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GLP-2 as Beneficial Factor in the Glucose Homeostasis in Mice Fed a High Fat Diet

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Glucagon like peptide-2 (GLP-2) is a gastrointestinal hormone released in response to dietary nutrients, which acts through a specific receptor, the GLP-2 receptor (GLP-2R). The physiological effects of GLP-2 are multiple, involving also the intestinal adaptation to high fat diet (HFD). In consideration of the well-known relationship between chronic HFD and impaired glucose metabolism, in the present study we examined if the blocking of the GLP-2 signaling by chronic treatment with the GLP-2R antagonist, GLP-2 (3–33), leads to functional consequences in the regulation of glucose metabolism in HFD-fed mice. Compared with animals fed standard diet (STD), mice at the 10th week of HFD showed hyperglycaemia, glucose intolerance, high plasma insulin level after glucose load, increased pancreas weight and β cell expansion, but not insulin resistance. In HFD fed mice, GLP-2 (3–33) treatment for 4 weeks (from the 6th to the 10th week of diet) did not affect fasting glycaemia, but it significantly increased the glucose intolerance, both fasting and glucose-induced insulin levels, and reduced the sensitivity to insulin leading to insulin-resistance. In GLP-2 (3–33)-treated HFD mice pancreas was significantly heavier and displayed a significant increase in β-cell mass in comparison with vehicle-treated HFD mice. In STD mice, the GLP-2 (3–33) treatment did not affect fasted or glucose-stimulated glycemia, insulin, insulin sensitivity, pancreas weight and beta cell mass. The present study suggests that endogenous GLP-2 may act as a protective factor against the dysregulation of the glucose metabolism that occurs in HFD mice, because GLP-2 (3-33) worsens glucose metabolism disorders.
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Glucagon-like peptide-2 (GLP-2) is a 33- amino acid proglucagon-derived peptide, related in sequence to glucagon-like peptide-1 (GLP-1) and co-secreted in response to nutrients, and specifically fat and carbohydrates, from intestinal endocrine L cells (for review see Marathe et al., 2013; Baldassano and Amato, 2014; Drucker and Yusta, 2014). While GLP-1 is one of the most potent insulinotropic substances known, GLP-2 mainly acts to maintain intestinal homeostasis and to enhance barrier function (Holst, 2004; Janssen et al., 2013). GLP-2 also promotes the rapid stimulation of hexose transport (Cheeseman et al., 1997), reduces short-term food intake (Tang-Christensen et al., 2000; Baldassano et al., 2012) and can modulate the gastrointestinal functions, such as gastric emptying, intestinal motility, and intestinal enteric secretion (Wøjdemann et al., 1998; Nagell et al., 2004; Amato et al., 2009; Baldassano et al., 2009; Amato et al., 2010; Cinci et al., 2011). The peptide acts through a specific G protein-coupled receptor (GLP-2R) that is expressed in central and enteric neurons, vagal sensory neurons, pancreatic α cells, enteroendocrine cells and myofibroblasts (Lovshin et al., 2004; Ørskov et al., 2005; Guan et al., 2006; Nelson et al., 2007; Baldassano et al., 2009).

GLP-2 has been reported to play a beneficial role in obesity condition (Cani et al., 2009). In general, obesity may be due to an high fat diet (HFD), nutritional condition that may lead to metabolic syndrome which is defined as a cluster of obesity, glucose intolerance, insulin resistance, hypertension, and dyslipidemia (Buettner et al., 2007; Shin et al., 2013). GLP-2, in genetically obese mice, lowers endotoxemia, reduces gut permeability, and decreases systemic and hepatic inflammation, oxidative stress and macrophage infiltration markers (Cani et al., 2009). We showed that GLP-2 is involved in the regulation of the small intestine morphological changes following chronic HFD (Baldassano et al., 2013). Indeed, the chronic treatment with the GLP-2R antagonist, GLP-2 (3–33), reduces the increase in crypt–villus height and in the cell number per villus in HFD mice. However, the functional consequences of the blocking of the GLP-2 signaling by GLP-2 (3–33) in this animal model, if any, have not been explored yet. It is likely to hypothesize that changes in mucosal morphology induced by GLP-2R antagonist chronic treatment are associated with changes in metabolic homeostasis, in consideration of the key role played by the gut for disposal of nutrient.

Thus, our aim was to investigate the role of endogenous GLP-2 signaling in the glucose homeostasis in HFD-fed mice by chronic treatment with the GLP-2R antagonist, GLP-2 (3–33).

