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GPR39 Signaling Is Stimulated by Zinc Ions But Not by Obestatin

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GPR39 is an orphan member of the ghrelin receptor family that recently was suggested to be the receptor for obestatin, a peptide derived from the ghrelin precursor. Here, we compare the effect of obestatin to the effect of Zn^{2+} on signal transduction and study the effect of obestatin on food intake. Although Zn^{2+} stimulated inositol phosphate turnover, cAMP production, arrestin mobilization, as well as cAMP response element-dependent and serum response element-dependent transcriptional activity in GPR39-expressing cells as opposed to mock-transfected cells, no reproducible effect was obtained with obestatin in the GPR39-expressing cells. Moreover, no specific binding of obestatin could be detected in two different types of GPR39-expressing cells using three different radioiodinated forms of obestatin. By quantitative PCR analysis, GPR39 expression was readily detected in peripheral organs such as duodenum and kidney but not in the pituitary and hypothalamus, *i.e.* presumed central target organs for obestatin. Obestatin had no significant and reproducible effect on acute food intake in either freely fed or fasted lean mice. It is concluded that GPR39 is probably not the obestatin receptor. In contrast, the potency and efficacy of Zn^{2+} in respect of activating signaling indicates that this metal ion could be a physiologically relevant agonist or modulator of GPR39. (*Endocrinology* 148: 13–20, 2007)

IN 1997, GPR39 and GPR38 were cloned as two novel members of the growth hormone secretagogue (GHS) and neurotensin family of 7TM, G protein-coupled receptors (1). The endogenous ligand for the GHS receptor, which originally had been discovered as the molecular target for artificial growth hormone-releasing peptides and nonpeptide compounds (2), was subsequently identified to be a novel hormone, ghrelin, and the GHS receptor is consequently now called the ghrelin receptor (3). Somewhat surprisingly, ghrelin turned out to be mainly a gastric hormone, which is secreted in the fasting state and has multiple potential functions in the body, of which the most well studied has been its role as a hunger signal from the gastrointestinal (GI) tract to the central nervous system (4–6).

Originally, GPR39 was described as being widely expressed in the body; for example, in multiple regions of the central nervous system as well as in various peripheral organs (1). However, although GPR38 was already deorphanized in 1999 as the receptor for motilin (7), which is an important peptide regulator of GI tract motility, GPR39 has not yet been subject to much attention. During a systematic screening of members of the ghrelin receptor family, we discovered that GPR39, like the ghrelin receptor and the neurotensin 2 receptor, was signaling with high constitutive activity through various Gq and G12/13 signaling pathways resulting in, for instance, increased inositol phosphate (InsP) turnover, as well as activation of cAMP response element (CRE) and serum response element (SRE) transcriptional activity (8). Interestingly, GPR39 signaling could be further stimulated by micromolar concentrations of zinc ions (8).

Recently, Zhang *et al.* (9) reported that a 23-amino-acid, carboxy-amidated peptide named obestatin bound with high affinity to GPR39 and through this receptor-stimulated cAMP production and SRE transcriptional activity with high potency. Through the development of a specific RIA, obestatin had been discovered as a second peptide product from the ghrelin precursor in analogy with the various peptide products being derived from, for example, the proopiomelanocortin precursor through tissue-specific differential processing (9). In animal experiments, obestatin was shown to have opposite effects compared with ghrelin because it was able to reduce food intake and decrease body weight gain, as well as decrease gastric emptying (9). Quantitative PCR (QPCR) analysis was used to support the notion that the target for obestatin was GPR39 centrally expressed, for example, in the hypothalamus and peripherally in the GI tract (9). Thus, the conclusion was that ghrelin and obestatin are two peptide products derived from a common precursor but having opposite effects, mediated through two different members of the same receptor family expressed in the same tissues, the ghrelin receptor and GPR39 (9, 10).

In the present study, we compare the effect of Zn^{2+} with that of obestatin on the signaling of GPR39 expressed het-

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Abbreviations: BRET, Bioluminescence resonance energy transfer; CRE, cAMP response element; DMF, dimethylformaldehyde; GHS, growth hormone secretagogue; GI, gastrointestinal; HOBt, 1-hydroxybenzotriazole; InsP, inositol phosphate; QPCR, quantitative PCR; SRE, serum response element; TFA, trifluoroacetic acid.

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erologously in various cell lines, and we measure the GPR39 expression in the presumed target organs for obestatin, *i.e.* the hypothalamus and the GI tract, and test the effect of obestatin on acute food intake in mice.

Materials and Methods

Ligands used for assays

$ZnCl_2$ and $NiCl_2$ were purchased from Sigma Chemical Co. (St. Louis, MO) and diluted in distilled water. Obestatin peptides were synthesized as described below and were dissolved and further diluted in 0.1 mM acetic acid containing 0.1% BSA. A batch of human obestatin peptide was also kindly provided by Aaron Hsueh (Stanford University, Stanford, CA) and according to his protocol dissolved to a 1 mM stock solution in 30% acetic acid, from which standards were prepared by further dilution in 0.1 mM acetic acid containing 0.1% BSA.

