Next, glucose-free incubation was performed for 45 min in Krebs-Ringer phosphate buffer (8). Cells were then incubated with 0, 1, 10, or 100 nM insulin for 15 min, and 2-deoxy-D-[^{3}H]glucose uptake during the subsequent 4 min was measured as described previously (3). Each treatment combination (virus and insulin) was examined twice.

Glycogen synthesis assay. 3T3-L1 adipocytes in 24-well culture plates were infected with adenovirus at an MOI of 100 in DMEM containing 4.5 g/l glucose and 10% FCS. At 40 h after infection, the cells were washed once with serum-free DMEM containing 1 g/l glucose and 0.2% BSA and then incubated with the same medium for 5 h. The cells were then incubated with 200 μl of the same medium containing 1.5 μCi/ml of D-[U-^{14}C]glucose (230–370 mCi/mmol) and stimulated with 0, 0.1, 1, or 100 nM insulin for 3 h. After insulin stimulation, the cells were washed twice with ice-cold PBS and incubated with 200 μl of 10 N KOH at 4°C for 3 h. The cells were then scraped off, collected, and boiled with 2 mg of glycogen for 30 min. The lysate was mixed with 800 μl of ethanol and incubated at −20°C overnight. Tubes were centrifuged at 15,000 rpm for 20 min, and the supernatant was discarded. The glycogen pellets were rinsed once with 80% ethanol, dissolved in 200 μl of water, mixed with 800 μl of ethanol, and incubated again at −20°C overnight. The tubes were centrifuged, the pellets were dissolved in 200 μl of 0.1 N HCl and mixed with ACS II (Amersham Biosciences, Piscataway, NJ), and the incorporated ^{14}C was quantified with a liquid scintillator. Each treatment combination (virus and insulin) was examined twice.

Statistical analysis. Figures 1–8 show means ± SE. To analyze the results of the experiments, Student's unpaired *t*-test or two-way ANOVA with replication was used to demonstrate significant differences. With two-way ANOVA, mainly viral and growth hormone stimulation factors were assessed.

RESULTS

Transient CTMP overexpression enhanced Akt phosphorylation and activation in COS-1, HepG2, HEK293, and HeLa cells under both unstimulated and stimulated conditions. We created an expression vector as well as an adenovirus to express amino-terminally FLAG-tagged CTMP in cultured cells. In our subsequent experiments, COS-1, HeLa, and HepG2 cells and 3T3-L1 adipocytes were infected with this virus. We confirmed an ~22-kDa single band in the samples from these infected cells, using either anti-FLAG antibody or anti-CTMP antibody (Fig. 1*A*, *top*).

To investigate whether CTMP influences the phosphorylation state of Akt, we infected COS-1 cells with various titers of CTMP or LacZ (control) virus at an MOI of 3 and evaluated phosphorylations of Akt at Thr^{308} and Ser^{473} by immunoblotting with phospho-specific antibodies. As shown in Fig. 1*A*, CTMP enhanced endogenous Akt phosphorylations at both sites, in a viral dose-dependent manner, in the basal state, while control LacZ virus had no effect. The maximal level of Akt phosphorylation by CTMP overexpression was comparable with that induced by EGF stimulation. EGF stimulation had a small additional effect on Akt phosphorylation in CTMP-overexpressing cells (Fig. 1*A*, *right 2 lanes*), suggesting that high CTMP expression could induce nearly maximal Akt phosphorylation.

Subsequently, to confirm this phenomenon, we investigated the effects of CTMP overexpression on Akt phosphorylation by expressing CTMP in other types of cultured cells such as HepG2 and HeLa cells and 3T3-L1 adipocytes. In HepG2 cells, which are insulin-sensitive cells, CTMP produced a similar enhancement of Akt phosphorylation (Fig. 1*B*). Infection of HeLa cells produced a similar result (data not shown).

To rule out the possibility that the difference in overexpression systems between plasmid transfection and adenoviral gene transfer was responsible for the different results, CTMP was transiently overexpressed in HEK293 and COS-1 cells (Fig. 1, *C* and *D*, respectively) with an expression plasmid containing CTMP cDNA and the calcium phosphate method. In HEK293 cells, CTMP overexpression increased Akt phosphorylation as shown by immunoblotting with phospho-specific antibodies under both basal and vanadate-treated conditions (Fig. 1*C*, *left*). The phosphorylation of GSK-3β was also markedly increased by CTMP overexpression. Indeed, Akt kinase activity was increased by CTMP overexpression under both basal and vanadate-treated conditions (Fig. 1*C*, *right*). Very similar effects were also observed in COS-1 cells transfected with the CTMP expression plasmid (Fig. 1*D*). These results strongly suggest that CTMP overexpression increases Akt phosphory-

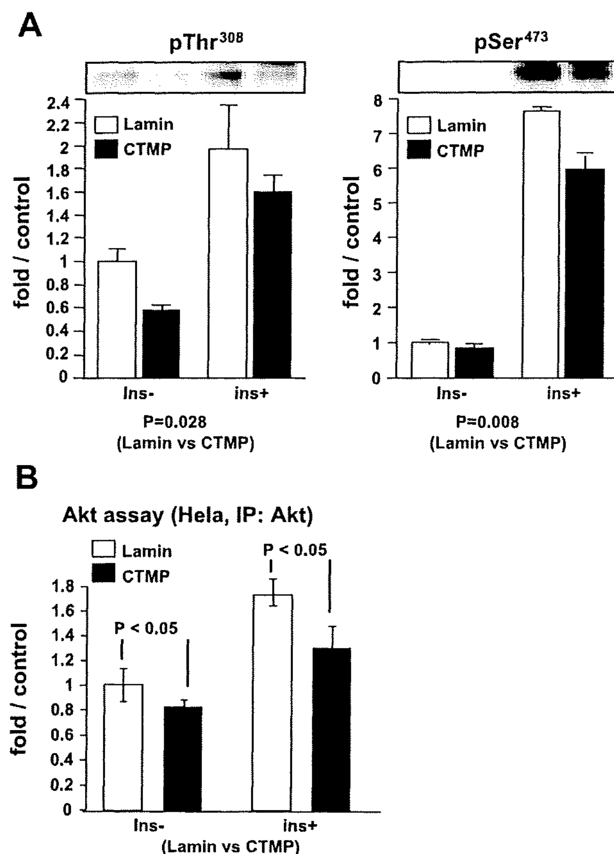

Fig. 4. CTMP small interfering RNA (siRNA) in HeLa cells. HeLa cells were transfected with siRNA of lamin (control) or CTMP, and Akt phosphorylations were measured 48 h after transfection. *A*: Akt phosphorylations at Thr^{308} and Ser^{473} were assayed by Western blotting, and representative bands are shown at *top* (Thr^{308}, *left*; Ser^{473}, *right*). Phosphorylation levels at Thr^{308} and Ser^{473} were quantified in experiments conducted in triplicate, and mean ± SE fold increases over LacZ without insulin are shown at *bottom*. Akt phosphorylations at Thr^{308} and Ser^{473} were significantly decreased by siRNA of CTMP. *B*: Akt kinase activities were quantified in experiments conducted in triplicate, and values are mean ± SE fold increases over LacZ without insulin.

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lation and activity, irrespective of the transfection method or cell types.

CTMP expression induced phosphorylations of Foxo1 and GSK-3β in HeLa cells. In the following experiments, we investigated the effects of CTMP on signals downstream from Akt. First, we examined phosphorylations of well-known Akt substrates, Foxo1(5, 31) and GSK-3β (11, 49), using their respective phospho-specific antibodies. Insulin induced both Foxo1 and GSK-3β phosphorylation in HeLa cells overexpressing Akt (Fig. 2, *lane 3*). Co-overexpression of CTMP enhanced the phosphorylation of both Foxo1 and GSK-3β in the basal state, compared with that of LacZ (Fig. 2, *lane 2*), although no significant difference was observed in the presence of insulin stimulation.

Stable overexpression of CTMP enhances phosphorylations of Akt and GSK-3β in NIH3T3 cells. To exclude the possibility that the difference between stable and transient overexpressions is responsible for the different results, CTMP was stably overexpressed in NIH3T3 cells by expression plasmid transfection followed by G-418 selection. As shown in Fig. 3*A*, two cell lines overexpressing CTMP (CTMP1 showing higher expression level than CTMP2) were prepared, and the expression level of Akt was confirmed to be unchanged. While basal Akt phosphorylation was not significantly altered in either CTMP-overexpressing cell line (Fig. 3*B*, *left 3 lanes*), Akt phosphorylation in response to vanadate stimulation was markedly enhanced in the CTMP-overexpressing cells (Fig. 3*B*, *right 3 lanes*). The phosphorylation of GSK-3β was also demonstrated to be significantly increased by stable CTMP overexpression under both unstimulated and vanadate-stimulated conditions (Fig. 3*C*). These results indicate that stable overexpression of CTMP increases Akt activity under vanadate-stimulated conditions.

siRNA of CTMP inhibited Akt phosphorylation. Next, we suppressed endogenous CTMP expression with siRNA to examine its physiological functions. The mRNA level of CTMP was determined by real-time PCR and standardized with that of GAPDH. siRNA decreased CTMP transcription to approximately one-fifth of that in HeLa cells transfected with lamin siRNA [lamin 1.00 ± 0.27, CTMP 0.21 ± 0.01 (arbitrary units); $P = 0.002$], although inhibition of CTMP protein expression could not be examined because the CTMP antibody was not sufficiently sensitive to detect endogenous CTMP protein in HeLa cells. Under these conditions, phosphorylations of Akt were partially but significantly suppressed at both Thr^{308} and Ser^{473} sites by CTMP siRNA to a greater extent than by lamin siRNA, as shown in Fig. 4*A*, *top*. Akt kinase activity was also slightly but significantly suppressed by treatment with CTMP siRNA (Fig. 4*B*).

CTMP enhanced membrane localization of Akt without affecting PI3-kinase activity. To elucidate the mechanism underlying the positive effect of CTMP on Akt activation, we prepared the membrane fraction of COS-1 cells overexpressing CTMP or control LacZ by adenoviral gene transfer at an MOI of 3. As shown in Fig. 5*A*, *left*, immunoblotting revealed that overexpression of CTMP markedly increased the amount of Akt in the membrane fraction in the basal state and also slightly but significantly increased that in the EGF-stimulated state.

