Ischemic Preconditioning Attenuates Myocardial Apoptosis through Regulation of Hypoxia Inducible Factor-1αand Heat Shock Protein 70

Yun-Wen Peng, Catherine Pawloski, Ian M. Charpie and John R. Charpie*

Department of Pediatrics & Communicable Diseases, University of Michigan Medical School, University of Michigan, Ann Arbor, MI 48109-4204, USA

Abstract: Ischemic preconditioning (IPC) increases expression of several cardioprotective genes and attenuates myocardial dysfunction after ischemia-reperfusion (IR) injury. However, the precise cellular mechanisms by which IPC confers myocardial protection are incompletely understood. We hypothesized that the beneficial effect of IPC in the heart is due to upregulation of two key transcription factors, Hypoxia Inducible Factor-1α (HIF-1α) and Heat Shock Protein 70 (HSP70). In this study, neonatal rat cardiomyoblasts (H9c2 cells) were subjected to IPC (four cycles of 15-min hypoxia/15-min reoxygenation), followed by 12-hr hypoxia-reoxygenation (HR). HIF-1α and HSP70 expression were measured by ELISA and immunoblot. Apoptosis was assessed by DNA fragmentation and caspase-3 activity. The results showed that IPC induced HIF-1α and HSP70 expression and attenuated apoptosis after 12-hr HR. Pretreatment with DMOG, an HIF-1α activator, showed a similar protective effect as IPC. An HIF-1α inhibitor (CAY10585) or HSP70 inhibitor (KNK437) decreased IPC-induced HIF-1α or HSP70 expression, respectively, and abrogated the anti-apoptotic effect of IPC. In summary, IPC is associated with increased HIF-1α and HSP70 expression and a subsequent decreased apoptosis in neonatal cardiomyoblasts exposed to HR. These results suggest that HIF-1α and HSP70play important roles in IPC-induced cardioprotection, and these endogenous transcription factors may provide a novel therapeutic target to prevent myocardial IR injury *in vivo*.

Keywords: Ischemic preconditioning**,** hypoxia reoxygenation, H9c2 cells, transcription factor, cardioprotection.

1. INTRODUCTION

Open heart surgery for repair of congenital heart disease necessitates a period of myocardial ischemia-reperfusion (IR) due to aortic cross-clamping and cardioplegic arrest. Ischemia-reperfusion can cause injury, in part, due to excess production of oxygen-derived free radicals including superoxide anion and peroxynitrite. These free radicals cause irreversible damage to DNA, RNA, and proteins, leading to cellular dysfunction and death [1]. Ischemic preconditioning (IPC) is an experimental technique in which tissues are subjected to repeated, short, episodes of sublethal IR that confer protection against prolonged, potentially lethal ischemic injury. IPC was initially described in the mid 1980's as an endogenous protective mechanism in myocardium, and now has been demonstrated in multiple tissues including brain, liver, and kidney [2, 3]. Endogenous defense mechanisms, such as antioxidant enzymes and heat shock proteins (HSPs), are activated by IPC and thus attenuate damage caused by oxidative stress. Thus far, the existing literature suggests that the cardioprotective effects of IPC against IR injury are a consequence of a change in the redox state of cardiomyocytes [4, 5].

During IPC, several proteins are up-regulated including hypoxia inducible factors (HIF). Members of the HIF family regulate a variety of genes that affect a myriad of cellular processes including metabolism, angiogenesis, cell survival, and oxygen delivery, all of which are important for normal cardiac function. HIF-1α, in particular, is a transcription factor that regulates expression of several "hypoxia genes" such as erythropoietin, glucose transporters, glycolytic enzymes, and growth factors including platelet–derived growth factor B and vascular endothelial growth factor. HIF-1α is a key regulator of the cellular hypoxic response that, upon oxygen deprivation, leads to an accelerated ATP production in an oxygen-independent manner. HIF-1α has been proposed to be an important contributor to IPC-induced cellular protection [6, 7].

