

ORIGINAL ARTICLE

Neutralizing circulating ghrelin by expressing a growth hormone secretagogue receptor-based protein protects against high-fat diet-induced obesity in mice

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Ghrelin is a stomach-derived peptide hormone that stimulates appetite and promotes adiposity through binding to the growth hormone secretagogue receptor (GHS-R1a). Administration of ghrelin in rodents increases weight gain due to stimulating food intake and reducing fat utilization. Therefore, reducing circulating ghrelin levels holds the potential to reduce weight gain. We developed a GHS-R1a-fusion constructs of a decoy protein containing the ligand-binding domains of the ghrelin receptor. Intramuscular injection of the GHSR/Fc plasmid decreased circulating levels of acylated-ghrelin. When challenged with the high fat diet, treated mice displayed reduced weight gain compared with controls, which was associated with reduced fat accumulation in the peritoneum but not lean mass. Quantitative PCR with reverse transcription showed increased PPAR γ and hormone sensitive lipase transcripts levels in adipose tissue of treated animals, illustrating a preference for increased fat utilization. Intra-peritoneal glucose tolerance and insulin tolerance tests showed improved glucose clearance and insulin sensitivity in GHSR/Fc treated animals. We suggest that *in vivo* expression of the GHSR-based fusion protein prevents diet-induced weight gain, altering adipose gene expression and improving glucose tolerance. These findings, while confirming the role of ghrelin in peripheral energy metabolism, suggest that a strategy involving neutralization of the circulation ghrelin by intramuscular injection of the GHSR1/Fc fusion construct may find clinical application in the treatment of obesity.

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INTRODUCTION

Excessive adiposity is a risk factor for many chronic diseases, including type 2 diabetes mellitus, cardiovascular disease and cancer.¹ Studies have shown that even a modest reduction in weight has a positive impact on cardiovascular risk factors^{2,3} and is associated with a reduced risk for both developing type 2 diabetes mellitus and diabetes-associated complications.⁴

Lifestyle interventions aimed at reducing calories and increasing physical activity through behavioral changes are currently recommended as the first-line approach for weight management. However, these strategies alone are less successful when compared to pharmacological interventions for maintained weight loss (6–12 months).⁵ Unfortunately, most of the drugs approved for the treatment of obesity have been withdrawn from use due to their side effects.⁵ Recently, targeting of gut hormones for the treatment of obesity has garnered interest.⁶ Infusion of glucagon-like peptide 1 and peptide YY are able to reduce appetite by acting on feeding regions of the brain in humans.⁷ Another promising candidate is the stomach-derived peptide hormone ghrelin. Ghrelin levels peak in circulation during energy depleted states leading to activation of the appetite stimulating neuropeptide Y and Agouti gene-related peptide neurons within the arcuate nucleus of the hypothalamus.⁸ This action occurs via

ghrelin binding to the growth hormone secretagogue receptor (GHS-R1a).⁹ In addition to appetite, ghrelin promotes the differentiation of adipocytes and the preference for storage of calories in adipose tissue.^{10,11}

The ghrelin peptide is derived from proghrelin, which is a precursor peptide proteolytically cleaved to produce acylated ghrelin (AG),¹² unacylated ghrelin (UAG)¹² and obestatin.¹³ However, *in vitro* studies have shown that both UAG¹⁴ and obestatin¹³ are unable to bind to GHS-R1a. The GHS-R1a is a G-protein-coupled receptor,¹⁵ and is activated through the binding of its only known endogenous ligand, AG.¹⁴ A recent study suggested that AG binds to GHS-R1a in the second extracellular loop (EC2), which forms a hydrophobic pocket, allowing the lipophilic acylated side chain of ghrelin to be stabilized during the binding.¹⁶

Depleting circulating ghrelin holds the potential to reduce caloric intake and promote fat energy utilization. As such, we constructed mammalian expression plasmid vectors encoding the ligand-binding domains of the GHS-R1a, specifically the N-terminal (Nt), and/or the first, second extracellular domain (EC1, EC2) and fused with a human IgG constant regions (Fc), forming GHSR/Fc (Figure 1a). *In vivo* expression of these fusion proteins was achieved through plasmid-intramuscular injection and

