Chapter 18
Therapeutic Effects of Ischemic-Preconditioned Exosomes in Cardiovascular Diseases

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18.1 Introduction

Cardiovascular disease (CVD) has been an immense health and economic burdens globally for years. From 2003 to 2013, death rates attributable to CVD declined 28.8%. In the same 10-year period, the actual number of CVD deaths per year declined by 11.7%. Yet in 2013, CVD still accounted for 30.8% (800,937) of all 2,596,993 deaths, or ≈1 of every 3 deaths in the United States [1].

Acute myocardial infarction (MI) as the hallmark of CVD has been considered as the leading cause of mortality worldwide. For now, percutaneous intervention is the most effective strategy to save dying myocardium. However, the reperfusion of acute ischemic myocardium itself is able to cause cardiomyocyte death. The

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underlying oxidative stress, intracellular Ca^{2+} overloading, rapid change in PH, inflammatory reaction and mitochondrial dysfunction all put the myocardium in danger [2]. Ischemic preconditioning (IPC) is a phenomenon that produce resistance to loss of blood supply by creating intermittent short episodes of ischemia. Having been reviewed detailed, IPC might be a potential treatment for ischemic/reperfusion (IR) injury [3–5]. A new concept that extracellular vesicles encompassing exosomes participates in the ischemic preconditioning has been brought out [6]. In this chapter, we summarize the protective effects of IPC exosomes in CVD and the most relevant discoveries in basic science.

18.2 Ischemic Preconditioning

As early as 1990s, it was hypothesized that episodes of brief ischemia would precondition the myocardium for the following ischemia. It is a strategy of creating brief short cycles of non-lethal ischemia-reperfusion stimulus and followed by persistent ischemia. It is expected that IPC would initiate a cardioprotective phenotype and render the myocardium resistant to a subsequent more severe sustained ischemic insult. The principle is to increase the myocardium tolerance to ischemia in various pathways.

To prove this, an ischemic model on the dogs was created. One group was treated with four 5 min circumflex occlusions, each separated by 5 min of reperfusion, followed by a sustained 40 min occlusion. The other group got a single 40 min occlusion. Results shown that preconditioned group had a limited infarct size to 25% of that seen in the non-preconditioned group [7]. Encouraging by this, another similar study was carried out to testify whether this protective effect also works in the remote virgin myocardium. Conclusions agree with the hypothesis and imply that preconditioning may be mediated by factors transported throughout the heart during brief ischemia/reperfusion [8]. Later, several studies found that short periods of ischemia and reperfusion of a tissue can protect a distant tissue against subsequent ischemia [9–13]. Furthermore, a reduction in the coronary resistance and subsequent increase in coronary artery flow was observed in a model exposed to intermittent ischemic conditioning [14]. Similar results were also obtained in human study [15]. With these evidence, remote ischemic preconditioning (RIPC) has been increasingly accepted as an effective method to improve cardiac function after IR injury. Some studies suspected that it is opioid receptor dependent [9, 16], while others support that the activity of a vagal pre-ganglionic neurons is essential in the remote ischemic preconditioning [17]. With all the evidence, the role of RIPC in IR injury is strongly supported [18]. Apart from that, myocardial postconditioning has been shown to benefit in reducing myocardial infarct size [19]. In spite of this, disagreement still exist. Researchers have been arguing that RIPC does not decrease ischemia-associated mortality, nor it reduce major adverse cardiovascular events [20].