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Materials and Methods

Animals

The animal studies were approved by Ministero della Sanita' (Rome, Italy). All of the animal procedures for the care and use of laboratory animals were in conformity with the Italian D.L. no. 116 of 27 January 1992 and subsequent variations and the recommendations of the European Economic Community (86/609/ECC).

Male C57BL/6J (B6) mice, purchased from Harlan Laboratories (San Pietro al Natisone Udine, Italy), 4 weeks of age, were housed under standard conditions of light (12 h light:12 h darkness cycle) and temperature (22–24°C), with free access to water and food. After acclimatization (1 week), the animals were weighed and divided in two groups, which were fed a standard diet (STD) (4RF25, Mucedola, Milan, Italy), or a high fat diet (HFD) (PF4051/D, Mucedola) for 10 weeks. Throughout the 10 weeks of the study, food intake was measured daily while body weight was measured once every 3 days. Initially, 6 animals of each group were used to characterize the glucose metabolic state of mice fed differently for 10 weeks.

Peptide

Synthetic GLP-2 (3–33) was provided by Caslo Laboratory (Lyngby, Denmark). Purity ($\geq$95%) and correctness of structure were confirmed by mass, sequence, and HPLC analysis.

Glucose and insulin tolerance tests

Intraperitoneal glucose tolerance test (IPGTT) and insulin tolerance test (ITT) were carried out in mice fasted for 6 h with free access to water. For IPGTT, the mice were injected intraperitoneally (i.p.) with glucose (2 g/kg body weight) (D-glucose, Sigma Aldrich, Milan, Italy) in 0.9% saline. Because gastric emptying rate, glucose absorption and incretin effects are major factors determining the insulin secretion response to the glucose oral, we ruled out the oral glucose tolerance test. For ITT mice were given an i.p. injection of insulin (1.2 U/kg body weight) (Insuman Rapid, Sanofi Aventis, Italy) in 0.9% saline. Blood glucose was measured up to 120 min (0, 15, 30, 60, 90, 120) by tail vein using a glucometer (GlucoMen LX meter, Menarini, Italy).

For measurement of plasma insulin blood samples were collected during the IPGTT by tail vein puncture at 0, 15, 60, and 120 min, immediately transferred into chilled tubes containing a final concentration of 1 mg/ml EDTA, and centrifuged at 825 g at 4° C for 10 min to obtain plasma, which was stored at −80°C until analysis.

Quantification of plasma insulin was carried out by ELISA kit for mouse (Alpco diagnostics, Salem, NH) according to the manufacturer's instructions. The experimental detection limit of the assay was 0.1 ng/ml. Glucose and insulin tolerance tests and biochemical analysis were conducted before and after chronic treatment with GLP-2 (3–33).

Tissue processing

The pancreases were removed, weighted and subsequently were cut into approximately 10 pieces, fixed in formalin and embedded in paraffin for histological and immunomorphological analyses.

Histological analysis

Samples of total pieces of pancreas were processed for light microscopy examination. Sections with a thickness of 5 μm were obtained from paraffin blocks and were stained with hematoxylin and eosin. After the slides were observed using an automated Leica DM5000 B microscope (Leica, Milan, Italy) connected to a high-resolution camera, Leica DC300 F (Leica), to measure total pancreas area and islet area.

Immunomorphological analyses

The immunomorphological analyses were performed by immunofluorescence experiments. Five-micrometer sections were dewaxing in xylene, rehydration in ethanol, washing in phosphate-buffered solution (PBS), incubated with unmasking solution (tri-sodium citrate 10 mM, 0.05% Tween 20) for 10 min at 60°C and treated with blocking solution (3% albumin bovine serum in PBS) for 30 min. Then, the sections were incubated with the primary antibody, rabbit anti-insulin (Insulin H-86, code sc-9168, Santa Cruz Biotecnology, Europe) diluted 1:50, overnight at 4°C. Sections were rinsed twice for 10 min in PBS and thereafter incubated with secondary antibody with specificity for rabbit-IgG conjugated with fluorescein isothiocyanate (FITC; diluted 1:200; Sigma-Aldrich, Milan, Italy). Nonimmune rabbit serum was used for negative controls.

The sections were examined using an automated Leica DM5000 B microscope (Leica, Milan, Italy) connected to a high-resolution camera, Leica DC300 F (Leica). Images were processed using ImageJ software (ImageJ 1.43u, National Institute of Mental Health, Bethesda, Maryland). The number of positive pixels indicative of insulin staining was summed with the use of the optimixed positive pixel count algorithm and normalized per total pancreas area for each mouse. Total β cell mass was calculated by multiplying this value by the weight of the pancreas (Bahrami et al., 2010). A total of six pancreas from each group of animals was analyzed. The examination and the computer analysis of the histological sections were performed without knowledge of the origin of the tissue samples.

