·基础研究·

Repression of SIRT5 is Involved in RIP140-mediated Metabolic Dysregulation in Cardiomyocytes

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Abstract: 【Objective】 To investigate the effect of desuccinylase Sirtuin5 (SIRT5) on receptor-interacting protein 140 (RIP140)-mediated metabolic dysfunction in cardiomyocytes. 【Methods】 RIP140 was overexpressed by Adenovirus infection and SIRT5 was overexpressed by plasmid transfection. RIP140 and SIRT5 were knocked down by siRNA interference. The expression of RIP140 and SIRT5 were measured by qRT-PCR and western blot. The transcription levels of mitochondrial DNA-encoded genes were detected by qRT-PCR. Mitochondrial membrane potential was detected by tetramethylrhodamine ethyl ester (TMRE) fluorescence anlysis. Cellular oxygen consumption and ATP production were investigated by assay kits. All data are from at least three independent experiments. 【Results】 RIP140 overexpression significantly attenuated SIRT5 expression (P<0.05), whereas knockdown of endogenous RIP140 elevated SIRT5 expression (P<0.05) in cardiomyocytes. Superabundant RIP140 also induced hypersuccinylation of mitochondrial proteins, suggesting RIP140 could repress the desuccinylase activity of SIRT5. Moreover, SIRT5 overexpression reversed RIP140-mediated mitochondrial dysfunction and energy metabolic impairment, such as repression of mitochondrial DNA-encoded genes (P<0.05), decrease of mitochondrial membrane potential (P<0.05), as well as reduction of cellular oxygen consumption (P<0.05) and ATP production (P<0.05). Furthermore, the regulation of RIP140 on SIRT5 was dependent on the peroxisome proliferator-activated receptor α (PPARα) in cardiomyocytes. 【Conclusion】 RIP140 induces mitochondrial dysfunction and metabolic impairment through repression of SIRT5 in cardiomyocytes.

Key words: SIRT5; RIP140; mitochondrial metabolism; cardiac metabolic dysregulation.

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RIP140通过抑制SIRT5表达介导心肌细胞能量代谢紊乱

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摘 要:【目的】探讨去琥珀酰化酶Sirtuin5(SIRT5)对受体相互作用蛋白140(RIP140)诱导的心肌线粒体能量代谢紊乱的影响。【方法】利用腺病毒感染心肌细胞诱导RIP140过表达,利用质粒转染诱导SIRT5基因过表达,siRNA干扰敲低RIP140、SIRT5表达;qRT-PCR和Western blot检测SIRT5、RIP140的表达变化;qRT-PCR检测线粒体编码基因的表达情况,TMRE染色法评估线粒体膜电位,试剂盒检测细胞耗氧和ATP生成情况。所有数据均来自至少3次的独立实验。【结果】过表达RIP140明显抑制SIRT5的表达(P<0.05);敲低内源性RIP140能增加SIRT5的表达(P<0.05)。过表达RIP140亦能诱导

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线粒体蛋白高度琥珀酰化，抑制SIRT5的去琥珀酰化酶活性。除此，RIP140能诱导线粒体功能紊乱和能量代谢损伤作用，表现为线粒体编码基因的表达抑制（$P<0.05$），线粒体膜电位降低（$P<0.05$），细胞耗氧和ATP合成减少（$P<0.05$），而过表达SIRT5能抑制RIP140介导的上述能量代谢损伤（$P<0.05$）。此外，SIRT5受RIP140的负性调控作用依赖于过氧化物增殖体激活受体α（PPARα）。【结论】RIP140通过抑制SIRT5诱导心肌细胞线粒体功能紊乱和能量代谢损伤。

关键词：SIRT5；RIP140；线粒体代谢；心肌代谢紊乱

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The heart is a highly energy-consuming organ, requiring a continuous supply of ATP synthesis to maintain its contractile function [1]. The majority of ATP is produced by the mitochondria in eukaryotic cells through fatty acid oxidation and oxidative phosphorylation [2]. In recent years, mitochondria are well-recognized as a vital player in cardiovascular diseases such as myocardial infarction and cardiomyopathies [2]. The repression of mitochondrial biogenesis including the damage or deletion of mitochondrial DNA (mtDNA), as well as the alternations of mitochondrial structure and function, can lead to deficiency of ATP generation during heart failure [3]. The sirtuins (SIRT) is an evolutionarily conserved family of NAD^+-dependent enzymes involved in the dynamic regulation of cellular physiology, especially the maintenance of metabolic homeostasis [4]. Among the seven sirtuins (SIRT1-7), SIRT3-5 are located in the mitochondria and are mainly involved in regulating energy metabolism [5]. SIRT4 is reported to exacerbate cardiac hypertrophy and cardiac dysfunction in response to Ang II [6]. SIRT3 is considered to be the major deacetylase in the mitochondria, since SIRT3-deficient mice exhibit significant mitochondrial protein hyperacetylation in mitochondrion-rich tissues, such as liver and heart [7]. Apart from SIRT3, SIRT5 also plays a positive role in regulating cardiomyocyte metabolism and function [8]. SIRT5, predominantly expressed in heart, brain, liver and kidney, has a strong desuccinylationactivity but a weak deacetylating activity [8]. It was reported that protein lysine succinylation was predominantly accumulated in the heart of SIRT5 KO mice [8], and that knockout of SIRT5 exacerbated ischemia/reperfusion injury [9]. RIP140 acts as a key upstream regulator of energy expenditure, suppressing the expression of gene clusters involved in substrates metabolism [10]. In the heart, RIP140 impairs mitochondrial structure and biogenesis, and finally weakens mitochondrial oxygen consumption and ATP synthesis [11, 12]. Though the effect of RIP140 on cardiac metabolic regulation is well established, the regulatory mechanisms of RIP140 are poorly defined. Recent research demonstrated that SIRT5 is regulated by PGC-1α [13], which shares lots of common downstream targets with RIP140 [11]. Whether SIRT5 could inhibited by RIP140 opposing PGC-1α in cardiomyocytes deserves further investigation. In this study, we found the expression and desuccinylase activity of SIRT5 were repressed by RIP140 in neonatal rat cardiomyocytes. Furthermore, suppression of SIRT5 participated in RIP140-induced mitochondrial dysfunction. These findings point out that SIRT5 is a novel target of RIP140 that controls cardiac mitochondrial metabolism.

1　Materials and methods

1.1　Cell culture

Primary culture of neonatal rat cardiomyocytes (NRCM) were isolated from the hearts of 1 to 3-day-old Sprague-Dawley (SD) rats (SPF grade, from the Experimental Animal Center of Sun Yat-sen University) and cultured in DMEM with 10% fetal bovine serum and 0.1 mmol/L bromodeoxyuridine at 37 ℃ in a humidified atmosphere (5% CO_2, 95% air) as previously described [12].

1.2　Adenovirus infection

Adenovirus expressing RIP140 (Ad-RIP140, encoding rat full-length sequence of RIP140) and control adenovirus (Ad-GFP) were performed with pAdEasy system, as previously described [11]. NRCM

were exposed to adenovirus at the infection of MOI 60 for 36 h or 48 h before they were harvested for RNA or protein extracts, respectively.