18.3 Mechanism of IPC

Several systems have been proven to participate in this process, including ATP-sensitive potassium channels, reactive oxygen species, nitric oxide and various protein kinases [21]. In an ischemic rat model, remote ischemic preconditioning (RIPC) group was treated with four cycles of 5 min of limb ischemia. Followed by 5 min of reperfusion and subjected to 45 min of sustained ischemia by occluding the left coronary artery. Controlled group were treated just with 45 min of sustained artery occlusion. Mitochondrial ATP-sensitive K(+) (K(ATP)) channels were identified as an effector mechanisms for remote preconditioning [22]. Comparable conclusion was also made in a study for modulation of K(ATP) channels in endothelial IPC in human [23]. Another well explored mechanism is the regulation of inflammatory response during IPC. It has been proven that RIPC stimulus modifies human inflammatory gene expression, leading to cardioprotective effect due to affecting the inflammatory process [24]. Circulating cytokines and hypoxia induced factor-1α were found to be influenced as well [25]. Other factors, including oxygen radicals [26], neurotransmission [27–29], cannabinoids [30], nitric oxide synthase [31], connexin 43 phosphorylation [32], mitogen-activated protein kinases (MAPKs) [33], miR-144 [34] and phosphatidylinositol-3-kinase system [35] are all testified. However, little is known about the role of exosomes in IPC. Exosomes has recently been gaining attention with regards to its inter-cellular communication during IR injury. It contains nucleic acid and other important messenger factors. Understanding the underlying mechanism will help us understand how the heart respond to injury and stress at a deeper level.

18.4 Exosomes

Exosomes are small microvesicles (EV) that are released from late endosomal compartments of cells [36]. They are 40–199-nm vesicles released during reticulocyte differentiation as a consequence of multivesicular endosome fusion with the plasma membrane. They have been isolated from diverse body fluids, including semen, saliva, breast milk, amniotic fluid, ascites fluid, cerebrospinal fluid, and bile. EVs can be secreted and specifically taken up by other cells, mediating intercellular signal exchange [37]. Similarly to cytokines that constitute a network of communication, EVs may also exert their functions in a network, affecting distal organs [38]. In a study, rat's heart was exposed to 3 × 5–5 min global ischemia and reperfusion or 30 min aerobic perfusion. The presence or absence of EVs was confirmed by dynamic light scattering, the EV marker HSP60 based on Western blot, and electron microscopy. It was found that IPC markedly increased EV release from the heart, indicating that EV is necessary for cardioprotection by RIPC [37]. mRNA intended for both small and large ribosomal subunits as well as mRNA coding for proteins involved in mitochondrial energy generation are found in the cardiomyocyte-derived EVs, which implies EVs might participate in some protein production in the targeted cells.

These EVs, proven to belong to the exosome family, could be denoted "cardiosomes". Microscopic findings suggested its role in metabolism of microenvironment [39]. Furthermore, by introducing the exosomes from the newts to the rat's heart tissue, new proliferation of the rat cardiomyocyte and improvement in its function were observed [40]. These evidence confirm that exosome is closely associated with cardiac restoration.

18.5 Exosomes and IPC

Ischemic preconditioning effects can be transferred to nonpreconditioned animals via whole blood transfusion [41] or directly cell implantation [40]. These findings suggested a humoral mechanism for preconditioning at a distance. Exosomes contain many unique features like surface proteins/receptors, lipids, mRNAs, microRNAs, transcription factors and other proteins [41]. Stimulated by RIPC, exosomes acutely activate pro-survival kinases that rapidly prepare the heart against ischemia-reperfusion injury [42]. For now, it has been well established that exosome play an essential role in tumor and infection disease, but increasing studies proposed that it is also crucial for cardioprotection during IR injury.

Cells from different organ systems are able to produce exosomes and working actively in RIPC.

18.6 Cardiogenic Exosomes

It was discovered that human cardiomyocytes can produce exosomes-like vesicles via multivesicular endosome (MVE)-dependent pathway [43]. Released from the injured tissue, it carries signaling molecules to activate tissue repair. Isolated cardiac progenitor cells (CPC)-exosomes are found to express cardiac transcription factor, GATA4 and could be recognized by cardiac cells by H9C2. *In vivo* study demonstrated that exosomes from CPC could inhibit apoptosis induced by IR injury [44]. Emerging evidence demonstrated that exosomes participate in RIPC-induced cardioprotection. Coronary perfusates from preconditioned hearts contained more EVs than perfusates isolated from control. Correlating to the result that preconditioned group had smaller infarct size than the control group, it is concluded that the release of EVs from the heart after preconditioning stimuli is increased and that EVs are responsible for the transmission of remote conditioning signals for cardioprotection [37].

