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Oligoclonal Antibody Targeting Ghrelin Increases Energy Expenditure and Reduces Food Intake in Fasted Mice

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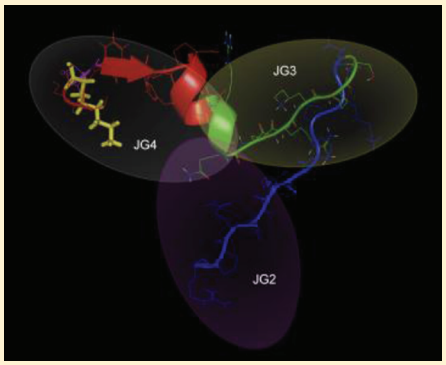
ABSTRACT: Ghrelin, an enteric peptide hormone linked to the pathophysiology of obesity has been a therapeutic target of great interest over the past decade. Many research efforts have focused on the antagonism of ghrelin's endogenous receptor GHSR1a, which is found along ascending vagal afferent fibers, as well as in the arcuate nucleus of the hypothalamus. Additionally, peptidic inhibitors of ghrelin *O*-acyltransferase, the enzyme responsible for the paracrine activation of ghrelin, have recently been studied. Our research has taken an alternative immunological approach, studying both active and passive vaccination as a means to sequester ghrelin in the periphery, with the original discovery in rat of decreased feed efficiency and adiposity, as well as increased metabolic activity. Using our previous hapten designs as a stepping-stone, three monoclonal antibodies (JG2, JG3, and JG4) were procured against ghrelin and tested *in vivo*. While mAb JG4 had the highest affinity for ghrelin, it failed to attenuate the orexigenic effects of food deprivation on energy metabolism or food intake in mice. However, animals that were administered a combination of JG3:JG4 (termed a doublet) or JG2:JG3:JG4 (termed a triplet) demonstrated higher heat dispersion and rate of respiration (higher CO_2 emission and O_2 consumption) during a 24 h fast refeed. Mice administered the triplet cocktail of JG2:JG3:JG4 also demonstrated decreased food intake upon refeeding as compared to control animals. Recently, Lu and colleagues reported that a passive approach using a single, high affinity *N*-terminally directed monoclonal antibody did not abrogate the effects of endogenous ghrelin. Our current report corroborates this finding, yet, refutes that a monoclonal antibody approach cannot be efficacious. Rather, we find that a multiple monoclonal antibody (oligoclonal) approach can reproduce the underlying logic to previously reported efficacies using active vaccinations.



KEYWORDS: *ghrelin, monoclonal antibodies, passive vaccination, active vaccination, metabolism, food intake*

■ INTRODUCTION

Obesity, defined as a body mass index of 30 kg/m^2 or greater, is an increasingly prevalent global health concern that carries increased risk of morbidity and mortality.[1,2] Many hormones and regulatory pathways, both energy homeostatic and non-homeostatic, are involved in the pathophysiology of obesity, a complex, multifactorial medical issue.[3] One hormone implicated in the control of food intake and perhaps in some forms of obesity is ghrelin (Ghr).[4]

Ghrelin, a 28 amino acid residue enteric peptide hormone, was discovered in 1999 by Kojima and others while seeking the endogenous ligand for the growth hormone secretagogue receptor, GHS-R1a. The enteric expression of Ghr, produced in the stomach by X/A oxyntic mucosal cells, correlates negatively with blood glucose levels, food intake levels, and leptin levels.[4] Plasma Ghr levels rise preprandially and decline postprandially, consistent with the hypothesized role of Ghr in hunger and meal initiation.[5] Patients with Prader–Willi syndrome, a genetic form of obesity, show 4–5-fold elevations in circulating ghrelin as compared to obese controls; the elevated ghrelin levels are proposed to drive the voracious hyperphagia seen in children with this disorder.[6,7] Caloric restriction (e.g., dieting) or fasting also increases circulating ghrelin levels, a putative counter-regulatory mechanism that impedes maintenance of weight loss.[8] Thus, ghrelin and the biochemical pathways it impacts are putative therapeutic targets for the control of appetite and/or body weight.[5]

Ghrelin also influences energy homeostasis by reducing energy expenditure. For example, Yasuda and colleagues demonstrated that centrally administered ghrelin decreases sympathetic nerve activity in rat brown adipose tissue (BAT), which contributes to energy expenditure and thermogenesis via uncoupling protein 1 (UCP1).[9] Conversely, young ghrelin-null mutant mice showed increased whole-body energy expenditure.[10,11] Systemic administration of proghrelin, the ghrelin precursor, also reduced respiratory quotient in mice, indicating decreased utilization of lipid as a fuel substrate.[12]

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The octanoylation of the serine-3 residue of Ghr by ghrelin *O*-acyltransferase enzyme (GOAT) is required for its high affinity and agonist activity at GHS-R1a, a 7 transmembrane domain receptor found primarily in the pituitary, hypothalamus, and hippocampus. Octanoylated Ghr binding to GHS-R1a leads to trimerization of the G-protein receptor and downstream coupling to the phosphoinositidase C-linked $G\alpha_9$/$G\alpha_{11}$ proteins, which in turn upregulate intracellular Ca^{2+} levels.[13] GHS-R1a appears to be the primary target via which ghrelin induces growth hormone (GH) secretion and stimulates appetite,[3] leading to increased caloric intake, body weight, and adiposity.[14] Other molecular targets of Ghr have also been suggested[15] (AMPK) and may play additional roles in the peptide's regulatory effects on metabolism and energy homeostasis. Acylated Ghr comprises only about 10% of the Ghr in circulation (~117 ± 37 fmol/mL),[16] and *des*-acyl Ghr, while inactive at GHS-R1a, might be active at other molecular targets.[15]

