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Insulin sensitivity : modulation by the gut-brain axis

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Chapter 7

Ghrelin differentially affects hepatic and peripheral insulin sensitivity in mice

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ABSTRACT

Aims/hypothesis. The present study was conducted to evaluate the effects of ghrelin on insulin's capacity to suppress endogenous glucose production (EGP) and promote glucose disposal (GD) in mice. To establish if the GHS-receptor can mediate the putative impact of ghrelin on insulin action, we also determined the metabolic effects of GHRP-6, a specific GHS-R agonist. In addition, we explored the biological significance of des-ghrelin in this experimental context.

Methods. Vehicle, ghrelin, GHRP-6, des-ghrelin or the combination of des-ghrelin and ghrelin were infused i.v. for 3 hours. Simultaneously, EGP and GD were measured by ^{14}C -glucose dilution during a hyperinsulinaemic euglycaemic clamp. Tissue specific glucose uptake in muscle and adipose tissue were measured using ^3H -2-deoxy-glucose.

Results. During hyperinsulinaemia, GD was 31% higher in ghrelin-treated mice compared with vehicle (77 ± 16 vs 59 ± 8 $\mu\text{mol/kg/hour}$ respectively, $P<0.05$), which was in accordance with enhanced 2-DG uptake in muscle in ghrelin-treated animals. In contrast, EGP was less effectively suppressed by insulin during ghrelin infusion (by 46 ± 22 vs 71 ± 11 % in controls, $P<0.05$). GHRP-6 did not affect insulin action. Des-ghrelin hampered insulin's capacity to inhibit EGP, whereas it did not affect GD. The restraining effects of des-ghrelin and ghrelin on hepatic insulin action were abolished by simultaneous administration of both peptides.

Conclusions/interpretation. Ghrelin hampers insulin's capacity to suppress EGP, whereas it reinforces insulin action on GD, independently of food intake and body weight. These metabolic effects are unlikely mediated by the GHS-receptor. Furthermore, simultaneous administration of des-ghrelin abolishes the inhibitory effect of ghrelin on hepatic insulin action.

INTRODUCTION

Ghrelin is a small 28 amino acid peptide, that is primarily produced by the stomach and binds to the growth hormone secretagogue (GHS) receptor (GHS-R) [1;2]. It circulates in two major forms: n-octanoyl-ghrelin (ghrelin), which contains an n-octanoyl modification at serine-3, and des-octanoyl or unacylated ghrelin (des-ghrelin) [3]. Although des-ghrelin does not bind to the GHS-R [1;4], it may be biologically active [5].

Ghrelin is a component of the gut-brain-axis involved in the control of energy balance. It stimulates food intake in rodents and man [5-8], where peak levels in plasma occur just before each meal and fall rapidly after re-feeding, suggesting that it serves to initiate food consumption [9]. To enhance appetite, ghrelin acts in the hypothalamus where it promotes neuropeptide Y (NPY) and orexin gene expression and inhibits pro-opiomelanocortin (POMC)/ α -melanocyte stimulating hormone (α MSH) expression via activation of the GHS-R [10-16]. We recently showed that intracerebroventricular infusion of NPY hampers insulin's capacity to suppress endogenous glucose production (EGP) [17], whereas central injections of melanotan II, a synthetic analog of α MSH, reinforces insulin action on glucose disposal (GD) [18]. In concert, these findings led us to hypothesize that ghrelin might adversely affect insulin sensitivity through activation of the GHS-R. To address this hypothesis, we intravenously administered vehicle, ghrelin, or growth hormone releasing peptide 6 (GHRP-6, a specific agonist of the GHS-R), and measured EGP and GD by 14 C-glucose dilution during a hyperinsulinemic clamp in mice.

Until recently, acylation of the serine-3 residue of the ghrelin molecule was believed to be required for bioactivity. However, recent data suggest that des-ghrelin may counteract ghrelin in the control of energy balance [19], and in vitro experiments revealed opposing effects of ghrelin and des-ghrelin on glucose production by primary hepatocytes [20]. To further explore the role of des-ghrelin in the regulation of fuel flux, we studied the effects of this peptide in the same experimental context.

MATERIAL AND METHODS

Animals

Male C57BL/6J mice were housed in a temperature-controlled room on a 12-hour light-dark cycle, with free access to water and chow diet. All animal experiments were performed in accordance with the regulations of Dutch law on animal welfare and the institutional ethics committee for animal procedures approved the protocol.

