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(3/3)
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計畫類別：■個別型計畫　　□整合型計畫
計畫編號：NSC　93－2320　－B　－040　－053　－
執行期間：93　年　8 月　1　日至　94 年　7 月　31 日

計畫主持人：林庭慧
共同主持人：

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執行單位：中山醫學大學 生物醫學科學學系

中　華　民　國　　94 年　　10 月　23　日

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中文摘要

關鍵詞: 一氧化氮, 前列腺素E_2, 環腺甘酸, 腎臟絲球體細胞

一氧化氮與前列腺素E_2在免疫及發炎反應中扮演重要角色。一氧化氮由一氧化氮合成脢催化產生。一氧化氮合成與前列腺素E_2訊息路徑之間的交互作用，涉及複雜機轉。在此份研究計劃中，我們探討一氧化氮與前列腺素E_2及其之間交互作用在腎臟絲球體細胞中環腺甘酸訊息路徑所扮演之角色，以瞭解二者之間的交互作用，如何誘發腎絲球體腎炎之致病機轉。我們的實驗結果顯示，在腎臟絲球體細胞中，外加入前列腺素E_2及環腺甘酸可以加強內毒素及干擾素誘發之一氧化氮生成。此外，在腎臟絲球體細胞中加以一氧化氮捐贡者(GEA 3162)，導致了前列腺素E_2誘發之下游環腺甘酸反應下降。

英文摘要

Keywords : nitric oxide (NO), prostaglandin E_2 (PGE_2), cAMP, glomerular mesangial cells.

The role of nitric oxide (NO) and prostaglandin E_2 (PGE_2) in mediating immune response have been well documented. NO, generated from L-arginine, is the product of nitric oxide synthase (NOS). The interaction between NO biosynthesis and PGE_2 signaling pathway was intricate. Preliminary data indicated both PGE_2 and dibutyryl cyclic AMP (db-cAMP), a cell-permeable derivative of cAMP, stimulated the production of nitric oxide (NO) and the expression of inducible nitric oxide synthase (iNOS) in the SV40-transformed mouse mesangial (MES-13) cells. Incubation of MES-13 cells with db-cAMP alone did not produce NO and induce iNOS expression. The combination of lipopolysaccharide (LPS) with db-cAMP greatly enhanced NO production in a synergistic pattern, and led to induction of iNOS expression, both in mRNA and protein level. Prostaglandin E_2 (PGE_2), known to be an intracellular physiological trigger of cAMP formation, stimulated no inducible iNOS when added alone or with LPS. Induction of NO production and iNOS expression by PGE_2 were only observed in response to LPS + interferon-gamma (IFN-gamma) in MES-13 cells. Our data indicated PGE_2 as a co-stimulatory factor amplifying LPS + IFN-gamma -induced iNOS gene expression and subsequent NO synthesis in MES-13 cells.

It has been well established that NO activates the enzyme guanylyl cyclase, but little has been reported about the effects of NO on other important second messengers, such as cAMP. Preincubation of the NO donor, GEA 3162 in MES-13 cells, inhibited PGE_2-induced cAMP accumulation. The attenuated response of PGE_2- stimulated cAMP accumulation was observed in the presence of 3-isobutyl-1-methylxanthine, a phosphodiesterase (PDE) inhibitor. This indicated the inhibitory effect of GEA 3162 on PGE_2- stimulated cAMP accumulation

was independent of PDE activity. Although NO reportedly acts by reducing AC activity in some cells, GEA 3162 did not inhibit forskolin-induced cAMP accumulation in MES-13 cells and coincubation of MES-13 cells with the sulfhydryl-reducing agent, dithiothreitol (DTT), did not reverse the inhibitory response. This suggested that the main effect of NO might not interact directly with adenylyl cyclases.

二、緣由與目的

The present study is designed to understand the interaction of NO biosynthesis and PGE_2 signaling pathway in SV40-transformed mouse mesangial (MES-13) cells. Although the role of PGE_2 on NO biosynthesis has been well-defined, the effect of NO donor on PGE_2 signaling pathway were poorly understood. By understanding the effect of NO on the PGE_2 signaling pathway in MES-13) cells, the physiological roles of NO and its interaction with PGE_2 in renal ingfammatory status will be elucidated.

