Circulating ghrelin concentrations are lowered by intracerebroventricular insulin

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Abstract

Aims/hypothesis Ghrelin is a peptide that is mainly produced by the stomach and stimulates food intake, adiposity and weight gain. Previous studies have documented that plasma levels of ghrelin are reduced by insulin, but the mechanisms that mediate this effect are unclear.

Methods To determine whether phosphatidylinositol 3-kinase (PI(3)K) and/or mitogen-activated protein kinase (MAPK) are involved in this insulin action, we tested the intracerebroventricular (i.c.v.) effect of specific inhibitors of PI(3)K (LY294002 and wortmannin) and MAPK (PD98059 and UO126) on the insulin-mediated reduction of ghrelin levels in rats.

Results Intracerebroventricular treatment with insulin reduced ghrelin levels. Inhibition of PI(3)K specifically blocked the insulin-induced reduction in ghrelin concentration, whereas inhibition of MAPK had no effect on insulin-mediated actions. Moreover, pretreatment with i.c.v. PI(3)K inhibitors blocked the reduction of ghrelin levels after OGTT-induced hyperglycaemia and hyperinsulinaemia.

Conclusions/interpretation These data demonstrate that changes in insulin action in the central nervous system regulate circulating ghrelin levels and that PI(3)K is a specific mediator of this action.

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Keywords CNS · Ghrelin · Insulin

Abbreviations

CNS central nervous system
i.c.v. intracerebroventricular(ly)
ESM electronic supplementary material
IR insulin receptor
MAPK mitogen-activated protein kinase
PI(3)K phosphatidylinositol 3-kinase

Introduction

Ghrelin is a peptide that is produced mainly by the stomach and stimulates food intake, adiposity and weight gain [1]. In cross-sectional studies, fasting plasma ghrelin and insulin were inversely correlated. Moreover, McCowen et al. suggest that whole-body insulin responsiveness plays a role in meal-related ghrelin inhibition [2], although the target tissues and the mechanisms that mediate the insulin-induced reduction in circulating ghrelin levels remain to be clarified.

Recently it has been reported that different insulin responses are mediated by the effects of insulin on the central nervous system (CNS), including the control of food intake, thermogenesis and, at least in part, hepatic glucose output [3, 4]. Thus, we hypothesised that the ability of insulin to suppress ghrelin may be mediated centrally.

Insulin receptors (IR) are expressed in most tissues of the body, including liver, muscle, fat and neurons of the CNS [3, 5]. The IR is a protein tyrosine kinase, which, when activated by insulin binding, phosphorylates intracellular

protein substrates, including IRS and Src homology collagen [4]. Following tyrosine phosphorylation, IRSs act as docking proteins for several Src homology 2 domain-containing proteins, including phosphatidylinositol 3-kinase (PI(3)K) and growth factor receptor-binding protein 2 [4, 5]. Downstream of PI(3)K, activation of a serine/threonine kinase, Akt, occurs. In contrast, downstream of growth factor receptor-binding protein 2 activation of the mitogen-activated protein kinase (MAPK-ERK) occurs, which is important for the regulation of gene expression and cell growth [4].

The aim of the present study was to examine the molecular mechanisms involved in the effects of intracerebroventricular (i.c.v.) insulin on circulating ghrelin levels. First, we investigated whether i.c.v. administration of insulin affects ghrelin levels. Next, to examine whether PI(3)K and/or MAPK are involved in this action of insulin, we tested the effects of i.c.v. administration of specific inhibitors of PI(3)K and MAPK on the insulin-mediated reduction of ghrelin levels.

Materials and methods

Animals

Animals, measurement of food intake, surgical procedures, and tissue extraction are described in the electronic supplementary material (ESM).

Treatments and measurement of hormones

Rats deprived of food for 48 h with free access to water were injected i.c.v. (3 μ l bolus injection) with inhibitors of PI(3)K (LY294002, 50 μ mol/l and wortmannin, 100 nmol/l; Calbiochem, San Diego, CA, USA) or of MAPK (PD98059, 5 μ g and UO126, 7 μ g; LC Laboratories, Woburn, MA, USA), or their vehicle, 30 min before insulin (i.c.v.) or glucose (orally). In an OGTT, glucose (2 g/kg body weight) was administered through a gastric catheter. Blood samples were then collected and centrifuged and the plasma obtained was analysed for measurement of glucose, insulin and ghrelin levels. Total ghrelin and insulin were measured using a commercial RIA (Linco Research, St. Charles, MO, USA).

