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# Effects of *N*-n-butyl Haloperidol Iodide on Myocardial Ischemia/Reperfusion Injury and *Egr-1* Expression in Rat

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**Abstract** We have previously shown that *N*-n-butyl haloperidol iodide ($F_2$) derived from haloperidol reduces ischemia/reperfusion-induced myocardial injury by blocking intracellular $Ca^{2+}$ overload. This study tested the hypothesis that cardio-protection with $F_2$ is associated with an attenuation in the expression of early growth response gene 1 (*Egr-1*). In an *in vivo* rat model of 60 min coronary occlusion followed by 180 min of reperfusion, treatment with $F_2$ significantly reduced myocardial injury evidenced by the reduction in release of plasma creatine kinase, myocardial creatine kinase isoenzyme and lactate dehydrogenase. In cultured neonatal rat cardiomyocytes of hypoxia for 3 h and reoxygenation for 1 h, $F_2$ treatment attenuated necrotic and apoptotic cell death, as demonstrated by electron microscopy. Concomitant with cardio-protection by $F_2$, the increased expression levels of *Egr-1* mRNA and protein were significantly reduced in myocardial tissue and cultured cardiomyocytes as detected by reverse transcription-polymerase chain reaction, immunohistochemistry and immunocytochemistry. In conclusion, these results suggest that the protective effect of $F_2$ on ischemia/reperfusion- or hypoxia/reoxygenation-induced myocardial injury might be partly mediated by downregulating *Egr-1* expression.

**Key words** *N*-n-butyl haloperidol iodide; ischemia/reperfusion injury; early growth response gene-1; myocardium; cardiomyocyte

Ischemia/reperfusion (I/R) in the heart initiates a series of rapid inductions of pathological changes resulting in inflammatory and endothelial cell-cell interactions, cardiac dysfunction and cardiomyocyte cell death [1–3]. Therapeutic strategies focusing on attenuation of I/R-mediated cellular events, such as depletion of inflammatory cells from circulation, prevention of abnormal cell-cell interactions and reduction of intracellular $Ca^{2+}$ overload, have shown attenuation in myocardial injury [4,5]. Recently, more attention has been placed on the molecular mechanisms in myocardial I/R injury [6,7].

Early growth response gene 1 (*Egr-1*), a member of the zinc finger family of transcription factors, is one of the immediate-early genes that responds to stimulation, and the upregulation of its expression level has been demonstrated in the heart, lung, gut and kidney after I/R [8]. With *Egr-1*-null mice or antisense *Egr-1* oligodeoxyribonucleotide, previous studies have showed that *Egr-1* might be a master switch in the pathogenesis of I/R injury due to its coordinating upregulation of divergent gene families underlying the pathophysiological event of I/R [9, 10]. In addition, other studies have reported that overexpression of *Egr-1* is triggered by elevated intracellular $Ca^{2+}$ [11]. Therefore, it is reasonable to speculate that an attenuation of intracellular $Ca^{2+}$ overload has the potential to reduce myocardial injury by reducing *Egr-1* expression after I/R.

Haloperidol, an antipsychotic compound, has been shown to possess vasodilatory and cardioprotective effects [12,13]. However, the extrapyramidal side effects of haloperidol have limited its potential clinical application. We have synthesized a series of quaternary ammonium salt derivatives of haloperidol and screened as *N*-n-butyl

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haloperidol iodide ($F_2$) (**Fig. 1**), which was granted a Chinese national invention patent (No. ZL96119098.1). As we reported previously, $F_2$ has vasodilatory effects, but without haloperidol-induced side-effects [14]. In addition, $F_2$ reduces myocardial I/R injury and preserves ATPase activity, largely mediated by blocking intracellular $Ca^{2+}$ overload [15]. However, we do not know whether these protective effects elicited by $F_2$ are also related to a modulation in molecular changes, for example, *Egr-1* expression after I/R. Therefore, this study tested the hypothesis that $F_2$ reduces tissue and cell damage by modulating expression of *Egr-1* mRNA and protein in an *in vivo* rat model of I/R as well as in cultured neonatal rat cardiomyocytes of hypoxia (H) and reoxygenation (Re). The hypothesis is based on previous reports of *in vitro* and *in vivo* observations, which showed that intracellular $Ca^{2+}$ overload-mediated *Egr-1* expression causes inflammation, vascular hyperpermeability and induction of myocardial injury [4,9–11] and that $F_2$ has the capability to reduce intracellular $Ca^{2+}$ overload [15].

