Biochemical Pharmacology 79 (2010) 1428–1436

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

N-*n*-Butyl haloperidol iodide protects against hypoxia/reoxygenation-induced cardiomyocyte injury by modulating protein kinase C activity

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ARTICLE INFO

Article history:
Received 8 November 2009
Accepted 19 January 2010

Keywords:
Apoptosis
Cardiomyocytes
Early growth response-1
Hypoxia/reoxygenation
Inflammation
Protein kinase C

ABSTRACT

N-*n*-Butyl haloperidol iodide (F_2), a novel compound derived from haloperidol, protects against the damaging effects of ischemia/reperfusion (I/R) injury *in vitro* and *in vivo*. We tested whether the myocardial protection of F_2 on cardiomyocyte hypoxia/reoxygenation (H/R) injury is mediated by modulating protein kinase C (PKC) activity in primary cultured cardiomyocytes. Primary cultures of ventricular cardiomyocytes underwent 2-h hypoxia and 30-min reoxygenation. Total PKC activity was measured, and the translocation pattern of PKCα, βII, δ and ε isoforms was assessed by fractionated western blot analysis. We investigated the association of PKC isoform translocation and H/R-induced injury in the presence and absence of the specific inhibitors and activator. Measurements included cell damage evaluated by creatine kinase (CK) release, and apoptosis measured by annexin V-FITC assay. In primary cultured cardiomyocytes exposed to H/R, PKCα, δ and ε were translocated, with no change in PKCβII activity. Total PKC activity, CK release and apoptosis were increased after H/R. Treatment with the conventional PKC inhibitor Gö6976 reduced early growth response-1 (Egr-1) protein expression and attenuated apoptosis. The PKCε inhibitor peptide εV1–2 increased H/R injury without influencing Egr-1 expression. Pretreatment with F_2 inhibited translocation of PKCα, increased translocation of PKCε, and relieved the CK release and apoptosis. The protection of F_2 was blocked in part by the conventional PKC activator thymeleatoxin (TXA) and εV1–2 peptide. F_2 significantly alleviated H/R-induced injury, which might be attributed to the combined benefits of inhibiting PKCα and activating PKCε.

1. Introduction

Haloperidol (Hal) (Fig. 1A), a classical anti-psychotic butyrophenone drug, has been shown to possess cardiohemodynamic effects and electrophysiological action. Hal at a clinical dose relieved symptoms of unstable angina pectoris and ameliorated ischemic changes seen on electrocardiography in patients [1], but large-sample studies are hampered by its extrapyramidal adverse reactions. Therefore, we used the piperidine group of Hal to modify and screen 1 compound, *N*-*n*-butyl haloperidol iodide (F_2) (Fig. 1B), which was found to maintain the relaxation of the coronary artery but have no extrapyramidal side reactions [2]. The compound has been patented. Our previous studies showed that F_2 could antagonize myocardial ischemia/reperfusion (I/R) injury in rats and rabbits. Its cardioprotective mechanism might be associated with the inhibition of Ca^{2+} overload by blocking the calcium channels of ventricular myocytes [3,4] and suppressing the expression of early growth response-1 (Egr-1) [5,6]. This mechanism, in turn, functions as a master switch orchestrating the expression of diverse gene families to elicit a pathological response to hypoxia, I/R, and vascular stress [7].

The protein kinase C (PKC) family of highly homologous enzymes is a group of closely related serine–threonine protein kinases, further classified as the conventional PKCs (α, βI, βII, and γ), the novel PKCs (δ, ε, θ, and η) and the atypical PKCs (ζ, ι/λ). Differences in substrate specificity between the PKC isoforms suggest that a particular PKC isoform within a single cell may regulate specific physiological responses [8]. PKC activation includes translocation of the enzyme from the cytosol to the membrane, which is implicated in numerous cellular signal-transduction processes affecting a variety of functions [9,10]. A number of reports have described PKC activation associated with ischemic preconditioning (IPC) models and acute cardiac injuries [11,12] or chronic cardiac diseases [13]. Myocardial

Abbreviations: CK, creatine kinase; DMSO, dimethylsulfoxide; Egr-1, early growth response-1; ERK, extracellular-signal-regulated protein kinase; F_2, *N*-*n*-butyl haloperidol iodide; Hal, haloperidol; H/R, hypoxia/reoxygenation; I/R, ischemia/reperfusion; IP_3, inositol triphosphate; IPC, ischemic preconditioning; JNK, c-Jun N-terminal kinase; PI, propidium iodide; PKC, protein kinase C; PPI, phosphatidylinositol; TXA, thymeleatoxin.

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doi:10.1016/j.bcp.2010.01.021

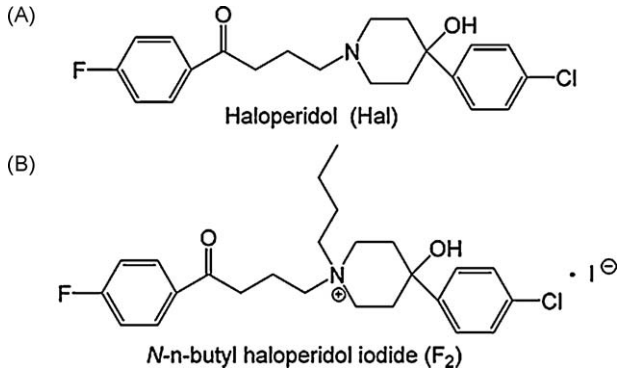

Fig. 1. Chemical structure of (A) haloperidol (Hal) and (B) *N*-*n*-butyl haloperidol iodide (F_2).

I/R or hypoxia/reperfusion (H/R) stimuli are also potent simulators of the translocation of PKCα, β, δ, and ε isoforms [14–17]. Pharmacologic- or gene therapy-based methods have shown inhibition of PKCα/β to enhance cardiac contractility and attenuate heart failure in a rat model of post-infarction cardiomyopathy [18,19]. Homozygous PKCβ-null mice and wild-type mice subjected to I/R and fed the PKCβ inhibitor ruboxistaurin showed increased protection against organ dysfunction and damage in disorders characterized by hypoxia or I/R injury [15,20]. In addition, the opposite roles of PKCδ and ε in cardiac ischemia and reperfusion-induced apoptosis and necrosis in the myocardium [21]. Selective activation of PKCε before and early during ischemia can boost the endogenous survival response, whereas inhibition of PKCδ at reperfusion can preserve cardiomyocytes that would otherwise undergo apoptotic and oncotic cell death [12,22,23]. PKCδ preferentially activates the cell-damaging c-Jun N-terminal kinase (JNK) pathway, whereas PKCε activates the cell-survival extracellular-signal-regulated protein kinase (ERK) pathway. Although cardiac PKC isozymes may have different functional roles with respect to the phenotype and physiological properties expressed by muscle cells, the identity of the individual isozymes that control cardiac gene regulation and trigger diverse responses in myocytes remains unknown.

