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Involvement of D-Asp in P450 aromatase activity and estrogen receptors in boar testis

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Summary. Mammalian testis contains D-aspartic acid (D-Asp), which enhances testosterone production. D-Asp, on other hand, also stimulates 17β-estradiol synthesis in the ovary of some lower vertebrates. We studied boar testis in order to determine if D-Asp intervenes in 17β-estradiol synthesis in the testis of those mammals which produce significant amounts of estrogens as well as testosterone. The boar testis contains D-Asp (40 ± 3.6 nmol/g tissue) which, according to immunohistological techniques, is localized mainly in Leydig cells, and, to a lesser extent, in sustentacular (Sertoli), peritubular and some germ cells. The enzyme P450aromatase is present in Leydig cells and few germ cells. In vitro experiments showed that the addition of D-Asp to testicular tissue extracts induced a significant increase of aromatase activity, as evaluated by testosterone conversion into 17β-estradiol. The enzyme's K_m was not affected by D-Asp (about 25 nM in both control and D-Asp added tests). On the basis of these results we suggest that, as in the ovary, D-Asp is involved in the local control of aromatase activity of boar testis and, therefore, it intervenes in the 17β-estradiol production. In the testis, the D-Asp targets are presumably the Leydig cells, which having also a nuclear estrogen receptor are, in turn, one of the putative targets of the 17β-estradiol that they produce (autocrine effect).

Keywords: P450arom – D-Asp – Estrogen receptors – 17β-estradiol synthesis – Boar testis

Introduction

The testis of several mammalian species is a source of estrogens, although the roles of these hormones in the male gonad are still not completely known. Either Leydig or germ cells appear to be the site of 17β-estradiol synthesis since they contain the cytochrome P450 aromatase (P450arom), the enzyme which catalyzes androgen conversion into estrogens. Moreover, both Leydig and some germ cells express the estrogen receptor (ER), an aspect that makes them a putative target of the hormone they produce (autocrine effect).

In several mammals, the male gonad, besides a high testosterone production, has also the capacity to synthesize 17β-estradiol, although at a low rate and in amounts different in the species so far studied. The estrogen hormone appears to be primarily in Leydig cells but also, to a lesser extent, in Sertoli and germ cells (Carreau et al., 1988, 1999). 17β-estradiol synthesis depends on the P450 aromatase, which catalyses the conversion of testosterone into estrogens (Simpson et al., 2002). In the adult testis, the enzyme has been mainly localised in Leydig cells, but also in germ cells and, to a lesser extent in Sertoli cells (Payne et al., 1976; Brodie and Inskter, 1993; Turner et al., 2002). The aromatase activity, moreover, appears to be under the control of modulators both at transcriptional and post transcriptional levels (Genissel et al., 2001).

In the gonads, a local putative modulator of sex hormone synthesis could be the amino acid D-aspartic acid (D-Asp) whose presence, in free form, has been documented in a variety of animal tissues, including the gonads (D'Aniello et al., 1998a). In the testis, D-Asp intervenes in testosterone synthesis. In adult male rats, in fact, the endogenous D-Asp content of testis is higher in sexually active animals and, according to the results of several in vivo and in vitro experiments, it induces a significant increase of testosterone output (D'Aniello et al., 1996, 1998a, 2000a, b; Nagata et al., 1999a). On the other hand, some studies carried out on seasonally breeding lower vertebrates (an amphibian and a reptilian species), have shown that in females the D-Asp content of the ovary significantly increases during the reproductive period,

concomitantly to the 17β-estradiol level increases both in the gonad and plasma, and, moreover, in the ovary of non-reproductive females D-Asp induces, in vivo and in vitro, a significant increase of 17β-estradiol synthesis and a decrease of its typical precursor, i.e. testosterone (Di Fiore et al., 1998b; Assisi et al., 2001). These findings, therefore, clearly confirm the role of D-Asp as a local regulator agent of sex hormone production by vertebrate gonads, but, at the same time, it has been proposed that D-Asp has a dual role linked to the sex, i.e. D-Asp stimulates androgen synthesis in the testis and 17β-estradiol synthesis in the ovary.

