Involvement of Agouti-Related Protein, an Endogenous Antagonist of Hypothalamic Melanocortin Receptor, in Leptin Action

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To understand the role of agouti-related protein (AGRP), an endogenous antagonist of hypothalamic melanocortin receptor, in leptin action, we produced a full-length recombinant AGRP and examined its effect on the satiety effect of leptin. We also studied leptin's regulation of hypothalamic AGRP mRNA expression. A single intracerebroventricular (ICV) injection of AGRP significantly increased cumulative food intake and body weight in a dose-dependent manner in rats. The leptin-induced inhibition of food intake and body weight was reversed by co-injection of AGRP in a dose-dependent manner. Hypothalamic AGRP mRNA expression was upregulated in leptin-deficient *ob/ob* mice and leptin receptor-deficient *db/db* mice and downregulated in lethal yellow *agouti* mice (KKA^y mice) with hyperleptinemia. A single ICV injection of leptin reversed the increased AGRP mRNA levels in *ob/ob* mice but not in *db/db* mice. In control mice and KKA^y mice, AGRP mRNA expression was upregulated during fasting, when plasma leptin concentrations were decreased. No significant increase in AGRP mRNA expression was noted during fasting in control mice and KKA^y mice treated with leptin. This study provides the first direct evidence that AGRP is a negative regulator of leptin action, and leptin downregulates hypothalamic AGRP production. Because leptin is shown to increase hypothalamic α-melanocyte stimulating hormone (α-MSH) production, our data suggest that its action via the hypothalamic melanocortin system is determined by the balance between the levels of its agonist and antagonist, α-MSH and AGRP. *Diabetes* 48:2028–2033, 1999

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aCSF, artificial cerebrospinal fluid; AGRP, agouti-related protein; AGRP-(83–132), AGRP-(83–132)-NH_2 fragment; α-MSH, α-melanocyte stimulating hormone; Arc, hypothalamic arcuate nucleus; A^y, lethal yellow agouti locus; BAT, brown adipose tissue; GLP-1, glucagon-like peptide-1 (7–36) amide; HPLC, high-performance liquid chromatography; IC_{50}, 50% inhibitory concentration; ICV, intracerebroventricular; IPTG, isopropyl-β-D-thiogalactopyranoside; MC3-R, MC4-R, and MC5-R, melanocortin receptor-3, -4, and -5; MC4-R$^{-/-}$ mice, mice with targeted disruption of MC4-R; NDP-MSH, Nle^4-D-Phe^7-α-MSH; POMC, proopiomelanocortin; UCP-1, uncoupling protein-1.

Leptin is an adipocyte-derived blood-borne satiety factor that acts directly on the hypothalamus, thereby regulating food intake and energy expenditure (1–3). However, the molecular mechanism of hypothalamic action of leptin has been poorly understood. Melanocortin peptides derived from the proopiomelanocortin (POMC) gene, especially α-melanocyte stimulating hormone (α-MSH), have been involved in the regulation of feeding behaviors (4,5). Leptin receptor is expressed in the POMC neurons in the hypothalamic arcuate nucleus (Arc) (6), where it increases POMC gene expression (7). Recently, we and others have demonstrated that a single intracerebroventricular (ICV) injection of leptin decreases food intake and body weight and increases mitochondrial uncoupling protein-1 (UCP-1) mRNA expression in the brown adipose tissue (BAT), which is abolished by co-injection of SHU9119, a potent antagonist of α-MSH (8,9). These findings suggest that satiety effect and sympathetic activation of leptin are mediated by the hypothalamic melanocortin system; leptin regulates food intake and energy expenditure at least in part via α-MSH. It has been recognized that lethal yellow *agouti* mice (10), a genetically determined rodent model of leptin resistance (11), develop a maturity-onset obesity with hyperleptinemia due probably to the antagonism of melanocortin receptor-4 (MC4-R) by ectopic expression of the agouti protein (12). Furthermore, targeted disruption of MC4-R in mice (MC4-R$^{-/-}$ mice) has resulted in obesity and hyperleptinemia similar to those of A^y/+ mice (13). Thus, a defect in the hypothalamic α-MSH/MC4-R signaling pathway might cause leptin resistance.