GLP-2 (3–33) treatment

After 6 weeks of feeding with the respective diets, a subgroup of mice on each diet was injected once a day i.p. with 100 μl of GLP-2 (3–33) (60 ng) or PBS (vehicle control) for 4 weeks, as previously reported (Baldassano et al., 2013). After 4 weeks period treatment, IPGTT and ITT and pancreatic analysis were carried out.

Statistical analysis

Results are shown as means ± S.E.M. The letter n indicates the number of experimental animals. The comparison between the groups was performed by ANOVA followed by Bonferroni's post-test or by Student's *t*-test when appropriate using Prism Version 6.0 Software (Graph Pad Software, Inc., San Diego, CA). Areas under the curve (AUC) values for the glucose and plasma insulin were calculated using the trapezoidal rule. A P value <0.05 was considered to be statistically significant.

Results

Impact of a chronic HFD on glycemic control, insulin sensitivity and pancreas morphology

After 10 weeks on high fat diet, mice had greater mass gain compared to STD mice, being the body weight 28 ± 0.8. and 24 ± 1.2 ($P < 0.01$, n = 12/group), respectively. The food intake was not different between groups (HFD: 2.33 ± 0.2 g/day and STD: 2.67 ± 0.3 g/day), but the energy intake was greater in HFD mice (14 ± 1.2 Kcal/day) than in STD mice (9.3 Kcal ± 1.0 Kcal/day $P < 0.05$; n = 12/group).

Mice on high fat diet displayed hyperglycemia. The basal fasting glucose level was 173.8 ± 5.9 mg/dl in HFD mice and 123.5 ± 12.8 mg/dl in STD mice ($P \leq 0.05$). HFD mice displayed also an impaired glycemic response following intaperitoneal glucose load. In the IPGTT the plasma glucose increased to a maximum after 15 min of glucose administration in both groups, but this maximun was significantly higher and remained more elevated in HFD mice than in STD mice (Fig. 1A). This is

