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An Acyl-Ghrelin Specific Neutralizing Antibody Inhibits the Acute Ghrelin-Mediated Orexigenic Effects in Mice

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Abstract

Ghrelin is a 28-amino acid peptide secreted mainly by the stomach. Acyl-ghrelin, which binds to and activates the growth hormone secretagogue receptor type 1a (GHS-R1a), is considered to be the active form for its orexigenic effects. It has been demonstrated that peripheral administration of ghrelin stimulates food intake and adiposity in rodents and humans. Accordingly, different approaches to antagonize ghrelin/GHS-R1a signaling have been pursued for the treatment of obesity. In the present study, we generated and characterized high affinity anti-acyl ghrelin specific monoclonal antibodies (mAbs). *In vitro*, the lead mAb (33A) displayed specific binding to acyl-ghrelin with an estimated $K_d < 100$ pM. In recombinant receptor cell-based assays, 33A dose-dependently inhibited the ghrelin-mediated calcium signal with an $IC_{50} \sim 3.5$ nM. *In vivo*, ghrelin dose-dependently stimulated food intake in mice, and this effect was fully blocked by a single injection of 33A. In a 4-week chronic study, 33A was shown to effectively bind to endogenous acyl-ghrelin; however, chronic administration of 33A did not affect food intake or body weight gain in a mouse model of diet-induced obesity (DIO). Our results indicate that peripheral neutralization of ghrelin can suppress appetite stimulated by a transient surge in ghrelin levels. The lack of long term long-term effects on body weight control by 33A suggests that compensatory mechanisms may contribute to the regulation of energy balance.

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Ghrelin is a hormone that is primarily produced in the gut and that has a unique posttranslational acylation modification at its serine-3 residue (Kojima et al., 1999; Nakazato et al., 2001). This acylation process is essential for the endocrine actions of ghrelin. Acyl-ghrelin, which accounts for about 10% of the total circulating ghrelin, activates its receptor, growth hormone secretagogue receptor type 1a (GHS-R1a), to stimulate food intake and growth hormone secretion (Kojima et al., 1999; Nakazato et al., 2001; Sun et al., 2004). In contrast, des-acyl ghrelin, which lacks the fatty acid modification, fails to bind or activate the GHS-R1a at physiological concentrations *in vitro* (Hosoda et al., 2000). Recent studies, however, indicated that des-acyl ghrelin may offset the inhibitory effect of acylated ghrelin on insulin secretion in humans (Broglia et al., 2004) and reduce food intake in rats (Chen et al., 2005). Therefore, to develop a pharmacological tool that selectively inhibits acyl-ghrelin is critical for evaluating the effects of blocking ghrelin/GHS-R1a pathway.

The physiological function of ghrelin as an orexigenic hormone has been shown to be independent of its effects on growth hormone secretion (Toshinai et al., 2001; Tschop et al., 2000). The ghrelin receptor is highly expressed in the arcuate nucleus (Arc) of hypothalamus, and in neurons producing neuropeptide Y (NPY) and Agouti-related protein (AgRP), two well known orexigenic peptides. Ghrelin administration activates NPY/AgRP neurons and increases hypothalamic NPY mRNA expression (Chen et al., 2004). Moreover, administration of NPY and AgRP antagonists abolishes the ghrelin induced hyperphagic responses (Horvath et al., 2001; Schwartz et al., 2000) suggesting that ghrelin is part of the neural-network involved in the homeostasis of energy balance.

Circulating ghrelin levels have been shown to fluctuate with feeding status. Specifically, ghrelin concentrations rise before meals and fall rapidly after meals, suggesting a role for ghrelin in meal initiation (Cummings et al., 2001). Plasma ghrelin levels are negatively associated with body

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weight. In humans, ghrelin levels are reduced in obese subjects and rise following weight loss, reflecting a feedback mechanism to reduce appetite in the obese state and increase appetite in the normal state (Cummings et al., 2002; Tschop et al., 2000). Moreover, peripheral administration of ghrelin stimulates food intake and body weight gain in both rodents and humans (Tschop et al., 2000; Wren et al., 2001). Furthermore, people with Prader-Willi syndrome, a disease associated with hyperghrelinemia, are hyperphagic and obese (Erdie-Lalena et al., 2006). In contrast, decreased ghrelin levels have been associated with the weight loss that follows either partial or total gastrectomy (Takachi et al., 2006).

