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Effect of PKC-ζ mediating Ang Ⅱ-stimulated activation of CCDPK on rat cardiac fibroblast proliferation

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KEY WORDS heart; fibroblasts; cultured cells; Ca^{2+}-calmodulin dependent protein kinase; angiotensin Ⅱ; cell division; PD 98059; epidermal growth factor-urogastrone; phosphotransferases; phorbol 12, 13-dibutyrate

ABSTRACT

AIM: To investigate whether the effect of angiotensin (Ang) Ⅱ or epidermal growth factor (EGF) on cardiac fibroblast proliferation involved in activation of extracellular signal-regulated kinase (ERK) 1/2 or Ca^{2+}-calmodulin dependent protein kinase (CCDPK) mediated by protein kinase C (PKC)-ζ. METHODS: Relative activity of CCDPK was measured by Western blotting. DNA synthesis was assayed by [^{3}H] thymidine incorporation. RESULTS: PDBU caused no decrease in Ang Ⅱ- and 10 % FCS-stimulated CCDPK activity and DNA synthesis. In contrary, 65 % or 75 % EGF- or tetradecanoylphorbol acetate (TDPA, formally called PMA)-stimulated CCDPK activity and 38 % or 42 % [^{3}H] thymidine incorporation treated by PDBU were inhibited, respectively. Meanwhile 70 % and 72 % CCDPK activities induced by Ang Ⅱ and EGF were inhibited by PD 98059, respectively. CONCLUSION: PKC-ζ mediated Ang Ⅱ-induced activation of CCDPK and cardiac fibroblast proliferation.

INTRODUCTION

Cardiac interstitium fibrosis and hypertrophic myocytes were the two important pathologic reasons for pressure overload cardiac hypertrophy. However cardiac fibroblasts over-proliferation as well as secretion of large amounts of collagen protein is the main pathologic event. The abnormal proliferation of cardiac fibroblasts and deposition of the extracellular matrix (ECM) protein, collagen, associated with hypertensin and mycardial infarction, may adversely affect the performance of the heart[1] and could result in cardiac fibrosis, cardiac remodification, and further heart failure[2]. Ang Ⅱ has been considered as an important inducement for cardiac fibroblasts proliferation and hypertrophy of cardiac myocytes through autocrine or paracrine system[3]. Ang Ⅱ not only rapidly increases intracellular calcium and activates protein kinase C but also stimulates many of the same signal transduction events as growth factor including protein-tyrosine phosphorylation[4], stimulation of c-*fos*, c-*myc*[5], and Ca^{2+}-calmodulin dependent protein kinase (CCDPK)[6,7]. Meanwhile some of growth factors were often involved in these courses[8]. Therefore, insights into the signal transduction of Ang Ⅱ in cardiac fibroblasts is the most important in elucidating mechanism of cardiac fibrosis.

Recently, several studies have suggested that CCDPK were the most important component of transferring message in cell growth[9]. CCDPK are a family of serine/theronine protein kinase which have two isoforms: p42 and p44 activated as an early response to a variety of stimuli involved in cellular growth, transformation, and differentiation. It could be activated by growth stimuli in quiescent cells requiring phosphorylation of a dual specific protein kinase, MEK, which is itself regulated by MEK kinase and/or Raf kinase, and thereby to activate its substrates-nuclear transcription factors located at downstream signal cascade in nucleus and up-regulate the expression of those factors to perform their effects on biologic regulation on cell growth[10].

PD 98059, $C_{16}H_{13}NO_3$, M_r 267.28, 2-(2′-amino-3′-mehtoxyphenyl)-oxanaphthalen-4-one, an MEK1 inhibitor has been shown to act *in vivo* as a highly selective inhibitor of MEK1 activation and the CCDPK cascade[11], and inhibits differentiation of PC-12 cells[12] and

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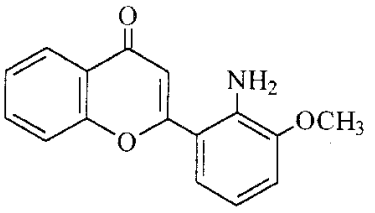
vascular smooth muscle cells proliferation induced by Ang Ⅱ and EGF.



