行政院國家科學委員會專題研究計畫 成果報告

蛋白質磷酸化對腎臟絲球體細胞中誘發型一氧化氮合成酶活性調控之探討

研究成果報告(精簡版)

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計 畫 主 持 人：林庭慧

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中 華 民 國 96 年 10 月 27 日

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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計畫主持人：林庭慧
共同主持人：

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中　華　民　國　　96 年　　10 月　23　　日

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中文摘要

關鍵字: 腎絲球細胞，誘發型一氧化氮合成酶，蛋白質磷酸化，蛋白分解。

中文摘要

一氧化氮在腎臟發炎反應中扮演重要角色，過量之一氧化氮生成會破壞腎臟機能，並促使腎小球腎炎之發生。許多的研究藉由調控誘發型一氧化氮合成腶來降低一氧化氮過量所造成之細胞毒害。大多數的研究報告都集中重點在轉錄層次上來調控誘發型一氧化氮合成腶；只有少數論文提及誘發型一氧化氮合成腶可在後轉譯層次上受到調控。我們的結果顯示，前列腺素 E2 抗拮劑(AH23848)可藉由減弱細胞內環腺甘酸含量及加速誘發型一氧化氮合成酶蛋白的分解，來抑制腎絲球細胞中產生的一氧化氮。前列腺素 E2 抗拮劑加速誘發型一氧化氮合成酶蛋白分解是透過蛋白質激酶 A，因為蛋白質激酶 A 的抑制劑亦可加速誘發型一氧化氮合成酶蛋白的分解。由於實驗結果亦顯示誘發型一氧化氮合成酶可被絲氨基酸/滋利氨基酸蛋白質激酶磷酸化，並且影響誘發型一氧化氮合成酶之活性。我們推測誘發型一氧化氮合成酶在後轉譯層次上所受到之調控與其蛋白質穩定度有關。

英文摘要

Excessive release of nitric oxide (NO) by mesangial cells contributes to the pathogenesis of glomerulonephritis. Prostaglandin E_2 (PGE_2) produced at inflammatory sites regulates the release of NO through its downstream signaling. In glomerular mesangial cells (MES-13 cells), PGE_2 modulated NO production mainly through EP4 receptor in a cAMP-dependent manner. Lipopolysaccharide and interferon-γ (LPS + IFNγ)-induced NO production, inducible nitric oxide synthase (iNOS) gene and protein expression were greatly inhibited by AH23848, an EP4 antagonist. Further investigation indicated that AH23848 attenuated endogenous cAMP accumulation in MES-13 cells and modulated NO production through declination of iNOS gene expression and acceleration of iNOS protein degradation. AH23848 downregulated the iNOS protein in MES-13 cells through protein kinase A (PKA) since KT5720, a PKA-specific inhibitor, reduced iNOS protein stability. A short exposure of activated MES-13 cells to okadaic acid augmented iNOS activity. The results of this study led us to speculate that cAMP might regulate iNOS-stimulated NO synthesis through posttranslational mechanisms. Attenuation of cAMP signaling and the phosphorylation status of the iNOS protein may account for the effect of AH23848 in accelerating iNOS protein degradation in MES-13 cells.

Keywords: glomerular mesangial cells; inducible nitric oxide synthase; protein phosphorylation; protein degradation.

二、緣由與目的

The present study is designed to understand whether cAMP may regulate iNOS-stimulated NO synthesis through posttranslational modification of iNOS protein in SV40-transformed mouse mesangial (MES-13) cells. The role of cAMP in iNOS-stimulated NO synthesis has been well-elucidated at the transcription and post-transcriptional level. On the other hand, the information regarding the role of cAMP in posttranslational modification of iNOS protein is meager. Understanding the regulation of iNOS enzyme activity at the post-translational level may provide a pharmacological strategy to attenuate NO production in treating glomerulonephritis.

