GLP-1-derived nonapeptide GLP-1(28–36)amide represses hepatic gluconeogenic gene expression and improves pyruvate tolerance in high-fat diet-fed mice

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1Division of Advanced Diagnostics, Toronto General Research Institute, University Health Network, Toronto, Ontario, Canada; 2Institute of Medical Science, University of Toronto, Toronto, Ontario, Canada; and 3Department of Physiology, University of Toronto, Toronto, Ontario, Canada

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Ip W, Shao W, Chiang YA, Jin T. GLP-1-derived nonapeptide GLP-1(28–36)amide represses hepatic gluconeogenic gene expression and improves pyruvate tolerance in high-fat diet-fed mice. Am J Physiol Endocrinol Metab 305: E1348–E1358, 2013. First published October 1, 2013; doi:10.1152/ajpendo.00376.2013.—Certain “degradation” products of GLP-1 were found to possess beneficial effects on metabolic homeostasis. Here, we investigated the function of the COOH-terminal fragment of GLP-1, the nonapeptide GLP-1(28–36)amide, in hepatic glucose metabolism. C57BL/6 mice fed a high-fat diet (HFD) for 13 wk were injected intraperitoneally with GLP-1(28–36)amide for 6 wk. A significant reduction in body weight gain in response to HFD feeding was observed in GLP-1(28–36)amide-treated mice. GLP-1(28–36)amide administration moderately improved glucose disposal during glucose tolerance test but more drastically attenuated glucose production during pyruvate tolerance test, which was associated with reduced hepatic expression of the gluconeogenic genes Pck1, G6pc, and Ppargc1a. Mice treated with GLP-1(28–36)amide exhibited increased phosphorylation of PKA targets, including cAMP response element-binding protein (CREB), ATF-1, and β-catenin. In primary hepatocytes, GLP-1(28–36)amide reduced glucose production and expression of Pck1, G6pc, and Ppargc1a, which was associated with increased cAMP content and PKA target phosphorylation. These effects were attenuated by PKA inhibition. We suggest that GLP-1(28–36)amide represses hepatic gluconeogenesis involving the activation of components of the cAMP/PKA signaling pathway. This study further confirmed that GLP-1(28–36)amide possesses therapeutic potential for diabetes and other metabolic disorders.

THE INCRETIN HORMONE GLUCAGON-LIKE PEPTIDE-1 (GLP-1) is secreted by intestinal endocrine L cells upon food intake and hormone regulation (2, 4, 25). GLP-1 stimulates insulin secretion in a glucose concentration-dependent manner and positively regulates pro-insulin gene expression (38) and β-cell survival, as well as proliferation of pancreatic β-cells (4, 25, 48). Extrapancreatic effects of GLP-1 have also been recognized, including the induction of satiety (47), central regulation of metabolism (18), improvement in cardiac and vascular function (6, 8), reduction in gastric emptying (24), and repression of hepatic glucose production (34), as reviewed in detail elsewhere (4, 48). Owing to its important function in controlling blood glucose homeostasis, two new categories of antidiabetic drugs have been developed in the past decade, namely GLP-1 receptor (GLP-1R) agonists and dipeptidyl peptidase-4 (DPP-4) inhibitors, both of which act to increase the activity of GLP-1 signaling to lower plasma glucose levels (4, 48).

The active form of GLP-1 is typically considered to be the GLP-1(7–36)amide and GLP-1(7–37) peptides, which are produced from the prohormone proglucagon via the cleavage by the prohormone convertase PC1/3 (11, 31). In the circulation, GLP-1(7–36)amide and GLP-1(7–37) have a very short half-life of around 2 min, as they are rapidly degraded by DPP-4 to GLP-1(9–36)amide or GLP-1(9–37). However, recent extensive studies have demonstrated that the previously considered inactive peptide GLP-1(9–36)amide may exert protective effects in the heart via both GLP-1R-dependent and -independent pathways (6, 26). Adding further to the complexity, GLP-1(7–36)amide or GLP-1(7–37) as well as GLP-1(9–36)amide or GLP-1(9–37) can also be cleaved by another peptidase, namely neutral endopeptidase (NEP) 24.11, near the COOH-terminal end to produce the nonapeptide GLP-1(28–36)amide [defined as GLP-1(28–36)a hereafter] (15). Recently, we and others have revealed the beneficial effects of GLP-1(28–36)a on pancreatic β-cells in vivo and in vitro (21, 37). In addition, this short fragment of GLP-1 was shown to mitigate the development of obesity and hepatic steatosis in mice in response to high-fat diet (HFD) consumption (45). Furthermore, GLP-1(28–36)a was suggested to target mitochondria and to attenuate glucose production in mouse primary hepatocytes, although the underlying mechanisms of these beneficial effects are currently unclear (42).

