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Review

Emerging role of non-coding RNAs and extracellular vesicles in cardioprotection by remote ischemic conditioning of the heart

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Remote ischemic conditioning of the heart (including pre-, per-, and post-conditioning) is a phenomenon where short episodes of non-lethal ischemia in the distant vessels within the heart or distant organs from the heart protects the myocardium against sustained ischemia/reperfusion injury. Several pathways have been proposed to be involved in the mechanisms of Remote ischemic conditioning. While triggers of Remote ischemic conditioning act in preconditioned areas, its mediators transduce protective signals via humoral or neuronal pathways to the heart. Remote ischemic conditioning is mediated via receptor and non-receptor signaling through secondary mediators, which transfer the signal within the cardiomyocyte and activate cardioprotective pathways that lead to higher resistance of the heart to ischemia/reperfusion. Apparently, identification of endogenous signal molecules involved in the mechanisms of Remote ischemic conditioning have therapeutic implications in the management of patients suffering from myocardial ischemia through the development of diverse beneficial effects. Recently, different non-coding RNAs such as microRNAs or long non-coding RNAs have been identified as emerging factors that trigger protective mechanisms in the heart. These non-coding RNAs are transferred to the heart via extracellular vesicles that exert remote cardioprotection. This review is intended to summarize the existing knowledge about the potential role of extracellular vesicles as humoral transmitters of Remote ischemic conditioning and emphasize the involvement of non-coding RNAs in the mechanism of cardioprotection by Remote ischemic conditioning.

Keywords

Remote ischemic conditioning; extracellular vesicles; non-coding RNA; microRNA; cardioprotection

1. Introduction

Remote ischemic preconditioning (RIPC) of the heart, a special type of ischemic preconditioning (IPC) was first discovered by Murry et al. (1986), but reported by Przyklenk et al. (1993). In this study, brief episodes of ischemia from one vascular bed were observed to protect remote intact myocardium from subsequent sustained coronary artery occlusion-induced ischemia/reperfusion (I/R) injury. While the classical IPC protects only those myocytes that are subjected to initial ischemia by coronary occlusion, the initial ischemic episodes in RIPC are induced in distant areas within the heart or distant organs. RIPC is believed to be a more applicable type of IPC for cardioprotection in humans and similar protective effects are achieved by remote per-conditioning (RIPerC) (Zhao et al., 2003) as well as by remote post-conditioning (RIPostC) (Zhao et al., 2003). Remote ischemic conditioning (RIC) is commonly used for cardioprotection that is achieved by short episodes of ischemia at remote organs before, during, or after sustained ischemia of the coronary artery.

During the past two decades, numerous experimental studies have documented the efficiency of RIC in protecting the heart from I/R injury. In these studies, cardioprotection was evoked by ischemic insults from different distant organs including the kidney (Diwan et al., 2008; Kant et al., 2008; Lang et al., 2006; Takaoka et al., 1999; Weinbrenner et al., 2002), small intestine (Gho et al., 1996; Schoemaker and van Heijningen, 2000), and liver (Barteková et al., 2003, 2004). Moreover, RIC is also induced by limitation of blood flow in the extremities (Birnbaum et al., 1997; Ferko et al., 2015; Oxman et al., 1997; Sharma et al., 2016), which seems to have potentially the highest relevance for use in humans (Kharbanda et al., 2001). For the purpose of developing pharmacological mimetics of RIC to maximize its clinical applicability, it is essential to uncover molecules that act as key players in cardioprotection afforded by RIC. Extensive research has been carried out and numerous mechanisms have been proposed that involve signal transduction of RIC. These mechanisms may be divided into three categories: (a) triggers of RIC activated in the preconditioned remote organ/tissue, (b) mediators of RIC responsible for transfer of cardioprotective signal from remote organ to the heart and subsequently within the heart, including various pre-receptor (humoral) and post-receptor mediators, and (c) targets or end-effectors of RIC within the heart tissue the modulation of which leads to enhanced resistance of the heart to the sustained

