Egr-1, a Central and Unifying Role in Cardioprotection from Ischemia-Reperfusion Injury?

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Key Words

Early growth response-1 • Calcium channel blocking • Antisense oligodeoxyribonucleotide • Ischemia-reperfusion • Hypoxia-reoxygenation • Myocardium • *N*-n-butyl haloperidol iodide

Abstract

Aims: Our previous studies have shown that *N*-n-butyl haloperidol iodide (F_2) can antagonize myocardial ischemia/reperfusion (I/R) injury by blocking intracellular Ca^{2+} overload and suppressing Egr-1 overexpression. The present study is to investigate the relation between the reduction of Ca^{2+} overload and the inhibition of Egr-1 overexpression. **Methods:** The Sprague-Dawley rat myocardial I/R model and cultured cardiomyocyte hypoxia-reoxygenation (H/R) model were established. Administration of Egr-1 antisense oligodeoxyribonucleotide (AS-ODN) only or combining with F_2 , Egr-1 protein expression was examined by Western-blot analyses. Hemodynamic parameters, creatine kinase (CK) and lactate dehydrogenase (LDH), superoxide dismutase (SOD) and malondialdehyde (MDA), myeloperoxidase (MPO), cardiac troponin I (cTnI), and tumor necrosis factor- α (TNF- α) were measured to assess the degree of injury and inflammation of myocardial tissues and

cells. **Results:** Treatment with Egr-1 AS-ODN significantly reduced Egr-1 protein expression and attenuated injury and inflammation of myocardium caused by I/R or H/R evidenced by the amelioration of hemodynamics, the decrease in leakage of CK, LDH, cTnI, the increase in MDA generation, the decrease in SOD activity, the reduction of MPO activity in myocardial tissues and release of TNF- α from cultured cardiomyocytes. Treatment with F_2 combined with Egr-1 AS-ODN, the inhibition of Egr-1 protein expression and inflammation (MPO activity and TNF- α level) were not enhanced, but the protection from myocardial I/R (or H/R) injury was significantly increased in hemodynamics and cytomembrane permeability relative to the using of Egr-1 AS-ODN only. **Conclusion:** These data suggest that the inhibition of Egr-1 overexpression cannot involve all mechanisms of cardioprotection from I/R injury.

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Introduction

Rapidly initiating reperfusion is the most effective treatment to reduce infarct size resulting from ischemia.

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However, reperfusion has the potential to introduce additional lethal injury that is not evident at the end of ischemia. Ischemia-reperfusion (I/R) injury represents a clinically relevant problem associated with thrombolysis, angioplasty, organ transplantation and coronary bypass surgery. Such injury is expressed physiologically as endothelial and microvascular dysfunction, impaired blood flow, metabolic dysfunction, cellular necrosis, and apoptosis [1]. The mechanism of I/R injury remains incompletely understood, although the essential factors, including (i) cytosolic and mitochondrial Ca^{2+} -overload, (ii) release of reactive oxygen species (ROS), (iii) an acute inflammatory response, and (iv) shift in substrate use, have been well documented to explain it [2]. At present, more research has been tended to focus on the precise intracellular signaling pathway and elements responsible for tissue injury in I/R [3-7]. It was reported that I/R condition in many organs causes overexpression of Egr-1, which indicates that Egr-1 is involved in I/R injury [8-13]. Basing on results of a model of lung I/R, Yan et al proposed that Egr-1 could be a “master switch” to trigger expression of pivotal regulators of inflammation, coagulation and vascular hyperpermeability, which are thought to be the central pathogenesis of I/R injury [8].

