Stimulation of steroidogenic acute regulatory protein (StAR) gene expression by D-aspartate in rat Leydig cells

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Abstract D-aspartate and human chorionic gonadotropin act synergistically to increase testosterone production in purified rat Leydig cells, and D-aspartate stimulates testosterone synthesis even in the absence of human chorionic gonadotropin stimulation. In addition, D-aspartate enhances steady-state cellular mRNA and protein levels of steroidogenic acute regulatory protein, which is a key regulatory factor in gonadal and adrenal steroidogenesis. D-aspartate therefore appears to increase testosterone production in rat Leydig cells by stimulating steroidogenic acute regulatory protein gene expression. To our knowledge, this is the first report demonstrating a direct effect of D-aspartate on gene expression in mammalian cells.

Key words: D-aspartate; Steroidogenic acute regulatory protein; StAR; Testosterone; Leydig cell

1. Introduction

The presence of D-aspartate (D-Asp) has been demonstrated in a wide variety of mammalian tissues [1,2]. Levels of this unique amino acid are transiently increased during tissue development [1,2]. We recently demonstrated that D-Asp is localized in specific cell types in developing tissues [3–5] and changes its localization within the organs as well as its intracellular distribution during development [6]. Our recent study also indicated that D-Asp is actually synthesized in mammalian cells [7]. These results suggested that D-Asp has some physiological functions in mammalian cells. Indeed, D-Asp suppresses norepinephrine-induced melatonin secretion in cultured rat pinealocytes, which contain significant levels of the amino acid in vivo [8].

In adult rat testis, approximately 30% of the total Asp content is in the D-form [3,9]. Our previous report demonstrated that D-Asp increases human chorionic gonadotropin (hCG)-induced testosterone production in purified rat Leydig cells [10]. In these cells, intracellular cholesterol is transported to the inner mitochondrial membrane, where cholesterol is converted to pregnenolone by cytochrome P450 side-chain cleavage (P450scc) as the first step in testosterone synthesis. The process of cholesterol delivery to the inner mitochondrial membrane is the rate-limiting step for testosterone synthesis, which is activated in response to hCG stimulation by a cAMP signal transduction pathway [11,12]. D-Asp increases testosterone production without affecting the cAMP content of the cells, and its point of action was traced to the rate-limiting process of cholesterol delivery. L-Asp, D,L-glutamate and D,L-asparagine were all unable to substitute for D-Asp, which is presumably to be taken up into the cells to increase steroid hormone production [10].

Steroidogenic acute regulatory protein (StAR) is a key regulatory protein which plays an essential role in gonadal and adrenal steroidogenesis. It is assumed to enhance the rate-limiting step, cholesterol delivery to the inner mitochondrial membrane [11,13]. Mutations in the StAR gene are responsible for congenital lipoid adrenal hyperplasia, an autosomal recessive disease in which adrenal and gonadal steroid synthesis is markedly impaired [14]. StAR mRNA is specifically expressed in steroidogenic tissues such as the testis, ovary and adrenal gland [15,16]. In these tissues, StAR expression is stimulated by trophic hormones such as adrenocorticotropic hormone, luteinizing hormone and follicle-stimulating hormone [12]. Transfection of steroidogenic and non-steroidogenic cells with StAR cDNA directly increased steroid production in the absence of hormone stimulation [17,18].

In this report, we demonstrate that D-Asp and hCG act synergistically to increase testosterone production in purified rat Leydig cells, and that D-Asp stimulates testosterone synthesis even in the absence of hCG stimulation. Furthermore, D-Asp enhances steady-state levels of StAR mRNA and protein. D-Asp therefore appears to increase testosterone production by stimulating StAR gene expression in rat Leydig cells. To our knowledge, this is the first report demonstrating an effect of D-Asp at the genetic level in mammalian cells.