Agouti-related protein (AGRP) was originally identified in 1997 using an expressed sequence tag database (14,15). Human preproAGRP is a 132–amino acid protein with a putative signal sequence and is 25% identical to human agouti protein. The mature AGRP is thought to be a 108–amino acid cysteine-rich carboxyl terminus, which is produced on cleavage of the signal sequence from its precursor. While expression of the agouti protein is restricted normally to the skin (10), AGRP is expressed in the Arc (14,16) and is shown to be a potent antagonist of MC3-R and MC4-R (15,17,18). It is, therefore, conceivable that AGRP can antagonize leptin action by antagonism of MC4-R. It is also reported that AGRP is upregulated in the Arc from leptin-deficient *ob/ob* mice and leptin receptor–deficient *db/db* mice (14,15), suggesting that leptin

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may, in turn, regulate hypothalamic AGRP production. All the findings suggest presence of complex interactions between leptin and AGRP. To elucidate the role of AGRP in leptin action, we produced the full-length recombinant AGRP and examined its effect on the satiety effect of leptin. We also studied leptin's regulation of hypothalamic AGRP mRNA expression.

RESEARCH DESIGN AND METHODS

Animals. Eight-week-old male Sprague-Dawley rats and 20-week-old male C57BL/6J *ob/ob* mice; C57BL/KsJ *db/db* mice and their respective control C57BL/6J and C57BL/KsJ mice; and KK and KKA^y mice (19) were purchased from Japan CLEA (Tokyo). Animals were housed in a temperature-, humidity-, and light-controlled room (12-h light/12-h dark cycle) and allowed free access to water and standard rat chow (CE-2, 352 kcal/100 g; Japan CLEA). All experimental procedures were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research.

Preparation of recombinant human AGRP and leptin and other peptides. Approximately 20 µg of total RNA from the human adrenal was reverse-transcribed by oligo (dT) priming and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Grand Island, NY), and the resulting single-stranded DNA was subjected to PCR with primers (sense, 5′-TTTAGCGCTCGATGGGT TTGGCTCCC-3′; antisense, 5′-TTTGTCGACCTAGGTGCGGCTGCAGGGATT-3′) selected to amplify sequences corresponding to nucleotides from +64 to +399 of the human AGRP cDNA (14) (GenBank U89485). The sense and antisense primers included *Eco* 47 III and *Sal* I restriction sites, respectively, to facilitate the subsequent subcloning. The PCR product was digested with *Eco* 47 III and *Sal* I and subcloned into the *Sma* I and *Sal* I sites of the pQE32 vector (QIAGEN, Hilden, Germany), which places the 6xHis coding sequences at the 5′ end of the insert (pQE32AGRP). All predicted junction sequences were verified by DNA sequencing. *Escherichia coli* JM109 was transformed with pQE32AGRP. Recombinant His-Tag/AGRP fusion protein was induced in the presence of 1 mmol/l isopropyl-β-D-thiogalactopyranoside (IPTG) and purified on an Ni-NTA agarose using the QIAexpress Kit (QIAGEN) according to the manufacturer's protocols. The purified protein was further fractionated by reverse-phase high-performance liquid chromatography (HPLC) using the RESOURCE RPC column (Amersham Pharmacia Biotech, Buckinghamshire, U.K.), and analyzed by 4 to 20% SDS-PAGE and silver staining. Protein concentrations were determined by the Bradford method (20).

Recombinant human leptin was prepared as described (21). α-MSH, Nle^4-D-Phe^7-α-MSH (NDP-MSH), and glucagon-like peptide-1 [7–36] amide (GLP-1) (22) were purchased from Peptide Institute (Minoh, Japan). SHU9119 was purchased from Phoenix Pharmaceuticals (Mountain View, CA). AGRP, leptin, α-MSH, and GLP-1 were dissolved in artificial cerebrospinal fluid (aCSF) (23) for the ICV injection.

Binding assays. NDP-MSH was labeled with ^{125}I and purified as described (24). The specific activity of [^{125}I]-NDP-MSH ranged from 250–1,000 µCi/mmol. Binding assays with rat hypothalamic membranes were performed as described with slight modifications (24). Hypothalami were removed from Sprague-Dawley rats and homogenized in a homogenizing buffer (50 mmol/l Tris-HCl, 140 mmol/l NaCl, 2.5 mmol/l $CaCl_2$, 1.2 mmol/l $MgCl_2$, 0.6% ascorbic acid, and 500 KIU/ml aprotinin, pH 7.2) and washed three times by centrifugation (40,000*g* for 15 min). The pellet was resuspended and incubated at 37°C for 2 h with a fixed concentration (2.0 nmol/l) of [^{125} I]-NDP-MSH and various amounts of unlabeled NDP-MSH, α-MSH, SHU9119, or AGRP in 0.2 ml binding buffer (the homogenizing buffer plus BSA [0.25%]). The samples were washed five times to terminate the reaction. Radioactivity was counted by the Packard autogamma scintillation spectrometer (Redondo Beach, CA), and data were analyzed by fitting it to the four parametric logistic functions using nonlinear least squares regression. Nonspecific binding was <5% of the total binding when determined using 3.0 nmol/l unlabeled NDP-MSH. All assays were performed in duplicate and repeated three times.

