

***N*-n-butyl Haloperidol Iodide Protects Cardiac Microvascular Endothelial Cells From Hypoxia/Reoxygenation Injury by Down-Regulating Egr-1 Expression**

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Key Words

Early growth response-1 • *N*-n-butyl haloperidol iodide • Cardiac microvascular endothelial cells • Hypoxia/reoxygenation

Abstract

Aims: Our previous studies have shown that *N*-n-butyl haloperidol iodide (F2) can antagonize myocardial ischemia/reperfusion (I/R) injury by down-regulating the early growth response (Egr)-1 expression, but the molecular mechanisms are not well understood. Because there is evidence implicating myocardial I/R injury is closely associated with endothelial dysfunction. The present study is to test the hypothesis that the protective effects of F2 on myocardial I/R injury is related closely with down-regulating Egr-1 expression on cardiac microvascular endothelial cells (CMECs). **Methods:** A model of cultured CMECs exposed to hypoxia/reoxygenation (H/R) was developed. With antisense Egr-1 oligodeoxyribonucleotide (ODN), the relationship between Egr-1 expression and endothelial H/R injury

was investigated. Egr-1 mRNA and protein expression were examined by real-time fluorescent quantitative PCR, immunocytochemical staining and Western-blot analysis. Lactate dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD), intercellular adhesion molecule-1 (ICAM-1), adherence of neutrophil and platelets, and cell viability were measured after H/R to evaluate the degree of endothelial injury. **Results:** Pretreatment with antisense Egr-1 ODN significantly reduced Egr-1 protein expression and attenuated injury of CMECs. Consistent with down-regulation of Egr-1 expression by F2, inflammation and other damage were significantly reduced as evidenced by a decrease of ICAM-1 expression, reduction of neutrophil and platelets adherence, increase in SOD, and decreases in MDA and LDH levels, resulting in the rise of cell viability. **Conclusions:** We demonstrate a protective effect of F2 in CMECs against H/R injury by down-regulating Egr-1 expression, which might be play a vital role in the pathogenesis of myocardial I/R injury.

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1015-8987/10/0266-0839\$26.00/0

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Introduction

The early growth response (Egr)-1 gene is one of the immediate-early genes and a member of zinc finger family of transcription factors. Its product, Egr-1 protein, with sequence-specific DNA-binding domains, binds to a GC-rich consensus site and activates expression of multiple downstream target genes, including interleukin 1 β , macrophage inflammatory protein, intercellular adhesion molecule 1 (ICAM-1), tissue factor (TF), plasminogen-activator inhibitor and vascular endothelial growth factor [1]. According to recent data, Egr-1 expression is up-regulated in many organs after ischemia/reperfusion (I/R) challenge. It has been reported that pharmacological down-regulation of the Egr-1 pathway could prevent hepatic I/R injury [2]. With Egr-1 knock-out or antisense oligodeoxyribonucleotide (ODN) technology, it well demonstrated that loss of Egr-1 could reduce expression of crucial regulators to block pathogenesis in I/R injury [1, 3]. These findings indicate that up-regulation of Egr-1 could be the common denominator in I/R injury in various organs and Egr-1 might be a good therapeutic target for I/R injury.

We and others have demonstrated increased Egr-1 expression in the heart following I/R [4, 5]. Myocardial tissue contains all different types of cells, including endothelial cells, cardiomyocytes and inflammatory cells (i.e., neutrophils and macrophages). Therefore, the analysis from such a tissue block can not differentiate cell types in response to Egr-1 expression. To resolve this issue, endothelial cells and cardiomyocytes should be isolated for determination of Egr-1 expression respectively. In addition, cardiac endothelial cells have been found to be more sensitive to and to play an important role during I/R injury [6, 7]. Dysfunction of endothelial cells occurs after reperfusion quickly, long before development of myocardial cell injury, which noticeably begins at 3 hours after reperfusion [8]. Furthermore, Egr-1 expression rapidly increased in bovine aortic endothelial cells subjected to hypoxia [9]. Nevertheless, whether Egr-1 has the similar activation in cardiac microvascular endothelial hypoxia/reoxygenation (H/R) injury has not been fully identified yet.

N-n-butyl haloperidol iodide (F2) is a new compound, which was synthesized by our laboratory and was granted a Chinese national invention patent (No. ZL96119098.1). Our previous studies have shown that the cardioprotective effect of F2 maybe associated with its ability to block calcium channels of ventricular myocytes [10-12]. We have also shown that F2 decreases the expression of Egr-

1 in both I/R myocardial tissues and H/R cardiomyocytes, correlating with improved myocardial function [5, 13]. It is uncertain whether down-regulation of Egr-1 expression by F2 in cardiac microvascular endothelial cells (CMECs) is critical to the alleviation of endothelial dysfunction and myocardial I/R injury. The purpose of this study is to elucidate the cause-effect relationship between Egr-1 overexpression and endothelial H/R injury, and investigate further the effects of F2 on injury and inflammation as well as Egr-1 mRNA and protein expression in H/R CMECs, and thereby discover the new mechanism of F2 to antagonize myocardial I/R injury.

