GLP-1(32-36)amide Pentapeptide Increases Basal Energy Expenditure and Inhibits Weight Gain In Obese Mice

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ABSTRACT

The prevalence of obesity-related diabetes is increasing world-wide. Here we report the identification of a pentapeptide, GLP-1(32-36)amide (LVKGRamide), derived from the glucoincretin hormone GLP-1, that increases basal energy expenditure, curtails the development of obesity, insulin resistance, diabetes, and hepatic steatosis in a diet-induced obese mice. The peptapeptide inhibited weight gain, reduced fat mass without change in energy intake, and increased basal energy expenditure independent of physical activity. Analyses of tissues from peptide-treated mice reveal increased expression of UCP-1 and UCP-3 in brown adipose tissue and increased UCP-3 and inhibition of acetylCoA carboxylase in skeletal muscle, findings consistent with increased fatty acid oxidation and thermogenesis. In palmitate-treated C2C12 skeletal myotubes GLP-1(32-36)amide activated AMP kinase and inhibited acetyl CoA carboxylase suggesting activation of fat metabolism in response to energy depletion. By mass spectroscopy the pentapeptide is rapidly formed from GLP-1(9-36)amide, the major form of GLP-1 in the circulation of mice. These findings suggest that the reported insulin-like actions of GLP-1 receptor agonists that occur independently of the GLP-1 receptor might be mediated by the pentapeptide, and the previously reported nonapeptide (FIAWLVKGRamide). We propose that by increasing basal energy expenditure GLP-1(32-36)amide might be a useful treatment for human obesity and associated metabolic disorders.

INTRODUCTION

The prevalence of obesity and associated diabetes and metabolic disorders has increased dramatically over the past 20 years. Obesity-related metabolic disorders are manifested as insulin resistance, diabetes, hypertension, hyper-lipidemia, hepatic steatosis, and accelerated atherosclerosis. Although the pathophysiology underlying these disorders remains unknown, insulin resistance appears to be a major contributor (1-4). Currently, effective treatments for obesity are not available. Here we report that a pentapeptide, GLP-1(32-36)amide [LVKGRamide], derived from the C-terminus of the glucoincretin hormone glucagon-like peptide-1 (GLP-1), increases basal energy expenditure, inhibits weight gain, reduces fat mass, and the development of insulin resistance and hepatic steatosis when administered to diet-induced obese mice.

GLP-1 receptor agonists are in use for the treatment of type 2 diabetes based on their stimulation of insulin secretion and a lowering of glucagon levels (5-6). The full-length receptor agonist GLP-1(7-36)amide, is rapidly inactivated in the circulation via cleavages by the endopeptidases diaminopeptidyl peptidase-4 (Dpp4) and a neutral endopeptidase (NEP 24.11) known as neprilysin (7,8). The cleavage of GLP-1(7-36)amide by
Dpp4 gives rise to GLP-1(9-36)amide devoid of insulin-releasing activities and NEP 24.11 cleaves GLP-1 into several small peptides (9). Earlier we proposed that cleavages of GLP-1 by the endopeptidase nepriysin generate a pentapeptide and a nonapeptide, GLP-1(28-36)amide (FIAWLVKGRamide) with insulin-like actions on insulin-responsive target tissues (10). GLP-1(9-36)amide, the product of cleavage of GLP-1 by Dpp4, exerts insulin-like and anti-oxidant actions on the heart (11-14) and vasculature (15) and suppresses glucose production both in isolated mouse hepatocytes (16) and in obese, insulin-resistant human subjects without effect on plasma insulin levels (17). The administration of GLP-1(9-36)amide or the nonapeptide to obese mice increases energy expenditure, curtails weight gain, and inhibits the development of insulin resistance, diabetes and hepatic steatosis (18,19). In obese mice the nonapeptide promotes glucose disposal and beta cell regeneration in streptozotocin-induced diabetic mice (21), protects beta cells against glucolipotoxic stress (22), and suppresses hepatic glucose production in obese mice (23). The nonapeptide appears to enter isolated insulin-resistant mouse hepatocytes in vitro, targets to mitochondria, and suppresses glucose production, reactive oxygen species, and restores cellular ATP levels that are depleted by oxidative stress (20, 22). The actions of the peptides appear to occur preferentially in obese, insulin-resistant compared to lean, insulin-sensitive human subjects (17) or mice (19) and occur by mechanisms independent of the GLP-1 receptor (10, 24). Because both peptides are major end-products of the proteolysis of GLP-1 by neprilysin (9) in addition to the nonapeptide, we investigated the actions of the pentapeptide (10), in diet-induced obese mice. We find that the pentapeptide increases basal energy expenditure, curtails weight gain, and inhibits the development of insulin resistance, diabetes, and hepatic steatosis independently of energy intake or physical activity. The expression of the uncoupling proteins UCP-1 and UCP-3 is increased in the brown adipose tissue, as is UCP-3 in skeletal muscle of obese mice in response to GLP-1(32-36)amide, findings consistent with an increase in basal energy expenditure. In addition, the activity of acetylCoA carboxylase is inhibited in the skeletal muscle of the peptide-treated mice indicative of an increase in fatty acid oxidation. In C2C12 skeletal myotubes in vitro GLP-1(32-36)amide activates AMPK and inhibits ACC further supporting increased fatty acid oxidation. These findings of novel energy-modulatory actions of the pentapeptide in obese mice suggest the possibility that it might be an effective treatments for obesity-related diabetes.

