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ScienceDirect
Theriogenology 67 (2007) 249–254
Theriogenology
www.theriojournal.com

D-Aspartic acid and nitric oxide as regulators of androgen production in boar testis

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Received 12 April 2006; received in revised form 23 June 2006; accepted 24 July 2006

Abstract

D-Aspartic acid (D-Asp) and nitric oxide (NO) are two biologically active molecules playing important functions as neurotrasmitters and neuromodulators of nerve impulse and as regulators of hormone production by endocrine organs. We studied the occurrence of D-Asp and NO as well as their effects on testosterone synthesis in the testis of boar. This model was chosen for our investigations because it contains more Leydig cells than other mammals. Indirect immunofluorescence applied to cryostat sections was used to evaluate the co-localization of D-Asp and of the enzyme nitric oxide synthase (NOS) in the same Leydig cells. D-Asp and NOS often co-existed in the same Leydig cells and were found, separately, in many other testicular cytotypes. D-Asp level was dosed by an enzymatic method performed on boar testis extracts and was 40 ± 3.6 nmol/g of fresh tissue. NO measurement was carried out using a biochemical method by NOS activity determination and expressed as quantity of nitrites produced: it was 155.25 ± 21.9 nmol/mg of tissue. The effects of the two molecules on steroid hormone production were evaluated by incubating testis homogenates, respectively with or without D-Asp and/or the NO-donor L-arginine (L-Arg). After incubation, the testosterone presence was measured by immunoenzymatic assay (EIA). These *in vitro* experiments showed that the addition of D-Asp to incubated testicular homogenates significantly increased testosterone concentration, whereas the addition of L-Arg decreased the hormone production. Moreover, the inclusion of L-Arg to an incubation medium of testicular homogenates with added D-Asp, completely inhibited the stimulating effects of this enantiomer. Our results suggest an autocrine action of both D-Asp and NO on the steroidogenetic activity of the Leydig cell.

Keywords: D-Aspartic acid; Nitric oxide; Testis; Testosterone

1. Introduction

D-Aspartic acid (D-Asp) and nitric oxide (NO) are two widely distributed molecules in animal tissues where they play important functional roles in several cellular processes, many of which are still to be defined. Both substances are present in male gonads of vertebrate species, where several lines of research indicate their putative implication in the regulation of androgen synthesis [1,2].

D-Asp, in fact, enhances testosterone production by acting both as an activator of the hypothalamus–pituitary–gonad axis and as a local regulator of gonad steroidogenesis [3,4]. A previous report demonstrated that D-Asp increases chorionic gonadotropin (hCG)-

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0093-691X/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2006.07.016

induced testosterone production in mammalian Leydig cells [5]. In these cells, intracellular cholesterol is transported to the inner mitochondrial membrane, where it is converted to pregnenolone by cytochrome P450 as the first step of testosterone synthesis [5].

In tissues, NO is synthesized from L-arginine by NO synthase (NOS) which requires calcium-calmodulin for activation [6]. L-Arginine is the only known physiological nitrogen donor for NOS-catalyzed reactions. NO has been shown to regulate the biology and physiology of the reproductive system [7]. Testicular cells are well equipped with a NO-cGMP pathway, which may significantly participate in the regulation of testicular functions such as spermatogenesis and steroidogenesis [8–11]. NO is mainly expressed in Leydig cells where it regulates the concentration of testosterone by acting in an autocrine/paracrine fashion [12–14]. In fact, NO is involved in testicular testosterone synthesis causing a significant decrease of androgen production [8,9].

The effects of D-Asp and NO on testicular testosterone production have only been studied separately. This is the first study describing the effects of both molecules on mammalian steroidogenesis. In fact, the contrasting effects of D-Asp and NO on testicular testosterone production suggests that these agents are in some way linked in local regulation of steroidogenesis, i.e. the D-Asp stimulatory activity on androgen production could be contrasted by NO and *vice versa*. To verify this assumption, we carried out our study on the boar, which was chosen because of its abundance in Leydig cells.

The level of D-Asp and the activity of the enzyme nitric oxide synthase (as an index of NO putative production) were evaluated by specific biochemical assays and both molecules were co-localized in the same Leydig cells by means of a double labeling immunofluorescent technique. The separated and combined effects of D-Asp and NO on endogenous testosterone production were, finally, evaluated by *in vitro* tests.

