STUDIES OF THE ROLE OF GPR54-KISSPEPTIN SIGNALING IN ENDOCRINE FUNCTION OF PRIMATE TESTES

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Dedicated to my loving parents

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic Hormone
CNS	Central Nervous System
FSH	Follicle Stimulating Hormone
GPR 54	G-protein Coupled Receptor 54
GnRH	Gonadotropin Releasing Hormone
GABA	γ-amino butyric acid
GHRH	Growth Hormone Releasing Hormone
hCG	Human Chorionic Gonadotropin
HPG axis	Hypothalamic-pituitary Gonadal Axis
HPT axis	Hypothalamic-pituitary Testicular Axis
LH	Luteinizing Hormone
LHRH	Luteinizing Hormone Releasing Hormone
NPY	Neuropeptide Y
RIA	Radio-immuno Assay

ABSTRACT

Reproductive functions are tightly regulated by the hormones of hypothalamus and anterior pituitary; together with gonadal hormones they form the so called hypothalamic pituitary gonadal axis. Recently, kisspeptin peptide along with its seven transmembrane G protein coupled receptor, GPR54, was identified in mammals as a central gatekeeper of the reproductive cascade. However all recent work in rodents and primates has focused on central effects of kisspeptin administration. Demonstration of presence of GPR54 receptor in testes raises the possibility of direct action of kisspeptin on the distal component of the reproductive axis. Therefore, in the present study we analyzed direct testicular action of kisspeptin in the adult intact male rhesus monkey, a representative higher primate. The paradigm we used to examine the hypothesis was that of pituitary gonadotropin-clamped monkey model with pretreatment with acyline, a GnRH receptor antagonist. Since effect of kisspeptin administration on testosterone levels in adult male monkey has not been reported, a corollary objective of the study was to characterize changes in testosterone levels following peripheral kisspeptin administration.

Four adult intact male rhesus monkeys (*Macaca mulatta)*, maintained under standard colony conditions of feeding and management, were used in this study. The animals were habituated to chair restraint prior to experiments in order to study them without sedation. Animals were implanted with iv cannula to gain continuous access to venous circulation for drug administration and blood sampling. Animals were assigned to receive iv saline (0.9 % NaCl), kisspeptin-10

(50 ug) and kisspeptin-10 (50 ug) with acyline pretreatment (60 ug/kg and 120 ug/kg BW, sc, morning and evening, respectively). Endocrine effects of kisspeptin on the testes were examined by monitoring plasma testosterone levels. In addition effect of kisspeptin administration on plasma glucose and cortisol levels was also studied because of presence of GPR54 receptor on pancreas and adrenal gland. Testosterone and cortisol concentration were measured by specific radioimmunoassays. Plasma glucose levels were measured by using blood glucose strip test in sensocard blood monitoring system.

The peripheral administration of kisspeptin but not vehicle caused a robust increase in plasma testosterone levels 30 minutes post injection that lasted for the next 180 minutes. However, this dramatic increase of testosterone was abolished when kisspeptin was administered to acyline pretreated animals. Plasma cortisol levels of kisspeptin treated animals were moderately low as compared to the vehicle treated animals. Plasma glucose levels were not affected by kisspeptin administration.

These studies suggest that peripheral kisspeptin administration induces a robust acute stimulation of testosterone secretion in adult intact male monkeys. However, such an effect is not produced directly at the testicular Leydig cell level. Rather our results demonstrate that the primate hypothalamic-pituitary-testicular axis is strongly stimulated by kisspeptin, through an action of the peptide at a site afferent to GnRH neurons. A role of kisspeptin on other testicular functions like spermatogenesis, however, still cannot be excluded. Also present study provides a rationale to further asses the involvement of kisspeptin-GPR54 signaling in affecting primate hypothalamic response to stress.

INTRODUCTION

Reproductive functions are tightly regulated by hormones of hypothalamus (gonadotropin releasing hormone) and anterior pituitary (gonadotropins). Together these hormones govern the gametogenic and endocrine activities of gonads.

Gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) are synthesized in gonadotropes cells of the anterior pituitary (Nakane, 1970). The stimulation of gonadotropes is in turn regulated by gonadotropin releasing hormone (GnRH), a decapeptide, synthesized and stored in specific neurosecretory neurons present largely in medial basal hypothalamus (Silverman et al., 1990). GnRH neurons are only handful in numbers with 1,000-3,000 neurons consistently found across mammalian species and these neurons are diffusely spread throughout the diagonal band of broca, septum, organum vasculosum of lamina terminalis, preoptic area and mediobasal hypothalamus (Silverman et al., 1994). GnRH enters pituitary portal vasculature and travels to the pituitary to signal the synthesis and secretion of the pituitary gonadotropins (Silverman et al., 1994). Though GnRH neurons are diffusely spread but surprisingly they fire synchronously to produce the intermittent episodes of the hormone release in median eminence (Sisk and Foster, 2004). It may thus be viewed that GnRH is the primary regulator of reproductive functions and its release drives the subsequent pituitary gonadotropin secretion and then gonadal functions.

An important component of the regulation of gonadotropins is in fact the modulation of GnRH secretion from within the hypothalamus (Evans, 1999). The release of GnRH in timely and concentration-regulated fashion is, in part, achieved by mechanisms mediated by peptides and neurotransmitters of the hypothalamus (Kalra and Crowley, 1992; Xu et al., 1996; Johnston et al., 1992; Vijayan and McCann, 1979; Kalra, 1993; Terasawa, 1995; Rossmanith et al, 1996; Levine, 1997).

Although, these regulatory peptides are known to be synthesized in hypothalamus and act on GnRH neurons, there is also circumstantial evidence for a pituitary site of action, provided by the confirmed transfer of peptides from the hypothalamus to the anterior pituitary via the portal blood (Evans, 1999). Most of these peptides have been detected in median eminence before departure from the hypothalamus region. Observation of localization or measurement in the median eminence region of galanin (Lopez et al., 1991), NPY (McDonald et al., 1987; Sahu et at., 1989; Prasad et al., 1993; Zimmerman and Antunes, 1976), neurotensin (Watanabe and Takebe, 1993), oxytocin (Zimmerman and Antunes, 1976; Silverman, 1976), PACAP (Mikkelsen et al., 1995; Koves et al., 1990; Vigh et al., 1991) and substance P (Brown et al., 1990; Parnet et al., 1990) have been reported. But the presence of the peptides in the portal blood, in transport to the anterior pituitary gland subsequent to their release from the median eminence, is a crucial, although not a definitive observation that is necessary before it is possible to suggest a hypophysiotropic activity of these peptides (Evans, 1999).

Other than peptides, acting as neurotransmitters, single or double amino acid derived neuromediators also act as regulator to GnRH neurons. These include catecholamine, serotonin, histamine, γ-amino butyric acid (GABA) and glutamate. Out of these GABA is a more important regulator of GnRH release

(Vincent et al., 1982; Han et al., 2004). The infusion of GABA in regions containing GnRH neuron inhibits the pulsatile release of LH in ovariectomized rats (Lamberts et al., 1984) suggesting an inhibitory role for GABA in the regulation of GnRH/LH release in preoptic area/anterior hypothalamus region. In contrast to inhibitory effect of GABA on LH secretion, an excitatory role in the regulation of GnRH release was suggested by the studies in which GABA was infused in the vicinity of the median eminence (Vijayan and McCann, 1978). Furthermore, GABA and $GABA_A$ agonists stimulate GnRH secretion from median eminence fragment in vitro (Nikolarakis et al., 1988).

