

Letter to the Editor



Relaxin Inhibit Cardiac Fibrosis Induced by Phorbol 12-myristate 13-acetate *

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Relaxin is known to inhibit cardiac fibrosis. However, it is unclear whether relaxin could regulate the effects of Phorbol 12-myristate 13-acetate (PMA, PKC activator) on cardiac fibrosis. So the influence of relaxin on the cell proliferation and collagen expression induced by PMA in cultured cardiac fibroblasts was studied. It showed that PMA significantly increased cardiac fibroblasts proliferation, Type I pro-collagen protein expression, Type I pro-collagen mRNA expression, and rhRLX absolutely significantly decreased PMA induced effects on cardiac fibroblasts proliferation and Type I pro-collagen expressions, indicating that relaxin could inhibit cardiac fibrosis induced by PMA.

The insulin-related peptide relaxin (RLX) has long been regarded as a central hormone of human pregnancy that contributes to protect cardiac structure and function. The leucine-rich G protein-coupled receptors 7 and 8 (LGR7 and LGR8) were identified as relaxin receptors. It is important that relaxin has been shown to inhibited or reversed heart fibrosis *in vitro* and *vivo*. In cardiac fibroblasts, relaxin inhibits transforming growth factor beta (TGF- β), AngII, and high glucose-induced cell proliferation and collagen production and promotes matrix degradation^[1-2]. Relaxin has also inhibited or reversed fibrosis *in vivo* animal model, such as spontaneously hypertensive rat model, transgenic animals overexpressing β 2 adrenergic receptors and the streptozotocin (STZ)-treated mRen-2 rat model of diabetes^[3-4]. Male relaxin-deficient mice demonstrate an age-related progression of cardiac fibrosis^[5]. Relaxin suppresses atrial fibrillation by reversing fibrosis in spontaneously hypertensive rat hearts^[6]. These findings demonstrate that relaxin is a potent antifibrotic agent, that has important therapeutic potential for the treatment of cardiac disorders associated with fibrosis. Recently, clinical

study showed treatment of acute heart failure with serelaxin was associated with dyspnoea relief and 180-day mortality reduced^[7]. However, the mechanisms underlying the beneficial effects of relaxin have not been fully elucidated.

Protein kinase C isozymes (PKCs), a family of serine-threonine kinases, involved in the differentiation and proliferation of a variety of cells and in a variety of intracellular signal transduction and regulation. The PKC family of isozymes is divided into three major groups: the conventional PKC isozymes (c-PKC, α , β I, β II), the novel PKC isozymes (n-PKC, δ , ϵ , η , and θ), and the atypical PKC members (ζ and λ PKC). Phorbol 12-myristate 13-acetate (PMA) is a activator of c-PKC and n-PKC. PKC may be a common pathway of cardiac fibrosis. *In vitro*, protein kinase C isozymes have effects on cardiac fibroblast proliferation. *In vivo* animal model, it is confirmed that PKC is involved in the process of myocardial fibrosis. Short-term PMA treatment activate PKC; However, long-term PMA treatment of neonatal cardiac fibroblasts resulted in downregulation of PKC isoenzymes because PKC is excessive consumed.

Relaxin mediates cardiac myofilament function through a PKC-dependent pathway^[8], which demonstrate the effects of relaxin on cardiac function may be associated with PKC. cPKC and nPKC in the PKC family have close relationship with cardiac fibrosis. PMA is the activator of cPKC and nPKC, so PMA was selected as PKC activator in the study. In this study, Cardiac fibroblasts were subjected to short-term PMA treatment (30 min) in the absence or presence of rhRLX over 72 h, cell proliferation and collagen synthesis were investigated. Moreover, relaxin and LGR7 mRNA expression regulated by PMA were studied.

Using cultured cardiac fibroblasts of neonatal Sprague-Dawley rats as a model, groups were

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divided into: control group; PMA group (PMA, 0.4 µg/mL, 0.2 µg/mL, 0.1 µg/mL); PMA add RLX group (recombinant human relaxin, 100 ng/mL). The cell proliferation was measured by MTT and the expression of pro-collagen I by western blot and real time RCR. Samuel et al. and we previously demonstrated that relaxin alone had no marked effect on proliferation and activation of cardiac fibroblasts^[1-2], so there is no control for the effects of rhRLX in the absence of PMA treatment in this study. The data were expressed as the standard error of the mean, and the differences in multiple groups were compared with the ANOVA method. The inter-group difference was tested by SNK, and $P < 0.05$ was considered statistically significant.