2. Material and methods

2.1. Animals and sampling

Six adult boars provided by a local slaughterhouse (Afragola, Naples) were used for this investigation. The methods of dissection and the rearing conditions were in accordance with Italian law (D.L.vo 116/92). Soon after death of the animals, the testes were rapidly dissected out. From each animal one testis was fixed in Bouin's fluid for 12–24 h or in 4% buffered paraformaldehyde, pH 7.4, for 8–12 h, and processed for histology and immunohistochemistry, while the other was frozen in liquid nitrogen and kept at −80 °C until used for D-Asp and NO biochemical determination and for *in vitro* tests.

2.2. Immunofluorescence

The presence of D-Asp and NO in boar testis was investigated by the immunofluorescence method. Seven randomly chosen sections of testes for each animal were examined. Sections were air-dried at room temperature, washed in PBS and preincubated with PBS plus 3% normal goat serum. In the specific step a polyclonal rabbit antiserum against D-Asp (a kind gift by Dr. A. D'Aniello, Zoological Station, Naples) was used in combination with a mouse monoclonal antiserum against the neuronal isoform of nitric oxide synthase (NOS) (Charles River, 91278). They were incubated overnight at room temperature at the dilutions of 1:1000 and 1:300, respectively. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson Immuno. Res. Lab., 111095045) and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (Jackson Immuno. Res. Lab., 115025146) were simultaneously applied on sections for 1 h at room temperature, as secondary antibodies. Sections were observed by means of a Nikon Eclipse E600 microscope equipped for reflected fluorescence.

Specificity of immunofluorescence for both D-Asp and NO was obtained: (1) without first antibody (substituted with 0.1 M phosphate-buffered saline or no-immune serum), but with the second antibody; (2) without the second antibody but with the first one. These controls were negative.

Photographs were taken using Kodak Ektachrome P1600 film.

The scoring of immunoreaction was performed on seven randomly chosen sections from each animal.

2.3. Quantitative determination of testicular D-Asp

Tissue samples were homogenized with 0.5 M perchloric acid (PCA) in a 1:10 ratio and centrifuged at 30,000 × *g* for 20 min. Supernatants were brought to pH 7.5–8.5 by adding 5 M KOH, cooled for 30 min at 0 °C; the potassium perchlorate precipitate was removed by centrifugation. Supernatants were adjusted to a pH of about 2.5 with 1 M HCl, and the amino acids were purified on a cation exchange column (AG 50W-X8 resin, hydrogen ionic form, 200–400 mesh, Bio-Rad). Samples were loaded on columns (1 cm × 3 cm) equilibrated with 0.01 M HCl, and after a washing with 10 ml 0.01 M HCl,

were eluted with 8 ml of 4 M NH_4OH. The elutes were dried by evaporation in small Petri dishes on a hot plate at 40–60 °C under a hood. The dry elutes were dissolved in 1 ml of 0.01 M HCl. They were then purified by slowly passing them, by means of a syringe, through a Sep-Pak C-18 cartridge (300 mg; Waters, Milan, Italy) which had been previously activated with methanol or acetonitrile and washed with distilled water. To recover all the amino acids from these elutes, the cartridge was eluted twice with 2 ml of 0.01 M HCl. The resulting elutes of each column were combined, and either dried using a Savant centrifuge or left to evaporate in small Petri dishes at 40–50 °C under the hood. The dry residues were then dissolved in 200 μl 0.01 M HCl and analyzed for D-Asp content. The assay procedure is identical to that previously described by Di Fiore et al. [15]. A standard curve was obtained by applying the enzymatic method to D-Asp solutions of known concentrations.

2.4. Determination of NO activity

NO was assessed, according to Meli et al. [16], by measuring tissue nitrite production as an indicator of NO synthesis. Frozen samples of testes were thawed and rinsed in cold PBS buffer (0.1 M, pH 7.4), weighed and homogenized (1:2, w/v) in the same ice-cold buffer. The suspensions were centrifuged at $3500 \times g$ for 15 min and aliquots of 100 μl of supernatant were mixed with aliquots of 100 μl of Griess reagent containing 1% sulphanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid. The suspensions were kept at room temperature for 10 min and then their absorbance at 550 nm was measured in a microplate reader (Titertek multiskan MCC). Solutions of sodium nitrite were used as standards. Nitrite concentration in tissues was calculated by comparing their OD with those of the standard solutions. The results were expressed as nmol of nitrite (NO_2^-) released by 1 mg of fresh tissue.

