

行政院國家科學委員會專題研究計畫成果報告

— 一氧化氮與內皮素之間於新生鼠心臟細胞的交互作用

Interaction between nitric oxide and endothelin-1 in neonatal rat cardiomyocytes

計畫編號：NSC 89-2316-B-002-028

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一、中文摘要

內皮素會造成心肌細胞的肥大，而於此過程中，心血管系統所釋放的一氧化氮扮演著某種程度的對抗作用。本研究，在於探討內生性一氧化氮對抗內皮素所誘發 *c-fos* 基因表現的作用機轉。實驗結果顯示：內皮素經由活化其乙型接受器，會刺激一氧化氮合成酵素，造成一氧化氮生成增加。經由一氧化氮貢獻劑如 SNAP、SIN-1 及一氧化氮清除劑 PTIO 的運用，以北方點墨法搭配報告者基因轉染的方法，顯示一氧化氮可對抗內皮素所誘發 *c-fos* 的基因表現；另外，以不同的負向突變基因 Ras (RasN17), Raf-1 (Raf301), 或 mERK，轉染入心肌細胞皆可對抗內皮素所誘發 *c-fos* 的基因表現，顯示內皮素誘發 *c-fos* 的基因表現是經由 Ras/Raf/ERK 的訊息路徑；直接觀察一氧化氮對內皮素所激活 ERK 的作用，一氧化氮亦可對抗內皮素的激活 ERK 的活性；猶有甚者，一氧化氮尚且可對抗內皮素所增加乙型重鍊肌蛋白基因啟動子活性的作用。縱合觀之，內皮素會造成心肌細胞一氧化氮生成增加，而一氧化氮可進一步對抗內皮素的激活 ERK 的活性，造成對抗內皮素所誘發 *c-fos* 的基因表現。

關鍵詞：內皮素、心肌細胞、一氧化氮、Ras/Raf/ERK 訊息路徑、*c-fos*

Abstract

Endothelin-1 (Et-1) treatment of cardiac myocytes (CM) induces cardiac hypertrophy. Cardiovascular release of nitric oxide (NO) may play a role during cardiac hypertrophy. The present study examined the protection mechanism of endogenously released NO on

c-fos induction by Et-1 in neonatal rat CM. CM exposed to Et-1 induced NO release. Et-1 stimulated nitric oxide synthase (NOS) activity led to NO production that was attenuated by treating CM with an antagonist to endothelin B receptor. CM treated with a NO donor, S-nitroso-N-acetylpenicillamine (SNAP) or 3-morpholinosydnonimine (SIN-1), inhibited Et-1-induced *c-fos* expression. Conversely, CM treated with a NO scavenger, 2-phenyl-4,4,5,5-tetramethyl-imidazole-1-oxyl-3-oxide (PTIO), augmented Et-1-induced *c-fos* expression. The attenuation of NO on *c-fos* expression was shown by reducing either *c-fos* mRNA levels or *c-fos* promoter activities using a chimera containing the *c-fos* promoter region (-2.25 kb) ligated to a reporter gene CAT. In contrast to the enhanced promoter activity in CM after PTIO treatment, attenuated Et-1-induced *c-fos* promoter activity was shown in CM treated with a NO donor. CM cotransfected with a dominant negative mutant of Ras (RasN17), Raf-1 (Raf301), or a catalytically inactive mutant of extracellular signal-regulated kinase (ERK)-2 (mERK) inhibited Et-1-induced *c-fos* promoter activity, indicating Ras/Raf/ERK pathway was involved. NO modulation of this signaling pathway was shown by its inhibitory effect on Et-1-induced ERK activity. CM treated with NO resulted in a decrease of Et-1-induced binding of nuclear proteins to the AP-1 binding sequences. Furthermore, CM treated with a NO donor significantly suppressed Et-1-induced β -myosin heavy chain promoter activities. These results indicate that CM under Et-1 treatment increases NO levels and the increased NO attenuated Et-1-induced *c-fos* expression *via* the ERK signaling pathway. These findings support the importance of NO as a negative regulator in Et-1-induced gene expression and cardiac hypertrophy.