Synthesis of obestatin

The 23-amino-acid carboxy-amidated human obestatin peptide FNAPFDVGIKLSGVQYQQHSQAL-NH2 and the corresponding rat obestatin FNAPFDVGIKLSGAQYQQHGRAL-NH2 were synthesized by automated multiple solid-phase peptide synthesis (Syro, MultiSynTech, Bochum, Germany) by the orthogonal Fmoc/tert-butyl strategy. Nα-Fmoc-protected amino acids, 1-hydroxy-benzotriazole (HOBt), and the 4-(2′,4′-dimethoxyphenyl)-phenoxy (rink amide) resin were purchased from Novabiochem (Schwalbach, Germany). *N,N′*-diisopropylcarbodiimid was obtained from Sigma-Aldrich (Taufkirchen, Germany). Solvents were purchased as previously described (11). For side chain protection of the amino acids, the following protection groups were chosen: tert-butyl for Ser and Tyr; tert-butyloxy for Asp; trityl for Asn, Gln, and His; and tert-butyloxycarbonyl for Lys. To obtain the peptide amide, the Rink amide resin was used (30 mg, loading capacity 0.45 mmol/g). Fmoc-amino acids were dissolved in 0.5 M HOBt/dimethylformaldehyde (DMF) and Fmoc-Phe-OH in 0.5 M HOBt/*N*-methylpyrrolidone, respectively, and were coupled twice for 40 min each after activation with *N,N′*-diisopropylcarbodiimid (11). Amino acids and activation reagents were used in a 10-fold excess. The removal of the Fmoc group was carried out with 40% piperidine in DMF for 3 min and a second incubation with 20% piperidine in DMF within 10 min. For the removal of all acid labile protecting groups and the cleavage of the peptide from the resin, a mixture of trifluoroacetic acid (TFA)/thioanisole/*p*-thiocresol [90:5:5 (vol/vol)] was applied for 3 h. The peptide was precipitated from ice-cold diethyl ether, centrifuged at 4 C, and the supernatant was decanted. The peptide was resuspended in fresh ether, centrifuged again four times, and was finally dissolved in water/tert-butanol [3:1 (vol/vol)] and lyophilized. Purity of the peptides was more than 95% according to analytical reversed-phase HPLC on a RP18-column (Vydac, 4.6 × 250 mm; 5 μm, 300 Å) eluted with a linear gradient of 10–60% B in A [A, 0.1% (vol/vol) TFA in water; B, 0.08% (vol/vol) TFA in acetonitrile] over 30 min and a flow rate of 0.6 ml/min. Identity of the peptides was proven by matrix-assisted laser desorption ionization mass spectrometry on a Voyager-DE RP workstation (Applied Biosystems, Darmstadt, Germany) (rat obestatin theoretical mass, 2516.87; M + Hexp., 2518.3; human obestatin theoretical mass, 2546.89; M + Hexp., 2548.2).

cDNA, transfection, and tissue culture

Human GPR39 receptor cDNA was cloned from a human stomach cDNA library, and its sequence was confirmed (corresponding to accession no. AAC26082). COS-7 cells were grown in DMEM 1885 supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.01 mg/ml gentamicin. COS-7 cells were transfected with 40 μg/175 cm^2 GPR39 receptor or FLAG-tagged GPR39 cDNA in pcDNA3 vector using a calcium phosphate precipitation method with chloroquine addition as previously reported (12). HEK293 cells were grown in DMEM 31966 with high glucose supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.01 mg/ml gentamicin. HEK293 cells were transfected with Lipofectamine 2000 (no. 11668-019, Invitrogen, Carlsbad, CA).

Phosphatidylinositol turnover

One day after transfection, COS-7 cells were incubated for 24 h with 5 μCi [^{3}H]-myo-inositol (Amersham, Little Chalfont, UK; catalog no. PT6-271) in 1 ml medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.01 mg/ml gentamicin per well. Cells were washed twice in buffer and were incubated in 0.5 ml buffer supplemented with 10 mM LiCl at 37 C for 30 min. After stimulation for 45 min at 37 C with Zn^{2+} or obestatin, the cells were extracted with 10 mM formic acid followed by incubation on ice for 30 min. The resulting supernatant was purified on Bio-Rad (Hercules, CA) AG 1-X8 anion-exchange resin to isolate the negatively charged InsPs. After application of the cell extract to the column, the columns were washed twice with buffer (60 mM sodium formate and 100 mM formic acid) to remove glycerophosphoinositol. InsP1, InsP2, InsP3, and InsP4 were eluted by addition of elution buffer (1 mM ammonium formate, 100 mM formic acid). Determinations were made in duplicates. The columns were regenerated by addition of 3 ml regeneration buffer (3 M ammonium formate, 100 mM formic acid) and 5 ml water.

cAMP assay

One day after transfection, COS-7 cells (2.5×10^5 cells per well) were incubated for 24 h with 2 μCi [^{3}H]adenine (Amersham; catalog no. TRK 311) in 1 ml DMEM 1885 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.01 mg/ml gentamicin. Cells were washed twice and incubated for 15 min at 37 C in 1 ml freshly prepared binding buffer supplemented with 1 mM isobutylmethylxanthine (Sigma; no. I5879), 40 μg/ml bacitracin, and with various concentrations of ligands or with 50 μM forskolin. After incubation, cells were placed on ice, medium was removed, and cells were lysed with 1 ml 5% (wt/vol) trichloroacetic acid, supplemented with 0.1 mM cAMP and 0.1 mM ATP for 30 min. The lysis mixtures were loaded onto a Dowex 50W-X4 (Bio-Rad; 142-1351) column (Bio-Rad; poly-prep columns, 731-1550), which was washed with 2 ml water and placed on top of alumina columns (Sigma; catalog no. A9003) and washed again with 10 ml water. The columns were eluted with 6 ml 0.1 M imidazole (Sigma; catalog no. I0125) into a 15-ml Optiphase Highsafe scintillator (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Columns were reused up to 10 times. Dowex columns were regenerated by adding 10 ml 2 N HCl followed by 10 ml water and the alumina columns by adding 2 ml 1 M imidazole, 10 ml 0.1 M imidazole, and finally 5 ml water. Determinations were made in triplicate.

