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Ghrelin activates neuronal constitutive nitric oxide synthase in pancreatic islet cells while inhibiting insulin release and stimulating glucagon release

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SUMMARY

In view of our previous data, showing that ghrelin and nitric oxide (NO) display apparently parallel effects on insulin secretion (inhibitory) and glucagon secretion (stimulatory), we have now investigated the effect of ghrelin on islet hormone secretion in relation to its effect on NO synthase (NOS) isoenzymes in isolated rat pancreatic islets. Dose-response studies revealed that ghrelin at concentrations of 0.01-1 μmol l^{-1} inhibited insulin secretion stimulated by 8.3 mmol l^{-1} glucose, while ghrelin at concentrations lower than the physiological range (0.01 pmol l^{-1}- 1 nmol l^{-1}) were without effect. In contrast, glucagon secretion was stimulated by 1.0 nmol l^{-1}- 1 μmol l^{-1} ghrelin. These effects of ghrelin on insulin and glucagon secretion were accompanied by increased NO production through activation of neuronal constitutive NOS (ncNOS). Ghrelin had no appreciable effect on the activity of inducible NOS (iNOS) in the islets. Addition of an NO scavenger (cPTIO) or the NOS inhibitor L-NAME to the incubation medium prevented the effects of ghrelin on hormone secretion from isolated islets.

The present results confirm our previous data showing that ghrelin inhibits insulin and stimulates glucagon secretion from pancreatic islets of the mouse and we now show similar effects in rat islets. The effects of ghrelin were accompanied by an increased rate of NO production. Conceivably, ncNOS activation partly accounts for to the inhibitory effect of ghrelin on insulin secretion and the stimulatory effect of ghrelin on glucagon secretion.

1. Introduction

Ghrelin secreted from the A-like cells in the oxyntic mucosa of the stomach [1-3] is a powerful stimulator of growth hormone release [1]. There is much evidence also that ghrelin is involved in the regulation of food intake and energy homeostasis [4]. In addition, ghrelin has been reported to affect both insulin and glucagon secretion *in vitro* and *in vivo* [5-7]. Exactly how ghrelin influences islet hormone secretion is still unknown, however, and both stimulatory and inhibitory and no effects are on record [5-7].

We have previously shown that ghrelin at low concentrations suppressed and at high, supraphysiological concentrations stimulated glucose-induced insulin secretion from isolated mouse islets [5]. Glucagon secretion, on the other hand, was stimulated by a wide range of ghrelin concentrations [5]. The signals that are responsible for the ghrelin-evoked effects on glucagon and insulin secretion have not yet been identified. We have found repeatedly that stimulated production of NO by activation of islet NOS has an inhibitory effect on glucose-induced insulin release and an amplifying effect on glucagon secretion [8-17]. In view of the parallelism between the effect of ghrelin and the effect of stimulated NO production [11-14] we were compelled to explore the possibility that ghrelin-induced effects on NO production account for the effects of ghrelin on islet hormone secretion. Most data speak in favour of NO being a negative modulator of stimulated insulin secretion (following glucose, L-arginine and cholinergic activation) [8-18], although insulin release has been reported to actually be promoted by NO under certain conditions (various insulin-cell lines, low to medium glucose and low concentrations of NO) [15, 19-21].

It is widely accepted that NO functions as an intracellular messenger molecule in the regulation of insulin and glucagon secretion although the details of the signalling pathway remain uncertain [8-21]. The formation of NO in pancreatic islet cells is catalysed by a constitutively expressed neuronal NO synthase (ncNOS), the activity of which is

Ca^{2+}/calmodulin-dependent, and/or by an inducible NOS (iNOS) the activity of which is Ca^{2+}/calmodulin-independent [22]. The expression of iNOS in pancreatic islets can be induced by a variety of inflammatory agents and cytokines [23-26]. Interestingly, however, we showed recently that iNOS activity can be induced also by hyperglycaemia [12] and by hyperlipidaemia [13, 14] in islets from normal healthy animals.

