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D-Aspartate stimulation of testosterone synthesis in rat Leydig cells

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Abstract D-Aspartate increases human chorionic gonadotropin-induced testosterone production in purified rat Leydig cells. L-Aspartate, D-,L-glutamate or D-,L-asparagine could not substitute for D-aspartate and this effect was independent of glutamate receptor activation. Testosterone production was enhanced only in cells cultured with D-aspartate for more than 3 h. The increased production of testosterone was well correlated with the amounts of D-aspartate incorporated into the Leydig cells, and L-cysteine sulfinic acid, an inhibitor of D-aspartate uptake, suppressed both testosterone production and intracellular D-aspartate levels. D-Aspartate therefore is presumably taken up into cells to increase steroidogenesis. Intracellular D-aspartate probably acts on cholesterol translocation into the inner mitochondrial membrane, the rate-limiting process in steroidogenesis.

Key words: D-Aspartate; Testosterone; Steroidogenesis; Leydig cell; Human chorionic gonadotropin; Glutamate transporter

1. Introduction

A variety of D-amino acids have been found in the mammalian body and widespread and transient appearance of D-aspartate (D-Asp) has been observed in various mammalian tissues [1,2]. D-Asp levels transiently increase during the development of human [3] and rat brains [4], the rat retina and the chicken embryonic brain [5]. In the brain of patients with Alzheimer disease, D-Asp levels are significantly decreased [6]. D-Asp is also found in a variety of neuroendocrine and endocrine organs such as the testis [7,8], pineal [9,10], adrenal and pituitary glands [4,8] and the time periods of maximal D-Asp content correspond well with the morphological and functional maturation of these organs. Immunohistochemical studies with anti-D-Asp antiserum previously prepared in this laboratory demonstrated that D-Asp appears and becomes localized in specific cell types of these organs at distinct periods of development [7,11,12]. In the differentiating neurons of the rat central nervous system D-Asp appears to change its intracellular localization during neurogenesis [13]. In the rat brain, D-Asp has affinity for the glutamate (Glu) binding site of NMDA receptors [1], the activation of which is conceivably involved in ontogeny of the brain [14]. Our recent study indicated that D-Asp is actually synthesized in mammalian cells [15]. These lines of evidence suggested that D-Asp has some physiological role as a novel type of messenger in mammals. We demonstrated in other recent reports that high contents of D-Asp (as high as 30–40% of total Asp) are present in the cytoplasm of pinealocytes of the rat pineal gland [9–11]. Although synthesis of D-Asp is minimal to non-existent in cultured rat pinealocytes, exogenous D-Asp is efficiently taken up into these cells [16] and released in response to norepinephrine stimulation. This results in the concomitant suppression of norepinephrine-induced melatonin secretion, presumably via D-Asp stimulation of Glu receptors [16].

In adult rat testis, approximately 30% of total Asp is in the D-form [7,8]. The D-Asp content is relatively low in the testis of 3 weeks old rats, but increases between the age of 6 and 10 weeks and then remains constant until the age of 40 weeks [7]. D-Asp is localized in a region rich in elongate spermatids, the most mature form of germ cells [7]. In isolated rat testis, D-Asp was suggested to be involved in testosterone synthesis using a crude mixture of testicular cells [17]. As a further step towards clarifying the functional significance of D-Asp in steroidogenesis, we have purified Leydig cells from rat testis and studied the direct effects of D-Asp on human chorionic gonadotropin (hCG)-stimulated steroidogenesis.

2. Materials and methods

2.1. Animals and reagents

Adult male Sprague-Dawley rats (10–12 weeks old) were purchased from Charles River Japan (Kanagawa, Japan). Collagenase (type II), testosterone, BSA, hCG, L-cysteine sulfinic acid (LCSA), 22(*R*)-hydroxycholesterol, dibutyryl cAMP, D-Asp and other amino acids were obtained from Sigma (St. Louis, MO, USA). Glu receptor agonists, AMPA, NMDA and quisqualate were from Sigma, kainate from Wako (Osaka, Japan), DCG-IV and L-AP4 were from Tocris Cookson (Ballwin, MO, USA). HEPES was from Nacalai Tesque (Kyoto, Japan). Dulbecco's modified Eagle's medium:F-12 (1:1) (DMEM/F-12), Medium 199 (M199), penicillin and streptomycin were purchased from Gibco-BRL (New York, USA). Percoll was a product of Pharmacia (Uppsala, Sweden). 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was from Wako. Pirkle-type chiral column, OA2500(S), was kindly donated by Sumika Chemical Analysis Service (Osaka, Japan). Rabbit antiserum for testosterone-3(*E*)-carboxymethyloxime-BSA conjugate and testosterone-3(*E*)-carboxymethyloxime-peroxidase conjugate were obtained from Cosmo Bio (Tokyo, Japan). Microplates coated with goat antiserum raised against rabbit IgG were from Eiken Chemical (Tokyo, Japan).