PD 98059 PD 98059

Protein kinase C (PKC)-ζ has been shown to activate MEK kinase and CCDPK *in vitro*[14] and *in vivo*[15]. It represents an atypical PKC isoform which does not bind with Ca^{2+} and can not be activated by diacylglycerol or phorbol 12-myristate 13-acetate[16]. PKC-ζ could mediate Ang Ⅱ but not platelet driver growth factor (PDGF) or tetradecanoylphorbol acetate (TDPA, formally called PMA) activation of CCDPK in vascular smooth muscle cells (VSMC)[17]. But it is not clear in cardiac fibroblast. In the present study, we studied if PKC-ζ mediated Ang Ⅱ-induced activation of CCDPK and cardiac fibroblast proliferation.

MATERIALS AND METHODS

Chemicals [Sar^1]angiotensin Ⅱ, PDBU, TDPA, anti-mitogen activated-protein kinase antibody, and PD 98059 were purchased from New England Biolabs In. Glass-fiber filter were purchased from Whatman Co. Western blot chemiluminescence reagent plus was purchased from NEN^{TM} Life Science Products.

Cell culture[6] Cardiac fibroblasts were isolated from heart ventricles of 1－3 d neonatal rat (Supplied by the Animal Center of Hu-nan Medical University, Grade Ⅱ, Certificate № 20-009) and maintained in M199 supplemented with 10 % fetal calf serum. The fibroblastic nature of cells was determined by immunohistochemistry staining with anti-human factor Ⅷ for endothelial cells (the positive rate 3 %), anti-desmin for muscle cells (the positive rate 2 %), and anti-vimentin for fibroblasts (the positive rate 95 %). Passages 2 to 3 cardiac fibroblasts at 70 %－80 % confluence were growth arrested by incubation in serum-free medium for 24 h prior to use.

Measurement of DNA synthesis[7] Cell proliferation in terms of DNA synthesis was determined by measuring [^{3}H]thymidine incorporation. Cardiac fibroblasts were plated in 24-well plates at 7.5×10^8 cells/m^2. After incubation in serum-free medium for 24 h, cells were stimulated with agonists for 18 h, labelled with [^{3}H] thymidine 74 MBq/well for 6 h, were washed with cold PBS(－), trypsinized, resuspended in 10 % trichloroacetic acid (TCA), and vortexed vigorously to lyse the cells. The cell lysate was vacuum-filtered through a glass-fiber filter. After washing with cold 10 % TCA followed by 95 % ethanol, the filter was dried. The radioactivity of incorporated [^{3}H] thymidine was measured in a liquid scintillation counter (Beckman). Independent experiments were performed 5 times.

Preparation of cell lysates[18] To investigate effect of PDBU on cardiac fibroblast, the cultured neonatal rat cardiac fibroblasts were pretreatment with PDBU (1 μmol·L^{-1}, 24 h), cells were then stimulated by Ang Ⅱ (10 μmol·L^{-1}, 5 min), EGF (10 μmol·L^{-1}, 10 min), 10 % FCS (10 min) and TDPA (200 μmol·L^{-1}, 5 min), respectively, and meanwhile to investigate if the activation of CCDPK stimulated by Ang Ⅱ or EGF is mediated by MEK in cardiac fibroblasts, the cells were pretreated with a specific inhibitor of MEK：PD 98059 (30 μmol·L^{-1}, 30 min), and then were stimulated by Ang Ⅱ and EGF, respectively. After the treatment above, the cells were washed with PBS(－), and were lysed in 0.1 mL of lysis buffer containing (mmol·L^{-1})：NaCl 50, NaF 50, sodium pyrophosphate 50, egtazic acid 5, edetic acid 5, Na_3VO_4 2, phenylmethylsulfonyl fluoride 0.5, and HEPES 10 at pH 7.4, along with 0.1 % Triton X-100 and leupeptin 10 mg·L^{-1}. Cell lysates were frozen on ice, scrapped, and sonicated, and centrifuged at 18 000×*g* for 15 min (4 ℃), protein concentration were estimated by the Bradford method[19].

Western blot analysis[20] The cell lysates containing 10 μg extracted protein were added in loading buffer (tris/HCl 0.33 mol, SDS 10 % [wt/vol], glycerol 40 % [vol/vol], dithiothreitol 20 % [vol/vol], bromophenol blue 0.1 % [wt/vol]) and were subjected to SDS-PAGE in a 10 % SDS gel, and the protein were then transferred to PVDF membrane, which was then blocked for 1 h with 5 % BSA in PBST (Na_2HPO_4 80 mmol·L^{-1}, NaH_2PO_4 20 mmol·L^{-1}, NaCl 100 mmol·L^{-1} containing 0.05 % Tween-20). The blots were incubated for 1 h at 25 ℃ with the primary monoclonal antibodies of ERK1/2 at a 1：1000 dilution, followed by incubation for 1 h with secondary antibody (horseradish peroxidase conju-

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gated) at a 1∶10 000 dilution. Immunoreactive bands of p42 and p44 ERK1/2 were visualized by using enhanced chemiluminescence reagents. Quantification of p42 and p44 ERK1/2 activity was made by laser scanning densitometry (Pharmacia LKB) of autoradiographs.