These data, consequently, pose an interesting question, i.e. in those mammalian testes that produce either testosterone or 17β-estradiol, does D-Asp exert any role in the 17β-estradiol synthesis? In other words, in the testes does D-Asp regulate, besides testosterone production, also the aromatase activity which converts testosterone into 17β-estradiol? To address this question, we choose to study boar testis which, among mammals, is known to produce a fairly high amount of estrogens (Raeside et al., 1993; Conley et al., 1996). In the testes of adult specimens we, therefore, investigated the aromatase localization and in vitro activity, the D-Asp level, localization and in vitro activity, and the ER localization.

The ER localization study was carried out in order to evaluate if, as in other mammalian species, testicular estrogen targets can be demonstrated also in the pig testis. As it is well known, the biological estrogen actions are mediated by their specific receptors that belong to the family of nuclear hormone receptors (Green et al., 1986; Kuiper et al., 1996). In rodents, the ER presence has been reported in the Leydig cells of adult male testis (Mowa and Iwanaga, 2001; Zhou et al., 2002). In these animals, ER expression, moreover, has been noticed also in Sertoli and germ cells (Saunders et al., 1998).

Materials and methods

Testis samples

Adult boar testes were obtained from slaughter. Tissue samples were fixed by immersion in Bouin' s fluid for 12–24 h or buffered in 4% paraformaldehyde, pH 7.4, for 8–12 h at room temperature. After washing in tap water, the samples were processed for paraffin embedding in vacuum or were immersed in phosphate-buffered saline (PBS, pH 7.4, 0.01 M) containing 0.1% sodium azide and 20% sucrose, as a cryoprotectant, for 24–48 h. Both paraffin-embedded and cryoprotected samples were cut 8–10 mm thick. This material was utilised to perform immunohistochemical and immunofluorescent stainings. Other testis samples were kept at −80 °C and utilised to carry out the in vitro aromatase determinations.

Immunohistochemical methods

The immunohistochemical staining for aromatase and D-Asp demonstration was performed through the peroxidase antiperoxidase (PAP) method of Sternberger (1979). Sections were rinsed in distilled water, after dewaxing if necessary, and treated for 20 min with 3% H_2O_2. Then they were rinsed in PBS (pH 7.4), containing 0.2% Triton X-100 and 0.1% bovine serum albumin and incubated with normal goat serum for 30 min. The primary antibodies were rabbit polyclonal immunoglobulin raised against D-Asp (Inst. Bas. Med. Sci., Oslo), or human P450 aromatase (Biogenesis, 719100). They were poured on sections and incubated in a humid chamber for 24 h at 4 °C at dilutions of 1:800 and 1:1000, respectively. A secondary antiserum raised in goat and, thereafter, a PAP complex was applied on sections for 30 min at room temperature. The sites of the immunoreactive reactions were visualized using a H_2O_2 activated solution of 3,3-diaminobenzidine tetrahydrochloride in Tris buffer (10 mg in 15 ml). Sections were lightly counterstained with hematoxylin in order to visualize the structural details.

The immunocytochemical reaction for estradiol receptors was carried out using the Abbott ER-ICA monoclonal test (Abbott spa, Rome, Italy). The specificity of immunohistochemical reactions was checked by incubating sections with primary antisera previously absorbed with an excess of the relative antigens. Such sections resulted always unstained. All sections were observed by means of a Leitz Laborlux microscope equipped for reflected fluorescence. FITC was visualized by a filter set containing a 450–490 nm band pass excitation filter and a 515/20 nm band pass barrier filter. Photographs were taken using Kodak 64 Iso film for normal light and Kodak 1600 Iso technical Pan b/w film for fluorescent light.

Quantitative determination of testicular D-Asp

Testis 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 the addition of 5 M KOH, cooled for 30 min at 0 °C, and 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 × 3 cm) equilibrated with 0.01 M HCl, and after a washing with 10 ml 0.01 M HCl, the amino acids 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 40–50 °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 on 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. In order to recover all the amino acids from these elutes, the cartridge was eluted two times with 2 ml of 0.01 M HCl. The combined elutes of each column were 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, using the assay procedure previously described by Di Fiore et al. (1998b). A standard curve was obtained by applying the enzymatic method to D-Asp solutions of known concentrations.

