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Role of miRs in Cardiac Preconditioning

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Abstract

Preconditioning of the heart by sub-lethal ischemia, mild heat shock or hypoxia have evolved as powerful experimental tools to discover novel signaling mechanisms in cardioprotection. The ultimate goal is to determine novel therapeutic targets for potential application in humans in protecting the heart against ischemia-related injuries. In recent years, there has been a tremendous interest in understanding the role of small non-coding RNAs, microRNAs (miRs), in cardiovascular diseases. miRs have been recognized as regulators of gene expression by destabilization and translational inhibition of target mRNAs. Studies have shown that several miRs including miR-1, miR-133, miR-21, miR-126, miR-320, miR-92a and miR-199a are regulated after preconditioning and play an active role in protection of the heart against ischemia/reperfusion injury. These miRs also drive the synthesis of important cardioprotective proteins including HSP-70, eNOS, iNOS, HSP-20, Sirt-1 and HIF-1 α . We believe that identification and targeted delivery of miR(s) in the heart could have an immense therapeutic potential in reducing myocardial infarction in patients suffering from heart disease.

Keywords

Myocardial preconditioning; Ischemia-reperfusion injury; miRs; Hypoxia; Heat shock; Nitric oxide synthase; Heat shock proteins

1. Introduction

Heart disease remains a leading cause of morbidity and mortality in the United States and world-wide. Over the past 30 years, enormous progress has been made in our understanding of the basic biochemical and molecular mechanisms that underlie the development of ischemia/reperfusion (I/R) injury. Ischemic preconditioning (PC) is an experimental manipulation that exploits the endogenous protective mechanism activated by mild ischemic stress resulting in an enhanced ability of the heart to cope with another similar or greater stress. The phenomenon of PC was first described by Murry et al. (1) and remains as one of

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the major fundamental discoveries in the field of ischemic biology. The protective effects of PC occur in 2 distinct phases: an early phase that develops rapidly after the stimulus but dissipates within 2 to 3 hours and a late phase that becomes apparent 12 to 24 hours later and persists for ≈ 72 hours (2). The range of protection is different in the two phases. The early phase is very effective in limiting lethal I/R injury (i.e. infarction) but does not protect against reversible post-ischemic contractile dysfunction (myocardial “stunning”). The late phase protects against both infarction and stunning, although it is less powerful than the early phase in limiting infarct size. The late phase of PC is likely to have greater clinical relevance, because it has a broader range of protection, and since its duration is 30–40 times greater than that of the early phase (3). To date, intensive research has led to considerable progress in understanding the fundamental nature of PC and many of the potential pathways involved. As shown in Figure 1, mild, sub-lethal ischemic stress causes the release of endogenous triggers including adenosine, bradykinin and opioids. The 3 triggers bind to Gi protein-coupled receptors (Gi-PCR). The additive trigger theory requires that binding of the 3 triggers by their respective receptors results in signaling that converges on a common target. It is thought that protein kinase C (PKC) is the common target because protection afforded by PC and all of the triggers can be eliminated by PKC inhibitors as reviewed by Yang et al. (4). PKC and probably other kinases, result in the activation of cytoplasmic and normally dormant stress responsive transcription factors, such as NF- κ B, STAT 1 and STAT 3, heat shock transcription factor-1 (HSF-1) and possibly many other transcriptional regulators. This process culminates in the upregulation of cardioprotective genes, leading to the synthesis of new proteins including HSP-70, MnSOD, hemeoxygenase (HO-1), and inducible nitric oxide synthase (iNOS). These proteins, either individually or in concert, lead to the protection afforded by late PC. Such identification of the cellular basis of preconditioning has provided a solid conceptual scaffold for developing novel therapeutic strategies. In fact, targeting these pathways with pharmacological agents including adenosine receptor agonists (5,6,7,8,9), nitric oxide donors (10,11,12), bradykinin (13,14,15), p38 MAP kinase activator, anisomycin (16,17), chemical inducer of hypoxia-like responses, cobalt chloride (18), ATP-sensitive or Ca^{2+} -activated potassium channel openers including diazoxide (19,20), and phosphodiesterase-5 inhibitors (21,22) have also been shown to exhibit preconditioning-like cardioprotective effects. Despite the intense effort and multiple clinical trials, thus far, none of these agents has yet translated into clinical use (23). Accordingly, there is a need for discovering new mediators of preconditioning that afford protection during periods of ischemic injury.