Potent sites of central action for exogenous Ghr to induce food intake include the arcuate nucleus (ARC) of the hypothalamus[16] and the caudal hindbrain in the vicinity of the fourth ventricle.[17] However, peripheral Ghr can also signal the central nervous system (CNS) directly, via the afferent fibers of the ascending vagus nerve, the primary neuroanatomic linkage between the alimentary tract and the nucleus of the solitary tract in the hindbrain. Surgical deafferentiation of the gastric vagus blocks the ability of peripheral Ghr administration to increase food intake without attenuating the orexigenic effects of central Ghr administration.[18] Peripheral Ghr signals through the vagus nerve to the AZ noradrenergic cell groups of the nucleus tractus solitarius (NTS)[19] leading to increased expression of the anabolic molecules neuropeptide Y (NPY) and agouti-related protein (AgRP) in the ARC.[20] Thus, enteric Ghr might modulate energy homeostasis via both peripheral (vagus) and central (endocrine permeation of blood−brain barrier) sites of action. GOAT, which has been shown to be produced within the CNS in addition to the stomach,[21] also may activate Ghr to act locally in paracrine or autocrine fashion.

In light of the reviewed findings, ghrelin and its receptor have been key targets for weight modulation therapies in the past.[22,23] For example, small molecule antagonists of GHS-R1a have been shown to reduce food intake and whole-body weight in part by increasing the oxidation of body fat stores.[22] Using an approach to block endogenous ghrelin directly, Becskei and colleagues created an anti-ghrelin RNA-spiegelmer (NOX-B11-3) that blocks ghrelin induced c-Fos neuronal activation in the ARC.[24] Helmling and others previously used the same approach creating a RNA-spiegelmer that suppressed ghrelin-induced GH release in rats.[25]

Using an immunopharmacotherapy approach, we and others have found that active immunization to generate a polyclonal response against ghrelin reduced weight gain and/or adiposity in multiple species.[26−28] For example, in male Wistar rats, we reported that two haptens against acylated ghrelin, including Ghr1 (residues 1−10) and Ghr3 (full length, residues 1−28), conjugated to keyhole limpet hemocyanin (KLH), led to decreased feed efficiency, relative adiposity, and body weight gain[26] in direct relation to the affinity of the anti-ghrelin polyclonal response. Vizcarra and colleagues detailed that, in growing pigs, feed intake was decreased by more than 15% in actively immunized subjects, and that overall weight gain in treated pigs was 10% less than control animals.[27] Finally, we recently found in adult mice that active immunization against ghrelin reduced adiposity, curbed spontaneous/deprivation-induced food intake, and facilitated net weight loss after food deprivation and refeeding.[28] Our group further demonstrated that passive immunization with GHR-11E11, a catalytic antibody that can degrade the ester moiety on the serine-3 residue of octanoylated ghrelin, converting acyl ghrelin to "inactive" *des*-acyl ghrelin (k_{cat}/K_M of 9 M^{-1} s^{-1}), induced a higher metabolic rate during fasting as compared to mice immunized with a control antibody.[29] Following our research leads, Lu and colleagues developed a high-affinity *N*-terminally directed monoclonal antibody (mAb) against acyl-ghrelin. Passive administration of this single mAb only blocked exogenous ghrelin-induced food intake; however, it failed to modulate spontaneous food intake or body weight gain in obese mice over a 4-week study.[30]

The negative of findings of Lu and colleagues could potentially be interpreted in one of two broad ways. One interpretation might be that endogenous ghrelin does not play a pertinent, nonredundant role in energy homeostasis, a view based largely on observations that adult ghrelin-null mice show normal food intake, energy expenditure, and body weight when fed chow *ad libitum*.[11,31,32] An alternative interpretation is that ghrelin does play an essential role, but that the passive immunization approach used by Lu and colleagues was insufficient to abrogate the peptide's function. Given the reported inability of passive immunization with a single, high-affinity mAb to alter energy homeostasis substantively[30] and the ability of active immunization, which generates a polyclonal response to modulate aspects of energy homeostasis in multiple species,[26−28] we hypothesized that monoclonal (i.e., single epitope) targeting might be insufficient to abrogate ghrelin's action and that an oligoclonal (i.e, multiple epitope) approach might be crucial to previous findings seen with active immunization. Indeed, others have previously shown synergy between oligoclonal antibodies in neutralizing botulinum neurotoxin (BoNT), tetanus toxin, or HIV infection.[33−36] Herein, we confirm Lu et al's finding that acute administration of a high-affinity, *N*-terminally directed mAb against ghrelin per se is insufficient to alter energy expenditure or food intake in mice. We demonstrate, however, that synergistic combinations of pairs or triplets of anti-ghrelin mAbs increased energy expenditure and/or reduced refeeding in fasted mice, suggesting that oligoclonal (or polyclonal) immunosequestration of peripheral ghrelin is required to attenuate its biological action on energy homeostasis.

MATERIALS AND METHODS

Synthesis and Screening of Anti-Ghrelin Antibodies. All haptens were prepared on a 1.0 mmol scale using solid-phase peptide synthesis.[26] The first hapten was composed of the first 10 amino acids of ghrelin (*N*-terminus), with an additional cysteine residue appended at the *C*-terminus. The immunogenic carrier protein keyhole limpet hemocyanin (KLH) was conjugated to the 10-mer using a sulfosuccinimidyl-4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate (SMCC) linker at the *C*-terminal cysteine. The antibody generated from this hapten was labeled JG4 1C4. Monoclonal antibody JG3 8H11 was elicited against full-length ghrelin−KLH, but wherein the octanoylated ester was abbreviated to a butyryl ester due to insolubility issues. JG2 7B4 monoclonal antibody was elicited against ghrelin amino acids 13-28-KLH. All the haptens and substrates were prepared as *C*-terminal amides. The subsequent monoclonal antibodies

were purified from ascites using ion-exchange and protein G affinity chromatography.