Measurement of aromatase activity

Aromatase activity was measured by evaluating the in vitro conversion rate of testosterone to 17β-estradiol performed by acetone powder extracts of fresh tissue, as previously reported (Di Fiore et al., 1998a). Acetone powder was prepared by homogenizing testis samples with cold acetone (1:3 w/v), and the suspension was centrifuged at 3000 *g* for 10 min. The extraction was repeated three times. The pellets were freed of acetone traces under a nitrogen flow and stored at −20 °C

until use. The testicular extract was suspended in Krebs-Ringer nutrient medium (1:1 w/v), supplemented with antibiotics (penicillin 50 IU/l, streptomycin 50 IU/l and nistatin 100 IU/l). Different tests were performed using increasing concentrations of testosterone (0–35 μM) with or without D-Asp (0–75 mM) at different incubation times (0–3 h). Preliminary tests were performed to ascertain the better incubation temperature and time. On the basis of these results, an incubation of 1 h at 37 °C was chosen for all subsequent experiments. Usually one mL of the testicular extract suspension was distributed in each well of a multi-well plate. To the first set of wells were added increasing amounts of testosterone (0–35 μM) dissolved in 100 μl of NADPH solution (3 mg/ml). A parallel set received, in addition, D-Asp (0–75 mM) dissolved in 100 μl of physiological saline solution (0.7% NaCl, pH 7.4). A third set contained only the sample suspension and saline (100 μl). The suspensions were incubated, in a shaking bath, as reported before and then rapidly frozen. The well contents were then extracted three times with ether. Solvents were pooled and air dried. Determination of 17β-estradiol was carried out on the residues using an enzyme immunoassay kits (EIA) (Biochem Immuno Systems). The following limits of detection were observed for 17β-estradiol: sensitivity 6 pg, intra-assay variability 5.3%, inter-assay variability 7.5%.

Determination of proteins

The protein content was determined according to the method of Lowry et al. (1951), using BSA as a standard.

Statistical analysis

Data of biochemical assays were analysed by a one way ANOVA method followed by Duncans' multi-range test. Values were expressed as mean ± SE. Kinetics was analysed using linear regression analysis.

Results

In the testes of the examined boars (six adults), active spermatogenesis was going on in seminiferous tubules. As generally described in the morphology of boar male gonads, numerous Leydig cell were dispersed in clusters between seminiferous tubules where they were readily distinguishable from other interstitial cellular components because of their larger size, polygonal shape and a bulky, round central nucleus.

Figure 1A shows the results of an immunoreactivity test (IR) for aromatase. A positive reaction is localized mainly in Leydig cells. Figure 1B shows the IR for D-Asp. IR positive substances are localized in the majority of Leydig cells, and, in addition, in several spermatogonia, and in peritubular and in Sertoli cells. It is evident that in Leydig cells immunoreactive substances are present both in the nucleus and in the cytoplasm. On the contrary, the few positive spermatogonia show immunoreactivity localized in the cytoplasm. D-Asp IR was also detected in rare peritubular cells and in the irregular cytoplasmic profile of the Sertoli cells. Figure 1C shows that estradiol receptor (ER) immunoreactivity was mainly found in numerous nuclei of the Leydig cells.