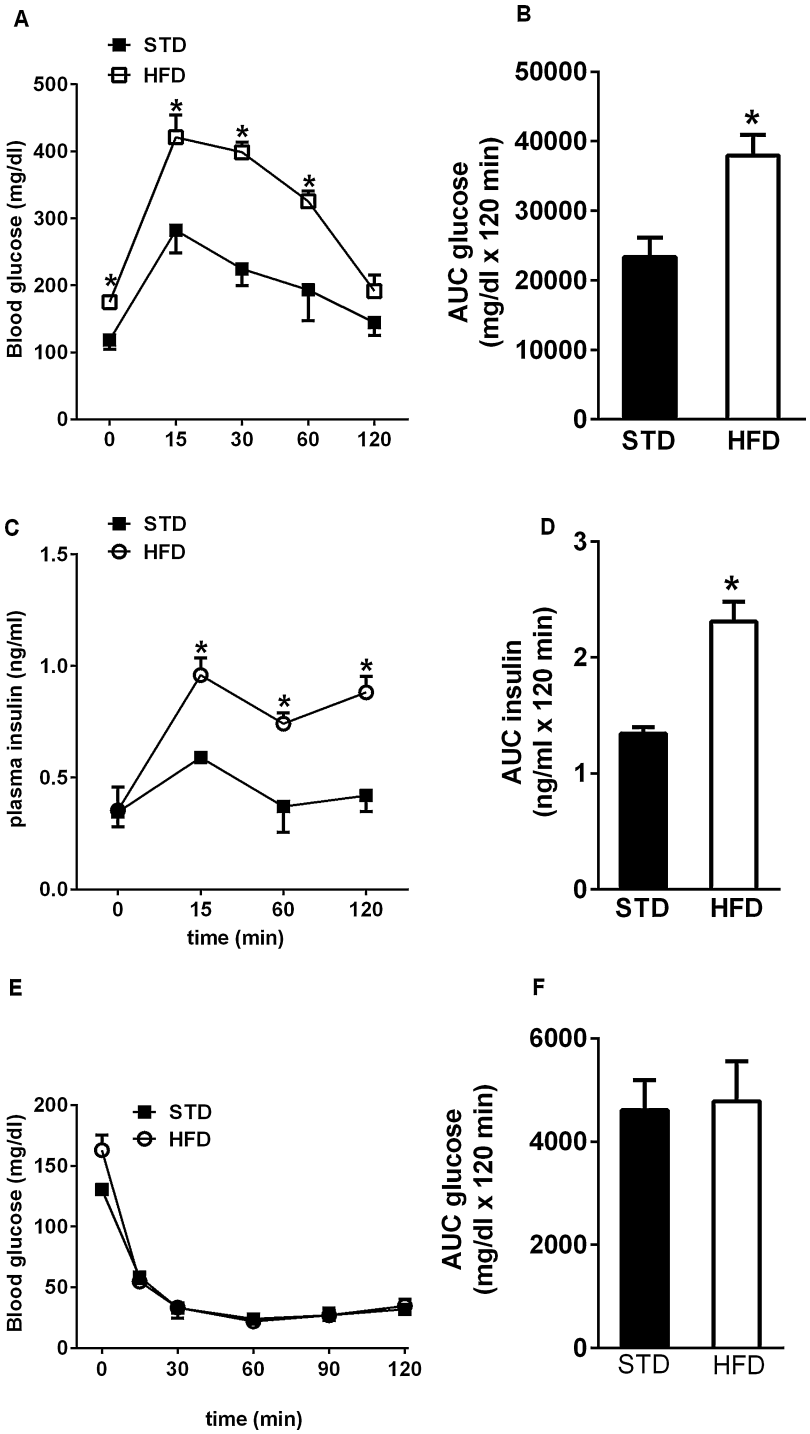

Fig. 1. Glucose tolerance, plasma insulin and insulin sensitivity in standard diet (STD)- and high fat diet (HFD) fed mice. (A) Blood glucose concentration during intraperitoneal glucose tolerance test (IPGTT). (B) AUC for blood glucose concentrations during IPGTT. (C) Plasma insulin during IPGTT. (D) AUC for plasma insulin during IPGTT. (E) blood glucose concentrations during insulin tolerance test (ITT). (F) AUC for blood glucose concentrations during ITT. To measure glucose tolerance and plasma insulin mice were given an intraperitoneal injection of glucose (2 g/kg body weight) while to measure insulin sensitivity mice were given an intraperitoneal injection of insulin (1.2 U/kg body weight). Data are mean values ± S.E.M. (n = 6 mice/group). $^{*}P \leq 0.05$.

also illustrated by the measurement of the glucose area under the curve (AUC) (Fig. 1B).

Plasma insulin level in fasted states was similar between the two groups of animals while it was more elevated in HFD mice during the course of the intraperitoneal glucose load (Fig. 1C), as reflected by the AUC for insulin (Fig. 1D).

HFD mice did not lose insulin sensitivity. No difference in glucose excursion was detected after intraperitoneal

administration of exogenous insulin in HFD compared with STD mice (Fig. 1E and F).

In HFD mice pancreas was heavier (+44% $P < 0.05$). Moreover HFD mice showed larger pancreatic islets (islet size varied from 13,392.5 ± 936.91 μm^2 in STD to 19,960 ± 767.023 μm^2 in HFD mice, + 49% $P < 0.05$) and significantly greater β cell mass than STD mice (Fig. 2).

Effects of GLP-2 (3-33) treatment on glucose tolerance and plasma insulin in HFD Mice

In HFD mice, the chronic treatment with GLP-2R antagonist, GLP-2 (3–33) (60 ng), did not modify food intake/die and body weight (Fig. 3). Moreover, the GLP-2 (3–33) treatment did not affect fasting glycemia, but it significantly reduced glucose tolerance. Blood glucose levels after intraperitoneal glucose load were significantly increased in GLP-2 (3–33) treated mice (Fig. 4 A and B). Moreover, the chronic treatment affected the plasma insulin levels in both fasted and glucose stimulated states. In GLP-2 (3–33) treated mice, the insulin level was significantly increased both in fasted state and in response to intraperitoneal glucose load at all-time points (Fig. 4C and D).

Effects of GLP-2 (3–33) treatment on insulin sensitivity in HFD Mice

The chronic treatment with GLP-2 (3–33), (60 ng) significantly reduced the sensitivity to insulin. In GLP-2 (3–33) treated mice the levels of blood glucose decreased less in comparison with PBS-treated HFD animals after intraperitoneal exogenous administration of insulin (fig. 4 E and F).

Effects of GLP-2 (3–33) treatment on pancreas in HFD Mice

In HFD mice, the chronic treatment with GLP-2 (3–33) affected pancreatic islets. We found that pancreas was significantly heavier and displayed significant increase in β-cell mass in HFD GLP-2 (3–33)-treated mice (Fig. 5).