RESEARCH DESIGN AND METHODS

Peptide synthesis

GLP-1(LVKGRamide) was synthesized by solid phase methods. Peptide was purified to single component on HPLC and by Mass spectroscopy the product molecular weight was 571.38.
Animal studies
C57Bl/6J male mice of 6 to 9 weeks of age were obtained from Jackson Laboratories, Bar Harbor, ME. Mice were housed 4 per cage under a light/dark cycle of 12 hours with free access to food and water, except when noted. Mice were fed a regular laboratory chow (LFD) (10 Kcal % fat, Research diets, # D12450B) or a high-fat diet (HFD) (60 Kcal % fat, Research Diets, #D12492) throughout the studies. All procedures were approved by the MGH Institutional Animal Care Use Committee.

Formation of GLP-1(32-36)amide from GLP-1(9-36)amide in plasma
Mice of 21 weeks of age on a LFD or HFD for 15 weeks, were given intraperitoneal injections of 500 µg of GLP1(9-36)amide. Blood samples were collected from the tail vein at 0, 5, 15 minutes and by cardiac puncture at 30 minutes following the administration of GLP1(9-36)amide. Plasmas were analyzed for the formation of GLP1(32-36)amide. Peptide extraction was performed by acetonitrile precipitation (25, 26) and detection by liquid chromatography-mass spectroscopy with an Agilent 1200 HPLC and Applied Biosystems Qtrap 4000 mass spectrometer with a Turbo Spray ion source.

GLP-1(32-36)amide infusion studies
At 6 to 10 weeks of age mice were placed on a HFD for 7 to 22 weeks. At 13 to 28 weeks of age mice were randomized by average body weight (vehicle group: 38.1 g and peptide treated group: 37.3 g) and were implanted with mini-osmopumps (Alzet #1004) to infuse vehicle (0.154 M NaCl/0.2% human serum albumin) or GLP-1(32-36)amide (50-70 nmols/Kg BW/day) over 12 to 19-weeks [19]. During the infusion studies body weights and food intake were monitored weekly and twice weekly, respectively. Fat and lean body mass was assessed on week 15 of the pentapeptide infusions using a whole-body dual X –ray scanner (Lunar Piximar, GE Medical Systems, Wauwatosa, WT).

Feed efficiency
Feed Efficiency (FE) (27) was evaluated during the infusions of vehicle or peptide as described (19).

Liver histology and triglyceride content and plasma triglyceride levels.
Tissue staining (H&E) of livers, and levels of triglycerides in liver extracts and plasmas (28) of mice at the end of the 15-weeks infusion and 32 weeks on high fat were determined as described earlier (18).

Indirect calorimetry, and locomotor activity
Mice at 30 weeks on HFD and 12 weeks infusions with vehicle or GLP-1(32-36)amide were single caged for 5 days before placing them into individual metabolic chambers for 2 days for acclimatization, with free access to food and water. Oxygen consumption was determined by indirect calorimetry (TSE systems, Inc). Oxygen consumption was measured at 5-minute intervals for a total of 120 hours. The results presented correspond to the last 6 hours of the light cycle and 12 hours of the dark cycle. Loco-motor activity was determined by counting infrared light beam breaks (InfraMot Phenomaster/Labmaster cage system). Results are presented for the last 6 hours of the light cycle and complete dark cycle and the average activity during both dark and light phases.

**Fasting plasma glucose and insulin, glucose and insulin tolerance tests**

Plasma glucose and insulin levels were obtained in fasted (16 hrs) mice fed a HFD for 29 weeks and treated with pentapeptide or vehicle for 12 weeks. Glucose tolerance tests were performed on mice at 24 weeks on HFD and 16 week infusions with vehicle or GLP-1(32-36)amide and were fasted overnight (16 hrs). Glucose (2g/Kg lean mass) was administrated by intraperitoneal injection. Blood samples were drawn from the tail vein at 0, 10, 30, 60, 120 and 140 minutes following glucose administration. Insulin tolerance tests were done on mice at 38 weeks on HFD and 19 week infusions with either vehicle or GLP-1(32-36)amide. Mice were fasted for 4 hours and given 0.75 U/Kg lean mass insulin by intraperitoneal injection. Blood glucose was monitored at 0, 15, 30, 45 and 60 minutes following insulin injection. Blood draws were obtained by tail nick and plasma glucose was measured using a glucometer OneTouch UltraMini glucose meter (LifeScan, Johnson & Johnson Company, Philadelphia, PA). Fasting insulin levels were determined using the rat/mouse Elisa kit (Crystal Chem, Downers, IL). Area under curve (AUC) was determined by standard methods.

**C2C12 myotube studies**

C2C12 skeletal muscle cells were obtained from ATTC (Manassas, VA) and cultured in growing media (DMEM media: 25mM glucose, 10% FBS and 1% PS) until differentiation when cells were cultured in differentiation media (DMEM: 25mM glucose, 2% HS and 1% PS). Experiments were carried out at day 5 of differentiation. C2C12 myotubes were fasted for 6 h (no serum added) before incubation with GLP-1(32-36)amide (100 pM) and/or 0.2 mM palmitate: 0.034 mM BSA (5:1) for 16 h in fasting media.

