Direct Action of Genistein on the Hypothalamic Neuronal Circuits in Female Rats

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ABSTRACT : Mammalian reproduction is regulated by a feedback circuit of the key reproductive hormones such as GnRH, gonadotropin and sex steroids on the hypothalamic-pituitary-gonadal axis. In particular, the onset of female puberty is triggered by gain of a pulsatile pattern and increment of GnRH secretion from hypothalamus. Previous studies including our own clearly demonstrated that genistein (GS), a phytoestrogenic isoflavone, altered the timing of puberty onset in female rats. However, the brain-specific actions of GS in female rats has not been explored yet. The present study was performed to examine the changes in the activities of GnRH neurons and their neural circuits by GS in female rats. Concerning the drug delivery route, intracerebroventricular (ICV) injection technique was employed to eliminate the unwanted actions on the extrabrain tissues which can be occurred if the testing drug is systemically administered. Adult female rats (PND 100, 210-230 g BW) were anaesthetized, treated with single dose of GS (3.4 ㎍/animal), and sacrificed at 3 hrs post-injection. To determine the transcriptional changes of reproductive hormone-related genes in hypothalamus, total RNAs were extracted and applied to the semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). ICV infusion of GS significantly raised the transcriptional activities of enhanced at puberty1 (EAP-1, $p<0.05$), glutamic acid decarboxylase (GAD67, $p<0.01$) which are known to modulate GnRH secretion in the hypothalamus. However, GS infusion could not change the mRNA level of nitric oxide synthase 2 (NOS-2). GS administration significantly increased the mRNA levels of *KiSS*-1 ($p<0.001$), GPR54 ($p<0.001$), and GnRH ($p<0.01$) in the hypothalami, but decreased the mRNA levels of LH-β ($p<0.01$) and FSH-β ($p<0.05$) in the pituitaries. Taken together, the present study indicated that the acute exposure to GS could directly activate the hypothalamic GnRH modulating system, suggesting the GS's disrupting effects such as the early onset of puberty in immature female rats might be derived from premature activation of key reproduction-related genes in hypothalamus-pituitary neuroendocrine circuit.

Key words : Genistein (GS), Intracerebroventricular injection, GnRH, Neuroendocrine circuits, Female rats.

INTRODUCTION

Mammalian reproduction is regulated by a feedback circuit of the key reproductive hormones such as GnRH, gonadotropin and sex steroids on the hypothalamic-pituitary-gonadal (H-P-G) axis. In particular, the onset of female puberty is triggered by gain of a pulsatile pattern and increment of GnRH secretion from hypothalamus (Ebling, 2005). Though the hypothalamic GnRH secretion is a pivotal CNS signal in the regulation of puberty onset, presence of novel up-stream modulations such as kisspeptin input are now evident. Furthermore, the activity of GnRH neurons is also modulated by many environmental factors (i.e., photoperiod and chemicals) and internal cues (i.e., nutritional state and stress) (Rasier et al., 2006; Ahmed et al., 2009).

Great interest and concern about possible health threats posed by endocrine-disrupting chemicals (EDCs) have been on the rise not only in scientific societies but in public and governments. Ample of evidence is available that EDCs have adverse effects on reproduction, cancer formation, and preprogrmming of adult-onset diseases (Diamanti-Kandarakis et al., 2009). In this context, genistein (GS), a phytoestrogenic isoflavone, is unique because of its beneficiary effects particularly in aged women (Fitzpatrick, 2003). Previous studies including our own, however, indicated

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that GS also modified the female reproductive physiology including puberty onset in rats(Kouki et al., 2003; Lee & Lee, 2006a,b; McClain et al., 2007).

Since GS has weak estrogenic potential so any cells which have estrogen receptors can be activated by GS. Nonetheless, the brain-specific actions of GS on the reproduction has not been explored yet in rodents. The present study was performed to examine the changes in the activities of GnRH neurons and the related neuroendocrine circuits by GS exposure in female rats. Semi-quantitative RT-PCRs were done to measure the mRNA levels of enhanced at puberty1 (EAP1), glutamic acid decarboxylase (GAD67), nitric oxide synthase (NOS), *KiSS*-1, GPR54, GnRH in hypothalamus and common alpha subunit of glycoprotein hormone (Cα), LH beta subunit (LHβ), FSH beta suunit (FSHβ) in pituitary. Concerning the drug delivery route, intracerebroventricular (ICV) injection technique was employed to eliminate the unwanted actions on the extrabrain tissues which can be occurred if the testing drug is systemically administered.