Treatment with PMA (0.4 µg/mL, 0.2 µg/mL, 0.1 µg/mL) significantly increased cells proliferation. The cardiac fibroblasts proliferation in the PMA 0.4 µg/mL group was not statistically higher than the PMA 0.2 µg/mL group (Figure 1A). PMA treatments significantly dose-dependent increase in Type I procollagen mRNA expression, with maximal effectiveness at approximately 0.4 µg/mL (Figure 1B), PMA (0.4 µg/mL) increased Type I procollagen protein expression by 15% ($P < 0.05$; Figure 1C). This study has demonstrated that PMA (cPKC and nPKC activator) significantly promoted cardiac fibrosis. Regardless of the dose of PMA, rhRLX (100 ng/mL) significantly inhibited the PMA ($P < 0.01$; Figure 1A) mediated effects on cardiac fibroblasts proliferation. RhRLX treatment normalized the cardiac fibroblasts proliferation which stimulated by PMA ($P > 0.05$, versus control). The ability of rhRLX to modulate pro-collagen I mRNA level was investigated in the presence of PMA, which stimulated procollagen I mRNA expression in a dose-dependent manner. rhRLX (100 ng/mL) marked decreased the PMA- (by 50%-60%, $P < 0.01$) induced effects on pro-collagen I mRNA expression over a 72-h period (Figure 1B). After treated with PMA (0.4 µg/mL) for 30 min, cardiac fibroblasts were subjected in the absence or presence of rhRLX over 72 h. rhRLX significantly inhibited pro-collagen I protein expression stimulated with PMA (Figure 1C). Previous studies have demonstrated that rhRLX reverses cardiac fibrosis and have no effect upon normal heart *in vitro* and *vivo*, including our study^[1-2]. The present study further confirmed that relaxin might form a class of anti-fibrosis agent in pathological condition, including heart disease.

As shown in Figure 2, using real-time RT-PCR, PMA significant increased in relaxin and relaxin