Egr-1 is activated in response to cardiac hypoxia and I/R [24,25]. With use of antisense Egr-1 oligodeoxynucleotide, our previous study *in vivo* and *in vitro* demonstrated that overexpression of Egr-1 could be responsible for myocardial I/R injury [5,6,26]. Recent studies show that activated PKC is a critical upstream regulator of Egr-1 [27–29]. For example, hypoxia induces Egr-1 expression in endothelial cells via the PKCα-dependent pathway [30]. Egr-1 expression and ERK and JNK phosphorylation after lung I/R is reduced in PKCβ-knockout mice or wild-type mice fed the inhibitor of PKCβ, especially the βII isoform [31,32].

We aimed to test whether F_2 reduces cell damage by modulating PKC activity in primary cultured cardiomyocytes induced by H/R. Further, we aimed to investigate the mechanisms underlying inhibition of Egr-1 expression by F_2 in a cardiomyocyte H/R model. Specifically, we examined the PKC isoforms α, βII, δ and ε, which predominate in myocardium. The H/R model is often used to study the effects of I/R *in vitro*. The use of primary cardiomyocyte cultures allowed us to eliminate the confounding effects of administering PKC inhibitors *in vivo* and inhibition of PKC on multiple cell types in numerous organs.

2. Materials and methods

2.1. Experimental model

Adult Sprague–Dawley rats (200–250 g) were obtained from the Laboratory Animal Breeding and Research Center (Shantou, China). Care of rats in this investigation conformed to *The Guide for the Care of 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.

2.2. Cell culture

Ventricular cardiac myocytes from 2-day-old Sprague–Dawley rats were cultured by gentle, serial trypsinization. A preplating step was included to reduce the number of contaminating non-myocytes. Myocytes represented 90–95% of total adhering cells. The dispersed cells were plated in M-199 medium containing 10% fetal bovine serum for 30 min to remove non-cardiomyocytes, then 2.5×10^6 cells/ml (10 ml/dish) were placed in 100-mm culture dishes and maintained at 37 °C in a 5% CO_2 incubator with 0.1 mM 5-bromo deoxyuridine for the first 4 days. Experiments were performed on days 5 or 6 of culture.

2.3. Hypoxia/reoxygenation procedure

Hypoxia was induced by replacing the initial culture medium with buffer pH 6.2: 137 mM NaCl, 12 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2 \cdot H_2O$, 4 mM HEPES, 20 mM Na lactate [33]. Cardiomyocytes were incubated in an air-tight chamber gassed with pure N_2 at 37 °C for 2 h. The buffer was then replaced with fresh oxygenated culture medium, and the dishes were transferred into a normoxic incubator for 30 min of reoxygenation.

2.4. Chemicals and experimental protocols

N-*n*-Butyl haloperidol iodide (F_2) was synthesized by our laboratory. The conventional PKC activator thymeleatoxin (TXA) and the phospholipase-C inhibitor U73122 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The conventional PKC inhibitor Gő6976 and myristoylated PKCε inhibitory peptide εV1–2 were from Biomol (Plymouth Meeting, PA, USA). Other chemical and regents were from local commercial sources. Gő6976, TXA and εV1–2 were dissolved in dimethylsulfoxide (DMSO). U73122 was dissolved in ethanol. The final DMSO or ethanol concentration in solution was ≤0.1%, and the concentration had no effect on viability of normal primary cardiomyocytes and H/R-induced primary cardiomyocytes.

After 5 or 6 days of cell culture in normoxic medium, cardiomyocytes were randomly divided into eight groups for treatment: control (incubated under normoxic condition); H/R; simulated H/R pretreated with F_2 (1×10^{-6} mol/L), Gő6976 (1×10^{-6} mol/L), or εV1–2 (1×10^{-5} mol/L) for 30 min; simulated H/R pretreated with TXA (1×10^{-6} mol/L), εV1–2, or U73122 (1×10^{-5} mol/L) exposed to F_2 for 10 min.

2.5. Protein extraction

Cardiomyocytes were homogenized in 200 μl ice-cold lysis buffer (25 mM Tris–HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton X-100, 10 mM β-mercaptoethanol, 1 μg/ml leupeptin, 1 μg/ml aprotinin). The homogenized samples were allowed to sit on ice for 20 min, then, were centrifuged at 10,000 × *g* for 10 min at 4 °C; the supernatant was saved. The supernatant represented both cytosolic and soluble membrane protein and corresponded to the total protein. The subcellular distribution of PKC isoforms in cell fractions was determined as described [14]. Briefly, cardiomyocyte cultures were washed with phosphate buffered saline (PBS) and then immediately lysed in 0.5 ml ice-cold homogenization buffer (20 mM Tris–HCl, pH 7.5, 2 mM EDTA,

2 mM EGTA, 6 mM β-mercaptoethanol, 50 μg/ml aprotinin, 48 μg/ml leupeptin, 5 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium vanadate, and 50 mM NaF), sonicated, and centrifuged at 100,000 × *g* for 1 h. The supernatant was removed (soluble fraction) and the pellet was resuspended in 0.3 ml homogenization buffer containing 1% Triton X-100 to solubilize particulate proteins. After being shaken on ice for 30 min, Triton X-100 insoluble proteins were removed by centrifugation at 10,000 × *g* for 10 min, and the supernatant was saved (particulate fraction).

