Effect of 6-Hydroxydopamine (6-OHDA) on the Expression of Testicular Steroidogenic Genes in Adult Rats

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ABSTRACT : A neurotoxin, 6-hydroxydopamine (6-OHDA) has been widely used to create animal model for Parkinson's disease (PD). The present study was undertaken to examine whether depletion of brain dopamine (DA) stores with 6-OHDA can make alteration in the activities of the testicular steroidogenesis in adult rats. Young adult male rats (3 months old) were received a single dose of 6-OHDA (200 ㎍ in 10 ㎕/animal) by intracerebroventricular (icv) injection, and sacrificed after two weeks. The mRNA levels of steroidogenesis-related enzymes were measured by qRT-PCRs. Serum testosterone levels were measured by radioimmunoassay. Single icv infusion of 6-OHDA significantly decreased the mRNA levels of CYP11A1 (control:6-OHDA group=1:0.68±0.14 AU, $p<0.05$), CYP17 (control:6-OHDA group=1:0.72±0.13 AU, $p<0.05$). There were no changes in the mRNA levels of 3β-HSD (control:6-OHDA group=1:0.84±0.08 AU) and 17β-HSD (control: 6-OHDA group=1:0.63±0.20 AU), though the levels tended to be decreased in the 6-OHDA treated group. Administration of 6-OHDA decreased significantly the mRNA level of StAR when compared to the level of saline-injected control animals (control:6-OHDA group=1:0.72±0.08 AU, $p<0.05$). Treatment with single dose of 6-OHDA remarkably lowered serum testosterone levels compared to the levels of control group (control:6-OHDA group=0.72±0.24:0.13±0.03 ng/㎖, $p<0.05$). Taken together with our previous study, the present study demonstrated that the activities of hypothalamus-pituitary-testis hormonal axis could be negatively affected by blockade of brain DA biosynthesis, and suggested the reduced reproductive potential might be resulted in the animals. More precise information on the testicular steroidogenic activities in PD patients and PD-like animals should be required prior to the generalization of the sex steroid hormone therapy to meet the highest standards for safety and efficacy.

Key words : 6-hydroxydopamine (6-OHDA), Parkinson's disease(PD), Testis, Steroidogenic activities, Rat.

INTRODUCTION

With the elderly population rising sharply, rapid shift toward aging society is now evident. Concomitantly, the increased incidence of chronic neurodegenerative diseases like Alzheimer's disease and Parkinson's disease (PD) is becoming a serious social issue (Fratiglioni & Qiu, 2009). Large portion of male patients with such neurological disorders have been reported to exhibit lowered serum testosterone (Ready et al., 2004; Cherrier, 2009). On the other hand, though the rate can vary between individuals, both cross-sectional and longitudinal studies have revealed that the serum testosterone levels decline with normal aging even in healthy men (Vermeulen, 1991; Harman et al., 2001). Therefore, exploring the possibility of the cause-and-effect relationship between PD and serum testosterone deficiency in male could be an intriguing subject.

Intrabrain application of 6-hydroxydopamine (6-OHDA), a neurotoxic drug which is known to result in depletion of central dopamine (DA) stores, is widely used to create animal model for PD (Kostrzewa & Jacobowitz, 1974; Deumens et al., 2002). In the previous study, we demonstrated that the single intracerebroventricular (icv) administration of 6-OHDA significantly lowered the mRNA levels of gonadotropin-releasing hormone (GnRH) and corticotropin

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releasing hormone (CRH) in the hypothalami and the mRNA levels of common alpha subunit of glycoprotein homones (Cgα), LH beta subunit (LH-β), and FSH beta subunit (FSH-β) in the pituitaries (Heo et al., 2009). Such altered neuroendocrine function in rat PD model is not surprising because the corelation between central DA depletion and the elevated serum PRL has been well-established in PD patients (Thorner & Login, 1981).

In the present study, we hypothesized that the central DA depletion might cause the declined activities of hypothalamus-pituitary-testis (HPT) hormonal axis resulting the lowered serum testosterone level in adult, not aged, rats. It is plausible that the chronically reduced hormonal signals in hypothalamus-pituitary circuit will lead to poor testicular responses and eventually gradual decrement in HPT axis activities as shown in aged animals. To address this hypothesis, we employed the 6-OHDA injection rat model which was used in the previous study (Heo et al., 2009), and focussed on the transcriptional activities of the testicular steroidogenic enzymes and the related factor.