Binding Analysis by BIAcore. A BIAcore 3000 instrument (BIAcore, Uppsala, Sweden) was utilized to determine directly whether the mAbs interacted with full-length acyl-ghrelin and *des*-acyl ghrelin peptides or ghrelin fragments (Ghr 1−5 and 1−10, Peptides International, Inc.). Each tested mAb was immobilized onto a CM3 or CM5 chip using the NHS/EDC coupling method, as well as an in-line reference flow cell coupled with an unrelated mAb (NIC-1 9D9). All measurements were conducted in HEPES buffer (pH 7.6) containing 0.15 M NaCl, 3 mM EDTA, and 0.005% surfactant P20 (HBS-EP) running buffer at 25 °C with a flow rate of 30 μL/min. Acyl, *des*-acyl ghrelin, or ghrelin fragments were prepared in running buffer at various concentrations ranging from 1 nM to 50 μM and then injected over the mAb-immobilized flow cells. The binding response observed 2.5 min after the end-of-injection was used to define the binding capacity for each mAb against different concentrations of ghrelin peptides. A response ten times higher than the standard deviation of buffer injections was considered "positive" binding.

Kinetic Analysis by BIAcore. To determine the kinetic constants of each defined mAb against acyl-ghrelin or ghrelin fragments, mAbs immobilized onto a CM3 chip were targeted at 3000 RU (for acyl-ghrelin), or a CM5 chip at 6,000 RU (for Ghr 1−10) and 10,000 RU (for Ghr 1−5) respectively, in order to acquire similar R_{max} values across samples. An in-line reference flow cell was immobilized with an unrelated nicotine mAb (NIC-1 9D9) at the same level as the ghrelin mAbs. Ghrelin was diluted in running buffer so as to obtain a series of concentrations ranging from 0.2 nM to 4 μM. At least 5 different concentrations of ghrelin were injected into the flow cell for repeated analysis with double references,[37] and the interaction between soluble ghrelin and mAb was recorded on a sensorgram. The kinetic data were evaluated via fitting the experimental data with BIAevaluation software (v. 4.1) using an appropriate kinetic model. Kinetic constants, including the association and dissociation equilibrium rate constants (K_a and K_d) and association and dissociation rate constants (k_{on} and k_{off}), were calculated separately for each mAb. All BIAcore kinetic analyses were double-referenced, i.e., with a reference flow cell and blank buffer run.

Epitope Mapping by BIAcore. To reveal the steric relationships between antigenic sites on acyl-ghrelin that were potentially recognized by the defined mAbs, a pairwise epitope mapping experiment was performed using surface plasmon resonance (SPR) technology. For this, each defined mAb was immobilized onto a CM5 chip via NHS/EDC amine-coupling, followed by the injection of a saturating concentration (10 μM) of acyl-ghrelin. Once the surface was saturated, a secondary mAb was injected, and binding activity was observed in real-time via the sensorgram. This procedure was repeated for all mAbs with all possible binary combinations undertaken, and the binding signal for each pair of mAbs was recorded in a reactivity pattern matrix for further analysis.[38] All BIAcore epitope mapping analyses were double-referenced to a reference flow cell (with the unrelated antinicotine antibody) and blank cell.

Animal Protocols and Antibody Administration. All mouse studies adhered to the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute. Twelve adult male C57BL/6J mice (25.0−28.3 g) were purchased from Charles River Laboratories (Frederick, MD) for each of 4 replicate experiments (n = 48 total). Mice were housed in single cages in a temperature-controlled vivarium (60% humidity, 22 °C) with a 12 h light/12 h dark cycle (6:30 am to 6:30 pm) with *ad libitum* access to water and standard pelleted chow diet (LM-485 Diet 7012; Harlan Teklad, Madison, WI) for 2−3 weeks prior to antibody injection. Antibodies were prepared in phosphate buffered saline (pH 7.4) and injected subcutaneously in 130 μL volumes to obtain final total administered antibody doses of 15 mg/kg (5 mg/kg per mAb for triplet injection, 7.5 mg/kg each for doublets). mAbs were injected five days prior to calorimetric experiments to allow adequate distribution *in vivo*.[39]

Metabolic and Food Intake. Subjects were singly housed and acclimated (>72 h) to indirect calorimetry chambers where O_2 consumption, CO_2 emission, and heat dissipation were measured using a computer-controlled, open-circuit system (Oxymax System) that was part of an integrated Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH). The Plexiglas test chambers (20 × 10 × 12.5 cm) had a stainless steel elevated wire floor and were equipped with a sipper tube delivering water and a food tray connected to a balance. Room air was passed through chambers at a flow rate of 0.5 L/min. Exhaust air from each chamber was sampled at 20 min intervals for 30 s. Sample air was sequentially passed through O_2 and CO_2 sensors (Columbus Instruments) for determination of O_2 and CO_2 content, from which measures of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were estimated. Outdoor air reference values were sampled after every four measurements. Gas sensors were regularly calibrated with primary gas standards containing known concentrations of O_2, CO_2, and N_2 (Airgas Puritan Medical, Ontario, CA). Respiratory exchange ratio (RER) was calculated as the ratio of carbon dioxide production (VCO_2) to oxygen consumption (VO_2). Energy expenditure measures (VO_2, VCO_2, and heat formation $[(3.815 + 1.232 \times \text{RER}) \times VO_2$ (in liters)$]$ were corrected for estimated effective metabolic mass per Kleiber's power function. Data were recorded under ambient room temperature (24−26 °C). During acclimation, powdered chow and water were available *ad libitum*. Subjects were then subjected to a 24 h fast beginning from the onset of the light cycle, during which water remained available. At the onset of the next light cycle, mice were allowed to refeed for 6 h, during which food intake was automatically measured by a computer-monitored scale bearing the food tray (0.1 g precision). Data were graphed using Prism software (GraphPad Software Inc., San Diego, CA). All values were plotted as the mean ± standard error of the mean (SEM).