Materials and Methods

Animals

Neonatal and adult Sprague-Dawley (SD, Grade α , Certificate No. 2007100801) rats of either sex were used in our experiments. All animals were treated in compliance with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and followed the rules of the National Animal Protection of China. The study was approved by the Institutional Animal Care and Use Committee of Shantou University Medical College.

Cell culture and preparation of H/R

Primary culture of neonatal rat CMECs and H/R model were carried out by previously described methods [14]. In short, the isolated cells from 1-4 days neonatal rats were purified and cultured on dishes coated with 1% gelatin (Sigma, USA). Cells reached confluence within 4-5 days and displayed almost uniform "cobblestone" morphology (Fig. 1A). All experiments were carried out on cells at passages 3-5. 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 CMECs 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 dishes were transferred into a normoxic incubator (95% air-5% CO₂) for 2 h of reoxygenation.

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

Antisense and scrambled-sequence phosphorothioate Egr-1 ODNs of 15 base-pair length were commercially synthesized. Sequence of antisense Egr-1 ODN was 3'-TAC CGT CGC CGG TTC -5' as described previously [16]. For control experiments, scrambled-sequence Egr-1 ODNs (3'-TCG TGC CGC TGC CAT -5') were used [1]. 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.

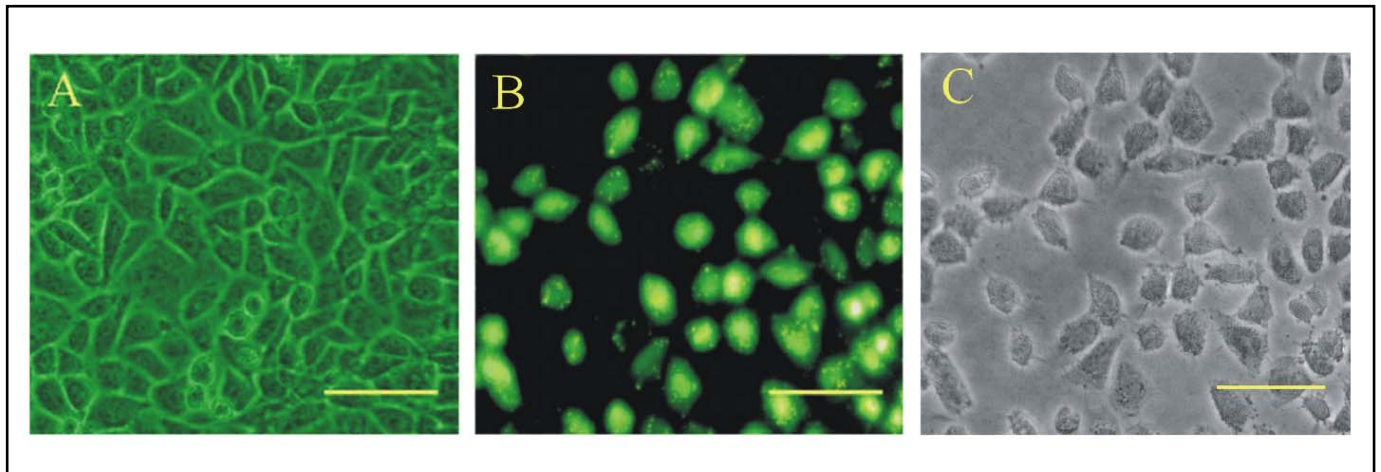


Fig. 1. (A) Confluent endothelial monolayer with cobblestone morphology was observed by inverted microscope. (B) FITC-labeled antisense Egr-1 ODN was observed in the cytoplasm and mainly in nuclei of cultured CMECs by fluorescence microscopy (The ODN uptake was analyzed 24 h after administration with FITC-labeled Egr-1 AS-ODN). (C) Corresponding field of photo B was observed by phase-contrast microscopy (Bar=100 μ m).

Experimental protocols

After reaching 90-95% confluence, cells were cultivated in serum-free Medium 199 for 24 h. CMECs were randomly divided into one of seven groups: Control (untreated), H/R, Lip-H/R (H/R pretreated with LipofectamineTM2000 Reagent), AS-H/R (H/R pretreated with antisense Egr-1 ODN), Sc-H/R (H/R pretreated with the scrambled-sequence Egr-1 ODN), F2 [H/R pretreated with F2 at a dose of 1 μ M dissolved in polyethylene glycol 400 (PEG)], PEG (H/R pretreated with PEG). CMECs of the Control group were incubated under normoxic conditions for 5 h. Cells of other groups underwent 3 h of hypoxia plus 2 h of reoxygenation. ODNs at a dose of 4×10^{-7} mol/L respectively were transfected into cultured cell 24 h before hypoxia by using LipofectamineTM2000. F2 or an equal volume of PEG was added to cells 5 min before H/R. After reoxygenation, culture media from all groups was collected for measurement of lactate dehydrogenase (LDH) release. CMECs from all groups were harvested and levels of superoxide dismutase (SOD), malondialdehyde (MDA), membrane-bound ICAM-1 (mICAM-1), Egr-1 mRNA, and Egr-1 protein were measured.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (PCR)

