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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利用過量表現多巴胺受器之第三胞內環於NCI-H259細胞株來確認何

者多巴胺受器為調節皮質醛酮的分泌

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中文摘要

關鍵詞: 多巴胺 皮質醛酮 轉殖

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，第二型的多巴胺受器拮抗劑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。最近吾人以逆轉化-多聚酶鏈鎖反應法，和原位雜交的研究方法（NSC-88-2341-B002-234）發現，DR2 和 DR4 都表現在腎上腺的髓質和皮質，但 DR3 則無。同時在皮質醛酮瘤也有這兩種 DR 的表現。因此，推測這兩種 DR 對皮質醛酮的調節有重要的角色。

由於多巴胺受器的作用是經由其胞內第三環(third intracytoplasmic loop, I3L）和 G 蛋白結合，增加或抑制 adenylyl cyclase 進而影響 cAMP 的產生。而不同亞型的 DR 受器與不同的 G 蛋白結合。一些證據顯示 DR2 類的受器，是經由與 Gi 蛋白質結合，而產生抑制 adenylyl cyclase；或者經由改變細胞內鈣離子濃度，而達到生理作用。此研究中，吾人以 DR2 和 DR4 其胞內第三環的一段基因（各有），選殖在 pCEP4 的質體，轉移至人類腎上腺癌細胞株（adrenocortical carcinoma cell line, NCI-H295R），造成此段基因的過量表現。結果發現過量表現 DR4 之 I3L 的細胞，其基礎的皮質醛酮分泌比原始細胞者高出 5-10 倍，且對血管張力素的刺激也有過度的增加。反之，過量表現 DR2 之 I3L 的細胞，基礎的皮質醛酮分泌降低，且對血管張力素反應較不明顯。此結果顯示，DR4 對皮質醛酮的分泌有刺激作用，而 DR2 則有抑制作用。這個結果和吾人最近使用較具特異性的多巴胺受器拮抗劑的研究結果吻合。

英文摘要

Key words: *dopamine receptor, aldosterone, transfection,*

There are evidences that aldosterone secretion is subjected to a dopaminergic inhibitory mechanism. Administration of dopaminergic antagonists, such as metoclopramide, causes a rise in plasma aldosterone level in several animal species as well as human. In addition, dopamine can decrease aldosterone secretion stimulated by angiotensin II or high K level. We recently demonstrated that both D2 and D4 expressed in the adrenal gland, both the cortex and medullae, although D4 seemed to express more abundantly and constantly. The messages in the cortex was mainly localized in the ZG. Furthermore, aldosterone-producing adenoma also possessed both DR. Therefore, these DR may play an important role in aldosterone secretion.

D2-like receptor is coupled with a G protein via the third intracytoplasmic loop (I3L). Inhibition of adenylyl cyclase (AC) 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 cloned the I3L of D2 and D4 into pCEP4 vector and transfected the clones into adrenocortical carcinoma cell line, NCI-H295R. Over-expression of I3L of D4 in these cells resulted in a significant elevation of the basal aldosterone level as compared with the native cells. In contrast, overexpression of I3L of D2 led to a lower level of the basal aldosterone level. Exaggerated response of aldosterone secretion to angiotension II (1μM) was also observed in cells transfected with D4 I3L, but blunt response in cells transfected with D2 I3L. These results are compatible with our recent pharmacological data and indicate that D4 can stimulate, while D2 inhibits, aldosterone secretion from NCI-H295R cells.

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 (MCP), 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 MCP-induced aldosterone secretion [Rizzi, et al., 1997], dopamine binding sites in the adrenal glomerulosa has been well recognized [Bevilacqua, et al., 1982; 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 et al, 1986; 1988; Stern et al., 1986; Amenta, 1994; Amenta & Ricci , 1995). However, the subtype of DR has not been determined at molecular level. We have recently demonstrated that both D2 and D4 receptors express in the adrenal cortex, especially in the zona glomerulosa [Wu et al., 2001]. Furthermore, both receptors modulate aldosterone secretion, although with opposite effects.