Arrestin recruitment assay

The stop codon of GPR39 was removed and subcloned into a 5′-position of renilla luciferase cDNA. The GPR39-RLuc was transiently expressed in a HEK293 cell line stably expressing a [R393E;R395E] mutant of human β-arrestin-2, which is N-terminally tagged with GFP2 (GFP2-βarr2[R393E;R395E]) as previously described (13). All cDNA clones were verified by DNA sequencing. Bioluminescence resonance energy transfer (BRET) 2 measurements were performed as previously described (14) using a Mithras LB 940 plate reader (Berthold Technologies, Bad Wildbad, Germany). Briefly, after harvesting, 180 μl resuspended cells were distributed in 96-well microplates (white Optiplate; PerkinElmer Life and Analytical Sciences, Boston, MA), resulting in a density of approximately 200,000 cells per well. Agonist was added manually, and substrate addition was performed with an injector that injected the substrate DeepBlueC (PerkinElmer Life and Analytical Sciences) (final concentration, 5 μM, 2 sec) before reading. The optimal reading time after agonist addition was determined to be 5 min. The signals detected at 400 and 515 nm were measured sequentially, and the 515:400 ratios were calculated.

CRE and SRE reporter assay

HEK293 cells (3×10^4 cells/well) seeded in 96-well plates were transiently transfected. In the case of the CRE reporter assay, the cells were transfected with a mixture of pFA2-CREB and pFR-Luc or SRE-Luc reporter plasmid (PathDetect CREB Trans-Reporting System, Stratagene, La Jolla, CA) and the indicated amounts of receptor DNA. After transfection, the cells were maintained in low serum (2.5%) throughout

the experiments and were treated with the respective inhibitors of intracellular signaling pathways. One day after transfection, cells were treated with the respective ligands in an assay volume of 100 μl medium for 5 h. The assay was terminated by washing the cells twice with PBS and addition of 100 μl luciferase assay reagent (LucLite, Packard, Johannesburg, South Africa). Luminescence was measured in a TopCounter (Top Count NXTTM, Packard) for 5 sec. Luminescence values are given as relative light units.

Competition binding assays

Two protocols were followed. Transiently transfected COS-7 cells were transferred to culture plates 1 d after transfection at a density of 250×10^3 cells per well. Two days after transfection, competition binding experiments were performed for 3 h at 4 C using 25 pM ^{125}I-obestatin (Amersham). Binding assays were performed in 0.5 ml 50 mM HEPES buffer (pH 7.4) supplemented with 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.1% (wt/vol) BSA, 40 μg/ml bacitracin. Nonspecific binding was determined as the binding in the presence of 1 μM unlabeled obestatin. Cells were washed twice in 0.5 ml ice-cold buffer, 0.5–1 ml lysis buffer (8 M urea, 2% NP40 in 3 M acetic acid) was added, and the bound radioactivity was counted. Determinations were made in duplicate. CHO-K1 cells (no. CRL-9618, American Type Culture Collection, Manassas, VA) were grown to near confluency in HAM F12 medium and transfected with GPR39-pcDNA3.1a plasmid using Lipofectamine 2000 (Invitrogen; catalog no. 11668-019). The transfected cells were diluted and treated with geneticin (1 mg/ml) for 1 wk, whereupon stably transfected clones were selected (GPR39-CHO). GPR39-CHO cell clones were seeded in 24-well tissue culture plates precoated with poly-L-lysine (0.1 mg/ml)/2.5×10^5 cells per well after overnight incubation (HAM F12 medium, 37 C, 5% CO_2). The cells were washed with fresh HAM F12 medium and incubated in triplicate for 2.5 h at either 4 or 25 C with 0.25 ml incubation medium (HAM F12 supplemented with either 50 pM or 1 nM ^{125}I-[Tyr^{16}]-human obestatin or ^{125}I-Bolton-Hunter-labeled human obestatin). Tracers were prepared in-house (Novo Nordisk A/S, Maaloev, Denmark) from human obestatin (Bachem, Bubendorf, Switzerland; H-6365 lot no. 3000906) and HPLC purified in-house to a specific activity of 2200 Ci/mmol. Nonspecific binding was estimated by inclusion of 1 μM nonlabeled human obestatin. The assays were terminated by aspiration of the incubation medium followed by a wash with 0.25 ml ice-cold HAM F12 medium. The cells were lysed with 0.25 ml 0.1 N NaOH, and the radioactivity of the lysate was quantified in a γ-counter.