Statistical Analysis. Data were analyzed by two-way analysis of covariance (ANOVA), with hapten group as a between-subject factor (e.g., vehicle vs doublet AB vs doublet AC vs doublet BC), time as a repeated measure (where applicable), and cohort as a covariate. To ensure reproducibility of findings, subjects were run in 4 independent cohorts per experiment (2×), balanced for treatment group. Significant omnibus tests ($p < 0.05$) were further interpreted by simple effects analysis and by ANCOVAS comparing individual hapten groups to the vehicle condition. Results are expressed as mean ± SEM. The statistical package used was Systat 12.0 (SPSS, Chicago, IL).

RESULTS

Monoclonal Antibodies Bind with High Specificity to Acyl-ghrelin. Monoclonal antibody affinity to full-length acyl-ghrelin was initially tested by ELISA.[26] JG4 1C4 had the highest affinity to the peptide with an estimated K_d of 6.2 nM. JG3

Table 1. Kinetic Analysis of mAbs against Acyl-ghrelin and Truncated Ghrelin Peptides by BIAcore[a]

			full length acyl-Ghr			Ghr 1−5			Ghr 1−10		
mAb	hybridoma	isotype	k_{on}	k_{off}	K_d	k_{on}	k_{off}	K_d	k_{on}	k_{off}	K_d
JG2	7B4	κγ3	9.78E^{5}	1.20E^{-2}	1.23E^{-8}	nd[b]	nd	>5.00E^{-5}	nd	nd	>5.00E^{-5}
JG3	8H11	κγ2α	9.92E^{3}	2.67E^{-4}	2.69E^{-8}	nd	nd	>5.00E^{-5}	nd	nd	>5.00E^{-5}
JG4	1C4	κγ2α	1.02E^{7}	7.92E^{-4}	7.76E^{-11}	5.43E^{6}	5.46E^{-3}	1.01E^{-9}	4.90E^{6}	6.67E^{-4}	1.36E^{-10}

[a] k_{on} rates represented as (1/M-s). k_{off} rates represented as (1/s). K_d values represented in M. [b] nd: Values not determined due to affinities greater than 50 μM.

8H11 and JG2 7B4 mAbs had relative affinities estimated by ELISA of 50 nM and 800 nM respectively. Noteworthy was that mAb JG4 failed to bind *des*-acyl-ghrelin up to 3 μM, whereas the other two mAbs demonstrated binding as potent as 3 nM for *des*-acyl Ghr (BIAcore end point analysis, data not shown). A more accurate determination of antibody affinity/specificity was undertaken by BIAcore due to the instrument's ability to monitor real time kinetics as well as its sensitivity to low vs high affinity antibodies.[40−42] JG4, JG2, and JG3 had relative K_d's of 77.6 pM, 12.3 nM, and 26.9 nM for acyl-ghrelin, confirming mAb JG4 to have the highest affinity to the peptide. Kinetic analysis through BIAcore provided kinetic association and dissociation constants (Table 1). JG4 and JG2 bind full-length acyl-ghrelin fitting a 1:1 binding with mass transfer model and a 1:1 Langmuir binding model. JG3 however, has relatively slow k_{on}/k_{off} rates and as such fits a two state reaction model in which a conformational change of the peptide is hypothesized to occur during the association phase.

To examine epitope fidelity, the truncated peptides Ghr 1−5 and Ghr 1−10 were tested. As anticipated, only mAb JG4 presented binding for these *N*-terminal fragments with relative affinities of 1.01 nM and 136.0 pM respectively. These BIAcore analyses further validate JG4's recognition of the octanoylated serine-3 residue with high specificity to the *N*-terminus of acyl-ghrelin.

Through simple epitope mapping it was determined that JG4 and JG2 recognize distinct binding epitopes on ghrelin, whereas JG3 must recognize a "conformational epitope" that may overlap with the recognition sequence for JG2 and JG4. Specifically, initial binding of JG3 to ghrelin did not exclude the subsequent binding of JG2 or JG4 mAbs to the ghrelin/JG3 complex. However, initial binding of either JG2 or JG4 to ghrelin excluded the subsequent binding of JG3 to the JG2− or JG4−ghrelin complex. These data suggest that, upon JG3 binding to ghrelin, a conformational change occurs, perhaps in which the naturally extended secondary structure of ghrelin folds into a more stable loop/supercoil.[43] Conversely, the initial binding of JG2 or JG4 may prevent the linear ghrelin from folding, which is hypothesized to be crucial for forming a stable complex with JG3 (see Figure 1).

JG3:JG4 Doublet Uniquely Alters Whole-Body Metabolism in Fasted and Refeeding Mice. Due to the high binding affinity of JG4 1C4 to acyl-ghrelin, we began calorimetry experiments by injecting mice subcutaneously with only mAb JG4 at a dose of 15 mg/kg. Initial calorimetric and food intake studies demonstrated no significant differences between monoclonal antibody JG4 1C4 and the nicotine control antibody NIC-1 9D9 in male C57BL/6J mice for any of the whole-body metabolism measures (heat, VO_2, VCO_2, and respiratory exchange ratio [RER]) at any time point of the fast or refeeding stages (data not shown). JG4 1C4 also did not alter cumulative food intake after the 24 h fast at any time point through 6 h (data not shown). Based on our positive active immunization findings, we were led to ask whether an oligoclonal antibody response might better enable acyl-ghrelin