In the current study, we aimed to determine the biological effects of GLP-1(28–36)a in the liver using the HFD-induced obese mouse model and investigate the mechanism by which GLP-1(28–36)a represses hepatic glucose production and gluconeogenic gene expression both in vivo and in isolated mouse primary hepatocytes.

MATERIALS AND METHODS

Reagents. The nonapeptide GLP-1(28–36)amide (sequence: FIAWLVKGR-amide) was synthesized by Biomatik (Wilmington, DE) and assessed to be >98% valid peptide by HPLC and mass spectrometry analyses (37). The adenylyl cyclase activator forskolin and the protein kinase A (PKA) inhibitor H89 were obtained from Sigma-Aldrich (St. Louis, MO).

Animals. Male C57BL/6 mice were purchased from Charles River Laboratories (St. Laurent, QC, Canada) and housed on a 12:12-h light-dark cycle at ambient room temperature with free access to food and water. At the age of 8 wk, mice were separated into individual cages and placed on a HFD (Bio-Serv, Frenchtown, NJ) consisting of 60% calories derived from fat. Following a HFD feeding period of 13 wk, administration of PBS (vehicle) or GLP-1(28–36)a (18.5 nmol/kg body wt) was commenced via intraperitoneal (ip) injections daily.
between 4 and 6 PM while maintaining HFD feeding. Body weight and food weight were measured weekly for 4 wk. Food weight was converted into energy using the indicated conversion factor of 5.49 kcal/g. Feeding efficiency was calculated as a ratio of body weight gain to energy consumption. At 4 and 5 wk following the commencement of injections, the intraperitoneal glucose and pyruvate tolerance tests, respectively, were performed. Mice were euthanized for collection of tissues 6 wk following the beginning of injections. The final ip injections were administered the day before euthanization. All animal experiments were performed in accordance with the Guide for Care and Use of Experimental Animals (University Health Network, Toronto, ON, Canada), and all experimental animal protocols were approved by the Institutional Animal Care and Use Committee of the University Health Network.

**Intraperitoneal glucose and pyruvate tolerance tests.** Mice were fasted for 16 or 6 h prior to ip injection of glucose (1 g/kg body wt) or sodium pyruvate (2 g/kg body wt), respectively (51). Plasma glucose was measured in blood samples collected from the tail vein by a glucometer (Roche Accu-Chek).

**Insulin measurement.** Plasma insulin levels were measured using the Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem, Downers Grove, IL) according to the manufacturer’s instructions.

**Isolation of mouse primary hepatocytes and cell culture.** Primary hepatocytes were generated according to the described method, with some minor modifications (52). Briefly, the hepatic portal vein was cannulated with a 25G Vacutainer butterfly needle in anaesthetized (5% isoflurane) 6- to 8-wk-old chow-fed male C57BL/6 mice. Anterograde perfusion of the liver with Hanks’ Balanced Salt Solution was performed using a peristaltic pump (Fisher Scientific, Toronto, ON, Canada), followed by digestion with DMEM containing 5.5 mmol/l glucose, 15 mmol/l HEPES, 1% penicillin-streptomycin, and type IV collagenase (100 collagen digestion units/ml, Sigma/Aldrich). Hepatocytes were extracted from digested livers, filtered through a 70-μm membrane, washed three times in DMEM, and resuspended in DMEM containing 25 mmol/l glucose, 1 mmol/l sodium lactate, 15 mmol/l HEPES, 1% penicillin-streptomycin, 100 mmol/l dexamethasone, and 10% fetal bovine serum (FBS) prior to 240,000 cells/well being seeded in 12-well plates coated with type I collagen (BD Biosciences, Franklin Lakes, NJ). After 4 h, the media were replaced with 15 mmol/l sodium lactate, 5 mmol/l glucose, 5 mmol/l HEPES, 1% penicillin-streptomycin, and 10 mmol/l dexamethasone. All experiments were performed on the following day after seeding. The human hepatic carcinoma cell line HepG2 was cultured in normal DMEM containing 25 mmol/l glucose, 1% penicillin-streptomycin, and 10% FBS, as described previously (17).

