The hypothalamic neuropeptide 26RFa acts as an incretin to regulate glucose homeostasis

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Abstract

26RFa is a hypothalamic neuropeptide that promotes food intake. 26RFa is up-regulated in obese animal models and its orexigenic activity is accentuated in rodent fed a high fat diet, suggesting that this neuropeptide might play a role in the development and maintenance of the obese status. As obesity is frequently associated with type 2 diabetes, we investigated whether 26RFa may be involved in the regulation of glucose homeostasis. In the present study, we show a moderate positive correlation between plasma 26RFa levels and plasma insulin in diabetic patients. Plasma 26RFa concentration also increases in response to an oral glucose tolerance test. In addition, we found that 26RFa and its receptor GPR103 are present in human pancreatic β cells as well as in the gut. In mice, 26RFa attenuates the hyperglycemia induced by a glucose load, potentiates insulin sensitivity and increases plasma insulin concentrations. Consistent with these data, 26RFa stimulates insulin production by MIN6 insulinoma cells. Finally, we show, using in vivo and in vitro approaches, that a glucose load induces a massive secretion of 26RFa by the small intestine. Altogether, the present data indicate that 26RFa acts as an incretin to regulate glucose homeostasis.

Key words: 26RFa, incretin, glucose homeostasis, insulin, β cell, pancreatic islet, gastrointestinal tract.
Introduction

26RFa and its N-extended form 43RFa (also referred to as QRFPs), are RFamide peptide discovered in the brain of various vertebrate species and identified as the cognate ligands of the human orphan G protein-coupled receptor GPR103 (1-6). Neuroanatomical observations revealed that 26RFa- and GPR103-expressing neurons are primarily localized in hypothalamic nuclei involved in the control of feeding behaviour (1, 2, 5, 7). Indeed, i.c.v. administration of 26RFa or 43RFa stimulates food intake (1, 5, 8, 9), and the neuropeptide exerts its orexigenic activity by modulating the NPY/POMC system in the arcuate nucleus (9). Chronic injection of 43RFa induces a significant increase in mice body weight and fat mass which is associated with a hyperphagic behaviour (8), and the orexigenic activities of 26RFa and 43RFa are more robust in rodents fed a high fat diet (8, 10). Consistent with these observations, expression of prepro26RFa mRNA is up-regulated in the hypothalamus of genetically obese \textit{ob/ob} and \textit{db/db} mice, and rodents submitted to a high fat diet (5, 10). Altogether, these observations support the notion that 26RFa could play a role in the development and maintenance of the obese status.

Obesity is frequently associated with type II diabetes which is characterized by chronic hyperglycemia induced by impaired insulin secretion and increased insulin resistance (11-13). Accumulating evidence support the peripheral role of neuropeptides controlling feeding behaviour such as neuropeptide Y (NPY), orexins, ghrelin, corticotropin-releasing factor (CRF) or apelin, in the regulation of glucose homeostasis (14-18), coining the new concept that hypothalamic neuropeptides may serve as a link between energy and glucose homeostasis, and identifying them therefore as potential therapeutic targets for the treatment of diabetes and obesity (19, 20).

In the present study, we have investigated the possible involvement of 26RFa in the regulation of glucose homeostasis. We notably show an increase in plasma 26RFa levels in
diabetic patients, and during an oral glucose tolerance test. We also found that 26RFa attenuates glucose-induced hyperglycemia, potentiates insulin sensitivity, increases plasma insulin concentrations, and that a glucose challenge induces a massive secretion of 26RFa by the gut.

**Research Design and Methods**

**Patients**

161 subjects were recruited in the Department of Endocrinology, Diabetes, and Metabolic Diseases of the University Hospital of Rouen. Patients were divided into four groups according to their weight and glucose tolerance. Obese patients (BMI>30), and type 1 and type 2 diabetic patients (DT1, DT2) were defined according to the ADA criteria. Clinical examination did not reveal any abnormalities. Healthy subjects (HS) underwent standard endocrine tests to exclude any metabolic abnormalities. All of the subjects included in the study gave a written informed consent according to our Ethical Committee instructions.