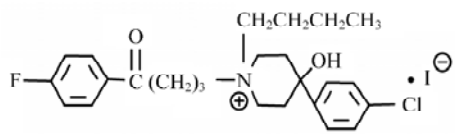

**Fig. 1 Chemical structure of *N*-n-butyl haloperidol iodide ($F_2$)**

## Materials and Methods

### Surgical preparation of animals and experimental protocol

Male adult Sprague-Dawley rats weighing 200–250 g (Grade II, Certificate No. 2003A054) provided by the Experimental Animal Center of Medical College, Shantou University (Shantou, China), were anesthetized with an initial intraperitoneal injection of sodium pentobarbital (40–50 mg/kg). The animals were ventilated with a rodent respirator. The right femoral artery was cannulated for blood sampling. The chest was opened using a left thoracotomy through the fifth intercostal space. After pericardiotomy, a silk suture was placed under the left anterior descending (LAD) coronary artery, 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 LAD occlusion. Constant body temperature was maintained between 37 ºC and 38 ºC by a heating pad.

All rats were randomly assigned into four groups: sham ($n$=10), the ligature was placed under the LAD without occlusion for a total of 4 h of the experimental period; I/R control ($n$=10), heart was subjected to 60 min LAD occlusion followed by 180 min of reperfusion; $F_2$ ($n$=10), $F_2$ at a dose of 2 mg/kg dissolved in 100 μl of polyethylene glycol (PEG) 400 and 0.2 ml of saline was injected through the sublingual vein 5 min before ischemia; and vehicle ($n$=10), 100 μl of PEG and 0.2 ml of saline without $F_2$ was given through the sublingual vein 5 min before ischemia. At the end of the experiment, blood was taken from the carotid artery for analyzing activities of creatine kinase (CK), creatine kinase MB isoenzyme (CK-MB) and lactate dehydrogenase (LDH). The area-at-risk myocardium was excised for immunohistochemical examination, or stored at –70 ºC for determining *Egr-1* mRNA.

### Activities of plasma CK, CK-MB and LDH

At the end of the experiment, the arterial blood sample (2 ml) was centrifuged at 2500 *g* for 3 min. The plasma was transferred to a microcentrifuge tube and stored at –20 ºC until analysis. Activities of CK, CK-MB and LDH were detected using CK detection kit (Shanghai Fosum Long March Medical Science, Shanghai, China), LDH detection kit (Shanghai Fosum Long March Medical Science) and CK-MB detection kit (Randox Laboratories, Antrim, UK) by automatic analyzer (Model 7060; Hitachi, Hitachi, Japan).

### Isolation of neonatal rat cardiomyocytes and preparation of H/Re

Neonatal rat ventricular myocytes were isolated from 1–4-day-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% fetal calf serum for 30 min to remove non-cardiomyocytes [16]. The isolated cardiomyocytes at a density of 2.5×$10^6$ cells per well were then cultured in the medium with 0.1 mM 5-bromo deoxyuridine in an incubator with 5% $CO_2$ at 37 ºC to further inhibit the growth of non-cardiomyocytes for the first 3 d. Hypoxia was induced by replacing the initial culture medium with pH 6.2 buffer (137 mM NaCl, 12 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2{\cdot}H_2O$, 4 mM HEPES and 20 mM Na lactate) [16], and the cardiomyocytes were incubated in an air-tight chamber gassed with pure $N_2$ at 37 ºC 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 d of cell culture in normoxic medium, the cardiomyocytes were randomly divided into four groups: sham ($n$=5), the cardiomyocytes were seeded on the plate for a total of 4 h of normoxic incubation; H/Re control ($n$=5), the cardiomyocytes underwent 3 h of H and 1 h of Re; $F_2$ ($n$=5), $F_2$ dissolved in PEG ($1\times10^{-4}$ M) was added into the cells at a concentration of $1\times10^{-6}$ M before H/Re; and vehicle ($n$=5), an equal volume of PEG without $F_2$ was added into the cells before H/Re.