三、Results (結果)

Effects of PGE_2 agonists and antagonists and the COX-2 inhibitor on LPS + IFNγ-induced NO production, iNOS mRNA and protein expressions in MES-13 cells

NO production in the presence of test compounds was measured in MES-13 cells. The combination of 1μg/ml LPS + 5 ng/ml IFNγ showed a significant increase in NO production after 18 h of incubation (6.4 ± 0.14 vs. 1.34 ± 0.11 μmole/L of the control). Effects of specific PGE_2 agonists on NO production in MES-13 cells were determined (data not shown). The addition of 10^{-6} M PGE_2 into the LPS + IFNγ mixture slightly enhanced NO production to 7.38 ± 0.06 μmole/L. Moreover, the addition of 10^{-6} M 11-deoxy PGE_1 to the LPS + IFNγ mixture stimulated NO production to 7.45 ± 0.09 μmole/L. On the contrary, at 10^{-6} M, neither 17-phenyl trinor PGE_2, butaprost, nor sulprostone showed any effect on NO production. To confirm the roles of different EP receptors in endogenous PGE_2-mediated NO production, the effects of selective antagonists of PGE_2 receptors on NO production were determined. The addition of 10 μM SC19220 (an EP1 antagonist) and 10 μM AH6809 (an EP2 antagonist) showed no effect on NO production, while 10 μM AH23848, an EP4 antagonist, significantly attenuated NO production from 6.4 ± 0.14 to 5.5 ± 0.18 μmole/L (data not shown). Moreover, the NO concentration in the medium was measured in the presence of NS-398, a selective cyclooxygenase 2 (COX-2) inhibitor. NS-398 at 10 μM significantly reduced the LPS + IFNγ-induced NO production from 6.4 ± 0.14 to 3.23 ± 0.15 μmole/L. The extent of NO inhibition in the presence of the COX-2 inhibitor was close to the effect of 30 μM AH23848, which reduced the LPS + IFNγ-induced NO production from 6.4 ± 0.14 to 3.48 ± 0.91 μmole/L (Fig. 1). Effects of the EP4 agonists and antagonists, and a COX-2 inhibitor on iNOS mRNA were measured. (data not shown), the expression of iNOS mRNA was significantly induced with the addition of LPS + IFNγ, but insignificant enhancement of mRNA was observed with further addition of either PGE_2 or 11-deoxy PGE_1. Conversely, addition of AH23848 and NS-398 to the LPS + IFNγ mixture markedly attenuated iNOS mRNA expression (data not shown). Further investigation of the effect of EP4 agonists and antagonists, and the COX-2 inhibitor on iNOS protein was performed. LPS + IFNγ induced the relative amount of iNOS protein in MES-13 cells. Again, co-supplementation of LPS + IFNγ with PGE_2 or 11-deoxy PGE_1 only slightly enhanced iNOS protein amount (data not shown). On the other hand, addition of AH23848 and NS-398 to the LPS + IFNγ mixture markedly attenuated 50 % of iNOS protein amount. These data indicated that exogenous PGE_2 vaguely enhanced NO production and iNOS protein expression, while blocking endogenous PGE_2 with either an EP4 antagonist or a COX-2-specific inhibitor significantly reduced the NO concentration and attenuated the iNOS mRNA and protein levels.

The EP4 receptor antagonist, AH23848, downregulated NO production through attenuation of cAMP signaling in MES-13 cells

