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Effects of *dl*-praeruptorin A on nucleus factor- κ B and tumor necrosis factor- α expression in ischemia-reperfusion hearts¹

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KEY WORDS *dl*-praeruptorin A; myocardial; ischemia; reperfusion injury; nucleus factor- κ B; tumor necrosis factor- α

ABSTRACT

AIM: To study the effects of *dl*-praeruptorin A (Pd-Ia) on nucleus factor- κ B (NF- κ B) activity and tumor necrosis factor- α (TNF- α) expression in ischemia-reperfusion (I/R) myocardium. **METHODS:** Langendorff's isolated rat heart was subjected to a 10-min ischemia followed by a 30-min reperfusion. NF- κ B activity in nucleus was analyzed by Sandwich Enzyme-Linked Immunosorbent Assay (ELISA). TNF- α level in cytoplasm was measured by radioimmunoassay. Infiltration of neutrophils was observed using Hematoxylin-Eosin staining under optical microscope. **RESULTS:** Pd-Ia 1.0 μ mol/L with 30-min preventive perfusion decreased NF- κ B activity from 0.98 ± 0.13 to 0.65 ± 0.17 ($P < 0.05$ vs solvent) and down-regulated TNF- α expression from 13.7 ± 6.1 μ g/L to 9.4 ± 2.7 μ g/L ($P < 0.01$ vs solvent) under conditions with increase of coronary flow, negative inotropic action, inhibition of creatine kinase and without chronotropic action, whereas, infiltration of neutrophils was mild. **CONCLUSION:** Pd-Ia inhibited NF- κ B activity in I/R myocardium and led to down-regulation of TNF- α expression, which might be one of molecular mechanisms of Pd-Ia in cardioprotection.

INTRODUCTION

Ischemia-reperfusion (I/R) injury commonly occurred during thrombolytic therapy for myocardial infarction, restoration of blood flow after cardioplegic arrest in cardiovascular surgery, and heart transplantation. It was a complicated pathophysiological process, involving in calcium overload, free radical

production, metabolic abnormalities, and inflammatory reaction, *etc.* Along with development of modern molecular biology, the expression and regulation of gene in myocardium I/R injury were focused on nuclear transcription factor- κ B (NF- κ B) and inhibitory κ B (I κ B)^[1-4].

NF- κ B belongs to a rel/NF- κ B protein family and found primarily in immune, inflammation, and cellular defenses, which played very important roles in regulating multiple immediate-early gene expressions. The reasons were probably related to impact on the process of inflammatory reaction, cellular apoptosis and necrosis, ischemic preconditioning, and ventricular remodeling, *etc.*, especially involved in positive and negative feedback, and cross-regulation in NF- κ B activation^[5-9]. However, the exact mechanism was unclear, thus, it is necessary

¹ Project partly supported by the Key Foundation of the Ministry of Public Health of China, No 88402257.

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Received 2002-11-26

Accepted 2003-05-02

to further explore the molecular mechanisms of NF- κ B activation in myocardial I/R injury.

Tumor necrosis factor- α (TNF- α), an important inflammatory cytokine, was regulated by NF- κ B and counteracted on NF- κ B activation^[10]. Sometimes, improper activation of NF- κ B could induce excessive inflammatory responses and injury. Therefore, modulation of NF- κ B activation might dedicate a new target in alleviation of myocardium I/R injury^[11-13].

Our previous studies had proved that *dl*-praeuropterin A (Pd-Ia), a Ca²⁺-influx blocker^[14-16] and K⁺-channel opener^[17], isolated from a traditional Chinese medicine "Baihua Qianhu", had cardioprotective effects and relieved inflammatory reaction and apoptosis in I/R myocardium through inhibition of interleukin-6 (IL-6) and Fas, bax, bcl-2 protein expression^[18]. However, the effects of Pd-Ia on NF- κ B activation and TNF- α expression in I/R myocardium had not been investigated. In the present study, the influences of Pd-Ia on these two factors were firstly observed and aimed to offer a new target of therapy for ischemic disease.

