RRPC89101346 (5 .P)

行政院國家科學委員會補助專題研究計畫成果報告

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※ 關於大白鼠腦下腺細胞中D-天門冬胺酸之生合成的研究 ※

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計畫類別：☑個別型計畫　□整合型計畫

計畫編號：NSC 89－2320－B－038－072－

執行期間： 89 年 09 月 01 日 至 90 年 07 月 31 日

計畫主持人：李仁愛

共同主持人：

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執行單位：台北醫學大學藥學系

中 華 民 國 90 年 09 月 01 日

行政院國家科學委員會專題研究計畫成果報告

關於大白鼠腦下腺細胞中 D-天門冬胺酸之生合成的研究

Biosynthesis of D-aspartate in rat pituitary cells

計畫編號：NSC 89-2320-B-038-072

執行期限：89 年 09 月 01 日至 90 年 7 月 30 日

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一、中文摘要

由我們先前的研究得知 D-天門冬胺酸在大白鼠腦下腺中分佈於催乳激素分泌細胞中[1)]，為探討腦下腺是否自行合成 D-天門冬胺酸以及 D-天門冬胺酸對催乳激素的分泌是否造成影響，本研究使用能分泌催乳激素的 GH_3 細胞株，以高效能液相層析儀及免疫細胞化學染色法來分析，結果觀察到於細胞培養過程中 D-天門冬胺酸的含量之增加及 D-天門冬胺酸在此細胞中的分佈情形；另外也發現隨著 D-天門冬胺酸添加量的增加，促甲狀腺激素分泌激素所促進之催乳激素分泌量也隨之增加。由上可知 D-天門冬胺酸在腦下腺中被生合成且可能在腦下腺中擔任訊息傳遞者的角色。

關鍵詞：D-天門冬胺酸、腦下腺、催乳激素、促甲狀腺激素分泌激素、GH_3 細胞株

Abstract

D-Aspartate (D-Asp) is localized in prolactin (PRL)-containing cells of the rat anterior pituitary gland [1)]. In order to determine whether D-Asp is actually produced by the anterior pituitary gland and whether it plays a physiological role in PRL function, a PRL-secreting clonal strain of rat pituitary tumor cells (GH_3) was employed in this study. HPLC analysis and immunocytochemical staining detected the presence and synthesis of D-Asp in the cytoplasm of these cells. In addition, thyrotropin-releasing hormone-stimulated PRL secretion was increased in a dose-dependant fashion by D-Asp from these cells. These results suggest that the anterior pituitary gland synthesizes D-Asp and that D-Asp acts as a messenger in this gland.

Keywords: D-aspartate; pituitary gland; prolactin; thyrotropin-releasing hormone; GH_3 cells

二、緣由與目的

D-Aspartate (D-Asp) is now known to be involved in several biological activities in the mammalian body: D-Asp suppresses melatonin synthesis in cultured rat pinealocytes [2)] and the isolated rat pineal gland [3)], and stimulates testosterone synthesis in isolated rat Leydig cells [4)] by increasing the expression of Steroidogenic Acute Regulatory protein (StAR) [5)]. D-Asp is found within specific cell types during development, and displays alternative intracellular and tissue locations during development [1,6-10)]. These results suggest that D-Asp functions as a novel type of messenger for cell regulation in mammals. Recently, a serine (Ser)-specific racemase was cloned from rat brain, suggesting that it might be responsible for the synthesis of D-Ser in the mammalian body [11)]. We reported that D-Asp was synthesized in mammalian cells (pheochromocytoma 12 cells), but the precise synthetic pathway was not elucidated [12)]. Moreover, our results supported the notion that D-Asp is synthesized by the mammalian body, but failed to identify the primary tissue(s) responsible for D-Asp synthesis. In our recent report, we suggested that the rat anterior lobe was the site of D-Asp synthesis and that D-Asp acted as a messenger in a paracrine or autocrine fashion in this tissue [13)]. An immunohistochemical study with anti-D-Asp antiserum demonstrated that D-Asp was localized to prolactin (PRL)-containing cells or some other very closely related type of cells in the anterior lobe of the