The EP4 receptor utilized cAMP as a second messenger to transmit the signal of

PGE_2 in MES-13 cells. To further confirm the role of cAMP in PGE_2-modulated NO synthesis in MES-13 cells, we examined the effect of cAMP on LPS + IFNγ-induced NO production. The NO concentration in the medium was measured in the presence of dibutyryl cAMP (db-cAMP), an analogue of cAMP. db-cAMP at 0.2 mM enhanced NO production from 6.4 ± 0.14 to 7.32 ± 0.08 μmole/L (data not shown). Moreover, co-supplementation of LPS + IFNγ with db-cAMP enhanced iNOS mRNA (data not shown). These observations demonstrated that the cAMP-mediated signaling cascade is responsible for the role of PGE_2 in regulating iNOS activity in MES-13 cells. Moreover, endogenous cAMP levels in LPS + IFNγ-stimulated MES-13 cells were compared between AH23848-treated and untreated groups. The endogenous cAMP level in LPS + IFNγ non-stimulated MES-13 cells (i.e., the control) was 385 ± 27 fmole/well containing 2.5×10^5 cells (Fig. 2). Stimulation of MES-13 cells with LPS + IFNγ for 18 h increased the endogenous cAMP level to 908 ± 26 fmole/well, while co-incubation of AH23848 with LPS + IFNγ decreased the endogenous cAMP level to 566 ± 32 fmole/well. These results indicate that through attenuation of cAMP signaling, AH23848 downregulated NO production in MES-13 cells.

AH23848 modulated iNOS protein stability

To determine whether AH23848 modulates iNOS protein stability, a pulse-chase experiment was performed (Fig. 3). MES-13 cells were treated with 1μg/ml LPS + 5 ng/ml IFNγ in the absence or presence of 30 μM AH23848 for 18 h. The culture medium was then removed and replaced with starvation medium containing no methionine for 45 min. Pulse medium containing L-[^{35}S] methionine (100 μCi/dish) was incubated with cells for 3 h followed by chase medium containing no labeled methionine. The cell lysate was then collected at the indicated time points (1, 3, and 6 h), and labeled iNOS protein was detected by immunoprecipitation, SDS/PAGE and autoradiography. As shown in Fig. 3, 78 ± 8 %, 60 ± 3 %, and 29 ± 8 % of L-[^{35}S] methionine-labeled iNOS proteins remained in AH23848-untreated MES-13 cells collected at 1, 3, and 6 h, respectively, while only 65 ± 4 %, 27 ± 12 %, and 9 ± 9 % of labeled iNOS proteins remained in AH23848-treated MES-13 cells collected in the same periods. These results revealed that iNOS proteins from AH23848-treated MES-13 cells decreased more rapidly than those of AH23848-untreated groups.

KT5720 inhibited NO production and iNOS protein expression through a decrease in iNOS protein stability

The role of PKA, the downstream signaling molecule of cAMP, in iNOS-stimulated NO synthesis was further investigated. The addition of 10 μM H-89, 50 μM H-8, and 1 μM KT5720 attenuated LPS + IFNγ-induced NO production from 7.39 ± 0.65 to 3.78 ± 0.47, 1.04 ± 0.38, and 3.31 ± 0.55 μmole/L, respectively (Fig. 4, upper panel). Moreover, the addition of H-89, H-8, and KT5720 diminished LPS + IFNγ-induced iNOS protein expression (Fig. 4, lower panel). There is no doubt as to the participation of PKA in the LPS + IFNγ-induced NO production in MES-13 cells. Whether PKA modulates iNOS protein stability was further investigated by pulse-chase experiment. As shown in Fig.5, 78 ± 8 %, 60 ± 3 %, and 29 ± 8 % of L-[^{35}S] methionine-labeled iNOS proteins remained in untreated MES-13 cells collected at 1, 3, and 6 h, respectively, while only 56 ± 7 %, 24 ± 10 %, and 1±0% of L-[^{35}S] methionine-labeled iNOS proteins remained in KT5720-treated MES-13 cells. Taken together, these results indicate that iNOS proteins from KT5720-treated MES-13 cells decreased more rapidly than those of untreated groups.

