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Decrease in hypothalamic *Kiss1* and *Kiss1r* expression: a potential mechanism for fasting-induced suppression of the HPG axis in the adult male rhesus monkey (*Macaca mulatta*)

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Abstract

Fasting suppresses functioning of the hypothalamic-pituitary-gonadal (HPG) axis by mechanisms that are incompletely understood. In 2003, hypothalamic kisspeptin-Kiss1r signaling was discovered to play a significant role in regulating the HPG axis. We have recently shown that in adult male macaques, short-term fasting attenuates the response of the HPG axis to an exogenous kisspeptin challenge. In the present study, we explored the mechanism underlying this attenuated response by examining the modulation of the hypothalamic expression of *Kiss1* and *Kiss1r* under short-term fasting and normal feeding conditions in the adult male macaques. Hypothalamic mRNA was extracted from normal fed (n=3) and 48-h fasted (n=3) monkeys. *Kiss1, Kiss1r, and GnRH1* mRNA was quantified by reverse transcription followed by real-time polymerase chain reaction. In addition, blood samples were collected for measurement of plasma concentrations of glucose, cortisol, leptin, and testosterone. In contrast to fed animals, plasma glucose, leptin, and testosterone levels decreased and cortisol levels increased in fasted animals. The hypothalamic expression of *Kiss1* and *Kiss1r* mRNA was significantly lower ($P<0.05$) in fasted monkeys compared to fed monkeys while hypothalamic *GnRH1* mRNA expression was comparable between the two groups. Thus, our results demonstrate that expression of hypothalamic *Kiss1* and *Kiss1r* decrease after a short-term fasting in monkeys. This decrease may contribute to the suppression of the HPG axis during fasting conditions in primates. In addition, our finding of lower expression of *Kiss1r* in fasted monkeys provides an explanation for the attenuation in the HPG axis response to peripheral kisspeptin challenge during short-term fasting.

Keywords

Fasting; HPG axis; GnRH1; Kiss1; Kiss1r; Testosterone; Monkey

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Introduction

Kisspeptin is a family of peptide hormones which belongs to RFamide superfamily of peptides [1,2]. All members of kisspeptin family are encoded by the *Kiss1* (non-human)/ *KISS1* (human) gene [1]. All kisspeptins act through a G protein couple receptor, GPR54 (also named as AXOR12 or hOT7T175), which is now consensually familiarized as KISS1R/ Kiss1r [1-4]. Kisspeptin is involved in many important physiological processes including a prominent role in the regulation of reproduction [5-10].

Kisspeptin is a powerful stimulator of the hypothalamic-pituitary-gonadal (HPG) axis in several vertebrate species including mammals [11-16]. Kisspeptin exerts its stimulatory effect by increasing signaling through GnRH receptor because pre-treatment with acyline, a GnRH receptor antagonist, blocks kisspeptin induced stimulation of the HPG axis [13,15,17]. *Kiss1r* is expressed in several regions of hypothalamus including GnRH neurons in several mammalian species [18-20]. Similarly, many studies have demonstrated *Kiss1* expression in the hypothalamus of non-primate [14,21], non-human primate [15], and human [3] species.

The hypothalamic expression of *Kiss1* and *Kiss1r* mRNA responds to both external and internal cues including steroid milieu, seasonal variation and metabolic status [22-25]. In the rat, short-term fasting decreases hypothalamic *Kiss1* and increases *Kiss1r* mRNA expression [26]. In contrast, in the mouse, short-term fasting decreases expression of both *Kiss1* and *Kiss1r* genes [27]. Like wise, diabetic rats (by STZ injection) display decreased expression of *Kiss1* mRNA in the hypothalamus [28]. Lactation is also known to significantly reduce expression of hypothalamic *Kiss1* in rats [29].

Quantification of hypothalamic *Kiss1* and *Kiss1r* expression in states of energy imbalance in primates has not yet been examined. Recently, we demonstrated that short-term fasting cause attenuation in the response of the HPG axis to exogenous kisspeptin administration in the adult male rhesus monkey [30] but the mechanism underlying this attenuated response is unclear. Therefore, the purpose of this study was to examine hypothalamic *Kiss1r* mRNA expression under feeding *ad libitum* and short-term fasting conditions in the adult male rhesus monkey. We hypothesized that *Kiss1* and *Kiss1r* mRNA contents will be lower in fasted monkeys as compared to fed *ad libitum* monkeys.