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 (Bevilacqua, et al., 1982; Missale et al, 1986; Gallo-Payet, et al. 1991; Aherne ert al., 1997). 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 et al, 1990; 1991). The fresly 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, or low affinity of the anatgonists to a specific DA receptor, eg. D4 or D5 (MacDonald, 1991). The subtype of DR which accounts for the inhibitory effect of MCP is not elucidated. MCP is a nonselective DA antagonist with high affinity to D2 and D3 receptors. However, it also displays high

selectivity to D1, D4 and D5 receptors (Rizzi, et al., 1997).

Therefore, a molecular approach is mandatory to elucidate the dopaminergic regulation of aldosterone secretion. It has been recognized that the third intra-cytoplasmic loop (I3L) is important to exert the effects of dopamine receptors. 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 adenylyl cyclase (AC), the D2S receptor isoform displayed higher affinity to inhibit AC than D2L (Dal Toso et al., 1989; Montmayeur & Borrelli, 1991). Attempts to identify the preferred G protein □-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, 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. However, because the different amino-acid sequences of the I3L of these DR, it is highly suggested that they may couple different G-proteins.

In the present study, we cloned the I3L of human D2 and D4 receptors into an expression vector and transfected them to H259R cells. The transfected cells were thus used to examine the dopaminergic regulations of aldosterone secretion.

Materials and Methods

NCI-H259 cells culture

By RT-PCR we have found that this cell line has the expression of human D2 and D4 receptors (Wu et al., 2001).

1. NCI-H295R cells, obtained from the American Type Culture Collection (Rocville, MD) were maintained in Dulbeco's Modified Eagle's Medium/Ham's F12 (1:1) supplemented with HEPES (15mM), insulin (5ug/mL), transferrin (5ug/mL), selenium (5ng/mL), bovine serum albumin (1.25mg/mL), linoleic acid (5.35ug/mL), fetal bovine serum (2.5%), glutamine (2mM), and antibiotics (penicillin/streptomycin). Cells were grown in 75 cm2 flasks at 37C under atmosphere of 5% CO2/95% room air. The culture medium (DMEM/F12) includes fetal bovine serum (2.5%), glutamine (2mM), and antibiotics (penicillin / streptomycin). Cells used for the described experiments were routinely maintained as monolayer cultures. When studied, the cell medium was removed and replaced with serum-free medium (DMEM/F12 containing

antibiotics and 0.01% bovine serum albumin), and cells were cultured for a another 24 h before they were treated with 1□M angiotensin II. Dopamine was added to blunt the stimulating effect of AII.

2. The aldosterone concentrations of the cell medium will be measured by radioimmunoassay.

Cloning the I3L (the third intracytoplasmic loop) of DR

1. The I3L of D2 and D4 will be amplified by RT-PCR from NCI-H259 cells.

RNA extraction and reverse transcriptase polymerase chain reaction

Total RNA was isolated from NCI-H259 cells by using cesium chloride gradient method. cDNA was synthesized and amplified with the use of specific primers for D2DR :D2I3a with a BamHI cutting site: TTTGGATCCCAAGATCTACATTGTC CTCC; D2I3b with a HindIII cutting site: TTTAAGCTTTCTGAGTGGCTTTCTT CTC), D4DR: D4I3a with a BamHI cutting site: ATTGGATCCGGCCACGTTCCGC GGCCTGC; D4I3b with a HindIII cutting site: ATTAAGCTTACCTCATGGCCTT GCGCTCC.

No genomic DNA was amplified when using nontranscribed mRNA. The amplified I3L DNA will be sequenced to confirm the nucleotide sequences.

2. Construct of I3L clone

The aliquot of the NCI-H295R cDNA was amplified by PCR (Pfx, Gibco-BRL) with D2 specific primers (D2I3a and D2I3b). The sequence corresponds to the D2 third intracytoplasmic loop. The fragment is cloned into pRSET-B. Then, the D2I3 loop accompanied with a His-tag in pRSET-B fragment is amplified by PCR with the primers (SETa: ATTGCGGCCGCTATGCGGGGTTCTCATCATC, and SETb: ATTGCGGCCGCTAGTTATTGCTCAGCGGTGG), The product is then digested and cloned into pCEP4, a vector with a CMV promotor. The D4I3-pCEP4 construct was obtained by similar method.