**Metabolic parameters in human**

Plasma 26RFa levels were measured using a specific radioimmunoassay (RIA) (21). Blood glucose was measured using a glucose oxidase activity test (LX20 Beckman Coulter, Villepinte, France). HbA1c was analysed by HPLC (G7 HPLC Analyzer, Tosoh, Lyon, France). Plasma hormone levels were measured using the following assays: plasma insulin (Elecys for automated insulin Assay COBAS 6000CE, Roche Diagnostics, Meylan, France), GIP (Elisa-assay, Millipore, St Charles, Missouri, USA), and GLP-1 (Elisa Epitope Diagnostics, San Diego, CA USA). Plasma ghrelin was assayed using a commercial kit (# EK-031-30, Phoenix, Belmont, Ca).
Homeostasis Model Assessment of Insulin Resistance (HOMA-IR index) was calculated as the ratio $\frac{\text{fasting glucose (mmol L}^{-1}) \times \text{fasting insulin (}\mu\text{U mL}^{-1})}{22.5}$ (22).

**Oral glucose tolerance test in human**

Nine healthy volunteers recruited for a previous study (23), one patient with gastroparesis, and another one with a total gastrectomy, underwent a 75-g oral glucose tolerance test (OGTT) after a 12-h fasting period. 26RFa, glucose, insulin, GIP, GLP-1 and ghrelin concentrations were measured in fasting blood samples obtained at 0, 30, 60, 120, 150 and 180 min after oral glucose loading.

**Immunohistochemical procedure**

Deparaffinized sections (15-µm thick) of human and mouse tissues were used for immunohistochemistry. All the tissue procurement protocols (for human) were approved by the relevant institutional committees (University of Rouen) and were undertaken under informed consent of each patient and all of the participants. Tissue sections were incubated for 1 h at room temperature with either rabbit polyclonal antibodies against 26RFa (24) diluted 1:400, or GPR103 (#NLS1922; Novus Biologicals, Littletown, CO) diluted 1:100, or EM66 (25) diluted 1:200, or mouse monoclonal antibodies against insulin (Sigma-Aldrich, Saint-Quentin Fallavier, France) diluted 1:1000. The sections were incubated with a streptavidin-biotin-peroxydase complex (Dako Corporation, Carpinteria, CA), and the enzymatic activity was revealed with diaminobenzidine. The slices were then counterstained with hematoxylin. Observations were made under a Nikon E 600 light microscope.

**Blood glucose and insulin measurements in mice**

For i.p. glucose tolerance test and glucose-stimulated insulin secretion assay, mice (male
C57BL/6, 20-23 g) were fasted for 16 h with free access to water and then injected i.p. with glucose (2 g/kg) and 26RFa (500 µg/kg) or PBS solution. For measurements of basal glycemia, mice were fed ad libidum and injected i.p. with 26RFa (500 µg/kg) or PBS solution. Blood plasma glucose concentrations were measured from tail vein samplings at various times using an AccuChek Performa glucometer (Roche Diagnostic, Saint-Egreve, France). Plasma insulin concentrations were determined using an ultrasensitive mouse insulin ELISA kit (Mercodia, Uppsala, Sweden). For insulin tolerance test, mice were fasted for 6 h and injected i.p. with 0.75 units/kg body weight of human insulin (Eli Lilly, Neuilly-sur-Seine, France) and 26RFa (500 µg/kg) or PBS. For i.v. glucose tolerance test, mice were catheterized in the tail vein under anesthesia, 24 h prior to the test. For oral and i.v. glucose tolerance test, mice were fasted for 16 h before the test with free access to water. Plasma samples were obtained from decapitation 30 or 120 min after a 2-g/kg oral glucose load or 3 or 30 min after a 1-g/kg i.v. glucose load and assayed for 26RFa.

**Insulin secretion by MIN6 cells**

Mouse insulinoma cells (MIN6), a kind gift from Dr J. Miyazaki (26), were grown in DMEM containing 25 mM glucose and supplemented with 15% heat-inactivated fetal bovine serum in a humidified atmosphere of 5% CO2, 95% air at 37° C. Before the experiments, the culture medium was removed and MIN6 cells (3x10^5 cells/well) were preincubated in a Krebs-Ringer bicarbonate buffer containing 0.2% BSA and 2.8 mM glucose (low glucose, LG) for 1 h at 37° C (period P1) to evaluate basal insulin secretion. The culture medium was then removed and replaced by the same culture medium (LG) or a culture medium with 16.7 mM of glucose (high glucose, HG) added or not with 26RFa and the GLP-1 analogue exenatide (10^-6 M each) for 1 h (period P2). Insulin secretion in each well was expressed as the ratio between secretion during the P2 period/secretion during the P1 period.
Quantitative PCR

Total RNA from mice various tissues and MIN6 cells was isolated as previously described (27). Relative expression of the 26RFa, GPR103, NPFF2 receptor (NPFF2), insulin receptor (INS-R), GLUT-2 and GLUT-4 genes was quantified by real-time PCR with appropriate primers (Table I). GAPDH or β-actin were used as internal controls for normalization. PCR was carried out using Gene Expression Master Mix 2X assay (Applied Biosystems, Courtaboeuf, France) in an ABI Prism 7900 HT Fast Real-time PCR System (Applied Biosystems). The purity of the PCR products was assessed by dissociation curves. The amount of target cDNA was calculated by the comparative threshold (Ct) method and expressed by means of the 2-ΔΔCt method.

