ISSN 1671-4083
Shanghai Institute of Materia Medica
Chinese Academy of Sciences
http://www.ChinaPhar.com

Effects of *dl*-praeruptorin A on interleukin-6 level and Fas, bax, bcl-2 protein expression in ischemia-reperfusion myocardium[1]

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KEY WORDS *dl*-praeruptorin A; myocardium; ischemia; reperfusion injury; apoptosis; interleukin-6

ABSTRACT

AIM: To investigate the effects of *dl*-praeruptorin (Pd-Ia) on interleukin-6 (IL-6) level and apoptosis-related protein expression in ischemia-reperfusion myocardium. **METHODS:** Left anterior descending coronary artery was subjected to 30 min ischemia followed by 120 min reperfusion in open-chest anesthetized rats. Serum IL-6 level was measured by radioimmunoassay. Apoptosis-related protein Fas, bax, and bcl-2 expression was detected by immunohistochemistry and computer image analysis system. Infiltration of neutrophils was observed using Hematoxylin-Eosin staining under optical microscope. **RESULTS:** Pd-Ia 2.0 mg·kg^{-1} iv lowered serum IL-6 level and Fas, bax, bcl-2 expression under conditions with hypotension and without changes on heart rate, but increased the ratio of bcl-2/bax. There existed a close linearity and positive correlation between IL-6 level and Fas, bax, bcl-2 expression. Whereas, the infiltration of neutrophils was mild. **CONCLUSION:** Pd-Ia elicits a novel target in the therapeutic prevention of postischemic cardiomyocyte death. The reason might be associated with modulating the expression of some immediate-early genes including IL-6, *Fas*, *bax*, and *bcl*-2 in ischemia-reperfusion myocardium.

INTRODUCTION

A number of studies have shown that ischemia-reperfusion induce inflammatory cytokine gene expression such as tumor necrosis factor-α (TNF-α)[1,2], interleukin (IL)-β, IL-6, IL-8, interferon-γ[3,4], and intercellular adhesion molecule-1[5] in myocardium. These overexpressed myocardial cytokines may play a critical role in the progress of myocardial dysfunction, including ischemia-reperfusion injury (MIR), vascular wall remodeling[6], heart failure[7], and cardiac hypertrophy[8]. Recent evidence suggested that locally produced TNF could also contribute to postischemic myocardial dysfunction *via* direct depression of

[1] Project partly supported by the Key Foundation of the Ministry of Public Health of China, № 88402257.
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Received 2001-12-04 Accepted 2002-05-30

contractility and induction of cardiomyocyte apoptosis[9]. However, the molecular mechanism of controlling and regulating the expression of these immediate-early genes during MIR has remained unclear. As cardiomyocyte apoptosis is one of the major contributors to the pathogenesis, accordingly, prevention of it may be a reasonable therapeutic strategy.

In our previous studies, a pyranocoumarin component *dl*-praeruptorin (Pd-Ia) isolated from Baihua Qianhu, a traditional Chinese medicine (*Peucedanum praeruptorum* Dunn), as a Ca^{2+}-influx blocker[10-12] and K^+-channel opener[13], displays myocardial protection[14-16]. In the present study, we mainly investigated the effects of Pd-Ia on serum IL-6 level and Fas, bax, bcl-2 protein expression in ischemia-reperfusion myocardium of rats, in order to further explore the activity and mechanism of Pd-Ia.

MATERIALS AND METHODS

Drugs and reagents Pd-Ia was presented friendly by Prof OKUYAMA T (Department of Pharmacognosy and Phytochemistry, Meiji College of Pharmacy, Tokyo 154, Japan); ^{125}I-interleukin-6 radioimmunization kit was produced by 301 Radioimmunization Research Center, Beijing, China; Rabbit anti-rat monoclonal antibodies (including Fas, bax, and bcl-2) were prepared by Santa Cruz Biological Technology Co; Instant-type SABC (Strept-Avidin-Biotin-enzyme Complex) immunohistochemistry kit was provided by Wuhan Boster Biological Technology Co.

Experimental protocol Forty-seven healthy male Wistar rats of both sexes, weighing 274 g±16 g were randomly divided into 7 subgroups (n=6-7). Group I, sham operation control; Group II, operation control; Group III, solvent control (PEG400); Group IV, positive control (nifedipine); Group V, Pd-Ia 0.5 mg·kg^{-1}; Group VI, Pd-Ia 1.0 mg·kg^{-1}; Group VII, Pd-Ia 2.0 mg·kg^{-1}.

