The Protective Effect of Egr-1 Antisense Oligodeoxyribonucleotide on Myocardial Injury Induced by Ischemia-reperfusion and Hypoxia-reoxygenation

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Key Words

Antisense oligodeoxyribonucleotide • Early growth response-1 • Hypoxia-reoxygenation • Ischemia-reperfusion • Myocardial

Abstract

Aims: Our previous studies have shown that myocardial ischemia-reperfusion (I/R) injury is related closely with early growth response (Egr)-1 overexpression. The present study is to confirm thoroughly the effects of Egr-1 on the occurrence and development of myocardial I/R injury. **Methods:** The Sprague-Dawley rat myocardial I/R model and cultured cardiomyocyte hypoxia-reoxygenation (H/R) model were established. The synthesized Egr-1 antisense oligodeoxyribonucleotide (AS-ODN) was transfected into myocardial tissues and cells. Hemodynamic parameters, myeloperoxidase (MPO), cardiac troponin I (cTnI), tumor necrosis factor- α (TNF- α), morphology, spontaneous beat and cell viability were measured to assess the degree of injury and inflammation of myocardial tissues and cells. **Results:** *In vivo*, Egr-1 AS-ODN significantly attenuated injury

and inflammation of myocardial tissues caused by I/R evidenced by the amelioration of hemodynamics and the reduction in MPO activity. *In vitro*, Egr-1 AS-ODN significantly relieved injury and inflammation of cultured cardiomyocyte caused by H/R evidenced by the improvement in morphology, structure and beat as well as the decrease in leakage of cTnI and release of TNF- α from cultured cardiomyocyte. **Conclusions:** These data suggest that Egr-1 plays a vital role in the pathogenesis of myocardial I/R injury and Egr-1 AS-ODN could protect the myocardium from I/R injury.

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Introduction

The early growth response (Egr)-1 gene belongs to the category of immediate early genes. Egr-1 protein (also called NGFI-A, Zif268, Krox-24 and TIS8) encoded by Egr-1 gene is an 80-82 kDa transcription regulatory factor. The Egr-1 protein contains three zinc finger DNA-binding

domains and binds to a G/C-rich consensus sequence, GCG (G/T) GGG CG. Egr-1 binding domains are present 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, tissue factor, plasminogen-activator inhibitor and platelet-derived growth factor (PDGF)-A, et al [1-4]. Therefore, although Egr-1 function was initially thought to be only associated with growth and differentiation, 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 [1, 5]. Moreover, Egr-1 has been termed as an immediate-early response protein due to its brisk kinetics of induction and decay, but it can exert a commanding influence on long-term cellular homeostasis by regulating the expression of multiple downstream target genes. Egr-1 becomes a manifest in response to environmental challenge and acts as an important pathogenetic factor.

Myocardial ischemia-reperfusion (I/R) injury is caused by blockage of coronary blood flow followed by restoration of blood flow, resulting in cardiac contractile dysfunction and arrhythmias, as well as other irreversible cardiomyocyte damage. It represents a clinically relevant problem associated with thrombolysis, angioplasty, organ transplantation and coronary bypass surgery. The research about myocardial I/R injury is an important area in cardiology. Although the essential factors, including leukocytic adherence and invasion, activation of coagulant and oxidative stress, have been implicated in the pathogenesis of the tissue injury [6, 7], but the molecular basis for this pathological process have not been clearly identified. It was reported that I/R condition in many organs including heart causes overexpression of Egr-1, which indicates that Egr-1 is involved in I/R injury [2, 8-12]. Furthermore, Egr-1 has a well-established pivotal role in pathogenesis of lung I/R injury [2, 3]. Nevertheless, whether Egr-1 has the similar role in myocardial I/R injury has not been fully identified yet.

Our preliminary study had shown that Egr-1 antisense oligodeoxynucleotide AS-ODN could relieve myocardial injury as evidenced by the inhibition in leakage of CK and LDH, and the reduction in oxidative stress by the inhibition of Egr-1 protein expression [13]. The purpose of this study is to investigate further the effects of Egr-1 AS-ODN on morphology, function and inflammation of myocardial tissues or cultured cardiomyocytes in I/R model *in vivo* and *in vitro*, which will elucidate the central role of Egr-1 in

occurrence and development of myocardial I/R injury.