2.2. Preparation of Leydig cells

Purification of rat Leydig cells was carried out according to the method of Sun et al. [18] with some modifications. Two decapsulated rat testes were dispersed by shaking (140–150 cycles/min) for 20 min at 34°C in 3 ml of M199-BSA (M199 including 25 mM HEPES, pH 7.3, 0.1% BSA, 100 U/ml penicillin and 100 μg/ml streptomycin) containing 0.05% collagenase. After incubation, the dispersed tissues were diluted with M199-BSA and the solution was filtered through a nylon mesh (Falcon, 70 μm). Interstitial cells were precipitated by centrifugation of the filtrate and resuspended in 2 ml of M199-BSA. Leydig cells were purified on a discontinuous gradient of Percoll in M199-BSA. The gradient was composed of successive layers of Percoll with average densities of 1.09, 1.07, 1.05 and 1.035 g/ml. The interstitial cells were layered on the gradient and centrifuged at $800\times g$ for 20 min. Cells contained in the layer of Percoll of average density of

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Abbreviations: hCG, human chorionic gonadotropin

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1.07 were then collected, diluted with two volumes of M199-BSA and centrifuged at 150×*g*. Precipitated cells were then washed once and resuspended in DMEM/F-12-BSA (DMEM/F-12 including 25 mM HEPES, pH 7.3, 0.1% BSA, 100 U/ml penicillin and 100 μg/ml streptomycin). Cell viability determined by a trypan blue exclusion test was more than 90%. The purity of the resulting Leydig cell preparation as determined by 3β-hydroxysteroid dehydrogenase was more than 85%.

2.3. Leydig cell culture

Purified Leydig cells were plated in 48-well culture plates (Coster, Cambridge, MA, USA) and cultured in DMEM/F-12-BSA at 37°C under 5% CO_2-95% air. The cells were cultured in the presence of different concentrations of D-Asp for different time intervals and then incubated with hCG (5 mIU/ml) or other compounds for 2 h. After the incubation, media were collected and centrifuged at 600×*g* for 10 min. The supernatants were stored at −20°C until assayed for testosterone. Cell viability was determined by staining the cells with crystal violet according to the method of Kueng et al. [19] with some modifications.

2.4. Enzyme immunoassay (EIA) of testosterone

Amounts of testosterone in culture media were measured by EIA following the method of Marcus and Durnford [20] with some modifications. Intraassay and interassay variations were 8.3% and 11.3%, respectively.

2.5. Amino acid analysis

Samples for amino acid analysis were prepared by following the method described in our previous reports [7,10,16]. The wells were washed with PBS(−) and 10 volumes of methanol were added for the extraction of cellular amino acids. The amino acids extracted were then quantitated fluorometrically by HPLC.

2.6. Statistic analysis

Results are expressed as mean ± S.D. Significant differences between groups were determined by Duncan's multiple range test.

