Hypoxic Preconditioning Increases Survival of Cardiac Progenitor Cells via the Pim-1 Kinase-Mediated Anti-Apoptotic Effect

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Background: Stem cells transplanted to the ischemic myocardium usually encounter massive cell death within a few days after transplantation, and hypoxic preconditioning (HPC) is currently used as a strategy to prepare stem cells for increased survival and engraftment in the heart. The purpose of this study is to determine whether Pim-1 kinase mediates any beneficial effects of HPC for human cardiac progenitor cells (CPCs).

Methods and Results: Human CPCs were isolated from an adult heart auricle and were purified by magnetic-activated cell sorting using c-kit magnetic beads; they were hypoxic preconditioned for 6 h. Both Pim-1 and p-Akt were determined. CPCs were assigned to one of the following groups: (1) control (without HPC); (2) HPC; or (3) HPC+I (Pim-1 inhibitor). HPC can promote the survival of CPCs. HPC enhances the expression of Pim-1 kinase in a time-dependent manner, which causes a reduction of proapoptotic elements (cytochrome c and cleaved caspase-3) and the preservation/modulation of important components of the mitochondria (Bcl-2, Bcl-XL and p-Bad), and attenuates mitochondrial damages. All of these protective effects were blocked by a Pim-1 inhibitor.

Conclusions: Pim-1 plays a pivotal role in the protective effect of HPC for CPCs, and the promotion of the expression of Pim-1 in CPCs can as serve part of molecular therapeutic interventional strategies in the treatment of cardiomyopathy damage by blunting CPC death. (Circ J 2014; 78: 724–731)

Key Words: Cardiac progenitor cells; Hypoxic; Mitochondria; Pim-1; Preconditioning

Ischemic heart disease, such as myocardial infarction, is one of the most important diseases causing death worldwide. At present, the main clinical treatment is palliative, and is aimed at preserving the function of the remaining cardiac myocytes. Cell transplantation offers the potential to effectively reverse cardiac damage and restore cardiac function. Mounting evidence suggests the existence of cardiac stem cell-like populations in adult hearts that can be self-renewing, clonogenic and multipotent, and give rise to cardiac myocytes, smooth muscle and endothelial cells in vivo and in vitro, which has undermined the notion of the adult heart as being a terminally differentiated organ without self-renewing potential. It is now recognized that resident cardiac progenitor cells (CPCs), being c-kit+ cells, exist in the adult mammalian heart, including mouse, rat and human, which creates a whole new field of research for heart diseases and myocardial repair.

Various stem/progenitor cells, including CPCs, show positive effects on cardiac cell therapy; however, many barriers must be overcome for these cells to be useful as a therapeutic agent to clinically treat patients, and the primary one is to find some methods for improving stem/progenitor cells survival and long-term engraftment after transplantation. Therefore, techniques that enhance the recruitment and retention of transplanted CPCs are crucial to adequately replenish the resident progenitor cell pool and maximize its regenerative potential. Tissue kallikrein-modified mesenchymal stem cells were more resistant to hypoxia-induced apoptosis and provided enhanced protection against ischemic cardiac injury after myocardial infarction. Hypoxic preconditioning (HPC) might be a novel approach for enhancing the therapeutic benefit of CPC therapy. Although HPC in the myocardium has been investigated for many years, its physiological mechanism is still not completely understood. Hypoxia increases Pim-1 in a hypoxia inducible factor-1α-independent manner in cancer cells. Pim-1, a proto-oncogenic serine/threonine kinase, was originally discovered as the proviral integration site for the Moloney murine leukemia virus, which regulates apoptosis and the cell cycle by phosphorylating target proteins.
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These samples were minced and seeded onto the surface of uncoated Petri dishes for a 2–3 week culture. Cells outgrown from the tissue specimens were analyzed by flow cytometry at passage P 0. C-kit + CPCs can be isolated from the entire cell population by magnetic-activated cell sorting with CD117 (c-kit) magnetic beads (Miltenyi Biotec Inc, Auburn, CA, USA) according to the manufacturer’s protocol.

HPC of CPCs In Vitro

C-kit + CPCs were divided into 5 groups: normoxia, hypoxia for 3 h, 6 h, 12 h and 24 h, respectively. Hypoxia was achieved by placing the cells in a Modular Incubator Chamber (Billumps-Rothenberg, Del Mar, CA, USA) according to the manufacturer’s instructions. After a brief time spent in the chamber, the cells were flushed with a mixture of 0.1% O2, 5% CO2 and 94.9% N2 for 5 min. The chamber was then closed and the cells incubated at 37°C for various lengths of time.

