RESEARCH

GLP-1 signaling is required for improvement of glucose tolerance by osteocalcin

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Abstract

Osteocalcin is a bone-derived hormone that in its uncarboxylated form (GluOC) plays an important role in glucose and energy metabolism by stimulating insulin secretion and pancreatic β -cell proliferation through its putative receptor GPRC6A. We previously showed that the effect of GluOC on insulin secretion is mediated predominantly by glucagon-like peptide-1 (GLP-1) released from intestinal endocrine cells in response to GluOC stimulation. Moreover, oral administration of GluOC was found to reduce the fasting blood glucose level, to improve glucose tolerance, and to increase the fasting serum insulin concentration and β -cell area in the pancreas in wild-type mice. We have now examined the effects of oral GluOC administration for at least 4 weeks in GLP-1 receptor-knockout mice. Such administration of GluOC in the mutant mice triggered glucose intolerance, enhanced gluconeogenesis and promoted both lipid accumulation in the liver as well as adipocyte hypertrophy and inflammation in adipose tissue. Furthermore, inactivation of GLP-1 receptor signaling in association with GluOC administration induced activation of the transcription factor FoxO1 and expression of its transcriptional coactivator PGC1 α in the liver, likely accounting for the observed upregulation of gluconeogenic gene expression. Our results thus indicate that the beneficial metabolic effects of GluOC are dependent on GLP-1 receptor signaling.

Key Words

- ▶ glucagon-like peptide-1 (GLP-1)
- ▶ GLP-1 receptor
- ▶ osteocalcin
- ▶ gluconeogenesis
- ▶ glucose tolerance

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Introduction

The number of individuals with type 2 diabetes has increased greatly over the past several decades (Mathers & Loncar 2006). Given that diabetes is associated with an ~75% increase in mortality rate in adults (Gregg *et al.* 2018), interventions to reverse this disease and its related disorders are of particular clinical importance. Many recent studies have revealed that the osteoblast-derived hormone osteocalcin in its uncarboxylated form

(GluOC) is able to reverse the diabetic condition by affecting multiple aspects of systemic glucose and energy metabolism (Lee *et al.* 2007a, Ferron *et al.* 2008, 2010, Fulzele *et al.* 2010) in a manner dependent on interaction with its putative receptor GPRC6A (Oury *et al.* 2011). Studies with wild-type (WT) and various genetically modified mice have shown that the metabolic effects of GluOC are primarily dependent on its ability to promote

pancreatic β -cell function either directly (Ferron *et al.* 2008, Wei *et al.* 2014) or indirectly through stimulation of the secretion of glucagon-like peptide-1 (GLP-1) from intestinal endocrine cells (Mizokami *et al.* 2013, 2014).

GLP-1 is a member of the incretin family of hormones that are secreted from enteroendocrine cells and which stimulate insulin secretion in a glucose-dependent manner as well as suppress glucagon secretion (Baggio & Drucker 2007). In addition to its insulinotropic action, GLP-1 has various extrapancreatic effects such as attenuation of hepatic lipid accumulation, prevention of cardiovascular disease, and amelioration of obesity (Xiaokun *et al.* 2006, Lee *et al.* 2007b, 2012, Seino & Yabe 2013).

The potential of GluOC as a therapeutic agent has been tested in mouse models of obesity. Recombinant GluOC administered continuously via a s.c. osmotic pump not only reduced fat mass but also improved glucose metabolism by ameliorating both glucose intolerance and insulin resistance (Ferron *et al.* 2008). Daily intraperitoneal injection of GluOC, a more practical mode of administration, was as effective as was delivery with the osmotic pump. In addition to improving glucose handling, intermittent GluOC injections reversed hepatic steatosis induced by a high-fat diet and increased the number of mitochondria in skeletal muscle, indicative of an increase in energy expenditure (Ferron *et al.* 2012). We have also shown that oral administration of GluOC, the most common and safe route of drug administration, was as effective as intraperitoneal injection with regard to inducing an improvement in glucose tolerance and reduction in adipocyte size. These effects of oral GluOC were abolished by prior s.c. administration of exendin(9–39), an antagonistic peptide of the GLP-1 receptor (GLP-1R), suggestive of a requirement for GLP-1R signaling (Mizokami *et al.* 2013, 2014). However, the role of GLP-1R signaling in the beneficial effects of GluOC has remained unclear.

We have now examined the contribution of GLP-1R signaling to the improvement in glucose handling induced by oral administration of GluOC with the use of GLP-1R-knockout (KO) mice (Scrocchi *et al.* 1996). Daily administration of GluOC in the mutant mice for at least 4 weeks induced glucose intolerance, insulin resistance, and adipocyte hypertrophy, likely as a result of the promotion of gluconeogenesis in the liver and inflammation in adipose tissue. Our results thus indicate that GLP-1R signaling is necessary for the beneficial actions of GluOC and that, in the absence of GLP-1R signaling, administration of GluOC adversely affects glucose handling rather than being without effect.