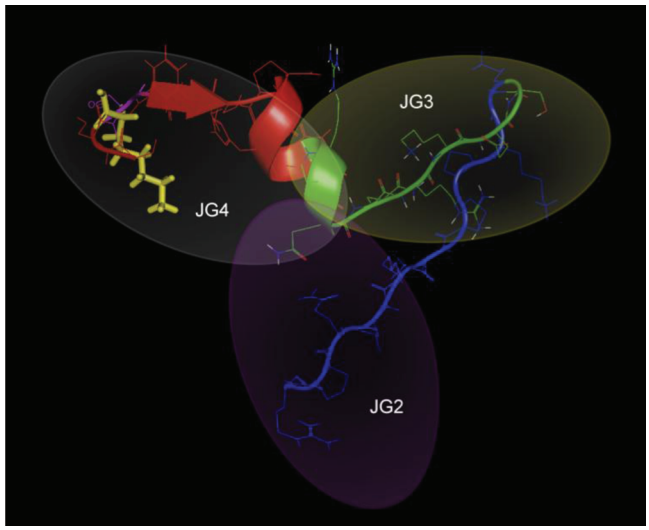

Figure 1. Shown is the secondary structure of full-length acyl-ghrelin with potential binding sites of mABs JG2, JG3, and JG4, inferred from BIAcore analysis, highlighted as oval regions. mAB JG4 recognizes the *N*-terminus of ghrelin, specifically the acylated side chain found on serine-3. mAB JG3 recognizes an internal binding site on ghrelin, possibly at the loop structure found from residues serine-18−lysine-20, and mAB JG2 recognizes the *C*-terminus of ghrelin. Structure shown modified from PDB 1P7X.

sequestration, as has been observed for other systems.[26] Thus, monoclonal antibodies were further examined as doublets JG2:JG4, JG3:JG4, and JG2:JG3 at 7.5 mg/kg per antibody.

Main effects of doublet indicated that the ghrelin doublet cocktails elicited effects on energy expenditure (heat; $F(3,30) = 3.570$, $p < 0.025$; VCO_2; $F(3, 30) = 4.003$, $p < 0.016$; VO_2; $F(3, 30) = 2.431$, $p < 0.085$); and respiratory exchange ratio (RER; $F(3, 30) = 3.642$, $p < 0.024$). Pairwise comparisons showed that subjects injected with doublet JG3:JG4 demonstrated significantly higher heat dissipation (kcal/h·kg$^{0.75}$) (heat; $F(1,14) = 8.565$, $p < 0.011$), with increased CO_2 emission (VCO_2; $F(1,14) = 6.898$, $p < 0.020$) and O_2 consumption (VO_2; $F(1,14) = 4.511$, $p < 0.052$), as well as greater respiratory exchange ratio (RER; $F(1,14) = 7.511$, $p < 0.016$) during the first 14 h of the fast as compared to nicotine control mAb subjects (Figure 2). In contrast, mice treated with JG2:JG4 showed a main effect only on heat dissipation (heat; $F(1,14) = 6.756$, $p < 0.021$), however, pairwise analysis of individual time bins showed no significance. The JG2:JG3 doublet did not differ from control mAb-treated mice within any time-bin of the fast on the metabolic measures.

During the refeeding stage, mice injected with doublet JG3:JG4 demonstrated significant increase in heat dissipation during hour 2 as well as increased CO_2 emission during hours 2 and 6 (Figure 3, heat; $F(1, 14) = 8.869$, $p < 0.010$; VCO_2; $F(1, 14) = 6.601$, $p < 0.022$), suggesting the continuation of relative increases in energy expenditure even during energy

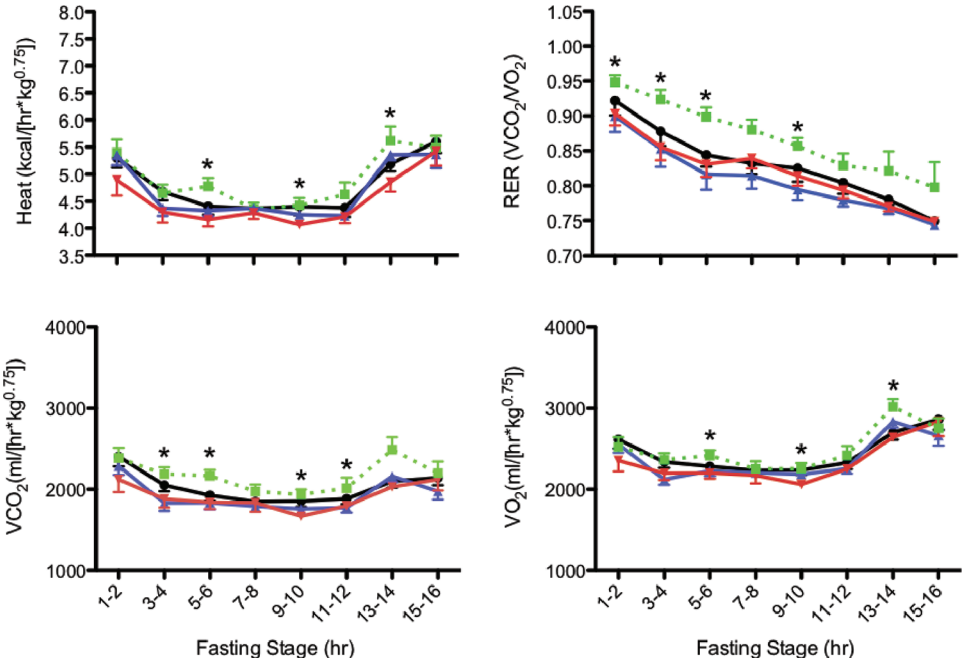

Figure 2. Shown are the rate of energy expenditure (heat, top left), respiratory exchange ratio (RER, top right), the rates of carbon dioxide production (VCO_2, bottom left) and oxygen consumption (VO_2, bottom right) in food deprived, antibody-treated, adult male C57BL/6J mice as recorded in open-circuit indirect calorimetry chambers. Data are expressed in 2 h bins as $M \pm$ SEM during the first 16 h of fasting beginning at light onset. Mice received subcutaneous administration (15 mg/kg total dose) of ghrelin mAbs in doublets (black ●) JG2:JG4, (green ■) JG3:JG4, and (blue ▲) JG2:JG3 (n = 6) or (red ▼) a nicotine control Ab (n = 6, NIC-1 9D9) 4 days prior to data collection; *, $p < 0.05$ vs control Ab-treated mice. Dark onset begins at hours 13−14 of the fasting stage.