2.5. In vitro tests

Frozen testis samples were thawed and homogenized, 1:1 (w/v), with Krebs-Ringer nutrient medium (0.11 M NaCl, 0.046 M KCl, 0.019 M $CaCl_2$, 0.066 M $MgCl_2$, 0.025 M $NaHCO_3$, 0.014 M NaH_2PO_4), supplemented with: 0.015 M glucose, 2 mg/ml bovine serum albumin, antibiotics (50 IU/l penicillin, 50 IU/l streptomycin and 100 IU/l nistatin) and 0.1 mg/ml NADPH.

In the first group of experiments, this suspension was distributed, 3 ml each, into multi-well plates and incubated, using a shaking bath, with or without 300 μl of D-Asp 0.5 M (final concentration 50 mM) at 37 °C for 2 h. Parallel sets of suspensions were incubated with or without 300 μl of the NO-donor L-Arginine (L-Arg) 1.0 mM (final concentration 1.0 mM) at the same temperature and time. These conditions were chosen on the basis of preliminary tests, performed using increasing concentrations of D-Asp (0–75 mM) and of L-Arg (0.5–5.0 mM), at different incubation times (0–3 h). A third set of suspensions contained the sample and saline (100 μl) alone. A rapid freezing ended incubations.

In a second group of *in vitro* experiments, suspension aliquots (3 ml each) were incubated, into multi-well plates with D-Asp (final concentration 50 mM) at 37 °C. Some of the incubations ended after 1 h, the others were incubated for another hour, with or without added L-Arg (final concentration 1.0 mM).

Both the two series of experiments were repeated three times.

All well contents, coming from the first and the second experiments, were extracted three times with 5 ml of ethyl ether. The ether phases of the three extractions were pooled and dried in air. Testosterone determination was carried out on the residues by the enzyme immunoassay Kits (EIA) (Biochem Immuno Systems). The following limits of detection were observed: sensitivity was 6 pg (intra-assay variability 5.3%, inter-assay variability 7.5%).

2.6. Statistical analysis

Data coming from *in vitro* tests were compared by analysis of variance followed by Duncan's test for multi-group comparison and Student's *t*-test for between-group comparison. All data were expressed as mean ± standard deviations. The level of significance was taken at $p < 0.01$ and $p < 0.05$.

3. Results

3.1. Immunofluorescence

Histological observation showed that the seminiferous tubules of the examined testes were undergoing active spermatogenesis. As generally described for boar male gonad, numerous Leydig cells, isolated or clustered, were contained in the interstitial spaces, where they were readily distinguishable because of their large size, polygonal shape and bulky, central nucleus.

D-Asp and NOS immunoreactions were often found in the same Leydig cells (Fig. 1a and b), Both took on the shape of granules packed in clusters that filled a large portion, if not the entire, cytoplasm. The finding of