2. MicroRNAs (miRs) and their role in Cellular Processes

The miRs are a family of small RNA with an average length of 24 nucleotides, which is too short to code for any protein. The first animal miR was described in 1993 as a regulator of developmental timing in *C. elegans* (24). Initially, they were considered as by-products of mRNA transcription. However, in recent years, miRs have emerged as novel regulators of gene expression. They are transcribed by RNA polymerase II as primary transcripts (pri-miRs) (25), though some miRs that are interspersed among repetitive DNA elements like Alu repeats can also be transcribed by RNA polymerase III (26). The pri-miRs are subsequently processed by Drosha and DGCR8 (Pasha) complex resulting in a 70 nucleotide

long stem loop structure called precursor-miR (pre-miR). From the nucleus, the pre-miRs are then exported to the cytoplasm by exportin-5, where they are processed by Dicer, an RNase III enzyme, and bound to miR-induced silencing complex (RISC) to form the mature miRs of 18–23 nucleotide length. The key proteins in the RISC complex are Argonaute 2 and transactivation-responsive RNA-binding protein. The mature miR along with the RISC bind to complementary sites in the mRNA transcripts and negatively regulate gene expression (27,28). miRs that bind to mRNA targets with imperfect complementarity regulate the target gene at the level of protein translation, while miRs that bind to their mRNA targets with perfect complementarity induce target mRNA cleavage (29).

Several recent studies suggest that miRs participate in many cellular processes, such as apoptosis (30,31,32), fat metabolism (33), cell differentiation (34,35,36), tumorigenesis (37), cardiogenesis (38,39,40,41) and angiogenesis (42). miRs are also critically involved in the pathological processes of adult hearts, including cardiac hypertrophy (43,44,45), arrhythmogenesis (46) and heart failure (47). Among the miRs expressed in the heart, miR-1 and miR-133 seem to be cardiac-specific during development and in adulthood (48). miR-138 was shown to be involved in cardiac patterning in zebrafish (49). This information is very useful given that chamber-specific gene expression and patterning events are highly conserved across species including humans (49). miR-138 normally suppresses atrioventricular canal gene expression and disruption of its function led to the expansion of atrioventricular canal gene expression into the ventricle and resulted in failure of cardiomyocyte maturation.

Recent studies have shown that circulating levels of vascular and inflammation-associated miRs are significantly downregulated in patients with coronary artery disease (50). Levels of miR-126, miR-17, miR-92a, and the inflammation associated miR-155 were significantly reduced in patients with coronary artery disease compared with healthy controls. Likewise, the smooth muscle-enriched miR-145 was significantly reduced. In contrast, cardiac muscle-enriched miRs (miR-133a, miR-208a) tended to be higher in patients with coronary artery disease. miR-1 was found to be upregulated in response to I/R injury in the rat heart (51) and in a rat model of myocardial infarction (52). Also, oxidative stress increases miR-1 levels and subsequently decreases heat shock proteins including HSP-60 and HSP-70 (53). The muscle-specific miRs; miR-1 and miR-133, produce opposing effects on apoptosis by targeting HSP-60, HSP-70 and caspase-9 in cardiomyocytes. Overexpression of miR-1 induced apoptosis and augmented hydrogen peroxide (H₂O₂)-induced apoptosis in H9c2 cells (54). In contrast, inhibiting miR-1 conferred resistance of cells to H₂O₂. miR-320 has been shown to be involved in the regulation of I/R injury via targeting HSP-20 (55). Transgenic mice with cardiac-specific overexpression of miR-320 had a larger myocardial infarct size following I/R injury, whereas inhibiting miR-320 with antagomir-320 significantly reduced infarct size (54). Recently, Bonauer et al. (56) reported that miR-92a levels were increased 24 h after coronary artery occlusion in mice. The injection of antagomir-92a after coronary artery occlusion in mice improved left ventricular function, reduced myocardial infarct size, attenuated apoptosis, and increased angiogenesis in the border zone. These studies showed that miR-92a targeted Sirt1, a class III histone deacetylase and longevity gene. Therefore, inhibiting miR-92a may potentially be important in reducing infarct size and improving remodeling and neovascularization after

myocardial infarction. Another miR that is widely expressed in the heart and seems to play a key role in angiogenesis is miR-126. Knock-down of miR-126 during the embryonic development of zebrafish caused severe defects in vascular development, including collapsed blood vessels and cranial bleeding (57). Interestingly, miR-126 mutant mice with successful embryonic development demonstrated limited angiogenesis and decreased survival after myocardial infarction by coronary artery ligation.