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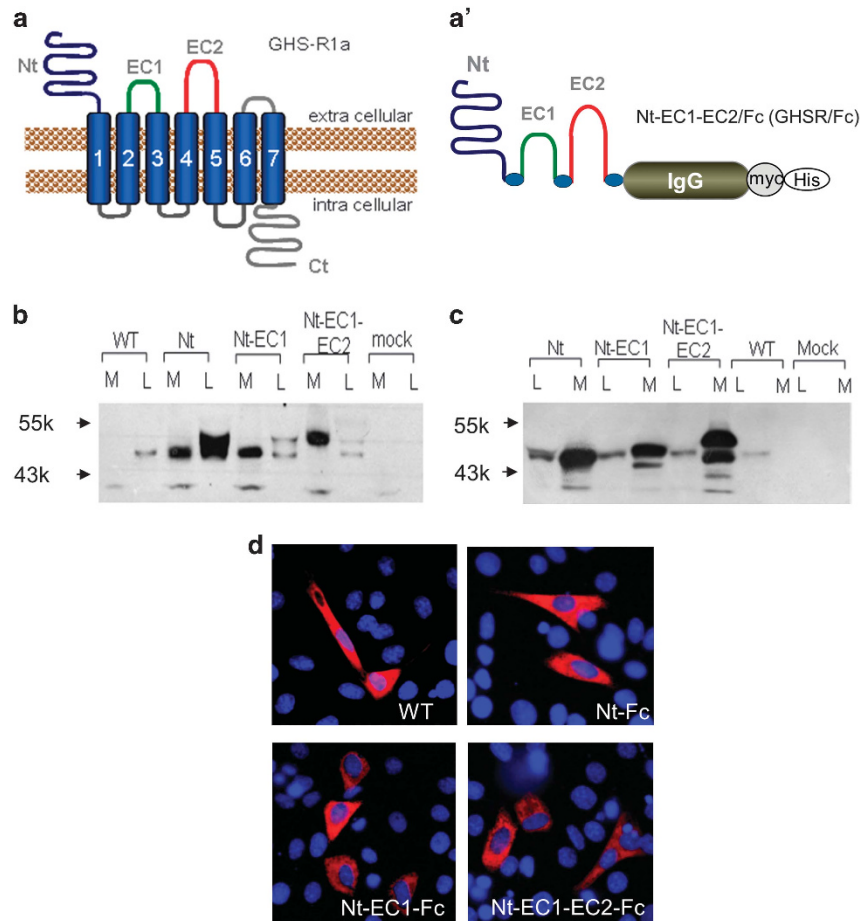


Figure 1. Validation of GHSR related constructs. Illustration of the full length (WT) (a) and extracellular domain (GHSR/Fc) constructs (a'). Domains are identified as N-terminal (Nt), extracellular domains 1 and 2 (EC1, EC2) and human immunoglobulin IgG2 constant region (Fc). Western blots were conducted using anti-Myc tag in cell lysates (L) and culture media (M) from L6 cells (b), and CHO (c) cells transfected with GHSR/Fc-based constructs. Fluorescent immunocytochemistry using anti-human-Fc IgG was conducted in L6 muscle cells transfected with WT and GHSR/Fc plasmids (d, transfected cells shown in red).

subsequent electroporation in gastrocnemius muscle of mice. The fusion constructs omitted the sequence motif corresponding to the transmembrane domains of the receptor, allowing the production of GHSR/Fc that is secretable. We found that *in vivo* expression of GHS-R1a fusion construct (Fc) containing the extracellular-binding loops 1 and 2 (GHSR/Fc) caused a reduction in AG but, not UAG levels. This fusion construct protected mice from high fat diet (HFD)-induced weight gain, which was associated with altered adipose gene expression profile and improved glucose clearance and insulin sensitivity. Our observations suggest that the GHSR/Fc fusion construct may find clinical use in treating obesity.

RESULTS

Validation of GHSR constructs

We first examined the expression and secretion of the fusion proteins under *in vitro* conditions by the transient transfection of the plasmid vectors (Figure 1a) into L6 rat skeletal muscle cell line. At 48 h after transfection, the medium and the cells were harvested separately and extracted proteins were subjected to western blot analysis using anti-Myc tag antibodies. The expression of the fusion constructs (Nt-, Nt-EC1 and Nt-EC1-EC2, denoted as GHSR/Fc) was consistently detected in both the cell lysate and culture media whereas the wild type (WT)/Fc, which contains the transmembrane domains, was only found in the cell lysate

(45kD; Figure 1b). Similar results were also obtained in the culture media or cell lysate in CHO cells transfected with the fusion constructs (Figure 1c). Immunocytochemistry experiments using anti IgG-Fc antibodies showed that Nt-EC1-EC2 (GHSR/Fc), Nt/Fc, EC1/Fc and WT/Fc were detected in the transfected L6 cells (Figure 1d), suggesting that these fusion proteins can be produced in the mammalian expression system *in vitro*.

Effects of *in vivo* expression of GHSR/Fc in mice

To examine the impact of GHSR gene therapy on energy intake and weight gain, food consumption and body weight were measured in mice injected with the GHSR-based constructs and control mice (injected with the Fc empty vector) fed on a HFD. We found that mice treated with the GHSR/Fc construct gained significantly less body weight compared with the control animals (Figure 2a). The weight differences began at 30 days post injection (21.0 ± 0.195 g vs 23.8 ± 0.433 g in control, $P < 0.05$) and continued until the termination of the experiment at 54 days post the first injection (23.5 ± 1.36 g vs 29.6 ± 0.434 g in control, $P < 0.001$), (Figure 2a). Mice that received Nt/Fc, Nt-EC1/Fc or WT/Fc injections all showed a modest reduction in weight gain that did not reach statistical significance. Only the GHSR/Fc caused a significant reduction in diet-induced weight gain, and this was the focus of the remaining analysis.

Since ghrelin was previously shown to have a potent orexigenic effect, the daily food and water consumption was examined.

