RANTES (CCL5) reduces glucose-dependent secretion of glucagon-like peptides 1 and 2 and impairs glucose-induced insulin secretion in mice

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Pais R, Zietek T, Hauner H, Daniel H, Skurk T. RANTES (CCL5) reduces glucose-dependent secretion of glucagon-like peptide 1 and 2 and impairs glucose-induced insulin secretion in mice. Am J Physiol Gastrointest Liver Physiol 307: G330–G337, 2014. First published May 29, 2014; doi:10.1152/ajpgi.00329.2013.—Type 2 diabetes is associated with elevated circulating levels of the chemokine RANTES and with decreased plasma levels of the incretin hormone glucagon-like peptide 1 (GLP-1). GLP-1 is a peptide secreted from intestinal L-cells upon nutrient ingestion. It enhances insulin secretion from pancreatic β-cells and protects from β-cell loss but also promotes satiety and weight loss. In search of chemokines that may reduce GLP-1 secretion we identified RANTES and show that it reduces glucose-stimulated GLP-1 secretion in the human enteroendocrine cell line NCI-H716, blocked by the antagonist Met-RANTES, and in vivo in mice. RANTES exposure to mouse intestinal tissues lowers transport function of the intestinal glucose transporter SGLT1, and administration in mice reduces plasma GLP-1 and GLP-2 levels after an oral glucose load and thereby impairs insulin secretion. These data show that RANTES is involved in altered secretion of glucagon-like peptide hormones most probably acting through SGLT1, and our study identifies the RANTES-receptor CCR1 as a potential target in diabetes therapy.

glucagon-like peptide-1; RANTES; cAMP; sodium/glucose transporter-1

RANTES (regulated on activation; normal T cell expressed and secreted) belongs to the C-C subfamily of chemokines and has been shown to be predictive for the development of future metabolic alterations (21). It promotes the recruitment and activation of monocytes (28), lymphocytes (41), mast cells (8), and eosinophils (9) via specific receptors of the G protein-coupled receptor (GCPR) family, namely CCR1, CCR3, and CCR5 (44), which predominantly signal through Gi/o (45). RANTES is secreted from platelets, macrophages, eosinophils and fibroblasts but has also been shown to be secreted from the adipose tissue (43). Recently, RANTES was described as constitutively expressed in mouse and human pancreatic islets with differences in cellular localizations between the two species (26). In adipose tissue RANTES gene expression and secretion positively correlates with adipocyte size (43), and plasma levels are increased in obese human subjects and mice (19) (50) associated with impaired glucose tolerance. In type 2 diabetic humans (24) as well as in nonobese diabetic (NOD) mice as model of spontaneous diabetes in the mouse, plasma levels are also increased (6).

The incretin hormone glucagon-like peptide 1 (GLP-1), which is secreted by enteroendocrine L-cells in response to glucose (36) and other nutrients (10, 47, 48), potentiates glucose-stimulated insulin secretion (32), enhances β-cell growth and survival, inhibits glucagon release, delays gastric emptying, and suppresses food intake (11). GLP-1 thus has antidiabetic effects. This has led to new therapies for type 2 diabetes treatment using GLP-1 mimetics and inhibitors of the GLP-1 degrading enzyme dipeptidyl-peptidase IV (DPP IV) (12).

While RANTES levels are elevated in human type 1 and type 2 diabetes (33, 34), GLP-1 concentrations are reduced (31, 46), but no interaction between the two proteins has been described. Although RANTES expression in intestine has been described in inflammatory bowel disease (3), no one has assessed RANTES effects on enteroendocrine cells such as GLP-1 secreting L-cells in the gut. In the present study, we aimed to elucidate if elevated RANTES levels could be responsible for the impaired GLP-1 response observed in diabetes. Therefore we determined the presence of the RANTES receptor CCR1 in murine intestine and the human L-cell model NCI-H716, tested the effects of recombinant RANTES on glucose-stimulated GLP-1 secretion in vitro, and assessed the effects of RANTES on glucose-dependent gut hormone and insulin secretion in vivo in mice. Furthermore we investigated the intracellular mechanisms through which RANTES acts.