3. Role of miRs in Ischemic Preconditioning

Although a number of cardioprotective proteins have been implicated in PC, the use of pharmacologic agents to promote or inhibit these potential therapeutic targets has not been without limitations due to several reasons, including off-target drug effects. Therefore utilizing miRs as a target for therapy is more promising since these small RNAs have known sequences, which may reduce the occurrence of non-specific adverse effects. In a recent study, we examined the role of miRs induced by PC in protection against myocardial I/R injury (58). We induced miRs by short bursts of global ischemia and reperfusion in the isolated-perfused heart. This PC protocol caused a significant induction of multiple miRs including miR-1 ($162 \pm 13\%$), miR-21 ($118 \pm 6\%$), and miR-24 ($46 \pm 12\%$) (Figures 2B and 2C). PC stimulus (2 bursts of 30 sec global ischemia followed by 90 sec reperfusion, Figure 2A) reduced infarct size from $29.7 \pm 2.1\%$ in the control group to $9.1 \pm 1.8\%$ in the PC group (69.3% reduction, mean $\pm$ SEM, $P < 0.05$). Interestingly, similar brief episodes of hypoxia have been shown to increase miR-21 in isolated myocytes as well (59). To determine the cause-and-effect relationship between PC-induced endogenous miRs and cardioprotection, we injected the pool of extracted miRs from the non-preconditioned and preconditioned hearts into the left ventricular wall in situ in a separate set of mice (miR-injected group). The direct injection approach ensured the delivery of miRs into the risk zone (area to be subjected to ischemia) of the heart. Forty-eight hours later, the mice were subjected to I/R injury *in vivo*. PC-derived miRs produced a protective phenotype with significantly lower infarction ($18.8 \pm 2.5\%$) as compared to saline-injected controls ($37.5 \pm 2.2\%$) or miRs prepared from non-PC hearts ($39.3 \pm 2.3\%$). There was no difference in infarct size between the saline-injected controls versus non-IPC miR-treated hearts (Figure 2D). In order to confirm uptake of exogenous miR-21 following injection in LV wall, RT-PCR was performed and endogenous U1A small nuclear RNA (RNU1A) was used as control (Figures 2E and 2F).

To gain further insight into the mechanisms underlying miR-induced protection, we probed several target protective proteins that are implicated in PC. As shown in Figure 3A, we observed an induction of eNOS mRNA ($61 \pm 6.7\%$), however, iNOS mRNA was unchanged. Western blot analysis showed significant up-regulation of eNOS protein ($92.0 \pm 8.1\%$) and HSF-1 ($42.7 \pm 3.0\%$) after IPC-miR treatment. HSP-70 was also significantly increased ($102.3 \pm 8.9\%$) 48 hours after PC-miR treatment. Again, similar to mRNA, iNOS protein was not changed (Figures 3B and 3C).

The role of NO derived from eNOS in the late phase of PC has been suggested previously (60). Although the critical role of iNOS has also been well documented in the late phase of PC (61) and pharmacological preconditioning (22,62), there was no upregulation of this

