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The neuropeptide 26RFa (QRFP) is a key regulator of glucose homeostasis and its activity is markedly altered in obese/hyperglycemic mice

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Running head: the neuropeptide 26RFa and chronic hyperglycemia

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ABSTRACT

Recent studies have shown that the hypothalamic neuropeptide 26RFa regulates glucose homeostasis by acting as an incretin, and increasing insulin sensitivity. In this study, we further characterized the role of the 26RFa/GPR103 peptidergic system in the global regulation of glucose homeostasis using a 26RFa receptor antagonist, and also assessed whether a dysfunction of the 26RFa/GPR103 system occurs in obese hyperglycemic mice. Firstly, we demonstrate that administration of the GPR103 antagonist reduces the global glucose-induced incretin effect and insulin sensitivity whereas, conversely, administration of exogenous 26RFa attenuates glucose-induced hyperglycemia. Using a mouse model of high-fat diet-induced obesity and hyperglycemia, we found a loss of the anti-hyperglcemic effect and insulinotropic activity of 26RFa, accompanied with a marked reduction of its insulin-sensitive effect. Interestingly, this resistance to 26RFa is associated with a down-regulation of the 26RFa receptor in the pancreatic islets, and insulin target tissues. Finally, we observed that the production and release kinetics of 26RFa after an oral glucose challenge is profoundly altered in the high-fat mice. Altogether, the present findings support the view that 26RFa is a key regulator of glucose homeostasis whose activity is markedly altered under obese/hyperglycemic conditions.

Key words: 26RFa; incretin; glucose homeostasis; diabetes; obesity

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INTRODUCTION

The neuropeptide 26RFa, also referred to as QRFP (for pyroglutamilated RFamide peptide), is the latest member of the RFamide peptide family discovered (5,9,16). 26RFa and its N-extended form 43RFa were identified as the cognate ligands of the human orphan G-protein-coupled receptor, GPR103 (6,9,16,30). This peptidergic system is abundantly expressed in the hypothalamus and is involved in the control of feeding behaviour (3,4,5,30). Indeed, 26RFa (or 43RFa) stimulates food intake (5,20,21,30), and the neuropeptide exerts its orexigenic activity via a modulation of the NPY/POMC network in the arcuate nucleus (20). These observations have been recently confirmed by data obtained from 26RFa-deficient mice showing that the knockout (KO) mice are lean and hypophagic, and that, under a high-fat (HF) diet, the 26RFa KO mice show a lower weight gain than the wild-type animals (22). In line with this, a more sustained orexigenic activity of 26RFa/43RFa has been reported in wild type mice submitted to a HF diet (21,27). Moreover, the expression of prepro26RFa mRNA is up-regulated in the hypothalamus of genetically obese *ob/ob* and *db/db* mice, and rodents submitted to a HF diet (27,30). Altogether, these findings support the notion that 26RFa could play a role in the development and maintenance of the obese status (7).

Accumulating evidence over the last decade supports the role of neuropeptides controlling feeding behavior such as neuropeptide Y (NPY), orexins, ghrelin, corticotropin-releasing factor (CRF) or apelin, in the peripheral regulation of glucose homeostasis (14,15,23,32,33). This has led to the new concept that hypothalamic neuropeptides may serve as a link between energy and glucose homeostasis, and therefore, they represent potential therapeutic targets for the treatment of diabetes and obesity (28,29). In this context, the potential role of 26RFa in the control of glucose homeostasis was investigated. It was found that 26RFa/43RFa and GPR103 are expressed in pancreatic islets, as well as in various insulin-secreting cell lines

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(11,26), and that 26RFa and 43RFa prevent cell death and apoptosis of β cell lines and isolated human pancreatic islets (11). It has been shown that 26RFa is abundantly expressed by the gut and that i.p. administration of the neuropeptide attenuates glucose-induced hyperglycemia by increasing plasma insulin via a direct insulinotropic effect on the pancreatic β cells, and also by increasing insulin sensitivity (26). Furthermore, it was reported that an oral glucose challenge induces a massive secretion of 26RFa by the gut into the blood, strongly suggesting that this neuropeptide plays an important role in the regulation of glycemia by acting as an incretin (26).

The incretin effect is known to be markedly reduced in diabetic patients (18), although most studies have reported that levels of GLP-1 and gastric inhibitory polypeptide (GIP) in response to oral glucose were similar in diabetic and non-diabetic subjects (8,13,18). This incretin defect is notably characterized by a decreased, albeit partially preserved, insulinotropic potency of GLP-1 and an almost complete loss of insulin secretion to GIP (18). In this context, the objective of the present study was twofold: (1) to further characterize the role of 26RFa/GPR103 peptidergic system in the global regulation of glucose homeostasis (via an oral glucose tolerance test) using a 26RFa receptor antagonist (namely 25e) (10); and (2) to determine whether a dysfunction of the 26RFa/GPR103 system occurs in obese/hyperglycemic conditions.

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MATERIAL AND METHODS

Animals. Male C57Bl/6 mice (Janvier laboratory, Le Genest-Saint-Isle, France), weighing 22–25 g, were housed at 5 animals per cage, with free access to standard diet (U.A.R., Villemoisson-sur-Orge, France) and tap water. They were kept in a ventilated room at a temperature of 22±1°C under a 12-h light/12-h dark cycle (light on between 7 h and 19 h). All the experiments were carried out between 09.00 h and 18.00 h in testing rooms adjacent to the animal rooms. Animal manipulations were performed according to the European Community Council Directive of November 24th 1986 (86:609: EEC), and were conducted by authorized investigators.

High-fat (HF) model. Mice were submitted to a dietary challenge at two months of age by using a standard chow (U8200G10R; energy density: 2 830 kcal/kg; Scientific Animal Food & Engineering (SAFE), Augy, France) or a HF diet (U8978; energy density: 5 283 kcal/kg; SAFE). Experiments were performed after three months of dietary challenge. Our Institutional Animal Use and Care Committee (CENOMAX, Agreement of the Ministry of Research n°54) approved the experimental procedures (Agreement n°: #11752).

Peptides and chemicals. Mouse 26RFa (ASGPLGTLAEELSSYSRRKGGFSFRF-NH_2) was synthezised (0.1 mmol scale) by the solid phase methodology on a Rink amide 4-methylbenzhydrylamine resin (Biochem, Meudon, France) by using a 433A Applied Biosystems peptide synthesizer and the standard fluorenylmethoxycarbonyl (Fmoc) procedure. The synthetic peptide was purified by reversed-phase HPLC on a 2.2 × 25-cm Vydac 218TP1022 C_{18} column (Alltech, Templemars, France) by using a linear gradient (10-50% over 50 min) of acetonitrile/trifluoroacetic acid (99.9 : 0.1 ; v/v) at a flow rate of 10 mL/min. Analytical HPLC, performed on a Vydac 218TP54 C_{18} column (0.46 × 25-cm), showed that the purity of the peptides was greater than 99%. The identity of the synthetic

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peptide was confirmed by mass spectrometry on a JEOL model AX-500 mass spectrometer. The GPR103 antagonist 25e was synthesized as previously described (10). Insulin and exenatide were purchased from Eli Lilly (Neuilly-sur-Seine, France).

Blood glucose and insulin measurements in mice. For i.p. glucose tolerance test and glucose-stimulated insulin secretion assay, mice were fasted for 16 h with free access to water and then injected i.p. with glucose (1.5 g/kg) and 26RFa (500 µg/kg) or PBS solution. For measurements of basal glycemia, mice were fed ad libitum 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 AlphaLISA insulin detection kit (ref AL204c, Perkin Elmer, Courtabeuf, France). 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 the GPR103 antagonist 25e (5 mg/kg), or PBS. For oral glucose tolerance test, mice were fasted for 16 h before the test with free access to water and injected i.p. with the GPR103 antagonist 25e (5 mg/kg) or PBS 30 min before a 2 g/kg oral glucose load. For the experiments with 26RFa, the peptide (500 µg/kg) was injected (i.p.) just before the glucose load. Blood plasma glucose and insulin concentrations were measured from tail vein samplings at various times and plasma samples for 26RFa concentration measurements were obtained from decapitation just before or 30 and 120 min after the glucose challenge, and then assayed for 26RFa.