Under urethane-chloralose anaesthesis, open-chest, and artificial ventilation, left anterior descending coronary artery of rat was dissected. A 5-0 silk suture was passed around the vessel and an occlusive snare was placed around it. Thirty minutes ischemia followed by 120 min reperfusion was produced as reported previously[14]. Mean carotid arterial pressure (BP) and heart rate (HR) were measured using 8-channel recorder (RM-6000, Nihon Kohden).

A bolus of Pd-Ia was injected iv while onset of reperfusion. Blood sample was drawn out from right ventricular chamber at 120 min after reperfusion. Serum IL-6 level was determined by radioimmunoassay. Partial myocardium from area subjected to MIR was scissored snap-frozen, and sliced by freezing microtome. Fas, bax, and bcl-2 expression was surveyed by immunohistochemistry and computer image analysis system[17]. Chromogenic reaction was performed with 3',3-diaminobenzidine 0.5 g/L (25 ℃±1 ℃, 10 min), counterstained in hematoxylin and observed under light microscopy. Brown staining in epicyte or cytoplasm was evaluated as positive expression, which was examined by calculating the ratio of mean optic density and area in positive stain, namely, positive expressive index (PEI).

Infiltration of neutrophil was examined by Hematoxylin-Eosin staining under optical microscope.

Statistical analysis All data was expressed as mean±SD. Unpaired student's t-test was used to assess statistical significance of differences between Pd-Ia and various related control groups. Comparison of parameters before and after administration of drugs was performed using one-way ANOVA analysis of variance. P<0.05 was considered to be significant.

RESULTS

Effects of Pd-Ia on serum IL-6 level Pd-Ia decreased serum IL-6 level at higher dose of 2.0 mg/kg markedly [(116±11) ng/L *vs* (153±15) ng/L in solvent control, P<0.05]. This decrease was similar to that of nifedipine. IL-6 level in operation control was higher than that of sham control, suggesting that MIR induced the overexpression of IL-6, but no significant change was found in solvent control (Tab 1).

Effects of Pd-Ia on Fas, bax, and bcl-2 expression Pd-Ia inhibited Fas, bax, and bcl-2 expression (Fig 1). At the dose of Pd-Ia 2.0 mg·kg^{-1}, PEI of Fas, bas, and bcl-2 were decreased significantly (3.4 %±

Tab 1. Effects of Pd-Ia on serum IL-6 level and Fas, bax, bcl-2 expression in ischemia-reperfusion myocardium of rats. PEI: positive expressive index; Sham: sham control; Oper: operation control; Sol: solvent control; Nif: nifedipine control. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* solvent group. $^{f}P<0.01$ *vs* sham group.

Group	Dose/mg·kg^{-1}	*n*	IL-6/ng·L^{-1}	PEI/% Fas	PEI/% bax	PEI/% bcl-2	bcl-2/bax
Sham	-	7	122± 6	1.9±0.5^{c}	1.4±0.3^{c}	1.8±0.5^{c}	1.35±0.11^{c}
Oper	-	6	152±18	7.0±2.4^{f}	5.9±1.4^{f}	5.7±1.0^{f}	0.96±0.11^{f}
Sol	-	7	153±15	6.7±1.5^{f}	6.0±1.0^{f}	5.4±0.9^{f}	0.92±0.09^{f}
Nif	5.0	6	124±19^{b}	3.6±0.6^{c}	1.8±0.4^{c}	3.6±0.6^{b}	2.05±0.22^{c}
Pd-Ia	0.5	7	129±18	3.8±0.7^{b}	3.5±0.4^{c}	4.2±1.0	1.16±0.17
	1.0	7	120±16^{b}	3.5±0.7^{c}	2.8±0.3^{c}	3.4±0.9^{c}	1.28±0.23^{c}
	2.0	7	116±11^{b}	3.4±0.9^{c}	2.1±0.4^{c}	2.9±0.7^{c}	1.42±0.06^{c}

