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Obesity and mild hyperinsulinemia found in neuropeptide Y-Y1 receptor-deficient mice

(uncoupling protein/insulin/weight gain)

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ABSTRACT **To elucidate the role of neuropeptide Y (NPY)-Y1 receptor (Y1-R) in food intake, energy expenditure, and other possible functions, we have generated Y1-R-deficient mice (Y1-R$^{-/-}$) by gene targeting. Contrary to our hypothesis that the lack of NPY signaling via Y1-R would result in impaired feeding and weight loss, Y1-R$^{-/-}$ mice showed a moderate obesity and mild hyperinsulinemia without hyperphagia. Although there was some variation between males and females, typical characteristics of Y1-R$^{-/-}$ mice include: greater body weight (females more than males), an increase in the weight of white adipose tissue (WAT) (approximately 4-fold in females), an elevated basal level of plasma insulin (approximately 2-fold), impaired insulin secretion in response to glucose administration, and a significant changes in mitochondrial uncoupling protein (UCP) gene expression (up-regulation of UCP1 in brown adipose tissue and down-regulation of UCP2 in WAT). These results suggest either that the Y1-R in the hypothalamus is not a key molecule in the leptin/NPY pathway, which controls feeding behavior, or that its deficiency is compensated by other receptors, such as NPY-Y5 receptor. We believe that the mild obesity found in Y1-R$^{-/-}$ mice (especially females) was caused by the impaired control of insulin secretion and/or low energy expenditure, including the lowered expression of UCP2 in WAT. This model will be useful for studying the mechanism of mild obesity and abnormal insulin metabolism in noninsulin-dependent diabetes mellitus.**

Neuropeptide Y (NPY) is an important regulator in the central and peripheral nervous system (1, 2). It modulates numerous physiological processes, including feeding behavior, anxiety, blood pressure, and circadian rhythm (3–8). Among its various biological functions, that of controlling feeding has been attracting the attention of researchers and clinicians who study obesity and diabetes seeking new therapies. NPY has been proven to be one of the major affecters of leptin in a study of NPY-null *ob/ob* mice, which exhibited attenuated obesity to some extent (9). Studies of various organs and cell types using peptide fragments of NPY have indicated that multiple NPY receptor subtypes exist. NPY receptors have been classified into at least six receptors: Y1, Y2, Y3, Y4, Y5, and Y6 (10). Though pharmacological studies using various NPY analogs have suggested that it is quite likely that the NPY-Y1 (Y1-R) (11, 12) and/or the NPY-Y5 receptors (Y5-R) (13) are involved in the control of feeding, there is some controversy about which of them is the key molecule. Another interesting point regarding Y1-R is its wide spectrum of expression in various tissues, including pancreas, lung, kidney, heart, bone marrow cells, skeletal muscle, and so on (14). We are very interested in the as yet undiscovered biological functions of Y1-R in various tissues. To address these questions, we have investigated the influence of Y1-R deficiency on feeding behavior, energy expenditure, and other possible functions by creating Y1-R$^{-/-}$ mice. Contrary to our expectation that the lack of NPY signaling through Y1-R would result in impaired feeding and weight loss, Y1-R$^{-/-}$ mice exhibited mild obesity without any change in feeding behavior, mild hyperinsulinemia, impaired insulin secretion in response to hyperglycemia, and a significant decrease in uncoupling protein (UCP) 2 expression in white adipose tissue (WAT).

MATERIALS AND METHODS

Targeting Vector Constructs. Upon screening of a mouse 129/SVJ genomic library (Stratagene) with the mouse Y1-R cDNA as a probe, we obtained five positive clones (14). To construct the targeting vector, one of these clones, a 10.5-kbp fragment containing exons 2 and 3, was used. A *Hin*dIII–*Xho*I fragment (3.0 kbp) containing exons 2 and 3 was inactivated by substitution with a neomycin-resistance gene driven by the thymidine kinase (TK) gene promoter. The resulting targeting vector contained 5′ and 3′ homology regions of 4.0 and 3.5 kbp, respectively. The TK gene for negative selection also was ligated to the 5′ end of the targeting vector (Fig. 1*A*).

Embryonic Stem (ES) Cell Line and Cell Culture. The ES cell line E14–1 was transfected with the linearized targeting vector, and selection was carried out with G418 (300 μg/ml, GIBCO) and Gancyclovir (2 μM, Syntex Cytovene). After 6 days, drug-resistant clones were individually expanded, and their genomic DNAs were prepared for Southern blot analysis. The targeted clones of the ES cells were selected by PCR using the primers of neomycin-resistance gene (5′-CGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGC-3′) and the upstream sequence of the left side arm (5′-CTAAGCAGCTTGCCTCAAGAACTTTTGGTTTCCTC-3′), and by Southern blot analysis using the 1-kbp *Bam*HI–*Kpn*I fragment as a probe (Fig. 1*A*).

Production of Germ-Line Chimeras. The targeted ES cells were injected into blastocysts of C57BL/6 mice, which then were transplanted into the uteri of pseudopregnant foster mothers. The resulting chimeric mice, which contain a significant contribution from the targeted ES cells, were crossed with C57BL/6 females to obtain the germ-line transmission of the mutant Y1-R allele.