Keywords: Endothelin-1, cardiomyocyte, nitric oxide, Ras//Raf/ERK

signaling pathway, *c-fos*

二、緣由與目的

Endothelin-1 (Et-1), a 21-amino acid peptide, is the most potent long-lasting vasoconstrictor (Yanagisawa et al., 1988). It is also a hypertrophy-promoting factor for various cells including cardiomyocytes (Wang et al., 1992 and 1993; Yamazaki et al., 1996). Cardiomyocytes contain EtA and EtB receptors (Kanno et al., 1993). We reported previously that Et-1 stimulated hypertrophic response by stimulating α - and β -myosin heavy chain gene expression in cardiomyocytes (Wang et al., 1992). We further showed that Et-1 induced *c-fos* expression and this induction was mediated via EtA receptor in cardiomyocytes (Cheng et al., 1999). In contrast, Et-1 triggering the release of NO from endothelial cells is mediated via EtB receptor (Tsukahara et al., 1994). The activation of EtA receptor in cardiomyocytes leads to the activation of phospholipase C and protein kinase C that result in an activation of mitogen-activated protein kinases (MAPK) (Clerk et al., 1994).

The MAP kinases are a family of serine-threonine kinases, which include the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs/stress-activated protein kinases), and p38 MAPK. The ERKs that have been well studied are generally activated by mitogenic stimuli *via* a Ras/Raf1/MEK1 signaling cascade. This leads to activation of transcription factors such as Elk1 and *c-fos*. The signaling system leading to the activation of MAPK is subjected to diverse and complex regulation and the upstream of this kinase cascade has not been fully defined (Force and Bonventre, 1998; Schorb et al., 1995; Duff et al., 1995). Recent studies suggested that the imbalance of the intracellular oxidative and reductive status affected MAPK signaling pathways (Powis et al., 1997; Aikawa et al., 1997). When cells exposed to mitogens or cytokines, the intracellular redox status was altered by changing the oxidation-reduction state of essential thiol group in protein(s) (Lander et al., 1995; Sundaresan et al., 1995; Kamata et al., 1996). This redox-sensing mechanism underlying cellular responses is at least partly controlled at the level of transcription. Little is known of the molecular basis of such redox regulation in cells.

It is well documented that nitric oxide

(NO) or related molecules modulate cellular response. NO exerts its action by stimulating soluble guanylate cyclase, leading to an increase of cyclic GMP levels that activate cyclic GMP-dependent protein kinases (Schmidt and Walter, 1994). In addition, NO is capable of reacting with oxygen radicals such as superoxide anion to form peroxynitrate and the consequence of diminished NO may contribute to the pathogenesis of atherosclerosis, hypertension-related complication (Lander et al., 1997; Akaike et al., 1998). Furthermore, recent studies have shown that NO or related molecules covalently modify cysteine residues in proteins and such modification alters protein function and signaling amplitude ().

Constitutive NO synthase (NOS3) and increased NO production were noticed during pathological states such as myocardial failure (Balligand et al., 1993; Haywood et al., 1996). It has been shown that NO antagonizes the actions of Et-1 in many cell types (Rizvi and Myers, 1997). Calderone et al (1998) reported that NO inhibited the growth-promoting effects of norepinephrine in cardiomyocytes. NO appears to attenuate the mitogenic effects despite the detailed mechanisms are not clear. The present study examined whether NO production was regulated by Et-1 in cardiomyocytes and the role of NO on Et-1-induced *c-fos* gene expression. We report that cardiomyocytes exposed to Et-1 increased NO production that was mediated via EtB receptor. The released NO consequently attenuated Et-1-induced *c-fos* expression *via* the ERK signaling pathway. The inhibition of NO on Et-1-induced cardiac effects results in a decrease of β -myosin heavy chain transcriptional activity. Present results support the importance of NO derived from cardiomyocytes as a negative regulator for Et-1-induced cardiac hypertrophy.