We have previously suggested that an increased intracellular NO level in the islets inhibits insulin release stimulated by glucose [8-12, 15, 16]. In contrast, we have found repeatedly that NO stimulates glucagon secretion [8, 9, 15, 17, 27]. Since the activity of islet ncNOS and/or iNOS can be modified by a variety of regulatory peptides and neurotransmitters [14, 26, 27], the aim of the present investigation was to study how ghrelin affects ncNOS and iNOS in isolated pancreatic islets in an attempt to see whether the effects of ghrelin on insulin and glucagon secretion might be accompanied by changes in the formation of NO.

2. Methods

2.1. Drugs and chemicals

Rat ghrelin-28 was a kind gift from Dr. N. Yanaihara and Dr. C. Yanaihara at the Yanaihara Institute, Shizuoka, Japan. Collagenase (CLS-4) from Worthington Biochemicals (Freehold, NJ, USA) was used to prepare the pancreatic islets. Bovine serum albumin (BSA) was from ICN Biomedical (High Wycombe, UK). N^G-nitro-L-arginine methylester (L-NAME) (a NOS inhibitor) [16, 22] and 2- (4-carboxyphenyl)-4,4,5,5- tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) (an NO scavenger) [19] were from Sigma Chemicals, St Louis, MO, USA. All other chemicals were from British Drug Houses (Poole, UK) or Merck (Darmstadt, Germany). Radioimmunoassay kits for determination of insulin were obtained from Diagnostika

(Falkenberg, Sweden) while those for glucagon determination were from Eurodiagnostica (Malmö, Sweden).

2.2. Animals

Freely fed female rats of the Sprague-Dawley strain (B&K, Sollentuna, Sweden), weighing 200-225 g were used. They were given a standard pellet diet (B&K) and tap water *ad libitum*. The experiments were approved by the local animal welfare committee, Lund, Sweden.

2.3. Experimental protocol

Pancreatic islets were isolated from rats, killed by cervical dislocation. A collagenase solution was injected into the bile-pancreatic duct, followed by excision of the pancreas and isolation of the islets by a standard digestion procedure [8, 28]. Freshly isolated islets were preincubated for 30 min at 37 °C in Krebs-Ringer bicarbonate (KRB) buffer, pH 7.4, supplemented with 10 mmol l^{-1} Hepes, 0.1% bovine serum albumin and 1 mmol l^{-1} glucose. Each incubation vial was gassed with 95% O_2 and 5% CO_2 to obtain constant pH and oxygenation. After preincubation, the buffer was changed to a medium containing 8.3 mmol l^{-1} of glucose in the presence of different concentrations of ghrelin. When only hormone secretion was measured we used 12 islets in a volume of 1 ml while 225 islets/1.5 ml were used when both NOS activities and hormone secretion were determined. The islets were incubated in a metabolic shaker (30 cycles per min) at 37°C. After 90 min of incubation, aliquots of the medium were removed for assay of insulin and glucagon.

2.3.1. Assay of islet NOS activities.

Preincubation and incubation of freshly isolated islets were performed as stated above. After incubation, aliquots of the medium were removed for determination of insulin and glucagon,

whereafter the islets were washed and collected in 200 μl buffer, containing 20 mmol l^{-1} HEPES, 0.5 mmol l^{-1} EDTA and 1 mmol l^{-1} DL-dithiothreitol, and thereafter stored at -20°C. On the day of the assay, the islets were sonicated on ice and the buffer solution was enriched with 0.45 mmol l^{-1} $CaCl_2$, 2 mmol l^{-1} NADPH, 25 U/ml calmodulin, and 0.2 mmol l^{-1} L-arginine. For the determination of iNOS activity both Ca^{2+} and calmodulin were omitted. The homogenate was incubated at 37°C under constant air bubbling with air, 1.0 ml/min for 2 h. Aliquots of the incubated homogenate (200 μl) were then passed through an 1 ml Amprep CBA cation-exchange column for determination of L-citrulline by high performance liquid chromatography (HPLC). The method has been described in detail [8, 12]. Since L-citrulline and NO are generated in equimolar amounts, and since L-citrulline is stable whereas NO is not, L-citrulline is the preferred parameter when measuring NO production. Protein was determined according to Bradford [29] on samples from the original homogenate.