Statistical analysis Data were presented as $\bar{x} \pm s$ for all experiments repeated at least 4 times. Significant difference was determined by t test.

RESULTS

Effect of PD 98059 on Ang Ⅱ-activated CCDPK in cardiac fibroblasts The results showed that 70 % and 72 % CCDPK activities induced by Ang Ⅱ (10 nmol·L^{-1}, 5 min) and EGF (10 nmol·L^{-1}, 10 min), respectively, were inhibited by PD 98059 (Fig 1, Tab 1). This suggested that both Ang Ⅱ and EGF activated MEK.

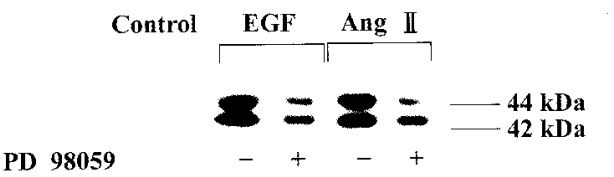

Fig 1. Western blotting of PD 98059 on CCDPK activity induced by Ang Ⅱ and EGF in cardiac fibroblasts.

Tab 1. Effect of PD 98059 on CCDPK activity in rat cardiac fibroblast induced by Ang Ⅱ and EGF. $n = 5$ independent experiments, $\bar{x} \pm s$. $^{c}P < 0.01$ *vs* Ang Ⅱ group. $^{f}P < 0.01$ *vs* EGF group.

Treatment	Relative activity (AU/mm²)
Control	**0**
Ang Ⅱ	2.62 ± 0.31
Ang Ⅱ + PD 98059	0.78 ± 0.07[c]
EGF	2.68 ± 0.28
EGF + PD 98059	0.76 ± 0.10[f]

Effect of PDBU treatment on CCDPK activation and [³H] thymidine incorporation in cardiac fibroblast The results showed that PDBU caused no significant decrease in Ang Ⅱ- and 10 % FCS-stimulated CCDPK activity, in contrary, inhibited EGF and TDPA-stimulated CCDPK activity (decreased by 65 % and 75 %, respectively) (Fig 2 and Tab 2).

PDBU did not inhibit [³H]-thymidine incorporation in cardiac fibroblasts induced by Ang Ⅱ or 10 % FCS (Tab 3). However, EGF and TDPA-induced [³H]-thymidne incorporation were suppressed significantly by pretreatment with PDBU (decreased by 38 % and 42 %, respectively).

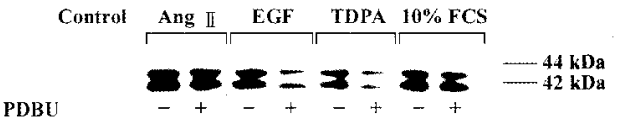

Fig 2. Western blotting of PDBU on CCDPK activity induced by Ang Ⅱ, EGF, TDPA, and FCS.

Tab 2. Effect of PDBU on CCDPK activity in rat cardiac fibroblast induced by Ang Ⅱ, EGF, TDPA, and 10 % FCS. ($n = 5$ independent experiments, $\bar{x} \pm s$). $^{a}P > 0.05$ *vs* Ang Ⅱ group. $^{f}P < 0.01$ *vs* EGF group. $^{i}P < 0.01$ *vs* TDPA group. $^{j}P > 0.05$ *vs* FCS group.

Treatment	Relative activity (AU/mm²)
Control	**0**
Ang Ⅱ	2.72 ± 0.18
Ang Ⅱ + PDBU	2.73 ± 0.29[a]
EGF	2.49 ± 0.15
EGF + PDBU	0.87 ± 0.13[f]
TDPA	1.97 ± 0.20
TDPA + PDBU	0.49 ± 0.10[i]
FCS	2.72 ± 0.39
FCS + PDBU	2.45 ± 0.24[j]

Tab 3. Effect of PDBU treatment on [³H] thymidine incorporation in rat cardiac fibroblast induced by Ang Ⅱ, EGF, TDPA and 10 % FCS. ($n = 5$ independent experiments, $\bar{x} \pm s$) $^{a}P > 0.05$ *vs* Ang Ⅱ group. $^{f}P < 0.01$ *vs* EGF group. $^{i}P < 0.01$ *vs* TDPA group, $^{j}P > 0.05$ *vs* FCS group.

