The Protective Effects of *N*-n-butyl Haloperidol Iodide on Myocardial Ischemia-Reperfusion Injury in Rats by Inhibiting Egr-1 Overexpression

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Key Words

Antisense oligodeoxyribonucleotide • Early growth response gene-1 • Hypoxia/reoxygenation • Ischemia/reperfusion • Myocardial • *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. The present study is to test the hypothesis that the protective effects of F_2 on myocardial I/R injury is mediated by downregulating Egr-1 expression. **Methods:** The Sprague-Dawley rat myocardial I/R model and cardiomyocyte hypoxia/reoxygenation (H/R) model were established. With antisense Egr-1 oligodeoxyribonucleotide (ODN), the relationship between Egr-1 expression and myocardial I/R injury was investigated. Hemodynamic parameters, 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. Egr-1 mRNA and protein expressions were examined by Northern-blot and Western-blot analyses. **Results:** Treatment with antisense Egr-1 ODN significantly reduced Egr-1

protein expression and attenuated injury of myocardial tissues and cells. Meanwhile, treatment with F_2 significantly inhibited the overexpression of Egr-1 mRNA and protein in myocardial tissues and cells. Consistent with downregulation of Egr-1 expression by F_2 , inflammation and other damages were significantly relieved evidenced by the amelioration of hemodynamics, the reduction in myocardial MPO activity as well as the decrease in leakage of cTnI and release of TNF- α from cardiomyocyte. **Conclusions:** These results suggested that the overexpression of Egr-1 was causative in myocardial I/R or H/R injury, and F_2 could protect myocardial tissues and cells from I/R or H/R injury, which was largely due to the inhibition of Egr-1 overexpression.

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Introduction

N-n-butyl haloperidol iodide (F_2) (Fig.1) is a new compound, which was synthesized by our laboratory and was granted a Chinese national invention patent (No. ZL96119098.1). Our own studies have shown that F_2 can antagonize myocardial ischemia/reperfusion (I/R) injuries in the rat and rabbit, and its cardioprotective mechanism

might be associated with the blocking calcium channels of ventricular myocytes and vascular smooth muscle cells [1-5].

The *egr-1* gene, also known as NGFI-A, krox24, or TIS8, is one of the immediate-early genes and a member of the zinc finger family of transcription factors. Its product, EGR-1, is a nuclear protein with sequence-specific DNA binding activity. Because EGR-1 can recruit expression of multiple downstream target genes, it is believed to act as an intracellular “third messenger” [6-8]. According to the recent data, levels of Egr-1 expression are upregulated in the heart [9, 10], lung [11, 12], gut [13] and kidney [14] after I/R challenge. 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 [11]. Likewise, other studies have reported that drugs targeting the Egr-1 could prevent hepatic I/R injury by inhibiting expression of Egr-1 [15, 16]. These findings indicate that the overexpression of Egr-1 could be the common denominator in I/R injury in various organs and Egr-1 might be a good therapeutic target in the I/R conditions. Up to now, although the Egr-1 upregulation was also demonstrated in the heart after I/R according to our and other results, its role in myocardial I/R injury has not been well-established [9, 10, 17].

Our preliminary study had shown that F₂ could decrease the overexpression of Egr-1, which was associated with its cardioprotective effects [17]. But it is uncertain whether downregulating Egr-1 expression of F₂ is a cause or effect of the altered degree of myocardial I/R injury. The purpose of this study is to elucidate the cause-effect relationship between Egr-1 overexpression and myocardial I/R injury, and investigate further the effects of F₂ on injury and inflammation of myocardial tissues and cells as well as Egr-1 mRNA and protein expression in myocardial tissues and cultured cardiomyocytes caused by I/R or hypoxia/reoxygenation (H/R), and thereby discover the new mechanism of F₂ to antagonize myocardial I/R injury.