enzyme in the PC-miR-treated hearts. The induction of HSPs by stressful stimuli such as elevated temperature or ischemia (63) is mediated by the heat shock transcription factor (HSF-1). It is known that the cytoplasmic HSF-1 monomer forms a trimer and moves to the nucleus where it binds to its target sites (known as heat shock elements) in the regulatory regions of the HSP genes. Following HSF-1 phosphorylation, it induces expression of HSP-70 that could protect hearts against ischemic injury during the late phase of PC (64). A similar cardioprotective effect of this protein was observed with direct myocardial gene transfer of HSP-70 as reported previously (65). PC-miRs induced HSF-1 and HSP-70 which may have played a role in ischemic tolerance observed in the heart. However, the specific miR responsible for the increased synthesis of HSF-1/HSP-70 or eNOS was not clear from these studies. miR-1 has been linked to post-transcriptional repression of HSP-60 and HSP-70 in H9c2 cells (53) which is in contrast to the upregulation of HSP-70 synthesis observed in our study. Also, eNOS introns contain miRs which regulate eNOS expression (66). Classically, exon-based siRNA inhibits gene expression by increasing the mRNA degradation (67), which requires perfect sequence match. These studies (66) showed that intron-based miR or siRNA can gene-specifically suppress eNOS expression. Alternatively, it was suggested that some miRs could exert translational inhibition via imperfect sequence match at 3' UTR.

The significance of miR-21 in PC is supported by a recent study by Dong et al. (68). These authors showed that miR-21 was downregulated in the infarcted areas but upregulated in the border areas of the heart after ischemia. Furthermore, PC inhibited the downregulation of miR-21 in the infarcted areas and overexpression of miR-21 reduced cell apoptosis in the border and the infarcted areas, decreased myocardial infarct size, and improved left ventricular remodeling at two weeks following infarction.

The role of phosphatidylinositol-3-kinase PI3K/Akt signaling has been well established in cardioprotection against I/R injury. Akt is an anti-apoptotic protein that has been implicated in the negative regulation of Fas ligand. PC (induced by sublethal ischemia) or pharmacologic agents has been shown to induce Akt phosphorylation, which is required for cardioprotection mainly through attenuation of apoptosis. Sayed et al (59) demonstrated that miR-21 is a downstream effector of Akt and partly mediates its anti-apoptotic effects. Transgenic mice overexpressing miR-21 had smaller infarct size following ischemia and ameliorated heart failure. Another interesting finding in this study was that Akt-mediated suppression of Fas ligand during hypoxia in cardiomyocytes was reversed upon knock-down of miR-21, as was its effect on caspase-8 activity. These studies provide direct evidence that miR-21 mediates Akt-induced attenuation of apoptosis.

4. Role of miRs in Heat Shock Preconditioning

Mild heat-shock has been shown to improve myocardial survival after subsequent prolonged I/R injury (69). The specific mechanisms underlying heat-shock protection include synthesis of HSPs (70), antioxidant defenses (71), and enhanced mitochondrial respiration (72). In addition, it has been shown that heat-shock protects by opening of mitochondrial K_{ATP} channels (73) and causes resistance to opening of mitochondrial permeability transition pore (74), which may contribute to heat-shock protection against cellular injury through

inhibition of apoptosis. Recently, we tested the hypothesis that miRs induced by heat shock stimulus may also play role in protection against I/R injury in the heart. In this study (75), we induced endogenous miRs through heat-shock and injected them into non-heat-shocked mice. Similar to the PC model described above, this unique experimental design took advantage of testing the role of endogenously induced miRs in protection against I/R injury. Mice treated with the mixture of miRs isolated from mildly heat-shocked mice demonstrated improved ischemic tolerance. Infarct size was reduced significantly from $40 \pm 2.7\%$ (percentage of total risk area, mean $\pm$ S.E.M.) in the non-heat-shocked controls to $18.5 \pm 3.8\%$ in mice treated with miRs (Figure 4). Moreover, chemically synthesized exogenous miR-21 also reduced infarct size by 64% ($P < 0.05$ versus control). The miR-21-induced protection was totally abolished when mice were co-treated with miR-21 antisense. miR treatment caused profound changes in several apoptotic related genes as determined by gene microarray analysis. In particular, caspase family members 1, 2, 8 and 14 were suppressed in the hearts treated with miR from heat-shocked mice. Except for BNIP-3, most of the pro-apoptotic genes including Bid (BH3 interacting domain death agonist), Bcl-10 (B-cell leukemia/lymphoma 10), Cidea (cell death-inducing DNA fragmentation factor, alpha subunit-like effector A), Ltbr (lymphotoxin B receptor), Trp53 (transformation related protein 53), Fas (TNF receptor superfamily member) and FasL (Fas ligand, TNF superfamily, member 6), were repressed. On the other hand, the anti-apoptotic genes, Bag-3 (Bcl-2-associated athanogene) and Prdx2 (Peroxiredoxin 2) were increased.