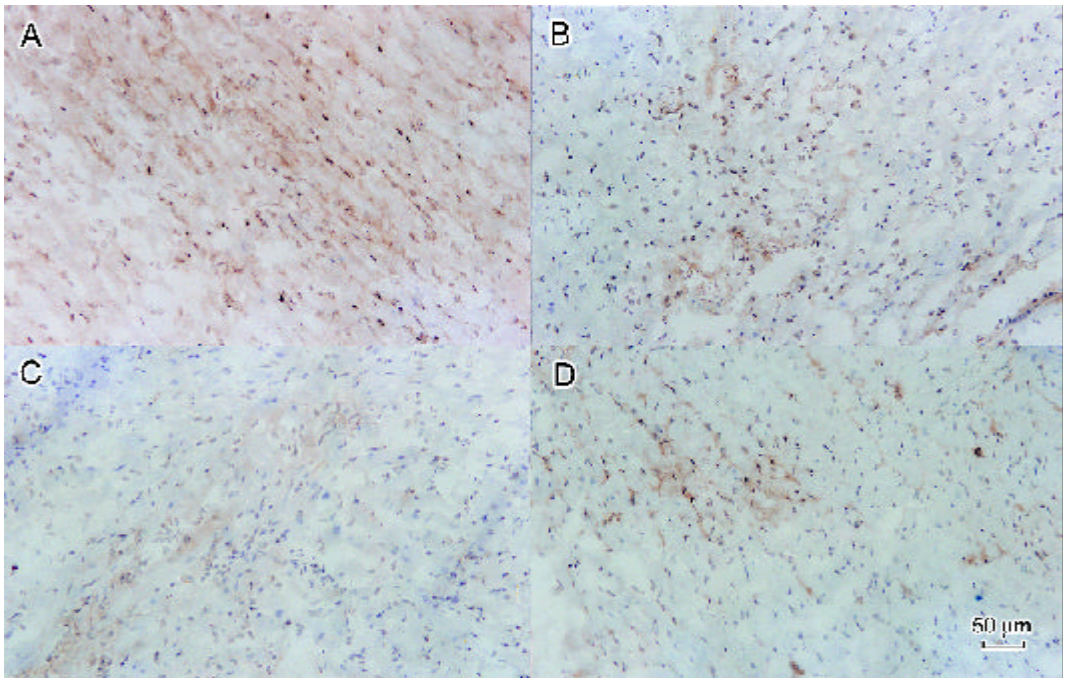

Fig 1. Effects of Pd-Ia on Fas (b), bax (c), and bcl-2 (d) protein expression in left ventricular myocardium subjected to 30 min ischemia followed by 120 min reperfusion in open-chest anesthetized rats treated by a bolus injection intravenously of solvent (PEG 400) solution 1 mL/kg (a) or *dl*-praeruptorin A 2.0 mg/kg (b, c, and d) immediatedly before reperfusion. Epicyte or cytoplasma brown staining indicates positive expression. ×200.

0.9 %, 2.1 %±0.4 %, and 2.9 %±0.7 % *vs* 6.7 %±1.5 %, 6.0 %±1.0 %, and 5.4 %±0.9 % in solvent control, $P<0.01$), in contrast, it increased the ratio of bcl-2/bax (1.42±0.06 *vs* 0.92±0.09 in solvent control, $P<0.01$). The attenuation was a little similar but weak to that of nifedipine. Fas, bax, bcl-2 expression in operation control was markedly higher than sham control ($P<0.01$), whereas the ratio of bcl-2/bax was lower ($P<0.05$, Tab 1), indicating that MIR assuredly lead to apoptosis-related protein overexpression.

Correlation analysis between serum IL-6 level and Fas, bax, and bcl-2 expression There existed a

close linearity and positive correlation between serum IL-6 level and Fas, bax, bcl-2 expression (Fig 2). The correlation coefficients (*r*) were 0.8324, 0.7429, and 0.7423 ($P<0.01$), respectively.

