

Kisspeptin stimulation of insulin secretion: mechanisms of action in mouse islets and rats

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Abstract

Aims/hypothesis Kisspeptin is a novel peptide identified as an endogenous ligand of the G-protein-coupled receptor 54 (GPR-54), which plays a crucial role in puberty and reproductive function. High levels of GPR-54 and kisspeptin have been reported in the pancreas and we have previously shown that kisspeptin potentiates glucose-induced insulin release from isolated islets, although the mechanisms underlying this effect were unclear.

Methods Insulin secretion from isolated mouse islets was measured to characterise the effects of kisspeptin. The effects of kisspeptin on both p42/44 mitogen-activated protein kinase (MAPK) phosphorylation and intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in mouse islets were also investigated. Furthermore, kisspeptin was administered to rats in vivo and effects on plasma insulin levels measured.

Results In the current study, kisspeptin induced a concentration-dependent potentiation of glucose-induced (20 mmol/l) insulin secretion from mouse islets, with maximal effects at 1 $\mu\text{mol/l}$, but had no effect on insulin secretion at a substimulatory concentration of glucose (2 mmol/l). Activation of GPR-54 by kisspeptin also caused reversible increases in $[\text{Ca}^{2+}]_i$ in Fura-2 loaded dispersed islet cells.

The kisspeptin-induced potentiation of glucose-induced insulin secretion was completely abolished by inhibitors of phospholipase C and p42/44 MAPK, but not by inhibitors of protein kinase C or p38 MAPK. Intravenous administration of kisspeptin into conscious, unrestrained rats caused an increase in circulating insulin levels, whilst central administration of kisspeptin had no effect, indicating a peripheral site of action.

Conclusions/interpretation These observations suggest that neither typical protein kinase C isoforms nor p38 MAPK are involved in the potentiation of glucose-induced insulin release by kisspeptin, but intracellular signalling pathways involving phospholipase C, p42/44 MAPK and increased $[\text{Ca}^{2+}]_i$ are required for the stimulatory effects on insulin secretion. The observation that kisspeptin is also capable of stimulating insulin release in vivo supports the conclusion that kisspeptin is a regulator of beta cell function.

Keywords Beta cell · GPR-54 · Insulin · Islet · Kisspeptin

Abbreviations

$[\text{Ca}^{2+}]_i$	Intracellular Ca^{2+} concentration
DAG	Diacylglycerol
GPR-54	G-protein-coupled receptor 54
i.c.v.	Intracerebroventricular
MAPK	Mitogen-activated protein kinase
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate

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Introduction

Kisspeptins are a family of peptides encoded by the *KISS1* gene which have been identified as the endogenous ligands

for the G-protein-coupled receptor 54 (GPR-54). *KISS1* was originally identified in breast cancer and melanoma cell lines as a metastasis suppressor gene involved in the inhibition of tumour progression [1–3]. It has since been shown to play a pivotal role in the control of the hypothalamo–pituitary–gonadal axis, where it regulates the onset of puberty [4, 5]. Studies using fasted or obese animals have also suggested that hypothalamic kisspeptin may play an important role in mediating the inhibitory effects of nutritional status on reproductive function, implying a possible role as a whole-body energy sensor [6, 7]. However, the functions of kisspeptin and GPR-54 are not confined to hypothalamic neurones, since both are expressed in various peripheral tissues including, at particularly high levels, in the pancreas [3]. We have recently identified the site of pancreatic kisspeptin and GPR-54 content as being exclusively in the endocrine pancreas, consistent with a peripheral role in whole-body fuel homeostasis [8]. We have also demonstrated that in addition to its hypothalamic effects, kisspeptin stimulates the release of insulin from isolated mouse and human islets [8]. The established role of kisspeptin in mediating the effects of altered nutritional status on reproductive function, combined with the ability of kisspeptin to stimulate insulin release and the importance of pancreatic beta cells as regulators of fuel homeostasis, suggests a potential physiological role for kisspeptin in regulating islet function. The present study was undertaken to further characterise the stimulatory effect of kisspeptin on insulin release, and to determine whether the kisspeptin and GPR-54 system represents a novel regulator of beta cell function.