Real-time QPCR

Real-time QPCR was performed using the Mx3000P (Stratagene), with the SYBR Premix Ex Taq (Takara, Gennevilliers, France), using the following primer sets: GPR39, (5′-AGTGAGGAGAGCCGGACAG-3′ and 5′-CAGTCATGTTTGGGTTTTGC-3′); ghrelin receptor, (5′-AAGATGCTTGCTGTGGTGGT-3′ and 5′-AAAGGACACCAGGTTGCAGT-3′); and β-actin, (5′-TTCTACAATGAGCTGCGTGTG-3′ and 5′-GGGGTGTTGAAGGTCTCAAA-3′). β-Actin was used as a reference gene, and the maximum expression was arbitrarily set to one, thus showing the relative expression in various tissues. The specificity of the primers was evaluated both through melting curve analyzes and sequencing of amplified products. Furthermore, QPCRs were performed on standard dilutions to verify the efficiency of the QPCR were between 95 and 105%. RNA from rat tissues (kidney, duodenum, lung, whole pituitary, and whole hypothalamus) was extracted using the RNeasy Lipid Tissue Mini kit (QIAGEN, Valencia, CA), followed by cDNA synthesis using the ImProm-II Reverse Transcription System (Promega, Madison, WI).

Food intake studies in mice

Food intake studies in mice were performed in lean, individually housed C57BL/6J mice (arriving 8 wk old from Harlan Ltd., Bicester, UK) weighing approximately 25 g kept on a normal-phase light-dark cycle (light on, 0700 h; off, 1900 h). Two regimes were probed. The first group was freely fed lean mice, which included four groups of animals (n = 10–11) having free access to a standard pelleted rodent diet (Harlan Teklad Global 2018 diet) and tap water and habituated to a daily presentation for 4 h of a wet mash diet (1 part powdered chow:1.5 parts tap water), which ensured a high level of food intake over the initial period studied. On the day of the experiment, the animals received, 0.5 h before presentation of the wet mash an, ip injection of 1) vehicle, 2) 0.25 mg/kg obestatin (~100 nmol/kg), 3) 2.5 mg/kg obestatin (~1000 nmol/kg), or 4) 10 mg/kg sibutramine. Human obestatin (from the University of Leipzig) was dissolved directly in saline. After 4 h, the wet mash was replaced with a known quantity of standard pellets. The second group was fasted lean mice. Animals were fasted for 16 h before the experiment. Four groups (n = 10–12) were treated with vehicle, obestatin, or sibutramine as described above for the freely fed animals. However, in this study, chow was replaced 30 min after dosing. Regime 1 was probed twice, regime 2 was probed once in the same group of animals with at least 7 d of washout period in between, and animals were allocated randomly to different groups in each experiment. Data were analyzed by ANOVA.

Results

InsP production

Previously, we have found that GPR39 signals effectively through the Gq pathway (8). As shown in Fig. 1A, Zn^{2+} stimulated InsP production in a dose-dependent manner with an EC_{50} of 22 ± 4 μM in COS-7 cells transiently transfected with GPR39 but not in mock-transfected cells (data not shown). In contrast, obestatin in concentrations up to 1 μM, which is a very high concentration for a peptide hormone or neuropeptide, did not affect InsP production in the GPR39-transfected cells in experiments performed in parallel with the Zn^{2+} experiments (Fig. 1A). Similar results, *i.e.* positive for Zn^{2+} and negative for obestatin, were obtained when either the wild-type GPR39 or an N-terminally FLAG-tagged GPR39 receptor were used (data not shown). Obestatin was equally ineffective in stimulating InsP production when coadministered with Zn^{2+} at a dose resulting in approximately 30% stimulation of InsP production.

cAMP production

Zhang *et al.* (9) reported that GPR39 could signal through the Gs pathway. This observation was confirmed because Zn^{2+} in the GPR39-transfected COS-7 cells stimulated cAMP production in a dose-dependent manner, with an EC_{50} of 7.3 ± 2 μM (Fig. 1B), but not in mock-transfected cells (data not shown). Obestatin, however, did not alter the cAMP production in experiments performed in parallel (Fig. 1B). The high constitutive activity of GPR39 observed in many other signaling pathways was not observed for cAMP production (data not shown).

Arrestin mobilization assay

GPR39 fused to renilla luciferase was transiently transfected into HEK293 cells that stably express a GFP-labeled arrestin-2. As shown in Fig. 1C, Ni^{2+}, in a dose-dependent fashion, stimulated surface mobilization of arrestin as reflected in the increase in BRET signal between the luciferase-labeled GPR39 and the GFP-labeled arrestin. In contrast, neither human nor rat obestatin showed any effect on arrestin mobilization in concentrations up to 10 μM in the GPR39-transfected HEK293 cells (Fig. 1C).

CRE transcriptional activity

As measured in a luciferase reporter assay, Zn^{2+} stimulated CRE transcriptional activity in GPR39-transfected