三、結果

Effects of Et-1 on NO synthesis and NOS activity

The effect of Et-1 on NO generation and NOS activity in cardiomyocytes were analyzed. Exposure of cardiomyocytes to Et-1 (10 nM) induced NO generation, with a maximal increase of 2-fold at 30 min after stimulation (Figure 1A). To specify whether the Et-1 receptor subtypes that were responsible for the generation of NO in cardiomyocytes, we pretreated

cardiomyocytes with Et-1 receptor type A antagonist (BQ485) or receptor type B antagonist (BQ788) before the Et-1 exposure. As shown in Figure 1B, BQ788 (100 nM) significantly inhibited the Et-1-induced generation of NO. In contrast, BQ485 (100 nM) had no significant effect on Et-1-induced generation of NO. Treatment of cardiomyocytes with Et-1 receptor type B agonist IRL1620 (100 nM) alone also induced NO generation. These results suggested that an increase of NO generation upon Et-1 treatment was mediated mainly through the binding of Et-1 to Et-1 receptor type B. All three NOS isoforms characterized to date depend upon calmodulin activation, but constitutive NOS are distinguished from inducible NOS by their calcium sensitivity for the binding of activator calmodulin and enzymatic activity within the physiological range for intracellular calcium (Nathan and Xie, 1994). A constitutive NO synthase activity was detected and quantified in whole extracts of cardiomyocytes, and this activity was dependent upon the presence of calcium (Fig 1C). Exposure of cardiomyocytes to Et-1 (10 nM) increased NOS activity, with a maximal increase of 2-fold at 30 min after stimulation (Figure 1C). To specify whether the Et-1 receptor subtypes that were responsible for the increase of NOS activity in cardiomyocytes, we pretreated cardiomyocytes with Et-1 receptor type A antagonist (BQ485) or receptor type B antagonist (BQ788) before the Et-1 exposure. As shown in Figure 1D, BQ788 (100 nM) significantly inhibited the Et-1-increased NOS activity. In contrast, BQ485 (100 nM) had no significant effect on Et-1-increased NOS activity. Treatment of cardiomyocytes with Et-1 receptor type B agonist IRL1620 (100 nM) alone also increased NOS activity.

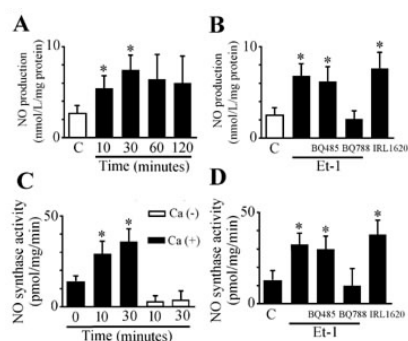


Figure 1: Effects of Et-1 on NO synthesis and NOS activity.

NO Modulates Et-1-Induced *c-fos* Gene

Expression in cardiomyocytes

To explore whether NO modulates the induction of *c-fos* gene in Et-1-treated cardiomyocytes. Cardiomyocytes were preincubated with an NO donor, *ie*, SNAP or SIN-1, for 30 minutes before being treated with Et-1 in the presence of the NO donor. As shown in Figure 2, SNAP treatment of cardiomyocytes at a concentration of 100 $\mu\text{mol/L}$ significantly suppressed Et-1-induced *c-fos* mRNA expression. Similarly, treatment of cardiomyocytes with another NO donor, SIN-1 (100 $\mu\text{mol/L}$), also significantly attenuated Et-1-induced *c-fos* expression. When cardiomyocytes were pretreated with PTIO, an NO scavenger, for 1 hour and then treated with Et-1 in the presence of the agent. In contrast to the inhibitory effect by the NO donor, PTIO treatment of cardiomyocytes at a concentration of 100 $\mu\text{mol/L}$ significantly augmented Et-1-induced *c-fos* mRNA levels (Figure 2). These results suggest that Et-1-induced *c-fos* expression is modulated by NO.