2.3.2. Determination of insulin and glucagon

Insulin and glucagon were determined by radioimmunoassay [30-32].

2.4. Statistics

Results were expressed as means ± SEM. The level of significance for the difference between sets of data was assessed using Student's unpaired *t*-test or analysis of variance followed by Tukey-Kramer's test whenever appropriate. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of ghrelin on insulin and glucagon secretion from isolated pancreatic islets

Figure 1 shows the effects of different concentrations of ghrelin on insulin and glucagon secretion at 8.3 mmol l^{-1} glucose. Ghrelin concentrations of 10-1000 nmol l^{-1} inhibited

glucose-stimulated insulin release, while concentrations lower than the physiological range (indicated by the shadowed area) were without effect. In contrast, glucagon secretion was stimulated by ghrelin over a wide concentration range (1.0 nmol l^{-1}-1000 nmol l^{-1}), embracing concentrations that were both lower and higher than the concentration measured in blood (Fig. 1).

3.2. Effect of ghrelin on islet NOS activities in relation to insulin and glucagon secretion

Islet activities of ncNOS and iNOS as well as total NOS were determined after incubation at 8.3 mmol l^{-1} glucose in the absence and presence of ghrelin. We used concentrations of ghrelin that inhibited glucose-stimulated insulin release, i.e., 0.01 and 1 μmol l^{-1}, (see Fig. 1). Figure 2 shows that ghrelin increased islet ncNOS-catalysed NO production, while iNOS activity was almost undetectable and not affected by ghrelin. Total NOS activity was increased by ghrelin (Fig. 2a). In parallel with the increased ncNOS-catalysed NO production, the glucose-stimulated insulin secretion was suppressed in the presence of ghrelin (0.01 and 1 μmol l^{-1}) while glucagon secretion was increased (Fig. 2b and 2c).

3.3. Effect of ghrelin on insulin and glucagon secretion in the presence of the NO scavenger cPTIO or the NOS inhibitor L-NAME

To further elucidate the possible importance of ghrelin-stimulated ncNOS activity on the mechanisms of action of ghrelin on insulin and glucagon secretion we incubated isolated islets in the presence of the NO scavenger cPTIO or the NOS inhibitor L-NAME at 8.3 mmol l^{-1} glucose in the absence and presence of ghrelin. Figure 3 shows that neither cPTIO (200 μmol l^{-1}) nor L-NAME (5 mmol l^{-1}) had any appreciable effect on insulin or glucagon secretion in the absence of ghrelin at this modest glucose concentration. Ghrelin suppressed insulin release and stimulated glucagon release, effects that were prevented by addition of cPTIO or L-NAME to the incubation medium (Fig. 3).

4. Discussion

In a series of studies it has been shown that NO (generated by islet ncNOS) is a powerful inhibitor of glucose-stimulated insulin release [8-12, 16, 33] and an important stimulator of glucagon release [8, 9, 15, 17, 27]. We have recently found ghrelin to exert similar actions on islet hormone release, *i.e.* to inhibit insulin and stimulate glucagon release [5]. For this reason we decided to examine the possibility that ghrelin acts by influencing the activity of NOS in the pancreatic islet cells. Indeed, the present data speak in favour of NO mediating the effects of ghrelin on the insulin and glucagon cells. We have shown previously, by conventional and confocal immunohistochemistry, that both these cell types harbour ncNOS [34-37], the immunostaining being uniformly distributed among insulin and glucagon cells [34].