5. Role of miRs in Hypoxic Preconditioning

The myocardial endogenous adaptation to hypoxia leading to resistance against subsequent lethal injuries appears to be an inherent part of the “late phase of preconditioning” (76). Incessant or chronic systemic hypoxia can elicit a complex adaptive response that leads to enhanced tolerance to myocardial I/R injury (77,78). Hypoxia-inducible factor 1 α (HIF-1 α) has been shown to be an essential mediator of the acute systemic hypoxia-induced cardioprotection (79). HIF-1 is a heterodimeric α/β transcription factor that mediates tissue responses to hypoxia (80). HIF-1 is essential in the transcription of numerous genes that are implicated in oxygen homeostasis, especially in response to diminished oxygen tension. Prolyl hydroxylases (PHDs) hydroxylate HIF-1 during normoxia, which allows von Hippel-Lindau to bind and ubiquitinate HIF-1, marking it for proteasomal degradation. This process is inactivated during hypoxia, thus, permitting rapid accumulation of HIF-1. Three PHD isoforms, PHD1, PHD2 and PHD3 have been identified (81,82). PHD2 is ubiquitously expressed and exhibits the highest specific activity toward HIF-1 α (83). Both PHD2 inhibition and hypoxia stabilize HIF-1 α protein and lead to increased levels of active HIF-1 α/β heterodimers in cell nuclei (84). Previous studies from our laboratory have demonstrated that HIF-1 α stabilization by pharmacological inhibition of PHD2 exerted a cardioprotective effect against I/R injury in rabbits (85). Furthermore, *in vitro* and *in vivo* PHD2 silencing using a siRNA strategy produced transcriptionally active HIF-1 and normoxic activation of HIF-1 and attenuated reperfusion injury via an iNOS-dependent pathway (86). Recently, Rane et al (87) reported that HIF-1 α is a predicted target of miR-199a and is rapidly induced by hypoxia. The luciferase activity test showed that miR-199a interacted with the 3'-UTR of HIF-1 α and that inhibiting miR-199a induced its

expression in cardiomyocytes. Both hypoxic preconditioning and silencing of miR-199a were associated with upregulation of iNOS and Bcl-2, whereas overexpression of miR-199a prevented upregulation of iNOS during hypoxic preconditioning. Overexpression of miR-199a reduced Sirt1 levels, whereas silencing miR-199a by antisense oligonucleotide or by downregulating miR-199a by hypoxic preconditioning increased Sirt1 levels. miR-199a induced protection against I/R injury both by decreasing suppression of translation of HIF-1 α and by enhancing its stability via Sirt1-induced inhibition of PHD2. These studies suggest that miR-199a could be a valuable target for inducing PC through regulation of PHD2/HIF-1 during normoxia and hypoxia.

6. Summary

In addition to the extensively studied signaling mechanisms, the induction of miRs following PC, hypoxia and sub-lethal heat shock clearly suggest that these small RNAs are novel regulators of cardioprotective gene expression against I/R injury. Among several miRs, miR-1, miR-21, miR-92a, miR-133, miR-138, miR-126, miR-199, and miR-320 appear promising and seem to play key roles in cardiac cell fate decisions including cardiac conduction, regulation of cardiac patterning, management of angiogenesis, and protection against ischemia as shown in Figure 5. These miRs could work independently or in concert to achieve certain outcomes, and understanding their exact mechanism of action as well as their downstream targets is necessary for safe intervention. Specific miRs are important in controlling I/R injury and therefore could be potential therapeutic targets for reducing ischemia-related cardiovascular diseases. Altering miRs by developing stable miR mimetics and/or antagomirs is likely to emerge as an alternative and safe method for conferring immediate and long-lasting protection against I/R injury. However, there are a number of important issues that need to be addressed before these miR-modifying drugs can be used routinely in the clinical setting. These include the efficiency of delivery of miR-based agents to the desired tissue or site. For example, novel techniques such as nanoparticles can be employed and tagged to home to the desired organ, such as the heart in order to ensure a local effect. It is also possible to tag antogomirs with a glycoconjugate, which can then act as a docking agent for the tagged molecules to reach their target site. Despite these technical challenges, the future of miR-based targeted therapy for cardioprotection appears promising.