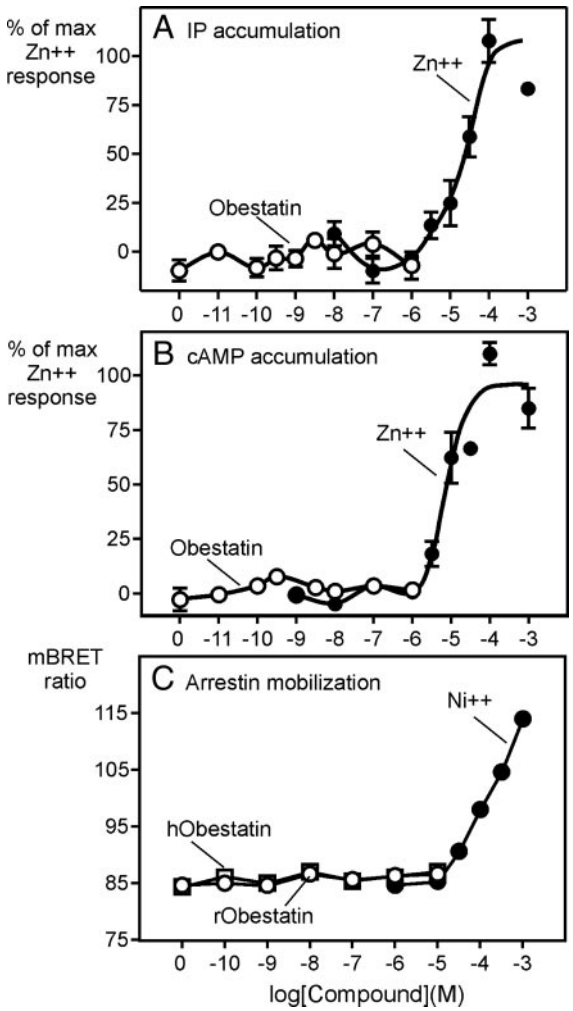

FIG. 1. GPR39-mediated signaling through Gq/phospholipase C as measured by InsP accumulation (A), Gs/adenylate cyclase as measured by cAMP production (B), and arrestin as measured by BRET (C). InsP and cAMP experiments were performed on transiently transfected COS-7 cells as parallel dose-response experiments with $ZnCl_2$ (*black circles*) or human obestatin (*white circles*). Data are presented as percentage of maximum Zn^{2+}-induced stimulation above basal, mean ± SE, for five independent experiments performed in duplicates. EC_{50} for Zn^{2+} stimulation of InsP turnover was 22 ± 4 μM and for cAMP turnover was 7.3 ± 2 μM. Similar results were obtained with different GPR39 clones in different vectors, with and without a FLAG tag and with different obestatin peptide batches and in transiently transfected CHO cells. Arrestin surface mobilization shown in C was measured in HEK293 cells by BRET between luciferase fused to the C-terminal tail of GPR39 and GFP fused to the C-terminal tail of an arrestin mutant (see *Materials and Methods*).

HEK293 cells but not in mock-transfected cells (Fig. 2, A and C). Two representative experiments out of seven where obestatin was probed in parallel with Zn^{2+} as agonist for CRE-controlled transcriptional activity in both GPR39 and mock-transfected cells are shown in Fig. 2, B and D compared with A and C. The previously reported constitutive signaling activity of GPR39 through the CRE pathway (8) is reflected in the high, fluctuating basal CRE activity observed in the GPR39-transfected cells as opposed to the mock-transfected cells (Fig. 2, A–D).

SRE transcriptional activity

As measured in an SRE-luciferase reporter assay, Zn^{2+} also stimulated SRE transcriptional activity in the GPR39-transfected HEK293 cells as opposed to mock-transfected cells (Fig. 2, E and G). Two representative experiments out of seven where obestatin was probed in parallel with Zn^{2+} as agonist for SRE-controlled transcriptional activity in GPR39 and mock-transfected cells are shown in Fig. 2, F and H compared with E and G. Once again, note that the previously reported constitutive signaling activity of GPR39 through the SRE pathway (8) is reflected in the high, fluctuating basal SRE activity observed in the GPR39-transfected cells as opposed to the mock-transfected cells (Fig. 2, E–H).

Radiolabeled obestatin binding

Three different obestatin tracers were used, two of which were made by oxidative iodination and one by Bolton-Hunter iodination, based on two differently synthesized peptides (for details, see *Materials and Methods*). However, none of these obestatin tracers showed any specific binding to the transiently transfected COS-7 or the stably transfected CHO cells expressing the GPR39 receptor. In fact, even the unspecific binding of the obestatin tracer was very low, *i.e.* less than 1%. QPCR analysis and signal transduction assays using Zn^{2+} as a positive control demonstrated that the transfected cells did express the GPR39 receptor.

QPCR analysis of GPR39 expression in tissues

High-level expression of GPR39 could be detected in peripheral organs of the rat such as the duodenum and kidney (Fig. 3). However, in the hippocampus and the pituitary, the GPR39 expression was below the detection limit of the employed QPCR method (Fig. 3). Importantly, detection of high level expression of the ghrelin receptor in the hypothalamus and pituitary demonstrated that the RNA preparations from these tissues were of adequate quality.

Effect of obestatin on acute food intake in mice

In an initial experiment on freely fed mice, a nonsignificant tendency toward a suppressive effect of obestatin on the wet mash intake over the first 4 h was observed (Fig. 4A). However, no effect was observed at 24 h, whereas the positive control, sibutramine, decreased food intake significantly at all time points ($P < 0.01$, Dunnett's two-tailed test). When the experiment was repeated, no tendency was observed for obestatin suppressing food intake, whereas the positive control sibutramine again had the expected significant effect (Fig. 4B). To try to mimic the experimental setup used by Zhang *et al.*, the effect of obestatin on acute food intake was also probed in 16-h-fasted mice, but with the same lack of effect (Fig. 4C). In this case, only a nonsignificant tendency to a suppressive effect was observed for the positive control, sibutramine, conceivably because of the stronger hunger drive in the fasted animals.