Materials and methods

Animals and GluOC administration

All animal experiments were approved by the Animal Ethics Committee of Kyushu University (approval no. A29-074). GLP-1R KO mice were kindly provided by Dr D J Drucker. They were originally generated from CD-1 morulae (Scrocchi *et al.* 1996) and were backcrossed with C57BL/6J mice (The Jackson Laboratory) for at least six generations, with C57BL/6J mice serving as controls. All mice were maintained in a specific pathogen-free facility under a 12-h-light, 12-h-darkness cycle and with free access to normal chow (CRF-1; Oriental Yeast, Tokyo, Japan) and water. Recombinant mouse GluOC was prepared as described previously (Mizokami *et al.* 2013). The experimental protocol is shown in Fig. 1A. ZSTK474 (Chem Scene, Monmouth Junction, NJ, USA) and ZLN005 (Cayman Chemical) were dissolved in saline containing 5% dimethyl sulfoxide and 0.5% hydroxypropylcellulose (Fujifilm Wako, Osaka, Japan) and were administered orally at respective doses of 10 mg/kg or 15 mg/kg daily for 8 days, with or without GluOC (10 μ g/kg), to female WT mice at 8 weeks of age.

Serum analysis

Serum concentrations of GLP-1 (Fujifilm Wako Shibayagi, Gumma, Japan) and insulin (Mercodia, Uppsala, Sweden) were measured with an ELISA kit. Dipeptidyl peptidase-IV inhibitor (Merck-Millipore) was added immediately to blood samples to a final concentration of 100 μ mol/L for GLP-1 measurement. The serum levels of triglyceride, total cholesterol, and nonesterified fatty acids (NEFAs) were measured by Nagahama Institute for Biochemical Science (Oriental Yeast, Shiga, Japan).

Metabolic assessment

A glucose tolerance test (GTT), pyruvate tolerance test (PTT), and assessment of glucose-stimulated insulin secretion (GSIS) were performed after mice had been deprived of food for 20 h. Glucose (2 g/kg) or sodium pyruvate (1.5 g/kg) was injected intraperitoneally, and the blood glucose concentration was measured at various times thereafter with Free Style Lite Blood Glucose test strips (Abbott Laboratories). An insulin tolerance test (ITT) was performed after mice had been deprived of food for 4 h. Insulin (0.3 U/kg) (Humulin R; Eli Lilly) was injected intraperitoneally, and the blood glucose concentration was measured at various times thereafter. The serum

Figure 1

Long-term oral administration of GluOC induces glucose intolerance in GLP-1R KO mice. (A) Experimental protocol. Female GLP-1R KO or WT mice at 8 weeks of age were fed a normal diet and subjected to daily (except for Sundays) oral administration of either saline (control) or GluOC (10 µg/kg, freshly diluted in saline) at 08:30 h for 4–7 weeks, beginning at 8 weeks of age. GSIS, glucose-stimulated insulin secretion. (B) Body weight of mice during the first 4 weeks of GluOC or saline administration. (C) The serum GLP-1 concentration of GLP-1R KO mice at 10 weeks of age was measured after deprivation of food for 5 h. Blood was collected from the orbital plexus before and 20 min after GluOC administration. (D) Blood glucose concentration of mice at 14 weeks of age either in the fed state or after 20 h of food deprivation. (E and F) WT (E) and GLP-1R KO (F) mice were subjected to an intraperitoneal GTT (left panels) after daily GluOC or saline administration for 4 weeks as well as to an ITT (right panels) after such treatment for 5 weeks. The area under the curve (AUC) is shown in each inset. All data are means $\pm$ S.E.M. for eight to ten mice per group. * P < 0.05, ** P < 0.01 for the indicated comparisons or versus the corresponding value for saline (control) as determined by Student's *t* test (C and D) or by two-way ANOVA followed by Bonferroni's *post hoc* test (E and F). ns, not significant.

insulin concentration was determined with an ELISA kit as described earlier.

Histomorphometry

Gonadal white adipose tissue (WAT), liver, and pancreas were excised, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at a thickness of 6 µm. WAT and liver sections were stained with Mayer's hematoxylin and Eosin Y (Muto Pure Chemicals, Tokyo, Japan). The size of adipocytes in sectional areas of WAT as well as the area of white spots (indicative of lipid droplets) in sections of the liver were evaluated using BZ-II Analyzer (Keyence, Osaka, Japan). Histomorphometric analysis of the pancreas was performed as described previously (Mizokami *et al.* 2014). Islet area was measured as the surface area positive for

insulin with the use of a BZ-II Analyzer (Keyence). WAT sections were similarly subjected to immunostaining with antibodies to F4/80 (1:500 dilution) (ab111101; Abcam).

Gene expression analysis

Total RNA was extracted from gonadal WAT and liver with the use of an RNeasy Mini Kit (Qiagen) and was subjected to RT with High-Capacity cDNA RT Kit (Life Technologies). The resulting cDNA was subjected to real-time PCR analysis with KOD SYBR qPCR Mix (Toyobo, Osaka, Japan) in a Takara PCR Thermal Cycler Dice Gradient instrument (Takara Bio). Primer sequences are listed in Supplementary Table 1 (see section on [supplementary materials](#) given at the end of this article).