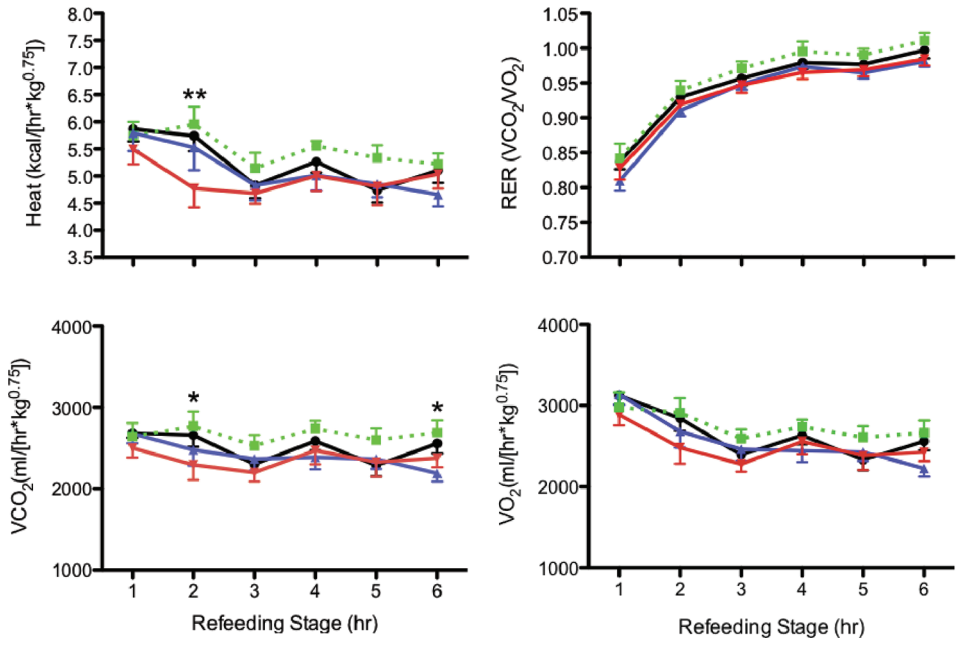

Figure 3. Shown are the rate of energy expenditure (heat, top left), respiratory exchange ratio (RER, top right), the rates of carbon dioxide production (VCO_2, bottom left) and oxygen consumption (VO_2, bottom right) in antibody-treated, adult male C57BL/6J mice refeeding after a 24 h fast as recorded in open-circuit indirect calorimetry chambers. Data are expressed in 1 h bins as $M \pm$ SEM during the first 6 h of refeeding beginning at light onset. Mice received subcutaneous administration (15 mg/kg total dose) of ghrelin mAbs in doublets (black ●) JG2:JG4, (green ■) JG3:JG4, and (blue ▲) JG2:JG3 (n = 6) or (red ▼) a nicotine control Ab (n = 6, NIC-1 9D9) 5 days prior to data collection; *, $p < 0.05$, **, $p < 0.01$ vs control Ab-treated mice.

repletion. During refeeding, a trend was observed in VO_2 consumption across groups (VO_2; $F(3, 30) = 2.498$, $p < 0.079$), whereas, respiratory exchange ratio no longer differed reliably between groups (RER; $F(3, 30) = 1.830$, $p < 0.163$). Caloric intake across the 6 h period was not significantly different between any of the doublet groups vs the nicotine control group (Figure 4; intake; $F(3, 29) = 0.387$, $p < 0.763$).

mAb Triplet Cocktail Alters Whole-Body Metabolism and Reduces Caloric Intake in Refeeding Fasted Mice. When mAbs were administered as a "cocktail" combination of

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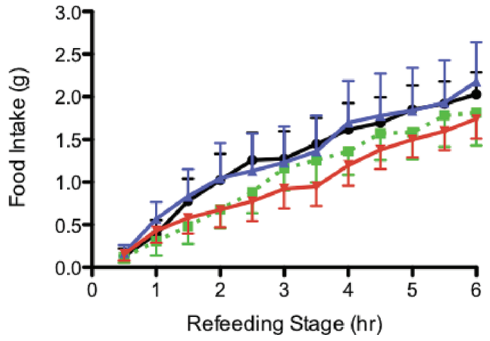

Figure 4. Food intake in 24 h food-deprived, antibody-treated adult male C57BL/6J mice as recorded in open-circuit indirect calorimetry chambers. Data are expressed in 30 min bins as $M \pm$ SEM during the first 6 h of refeeding beginning at light onset. Mice received subcutaneous administration (15 mg/kg total dose) of ghrelin mAbs in doublets (black ●) JG2:JG4, (green ■) JG3:JG4, and (blue ▲) JG2:JG3 ($n = 6$) or (red ▼) a nicotine control Ab ($n = 6$, NIC-1 9D9) 5 days prior to data collection.