Hyperinsulinaemic euglycaemic clamp study

Mice were fasted for 4 hours with food withdrawn at 05.00 a.m. Hyperinsulinaemic euglycaemic clamp studies were started at 9.00 a.m. as described earlier [21;22]. During the experiments, mice were sedated with 6.25 mg/kg Acepromazine (Sanofi sante animale, Libourne Cedex, France) 6.25 mg/kg Midazolam (Roche, Mijdrecht, the Netherlands), and 0.3125 mg/kg Fentanyl (Janssen-Cilag, Tilburg, the Netherlands).

Vehicle, ghrelin, or GHRP-6 (peptides from PhoenixEurope GmbH, Karlsruhe, Germany) were administered in a primed (0 µg, 0.6 µg, 0.26 µg) continuous (0 µg/h, 1 µg/h, 0.43 µg/h) i.v. infusion during the whole experiment (basal and hyperinsulinemic period). The doses of ghrelin, and GHRP-6 were based on previous reports [6;7;23]. First, basal rates of glucose production/uptake were measured by giving a primed (p) continuous (c) infusion of ¹⁴C-glucose (p: 0.7 µCi, c: 1.2 µCi/h) (Amersham, Little Chalfont, U.K.) for 80 min. Subsequently, insulin was administered in a primed (4.1 mU), continuous (6.8 mU/h) i.v. infusion for 2 to 3 hours aimed at steady state circulating insulin levels of approximately 6 ng/ml. A variable infusion of 12.5% D-glucose was used to maintain euglycaemia (measured via tail bleeding, Freestyle, TheraSense, Disetronic Medical Systems BV, Vianen, The Netherlands). Blood samples (75 µl) were taken during the basal period (after 60 and 80 minutes) and during the clamp period (when glucose levels were stable and 20 and 40 minutes later) for determination of plasma glucose, NEFA, insulin and ¹⁴C-glucose specific activities.

To assess tissue-specific insulin-mediated glucose uptake, 2-deoxy-D-[³H]glucose (2-DG; Amersham Little Chalfont, U.K.) was administered as a bolus (1 µCi) 40 minutes before the end of the clamp experiment. At the end of the clamp, mice were killed, and skeletal muscle (hindlimb) and adipose tissue were isolated and frozen in liquid nitrogen for subsequent analysis.

Additional studies with des-ghrelin

In addition, a hyperinsulinaemic euglycaemic clamp study was performed while des-ghrelin or a combination of ghrelin en des-ghrelin were administered. Des-ghrelin, or the combination of ghrelin and des-ghrelin were administered in a primed (0.6 µg or 0.6 and 0.6 µg) continuous (1 µg/h or 1 and 1 µg/h) i.v. infusion during the whole experiment (basal and hyperinsulinemic). The doses of des-ghrelin and the combination of ghrelin and des-ghrelin were based on a previous report [23]. Hyperinsulinaemic euglycaemic clamps were performed as stated above and randomised with these other groups.

Analytical procedures

Plasma levels of glucose and NEFA were determined using commercially available kits (Instruchemie, Delfzijl, The Netherlands and Wako, Neuss, Germany). Plasma insulin concentration was measured by a mouse insulin ELISA (Mercodia, Uppsala, Sweden). Total

plasma ^{14}C -glucose was determined in 7.5 μl plasma and in supernatants after trichloroacetic acid (20%) precipitation and water evaporation to eliminate tritiated water.

Tissue analysis

For determination of tissue 2-DG uptake, the homogenate of muscle and adipose tissue was boiled, and the supernatant was subjected to an ion-change column to separate 2-DG-6-phosphate from 2-DG as described previously [21;24].

Calculations

Turnover rate of glucose ($\mu\text{mol}/\text{min}/\text{kg}$) was calculated during the basal period and in steady-state clamp conditions as the rate of tracer infusion (dpm/min) divided by the plasma specific activity of ^{14}C -glucose (dpm/ μmol). The ratio was corrected for body weight. During the hyperinsulinemic clamp EGP was calculated as the difference between the tracer-derived rate of glucose appearance and the glucose infusion rate.

Tissue-specific glucose uptake in muscle and adipose tissue was calculated from tissue 2-DG content, corrected for plasma specific activity and expressed as $\mu\text{mol}/\text{g}$ tissue.

Statistical analysis

Differences between groups were determined by Kruskal-Wallis non-parametric test for k independent samples. When significant differences were found, Mann-Whitney non parametric test was used as follow-up test to determine differences between experimental groups and the control group. A p -value < 0.05 was considered statistically significant. All values shown represent mean $\pm$ SD.

