Kisspeptin-13 inhibits insulin secretion without affecting glucagon or somatostatin release: study in the perfused rat pancreas

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Abstract

Kisspeptins are a family of peptides encoded by the KISS1 gene, which binds to G-protein-coupled receptor (GPR54), an orphan GPR54 related to galanin receptors. Endogenous forms composed of 54, 14, and 13 amino acids have been identified. Kisspeptin and GPR54 mRNAs have been detected in pancreatic B and A cells. Furthermore, kisspeptin-54 has been shown to slightly stimulate the last phase of glucose-induced insulin secretion in mouse and human islets and to inhibit insulin release in MIN6 cells. We have investigated the effect of kisspeptin-13 on insulin, glucagon, and somatostatin secretion. The study was performed in the perfused rat pancreas. Glucose, arginine, carbachol, and exendin-4 were used as secretagogues. Hormones were measured by RIA. Kisspeptin-13 reduced

glucose-induced insulin secretion in a dose-dependent manner ($IC_{50}=1.2$ nM) and inhibited the insulin responses to both carbachol and exendin-4. Kisspeptin-13 blocked arginine-induced insulin secretion without affecting the glucagon or somatostatin responses to this amino acid, thus indicating that kisspeptin-13 influences B cells directly, rather than through an A- or D-cell paracrine effect. The reduction of the insulin response to exendin-4 induced by kisspeptin-13 was also observed in pertussis toxin-treated rats, thus suggesting an inhibition independent of G_i proteins. In view of the potent insulinostatic effect of kisspeptin-13, it is tempting to speculate that kisspeptins may be implicated in the regulation of B-cell secretion.

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Introduction

Kisspeptins are a family of peptides encoded by the KISS1 gene (Kotani *et al.* 2001, Muir *et al.* 2001, Ohtaki *et al.* 2001). KISS-1 was originally identified as a putative human metastasis suppressor gene in melanomas and breast cancer cells that acts without affecting tumorigenicity (Lee *et al.* 1996). Its expression has also been associated with antimetastatic activity in papillary thyroid (Ringel *et al.* 2002), esophageal (Ikeguchi *et al.* 2004), and bladder carcinoma (Sánchez-Carbayo *et al.* 2003).

The predicted KISS1 protein consists of 145 amino acids processed to a 54 amino acid peptide and subsequently amidated at the C-terminus. This peptide has recently been identified by three different groups as the endogenous ligand for an orphan G-protein receptor (GPR54; also known as AXOR-12; Kotani *et al.* 2001, Muir *et al.* 2001, Ohtaki *et al.* 2001) and was termed metastatin (Ohtaki *et al.* 2001) or kisspeptin (Kotani *et al.* 2001, Muir *et al.* 2001). Endogenous forms composed of 14 and 13 amino acids have also been identified (Kotani *et al.* 2001). Kisspeptin-10, the common C-terminal decapeptide shared by these forms, is the minimum sequence necessary for GPR54 receptor activation

(Kotani *et al.* 2001, Muir *et al.* 2001) and is secreted by cultured human trophoblasts (Bilban *et al.* 2004).

GPR54 is an orphan G protein-coupled receptor structurally related to galanin receptors, originally cloned from rat brain (Lee *et al.* 1999). Tissue distribution of GPR54 and its cognate ligand precursor, KISS1, often coincides. The levels of the transcripts of both are the highest in the placenta (Lee *et al.* 1996, Kotani *et al.* 2001, Ohtaki *et al.* 2001). Moreover, both KISS1 and GPR54 are widespread throughout the CNS (higher levels are reported in hypothalamus and pituitary; Lee *et al.* 1996). The hypothalamic KISS1/kisspeptin/GPR54 system has been shown to play a role as a major gatekeeper of gonadotropin-releasing hormone (GnRH) neurons and the reproductive axis (Messenger 2005, Castellano *et al.* 2006, Fernández-Fernández *et al.* 2006, Tena-Sempere 2006).

KISS1 is also expressed in peripheral tissue, testis, pancreas, liver, and small intestine, and kisspeptin receptor is highly expressed in pancreas, with lower expression in spleen, testis, peripheral blood lymphocytes, and adipose tissue (Kotani *et al.* 2001, Ohtaki *et al.* 2001, Harms *et al.* 2003).

Hauge-Evans *et al.* (2006) have recently demonstrated the presence of kisspeptin and GPR54 mRNAs in both pancreatic B and A cells and GPR54 expression in MIN6

and α TC1 pancreatic cell lines. Kisspeptin-54 has been shown to stimulate the late phase of glucose-induced insulin secretion in mouse and human islets and to inhibit insulin secretion from MIN6 cells.