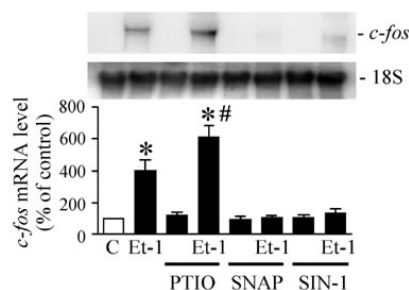


Figure 2: NO Modulates Et-1-Induced *c-fos* mRNA

To further determine whether the modulation of NO in Et-1-induced *c-fos* expression is a transcriptional event, an *c-fos* promoter construct containing the *c-fos* promoter region (-2.25 kb) and the reporter gene chloramphenicol acetyltransferase were transiently transfected into cardiomyocytes. As shown in Figure 3, cardiomyocytes exposed to 24 hours of Et-1 (10 nM) significantly increased *c-fos* promoter activity by 2.4-fold compared with untreated cells. The addition of both SNAP and SIN-1 to cardiomyocytes completely abolished this increased *c-fos* promoter activity. Conversely, treatment of cardiomyocytes with PTIO enhanced this promoter activity. These results together suggest that NO modulation of *c-fos* induction by Et-1 involves transcriptional regulation.

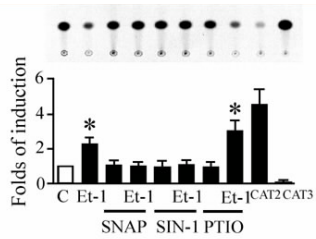


Figure 3: NO Modulates Et-1-Induced *c-fos* promoter activity

NO Regulates Et-1-Induced AP-1 Binding

The Et-1-induced *c-fos* gene expression was concordant with an increase in activator protein-1 (AP-1) activity. Using the electrophoretic mobility shift assay, the transcription factor AP-1 DNA binding activity was stimulated by Et-1 treatment for six hours (Figure 4). The Et-1-stimulated AP-1 binding was also blocked by the NO donor, SNAP or SIN-1, and enhanced by NO scavenger, PTIO, indicating that intracellular NO level is involved in the modulation of Et-1-stimulated AP-1 activity. This binding was obviously specific to AP-1, because it was abolished by coincubation of nuclear proteins with c-Fos and c-Jun antibodies. These results indicate that NO inhibits the AP-1 protein induced by Et-1.

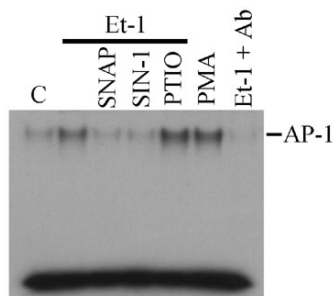


Figure 4: NO Regulates Et-1-Induced AP-1 Binding

Et-1-Induced *c-fos* Gene Expression Is Mediated via the Ras/Raf/ERK Pathway

To dissect the signaling pathway in regulating *c-fos* induction by Et-1, we used the dominant negative mutants of Ras (RasN17) and Raf-1 (Raf301) and a catalytically inactive mutant of ERK2 (mERK), all of which are associated with the Ras/Raf/ERK pathway, to examine the effect of these mutants on the induction of *c-fos* by Et-1. As shown in Figure 5, cardiomyocytes that were cotransfected with the empty vector control PSR revealed no effect on Et-1-induced *c-fos* promoter activity. However, cotransfection of the cells with RasN17, Raf301, or mERK resulted in a significant inhibition in Et-1-induced *c-fos* promoter

activity. Consistently, cardiomyocytes treated with a specific inhibitor to mitogen-activated protein kinase kinase (MEK), ie, PD98059, attenuated Et-1-induced *c-fos* promoter activity. In contrast, cardiomyocytes cotransfected with a dominant positive mutant of Ras (RasL61) or MEK1 greatly increased their *c-fos* promoter activity. These results suggest that the Ras/Raf/ERK signaling pathway is involved in Et-1-induced *c-fos* gene expression in cardiomyocytes. ERKs are the upstream regulators of Et-1-induced *c-fos* gene expression.

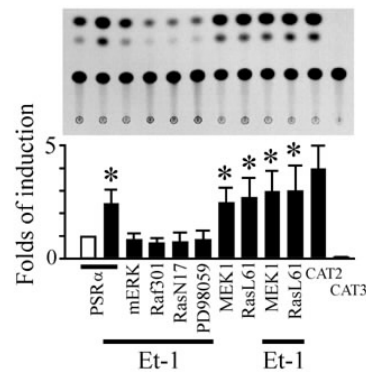


Figure 5: Et-1-Induced *c-fos* Gene Expression Is Mediated via the Ras/Raf/ERK Pathway