1.3 Plasmid transfection and siRNA interference

Plasmid of SIRT5, as well as siRNA of SIRT5 and PPARα were purchased from Genepharma (China).NRCM were transiently transfected with plasmid (2 mg/L) or siRNA (100 nmol/L) using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. The plasmid of SIRT5 encodes the rat full-length sequence of SIRT5. The sequences of siRNAs were shown in Table 1.

1.4 Quantitative real-time PCR

Total RNA was extracted in Trizol reagent (Invitrogen, USA) and 1 μg was reverse transcribed using Thermo RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The quantitative PCR reactions were performed with SYBR Green qPCR kit (Toyobo, Japan). The RNA levels of target genes were normalized against the β-actin control levels. The primer sequences were presented in Table 2.

Table 1 Oligo sequences used for RNA interference

Target gene	RNA Oligo Sequence
SIRT5	Sense,5′-CCAACAGAUUCAGGUUUCATT-3′;Antisense,5′- UGAAACCUGAAUCUGUUGGTT-3′
RIP140	Sense,5′-GCCGUAGAUAAUGCCAAUATT-3′;Antisense,5′- UAUUGGCAUUAUCUACGGCTT-3′
PPARα	Sense,5′-GCCUGGCCUUCUAAACAUATT-3′;Antisense,5′- UAUGUUUAGAAGGCCAGGCTT-3′
Negative Control	Sense,5′-UUCUCCGAACGUGUCACGUTT-3′;Antisense,5′- ACGUGACACGUUCGGAGAATT-3′

Table 2 Primer sequences used for real-time PCR

Target gene	Sequence
SIRT3	Forward: 5′- AAGACATACGGGCTGACG-3′,Reverse:5′- GATCTGCCAAGGCGAAAT-3′
SIRT4	Forward: 5′- TTGATTTCATCCGCAGTG-3′,Reverse:5′- CCCAAGTTTCTCCCAGTT-3′
SIRT5	Forward: 5′- AACGCAAAGCACATAGTCAT-3′,Reverse:5′- AAGCAAAGGCCAGAGGAGT-3′
RIP140	Forward: 5′- GCCACAGTCAAGCAAACTGG-3′,Reverse:5′- AGGAACACCGCACATTGGAT-3′
Cyt b	Forward : 5′- GCAGCTTAACATTCCGCCCAATCA-3′,Reverse: 5′- TGTTCTACTGGTTGGCCTCCGATT-3′
ND-1	Forward : 5′- AAGCGGCTCCTTCTCCCTACAAAT-3′,Reverse: 5′- GAAGGGAGCTCGATTTGTTTCTGC-3′
mt-co1	Forward : 5′- AAGGTTTGGTCCTGGCCTTA-3′,Reverse: 5′- GCAAGGCGTCTTGAGCTAT-3′
β-actin	Forward: 5′- TCGTGCGTGACATTAAAGAG-3′,Reverse:5′- ATTGCCGATAGTGATGACCT-3′

1.5 Western blot analysis

Total protein extracts were prepared as previously described[12]. Mitochondrial extracts were prepared using the Cytoplasmic and Mitochondrial Protein Extraction Kit (Sangon Biotech, China). 50 μg of total protein extracts (or 15ug of mitochondrial protein extracts) were separated by electrophoresis on SDS-polyacrylamide gels, transferred to PVDF membranes (Millipore, USA), incubated with primary antibodies, followed by appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies and later detected by the LAS4000 imager (GE Healthcare, USA). The intensity of blots was quantified by NIH Image J software. Rabbit polyclonal antibodies against RIP140 (1∶800, Abcam), SIRT5 (1∶2500, Abcam), PPARα (1∶1000, Abcam), and succinyl-lysine (1∶1000, PTM Biolab) were used as primary antibodies. Mouse monoclonal antibodies against α-Tubulin (1∶5000, Sigma) and COX-IV (1∶1000, Cell Signaling Technology) served as loading control

of the whole cell lysis and mitochondria lysis, respectively.

1.6 Measurement of mitochondrial membrane potential

After incubation in DMEM with 10 nmol/L tetramethylrhodamin ethyl esters (TMRE, Invitrogen) at 37 ℃ for 30 min, NRCM in 48-well plate were washed with PBS and incubated in fresh DMEM. The images from randomly selected fields (60 for each group) were captured by High Content Screening system (Thermo Fisher Scientific, USA). Mitochondrial membrane potential was measured and analyzed in vHCS view software.

1.7 Oxygen consumption assay

NRCM were cultured in a 96-well clear bottom plate. Measurements were performed in fresh DMEM containing 6.25% MitoXpress® Xtra reagent (Luxcel Biosciences, Ireland) covered with pre-warmed Mineral Oil, using a fluorescence plate reader-FLUOstar Omega (BMG Labtech, Germany) kinetically for 150 minutes at 37° C. Excitation and emission wavelengths were 380 and 650 nm, respectively. Oxygen consumption was determined by the average slope of relative fluoresce unit (RFU) as recommended.

1.8 Measurement of intracellular ATP levels

ATP levels were measured with the ATP Assay Kit (Beyotime, China). According to the manufacturer's recommendations, NRCM cultured in 6-well plate were harvested and lysed in 200 μL ATP lysate buffer (1∶10). To determine intracellular ATP levels, 100 μL harvested protein supernatant was added to each well, mixed and intermediately (2 s) read. ATP concentration was measured by 10 s RLU value and normalized by protein concentrations.

1.9 Statistical analysis

Data were presented as mean±SEM from at least three independent experiments. Statistical analysis was performed with Graphpad Prism 5.0 software. Comparison between two groups was performed by two-tailed unpaired Student's t-test. Differences among groups were tested by one-way ANOVA followed by the Bonferroni post hoc test. In all cases, differences were considered statistically significant at values of $P<0.05$.

2 Results

2.1 SIRT5 was negatively regulated by RIP140 in NRCM

To explore the regulation of the mitochondrial sirtuins by RIP140, we transduced NRCM via Ad-RIP140 or Ad-GFP adenoviral infection and measured the mRNA expression of the SIRT3-5 (n=10). The mRNA levels of SIRT3 (0.77±0.047 folds of control group, t=3.66, P=0.0018 vs control group) and SIRT5 (0.62±0.036) folds of control group, t=7.32, $P<0.001$ vs control group) were decreased by RIP140, though no change of SIRT4 was observed (Fig. 1A). Notably, SIRT5 was reduced more obviously than SIRT3. Moreover, SIRT5 protein expression was consistently attenuated by RIP140 overexpression (0.71±0.061 folds of control group, t=3.76, P=0.0056 vs control group, Fig. 1B). Conversely, the siRNA-mediated RIP140 knockdown increased SIRT5 mRNA (1.49 ± 0.11 folds of NC group, t=3.23, P=0.012 vs NC group) and protein expression (1.63±0.14 folds of NC group, t=3.14, P=0.014 vs NC group) in NRCM (Fig. 1C&D). These observations indicate that SIRT5 was negatively regulated by RIP140 in NRCM.