Inhibition of the ghrelin/GHS-R1a signaling pathway represents a potential approach for treating obesity. Genetic deletion of ghrelin or GHS-R1a results in a modest weight reduction in mice that were fed a high fat diet (Wortley et al., 2005; Zigman et al., 2005). Additionally, intracerebroventricular (i.c.v.) injection of anti-ghrelin polyclonal IgG in rats dose-dependently inhibited fast-induced feeding and suppressed dark phase food intake (Nakazato et al., 2001). Administration of a small molecule GHS-R1a antagonist effectively reduced food intake and promoted body weight loss in diet-induced obese (DIO) mice (Esler et al., 2007). Furthermore, ghrelin neutralization with a ribonucleic acid Spiegelmer reduced obesity in DIO mice (Shearman et al., 2006). Recently, ghrelin-O-Acyltransferase, an enzyme that attaches octanoate to serine-3 of ghrelin, has been identified (Yang et al., 2008). It has been proposed that inhibition of this enzyme would block the ghrelin action and such an inhibitor would be very specific since ghrelin is the only known protein to be modified by acylation. A similar approach can be applied to selectively block the activity of ghrelin with an antagonist that only recognizes acylated ghrelin.

In the current study, we report the generation and characterization of 33A, a high affinity acyl-ghrelin specific monoclonal antibody (mAb). The aim of the study was to determine the effects of

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peripheral injection of 33A on ghrelin-induced food intake in mice and to assess its utility as a tool to investigate the effects of peripheral neutralization of the ghrelin signal on energy balance.

Materials and Methods

Animals and Drugs. All mouse studies were conducted at Amgen Inc. and approved by the Institutional Animal Care and Use Committee. Male C57BL/6 mice were purchased from Charles Rivers Laboratories, Frederick, MD. Mice were single-housed in a temperature-controlled environment with a 12-h light and 12-h dark cycle (06:30-18:30), and had free access to a standard chow diet (Harlan Teklad, Madison, WI) for at least 2 weeks before injection of the antibody. Compounds including ghrelin and antibodies were formulated in PBS and injected i.p. in 100 μ l volume at the indicated dose level.

Antibody Generation and Screening. The antigen was composed of the first 11 amino acids of human ghrelin coupled with KLH. The immunization procedures were performed as described previously (Kilpatrick et al., 1997). Briefly, over the course of 11 days, a total of 5 immunizations were carried out at subcutaneous sites proximal to draining lymph nodes in 8-10 week old BDF1 mice. After the final series of injections, cell suspensions of lymphocytes harvested bilaterally from popliteal, superficial inguinal, axillary, and brachial lymph nodes were fused with SP2/0.AG14 cells at a ratio of 2.5:1 by electro fusion. Fusion and hybridoma selection were performed as described by Kohler and Milstein (Kohler and Milstein, 1975).

Antibody Binding Assay. Serial dilutions of biotin-labeled human full-length acyl- or des-acyl ghrelin (1 μ M-0.45 nM) were coated onto a Reacti-bind Neutravidin plate (Pierce, Rockford, IL). Antibody 33A (100 μ l at 5 μ g/ml) was added to the plate and incubated for 45 min. Bound antibody was detected with HRP-conjugated goat anti-mouse Fc (Pierce) coupled in the presence of the chromogenic substrate 3,3', 5,5'-tetramethylbenzidine (TMB, Sigma, St. Louis, MO). The absorbance was read at 450 nm.

BIACore Measurements. BIACore measurements were carried out on a BIACore 2000 instrument (BIACore Inc., Piscataway, NJ). Immobilization of Neutravidin to the CM5 sensor chip surface was performed according to the manufacturer's instructions. To perform a solution equilibrium binding analysis of 33A by BIACore, 33A (1 nM) was incubated with varying concentrations (0.03 nM-1000 nM) of human ghrelin in PBS with 0.005% P-20 and 0.1 mg/ml ml BSA. Incubations were performed at room temperature for at least 5 h to allow samples to reach equilibrium. The incubated samples were then injected over the immobilized surfaces. The dissociation equilibrium constant (K_d) was obtained from a nonlinear regression analysis of the competition curve using a one-site homogeneous binding model (KinExA Pro software, Sapidyne Instruments Inc., Boise, ID).

Epitope Mapping. The antibody binding site was mapped using an ELISA-based competition study using previously described methodology (Wang and Jin, 1998). Ghrelin fragments were custom-synthesized by Phoenix Pharmaceuticals, Inc. Burlingame, CA. Biotin-labeled full length human ghrelin (2 nM) was coated onto a Reacti-bind Neutravidin-coated plate (Pierce) and incubated with 100 μ l of 33A at 5 μ g/ml and 100 μ l of ghrelin fragments (Ghr aa 1-11, Ghr aa 1-7, and Dap-Ghr aa 1-7) at 2 μ M for 1 h at room temperature. Bound antibody was detected as described above (antibody binding assay).