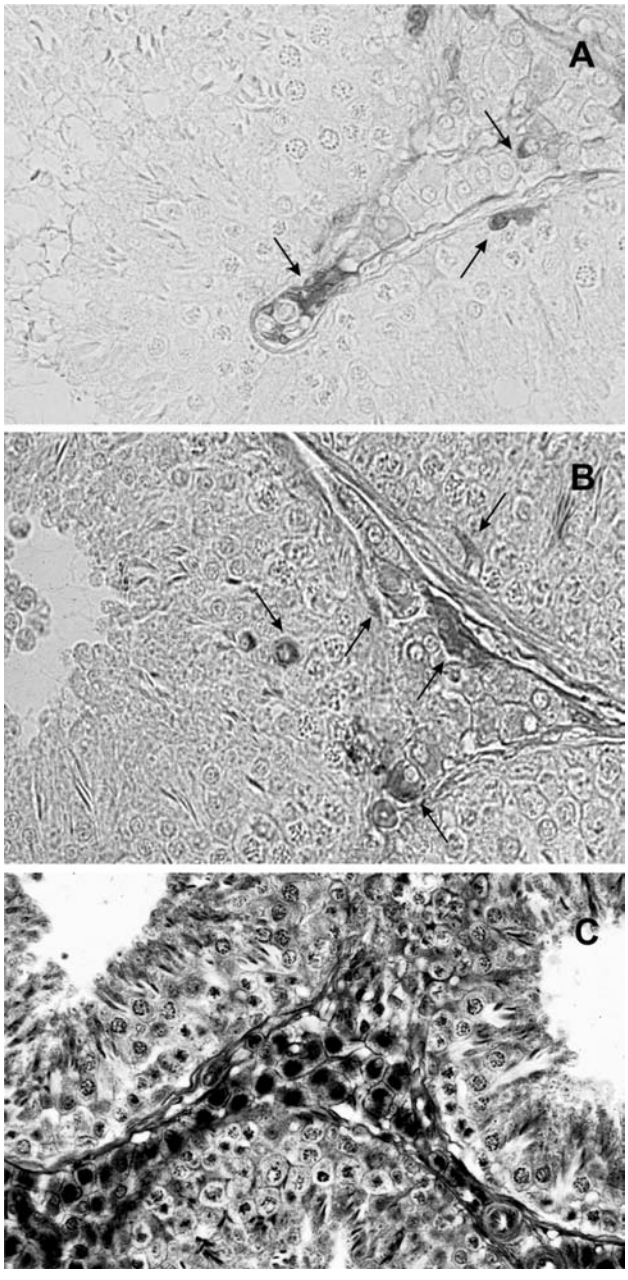

Fig. 1. Sections from boar testis. Immunoreactivity to aromatase enzyme in the cytoplasm of the clustered Leydig cells and in isolated germ cells (**A**); D-Asp immunoreactive materials in: the cytoplasm of the clustered and isolated Leydig cells, in peritubular cells, in Sertoli cells, tipically elongated and irregular in shape, in reactive spermatogonia (**B**); ER immunoreactivity in nuclei of Leydig cells (**C**). Original magnification: ×510

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

The upper panel of Fig. 2 reports the results of aromatase activity evaluated in vitro in presence of increasing amounts of testosterone substrate (1.4 to 7.0 μM), of a