Total cellular RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer's instructions. Quantitative real-time PCR was performed using SYBR green PCR master mix (Applied Biosystems) and carried out using an ABI Prism 7300 Sequence Detection System (Applied Biosystems). Primer sequences for rat β -actin were (forward) 5' -CCA ACC GTG AAA AGA TGA CCC -3'; (reverse) 5' -AAT GCC AGT GGT ACG ACC AGA G -3'; and for rat Egr-1 (forward) 5' -CAA GGG TGG TTT CCA GGT T -3'; (reverse) 5' -GGC TGG GTT TGA TGA GTT -3'. As an internal control and for normalization purposes, level of mRNA expression for β -actin, a housekeeping

gene, was measured simultaneously with Egr-1 expression for each sample. Results are presented as relative amounts of Egr-1 mRNA versus the β -actin mRNA value for the same sample. The cycling protocol was 3 min 95°C initial denaturation, 30 s 94°C, 30 s 60°C, for 30 cycles. Melting curve analysis was performed to ensure that only one specific product was amplified.

Western blot analysis

Total protein extraction and protein concentration determination were performed as previously described [13]. Forty micrograms of total protein was subjected to SDS-PAGE (10%) followed by electrophoretic transfer to nitrocellulose membranes (Amersham, Biosciences, Piscataway, NJ, USA). Immunoblotting was performed with a primary rabbit anti-rat Egr-1 antibody (1:200 dilution; Santa Cruz Biotechnology, Inc.) and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:2000 dilution). Blots were then re-probed with β -actin (1:2000, Sigma-Aldrich Inc. USA) to confirm equal loading of samples.

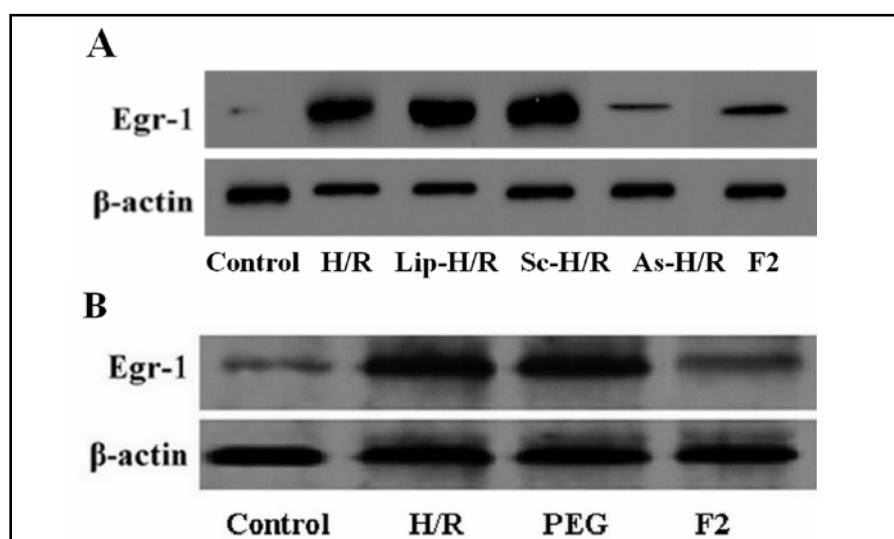
Immunocytochemistry

For immunocytochemical analysis, CMECs grown on coverslips were fixed in ice-cold propanone. All coverslips were first stained with primary antibodies, rabbit anti-rat Egr-1 IgG (1:200, Santa Cruz Biotechnology Inc., USA). Secondary antibody biotinylated, Streptavidin Biotin Complex (SABC) and substrates were applied according to the manufacturer's protocols of the High-SABC immunohistochemistry kit (Boster Biological technology LTD, China). Then the coverslips were counterstained with hematoxylin. Egr-1-immunopositive cells were counted under a bright field microscope.

Isolation of neutrophils and adhesion assay

Adult rats (weighing 250-300g) were anesthetized and blood was obtained by an intracardiac puncture and stored in

Fig. 2. Egr-1 expression was significantly inhibited by antisense Egr-1 ODN (A) and F2 (B) by Western blot analysis (n=5). * $P<0.05$ vs. Control group; ** $P<0.01$ vs. Control group; †† $P<0.01$ vs. H/R group.



heparin-coated tubes [17]. Diluted blood mixture was slowly layered onto discontinuous Ficoll-Hypaque gradients. After centrifugation at room temperature (1400 rpm for 30 min) cells were washed in HBSS, and erythrocytes were lysed in 0.84% NH_4Cl . Remaining, cells were resuspended in Medium 199. Isolated cells consisted of $91.6 \pm 0.9\%$ neutrophil with $95 \pm 0.7\%$ viability by trypan blue dye exclusion [18].

