Loss of the miR-144/451 cluster impairs ischaemic preconditioning-mediated cardioprotection by targeting Rac-1

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Aims

While a wealth of data has uncovered distinct microRNA (miR) expression alterations in hypertrophic and ischaemic/reperfused (I/R) hearts, little is known about miR regulation and response to ischaemic preconditioning (IPC).

Methods and results

We analysed miRs in murine hearts preconditioned with six cycles of 4 min ischaemia via coronary artery occlusion, followed by 4 min reperfusion in vivo. Both miRs within the miR-144/451 cluster were the most elevated among a cohort of 21 dysregulated miRs in preconditioned hearts, compared with shams. To investigate the significance of this finding, we examined IPC-mediated cardioprotection within a miR-144/451-knockout (KO) mouse model. Wild-type (WT) hearts exposed to IPC followed by I/R (30 min/24 h) showed a smaller infarction size compared with mice treated with I/R alone. In contrast, IPC failed to protect miR-144/451-KO hearts against infarct caused by I/R treatment. Thus, the miR-144/451 cluster is required for IPC-elicited cardioprotection. Rac-1, a key component of NADPH oxidase, was mostly up-regulated in KO hearts among three bona fide targets (Rac-1, 14-3-3ζ, and CUGBP2) for both miR-144 and miR-451. Accordingly, reactive oxygen species (ROS) levels were markedly increased in KO hearts upon IPC, compared with IPC-WT hearts. Pre-treatment of KO hearts with a Rac-1 inhibitor NSC23766 (20 mg/kg, ip) reduced IPC-triggered ROS levels and restored IPC-elicited cardioprotection. Using antagomiRs, we showed that miR-451 was largely responsible for IPC-mediated cardioprotection.

Conclusion

Loss of the miR-144/451 cluster limits IPC cardioprotection by up-regulating Rac-1-mediated oxidative stress signalling.

Keywords

MicroRNA-144 • MicroRNA-451 • Ischaemic preconditioning • Myocardial ischaemia/reperfusion • Rac-1 • Oxidative stress

1. Introduction

Ischaemic preconditioning (IPC) provides strong protective effects against cardiac ischaemia/reperfusion (I/R)-induced injury.¹ While IPC is cardioprotective in humans, clinical applicability is limited because intervention must be performed prior to acute myocardial infarction.² Therefore, it is important to identify innate mediators or end effectors as substitutive agents for mimicking IPC’s protection.

Several signalling pathways and numerous mediators/end effectors have been elucidated to modulate IPC so far, including PI3-kinase-Akt, AMP-activated kinase (AMPK)-mediated sarcolemmal K_{ATP} channels, 3'-phosphoinositide-dependent kinase-1 (PDK1), nitric oxide, protein kinases C/G, adenosine A1 receptors, oxygen-derived free radicals, cyclooxygenase-2 (COX2), and heat-shock proteins.¹⁻⁵ However, the translation of IPC-mimetics into the clinical setting has been disappointing,⁶ suggesting that the mechanisms underlying
IPC-elicted cardioprotection are difficult to manipulate and not fully understood.

MicroRNAs (miRs) are endogenous non-coding small RNA molecules that regulate gene expression post-transcriptionally through pathways that may represent therapeutic targets for human heart disease. Expression patterns of miRs are altered distinctly during cardiac remodelling and heart failure. Our laboratory and other groups have observed that miRs function as prompt stress-regulated molecular switches of gene expression in the myocardium. However, the dynamics and physiology of miR expression in IPC are largely unknown.

In this study, we discovered profound alterations of miR expression in murine hearts subjected to IPC. Among numerous dysregulated miRs, those within the miR-144/451 cluster were the most significantly up-regulated by IPC. While our recent observations show that both miR-144 and miR-451 confer protection against simulated I/R-triggered cell death in adult cardiomyocytes, it is unclear whether the miR-144/451 cluster is required for IPC-elicted cardioprotection. Therefore, we hypothesize that loss of the miR-144/451 cluster will largely offset IPC-induced protective effects in animal hearts. This hypothesis will be tested in the present study using an miR-144/451 knockout (KO) mouse model and antagoniR-treated mice.