2.6. Detection of PKC activity

The PepTag assay [34] uses a brightly colored, fluorescent peptide substrate that is highly specific to PKC. Phosphorylation by PKC changes the net charge of the substrate from +1 to −1, thereby allowing the phosphorylated and nonphosphorylated versions of the substrate to be separated on an agarose (0.8%) gel. The phosphorylated species migrates toward the positive electrode, whereas the nonphosphorylated substrate migrates toward the negative electrode. The phosphorylated peptide in the band can then be visualized under UV light. Protein from the total, soluble, and particulate cellular fractions was incubated with PKC reaction mixture (25 μl) according to the manufacturer's protocol (Promega, Madison, WI, USA) at 30 °C for 30 min. The reactions were stopped by placing the tubes in a boiling water bath for 10 min. After adding 80% glycerol (1 μl), the samples were loaded onto an agarose gel (0.8% agarose in 50 mM Tris–HCl, pH 8.0), then separated on the agarose gel in the same buffer at 100 V for 25 min, and the bands were visualized under UV light.

2.7. Western blot analysis

Protein from the total, soluble, and particulate cellular fractions underwent protein immunoblotting as described previously with minor modifications [35]. Equal amounts of protein were separated on 8% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked by 1-h incubation at room temperature in a Tris-buffered saline solution (TBS-T: 20 mM Tris, pH 7.6, 135 mM NaCl, and 0.05% Tween) containing 5% nonfat dry milk. Membranes were probed with anti-PKCα, PKCβII, PKCδ and PKCε and anti-Egr-1 antibodies (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight. After the primary antibody incubation, the membrane was washed 3 times with TBS-T. The appropriate secondary antibody, horseradish peroxidase-labeled goat-anti-rabbit IgG, was then added to the membrane according to the vendor's recommendation (1:2000 dilution; Boster Biotech, Wuhan, China) and incubated for 2 h at room temperature. The membrane was again washed 3 times with TBS-T and once with TBS. The bound antibodies were detected by use of SuperSignal western blotting kits (Pierce Biotech, Rockford, IL, USA). Densitometric analysis of western blots involved use of Quantity One® Software (v4.5.2, Bio-Rad, Hercules, CA, USA).

2.8. Measurement of CK activity

Cellular damage was evaluated by measuring creatine kinase (CK) release in culture medium. After H/R treatments, where indicated, a spectrophotometric CK enzyme assay was performed with the test kits (Jiancheng Bioengineering Institute, Nanjing, China) and Multiskan Spectrum Microplate Spectrophotometer (Thermo Labsystems, Finland).

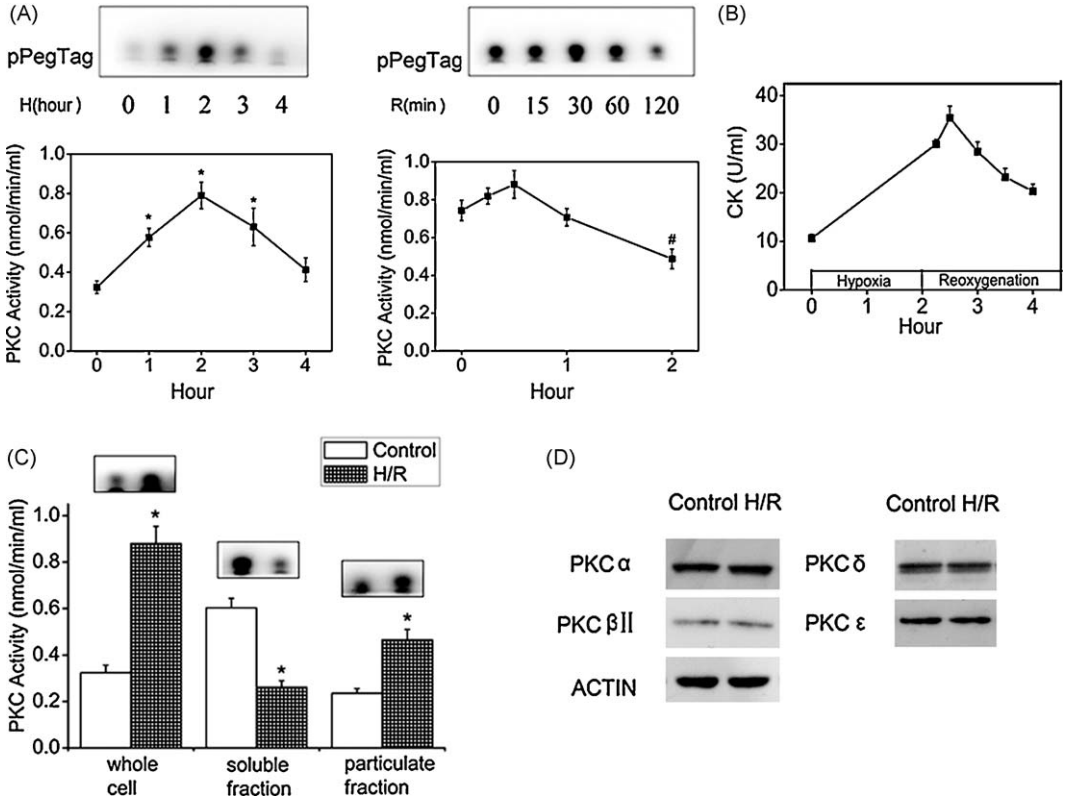

Fig. 2. Activity of total protein kinase C (PKC) and intracellular distribution after hypoxia/reoxygenation (H/R) as measured by PepTag® Non-Radioactive Protein Kinase Assay and fractionated western blot analysis. (A) Time course of activation of total PKC activity induced by hypoxia and reoxygenation, respectively. Values are mean ± S.E.M. (n = 10). $^{*}P < 0.05$ vs. control cells; $^{\#}P < 0.05$ vs. hypoxia cells. (B) Time course of creatine kinase (CK) release by H/R-induced cell damage. Values are mean ± S.E.M. (n = 15). (C) Total amount of PKC activity in the whole cell, soluble fraction and the particulate fractions after 2-h hypoxia and 30-min reoxygenation. Values are mean ± S.E.M. (n = 10). $^{*}P < 0.05$ vs. control cells. (D) Total protein levels of individual PKC isoforms exposed to H/R in primary cultured cardiomyocytes.