2. Materials and methods

2.1. Materials

hCG and D-Asp were obtained from Sigma Chemical Co. (St. Louis, MO, USA). [α-^{32}P]-dCTP (110 TBq/mmol) was a product of Amersham Pharmacia Biotech Inc. (Piscataway, NJ, USA). Oligonucleotide primers were prepared by Sawady Technology Inc. (Tokyo, Japan). Restriction enzymes and Taq DNA polymerase were purchased from TAKARA (Kyoto, Japan). Horseradish peroxidase-conjugated goat anti-rabbit IgG was a product of Seikagaku Corporation (Tokyo, Japan).

2.2. Rat Leydig cell preparation and culture

Rat Leydig cells were prepared and cultured as described previously [10]. The purified Leydig cells were cultured in the presence or absence of 200 μM D-Asp for 16 h, followed by incubation with varying concentrations of hCG for 2 h before being removed from the culture media by centrifugation at 600×*g*. The media were then assayed for testosterone content.

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Abbreviations: D-Asp, D-aspartate; EIA, enzyme immunoassay; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; hCG, human chorionic gonadotropin; StAR, steroidogenic acute regulatory protein

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2.3. Enzyme immunoassay (EIA) of testosterone

Testosterone in the culture media was measured by enzyme immunoassay as described previously [10].

2.4. Preparation of StAR probe

A 224 bp rat StAR cDNA fragment was amplified by PCR using primers (sense: AACACTCTACAGTGACCAGGAGCTGT, antisense: GGTCCACCAGTTCTTCATAG) which correspond to the rat StAR cDNA sequences nt192–217 and nt415–396 [19] and cloned into a pT7Blue-2 T-vector (Novagen, Madison, WI, USA). The resulting insert was extracted and purified using a QIAEX II kit (QIAGEN Inc., Valencia, CA, USA), and labeled with 1.85 MBq of [α-^{32}P]-dCTP (10^9 dpm/μg) using a DNA labeling kit (Ready To Go, Amersham Pharmacia Biotech), according to the respective manufacturers' instructions. Labeled probes were separated from free nucleotides with G50 spin columns (ProbeQuant, Amersham Pharmacia Biotech).

2.5. RNA extraction and Northern blot analysis

Total RNA was extracted from cultured rat Leydig cells with ISOGEN reagent (Wako Chemical Ind., Osaka, Japan), a mono-phasic solution of phenol and guanidine isothiocyanate. Approximately 30 μg of total RNA was separated by electrophoresis on 1.0% agarose/18% formaldehyde/MOPS gels and transferred to Hybond N$^-$ nitrocellulose membranes (Amersham Pharmacia Biotech). After 1 h of prehybridization at 65°C, filters were hybridized with the labeled probes (rat StAR probe or rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA) at $1–2\times10^6$ dpm/cm^2 for 2 h at 65°C in hybridization buffer (Rapid hyb buffer, Amersham Pharmacia Biotech). Following hybridization, membranes were rinsed with 2×SSPE, 0.1% SDS and washed in 0.1×SSPE, 0.1% SDS and then exposed to Kodak X-OMAT AR films at −80°C for 1–2 days. Intensities of autoradiographic bands were estimated by densitometric scanning.

2.6. Western blot analysis

Cultured Leydig cells were solubilized in the well with 0.1% SDS and cellular proteins were separated on 12.5% SDS/polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. The membranes were incubated for 1 h at room temperature (R.T.) with a rabbit polyclonal antibody raised against a ten amino acid peptide containing residues #88–98 of the 30 kD StAR protein [18]. The antibody was kindly donated by Prof. D.M. Stocco, Texas Tech University, TX, USA. Subsequently the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 1 h at R.T. and bound antibody was detected by chemiluminescence using a ECL detection kit (Amersham Pharmacia Biotech) and Kodak X-OMAT AR films.

2.7. Statistical analysis

Results were expressed as mean ± standard deviation. Significant differences between groups were determined by Duncan's multiple-range test.