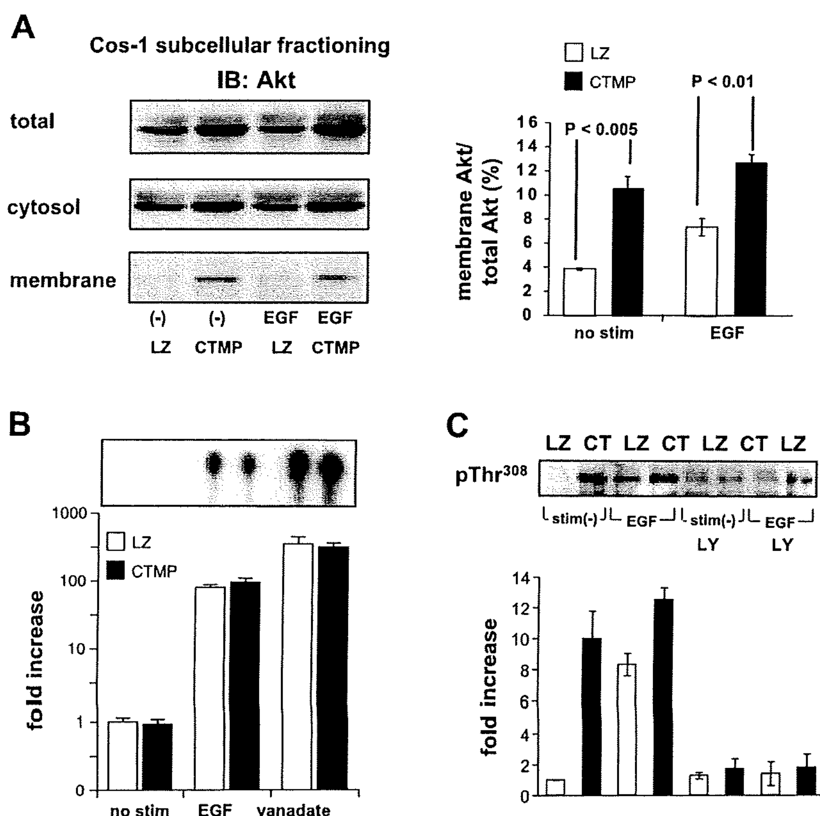

Fig. 5. Mechanisms of CTMP effect on Akt. *A*: COS-1 membrane fraction was purified with ultracentrifugation and then electrophoresed and immunoblotted (IB) with anti-Akt antibody. CTMP markedly increased membrane-localized Akt, especially in the basal state. The calculated ratio of membrane Akt to total Akt is shown at *right*. The experiment was done in triplicate. The graph shows means ± SE, and 1 band representative of the 3 is presented. *B*: COS-1 cells were stimulated without or with 50 ng/ml EGF or 100 nM orthovanadate, and anti-phosphotyrosine-immunoprecipitated phosphatidylinositol 3-kinase (PI3-kinase) activity in the cell lysate was assayed. The graph shows the fold increase ± SE over LacZ without stimulation on a logarithmic scale. CT, CTMP. Each experiment was done 3 times, and 1 spot representative of the 3 is shown at *top*. PI3-kinase activity was unchanged by CTMP expression, regardless of stimulation. *C*: COS-1 expressing LacZ or CTMP (CT) were stimulated without or with EGF for 10 min and then incubated without or with 20 nM LY-294002 (LY) for 2 min. Akt phosphorylations at Thr^{308} were quantified in experiments conducted in triplicate, and representative bands are shown at *top*. The graph shows mean ± SE fold increases over LacZ without insulin. The enhancing effect of CTMP on Akt phosphorylations was reversed within 2 min of LY-294002 incubation.

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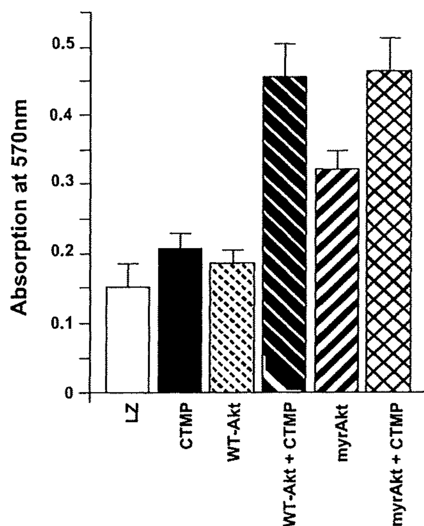
The ratio of membrane Akt to total Akt was calculated and is shown in Fig. 5*A*, *right*.

To exclude the possibility that the effects of CTMP on Akt are mediated by PI3-kinase activation that is upstream from Akt, we assayed the PI3-kinase activity of anti-phosphotyrosine immunoprecipitants from LacZ- or CTMP-expressing COS-1 cells. As shown in Fig. 5*B*, PI3-kinase activity did not differ between samples from LacZ-expressing cells and those expressing CTMP.

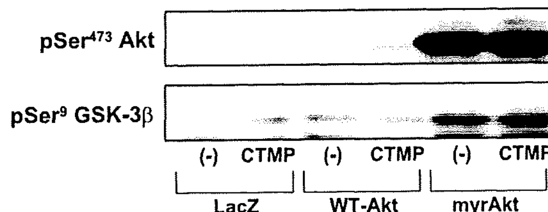
However, it was shown that treatment with LY-294002 obviously attenuated the Akt phosphorylation induced by either CTMP overexpression or EGF stimulation within 2 min (Fig. 5*C*). Thus it is likely that CTMP does enhance Akt phosphorylation, but at least basal level PI3-kinase activity is necessary.

CTMP expression with Akt rescued HeLa cells from UV-B irradiation-induced apoptosis. One of the well-known functions of Akt is antiapoptosis. Therefore, we investigated whether CTMP overexpression produces an antiapoptotic effect on cultured cells. HeLa cells, 12 h after adenoviral infection, were irradiated with UV-B. Cellular viability after irradiation was assayed with the MTT assay. As shown in Fig. 6, expression of CTMP alone tended to increase cellular viability



comparison		p value
LZ	CTMP	0.0002
LZ	WT-Akt	NS
LZ	WT-Akt + CTMP	<0.0001
LZ	myrAkt	0.0002
CTMP	WT-Akt + CTMP	0.0002
WT-Akt	WT-Akt + CTMP	<0.0001
myrAkt	myrAkt + CTMP	0.0514

Fig. 6. 4,5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay of UV-B-irradiated HeLa cells. HeLa cells in 96-well culture plates were infected with adenovirus in 6 combinations: LacZ (LZ), CTMP, wild-type (WT) Akt, both WT Akt and CTMP, myr-Akt, and both myr-Akt and CTMP. The MOI was 10 for each virus. At 12 h after infection cells were irradiated with UV-B, and 8 h after irradiation MTT was added. MTT uptake was assayed by absorption at 570 nm. Some of the *t*-test results are shown in the table (*bottom*).



comparison		p value
LZ	CTMP	0.0002
LZ	WT-Akt	<0.0001
CTMP	WT-Akt + CTMP	<0.0001
WT-Akt	WT-Akt + CTMP	0.0002
myrAkt	myrAkt + CTMP	NS

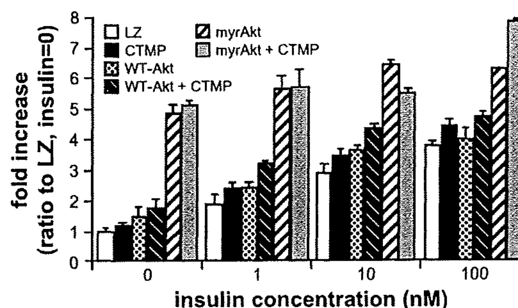


Fig. 7. 2-Deoxyglucose uptake assay in 3T3-L1 adipocytes. 3T3-L1 adipocytes were infected with adenovirus in 6 combinations, as described in Fig. 5, at an MOI of 100 for each virus. The phosphorylations of Akt and GSK-3β were investigated for each of these combinations and are shown at *top*. The blot is representative of 4 independent experiments, and the CTMP-induced increase in Akt phosphorylation was observed when WT-Akt was compared with WT-Akt + CTMP. A CTMP-induced increase in GSK-3β phosphorylation was observed in the comparison between LacZ and CTMP. No such significant differences were seen under the insulin-stimulated conditions (data not shown). 2-deoxy-D-[^{3}H]glucose uptakes with 0, 1, 10, and 100 nM insulin stimulation for 15 min were assayed twice each and are presented graphically at *bottom*. Repeated-measures 2-way ANOVA was conducted to detect the significance of differences in glucose uptake between pairs of viral conditions, and several of the results are shown in the table (*middle*).

compared with LacZ, but the difference was not significant. When Akt was coexpressed, CTMP showed a marked and significant antiapoptotic effect. myr-Akt, a well-known constitutively active type of Akt, also showed an apparent antiapoptotic effect.

CTMP expression in 3T3-L1 adipocytes modestly enhanced glucose uptake. Akt activation is known to induce glucose uptake of adipocytes via translocation of GLUT4 to the plasma membrane. We investigated the effect of CTMP overexpression on the glucose transport activity of 3T3-L1 adipocytes (Fig. 7). The CTMP overexpression level in 3T3-L1 adipocytes is much lower, even with the adenoviral transfer system, than in other cell lines such as COS-1 or HepG2. Thus slightly increased Akt phosphorylation was observed only in the WT Akt-overexpressing cells in the basal state. Similarly, slightly increased GSK-3β phosphorylation was observed only in LacZ cells under basal conditions. While myr-Akt induced a fivefold increase in glucose uptake in the absence of insulin stimulation (30), CTMP overexpression induced a relatively mild (1.2-fold over that of LacZ) but significant increase in uptake. Insulin

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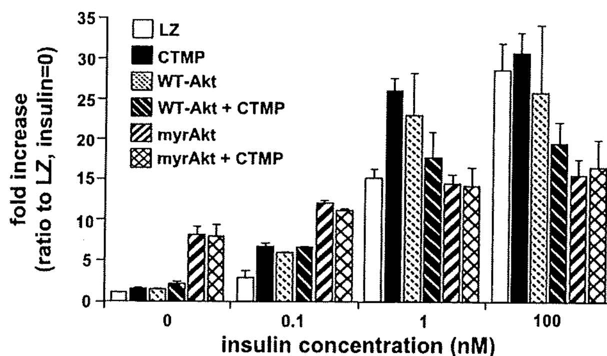
dose-dependently increased glucose uptake. Comparison of both CTMP and WT Akt overexpression with Akt overexpression alone, in response to stimulation with each of the indicated concentrations of insulin, showed the additional augmenting effect of CTMP on glucose uptake to be significant, although also of a modest degree (20% increase). This observation suggests that the effects of CTMP and Akt on glucose uptake are additive and more significant than that of the LacZ control.

CTMP expression in 3T3-L1 adipocytes enhanced glycogen synthesis. Since Akt is also known to enhance glycogenesis via inhibition of GSK-3β, the effect of CTMP on glycogenesis in 3T3-L1 adipocytes was investigated (Fig. 8). With insulin stimulation, glycogen synthesis increased in a dose-dependent manner in control LacZ-infected cells. Since the effect of CTMP plus Akt was observed to differ between low-dose and high-dose insulin conditions, statistical analyses were performed separately for the low- and high-dose groups. CTMP expression significantly enhanced glycogen synthesis. The magnitude of this enhancement was high when cells were stimulated with low concentrations of insulin (0.1 and 1 nM). However, the synergistic effect of CTMP and WT Akt overexpressions on glycogen synthesis was observed only in the nonstimulated state. On the other hand, constitutively active Akt (myr-Akt) markedly increased glycogen synthesis in the basal state. However, when stimulated with a high concentration of insulin (100 nM), myr-Akt expression suppressed glycogen synthesis. This finding suggests that when the Akt signaling pathway is highly stimulated for a long period, some negative feedback suppression(s) is exerted on the glycogen synthetic pathway (insulin desensitization). Comparing 3T3-L1 adipocytes expressing solely WT Akt and those coexpressing WT Akt and CTMP, glycogen synthesis was higher in the latter when the cells were unstimulated or stimulated with a low concentration of insulin (0 or 0.1 nM). In contrast, glycogen synthesis was higher in the former when cells were stimulated with higher concentrations of insulin (1 and 100 nM). This is attributable to the same desensitization mechanism.



comparison		p value
LZ	CTMP	0.0001
LZ	WT-Akt	0.0027
CTMP	WT-Akt + CTMP [ins=0, 0.1]	NS
CTMP	WT-Akt + CTMP [ins=1, 100]	0.0003
WT-Akt	WT-Akt + CTMP [ins=0, 0.1]	0.0395
WT-Akt	WT-Akt + CTMP [ins=1, 100]	0.0123
myrAkt	myrAkt + CTMP	NS

Fig. 8. Glycogen assay in 3T3-L1 adipocytes. 3T3-L1 adipocytes were infected with adenovirus in 6 combinations, as described in Fig. 5, at an MOI of 100 for each virus. [U-^{14}C]glucose incorporations into glycogen with 3 h of 0, 0.1, 1, or 100 nM insulin stimulation were each assayed twice. Repeated-measured 2-way ANOVA was conducted to detect the significance of differences between pairs of viral conditions, and several of the results are shown in the table. The comparison between CTMP and WT-Akt + CTMP, and that between WT-Akt and WT-Akt + CTMP, showed the effects of interactions. Therefore, data obtained with the lower (0, 0.1 nM) and higher (1, 100 nM) doses of insulin were analyzed separately for these 2 cases.