Functional Activity of Neutralizing Antibodies. CHOD⁻ cells stably expressing the human GHS-R1a and aequorin reporter genes (Euroscreen S.A., Brussels Belgium) were used to measure ghrelin-induced mobilization of intracellular calcium (Howard et al., 1996; Stables et al., 1997). Cells were plated the night before the assay at a density of 3×10^4 cells/well in a 96-well assay plate (Corning, Inc., Corning, NY). They were then loaded with 15 μ M coelenterazine (Invitrogen Corp. Carlsbad, CA) in assay buffer (Ham's F12, 30 mM HEPES, 0.1% BSA) for 2 h.

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Serial dilutions of antibodies (10^{-6} M – 10^{-9} M) were incubated with 10 nM of ghrelin (Phoenix Pharmaceuticals, Inc.) for 1 h before being added to the cells. Calcium flux was measured using a luminescence imaging plate reader (Amgen Inc., Thousand Oaks, CA). The neutralizing activity of the tested antibodies (EC_{50}) was calculated from the value of peak luminescence.

Effect of 33A on Ghrelin-Induced Food Intake in Mice. Mice were continuously acclimated for 7 days to daily saline injection and were fed chow pellets in a Petri dish. At the end of the acclimation period, mice were randomly assigned to treatment groups based on their body weights. Two h before starting the measurement of food intake (7:00), mice were injected i.p. with vehicle, control IgG (6 nmol), or 33A (at doses of either 3 or 6 nmol). At 9:00, mice were injected with 3 nmol ghrelin or vehicle and immediately placed into new cages with free-access to a known amount of chow pellets in a Petri dish. Food intake was monitored by weighing the food at 0.5, 1, 2, and 3 h after the ghrelin injection. Mice were sacrificed and trunk blood was collected 4 h after vehicle or ghrelin injections.

Effect of 33A on Fasting-Induced Refeeding in Mice. After the 7-day acclimation period, at 21:00, mice were injected i.p. with vehicle, control IgG (100 μ g), or 33A (at doses of either 10 or 100 μ g). Mice were then fasted for 12 h and the next morning, at 9:00, mice were refeed with a known amount of food. Food was weighed 1, 2, 4, 6, 8, and 24 h after the initiation of the refeeding. Mice were sacrificed and trunk blood was collected 24 h after the refeeding period.

Chronic Administration of 33A in DIO Mice. DIO mice were prepared by feeding 4 week old C57BL/6 male mice a high fat-diet (HFD) with 60% of the calories derived from fat enriched with saturated fatty acids (D12492 Research Diets, Inc., New Brunswick, NJ, USA). Following 12 weeks of the diet period, DIO mice were stratified into control and treatment groups based on body weight. They were ip injected with control IgG or ghrelin neutralizing antibody 33A at

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15mg/kg every three days for 4 weeks. Mice were continually fed HFD during the drug-treatment period. Body weight was recorded every three days at the time of injection, and food intake was measured every 6 days throughout the study. Three days after the last injection, blood was collected from each mouse to measure plasma ghrelin and antibody levels.

Plasma Ghrelin Measurements. Plasma ghrelin was measured using an ELISA kit from Linco Research, St. Charles, MO. Blood samples were collected 36 h after peptide, antibody, or IgG injections and immediately transferred to chilled tubes containing Na₂EDTA (1.25 mg/ml) and aprotinin (2 µg/ml). Plasma was separated by centrifugation at 2,000 rpm for 15 min at 4°C. Hydrogen chloride was added to the samples at a final concentration of 0.1 N immediately after separation of the plasma. The concentrations of acyl- and des-acyl ghrelin were measured according to the manufacturer's protocol. To measure bound acyl-ghrelin levels, bound ghrelin was dissociated from 33A by acid treatment (0.15N HCl) and 33A was then precipitated using ethanol (61% v/v) (Koranyl et al., 1973). Samples were centrifuged at 5000 rpm for 30 min at 4°C to remove the precipitated 33A. The resulting supernatant was transferred to a fresh tube and lyophilized in a SpeedVac. The lyophilized plasma was then resuspended with Linco ELISA assay buffer and the ghrelin level was measured according to the manufacturer's protocol.

Plasma 33A Levels. Acyl-ghrelin (0.5µg/ml) was first coated on an ELISA plate, and plasma samples from treated animals were incubated on the plate for 2 h. The 33A was detected with HRP-conjugated goat anti-mouse Fc (Pierce) coupled in the presence of the chromogenic substrate 3,3', 5,5'-tetramethylbenzidine (TMB, Sigma, St. Louis, MO). The absorbance was read at 450 nm.

Statistical Analysis. Data analysis and EC₅₀ values was estimated using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA.). All values are expressed as the mean ± S.E.M.

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Comparisons across groups were done using One Way Analysis of Variance (ANOVA). When the ANOVA yielded a significant difference, post hoc analysis was done using Turkey's HSD.