Induction and accumulation of HSPs represent another biologically important cellular response to external stress. HSPs are molecular chaperones that enhance the survival of cells exposed to different stressors including heat and ischemia. HSPs are a unique group of proteins that have been grouped into families based upon their sequence and molecular masses [8]. The induction of HSP70 has been implicated in myocardial protection, and has been reported in response to a variety of cardiac insults such as IR and heart failure *in vivo* and *in vitro*. Recently, strong evidence for a role of HSP70 in myocardial protection against ischemic injury was established in

*Address correspondence to this author at the Department of Pediatrics & Communicable Diseases, University of Michigan Medical School, University of Michigan, 1540 E. Hospital Drive, Ann Arbor, MI 48109-4204, USA; Tel: 734-936-8993; Fax: 734-936-9470; E-mail: jcharpie@umich.edu

E-ISSN: 2311-052X/14

transgenic animal models [9]. The cellular and temporal pattern of HSP70 induction varies depending on the nature and severity of cardiac injury and has also been found to match the known histopathology for various cardiac injuries. Thus, HSP70 induction is a marker of cellular stressin the cardiovascular system [10, 11].

This study aimed to investigate the roles of HIF-1α and HSP70 in IPC-induced myocardial protection in an experimental model of HR injury in neonatal rat cardiomyocytes. We hypothesize that increased levels of HIF-1α and HSP70 confer myocardial protection during IPC.

2. MATERIALS AND METHODS

2.1. Cell Culture and Treatment

Embryonic rat heart derived H9c2 (2-1) cells (ATCC, Manassas, VA) were maintained in Dulbecco's modified eagle medium (DMEM;Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS;Gibco) and antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin; Gibco) at 37° C in a 95% room air/5% CO_2 incubator. To investigate the role of HIF-1α on cardiomyocytes, H9c2 cells were pretreated with 1.0 mM of dimethyloxalyl glycine (DMOG;Cayman Chemical, Ann Arbor, MI) for 24 hours or 60 µM of CAY10585 (4-hydroxy-3-[[2-(4-tricyclo [3.3.1.13,7] dec-1-ylphenoxy)acetyl]-benzoic acid, methyl ester; Cayman Chemical) for 16 hours in some experiments. DMOG, an indirect HIF-1α activator, increases HIF-1α through competitive inhibition with prolyl hydroxylases (PHDs). It is well known that degradation of HIF-1α under normoxic condition is mediated by PHDs that function as oxygen sensors, and that their inhibition due to lack of oxygen leads to HIF-1α accumulation and transactivation of its target genes [12]. CAY10585, an aryloxyacetylaminobenzoic acid analogue and a new class of HIF-1α inhibitor, works *via* inhibition of HIF-1α activation and gene transcriptional activity [13]. To investigate the role of HSP70, H9c2 cells were pretreated with 50 µM of KNK437 (N-formyl-3,4-methyl-enedioxy-benzylidine-γ-butyrolactam, Calbiochem, Billerica, MA) for 6 hours. KNK437, a benzylidene lactam compound, is a novel inhibitor of thermal tolerance and blocks the induction of HSP70 (primary target), HSP 72, and HSP105 [14].

2.2. HR and IPC Procedures

Hypoxia-reoxygenation (HR) is a well-established *in vitro* cell injury model that is often used to mimicIR injury. For hypoxic conditions, H9c2 cells were grown to the desired confluence, the growth medium was replaced with glucose-free, serum-free medium containing 2% oxyrase (Oxyrase, Mansfield, OH), and the cells were transferred to a hypoxic chamber. A hypoxic environment (< 3% O_2) was achieved using a modular incubator chamber gassed with 95% N_2/5% CO_2. Oxygen concentration was constantly monitored during incubation using a microprocessor-based oxygen sensor. Subsequently, media was replaced with standard culture media during a 2 h period of room air re-oxygenation. For IPC, cultured H9c2 cells were subjected to 4 cycles of 15 minutes hypoxia followed by

