

## Anti-Melanocortin-4 Receptor Autoantibodies in Obesity

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**Background:** The melanocortin-4 receptor (MC4R) is part of an important pathway regulating energy balance. Here we report the existence of autoantibodies (autoAbs) against the MC4R in sera of obese patients.

**Methods:** The autoAbs were detected after screening of 216 patients' sera by using direct and inhibition ELISA with an N-terminal sequence of the MC4R. Binding to the native MC4R was evaluated by flow cytometry, and pharmacological effects were evaluated by measuring adenylyl cyclase activity.

**Results:** Positive results in all tests were obtained in patients with overweight or obesity (prevalence, 3.6%) but not in normal weight patients. The selective binding properties of anti-MC4R autoAbs were confirmed by surface plasmon resonance and by immunoprecipitation with the native MC4R. Finally, it was demonstrated that these autoAbs increased food intake in rats after passive transfer via intracerebroventricular injection.

**Conclusion:** These observations suggest that inhibitory anti-MC4R autoAbs might contribute to the development of obesity in a small subpopulation of patients. (*J Clin Endocrinol Metab* 94: 793–800, 2009)

In previous experiments we have shown that active immunization of rats against a 15-amino acid sequence of the N-terminal (NT) domain of the melanocortin-4 receptor (MC4R) results in a mild form of obesity and insulin resistance (1), which was more pronounced under a high-fat diet (2). These findings are consistent with observations by other authors that the NT domain of the MC4R is essential for the function of the MC4R and is involved in the maintenance of its constitutive activity (3). In further *in vitro* experiments, we could demonstrate that anti-MC4R antibodies (Abs) acted as inverse agonists and as non-competitive antagonists (1). The passive transfer of purified Abs

*in vivo* by intracerebroventricular (icv) injection in rats induced an increase in food intake (1).

These experimental findings prompted us to hypothesize that autoAbs against the MC4R might exist in a subpopulation of obese patients and contribute to the development of their disease. It has been reported that autoAbs against peptides such as  $\alpha$ -MSH may be involved in the pathogenesis of eating disorders (4, 5). Inhibitory autoAbs against the MC4R would be expected to increase appetite and might thereby be of pathophysiological significance (6–9). In this paper we report for the first time the presence of inhibitory autoAbs against the MC4R in human sera. The fact that such Abs were only found in patients with over-

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Abbreviations: Ab, Antibody; AC, adenylyl cyclase; autoAbs, autoantibodies; BMI, body mass index; FACS, fluorescence-activated cell sorting; HEK-293, human embryonic kidney 293; hMC4R, human MC4R; icv, intracerebroventricular; MC4R, melanocortin-4 receptor; NT, N-terminal; NW, normal weight; OB, obese; OW, overweight.

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**TABLE 1.** Characteristics of patient population

	OB (n = 80)	OW (n = 60)	NW (n = 76)
Age (yr)	62.6 ± 13.9	67.4 ± 14.7	59.8 ± 16.7
Females/males (n)	49/31	27/33	46/30
BMI (kg/m <sup>2</sup> )	35.9 ± 4.3	26.9 ± 1.4	22.0 ± 1.7

Data are presented as mean ± sd.

weight or obesity and blocked the activity of the MC4R suggests that such anti-MC4R autoAbs might contribute to the pathophysiology of obesity.

## Patients and Methods

### Human sera

Sera were obtained from 129 patients at the Stadtklinik, Medizinische Klinik I, Baden-Baden, Germany, who suffered from metabolic diseases (e.g. type 2 diabetes) but were free from acute illness and had different body mass index (BMI): obese (OB), BMI ≥ 30 kg/m<sup>2</sup>, n = 34; overweight (OW), 25 ≤ BMI < 30 kg/m<sup>2</sup>, n = 43; and normal weight (NW), BMI < 25 kg/m<sup>2</sup>, n = 52. Additional sera were obtained from 84 patients consecutively admitted to the University Hospital, Strasbourg, France. These patients also suffered from metabolic diseases including disorders with an autoimmune background (e.g. type 1 diabetes, Hashimoto's disease) and had different BMI: OB, n = 46; OW, n = 17; and NW, n = 24. Basic information on the total patient population is summarized in Table 1. The sera were coded, and all screening experiments were done in a fully blinded way. The studies were approved by the local ethical committees of France and Germany.

### NT peptides

Peptides corresponding to the NT domain of the human MC3R and MC4R (FSNQSSSGFCEQVFIKPEV and KTSLLHLWNRSSHGLHG, respectively) were designed and synthesized as described previously (1, 10).

### Direct and inhibition ELISA

The direct ELISA procedure has been described previously (11). To confirm the signal observed in the direct ELISA, an inhibition ELISA was used to titrate the positive sera preincubated with increasing concentrations ( $2 \times 10^{-12}$  to  $2 \times 10^{-6}$  M) of the MC4R NT peptide in solution. Specificity was defined as an inhibition of at least 20% (11).