DISCUSSION

Akt is activated by 3-phosphoinositides and PDKs, but there exist several proteins that bind to Akt and modulate its activation state (2, 4, 14, 34, 40). CTMP was reportedly shown to bind to the carboxy terminus of Akt and to inhibit its phosphorylation and activation. However, our repeated careful experiments demonstrated that CTMP enhanced the phosphorylation and activation of Akt and its downstream signal pathways, regardless of whether a transient or a stable expression system was used. Furthermore, siRNA-mediated suppression of CTMP inhibited Akt phosphorylation. However, this suppression was minimal, possibly suggesting that the presence of a substantial basal level of Akt is required for CTMP to be functional in HeLa cells. These findings are quite contrary to those of a previous report (34), but this discrepancy is not attributable to differences in the cell line or the method used for transfection, because we utilized various cell lines and obtained essentially the same results. Thus we cannot explain the different conclusions.

CTMP is reportedly located mainly on the plasma membrane. Indeed, we found that overexpression of CTMP markedly enhances membrane localization of Akt. From this finding, we speculate that the mechanism underlying the enhancing effect of CTMP on Akt phosphorylation involves translocation of Akt to the plasma membrane. It is well established that, with targeting to the membrane, Akt conformational change occurs such that Thr^{308} and Ser^{473} are presented to the outside of the Akt molecule and phosphorylated by PDKs (36).

In the aforementioned previous report, the authors suggested that CTMP on the plasma membrane binds to Akt in the basal state and that CTMP binding to Akt does not completely block Akt phosphorylation in the stimulated state, but rather makes it more difficult. After phosphorylation is achieved, Akt would presumably disassociate from CTMP. However, this theory is somewhat difficult to understand, because it is unclear how CTMP suppresses Akt activation in the stimulated state, despite dissociating from Akt in that state.

Our interpretations appear to be more reasonable and are easier to understand: Akt is located mainly in the cytosol in the basal state, and CTMP, which is always on the plasma membrane, recruits Akt from the cytosol to the plasma membrane, leading to the phosphorylation of Thr^{308} and Ser^{473} of Akt by PDKs. Indeed, CTMP-induced membrane translocation of Akt was observed in the presence of wortmannin. We also confirmed that CTMP does not influence PI3-kinase activity, suggesting that the effects of CTMP on Akt are direct. However, the PI3-kinase inhibitor LY-294002 dephosphorylates CTMP-induced Akt phosphorylation. This finding indicates that CTMP-induced Akt phosphorylation is maintained by basal PI3-kinase activity and/or basal concentrations of 3-phos-

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phoinositides. In addition, as shown in Fig. 5, the total amount of Akt may be slightly increased. We speculate that CTMP induces translocation of Akt to the membrane, and that membrane-bound Akt thereby becomes susceptible to phosphorylation by upstream kinases such as PDK-1, which requires PI3-kinase activation, and phosphorylation of Thr^{308} and Ser^{473} by PDKs. It is also likely that CTMP increases the stability of Akt, possibly because of the increased amount of Akt.

To evaluate whether CTMP influences the antiapoptotic function of Akt, we combined UV-B irradiation (20) and MTT assay (15) in HeLa cells. The result showed clearly that CTMP enhances antiapoptosis, especially with Akt coexpression. In the previous report, the authors showed stable expression of CTMP in AKT8 tumor cells to inhibit tumor growth. Their experiment was designed to observe tumor growth, which is a more integrated cellular process than a specific antiapoptotic function. Moreover, AKT8 cells highly express constitutively active Akt, and the apoptotic signal in these cells may differ from that in physiological cells. On the other hand, our experiment induced relatively short-term CTMP expression in HeLa cells, in which Akt signaling would be nearer to physiological conditions. Therefore, we believe that our results reflect the physiological function of CTMP, at least as regards the antiapoptotic effect.

Akt reportedly plays critical roles in insulin-induced glucose metabolism, i.e., glycogen synthesis and glucose uptake. As for glycogen synthesis, Akt has been established as directly phosphorylating and inactivating GSK-3β, which results in activation of glycogen synthase. As for glucose uptake, constitutively active Akt reportedly induces GLUT4 translocation, thereby increasing glucose uptake. Our experiments revealed that CTMP enhances both of these pathways, indicating that CTMP may function as an insulin-sensitizing molecule for glucose metabolism, possibly in relation to insulin sensitivity. In the glycogen synthesis assay, expression of myr-Akt, or coexpression of CTMP and Akt, induced insulin desensitization. This phenomenon may be attributable to glycogen synthesis not being regulated solely by the insulin/Akt/GSK-3β/glycogen synthase pathway but also by the insulin/protein phosphatase-1 pathway (10, 42), which may be suppressed by chronic Akt activation. At a minimum, the desensitization occurred in response to both myr-Akt expression and Akt-CTMP coexpression, which is consistent with our finding that CTMP strongly activates coexpressed Akt. On the other hand, the effects of CTMP on insulin-induced glucose uptake were modest, although statistically significant. From this finding, we speculate that CTMP leads to Akt activation mainly on the plasma membrane, while for efficient GLUT4 translocation activation of Akt in other intracellular compartments may be critical.

Recently, we (2) and another group (17) have identified a novel 200-kDa protein that binds to the carboxy terminus of Akt and markedly enhances Akt phosphorylation. This protein was termed Akt phosphorylation enhancer (APE), or Girdin. We have the impression that APE/Girdin exerts more potent activity, markedly increasing Akt phosphorylation, although exact comparison is difficult. Taking into consideration that both APE/Girdin and CTMP bind to the carboxy terminus of Akt, the mechanisms underlying the increases in Akt phosphorylation may be similar. We speculate that their binding to the carboxy terminus of Akt would induce conformational changes in Akt, thereby possibly making Akt more easily accessible to PDK-1 and PDK-2. Interestingly, APE/Girdin binds to actin, and CTMP is located at the plasma membrane. Thus both APE/Girdin and CTMP enhance Akt activity by modifying the conformation of the Akt carboxy terminus, but the former may function by interacting with the actin network and the latter at the plasma membrane. Further work is necessary to elucidate the similarities and differences in these proteins.

In summary, our experimental findings on CTMP overexpression and suppression in various cell systems allow us to draw the conclusion that CTMP enhances Akt phosphorylation and activation. The mechanism appears to involve membrane-localized CTMP recruiting Akt from the cytosol to the plasma membrane. CTMP-induced Akt activation results in phosphorylation of Akt substrates. It also activates multiple downstream Akt pathways, including antiapoptotic, glycogen synthetic, and glucose uptake processes. Therefore, CTMP may be involved in cellular antiapoptotic mechanisms and insulin sensitivity.

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Social Isolation Affects the Development of Obesity and Type 2 Diabetes in Mice

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Social isolation is associated with increased risks of mortality and morbidity. In this study, we show that chronic individual housing accelerated body weight gain and adiposity in KK mice but not C57BL6J mice, and fully developed diabetes in KKA^y mice. Individually housed KK and KKA^y mice increased body weight gain over the initial 2 wk without increased daily average food consumption compared with group-housed animals. The individually housed KK and KKA^y mice then gradually increased food consumption for the next 1 wk. The chronic social isolation-induced obesity (SIO) was associated with hyperleptinemia and lower plasma corticosterone and active ghrelin levels but not hyperinsulinemia. Elevated plasma leptin in the SIO suppressed expression of 5-HT2C receptor in white adipose tissue. The SIO was also associated with decreased expression of β3-adrenergic receptors in white adipose tissue and hypothalamic leptin receptor, which might be secondary to the enhanced adiposity. Interestingly, social isolation acutely reduced food consumption and body weight gain compared with group-housed obese db/db mice with leptin receptor deficiency. Social isolation-induced hyperglycemia in KKA^y mice was associated with increased expression of hepatic gluconeogenetic genes independent of insulin. These findings suggest that social isolation promotes obesity due to primary decreased energy expenditure and secondary increased food consumption, which are independent of the disturbed leptin signaling, in KK mice, and develops into insulin-independent diabetes associated with increased expression of hepatic gluconeogenetic genes in KKA^y mice. Thus, social isolation can be included in the environmental factors that contribute to the development of obesity and type 2 diabetes. (*Endocrinology* 148: 4658–4666, 2007)

SOCIAL ISOLATION OR lack of social support is associated with increased risks for mortality (1, 2) and negative health outcomes, including heart disease, hypertension, stroke, and arthritis (3–7). Excess body weight during midlife is also associated with an increased risk factor of mortality and the negative health outcomes (8). However, interesting aspects of the relationship between social isolation and development of obesity remain to be resolved. Although individually housed Swiss CD-1 mice and Wistar rats do not grow as fast as group-housed ones (9–11), animal models of social isolation-induced obesity (SIO) and type 2 diabetes have yet to be identified.

The KK mice have long been included in the group of animals that become obese and develop diabetes, as have the A^y mice. The A^y yellow mice are known to become obese, and when bred with the KK mice, the development of diabetes is more pronounced. To determine the effects of social isolation on the development of obesity and type 2 diabetes, we examined the effects of individual and group housing on body weight, adipose tissues, plasma hormone levels, and expression of genes involved in the regulation of energy homeostasis in C57BL6J, KK, and KKA^y mice.

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Abbreviations: β3-AR, β3-Adrenergic receptor; BAT, brown adipose tissue; CNS, central nervous system; Fbp, fructose bisphosphatase; G6Pase, glucose-6-phosphatase; MC, melanocortin; PEPCK, pyruvate carboxykinase; SIO, social isolation-induced obesity; SOCS-3, suppressor of cytokine signaling 3; UCP, uncoupling protein.

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Materials and Methods

General procedures

Four-week-old male C57BL6J mice, KK, and KKA^y mice and 8-wk-old db/db mice were purchased from Japan CLEA (Tokyo, Japan). After the arrival of the animals, all mice were group-housed and acclimated to the colony for 1 wk before the experiment. Before the experiment, they were all housed (three to four mice per cage) with free access to water and chow pellets in a light (12 h on/12 h off; lights off at 2000 h)- and temperature (20–22 C)-controlled environment. One week later, animals were randomly transferred to individually housed conditions. Mice were housed in groups of three to four per cage (21.5 × 32 × 14 cm) or individually for 3 wk preceding decapitation. Their body weights were measured every 7 d in the morning for 3 wk. The average amounts of daily food consumption per week were evaluated in 5- to 8-wk-old animals.

Administration of drugs

Four-week-old C57BL6J mice were individually housed in standard mouse cages with free access to food and water for 1 wk before testing. Mice were injected ip with saline or leptin (5 mg/kg). They were not fed chow pellets. Sixty minutes later, the animals were decapitated and the epididymal white adipose tissue was removed for RNA extraction. The experiment was performed between 1000–1200 h. The dose of leptin (5 mg/kg) was selected based on the evidence that leptin induced hypophagia and decreases in body weights in 5-HT2C receptor mutant and wild-type mice (12).

