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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※　血管收縮素訊息路徑及神經鏈基因表現在人類正常肺細胞及肺癌細胞之研究(2/3)
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計畫主持人：林庭慧

共同主持人：

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□國際合作研究計畫國外研究報告書一份

執行單位：中山醫學大學 生命科學系

中　華　民　國　　　93 年　　　5 月　23　　日

行政院國家科學委員會專題研究計畫成果報告

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中文題目

前列腺激素 E2 在 M-1 細胞株中加強細胞素誘發之一氧化氮生成.

中文摘要

關鍵詞: 血管收縮素, 前列腺激素 E2, 環腺甘酸, 鈣離子, 一氧化氮, M-1 細胞株.

前期報告中文摘要

在去年之成果報告中，我們探討血管收縮素在正常肺細胞株 MRC-5 及小細胞肺癌 H-209 是否進行正常之訊息傳遞路徑。利用環腺甘酸分析組套，分析經過血管收縮素刺激之後，在 MRC-5 細胞中，我們無法偵測出環腺甘酸含量之上升，表示血管收縮素在 MRC-5 細胞中並未進行第二亞型受體驅動之正常訊息傳遞路徑。我們也同時研究煙草中之致癌物，benzopyrene, 對血管收縮素及相關神經鏈在此二株細胞中之基因表現。我們發現，在 MRC-5 中，全然無血管收縮素及其他神經鏈之基因表現。但在 H-209 中, 雖無 AVP 及 V1a receptor 之基因表現，但卻有 V2 receptor, CCK-B 以及 GRP 此二神經鏈之基因表現。然而，使用不同濃度之 benzopyrene,分別處理不同時間卻看不到 V2 receptor, CCK-B 以及 GRP 在基因表現上有何差異。Benzopyrene 對 V2 receptor, CCK-B 以及 GRP 在 H-209 中之基因表現可能並無直接影響。在過去一年中，雖然原來之計劃結果不如預期。 我們卻在另一腎臟集尿管細胞株(M-1 cells)中發現了有趣之實驗結果，撰寫在這份進度報告中。

本期報告中文摘要

M-1 cells 是腎臟集尿管細胞株，負責腎臟水份再吸收作用。血管收縮素及前列腺激素 E2 負責腎臟水份再吸收作用眾所周知。利用反轉錄胺聚合連鎖反應 ，雖然某些血管收縮素及前列腺激素 E2 受體可被偵測。但利用這些受體專一性高之促進劑及抗拮劑，我們發現在腎臟集尿管細胞株(M-1 cells)中，只有前列腺激素 E2 經過其第四亞型受體誘發之環腺甘酸訊息路徑是進行的。血管收縮素在 M-1 細胞中並未進行其第二亞型受體驅動之正常訊息傳遞路徑。此外，前列腺激素 E2 經過其第一亞型及第三亞型受體所激發之訊息路徑在 M-1 細胞中亦是無法被偵測。由於前列腺激素 E2 乃透過其第一亞型及第三亞型受體激發之訊息路徑來降低細胞內環腺甘酸之量進而抑制水份再吸收。而今第一亞型受體激發之促進細胞內鈣離子濃度在 M-1 細胞中無法被偵測，且第一亞型受體及第四亞型受之間並無交談(no cross talk) 。因此，何種訊息路徑在 M-1 細胞中是進行來抗衡水份再吸收是我們研究之重點。我們發現一氧化氮(nitric oxide) 會是在 M-1 細胞中, 抗衡前列腺激素 E2 誘發水份再吸收之重要因子。

英文題目

PGE2 enhances cytokines-elicited nitric oxide production in M-1 mouse CCD cells

英文摘要

Keywords : Arginine vasopressin, PGE2, cyclic AMP, calcium, nitric oxide, M-1 cells