### Ig precipitation

IgG fractions were prepared from 500 μl of serum samples by ammonium sulfate precipitation at a final saturation of 33%. After precipitation, sera were placed on ice for 1 h and centrifuged at 10,400 × g during 20 min. The pellets were resuspended with 300 μl of PBS and dialyzed at 4 C against PBS for 96 h.

### Human embryonic kidney 293 (HEK-293) cell culture

HEK-293 cells overexpressing the human MC4R (HEK-hMC4R) were cultured in DMEM (Sigma, St. Louis, MO) completed with 10% fetal calf serum (Biococoncept, Allschwil, Switzerland), 1% penicillin/streptomycin (GIBCO, Grand Island, NY), and 600 μg/ml G418 antibiotic (Sigma) in a humidified atmosphere containing 5% CO<sub>2</sub> at 37 C.

### Fluorescence-activated cell sorting (FACS)

To check whether the Abs present in the IgG fraction were able to recognize the hMC4R, flow cytometry assays were performed as described previously (12). Twenty-eight IgG fractions from direct ELISA positive sera were tested.

### Adenylyl cyclase (AC) assay

To assess the ability of autoAbs to modulate the MC4R activity, AC assays were performed on HEK-hMC4R cells. In addition, purified anti-MC4R autoAb fractions from two patients, A69 and B6, were tested. IgG fractions were considered as positive when the AC activity was significantly decreased below basal levels in the absence of α-MSH or when the EC<sub>50</sub> values were significantly shifted to the right in the presence of α-MSH. All data were expressed as mean ± SEM.

### Purification of anti-MC4R autoAbs

The anti-MC4R autoAbs were affinity-purified by using NT-peptide coupled via the N terminus to a cyanogen bromide-activated Sepharose 4B column (Amersham Biosciences, Uppsala, Sweden) according to manufacturer's instructions. Sera were loaded on the column at 4 C. The Abs were eluted with 0.2 M glycine (pH 2.7), collected in tubes containing 1 M Tris buffer (pH 8.0), subsequently dialyzed against PBS overnight at 4 C, and stored at –20 C.

### Depletion of IgG and anti-MC4R autoAbs

The serum of an individual OW patient (A57) was used in these experiments. It was incubated overnight with cyanogen bromide-activated Sepharose beads (Amersham Biosciences) saturated with glycine. Subsequently, the serum was incubated overnight with protein A/G agarose or NT-peptide conjugated to Sepharose beads. The supernatant was precipitated and tested in an AC assay.

### Surface plasmon resonance experiments

Assays were performed in the BIACORE 3000 system as described previously (1). Kinetic parameters of the interaction between anti-MC4R autoAbs from patients A69 and B6 were measured at 25 C in two series of experiments. First, five different concentrations (0.25–4 μM) of purified anti-MC4R autoAbs from patient A69 were injected at a flow rate of 30 μl/min for 300 sec on the immobilized MC4R NT peptide and MC3R NT peptide as control, followed by a dissociation phase of 400 sec. Subsequently, five different concentrations (0.125–1 μM) of purified anti-MC4R autoAbs from patient B6 were injected at a flow rate of 30 μl/min for 300 sec on the immobilized NT peptides, followed by a dissociation phase of 400 sec. The kinetic parameters were calculated using BIAevaluation software 4.1 (GE Healthcare, Uppsala, Sweden). All RU values obtained with the control MC3R NT peptide were subtracted from those obtained with the MC4R NT peptide to compensate for nonspecific binding.

### Membrane preparations

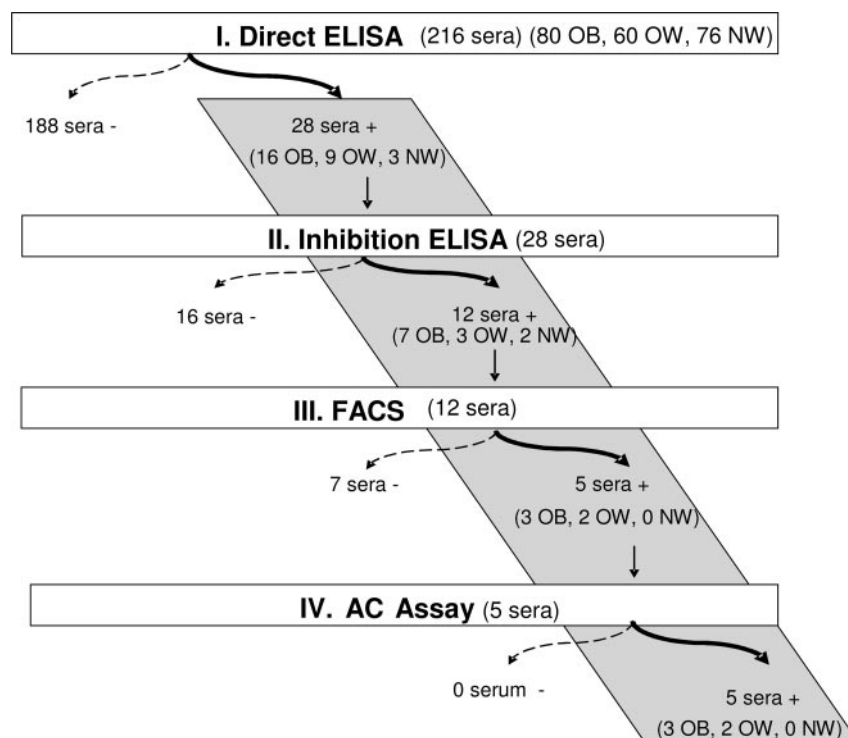
HEK-293 cells stably transfected with the hMC4R or hypothalamic from male Sprague-Dawley rats were prepared as described previously (13).