Blood chemistries

Plasma ghrelin and adiponectin levels were measured by ELISA using an active ghrelin ELISA kit and des-acyl ghrelin ELISA kit (Mitsubishi Kagaku Iatron Inc., Tokyo, Japan) and mouse adiponectin ELISA kit (Ootsuka Inc., Tokyo, Japan). For the ELISA of active ghrelin, 1 N hydrogen chloride was added to the samples at a final concentration of 0.1 N immediately after plasma separation. Plasma leptin, insulin, and corticosterone levels were measured using mouse leptin (Linco, St. Louis, MO), rat insulin (Linco), and rat corticosterone (ICN Biomedicals,

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Costa Mesa, CA) RIA kits, respectively. Blood glucose levels were measured using glucose strips (blood glucose monitoring system; FreeStyle; KISSEI, Tokyo, Japan).

The animal studies were conducted under protocols in accordance with the institutional guidelines for animal experiments at Tohoku University Graduate School of Medicine.

Real-time quantitative RT-PCR

Total RNA was extracted from the epididymal white adipose tissue, brown adipose tissue (BAT), and soleus skeletal muscle by the acid-isolated guanidium thiocyanate-phenol-chloroform method and was isolated from mouse liver and hypothalamic tissue using the RNeasy Midi kit (Qiagen, Hilden, Germany) according to the manufacturer's directions. cDNA synthesis was performed using a Super Script III First-Strand Synthesis System for RT-PCR Kit (Invitrogen, Rockville, MD) using 1 μg total RNA. cDNA synthesized from total RNA was evaluated in a real-time PCR quantitative system (Light Cycler Quick System 350S; Roche Diagnostics, Mannheim, Germany). The primers used are listed in Table 1.

The relative amount of mRNA was calculated using β-actin mRNA as the invariant control. The data are shown as the fold change of the mean value of the control group, which received saline.

Data are presented as the mean values ± SEM (n = 5–8). Comparisons between the two groups were performed using two-tailed unpaired Student's *t* test. Comparisons among more than two groups were done by ANOVA using Bonferroni's test. The presence of a linear correlation was assessed using a parametric (Pearson's) correlation test. A *P* value of less than 0.05 was considered statistically significant.

TABLE 1. The primers used for real-time RT-PCR

Gene	Primer	Sequence
LepR	Sense	CTGAATTTCCAAAAGCCTGA
	Antisense	AAGCTGTATCGACACTGATTTC
MC4R	Sense	GAGGTGTTTGTGACTCTGGG
	Antisense	GAACATGTGGACATAGAGAG
5-HT2CR	Sense	CTGAGGGACGAAAGCAAAG
	Antisense	CACATAGCCAATCCAAACAAAC
5-HT1BR	Sense	TGCCTGCTGGTTTCACAT
	Antisense	GCGCACTTAAAGCGTATCA
SOCS-3	Sense	GCGGGCACCTTTCTTATCC
	Antisense	TCCCCGACTGGGTCTTGAC
β3-AR	Sense	ATGGCTCCGTGGCCTCAC
	Antisense	CCCAACGGCCAGTGGCCAGTCAGCG
UCP-1	Sense	GACAGTACCCAAGCGTACCAA
	Antisense	CATGATGACGTTCCAGGACC
UCP-2	Sense	GTTCCTCTGTCTCGTCTTGC
	Antisense	GGCCTTGAAACCAACCA
UCP-3	Sense	GTTGCTGGAGTCTCACCTGT
	Antisense	TCTTCAGCATACAGTGCAGA
PPARα	Sense	CGGGTAACCTCGAAGTCTGA
	Antisense	CTAACCTTGGGCCACACCT
PPARγ	Sense	CTGCTCAAGTATGGTGTCCATGAG
	Antisense	GAGGAACTCCCTGGTCATGAATC
PPARδ	Sense	GCTGCTGCAGAAGATGGCA
	Antisense	CACTGCATCATCTGGGCATG
G6Pase	Sense	TGCAAGGGAGAACTCAGCAA
	Antisense	GGACCAAGGAAGCCACAATG
Fbp1	Sense	TCTGCACCGCGATCAAAG
	Antisense	GTTGAGCCAGCGATACCATAGAG
Fbp2	Sense	AGAAAGACCACGGAGGACGA
	Antisense	CCCGCAGCCACGATGT
PEPCK	Sense	AGCGGATATGGTGGGAAC
	Antisense	GGTCTCCACTCCTTGTTC
β-actin	Sense	TTGTAACCAACTGGGACGATATGG
	Antisense	GATCTTGATCTTCATGGTGCTAGG

Results

Changes in body weight and adipose tissue weight in individually and group-housed C57BL6J mice and KK mice

There were no significant differences in body weight change between individually housed and group-housed C57BL6J mice for 3 wk in 6- to 8-wk-old animals (Fig. 1A), whereas the increases in body weight were significantly greater in individually housed KK mice than those in group-housed ones after 6 wk of age (Fig. 1B). Epididymal white adipose tissue and BAT weight significantly increased in 8-wk-old individually housed KK mice compared with group-housed ones, whereas there were no differences between individually housed and group-housed C57BL6J mice (Fig. 1, C and D). These findings indicate that chronic social isolation accelerates body weight gain and adiposity in KK mice but not C57BL6J mice.

Plasma chemistries in individually housed and group-housed KK mice

To determine the characteristics of obesity induced by chronic social isolation, we examined blood chemistries in individually housed and group-housed 8-wk-old C57BL6J and KK mice. Plasma leptin levels were significantly elevated in individually housed 8-wk-old KK mice compared with group-housed ones (15.5 ± 1.1 *vs.* 6.01 ± 0.55 ng/ml, $P < 0.05$). There were no significant differences in plasma insulin levels between the individually housed and group-housed 8-wk-old KK mice (5.34 ± 0.94 *vs.* 3.90 ± 0.62 ng/ml). Plasma corticosterone and active ghrelin, but not des-acyl ghrelin, levels were significantly decreased in the individually housed 8-wk-old KK mice compared with group-housed ones (corticosterone, 23.9 ± 2.7 *vs.* 56.3 ± 11.9 ng/ml, $P < 0.05$; active ghrelin, 8.72 ± 0.94 *vs.* 14.88 ± 1.37 fmol/ml, $P < 0.05$; and des-acyl ghrelin, 258 ± 21.0 *vs.* 288 ± 30.17 fmol/ml). There were no significant differences in the plasma adiponectin or blood glucose levels between the individually housed and group-housed 8-wk-old KK mice (adiponectin, 7.12 ± 0.29 *vs.* 7.62 ± 0.59 μg/ml; glucose, 156 ± 9 *vs.* 160 ± 5 mg/dl). These hormonal and metabolic alterations induced by social isolation in KK mice were not found in C57BL6J mice (data not shown). These findings suggest that chronic SIO is not due to hyperinsulinemia or hypercorticosteronemia, and is not associated with decreases in plasma adiponectin or des-acyl ghrelin levels.

Altered expression of genes involved in energy homeostasis and daily food consumption of individually housed and group-housed KK mice

To further determine the characteristics of chronic SIO associated with hyperleptinemia in the individually housed and group-housed 8-wk-old KK mice (Fig. 2A), we examined the expression of hypothalamic leptin receptor (Ob-Rb; *LepR*), melanocortin (MC)-4 receptor, and 5-HT2C receptor, which are involved in the central regulation of feeding behavior and energy homeostasis (13). Hypothalamic *LepR* mRNA levels were significantly decreased in the individually housed 8-wk-old KK mice compared with group-housed mice (24% decrease), although there were no significant dif-

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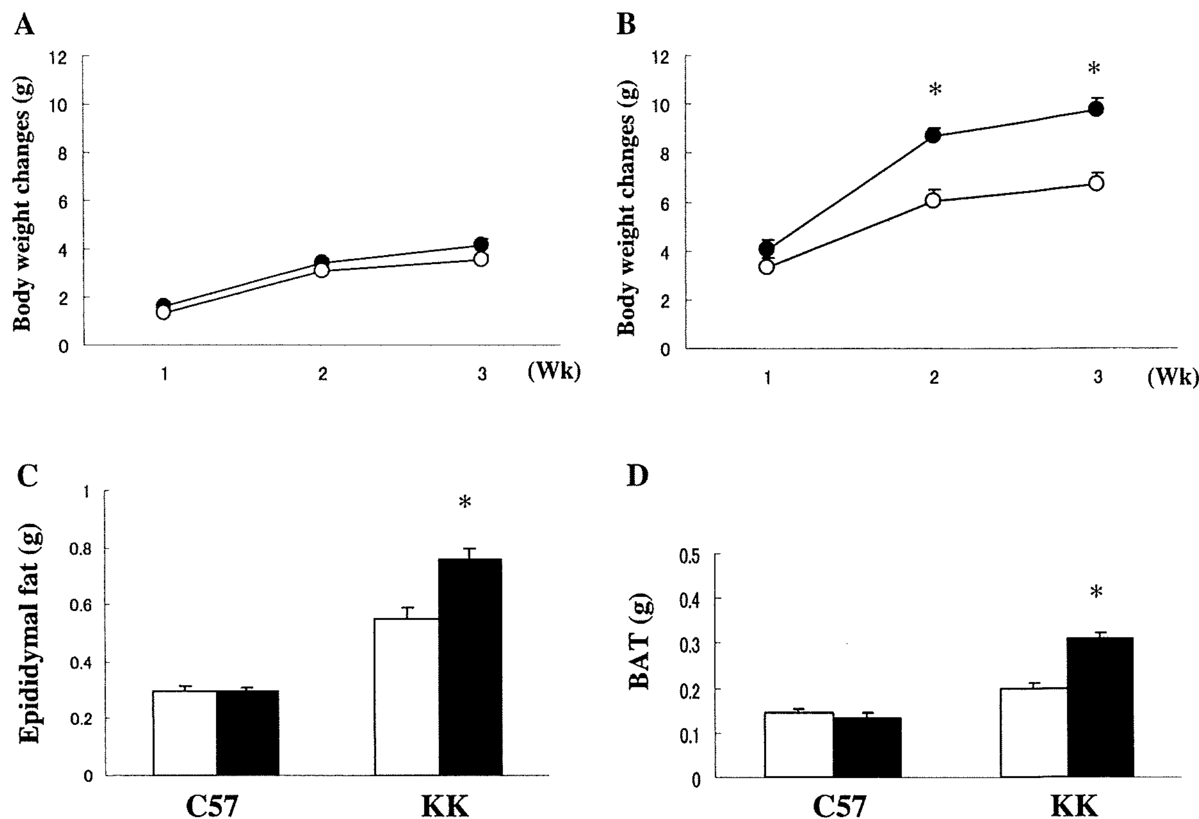

FIG. 1. Body weight changes for 3 wk from the period of 5 to 8 wk old in individually housed (*solid circles*) and group-housed (*open circles*) C57BL6J mice (A) and KK mice (B). Epididymal white adipose tissue (C) and BAT weight (D) in individually housed (*filled bars*) and group-housed (*open bars*) 8-wk-old C57BL6J mice and KK mice as described in *Materials and Methods*. Basal body weight: individually housed and group-housed C57BL6J mice were 18.5 ± 0.2 and 18.3 ± 0.2 g, respectively; individually housed and group-housed KK mice were 22.0 ± 0.2 and 22.0 ± 0.2 g, respectively. Data are presented as the mean values ± SEM (n = 8). C57, C57BL6J mice; KK, KK mice. *, $P < 0.05$.

ferences in hypothalamic MC-4 receptor or 5-HT2C receptor mRNA levels (Fig. 2A). In addition, there were no significant differences in mRNA levels of hypothalamic suppressor of cytokine signaling (SOCS)-3, which is related to the central leptin resistance (14–16), between the two groups (Fig. 2A).

