Egr-1, the Potential Target of Calcium Channel Blockers in Cardioprotection with Ischemia/Reperfusion Injury in Rats

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Key Words

Calcium channel blocker • Early growth response gene-1 • Ischemia/reperfusion • Hypoxia/reoxygenation • Myocardium, cardiomyocyte

Abstract

Aims: In this study, we tested whether Egr-1 is a potential target of calcium channel blockers in cardioprotection with I/R injury. **Methods:** We treated rats in vivo I/R and rat cultured cardiomyocytes in vitro hypoxia/reoxygenation (H/R) models with three types of classical calcium channel blockers (verapamil, diltiazem and nifedipine). Activity of creatine kinase (CK), lactate dehydrogenase (LDH), myeloperoxidase (MPO) superoxide dismutase (SOD) and level of malondialdehyde (MDA) in plasma and culture medium were measured to assess the degree of injury and inflammation of myocardial tissues and cells. Egr-1 mRNA and protein expressions were examined by RT-PCR and Western-blot analyses. **Results:** Calcium channel blockers (verapamil, diltiazem and nifedipine) significantly attenuated myocardial injury, as shown by reduced release of CK and LDH, preserved SOD activity and decreased MDA production and MPO activity. Concomitant with cardioprotection by calcium channel blockers, the

mRNA and protein expression of Egr-1 increased with I/R and H/R injury was significantly reduced in myocardial tissue and cultured cardiomyocytes. **Conclusions:** These results suggested that the cardioprotective effects of calcium channel blockers with I/R or H/R injury might be mediated by downregulating Egr-1 expression. Egr-1 might be the potential target of calcium channel blockers in cardioprotection with ischemia/reperfusion injury.

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Introduction

The rationale for quickly reperfusing the myocardium after myocardial infarction is to salvage the ischemic myocardium. The development of revascularization techniques, such as percutaneous coronary intervention and thrombolytic therapy, have helped in this endeavor [1]. However, reperfusion itself may cause additional damage, called “myocardial reperfusion injury” [2]. Myocardial calcium (Ca²⁺) overload could be responsible, at least in part, for reperfusion injury. Ca²⁺ overload during reperfusion may occur by several mechanisms. One mechanism is related to increased Ca²⁺ influx through

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1015-8987/09/0242-0017\$26.00/0

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Ca²⁺ channels. The second mechanism is the reduced Ca²⁺ reuptake by the sarcoplasmic reticulum. Thirdly, Ca²⁺ entry via Na⁺-Ca²⁺ exchanger (NCX) is believed to importantly contribute to intracellular Ca²⁺ overload under the condition of ischemia and reperfusion. During ischemia, intracellular Na⁺ concentrations rise, in part because intracellular acidosis activates Na⁺/H⁺ exchange, which promotes Na⁺ influx in exchange for H⁺ efflux. The elevated levels of intracellular Na⁺ alter the driving force on NCX, so that it functions in reverse-mode to remove Na⁺ from the cell in exchange for Ca²⁺ [3, 4]. Therefore, the great accumulation of Ca²⁺ during reperfusion of myocardial cells, found to be irreversibly injured during ischemia [5-7].

Calcium channel blockers are well known for their cardioprotective effects, such as reduced mortality after myocardial infarction in humans and animals, reduced infarct size after ischemia/reperfusion injury and prevention of cardiac remodeling after myocardial infarction [6]. In our previous studies, we demonstrated that our synthesized compound *N*-n-butyl haloperidol iodide F₂, Chinese patent No. ZL96119098.1) protected the heart against ischemia/reperfusion injury in different animal models [8, 9]. Its cardioprotective mechanism might be associated with blocking calcium channels and decreasing intracellular free calcium in ventricular myocytes [10].

The gene early growth response 1 (*egr-1*), one of the immediate-early genes, is a member of the zinc finger family of transcription factors. Because *Egr-1* can recruit the expression of multiple downstream target genes it is believed to act as an intracellular “third messenger” [11-13]. *Egr-1* could be a master switch to trigger the pathogenesis of lung ischemia/reperfusion injury [14], and drugs targeting the *Egr-1* were found to prevent hepatic ischemia/reperfusion injury by inhibiting expression of *Egr-1* [15, 16]. These findings indicate that the overexpression of *Egr-1* could be the common denominator in ischemia/reperfusion injury in various organs, so it might be a good therapeutic target in ischemia/reperfusion conditions. Our previous study involving antisense *Egr-1* oligodeoxyribonucleotides suggested that overexpression of *Egr-1* was a causal factor in myocardial ischemia/reperfusion or hypoxia/reoxygenation injury, and F₂ could protect myocardial tissues and cells against ischemia/reperfusion or hypoxia/reoxygenation injury, which was largely due to the inhibition of *Egr-1* overexpression [17, 18].