CPCs damage or apoptosis was induced via prolonged hypoxia for 48 h along with serum-free DMEM.

Methods

Isolation and Culture of Human CPCs

We isolated human CPCs from adult human heart tissue that was removed during cardiosurgery, such as, valve replacement surgery. The present study is in accordance with the approval of the Southeast University Medical Ethics Committee (Approval No: 2012ZD11KY28.0). In general, we used the human heart auricle (appendix of the atrium). These surgical samples, which were approximately 0.4–2 g, can provide an excellent source of CPCs.

Reagents Treatment

CPCs were incubated with Quercetagetin (Calbiochem, San Diego, CA, USA), a specific Pim-1 activity inhibitor, at 10 μmol/L.

Apoptosis Assay

Apoptosis assays were performed using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Pharmingen, San Diego, USA) according to the manufacturer’s instructions. Briefly, cells were collected and resuspended in binding buffer.

Myocardial repair is significantly enhanced by genetic engineering of CPCs with Pim-1 kinase, which is a crucial facet of cardioprotection downstream of Akt. Akt is a nodal signaling kinase that influences multiple cellular processes including metabolism, cycling, cell growth and apoptosis, which exerts cardioprotective effects in concert with another serine/threonine kinase called Pim-1 that lies downstream of nuclear Akt accumulation. Primary downstream targets of Pim-1 include molecules responsible for the regulation of cellular survival and mitotic activity. Pim-1 overexpression in cardiomyocytes results in enhanced cell survival, whereas a loss of Pim-1 results in increased apoptotic cell death.

It has been reported that HPC enhanced the benefit of CPCs therapy for the treatment of myocardial infarction. However, the mechanism has not been fully elucidated. In this study, the results demonstrate that exposure of human CPCs to hypoxia enhances the expression of Pim-1, which can promote human CPCs survival.

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Western Blotting
Protein extracts from CPCs were separated by SDS-PAGE on 10% (for Pim-1, total and phosphorylated Akt, Bcl-2, Bcl-XL, phosphorylated Bad, cleaved caspase-3 and GAPDH) or 12% (for cytochrome c) gels and transferred to a PVDF membrane (Millipore Corporation, Bedford, USA). The membranes were incubated overnight with the following primary antibodies (1:1000, all from Santa-Cruz Biotechnology, Santa Cruz, USA). Immunoblotted proteins were visualized by using an ImmunoStar HRP Substrate Kit (BioRad, Hercules, USA) and quantified by using Kodak Image Station 440CF. To confirm equal protein loading, blots were stripped with 0.4 mol/L NaOH and then re-blotted with an anti-α-actin antibody (Santa-Cruz Biotechnology, Santa Cruz, USA) for both cytosolic and mitochondrial frac-

Annexin V-FITC and propidium iodide were added, and the reaction was incubated in the dark for 15 min. Cells were analyzed using a FACS for flow cytometry.

Transwell Migration Assay
For migration of cells through transwell filters with 8-μm pores (Fisher Scientific, Pittsburgh, USA), cells (1 clone per construct) were plated in triplicate, 5×10^4 cells per transwell insert in serum-free DMEM. Cells settled for 15 min, then, lower chambers were filled with DMEM containing 10% fetal bovine serum. After 4 h, cells were fixed, stained with crystal violet and counted. The data represent the average number of cells per field for 3 independent experiments.
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A plasticizer, dibutyl phthalate, was added at 0.5%. Thin sections (70 nm) were cut using a Leica Ultracut UCT, stained with uranyl acetate and lead citrate, and examined in a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan) equipped with a Mega-View-III digital camera and a Soft-Imaging-System (SIS) for the computerized acquisition of images.

**Measurement of the Mitochondrial Membrane Potential**
The mitochondrial membrane potential ($\Delta\psi_m$) was measured with a Mitochondrial Membrane Potential Assay Kit with JC-1 (Beyotime Biotechnologies, Shanghai, China) according to the manufacturer’s instruction. Briefly, 50,000 cells were collected by trypsinization and incubated with JC-1 for 20 min at 37°C in the dark. The stained cells were washed with ice-cold working solution twice and then analyzed by flow cytometry (FACSCalibur, BD Biosciences, Mountain View, USA) and CELLQuest software (FACSCalibur, BD Biosciences, Mountain View, USA). Twenty thousand cells were analyzed in each measurement. JC-1 aggregates in the polarized mitochondrial matrix and forms J-aggregates, which emit red fluorescence at 590 nm when excited at 585 nm. However, JC-1 cannot aggregate in the depolarized mitochondrial matrix and exists as JC-1 monomers, which emit green fluorescence at 530 nm when excited at 515 nm. Mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio.