Materials and Methods

Preparation of Sense, antisense and scrambled-sequence Egr-1 oligodeoxyribonucleotides (ODNs)

Antisense, sense and scrambled-sequence phosphorothioate Egr-1 ODNs of 15 base-pair length were commercially synthesized. The sequence of antisense Egr-1 ODN was 3'-TAC CGT CGC CGG TTC -5' as described

Fig. 1. Chemical structure of N-n-butyl haloperidol iodide (F₂).

previously [18]. For control experiments, sense Egr-1 (3'-GAACCGGCGACGGTA -5') and scrambled-sequence Egr-1 ODNs (3'-TCGTGCCGCTGCCAT -5') were used [18]. All phosphorothioate ODNs were commercially synthesized and purified by using high-performance liquid chromatography (Takara Biotechnology Co., Ltd, China). FITC-labeled antisense ODN was used to check the cellular uptake of ODN.

Surgical preparation of animal and experimental protocol

All animals were treated in compliance with ‘The Guide for the Care of Use of Laboratory Animals’ published by the US national Institute of Health (NIH Publication No. 85-23, revised 1996) and followed the rules of National Animal Protection of China.

Male adult Sprague–Dawley rats weighing 200-250g were anesthetized with an initial intraperitoneal injection of sodium pentobarbital (40-50 mg/kg). The animals were ventilated with oxygen-enriched room air using a rodent respirator with 60 breaths per minute and the tidal volume was set to 8ml. The right femoral artery was cannulated for monitoring arterial pressure and blood sampling. The left ventricular pressure was measured via a polyethylene catheter inserted into the left ventricle through the right common carotid artery. The chest was opened via a left thoracotomy through the fifth intercostal space. After pericardiotomy, a silk suture was placed under the left anterior descending coronary artery (LADCA), 2-3 mm from its origin, and the ends of the tie were threaded through a small plastic tube to form a snare for reversible LADCA occlusion.

All rats were randomly assigned into one of seven groups: Sham, Control (I/R control), AS-I/R (I/R treated with antisense Egr-1 ODN), S-I/R (I/R treated with sense Egr-1 ODN), Sc-I/R (I/R treated with scrambled-sequence Egr-1 ODN), F₂ [I/R treated with F₂ at a dose of 2mg/kg dissolved in 33% (V/V) polyethylene glycol 400 (PEG)], PEG (I/R treated with 33% 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 the suture was left untied. The ODNs at the dose of 4 mg/kg respectively were given through the sublingual vein 24 h before ischemia. F₂ or or equal volume of PEG was administered 5 min before ischemia. Hemodynamic parameters were measured with BL-420 Biological Experimental System (Chengdu Technology & Market Co, LTD, China) at baseline, 60 min of ischemia and 180 min of reperfusion. At the end of experiment, blood was taken

Fig. 2. The ODN uptake of myocardial tissues and cultured cardiomyocytes was analyzed 24h after administration with FITC-labeled antisense Egr-1 ODN. Fluorescence was detected in the cytoplasm and nuclei of myocardial tissues (B), while mainly in the nuclei of cultured cardiomyocytes (D) by fluorescence microscopy. Photo A and C were corresponding field of photo B and D respectively by phase-contrast microscopy (Bar=100 μ m).

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 mRNA and protein.

Isolation of neonatal rat cardiomyocytes and preparation of H/R

Neonatal rat ventricular myocytes were isolated from 1- 4 days old Sprague-Dawley rats. The hearts were rapidly excised, minced and dissociated with 0.1% trypsin. The dispersed cells were plated in the medium with 15% FBS for 30 min to remove non-cardiomyocytes [19]. The isolated cardiomyocytes at a density of 2.5×10^6 cells per well were then cultured in the medium with 5-Bromo deoxyuridine (0.1mmol/L) to further inhibit the growth of non-cardiomyocytes for the first 3 d [20]. Hypoxia was induced by replacing the initial culture medium with pH 6.2 buffer (in mM): 137 NaCl, 12 KCl, 0.49 MgCl_2 , 0.9 $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 4 HEPES, 20 Na lactate [19], and the cardiomyocytes were incubated in an air-tight chamber gassed with pure N_2 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% CO_2) for 1 h of reoxygenation.