ICV injection into Sprague-Dawley rats. A stainless steel ICV cannula (outer diameter 1.09 mm) (Becton Dickinson, Sparks, MD) was implanted under anesthesia in the skull of rats 5 days before the injection experiment, using coordinates (6.5 mm anterior to the lambdoidal suture; ±1.4 mm lateral to the midline; 4.5 mm from the dural surface) (21, 25). The ICV cannula placement was confirmed in all rats by introducing Evans blue after the experiments (21). Only the animals that showed the correct ICV cannula placement were included in the present study. For the ICV injection of AGRP alone, cumulative food intake and body weight change were measured during the 5 h after ICV injection of AGRP at the early light phase (10:00). For the ICV co-injection of AGRP and α-MSH, leptin, or GLP-1, cumulative food intake and body weight change were measured during 5 h at the onset of the dark phase after a single ICV injection of AGRP (0.15 to 1.5 µg/10 µl per rat) or aCSF (10 µl) (18:30), followed by the treatment with or without α-MSH (5.0 µg), leptin (2.0 µg), or GLP-1 (10 µg) (19:00).

Total RNA extraction and Northern blot analysis. Total RNA was extracted from hypothalami of genetically obese *ob/ob* and *db/db* mice, their control C57BL/6J and C57BL/KsJ mice, and KK and KKA^y mice at 9:00 when fed ad libitum or after fasting (72 h for *ob/ob*, *db/db*, 48 h for KKA^y mice, and 24 h for C57BL/6J, C57BL/KsJ, and KK mice). Hypothalamic RNA was extracted from *ob/ob* and *db/db* mice that received a single ICV injection of leptin (1.0 µg/1 µl per mouse) through a 27-gauge microsyringe placed in an appropriate position 6 h before they were killed. Hypothalamic RNA was also extracted from C57BL/6J, KK, and KKA^y mice that received an ICV injection of leptin (1.0 µg/1 µl per mouse) during fasting. Northern blot analysis was performed (26) with the ^{32}P-labeled mouse AGRP cDNA fragment as a probe. A human β-actin genomic probe (Wako Pure Chemical, Osaka, Japan) was used to monitor the amount of total RNA in each sample. Actin transcripts were roughly equivalent among different RNA samples (data not shown).

Radioimmunoassay for mouse leptin. Blood was sampled from the retro-orbital sinus of mice when fed ad libitum or after fasting. Plasma leptin concentrations were determined using the radioimmunoassay for mouse leptin (Linco Research Immunoassay, St. Louis, MO).

Statistical analysis. All data were expressed as means ± SE. Statistical significance of differences in mean values was assessed by Duncan's multiple-range test following one-way analysis of variance.

RESULTS

Preparation of recombinant human AGRP. Recombinant His-Tag/AGRP fusion protein was bacterially expressed with the IPTG induction for 3 h. The crude lysates were purified with Ni-NTA agarose and reverse-phase HPLC and evaluated by SDS-PAGE and silver staining (Fig. 1*A*). A single band with a molecular mass of ~20 kDa was detected with the IPTG induction. The molecular weight was roughly consistent with that calculated from the full-length AGRP and 6xHis His-Tag. No protein bands were detected in the absence of IPTG (data not shown).

Binding assays. Using rat hypothalamic membranes, we examined displacement curves of [^{125}I]-NDP-MSH by unlabeled NDP-MSH, α-MSH, SHU9119, and the full-length AGRP (Fig. 1*B*). Hypothalamic binding of [^{125}I]-NDP-MSH (2.0 nmol/l) was displaced completely by unlabeled NDP-MSH and α-MSH (1 and 100 nmol/l, respectively), indicating the presence of intact α-MSH binding sites in the hypothalamic membrane preparations. The 50% inhibitory concentration (IC_{50}) values calculated for NDP-MSH, α-MSH, and SHU9119 were 0.44, 94.0, and 0.13 nmol/l, respectively. The IC_{50} value for AGRP was 190 nmol/l. Recombinant AGRP at the dose of 1 µmol/l displaced completely the NDP-MSH binding to the rat hypothalamic membrane preparations, in which MC3-R, MC4-R, and MC-5R are expressed, suggesting that it can