**Real-time RT-PCR analysis.** Total RNA isolation and cDNA synthesis were performed as described previously (17). Real-time RT-PCR analysis was performed using the iTag Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) and the 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA). Specific primers for RT-PCR in this study are listed as follows: Actb forward, 5′-TCTAGTGAAGTGACGTGTA-3′; Actb reverse, 5′-CTTACAGCA-TTTGGCGTG-3′; Pck1 forward, 5′-CATAACGGTCTGGACTTC-3′; Pck1 reverse, 5′-GAATGGGATGACATACATGGTGCG-3′; G6pc forward, 5′-CCTGGGGCTGCTGGTGGC-3′; G6pc reverse, 5′-AAGACCCACAAATGGAGGCT-3′; Crebl forward 5′-GTGAGGAAGGTCTGTACCC-3′; Crebl reverse, 5′-ACCTGGGCTATGGTCGAT-3′; Pparα forward, 5′-GTCCTCTCCTCATGCGAC-3′; Pparα reverse, 5′-TAGCTGAGCTGCGTGCG-3′.

**Western blotting and antibodies.** Whole cell lysates were prepared from liver tissue or cultured cells and subjected to SDS-PAGE, as described previously (17). Protein concentration was measured using the Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL) for chemiluminescent detection. Densitometric analysis was performed using Image J.

**Glucose production assay.** Primary hepatocytes were depleted of glycogen by incubating in glucose-free DMEM for 2 h. Cells were then washed with PBS and incubated in glucose production buffer (DMEM without glucose, FBS, or phenol red and supplemented with 10 mmol/l HEPES, 10 mmol/l dexamethasone, 1% streptomycin-penicillin, 2 mmol/l sodium pyruvate, and 20 mmol/l sodium lactate) for 2 h. The medium was collected for measurement of glucose using a glucose assay kit provided by Sigma-Aldrich, followed by normalization to cellular protein content.

**CAMP assay.** The collection and measurement of cAMP from hepatocytes was performed using the cyclic AMP Enzyme Immunoassay Kit (Cayman Chemical, Ann Arbor, MI), following the directions of the manufacturer (37).

**Luciferase reporter analysis.** Transfection of 2 μg/well PEPCk(−595) to +57 bp)-LUC plasmid DNA construct (17) was achieved using 3 μg of polyethyleneimine (Sigma) in 12-well plates. Luciferase (LUC) reporter analyses were performed using firefly luciferin substrate (BioShop, Burlington, ON, Canada), as described previously (49).

**Statistical analysis.** Quantitative results are expressed as means ± SE. Significance was determined using the Student t-test or one-way ANOVA with Bonferroni post hoc test as appropriate for single or multiple comparisons. Differences were considered statistically significant when P < 0.05.

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**RESULTS**

GLP-1(28–36)AMIDE REPRESSION HEPATIC GLUCONEOGENESIS

GLP-1(28–36) reduces body weight gain in response to HFD feeding. Thirteen weeks after the onset of HFD feeding, the body weight of mice surpassed 40 g (Fig. 1A). These HFD-induced obese mice were then subjected to either PBS vehicle or GLP-1(28–36) ip injection. In agreement with a previous report by Tomas et al. (45), mice treated with GLP-1(28–36) were lower in body weight after 4 wk of treatment compared with their vehicle-control-injected counterparts, although the difference was not statistically significant (Fig. 1A).

Body weight gain, however, was significantly reduced with GLP-1(28–36) treatment (Fig. 1B). In our study setting, a difference in energy consumption between the two groups of mice was not observed (Fig. 1, C and D), which is in contrast to the report by Tomas et al. (45) in which GLP-1(28–36) was administered via osmotic pumps 4–7 wk after HFD feeding. A reduced gain in body weight despite similar intake of energy demonstrates the lower feeding efficiency of the GLP-1(28–36)-treated mice (Fig. 1E). Thus, GLP-1(28–36) administration rendered the mice resistant to the further development of obesity in response to HFD consumption.