3. Results

3.1. D-Asp effect on testosterone synthesis in rat Leydig cells

In a previous report [10], we demonstrated that D-Asp increases hCG-induced testosterone synthesis in a dose-dependent manner in purified rat Leydig cells. In the present study, we examined the effect of D-Asp on the testosterone synthesis induced by varying doses of hCG. D-Asp (200 μM) stimulated testosterone production to a similar extent in cells exposed to a wide range of hCG concentrations (Fig. 1A). D-Asp and hCG therefore appear to have synergistic rather than additive effects on testosterone synthesis. In addition, D-Asp but not L-Asp stimulated testosterone production even in the absence of hCG stimulation (Fig. 1B). The increase in testosterone synthesis was observed in cells treated with D-Asp for 16 h but not in cells treated for 1 h (Fig. 1B). The effect of D-Asp on hCG-induced testosterone synthesis was also time-dependent and required more than 3 h exposure to D-Asp to become apparent [10]. These lines of evidence suggest that D-Asp acts at a site(s) distinct from the stimulatory pathway activated by hCG.

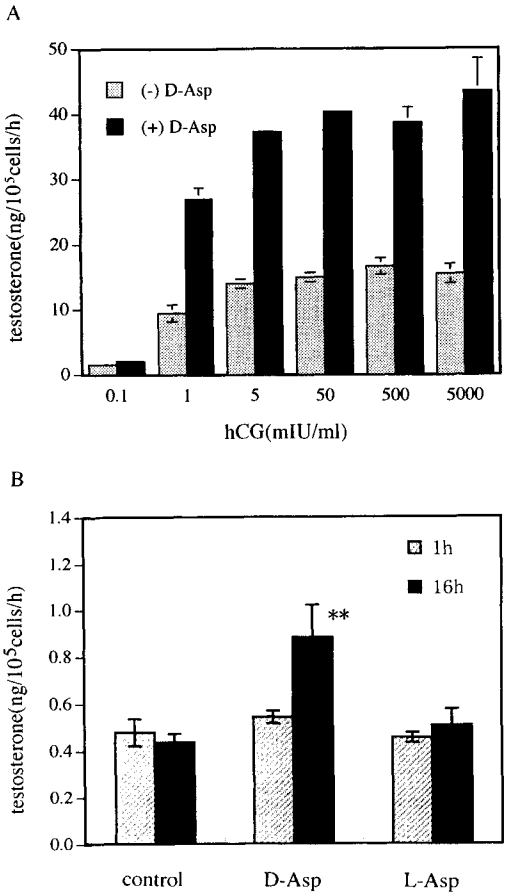

Fig. 1. D-Asp effect on testosterone synthesis in rat Leydig cells. A: Purified rat Leydig cells were cultured for 16 h in the presence or absence of 200 μM D-Asp and then incubated with the indicated concentrations of hCG for 2 h before testosterone secreted into the medium was assayed by EIA. Data are shown as mean ± S.D., $n=3$. B: Purified Leydig cells were cultured for the indicated time periods in the presence or absence of 200 μM D- or L-Asp and then incubated for 2 h without stimulation by hCG before testosterone secreted into the medium during this incubation was assayed by EIA. Results which are significantly different from controls are indicated by asterisks ($P<0.01$, $n=3$).

3.2. D-Asp effect on StAR gene expression in rat Leydig cells

We previously demonstrated that D-Asp is taken up into rat Leydig cells to increase the rate of cholesterol transport to the inner mitochondrial membrane [10]. Cytochrome P450 side-chain cleavage (P450scc) at this site catalyzes the first step in steroid hormone synthesis. The process of translocation to the inner mitochondrial membrane is the rate-limiting step for steroid synthesis, and StAR is the key regulatory protein in this process [11,13].