2.9. Annexin V-FITC apoptosis assay

Apoptosis was assessed by the Annexin V-FITC Apoptosis Detection kit (Bipec Biopharma Corp., Cambridge, MA, USA) according to the manufacturer's protocol. Briefly, cells were washed with ice-cold PBS, then resuspended in 400 μl of binding buffer at 1×10^6 cells/ml. An amount of 5 μl annexin V-FITC stock solution was added to the cells, and cells were incubated in the dark for 15 min at room temperature. Immediately after mixing with 10 μl propidium iodide (PI) solution for another 5 min in the dark, the samples were analyzed by flow cytometry with use of a FACSort Flow Cytometer (Becton-Dickinson, Franklin Lake, NJ, USA). Approximately 10,000 cells were counted in each of the samples, and data were analyzed by use of WinMDI software (v2.9, Bio-Soft Net).

2.10. Statistical analysis

All data are expressed as mean ± S.E.M. Student's unpaired *t*-test was used to compare differences between two groups. One-way analysis of variance (ANOVA) followed by Student–Newman–Keuls's test was used to compare the differences among more than two groups. A $P < 0.05$ was considered statistically significant.

3. Results

3.1. PKC activity and PKC isoform expression in primary cardiomyocytes after H/R

The first series of experiments investigated the activation of PKC during H/R in primary cultured cardiomyocytes. Total PKC activity significantly increased after the onset of hypoxia, and peaked at 2 h of hypoxia, remained significantly activated at up to 60 min of reoxygenation, then returned to control values after 2 h of reoxygenation (Fig. 2A). Cell damage was determined by release of CK enzyme, which increases with necrosis in cultured cells. CK activity significantly increased after hypoxia and remained elevated even after 30 min of reoxygenation (Fig. 2B). H/R caused an increase of total PKC activity, which was also involved in translocation of the enzyme. The maximal PKC activity was increased in the particulate fraction (Fig. 2C) after H/R (2 h/30 min), with a significant decrease of PKC activity in the soluble fraction. However, the total protein levels of PKCα, PKCβII, PKCδ and PKCε did not change after H/R (Fig. 2D).

3.2. Effect of F_2 on H/R-induced selective translocation of PKC isoforms

Given that translocation of PKC isoforms from the soluble to particulate fraction is the hallmark of PKC activation, we next examined the translocation pattern of PKC isoforms in response to H/R in the presence and absence of F_2. All but PKCβII translocated in response to H/R. Compared to control cells, H/R-treated cells showed a significant increase in PKCα and ε activity associated with the particulate fraction, but a decrease of PKCδ in the particulate fraction (Fig. 3A). In fact, PKCβII showed significant translocation from the soluble to the particulate fraction after 3-h hypoxia then 30-min reoxygenation (Fig. 3B).

We next tested whether F_2 specifically alters the subcellular distribution of individual PKC isoforms. As compared with cells with H/R alone, primary cardiomyocytes treated for 30 min with F_2 then H/R showed a significant increase in the translocation of PKCε

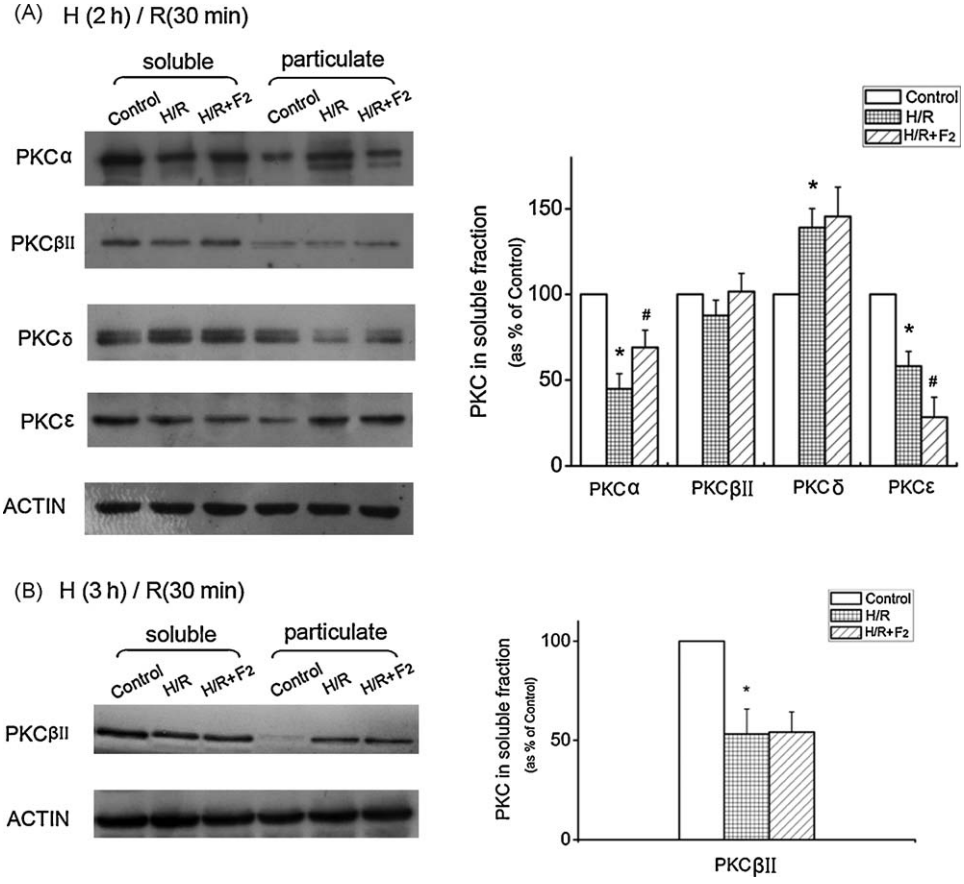

Fig. 3. (A) Primary cultured cardiomyocytes exposed to hypoxia/reoxygenation (H/R) showing translocation of PKCα, δ, PKCε, and PKCβII. *N*-*n*-Butyl haloperidol iodide (F_2; 1 μM) influenced H/R-induced translocation of PKCα and ε. (B) Effect of F_2 on translocation of PKCβII induced by 3-h hypoxia followed by 30-min reoxygenation in primary cultured cardiomyocytes. Immunoblots in the left panel underwent densitometric analysis, and percentage translocation from soluble to particulate fraction was determined. Results are percentage of control cells. Values are mean ± S.E.M. of five independent experiments. $^{*}P < 0.05$ vs. control cells; $^{\#}P < 0.05$ vs. H/R cells.