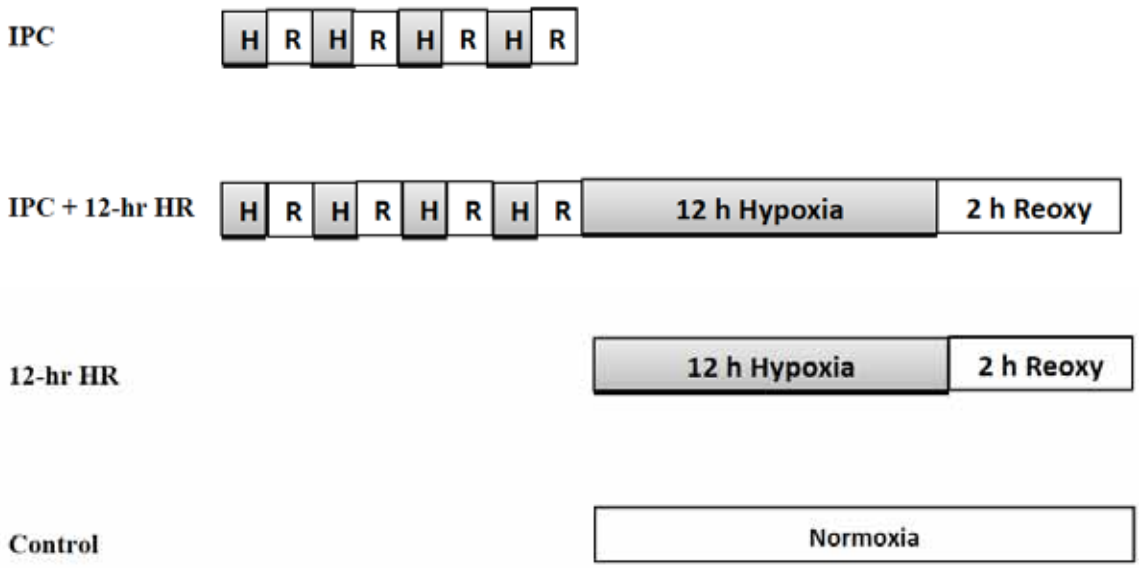

Figure 1: HR and IPC procedure.

H representing 15 minutes of hypoxia; R representing 15 minutes of reoxygenation.

15 minutes re-oxygenation in glucose-free, serum-free medium with Oxyrase (Figure **1**). For the control samples, cells were cultured in complete medium at 37°C in a 95% room air/5% CO_2 incubator. After the treatment, the cells were harvested and analyzed. No cytotoxic effects on H9c2 cells were observed when they were incubated in medium containing vehicles.

2.3. Nuclear Extraction and HIF-1α Enzyme-Linked Immunosorbent Assay (ELISA)

Culture medium was removed and cells were washed with PBS (Gibco). Nuclear protein extraction reagent was added to extract nuclear protein according to the suggested manufacturer's protocol (Nuclear Extraction Kit, Cayman Chemical). Briefly, the cells were resuspended in ice-cold hypotonic buffer. Addition of detergent (10% Nonidet-P40) disrupted the cytoplasmic membranes and allowed access to the cytoplasmic fraction while maintaining the integrity of the nuclear membrane. After separation of the cytoplasmic fraction from the nuclei by brief centrifugation, the pelleted nuclei were lysed in ice-cold extraction buffer containing a mixture of protease and phosphatase inhibitors. The protein concentration in the nuclear fraction was determined by the BCA protein assay reagents (BCA protein assay kit, Thermo, Rockford, IL).

Nuclear extracts were analyzed by HIF-1α Transcription Factor Assay kit (Cayman Chemical) to determine the amount of active HIF-1α present in the samples. All procedures were performed according to the manufacturer's instructions. Briefly, a specific double stranded DNA (ds DNA) sequence containing the HIF-1α response element (5'-ACGTG-3') was immobilized to wells of a 96-well plate. HIF-1α in the nuclear extracts bound specifically to the HIF-1α response element. The HIF transcription factor complex was detected by addition of a specific primary antibody directed against HIF-1α. A secondary antibody conjugated to horseradish peroxidase (HRP) was added to provide a sensitive colorimetric readout at 450 nm. Data were normalized to protein content and presented as fold of HIF-1α expression in normoxic conditions.