Small Interfering RNA

Mouse Qrfpr Silencer Select Predesigned siRNA (SC-60730) (GPR103 siRNA(m)) and Silencer Negative Control siRNA (SC-37007) were purchased from Santa Cruz Biotechnology (Dallas, TX), and AMAXA cell line nucleofector KIT-V was purchased from LonZa (Basel, Switzerland). Mouse insulinoma cells (MIN6) were transfected with 10 µM control or GPR103 small interfering RNAs (siRNAs) using AMAXA cell line nucleofector KIT-V, according to the manufacturer's instructions. 48 h after transfection, cells were submitted to Q-PCR and insulin secretion experiments. Efficiency of transfection was assessed by RT-PCR.

Expression and secretion of 26RFa by intestine fragments

Three consecutive mice samples of duodenum, proximal jejunum and ileum were collected. For each intestinal segment, one sample was immediately frozen in liquid nitrogen for Q-PCR
experiments and two samples were mounted in Ussing chambers with an exchange surface of 0.07 cm², as previously described (28). Glucose-free DMEM (1 ml) was applied at the apical and serosal sides of the intestinal segments. At the apical side, DMEM supplemented with either glucose 2.8 mM or 16.7 mM was applied. At the serosal side, only DMEM with glucose 3 mM was applied. After 3h at 37°C, media from apical and serosal sides were collected and assayed for 26RFa.

Statistical analysis

Statistical analysis was performed with Statistica (5th version). A student t-test or ANOVA for repeated measures were used for comparisons between two groups. A post-hoc comparison using Tukey HSD was applied according to ANOVA results. Statistical significance was set up at p < 0.05.

Results

Fasting plasma 26RFa levels in obese and diabetic patients

The phenotypic characteristics of the 4 groups of subjects studied are reported in figure 1A. Circulating levels of 26RFa in fasting condition was significantly enhanced in obese patients (466 ± 37 pg/ml) and DT2 subjects (488 ± 48 pg/ml) vs HS (338 ± 42 pg/ml; p<0.05) (Fig. 1B). DT1 patients showed plasma 26RFa levels (494 ± 68 pg/ml) similar to those of DT2 subjects (Fig. 1B). No correlation was found between plasma 26RFa levels and age, BMI, waist circumference and fasting blood glucose. Conversely, a moderate positive correlation was found between circulating 26RFa and fasting plasma insulin (r=0.37, p=0.0015) (Fig. 1C), and the insulin resistance marker HOMA-IR (r=0.54, p<0.0001) (Fig. 1D).

Oral glucose tolerance test in human
An oral glucose tolerance test (OGTT) with 75 g glucose was performed in 9 healthy volunteers. Blood glucose and insulin as well as GLP-1 and GIP showed a rapid elevation 30 min after the glucose load (Fig. 2A, B). By contrast, plasma 26RFa levels were stable during the first 90 min of the test but increased significantly 120 min after the glucose load (p<0.01, Fig. 2A, B). The amplitude of the rises was much higher for GLP-1 (423 ± 57 %) and GIP (887 ± 229 %) than for 26RFa (182 ± 23 %) (Fig. 2B). Ghrelin showed a plasma profile totally different from that of 26RFa with a rapid decline at 30 min and then levels remained stable until the end of the test (Fig. 2B). In the patient with gastroparesis, the 26RFa peak was delayed as compared to the HS group (210 min vs 120 min) (Fig. 2C). Conversely, in the gastrectomized patient, the 26RFa rise occurred earlier than in the healthy volunteers (90 min vs 120 min) (Fig. 2D).

Localization of 26RFa and GPR103 in the human pancreas and gastrointestinal tract

Treatment of human pancreas sections with the 26RFa and GPR103 antibodies revealed that the neuropeptide and its receptor were specifically localized in the endocrine islets and were present within the same islets (Fig. 3A, B). Treatment of consecutive sections with the 26RFa or GPR103 antibodies, and the insulin antibodies revealed that the histological distribution of 26RFa- and GPR103-like immunoreactivity (LI) was similar to that of insulin (Fig. 3C-F).