NO Regulates Et-1-Induced ERK Phosphorylation and Activity in cardiomyocytes

Given that we showed that the ERK signaling pathway is involved in Et-1-induced *c-fos* expression and that NO modulates Et-1-induced *c-fos* expression, we further investigated whether NO modulates the activation of ERK in Et-1-treated cardiomyocytes. We first examined the ERK1/ERK2 phosphorylation in cardiomyocytes exposed to Et-1 (10 nM) in the presence of the NO donor or NO scavenger. As shown in Figure 6, treatment of Et-1 to cardiomyocytes for 15 minutes induced rapid phosphorylation of ERK1/ERK2 by 4.7-fold. Cardiomyocytes preincubated with an NO donor, SNAP, significantly inhibited Et-1-induced ERK1/ERK2 phosphorylation by 80%. In the same experiment, PTIO treatment of cardiomyocytes augmented Et-1-induced ERK1/ERK2 phosphorylation by 40% above that by Et-1-treatment only (Figure 6).

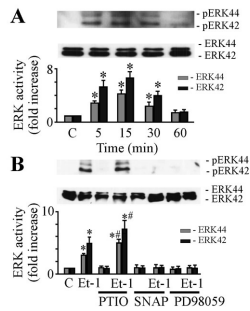


Figure 6: NO Regulates Et-1-Induced ERK Phosphorylation

NO modulation of the ERK signaling pathway was further elucidated by its inhibitory effect on Et-1-induced ERK activity. Cardiomyocytes exposed to Et-1 rapidly induced ERK activity by 3.4-fold, as indicated by an increase of ³²P-labeled phosphorylation of MBP by ERK (Figure 7). Treatment of cardiomyocytes with SNAP significantly attenuated this ERK activation by 90%. In contrast, PTIO treatment of cardiomyocytes enhanced this ERK activation by 50% (Figure 7). Taken together, these findings imply that NO modulates Et-1-induced *c-fos* gene expression *via* its inhibitory effect on the ERK signaling pathway in cardiomyocytes.

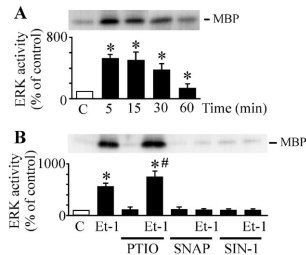


Figure 7: NO Regulates Et-1-Induced ERK Activity

The activation of β myosin heavy chain promoter (β -MHC) by Et-1 is modulated by NO

The hypertrophic responses in cardiac myocytes are characterized by the induction of a fetal gene program (Komuro and Yazaki, 1993). To get insight into the relation between Et-1 and NO, we examined whether NO modulates Et-1-induced expression of cardiac fetal genes. Incubation with Et-1 (10 nM) for 48 h increased β -MHC promoter activities by 2.3-fold as compared with control level (Figure 8). When cardiac myocytes were pre-incubated with a NO donor, DEA-NO (100 μ M) for 60 min, Et-1-induced activation of β -MHC promoter activities was significantly suppressed.

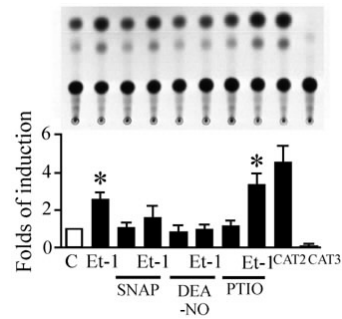


Figure 8: The activation of β myosin heavy chain promoter (β -MHC) by Et-1 is modulated by NO

四、討論

Cardiac myocyte hypertrophy is a central feature of all types of heart muscle failure. Hypertrophic stimuli reach the nucleus via multiple signaling pathways within cardiac myocytes and elicit changes in cardiac gene expression. Et-1 is one of the local factors that play important roles in the development of heart failure. Further elucidation of the precise mechanisms by which this central pathway modulates the hypertrophic response may provide novel therapeutic approaches to human heart failure. Many hypertrophic stimuli, including Et-1 stimulation, have been shown to activate the Ras-mitogen-activated protein kinase pathway, also known as the ERK pathway (Post et al., 1996; Fuller et al., 1998). ERK1/2 are one element in a series of kinases that serve to connect the nucleus with cytosolic events. The present study demonstrated that inhibition of the ERK cascade with PD98059 or mERK blocked the NO modulation of Et-1-induced *c-fos* gene expression. These findings demonstrate that ERK1/2 plays a upstream role of NO modulation of Et-1-induced *c-fos* gene expression signaling of cardiac myocytes.