Experimental protocols

After 5 – 7 days of cell culture in normoxic medium, the cardiomyocytes were randomly divided into one of eight groups: Sham, Control (H/R control), Lip-H/R (H/R treated with LipofectamineTM2000 Reagent), AS-H/R (H/R treated with

antisense Egr-1 ODN), S-H/R (H/R treated with sense Egr-1 ODN), Sc-H/R (H/R treated with scrambled-sequence Egr-1 ODN), F_2 (H/R treated with F_2 at a dose of 1×10^{-6} mol/L dissolved in PEG), PEG (H/R treated with 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. The ODNs at the dose of 4×10^{-7} mol/L were transfected into cultured cell 24 h before hypoxia by using LipofectamineTM2000 Reagent. F_2 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 tumor necrosis factor- α (TNF- α). The cardiomyocytes were collected to measure the levels of SOD, MDA and Egr-1 mRNA and 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 creatine kinase (CK) and lactate dehydrogenase (LDH) in plasma and culture medium were determined using the test kits (Jiancheng Bioengineering Institute, Nanjing, China).

Activity of superoxide dismutase (SOD) and level of malondialdehyde (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

Table 1. Enzyme activities and MDA level in plasma and 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 myocardial tissues. All values are expressed as mean±S.E.M. (n=8). *P<0.05 vs. Sham group; † P<0.05 vs. Control group.

Group	CK	LDH	SOD	MDA
Sham	60±3	21186±726	231±11	3.9±0.2
Control	134±6*	83836±1630*	120±9*	9.6±0.4*
S-I/R	139±4*	80942±2972*	114±10*	9.2±0.3*
Sc-I/R	137±7*	79575±2003*	127±9*	9.0±0.5*
AS-I/R	90±7*†	47054±3462*†	158±10*†	5.4±0.2*†

Table 2. Enzyme activities and MDA level in culture medium and cultured cardiomyocytes of different groups *in vitro*. 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 culture medium. SOD and MDA were from cardiomyocytes. All values are expressed as mean±S.E.M. (n=10). *P<0.05 vs. Sham group; † P<0.05 vs. Control group.

Group	CK	LDH	SOD	MDA
Sham	11.3±1.1	61±3	131±6	0.37±0.03
Control	32.0±2.0*	263±10*	70±4*	1.28±0.06*
Lip-H/R	30.2±1.7*	271±14*	74±6*	1.30±0.09*
S-H/R	30.1±1.6*	266±11*	79±4*	1.35±0.08*
Sc-H/R	31.8±1.7*	284±11*	77±6*	1.35±0.08*
AS-H/R	21.8±0.7*†	107±5*†	106±5*†	0.69±0.04*†

xanthine oxidase method and the level of MDA using thiobarbituric acid stain method.

Myocardial tissue myeloperoxidase (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 µmol 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 µl) 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 assessed using ELISA kit (Bender Medsystems Inc, Austria). Briefly, standards (50 µl) and samples (100 µl) were added to a 96-well plate coated with capture antibody. Then, Bio-conjugate anti-rat TNF-α antibody was added to all wells. After incubating for 2h, each well was washed four times with PBS-T (PBS containing 0.1% Tween 20), and then incubated with avidin-HRP for 1h. After thorough washing, each well was incubated

with substrate solution for 15min. Reaction was stopped by 1M phosphoric acid. Plate was read at 450nm and the concentration of TNF-α was calculated according to the standard curve.

Northern-blot analysis

Total RNA extracted from myocardial tissue (30 µg) or cultured cardiomyocyte (20 µg) was denatured at 50°C in the presence of glyoxal, separated by electrophoresis in 1.5% agarose gel and blotted onto Hybond-N membrane. Blots were hybridized with the random-primed ³²P-labeled cDNA Egr-1 probe at 42°C overnight (Takara Biotechnology Co., Ltd, China). Membranes were then stripped and rehybridized with ³²P-labeled β-actin as an internal control for RNA loading.

