

Full Paper

Markedly Reduced White Adipose Tissue and Increased Insulin Sensitivity in *Adcyap1*-Deficient Mice

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Abstract. Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide implicated in several metabolic functions, including insulin secretion and sympathoadrenal activation. To clarify the roles of PACAP in maintenance of whole-body glucose and lipid homeostasis, the impact of the deletion of PACAP on glucose homeostasis, body weight, and adipose tissue mass was examined by comparing mice lacking the *Adcyap1* gene encoding PACAP (*Adcyap1*^{−/−}) with wild-type littermate controls. *Adcyap1*^{−/−} mice showed significant hypoinsulinemia, although being normoglycemic, and lower body weight as well as reduced food intake. They also showed greatly reduced white adipose tissue mass, in which the mRNA expression of adipocyte fatty acid-binding protein (aP2), a marker of adipocyte differentiation, was decreased. Glucose and insulin tolerance tests revealed increased insulin sensitivity in *Adcyap1*^{−/−} mice. In accordance with these observations, plasma levels of resistin, an adipocytokine implicated in insulin resistance, were decreased in *Adcyap1*^{−/−} mice. After a high-fat dietary challenge for six weeks, *Adcyap1*^{−/−} mice still showed lower body weights and increased insulin sensitivity. These results indicate the crucial roles of PACAP in energy metabolism, including lipid metabolism, and in the regulation of body weight, raising the possibility that the PACAP-signaling pathway that favors energy storage could be a therapeutic target for obesity.

Keywords: adipose tissue, body weight, insulin sensitivity, pituitary adenylate cyclase-activating polypeptide (PACAP), resistin

Introduction

Body weight and the storage of energy in adipose tissue are determined by the interaction between genetic, environmental and psychosocial factors which ultimately act by changing the energy balance, that is, the long-term balance between energy intake and expenditure (1). Studies on the genetics behind treating disorders of

human body-weight regulation and adiposity have made remarkable progress. Since increased adipose tissue is implicated as a primary mechanism underlying the metabolic syndrome that comprises obesity, insulin resistance, glucose intolerance, and dyslipidemia, a growing number of potential new molecular drug targets for treating metabolic syndrome and its components have been found (1, 2). However, since existing medications for these conditions are of limited efficacy, it continues to be important to identify new genes and new regulatory pathways for drug discovery.

Pituitary adenylate cyclase-activating polypeptide (PACAP) (3) is a pleiotropic neuropeptide that belongs

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to a family of structurally related peptides including vasoactive intestinal peptide (VIP), secretin, glucagon, glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic peptide (GIP), and growth hormone-releasing hormone (GHRH) (4–8). PACAP acts as a neurotransmitter or neuromodulator and exerts a plethora of peripheral and central effects influencing whole-body glucose and energy homeostases, including being insulinotropic (9); having insulin-sensitizing properties; influencing thermoregulation, the sympatho-adrenal response (10), and hormonal regulation of lipid and carbohydrate metabolism (11); and participating in the regulation of higher brain functions such as neuropsychological functioning (12, 13).

Our recently developed *Adcyap1*^{−/−} mice, lacking the *Adcyap1* gene encoding PACAP, had profound phenotypes including behavioral abnormalities (12–14). *Adcyap1*^{−/−} mice on a mixed 129/Ola and C57/Bl6 genetic background showed a high pre-weaning mortality rate. Three different knockout lines have been developed separately from our colony (10, 11, 15). The mutant mice prepared by Sherwood's group also showed a greater mortality within the second postnatal week in a wasted state with microvesicular fat accumulation in the liver, skeletal muscle, and heart; and the mice exhibited impaired thermoregulation (11, 16). The mutant mice made by Eiden's group showed a profound insulin-induced hypoglycemia associated with an increased mortality, probably due to impaired long-term secretion of epinephrine (10).

Mortality in *Adcyap1*^{−/−} mice seemed to be even much more severe on a C57/Bl6 background than when using the hybrid 129/Ola and C57/Bl6 background (unpublished observation), hampering the study of PACAP in the adult stage. By contrast, when the null mutation was transferred onto a CD1 (ICR) mouse background, nearly 100% of the mutants survived and still exhibited most of the knockout phenotypes including prominent psychomotor abnormalities (12, 13, 17), enabling us to assess the long-term metabolic consequence of PACAP deficiency.