Western blotting analysis

Western blotting of hypothalamus samples is described in the ESM.

Statistical analysis

Where appropriate, the results are expressed as mean $\pm$ SEM, accompanied by the number of rats used in the experiment. Comparisons among groups were made using parametric two-way ANOVA. Further comparisons were made using the Newman–Keuls test. A *p* value of less than 0.05 was considered statistically significant.

Results

Effect of specific inhibitors of PI(3)K and MAPK on insulin-mediated reduction of circulating ghrelin levels

The effect of insulin on the control of food intake was studied in rats fasted for 6 h by measuring the total food intake over 12 h after a single i.c.v. injection of insulin just before the onset of the dark cycle. Insulin induced a reduction of 26% in the 12-h food intake compared with the vehicle-treated group (Fig. 1a), without changes in peripheral blood glucose and plasma insulin levels during 120 min (data not shown). Figure 1b shows a clear increase in insulin-stimulated IR phosphorylation, which was maximal at 15 min and then declined sharply. Similarly to IR tyrosine phosphorylation, there was a great increase in insulin-stimulated Akt phosphorylation in the hypothalamus, which peaked at 15 min before declining (Fig. 1b). We also observed that i.c.v. treatment with insulin reduced ghrelin levels at 15 min and that this effect persisted until 120 min after the i.c.v. infusion (Fig. 1c).

Next, to investigate whether PI(3)K and/or MAPK were involved in this action of insulin, we tested the effect of specific inhibitors of PI(3)K and MAPK administered i.c.v. on the insulin-mediated reduction of ghrelin levels. We found that inhibition of PI(3)K specifically blocked the insulin-induced reduction of the ghrelin concentration, whereas the administration of inhibitors of MAPK with insulin or vehicle did not affect insulin-mediated actions. The decrease in circulating ghrelin levels induced by i.c.v. administration of insulin after 120 min was 34 $\pm$ 5% in the presence of vehicle, 23 $\pm$ 2% in the presence of PD98059 (*p*<0.05) and 26 $\pm$ 3% in the presence of UO126 (data not shown), with no alterations in the presence of LY294002 or wortmannin. Plasma glucose was not affected by i.c.v. administration of insulin or vehicle.

Effect of specific inhibitors of PI(3)K and MAPK on the OGTT-mediated reduction of circulating ghrelin levels

There were increases in the plasma glucose and plasma insulin concentrations in the first 30 min after glucose intake, which returned almost to basal levels at 120 min

Fig. 1 **a** Rats fasted for 6 h were injected i.c.v. with vehicle (–, 3 μ l) or insulin (+, 3 mU) and were immediately exposed to food for 12 h. Data are mean±SEM; $n=8$. * $p < 0.05$, relative to controls. **b** Insulin-induced IR tyrosine phosphorylation and serine phosphorylation of Akt in hypothalamus of rats. Hypothalamic extracts from rats (without i.c.v. insulin infusion, time 0) or treated with insulin (15, 30, 60, 90 and 120 min after i.c.v. infusion) were homogenised and the proteins were separated on SDS-PAGE gels. Tissue extracts were immunoprecipitated with anti-IR antibody and immunoblotted with anti-phosphotyrosine or with phosphoserine-specific Akt antibodies. A Western blot representative of each group is shown. **c** Time course of plasma ghrelin levels in rats in response to i.c.v. insulin and to insulin in the presence of PI(3)K (LY294002, 50 μ mol/l; i.c.v.) and MAPK (PD98059, 5 μ g; i.c.v.) inhibitors. Open triangle, vehicle + vehicle; filled triangle, vehicle + insulin; square, LY294002 + insulin; filled circle, PD98059 + insulin. Data are mean±SEM of nine animals in each group. * $p < 0.05$

post OGTT (Fig. 2a). In parallel, there was a clear increase in IR phosphorylation, which was maximal at 15 min and then declined sharply. Similarly to IR tyrosine phosphorylation, there was a great increase in OGTT-stimulated Akt

phosphorylation in the hypothalamus, which peaked at 15 min and then declined (Fig. 2b). As shown in Fig. 2c, plasma ghrelin levels were reduced (by $27 \pm 4\%$) 120 min after oral intake of glucose. We observed that only the infusion of PI(3)K inhibitors into the lateral cerebral ventricle blocked the reduction of circulating ghrelin levels following the hyperglycaemia and hyperinsulinaemia in-

