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# Comparison of the effects of peripherally administered kisspeptins

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ABSTRACT

Kisspeptins are structurally closely related peptides derived from the *Kiss1* gene that have been demonstrated to stimulate the hypothalamo-pituitary gonadal axis. The natural peptide products derived from post-translational processing of the kisspeptin precursor have not been elucidated. We examined the acute effect on serum levels of free testosterone in the adult male mouse after systemic administration of kisspeptins with different lengths of both human and mouse origin. Mouse kisspeptin-10 and -52 dose-dependently increased serum testosterone, and both peptides showed similar potency and efficacy. Human kisspeptin-10 and kisspeptin-54 evoked robust increase in serum testosterone, with the same potency as for mouse kisspeptins. Other members of the RFRP family of peptides, i.e. RFRP-1 and -3 were inactive. Time-course experiments revealed that the longer forms had a slower onset of action, and the long human form also a more prolonged effect. The effect of the peripherally administered mouse kisspeptin-10 could be totally blocked by the GnRH antagonist acyline. Finally, peripherally administered mouse kisspeptin-10 had no effect on Fos induction in GnRH cells. These data show that all peptides tested are active and supports the concept that their effect is mediated by a target upstream of the pituitary, such as the median eminence.


## 1. Introduction

It is well-established that the peptides derived from the metastasis suppressor gene transcript *Kiss1* are involved in regulation of gonadotrophins [1–3]. To date a number of structurally related peptides named kisspeptins have been identified in several species and their actions are mediated via the G-protein coupled receptor GPR54 (also termed AXOR12 or hOT7T175) [4–6]. The human *Kiss1* gene encodes a 145 amino acid precursor peptide that is likely cleaved into a C-terminal 54 amino acid product known as human kisspeptin-54 (also named metastin) [7]. In rodents, the kisspeptin precursor is posttranslationally spliced into a 52 amino-acid peptide (murine kisspeptin-52), which has several sequence differences to the human kisspeptin-54 sequence including a tentative disulfide bridge between positions 4 and 16 [8]. Further, the C-terminal amino acid residue differs between human and mouse: in rodents the extreme C-terminal residue is a tyrosine (Y) while a phenylalanine (F) is representative in man. The biological activity of kisspeptins is determined by the C-terminal end of the peptide, because shorter fragments such as kisspeptin-10, -13, and -14 also bind to GPR54 with the same affinity as the longer forms [8–10]. The concept has evolved that kisspeptin/GPR54 is primarily located in the hypothalamus, where kisspeptins operate as essential transmitters for the activation of GnRH neurons at puberty and the kisspeptin/GPR54 neurons are regulated by gonadal and metabolic signals [11–13].

The strong effect of kisspeptin is documented after intracerebral injection of kisspeptin-10 leading to a dramatic and potent effect on serum levels of FSH, LH and testosterone [3,13–17]. Central kisspeptins act directly on GnRH cells, because GPR54 is expressed and intracerebroventricular administration of mouse kisspeptin-52 can induce c-Fos in these neurons [18]. Peripheral administration of kisspeptin produces consistently a release of LH and testosterone [17,19], and it is considered that this effect involves GnRH neurons, although a direct action in primate testis has also been suggested [19,20]. One objective of the present study was therefore to define the site of action of peripherally administered kisspeptin and in particular to study whether GnRH neurons in the preoptic area were directly affected by peripheral administration of kisspeptin. This was carried out by determining whether c-Fos was induced in GnRH neurons after peripheral administration of kisspeptin.

Chronic central administration of the peptide to immature rats induces the activation of the gonadotrophic axis [14], and increases sexual activity in hamsters kept under a short photoperiod [21]. The kisspeptinergic system is a regulator of puberty and critically involved in the complex cascade of sex-specific development and maturation associated with puberty [22].

However, the exact nature of the peptides derived from the kisspeptin precursor is not known. While it is generally believed that

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all kisspeptins are of equal potency and efficacy *in vitro*, it may not be the same *in vivo*, because the different peptides may display differences in their dynamics when released. The other aim of this study was to create an understanding of the dose-reponse-dependent effect on circulating testosterone for short and long forms of both murine and human kisspeptins after systemic administration in mice.

## 2. Materials and methods

### 2.1. Peptides, animals, and dosing

Mouse and human kisspeptin-10 (mouse and human Kiss1 (110-119)-$NH_2$) were custom synthesized by Caslo Aps (Lyngby, Denmark), mouse kisspeptin-52 (mouse Kiss1 (68-119)-$NH_2$ with a Cys4–Cys18 disulfide bridge) was custom synthesized by Abgent (Abingdon, Oxfordshire UK) and human kisspeptin-54 (human Kiss1 (68-121)-$NH_2$) was custom synthesized by GenScript (Piscataway, New Jersey, USA). All other peptides used in the present experiments were purchased from Phoenix Pharmaceuticals (Belmont, California, USA). All peptides were more than 95% pure as revealed by HPLC and the peptides were dissolved immediately prior to use. Acyline, a selective GnRH receptor antagonist [23] was kindly provided by Drs. John Amory and William J. Bremner (University of Washington, Seattle, WA., USA).

Adult male NMRI mice (Taconic, Ll Skensved) weighing 30–32 g were housed five animals per cage. The animals were kept under 12:12 light: dark cycle, in a temperature controlled room, with free access to food and water and acclimatized in the same cage for at least 7 days before the experiment. All experiments were conducted in accordance with the Declaration of Helsinki, the Danish National Guide for Care and Use of Laboratory animals and the European Communities Council Directive (86/609/EEC; license number 2005/561-962).