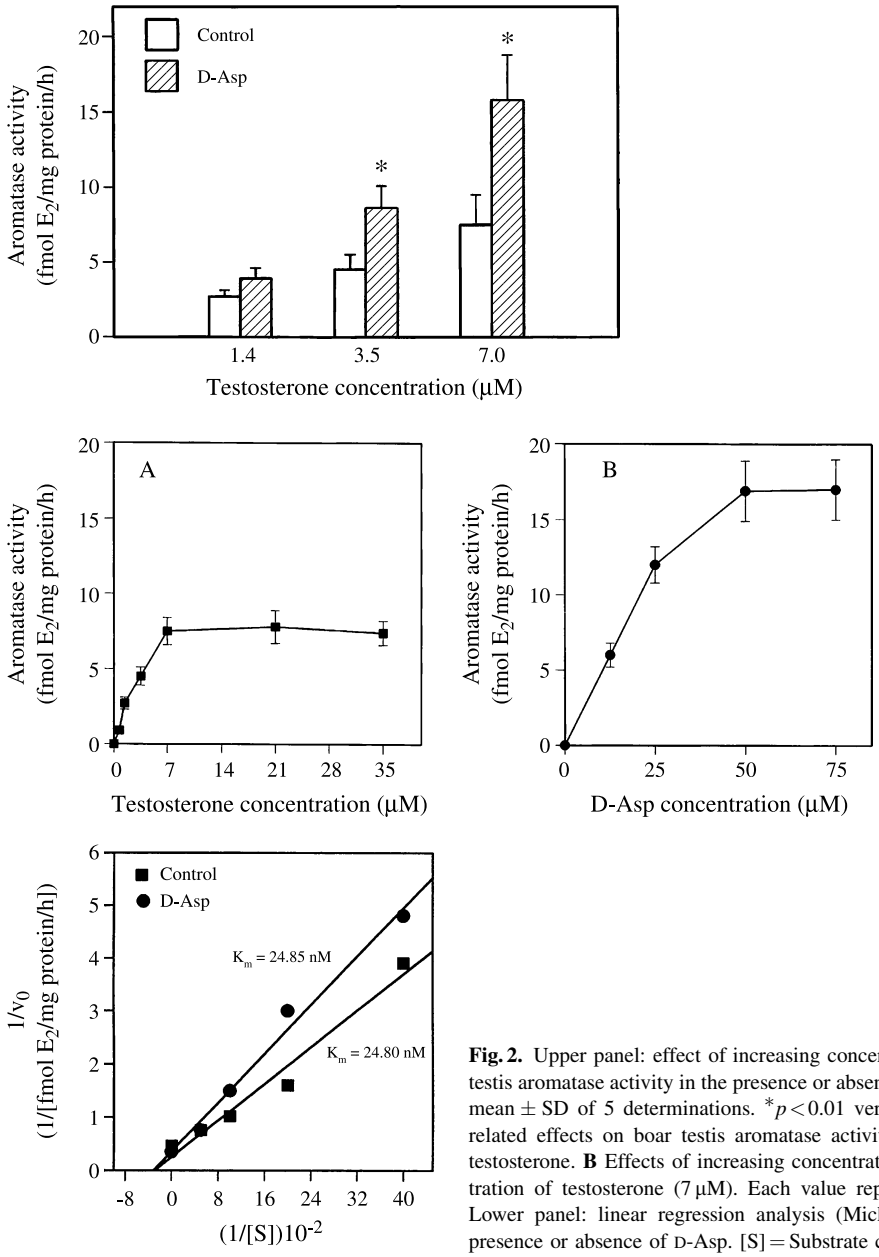

Fig. 2. Upper panel: effect of increasing concentrations of testosterone (1.4–7.0 μM) on boar testis aromatase activity in the presence or absence of 50 mM D-Asp. Each value represents the mean ± SD of 5 determinations. $^{*}p < 0.01$ versus respective controls. Middle panels: dose-related effects on boar testis aromatase activity. **A** Effects of increasing concentrations of testosterone. **B** Effects of increasing concentrations of D-Asp evaluated at saturating concentration of testosterone (7 μM). Each value represents the mean ± SD of 5 determinations. Lower panel: linear regression analysis (Michaelis-Menten plot) of aromatase activity in presence or absence of D-Asp. [S] = Substrate concentration (testosterone)

fixed amount of D-Asp (50 mM) and boar testis extract. In controls, devoid of D-Asp, as expected, the enzyme activity increases linearly as testosterone concentration increases (1.4 to 7.0 μM), whereas in wells with added D-Asp, the aromatase activity depended on substrate concentration. It is evident that 17β-estradiol production occurred at a higher rate when substrate was 3.5 and 7.0 μM ($p < 0.01$ versus respective control), becoming enhanced 2 times at testosterone saturating point (7.0 μM).

The middle panel of Fig. 2 reports the aromatase activity in presence of increasing amounts of testosterone substrate (A) and D-Asp (B). The aromatase activity was evaluated on the basis of 17β-estradiol production coming from exogenous testosterone (0–35 μM) conversion (A). In response to increasing concentrations of testosterone, there was a linear increase of 17β-estradiol production in the range of 0–7.0 μM. For this reason the 7.0 μM testosterone concentration was chosen to evaluate the effects of D-Asp on aromatase activity. As shown in Fig. 2B, the addition of increasing amounts of D-Asp (0–75 mM) to incubation wells caused a dose-related increase of aromatase activity in the range of 0–50 mM.

The lower panel of Fig. 2 shows Michaelis-Menten plots analysed using linear regression analysis. Similar K_m values very found: 24.80 nM for control and 24.85 nM for D-Asp, respectively.

Discussion

The purpose of this study has been primarily to get information on the involvement of D-aspartic acid (D-Asp) on estrogen synthesis in mammalian testis. It is well established that in several mammals the gonad produces, besides testosterone, some variable amounts of estrogens, which, according to aromatase distribution, are synthesized primarily in Leydig and, to a lesser extent, in Sertoli and germ cells (Brodie and Inkster, 1993; Nitta et al., 1993; Raeside et al., 1993; Tsubota et al., 1993; Carreau et al., 1999; Carreau, 2001). In adult testis, moreover, estrogen receptors (ER α and β) have been localized mainly in Leydig, but also in Sertoli and germ cells, proposing these cells as putative estrogen targets (Saunders et al., 1998; Mowa and Iwanaga, 2001; Zhou et al., 2002). Our immunoreactive tests (IR) for ER localization in boar testis confirm the ER localization in the nuclei of many Leydig cells. The role of estrogens in the physiology of male gonad, however, is still debated, although it has been supposed that in mature testis this steroid can impair Leydig cell androgen production, intervenes in the regulation of fluid reabsorption in efferent ducts (Hess, 2000) and operates as potent germ cell survival factors (Pentikainen et al., 2000).