Insulin secretion by the EndoC-βH1 cells. Human β cells (EndoC-βH1), a kind gift from Dr R. Scharfmann (INSERM U1016, Institut Cochin, Paris, France), were grown in DMEM containing 2.8 mM glucose and supplemented with 2% heat-inactivated fetal bovine serum, 2-mercaptoethanol (50 µM), nicotinamide (10 mM), selenite sodium (6.7 ng/ml), transferin (5.5 µg/ml), penicillin (100 mg/l) and streptomycin (100 mg/l), in a humidified atmosphere of 5%

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CO2, 95% air at 37° C. Concurrently, some wells containing the EndoC-βH1cells were incubated in the same medium containing a solution of palmitate (10 mM, P9767-5G, Sigma Aldrich) for four days. Before the experiments, the culture medium was removed and EndoC-βH1 cells ($4.3x10^5$ 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 16.5 mM of glucose (high glucose, HG) added or not with 26RFa (10^{-6} M) and the GLP-1 analogue exenatide (5 10^{-7} M) for 1 h (period P2). Insulin secreted in the culture medium was measured using a commercial ELISA kit (Mercodia Insulin ELISA, Uppsala, Sweden) and was expressed as the ratio between secretion during the P2 period/secretion during the P1 period.

Immunohistochemical procedure. Deparaffinized sections (15-µm thick) of duodenum and pancreas of control or HF mice were used for immunohistochemistry. Tissue sections were incubated for 1 h at room temperature with rabbit polyclonal antibodies against 26RFa (3) diluted 1:400 or GPR103 (#NLS1922; Novus Biologicals, Littletown, CO) diluted 1:100. The sections were incubated with a streptavidin-biotin-peroxydase complex (Dako Corporation, Carpinteria, CA), and the enzymatic activity was revealed with diaminobenzidine. Some slices were then counterstained with hematoxylin. Observations were made under a Nikon E 600 light microscope.

Quantitative PCR. Total RNA from the skeletal muscle, adipose tissue, liver and duodenum of HF and control mice, and from EndoC-βH1 cells was isolated as previously described (25). Relative expression of the 26RFa, GPR103, GLP-1 receptor, GLUT-1 and GLUT-2 genes was quantified by real-time PCR with appropriate primers (Table 1). GAPDH or cyclophilin A 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

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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.

26RFa radioimmunoassay. Quantification of 26RFa in plasma and tissue samples was carried out using a specific radioimmunoassay (RIA) set up in the laboratory that has been previously described in detail (3). For the RIA procedure, each plasma sample was diluted (1:1) in a solution of water/TFA (99.9:0.1; v/v). The duodenum fragments were immersed for 15 min in boiling 0.5 M acetic acid, homogenized at 4°C using a glass potter homogenizer and centrifuged (6,000 *g*, 4°C, 30 min). Diluted plasma and supernatant fluid of tissue extracts were pumped at a flow rate of 1.5 ml/min through one Sep-Pak C_{18} cartridge. Bound material was eluted with acetonitrile/water/TFA (50:49.9:0.1; v/v/v) and acetonitrile was evaporated under reduced pressure. Finally, the dried extracts were resuspended in PBS 0.1 M and assayed for 26RFa.

Statistical analysis. Statistical analysis was performed with Statistica (5^{th} 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 $p < 0.05$.

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RESULTS

Consequence of blocking 26RFa action on glucose and insulin tolerance in control mice. Administration of the 26RFa receptor antagonist, 25e, 30 min before an oral glucose tolerance test resulted in a significant increase of the glycemia between 30 and 60 min after the oral glucose challenge (Fig. 1*A*), as assessed by the calculation of the areas under the curve (Fig. 1*A insert*). Plasma insulin concentrations tended to be slightly higher in animals that received the GPR103 antagonist, but without reaching the statistical significance (Fig. 1*B*).

Administration of 25e during an insulin tolerance test significantly reduced insulin sensitivity from 15 to 90 min after the i.p. injection of insulin ($p<0.05$-$p<0.001$) (Fig. 1*C*) as assessed with a statistical difference of the areas under the curve of $p=0.07$ between 25e-treated mice and animals that did not receive the GPR103 antagonist (Fig. 1*C insert*).

Effect of a 26RFa injection on blood glucose and plasma insulin levels following an oral glucose challenge.

We completed the experiments using the 26RFa receptor antagonist, by performing oral glucose tolerance tests with administration of exogenous 26RFa. In these experiments, the hyperglycemic effect induced by the glucose challenge was significantly reduced by the i.p. injection of 26RFa (Fig. 1*D*), but without any significant alteration of plasma insulin levels (Fig. 1*E*).

Effect of 26RFa on basal glycemia, glucose-induced hyperglycemia and insulin tolerance. The high-fat (HF) diet challenge induced a significant ($p<0.001$) weight gain right from the first month of treatment (Fig. 2*A*). After three months, the HF mice exhibited a weight of 43.00 ± 0.65 g, whereas mice fed a standard chow weighted 27.00 ± 0.85 g (Fig. 2*A*). Similarly, plasma glucose was significantly increased ($p<0.01$) after one month of HF diet (Fig. 2*B*). After 3 months, the HF mice exhibited a fasting glycemia of 2.11 ± 0.06 g/l,

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whereas that of the mice fed a standard chow at the same time was 1.16 ± 0.05 g/l ($p<0.001$; Fig. 2*B*).

The impact of 26RFa was investigated on basal glycemia in mice fed a standard chow and in HF mice. As illustrated in figure 2*C*, *D*, i.p. administration of 26RFa did not affect significantly basal plasma glucose levels during the 90-min period of the test in the two diet conditions. By contrast, an IPGTT performed in the two groups of mice revealed that 26RFa significantly attenuated ($p<0.05$) the hyperglycemia induced by an i.p. glucose challenge in mice fed a standard chow during the first 30 min of test, whereas this anti-hyperglycemic effect of the neuropeptide was totally abolished in HF mice (Fig. 2*E*, *F*). I.p. injection of 26RFa also significantly potentiated ($p<0.05$) insulin-induced hypoglycemia during the 90 min following the insulin challenge in mice fed a standard chow as illustrated by the quantification of the areas under the curves (Fig. 3*A*, *C*). By contrast, the beneficial effect of 26RFa on insulin sensitivity was strongly attenuated in HF mice (Fig. 3*B*, *C*). Quantification of GPR103 mRNA in insulin target tissues revealed a significant decrease ($p<0.05$) in the expression of the 26RFa receptor in the muscle and the adipose tissue of HF mice (Fig. 3*D*). No expression of GPR103 was detected in the liver of the two groups of animals (Fig. 3*D*).

Consequence of blocking 26RFa action on glucose and insulin tolerance in HF mice. Administration of the GPR103 antagonist 25e during an oral glucose tolerance test in HF mice had no significant effect on plasma glucose and insulin levels (Fig. 4*A, B*) in contrast to what was observed in mice fed a standard chow (Fig. 1*A, B*). i.p. injection of 25e had no significant impact on insulin sensitivity during an insulin tolerance test in HF mice (Fig. 4*C*) in contrast to the insulin-resistant effect of the GPR103 antagonist observed in mice fed a standard chow (Fig. 1*C*). In addition, administration of 26RFa had no effect on the hyperglycemia induced by the oral glucose load (Fig. 4*D*) nor on plasma insulin levels (Fig. 4*E*).