### Immunoblotting

Membrane proteins were denatured for 5 min by boiling in loading buffer without β-mercaptoethanol, subjected to electrophoresis on a 10% polyacrylamide gel containing sodium dodecyl sulfate, and finally electrotransferred onto nitrocellulose membranes. Human IgG fractions diluted 1:5000 in PBS-Tween 3% nonfat dry milk were applied to the membrane and revealed using peroxidase conjugated goat antihuman Ab (Jackson ImmunoResearch Laboratories, San Diego, CA) (1:5000) and ECL plus™ reagent (Amersham Bioscience).

### Immunoprecipitation

MC4R membrane preparations (10 μg) were incubated with purified human anti-MC4R autoAbs (25 μg) overnight at 4 C. The mixture was immunoprecipitated with 25 μl of protein A/G agarose. The immunoprecipitated samples were loaded on a 4–15% gradient gel (Bio-Rad, Hercules, CA) and transferred onto nitrocellulose membranes. The presence of MC4R was detected by using polyclonal anti-MC4R Abs (Ab-

**TABLE 2.** Sequential screening steps and numbers of sera tested

Step I (direct ELISA with NT peptide) was designed to detect the presence of anti-NT IgG. Step II (inhibition ELISA with NT peptide) was used to confirm the signal obtained in step I. Step III (FACS) was designed to determine whether the Abs detected in steps I and II recognized the native MC4R. Step IV (AC assay) was designed to assess the functional activity of the Abs. Results are presented as numbers of positive sera from OB, OW, and NW individuals.

cam, Cambridge, UK) in the standard procedure described in the One-Step Complete IP-Western kit (Genscript, Piscataway, NJ).

### Icv injection in rats

Male Sprague-Dawley rats (275–325 g) with a chronically implanted catheter in the third cerebral ventricle were used (1). Purified autoAbs from patient A69 or B6 were slowly (1 min) injected icv at 0900 h at a dose of 0.1  $\mu$ g or 1.0  $\mu$ g in a volume of 2  $\mu$ l using a Hamilton syringe. These doses were selected based on the results of comparative *in vitro* studies. Control rats received BSA. After the injection of autoAbs and BSA, food intake was continuously recorded during the following 48 h using an automatic food intake apparatus (TSE Systems, Bad Homburg, Germany).

### Statistical analysis

AC assay results were analyzed by Fisher test using GraphPad Prism 4 software (GraphPad Software Inc., La Jolla, CA). Proportions were analyzed using proportion z-test. Kruskal-Wallis test was used for the analysis of ELISA optical density and for percentage of fluorescent cells in FACS experiments.

## Results

### Patient characteristics and screening procedure

The patient characteristics and the results of the sequential screening of patients' sera are shown in Tables 1 and 2, respectively.

### Immunoreactivity against the NT peptide

Among the 216 sera collected from patients with metabolic diseases, 28 (13%) contained IgG Abs that interacted with the NT

peptide of the MC4R in a direct ELISA. Sixteen of these positive sera were from OB, nine from OW, and three from NW individuals (Fig. 1A). The prevalence of circulating anti-NT autoAbs as determined by direct ELISA was 20% in OB, 15% in OW, and 3.9% in NW individuals. The median OD<sub>450nm</sub> values were significantly higher in OB and OW than in NW individuals (Fig. 1B).

The positive signal obtained in 28 sera by direct ELISA was confirmed for 12 sera by using inhibition ELISA. Seven of these sera came from OB, three from OW, and two from NW individuals (Fig. 1C). The prevalence of circulating anti-NT autoAbs as determined by inhibition ELISA was 8.8% in OB, 5% in OW, and 2.6% in NW individuals.