Egr-1 expression is induced by Ca²⁺ stimulation, and its up-regulation is Ca²⁺ dependent [19, 20]. Data from our laboratory suggest that the cardioprotective effect of

F₂ on myocardial ischemia/reperfusion injury is associated with its characteristics as a calcium channel blocker as well as a downregulator of *Egr-1* overexpression mediated by Ca²⁺ overload [17]. However, the effect of the classical calcium channel blockers on *Egr-1* overexpression in ischemia/reperfusion injury has not been well established. In addition, the traditional viewpoint of the mechanism of calcium antagonism in cardioprotection with ischemia/reperfusion injury is antagonism to Ca²⁺ overload. Nevertheless, *egr-1* has not been reported as a potential target of calcium channel blockers in cardioprotection with myocardial ischemia/reperfusion injury.

We aimed to investigate the effects of the three types of classical calcium channel blockers (verapamil, diltiazem and nifedipine) on ischemia/reperfusion or hypoxia/reoxygenation injury of myocardial tissues and cultured cardiomyocytes, as well as *Egr-1* mRNA and protein overexpression caused by ischemia/reperfusion or hypoxia/reoxygenation stimulation to investigate a possible crucial mechanism of calcium channel blockers in myocardial ischemia/reperfusion injury.

Materials and Methods

Preparation of animals

All animals were treated in compliance with “The Guide for the Care of Use of Laboratory Animals” 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.

Male adult Sprague-Dawley rats weighing 200-250g were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg), then ventilated with oxygen-enriched room air by use of a rodent respirator for 60 breaths/min; the tidal volume was set to 8 ml. The chests of rats were opened via a left thoracotomy through the fifth intercostal space. After pericardiotomy, a silk suture was placed under the left anterior descending coronary artery, 2-3 mm from its origin, and the ends of the suture were threaded through a small plastic tube to form a snare for reversible artery occlusion [21].

Rats were randomly divided into sham group and 5 groups of ischemia/reperfusion for treatment: control group (ischemia/reperfusion alone), vehicle group (0.1% v/v DMSO), verapamil (0.5 mg/kg) group, diltiazem (0.75 mg/kg) group, and nifedipine group (0.2 mg/kg dissolved in DMSO). Hearts of the ischemia/reperfusion rats underwent 60-min left anterior descending coronary artery occlusion, then 180-min reperfusion. Hearts of the sham rats underwent the same surgical procedure, but the suture was left untied. Drugs or DMSO were administered by sublingual intravenously 5 min before ischemia. At the end of experiment, blood was taken from the carotid artery for creatine

Group	CK	LDH	SOD	MDA	MPO
Sham	26±7	20757±6609	221±38	1.14±0.22	0.90±0.22
Control	131±23 [*]	100220±10005 [*]	107±19 [*]	5.44±0.70 [*]	4.79±1.12 [*]
Vehicle	129±21 [*]	98705±10965 [*]	105±17 [*]	5.47±0.79 [*]	4.73±1.02 [*]
Verapamil	61±10 [†]	40104±7439 [†]	173±25 [†]	2.37±0.32 [†]	2.16±0.47 [†]
Diltiazem	63±13 [†]	42901±7941 [†]	169±29 [†]	2.44±0.35 [†]	2.23±0.56 [†]
Nifedipine	70±15 [†]	46246±8148 [†]	161±31 [†]	2.52±0.40 [†]	2.88±0.69 [†]

Table 1. Enzyme activities and malondialdehyde level in plasma and myocardial tissues of different groups *in vivo*. CK, creatine kinase (U/ml); LDH, lactate dehydrogenase (U/mL); SOD, superoxide dismutase (U/mg prot); MDA, malondialdehyde (nmol/mg prot); MPO, myeloperoxidase activity (U/g). CK and LDH from plasma, SOD, MDA, and MPO from the area at risk myocardial tissues in a rat I/R model of 60 min coronary occlusion followed by 180 min of reperfusion. All values are expressed as mean ± S.D. (n=15). ^{*}*P*<0.01 vs. Sham group; [†]*P*<0.05 vs. Control group.

kinase (CK) and lactate dehydrogenase (LDH) measurement. The ischemic myocardium was excised for measurement of myeloperoxidase (MPO), superoxide dismutase (SOD) and malondialdehyde (MDA), or stored at -70°C for determining levels of *Egr-1* mRNA and protein.