2.2 RIP140 overexpression induced hypersuccinylation of mitochondrial proteins in NRCM

As SIRT5 is expected to regulate cellular functions through enzymatic protein modifications, we investigated the succinylation activity of SIRT5 in total protein and mitochondrial protein of NRCM. As shown in Fig. 2A, the succinylation level in the mitochondrial fraction of cardiomyocytes was decreased by overexpression of SIRT5 but was increased by knockdown of SIRT5. What's more, succinylation level of mitochondrial proteins was increased in NRCM infected with RIP140 adenovirus, which could be reversed by overexpressing SIRT5 simultaneously (Fig.2B). These observations suggest that RIP140 not only decreases the expression of SIRT5 but also enhances the succinylation level of mito-

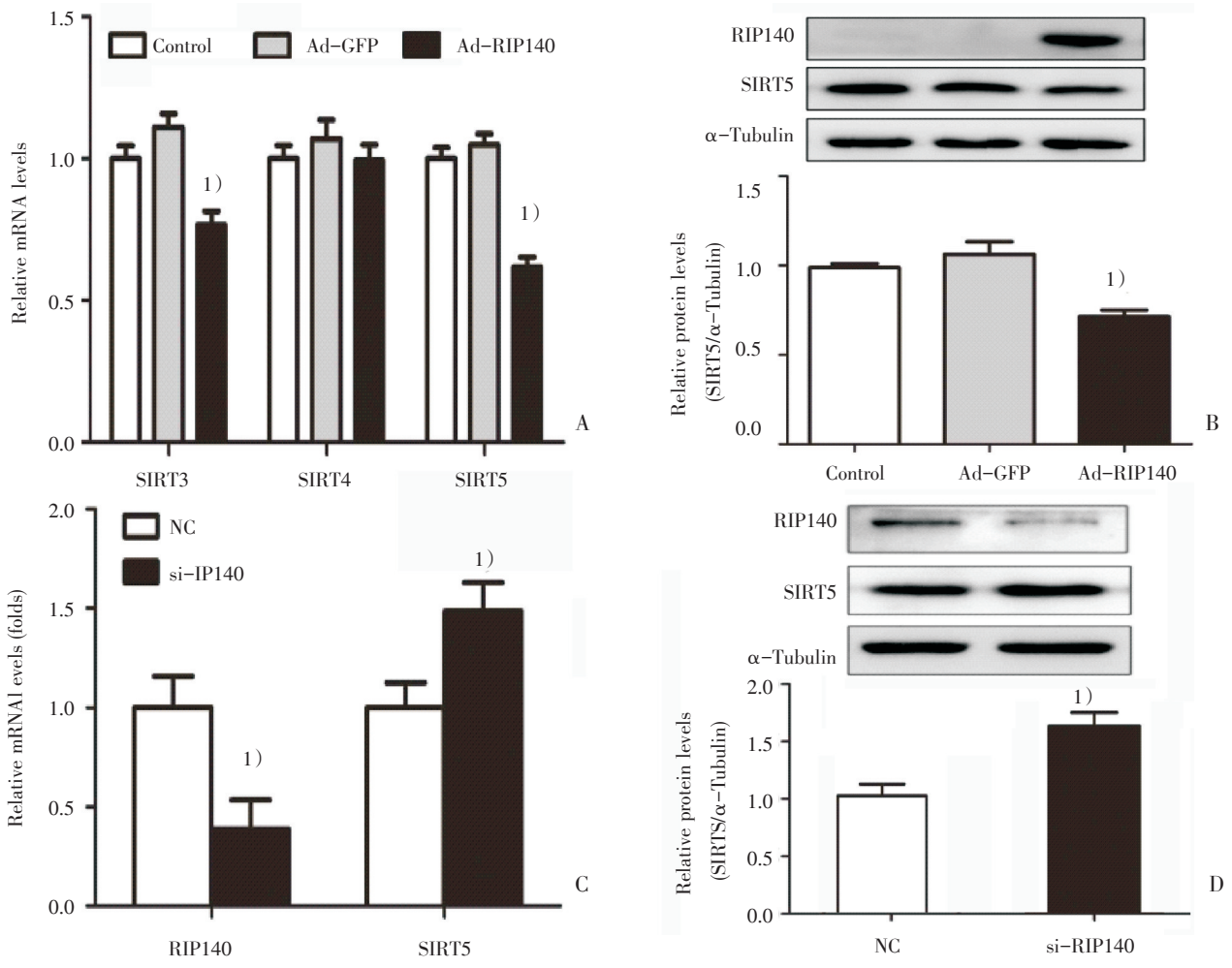

Fig.1 SIRT5 was negatively regulated by RIP140 in NRCMs

(A) NRCMs were transduced with Ad-RIP140 or control Ad-GFP adenoviruses (MOI 60) for 36 h, and quantitative RT-PCR was performed to measure the mRNA of mitochondrial sirtuins. n=10. (B) Western blot analysis showed the protein expression of SIRT5 in cardiomyocytes transduced with RIP140 adenoviruses (MOI 60) for 48 h. n=5. Data of (A) and (B) were shown as mean ± SEM. 1)P< 0.05 as compared to the Control group. (C) Quantitative RT-PCR analysis showed that SIRT5 was induced after transfected cardiomyocytes with siRNA (100 nmol/L) of negative control (NC) or RIP140 for 48 h. n=5. (D) Western blot analysis showed the protein expression of RIP140 and SIRT5 in cardiomyocytes transfected with siRNA (100 nmol/L) of RIP140 and negative control (NC) for 72 h. n=5. Data of(C) and (D) were shown as mean ± SEM. 1)P<0.05 ascompared to the NC group.

chondrial proteins.

2.3 SIRT5 prevented RIP140-induced impairment of mitochondrial biogenesis and mitochondrial function

Mitochondrial biogenesis in cardiomyocytes was assessed by copy numbers of mitochondrial DNA-encoded genes, including cytochrome b (Cyt b), NADH dehydrogenase subunit 1 (ND1), and mitochondrially encoded cytochrome C oxidase I (mt-co1) by quantitative RT-PCR. NRCM with superabundant RIP140 showed a significant decrease in the ratios of Cyt b/β-actin (0.54 ± 0.10 folds of Ad-GFP group), ND1/β-actin (0.51±0.11 folds of Ad-GFP group) and mt-co1/β-actin (0.31± 0.098 folds of Ad-GFP group), which was reversed by overexpression of SIRT5 (1.38±0.11 folds of Ad-GFP group, F=24.43, P<0.001, t=7.24, P<0.05 vs Ad-RIP140 for Cyt b; 1.49 ± 0.12 folds of Ad-GFP group, F=22.79, P<0.001, t=6.95, P<0.05 vs Ad-RIP140 for ND1; 1.49 ± 0.11 folds of Ad-GFP group, F=28.65,