Excitatory amino acids are the other class of neuromodulators affecting GnRH neurons, which include glutamate, aspartate, glycine and taurine of which glutamate is important in the context of LH regulation. It has been shown to regulate LH secretion in rats (Schainker and Cicero, 1980; Tal et al., 1983; Bourguignon et al., 1989), mice (Saitoh et al., 1991), sheep (Estienne et al; 1989), bull calves (Shahab et al; 1993) and monkeys (Wilson and Knobil, 1982; Gay and Plant, 1987; Plant et al., 1989; Medhamurthy et al., 1990).

In addition to classical GnRH-gonadotropin regulation of testicular endocrine function there is also paracrine regulation evident in testes (as reviewed by Saez, 1994 and Weinbaur et al., 1997). This local control includes different types of growth factors like nerve growth factor, insulin like growth factor and neuropeptides like opioids, oxytocin and vasopressin, GHRH, GnRH, ACTH, corticotropic-releasing hormone and β-endorphin. Additionally, local factors like prostaglandins, endothelin and interleukins are also shown to be expressed in the testis. For majority of these factors the physiological relevance in vivo and

their real meaning for the testicular function remains unknown. It is probable that factors produced locally are important for the modulation of gonadotropin activity in the testis.

Recently another peptide kisspeptin involved in central regulation of GnRH has been identified (Muir et al., 2001; Ohtaki et al., 2001; Kotani et al., 2001). The discovery of the role of kisspeptin and its receptor, a G protein coupled receptor i.e., GPR54 in puberty is the most exciting finding made in the field of reproductive biology since the discovery of GnRH in the 1970s (Lee et al., 2001). Since the discovery of GnRH, many new neurotransmitters and neuropeptides have been shown to play a role in the regulation of GnRH neurons but none of them has such a dramatic effect as kisspeptin's.

KiSS-1 the gene that encodes kisspeptin peptides was discovered in experiments designed to determine the gene responsible for the antimetastatic effect of human chromosome 6 (Lee et al., 1996). *KiSS-1* is actually located on chromosome 1 (iq32), although elements on chromosome 6 are thought to regulate *KiSS-1* expression from upstream (Miele et al., 1996; Weat et al., 1998; Goldberg et al., 2003). *KiSS-1* is expressed in the central nervous system, pituitary, testes, ovaries, pancreas and intestine but is most concentrated in placenta (Muir et al., 2001; Ohtaki et al., 2001; Terao et al., 2004).

KiSS-1 encodes a 145-amino-acid peptide that is proteolytically cleaved into a family of peptides referred to as kisspeptins (West et al., 1998). The most abundant of which is an amidated 54-amino-acid protein, kisspeptin-54, also known as metastin (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). Truncated forms of the KiSS-1 peptide, 13 and 14 amino acids long, and sharing a common C-terminus with the 54-residue peptide, were also isolated from human

placental extracts (Kotani et al., 2001). The peptides were named kisspeptins, although kisspeptin-54 is also known as metastin in deference to its antimetastatic activity. The kisspeptins belong to the RF-amide peptide family, a loosely defined group of peptides with an arginine-phenylalanine amide structure at their carboxy terminals (Dockery et al., 2004). Two specific antibodies have shown that kisspeptide-54 is present in plasma at very low concentration in both sexes (Horikoshi et al., 2003).

The C-terminal decapeptide, common to all the kisspeptins, is the minimum sequence necessary for receptor activation (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001) and it has subsequently been shown that cultured first trimester trophoblast secrete this kisspeptin-10 in addition to the 13-, 14- and 54- residue forms (Bilban et al., 2004). These endogenous forms of kisspeptin have been reported to have a similar affinity and efficacy in vitro (Kotani et al., 2001); although some studies have suggested that the shorter fragments are more efficacious (Muir et al., 2001; Ohtaki et al., 2001). The antimetastatic effects of kisspeptin-54 were shown to be mediated via GPR54. The kisspeptin-54 inhibits chemotaxis and invasion of chinese hamster ovary cells transfected with GPR54 in vitro, and attenuates pulmonary metastasis of GPR54-transfected melanoma in vivo (Ohtaki et al., 2001; Hori et al., 2001).

GPR54 was initially isolated as an orphan receptor showing significant homology to the galanin receptors, but further it was shown that it does not bind with radiolabelled galanin (Lee et al., 1999). Three groups almost simultaneously discovered that the 54-amino acid carboxy-terminally amidated peptide product of the human KiSS-1 gene activated the human orthologue of the rat G-protein coupled receptor, GPR54. These three groups included Marc Parmentier's group

from Belgium (Kotani et al, 2001), David Harrison's group from United Kingdom (Muir et al, 2001) and Masahiko Fujino's group from Japan (Ohtaki et al, 2001). These simultaneous discoveries in 2001 suddenly expanded the curiosity about *KiSS-1* and GPR54 system. *GPR54* gene has been demonstrated to be located on chromosome 19 (de Roux et al, 2003). *GPR54* and its human orthologue (also known as AXOR12 or hOT7T175) have a similar, but not identical, expression pattern to *KiSS-1* with receptor expressed in CNS, placenta, pituitary, liver, intestine, pancreas and testes (Funes et al., 2003; Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001; Lee et al., 1999; Terao et al., 2004).

GPR54 is a 398 amino acid G-protein-coupled receptor with a short extracellular domain, seven transmembrane domains linked by extracellular and intracellular loops and an intracellular domain (Lee et al., 1999). Potential N-glycosylation sites are present within the extracellular domain as well as phosphorylation sites within the intracellular domain. It shows 40% homology with galanin receptors. It is mainly coupled to phospholipase-C beta but it may also activate other transduction pathways such as phospholipase A2 (Kotani et al., 2001). MAP kinases were shown to be activated by GPR54 in chinese hamster ovary cell lines (Kotani et al., 2001). As described earlier, the ligand of GPR54 is Kisspeptin-54 derived from KiSS-1 gene by a complex post translational process (Harms et al., 2003).

The kisspeptin/GPR54 system is well suited to regulate neuroendocrine function. Both kisspeptin and GPR54 expression are also highly expressed in the hypothalamus, with kisspeptin-immunoreactive cell bodies located in the arcuate, dorso-medial, paraventricular and ventromedial nuclei, and kisspeptin immunoreactive fibers projecting to region including the arcuate and dorsomedial nuclei and the preoptic area, the retrochiasmatic area and the zona incerta

(Brailoiu et al., 2005). In situ hybridization has shown that GPR54 is synthesized in the arcuate and dorsomedial nuclei, the lateral, anterior and ventromedial hypothalamic areas and the medial preoptic area (Brailoiu et al., 2005; Shahab et al., 2005). *GPR54* is also highly expressed in the pituitary (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001; Lee et al., 1999; Terao et al., 2004).