三、Results (結果)

A series of experiments have been conducted to determine the role of cAMP signaling pathway involving PGE_2 in MES-13 cells. First, we demonstrated mRNA expression of EP1, EP2 and EP4 receptors but not EP3 receptor in MES-13 (Fig. 1). The level of cAMP of non-treated MES-13 cells (i.e., control) was 117 ± 13 fmoles / well containing 2×10^5 cells as compared with 1184 ± 100 fmoles / well in cells treated with PGE_2 (10^{-6} M) (Fig. 2). Butaprost, a selective EP2 agonist did not alter cAMP level (142 ± 9 fmoles / well), whereas 11-deoxy- PGE_1, an agonist for both EP2 and EP4, markedly increased cAMP to 890 ± 76 fmoles / well. Cellular Ca^{2+} was not affected by PGE_2 or EP1 agonist (data not shown). Together these data suggest that EP4 is the main PGE_2 receptor operating in MES-13 cell.

The effect of PGE_2 on NO production was then investigated in MES-13 cells. The results showed that exogenously added LPS+ IFNγ stimulated NO production with an increase to 5.27 ± 0.85 μmoles/L from 0.11 ± 0.1 μmoles/L of the control after 18 hours incubation (Table 1). Addition of 10^{-6} M PGE_2 into the LPS+ IFNγ mixture in MES-13 cells incubated for 18h markedly enhanced NO production to 6.45 ± 0.65 μmoles/L (Table 1). 11-deoxy PGE_1 at 10^{-6} M added to the LPS+ IFNγ mixture also further stimulated NO production to 6.69 ± 0.36 μmoles/L (Table 1). The expression of iNOS was significantly induced after 18 hours of incubation with LPS+IFNγ and was further enhanced in medium treated PGE_2 (Fig. 3b). Fig. 3a shows relative amounts of iNOS mRNA with the value of non-treated cells set arbitrarily at 1. The LPS+IFNγ (L) increased relative amount of iNOS mRNA to 3.7, whereas co-supplement of the LPS+IFNγ with PGE_2 (LIP) further increased the value to 5.2. The data indicated PGE_2 enhanced NO production and iNOS mRNA expression induced by LPS+IFNγ in MES-13 cell. This effect of PGE_2 was mainly through EP4 receptor since addition of 10^{-6} M 11-deoxy PGE_1, an agonist of EP4, also enhanced LPS+ IFNγ-stimulated NO production and iNOS gene expression (Table 1; Fig 4b). Fig. 4a depict relative amounts of iNOS mRNA with the value of non-treated cells set arbitrarily at 1. The LPS+IFNγ (L) increased relative amount of iNOS mRNA to 4.7, whereas co-supplement of the LPS+IFNγ with 11-deoxy PGE_1 (LI-11) further increased the value to 7.2. The data therefore,

clearly demonstrated that exogenous PGE_2 enhanced LPS+IFNγ-stimulated NO production and iNOS gene expression in MES-13 cells through EP4-mediated signaling.

Numerous studies indicated that various cytokines not only induced NO production but also regulated COX gene expression and PGE_2 production differentially. Moreover, several reports suggested that the effect of PGE_2 on NO production may be mediated through modulation of COX-2. Whether COX gene expression was upregulated and endogenous PGE_2 was produced under the treatment of LPS+IFNγ in MES-13 cells will be investigated in the present proposal. In addition, the effect of COX inhibitors on the NO production and iNOS gene expression in LPS+IFNγ-treated MES-13 cells will be further elucidated. Whether NO reaching certain threshold exerts feedback effect on PGE_2 biosynthesis and PGE_2 signaling will be examined. We have demonstrated a NO donor, GEA3162, inhibits PGE_2-stimulated cAMP accumulation in a dose-dependent manner in MES-13 cells (Fig.5). Whether the cytokines-induced NO or NO donors exert any effect on COX gene expression and PGE_2 biosynthesis remains to be determined.

The mechanism responsible for the inhibitory effect of NO donor on the PGE_2-evoked cAMP accumulations in MES-13 cells is independent of cGMP. As shown in Fig.6, 8-bromo-cGMP did not mimic NO response and exerted no inhibitory effect on PGE_2-stimulated cAMP accumulation. It has been reported that NO inhibited certain types of ACs, particularly AC5 and AC6. We have identified the distribution of AC isozymes and their expression level in MES-13 cells using RT-PCR. As shown in table 2, of 31 colonies sequenced, 24 (77%) was identified as AC7, 4 (13%) as AC6, 3 (10%) as AC9. Thus, AC7 represented the predominant AC isozyme in MES-13 cells. Since GEA3162 did not alter the forskolin-stimulated cAMP accumulations in MES-13 cells (Fig.7), these data indicated the inhibitory effect of NO donor, GEA-3162, on PGE_2-stimulated cAMP accumulations was not through interaction with AC. Thus, the inhibitory effect of NO on PGE_2-stimulated cAMP accumulation in MES-13 cells occurs neither via cGMP nor a direct effect on AC. The mechanism responsible for the inhibitory effect of NO donor on the PGE_2-evoked cAMP accumulations will be further determined in the proposed study.