**Results**

**HPC Induces Pim-1 Expression**
We first verified the effect of HPC on the expression of Pim-1 in human CPCs. A time-course of the response of the Pim-1 protein to HPC (6 h) was conducted at 0, 4, 8, 12 and 24 h and compared with cells cultured under normoxia conditions. In the NPC group, protein expression of Pim-1 was very low or absent (Figure 1). Pim-1 protein expression was upregulated by HPC in a time-dependent manner (Figure 1). A P value of <0.05 (2-tailed) was considered statistically significant.
The effect of Pim-1 on cell anti-apoptosis was assessed by flow cytometry analysis after being labeled with annexin V and propidium iodide. Cells apoptosis was induced via prolonged hypoxia for 48 h along with serum deprivation. It showed a reduction in apoptosis on CPCs that were under HPC for 6 h compared with control CPCs cultured in normoxic conditions. The protective effects of HPC were largely blocked when preconditioned CPCs were incubated with Quercetagetin (Figures 2B, D).

Pim-1 Enhances the Expression of Proteins Related to Anti-Apoptosis

HPC enhanced the expression of anti-apoptotic proteins Bcl-2 (Figure 3A) and Bcl-XL (Figure 3B) significantly, both of which are Bcl-2 family members. No significant difference among groups was found in the expression of total Bad, but HPC resulted in increased phosphorylation of Bad at serine 112 (Figure 3C) significantly. All of them were significantly reduced by Quercetagetin, and there was no significant difference between this group and the control group, whereas the HPC-induced expression of Pim-1 was not affected by Quercetagetin (Figure 3).

Pim-1 Prevented the Release of Cytochrome c From Mitochondria

Cytochrome c is released following mitochondrial membrane permeabilization. Pro-apoptotic stimuli induced mitochondrial membrane permeabilization and promoted the release of Cytochrome c in the cytosol leading to the activation of pro-apoptotic factors, such as caspase-3. As shown in Figure 3, HPC resulted in reduced cytosolic content of cytochrome c. HPC-induced reduction was abolished by Quercetagetin, and there was no significant difference between this group and the control group, but the expression of Pim-1 induced by HPC was not affected by Quercetagetin (Figure 3).

Pim-1 Promotes Cell Migration and Decreases the Apoptosis of CPCs

To confirm the effects of Pim-1 on cell migration, the transwell assay was used to assess migration as the number of cells that migrated through 8-μm pores. Representative images are shown in Figure 2A. Figure 2C indicates the average number of cells per field. HPC significantly increased migration compared with control cells, but Quercetagetin reduced migration to control levels. These results indicate that Pim-1 plays an important role in the migration of human CPCs induced by HPC.
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Discussion

HPC enhances the benefit of CPC therapy for the treatment of myocardial infarction because HPC activates the survival mechanisms inside the cell and prepares cells for transplantation. After performing hypoxia time-course experiments, 6 h of HPC is considered as the optimal HPC duration, which is similar to the findings of previous studies. This study indicates that human CPCs are a population of progenitor cells that retain their capacity for self-renewal and clonogenic expansion in vitro and can differentiate into cardiomyocytes. More importantly, we find that HPC increases human CPCs anti-apoptosis and migration capacity in vitro, and the beneficial effects generated from HPC of CPCs is mediated by the Akt/Pim-1 axis signaling pathway and represents a Pim-1 dependent manner.

Akt is a nodal-signaling kinase that influences multiple cellular processes including metabolism, cycling, cell growth and apoptosis, which exerts cardioprotective effects in concert with Pim-1, which lies downstream of nuclear Akt accumulation. Pim-1 expression inhibits pathological damage and remodeling resulting from myocardial infarction and pressure overload-induced hypertrophy. The present study indicates, for the first time, that HPC enhances the expression of p-Akt in a time-dependent manner, and the expression of Pim-1 has the same change trend with p-Akt, but the expression level of Pim-1 in normal human CPCs is very low. Transwell migration and apoptosis assays indicate that HPC can promote human CPCs migration and decrease the apoptosis of human CPCs, and these beneficial effects can be blocked by Quercetagetin.
a specific Pim-1 activity inhibitor. Further studies show that HPC enhances the expression of anti-apoptotic members Bcl-2, Bcl-XL and phosphorylation of Bad at serine 112 significantly, prevents the release of cytochrome c from mitochondria, reduces the cleaved caspase-3 cytosolic level, elevates mitochondrial membrane potential, and attenuates mitochondria damage significantly; however, all of these protective effects are blocked by Quercetagetin. These results suggested that Pim-1 played a pivotal role in the protective mechanisms of HPC.