Confluent CMECs grown in 96-well plates were exposed to H/R. For measuring neutrophil adhesion, CMECs were washed with Hank's balanced salt solution (HBSS), and then 100 μl neutrophils (1×10^6 cells/mL) were added to each well. After 1 h of contact, unbound neutrophils were removed by four washings with PBS. Adherent neutrophils were collected and lysed with Triton X-100 (0.1 % v/v) (Sigma, USA). Myeloperoxidase (MPO) in adherent neutrophil fraction was measured as described with slight modify [19]. Briefly, MPO was assayed by the increase in optical density at 460 nm using commercial test kits (Jiancheng Bioengineering Institute, Nanjing, China). Adhesion was expressed as percentage of adherent neutrophils relative to the total number of neutrophils added at the beginning of the experiment [20].

Preparation of Rat Platelets and the interaction with CMECs

Whole blood was obtained as described above and immediately spun in a refrigerated centrifuge at 900 rpm for 10 min. Supernatant was collected and centrifuged again at 3000 rpm for 10 min to obtain platelets [17]. Then, platelets were resuspended in Medium 199 and counted (>95% pure).

CMECs were grown to confluence on glass coverslips [21]. After H/R stimulation, platelets were added to CMECs (platelets:CMECs=300:1) and incubated for 90 min at 37°C . Cells were washed extensively to remove unbound platelets and then fixed in glutaraldehyde (2.5% in PBS) overnight at 4°C . Fixed cells were subjected to serial dehydration with 25, 50, 75, 95, and 100% ethanol for 15 min each, and then exposed to fresh 100% ethanol for 1 h. Dried specimens were mounted onto copper stubs, and gold coated [22]. All samples were examined using a scanning electron microscope (JSM-6360LA; JEOL Ltd., Tokyo, Japan).

Measurements of mICAM-1 protein expression

Confluent CMECs grown in 6-well plates were exposed to H/R. After H/R stimulation, CMECs were washed extensively and collected via trypsinization and the total cellular proteins were extracted [14]. Total cellular protein extraction was assayed for mICAM-1 using a commercially available ELISA kit (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. Results were read immediately at 450 nm. Sensitivity of the assays was 2 pg/mL for mICAM-1.

Determination of LDH, MDA, and SOD

LDH, an indicator of cell injury, was detected by chromatometry with an assay kit (Jiancheng Bioengineering Institute, Nanjing, China). Briefly, after H/R stimulation, culture supernatant (50 μl) was gently aspirated and saved for LDH determination. After reaction, each sample was detected and the absorbance was read at wavelength 440 nm. LDH could catalyze the synthesis of pyruvic acid from lactic acid and pyruvic acid then reacted to form 2,4-dinitrophenyl-hydrazine. The latter showed brownish red color in basic solution.

According to the commercial test kits (Jiancheng Bioengineering Institute, Nanjing, China), the cellular protein was measured spectrophotometrically by using Coomassie bright-blue (CBBG250) stain method. The content of MDA, a compound produced during lipid peroxidation, was determined using the thiobarbituric acid stain method. SOD, an important anti-oxidant enzyme that plays a pivotal role in preventing cellular damage caused by ROS, was measured by spectrophotometry using the xanthine oxidase method [13]. After reaction, each sample was detected and the absorbance was read at wavelength 550 nm.

Cell viability

Cell viability was determined by trypan blue dye exclusion test as follows: attached CMECs were digested with 0.1% trypsin and 0.02% EDTA (V/V 1:1). Detached cells were collected in cold culture medium with 10% calf serum. Cell suspensions (900 μl) were stained with 0.4% trypan blue (100 μl). After 5 min, viable (blue-negative) and nonviable (blue stained) cells were counted with a hemacytometer. The viability of