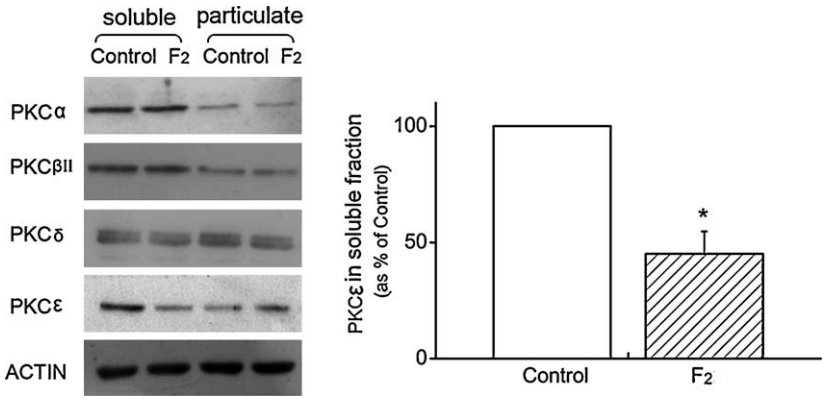

Fig. 4. Primary cultured cardiomyocytes were untreated or exposed to *N*-*n*-butyl haloperidol iodide (F_2; 1 μM) to examine translocation of PKC isoforms. Immunoblots in the left panel underwent densitometric analysis, and percentage translocation from the soluble to particulate fraction was determined. Results are percentage of control cells. Values are mean ± S.E.M. of five independent experiments. $^*P < 0.05$ vs. control cells.

from the soluble to particulate fraction, with a decrease in the translocation of PKCα from the soluble to particulate fraction. Samples with the two fractions probed with antibodies specific for PKCβII and PKCδ showed no difference in translocation as compared with H/R-induced cells. Also, F_2 had no effect on translocation of PKCβII induced by 3-h hypoxia then 30-min reoxygenation (Fig. 3B).

PKC, specifically PKCε, has been implicated as mediating IPC. Therefore, we examined whether treating normal primary cardiomyocytes with F_2 could stimulate the translocation of PKC isoforms. Pretreatment with F_2 produced a significant increase of PKCε activity in the particulate fraction and a decrease of PKCε activity in the soluble fraction as compared with control cells (Fig. 4). However, F_2 had no effect on other isoforms of PKC (α, βII, δ) in the particulate or soluble fractions in normal primary cardiomyocytes.

3.3. PKCα is involved in up-regulation of Egr-1 expression and cell damage induced by H/R

H/R-induced translocation of PKCα and PKCε from the soluble to particulate fraction was inhibited by the isozyme-selective

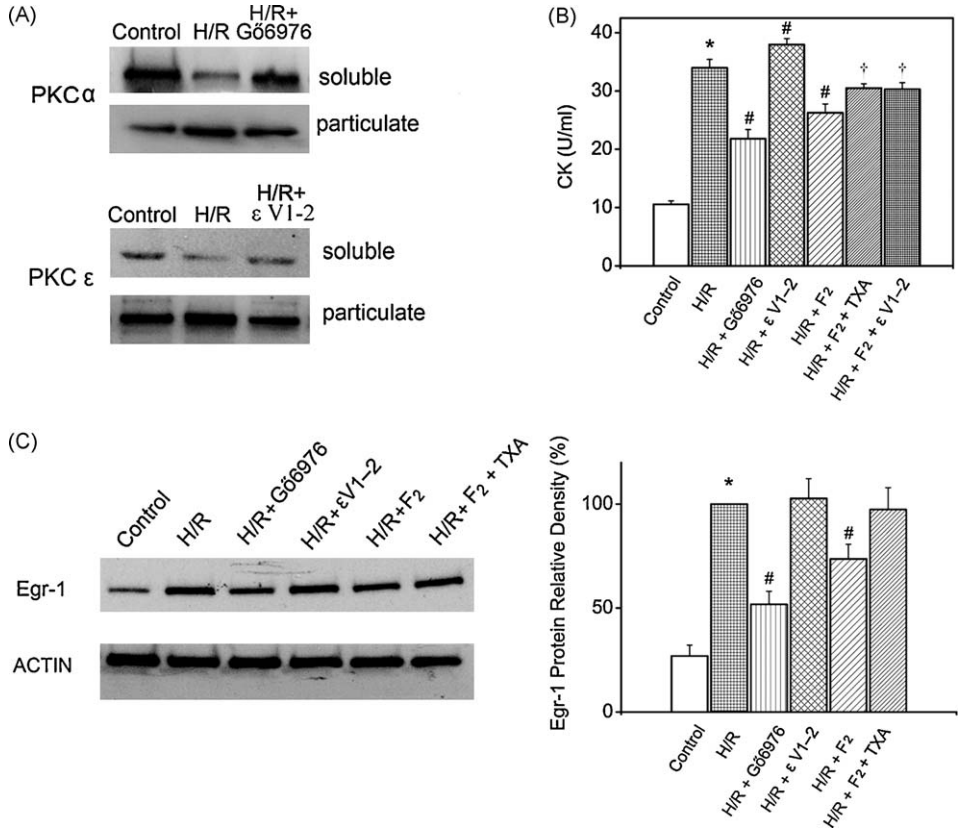

Fig. 5. (A) Hypoxia/reoxygenation (H/R)-induced translocation of PKCα and PKCε from the soluble to particulate fraction inhibited by the isozyme-selective inhibitor Gő6976 and εV1–2 peptide, respectively. (B) Effect of *N*-*n*-butyl haloperidol iodide (F_2; 1 μM), inhibitor and activator of PKC specific isoforms on release of CK in cultured cardiomyocytes. Values are mean ± S.E.M. ($n = 15$). $^*P < 0.05$ vs. control cells; $^{\#}P < 0.05$ vs. H/R cells; $^{\dagger}P < 0.05$ vs. F_2 group. (C) Western blot analysis of effect of F_2 (1 μM), inhibitor and activator of PKC specific isoforms on protein levels of early growth response-1 (Egr-1) in cultured cardiomyocytes. Immunoblots in the left panel underwent densitometric analysis. Results are percentage of control cells. Values are mean ± S.E.M. of five independent experiments. $^*P < 0.05$ vs. control cells; $^{\#}P < 0.05$ vs. H/R cells.