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Effect of 26RFa on insulin production. The effect of 26RFa on insulin production was investigated *in vivo* and *in vitro*. An acute glucose-stimulated insulin secretion test showed that injection of 26RFa significantly stimulated glucose-induced insulin production in mice fed a standard chow ($p<0.05$; Fig. 5*A*), whereas this insulinotropic effect of the neuropeptide was totally abolished in HF mice (Fig. 5*B*). Concurrently, the impact of 26RFa on insulin secretion by pancreatic β cells was examined using the human insulin-secreting cell line, EndoC-βH1, that were treated with palmitate to mimic conditions of lipotoxicity. As illustrated in figure 5*C*, addition of 26RFa in the culture medium significantly stimulated insulin secretion by untreated EndoC-βH1 cells incubated in LG ($p<0.05$) or HG ($p<0.01$) conditions. The GLP-1 analogue, exenatide, used as a positive control, also significantly stimulated insulin secretion ($p<0.01$) by the untreated EndoC-βH1 cells (Fig. 5*C*). Conversely, when the cells were treated for 96 h with palmitate, the insulinotropic effect of 26RFa was totally abolished whatever the glucose concentration in the culture medium (Fig. 5*D*). By contrast, exenatide still significantly stimulated insulin secretion by palmitate-treated cells ($p<0.01$, Fig. 5*D*). Comparison of insulin secretion between native and palmitate-treated EndoC-βH1 cells revealed that, in non-stimulated conditions, basal insulin release was not affected in palmitate-treated cells (Fig. 5*E*). By contrast, under stimulatory conditions (26RFa or exenatide), insulin secretion was dramatically altered in palmitate-treated cells as compared to native cells (Fig. 5*E*). Finally, expression of GLUT-1, GLUT-2, the GLP-1 receptor and GPR103 was evaluated in native and palmitate-treated EndoC-βH1 cells (Fig. 5*F*). GLUT-1 and the GLP-1 receptor mRNA levels were not altered by the lipotoxic treatment of the cells (Fig. 5*F*). By contrast, expression of GLUT-2 was significantly decreased ($p<0.05$) in palmitate-treated cells and that of GPR103 was also lowered ($p=0.06$; Fig. 5*F*). Consistent with this latter observation, immunohistochemical observation of pancreas of mice fed a

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267 standard chow and HF mice revealed a decrease in the intensity of the GPR103 labeling in the
268 pancreatic islets of HF mice (Fig. 5*G*, *H*).

269 *Effect of the high fat diet on gut 26RFa production.* We first investigated the expression
270 and distribution of 26RFa in the duodenum of HF mice and mice fed a standard chow using a
271 Q-PCR approach combined with immunohistochemistry (Fig. 6*A*, *B*). The expression of
272 26RFa was significantly lowered ($p<0.01$) in the duodenum of HF mice as compared to mice
273 fed a standard chow (Fig. 6*A*). In agreement with this finding, the immunohistochemistry
274 revealed an important decrease in the number of enterocytes immunostained with the 26RFa
275 antibody in the duodenum of HF mice (Fig. 6*B*).

276 The impact of a 2 g/kg oral glucose load on plasma glucose, insulin and 26RFa, and on
277 26RFa content in the duodenum was investigated in HF mice and mice fed a standard chow
278 (Fig. 6*C*-*G*). In mice fed a standard chow, the oral glucose challenge induced an important
279 increase of the glycemia at 30 min, followed by a decrease at 120 min (Fig. 6*C*). The HF mice
280 exhibited a similar glycemic profile although plasma glucose levels were significantly higher
281 all along the test ($p<0.05$-0.01; Fig. 6*C*). Measurement of plasma insulin during the OGTT
282 indicated that insulinemia was always higher in HF mice as compared to mice fed a standard
283 chow, as assessed by the point by point statistical analysis ($p<0.05$-0.001) and the
284 measurement of the area under the curve ($p<0.01$) (Fig. 6*D*). Plasma 26RFa profile in mice
285 fed a standard chow was very similar to that observed for glycemia and insulinemia, with an
286 increase of plasma 26RFa levels observed at 30 min followed by a decrease at 120 min (Fig.
287 6*E*). In HF mice, plasma 26RFa concentration was similar to that of mice fed a standard chow
288 during the first 30 min of the test, but the obese/hyperglycemic mice exhibited a dramatic rise
289 in plasma 26RFa levels at 120 min ($p<0.05$) that was not observed in mice fed a standard
290 chow (Fig. 6*E*), as underlined by the significant difference of the areas under the curves (Fig.
291 6*F*). Quantification of 26RFa content in the duodenum showed a significant decrease ($p<0.01$)

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of the neuropeptide production in HF mice under basal condition (at T0) (Fig. 6*G*). During the oral glucose tolerance test, 26RFa content in the duodenum exhibited a profile closely related to that of plasma 26RFa in the two groups of mice with, in particular, a highly significant rise of duodenal 26RFa production ($p<0.001$) 120 min after the oral glucose challenge in HF mice (Fig. 6*G*).

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DISCUSSION

In the first part of the present study, we took advantage of the first disclosed GPR103 antagonist (10) that can be used as an *in vivo* tool to decipher the role of 26RFa in glucose homeostasis. We have previously demonstrated that 26RFa could be considered as a novel incretin (26). This conclusion was based on experiments carried out by our team showing that 26RFa is secreted by the gut in response to an oral glucose load, and that the neuropeptide stimulates insulin secretion from pancreatic β cells, leading to an anti-hyperglycemic effect (26). However, without a specific antagonist molecule, it is difficult to isolate the proper role of 26RFa on the glycemic status from the well-known effects of the other incretins such as GLP-1 and GIP during an oral glucose challenge, which is recognized as the gold standard test to assess the effects of incretin hormones. Here, we further characterized the role of 26RFa in the global regulation of glucose homeostasis using a GPR103 antagonist with DMPK properties suitable for *in vivo* experiments, namely 25e (10). In our set of experiments, administration of 25e induced a robust hyperglycemic effect during an oral glucose load in mice fed a standard chow. We also found that administration of the 26RFa receptor antagonist significantly reduces insulin sensitivity, confirming that one mechanism of action of the 26RFa/GPR103 system to control glucose homeostasis is to increase insulin sensitivity in addition to its incretin activity (26). There was a tendency for 25e to increase plasma insulin levels during a glucose challenge. We hypothesize that this may reflect the occurrence of a compensatory mechanism to counteract the insulin-resistant effect of the GPR103 antagonist. However, this increase of insulin secretion is not sufficient to maintain normoglycemia, suggesting that blockade of 26RFa action also alter the insulin secreting capacity of the pancreatic islets. Conversely, we show that exogenous administration of 26RFa during an oral glucose load significantly reduces the hyperglycemia without any significant alteration of the

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plasma insulin levels. In this experiment, it is important to note that, in fact, we have analyzed the effects of both exogenous and endogenous 26RFa. Thus, it seems that exogenous administration of 26RFa could potentiate the antihyperglycemic effect already induced by endogenous 26RFa production. We may speculate that the decrease of the glycemia induced by exogenous 26RFa is rather linked to an alteration of the insulin sensitivity than insulin secretion since no modification of plasma insulin levels was observed. Together, these findings confirm our previous data (26) and support the view that the 26RFa/GPR103 system plays a crucial role to sustain normoglycemia in stimulatory conditions.