$P<0.001$, $t=7.66$, $P<0.05$ vs Ad-RIP140 for mt-co1, Fig.3A). Since healthy mitochondria maintains an electrochemical membrane gradient that drives ATP production, mitochondrial membrane potential was assessed using sensitive fluorescent probe TMRE. As shown in Fig.3B (0.46±0.089 folds of Ad-GFP group in Ad-RIP140 group, 1.42±0.11 folds of Ad-GFP group in Ad-RIP140 combined with overexpressed SIRT5 group, $F=30.23$, $P<0.001$), SIRT5 overexpression significantly prevented RIP140-induced decrease of TMRE fluorescence ($t=7.95$, $P<0.05$ vs Ad-RIP140), although SIRT5 knockdown did not aggravate the impairment. Indeed, overexpression of SIRT5 alone in NRCM also increased copy numbers of mtDNA (1.60±0.16 folds of control group, $t=3.07$, $P=0.037$ for Cyt b; 1.69 ±0.14 folds of control group, $t=4.25$, $P=0.013$ for ND1; 1.66±0.10 folds of control group, $t=5.34$, $P=0.0059$ for mt-co1. Fig. 4A) and mitochondrial membrane potential (1.72±0.16 folds of control group, $t=3.82$, $P=0.019$, Fig.4B), in line with the study in C2C12 cells [14]. We next sought to explore the cellular oxygen consumption and ATP production. A significant loss of oxygen consumption was observed in NRCM treated with Ad-RIP140 (0.41±0.083 folds of Ad-GFP group), which was reversed by overexpression of SIRT5 (0.88±0.040 folds of Ad-GFP group, $F=23.06$, $P<0.001$, $t=4.80$, $P<0.05$ vs Ad-RIP140, Fig.3C). Besides, oxygen consumption rate was increased in NRCM overexpressed SIRT5 (2.37±0.31 folds of control group, $t=4.39$, $P=0.012$ vs control group) but decreased by SIRT5 knockdown (0.69±0.11 folds of control group, $t=2.97$, $P=0.041$ vs control group, Fig. 4C). Consistently, a significant loss of ATP production was observed in NRCM transduced with Ad-RIP140 adenovirus (0.62 ± 0.063 folds of Ad-GFP group), which could be reversed by overexpression of SIRT5 (2.58 ± 0.13 folds of Ad-GFP group, $F=118.0$, $P<0.0001$, $t=16.12$, $P<0.05$ vs Ad-RIP140, Fig. 3D). Compared to Ad-RIP140 group, simultaneously overexpression of SIRT5 raised the intracellular ATP content by about 2.58-fold. Furthermore, SIRT5 overexpression enhanced

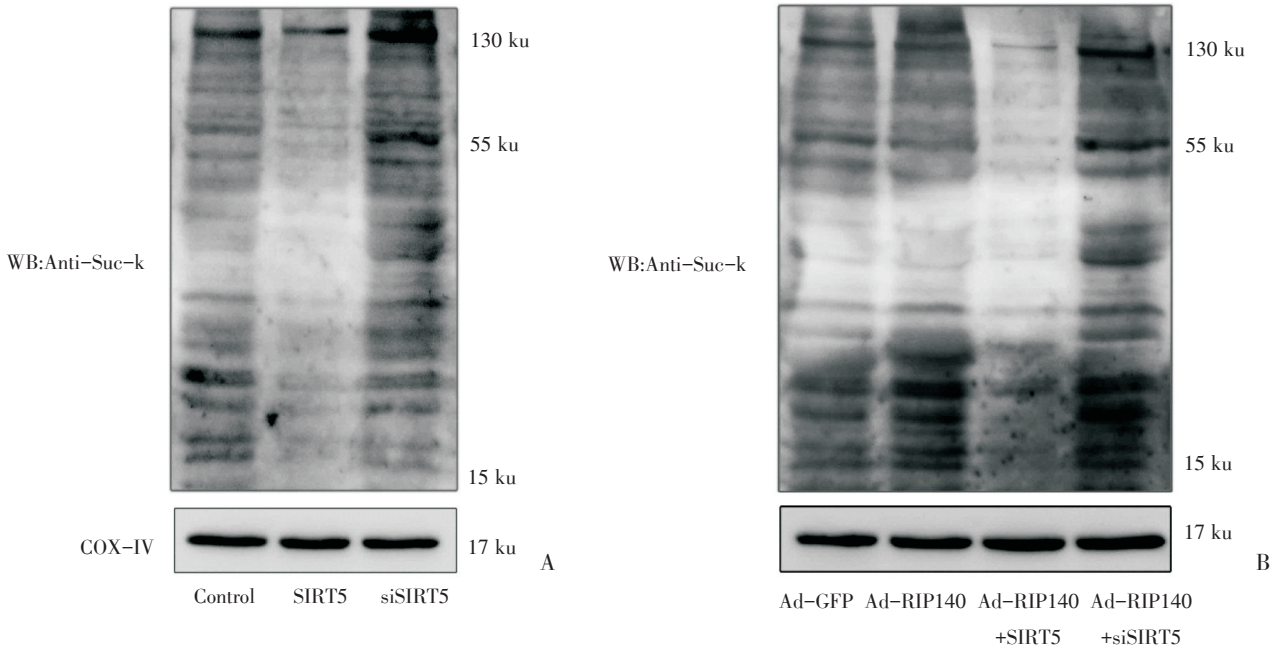

Fig.2　RIP140 overexpression induced hypersuccinylation of mitochondrial proteins in neonatal rat cardiomyocytes

(A) Western blot analysis showed the succinylation status of mitochondrial proteins in cardiomyocytes transfected with SIRT5 plasmids (2 mg/L) or siRNA (100 nmol/L) for 72 h. $n=3$. (B) Succinylation status of mitochondrial proteins were detected in cardiomyocytes treated with Ad-RIP140 (MOI 60, 66 h), combined with SIRT5 plasmid (2 mg/L, 72 h) or siRNA (100 nmol/L, 72 h). $n=3$

cellular ATP production (3.03±0.27 folds of Control group, t=7.05, P= 0.0021 vs Control group) while knockdown of SIRT5 did not affect ATP levels (t= 0.65, P=0.55 vs Control group, Fig. 4D). However, the knockdown of SIRT5 did not aggravate RIP140-mediated impairment in ATP production and oxygen consumption. These results revealed that SIRT5 overexpression could inhibit RIP140-induced mitochondrial dysfunction and energy metabolic dysfunction.