DNA and RNA Analyses. Genomic DNA was prepared from the tail of agouti offspring for genotyping by PCR and Southern blot analyses. The primer sequences were: wild-type primer 1 (P1), 5′-GCGACTGGGCTAGCCTGCTTCAGAGACGTCTTGGA-3′; wild-type primer 2 (P2), 5′-GCCACCTGCCCTTG-

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Abbreviations: NPY, neuropeptide Y; Y1-R, Y1 receptor; WAT, white adipose tissue; UCP, uncoupling protein; ES, embryonic stem; BAT, brown adipose tissue.

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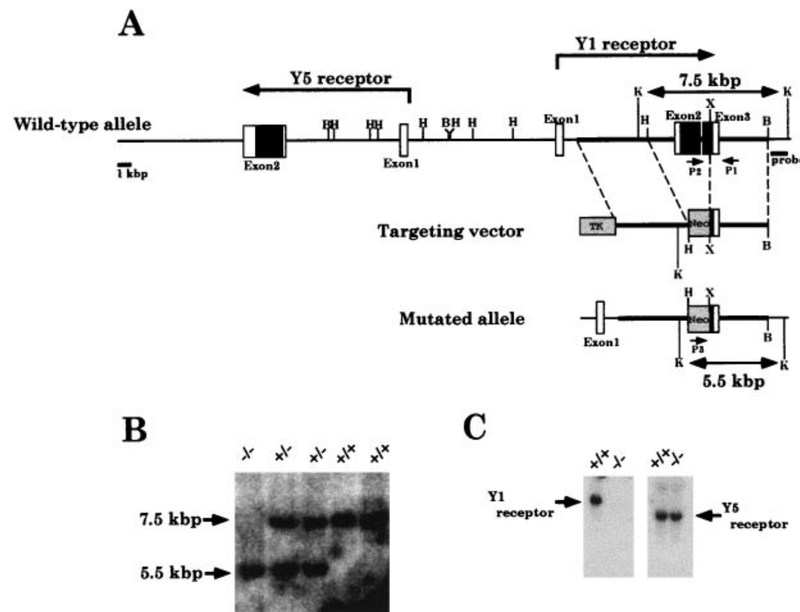


FIG. 1. Disruption of Y1-R gene in mice. (*A*) Partial restriction map of the allele of wild-type Y1-R and Y5-R genes (*Top*), the targeting construct (*Middle*), and the predicted homologous recombinant allele (*Bottom*). The closed and open boxes indicate coding and noncoding regions in the exons, respectively. Pertinent restriction enzyme sites are noted (B, *Bam*HI; H, *Hin*dIII; X, *Xho*I; K, *Kpn*I). P1, P2, and P3 indicate the positions of the PCR primers used in genotyping. (*B*) Southern blot analysis of DNA from the tails of Y1-$R^{+/+}$, Y1-$R^{+/-}$, and Y1-$R^{-/-}$ mice using the 3′ probe indicated in *A*. Wild-type (7.5 kbp) and recombinant (5.5 kbp) *Kpn*I-digested fragments were identified. (*C*) Northern blot analysis of Y1-R and Y5-R mRNA expressions in Y1-$R^{+/+}$ and Y1-$R^{-/-}$ mouse brain. Y1-R and Y5-R mRNAs were identified by using Y1-R and Y5-R cDNAs as probes, respectively (14, 15).

GCTGTGATATTCACCTTGGCT-3′; and mutant primer 3 (P3), 5′-GCCCATTCGCCGCCAAGCTCTTCAGCAATAT-CACG-3′ (Fig. 1*A*). Total RNAs were isolated from brain, brown adipose tissue (BAT), WAT, and skeletal muscle by using an RNA isolation kit (Stratagene). Northern blot analysis of the Y1-R and Y5-R mRNAs in the brain was carried out by using the exon 2 region of each receptor as a probe. The Y1-R and Y5-R probes were generated by PCR using the primers 5′-GAACTC-AACTCTGTTCTCCAAGGTTG-3′ and 5′-GTTGCAGGTG-GCAATGATCTGGTG-3′ for Y1-R, and the primers 5′-CCTGATTGGGCTCTATACATTTGTAA-3′ and 5′-CT-GAGCATCTGAGCTTTCTTCAGG-3′ for Y5-R, respectively. Mitochondrial UCP1, UCP2, and UCP3 mRNAs in the BAT, WAT, and skeletal muscle were detected by standard Northern blot techniques. The UCP1 probe was generated from mouse skeletal muscle cDNAs by PCR using two primers (5′-AG-GTCGTGAAGGTCAGAATGCAAGC-3′ and 5′-TTATGTG-GTACAATCCACTGTCTGCC-3′). The UCP2 and UCP3 cDNAs (the entire ORFs) were donated by J.-L. Chen and N. Zhang (Tularik, South San Francisco) and were used as probes.