MATERIALS AND METHODS

Drugs and reagents Pd-Ia extracted from the root of *Peucedanum praeruptorum* Dunn, were provided by Prof OKUYAMA T (Department of Pharmacognosy and Phytochemistry, Meiji College of Pharmacy, Tokyo 154, Japan), diltiazem (Fanabe Seiyaku), goat polyclonal IgG of NF- κ B p65 and rabbit polyclonal IgG of NF- κ B p65 (Santa Cruz, USA), goat antirabbit HRP-IgG (HuaMei, China), 96-well microtiter plates (Costar, USA), TNF- α radioimmunoassay kit (Chinese Atomic Energy Research Institute, China), LDH, and CK assay kits (Jian Cheng Biology Research Institute, Nanjing) were used in this study.

Heart perfusion Thirty-five Wistar rats (200-400 g, Grade II, Certificate No 0001) of both sexes supplied by Laboratory Animal Center of China Medical University were divided randomly into 5 groups: sham, solvent, diltiazem 1.2 μ mol/L, Pd-Ia 0.2 μ mol/L, Pd-Ia 1.0 μ mol/L, and Pd-Ia 3.0 μ mol/L, respectively. All experiments were performed in accordance with Guidelines for Animal Experimentation established by the Center of Liaoning Experimental Animal Administration.

Langendorff's isolated perfused heart was performed as follows^[19]: perfused pressure, 47.8 mmHg; Krebs-Henseleit solution [(in mmol/L) NaCl 119, KCl

4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25, and glucose 10 (pH 7.4)] warmed at 38 °C by circulated water from the thermostat and equilibrated with 95 % O₂/5 % CO₂. After equilibrium for 15-30 min (reached to stable conditions), the heart was perfused with drug solutions firstly for 30 min, and then, subjected to a 10-min ischemia followed by a 30-min reperfusion. Meanwhile, myocardial contraction (MC), heart rate (HR) and coronary flow (CF) were examined before ischemia and 10, 20, and 30 min after reperfusion, respectively. The activities of lactate dehydrogenase (LDH) and creatine kinase (CK) were detected through the perfusion fluids collected. Left ventricles wrapped with tin foil paper were frozen in liquid nitrogen immediately at the end of experiment. All accumulated myocardial samples were preserved in -85 °C superlow temperature refrigerator for the purposes of future wax section preparation and protein isolation.

Isolation of proteins in nucleus and cytoplasm

About 0.1 g myocardial samples plus 0.8 mL ice-cold hypotonic buffer A [HEPES 10 mmol/L, pH 7.9, KCl 10 mmol/L, edetic acid 0.1 mmol/L, dithiothreitol (DTT) 1 mmol/L, protease inhibitor phenylmethylsulfonyl fluoride (PMSF) 0.5 mmol/L, and phosphatase inhibitor NaF 50 mmol/L], were homogenized. The homogenates were centrifuged (15 min, 100 \times g, 4 °C) in order to eliminate unbroken tissue. After ice-incubation for 20 min, the supernatants were vortexed for 30 s and centrifuged (15 min, 5000 \times g, 4 °C). Supernatants containing cytoplasmic proteins were stored at -85 °C. The remainder were mixed with ice-cold hypertonic salt buffer B (the same as buffer A mentioned above, but NaCl 0.4 mol/L replaced KCl 10 mmol/L). After adding 10 % nonidet P-40, 50 μ L, it was ice-incubated for 20 min and centrifuged (15 min, 14 000 \times g, 4 °C). Then, the nuclear proteins in supernatants were obtained and stored at -85 °C. The total proteins were determined by Coomassie blue staining assay.

Sandwich enzyme-linked immunosorbent assay (ELISA) The 96-well microtiter plates were coated with goat polyclonal IgG of NF- κ B p65 (1:500, 100 μ L per well, 4 °C) for a night. Next morning, after washing three times with PBS, the plates were filled with bovine serum albumin 0.6 mmol/L (100 μ L per well) and stayed at 4 °C passing the night again. After the plate washing, the test samples (1:50) were added and incubated at 37 °C for 2 h (the same as belows), then, rabbit polyclonal IgG of NF- κ B p65 (1:500) and sheep anti-rabbit HRP-IgG (1:1000) were added, respectively.