### Transmission electron microscopy

The attached cardiomyocytes were digested with 0.25% trypsin and 0.2% EDTA (*V*/*V*, 1:1). After they were washed twice with phosphate-buffered saline, the cell suspensions were centrifuged at 250 *g* for 20 min. The mass of cells was extracted carefully and cut into small pieces. The samples were fixed with 2.5% glutaraldehyde followed by postfixation with osmium tetroxide for 1 h, stained with 2% aqueous uranyl acetate, and dehydrated in a graded series of ethanol. After infiltration and polymerization, the samples were cut into ultra-thin sections. Lastly, the sections were stained with lead citrate and observed using a transmission electron microscope (Hitachi 300; Hitachi).

### Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from tissue or cultured cells using Trizol reagent (Invitrogen, Carlsbad, USA). RNA was quantified by the ratio of the absorbance at wavelengh 260 nm to that at wavelength 280 nm (more than 1.8). RT-PCR amplification was carried out with RevertAid™H minus first-strand cDNA synthesis kit (MBI Fermentas, Vilnius, Lithuania) and PCR using a *Taq* DNA polymerase kit (MBI Fermentas). The sequences of forward and reverse primers for rat *Egr-1* were 5′-GCAACACTTTGTGGCCTGAA-3′ and 5′-GAGTTGGGACTGGTAGGTGT-3′, respectively, yielding a 512 bp product. The forward and reverse primers of β-actin, used as the control, were 5′-GTGGGTATGGGTCAGAAGGA-3′ and 5′-AGCGCGTAACCCTCATAGAT-3′, respectively, yielding a 380 bp product. PCR conditions were as follows: 30 cycles, denaturating at 94 ºC for 45 s, annealing at 52 ºC for 60 s and extending at 70 ºC for 60 s, with initial heating at 95 ºC for 5 min and final extending at 70 ºC for 10 min. The PCR products were separated on 1.2% agarose gels and stained with ethidium bromide. The band relative densities of mRNA were analyzed by Fluorchem 8900 (Alpha Innotech, Miami, USA).

### Immunohistochemistry and immunocytochemistry

For immuohistochemical analysis, the tissue block was initially immersed in 4% paraformaldehyde for 6 h then transferred to 30% sucrose overnight. The tissue blocks were dehydrated in 30% sucrose, washed, embedded and frozen in optimal cutting temperature compound. Cryosections of the tissue blocks (10 μm) were cut using a Leica CM 100 cryostat (Leica, Wetzlar, Germanny). For immunocytochemical analysis, cardiomyocytes grown on coverslips were fixed in ice-cold propanone. All sections or coverslips were first stained with primary rabbit antibodies against rat *Egr-1* immunoglobulin G (1:200; Santa Cruz Biotechnology, Santa Cruz, USA). Secondary antibody biotinylated streptavidin biotin complex and substrates were applied according to the manufacturer's protocols of the High-SABC immunohistochemistry kit (Wuhan Boster Biological Technology, Wuhan, China). The *Egr-1*-immunopositive cells were counted in the optic microscope at 400× magnification.

### Statistical analysis

Data are shown as the mean±standard error of the mean. 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

### Changes in enzymatic activities in plasma

A comparison in levels of plasma CK, CK-MB and LDH among all groups at the end of the experiment is shown in **Table 1**. Compared with the sham group, the plasma CK, CK-MB, and LDH activities in the I/R control group were

**Table 1 Activities of creatine kinase (CK), creatine kinase MB isoenzyme (CK-MB) and lactate dehydrogenase (LDH) in all experimental groups in this study ($n$=10)**

| Group | CK (U/L) | CK-MB (U/L) | LDH (U/L) |
|---|---|---|---|
| Sham | 1245±72 | 2020±217 | 671±37 |
| I/R control | 3388±367** | 5018±414** | 1343±103** |
| Vehicle | 3131±263** | 5501±522** | 1442±68*** |
| $F_2$ | 1831±201†‡ | 2682±304†‡ | 937±51*†‡ |