The cytoplasmic mitogen-activated protein kinase (MAPK) cascade is a process through which extracellular stimuli can be transmitted from the cell surface to the nucleus (Cook et al., 1993). *C-fos* is an immediate-early gene encoding a DNA-binding protein of the AP-1 family, which can function in the transcription activation of many other gene (). MAPK, including ERK1/ERK2 and *c-fos* are all rapidly and transiently activated by Et-1 (Figure 5 and 6; Cheng et al., 1999). We reported here that the Et-1-induced activation of ERK1/ERK2 and the expression of *c-fos* are highly sensitively to the NO pretreatment. By using a MAPK kinase inhibitor, the involvement of MAPK in the NO modulation of Et-1-induced *c-fos* gene expression is also demonstrated.

The immediate proto-oncogene *c-fos* is normally expressed at a minimal level in the mammalian heart. Increased proto-oncogene expression has been implicated in the development of cardiac hypertrophy (Barka et al., 1987). Induction of *c-fos*, a transcriptional factor that interacts with *cis*-regulatory element AP-1 in many genes, has important consequences for downstream gene expression that may determine the phenotypic response to Et-1. The activation of *c-fos* transcription is thought to be mediated by the activation of MAPK (Saklatvala et al., 1993). In cardiomyocytes, Et-1 has been shown to activate two closely related MAPK isoforms, p42MAPK and p44MAPK (Post et al., 1996) *via* a Ras-dependent pathway.

Exogenous NO has been reported to inhibit protein kinase C gene expression and its activity in a variety of cells. Jun et al. (1994) reported that lipopolysaccharide- or phorbol 12-myristate 13-acetate-induced NO or the NO donor sodium nitroprusside inhibited the expression of the protein kinase C delta gene in murine peritoneal macrophages. Studer et al. (1996) reported that the NO donor S-nitroso-N-acetylpenicillamine inhibited basal protein kinase C activity in mesangial cells. The modulation of protein kinase C activity is important for cellular proliferation and protein synthesis. We investigated whether the Et-1 increased expression of the intermediary signaling element mitogen-activated protein kinase, which is downstream of protein kinase C, and the proto-oncogen *c-fos* were modulated by NO treatment. Various growth factors, including Et-1, are known to activate mitogen-activated protein kinase and *c-fos* (Jones et al., 1995; Cheng et al., 1999). We observed that mitogen-activated protein kinase and *c-fos* gene expression were increased in cardiomyocytes with Et-1 stimulation.

The present study implies that NO regulates Et-1-induced *c-fos* expression in cardiomyocytes via the inhibition of the ERK signaling pathway. However, the exact target molecule(s) of this NO modulation has not been defined. NO activates guanylate cyclase to produce cGMP, which affects various targets, including cGMP-dependent protein kinase (Schmidt et al., 1993). The Ras/Raf/ERK signaling pathway is inhibited by the cGMP-dependent protein kinase via the phosphorylation of c-Raf kinase on Ser43 (Suhasini et al., 1998). In the present study, cardiomyocytes treated with KT5823, a cGMP-dependent protein kinase inhibitor,

did attenuate the inhibitory effect of NO on Et-1-induced promoter activity (data not shown). This implies that the inhibitory effect of NO on Et-1-induced *c-fos* induction is mediated via the cGMP-dependent protein kinase.

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附件：封面格式

行政院國家科學委員會補助專題研究計畫成果報告

一氧化氮與內皮素之間於新生鼠心臟細胞的交互作用

計畫類別： 個別型計畫 整合型計畫

計畫編號：NSC 89 - 2316 - B - 002 - 028 -

執行期間：88年08月01日至89年07月31日

計畫主持人：陳 錦 澤

共同主持人：

計畫參與人員：

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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：國立台灣大學醫學院內科

中 華 民 國 89 年 10 月 30 日