Methods

Materials Mouse kisspeptin-10 was supplied by Phoenix Pharmaceuticals (Karlsruhe, Germany). The protein kinase C (PKC) inhibitor Gö6976, the phospholipase C (PLC) inhibitor U73122, the p42/44 mitogen-activated protein kinase (MAPK) inhibitor PD98059 and the p38 MAPK inhibitor SB203580 were from Calbiochem (Nottingham, UK). All inhibitors were dissolved in DMSO such that the final DMSO concentration in incubation buffers was 0.1% (vol./vol.). The relevant controls received 0.1% DMSO (vol./vol.) alone. Fura-2/AM, collagenase, fetal calf serum, penicillin, glutamine and streptomycin were from Sigma (Poole, UK). RPMI was supplied by Invitrogen (Paisley, UK). The antibodies against total p42/44 MAPK (1:2,000) and against phosphorylated p42/44 MAPK (1:2,500) were obtained from Promega (Southampton, UK). Ketamine was supplied by Pharmacia & Upjohn (Crawley, UK) and rompun by Bayer (Leverkusen, Germany). Fluid swivels

and spring tethers for i.v. cannulation were from Instec Laboratories (Boulder, CO, USA). Blood glucose meters were from Boehringer Mannheim (Mannheim, Germany).

Islet isolation and maintenance Islets of Langerhans were isolated from male ICR mice (Harlan, Bicester, UK) by collagenase digestion of the exocrine pancreas and incubated at 37°C in RPMI (supplemented with 10% [vol./vol.] fetal calf serum, 2 mmol/l glutamine and 100 U/ml penicillin/0.1 mg/ml streptomycin) for at least 24 h before use.

Insulin secretion in vitro The effects of kisspeptin on insulin secretion from mouse islets were assessed using either a temperature-controlled perfusion apparatus, essentially as described previously [9], or in static incubations of islets. For perfusion experiments, groups of 40 islets were perfused with a bicarbonate-buffered physiological salt solution (0.5 ml/min, 37°C, [10]) containing 2 mmol/l glucose, 2 mmol/l CaCl_2 and 0.5 mg/ml BSA. Samples of perfusate were taken at 2 min intervals and stored at –20°C until assayed for insulin content.

For static incubations islets were pre-incubated for 2 h in RPMI containing 2 mmol/l glucose. Groups of three islets were transferred into 1.5 ml Eppendorf tubes and incubated at 37°C for 1 h in physiological salt solution as described above, supplemented with agents of interest. After 1 h, samples of the incubation medium were taken and stored at –20°C until assayed for insulin content.

The insulin content of perfusion and static incubation samples was determined using an in-house radioimmunoassay [11].

Calcium microfluorimetry Islets were dissociated in 0.02% (wt/vol.) EDTA, cells were seeded onto acid–ethanol-washed glass cover slips (100,000 cells/cover slip) and allowed to adhere overnight in RPMI under standard tissue culture conditions. Changes in intracellular calcium were measured by calcium microfluorimetry using Fura-2/AM, as described previously [12]. Cells were perfused with a salt solution supplemented with 2 mmol/l glucose and substances of interest. Fluorescence data were collected every 3 s and expressed as a 340:380 ratio.

Measurement of MAPK activation Groups of 200 islets were incubated (37°C, 5 min) in a physiological salt solution containing either 2 mmol/l or 20 mmol/l glucose in the presence or absence of 1 $\mu\text{mol/l}$ kisspeptin, then cooled and pelleted by centrifugation (10,000 g, 1 min). The supernatant fraction was discarded and protein extracts were prepared as described [13]. Proteins were separated by PAGE, transferred to membranes and immunoprobed for p42/44 MAPK and for phosphorylated (activated) MAPK, as described previously [13].

Insulin secretion in vivo Adult Wistar rats (B&K, Battlesbridge, UK), were housed under controlled conditions (14:10 h light:dark; lights on at 07:00; temperature $22\pm 2^\circ\text{C}$) and provided with food and water ad libitum. All animal procedures were undertaken in accordance with the UK Home Office Regulations. All surgical procedures were carried out with ketamine (100 mg/kg i.p.) and rompun (10 mg/kg i.p.) anaesthesia. Rats were chronically implanted with an intracerebroventricular (i.c.v.) guide cannula, positioned into the left lateral cerebral ventricle as described previously [14]. Following a 10 day recovery period rats were fitted with two indwelling cardiac catheters via the jugular veins [14]. The catheters were exteriorised at the back of the head and secured to a cranial attachment: the rats were fitted with a 30 cm long metal spring tether. The distal end of the tether was attached to a fluid swivel, which allowed the rat freedom to move around the enclosure. Experimentation commenced 3 days later.