Several lines of evidence supported the role of GPR54 as gatekeeper of the reproductive cascade. In human loss-of-function point mutation and deletions within the coding sequence of the GPR54 gene were identified in patients with idiopathic hypogonadotropic hypogonadism, a condition characterized by the absence of spontaneous pubertal development, low sex steroids and inappropriate low gonadotropins (Seminara et al., 2003; de Roux et al., 2003). Mice carrying null mutations of GPR54 (GPR54-/-) recapitulated the human phenotype and have provided clues that the defect was at the level of GnRH processing or secretion (Seminara et al., 2003; Funes et al., 2003). In the brain, GPR54 may act as neuromodulator of the gonadotropic axis and this function may be considered as main biological function of GPR54 pertaining to reproduction in mammals. Central or peripheral administration of kisspeptin stimulates the hypothalamic-pituitary gonadal axis. Central (i.c.v) injection of kisspeptin-10 or kisspeptin-54 potently increases circulating concentration of LH and FSH in both male and female, prepubertal and adult rodents (Gottsch et al., 2004; Thompson et al., 2004; Navarro et al., 2004; Navarro et al., 2005a; 2005b). Kisspeptin-10 (i.c.v and i.v) has been shown to potently stimulate LH release in agonadal juvenile male monkeys (Shahab et al., 2005).

In addition, icv and ip kisspeptin-10 has been shown to raise circulating testosterone in adult male rats (Thompson et al., 2004). Subcutaneous kisspeptin-54 stimulated LH and FSH release in adult male rats and in prepubertal female

rats, with or without priming with pregnant mare serum gonadotropin induced ovulation (Matsui et al., 2004). The kisspeptin appears to have a more potent effect on LH release than FSH release (Thompson et al., 2004, Navarro et al., 2005). Chronic central kisspeptin administration to females rats can induce puberty, as assessed by advanced vaginal opening, increased uterus mass and increased circulating LH and estrogen concentration. This precocious activation of reproductive axis occurs even in the models of leptin insufficiency (Navarro et al., 2004).

The effects of kisspeptin on the HPG axis are mediated via GPR54, as peripheral administration of kisspeptin to GPR54-/- mice has no effect on circulating gonadotropins (Messager et al., 2005). Kisspeptin appears to stimulate the HPG axis by the release of GnRH. The central and peripheral action of kisspeptin on the HPG axis is blocked by GnRH antagonists in rodents and monkeys (Gottsch et al., 2004; Matsui et al., 2004; Irwig et al., 2004; Shahab et al, 2005). Furthemore, Peripheral kispeptin-54 or central kisspeptin-52 has been shown to induce c*Fos* immunoreactivity in the majority of GnRH neurons in the rat hypothalamus (Matsui et al., 2004; Irwig et al., 2004). Following peripheral administration of kisspeptin, evidence that the greatest neural activation was seen in the interior preoptic areas and medial basal hypothalamus (Matsui et al., 2004) suggested that kisspeptin directly stimulate GnRH neurons. Furthermore, Kisspeptin-10 stimulates the release of GnRH from ex vivo hypothalamic slices (Thompson et al., 2004) and icv injection of kisspeptin in sheep increases GnRH concentration in cerebrospinal fluid (Messager et al., 2005). Also GPR54 is present in the medial preoptic area (Lee et al., 1999), which is home to a dense population of GnRH synthesizing cell bodies (Merchenthaler et al., 1989; Silverman et al., 1987). The GPR54 receptor was first localized to hypothalamic GnRH neuron in a cichlid fish (Parhar et al., 2004).

GPR54 is also shown to be colocalized with GnRH neurons in the mouse and monkey hypothalamus (Han et al., 2005; Shibata et al., 2005). It therefore appears likely that the action of kisspeptin on GnRH neuron is direct. Although kisspeptin may signal directly to GnRH neurons but interestingly kisspeptin was recently shown to colocalize with GnRH neurons in ovine brain indicating an autocrine role of kisspeptin on GnRH release in sheep (Pompolo et al., 2005). It may also be likely that kisspeptin may be co-secreted with GnRH into the hypophyseal portal blood to act on pituitary. The latter possibility appears to be supported by recent demonstration that rat hypothalamic fragments secrete kisspeptin (Nazian., 2005).

Though GPR54 is highly expressed in the pituitary (Kotani et al., 2001; Muir et al., 2001), kisspeptin has no effect on LH or FSH release from adult male rat anterior pituitary fragments (Thompson et al., 2004). However, Navarro et al. (2005a and b) have shown that kisspeptin is capable of releasing LH and enhancing GnRH stimulated FSH release from static incubation of pituitary tissue from prepubertal male rats. It is unclear whether the discrepancies between these findings are due to difference in technique or because the rats used were prepubertal (Navarro et al., 2005a; 2005b) or adult (Matsui et al., 2004). Although a direct anterior pituitary action is extremely possible, it appears unlikely to constitute the major pathway by which kisspeptin stimulates the hypothalamic-pituitary gonadal axis.

The effects of central and peripheral kisspeptin on testosterone release in rodents or humans are most likely mediated via GnRH and gonadotropin release. However, GPR54 is also expressed in the testis (Kotani et al., 2001; Ohtaki et al., 2001) raising the possibility of direct testicular site of action of kisspeptin. As a matter of fact both *KiSS-1* and *GPR54* were found in the human testis (Ohtaki et al., 2001; Kotani et al., 2001). High levels of *KiSS-1* gene were observed in testes

whereas for *GPR54* moderate expression was found in human testes (Ohtaki et al., 2001). Parenthetically, many neuropeptides like GnRH, GHRH and oxytocin have been shown to be present in testis and likely to be involved in a local/paracrine regulation of testicular endocrine function. Present experiment, therefore, was designed to determine the direct testicular effect of kisspeptin in an adult monkey model in which hypothalamic-pituitary axis was blocked by pre-administering GnRH receptor antagonist. In this paradigm if kisspeptin causes an increase in testosterone level then it would indicate an endocrine effect of kisspeptin at testicular level in primates.

Additionally, we examined the effect of kisspeptin on blood glucose levels as GPR54 has been also shown to be expressed in pancreas (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). Cortisol levels were monitored also during the course of this study to check whether the animals were stressed by chair restraint procedures and to detect a possible activation of the hypothalamic-pituitary-adrenal axis by kisspeptin administration.

MATERIAL AND METHODS

Animals

Four adult intact male rhesus monkeys (*Macaca mulatta*), weighing 6.0-8.0 kg were used in this study. The animals were housed in individual cages, under standard colony conditions and were fed with monkey food at 1300-1330 hours daily and supplemented with fresh fruits in the morning. Water was available *ad libitum*.

Chair-restraint Training

The monkeys were trained for chair-restraint prior to initiation of the experiment in order to sample these animals without sedation or anesthesia. Under ketamine sedation (5mg/kg BW, im) animals were affixed to a primate chair. After recovery from sedation the animals were allowed to sit on the chair for gradually increasing periods. The animals were habituated to chair restraint in a period of couple of months.

Catheterization

To permit sequential withdrawal of the blood samples and iv administration of drugs, the animals were anesthetized with ketamine hydrochloride (Ketamax, Rotexmedica, Trittau, Germany), 5-10mg/kg BW, im), and a taflon cannula (Vasocan Branule, B. Braun Melsungen AG, Belgium; 0.8mm/22G O.D) was inserted in the sephanous vein ~ 30 min before initiation of sampling and the animals were restrained to the chair. The free end of the cannula was attached to a syringe via a butterfly tubing 20G and 300mm length. Blood sampling and infusion of drugs were carried out when the animals had fully regained consciousness.