GLP-1(28–36) improves pyruvate tolerance in HFD-fed mice. To determine whether GLP-1(28–36) administration affects carbohydrate metabolism in the diet-induced mouse model of type 2 diabetes, we performed two tolerance tests. Mice treated with GLP-1(28–36) showed significantly reduced plasma glucose levels 10 min after an ip glucose challenge was delivered (glucose tolerance test) (Fig. 2A). However, there was no significant difference in the glucose area under the curve within the 90-min experimental period, arguing against the existence of an effect of GLP-1(28–36) on modulating whole body glucose disposal, under the dosage, route, or period parameters of GLP-1(28–36) administration used in current study (Fig. 2B). On the other hand, we observed a profound effect of GLP-1(28–36) on the tolerance of obese
mice to a challenge of pyruvate, a major substrate for hepatic gluconeogenesis, as the mice treated with GLP-1(28–36)a displayed significantly reduced plasma glucose levels at both the 10- and 90-min time points following pyruvate challenge (Fig. 2C). Thus, the time at which we terminated the experiment represents a point when GLP-1(28–36)a exerts its repressive effect on hepatic glucose production ahead of its overall effect on glucose homeostasis, which was observed by Tomas et al. (45) in long-term treatment. Because plasma insulin levels in these two groups of mice were comparable, the improvement in pyruvate tolerance by GLP-1(28–36)a administration in this HFD-induced obese mouse model is unlikely due to a secondary effect in pancreatic β-cells (Fig. 2D).

GLP-1(28–36)a represses hepatic gluconeogenesis. Improved pyruvate tolerance in GLP-1(28–36)a-injected mice suggests that this nonapeptide may repress hepatic glucose production. Hence, we assessed the expression of hepatic gluconeogenic genes in the two groups of mice following 6 wk of ip injections. Indeed, the mRNA levels of Pck1 and G6pc, which encode the two rate-limiting enzymes of gluconeogen-
GLP-1(28–36)amide represses hepatic gluconeogenesis

We then conducted in vitro assessments of the effect of GLP-1(28–36)amide on hepatic gluconeogenesis. Mouse primary hepatocytes were isolated from adult chow-fed C57BL/6 mice, followed by vehicle (acetonitrile) or GLP-1(28–36)amide treatment. Hepatocytes treated with GLP-1(28–36)amide for 4 h secreted significantly less glucose into the surrounding media (Fig. 3D). In addition, we observed a significant reduction of Pck1 and G6pc mRNA levels following GLP-1(28–36)amide treatment (Fig. 3E), in agreement with our in vivo findings (Fig. 3A). The profound repressive effect of GLP-1(28–36)amide on Pck1 and G6pc mRNA expression as well as PEPCK levels was also evident when we performed the same examinations in the human hepatic HepG2 cell line (Fig. 3, F and G). In addition, GLP-1(28–36)amide repressed the expression of the PEPCK-luciferase fusion gene construct (17) when it was transfected into HepG2 cells (Fig. 3H). Furthermore, GLP-1(28–36)amide treatment attenuated forskolin-stimulated gluconeogenic gene expression in primary hepatocytes (Fig. 3f). The repressive effect of GLP-1(28–36)amide on gluconeogenesis observed in the in vitro setting further supports the notion for the existence of a direct effect of this nonapeptide in hepatocytes independent of insulin or extrahepatic mechanisms.

GLP-1(28–36)amide activates cAMP/PKA signaling. In pancreatic β-cells, GLP-1 is known to exert its incretin effect via cAMP/PKA activation. In hepatocytes, the cAMP/PKA signaling pathway is also importantly involved in the regulation of gluconeogenesis in response to fasting and fed cues in which the activation of cAMP/PKA typically leads to a stimulation, but not the repression, of hepatic gluconeogenesis (1). Here, we assessed the effect of GLP-1(28–36)amide on the cAMP/PKA signaling cascade. Increased levels of the phosphorylation of known targets of PKA, including cAMP response element-binding protein (CREB; Ser133), activating transcription factor-1 (ATF-1; Ser63), and β-catenin (Ser675) were observed in the livers of mice treated with GLP-1(28–36)amide (Fig. 4, A and B). Moreover, protein levels of total CREB were also increased significantly following GLP-1(28–36)amide administration (Fig. 4, A and B), although we did not detect appreciable changes in Creb1 mRNA levels (Fig. 4C). Nevertheless, phosphorylated CREB (Ser133) levels remained significantly enhanced when normalized to total CREB levels (Fig. 4D).