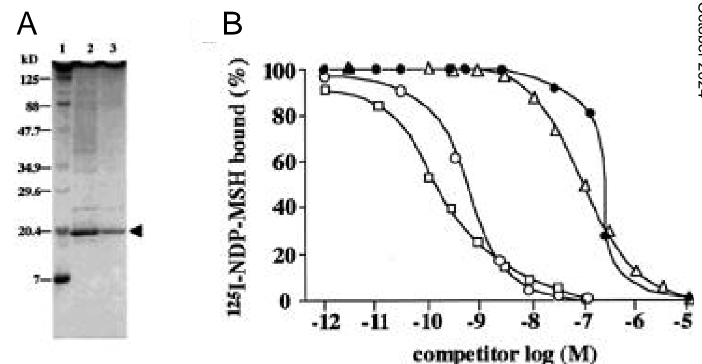

FIG. 1. *A*: SDS-PAGE and silver staining analysis of the proteins from the serial steps of the purification procedure. *Lane 1*, size marker (prestained SDS-PAGE standards; Bio-Rad Laboratories, Hercules, CA); *lane 2*, proteins purified on an Ni-NTA agarose; *lane 3*, proteins purified and fractioned by reverse-phase HPLC. *B*: Displacement curves of [^{125}I]-NDP-MSH (2.0 nmol/l) by NDP-MSH (○), α-MSH (△), SHU9119 (□), and AGRP (●) using rat hypothalamic membrane preparations.

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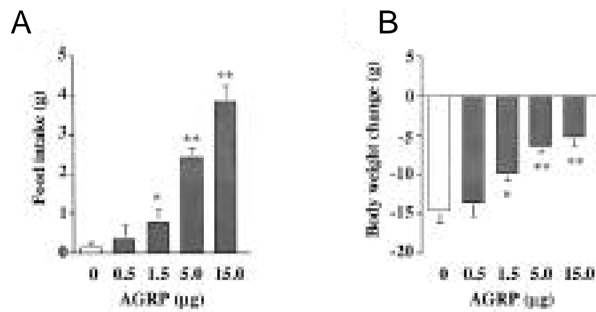

FIG. 2. Effects of a single ICV injection of AGRP on food inake (*A*) and body weight change (*B*) in rats. □, aCSF; ■, AGRP at doses of 0.5, 1.5, 5.0, and 15.0 μg. Values are means ± SE (n = 8–10). *P < 0.05, **P < 0.01 compared with aCSF-treated groups.

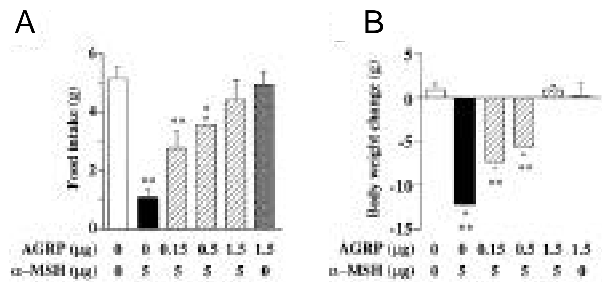

FIG. 3. Effects of a single ICV co-injection of AGRP and α-MSH on food inake (*A*) and body weight change (*B*) in rats. □, aCSF; ■, 5.0 μg α-MSH alone; ▨, 5.0 μg α-MSH plus AGRP at doses of 0.15, 0.5, and 1.5 μg; ▩, 1.5 μg AGRP alone. Values are means ± SE (n = 8–10). *P < 0.05, **P < 0.01 compared with aCSF-treated groups.

antagonize the agonist binding to each of the melanocortin receptor subtypes.

ICV injection of AGRP in Sprague-Dawley rats. Cumulative food intake of vehicle-treated animals during 5 h at the early light phase was significantly small compared with that of untreated control animals at the early dark phase (0.1 ± 0.1 vs. 5.5 ± 0.6 g, n = 8–10, P < 0.01). A single ICV injection of AGRP (1.5–15.0 μg) increased significantly and dose-dependently cumulative food intake in rats compared with vehicle-treated groups (Fig. 2*A*). Treatment with 15.0 μg AGRP increased cumulative food intake ~30-fold relative to vehicle-treated groups (P < 0.01).

Body weight of vehicle-treated rats was reduced during 5 h at the early light phase (–14.7 ± 1.5 g) but increased during 5 h at the onset of the dark phase (0.8 ± 0.5 g). In rats that received a single ICV injection of AGRP (1.5–15.0 μg), body weight was increased significantly and dose-dependently compared with vehicle-treated groups during 5 h at the early light phase (Fig. 2*B*). In rats that received a 1.5 μg AGRP treatment, body weight reduction was inhibited by 34% compared with vehicle-treated controls (–14.7 ± 1.5 vs. –9.7 ± 1.0 g, P < 0.05). Treatment with 15.0 μg AGRP caused a significant inhibition of body weight reduction by 72% relative to vehicle-treated groups (P < 0.01).