**Western immunoblot analysis**

Cells lysates of C2C12 myotubes (15 µg), gastrocnemius muscle homogenates (15 µg) and brown adipose tissue homogenates (10 µg) from in vivo mouse studies were resolved by SDS-PAGE and proteins were analyzed by the Western immunoblot procedure as described (16). See Figure 5 legend.
**ROS determination**

C2C12 myoblasts were seeded in 12 well fluorescence plates grown until confluence and differentiated to myotubes for 5 days. ROS levels were determined in palmitate-treated myotubes as described (20). See Figure 6 legend.

**Quantitative RT-PCR assays of mRNA levels**

Mouse tissues were removed and immediately submerged in cold RNA later (Qiagen, CA, USA) or frozen in liquid nitrogen. Total RNA was isolated and analyzed by quantitative PCR (CFX 384, BioRad, Richmond, CA) as described (16). The primers used for QPCR are available upon request.

**Statistics and Data Analyses**

Data are presented as mean ± standard error of the mean. Statistical analyses were performed using Student’s t-test. P values of less than 0.05 were considered to be statistically significant.

**RESULTS**

**Formation of GLP-1(32-36)amide**

GLP-1(7-36)amide is rapidly cleaved in vivo (1-2 min) by dipeptidyl peptidase-4 (Dpp4) to GLP-1(9-36)amide, a peptide devoid of significant insulinotropic activities (8) and is the major form of GLP-1 in the circulation. GLP-1(9-36)amide has insulin-like actions on heart, vasculature, and liver (10, 16-19, 24, 28). Additional cleavages of GLP-1 occur by the endopeptidase (NEP 24.11) in vitro resulting in the formation of the nona and pentapeptides, GLP-1(28-36)amide and GLP-1(32-36)amide as major products, respectively [9] (Figure 1A). To examine the formation of the pentapeptide by cleavage from GLP-1(9-36)amide in lean and obese mice we administered GLP-1(9-36)amide by intraperitoneal injection and collected blood samples at 0, 5, 15, 30 min for analyses of the levels of the peptides by semi-quantitative liquid chromatography-mass spectroscopy. GLP-1(9-36)amide appears in plasma within 5 and was undetectable by 30 minutes, respectively, after the injection of GLP-1(9-36)amide (Figure 1B). At 5 min the plasma concentrations of GLP-1(9-36)amide, GLP-1(28-36)amide, and GLP-1(32-36)amide in lean mice were 3.3 μM, 4 μM (data not shown), and 80 nM, respectively. (Figure 1B). The plasma levels of the nona and pentapeptides are 5 to 10-fold lower in the obese compared to the lean mice.

GLP-1(32-36)amide prevents the development of diet-induced-obesity and hepatic steatosis in high fat-fed mice
At 6 to 10 weeks of age mice were placed on a HFD for 17 to 18 weeks before beginning the continuous infusions of vehicle or GLP-1(32-36)amide for 12 to 16 weeks. The infusion of GLP-1(32-36)amide for 12 weeks attenuated the development of DIO (Figure 2A). Mice receiving GLP-1(32-36)amide for 10 weeks gained less weight compared to vehicle control at which time the peptide-treated mice began to lose weight (Figure 2B). The average weekly change in body weight gain of mice infused with the pentapeptide was 50% less than that of mice receiving control vehicle over the ten-week infusion period (Figure 2C). Caloric intakes in peptide and vehicle treated mice were similar (Figure 2D). The feed efficiency (FE) indicated that 80% less of the caloric intake went into body weight in mice treated with the pentapeptide compared to vehicle (vehicle control, 0.016 +/- 0.0018; pentapeptide treated, 0.003 +/- 0.00075; p = 0.06).

Measurement of body fat and lean mass by DXA between vehicle and GLP-1(32-36)amide mice treated for 16 weeks showed a 40% reduction in fat mass in the GLP-1(32-36)amide infused mice compared to vehicle and no significant changes in lean mass (Figure 2E and F). GLP-1(32-36)amide decreases liver fat accumulation when compared to vehicle as shown in the H&E staining liver tissue sections (Figure 2G). The triglyceride contents in livers of mice receiving GLP-1(32-36)amide decreased by 60% when compared to vehicle, and were similar to that of mice fed a regular chow diet; 21.13 ± 5.6 mg/ml in livers of mice infused with GLP-1(32-36)amide compared to 21.6 ± 7 mg/ml protein in mice fed regular chow (Figure 2H). Moreover, the pentapeptide reduced both plasma glycerol levels; 0.12 ± 0.025 mg/ml compared to 0.25 ± 0.04 mg/ml vehicle control (p< 0.02), and triglyceride levels; 0.33± 0.06 mg/ml compared to 0.65 ± 0.06 vehicle control (p< 8E-04) (Table 1).