Results

Ghrelin Monoclonal Antibody 33A Selectively Binds to Acyl-Ghrelin. Mouse mAbs were generated by immunizing mice with the N-terminal fragment of the acyl-ghrelin. To investigate the binding specificity of the mAbs, we performed an ELISA. As shown in Fig. 1A, Ghrelin antibody 33A bound to acyl-ghrelin with an EC_{50} ~2 nM. 33A failed to bind to desacyl-ghrelin at concentrations up to 1 μ M. The specificity of 33A to acyl-ghrelin was further confirmed in the BIAcore based solution equilibrium assay. The dissociation constant (K_d) was estimated to be < 100 pM (Fig. 1B). An ELISA based epitope mapping was used to further define the critical residues of ghrelin for 33A binding. As shown in Fig. 1C, the addition of the N-terminal acyl-ghrelin fragment (aa 1-7) completely abolished the 33A binding to the full-length ghrelin, suggesting that 33A epitope was located within the N-terminal first 7 amino acids. Moreover, replacement of the fourth amino acid with alanine reduced the interaction between 33A and ghrelin (Fig. 1C). Importantly, Dap-ghrelin, in which the serine 3 residue with octanoic acid was replaced with diaminopropionic acid (Dap) (Bednarek et al., 2000) failed to compete with the 33A binding to acyl-ghrelin. The results indicated that the acyl group attached to the serine 3 residue and its neighboring phenylalanine are critical residues for the binding of 33A to ghrelin.

Ghrelin Monoclonal Antibody 33A Inhibited Ghrelin-Induced Calcium Mobilization *In Vitro*. After establishing that 33A selectively bound acyl-ghrelin, the antagonism of ghrelin-induced calcium mobilization was examined in an aequorin-based functional assay. As shown in Fig. 2, 33A dose-dependently inhibited both human and mouse acyl-ghrelin mediated calcium signals with similar IC_{50} values. Unsurprisingly, 33A showed similar antagonistic activity against both species due to the fact that the N-terminal region of human ghrelin (amino acids 1-10) is 100% identical to the same region of mouse ghrelin.

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33A Inhibited Ghrelin-Mediated Food Intake. Peripheral administration of acyl-ghrelin in mice induced robust and transient increases in food intake (Fig. 3A). Most of the orexigenic effects occurred 30 min after ghrelin injection (Fig. 3A). To investigate the neutralizing activity of 33A to acyl-ghrelin *in vivo*, we tested whether 33A would block ghrelin-induced food intake in mice. As demonstrated in Fig. 3B, 33A at doses of 3 and 6 nmol inhibited ghrelin (3 nmol) induced food intake by 90% and 100%, respectively; while vehicle or control IgG (6 nmol) had no effect on ghrelin induced food intake. These results confirmed the neutralizing activity of 33A to ghrelin *in vivo*, and provided a useful pharmacological tool to investigate the role of ghrelin in energy intake under various nutritional statuses.

33A Had No Effect on Food Intake After Fast-Refeeding Challenge. Since ghrelin levels are elevated during fasting (Cummings et al., 2001), it has been proposed that ghrelin is an important meal-initiation hormone. We therefore investigated whether 33A would block fasting-induced food intake followed by refeeding in mice. Control IgG (100 µg) or one of two doses of 33A (10 µg or 100 µg) was administered 12 h before refeeding the mice to allow enough time for the mAb to accumulate in the circulation. Food intake was monitored for 24 h during the course of refeeding (Fig. 4). The difference in accumulated food intake between 33A and control IgG treated mice was not statistically significant at any of the time points measured.

Plasma Ghrelin Levels Increased after 33A Administration. To study the effects of 33A on plasma ghrelin levels, mice were exposed to a fast-refeeding challenge as previously described. 33A increased plasma acyl-ghrelin by approximately 8-fold, suggesting that the neutralization of ghrelin by 33A induced a compensatory secretion of acyl-ghrelin (Fig. 5A). The plasma des-acyl ghrelin level was not affected with 33A administration (Fig. 5B).

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Chronic Administration of 33A Had No Effects on Body Weight and Food Intake in DIO

Mouse Model. To test the long-term effects of 33A, DIO mice were ip injected with 33A or IgG controls (15 mg/kg every 3 days) for 4 weeks. Mice treated with antibody 33A showed similar body weight gain when compared with mice treated with control IgG (Figure 6A). The cumulative food intake calculated by combining the amount of food consumed from all previous days was also not different in mice treated with 33A compared with the control animals (Figure 6B). At the end of the 4-week treatment period, 99% of acyl-ghrelin in the plasma was bound to 33A (Fig. 6C). Furthermore, free 33A levels in the plasma were ~15,000pmol/ml, approximately 500,000 fold higher than the circulating acyl-ghrelin levels (Fig. 6D).