Fig. 2 demonstrates that D-Asp increases hCG-induced StAR gene expression in rat Leydig cells. hCG induces StAR expression by increasing transcription of the gene through a cAMP second messenger pathway [20,21]. Northern blot analysis demonstrated that the steady-state level of StAR mRNA is increased by hCG treatment, and prior treatment with D-Asp further increased the StAR mRNA level approximately 3.5-fold relative to hCG treatment without D-Asp.

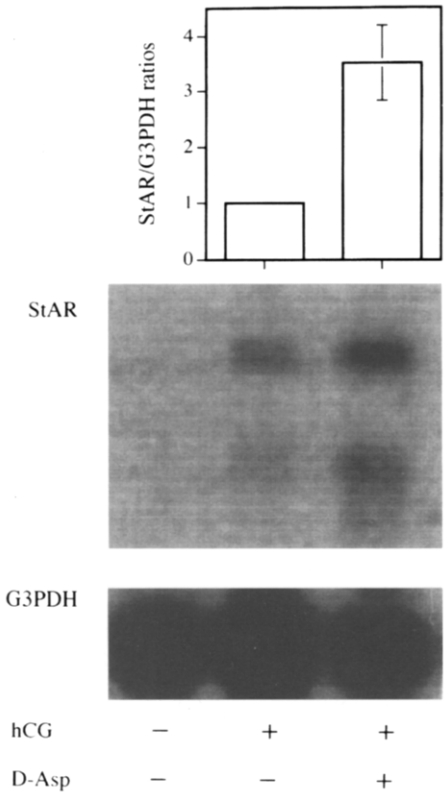

Fig. 2. Northern blot analysis. Purified Leydig cells were cultured for 16 h in the presence or absence of 200 μM D-Asp and then stimulated with 5 mIU/ml hCG or incubated without hCG. Total RNA (30 μg) was prepared from the cells, separated on formaldehyde-denaturing gels and transferred to nylon membranes. Blots were hybridized with [^{32}P]-labeled rat StAR and G3PDH DNA probes. Lower panel: Representative Northern blot. Two major transcripts (3.8 and 1.7 kb) [26] are evident. Upper panel: The intensities of the autoradiographic bands were estimated and expressed as StAR/G3PDH ratios. The data presented relate to the 3.8 kb transcript and are shown as mean ± S.D. ($n = 6$).

Moreover, StAR mRNA levels were also increased by D-Asp treatment even in the absence of hCG stimulation (data not shown).

In Fig. 3, Western blot analysis was carried out using total protein from the Leydig cells. The protein levels detected by anti-StAR antibody were increased significantly by hCG stimulation, and D-Asp pretreatment further increased the protein level approximately 1.9-fold. These lines of evidence indicate that D-Asp enhances StAR gene expression at both mRNA and protein levels, resulting in the increased production of testosterone in rat Leydig cells.

4. Discussion

This study demonstrated that D-Asp and hCG synergistically up-regulate the production of testosterone in rat Leydig cells, apparently by increasing StAR gene expression. D-Asp alone increases testosterone production and enhances StAR expression, even in the absence of hCG stimulation. To our knowledge, this is the first report of a direct effect of D-Asp on mammalian gene expression.

In Leydig cells, hCG increases testosterone production via activation of adenylate cyclase [12]. The increased levels of cAMP up-regulate several steroidogenic genes, including StAR, and the induced StAR protein is assumed to facilitate by as yet undefined mechanism the translocation of cholesterol to the inner mitochondrial membrane, which is a rate-limiting process in steroid synthesis [13]. We have previously demonstrated that D-Asp increases hCG-induced testosterone production without affecting cAMP levels [10]. Because D-Asp increases dibutyryl cAMP-induced testosterone synthesis, but does not affect steroid synthesis after 22(*R*)-hydroxycholesterol supply, it appears that D-Asp acts primarily on the process of cholesterol translocation to the inner mitochondrial membrane [10]. The present results are consistent with these earlier observations.