The early growth response (Egr)-1 gene belongs to the category of immediate early genes, which encodes an 80-82 kDa nuclear protein. The Egr-1 protein is a transcription regulatory factor, which contains three zinc finger DNA-binding domains in promoter regions of many genes including tumor necrosis factor- α (TNF- α), interleukin-1 β , intercellular adhesion molecule-1 (ICAM-1), macrophage inflammatory protein-2, monocyte chemotactic protein-1, and tissue factor, et al [8, 14, 15]. Egr-1 function was initially thought to be only associated with growth and differentiation, but later studies provided a new perspective on Egr-1 biological role. It might be a master regulator of genes involved in cellular response to diverse extracellular stimulations [16, 17]. Egr-1 has a well-established pivotal role in the pathogenesis of lung I/R injury [8, 15]. Furthermore, our previous study also confirmed that Egr-1 might play a vital role in the pathogenesis of myocardial I/R injury [18].

N-n-butyl haloperidol iodide (F_2), a novel compound of calcium channel blocker, was synthesized by our laboratory and granted a Chinese national invention patent (No. ZL 96119098.1) [19]. Our previous studies showed that F_2 could antagonize myocardial I/R injuries in rat and rabbit, and its cardioprotective mechanism might be associated with the inhibition of Ca^{2+} overload by blocking calcium channels of ventricular myocytes and the

suppression Egr-1 overexpression [20-23]. Ca^{2+} , as we know, is an important “second messenger”. And Egr-1 protein is believed to act as an intracellular “third messenger” owing to its recruiting expression of multiple downstream target genes [24-26]. It has been reported that Egr-1 expression is induced by Ca^{2+} stimulation and its upregulation is Ca^{2+} -dependent [27, 28]. The Ras/Raf-1/ERK $_{1/2}$ pathway mediated by the Ca^{2+} /protein kinase C, Ca^{2+} /calmodulin and Ca^{2+} / calcineurin pathways, and the Ca^{2+} /MAPK pathway are involved in the activation and expression of Egr-1 [11, 28-32]. We also found that three types of classical calcium antagonists (verapamil, diltiazem and nifedipine) could down-regulate Egr-1 overexpression induced by I/R stimulation [33]. These would seem to imply that Ca^{2+} and Egr-1 are two important links in the signal pathway of I/R injury. As Yan et al demonstrated, Egr-1 activation played a central and unifying role in the pathogenesis of ischemic tissue damage [8]. The cardioprotection of reducing Ca^{2+} overload seems to be ultimately via suppression of Egr-1 expression. So we want to know whether the protection of F_2 on myocardial I/R injury is only through suppressing Egr-1 overexpression and whether the inhibition of Egr-1 overexpression can involve all mechanisms of cardioprotection from I/R injury including reduction of Ca^{2+} overload. The purpose of this study is to investigate the relation between the reduction of Ca^{2+} overload and the inhibition of Egr-1 overexpression.

Materials and Methods

Preparation of Egr-1 antisense oligodeoxyribonucleotide (AS-ODN)

Egr-1 antisense Phosphorothioate oligodeoxyribonucleotide of 15 base-pair length were commercially synthesized (Takara Biotechnology Co., Ltd, China). The sequence of Egr-1 AS-ODN was 3'-TAC CGT CGC CGG TTC -5' [15]. As reported previously [23], FITC labeled Egr-1 AS-ODN was found in the cytoplasm and nuclei of myocardial tissues, while mainly in the nuclei of cultured cardiomyocytes, indicating that Egr-1 AS-ODN had successfully been uptaken up.

Preparation of myocardial I/R

All animals were treated in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and experimental protocols were approved by the Laboratory Animal Ethics Committee of our institution.