Effects of GLP-2 (3–33) treatment on glucose metabolic parameters in STD-fed mice

In STD mice, the GLP-2 (3–33) treatment did not affect fasted or glucose-stimulated glycemia, insulin, insulin sensitivity, pancreas weight and beta cell mass (data not shown).

Discussion

Our study shows that endogenous GLP-2 is not essential in the control of glucose homeostasis or pancreas endocrine function under normal conditions, but it can participate as a favorable factor to the maintenance of glucose in HFD mice. Indeed, the blockade of the GLP-2R signaling accelerates the process leading to insulin resistance in HFD mice.

Up to date the GLP-2 action on glucose homeostasis has been scarcely explored and the importance of GLP-2R signaling is not clear (Guan, 2014). In fact, GLP-2R global deficiency is

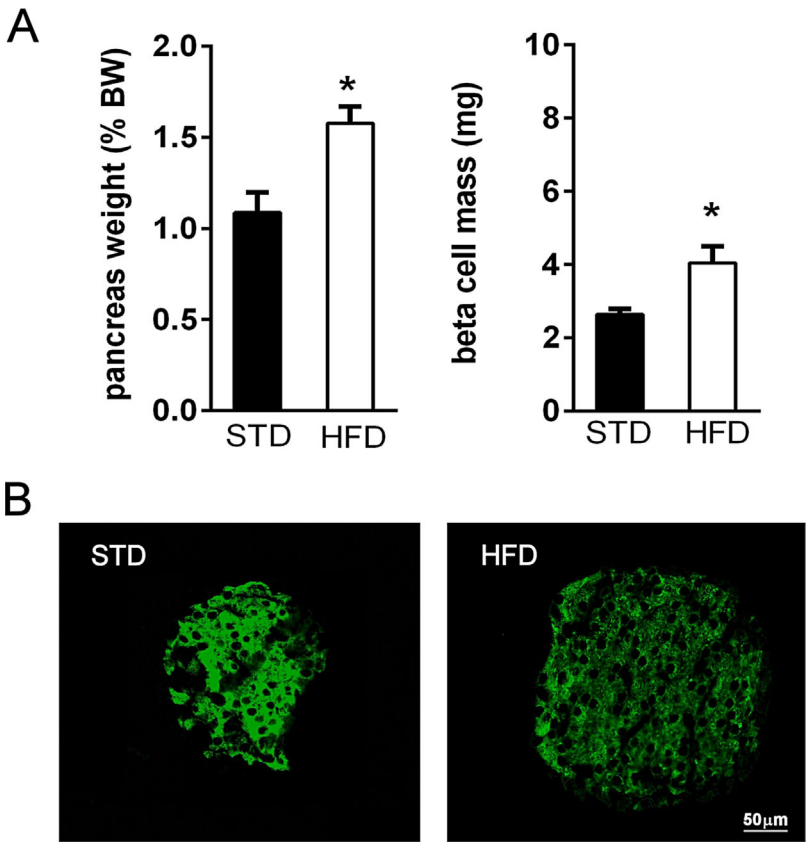

Fig. 2. (A) Pancreas weight and β cell mass in mice fed a standard diet (STD) or high fat diet (HFD). Data are mean values ± S.E.M. (n = 6 mice/group). *$P \leq 0.05$. (B) Representative immunofluorescence staining of insulin from pancreatic sections of mice fed a standard diet or a high fat diet. Scale bar 50 μm.

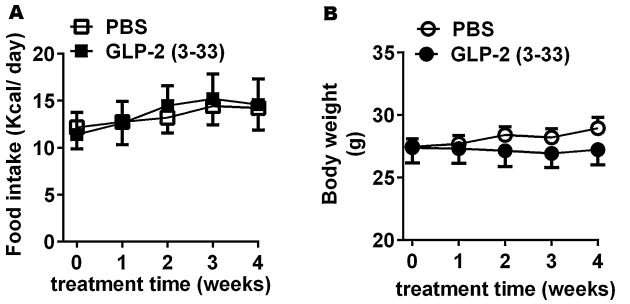

Fig. 3. Effects of chronic treatment with GLP-2 (3–33) (60 ng) on food intake and body weight in mice fed and high fat diet (HFD). (A) Time course of the effect on food intake during 4 weeks of GLP-2 (3–33) treatment. (B) Time course of the effect on body weight during 4 weeks of GLP-2 (3–33) treatment. Data are mean values ± S.E.M. (n = 6 mice/group).

not critical for glucose homeostasis in normal or lean diabetic mice, while it accentuates hyperglycemia and impaired glucose tolerance in genetic (ob/ob) obese mice (Bahrami et al., 2010). However, recent evidence have shown that mice lacking GLP-2R in pro-opiomelacortin (POMC) neurons of hypothalamic arcuate nucleus display glucose intolerance and hepatic insulin resistance suggesting that GLP-2 can function as a key neural transmitter in the hypothalamic-brainstem neurocircuits to fine-tune glucose homeostasis (Shi et al., 2013). In addition, GLP-2 is considered as a key signal to contribute to glycemic improvement after bariatric surgery, particularly Roux-en-Y gastric bypass (RYGB) (Saeidi et al., 2013).