Leptin increases central sympathetic outflow to white adipose tissue via β3-adrenergic receptor (β3-AR), leading to increased lipolysis (13, 17). Mice with a null mutation of the β3-AR gene have a mild increase in fat stores at an early age (18). A disturbance of sympathetic neural action on adipose tissues by β3-AR results in increased fat stores without hyperphagia (13, 17, 18). The present study demonstrates that the mRNA levels of β3-AR in epididymal white adipose tissue were significantly decreased in the 8-wk-old individually housed KK mice compared with the group-housed ones (Fig. 2B).

The expression of 5-HT2C receptors appears to be restricted to the central nervous system (CNS) (19). Interestingly, the present study demonstrates that the 5-HT2C receptor is expressed in epididymal white adipose tissue, and the mRNA levels of the 5-HT2C receptor but not the 5-HT1B receptor in epididymal white adipose tissue were significantly decreased in 8-wk-old individually housed KK mice compared with the group-housed animals (Fig. 2C). Plasma leptin levels and the 5-HT2C receptor mRNA levels in white adipose tissue were inversely correlated ($r = -0.84$, $P = 0.0012$) (Fig. 2D).

There were no significant differences in average daily food consumption between the two groups for the initial 2 wk, and then individually housed KK mice significantly increased food consumption compared with the group-housed KK mice for the next 1 wk (Fig. 2E).

Effects of leptin on expression of 5-HT2C receptor in white adipose tissue

To further determine the effects of leptin on 5-HT2C receptor expression in white adipose tissue, we examined exogenous administration of leptin on 5-HT2C receptor mRNA levels in epididymal white adipose tissue in C57BL6J mice. Intraperitoneally administration of leptin (5 mg/kg) dramatically decreased 5-HT2C receptor but not 5-HT1B receptor mRNA levels in epididymal white adipose tissue compared with saline controls (Fig. 2F). These findings suggest that leptin down-regulates the expression of 5-HT2C receptor in white adipose tissue independent of feeding behavior.

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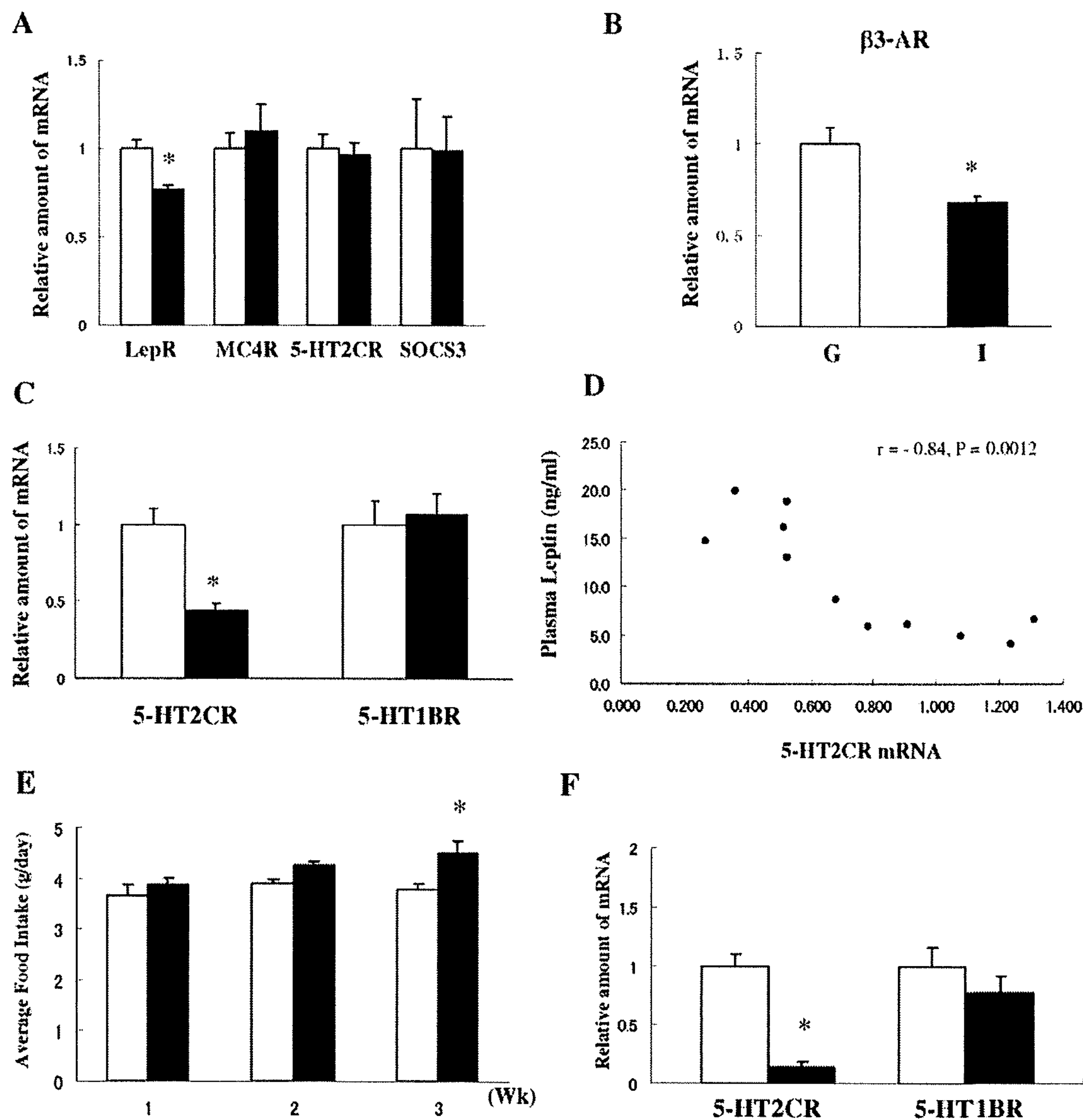

FIG. 2. Expression of *LepR*, *MC4R*, *5-HT2CR*, and *SOCS-3* genes in the hypothalamus (A), and expression of *β3*-AR gene (B) and *5-HT2C* receptor gene (C) in the epididymal white adipose tissue, and the relationship between plasma leptin levels and *5-HT2C* receptor gene expression in epididymal white adipose tissue (D), and average daily food consumption per week of 8-wk-old individually housed (*filled bars*) and group-housed (*open bars*) KK mice (E) as described in *Materials and Methods*. Effects of leptin on 5-HT2C receptor and 5-HT1B receptor mRNA levels in the epididymal white adipose tissue (F) of 5-wk-old C57BL6J mice, as described in *Materials and Methods*. Data are presented as the mean values ± SEM (n = 5–6). LepR, Leptin receptor; MC4R, MC-4 receptor, 5-HT2CR, serotonin 5-HT2C receptor; I, individually housed animals; G, group-housed animals. *, $P < 0.05$.

Altered expression of uncoupling proteins (UCPs) in adipose tissues and skeletal muscle of individually housed and group-housed KK mice

UCPs on the mitochondrial inner membrane are effectors for adaptive thermogenesis (20). The expression of UCP-1 in BAT and the expression of UCP-2 in white adipose tissue has been suggested to increase in response to high-fat diet (21). The present study demonstrates that UCP-2 mRNA levels in epididymal white adipose tissue were significantly decreased in individually housed 8-wk-old KK mice compared with group-housed mice, and there were no significant differences in UCP-1 mRNA levels in BAT, UCP-2 mRNA levels in the liver or the soleus muscle between the 8-wk-old individually housed and group-housed KK mice (Table 2).

Altered expression of PPARs in adipose tissue, skeletal muscle and liver of individually housed and group-housed KK mice

The nuclear receptor peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily that function as fatty acid-activated transcription factors (22). Among three related PPAR family members: PPARα, PPARγ, and PPARδ, the expression of PPARγ and PPARα in epididymal white adipose tissue and skeletal mus-

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TABLE 2. Altered UCP-1 mRNA levels in the BAT, UCP-2 mRNA levels in the epididymal white adipose tissue (WAT) and soleus muscle, and UCP-3 mRNA levels in the soleus muscle of individually housed and group-housed 8-wk-old KK mice

		G	I
UCPs			
BAT	UCP-1	1 ± 0.08	1.10 ± 0.05
WAT	UCP-2	1 ± 0.10	0.61 ± 0.21[a]
Muscle	UCP-2	1 ± 0.12	0.93 ± 0.14
	UCP-3	1 ± 0.20	1.10 ± 0.21
PPARs			
WAT	PPARδ	1 ± 0.06	0.80 ± 0.07
	PPARα	1 ± 0.10	1.20 ± 0.10
	PPARγ	1 ± 0.12	1.20 ± 0.12
Muscle	PPARδ	1 ± 0.06	1.00 ± 0.16
	PPARα	1 ± 0.06	0.90 ± 0.08
	PPARγ	1 ± 0.10	0.66 ± 0.05[a]

Altered PPARδ, PPARα, and PPARγ mRNA levels in the WAT and soleus muscle of individually and group housed 8-wk-old KK mice, as described in *Materials and Methods*. Data are presented as the mean values ± SEM (n = 6). I, Individually housed KK mice; G, group housed KK mice.

[a] $P < 0.05$.

cle is reportedly increased in response to high-fat diet (23–25). The present study demonstrates that there were no differences in either PPARγ or PPARα mRNA levels in the epididymal white adipose tissue or the soleus muscle between individually housed and group-housed 8-wk-old KK mice (Table 2).

PPARδ enhances fatty acid catabolism and energy uncoupling in white adipose tissue and/or skeletal muscle, leading to prevention of diet-induced obesity (26, 27). However, the present results demonstrate that there were no significant differences in PPARδ mRNA levels in the epididymal white adipose tissue or the soleus muscle between individually housed and group-housed 8-wk-old KK mice (Table 2).

The increased expression of PPARγ and PPARα in the liver is a common characteristic of obese rodents including ob/ob mice, db/db mice, and obese 5-HT2C receptor mutant mice (28). The present study also demonstrates that PPARγ and PPARα mRNA levels in the liver were significantly increased in 8-wk-old individually housed KK mice compared with group-housed ones (Fig. 3, A and B). There were no significant differences in expression of gluconeogenetic genes such as glucose-6-phosphatase (G6Pase), fructose bisphosphatase (Fbp) 1, and Fbp2 in the liver of 8-wk-old individually housed and group-housed KK mice (Fig. 3C). These findings support the finding that there were no differences in blood glucose levels between the individually housed and group-housed KK mice. Altered expression of these genes was not found between the 8-wk-old individually housed and group-housed C57BL6J mice (data not shown).

