Peripheral Administration of RF9 Does Not Affect Hypothalamic-Pituitary-Gonadal Axis in Normal Fed Adult Male Macaque

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Abstract

Stress represses hypothalamic-pituitary-gonadal axis (HPG-axis) but RF9, a synthetic peptide, rescues such repression. To assess the role of RF9 in regulating HPG-axis under normal physiological conditions in higher primates, RF9 was administered to intact adult male rhesus monkeys and response of the HPG-axis was examined by measuring plasma testosterone as an end parameter of the axis. Control group (n=4) received normal saline whereas the treated group (n=4) received RF9. On the first day of experiment, four bolus injections of normal saline (1ml/animal) were administered intravenously at 2-hour interval to the control monkeys. Similarly, on the second day of experiment, treated group received four iv bolus injections of RF9 (0.1mg/kg BW) at 2-hour interval. Serial blood samples were collected at 20 min interval during a 6-hour period which started just after first saline/RF9 injection. Plasma testosterone levels were measured by using a specific EIA. Overall means of plasma testosterone levels and plasma testosterone area under curve (AUC) and overall mean testosterone and mean testosterone AUC in short time windows following each injection of RF9 and saline were comparable between the groups. Our results demonstrate that RF9 has no role in regulating HPG-axis under normal physiological conditions in adult male monkeys.

Introduction

Reproduction is regulated by a complex signaling interplay, existing in the three components of hypothalamic-pituitary-gonadal axis (HPG-axis) including the hypothalamus, the pituitary, and the gonads [1-4]. Hypothalamic gonadotropin releasing hormone (GnRH) regulates the secretion of pituitary luteinizing hormone (LH) and follicle stimulating hormone (FSH) that in turn regulate gametogenesis and steroidogenesis in gonads [5, 6]. Gonadotropin inhibitory hormone (GnIH) and kisspeptin are the afferent regulators of GnRH secretion. GnIH is the inhibitor while kisspeptin is the elicitor of GnRH secretion and reproduction [7, 8]. Mammalian orthologs of GnIH are termed as RFamide related peptide-1 (RFRP-1) and RFRP-3 as they have RFamide motif at their C-terminus. We have reviewed that stress represses reproductive axis through up-regulating GnIH and down-regulating kisspeptin expression [7, 8]. Importantly, one of our previous studies showed that RF9 rescues stress induced repressed HPG-axis [7-9].

Similarly, RF9 administration has also increased gonadotropin expression under normal physiological conditions in various animal models [10, 11]. Originally, RF9 was developed as an antagonist of the neuropeptide FF receptors (NPFFRs) [NPFF1R or GPR147 and NPFF2R or GPR74], activated by various neuropeptides including GnIH [12]. NPFF1R or GPR147 has been reported as a receptor for mammalian GnIH [13, 14]. However, recent studies showed that RF9 not only antagonizes NPFF mediated hyperalgesia but similar to NPFF, it stimulate food intake [12, 15]. Importantly, RF9 acts as an agonist of kisspeptin receptor (GPR54) on GnRH neurons and stimulate GnRH firing rate independent of RFRP-3 [16, 17]. Foregoing data collectively suggest that RF9 is involved in the regulation of HPG-axis during stress
conditions. However, a functional role of RF9 in normal healthy higher primates has not been investigated. In light of above evidences, we tried to determine the role of RF9 in the regulation of HPG-axis in normal fed adult male rhesus monkeys. In the present study, the effects of RF9 administration on HPG-axis were indirectly monitored by tracking changes in the levels of plasma testosterone which is the terminal endocrine product of the axis.

Materials and Methods

I Animals

Eight adult intact male rhesus monkeys (Macaca mulatta), weighing on average±SEM 10.38±0.24 kg were used in this cross sectional study. The animals were housed in individual cages, under standard colony conditions in the Department’s Primate Facility. Our previous studies have described the housing, feeding, chair-restraint-training, and sedation of the animals [18-20].

II Pharmacological Agents

RF9 (1-Adamantanecarbonyl-Arg-Phe-NH$_2$), a trifluoroacetate salt, was synthesized by Chinapeptides (Shanghai, China). Working solutions of RF9 were prepared in normal saline (0.9% NaCl). Heparin and ketamine (Rotex medica, Trittau, Germany) were purchased locally.