Reagents

Human kisspeptin-10 (112-121) (Phoenix pharmaceuticals, Belmont, CA. USA) and GnRH receptor antagonist acyline was kindly provided by Dr. Tony Plant, University of Pittsburgh, USA. LHRH was purchased from Sigma Chemical Company, St Louis, MO. USA; catalogue # L-7134. hCG (Pregnyl®, N.V Organon Oss Holland) and heparine (Rotexmedica, Germany) purchased locally. Working solutions of kisspeptin, LHRH and hCG were made in normal saline while acyline was dissolved in 5% aqueous mannitol.

General Experimental Design

Actual experiment comprised of 3 days of blood sampling. All four animals were used on each day of sampling. A total of 13 blood samples (~ 2.5ml/sample at 30min intervals) were taken from each animal on each day of sampling. Sampling started at 0900-0930 hours, and samples were obtained at 30-min intervals for 30min before injections (-30 and 0min) and for 360min thereafter. Kisspeptin or normal saline as vehicle was administered immediately after taking 0min sample. Intravenous hCG (50 IU) and GnRH (1ug) were used as a positive control to examine the responsiveness of testicular tissue and pituitary and to check the efficacy of acyline treatment, respectively. hCG was given after 240min sample while GnRH was given after 300min sample in all experiments. Samples were taken in hepranized syringes and immediately transferred to culture tubes kept on ice. After completion of sampling, tubes were centrifuged at 3000 rpm and plasma was extracted and stored at -15 °C until assayed. After each sample approx 3 ml of normal saline containing 5 IU of heparin was injected to compensate the lost blood volume to prevent hypovolumic shock to the animals.

At day 1st of sampling, vehicle (normal saline, 1ml) was administered iv as a control after 0min sample, hCG was administered after 240min of sampling and GnRH was administered after 300 min of sampling.

At 2nd day of sampling which was 2 days after the first day of sampling, kisspeptin (50ug, iv) was administered after 0 min of sampling, hCG and GnRH was given as described earlier.

A day after the kisspeptin administration bleed, the animals received two doses of GnRH receptor antagonist acyline subcutaneously one the morning (60ug) and one in the evening (120ug). No samples were taken at this day.

At 3rd day of the sampling (24 hour after acyline treatment), animals were administered kisspeptin after 0 min sample and hCG and GnRH were injected after 240 & 300 min of samples, respectively.
In all experiments, kisspeptin was administered as a iv bolus in dose of 50ug/animal. All kisspeptin injections were given between 1000 and 1030 hours.

In the original experiment, no effect of GnRH on testosterone secretion was evident likely due to masking by hCG administration. Therefore, to verify the efficacy of acyline a separate study was carried out on the same group of animals (n=4). This experiment was performed after a month of original study. All four animals were bled twice. First vehicle pretreated animals were given an iv bolus of GnRH (1ug). Blood samples were obtained at 30-min intervals for 30 min before injections (30 and 0 min) and for 60 min thereafter the administration of GnRH. On 2nd day, animals were given iv GnRH bolus (1ug) 24hour following acyline treatment as mentioned above.

Blood levels of cortisol in vehicle and kisspeptin administered animals were analyzed to access the stress state of animals during the experimental procedure and also to determine any effect of kisspeptin on cortisol secretion. Similarly basal glucose changes were also monitored to asses of metabolic state of chair restraint monkeys and affect, if any of kisspeptin treatment on glucose levels.

Radioimmunoassay of Hormones

Plasma testosterone and plasma cortisol concentrations were determined by using specific solid phase competitive radioimmunoassays (RIA). The testosterone and cortisol RIA kits were purchased from Immunotech (Prague, Czech Republic). The assays were done according to the instructions given by the manufacturer. Tubes for testosterone were incubated at 37°C while tubes for cortisol were incubated at 24°C with shaking (400rpm). Then tubes were carefully decanted and placed in a Beckman Gamma counter (Gamma 5500) for counting in bound radioactivity. The counting time for each tube was one minute.

For testosterone the sensitivity of the assay was 0.025 ng/ml and intra- and inter assay coefficients of variation were 14.8% and 15%, respectively. For cortisol the analytical sensitivity of the assay was 10nM and intra- and inter assay coefficient of variation were 5.8% and 9.3%, respectively.

Blood Glucose Determination

Venous blood glucose concentrations were measured each half an hour using SensoCard in vitro blood glucose monitoring system. This system comprised of blood glucose test strips and blood glucose meter. The meter indicates blood glucose concentration by checking the reaction between chemical reagents and the blood drop on test strip. Reaction triggers a current generation in

the test strips reagent zone and this current is conducted to the meter. Current is in correlation with blood glucose concentration.

Statistical Analysis

All data presented are mean ± SEM. One way analysis of variance (ANOVA) was used to analyze differences between plasma testosterone, plasma cortisol and blood glucose concentrations after vehicle, kisspeptin and acyline+kisspeptin administrations. Student's t test was employed to determine differences between means of pre and post treatment values. Statistical significance was set at $P \leq 0.05$.

RESULTS

Effect of peripheral administration of kisspeptin-10 on plasma testosterone in adult male monkeys

Peripheral administration of 50ug kisspeptin-10 to all four adult male monkeys induced a robust increase in plasma testosterone levels (Fig. 1). Within 30 min after peripheral administration of kisspeptin-10, plasma testosterone level increased by 2 folds and remained elevated for 2-3 hours. Vehicle administered did not affect testosterone levels. Acyline pretreatment abolished the stimulatory action of peripheral administered kisspeptin on plasma testosterone secretion (Fig. 1). That the testicular tissue was responsive in terms of testosterone secretion was evident by hCG administration which caused a sudden increase in plasma testosterone levels in all three treatment groups (Fig. 1). However, effect of GnRH bolus aimed to check the responsiveness of the pituitary could not be determined because of already elevated testosterone levels following hCG. Mean ± SEM testosterone concentration in all three treatment groups are shown in Fig. 2. The comparison between mean pre-treatment (- 30-0min) and post-treatment (30-240 min) plasma testosterone values showed a significant difference in kisspeptin treatment group (Fig. 3). The pre-treatment and post-treatment plasma testosterone values in vehicle and acyline+kisspeptin treatments fail to show any significant difference (Figs. 3 and 4).