The second objective of the present study was to investigate whether an obese/chronic hyperglycemic status is associated with a dysfunction of the 26RFa/GPR103 system. For this purpose, we submitted mice to a HF diet for a period of three months. Measurement of weight and glycemia revealed that the HF mice were actually obese and intolerant to glucose. 26RFa did not affect basal plasma glucose levels neither in the HF mice nor in control animals. This observation is consistent with the incretin status of the neuropeptide previously reported (26). By contrast, we found that the anti-hyperglycemic effect of 26RFa observed during a glucose tolerance test in control mice was lost in HF mice. We previously showed that the anti-hyperglycemic effect of 26RFa was the result of both increased insulin sensitivity in target tissues and a direct stimulation of insulin production by the pancreatic β cells (26). Here, we show that during an insulin tolerance test, the beneficial effect of 26RFa on insulin sensitivity is significantly attenuated. This was probably due to the fact that the expression of GPR103 mRNA was down-regulated in muscle and adipose tissues of HF mice compared to controls.

In the *in vivo* experiments, we found that the stimulatory effect of 26RFa on insulin production was totally lost in the HF mice. We have previously shown that 26RFa exerts a direct insulinotropic effect on pancreatic β cells via activation of GPR103 (26). In this study, we used a human β pancreatic cell line that expresses GPR103 (i.e., EndoC-βH1cells) to

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348 further explore the direct effects of 26RFa on insulin secretion. We show that 26RFa robustly
349 stimulates insulin release by these cells. Treatment of the EndoC-βH1cells with palmitate, that
350 mimics conditions of lipotoxicity observed in diabetes, totally abolished the insulinotropic
351 effect of 26RFa. By contrast, the GLP-1 analogue, exenatide, was still able to stimulate
352 insulin release (albeit with a reduced potency) under the same lipotoxic conditions. This latter
353 observation is in agreement with previous studies showing a reduction of GLP-1-induced
354 insulin secretion under conditions of lipo and glucotoxicity induced by a HF diet (12).
355 Finally, we show that the expression of the 26RFa receptor is lowered in the EndoC-βH1cells
356 cultured in lipotoxic conditions as well as in the pancreatic islets of HF mice, suggesting that
357 this decreased expression of GPR103 in β-cells is responsible, at least in part, for the loss of
358 the insulinotropic effect of 26RFa in obese/hyperglycemic conditions. In accordance with
359 these findings, a loss of GIP insulinotropic effect has also been reported in type 2 diabetes
360 (13,19) and down-regulation of the GIP receptor has been proposed as one of the mechanisms
361 underlying the lack of GIP activity in type 2 diabetic patients (1, 17, 24). Similarly, a down-
362 regulation of the GLP-1 receptor has been described in pancreatic β cells under diabetic
363 conditions that may underlie the reduced incretin effect of GLP-1 in diabetes (17). However,
364 some studies suggest that the loss or altered incretin effect in type 2 diabetes is a consequence
365 of the diabetic state and not a primary event and that the incretin defect is mainly a
366 consequence of general β cell dysfunction (8, 13, 17, 18). Together, these observations
367 support the idea that the altered insulinotropic activity of the incretins in diabetes is probably
368 the result of a down-regulation of their receptor expression in β-cells associated with a
369 dysfunction of insulin synthesis and production.

370 We have previously found that the enterocytes of the duodenum are the main source of
371 peripheral 26RFa and that its release to the general circulation is increased after an oral
372 glucose challenge (26). Here, we show that, after an overnight fasting, the expression and

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production of 26RFa in the duodenum is significantly decreased in HF mice. This finding, together with the low expression of the 26RFa receptor in insulin target tissues in HF mice, suggests that the 26RFa/GPR103 peptidergic system is markedly altered under obese/chronic hyperglycemic condition. However, we concurrently show that, under stimulatory conditions (an oral glucose load), the duodenum of HF mice keeps its ability to produce and secrete large amounts of 26RFa in the general circulation and is even over-stimulated as compared to control mice. Indeed, in HF mice, plasma 26RFa levels still increase 2 h after the oral glucose load whereas, in mice fed a standard chow, they return to basal values as previously reported (26). Interestingly, this plasma 26RFa profile during the OGTT in HF animals follows that of duodenal 26RFa production suggesting that the duodenum is the main source of circulating 26RFa. In addition, these high plasma 26RFa levels 2 h after glucose ingestion might reflect the occurrence of a mechanism of action involving 26RFa production by the gut, aiming at decreasing glycemia. In this context, it would be interesting to investigate whether insulin, whose plasma levels also remain high 2 h after the oral glucose challenge in HF mice, could regulate 26RFa production by the gut as the hormone has already been reported to be able to regulate glucose intestinal absorption, in addition to its peripheral effects (31). It is noteworthy that this discrepancy in the plasma 26RFa profile during an oral glucose tolerance test between normal and chronic hyperglycemic conditions is not found for GLP-1 and GIP (2) and thus seems to be a specificity of 26RFa that deserves further investigation.

Finally, we show that administration of the 26RFa receptor antagonist 25e or 26RFa in obese/hyperglycemic mice has no effect on the glycemic response during an oral glucose tolerance test or an insulin tolerance test, nor an insulin secretion during an acute glucose-stimulation insulin secretion test. This observation is in agreement with the blunted 26RFa response we have observed in HF mice and can be linked to the decreased expression of GPR103 in insulin target tissues and in β pancreatic cells of the HF mice.

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In conclusion, we have confirmed, using a 26RFa receptor antagonist, that the neuropeptide 26RFa is a key regulator of glucose homeostasis by acting as an incretin and improving insulin tolerance. Our data also reveal a loss of the incretin and insulin-sensitive effect of 26RFa in obese/chronic hyperglycemic conditions that is associated with a down-regulation of its receptor. Together, these observations strongly suggest that dysfunction of the 26RFa/GPR103 system participate to the general reduced incretin effect and insulin intolerance observed in diabetes.

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DISCLOSURES

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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. and N.C. contributed to the study design and interpretation and wrote the manuscript. MA. LS., J.B., A.A., M.P., J.M. and M.M. contributed to the in vivo experiments on mice. J.B., MP. H.B., M.C. and P.D. contributed to the in vitro experiments on cell lines. S.C. and M.P. contributed to the immunohistochemical experiments. J.B., M.P., V.B. and H.B. contributed to the PCR experiments. A. B. and E. N. performed the insulin assays. J. L. and A. N. produced 26RFa and 25e. H.L. and Y. A. revised and approved the final version of the manuscript. 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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LEGENDS TO FIGURES

Figure 1: Consequence of blocking 26RFa action on glucose and insulin tolerance in control mice. (***A, B***) i.p. injection of the GPR103 antagonist 25e at a dose of 5 mg/kg significantly increases the blood glucose level during an oral glucose tolerance test (*A, insert*). During the same test, plasma insulin concentrations tend to be higher all along the test in animals that received the GPR103 antagonist (*B*), although statistical analysis does not reveal any significant difference between treated and non-treated animals (*B insert*). (***C***) Administration of 25e during an insulin tolerance test significantly reduces insulin sensitivity from 15 to 90 min after the i.p. injection of insulin. (***D, E***) i.p. administration of 26RFa (500 µg/kg) reduces significantly the hyperglycemia induced by a 2-g/kg oral glucose challenge (*D*), but without affecting plasma insulin levels (*E*). (***Inserts***) Calculation of the areas under the curves. Data represent means ± SEM of 3 independent experiments (n=10-15/group). *, $p<0.05$; **, $p<0.01$; *** $p<0.001$ *vs* non-treated animals.

Figure 2: Effect of 26RFa on basal glycemia and glucose-induced hyperglycemia. (***A, B***) A 3-month high fat diet induces a highly significant weight gain (*A*) and increase of glycemia (*B*) right from the first month of treatment as compared to mice fed a standard chow. (***C, D***) 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 neither in mice fed a standard chow (*C*) nor in HF mice (*D*). (***E, F***) Effect of 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 (1.5 g/kg) throughout the duration of the test in mice fed a standard chow (*E*). In HF mice, the anti-hyperglycemic effect of 26RFa during the glucose tolerance test is totally abolished (*F*). Calculation of the areas under the curves reveal

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no significant difference between the PBS and the 26RFa groups whatever the type of diet given (*inserts*). Data represent means ± SEM of 5 independent experiments (n=20/group). ns, non significant; *, p<0.05; **, p<0.01; ***, p<0.001 *vs* mice fed a standard chow.