III Venous Catheterization

To permit sequential withdrawal of blood samples and iv administration of vehicle/RF9, the animals were anesthetized with ketamine (5 mg/kg BW, im). A Farcocath iv cannula (0.9mm/22G; Medical industries SAE, Alexandria, Egypt) was inserted in the saphenous vein. The distal end of the cannula was attached to the syringe via a butterfly tube (length 300mm, volume 0.29ml 20GX3/4”, JMS, Singapore). Experiment was not initiated until the animals became fully active.

IV Treatment

The animals were randomly divided into two experimental groups (n=4). Control group received normal saline (0.9% NaCl) as vehicle containing 0.0012% dimethyl sulfoxide (DMSO). Treated group received RF9, dissolved in normal saline containing 0.0012% DMSO. On the first day of experiment, control group (n=4) received intravenously four bolus injections of normal saline (1ml/animal) containing DMSO at 2-hr interval and was subjected to a 6-hr serial blood sampling at 20 min interval. Blood sampling was initiated 60 min before injection of the first saline bolus. On the second day of experiment, treated group animals (n=4) were similarly given four iv bolus injections of RF9 (0.1mg/kg BW) at 2-hr interval and bleed as above. The experiment was approved by the Departmental Committee for Care and Use of Animals.

V Blood Sampling

Blood samples (1.5ml) were collected in heparinised (lithium-salt) syringes (Becton Dickinson Pvt Ltd, Lahore, Pakistan). Following withdrawal of each sample, an equal volume of heparinised normal saline was injected to avoid hypovolemic stress. The blood samples were centrifuged within 60 min after completion of the bleed, at 3500 rpm at 4°C in a Kokusan refrigerated centrifuge (Model H-103RS, Kokusan enshinki. Co. Ltd., Tokyo, Japan) for 15 min. Blood plasma was separated and stored at -20°C until hormonal analysis.

VI Enzyme Immunoassay (EIA) of Testosterone

Plasma testosterone concentrations were determined by using an EIA kit for human testosterone (MicroLISA, Amgenix Inc, San Jose, CA, USA). The assay was carried out as described in the protocol provided with the kit. The sensitivity of testosterone assay was 0.05ng/ml. Inter- and intra-assay coefficients of variation based on quality control samples were <9% and <10%, respectively.

VII Statistical Analyses

All the data are presented as mean±SEM. Area under the curve (AUC) which is the measure of hormone secreted over a period of time was calculated for testosterone using a trapezoidal method. Mean of plasma testosterone levels and plasma testosterone AUC after saline and RF9 injections were compared by using unpaired t-test. Variation in the mean testosterone concentration after saline and RF9 administrations during a 6-hr serial sampling period was measured by using repeated measures one-way ANOVA followed by post hoc Dunnett’s multiple comparison test. Statistical significance was set at P<0.05. Data were analysed by using GraphPad Prism version 5.01 (GraphPad Software Inc., San Diego, CA, USA).

Figure 1: Pattern of changes in mean±SEM plasma testosterone levels in adult male rhesus monkey (n=4) before and after A) RF-9 administrations and B) saline administrations. Arrows indicate the time of administration of iv bolus RF-9 or saline injections. One-way ANOVA with repeated measures showed no significant changes in plasma testosterone levels at different time points in both cases.
Results

Pattern of changes in mean plasma testosterone levels during a 6-hr period in RF9 and saline treated monkeys is shown in (Figure 1). One-way ANOVA with repeated measures did not reveal any significant change in the mean testosterone levels in both RF9 and saline treated animals. Comparison of mean testosterone AUC and mean testosterone levels observed in post RF9 and saline treatment period is given in (Figure 2). Unpaired t test analyses indicated that the mean testosterone AUC and mean testosterone levels were statistically comparable in RF9 and saline injected monkeys. A comparison of mean testosterone AUC and mean testosterone levels in small time windows, each after one hour of four consecutive injections of RF9 and saline, is given in (Figure 3). The mean testosterone AUC and mean testosterone responses to each of four RF9 or saline injections remained unchanged as revealed by one-way ANOVA with repeated measures.

Figure 2: A) Comparison of mean±SEM AUC for plasma testosterone and B) mean±SEM plasma testosterone concentrations during a 6-hr period, during which monkeys (n=4) received four iv bolus injections of RF-9 and saline. Unpaired t test analyses revealed no significant differences in the mean level in both cases.