Discussion

Obesity and its associated complications are among the most prevalent global health problems. A therapeutic strategy being actively pursued is to block the orexigenic pathways (Foster-Schubert and Cummings, 2006). Most of the appetite-stimulating molecules, such as neuropeptide Y (NPY), AgRP, melanin-concentrating hormone (MCH), and endocannabinoids, are produced in the specific regions of the brain that are involved in the regulation of energy balance. Ghrelin is the only peripheral orexigenic peptide known to date, and its orexigenic activity is dependent on the acylation of the peptide. Considerable evidence suggests that ghrelin contributes to the hunger signal during meal initiation (Cummings et al., 2001; Drazen et al., 2006). The relative importance of ghrelin in the overall regulation of energy balance, however, is still unclear. Ghrelin knock out (KO) mice display minimal body-weight changes on standard chow diet (Sun et al., 2004). Nevertheless, the KO mice do not address the specific role of acyl-ghrelin in energy balance, as both forms of ghrelin are eliminated, and some studies suggest that des-ghrelin counteracts the metabolic actions of acylated ghrelin (Broglia et al., 2004). Additionally, ghrelin KO mice may have developed adaptive mechanisms in their neuroendocrine pathways in order to compensate the lack of ghrelin signal since conception. Thus, a pharmacological agent that selectively blocks the acyl-ghrelin mediated signal in adult animals is a useful tool to elucidate the function of acyl-ghrelin in energy homeostasis.

Nakazato *et al.* generated a polyclonal antibody against ghrelin (Nakazato et al., 2001). To date, this is the only anti-ghrelin antibody that has been reported. Although this study showed that i.c.v. injection of the anti-ghrelin IgG suppressed starvation-induced feeding compared with the preimmune IgG (Nakazato et al., 2001), the specificity and selectivity of this antibody were not characterized. In the current study, we showed that 33A preferentially binds to the acylated form of ghrelin with high affinity, and not to des-acyl ghrelin. Two independent *in vitro* assays, ELISA and BIAcore, were used to establish the binding preference of 33A. Moreover, 33A blocked the

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increase in food intake induced by exogenous administration of acyl-ghrelin, demonstrating that 33A antagonized activity associated with acylated ghrelin in mice.

During the fast-refeeding challenge, 33A did not influence the food intake, as shown by the fact that mice treated with 33A consumed amounts of food similar to that consumed by the control IgG group. It is noteworthy that plasma acylated ghrelin increased about 8 fold with 33A treatment and des-acylated ghrelin level remained unchanged. Therefore the acylated/total ghrelin ratio increased by 7 fold in mice treated with 33A. These results suggested that blockage of peripheral ghrelin induced compensatory increases in acylated ghrelin levels by an unknown mechanism. This scenario is reminiscent of the observations that patients with chronic atrophic gastritis have higher plasma acylated ghrelin levels and increased acylated/total ghrelin ratio (Campana et al., 2007). In these patients, loss of ghrelin-producing cells induced a compensatory increase in plasma active ghrelin. Whether this is mediated by an increase in the acylating process remains to be determined.

Consistent with the lack of phenotype in ghrelin KO mice, chronic antagonizing peripheral ghrelin using 33A did not reduce food intake or body weight gain in DIO mice; therefore, the pharmacological significance of anti-ghrelin approaches appears to be limited, at least in rodent models. Due to the excess amount of 33A administered in mice and the estimated half-life of 6-8 days for monoclonal antibodies (Roopenian et al., 2003), the neutralizing capability of 33A had far exceeded the endogenously produced acyl-ghrelin. This is demonstrated by the fact that 99% of plasma acyl-ghrelin was bound to 33A. Several factors, therefore, may have contributed to the lack of the 33A effect during the chronic treatment. First, increase of ghrelin levels in the brain induced by fasting cannot be attenuated by peripheral injection of 33A. Although ghrelin is mainly expressed and secreted from the stomach, it is also found to be expressed in the hypothalamus (Nakazato et al., 2001), which is the major site of its orexigenic action (Cowley,

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2003; Cowley et al., 2003). Ghrelin-producing neurons in the hypothalamus are located within and adjacent to the Arc and interact with NPY/AgRP neurons through GHS-R. Secretion of ghrelin in the hypothalamus is highly regulated by nutritional status such as feeding, fasting, and exposure to a high fat diet. Recent studies show that hypothalamic fatty acid metabolism mediates the orexigenic action of ghrelin (Lopez et al., 2008). In the context of food deprivation, the increased ghrelin level promotes feeding through AMP-dependent protein kinase-mediated modulation of hypothalamic fatty acid metabolism. Small molecule GHS-R1a antagonists with central nervous system exposure have been shown to decrease 24-h food intake by 17% in the fasted-refed model (Esler et al., 2007). Administration of 33A into the brain will address whether neutralizing acyl-ghrelin by a mAb in the central nervous system will result in improved energy balance. Second, food intake is regulated by a complex neuroendocrine network and redundant orexigenic pathways. Several neuropeptides including NPY, AgRP, and MCH have been known to induce anabolic and appetite stimulating functions. Inhibition of a single pathway might not have a significant impact to energy homeostasis. Strategies targeting more than one pathway might be required for significant reduction in energy intake.