2. Methods

2.1 The protocol for IPC in murine hearts

An equivalent set of 12 male mice (B6129SF2/J, 10–12 weeks old) were used: six were subjected to IPC treatment and six were sham-treated. All the animal experiments conformed to the Guidelines for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health, and approved by the University of Cincinnati Animal Care and Use Committee. Mice were anaesthetized intraperitoneally using sodium pentobarbital (50 mg/kg), and the adequacy of anaesthesia was confirmed by the absence of reflex response to foot squeeze. Mice in IPC groups underwent occcluder placement following by a series of six 4 min coronary occlusions interspersed with 4 min reperfusion. This protocol of IPC was selected because it has been proved highly effective by other laboratories in reducing mouse myocardial infarction size (by ~70–80%). At the end of these procedures, mice were sacrificed with an overdose of sodium pentobarbital (200 mg/kg) intraperitoneal injection and hearts were collected for isolation of total RNA.

2.2 miR array and analysis

Total RNA was isolated by using Trizol reagent (Invitrogen). The RNAs were fluorescently labelled with Cy3 or Cy5 using the two-step fluorescent labelling process. Microarray data pre-processing and analysis were performed using R statistical software and the limma package of Bioconductor, as detailed in Supplementary material online.

2.3 Ex vivo IPC and I/R

The mouse heart was perfused in a retrograde fashion with KH buffer at a constant pressure of 100 cmH₂O at 37°C. KH buffer was oxygenated with 95% O₂/5% CO₂ and maintained at pH 7.4. After equilibrium perfusion (10 min) or IPC (three cycles of 5 min of ischaemia and 5 min of reperfusion), perfused mouse hearts were subjected to 20 min of no-flow ischaemia, followed by 40 min of reperfusion. Sham groups were equilibrated for 40 min before 20 min of no-flow ischaemia and 40 min of reperfusion. The left ventricular end-diastolic pressure, left ventricular developed pressure (LVDP), maximum rate of contraction (-dP/dt), and maximum rate of relaxation (-dP/dt) were monitored during this process, as we described previously.

2.4 Cardiac injury analysis

Cardiac injury was assessed by measuring lactate dehydrogenase (LDH) release. In situ DNA fragmentation was assessed using the DeadEnd Fluorometric TUNEL system (Promega), followed by staining with an anti-α-sarcenomic actin antibody (Sigma-Aldrich) and 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen). For more accurate quantification of apoptosis in I/R hearts, DNA fragmentation was determined by a cell-death-detection ELISA kit (Roche Applied Science). Caspase-3 activity was determined in cardiac lysates (100 µg) using Caspase-3/CPP32 Fluorometric Assay kit (BioVision). In vivo myocardial infarct size was determined by perfused hearts with 1% triphenyl tetrazolium chloride. All the details are included in Supplementary material online.

2.5 Generation and characterization of miR-144/451-KO mice

The miR-144/451-KO mouse model was generated as described previously. Primers for genotyping of miR-144/451-KO mice are as follows: forward, 5′-TTT TGC CTG TAATCTGTATCCCTAAGGA-3′; reverse 1, 5′-GGG TAC CCA GAC TAG TAC ATC ATC TAT A-3′; reverse 2, 5′-ATC CCC TCG AGC CTA ATA ACT TC-3′. Wild-type (WT) mice will display one band of 290 bp DNA fragment; KO mice will exhibit one band of 156 bp DNA fragment; and heterozygous will reveal both bands of 290 and 156 bp DNA fragment (see Supplementary material online, Figure S1).

2.6 Treatment of mice with antagoniRs

AntagoniRs were synthesized by Dharmaco (www.dharmacon.com). Sequences are: 5′-a,uccacuacucuuacuag,uu,a-Chol-3′ (AntagomiR-144); 5′-a,uccacacucuuacucuaag,uu,u,a-Chol-3′ (AntagomiR-451); 5′-u,uccacacacacucucucug,aa,a-Chol-3′ (antagomiR mutant as a control). Lower case letters represent 2′-O-methyl-modified oligonucleotides, subscript ‘s’ represents a phosphorothioate linkage, and ‘Chol’ represents linked cholesterol. AntagoniR oligonucleotides were deprotected, desalted, and purified by high-performance liquid chromatography. B6129SF2 male mice (6 weeks old) received antagoniR-144, −451, or mutant antagoniR, or a comparable volume of saline (200 µL) through three consecutive daily tail vein injections via (3 × 40 mg/kg body weight). The levels of miRs (miR-144 and -451) were determined by qRT–PCR, as described above. Ex vivo ischaemia preconditioning followed by I/R was performed at the third day after final injection.