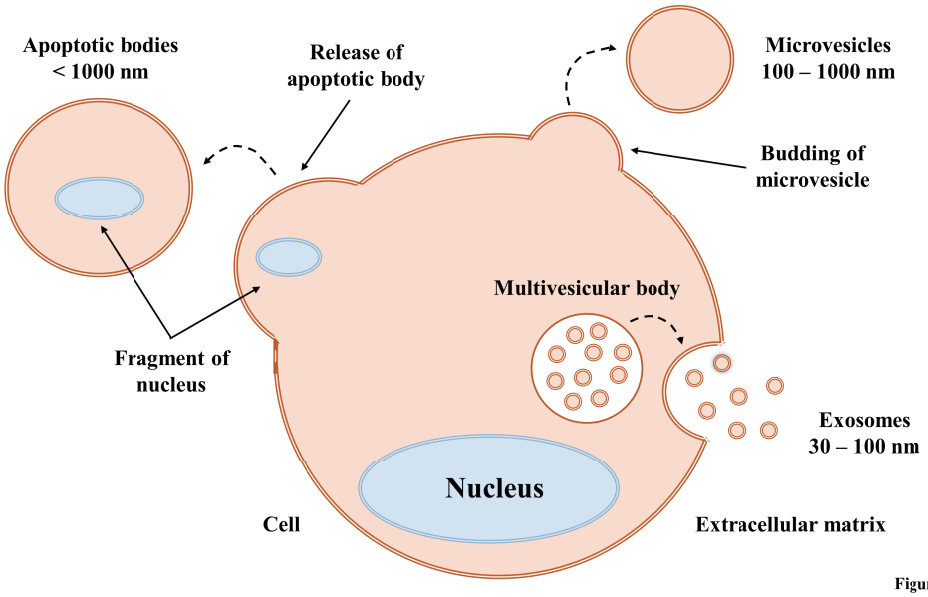

Figure 1. Biogenesis of different types of extracellular vesicles. There are 3 types of extracellular vesicles. First group is exosomes which, are the smallest particles with size about 30-100 nm. These are created inside of the cell and are stored in multivesicular bodies. When the shipment of exosomes is ready, multivesicular bodies fuse with the cytoplasmic membrane and exosomes with its cargo are released into the extracellular matrix. Second type is microvesicles, which have size around 100 - 1000 nm. These vesicles are created differently than exosomes. Their biogenesis includes budding of cytoplasmatic membrane and release of microvesicle to the outer space of the cell. Last group is apoptotic bodies, which are usually the biggest group of these particles, with size above 1000 nm. They are released in an event of programmed cell death (apoptosis). In this case nucleus of the cell with genetic material and another organelles are fragmented to smaller parts and cell is disintegrated to small particles, apoptotic bodies, this way the cell undergoes its destruction without inflammation.

I/R injury. It should be mentioned that it is believed that mechanisms of both classical IPC and RIC are, at least partially, universal; thus the same molecules may be involved in both types of cardioprotection. Additionally, it should be noted that triggers of RIC may be the same substances as those responsible for classical IPC; however, the exact molecules triggering the cardioprotective mechanism of RIC have not been thoroughly identified. Potential candidates include molecules such as nitric oxide (NO) (Tokuno et al., 2002), opioids (Randhawa and Jaggi, 2017), or adenosine (Randhawa and Jaggi, 2016).

Transfer of cardioprotective signals from remote organs to the heart may be mediated via humoral factors or neural pathway while there is some evidence for the involvement of both (Pickard et al., 2016). Among humoral factors, the involvement of circulating proteins such as cytokines (Gedik et al., 2017) and heat shock proteins (Maciel et al., 2017) have been proposed to be involved in RIC signal transfer via blood circulation to the heart. RIC may play a crucial role in positive changes in mitochondrial function because the impairment of mitochondrial respiration has been found as one of the principal factors that lead to depressed heart function from I/R injury (Boengler et al., 2018; Ferko et al., 2014). There are different membrane-bound and intracellular proteins, that are essential for heart function.Ion pumps and sarcomeric proteins are considered to be important targets for different types of cardioprotection due to RIC, as well as function impairment, which has been shown to contribute to depressed heart function due to I/R (Abdul-Ghani et al., 2014; Belliard et al., 2016; Garcia-Dorado et al., 2006; Steinberg, 2013).

Recently, extracellular vesicles, particularly exosomes, have been identified as potential carriers of cardioprotective signals of RIC (Giricz et al., 2014; Minghua et al., 2018). In line with these findings, the main goal of the current review is to summarize the most recent knowledge about the potential involvement of extracellular vesicles in remote cardioprotection. Furthermore, different types of non-coding RNAs (ncRNAs) have been recently identified as new players in cardioprotection. Notably, ncRNAs may be up- or downregulated within the heart tissue due to various triggers; however, certain RNAs have also been identified to be involved in the cargo of extracellular vesicles (Bei et al., 2017; Chevillet et al., 2014; Sluijter et al., 2018). This makes ncRNAs as prime candidates for humoral as well as intracellular mediators involved in the cardioprotective mechanism of RIC. In addition to the potential role of extracellular vesicles, another goal of the present review is to describe the current knowledge for the involvement of various ncRNAs, particularly microRNAs, in the RIC phenomenon.

2. Role of extracellular vesicles and vesicular ncRNAs in RIC

2.1 Extracellular vesicles

Extracellular vesicles are lipid bilayer-coated particles secreted by most cell types into the extracellular space and subsequently

