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Glucose and Weight Control in Mice with a Designed Ghrelin O-Acyltransferase Inhibitor

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Abstract

Ghrelin is a gastric peptide hormone that stimulates weight gain in vertebrates. The biological activities of ghrelin require octanoylation of the peptide on Ser3, an unusual post-translational modification that is catalyzed by the enzyme ghrelin O-acyltransferase (GOAT). Here, we describe the design, synthesis, and characterization of GO-CoA-Tat, a peptide-based bisubstrate analog that antagonizes GOAT. GO-CoA-Tat potently inhibits GOAT in vitro, in cultured cells, and in mice. Intraperitoneal administration of GO-CoA-Tat improves glucose tolerance and reduces weight gain in wild-type mice but not in ghrelin-deficient mice, supporting the concept that its beneficial metabolic effects are due specifically to GOAT inhibition. In addition to serving as a research tool for mapping ghrelin actions, GO-CoA-Tat may help pave the way for clinical targeting of GOAT in metabolic diseases.

The persistent rise in the proportion of overweight individuals in Western society over the past 30 years has been associated with substantial excess morbidity and is widely recognized as a major public health concern. To address this problem, intensive efforts are underway to

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Materials and Methods

Figs. S1 to S10

References

clarify nutrient-hormone interactions contributing to weight gain. Starting with the isolation of leptin (1), a series of hormones acting centrally and peripherally to influence body mass have been discovered. Among these, the gastric peptide hormone acyl ghrelin has generated considerable interest as an important stimulus for weight gain (2–5) and modulator of glucose homeostasis (6–8). Various strategies in therapeutic development have been devised to antagonize acyl ghrelin (9,10), although none has yet emerged as clinically beneficial. Acyl ghrelin has an unusual Ser3 octanoylation; only acylated ghrelin can bind and activate the growth hormone secretagogue receptor (GHSR-1a). The cDNA for the enzyme responsible for this esterification, GOAT, has recently been cloned (11,12). GOAT has been suggested as a potential therapeutic target for modulating weight gain and glucose control, but this has not yet been directly tested (9,13). An acyl ghrelin product analog Dap-ghrelin blocks GOAT activity in a microsomal assay (14).

We designed bisubstrate analog GO-CoA-Tat based on the theory that if GOAT uses a ternary complex mechanism which templates octanoyl-CoA and ghrelin peptide, then linking the two substrates with a non-cleavable bridge could combine the binding energies of the individual ligands without the entropic loss associated with forming the ternary complex (Fig. 1A). A related strategy has been used for other peptide modifying enzymes including histone acetyltransferases (HAT) and protein kinases (15,16). Since we were uncertain about the ghrelin peptide length needed for recognition by GOAT, we selected amino acids 1-10 for coupling to octanoyl-CoA, to maximize inclusion of highly conserved ghrelin residues. An 11-mer HIV Tat-derived peptide sequence was also attached to the C-terminus via an amino-hexanoyl linker to enhance cell penetration. Synthesis of this tripartite compound, GO-CoA-Tat (1), was performed using a solid phase strategy (Fig. 1B). A set of related analogs (2–6) with different peptide lengths and individual deletion of CoA, octyl, and Tat, respectively, were also synthesized (Fig. 2A) (17).

To analyze the cellular effects of GO-CoA-Tat, we generated two human cell lines [in HeLa (epithelial) and HEK (embryonic kidney)] that stably express GOAT and preproghrelin (see fig. S1A) and show robust production of ghrelin in both its acyl and desacyl forms when grown in medium supplemented with octanoic acid. GO-CoA-Tat but not control compound D4-Tat (tetra-aspartate, to simulate the negative CoA charge, similarly linked to the Tat peptide) inhibited the production of acyl ghrelin but not desacyl ghrelin with an $IC_{50} \sim 5 \mu M$ (Fig. 1 and fig. S1). Interestingly, maximal inhibition was achieved only after 24 h of exposure to compound in both preproghrelin/GOAT-transfected HeLa and HEK cells (Fig. 1 and fig. S1). The slow kinetics might result from either atypical enzymatic characteristics or pre-formed acyl ghrelin stores. To further investigate this delay, we tested GO-CoA-Tat in vitro with recombinant microsomal GOAT (Fig. 2C and fig. S2) using a radioactive assay (14). Virtually complete GOAT inhibition was achieved with 100 nM GO-CoA-Tat within 5 min (Fig. 2C and fig. S2), suggesting that the delay in reduction of cellular acyl ghrelin levels may reflect a significant preexisting intracellular reservoir. We also showed that two chemically modified versions of the inhibitor, GO-CoA-Tat-F4BP and GO-CoA-Tat-L5BP, in which Phe4 or Leu5, respectively, is replaced with a photoreactive amino acid benzoyl-phenylalanine and each is tagged with a biotin group, can covalently cross-link to recombinant solubilized or microsomal GOAT, providing evidence for direct binding of GO-CoA-Tat (Fig. 2D and fig. S2C). This cross-linking interaction appears to be specific, as it can be blocked by GO-CoA-Tat (Fig. 2D and fig. S2C).