Measurement of Body Weight, Food Intake, and Body Temperature. The mice were housed individually and fed with powdered CRF-1 (Oriental Yeast, Osaka), which is a standard chow for mice. Body weight and food consumption were measured once a week from the fourth to the 24th weeks after birth. Feeding efficiency was determined according to the method described by Ohki-Hamazaki *et al.* (16). Mice (6–8 weeks) were fed an amount of CRF-1 equal to 5% of body weight, and their body weights were measured every day for 4 days. A Pocket Scanner system (BioMedic, Seaford, DE) was used to measure body temperature. Microcapsules containing a temperature sensor were placed in the infrasplenic space of female mice 13–19 weeks old. To minimize artificial changes in body temperature caused by fear of the sensor probe, we accustomed the mice to the probe by frequently placing it in them for 2 weeks before the actual measurement.

Blood Analysis. Blood glucose levels were measured by the hexokinase/G6PD method; plasma triacyl glycerol was determined by the modified method of Esders and Goodhue using a Monarch analysis system (Eli Lilly); and plasma insulin levels were determined by ELISA (Morinaga, Tokyo, Japan).

Glucose Tolerance Test. For this analysis, 16 wild-type (six males and 10 females) and 18 deficient (eight males and 10 females) mice at 29 weeks were used. Half of the wild-type and deficient mice were given 30% glucose/water instead of water for 2 weeks, and the others were given plain water. After 2 weeks (31 weeks of age), the glucose and insulin levels in their plasma were measured. At the same time, urine was collected from all of them, and their glucose levels were measured by an UIEACE-Kc (Terumo, Somerset, NJ).

Pathological Examination. Necropsies were performed on mice. After the weights of major tissues, such as heart, thymus, liver, kidney, spleen, testis, and ovary, were measured, the ratio of the each tissue weight relative to the body weight was calculated. These tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4–5 microns, and then stained with hematoxylin and eosin. These preparations were examined under a light microscope.

RESULTS

Generation and Characterization of Y1-R-Deficient Mice. The Y1-R gene is located in the 8B3-C2 region of mouse chromosome and consists of three exons (14, 15). We disrupted the Y1-R gene by replacing part of an internal exon with a thymidine kinase-neomycin-resistance cassette. The targeting strategy for this disruption is shown in Fig. 1*A*. This DNA construct was introduced into E14–1 cells, and transfectants were selected with G418. A Southern blot analysis revealed that three clones from among the G418-resistant colonies had undergone homologous recombination. Cells from the these targeted clones were injected into C57BL/6 blastocysts, and the embryos were reimplanted into foster animals to produce chimeric mice. Male chimeras containing a substantial number of 129/SVJ cells were bred to C57BL/6 females to produce F_1 129/B6 offspring containing the targeted Y1-R allele. Heterozygous F_1 mice were interbred, and their offspring were genotyped by PCR using the primers shown in Fig. 1*A* (data not shown) and by Southern blot analysis (Fig. 1*B*). A Northern blot analysis showed the 4.0-kb Y1-R mRNA to be

present in the brains of wild-type (Y1-$R^{+/+}$) mice, but absent in homozygous mutant (Y1-$R^{-/-}$) mice (Fig. 1*C*). Because mouse Y1-R and Y5-R genes are located close to each other (15), we were concerned that the disruption of Y1-R might affect the expression of Y5-R. Indeed, the 5′ noncoding exon of the mouse Y1-R gene is about 10 kbp upstream from the transcriptional initiation site of the Y5-R gene and has an opposite orientation (Fig. 1*A*). However, our concerns proved baseless, and we found the expression of the Y5-R gene in the brain of Y1-$R^{-/-}$ mice to be intact (Fig. 1*C*). The distribution of genotypes among F_2 progeny was in accordance with the Mendelian rule (Y1-R +/+:+/−:−/− = 31:56:25). The Y1-$R^{-/-}$ mice were viable and fertile. Their major tissues, such as liver, kidney, heart, thymus, spleen, and testis, exhibited neither macroscopic nor microscopic change. No abnormality, such as monocytes, lymphocytes, neutrophils, eosinophils, basophils, reticulocytes, or platelets, was found in the blood cell composition in spite of the high expression of the Y1-R gene in bone marrow cells (14).

Body Weights of Y1-R-Deficient Mice. The group of Y1-$R^{-/-}$ mice started to become heavier, on average, than Y1-$R^{+/+}$ mice after 9 weeks; and this weight gain was more pronounced in females (Fig. 2*B*). Indeed, the body weight of Y1-$R^{-/-}$ females was 26.9% ($P < 0.05$) greater than that of Y1-$R^{+/+}$ mice at 24 weeks (Fig. 2*B*). In males, although the slight increase in body weight of Y1-$R^{-/-}$ mice at 24 weeks is not statistically significant (Fig. 2*A*), the rate at which Y1-$R^{-/-}$ males gained weight between 6 and 24 weeks was significantly greater than that of Y1-$R^{+/+}$ mice (Fig. 2*C*). We compared the weight of the fatty tissues of 31-week-old Y1-$R^{-/-}$ and Y1-$R^{+/+}$ mice. The weights of the ovarian and retroperitoneal fat pads of Y1-$R^{-/-}$ females were approximately 3.9-fold and 4-fold greater than those of the control mice, respectively (Fig. 2*F*). In contrast, the weights of the epedidymal and retroperitoneal fat pads of Y1-$R^{-/-}$ males were almost the same as those of Y1-$R^{+/+}$ mice (Fig. 2*E*).