4.1. Insulin secretion

In our recent study [5] of the *in vivo* effects of ghrelin on glucose-stimulated insulin secretion we found that ghrelin inhibited the insulin response, which is mainly transduced through the Ca^{2+}/calmodulin pathway. Further, ghrelin was found to inhibit the insulin response to the cholinergic agonist carbachol (the phospholipase C pathway), while it enhanced the insulin response to IBMX (the cyclic AMP pathway). These results suggest that the inhibitory effect of ghrelin on stimulated insulin release might, at least in part, be exerted through NO, since both glucose and carbachol stimulated NOS activities, whereas cyclic AMP had an inhibitory effect on the NOS isoenzymes [8-12, 14, 26, 27]. In this context it should be noted that high, unphysiological doses of ghrelin stimulated basal insulin secretion *in vivo* both in the rat [38] and in the mouse [5]. This observation was further corroborated by our finding [5] that very high concentrations of ghrelin stimulated insulin release *in vitro* from mouse islets. However, as shown in Fig. 1 the stimulatory effect of ghrelin could not be reproduced in isolated rat

islets. Conceivably, species differences manifested at supraphysiological concentrations of ghrelin may account for this difference. In this context it should be noted that other authors [39] have reported that ghrelin at 1 pmol l^{-1} might have a slight stimulatory effect on glucose-induced insulin release from isolated rat islets. Such an effect was not observed in the present study. The reason for this discrepancy is unclear.

In the present study we used a concentration of glucose (8.3 mmol l^{-1}), which has a modest stimulatory effect on insulin release and does not by itself increase either ncNOS or iNOS activity in the islets; at least 10 mmol l^{-1} of glucose is required to activate the enzymes [12]. Our observation that ghrelin, at concentrations that inhibited glucose-stimulated insulin release, raised the ncNOS activity agrees with the finding that NO suppresses glucose-stimulated insulin secretion [8-16]. Further, our results may suggest that one possible mechanism of action of ghrelin is to increase the sensitivity of ncNOS to glucose so that the enzyme is activated already at glucose concentrations lower than 10 mmol l^{-1}. There was a graded increase in NO production in the islets after addition of ghrelin at concentrations of 0.01 μmol l^{-1} and 1 μmol l^{-1}, although insulin release was suppressed to approximately the same degree by both concentrations. Hence, activation of ncNOS may not be the sole effect of ghrelin on the insulin cell. In this context it should be emphasized that recent evidence from an electron microscopic study [18] indicates that ncNOS has a preferential localization to the secretory granules in the insulin cell, an observation which supports the view that NO contributes to the regulation of storage and secretion of insulin.

The present results show that the negative impact of ghrelin on insulin release can be prevented by addition of either the NO scavenger cPTIO or the NOS inhibitor L-NAME to the incubation medium. Hence, these data provide evidence - albeit indirect- for the view that ncNOS-derived NO mediates the effect of ghrelin on the insulin cell. The mechanism by

which NO impairs insulin release is unknown, but we have suggested [8-12, 16, 27, 37, 40] that NO acts through S-nitrosylation of the glutathione system and/or important regulatory proteins in the stimulus-secretion coupling.

4.2. Glucagon secretion

Already in 1994 we proposed that NO is a physiological stimulator of glucagon secretion [40]. Later on we demonstrated by immunocytochemistry that the glucagon cells are rich in ncNOS, while iNOS could be observed only in a few scattered glucagon cells after provocation with lipopolysaccharides (endotoxin) [34]. Moreover, we have shown repeatedly that various NOS inhibitors suppress glucagon release stimulated by L-arginine or carbachol both *in vivo* and *in vitro* [8, 9, 13-15, 17, 27, 40], in support of the view that NO stimulates glucagon secretion. Indeed, a stimulatory effect of NO on glucagon release could be demonstrated by showing that addition of gaseous NO to incubated islets stimulated glucagon release [41]. Recently Mori *et al.* [42], using a cultured glucagon cell line, confirmed our observation that NO stimulates glucagon release [8, 9, 13-15, 17, 27, 40].