GO-CoA-Tat appears to be a selective GOAT antagonist since at 10 μM , it showed less than 15% inhibition of three acetyl-CoA utilizing enzymes in vitro including p300 HAT, PCAF HAT, and serotonin N-acetyltransferase (fig. S3A). Moreover, GO-CoA-Tat appears non-toxic to cell viability in the concentration range studied and does not antagonize the GHSR-1a receptor (fig. S3, B to G). A broader analysis of GO-CoA-Tat (1) and analogs (2–

6) reveals a requirement for at least 10 ghrelin residues, as well as the CoA, octanoyl, and Tat components, respectively, for the most potent cellular inhibition of cellular GOAT (Fig. 2, A and B). These results are consistent with GO-CoA-Tat behaving as a bona fide bisubstrate analog in antagonizing GOAT activity. Furthermore, the requirement for the Tat sequence for inhibitory activity (see compound 6, Fig. 2B) argues that cell penetration is critical, and the compound is not acting on a cell surface receptor.

To examine whether GO-CoA-Tat blocks acyl ghrelin production in mice, we explored the effect of intraperitoneally delivered GO-CoA-Tat at 11 $\mu\text{mol/kg}$ (40 mg/kg) in wild type (wt) C57BL6 animals on a medium chain triglyceride (MCT) diet (13). Treatment with GO-CoA-Tat, but not D4-Tat control or vehicle, led to decreased serum levels of acyl ghrelin (Fig. 3A and fig. S4, C and D), with maximum inhibition 6 h after administration (Fig. 3A). There was no significant effect on serum levels of desacyl ghrelin (Fig. 3B). Since the average ghrelin levels were found to vary considerably among mice, the statistical significance was most clear when acyl ghrelin was expressed as a percent of the total (Fig. 3C). These results strongly suggest that GO-CoA-Tat targets GOAT in vivo.

We next examined the effect of GO-CoA-Tat on weight gain. Wt mice were fed an MCT diet over a one month period. These mice were treated every 24 h with GO-CoA-Tat (11 $\mu\text{mol/kg}$ IP) and monitored daily for body mass. In addition, the mice were subjected to quantitative magnetic resonance (QMR) spectroscopy to evaluate the animals' fat and lean mass (13). These experiments showed that chronic GO-CoA-Tat treatment in mice prevented the significant weight gain observed in vehicle-treated mice on an MCT-rich high fat diet (Fig. 3D). Moreover, the QMR measurements showed that, relative to controls, the GO-CoA-Tat treated animals displayed significantly lower fat mass, but not lean mass (Fig. 3E and fig. S4, A and B).

To investigate the potential for GO-CoA-Tat induced generalized toxicity as a source of weight loss, we assessed the blood chemistries and blood cell counts in the animals after one month of GO-CoA-Tat treatment. These analyses showed no evidence of liver, renal, pancreas, or bone marrow toxicities that could account for weight loss (fig. S5). Importantly, GO-CoA-Tat treated mice displayed lower blood glucose as well as lower IGF-1 levels, which is consistent with endogenous acyl ghrelin modulating the somatotrophic axis (fig. S5).