Food Consumption. We measured the volume of food intake of Y1-$R^{-/-}$ and Y1-$R^{+/+}$ mice on a weekly basis for 20 weeks. No statistically significant increase in food intake was observed in Y1-$R^{-/-}$ mice in spite of the increase in body weight, even though murine models of obesity generally exhibit hyperphagia (Fig. 2*D*).

Analyses of Serum Glucose, Insulin, and Triacyl Glycerol. Blood was collected from mice given food and water when they were 11, 15, 24, and 31 weeks of age, and the blood glucose, plasma insulin, and triacyl glycerol levels were measured. As shown in Fig. 3 *A* and *B*, both male and female mutant mice exhibited moderate hyperinsulinemia at all of the ages we examined, even though there was no significant difference in blood glucose or triacyl glycerol levels between mutant and wild-type mice (data not shown). In mice at the age of 31 weeks, we also found no change in plasma cholesterol, serum T3 and T4 levels between mutant and control mice (data not shown). Furthermore, to determine the mechanism of the mild hyperinsulinemia exhibited in Y1-$R^{-/-}$ mice, we performed oral glucose tolerance tests. In these tests, 29-week-old wild-type and deficient mice were given a 30% glucose solution instead of plain water for 2 weeks. At 31 weeks, neither Y1-$R^{-/-}$ nor Y1-$R^{+/+}$ mice showed glycosuria (data not shown) or marked hyperglycemia (Fig. 4*B*). In Y1-$R^{+/+}$ mice the administration of the 30% glucose solution led to the significant increase in the insulin levels by 2.1-fold (males) and 3.6-fold (females) compared with Y1-$R^{+/+}$ mice given plain water, whereas the slight change in the insulin level was observed in Y1-$R^{-/-}$ mice (Fig. 4*A*). To estimate the capability of insulin secretion in response to glucose administration, the ratio of the insulin level to the plasma glucose level was calculated. As shown in Fig. 4*C*, the administration of the 30% glucose solution led to the increase in the insulin/glucose ratio in Y1-$R^{+/+}$ mice by 1.5-fold (males) and 3.3-fold (females) compared with Y1-$R^{+/+}$ mice given plain water. On the other hand, these significant changes in the insulin/glucose ratio were not observed in Y1-$R^{-/-}$ mice given the glucose solution (Fig. 4*C*). Although these results are not statistically significant, it is likely