26RFa-LI was also investigated in the human gut. In the antral part of the stomach, numerous epithelial cells of the gastric glands were labeled with the 26RFa antibodies (Fig. 3G). In the duodenum, 26RFa-LI was detected in most of the enterocytes and goblet cells (Fig. 3H). In the ileum and the colon, the distribution of 26RFa-LI was very similar to that observed in the duodenum although the number of 26RFa-labeled cells was much lower than in the duodenum (Fig. 3I, J).
Effect of 26RFa on glucose homeostasis in mice

An IPGTT, performed in mice, revealed that 26RFa significantly attenuated (p<0.01 and p<0.001) the hyperglycemia induced by an i.p. glucose challenge all throughout the duration of the test (Fig. 4A). Concurrently, during an insulin tolerance test, 26RFa significantly potentiated (p<0.05 and p<0.01) insulin-induced hypoglycemia between 30 min and 60 min after the insulin challenge (Fig. 4B). By contrast, 26RFa did not affect basal plasma glucose levels during the 90-min period of the test (Fig. 4C). Finally, an acute glucose-stimulated insulin secretion test revealed that i.p. injection of 26RFa significantly stimulated (p<0.05 and p<0.001) glucose-induced insulin production (Fig. 4D).

Effect of 26RFa on insulin secretion by MIN6 cells

We, then, searched for an eventual direct effect of 26RFa on β cell insulin secretion. For this, we used the mouse insulinoma cell line, MIN6. Q-PCR experiments indicated that MIN6 cells expressed both 26RFa and GPR103 mRNA but not NPFF2, the other RFamide receptor that 26RFa can bind to, whatever the glucose concentration applied in the culture medium (Fig. 5A). GPR103, 26RFa, INS-R and GLUT-2 were also expressed in cells transfected with non-silencing siRNA (Fig. 5B). Transfection of the cells with siRNA to GPR103 resulted in a total loss of GPR103 expression whereas the expression of the other genes was not altered (Fig. 5B). Both 26RFa and exenatide (10⁻⁶ M each) induced a highly significant increase of insulin release by siControl cells cultured in a LG medium. Incubation of the cells in a HG medium resulted also in a significant increase of insulin secretion (Fig. 5C). Addition of 26RFa in this latter condition of cell culture did not alter insulin production whereas exenatide strongly stimulated insulin secretion (Fig. 5C). No additive effects of 26RFa and exenatide was observed in both LG and HG condition (Fig. 5C). Invalidation of GPR103 expression resulted in a total loss of 26RFa-induced insulin secretion whereas the stimulatory effect of exenatide
in both LG and HG medium was maintained (Fig. 5D). Addition of 26RFa to exenatide did not significantly alter exenatide-induced insulin secretion (Fig. 5D).

Expression of GPR103 in mice insulin-sensitive tissues

Q-PCR realized in insulin target tissues revealed that GPR103 mRNA was expressed in the striated muscle, the liver and the adipose tissue, with higher levels in the liver (p<0.001; Fig. 5E). Expression of the insulin receptor (INS-R) and the glucose transporter GLUT-4 was also investigated in the same tissues. Expression of INS-R was significantly higher (p<0.001) in the liver as compared to the muscle and the adipose tissue (Fig. 5E). Expression of GLUT-4 was not detected in the liver and was much lower (p<0.001) in the adipose tissue as compared to the muscle (Fig. 5E).

Effect of glucose on 26RFa secretion by the mouse gastrointestinal tract

We finally investigated whether glucose could induce 26RFa release from the gut in mice. As illustrated in figure 6A, 26RFa mRNA was actually expressed in the duodenum, jejunum and ileum of the mouse, with higher levels in the duodenum (p<0.05; Fig. 6A). Concurrently, immunohistochemical experiments revealed the presence of intensely 26RFa-labeled cells in the mouse upper and lower intestine (Fig. 6B). In the duodenum, scattered positive cells were found in the epithelium (Fig. 6B1). In the ileum, the localization of clusters of 26RFa-positive cells suggested that these cells may correspond to paneth cells (Fig. 6B2). Treatment of consecutive jejunum sections with either the 26RFa antibodies or the EM66 (a fragment of the chromogranin, secretogranin II) antibodies revealed that the two antibodies label the same cells (Fig. 6B3 and 4), indicating that the 26RFa-containing cells probably correspond to neuroendocrine cells. Oral administration of glucose induced a highly significant increase...
(p<0.001) in plasma 26RFa levels 30 min after the glucose challenge that corresponded to the plasma glucose peak (Fig. 6C). By contrast, i.v. administration of glucose did not significantly alter plasma 26RFa levels all along the test (Fig. 6D). We also used a model of Ussing chambers in which we exposed the luminal (apical) side of intestine fragments to LG or HG concentrations, and we measured 26RFa levels in both the apical and basal sides of the intestine fragments. Duodenal fragments exposed to HG released massive amounts (p<0.01) of 26RFa in the basal chamber as compared to duodenum slices exposed to LG (Fig. 6E). In contrast, we did not observe any alteration of 26RFa flows in jejunum and ileum fragments treated with LG vs HG (Fig. 6E).