3. Results

3.1. Specific stimulation of testosterone synthesis by D-Asp in rat Leydig cells

In purified rat Leydig cells, D-Asp directly increased hCG-induced testosterone synthesis. When purified rat Leydig cells were precultured overnight (21 h) in the presence of D-Asp, hCG-induced testosterone synthesis during the subsequent 2 h incubation increased approximately four fold compared to control cells, cultured in the absence of D-Asp (Fig. 1). An increase in testosterone synthesis was dependent upon the culture period with D-Asp. After 1 h of culturing with D-Asp, testosterone synthesis was not yet enhanced, but after 3 h of culturing with D-Asp, testosterone synthesis was considerably stimulated (Fig. 1). As described previously [21], longer culturing of cells with D-Asp resulted in decreased testosterone synthesis (Fig. 1), though freshly isolated and purified Leydig cells showed high levels of testosterone synthesis in response to hCG induction. This may have been due to decreased hCG sensitivity brought about by longer culturing, perhaps caused by a decrease in receptor number and/or signal transduction, since the basal synthetic rate of testosterone (without induction by hCG) was similar between 1 h and overnight cultures (1 h: 0.25 ± 0.01 ng/10^5 cells/h, overnight: 0.24 ± 0.02 ng/10^5 cells/h). However, the increased synthesis of testosterone by D-Asp was not ascribable to preserved sensitivity to hCG in the cells, since cAMP content after hCG challenge was the same between the cultures with and without D-Asp (data not shown). In addition, crystal violet staining demonstrated that numbers of cells attached to the wells were almost the same after preculturing in the presence or absence of D-Asp (the absorbance of the stained cells precultured with D-Asp was 117% compared to that without D-Asp). These results indicated that culturing with exogenous D-Asp increases hCG-induced testosterone production in rat Leydig cells after more than 3 h. In the following experiments the cells were precultured with D-Asp for 16 h, since this resulted in the highest increase in testosterone production compared to control cells.

D-Asp is known to act as an agonist for Glu receptors [1].

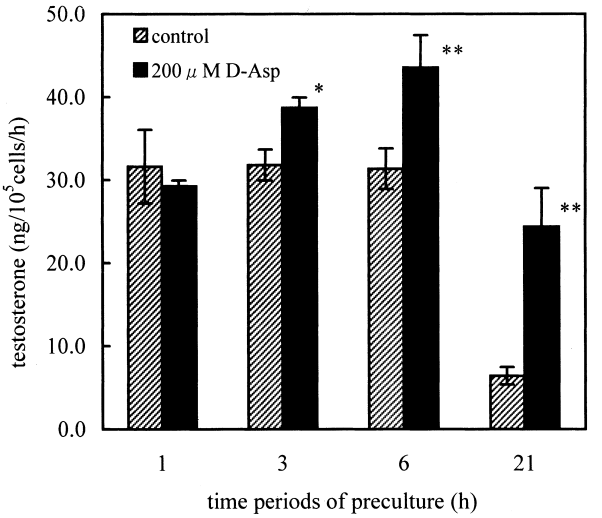

Fig. 1. Time-dependent increase of testosterone synthesis by D-Asp in rat Leydig cells. Purified Leydig cells were cultured for the indicated time periods in the presence or absence (control) of 200 μM of D-Asp. The control medium (without addition of D-Asp) contained approximately 25 nM of D-Asp. Subsequently the cells were incubated in the medium containing 5 mIU/ml of hCG for 2 h and testosterone in the medium was assayed by EIA. An increase in testosterone synthesis was observed at similar levels in the cultures for 16 h up to 24 h. Significantly different compared to control, $*P < 0.05$, $n = 3$; $**P < 0.01$, $n = 3$.

Table 1
Stereospecificity of D-Asp stimulation of testosterone synthesis in rat Leydig cells

	Testosterone (ng/10^5 cells/h)
Experiment I	
Control	6.7 ± 0.6
D-Aspartate	19.6 ± 2.2**
L-Aspartate	7.7 ± 0.3
D-Glutamate	7.3 ± 0.9
L-Glutamate	6.4 ± 1.0
Experiment II	
Control	5.9 ± 0.3
AMPA	7.8 ± 0.4
NMDA	6.0 ± 0.5
Kainate	6.6 ± 0.4
Experiment III	
Control	6.5 ± 0.3
Quisqualate	8.5 ± 1.2
DCG-IV	8.9 ± 1.4
L-AP4	5.0 ± 0.3

Purified rat Leydig cells were precultured with various compounds as indicated (200 μM each) for 16 h. Subsequent incubation with hCG and assay of testosterone were the same as in Fig. 1. 500 μM of synthetic agonists were also examined with similar results. Agonists for ionotropic Glu receptors and metabotropic Glu receptors were assayed in Exp. II and Exp. III, respectively. Significantly different compared to control: $**P < 0.01$, $n = 3$.