Materials and Methods

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

Egr-1 antisense, sense and scrambled-sequence phosphorothioate ODNs of 15 base-pair length were commercially synthesized (Takara Biotechnology Co., Ltd, China). The sequence of Egr-1 antisense ODN (AS-ODN) was 3'-TAC CGT CGC CGG TTC -5' as described previously [3]. For control experiments, Egr-1 sense ODN (S-ODN) (3'-GAA CCG GCG ACG GTA -5') and scrambled-sequence ODN (Sc-ODN) (3'-TCG TGC CGC TGC CAT -5') were used [3]. FITC-labeled AS-ODN was used to check the cellular uptake of ODN.

Preparation of myocardial I/R

All animals were treated in compliance with the rules of National Animal Protection of China. Adult male Spargue-Dawley rats (weighing 200-250g, Grade II, Certificate 2007A024) were provided by the Experimental Animal Center of Medical College, Shantou University (Shantou, China). Myocardial I/R model of rat was performed as previously described [13]. 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: Sham, Control (I/R control), AS-I/R (I/R treated with Egr-1 AS-ODN), S-I/R (I/R treated with Egr-1 S-ODN) and Sc-I/R (I/R treated with Egr-1 Sc-ODN). 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 ODNs at the dose of 4 mg/kg respectively were given through the sublingual vein 24 h before ischemia. At the beginning of experiment, a cannula was inserted into the right femoral artery, and then hemodynamic parameters were measured by 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, the ischemic myocardium was excised for level of myeloperoxidase (MPO).

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 [13]. 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 d to eliminate non-cardiomyocytes [14].

After 5-7 days of cell culture in normoxic medium, the cardiomyocytes were randomly divided into one of six groups: Sham, Control (H/R control), Lip-H/R [H/R treated with Lipofectamine™2000 Reagent (Lip)], AS-H/R (H/R treated with Egr-1 AS-ODN), S-H/R (H/R treated with Egr-1

Time	Group	HR	SP	MAP	LVSP	+dp/dt _{max}	-dp/dt _{max}
Ischemia	Sham	365±12	133±7	82±3	148±6	9000±329	5836±319
	Control	319±17*	88±5*	62±3*	111±2*	5776±320*	3704±292*
	S-I/R	311±15*	85±6*	61±4*	106±3*	5796±341*	3784±317*
	Sc-I/R	318±17*	88±6*	65±5*	107±4*	5599±375*	3796±342*
	AS-I/R	377±15†	97±4	74±4†	123±5†	6629±493	4501±324
Reperfusion	Sham	337±9	127±5	82±2	141±4	7809±373	5487±245
	Control	262±21*	71±3*	52±4*	93±3*	3922±248*	2828±239*
	S-I/R	267±25*	72±4*	52±5*	94±4*	4114±272*	2896±282*
	Sc-I/R	245±20*	70±5*	49±4*	92±5*	3959±299*	2735±337*
	AS-I/R	358±15†	83±5†	63±5†	105±7†	5357±446†	3814±354†

Table 1. Hemodynamic parameters in different groups during the course of the experiment. HR, heart rate (beats/min); SP, systolic pressure (mmHg); MAP, mean-arterial 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.

S-ODN) and Sc-H/R (H/R treated with Egr-1 Sc-ODN). 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·H₂O, 4 HEPES, 20 Na lactate [15], 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 ODNs at the dose of 4×10⁻⁷ mol/L were transfected into cultured cells 24 h before hypoxia by using Lip. At the end of reoxygenation, the culture medium was collected for levels of cardiac troponin I (cTnI) and TNF-α.

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 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 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.

Assessment of morphologic and beat of cultured cardiomyocytes

The cardiomyocytes in the dishes were observed in the inverted microscope for their morphology and beat. Meanwhile, the cells grown on coverslips were fixed with ice-cold acetone, and then stained routinely with hematoxylin-eosin (H-E). The stained cells were observed and photographed in the optic microscope for the morphology.

Cell viability

Cell viability was determined by the trypan blue dye exclusion method as follows: the attached cardiomyocytes were digested with 0.1% trypsin and 0.02% EDTA (V/V 1:1). Free cells were collected in cold culture medium with 15% calf serum. The cell suspensions (900uL) were fixed with 0.4% trypanblau (100uL). After stained for 3 min, the viable (blue stained) and nonviable (blue-negative) cells were counted according to the standard procedure for white blood cell count. The viability of cardiomyocytes was expressed as a percentage of viable cells/total cells.

Statistical analysis

All data are shown as the mean ±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 as statistically significant.

Fig. 1. 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=8. *P<0.05 vs. Sham group; †P<0.05 vs. Control group.

Table 2. 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=6). Lip, Lipofectamine™2000 Reagent. Before being transfected into cultured cells, the ODNs were mixed with Lip to form the ODN-Lip complexes. All values are expressed as mean $\pm$ S.E.M. *P<0.05 vs. Sham group; †P<0.05 vs. Control group.

