行政院國家科學委員會專題研究計畫成果報告

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※ 過量表現第四型多巴胺受器之第三胞內環於 NCI-259 細胞株 ※

※ 引起皮質醛酮分泌增加的機制探討 ※

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計畫編號：NSC 90－2314－B－002－221－

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計畫主持人：　吳寬墩　教授

計畫參與人員：研究助理 林欣怡

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- □出席國際學術會議心得報告及發表之論文各一份
- □國際合作研究計畫國外研究報告書一份

執行單位：國立臺灣大學醫學院內科

中　華　民　國　92　年　04　月　09　日

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中文摘要

許多臨床及實驗室的證據顯示，皮質醛酮的分泌受到多巴胺的抑制。而這種調節可能是經由類似第二型的多巴胺受器（dopamine receptor, DR）的作用。過去的研究證明，第二型的多巴胺可以抑制血管張力素 II（angiotensin II, AII）或鉀離子刺激皮質醛酮分泌。但是類似第二型的多巴胺受器已知有三種: D2, D3, D4，目前仍不清楚這種抑制作用是經由何種亞型的 DR？最近吾人發現，D2 和 D4 都表現在腎上腺的髓質和皮質，但 D3 則無。進一步以多巴胺受器的拮抗劑發現，D2 和 D4 對於皮質醛酮的分泌皆有影響，但作用似乎相反；但目前仍無一種藥物是對某種亞型的 DR 有很好的特異性。

由於這些 DR 的作用是經由其胞內第三環（third intracytoplasmic loop, I3L）和 G 蛋白結合，而發生訊息傳遞，且不同亞型的 DR 受器與不同的 G 蛋白結合，因此吾人將 D2 和 D4 的 I3L 轉染於可分泌皮質醛酮的腎上腺癌細胞株（NCI-H295R），並挑選過量表現 D2I3L 或 D4I3L 的單株細胞培養之。結果發現，過量表現 D4I3L 的細胞其基礎的皮質醛酮濃度明顯高於 H295R 的分泌（90.5±7.8 *vs.* 1.2±0.2 ng/dl/mg.protein, $p<0.0001$），反之，過量表現 D2I3L 的細胞則無(1.0±0.2 ng/dl/mg.protein)。 以 1 M 的 A-II 刺激時，後者分泌皮質醛酮能力與 H295R 細胞明顯降低(3.0±0.8 *vs.* 18.2±1.5 ng/dl/mg.protein)，而 D4I3L 細胞對 A-II 有明顯的反應(548.0±23.4 ng/dl/mg.protein)。吾人進一步以測定胞內鈣離子濃度，並無發現這些轉染 I3L 的細胞鈣離子濃度有所差異。以碎片箝制法（patch clamp）在 H295R 細胞發現一個很小的向內電流，並輕微受 A-II 的影響，雖然 D4I3L 細胞也有這個現象，但和 H295R 沒有顯著的不同。不過由於轉染 I3L 細胞不太穩定，因此無法更進一步實驗。吾人同時發現這些轉染的細胞經過數代(約 5-7 次)後，I3L 就不再表現。

綜合以上的結果，吾人認為 D2 和 D4 對皮質醛酮的分泌皆具調節功能，這種調節機轉可能是經由胞內第三環與 G 蛋白結合而發生的；D2 可能具有抑制，而 D4 有刺激皮質醛酮分泌的作用。

關鍵詞：多巴胺受器，皮質醛酮，轉染，G 蛋白

ABSTRACT

There are evidences that aldosterone secretion is subjected to a dopaminergic inhibitory mechanism. Dopamine can decrease aldosterone secretion stimulated by angiotensin II or high K level. This modulation is thought to be mediated via a dopamine2-like receptor. However, the subtype of DR is not completely elucidated. We recently demonstrated that both D2 and D4, but not D3, expressed in the adrenal gland, both the cortex and medullae.

Due to the lack of specific dopamine antagonists to the subtype of DR, the importance of which subtype of DR responsible for aldosterone secretion may only be resolved by molecular biology.