**Discussion**

Incretins are peptides released by the gut after ingestion of glucose or nutrients that act directly on pancreatic β cells to stimulate insulin secretion and lower plasma glucose. Until now, two incretins had been identified, i.e. glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) (29). Here, we provide evidence that 26RFa, a neuropeptide of 26 amino acids, initially discovered as an orexigenic molecule in the hypothalamus (1), is produced in abundance in the gut and acts as an incretin hormone.

We first investigated plasma 26RFa levels in obese patients with or without type 2 diabetes. The two groups show higher concentrations of circulating 26RFa as compared to healthy subjects. This finding is consistent with previous data obtained in obese animal models showing an up-regulation of the 26RFa system in genetically obese mice and rats fed a high fat diet (5, 8, 10), and suggests that, in human as in rodents, up-regulation of the 26RFa system may play a role in the development and maintenance of the obese status. However, high plasma 26RFa levels were also detected in patients with type 1 diabetes that were not obese. Correlation analysis revealed a moderate, but significant, positive correlation between
plasma 26RFα and plasma insulin, and the insulin resistance marker HOMA-IR. Altogether, these observations suggested a possible link between the 26RFα system and glucose homeostasis. We thus examined the evolution of plasma 26RFα levels during an OGTT in healthy subjects. Our data reveal a significant increase in plasma 26RFα during the test, indicating that an oral glucose load influences the concentrations of circulating 26RFα.

Comparison of the plasma 26RFα profile with those of GIP and GLP-1 during the OGTT reveals that the 26RFα response to glucose challenge is delayed as compared to those of the two incretins, and that the amplitude of the 26RFα peak is much lower than those of GIP and GLP-1. This suggests that the mechanism of action of 26RFα to regulate glucose homeostasis is different from those of GIP and GLP-1 in human.

Immunohistochemical examination of human pancreatic slices indicates that 26RFα and its receptor are present in endocrine islets but virtually absent in the exocrine pancreas. Our data also show that 26RFα and GPR103 are expressed by the insulin-producing β cells, suggesting an effect of the neuropeptide on β cell activity. Consistent with this hypothesis, a recent study reports that 26RFα and GPR103 are expressed in the pancreatic islets, and that 26RFα and its N-extended form, 43RFα, prevent β cell death and apoptosis (30). The present study shows that 26RFα and its receptor are present in the same pancreatic islets, indicating that 26RFα may regulate β cell activity via an autocrine mechanism.

26RFα is a neuropeptide initially known to be produced by hypothalamic neurons (1, 4, 5) that cannot be responsible for the robust concentrations of the peptide detected in the blood (present data, 22). The observation that oral glucose load results in an increase of plasma 26RFα, as observed for incretins, suggested that the gastrointestinal tract may produce 26RFα. Indeed, our immunohistochemical investigations reveal that 26RFα is produced in abundance in the stomach and small intestine and in lower amounts in the colon, indicating that the gut is probably an important source of circulating 26RFα.
We thus carried out several experiments in mice which revealed first that i.p. administration of 26RFa during a glucose tolerance test significantly attenuates glucose-induced hyperglycemia. In contrast, the neuropeptide does not affect glucose basal levels, suggesting that 26RFa exerts rather an anti-hyperglycemic effect than a hypoglycemic effect.

Concurrently, we show that 26RFa enhances insulin sensitivity during an insulin tolerance test, and increases insulin production during an acute glucose-stimulated insulin secretion test, indicating that the anti-hyperglycemic action of the neuropeptide is probably the result of both increased insulin sensitivity in target tissues and stimulation of insulin production. In agreement with this latter hypothesis, we found that 26RFa stimulates insulin secretion by the MIN6 mouse insulinoma cell line which expresses GPR103, and that invalidation of GPR103 totally abolishes 26RFa-induced insulin release. In addition, we show that NPFF2, the other RFamide receptor that 26RFa and 43RFa can bind to (6), is not expressed by MIN6 cells.