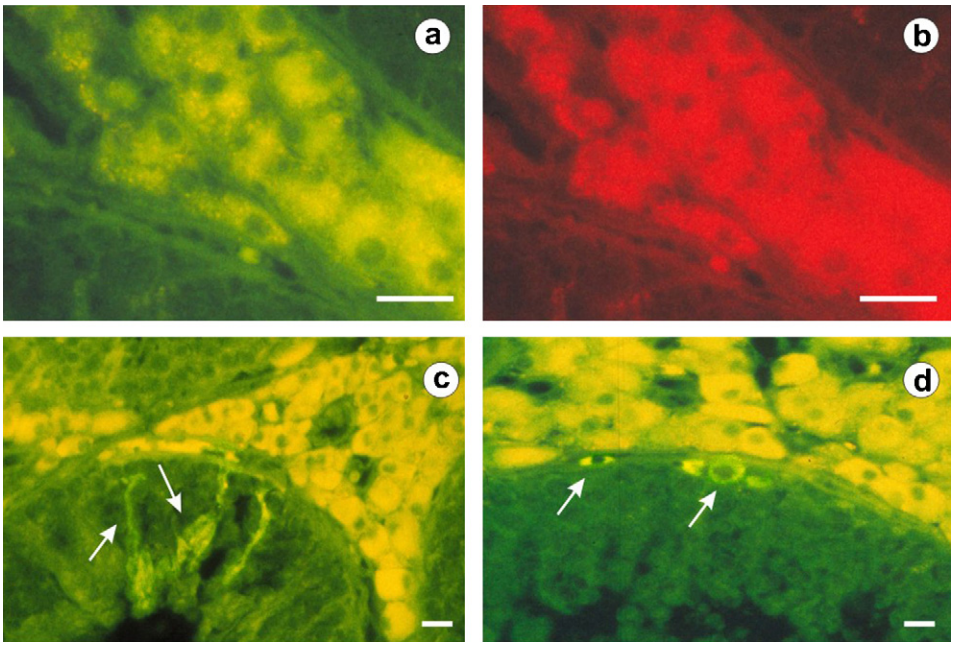

Fig. 1. D-Asp- (a, c, and d) and NOS-immunoreactivity in cytotypes of the boar testis. (b) A single section showing a cluster of Leydig cells containing both D-Asp (a) and NOS (b). (c) Three elongated Sertoli cells arising from the basis of the germinative epithelium up to the tubular lumen. (d) Two clearly stained peritubular cells at the boundary of a seminiferous tubule. Double labeling immunofluorescent technique utilizing FITC (a, c, and d) and TRITC (b) as fluorochromes. Bars: 10 μm.

interstitial spaces entirely stuffed with D-Asp- and/or NOS-reactive Leydig cells was common.

NOS-positive cytotypes other than Leydig were also found elsewhere in the testis but their description is omitted here because their presence has already been described. Instead of D-Asp presence in other testicular compartments is now described in Sertoli (Fig. 1c) and peritubular (Fig. 1d) cells which were rare, mainly when compared to the number of D-Asp-positive Leydig cells, but always clearly stained.

3.2. Quantitative determination of testicular D-Asp

The biochemical D-Asp determination indicated that this amino acid content in the boar mature testis was 40 ± 3.6 nmol/g of fresh tissue.

3.3. Determination of NO activity

The biochemical measurement of NO_2^- tissue content, performed by the Meli's method, was 155.25 ± 21.9 nmol/mg.

3.4. In vitro tests

Fig. 2 shows the results from an *in vitro* experiment in which D-Asp and/or L-Arg were added to a medium containing boar testis homogenates. It is evident that D-Asp induced a significant ($p < 0.01$) increase of testosterone. On the contrary, L-Arg causes a significant ($p < 0.05$) decrease of testosterone. The simultaneous presence of the two substances did not significantly modify the testosterone content, as evaluated in the controls.

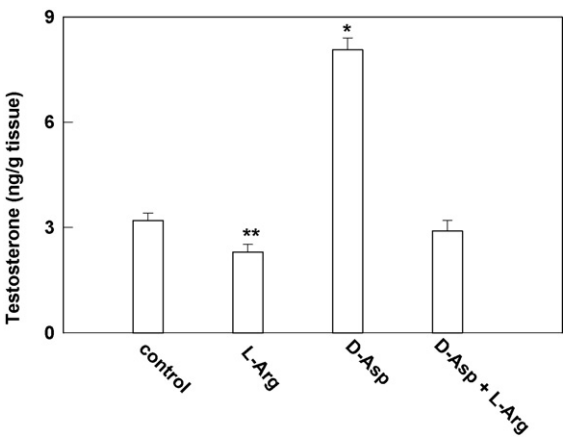

Fig. 2. *In vitro* experiments showing the effects of the addition of D-Asp and/or L-Arg to boar testicular tissue on testosterone concentrations. Each value represents the mean ± S.D. of three different determinations. $^{*}p < 0.01$; $^{**}p < 0.05$ vs. control.

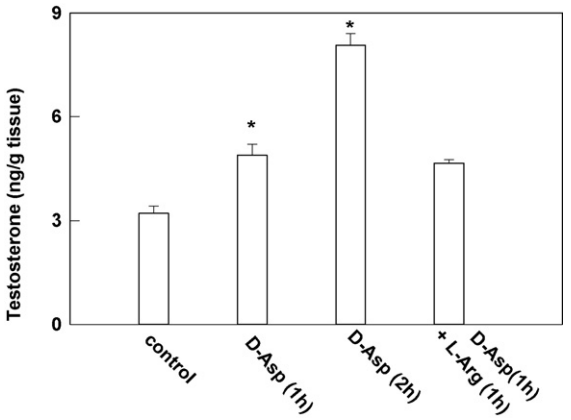

Fig. 3. *In vitro* experiments illustrating the effects of the addition of D-Asp at 1 and 2 h as well as the addition of D-Asp plus L-Arg for 1 h to boar testicular tissue on testosterone concentrations. Each value represents the mean ± S.D. of three different determinations. $^{*}p < 0.01$.