Table 1 Effect of LPS (1 mg/ml), IFNγ (5 ng/ml), PGE_2 (10^{-6}M), 11-deoxy PGE_1 (10^{-6} M) on the nitrite production in MES-13 cells.

Addition	Nitrite, μM
None (control)	0.11 ± 0.10
LPS (1 mg/ml) + IFNγ (5 ng/ml)	5.33 ± 0.16*
LPS (1 mg/ml) + IFNγ (5 ng/ml)+ PGE_2 (10^{-6}M)\	6.00 ± 0.15≠
LPS (1 mg/ml) + IFNγ (5 ng/ml)+ 11-deoxy PGE_1 (10^{-6}M)	6.69 ± 0.36≠ \

Data are expressed as means ± S. D. for six samples. MES-13 cells were seeded on 10 cm plates and treated for 18 hours in medium (control) and LPS (1 mg/ml) + IFNγ (5 ng/ml). Either 10^{-6} M PGE_2 or 11-deoxy PGE_1 was coincubated with LPS+ IFNγ 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. LPS+ IFNγ. Statistical analysis was performed by One Way Analysis of Variance (Fisher LSD Method) and a *p* value ＜ 0.05 was considered statistically significant.

Table 2 Distribution of AC isozymes in MES-13 cells

Using RT-PCR, degenerate primers (ACXf-ACEr) amplified a PCR fragment around 243 bp in MES-13 cells. The 243 bp PCR products were subcloned to pCR^{TM}II vector (*Invitrogen*) and each clone was identified by DNA sequence analysis. The sequencing of 31 individual colonies confirmed the presence of mRNA for AC7, AC6 and AC9 isozymes in MES-13 cells. The values are the percentages of total 31 clones expressing each AC isoform.

Number of samples	AC subtypes		
31	AC7	AC6	AC9
	77 %	13 %	10 %

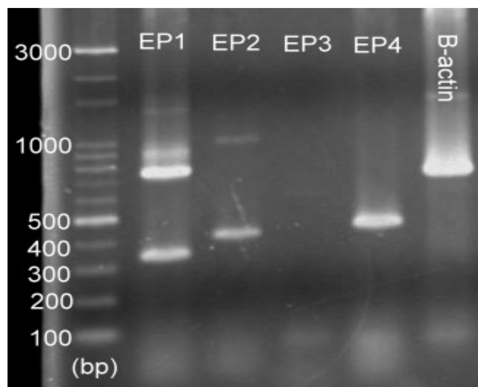

Fig. 1 Measurement of EP1, EP2 and EP4 receptor mRNA in MES-13 glomerular mesangial cells

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Total RNA prepared by tri-reagent was used to detect mRNA coded for EP1, EP2, EP3 and EP4 by RT-PCR. As shown in line 1, two PCR fragments (336 bp and 700 bp) were amplified in MES-13 cells. The size of 336 bp was confirmed to be EP1 as revealed by DNA sequencing. The distribution of all EP1, EP2 and EP4 receptors were confirmed in this cell line.

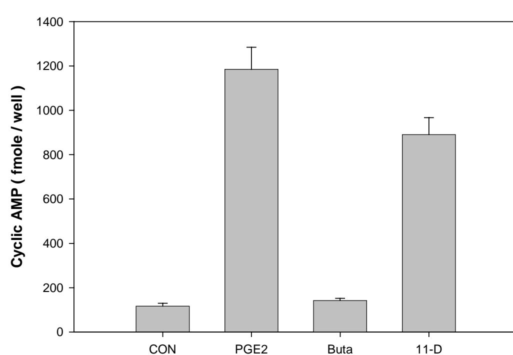
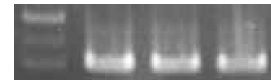


Fig. 2 Effect of specific PGE_2 agonists on cAMP accumulation in MES-13 glomerular mesangial cells. Prior to stimulation, cells (approximately 2×10^5 cells /well) were preincubated for 10 min at 37^0C in medium containing 0.5 mM IBMX. The individual agonists for each PGE_2 receptor were added in the 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 means ± S. D. for triplicate samples of a typical experiment. The experiments were repeated twice with similar results. CON = control; Buta = butaprost; 11-D = 11-deoxy- PGE_1.