inhibitor Gő6976 and εV1–2 peptide, respectively (Fig. 5A). Release of the cytosolic enzyme CK is a measure of membrane leakiness, which increases with necrosis in cultured cells. After H/R, the level of CK release increased significantly in primary cultured cardiomyocytes as compared with control cells. Gő6976, a cPKC inhibitor, and F_2 could minimize the H/R-induced leakage of CK out of myocardial cells. However, compared with cells treated with H/R alone, those pretreated with the PKCε inhibitor peptide εV1–2 and H/R showed increased CK release. Also, protection of F_2 was blocked in part by the PKC activator TXA and εV1–2 peptide (Fig. 5B). Compared with control cells, cells with H/R showed significantly increased expression of Egr-1 protein but significantly inhibited expression with Gő6976 and F_2 treatment. Pretreatment with εV1–2 peptide conferred no alteration in protein level of Egr-1. Also, inhibition of Egr-1 expression by F_2 was abolished in part by TXA (Fig. 5C).

3.4. Effect of F_2 on H/R-induced apoptosis is associated with inhibition of PKCα and activation of PKCε

Two-hour hypoxia then 30-min reoxygenation caused early apoptosis in primary cultured cardiomyocytes (Fig. 6). F_2 pretreatment significantly reduced H/R-induced apoptosis. Cardiomyocytes pretreated with the PKCε inhibitor peptide εV1–2 before H/R showed a significant increase in cell death as compared with H/R alone. However, in cells with εV1–2 peptide introduced first, then F_2, then H/R, the F_2 protective effect was abolished. For PKCα. Gő6976 could decrease apoptosis, and the protective effect of F_2 was also blocked in part by TXA (Fig. 6).

3.5. The molecular mechanism of F_2 on PKCε translocation is associated with a phosphoinositol (PI) signaling pathway.

To determine whether the effect of F_2 on PKC translocation is by interfering with the PI lipid signaling pathway, we used the inhibitor of phospholipase-C, U73122, which inhibits the hydrolysis of phosphatidylinositol (PPI) to inositol triphosphate (IP_3). Western blot assay showed that the effect of F_2 on activation of PKCε translocation to the particulate fraction was inhibited in normal or H/R-induced cardiomyocytes (Fig. 7).

4. Discussion

We tested whether the myocardial protection of F_2 on cardiomyocyte H/R injury is mediated by modulating PKC activity, especially its isoforms, in primary cultured cardiomyocytes. H/R

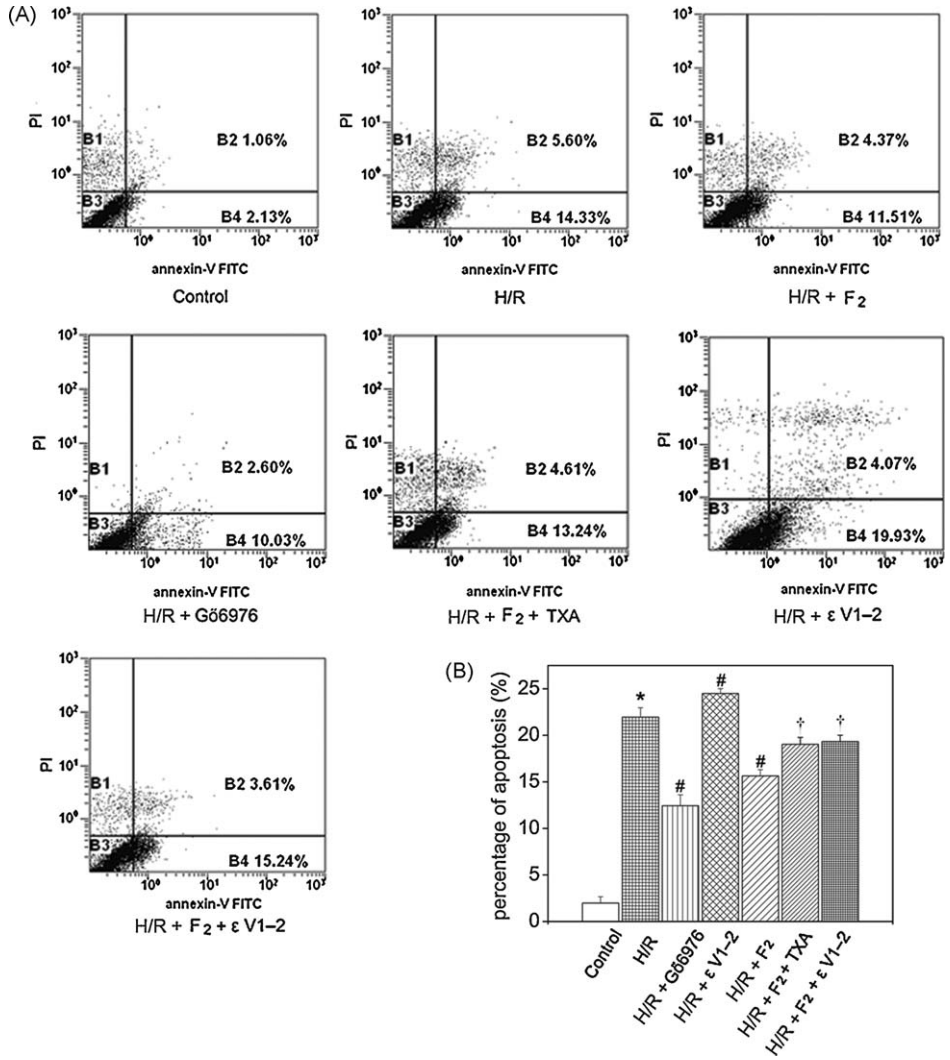

Fig. 6. Effect of *N*-*n*-butyl haloperidol iodide (F_2; 1 μM), inhibitor and activator of PKC specific isoforms on H/R-induced apoptosis in primary cultured cardiomyocytes. Apoptotic cell death was measured by staining with (A) annexin V-FITC/PI; percentage apoptosis from different experimental groups is shown in the upper panel. Values are mean ± S.E.M. ($n = 10$). $^{*}P < 0.05$ vs. control cells; $^{\#}P < 0.05$ vs. H/R cells; $^{\dagger}P < 0.05$ vs. F_2 cells.