MATERIAL AND METHODS

Animals

Six adult intact male rhesus monkeys (*Macaca mulatta*), 6-9 years old, weighing 8.3 –14.7 kg were used in this study. The animals were housed in individual cages, in the same room, under temperature (25±3°C)- and light (06:00-18:00)- controlled conditions in the Department of Animal Sciences Primate Facility. The animals were fed daily with fresh fruits (0900-0930 h), hard boiled eggs (1100 h) and bread (1300-1330 h). Water was available *ad libitum*. All animal experiments were approved by the Departmental Committee for Care and Use of Animals.

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Hypothalamic tissues and blood samples collection

Hypothalamic tissues were obtained from six monkeys. Three of the animals were 48 hours fasted and three were normal fed at the time of euthanasia. The animals were sacrificed after intramuscular injection of ketamine hydrochloride (Ketler, Astarapin, Germany 10 mg/kg BW i.m.) and followed by a 3-5 ml intravenous injection. The brain was removed from the cranium immediately. The hypothalami (MBH and POA) were then extracted out from the brain using dissection scheme described for the rhesus monkey before [31]. Each hemi-hypothalamus was transferred to TRIzol (1ml; Invitrogen) filled tubes and placed on −180°C until RNA extraction. In addition, blood samples were taken from these animals for hormones analysis.

Real-Time PCR

Kiss1, Kiss1r, and *GnRH1* mRNA expression in hypothalamic tissues from male rhesus monkeys were quantified by real-time PCR. After reverse transcription of total RNA, 1 µl of 10 ng/µl cDNA was used for real-time PCR. Quantity of each gene mRNA expression was determined from a relative standard curve. Relative standard curves were generated by diluting one hypothalamic sample, having highest mRNA expression, 1:0, 1:1, 1:2, 1:4, 1:8 times. Each sample was run in triplicate along with a positive control (rhesus monkey normal tissue placental cDNA; BioChain Institute, Hayward, CA 94545, USA) and a negative control. The threshold cycle number (CT) from each sample was referred to standard curve to estimate the corresponding RNA content, and each RNA value was then normalized for procedural losses by using the ß-actin RNA values estimated from the relative standard curve.

The *ACTB, GnRH1, Kiss1r,* and *Kiss1* primers and fluorescent probes used were designed to target a segment comprised within the cloned cDNA. All primers and fluorescent probes for real-time PCR were selected by use of the program Primer3 (Primer3 Web site; [32]). The accession number of genes used for primers and probes selection are; *Kiss1* (AY823262), *Kiss1r* (AY823261), *GnRH1* (S75918), and *ACTB* (AY369786). The primer sequences (Integrated DNA Technologies) for *Kiss1r* were 5′ forward (5′-CTCGCTGGTCATCTACGTCA-3′) and 3′ reverse (5′-CGAACTTGCACATGAAATCG-3′). The internal fluorescent oligodeoxynucleotide probe sequence was 5′-ACAAGCCGATGCGGACCGTGA-3′ (Integrated DNA Technologies) and was covalently linked to the fluorescent dye HEX at the 5′ end and to the quencher dye Black Hole Quencher-1 at the 3′ end. The *Kiss1* real-time PCR primers used were 5′ forward (5′-CTGGAATCCCTGGACCTCTC-3′) and 3′ reverse (5′-TTGTAGTTCGGCAGGTC CTT-3′). The internal fluorescent oligodeoxynucleotide probe was 5′-GAGGAAGCCGTCTGCTACTG-3′. The *GnRH1* real-time PCR primers used were 5′ forward (5′-AGATGCCGAAAATTTGATGG-3′) and 3′ reverse (5′-TTTCCAGAGCTCCTTTCAGG-3′). The internal fluorescent oligodeoxynucleotide probe was 5′-AACTGGCAGAAACCCAACAC-3′. The *ACTB* real-time PCR primers used were 5′ forward (5′-GCTTCTAGGCGGACTGTGAC-3′) and 3′ reverse (5′-CACCTTCACCGTTCCAGTTT-3′). The internal fluorescent oligodeoxynucleotide probe was (5′-GATGAGATTGGCATGGCTTT-3′).

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Real-time PCRs were performed in a total volume of 20 μl, each reaction containing 1 μl of the reverse transcribed sample or 1, 0.5, 0.25, 0.125, 0 dilution of one of hypothalamic sample, 10 μl of AmpliTaq Gold PRC Master Mix (Applied Biosystems/Roche Molecular System), 0.8 μl of each primers and probe, 0.3 μl of reference dye, and 5.3 μl DEPC water. The real-time PCR program consisted of an initial annealing period of 2 min at 50°C, followed by 10 min of denaturing at 95°C and 40 cycles of 15 sec at 95°C and 1 min at 60°C.