We then determined the direct in vitro effect of GLP-1(28–36)amide on cAMP/PKA signaling in mouse primary hepatocytes. Indeed, 4-h treatment of GLP-1(28–36)amide stimulated the phosphorylation of CREB (Ser133), ATF-1 (Ser63), and β-catenin (Ser675) in primary hepatocytes (Fig. 5, A and B), although the stimulation on CREB and ATF-1 phosphorylation was less substantial compared with the effect of forskolin, a chemical commonly utilized as an activator of adenyl cyclase. Furthermore, we noticed a repressive effect of forskolin on total CREB levels, which was not observed upon GLP-1(28–36)amide treatment (Fig. 5, A and B). When CREB Ser133 phosphorylation levels were normalized to total CREB levels, the activations by forskolin and GLP-1(28–36)amide were 9.4- and 1.6-fold, respectively (Fig. 5C). Again, we did not observe any effect of GLP-1(28–36)amide on Creb1 mRNA levels (Fig. 5D). Surprisingly, the stimulatory effects of forskolin and GLP-1(28–36)amide on cytoplasmic cAMP levels in primary hepatocytes were comparable (Fig. 5E). In the HepG2

Fig. 2. GLP-1(28–36)amide improves pyruvate tolerance in HFD-fed mice. A–C, mice treated with either PBS vehicle (n = 4) or GLP-1(28–36)amide (n = 3) were starved for 16 or 6 h prior to ip injection of glucose (1 g/kg) or pyruvate (2 g/kg) for glucose (A) and pyruvate tolerance tests (C) after 4 and 5 wk of injections, respectively, with area under the curve (AUC) for glucose tolerance test shown (B). D: fasting plasma insulin levels were measured. *P < 0.05. IPGTT, intraperitoneal glucose tolerance test.
Fig. 3. GLP-1(28–36)a reduces hepatic gluconeogenic gene expression. A and B: liver tissue was collected following 6 wk of treatment of either PBS vehicle (n = 4) or GLP-1(28–36)a (n = 3) for measurement of Pck1 and G6pc levels by real-time RT-PCR (A) and phospho-enolpyruvate carboxykinase (PEPCK) levels by Western blotting (B). C: densitometric quantification of PEPCK normalized to β-actin. D and E: primary hepatocytes isolated from chow-fed C57BL/6 mice were treated with acetonitrile (vehicle) or 100 nmol/l GLP-1(28–36)a (n ≥ 3), followed by measurement of glucose output after 4 h (D) or Pck1 and G6pc mRNA expression after 8 h (E). F and G: HepG2 cells were treated with acetonitrile (vehicle) or 100 nmol/l GLP-1(28–36)a (n ≥ 3) for 8 h, followed by measurement of Pck1 and G6pc mRNA expression by real-time RT-PCR (F) and for measurement of PEPCK protein levels by Western blotting (G). H: HepG2 cells were transfected with the PEPCK-luciferase (~593 to +67 bp) reporter construct. Forty-eight hours after the transfection, cells were treated with acetonitrile (vehicle) or 100 nmol/l GLP-1(28–36)a for 4 h (n ≥ 3), followed by cell harvesting and luciferase activity assessment. I: mouse primary hepatocytes were treated with acetonitrile (vehicle), 100 nmol/l GLP-1(28–36)a, 25 μmol/l forskolin (FSK), or both GLP-1(28–36)a and FSK for 8 h (n ≥ 3), followed by RNA extraction and the measurement of Pck1 and G6pc expression by real time RT-PCR. *P < 0.05.
cell line, the phosphorylation of CREB (Ser\textsuperscript{133}), ATF-1 (Ser\textsuperscript{63}), and β-catenin (Ser\textsuperscript{675}) was elevated in a time-dependent manner following GLP-1(28–36)a treatment, with statistical significance observed at the 120- and 240-min time points (Fig. 5, F and G). No notable differences in the phosphorylation status of FoxO1 (Ser\textsuperscript{256}), AMP-activated protein kinase (AMPK; Thr\textsuperscript{172}), Akt (Ser\textsuperscript{473}), or GSK-3β (Ser\textsuperscript{21/9}) could be detected in primary hepatocytes upon treatment with GLP-1(28–36)a (data not shown).