MATERIALS AND METHODS

Cell Culture

All cell culture materials were purchased from PAA Laboratories (Pasching, Germany) unless otherwise indicated. The human enteroendocrine NCI-H716 (ATCC, no. CCL-251) was obtained from the American Type Cell Culture Collection. Cells were grown in suspension in RPMI 1640 medium containing 2 mM glutamine supplemented with 10% (vol/vol) fetal bovine serum, 1 mM sodium pyruvate, 100 IU/ml penicillin, and 100 mg/ml streptomycin and maintained in a humidified atmosphere containing 5% CO2/air at 37°C. For GLP-1 secretion studies, cAMP measurement assays and immunostainings, cells were seeded on Matrigel (BD Biosciences, Heidelberg, Germany).

Secretion of GLP-1 from NCI-H716 Cells

NCI-H716 cells were seeded at a density of 4 × 105/ml in 24-well culture plates. For the experiments, growth medium was replaced, and cells were washed twice with PBS and then treated with or without 10 nM recombinant human (rh) RANTES (Immunotools, Friesoythe, Germany) dissolved in fresh medium for 2, 6, 12, and 24 h. Cells were then incubated with or without 500 mM glucose (22) and RANTES in PBS, 1 mM CaCl2, pH 7.2, supplemented with dipeptidyl peptidase IV inhibitor (20 μM/ml, Millipore, Schwalbach, Germany) for 1 h at
37°C as previously conducted by Jang et al. (22). Appropriate osmotic control experiments were done using 500 mM of a nonmetabolizable sugar 2-deoxy-glucose. Supernatants were collected and centrifuged at 1,000 g for 10 min at 4°C. Cells were homogenized and protein concentration per well was measured by Bradford assay (Bio-Rad, Munich, Germany). GLP-1 (active) was assayed using an ELISA (Millipore) and normalized to protein content.

**Effect of Met-RANTES on GLP-1 Secretion**

Met-RANTES (20 nM, R and D Biosystems), the CCR1 receptor antagonist (35), was added with or without 10 nM RANTES to cells for 2 h before stimulation with glucose. GLP-1 secretion was performed as described before.

**Immunofluorescence and Confocal Microscopy**

**Cells.** NCI-H716 cells grown on sterilized glass coverslips were fixed with 4% (vol/vol) paraformaldehyde (PFA) in Tris-buffered saline (TBS) for 15 min at room temperature. Cells were washed twice with TBS and incubated in blocking solution [3% BSA (wt/vol) in TBS] for 30 min at room temperature. This was followed by an overnight incubation with primary antibodies, anti-CCR1 (1:50, sc-7934, Santa Cruz Biotechnology, Heidelberg, Germany) or anti-GLP-1 (1:50, sc-7782, Santa Cruz Biotechnology) followed by 1 h incubation with appropriate secondary antibodies conjugated with FITC, Cy5, or Cy3 (used at 1:150 dilution; Jackson Laboratories Immuno Research, West Grove, PA) and DAPI for nuclear staining. The coverslips were then washed twice with TBS before mounting on glass slides using Dako mounting medium. The cells were visualized with a laser scanning spectral confocal microscope (Leica TCS SP2, spectral confocal and multiphoton system).

**Tissue.** Mouse intestinal tissue was fixed overnight at 4°C in 4% (vol/vol) PFA in TBS, dehydrated in graded concentrations of ethanol and xylene at 40°C, embedded in paraffin (Paraplast embedding medium), and sectioned into 5- to 6-μm thick sections and dried overnight at room temperature on Superfrost Plus micro slides (Menzel, Braunschweig, Germany). Subsequently, sections were dewaxed in xylene and rehydrated in descending concentrations of ethanol in water. For demasking of epitopes, the sections were incubated in 10 g/l of 0.1 M HCl was added and incubated at room temperature for 20 min. Cells were scraped off the surface with a cell scraper. The mixture was then added to the cells in one dish resulting in a final concentration of 5 nM for the siRNAs. Cells were examined 24, 48, and 72 h after transfection by Western blot.