2.4 RIP140 repressed SIRT5 in a PPARα-dependent manner

PPARα is known as an important role in regulation of cardiac energy metabolism. What's more, RIP140 repressed expression of PPARα, resulting in downregulation of metabolic enzymes in heart [12, 15]. Thus, we further explored if PPARα participated in

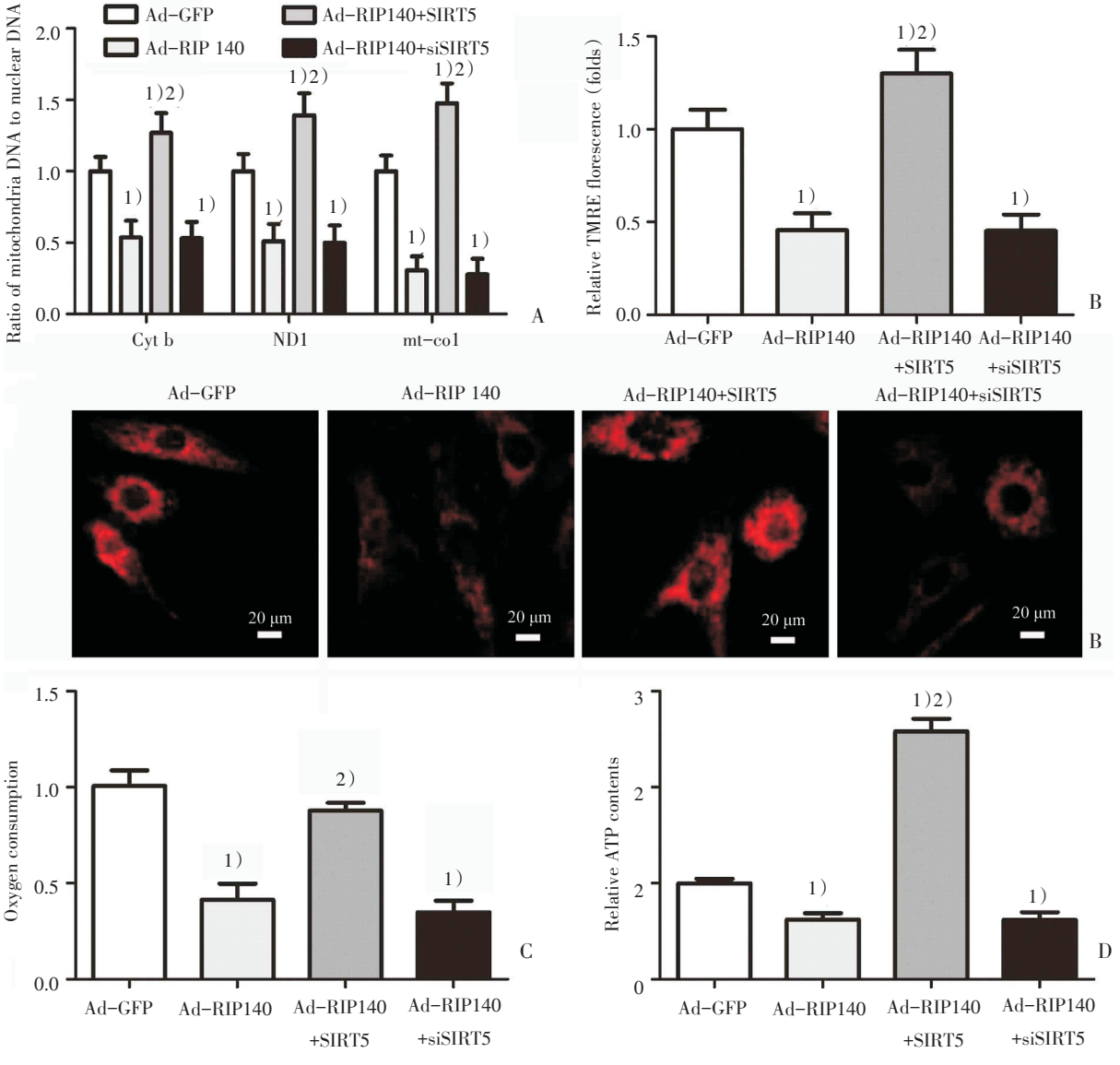

Fig.3 SIRT5 reversed down-regulation of key metabolic genes and protected against down-regulation of mitochondrial biogenesis and function by RIP140

Cardiomyocytes were treated with Ad-RIP140 (MOI 60, 42 h), simultaneously with SIRT5 plasmid (2 mg/L, 48 h) or siRNA (100 nmol/L, 48 h). (A)The mRNA expressions of mitochondrial DNA-encoded genes were measured by quantitative real-time PCR. n=4. (B) Alternations of mitochondrial membrane potentialwas detected by TMRE fluorescence analysis. n=4. (C)Relative oxygen consumption rate and (D) relative ATP content was measured by assay kits. n=4.Data were shown as mean±SEM. 1)P<0.05 as compared to the Ad-GFP group. 2) P<0.05 as compared to the Ad-RIP140 group.