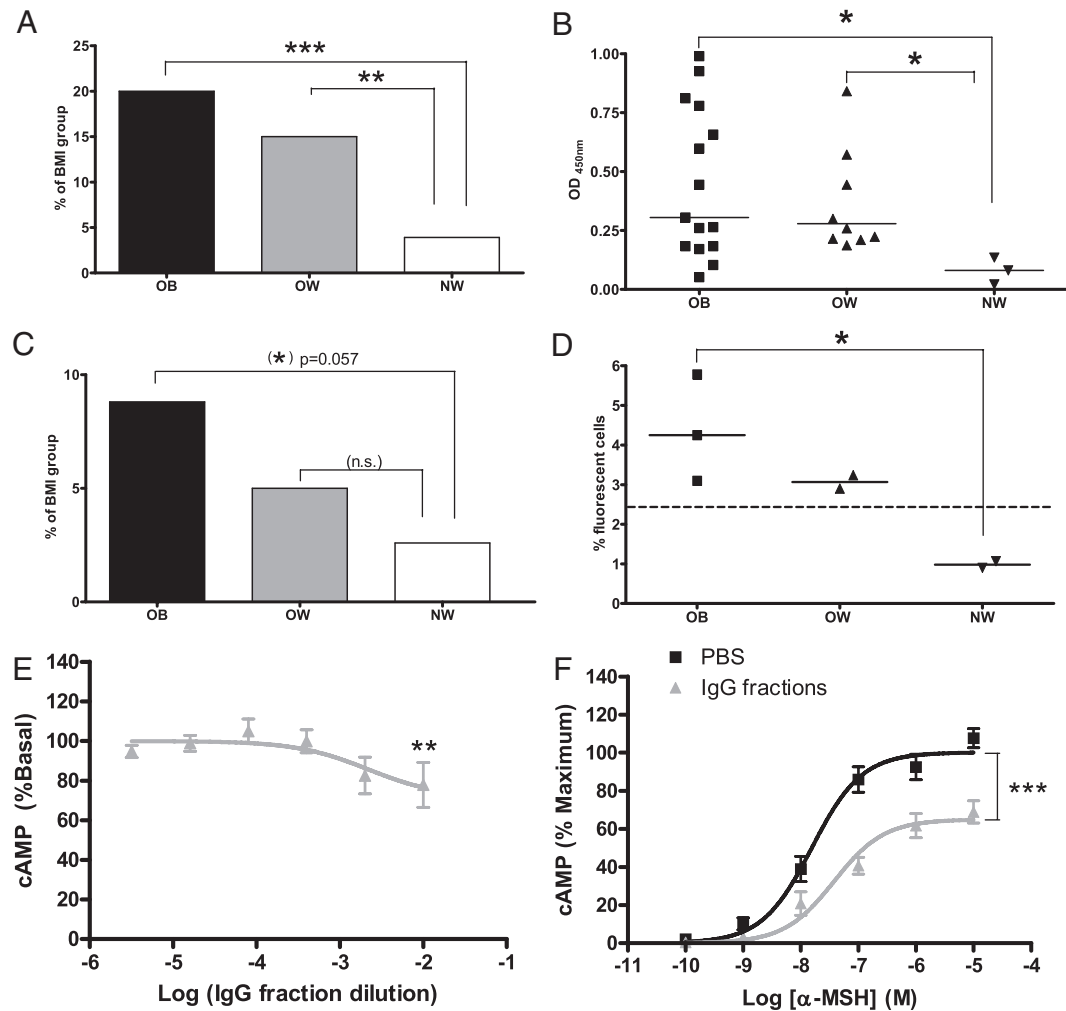
### Immunoreactivity against the MC4R

IgG fractions from the 12 sera containing anti-NT autoAbs were tested for their ability to bind to HEK-hMC4R cells in FACS experiments. Five of the 12 IgG fractions labeled HEK-293 cells. Three came from OB and two from OW individuals (Fig. 1D). Three of these autoAbs belonged to the German and two to the French patient population. The prevalence of circulating anti-MC4R autoAbs was 3.8% in OB and 3.3% in OW individuals. No such autoAbs were found in sera of NW individuals.

### Pharmacological activity

#### AC assay

Under basal conditions, the IgG fractions tended to reduce cAMP production; the changes were small but significant ( $P <$



**FIG. 1.** Results of screening assays. A, Results of direct ELISA presented as percentage of positive sera in each BMI group. The prevalence of positive sera was approximately four times higher in OB and OW than in NW individuals (\*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; proportion z-test). B, Optical density in positive sera. The median  $OD_{450nm}$  values were higher in sera from OB and OW than from NW individuals (\*,  $P < 0.05$ , Kruskal-Wallis test). C, Results of inhibition ELISA presented as percentage of positive sera in each BMI group. The prevalence of positive sera was approximately four times higher in OB [(\*)  $P = 0.057$ ; proportion z-test] than in NW individuals. D, Percentage of fluorescent cells detected in positive sera from FACS experiments; the dotted line indicates the calculated threshold. The number of fluorescent cells was higher in OB than in NW individuals (\*,  $P < 0.05$ , Kruskal-Wallis test). E, Effects of all FACS-positive IgG fractions ( $\blacktriangle$ ) on cAMP production in HEK-hMC4R cells under basal conditions. There was a concentration-dependent reduction in cAMP production, suggesting an inverse agonist activity (\*\*,  $P < 0.01$ , F-test). F, Effects of a 1/100 dilution of all FACS-positive IgG fractions ( $\blacktriangle$ ) on cAMP production upon treatment with increasing concentrations of the MC4R agonist  $\alpha$ -MSH. Control experiments were performed with PBS ( $\blacksquare$ ). The IgG fractions shifted the concentration-response curve significantly (\*\*\*,  $P < 0.001$ , F-test) to the right and decreased the maximum effects of  $\alpha$ -MSH, suggesting a noncompetitive antagonist activity. Mean  $\pm$  SD of five different sera measured in triplicate.