Here, we have examined the possible alterations in metabolic functions, including glucose homeostasis as well as body weight and white adipose tissue mass in *Adcyap1*^{−/−} mice.

Materials and Methods

Animals

All animal care and handling procedures were approved by the Animal Research Committee of Osaka University. The generation of *Adcyap1*^{−/−} mice using a gene targeting technique has been reported previously

(12). The null mutation was backcrossed onto a CD1 mouse background 6 times. Wild-type and *Adcyap1*^{−/−} mice obtained from the intercross of heterozygous animals were used in this study. Mice were housed in a temperature (23 ± 1°C) and light-controlled room with a 12-h light / 12-h dark cycle (lights on from 8:00 AM to 8:00 PM) and allowed free access to water and food. Food intake was measured by subtracting uneaten food from the initially premeasured food.

Histological analysis of adipose tissues and islet

After mice were anesthetized and perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS), epididymal white adipose tissue was carefully removed, weighed, and cut into 50-μm sections, which were then stained with hematoxylin-eosin. The diameter of adipocytes was determined by digital image analysis using the Scion Image program (Scion Corp., Frederick, MD, USA). Islet histomorphometry was performed as described previously (18).

Reverse transcription-polymerase chain reaction analysis

Total RNA was extracted from epididymal white adipose tissue by using the SV Total RNA Isolation System (Promega, Madison, WI, USA), and semi-quantitative real-time reverse transcription-polymerase chain reaction was performed by using the DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland) and the following primers for adipocyte fatty acid-binding protein (aP2): 5'-TGA TGC CTT TGT GGG AAC CT-3' (sense) and 5'-GCT TGT CAC CAT CTC GTT TTC TCT-3' (antisense). The primers used for GAPDH as an internal control were as follows: 5'-CTC ATG ACC ACA GTC CAT GC-3' (sense) and 5'-CAC ATT GGG GGT AGG AAC AC-3' (antisense).

Measurement of plasma resistin, glucose, insulin, and triglyceride levels, and glucose and insulin tolerance tests

Plasma resistin levels were determined as described previously (19). Plasma glucose, insulin, and triglyceride levels were determined by using the Glucose CII-Test (Wako Pure Chemical Industries, Osaka), Sensitive Rat Insulin RIA kit (Linco Research, St. Louis, MO, USA), and Triglyceride G-test (Wako), respectively. Glucose and insulin tolerance tests were performed as described previously (18).

High-fat diet study

Mice of each genotype were divided at random into 2 groups: the normal diet group was fed with CE-2 (342 kcal/100 g) (CLEA Japan, Inc., Tokyo) and the

high-fat diet group was fed with CE-2 containing 20% beef tallow (429 kcal/100 g) (CLEA Japan) between 6 – 12 weeks of age.

Statistics

Statistical analyses of the results were performed with ANOVA followed by Fisher's PLSD or Student's *t*-test, where appropriate. All values are expressed as the mean \pm S.E.M.

Results

Mendelian segregation of *Adcyap1*^{-/-} mice in a CD1 mouse background

Previous studies showed that the PACAP deficiency is associated with high infant mortality in a mixed 129/Ola-C57/Bl6 mouse background and that this mortality is greatly reduced when a CD1 background is used (12, 17). Indeed, in the present study, virtually all *Adcyap1*^{-/-} mice that had >98% CD1 genetic background survived. Mendelian segregation of genotypes from heterozygous breeding (*n* = 241) was observed at 3 weeks of age (genotype distribution, 24.9%, 51.0%, and 24.1% for wild-type, heterozygous, and *Adcyap1*^{-/-} mice, respectively), indicating no significant selective loss of *Adcyap1*^{-/-} pups. *Adcyap1*^{-/-} mice were still alive until at least 13 weeks of age.