RESULTS

Plasma parameters

Body weight, plasma glucose, NEFA and insulin concentrations in basal and hyperinsulinemic conditions are shown in Table 1. Body weight did not differ between the groups of mice. Basal glucose levels were significantly lower in the group that received GHRP-6 compared to the control group. Basal insulin and NEFA levels were not different between groups. Moreover, in steady state hyperinsulinemic conditions, plasma glucose, NEFA and insulin concentrations were not different between groups.

Glucose turnover

In basal conditions, EGP (and thereby whole body GD) was significantly lower in animals that received GHRP-6 compared to control animals (control: 44 ± 9 $\mu\text{mol}/\text{kg}/\text{hour}$, ghrelin: 40 ± 7

Table 1. Body weight and plasma parameters in mice that received ghrelin (n=9), des-ghrelin (n=8), ghrelin and des-ghrelin together (n=7), GHRP-6 (n=9) or vehicle (n=8).

	<i>Body weight (g)</i>	<i>Glucose (mmol/l)</i>		<i>Insulin (ng/ml)</i>		<i>NEFA (mmol/l)</i>	
		Basal	Hyperinsulinemic	Basal	Hyperinsulinemic	Basal	Hyperinsulinemic
Control	27.1 ± 1.8	5.8 ± 0.73	8.8 ± 1.2	0.93 ± 0.63	6.2 ± 1.5	1.0 ± 0.2	0.5 ± 0.2
Ghrelin	26.6 ± 2.2	5.9 ± 0.84	8.8 ± 0.74	0.91 ± 0.73	5.8 ± 2.1	1.0 ± 0.3	0.4 ± 0.3
GHRP-6	26.3 ± 1.5	4.8 ± 0.48**	8.5 ± 1.8	1.0 ± 0.50	6.8 ± 2.6	1.0 ± 0.2	0.3 ± 0.1
Des-ghrelin	26.9 ± 2.2	6.3 ± 1.0	7.8 ± 0.83	1.1 ± 0.38	5.7 ± 1.9	1.0 ± 0.3	0.4 ± 0.1
Des-ghrelin and ghrelin combined	27.6 ± 2.4	4.9 ± 0.90*	7.6 ± 1.1	0.87 ± 0.58	6.6 ± 1.8	1.0 ± 0.2	0.4 ± 0.1

Values are expressed as means ± sd, * $p < 0.05$ compared to control, ** $p < 0.01$ compared to control

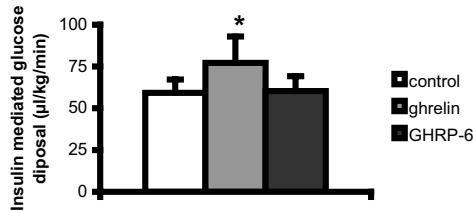


Fig 1. Insulin mediated glucose disposal (μmol/kg/min) in mice that received ghrelin (n=9), GHRP-6 (n=9) or vehicle (n=8). Data are means ± SD. *p<0.05 compared to control mice.

GD rate was significantly higher in animals that received ghrelin compared to the control group (control: 59±8 μmol/kg/hour, ghrelin: 77±16 μmol/kg/hour, GHRP-6: 60±9

μmol/kg/hour, GHRP-6: 33±4 μmol/kg/hour; p<0.01). During the hyperinsulinemic period, the rate of glucose infusion necessary to maintain euglycemia was significantly higher in animals that received ghrelin than in control mice (control: 91±18 μl/hour, ghrelin: 136±27 μl/hour, GHRP-6: 117±31 μl/hour, p<0.01). Accordingly,

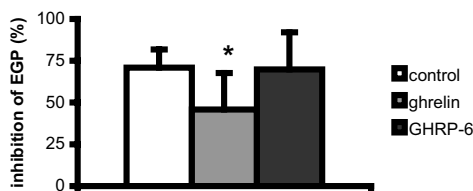


Fig 2. Inhibition of EGP (%) by insulin in mice that received ghrelin (n=9), GHRP-6 (n=9) or vehicle (n=8). Data are means ± SD. *p<0.05 compared to control mice.

In contrast, hyperinsulinaemia suppressed EGP significantly less in animals that received ghrelin compared to controls (control: 71±11 %, ghrelin: 46±22 %, GHRP-6: 70±22 %; p<0.05, see figure 2).