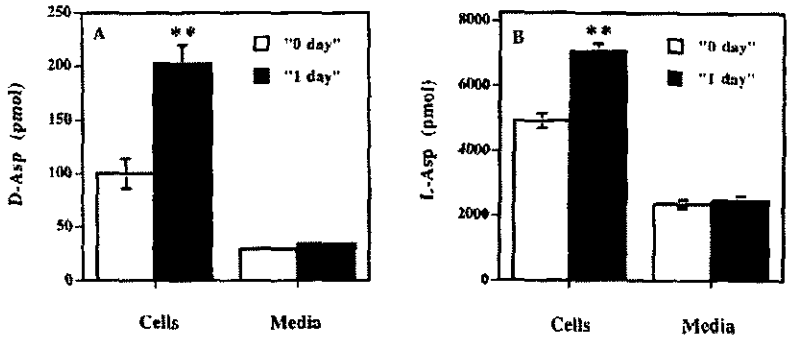

FIG. 1. Increase in cellular D-Asp content during the culture of GH_3 cells. GH_3 cells (8×10^5 cells) were inoculated into 6-well plates and cultured in 2 ml of DMEM. After preculture for 1 day to allow the cells to attach to poly-L-lysine-coated plastic plate, the medium was replaced with fresh medium and the culture was restarted. Then aliquots of medium were removed *immediately after medium replacement* ("0 day" culture) and after one day of culture ("1 day" culture) to measure D-Asp contents (see Materials and Methods for details). The results (mean ± SD, $n = 6$ independent cultures) were shown in the figures as well. **Significantly *different* compared to "0 day," $P < 0.01$, $n = 6$.

gland [1]. Therefore, D-Asp appears to be involved in PRL function in the rat pituitary gland.

In this report, we demonstrate that D-Asp is actually produced in a PRL-synthesizing clonal strain of a rat pituitary tumor cell, GH_3. Moreover D-Asp was found to increase the thyrotropin-releasing hormone (TRH)-stimulated secretion of PRL in these cells. These results strongly suggest that D-Asp is an important messenger in mammals, whose production in the anterior lobe of the rat pituitary gland results in increased secretion of PRL.

三、結果與討論

In the anterior lobe of the rat pituitary gland, at least six different types of cells are represented, five of which produce and secrete different types of hormones such as growth hormone, PRL, ACTH, thyroid stimulating hormone and gonadotropic hormone [1,14]. D-Asp is specifically present in the cytoplasm of PRL-containing cells or some other very closely related type of cells[1]. Recently we proposed that D-Asp is produced in the rat pituitary gland and acts as an messenger in a paracrine or autocrine fashion in the tissue [13]. In order to address the question whether D-Asp is produced in the pituitary gland, we investigated D-Asp synthesis in GH_3 cells, a clonal strain of rat pituitary tumor cells which produces and secretes PRL and growth hormone [15,16]. In Fig. 1, the D,L-Asp levels were determined in GH_3 cells and the media during culture. D- and L-Asp contents were significantly increased in the cells, while the contents in the media were unchanged during 1 day culture. This result indicated that D-Asp is actually synthesized in the culture of GH_3 cells, since this amino acid is not supplemented during the culture. Cellular D-Asp contents at "0 day" and "1 day" were approximately 2.0% and 2.9% of total Asp (D+L), respectively.

The Asp fraction obtained from GH_3 cells was separated on another chiral column of an opposite configuration and the peak in the chromatogram was confirmed to be that of D-Asp, and not that of other possible contaminants (data not shown). By this type of column, the elution order of D,L-Asp was reversed and therefore the peak can be identified as D-Asp, as described in our previous report [12].

The localization of D-Asp in GH_3 cells was examined with anti-D-Asp antiserum which was prepared and characterized previously in our laboratory [5,8]. As shown in Fig. 2, D-Asp immunoreactivity (IR) is apparent over the entire cytoplasm of GH_3 cells. However, its intensity between the cells was somewhat different as previously observed in PC_{12} cells [12], while details on the regulation of the cellular D-Asp content still remain to be known. Preabsorption of the antiserum with D-Asp hapten abolished IR and preimmune serum revealed no IR (data not shown).

GH_3 cells secrete increased amounts of PRL in responce to a variety of physiological secretagogues such as epidermal growth factor [17], insulin [18], estradiol [19], vasoactive intestinal peptide [20] and thyrotropin-releasing hormone (TRH) [21-23]. In Fig. 3, the effect of D-Asp on TRH-stimulated release of PRL from GH_3 cells were investigated. GH3

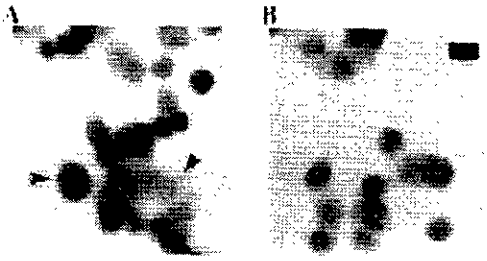

FIG. 2. Immunocytochemical staining of GH_3 cells with anti-D-Asp antibody. GH_3 cells cultured in DMEM for 4 days were probed with anti D-Asp antiserum (a) and preabsorbed antiserum (b) by the method described under Materials and Methods. The arrowheads indicate two cells with different intensities. Bar, 10 μm.

cells were incubated for 30 min with 0, 10 and 50μM of D-Asp in the presence or absence of 1μM TRH and subsequently PRL level in the medium were quantitated by enzyme immunoassay. As shown in Fig. 3, PRL secretion was increased in the cells which were incubated with D-Asp and the effect was dose-dependent on D-Asp treatment (the result with 50μM D-Asp + 1μM TRH was statistically significant from that with 1μM TRH).