Adult male Spargue-Dawley rats weighing 250-300g were provided by the Experimental Animal Center of Shantou University Medical College. Myocardial I/R model of rat was performed as previously described [23]. In brief, after

anesthetization, thoracotomy and pericardiotomy, the left anterior descending coronary artery (LADCA) was occluded reversibly by a silk suture. All rats were randomly assigned into one of five groups ($n=15$ each group): Sham, Control (I/R control), AS (I/R treated with Egr-1 AS-ODN), AS+F₂ (I/R treated with Egr-1 AS-ODN and F₂) and PEG [I/R treated with solvent of 33% (V/V) polyethylene glycol 400 (PEG)]. Hearts of I/R were subjected to 60 min LADCA occlusion followed by 180 min of reperfusion. Rats of the Sham group were subjected to the same surgical procedure, except that the suture was left untied. The AS-ODN at the dose of 4 mg/kg respectively was given through the sublingual vein 24 h before ischemia. F₂ (at a dose of 2 mg/kg dissolved in 33% PEG) or equal volume of PEG was administered 5 min before ischemia. At the beginning of experiment, two cannulae were inserted into the right femoral artery and the left ventricle, respectively. Then the hemodynamic parameters were measured by BL-420 Biological Experimental System (TME Technology Co., Chengdu, China) at baseline, 60 min of ischemia and 180 min of reperfusion. At the end of experiment, blood was taken from the carotid artery for creatine kinase (CK) and lactate dehydrogenase (LDH) measurements. The ischemic myocardium was excised for levels of myeloperoxidase (MPO), superoxide dismutase (SOD) and malondialdehyde (MDA), or stored at -70°C for determining the levels of Egr-1 protein.

Primary culture of ventricular myocytes and preparation of hypoxia-reoxygenation (H/R)

Primary culture of neonatal rat ventricular myocytes and H/R model were carried out by previously described methods [23]. In short, the isolated cells from 1-4 days neonatal rats were purified by preplating 30 min at 37°C and adding 5-Bromo deoxyuridine into the medium for the first 3 days to eliminate non-cardiomyocytes [34]. After 5-7 days of cell culture in normoxic medium, the cardiomyocytes were randomly divided into one of five groups: Sham, Control (H/R control), AS (H/R treated with Egr-1 AS-ODN), AS+F₂ (H/R treated with Egr-1 AS-ODN and F₂) and Lip+PEG [H/R treated with Lipofectamine™2000 Reagent (Lip) and solvent of PEG]. The cardiomyocytes of Sham group were incubated under normoxic condition for 4 h. The cells of other groups underwent 3 h of hypoxia and 1 h of reoxygenation as described below. Hypoxia was induced by replacing the initial culture medium with pH 6.2 buffer (in mM): 137 NaCl, 12 KCl, 0.49 MgCl₂, 0.9 CaCl₂, 4 HEPES, 20 Na lactate [35], and the cardiomyocytes were incubated in an air-tight chamber gassed with pure N₂ at 37°C for 3 h. The buffer was then replaced with fresh oxygenated culture medium and the dishes were transferred into a normoxic incubator (95% air-5% CO₂) for 1 h of reoxygenation. The AS-ODN at the dose of 4×10^{-7} mol/L and Lip were transfected into cultured cells 24 h before hypoxia. F₂ (at a dose of 1×10^{-6} mol/L dissolved in 33% PEG) or equal volume of PEG was added into the cells before H/R. At the end of reoxygenation, the culture medium was collected for enzyme assays (CK, LDH), levels of cardiac troponin I (cTnI) and TNF- α . The cardiomyocytes were collected to measure the levels of SOD, MDA and Egr-1 protein.

Levels of CK and LDH in plasma and culture medium

The arterial blood sample (2 ml) was centrifuged at 2500 g for 10 min. The plasma was transferred to a microcentrifuge tube and stored at -20 °C until analysis. Levels of CK and LDH in plasma and culture medium were determined using the test kits (Jiancheng Bioengineering Institute, Nanjing, China).

Activity of SOD and level of MDA in myocardial tissues and cultured cardiomyocytes

According to the commercial test kits (Jiancheng Bioengineering Institute, Nanjing, China), the tissue protein was measured spectrophotometrically by using Coomassie bright-blue (CBBG250) stain method, the activity of SOD using xanthine oxidase method and the level of MDA using thiobarbituric acid stain method.