ICV co-injection of AGRP and α-MSH in Sprague-Dawley rats. To examine whether recombinant AGRP can antagonize the satiety effect of α-MSH in vivo, we examined the effect of a single ICV co-injection of AGRP and α-MSH on food intake and body weight in rats during 5 h at the onset of the dark phase. Following a single ICV injection of 5.0 μg α-MSH, cumulative food intake was reduced by ~75% relative to vehicle-treated controls (5.1 ± 0.4 vs. 1.3 ± 0.9 g, n = 8–10, P < 0.01) (Fig. 3*A*). By co-injection of AGRP (0.15–1.5 μg), the α-MSH-induced inhibition of food intake was reversed in a dose-dependent manner. Co-injection of 1.5 μg AGRP abolished completely the α-MSH-induced decrease in cumulative food intake relative to vehicle-treated rats (4.4 ± 0.7 vs. 5.1 ± 0.4 g).

Body weight gain was also inhibited significantly in rats treated with 5.0 μg α-MSH relative to vehicle-treated rats during 5 h at the onset of the dark phase (–12.9 ± 0.6 vs. 0.9 ± 0.7 g, P < 0.01) (Fig. 3*B*). Treatment with AGRP (0.15–1.5 μg) also reversed the α-MSH-induced inhibition of body weight change in a dose-dependent manner. Co-injection of 1.5 μg AGRP abolished completely the α-MSH-induced inhibition of body weight change relative to vehicle-treated rats (1.0 ± 0.4 vs. 0.9 ± 0.7 g).

In this study, treatment with 1.5 μg AGRP alone did not affect food intake and body weight in rats relative to vehicle-treated rats during 5 h at the onset of the dark phase.

ICV co-injection of AGRP and leptin in Sprague-Dawley rats. To elucidate the possible antagonism of the satiety effect of leptin by AGRP, we examined the effect of a single ICV co-injection of AGRP and leptin on food intake and body weight in rats during 5 h at the onset of the dark phase. Following a single ICV injection of 2.0 μg leptin, cumulative food intake was reduced by ~81% relative to vehicle-treated rats during 5 h at the onset of the dark phase (1.0 ± 0.2 vs. 5.1 ± 0.4 g, n = 8–10, P < 0.01) (Fig. 4*A*). By co-injection of AGRP (0.15–1.5 μg), inhibition of food intake with 2.0 μg leptin was reversed in a dose-dependent manner. Co-injection of 1.5 μg AGRP abolished the leptin-induced decrease in cumulative food intake relative to vehicle-treated rats (3.9 ± 0.3 vs. 5.1 ± 0.4 g, P > 0.05).

During 5 h at the onset of the dark phase, body weight gain was inhibited significantly in rats treated with 2.0 μg leptin relative to vehicle-treated rats (–11.9 ± 1.0 vs. 0.9 ± 0.7 g, P < 0.01) (Fig. 4*B*). Treatment with AGRP (0.15–1.5 μg) also reversed the leptin-induced inhibition of body weight change in a dose-dependent manner. Co-injection of 1.5 μg AGRP abolished the leptin-induced inhibition of body weight change relative to vehicle-treated rats (–0.9 ± 0.9 vs. 0.9 ± 0.7 g, P > 0.05).

To assess the specificity of AGRP to reverse anorexia induced by leptin, we also examined the effect of the ICV co-injection of AGRP and GLP-1 (22) on food intake and body weight. Co-injection of AGRP did not affect the GLP-1–induced inhibition of food intake and body weight change

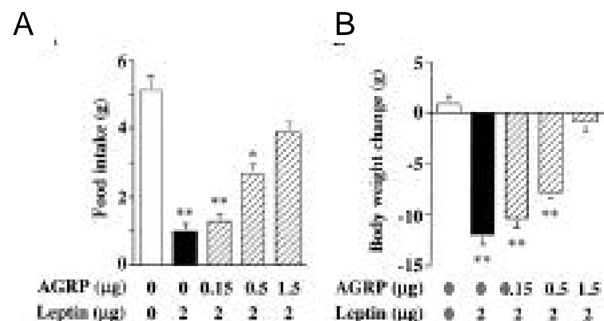

FIG. 4. Effects of a single ICV co-injection of AGRP and leptin on food inake (*A*) and body weight change (*B*) in rats. □, aCSF; ■, 2.0 μg leptin alone; ▨, 2.0 μg leptin plus AGRP at doses of 0.15, 0.5, and 1.5 μg. Values are means ± SE (n = 8–10). *P < 0.05, **P < 0.01 compared with aCSF-treated groups.