Plasma insulin, glucose, and triglyceride levels; islet histomorphometry; and food intake in *Adcyap1*^{-/-} mice

The levels of plasma insulin, glucose, and triglyceride were determined in 12-week-old *Adcyap1*^{-/-} and wild-type mice in a fed state (Table 1). Although there were no significant differences in plasma glucose and triglyceride levels between the *Adcyap1*^{-/-} and wild-type mice, plasma insulin levels were significantly lower in *Adcyap1*^{-/-} as compared to wild-type mice (1.10 ± 0.14 vs 2.25 ± 0.35 ng/ml, $P < 0.01$).

Histomorphometric analysis of islets showed that the mean islet area slightly increased in *Adcyap1*^{-/-} mice as compared to wild-type mice, although islet number per pancreatic area did not significantly differ between the two groups (Table 1).

In accordance with our previous observation (20), food intake was significantly reduced in *Adcyap1*^{-/-} mice (*Adcyap1*^{-/-}, 4.83 ± 0.25 g/day; wild-type, 5.73 ± 0.20 g/day; *n* = 9 – 11/genotype, 13 weeks of age, $P < 0.05$).

Reduced white adipose tissue mass in *Adcyap1*^{-/-} mice

Nakata et al. (21) demonstrated that the specific PACAP receptor (PAC₁ receptor) is expressed in rat fat tissue and 3T3-L1 adipocytes and that PACAP enhances insulin-induced glucose uptake by 3T3-L1 cells. We have therefore examined the effects of the PACAP deficiency on adipose tissue mass in *Adcyap1*^{-/-} mice. Heart and liver weights were not different between wild-type and *Adcyap1*^{-/-} mice, whereas the weight of epididymal, retroperitoneal, and abdominal subcutaneous white adipose tissues were significantly lower in *Adcyap1*^{-/-} mice as compared to wild-type mice (Fig. 1A). Although body weights of the *Adcyap1*^{-/-} mice were also significantly lower than those of the wild-type mice, the ratio of each adipose tissue weight to total body weight was still significantly lower in *Adcyap1*^{-/-} mice as compared to wild-type mice (Fig. 1B).

Histology of adipose tissue and plasma resistin levels in *Adcyap1*^{-/-} mouse

Histological examination of epididymal adipocytes revealed that adipocytes with an area ranging from 500 – 1500 μm^2 tended to be more prevalent in *Adcyap1*^{-/-} as compared to wild-type mice, although this did not reach a significant difference (Fig. 2: A and B). The mRNA expression of aP2, a marker of adipocyte differentiation (22), was lower in *Adcyap1*^{-/-} adipose tissue (Fig. 2C).

Table 1. Plasma insulin, glucose, and triglyceride levels and islet histomorphometry in *Adcyap1*^{-/-} mice

	Wild-type	<i>Adcyap1</i> ^{-/-}	
Plasma parameters			
Insulin (ng/ml)	2.25 ± 0.35	1.10 ± 0.14	$P < 0.01$
Glucose (mg/dl)	175.1 ± 4.0	170.6 ± 3.9	N.S.
Triglyceride (mg/dl)	177.5 ± 12.7	147.6 ± 15.7	N.S.
Islet histomorphometry			
The mean islet area (mm^2)	0.025 ± 0.002	0.036 ± 0.003	$P < 0.05$
The number of islets (per mm^2 total pancreatic area)	0.226 ± 0.026	0.208 ± 0.009	N.S.

The three plasma parameters were determined in 12-week-old wild-type and *Adcyap1*^{-/-} mice (*n* = 14/genotype). The mean islet area and the number of islets per square millimeter of total pancreatic area were determined in 10.8 \pm 0.5-week-old wild-type and *Adcyap1*^{-/-} mice (*n* = 5/genotype). N.S., not significant.