Transfection

NCI-H295R cells were plated at 50-60% confluent on 24-well plates with DMEM/F12 + 2.5% FBS. The cells are transfected with D2I3-pCEP4 and D4I3-pCEP4 individually by Qiagen Transfection Kit. The successfully transfected cells were selected by adding ____ in the media.

Results and Discussion

Over-expression of D2I3L and D4I3L in the transfected cells

To differentiate the native D2 and D4 receptors from the transfected intra-cytoplasmic loops of D2 and D4, primers flanking the I3L were used. For D2 receptor, D2I3a and D2I3b were used to amplified the transfected I3L; for D4, D4I3a and D4I3b were the primers. To examine the expression of the native D2 and D4 receptors, one flanking primer was used: for D2, the primers were D2I3a and 5'-3'; for D4, the primers were 5'-3' and D4I3b.

As shown in Fig 1, there was no difference in the expression of the native D2 or D4 receptors between the transfected D2 and D4 cells and the untransfected H259R cells. Significant amounts of D2 and D4 I3L were found in the transfected D2 and D4 cells, respectively. However, the expression of the transfected I3L decreased significantly after more than 10 passing of the cells (data not shown). Therefore, the experiments were done in transfected cells passed less than 10.

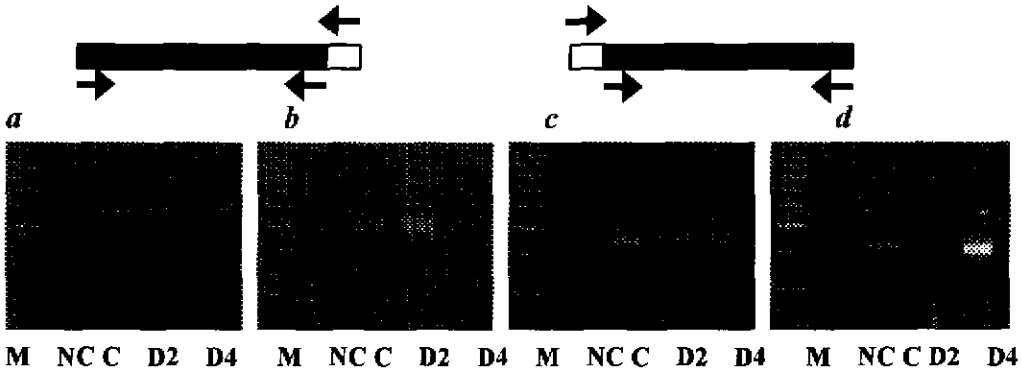

Fig 1. Expression of the third intra-cytoplasmic loop (black bars) of human D2 and D4 receptors in the H295R cells. The products of panels a and b were those amplified with D2 primers, and panels c and d with D4 primers, respectively. The products of panels a and c were those amplified with the primers (black arrows) within the I3L, and those of panels b and d amplified with one primer flanking the I3L (dashed arrows). M, 100-bp marker; NC, no template; C, H259R cells; D2, I3L of D2-transfected cells; D4, I3L of D4-transfected cells.

The basal aldosterone levels from the transfected D2 and D4 cells

Significant high levels of basal aldosterone secretion from the transfected D4 cells were noted; a 10-50 fold increase as compared with the H259R cells (90.5±7.8 *vs.* 1.2±0.2 ng/dl/mg.protein, $p<0.0001$) (Fig 2). In contrast, there was no significant change of the basal aldosterone secretion from the transfected D2 cells (1.0±0.2 ng/dl/mg.protein).

Our previous study shows that dopamine may increase or inhibit aldosterone secretion via D4 or D2 receptors, respectively (Wu et al., 2001). The present results further confirm the observations and indicate that the effect of D4 receptor is mediated through the third intra-cytoplasmic domain.

Responses to angiotensin II and dopamine

After incubation with 1 μM dopamine or 1 μM clozapine for 24 hours, there was no significant change of aldosterone secretion from either H259R cells or D2-I3L transfected cells (Fig 2). Dopamine alone had mildly decreased the aldosterone secretion from D4-I3L transfected cells, but no effect on H259R cells or D2-I3L cells.

Addition of 1μM angiotensin II (A-II) significantly increased aldosterone secretion from all the cells. However, the increment was less from the D2-I3L transfected cells. The increase of aldosterone secretions from D2-I3L and D4-I3L cells were slightly decreased by dopamine and further decrease was observed when 1 μM clozapine was added.