Figure 3: Effect of 26RFa on insulin tolerance. (***A-C***) i.p. administration of 26RFa (500 µg/kg) during an insulin tolerance test significantly potentiates insulin-induced hypoglycemia during the 90 min following the insulin load (0.75 Units/kg) in mice fed a standard chow (*A*) as illustrated by the quantification of the areas under the curves (*C*) whereas, in HF mice, the beneficial effect of 26RFa on insulin sensitivity is strongly attenuated (*B, C*). Data represent means ± SEM of 5 independent experiments (n=20/group). **(*D*)** Expression of GPR103 mRNA was determined by quantitative polymerase chain reaction and adjusted to the signal intensity of GAPDH in the muscle, the adipose tissue and the liver. GPR103 mRNA levels are significantly lowered in the muscle and adipose tissue of HF mice as compared to those measured in mice fed a standard chow. No expression of GPR103 is detected in the liver of control or HF mice. Data represent means ± SEM of 3 independent experiments (n=6-9/group). *, p<0.05 *vs* mice fed a standard chow.

Figure 4: Consequence of blocking 26RFa action on glucose and insulin tolerance in HF mice. (*A, B*) Administration of the GPR103 antagonist 25e (5 mg/kg) during an oral glucose tolerance test in HF mice has no significant effect on plasma glucose (*A*) and insulin levels (*C*), as assessed by the calculation of the areas under the curves (*inserts*). **(*C*)** Similarly, i.p. injection of 25e does not alter insulin sensitivity during an insulin tolerance test in HF mice. **(*D, E*)** i.p. administration of 26RFa (500 µg/kg) has no significant effect on the hyperglycemia induced by an oral glucose challenge in HF mice (2 g/kg) (*D*), nor on plasma

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insulin levels (*E*). **(*Inserts*)** Calculation of the areas under the curves. Data represent means ± SEM of 3 independent experiments (n=10-12/group).

Figure 5: Effect of 26RFa on insulin production. (*A, B*) 26RFa (500 µg/kg) stimulates significantly glucose-induced insulin production during an acute glucose (1.5 g/kg)-stimulated insulin secretion test in mice fed a standard chow (*A*), whereas injection of the neuropeptide has no effect on plasma insulin in HF mice (*B*). Data represent means ± SEM of 5 independent experiments (n=20/group). **(*C, D*)** Effect of 26RFa (10^{-6} M) on insulin secretion by the human pancreatic β cell line EndoC-βH1non treated or treated by palmitate for 96 h. 26RFa significantly stimulates insulin secretion in untreated EndoC-βH1 cells incubated in a low glucose (LG) or a high glucose (HG) medium (*C*). Conversely, when the cells are treated with palmitate, the insulinotropic effect of 26RFa is abolished whatever the glucose concentration in the culture medium (*D*). Data represent means ± SEM of 4 independent experiments (n=10/condition). **(*E*)** Comparison of insulin secretion between native and palmitate-treated EndoC-βH1 cells. In non-stimulatory conditions, insulin release is not affected in palmitate-treated cells as compared to native cells. By contrast, under stimulatory conditions (26RFa or exenatide), the secreting response of the cells is significantly altered by the palmitate treatment. Data represent means ± SEM of 4 independent experiments (n=10/condition). **(*F*)** Expression of the glucose transporters GLUT-1 and GLUT-2, the GLP-1 receptor (GLP1R) and GPR103 mRNA was determined in native EndoC-βH1 cells and in palmitate-treated EndoC-βH1 cells by quantitative polymerase chain reaction and adjusted to the signal intensity of cyclophilin A. GLUT-1 and the GLP1R mRNA levels are not altered by the lipotoxic treatment of the cells whereas the expression of GLUT-2 and GPR103 is decreased in palmitate-treated cells. Data represent means ± SEM of 3 independent experiments (n=6-9/group). *, $p<0.05$; **, $p<0.01$. **(*G, H*)** Immunohistochemical labeling of

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pancreatic islets of control and HF mice with GPR103 antibodies. Representative photomicrographs of pancreatic islets showing a robust decrease in the intensity of the GPR103 labeling in the pancreatic islets of HF mice (*H*) as compared to the pancreatic islets of mice fed a standard chow (*G*). Scale bars: 50 µm.

Figure 6: Effect of the high fat diet on gut 26RFa production. (*A, B*) Expression and distribution of 26RFa in the duodenum of control and HF mice. Expression of 26RFa mRNA in the duodenum was determined by quantitative polymerase chain reaction and adjusted to the signal intensity of GAPDH, and its distribution was determined by immunohistochemistry. The expression of 26RFa is significantly lower in the duodenum of HF mice as compared to mice fed a standard chow (*A*). In agreement with this finding, the immunohistochemistry reveals an important decrease in the number of enterocytes immunostained with the 26RFa antibody in the duodenum of HF mice in comparison to duodenal slices of mice fed a standard chow (*B*). Scale bars: 50 µm. **(*C-G*)** Impact of a 2-g oral glucose load on plasma glucose, insulin and 26RFa, and on 26RFa content in the duodenum of HF mice and mice fed a standard chow. During an oral glucose challenge, the HF mice exhibit a similar glycemic profile to that of mice fed a standard show, although their plasma glucose levels are significantly higher all along the test (*C*). Measurement of plasma insulin during the same test reveals that insulinemia is always higher in HF mice as compared to mice fed a standard chow, as assessed by the point by point statistical analysis and the calculation of the areas under the curves (*D*). In HF mice, plasma 26RFa concentration is similar to that of mice fed a standard chow during the first 30 min of the test, but the obese/diabetic mice exhibit a dramatic rise in plasma 26RFa levels at 120 min that is not observed in mice fed a standard chow (*E*), as underlined by the significant difference of the areas under the curves (*F*). Quantification of 26RFa concentration in the duodenum reveals

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lower duodenal 26RFa content in HF mice under basal condition (*G*). During the oral glucose tolerance test, 26RFa content in the duodenum exhibits a profile closely related to that of plasma 26RFa in mice fed a standard chow as well as in HF mice with, in particular, a significant rise of duodenal 26RFa production 30 and 120 min after the oral glucose challenge in obese/diabetic animals (*G*). Data represent means ± SEM of 5 independent experiments (n=18-20/group). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ *vs* mice fed a standard chow. §, $p<0.05$; §§, $p<0.01$ *vs* T0 min.