Discussion

In the present study, we found robust stimulatory effects of Zn^{2+} on GPR39 signaling, whereas we were unable to

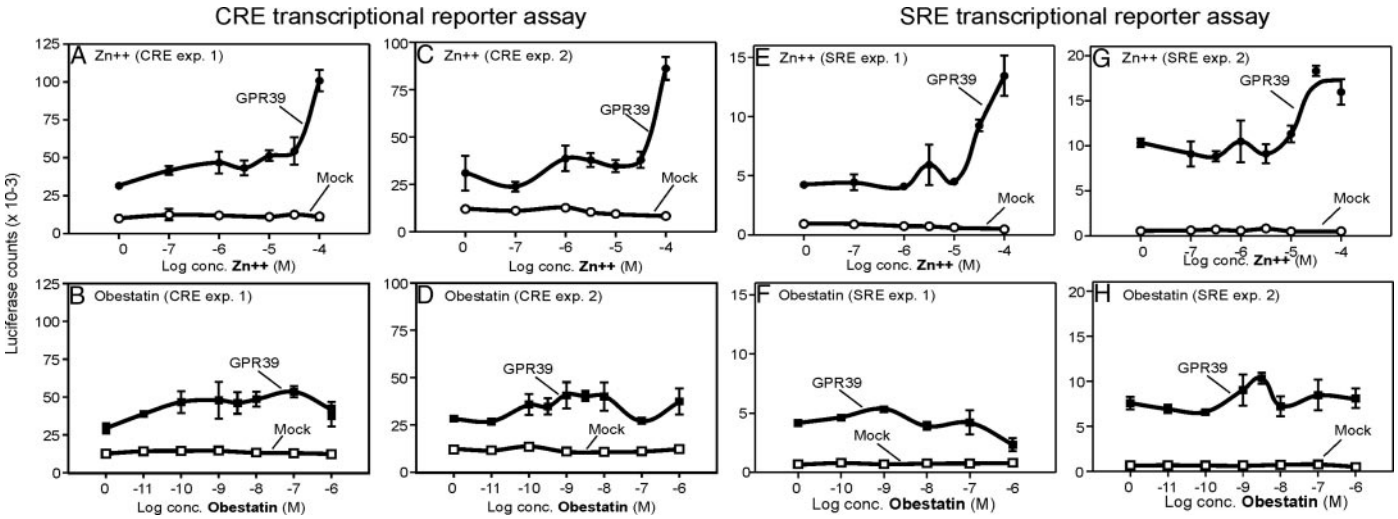

FIG. 2. GPR39-mediated activation of CRE (A–D) and SRE (E–H) transcriptional activity in response to Zn^{2+} and to obestatin. HEK293 cells were transiently transfected with the FLAG-tagged human GPR39 cDNA plus the reporter cDNA for either CRE or SRE transcriptional activity (see Materials *and Methods*). A and C, CRE activity during dose-response curves for Zn^{2+} ($ZnCl_2$) in two representative experiments; B and D, Dose-response curves for human obestatin performed in parallel experiments. Data are representative experiments of seven independent experiments performed in triplicate giving an average EC_{50} value for Zn^{2+} in stimulating CRE activity of 37 ± 8 μM. E and F, SRE activity during dose-response curves for Zn^{2+} in two representative experiments; F and H, dose-response curves for human obestatin performed in parallel experiments. Data are representative experiments of seven independent experiments performed in triplicate, giving an average EC_{50} value for Zn^{2+} in stimulating SRE activity of 52 ± 14 μM. Similar results were obtained with wild-type GPR39 receptor, *i.e.* without the FLAG tag.

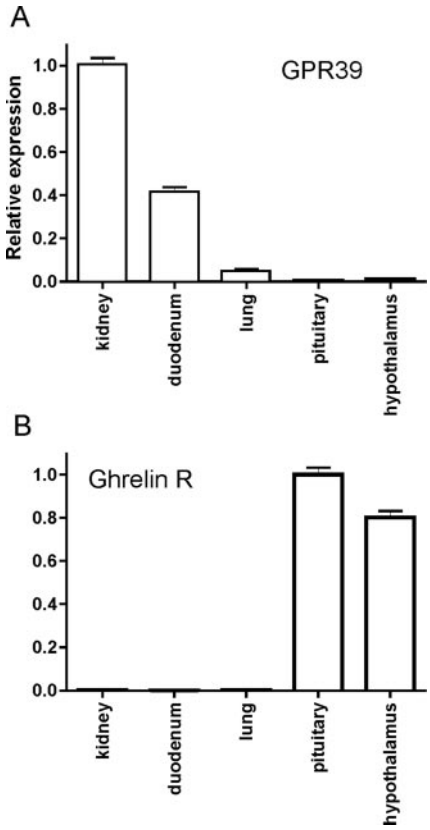

FIG. 3. Real-time QPCR analysis of GPR39 and ghrelin receptor expression in rat kidney, duodenum, lung, pituitary, and hypothalamus. For each receptor, the tissue with the highest level of expression has been set to 1.0, *i.e.* kidney for GPR39 and pituitary for the ghrelin receptor. For comparison of relative expression levels in the tissues, β-actin was used as a reference gene.

confirm the reported agonistic effects of obestatin on this receptor (9). In accordance with the negative signaling results, we did not observe any specific binding of radioiodinated obestatin to GPR39-expressing cells. Moreover, in agreement with a recent in situ study (15), our Q-PCR analysis failed to detect significant expression of GPR39 in the hypothalamus, *i.e.* a proposed main central target organ for obestatin (9). Finally, no significant and reproducible effect was observed for obestatin on acute food intake in mice. These observations indicate that GPR39 is not the obestatin receptor but that Zn^{2+} could be an endogenous agonist and modulator of GPR39 signaling.