2.4. Protein Extraction and Immunoblot Analysis

Cells were extracted with RIPA lysis buffer (Pierce, Rockford, IL) and Complete Protease Inhibitor cocktail (Roche Diagnostic, Indianapolis, IN). Protein concentrations were determined by the BCA method (Pierce). Cell lysates were separated by 7.5% (for HIF-1α) or 12% (for HSP70) SDS polyacrylamide gel electrophoresis and proteins were transferred to nitrocellulose membranes (Bio-Rad Lab., Hercules, CA). The membranes were blocked with 3% BSA and then incubated overnight at 4º C with primary antibody against HIF-1α (1:750, Pierce) or HSP70 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA). After washing, the blots were incubated with a specific horseradish peroxidase-conjugated secondary antibody (1:2500, Santa Cruz Biotechnology). Detection was made with enhanced chemiluminescence detection system (ECL) from Pierce. Membranes were also probed with an antibody to actin or GAPDH (1:200, Santa Cruz Biotechnology) to control for protein loading conditions. Blot images were quantified with Image J software (NIH, Bethesda, MD). The results were normalized to actin or GAPDH intensity levels.

2.5. DNA Fragmentation Analysis

Cardiomyocyte apoptosis was analyzed by detection of DNA fragmentation (Cell Death Detection ELISA, Roche Applied Science). Samples containing nucleosomes were placed into an anti-histone-coated microplate. Subsequently, a mixture of anti-DNA-peroxidase (POD), which reacts with the DNA-part of the nucleosomes, was added and incubated. After washing, the amount of nucleosomes was quantified by the POD retained as an immunocomplex. POD was determined photometrically with 2,2'-azino-di(3-ethylbenzthiazoline-sulphoneate) (ABTS) as substrate, and then optical density was read with a microplate reader at 405 nm.

2.6. Caspase-3 Activity Assay

Caspase-3 activity was assessed by a fluorescence assay utilizing a specific substrate, N-Ac-DEVD-N'-MC-R110 (Caspase-3 Fluorescence Assay Kit, Cayman Chemical). After treatment, the cells were washed with ice-cold PBS and collected by trypsinization. The cell pellet was resuspended in lysis buffer and incubated for 30 min. The cells were lysed at room temperature for 30 min, and the supernatants were assayed for caspase-3 activity. After addition of active caspaspe-3 substrate, samples were incubated for 30 min at 37ºC and assayed for fluorescence intensity (excitation=485 nm; emission=535 nm). The fluorescence activity was normalized to total protein content for each sample.

2.7. Statistical Analysis

All data were reported as Mean ± S.E. from at least three independent experiments performed in duplicate.

The significance of differences between two groups were evaluated by an unpaired Student's t-test (GraphPad Prism, v6), followed by Bonferroni Correction. A value of $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. IPC Treatment Enhanced HIF-1α Expression in H9c2 Cells

Several different IPC protocols were evaluated in this study (data not shown). H9c2 cells exposed to four cycles of 15 minutes hypoxia followed by 15 minutes re-oxygenation demonstrated consistent myocardial protection and technical feasibility. Therefore, we used four cycles of 15 min/15 min HR as our IPC protocol in all subsequent experiments. Furthermore, we performed dose-response studies for DMOG (0.25 to 5.0 mM) and CAY10585 (6 to 60 μM). We found that 1 mM of DMOG was the lowest concentration that generated the maximum increase in HIF-1α expression, and 60 μM of CAY10585 showed the greatest inhibitory response. Based on these data, 1 mM of DMOG and 60 μM of CAY10585 were used for the remaining experiments.

As shown in Figure **2A**, HIF-1α level increased significantly in response to IPC treatment compared to normoxic control cells. Pretreatment of normoxic cells with1 mM DMOG (an HIF-1α activator) also increased HIF-1α level (1.81 ± 0.02-fold, $p<0.001$) to a similar extent as IPC. However, pretreatment with the HIF-1α inhibitor CAY10585 (60 μM) prior to IPC significantly reduced HIF-1α compared to IPC alone (0.96 ± 0.07 *vs.* 1.92 ± 0.15-fold respectively, $p<0.05$).