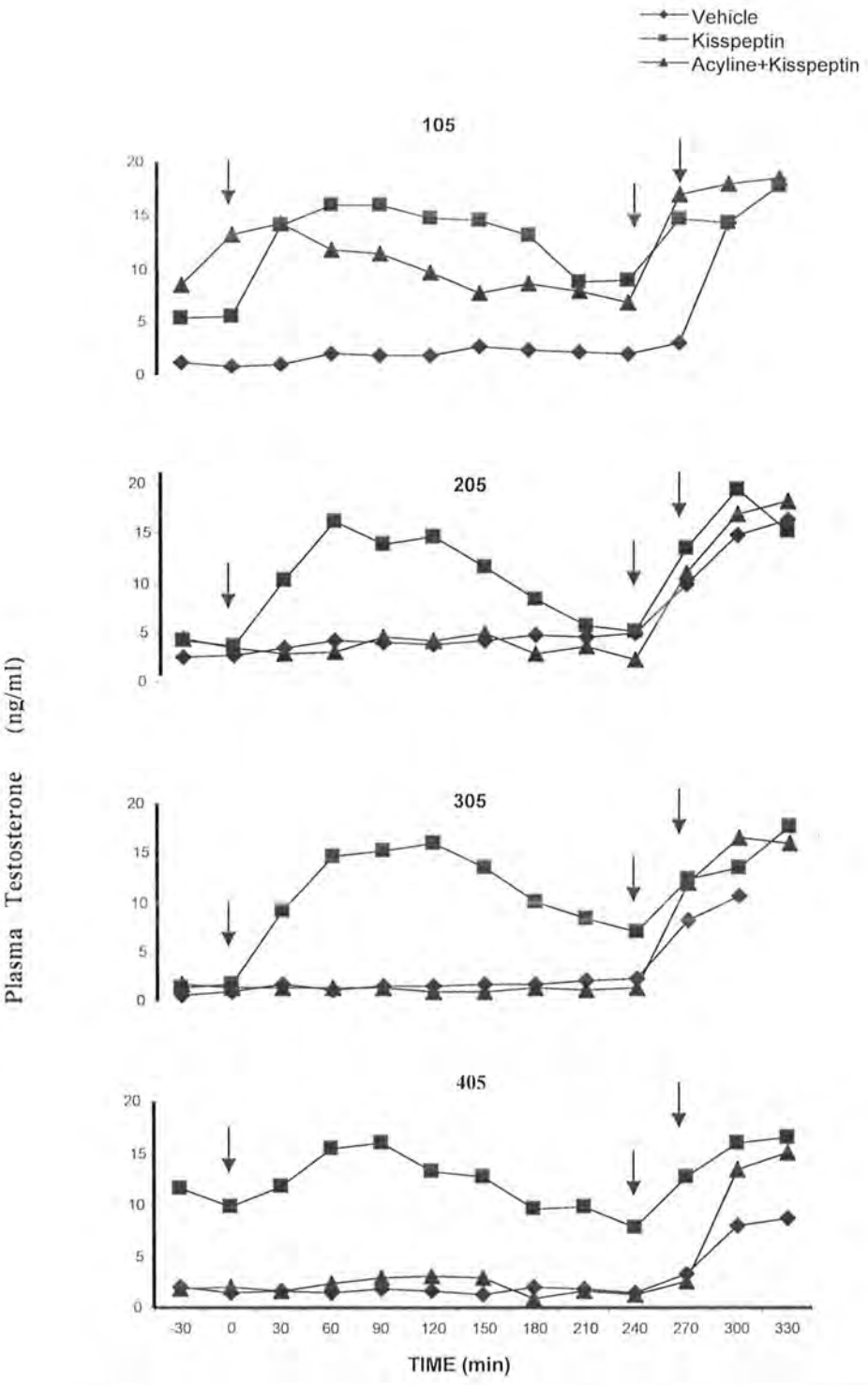

Fig.1. Plasma testosterone levels of individual animals receiving Vehicle, Kisspeptin (50ug) and Acyline (60ug/kg b.w, 120ug/kg b.w)+Kisspeptin (50ug) treatments. First arrow indicates the time of kisspeptin/vehicle, second arrow indicates the time of hCG (50 IU) administration, and third arrow shows time of GnRH bolus (1ug).

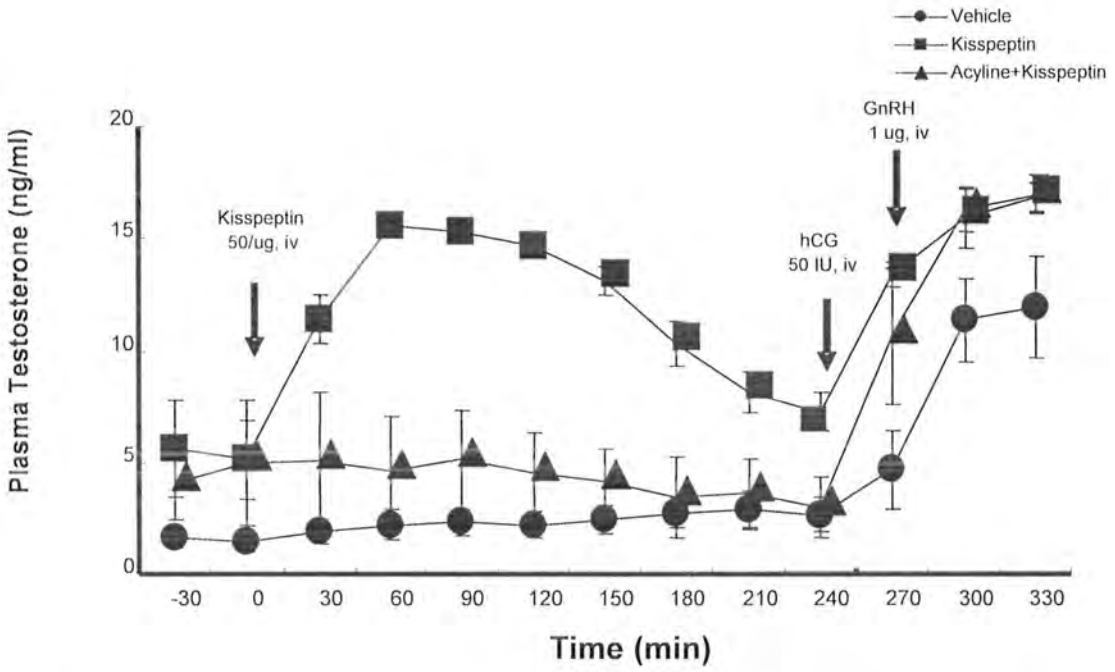

Fig.2. Mean (± SEM) plasma testosterone concentrations in adult intact male rhesus monkeys (n=4) before and after the administration of kisspeptin. The animals were challenged with hCG and GnRH (arrows) immediately after 240 min and 300 min time points, respectively, to access responsiveness of the testis and pituitary.