Protein extraction and immunoblot analysis

Mice were deprived of food for 5 h, after which the liver was excised and homogenized in a solution containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 2 mmol/L EDTA, 1% Triton X-100, and phosphatase (Nacalai Tesque, Kyoto, Japan) and protease (Sigma-Aldrich) inhibitor cocktails. The homogenate was centrifuged at 20,000 *g* for 30 min at 4°C, and the resulting supernatant was saved for analysis. For cell fractionation experiments, mouse hepatoma Hepa1-6 cells (RCB1638; RIKEN BRC, Tsukuba, Japan) were suspended in a solution containing 0.25 mol/L sucrose, 10 mmol/L Hepes-NaOH (pH 7.4), 25 mmol/L KCl, 2.5 mmol/L magnesium acetate, and digitonin (100 mg/mL), maintained at room temperature for 5 min, and then centrifuged at 20,000 *g* for 20 min at 4°C to yield a crude cytosolic fraction (supernatant) and crude membrane pellet. Immunoblot analysis was performed as described previously (Otani *et al.* 2015). In brief, equal amounts of protein were fractionated by SDS-PAGE, and blotted with antibodies to phosphorylated or total forms of FoxO1, CREB, or Akt (all from Cell Signaling Technology) as well as with those to PGC1 α (Novus Biologicals) and β -actin (Santa Cruz Biotechnology).

Explant culture of mouse liver and epididymal WAT

Liver and epididymal WAT were removed from mice at 8 weeks of age into ice-cold PBS, rinsed, cut into 2-mm pieces, and cultured overnight in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum. The tissue explants were incubated in the absence or presence of GluOC and exendin-4 (Bachem, Bubendorf, Switzerland) for 24 h and then frozen in liquid nitrogen for immunoblot analysis as described earlier for fresh liver.

Cell culture and HTRF assay

Hepa1-6 cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (0.1 mg/mL). Confluent cells were deprived of serum for 24 h before incubation for 30 min at 37°C in the absence or presence of GluOC, exendin-4 (Bachem), or exendin(9–39) (Bachem). The cells were then lysed and assayed for phospho-FoxO1 (Ser²⁵⁶) and total FoxO1 with the use of homogeneous time-resolved fluorescence (HTRF) kits (Cisbio, Codolet, France) and a 2030 Arvo X Multilabel plate reader (PerkinElmer), with excitation at 330 nm and emission at

620 nm (donor) and 665 nm (acceptor). The 665/620 nm emission ratio was calculated.

Statistical analysis

Quantitative data are presented as means $\pm$ s.e.m. Statistical analysis of measurements at single time points was performed with the two-tailed unpaired Student's *t* test. Two-way ANOVA for repeated measurements and the Bonferroni *post hoc* test were performed with the use of Prism software version 6.0 (GraphPad Software). A *P* value of <0.05 was considered statistically significant.

Results

Oral GluOC administration induces glucose intolerance in GLP-1R KO mice

We previously showed that acute oral administration of GluOC triggers GLP-1 secretion in female WT mice, with its long-term administration increasing serum GLP-1 and insulin levels as well as improving glucose tolerance (Mizokami *et al.* 2013, 2014). These effects of oral GluOC were abolished by prior s.c. administration of the GLP-1R antagonist exendin(9–39), suggesting a role for GLP-1R signaling (Mizokami *et al.* 2014). To explore further the role of GLP-1R signaling in GluOC-mediated regulation of glucose homeostasis, we orally administered GluOC (10 μ g/kg) or saline daily to female GLP-1R KO mice beginning at 8 weeks of age and examined its metabolic effects at various times thereafter (Fig. 1A). Daily food intake during the initial week of GluOC administration did not differ substantially between WT (*n*=8) and GLP-1R KO (*n*=9) mice (1.8 ± 0.06 and 1.69 ± 0.03 g/day, respectively). Administration of GluOC had no effect on the body weight of WT or GLP-1R KO mice from 8 to 12 weeks of age (Fig. 1B). We previously showed that oral administration of GluOC resulted in a marked increase in the circulating GLP-1 level in WT mice compared with that apparent in animals treated with saline (60.2 ± 7.4 vs 18.4 ± 3.5 pg/mL) (Mizokami *et al.* 2013). We also observed a similar GluOC-induced increase in the serum GLP-1 concentration in GLP-1R KO mice at 10 weeks of age (Fig. 1C). Administration of GluOC for 4 weeks had no effect on blood glucose concentration in WT or the mutant mice in the fed state. In contrast, it lowered the blood glucose level of WT mice in the fasted state, consistent with previous observations (Mizokami *et al.* 2014), whereas it increased that in food-deprived GLP-1R KO mice (Fig. 1D).

To evaluate further the impact of GLP-1R deficiency on glucose metabolism, we performed an intraperitoneal GTT and ITT at 4 and 5 weeks, respectively, after the onset of GluOC administration. Daily oral administration of GluOC improved glucose handling without affecting insulin sensitivity in WT mice (Fig. 1E), consistent with our previous findings (Mizokami *et al.* 2014). However, such GluOC administration in GLP-1R KO mice induced glucose intolerance and impaired the hypoglycemic response to insulin (Fig. 1F).