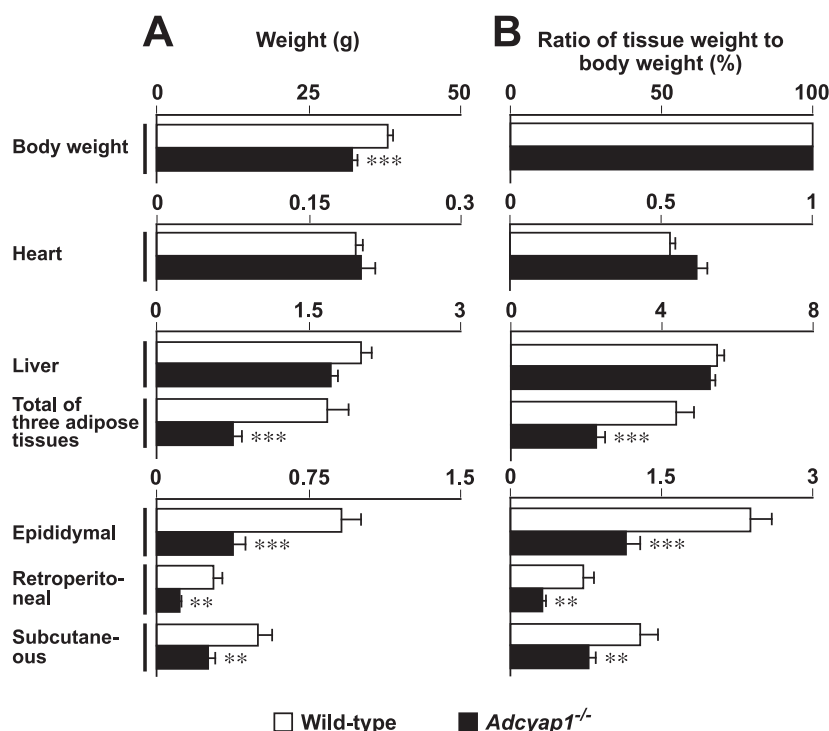


Fig. 1. Reduced white adipose tissue weight in *Adcyap1*^{-/-} mice. Body weight and weights of heart, liver, and white adipose tissues (A), as well as their percentage ratio to body weight (B), were determined in 12-week-old wild-type (open bars) and *Adcyap1*^{-/-} (closed bars) mice (heart and liver, $n = 8$ /genotype; the other, $n = 11$ /genotype). ** $P < 0.01$, *** $P < 0.001$ vs wild-type mice.

The plasma levels of resistin, an adipocytokine implicated in insulin resistance (19, 23), were decreased in *Adcyap1*^{-/-} mice (Fig. 2D). The plasma adiponectin and leptin levels and tumor necrosis factor- α (TNF α) mRNA expression in epididymal white adipose tissue did not significantly differ between the two groups (data not shown).

Glucose and insulin tolerance tests

To explore whether the reduced adipose mass is associated with an increased insulin sensitivity in *Adcyap1*^{-/-} mice, glucose and insulin tolerance tests were performed. The plasma glucose levels after injecting 2 g/kg body weight glucose (Fig. 3A) or 0.5 U/kg porcine insulin (Fig. 3B) were significantly lower in *Adcyap1*^{-/-} as compared to wild type mice, indicating increased insulin sensitivity in *Adcyap1*^{-/-} mice.

Changes in body weight and adipose tissue mass in mice fed on a high-fat diet

The body weight of *Adcyap1*^{-/-} mice was already slightly lower, by approximately 8% ($P < 0.05$) at 4 weeks of age, and this difference was maintained until at least 12 weeks of age (the difference at 12 weeks, approximately 8%, $P < 0.001$; Fig. 4). To examine the role of PACAP in high-fat diet-induced adiposity, we examined body weight gain and white adipose tissue mass in mice fed either a normal or high-fat diet containing 20% beef tallow. Body weights were measured weekly between

4 and 12 weeks of age and mice were challenged with a high-fat diet between 6 and 12 weeks of age (Fig. 4). Although *Adcyap1*^{-/-} mice still showed lower body weight than wild-type mice under the high-fat diet ($P < 0.001$), both genotype groups gained relatively similarly more body weight than those on a normal diet (both genotypes, $P < 0.001$). Intakes of high-fat diet was not significantly changed between both genotype groups (*Adcyap1*^{-/-}, 5.20 ± 0.30 g/day; wild-type, 5.65 ± 0.40 g/day; the difference was not significant).