0.01, F-test) (Fig. 1E). When fixed dilutions of the IgG fractions were studied in the presence of increasing concentrations of  $\alpha$ -MSH, the concentration-response curve was significantly ( $P < 0.001$ , F-test) shifted to the right, and the maximum effect of  $\alpha$ -MSH was reduced (Fig. 1F). No IgG fraction from NW individuals had such an effect (data not shown). The prevalence of inhibitory autoAbs against the hMC4R in the combined OB/OW population was 3.6%.

### Specificity of the pharmacological activity

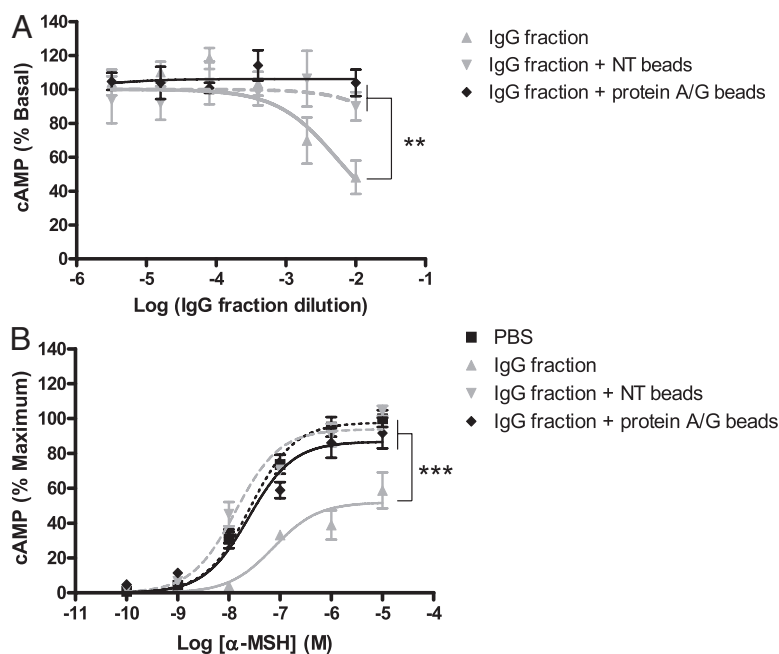
For these experiments, an anti-MC4R autoAb fraction from the serum of a single OW individual was studied in HEK-hMC4R cells. The anti-MC4R autoAb fraction inhibited basal AC activity in a concentration-dependent manner, *i.e.* it acted as an inverse agonist (Fig. 2A). General and specific IgG depletion sig-

nificantly decreased the inverse agonist activity, suggesting that it was due to the presence of anti-MC4R autoAbs (Fig. 2A).

AC activity was also measured in the presence of increasing concentrations of  $\alpha$ -MSH. Under these conditions, the anti-MC4R autoAb fraction shifted the concentration-response curve to the right and decreased the maximum efficacy of  $\alpha$ -MSH, *i.e.* it acted as a noncompetitive antagonist (Fig. 2B). General or specific IgG depletion restored the full effects of  $\alpha$ -MSH, suggesting that the blockade was due to the presence of anti-MC4R autoAbs (Fig. 2B).

### Comparison of autoAbs from two individual patients

Two patients' sera were selected for comparative studies, and anti-MC4R autoAbs were purified by using specific immunoadsorption. Patient B6 serum was positive in all four screening tests, whereas patient A69 serum was positive in the direct and inhi-



**FIG. 2.** General and specific IgG depletion. A, Effects of an IgG fraction (44 mg/ml) from a single OW individual (▲) on cAMP production in HEK-hMC4R cells under basal conditions. The depletion of IgGs with either NT- (▼) or protein A/G- (◆) coated beads eliminated the inverse agonist activity (\*\*,  $P < 0.01$ , z-test). B, Effects of a 1:100 dilution of an IgG fraction from a single OW individual (▲) on cAMP production upon treatment with increasing concentrations of the MC4R agonist  $\alpha$ -MSH. Control experiments were performed with PBS (■). The IgG fraction shifted the concentration-response curve to the right and decreased the maximum effects of  $\alpha$ -MSH. The depletion of IgGs with either NT- (▼) or protein A/G- (◆) coated beads eliminated the noncompetitive antagonist activity (\*\*\*,  $P < 0.001$ , z-test). Mean  $\pm$  sd of triplicate measurements.

bition ELISA but negative in the FACS and AC assays. Thus serum B6 contained autoAbs that recognized both the NT peptide and the native hMC4R, whereas serum A69 contained autoAbs that recognized only the NT peptide.