353bp (iNOS)

330 bp (GAPDH)



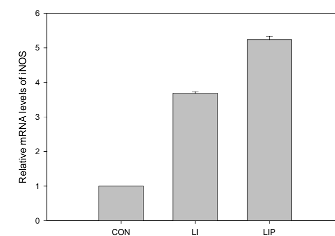


Fig. 3 Effects of LPS+IFNγ and PGE_2 on mRNA for inducible nitric oxide synthase (iNOS) in MES-13 glomerular mesangial cells.

For gene expression of inducible nitric oxide synthase (iNOS), MES-13 cells (4×10^5 cells) were loaded on 10 cm plate the day before the addition of stimulators. Cells were treated with or without LPS (1 μg/ml) and IFN-γ (10 ng/ml) for 18 hours in the presence or absence of 10^{-6}M PGE_2. The expression of iNOS mRNA was analyzed by semi quantitative RT-PCR. GAPDH mNRA was used as an internal control. Panel a presents the relative levels of iNOS mRNA. Panel b depicts RT-PCR amplification of iNOS in RNA extracts. The relative mRNA levels of iNOS were

quantified by scanning densitometry (Zero-Dscan) of the band intensities and normalized with GAPDH mRNA level. The increase in iNOS mRNA levels was expressed based the control group which was arbitrarily set at 1. Data are the means ± S. D. for duplicate samples of two typical experiment. C = control; L = LPS+IFNγ; LIP= LPS+IFNγ +PGE_2.

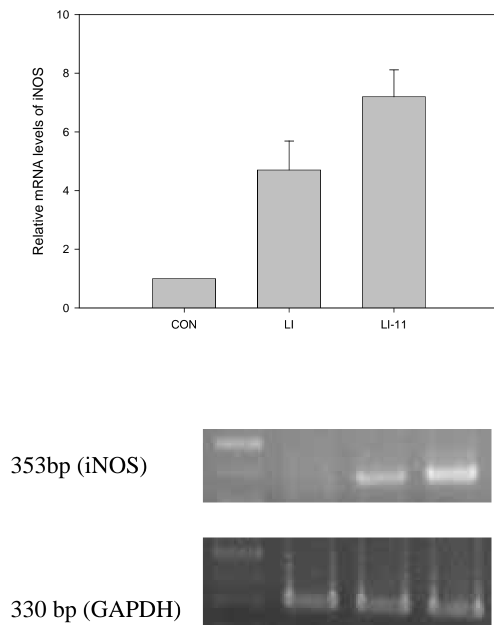


Fig. 4 Effects of 11-deoxy PGE_1 on mRNA for inducible nitric oxide synthase (iNOS) in MES-13 glomerular mesangial cells.

Cells were treated with or without LPS (1 μg/ml) and IFN-γ (10 ng/ml) for 18 hours in the presence or absence of 10^{-6}M 11-deoxy PGE_1. The expression of iNOS mRNA was analyzed by semi quantitative RT-PCR. GAPDH mNRA was used as an internal control. Panel a presents the relative levels of iNOS mRNA. Panel b depicts RT-PCR amplification of iNOS in RNA extracts. The relative mRNA levels of iNOS were quantified by scanning densitometry (Zero-Dscan) of the band intensities and normalized with GAPDH mRNA level. The increase in iNOS mRNA levels was expressed based the control group which was arbitrarily set at 1. Data are the means ± S. D. for duplicate samples of two typical experiment. C = control; LI = LPS+IFNγ; LI-11= LPS+IFNγ +11-deoxy PGE_1.

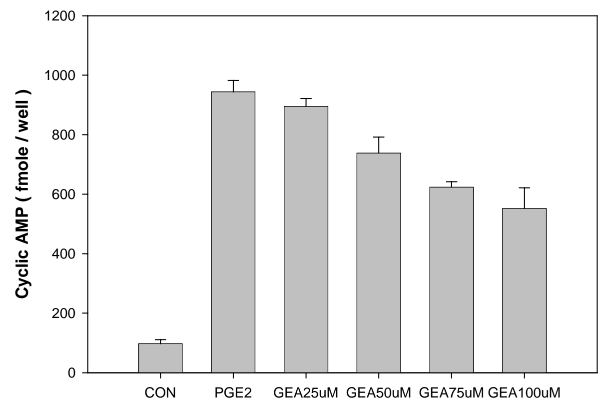


Fig. 5 Effects of GEA 3162 (25 μM-100μM) on the PGE_2-stimulated cAMP accumulations in MES-13 glomerular mesangial cells. MES-13 cells were coincubated with different concentration of GEA 3162 and 10^{-6}M PGE_2 in medium for 10 min. The amount of cAMP extracted was quantified by cAMP biotrak enzymeimmunoassay (EIA) system. Data are the means ± S. D. for triplicate samples of a typical experiment. The experiments were repeated twice with similar results.