In parallel, after 6 weeks of a high-fat diet, weights of epididymal, retroperitoneal, and abdominal subcutaneous white adipose tissues in both genotype groups were significantly increased as compared to mice fed on a normal diet (total of the three adipose tissues, wild-type, 1.9-fold; *Adcyap1*^{-/-}, 2.6-fold), while there was no significant change in the weight of the heart (Fig. 5).

Insulin sensitivity after the high-fat dietary challenge

To examine insulin sensitivity in mice fed with the high-fat diet, glucose and insulin tolerance tests were performed. As shown in Fig. 6, plasma glucose levels after the glucose or insulin injection were still significantly lower in *Adcyap1*^{-/-} mice as compared to wild-type mice (glucose tolerance test, $P < 0.001$; insulin tolerance test, $P < 0.001$). The plasma resistin levels were also decreased in *Adcyap1*^{-/-} mice (*Adcyap1*^{-/-}, 32.4 ± 3.1 ng/ml; wild-type, 45.3 ± 4.2 ng/ml; $n = 5 - 6$ /genotype, $P < 0.01$).

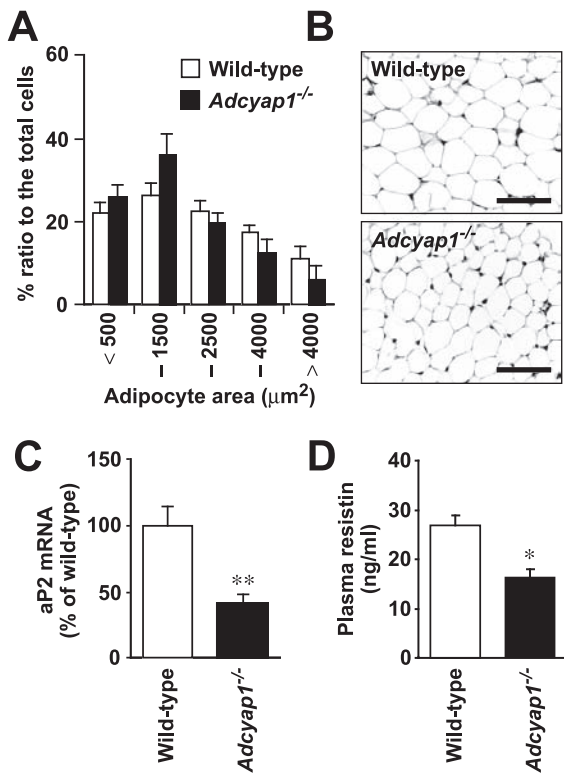


Fig. 2. The morphology of epididymal adipocytes, aP2 mRNA levels, and plasma resistin levels. A: The area histogram of epididymal adipocytes in wild-type (open bars) and *Adcyap1*^{-/-} (closed bars) mice ($n = 6$ /genotype). B: Hematoxylin and eosin staining in sections of epididymal adipose tissue. Scale bars, 100 μm . C and D: aP2 mRNA expression levels in adipose tissues (C) and the plasma resistin levels (D) in wild-type (open bars) and *Adcyap1*^{-/-} (closed bars) mice (aP2 mRNA, $n = 5 - 7$ /genotype; resistin, $n = 6$ /genotype). * $P < 0.05$, ** $P < 0.01$ vs wild-type mice.

Discussion

The present study shows that PACAP deficiency leads to reduced body weight concomitant with a greatly decreased white adipose tissue mass and increased

insulin sensitivity in adult mice. Recently, Nakata et al. (20) have shown that *Adcyap1*^{-/-} mice have a reduced food intake and that PACAP directly activates orexigenic neuropeptide Y neurons, as a likely reason for this reduced food intake. Indeed, neuropeptide Y mRNA levels were significantly lower in the arcuate nucleus (a feeding center) of *Adcyap1*^{-/-} mice. However, the situation is unlikely to be this simple. The direct catabolic and anabolic effects of PACAP on lipid metabolism has been reported in primary rat adipocytes, in which PACAP as well as isoproterenol, induced lipolysis, whereas in the presence of higher levels of insulin, they potentiated the anabolic effects of insulin (24). These dual effects of PACAP have been postulated to be of physiological importance during fasting and postprandial phases.