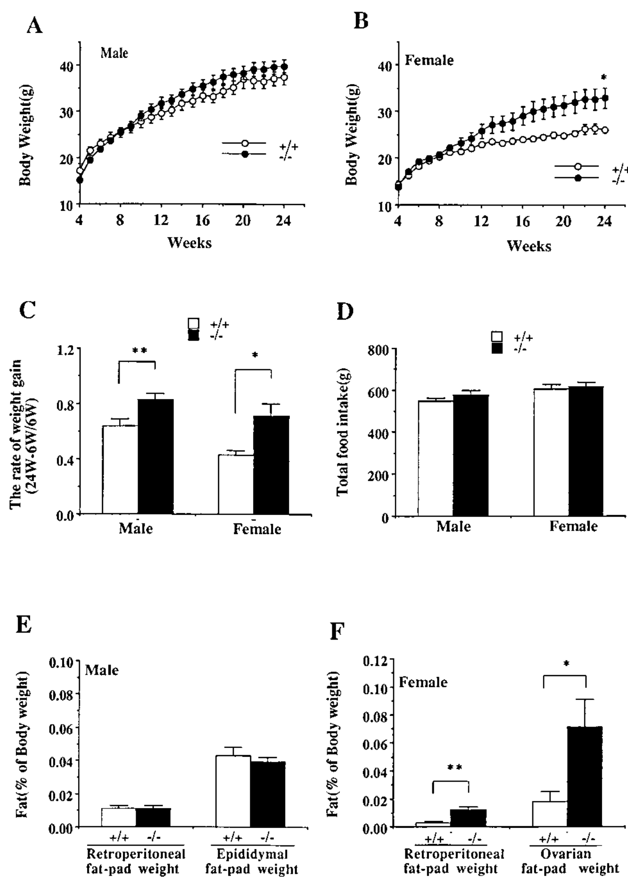


FIG. 2. Body weight, food consumption, and adipose tissue mass of Y1-$R^{+/+}$ and Y1-$R^{-/-}$ mice. (*A* and *B*) Growth curves of Y1-$R^{+/+}$ (males: $n = 8$; females: $n = 7$) and Y1-$R^{-/-}$ (males: $n = 10$; females: $n = 8$) mice from 4 to 24 weeks of age (*A*: males; *B*: females). Each data point is the mean ± SE, and * indicate values of statistical significance ($P < 0.05$), as determined by two-tailed unpaired Student's *t* tests. (*C*) The rate of weight gain between 6 and 24 weeks of Y1-$R^{-/-}$ and Y1-$R^{+/+}$ mice. Each data point is the mean ± SE, and * indicate values of statistical significance (*, $P < 0.05$, **, $P < 0.01$), as determined by two-tailed unpaired Student's *t* tests. (*D*) The food intake of Y1-$R^{+/+}$ (males: $n = 8$; females: $n = 7$) and Y1-$R^{-/-}$ (males: $n = 10$; females: $n = 8$) mice were monitored over a 20-week period (4–24 weeks). All values are means ± SE. A two-tailed unpaired Student's *t* test was used for statistical analysis. (*E* and *F*) After measurement of body weight, the fat pads from the indicated sites were weighed (*E*: males; *F*: females). *, $P < 0.05$; **, $P < 0.01$.

that the responsiveness of Y1-$R^{-/-}$ mice to large amounts of glucose was impaired. Taken together, these results suggest that a Y1-R deficiency triggers abnormal regulation of insulin secretion.

Feed Efficiency in Y1-R-Deficient Mice. These findings raised the possibility that a Y1-R defect reduces energy expenditure and creates an abnormal energy balance, resulting in a moderate increase in body weight and excessive storage of fat. We then analyzed the rate of weight loss when food intake was restricted. Y1-$R^{-/-}$ and Y1-$R^{+/+}$ mice (6–8 weeks) were fed an amount of powdered CRF-1 equal to 5% of body weight for 3 days. Initially, the body weight of the Y1-$R^{-/-}$ mice (23.8 ± 1.7 g) was similar to that of the Y1-$R^{+/+}$ mice (21.1 ± 1.2 g, $P = 0.22$). After 3 days, the rate of weight loss was found to be significantly lower for Y1-$R^{-/-}$ mice than for $Y1R^{+/+}$ mice (Fig. 5). These results suggest that the Y1-$R^{-/-}$ mice expended less energy, resulting in moderate obesity. Because the Y1-$R^{-/-}$ mice started to expend less energy when they were young and in the preobesity stage, they were able to accumulate energy over a long period and become moderately obese.

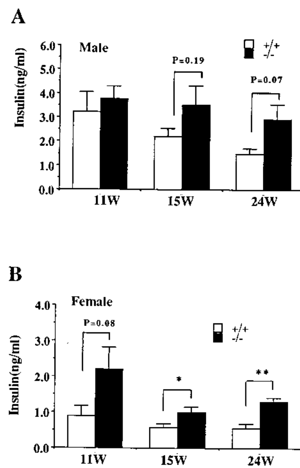


FIG. 3. Analysis of the plasma insulin for Y1-R$^{+/+}$ and Y1-R$^{-/-}$ mice. Insulin levels for each type of mouse at ages 11, 15, and 24 weeks (*A*: males; *B*: females). The blood was collected from Y1-R$^{+/+}$ (males: n = 8; females: n = 7) and Y1-R$^{-/-}$ (males: n = 10; females: n = 8) mice provided with food and water. All values are means ± SE. A two-tailed unpaired Student's t test was used for statistical analysis. *, $P < 0.05$; **, $P < 0.01$.

Thermoregulation in Y1-R-Deficient Female Mice. To assess whether thermogenesis was lower in mutant female mice, we measured their body temperature. When the ambient temperature was 23°C, the body temperature of mutant females (37.0 ± 0.1°C, n = 6) was essentially the same as that of the controls (36.9 ± 0.1°C, n = 7, P = 0.71) (Fig. 6*A*). Moreover, no abnormal thermogenesis was observed in mutant females exposed to a temperature of 4°C for 55 h (Fig. 6*B*), indicating that nonshivering thermogenesis remained unaffected in Y1-R-deficient mice.

It has been suggested that mitochondrial UCPs are involved in the control of body temperature through their ability to regulate thermogenesis (17). A consideration of the basis for energy expenditure in an organism has pointed to UCPs as an important site of facultative energy expenditure. To further investigate whether the lack of a functional Y1-R could be involved in changes in the expression of UCP genes, the expressions of the UCP1, UCP2, and UCP3 genes in BAT, WAT, and skeletal muscle were examined in mutant and wild-type female mice kept at a temperature of 23°C. As shown in Fig. 7 *A* and *B*, we found that the levels of UCP1 mRNA in BAT was higher in mutant mice than in the wild type, and that there was a dramatic decrease in UCP2 expression in the WAT of mutant mice. In addition, there was a moderate increase in the UCP2 mRNA levels of skeletal muscle in mutant females, although we found no significant change in UCP3 expression in that tissue.