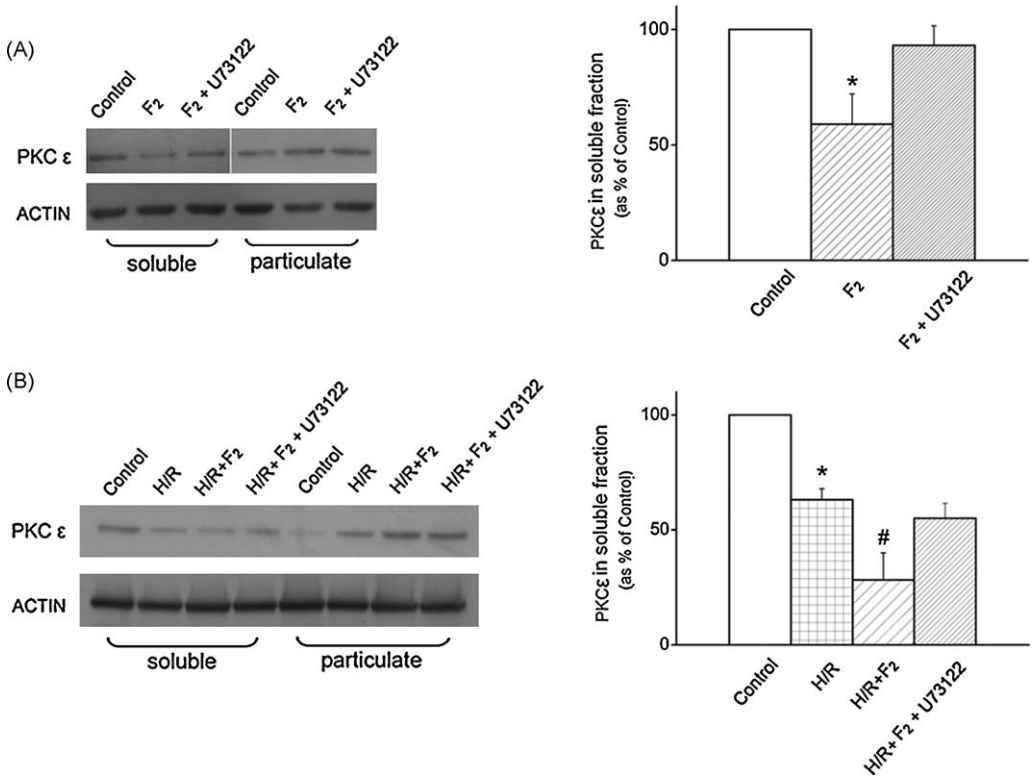

Fig. 7. Phospholipase-C inhibitor U73122 inhibited the effect of *N*-*n*-butyl haloperidol iodide (F_2; 1 μM) on the increase of PKCε translocation to the particulate fraction in normal (A) or H/R-induced myocytes (B). Immunoblots in the left panel underwent densitometric analysis, and percentage translocation from the soluble to particulate fraction was determined. Results are percentage of control cells. Values are mean ± S.E.M. of three independent experiments. $^{*}P < 0.05$ vs. control cells; $^{\#}P < 0.05$ vs. H/R cells.

caused the specific translocation of PKC isoforms in primary cardiomyocytes. Myocardial protection of F_2 depended on PKCα inhibition and PKCε activation. Inhibition of PKCα translocation was associated with downregulation of Egr-1 protein expression and attenuated cell damage. PKCε activation is necessary for cardioprotection against cardiomyocyte apoptosis.

Several studies have demonstrated that the H/R-stimulation component of myocardial I/R insult is sufficient to influence PKC activity. We found that stimulation of total PKC activity is associated with cell damage induced by H/R in cardiomyocytes. A similar effect of I/R inducing an increase in PKC activity was reported for the heart and brain [16,36]. One mechanism by which hypoxia and H/R may increase total PKC activity is by enhanced PKC protein expression. PKCδ and ε are increased in expression during prolonged periods of ischemia [17], but less is known about the reperfusion period. Additionally, the large increases in PKC activity likely cannot be explained by increased protein expression alone. PKC must be altered by other intracellular mechanisms. For example, once activated, PKC translocates from the cytoplasm to the membrane. Furthermore, PKC itself is phosphorylated on activation. In that experiment, as in ours, the protein levels of each PKC isoform were not altered by H/R stimulation. Thus, the increase in total PKC activity as a consequence of H/R appears to result from a posttranslational process. Our translocation studies further support this finding. Of the four isoforms we studied, PKCα, δ and ε but not βII were translocated on H/R (2 h/30 min). Therefore, individual PKC isoforms could be altered in the physiological or pathological setting by posttranslational mechanisms. We found a significant increase in the translocation of PKCβII from the soluble to particulate fraction in cardiomyocytes after H/R for 3 h/30 min but not 2 h/30 min as compared with control cells. This discrepancy may be explained by different environments between different times of the hypoxia model, different *in vivo* and *in vitro* systems, and different injury between H/R and I/R.

A number of reports suggested that inhibition of the conventional Ca^{2+}-dependent PKCα improves myocardial β-adrenergic receptor signaling and ventricular function in myocardial protection and recovery of function resulting from decreased G protein-coupled receptor kinase 2 activity after cardioplegic arrest, cold preservation, and normothermic reperfusion with cardiac transplantation [18,19]. Also, PKCα activation appears to be a major determinant of an increase in cell permeability by interacting with cytoskeletal proteins [37]. There is also evidence that Ca^{2+}-dependent PKCβ may be a potent activator of JNK and proapoptotic signaling in the myocardium subjected to I/R *in vivo*. Deletion of PKCβ and its pharmacological antagonism in a murine model of occlusion–reperfusion of the LAD coronary artery conferred a highly protective effect [15]. Recent studies show that PKC isoforms, particularly PKCα and βII, are critical upstream regulators of Egr-1 in response to hypoxia and I/R injury. A growing body of evidence indicates that the novel Ca^{2+}-independent PKCε and PKCδ isoforms have opposite effects in cardiac ischemia: PKCε activation mediates cardioprotection from cell damage caused by ischemia, and PKCδ activation mediates reperfusion-induced cell damage. Mochly-Rosen and co-workers [38] showed that acute activation of PKCε before organ transplantation mimics IPC and increases organ survival. That activation of PKCδ is deleterious to myocardial function is not a new concept. Myocardial I/R causes translocation of PKCδ to mitochondria, which in turn affects the activity of downstream apoptotic factors through the release of cytochrome *c*, and induces cardiomyocyte apoptosis and necrosis [22]. Therefore, different PKC isoforms have distinct temporal and opposing roles in regulating myocardial damage induced by I/R. The increase in activity of different seemingly counteractive kinases may be the result of endogenous mechanisms altering the balance between cell survival and cell death and limiting the degree of injury to I/R-induced stimuli. We therefore suggest that selective inhibition and/or activation of individual PKC isoforms

may provide a novel approach for preventing or arresting the progression of cardiomyopathic abnormalities.