Both arginine vasopressin (AVP) and prostaglandin E2 (PGE2) are involved in water reabsorption in renal tubular cells. In the present study, the downstream signaling of AVP and PGE2 were investigated in a M-1 cell line, a cortical collecting duct (CCD) cell line derived from a mouse harboring temperature-sensitive SV 40 large T-antigen gene. RT-PCR detected the gene expression of V2, VACM-1, EP1, EP2, EP3 and EP4 receptors in M-1 cells. Further analysis of the downstream signaling of these receptors using selective agonists and antagonists indicated the PGE2 signaling pathway, but not AVP, was detectable in M-1

cells. EP4, but not EP2, was the main receptor subtype that coupled to Gs proteins to increase the cAMP level and increase water reabsorption in M-1 cells. Regarding the inhibitory effect of PGE2 on water and salt reabsorption, traditional EP3 receptors coupling to Gi proteins and EP1 receptors signaling via increased intracellular calcium were undetectable in M-1 cells. A novel inhibitory mechanism of PGE2 on water reabsorption signaling through EP4 receptors involving nitric oxide (NO) was speculated in M-1 cell**s.** NO plays important roles in the regulation of nephron transport, renal salt and fluid reabsorption and is known to attenuate antidiuretic hormone-stimulated osmotic water permeability in CCD. PGE2 or dibutyryl cAMP (db-cAMP), a cell-permeable derivative of cAMP, caused no significant NO production when added alone in M-1 cells. On the other hand, in the presence of cytokines mixture consisting of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and interferonγ (IFN-γ), PGE2 enhanced NO production and inducible nitric oxide synthase (iNOS) expression. We suggest PGE2 exerts biphasic effects on the water reabsorption in M-1 cells, depending on the presence of cytokine mixtures. PGE2 alone, signaling through EP4, increased cAMP level and enhanced water reabsorption in M-1 cells. Under circumstance such as renal injury or inflammation, when the cytokines was produced in M-1 cells, PGE2 enhances NO production. NO then acts as a blocker to counteract the effect of PGE2 and cAMP to inhibit water reabsorption.

二、 Results (結果)

Effect of specific AVP and PGE2 agonists on cAMP accumulation in M-1 cells

To determine potential involvement of cAMP signaling pathway in AVP and PGE2, response of cAMP to specific agonists for AVP, PGE2 receptors was measured in M-1 cells. The level of cAMP of non-treated M -1 cells (i.e., control) was 180 ± 37 fmoles / well containing 2×10^{-5} cells (Fig. 1). The levels in cells treated with 10^{-6} M AVP (i.e., [Arg^8] vasopressin), or ddAVP (i.e.,[deamino-Cys, Arg^8] vasopressin) were not different from that of the non-treated control. Similarly, an antagonist of V2 receptor, i.e., [$Adamantaneacetyl^1$,o-Et-D-Try^2,Val^4,Amino $butyryl^6$, $Agr^{8,9}$]-Vasopressin (ANT) had no effect on cAMP as compared with that of the control. Thus, AVP-mediated process may not involve cAMP signaling pathway in M-1 cells. Conversely, addition of PGE2 (10^{-6} M) increased cAMP level to 1485 ± 134 fmoles / well, approximately 8-fold of the control. Butaprost, a selective EP2 agonist did not alter cAMP level (235 ± 23 fmoles / well), whereas 11-deoxy-PGE1, an agonist for both EP2 and EP4, markedly increased cAMP to 1299 ±189 fmoles / well. Sulprostone, a specific agonists for EP3, did not change cAMP level (179 ±16 fmoles / well). Apparently, EP4 was the main receptor subtype responsible for cAMP production in M-1 cells. There was a slight but significant increase in cAMP by EP1 agonist (i.e., 17-phenyl trinor PGE2) (314 ± 23 fmoles / well). The results suggest that cAMP-increasing potency was the highest with PGE2 followed in decreasing order by 11-deoxy PGE1 and 17-phenyl trinor PGE2 (Fig. 1).