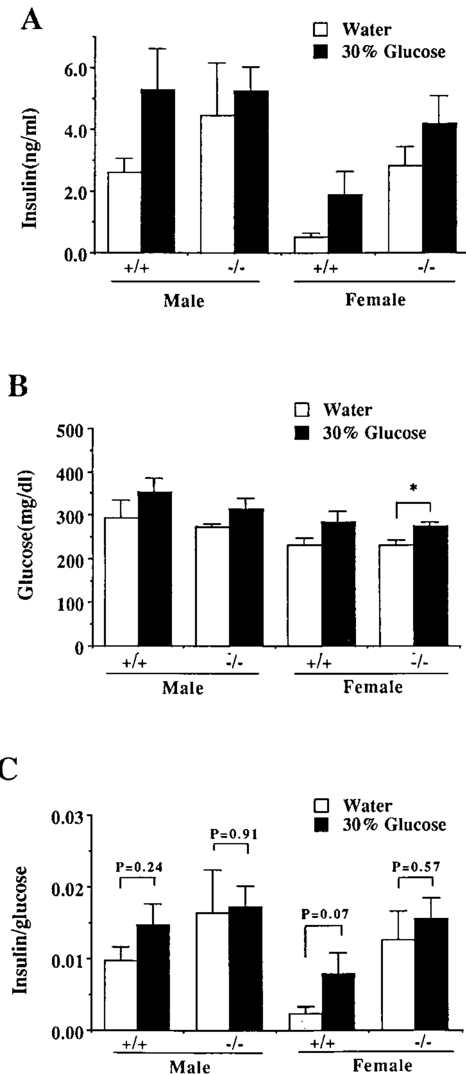


FIG. 4. Glucose tolerance tests for Y1-R$^{+/+}$ and Y1-R$^{-/-}$ mice at 31 weeks of age. Half of wild-type (three males and five females) and deficient (four males and five females) mice (29 weeks of age) were given a 30% glucose solution instead of plain water for 2 weeks. Insulin (*A*) and glucose (*B*) levels with or without the administration of 30% glucose, respectively (*, $P < 0.05$). (*C*) The effect of the administered glucose on insulin secretion. The ratio of plasma insulin level (*A*) to blood glucose level (*B*) (insulin/glucose ratio) was estimated to determine the capability of insulin secretion in response to glucose administration. All values are means ± SE. A two-tailed unpaired Student's t test was used for statistical analysis.

DISCUSSION

NPY receptor antagonists have been developed for therapeutic intervention against obesity, because pharmacological studies using various NPY analogs suggest that feeding is mediated by Y1-R and/or Y5-R (11–13). However, it is not yet fully understood which NPY receptor is important in feeding control because of the many inconsistent results on the relationship between Y1-R/Y5-R and feeding. Recent reports indicate that Y1-R antagonists successfully inhibits NPY-induced feeding in rats, whereas Y5-R antagonists do not (18–20).

In the present study, our data suggest that the lack of NPY signaling through Y1-R results in moderate obesity and mild hyperinsulinemia without hyperphagia. The degree of obesity found in Y1-R$^{-/-}$ mice was as mild (≈27%) as that found in mice deficient of bombesin receptor subtype 3 (16) or metallothionein I and II (21). Although the latter types exhibited hyperphagia, the food intake of Y1-R$^{-/-}$ mice remained unchanged. So, the question is: what happens to the signal pathway of leptin/NPY in feeding control when mice lose Y1-R? One possible reason that feeding is unaffected might be that other NPY receptors, such as Y5-R, compensate for the Y1-R deficiency. This theory will be tested by generating Y5-R-null Y1-R$^{-/-}$ mice. Another possibility is that the role of the leptin/NPY pathway in feeding control is rather minor relative to that of many other factors such as orexins (22) and cocaine- and amphetamine-regulated transcript (23), so that we might not see any change in feeding behavior in Y1-R$^{-/-}$ mice. This speculation is consistent with the result that NPY$^{-/-}$ mice exhibit normal food intake (24). There is an inconsistency between the results showing the successful inhibition of feeding by Y1-R antagonists and the negative results on feeding for Y1-R$^{-/-}$ mice. One explanation is that the Y1-R deficiency existed even before birth and might have been compensated by other mechanisms as the mice grew, whereas the challenge from the Y1-R antagonist can only inhibit feeding effectively when NPY signaling is used as a main route in feeding

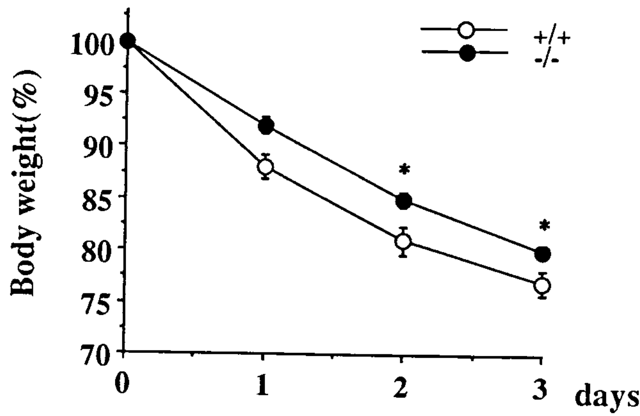


FIG. 5. Feeding efficiency of Y1-R$^{+/+}$ and Y1-R$^{-/-}$ mice. To analyze the rate of weight loss when food was restricted, Y1-R$^{-/-}$ (n = 6) and Y1-R$^{+/+}$ (n = 10) mice at 6–8 weeks of age were fed an amount of CRF-1 powder equal to 5% of body weight. All values are means ± SE. A two-tailed unpaired Student's t test was used for statistical analysis. *, $P < 0.05$.

control. The conditional knockout of Y1-R would help to clear up this ambiguity.