To gain further insight into the effect of kisspeptin on islet cell secretion, we have investigated the effect of kisspeptin-13 on insulin, glucagon, and somatostatin secretion. The study was performed in the isolated perfused rat pancreas.

Animals, materials, and methods

Animals

Male Wistar rats (200–225 g body weight) from our inbred colony were used as donors. Animals were maintained in accordance with the guidelines established by the European Union (86/609). All experiments were performed between 0900 and 1200 h. This is a period of minimal baseline feeding and activity for rodents. Rats had free access to food and water until they were used in the experiments. Feeding activity in rats shows a diurnal rhythmicity. Under *ad libitum* conditions, rats consume most of their food during the dark phase, in which feeding activity is bimodal, with peaks at the beginning (dusk feeding peak) and toward the end (dawn feeding peak) of the night (Meguid *et al.* 1992, Varma *et al.* 1999).

Experimental protocol

Each rat was anesthetized with sodium pentobarbital (50 mg/kg, i.p.), after which the pancreas was dissected and perfused *in situ*, as previously described (Silvestre *et al.* 1986). Effluent samples were collected from the portal vein, without recycling, at 1-min intervals (flow rate, 2 ml/min), and frozen at -20°C until the time of assay. The perfusion medium consisted of a Krebs–Henseleit buffer: 115 mM NaCl, 4.7 mM KCl, 2.6 mM CaCl_2 , 1.19 mM H_2HPO_4 , 1.19 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 24.9 mM HNaCO_3 (gas phase 95:5, $\text{O}_2:\text{CO}_2$; pH 7.4), supplemented with 4% (w/v) dextran T-70 (Pharmacia LKB Biotechnology, Uppsala, Sweden), 0.5% (w/v) Cohn fraction V bovine albumin (Sigma–Aldrich), and glucose (5.5 mM; Sigma–Aldrich). Kisspeptin-13 was purchased from Bachem AC (Switzerland) and tested at graded concentrations (1 μM , 100 nM, 10 nM, 1 nM, and 0.1 nM).

All substances studied were dissolved in 0.9% NaCl containing 0.1% BSA (Cohn Fraction V, Sigma–Aldrich). These solutions were prepared daily, immediately before experiments.

After a 35-min equilibration period, baseline samples were collected for 5 min and, at zero time, kisspeptin-13 was infused through a sidearm cannula. Stimulation of hormone secretion was induced by increasing the perfusate glucose concentration from 5.5 to 9 mM and by infusing 10 mM arginine, 10 μM carbachol, or 1 nM exendin-4. The sequence of addition of compounds to the perfusate is indicated in the corresponding figures. In control experiments, an identical volume of buffer was infused.

Pertussis toxin treatment

Treatment with pertussis toxin (PTX) from *Bordetella pertussis* (Sigma–Aldrich) was performed by injecting a single dose (3 $\mu\text{g}/100\text{ g b.w.}$, i.p.) 3 days before perfusions. This PTX treatment has been found to effectively ADP-ribosylate and inactivate G_i -protein coupled to adenylate cyclase (Ui *et al.* 1984, Komatsu *et al.* 1994, Silvestre *et al.* 1994, Egido *et al.* 2007). Control rats were subjected to a sham treatment consisting of injection of the corresponding buffer (50% glycerol, 0.5 M NaCl, 0.05 M Tris–glycine, pH 7.5).

Hormone determination

Insulin, glucagon, and somatostatin were analyzed by RIA using the charcoal separation method (Herbert *et al.* 1965, Faloona & Unger 1974, Harris *et al.* 1978). Experiments were randomized and all samples for each series of experiments were analyzed within the same assay.

Insulin

Anti-pig insulin serum (I8510, Sigma–Aldrich) and rat insulin standards (Novo Nordisk, Bagsvaerd, Copenhagen, Denmark) were employed. The inter- and intra-assay variations were 5.3 and 4% respectively.

Glucagon

Anti-glucagon serum (30 K) was kindly donated by R H Unger (University of Texas, Health Sciences Center, Dallas, TX, USA). The inter- and intra-assay variations were 6.4 and 4.5% respectively.

Somatostatin

Anti-somatostatin serum (80C) was also donated by R H Unger (University of Texas, Health Sciences Center). The inter- and intra-assay variations were 5.4 and 4.6% respectively.

Statistical analysis

Results are expressed as the mean $\pm$ S.E.M. Insulin response was calculated as the integrated area of the curve above the mean preinfusion level (average of all the baseline levels for each perfusion) using the trapezoidal method. The normal distribution of our data was demonstrated by the Kolmogorov–Smirnov test (Siegel 1978). Differences between values were tested for significance by repeated-measures ANOVA in each group and by the unpaired Student's *t*-test.