GLP-1(28–36)a represses the expression of peroxisome proliferator-activated receptor-γ coactivator-1α. Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) can be upregulated by CREB-mediated cAMP signaling, and it serves as a transcriptional coactivator for PEPCK and glucose-6-phosphatase in concert with CREB and FoxO1 (14). We found that the mRNA level of the gene for PGC-1α, Ppargc1a, was significantly lower in the livers of GLP-1(28–36)a-injected mice compared with that of the vehicle-treated mice (Fig. 6A).

This downregulatory effect of GLP-1(28–36)a on Ppargc1a mRNA levels was then verified in vitro in mouse primary hepatocytes (Fig. 6B). In contrast, forskolin stimulated Ppargc1a mRNA expression levels in primary hepatocytes, whereas GLP-1(28–36)a attenuated the stimulatory effect of forskolin (Fig. 6B). These observations collectively suggest that GLP-1(28–36)a may downregulate hepatic gluconeogenesis via a mechanism upstream of Ppargc1a.

Inhibition of PKA attenuates the effect of GLP-1(28–36)a on gluconeogenic gene expression. To further explore the involvement of cAMP/PKA signaling in mediating the repressive effect of GLP-1(28–36)a on hepatic gluconeogenic gene expression, we assessed the effect of PKA inhibition in mouse primary hepatocytes. Pretreating primary hepatocytes with the PKA inhibitor H89 attenuated the ability of 4-h treatment of GLP-1(28–36)a or forskolin to stimulate CREB (Ser\textsuperscript{133}), ATF-1 (Ser\textsuperscript{63}), and β-catenin (Ser\textsuperscript{675}) phosphorylation (Fig. 7, A and B). The pretreatment of primary hepatocytes with the same dosage of H89 also blocked or significantly attenuated the repressive effect of GLP-1(28–36)a on Pck1 and Ppargc1a expression levels, whereas the effect on restoring G6pc expression was not significant (Fig. 7C). In the HepG2 cell line, inhibition of PKA also blunted the stimulatory effect of GLP-1(28–36)a on PKA target phosphorylation (Fig. 7D).

**DISCUSSION**

Although NEP 24.11 has been known for nearly two decades to cleave GLP-1 into GLP-1(28–36)a (15), the potential biological function or “pharmacological” effect of this nonapep-
Investigations have shown that GLP-1(28–36)α prevented obesity and hepatic steatosis in the HFD-induced diabetic mouse model (42). The present study characterized the biological actions and initiated the exploration of the underlying mechanism of GLP-1(28–36)α in hepatocytes. We verified its effect on attenuating body weight gain and revealed for the first time the improvement of tolerance to a challenge of pyruvate independent of plasma insulin elevation. Although we cannot exclude the potential contribution of reduced adipose tissue to a reduction in hepatic glucose production, we assessed the direct effect of GLP-1(28–36)α on hepatic gluconeogenic gene expression. In both in vivo and in vitro settings, GLP-1(28–36)α repressed the expression of two gluconeogenic enzymes and the gluconeogenic transcriptional coactivator PGC-1α.

Despite great technical difficulty, an effort has been made to determine whether the incretin hormone GLP-1 also possesses p-catenin (Ser675), p-CREB (Ser133), p-A T/1 (Ser63), CREB, β-actin.

**Table 1.**

- **Column A:**
  - p-CREB (Ser133)
  - p-ATF-1 (Ser63)
  - p-β-catenin (Ser675)
  - CREB
  - β-actin

- **Column B:**
  - p-CREB (Ser133)
  - p-ATF-1 (Ser63)
  - p-β-catenin (Ser675)
  - CREB

- **Column C:**
  - p-CREB (Ser133)
  - p-ATF-1 (Ser63)
  - p-β-catenin (Ser675)
  - CREB

- **Column D:**
  - Creb1 mRNA levels (fold)
  - p-CREB (Ser133) density (fold)
  - p-ATF-1 (Ser63) density (fold)
  - p-β-catenin (Ser675) density (fold)