2.7 Measurement of oxidative stress

The levels of reactive oxygen species (ROS) were measured using an oxidation-sensitive fluorescent probe, CM-H2DCFDA (Invitrogen) methods. The heart homogenate was incubated with 10 µM CM-H2DCFDA for 30 min at 37°C. Then, the ROS generation was measured by the fluorescence intensity in each well at an excitation wavelength of 495 nm and an emission wavelength of 530 nm. NADPH oxidase activity was assessed in heart homogenates by lucigenin-enhanced chemiluminescence (50 µg of protein, 100 µM NADPH, 5 µM lucigenin) with a multilabel counter (Victor3 Wallac). The NADPH oxidase activity was determined as the difference between wells without diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase, and with DPI.

2.8 Western blot analysis

Protein samples were extracted from hearts with the procedures as described in detail elsewhere. Equal amounts of protein were subjected to SDS–PAGE. A primary antibody against Rac-1, or CUGBP2, or...
14-3-3ζ was used (1:250 of dilution, Santa Cruz Biotechnology, CA, USA). α-Actin (1:1000 of dilution, Sigma-Aldrich) was performed as an internal control.

2.9 Statistical analysis
All values are expressed as mean ± SD. Student’s t-test was used for two-group comparisons; a value of \( P < 0.05 \) was considered statistically significant.

3. Results
3.1 miRNA expression profile in IPC murine hearts
Myocardial miRs actively regulate physiological and pathological stress.7–16 To identify miRs associated with IPC, we performed microarray analysis in murine hearts subjected to in vivo IPC with six cycles of 4 min ischaemia, via coronary artery occlusion, followed by 4 min reperfusion. Among 477 miR probes, we identified 21 miRs that were differentially expressed in IPC hearts compared with sham groups (Figure 1A; see Supplementary material online, Table S1; \( n = 6, \ P < 0.005 \)). These aberrant miRNAs include up-regulation of miR-144, -451, -762, -551b, -763, -142-3p, -294*, -706, and -290-5p, and down-regulation of miR-1, -805, -133a*, -467b*, -466b-5p, -142-3p, -675-5p, -466a-5p, -466i, -297b-3p, -574-5p, -200a, and -27b (Figure 1A). Notably, miR-1, -144, and -451 were the most significantly dysregulated miRs (Figure 1B), which was further validated by real-time stem-loop PCR (RT-siPCR) (Figure 1C). To exclude in vivo contamination with erythrocyte cells that express high levels of miR-144/451, we isolated total RNA from ex vivo Langendorff-perfused hearts upon IPC with three cycles of 5 min I/R (Figure 1D) and performed RT-siPCR to determine the levels of miR-144 and -451. Consistently, both miR-144 and miR-451 were increased by 1.5-fold in ex vivo IPC hearts, compared with the sham group (Figure 1D). Considering that a number of mediators and multiple signalling pathways are involved in IPC and thousands of proteins fine-tuned by miRs, these results suggest that miRs may have causal contributions to the cardioprotection of IPC. Actually, numerous studies have suggested that decreased miR-1 may partly contribute to cardioprotection against I/R injury.21–24 Our recent studies also showed that miR-144/451 protect cardiomyocytes against simulated I/R.16 However, whether the miR-144/451 cluster is required for IPC-elicited cardioprotection remains unknown. Thus, we employed an miR-144/451-KO mouse model and an antagoniR-mediated knockdown approach to address this issue.