Results

Figure 1 shows the effect of 1 μM kisspeptin-13 on basal and on arginine-induced insulin (Fig. 1A), glucagon (Fig. 1B),

Figure 1 Effect of 1 μ M kisspeptin-13 on the (A) insulin, (B) glucagon, and (C) somatostatin responses to 10 mM arginine at 5.5 mM glucose. Solid line ($n=6$): control, arginine alone. Broken line: arginine + kisspeptin-13 ($n=7$). Means $\pm$ S.E.M.

and somatostatin (Fig. 1C) secretion. Infusion of 1 μ M kisspeptin-13 did not significantly alter basal insulin, glucagon, or somatostatin secretion at a constant glucose concentration (5.5 mM). At 1 μ M, kisspeptin-13 blocked both phases of the arginine-induced insulin response (incremental areas: first phase: 14 ± 7 ng/5 min versus 144 ± 80 ng/5 min in controls; $P < 0.01$; second phase: 9.4 ± 11 ng/15 min versus 298 ± 58 ng/15 min in controls; $P < 0.01$) and did not significantly alter the glucagon or somatostatin responses elicited by this amino acid (incremental areas: 14 ± 4 ng/20 min versus 15.7 ± 1.3 ng/20 min in control perfusions, $P = 0.757$; and 397 ± 119 pg/20 min versus 479 ± 106 pg/20 min in control perfusions, $P = 0.71$ respectively). Furthermore, ANOVA has demonstrated that basal insulin secretion was not affected by a prolonged (20 min) infusion of 1 μ M kisspeptin-13 ($F = 1.53$; NS; data not shown).

Figure 2 shows the effect of graded concentrations of kisspeptin-13 on glucose-stimulated insulin secretion. As shown in Fig. 2A, infusion of kisspeptin-13 at 1 μ M did not significantly alter basal insulin secretion at a constant glucose concentration (5.5 mM), but suppressed both phases of the insulin response induced by increasing the perfusate glucose concentration from 5.5 to 9 mM (incremental areas: first phase: -1.7 ± 1.7 ng/5 min versus 41.6 ± 5.1 ng/5 min in controls; $P < 0.01$; second phase: 0.1 ± 5.8 ng/15 min versus 103 ± 27 ng/15 min in controls; $P < 0.01$). The insulin response to glucose observed in control perfusions (incremental area: 145 ± 32 ng/20 min) was significantly inhibited by kisspeptin-13 at 100 nM (incremental areas: 4.2 ± 8 ng/20 min; $P < 0.01$; Fig. 2B) and at 10 nM (incremental areas: 38 ± 16 ng/20 min; $P < 0.025$; Fig. 2C). At 1 nM, kisspeptin-13 reduced glucose-induced insulin secretion (incremental area: 77 ± 28 ng/20 min), although the difference was not statistically significant (Fig. 2D). At 0.1 nM, kisspeptin-13 did not significantly affect glucose-induced insulin secretion (incremental areas: 151 ± 63 ng/20 min versus 145 ± 33 ng/20 min in controls; $P = 0.918$; Fig. 2E). The dose-response curve was fitted to a sigmoidal curve ($R^2 = 0.99$) with an IC_{50} of 1.2 nM (Fig. 3).

Figure 4 shows that infusion of kisspeptin-13 at 10 nM inhibited the insulin response to 10 μ M carbachol (incremental areas: 124 ± 24 ng/20 min versus 465 ± 85 ng/20 min; $P < 0.01$).

Finally, we examined the effect of kisspeptin-13 on the insulin response to exendin-4 both in PTX-treated and untreated rats (Fig. 5). As shown in Fig. 5A, in pancreases isolated from untreated rats, infusion of 10 nM kisspeptin-13 blocked the insulin response to exendin-4 (incremental areas: 19 ± 6.7 ng/15 min versus 80 ± 19 ng/15 min in controls; $P < 0.05$). The inhibitory effect of kisspeptin-13 on the insulin response to exendin-4 was also observed in pancreases from PTX-treated rats (incremental areas: 15 ± 8.7 ng/15 min versus 88 ± 13 ng/15 min in controls; $P < 0.05$; Fig. 5B).