D2-like receptor is coupled with a G protein via the third intracytoplasmic loop (I3L). Inhibition of adenylyl cyclase via D2 and D4 has been well demonstrated. It has been shown that different isoforms of DR preferentially bind their specific G proteins. In this study, we transfected the I3L of D2 and D4 into human adrenocortical carcinoma cells (NCI-H295R) to overexpress either D2I3L or D4I3L. A single clone of the D2I3L or D4I3L cells were cultured for experiments. The basal secretion of aldosterone from the D4I3L cells was much higher than that from the native H295R cells (90.5±7.8 *vs.* 1.2±0.2 ng/dl/mg.protein, p<0.0001), whose aldosterone secretion was not different from those of D2I3L cells (1.0±0.2 ng/dl/mg.protein). The latter also had a blunt response to 1 μM A-II (3.0±0.8 ng/dl/ mg.protein) as compared with the native H295R and D4I3L cells (18.2±1.5 and 548.0±23.4 ng/dl/ mg.protein, respectively). The intracellular calcium levels among these three cell lines were not different. Patch-clamp study revealed a small inward current, which could be slightly amplified by adding 1μM A-II. No significant difference was observed when the D4I3L cells were studied. However, further studies could not be done due to the poor vitality of the cells. We also observed that the expression of I3L was lost after 4-5 passages of the transfected cells.

In summary, by transfection of the third intracytoplasmic loop of D2 and D4 receptors, we demonstrated that D2 and D4 could modulate the aldosterone levels, probably via the G protein-binding domain. Overexpression of D2I3L inhibits, whereas D4I3L increases, aldosterone secretion.

Keywords: dopamine receptor, aldosterone, transfection, G protein

INTRODUCTION

There are evidences that aldosterone secretion is subjected to a dopaminergic inhibitory mechanism [McKenna et al., 1979; Carey et al., 1980; Ganguly, 1984; Porter, et al., 1992]. Administration of dopaminergic antagonists, such as metoclopramide, causes a rise in plasma aldosterone level in several animal species as well as human [Fraser et al., 1989]. Although there is argumentation of the mechanism of metoclopramide-induced aldosterone secretion [Rizzi, et al., 1997], dopamine binding sites in the adrenal glomerulosa has been well recognized [Stern, et al., 1986; Missale, et al., 1986].

In pharmacological and autoradiographic studies, D2-like receptor in the adrenal cortex has been well demonstrated (Missale, 1986; 1988; Stern 1986; Amenta, 1994). However, the subtype of DR has not been determined at molecular level. In autoradiographic study, DR has been shown localized mainly in the zona glomerulosa (ZG). The DR was shown to have the highest affinity to clozapine (Amenta et al, 1994; Amenta & Ricci, 1995), which binds to the D4 receptor with an affinity 10 times higher than to D2 and D3 receptors (Van Tol, et al., 1991). We have recently observed that both human D2 and D4 receptors expressed in the adrenal medullae as well as adrenal cortex. The DR was mainly localized in the ZG. In addition, D2 and D4 were also expressed in aldosterone-producing adenoma (Wu et al., 2001). The roles of D2 and D4 in the regulation of aldosterone secretion were further suspected by using dopaminergic antagonists (Wu et al., 2001). However, the lack of specificities of dopaminergic antagonists for D2 and D4 makes the differentiation difficult.

It has been recognized that DR can influence the activity of adenylyl cyclase

(AC). D2-like receptors can inhibit AC whereas D1-like receptors are associated with stimulation of AC. Whether D3 and D4 receptors have their preferred G-proteins remains clarified. The G-protein binding dormant was thought localized in the third intracytoplasmic loop (I3L). Due to the different amino-acid sequences of the I3L of these DR, it is highly suggested that they may couple different G-proteins. In this study, we used a molecular approach to investigate whether both D2 and D4 modulate aldosterone secretion. We transfected the I3L of human D2 and D4 into human adrenocotical carcinoma cells (NCI-H295R) to overexpress the I3L of either D2 or D4 cells.

RESULTS

The sequences of D2I3L and D4I3L after cloning to were confirmed as well as the opening frames. The transfection efficiency was approximately 20%. We had observed that the expression of the transfected I3L was not existent after cell passages for more than 5 times.

The basal ALD level of D4I3L cells was higher than that from native H295R cells (90.5±7.8 *vs.* 1.2±0.2 ng/dl/mg.protein, $p<0.0001$, Fig 1). However, D2I3L cells secreted a similar level of ALD (1.0±0.2 ng/dl/mg protein) to that of H295 cells. After adding 1 μM A-II, the ALD secretion from the native H295R and D4I3L cells was significantly increased (18.2±1.5 and 548.0±23.4 ng/dl/mg protein, respectively). In contrast, the response to A-II was blunt in D2I3L cells (3.0±0.8 ng/dl/mg protein). Dopamine, 1 μM, alone had no significant effect on ALD secretion from either D2I3L or D4I3I cells. In the presence of A-II, dopamine had a mild inhibition of aldosterone secretion from D4I3L cells ($p=0.062$).