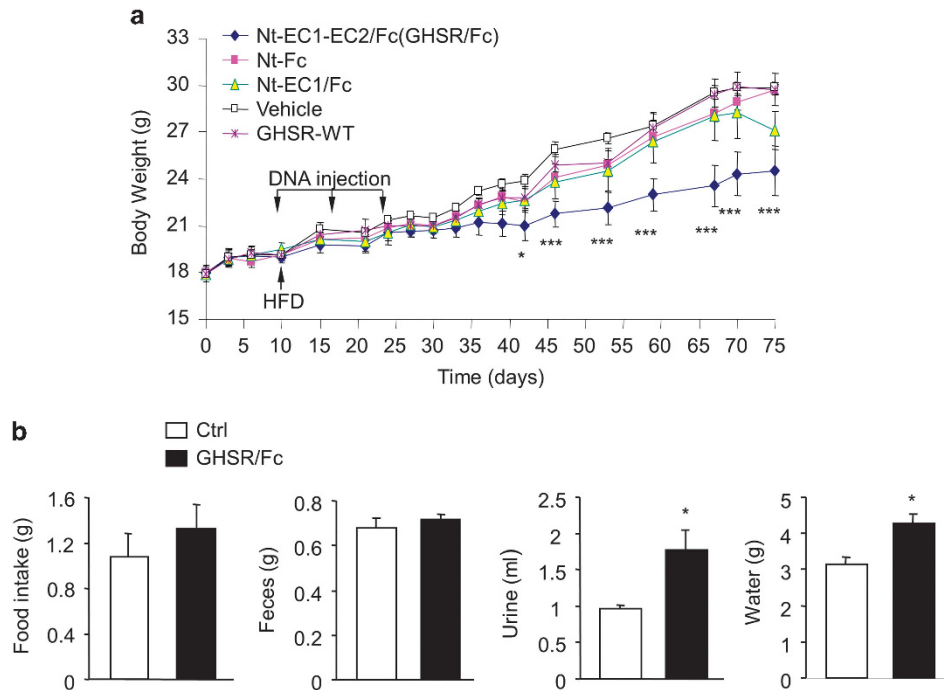


Figure 2. Effect of GHSR/Fc on weight gain and metabolic parameters. Mice received three intramuscular injections of indicated plasmids, and were placed on a HFD the second day after the first gene transfer. The body weight was monitored during the feeding course (a). Food and water intake as well as urine and feces excretion were measured at the end of the experiment (b). Data are Mean \pm s.e.m., * $P < 0.05$, *** $P < 0.0001$, ($n = 5$).

As shown in Figure 2b, there was no difference in food intake between control and GHSR/Fc treated mice. Treatment with GHSR/Fc increased water intake and urine.

In vivo expression of the GHSR/Fc was verified by western blot analysis. Western blotting of whole gastrocnemius muscle detected a 55-kDa band corresponding to the GHSR/Fc fusion protein in each of the GHSR/Fc mice treated mice (Figure 3a). Furthermore, western blot analysis of plasma showed the presence of the 55-kDa band in GHSR/Fc treated mice but not in vehicle-injected control mice (Figure 3b). To confirm the ghrelin neutralizing effect of this treatment, the levels of AG and total ghrelin (primarily UAG) were measured in circulation. Figure 3c demonstrates that expression of GHSR/Fc did not alter the levels of total ghrelin (Figure 3c 140.9 ± 30.2 pg ml⁻¹ vs 140.8 ± 37.94 pg ml⁻¹ in control) but significantly reduced the levels of AG (Figure 3c, 62.3 ± 9.8 pg ml⁻¹ vs 32.3 ± 5.6 pg ml⁻¹ in control, $P < 0.05$). Finally, to address if reduced AG levels had an effect on the growth hormone pathway, plasma levels of the GH-dependent insulin like growth factor-1(IGF-1) were examined. No difference was found for IGF-1 levels between the GHSR/Fc treated and control mice (Figure 3d).

Effects of GHSR/Fc on adipose tissue

To determine the source of the reduced weight gain in the treated animals, fat pad and lean tissue (gastrocnemius and soleus muscles) were investigated. Visually, the amount of white adipose tissue (WAT) found in the peritoneum of treated mice was less than the control (Figure 4a). This difference was quantified by the weighing of various fat pads. Several fat stores were significantly smaller in the treated mice including retroperitoneal (0.476 ± 0.12 g vs 0.948 ± 0.119 g in control, $P < 0.001$), peri-renal (0.44 ± 0.19 g vs 0.734 ± 0.011 g in control, $P < 0.05$) and inguinal fat pads (0.396 ± 0.067 g vs 0.722 ± 0.56 g in control, $P < 0.05$; Figure 4b). Lean mass was not affected by treatment as determined by the equal gastrocnemius muscle weight (0.131 ± 0.016 g vs 0.109 ± 0.011 g in control; Figure 4c).

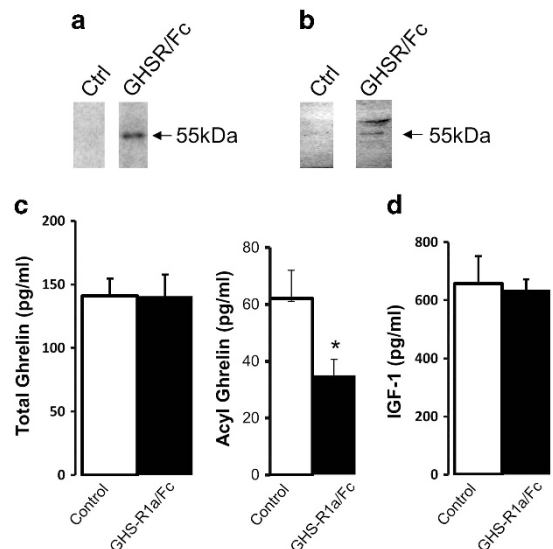


Figure 3. Western blots were conducted using anti-Myc tag in gastrocnemius muscle tissue lysate (a) and blood plasma (b) at the end of the study. Circulating levels of total (AG and UAG), acyl specific (AG) (c) and IGF-1 (d) were assayed at the end of the study. Data are mean \pm s.e.m., * $P < 0.05$.