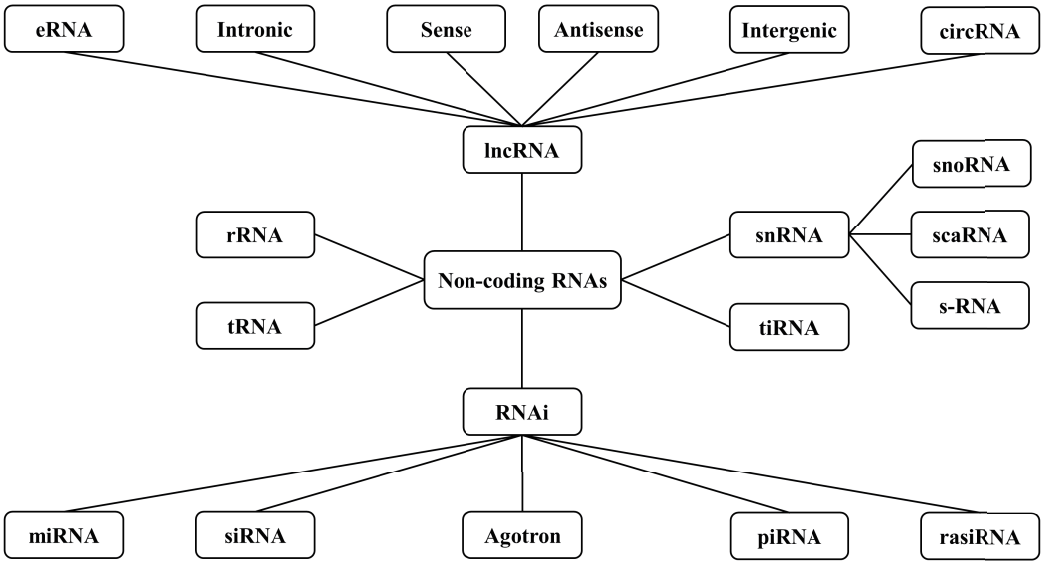

Figure 2. Subfamilies of Non-coding RNAs. Non-coding RNAs are divided into several subfamilies according to their length and functions. Abbreviations: lncRNA, long noncoding RNA; snRNA, small nuclear RNA; rRNA, ribosomal RNA; tRNA, transfer RNA; tiRNA, tRNA derived stress induced small RNA; eRNA, enhancer RNA; circRNA, circular RNA; snoRNA, small nucleolar RNA; scaRNA, small cajal RNA; s-RNA, RNA derived small RNA; miRNA, micro RNA; siRNA, small interfering RNA; piRNA, piwi interacting RNA; rasiRNA, repeat associated small interfering RNA; RNAi, RNA interference – process in which RNAs inhibit gene expression or translation by neutralizing targeted mRNAs.

into the circulation. In addition to plasma, these can be found in most body fluids including urine, saliva, breast milk, and cerebrospinal fluid (Akers et al., 2015; Fujita and Nonomura, 2018; Nair et al., 2018; Zempleni et al., 2017). Several subpopulations of extracellular vesicles are based on size and origin. Exosomes are the smallest particles ranging from 30-100 μm in diameter and created inside the cell and released from multivesicular bodies called microvesicles, also termed microparticles, which are approximately 100-1000 μm in diameter. These microvesicles are created by budding of the cytoplasmic membrane and released in the outer space of the cell (Andaloussi et al., 2013; van der Merwe and Steketee, 2017). Recently, extracellular vesicles have been termed to be small and large extracellular vesicles rather than exosomes and microvesicles. The terms exosomes and microvesicles should be used if the correct origin of the particular extracellular vesicle is proven (Jeppesen et al., 2019; Mateescu et al., 2017). Certain investigators have suggested a third category of extracellular vesicles, apoptotic bodies, sized over 1000 μm; however, these differ from exosomes and microvesicles both in genesis within the cell and function. Apoptotic bodies are not considered to serve as humoral factors for intercellular communication (Akers et al., 2013). A schematic representation of the extracellular vesicle types and genesis are shown in Fig. 1.

Initially, extracellular vesicles were thought to be "platelet dust"; however, it is now observed that these vesicles possess various biological functions that are considered as novel mediators of cell-to-cell communication (Hargett and Bauer, 2013). As discussed previously, extracellular vesicles are released by various cells that are consequently taken up by other neighboring or remote cells that exert paracrine or endocrine effects. Thus, constituents of extracellular vesicles have been shown to act as humoral factors that affect cells and tissues that make these candidates humoral mediators of RIC. In addition, these have been suggested to be biomarkers of certain diseases and are detectable in body fluids. However, standardization of the current methods for extracellular vesicle detection and characterization is needed before using these as a true diagnostic tool (Bei et al., 2017; Li et al., 2017; Raeven et al., 2018; Sluijter et al., 2018). Nonetheless, it is becoming clear that extracellular vesicles are involved in physiological processes in cardiac cells and intercellular communication and believed to play an essential role in the progression of different types of cardiovascular disease participating in the regulation of cardiomyocyte hypertrophy and apoptosis, as well as cardiac fibrosis and angiogenesis (Bang et al., 2014; Waldenström and Ronquist, 2014). Extracellular vesicles have also been suggested to be involved in the pathophysiology of certain cardiomyopathies such as peripartum cardiomyopathy and cardiomyopathy induced by diabetes or sepsis (Ailawadi et al., 2015). Furthermore, these entities have been indicated to be associated with several comorbidities of endothelial dysfunction underlying heart failure including obesity and hypertension (Gohar et al., 2018).

2.2 Role of Non-coding RNAs in RIC

Extracellular vesicles are widely enriched with different RNAs including small non-coding microRNAs (miRNAs). The role of these ncRNAs in the regulation of the cardiovascular system and cardioprotection is of great interest. A wide family of ncRNAs

includes various types of RNAs, which in contrast to messenger RNAs (mRNAs), are not translated into proteins. Important types of ncRNAs are ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs); however, there is emerging evidence for functional importance of other types of ncRNAs, mostly miRNAs, long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs). As it is shown in Fig. 2 the ncRNA family consists of numerous types of RNA molecules that are divided into several subfamilies.

NcRNAs have been shown to mediate pathophysiological processes in the cardiovascular system and serve as biomarkers of different types of cardiovascular disease (Ágg et al., 2018; Devaux et al., 2015; Devaux, 2017; Gomes et al., 2017, 2018). The role of ncRNAs has been long proven in cardioprotection; however, the mechanism of how they reach remote target cells or even other organs has only been recently investigated. Although ncRNAs are generally believed to be more stable in the extracellular space than mRNAs due to relative resistance to breakdown by RNAses (Shvedova et al., 2016), stability is further improved by transport in the extracellular vesicles. The most studied subfamily of non-coding RNAs related to cardiac function, disease, and cardioprotection are the miRNAs. Regarding cardioprotection, expression of several miRNAs, e.g. miR-1, miR-21 and miRNA-125b*, have been identified to be significantly changed due to ischemic pre- or post-conditioning (Cheng et al., 2010; Duan et al., 2012; Perrino et al., 2017; Varga et al., 2014). Importantly, several mi-RNAs altered by I/R were significantly affected by preconditioning, post-conditioning, or both Moreover, transfection of selected mimics of mi-RNAs into cardiac myocytes subjected to simulated I/R showed significant cytoprotective effect (Varga et al., 2014). Protective miRNAs, also termed protectomiRs e.g. miRNA-125b*, have been proposed to be promising protectomiR for cardioprotection against I/R injury (Varga et al., 2018).