GLP-1(32-36)amide attenuates the development of fasting hyperglycemia, hyperinsulinemia, and improves insulin sensitivity in HFD mice

Before beginning the treatment of DIO mice the fasting glucose and insulin levels were similar amongst the two pre-treatment groups of mice: vehicle mice (glucose: 4.8 ± 1.08 mM, insulin: 209.2 ± 89.17 pM) and peptide-treated mice (glucose: 4.3 ± 0.2 mM, insulin: 116.7 ± 38 pM). The infusions of GLP-1(32-36)amide for 12 weeks attenuated the progression of fasting hyperglycemia and hyperinsulinemia (Table 1).

To further determine the actions of GLP-1(32-36)amide on glucose homeostasis in DIO mice, intraperitoneal glucose and insulin tolerance tests were performed in mice receiving continuous infusions of the pentapeptide for 10 weeks. Mice fed a high fat diet for 24 weeks exhibited impaired glucose tolerance following the glucose challenge. The infusion of GLP-1(32-36)amide for 16-weeks resulted in an improvement in the glycemic excursion over the 140 minutes of the glucose tolerance test (left panel Figure 3A). Fasting plasma glucose and insulin levels at the beginning of the glucose tolerance challenge were reduced in GLP-1(32-36)amide infused mice, glucose: 169.16±7.5 mg/dl compared to 221.5±8.6 mg/dl vehicle control (p<4.3 E-04)
and insulin: $409.7 \pm 65.2$ pg/ml compared to $654.8 \pm 129$ pg/ml vehicle control. Mice receiving pentapeptide showed a lower AUC indicating improved glycemic control (upper right panel Figure 3). Plasma insulin concentrations 10 minutes after glucose administration were higher in mice receiving GLP-1(32-36)amide (lower right panel Figure 3), a finding consistent with an increase in the insulin/glucose ratio in GLP-1(32-36)amide infused mice (pentapetide: $4.7 \pm 1.78$ compared to vehicle $1.5 \pm 0.52$, $p<0.048$). Likewise, mice fed a HFD for 38-weeks and receiving GLP-1(32-36)amide for 19-weeks, showed a more pronounced glucose-lowering response compared to that of vehicle-treated mice when challenged with an insulin tolerance test. These results suggest that mice infused with the pentapeptide are more insulin responsive when compared to the glucose response of vehicle-treated mice (right panel Figure 3B). The AUC glucose following insulin administration was lower in mice receiving GLP-1(32-36)amide than in vehicle-treated mice (left panel Figure 3B). Thusly, GLP-1(32-36)amide administration protects the obese mice from the development of insulin resistance.

GLP-1(32-36)amide increases basal metabolic rate

To determine whether the attenuation of weight gain by GLP-1(32-36)amide was associated with changes in energy homeostasis, indirect calorimetry was performed in mice fed a HFD for 30-weeks and at the end of 12-weeks of treatment with GLP-1(32-36)amide. GLP-1(32-36)amide increased both total body and lean body resting oxygen consumption during the light and dark cycles (Figure 4A left and 4B left panels, respectively). Total body oxygen consumption rates increased by 22% and 33% over the light and dark phases, respectively (Figure 4A, right panel). No differences were observed between control and pentapeptide-treated mice in either energy intake (data not shown) or in physical activity (Figure 4C). Thus, these findings indicate that the regulatory effects of GLP-1(32-36)amide on energy homeostasis are associated with an increase in basal energy expenditure.

GLP-1(32-36)amide increases the expression of uncoupling proteins in brown adipose tissue (BAT) and increases fatty acid oxidation in skeletal muscle

Because of the finding of an increase in basal energy expenditure in the pentapeptide-treated mice we examined BAT, a known thermogenic tissue (30, 31) for effects of GLP-1(32-36)amide on the expression of uncoupling proteins. The protein expression of both UCP-1 and UCP-3 in BAT was significantly increased in mice infused with GLP-1(32-36)amide for 15 weeks and on a high fat diet for 32 weeks when compared to obese vehicle control mice (Figure 5A and B). Likewise, mRNA expression for both UCP-1 and UCP-3 was increased by ~1.5 to 2-fold in mice receiving pentapeptide (Figure 5C). The expression of the transcriptional co-activator PGC-1α, known to modulate the expression of UCP-1, oxidative metabolism, and thermogenesis in brown
adipose tissue [32], was increased in BAT of mice receiving the pentapeptide. Likewise, the expression of PPARα, known to have important actions in the modulation of fatty acid oxidation [33], was increased (Figure 5C). Cytochrome C expression was also increased, although the increase did not reach statistical significance (Figure 5C). Because skeletal muscle accounts for 20% of overall energy metabolism [34], we explored the role of skeletal muscle on energy homeostasis in obese mice treated with the pentapeptide. The ratio of the phosphorylated to total Acetyl-CoA carboxylase (pACC/ACC) was increased in gastrocnemius skeletal muscle of mice receiving GLP-1(32-36)amide for 16 weeks and on HFD for 24 weeks when compared to vehicle-treated mice (Figure 5D and E). No changes were observed in skeletal muscle for UCP-3 protein (Figure 5D), although UCP-3 mRNA was increased (Figure 5F). Muscles of GLP-1(32-36)amide-treated mice showed either no change or a moderate increase in the transcription of genes associated with oxidative metabolism and/or mitochondrial biogenesis, such as PGC-1α, PPARα/γ, COX4i1 and COX7a1 (Figure 5F). The expression of SCD-1 however was substantially decreased suggesting a decrease in muscle adiposity, possibly due to an increase in fatty acid metabolism (Figure 5F). Increased fatty acid oxidation in skeletal muscle might provide the fuel required to fulfill the energetic demands imposed by the increased energy expenditure in mice treated with the pentapeptide.