The $[Ca2+]_i$ of the native H295 cells was 89.2±9.2 nM, which was not different from those of D4I3L cells (58.3±15.1 nM). Due to the poor vitality, no measurement of $[Ca2+]_i$ was done for D2I3L cells. A-II (1 μM) raised $[Ca2+]_i$ by 6 folds in H295R as well as D4I3L cells. Dopamine had no effect on $[Ca2+]_i$ in either H295R or D4I3L cells.

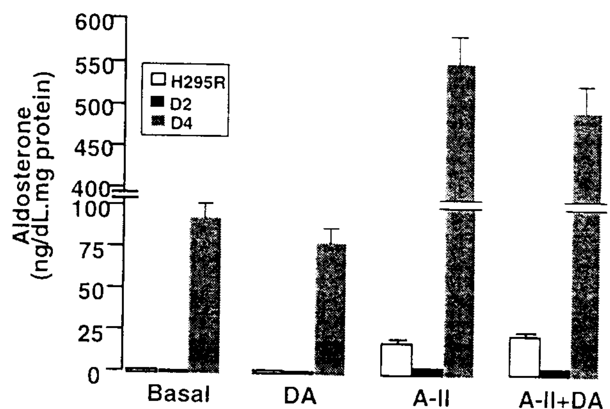

Fig. 1 The aldosterone secretion from H295R (white bar), D2I3L (black bar) and D4I3L (gray bar). Each data is the mean and SEM from 3 experiments. DA, 1 μM dopamine; A-II, 1 μM angiotensin II.

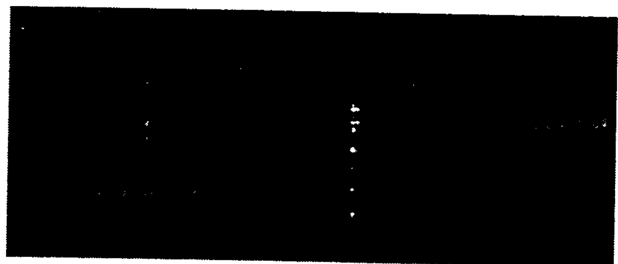

Fig 2. RT-PCR of Gi1a (lanes 1-4), Gi2a (lanes 5-8) and Gi3a (lanes 9-12) proteins in NCI-H295R cells (lanes 2, 6, and 10), D2-I3L transfected cells (lanes 3, 7, and 11) and D4-I3L transfected cells (lanes 4, 8 and 12). Lanes 1, 5 and 9, no template.

Whole cell patch clamp was done only for D4 and H295R cells. In H295R cell, an inward current was detected and was increased mildly after 10 μM A-II. In D4I3L cells, although a similar inward current was measured, no significant current change was found(<10pA) after adding A-II.Further analysis was difficult because of (1) small current, (2) variable cell condition, especially in D4I3L cells (variable in size and morphology), and (3) small available sample number.

Because Gi proteins are speculated to couple with D2-like receptors, we did RT-PCR to study whether the expression of alpha subunits of Gi proteins was altered by tranasfection of D2I3L and D4I3L. As shown in Fig. 2, the expressions of Gi1a, Gi2a and Gi3a were not significantly different between the native H295R and I3L transfected cells. However, more precise measurements of the messages of these proteins are necessary to elucidate the speculation.

DISCUSSION

In the present study, we demonstrated that overexpression of the I3L of human D2 and D4 receptors can modulate the aldosterone secretion. In spite of belonging to D2-like receptor, D2 and D4 have opposite effects on aldosterone secretion. This observation was compatible with our previous pharmacological experiments on H295R cells (Wu et al., 2001). These data show that dopamine can increase aldosterone secretion via D4 receptor, whereas decreasing aldosterone secretion via D2 receptor.