In summary, we generated a high-affinity mAb to acyl-ghrelin and demonstrated the potent antagonistic activities of the mAb in in vitro and mouse models. The neutralizing mAb provided a useful tool to study the role of ghrelin in short-term changes in nutritional status and in a chronic model of diet-induced obesity. Our study indicates that antagonizing the peripheral ghrelin pathway alone may not be effective for treating obesity; therefore, combination therapies that block multiple orexigenic pathways may be needed to evaluate the potential of anti-ghrelin therapies. Additional studies are needed to provide further insight in understanding of the contributions of the ghrelin pathway in overall energy intake and body weight regulation.

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Footnotes

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Legends of Figures

Fig. 1. Ghrelin monoclonal antibody 33A selectively binds acyl-ghrelin. A, Antibody binding study, Biotin-labeled acyl- (■) or des-acyl ghrelin (◆) was coated onto a Neutravidin plate and incubated with 33A as described in the Materials and Methods section. B, BIACore measurement. 33A was incubated with varying concentrations of human ghrelin and injected onto BIACore surface. The dissociation K_d was obtained from a nonlinear regression analysis of the competition curve using a one-site homogeneous binding model. C, Epitope mapping. Thousand-fold excess of different lengths of ghrelin fragments (Ghr1-11 and Ghr1-7) competed for 33A with biotin-labeled full length ghrelin coated on the plate. Ala# indicates the position of amino acid replacement with alanine. Dap-ghr1-7 indicates serine 3 residue with octanoic acid replaced with Dap. Data represent mean \pm S.E.M.

Fig. 2. Antibody 33A neutralized ghrelin-induced calcium mobilization in an Aequorin cell-based assay. CHO δ cells stably expressed the human GHS-R1a and aequorin reporter genes. Serial dilutions of 33A (10^{-6} M to 10^{-9} M) were incubated with 10 nM of human ghrelin (□) or mouse ghrelin (▲) for 1 h before adding to the cells. Serial dilutions of mouse IgG (■) (10^{-6} M to 10^{-9} M) were incubated with 10 nM of human ghrelin. Calcium flux was measured using a luminescence imaging plate reader. The neutralizing activity of antibodies (EC_{50}) was calculated from the value of peak luminescence.

Fig. 3. Antibody 33A inhibited ghrelin-mediated food intake. A, vehicle, 1 nmol, or 3 nmol of ghrelin was injected and food intake was measured at 0.5, 1, 2, and 4 h. B, vehicle/ghrelin (black bars), control IgG (diagonal bars, 6 nmol), or 33A (dotted bars, 3 nmol; dotted vertical bars, 6 nmol) was injected i.p. 2 h before the administration of 3 nmol ghrelin. One group of mice (open

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bars) was injected with vehicle/vehicle following the same schedules as the treated groups described above. Food intake was measured 0.5, 1, and 2 h after the administration of ghrelin or vehicle. Data represent mean \pm S.E.M., n = 10 per group, *: P < 0.05 vs IgG/ghrelin-treated group.

Fig. 4. Antibody 33A had no effect on food intake during a fast-refeeding challenge. Mice were injected with control IgG (open bars), 10 μ g of 33A (striped diagonal bars), or 100 μ g of 33A (black bars) and then subjected to a 12-h fast. Food intake was measured 1, 2, 4, 8, and 24 h after the refeeding was initiated.

Fig. 5. Plasma acylated ghrelin levels are increased in 33A-treated mice. A, Acyl-ghrelin, B, Des-acyl ghrelin, Mice were injected with IgG (open bars), 10 μ g of 33A (striped diagonal bars), or 100 μ g of 33A (black bars). Refeeding was initiated 12 h after the antibody injection. Blood was collected after a 24 h refeeding period, and plasma levels of acylated ghrelin and des-acylated ghrelin were measured. Data represent mean \pm S.E.M., *: P < 0.05 compared with control IgG-treated group.

Fig. 6. The Effects of 33A Chronic Administration in DIO Mice. DIO mice were ip injected with 33A or IgG controls (15 mg/kg every 3 days) for 4 weeks. The cumulative body weight change (A) and the cumulative food intake (B) were measured. Three days after the last injection, blood samples were collected and plasma free and 33A-bound acyl-ghrelin levels (C) and plasma unbound 33A levels (D) were measured. Data represent mean \pm S.E.M.