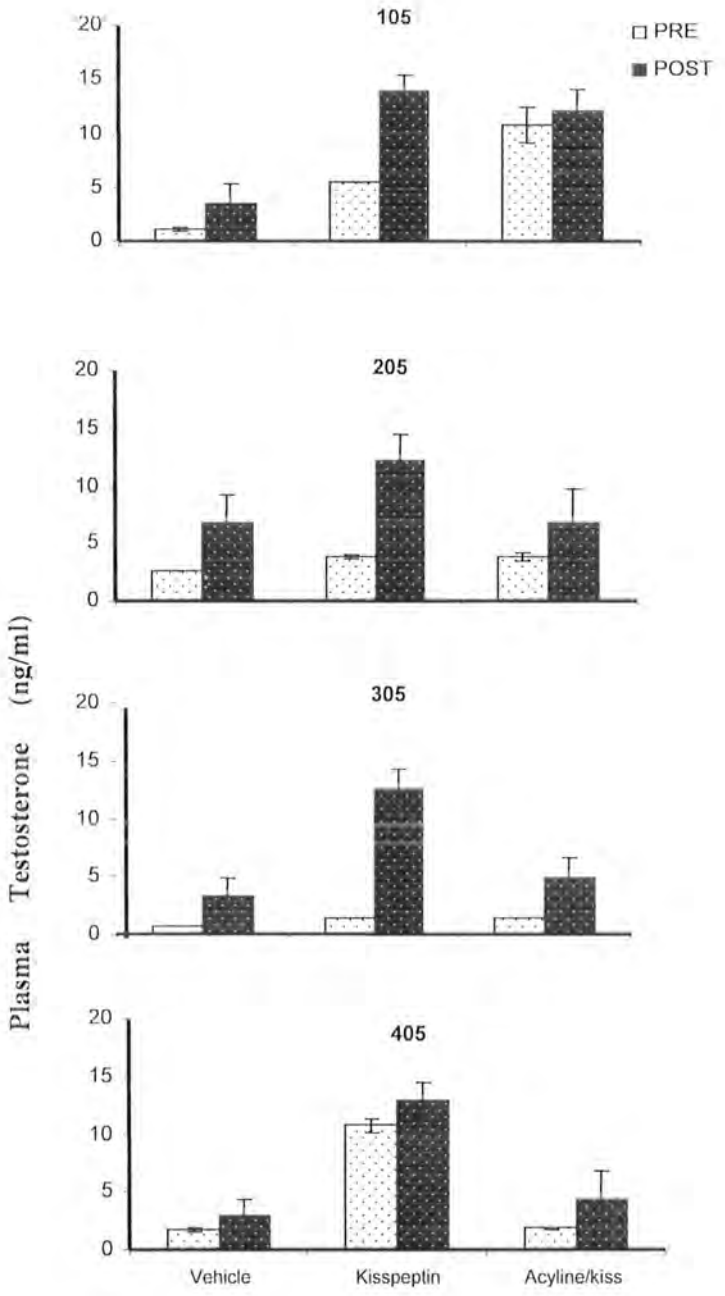

Fig. 3. Comparison of mean plasma testosterone levels observed in pre- (-30-0min) and post-kisspeptin (30-240min) administration periods in individuals male rhesus monkeys.

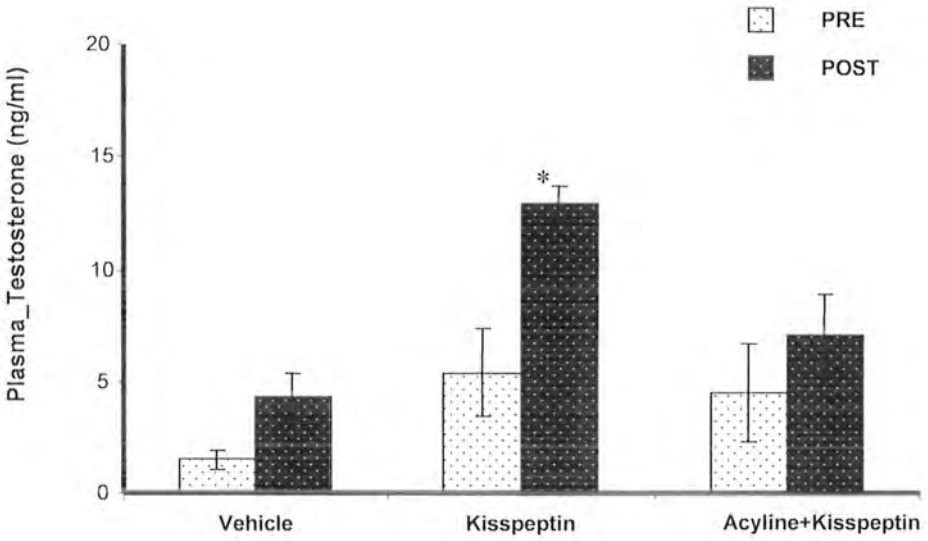

Fig. 4. Comparison between mean ± SEM pre and post treatment values of plasma testosterone in adult male rhesus monkeys (n=4). * $P \leq 0.05$

Efficacy of GnRH receptor antagonist (Acyline) in adult male monkeys

Peripheral administration of 1ug GnRH to adult male monkeys induced a robust discharge of plasma testosterone in animals given vehicle. On the other hand acyline pretreatment completely blocked this action of GnRH. This increase of testosterone levels observed in vehicle treated animals was 5 to 10 folds as compare to that in acyline treatment (Fig. 5). This confirmed the efficacy of acyline as an efficient GnRH receptor antagonist acting at the pituitary level.

Effect of peripheral administration of kisspeptin-10 on plasma cortisol in adult male monkeys

Peripheral administration of kisspeptin-10 to adult male monkeys induced a slight decrease in plasma cortisol level when compared to the concentrations observed after vehicle administration (Fig. 6). However, difference between mean post-vehicle and post-kisspeptin cortisol levels did not attain full statistical significance (P=0.07) (Fig. 7).

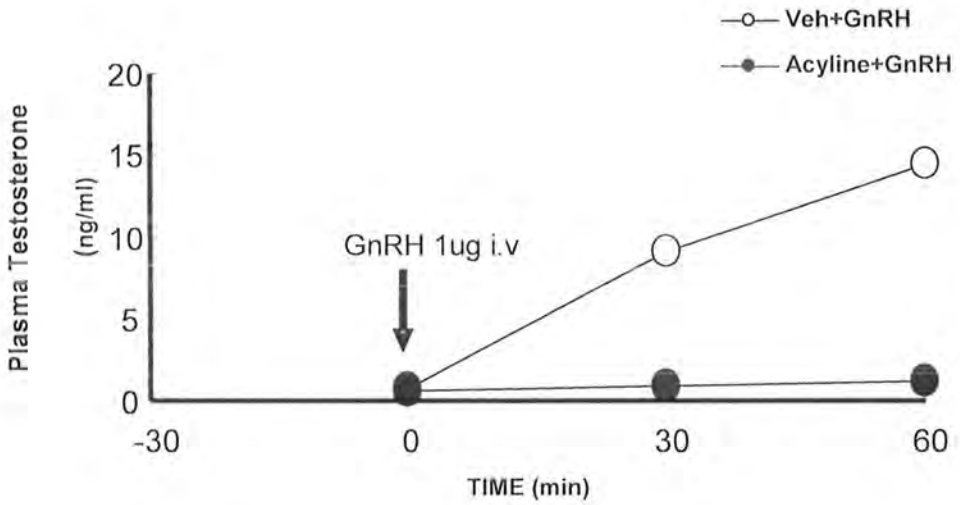

Fig. 5. Efficacy of GnRH receptor antagonist (acyline) in suppressing GnRH action in adult male rhesus monkeys (n=4). While GnRH induced a significant stimulation of testosterone in animals pretreated with vehicle, it had no such effect where animals were pretreated with acyline. Arrow indicates the time of GnRH administration.