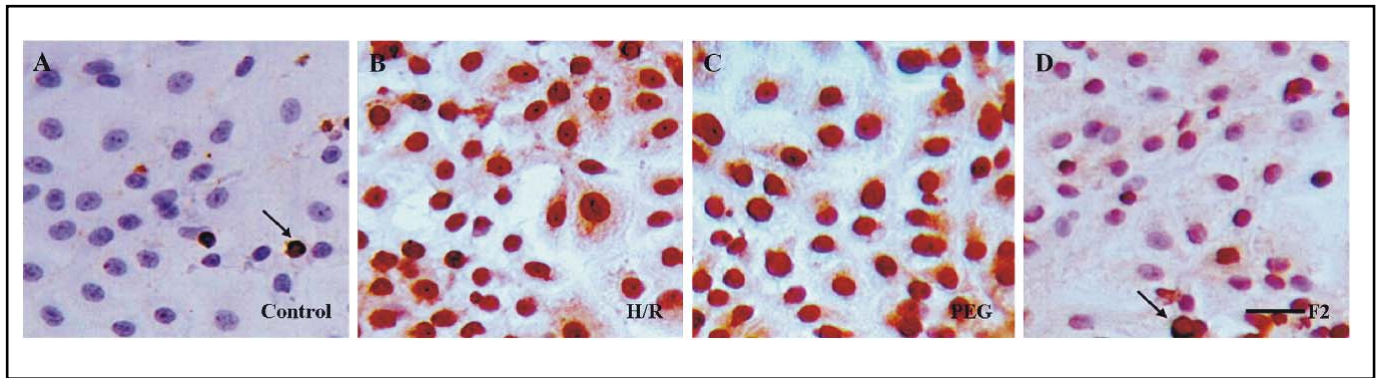


Fig. 3. Immunocytochemical analysis. Egr-1 was weakly expressed in the Control group (A). In the H/R (B) and PEG (C) groups, strong expression was detected. In F2 group (D), expression of Egr-1 was decreased significantly. Brown staining (arrows) indicates positive expression of Egr-1 (n=5), Bar=50 μ m.

CMECs was expressed as a percentage of viable cells/total cells.

Statistical analysis

Results are expressed as mean \pm S.E.M. Statistical analysis was carried out using one-way ANOVA, followed by a Student-Newman-Keuls test, with $P < 0.05$ considered as being statistically significant.

Results

Uptake of antisense Egr-1 ODN

FITC-labeled antisense Egr-1 ODN was found in the cytoplasm and mainly in the nuclei of cultured CMECs, indicating that antisense Egr-1 ODN had successfully been transfected (Fig. 1B and C).

Effects of antisense Egr-1 ODN and F2 on levels of Egr-1 mRNA and protein expression

Compared with H/R group, expression levels of Egr-1 protein in Control group showed significantly low level determined by Western blot assay. But Egr-1 expression induced by H/R significantly inhibited when antisense Egr-1 ODN and F2 were treated. These changes were not altered by PEG, and there were no differences between Lip-H/R and Sc-H/R groups (Fig. 2A and B).

To further confirm the results from Western blot, immunocytochemical analysis was performed. In Control groups, few cells expressed Egr-1 staining (Fig. 3A). Stimulation of H/R showed a dramatic increase in the number of CMECs expressing Egr-1 protein (Fig. 3B), which was not altered by PEG (Fig. 3C). Egr-1 induction was significantly inhibited by F2 (Fig. 3D).

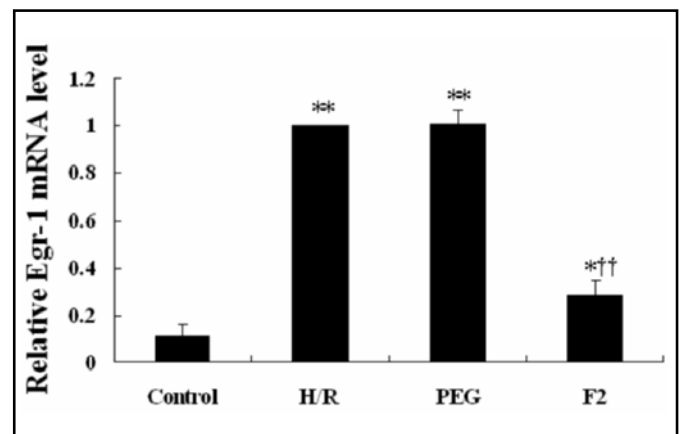


Fig. 4. Measurement of Egr-1 mRNA level in cultured CMECs by quantitative real-time PCR analysis (n=5). F2 significantly inhibited Egr-1 mRNA expressing * $P < 0.05$ vs. Control group; ** $P < 0.01$ vs. Control group; †† $P < 0.01$ vs. H/R group.

Similarly, examination of Egr-1 mRNA determined by quantitative real-time PCR analysis revealed H/R mediated induction of Egr-1 mRNA was reduced by F2 (Fig. 4).

Effects of antisense Egr-1 ODN and F2 on changes of enzymatic activities

To characterize the protective effects of F2, the activities of LDH, MDA, and SOD were quantified. Compared with Control groups, H/R groups showed a higher level of both LDH and MDA, and showed a decreased level of SOD after reoxygenation. However, antisense Egr-1 ODN and F2 could significantly maintain the activity of SOD, reduce the product of MDA,

Group	LDH	SOD	MDA
Control	1220.57±84.80	114.30±8.04	0.41±0.04
H/R	3366.84±158.26**	64.29±5.038**	1.37±0.11**
Lip-H/R	3306.21±162.26**	63.48±5.14**	1.42±0.13**
Sc-H/R	3493.97±152.02**	65.22±5.90**	1.35±0.10**
AS-H/R	1618.74±125.38**††	99.073±8.31**††	0.64±0.07**††
F2-H/R	1755.56±168.90**††	89.88±8.49**††	0.80±0.08**††

Table 1. Enzyme activities and MDA level in culture medium and CMECs of different groups *in vitro*. LDH, lactate dehydrogenase (U/L); SOD, superoxide dismutase (U/mg prot); MDA, malondialdehyde (nmol/mg prot). LDH was from plasma. SOD and MDA were from CMECs. All values are expressed as mean ± S.E.M. (n=9). ***P*<0.01 vs. Control group; ††*P*<0.01 vs. H/R group.

and minimize LDH release as compared with H/R groups respectively. There were no differences among H/R, Lip-H/R and Sc-H/R groups (Table 1). Therefore, the cytoprotective effects of F2 can be explained in part by its ability to reduce Egr-1 induction.