MATERIALS AND METHODS

1. Animals and Treatments

Female Sprague Dawley rats weighing 150 g were purchased from Han-Lim Animal (Gyunggi-do, Korea) and reared in our animal facility under conditions of 12-h light/dark cycle (lights on at 07:00h) and constant temperature of 22±1℃. All protocols involved in this study were approved by the Institutional Animal Care and Use Committee at the Sangmyung university in accordance with guidelines established by the Korea Food and Drug Administration (KFDA).

2. ICV Injection

Rats (weighing 210-230 g) were anaesthetized with Zoletil50 (5 ㎎/㎏, ip injection; Virbac, France) and placed in a stereotaxic apparatus (Stoelting. USA). A single dose of GS (3.4 ㎍/10 ㎕; LC laboratories, USA) or vehicle (0.9% saline solution with 0.3 ㎍/㎕ ascorbic acid) was injected in the third ventricle (coordinates: 0.92 ㎜ posterior and 1.4 ㎜ right to bregma, and 3.2 ㎜ below the dura). Microinjection was made through a 10 ㎕ Hamilton microsyringe, and the volume of fluid injected over 5 sec was 0.5 ㎕. The dose was selected according to the previous study (Misztal et al., 2008). Animals were sacrificed at 3 hours post-injection. Tissues were immediately removed, homogenized in solution D (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarkosyl and 0.1 M 2-mercaptoethanol) and stored at －70℃ until used for RNA extraction.

3. Total RNA Preparation and RT-PCR Analyses

Total RNAs were isolated from hypothalamic samples using the single-step, acid guanidinum thiocyanate-phenol-

Table 1. Primer sets for semi-quantitative RT-PCR analyses

Gene	Nucleic acid sequence	Product size	AT (℃)
EAP-1	F 5'-AGC CCC AAC TCA TCC TCA G R 5'-ACG CTC CTG GTC TGT GCT C	380 bp	63
GAD67	F 5'-CAG CCA GAC AAG CAG TAT GA R 5'-GAG ATG ACC ATC CGG AAG AA	404 bp	60
NOS-2	F 5'-ACA CAC AGC GCT ACA ACA TC R 5'-ACA GAA GCA AAG AAC ACC GC	427 bp	69
GPR54	F 5'-ACT GTC AGC CTT AGC ATC TG R 5'-TGC TGT AGG ACA TGC AGT GA	599 bp	63
KiSS-1	F 5'-ACT GGC AAA AAT GGC ACC TG R 5'-AGT TGT AGG CTG ACA TGT CC	272 bp	66
GnRH	F 5'-CGC TGT TGT TCT GTT GAC TG R 5'-GCT TCC TCT TCA ATC AGA CG	234 bp	61
Cα	F 5'-ATA CTT CTC CAA GCT GGG TG R 5'-CGA CAC TCA GTG CCA TCG CA	294 bp	60
LH-β	F 5'-AGA TGG ACA GCC TTG TGA CC R 5'-AGG ACT GCT AGC AGC ACT GT	425 bp	69
FSH-β	F 5'-CCA TGA TGA AGT CGA TCC AG R 5'-CTT ATG GTC TCG TAC ACC AG	304 bp	63
GAPDH	F 5'-CCA TCA CCA TCT TCC AGG AG R 5'-CCT GCT TCA CCA CCT TCT TG	570 bp	50

F, forward; R, reverse; AT, annealing temperature.

chloroform extraction method (Chomczynski & Sacchi, 1987). Total RNAs were used in RT-PCR reactions carried out with Maxime™ RT PreMix (Intron, Korea) and Accu-Power PCR Premix (Bioneer, Korea) according to the manufacturer's instructions. Sequences of the gene primer sets and the annealing temperatures are given in Table 1. As internal control, parallel amplification of GAPDH mRNA was carried out in each sample. PCR-generated cDNA fragments were resolved in 1.5% agarose gels and visualized by ethidium bromide staining. Quantification of the PCR products was performed by densitometric scanning using an image analysis system (Imager Ⅲ-1D main soft ware, Bioneer, Korea), and the values of the specific targets were normalized to those of GAPDH to express arbitrary units (AU) of relative expression.

4. Statistical Analysis

Statistical analysis was performed using Student's *t*-test. Data were expressed as means±S.E., and p value < 0.05 denoted the statistically significant difference.