Experiments commenced between 09.30 and 10.30 hours. Blood samples (0.25 ml) were drawn manually from one of the catheters to determine blood glucose concentrations, measured using a RefloLux S blood glucose monitor, and to obtain plasma samples that were stored for later insulin radioimmunoassay. Blood samples taken immediately before and after kisspeptin administration were assayed to determine the increase in circulating levels of kisspeptin using a commercially available human kisspeptin ELISA (Phoenix Pharmaceuticals). After 30 min, animals were injected with either 37.5 nmol kisspeptin i.v. or 3.75 nmol kisspeptin i.c.v., based on kisspeptin concentrations used in previous studies to stimulate plasma luteinising hormone release [15, 16]. Blood samples were taken at the following time points, -30, -5, +15, +30, +60, +120 and +240 min relative to the injection of kisspeptin.

Statistical analysis Secretion data are expressed as means \pm SEM. Statistical analyses were performed using ANOVA or Student's *t* tests as appropriate and $p < 0.05$ was considered significant.

Results

Effect of kisspeptin on insulin secretion from mouse islets in vitro Kisspeptin potentiated glucose-induced insulin secretion from isolated, perfused mouse islets in a reversible manner, as shown in Fig. 1a. Increasing the glucose concentration in the perfusate from a basal of 2 mmol/l glucose to a maximal stimulatory concentration of 20 mmol/l glucose initiated a biphasic insulin secretory response and the addition of 1 $\mu\text{mol/l}$ kisspeptin during the stable second phase of glucose-induced secretion induced an approximately three-

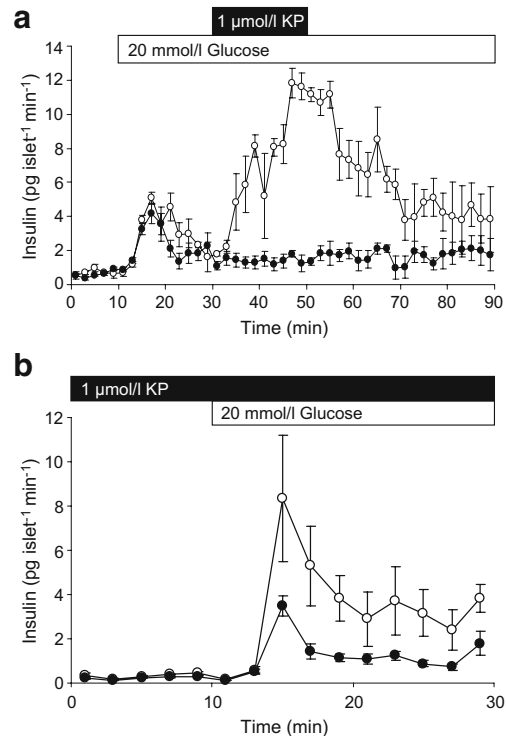


Fig. 1 Kisspeptin enhances glucose-induced insulin secretion from mouse islets. **a** Increasing the glucose concentration from 2 mmol/l (0–10 min) to 20 mmol/l (white bar) induced a biphasic stimulation of insulin secretion from mouse islets. Exposure to kisspeptin (1 $\mu\text{mol/l}$, 30–50 min, white circles) enhanced the second, plateau phase of insulin secretion over that induced by 20 mmol/l glucose alone (black circles). Following withdrawal of kisspeptin, insulin secretion returned to the previous glucose-stimulated plateau levels. Mean \pm SEM, $n=8$. **b** Exposure to kisspeptin (1 $\mu\text{mol/l}$, 0–30 min, white circle) had no effect on insulin secretion at basal glucose levels (2 mmol/l, 0–10 min), but enhanced both the first and second phases of glucose-induced insulin release compared with release from islets treated with glucose alone (black circle). Mean \pm SEM, $n=8$. KP, kisspeptin

fold increase in insulin release ($p < 0.01$ vs second-phase glucose-induced secretion). Following the removal of kisspeptin, insulin secretion returned to the second-phase, glucose-induced levels. Figure 1b shows similar experiments in which the perfused islets were exposed to kisspeptin (1 $\mu\text{mol/l}$) at the same time as 20 mmol/l glucose. The presence of kisspeptin significantly ($p < 0.01$) enhanced the first rapid phase of glucose-induced insulin secretion in addition to inducing a maintained elevation in the second, plateau phase of the secretory response.

