Rapamycin suppresses hypoxia/reoxygenation-induced islet injury by up-regulation of miR-21 via PI3K/Akt signalling pathway

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Abstract

Objectives: Increasing evidences indicate that microRNAs may play a critical role in the regulation of hypoxia/reoxygenation (H/R) injury, and their expression is associated with mTORC activity. We propose that rapamycin modulates H/R-induced islets injury by regulating microRNA expression.

Materials and methods: We investigated whether rapamycin treatment could alter the expression profile of miRNAs in islets. Furthermore, we assessed the islet apoptosis and function after H/R or syngeneic islet transplantation.

Results: We found that rapamycin treatment significantly decreased H/R-induced islet apoptosis, and improved islet function *in vivo* and *in vitro*, and that miR-21 gene transcription is controlled by rapamycin. When the PI3k/Akt signalling pathways was blocked by wortmannin, the up-regulative effects of rapamycin on miR-21 expression were inhibited *in vitro*. Furthermore, our study clearly demonstrates that miR-21 is essential for the rapamycin-mediated protection islets against H/R injury.

Discussion: Our findings indicate that up-regulation of miR-21 function in islets by treatment with rapamycin or overexpression of the miR-21 could represent a potential new therapy for the treatment of H/R injury.

Conclusion: The results of this study clearly suggest that rapamycin exerts its inhibitory effects on islets H/R injury by inducing miR-21 expression via PI3K/Akt.

1 | INTRODUCTION

Islets are exceptionally susceptible to ischaemia/reperfusion injury, an increased incidence of primary graft non-functionality, and β -cell death during a transplant procedure.¹ Hypoxia/reoxygenation (H/R) is the leading cause of β -cell death during islet preparation, implantation and revascularization, with the highest percentage of islet graft loss and dysfunction occurring just days after transplantation.^{1–3} The reduction and the repair of the ischaemia/reperfusion injury at its early stage of transplantation has become the key link that could prolong the graft function and survival and improve the success rate of clinical islet transplantation.⁴

Rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR), has been widely used as an immunosuppressive agent for the

prophylaxis of allograft rejection.⁵ The mTOR is an evolutionally conserved serine/threonine kinase that integrates cellular and environmental cues, including growth factors, nutrients, cellular energy, as well as immune stimulations, Toll-like-receptors, and cytokine receptors,⁶ to regulate cell growth, metabolism and apoptosis. In contrast to its immune suppressive effect, mTOR inhibition was found recently that the beneficial early microcirculation^{7,8} and anti-apoptosis activity⁸ might contribute to its therapeutic potential during ischaemia/reperfusion injury. Recently, several studies have demonstrated that rapamycin protects against myocardial,⁹ hepatic,¹⁰ pancreatic,⁸ encephalic⁷ and renal¹¹ apoptosis/necrosis during ischaemia/reperfusion.

MicroRNAs (miRNAs), a novel class of endogenous, non-coding, single-stranded RNAs, have emerged as a group of important regulators via degradation or translational inhibition of their target mRNAs.^{12,13}

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Increasing evidences indicate that miRNAs are involved in the regulation of H/R injury.^{13–15} For example, miR-21 protected cells against hypoxia/reoxygenation injury through its target programmed cell death 4 (PDCD4).¹⁴ miR-320 was involved in the regulation of H/R injury and knockdown of endogenous miR-320 provided protection against H/R-induced cells apoptosis *via* antithetical regulation of Hsp20.¹⁵

Emerging evidence suggests that miRNA expression is associated with mTORC activity.¹⁶ Although the role of miRNAs in the regulation of H/R injury has been documented, few studies have reported the cellular consequences of targeted re-expression of specific miRNAs. Therefore, we hypothesized that rapamycin might inhibit the H/R-induced islets injury *via* regulation of miRNA biogenesis. In this study, we investigated whether rapamycin treatment could alter the expression profile of miRNAs in islets, and we assessed the cellular consequences of this treatment. Our data demonstrate that rapamycin exerts its effects on the H/R-induced islets injury by inducing miR-21 expression.