Myocardial tissue MPO activity

According to the manufacturer's kit instruction (Jiancheng Bioengineering Institute, Nanjing, China), the MPO activity of the myocardial homogenate was measured. One unit of MPO activity is defined as the quantity of enzyme degrading 1 μ mol peroxide/min at 37°C. The data was expressed as MPO activity per gram of tissue.

Measurements of cTnI and TNF- α levels in culture medium

The release of cTnI was detected from culture medium at the end of reoxygenation. Culture medium (500 μ l) was collected, and the levels of cTnI were measured using the two-site sandwich immunoassay kit (Bayer Corp., USA) by ACS:180 Automated Chemiluminescence System (Bayer Corp., USA). The concentration of TNF- α in culture media was determined by ELISA with a commercially available ELISA kit (Bender Medsystems Inc, Austria) according to the manufacturer's instructions. All samples and standards were measured in duplicate.

Western-blot analysis

Total protein extracts were prepared from myocardial tissue or cultured cells using cell lysis buffer containing a protease inhibitor cocktail (aprotinin, leupeptin, pepstatin A, and PMSF). The protein concentration was determined by the Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amount of total protein (60 μ g for tissue and 80 μ g for cells) was subjected to SDS-PAGE (10%) followed by electrophoretic transfer to nitrocellulose membranes. Nonspecific binding was blocked by incubation of membranes with milk overnight at 4°C. The blot was incubated with a rabbit anti-rat Egr-1 antibody (Santa Cruz Biotechnology Inc., USA) in 1:200 dilution for tissues and 1:100 dilution for cells at room temperature for 3 h. The blots were then washed with TBS-T, and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000) at room temperature for 2 h. Detection of immunoreactive bands was performed by Western blotting chemiluminescence luminol reagents (Santa Cruz Biotechnology Inc., USA). Blots were then reprobed with β -actin (1:2000, Sigma-Aldrich Inc. USA) to confirm equal loading of samples.

Fig. 1. Western blot analysis of Egr-1 protein in myocardial tissues and cultured cardiomyocytes. Quantitative densitometric data were expressed as a percentage of that in Control group. All values are expressed as mean $\pm$ S.E.M. of five independent experiments. * P <0.05 vs. Sham group; † P <0.05 vs. Control group.

Time	Group	HR	SAP	DAP	LVSP	+dP/dtmax	-dP/dtmax
Ischemia	Sham	365 $\pm$ 10	134 $\pm$ 5	61 $\pm$ 6	149 $\pm$ 4	9027 $\pm$ 367	5862 $\pm$ 367
	Control	321 $\pm$ 14*	86 $\pm$ 4*	50 $\pm$ 2*	110 $\pm$ 2*	5718 $\pm$ 258*	3693 $\pm$ 240*
	PEG	316 $\pm$ 13*	86 $\pm$ 4*	51 $\pm$ 3*	106 $\pm$ 3*	5668 $\pm$ 279*	3764 $\pm$ 245*
	AS	373 $\pm$ 12	97 $\pm$ 3*†	64 $\pm$ 3†	122 $\pm$ 4*	6628 $\pm$ 373*	4509 $\pm$ 288*
	AS+F ₂	342 $\pm$ 10	110 $\pm$ 3*†	63 $\pm$ 4†	129 $\pm$ 3*†	6681 $\pm$ 371*	4674 $\pm$ 318
Reperfusion	Sham	344 $\pm$ 8	129 $\pm$ 3	63 $\pm$ 3	142 $\pm$ 4	7975 $\pm$ 259	5518 $\pm$ 200
	Control	264 $\pm$ 17*	71 $\pm$ 3*	40 $\pm$ 2*	93 $\pm$ 2*	3928 $\pm$ 195*	2852 $\pm$ 190*
	PEG	254 $\pm$ 19*	70 $\pm$ 3*	41 $\pm$ 3*	93 $\pm$ 4*	3970 $\pm$ 232*	2681 $\pm$ 216*
	AS	354 $\pm$ 12†	84 $\pm$ 4*	51 $\pm$ 2*†	107 $\pm$ 4*†	5381 $\pm$ 338*†	3840 $\pm$ 269*†
	AS+F ₂	316 $\pm$ 11†	102 $\pm$ 4*†#	59 $\pm$ 3†	119 $\pm$ 3*†#	6140 $\pm$ 261*†	4556 $\pm$ 293†