Recently, Marsh *et al.* (25) reported that mice lacking Y5-R developed mild late-onset obesity characterized by increased body weight, food intake, and adiposity. They also showed that food intake induced by intracerebroventricular administration of NPY was much lower in Y5-R$^{-/-}$ mice. Although both Y1-R- and Y5-R-deficient mice developed higher body weight and increased body fat compared with wild-type mice, different mechanisms seemed to be involved because Y5-R$^{-/-}$ mice exhibited hyperphagia, whereas Y1-R$^{-/-}$ mice did not. The development of obesity in Y1-R$^{-/-}$ and Y5-R$^{-/-}$ mice also displayed sex-dependent variations. The late-onset obesity was most pronounced in female Y1-R$^{-/-}$ and male Y5-R$^{-/-}$ mice. With respect to the gender difference in the development of obesity, it was reported that female $\beta 3$ adrenergic receptor-deficient mice tend to have more body fat than male mice (26). The difference in fat storage between males and females might be caused by a difference in hormones. Especially in females, these findings raise the possibility that a defect in NPY signaling through Y1-R might be a contributory trigger for reduced energy expenditure and abnormal energy balance, resulting in a moderate increase in body weight and the excessive storage of body fats.

UCPs play an important role in generating heat and burning calories by creating a pathway that allows the dissipation of the proton electrochemical gradient across the mitochondrial inner membrane of mammalian tissues. This process protects organisms against cold and regulates energy balance (27). UCPs have been classified into at least three types: (*i*) UCP1, by virtue of its unique expression in BAT, may primarily mediate cold-induced thermogenesis to maintain body temperature (28, 29); (*ii*) UCP2 is expressed in many tissues, including sites not thought to

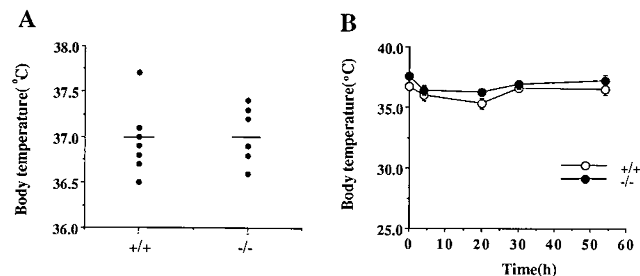
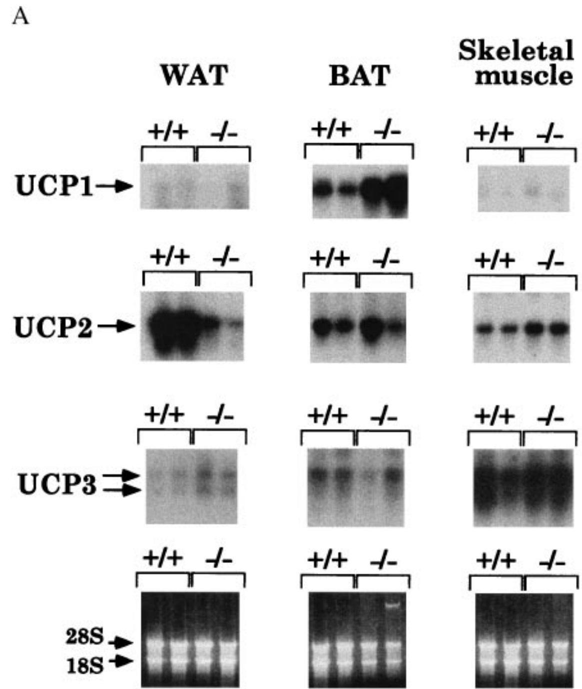


FIG. 6. Thermoregulation in female deficient mice. (*A*) Thermoregulation at an ambient temperature of 23°C [Y1-R$^{-/-}$ (n = 6) and Y1-R$^{+/+}$ (n = 7)]. (*B*) Thermoregulation during cold (4°C) exposure [Y1R$^{-/-}$ (n = 6) and Y1-R$^{+/+}$ (n = 7)].



