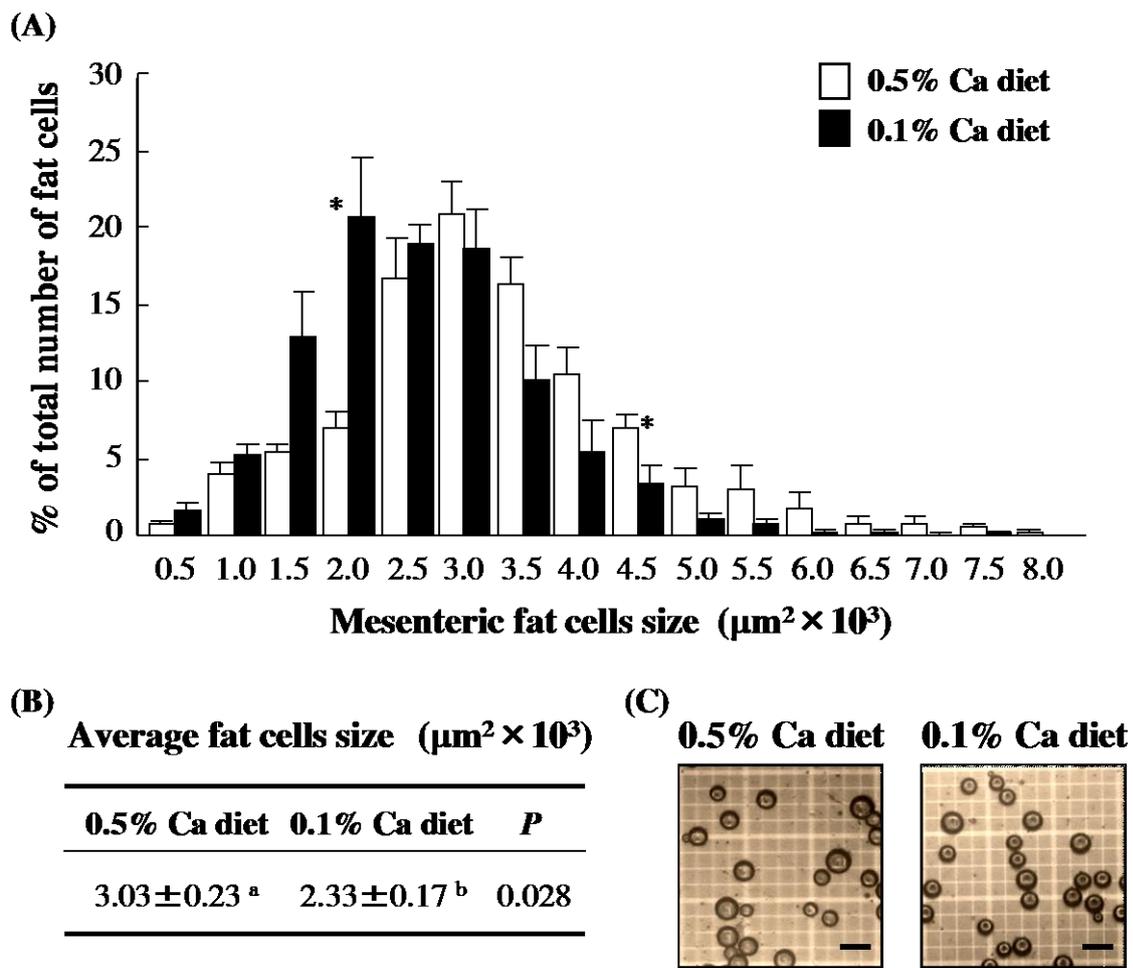


Figure 3

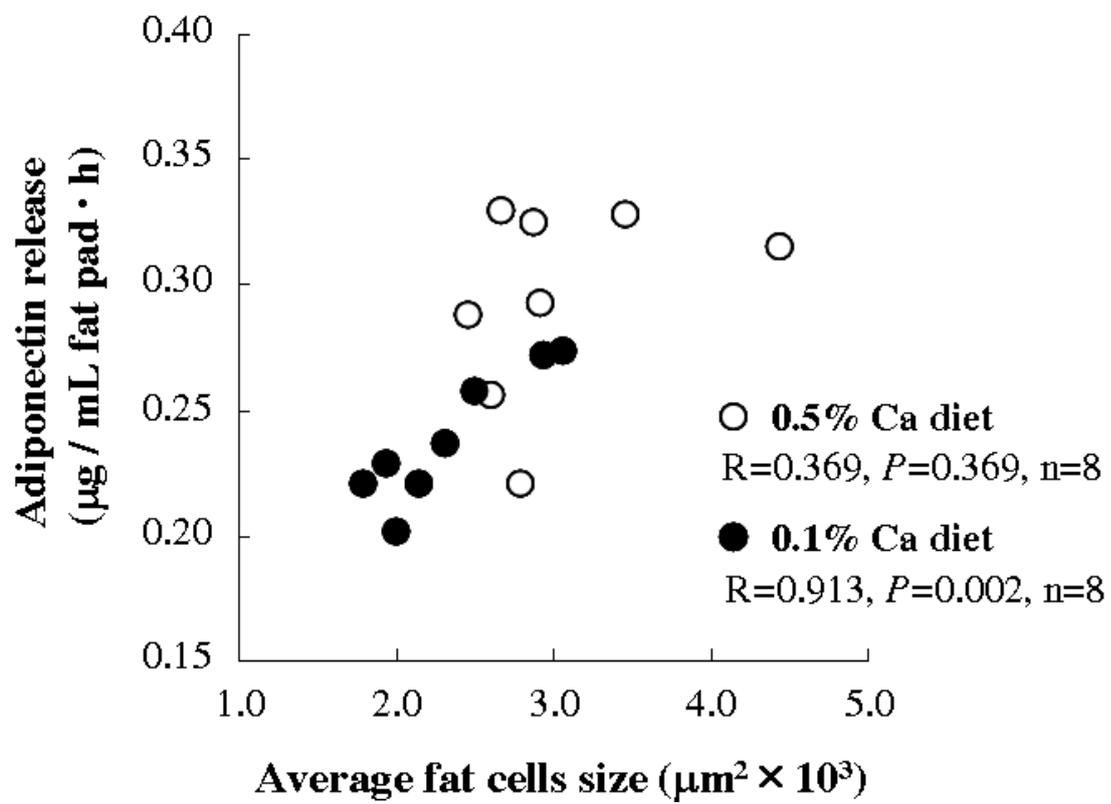


Figure 4