Effects of GluOC administration on β -cells in GLP-1R KO mice

Given that impairment of glucose-stimulated insulin secretion can result in apparent glucose intolerance, we examined serum insulin levels after intraperitoneal injection of glucose in mice subjected to daily GluOC or saline administration for 6 weeks (Fig. 1A). The fasting serum insulin concentration was increased by long-term GluOC administration in WT mice (Fig. 2A), consistent with our previous finding (Mizokami *et al.* 2014), but not in GLP-1R KO mice (Fig. 2A). The fasting serum insulin level was increased by intraperitoneal glucose injection in all four groups of mice (Fig. 2A). However, it was significantly smaller in GluOC-treated GLP-1R KO mice compared with their WT counterparts after glucose injection. To determine whether this lower circulating insulin level of the KO mice was due to a decrease in islet mass, we subjected pancreatic sections to histomorphometric analysis. Representative sections immunostained for insulin are shown in Fig. 2B. There was no significant difference in average islet area between control WT and GLP-1R KO mice (Fig. 2B), similar to previous findings (Scrocchi *et al.* 1996, Moffett *et al.* 2014). On the other hand, GluOC administration induced an ~1.5-fold increase in islet area in WT mice (Fig. 2B), consistent with our previous data (Mizokami *et al.* 2014), whereas it had no effect on this parameter in GLP-1R KO mice (Fig. 2B).

GluOC administration induces adipose tissue inflammation in GLP-1R KO mice

We previously showed that GluOC administration reduces adipocyte size in WT mice (Otani *et al.* 2015, Yasutake *et al.* 2016). However, the size of adipocytes in gonadal WAT of GLP-1R KO mice was increased to a small but significant extent by GluOC administration (Fig. 3A). The serum concentration of triglyceride was

Figure 2

Effects of GluOC administration on β -cells in GLP-1R KO mice. (A) Serum insulin concentration before and 15 min after intraperitoneal glucose injection in WT and GLP-1R KO mice treated with GluOC or saline for 6 weeks. (B) Representative immunostaining for insulin in pancreatic sections from WT or GLP-1R KO mice treated with GluOC or saline for 7 weeks (left) as well as quantification of average islet area in such images (right). The sections were counterstained with hematoxylin. Bars, 100 μ m. Islets in at least five sections were examined for each mouse. All data are means $\pm$ s.e.m. for eight to ten mice per group. * $P < 0.05$ (Student's *t* test).

reduced and that of NEFAs was increased by GluOC administration in WT mice (Fig. 3B), with these changes possibly being a result of the upregulation of triglyceride hydrolysis by GluOC (Otani *et al.* 2018). On the other hand, the serum triglyceride concentration in GLP-1R KO mice was lower than that in WT mice and was not affected by GluOC administration, whereas the serum NEFA concentration in the mutant mice was lowered by GluOC treatment, suggestive of possible downregulation of triglyceride hydrolysis (Fig. 3B). Total cholesterol levels were not altered by GluOC administration in animals of either genotype (Fig. 3B). To explore further the effects of GluOC administration on adipose tissue, we examined the expression of various genes related to adipocyte function or inflammation in gonadal WAT (Fig. 3C). Expression of the genes for fatty acid synthetase (FAS), sterol response element-binding protein 1c (SREBP1c), and peroxisome proliferator-activated receptor γ (PPAR γ) was unaffected by GluOC treatment in WT or GLP-1R KO mice, suggesting that adipogenesis was not altered by GluOC. Expression of genes for adipokines such as adiponectin, leptin, and

Figure 3

GluOC administration induces adipose tissue inflammation in GLP-1R KO mice. (A) Gonadal WAT from GLP-1R KO mice treated with GluOC or saline for 7 weeks was subjected to hematoxylin-eosin staining. Bars, 50 μm. The average adipocyte area was determined for 50 adipocytes per slide, with at least three sections being examined for each mouse. (B) Serum triglyceride, NEFA, and total cholesterol concentrations of WT and GLP-1R KO mice treated as in (A). (C) Quantitative RT-PCR analysis of relative mRNA abundance for the indicated proteins in gonadal WAT of WT and GLP-1R KO mice treated as in (A). Results were normalized by the amount of β-actin mRNA. Abbreviations not defined in text: HSL, hormone-sensitive lipase; ATGL, adipose triglyceride lipase; Adipoq, adiponectin; IL, interleukin; MCP-1, monocyte chemoattractant protein-1. Data in (A) to (C) are means ± s.e.m. for eight to ten mice per group. **P* < 0.05 versus corresponding value for saline-treated (control) mice (Student's *t* test). (D) Immunohistochemical staining of the macrophage marker F4/80 in epididymal WAT sections from GLP-1R KO mice treated as in (A). Arrowheads indicate F4/80-positive macrophages. Bars, 100 μm. Quantification of CLSs in two sections from each of four to six mice per group is also shown. Data are means ± s.e.m. **P* < 0.05 (Student's *t* test). (E) Explants of epididymal WAT from WT or GLP-1R KO mice were stimulated with GluOC (10 ng/mL) for 24 h in the absence or presence of exendin-4 (10 nmol/L) and were then subjected to immunoblot analysis with antibodies to total or phosphorylated (p) forms of FoxO1 as well as with those to β-actin (loading control). A representative blot as well as quantitative data for the relative phospho-FoxO1/total FoxO1 ratio determined by densitometry (means ± s.e.m. for four mice of each genotype) are shown. **P* < 0.05, ***P* < 0.01 (Student's *t* test).

resistin was also unchanged after GluOC administration in mice of either genotype. In contrast, GluOC administration increased expression of the gene for the lipolysis-related protein perilipin in WAT of WT mice but not in that of the KO mice, indicating that lipolysis is upregulated by GluOC administration in WT animals, consistent with the observed changes in serum triglyceride and NEFA levels (Fig. 3B). Furthermore, GluOC administration increased expression of the genes for the inflammatory markers F4/80 and tumor necrosis factor-α (TNF-α) in WAT of GLP-1R KO mice, whereas expression of the TNF-α gene in WAT of WT mice was downregulated by GluOC treatment (Fig. 3C), consistent with our previous data (Kawakubo-Yasukochi *et al.* 2016). The number of crownlike structures (CLSs), which reflect macrophage infiltration around

dead adipocytes, was also significantly increased in WAT of GluOC-treated GLP-1R KO mice (Fig. 3D).