Isolation of neonatal rat cardiomyocytes and preparation of hypoxia/reoxygenation

Neonatal rat ventricular myocytes were isolated from 1 to 4-day-old Sprague-Dawley rats. Hearts were rapidly excised, minced and dissociated with 0.1% trypsin. The dispersed cells were plated in medium with 15% FBS for 30 min to remove non-cardiomyocytes [22]. The isolated cardiomyocytes at 2.5×10^6 cells per well were cultured in medium with 5-Bromo deoxyuridine (0.1 mmol/L) to further inhibit the growth of non-cardiomyocytes for the first 3 days. Hypoxia was induced in cardiomyocytes 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] [22], and incubation in an air-tight chamber with pure N₂ gas at 37°C for 3 h. The buffer was then replaced with fresh oxygenated culture medium, and dishes were transferred to a normoxic incubator (95% air-5% CO₂) for 1 h of reoxygenation.

Treatment of cardiomyocytes

After 5-7 days of cell culture in normoxic medium, cardiomyocytes were randomly divided into sham group and 5 groups of hypoxia/reoxygenation for treatment: control group (hypoxia/reoxygenation alone), vehicle group (0.1% v/v DMSO), verapamil (2.0×10^{-6} mol/L) group, diltiazem (1.0×10^{-5} mol/L) group, and nifedipine group (1.0×10^{-5} mol/L dissolved in DMSO). Cardiomyocytes of the sham group were incubated under the normoxic condition for 4 h. The cells of other groups underwent

3 h of hypoxia and 1 h of reoxygenation. Drugs or an equal volume of DMSO was added into the cells before hypoxia/reoxygenation. At the end of reoxygenation, the culture medium was collected for assay of enzymes (CK, LDH). 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 CK and LDH in plasma and culture medium were determined by use of commercial test kits (Jiancheng Bioengineering Institute, Nanjing, China).

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

With use of commercial test kits (Jiancheng Bioengineering Institute, Nanjing, China), tissue protein was measured spectrophotometrically by the Coomassie bright-blue (CBBG250) staining method. The activity of SOD was analyzed by the xanthine oxidase method and level of MDA by the thiobarbituric acid staining method. MPO activity of myocardial homogenates was measured by commercial test kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. One unit of MPO activity is defined as the quantity of enzyme degrading 1 μmol peroxide/min at 37°C. The data are expressed as MPO activity per gram of tissue.

RT-PCR assay

Total RNA was extracted from tissue or cultured cells by use of Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was quantified by the ratio of the absorbance at wavelength

Group	CK	LDH	SOD	MDA
Sham	12.64±2.89	3019.84±297.4	29.15±7.63	0.20±0.05
Control	57.18±9.32 [*]	6940.04±407.7 [*]	15.08±2.08 [*]	0.99±0.29 [*]
Vehicle	55.04±5.16 [*]	7003.74±570.3 [*]	16.14±2.29 [*]	1.02±0.28 [*]
Verapamil	23.12±5.07 [†]	3855.27±233.3 [†]	26.99±8.03 ^{††}	0.41±0.11 ^{††}
Diltiazem	26.48±6.15 [†]	4185.66±316.7 [†]	25.26±7.19 ^{††}	0.44±0.14 ^{††}
Nifedipine	32.36±7.40 [†]	4564.02±402.4 [†]	20.80±6.37 [†]	0.61±0.18 ^{††}

Table 2. Enzyme activities and malondialdehyde level in culture medium and cultured cardiomyocytes of different groups *in vitro*. CK, creatine kinase (U/ml); LDH, lactate dehydrogenase (U/mL); 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.D. (n=15). ^{*}P<0.01 vs. Sham group; [†]P<0.05, ^{††}P<0.01 vs. Control group.

260 nm to that at wavelength 280 nm (more than 1.8). RT-PCR amplification involved use of RevertAid (MBI Fermentas, Vilnius, Lithuania) H minus first-strand cDNA synthesis and PCR with a *Taq* DNA polymerase kit (MBI Fermentas). The sequences of forward and reverse primers for rat *Egr-1* were 5'-GCA ACA CTT TGT GGC CTG AA-3' and 5'-GAG TTG GGA CTG GTA GG TGT-3', respectively, yielding a 512-bp product [23]. The sequences of forward and reverse primers of β -actin, used as the control, were 5'-GTG GGT ATG GGT CAG AAG GA-3' and 5'-AGC GCG TAA CCC TCA TAG AT-3', respectively, yielding a 380-bp product. PCR conditions were as follows: 30 cycles, denaturing at 94°C for 45 s, annealing at 52°C for 60 s and extension at 70°C for 60 s, with initial heating at 95°C for 5 min and final extension at 70°C for 10 min. The PCR products were separated on 1.2% agarose gels and stained with ethidium bromide. The relative densities of mRNA bands were analyzed by use of Fluorchem 8900 (Alpha Innotech, Miami, FL, USA).