MATERIALS AND METHODS

1. Animals and Treatments

Male Sprague Dawley rats weighing about 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 07:00 h) and constant temperature of 22±1℃. All protocols involved in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at the Sangmyung university in accordance with guidelines established by the Korea Food and Drug Administration (KFDA).

2. ICV Injection

Rats (weighing 250-300 g; about 3 months old) were anaesthetized with Zoletil-50 (5 ㎎/㎏, ip injection; Virbac, France) and placed in a stereotaxic apparatus (Stoelting. USA). A single dose of 6-OHDA (200 ㎍ in 10 ㎕ saline/rat; Sigma-Aldrich, USA) or vehicle (0.9% saline solution with 0.3 ㎍/㎕ ascorbic acid) was injected in the right lateral ventricle (LV) (coordinates: anterior-posterior, 0.4 ㎜ poeterior to bregma; lateral, 1.4 ㎜ right to bregma; dorsoventral, 3.2 ㎜ below the dura; Paxinos & Watson, 1998). Microinjection was made through a 10 ㎕ Hamilton microsyringe, and the volume of fluid injected over 1 min was 1.0 ㎕. The injection site and the dose were selected according to the previous studies which were shown significant depletion of CA contents and lowered expression of hypothalamus-pituitary reproductive hormones (Selvage et al., 2004; Monda et al., 2007; Heo et al., 2009). After two weeks of recovery, animals were sacrificed at 1,800 hour. The testes were immediately removed, homogenized in solution D (4M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarkosyl and 0.1M 2-mercaptoethanol) and stored at −70℃ until used for RNA extraction. Sera were collected and stored to −20℃ until used for steroid hormone assay.

3. Total RNA Preparation and RT-PCR Analyses

Total RNAs were isolated from hypothalamic samples using the single-step, acid guanidinum thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi, 1987). Total RNAs were used in RT-PCR reactions carried out with Maxime™ RT PreMix (Intron, Korea) and AccuPower 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.

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

Gene	Accession number	Primer sequence	Product size(bp)	AT(℃)
GAPDH	NM_017008	F 5'-CCATCACCATCTTCCAGGAG R 5'-CCTGCTTCACCACCTTCTTG	576	50
CYP11A1	NM_017286.2	F 5'-CATCAAGGAGACACTGAGAC R 5'-GCATCAGGATGAGGTTGAAC	368	60
CYP17	NM_012753.1	F 5'-CACCAACTTTCAATGACCGG R 5'-GGGTCGATCAGAAAGACTAC	442	60
3β-HSD	NM_017265.4	F 5'-CCACTTGGTCACACTGTCAA R 5'-CTGAGGCATAACTACCTGTG	367	60
17β-HSD	BC061543.1	F 5'-GGAACAGATCCCAGAATGAC R 5'-ACAGACATGACCAACACTGG	498	60
StAR	AB001349.1	F 5'-AAACTGACTCCAGAGTGCTC R 5'-TGTCCTTCACTGTCAGCTTC	522	60

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

4. Testosterone Radioimmunoassay(RIA)

The total testosterone levels in serum were measured using commercially available radioimmunoassay (RIA) kits (Diagnostic Systems Laboratories, Inc., Webster, TX, USA). The intra-assay coefficient of variation for total T in serum was 3.5% (n=6). The inter-assay coefficient of variation for total T in serum was 4.5% (n=6).

5. 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 the testicular steroidogenic enzymes; P45011a1 (CYP11A1, often referred to as P450scc), 17α-hydroxylase (CYP17), 3β-hydroxysteroid dehydrogenase (3β-HSD, also referred to as Δ-5-4 isomerase) and 17β-hydroxysteroid dehydrogenases (17β-HSD). Single ICV infusion of 6-OHDA significantly decreased the mRNA levels of CYP11A1 (control:6-OHDA group=1:0.68±0.14 AU, p<0.05, Fig. 1A), CYP17 (control: 6-OHDA group=1:0.72±0.13 AU, p<0.05, Fig. 1B). There were no changes in the mRNA levels of 3β-HSD (control: 6-OHDA group=1:0.84±0.08 AU) and 17β-HSD (control: 6-OHDA group=1:0.63±0.20 AU), though the levels tended to be increased (Fig. 1C and 1D, respectively).