Hormone and blood glucose measurements

Plasma T and cortisol concentrations were determined by specific RIAs. The T and cortisol RIA kits were purchased from Immunotech Marselle Cedex 9, France and Immunotech Prague 10, Czech Republic, respectively. The RIAs were done as per manufacturer instructions. The bound radioactivity was determined by using Beckman Gamma counter (Gamma 5500). Sensitivity of the T assay was 0.025 ng/ml and intra- and inter-assay coefficients of variation were both below 15%. Sensitivity of the cortisol assay was 0.82 μg/dl and the intra-assay coefficient of variation was below 6%. Leptin was measured using ELISA kits (AssayMax Human ELISA; Assaypro 41 Triad south drive St. Charles, MO). The sensitivity of the leptin assay was < 150 pg/ml; the intra- and inter-assay coefficients of variation were below 8%. Glucose concentrations were measured in drops of whole blood by using Oncall-plus blood glucometer (ACON laboratories, San Diego, CA, USA).

Statistical analysis

Statistical comparisons for the mean glucose, cortisol, leptin, and T levels under fasting and fed conditions were made by paired student's t tests. *Kiss1*, *Kiss1r*, and *GnRH1* mRNA contents were also compared by paired student's t tests. All data are presented as mean (±SEM). Results were considered statistically significant at $P<0.05$.

Results

Mean basal plasma glucose, cortisol, leptin, and T are shown in figure 1. As compared to fed monkeys, mean plasma glucose and testosterone levels decreased and cortisol levels increased in fasted animals.

The comparison of hypothalamic *Kiss1*, *Kiss1r*, and *GnRH1* mRNA contents under fed and fasting conditions are illustrated in figures 2-4. The hypothalamic expression of *Kiss1* mRNA was significantly lower ($P<0.01$) in fasted monkeys compared to fed monkeys (fig. 2). Similarly, the hypothalamic expression of *Kiss1r* mRNA was also significantly ($P<0.03$) lower in fasted monkey compared to fed monkeys (fig.3). While no difference was observed between hypothalamic *GnRH1* mRNA contents of fed and fasted animals fig.4.

Discussion

Kisspeptin has been hypothesized to serve as a major conduit for relaying information regarding metabolic status to centres of the brain that control the HPG axis [25]. Conditions of energy imbalance suppress the HPG axis, in part, through a decrease in the hypothalamic expression of *Kiss1* and or *Kiss1r* mRNA [25,27,33]. To the best of our knowledge, there is

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no study available on the quantification of hypothalamic *Kiss1* and *Kiss1r* mRNA in metabolically deficient conditions like fasting, in primates. In the present study, we quantified hypothalamic *Kiss1* and *Kiss1r* mRNA under normal feeding and short-term fasting conditions in the adult male rhesus monkey. Our results demonstrated that the expression of hypothalamic *Kiss1* and *Kiss1r* mRNA was significantly lower in the fasted animals as compared to fed animals. In contrast, no significant difference was observed between the hypothalamic *GnRH1* mRNA content of fed and fasted animals.

This decrease in hypothalamic *Kiss1* expression may contribute to the suppression of the HPG axis during fasting conditions in primates. Recently, a large number of studies in rodents demonstrated that lower expression of hypothalamic *Kiss1* mRNA was associated with reproductive dysfunctions in metabolic conditions of energy imbalance [reviewed in 25]. While exogenous administration of kisspeptin reversed the effect of energy imbalance on the HPG axis by significantly increasing the reproductive hormones secretion [25]. In the adult male rhesus monkey, recently, we have shown that exogenous kisspeptin averted the effect of short-term fasting on the HPG axis [30]. Kisspeptin administration significantly increased plasma T secretion in fasted monkeys in the latter study. Taken together, these findings suggest that low expression of *Kiss1* in fasting conditions can be a factor that may lead to decrease in the endogenous drive of kisspeptin to the HPG axis while exogenous kisspeptin may compensate for this decrease in the endogenous drive. However in our earlier study [30], several aspects of T response to KP were significantly reduced in fasted monkeys. Mean plasma T concentrations as well as T AUC for the 3 h after KP administration period was significantly reduced in fasted animals indicating lesser T response to KP stimulation. Similarly, the time taken to elicit first significant rise in T secretion following administration of KP was significantly greater in fasted animals, indicating that T response to KP administration was significantly delayed by fasting [30]. The mechanism for this attenuation in the HPG axis response to peripheral kisspeptin challenge was not clear. However, the causation of the attenuation was of hypothalamic origin as pituitary response to GnRH and testicular response to hCG were comparable in fed and fasted monkeys [30,34-36]. Our finding in the present study that the hypothalamic *Kiss1r* mRNA expression is also significantly decreased, therefore, provides an explanation for the attenuation in the HPG axis response to peripheral kisspeptin challenge during short term fasting condition in the adult male rhesus monkey.