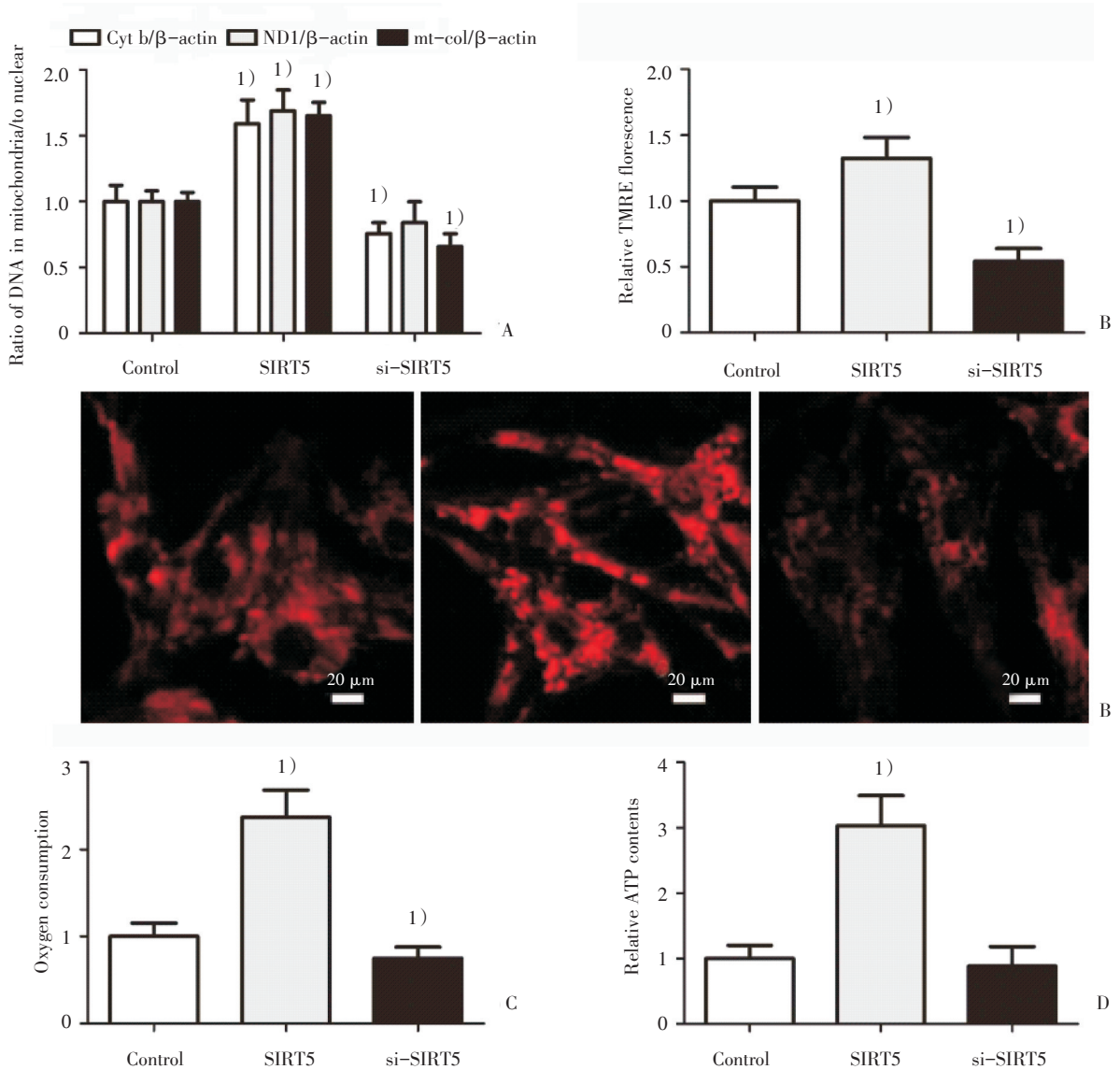

Fig.4　SIRT5 augmented mitochondrial function in cardiomyocytes

NRCM were transfected with SIRT5 plasmid (2 mg/L) or siRNA knockdown (100 nmol/L) for 48 h. (A) The mRNA expressions of mitochondrial DNA-encoded genes were measured by quantitative real-time PCR. n=3. (B) Alternations of mitochondrial membrane potentialwas detected by TMRE fluorescence analysis. n=3. (C) Relative oxygen consumption and (D) Relative ATP content were measured by assay kits. n=3. Data were shown as mean ± SEM. Statistical analysis was performed by two-tailed unpaired Student's t-test between two groups. 1) P<0.05, as compared to the Control group.

the repression of SIRT5 by RIP140. Firstly, NRCM were treated with a special PPARα agonist Wy14643 at indicated exposure time and dose, and the protein expression of SIRT5 was measured. The results showed that PPARα elevated SIRT5 protein expression levels in a time- and concentration-dependent manner (Fig.5A&B). SIRT5 protein expression increased to (2.22±0.095) folds for 24 h (t=8.82, P=0.0009 vs 0 h group) and to (2.57±0.10) folds for 48 h (t=11.02, P=0.0004 vs 0 h group) in NRCM treated with 25 μmol/L of Wy14643. SIRT5 protein expression increased to (2.20±0.089) folds in NRCM treated with 25 μmol/L of Wy14643 (t=7.38, P<0.001 vs 0 μmol/L group) and to (2.36±0.11) folds in NRCM treated with 50 μmol/L of Wy14643 (t=7.77, P<0.001 vs 0 μmol/L group). In contrast, knockdown of PPARα decreased the protein expression of SIRT5 in NRCM (0.47±0.095 folds of NC

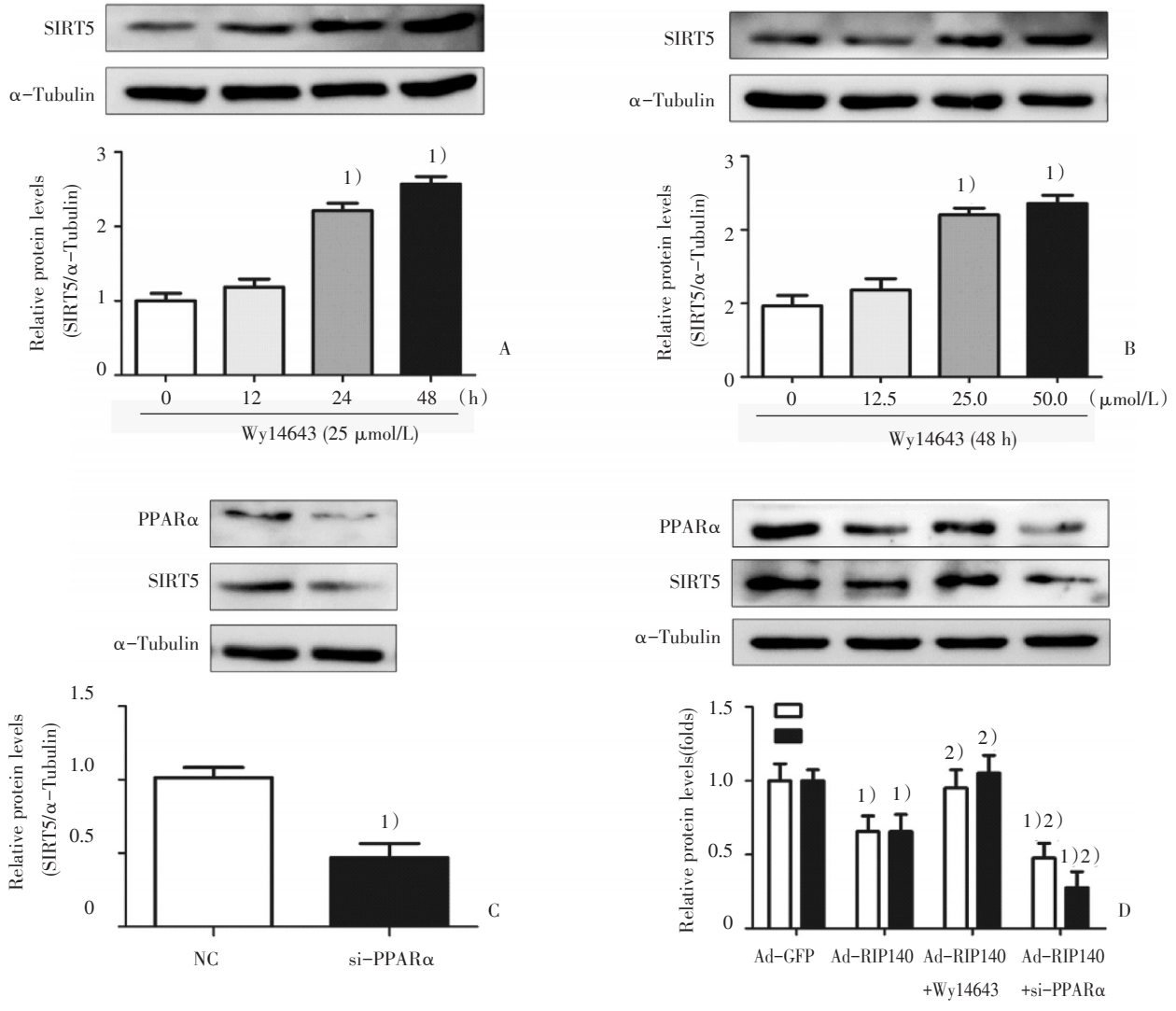

Fig.5 RIP140 inhibited SIRT5 in a PPARα-dependent manner

(Aand B) Western blot analysis showed the protein expression of SIRT5 in NRCM treated with 25 μmol/L of Wy14643 (PPARα agonist) for indicated time (left panel) or with indicated concentrations of Wy14643 (PPARα agonist) for 48 h (right panel). n=3. (C) Western blot analysis showed the protein expression of SIRT5 and PPARαin Cardiomyocytes transfected with siRNA(100 nmol/L) of PPARα or negative control (NC) for 72 h. n =4. (D) Cardiomyocytes were infected with Ad-RIP140 (MOI 60, 48 h), simultaneously treated withWy14643 (PPARα agonist) (25 μmol/L, 48 h) orPPARαsiRNA(100 nmol/L, 72 h). The levels of protein of SIRT5 and PPARαwere measured. n=4. Data were shown as mean ± SEM. 1) P<0.05as compared to the Ad-GFP group. 2)P<0.05 as compared to the Ad-RIP140 group.