In mammalian male gonad, Leydig cells, and to a lesser extent, Sertoli and germ cells have been found to express the cytochrome P450 aromatase, which converts testosterone into 17β-estradiol. As Leydig cell concerns, the enzyme has been demonstrated in rat (Brodie and Inkster, 1993), horse (Eisenhauer et al., 1994), adult ram (Bilinska et al., 1997; Schmalz and Bilinska, 1998), the bank vole (Bilinska et al., 2000), pig (Conley et al., 1996), and in the cultured rat Leydig cells (Levallet et al., 1998). Moreover, it has been proposed that the aromatase activity is under the control of modulators both at trascriptional and post-trascriptional levels (Genissel et al., 2001). In boar testis, the majority of Leydig cells express aromatase immunoreactivity, confirming the previous observations which indicate that the Leydig cells are one of the main sources of estrogens in the testis. This aspect is particularly evident in the boar testis, which is characterized by a relatively abundant interstitial tissue and a sustained estrogen production (Raeside et al., 1993; Conley et al., 1996; Saez et al., 1989).

D-Asp is an usual component of many animal tissues, including the gonads, where this amino acid level generally increases along with the physiologically activity (Di Fiore et al., 1998b; Assisi et al., 2001; Raucci et al., 2004, 2005). In rat testis, D-Asp immunoreactivity has been noticed in Leydig and Sertoli cells (D'Aniello et al., 1996) and in germ cells (Sakai et al., 1998). In several mammalian species, moreover, D-Asp enhances, in vivo and in vitro, testosterone production by the gonad, a property which has assigned to this amino acid a role as a local regulatory agent of steroid synthesis (D'Aniello et al., 1996, 1998b; Nagata et al., 1999a). These findings are consistent with the results of our biochemical and immunohistochemical studies, since the boar testis has been shown to contain D-Asp in amounts similar to that registered in other mammalian testis and this amino acid, according to immunohistochemical tests, is present mainly in Leydig cells. In addition to its stimulatory effect on testosterone production, D-Asp has been proposed to intervene also on 17β-estradiol synthesis in the ovary. Recent studies carried out on the ovary of several lower Vertebrate species, in fact, indicate that D-Asp stimulates the local aromatase activity (Di Fiore et al., 1998b; Assisi et al., 2001).

In boar testis, the localization of D-Asp-IR and P450arom-IR into many Leydig cells, and, moreover, the results of the in vitro experiments support the hypothesis that D-Asp, as in the ovary, induces 17β-estradiol synthesis. In fact, our results showed that the 17β-estradiol production by testicular tissue samples depended on testosterone substrate and was enhanced by D-Asp. This effect was higher at substrate saturation point when the increase of 17β-estradiol production was two times greater than in the controls. Furthermore, our study of aromatase catalytic properties, using linear Michaelis-Menten plots, showed that, although the enzyme activity rate was higher in presence of D-Asp, its K_m values was very similar in control and D-Asp added samples, suggesting that the D-Asp effect is performed on the same enzyme species.

These findings on boar testis, therefore, suggest a local intervention by D-Asp on aromatase activity and the related 17β-estradiol production, in addition to the well documented effect on androgen production. At present, the mechanism of action of D-Asp on aromatase remains unknown. However, since an ex novo synthesis of the enzyme in in vitro experiments seems untenable, it should be hypothesized the existence of a cytoplasmatic control

system of aromatase activity, which can be influenced by D-Asp. It is not known, moreover, if this mechanism is similar to that operating in D-Asp-dependent androgen synthesis which involves a regulatory protein which intervenes in some of the first steps of steroidogenesis. D-Asp induces both a de novo synthesis of this protein but also a post-transcriptional activation of the inactive form (Nagata et al., 1999b). Future studies will be focused in order to clarify if similar mechanisms regulated the effects on aromatase by D-Asp.