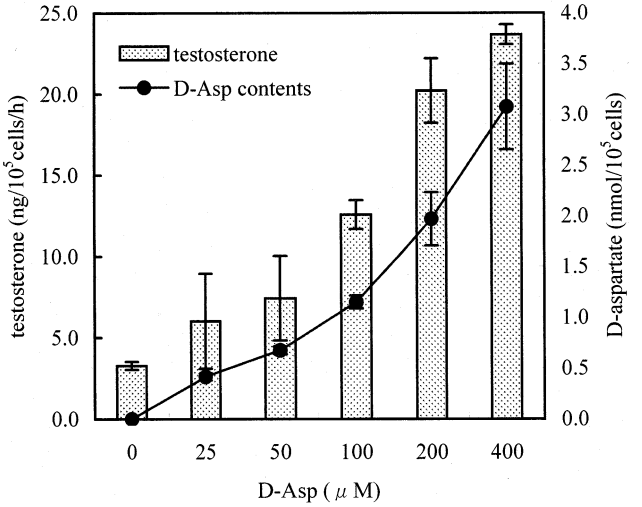

Fig. 2. Effect of various doses of D-Asp on testosterone synthesis and D-Asp contents in Leydig cells. Purified Leydig cells were cultured with the indicated doses of D-Asp for 16 h. Subsequently the cells were incubated in the medium containing 5 mIU/ml of hCG for 2 h as in Fig. 1. Testosterone in the medium and cellular D-Asp content after culturing for 16 h were determined ($n=3$) as in Section 2. The increase of testosterone production was also observed in a range of hCG concentrations from 0.2 to 125 mIU/ml and in a time period of 5 h after the addition of hCG.

Our previous report suggested that activation of the receptor by D-Asp is involved in the suppression of melatonin secretion in rat pineal gland [16]. Therefore D-Asp also may participate in the increased synthesis of testosterone in rat Leydig cells through activation of the receptors. However, this is not the case. L-Asp, D-,L-Glu (Table 1), D- or L-asparagine (data not shown) could not substitute for D-Asp and various synthetic agonists for the subtypes of Glu receptors were not effective as a substitute for D-Asp. Furthermore, the stimulation by D-Asp was not affected in the presence of these receptor agonists or antagonists (data not shown). These results indicated that the increase in testosterone production by D-Asp is independent of activation of Glu receptors in rat Leydig cells.

3.2. Uptake of D-Asp in rat Leydig cells

In the following studies we addressed the issue of whether prior incorporation of D-Asp into the Leydig cells and subsequent culturing are necessary for the increase in testosterone production. Fig. 2 indicates that the amounts of testosterone produced were dependent on the doses of exogenous D-Asp. The amounts of D-Asp taken up into the cells also increased depending on the doses of D-Asp and its amounts in the cells were well correlated with the amounts of testosterone synthesized (Fig. 2). D-Asp was presumably incorporated into the cells via the L-Glu transporter which has an affinity for D-Asp in addition to L-Glu and L-Asp [22,23]. D-Asp accumulated at a high level in the cells probably due to a very low level of degradation by D-Asp-oxidase in the testis [24]. Our preliminary experiments performed with RT-PCR revealed that a major subtype of Glu transporters expressed in rat Leydig cells is GLAST [25], which is efficiently inhibited by LCSA [26]. As demonstrated in Fig. 3, D-Asp incorporation into the cells and stimulation of testosterone production by D-Asp were concomitantly suppressed depending on the doses of LCSA. D-Asp content in the cells was again well correlated with testosterone production. LCSA itself had almost no effects on the production of testosterone without D-Asp, although it brought about a weak increase in testosterone synthesis at some doses (Fig. 3).

The decrease in the cellular content of D-Asp and the drop in testosterone production elicited by LCSA were both restored by the simultaneous addition of a higher dose of D-Asp (in cells precultured with 200 μM of D-Asp, the cellular content of D-Asp and testosterone production were suppressed by 50 μM LCSA to 50% and 60%, respectively, but could be completely restored by the addition of 400 μM D-Asp). It is interesting that when the cells were cultured without D-Asp for 24 h prior to culturing with D-Asp for 16 h, testosterone synthesis no longer increased, though basal testosterone production remained constant after 24 h of culture. Uptake of D-Asp also significantly decreased in these cells precultured for 24 h. These lines of evidence suggested that D-Asp must be taken up into fresh Leydig cells for the stimulation of testosterone production.