HIF-1α protein expression was also increased in IPC-treated cells compared to the normoxic control cells (0.87 ± 0.06 *vs.* 0.47 ± 0.02 relative units, $p<0.01$), as detected by immunoblot analysis (Figure **2B**). Similarly, DMOG treatment increased the level of HIF-1α protein expression compared to control (1.06 ± 0.06 *vs.* 0.47 ± 0.02 relative units, $p<0.01$). In contrast, HIF-1α protein expression was attenuated after pretreatment with CAY10585 prior to IPC compared to IPC alone (0.56 ± 0.09 *vs.* 0.87 ± 0.06 relative units, $p<0.05$).

In summary, IPC significantly increases HIF-1α expression in neonatal cardiomyocytes to a similar degree as a known HIF-1α activator. Furthermore, the increase in HIF-1α expression in response to IPC is attenuated by an HIF-1α inhibitor.

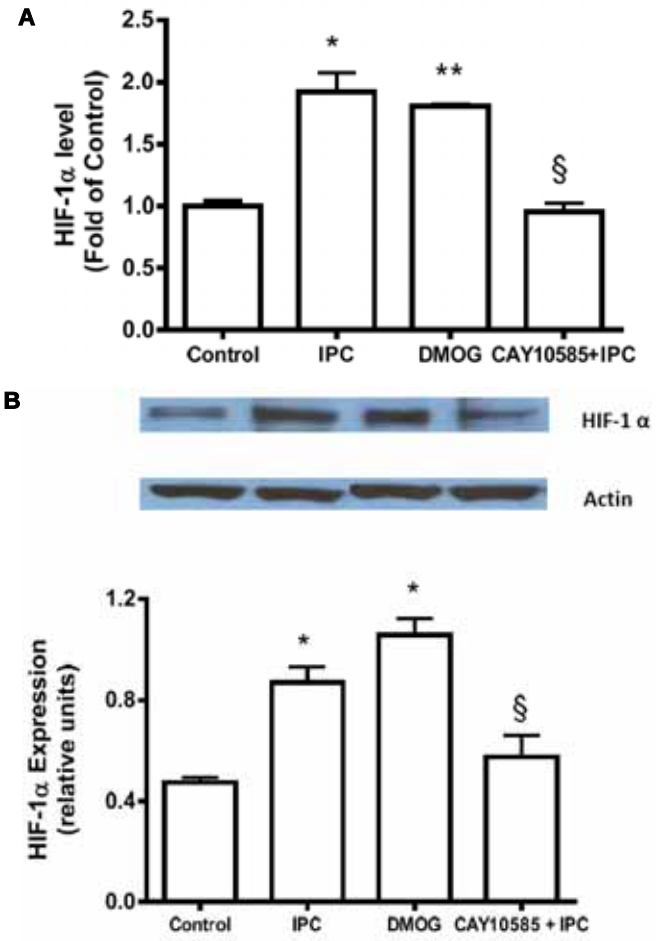

Figure 2: Effect of IPC on HIF-1α expression in H9c2 cells.

H9c2 cells were treated by IPC, DMOG (1.0 mM) or IPC + CAY10585 (60 μM). Protein levels of HIF-1α were measured by ELISA (**A**) or analyzed by immunoblot analysis (**B**). The results are Mean ± SE of four or five different cell cultures**.** *p<0.01, **p<0.001 *vs.* control, §p<0.05 *vs.* IPC.

3.2. IPC Treatment Increased HSP70 Levels

As shown in Figure **3**, HSP70 protein expression was dramatically increased in IPC-treated cells compared to normoxic control cells (0.69±0.04 *vs.* 0.10± 0.02 relative units, $p<0.001$). However, pretreatment with KNK437 (50 μM), an HSP70 inhibitor, significantly reduced HSP70 expression compared to IPC alone (0.50 ± 0.03 *vs.* 0.69 ± 0.04 relative units, $p<0.05$). These results suggest that HSP70 protein expression is upregulated by IPC treatment in neonatal cardiomyocytes.

3.3. IPC-Mediated Upregulation of HIF-1α and HSP70 Attenuated HR-Induced Apoptosis

To examine how IPC-mediated upregulation of HIF-1α and HSP70 expression influence HR-induced cardiomyocyte injury, DNA fragmentation and caspase-3 activity were measured as indices of cell apoptosis.