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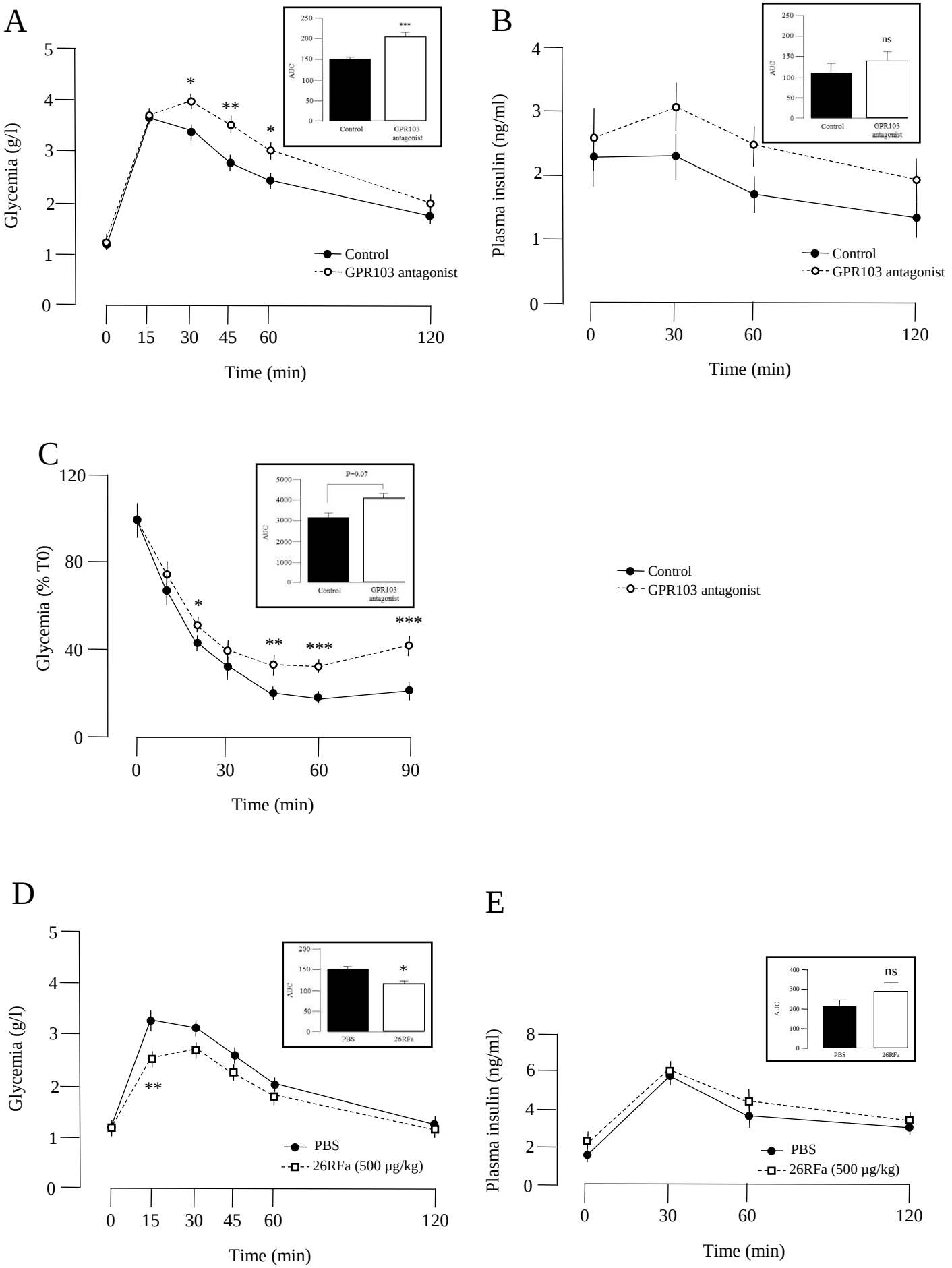


Figure 1

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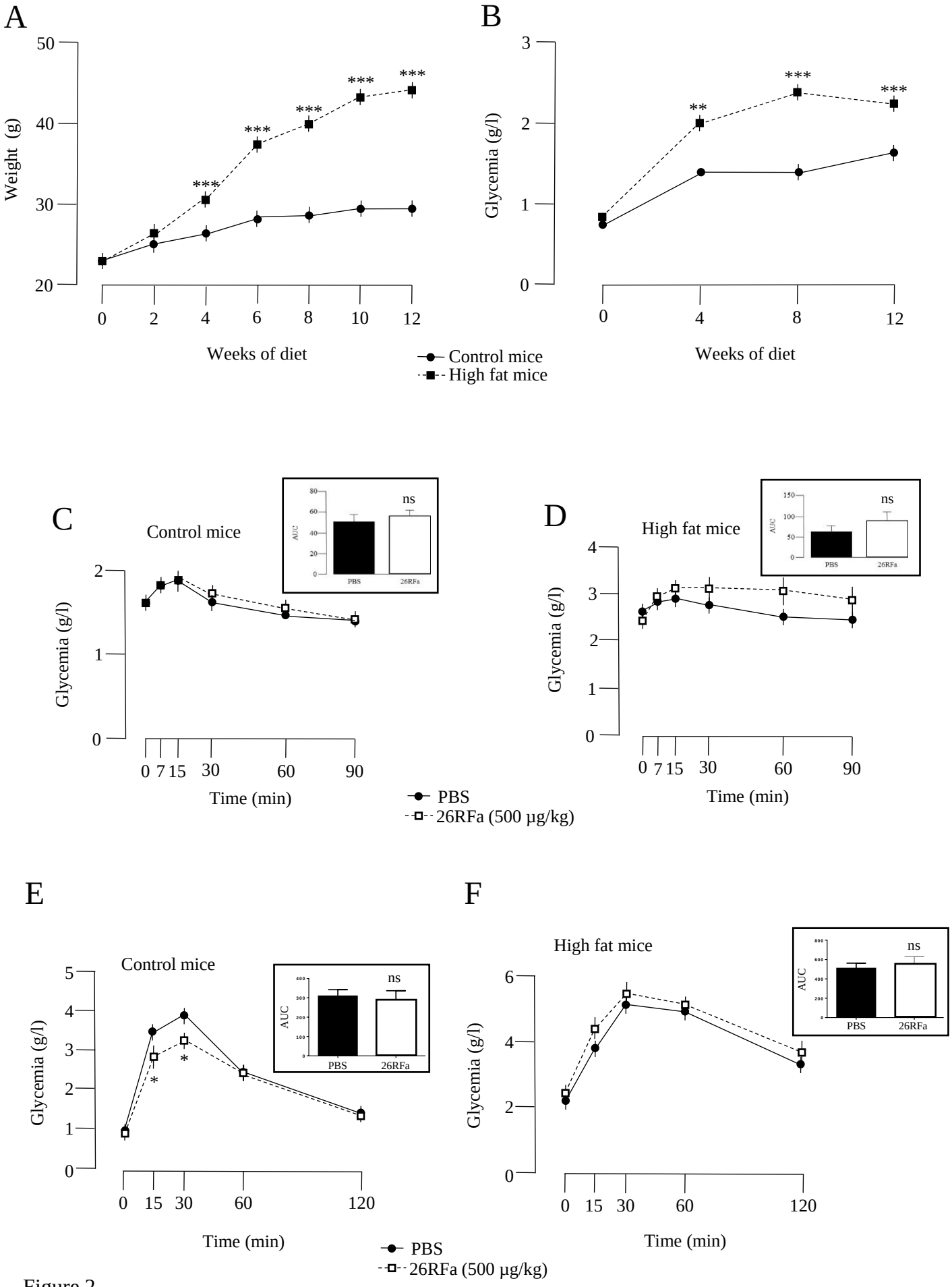