**Fig. 5.** GLP-1(28–36)α stimulates the cAMP/PKA signaling pathway in primary hepatocytes. A: primary hepatocytes isolated from chow-fed C57BL/6 mice were treated with acetonitrile (vehicle), 25 μmol/l FSK, or 100 nmol/l GLP-1(28–36)α for 4 h (n ≥ 3) for analysis of PKA target phosphorylation in whole cell lysates by Western blotting. B: quantification of protein phosphorylation in A normalized to β-actin. C: quantification of phosphorylated CREB (Ser133) normalized to total CREB. D: mouse primary hepatocytes were cultured and treated for 8 h (n ≥ 3), followed by measurement of Creb1 mRNA expression.

**Fig. 6.** HepG2 cells were treated with 100 nmol/l GLP-1(28–36)α for the indicated times (n ≥ 3). Cells were harvested for measurement of PKA target phosphorylation by Western blotting with indicated antibodies. G: quantification of protein phosphorylation levels in F normalized to β-actin. *P < 0.05.
an insulin-independent effect on hepatic glucose production. An early report in 1994 showed that infusion of GLP-1 resulted in lower rates of hepatic glucose production in human subjects (16). However, this outcome was attributed entirely to the incretin effect of GLP-1 (16). Other studies attempted to rule out the pancreatic effects of GLP-1 by utilizing the pancreatic clamp technique in which somatostatin is co-infused. When the pancreatic effects of GLP-1 were eliminated, insulin-independent effects of GLP-1 on peripheral glucose turnover could not be demonstrated clearly in several investigations (19, 31, 41). However, one study showed that GLP-1 produced a 17% decrease in glucose appearance, with no significant effect on glucose disappearance, suggesting that GLP-1 suppresses hepatic glucose production independent of its action on pancreatic hormones (34). In accord with this report, a very recent human pancreatic clamp study demonstrated that infusion of physiological postprandial levels of GLP-1 reduced hepatic glucose production by 27% but had no effect on whole body glucose disposal (36). The in vivo and in vitro repressive effect of GLP-1 (28–36) amid on hepatic gluconeogenic gene expression observed in the current study supports the notion that a cleavage product of GLP-1, GLP-1 (28–36) amid, may possess the ability to reduce hepatic gluconeogenesis.

Another interesting question is whether GLP-1 reduces hepatic glucose production via direct activation of its receptor on hepatocytes or indirectly through a neurally mediated pathway or via a yet-to-be determined alternative mechanism. Early investigations indicated the lack of GLP-1R in the liver (4, 7), whereas a few recent studies have suggested the existence of a functional GLP-1R on hepatocytes (3, 12, 13, 39). A very recent study pointed out that antibodies utilized for GLP-1R detection are unreliable because they generated false-positive signals (32). Regardless of these controversies, we present evidence here that GLP-1 (28–36) amid represses hepatic gluconeogenic gene expression directly in primary hepatocytes, leading to reduced glucose output. Administration of this peptide in diet-induced obese mice reduced hepatic gluconeogenic gene expression.

NEP 24.11 efficiently cleaves GLP-1 (7–36) amid or GLP-1 (9–36) amid into GLP-1 (28–36) amid (15) or the even smaller COOH-terminal peptide GLP-1 (32–36) amid (44). When the NEP 24.11 inhibitor candoxatril was administered in pigs undergoing GLP-1 infusion, glucose tolerance improved, likely through the improvement of GLP-1 pharmacokinetics (33). NEP 24.11 inhibition also led to improved vascular and neural complications in HFD-induced and streptozotocin-induced diabetic rats (9, 10, 30). These observations collectively suggest that the prevention of NEP 24.11-mediated GLP-1 degradation confers benefits to glucose homeostasis, which is conceptually in contradiction to the notion that GLP-1 (28–36) amid exerts beneficial effects in glucose metabolism. A potential explanation would be that the full-length GLP-1 and its “degradation” COOH-terminal fragments execute their beneficial function via different molecular mechanisms with and without the involvement of GLP-1R. This could be due to the utilization of a yet-to-be identified receptor or receptor-independent mechanism such as mitochondrial targeting (42). Because the method for specific detection of circulating endogenous GLP-1 (28–36) amid has yet to be developed, we may propose only that GLP-1 (28–36) amid possesses pharmacological effects. Determination of the physiological function of GLP-1 COOH-terminal fragments requires further investigations.