Available evidences suggest that the HPG axis remains intact during fasting conditions. Testicular response to hCG and pituitary response to GnRH remained normal during short-term fasting conditions [30,34-36]. Similarly, hypothalamic *GnRH1* mRNA and contents are not affected by fasting-related metabolic deficiency [37-40]. Our present results also showed that *GnRH1* expression was comparable between fed and fasted monkeys, suggesting that suppression of the HPG axis during fasting conditions did not involve modulation of *GnRH1* mRNA expression.

The metabolic signal generated by fasting condition that may cause decreased expression of hypothalamic *Kiss1* and *Kiss1r* mRNA is not known. The negative energy balance and the stress can be important potential factors in this regard. The design of the present study precluded distinction between the two. Literature indicates role of hormones of both

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negative energy balance and stress on hypothalamic *Kiss1 expression* [27,33,41-43]. Recently, leptin has been shown as a major factor responsible for the regulation of *Kiss1* expression [33]. Similarly, cortisol has also been shown to modulate the expression of *Kiss1*and *Kiss1r* [41,42], while ghrelin has been implicated more recently to decrease expression of hypothalamic *Kiss1* [43]. Preliminary findings showed that insulin did not affect *Kiss1* expression [27]. Fasting leads to hypoleptinema but increased levels of cortisol and ghrelin. Therefore, it is possible that hormonal signals of negative energy balance and stress or both entrain reduction in expression of hypothalamic *Kiss1* and *Kiss1r* in monkeys.

In summary, our results show that the hypothalamic expression of *Kiss1* and *Kiss1r* mRNA is decreased during short-term fasting conditions in the adult male macaque. In contrast, no change in the hypothalamic expression of *GnRH1* is observed. Our findings provide an explanation for the earlier observed attenuation in the monkey HPG axis response to peripheral kisspeptin challenge after short-term fasting. The decrease in hypothalamic *Kiss1* and *Kiss1r* expression may be one of the major contributing factors in causing suppression of the HPG axis during fasting conditions in primates.

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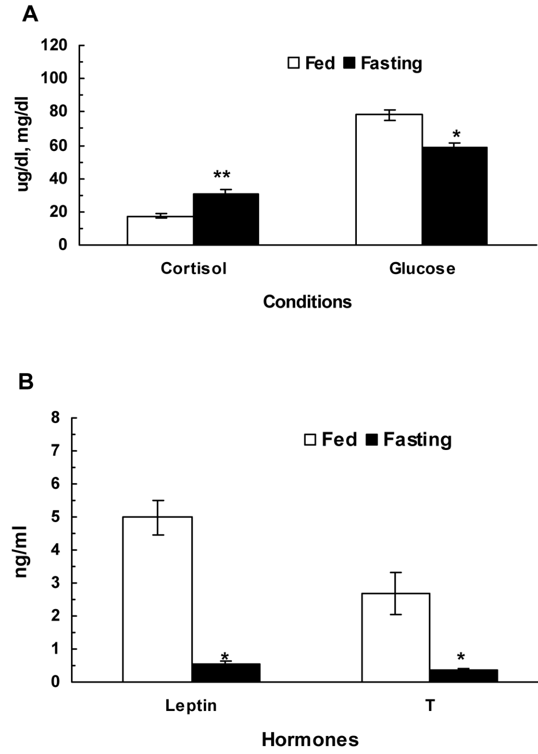

Figure 1.
Comparison of overall mean (±SEM) basal plasma cortisol (μg/dl) and glucose (A), leptin (ng/ml) and T (ng/ml) (B) concentrations in normal fed and 48-h fasted adult male rhesus monkeys (n=3). Fasting resulted in a significant reduction in mean plasma leptin and T levels (*P<0.01) while it significantly (**P<0.01) increased plasma cortisol levels (n=3). Blood glucose levels also significantly decreased in fasted animals.