#### Surface plasmon resonance with the NT peptide

Purified anti-MC4R autoAbs from patient A69 in solution were tested for binding to the immobilized NT peptide. Specific binding was obtained at a relatively high  $K_D$  ( $1.2 \times 10^{-5}$  M). When tested under the same conditions, purified anti-MC4R autoAbs from patient B6 showed specific binding with approximately 20 times higher affinity ( $K_D = 6.0 \times 10^{-7}$  M).

#### Immunoprecipitation of the native MC4R

The interaction of the anti-MC4R autoAbs with the native human or rat MC4R was assessed in immunoprecipitation experiments. The autoAbs from patient B6 precipitated both human and rat MC4Rs (Fig. 3). In contrast, autoAbs from patient A69 failed to precipitate the MC4R in membrane preparations from either HEK-hMC4R cells or rat hypothalami (Fig. 3).

#### In vitro effects on AC activity

Purified anti-MC4R autoAbs were tested in the AC assay to assess their pharmacological effects on the stimulation of MC4R activity by  $\alpha$ -MSH. Purified autoAbs from patient B6 decreased significantly the maximum effect of  $\alpha$ -MSH (Fig. 4A). In contrast, autoAbs purified from serum of patient A69 were inactive (Fig. 4A).

#### In vivo effects on food intake

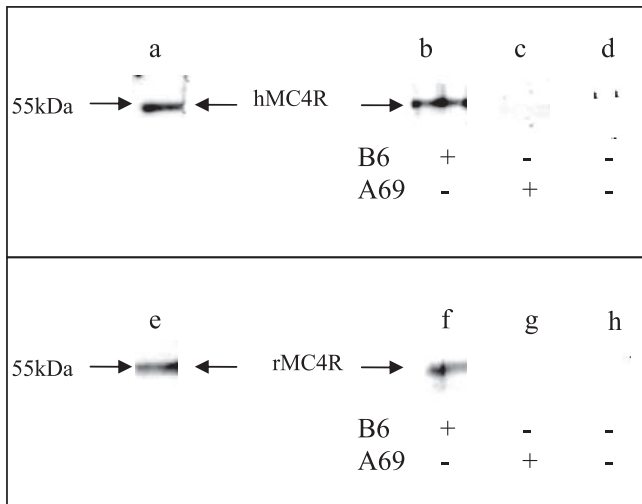
Purified anti-MC4R autoAbs were tested *in vivo* by a single injection of a dose of 0.1 or 1  $\mu$ g into the third cerebral ventricle of rats. Food intake was continuously measured over the subsequent 48 h. A dose of 0.1  $\mu$ g was not effective (data not shown). In rats treated with 1  $\mu$ g autoAbs from patient B6, 48-h food intake was 10% higher than in those treated with autoAbs from patient A69 or in controls receiving BSA (Fig. 4B).

#### Discussion

The existence of autoAbs against G protein-coupled receptors is becoming widely recognized, and the number of reports on diseases mediated by autoAbs is increasing (14–16). However, the detection of autoAbs is fraught with methodological issues, and the proof of a causal relationship between autoAbs and symptoms requires the demonstration of biological activity in functional assays (17, 18). In our studies on human blood samples, we could take advantage of our previous experience with MC4R immunization (1, 2). We chose a direct ELISA followed by an inhibition ELISA as the initial assays to detect possible autoAbs directed against the NT domain of the MC4R. Because in both assays the NT peptide was used as the bait, Abs binding to any conformation of this 15-amino acid sequence would give a positive signal. To detect only autoAbs that recognized the NT sequence in its native conformation, cells expressing the hMC4R were studied by using FACS. Finally, AC activity measurements were used to assess the pharmacological properties of these autoAbs.

At each step in the screening procedure, the prevalence of positive sera was higher in OB and OW patients than in NW patients. This difference in prevalence was 17.8% in OB/OW *vs.* 3.9% in NW individuals after the first ELISA test. Moreover, positive sera showed higher anti-NT immunoreactivity as indicated by the higher OD values. The difference between the prevalence of positive sera in OB/OW *vs.* NW individuals was smaller in the following inhibition ELISA (7.1 *vs.* 2.6%). In the subsequent FACS assay in which binding of purified IgG fractions to the native MC4R was determined, only IgG fractions from sera of OB or OW patients gave positive results (5 of 140, prevalence in OB/OW patients: 3.6%). All IgG fractions that were positive in the FACS assay showed inhibitory activity in AC assays. IgG fractions from NW patients had no such effects.