Treatment	[³H] Thymidine incorporation (Bq/well)
Control	**443 ± 19**
Ang Ⅱ	573 ± 65
Ang Ⅱ + PDBU	526 ± 53[a]
EGF	609 ± 35
EGF + PDBU	379 ± 42[f]
TDPA	683 ± 18
TDPA + PDBU	399 ± 38[i]
FCS	608 ± 48
FCS + PDBU	653 ± 65[j]

DISCUSSION

CCDPK could integrate messages transferred from several systems of response such as receptor of tyrosine kinase, receptor of coupling with G protein, and play an important role in the end common pathway or confluence of transduction of several growth messengers[21]. Stimulation of CCDPK requires phosphorylation of a dual specific protein kinase, MAP kinase kinase or MEK, which is itself regulated by MEK kinase and/or Raf kinase. PD 98059, a specific inhibitor of MEK, could suppressed CCDPK activity induced by Ang Ⅱ or EGF in cultured cardiac fibroblasts in our studies. This suggested that MEK mediated AngII or EGF-induced activation of CCDPK.

The present studies showed that TDPA, a special agonist of PKC-induced activation of CCDPK and cardiac fibroblast proliferation was inhibited significantly by PDBU. This suggested that the inhibition of PDBU on CCDPK activity and cell proliferation be resulted from down-regulation of PKC. Although PKC could be activated by Ang Ⅱ, PDBU down-regulated PKC did not inhibit Ang Ⅱ-stimulated activation of CCDPK, it is contrary to EGF in cultured cardiac fibroblasts. PKC has been suggested to be both "upstream" and "downstream" of CCDPK in signal transduction cascades[22,23]. PDBU could deplete PKC-α, β, δ, ε, isoforms but not ζ and PKC-ζ mediates Ang Ⅱ activation CCDPK in VSMC[17]. Therefore it suggested that G-protein-coupled AT_1 receptors mediated Ang Ⅱ activation of CCDPK in cardiac fibroblast is dependent on PKC-ζ, however, protein-tyrosine-kinase mediated EGF activation of CCDPK is independent on PKC-ζ, but not excluded other PKC isoforms involving in EGF-stimulated activation of CCDPK. DNA synthesis induced by EGF was declined correspondingly, but not for Ang Ⅱ. This indicated that the proliferation of cardiac fibroblast, the common terminal biologic effect induced by Ang Ⅱ or EGF was mediated by CCDPK through different isoforms of PKC. FCS-induced CCDPK activity and cell proliferation could not be inhibited by PDBU, this may be the reason for that the content of FCS is more complex and exists a certain unknown factor to be able to activate PKC-ζ.

These results suggest that PKC-ζ mediated Ang Ⅱ-induced activation of CCDPK and cardiac fibroblast proliferation, but EGF-induced activation of CCDPK and cardiac fibroblast proliferation was mediated by other isoforms of PKC.

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蛋白激酶 C-ζ 介导血管紧张素Ⅱ激活的 CCDPK 对大鼠心肌成纤维细胞增殖的影响

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关键词 心脏；成纤维细胞；培养的细胞；Ca^{2+}-钙调节蛋白依赖性蛋白激酶；血管紧张素Ⅱ；细胞分裂；PD 98059；表皮生长因子-尿抑胃素；磷酸转移酶类；佛波醇 12，13-二丁酸盐

目的：探讨血管紧张素Ⅱ（Ang Ⅱ）和表皮生长因子（EGF）是否经不同的蛋白激酶 C（PKC）亚基介导而激活 CCDPK 产生促培养新生大鼠心肌成纤维细胞增殖. 方法：Western Bloting 和 [^{3}H] thymidine 参入法. 结果：PDBU 对 Ang Ⅱ 诱导的 CCDPK 活性和 DNA 合成速率无显著影响. 而 EGF 诱导的 CCDPK 活性下降了65 %，DNA 合成速率下降了38 %. PD 98059 对 Ang Ⅱ 和 EGF 诱导的 CCDPK 活性分别下降了70 % 和72 %. 结论：PKC-ζ 介导了 Ang Ⅱ 对 CCDPK 的激活及心肌成纤维细胞的增殖效应.

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