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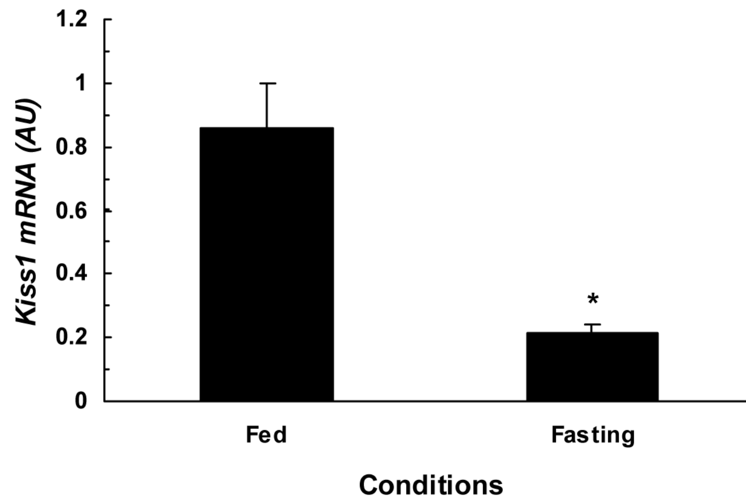

Figure 2.

Comparison of mean (±SEM) hypothalamic *Kiss1* mRNA contents in fed (1.27, 2.23, 1.64 AU) and 48-h fasted (0.47, 0.50, 0.08 AU) adult male monkeys (n=3). Hypothalamic *Kiss1* mRNA contents significantly (**P*<0.01) reduced in fasted monkeys as compared to fed ad libitum monkeys.

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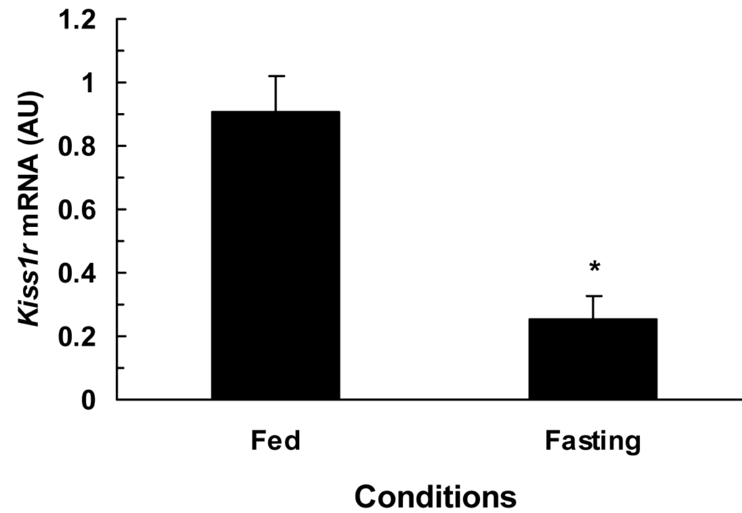

Figure 3.

Comparison of mean (±SEM) hypothalamic *Kiss1r* mRNA contents in fed (0.68, 0.99, 1.05 AU) and 48-h fasted (0.39, 0.22, 0.15 AU) adult male rhesus monkeys (n=3). Hypothalamic *Kiss1r* mRNA contents were significantly (*P<0.03) reduced in fasted monkeys as compared to fed *ad libitum* monkeys.

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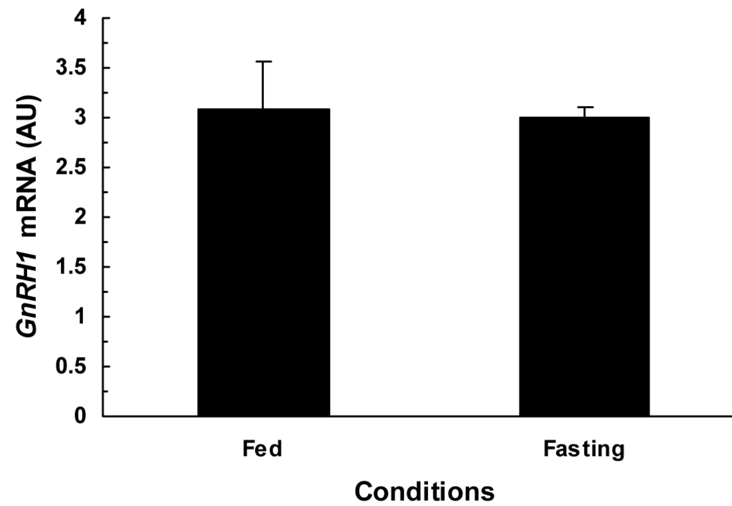

Figure 4.

Comparison of mean (±SEM) hypothalamic *GnRH1* mRNA contents in fed (2.34, 4.00, 2.88 AU) and 48-h fasted (2.97, 3.20, 2.82 AU) adult male monkeys (n=3). Hypothalamic *GnRH1* mRNA contents were comparable between fasted and fed ad libitum monkeys.