Secondly, the transcriptional activity changes of steroidogenic acute regulatory protein (StAR) in the testis were measured. StAR is a novel mitochondrial cholesterol transport which is expressed abundantly in steroid producing tissues (Lavoie & King, 2009). 6-OHDA administration decreased significantly the mRNA level of StAR when compared to the level of saline-injected animals (control: 6-OHDA group=1:0.72±0.08 AU, p<0.05, Fig. 2).

Thirdly, the serum testosterone levels were measured using radioimmunoassay. Treatment with single dose of 6-OHDA remarkably lowered serum testosterone level compared to the level of control group (control:6-OHDA group =0.72±0.24:0.13±0.03 ng/㎖, p<0.05, Fig. 3).

DISCUSSION

As life expectancy has been increased, the numbers of patients who suffer from chronic neurodegenerative diseases

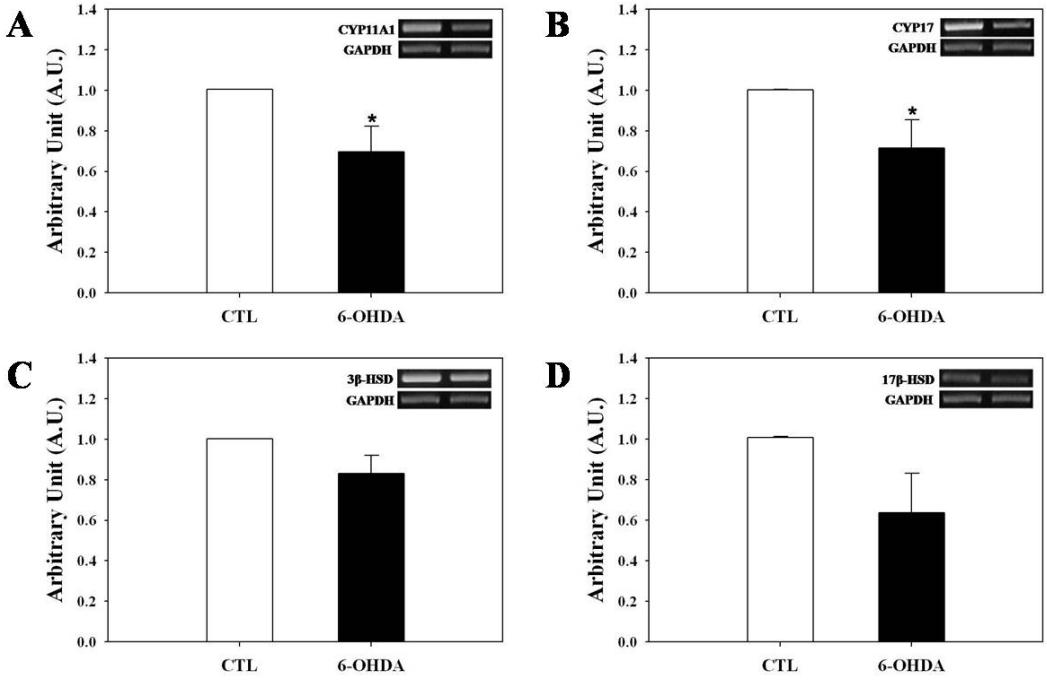

Fig. 1. Semi-quantitative RT-PCR analyses of CYP11A1, CYP17, 3β-HSD and 17β-HSD expression in the testes. RT-PCR analyses showing the significantly lowered mRNA levels of CYP11A1 (A) and CYP17 (B) when the rats were treated with single dose of 6-OHDA (200 μg in 10 μℓ saline/rat) by icv injection and were sacrificed at 2 weeks after the neurotoxin administration. The mRNA levels of 3β-HSD (C) and 17β-HSD (D) in testes were not changed. GAPDH RT-RCR was used as an internal control. Semi-quantitative RT-PCRs were carried out as described in 'Materials and Methods'. A typical pattern (inset gel photo) of RT-PCR results are presented from the five replicated experiments. Values are expressed as mean±S.E.. *, Significantly different from control group, $P<0.05$.