2 | MATERIALS AND METHODS

2.1 | Animals

Male C57BL/6 mice were purchased from Dossy Biological Technology Co., Ltd (Chengdu, China). For experiments, adult mice (8-week old, weighing 22 ± 3 g) were kept in ventilated cages. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of West China Hospital, Sichuan University. All surgery was performed under sodium pentobarbital anaesthesia, and all efforts were made to minimize suffering.

2.2 | Islet isolation

Male C57BL/6 islets were isolated using established methods.¹⁷ Briefly, the pancreas was distended with 2.5 mL of a 0.5 mg/mL collagenase XI (Sigma, St. Louis, MO, USA) solution and digested for 17 minutes at 37°C in a water bath. The islets were purified by density gradient centrifugation. The islets were collected and washed with Hank's solution containing 1% bovine serum albumin (BSA; Roche, Basel, Switzerland), then stained with dithizone (DTZ; Sigma) and counted under a microscope.

2.3 | Transfection of miRNA mimics and specific anti-miRNAs

Islets were cultured in six-well plates and transfected with synthetic miR-21 (miR-21 mimic) or miRNA scrambled controls (containing a sequence from GFP) at a final concentration of 25 nM using Oligofectamine (Invitrogen, Carlsbad, CA, USA) as described in Anping et al.¹⁸ Islets were transfected with anti-miR21 (miRNA inhibitors) or an anti-miRNA control at a final concentration of 250 nM using the

DharmaFECT3 transfection reagent (Dharmacon).¹⁸ Chemically synthesized miRNA-21 inhibitors, mimics and negative controls were obtained from Dharmacon or ABM.

2.4 | Hypoxia-reoxygenation culture

Islets were cultured under hypoxic conditions (1% O₂, 5% CO₂ and 94% N₂) for 8 hours in a six-well plate with or without rapamycin (Rapamune; Wyeth Europe, Taplow, UK) or/and the PI3K/AKT phosphorylation inhibitor wortmannin (10 µM; Sigma). Next, they were transferred to ordinary conditions and cultured for a further 16 hours. The plates were analysed immediately.

2.5 | Determination of islet cell apoptosis

The islet cell apoptosis was determined using established methods.¹⁹ Briefly, the dissociated islet cells were incubated with propidium iodide (PI) and annexin V-FITC (annexin V). The dual-parameter flow cytometry was used to determine the number of apoptotic cells (annexin V/PI double-positive cells). In addition, the activity of caspase-3 in islets was examined by Western blot analysis.

2.6 | Glucose-stimulated insulin secretion (GSIS) and insulin content measurement

Handpicked islets were subjected to a static glucose challenge in Krebs-Ringer bicarbonate buffer (KRBB) (pH 7.35) containing 10 mmol/L HEPES and 0.5% BSA (Sigma). After conditioning, the islets were incubated in KRBB containing low (2.8 mmol/L) and high (20 mmol/L) glucose concentrations for 1 hour. At the end of the glucose challenge, the insulin levels were measured by an enzyme-linked immunosorbent assay (ELISA) (Linco Research, St Charles, Missouri, USA). The stimulation index (SI) for each experimental condition was calculated as the ratio of insulin released in high versus low glucose concentrations.

2.7 | MicroRNA microarray

MicroRNAs were identified by miRNA microarray analysis (Agilent Technologies, Santa Clara, CA, USA). Standard methods were used to extract, label and hybridize triplicate samples. Microarray results were analysed using Partek. Briefly, islets, cultured in RPMI-1640 medium (Gibco, Invitrogen Grand Island, NY, USA) containing 10% FBS, were treated with rapamycin (10 nM, 24 hours) or vehicle control. Isolated RNA was applied to a assays 350 miRNAs by ligating two complementary tagged DNA probes to each target miRNA and hybridizing the complexes to a nitrocellulose membrane. Each target was assayed in duplicate and normalized to the expression of RNU48.

2.8 | Quantitative real-time PCR

Quantitative real-time PCR were performed using established methods.¹⁸ Briefly, RNA was purified using RNeasy (Qiagen, Hilden,

Germany) or miRVANA (Ambion Austin, TX, USA) kits with DNase digestion on RNeasy columns. Complementary DNA (cDNA) was generated using the High-Capacity cDNA Reverse Transcription Kit (Roche). An iQ5 PCR machine (Bio-Rad Hercules, CA, USA) was used to obtain quantitative measurements of the changes in gene expression levels.