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Figure 1.

Outline of the mechanisms of ischemic preconditioning: Sub-lethal ischemic stress causes the release of triggers including adenosine, bradykinin and opioids.

The 3 triggers bind to Gi protein coupled receptors (GiPCRs). Protein kinase C (PKC) is the common target of activation of GiPCRs because protection afforded by PC and all of the triggers could be eliminated by PKC inhibitors. The GiPCR activation also triggers PI3 kinase activation which causes phosphorylation of Akt. Phosphorylated Akt subsequently activates endothelial nitric oxide synthase (eNOS) to produce nitric oxide (NO), which then stimulates guanylyl cyclase (GC) to produce cGMP which in turn stimulates PKG. PKG has a role in opening of the mitochondrial K_{ATP} channel that eventually leads to an early preconditioning effect. The PKC (and other kinases) activate transcription factors which lead to the upregulation of cardioprotective proteins including HSP-70, MnSOD, hemeoxygenase (HO-1), inducible nitric oxide synthase (iNOS) which are responsible for the late phase of PC.

Figure 2.

Induction of miRs by ischemic preconditioning (PC) and Effect of miR on myocardial infarct size following ischemia/reperfusion. (A) Infarct size reduction following IPC ($n = 6/\text{group}$). (B) Gel electrophoresis image of RT-PCR products of miRNAs. (C) Average normalized results showing increase in miR-1, miR-21 and miR-24 following IPC. The results are means $\pm$ SEM from 3 independent hearts. (D) Reduction of myocardial infarct size (% of risk area) following direct delivery of IPC-miRNA into the heart. * $P < 0.05$ versus saline control and non-IPC miRNA treated hearts. (E) Uptake of miR-21 following injection in LV wall. Gel electrophoresis image of the RT-PCR for miR-21. (F) Average normalized changes in miR-21. Endogenous U1A small nuclear RNA (RNU1A) was used as control. (From Yin, Salloum and Kukreja 2009 with permission).

Figure 3.

Effect of miRs on induction of cardioprotective proteins. (A) RT-PCR showing eNOS and iNOS mRNA levels. A significant increase in eNOS was noted 2 h following treatment with IPC-miR. (B) Representative western blot showing expression of eNOS, iNOS, HSF-1 at 4 h and HSP 70 at 48 h following treatment with IPC-miR or non-IPC-miR control. (C) Bar diagram showing average % increase in proteins shown in 'B'. Results are means $\pm$ SEM from 3 independent hearts per group. (From Yin, Salloum and Kukreja 2009 with permission).

Figure 4.

Effect of miR-21 and its inhibitor on cardiac infarct size following ischemia/reperfusion.

Top: bar diagram showing a significant reduction of infarct size compared to the non-treated controls. Chemically synthesized miR-21 also reduced infarct size significantly. The miR-induced infarct size reduction was completely abolished by miR-21 inhibitor. Bottom: representative images of mouse heart slices stained with 10% of triphenyl tetrazolium chloride (TTC). Heart samples were selected from the following experimental groups: Control, miRs, miR-21 and miR-21 inhibitor. Note that the infarct area is significantly larger (as shown by the increased pale area) in the control and miR-21 inhibitor-treated mice as compared to the miRs and miR-21 groups. The hearts were subjected to 20 min of global ischemia and 30 min of reperfusion 24 h following miR injection. Infarct size was measured at the end of ischemia/reperfusion using tetrazolium staining. (From Yin, Wang and Kukreja 2008 with permission).

Figure 5.

Schematic diagram showing the potential role of various miRNAs in the cardiovascular system. As mentioned in the text, miR-1 and miR-133 play a key role in cardiogenesis; miR-138 is involved in cardiac patterning; several miRNAs including miR-155 are associated with inflammation; miR-126 is essential for angiogenesis; miR-21 induces a preconditioning-like cardioprotective effect against ischemia/reperfusion injury and attenuates apoptosis; downregulation of miR-320 causes induction of HSP-20 leading to cardioprotection against ischemia; downregulation of miR-199a stabilizes HIF-1α through suppression of PHD2 and results in cardioprotection against ischemia/reperfusion injury.