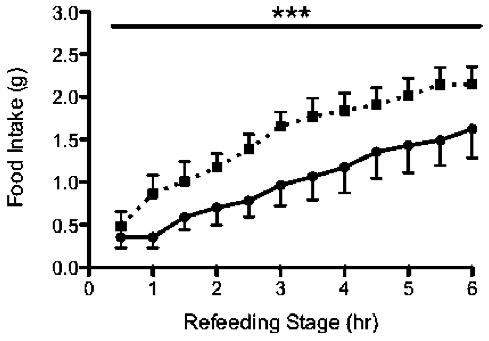

Figure 6. Food intake in 24 h food-deprived, antibody-treated adult male C57BL/6J mice as recorded in open-circuit indirect calorimetry chambers. Data are expressed in 30 min bins as $M \pm$ SEM during the first 6 h of refeeding beginning at light onset. Mice received subcutaneous administration (15 mg/kg total dose) of ghrelin mAbs in triplet (●) Ghr mAbs JG2, JG3, and JG4 ($n = 6$) or (■) a nicotine control Ab ($n = 6$, NIC-1 9D9) 5 days prior to data collection; ***, $p < 0.0001$ vs control Ab-treated mice.

all three antibodies (but still at a total dose of 15 mg/kg), significant increases in heat dissipation and O_2 consumption were noted during hours one and two of the refeeding stage (Figure 5; heat hour 1; $F(1,8) = 7.523$, $p < 0.025$; heat hour 2; $F(1, 8) = 6.319$, $p < 0.036$; VO_2 hour 1; $F(1, 8) = 8.189$, $p < 0.021$; VO_2 hour 2; $F(1,8) = 8.136$, $p < 0.021$). The mice administered the cocktail also demonstrated significantly less cumulative caloric intake during the first 6 h of the refeeding stage as compared to the nicotine control mice (Figure 6; intake; $F(1, 15) = 1.751$, $p < 0.0001$). Significant reductions in intake were evident as early as 30 min following the return of food to the chambers.

■ DISCUSSION

With health complications attributable to obesity rising at an alarming rate,[44] ghrelin systems have been targeted as a potential means to facilitate weight loss. Ghrelin, the only peripheral octanoylated, orexigenic peptide known to date, has been hypothesized to promote meal initiation[5] and reduce energy expenditure,[9−11] especially as a feedback response to negative energy balance.[45] As such, pharmacologic agents that target ghrelin, its endogenous receptor, or its post-translational octanoylation may be useful not only for understanding the biology of ghrelin but also for facilitating weight loss by blunting deprivation-induced food intake or by disinhibiting energy expenditure from the "thriftiness" response to undernutrition.[46]

Previously, GHS-R1a-mediated antagonists such as isoxazole carboxamides developed by Abbott have demonstrated modestly potent inhibition of the receptor with an IC_{50} of 130 nM; however, the compounds suffered from poor pharmacokinetic profiles due to less than 5% oral bioavalibility

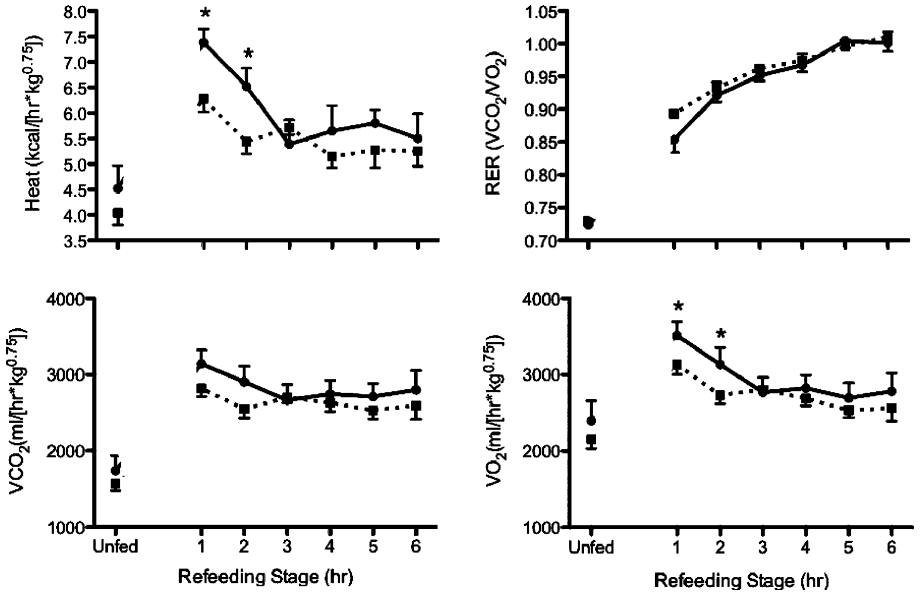

Figure 5. Shown are the rate of energy expenditure (heat, top left), respiratory exchange ratio (RER, top right), the rates of carbon dioxide production (VCO_2, bottom left) and oxygen consumption (VO_2, bottom right) in antibody-treated, adult male C57BL/6J mice as recorded in open-circuit indirect calorimetry chambers. Data are expressed in 1 h bins as $M \pm$ SEM during the last hour of the fasting stage ("Unfed") and the first 6 h of refeeding beginning at light onset. Mice received subcutaneous administration (15 mg/kg total dose) of ghrelin mAbs in triplet (●) Ghr mAbs JG2, JG3, and JG4 ($n = 6$) or (■) a nicotine control Ab ($n = 6$, NIC-1 9D9) 5 days prior to data collection; *, $p < 0.05$ vs control Ab-treated mice.

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in rats.[23] Ghrelin *O*-acyltransferase inhibitors,[47] such as GO-CoA-Tat developed by Barnett and colleagues, prevented significant weight gain in mice fed a MCT-rich, high fat diet. However, GOAT modulation is a relatively new approach, and only two peptide-based inhibitors have been established to date with minimal *in vivo* modeling.[48,49]

Here, rather than using a small molecule or GOAT enzyme inhibitor pharmacological approach, we used immunopharmacotherapy, a strategy we have applied successfully in other fields such as drug abuse and dependence.[50−52] In the present study, we targeted acyl-ghrelin in mice by generating and administering multiple monoclonal antibodies created against three distinct haptens. All mAbs bound with high specificity to the active octanoylated form of ghrelin *in vitro* with mAb JG4 1C4 having the highest affinity to the peptide (77.6 pM). JG4 was procured against a hapten containing only the first ten residues of acyl-ghrelin along with a *C*-terminal cysteine used for conjugation to the carrier protein KLH. Correspondingly, JG4 binds with high affinity to the *N*-terminus of full-length acyl-ghrelin as well as acyl-ghrelin fragments 1−5 and 1−10. In contrast, JG2 mAb was prepared against a hapten containing the *C*-terminal ghrelin residues 13−28 and binds both acyl and *des*-acyl forms of ghrelin *in vitro*, presumably at their *C*-terminus. mAb JG3 was created against the full-length peptide and, like JG2, complexes with both *des*-acyl-ghrelin and acyl-ghrelin. Unlike JG2, however, JG3 appears to recognize internal peptide binding sites, as indicated by kinetic and epitope mapping BIAcore analysis.