We previously showed that GluOC activates the transcription factor CREB (cAMP response element-binding protein) and increases the expression of the transcription factor FoxO1 (forkhead box protein O1) in 3T3-L1 adipocytes (Otani *et al.* 2015, 2018). On the other hand, GLP-1R signaling inhibits the action of FoxO1 by inducing its nuclear exclusion via activation of phosphatidylinositol 3-kinase (PI3K)–Akt signaling in pancreatic β-cells (Buteau *et al.* 2006). Given that FoxO1 plays an important role in regulating the expression of inflammation-related genes (Nakae *et al.* 2008), we examined the effects of GluOC in the absence or presence of the GLP-1R agonist exendin-4 on the expression

and phosphorylation state of FoxO1 in epididymal WAT explants (Fig. 3E). The abundance of FoxO1 was greater in WAT from the mutant mice than in that from WT mice, although the reason for this difference is unclear. GluOC treatment increased the expression of FoxO1 in WAT from mice of each genotype. GluOC also induced the phosphorylation of FoxO1, and this effect tended to be enhanced by exendin-4 in WAT of WT mice. However, in the presence of GluOC with or without exendin-4, FoxO1 phosphorylation levels were significantly lower in WAT from GLP-1R KO mice than in that from WT mice. Together, these findings suggested that oral GluOC administration in GLP-1R KO mice suppressed lipolysis and thereby triggered adipocyte hypertrophy and inflammation in WAT in part by preventing the nuclear exclusion and thereby promoting the activation of FoxO1.

GluOC administration promotes gluconeogenesis in GLP-1R KO mice

Given that hepatic steatosis is associated with insulin resistance, we subjected liver sections prepared from WT and GLP-1R KO mice to histological analysis.

Lipid accumulation was not apparent in the liver of mice of either genotype fed a normal diet (data not shown). We therefore examined animals fed a high-fat, high-sucrose diet after lactation, with administration of GluOC or saline from 8 to 15 weeks of age. Lipid accumulation was detected in the liver of both WT and GLP-1R KO mice, but whereas that in WT mice was markedly decreased by GluOC administration (Fig. 4A), consistent with previous observations (Ferron *et al.* 2012, Gupte *et al.* 2014), that in GLP-1R KO mice was increased (Fig. 4A).

Quantitative RT-PCR analysis of mice fed a normal diet revealed that expression of the genes for the gluconeogenic enzymes PEPCK (phosphoenolpyruvate carboxykinase) and G6PC (catalytic subunit of glucose-6-phosphatase) and for PPAR γ coactivator 1 α (PGC1 α), a coactivator for various transcription factors including FoxO1 in the activation of gluconeogenic genes (Altarejos & Montminy 2011), was significantly increased specifically in the liver of GluOC-administered GLP-1R KO mice (Fig. 4B). Consistent with these results, a PTT in mice treated with GluOC or saline for 4 weeks revealed that GluOC increased hepatic gluconeogenesis in GLP-1R KO mice but not in WT mice (Fig. 4C). These findings may account

Figure 4

GluOC administration promotes gluconeogenesis in GLP-1R KO mice. (A) Representative hematoxylin-eosin staining of liver sections from WT and GLP-1R KO mice fed a high-fat, high-sucrose diet after weaning and treated with GluOC or saline for 7 weeks (left). Bars, 100 μ m. The lipid area was measured in at least three sections for each mouse (right). (B) Quantitative RT-PCR analysis of relative mRNA abundance for the indicated proteins in the liver of WT or GLP-1R KO mice fed a normal diet and treated for 7 weeks with GluOC or saline. Results were normalized by the amount of β -actin mRNA. (C) PTT for WT and GLP-1R KO mice fed a normal diet and treated for 4 weeks with GluOC or saline. All data are means $\pm$ S.E.M. for eight to ten mice per group. * P < 0.05 versus corresponding value for saline-treated (control) mice as determined by Student's t test (A and B) or by two-way ANOVA followed by Bonferroni's *post hoc* test (C).

for the increased fasting blood glucose level (Fig. 1D) and glucose intolerance (Fig. 1F) induced in the KO mice by GluOC administration.