Figure 2

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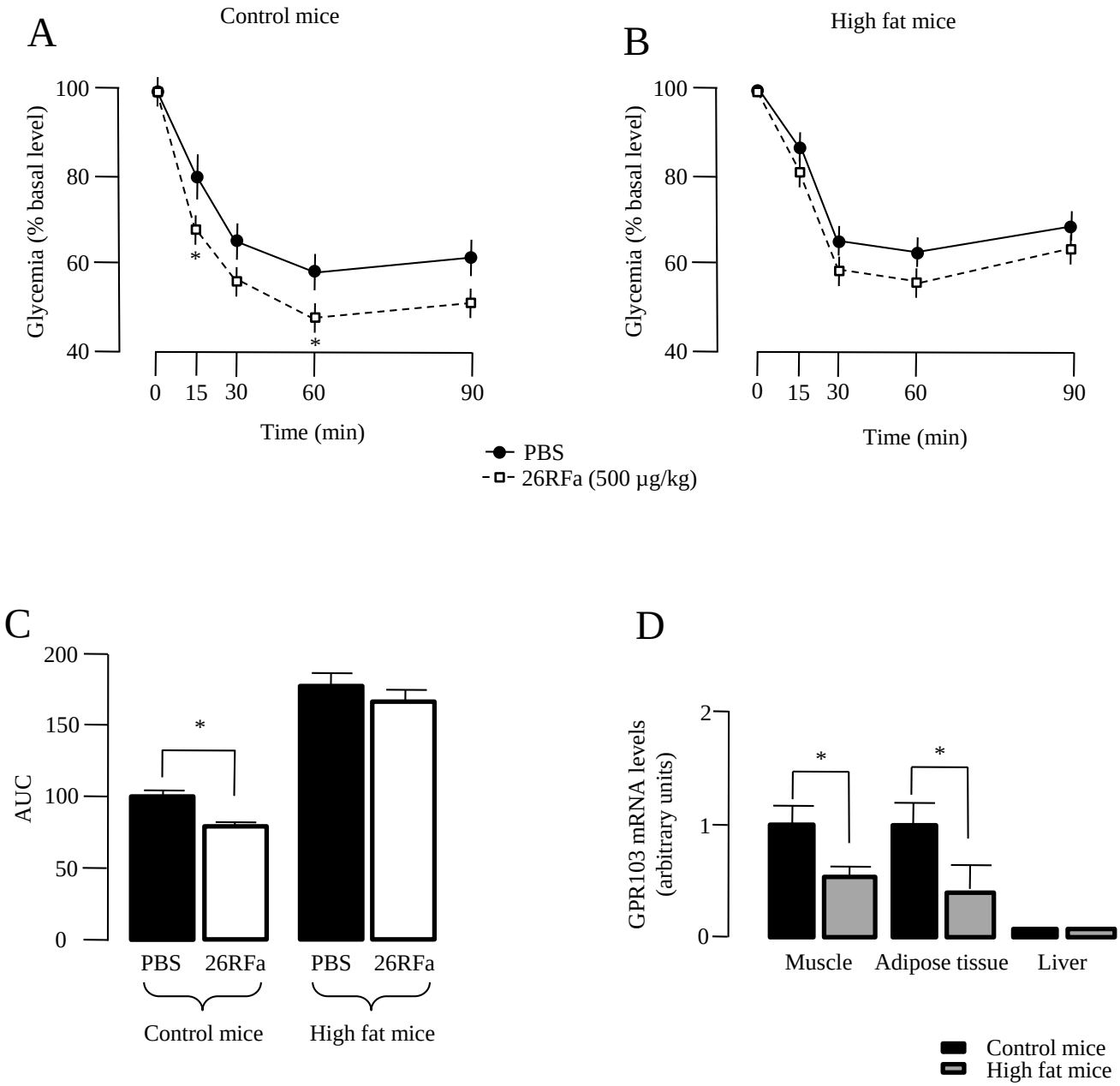


Figure 3

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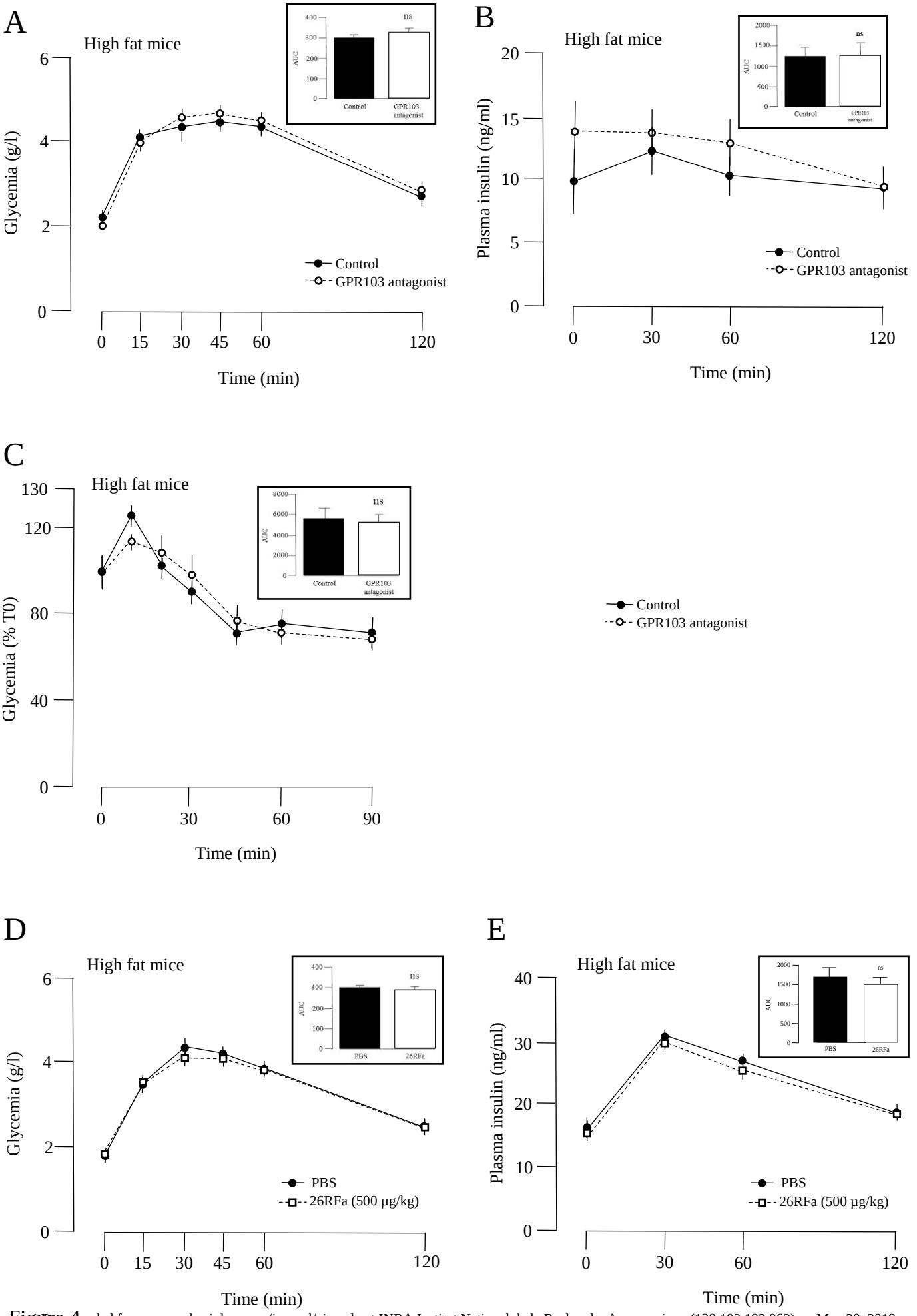