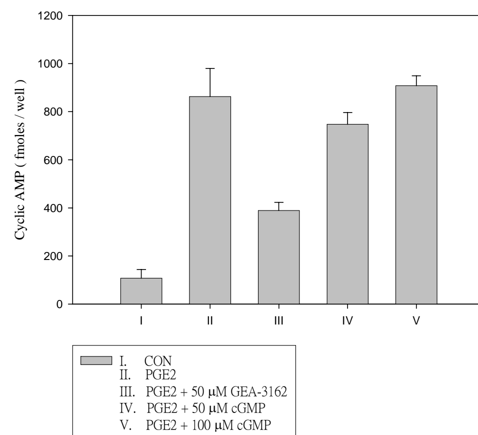


Fig. 6 Effect of 8-bromo-cGMP on the PGE_2-stimulated cAMP accumulations in MES-13 glomerular mesangial cells. MES-13 cells were coincubated with 50 μM GEA 3162 and 10^{-6} M PGE_2. In parallel condition, 50 μM GEA 3162 was replaced with 8-bromo-cGMP (50 μM, 100 μM) and incubated with 10^{-6} M PGE_2 in medium at 37^0C for 10 min. The amount of cAMP extracted was quantified by cAMP biotrak enzymeimmunoassay (EIA) system. Data are the means ± S. D. for triplicate samples of a typical experiment. The experiments were repeated twice with similar results.

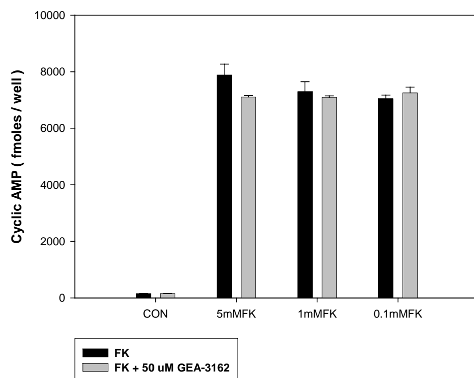


Fig. 7 Effects of GEA 3162 (50 μM) on the forskolin-stimulated cAMP accumulations in MES-13 glomerular mesangial cells. MES-13 cells were incubated with 50 μM GEA 3162 in medium at 37^0C for 10 min with the addition of different concentration of forskolin (0.1mM, 1 mM, 5 mM). The amount of cAMP extracted was quantified by cAMP biotrak enzymeimmunoassay (EIA) system. Data are the means ± S. D. for triplicate samples of a typical experiment. The experiments were repeated twice with similar results.

四 、討論 (Discussion)

Our preliminary data indicated PGE_2 enhanced LPS+IFNγ-induced NO production and iNOS gene expression through increased intracellular cAMP level via EP4 receptor in MES-13 cells. It is well known that the effect of PGE2 on NO production via EP4 was through activation of cAMP-dependent protein kinase A (i.e. PKA) and subsequently, the cAMP-response element binding protein (CREB). Through binding to the cAMP-response element (CRE) located within the enhancer region of iNOS gene, the enhanced iNOS gene expression at the transcriptional level increased the LPS+IFNγ-induced NO production. Whether PKA exerts any post-translational effect on iNOS protein and iNOS activity lacked information. Investigation of the potential PKA phosphorylation on the iNOS protein should be determined.

Preincubation of the NO donor, GEA 3162 in MES-13 cells, inhibited PGE_2-induced cAMP accumulation. The mechanism responsible for the inhibitory effect of NO donor on the PGE_2-evoked cAMP accumulations in MES-13 cells is independent of PDE, cGMP and adenylyl cyclase. Whether the mechanism is due to the effect of GEA 3162 on the gene expression of PGE_2 receptors or altering on the binding affinity of PGE_2 toward EP4 receptor should be determined in MES-13 cells.

五、參考文獻 (References)

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