GLP-1R signaling suppresses GluOC-induced activation of FoxO1 and PGC1 α

The expression of gluconeogenic genes is regulated by the transcription factor CREB. CREB thus promotes gluconeogenesis by binding to the promoters of the PEPCK and G6PC genes as well as by increasing the expression of PGC1 α and members of the nuclear receptor subfamily 4 group A (NR4A) family of orphan nuclear hormone receptors (Herzig *et al.* 2001, Pei *et al.* 2006, Altarejos & Montminy 2011). Under conditions of prolonged food deprivation, PGC1 α acts as a coactivator for FoxO1, which also binds to the promoters of the PEPCK and G6PC genes, to further promote gluconeogenesis (Haeusler *et al.* 2010, Altarejos & Montminy 2011). We showed that GluOC increased FoxO1 expression in WAT explants from WT or GLP-1R KO mice, and that GLP-1R signaling appeared to trigger the phosphorylation-dependent nuclear exclusion of FoxO1 in WT but not in GLP-1R KO mice (Fig. 3E). Our observation that GluOC administration increased expression of the PGC1 α gene in the liver of GLP-1R KO but not WT mice (Fig. 4B) thus suggested that GluOC might stimulate gluconeogenesis by activating FoxO1 and PGC1 α in the KO mice and that GLP-1R signaling suppresses these effects. We therefore examined the phosphorylation state of FoxO1 in the liver (Fig. 5A). GluOC administration tended to increase the phosphorylation of FoxO1 in WT mice and to reduce it in GLP-1R KO mice, whereas the abundance of FoxO1 tended to be higher in the mutant mice. The ratio of phospho-FoxO1/total FoxO1 was significantly lower in GluOC-treated GLP-1R KO mice than in GluOC-treated WT mice. GluOC administration promoted CREB phosphorylation in the liver of mice of both genotypes (Fig. 5A).

We then studied the mouse hepatoma cell line Hepa1-6 to investigate further the relation between GLP-1R and GluOC signaling pathways. These cells were confirmed to express GLP-1R and the putative GluOC receptor GPRC6A by immunoblot analysis (data not shown). The GLP-1R agonist exendin-4 triggered FoxO1 phosphorylation in Hepa1-6 cells, whereas GluOC had no such effect (Fig. 5B). The exendin-4-induced phosphorylation of FoxO1 was abolished by the GLP-1R antagonist exendin(9–39), and the additional presence of GluOC further diminished the phosphorylation level of FoxO1 (Fig. 5B). We also found that PGC1 α was predominantly located in the

membrane fraction of Hepa1-6 cells and that stimulation with GluOC increased the abundance of PGC1 α in a concentration-dependent manner (Fig. 5C). This effect of GluOC was abolished in the presence of exendin-4 (Fig. 5C). Consistent with these findings, GluOC also induced PGC1 α expression in liver explants from WT mice, and this effect was attenuated by exendin-4 (Fig. 5D).

Figure 5

Lack of GLP-1R signaling promotes GluOC-induced FoxO1 and PGC1 α activity. (A) The liver of WT or GLP-1R KO mice that had been treated with GluOC or saline for 7 weeks was subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of FoxO1 and CREB as well as with those to β -actin (loading control). A representative blot as well as quantitative data for relative FoxO1 abundance and the relative phospho-FoxO1/total FoxO1 ratio determined by densitometry are shown. Quantitative data are means $\pm$ S.E.M. for five mice of each genotype. * P < 0.05 (Student's t test). (B) Hepa1-6 cells were incubated in the absence or presence of GluOC (10 ng/mL), exendin-4 (10 nmol/L), or exendin (9–39) (100 nmol/L) for 30 min, after which the relative ratio of Ser²⁵⁶-phosphorylated to total forms of FoxO1 was determined by HTRF assays. The effect of insulin (100 nmol/L) was examined as a positive control. Data are means $\pm$ S.E.M. from four independent experiments. † P < 0.05 for the indicated comparisons; * P < 0.05 versus the value for no addition (Student's t test). (C) Immunoblot analysis of PGC1 α in cytoplasmic (S, supernatant) and membrane (P, pellet) fractions prepared from Hepa1-6 cells after incubation for 48 h in the absence or presence of GluOC or exendin-4. The inhibitory effect of exendin-4 on GluOC-induced PGC1 α expression was concentration dependent (data not shown). The blot is representative of five independent experiments. (D) Immunoblot analysis of PGC1 α in explants of the liver from WT mice incubated in the absence or presence of GluOC (10 ng/mL) or exendin-4 (10 nmol/L) for 48 h. The blot is representative of three independent experiments.

Together, these results suggested that GLP-1R signaling suppresses GluOC-mediated promotion of the gluconeogenic program at the level of PGC1 α expression and FoxO1 activity.

GLP-1R signaling inhibits GluOC-mediated upregulation of gluconeogenesis

We next examined whether GluOC triggers up-regulation of gluconeogenesis in the absence of PI3K-Akt signaling. We treated WT mice with the PI3K inhibitor ZSTK474 in the absence or presence of GluOC, and then determined the effects of such treatment on gluconeogenesis. Daily oral administration of ZSTK474 for 8 days resulted in marked inhibition of Akt phosphorylation in the liver (Fig. 6A). GluOC administration appeared to promote gluconeogenesis as evaluated by the PTT in mice treated with ZSTK474 (Fig. 6B), similar to its effect in GLP-1R KO mice (Fig. 4C), whereas it had no such effect in mice treated with vehicle (Fig. 6B). We also examined the effect of PGC1 α up-regulation on gluconeogenesis. Daily administration of ZLN005, a transcriptional activator of PGC1 α expression, markedly increased the abundance of PGC1 α in the liver of WT mice (Fig. 6C) as well as promoted gluconeogenesis in these mice as revealed by a PTT (Fig. 6D). These results thus suggested that increased PGC1 α expression as well as attenuation of PI3K-Akt signaling in the liver contribute to up-regulation of gluconeogenesis in GLP-1R KO mice treated with GluOC.