Table 1. Hemodynamic parameters in different groups during the course of the experiment. HR, heart rate (beats/min); SAP, systolic arterial pressure (mmHg); DAP, diastolic arterial pressure (mmHg); LVSP, left ventricular systolic pressure (mmHg); positive dP/dt_{max} (mmHg/s); negative dP/dt_{max} (mmHg/s). Ischemia: 60 min of ischemia; Reperfusion: 180 min of reperfusion. All values are expressed as mean $\pm$ S.E.M. (n =15). * P <0.05 vs. Sham group; † P <0.05 vs. Control group; # P <0.05 vs. AS group.

Statistical analysis

The band relative density of protein was analyzed by Fluorchem™ software (Alpha Innotech, USA). Data are expressed as mean $\pm$ S.E.M. The significance of differences was determined by using One-way ANOVA, followed by Student-Newman-Keuls test. P <0.05 was considered statistically significant.

Results

Effect of Egr-1 AS-ODN and F₂ on change of Egr-1 protein expression induced by I/R or H/R

As shown in Fig. 1, compared with the respective Sham groups, expression levels of Egr-1 protein in

myocardial tissues and cultured cardiomyocytes of Control group were significantly increased about 5.6- and 9.4-fold. These changes were not altered by PEG or Lip+PEG, but were significantly inhibited by 60% and 69% when Egr-1 AS-ODN was transfected into the animals and cultured cells. However, there was no difference between AS and AS+F₂ group in the Egr-1 protein expression level.

Effects of Egr-1 AS-ODN and F₂ on hemodynamic variables

Hemodynamic parameters measured throughout the experiments are summarized in Table 1. There were no differences between groups in all baseline hemodynamic

Group	CK	LDH	SOD	MDA
Sham	24 ± 2	18873 ± 1373	196 ± 5	2.5 ± 0.2
Control	124 ± 6*	84587 ± 1473*	97 ± 2*	8.7 ± 0.5*
PEG	120 ± 5*	82260 ± 1551*	99 ± 4*	9.1 ± 0.5*
AS	90 ± 4*†	46784 ± 2326*†	118 ± 3*†	5.8 ± 0.3*†
AS+F2	59 ± 3*†#	36850 ± 2667*†#	142 ± 6*†#	4.6 ± 0.4*†#

Table 2. Enzyme activities and MDA level in plasma or myocardial tissues of different groups *in vivo*. CK, creatine kinase (U/ml); LDH, lactate dehydrogenase (U/L); SOD, superoxide dismutase (U/mg prot); MDA, malondialdehyde (nmol/mg prot). CK and LDH were from plasma. SOD and MDA were from ischemic myocardial tissues. All values are expressed as mean ± S.E.M. (n=15). * $P < 0.05$ vs. Sham group; † $P < 0.05$ vs. Control group; # $P < 0.05$ vs. AS group.

Group	CK	LDH	cTnI	SOD	MDA
Sham	14.2 ± 1.1	5961 ± 102	2.17 ± 0.04	27.0 ± 0.9	0.26 ± 0.03
Control	30.3 ± 2.4*	9865 ± 105*	4.78 ± 0.14*	16.7 ± 0.8*	1.09 ± 0.07*
Lip+PEG	31.0 ± 2.4*	8578 ± 298*	5.07 ± 0.25*	14.1 ± 1.0*	0.91 ± 0.04*
AS	23.7 ± 2.3*†	7454 ± 79*†	3.13 ± 0.12*†	22.1 ± 1.9*†	0.64 ± 0.03*†
AS+F2	17.9 ± 1.4*†#	6882 ± 128*†#	2.60 ± 0.05*†#	25.4 ± 1.3*†#	0.42 ± 0.03*†#