**RNA Isolation and RT-PCR**

Total RNA was extracted from NCI-H716 cells and mouse intestinal mucosa by using TRIzol (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s instructions. Intact RNA was confirmed by running an RNA gel. Twenty microliters of cDNA was synthesized from 5 μg of RNA using a commercially available kit (Ambion, Applied Biosystems, Darmstadt, Germany). The PCR reaction was carried out in a Roche Light Cycler for 35 cycles using primers for CCR1 as described (30).

**SDS PAGE and Western Blotting**

Proteins (10 μg/lane) were subjected to electrophoresis on a 10% (wt/vol) polyacrylamide gel. Semidry transfer of protein was done onto a nitrocellulose membrane. After transfer was complete, the membrane was incubated in blocking solution, 3% BSA in TBS for 1 h at room temperature. The membranes were incubated with anti-CCR1 rabbit polyclonal antibodies (1:200), and anti-β-actin goat polyclonal antibodies (1:200, Santa Cruz Biotechnology) in TBS with 0.1% (vol/vol) Tween 20 (TBS-T) overnight with gentle agitation at 4°C. After three washes in TBS-T, the membranes were incubated with secondary antibodies labeled with IRDye infrared dyes, IRDYE 800-conjugated goat anti-rabbit IgG (LI-COR Biosciences) diluted in TBS-T (1:20,000) for 1 h at room temperature with gentle agitation. At the end of the incubation, the membranes were visualized and analyzed by the ODYSSEY Infrared Imaging System (LI-COR Bioscience).
a cAMP-acetylcholinesterase conjugate (tracer) for a limited number of cAMP-specific rabbit antibody binding sites.

Protein Kinase A Assay

Five-centimeter-long everted mouse intestine segments were incubated for 30 min at 37°C in Krebs buffer containing 50 mM glucose in the absence (control) or presence of 100 nM RANTES. Mucosa from each segment were scraped and homogenized. Samples were centrifuged at 150,000 g for 10 min at 4°C. Protein kinase A (PKA) activity was detected and quantified in the supernatant using a commercially available kit (Enzo Life Sciences, Lorrach, Germany).

Ex Vivo 1-O-Methyl α-D-Glucopyranoside Transport Experiments

Groups of four everted intestinal rings of 1 cm in length from murine proximal small intestine were incubated at 37°C for 3 min in the absence (control) and the presence of 100 nM RANTES and 0.5 mM of the SGLT1 (sodium/glucose-transporter 1) inhibitor phloridzin followed by incubations for 2 min in Krebs buffer containing 1 mM of the SGLT1-specific substrate 1-O-methyl α-D-glucopyranoside (α-MDG) and radiotracer [1-14C]-α-MDG (American Radiolabeled Chemicals, St Louis, MO) or containing 1 mM glycyl-sarcosine (Gly-Sar) with radiotracer [14C]Gly-Sar (Amersham Biosciences, Freiburg, Germany) for measuring SGLT1-mediated glucose and PEPT1-mediated Gly-Sar transport in the absence or the presence of RANTES. After incubation, rings were washed twice in ice-cold Krebs solution, and radioactivity incorporated in the tissue was quantified by liquid scintillation.

In Vivo Mice Experiments

Male C57BL/6 mice (16–17 wk old) were maintained at 22 ± 1°C and a 12:12-h light/dark cycle with access to tap water and fed a chemically defined diet (S5745-E702, Ssniff, Soest, Germany) ad libitum. All procedures applied were conducted according to the German guidelines for animal care and approved by the state ethics committee under reference number 55.2-1-54-2532-67-11. To study the effect of RANTES in mice, animals received an intraperitoneal injection of RANTES (10 μg in 200 μl PBS) for four consecutive days; controls received the same volume of PBS. On the day of the experiment, mice were fasted for 6 h, and fasting blood glucose was measured from the tail vein with a glucometer (FreeStyle Lite, Abbott Diabetes Care, Oxon, UK). Mice were administered a glucose bolus (6 g/kg) as conducted by Gorboulev et al. (20) or water immediately after the last intraperitoneal injection by gavage, and blood glucose was measured 10 min later. Mice were anesthetized with isoflurane and blood samples were obtained from the retro-orbital sinus. DPP IV inhibitor (at 20 μl/ml) was added to the blood within 30 s and centrifuged at 4°C at 1,200 g for 10 min. Plasma obtained was used to measure active GLP-1 (Millipore), total GLP-2 (Yanaihara Institute, Japan), total GIP (gastric inhibitory polypeptide; Millipore), insulin (Crystal Chem), and RANTES (R&D systems) by ELISAs.