3.2 Characterization of an miR-144/451-KO mouse model
It has been predicted that miR-144 has hundreds of potential targets and miR-451 has much fewer assumed targets (http://www.mirdb.org/; http://www.mirbase.org). Nonetheless, miR-144/451-deficient mice bred normally, presented a normal Mendelian ratio, and displayed no obvious physical abnormalities.20,25 Here, we evaluated the long-term and global consequence of miR-144/451 KO in adult hearts and observed that miR-144/451-null hearts (Figure 2A) exhibited similar rates of contraction (\(+\text{dP/dt}\)) and relaxation (\(-\text{dP/dt}\)), and LVDP compared with WT hearts, as measured by Langendorff preparations (Figure 2B). In addition, the ratio of heart weight/body weight (Figure 2C), cardiomyocyte cross-sectional area (Figure 2D), and the capillary density (Figure 2E) were not affected by loss of miR-144/451. Thus, ablation of the miR-144/451 cluster did not alter normal cardiac growth and contractile function.

3.3 Ablation of miR-144/451 impairs IPC efficacy on post-I/R functional recovery
Next, we determined the effects of miR-144/451 loss on IPC-induced cardioprotection against I/R injury. To minimize secondary effects caused by in vivo neurohumoral factors and inflammatory components during IPC-I/R, we used an isolated perfused heart preparation. Both KO and WT hearts were subjected to IPC with three cycles of 5 min I/R, followed by 20 min global no-flow ischaemia/40 min reperfusion. Sham operations consisted of 30 min flow succeeded by I/R (20/40 min). In WT hearts, IPC groups exhibited significantly better functional recovery than sham hearts (Figure 3A–C), as evidenced by increased rates of contraction (\(+\text{dP/dt}\); Figure 3A), relaxation (\(-\text{dP/dt}\); Figure 3B), and LVDP (Figure 3C). These results are consistent with previous reports that IPC improves the post-I/R recovery of cardiac function.26,27 However, in miR-144/451-KO hearts, the degree of recovery in contractile function (\(+\text{dP/dt}\) and LVDP) was similar between IPC and sham operations (Figure 3A–C). These data indicate that ablation of miR-144/451 negates IPC-triggered cardioprotective effects on post-I/R functional recovery.

Given the marked loss of IPC efficacy on the recovery of myocardial function, we then examined the consequence of miR-144/451 deficiency in post-I/R cellular damage. The extent of necrotic and apoptotic cell death was determined in both KO and WT hearts upon IPC-I/R or sham-I/R. As shown in Figure 3D, IPC significantly reduced the release of LDH (a biochemical marker for necrotic cell death) by ~22% from WT hearts, compared with sham samples (\(n = 11, \ P < 0.05\)). In contrast, the levels of LDH released from KO hearts were not different between IPC and sham operations (Figure 3D). Likewise, the number of TUNEL-positive nuclei was significantly decreased by ~64% in the WT myocardium upon IPC, relative to sham (\(n = 5, \ P < 0.01\); Figure 3E). Additionally, heart lysates from a subset of experimental animals were assayed for histone-associated DNA fragmentation, which exhibited a ~56% decrease in WT hearts upon IPC over sham operation (\(n = 5, \ P < 0.01\); Figure 3F). Moreover, the activity of caspase-3 was reduced by ~14% in IPC-WT hearts compared with sham WTs (\(n = 5, \ P < 0.05\); Figure 3G). However, all three assays (DNA fragmentation, TUNEL assay and caspase-3 activity) demonstrated that IPC had no cellular protective effects against subsequent I/R injury in miR-144/451-null hearts, as evidenced by the similar degree of cardiomyocyte damage between IPC and sham groups (Figure 3E–G). Therefore, the miR-144/451 cluster appears essential in IPC-triggered cardioprotection against I/R injury.

In addition, we noticed that ablation of the miR-144/451 cluster not only inhibited IPC efficacy, but also significantly depressed functional recovery during reperfusion in the absence of IPC (Figure 3A–C). Similarly, the degree of cellular damage (LDH release, myocardial apoptosis, DNA fragmentation, and Caspase-3 activity) was aggravated in miR-144/451-deficient hearts upon sham I/R, compared with WT hearts (Figure 3E–G). These results suggest that ablation of the miR-144/451 cluster exacerbates myocardial injury after I/R.
miRNA expression profile in IPC hearts. (A) A heat-map of the up-regulated and down-regulated miRNAs in murine hearts subjected to six cycles of 4 min LAD occlusion and 4 min reperfusion, compared with sham groups. All of the miR array raw data are available in Supplementary material online, Table S1. (B) Microarray data are summarized by a volcano plot graph, which displays both fold-change and t-test criteria (log odds). miR-1, -144, -451, and -762 are the most significantly dysregulated miRs in IPC hearts compared with shams. (C) Alterations in expression levels of miR-1, -144, and -451 were validated by qRT–PCR (normalized to control U6). (D) Both miR-144 and miR-451 were significantly up-regulated in murine hearts upon ex vivo IPC (n = 6, *P < 0.05).
3.4 KO of the miR-144/451 cluster negates IPC efficacy on infarction size in vivo