3.3. D-Asp effect on the testosterone synthetic pathway

In response to hCG stimulation, receptor-coupled adenylate cyclase is activated [27] and increased levels of cAMP stimulate testosterone production by an undefined mechanism in Leydig cells [28–30]. Therefore, dibutyryl cAMP can substitute for hCG in the induction of testosterone synthesis. Fig. 4 indicates that D-Asp increased dibutyryl cAMP-induced testosterone synthesis similarly to hCG. As described above, cAMP contents after hCG challenge were not affected by preculturing the cells with D-Asp. These results suggested that the effect of D-Asp on testosterone synthesis is beyond cAMP formation.

The first step of testosterone synthesis is the conversion of cholesterol to pregnenolone, which is catalyzed by P-450 side-chain cleavage (P-450scc) in inner mitochondrial membranes [28–30]. The overall rate of testosterone synthesis is limited by

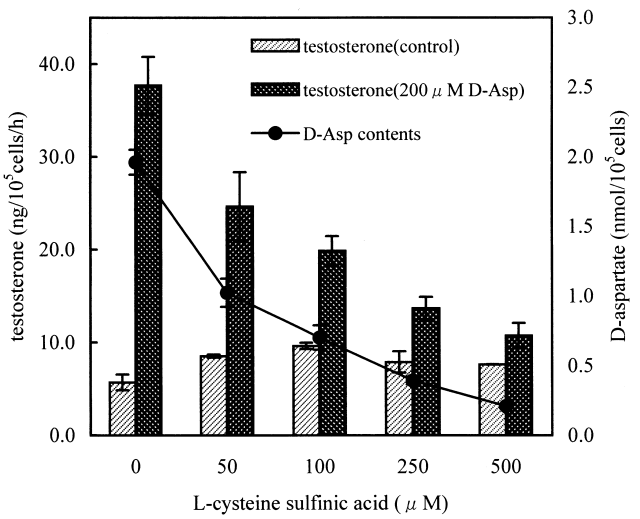

Fig. 3. Effect of LCSA on testosterone synthesis and Asp contents in Leydig cells. Purified Leydig cells were incubated with indicated doses of LCSA for 10 min prior to the addition of 200 μM D-Asp and cultured for 16 h. Control cells received no D-Asp. Subsequent incubation with hCG and assay of testosterone were the same as in Fig. 1. The amounts of testosterone synthesized and D-Asp contents in the cells after 16 h of culturing with D-Asp and LCSA were determined ($n=3$) as in Section 2.

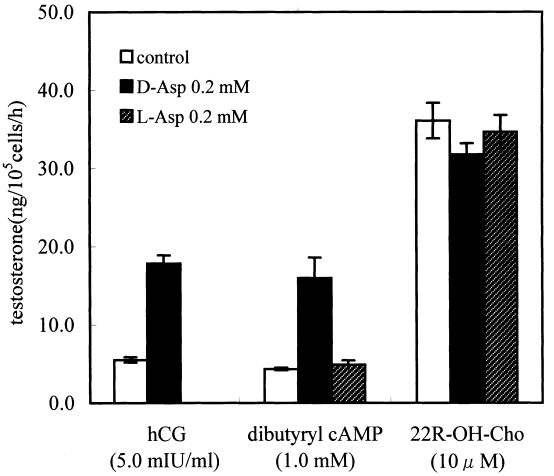

Fig. 4. Effects of dibutyryl cAMP and 22(*R*)-hydroxycholesterol on testosterone synthesis in Leydig cells. Purified Leydig cells were cultured with 200 μM of D- or L-Asp for 16 h. Subsequently the cells were incubated in the medium containing hCG (5 mIU/ml), dibutyryl cAMP (1.0 mM) or 22(*R*)-hydroxycholesterol (10 μM), respectively for 2 h and the amounts of testosterone synthesized were measured ($n = 3$).

the availability of cholesterol to the intramitochondrial P-450scc. To surmount this limitation, 22(*R*)-hydroxycholesterol, a freely accessible substrate to the side-chain cleavage reaction, can be supplied to attain maximal testosterone synthesis by the steroidogenic cascade [31]. In order to investigate the effect of D-Asp on the ultimate capacity of P-450scc and subsequent steroidogenic cascade, testosterone synthesis was measured after supplying 22(*R*)-hydroxycholesterol to the cells. As in Fig. 4, similar amounts of testosterone were produced in control and D-Asp-treated cells in the presence of the cholesterol derivative, indicating that D-Asp does not affect the P-450scc and subsequent enzymatic steps. These lines of evidence imply that the effect of D-Asp is beyond cAMP formation and prior to the side-chain cleavage by P-450scc, possibly supply of cholesterol to the P-450scc.