Since reduced caloric consumption was not responsible for the reduction in WAT, alterations in WAT mRNA expression in the context of several metabolic genes were examined. Visceral WAT was examined for the adipokine leptin (a hormone that signals in response to fat cell anabolism), hormone sensitive lipase (HSL; an enzyme catalyzing the breakdown of triglycerides to fatty acids) and PPAR γ (a nuclear receptor that promotes adipogenesis) mRNA using quantitative RT-PCR. Treated animals had lower levels of

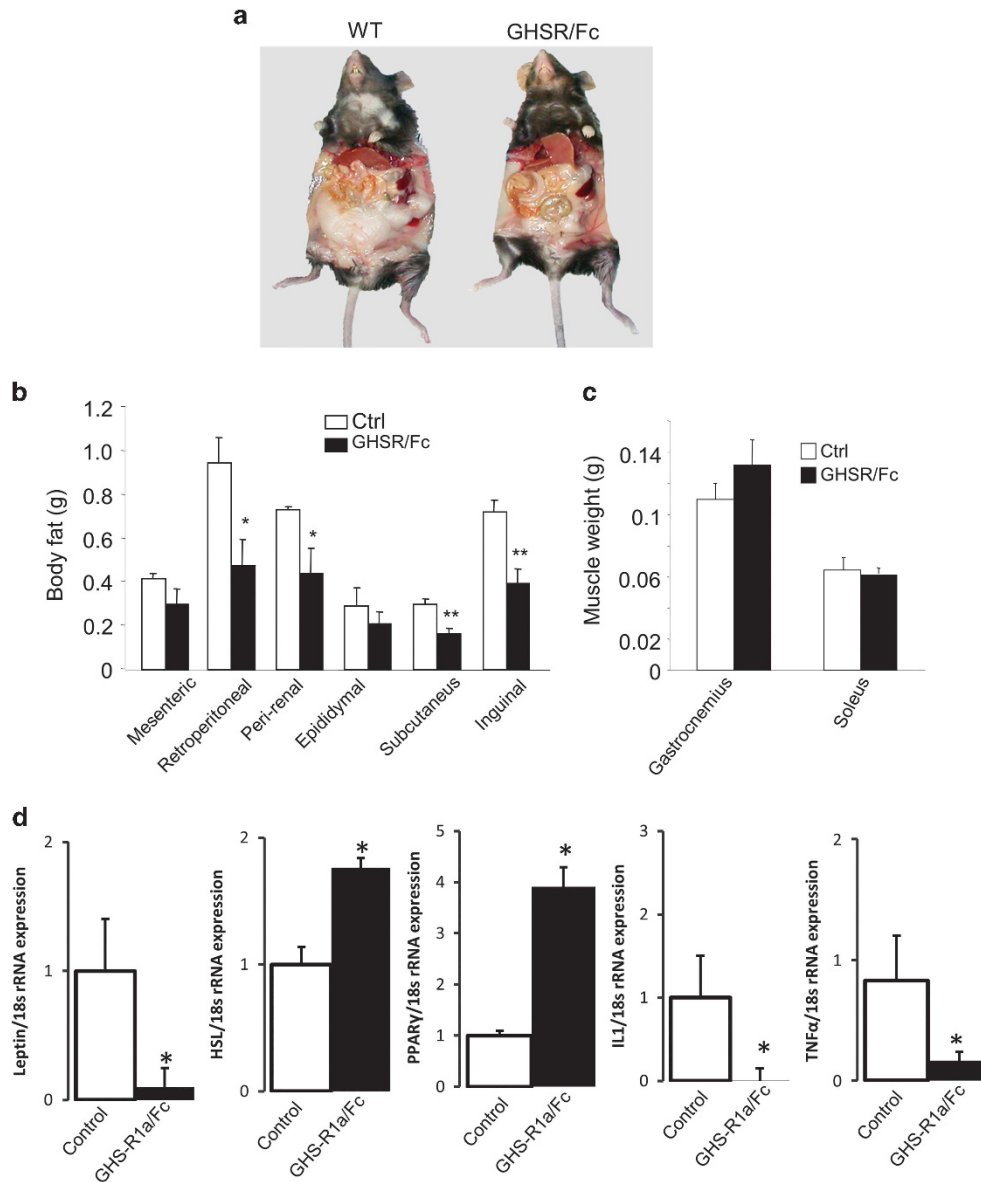


Figure 4. Effect of GHSR/Fc on adipose tissue. Post experimentally, peritoneal fat pads were observed from sacrificed GHSR/Fc treated and control mice (a). Fat pad mass (b) and gastrocnemius muscle mass (c) were quantified for indicated stores in GHSR/Fc and control mice (b). mRNA Transcripts levels of leptin, HSL, PPAR γ , IL-1 and TNF α were quantified by real time RT-PCR relative to 18 s ribosomal RNA by using the standard curve method (d). Data are Mean \pm s.e.m., * $P < 0.05$, ** $P < 0.001$, ($n = 5$).

leptin gene expression in WAT (0.10 ± 0.14 fold of control, $P < 0.05$) while HSL mRNA (1.76 ± 0.07 fold of control, $P < 0.05$) and PPAR γ mRNA (3.91 ± 0.378 fold of control, $P < 0.05$) expression were elevated (Figure 4d). Furthermore, mRNA levels of adipose derived pro-inflammatory cytokines known to be elevated in obesity were examined in WAT. Both interleukin-1 (IL-1) and tumor necrosis factor α (TNF α) were reduced in GHSR/Fc compared to control ($2.9 \pm 1.6\%$ of control for IL-1, and $15 \pm 7\%$ of control for TNF α , $P < 0.05$).