In C57BL/6J mouse model a chronic exposure to HFD induces obesity and a progressive deterioration of metabolic control, characterized by hyperglycemia, hyperinsulinemia, and insulin resistance (Surwit et al., 1988; Lee et al., 1995; Ahrén et al., 1997). Accordingly, this animal model is used in studies on pathophysiology of impaired glucose and type II diabetes (Winzell et al., 2007) and for the development of new treatments (Reimer et al., 2002). The high fat-fed mouse model is a dynamic model in which compensatory adaptations may change by time (Winzell et al., 2007), then we focused our study on glucose control at 10 weeks of HFD to establish the consequence of GLP-2 (3–33) treatment. In our experiments, mice at the 10th week of HFD showed hyperglycemia, glucose intolerance and high plasma insulin level after glucose load. However, they did not present reduced insulin sensitivity, at least at the maximal dose we tested, in according to previous studies (Lamont and Drucker, 2008; Baharami et al., 2010). Likewise, we detected increased pancreas weight and β cell expansion. Increase in islets mass, which begins early in HFD exposure, coincident with the onset of hyperglycemia and glucose intolerance, but before the onset of insulin resistance has been reported (Stamateris et al., 2013). Therefore, the higher increase in insulin secretion in response to glucose load in HFD mice may be correlate with increased pancreatic beta cell mass, consistent with previous study (Collins et al., 2010; Fraulob et al., 2010; Li et al., 2011; Wu et al., 2013).

After having characterized the metabolic state of mice after 10 weeks of HFD feeding, we asked whether endogenous GLP-2 was involved in the glucose homeostasis. To reach this goal, we blocked the signal mediated by the GLP-2R by treating the animals for 4 weeks (from the 6th to the 10th week of diet) with the GLP-2R antagonist, GLP-2 (3–33). The dose, the route of administration and treatment period were based on previous studies describing the intestinotrophic properties of the GLP-2 in mouse (Iakoubov et al., 2009; Baldassano et al., 2013). On the other, the GLP-2R antagonist has enabled experiments delineating the importance of endogenous GLP-2 action in vivo (Nelson et al., 2008; Shin et al., 2005).

The first finding was that the blockade of the GLP-2R signaling deteriorates glucose control only in HFD mice, as suggested by increased glucose intolerance, significantly higher insulin levels in both fasted state and after glucose load and less sensitivity to exogenous insulin in comparison with HFD mice treated with PBS. In contrast, the chronic treatment with GLP-2 (3–33) did not affect glycemic parameters, glucose tolerance, insulin sensitivity or pancreas weight and beta cell mass in STD mice, ruling out a crucial role for the endogenous GLP-2 in glucose homeostasis in normal conditions. Indeed, GLP-2R deletion only in pro-opiomelanocortin neurons impairs postprandial glucose tolerance and hepatic insulin resistance (Shi et al., 2013) indicating that GLP-2 is involved in the control of glucose homeostasis by acting centrally. Although we did not detect evidence for significant changes in glucose metabolic parameters in STD mice, we have no indication if GLP-2R located in central neurons are blocked in our experimental protocol and we cannot exclude the possibility that central nervous system GLP-2 is important for the maintenance of glucose homeostasis.

Data from our experiments allow us to hypothesize that endogenous GLP-2 would act as a protective factor against the dysregulation of the glucose metabolism that occurs in HFD mice, because GLP-2 (3–33) exacerbates glucose metabolism disorders. Our results are in line with previous studies which do not support a role for endogenous basal GLP-2R signaling in the control of glucose homeostasis or islet function under normal or lean diabetic mice, but elimination of GLP-2R signaling in genetically obese mice impairs the normal islet adaptative response required to maintain glucose homeostasis (Bahrami et al., 2010). Indeed, the Authors did not observed any difference in glucose homeostasis in GLP-2R$^{+/+}$ versus GLP-2R$^{-/-}$ mice fed a high fat diet for 5 months (Bahrami et al., 2010). Compensation in transgenic model is common and the mouse model apart from the elimination of GLP-2R signaling was also leptin-deficient. Thus, the discrepancy with our result may be due to compensatory factors that could mask the effect of loss of endogenous GLP-2R signaling. Alternatively, difference in the period of diet (10 weeks vs. 5 months) may also account for differences between our data and the finding reported by Baharami et al. (2010). The diabetes/obesity syndrome worsens with time and with increasing obesity (Collins et al., 2004) and the beneficial effect exerted by GLP-2R activation could be hampered. Indeed, within 16 weeks of high-fat diet, the diabetes/obesity syndrome is completely reversible at this stage in these mice (Parekh et al., 1998) suggesting that the regulatory mechanisms are still working.