Fig. 3 reports the results of parallel tests in which boar testis homogenates where initially exposed to D-Asp and incubated for 1 h. Thereafter, some of them were stopped, whereas the others, with or without L-Arg, were incubated for another hour. D-Asp induced the expected testosterone increases after an hour of incubation ($p < 0.01$). In the following hour of incubation the testosterone still increased in the plates bearing D-Asp, while in those with L-Arg, the D-Asp-dependent testosterone increase appeared to be abolished.

4. Discussion

Although the control of the endocrine activity of Leydig cells in vertebrate testis has long been known to depend mainly on the gonadotropin LH secreted by the pituitary, further observations have shown that this function is modulated in the gonad itself through several local agents which intervene on Leydig cell activity as autocrine and/or paracrine hormones [17,18]. Recent studies have shown that two of these molecules could be D-aspartic acid (D-Asp) and nitric oxide (NO) since both appear to intervene on steroidogenesis, although with opposite effects.

Free D-Asp has been found in the testis of several mammalian and non-mammalian species [1,4]. In seasonally breeding amphibian and reptilian species its testicular level significantly increases during the reproductive period and matches the increase of testosterone both in the gonad and plasma [19,20]. In the present study free D-Asp is contained at a concentration of 40 ± 3.6 nmol/g fresh tissue, a similar level to that found in the testis of the other mammalian species [4]. The addition, moreover, of D-Asp to homogenates of boar testis *in vitro*, results in temporary but consistent increases in testosterone production. On the whole, these findings indicate that, besides a stimulatory effect on hypothalamus–pituitary–testis axis [4], D-Asp can also act locally on steroidogenesis. In the testis, the main target of D-Asp should be the Leydig cells, an assumption supported by the demonstration of intense D-Asp immunoreactivity in many of them, yet other cytotypes have been shown to contain this enantiomer: i.e., peritubular and Sertoli cells as well as spermatogonia and spermatids. The putative D-Asp function in these cells is unknown [1,21]. The mechanism for D-Asp local action on Leydig cell steroidogenesis has been proposed by Nagata et al. [5]. Acting on the gene, D-Asp induces the synthesis of StAr protein believed to facilitate the translocation of cholesterol to the inner mitochondrial membrane. This makes the steroid available for the cytochrome $P450_{SCC}$ enzyme system that operates the side-chain cleavage of cholesterol. The resulting pregnenolone migrates to the cytoplasm where, in the microsomal compartment, it is converted into testosterone. Cytochrome $P450_{SCC}$ activity is a rate-limiting step in steroidogenesis.

Several pieces of evidence indicate that the free radical NO plays an important role in male gonad endocrine activity. Immunohistochemical studies have revealed that NOS, the enzyme which synthesizes this molecule, is present in many testis cytotypes, especially the Leydig cells and, to a lesser extent, peritubular, Sertoli, endothelial and macrophage cells and in spermatogonia, spermatocytes and spermatids as well [10,11,22]. Nitric oxide generating systems, moreover, appear to exert inhibition on testosterone production and on sperm motility/apoptosis and most likely also modulates the contraction of peritubular myofibroblasts [8,13,14,23–25]. Functional studies on cultured Leydig cells [8,9] and other steroid producing cells [26], finally, have shown that the $P450_{SCC}$ catalyzed conversion of cholesterol to pregnelonone is, probably, the steroidogenic step inhibited by NO.

In the present study, the above reported immunohistochemical results have found support in our study on boar testis which, moreover, appears to potentially produce 155.25 ± 21.90 nmol of NO/mg fresh tissue. Furthermore, immunohistochemical tests on boar testis have indicated that both D-Asp and NO can reside in the same Leydig cell, supporting their local regulation of androgen synthesis. This relationship is supported by the observation that in *in vitro* tests, the stimulatory effect on testosterone production exerted by D-Asp is completely prevented when a NO generating system is

added to incubation media. According to previous studies, it could be hypothesized that although the availability of cholesterol induced by the D-Asp dependent StAr protein increases, the inhibition of the cytochrome $P450_{SCC}$ system by NO renders this substrate unusable and, therefore, interrupts testosterone synthesis.

In conclusion, the data reported suggest that involvement of regulatory factors as well as D-Asp and NO significantly contribute to testis endocrine signaling and regulation.

Acknowledgement

This work was supported by a MIUR grant (cofin PRIN 2004, prot. 2004051955).

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