2.3 Role of exosomes in RIC

There is a growing body of evidence for the role of extracellular vesicles in cardioprotection including that afforded by RIC (Davidson et al., 2018; Giricz et al., 2014; Sluijter et al., 2018). It has been shown that circulating extracellular vesicles, even from non-treated animals, reduce infarct size; however, origin was not proven in this study (Vicencio et al., 2015). Thus, there is the question: what kinds of cells release these extracellular vesicles with cardioprotective signals? This crucial question has not been answered in sufficient detail; however, these particles released from various cells, mainly different types of stem cells and progenitor cells, were proposed to have a therapeutic effect in different cardiovascular diseases such as acute myocardial infarction, stroke, or pulmonary hypertension (Radosinska and Bartekova, 2017; Safari et al., 2016; Singla, 2016; Suzuki et al., 2016; Tsao et al., 2014; Yuan et al., 2018). Experimental studies have demonstrated the efficacy of mesenchymal stem cells (MSCs)-derived exosomes for the treatment of I/R injury and myocardial infarction (MI) as manifested by infarct size reduction and improved recovery of cardiac function (Cui et al., 2017; Lai et al., 2010; Yu et al., 2015; Zhu et al., 2018). Beneficial effects of MSC-derived exosomes in reducing cardiac I/R injury have been confirmed by meta-analysis (Zhang et al., 2016). Cardiac progenitor cell (CPC)-derived extracellular vesicles are also proposed to exert cardioprotective effects in MI. It has been shown that treatment with human CPC-derived extracellular vesicles improved post-MI recovery of cardiac function associated with suppression of cardiomyocyte apoptosis and stimulation of angiogenesis (Barile et al., 2014). In addition, *in vivo* delivery of mouse CPC-exosomes inhibited cardiomyocyte apoptosis in acute myocardial I/R in mice (Chen et al., 2013). Moreover, CPC-derived particles exert similar protective effects in preserving cardiac function after MI compared to parent cells indicating that these are key paracrine mediators effect CPCs (Kervadec et al., 2016). Recently, exosomes secreted by normoxic and hypoxic cardiosphere-derived cells (CDCs) have been shown to exert anti-apoptotic effects in human embryonic stem cell-derived cardiomyocytes suggesting therapeutic potential of CDC-exosomes for the treatment of ischemic heart disease (Namazi et al., 2018). Additionally, microvesicles from endothelial differentiated medium-preconditioned adipose-derived stem cells (ASC) have been shown to promote angiogenesis, via the delivery of miRNA-31 to vascular endothelial cells that target and suppress the antiangiogenic inhibiting-factor HIF-1. This suggests a potential microvesicles-based angiogenic therapy for ischemic disease (Kang et al., 2016).

In addition to the cardioprotective potential of different stem and progenitor cells derived from extracellular vesicles, ischemia-induced release of these particles into circulation and subsequent protective effects on the heart subjected to I/R injuryhas recently been documented as potential cardioprotective mechanism of RIC. Giricz et al. (2014) demonstrated that ischemic preconditioning (IPC) evoked by 3 cycles of 5 min ischemia and 5 min reperfusion in isolated and perfused rat hearts increased the amount of extracellular vesicles released into coronary perfusates. Furthermore, perfusates from preconditioned hearts depleted of extracellular vesicles did not decrease infarct sizes in recipient hearts exposed to I/R indicating that these particles are necessary for cardioprotection by RIPC. Another study focused on their role in RIPC has documented that exosomes derived from preconditioned mouse MSCs by exposition to two cycles of anoxia (30 min) and intermittent re-oxygenation (10 min), and then injected along the border between infarct zone and normal myocardium after coronary occlusion in mouse hearts, decreased fibrosis and infarct sizes in these hearts (Feng et al., 2014).

Data on mediators carried by extracellular vesicles are rapidly increasing with non-receptor-mediated mechanisms such as vesicular transfer of nucleic acids (including ncRNAs or functional enzymes) and receptor-mediated mechanisms that are implicated in the cardioprotective effects of these particles. However, this section is focused mainly on the role of ncRNAs carried by extracellular vesicles in the mechanism of RIC. In a study of Feng et al. (2014), preconditioned MSC-derived exosomes that induced cardioprotection were enriched with miRNA-22 and mobilized to cardiomyocytes where apoptosis was reduced due to ischemia. The anti-apoptotic effects of miR-22 were mediated by direct targeting of methyl-CpG-binding protein 2 (Mecp2) suggesting that beneficial effects of RIPC via preconditioned MSC-derived exosomes may be mediated through miR-22 targeting Mecp2. The potential role of exosomes in RIC mechanisms was further supported by a study of Yamaguchi et al. (2015) in which the authors showed that remote ischemic post-conditioning (RIPostC) maintained by 5 cycles of 5 min bilateral hind limb ischemia and 5 min of reperfusion once a day for 4 weeks after MI in rats. This study showed that