Figure 2 Effect of graded doses of kisspeptin-13 on the insulin response to glucose. Solid line ($n=9$): control, glucose alone. Broken line: glucose+kisspeptin-13. (A) 1 μ M kisspeptin-13 ($n=6$). (B) 100 nM kisspeptin-13 ($n=5$). (C) 10 nM kisspeptin-13 ($n=7$). (D) 1 nM kisspeptin-13 ($n=4$). (E) 0.1 nM kisspeptin-13 ($n=4$). Means $\pm$ S.E.M.

Discussion

The foregoing results demonstrate that, in pancreases isolated from normal rats, infusion of kisspeptin-13 dose dependently inhibits glucose-induced insulin secretion. This inhibitory effect of kisspeptin-13 was observed when insulin secretion was stimulated by an increase in the perfusate glucose concentration (from 5.5 to 9 mM), resembling that occurring in normal subjects upon consuming a

carbohydrate-rich meal; however, no effect of kisspeptin-13 on basal insulin secretion was observed (with constant infusion of 5.5 mM glucose).

Hauge-Evans *et al.* (2006) have recently shown that 1 μ M kisspeptin induced a slight, but significant, inhibitory effect on insulin secretion from MIN6 cells, while it potentiated the late phase of glucose-induced insulin secretion from mouse and human perfused islets. The discrepancy between our findings and those of the above-mentioned authors may be due to the

Figure 3 Dose-response curve corresponding to the inhibitory effect of kisspeptin-13 on the glucose-induced insulin response in the perfused rat pancreas. Hormone responses were calculated as the incremental areas under the curve. Means $\pm$ S.E.M.

different experimental protocols employed in these investigations. The isolated rat pancreas may be more sensitive to several secretagogues and inhibitors of insulin secretion than islets obtained by collagenase digestion of the pancreas. On the other hand, in the present study, we have employed kisspeptin-13, the C-terminal fragment of kisspeptin, a fact that might also explain the discrepancies observed with regard to the reported effect of kisspeptin on glucose-induced insulin secretion. Concerning this point, it should be pointed out that, in cells

Figure 4 Effect of 10 nM kisspeptin-13 on the insulin response to 10 μ M carbachol at 5.5 mM glucose. Solid line ($n=4$): control, carbachol alone. Broken line ($n=5$): carbachol + kisspeptin-13. Means $\pm$ S.E.M.

Figure 5 Effect of 10 nM kisspeptin-13 on the insulin response to 1 nM exendin-4 at 5.5 mM glucose. (A) Control rats. Solid line ($n=7$): control, exendin-4 alone. Broken line ($n=5$): exendin-4 + kisspeptin-13. (B) PTX-treated rats. Solid line ($n=11$): control, exendin-4 alone. Broken line ($n=8$): exendin-4 + kisspeptin-13. Means $\pm$ S.E.M.

overexpressing GPR54, all kisspeptin fragments have been found to have a similar affinity and efficacy for this receptor, thus indicating that the C-terminal portion of the kisspeptin molecule is responsible for the activation of GPR54 (Kotani *et al.* 2001). However, other studies have found that kisspeptin-54 shows lower agonistic potency toward AXOR-12/GPR54 than kisspeptin-13 (Muir *et al.* 2001).

In our experimental model, the insulinostatic effect of kisspeptin-13 on glucose-induced insulin secretion is dose dependent, and the IC_{50} (1.2 nM) is comparable with the EC_{50} value (1.38 nM) reported for binding of kisspeptin-13

to rat GPR54 (Kotani *et al.* 2001). The inhibition of insulin secretion by kisspeptin-13 in the nanomolar range resembles the effect induced by other insulinostatic peptides – galanin (Sharp *et al.* 1989, Drews *et al.* 1994), somatostatin (Schuit *et al.* 1989), and 26RFa (Egido *et al.* 2007).

Kisspeptin-13 has been detected in peripheral plasma (Horikoshi *et al.* 2003, Panidis *et al.* 2006), where its levels correlate negatively with fasting plasma insulin levels (Panidis *et al.* 2006). Plasma kisspeptin concentrations are in the picomolar range, much lower than the concentrations employed in this study. However, kisspeptin has also been found in the pancreas (Kotani *et al.* 2001, Harms *et al.* 2003, Masui *et al.* 2004), and it can be assumed that in addition to the kisspeptin reaching the B cell through the systemic circulation, the local production of this peptide would augment its intra-islet concentration. Interestingly, plasma kisspeptin levels are dramatically elevated during pregnancy (up to 7000-fold higher than the normal value). This increase is in accord with the progression of pregnancy and seems to be related to the placental production of this family of peptides (Horikoshi *et al.* 2003).