The effects of kisspeptin on glucose-induced insulin secretion from perfused mouse islets (Fig. 1) were consistent with the results of experiments using static incubations of mouse islets, as shown in Fig. 2. Thus, kisspeptin (100 pmol/l–1 $\mu\text{mol/l}$) had no effect on insulin secretion in the presence of a substimulatory concentration of glucose (2 mmol/l), but potentiated 20 mmol/l glucose-induced

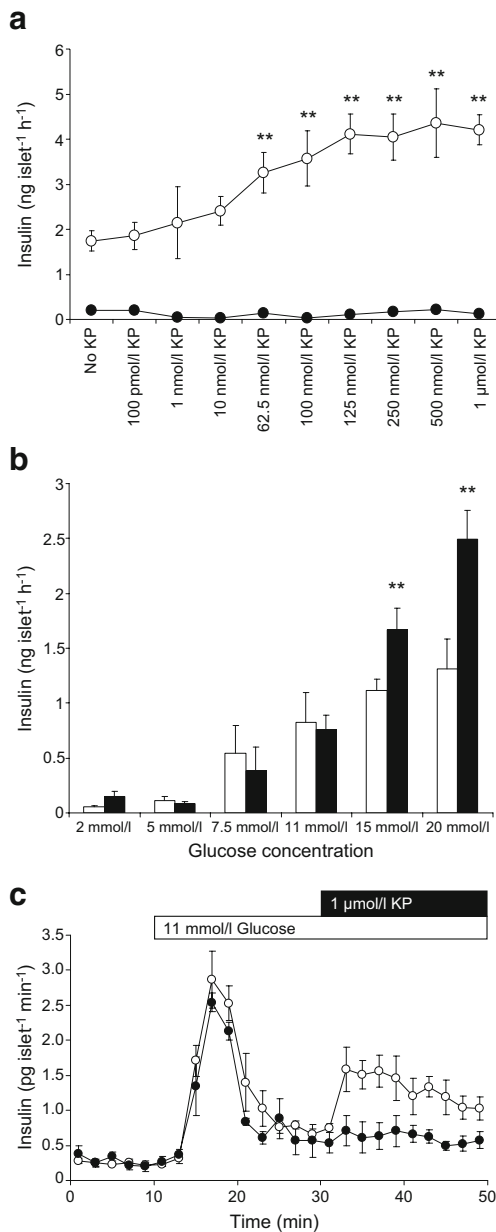


Fig. 2 In vitro concentration and glucose dependency of the effects of kisspeptin on insulin secretion. **a** In the presence of 20 mmol/l glucose (white circles), insulin release was potentiated in a kisspeptin-concentration-dependent manner. Kisspeptin concentrations of 62.5 nmol/l or higher were required for a significant effect on insulin secretion. At 2 mmol/l glucose (black circles), kisspeptin had no effect at any concentration tested. Mean±SEM, $n=9$, $**p<0.01$ vs 20 mmol/l glucose alone. **b** In static incubation experiments, kisspeptin significantly potentiated insulin release at glucose levels of 15 mmol/l and higher in vitro. Results from systems with 100 nmol/l kisspeptin are shown in black, and those from systems without kisspeptin are shown in white. Mean±SEM, $n=9$, $**p<0.01$ vs absence of kisspeptin. **c** In perfusion experiments, exposure to kisspeptin (1 μmol/l, 30–50 min) enhanced the second, plateau phase of insulin secretion compared with that induced by 11 mmol/l glucose alone. Results for islets exposed to kisspeptin are shown as white circles; results for control islets, exposed to glucose only, are shown as black circles. Mean±SEM, $n=8$. KP, kisspeptin

insulin secretion in a concentration-dependent manner, significantly stimulating insulin release at concentrations of 62.5 nmol/l and above (Fig. 2a). The stimulatory effects of kisspeptin were dependent on the presence of a stimulatory concentration of glucose, as shown in Fig. 2b and c. In static incubation experiments (Fig. 2b) kisspeptin (100 nmol/l) had no significant effects on insulin secretion at glucose concentrations lower than 15 mmol/l (Fig. 2b) but perfusion experiments, which allow a more sensitive assessment of changes in the rate of insulin secretion, revealed that kisspeptin (1 μmol/l) enhanced insulin secretion at a glucose concentration of 11 mmol/l which is within the physiological postprandial range for a mouse (Fig. 2c).

Intracellular mechanisms involved in kisspeptin-induced insulin secretion The intracellular signalling pathways involved in the kisspeptin-induced potentiation of insulin release were investigated using pharmacological inhibitors of effector systems known to be important in beta cells. Figure 3a shows that the kisspeptin-induced potentiation of glucose-stimulated insulin release was inhibited by both the PLC inhibitor, U73122 (10 μmol/l), and the p42/44 MAPK inhibitor, PD98059 (50 μmol/l). In contrast, inhibitors of p38 MAPK, SB203580 (50 μmol/l), and of diacylglycerol (DAG)-dependent PKC isoforms, Gö6976 (1 μmol/l), had no effect on kisspeptin-induced insulin secretion. The possible role of DAG-dependent PKC isoforms in mediating the effects of kisspeptin was further investigated using islets in which PKC activity was downregulated by 24 h treatment with 500 nmol/l 4β-Phorbol 12-myristate 13-acetate (PMA) [17, 18], shown in Fig. 3b. PKC downregulation was demonstrated by the loss of subsequent PMA-induced insulin secretory responses in islets pre-treated with 4β-PMA, compared with control islets treated with the inactive phorbol ester, 4α-PMA. In contrast, the 4β-PMA-treated islets showed identical secretory responses to control islets treated with the inactive 4α-PMA when challenged with 20 mmol/l glucose alone or with 1 μmol/l kisspeptin in the presence of 20 mmol/l glucose ($108\pm5\%$ and $112\pm11\%$ vs 4α-PMA treated islet responses, respectively, $n=9$, $p>0.2$).