In the present study, the dependence of the pharmacological effects of the autoAb fractions on the presence of IgGs was demonstrated *in vitro*. For this purpose, an anti-MC4R autoAb fraction from a single OW patient was used. Under control conditions, this fraction showed pharmacological activity as an inverse agonist and a noncompetitive antagonist of the MC4R. After general depletion of all IgG or specific depletion of anti-MC4R



**FIG. 3.** Immunoprecipitation of the human and rat MC4R (hMC4R and rMC4R, respectively). Lanes a and e show the hMC4R (prepared from HEK-hMC4R cells) and the rMC4R (prepared from rat hypothalami) as detected by commercially available polyclonal anti-MC4R Abs. Lanes b and c show the results obtained with hMC4R and purified anti-MC4R autoAbs from patients B6 or A69, whereas lanes f and g show the corresponding results with rMC4R. Lanes d and h show control experiments without Abs. The purified anti-MC4R autoAbs from patient B6 precipitated both the hMC4R (b) and the rMC4R (f), whereas those from patient A69 were inactive.

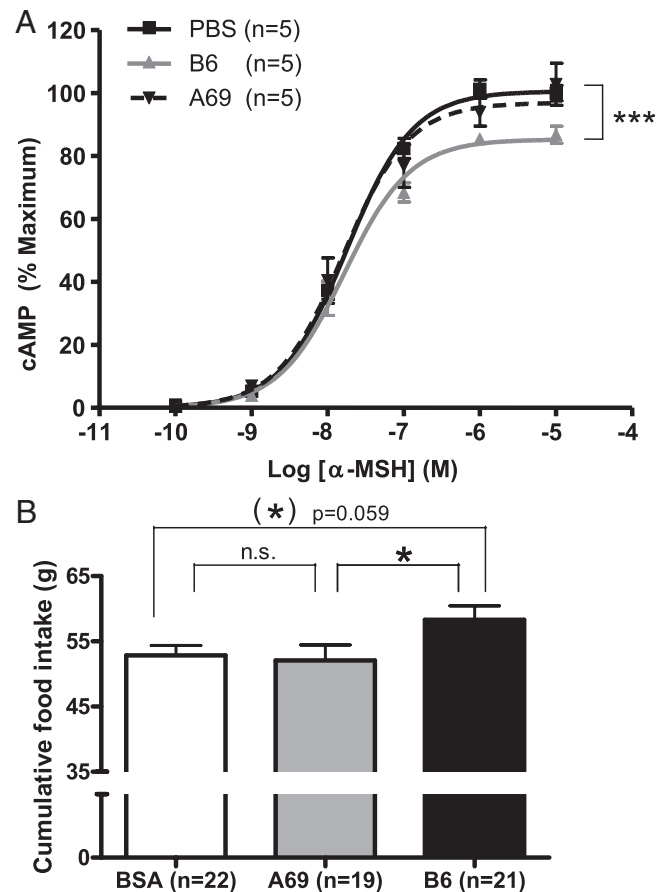
IgGs from this fraction, its pharmacological activity was lost. These findings strongly suggest that the inhibitory effects of this fraction on the MC4R were due to the fact that it contained anti-MC4R autoAbs.

For a detailed evaluation of the physicochemical, biochemical, and pharmacological properties of autoAbs, sera of two OB patients were compared. One serum (B6) was positive in all four screening steps, whereas the other one (A69) was positive in the initial two ELISA tests but negative in the subsequent FACS and AC assays. Serum B6 was therefore considered to contain inhibitory autoAbs against the native hMC4R, whereas serum A69 served as a control that was supposed to contain autoAbs against the NT peptide.

Surface plasmon resonance revealed binding to the NT domain for both sera, but autoAbs from patient B6 had a much higher affinity than those from patient A69. The dissociation constant for B6 was in the range of that observed with neutralizing Abs against other peptidic antigens (19). The difference in affinity between B6 and A69 may explain why only B6 autoAbs gave positive results in the tests using the native MC4R.

Immunoprecipitation is widely regarded as the most reliable method to demonstrate the existence of autoAbs (18). Artifacts are minimized by the fact that an antigen must be coprecipitated with an Ab before it is revealed by Western blotting. This procedure ensures that a specific binding step is followed by the specific identification of the bound molecule. Anti-MC4R autoAbs from patient B6 coprecipitated human and rat MC4Rs, whereas those from patient A69 were inactive. This result strongly suggests that B6 autoAbs specifically and strongly interacted with the NT domain of the MC4R in its native configuration.