Figure 1.

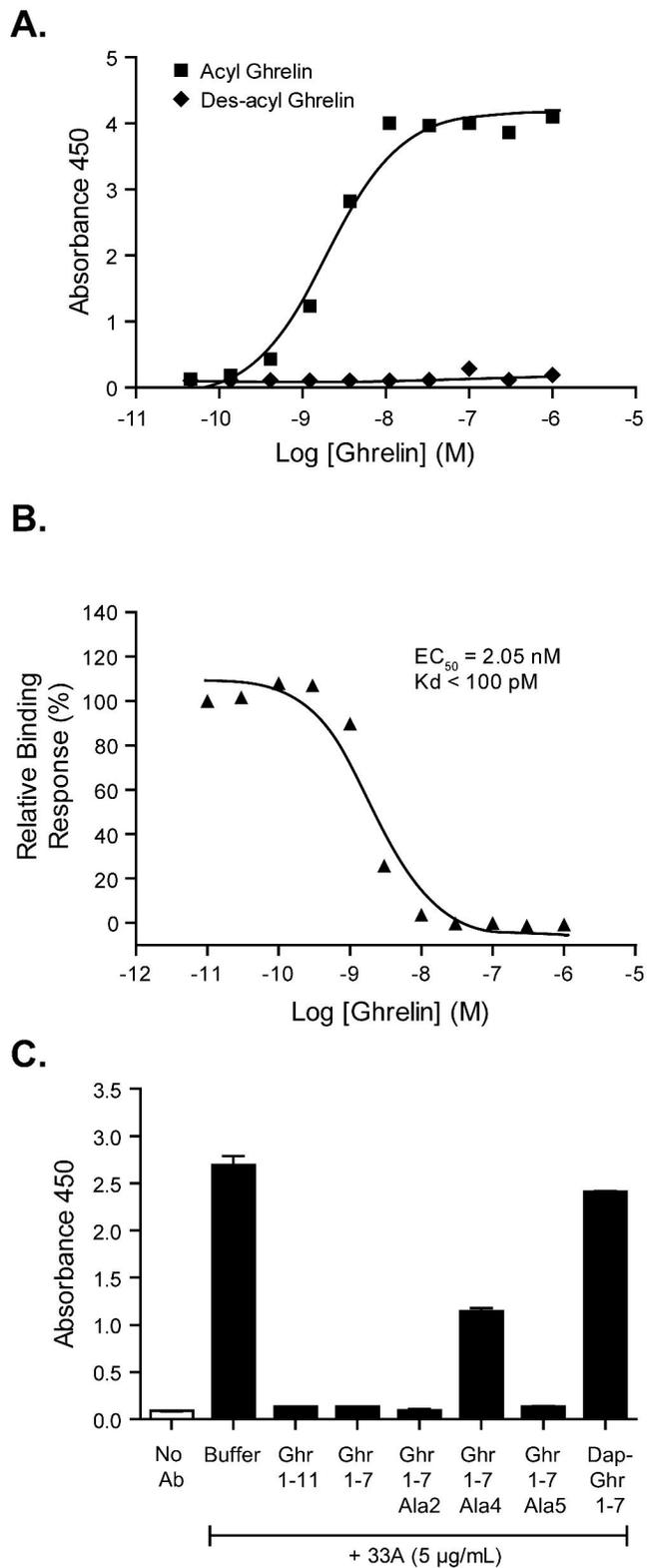


Figure 2.

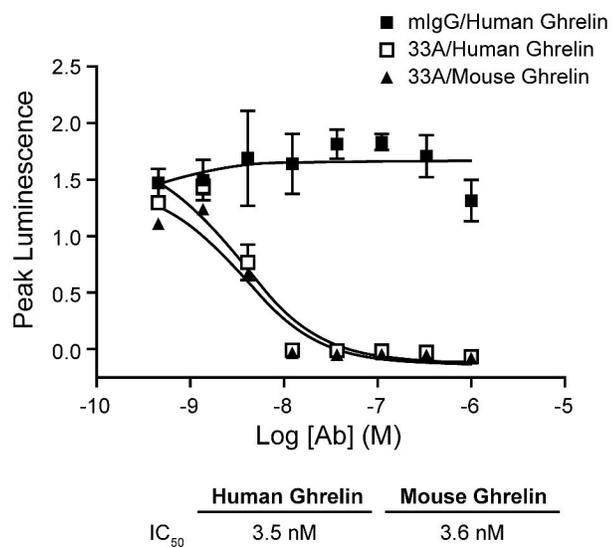


Figure 3.