Mean±standard error of the mean. *$P$<0.05, ** $P$<0.01 and *** $P$<0.001 versus sham group; † $P$<0.05 versus ischemia/reperfusion (I/R) control group; ‡ $P$<0.05 versus vehicle group.

distinctly increased by 2.7-fold, 2.5-fold and 2.0-fold, respectively. A similar pattern in changes of these parameters in the vehicle group was also demonstrated (2.5-fold, 2.7-fold and 2.1-fold, respectively). Administration of $F_2$ distinctly attenuated changes in plasma CK, CK-MB and LDH with an increase of 1.5-fold, 1.3-fold and 1.4-fold, respectively, which were statistically different to those in the I/R control group.

### Changes in cardiomyocyte ultrastructure

In the sham group, the shapes of organelles were normal and the chromatin was distributed evenly in the nucleus of cardiomyocytes [**Fig. 2(A)**]. Relative to normal cardiomyocytes, H/Re caused significant morphological changes with two distinct types of cell death: necrosis and apoptosis [**Fig. 2(B)**]. Necrosis was characterized by cell swelling, membrane structure destruction and organelle breakdown, whereas apoptosis was distinguished as chromatin condensation and margination. At the same time, degeneration characterized by sparse cytoplasm and swollen or vacuolized mitochondria were observed in many cardiomyocytes. Addition of PEG without $F_2$ had no effect on changes in cardiomyocyte ultrastructure [**Fig. 2 (C)**], which showed similar morphological changes as those in the I/R control group. However, these changes in cardiomyocyte ultrastructure were markedly inhibited when $F_2$ was added to the cells before H/Re. The necrotic and apoptotic cells were barely observed. Although vacuolar mitochondria were also observed, the cytoplasm was rich in organelles [**Fig. 2(D)**].

### Levels of *Egr-1* mRNA in myocardial tissue and cultured cardiomyocytes

Levels of *Egr-1* mRNA in the four groups are shown in **Fig. 3**. Relative to the sham group, levels of *Egr-1* mRNA in myocardial tissue and cultured cardiomyocytes in the I/R (H/Re) control group were significantly increased at the

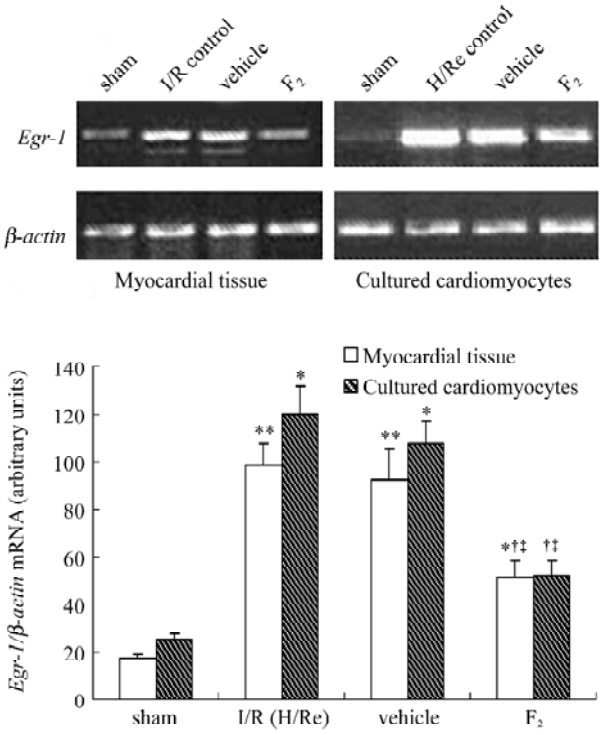


**Fig. 3 Levels of early growth response gene 1 (*Egr-1*) mRNA in myocardial tissue and cultured cardiomyocytes detected by reverse transcription-polymerase chain reaction**

Quantitative densitometric data are expressed as percentage of *β-actin*. All values are expressed as mean±standard error of the mean from five separate experiments. * $P<0.05$ and ** $P<0.01$ versus sham group; † $P<0.05$ versus ischemia/reperfusion (I/R) hypoxia or reoxygenation (H/Re) control group; ‡ $P<0.05$ versus vehicle group. $F_2$, *N*-n-butyl haloperidol iodide.