Data Analysis

GraphPad Prism version 4.01 for windows (GraphPad Software) was used to draw graphs and for statistical analysis. All data are expressed as means ± SE. Statistical significance between experimental groups was assessed by ANOVA and or Student’s t-test as indicated.

RESULTS

Effect of rh RANTES on GLP-1 Secretion from NCI-H716 Cells

To test the effect of RANTES on GLP-1 secretion, we used the human L-cell model NCI-H716, which has been shown to
respond to a range of secretagogues (25, 38, 39), NCI-H716 cells showed a positive staining for GLP-1 (data not shown). Pretreatment of the cells with 10 nM RANTES significantly reduced GLP-1 secretion following glucose stimulation by 63.4 ± 12.4%, 57.5 ± 7.5%, 52.8 ± 27.5%, and 54.3 ± 21.0% after 2, 6, 12, and 24 h of treatment (Fig. 1) compared with cells not exposed to RANTES. Basal GLP-1 levels were unaffected by RANTES treatment (data not shown).

Expression of the RANTES Receptor in NCI-H716 Cells and Mouse Small Intestine

The RANTES receptor CCR1 has previously been shown to be expressed in colonic tissues of humans and rats (1, 3). We identified the mRNA transcript for CCR1 by real-time PCR on NCI-H716 cells (Fig. 2A), and mRNA of CCR1 was also detected in mouse intestine (data not shown). CCR1 protein was also identified by Western blot and immunofluorescence (IF) as shown in Fig. 2, B and C, in the NCI-H716 cells, indicating that the RANTES receptor is present on enteroendocrine GLP-1 secreting L-cells. In mouse small intestine, CCR1 was identified in L-cells identified by GLP-1 positive staining as well as in some other cells (Fig. 2D).

Effect of Met-RANTES on GLP-1 Secretion

Since maximal inhibition of GLP-1 secretion was already seen after 2 h incubation of the NCI-H716 cells with RANTES with no improvement using longer incubation periods (see Fig. 1), further cell culture experiments were done using this time point.

To confirm the effects of RANTES on glucose-stimulated GLP-1 secretion, we used Met-RANTES, which binds with high affinity to the CCR1 receptor but not does activate it, and examined if the impairment of GLP-1 secretion caused by RANTES would be alleviated by this treatment. NCI-H716 cells were preincubated with RANTES ± Met-RANTES for 2 h followed by 1 h stimulation with glucose. Antagonism with Met-RANTES resulted in complete recovery of GLP-1 secretion compared with untreated cultures (Fig. 3A). These data confirm the specific involvement of RANTES in the course of disturbances in GLP-1 secretion.

siRNA Silencing of CCR1 and Its Effect on GLP-1 Secretion

Further confirmation of the role of CCR1 as a target of RANTES in the course of GLP-1 disturbance was shown by CCR1 downregulation using small interfering RNA in the NCI-H716 cells. Western blot analysis was performed on the cells following 1, 2, and 3 days after transfection and on untransfected (U) and scrambled siRNA transfected (M) cells. A significant reduction of CCR1 protein levels by 80.6 ± 6.6% was obtained after 1 day posttransfection, which recovered on the 2nd and 3rd days (Fig. 3B). CCR1 siRNA-treated cells (T) after 1 day posttransfection were used and showed a significant improvement in GLP-1 secretion upon glucose stimulation accounting for 48.3 ± 12.0% more than in untransfected cells treated with RANTES (U) (Fig. 3C). Mock transfected cells (M) did not display any such improvement.