In conclusion, our observations suggest that 17β-estradiol production by boar Leydig cells is under a complex control which includes the amino acid D-Asp that operates locally by inducing an enhancement of the aromatase activity, which in turn generates estrogens. The latter probably increases the presence of estradiol receptors and, therefore, makes possible a 17β-estradiol local autocrine effect.

References

Assisi L, Botte V, D'Aniello A, Di Fiore MM (2001) Enhancement of aromatase activity by D-aspartic acid in the ovary of lizard *Podarcis s. sicula*. Reproduction 121: 803–808

Bilinska B, Lesniak M, Schmalz B (1997) Are ovine Leydig cells able to aromatize androgens? Reprod Fertil Dev 9: 193–199

Bilinska B, Schmalz-Fraczek B, Sadowska J, Carreau S (2000) Localization of cytochrome P450 aromatase and estrogen receptors alpha and beta in testicular cells. An immunohystochemical study of the bank vole. Acta Histochem 102: 167–181

Brodie A, Inskter S (1993) Aromatase in the human testis. J Steroid Biochem Mol Biol 44: 549–555

Carreau S (2001) Germ cells: a new source of estrogens in the male gonad. Mol Cell Endocrinol 178: 65–72

Carreau S, Genissel C, Bilinska B, Levallet J (1999) Sources of oestrogen in the testis and reproductive tract of the male. Int J Androl 22: 211–223

Carreau S, Papadopoulos V, Drodowsky MA (1988) Stimulation of adult rat Leydig cell aromatase activity by a Sertoli cell factor. Endocrinology 122: 1103–1109

Conley AJ, Corbin CJ, Hinshelwood MM, Liu Z, Simpson ER, Ford JJ, Harada N (1996) Functional aromatase expression in porcine adrenal gland and testis. Biol Reprod 54: 497–505

D'Aniello A, Di Fiore MM, Fisher GH (1998a) Occurrence of D-aspartic acid in animal tissues and its role in the nervous and endocrine systems. Trends Comp Biochem Physiol 4: 1–21

D'Aniello A, Di Fiore MM, D'Aniello G, Colin FE, Lewis G, Setchell BP (1998b) Secretion of D-Aspartic acid by the rat testis and its role in endocrinology of the testis and spermatogenesis. FEBS Lett 436: 23–27

D'Aniello A, Di Fiore MM, Fisher GH, Milone A, Seleni A, D'Aniello S, Perna AF, Ingrosso D (2000a) Occurrence of D-Aspartic acid and N-Methyl-D-Aspartic acid in rat neuroendocrine tissues and their role in the modulation of luteinizing hormone and growth hormone release. FASEB J 14: 699–714

D'Aniello G, Tolino A, D'Aniello A, Errico F, Fisher GH, Di Fiore MM (2000b) The role of D-aspartic acid and N-Methyl-D-Aspartic acid in the regulation of prolactin release. Endocrinology 141: 3862–3870

D'Aniello A, Di Cosmo A, Di Cristo C, Annunziato L, Petrucelli L, Fisher GH (1996) Involvement of D-aspartic acid in the synthesis of testosterone in rat testes. Life Sci 59: 97–104

Di Fiore MM, Assisi L, Botte V (1998a) Aromatase and testosterone receptor in the liver of the female green frog, *Rana esculenta*. Life Sci 62: 1949–1958

Di Fiore MM, Assisi L, Botte V, D'Aniello A (1998b) D-Aspartic acid is implicated in the control of testosterone production by the vertebrate gonad. Studies on the female green frog, *Rana esculenta*. J Endocrinol 157: 199–207

Eisenhauer KM, McCue PM, Nayden DK, Osawa Y, Roser JF (1994) Localization of aromatase in equine Leydig cells. Dom Anim Endocrinol 11: 291–298

Genissel C, Levallet J, Carreau S (2001) Regulation of cytochrome P450 aromatase gene expression in the adult rat Leydig cells: comparison with estradiol production. J Endocrinol 168: 95–105

Green S, Walter P, Kumar V, Krust A, Bornert JM, Argos P, Chambon P (1986) Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature 320: 134–139

Hess RA (2000) Oestrogen in fluid transport in efferent ducts of the male reproductive tract. Rev Reprod 5: 84–92