Figure 3c,d shows that 20 mmol/l glucose induced a rapid (5 min) increase in phosphorylated p42/44 MAPK in islets when compared with those maintained in 2 mmol/l glucose. However, kisspeptin (1 μmol/l) had no detectable effect on p42/44 MAPK activation at either glucose concentration. In dispersed mouse islet cells, 1 μmol/l kisspeptin caused an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) when administered either at 2 mmol/l or 20 mmol/l glucose, although a greater proportion of cells responded at 20 mmol/l glucose (Fig. 4; 12/67 cells [18%] at 2 mmol/l glucose; 40/67 cells [60%] at 20 mmol/l glucose; data from seven separate experiments). Only cells that exhibited an increase in $[Ca^{2+}]_i$ in response to both 20 mmol/l glucose

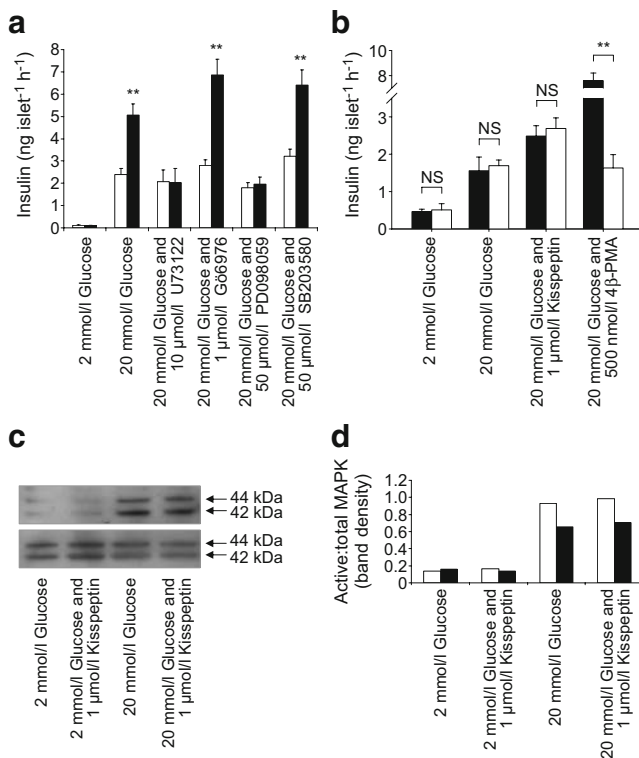


Fig. 3 Intracellular signalling pathways mediating the effects of kisspeptin in islets. **a** The PLC inhibitor, U73122, and the p42/44 MAPK inhibitor, PD098059, both completely blocked the potentiation of glucose-stimulated insulin release caused by kisspeptin in mouse islets. The PKC inhibitor, Gö6976, and the p38 inhibitor, SB203580, had no inhibitory effect on the kisspeptin potentiation of insulin release. Results from systems with 100 nmol/l kisspeptin are shown in black, and those from systems without kisspeptin are shown in white. Mean±SEM, $n=9$, $*p<0.01$ vs 20 mmol/l glucose without kisspeptin. **b** PKC downregulation by 24 h incubation in the presence of 500 nmol/l 4β-PMA (white bars) had no effect on insulin release from isolated islets stimulated by 20 mmol/l glucose or 1 µmol/l kisspeptin compared with release from islets incubated for 24 h with inactive 4α-PMA (black bars). However, 24 h incubation in 4β-PMA completely blocked the stimulation of insulin release caused by acute exposure to 500 nmol/l 4β-PMA. Mean±SEM, $n=9$, $**p<0.01$ vs 4α-PMA controls. **c** Phosphorylated p42/44 MAPK immunoreactivities (upper panel) and total p42/44 MAPK immunoreactivities (lower panel) were detected in extracts of mouse islets incubated for 5 min at 37°C. Arrows show molecular mass calculated from the gel migration positions of proteins of known molecular mass. The figure shows one experiment that was reproduced three times using different samples. **d** Band densities were quantified for both p42 (white bars) and p44 (black bars) MAPK and expressed as a ratio of active:total MAPK

and 100 µmol/l tolbutamide, which were most likely to be beta cells, were included in the analysis.