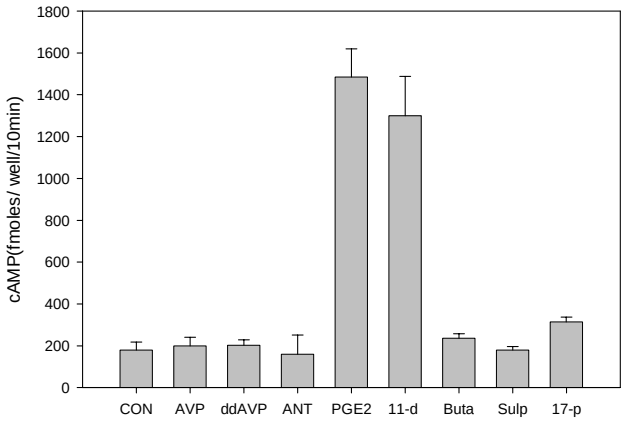


Fig. 1. Effect of 10^{-6} M AVP, ddAVP, antagonist of V2 receptor, PGE2, 11-deoxy PGE1, butaprost, sulprostone and 17-phenyl trinor PGE2 on cAMP production in M-1 cells. Prior to stimulation, cells (approximately 2×10^5 cells /well) were preincubated for 10 min at 37^0C in medium containing 0.5 mM IBMX. The individual agonists and antagonists for each receptor were added in the medium containing 0.5 mM IBMX. The cells were then incubated

at 37^0C for 10 min. The amount of cAMP extracted from each well was quantified by cAMP biotrak enzymeimmunoassay (EIA) system. Data are the mean ± S.D from one representative experiment done in triplicate; the experiments were repeated twice with similar results. CON=control; AVP= arginine vasopressin; ddAVP=[deamino-Cys, Arg^8] vasopressin, ANT=V2 antagonist; 11-d = 11-deoxy PGE1; Buta=butaprost; Sulp=sulprostone; 17-p=17-phenyl trinor PGE2.

Inhibitory effect of ionomycin and 17-phenyl trinor PGE2 on PGE2/11-deoxyPGE1-induced cAMP in M-1 cells

Ionomycin, a calcium ionophore, rapidly increased intracellular calcium in M1-cells (data not shown). Addition of either AVP or PGE2 had no effect on intracellular calcium in M-1 cells (data not shown). PGE2 and 11-deoxy PGE1 again markedly increased cAMP levels (Fig. 2a). Ionomycin decreased cAMP induced by PGE2 by 50% (3110 ±108 fmoles/ well vs. 1510 ± 69 fmoles/ well) and by 11-deoxy PGE1 by 40% (1983 ±238 fmoles/ well vs. 1174 ± 248 fmoles/ well) (Fig.2a). The results suggested that the inhibition of ionomycin on PGE2-evoked cAMP was mainly through EP4 receptor. Addition of 17-phenyl trinor PGE2 did not attenuate cAMP levels increased by PGE2 or 11-deoxy PGE1 alone in M-1 cells (Fig.2b). It should be noted that 17-phenyl trinor PGE2 did not affect intracellular calcium in M-1 cells (data not shown).

Fig.2 (a)

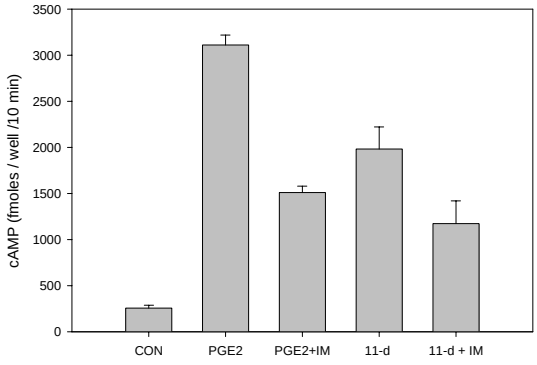


Fig.2 (b)