Similar findings of increased muscle oxidative phosphorylation were observed in C2C12 myotubes. The incubation of myotubes with GLP-1(32-36)amide (100 pM) for 16 hours increased both the ratio between phosphorylation of AMPK and the total expression of AMPK (pAMPK/AMPK) and the phosphorylation (inhibitory) of its downstream target acetylCoA carboxylase (ACC) without differences in the expression of total ACC (Figure 6A-C). Likewise, GLP-1(32-36)amide restored the decreased phosphorylation of both AMPK and ACC phosphorylation in C2C12 myotubes in which oxidative metabolism had been impaired by pre-treatment of the myotubes with palmitate for 16 hours (Figure 6C-D). In addition, GLP-1(32-36)amide reduces intracellular levels of reactive oxygen species (ROS) induced in C2C12 myotubes by their treatment with palmitate (Figure 6E).

**DISCUSSION**

Herein we describe the identification and the partial characterization of the actions of a pentapeptide, GLP-1(32-36)amide, LVKGRamide, derived from the C-terminus of the gluco-regulatory hormone GLP-1. The pentapeptide appears to arise in the circulation via the cleavage of GLP-1 by an endopeptidase such as neprilysin (NEP 24.11) (9), or a related endopeptidase. Mass spectrometry detected the appearance of the pentapeptide in plasma following the intraperitoneal injection of GLP-1(9-36)amide in mice. The pentapeptide
was postulated to exist in vivo based on the evidence that, neprilysin generates the pentapeptide from GLP-1 (9) and a candoxatril-sensitive endopeptidase, decreases levels of infused GLP-1 in the circulation of pigs (29).

The pentapeptide is biologically active when infused into DIO mice and when added to cultured C2C12 myotubes. Pentapeptide infusions in DIO mice increase basal energy expenditure without effect on energy intake, inhibit weight gain, and the development of insulin resistance and hepatic steatosis. Several observations indicate that increased energy expenditure is a major action of the pentapeptide. Pentapeptide-treated mice showed a 20-30% increase in oxygen consumption without significant changes in physical activity or food intake. Food intake was no different among vehicle control and treated group. The feed efficiency showed that approximately 80% less of the caloric intake went into body weight in mice infused with the pentapeptide compared to vehicle control mice indicating the existence of a substantial dissipation of the intake of caloric energy in pentapeptide-treated mice. Furthermore the pentapeptide increases the expression of the uncoupling proteins UCP-1 and UCP-3 in BAT, and decreases the activity of ACC in skeletal muscle of mice indicative of increased fatty acid oxidation (35, 36). The physiological mechanisms of GLP-1(32-36)amide on the inhibition of weight gain differ from those of GLP-1 receptor agonists. Receptor agonists inhibit energy intake with little or no effects on energy expenditure (37-40). Notably, basal energy expenditure is increased in mice lacking the GLP-1 receptor (41) suggesting a lowering of energy expenditure in response to receptor activation.

Although the precise mechanisms by which GLP-1(32-36)amide promotes energy expenditure remain unknown, one mechanism might be the uncoupling of mitochondrial respiration. Uncoupling dissipates energy in the form of heat (thermogenesis), reduces mitochondrial energy overload and membrane charge, and the formation of ROS (oxidative stress) (42). The findings of increased UCP-1 and UCP-3 expression in BAT and UCP-3 expression in skeletal muscle of mice treated with GLP-1(32-36)amide is consistent with an uncoupling of respiration. We suggest that the activation UCPs in BAT, and the resulting increased energy dissipation, contributes to the metabolic phenotype observed in the pentapeptide-treated mice. The transplantation of BAT tissue into either normal or DIO mice improves glucose tolerance, increases insulin sensitivity, and lowers body weight and fat mass (43).

A notable finding was the normalization of the hepatic triglyceride content in obese mice infused with the pentapeptide to levels found in lean mice fed a normal chow diet. This finding suggests that the peptide prevented, or possibly reversed, the development of hepatic steatosis. Although we did not measure triglyceride levels in the livers of the obese mice prior to starting the infusions of the pentapeptide, it is likely that the mice had fatty livers after 17 weeks on the high-fat diet, the time that the infusions began. Male C57bl/6 mice characteristically develop simple hepatic steatosis and elevated levels of liver triglycerides by at least 12 weeks on the high fat diet (60 kCal fat) used in our studies (18).
In studies of cultured C2C12 skeletal myotubes the pentapeptide activates the energy sensor AMP kinase (AMPK), inhibits acetylCoA carboxylase (ACC), and prevents an increase in levels of reactive oxygen species (ROS) in response to palmitate treatment. AMPK phosphorylates and inhibits ACC. ACC regulates the anabolism and catabolism of fatty acids via the formation of malonylCoA, both a substrate for the synthesis of fatty acids and an inhibitor of fatty acid oxidation. Inhibition of ACC reduces malonylCoA levels and relieves inhibition of carnitine palmitoyl transferase-1 required for the transport and oxidation of fatty acids in mitochondria. AMPK-mediated inhibition of ACC activity by the pentapeptide and enhanced fatty acid oxidation might account for the reduction in body fat mass observed in response to the pentapeptide.