Western blot analysis

Total protein extracts were prepared from myocardial tissue or cultured cells with use of 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 amounts of total protein (60 μ g for tissue and 80 μ g for cells) underwent 10% SDS-PAGE, then were electrophoretically transferred to nitrocellulose membranes. Nonspecific binding was blocked by incubation of membranes with skim milk overnight at 4°C. The blot was incubated with a rabbit anti-rat *Egr-1* antibody (1:200 dilution for tissues and 1:100 dilution for cells; Santa Cruz Biotechnology, Santa Cruz, CA, USA) 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 involved western blot analysis chemiluminescence luminol reagents (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were then reprobed with β -actin (1:2000, Sigma-Aldrich, USA) to confirm equal loading of samples.

Statistical analysis

The relative densities of mRNA or protein were analyzed by use of FluorchemTM software (Alpha Innotech, USA). Values are expressed as mean ± S.D. Comparisons among groups involved ANOVA (single factor), followed by Student-Newman-Keuls test with use of Microsoft Excel (2003). A *P* < 0.05 was considered statistically significant.

Results

Effect of calcium channel blockers on enzyme activities induced by ischemia/reperfusion or hypoxia/reoxygenation

After ischemia/reperfusion or hypoxia/reoxygenation, the CK and LDH activity and MDA product were significantly higher and SOD activity was significantly lower than that in sham group (Tables 1, 2). However, treatment with verapamil, diltiazem and nifedipine could attenuate the leakage of CK and LDH from myocardial cells caused by ischemia/reperfusion or hypoxia/reoxygenation, preserve the activity of SOD, and reduce the production of MDA.

Fig. 1. RT-PCR of *Egr-1* mRNA in myocardial tissue and cultured cardiomyocytes. Line 1 Sham group, Line 2 Control group, Line 3 Vehicle group, Line 4 Verapamil group, Line 5 Diltiazem group, Line 6 Nifedipine group. Quantitative densitometric data are expressed as a percentage of β -actin. All values are expressed as mean $\pm$ S.D. of five independent experiments. # P <0.05 vs. Sham group; * P <0.05 vs. Control group.

Effect of calcium channel blockers on myocardial tissue MPO activity

MPO activity was low in the sham group at the end of the observation period (Table 1). However, in the control group, MPO activity was markedly increased relative to the sham group. Treatment with verapamil, diltiazem and nifedipine significantly reduced ischemia/reperfusion-increased MPO activity in the ischemic myocardium as compared with ischemia/reperfusion alone. These data suggest that calcium channel blockers reduced neutrophil accumulation in the ischemia/reperfusion myocardium.

Effect of calcium channel blockers on levels of Egr-1 mRNA and protein in myocardial tissues and cultured cardiomyocytes

RT-PCR showed the level of *Egr-1* mRNA in myocardial tissues and cultured cardiomyocytes in the control group significantly increased as compared with that in sham group (Fig. 1). Treatment with verapamil, diltiazem and nifedipine significantly reduced the mRNA level of *Egr-1* in tissue and cells. Western blot analysis revealed the protein level of *Egr-1* in tissue and cardiomyocytes significantly increased as compared with that in sham group and significantly reduced with verapamil, diltiazem and nifedipine treatment (Fig. 2).

Discussion

In recent decades, two major theories supported by most experimental evidence suggested that Ca^{2+} overload (Ca^{2+} hypothesis) and the generation of oxygen-derived free radicals (oxyradical hypothesis) are responsible for the myocardial ischemia/reperfusion injury. With the exploration of the pathogenic mechanism in ischemia/reperfusion injury, inflammation was found to be the cause of the main pathological changes. The pathogenesis of coagulation, inflammation and vascular permeability in the ischemia/reperfusion condition is mediated by some inflammation-related genes such as interleukin 1- β , macrophage inflammatory protein, intercellular adhesion molecule 1, tissue factor, plasminogen-activator inhibitor and vascular endothelial growth factor [14, 22, 23]. These genes are the downstream target genes coordinately upregulated by the activation and expression of *Egr-1*, caused by ischemia/reperfusion stimulation. Thus, *Egr-1* was considered to have a central and unifying role in the pathogenesis of ischemia/reperfusion injury [14].