Effect of RANTES on Intracellular cAMP

An elevation of intracellular cAMP level was demonstrated to increase GLP-1 secretion in several models of intestinal enteroendocrine cells (EEC), and PKA was described to mediate the stimulatory effect of cAMP on GLP-1 secretion (5, 18, 39, 42). RANTES treatment for 5 min caused a dose-dependent reduction in basal intracellular cAMP levels in NCI-H716 cells accounting for 32.7 ± 9.7% at 10 nM RANTES.
This concentration also significantly reduced the glucose- and forskolin-induced increase in intracellular cAMP by 48.5\% and 88.1\%, respectively (Fig. 4, B and C). Also RANTES incubation along with glucose significantly reduced relative PKA activity in mouse intestinal tissues by 40.4\% after 30 min of incubation, and these results are in line with those obtained for changes in cAMP.

**Effect of RANTES on SGLT1 Transport Function**

The intestinal sodium-dependent glucose transporter SGLT1 has been demonstrated to be pivotal for glucose-induced GLP-1 secretion (20, 29, 40). We therefore studied whether the RANTES-induced impairment of GLP-1 secretion might be linked to SGLT1 transport activity. Specificity for SGLT1-mediated transport was demonstrated in mouse intestinal tissue by use of 0.5 mM of the SGLT1-specific inhibitor phloridzin, which reduced uptake of the nonmetabolizable SGLT1 substrate α-MDG by 89.9\% as shown in Fig. 5 A. RANTES pretreatment of mucosal tissues resulted in a dose-dependent reduction of SGLT1-mediated α-MDG uptake with a maximal effect for inhibition by 61.7\% compared with controls.
RANTES (100 nM), however, had no effect on the uptake of the nonmetabolizable radiolabeled dipeptide Gly-Sar as a model substrate for the proton coupled di-/tripeptide transporter PEPT1 (Fig. 5B), which indicates that the inhibitory effect of RANTES is specific to the SGLT1 transporter.

**Blood Glucose, Insulin, and Gut Hormone Levels in Mice Treated with RANTES**

To test if RANTES would show the same inhibitory effect on GLP-1 secretion in mice as was seen in the NCI-H716 cells, we injected mice with RANTES via the intraperitoneal route and, soon after, gavaged them with glucose bolus. In addition to GLP-1, plasma GLP-2, GIP, insulin as well as blood glucose were measured, and circulating RANTES levels were also assessed. RANTES levels at day 4 increased to 432.6 ± 71.3 pg/ml in RANTES-injected mice compared with saline-injected controls with 25.6 ± 1.8 pg/ml. Fasting blood glucose levels remained unchanged between groups, and after glucose gavage, were slightly (25.7 ± 19.3%) but not significantly reduced in RANTES-treated mice. Glucose stimulated ~3-fold circulating active GLP-1, and this was completely abolished by RANTES treatment (Fig. 6A). The glucose-dependent increase in plasma GLP-2, cosecreted with GLP-1, was as well blunted by RANTES administration (Fig. 6B), whereas GIP levels were slightly but not significantly reduced by RANTES (Fig. 6C). In contrast to control mice, RANTES-treated mice also failed to sufficiently increase insulin levels after the glucose bolus (Fig. 6D).

**DISCUSSION**

Diminished GLP-1 secretion is observed in diabetic subjects, and incretin-based therapies were shown to improve metabolic control by restoring normal glycemia in patients with type 2 diabetes (13, 14). The causes of the impaired GLP-1 secretion in response to food intake are poorly understood. Cytokines like leptin (2) and more recently IL-6 were shown to influence GLP-1 secretion (17). The chemokine RANTES, which is also secreted from adipocytes like leptin, was shown to be elevated in diabetic subjects, but no link between RANTES and GLP-1 has been described so far.