The pentapeptide increased the transcriptional expression of UCP3 in C2C12 myotubes and the skeletal muscle. Although the role of UCP3 in skeletal muscle is controversial (44) it is believed that UCP3 serves as a fatty acid transporter (30, 31). The transport of fatty acids from the mitochondrial matrix to the outside of the inner membrane captures protons that are then carried retrograde back through the membrane to the matrix, thereby causing a proton leakage and energy dissipation (31,43).

We speculate that the de novo lipogenesis pathway might be modulated by GLP-1(32-36)amide. The findings of increased basal energy expenditure, activation of AMPK, and increased fatty acid oxidation in pentapeptide-treated obese mice, coupled with an enhanced glucose disposal in hyperglycemic dogs in response (45), raises the possibility that GLP-1(32-36)amide might act via AMPK-responsive pathways similar to those activated by leptin that induce de novo lipogenesis in skeletal muscle (35). De novo lipogenesis would explain the findings of the effects of GLP-1(32-36)amide on brown adipose tissue and skeletal muscle as de novo lipogenesis, mediated by AMPK, links glucose uptake, fatty acid synthesis and oxidation, with futile substrate cycling and facultative thermogenesis (32).

In summary, the findings described here suggest that GLP-1(32-36)amide might be an effective treatment for obesity and its related metabolic disorders in humans. In mice the pentapeptide appears to modulate mitochondrial energy metabolism by increasing energy expenditure and fat metabolism resulting in a reduction in rates of weight gain, body fat mass, oxidative stress, and insulin resistance. The pentapeptide is a product of the GLP-1 hormone naturally produced in the body. No adverse effects were observed in mice receiving continuous infusion of the the pentapeptide for up to 16 weeks, suggesting that the pentapeptide might have a favorable safety profile, as well as efficacy in curtailing weight gain and possibly even promoting weight loss in humans. Whether or not the pentapeptide is active in humans, as well as in mice and dogs, awaits the results of clinical trials.

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**Author contributions.** ET conceived of and carried out the experiments, evaluated data, and prepared the figures and the manuscript. VS and KM participated in the experimental work, and in the analysis of the data. AK synthesized, purified, and characterized the pentapeptide. PE and WWB conducted and planned the mass spectroscopy experiments. JFH conceived of experiments, evaluated data, and contributed to the manuscript. JFH is the guarantor of this work and, as such, had full access to all of the data in the study and takes full responsibility for the integrity of the data and the accuracy of the data analyses.

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**REFERENCES**

1. Tateya S, Kim F, Tamori Y. Recent advances in obesity-induced inflammation and insulin resistance. Front Endocrinol (Lausanne) 2013;4:93:1-14
20. Tomas E, Stanojevic V, Habener, JF. GLP-1-Derived Nonapeptide GLP-1(28-36)amide targets to mitochondria and suppresses glucose production and oxidative stress in isolated mouse hepatocytes. Regul Pept 2011;167:177-184
27. Parekh PI, Petro AE, Tiller JM, Feinglos MN, Surwit RS. Reversal of diet-induced obesity and diabetes in C57BL/6J mice. Metabolism 1998;47:1089-1096
34. Rofle DF, Brown GC. Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiol Rev 1997;77:731-758

FIGURE LEGENDS

Figure 1. GLP-1 (32-36)amide pentapeptide derived from GLP-1(9-36)amide. A. Formation of the C-terminal peptides GLP-1(9-36)amide, GLP-1(28-36)amide, and GLP-1(32-36)amide. B. Intraperitoneal administration of GLP-1(9-36)amide (500 ug) to mice results in the formation and appearance of GLP-1(32-36)amide in the plasma. Data obtained by a semi-quantitative LC-MS assay (See Methods).

Figure 2. Continuous infusions of GLP-1(32-36)amide prevent the development of diet-induced obesity and attenuate the development of hepatic steatosis in mice fed a high fat diet (HFD). A. Photographs of
representative mice infused with GLP-1(32-36)amide for 13–weeks. B. Curtailment of weekly body weight gain in mice fed a HFD in response to 12-week infusions of GLP-1(32-36)amide (n=6 per group), *p<0.05, weeks-5-8 ** p<0.04, week-9 *** p< 0.03, week-10 # p< 0.01 peptide versus vehicle. C. Weekly incremental mean changes in body weights of data shown in (B), *p< 0.04 peptide vs. vehicle (n=6 per group). D. Energy intake during 10-week period of vehicle and peptide infusions (n=6 per group). F. Body composition of mice after 16-week infusions of vehicle and GLP-1-(32-36)amide (n=6 per group), *p< 0.008 peptide versus vehicle. G. Fat mass expressed as % of vehicle control from (F), *p< 1.3E-03 peptide versus vehicle. H. Triglyceride content of samples of livers in mice infused with GLP-1(32-36)amide for 15-weeks (n=6 per group). Values are represented as % vehicle control, *p< 0.005 peptide versus vehicle. Liver triglyceride levels in HFD-fed mice receiving peptide infusions decrease to levels similar to those seen in mice fed a regular chow diet (21.5 ± 7.04).