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TABLE 1
Body weight and plasma leptin concentrations in 20-week-old male mice

		Body weight (g)	Leptin (ng/ml)	
Background	Genotype	Feeding	Feeding	Fasting
C57BL/6J	+/+	24.8 ± 0.1	3.7 ± 1.1	0.8 ± 0.2
	ob/ob	48.9 ± 0.1	ND	ND
C57BL/KsJ	*db/db*	46.1 ± 3.1	ND	ND
KK	+/+	26.2 ± 1.3	6.3 ± 0.5	2.1 ± 0.4
	A^y/+	37.5 ± 1.5	77.0 ± 2.9	10.9 ± 1.9

Data are means ± SE (n = 5). Periods of fasting are described in METHODS. ND, not determined.

(data not shown), indicating that AGRP can specifically antagonize the satiety effect of leptin.

Regulation of hypothalamic AGRP mRNA expression by leptin. Table 1 shows body weight and plasma leptin concentrations in 20-week-old male mice used in this study.

Northern blot analysis identified a single mRNA species 0.7 kb in size in mouse hypothalami (Fig. 6*A*). Expression of AGRP mRNA was increased in hypothalami from *ob/ob* and *db/db* mice compared with the control C57BL/6J mice, which is consistent with previous reports (14,15). There was no significant difference in AGRP mRNA expression between the control C57BL/6J and C57BL/KsJ mice (data not shown). Increased AGRP mRNA expression in *ob/ob* mice was reversed completely by a single ICV injection of leptin (1.0 µg) for 5 h (Fig. 6*C*). On the other hand, no significant changes in AGRP mRNA levels were noted in *db/db* mice after the ICV injection of leptin.

We examined hypothalamic AGRP mRNA expression in mice during fasting (Fig. 7). Plasma leptin concentrations in C57BL/6J mice after fasting as indicated were decreased (Table 1). After 24-h fasting, AGRP mRNA expression was increased significantly in the hypothalamus from C57BL/6J mice, which is consistent with a previous report (16). No significant changes in AGRP mRNA levels were noted in *ob/ob* and *db/db* mice after 72-h fasting. As in C57BL/6J mice, AGRP mRNA expression was increased significantly in the hypothalamus from C57BL/KsJ mice after 24-h fasting (data not shown). We also examined hypothalamic AGRP mRNA expression in KK and KKA^y mice. No significant amount of AGRP mRNA was detected in the hypothalamus from KKA^y mice with hyperleptinemia, which is shown to be resistant to leptin (11). This result is in striking contrast to *ob/ob* and *db/db* mice (Fig. 6*B*). However, as in C57BL/6J mice, hypothalamic AGRP mRNA expression was induced in KKA^y mice during 48-h fasting (Fig. 8*A* and *B*), when plasma leptin concentrations were decreased (Table 1).

To elucidate the possible role of leptin deficiency in increased AGRP mRNA expression during fasting in these animals, we examined hypothalamic AGRP mRNA expression in C57BL/6J and KKA^y mice that received leptin before and after 24- or 48-h fasting. After fasting, no significant increase of AGRP mRNA expression was observed in either C57BL/6J or KKA^y mice that received leptin. AGRP mRNA expression was also induced in KK mice during 24-h fasting, when plasma leptin concentrations were decreased, and the induction was inhibited by a single ICV injection of leptin (data not shown).

DISCUSSION

This study demonstrates that a single ICV injection of the full-length AGRP alone can acutely increase food intake and body weight in rats. This finding is consistent with previous reports that chronic overexpression of AGRP leads to obesity in trangenic mice (15,27). It is likely that AGRP exerts its orexigenic effect through the inhibition of the α-MSH binding at MC4-R and/or independent of the α-MSH binding at MC4-R. Whatever the mechanism, the AGRP expressed in the Arc may have a tonic inhibitory effect on food intake and body weight under physiologic conditions.

The present study represents the first demonstration that AGRP can antagonize the satiety effect of leptin. These observations suggest that AGRP, an endogenous negative regulator of the hypothalamic melanocortin system, constitutes a novel regulatory mechanism of leptin action within the hypothalamus. Antagonism of leptin action by AGRP should be attributable mostly to its ability to block the hypothalamic α-MSH/MC4-R signaling activated by leptin (5). The discussion above also supports the notion that the hypothalamic melanocortin system plays a pivotal role in leptin action. Therefore, AGRP may be one of the candidate molecules of leptin resistance, and thus the possible leptin resistance may

