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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在類成骨細胞中環氧化酵素的表達受到胰磷酯質酵素 A_2 及 Staurosporine 調控的機轉

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計畫主持人：袁俊傑

計畫參與人員：王秋雅、雷慧菁

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主持人：袁俊傑　國立交通大學生物科技系

一、中文摘要

磷脂酵素 A_2 刺激第二型前列腺素合成酵素的生合成的能力可因 staurosporine 的存在具有加成的效果。我們由西方墨點與北方墨點分析的結果，進一步發現 staurosporine 本身即可刺激第二型前列腺素合成酵素在細胞中大量的生合成。為了解轉錄因子在此一作用的角色，我們逐步移除第二型前列腺素合成酵素的啟動子辨識序列，從而發現-188 到+70 的片段在 staurosporine 刺激第二型前列腺素合成酵素的表現中扮演著重要的角色。這一段序列中含有三個啟動子辨識區，即 AP-2、NF-IL6 與 CRE。進一步經由定位突變後，證實 AP-2 及 NF-IL6 可經 staurosporine 刺激而活化，並進而造成第二型前列腺素合成酵素在老鼠成骨細胞中的表現。以 20nM staurosporine 刺激後，發現含有質體 GLB/NA、GLB/NC 及 GLB/AC 的細胞，其蟲螢光素酵素的活性在八小時後達到最高，而在十小時後則急速降低。此一現象不因激酵素 C 的抑制劑 GF 109203X 的加入而有所改變，證明 staurosporine 激發第二型前列腺素合成酵素之生合成與激酵素 C 的訊息傳遞路徑無關。

關鍵詞：胰磷酯質酵素 A_2、胰磷酯質酵素 A_2 受體、NF-IL6、Staurosporine、離氨酸激酵素、環氧化酵素

Abstract

The induction of cyclooxygenase-2 (COX-2) is known to play a pivotal role in a broad range of physiological and pathological processes. The expression of COX-2 gene is regulated by many extracellular stimuli, including growth factors, cytokines as well as tumor promoters. Recently, we found that staurosporine, a potential anti-tumor drug, could up-regulate the expression of COX-2 gene in mouse osteoblast-like cell line, MC3T3-E1. The ability of staurosporine to induce the expression COX-2 gene was investigated by using luciferase reporters under the control of various COX-2 core promoter regions. Two promoter regions corresponding to AP-2 and NF-IL6, respectively, were identified to be responsible for the staurosporine-mediated COX-2 upregulation. AP-2 and NF-IL6 could be activated by staurosporine in a time- and dose-dependent manner. The luciferase activity can be induced at as low as 5 nM staurosporine and reached maximum at about 20 nM. In contrast, the very selective protein kinase C inhibitor GF109203X did not stimulate the activation of both AP-2 and NF-IL6 as measured in the luciferase reporter assay. Mutagenesis analysis further confirmed that both NF-IL6 and AP2 are involved in the staurosporine-induced COX-2 gene upregulation.

Keywords: PLA2-I, PLA2-I receptor, NF-IL6, Staurosporine, Tyrosine kinase, Cyclooxygenase-2,

二、緣由與目的

Staurosporine, a microbial antifungal alkaloid of *Streptomyces staurospores*, is a potent inhibitor of protein serine/threonine

kinases [1,2]. It has been widely used in exploring the physiological functions of many protein serine/threonine kinases, including protein kinase C (PKC). However, several lines of evidence have suggested that staurosporine could induce cellular responses through mechanisms other than inhibiting protein kinases. In rat fibroblast, for example, the expression of collagenase could be increased by nanomolar range staurosporine after depleting PKC by prolonged treatment with phorbol ester [3]. Staurosporine was also reported to induce the translocation of PKC to membrane and, subsequently, upregulated the expression of urokinase-type (u-PA) plasminogen activator in porcine epithelial cell line [4]. In nitric oxide synthase was shown to be upregulated by staurosporine but not by selective PKC inhibitors, calphostin C and Ro 31-8820 [5]. Furthermore, staurosporine was found to stimulate neurite outgrowth in a variety of cells, including PC12 cells [6], SH-SY5Y neuroblastoma cells [7], and dorsal root ganglia from chick embryo [8]. Furthermore, staurosporine was shown to activate NF-êB in many cell lines, including vascular smooth muscle cells [5], HL60 [9], EL4 [10], and human keratinocytes [11]. The activation of NF-êB resulted in the induction of nitric oxide synthase, and cytokines, i.e., interleukin-2 and -8, respectively.