Altogether, these findings indicate that 26RFa is able to stimulate insulin release by insulin-secreting cells via the activation of GPR103. Consistent with this finding, it has been recently reported that 43RFa, the N-extended form of 26RFa, stimulates insulin secretion by human pancreatic islets and INS-1E β cells, and that this effect is exerted via GPR103 (30). However, in the same study, the authors report that 26RFa inhibits insulin secretion via a distinct signaling pathway than GPR103, an observation previously made in rat perfused pancreas (31), and suggest that NPFF2 may mediate the insulinostatic activity of 26RFa. Here, we show that MIN6 cells do not express NPFF2 that might explain the discrepancy between our data and those found in INS-1 cells. However, at present, it is not known whether INS-1 cells or human pancreatic islets express NPFF2 and the effects of activation of NPFF2 on insulin secretion remain unknown as well.

The increased insulin sensitivity induced by 26RFa led us to investigate the expression of the 26RFa receptor in insulin target tissues. We found that GPR103 is co-expressed with the
insulin receptor and the glucose transporter, GLUT-4, in the muscle, liver and adipose tissue, with a higher expression in the liver, suggesting that 26RFa may play a role in glucose uptake, a hypothesis that awaits further study. Interestingly, in a previous study, GPR103 has also been detected in epididymal fat pads and shown to be elevated by 16-fold in high fat mice (32). In addition, these authors showed, using 3T3-L1 adipocyte cells, that 26RFa elicits a dose-dependent increase in fatty acid uptake and increases the expression of genes involved in lipid uptake as well as triglyceride accumulation (32).

One main feature of incretins is to be released by the gut under an oral glucose challenge (33). We thus investigated the possibility that 26RFa may be released by the gut after glucose ingestion in mice. We confirmed the presence of 26RFa in the gut, with a higher expression of the neuropeptide in the duodenum as previously observed in human (present study). We found that an oral glucose load results in a massive increase in plasma 26RFa levels 30 min after glucose ingestion and that, conversely, i.v. administration of glucose does not affect plasma 26RFa. Consistent with this finding, we show that application of high glucose concentrations at the apical side of duodenal fragments induces an important release of 26RFa at the serosal side of the duodenal slices, indicating that glucose ingestion induces an important secretion of 26RFa from the duodenum to the blood. Supporting this view, we show that, in a human patient with delayed gastric emptying, the rise in plasma 26RFa levels during an OGTT is delayed by 90 min as compared to healthy subjects. Conversely, in a patient who underwent a total gastrectomy, the 26RFa peak occurs earlier at 90 min.

The present study provides evidence that 26RFa meets the criteria of an incretin hormone. However, 26RFa also shows differences with GIP and GLP-1. For instance, during an OGTT in human, the 26RFa response to the oral glucose load is delayed as compared to the GIP and GLP-1 peaks that overlap with those of glucose and insulin. At present, the physiological relevance of this delayed 26RFa response in human remains unclear. One hypothesis could be
that the aim of the delayed 26RFa increase is to sustain the GIP and GLP-1 insulinotropic action that, however, deserves further investigation.

In conclusion, we report for the first time that obesity and diabetes in human are associated with elevated plasma levels of the orexigenic neuropeptide 26RFa. We also show that 26RFa produced by the gut is released in the blood after a glucose challenge and reduces glucose-induced hyperglycemia via, at least in part, a direct stimulatory effect on insulin secretion by pancreatic β cells. Taken together, these findings strongly suggest that 26RFa is a novel incretin that regulates glucose homeostasis.

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Conflict of interest statement. The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Author Contributions. G.P., H.L., Y.A. and N.C. contributed to the study design and interpretation and wrote the manuscript. L.J., A.A., M.C., and J.L. contributed to the in vivo experiments on mice. L.J., M.O., M.M., C.B. and J. KC. Contributed to the in vitro experiments on cell lines. M.P. and F.G. contributed to the immunohistochemical
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helped to the analysis of the data, and with the discussion. N.C. is the guarantor of this work
and, as such, had full access to all the data in the study and takes responsibility for the
integrity of the data and the accuracy of the data analysis.

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Figure Legends

Figure 1. Evolution of plasma 26RFa concentrations in obese and diabetic patients. (A) Phenotypes of the patients included in the study. (B) Plasma 26RFa levels were measured, using a specific radioimmunossay for human 26RFa, in the four groups of patients after an overnight fasting. Obese patients without or with type 2 diabetes show a significant increase in plasma 26RFa concentrations (p<0.05) as compared to HS. Type 1 diabetic patients also show an increase in circulating 26RFa that does not reach the level of significativity (p=0.057). Data represent means ± SEM of 5 independent experiments. *, p<0.05. (C and D) Correlation analysis revealed that plasma 26RFa is positively correlated with fasting insulin (r=0.37, p=0.0015) (C) and the insulin resistance marker HOMA-IR (r=0.54, p<0.0001) (D).