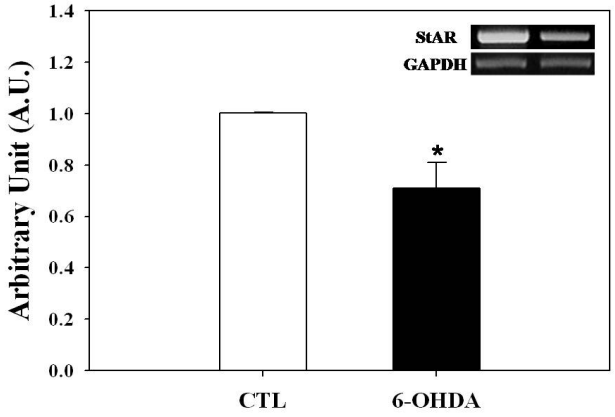

Fig. 2. Semi-quantitative RT-PCR analysis of StAR expressions in the rat testes. RT-PCR analysis showing the significantly lowered testicular StAR expression in the rats which were treated with 6-OHDA by icv injection when compared with saline-treated control rats. Values are expressed as mean±S.E. (n=5). *, Significantly different from control group, $P<0.05$.

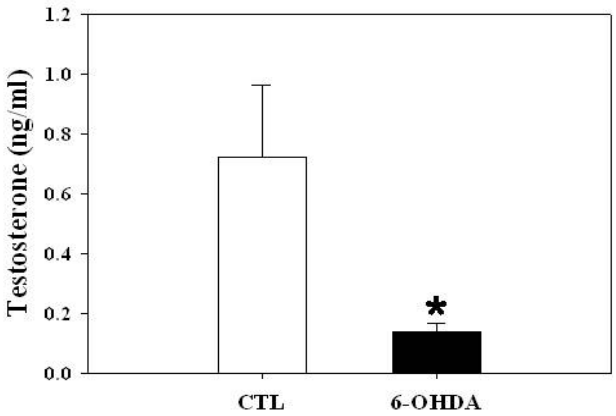

Fig. 3. Serum testosterone levels detected by specific RIA. The trunk bloods were collected and centriguged (1,000×g, for 20 min, at 4℃). The resulting sera were kept at freezer (−20℃) before use. Specific testosterone RIAs were carried out as described in 'Materials and Methods'. Values are expressed as mean±S.E.(n=5). *, Significantly different from control group, $P<0.05$.

such as Alzheimer's disease and PD is inevitably increasing. In modern nations, numerous factors including stress, body accumulation of environmental contaminants (e.g. mercury) and high fat diet are thought to accelerate the initiation and progress of such diseases. Apart from the various neurological symptoms, many patients with neurodegenerative diseases, in particular PD, show decreased sexual desire (libido) and poor sexual performance (Bronner et al., 2004). PD patients frequently show the deficiency of serum testosterone (Reddy et al., 2004). On the other hand, testosterone deficiency is also found in 20-25% of 'healthy' men over the age 60 years and may have similarities with non-motor symptoms of PD like apathy and fatigue (Harman et al., 2001). At this moment, one can only say that there is a strong correlation between low serum testosterone level and non-motor PD symptoms. Previously, we demonstrated that the central DA depletion lead to declined transcriptional activities of hypothalamus-pituitary reproductive hormone circuit (i.e. GnRH-gonadotropins) using rat PD model with single dose of 6-OHDA administration (Heo et al., 2009). We simultaneously found the remarkable changes in the activities not only of hypothalamus-pituitary stress modulating circuit (i.e. CRH-ACTH) but of adrenal steroidogenic enzymes (data not shown). These findings allowed us to hypothesize that the activities of some factors which are responsible for sex steroid biosynthesis in testis could be significantly altered when brain DA stores are depleted. In the present study, we demonstrated that the reductions in the transcriptional activities of the specific testicular steroidogenic factors and serum testosterone in the 6-OHDA injected young adult rats, confirming our hypothesis. Based on these results, we can postulate that PD might be a crucial reason for weakening the overall activities of HPT hormonal axis.