To further determine the consequence of miR-144/451 deficiency in vivo, we subjected WT and KO hearts to in vivo IPC with six cycles of 4 min ischaemia via coronary artery occlusion and 4 min reperfusion followed by 30 min ischaemia/24 h reperfusion. The sham operation surgery consisted of 1 h open chest followed by I/R (30 min/24 h). In sham operation groups, the infarction size (ratio of infarct-to-risk region) was 25.1 ± 2.5% in WT hearts (n = 6) and was increased to 43.6 ± 6.3% in miR-144/451-KO hearts (n = 7, P < 0.001). In contrast, upon IPC, the myocardial infarction size was dramatically reduced to 5.9 ± 1.7% in WT hearts and showed no significant reduction in miR-144/451-null hearts (40.7 ± 3.4%) (Figure 3H and I). Importantly, the region at risk was not significantly different between groups under sham and IPC conditions (Figure 3J). These results are consistent with our ex vivo findings that loss of the miR-144/451 cluster not only offsets IPC efficacy, but also significantly deteriorates I/R-induced myocardial damage.

3.5 Rac-1 is a major efficacy target responsible for miR-144/451-mediated action

Currently, dissecting the mechanisms by which miRs exert their physiological effects represents the utmost challenge, as most miRs are predicted to target hundreds of genes. Nonetheless, a growing number of studies suggest that most targets of miR may have only minor effects on a specific phenotype, and the strong repression of one or a few targets might have a major impact on a specific biological process.28,29 Rasmussen et al.25 recently observed that Rac-1 was the most up-regulated gene (3.5-fold) among a cohort of 588 up-regulated genes in miR-144/451-null erythroblasts. Whether Rac-1 is a major target for miR-144/451 in the heart is unknown. Furthermore, CUGBP2 and Ywhaz (14-3-3z) are also validated targets for both miR-144 and miR-451.16,20 Interestingly, 14-3-3z has been shown to act upstream of Rac-1,30 and CUGBP2 negatively regulates the expression of COX2, a known cardioprotective protein, which may be involved in cardiac Rac-1 signalling.31,32 Put together, these data suggest that divergent targets of miR are functionally related, and Rac-1 seems to be a nodal target for the miR-144/451 cluster (Figure 4A). Hence, we first validated that both miR-144 and miR-451 directly recognized the 3′-UTR of mouse Rac-1 by the luciferase reporter assay, but miR-144 was less effective targeting Rac-1 3′-UTR than miR-451 (see Supplementary material online, Figure S2). Next, we measured protein levels of Rac-1, CUGBP2, and 14-3-3z in miR-144/451 KO hearts and WTs. Immuno-blot analysis revealed that both miR-144 and miR-451 directly recognized the 3′-UTR of mouse Rac-1 by the luciferase reporter assay, but miR-144 was less effective targeting Rac-1 3′-UTR than miR-451 (see Supplementary material online, Figure S2). Next, we measured protein levels of Rac-1, CUGBP2, and 14-3-3z in miR-144/451 KO hearts and WTs. Immuno-blot analysis revealed that both miR-144 and miR-451 directly recognized the 3′-UTR of mouse Rac-1 by the luciferase reporter assay, but miR-144 was less effective targeting Rac-1 3′-UTR than miR-451 (see Supplementary material online, Figure S2). Next, we measured protein levels of Rac-1, CUGBP2, and 14-3-3z in miR-144/451 KO hearts and WTs. Immuno-blot analysis revealed that both miR-144 and miR-451 directly recognized the 3′-UTR of mouse Rac-1 by the luciferase reporter assay, but miR-144 was less effective targeting Rac-1 3′-UTR than miR-451 (see Supplementary material online, Figure S2). 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Given that Rac-1 is known to activate the NADPH oxidase (Nox) contributing to a large amount of ROS generated in the heart,\textsuperscript{33} we next measured the content of ROS and the activity of Nox in miR-144/451-null hearts and WTs. Surprisingly, both the ROS level and the Nox activity in miR-144/451-KO hearts were similar to WT hearts under sham conditions (Figure 4D and E). This suggests that the myocardium has a compensatory homeostatic capacity in maintaining the normal redox balance. However, upon three cycles of brief I/R (5/5 min) \textit{ex vivo}, miR-144/451-null hearts revealed a 1.9-fold increase in the Nox activity, whereas WT hearts exhibited a 1.5-fold elevation (Figure 4D). Likewise, ROS levels were increased by 1.7-fold in miR-144/451-null hearts, whereas WT hearts showed an increase of only 1.2-fold (Figure 4E). Accordingly, while the LDH release was increased in both miR-144/451-KO hearts and WT hearts upon IPC, the degree of increase was more pronounced in KOs than WTs (Figure 4F). These data indicate that ablation of the miR-144/451 cluster results in an excessive generation of ROS during IPC and consequently leads to myocyte death, which contributes to the loss of IPC efficacy.