Finally, substrate enzyme OPD 2.0 mmol/L (containing 0.006 % H₂O₂) were added and incubated for 1 h, and citric acid 2.0 mol/L (50 μL per well) was added to end the reaction. NF-κB was evaluated with absorbance (*A*) value detected by microplate photometer at λ_{450 nm}^[20].

Radioimmunoassay Cytoplasmic TNF-α was measured according to instruction.

Pathologic observation Myocardium was stained by Hematoxylin-Eosin's method, and infiltration of inflammatory cells was observed under optics microscope.

Statistical analysis All data were expressed as mean±SD. One-way ANOVA analysis was used by SPSS software to assess statistical significance between drug groups and various related control groups. *P*<0.05 was considered to be significant. The relationship between TNF-α and NF-κB was estimated by correlation analysis.

RESULTS

Effects on cardiohemodynamics Pd-Ia (0.2, 1.0, and 3.0 μmol/L) dose-dependently decreased MC (*P*<0.05 vs solvent), and the mean inhibitory rates were 33 %±20 %,

55 %±20 % and 57 %±30 %, respectively. Also Pd-Ia reduced HR slightly, but there was no statistic significance (*P*>0.05). Pd-Ia expressed a dual action to CF, it increased CF at lower dose (0.2 μmol/L), but decreased it at higher dose (3.0 μmol/L), (*P*<0.05 vs solvent). Diltiazem had similar cardiohemodynamic effects to Pd-Ia (0.2 μmol/L), but its inhibition on HR was markedly significant compared with Pd-Ia, the mean inhibitory rate reached to 45 %±26 % (*P*<0.01 vs sham, Tab 1).

Effects on NF-κB activity Pd-Ia in all three doses decreased NF-κB activity markedly, for example, at doses of 0.2 and 1.0 μmol/L, NF-κB activity was inhibited from 0.98±0.13 to 0.60±0.14 and 0.65±0.17, respectively (*P*<0.05 vs solvent). Diltiazem had the similar effect to Pd-Ia, the activity was inhibited from 1.3±0.6 to 1.01±0.14 (*P*<0.05 vs sham), but it seemed weak in potency compared with Pd-Ia.

Effects on TNF-α expression Pd-Ia (1.0 μmol/L, 3.0 μmol/L) decreased TNF-α from (13.7±6.1) μg/L to (9.4±2.7) μg/L and (10.3±2.2) μg/L, respectively (*P*<0.01 vs solvent). However, diltiazem had no significant changes on TNF-α (*P*>0.05 vs sham).

Tab 1. Effects of Pd-Ia on cardiohemodynamics in isolated rat hearts subjected to a 10-min ischemia followed by a 30-min reperfusion. Mean±SD. ^b*P*<0.05, ^c*P*<0.01 vs sham. ^e*P*<0.05, ^f*P*<0.01 vs Sol.

Group	Conc/ μmol·L ⁻¹	<i>n</i>	Parameters	Reperfusion time/min			
				0	10	20	30
Sham	-	6	MC	2.4±0.9	1.1±1.3	1.0±1.1	0.95±1.02
			HR	226±64	146±38	166±43	168±43
			CF	8.8±1.4	5.9±2.4	5.8±2.1	5.5±2.0
Sol	-	4	MC	2.8±1.4	0.4±0.3	0.4±0.4	0.4±0.3
			HR	184±53	134±59	155±59	167±59
			CF	7.4±2.1	5.0±2.7	4.8±2.8	4.5±2.6
Dil	1.2	5	MC	2.5±1.6	0.7±0.6	0.7±0.5	0.6±0.4
			HR	125±77 ^c	151±35	182±23	192±30
			CF	8.4±1.6	7.6±1.5 ^e	7.3±1.5 ^e	6.7±1.5 ^e
Pd-Ia	0.2	6	MC	1.4±0.4 ^e	0.13±0.05 ^b	0.22±0.19	0.27±0.32
			HR	172±49	112±25	155±36	150±21
			CF	7.5±2.8	5±4	5±3	5±3
	1.0	5	MC	1.2±0.5 ^{bc}	0.22±0.11 ^b	0.28±0.08	0.24±0.09
			HR	183±51	132±92	185±53	198±62
			CF	6.9±2.8	4.2±1.7	4.0±1.3	3.8±1.2
	3.0	5	MC	1.1±0.8 ^{bc}	0.24±0.17	0.4±0.4	0.3±0.3
			HR	186±83	106±47	119±51	136±48
			CF	4.0±2.1 ^{ce}	2.9±1.2 ^b	2.7±1.3 ^b	2.5±1.1 ^e