Fig. 2 **a** Plasma glucose (open square) and plasma insulin (filled circle) concentrations in rats during OGTT (2 g/kg body weight). Data are mean±SEM; $n=8$. **b** IR tyrosine phosphorylation and serine phosphorylation of Akt in hypothalamus of rats in response to OGTT. At the indicated time after the OGTT (2 g/kg body weight), hypothalamic extracts were homogenised and the proteins were separated on SDS-PAGE gels. Tissue extracts were immunoprecipitated with anti-IR antibody and immunoblotted with anti-phosphotyrosine or with phosphoserine-specific Akt antibodies. A representative Western blot of each group is shown. **c** Time course of plasma ghrelin levels in rats in response to OGTT and to OGTT in the presence of PI(3)K (LY294002, 50 μ mol/l; i.c.v.) and MAPK inhibitors (PD98059, 5 μ g; i.c.v.). Open square, vehicle + vehicle; filled square, vehicle + OGTT; open circle, LY294002 + OGTT; filled triangle, PD98059 + OGTT. Data are mean±SEM of nine animals in each group. * $p < 0.05$

duced by the OGTT. Administration of inhibitors of MAPK with insulin or vehicle did not affect insulin-mediated actions. The OGTT-induced decrease in circulating ghrelin levels was $25 \pm 12\%$ in the presence of PD98059 and $24 \pm 9\%$ in the presence of UO126 ($p < 0.05$); these values were not altered in the presence of LY294002 or wortmannin.

Discussion

In this study we showed that i.c.v. infusion of insulin did not change peripheral blood glucose or plasma insulin levels, but reduced ghrelin levels. Inhibition of hypothalamic PI(3)K specifically blocked the insulin- and OGTT-induced reduction in ghrelin concentration, whereas the inhibition of MAPK had no effect on insulin-mediated actions.

There is evidence that the type of macronutrient ingested has a marked effect on postprandial ghrelin concentrations, carbohydrates having the most pronounced effects [6, 7]. Our data regarding ghrelin levels during the OGTT are in accordance with previous data showing that insulin is a key regulator of the postprandial ghrelin suppression response [8], although some controversy surrounds this topic [9]. To date, studies (at least in humans) indicate that supraphysiological or prolonged peripheral insulin administration affects the plasma ghrelin concentration [10]. However, physiological short-term peripheral insulin elevation had no effect on ghrelin levels in most of these studies [9, 10]. Our data show that during the OGTT the elevations in the plasma insulin level are able to induce insulin signalling in the hypothalamus, as demonstrated by increased IR tyrosine phosphorylation and Akt serine phosphorylation in this tissue. In addition, we also showed that i.c.v. administration of inhibitors of PI(3)K blocks the suppressor effect of insulin on ghrelin levels during OGTT, suggesting that this physiological postprandial effect of insulin is centrally mediated, through the IRS/PI(3)K pathway.

The physiological relevance of reduced ghrelin levels in vivo is not well established. Recently, different studies have shown that mice deficient in ghrelin or ghrelin receptors display normal regulation of food intake. However, these mice were resistant to diet-induced obesity when fed a high-fat diet, suggesting that ghrelin and/or ghrelin-responsive pathways may have an important role in metabolic adaptation to nutrient availability [11, 12].

Ghrelin is primarily produced in the mucosal endocrine cells of the stomach [13], but it is also synthesised in the hypothalamic arcuate nucleus [1]. We have demonstrated that insulin acts mainly in the arcuate nucleus to reduce food intake [3]. Together, these data and the results presented here

suggest that the reduction in circulating ghrelin, observed after i.c.v. insulin infusion, may be mediated by the hypothalamus, probably through a hypothalamus–stomach neuronal pathway, but we cannot exclude a direct effect on the hypothalamus (arcuate nucleus), which also produces ghrelin.

In conclusion, our data provide evidence that changes in the action of insulin in the CNS regulate circulating ghrelin levels. The finding that i.c.v. insulin does not alter plasma insulin levels indicates that these effects are due solely to the central neural action of insulin. Our data also reveal a key role for PI(3)K in the control of the suppressive effect of insulin on ghrelin levels.

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