### 3.6 Inhibition of Rac-1 restores IPC-efficacy in miR-144/451-null hearts

We hypothesized that up-regulation of Rac-1 leads to increased ROS in miR-144/451-null hearts. Thus, we tested whether pre-inhibition of Rac-1 restores IPC-induced cardioprotective effects in miR-144/451-KO mice. We pre-treated mice with an Rac-1 inhibitor NSC23766 (20 mg/kg, ip injection) or saline for 30 min, followed by \textit{ex vivo} IPC with three cycles of brief I/R and subsequent 20 min ischaemia/40 min reperfusion (Figure 5A). A subset of samples were collected at 30 min treatment of NSC23766 to validate the efficacy of Rac-1 inhibition. As shown in Figure 5B and C, the Nox activity was reduced by \textasciitilde35\% and the ROS level was decreased by \textasciitilde12\% in NSC23766-treated miR-144/451-null hearts, compared with the saline treatment (n = 4, P < 0.05). As expected, pre-inhibition of Rac-1 by NSC23766 significantly improved contractile functional recovery in miR-144/451-KO hearts, as evidenced by a 74\% increase in contraction (+dP/dt) recovery (Figure 5D) and 71\% increase in relaxation (–dP/dt) recovery, relative to saline controls (Figure 5E) (n = 6, P < 0.05). Similarly, in NSC23766-pre-treated miR-144/451-null hearts, IPC-I/R-triggered release of LDH was reduced by 31\% (Figure 5F), the degree of DNA fragmentation was dampened by 43\% (Figure 5G), and the caspase-3 activity was suppressed by 16\% (Figure 5H), relative to saline-treated KO hearts (n = 6, P < 0.05). Collectively, these results further indicate a causal relationship between the miR-144/451-mediated action and their modulated target Rac-1 in the heart.

### 3.7 Effects of separate miR-144/451-knockdown on IPC-triggered cardioprotection

Our laboratory and others have recently demonstrated that the miR-144/451 cluster is controlled by GATA-1 in blood cells\textsuperscript{34} and GATA-4 in cardiomyocytes.\textsuperscript{16} While both miRs are transcribed on a single precursor RNA, the content of mature miR-451 is at least 15\% higher than miR-144 in the murine heart (see Supplementary material online, Table S1). Our \textit{in vitro} data also indicate that miR-451 exerted a greater impact on myocyte survival than miR-144.\textsuperscript{16} These observations led us to hypothesize that within the miR-144/451 cluster, miR-451 plays a pivotal role in modulating IPC-efficacy. To test this hypothesis, we administered WT mice (B6/129F1, 6 weeks old, male) with either anti-miR-144 or anti-miR-451 via three consecutive daily tail vein injections (3 \times 40 mg/kg) (Figure 6A). Mutated antisense oligonucleotides were used as controls. Three days after the last administration of antisense miRNAs, stem-loop RT–PCR showed a 59\% 2\% reduction in miR-144 expression in the anti-miR-451-treated heart tissue (Figure 6B) and a 71\% 1\% reduction in miR-451 expression in anti-miR-451-treated hearts (Figure 6C). In contrast, the mutated anti-miR control had no effect on the expression level of either miR-144 or miR-451 compared with the saline treatment (Figure 6B and C). These results indicate that injection of anti-miR efficiently down-regulates the expression of its respective miR in the heart.