Development of diabetes in individually housed and group-housed KKA^y mice

A^y mice have dominant alleles at the agouti locus (*A*), which produces ectopic expression of the agouti peptide, an antagonist of the hypothalamic MC-4 receptors and MC-3

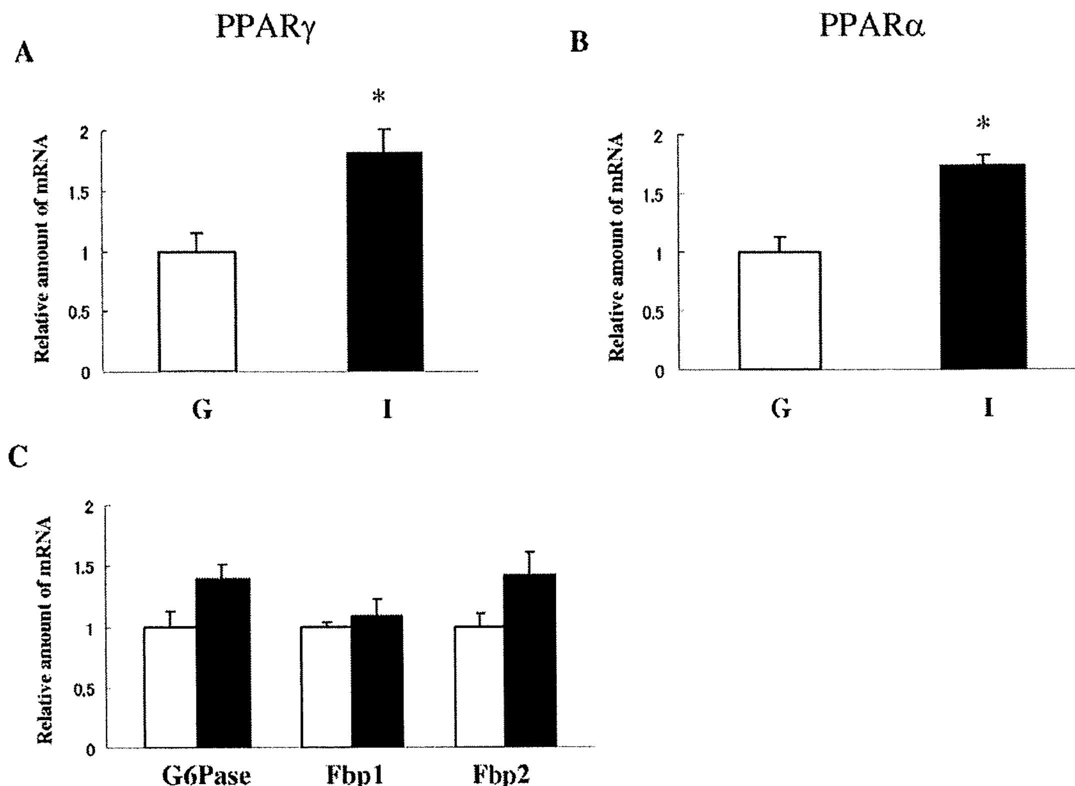

FIG. 3. Altered PPARγ (A), PPARα (B), and GP6ase, Fbp1, and Fbp2 (C) mRNA levels in the liver of 8-wk-old individually housed (*filled bars*) and group-housed (*open bars*) KK mice, as described in *Materials and Methods*. Data are presented as the mean values ± SEM (n = 5–6). I, Individually housed animals; G, group-housed animals. *, $P < 0.05$.

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receptors, and display hyperphagia, obesity, and diabetes (29–31). Individually housed 8-wk-old KKA^y mice displayed hyperglycemia in association with increased body weight, epididymal white adipose tissue weight, and plasma leptin levels compared with group-housed KKA^y mice (Fig. 4, A–E), whereas there were no significant differences in plasma insulin or adiponectin levels between individually housed and group-housed KKA^y mice (Fig. 4, F and I). The plasma active ghrelin levels were remarkably decreased (Fig. 4G), and des-acyl ghrelin levels were slightly decreased in

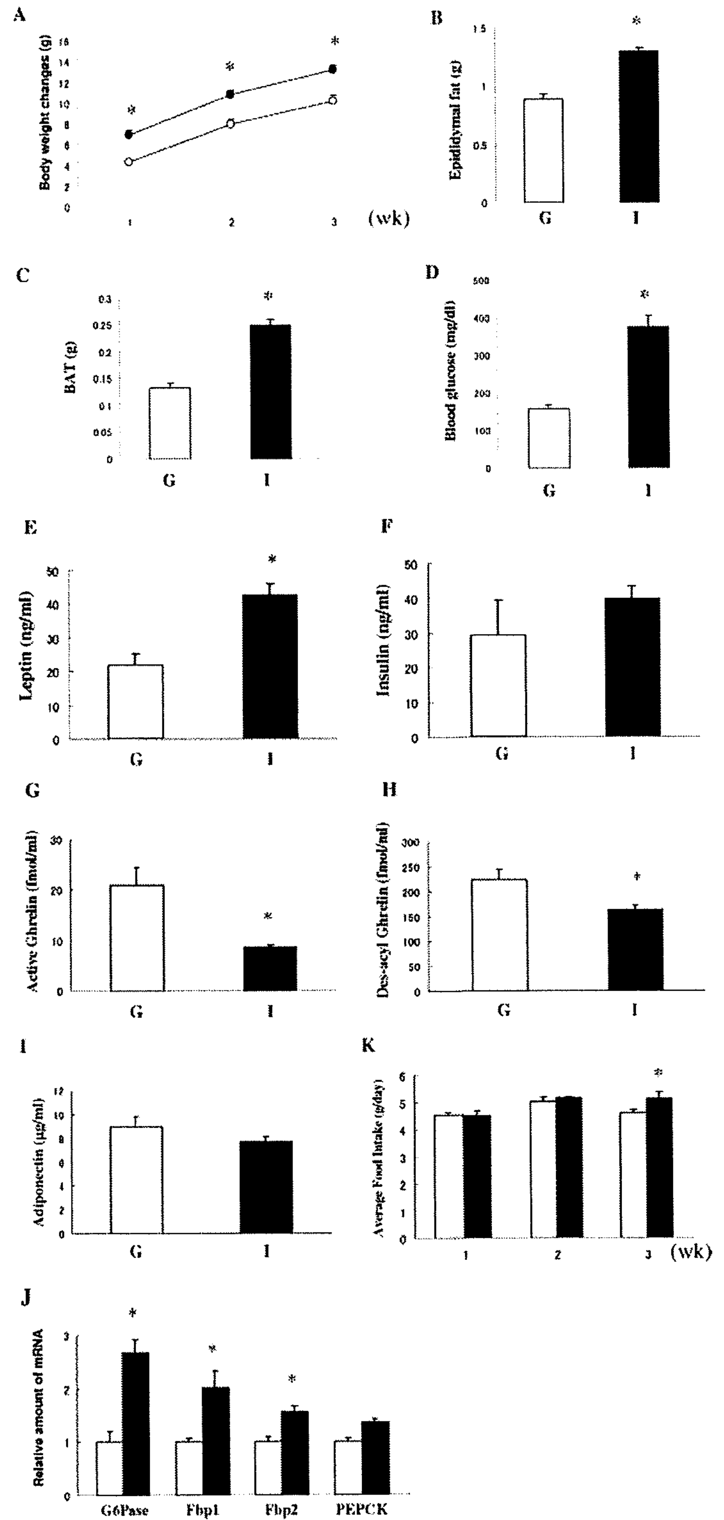

FIG. 4. Body weight changes for 3 wk in the period from 5- to 8-wk-old individually housed (*solid circles*) and group-housed (*open circles*) KKA^y mice (A). Epididymal white adipose tissue (B) and BAT weight (C), blood glucose (D), plasma leptin (E), insulin (F), active ghrelin (G), des-acyl ghrelin (H), and adiponectin levels (I), and GP6ase, Fbp1, Fbp2, and PEPCK mRNA levels (J) in the liver of 8-wk-old individually housed (*filled bars*) and group-housed (*open bars*) KKA^y mice as described in *Materials and Methods*. Average daily food consumption for the week that transpired (K) in the period from 5- to 8-wk-old individually housed (*solid circles* and *filled bars*) and group-housed (*open circles* and *open bars*) KKA^y mice. Basal body weight: individually housed and group-housed KKA^y mice 24.5 ± 0.2 and 24.5 ± 0.2 g, respectively. Data are presented as the mean values ± SEM (n = 6–8). I, Individually housed animals; G, group-housed animals. *, $P < 0.05$.

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individually housed KKA^y mice compared with group-housed KKA^y mice (Fig. 4H). Individually housed 8-wk-old KKA^y mice exhibited increased expression of the hepatic G6Pase, Fbp1, and Fbp2 genes, which are involved in gluconeogenesis (32), whereas there were no significant effects on hepatic pyruvate carboxykinase (PEPCK) mRNA levels (Fig. 4J). These findings suggest that chronic social isolation can fully develop into insulin-independent diabetes associated with increased hepatic gluconeogenetic genes in addition to obesity in KKA^y mice. There were no significant differences in average daily food consumption between the two groups for the initial 2 wk, and then the individually housed KKA^y mice slightly increased food consumption compared with the group-housed animals for the next 1 wk (Fig. 4K).

Effects of social isolation on food consumption and body weight gain in db/db mice

To further determine the physiological role of the decreased expression of the hypothalamic *LepR* gene, we examined body weight gain and daily food consumption in individually housed and group-housed obese db/db mice. Body weight gain was significantly lower in the individually housed than group-housed db/db mice after 9 wk of age (Fig. 5A). In addition, daily food consumption was relatively lower in individually housed db/db mice than the group-housed animals (Fig. 5B), and the average amount of daily food consumption per week in the 9- to 10-wk-old animals was significantly lower in individually housed than group-housed db/db mice (Fig. 5C).

Discussion

The present study demonstrates that chronic individual housing accelerated body weight gain and adiposity in KK and KKA^y mice but not C57BL6J mice. First, the social isolation-induced body weight gain in the KK strains occurred without increased food consumption, suggesting that decreased energy expenditure primarily contributes to the accelerated body weight gain. Subsequently, the SIO developed in association with slightly increased food consumption.

The SIO displays certain characteristics distinct from the general features of diet-induced obesity. The first reason in support of this is based on the result that despite lower active ghrelin, there were no differences in plasma des-acyl ghrelin levels between the individually housed and group-housed KK mice. We previously reported that hyperphagia decreases plasma des-acyl ghrelin, but not active ghrelin, levels in mice (31). The second reason is based on the result that hepatic UCP-2 gene expression was not increased in the individually housed KK mice. UCP-2 gene expression in the liver is increased in hyperphagic 5-HT2C receptor mutant mice (33). The third reason is based on the result that UCP-1 expression in BAT and UCP-2 expression in white adipose tissue were not increased in the individually housed KK mice. The UCP-1 expression in BAT and UCP-2 expression

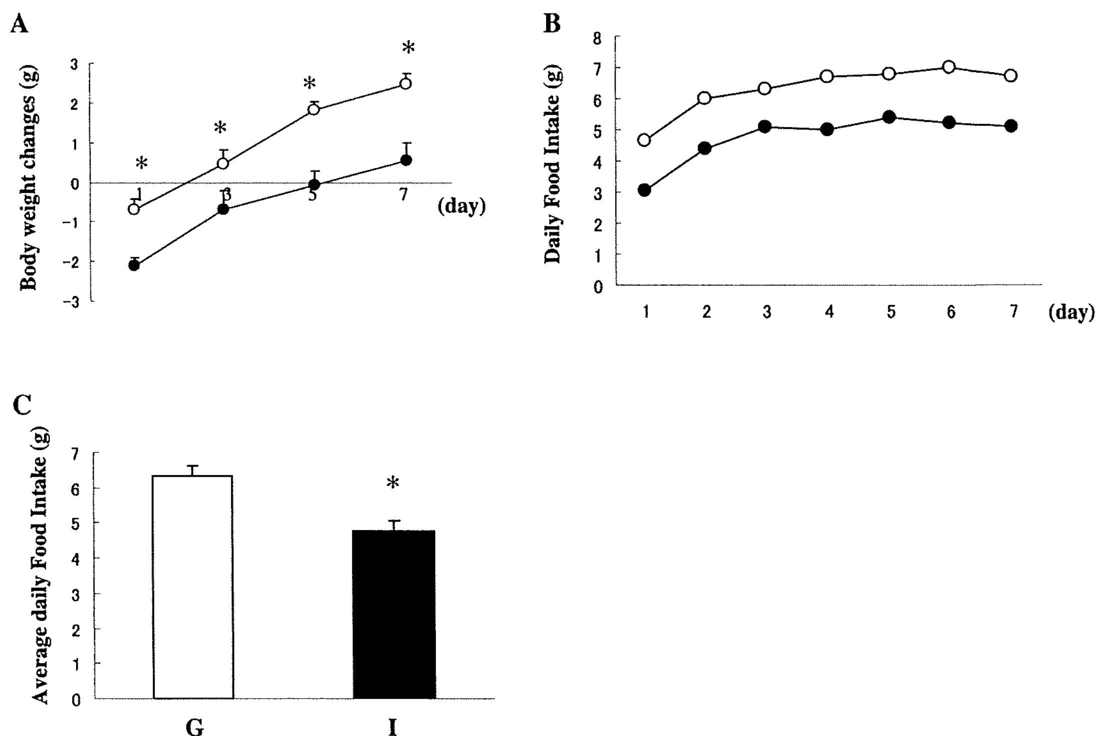

FIG. 5. Body weight changes (A), daily food consumption (B), and average daily food consumption for the week that transpired (C) in the period from 9- to 10-wk-old individually housed (*solid circles* and *filled bars*) and group-housed (*open circles* and *bars*) db/db mice. Basal body weights of the individually housed and group-housed db/db mice were 41.3 ± 0.3 and 41.5 ± 0.4 g, respectively. Data are presented as the mean values ± SEM (n = 7). I, Individually housed animals; G, group-housed animals. *, $P < 0.05$.