To further understand the effects of GO-CoA-Tat, we studied its effects on body weight and adiposity in ghrelin knockout mice. In contrast to its behavior in wt mice, GO-CoA-Tat did not significantly alter weight, fat, or lean mass in ghrelin knockout mice (Fig. 3, F and G, and fig. S4B). In a separate long term treatment study, we confirmed that during the course of one month of GO-CoA-Tat administration, ghrelin knockout mice gained more weight than otherwise genetically and age-matched wt mice (fig. S6A). Since initial body weights in ghrelin knockout mice were reduced compared to wt mice (19.7 g vs. 21.5 g), these data suggest that the GO-CoA-Tat treatment in the wt animals induces functional loss of ghrelin, bringing wt and knockout animals closer together. The food intake in these GO-CoA-Tat-treated wt and ghrelin knockout mice was similar (fig. S6B) suggesting that the weight differences might be caused by differences in metabolic activity as suggested by recent GOAT knockout studies (13), although we have not directly tested this. Finally, in an additional wt mouse study, we showed that GO-CoA-Tat slowed weight gain in wt mice fed a high fat diet. In that study we observed a relative reduction of fat mass without a change in lean mass, but no effect on food intake (fig. S7). Taken together, these studies suggest that GO-CoA-Tat can specifically reduce acyl ghrelin via GOAT inhibition and thereby prevent weight gain in mice.

It has been reported that acyl ghrelin can influence glucose homeostasis and insulin secretion in pancreatic islet cells (6–8,18–20), although the precise impact has varied among different studies (8,13,20–22). We pre-treated human islet cells with GO-CoA-Tat and showed that these cells displayed a statistically significant increase in insulin response to a glucose challenge when exposed to GO-CoA-Tat (fig. S8). These results suggest that acyl ghrelin plays a direct role in blunting insulin response, similar to what has recently been reported in humans (22). To investigate this in vivo, we studied wt mice that received an intraperitoneal glucose challenge of 2.5 g/kg after pre-treatment with GO-CoA-Tat. These mice show a significant increase in insulin response (Fig. 4A) that was accompanied by a reduction in blood glucose (Fig. 4B). We repeated this glucose challenge in ghrelin knockout animals and, under these conditions, GO-CoA-Tat did not have a significant effect compared either to vehicle or to its impact on wt animals analyzed in parallel (fig. S9). These data support the hypothesis that GO-CoA-Tat's effects on glucose regulation are mediated by acyl ghrelin inhibition. It is interesting that glucose tolerance tests in GOAT knockout vs. wt mice have shown mixed results (13) which may suggest that the acute pharmacologic action of acyl ghrelin inhibition is important for the insulin and glucose response observed here.

To further investigate the connection between GOAT inhibition and insulin regulation, we studied pancreatic islets isolated from mice treated with GO-CoA-Tat. As shown in Fig. 4, the insulin-producing β -cells stained positive for GHSR and islets exhibited a small population of ghrelin expressing cells, which are distinct from β -cells (Fig. 4C). QRT-PCR of islets isolated from mice treated with GO-CoA-Tat demonstrated a 20-fold reduction in *UCP2* mRNA (encoding uncoupling protein 2, a suppressor of insulin secretion) levels as compared to islets isolated from D4-tat treated mice (Fig. 4D) but no change in levels of insulin, ghrelin, or GHSR mRNAs. Additionally, QRT-PCR showed non-statistically significant effects on *UCP2* in the gastric fundus (Fig. 4E). Taken together, these data point to a tissue-specific role for GOAT inhibition in suppressing *UCP2* levels and enhancing insulin release in response to glucose. That GOAT inhibition modulates *UCP2* levels so dramatically further substantiates the connection of acyl ghrelin to obesity and type 2 diabetes (23–25).

Directly targeting the biosynthesis of the active acyl ghrelin hormone offers several potential advantages over receptor antagonists. First, these enzyme inhibitors may not need to cross the blood-brain barrier unlike acyl ghrelin receptor blockers, for which many of the key sites of action are in the brain (2,26). Second, receptor antagonists may drive higher acyl ghrelin formation and increase acyl/desacylin ghrelin ratios (21,26–28) that could be blunted by targeting the biosynthetic pathway. Finally, targeting an enzyme may have advantages over targeting an abundant receptor. Although GOAT inhibitor GO-CoA-Tat has some limitations as a peptide-based agent, we anticipate that future synthetic derivatization will be able to further maximize its pharmacodynamic and/or pharmacokinetic properties. In summary, this report lays the foundation for an approach to pharmacologic management of metabolic disorders through targeted GOAT blockade. We note that this strategy also has potential application to the targeting of other GOAT-related mBOAT (membrane bound O-acyl transferase) family members implicated in lipid metabolism and in important cancer-related signaling pathways such as hedgehog and Wnt (29).