2.9 | Western blot analyses

Western blot analyses were performed as described previously.²⁰ Briefly, total cell lysates were separated on SDS-PAGE gels, transferred to PVDF membranes (Millipore Bedford, MA, USA), immunoblotted with antibodies, and visualized using an enhanced chemiluminescence

detection system (Amersham Biosciences, Piscataway, NJ, USA). Protein expression was quantified by densitometry using ImageJ gel analysis software (rsbweb.nih.gov/ij). All values were normalized to GAPDH.

2.10 | Syngeneic islet transplantation

Eight-week-old C57BL/6 male mice were used as donors and recipients. Syngeneic recipients were rendered diabetic by intravenous administration of 200 mg/kg streptozotocin (STZ; Sigma). The animals were declared diabetic after two consecutive blood glucose measurements >250 mg/dL. Each recipient was transplanted with suboptimal (100 islets/recipient) or marginal mass (200 islets/recipient) syngeneic

FIGURE 1 Rapamycin leads to decreased islet apoptosis and improves islet function and survival in vitro and in vivo. The islets were treated with 10 μ M rapamycin or vehicle in serum-free media for 24 h under normoxic conditions or 24 h under hypoxic-reoxygenation conditions (n=5-6). The relative change effect (A) of rapamycin on the expression of caspase-3 (B) was quantified by Western blot analysis (B). The apoptotic rate (C) of islet cells (annexin V/PI double-positive cells), representative pictures (D) of islet apoptosis by FCM assay, and the function of GSIS (E) of islets. All treatments in this figure were carried out in triplicate, * $P < .05$. Suboptimal and marginal mass islet grafts were transplanted under the kidney capsule in syngeneic diabetic recipients. The probability (F) to reach euglycaemia (<200 mg/dL) within 14 d in suboptimal syngeneic islets for transplantation (100 islets/recipient) (n=15), and IPGTT profiles (G) at 7 d post-transplant in marginal mass (200 islets/recipient) syngeneic islets (n=5-6). Transplant recipients were given rapamycin or vehicle administered via gavage (1 mg/kg body) once a day after transplantation

islets. These syngeneic islets were inserted under the kidney capsule as described in Xiaojiong et al.¹⁷ Transplant recipients were given rapamycin or vehicle administered *via* gavage (1 mg/kg body) once a day after transplantation. Recipients of suboptimal mass syngeneic islets had their blood glucose monitored approximately every other day for 14 days. Recipients of marginal mass syngeneic islets were subjected to a fasting intraperitoneal glucose tolerance test (IPGTT) on post-operative day 7.

2.11 | Statistical analyses

All data are expressed as the mean $\pm$ SD. The differences between mean values were determined by the Student's *t*-test for singular comparisons and by the one-way ANOVA for multiple comparisons. The area under the curve was determined using the trapezoidal rule. Kaplan-Meier survival plots were analysed by the log-rank test. All statistical analyses were performed with SPSS using *P* < .05 to achieve significance.

3 | RESULTS

3.1 | Rapamycin protects islets from H/R injury

To determine the potential protective effects of rapamycin on H/R-induced islets injury, the islets were cultured with rapamycin (10 μ M) or vehicle in H/R for 24 hours, islet apoptosis was determined by a flow cytometry assay, the activity of caspase-3 was examined by Western blot analysis, and the islets function was determined by GSIS. The results showed that treatment of islets with rapamycin markedly suppressed H/R-induced cleaved caspase-3 expression (Figure 1A,B). Moreover, flow cytometry revealed a dramatic reduction of H/R-induced apoptosis in islets treated with rapamycin (Figure 1C,D). Consistent with a suppressive effect of rapamycin on H/R-induced apoptosis, GSIS revealed a dramatic elevation of H/R-induced decreasing the islets function in islets treated with rapamycin compared to vehicle control (Figure 1E). Next, we investigated whether rapamycin could prevent the early loss of transplanted syngeneic islets. We transplanted suboptimal or marginal mass islets into diabetic mice to detect islet survival and function post-transplant using blood glucose monitoring and IPGTT. The ability to reach euglycaemia (<200 mg/dL) within 14 days in suboptimal mass islet transplantation was as follows: 9 of 15 mice (60%) for the rapamycin group and 4 of 15 mice (26.7%) for the vehicle control group (Figure 1F). Rapamycin-treated animals exhibited a decrease in peak blood glucose values compared with control animals during IPGTT (Figure 1G). When the area under the curve (AUC) was calculated, the rapamycin-treated animals had a significantly lower AUC. These results suggest that rapamycin protects islets from H/R injury.