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA (1996) Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93: 5925–5930

Levallet J, Bilinska B, Mittre H, Genissel C, Fresnel J, Carreau S (1998) Expression and immunolocalization of functional cytochrome P450 aromatase in mature rat testicular cells. Biol Reprod 58: 919–926

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with Folin phenol reagent. J Biol Chem 193: 265–275

Mowa CN, Iwanaga T (2001) Expression of estrogen receptor-alpha and -beta mRNAs in the male reproductive system of the rat as revealed by in situ hybridization. J Mol Endocrinol 26: 165–174

Nagata Y, Homma H, Lee J-A, Imai K (1999a) D-Aspartate stimulation of testosterone synthesis in rat Leydig cells. FEBS Lett 444: 160–164

Nagata Y, Homma H, Matsumoto M, Imai K (1999b) Stimulation of steroidogenic acute regulatory protein (StAR) gene expression by D-aspartate in rat Leydig cells. FEBS Lett 454: 317–320

Nitta H, Bunick D, Hess RA, Janulis L, Newton SC, Millette CF, Osawa Y, Shizuta Y, Toda K, Bahr JM (1993) Germ cells of the mouse testis express P450 aromatase. Endocrinology 132: 1396–1401

Payne AH, Kelch RP, Musich SS, Halpern ME (1976) Intratesticular site of aromatization in the human. J Clin Endocrinol Metab 42: 1081–1087

Pentikainen V, Erkkila K, Suomalainen L, Parvinen M, Dunkel L (2000) Estradiol acts as a germ cell survival factor in the human testis in vitro. J Clin Endocrinol Metab 85: 2057–2067

Raeside JI, Wilkinson CR, Farkas G (1993) Ontogenesis of oestrogen secretion by porcine fetal testes. Acta Endocrinol 128: 549–554

Raucci F, Assisi L, D'Aniello S, Spinelli P, Botte V, Di Fiore MM (2004) Testicular endocrine activity is upregulated by D-aspartic acid in the green frog, *Rana esculenta*. J Endocrinol 182: 365–376

Raucci F, D'Aniello S, Di Fiore MM (2005) Endocrine roles of D-aspartic acid in the testis of lizard *Podarcis s. sicula*. J Endocrinol 187: 347–359

Saez JM, Sanchez P, Berthelon MC, Avallet O (1989) Regulation of pig Leydig cell aromatase activity by gonadotropins and Sertoli cells. Biol Reprod 41: 813–820

Sakai K, Homma H, Lee J-A, Fukushima T, Santa T, Tashiro K, Iwatsubo T, Imai K (1998) Localization of D-aspartic acid in elongate spermatids in rat testis. Arch Biochem Biophys 351: 96–105

Saunders PT, Fisher JS, Sharpe RM, Millar MR (1998) Expression of oestrogen receptor beta (ER beta) occurs in multiple cell types, including some germ cells, in the rat testis. J Endocrinol 156: R13–R17

Schmalz B, Bilinska B (1998) Immunolocalization of aromatase and estrogen receptors in ram Leydig cells. Ginekol Pol 69: 512–516

Simpson ER, Clyne C, Rubin G, Boon WC, Robertson K, Britt K, Speed C, Jones M (2002) Aromatase – a brief overview. Annu Rev Physiol 64: 93–127

Sternberger LA (1979) Immunocytochemistry, 2nd ed. J Wiley, New York

Tsubota T, Nitta H, Osawa Y, Mason JI, Kita I, Tiba T, Bahr JM (1993) Immunolocalization of steroidogenic enzymes, P450scc, 3 beta-HSD, P450c17, and P450arom in the Hokkaido brown bear (*Ursus arctos yesoensis*) testis. Gen Comp Endocrinol 92: 439–444

Turner KJ, Macpherson S, Millar MR, McNeilly AS, Williams K, Cranfield M, Groome NP, Sharpe RM, Fraser HM, Saunders PT (2002) Development and validation of a new monoclonal antibody to mammalian aromatase. J Endocrinol 172: 21–30

Zhou Q, Nie R, Prins GS, Saunders PT, Katzenellenbogen BS, Hess RA (2002) Localization of androgen and estrogen receptors in adult male mouse reproductive tract. J Androl 23: 870–881

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