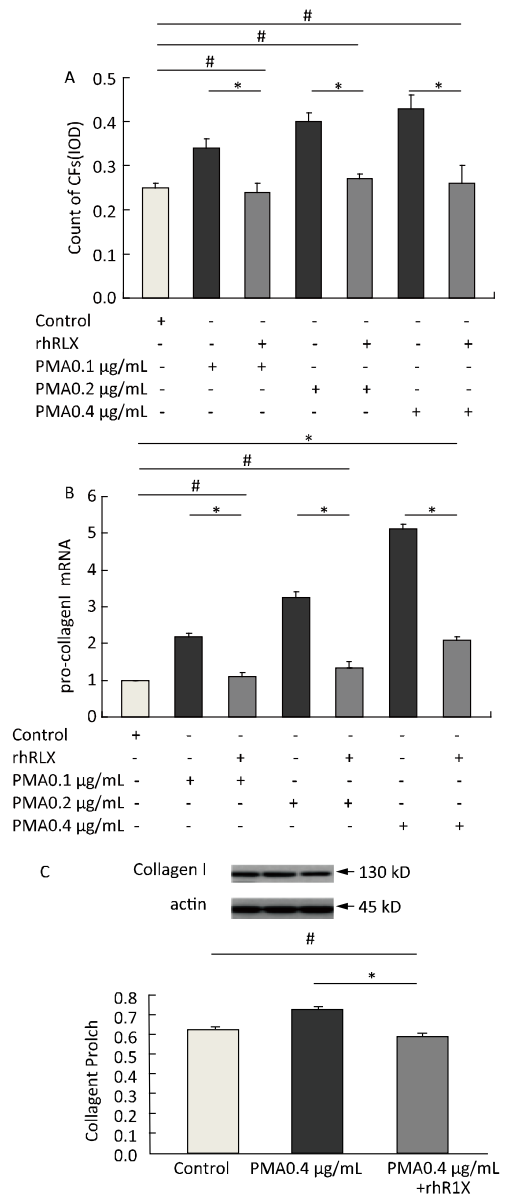


Figure 1. A, Effect of PMA and relaxin on cardiac fibroblasts proliferation. Neonatal cardiac fibroblasts were incubated with PMA of different dose for 30 min. The cellular proliferation was measured with MTT method. The results were shown in Figure 1A expressed as means±SE (n=8). $P < 0.05$, # $P > 0.05$. B, Effect of PMA and relaxin on Type I procollagen mRNA expression. Type I procollagen mRNA expression of cultured CFs was measured with real time PCR. The results were shown in Figure 1B expressed as means±SE (n=3). $P < 0.05$. C, Effect of PMA and relaxin on Type I collagen protein expression. Type I collagen protein expression was measured with Western blot. The results were shown in Figure 1C expressed as means±SE (n=3). $P < 0.05$.

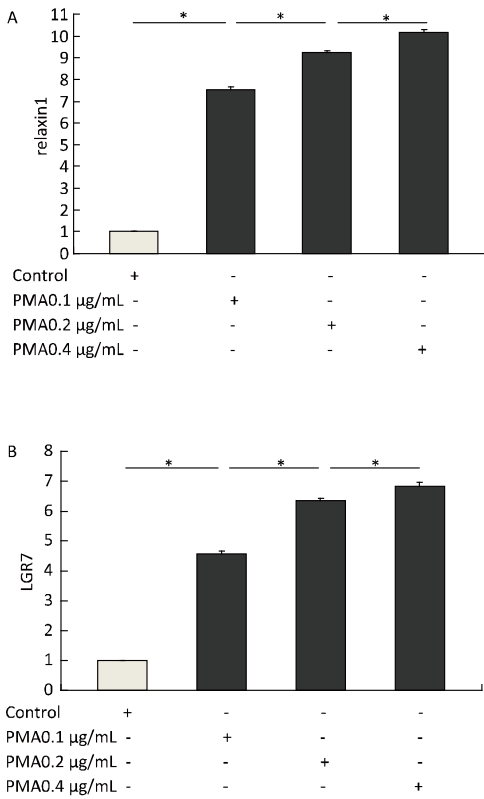


Figure 2. A, Effect of PMA on relaxin1 mRNA expression. The results were shown in Figure 2A expressed as means±SE (n=3). *P<0.05. B, Effect of PMA on LGR7 mRNA expression. The results were shown in Figure 2B expressed as means±SE (n=3). *P<0.05.

receptor (LGR7) mRNA expressions, when cardiac fibroblasts were treated for 30 min. A lower dose of PMA resulted in a marked stimulation of relaxin and LGR7 mRNA expressions. The higher dose of PMA, the stronger stimulation in expression of relaxin and LGR7 mRNA. Failing heart is an organ producing relaxin^[9]. PMA regulate relaxin secret by porcine luteal cells^[10]. In the study, Treatment with PMA upregulated the mRNA expression of relaxin and LGR7 in cardiac fibroblasts, which demonstrated that neonatal cardiac fibroblasts expressed relaxin and its receptor genes; In pathological conditions, relaxin and its receptor expressions may change. Although relaxin and its receptor regulated, cardiac fibroblast proliferation and collagen expression induced PMA was still increased. It was indicated that endogenous relaxin and its receptor upregulation induced by PMA did not play anti-heart fibrosis action.

PMA increased cardiac fibroblast proliferation and Type I procollagen gene and protein expressions,

rhRLX fully inhibited cardiac fibroblast proliferation and partially inhibited Type I collagen expression induced by PMA (a cPKC and nPKC activator). It was possible that cPKC and nPKC participated in the process of cardiac fibrosis and relaxin antagonized cardiac fibrosis associated with cPKC and nPKC. PKC isoforms may play different roles in the development of cardiac fibrosis, even if the same PKC isoform play different role in different pathophysiological state. In order to determine the role of PKC pathway by which relaxin inhibit cardiac fibrosis, future work is required to determine (1) the effects of relaxin on cardiac fibrosis induced by PKC isozyme selective activator, (2) the relationship of endogenous relaxin expression and secretion and PKC isoforms activation in pathological condition of heart.

In conclusion, this study indicated relaxin could antagonize cardiac fibrosis induced by PMA (PKC activator); PMA stimulated endogenous relaxin and its receptor upregulation, although this increase can not suppress cardiac fibrosis.

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