The central role of mitochondria in the development of apoptosis is now well-established. In response to a variety of stress signals to cells, mitochondria undergo dramatic changes in function that ultimately result in the release of several pro-apoptotic factors including cytochrome c, the important one, to trigger the activation of caspase cascade programmed cell death. Data has documented the anti-apoptotic actions of the serine/threonine kinase Akt, which acts in part through protecting mitochondrial structure and function, and the cardioprotective action of Akt depends on downstream induction of Pim-1 kinase, at least in part. A loss of mitochondrial transmembrane potential and mitochondrial swelling are the initial manifestation of mitochondrial damage, which cause the formation of mitochondrial transition pores and production of reactive oxygen species that are early events in cells destined to undergo programmed cell death. Subsequently, cytochrome c is released from the mitochondria, which results in the activation of caspases, such as caspase-3, and cell death. This important release of cytochrome c occurs at the onset of mitochondrial dysfunction. So, release of cytochrome c is shown to be an early event in the induction of the apoptotic cascade, acting upstream of caspase activation and aggravating mitochondrial depolarization.

Cytochrome c is located in the mitochondrial intermembrane space and released into the cytosol in consequence to the apoptotic stimulus, which consequently activates an apoptotic protease-activating factor and mediates caspase cascade programmed cell death. Thus, cytochrome c released from the intermembrane space is supposed to be the determining factor in the final step to apoptosis. In our study, the release of cytochrome c was prevented by the overexpression of Pim-1. Pim-1 exerts a protective effect of the myocardium through induction of Bcl-2 and Bcl-xL protein levels and phosphorylation/inactivation of Bad. Proteins including Bcl-2, Bcl-XL and Bad that are all members of the Bcl-2 family that can modulate several steps of cell programmed death, playing a central role in controlling the mitochondrial apoptosis pathway. Both Bcl-XL and Bcl-2 not only promote cell survival by binding and inhibiting pro-apoptotic Bcl-2 family members, but they also prevent cytochrome c release by regulating the mitochondrial membrane potential. Mitochondrial membrane potential decrease is proposed as an early irreversible event during apoptosis. The loss of mitochondrial transmembrane potential promotes the release of apoptogenic factors such as cytochrome c that facilitate the activation of downstream caspases, thereby promoting apoptotic cell death. In this study, Pim-1 overexpression in human CPCs protected the mitochondrial transmembrane potential in the face of factors inducing cell apoptosis. The pro-apoptotic group of Bcl-2 family proteins, such as Bad, are activated under stress conditions and elicit mitochondrial outer membrane permeabilization, resulting in the release of apoptotic molecules, such as cytochrome c, subsequent caspase-3/caspase-9 activation, and the development of apoptosis. Pim-1 kinase has been demonstrated to phosphorylate Bad specifically at serine 112, leading to its inactivation and thereby preventing apoptotic cell death. Phosphorylation of Bad in combination with residual Bcl-2 and Bcl-XL can supply enough of a protective effect to inhibit CPCs apoptosis. This study showed that Pim-1 overexpression also enhanced the phosphorylation/inactivation of Bad, which decreased the release of cytochrome c and cleaved caspase-3.

Conclusions

In conclusion, this study suggests that the anti-apoptotic effect is the central aspect of HPC protection, and HPC can promote cell migration as well. Many protective kinases play roles in these protective mechanisms. In particular, we report, for the first time, the involvement of Pim-1 in the protection for CPCs of HPC, elucidating a new aspect of signaling with implications for the regulation of cell survival. HPC enhances the expression of Pim-1 kinase, which causes a reduction of pro-apoptotic elements (cytochrome c and cleaved caspase-3), preservation/modulation of important components of the mitochondria (Bcl-2, Bcl-XL and p-Bad) and attenuates mitochondrial damages. Thus, Pim-1 plays a pivotal role in the protective effect of HPC for CPCs, and a promotion of the expression of Pim-1 in CPCs can serve as a molecular therapeutic interventional strategy in the treatment of cardiomyopathy damage by blunting CPCs death.

References

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2009; 120: 2077 – 2087.

Supplementary Files

Supplementary File 1

Figure S1. (A) Influence of different durations of the HPC to human CPCs (hCPCs) apoptosis rate. (B) Pim-1 mRNA level measured by qRT-PCR after HPC.

Supplementary File 2

Movie S1. Human cardiac progenitor cells (CPCs) differentiated into beating cardiomyocytes in vitro (×80 magnification).

Please find supplementary file(s): http://dx.doi.org/10.1253/circj.CJ-13-0841