Mitochondrial enzyme CYP11A1 is a member of the cytochrome P450 superfamily, and catalyzes the vary first step in all steroid hormone production; the conversion of cholesterol to pregnenolone. A hydroxylase CYP17 is also one of cytochrome P450 superfamily that acts upon pregnenolone and progesterone to add a hydroxyl group at carbon 17 of the steroid D ring. 3β-HSD catalyzes the oxidative conversion of delta 5-3 beta-hydroxysteroids to the delta 4-3-keto configuration, and is responsible for the conversion of pregnenolone to progesterone, 17-alpha-pregnenolone to 17-alpha-progesterone, and androstenediol to testosterone. 17β-HSD, a member of alcohol oxidoreductases group, catalyzes the dehydrogenation of 17-hydroxysteroids in steroidogenesis (for review, Lavoie & King, 2009). In the present study, the mRNA levels of CYP11A1, CYP17 and StAR were significantly decreased while the levels of 3β-HSD and 17β-HSD were not changed in the 6-OHDA injected rats. Our findings suggest the chronic central DA depletion may reduce the testicular steroid hormone biosynthesis by lowering the conversion rates at the early stage of the steroidogenesis particularly at 21-carbons progestagens formation step. Whether the icv 6-OHDA administration can alter biosynthesis of other gonadal (19-carbons androgens and 18-carbons estrogen) and adrenal steroid (21-carbons glucocorticoids and/or mineralocorticoids) in male and female will be intriguing question.

Intraperitoneal administration of 6-OHDA could bring about a sort of chemical sympathectomized state in pheripheral tissues without any effect on central catecholamine neurons because 6-OHDA cannot cross the blood-brain barrier (BBB) (Kostrzewa & Jacobowitz, 1974). In contrast, direct action of the icv administered 6-OHDA should be strictly restricted in the brain since the amount of locally injected 6-OHDA is generally so small and it will be subjected to copious dilution in systemic circulation. Therefore, we could draw four explanations for the lowered testicular steroidogenic activity in the centrally DA-depleted animals by icv 6-OHDA administration. First, the icv 6-OHDA treatment may directly change the activities of HPT hormonal axis by sequential suppression of catecholaminergic inputs, GnRH, gonadotropins and sex steroids in both hormone production and secretion. Second, the neurotoxin injection may alter the hypothalamus-pituitary-adrenal (HPA) feedback loop, and then the activities of

HPT axis are gradually lowered by hormonal signals from HPA axis. The concept that the cross-talk between HPA and HPT axis, which can lead profound impacts on a broad spectrum of homeostasis if not regulated properly, has been supported by ample of evidence (Mastorakos et al., 2006). Thirdly, certain brain inputs may modulate the levels of humoral signals, not the endocrine but immunological factors (e.g. IL-1β) in systemic blood flow, then these blood-borne factors can alter the steroidogenic activity in testis (Turnbull & Rivier, 1995). Indeed, icv 6-OHDA administration increase the central and peripheral immune activities (Lane et al., 2008; Pacheco-López et al., 2003). It is well documented that cytokines regulate, negatively in general, steroidogenesis at the level of the gonads and adrenal (Bornstein et al., 2004). Finally, some brainstem neurons seem to play a role in regulating testosterone secretion via a direct neural pathway between the hypothalamus and the testes in rats. Intratesticular injection of the transganglionic tracer pseudorabies virus revealed that the paraventricular nucleus (PVN) of the hypothalamus as a component of this neural link especially altering Leydig cell responsiveness to gonadotropin (Selvage & Rivier, 2003; Selvage et al., 2006). This hypothalamic-testicular neural pathway is influenced by brain catecholamines, since intrabrain injection of 6-OHDA significantly reversed the inhibitory effect of icv EtOH or IL-1β on the gonadotropin-responsiveness in the rat testis (Salvage et al., 2004).

There has been a growing interest in poorer quality of life in PD patients in an aging society (Lees et al., 2009). Besides motor and cognitive symptoms, sexual dysfunction such as erectile dysfunction, sexual dissatisfaction, and low sexual desire is common in male PD patients (Bronner et al., 2004). Thus, testosterone supplement therapy for male PD patients could be promising because it is considered to have not only neuroprotective effect but potential for improving sexual function (Cherrier, 2009; Vermeulen, 1991; Okun et al., 2002; Farmer, 2004). In this context, more precide information on the testicular steroidogenic activities in PD patients and PD-like animals should be required prior to the generalization of the hormone therapy to meet the highest standards for safety and efficacy.

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