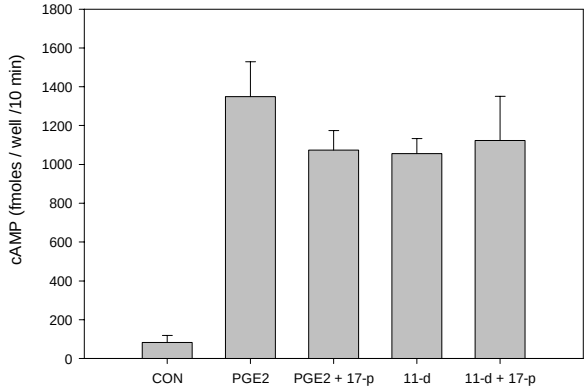


Fig.2. Effect of ionomycin and 17-phenyl trinor PGE2 on either PGE2 or 11-deoxy PGE1-induced cAMP production in M-1 cells. (a) The medium containing 0.5 mM IBMX was incubated with 400 nM ionomycin when either 10^{-6} M PGE2 or 10^{-6} M 11-deoxy PGE1 were added in M-1 cells. The PGE2-induced cAMP accumulations in M-1 cells were then measured. (b) 10^{-5} M 17-phenyl trinor PGE2, a specific agonist of EP1, was coincubated with 10^{-6} M PGE2 or 10^{-6} M 11-deoxy PGE1. No significant change of either PGE2 or 11-deoxy PGE1-induced cAMP accumulations was observed. Data are the mean ± S.D. from one representative experiment done in triplicate; the experiments were repeated twice with similar results. CON=control; 11-d = 11-deoxy PGE1; 17-p=17-phenyl trinor PGE2. IM = ionomycin.

Cytokines stimulate NO production and iNOS expression in M-1 cells

Nitric oxide production in the presence of various test compounds was first measured in M-1 cells. LPS, db-cAMP, TNF-α, IL-1β, IFN-γ alone or combination of LPS with db-cAMP or TNF-α, (IL)-1β, IFN-γ, did not alter NO production (data not shown). Effects of a mixture of TNF-α, IL-1β, and IFN-γ on NO production in M1-cells incubated for 24h, 48h and 72h were then determined. The mixture of the cytokines did not change NO production after 24h of incubation but appeared to increase the production after 48h-incubation (data not shown). However, there was significant increase in NO production by the cytokine mixture in M-1cells incubated for 72h (2.74 ± 0.06 μmoles/L vs. 1.02± 0.03 μmoles/L of the control) (Table 1). Addition of 5 mM db-cAMP into the cytokine mixture in M-1 cells incubated for 72h markedly enhanced NO production to 8.37 ± 0.32 μmoles/L (Table 2). PGE2 at 10^{-6} M added to the cytokine mixture also further stimulated NO

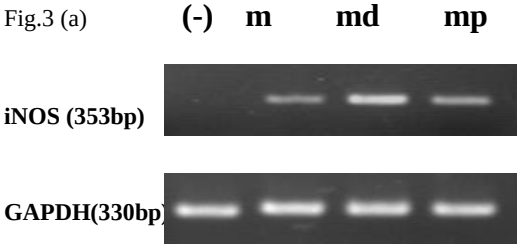
production to 3.47 ± 0.13 μmoles/L (Table 2). To the contrary, AVP at 10^{-6} M had no effect on NO production (data not shown). The effect of the cytokine mixture alone or in combination with db-cAMP or PGE2 on iNOS mRNA was determined. Figure 5 shows relative amounts of iNOS mRNA with the value of non-treated cells set arbitrarily at 1. The cytokine mixture increased relative amount of iNOS mRNA to 4.5, whereas co-supplement of the cytokine mixture with db-cAMP further increased the value to 9.8 (Fig. 3). Addition of PGE2 to the cytokine mixture also further increased iNOS mRNA value to 7.0 (Fig. 3). The data clearly documented that db-cAMP or PGE2 enhanced NO production and iNOS mRNA expression induced by cytokine mixtures.

Table 1. Effect of cytokines mixture, db-cAMP and PGE2 on the nitrite production in M-1 cells.

Addition	Nitrite, μM	
None (control)	1.02 ± 0.03	n= 5
Cytokine mixture	2.74 ± 0.06*	n= 5
Cytokine mixture + db-cAMP	8.37 ± 0.32#	n=5
Cytokine mixture + PGE2	3.47 ± 0.13#	n=5

Data are expressed as mean ± SEM. n: number of experimental observations. M-1 cells were seeded on 10 cm plates and treated for 72 hours in medium without dexamethasone (control) and cytokines mixture containing TNF-α (25 ng/ml), IL-1β (1ng/ml) and IFN-γ (10 ng/ml). Either 5mM db-cAMP or 10^{-6} M PGE2 was coincubated with cytokine mixture in the parallel sets of experiments. Nitrite was assayed from the supernatant of the medium as described in the MTERIALS AND METHODS. *$p<0.05$ vs. control; #$p<0.05$ vs. cytokine mixture. Statistical analysis was performed by One Way Analysis of Variance (Fisher LSD Method) and a *p* value < 0.05 was considered statistically significant.