Figure 2. Plasma 26RFa profile during an oral glucose tolerance test in human. (A) Plasma glucose, insulin and 26RFa levels were measured in 9 healthy volunteers during 180 min after a 75-g glucose load, with samplings every 30 min. As expected, a peak of plasma glucose and insulin occurs 30 min after the glucose load. In contrast, plasma 26RFa levels remain stable during the first 90 min of the test, but increase dramatically at 120 min and then decrease slowly until the end of the experiment. **, p<0.01. (B) Comparison of the plasma 26RFa profile during the OGTT with those of the two incretins glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), and with that of the other orexigenic neuropeptide ghrelin. A quick rise of GLP-1 and GIP is observed 30 min after the glucose load, similar to those of glucose and insulin, in contrast to 26RFa that peaks only at 120 min. Ghrelin levels decreased at 30 min and then remained stable until the end of the test. (C) Plasma glucose, insulin and 26RFa levels were measured every 30 min during 300 min in a patient with gastroparesis. Plasma glucose rises at 30 min whereas the insulin peak occurs 90
min after the glucose load. Plasma 26RFa levels increase only 210 min after the glucose challenge. (D) Plasma glucose, insulin and 26RFa levels were measured during 180 min, with samplings every 30 min, in a subject who underwent a total gastrectomy. Plasma glucose and insulin levels increase at 60 min after the glucose load. The increase in plasma 26RFa occurs 90 min after the glucose challenge. Data represent the values of a single patient for (C) and (D).

Figure 3. Immunohistochemical distribution of 26RFa and its receptor, GPR103, in the human pancreas and gastrointestinal tract. (A-B) Photomicrographs of pancreas sections showing that 26RFa and its receptor are specifically localized in the endocrine islets and are present within the same islets. (C-F) Photomicrographs of pancreas consecutive sections treated with 26RFa or GPR103, and insulin antibodies revealing that the distribution of 26RFa and its receptor are similar to that of insulin. (G) Photomicrographs of the antral part of the stomach showing that numerous epithelial cells of the gastric glands are labeled with the 26RFa antibodies. (H) Photomicrographs of the duodenum showing that numerous enterocytes and goblet cells of the villosities are labeled with the 26RFa antibodies. (I and J) Photomicrographs of ileum (I) and colon (J) slices showing that the distribution of 26RFa is very similar to that observed in the duodenum with the exception that the number of immunoreactive cells is much lower than in the duodenum (arrows). Scale bars: (A-F and H), 50 µm; (G), 200 µm; (I, J), 25 µm.

Figure 4. Effects of 26RFa on plasma glucose and insulin levels in mice. (A) Effect of intraperitoneal (i.p.) administration of 26RFa (500 µg/kg) during a glucose tolerance test. 26RFa significantly attenuates the hyperglycemia induced by the i.p. injection of glucose (2 g/kg) throughout the duration of the test. (B) Effect of i.p. administration of 26RFa (500
µg/kg) during an insulin tolerance test. 26RFa significantly potentiates insulin-induced hypoglycemia between 30 and 60 min after the i.p. insulin load (0.75 Units/kg). (C) Effect of i.p. administration of 26RFa (500 µg/kg) on basal plasma glucose levels. 26RFa does not alter basal plasma glucose during the 90 min following its injection. (D) Effect of i.p. administration of 26RFa (500 µg/kg) during an acute glucose-stimulated insulin secretion test. 26RFa significantly stimulates glucose-induced insulin production 15 and 30 min following the glucose load (2 g/kg). Data represent means ± SEM of 3 independent experiments (n=7/experiment). *, p<0.05; **, p<0.01; ***, p<0.001.