In order to analyse the dose-dependent effect of kisspeptins on circulating testosterone, the mice ($n$=5) received an intraperitoneal (i.p.) injection of 10 ml/kg doses of 0.1; 0.3; 1.0; 3.0; 10; and 30 nmol/kg of all kisspeptins tested in this investigation. The peptides were dissolved in sterile 0.9% saline with 0.5% bovine serum albumin. For the dose–response study, the mice were returned to their home cages immediately after the injection and trunk blood was collected after decapitation 30 min after the treatment. The blood was collected in centrifuge tubes centrifuged for 10 min at 3000 rpm and serum was collected and stored at −20 °C until assaying. The entire dose–response experiment was replicated.

In a separate experiment, the effect of different peptides with an RF (Arg-Phe)-$NH_2$ domain were tested for their ability to enhance testosterone concentrations. These include RFRP-1, RFRP-3 and the RFRP analogues RF-amide and YLPLRF-amide. These peptides were administered in a concentration of 30 nmol/kg and the animals ($n$=5/group) decapitated 30 min after.

For the time-course study, the mice ($n$=5) received one injection of 30 nmol/kg of one of the four peptides earlier tested in the study and returned to their home cage. At various time points (5, 10, 15, 30, 60, 90, 120 min, or 240 min) the mice were decapitated and trunk blood collected for analysis.

In order to investigate the relationship between activation of GPR54 and inhibition of the GnRH receptor, combinations of acylin (50 µg/animal i.p.) or vehicle were given 15 min before a single dose of 30 nmol/kg mouse kisspeptin-10. Again, trunk blood was collected 30 min after the injection of the kisspeptin.

### 2.2. Radioimmunoassay

Serum aliquots were stored at −20 °C until hormone levels were determined. Free serum testosterone was measured using a direct RIA kit (DPC coat-a-count RIA method; Siemens Medical Solutions, Mölndal, Sweden). This method used a 125-iodinated-labeled testosterone analogue, which does not bind to plasma proteins, and a testosterone-specific antiserum immobilised to the wall of a polypropylene tube. The samples were assayed directly in duplicates without prior extraction, and as about 1% of serum testosterone is found in a free form [24], the concentrations determined reflect this compared to measurements of the total testosterone levels.

### 2.3. Animals and Fos immunocytochemistry

For immunocytochemistry, mice ($n$=7) were injected i.p. with 30 nmol/kg mouse kisspeptin-10 and 60 min after they were deeply anesthetized with mebumal and a blood sample was taken before the animals were perfused with physiological saline until the blood was flushed. The animal was fixed by vascular perfusion with 4% formaldehyde-buffer, and the brains were removed, sectioned in series of four, and processed as described previously [25].

In order to visualize neurons in the brain expressing both Fos and GnRH-immunoreactivity, sections were first processed for Fos by means of the avidin–biotin immunohistochemical procedure [26,27]. The sections were incubated in a dilution with a rabbit serum against Fos [26,27], immunoreacted and reaction product visualized in a solution of 0.0625% diaminobenzidine, 2.5% nickel ammonium sulfate in the acetate buffer containing 0.03% hydrogen peroxide, for 6 min. The heavy metal-intensification of diaminobenzidine produced black staining of the Fos labeled nuclei. After several washings in PBS, the Fos-stained sections were incubated with a rabbit antiserum against GnRH (Chemicon, Temicula, cat#AB1567) diluted 1:1000 and stained with the same avidin–biotin method described above, but here the reaction product was visualized in 0.05% diaminobenzidine with 0.05% $H_2O_2$ in PBS buffer for 7 min and then washed twice in PBS buffer. The sections were mounted on gelatinised glass slides, dried, and coverslipped in Depex®.

All sections of the mouse forebrain that contained GnRH-positive neurons were analysed under a bright field illumination. All GnRH-immunoreactive neurons were counted in all available sections from one series by an observer unaware of the treatment group. Double-labeled neurons characterized by a black-stained nucleus within a brown bipolar neuron were counted, and for each animal the percentage of totally observed GnRH-immunoreactive neurons that co-expressed nuclear Fos staining were calculated.

## 3. Results

The dose–response effects of long- and short forms of mouse and human kisspeptins on free testosterone levels in serum were studied after intraperitoneal administration of different peptides to adult male mice determined using a radioimmunoassay. This assay detects only testosterone in its free form that accounts for only approximately 1% of the total testosterone in plasma [24]. As illustrated in Fig. 1, all four peptides produced a strong and dose-dependent effect on circulating free testosterone levels. The concentration of testosterone was increased by a dose of 3 nmol/kg of all kisspeptins tested. The maximal increase in testosterone was achieved by a dose of 10 nmol/kg and increasing the dose to 30 nmol/kg did not increase serum testosterone further. The dose–response curves were only slightly different from each other, though in general, the potency as $ED_{50}$ is roughly estimated to be around 3 nmol/kg. However, the potency of the long human form, human kisspeptin-54 (Fig. 1D), was slightly but not significantly higher than for the other three peptides tested.

We also examined the biological effect of other peptides with an RFamide C-terminal domain in the same assay. Other endogenous members of the RFamide family of peptides, such as RFRP-1 and RFRP-3 (mammalian orthologues of the avian gonadotrophin inhibitory hormone), as well as two other non-natural peptide fragments with an RFamide C-terminal domain were all found to be ineffective (Fig. 2).