A phosphatase inhibitor augmented iNOS activity in MES-13 cells

The effects of phosphatase inhibitors on iNOS activity were assessed by measuring the conversion of L-arginine into citrulline. MES-13 cells were stimulated with LPS + IFNγ for 16 h, followed by the addition of either a tyrosine phosphatase inhibitor (10 μM sodium orthovanadate) or a serine/threonine phosphatase inhibitor (20 nM okadaic acid) over a period of 15~120 min as indicated in Fig.6. Total cellular proteins were collected, and the conversion of L-arginine into citrulline was measured. iNOS activities measured in the presence of LPS + IFNγ are presented as 100%. A short exposure of MES-13 cells to 10 μM sodium orthovanadate for 15 and 30 min increased the iNOS activity by about 10%. When the incubation times were prolonged to 60 and 120 min, a 30% increment in iNOS activity was observed. For the effect of 20 nM okadaic acid, 25%~30% augmentation of iNOS activity was also observed at 60 and 120 min of incubation. Thus, the phosphatase inhibitors augmented iNOS activity.

Fig. 1 Effect of specific EP4 agonists and antagonists, cAMP analogue, a COX-2 specific inhibitor, on LPS + IFNγ-induced NO production in MES-13 cells.

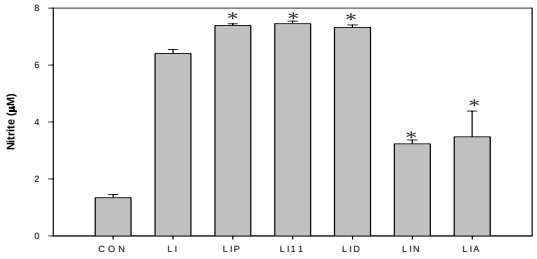


Fig. 2 AH23848 attenuated endogenous cAMP accumulation in MES-13 cells.

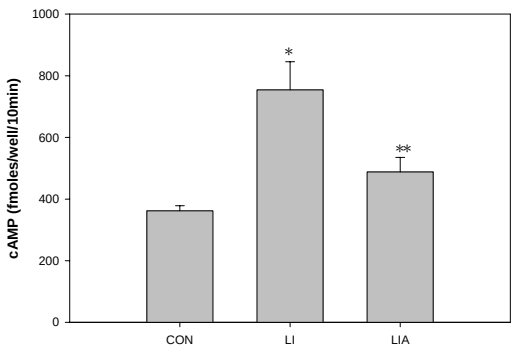


Fig. 3 AH23848 accelerated iNOS protein degradation in MES-13 cells as revealed by pulse-chase study.

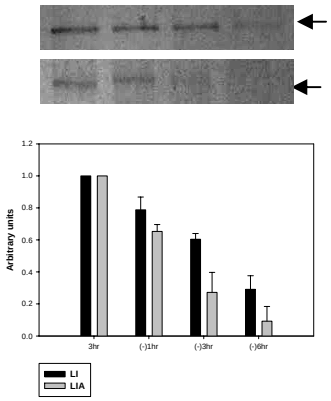


Fig. 4 Effect of PKA inhibitors on LPS + IFNγ-induced NO production and iNOS protein expression in MES-13 cells.

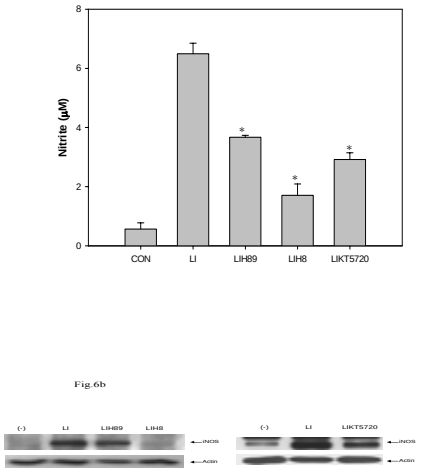


Fig. 5 KT5720 accelerated iNOS protein degradation in MES-13 cells as revealed by pulse-chase study.