RESULTS

Firstly, we measured the transcriptional activities of EAP-1, GAD67 and NOS-2 which are known to act as upstream modulator of GnRH neuronal activities in the hypothalamus. ICV infusion of GS significantly raised the transcriptional activities of EAP-1 (1:1.45±0.18 AU, $p<0.05$, Fig. 1A) and GAD67 (1:1.24±0.05 AU, $p<0.01$, Fig. 1B). Thee was no change in the mRNA level of NOS-2 in the GS-treated group compare to control group (1:1.14±0.12 AU, Fig. 1C).

Secondly, the activity changes of kisspeptin-GnRH system in hypothalamus were assayed. GS administration enhanced significantly the mRNA levels of *KiSS*-1 (1:3.01±0.35 AU, $p<0.001$, Fig. 2A) and its receptor GPR-54 (1:1.29± 0.08 AU, $p<0.001$, Fig. 2B). GnRH gene expression was also significantly increased in GS-treated group compared to control group (1:1.32±0.10 AU, $p<0.01$, Fig. 2C).

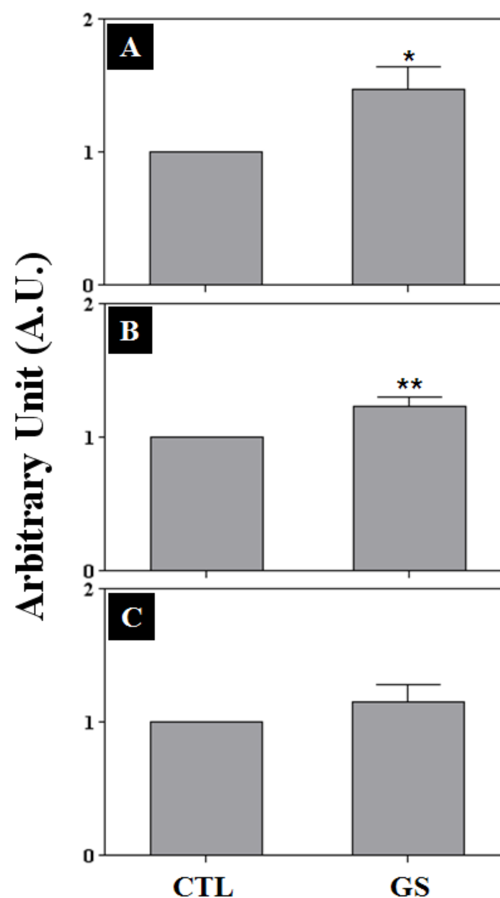

Fig. 1. Effects of GS ICV infusion on the expression of GnRH upstream modulators in the rat hypothalami. GS (3.4 ㎍/10 ㎕/animal) was ICV injected into adult female rats, and the animals were scrifieced at 3 hours post-injection. Control rats were received a same volume of saline. A, The relative ratio of EAP-1 transcript levels in hypothalamus of each groups, respectively; B, The relative ratio of GAD67 transcript levels in hypothalamus of each groups, respectively; C, The relative ratio of NOS-2 transcript levels in hypothalamus of each groups, respectively. Semi-quantitative RT-PCR was carried out as described in 'Materials and Methods'. Bars are mean±S.E. (n=6). *, Significantly different from control group, $p<0.05$; **, Significantly different from control group, $p<0.01$; CTL, control (vehicle).

Thirdly, as downstream targets of GnRH, transcriptional activities of pituitary gonadotropin subunits were measured. There was no change in the mRNA level of Cα in the