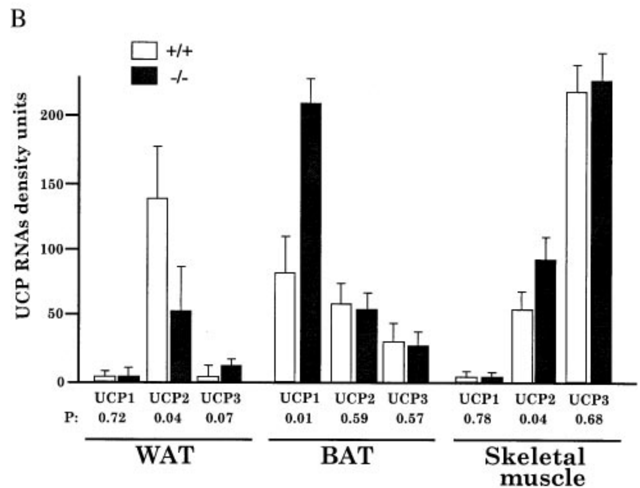


FIG. 7. (*A*) Northern blot analysis of UCP1, UCP2, and UCP3 mRNAs in BAT, WAT, and skeletal muscle. Total RNA was isolated from the BAT, WAT, and skeletal muscle of Y1-R$^{+/+}$ and Y1-R$^{-/-}$ female mice (18–45 weeks old) kept at 23°C by using the guanidinium thiocyanate method. Five micrograms of total RNA per lane was used for these experiments. (*B*) Bar graph showing the comparison of mRNA levels of UCP1, UCP2, and UCP3 in WAT, BAT, and skeletal muscle of Y1-R$^{+/+}$ and Y1-R$^{-/-}$ female mice. Data are presented as the mean ± SE of estimates for six female mice for WAT and BAT and three mice for skeletal muscle. A two-tailed unpaired Student's t test was used for statistical analysis.

mediate adaptive thermogenesis (30); and (*iii*) UCP3 is distinguished from UCP1 and UCP2 by its abundant and preferential expression in skeletal muscle and BAT in rodents (31, 32). As shown in Fig. 7 *A* and *B*, we found higher levels of UCP1 mRNA in the BAT of mutant female mice than in that of the wild type, whereas there was a dramatic reduction in UCP2 expression in the WAT of mutant mice. These results taken together suggest that a deficiency in NPY signaling via the Y1-R may be attributable to changes in the expressions of UCP genes. Recently, the inhibitory effects of hypothalamic NPY on both BAT thermogenic activity and sympathetic nerve activity to BAT were reported (33–37). The up-regulation of the UCP1 expression in BAT of Y1-R$^{-/-}$ mice shown in this report might be the result of

the deficiency of the inhibitory effects of NPY through Y1-R on BAT activity. Moreover, Enerback *et al.* (38) reported that UCP1-deficient mice did not develop obesity because of the compensation of UCP2 up-regulation for the loss of UCP1 in BAT. In Y1-R$^{-/-}$ mice, UCP2 in WAT can become part of a compensatory mechanism for sustainment of the body temperature in the Y1-R$^{-/-}$ mice, then its expression might be down-regulated. Although we have no evidence that UCP2 normally is involved in burning away extra calories and/or that it is dysfunctional in the obese, the significant decrease in the expression of UCP2 in WAT and the higher feeding efficiency (Fig. 5) of the Y1-R-deficient females might result in a lower expenditure of body energy and the development of mild obesity.

Obesity and noninsulin-dependent diabetes mellitus are thought to be tightly linked. Indeed, Y1-R$^{-/-}$ mice showed moderate obesity and impaired control of insulin secretion. The degree of hyperinsulinemia and impaired insulin secretion in response to the administration of a large amount of glucose that was found in Y1-R$^{-/-}$ mice is comparable to those found in insulin-receptor substrates 2 (39), bombesin receptor subtype 3, and metallothionein-deficient mice. However, the typical symptoms of diabetes mellitus (DM), such as glucosuria and hyperglycemia, did not appear in Y1-R$^{-/-}$ mice until 31 weeks. It would be worth trying to feed them on a high-calorie diet to promote the full development of DM. What is the mechanism of the impaired insulin secretion? It is believed that NPY has an inhibitory activity to glucose-induced insulin secretion via the Y1-R, which is expressed in the pancreas (40–42). One attractive explanation is that basal insulin secretion is increased when the NPY signaling via Y1-R is canceled. To confirm this hypothesis, we need to do an experiment in which Y1-R-specific antagonist is given to mice to see whether the insulin secretion is increased or not. Another possibility is that impaired insulin secretion is caused by desensitization to insulin because of the obesity. Because the impaired insulin secretion starts to manifest itself when the mice are young and in the preobesity stage, it is likely that impaired insulin secretion is a direct effect of the absence of Y1-R in the pancreas. Many molecules such as glucokinase are believed to be involved in the control of insulin secretion (43). To study its mechanism and the interaction between Y1-R and another molecules in insulin secretion, for example, a cross between Y1-R$^{-/-}$ mice and glucokinase$^{-/+}$ mice (44) would be helpful.

In conclusion, Y1-R-deficient mice showed moderate obesity and mild hyperinsulinemia without hyperphagia. This model could be used to study the mechanism of mild obesity and borderline cases of noninsulin-dependent diabetes mellitus, which could lead to the development of therapies focusing on NPY-Y1-R signaling.

Note Added in Proof. After submission of this manuscript, Pedrazzini *et al.* (45) reported mice deficient for the expression of Y1-R. Those mice also showed a mild late-onset obesity (more pronounced in females than in males), hyperinsulinemia, and complete absence of NPY-mediated vasoconstriction.

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