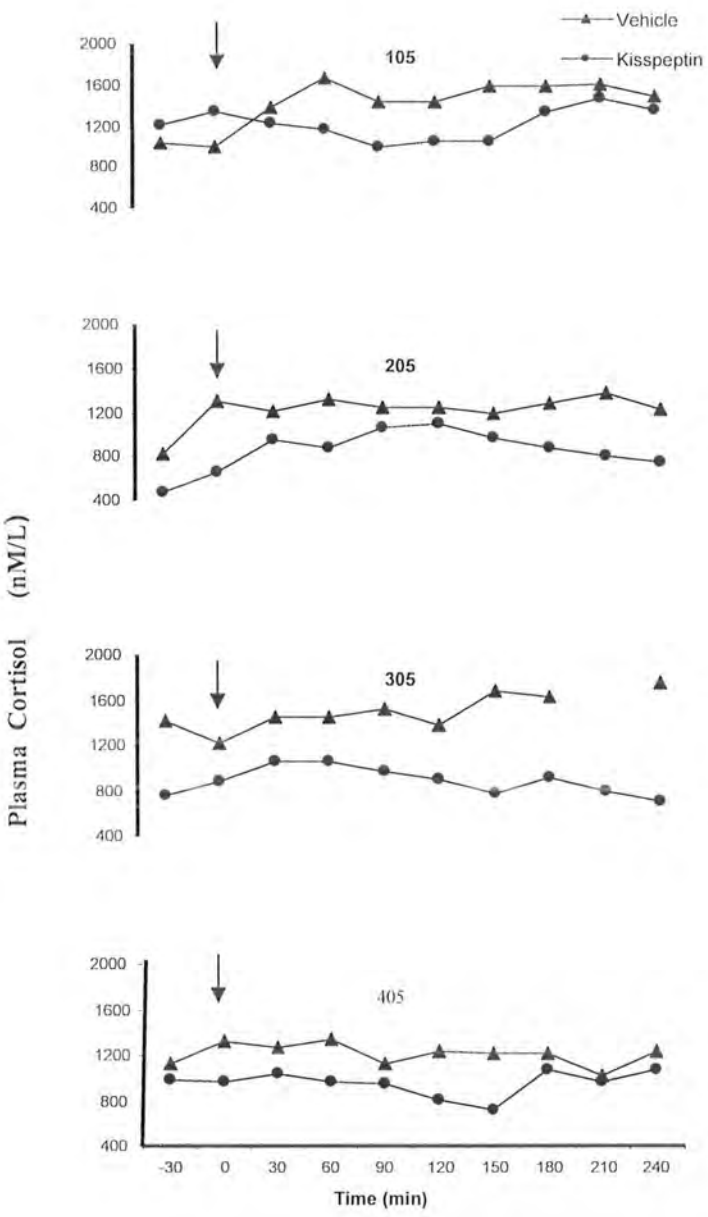

Fig. 6. Blood cortisol concentrations in vehicle and kisspeptin treated individual male rhesus monkeys. Arrows indicate the time of administration of the relative treatment.

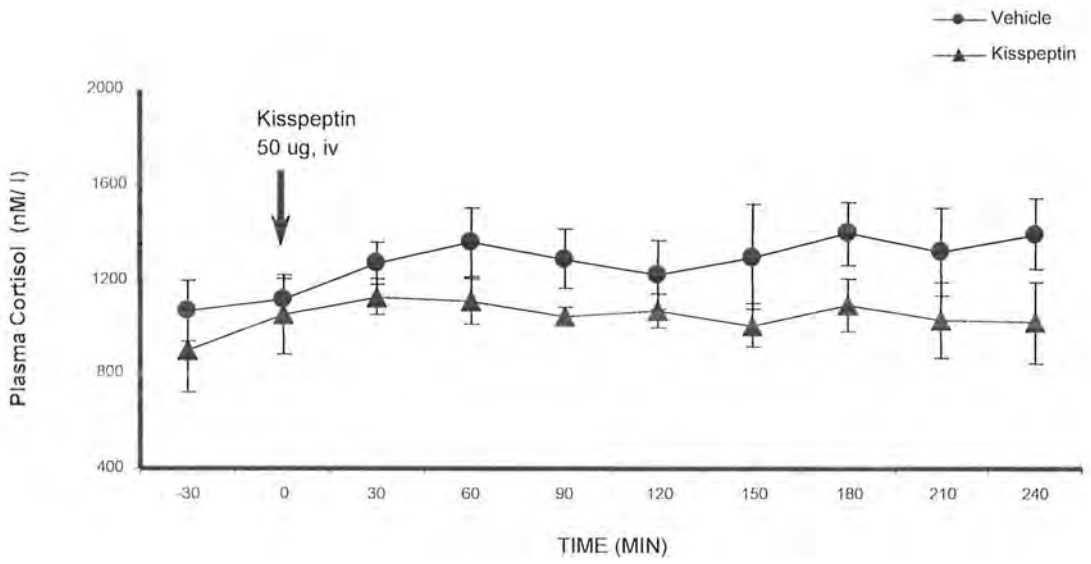

Fig. 7. Mean (± SEM) plasma cortisol concentration after kisspeptin administration in adult male rhesus monkeys (n=4). Mean cortisol levels in the post-kisspeptin period appear to be less than the levels observed in post vehicle period.

Effect of peripheral administration of kisspeptin-10 on blood glucose in adult male monkeys

Peripheral administration of kisspeptin-10 to adult male monkeys induced a slight increase in blood glucose levels. However, a similar trend was also apparent following vehicle treatment (Fig. 8). However analysis of mean blood glucose levels identified a passive stimulatory effect of kisspeptin on glucose levels (Fig. 9).

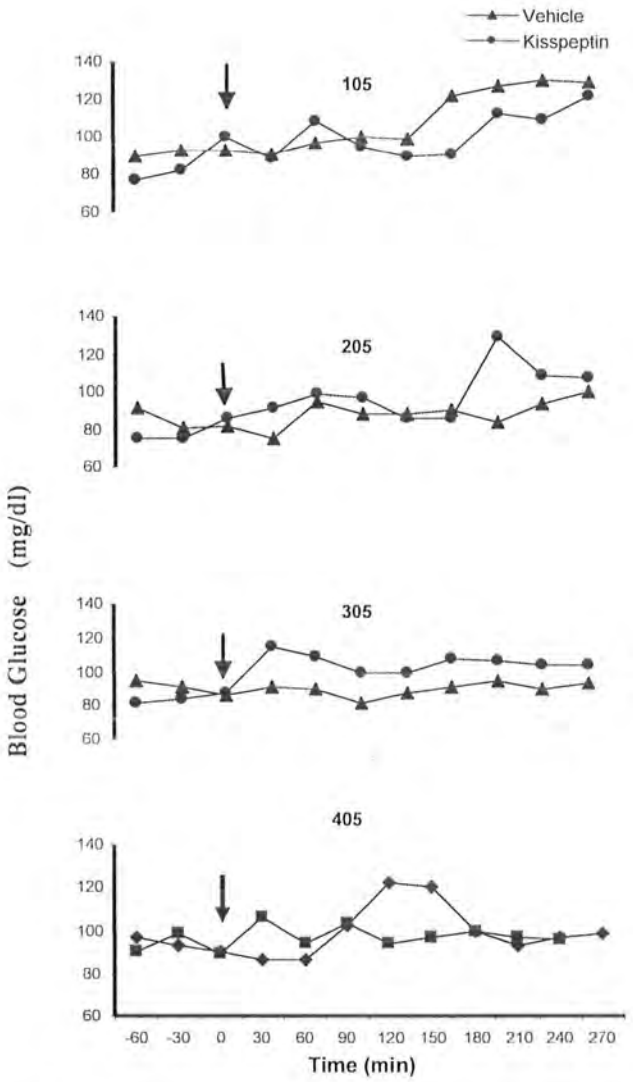

Fig. 8. Effect of kisspeptin on blood glucose concentration in individual male rhesus monkeys. Arrows indicate the treatment time.