Tissue-specific glucose uptake

Insulin-mediated 2-DG uptake in muscle tissue was higher in ghrelin-treated animals, compared to the control group (muscle: control: 8.6 ± 4.4; ghrelin: 26 ± 21; GHRP-6: 7.1 ± 3.4 μmol/g tissue, p<0.05). Insulin-mediated 2-DG uptake in adipose tissue tended to be higher in ghrelin-treated animals compared to the

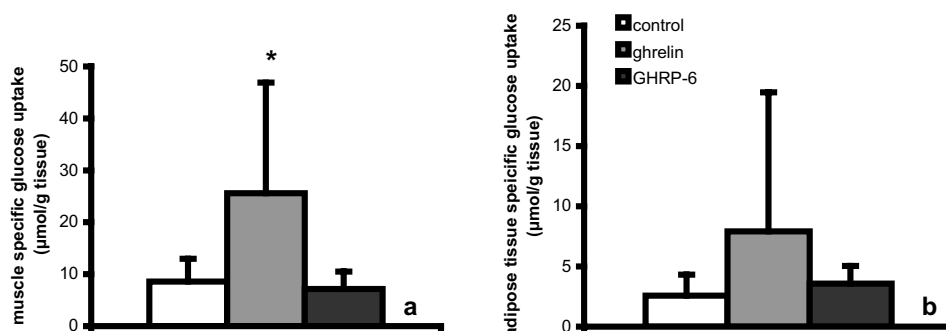


Fig 3 a) Muscle-specific glucose uptake (μmol/g) under hyperinsulinemic conditions in mice that received ghrelin (n=8), GHRP-6 (n=9) or vehicle (n=5). **b)** Adipose tissue-specific glucose uptake (μmol/g) under hyperinsulinemic conditions in mice that received ghrelin (n=8), GHRP-6 (n=9) or vehicle (n=5). Data are means ± SD. *p<0.05 compared to control mice.

control group, although this difference did not reach statistical significance (adipose tissue: control: 2.6 ± 1.7 ; ghrelin: 7.9 ± 11 ; GHRP-6: 3.6 ± 1.5 $\mu\text{mol/g}$ tissue, $p=0.09$) (see figure 3a and b).

Additional studies with des-ghrelin.

Plasma parameters

Body weight, plasma glucose, NEFA and insulin concentrations in basal and hyperinsulinemic conditions are shown in Table 1. Body weight did not differ between the groups of mice. Basal glucose levels were significantly lower in the group that received the combination of ghrelin and des-ghrelin compared to the control group. Basal insulin and NEFA levels were not different between groups. Moreover, in steady state hyperinsulinemic conditions, plasma glucose, NEFA and insulin concentrations were not different between groups.

Table 2. Glucose turnover and tissue specific glucose uptake in mice that received des-ghrelin (n=8) ghrelin and des-ghrelin together (n=7), or vehicle.

	control	des-ghrelin	ghrelin and des-ghrelin combined
basal EGP ($\mu\text{mol/kg/min}$)	44 ± 9	48 ± 9	$27 \pm 7^{**}$
glucose infusion rate ($\mu\text{l/hour}$)	91 ± 18	70 ± 30	104 ± 19
insulin mediated glucose disposal ($\mu\text{mol/kg/min}$)	59 ± 8	54 ± 15	50 ± 17
inhibition of EGP (%)	71 ± 11	$47 \pm 13^{**}$	75 ± 21
muscle specific glucose uptake ($\mu\text{mol/g}$ tissue)	8.6 ± 4.4	11 ± 6.1	10 ± 9.1
adipose tissue specific glucose uptake ($\mu\text{mol/g}$ tissue)	2.6 ± 1.7	2.6 ± 1.1	2.1 ± 1.3

Values are expressed as means $\pm$ SD, ** $p<0.01$ compared to control

Glucose turnover and tissue specific glucose uptake

Basal EGP, Glucose infusion rate, insulin mediated GD, inhibition of EGP, and tissue specific glucose uptake are shown in Table 2. During basal conditions, EGP (and thereby whole body GD) was significantly lower in animals that received the combination of ghrelin and des-ghrelin compared to the control animals. During the hyperinsulinemic period, the rate of glucose infusion necessary to maintain euglycemia, insulin stimulated GD, and tissue specific glucose uptake did not differ between the groups. In contrast, hyperinsulinaemia suppressed EGP significantly less in animals that received des-ghrelin compared to controls.

DISCUSSION

This study shows that intravenous administration of ghrelin reinforces insulin action on GD, whereas it hampers insulin's capacity to inhibit EGP. In contrast, administration of GHRP-6 did not affect insulin action. Des-ghrelin hampered insulin's capacity to inhibit EGP, whereas it did not affect GD. The restraining effects of des-ghrelin and ghrelin on hepatic insulin action were abolished by simultaneous administration of both peptides.