To confirm further that the chronic treatment with GLP-2 (3–33) more rapidly leaded the mice to a pre-diabetic stage we also evaluated β cell mass. We found, in conjunction with the reduced glucose tolerance and increased plasma insulin secretion, that the blockade of the GLP-2R signaling further increases β cells mass in HFD mice, suggesting that mice are still in a pre-diabetic stage. We interpreted the increase in β cell mass induced by GLP-2R blockade as being the consequence of the islet adaptation to the potent stimuli exerted on hyperglycemia and hyperinsulinemia. Thus, the blockade of the GLP-2 signaling indirectly affects β cell mass through impairing glucose and insulin levels. Indeed, it is known that high plasma levels of insulin and glucose increase β cell mass in rodents (Flier et al., 2001; Jetton et al., 2005; Jetton et al., 2008; Levitt et al., 2011; Stamateris et al., 2013).

On the basis of our results, we cannot establish at which level the endogenous peptide exerts its action, but we can just

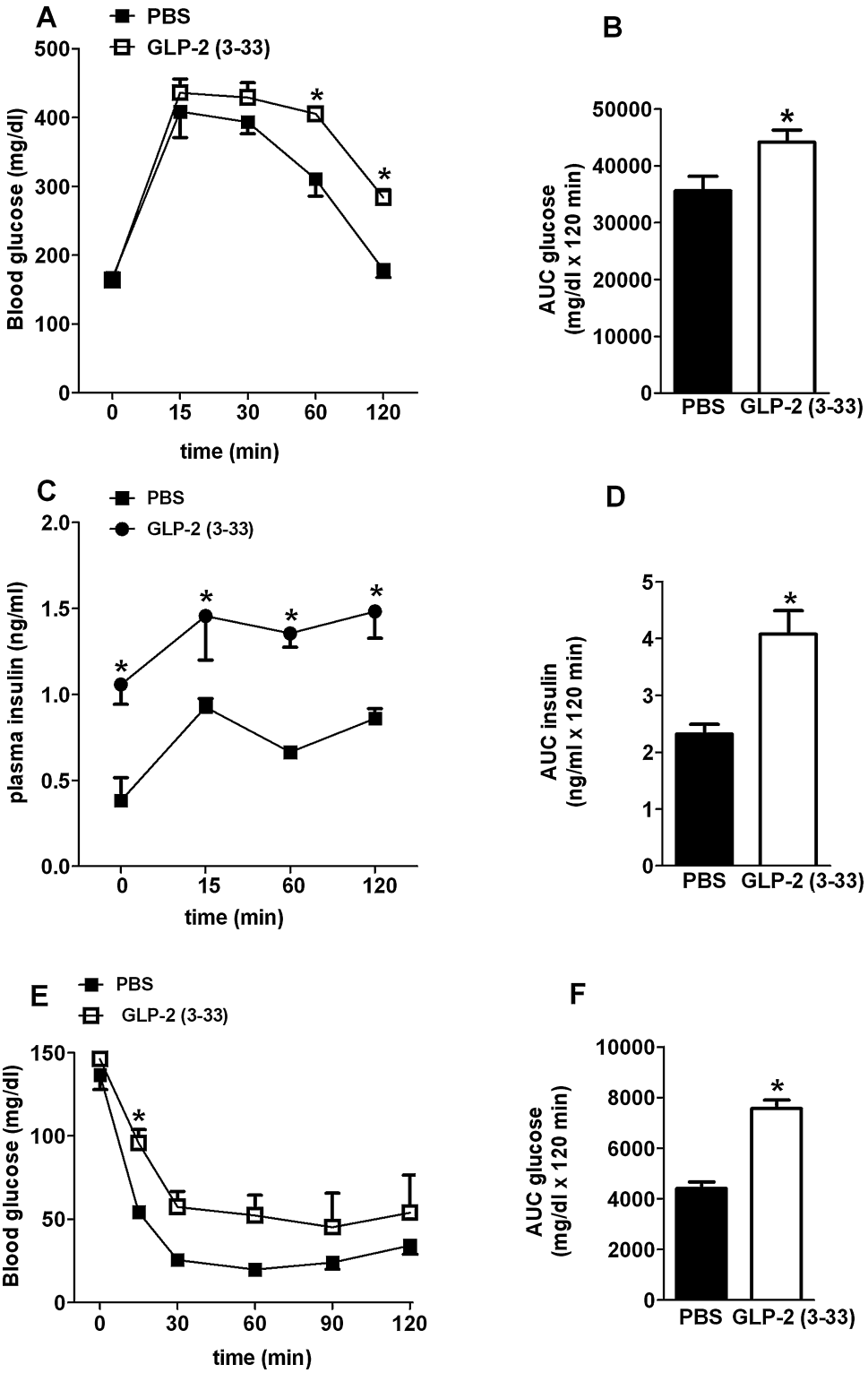

Fig. 4. Effects of chronic treatment with GLP-2 (3-33) (60 ng) on glucose tolerance, plasma insulin and insulin sensitivity in mice fed and high fat diet (HFD). (A) Blood glucose concentration during intraperitoneal glucose tolerance test (IPGTT). (B) AUC for blood glucose concentrations during IPGTT. (C) Plasma insulin during IPGTT. (D) AUC for plasma insulin during IPGTT. (E) Blood glucose concentrations during insulin tolerance test ITT. (F) AUC for blood glucose concentrations during ITT. To measure glucose tolerance and plasma insulin mice were given an intraperitoneal injection of glucose (2 g/kg body weight) while to measure insulin sensitivity mice were given an intraperitoneal injection of insulin (1.2 U/kg body weight). Data are mean values ± S.E.M. (n = 6 mice/group). $^{*}P \leq 0.05$.

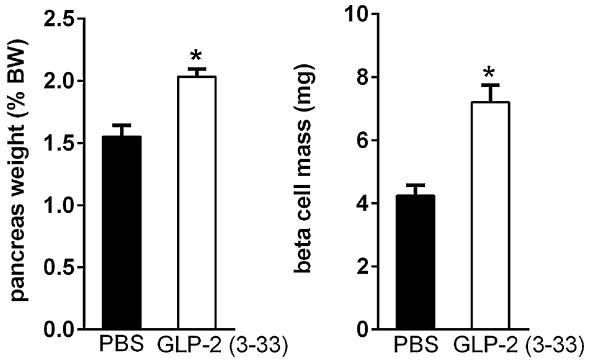

Fig. 5. Pancreas weight and β cell mass in mice fed high fat diet (HFD) after chronic treatment (for 4 weeks) with GLP-2 (3–33) (60 ng) or vehicle (PBS). Data are mean values ± S.E.M. (n = 6 mice/group). $^{*}P \leq 0.05$.