Table 3. Enzyme activities, cTnI and MDA levels in culture medium or cultured cardiomyocytes of different groups *in vitro*. CK, creatine kinase (U/ml); LDH, lactate dehydrogenase (U/L); cTnI, cardiac troponin I (ng/ml); SOD, superoxide dismutase (U/mg prot); MDA, malondialdehyde (nmol/mg prot). CK, LDH and cTnI were from culture medium. SOD and MDA were from cardiomyocytes. All values are expressed as mean ± S.E.M. (n=12). * $P < 0.05$ vs. Sham group; † $P < 0.05$ vs. Control group; # $P < 0.05$ vs. AS group.

parameters (data not shown) and no significant change for hemodynamics in the Sham group throughout the experimental period. But LADCA occlusion significantly decreased heart rate (HR), systolic arterial pressure (SAP), diastolic arterial pressure (DAP), left ventricular peak systolic pressure (LVSP) and positive and negative $dP/dt_{\max}$. These parameters further declined at the end of 180 min of reperfusion. Changes in hemodynamic parameters followed the same pattern in the PEG group as those in the Control group. However, at the end of reperfusion changes in these parameters were significantly alleviated by Egr-1 AS-ODN. Furthermore, the alleviative effects, especially in SAP and LVSP, were enhanced when combining F₂ with Egr-1 AS-ODN.

Effects of Egr-1 AS-ODN and F₂ on changes of membrane permeability and ROS induced by I/R or H/R

Membrane permeability (reflected by the leakage of CK, LDH, cTnI out of myocardial cells) and ROS (reflected by the SOD activity and MDA level) comparisons among groups were shown in Table 2 and 3. After reperfusion or reoxygenation, the leakage of CK, LDH, cTnI from cardiomyocytes and the product of MDA increased significantly and the activity of

SOD decreased significantly in cardiomyocytes, as compared with the respective Sham groups. However, Egr-1 AS-ODN could maintain the activity of SOD, reduce the product of MDA, and attenuate the leakage of CK, LDH and cTnI out of myocardial cells caused by I/R or H/R. A further decrease of CK, LDH, cTnI and increase of SOD were observed in the group treated with Egr-1 AS-ODN and F₂ combination compared with the group treated with Egr-1 AS-ODN only.

Effect of Egr-1 AS-ODN and F₂ on MPO activity in myocardial tissue and TNF-α level in culture medium

The MPO activities (n=15) in myocardial tissue and TNF-α levels (n=8) in culture medium in five groups are shown in Fig. 2. MPO activity and TNF-α level were low in the Sham group, but showed an increase of about 3.6 and 13.2 times in the Control groups. These increased changes were not altered by PEG or Lip+PEG. Administration of Egr-1 AS-ODN significantly reduced MPO activity and TNF-α level by 40.3% and 31.3% compared with Control group. However, a small further decrease of MPO activity and TNF-α level but no significant difference was observed in AS+F₂ group compared with AS group.

Fig. 2. The MPO activity in ischemic myocardial tissues and TNF- α level in supernatant of cultured cardiomyocytes. MPO, myeloperoxidase (U/g, $n=15$). TNF- α , tumor necrosis factor- α (pg/ml, $n=8$). The values are expressed as mean $\pm$ S.E.M. * $P<0.05$ vs. Sham group; † $P<0.05$ vs. Control group.

Discussion

The present study *in vivo* and *in vitro* demonstrated unanimously that the inhibition of Egr-1 overexpression is beneficial for myocardial injury induced by I/R (or H/R), and that the effect of F₂ protection from myocardial I/R (or H/R) injury is beyond that of Egr-1 suppression.