Effect of peripheral and central kisspeptin on insulin secretion in vivo When administered via an i.v. injection, 37.5 nmol kisspeptin produced an increase of 11.8 ng/ml (9 nmol/l) in circulating plasma kisspeptin levels and caused an approximately fourfold increase in plasma insulin lasting for longer than 90 min in conscious adult rats

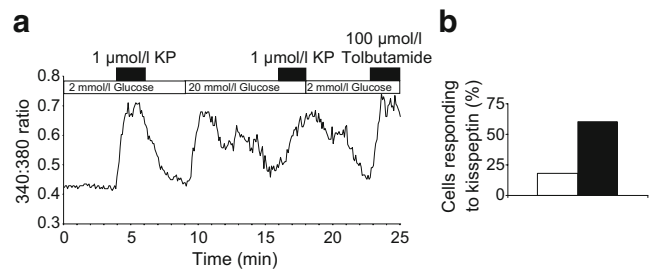


Fig. 4 Kisspeptin effects on intracellular calcium in islet cells. **a** Administration of 1 µmol kisspeptin increases $[Ca^{2+}]_i$ in dispersed mouse islet cells that respond to both 20 mmol/l glucose and 100 µmol/l tolbutamide. The trace is representative of seven separate experiments. **b** Percentage of cells that exhibited an increase in $[Ca^{2+}]_i$ in response to 1 µmol/l kisspeptin at either 2 mmol/l glucose (white bar) or 20 mmol/l glucose (black bar) KP, kisspeptin

without any significant change in blood glucose levels (Fig. 5a). In contrast, kisspeptin had no significant effect on plasma insulin when administered centrally via an i.c.v. cannula (Fig. 5b).

Discussion

The role of hypothalamic kisspeptin in the reproductive axis is well established, but the effects of kisspeptin in peripheral tissues remain relatively unknown. The results presented here confirm our previous preliminary report that

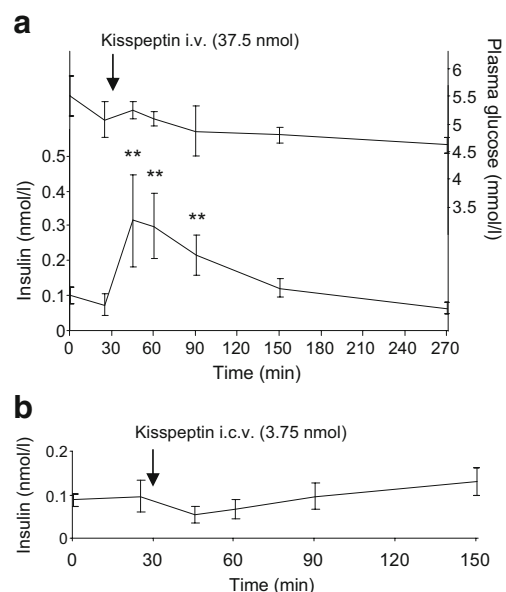


Fig. 5 In vivo effects of kisspeptin on insulin secretion. **a** Intravenous administration of kisspeptin caused a rapid increase in plasma insulin levels lasting over 90 min, but caused no significant change in plasma glucose. **b** Intracerebroventricular administration of kisspeptin had no effect on plasma insulin. Mean±SEM, $n=5-7$, $**p<0.01$ vs pre-treatment control values

exogenous kisspeptin potentiated glucose-induced insulin secretion from isolated mouse islets [8], and further characterise this action. In the present study, kisspeptin enhanced both the first and second phases of the glucose-induced secretory response, but did not initiate a secretory response in the absence of a stimulatory concentration of glucose, consistent with the mode of action of many receptor-operated agonists that modulate the amplitude of nutrient-induced insulin secretory responses. The stimulatory effects of kisspeptin on insulin secretion were fully reversible, again consistent with a receptor-mediated action, and the islets were subsequently able to maintain glucose-induced insulin secretion suggesting that kisspeptin had no deleterious effects on the beta cells.