Recently, staurosporine was found to induce the expression of cyclooxygenase-2 (COX-2, EC 1.14.99.1) [12,13]. Cyclooxygenase is the key enzyme in the pathway of the conversion of arachidonic acid to prostanoids [14-15], which are important cellular local mediators of a variety of biological functions, including proliferation, ovulation, inflammation, muscle contraction, electrolytes re-absorption, and bone metabolism. Two distinct COX isozymes, i.e., COX-1 and COX-2, have been identified and cloned in many species. The COX-1 is generally considered as a constitutive enzyme in a wide variety of tissues and is thought to be involved in the housekeeping functions. The COX-2 gene has been characterized as an early response gene that is strongly induced at sites of inflammation [15-16]. In addition to inflammation, elevated COX-2 expression has been associated with cell growth regulation and carcinogenesis [17-20]. The overexpression of COX-2 was shown to potentiate the tumorigenesis of cells [21]. Therefore, it is postulated that the tumor promoting activity of staurosporine is correlated with its stimulatory effect on the expression of COX-2 [13].

The molecular mechanism underlying staurosporine-induced COX-2 expression is unknown so far. Although the promoter region of the COX-2 gene also contains a NF-êB cis-responding element [22], it does not rule out the involvement of the other transcription factors, such as NF-IL6, AP2, ATF/CRE, and E-box [22-24], in the regulation of COX-2 gene. In this study, we used MC3T3-E1 cell line as a model to investigate the stimulatory effect of staurosporine on the expression of COX-2 gene. MC3T3-E1 is an osteoblast-like cell line established from newborn mouse calvaria. The cells differentiate into osteoblast and show calcification in vitro. The preliminary results showed that COX-2 gene could also be induced by staurosporine in MC3T3-E1 cells. Interestingly this induction is through the activation of transcription factors, AP2 and NF-IL6.

三、結果與討論

Stimulation of cyclooxygenase-2 synthesis in MC3T3-E1 cells by staurosporine – Previous studies have shown that porcine phospholipase A_2-I ($pPLA_2$-I) could induce the increasing of both mRNA and protein levels of COX-2 in MC3T3-E1 cells [20]. In an effort to investigate the signaling pathway involved in $pPLA_2$-I -induced COX-2 gene upregulation, we found that staurosporine could potentiate the stimulatory effect of $pPLA_2$-I. Upon treatment with 50 nM staurosporine, the luciferase reporter vector GLB/18 transfected cells exhibited 10-(without 50 nM $pPLA_2$-I) and 24-fold (with 50 nM $pPLA_2$-I) higher luciferase activity, respectively, compared with that of staurosporine-untreated cells. This result

suggests that staurosporine cannot only potentiate the stimulatory effect of $pPLA_2$-I but also induces the upregulation the expression of COX-2 gene by itself along. The Northern and Western analyses of staurosporine-treated MC3T3-E1 cells further confirmed this observation. As shown in Fig. 2A, the mRNA level of COX-2 gene significantly increased in MC3T3-E1 cells in as early as 30-min post-treatment with $pPLA_2$-I, staurosporine or both reagents. The amount of mRNA, however, was greatly increased in the presence of both 50 nM staurosporine and 50nM $pPLA_2$-I. The similar result was also observed in the protein level of COX-2 before and after treatment. These results clearly show that staurosporine along is sufficient to induce the expression of COX-2 gene in MC3T3-E1 cells. In contrast, staurosporine did not induce COX-1 in MC3T3-E1 cells (data not shown).

Staurosporine but not GF109203X induced the luciferase activity – Although the stimulatory effect of staurosporine on COX-2 gene expression has been demonstrated, the molecular mechanism underlying this process is not clear so far. Since staurosporine was shown to actively regulate the activity of protein kinase C (PKC) in many cell lines, we first hypothesized that PKC might participate in the process of staurosporine-induced COX-2 gene expression. Therefore, a more selective PKC inhibitor GF109203X was used in replace of staurosporine to stimulate GLB/18-W transfected MC3T3-E1 cells. As shown in Fig. 3A, the luciferase activity of GLB/18-W-transfected cells remained low with the treatment of various amounts of GF109203X (0-500 nM). Similarly, GF109203X at 200 nM could not change the stimulatory effect of staurosporine on the GLB/18-W-transfected cells (data not shown). These observations indicate that PKCs are not involved in the process of staurosporine- mediated COX-2 gene upregulation.