Figure 3. GLP-1(32-36)amide administration improves insulin sensitivity in diet-induced obese mice. A. Plasma glucose levels during an intraperitoneal glucose tolerance test (ipGTT) in mice fed a HFD for 24-weeks and 16-weeks continuous infusions of vehicle or GLP-1(32-36)amide (n=5-6 per group), #p< 2.8 E-04, *p< 0.0058, **p< 0.0028, ###p< 1.9E-05, ###*p< 0.0018, **##*p<0.0014 peptide versus vehicle, Right upper panel: quantification of area under the curve (AUC) for the total glycemic excursions (n=5-6 per group), *p<0.046 , Right lower panel: Plasma insulin concentrations obtained 10 minutes after glucose administration (n=5-6 per group). B. Plasma glucose levels during an insulin tolerance test (ITT) in mice fed a HFD for 38-weeks and 19-weeks continuous infusions of vehicle or GLP-1(32-36)amide (n=4 per group), ***p< 1.2E-03, **p< 2.05E-03, ****p< 0.09E-03, *p< 1.3E-03 peptide versus vehicle, and Right panel: quantification of AUC for the total glycemic excursions (n=4 per group), *p< 0.02.

Figure 4. Increased energy expenditure in mice infused with GLP-1(32-36)amide and no changes in physical activity during the dark cycle A. Whole body oxygen consumption (VO2) in mice fed a very high fat diet for 30 weeks and 12 week infusions of peptide (n=2-3 per group), Left panel: oxygen consumption was measured during the light and dark cycles, Right panel: total oxygen consumption over the light and dark phases, *p< 5.9E-43, **p< 4 E-66 peptide vs. vehicle control. B. Lean oxygen consumption (n=2-3 per group), Left panel: oxygen consumption was measured during light and dark cycles, Right panel: total oxygen consumption over light and dark phases, p< 1 E-20, **p< 1 E-35 peptide versus vehicle. D. Assessment of physical activity, Left panel: physical activity was measured during the light and dark cycles (n=2-3 per group), Right panel: total physical activity over the light and dark cycles, *p< 0.04 peptide versus vehicle. The results correspond to the last 6 hours of the light cycle and 12 hours of the dark cycle (A, B left panel) and the average values throughout the light and dark phases (A, B right panel).
**Figure 5.** GLP-1(32-36)amide induces changes in the expression and/or phosphorylation of genes associated with thermogenesis and oxidative metabolism in brown adipose tissue and skeletal muscle. **A.** UCP-1 and UCP-3 protein expression are increased in brown adipose tissue (BAT) of mice receiving GLP-1(32-36)amide for 15 weeks and fed a high fat diet for 32 weeks as shown in Western blot analysis (n=4-6 per group). **B.**

Quantification of the Western blot images (A) of UCP-1, upper panel, and UCP-3, lower panel (n=4-6 per group), *p* < 0.028, **p* < 0.03 peptide versus vehicle. **C.** Quantitative polymerase chain reaction (qPCR) showed enhanced expression of genes involved in thermogenesis and oxidative phosphorylation in brown adipose tissue of mice infused with GLP-1(32-36)amide for 15 weeks (n=6 per group), *p* < 0.04, **p* < 0.02, ***p* < 0.01 peptide versus vehicle. **D.** Phosphorylation of acetylCoA carboxylase (ACC) at Ser79 compared to total expression of ACC is enhanced in skeletal muscle of mice infused with GLP-1(32-36)amide for 16 weeks and 24 weeks on a high fat diet as shown in Western blot analysis (n=5-7 per group). No differences in UCP-3 protein levels were observed in skeletal muscle of mice receiving peptide compared to vehicle. **E.** Western blot images quantification (C) ratio between phospho-ACC and total ACC (pACC/ACC), *p* < 0.04 peptide versus vehicle. **F.**

qPCR analysis of oxidative metabolism genes in gastrocnemius skeletal muscle of mice infused with GLP-1(32-36)amide for 15 weeks (n=6 per group), *p* < 0.05, **p* < 0.02.

Western immunoblots were assayed with Anti-phospho-acetyl CoA carboxylase (Ser-79) from Millipore (Billerica, MA), Phospho-AMPK(alpha) (Thr-172) and AMPKalpha from Cell Signaling (Danvers, MA), Anti-phospho-acetyl CoA carboxylase (Ser-79) from Millipore (Billerica, MA), Actin from Sigma, UCP-1 and UCP-3 from Abcam (Cambridge, MA) and Tubulin (Santa Cruz Biotechnology) followed by secondary antibodies conjugated to horseradish peroxidase (Amersham Biosciences or Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Protein bands were visualized by enhanced chemiluminescence and quantified by laser densitometry in the linear range. Results from cell culture and in vivo studies were normalized by actin and tubulin respectively. Vertical lines are inserted to mark sites where the source autoradiographs were spliced to remove lanes of the intervening samples. Two lanes were removed in A because of gel loading artefacts. Four lanes were removed from the right of D because they contained samples unrelated to the subject matter of this report. All sample lanes were run on the same gel at the same time and all immunoblot analyses were conducted from the same samples.