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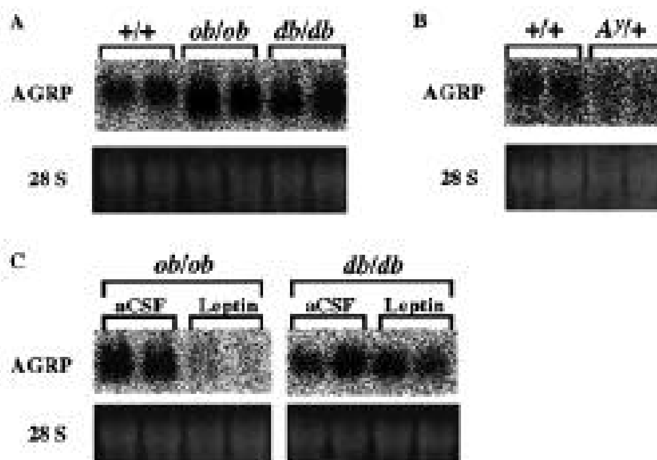

FIG. 5. Northern blot analysis of AGRP mRNA in the hypothalamus from C57BL/6J mice (+/+), C57BL/6J *ob/ob* mice (*ob/ob*), and C57BL/KsJ *db/db* mice (*db/db*) (*A*), KK mice (+/+) and KKA^y mice (A^y/+) (*B*), and C57BL/6J *ob/ob* mice (*ob/ob*) and C57BL/KsJ *db/db* mice (*db/db*) treated with aCSF or 1.0 µg leptin (*C*). Representative blots are shown. Total RNA (10 µg/lane) is analyzed in each lane.

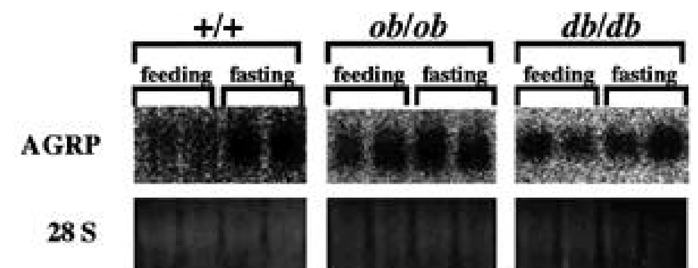

FIG. 6. Northern blot analysis of AGRP mRNA in the hypothalamus from C57BL/6J mice (+/+), C57BL/6J *ob/ob* mice (*ob/ob*), and C57BL/KsJ *db/db* mice (*db/db*) when fed ad libitum or after fasting. Representative blots are shown. Total RNA (10 µg/lane) is analyzed in each lane.

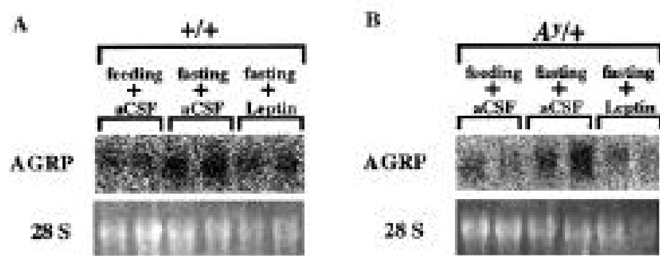

FIG. 7. Northern blot analysis of AGRP mRNA in the hypothalamus from C57BL/6J mice (+/+) (*A*) and KK mice (A^y/+) (*B*) when fed ad libitum or after fasting or having received leptin before and after fasting. Representative blots are shown. Total RNA (10 µg/lane) is analyzed in each lane.

contribute to the pathogenesis of obesity found in transgenic mice with overexpression of AGRP (15,27).

In this article, the reduction of body weight was more than that of food intake in rats treated with leptin alone (Fig. 4). This finding is consistent with the notion that leptin can increase energy expenditure as well as decrease food intake, which should contribute to a substantial reduction of body weight (1,2). A single ICV co-injection of AGRP completely abolished the leptin-induced decrease in body weight. These observations suggest that AGRP can also block the leptin-induced increase in energy expenditure. AGRP alone also seems to decrease energy expenditure because it can increase body weight more than food intake (Fig. 2). We have demonstrated that a single ICV injection of SHU9119 abolishes the leptin-induced increase in UCP-1 mRNA expression in the BAT, suggesting that leptin-induced thermogenesis and thus energy expenditure are mediated at least partly through the hypothalamic melanocortin system (9). Thus, AGRP can regulate negatively both the satiety effect and the sympathetic activation of leptin by antagonism of hypothalamic melanocortin receptors.