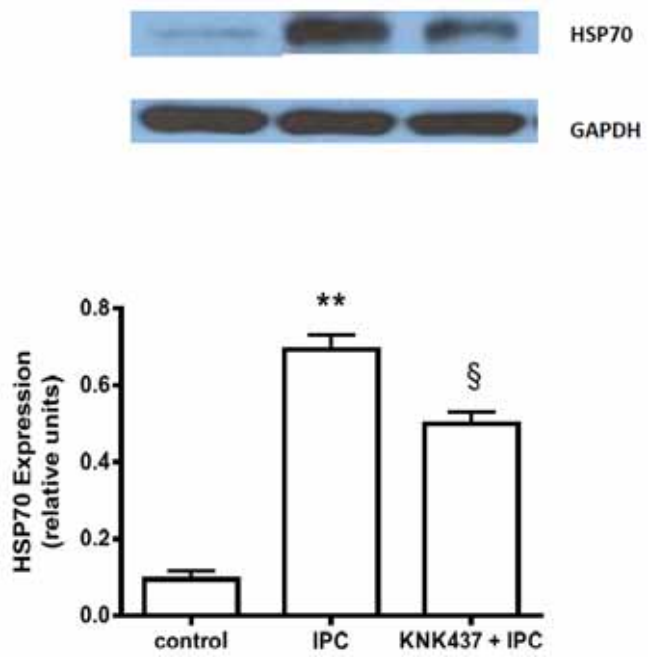

Figure 3: Effect of IPC on HSP70 expression in H9c2 cells.

H9c2 cells were treated by IPC or IPC + KNK437 (50 μM). Protein levels of HSP70 were analyzed by immunoblot analysis. The results are Mean ± SE of four different cell cultures. **p<0.001 *vs.* control, §p<0.05 *vs.* IPC.

Previously, we quantified apoptosis and necrosis by flow cytometry in H9c2 cells exposed to varying lengths of HR, and showed that 12 hours hypoxia and 2 hours reperfusion (12-hr HR) induced a maximal apoptotic response with little non-specific necrosis [15]. Therefore, we used 12-hr HR for all subsequent experiments.

As shown in Figure **4**, IPC alone did not significantly increase DNA fragmentation (1.29 ± 0.12-fold of control). However, exposure of H9c2 cells to 12-hr HR induced a nearly 13-fold increase in cell death, compared to normoxic control conditions (p<0.001). IPC treatment prior to 12-hr HR significantly reduced the amount of DNA fragmentation compared to 12-hr HR alone (7.80 ± 0.33 *vs.* 12.78 ± 0.53-fold respectively, p<0.001). Similar to the protective effects of IPC, pretreatment of H9c2 cells with the HIF-1α activator DMOG significantly attenuated cardiomyocyte apoptosis compared to 12-hr HR (9.23 ± 0.19 *vs.* 12.78 ± 0.53-fold respectively, p<0.001). Pretreatment of H9c2 cells with the HIF-1α inhibitor CAY10585 or the HSP70 inhibitor KNK437 prior to IPC abrogated the protective effects of IPC on DNA fragmentation (CAY10585 + IPC + HR: 11.89 ± 0.18-fold; KNK437 + IPC + HR: 10.03 ± 0.85-fold; IPC + HR: 7.80 ± 0.33-fold). These results suggest that both HIF-1α and HSP70 expression play important roles in the protective effects of IPC in neonatal cardiomyocytes exposed to 12-hr HR.