The molecular mechanism of D-Asp stimulation of StAR gene expression is still unclear at present. Whether the increase in StAR mRNA levels is due to enhanced transcription, increased message stability or both, remains to be elucidated. In the previous report [10] it was shown that D-Asp should be taken up into Leydig cells for the stimulation of steroidogenesis. Uptake of D-Asp through the L-glutamate transporter is known to induce several changes in cells, including intracellular acidification [22] and activation of Cl^- conductance [23], which may play a role in the increased StAR expression. Alternatively, a specific factor(s) may interact with D-Asp and cause the observed stimulation of StAR gene expression. Moreover, phosphorylation can enhance StAR activity [24] and D-Asp may have an effect on StAR phosphorylation in addition to its effect on StAR gene expression.

There is some discrepancy between the magnitude of the D-Asp effects on StAR mRNA (Fig. 2) and StAR protein (Fig. 3). StAR protein is synthesized as a large precursor including an N-terminal mitochondrial targeting sequence, and is sub-

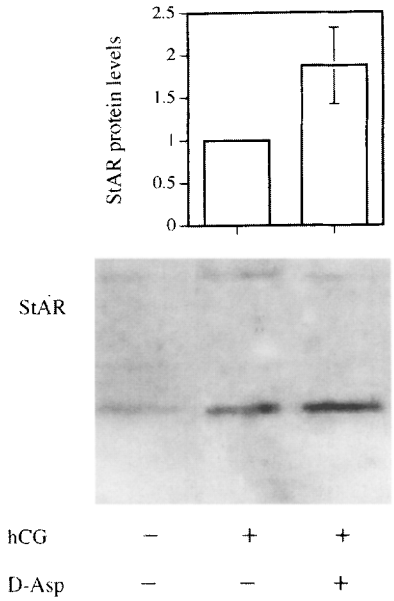

Fig. 3. Western blot analysis. Total cell homogenate (5 μg protein) was prepared from rat Leydig cells precultured in the presence or absence of 200 μM D-Asp for 16 h before being incubated with 5 mIU/ml hCG or without hCG for 2 h. Proteins were separated on 12.5% SDS/polyacrylamide gels and transferred to nitrocellulose membranes. Blots were probed with polyclonal antibody against StAR and bound antibody was detected by ECL. Lower panel: StAR protein with a molecular mass of approximately 30 kDa was detected [26]. Upper panel: The intensities of the bands were estimated and compared. The data are shown as mean ± S.D. ($n = 3$).

sequently imported by the mitochondria with concomitant cleavage of the N-terminal peptide resulting in appearance of the mature form [13]. However, mitochondrial importation is not required for StAR activity, since N-terminal deletion mutants of StAR have activities equivalent to that of wild-type StAR [17]. It is believed that the StAR precursor, not the mature form, is the form which is active outside the mitochondria and increases the rate of cholesterol translocation, possibly acting as a cholesterol transfer protein [25]. The mature form, which we detected in this study by Western blot, is thought to have no action on cholesterol transfer. StAR precursor protein was not detected in the present study, probably because it is much more labile than the mature form [17]. The apparent discrepancy between the increases in mRNA and protein levels of the mature form may be due to the labile nature of the precursor form. Similar discrepancies have been reported elsewhere [26,27].

Approximately 30% of total Asp is in the D-form in the rat testis [3,9]. D-Asp is mainly localized in the cytoplasm of elongate spermatids, the most mature of the germ cells, and is assumed to regulate steroidogenesis as a paracrine factor in the testis [3]. D-Asp is also present in other steroidogenic tissues such as the ovary, adrenal cortex and brain [1,28], where the levels of D-Asp are developmentally and spatially regulated [1,3,6]. Our results indicate that D-Asp may regulate steroid production through modulation of StAR gene expression in these steroidogenic tissues. Furthermore, D-Asp may play other, as yet undefined roles in other D-Asp-containing organs such as the pituitary, pineal gland and adrenal medulla, possibly by regulating the expression of genes other than StAR. The observations in this report should contribute to our understanding of the physiological functions of D-Asp in mammals.

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