Sham: sham control; Sol: solvent control; Dil: diltiazem control; Pd-Ia: *dl*-praueruptorin A group; *n*: number of rats; Conc: concentration; MC: myocardial contraction/cm; HR: heart rate/bpm; CF: coronary flow/mL·min⁻¹.

Effects on LDH and CK There was no difference in LDH between Pd-Ia groups and controls. However, at reperfusion 20 min, Pd-Ia (1.0 and 3.0 μmol/L) dose-dependently decreased CK from (73±16) kU/L to (34±6) kU/L ($P<0.05$ vs sham) and (24±4) kU/L, respectively ($P<0.01$ vs sham, Tab 2).

Tab 2. Effects of Pd-Ia on LDH and CK in isolated hearts of rat subjected to a 10-min ischemia followed by a 30-min reperfusion. $n=3$. Mean±SD. ^b $P<0.05$, ^c $P<0.01$ vs Sham. ^e $P<0.05$, ^f $P<0.01$ vs Sol.

Group	Conc/ μmol·L ⁻¹	Parameters	Reperfusion time/min		
			10	20	30
Sham	-	LDH	1047±274	581±71	504±217
		CK	120±54	73±16	45±13
Sol	-	LDH	109±39	310±170	403±147
		CK	58±15	42±18	36±9
Dil	1.2	LDH	636±142	574±126	450±63
		CK	35±16 ^b	18±4 ^c	15±10
Pd-Ia	0.2	LDH	479±178	681±185	148±48
		CK	43±8 ^b	42±5	37±5
	1.0	LDH	1133±507 ^e	209±84	624±561
		CK	23±10 ^c	34±6 ^b	22±2
	3.0	LDH	583±317	479±193	262±112
		CK	35±6 ^b	24±4 ^c	31±2

Conc: concentration; Sham: sham control; Sol: solvent control; Dil: diltiazem control; Pd-Ia: *dl*-praueruptorin A group; n : number of rats; LDH: lactate dehydrogenase/U·L⁻¹; CK: creatine kinase/kU·L⁻¹.

Correlation analysis between NF-κB activity and TNF-α expression There existed a linearity and positive correlation between NF-κB activity and TNF-α expression. The correlation coefficients (r) were 0.65 in solvent control and Pd-Ia group (1.0 μmol/L), respectively (Fig 2).

Effects on infiltration of inflammatory cells In controls, serious neutrophil infiltration was observed, and most nucleolus of neutrophils staining in blue or violet, or more seen indicates inflammatory cell infiltration, whereas, in Pd-Ia and diltiazem groups, they were alleviated markedly (Fig 3).

DISCUSSION

In this experiments, we found that Pd-Ia inhibited NF-κB activity in nucleus and led to down-regulation of TNF-α expression in cytoplasm under conditions

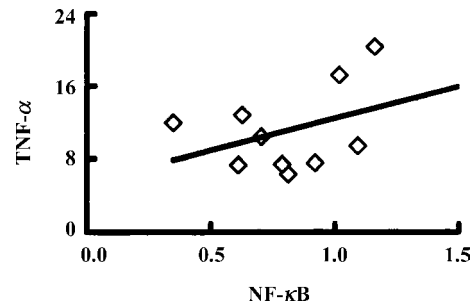


Fig 2. Correlativity analysis between NF-κB activity and TNF-α expression in solvent and Pd-Ia 1.0 μmol/L group during myocardial ischemia-reperfusion in isolated rat hearts. The correlation coefficients (r) were 0.65 respectively. Pd-Ia: *dl*-praueruptorin A; NF-κB: nuclear factor-κB; TNF-α: tumor necrosis factor-α.

