Theriogenology 81 (2014) 744–751

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

D-aspartate affects NMDA receptor-extracellular signal–regulated kinase pathway and upregulates androgen receptor expression in the rat testis

Alessandra Santillo [a], Sara Falvo [a], Paolo Chieffi [b], Lavinia Burrone [a], Gabriella Chieffi Baccari [a], Salvatore Longobardi [c], Maria Maddalena Di Fiore [a,*]

[a] Dipartimento di Scienze e Tecnologie Ambientali, Biologiche e Farmaceutiche, Seconda Università di Napoli, Caserta, Italy
[b] Dipartimento di Psicologia, Seconda Università di Napoli, Caserta, Italy
[c] Medical Liaison Office, Merck Serono S.p.A., Roma, Italy

ARTICLE INFO

Article history:
Received 27 May 2013
Received in revised form 28 November 2013
Accepted 9 December 2013

Keywords:
Testis
D-Aspartate
Testosterone
N-Methyl-D-Aspartic Acid receptor
Extracellular signal–regulated kinase
Androgen and estrogen receptor

ABSTRACT

Previous studies have demonstrated that D-aspartic acid (D-Asp) has a role in regulating the release and synthesis of testosterone in rats. In this study, we investigated the molecular pathway by which this amino acid triggers its action in the rat testis. We found expression of N-Methyl-D-Aspartic Acid (NMDA) receptor messenger RNAs for *NR1*, *NR2A*, and *NR2D* receptor subunits. After D-Asp administration, *NR1* and *NR2A* messenger RNA levels were significantly higher than those of controls, whereas *NR2D* levels remained unchanged. Expression of extracellular signal–regulated kinase (ERK) 1 protein was higher than that of ERK2 protein in the testis of both D-Asp–treated rats and controls. D-Asp administration increased testis levels of both phosphorylated ERK (P-ERK) 1 and 2. Using immunohistochemical technique, *NR1* and P-ERK 1 or 2 proteins were preferentially localized within the spermatogonia. Moreover, D-Asp administration increased both serum and testis testosterone levels but not estradiol levels. Finally, in D-Asp–treated rats, testicular androgen receptor protein levels were significantly increased, whereas both estrogen receptor α and P-450 aromatase levels were significantly decreased. Conclusively, our results, besides strengthening the evidence that D-Asp administration in rats induces testosterone synthesis, demonstrate for the first time that D-Asp (1) induces testicular NMDA receptor–ERK pathway, (2) upregulates androgen receptor expression, and (3) downregulates estrogen receptor expression.

1. Introduction

D-aspartic acid (D-Asp) is an endogenous amino acid present in the neuroendocrine tissues of vertebrates [1]. Several studies have demonstrated that D-Asp is concentrated in the rat endocrine glands, particularly in the pituitary gland and testes [2]. It is capable of eliciting the release of GnRH from the hypothalamus; the luteinizing hormone, growth hormone, and prolactin from the pituitary gland; and testosterone from the testis [2–4]. High concentrations of D-Asp have been recorded in the testicular parenchymal cells, interstitial fluid, venous blood plasma, rete testis, and epididymis [3]. A specific D-Asp localization in the rat testis has been observed in elongate spermatids [5], spermatozoa [6], and Leydig cells [7]. D-Asp levels increase in the testis immediately before birth and during sexual maturity, coincidently with testosterone synthesis [8]. Furthermore, an *in vitro* study carried out on the boar testis revealed that D-Asp enhances the activity of aromatase [9–11], the key enzyme that converts testosterone into 17β-estradiol [12].

* Corresponding author. Tel.: +39 0823 274527; fax: +33 0823 274571.
E-mail address: MariaM.DiFiore@unina2.it (M.M. Di Fiore).

0093-691X/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2013.12.009

The above evidence thus suggests that D-Asp plays a crucial role in reproduction, owing to its role either in the biosynthesis and release of sexual hormones or in the spermatogenesis.

Despite a growing body of literature on D-Asp and its involvement in reproduction, no investigation has yet been done on the particular molecular pathway underlying the action of D-Asp in the rat testis. Considering that numerous studies have demonstrated that D-Asp has a high affinity for N-Methyl-D-Aspartic Acid receptor (NMDAR) [13,14], in this study, we investigated the immunolocalization of *NR1* in the testis and the effects of *in vivo* D-Asp administration on the gene expression of the NMDAR subunits (*NR1*, *NR2A*, *NR2B*, *NR2C*, and *NR2D*) in the rat testis. Furthermore, because NMDAR activation induces phosphorylation of extracellular signal–regulated kinase (ERK), a signaling protein belonging to the Mitogen-activated protein kinases [15–18], we also investigated the immunolocalization of phosphorylated ERK (P-ERK) in the testis and the effects of D-Asp treatment on its protein expression. Finally, we evaluated whether D-Asp administration affects (1) both serum and testicular sex steroid (testosterone and 17β-estradiol) levels, (2) androgen receptor (AR) and estrogen receptor α (ERα) expression, and (3) P-450 aromatase expression.