Effects of F2 on levels of mICAM-1

In Control group, the expressions of mICAM-1 were low. Treatment with F2 reduced significantly mICAM-1 expression as compared with H/R groups respectively. There were no differences between H/R and PEG groups (Fig. 5A).

Effect of F2 on neutrophils adhesion and the interactions of platelets of CMECs

Light microscopy showed that significant adhesion of neutrophils to CMECs occurred after H/R, but not in Control groups. Compared with H/R groups, the MPO activity of adhesion of neutrophils was significantly diminished by F2. PEG had no effect on leukocyte adherence (Fig. 5B).

The interaction of platelets was performed by scanning electron microscopy. In Control groups, un-stimulated platelets did not aggregate and exhibited round, discoid form (Fig. 6A). H/R induced the formation of micro thrombosis consisting of platelets, which extended long pseudopodia and lost their round form (Fig. 6B). Compared with H/R groups, the aggregates were not affected by PEG (Fig. 6C), but were significantly diminished by F2 (Fig. 6D).

Viability of cultured CMECs

Relative to Control group, cell viability in the H/R group was significantly decreased. This change was not altered by PEG, but significantly reduced by F2 (Fig. 7).

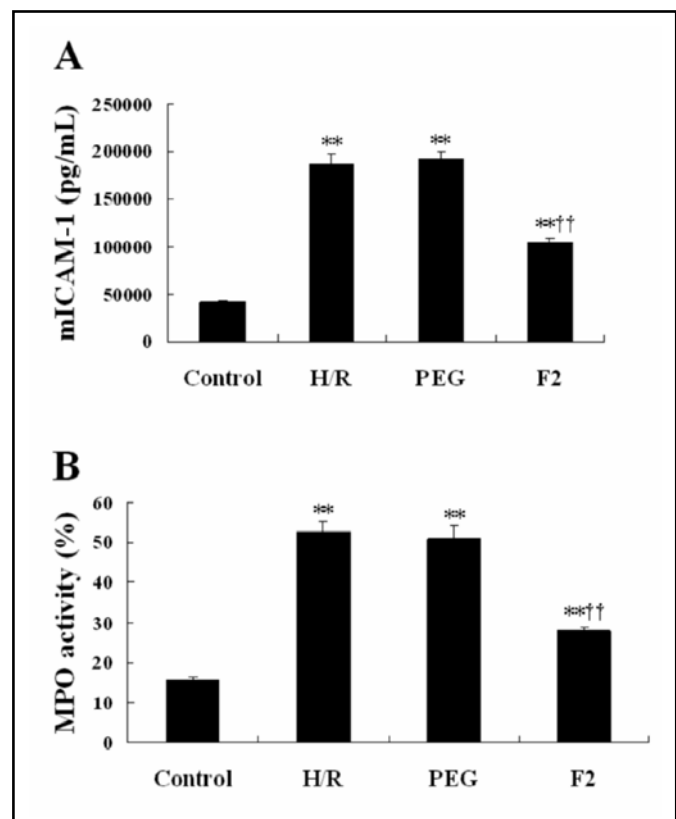
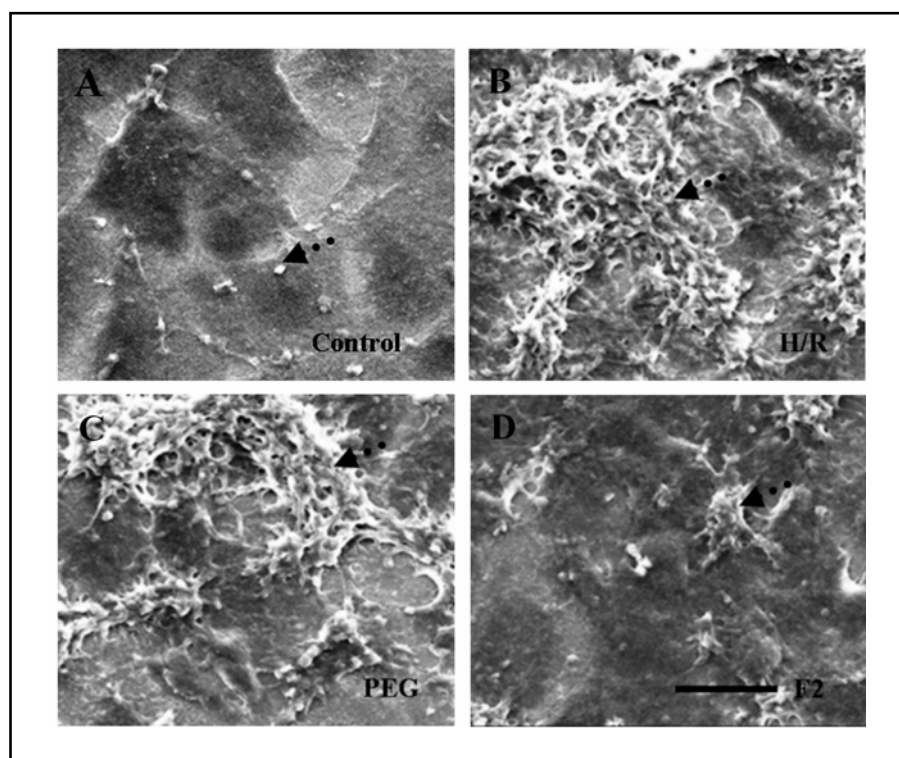