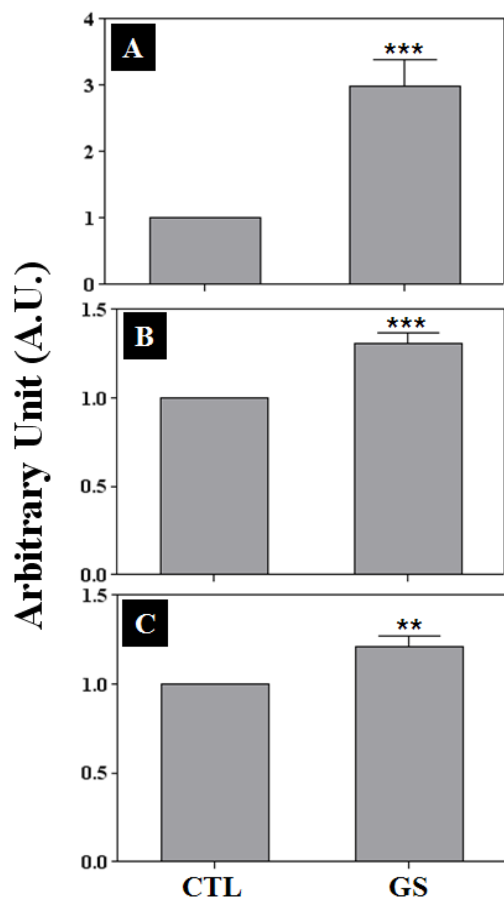

Fig. 2. Effects of GS ICV infusion on the expression of kisspeptin-GnRH system in the rat hypothalami. A, The relative ratio of *KiSS*-1 transcript levels in hypothalamus of each groups, respectively; B, The relative ratio of GPR54 transcript levels in hypothalamus of each groups, respectively; C, The relative ratio of GnRH transcript levels in hypothalamus of each groups, respectively. Semi-quantitative RT-PCR was carried out as described in 'Materials and Methods'. Bars are mean± S.E. (n=6). **, Significantly different from control group, $p<0.01$; ***, Significantly different from control group, $p<0.001$; CTL, control (vehicle).

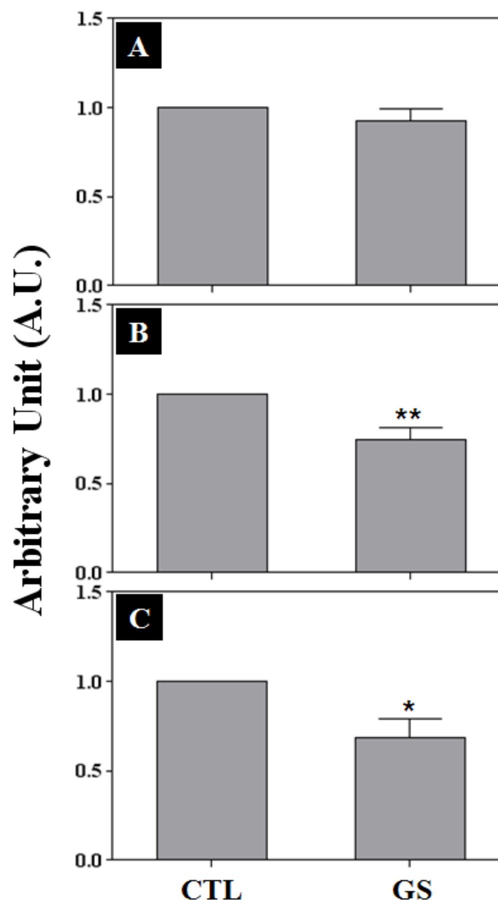

Fig. 3. Effects of GS ICV infusion on the expression of downstream targets of GnRH in the rat pituitaries. A, The relative ratio of Cα transcript levels in pituitary of each groups, respectively; B, The relative ratio of LH-β transcript levels in pituitary of each groups, respectively; C, The relative ratio of FSH-β transcript levels in pituitary of each groups, respectively. Semi-quantitative RT-PCR was carried out as described in 'Materials and Methods'. Bars are mean±S.E. (n=6). *, Significantly different from control group, $p<0.05$; **, Significantly different from control group, $p<0.01$; CTL, control (vehicle).

GS-treated group compare to control group (1:0.92±0.07 AU, Fig. 3A). However, ICV infusion of GS significantly decreased the transcriptional activities of LHβ (1:0.74±0.07 AU, $p<0.01$, Fig. 3B) and FSHβ (1:0.68±0.10 AU, $p<0.05$, Fig. 3C).

DISCUSSION

Lots of EDCs can interfere the H-P-G hormonal axis, resulting reproductive disorders such as abnormal development and early reproductive failure (Diamanti-Kandarakis et al., 2009). Recently, puberty onset in female children

tends to be progressively advanced, and a 'precocious puberty' which refers pubertal development in kids younger than age 8 years is frequently reported (Massart et al., 2006). Considerable portion of this phenomenon might be attributed to the increase of exposure to the EDCs which can alter the hormone actions in the regulation of GnRH-gonadotropin system (Parent et al., 2005). Among the EDCs, soy isoflavones are often referred to as weak estrogen and even as good EDC due to their beneficiary effects. Indeed GS, a member of isoflavones, is a relatively potent agonist for the recently cloned beta isoform of the estrogen receptor (ERβ); the nanomolar serum concentrations of free GS which can be easily achieved with daily intakes of soy-based meal in Asia (Messina et al., 2006). So one can postulate that GS intake may interfere the H-P-G hormonal axis during the critical or susceptive period of development. Our previous studies demonstrated the advanced puberty onset and activation of H-P-G axis in GS-treated female rats, supporting the idea (Lee & Lee, 2006a,b).