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in white adipose tissue are increased in responses to high-fat diet (21). The fourth reason is based on the result that the expression of PPARs in white adipose tissue and soleus muscle was not increased in the individually housed KK mice. The expression of PPARα and PPARγ in white adipose tissue and skeletal muscle is increased in responses to high-fat diet (23–25). The fifth reason is based on the result of hypothalamic SOCS-3 gene expression, because hypothalamic SOCS-3 has an inhibitory role in diet-induced obesity (14–16). Thus, it is possible that decreased energy expenditure rather than increased energy intake might primarily contribute to the social isolation-induced adiposity.

Circulating leptin signals the CNS, first to rapidly increase sympathetic outflow and then to inhibit food intake (13, 17). The sympathetic nervous system increases lipolysis and suppresses leptin expression in white adipose tissue through the β3-AR (13, 17). Thus, there is a negative feedback system between sympathetic nervous system stimulation and leptin production. Therefore, dysfunction of autonomic neural circuits between white adipose tissue and the CNS contributes to the development of obesity (13, 17). However, our present results demonstrate that social isolation decreased body weight gain in association with decreased daily food consumption in obese db/db mice, suggesting that disturbed leptin signaling does not contribute to the causes of SIO. Therefore, the decreased expression of hypothalamic *LepR* might be a secondary response to the enhanced adiposity induced by chronic social isolation. These findings suggest central neural mechanisms independent of leptin signaling contribute to the development of the SIO.

The central serotonin and leptin signaling contribute substantially to the regulation of feeding and energy homeostasis. The expression of 5-HT2C receptors appears to be restricted to the CNS (19). Mice with a null mutation of the 5-HT2C receptor gene elevate body weight, and are resistant to the anorexic effects of meta-chlorophenylpiperazine, indicating that 5-HT2C receptors contribute substantially to the serotonin regulation of body weight (19). Despite hyperphagia, 5-HT2C receptor mutant mice do not develop obesity until 6 months of age, because of increased physical activity (12, 34). Chronic hyperphagia and hyperactivity lead to a late onset obesity associated with hyperleptinemia in 5-HT2C receptor mutant mice because of decreased energy cost of physical activity (12, 34). 5-HT2C receptor has been suggested to regulate feeding behavior and physical activity rather than direct neural regulation of fat metabolism. Despite hyperactivity, pair-feeding, however, does not decrease body weight in 5-HT2C receptor mutants compared with wild-type mice (19). The present study demonstrates that white adipose tissue expresses serotonin 5-HT2C receptor, and the leptin-induced inhibition of 5-HT2C receptor expression in white adipose tissue might be an additive factor for the enhanced adiposity independent of feeding. The direct effects of 5-HT2C receptor gene on the white adipose tissue *in vivo* warrant further examination in the future.

Hepatic gluconeogenesis contributes to hyperglycemia in type 2 diabetes. Increased G6Pase, Fbp1, and Fbp2 genes involved in hepatic gluconeogenesis are associated with increased glucose production and blood glucose levels in db/db mice with insulin resistance and streptozocin-induced diabetic animals with insulin deficiency (32). Given our results, insulin-independent diabetes induced by chronic social isolation was also associated with increased expression of hepatic gluconeogenetic genes such as G6Pase, Fbp1, and Fbp2, but not PEPCK in KKA^y mice. PEPCK, a rate-limiting enzyme in the gluconeogenic pathway, is required for glucose synthesis from pyruvate, but is not required for glucose production from other carbon precursors such as glycerol. Given our results, an increased gluconeogenic pathway other than glucose synthesis from pyruvate might contribute to the chronic social isolation-induced hyperglycemia in the KKA^y mice. Hyperglycemia in individually housed KKA^y mice is due to hyperphagia, which decreases plasma des-acyl ghrelin levels (31). Agouti peptide, an endogenous MC-4 receptor antagonist, in addition to chronic social isolation, might induce hyperphagia, leading to the hyperglycemia in KKA^y mice.

In summary, these results suggest that social isolation promotes leptin-independent adiposity in KK mice and develops into insulin-independent diabetes associated with increased expression of hepatic gluconeogenetic genes in KKA^y mice. Thus, social isolation can be included in the environmental factors related to the development of obesity and type 2 diabetes, and group housing can apparently prevent or at least mitigate it.

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K.Non., K.Noz., and Y.O. have nothing to declare.

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OBSERVATIONS

Possible Relevance of HLA-DRB1*0403 Haplotype in Insulin Autoimmune Syndrome Induced by α-Lipoic Acid, Used as a Dietary Supplement

Insulin autoimmune syndrome (IAS) is characterized by frequent hypoglycemic attacks associated with the presence of autoantibodies to insulin in patients who have not received insulin injections. Approximately half of IAS patients have a medication history before onset, and over 90% of the agents are sulfydryl compounds such as methimazole, mercaptopropionyl glycine, or glutathione. In addition to these compounds, α-lipoic acid (ALA), which is widely used as a health supplement, is associated with a risk of IAS induction, as previously reported in *Diabetes Care* (1) and other journals (2,3). DRB1*0406 is reportedly the most common and DRB1*0403 the next most common HLA haplotype conferring susceptibility to IAS (4). As for ALA-induced IAS, all three reported cases have the DRB1*0406 but not the DRB1*0403 haplotype (1–3). However, we recently observed a case of IAS, possibly induced by ALA, in a patient who has the DRB1*0403 haplotype.

The patient, a 45-year-old woman, lapsed into hypoglycemic coma 1 month after starting to take ALA. She had not taken any of the other aforementioned sulfydryl compounds. She exhibited marked hyperinsulinemia (fasting plasma glucose 88 mg/dl, serum immunoreactive insulin 13,240 μU/ml, and serum C-peptide immunoreactivity 2.93 ng/ml). Antibodies to insulin were detected with an insulin binding ratio of 81.2%. Antibody affinity was low, while binding activity was high, as commonly observed in IAS. Based on these results, she was diagnosed as having IAS possibly induced by ALA. However, she has the DRB1*0403, not the DRB1*0406, haplotype.

This is the first report of a patient with ALA-induced IAS having the DRB1*0403 haplotype. Since the DRB1*0403 haplotype is reportedly associated with IAS induced by other sulfydryl compounds, it is likely to confer susceptibility to ALA-induced IAS. Although IAS was a relatively rare cause of hypoglycemia in the past, ALA has become more widely available as a dietary supplement for treating obesity and diabetes complications. Furthermore, in contrast to the very low prevalence of DRB1*0406 in ethnic groups other than East Asians, DRB1*0403 was found to be widely distributed across various populations (5). We should therefore be more aware of ALA-induced IAS.

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REVIEW

Avenues of Communication between the Brain and Tissues/Organs Involved in Energy Homeostasis

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Abstract. Obesity is a rapidly increasing public health concern worldwide as a major risk factor for numerous disorders, including diabetes, hypertension and heart disease. Despite remarkable advances in obesity research over the past 10 years, the molecular mechanisms underlying obesity are still not completely understood. To maintain systemic energy homeostasis, it is important that organs/tissues communicate metabolic information among each other. Obesity-related disorders can be thought of as resulting from dysregulation of this inter-tissue communication. This system has both afferent sensing components and efferent effecter limbs. The afferent signals consist of not only humoral factors, such as nutrients (glucose, fatty acids and amino acids) and adipocytokines (leptin, adiponectin and so on), but also autonomic afferent nerve systems. Both converge on brain centers, most importantly within the hypothalamus, where the signals are integrated, and the direction and magnitude of efferent responses are determined. The efferent elements of this physiological system include those regulating energy inputs and outputs, i.e. food intake and metabolic rates. In this review, we will summarize recent advances in research on metabolic information avenues to the brain, which are important for energy homeostasis.

Key words:

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THE worldwide prevalence of obesity, which is a major risk factor for numerous disorders, including diabetes, hypertension and heart disease, is increasing at an alarming rate, with major adverse consequences for human health [1]. Body weight is thought to be determined by the balance between energy intake and expenditure. However, alterations in daily food intake and physical activity do not rapidly affect body weight. Why is this? The most plausible explanation is the existence of systems which maintain energy homeostasis throughout the body. Energy homeostasis is maintained by multiple mechanisms that involve gathering information on the body's nutritional status and making appropriate behavioral and metabolic responses to changes in fuel availability. For such inter-organ/tissue communication, humoral factors, including insulin and adipocytokines, are known to be very important. In addition, we and other research groups have recently reported the autonomic nervous system to play an important role in conveying metabolic information. Using these systems, the brain obtains information on peripheral metabolic status and processes it to send signals which regulate metabolism in the periphery. In particular, the hypothalamus is a primary site of convergence and integration for redundant energy status signaling, which includes central and peripheral neural inputs as well as hormonal and nutritional factors. These pathways of inter-tissue communication are summarized in Fig. 1. Recent advances in this field are reviewed herein.

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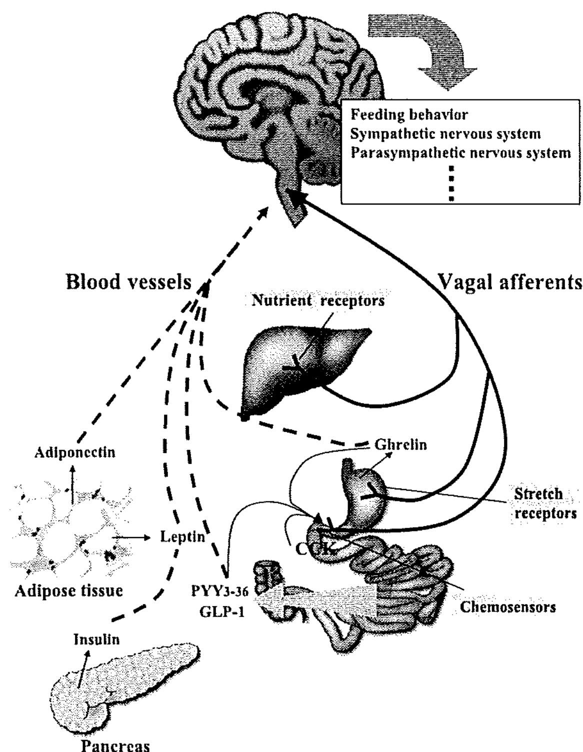

Fig. 1. Schematic presentation of inter-tissue communication (quoted from [31] with slight modification).