Little information is yet available about the biologically active concentrations of kisspeptin, and there are at least two possible sources of kisspeptin that may activate the beta cell GPR-54. Circulating levels of kisspeptin in humans are normally very low but increase markedly during pregnancy, and this circulating kisspeptin is assumed to be of placental origin [19]. Peak kisspeptin concentrations of approximately 2.5 nmol/l are detected during the third trimester in humans [19], but this is still somewhat lower than the concentrations of approximately 50 nmol/l that were required to stimulate insulin secretion from mouse islets in our *in vitro* experiments. To date there is no information about placental production or circulating levels of kisspeptin in mice, so it is possible that plasma kisspeptin levels during pregnancy reach higher levels in mice with their multiple placentas. Alternatively, it may be that islet GPR-54 is expressed to respond to locally released kisspeptin, as occurs in the hypothalamus, and our demonstration of the co-expression of kisspeptin and GPR-54 in islets is consistent with an intra-islet paracrine role for kisspeptin [8]. The concentration of a para/autocrine agent that will be achieved at its local sites of release and action is difficult to assess, but it is likely to be considerably higher than the concentration of the same agent in the peripheral circulation. This may explain why the potentiation of insulin release from isolated mouse islets required kisspeptin concentrations of around 50 nmol/l, which is considerably higher than the peripheral circulating concentrations of most peptide hormones.

Although the source of the kisspeptin that activates beta cell GPR-54 is uncertain, our results clearly demonstrate directly stimulatory effects of GPR-54 activation on nutrient-induced insulin secretion from isolated islets. The intracellular mechanisms involved in the effects of kisspeptin on beta cells are currently unknown, although a number of studies have investigated kisspeptin signalling in the hypothalamus [20, 21] and in tumour cell lines [1, 22]. Our experiments showed that kisspeptin had rapid stimulatory effects on beta cell cytosolic Ca^{2+} , and that the effects

of kisspeptin on insulin secretion were blocked by a PLC inhibitor, suggesting that kisspeptin potentiates glucose-induced insulin release from beta cells through GPR-54-linked PLC activation and increases in intracellular Ca^{2+} , most likely through the generation of inositol triphosphate and subsequent liberation of Ca^{2+} from the endoplasmic reticulum. This signalling pathway has previously been shown to play an important role in mediating the effects of kisspeptin on gonadotrophin-releasing hormone release in the hypothalamus [20], suggesting some overlap in the intracellular pathways involved in the effects of kisspeptin in different tissues. Increases in intracellular Ca^{2+} were not observed in all beta cells in response to kisspeptin administration, suggesting functional heterogeneity among islet beta cells at the level of GPR-54 production or its intracellular coupling. This is not too surprising since it is well known that individual beta cells are heterogeneous and differ in their sensory [23], biosynthetic [24, 25], intracellular Ca^{2+} [26, 27] and secretory [28] responses. PLC activation is also associated with the generation of DAG and the subsequent activation of DAG-sensitive isoforms of protein kinase C, an important mechanism in the stimulation of insulin release by some receptor-mediated stimuli [22], including acetylcholine [17, 18] and cholecystokinin [29]. However, our results do not support a role for this pathway in mediating the effects of kisspeptin on beta cells. An inhibitor of the DAG-sensitive PKC isoforms (Gö6976) had no effect on kisspeptin-dependent potentiation of glucose-induced insulin release from isolated islets, and downregulating islet content of DAG-sensitive PKC isoforms by prolonged exposure to 4 β -PMA [17, 18, 30] did not reduce the effects of kisspeptin on insulin secretion.

The p38 and p42/44 MAPK transduction cascades have been implicated in the effects of kisspeptin in hypothalamic neurones and in the CHO-K1 tumour cell line [1, 20], and the p42/44 MAPK pathway is important in mediating the effects of the calcium-sensing receptor on insulin release from beta cells [13]. Whilst the p38 MAPK pathway is involved in mediating the effects of kisspeptin on secretion of gonadotrophin-releasing hormone in the hypothalamus [20], the p38 inhibitor, SB203580, had no effect on kisspeptin-induced insulin secretion in our experiments, suggesting that the intracellular systems through which kisspeptin acts on cells may be tissue specific. However, the results of the current study demonstrated that a p42/44 MAPK inhibitor, PD98059, completely blocked the effects of kisspeptin on insulin release, suggesting an involvement of this pathway in beta cells. The effects of p42/44 MAPK activation appear to be permissive rather than causal for kisspeptin-induced insulin secretion. Thus, the presence of a stimulatory concentration of glucose enhanced p42/44 MAPK activation, as has been reported previously [31, 32], and pharmacological inhibition of this activation blocked

kisspeptin-induced insulin secretion *in vitro*. In contrast, kisspeptin had no effect on p42/44 MAPK phosphorylation irrespective of the ambient glucose concentration, suggesting that glucose-induced p42/44 MAPK activation is required for the potentiating effects of kisspeptin on insulin secretion, although p42/44 activation alone is insufficient to initiate a secretory response [31, 32]. These observations are consistent with a model in which nutrient-induced activation of p42/44 MAPK is not directly involved in the initiation of nutrient-induced insulin secretion, but enables the potentiation of nutrient-induced secretory responses by some receptor-operated stimuli, such as kisspeptin.