speculate about the mechanistic insight. We can rule out that GLP-2 controls glucose parameters indirectly by influencing food intake because it was not affected. A direct action on β cell function appears unlikely because the GLP-2R mRNA transcript was not detected in mouse islets but only in whole pancreas (Bahrami et al., 2010) or rat pancreatic alpha cells (de Heer et al., 2007). However, GLP-2 and GLP-2R expression could changes in obesity conditions, as previously reported (Rotondo et al., 2011; Baldassano et al., 2013). Moreover, the block of the GLP-2R might directly affect hepatic function and lipid accumulation, since the GLP-2R is expressed in mouse liver (El-Jamal et al., 2014). Hepatic steasosis has been reported HFD mice (de Meijer et al., 2010; Fraulob et al., 2010) and fatty liver is strongly associated with obesity and insulin resistance (Asrih and Jornayvaz, 2013). Another potential explanation for the effects of GLP-2 blockade on insulin resistance is related to GLP-2 ability to reduce gut permeability and consequently the leakage of bacterial endotoxins into the portal blood circulation (Benjamin et al., 2000). Previously studies have shown that the block of the GLP-2R can exacerbate inflammation in genetically obese mice (Cani et al., 2009) and endotoxemia and low-grade inflammation are associated with insulin resistance (Hotamisligil, 2006; Cani et al., 2007). Therefore, further studies are necessary to resolve this issue.

Anyway, the results support a potential protective and beneficial role of GLP-2 in HFD fed mice and in this view, there has been reported that the glutamine-induced GLP-2 secretion from ileal tissue is decreased in diabetic rats (Shan et al., 2013).

In conclusion, the findings of the present study suggest that endogenous GLP-2 is functionally important for the maintenance of glucose homeostasis in HFD mice because loss of the GLP-2R signaling worsens glucose control leading to insulin resistance.

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