Our studies of isolated islets revealed that ghrelin stimulates glucagon secretion and ncNOS-catalysed NO formation concomitantly. The suppressive effect of the NO scavenger cPTIO or the NOS inhibitor L-NAME on ghrelin-induced glucagon release reinforces our view that NO is a glucagon secretagogue [8, 9, 13-15, 17, 27, 40, 41]. Hence, we suggest that ghrelin activates NO production in the glucagon cell thereby stimulating the release of glucagon. Whether this action of ghrelin on the glucagon cell is important *in vivo* remains to be elucidated.

In the present study, ghrelin stimulated ncNOS activity in intact islets. It remains to show that ghrelin is capable of activating ncNOS in both insulin and glucagon cells. It cannot be

excluded that the enzyme activation is restricted to either the insulin cells or the glucagon cells, although ghrelin receptors have been demonstrated in both types of cells [43].

4.3. Concluding remarks

The present results of studies of isolated rat islets suggest that the effects of ghrelin on the endocrine pancreas might be exerted, at least in part, through enhanced ncNOS-catalysed formation of NO. The inhibitory effect of ghrelin on insulin secretion and the stimulatory effect of ghrelin on glucagon secretion coincide with increased NO formation through activation of islet ncNOS. Addition of a scavenger of NO (cPTIO) or an inhibitor of NOS (L-NAME) to the incubation medium prevented the effects of ghrelin on islet hormone secretion. We propose therefore that ghrelin may contribute to the control of insulin and glucagon secretion by stimulating ncNOS activity.

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Legends to Figures

Figure 1: Effect of increasing concentrations of ghrelin on insulin and glucagon release from isolated rat islets

Isolated islets were incubated in a medium, containing 8.3 mmol l^{-1} glucose. Insulin (a) and glucagon (b) release were measured after 90 min. Ghrelin concentrations ranged from 0.01 pmol l^{-1} to 1 μmol l^{-1}. Hatched area indicates the range of blood ghrelin concentrations in rats and mice during pre- and post-prandial conditions ([4,6,8,9,13,16,34,35 and own unpublished data]). Means ± S.E.M., 10-12 batches of islets at each point. Each batch contained 12 islets. Significant differences *versus* controls (8.3 mmol l^{-1} glucose without ghrelin) are denoted by *p< 0.05, **p<0.01, ***p<0.001.

Figure 2: Effects of ghrelin, 10 or 1000 nmol l^{-1}, on islet ncNOS and iNOS activities as well as on insulin and glucagon release from isolated islets

Isolated islets were incubated in a medium, containing 8.3 mmol l^{-1} glucose, in the presence of 10 or 1000 nmol l^{-1} ghrelin. Islet activities of NOS isoenzymes (ncNOS and iNOS) (a), insulin release (b) and glucagon release (c) were measured. Means ± S.E.M for 10-12 batches of islets at each point. Each batch contained 225 islets. Significant differences *versus* controls without ghrelin are denoted by *p< 0.05, **p<0.01, ***p<0.001.

Figure 3: Effect of ghrelin on insulin and glucagon release from isolated islets in the presence of cPTIO or L-NAME

Isolated islets were incubated in a medium containing 8.3 mmol l^{-1} glucose (G) in the presence of 1000 nmol l^{-1} ghrelin with or without cPTIO (200 μmol l^{-1}) or L-NAME (5 mmol l^{-1}). Insulin release (a) and glucagon release (b) were measured. Means ± S.E.M for 6-10

batches of islets at each point. Each batch contained 12 islets. Significant differences *versus* controls without ghrelin are denoted by ***$p<0.001$.