Discussion

Over a decade has passed since the initial finding that bone-derived GluOC regulates systemic glucose and energy metabolism (Lee *et al.* 2007a). Subsequent gain- or loss-of-function studies with mutant mice as well as studies based on delivery of GluOC by an osmotic pump or intraperitoneal injection in mice have established that GluOC indeed improves metabolic state (Ferron *et al.* 2008, 2010, 2012, Fulzele *et al.* 2010, Mizokami *et al.* 2014). We have also previously shown that oral administration of GluOC was as effective as delivery by osmotic pump or intraperitoneal injection with regard to its effects on glucose and lipid metabolism. We also showed that oral administration of GluOC triggers an increase in the serum concentration of GLP-1 and that the metabolic effects of oral GluOC are mediated at least in part by GLP-1 (Mizokami *et al.* 2014). In the present study, we made use of GLP-1R KO mice to investigate further the role

Figure 6
Role of PI3K and PGC1 α in the regulation of GluOC-induced gluconeogenesis. (A) The liver of WT mice treated orally with GluOC (10 μ g/kg) or ZSTK474 (10 mg/kg) for 8 days as indicated was subjected to immunoblot analysis with antibodies to total of Ser⁴⁷³-phosphorylated forms of Akt or to total or phosphorylated (p) forms of FoxO1. (B) PTT in WT mice treated as in (A). (C) The liver of WT mice treated orally with vehicle or ZLN005 (15 mg/kg) for 8 days was subjected to immunoblot analysis with antibodies to PGC1 α . (D) PTT in WT mice treated as in (C). All PTT data are means $\pm$ S.E.M. for four to six mice per group. * P < 0.05, ** P < 0.01 versus corresponding value for saline-treated (B) or vehicle-treated (D) mice as determined by two-way ANOVA followed by Bonferroni's *post hoc* test. (E) Proposed mechanism by which GLP-1R signaling regulates GluOC-induced upregulation of gluconeogenesis. GLP-1R signaling, which is enhanced by GluOC stimulation, attenuates PGC1 α expression as well as FoxO1 action, resulting in suppression of the expression of gluconeogenic genes.

of GLP-1R signaling in the effects of GluOC. Our results have revealed that the beneficial effects of GluOC are not apparent in the mutant mice. Instead, GluOC induced an increase in the fasting blood glucose level, glucose intolerance, insulin resistance, and fat accumulation in adipose tissue and the liver of GLP-1R KO mice. These findings indicate that the protective effects of GluOC against obesity and insulin resistance require GLP-1R signaling, and that GluOC actually has metabolically detrimental effects in the absence of GLP-1R signaling. Our findings are not specific to the oral route of GluOC administration, given that daily intraperitoneal injection of GluOC (30 μ g/kg) for 4 weeks in GLP-1R KO mice gave rise to glucose intolerance similar to that apparent after oral administration (Supplementary Fig. 1).

^{125}I -labeled recombinant or synthetic GluOC was found to be absorbed into the general circulation after oral administration in mice (Mizokami *et al.* 2013). Such absorption was also demonstrated by detection of an increase in the serum concentration of GluOC with an ELISA after its oral administration (Mizokami *et al.* 2014). Similar absorption of GluOC was detected in GLP-1R KO mice (Supplementary Fig. 2). The increase in circulating GluOC level is likely responsible for the harmful effects of oral GluOC observed in the mutant mice, given that oral administration of a pork bone extract containing GluOC showed no detrimental effects in the KO mice (data not shown) but was as effective as recombinant GluOC in terms of its beneficial metabolic effects in WT mice in the absence of an increase in serum GluOC level but with an increase in serum GLP-1 concentration (Mizokami *et al.* 2016). The putative GluOC receptor GPRC6A is present in both basal and apical membranes of enteroendocrine cells and triggers the secretion of GLP-1 from these cells (Mizokami *et al.* 2014), and we indeed found that oral administration of GluOC increased the serum concentration of GLP-1 in the GLP-1R KO mice.

Daily oral administration of GluOC in GLP-1R KO mice increased the fasting blood glucose level and triggered both glucose intolerance and insulin resistance, in marked contrast to its effects in WT mice (Mizokami *et al.* 2014). Glucose intolerance is generally the result of impairment of glucose-stimulated insulin secretion, insulin resistance, or glucose overproduction. With regard to insulin secretion, we found that the serum insulin level was not affected by GluOC in GLP-1R KO mice either before or after glucose injection, and that the islet area of the mutant mice was similarly unaffected by GluOC administration. Given that GLP-1R signaling in pancreatic β -cells both promotes cell proliferation and suppresses apoptosis (Drucker 2003, Hansotia & Drucker 2005), these results indicate that GluOC administration itself in the absence of GLP-1R signaling has little effect on insulin secretion and islet size. Effects on β -cells thus do not appear to contribute substantially to the glucose intolerance apparent in GluOC-treated GLP-1R KO mice.