In parallel, hearts from these anti-miR-treated mice were subjected to \textit{ex vivo} IPC with three cycles of 5 min I/R and a following I/R (20/40 min). We observed that knockdown of miR-451, but not miR-144, significantly impaired IPC-elicted cardioprotective effects, as evidenced by a 51\% reduction in contraction (+dP/dt) recovery (Figure 6D) and a 56\% decrease in relaxation (–dP/dt) recovery (Figure 6E) compared with saline-treated controls (n = 6, P < 0.005). These results were accompanied by an aggravation of myocardial damage, showing significant increases in the release of LDH (Figure 6F), and DNA fragmentation (Figure 6G) in anti-miR-451-treated hearts compared with controls.

In addition, oxidative stress was also determined in anti-miR-treated hearts upon IPC-I/R. We observed that the activity of Nox and ROS levels were significantly elevated by treatment with anti-miR-451, but not with anti-miR-144 or anti-miR controls,
Figure 4 Ablation of miR-144/451 activates the Rac-1-mediated oxidative stress signalling pathway. (A) 14-3-3ζ, Rac-1, and CUGBP2 are confirmed targets for both miR-144 and miR-451, and previous studies indicate that 14-3-3ζ and CUGBP2 act upstream of Rac-1. (B) Rac-1, but not 14-3-3ζ and CUGBP2, was significantly up-regulated in miR-144/451-null hearts compared with WTs. *P < 0.05 vs. WT, n = 6. (C) Protein levels of Rac-1 were significantly reduced in mouse hearts upon in vivo and ex vivo IPC, relative to shams. *P < 0.05 vs. sham, n = 6. (D) The activity of NADPH oxidase (Nox) and (E) the ROS levels were dramatically increased in IPC-miR-144/451-null hearts, compared with IPC-WTs, suggesting excessive oxidative stress, leading to elevation of LDH release (F). *P < 0.05 vs. sham; #P < 0.05 vs. IPC-WT, n = 5.
compared with saline-treated samples (Figure 6H and I) (n = 6, P < 0.005). Taken together, these data indicate that down-regulation of miR-451 is sufficient to offset IPC-mediated protective effects against later I/R challenge.

4. Discussion

In the present study, we demonstrate that loss of the miR-144/451 cluster largely negates IPC cardioprotection. Secondly, we elucidate that among potential targets, Rac-1 is a major efficacy target (or a nodal target) responsible for the miR-144/451-mediated action in the heart. Finally, our study unveils that the miR-144/451 cluster modulates the ROS production by targeting Rac-1, which offers a new regulatory mechanism for controlling myocardial oxidative stress.

It has been recognized that cellular ROS has dual functions: the physiological low level of ROS (i.e. generated by IPC) serves as a protective signal transducer in the heart; whereas excessive ROS produced under pathological conditions (i.e. myocardial I/R) are detrimental to cardiomyocytes. Accordingly, the present observations that the amount of ROS generated in miR-144/451-null hearts was increased by 42% more than WT hearts upon IPC may provide a mechanistic interpretation for the loss of IPC effects in the miR-144/451-deficient hearts. Notably, there are a number of sources (i.e. mitochondrial, xanthine oxidase, NADPH oxidase, and 5-lipoxygenase) to produce ROS, among which Rac-1 has been implicated as a critical stimulator of NADPH oxidase and ROS production in the heart. Indeed, our data have validated that Rac-1 is a bona fide target of miR-144/451. As a result, ablation of the miR-144/451 cluster significantly increased Rac-1 expression in the heart, leading to greater production of ROS than WTs. However, most predicted targets of miR-144/451 are functionally related to oxidative stress, whether other potential targets of miR-144/451 also contribute to IPC-elicited action remains to be determined in the future.