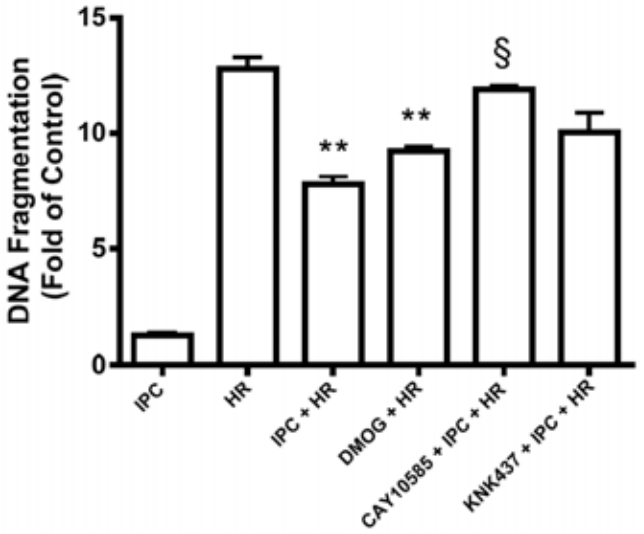

Figure 4: Effect of IPC on apoptosis in HR-treated H9c2 cells.

H9c2 cells were treated by IPC, HR, IPC + HR and combined with DMOG (1.0 mM) or CAY10585 (60 μM) or KNK437 (50 μM). Apoptosis was measured by DNA fragmentation analysis. Data are Mean ± SE of four independent experiments performed in duplicate. **p<0.001 *vs.* HR. §p<0.05 *vs.* IPC + HR.

To further confirm the roles of increased HIF-1α and HSP70 expression in the anti-apoptotic effects of IPC, we measured caspase-3 activity in H9c2 cells exposed to 12-hr HR. Caspase-3 is a member of the cysteine-aspartic acid protease family that has been identified as a key mediator of apoptosis in mammalian cells. As shown in Figure **5**, caspase-3 activity was not increased significantly by IPC alone (0.76 ± 0.08-fold). Following 12-hr HR, caspase-3 activity increased 4.09 ± 0.24-fold compared to normoxic control cells (p<0.001).

Consistent with the results of the DNA fragmentation assays, IPC prior to 12-hr HR significantly reduced caspase-3 activity compared to 12-hr HR alone (2.63 ± 0.15 *vs.* 4.09 ± 0.24-fold, p<0.001). Similarly, pretreatment with the HIF-1α activator DMOG also attenuated the increase in caspase-3 activity compared to 12-hr HR alone (1.71 ± 0.09 *vs.* 4.09 ± 0.24-fold, p<0.001). In contrast, addition of the HIF-1α inhibitor CAY10585 or HSP70 inhibitor KNK437 before IPC prevented the beneficial anti-apoptotic effects of IPC on caspase-3 activity (CAY10585 + IPC + HR: 3.88 ± 0.29-fold; KNK437 + IPC + HR: 4.31 ± 0.46-fold; IPC + HR: 2.63 ± 0.15-fold, p<0.05). In fact, pretreatment with CAY10585 or

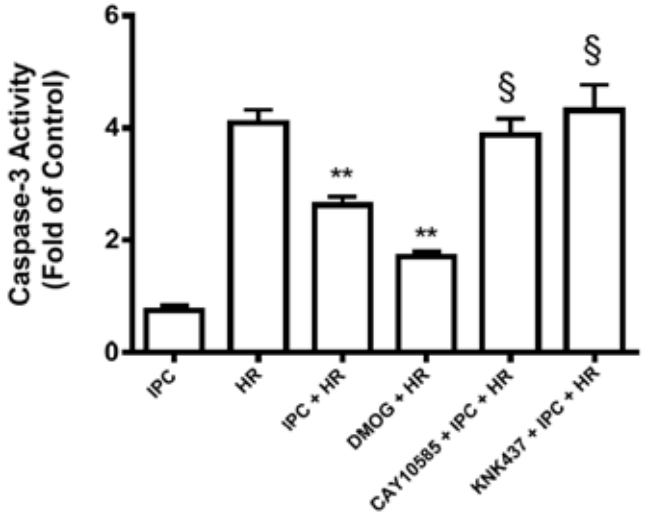

Figure 5: Effect of IPC on HR-treated H9c2 cells apoptosis as assessed by caspase-3 activity.

Cells were treated by IPC, HR, IPC + HR and pretreatment with DMOG (1.0 mM) or CAY10585 (60 μM) or KNK437 (50 μM). Data are Mean ± SE of four independent experiments performed in duplicate. **p<0.001 *vs.* HR, §p<0.05 *vs.* IPC + HR.