After a 6-week high-fat dietary challenge, although white adipose tissue mass in *Adcyap1*^{-/-} mice was increased to a similar extent as that in wild-type mice, increased glucose tolerance and insulin sensitivity in the *Adcyap1*^{-/-} mice were still observed relative to wild-type mice. These results suggest that adipose accumulation occurs normally in *Adcyap1*^{-/-} mice but that the PACAP deficiency led to protection against the development of insulin resistance, at least under the present high-fat dietary challenge.

In the present study, we could not determine the insulin levels in the course of glucose tolerance test because the insulin levels were fairly low and therefore a small amount of blood obtained from a tail vein was not enough to measure even by using the insulin radioimmunoassay kit with the highest sensitivity available. Such an examination and measurement of pancreatic insulin content in mice fed with a normal or high-fat diet are important and needs to be addressed in future studies.

Gray et al. (11) revealed that their PACAP knockout mice exhibited microvesicular fat accumulation in the

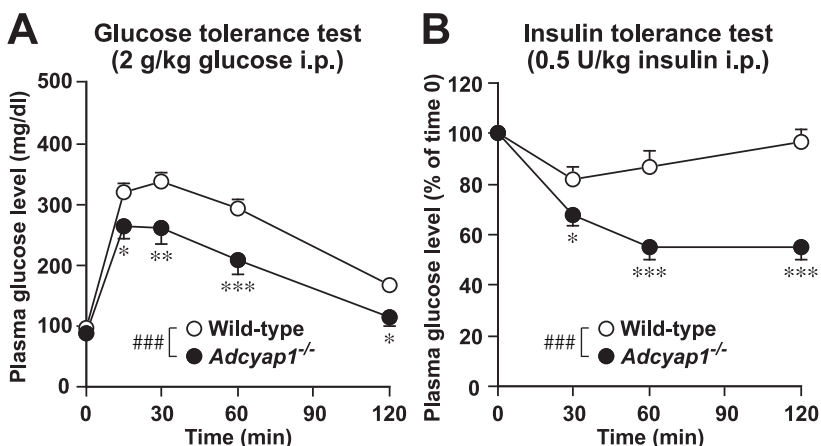


Fig. 3. Glucose and insulin tolerance tests. Glucose (2 g/kg body weight, A) and insulin (0.5 U/kg body weight, B) were intraperitoneally (i.p.) injected into 12-week-old wild-type (open circles) and *Adcyap1*^{-/-} (closed circles) mice, and their plasma glucose levels were determined ($n = 8 - 10$ /group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs wild-type mice at the same time; ### $P < 0.001$.

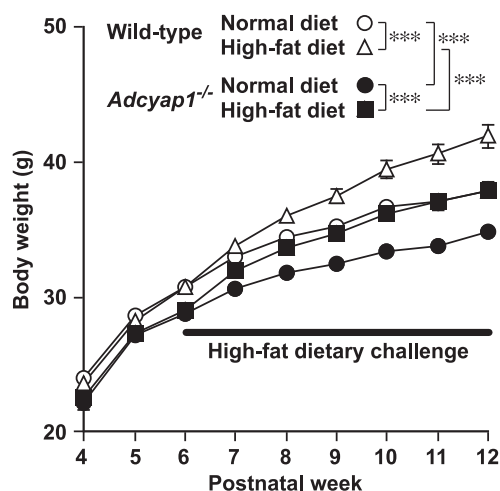


Fig. 4. Body weight changes in mice fed with a normal or high-fat diet. Wild-type (open symbols) and *Adcyap1*^{-/-} (closed symbols) mice were randomly divided into two groups; one group (triangles and squares) was fed the high-fat diet during the dietary challenge period between 6 and 12 weeks of age, while the control group (circles) was fed with normal diet throughout the experimental period. *n* = 36–44/group. ****P* < 0.001.