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 µg for tissue and 80 µg 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

Fig. 3. Measurement of Egr-1 protein in myocardial tissues and cultured cardiomyocytes by Western blot assay. Quantitative densitometric data were expressed as a percentage of that observed in Control group. All values are expressed as mean $\pm$ S.E.M of at least 5 separate experiments. * $P < 0.05$ vs. Sham group; † $P < 0.05$ vs. Control group.

Fig. 4. The MPO activity of area at risk myocardium in a rat model of 60 min coronary occlusion followed by 180 min of reperfusion. The values are expressed as mean $\pm$ S.E.M, $n = 10$. * $P < 0.05$ vs. Sham group; † $P < 0.05$ vs. Control group.

(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.

Statistical analysis

The band relative densities of mRNA or protein were analyzed by Fluorchem™ software (Alpha Innotech, USA). Data are shown as the 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.

Time	Group	HR	SP	LVSP	+dP/dt _{max}	-dP/dt _{max}
Baseline	Sham	361±8	156±4	192±5	11933±424	9750±481
	Control	373±10	149±9	186±4	10798±403	8282±341
	PEG	375±6	176±5	189±5	11333±452	9536±557
	F ₂	379±5	172±10	188±1	10332±603	8680±479
Ischemia	Sham	294±13	137±3	164±6	7784±385	5032±532
	Control	259±12*	116±8*	141±8*	6202±451*	3549±285*
	PEG	267±13*	121±6	137±8*	6472±507*	4042±508*
	F ₂	268±21*	127±9	156±10	6931±620*	4604±457†
Reperfusion	Sham	260±14	132±6	151±9	6523±384	4082±425
	Control	168±14*	76±8*	94±5*	3682±407*	1530±187*
	PEG	180±10*	84±10*	99±6*	3893±413*	1748±250*
	F ₂	209±16*	117±9†	128±6†	6046±429†	3806±441†

Table 3. Hemodynamic parameters in different groups during the course of the experiment. HR, heart rate (beats/min); SP, systolic pressure (mmHg); LVSP, left ventricular peak 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±S.E.M. (n=10). *P<0.05 vs. Sham group; † P<0.05 vs. Control group.

Results

Uptake of antisense Egr-1 ODN by myocardial tissues and cultured cardiomyocytes

FITC labeled antisense Egr-1 ODN was found in the cytoplasm and nuclei of myocardial tissues, while mainly in the nuclei of cultured cardiomyocytes (Fig. 2), indicating that antisense Egr-1 ODN had successfully been uptaken up.

Effects of antisense Egr-1 ODN on changes of enzymetic activities induced by I/R or H/R

Comparisons in levels of CK, LDH, SOD and MDA among groups were shown in Tables 1 and 2. After reperfusion or reoxygenation, the levels of CK, LDH and the product of MDA increased significantly and the activity of SOD decreased significantly in plasma, myocardial tissues, culture medium and cultured cells, as compared with the respective Sham groups. However, antisense Egr-1 ODN could maintain the activity of SOD, reduce

Group	cTnI	TNF-α
Sham	3.06±0.21	71±8
Control	4.81±0.22*	414±31*
PEG	4.89±0.27*	386±19*
F ₂	2.72±0.13†	210±22*†

Table 4. Levels of cTnI and TNF- α in supernatant of cultured cardiomyocytes of different groups. cTnI, Cardiac troponin I (ng/mL, n=10). TNF- α, Tumor necrosis factor-α (pg/mL, n=7). All values are expressed as mean±S.E.M. *P<0.05 vs. Sham group; † P<0.05 vs. Control group.

the product of MDA, and minimize the leakage of CK and LDH out of myocardial cells caused by I/R or H/R. There were no differences between Control, S-I/R and Sc-I/R groups nor between Control, Lip-H/R, S-H/R and Sc-H/R groups.

Fig. 5. Measurement of Egr-1 mRNA level in myocardial tissues and cultured cardiomyocytes by Northern blot. Quantitative densitometric data were expressed as a percentage of β -actin. All values are expressed as mean $\pm$ S.E.M of at least 4 separate experiments. * $P < 0.05$ vs. Sham group; † $P < 0.05$ vs. Control group.