Previously, we and others had demonstrated that active immunization targeting the *N*-terminal portion of ghrelin could reduce weight gain, increase energy expenditure and/or reduce food intake in multiple species.[26−28] In contrast, a previous study using passive immunization with a monoclonal antibody against ghrelin showed inefficacies in reducing food intake or body weight in mice over a four-week chronic study.[30] To reconcile these disparate findings, we hypothesized that a passive oligoclonal "cocktail" of the obtained mAbs might serve as a "minimal" polyclonal response as elicited by active immunization. That is, we considered that a combination of monoclonals might be effective in blunting fasting-induced reductions in energy expenditure and increases in food intake, wherein a singular monoclonal antibody had been ineffective. Indeed, consistent with the findings of Lu and colleagues (2009)[30], we found that passive immunization with a single specific, high-affinity (<100 pM) monoclonal antibody that targeted the ghrelin *N*-terminus (JG4) was ineffective in altering energy expenditure, fuel substrate utilization, or food intake in fasted mice. In contrast, a "doublet" treatment that combined JG4 with a particular *C*-terminally directed anti-ghrelin antibody (JG3) maintained greater energy expenditure and utilization of carbohydrate as a fuel substrate in fasted mice. Moreover, a "triplet" mAb cocktail combination that included the additional *C*-terminally directed antibody (JG2) that had been comparably ineffective in conjunction with JG3 or JG4 alone not only promoted increased energy expenditure but also reduced deprivation-induced food intake. The JG3:JG4 complex may have altered secondary structure of the ghrelin peptide, which could significantly reduce positive interaction at the GHSR1a,[53,54] due to JG3's ability to bind ghrelin only in a conformational manner as demonstrated by BIAcore analysis.

Growth hormone secretagogue receptors are expressed not only in the brain but also along the vagal afferent fibers.[18,55] As such, effective anti-ghrelin immunoneutralization may require that antibodies prevent not only BBB passage but also ligand−receptor interaction at the vagus nerve. If mAbs targeted not only the post-translationally modified serine residue that is key to GHSR1a signaling but also other potential recognition sites along the peptide that are critical for initially addressing the receptor, then a fully sequestered acyl-ghrelin might be prevented from forming the secondary structures necessary for lipid membrane interaction and subsequent GHSR1a activation.[56,57] Indeed, as is known for NPY and many other peptide-GPCR systems, amphiphilic stretches along the ligand hormone serve to first anchor the peptide onto the lipid bilayer and subsequently permit activation of the membrane bound receptor.[58,59] Thus, perhaps the present oligoclonal approach more effectively prevented ultimate signaling at the GPCR, by also interfering with appropriate peptide "recognition", rather than by relying only on immunoneutralization of "signaling" epitopes.

The mAb doublet JG3:JG4 bound ghrelin in a manner that increased relative energy expenditure and the relative utilization of carbohydrate as a fuel source in fasted C57BL/6J mice. The mechanism underlying the greater efficacy of the triplet vs doublet combination to modulate deprivation-induced food intake is uncertain, but perhaps relates to a greater ability of the triplet combination to interfere with ghrelin-signaling at the vagus. That is, perhaps the doublet combination is sufficient to disrupt BBB penetration by ghrelin and consequent hypothalamic GHSR1a stimulation, whereas only the triplet combination was sufficient to abrogate peripheral ghrelin signaling, which is known to contribute to ghrelin induced feeding.[18] Another possible explanation is that perhaps a lower threshold of cumulative GHS-R1a receptor stimulation is required to promote feeding responses as opposed to inhibiting energy expenditure. In this view, only the triplet cocktail combination was sufficient to reduce total ghrelin signaling below both thresholds of receptor stimulation.

Several limitations of the present immunoneutralization approach require mentioning. While monoclonal antibodies may be able to sequester acyl-ghrelin in the periphery, binding to CNS derived ghrelin would not be feasible, thus allowing for the evolution of a compensatory mechanism in which central GHS-R1a activation may depend predominantly on ghrelin produced and/or modified in the CNS. A peripheral compensation could also evolve in response to decreasing bioavailable levels of circulating acyl-ghrelin, leading to greater enteric ghrelin synthesis and secretion that potentially could surmount passive immunization or cause unanticipated, adverse local effects. Finally, any potential therapies involving mAbs would require multiple injections over the sustained period of treatment due to estimated half-lives of 6 to 8 days for IgG monoclonal antibodies in mice and 22 to 23 days in humans.[60]

In summary, we have created three monoclonal antibodies against serine-3 octanoylated ghrelin toward understanding the requirements for effective anti-ghrelin immunoneutralization and the effects of ghrelin immunopharmacotherapy on whole body metabolism and food intake in mice *in vivo*. We have concluded that an oligoclonal response is necessary to maintain greater whole body energy expenditure during fasting and subsequent refeeding over a 24 h period, as well as to reduce overall food intake upon refeeding. These results coincide with data from ourselves and others, whereby active vaccination against acyl-ghrelin decreased feed efficiency, relative adiposity, and body weight gain in three different species. Additional studies will further elucidate the role of immunopharmacotherapy against ghrelin and other metabolic targets in combating obesity through energy homeostasis pathways.

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