**Figure 6.** GLP-1(32-36)amide activates AMPK signaling, protects against palmitate-induced oxidative stress by restoring AMPK signaling, and reduces ROS levels in C2C12 myotubes. **A.** The incubation of C2C12 myotubes with GLP-1(32-36)amide (100 pM) for 16 hours increases the phosphorylation of AMPK at Thr-172 and its downstream target ACC at Ser-79 as shown in Western blot analysis. **B.** Quantification of the Western blot images of phospho-ACC (pACC) in the panel left panel and the ratio between phospho-AMPK and total AMPK (pAMPK/AMPK) in the right panel. Data are the means ± S.E, *p* < 0.05, #*p* < 3E-03 peptide versus
vehicle. C. GLP-1(32-36)amide (100 pM) restores the phosphorylation of ACC at Ser-79 reduced in palmitate-induced oxidative stress as shown in Western blot analysis. D. Quantification of the Western blot images of the phospho-ACC (pACC) [left panel] and ratio pAMPK/AMPK [right panel]. Data are the means± S.E, * p<0.05 peptide versus vehicle. E. Levels of ROS of C2C12 myotubes exposed to palmitate:BSA (0.2mM: 0.034mM) for 16 hours in the absence or presence of GLP-1(32-36)amide (100pM). Data are expressed as % production compared to palmitate:bsa, and are the means ± S.E, **p<7.6E-04 peptide versus vehicle.

For ROS determination at day 5 of differentiation cells were cultured in serum free media for 6 hours prior the incubation with 0.2 mM:0.034 mM palmitate:BSA for 16 hours in the presence or absence of GLP-1(32-36)amide (100pM) in fasting media. The following day cells were rinsed twice in PBS and loaded with 5μM MitoSOX reagent (Invitrogen,) in HBBS/Ca/Mg for10 minutes at 37°C. Cells then were washed in Hanks Balanced Salt Solution (HBBS)/Ca/Mg for 10 min and the same media was added during readings at 485/595nm.

Vertical lines are inserted to mark sites where the source autoradiographs were spliced to remove lanes of the intervening samples. Six and three lanes were removed from A and C, respectively, that contained concentrations of the pentapeptide apart from the focus of the study. All sample lanes were run on the same gel at the same time and all immunoblot analyses were conducted from the same samples.

**Table 1.** Continuous infusions of GLP-1(32-36)amide improve fasting plasma levels of glycerol and triglycerides, and reduces fasting plasma levels of glucose and insulin. Plasma glycerol and triglycerides levels after 16-week infusions of peptide in mice fed a HFD for 24-weeks were decreased to levels of mice fed a low fat normal chow diet (0.12 ± 0.02 and 0.58 ± 0.06 respectively) (n=6 per group), # p<0.02 and ## p< 0.008, peptide versus vehicle. Plasma glucose and insulin at 12-weeks infusions with GLP-1(32-36)amide and 29-weeks in HFD, * p< 1.3E-04 peptide versus vehicle.
A. DPP IV → NEP 24.11

GLP-1(7-36)a: HA EGTFTSDVSSYLEGQAAKE FIAW LVKGR-amide

GLP-1(9-36)a: EGTFTSDVSSYLEGQAAKE FIAW LVKGR-amide

GLP-1(28-36)a: FIAW LVKGR-amide

GLP-1(32-36)a: LVKGR-amide

B. Figure 1

- ○ Lean, IP GLP-1(9-36)a
- ■ Obese, IP GLP-1(9-36)a
- ▲ Obese, Vehicle
Figure 2 alternative
Figure 3

(A) ipGTT

(B) ITT
Figure 4

A. VO2 (total body: ml/h/kg)

B. VO2 (lean body: ml/h/kg)

C. Physical activity (beans/h)

Diabetes

Vehicle | GLP-1(32-36)a

Light cycle

Dark cycle

* p < 0.05

** p < 0.01
Figure 5

**A**

UCP-1

Tubulin

Vehicle GLP-1(32-36)a

UCP-3

Tubulin

Vehicle GLP-1(32-36)a

**B**

**BAT: UCP-1**

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**C**

**Relative mRNA**

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**D**

pACC

ACC

UCP-3

Tubulin

Vehicle GLP-1(32-36)a

**E**

**Muscle: pACC/ACC**

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**F**

**Relative mRNA**

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Figure 6

A

pACC
pAMPK
AMPK
β-actin

GLP-1(32-36)a: - + - +

B

pAMPK/AMPK

pACC
pAMPK
AMPK
β-actin

Vehicle GLP-1(32-36)a

Arbitrary units/β-actin

C

pACC
pAMPK
AMPK
β-actin

0.2mM pal:BSA: - + +

GLP-1(32-36)a: - - +

D

pAMPK/AMPK

pACC

Arbitrary units/β-actin

E

%ROS production compared to palmitate:BSA

GLP-1(32-36)a: - - +

Figure 6
Table 1. Fasting Plasma Glucose, Insulin, Glycerol, Triglyceride In Vehicle and GLP-1(32-36)amide-Treated DIO Mice

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<td>Plasma Insulin (pM)</td>
<td>296± 55.8</td>
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<td>Plasma Glycerol (mg/ml)</td>
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<td>Plasma Triglyceride (mg/ml)</td>
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<td>0.33± 0.02 ##</td>
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