3.2 | Rapamycin induces miR-21 expression in islets

To determine whether rapamycin regulates miRNA, we performed the miRNA microarray. Islets, cultured in RPMI-1640 containing 10%

FIGURE 2 Rapamycin induces miR-21 expression in islets. Islets were treated with rapamycin (10 nM, 24 h) or vehicle control, then rapamycin-regulated miRNA in islets cells identified by miRNA microarray (fold change >2). A, Nine miRNA were induced more than 2-fold, with miRs-21, 22, 24, 26a, 29b, 31, 181a, 199b and 221 being most highly increased, and two miRs-210 and 375 were down-regulated. The islets were treated with 10 nM rapamycin or vehicle in serum-free media for 24 h under normoxic conditions or 24 h under hypoxic-reoxygenation conditions (*n*=5-6). B, Real-time PCR was used to determine the relative miRNA expression levels in islets treated with vehicle or rapamycin. C, Time-course expression of the relative expression of miR-21 transcripts (Pri-miR-21), Pre-miR-221 or mature miR-221 normalized to GAPDH (for Pri-miR-221 or Pre-miR-221) or U6 small nuclear RNA (for mature miR-21) in islets treated with vehicle or rapamycin. All treatments in this figure were carried out in triplicate, **P* < .05

FBS, were treated with rapamycin (10 nM, 24 hours) or vehicle control. Nine miRNA were induced more than 2-fold, with miRs-21, 22, 24, 26a, 29b, 31, 181a, 199b and 221 being most highly increased

FIGURE 3 miR-21 is critical for rapamycin-mediated protection islets against H/R injury. The islets were transfected with the miR-21 mimic or a negative control mimic and subsequently cultured under hypoxia-reoxygenation for 24 h, islet apoptosis was determined by a flow cytometry assay (A), the activity of caspase-3 was examined by Western blot analysis (B), and the islets function was determined by glucose-stimulated insulin secretion (GSIS) (C). The islets were transfected with anti-miR-21 or a negative control and subsequently cultured with 10 μ M rapamycin under hypoxia-reoxygenation for 24 h. The expression of miR-21 was determined by real-time PCR (D). Islet apoptosis was determined by a flow cytometry assay (E), the activity of caspase-3 was examined by Western blot analysis (F), and the islets function was determined by GSIS (G). All treatments in this figure were carried out in triplicate, * $P < .05$

(Figure 2A). Two miRs-210 and 375 were down-regulated (Figure 2A). These miRNAs were further analysed by real-time PCR to determine whether treatment of islets with rapamycin for 24 hours could alter the expression of miRNA compared with untreated cells under ordinary conditions or H/R culture, which was expected to give more meaningful results. Both under ordinary and H/R conditions, miRNA 21, 24, 31 and 221 were induced by rapamycin 1.5-fold, and miR-210 was decreased 1.5-fold, consistent with the array results, with miR-21 being the most highly induced (Figure 2B). A time-course of the expression of miR-21 was examined after rapamycin treatment in islets. miR-21 was induced 2.6-fold after 12 hours of treatment with rapamycin and gradually decreased by 18 hours (Figure 2C). Importantly, robust induction of both the primary transcripts (pri-miR-21) and the intermediate product (pre-miR-21) of miR-21 was observed as early as 6 hours after rapamycin treatment, suggesting that miR-21 is likely to be transcriptionally induced by rapamycin (Figure 2C).