Fig.3 (a)



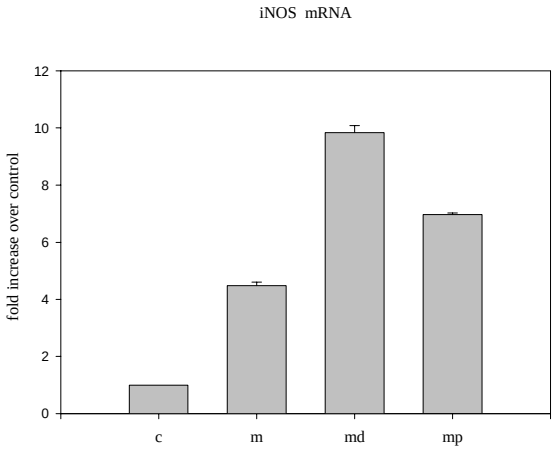


Fig.3. RT-PCR analysis of inducible nitric oxide synthase (iNOS) mRNA in M-1 cells. (a) Expression of inducible nitric oxide synthase (iNOS) mRNAs in M-1 cells. M-1 cells (4×10^5 cells) were loaded on 10 cm plate the day before the addition of stimulators. Cells were treated with or without cytokine mixtures containing TNF-α (10 ng/ml), IL-1-β (1 ng/ml) and IFN-γ (25 ng/ml) for 72 hours. PGE2 (10^{-6}M) and db-cAMP (5mM) were added to M-1 cells with cytokines mixture in parallel. RNA was prepared by tri-reagent. The expression of iNOS mRNA was analyzed by semi-quantitative RT-PCR. GAPDH mNRA was used as an internal control and its expression was the same in control and stimulated cells. (b) Quantitative analysis of the signals for the iNOS mRNA in (a). The relative mRNA levels of iNOS were quantified by scanning densitometry (Zero-Dscan) of the band intensities. The iNOS mRNA levels were normalized to GAPDH mRNA level. The increase in iNOS mRNA level was expressed as the fold increase over control. Representative of three independent experiments was shown. Data are the mean ± S.D. of one representative experiment done in duplicate. (-) = control; m = cytokines mixture containing TNF-a (10 ng/ml), IL-1-b (1 ng/ml) and IFN-g (25 ng/ml). md = cytokines mixture + db-cAMP; mp= cytokines mixture + PGE2.

四、討論 (Discussion)

RT-PCR detected EP1, EP2, EP3 and EP4 receptors in M-1 cells. Further characterization of downstream signaling using specific agonists of each receptor indicated cAMP, but not calcium signaling was operating in M-1 cells. Addition of PGE2, 11-deoxy PGE1 and 17-phenyl trinor PGE2 in M-1 cells increased cAMP accumulations in decreased order. Conversely, butaprost and sulprostone showed no effect on cAMP accumulations. Thus, PGE2 increased cAMP accumulations in M-1 cells mainly through EP1 and EP4, but not EP2 and EP3. Our data was consistent with the results presented by Nasrallah et al., (1). In their study, EP2 was

not expressed in M-1 cells and the Gi-protein-coupled EP3 receptor signaling was absent in M-1 cells. Our data agreed with their finding that it is the EP4 and not the EP2 receptor that couples to Gs in the collecting duct to increase cAMP. Also, from our study, the EP3 signaling was not operating in M-1 cells. Although they also confirmed the presence of EP1 receptors in M-1 cells, no further characterization of EP1 signaling was performed in their study. In the present study, the calcium signaling triggered from EP1 was investigated in M-1 cells. Addition of PGE2 or 17-phenyl trinor PGE2, a specific agonist of EP1, showed no calcium response in M-1 cells. Moreover, crosstalk between EP1 and EP4 was ruled out when compared with the inhibitory effect of ionomycin on PGE2/11-deoxy PGE1 evoked cAMP accumulations in M-1 cells. Thus, we concluded EP1 and EP3 signaling were undetectable and not involved in water reabsorption in M-1 cells. PGE2 exerts inhibitory effect toward AVP-induced water flow in CCD via different mechanism (2). Classic interpretation of the inhibitory effect of PGE2 on water reabsorption was mainly through pertussis toxin-insensitive and sensitive pathways, which were elicited via EP1 and EP3 receptors, respectively. In rabbit cortical collecting tubule, PGE2 acting at high concentrations (0.1- 10 microM) stimulates cAMP accumulations (2). At lower concentrations (0.1-100 nM), PGE2 inhibited AVP-stimulated osmotic water permeability by reducing cAMP generation via an inhibitory EP3 receptor(s) coupled to an inhibitory guanine nucleotide binging protein, Gi (2). PGE2 inhibit AVP-induced water flow dependent on extracellular calcium via EP1 receptors in rat medullary collecting tubule (3). It is evident that separate PGE2 receptors modulate water and sodium transport in rabbit cortical collecting duct (4). Due to the absence of EP1 and EP3 signaling in M-1 cells, we concluded the inhibitory effect of PGE2 on cAMP-dependent water reabsorption were not through either EP1 or EP3 signaling pathway in M-1 cells. In terms of the antagonist effect of PGE2 on water and salt reabsorption, certain mechanism signaling through EP4 receptor (or a mechanism involving NO) was speculated in M-1 cells.