Effect of neutralizing ghrelin on glucose metabolism ghrelin has been shown to affect glucose homeostasis.¹⁷ To determine the effect of GHSR/Fc treatment on glucose metabolism, intra-peritoneal glucose tolerance tests and insulin tolerance tests were performed on control and treated mice before and at the end of the study. Treated mice displayed improved glucose tolerance with significantly lower blood glucose at 10 min (14.5 ± 1.52 mmol l⁻¹ vs 18.9 ± 1.48 mmol l⁻¹ in control, $P < 0.01$) and 20 min (10.1 ± 0.968 mmol l⁻¹ vs 14.2 ± 0.989 mmol l⁻¹ in

control, $P < 0.05$) post glucose injection (Figure 5a). The area under the curve in the intra-peritoneal glucose tolerance tests was also significantly decreased in the treated mice (516 ± 32.2 vs 668 ± 22.1 in control, $P < 0.05$; Figure 5b). In insulin tolerance tests, the area under the glucose curve was lower in treated animals (180 ± 19.1 vs 230 ± 20.3 in control, $P < 0.05$; Figure 5c) indicating increased insulin sensitivity (Figures 5c and d). These results suggest that reduced circulating AG improves insulin sensitivity and glucose tolerance in mice on a HFD.

DISCUSSION

In this study we used an *in vivo* gene transfer method to administer GHSR1a-based fusion proteins as a 'decoy receptor' for circulating AG in mice. The fusion constructs were designed to incorporate the extracellular domains of the GHSR1a that interact with the acylated portion of ghrelin, fused with IgG Fc to improve the stability and half-life of the complex in circulation.¹⁸

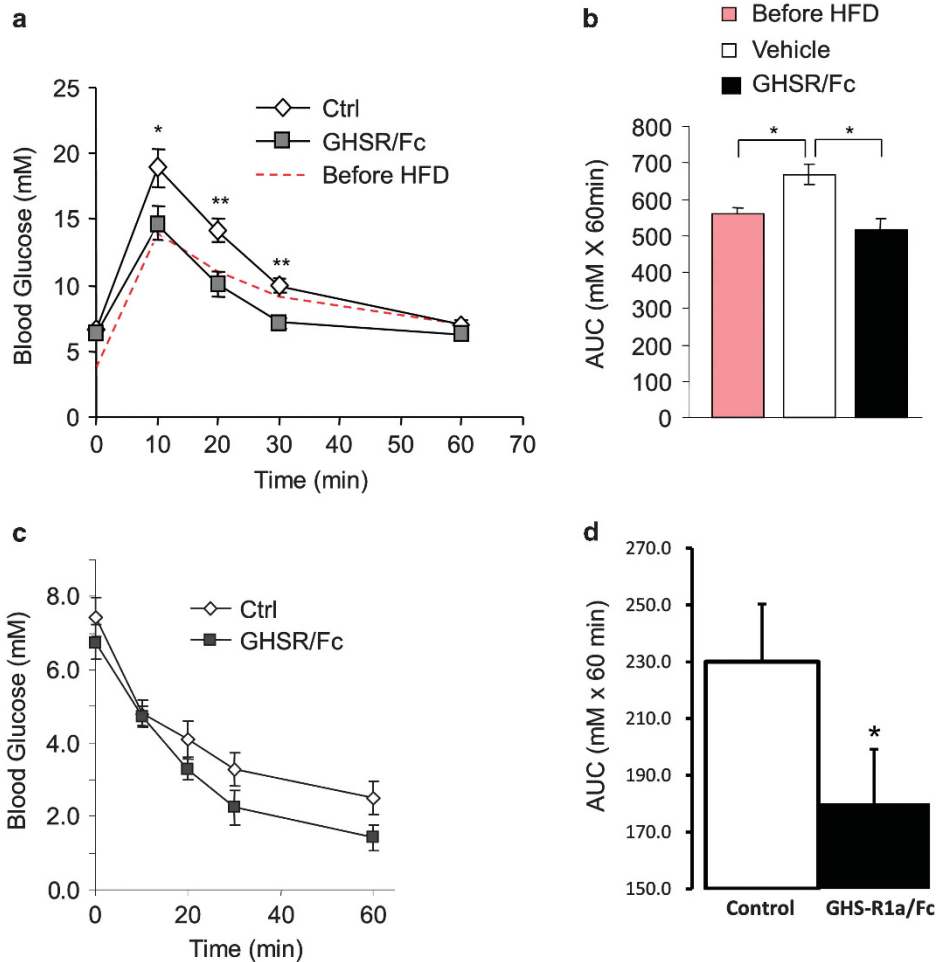


Figure 5. Intra-peritoneal glucose and insulin tolerance tests. Glucose tolerance test (IPGTT) were performed either prior to HFD feeding and before the metabolic cage assay (a). The area under the curve (AUC) for the glucose tolerance test was compared between GHSR/Fc and control mice (b). Insulin tolerance test (ITT) was conducted at the day before sacrifice the mice (c), and (AUC) is shown (d). Data are Mean \pm s.e.m., * $P < 0.05$, ** $P < 0.001$, ($n = 5$).