18.6.1 Mesenchymal Stem Cell (MSC) Derived Exosome

MSC is one type of adult stem cell that have great plasticity and has shown great potential for the replacement of damaged tissues such as bone, cartilage, tendon, and ligament [45]. In skeletal muscle, it has been proven that hypoxic preconditioned murine MSC enhanced muscle regeneration [46]. MSC are emerging as an extremely promising therapeutic agent for tissue regeneration and repair, proven by

animal models [47, 48]. Exosomes have been recognized as part of MSC's paracrine system that potentiates its cardioprotective effect. These exosomes carry various of miRNA and humoral factors to the target cells [49]. miR-22, miR-210, miR-21 and HIF-1α are found in exosomes isolated from preconditioned MSCs. miR-22, previously known as a critical regulator of cardiomyocyte hypertrophy and cardiac remodeling [50], was shown to protect ischemic hearts by targeting Mecp2 [6]. Preceding study established miR-210 exerts cytoprotective effects in cardiomyocytes [51]. It is elucidated that miR-210 as a potent negative regulator of stem cell apoptosis during ischemic preconditioning downstream of HIF-1α. During ischemic injury, MSC acts as a major source to deliver miR-210 to protect heart tissue [52]. More researches looking into the clinical therapeutic effect of MSC derived exosomes suggested the potential for using human embryonic stem-cell derived vascular cells on rescuing peri-scar border zone in myocardial infarction [53]. On the other hand, in an in *vitro* cardiac injury model, insulin-like growth factor 1 (IGF1) is proven to be part of the signal pathway in exosome-mediated cardiac repair [54].

18.7 Endothelial Cell Derived Exosome

Cardiac endothelial cells could also communicate and regulate myocardium by producing exosomes. Similarly, these exosomes are testified to have nearly twofold increase after preconditioning, and have more potent in reducing cardiac cell death [55]. What's more, endothelial derived exosomes are found to overexpress hypoxia-inducible factor-1 (HIF1) and higher contents of microRNAs. These factors increase tolerance of cardiac progenitor cells under hypoxic stress [56].

18.8 IPC and Proteasome

Proteasome protects against ischemic injury by removing damaged proteins. It is a major intracellular proteolytic system which degrades oxidized and ubiquitinated forms of protein intracellularly. One of important mechanism of cardiac injury during IR injury is the decrease in its function by oxidative modification and inhibition of fluorogenic peptide hydrolysis [57]. In recent studies, it has been proven that MSC derived exosomes ameliorates IR injury through proteomic complementation [58].

The combination of proteasome, ubiquitin, the ubiquitination machinery and the deubiquitinases, is called ubiquitin proteasome system (UPS). The major function of UPS is to prevent accumulation of non-functional, potentially toxic proteins. It contains one 20S subunit and two 19S regulatory cap units. The 20S proteasome is the central proteolytic structure which consists of two pairs of rings each contains seven subunits while the 19S subunit contains multiple ATPase active sites and ubiquitin binding sites. It confers selectivity for ubiquitin-conjugated proteins. Dysfunction of UPS was observed during IR injury, which could be one of the important factor contributing to the heart injury. Recent studies also revealed that IPC protect against ischemic injury by preserving UPS function [59]. IPC protects

UPS by diminishing oxidative damage to 19S regulatory subunits [60] and increasing the degradation of δPKC [61]. A way to quantify the cardioprotective effect from IPC could be to measure the postischemic levels of oxidized and/or ubiquitinated proteins. These levels could predict eventual cardiac function [62].