The identification of the mechanisms involved in PKC isoforms and H/R-induced injury in primary cardiomyocytes, demonstrated by pharmacological manipulation and biochemical means, is of biological relevance. The PKC isoform inhibitors and activators investigated were the PKCα inhibitor Gő6976 and activator TXA and PKCε inhibitor peptide εV1–2 for PKCα and ε, respectively. The PKCα inhibitor Gő6976 could minimize the H/R-induced increase in expression of Egr-1 protein and release of CK from myocardial cells and inhibit H/R-induced apoptosis. Although Gő6976 inhibition is a nonspecific effect for PKCα, PKCα was slightly more sensitive than PKCβI. We therefore suggest that the effect of Gő6976 on H/R injury is largely attributed to the action of PKCα. PKCα seems necessary to transduce some aspects of the Egr-1 signaling pathway involved in H/R-induced apoptotic and necrotic cardiomyocyte death. A growing body of evidence has indicated that the PKCε selective activator protects the isolated perfused rat heart against I/R injury. Thus, use of a cell culture model of hypoxic preconditioning revealed that the PKCε inhibitor peptide εV1–2 not only abolished cardioprotection but also inhibited the translocation of PKCε [39,40]. Our findings indicate that the εV1–2 peptide increased H/R-induced levels of CK release and apoptosis, which agrees with similar observations [41], but not Egr-1 expression. In addition, some reports suggested that PKCε is an effector in maintaining sustained rather than transient activity of the cell signaling pathway [42]. Therefore, PKCε is unlikely to play a major role in this Egr-1 induction, which supports that PKCε is necessary for cardioprotection by IPC in association with cell damage induced by H/R. These observations conclude a new facet in the biological role of PKCα and ε in response to hypoxia/ischemia and/or reperfusion.

We found that the quaternary ammonium salt derivatives of haloperidol, F_2, a new calcium antagonist that reduces cytosolic calcium levels, inhibited PKCα translocation and enhanced PKCε translocation. The inhibition of PKCα translocation by F_2 is consistent with reports that PKCα is Ca^{2+} dependent [43]. However, F_2 had no effect on the activation of Ca^{2+}-dependent PKCβII in our primary cardiomyocytes model. The PKCβ isoform may be marginally less Ca^{2+} dependent than Ca^{2+}-dependent PKCα and γ [44]. The activation of PKCε by F_2 is an important finding. F_2 altered the subcellular distribution of PKCε in untreated or cardiomyocytes under H/R. Individual components of the PI lipid signaling pathway that result in PKC activation could be influenced by hypoxia/ischemia and/or reperfusion. We suggest that, except for the calcium channel, the mode of action of F_2 is related to other pharmacological targets, which may interfere with the release of phospholipids from the plasma membrane or other intracellular lipid pools. Indeed, our data showed that the inhibitor of phospholipase-C, U73122, blocked the effect of F_2 on the increase of PKCε translocation in normal or H/R-induced primary cardiomyocytes. Of note, F_2 had no effect on activation of PKCδ in our primary cardiomyocyte model.

As we have demonstrated by electron microscopy previously, F_2 treatment attenuated necrotic and apoptotic cell death [6]. The cardioprotective mechanism of F_2 might be associated with inhibiting Ca^{2+} overload and suppressing Egr-1 expression. TXA was originally introduced as a highly selective activator of conventional PKCs. Our present data showed that inhibition of Egr-1 protein level by F_2 is blocked by the PKCα activator TXA, which is consistent with subsequent results that the protective effects of F_2 in reducing CK release and apoptotic cell death were abolished in part by TXA. F_2 could inhibit translation of PKCα but not PKCβII. So, we suggest that the cardioprotection of F_2 is mediated at least in part through PKCα inhibition. Egr-1 appears to be involved in the pathogenesis of ischemic myocardial tissue damage. That Egr-1-deficient mice display increased survival and lung function after pulmonary I/R demonstrates a causal relationship between Egr-1 and I/R damage. We therefore suggest that inhibition of Ca^{2+}-dependent PKCα redistribution by F_2, a new calcium antagonist, is associated with downregulation of Egr-1 protein expression and cell damage. In addition, decreased CK release and apoptosis with F_2 pretreatment but not Egr-1 expression were abolished in part by εV1–2 peptide, which supports that the cardioprotective mechanism of F_2 might be associated with activation of Ca^{2+}-independent PKCε and endogenous mechanisms, resulting in protection against H/R injury. Therefore, F_2 is cardioprotective to neonatal rat cardiac myocytes and is mediated at least in part through PKCα inhibition and PKCε activation.

In conclusion, improved efficacy with the use of F_2 in protecting against H/R-mediated apoptosis and inflammation injury is possibly attributed to the combined effects of inhibition of Ca^{2+}-dependent PKCα and Ca^{2+}-independent activation of PKCε. Because inhibition of PKCα was associated with downregulation of Egr-1 protein expression and cell damage, activation of PKCε is necessary for cardioprotection against cardiomyocyte apoptosis. Multidimensional therapeutic strategies such as those with F_2 can lead to a significant reduction in cardiac dysfunction after I/R injury and thus improve cardiac recovery.

Acknowledgements

This work was supported by NSFC-Guangdong Joint Funds (No. U0932005), the National Natural Science Foundation of China (No. 30672465), the Key Project of the Natural Science Foundation of Guangdong Province of China (No. 06118928) and the Research Fund for the Doctoral Program of Higher Education of China (No. 200805600003).

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