group, t=4.02, P=0.0070 vs NC group, Fig. 5C). These observations suggest that expression of SIRT5 could be regulated by PPARα. Secondly, to investigate whether PPARα participates in the repression of SIRT5 by RIP140, NRCM infected with Ad-RIP140 adenovirus were simultaneously treated with PPARα activator Wy14643 or transfected with PPARα siRNA. As shown in Fig. 5D, Wy14643 treatment reversed while PPARα siRNA exacerbated RIP140-mediated repression of SIRT5 (F=28.85, P< 0.001). The relative expression of SIRT5 in Ad-RIP140 combined with Wy14643 treatment groupapproximately reached the normal level (0.981±0.106)folds of Control (P<0.05 vs Ad- RIP140). In contrast, knockdown of PPARα by siRNA aggravate the RIP140-mediated repression of SIRT5 (0.393± 0.062 folds of Control, P<0.05 vs Ad- RIP140).Taken together, these results suggest that PPARα is involved in the

repression of SIRT5 by RIP140.

3 Discussion

As heart is a highly oxygen-consuming organ, abnormality of energy metabolism always results in cardiac dysfunction and even heart failure[1]. RIP140 is known as a deleterious regulator of cardiac mitochondrial function and metabolic homeostasis[11, 15-16]. RIP140 transgenic mice represented a great impact on the heart like cardiac fibrosis, atrial thrombosis, and cardiac hypertrophy[16]. Our published study reported that RIP140 were upregulated in overt heart failure and could accelerate the transition to heart failure in response to MI stress through repression of mitochondrial function[15]. Here, we demonstrated that RIP140 repressed the mitochondrial metabolism through down-regulating SIRT5 in NRCM, suggesting a novel mechanism of RIP140 in regulating the cardiac energy metabolism.

SIRT5 is reported to be induced by PGC-1α, which is a key factor regulating mitochondrial biogenesis and function[13]. Our previous study has shown that RIP140 and PGC-1α exert antagonistic role in regulating cardiac energy state sharing many targets[11]. RIP140 represses while PGC-1α promotes mitochondrial energy metabolism in cardiomyocytes[11]. In the present study, we found the expression and desuccinylase activity of SIRT5 were repressed by RIP140. As SIRT5 is induced by PGC-1α[13] and suppressed by RIP140, the functional antagonism in cardiac energy metabolism between RIP140 and PGC-1α is possibly associated with the regulation of SIRT5. Indeed, overexpression of SIRT5 augments mitochondrial function and energy metabolism which mimics the effect of PGC-1α[13], whileknockdown of SIRT5 mimics RIP140-mediated metabolic dysregulation in cardiomyocytes. What's more, these observations further confirm that RIP140 and PGC-1α exert antagonistic role in maintaining cardiac energy balance through SIRT5 pathway.

RIP140 is known to induce mitochondrial metabolic dysfunction[11], with the observations that superabundant RIP140 in cardiomyocytes decreased expression of ND1 (complex I), Cyt b (complex III) and mt-co1 (complex IV), as well as TMRE fluorescence intensity. The reversion in mtDNA copy number and TMRE fluorescence level indicated an improved functional state of mitochondrial respiration by SIRT5 overexpression. However, RIP140-mediated metabolic dysfunction can be only slightly deteriorated by knockdown of SIRT5, though knockdown of SIRT5 alone significantly repressed mitochondrial metabolism. In fact, energy metabolic homoeostasis have specific mechanisms to maintain a balance between energy production and expenditure[17]. Excessive consumption of energy or extremely energy repression may activate the adaptive pathway as a protective way. Therefore, once SIRT5 is repressed by RIP140 to a certain degree, the downstream dysfunction won't be exacerbated anymore through the SIRT5 pathway and we supposed that there may be other adaptive pathways to compensate. What's more, we observed overexpression of SIRT5 induced ATP production while knockdown of SIRT5 did not affect ATP level, lined with the study performed in HepG2 cells[13]. However, more than 20% reduction in ATP was observed in SIRT5 KO hearts compared with wild type after 24 h of fasting[8]. Besides, it's reported that ATP content was decreased in the overt heart failure but had no significant changes in its onset phase or myocardial infarction in rodent hearts[15]. These findings suggested us that ATP production depends mostly on the physiological and pathological status of the body. Thus, other metabolic pathway might also contribute to ATP synthesis in response to depletion of SIRT5 in physiological conditions.

SIRT5 is expected to regulate mitochondrial function mainly depending on its desuccinylase activity[8]. Succinylation is a widespread protein posttranslational modification and affects major metabolic pathways including fatty acid metabolism, oxidative phosphorylation, amino acid metabolism, TCA cycle, urea cycle, ketogenesis[8, 13, 18]. SIRT5 is recognized as the unique desuccinylase in mitochondria, hydrolyzing negatively charged lysine modifications[18]. In

this study, we observed SIRT5 desuccinylated the mitochondrial protein, in agreement with published data that SIRT5 is a global regulator of lysine succinylation in mitochondria[8]. It is revealed that mitochondria isolated from SIRT5$^{-/-}$ hearts exhibit considerable increases in global protein succinylation[8, 18]. Furthermore, we found superabundant RIP140 induced hypersuccinylation of mitochondrial proteins, which confirmed that SIRT5 is repressed by RIP140.

Our study has also revealed that RIP140 repressed SIRT3 in cardiomyocytes. It is well known that SIRT3 plays an important role in mitochondrial metabolism depending on its deacetylase activity[19]. Since SIRT3 acts as a deacetylase while SIRT5 is most likely a desuccinylase, they may play different role in the regulation of mitochondrial metabolism. Actually, SIRT3 and SIRT5 share many common substrates which are metabolic enzymes enriched in overlapping pathways including fatty acid β-oxidation, ketogenesis, and TCA cycle[20, 21]. Both SIRT3 and SIRT5 were reported to target VLCAD, a key fatty acid oxidation enzyme[21]. Furthermore, HMGCS2 is another substrate identified as substrate of both SIRT3[22] and SIRT5[18]. Numerous studies have revealed that SIRT3 depletion impairs mitochondrial function and abundant SIRT3 increases mitochondrial metabolism[23]. However, SIRT3$^{-/-}$ mice under basal condition appear phenotypically normal and show no significant change in fatty acid oxidation[23]. These observations suggest that energy homeostasis partially attribute to regulation of SIRT3 and other metabolic pathway might also contribute to the energy production in SIRT3$^{-/-}$ mice. As SIRT5 shares many common metabolic substrates with SIRT3, we speculated that SIRT5 may partially compensate for the lack of SIRT3 and SIRT3/5 act more like in an additive way. What's more, SIRT5 also has weak deacetylase activity by deacetylating proteins such as CPS1[24] and Cytochrome c[25], which are not deacetylating substrates of SIRT3. Thus, we speculated that SIRT3 and SIRT5 may act like in an additive way in regulating mitochondrial metabolism.