Fig. 5. (A) Level of mICAM-1 was detected by ELISA assay in culture CMECs (n=5). (B) MPO activities of neutrophils adhered to CMECs. Adhesion was expressed as the percentage of adherent neutrophils relative to the total number of neutrophils added at the beginning of the experiment (n=8). ***P*<0.01 vs. Control group; ††*P*<0.01 vs. H/R group.

Discussion

Although cardiomyocytes comprise almost 75% of the total tissue volume in the heart, cardiac endothelial

Fig. 6. Scanning electron micrograph of H/R-induced platelet adhesion to CMECs. In the Control group (A), unstimulated platelets did not aggregate and exhibited a round, discoid form. In the H/R (B) and PEG (C) groups, platelets aggregated and formed micro thrombi (arrow). Aggregates were significantly diminished by F2 (D) (Bar=10 μ m).



cells outnumber cardiomyocytes by 3:1 [23]. Endothelial cells in the heart also play an obligatory role in regulating and maintaining cardiac function. Many researches showed that cardiac endothelial cell plays an essential role with respect to cardiac metabolism, growth, contractile performance, and rhythmicity [23]. It is believed that hypoxia-ischemia initially causes damage to the endothelial cells of the myocardium [9]. Nevertheless, whether Egr-1 has the similar role in CMECs H/R injury has not been fully identified yet. Furthermore, it is uncertain whether the protective effects of F2 on myocardial I/R injury is related closely with the down regulation of Egr-1 expression on CMECs. So, an H/R model of cultured CMECs *in vitro* is needed to investigate the pathogenesis of myocardial I/R injury.

In order to elucidate the cause-effect relationship between Egr-1 overexpression and cellular injury, we studied the effects of Egr-1 AS-ODN on Egr-1 protein expression and changes of enzymetic activities induced by H/R. The present study demonstrated that Egr-1 AS-ODN exerted a great protective activity in H/R experiments after being transfected into CMECs by previously described methods, which successfully inhibited the expression of Egr-1 protein. It significantly improved cellular dysfunction demonstrated by the amelioration of SOD activity, the reduction in MDA level and LDH leakage *in vitro*. Meanwhile, Egr-1 AS-ODN relieved the CMECs injuries in morphology and structure as evidenced by in-

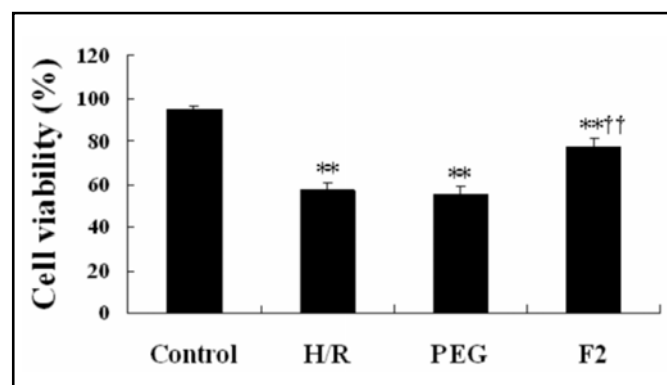


Fig. 7. Viability of cultured CMECs was determined by trypan blue dye exclusion and expressed as a percentage of viable cells/total cells (n=10). ** P <0.01 vs. Control group; †† P <0.01 vs. H/R group.

hibition of the changes of cell morphology in scanning electron micrograph and increase of cell viability determined by cytomembrane integrity as recently reported [14]. We initially investigated the expression of Egr-1 during different H/R times in cultured CMECs by Western blot assay. Egr-1 expression significantly increased after the onset of hypoxia within 15 minutes (data not shown), increased at 30 minutes, peaked at 3 hours, and remained significantly increased during the next two hours of reoxygenation [14]. Lo et al. employed bovine aortic endothelial cells with hypoxia and found that Egr-1 mRNA expression rapidly increased within 30 minutes [9]. These

results showed that Egr-1 expression of endothelial cells occurs more quickly than that of cardiomyocytes, which noticeably begins at 1 hour after reperfusion [13, 16]. According to the recent data, levels of Egr-1 expression are upregulated in various organs including the heart [24], lung [3, 25], gut [26] and kidney [27] after I/R challenge. Thus, Egr-1 was designated as having a central and unifying role in the pathogenesis of I/R without species- and tissue- specific influences [3].