We confirmed expression and secretion of the construct *in vitro* by plasmid transfection in L6 muscle and CHO cell lines. As expected, the full length WT/Fc was not detected in culture media as it possesses the hydrophobic transmembrane domains that would retain it in the plasma membrane. We further confirmed the expression in both the muscle and plasma from GHSR/Fc plasmid injected animals. To verify that the treatment led to altered ghrelin levels we measured both total (AG+UAG) and acylated (AG) levels in the circulation. Although there was no significant difference in the levels of total ghrelin, there was a significant reduction of AG in treated animals. Any differences in AG (5% of total ghrelin in circulation) occurring in the total ghrelin assay would likely be undetectable.^{14,19} The lack of difference in the total ghrelin assay confirms the specificity of the GHSR/Fc for binding only AG.

The impact of neutralizing ghrelin on energy homeostasis was examined by placing animals on a HFD. Thirty days after the HFD feeding, animals treated with GHSR/Fc had gained significantly less weight compared with empty vector treated mice on HFD. The reduced weight gain in the GHSR/Fc group was maintained until the end of the study at 74 days post gene transfer with a final weight gain reduction of over 20%. Our observations of reduced weight gain and reduced circulating AG in GHSR/Fc mice is in agreement with previous work examining the effects of ghrelin immunoneutralization.²⁰

We also designed constructs encoding for the N-terminal region of the GHSR1a (Nt/Fc) or the N-terminal and the first extracellular

loop (Nt-EC1/Fc). The effects of these constructs, while exerting some weight sparing effects, was moderate compared with GHSR/Fc. The GHSR/Fc was the one construct that incorporated all three of the extracellular domains of the GHSR1a (Nt, EC1 and EC2). Of particular importance was the incorporation of both the extracellular loop domains (EC1 and EC2) as these extracellular loops are thought to be the binding sites for AG to the GHSR1a.¹⁶

Despite the difference in weight gain, we did not observe any difference throughout the study in food consumed between the GHSR/Fc treatment and control groups. The GHSR/Fc mice showed an increased water consumption and urine output. Ghrelin's action on appetite occurs through binding with the GHSR1a in neurons within the arcuate nucleus of the hypothalamus.²¹ Although peripheral ghrelin administration has been shown to cross the blood-brain barrier and stimulate appetite,²² a population of ghrelin producing neurons also exists within the arcuate nucleus.²³ In the present study, due to the size of the GHSR/Fc protein, it is unlikely that it was able to cross the blood-brain barrier and neutralize hypothalamic ghrelin. Thus, our strategy may only be targeting peripheral ghrelin and its actions. In agreement with our study, ghrelin immunoneutralization studies also had no effect on food intake.²⁰ Furthermore, studies that examined both ghrelin and GHSR1a embryonic knockout mice showed no changes on feeding behavior. However, when challenged with a HFD, both knockout mice were more likely to utilize fat as their energy substrate.^{24,25} Consistent with these

previous observations, our finding was reduced fat pads primarily in the peritoneum.

Given that the food consumption was unchanged in GHSR/Fc treated mice, the reduced fat mass is likely the consequence of the reduction in AG which affected fat tissue metabolism. A recent study indicated that ghrelin acts directly on adipocytes to prevent lipolysis.²⁶ To determine the effect of reduced circulating ghrelin levels on adipose tissue, we measured the mRNA expression of fat metabolism genes in WAT. Leptin, which is produced during fat accumulation and adipocyte differentiation,²⁷ was lower in the GHSR/Fc treated animals. Of particular interest, HSL, a key enzyme in the catabolism of triglycerides to fatty acids,²⁸ was found to be elevated in the treated animals. This increased lipase expression is indicative of increased mobilization of fatty acids for energy substrate, which supports our finding that the treated animals were protected from adiposity while eating an HFD. Our finding also agrees with a previous indirect calorimetry study on ghrelin KO mice that suggested preferential use of fat as their energy substrate.²⁴

PPAR γ mRNA levels were also found to be elevated in the treated animals. Although activation of this nuclear receptor typically leads to the differentiation and growth of adipose tissue, some evidence suggests that increased PPAR γ may partition fat away from visceral stores.²⁹ This study shows that the most significant reduction on fat pad weight, in GHSR/Fc treated mice was in the visceral depots. These findings are supported by other studies that showed that low-dose ghrelin administration caused increased fat pad weight and altered adipocyte gene expression without an effect on feeding in mice.³⁰ Taken together, our data suggest that reducing circulating AG with GHSR-1a/Fc treatment leads to reduced fat stores and altered adipocyte gene expression.