RIP140 plays a key role in the regulation of a series of metabolic enzymes by co-repressing its partners, such as PPARs. PPARα is enriched in the heart and plays a prominent role in regulation of cardiac energy metabolism[26]. During the last decades, the protective role of PPARα in cardiovascular diseases such as cardiac hypertrophy through improving energy metabolism has been well illuminated[26]. Our published data also showed that RIP140 downregulated the protein expression of PPARα and deteriorated cardiac energy metabolism[12]. In this study, we found that PPARα selective agonist Wy14643 reversed the repression of SIRT5 caused by RIP140, whereas knockdown of PPARα aggravated the suppression of SIRT5 caused by RIP140. Although we only tested the influence of PPARα on SIRT5 at protein levels, it is revealed that the upstream promoter region of mouse SIRT5 possessed potential PPARα responsive elements[13]. These observations preliminarily suggest that RIP140 may repress SIRT5 in PPARα-dependent manner in cardiomyocytes.

In conclusion, the present study demonstrated that RIP140 repressed SIRT5 in a PPARα-dependent manner in cardiomyocytes. The repression of SIRT5 by RIP140 facilitated mitochondrial dysfunction and energy metabolic repression. An understanding of this regulatory process may provide novel therapeutic strategies for the treatment of cardiac energy metabolic derangement.

References

[1] Neubauer S. The failing heart--an engine out of fuel [J]. N Engl J Med, 2007, 356(11): 1140-1151.

[2] Murphy E, Ardehali H, Balaban RS, et al. Mitochondrial function, biology, and role in disease a scientific statement from the american heart association [J]. Circ Res, 2016, 118(12): 1960-1991.

[3] Bayeva M, Gheorghiade M, Ardehali H. Mitochondria as a therapeutic target in heart failure [J]. J Am Coll Cardiol, 2013, 61(6): 599-610.

[4] Gertz M, Steegborn C. Using mitochondrial sirtuins as drug targets: Disease implications and available compounds [J]. Cell Mol Life Sci, 2016, 73(15): 2871-2896.

[5] Newman JC, He WJ, Verdin E. Mitochondrial protein

acylation and intermediary metabolism: Regulation by sirtuins and implications for metabolic disease [J]. J Biol Chem, 2012, 287(51): 42436-42443.

[6] Luo YX, Tang X, An XZ, et al. SIRT4 accelerates Ang II-induced pathological cardiac hypertrophy by inhibiting manganese superoxide dismutase activity [J]. Eur Heart J, 2017, 38(18): 1389-1398.

[7] Lombard DB, Alt FW, Cheng HL, et al. Mammalian Sir2 homolog SIRT3 regulates global mitochondrial lysine acetylation [J]. Mol Cell Biol, 2007, 27(24): 8807-8814.

[8] Sadhukhan S, Liu XJ, Ryu D, et al. Metabolomics-assisted proteomics identifies succinylation and SIRT5 as important regulators of cardiac function [J]. P Natl Acad Sci USA, 2016, 113(16): 4320-4325.

[9] Boylston JA, Sun JH, Chen Y, et al. Characterization of the cardiac succinylome and its role in ischemia-reperfusion injury [J]. J Mol Cell Cardiol, 2015, 88(23): 73-81.

[10] Leonardsson G, Steel JH, Christian M, et al. Nuclear receptor corepressor RIP140 regulates fat accumulation [J]. P Natl Acad Sci USA, 2004, 101(22): 8437-8442.

[11] Chen Y, Wang Y, Chen J, et al. Roles of transcriptional corepressor RIP140 and coactivator PGC-1alpha in energy state of chronically infarcted rat hearts and mitochondrial function of cardiomyocytes [J]. Mol Cell Endocrinol, 2012, 362(1-2): 11-18.

[12] Zhang LK, Chen YF, Yue ZB, et al. The p65 subunit of NF-kappa B involves in RIP140-mediated inflammatory and metabolic dysregulation in cardiomyocytes [J]. Arch biochembiophys, 2014, 554(11): 22-27.

[13] Buler M, Aatsinki SM, Izzi V, et al. SIRT5 is under the control of PGC-1 alpha and AMPK and is involved in regulation of mitochondrial energy metabolism [J]. Faseb J, 2014, 28(7): 3225-3237.

[14] Polletta L, Vernucci E, Carnevale I, et al. SIRT5 regulation of ammonia-induced autophagy and mitophagy [J]. Autophagy, 2015, 11(2): 253-270.

[15] Chen YF, Chen SR, Yue ZB, et al. Receptor-interacting protein 140 overexpression impairs cardiac mitochondrial function and accelerates the transition to heart failure in chronically infarcted rats [J]. Transl Res, 2017, 180(9): 91-102.

[16] Fritah A, Steel JH, Nichol D, et al. Elevated expression of the metabolic regulator receptor-interacting protein 140 results in cardiac hypertrophy and impaired cardiac function [J]. Cardiovascular Res, 2010, 86(3): 443-451.

[17] Huss JM, Kelly DP. Mitochondrial energy metabolism in heart failure: A question of balance [J]. J Clin Invest, 2005, 115(3): 547-555.

[18] Rardin MJ, He WJ, Nishida Y, et al. SIRT5 regulates the mitochondrial lysine succinylome and metabolic networks [J]. Cell Metab, 2013, 18(6): 920-933.

[19] Anderson KA, Hirschey MD. Mitochondrial protein acetylation regulates metabolism [J]. EssaysBiochem, 2012, 52(11): 23-35.

[20] De Moura MB, Uppala R, Zhang YX, et al. Overexpression of mitochondrial sirtuins alters glycolysis and mitochondrial function in HEK293 cells [J]. Plos One, 2014, 9(8): e106028

[21] Zhang YX, Bharathi SS, Rardin MJ, et al. SIRT3 and SIRT5 regulate the enzyme activity and cardiolipin binding of very long-chain acyl-CoA dehydrogenase [J]. Plos One, 2015, 10(3): e0122297

[22] Shimazu T, Hirschey MD, Hua L, et al. SIRT3 Deacetylates mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase 2 and regulates ketone body production [J]. Cell Metab, 2010, 12(6): 654-661.

[23] Hirschey MD, Shimazu T, Goetzman E, et al. SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation [J]. Nature, 2010, 464(7285): 121-U137.

[24] Nakagawa T, Lomb DJ, Haigis MC, et al. SIRT5 deacetylates carbamoyl phosphate synthetase 1 and regulates the urea cycle [J]. Cell, 2009, 137(3): 560-570.

[25] Schlicker C, Gertz M, Papatheodorou P, et al. Substrates and regulation mechanisms for the human mitochondrial Sirtuins Sirt3 and Sirt5 [J]. J Mol Biol, 2008, 382(3): 790-801.

[26] Le K, Li R, Xu S, et al. PPARalpha activation inhibits endothelin-1-induced cardiomyocyte hypertrophy by prevention of NFATc4 binding to GATA-4 [J]. Arch-Biochem Biophys, 2012, 518(1): 71-78.

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