The precise mechanism of action by which kisspeptin-13 could reduce B-cell secretory activity is not known. We have found that, besides reducing glucose-induced insulin secretion, kisspeptin-13 inhibits the insulin response to secretagogues that act on the B cell via different mechanisms, i.e. arginine, which depolarizes the B cell (Flatt 2003), thus activating voltage-dependent Ca^{2+} channels; carbachol, which increases phospholipid turnover by activating phospholipase C (PLC; Zawulich 1996, Flatt 2003); and exendin-4, which activates the adenylate cyclase/cAMP system (Göke *et al.* 1993, Flatt 2003). These findings suggest that kisspeptin-13 might influence B cells by affecting a common distal step in the insulin secretory mechanism.

Kisspeptins have been shown to be the endogenous ligands for a heptahelical GPR54; Kotani *et al.* 2001, Muir *et al.* 2001, Ohtaki *et al.* 2001), which is found in the pancreas (Kotani *et al.* 2001, Masui *et al.* 2004). GPR54 mRNA expression has been detected in the MIN6 mouse insulinoma cell line and in the αTC1 mouse glucagon-secreting cell line (Hauge-Evans *et al.* 2006). Immunoreactive GPR54 was also found in human and mouse pancreatic islets with little or no immunoreactivity in the surrounding exocrine pancreas (Hauge-Evans *et al.* 2006). Thus, kisspeptin-13 might block insulin release by interacting with GPR54 present in pancreatic B cells. GPR54 couples primarily with $\text{G}_{\alpha_{q/11}}$ proteins, leading to PLC activation, phosphatidylinositol (PI) turnover, and increase in intracellular calcium and protein kinase C activity (Kotani *et al.* 2001, Muir *et al.* 2001, Ohtaki *et al.* 2001, Stafford *et al.* 2002, Becker *et al.* 2005). Activation of this pathway in the B cell is associated with an increase in insulin secretion, as has been reported for activation of muscarinic receptors by carbachol (Zawulich 1996, Gilon & Henquin 2001, Flatt 2003), and thus it cannot explain the insulinostatic effect of kisspeptin-13 found in the present study.

We have observed that the inhibitory effect of kisspeptin-13 on the insulin response to exendin-4 is maintained in PTX-treated rats, thus indicating that the insulinostatic effect of kisspeptin-13 is independent of PTX-sensitive G proteins. It should be mentioned that, in our perfused pancreas system, the same batch of PTX has been shown to be effective in preventing the inhibition of exendin-4-induced insulin secretion elicited by other insulinostatic peptides, such as 26RFa (Egido *et al.* 2007) or ghrelin (unpublished data).

We have found that kisspeptin-13, at a concentration that totally blocked insulin release (1 μM), failed to significantly modify either basal or arginine-stimulated glucagon output. In agreement with this, Hauge-Evans *et al.* (2006) have shown that, at the same concentration, kisspeptin failed to significantly modify glucagon secretion in the presence of either 2 or 20 mM glucose. The present study provides the first data on the effect of kisspeptin-13 on somatostatin release. The lack of effect of kisspeptin-13 on glucagon and somatostatin secretion would rule out the possibility that the insulinostatic effect of kisspeptin-13 be due to a paracrine mechanism mediated by A- or D-cell secretion.

Kisspeptins belong to a peptide family with a common Arg-Phe-NH₂ motif at their C-terminal end, and collectively termed RFamide-related peptides (RFPs; Kotani *et al.* 2001). Interestingly, other RFamide peptides have been shown to reduce insulin secretion at micromolar concentrations (Sorenson *et al.* 1984, Fehmann *et al.* 1990). Recently, using our perfused pancreas model, we have found that 26RFa, another RFamide peptide, behaves as a potent inhibitor of insulin secretion (Egido *et al.* 2007). The insulinostatic effect of 26RFa resembles that of kisspeptin-13. Both peptides dose dependently inhibited glucose-induced insulin secretion (with IC₅₀ at the nanomolar level) and reduced the insulin responses to arginine and exendin-4, thus suggesting that RFamide peptides may play a role in the control of B-cell secretion.

Conclusion

In view of the potent insulinostatic effect of kisspeptin-13, it is tempting to speculate that the kisspeptin family may be implicated in the regulation of B-cell secretion. The lack of effect of kisspeptin-13 on both glucagon and somatostatin secretion would indicate that it influences the B cell directly, rather than through an A- or D-cell paracrine effect.

As for the pathological relevance of our findings, it can be hypothesized that, by impairing insulin secretion, elevation of plasma kisspeptins could constitute a diabetogenic condition. Given the massive elevation of circulating kisspeptins during pregnancy, gestational diabetes might represent one of these conditions, a point worthy of investigation.

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