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Fig. 1. GO-CoA-Tat is a bisubstrate inhibitor that inhibits GOAT, lowering acyl ghrelin levels. **(A)** Mechanism-based design strategy. Lipid-enzyme interaction, not shown, may also be important. **(B)** Structure of GO-CoA-Tat and synthetic scheme for bisubstrate inhibitors that consist of three components: coenzyme A, octanoylated ghrelin peptide and a Tat peptide; Ahx-aminohexanoate. **(C)** Temporal inhibition of acyl but not desacyl ghrelin production by 6 μM GO-CoA-Tat in GOAT/preproghrelin-transfected HeLa cells. **(D)** Dose-response reduction of acyl but not desacyl ghrelin levels by GO-CoA-Tat in GOAT/preproghrelin-transfected HeLa cells after 24 h incubation.

Fig. 2.

GO-CoA-Tat targets GOAT directly in vitro and in a structure specific manner. **(A)** Structure of GO-CoA-Tat analogs (1–6). **(B)** Acyl and desacyl ghrelin levels after treatment with 6 μ M GO-CoA-Tat (1) and analogs (2–6) from GOAT/preproghrelin-transfected HeLa cells after 24 h. **(C)** In vitro acyltransferase inhibition assay (5 min reaction) with microsomal recombinant GOAT. **(D)** UV crosslinking of solubilized GOAT by biotin-tagged, benzophenylalanine analogs of GO-CoA-Tat (L5BP, F4BP) (5 μ M). Competitor is GO-CoA-Tat at 100 μ M. Immunoblots of cross-linked GOAT were visualized with streptavidin, loading was checked with anti-FLAG.

Fig. 3.

Effects of GO-CoA-Tat on blood ghrelin and body weight in mice. (A) Serum acyl ghrelin levels in WT C57BL6 mice on an MCT diet treated intraperitoneally with 11 μ mol/kg GO-CoA-Tat vs. D4-Tat control ($n = 5$) after 6, 12, and 24 h. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, std. errors shown). The changes in acyl ghrelin over 24 h in control animals are neither statistically significant ($P > 0.2$), nor reproducible in other experiments. (B) Serum desacyl ghrelin levels for experiment in Fig. 3A. (C) Percent acyl ghrelin for experiment in Fig. 3A. (D) Body weights in wt C57BL6 mice on an MCT diet treated with 11 μ mol/kg GO-CoA-Tat (red, $n = 5$) or vehicle (black, $n = 6$) for 1 mo (* $P < 0.05$; conventional ** and *** omitted for clarity, standard errors shown). (E) Fat mass in wt mice measured by QMR for experiment in 3D. (F) Body weights in ghrelin knockout C57BL6 mice on an MCT diet treated with 11 μ mol/kg GO-CoA-Tat (red, $n = 5$) or vehicle (black, $n = 5$) for 1 mo (standard errors shown). The larger error bars compared to data in Fig. 3D likely represent

the broader distribution of starting weights. Also note that the scales differ in the two panels, contributing to the larger error bars seen here. **(G)** Fat mass in ghrelin knockout mice measured by QMR for experiment in Fig. 3F.

Fig. 4. GO-CoA-Tat increases insulin, decreases glucose levels, and down-regulates islet cell *UCP2* mRNA. (A) C57BL/6 wt mice raised on normal mouse chow and treated with 8 μ mol/kg GO-CoA-Tat ($n = 4$) experienced a statistically significant increase in insulin secretion and (B) a statistically significant decrease in blood glucose as compared to control mice (treated with D4-Tat ($n = 4$)) when compound was administered 24 h prior to IP glucose challenge (2.5g/kg) (* $P < 0.05$, ** $P < 0.01$, std. errors shown). (C) Immunohistochemical staining of mouse islets. Left panel – insulin (green) ghrelin receptor (GHSR) (red) and cell nuclei stained with DAPI (blue). Middle panel – staining of islet for ghrelin (white) and insulin (green) demonstrates dual staining and ghrelin positive and insulin negative cells. Right panel – closeup of unmerged images in boxed area of middle panel. (D) QRT-PCR of islets and (E) gastric fundus isolated from mice treated with inhibitor 24 h prior to isolation and mRNA expression relative to control ($n = 3$).