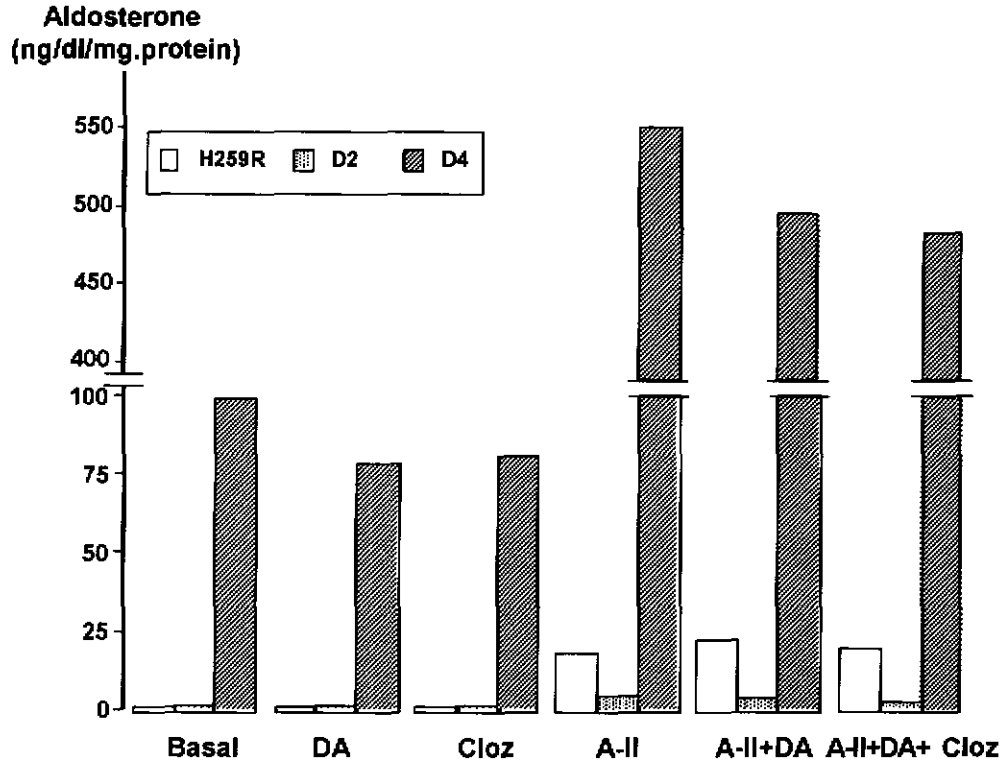

Fig 2. Basla aldosterone levels (ng/dl) from H259R cells (blank bar), D2-I3L (dotted bar) and D4-I3L (hatched bar) transfected H259R cells. Cells were also incubated in presence of 1μM dopamine (DA), 1 μM clozapine, or angiotensin II (A-II) for 24 hours.

References

Aherne AM, Vaughan CJ, Carey RM, O'Connell DP. (1997) Localization of dopamine D1A receptor protein and messenger ribonucleic acid in rat adrenal cortex. Endocrinology 138:1282-1288.

Amenta F, Chiandussi L, Mancini M, Ricci A, Schena M, Veglio F. (1994) Pharmacological characterization and autographic localization of dopamine receptors in the human adrenal cortex. Eur J Endocrinol 131: 91-96.

Amenta F, Ricci A. (1995) Autoradiographic localization of dopamine D2-like receptors in the rat adrenal gland. Clin Exp Hypert 17: 669-688.

Barili P, Zaccheo D, Amenta. (1996) Pharmacological chrarcterization and autoradiographic localization of dopamine receptors in the rat adrenal medulla. European Journal of Pharmacology 310: 129-135.

Bevilacqua M, Vago T, Sorza D, Norbiato G. (1982) Characterization of dopamine receptors by 3H-ADNT binding in calf zona glomerulosa. Biochem Biophys Res Commun 108: 1669-167, 1982.

Carey RM, Thorner MO, Ortt EM. (1980) Dopaminergic inhibition of metoclopramide-induced aldosterone secretion in man. J Clin Invest 66: 10-18.