Our in vitro and in vivo studies in mice identified a role of RANTES in reducing GLP-1 secretion. Circulating concentrations of RANTES in healthy humans range between 1 and 5 nM and increase to 20–30 nM in morbidly obese humans (49). NCI-H716 cells were thus treated with a dose of RANTES that was slightly higher than that found in normal healthy individuals, and this concentration already caused a dramatic reduction in glucose-stimulated GLP-1 output. We also demonstrated the presence of the RANTES receptor CCR1 in the enteroendocrine L-cell model NCI-H716 cells, and in mouse intestinal epithelium. RANTES secretion into the intestinal lumen has been shown (1), suggesting that RANTES can bind to its receptors (mainly CCR1) on the apical membrane. The specificity of RANTES effects on GLP-1 secretion from NCI-H716 cells was demonstrated by the use of Met-RANTES, which antagonized the RANTES effects.

cAMP is an important second messenger mediating downstream signaling following nutrient stimulation of enteroendocrine cells, including GLP-1-secreting L-cells. Elevation of cAMP has been repeatedly demonstrated to increase GLP-1 secretion in several models of intestinal enteroendocrine cells (5, 18, 39, 42). Furthermore, cAMP via CREB (cAMP response element-binding) can increase *proglucagon* gene expression in enteroendocrine cells (15). CCR1 is a Gαi-coupled receptor and was shown in primary mouse astrocytes to reduce intracellular cAMP levels and to inhibit PKA (51). PKA is a direct target of cAMP and has been proposed to mediate the stimulatory effect of cAMP on gut hormone secretion following activation of Gs-coupled receptors (37). We here show that...
RANTES reduced basal as well as glucose-stimulated changes in cAMP concentrations in the NCI-H716 cells. Furthermore, PKA activity was also reduced in mouse intestinal tissue treated with RANTES, which fits with the Gi-coupled nature of the CCR1 receptor. Thus we propose that elevated RANTES levels as seen in diabetes cause a reduction in intracellular cAMP levels and reduced PKA activity via the CCR1 receptor leading to impaired GLP-1 secretion from the L-cell into circulation.

Previous studies in mice have shown that glucose-dependent GLP-1 secretion is mediated by the rheogenic sodium-coupled glucose transporter (SGLT1), and phlorizin, as the most potent SGLT1 inhibitor, can completely block hormone secretion (20, 29). To assess SGLT1 function in the RANTES-dependent regulation of GLP-1 output, we used α-MGD, which is not transported by other glucose transporters such as GLUT2, which was described to be recruited from intracellular stores into the apical membrane at high luminal glucose loads (23). Our data collected in mouse intestine thus show that RANTES can dose-dependently reduce SGLT1-mediated α-MGD transport in vitro, and if RANTES reduces SGLT1 transport activity as our data suggest, a reduced glucose uptake into enteroendocrine cells would then lead to a reduced GLP-1 output.

In addition to GLP-1, plasma GLP-2 levels after glucose gavage also decreased in mice treated with RANTES. GLP-2 is cosecreted with GLP-1 from L-cells and acts as an intestinal growth factor and inhibits apoptosis (16). GLP-2 was also shown to increase rapidly intestinal hexose absorption via GLUT2 and SGLT1 (4, 7). These findings suggest the existence of a feedforward loop by which SGLT1-mediated glucose transport increases GLP-1 and GLP-2 secretion, with GLP-2 increasing, in turn, sugar uptake capacity, while GLP-1 cosecreted with GLP-1 from L-cells and acts as an intestinal output.

RANTES AND IMPAIRED GLP-1 SECRETION

To assess SGLT1 function in the intestine, we assessed the impact of RANTES on SGLT1 activity: mouse jejunal brush-border membrane vesicles (BMMVs) from control and RANTES-treated mice were incubated in the presence of 2-deoxy-D-glucose (2-DG), with or without RANTES. In BMMVs from control mice, basal 2-DG uptake was mediated by SGLT1, consistent with previous reports (11). However, in RANTES-treated mice, 2-DG uptake was significantly decreased, indicating a reduced SGLT1 activity. These findings suggest that RANTES interferes with the incretin effect, which is essential for a proper glucose homeostasis.

Interestingly, RANTES is a substrate of DPP IV with the truncated form of RANTES (33–68) shown to have a reduced ability to stimulate CCR1 (27). Whether inhibition of DPP IV in diabetes treatment affects indeed RANTES-mediated inhibition of GLP-1 output in the intestine in response to a meal should be a subject of future research. In summary, our study is the first to demonstrate an effect of RANTES on the secretion of GLP-1 from enteroendocrine cells in vitro and from mouse intestine in vivo, which makes the RANTES CCR1 receptor a putative target for diabetes management.

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