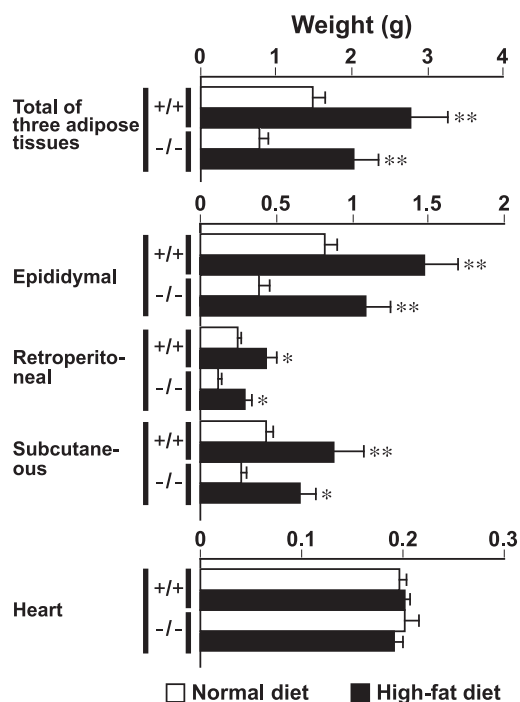


Fig. 5. Weights of white adipose tissues after the high-fat dietary challenge. Wild-type (+/+) and *Adcyap1*^{-/-} (-/-) mice were fed with either a high-fat diet between 6 and 12 weeks of age (closed bars) or normal diet throughout the experimental period (open bars). *n* = 7–8/group. **P* < 0.05, ***P* < 0.01 vs wild-type mice.

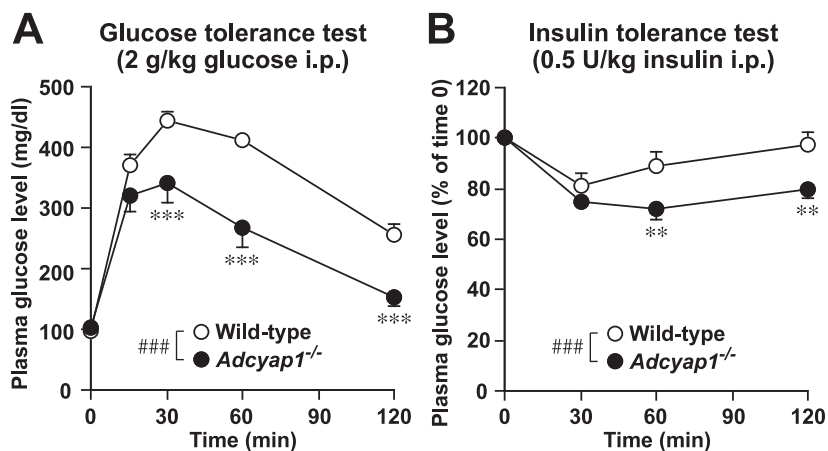


Fig. 6. Glucose and insulin tolerance tests after the high-fat dietary challenge. Glucose (2 g/kg body weight, A) and insulin (0.5 U/kg body weight, B) were intraperitoneally (i.p.) injected into 12-week-old wild-type (open circles) and *Adcyap1*^{-/-} (closed circles) mice fed with high-fat diet during the dietary challenge period between 6 and 12 weeks of age, and their plasma glucose levels were determined (*n* = 8–11/group). ***P* < 0.01, ****P* < 0.001 vs wild-type mice at the same time; ###*P* < 0.001.

liver, skeletal muscle, and heart at 6–8 days of age or at death, as well as elevated serum triglycerides and ketone bodies. Although they did not show the data, the depletion of subcutaneous fat deposits in wasted mutant mice at the time of death has been mentioned, suggesting the increased mobilization of fatty acids from adipose tissue. In the present study, our adult mutant mice on a CD1 background showed no statistically significant difference in, or even slightly lower, plasma triglyceride levels as compared to wild-type mice; nevertheless, they

did exhibit fatty atrophy. In addition, our mutant mice at an adult stage did not exhibit microvesicular fat accumulation in the liver and heart. Therefore, increased fatty acid mobilization seems unlikely to occur in our mutants, at least in their adult life.