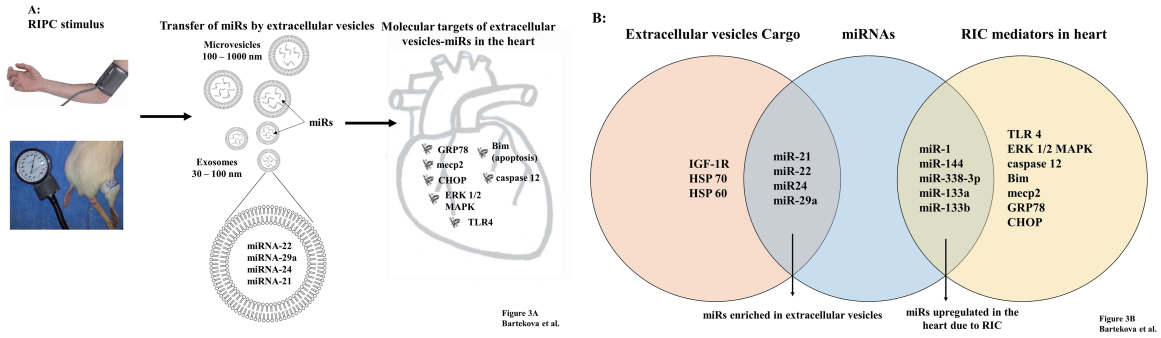

Figure 3. An overview of the role of microRNAs and extracellular vesicles in RIC. A: Extracellular vesicles-mediated mechanism of RIC: Conditioning stimuli in distant organ (limb) induce release of microvesicles and exosomes enriched with particular microRNAs (miR-22, miR-29a, miR-24, miR-21) to circulation. Extracellular vesicles are taken up by myocardial cells; and intracellular targets of extracellular vesicles -miRs, primarily various proteins (TLR-4, Bim, caspase 12, ERK1/2 MAPK, GRP78, CHOP), are up- or downregulated, finally leading to enhanced resistance of the heart to I/R injury. B: Pivotal role of microRNAs in RIC: There is proposed dual role of miRNAs in the mechanism of RIC; while certain miRNAs such as miR-21, miR-22, miR-24 or miR-29a are proposed to be involved in the cargo of extracellular vesicles and served as humoral mediators of RIC, other miRNAs such as miR-1, miR-144, miR-133 or miR-228-3p are upregulated within the heart due to RIC, thus being suggested as intracellular mediators of RIC.

the left ventricular ejection fraction (LVEF) significantly improved and attenuated MI-induced fibrosis that was associated with highly expressed miRNA-29a, a key regulator of tissue fibrosis, in the exosomes serum and the marginal area of the RIPostC group. In addition, miRNA-29a expression was significantly increased under hypoxic conditions in the differentiated C2C12 mouse myoblast cell line-derived exosomes. It is pointed out that insulin-like growth factor 1 receptor (IGF-1R), was highly expressed in serum exosomes, remote non-infarcted myocardium in RIPostC group, as well as in the C2C12-derived exosomes under hypoxic conditions. This data suggests that exosome-mediated transfer of miRNA-29a and IGF-1R may contribute to the beneficial effect of remote cardioprotection afforded by RIPostC.

Exosomal transfer of miRNA-24 has been proposed for exosome-mediated cardioprotection by RIPC. It has been shown that exosomes derived from the plasma of rats subjected to limb ischemia-induced RIPC are enriched with miR-24 in comparison to exosomes derived from non-preconditioned rats. In addition, plasma exosomes could be taken up by H9c2 cells, and it has been shown that miR-24-enriched exosomes released after RIPC reduced oxidative stress-induced injury and decreased apoptosis by downregulating expression of pro-apoptotic protein Bim in H_2O_2-treated H9c2 cells. *In vivo*, the miR-24 in RIPC-induced exosomes reduced cardiomyocyte apoptosis attenuated the infarct size, and improved heart function in rats subjected to MI evoked by a 45 min coronary occlusion and 24 hour reperfusion. Finally, the anti-apoptotic effect of miR-24 was counteracted by miR-24 antagomiRs or inhibitors both *in vitro* and *in vivo* suggesting that RIPC-induced exosomes could reduce apoptosis by paracrine transfer of miR-24. This further supports that exosomal miRNA-24 plays an important role in mediating the protective effects of RIPC (Minghua et al., 2018). The important role of miRNA cargo of extracellular vesicles in cardioprotection by RIPC has been supported by recently published human studies in coronary artery bypass (CABG) patients. In this study, the amount of 26 different miRNAs including the cardioprotective miRNA-21 was found to be increased in extracellular vesicles 5 minutes after RIPC. It was also observed that postoperative troponin I concentrations was also decreased (Frey et al., 2019). Interestingly, in addition to proposed pivotal role of certain miRNAs in remote cardioprotection mediated via exosomal transfer, rapid changes in mRNA content was found in cardiac extracellular vesicles due to IPC (Svennerholm et al., 2015). On the contrary, no specific qualitative or quantitative changes in DNA content were found in these particles after an *in vivo* myocardial IPC (Svennerholm et al., 2016).

Recently, it has been documented that co-culturing of primary adult rat cardiomyocytes with normoxic human umbilical vein endothelial cells (HUVECs) reduced cardiomyocyte death following simulated ischemia/reperfusion (SI/R); however, when extracellular vesicles were removed from the HUVEC-conditioned medium, the protection was lost. Furthermore, pre-incubation of cardiomyocytes with exosomes purified from HUVEC-conditioned medium reduced the cell death after SI/R while this protection was abolished by inhibitors of ERK1/2. It is pointed out that IPC induced exosome production from HUVECs as well as from isolated perfused rat hearts resulted in significantly higher protection against SI/R in cardiomyocytes suggesting that endothelial cells release exosomes that may contribute to RIPC via the activation of the ERK1/2 MAPK signaling pathway (Davidson et al., 2018). Finally, it has been documented that defective exosome-mediated communication may be the cause of failed cardioprotection by RIPC in rats with type 2 diabetes. It was shown that exosome-rich serum from normoglycemic rats attenuated hypoxia/reoxygenation-induced cell death in HL-1 cardiomyocytes while exosome-rich samples from Zucker diabetic fatty rats did not confer such protection in the HL-1 cells. Moreover, exosome-depleted serum from Zucker fatty rats was cytotoxic and exacerbated hypoxia/reoxygenation-induced cell death suggesting that the content or amount of extracellular vesicles released due to

cardioprotective interventions is modulated by metabolic derangements, thus contributing to the loss of RIPC-induced cardioprotection in metabolic co-morbidities (Wider et al., 2018).