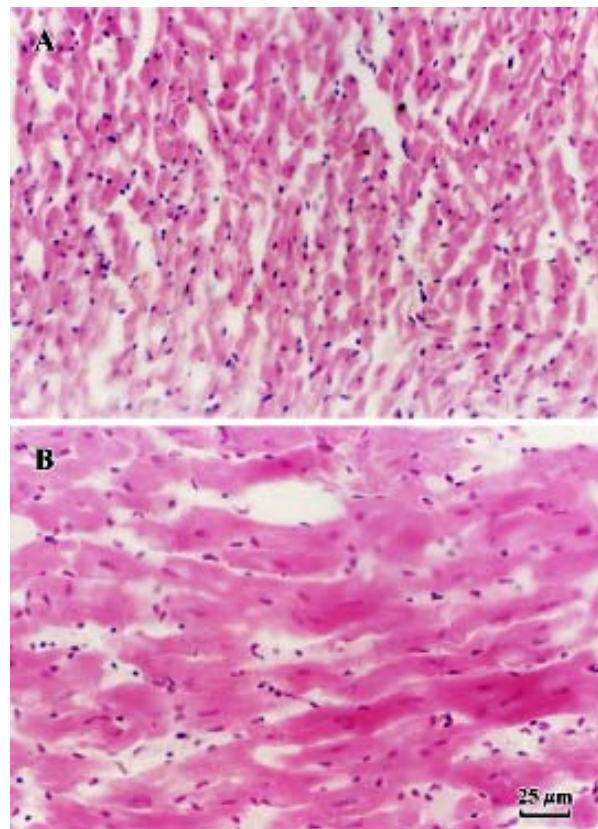


Fig 3. Micrograph demonstrating inflammatory cell infiltration in left ventricular myocardium subjected to a 10-min ischemia followed by a 30-min reperfusion in isolated rat hearts treated with a 30-min preventive perfusion of Pd-Ia 3.0 μmol/L (B) or K-H solution (A). ×400.

with increase of CF, negative inotropic action, and without chronotropic action. Also it relieved infiltration of neutrophils and lowered CK, all these would be beneficial to cardioprotection in myocardial I/R injury and corresponded with previous studies^[21,22].

It is well-known that modulation of NF- κ B activity can prevent myocardial I/R injury. NF- κ B exists as a latent cytoplasmic complex bound to I κ B, an inhibitory molecule to inhibit NF- κ B nuclear localization and DNA binding. Five I κ B isoforms are I κ B- α , I κ B- β , I κ B- γ , I κ B- δ , and I κ B- ϵ , respectively. Among them, I κ B- α phosphorylation could lead to an immediate NF- κ B activation, but I κ B- β was involved in a slow and persistent activation of NF- κ B, suggesting that I κ B- α might occurred in acute activation of NF- κ B. Conversely, the activation of NF- κ B could promote I κ B- α gene expression. Thus, the inhibition of NF- κ B mainly have two pathways: (1) inhibiting I κ B- α kinase activity to limit I κ B- α phosphorylation and NF- κ B activation^[23]; and (2) inducing I κ B- α gene expression. In this way, new-synthesized I κ B- α would rapidly supply the depletion of I κ B- α in cytoplasm in order to remodel NF- κ B complexes^[24, 25]. Pd-Ia inhibiting NF- κ B activation was probably involved in such two pathways, so it is necessary and valuable to explore further in these fields.

In addition, Pd-Ia reduced TNF- α over-expression in I/R myocardium, and there was a positive correlation between NF- κ B activity and TNF- α expression, suggesting that activation of NF- κ B might play important roles in up-regulation of TNF- α mRNA level and enhancement of TNF- α .

In conclusion, Pd-Ia attenuated NF- κ B activity in nucleus and TNF- α expression in cytoplasm, this might be one of its molecular mechanisms in cardioprotection. It displays a bright prospects in prevention and therapy of ischemic heart diseases and development of a new target of medicines for treatment of ischemic disease in future.

ACKNOWLEDGMENT We would especially thank to Experimental Center of China Medical University for expert technical assistances.

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