The expression of aP2 markedly increases during pre-adipocyte differentiation, contributing as a late marker of differentiation and it has also been detected in pre-adipocytes (22). Mice deficient in aP2 have been shown to develop dietary obesity but do not develop insulin

resistance or diabetes, implicating aP2 in the pathway that links obesity to insulin resistance (25). Markedly reduced aP2 mRNA levels in adipose tissue might be related to the increased insulin sensitivity in our *Adcyap1*^{-/-} mice. The adipocytokine resistin, a small cysteine-rich protein, is one of the major risk factors of insulin resistance in obesity (23). Recently, Nakata et al. (19) produced a new model of insulin resistance without obesity by transiently overexpressing resistin and showed that elevated levels of circulating resistin impaired glucose-induced insulin secretion, which could be ascribable to induction of insulin resistance in islets. Reduced plasma levels of resistin in *Adcyap1*^{-/-} mice, therefore, could lead to the increased insulin sensitivity.

Adcyap1^{-/-} mice showed normoglycemia in spite of marked hypoinsulinemia under the ad libitum-fed state, increased glucose disposal after a glucose challenge, and increased insulin-mediated glucose disposal. These results consistently show increased insulin sensitivity in *Adcyap1*^{-/-} mice, which is likely a consequence of the lean phenotype. Filipsson et al. (26) reported that PACAP has dual functions in regards to glucose homeostasis – PACAP injected intravenously inhibits insulin sensitivity and stimulates insulin secretion with no alteration in net glucose homeostasis. The former could be partly explained by PACAP-induced increase of catecholamine release from adrenal chromaffin cells (27). This possibility was confirmed in *Adcyap1*^{-/-} mice by Hamelink et al. (10). They showed that PACAP has a crucial role in biosynthesis and long-lasting secretion of epinephrine during insulin-induced hypoglycemia. Increased glucose tolerance and insulin sensitivity in *Adcyap1*^{-/-} mice might be due also to increased glucose metabolism in skeletal muscle. This possibility needs to be addressed in future studies.

PACAP binds to three types of G-protein-linked receptors: PACAP-selective (PAC₁) receptor and two receptors that are shared with VIP (VPAC₁ and VPAC₂) (4–6). PAC₁ receptor-deficient mice (28) and VPAC₂ receptor-deficient mice (29) show impaired glucose-induced insulin release; however the former show glucose intolerance, while the latter show normal glucose tolerance. In addition, in PAC₁ receptor-deficient mice, PACAP potentiates glucose-induced insulin secretion just slightly and clearly decreases the glucose elimination rate (28). Therefore, the PACAP-induced insulin resistance influencing net glucose homeostasis could be mediated to a greater extent by the VPAC₂ receptor as compared to the PAC₁ receptor. Furthermore, VPAC₂ receptor-deficient mice exhibit growth retardation in both genders and a slightly reduced fat mass (by 10%) in the female mutants, despite no histological abnormalities in adipose tissues (29). Therefore, VPAC₂

receptor-mediated PACAP signaling may be partly responsible for the decreased adipose tissue in *Adcyap1*^{-/-} mice. Collectively, an association between PACAP and multiple metabolic actions can be suggested, where the relative functions of the PACAP/VIP receptor subtypes may be responsible. Such a consideration might allow for the rational design of future therapeutic drugs for metabolic syndrome.

Our recently developed transgenic mice overexpressing PACAP in islet beta-cells exhibited increased glucose-induced insulin release and normal glucose tolerance, as well as an increase in BrdU-positive beta-cells after streptozotocin injection (30). We also crossed these transgenic mice with lethal yellow KKA^y mice, a genetic model for obesity-diabetes syndrome and showed that PACAP overexpression inhibited the islet hyperplasia seen in KKA^y mice (18). These results suggest the potential implication of PACAP in the long-term regulation of islet beta-cell mass and function. In line with this possibility, histological examination of islets showed that the mean islet area slightly increased in *Adcyap1*^{-/-} mice, although islet number per unit pancreatic area, and total islet mass also, did not significantly differ between *Adcyap1*^{-/-} and wild-type mice.

PACAP is structurally highly conserved during evolution, implying its vital importance (4, 5). This coincides with the findings showing PACAP deficiency leads to defects in many important biological processes. The present results further indicate the crucial roles of PACAP in metabolic processes, including lipid metabolism and in the regulation of body weight, raising the possibility that the PACAP-signaling pathway that favors energy storage could be a therapeutic target for obesity.

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