2. Materials and methods

2.1. Animals and experiments

Male Wistar rats, weighing 300 to 350 g, purchased from Charles River Laboratory (Lecco, Italy), were kept under regulated conditions of temperature (28 ± 2 °C) and lighting (12 hours light and 12 hours dark cycles). They received commercial food pellets ad libitum. The rats (n = 18) were divided into two groups: the first group was allowed to drink a solution consisting of 20 mM D-aspartate for 15 days; rats of the second group (control) were given fresh water for 15 days. At the end of the treatment, rats were first anesthetized by an intraperitoneal injection of chloral hydrate (40 mg/100 g body mass) and then decapitated. The trunk blood was collected, and the serum was separated and stored at −20 °C for later sex hormone determination. The testes were dissected out, weighed, and rapidly immersed both in Bouin's fluid and in liquid nitrogen for immunohistochemical and biochemical analyses, respectively. The experimental protocol and the housing conditions were in accordance with the Italian guidelines (Decreto legislativo 116/92) and authorized by the local Animal Care Committee (Servizio veterinario ASL 44, Prot. Vet. 22/95).

2.2. Specific determination of D-Asp

Testes from each animal were first homogenized (Ultra-Turrax T25 homogenizer) with 0.2 M Tris HCl, pH 8.2, in a ratio of 1:20. Tissue homogenate (100 μL) was supplemented with 20 μL of 0.5 M trichloroacetic acid and centrifuged at 15,000× *g* for 10 minutes. The supernatants were neutralized (to pH 6–8) using 1 M NaOH and the resulting sample was analyzed using high-performance liquid chromatography with the method described previously [19]. The areas of the peaks of amino acid standards were used to calculate the amounts of D-Asp contained in the testis of control and D-Asp–treated rats.

2.3. Immunohistochemistry

For detection of *NR1* and P-ERK 1 or 2 (Thr202/Tyr204) paraffin embedded sections (5 μm thick) were incubated in 1% normal goat serum (Sigma Chemical Corporation, St. Louis, MO, USA). Next, they were incubated overnight at 4 °C with the following rabbit polyclonal primary antibodies: *NR1* (1:50; n° sc-1467; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); P-ERK 1 or 2 (1:100; n° 9101; Cell Signaling Technology, Inc., Danvers, MA, USA). After washing in PBS, the sections were incubated for 1 hour at room temperature with biotinylated goat anti-rabbit antibody (1:500; Pierce, Rockford, IL, USA), followed by incubation for 1 hour with streptavidin (1:500; Pierce). Bound antibody was visualized using 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) and 0.3% H_2O_2 in Tris buffer (0.05 M, pH 7.6). For negative controls, the primary antibody was omitted.

2.4. RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was extracted from rat testes (n = 5, for each experimental group), using TRIzol standard protocol (Invitrogen Life Technologies, Carlsbad, CA, USA) and then treated for 30 minutes at 37 °C with DNase I (10 U per sample) (Amersham Bioscience, Buckinghamshire, UK) to eliminate any contaminations of genomic DNA. Total RNA purity and integrity were determined using spectrophotometry at 260/280 nm and electrophoresis on a denaturizing formaldehyde agarose gel. One microgram of the total RNA was reverse-transcribed using the SuperScript First-Strand Synthesis System kit (code 11904-018, Invitrogen Life Technologies). Specific primer sets were designed for quantitative real-time polymerase chain reaction (qRT-PCR) using primer 3 (http://frodo.wi.mit.edu/primer3). The sequences of used primers were reported in Table 1. As

Table 1
Primers for real-time polymerase chain reaction.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	GeneBank accession no.	Product size (bp)
NR1	CGGCTCTTGGAAGATACAGC	GTGAAGTGGTCGTTGGGAGT	U08261	150
NR2A	AAGGGGAGAGAGAGGCAAAG	CTGAGGCTCGATAGGGACAG	AF001423	97
NR2B	GTGAGAGCTCCTTTGCCAAC	GTCAGGGTAGAGCGACTTGC	NM_012574.1	101
NR2C	AGTTGGCAGCTGTGGTCTCT	CFAGGCCAAGCACAAAAAGC	U08259	102
NR2D	TAGTGTCAGTGCGCAGATCC	ACCATGAACCAGACGTAGCC	L31612	114
RPS12	AAATCGATCGAGAGGGGAAG	CTTGGCCTGAGATTCTTTGC	NM_031709.3	86