In addition, although KO/knockdown miR-144/451 in mouse hearts impairs IPC-mediated effects, we cannot exclude other miRs or molecules’ contributions to IPC-elicited protection. Actually, we have identified a cohort of 21 miRs significantly dysregulated in mouse hearts upon IPC in vivo. Notably, increased miR-21 and decreased miR-1 have been suggested to correlate with IPC in rat models. Furthermore, numerous molecules or signalling pathways in addition to miRs...
Figure 6 miR-451 plays a dominant role in the miR-144/451-mediated action. (A) A protocol for antagomiR treatment: WT mice (B6129SF2/J F2, 6 weeks old, male) received either antagomiR-144, antagomiR-451, or antagomiR control, or a comparable volume of saline (200 mL) through three consecutive daily tail vein injections (3 × 40 mg/kg body weight). Ex vivo IPC-I/R was performed at the third day after the final injection. (B and C) Stem-loop RT–PCR determined miR-144 or miR-451 expression in antagomiR-treated hearts. n = 4, *p < 0.001 vs. saline- and mutant-treated controls. (D and E) IPC-induced myocardial function recovery was impaired in antagomiR-451- but not in antagomiR-144-treated hearts; accordingly (F and G), myocardial injury (necrosis: LDH release and apoptosis: DNA fragmentation) was increased in antagomiR-451- but not in antagomiR-144-treated hearts. (H and I) Oxidative stress (Nox activity and ROS levels) was significantly activated in antagomiR-451- but not in antagomiR-144-treated hearts. *p < 0.05 vs. antagomiR control, n = 6.
have been elucidated to modulate IPC.\textsuperscript{1–3} For example, AMPK-controlled sarcolemmal KATP channels are proposed to be end effectors of preconditioning;\textsuperscript{3,5} PKD1 regulating mitochondrial membrane potential is essential for IPC of the myocardium;\textsuperscript{3} the JAK-STAT pathway, PI3K-Akt, COX2, and autophagy also play critical roles in IPC.\textsuperscript{36–39} Therefore, it may be impossible that intervention of one single pathway or mediator can fully simulate IPC effects in vivo. This may explain why the translation of IPC-mimetics into the clinical setting is disappointing. Nonetheless, the data present in this study may help us better understand the mechanisms underlying IPC and provide a molecular basis to development of novel strategies for the treatment of ischaemic heart disease.

There are several limitations in our study. First, we selected six cycles of 4 min ischaemia/4 min reperfusion as in vivo IPC protocol (a total duration of \(\sim 60\) min). This protocol could result in alterations of some protein levels, especially for miR-controlled proteins (i.e. Rac-1). However, such alterations may not occur in the heart upon less number of \(\Gamma\)R cycles. Furthermore, miR profiles identified in the present study may be different from those hearts upon the short duration of the IPC protocol. Nevertheless, a recent study by Lee et al.\textsuperscript{40} show that miR-144/451 are up-regulated by three-fold in murine brains at 3 h after 15 min ischaemia preconditioning, consistent with our present findings. Secondly, a chronic KO mouse model used in this study may yield compensatory effects that influence the proper assessment of IPC mechanisms. While we applied an Rac-1 inhibitor in this study may yield compensatory effects that influence the proper assessment of IPC mechanisms. While we applied an Rac-1 inhibitor in this study may yield compensatory effects that influence the proper assessment of IPC mechanisms.

In summary, our findings demonstrate that the miR-144/451 cluster may play a fundamental role in mediating IPC. Loss of the miR-144/451 cluster causes excessive generation of ROS during IPC via activation of Rac-1-mediated oxidative stress signalling, which results in myocardial death and thereby negates IPC-elicited cardioprotection. In addition, we identified that miR-451, but not miR-144, within the miR-144/451 cluster, plays a dominant role in this cluster-mediated phenotype. Future studies using a single miR (\(-144\) or \(-451\)) loss-of-function or gain-of-function model will be helpful in identifying the exact role for both partners within the miR-144/451 cluster in myocardial ischaemic tolerance and damage.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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