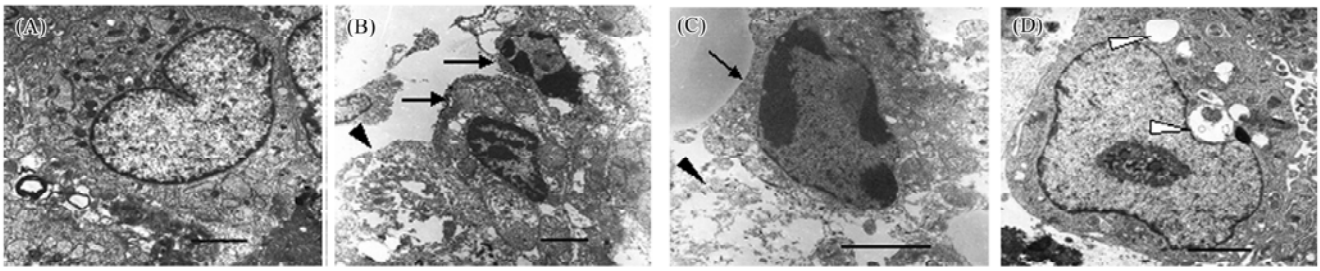


**Fig. 2 Ultrastructure of cultured neonatal rat cardiomyocytes**

(A) In the sham group, the shapes of organelle were normal. (B) In the hypoxia/reoxygenation (H/Re) control group (3 h of H followed by 1 h of Re), the necrotic (black arrow) and apoptotic cells (arrow heads) were detected. (C) In the vehicle group (H/Re treated with polyethylene glycol), apoptotic cells and breakdown organelles were detected. (D) In the *N*-n-butyl haloperidol iodide ($F_2$) group (H/Re treated with $F_2$, $1\times10^{-6}$ M), some mitochondrial vacuoles (white arrows) were detected in the cytoplasm. Experiments were repeated four times and similar results were obtained. Bar = 2 μm.

end of the experiment. These changes were not altered by PEG, but significantly reduced when $F_2$ was given to the animals or added to the cultured cells.

### Detection of Egr-1 protein expression in myocardial tissue and cultured cardiomyocytes

In the sham group, the weak immunostaining for *Egr-1* expression was detected in myocardium (5±1 per sight, $n$=4), but not in microvasculature [**Fig. 4(A)**]. In contrast, markedly enhanced *Egr-1* expression was observed in both myocardium and microvasculature in the I/R control group (29±3 per sight, $n$=4, $P$<0.05) [**Fig. 4(B)**]. These changes were not altered by PEG (30±3 per sight, $n$=4, $P$<0.05) [**Fig. 4(C)**], but significantly downregulated when $F_2$ was administered before I/R (16±2 per sight, $n$=4, $P$<0.05) [**Fig. 4(D)**]. In addition, *Egr-1* expression was also demonstrated in cultured cardiomyocytes. Relative to the sham group (7±1 per sight, $n$=4) [**Fig. 4(E)**], H/Re significantly increased *Egr-1* expression (27±4 per sight, $n$=4, $P$<0.05), localized mainly in the nucleus of cardiomyocytes [**Fig. 4(F)**], which was not altered by PEG (28±3 per sight, $n$=4, $P$<0.05) [**Fig. 4(G)**], but significantly inhibited by $F_2$ (13±2 per sight, $n$=4, $P$<0.05) [**Fig. 4(H)**], consistent with a down-regulation of *Egr-1* expression in myocardial tissue.

## Discussion

The present study demonstrates that $F_2$ reduces myocardial I/R injury in an *in vivo* model as evidenced by a reduction in leakage of myocardial enzymes such as CK, CK-MB and LDH. In cultured neonatal cardiomyocytes, $F_2$ also reduces H/Re-induced cell death. Consistent with attenuation in myocardial and cell injury by $F_2$, the expression levels of *Egr-1* mRNA and protein are significantly reduced.