The 20S subunit of the UPS is found to be attached to the cell plasma membrane and certain observations are interpreted as to suggest that they may be released into the extracellular medium [63]. Once released, they are recognized as circulating proteasomes. Study comparing the features of circulating proteasomes with those of proteasomes isolated from major blood cells found out that the subtype patterns of the circulating ones are clearly different [64]. Circulating proteasome is related to cell damage. Increased serum level is seen in various autoimmune disease [65].

18.9 Exosome and Proteasome

Several studies have been done to explore the correlation between exosome and proteasome. Profiled by mass spectrometry and antibody array, proteasomes of exosomes have been found to contain 857 unique gene products. A predominant feature of MSC exosome proteome is the presence of α and β chain of the 20S proteasome. Further work was done to explore the proteomic profiling of exosome. In *vivo* mouse myocardial infarction model was created by temporarily ligation of the LCA. Exosomes were injected in the treatment group before reperfusion. Proteins in the LCA ligated area was extracted, using a cell extraction buffer. Then sequenced protein analysis confirm the hypothesis that 20S proteasome exists in exosomes and could contribute to the cardioprotective activity. The presence of 20S proteasome in MSC exosomes further suggested that cells extruded 20S proteasome through exosomes [66]. Using the exosome as a carrier, we could deliver therapeutic proteasome specifically to different part of the organ systems.

18.10 Therapeutic Effect of Exosomes and Undetermined Questions

Exosomes have great impact on recipient cells. The distinct transmembrane proteins of exosomes directly interact with the receptors from the target cells [67]. This protein-receptor relationship makes exosomes as ideal carriers to deliver treatment or miRNA to specific cells. What's more, exosomes are non-immunogenic in nature, and have no accumulation in the liver. Based on this, exosomes are hypothesized as a promising medication-carrier [68]. Also, many aspects of exosome suggested itself as a novel means to identify cancer biomarkers for early detection and therapeutic targets, and therapeutic devices to ameliorate the progression of the disease [69].

Great interest has been raising on the remedial role of exosomes in coronary artery disease. Both in *vitro* and in *vivo* study have proven that MSC preserve cardiac function by paracrine system in which exosomes is fundamental. Since there is concern for potential tumor formation in *vivo* in stem cell therapy [70], the paracrine theory provide an alternative method for using MSC in treatment of coronary artery

disease. In addition, exosomes could accumulate in human atherosclerotic plaques, where they affect the physiologic balance [71]. The emergence of exosomes as alternative to cell therapy, opens a new insight into the treatment of cardiac disease. However, our knowledge of the transport, target cell biologic reaction and the complexity of interaction of pathways in exosomes remains immature. It is highly urgent to determine if exosomes from plasma after IPC would be more cardioprotective.

18.11 Summary

CVD has been considered as the number one killer worldwide. Tons of researches have been done to look into the ischemic process and mechanisms during ischemia. IPC is cardioprotective. Various factors, such as inflammatory factors, miRNA and proteins have been proved to play a role in mediating the cardioprotective effects of IPC. Increasing evidence suggested that exosome, a well-known messenger in cell-to-cell communication, is associated with IPC-related cardioprotection. Encouraged by this, exosomes are testified to apply to the injured heart tissue and was found that it improves cardiac function. These finding brings up a possible new treatment for CVD.

Traditional management for ischemia is timely effective restoration of blood flow. Besides that, cell and targeted molecular therapy have gained increasing interest as potential therapies. Large amount of cardiomyocytes dead during ischemia. Although emerging evidence support that human heart has the capacity to regenerate itself [72, 73], the endogenous proliferation rate of cardiomyocytes is too low to compensate for the loss of cardiomyocytes [54]. Stem cell based therapy solved this problem but still has its limitations, since it may also has the tumorigenic potential [70]. Based on this, the idea of exosome-centered therapy was developed and testified [74, 75]. However, more clinical studies need to be done to confirm the therapeutic effect of exosomes.

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