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

As shown in Fig. 3, compared with the respective Sham groups, expression levels of Egr-1 protein in myocardial tissues and cultured cardiomyocytes of Control group were significantly increased, but were significantly inhibited when antisense Egr-1 ODN was transfected into the animals and cultured cells. There were no differences between Control, S-I/R and Sc-I/R groups nor between Control, Lip-H/R, S-H/R and Sc-H/R groups.

Effects of F_2 on hemodynamic variables

Hemodynamic parameters measured throughout the experiments are summarized in Table 3. There were no differences between groups in all baseline hemodynamic parameters. LCA occlusion significantly decreased heart rate (HR), systolic pressure (SP), left ventricular peak

Fig. 6. Measurement of Egr-1 protein in myocardial tissues and cultured cardiomyocytes by Western blot assay. Quantitative densitometric data were expressed as a percentage of that observed in Control group. All values are expressed as mean $\pm$ S.E.M of at least 5 separate experiments. * $P < 0.05$ vs. Sham group; † $P < 0.05$ vs. Control group.

systolic pressure (LVSP) and positive and negative dp/dt. 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, except HR, were significantly alleviated by F_2 . No significant change in hemodynamics was detected in the Sham group throughout the experimental period.

Effect of F_2 on myocardial tissue MPO activity

The tissue MPO activities in the four groups are shown in Fig. 4. In the Sham group, MPO activity was low at the end of observation period. However, in the Control group, MPO activity was markedly increased relative to the normal tissue in the Sham group. Administration of F_2 significantly reduced MPO activity

in the ischemic myocardium compared with the Control group. These data suggest that F₂ reduced neutrophil accumulation in the ischemic/reperfused myocardium. No significant difference in MPO activity between the Control and PEG groups was found.

Effects of F₂ on levels of cTnI and TNF- α in culture medium

The levels of cTnI and TNF- α in culture medium were measured as shown in Table 4. Compared with the Control group, H/R caused significantly cTnI leakage and TNF- α release from cultured cardiomyocytes. Treatment with F₂, but not PEG, reduced significantly cTnI and TNF- α release from cultured cells.

Effects of F₂ on levels of Egr-1 mRNA and protein in myocardial tissues and cultured cardiomyocytes

Levels of Egr-1 mRNA in four groups are shown in Fig. 5. Relative to the respective Sham groups, levels of Egr-1 mRNA in myocardial tissues and cultured cardiomyocytes in the Control groups were significantly increased at the end of experiment. These changes were not altered by PEG, but significantly reduced when F₂ was administered to the animals and cultured cells.

Levels of Egr-1 protein in four groups are shown in Fig. 6. Consistent with changes in levels of Egr-1 mRNA in the Control groups, levels of Egr-1 protein in myocardial tissues and cultured cardiomyocytes were significantly increased relative to the respective Sham groups. Similarly, these changes were not altered by PEG, but significantly reduced with F₂.

Discussion

It has been reported that I/R condition caused expression and activation of Egr-1 which could coordinately upregulate downstream target genes such as interleukin 1 β , macrophage inflammatory protein, intercellular adhesion molecule 1 (ICAM-1), tissue factor, plasminogen-activator inhibitor and vascular endothelial growth factor [11, 18, 21]. The expressions of these representative genes mediate coagulation, inflammation and vascular permeability, which are the main pathological changes of I/R injury. Thus, Egr-1 was designated as having a central and unifying role in the pathogenesis of I/R injury [11].