It has been reported that the rapid activation of *Egr-1* is associated with I/R-induced tissue and cell injury [8,17–20]. Stimulation of *Egr-1* expression causes release of interleukin 1β, macrophage inflammatory protein 2, intercellular adhesion molecule 1, tissue factor, plasminogen-activator inhibitor 1, vascular endothelial growth factor and platelet-derived growth factor A [9,10,20,21]. The expressions of these representative genes mediate coagulation, inflammatory and vascular permeability, which are the main pathological changes associated with I/R injury. Thus, *Egr-1* has been designated a central and uni-

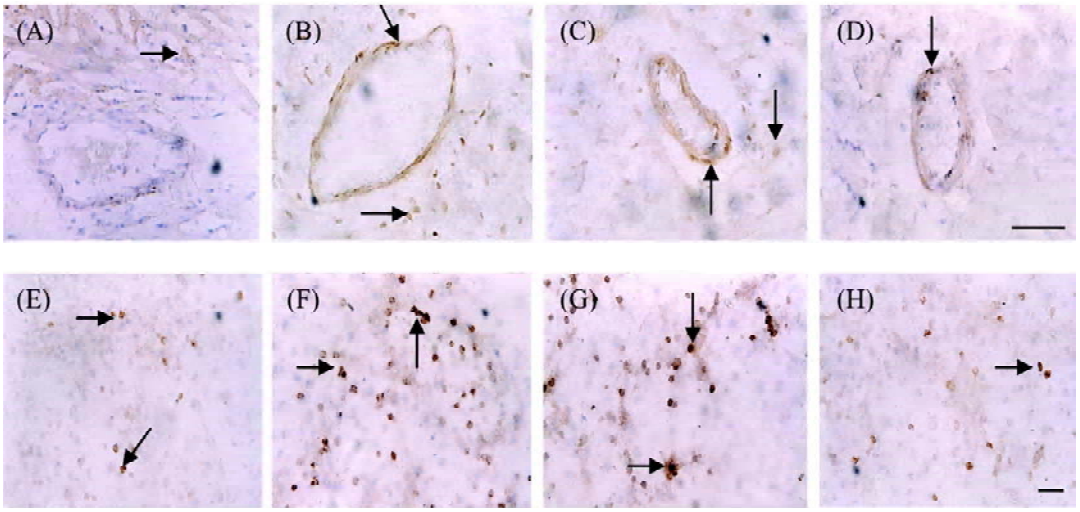


**Fig. 4 Early growth response gene 1 (*Egr-1*) expression detected by immunohistochemistry (A–D) and immunocytochemistry (E–H)**

(A) In myocardial tissue of the sham group, a weakly positive immunostaining of *Egr-1* was observed in myocardium. (B) In the ischemia/reperfusion (I/R) control group, strong expression was detected in myocardium and microvasculature. (C) In the vehicle group, the expression of *Egr-1* was the same as that of the I/R control group. (D) In the *N*-n-butyl haloperidol iodide ($F_2$) group, the expression of *Egr-1* was decreased compared with that of the I/R control and vehicle groups. (E) In cultured cardiomyocytes of the sham group, *Egr-1* was weakly expressed. (F) In the hypoxia and reoxygenation (H/Re) control group, strong expression was detected (mainly in the nucleus). (G) In the vehicle group, strong expression was also detected. (H) In the $F_2$ group, the expression of *Egr-1* was decreased compared with the H/Re control and vehicle groups. Brown staining (arrows) indicates the positive expression of *Egr-1*. Bar=50 μm.

fying role in the pathogenesis of I/R injury [10]. Combining these data with our results, we suggest that the alteration in the induction and activation of *Egr-1* might play a significant role in myocardial and cellular injury associated with I/R.