Group	cTnI	TNF- α
Sham	0.72 $\pm$ 0.05	76.10 $\pm$ 4.37
Control	3.10 $\pm$ 0.10*	427.70 $\pm$ 29.47*
Lip-H/R	3.00 $\pm$ 0.07*	470.75 $\pm$ 34.82*
S-H/R	2.97 $\pm$ 0.13*	451.21 $\pm$ 30.54*
Sc-H/R	3.00 $\pm$ 0.05*	424.75 $\pm$ 20.96*
AS-H/R	1.08 $\pm$ 0.07*†	285.77 $\pm$ 19.51*†

Results

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

As reported previously [13], FITC labeled Egr-1 AS-ODN was found in the cytoplasm and nuclei of myocardial tissues, while mainly in the nuclei of cultured cardiomyocytes (data not shown), indicating that Egr-1 AS-ODN had successfully been uptaken up.

Effects of Egr-1 AS-ODN on hemodynamic variables

Hemodynamic parameters measured throughout the experiments are summarized in Table 1. There were no differences between groups in all baseline hemodynamic parameters (data not shown). LADCA occlusion significantly decreased heart rate (HR), systolic pressure (SP), mean-arterial pressure (MAP), 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 S-I/R or Sc-I/R 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. No significant

change in hemodynamics was detected in the Sham group throughout the experimental period.

Effect of Egr-1 AS-ODN on myocardial tissue MPO activity

The tissue MPO activities in the five groups are shown in Fig. 1. 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 Egr-1 AS-ODN significantly reduced MPO activity in the ischemic myocardium compared with the Control group. These data suggest that Egr-1 AS-ODN reduced neutrophil accumulation in the ischemic/reperfused myocardium. No significant differences in MPO activity among the Control, S-I/R and Sc-I/R groups were found.

Effects of Egr-1 AS-ODN 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 2. Compared with the Control group, H/R caused significantly cTnI leakage and TNF- α release from cultured cardiomyocytes. Treatment with Egr-1 AS-ODN, but not S-ODN, Sc-ODN or Lip,

Fig. 2. The morphologic changes of cultured cardiomyocytes were detected by hematoxylin-eosin staining. (A) In Sham group, the pseudopodia of polygonal cultured cardiomyocytes were obviously detected. (B) In Control group, a majority of cells shrank and became round. The pseudopodia of cells were decreased. (C) In AS-ODN group, the morphology of most cells was normal, and their pseudopodia were abundant and clear. Experiments were repeated five times and similar results were obtained. Bar=50 μ m.

Fig. 3. The viability of cultured cardiomyocytes after 3 h of hypoxia followed by 1 h reoxygenation. Cell viability was determined by the trypan blue dye exclusion method and expressed as a percentage of viable cells/total cells. The values are mean $\pm$ S.E.M, n=10. *P<0.05 vs. Sham group; † P<0.05 vs. Control group.

reduced significantly cTnI and TNF- α release from cultured cells.

Effects of Egr-1 AS-ODN on morphology and beat of cultured cardiomyocytes

In the Sham group, the pseudopodia of polygonal cultured cardiomyocytes were obviously detected [Fig. 2(A)]. The spontaneous beat of cardiomyocytes was synchronized, rhythmical and powerful, and its rate was 85 ± 4 beats/min (range, 80-100 beats/min). After H/R, cardiomyocytes contracted, tending to get round in shape and its pseudopodia decreased [Fig. 2(B)], at the same time, arrhythmic beat and even cardiac arrest

were detected. However, these changes were markedly inhibited when Egr-1 AS-ODN, but not S-ODN, Sc-ODN or Lip, was transfected into the cells before H/R [Fig. 2(C)].

Effect of Egr-1 AS-ODN on viability of cultured cardiomyocytes

Viability of cardiomyocytes is shown in Fig. 3. Relative to the Sham group, cell viability in the Control group was significantly decreased. This change was not altered by S-ODN, Sc-ODN or Lip, but significantly reduced when Egr-1 AS-ODN was transfected into the cells.

Discussion

Egr-1 is an important transcription factor which can recruit multiple downstream target genes, especially many proinflammatory genes. Distinguished from extracellular “first messenger” and cytoplasmic “second messenger”, Egr-1 could act as intranuclear “third messenger” to translate extracellular signals into long-term cellular responses in signal transduction cascade [16-18]. According to recent data, Egr-1 expression is upregulated after I/R challenge in many organs such as lung, heart, gut and kidney [8-12]. In addition, it was reported that some drugs could prevent hepatic I/R injury by inhibiting Egr-1 expression [19, 20]. And what's more, with Egr-1 knock-out or AS-ODN technology, Yan et al. and Okada et al. well demonstrated that Egr-1 could be a master switch in pathogenesis of lung I/R injury due to triggering expression of crucial regulators in the main pathological processes of I/R injury [2, 3].