Figure 4

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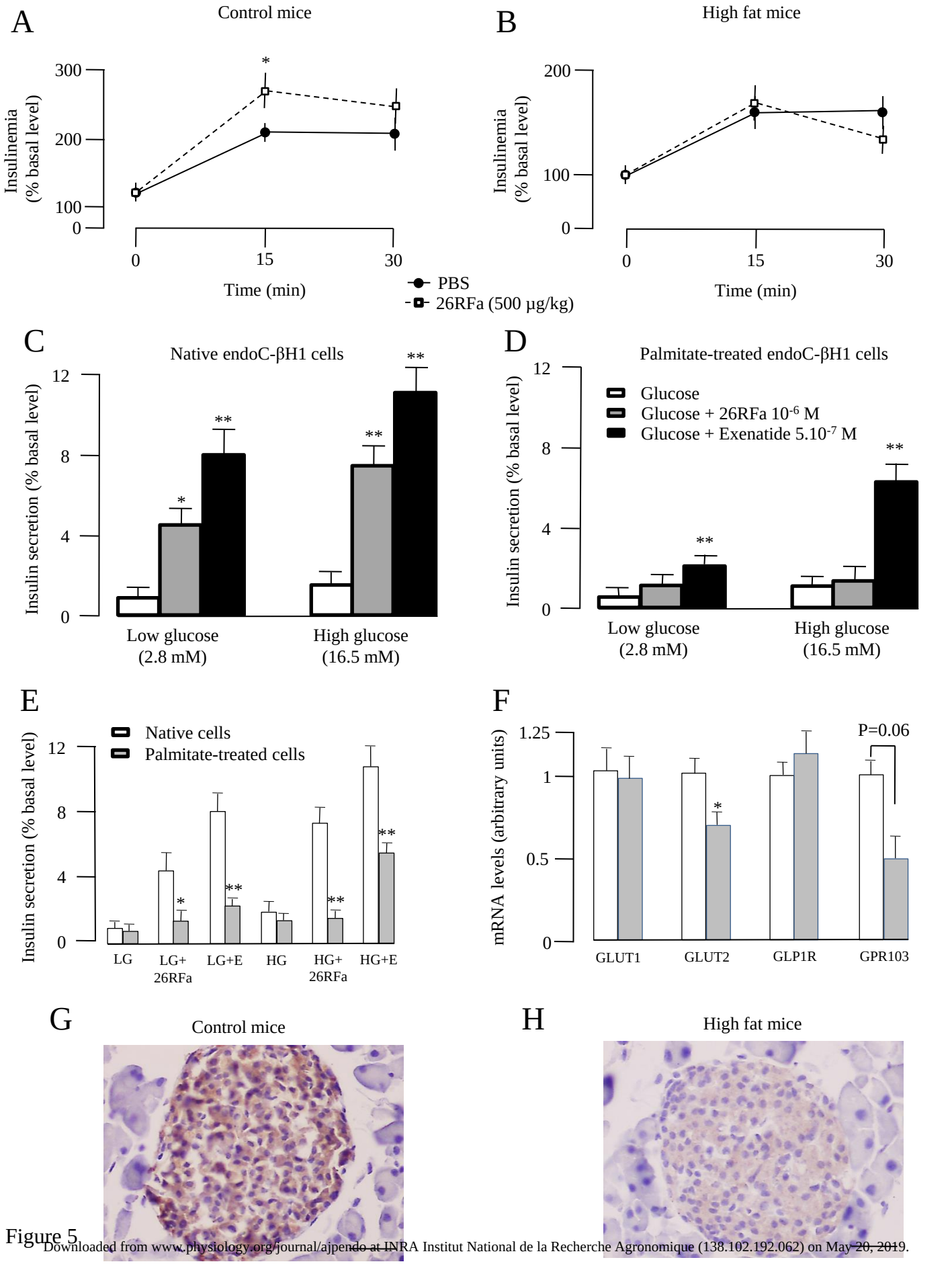


Figure 5

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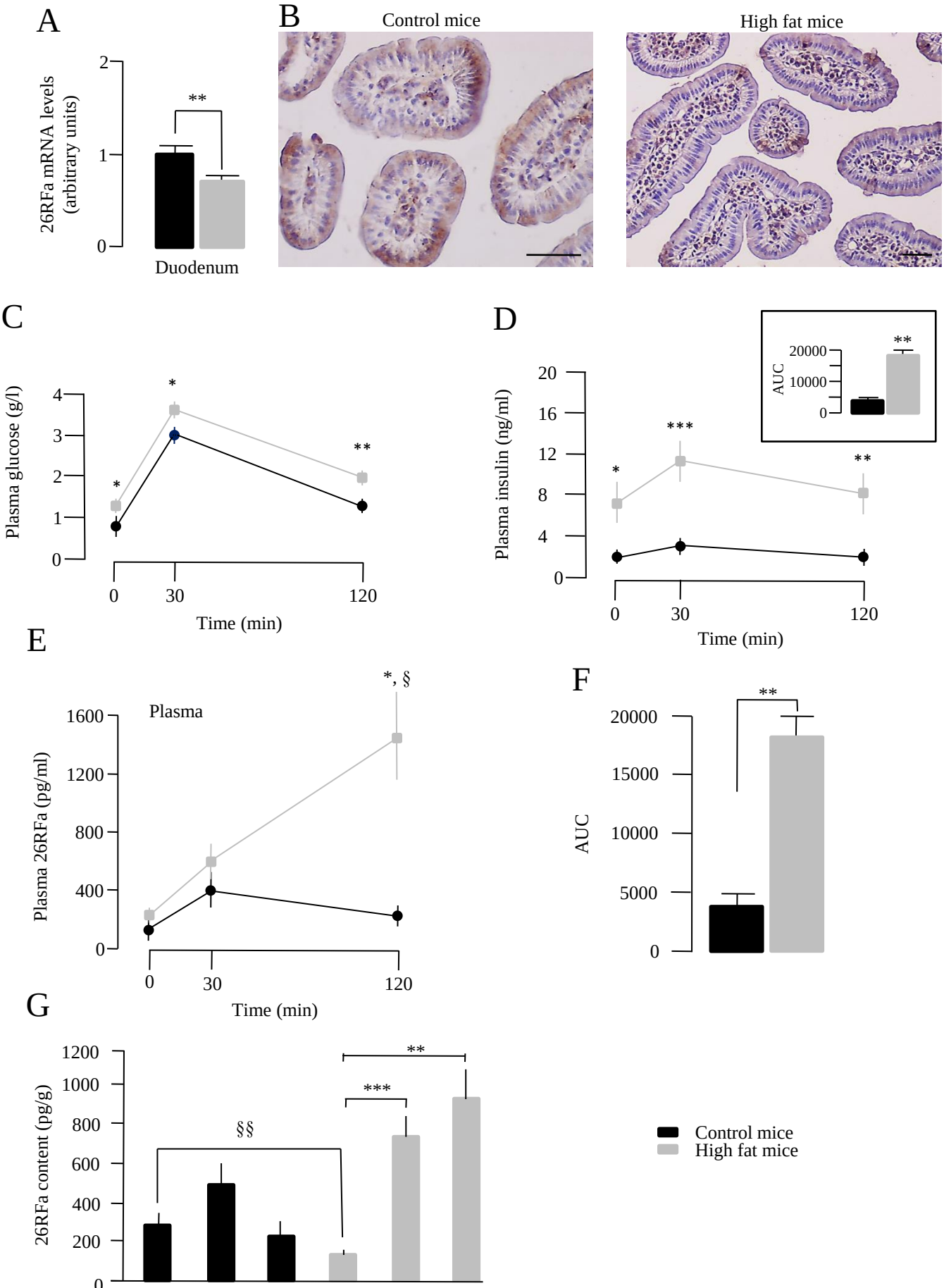


Figure 6

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Table 1 - Sequence of the primers used for the Q-PCR experiments

	Forward primer	Reverse primer
Human GPR103	GAAAGGCACCAGGGACTTGT	CCAGACCACACCTAGCATTGT
Human GLP-1 rec	TGAGCATAGGCTGGGGTGTT	CAGCCGGATAATGAGCCAGT
Human Glut-1	TGGCATCAACGCTGTCTTCT	AGCCAATGGTGGCATACACA
Humain Glut-2	CTAGTCAGGTGCATGTGCCA	AGGGTCCCAGTGACCTTATCT
Mouse GPR103	GGACGAAGGGCACCATCTT	CGTCACGATGCTCCAGAACA
Mouse 26RFa	TCTGCCGTCCTTACCATCTCA	TCTCAGGACTGTCCCAAAGGAG
Mouse GAPDH	TCCGGACGCACCCTCAT	CGGTTGACCTCCAGGAAATC
Human cyclophilin A	ATGGCACTGGTGGCAAGTCC	TTGCCATTCCTGGACCCAAA

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