2.4 Role of microvesicles in RIC

Microvesicles were also proposed to be involved in cardioprotective effects of RIPC. It has been documented that RIPC evoked by limb ischemia increased endothelium-derived (CD54 and CD146 positive) as well as pro-coagulant (Annexin V positive) microvesicles in both rats and humans. In the same study, RIPC decreased infarct size significantly after a 40 min myocardial ischemia followed by 2 h reperfusion in rats; however, the injection of RIPC-induced microvesicles did not decrease infarct size compared with MI alone. This suggests that these particles are not the biological vectors of RIPC (Jeanneteau et al., 2012). In contrast, recent studies by Ma et al. (2015) have shown that transfusion of microvesicles isolated from rats immediately, but not 6 hours after a hind limb ischemia-induced RIPC into recipient rats exposed to heart I/R injury, resulted in an increase in platelet-derived particles in blood associated with reduction in infarct size and improved functional recovery of hearts. The role of IPC-induced circulating microvesicles in cardioprotection against myocardial I/R injury was also confirmed in the study of Wang et al. (2016) where authors injected these isolated particles after coronary occlusion-induced IPC to recipient rats that were consequently exposed to MI and found that myocardial infarct size and cardiomyocyte apoptosis as well as LDH release were reduced. In addition, they found a decreased expression of Bax and activity of caspase 3 and increased expression of Bcl-2 and Bcl-2/Bax ratio after IPC-microvesicles treatment suggesting cardioprotective effects of IPC-microvesicles through inhibition of apoptosis induction. Finally, protective effects of circulating microvesicles derived from IPC on myocardial I/R injury have been reported recently in the study of Liu et al. (2018) where these particles isolated from blood after IPC elicited by three cycles of brief I/R due to coronary occlusion were intravenously injected to recipient rats 5 min before reperfusion in I/R injury. Microvesicles alleviated damage of myocardium and restored cardiac function after I/R manifested by increased heart rate and decreased ST-segment elevation, as well as reduced infarct size, decreased LDH activity, and decreased number of apoptotic cardiomyocytes. In addition, IPC-induced microvesicles decreased the activity of caspase 3 and the expression of endoplasmic reticulum stress (ERS) markers, GRP 78, CHOP, and caspase 12 suggesting the attenuation of ERS-induced apoptosis a novel cardioprotective mechanism of microvesicles-mediated remote cardioprotection.

2.5 General remarks on extracellular vesicles studies

It is apparent that extracellular vesicles play an important role in mediating remote cardioprotection evoked by RIC. However, there are several limitations in studies published so far including limitations on the methods used for the isolation and characterization of extracellular vesicles. Furthermore, isolation methods, as well as markers used for EV characterization, vary widely between studies, which may lead to uncertainties regarding the exact population of these particles studied (Onódi et al., 2018; Sódar et al., 2016; Théry et al., 2018). Thus, there should be some caution in data interpretation regarding biological functions of particular extracellular vesicles. In line with this view, a recent executed project highlighted the need for the better understanding of both extracellular vesicles isolation techniques and classification based on the biogenesis in RNA-oriented studies (Jeppesen et al., 2019). Distinct patterns of non-coding RNAs in different subclasses of extracellular vesicles are reported in this study suggesting that specification and stringent isolation of these particles is paramount in such experiments. Moreover, this study provided examples on RNAs previously believed to be part of the extracellular vesicles transcriptome to reside in the non-vesicular space, and thus, pointed the interest of the field to revisit previous viewpoints based on results of studies using outdated or inappropriate methods of their isolation (Jeppesen et al., 2019). Therefore, the data accumulated so far should be interpreted with caution as they should be verified by using methods indicated in the latest recommendations. Another limitation of RIC-extracellular vesicles studies is the lacking evidence of direct uptake of these particles by recipient cells in the heart despite their increased release due to RIC (Frey et al., 2019; Jeanneteau et al., 2012). In addition, identifying the exact cardioprotective molecules in the cargo of RIC-released extracellular vesicles is another challenge for the future. In fact, research in this field is still in its infancy, and therefore additional studies using advanced methods of their isolation and characterization are needed to fully uncover the exact role of extracellular vesicles in RIC.