4. Discussion

Here we demonstrate that D-Asp increases hCG-induced testosterone synthesis in purified rat Leydig cells. This increase in steroidogenesis was specific to D-Asp and the effect was independent of Glu receptor activation. Increased testosterone synthesis was observed only in cells precultured for more than 3 h with D-Asp. D-Asp is presumably to be taken up into the cells to increase steroidogenesis.

A recent study performed by our group [16] indicates that D-Asp taken up into cultured rat pinealocytes is released upon norepinephrine stimulation and concomitantly suppresses norepinephrine-induced secretion of melatonin. This suppression was also observed with L-Glu and L-Asp and these agonists assumed to activate the Glu receptor(s) and subsequently suppressed the activity of arylalkylamine *N*-acetyltransferase, a rate-limiting step in melatonin production [32–34]. By contrast, the stimulation of testosterone synthesis in rat testis is specific to D-Asp and independent of Glu receptor activation, as described in this study. The mechanism for increase of testosterone synthesis by D-Asp is apparently distinct from that of melatonin secretion suppression in the pineal gland.

In adult rat testis, the levels of D-Asp are as high as 30–40% of the total Asp content [7,8]. D-Asp levels show a strong relation with testosterone content in the testis during development [17]. In a crude mixture of rat testicular cells, D-Asp has been suggested to be involved in testosterone synthesis [17]. The testis consists of various types of cells [35] and testosterone synthesis in the testis is under control of autocrine factors as well as paracrine factors from these cells [36,37]. This study showed that D-Asp directly increases hCG-induced testosterone production in purified rat Leydig cells. Recently D-Asp has been reported to suppress testosterone production in isolated ovarian follicles of the green frog *Rana esculenta* [38]. In the gonads D-Asp presumably behaves as a paracrine factor for the regulation of steroidogenesis in spite of differential effects depending on species and sex.

We demonstrated by immunohistochemical and biochemical techniques that D-Asp is mainly localized in the cytoplasm of elongate spermatids, the most mature of the germ cells and at very low levels in interstitial cells (including Leydig cells) and Sertoli cells [7]. D-Asp contents in purified rat Leydig cells were actually very low (Fig. 2b), although its presence in Leydig and Sertoli cells has been reported [17]. During the final steps of germ cell maturation, elongate spermatids expel most of their cytoplasm and deposit it in the seminiferous tubules when they are released as free spermatozoa into the epididymides [39]. Since D-Asp content in the epididymides is very low compared to that in the testes [7], most of the D-Asp in the spermatids is assumed to remain in the seminiferous tubules and regulate steroidogenesis in Leydig cells in the interstitial spaces in a paracrine fashion.

In Leydig cells, steroidogenic pathways begin in the mitochondria with the conversion of cholesterol to pregnenolone [28–30]. Cholesterol is transported from intracellular sources (across the outer mitochondrial membrane) to the inner mitochondrial membrane where it is converted to pregnenolone by P-450scc. Pregnenolone is subsequently converted to testosterone in the steroidogenic cascade outside the mitochondria. The process of translocation to the inner mitochondrial membrane is the rate-limiting step for testosterone synthesis, which is activated in response to hCG stimulation through increased levels of cAMP. Recent investigations have revealed several components involved in cholesterol transfer, such as steroidogenic acute regulatory protein [28] and peripheral-type benzodiazepine receptor [29], a multiprotein channel that allows the transition of cholesterol. The results described in this study suggest that D-Asp acts on the process of cholesterol translocation to the inner mitochondrial membrane. The mechanisms of cholesterol movement into steroidogenic mitochondria are not yet defined and D-Asp may be involved in this key process of steroidogenesis. Further research on the mechanism of action of D-Asp should certainly provide valuable insight into the functional roles of D-Asp in endocrine organs.

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