Most EDC research has focussed on the systemic effects using basic toxicological tools after oral or ip administration, therefore limited information is available on the direct EDC effect (s) on the central nervous system (CNS) (Diamanti-Kandarakis et al., 2009). ICV injection technique was initially developed to circumvent the impermeability of the mammalian blood-brain barrier (BBB) to some exogenously administered drugs (Cook et al., 2009). In addition, ICV administration lenders to eliminate the unwanted actions on the extrabrain tissues which can be occurred if the testing drug is systemically administered. In case of GS, the most plausible route of exposure is oral intake as food or health drink, so this phytoestrogen's effects will be exerted systematically in all tissues which have functional estrogen receptors. In the present study we employed the ICV injection so the GS's (neuro) endocrine disrupting potential could be strictly limited in the CNS particularly around the regions of 3rd ventricle. To our knowledge this is the first demonstration of GS's direct action on the hypothalamic GnRH neuronal circuits in rodents. Only a handful of studies which monitored the effects of GS ICV infusion are available using ram system (Polkowska et al., 2004; Wójcik-Gładysz et al., 2005; Misztal et al., 2008).

The mammalian hypothalamic GnRH pulse generator is functional during perinatal period, and becomes dormant during infancy and adolescence, then gradually achieves a pulsatile secretory pattern and increments of GnRH discharge compatible with the activation of the pituitary-ovary hormonal axis, and puberty is eventually initiated (Plant, 1994). Since the establishment of H-P-G hormonal axis concept which is still highly valuable in explaining the control of reproductive physiology, steroid feedback inputs from gonad are thought to be most powerful signals on hypothalamic-pituitary neuroendocrine circuits. Numerous studies, however, revealed that the picture is not so simple but very complicated with involvement of many physiological factors. Recent findings indicate that the expression of EAP1, a nuclear transcription factor that activates GnRH expression and represses proenkephalin expression, increased at puberty in both nonhuman primate and rodent hypothalamus (Heger et al., 2007). The authors also demonstrated that inhibition of EAP1 in rodent hypothalamus using lentivirus mediated siRNAs delivery technique resulted in various reproductive disorders such are delayed puberty onset and abrupt estrous cycle. Our result that the ICV delivery of GS could activate EAP1 expression in rat hypothalamus is in good agreement with the previous reports. GAD67, a catalytic enzyme for GABA synthesis, is involved in suppression of GnRH before puberty in rhesus monkey (Mitsushima et al., 1996). In the present study, direct administration of GS significantly increased GAD67 expression in the hypothalamus. One plausible explanation is that there might be differences of GS action between prepubertal and adult rats, or GS's effect on hypothalamic GAD67 expression could be minor in overall regulation of hypothalamic reproductive function. We also found that GS infusion did not alter the NOS-2 expression in female rat hypothalamus. Though the role of nitric

oxide (NO) in the control of GnRH secretion is evident (Rettori et al., 1993; Rosselli et al, 1998), GS action seems not to be mediated by NO signaling pathway. Kisspeptin and its receptor GPR54 are now universally recognized as essential activators of GnRH-gonadotropic axis, with key roles in puberty onset and the control of gonadotropin secretion (Roa et al., 2008). Our findings that GS ICV infusion significantly increased the expressions of *KiSS*-1, GPR54 and GnRH in rat hypothalamus, support the current idea. It is intriguing that GS ICV infusion decreased pituitary LHβ and FSHβ expressions in the present study. This discordance between hypothalamic Kisspeptin-GnRH activities and pituitary gonadotropin expressions could be derived from the temporal gap during sequential activation of hypothalamus-pituitary neuroendocrine circuit.

Taken together, the present study indicated that the acute exposure to GS could directly activate the hypothalamic GnRH modulating system, suggesting the GS's disrupting effect such as the early onset of puberty in immature female rats might be derived from premature activation of key reproduction-related genes in hypothalamus-pituitary neuroendocrine circuit. Further studies will be needed to elucidate the precise action mechanism of GS on the hypothalamic neural circuits governing the female reproduction.

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