In this study, significant quantity of D-Asp is present in various mammalian tissues [24,25]. In the rat pineal gland and the rat testis, approximately 30-40% of total Asp is in the D-form [7,8,26,27]. Recent investigations revealed that this unique amino acid is involved in biological activities in these tissues. The pineal gland produces and secretes melatonin, an important pineal hormone. We have demonstrated that melatonin secretion from primary culture of the rat pinealocytes, parenchymal cells of the pineal gland was inhibited by D-Asp treatment [2]. In addition, our previous reports indicated that testosterone production by Leydig cells in the rat testis was enhanced by D-Asp treatment [4] by stimulating the gene expression of Steroidogenic Acute Regulatory protein (StAR), which is an essential factor for the production of the steroid hormone [5]. These activities were not observed with other D-amino acids [2] or stereospecific to D-isomer of Asp and not observed with the corresponding stereoisomer (L-Asp) [4].

In the rat pituitary gland, D-Asp concentration is approximately 2-4% of total Asp [26-28]. Immunohistochemical staining demonstrated that D-Asp is specifically localized in the cytoplasm of PRL-containing cells in the anterior lobe of the gland [1]. Furthermore, our recent study suggested that D-Asp is synthesized in the rat pituitary gland and acts as a messenger in a autocrine or paracrine fashion [3]. In this investigation, we used a PRL-secreting clonal strain of rat pituitary tumor cells, GH_3, and addressed the question whether D-Asp is synthesized in the cells and whether D-Asp has any biological role in PRL function. The results described here indicate that D-Asp is actually synthesized in GH_3 cells and increases TRH-stimulated PRL secretion from the cells. These lines of evidence support the proposal that D-Asp is produced and acts as an autacoid in the rat pituitary gland.

D-Asp is known to be an agonist for Glu receptors, similarly to L-Glu and L-Asp [3,24]. Several pituitary hormones are well documented to be secreted from the pituitary gland through the activation of Glu receptor(s) in the hypothalamus [28]. However, the presence of Glu receptor(s) are demonstrated in the anterior lobe of the rat pituitary gland [29-31]. These Glu receptors are functionally active [29] and present in various types of the anterior pituitary cells including PRL-containing cells [29,30]. In fact, hormone secretion is induced by direct action of Glu on the isolated anterior pituitary cells [32-36]. Glu activation of the receptors presumably elicits the increase of intracellular Ca^{2+} concentration [29], which results in the increased secretion of the anterior pituitary hormones including PRL [32-36]. D-Asp therefore possibly acts as agonist for the Glu receptor in the anterior lobe of the pituitary gland and our preliminary results indicated that L-Glu and L-Asp also enhanced TRH-stimulated PRL secretion from GH_3 cells [13]. Alternatively, it has been suggested that D-Asp in addition to L-Glu and L-Asp activates the pituitary cells through the mechanism involving a high affinity Glu transporter rather than Glu receptor [37,38]. The stimulatory mechanism of D-Asp on the PRL secretion remains to be elucidated.

Recent reports demonstrated that Glu receptor(s) is involved in the physiological reactions in the peripheral endocrine tissues such as the pineal gland [39] and the pancreas [40-41]. The secretion of melatonin [2-39], glucagon [42] and insulin [40,43] are modulated

through the activation of Glu receptor(s) and significant amounts of D-Asp are also found in these tissues [8,26,44]. In the anterior pituitary gland, D-Asp may be an endogenous and stimulatory transmitter via Glu receptor (s) for the secretion of PRL and possibly other hormones.

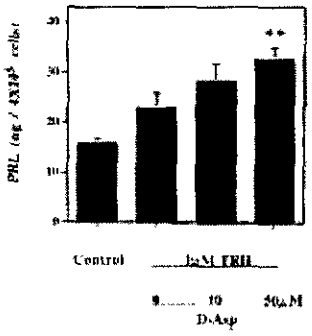

FIG. 3. Stimulation of TRH-stimulated prolactin secretion from GH_3 cells by D-Asp. GH_3 cells (4×10^5 cells per well) were cultured for 2 days in 1 ml of Ham's F-10 medium. The cells were then washed with serum-free DMEM and incubated at 37°C for 30 min in the presence of 1 μM TRH and various doses of D-Asp (0–50 μM). After incubation, PRL levels in the media were determined by enzyme immunoassay (see *Materials and Methods for details*). *Data are* shown as means ± SEM (n = 4 independent cultures). **Significantly different compared to the control, $P < 0.05$.

四、計畫成果自評

本研究按照預期計畫，成功地觀察到 D-天門冬胺酸在腦下腺 GH_3 細胞中的生合成，同時也觀察到其於 GH_3 細胞中的分佈情形，另外也發現隨著 D-天門冬胺酸之量的增加，TRH 促進 GH_3 細胞分泌催乳激素的作用也隨之增加，這些結果可推知 D-天門冬胺酸可能在腦下腺中擔任訊息傳遞者的角色。對於以後關於 D-天門冬胺酸的生理機能之探討很有助益。

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