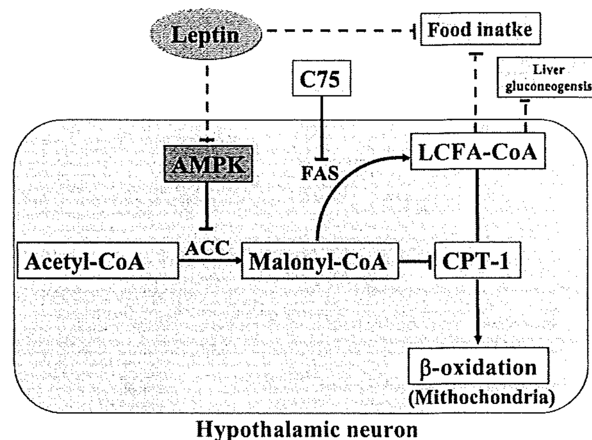

Fig. 2. Fatty acid metabolism, in the hypothalamic cells, which plays an important role in maintaining energy homeostasis (quoted from [65] with slight modification).

1. Brain inputs — humoral factors —

1) Nutrients

It is reasonable that essential nutrients, such as carbohydrates, lipids and proteins, mediate nutritional signals to the central nervous system by themselves. First, we will focus on the mechanism whereby these nutrients convey peripheral fuel status to the central nervous system.

a) Free fatty acids

The access of circulating free fatty acids to cerebrospinal fluids is generally proportional to the plasma fatty acid concentration [2, 3], indicating that the brain may acquire information regarding the peripheral metabolic state via cerebrospinal fatty acid levels. Fatty acid-sensitive neurons have been identified in the hypothalamus. For instance, an *in vitro* patch clamp study [4] showed that, among arcuate neurons, 13% of cells had increased electrical activity, while 6% had decreased activity when oleic acid was applied. Intracerebroventricular administration of oleic acid reportedly inhibits hepatic glucose production and food intake [5]. In addition, hypothalamic inhibition of carnitine:palmitoyl-CoA transferase-1 (CPT-1), an important mitochondrial enzyme that transfers long-chain fatty acyl-coenzyme A (LCFA-CoA) into mitochondria, decreases food intake and suppresses endogenous glucose production in the liver [6]. Furthermore, it was also reported that efferent vagal nerve signals from the brain to the liver are involved in hepatic gluconeogenesis in these experimental settings [7]. Hu *et al.* reported that central administration of C75, a potent inhibitor of fatty acid synthase (FAS), decreased food intake [8]. Since FAS inhibition increases malonyl-CoA and thus suppresses CPT1 activity, LCFA-CoA in hypothalamic neurons appears to be increased. Taken together, these results indicate that the cytosoplasmic LCFA-CoA concentration in hypothalamic neurons plays an important role in energy homeostasis (Fig. 2).

Leptin, an anorexigenic factor, reportedly decreases AMP-activated protein kinase (AMPK) activity in hypothalamic neurons [9], while ghrelin, an orexigenic factor, increases it [10]. AMPK is the downstream component of a kinase cascade that acts as a sensor of cellular energy charge, being activated by rising AMP coupled with falling ATP. AMPK phosphorylates and inhibits acetyl-CoA carboxylase (ACC), resulting in decreased malonyl-CoA levels. As malonyl-CoA inhibits CPT-1, AMPK activation decreases cytoplasmic LCFA-CoA levels. Thus, cytoplasmic LCFA-CoA in

hypothalamic neurons may be involved in appetite regulation by leptin and ghrelin.

b) Amino acids

Amino acids also seem to communicate energy status information from the periphery. Transport of amino acids across the blood-brain barrier has been demonstrated [11]. The levels of amino acids in cerebrospinal fluids are reflected by peripheral blood levels. Central administration of leucine increases hypothalamic mTOR (mammalian target of rapamysin) activity, and thereby decreases food intake and body weight [12]. mTOR is a highly conserved serine/threonine kinase, present in organisms from yeast to mammals, the activity of which is sensitive to levels of branched-chain amino acids, especially L-leucine [13, 14]. Thus, mTOR is known to be one of the energy sensors for amino acids conserved in throughout evolution in organisms and, in mammals, hypothalamic mTOR signaling appears to play an important role in regulating systemic energy metabolism. Leptin increases hypothalamic mTOR activity, and the inhibition of mTOR signaling blunts leptin's anorectic effect [12], although further studies are needed to clarify the role of mTOR in energy homeostasis.

c) Glucose

It is well known that increases in serum glucose affect glucose-sensing neurons in the hypothalamus, resulting in suppression of food intake and liver gluconeogenesis (glucostatic theory). Two populations of glucose-sensing neurons have been defined: those excited (in which electrical activity is increased; GE neurons) and those inhibited (decreased activity; GI neurons) as local glucose levels rise. Such neurons have mainly been characterized in the ventromedial hypothalamic nucleus (VMH) and the arcuate nucleus (ARC) [15]. Glucose sensing mechanisms in pancreatic β cells, which secrete insulin in response to rising blood glucose, have been well analyzed. The glucose sensor in pancreatic β cells involves mainly GLUT2, glucokinase and specific KATP channels. Analogously, glucose sensing mechanisms in glucose-responsive neurons have been proposed. Two recent studies found GLUT2 expression in the rat brain [16, 17]. In addition, glucokinase is expressed in the rat hypothalamus [18]. Expressions of both GLUT2 and glucokinase have also been demonstrated in the human hypothalamus [19]. Using calcium imaging and single cell RT-PCR in freshly dissociated neurons from the VMH, Kang *et al.* confirmed the presence of glucokinase and KATP channels in some glucosensitive neurons [20]. KATP channel activity represents a key step in the electrical activity of GE neurons in the ARC and VMH in response to glucose concentration changes [21].

2) Insulin

Insulin is a product of pancreatic β cells and is the master metabolic switch between the fed and fasted states, mediating metabolic fuel disposition and use. Therefore, it has been proposed that insulin itself might be the fuel status signal to the brain, but the precise mechanisms have long been unclear. The activation of insulin signaling in the ARC, in the absence of elevated systemic insulin, is sufficient to decrease food intake and blood glucose levels via substantial inhibition of endogenous glucose production (EGP) [22, 23]. A recent study revealed the central effects of insulin on the suppression of EGP to be mediated by the insulin receptor-insulin receptor substrate 2 (IRS2)-phosphatidylinositol 3OH kinase (PI3K) pathway, resulting in KATP channel activation in the ARC [24]. Inoue *et al.* reported that centrally administered insulin induces IL-6 production in the liver, followed by STAT3 activation, resulting in suppression of hepatic EGP [25]. The activation of insulin receptors in the brain, in particular the ARC of the hypothalamus, plays an important role in the regulation of glucose homeostasis and food intake.

3) Adipocytokines

a) Leptin

Leptin is produced mainly in adipocytes in proportion to fat stores; adequate leptin levels communicate the repletion of body energy stores to the central nervous system in order to suppress food intake and permit energy expenditure [26]. Leptin binds to leptin receptors (Ob-Rb) in the hypothalamus, resulting in activation of the JAK/STAT pathway [27, 28] and the IRS2/PI3K pathway [29]. In addition, Minokoshi *et al.* recently reported that leptin suppressed hypothalamic AMPK activity, leading to food intake suppression [9]. As stated above, leptin also activates mTOR signaling in the hypothalamus. Thus, leptin signaling involves at least four pathways, JAK/STAT, IRS2/PI3K, AMPK and mTOR. Complicated interactions

may exist among these four pathways.

In most individuals with ordinary obesity, circulating leptin levels are elevated, but the body does not adequately respond to this increased leptin with reduced food intake. This under-responsiveness to leptin in most forms of obesity has given rise to the idea that obesity is associated with, or even caused by, a state of relative leptin resistance similar to insulin resistance. The mechanisms underlying leptin resistance remain a matter of debate. From the therapeutic point of view, the mechanism underlying leptin resistance is an important issue which awaits clarification.

b) Adiponectin

There is a recent report [30] suggesting adiponectin to have central effects on energy metabolism. Intravenous administration of adiponectin increased the level in cerebrospinal fluid. In addition, central adiponectin administration increased systemic energy expenditure and reduced body weight, followed by decreased blood glucose and serum lipid levels. Detailed studies are needed to clarify the roles of adiponectin in communicating the peripheral metabolic state to the central nervous system.

2. Brain inputs — afferent nerve signals —

1) Innervation

a) Intra-abdominal innervation without white adipose tissues

First, the innervation of intra-abdominal tissues requires explanation. For example, the gut is innervated by both splanchnic (sympathetic) and vagal (parasympathetic) nerves. Detailed fiber count studies have revealed that the abdominal vagal nerve is comprised of approximately 75% afferent fibers, the splanchnic nerve 50%. Afferent signals from the gut to the brain are carried in vagal and splanchnic nerve pathways. Vagal afferents respond to specific luminal chemical stimuli, physiological levels of distention or nutrients, whereas splanchnic afferents convey information regarding noxious stimuli [31]. On the other hand, intrapelvic organs, urogenital organs and so on, are innervated by a pelvic nervous plexus, which consists of both sympathetic and parasympathetic nerves.

b) Innervation of intra-abdominal adipose tissues

White adipose tissues are also innervated by both efferent and afferent nerve fibers. As for the efferent sympathetic fibers, numerous reports have described functions, including lipolysis or β oxidation [32–34]. On the other hand, a recent study demonstrated white adipose tissues to be innervated by efferent parasympathetic nerve fibers [35], although their physiological functions remain to be elucidated. There are only a few articles focusing on the functions of afferent nerve fibers from white adipose tissues. Niijima [36] and Tanida *et al.* [37] used electrical firing measurements to show that leptin induces functional activation of afferent nerve fibers from epididymal white adipose tissues. In a more recent study, afferent nerve innervation in epidydimal white adipose tissues was demonstrated anatomically [38]. In addition, we recently reported the functional significance of afferent nerve signals from intra-abdominal adipose tissues which modulate hypothalamic leptin sensitivity, as described in detail below [39].

2) Signals transmitted by afferent autonomic nerve fibers

a) Signals from the gut

It has been reported that afferent autonomic nerve fibers convey signals carrying information about energy homeostasis [40–43]. Physiological distention of the gut as well as cholecystokinin (CCK) [44], PYY3-36 [45] and glucagon-like peptide-1 (GLP-1) [46] stimulate afferent vagal nerve fibers, resulting in food intake suppression. In contrast, ghrelin enhances food intake via the afferent vagus [47]. CCK is produced by mucosal enteroendocrine cells of the duodenum and jejunum and is secreted in response to the presence of food within the gut lumen. Sulfated CCK, which preferentially binds to CCK1 receptors on vagal afferent neurons, sends satiety signals to the brain; hence, vagotomy inhibits the anorectic effect of CCK [44]. GLP-1 and PYY3-36 secretions from enteroendocrine L cells are triggered by luminal nutrients. The mechanisms by which sugars activate L cells involve the closure of ATP sensitive potassium channels, resulting in depolarization of the cells, via a mechanism analogous to insulin secretion from β cells [48, 49]. Koda *et al.* [45] showed that peripheral administration of PYY3-36 stimulates vagal afferent nerves via a Y2 receptor which is expressed at nerve terminals. Abdominal