NO, another signaling molecule as important as cAMP, has been implicated in the regulation of nephron transport (5). Endothelial cell derived relaxing factor (i.e. NO) inhibits sodium transport and increases cGMP content in cultures mouse cortical collecting duct cells, which is the M-1 cells used in the present study (6). Inhibition of antidiuretic hormone-stimulated water permeability by NO in CCD was through activation of cGMP-dependent protein kinase, which in turn decreases intracellular cAMP (7). We showed *in vitro* study in M-1 cells, the cell-permeable cAMP analog, db-cAMP, greatly enhanced cytokines-induced NO production in a synergistic fashion. PGE2, as a cAMP-elevating agent, also enhances cytokines-induced NO production. Since PGE2 increased cAMP level through EP4 receptors in M-1 cells, the enhanced effect of PGE2 on cytokines-mixture induced NO formation might be through activation of EP4 receptors. The cAMP level triggered from the activation of EP4 receptor show less potency than db-cAMP in NO production. This may be due to the signals passing from receptor showed slight effect when compared with db-cAMP added exogenously. PGE2 or db-cAMP did not show any stimulatory effect on NO production when added alone. Only in the presence of three different cytokines: TNF , IL1 and IFN , either PGE2 or db-cAMP enhanced cytokines-induced NO production in M-1 cells in a synergistic fashion. Since NO inhibits AVP-induced water reabsorption in the collecting duct, it is speculated that PGE2 might inhibit AVP-stimulated water reabsorption by enhancing NO production due to the increase of cAMP level elicited through EP4 receptors in M-1 cells. The present results clearly documented that PGE2 enhanced cytokines-mixture induced NO formation and induced iNOS gene expression. PGE2 might counteract the effect of cAMP on water and salt reabsorption through NO. PGE2 alone stimulates cAMP accumulations through EP4 and enhanced water

reabsorption in M-1 cells. Under circumstance such as renal injury or inflammation, when the cytokines was produced in M-1 cells, PGE2 enhances NO production. NO then acts as a blocker to counteract the effect of PGE2 and cAMP to inhibit water reabsorption.

五、參考文獻 (References)

1. Nasrallah, R., Laneuville, O., Ferguson, S., and Hebert, R. L. Am J Physiol Renal Physiol, 281 (2001) F123-132.
2. Sonnenburg, W. K., and Smith, W. L. Journal of Biological Chemistry. 263 (1988) 6155-6160.
3. Aarab L, Montegut M, Siaume-Perez S, Imbert-Teboul M, and Chabardes D. European Journal of Physiology 423 (1993) 397-405.
4. Hebert RL, Jacobson HR, Fredin D, and Breyer MD. Am. J. Physiol. 34 (1993) F643-650.
5. Ortiz, P. A., and Garvin, J. L. Am J Physiol Renal Physiol, 282 (2002) F777-784.
6. Stoos, B. A., Carretero, O. A., and Garvin, J. L. J Am Soc Nephrol, 4 (1994) 1855-1860.
7. Garcia, N. H., Stoos, B. A., Carretero, O. A., and Garvin, J. L. Hypertension, 27 (1996) 679-683.