Reducing AG with GHSR/Fc treatment significantly improved glucose tolerance and insulin sensitivity in mice on HFD feeding. These findings are in agreement with several studies suggesting that ghrelin promotes glucose homeostasis through inhibiting insulin release from the pancreas.^{31,32} Because we did not observe any differences in circulating insulin levels, the improved glucose clearance may be a consequence of reduced hepatic glucose production in the GHSR/Fc treated animals. This is at least, in part, supported by previous investigation that demonstrated that ghrelin promotes glucose production in hepatocytes.³³ Moreover, the improved glucose tolerance may be a secondary effect to the reduced visceral adiposity in GHSR/Fc-mice. It is known that increased adiposity can lead to insulin resistance which is brought on by pro-inflammatory factors released from inflamed fat stores.³⁴

To determine the possible involvement of pro-inflammatory cytokines, we examined the mRNA expression of both IL-1 and TNF α in visceral adipose tissue. Both these genes had significantly less expression in GHSR/Fc treated animals, suggesting that improved insulin sensitivity in GHSR/Fc treated mice may be partially contributed by reduced pro-inflammatory cytokines in these mice. Therefore, our finding suggest that a strategy involving neutralization of circulating ghrelin, exemplified by the GHSR/Fc treatment, prevents weight gain and improves glucose tolerance. This may be beneficial in therapies for type 2 diabetes mellitus.

We examined if the GHSR/Fc treatment had any impact on other metabolic hormones. None of the hormones examined (glucagon-like peptide 1, insulin, peptide YY, pancreatic polypeptide and glucose insulinotropic peptide) were significantly altered by the expression of GHSR/Fc (data not shown). As ghrelin is a known GH secretagogue¹⁴ and GH has effects on glucose metabolism and insulin sensitivity,³⁵ the effects of ghrelin depletion on GH were examined. Since GH varies throughout the day in a pulsatile fashion a more stable measurement of GH levels can be obtained by measuring circulating IGF-1. Circulating IGF-1 levels were not affected by ghrelin neutralization and likely

were not responsible for the reduced weight and improved glucose parameters. This lack of effect is consistent with a previous report indicating that GH levels are unchanged in GHSR1a KO mice on HFD.²⁵

In summary, we developed a novel strategy using secretable GHSR1a-based fusion proteins to neutralize the circulating active ghrelin and hence reduce HFD-induced weight gain. Among those fusion constructs, we show that the GHSR/Fc which contains the Nt, EC1 and EC2 domains of GHSR1a is the most effective in reducing weight gain, improving insulin resistance and improving glucose tolerance in mice fed with HFD. This approach reduced body weight and adiposity, without affecting the appetite and, particularly, lean mass. The treatment also altered adipose gene expression, exemplified by increased fat catabolism and reduced pro-inflammatory cytokines genes, suggesting a shift to fat usage rather than storage in mice. This yields a secondary beneficial outcome exemplified by improved insulin sensitivity and improved glucose clearance. We thus suggest that GHSR/Fc fusion protein may have clinical application for the treatment and management of obesity and type 2 diabetes mellitus.

MATERIALS AND METHODS

Construct design

All ghrelin receptor constructs were designed based on the mouse growth hormone secretagogue receptor sequence (Gene ID: 208188). GHSR regions and mouse IgG Fc fragment were produced by PCR amplification. Primers used for amplification of the N-terminal region were (6mNtF) GCG GGG TAC CAT GTG GAA CGC GAC GCC A and (6mNtR) GCG AGT ACT CGC GGG GAA CAG TGG CAG CAG TTC, the first extracellular loop (6mEC1F) GCG AAG CTT TTC CAG TTT GTC AGC GAG AGC TGC ACC TAC GCC CCC AGC GAG ACC GTC ACC TGC and (6mEC1R) CGA AGC TTG CAG AGC AGG TCG CCG AAG TTC CAG GGC CGA TAC TGC CAG AGG CGC GCG GGG AAC AGT GGC AGC AGT TC, the second extracellular loop (6mEC2F) GCG ACG GAT CCC CGG GAC ACC AAC GAG TGC CGC GCC ACC GAG TTC GCT GTG CGC TCT CCC AGC GAG ACC GTC ACC TGC AAC and (6mEC2R) GCGGGGATCCGTG CCG TTC TCG TGC TCC ACG CCC ACC AGC ACG GCG TAG GTG CAG CTC TCG CTG AC and mouse IgG Fc fragment (mlgGF) GCG AGT ACT TGG CCC AGC GAG ACC GTC ACC TGC AAC and (mlgGR) GCG CTC GAG CAG GGA AGA AGT CTG TTA TCA TGC A. Each extracellular domain GHSR PCR product was cleaved with KpnI and Scal, whereas the mouse IgG Fc fragment was cleaved with Scal and XhoI. This was ligated into the pSecTag2B vector (Invitrogen, Mississauga, ON, Canada) at XhoI and Scal using T4 DNA ligase.

In vitro expression of the fusion constructs

To establish the expression and secretion of GHSR constructs, the rat skeletal muscle cell line L6 and the Chinese hamster ovary (CHO) cells were transfected with the designed plasmids. 40 μ g of cell extract and media were collected using standard methods and run out on 12% SDS-polyacrylamide gel electrophoresis gels. Following the end of experiments, the gastrocnemius muscle was collected and extracted from treated mice to examine the *in vivo* expression. Proteins were transferred to polyvinylidene fluoride membrane and probed anti-Myc (Millipore, Billerica, MA, USA) at 1:3000 at 4 C followed by secondary rabbit anti-mouse HRP at 1:5000 at room temperature (RT) for 1 hr.