Figure 3: A) Comparison of mean±SEM and B) mean±SEM AUC plasma testosterone levels observed during 0-60 min, 60-180 min, 180-300 min and 300-360 min windows following first, second, third and fourth injections of RF-9 or saline. Repeated measures ANOVA indicated non-significant differences in mean testosterone and mean testosterone AUC responses to RF-9 or saline injections.

Discussion

The present study was conducted to investigate the role of RF9 in the regulation of HPG-axis under normal physiological conditions in higher primates. This was done by administering RF9 to adult male rhesus monkeys and assessing the testosterone response as a terminal endocrine marker of the HPG-axis. We could not measure LH in the present study due to non-availability of the rhesus monkey LH assay. However, plasma testosterone levels strictly mimic LH levels and, therefore, can be used indirectly to provide a status of LH secretion. The main finding of the present study was that iv administration of RF9 had no effect on plasma testosterone levels in the adult male rhesus monkeys. This finding suggests that RF9 does not participate in minute to minute regulation of HPG-axis in the adult male rhesus monkeys. Our finding is contradictory to results observed in previous studies carried out in other species like ewe and rat [11, 21].

The dose of RF9 employed in the present study was based on our previous studies in which RF9 relieved fasting induced suppression of HPG-axis as evidenced by increasing plasma testosterone levels and stimulated HPA-axis by increasing plasma cortisol in normal fed adult male rhesus monkeys [9, 22]. These data suggest that the dose of RF9 used in the current study was effective. There can be a number of reasons of contradictory results observed in the present study in contrast to those found in previous studies. One very important fact is the species difference. Secondly, in the previous studies in rats and ewes, both central and systemic administration of RF9 was found to stimulate HPG-axis but in the current study, only systemic administration was assessed [10, 11]. As RF9 cannot cross blood brain barrier, therefore, it can also be speculated that systemic administration of RF9 might not have been effective in this study. Further studies are required to assess central administration of RF9 in primates. It is important to point out that...
initially RF9 was developed as an antagonist of neuropeptide FF receptors, stimulated by various peptides including GnIH.

Recent studies, however, have reported that FR9 has multiple and off-target actions and acts as an antagonist at some receptors and as agonist at others [8, 9, 12, 15, 16]. We recently demonstrated that RF9 stimulate HPA-axis in normal fed monkeys [22]. On the other hand, RF9 acts as an antagonist of NPFF receptor and thus relieves NPFF induced hyperalgesia but paradoxically, it also increases food intake like that of NPFF [12, 15]. Furthermore, it has been reported that RF9 acts as an agonist of kisspeptin receptor (GPR54) on GnRH neurons and increases GnRH neurons firing rate independent of GnIH [16, 17]. As RF9 acts both as an agonist and antagonist at different receptors, therefore it can be postulated that the concomitant activation and inhibition of different receptors by RF9 interact in such a way that it has no net effects on HPG-axis during normal fed conditions. Accordingly, RF9 stimulates GnRH secretion that positively regulates HPG-axis but it also increases cortisol in normal fed monkeys that inhibits reproduction [7, 8, 16, 22].

The primate HPG-axis has been shown to be greatly suppressed during stress, non-breeding season and lactation [9, 23-25]. Interestingly, a bolus injection of RF9 resulted in a rapid and sustained increase in plasma LH levels during anoestrus season in ewe [11]. Additionally, RF9 administration can rescue stress induced HPG-axis in adult male rhesus monkeys but have no effect on HPG-axis in normal fed monkeys (current study) [9]. During the anoestrus period, reproductive axis remains suppressed. Thus it appears probable that RF9 administration may stimulate reproductive axis during anoestrus and stress conditions. The foregoing evidences would, therefore, suggest that though RF9 may not be involved in regulation of the HPG-axis in normal conditions it may become important in conditions when the axis is suppressed. However, it is important to assess the effects of central administration of RF9 on HPG-axis in primates under normal physiological conditions.

In summary, present study revealed that brief repetitive systemic injections of RF9 did not affect plasma testosterone levels in healthy and normal fed adult male rhesus monkeys. This finding suggests that RF9 has no role in the regulation of HPG-axis under normal physiological conditions in the adult male monkeys. However, it remains possible that RF9 may become important in rescuing suppression of the HPG-axis in stress, lactation, non-breeding season and during prepuberty in higher primates. Because of multiple and off-target actions of RF9, it is recommended to design further studies for examining and exploring the exact mechanism of RF9 driven regulation of HPG-axis through its agonistic and antagonistic actions.

Data Availability

Not available.

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Conflicts of Interest

None.

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