The glucose infusion rate required to maintain euglycemia during insulin infusion was significantly higher in ghrelin treated animals, indicating that this peptide enhances whole body insulin sensitivity of glucose metabolism. In particular, ghrelin clearly stimulated insulin-mediated GD as determined by tracer dilution methodology, an observation that was in line with enhanced 2-DG uptake in muscle and adipose tissue during hyperinsulinaemia in ghrelin treated animals (although statistical significance was reached only for muscle). In contrast, ghrelin hampered inhibition of EGP by insulin. Our results do not fully confirm our *a priori* hypothesis. As previous papers have reported that ghrelin stimulates NPY release and inhibits POMC/ α -MSH neuronal activity in the arcuate nucleus [25], we expected to find ghrelin compromising insulin sensitivity of both liver and muscle/adipose tissue (we recently showed that central NPY hampers insulin's action on EGP, whereas melanocortin pathways promote insulin mediated GD [17;18]). However, the mechanistic routes whereby ghrelin impacts insulin action remain to be established. Intravenously administered peptide may act through central pathways, but also through activation of receptors on peripheral tissues (the GHS-R is present in various tissues like pancreas and liver [15;26]). Indeed, ghrelin blocks insulin's inhibitory effect on gene expression of key gluconeogenic enzymes in a hepatoma cell-line [26]. These data corroborate our own *in vivo* observation of ghrelin hampering insulin action on EGP. In contrast, i.v. administration of ghrelin appears to enhance insulin mediated glucose uptake, where we *a priori* hypothesised that it would block this action (through down-regulation of hypothalamic α -MSH signaling [18]). It is conceivable that ghrelin acts on myocytes and/or adipocytes directly to stimulate GD. We are not aware of any *in vitro* or *in vivo* data documenting ghrelin's effects on insulin action in muscle or adipose tissue. Thus, further studies are required to establish if ghrelin affects insulin action via central or peripheral mechanisms.

Administration of GHRP-6 did not affect insulin action. This observation agrees with a report indicating that, ghrelin stimulates glucose production by primary hepatocytes, but hexarelin does not [20], ghrelin promotes adipogenesis in bone marrow, whereas L163,255, a potent GHS-R agonist does not [27] and ghrelin inhibits preadipocyte cell proliferation via a

novel receptor subtype [28]. In concert, these data strongly suggest that ghrelin impacts insulin action and metabolism via an as yet unidentified receptor.

Des-ghrelin blocked insulin action on EGP as much as ghrelin did. In contrast, it did not affect insulin mediated GD. These data support the emerging view that n-octanoyl modification of the serine (3) residue of ghrelin is not absolutely required for bioactivity [19;20;23]. However, they apparently contradict a report suggesting that ghrelin and des-ghrelin have opposing (stimulatory vs. inhibitory, respectively) effects on glucose production by hepatocytes [20]. The contradiction could be explained by the fact that *in vitro* and *in vivo* administration of des-ghrelin may have differential effects, as des-ghrelin, like ghrelin, can act in the brain as well as in peripheral tissues [19;27]. Co-administration of des-ghrelin abolished the impact of ghrelin on hepatic insulin sensitivity, which accords with an *in vitro* study showing similar results in isolated hepatocytes [20]. Our findings corroborate mounting evidence indicating that des-ghrelin has potentially important biological effects. The receptor mediating des-ghrelin bioactivity remains to be identified.

The physiological relevance of the observed effects of ghrelin to decrease hepatic insulin sensitivity and increase peripheral insulin sensitivity remains to be established. Ghrelin secretion by (primarily) the stomach is significantly enhanced during fasting to stimulate appetite and initiate meal consumption [9]. In a previous study, we showed that fasting enhances insulin-mediated glucose uptake in mice [29]. The data we present here allow us to hypothesize that the rise of plasma ghrelin levels during fasting is involved in the physiology of this phenomenon. However, des-ghrelin concentrations clearly dominate the plasma profile of ghrelin-like peptides, at least in the human [30;31]. In view of the current evidence suggesting that des-ghrelin has metabolic effects of its own and indeed appears to interact with ghrelin in the control of metabolism, further studies are required to establish the role of ghrelin-peptides in the regulation of energy balance and fuel flux. Moreover, the receptor(s) mediating the metabolic signals conveyed by (des-)ghrelin need to be identified.

In conclusion, ghrelin differentially affects tissue-specific insulin action, hampering its ability to inhibit EGP while reinforcing its impact on GD. These effects occur acutely and they are not secondary to ghrelin's well-known influence on feeding and body weight. The GHS-R is not likely to mediate ghrelin's metabolic effects. Des-ghrelin also appears to modulate fuel flux and may counteract ghrelin in the control of glucose metabolism.

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