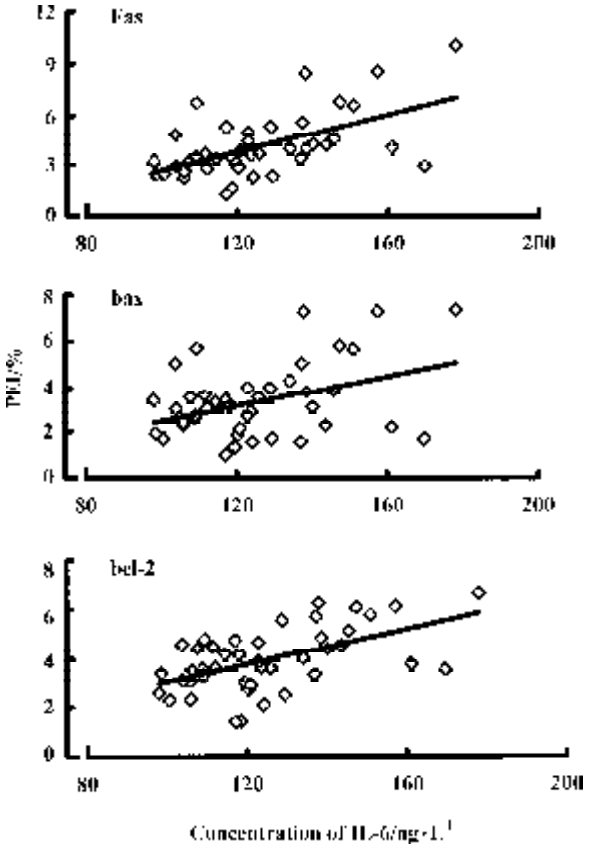

Fig 2. Correlativity analysis between serum IL-6 level and Fas, bax, bcl-2 protein expression in ischemia-reperfusion myocardium of rats treated iv with Pd-Ia 2.0 mg/kg. PEI: positive expression index.

Effects of Pd-Ia on infiltration of inflammatory cells The infiltration of neutrophils in Pd-Ia group displayed mild, and was not seen in sham control, whereas most appeared in operation and solvent controls (Fig 3).

Effects of Pd-Ia on BP and HR Pd-Ia decreased BP significantly, and the duration sustained over 60 min (Tab 2). The BP at maximal dose reached from 12.6 kPa±0.7 kPa to 9.4 kPa±1.2 kPa at 30 min after administration (25 %±6 % decreased, $P<0.01$ *vs* solvent group), but the potency was weaker than that of nifedipine (41 %±11 % decreased, $P<0.05$ *vs* nifedipine group). Furthermore, Pd-Ia had no influence on HR.

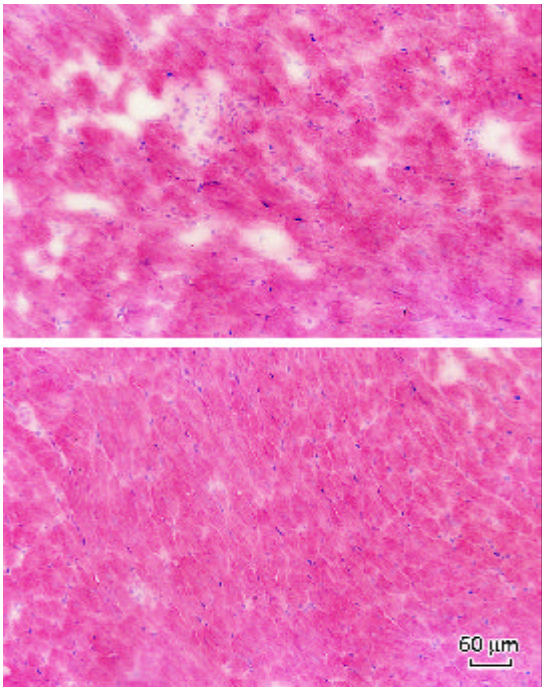

Fig 3. Micrograph demonstrating inflammatory cell infiltration using Hematoxylin-Eosin staining in left ventricular myocardium subjected to a 30 min ischemia followed by a 120 min reperfusion in open-chest anesthetized rats treated by a bolus injection intravenously of solvent (PEG 400) solution 1 mL/kg (upper) or *dl*-praeruptorin A 2.0 mg/kg (down) immediately before reperfusion. Most nucleolus of neutrophils staining in blue or violet, or more seen intraluminally indicates inflammatory cell infiltration. ×200.