Based on the relationship between Egr-1 overexpression and CMECs H/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 H/R. The results from Western-blot, immunocytochemistry, and quantitative real-time PCR analysis verified the former results, showing that F2 could down-regulate the overexpression of Egr-1 mRNA and protein in CMECs induced by H/R. Meanwhile, besides the reduction in enzyme release and ultrastructural changes just as recently reported [14], the cardioprotection with F2 was shown by the amelioration of the inflammation *in vitro* caused by H/R, demonstrated by a decrease in MPO activity due to accumulation and activation of neutrophil, the diminish of platelet adhesions and reduced release of ICAM-1, a pivotal inflammatory factor secreted by endothelial cell after stress. Combining with the literature, it is reasonable to speculate that the anti-inflammatory effect of F2 in CMECs is associated with its inhibition of the expression of Egr-1, the decrease of ICAM-1 release and the reduction of neutrophil and platelet adhesions to endothelial cells. ICAM-1 is one of the important downstream inflammatory genes of Egr-1. ICAM-1 was proposed as a marker of vascular inflammation and as an index to predict cardiovascular risk and future cardiovascular disease, presumably because of its ability to mediate adhesion of inflammatory cells to the endothelium [28, 29]. However, soluble ICAM-1 (sICAM-1) diminishes leukocyte adhesion to vascular endothelium in ischemia-reperfusion injury [30]. In contrast, mICAM-1 is involved in promotion of leukocyte adhesion and transendothelial migration. In the present study, expression of ICAM-1 was measured using the total protein extraction of CMECs (for measurement of mICAM-1), not the culture supernatants (for measurement of sICAM-1). We found that F2 significantly diminish the expression of mICAM-1, which promoted leukocyte adhesion. Leukocyte interaction with CMECs provokes mechanical obstruction of small vessels and releases inflammatory factor assaulting to cardiomyocytes [31], which

contributes to the first stage of I/R-induced tissue injury.

Egr-1 is also induced by oxidative damage, including oxidized lipids [32]. It was reported that MDA (the final product of lipid peroxidation) is a well-established mechanism of cellular injury and is used as an indicator of oxidative stress in cells and tissues [33]. SOD is an important protective enzyme, whose function is to detoxify reactive oxygen species (ROS). Nozik-Grayck E et al. well demonstrated that overexpression of extracellular SOD could prevent the early hypoxia-dependent upregulation of Egr-1 [34] whereas, Bek MJ et al. reported that overexpression of Egr-1 induces down-regulation of SOD and stimulates the generation of ROS [35]. In the present study, we found that H/R caused the down-regulation of SOD, an increase of MDA, and the overexpression of Egr-1 protein in CMECs. We found that antisense Egr-1 ODN and F2 reduced Egr-1 protein levels and attenuated the cellular injury in cytomembrane structure as evidenced by the reduction of LDH leakage, and in oxidative stress caused by ROS as evidenced by the increase of SOD activity and the decrease of MDA yield. These results suggested that the interaction among SOD, ROS and Egr-1 might occur during H/R injury of CMECs.

It is well recognized that TF, a downstream gene of Egr-1, is a vital inflammatory factor and can be induced at the surface of monocytes and endothelial cells after hypoxia which acts as a primary factor in the process leading to thrombosis [36]. The data from our laboratory suggested that inhibition of platelet adhesions by F2 was associated with the inhibition of Egr-1 expression in H/R injury. Therefore, we might deduce that antagonism to thrombosis of F2 might, in part, be a result of the down-regulation of Egr-1-mediated TF overexpression. However, the detailed signal transduction cascade between TF and Egr-1 needs further exploration.

In summary, this study provided new insight into the molecular mechanisms of F2 from myocardial I/R injury and illustrated a role for Egr-1 activation in the regulation of the thrombogenic potential of cardiac endothelial cells by mICAM-1. The data from our present and previous studies *in vivo* and *in vitro* further suggested that cardioprotection by F2 is associated with its chief inhibition of Egr-1 up-expression in CMECs, the suppression of mICAM-1 release, the attenuation of thrombosis and neutrophil activation, then decreases inflammatory factor assaulting to cardiomyocytes. Thus, pharmacologic inhibition of Egr-1 overexpression in endothelial cells might provide new therapeutic options to prevent thrombotic

complications in inflammatory processes such as atherosclerosis and “no reflow phenomenon” in cardiac I/R injury.

Acknowledgements

This study was supported by NSFC-Guangdong Joint Funds (No. U0932005), the Teamwork Projects funded

by Guangdong Natural Science Foundation of China (No.9351503102000001), the Research Fund for the Doctoral Program of Higher Education of China (No. 200805600003) and the Science and Technology Planning Project of Shantou (No. 2007). We gratefully appreciate Dr. Stanley Lin for English language editing. We also gratefully thank Prof. Wenhong Luo, Prof. Yucai Fu and Dr. Guanwu Li (Medical College, Shantou University) for their technical assistance and helpful advice.

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