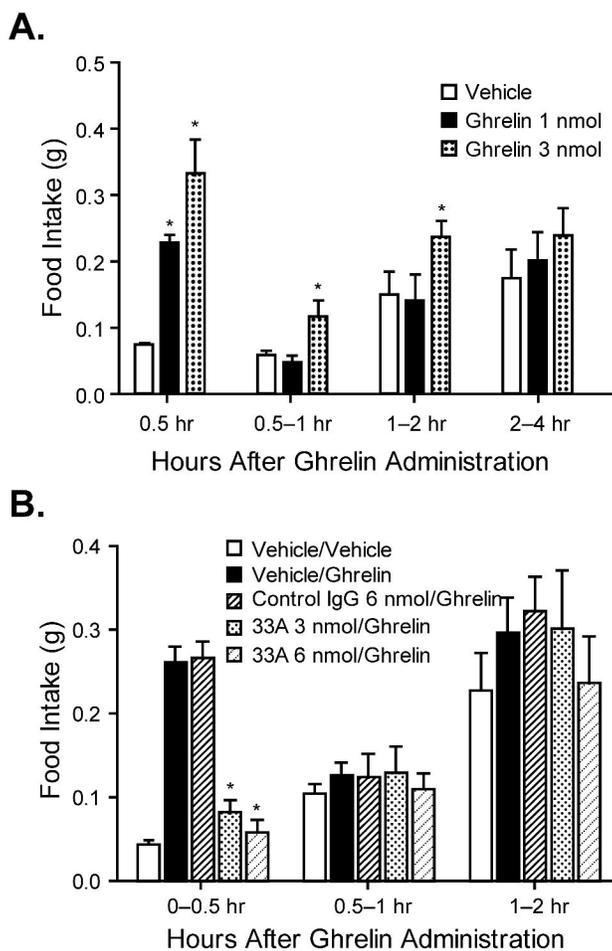


Figure 4.

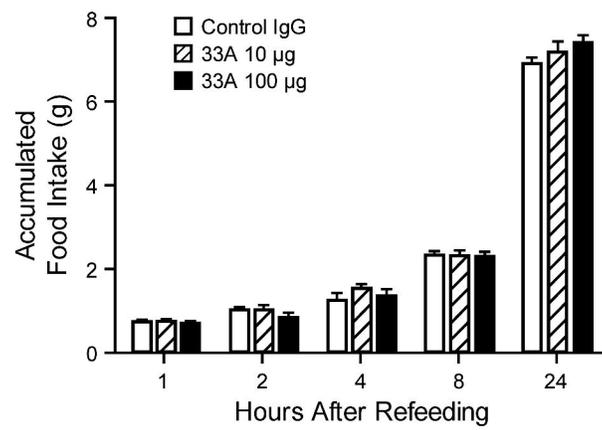


Figure 5.

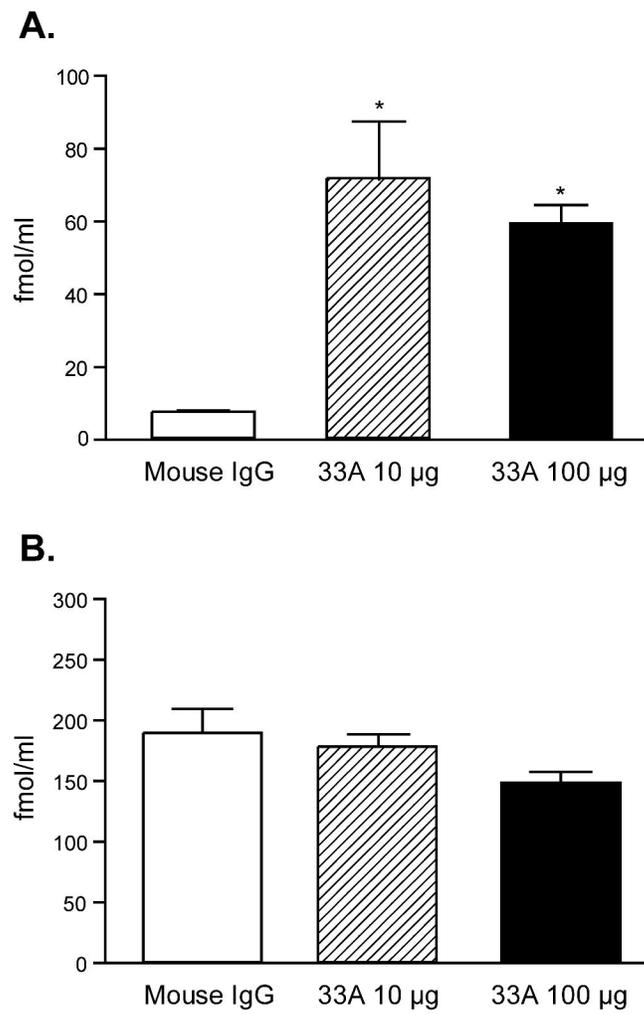


Figure 6