KNK437 followed by IPC + HR showed similar caspase-3 activity as HR alone. These data suggestthat in H9c2 cells, the cardioprotective effects of IPC are associated with increased HIF-1α and HSP70 expression.

4. DISCUSSION

Ischemia-reperfusion injury activates cell death pathways leading to organ dysfunction. During the past several years, new experimental evidence indicates that HIF-1α and HSP70 are upregulated in response to IR injury ostensibly to protect against IR-induced cell death [16-19]. Therefore, an improved understanding of the potential contributions of HIF-1α and HSP70 to IPC-mediated protection may aide in the design of novel therapeutic strategies to prevent myocardial IR injury. Because cardiac IPC results in profound increases in cardiomyocyte resistance to IR injury [20, 21], we hypothesized that IPC is associated with increased HIF-1α and HSP70 levels and subsequent cardioprotection. Our experimental results support this hypothesis.

HIF-1α serves as a key regulator of a number of genes responsible for hypoxic stress [22]. Some studies report that protection of tissues against sublethal ischemia by IPC is associated with elevated levels of the HIF-1α protein [23, 24]. During hypoxia, HIF-1α levels accumulate and, in turn, trigger increased expression of genes involved in glycolysis, glucose metabolism, mitochondrial function, cell survival, apoptosis, and other processes that can affect cell survival. In addition, a growing body of evidence has shown that HIF-1α plays an essential role in normal cardiac development, morphogenesis, and function [25-27]. Recently, data supporting a potential role for HIF-1α in IPC-mediated myocardial protection raised great interest due to the possible clinical implications [28-30].

In this study, we demonstrated that HIF-1α expression was significantly increased after IPC treatment by both ELISA and immunoblot analysis. Furthermore, in an experimental model of IR injury, cardiomyocyte apoptosis was significantly attenuated by IPC or pharmacological pretreatment with DMOG, a known stabilizer of HIF-1α protein expression. In contrast, HIF-1α expression and the beneficial anti-apoptotic effects of IPC were decreased following pretreatment with CAY10585, a novel small molecule inhibitor of HIF-1α accumulation and gene transcriptional activity. These findings support the hypothesis that increased HIF-1α expression is an important component of the protective effects of IPCin cultured neonatal cardiomyocytes.

The heat shock response is a highly conserved defense mechanism against tissue and cell stress injury [31, 32]. HSPs are categorized on the basis of their approximate molecular weights (from 10 to 150 kDa), and they display a wide range of functions including cytoprotection, and the intracellular assembly, folding, and translocation of oligomeric proteins. Members of the HSP family are induced in response to a number of stresses including sublethal heat and ischemia. In particular, HSP70 is a molecular chaperone that has shown to provide myocardial protection against IR-induced oxidant stress [33, 34]. Also, HSP70 has been shown to accelerate recovery of cardiac function following IR injury. These cytoprotective effects make HSP70 an attractive target for therapeutic interventions during open heart surgery [35-37].

Our experimental results showed that IPC dramatically increased HSP70 expression associated with a significant reduction in HR-induced apoptosis. Furthermore, KNK437, an HSP70 inhibitor, reduced HSP70 protein expression and abolished the anti-apoptotic effect of IPC as evidenced by marked increases in both DNA fragmentation and caspase-3 activity. These data support the hypothesis that HSP70 is substantially involved in IPC-mediated protection of cultured neonatal cardiomyocytes to IR injury.

In summary, the present study provides new evidence for the critically important contributions of HIF-1α and HSP70 to IPC-mediated protection against myocardial IR injury *in vitro*. These results may have important implications for the design of novel therapeutic agents to protect the heart from IR injury during cardiac surgery. Currently, we are in the process of extending these experimental observations to differentiated human cardiomyocytes in culture to investigate if these same pathways are operative in a human model of IPC.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

ACKNOWLEDGEMENTS

We thank Sunkyung Yu for statistical discussion and suggestion.

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Received on 15-01-2014 Accepted on 17-06-2014 Published on 14-07-2014

DOI: http://dx.doi.org/10.12970/2311-052X.2014.02.02.3