Why no effect and binding of obestatin to GPR39?

In principle, the reason could be differences in either the peptide or the receptor used in the present study and the previous report (9). Peptides from three different sources, which all have excellent track records in peptide synthesis, were used in the present study, *i.e.*, Amersham (used for one of the radio-iodinated tracers), Bachem (used for two other tracers and for unpublished, negative signal transduction assays; Stidsen C. *et al.*, unpublished observations), and University of Leipzig[1] (used for the signal transduction assays presented here). The obestatin peptides had the expected liquid chromatography-mass spectrometry quality control properties corresponding to the 23-amino-acid carboxyamidated obestatin peptide.[2] Also, the GPR39 receptor had the correct sequence and responded normally to Zn^{2+} (8),

[1] Institute of Biochemistry, Head Prof. Annette Beck-Sickinger (coauthor of the present paper).

[2] It should be noted that from a peptide chemical and a peptide synthesis point-of-view, obestatin is a rather simple peptide, which is easy to work with, as opposed to certain other peptides.

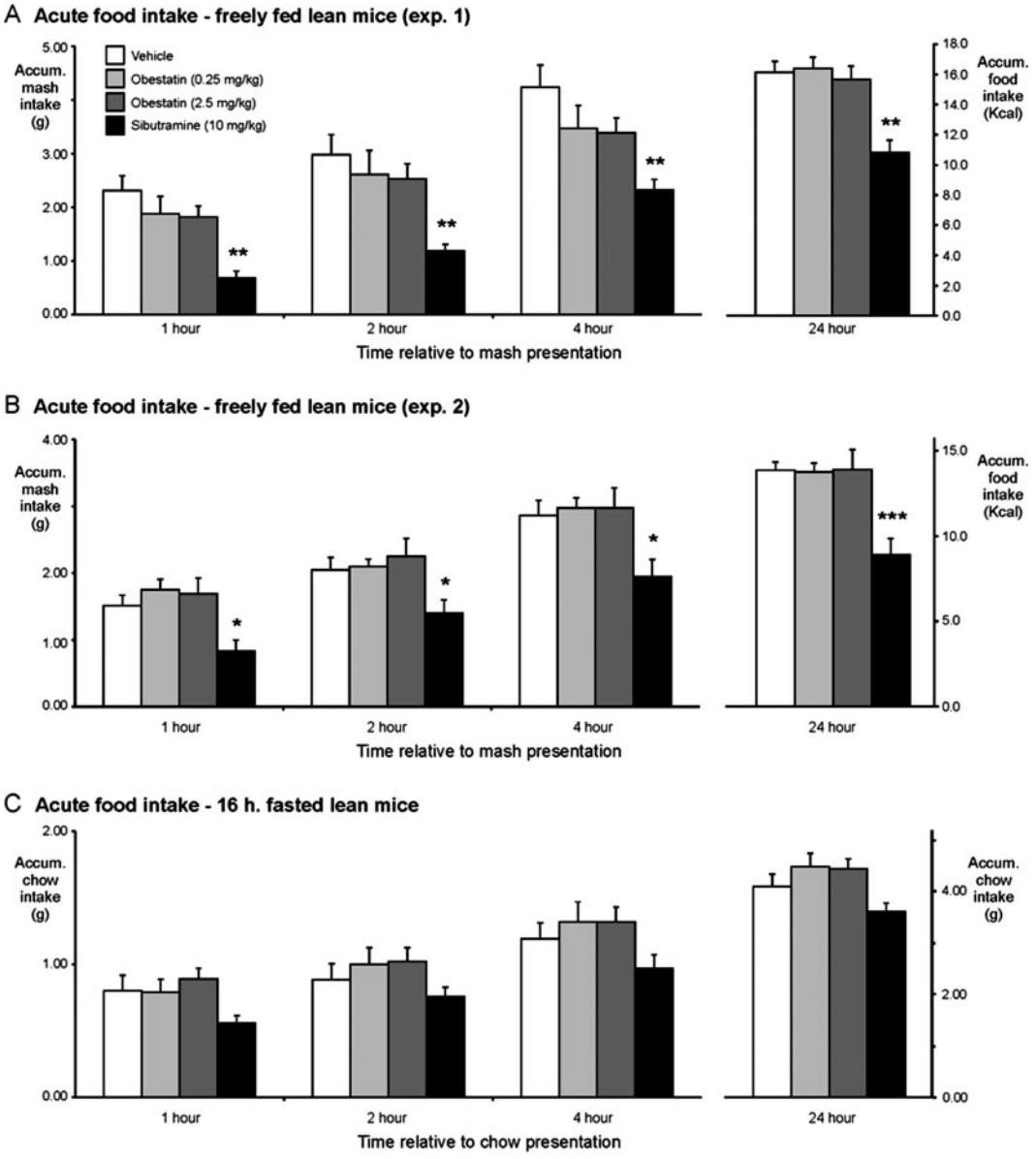

FIG. 4. Effect of acute ip administration of obestatin and positive control, sibutramine, on food intake in mice. A and B, Two independent experiments each including four groups of lean C57BL/6J mice, n = 10–11, which were treated with vehicle (*white columns*), 0.25 mg/kg obestatin ~ 100 nmol/kg (*light-gray columns*), 2.5 mg/kg obestatin ~ 1000 nmol/kg (*dark-gray columns*), or with 10 mg/kg sibutramine. Wet mash was presented to the animals 0.5 h after dosing. Accumulated food intake is shown as mean ± SE as mash intake (grams) for the 1–4-h periods and as kilocalories for the 24-h period. Data were analyzed by ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ Dunnett's test (two tailed) from vehicle. C, Four groups of mice treated as above but after a 16-h fasting period and receiving ordinary chow throughout the study instead of wet mash. The suppressive effect of sibutramine on food intake did not reach statistically significant levels in these fasted animals.