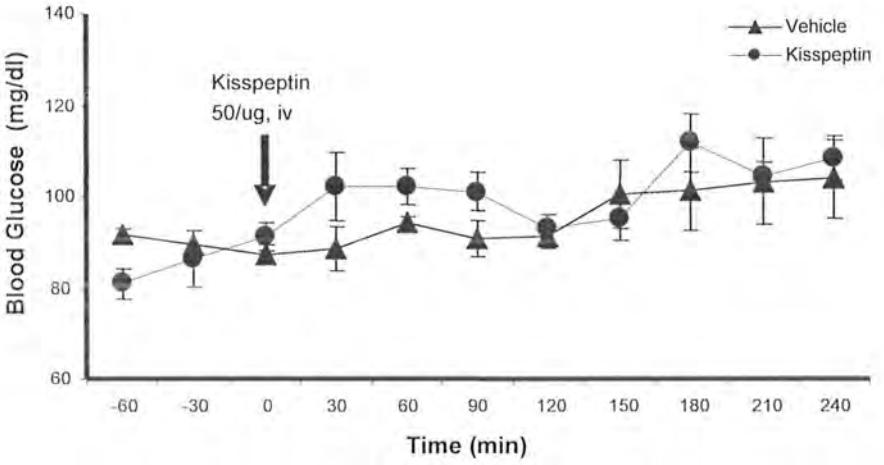

Fig. 9. Changes in mean (± SEM) blood glucose concentrations in adult male intact rhesus monkeys (n=4) given vehicle or kisspeptin. Arrow indicates the time of kisspeptin administration.

DISCUSSION

Recent studies have now established that kisspeptin-GPR54 signaling is important in regulation of reproductive axis in primates and rodents. However, all such studies have focused on central effects of kisspeptin. Locus of kisspeptin action on levels other than hypothalamus has not been systematically assessed in primates. In this regard, an action at pituitary level of kisspeptin is likely as indicated by presence of GPR54 in the pituitary. However ability of GnRH receptor antagonist to block actions of central as well as peripheral kisspeptin administration has been interpreted as that effect of kisspeptin on reproductive axis is mediated via GnRH release and is not a direct pituitary action. Furthermore, all primate studies have utilized castrated animals (Plant et al, 2006; Shahab et al, 2005; Seminara et al, 2006) and therefore terminal signal of HPT axis i.e., testosterone has not been assessed with regard to effect of kisspeptin administration. Human testes has been shown to express moderate level of GPR54 transcripts and interestingly a robust expression of *KiSS-1* (Ohtaki et al, 2001; Kotani et al, 2001; Muir et al, 2001). This observation would suggest that kisspeptin may have a local effect on primate testis. Parenthetically, many neuropeptides have been shown to be present in primate testis (Skinner , 1991; Saez , 1994) and implicated in paracrine regulation of the testicular function.

Therefore, in the current study we assessed a direct role of kisspeptin in testicular endocrine function particularly testosterone secretion in the rhesus monkey, a representative higher primate. The paradigm used to isolate local

action of kisspeptin was that of pituitary gonadotropin clamped monkey model with pretreatment with acyline, a GnRH receptor antagonist.

The seminal findings of the present study was that plasma testosterone concentrations in adult male rhesus monkeys determined under the influence of GnRH receptor antagonist were not found to be affected by peripheral kisspeptin-10 administration. This result contradicts our hypothesis that kisspeptin might have a direct local endocrine effect in terms of testosterone secretion at testicular level. Our finding of remarkable discharge of plasma testosterone after peripheral administration of kisspeptin-10 in male adult rhesus monkeys not treated with acyline is consistent with earlier observation though on LH secretion (Shahab et al, 2005) and therefore rules out the possibility that the dose of kisspeptin employed in the current study was inadequate. That the apparent lack of action of kisspeptin on the testis can attributed to unresponsiveness of Leydig cells was clearly ruled out as hCG administration in all clamped animals resulted in robust discharges of plasma testosterone. However, since GPR54 is expressed in testes (Ohtaki et al, 2001; Kotani et al, 2001; Muir et al, 2001) it remains likely that kisspeptin may have other cognate functions in the primate testis. Two possibilities stand out. First is that kisspeptin may affect the responsiveness of Leydig cell to LH stimulation as is the case with many locally expressed neuropeptides in the testis. Secondly, kisspeptin may be involved in regulating spermatogenesis. The latter appears to be supported by recent observations that kisspeptin affects cell cycle (Becker et al, 2005). During spermatogenesis, spermatozoa pass through cell divisions and different stages of cell cycle as well as undergo apoptosis and it could be possible that kisspeptin might exert an effect on spermatogenesis. We suggest that it is quite possible that kisspeptin at testicular level doesn't affect androgen production but rather exerts an action on other pathways or processes like spermatogenesis or manipulating testicular

response to gonadotropins. A concerted set of experiments utilizing in vitro pharmacological and immunocytochemical approaches and gene expression approaches will be helpful in elucidating local role of kisspeptin in the primate testis. Currently our results reaffirm that predominant pathway through which kisspeptin-GPR54 signaling affect reproductive axis in primates is through stimulation of GnRH neurons in the hypothalamus.

It was reported previously that GPR54 is expressed in pancreas (Kotani et al, 2001; Muir et al, 2001; Lee at al, 1999). Given endocrine effects of kisspeptin, it may be speculated that kisspeptin may also modulate pancreatic endocrine functions. However, our present study failed to demonstrate any significant affect of kisspeptin-10 on pancreatic endocrine function as indirectly inferred from no obvious modulation of blood glucose by kisspeptin administration in adult male monkeys.

An interesting preliminary observation we made during this study was that kisspeptin administration appeared to suppress the stress-related rise of plasma cortisol levels. Mean cortisol levels were lower in kisspeptin treated animals as compared to vehicle given animals. Although the animals used in the current study were habituated to chair restraint but some stress signals may still have been operational. It is possible that kisspeptin-10 acts at hypothalamic neurons important in stress responsiveness and may lessen activation of hypothalamic-pituitary-adrenal axis. Although we are not clear about the exact pathway through which kisspeptin-10 leads to reduced stress response but our these preliminary result does raise the interesting notion that kisspeptin-GPR54 signaling may also be involved in fine tuning of stress response in the primate hypothalamus. Clearly, however more studies are required to characterize and elucidate such a novel role of kisspeptin.

Taken together, our results suggest that kisspeptin administration induces a robust stimulation of testosterone secretion in adult male monkeys demonstrating that the HPT axis is strongly stimulated by kisspeptin. The effect is not localized at the testicular levels but secondary to activation of GnRH-gonadotropic axis. Our results also suggest a possible involvement of kisspeptin-GPR54 signaling in stress related regulation of hypothalamic-pituitary-adrenal axis.

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