A previous study has demonstrated the time-dependent expression of *Egr-1* in a murine model of lung I/R with progressively elevated *Egr-1* levels during 60 min of ischemia, and constant increasing expression during 180 min of reperfusion [10]. In the present study, *Egr-1* mRNA and protein expression were barely detected in animals and cardiomyocytes without ischemia (hypoxia) and reperfusion (reoxygenation) in the sham group. Although we did not observe the time-course in the change of *Egr-1* expression during I/R, there was a significant increase in the expression levels of *Egr-1* mRNA and protein at the end of 180 min of reperfusion or after 60 min of reoxygenation. To identify the potential source of changes in *Egr-1* expression, a recent study of I/R in gut has reported that depletion of intestinal resident macrophages prevents I/R injury by inhibiting *Egr-1* expression. CHEN *et al*. stated that activated resident macrophages in ischemic/reperfused gut express a high level of *Egr-1* and pro-inflammatory cytokines and thereby result in tissue damage by releasing reactive free radicals [8]. Myocardial tissue contains all different types of cells, including inflammatory cells (i.e., neutrophils and macrophages), endothelial cells and cardiomyocytes. Therefore, the analysis from such a tissue block can not differentiate cell types in response to *Egr-1* expression. To resolve this issue, cardiomyocytes were isolated for determination of *Egr-1* expression. Consistent with results obtained from an *in vivo* study, cardiomyocytes, which were subjected to H/Re, displayed high expression levels of *Egr-1* mRNA and protein in a comparable level from the tissue blocks of myocardium. These *in vitro* data strongly imply that cardiomyocytes are one of the main sources for *Egr-1* expression. However, we can not exclude the roles of other cells, such as endothelial cells and vascular smooth muscle cells, in expression of *Egr-1*, because positive immunohistochemical staining in coronary vessels was also detected after I/R.

A number of gene products that participate in the pathophysiological responses after I/R have been associated with enhanced expression of *Egr-1* [9,10,21]. However, the signaling pathways in activating *Egr-1* expression after I/R are not yet well defined. Recent studies have demonstrated the potential roles of protein kinase C (PKCβ), the Raf-MEK-Erk pathway and intracellular $Ca^{2+}$ [11,22–24]. PKC-null mice or wild-type mice treated with PKCβ inhibitor displayed increased survival compared with the control animals after single-lung I/R. Protection was associated with significant downregulation in *Egr-1* expression [22]. In a rat model of liver transplantation, the application of immunomodulator FTY720 has been shown to reduce hepatic damage and increase survival rates by inhibiting *Egr-1* expression-mediated pathways by the Raf-MEK-Erk pathway [23]. Mobilization of $Ca^{2+}$ has also been implicated in the induction of *Egr-1* expression with $Ca^{2+}$-ionophore or EDTA [11,24]. In the present study, the inhibition in overexpression of *Egr-1* mRNA and protein as well as protection with $F_2$ from *in vivo* and *in vitro* models accorded with these results. We have previously shown in isolated cardiomyocytes that $F_2$ inhibits intracellular $Ca^{2+}$ influx and overload, further maintains the integrity of the cell membrane, and minimizes ATP depletion [15]. In *in vivo* rat and rabbit models, $F_2$ reduced I/R myocardial injury [15,25]. Concomitant with the fact that *Egr-1* expression can be triggered by elevated intracellular $Ca^{2+}$ overload [11, 24] and $F_2$ reduces tissue and cell damage by inhibiting $Ca^{2+}$ overload [15], we conclude that myocardial and cardiomyocyte protection by $F_2$ might, in part, be a result of the inhibition of $Ca^{2+}$ overload-mediated *Egr-1* expression. However, direct demonstration in roles of *Egr-1* expression-induced myocardial injury and $Ca^{2+}$ overload-mediated *Egr-1* expression after I/R requires further investigation.

In summary, these data provide evidence that $F_2$ reduces myocardial injury in an *in vivo* model of I/R, demonstrated by the attenuation in release of enzymes. In cultured cardiomyocytes, $F_2$ preserves cellular ultrastructure, shown by the reduction in both necrosis and apoptosis after H/Re. In addition, $F_2$ decreases the expression levels of *Egr-1* mRNA and protein identified by RT-PCR, immunohistochemistry and immunocytochemistry in myocardium and cardiomyocytes. The data from present and previous studies in our laboratory [15,25] further suggest that cardioprotection by $F_2$ is associated with the inhibition of $Ca^{2+}$ overload and *Egr-1* expression after I/R. Therefore, an understanding of the molecular events that accompany myocardial I/R injury will enhance the development of therapeutic strategies for treatment of cardiac injury.

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