3.3 | miRNA-21 is essential for the rapamycin-mediated protection islets against ischaemia/reperfusion injury

We transfected islets with miR-21 mimic or a control mimic and examined the islet apoptosis and function after hypoxia-reoxygenation for 24 hours. Similar to rapamycin treatment, exogenous expression of miR-21 significantly suppressed islet apoptosis (Figure 3A),

down-regulated the activity of caspase-3 (Figure 3B), and elevated the islets function (Figure 3C). Next, RNA oligonucleotides targeted against miR-21 (anti-miR-21) were transfected into islets to inhibit the function of miR-21. Transfection of anti-miR-21 reduced both the basal and rapamycin-induced expression of miR-21 (Figure 3D). When miR-21 function was blocked by anti-miR-21, rapamycin did not decrease islet apoptosis (Figure 3E) or the expression levels of caspase-3 (Figure 3F), and not improve the islets function (Figure 3G) after H/R for 24 hours. Conversely, in islets transfected with the miR-21 mimic alone, significant inhibition of the islet apoptosis and amelioration of the islets function that was comparable to that observed in untransfected cells treated with rapamycin was observed after H/R for 24 hours (Figures 1A-E and 3A-C). These results indicate that rapamycin-dependent induction of miR-21 is essential for protecting H/R-induced islets injury.

3.4 | Rapamycin induces miR-21 expression through PI3K/Akt signalling

The mammalian (mechanical) target of rapamycin (mTOR) is an evolutionally conserved serine/threonine kinase, which regulates cell proliferation, metabolism and apoptosis. To determine whether rapamycin regulates miR-21 expression via a PI3K/Akt-dependent mechanism, the islets were treated with 10 mM rapamycin and the specific inhibitors of PI3K/Akt (10 μ M, Wortmannin) or vehicle control for H/R

FIGURE 4 Rapamycin-induced miR-21 expression through activation of PI3K/Akt signalling pathways. The islets were pre-treated with Wortmannin (10 μ M) for 1 h, then cultured with 10 μ M rapamycin under hypoxia-reoxygenation for 24 h, to determine miR-21 expression (A), islet function (B) and apoptosis (C), and caspase-3 (E), p-Akt (F) and p-S6 (G) expression (D). All treatments in this figure were carried out in triplicate, * $P < .05$

24 hours, to determine miR-21 expression, and islets function and apoptosis. The up-regulation effect of miR-21 on rapamycin-treated islet was greatly inhibited by Wortmannin treatment (Figure 4A); in the meantime, the protective effect of rapamycin on H/R-induced islets injury was counteracted by Wortmannin (Figure 4B-D). Additionally, we confirmed that Wortmannin inhibited Akt phosphorylation at the increased phosphorylation of ribosomal protein S6, a downstream target of mTORC, when islets were treated with rapamycin and Wortmannin. These results indicate that rapamycin may protect the islet from H/R-induced injury through up-regulation of miR-21 by mTORC/PI3K/Akt signalling pathways.

4 | DISCUSSION

A growing body of literature strongly suggests that mTOR may function as a key player in the cell growth, metabolism and apoptosis by integrating cellular and environmental cues, including growth factors, nutrients, cellular energy as well as immune stimulations, Toll-like receptors and cytokine receptors.⁶ In contrast to its immune suppressive effect, mTOR inhibition was found recently that the beneficial early microcirculation and anti-apoptosis activity might contribute to its therapeutic potential during H/R injury.^{7,8}

In recent years, several studies have demonstrated that rapamycin protects against myocardial,⁹ hepatic,¹⁰ pancreatic,⁸ encephalic⁷ and renal¹¹ apoptosis/necrosis during ischaemia and reperfusion. The organ protective effects of rapamycin were shown in kidneys and pancreases by improving microcirculation post ischaemia/reperfusion.¹⁰ Direct cytoprotective effects of rapamycin were demonstrated in cardiac ischaemia/reperfusion models and in vitro H/R injury model.⁸ More recently, rapamycin regulation of autophagy, protected tissue parenchymal cells, became the focus of its role in H/R injury.^{9,11}

Although rapamycin is considered to be important for the regulation of H/R injury, the regulatory mechanisms of H/R injury remain to be elucidated. Based on these important biological characteristics of rapamycin, we hypothesize that the inhibition of H/R injury progression via rapamycin is a potential treatment in islet transplantation procedures to decrease the incidence of islet injury. To examine the effects of rapamycin on the resistance to H/R-induced islets injury in vivo and in vitro, we induced islet apoptosis and injury by H/R in the presence or absence of rapamycin, which was incubated in vitro, to assess the insulin secretion capability of islets. Additionally, we performed kidney suboptimal or marginal mass syngeneic islet transplants in diabetic recipients, in the presence or absence of rapamycin, to assess transplantation efficiency and islet function. Our current study

clearly demonstrates that rapamycin treatment inhibits H/R-induced islets injury by up-regulating miRNA-21 expression.