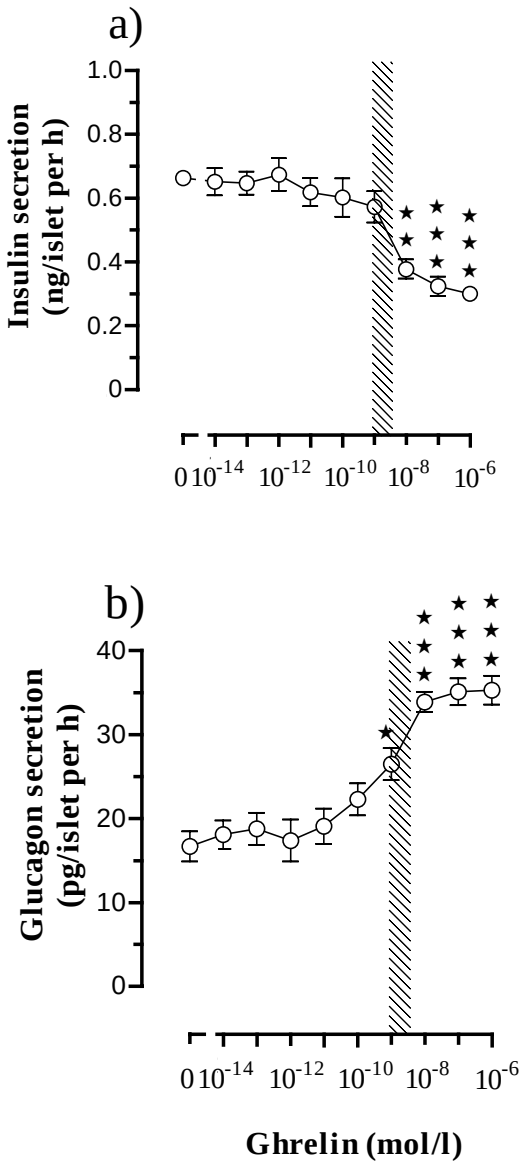
a)
Insulin secretion (ng/islet per h)
1.0
0.8
0.6
0.4
0.2
0
0 10-14 10-12 10-10 10-8 10-6
b)
Glucagon secretion (pg/islet per h)
40
30
20
10
0
0 10-14 10-12 10-10 10-8 10-6
Ghrelin (mol/l)

Fig. 1

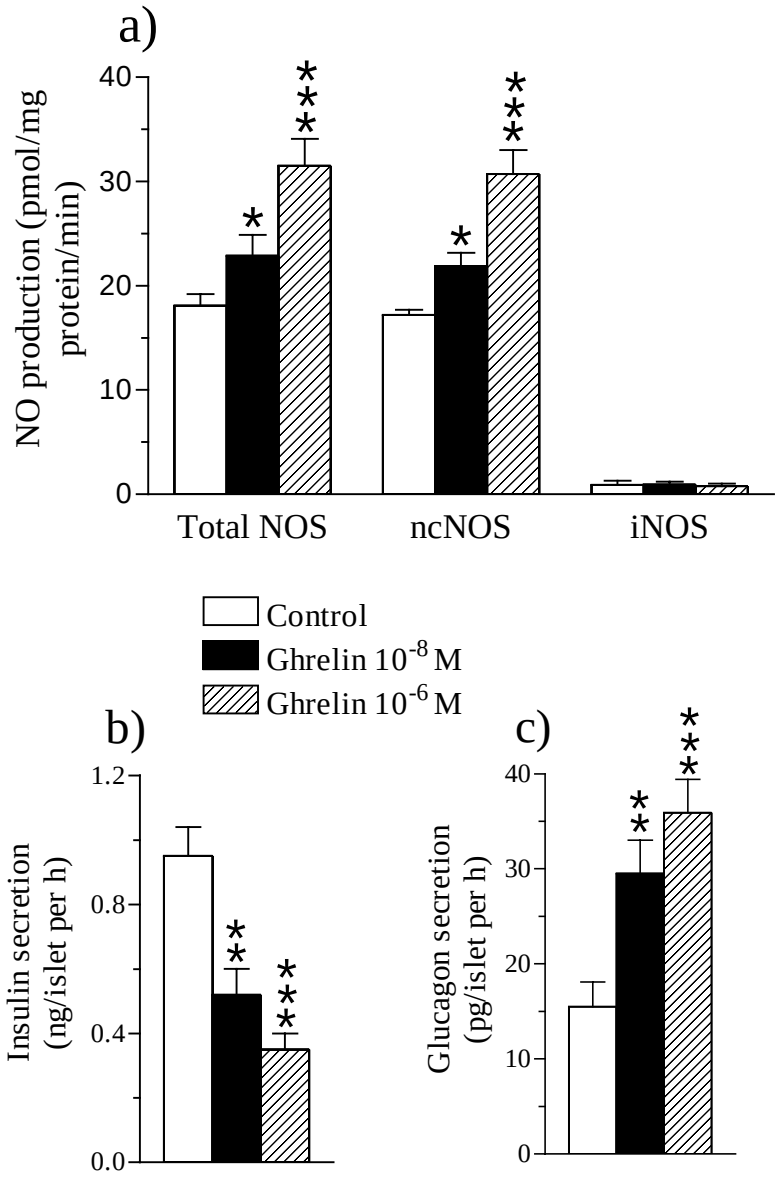
a)
NO production (pmol/mg protein/min)
40
30
20
10
0
*
***
*
***
Total NOS
ncNOS
iNOS
Control
Ghrelin 10-8 M
Ghrelin 10-6 M
b)
Insulin secretion (ng/islet per h)
1.2
0.8
0.4
0.0
**
***
c)
Glucagon secretion (pg/islet per h)
40
30
20
10
0
**
***