which here served as a convenient positive control. However, it should be noted that in the report from Zhang *et al.*, no constitutive activity was observed for GPR39 in the SRE reporter assay (see Fig. S5 in Ref. 8). This is in contrast to the strong constitutive activity previously reported for GPR39 through this signaling pathway (8), which was also observed in the present study (see difference between basal levels in GPR39-transfected *vs.* mock-transfected cells in Fig. 2, E–H). Dr. Aaron Hsueh kindly provided us with a sample of the GPR39 used in the original report (9). In our hands, this GPR39 cDNA was well expressed and displayed an equally high degree of constitutive activity as our own GPR39 cDNA (Holst, B., and T. W. Schwartz, unpublished observations). Importantly, two individual experiments are shown in Fig. 2 to illustrate that fluctuations in the high basal signaling activity of GPR39 in certain experiments perhaps could be interpreted as being a result of the exposure to obestatin, or in this case exposure to very low concentrations of Zn^{2+}. Nevertheless, at present, we will conclude that GPR39 probably is not the obestatin receptor.

Although there appears to be some difficulty in also reproducing some of the *in vivo* and *ex vivo* effects of obestatin, for example on food intake, body weight, gastric emptying, and gut contractility (16, 17), other groups have started to

report on various new effects of obestatin, for example on sleep (18). Thus, we would suggest that the endogenous receptor for obestatin should be searched for among the many 7TM receptors that still are orphan receptors.

GPR39 constitutive activity

As discussed above, like the structurally related ghrelin and neurotensin 2 receptors, GPR39 signals with high ligand-independent activity (8, 19, 20). For GPR39, this constitutive activity is observed in InsP turnover assays as well as in CRE- and especially in SRE-dependent transcriptional activity but not in MAPK signaling, despite the fact that GPR39 stimulates ERK1/2 MAPK phosphorylation very efficiently upon stimulation by Zn^{2+} (8). Similarly, in the present study, we observed that, although GPR39 stimulates cAMP production when exposed to Zn^{2+}, no reproducible constitutive activity was observed through the Gs signaling pathway. It should be noted that the degree of constitutive activity between different signal transduction pathways varies for other receptors, such as the ghrelin receptor, for which the ligand-independent signaling is particularly strong in the Gq-mediated phospholipase C pathway as reflected in the assays for InsP accumulation and in the CRE-dependent transcriptional activity (8).

Could Zn^{2+} be an endogenous ligand for GPR39?

Transition metal ions such as Zn^{2+} and Ni^{2+} stimulate GPR39 signaling in various pathways with an efficacy that is in the same order of magnitude as observed for the hormones ghrelin and motilin on their respective receptors in parallel experiments (8). In the body, specific transporters ensure that Zn^{2+} is stored in neuronal as well as certain endocrine secretory granules (21, 22), from where the metal ion is released during stimulation. The concentration of Zn^{2+} in for example the synaptic cleft has been estimated to reach more than 10^{-4} M (23). The potency of Zn^{2+} on GPR39 indicates that it could very well be a physiological agonist or modulator of GPR39 signaling. Previously, a naturally occurring metal ion site has been described in the NK3 receptor through which Zn^{2+} enhances agonist binding but only had marginal functional effects (24). In contrast, metal ion sites have been characterized in the wild-type melanocortin MC1 and MC4 receptors, where Zn^{2+} acts both as an efficient agonist and as an enhancer of the function of the endogenous agonist peptide, α-MSH, by increasing its potency and providing additive efficacy (25). On GPR39, Zn^{2+} is both a potent and efficacious agonist, indicating that GPR39 could act as a physiological sensor for changes in extracellular Zn^{2+} concentrations. It remains to be seen whether Zn^{2+} is the only natural ligand for GPR39 or whether the metal ion functions as an agoallosteric modulator (26), *i.e.* both being an agonist in itself and being an enhancer of the function of another, still unknown, endogenous hormone or transmitter.

Why no effect of obestatin on acute food intake?

In the original paper on obestatin, Zhang *et al.* (9) described rather robust suppressive effects of obestatin on food intake and body weight in mice. In an accompanying paper to the present study, Nogueiras *et al.* (16) report on difficulties in observing any effect of obestatin on food intake, body weight, energy expenditure, *etc.* as studied in a number of different rodent models. It is well known in the field that for certain orexigenic mechanisms, for example PYY3–36, there can be difficulties in showing robust, reproducible effects in rodents in certain laboratories (27), although the effect can be observed in other laboratories and in humans (28–30). Thus, for obestatin, the jury is still out. However, the present study does add a number to the list of studies, which have been unsuccessful in showing effects on food intake for obestatin (16).

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