In pharmacological and autoradiographic studies, D2-like receptor in the adrenal cortex has been well demonstrated (Missale, 1986; 1988; Stern 1986; Amenta, 1994). However, the subtype of DR has not been determined at molecular level. In autoradiographic study, DR has been shown localized mainly in the zona glomerulosa (ZG) and was shown to have the highest affinity to clozapine (Amenta et al. 1994; Amenta & Ricci, 1995) which binds to the D4 receptor with an affinity 10 times higher than to D2 and D3 receptors (Van Tol, et al., 1991).

Some in vitro studies failed to demonstrate the inhibitory effect of DA on aldosterone secretion. There are several explanations for these negative results. First, there are evidences that both D1 and D2 (or D2-like) receptors are expressed in the adrenal cortex (Missale et al. 1986; Gallo-Payet, et al. 1991). DA increases intracellular cAMP level via D1 receptor, but decreases it through D2 receptor (Missale et al., 1988; Gallo-payet, 1991). The inhibitory effect of DA on aldosterone secretion is significant only when the D1 receptor is blocked. Therefore, DA alone may have no effect on aldosterone secretion. Secondly, the expression of DR subtypes may change with different culture conditions (Gallo-Payet, 1990; 1991). The freshly isolated glomerulosa cells possess both D1 and D2 receptors, whereas in cultured conditions only D1 receptors exist. Thus, the dopaminergic effect on aldosterone may be opposite just because the cells are differently prepared. Thirdly, agonists or antagonists used in several experiments are usually nonselective to the subtypes of DA receptors. In those studies which failed to demonstrate the antidopaminergic action on aldosterone secretion may be due to the nonselective property of the antagonists (Warner et al., 1992), or low affinity of the anatgonists to a specific DA receptor, eg. D4 or D5 (MacDonald, 1991; Warner et al., 1992).

Therefore, as shown in the present study, a molecular approach may be the only way to resolve the problems in elucidating the

effects of DR on aldosterone secretion. The rationale to transfect the I3L, instead of the whole receptor molecule, is that this loop binds G proteins, which act as a signal transduction for DRs. Due to the different amino-acid sequences of the I3L of these DRs, it is highly suggested that they may couple different G-proteins. Why expression of I3L could modulate aldosterone secretion? We speculate that the overexpressed I3L of D2 or D4 receptors can bind their respective G-proteins and induce the signal transduction to exert their actions, mimicking the actions of whole dopamine receptors. However, we did not explore this issue in the present study due to the low yields of protein preparation.

Secretion of aldosterone is accompanied by an increase in [Ca2+]i. As shown in the present study, A-II increased intracellular calcium levels significantly. Although the basal aldosterone secretion was different between the native H295R and D4I3L cells, their basal [Ca2+]i were similar. In addition, the [Ca2+]i increased by A-II was not different. Therefore, the significant increase in aldosterone levels, either the basal or stimulated by A-II, from D4I3l cells may result from an increase in aldosterone synthesis rather than aldosterone secretion per se. Further study in aldosterone synthesis is needed to clarify this speculation.

It has been recognized that DR can influence the activity of AC. D2-like receptors can inhibit AC whereas D1-like receptors are associated with stimulation of AC. The D2 receptor exists as two alternatively spliced isoforms differing in the insertion of a stretch of 29 amino acids in the third intracytoplasmic loop (D2S and D2L). Although both isoforms inhibit AC, the D2S receptor isoform diplayed higher affinity to inhibit AC than D2L (Dal Toso et al., 1989; Montmayeur & Borrelli, 1991). Attempts to identify the preferred G protien a-subunit for D2S and D2L have led to conflicting results. One group suggested that the 29-amino acid insertion in the D2L receptor directs its interaction with Gi-2 (Guiramand et al., 1995, whereas another report showed that in transfected cell lines the D2S receptor signaled preferentially through Gi-2 and the D2L through Gi-3 (Senogles 1994). Whether D3 and D4 receptors have their preferred G-proteins remains clarified. In the present study, we examined the expression of the alpha subunits of Gi proteins. With RT-PCR, we did not find different expression of Gi1, Gi2 and Gi3 proteins between the native H295R and the I3L-transfected cells. The regulatory mechanism of Gi proteins in H295R cells is not understood. Whether the expression of the alpha subunit of G proteins can be regulated by binding their corresponding receptors remains exploration.

In summary, by transfection of the third intracytoplasmic loop of D2 and D4 receptors, we demonstrated that D2 and D4 could modulate the aldosterone levels, probably via the G protein-binding domain. Overexpression of D2I3L inhibits, whereas D4I3L increases, aldosterone secretion.

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