The staurosporine upregulates COX-2 in a time- and dose-dependent manner - The result showed that 50 nM staurosporine induces luciferase activity in GLB/18-W-transfected cells in a time-dependent manner. The induction of luciferase activity was first apparent at 4 h after the addition of staurosporine. The activity reached maximum after 8-hour incubation. After that, the stimulatory effect of staurosporine decreased rapidly and the luciferase activity returned to basal level at 24-hour. When incubate with various amounts (5-200 nM) of staurosporine, the GLB/18-W-transfected cells exhibited gradually increasing luciferase activity in the range of 0.5 to 20 nM. At 20 nM staurosporine the luciferase activity is about 6-fold higher than that of control. The stimulatory effect, however, decreased rapidly when the concentration of staurosporine in the medium was 100 nM or higher. These results suggest that staurosporine induces a transient, does-dependent activation on the expression of COX-2 gene.

The analysis of the promoter region of COX-2 gene – In previous studies, we have learned that the 5'-flanking region from –188 bp to +71 bp of COX-2 gene contains cis-responding elements, which may be activated by staurosporine. To rule out the involvement of other transcription factors in this process, we further analyzed the 5'-flanking region between -639 bp and +71 bp of COX-2 gene through series deletion. A variety of putative responding elements, including MEF2, NF-êB, SP1, AP2, NF-IL6, CRE, E-box, and TATA box, have been identified in this region [22-24]. The luciferase reporter vectors containing promoter regions covering various lengths of COX-2 gene were constructed. Each vector was transfected to MC3T3-E1 cells by lipofection, and the luciferase activity of the cell lysate was measure at 6 h after the addition of 20 nM staurosporine. Staurosporine did significantly induce the luciferase activity of cells transfected with GLB/60(-639 to +71 bp), GLB/40 (-478 to +71 bp), GLB/20 (-283 to +71 bp) and GLB/18 (-188 to +71), respectively. The luciferase activity marked decreased when the region between -188 bp and -133 bp was deleted. Interestingly, this region contains two cis-responding elements for NF-IL6 (-138 to -130 bp) and AP2 (-150

to -142 bp). This result is consistent with previous observation and suggests that both transcription factors NF-IL6 and AP2 may be activated by staurosporine.

Mutational analysis of the promoter region of COX-2 gene - Since the region between -188 bp and -133 bp contains responding elements for transcription factors, NF-IL6 and AP2, It will be interested to know which transcription factor may involve in the induction of COX-2 gene by staurosporine. To accomplish this we generated five luciferase reporter vectors containing mutated mouse COX-2 promoter region (-188 to +71 bp) through site-directed mutagenesis. These five reporter vectors were named GLB/18-NA (containing NF-IL6 and AP2 sites), GLB/18-NC (containing NF-IL6 and CRE sites), -AC (containing AP2 and CRE sites), -N (containing NF-IL6 only) and -A (containing AP2 only), respectively. Each vector was transfected into MC3T3-E1 cells and the luciferase activity was measured. The luciferase activity of all four mutants was induced significantly by 20 nM staurosporine to a similar level compared with that of wild type plasmid, GLB/18. These results suggest that both NF-IL6 and AP2 worked in combination in the staurosporine-induced cyclooxygenase-2 gene expression.

四、成果自評

The major goal of this project is to elucidate the molecular mechanism of staurosporine-mediated upregulation of cyclooxugenase-2 expression in MC3T3 cells. Although the whole picture of signaling pathway is still unclear, we reach following conclusion: i) staurosporine induces COX-2 gene expression activation of both AP-2 and NF-IL6, ii) staurosporine induces a dose- and time-dependent stimulatory response to COX-2 gene in MC3T3-E1 cells, and iii) the stimulatory effect of staurosporine on COX-2 gene is not through the inactivation nor activation protein kinase C.

These results are significant because staurosporine has been shown to induce differentiation of rat peochromocuytoma-derived PC-12 cells. The mechanism under lying this staurosporine-induced process is not clear so far. Since NF-IL6 has been shown to involve in the differentiation process in many cases. It is possible that staurosporine-induced PC-12 cell differentiation is also via the activation of NF-IL6. We will consult these results and develop new experiments to elucidate the signal transduction pathway of staurosporine in this event.

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