3. Role of non-vesicular ncRNAs in RIC

3.1 Non-extracellular vesicles miRNAs in RIC

In addition to extracellular vesicles-carried miRNAs, other miRNAs, particularly those produced within the heart tissue, have been shown to be changed due to RIC suggesting their role in cardioprotection. It has been documented that miRNA-1 was significantly downregulated in the heart tissue due to RIPC evoked by 3 cycles of 5-min limb ischemia in rats. Interestingly, the same miRNA was upregulated in classical IPC while infarct size was reduced in both conditioned groups suggesting that miRNA-1 is differentially regulated in various conditioning protocols (Duan et al., 2012). Unfortunately, such diverse results make the role of miRNA-1 in cardioprotection inconclusive. In a human study in patients undergoing RIPC (evoked by inflating a cuff on the upper arm for 5 min, 3 times) before CABG surgery has shown that myocardial tissue expression of miR-133a and miR-133b increased after aortic cross-clamping in both RIPC and control patient groups, while miR-1 was upregulated in the control group only. In addition, expression of miR-338-3p was higher in RIPC versus control. RIPC also preserved mitochondrial respiration throughout surgery and prevented patient hearts from postoperative atrial fibrillation in this study. The data suggests that RIPC preserves mitochondrial respiration and prevents upregulation of miR-1 during CABG surgery (Slagsvold et al., 2014a). Surprisingly, the same authors as in the previous study documented no changes in miRNA expression observed in left ventricular biopsies due to RIPC in the same patients undergoing this surgery (Slagsvold et al., 2014b). The potential role of miR-1 in RIPC has also been tested in an *in vivo* model of hind limb ischemia-induced RIPC followed by 35-min coronary artery occlusion in rats. It has been documented that miR-1 was downregulated by RIPC, I/R, as well as by RIPC

Table 1. Different types of the RIC-associated extracellular vesicles.

Type of extracellular vesicle	Conditioning procedure	Extracellular vesicle marker	Extracellular vesicle cargo	In-heart target	Reference
Exosomes + Microvesicles	IPC-preconditioned rat heart	HSP60	-	-	Giricz et al., 2014
Exosomes	preconditioned mouse MSCs	CD63	miRNA-22	Mecp2	Feng et al., 2014
Exosomes	Post-conditioned rat hind limb	CD9; HSP90	miRNA-29a; IGF-1R	-	Yamaguchi et al., 2015
Exosomes	RIPC by limb ischemia in rat	CD9; CD63; CD81	miRNA-24	Bim (apoptosis)	Minghua et al., 2018
Extracellular vesicles (non-defined)	RIPC by hind limb in humans	CD63	miRNA-21	-	Frey et al., 2019
Exosomes	IPC-conditioned HUVECs	CD63	-	ERK1/2 MAPK	Davidson et al., 2018
Microvesicles	RIPC by limb ischemia in rats	CD54; CD146; annexin V	-	-	Jeanneteau et al., 2012
Microvesicles (platelet origin) Microvesicle	RIPC by limb ischemia in rat	CD41; annexin V	-	-	Ma et al., 2015
	Coronary occlusion in rat, Microvesicle injected to recipient rats	CD45; CD61; CD144	-	GRP78, CHOP, caspase 12	Liu et al., 2018

Abbreviation: RIC – Remote ischemic conditioning; IPC –Ischemic preconditioning ; RIPC – Remote ischemic preconditioning; MSCs – Mesenchymal stem cells; Mecp2 – Methyl-CpG-binding protein 2.

followed by I/R after 2-hour reperfusion. In contrast, after 6-hour reperfusion, RIPC led to the upregulation of miR-1, while ischemia had no effect on miR-1 expression. This study has also shown an interaction between miR-1 and BDNF (brain-derived neurotrophic factor), a protein proposed to exert cardioprotective effects; however, protein levels of BDNF in rat hearts were not altered by either I/R or RIPC (Brandenburger et al., 2014).

Another miRNA, miR-144, has also been found, to be associated with cardioprotection by RIPC in an combined model of *in vivo* hind limb RIPC followed by global ischemia in isolated Langendorff-perfused mouse hearts. It was reported that RIPC increased, while I/R injury decreased miR-144 levels in the heart. Further, systemic treatment with miR-144 improved functional recovery of hearts and reduced infarct size similar to RIPC, and induced cardioprotective signaling by increased P-Akt, P-GSK3β and P-p44/42 MAPK, decreased p-mTOR, and induced autophagy. Conversely, administration of antagomiR-144 reduced myocardial levels of miR-144 and abrogated cardioprotection by RIPC, which also increased plasma levels of miR-144 in both mice and humans without any changes in amount of plasma exosomes or their miR-144 content. On the other hand, level of miR-144 precursor was significantly increased in exosomes, and miR-144 levels were increased in exosome-free serum (Li et al., 2014). In addition, the intravenous injection of miR-144 reduced left ventricular remodeling after myocardial infarction associated with reduced border zone fibrosis, inflammation and apoptosis in a mice *in vivo* MI model, where labeled miR-144 was found to be localized in the infarct and border zone and was taken up by cardiomyocytes and macrophages (Li et al., 2018). Moreover, the loss of miR-144 signaling has been shown to interrupt extracellular matrix remodeling post MI leading to compromised cardiac function (He et al., 2018). These data suggest that systemic release of miR-144 might play a pivotal role in the cardioprotection afforded by RIPC including its potent effects on post-MI remodeling.

It should be also pointed out that several miRNAs including miR-22 (Feng et al., 2014), miR-29a (Yamaguchi et al., 2015), and miR-24 (Minghua et al., 2018) have been proposed to be transferred in the cargo of extracellular vesicles and potentially exert cardioprotective effects in the target organ/heart. Potential involvement of miRNAs in RIPC-induced cardioprotection including those carried in extracellular vesicles is summarized in Fig. 3. Finally, RIPC-increased levels of certain miRNAs, particularly miR-21, have been documented to be associated with protection of other organs than heart, such as kidney protection in children with congenital heart disease undergoing cardiopulmonary bypass (Kang et al., 2018) or multiple organ protection against sepsis (Jia et al., 2017). These data suggest miR-21 being additional miRNA that may potentially be involved in the multi-organ protection afforded by RIPC. Taken together it could be pointed out that several miRNAs seem to be intimately involved in the cardioprotection evoked by RIPC. While certain miRNAs including miR-22, miR-29a and mir-24 are involved in the cargo of RIPC-released extracellular vesicles mediating the cardioprotective signal by humoral transport from preconditioned organ to the heart, expression of other miRNAs such as miR-1 or miR-144 is influenced by I/R and RIPC within the heart tissue suggesting these miRNAs to be post-receptor mediators/targets of RIPC in the heart.