DISCUSSION

Recent basic experimental and clinical evidence suggest that brief coronary occlusion followed by reperfusion leads to reversible myocardial dysfunction[18], whereas cardiomyocyte death during MIR is partially mediated by apoptosis[19,20]. In the present study, we have demonstrated that Pd-Ia reduced serum IL-6 level and Fas, bax, bcl-2 expression during MIR under conditions with hypotension and without changes on HR in rats. Meanwhile, the action of Pd-Ia was strongly similar but weaker to that of nifedipine, a calcium channel blocker. So we hypothesize that the mechanisms of action of Pd-Ia are probably associated with blockade of Ca^{2+} influx, inhibition of Fas-Fas ligands, and Ca^{2+}-

Tab 2. Effects of Pd-Ia on BP in ischemia-reperfusion myocardium of rats. Sham: sham control; Oper: operation control; Sol: solvent control; Nif: nifedipine control. T_0: time before drug; T_{30}: 30 min after drug; T_{60}: 60 min after drug. Mean±SD. $^cP<0.01$ *vs* solvent group. $^fP<0.01$ *vs* sham group. $^hP<0.05$ *vs* nifedipine group.

Group	Dose/mg·kg^{-1}	*n*	BP/kPa				
			T_0	T_{30}	$[(T_{30}-T_0)/T_0]$/%	T_{60}	$[(T_{60}-T_0)/T_0]$/%
Sham	-	7	16.2±1.8	16.2±2.6	0±5	15±4	-8±14
Oper	-	6	13.5±1.5	13.1±1.6	-3±6	12.5±2.1	-7±11
Sol	-	7	10.8±2.1	10.7±1.9	-1±8	9.8±1.0	-9±10
Nif	5.0	6	15.1±2.3	8.7±0.4^f	-41±11	8.9±0.9^f	-42±10
Pd-Ia	0.5	7	14.2±1.5	11.0±1.4^{cfh}	-22±8	11.3±1.7	-20±13
	1.0	7	14.7±1.6	11.1±0.7^{cfh}	-24±7	11.2±0.7	-24±7
	2.0	7	12.6±0.7	9.4±1.2^{cfh}	-25±6	9.5±1.2	-24±14

dependence, *etc*. Those are all capable of abolishing Ca^{2+}-overload of cytoplasm evoked by MIR[21], maintaining balance between intra- and extra-cellular calcium, remaining vasodilatation and stability of mitochondria[22], finally, leading to lower the occurrence of cardiomyocyte apoptosis. Thus, it is preferable to other vasodilators or calcium antagonists in cardiohemodynamic modulation[23] and cardioprotection during MIR. Secondly, the fact that Pd-Ia reduced Fas, bax, and bcl-2 expression also involved other factors such as fall of IL-6 level, alleviation of neutrophils infiltration, lowering heart load and myocardial oxygen consumption *via* indirect amelioration of cardio-hemodynamics, *etc*.

In conclusion, controlling and regulating the expression of some immediate-early genes including IL-6, Fas, bax, and bcl-2, exhibits a relievable or beneficial effect in ischemia-reperfusion myocardium. Pd-Ia elicits a novel target in the therapeutic prevention of postischemic cardiomyocyte death. It might be as a therapeutic agent to open a promising perspectives in prevention of MIR and cardioprotection.

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前胡甲素对缺血再灌注心肌 IL-6 水平及 Fas，bax，bcl-2 蛋白表达的影响[1]

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关键词 前胡甲素；心肌；缺血；再灌注损伤；细胞凋亡；白介素 - 6

目的：研究前胡甲素对缺血再灌注心肌 IL-6 水平及凋亡相关蛋白表达的影响．方法：麻醉开胸大鼠左前降枝冠状动脉蒙受 30 分钟缺血及 120 分钟再灌注．放射免疫法测定血清 IL-6 水平；免疫组化法和计算机图像分析系统检测凋亡相关蛋白 Fas，bax 及 bcl-2 的表达；苏木精一依红染色法染色并于光镜下观测嗜中性白细胞的浸润．结果：前胡甲素 2.0 $mg \cdot kg^{-1}$ iv，在降压和不影响心率的情况下，减少 IL-6 水平及 Fas，bax，bcl-2 蛋白的表达，但增加 bcl-2/bax 的比率．IL-6 水平及 Fas，bax，bcl-2 蛋白表达之间有密切的线性正相关，而嗜中性白细胞只有轻微浸润．结论：前胡甲素防治缺血后心肌细胞死亡出现新靶位，可能与机体在心肌缺血再灌注期间调控即早基因 IL-6，*Fas*，*bax*，*bcl-2* 的表达有关．

（责任编辑 韩向晖）