We then examined further whether systemic insulin resistance might have been responsible for the glucose intolerance of GluOC-treated GLP-1R KO mice. We found that GluOC triggered adipocyte hypertrophy in gonadal WAT and lipid deposition in the liver accompanied by an increase in proinflammatory gene expression in both tissues of the mutant mice. The expansion of adipocytes is closely associated with low-grade chronic inflammation characterized by production of proinflammatory cytokines

such as TNF- α that mediate insulin resistance (Hotamisligil *et al.* 1993, Hotamisligil 2006). Moreover, excess fat in the liver can also lead to inflammation that is associated with insulin resistance (Browning & Horton 2004, Maher *et al.* 2008, Zhang *et al.* 2013). The abundance of mRNAs for TNF- α and the macrophage marker F4/80 as well as the number of CLSs were markedly increased in WAT of GLP-1R KO mice by GluOC administration. Furthermore, FoxO1 function appeared to be increased in GluOC-treated WAT of GLP-1R KO mice as a result of an increase in its expression level and decrease in the extent of its phosphorylation. These observations suggest that GluOC administration induced inflammatory responses in adipose tissue and the liver that may have contributed to the insulin resistance and consequent glucose intolerance observed in GLP-1R KO mice.

We previously showed that stimulation of adipocytes with GluOC triggers intracellular cAMP accumulation and subsequent CREB activation (Otani *et al.* 2015), events shared with the signaling pathway for glucagon, a hormone that increases blood glucose levels (Altarejos & Montminy 2011). Activation of protein kinase A in the liver promotes CREB phosphorylation and consequent up-regulation of the expression of PGC1 α (Herzig *et al.* 2001) and FoxO1 (Wondisford *et al.* 2014). Under conditions of prolonged food deprivation, PGC1 α promotes the expression of gluconeogenic genes in hepatocytes in combination with FoxO1 (Herzig *et al.* 2001). On the other hand, GLP-1R signaling not only activates adenylate cyclase to produce cAMP in a similar manner to GluOC but also activates the protein kinase Akt, an event shared with the insulin signaling pathway, possibly leading to the phosphorylation and nuclear exclusion of FoxO1 (Kim & Egan 2008). Activation of FoxO1 has been proposed to be closely related to aspects of the pathogenesis of type 2 diabetes such as insulin resistance and excessive glucose production (Haeusler *et al.* 2014). For example, liver-specific ablation of FoxO1 in mice resulted in suppression of glucose production during fasting (Matsumoto *et al.* 2007). We have now found that GluOC administration reduced the level of FoxO1 phosphorylation in the liver of GLP-1R KO mice, whereas it tended to increase it in WT mice, probably as a result of the increase in serum GLP-1 levels. We also found that GluOC enhanced the inhibitory effect of the GLP-1R antagonist exendin (9–39) on the stimulation of FoxO1 phosphorylation induced by the GLP-1R agonist exendin-4 in Hepa1-6 cells. In addition, GluOC stimulated PGC1 α expression in Hepa1-6 cells as well as in liver explants in a manner sensitive to inhibition by exendin-4. Activation of PGC1 α

in the liver drives hepatic glucose production and is therefore a potential contributor to the development of obesity-related diabetes (Herzig *et al.* 2001, Finck & Kelly 2006, Altarejos & Montminy 2011). Indeed, the activity of PGC1 α is increased in the liver of several animal models of diabetes mellitus (Finck & Kelly 2006). Together, these findings suggest that hepatic glucose overproduction in the absence of the suppressive action of GLP-1 also contributes to the induction of glucose intolerance by GluOC in GLP-1R KO mice.

Adipose tissue-specific expression of a dominant negative form of FoxO1 in mice was shown to improve glucose tolerance and to downregulate TNF- α gene expression in WAT. Conversely, adipose tissue-specific expression of a constitutively active form of FoxO1 increased proinflammatory cytokine expression and induced systemic insulin resistance (Nakae *et al.* 2008). We recently showed that GluOC, especially at high concentrations, induced the expression of FoxO1 without affecting its phosphorylation in 3T3-L1 adipocytes (Otani *et al.* 2018). In contrast, GLP-1R signaling in adipocytes was found to suppress the expression of inflammatory cytokines such as TNF- α via inhibition of the nuclear factor (NF)-KB signaling pathway (Lee *et al.* 2012). The inflammatory phenotype of WAT induced by GluOC administration in GLP-1R KO mice may thus be due to enhanced FoxO1 activity and the absence of an anti-inflammatory effect of GLP-1R signaling.

In summary, we have here shown that the beneficial effects of GluOC on glucose and lipid metabolism are dependent on GLP-1R signaling and that GluOC itself has detrimental effects on glucose handling in the absence of such signaling, likely by inducing activation of FoxO1 and PGC1 α (Fig. 6E).

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-19-0288>.

Declaration of interest

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

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Author contribution statement

A.M. designed and performed most of the experiments, analyzed the results, and wrote the manuscript. S.M. performed the cell fractionation experiments. M.H. provided suggestions for experiments and data analysis as well as wrote the manuscript. S.M., J.G., T.K.-Y., T.O., H.T., and E.J. provided technical support, contributed to discussion, and reviewed the manuscript. A.M. and M.H. are the guarantors of this work and, as such, had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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