In contrast with the observed effects of kisspeptin to potentiate glucose-induced insulin secretion from isolated islets *in vitro*, a recent report suggests that kisspeptin inhibits the release of insulin from the perfused pancreas [33], raising the possibility that the effects of kisspeptin on islet function may be influenced by the *in vivo* environment. To determine whether the results obtained from our *in vitro* experiments in the present study are representative of the effects of kisspeptin on pancreatic islets *in vivo*, doses of kisspeptin previously shown to stimulate luteinising hormone release [15, 16] were administered to conscious, unrestrained rats. The results clearly demonstrated that exogenous kisspeptin stimulated insulin release *in vivo* in conscious rats, confirming the stimulatory effects on isolated islets *in vitro*. The immediate and sustained increase in plasma insulin levels induced by kisspeptin was not accompanied by detectable changes in blood glucose, presumably reflecting intact autoregulatory mechanisms in this model using non-anaesthetised, unrestrained animals. The unchanged levels of blood glucose also point to a direct effect of kisspeptin on pancreatic beta cells to stimulate insulin release, rather than a secondary effect through increasing blood glucose levels. Additionally, central administration of kisspeptin had no effect on plasma insulin, suggesting that the stimulation of insulin release by kisspeptin is mediated through a peripheral, as opposed to central, site of action. In both *in vivo* and *in vitro* experiments the effects of kisspeptin on insulin secretion were fully reversible, consistent with an effect mediated through the activation of a G-protein-coupled cell-surface receptor, such as GPR-54. The relatively prolonged effects of kisspeptin on insulin secretion *in vivo* and *in vitro* is in accordance with previous studies demonstrating sustained effects of kisspeptin in the hypothalamus for over 1 h after administration [15, 34]. Measurements using a commercially available assay suggested that the intravenous administration schedule used in our *in vivo* experiments produced increases in plasma kisspeptin (9 nmol/l) of the same order of magnitude as the physiological increases (2.5 nmol/l) reported in pregnant women [19], suggesting that there are circumstances where circulating kisspeptin

could influence islet function. However, some caution is required in interpreting these data, since plasma kisspeptin can be difficult to measure accurately [35]. In the current study we used a human kisspeptin assay because there are no available assays for rat kisspeptin and the inter-species specificity of the assay is unknown. Irrespective of the absolute concentrations of kisspeptin achieved in our *in vivo* experiments, the kisspeptin-induced increase in plasma insulin suggests that in the whole animal, as in isolated islets, kisspeptin has a stimulatory effect on insulin release. It is unclear why an inhibitory effect is seen in the perfused pancreas model [33], and further work is required to determine the factors responsible for differences between the observed effects in different models.

Our *in vitro* studies demonstrated that the stimulatory effects of kisspeptin on insulin secretion were dependent on the presence of a stimulatory concentration of glucose, consistent with the known mechanisms of many other receptor-operated agonists which potentiate nutrient-induced secretion but do not initiate a secretory response [36]. In contrast, kisspeptin administration *in vivo* markedly increased plasma insulin levels in animals with normal non-fasted blood glucose levels. There are several possible reasons for our *in vivo* observations. Thus, it is difficult to extrapolate from precisely defined experimental conditions *in vitro* to the much more complex *in vivo* environment in which, for example, islets are exposed simultaneously to many circulating nutrients and non-nutrient stimuli, and to the influence of intact innervation. In addition, it is difficult to compare the concentrations of kisspeptin to which the islets are exposed *in vitro* and *in vivo* without detailed knowledge of the pharmacokinetics of kisspeptin after single intravenous administration, so the observed differences in the degree of glucose-dependency may reflect differences in the concentrations of kisspeptin to which the islets have been exposed.

In conclusion, pancreatic islets express both kisspeptin and its receptor GPR-54, suggesting an important role in the control of islet function; this is supported by our *in vivo* and *in vitro* observations of the stimulatory effects of exogenous kisspeptin on insulin secretion. The known involvement of kisspeptin in puberty [4, 5] and pregnancy [37, 38], and in mediating the effects of nutritional status on reproductive function [6, 7] raises the possibility that kisspeptin plays multiple roles as a physiological regulator of whole body fuel homeostasis, at least partly by regulating islet function.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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