Dal Toso R, Sommer B, Ewart M et al. (1989) The dopamine receptor: two molecular forms generated by alternative splicing. EMBO J 8: 4025-4034.

Fraser R, Connell JMC, Inglis G, Kenyon CJ, & Tree M. (1989) The role of dopamine in the control of corticosteroid secretion and metabolism. J Steroid Biochem 32: 217-222.

Gallo-Payet N, Chouinard L, Balestre MN, Guillon G. (1990) Dual effects of doapmine in rat adrenal glomerulosa cells. Biochem Biophys Res Comm 172: 1100-1108.

Gallo-Payet N, Chouinard L, Balestre M-N, Guillon G. (1991) Mechanisms involved in the interaction of dopamine with angiotensin II on aldosterone secretion in isolated and cultured rat adrenal glomerulosa cells. Mol Cell Endocrinol 81: 11-23.

Ganguly A. (1984) Dopaminergic regulation of aldosterone secretion: how credible? Clinical Science 66: 631-637.

Guiramand J, Montmayeur JP, Ceraline J et al. (1995) Alternative splicing of the dopamine D2 receptor directs specificity of coupling to protiens. J Biol Chem 270: 7354-7358.

MacDonald TM. (1991) Metoclopramide, doperidone and dopamine in man: actions and interactions. European Journal of Clinical Pharmacology 40: 225-230, 1991.

McKenna TJ, Island DP, Nicholson WE, Liddle GW. (1979) Dopamine inhibits angiotensin stimulated aldosterone biosythesis in bovine adrenal cells. J Clin Invest

64: 287-291.

Missale C, Liberini P, Memo N, Carrura MO, Spano P. (1986) Characterization of dopamine receptors associated with aldosterone secretion in rat adrenal glomerulosa. Endocrinology 119: 2227-2232, 1986.

Missale R, Memo M, Liberini P, Spano P. (1988) Dopamine selectively inhibits angiotensin II- induced aldosterone secretion by interacting with D-2 receptors J Pharmacol Exp Ther 246: 1137-1143, 1988.

Montmayeur JP and BorrellI E. (1991) Transcription mediated by a cAMP-responsive promoter element is reduced upon activation of dopamine D2 receptors. Proc Natl Acad Sci USA 88:3135-3139.

Porter ID, Whitehouse BJ, Price GM, Hinson JP & Vinson GP. (1992) Effects of dopamine, high potassium concentration and field stimulation on the secretion of aldosterone by the perfused rat adernal gland. Journal of Endocrinology 133: 275-282.

Pupilli C, Lanzillotti R, Fiorelli G, Selli C, Gomez RA, Carey RM, Serio M, & Mannelli M. (1994) Dopamine D2 receptor gene expression and binding sites in adrenal medulla and pheochromocytoma. J Clin Endocrinol Metab 79: 56-61, 1994

Rizzi CA, Mierau J, Landinsky H. (1997) Regulation of plasma aldosterone levels by metoclopramide: a reappraisal of its mechanism from dopaminergic anatgonist to serotonergic agonism. Neuropharmacology 36: 763-768, 1997.

Senogles SE. (1994) The D2 dopamine receptor isoforms signal through distinct Gi□ proteins to inhibit adenylyl cyclase: A study with site-directed mutant Gi□ proteins. J Biol Chem 269:23120-23127.

Stern N, Eggena P, Chandler W, Tuck ML. (1989) Effects of central and peripheral dopamine antagonism on aldosterone secretion: evidence for adrenal mechanism. American Journal of Physiology 257: E588-E594, 1989.

Stern N, Ozaki L, Tuck ML. (1986) Evidence for dopaminergic binding and inhibitory sites in the human adrenal cortex. Metabolism 35: 1154-1158, 1986

Wu K-D, Chen Y-M, Chu J-S, Hsieh T-S, Hsieh B-S. (1995) Zona fasciculata-like cells determine the response of plasma aldosterone to metoclopramide and aldosterone synthase mRNA level in aldosterone-producing adenoma. J Clin Endocrinol Metab 80: 783-789.

Wu KD, Chen YM, Chu TS, Chen J, Hsieh BS. (2001) Expression and localization of human dopamine D2 and D4 receptors in pheochromocytoma and aldosterone-producing adenoma. *J Clin Endocrinol Metab* 86: 4460-4467.