The present study demonstrated that ischemia/reperfusion or hypoxia/reoxygenation caused the overexpression of *Egr-1* protein in myocardial tissues and cells both *in vivo* and *in vitro*. Such overexpression of

Fig. 2. Western blot analysis of Egr-1 protein in myocardial tissues and cultured cardiomyocytes. Line 1 Sham group, Line 2 Control group, Line 3 Vehicle group, Line 4 Verapamil group, Line 5 Diltiazem group, Line 6 Nifedipine group. Quantitative densitometric data were expressed as a percentage of that in Control group. All values are expressed as mean $\pm$ S.D. of five independent experiments. # P <0.05 vs. Sham group; * P <0.05 vs. Control group.

Egr-1 induced myocardial injury, as evidenced by increased activity of myocardial enzymes such as CK and LDH. In our previous study, we used the antisense, sense, and scrambled-sequence *Egr-1* oligodeoxyribonucleotides to indentify the caused-effect relationship between *Egr-1* overexpression/upregulation and the myocardial ischemia and reperfusion injury. The results showed that the antisense *Egr-1* oligodeoxyribonucleotides remarkably inhibited the expression of *Egr-1*. However, the expression of *Egr-1* was not inhibited by the sense and the scrambled-sequence *Egr-1* oligodeoxyribonucleotides [17]. In addition, our patent compound, *N*-n-butyl haloperidol iodide could inhibit the expression of *Egr-1* as well as blocked L-type calcium channel [10, 17]. The present study showed that calcium antagonists should downregulate the ischemia/reperfusion- or hypoxia/reoxygenation-induced overexpression of *Egr-1* mRNA and protein in myocardial tissue and cells. As well, calcium antagonists protected the myocardial tissues and cells against ischemia/reperfusion- or hypoxia/reoxygenation injury by attenuating CK and LDH leakage from cardiomyocytes. So, these two consistent studies suggested that the cardioprotection against ischemia/reperfusion injury of calcium antagonists was due to the inhibition of Egr-1 overexpression. Furthermore, the calcium antagonists significantly decreased MPO activity to weaken inflammation by attenuating the ischemia/reperfusion-induced accumulation and activation of neutrophils *in vivo*. Oxygen-derived free radicals not only

damaged the myofibrillar membrane and mitochondria and induced apoptosis and necrosis of myocytes but also resulted in inflammation, which was attributed to the myocardial ischemia/reperfusion injury. Our results suggest that the calcium antagonists strongly preserved the activity of SOD and decreased the production of MDA, the lipid peroxidation metabolite, for a beneficial effect on inflammation and myocardial ischemia/reperfusion injury. Although we could not confirm the direct antioxidation of the calcium antagonists or the interaction between the oxidation and the calcium antagonism, these results are consistent with other reports described the antioxidant properties of calcium antagonists as being due to either a direct scavenging effect or the preservation of the SOD activity [24-27]. Nevertheless, the antioxidant activity of calcium antagonists contributed to the cardioprotection with ischemia/reperfusion injury.

As compared to knowledge of the downstream target genes regulated by *Egr-1* mediating the ischemia/reperfusion injury, the signaling pathways activating *Egr-1* expression after ischemia/reperfusion are not yet well defined. It has previously been demonstrated that intracellular Ca^{2+} , the most important second transmitter, plays a potential role in the activation and expression of *Egr-1* [20]. The Ras/Raf-1/ERK_{1/2} pathway mediated by the Ca^{2+} /protein kinase C, Ca^{2+} /calmodulin and Ca^{2+} /calcineurin pathways, and the Ca^{2+} /MAPK pathway are involved in the activation and expression of *Egr-1* [20, 28-32]. Calcium antagonists are well known to

possess cardioprotection associated with the inhibition of Ca^{2+} overload in ischemia/reperfusion injury.

From our current study, cardioprotective effects of calcium channel blockers in myocardial injury might be due in part to the downregulation of *Egr-1* overexpression. However, the detailed mechanism of the Ca^{2+} overload antagonism and the downregulation of *egr-1* by calcium channel blockers, as well as the signal transduction cascade between Ca^{2+} and *Egr-1* needs further exploration.

Acknowledgements

This work is supported in part by research grants from the National Natural Science Found of China (No. 30672465), the Natural Science Foundation of Guangdong Province of China (No.06118928), and the Natural Science Foundation of Guangdong Province of China (No. 07300864). We gratefully appreciate Dr. Laura Heraty (BioMedEditing, USA) for refining the language.

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