3.2 Role of other ncRNAs in RIC

In addition to miRNAs, there is increasing evidence about the role of other types of non-coding RNAs, particularly circular RNAs (circRNAs) and long non-coding RNAs (lncRNAs) in cardiac function in both health and disease (Bei et al., 2018; Devaux et al., 2015; Devaux, 2017; Hobuß et al., 2019). Recently, it has been shown that changed expression of certain lncRNAs might be associated with cardioprotection against I/R injury (Kong et al., 2019; Zhang et al., 2018); however, there is no information

about the particular role of lncRNAs in remote cardioprotection by RIC. Furthermore, upregulation of several circRNAs such as Cdr1as (antisense circRNA to the cerebellar degeneration-related protein 1 transcript) (Geng et al., 2016), MFACR (mitochondrial fission and apoptosis-related circRNA) (Wang et al., 2017), MICRA (myocardial infarction-associated circRNA) (Salgado-Somoza et al., 2017) or circNCX1 (circRNA transcribed from the sodium/calcium exchanger-1 gene) (Li et al., 2018) have been shown to participate to cellular injury due to myocardial infarction and to regulate mitochondrial dynamics and apoptosis in the heart. These data suggest that circRNAs may be used as biomarkers to predict the outcome of MI or as targets for cardioprotective interventions. However, no data about potential association between circRNAs and cardioprotection by RIC have been published so far. Thus, in addition to the role of widely studied miRNAs in remote cardioprotection, there is an open field of research to discover potential role of other members of non-coding RNA family including lncRNAs or circRNAs in cardioprotection by RIPC.

4. Clinical applications of EVs and ncRNAs

Recently documented role of EVs and their cargo molecules, including several miRNAs, in the mechanisms of RIC clearly indicated that EVs are released into the circulation due to ischemic insult and also may mediate cardioprotection. Moreover, EVs are present in almost all body fluids and their number and content has been shown to be changed due to different pathological situations in cardiovascular system, not only due to IPC. In line with these observations, EVs and miRNAs have been proposed as potential biomarkers of cardiac injury as well as potential therapeutic tools for the treatment of cardiovascular diseases (Dickhout and Koenen, 2018; Zamani et al., 2019; Sluijter et al., 2018).

A number of circulating miRNAs including miR-1, miR-126, miR-133a/b, miR-145b, miR-199a, miR-208a/b and miR-499 were documented to be associated with different types and stages of cardiovascular disease such as acute coronary syndrome, myocardial infarction or heart failure, and considered as markers of cardiac injury or targets for therapy (Deddens et al., 2016; Jansen et al., 2014; Navickas et al., 2016; Vegter et al., 2016). However, biased selection of miRNAs in most studies and inconsistent results limit reliable identification of clinically useful miRNAs. Most probably, the majority of circulating miRNAs is transported by EVs (Gallo et al., 2012), but there is also evidence of EV-independent circulating miRNAs (Arroyo et al., 2011). Possible contamination of isolated EVs by other particles and proteins may decrease the prognostic value of EV-miRNAs as biomarkers. Moreover, release of EV due to RIC at distant organ from the heart point to difficulties in identifying the origin of detected circulating EVs which downgrade reliability of EV-based biomarkers from the view of their organ specificity. Thus, current technical limitations for EV isolation and characterization limit the use of EVs as biomarkers for cardiovascular disease.

In addition to biomarkers, EVs and EV-miRNAs were thought to be potentially used in the therapy of cardiovascular disease. Positive effects of RIC-induced EVs in myocardial injury support this view on EVs as therapeutic tools. In addition to use of EVs from defined cell types for regenerative therapies, EV could be potentially used also for drug delivery. However, the translation of EV into clinical therapies will require the categorization of EV-based therapeutics in compliance with existing regulatory frameworks. Moreover, safety and quality control as well as pharmacokinetics of EVs must be considered for pharmaceutical manufacturing of EVs before clinical use of EV-based therapies in the future (Lener et al., 2015; Morishita et al., 2017).

5. Conclusions

A vast array of miRNAs has been evidenced to mediate cardioprotection afforded by RIC via influencing canonical and newly proposed intracellular signaling mechanisms. However, their relative importance, interaction, kinetics, or translatability of their use into the clinics has been less studied. Hence it appears that there is a lack of ncRNA-based cardioprotective pharmacological solution to ischemic heart diseases. The emergence of extracellular vesicles, especially stem cell-derived particles, as sources or carriers of cardioprotective miRNAs has given the field of cardioprotection a badly needed boost. Although currently significant gaps exist in our knowledge on the role of extracellular vesicles in cardioprotection, their potential to contribute to future pharmacological solutions is plausible. However, the recently explored pitfalls and drawbacks of methodologies applied in their research have undermined the credibility of previously gathered information on the role of RNAs carried by extracellular vesicles. Moreover, the exact action of particular miRNA cargo of these particles in the recipient cell including their downstream molecular targets is still to be evaluated. Nevertheless, miRNAs, most likely vesicular miRNAs, continue to be rising stars for studies on cardioprotection by RIC (Table. 1). In addition, several non- extracellular vesicles miRNAs have been documented to be altered due to RIC within the heart, suggesting their role as post-receptor mediators of RIC. Finally, extensive studies are needed to establish, the role of lncRNAs or circRNAs in cardioprotection afforded by IPC or RIPC against I/R.

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Conflict of Interest

The authors declare no conflict of interest.

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