Our previous study demonstrated that I/R or H/R causes the overexpression of Egr-1 in myocardial tissues and cells [21]. In order to elucidate the cause-effect relationship between Egr-1 overexpression and myocardial I/R injury, we further studied the effects of Egr-1 AS-ODN on changes of enzymatic activities induced by I/R or H/R [13]. In above-mentioned study, we found that because of its direct inhibitory effect on Egr-1 protein Egr-1 phosphorothioate AS-ODN attenuated the myocardial I/R injury in cytomembrane structure as evidenced by the reduction of CK and LDH leakage, and in oxidative stress caused by reactive oxygen species (ROS) as evidenced by the increase of superoxide dismutase (SOD) activity and the decrease of malondialdehyde (MDA) yield [13]. In addition, Bhindi *et al* found that DNazymes targeting Egr-1 could attenuate myocardial neutrophil infiltration, increased ICAM-1 expression and myocardial infarct size following I/R [22], but as we all know, a series of damage in morphology, structure and function will appear in myocardial tissues and cells after I/R, so further evidences are needed to confirm the hypothesis that Egr-1 is a key contributor to myocardial I/R injury.

The present study demonstrated that Egr-1 AS-ODN exerted a great cardioprotective activity in I/R experiments after being transfected into myocardial tissues and cells by previously described methods [13], which successfully inhibited the expression of Egr-1 protein. It significantly improved myocardial

dysfunction demonstrated by the amelioration of hemodynamics *in vivo* and the spontaneous beat rhythm *in vitro*, and inhibited myocardial inflammation demonstrated by the decrease in MPO activity *in vivo* and in TNF- α release *in vitro*. Meanwhile, Egr-1 AS-ODN relieved the myocardial injuries in morphology and structure as evidenced by inhibition of the changes of cell morphology in microscope and increase of cell viability determined by cytomembrane integrity. Notably, besides the reduction in CK and LDH leakage as recently reported [13], the cardioprotection with Egr-1 AS-ODN was shown by the attenuation of the cTnI leakage from myocardial cells after H/R. cTnI leakage is accepted as the most specific and sensitive marker of myocardial injury and a breakdown signal of the contractile apparatus [23, 24], which accounted for abnormality in hemodynamics and spontaneous beat after I/R or H/R injury.

SOD is an important protective enzyme, whose function is to detoxify ROS. It was reported that overexpression of Egr-1 induces down-regulation of SOD and stimulates the generation of ROS [25], which was supported by our previous results as mentioned above [12]. Whereas, further studies are required to investigate whether Egr-1 down-regulates SOD after I/R challenge by competing with Sp1 [26-28], which is another transcription factor and a transcription enhancer of SOD, for overlapping binding site on SOD gene or not. It is well recognized that TNF- α , a downstream gene of Egr-1, is a vital inflammatory factor and secreted by mononuclear macrophage and myocardial cell after I/R [29]. More importantly, it is an early and central event because of its extensive actions on other cytokines and its comprehensive contribution to myocardial injury after I/R such as direct cytotoxicity, calcium dyshomeostasis and so on [29]. In all parameters we examined, although only SOD and TNF- α have direct relationship with Egr-1, considering their critical roles respectively in ROS toxicity and inflammation, which are fundamental and uniform pathophysiologic changes of myocardial I/R injury, the vital role of Egr-1 in the pathogenesis of myocardial I/R injury had been manifested to a great extent in this study. In addition, as expected, S-ODN, Sc-ODN and Lip 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.

In summary, this study showed that Egr-1 AS-ODN

could protect strikingly myocardium from I/R injury in different phases, from undetectable initial lesions such as cTnI leakage to final noticeable damage such as myocardial dysfunction. The data from our present and previous studies *in vivo* and *in vitro* demonstrated unanimously that Egr-1 might play a vital role in pathogenesis of myocardial I/R injury by up-regulation of harmful factor and down-regulation of protective factor. Therefore, comparing with existing remedy only to one aspect of I/R injury, the drug targeting Egr-1 would be a more perspective therapeutic strategy because of the Egr-1 ubiquitous transcriptional activity which is responsible for I/R-driven activation of multiple gene cascades.

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