Fig.2

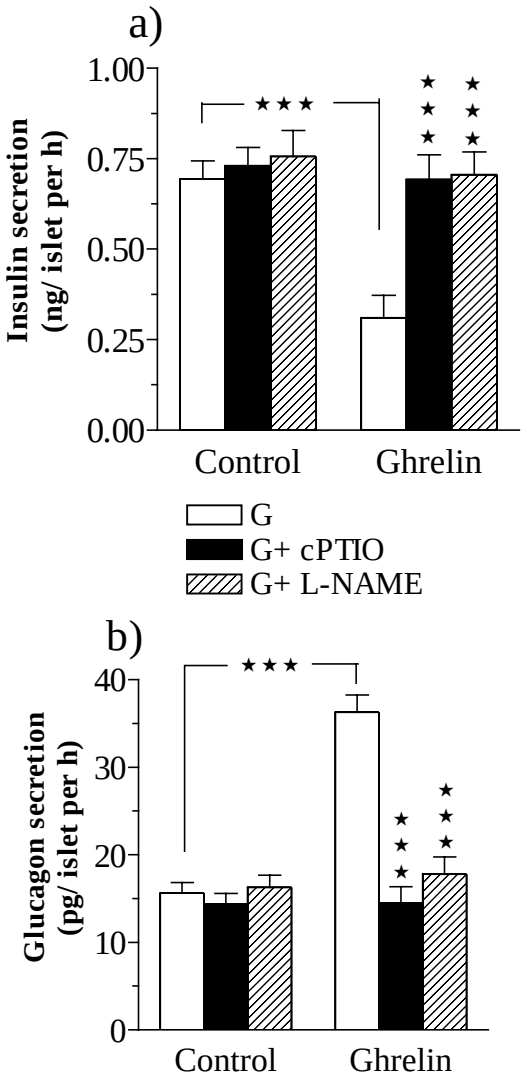
a)
Insulin secretion (ng/ islet per h)
1.00
0.75
0.50
0.25
0.00
Control
Ghrelin
G
G+ cPTIO
G+ L-NAME
b)
Glucagon secretion (pg/ islet per h)
40
30
20
10
0
Control
Ghrelin

Fig. 3