Finally, we performed with the human autoAbs the same type of *in vivo* experiment that had been done with rat and rabbit



**FIG. 4.** Pharmacological activity of anti-MC4R autoAbs *in vitro* and *in vivo*. A, Effects of anti-MC4R autoAbs (10 nM) from patient B6 (▲) or A69 (▼) on cAMP production upon treatment with increasing concentrations of the MC4R agonist α-MSH. Control experiments were performed with PBS (■). The anti-MC4R autoAbs from patient B6 significantly (\*\*\*) ( $P < 0.001$ , z-test) decreased the maximum efficacy of α-MSH, whereas those from patient A69 were inactive. Mean  $\pm$  SD of five measurements per curve. B, 48-h food intake in rats that received icv injections of purified anti-MC4R autoAbs from patients B6 and A69 or BSA (1  $\mu$ g in 2  $\mu$ l). Rats injected with anti-MC4R autoAbs from patient B6 (n = 21) showed a significant increase in food intake as compared with those injected with autoAbs from patient A69 (n = 19) or with BSA (n = 22). Data are presented as means  $\pm$  SEM [(\*)],  $P = 0.059$ ; \*,  $P < 0.05$ ; repeated measures two-way ANOVA with Bonferroni *post hoc* test].

anti-MC4R Abs (1, 2). After an icv injection in rats of autoAbs from patient B6, food intake gradually increased above the values seen after injection either of autoAbs from patient A69 or of BSA. The effects on food intake in the B6 group developed slowly and were significant by 48 h after injection. At that time, food intake was 10% higher in B6-treated than A69-treated rats. There was no difference between the A69 group and the BSA-treated control group. Compared with our previous studies with rat anti-MC4R Abs, the effect occurred in the same dose range (1  $\mu$ g), and its magnitude and time course were of the same order of magnitude as seen with rat or rabbit anti-MC4R Abs (1, 2).

Taken together, the serum samples from patient B6 gave positive results in four screening assays (direct and inhibition ELISA, FACS, and AC), in two additional *in vitro* experiments (surface plasmon resonance, immunoprecipitation), and in one *in vivo* study (food intake in rats). In all instances the effects were consistent with what would be expected from potent and specific inhibitory anti-MC4R autoAbs. We do not know whether the

autoAbs from all patients' sera with positive screening results show the same effects. However, it seems reasonable to assume that the serum from patient B6 was representative because it shared the same properties in the four screening tests with the other positive sera.

It can only be speculated how autoAbs against the MC4R are generated. Possible explanations are conformational similarities (antigen mimicry) of the NT sequence with exogenous infectious agents or with endogenous antigens generated via necrosis or degradation of related molecules (20, 21). Recent findings suggest that a potential source of antigens could be the gut flora (22, 23). This reservoir contains an enormous amount of structures with possible homology to endogenous molecules. The initiation and maintenance of an immune response depends on various not well-defined associated conditions (24, 25), and different environmental factors could have a modulating effect.

Circulating autoAbs against the MC4R can only become pathophysiologically active if they enter the brain. Thus an impaired function of the blood-brain barrier would be the prerequisite for the penetration of autoAbs into the brain tissue. This may occur more frequently than generally assumed because the tightness of the blood-brain barrier can be compromised not only by serious conditions such as trauma or epileptic seizures but also by more subtle disturbances such as psychological stress (26, 27). Whether the blood-brain barrier in the hypothalamic area is leaky or even absent is a matter of debate (28, 29). After acute systemic administration of Abs, a small fraction (0.1%) has been found to penetrate into brain tissue within 1 h (30). During chronic treatment, this percentage may be higher. Moreover, complete monoclonal Abs against amyloid  $\beta$ -peptide and F(ab')<sub>2</sub> fragments thereof have been shown to enter the central nervous system at therapeutically relevant levels (31, 32). Finally, our previous experiments in rats strongly suggest that Abs must have passed the blood-brain barrier because the actively immunized rats developed a phenotype that was consistent with blockade of hypothalamic MC4Rs (1, 33).

Because the anti-MC4R autoAbs observed in our present study reduced but did not completely eliminate the activity of the MC4R under basal and stimulated conditions, the functional disturbances caused by such autoAbs will probably be generally less pronounced than those seen in genetically mediated cases of impaired MC4R function. Conversely, it may be assumed that autoAbs will only play a significant pathophysiological role if they are directed against targets that are essential for the control of energy balance. AutoAbs against less important targets or against mediators that can be compensated for will probably not result in manifest disease.

The fact that the prevalence of autoAbs against the NT peptide or the MC4R segregated with BMI and that functionally active autoAbs were present in OW and OB but not in NW subjects strongly suggests a possible pathogenic role in the development of obesity. However, an association between the prevalence of autoAbs and BMI in a cross-sectional study does not prove a causal relationship. Additional studies are necessary to determine the precise prevalence of anti-MC4R Abs in OB patients and to prove their pathophysiological significance. If our present findings could be confirmed in such longitudinal studies, they could help to develop novel diagnostic tools and design

individualized treatment for a subpopulation of patients with obesity.

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