Numerous studies have revealed that degradation products of GLP-1 do exert biological activities. GLP-1 (9–36) amid increases myocardial glucose uptake and improves left ventricular performance in dogs with dilated cardiomyopathy (27) and mediates cytoprotection in cardiomyocytes through a pathway independent of GLP-1R (5, 6). In the brain, GLP-1 (9–36) amid rescues synaptic plasticity and memory deficits in a mouse model of Alzheimer’s disease (22). In the HFD-induced obese mouse model, GLP-1 (9–36) amid was also shown to repress glucose production and inhibit body weight gain (43, 46). To date, GLP-1 (28–36) amid has been shown to reduce body weight gain and hepatic steatosis in the HFD-induced mouse model (42, 45) and confer protective and proliferative benefits in pancreatic β-cells both in vitro and in the streptozotocin-induced type 1 diabetes model (21, 37). We have suggested that in pancreatic β-cells the beneficial effect of GLP-1 (28–36) amid is due at least partially to the activation of the cAMP/ PKA/β-catenin signaling pathway (37). As a cofactor of the stress signaling pathway effector FoxO, β-catenin is consid-
ered a positive stimulator of hepatic gluconeogenesis (20). As a cofactor of the Wnt signaling pathway effector TCF7L2, β-catenin activation may lead to the repression of hepatic gluconeogenesis, which has been suggested by a few recent studies (17, 28, 29).

Glucagon is secreted during fasting to enhance hepatic glucose production for energy supply to the rest of the body. Glucagon exerts its function by activating the cAMP/PKA signaling cascade, leading to the activation of CREB, which then serves to upregulate gluconeogenic gene expression in concert with TORC2, CBP, FoxO1, PGC-1α, and others (23). We show here that GLP-1(28–36)α activates the same cAMP/PKA/CREB pathway as glucagon but exerts the opposite effect on gluconeogenesis. Nevertheless, we found that GLP-1(28–36)α inhibited PGC-1α expression level, in contrast with the known stimulatory effect of glucagon and forskolin on PGC-1α expression (Fig. 6B) (50). The mechanism by which the hepatocyte differentiates between the signals from glucagon and GLP-1 or GLP-1(28–36)α is worthy of further investigation. Activation of a plasma membrane G protein-coupled receptor by glucagon or GLP-1 typically results in PKA target phosphorylation within 5–30 min (3). The relatively slow stimulation of PKA target phosphorylation by GLP-1(28–36)α that we observed in HepG2 cells (Fig. 5, F and G) leads us to speculate that the nonapeptide may activate a compartmentalized cAMP pathway in the mitochondria (3, 35). It is possible that activation of the cAMP pathway by glucagon at
the plasma membrane may result in increased gluconeogenesis, whereas activation of the cAMP pathway in the mitochondrial matrix by GLP-1(28–36)α may result in decreased gluconeogenesis (35, 42). In addition, although we did not observe any effect of GLP-1(28–36)α on AMPK phosphorylation (data not shown), we cannot completely exclude the possibility that GLP-1(28–36)α increases phosphorylated CREB via AMPK activation (40).

In summary, we have verified the repressive effect of GLP-1(28–36)α on body weight gain in the diet-induced obese model and revealed for the first time that this is associated with improved tolerance to pyruvate challenge in vivo. Our in vitro analyses suggest that GLP-1(28–36)α activates the cAMP/ PKA/CREB signaling pathway but has the unique ability to repress gluconeogenic gene expression and glucose output. These observations further support the notion that the nonapeptide GLP-1(28–36)α possesses therapeutic potential for diabetes and other metabolic disorders (21, 42, 45).

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DISCLOSURES
The authors declare no conflicts of interests, financial or otherwise.

AUTHOR CONTRIBUTIONS
W.I., W.S., Y.-t.C., and T.J. edited and revised the manuscript; W.I., W.S., Y.-t.C., T.J. analyzed the data; W.I., W.S., and T.J. interpreted the results of the analyses; W.I., W.S., Y.-t.C., and T.J. approved the final version of the manuscript; T.J. contributed to the conception and design of the research.

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