Because of AGRP's large size and multiple cysteine residues in its carboxyl terminus (14), it is important to address whether bacterially expressed AGRP is properly disulfide-linked and is functional in vivo. In the present study, using bacterially expressed AGRP, the IC_{50} values of AGRP and α-MSH are 200- to 400-fold higher than those of NDP-MSH in hypothalamic membrane preparations. Furthermore, ICV co-injection of AGRP (1.5 µg or ~0.1 nmol) completely abolishes decreased food intake and body weight by α-MSH (5 µg or ~5 nmol). There are two reports on the binding analysis of AGRP in cells expressing melanocortin receptors (MC3-R and MC4-R). A recent study with bacterially expressed AGRP, which is confirmed to be disulfide-linked (28), showed that the IC_{50} values of AGRP and α-MSH are 10- and 80-fold higher, respectively, than those of NDP-MSH in cells stably expressing MC4-R (18). In cells expressing MC3-R, the IC_{50} values of AGRP and α-MSH are 20-fold higher than those of NDP-MSH (18). Furthermore, with AGRP expressed in COS-7 cells (17), it was reported that the IC_{50} values of AGRP are several-fold higher than those of NDP-MSH in cells expressing MC3-R or MC4-R. On the other hand, there is only a report on the in vivo effect of AGRP using a synthetic human AGRP-(83–132)-NH2 fragment [AGRP-(83–132)] (29), which is confirmed to be disulfide-linked (30). It was reported that a single ICV injection of 1 nmol α-MSH significantly decreases food intake, which is abolished completely by co-injection of 1 nmol AGRP-(83–132). The lower binding affinity of AGRP than those reported previously (17,18) may be due to its potential incorrect folding (18). However, not only bacterially expressed AGRP but also synthetic α-MSH show a lower binding affinity in hypothalamic membrane preparations than in cells expressing melanocortin receptors. Thus, the lower binding affinity of AGRP might also be due to the difference in the melanocortin receptor preparations used. Furthermore, the potency of AGRP used in the present study to increase food intake and body weight is roughly comparable to that of AGRP-(83–132) (29), suggesting that it is functional in vivo.

This study demonstrates that central administration of leptin reverses completely the increased AGRP mRNA expression in *ob/ob* mice but not in *db/db* mice, thus providing direct evidence that leptin downregulates hypothalamic AGRP expression via leptin receptor in the central nervous system. On the other hand, AGRP mRNA expression is upregulated in C57BL/6J and KKA^y mice during fasting, when plasma leptin concentrations are decreased dramatically. This result is consistent with a previous report (16). No significant change in AGRP mRNA expression was noted in the above animals that received leptin during fasting. These observations suggest that leptin deficiency should contribute to the increased AGRP mRNA expression during fasting; leptin is a major negative regulator of AGRP mRNA expression during fasting. Since leptin receptor is expressed in the AGRP neurons (16), it is likely that leptin acts directly on the AGRP neurons and decreases its mRNA expression. In this report, KKA^y mice can respond to leptin in its inhibitory effect on hypothalamic AGRP mRNA expression, although they cannot do so in its satiety effect (11). Thus, AGRP mRNA expression may be a marker for central actions of leptin independent of the hypothalamic melanocortin system. In this regard, KKA^y mice will be a useful model for the elucidation of leptin actions independent of the hypothalamic melanocortin system (31).

Some of leptin's actions are thought to be mediated through the hypothalamic melanocortin system (8,9,31), which seems to be determined primarily by the balance between the levels of its agonist and antagonist, namely α-MSH and AGRP. Previous studies revealed that leptin causes a relatively small (1.5–2.0-fold) increase in hypothalamic POMC mRNA expression (7). On the other hand, this study shows that the increase or decrease in AGRP mRNA expression during fasting or leptin treatment, for instance, is as much as 3.0–5.0-fold. These observations suggest that leptin's actions mediated through the hypothalamic melanocortin system, mainly via MC4-R, are determined largely by its antagonist, AGRP, rather than by its agonist, α-MSH. This conclusion is consistent with the notion that physiologic signaling via MC1-R in the skin is regulated mainly by alteration in the levels of its antagonist, the agouti protein (10). In this regard, the elucidation of the role of AGRP in leptin action will give us a clue to understanding the mechanism of leptin resistance. Because of AGRP's potent antagonistic effect on leptin action, it is also tempting to speculate that the change in AGRP production in response to change in plasma leptin concentrations may represent one of the potentiating mechanisms of the metabolic consequences of the change in hypoleptinemia and hyperleptinemia.

In conclusion, the present study provides the first direct evidence that AGRP, an endogenous antagonist of the hypothalamic melanocortin receptor, is a negative regulator of

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satiety effect of leptin, whereas leptin, in turn, is a major negative regulator of hypothalamic AGRP mRNA expression. Our data also suggest that leptin action via the hypothalamic melanocortin system is determined by the balance between the levels of its agonist and antagonist, α-MSH and AGRP.

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