Myocardial I/R injury could bring about heart function decline and cytomembrane permeability-increasing. ROS generation is responsible for intracellular damage induced by I/R [36]. MDA is a marker of lipid peroxidation that occurs as a result of the damaging effects of ROS [37], and SOD is a cellular ROS scavenger. So myocardial I/R (or H/R) injury is evidenced by the decline of hemodynamic parameters, the rise of CK, LDH and cTnI leakage, the decrease of SOD activity and the increase of MDA yield in our experiment. The upregulation of Egr-1 expression after I/R (or H/R), which was consistent with myocardial injury, was inhibited by Egr-1 AS-ODN to result in the attenuation of myocardial injury (Table 1, 2, 3). It was reported that overexpression of Egr-1 induces down-regulation of SOD and stimulates the generation of ROS [27], which was supported by our experiment. These data also suggested that Egr-1 is a key contributor to myocardial I/R injury.

Egr-1 is an important transcription factor which can recruit multiple downstream target genes, especially many proinflammatory genes. TNF- α is an important downstream inflammatory gene of Egr-1 [17, 38], and it can release and activate inflammatory cells such as

neutrophil which can result in the increase of MPO activity. Egr-1 AS-ODN inhibited myocardial inflammation demonstrated by the decrease in MPO activity *in vivo* and in TNF- α release *in vitro* (Fig. 2). In addition, as expected, Lip and PEG had no effects on all indexes owing to their incapability of inhibiting Egr-1 protein expression, which indicated Egr-1 AS-ODN is valid and specific.

Our previous study had shown that F₂ could antagonize myocardial I/R injury by suppression the upregulated Egr-1 expression, but the inhibitory effect of F₂ on upregulated Egr-1 expression is weaker than that of Egr-1 AS-ODN [23]. The inhibition of Egr-1 protein expression by F₂ combined with Egr-1 AS-ODN was not more powerful than by Egr-1 AS-ODN only (Fig. 1). It is implied that Egr-1 AS-ODN is relatively sufficient for suppression the Egr-1 overexpression induced by I/R (or H/R). And it was supported by our experimental result that no significant difference was observed in the decrease of MPO activity and TNF- α level between AS+F₂ group and AS group (Fig. 2). Nonetheless, the protection from myocardial I/R (or H/R) injury of F₂ combined with Egr-1 AS-ODN was significantly enhanced in hemodynamics and cytomembrane permeability relative to the using of Egr-1 AS-ODN only (Table 1, 2, 3). Our previous studies showed that F₂ could block calcium channels of ventricular myocytes. It might be associated with the cardioprotection of F₂ from I/R injury resulted from inhibition of myocardial Ca²⁺ overload. It was suggested that the cardioprotection of inhibition of Ca²⁺ overload is in addition to that of

Egr-1 suppression, and the inhibition of Egr-1 overexpression cannot involve all mechanisms of cardioprotection from I/R injury.

Egr-1, as we know, can be activated by intracellular Ca^{2+} via a number of pathways [11, 28-32]. On the other hand, Egr-1 can negatively regulate expression of the sodium-calcium exchanger-1 [39] and endoplasmic reticulum Ca^{2+} -ATPase [40, 41] to inhibit the myocardial Ca^{2+} transport. Furthermore, Egr-1 activation can also be mediated by some signal pathways independent of intracellular Ca^{2+} , such as TPK-Ras-MAPK pathway [42-44], cAMP/PKA pathway [45, 46] and cGMP/PKG pathway [47]. To take our experimental results into consideration, it was suggested that Ca^{2+} overload and Egr-1 activation contributed to I/R injury independently although they are interrelated and interact on each other. Egr-1, is not a central and unifying

role in cardioprotection from ischemia-reperfusion injury.

In summary, this study showed that Egr-1 activation is not responsible for all pathogenesis of I/R injury though Egr-1 might play a vital role in pathogenesis of myocardial I/R injury.

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