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Antibodies as pharmacologic tools for studies on the regulation of energy balance

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Abstract

Objective: Active immunization in rats may serve several purposes: the production of a disease-like phenotype, the generation of pharmacologic tools, and the development of clinically useful therapies. We selected the melanocortin-4 receptor (MC4R) as a target because its blockade could provide a treatment for anorexia and cachexia.

Methods: We used a sequence of the N-terminal (NT) domain of the MC4R as an antigen. Rats immunized against the NT peptide produced specific MC4R antibodies (Abs) that were purified and characterized in vitro and in vivo.

Results: The Abs acted as inverse agonists and reduced under basal conditions the production of cyclic adenosine monophosphate in HEK-293 cells expressing the human MC4R. Rats immunized against the NT peptide developed a phenotype consistent with hypothalamic MC4R blockade, i.e., increased food intake and body weight, liver and fat-pad weights, hepatic steatosis, and increased plasma triacylglycerols. With a high-fat diet, plasma insulin levels were significantly increased. In separate experiments an increase in food intake was observed after injection of purified MC4R Abs into the third ventricle. When lipopolysaccharide was administered in NT-immunized rats the reduction of food intake was partly prevented in this model of cytokine-induced anorexia.

Conclusion: Our results show that active immunization of rats against the MC4R resulted in the generation of specific Abs that stimulated food intake by acting as inverse agonists of the hypothalamic MC4R. Pharmacologically active monoclonal MC4R Abs could be the starting point for the development of novel treatments for patients with anorexia or cachexia.

Keywords: Immunization; Melanocortin-4 receptor; Food intake; Anorexia; Lipopolysaccharide; G-protein–coupled receptor; Inverse agonist

Introduction

In the search for new treatments of anorexia and cachexia an immunologic approach offers several advantages. First, active immunization against a physiologically relevant target may result in a phenotype resembling a disease model. Second, the circulating antibodies (Abs) can be purified and used as pharmacologic tools. Third, the therapeutic potential of active or passive immunization can be evaluated in experimental models of human disease. Promising results in such models would be the starting point for the development of monoclonal Abs and derivatives thereof that could lead to clinically useful therapeutics.

We chose the melanocortin-4 receptor (MC4R) as a target for immunization experiments because it plays an important role in the regulation of energy balance [1–3]. Mutations of this receptor in humans result in severe forms of childhood obesity [4]. Conversely, data from experimental studies have suggested that MC4R blockade could be an effective treatment for anorexia and cachexia [5,6]. In several series of experimental studies in rats we have systematically evaluated the pathophysiologic and pharmacologic consequences of active and passive immunizations against the MC4R in rats [7]. When we started our experiments it was not clear whether it would be possible to generate Abs against an extracellular sequence of the MC4R and whether

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they would be functionally active. Furthermore, it was not known whether such Abs would gain access to the MC4R in the central nervous system. Our results demonstrated for the first time that such an immunologic approach is feasible and suggested that Abs could be used as possible therapeutic agents to treat anorexia and cachexia. In this mini-review we summarize our previous [7,8] and present [9] experimental work on this subject and discuss options for future developments.

Materials and methods

Preparation of antigen

Peptides corresponding to N-terminal residues 11–25 of the rat MC4R (NT peptide; KTSLHLWNRSSHGLHG) or to an unrelated control sequence (CO peptide; CANISREERREVFLSVPGL) were synthesized as described previously [10].

Animals

Male Sprague-Dawley rats, 6 wk old at the beginning of the experiments (initial body weight approximately 200 g), were obtained from Charles River Laboratories (L'Arbresle, France) and kept on a 12-h light/dark cycle (lights on from 0600 to 1800 h) in a room with constant temperature (22°C) and humidity (50%). Rats were housed individually 1 wk before the beginning of an experiment and were given free access to tap water and standard laboratory chow (NAFAG 3432, 3.0 kcal/g, 61.6% of total calories from carbohydrate, 24.8% from protein, and 13.6% from fat) or a high-fat diet (Research Diets, D12451, New Brunswick, NJ, USA; 4.7 kcal/g, 35% of total calories from carbohydrate, 20% from protein, and 45% from fat). All experiments were performed in accordance with Swiss regulations for animal experimentation.

Active immunization in rats

Under each of the two diet regimens rats were divided into three groups: an untreated control group (sham, $n = 5$), a group immunized with the NT peptide ($n = 5$), and a group immunized with the CO peptide ($n = 5$). Peptides were applied subcutaneously behind the neck (25 μg of peptide/rat in 0.2 mL of complete Freund's adjuvant for the first injection). Thirty days and 60 d later, rats received two booster injections of peptides in incomplete Freund's adjuvant. To estimate the efficacy of the immunization procedure, blood was collected before each injection by tail bleeding under isoflurane anaesthesia, and the presence of antipeptide Abs was assessed by enzyme-linked immunosorbent assay. Body weight and food intake were recorded three times per week at 0900 h.

At the end of the experiments rats were sacrificed by decapitation under isoflurane anesthesia at 0900 h. Blood was collected in tubes treated with ethylene-tetra-acetic acid after decapitation of the animals. The liver and epididymal and retroperitoneal fat pads were removed and weighed. Blood was centrifuged at 2000 $\times$ g for 20 min at 4°C. Plasma insulin was measured using the Insulin Rat Ultrasensitive Enzyme-Linked Immunosorbent Assay (DRG Instruments GmbH, Marburg, Germany). Plasma glucose was measured using a glucose RTU kit and triacylglycerols by means of a TG PAP 150 kit (both from Biomérieux, Marcy l'Etoile, France).

Active immunization in rabbits

Rabbits (female New Zealand White rabbits, initial weight approximately 2.5 kg; Grimaud, Rousays, France) were immunized using the same procedure as in rats (100 μg of NT peptide in 1 mL of complete Freund's adjuvant) but boosted only once 3 wk after the first immunization. Total blood was collected under isoflurane anaesthesia 6 wk after immunization.

Purification of Abs

The Abs from anti-NT rat or rabbit sera or anti-CO rat sera were purified using affinity chromatography. For this purpose the NT or CO peptides were coupled by their N-terminal ends to activated CNBr-Sepharose 4B (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. Sera were diluted 10 times in phosphate buffered saline and 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), subsequently dialyzed against phosphate buffered saline at 4°C overnight, and stored at −20°C.

Cell culture

HEK-293 cells expressing the human MC4R (hMC4R) were cultured in Dulbecco's Modified Eagle Medium (Sigma, St. Louis, MO, USA) containing 10% fetal calf serum (Bioconcept, Allschwil, Switzerland), 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA), and G418 at 600 μg/mL (Sigma) in a humidified atmosphere containing 5% CO_2 at 37°C.

Cyclic adenosine monophosphate assays

Cells were transferred to 24-well culture plates 72 h before treatment, washed for 4 h with culture medium (Dulbecco's Modified Eagle Medium, Sigma), and incubated for 1 h in Dulbecco's Modified Eagle Medium supplemented with 0.1% bovine serum albumin and 0.1 mM 3-isobutyl-1-methylxanthine (Sigma). Cells were treated with serial dilutions of purified rat or rabbit Abs for 30 min or preincubated with 25 μM for rat Abs or 0.1 μM for rabbit Abs (maximum possible concentrations) for 30 min and then