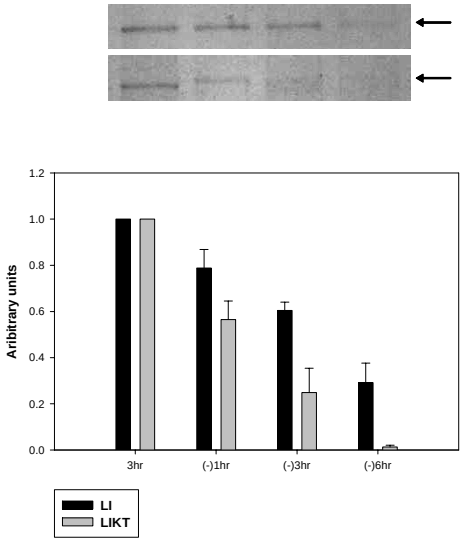
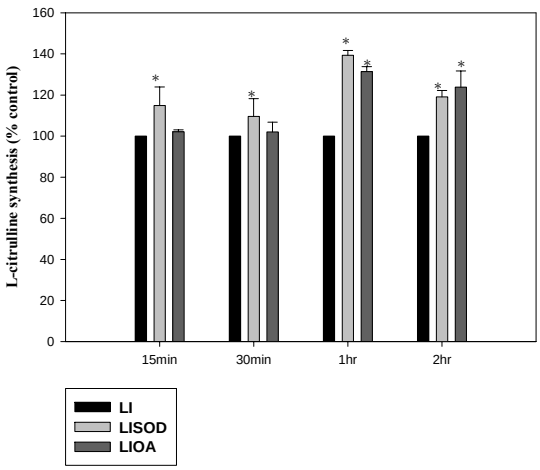


Fig. 6 Effects of phosphatase inhibitors on iNOS activity in MES-13 cells.



四 、討論 (Discussion)

As suggested by Won et al. [1], cAMP enhances iNOS protein expression by protecting the iNOS protein from degradation. This notion led to the following study to investigate whether cAMP plays a role in maintaining iNOS protein stability. Our data indicated that attenuation of cAMP signaling by AH23848 accelerated iNOS degradation as revealed by pulse-chase study. This indicated that reduction in intracellular cAMP level could lead to iNOS protein instability in MES-13 cells. A specific PKA inhibitor, KT5720, significantly inhibited LPS + IFNγ-induced NO production and iNOS protein expression in MES-13 cells. Pulse-chase study demonstrated that KT5720 accelerated iNOS degradation. This showed that activation of a downstream target of cAMP, i.e., PKA, is involved in maintaining iNOS protein stability. A short exposure of activated MES-13 cells to either okadaic acid or sodium orthovanadate augmented iNOS activity. Moreover, AH 23843 and KT 5720 decreased the signal recognized by PSAb, a phospho-(serine/threonine) PKA substrate antibody. This indicated the serine/threonine phosphorylation of iNOS protein was reduced when the EP4R-cAMP pathway was declined in activated MES-13 cells. Collectively, these data suggested that iNOS-stimulated NO synthesis could be modulated through posttranslational modification of the iNOS protein. PKA may stabilize the iNOS protein through serine/threonine phosphorylation and maintain iNOS in a configuration facilitating NO synthesis, such as by exposing the active site for substrate binding. Although no information regarding this speculation is available for iNOS, a stable dimeric structure resisting neuronal NOS (nNOS) from proteolysis indicates that protein conformation is an important determinant of NOS stability [2]. Whether phosphorylation sustains the iNOS protein in a precise configuration required for dimer formation is unknown. Nonetheless, destabilization of the dimeric structure of nNOS triggers its degradation through a ubiquitin-proteasomal pathway suggesting that iNOS stability may be determined through a similar structural

requirement. Although no direct evidence indicates a role of phosphorylation in protecting iNOS from degradation, an S714P mutant of iNOS, identified in Dahl/Rapp salt-sensitive rats, attenuated iNOS enzyme stability [3]. Accelerated ubiquitination and proteasome degradation of iNOS was found in an S714P mutant of iNOS [4]. Although the phosphorylation status of iNOS was unidentified in the S714P mutant, whether phosphorylation of iNOS protein protects the iNOS enzyme from degradation is certainly an interesting issue to be pursued in the future.

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