The present study demonstrated that I/R or H/R caused the overexpressions of Egr-1 protein in myocardial

tissues and cells. Moreover, the induction of Egr-1 after I/R or H/R was consistent with myocardial injury as evidenced by the rise of CK and LDH, the reduction in SOD activity and the increase in yield of MDA. Additionally, we found that only antisense Egr-1 ODN (but not sense Egr-1 ODN or scrambled-sequence Egr-1 ODN) reduced Egr-1 protein level and thereby attenuated the myocardial injury. Our results are in agreement with the study of Okada M *et al*, who employed lung I/R model with the same ODNs and found that Egr-1 have profound effects on lung I/R injury [11] and the study of Bhindi *et al*, where they found that DNazymes targeting Egr-1 could attenuate myocardial neutrophil infiltration, ICAM expression, and myocardial infarct size [22].

Bhindi *et al* recently hypothesized that Egr-1 upregulation contributes to myocardial I/R injury. Almost at the same time, we reported that the protective effect of F₂ on myocardial I/R injury was associated with the inhibition of Egr-1 overexpression [17]. Combining the present *in vivo* and *in vitro* study with these data, we also consider that Egr-1 could act as a trigger in the pathogenesis of myocardial I/R injury [22].

Based on the relationship between Egr-1 overexpression and myocardial I/R injury, new parameters and methods were used to further study the relationship between the cardioprotective effects of F₂ and its regulative effect on Egr-1 expression after I/R. The results from Western-blot and Northern-blot analysis verified the former results from reverse transcription-polymerase chain reaction, immunohistochemistry and immunocytochemistry, showing that F₂ could downregulate the overexpressions of Egr-1 mRNA and protein in myocardial tissue and cell induced by I/R or H/R. Meanwhile, besides the reduction in enzyme release and ultrastructural changes just as recently reported [17], the cardioprotection with F₂ was shown by the amelioration of the hemodynamics after I/R *in vivo* and the attenuation of the cTnI leakage from cultured cardiomyocytes after H/R *in vitro*. As we all know, cTnI is a highly sensitive and specific indicator of myocardial injury [23]. These results also showed concordance with our previous study, in which treatment with F₂ decreased myocardial infarct size following I/R. Furthermore, the present study showed that F₂ significantly weakened the inflammation *in vivo* or *in vitro* caused by I/R or H/R, demonstrated by a decrease in MPO activity due to accumulation and activation of neutrophil and the reduced release of TNF- α , a pivotal inflammatory factor secreted by cardiomyocytes after stress. Combining with the literature,

it is reasonable to speculate that the anti-inflammatory effect of F_2 is associated with its inhibition of the expression of Egr-1 and the secretion of TNF- α . TNF- α is an important downstream inflammatory gene of Egr-1 [16, 24], and it can in turn activate inflammatory cells such as neutrophil which can result in the increase of MPO activity. Based on these findings, it seems reasonable to speculate that the cardioprotective activity of F_2 in myocardial I/R might be associated with blocking Egr-1 expression/TNF- α secretion/neutrophil activation/ MPO release pathway.

According to the studies of Russo MW *et al* and Svaren J *et al*, Egr-1 transcriptional activity can be blocked by the corepressors NAB1/NAB2 [25, 26], thus high level of Egr-1 isn't equal to strong biological activity. However, in this study overexpression of Egr-1 was in concomitance with high level of TNF- α and low level of SOD. As TNF- α and SOD can be regulated positively and negatively [27] respectively by Egr-1, the results from this study can in turn prove the Egr-1 biological activity. Therefore, with antisense Egr-1 ODN, the present study *in vivo* and *in vitro* demonstrated unanimously that overexpression of Egr-1 could be responsible for myocardial I/R injury. Moreover, a series of beneficial effects of F_2 on I/R or H/R injury were largely due to the inhibition of Egr-1 overexpression.

It has been reported that Egr-1 expression is induced by Ca^{2+} stimulation and its up-regulation is Ca^{2+} -dependent [27, 28]. The data from our laboratory suggest that cardioprotection by F_2 is also associated with the inhibition of Ca^{2+} overload [4, 5] well-documented in I/R injury. Therefore, we deduce that antagonism to myocardial I/R injury of F_2 might, in part, be a result of the downregulation of Ca^{2+} overload-mediated Egr-1 overexpression. However, the detailed signal transduction cascade between Ca^{2+} and Egr-1 needs further exploration.

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