**Figure 5.** Effect and expression of 26RFa on the mouse insulinoma cell line MIN6. (A, B) Expression of 26RFa, GPR103, NPFF2, INS-R and GLUT-2 mRNA was determined in native MIN6 cells (A), and in cells transfected with nonsilencing siRNA (siControl) or transfected with siRNA to GPR103 (siGPR103) (B) by quantitative polymerase chain reaction and adjusted to the signal intensity of β-actin. Both 26RFa and GPR103 are expressed in native MIN6 cells grown in DMEN + 25 mM of glucose whereas NPFF2 mRNA is not detected (A). Incubation of the cells in a Krebs-Ringer medium complemented with glucose 2.8 mM for insulin secretion experiments does not modify this expression profile (A). GPR103, 26RFa, INS-R and GLUT-2 are also expressed in cells transfected with non silencing siRNA (B). Transfection of the cells with siRNA to GPR103 results in a total loss of GPR103 expression whereas the expression of the other genes is not altered (B). (C, D) Secretion of insulin by siControl or siGPR103 MIN6 cells incubated with a low glucose (LG) or a high glucose (HG) medium in the presence or absence of 26RFa (10⁻⁶ M) or the GLP-1 analogue exenatide (E, 10⁻⁶ M). In LG conditions, both 26RFa and exenatide induce a highly significant increase of insulin release by siControl cells (C). In HG condition, 26RFa does not alter insulin release whereas exenatide strongly stimulates insulin secretion (C). No additive effects of 26RFa and
exenatide are observed in both LG and HG condition (C). Invalidation of GPR103 expression results in a total loss of 26RFa-induced insulin secretion whereas the positive effect of exenatide is not affected (D). Addition of 26RFa to exenatide did not significantly alter exenatide-induced insulin secretion in both LG and HG conditions (D). Insulin mean basal level/well was 88±2 µU/ml. (E) Expression of GPR103, INS-R and GLUT-4 mRNA was determined by quantitative polymerase chain reaction and adjusted to the signal intensity of GAPDH in the muscle, the liver and the adipose tissue. GPR103 mRNA is expressed in the three tissues with higher levels detected in the liver. Expression of INS-R is significantly higher in the liver as compared to the muscle and the adipose tissue. Expression of GLUT-4 is not detected in the liver and is much lower in the adipose tissue as compared to the muscle.

Data represent means ± SEM of 3 independent experiments (n=5 per experiment). **, p<0.01; ***, p<0.001; ns, non significant.

**Figure 6.** Expression and secretion of 26RFa by the gastrointestinal tract in mice. (A) Expression of 26RFa mRNA was determined by quantitative polymerase chain reaction and adjusted to the signal intensity of GAPDH in duodenal, jejunal and ileum segments. 26RFa mRNA is expressed in the three regions of the mouse gastrointestinal tract with higher levels in the duodenum (n=6). (B) Immunohistochemical distribution of 26RFa in the mouse intestine. Intensely immunoreactive cells are found in the epithelium of the duodenum (1, arrows) and the ileum (2). In the ileum, 26RFa-positive cells are mostly found at the basis of the villosities and might thus correspond to Paneth cells (2, arrows). Treatment of consecutive jejunal sections with either the 26RFa (3) or the EM66 antibodies (4), which is a marker of neuroendocrine cells, indicates that the same cells are labeled by the two antibodies (3, 4, arrows). (C, D) Evolution of plasma 26RFa levels during an oral (C) and an intravenous (iv) (D) glucose tolerance test. A highly significant increase in plasma 26RFa concentration is
observed 30 min after the oral administration of glucose that corresponds to the plasma glucose peak (C). Conversely, no significant alteration of plasma 26RFa levels is detected after iv administration of glucose (D) (n=10 for each time point). (E) Secretion of 26RFa from duodenal, jejunal and ileal segments was evaluated in low glucose (LG, 2.8 mM) or high glucose (HG, 16.7 mM) conditions using a Ussing chambers model (n=8). Duodenal fragments exposed to HG concentrations at their apical pole release massive amounts of 26RFa in the basal chamber as compared to duodenal slices exposed to LG concentrations. In contrast, the 26RFa flow towards the basal compartment is not altered in the jejunum and the ileum in HG conditions vs LG conditions. Data represent means ± SEM of 3 independent experiments. *, p<0.05; **, p<0.01; ***, p<0.001; ns, non significant.
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HS, Healthy subjects; Obese, Obese patients; DT1, patients with type 1 diabetes; DT2, Obese subjects with type 2 diabetes

**Figure 1**
Figure 2

Plasma insulin (pmol/l) and glucose (mg/dl)

- Glucose
- Insulin
- 26RFa

Time (min)

0 30 60 90 120 150 180

**

Plasma 26RFa (pg/ml)

Time (min)

0 30 60 90 120 150 180

0

Plasma GLP-1 and GIP (% basal level)

Time (min)

0 30 60 90 120 150 180

0

Plasma 26RFa and ghrelin (% basal level)

Time (min)

0 30 60 90 120 150 180

0
Figure 4
Figure 5
Figure 6