Furthermore, our data demonstrate that miR-21 gene transcription is regulated by rapamycin. The rapamycin pathway is a well-known modulator of the expression of various protein-coding genes. To address this, we performed screens to determine whether rapamycin impacts miRNA levels in islets. The screens revealed a complex and previously unrecognized network of rapamycin-regulated miRNA in islets. Of the miRNA genes found to be regulated by rapamycin, miR-21 was the most significantly up-regulated. Using qRT-PCR to confirm our results, we demonstrate that miR-21 is significantly induced by rapamycin. Specifically, we found that miR-21 was induced 2.6-fold after 12 hours of treatment with rapamycin, and both the primary transcripts and intermediate products of miR-21 were observed as early as 6 hours after rapamycin treatment, suggesting that miR-21 expression is activated by rapamycin.

Currently, the PI3 kinase is known to control mTORC activities,⁶ and rapamycin protected livers from H/R via mTORC-Akt activation mechanisms.⁹ We further analysed rapamycin's effect on miR-21, demonstrating that miR-21 is induced by rapamycin via an PI3K/Akt-dependent mechanism. Our study suggests that treatment with rapamycin increased p-Akt and p-S6 expression levels in association with miR-21. Our data clearly showed that suppressing the PI3K/Akt signalling pathways by Wortmannin inhibited the caspase-3 and p-Akt expression in islets. Moreover, when the PI3K/Akt signalling pathways was blocked, the protective effects of rapamycin on H/R-induced islets injury were inhibited by Wortmannin. These results indicate that rapamycin may protect the islet from H/R-induced injury through up-regulation of miR-21 via PI3K/Akt signalling pathways.

miRNAs are endogenous, small RNA molecules that suppress expression of targeted mRNA.¹⁴ miR-21, one of the most extensively studied miRNAs, is importantly involved in processes relating to H/R injury.^{14,21} Up-regulation of miR-21 contributes to the protection of kidneys from subsequent H/R injury;²² however, knocking down miR-21 at the time of renal H/R injury has no effect on the H/R injury.^{23,24} miR-21 has also been reported to be involved with the protection of ischaemia/reperfusion injury of cardiac²⁵ and brain;²⁶ however, in some cases, it is unclear if that regulation is beneficial in islets H/R injury. We found that exogenous expression of miR-21 significantly suppressed the islet apoptosis and reduced the activity of caspase-3 after H/R for 24 hours. In addition, exogenous miR-21 significantly improves the islet function after H/R. When the basal and rapamycin-induced expression of miR-21 was inhibited by the transfection of anti-miR-21, we found that rapamycin did not decrease islet apoptosis and improve the islets function after H/R. These results confirm that miR-21 plays an essential role in the rapamycin-mediated protection of islets H/R injury. Previous studies have shown that miR-21 protect hypoxia/reoxygenation-induced cell apoptosis by its target gene, programmed cell death 4 (PDCD4)¹⁴ and prolyl hydroxylase domain protein 2 (PHD2).²⁷ But, few studies indicate the mechanism of miR-21 inhibiting islet apoptosis. Our subsequent research will to further explore the mechanism of miR-21

inhibiting islet apoptosis and confirmed the target of miR-21 on H/R-induced islets apoptosis.

The results of this study clearly suggest that rapamycin exerts its inhibitory effects on islets H/R injury by inducing miR-21 expression via PI3K/Akt. Our findings suggest that the up-regulation of miR-21 function in islets, either by treatment with rapamycin or by overexpression of the miR-21, represents a potential new therapy for the treatment of H/R injury of the mTORC-Akt pathway.

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CONFLICT OF INTEREST

No potential conflicts of interest relevant to this article were reported.

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