Immunofluorescence microscopy

To determine the localization and expression of transfected GHSR proteins cell lines were immunostained with anti IgG Fc antibodies which only detect the F chain of the transfected protein. L6 cells were grown on coverslips and were fixed for 1 hr at RT in 4% paraformaldehyde. They were then washed three times in PBS followed by 15 min of blocking in 5% normal horse serum. Cells were incubated with primary biotinylated anti mouse Fc in blocking solution for 2 hr at RT. Cells were then washed and treated with avidin conjugated Cy3 in blocking solution for 45 min in dark at RT. Coverslips were mounted on slides and visualized on a Zeiss Axioplan II microscope (Thornwood, NY, USA).

In vivo expression of GHSR/Fc in mice

C57/Bl6 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were housed under controlled light (12 h light/12 h dark) and temperature conditions, and had free access to food (normal rodent chow, or HFD where indicated) and water. All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the St Michael's Hospital Animal Care Committee.

In vivo expression of GHSR/Fc was achieved by intramuscular plasmid injection followed by an electroporation as described previously.¹⁸ Briefly, a total of 50 µg of plasmid DNA was injected (25 µg per leg) intramuscularly into gastrocnemius of 8-week-old male C57/Bl6 mice. An electrical current was applied using caliper electrodes (BTX, Holliston, MA, USA) on the muscle as follows; 8 pulses (pulse length 20 ms) with 1 s intervals at 200 V cm⁻¹. A conductive (aquasonic 100) gel was used to facilitate current delivery. Plasmid injection and electroporation were conducted once weekly for the first 3 weeks of the study course.

Food intake and body weight measurement

HFD (Research diets, North Brunswick, NJ, USA, containing 60% of kcal as fat) began on the day of the first DNA injection and continued until the end of the experiment 54 days later. Food consumption was measured by weighing of food basket in each cage every 3 days. Animals from each group were weighed individually every 3 days.

Glucose and insulin tolerance tests

Intra-peritoneal (ip) glucose and insulin tolerance tests were completed after 54 days of HFD. Animals were fasted overnight for 12 h prior to tests. For the intra-peritoneal glucose tolerance tests, a single bolus injection of glucose at 1.5 mg kg⁻¹ of mouse weight was administered ip. For the insulin tolerance tests, a single bolus injection of insulin at 0.75 U kg⁻¹ was injected ip. Tails tips were treated with topical anesthetic EMLA (AstraZeneca Canada Inc., Mississauga, ON, Canada) and blood samples were drawn from tail vein at 0, 10, 20, 30 and 60 min post injection. Blood samples (4–5 µl) were analyzed by the glucose oxidase method using the Bayer Acensia Elite XL glucometer (Bayer, Mississauga, ON, Canada).

Fat tissue collection

Post mortem analysis of fat tissues weight was completed by bi-lateral harvesting of fat pads and immediate weighing. The peritoneal fat tissue was snap frozen in liquid nitrogen for later RNA extraction.

Real-time PCR

Visceral WAT was collected at the end of the experiment. Total RNA was extracted using the Trizol (Invitrogen) extraction phenol chloroform precipitation method as per manufacturer's protocol. Samples were treated with DNAase (Invitrogen) and cDNA was produced using random hexamers under standard methods. Real-time PCR was conducted on the Bio-Rad CFX instrument (Bio-Rad, Mississauga, ON, Canada) using primers for leptin (forward: CCAAAACCTCATCAAGACC, reverse: TGCTCCACC ACCGAAACTC), hormone sensitive lipase (forward: TGCTCCACCA CCGAAACTC, reverse: TCTCCAGTTGAACCAAGCAGG TCA), PPAR γ (forward: GGAAAGACAACGGACAAATCAC, reverse: ATCCTTGGCCCTCTGAGATG, IL-1 (forward: TGCTGAAGCAGCTATGGCAA, reverse: TGTCGAGATTTGA AGCTG) and TNF α (forward: TGATCGTCCCAAAGGGAT, reverse: TTGCTACGACGTGGGCTAC). Data was analyzed by using Bio-Rad CFX manager software and relative expression was determined using the standard curve method with 18S as the normalization gene.

Hormone assays

Blood was collected at the end of the experiment in capillary tubes containing EDTA (Sarstedt, Nümbrecht, Germany) from ad-libitum fed mice. Plasma level of hormones involved in the regulation of energy metabolism was analyzed using the Milliplex hormone assay panel (Millipore) including; active glucagon-like peptide 1, insulin, peptide YY, pancreatic polypeptide and GIP (Millipore). Acylated ghrelin and IGF-1 plasma levels were analyzed by enzyme link immunoassays (Cayman chemical, Ann Arbor, MI, USA, and Millipore, Etobicoke, ON, Canada, respectively) and total ghrelin levels were measured using a radio-immunoassay (Phoenix Pharmaceuticals, Burlingame, CA, USA).

Statistical analysis

The relative changes in weight gain over time were analyzed using the two-way analysis of variance with Bonferroni post test to compare each group to the control group. Multiplex hormone assays analyzing each group were compared with the one way analysis of variance. Time points during intra-peritoneal glucose tolerance tests and insulin tolerance tests were examined by two-way analysis of variance. All other comparisons between the control and GHSR/Fc group were analyzed with the student's *t*-test.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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