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SIRT3 deficiency exacerbates ischemia-reperfusion injury: implication for aged hearts

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Porter GA, Urciuoli WR, Brookes PS, Nadtochiy SM. SIRT3 deficiency exacerbates ischemia-reperfusion injury: implication for aged hearts. Am J Physiol Heart Circ Physiol 306: H1602–H1609, 2014. First published April 18, 2014; doi:10.1152/ajpheart.00027.2014.—Ischemia-reperfusion (IR) injury is greatly complicated in aged hearts (6, 25). A number of observations draw a link among the loss of mitochondrial function, aging, and increased susceptibility of aged hearts to IR injury (6, 11, 25, 28, 58). However, the underlying mechanisms of this link remain ambiguous.

Within the aging field, a great deal of interest has been directed toward the silent information regulator of transcription (SIRT) family of proteins (4, 44). SIRTs are class III NAD+-dependent histone deacetylases involved in gene silencing (12a). Importantly, SIRT-mediated protein deacetylation has emerged as an important post-translational modification involved in signaling in several cell compartments (30). Although a direct role for nuclear/cytosolic SIRT1 in cardioprotection against IR injury has been reported (22, 39, 40), the role of other SIRTs in modulating IR injury is less clear.

Since mitochondria and metabolism are sensitive targets for damage during IR injury (36), the role of mitochondrial SIRTs in cardioprotection is of particular interest. SIRT3 is the major mitochondrial deacetylase (31), and several proteins implicated in regulating the response to IR have been identified as SIRT3 targets (47). They include cyclophilin D (18, 49), isocitrate dehydrogenase 2 (ICDH2) (59), Ku70 (52), MnSOD (46), and complex I (Cx I) activity with low O2 consumption rates compared with controls. In the Langendorff model, SIRT3kd adult hearts showed less functional recovery and greater infarct vs. WT, which recapitulates the in vitro results. In WT aged hearts, recovery from IR injury was similar to SIRT3+/− adult hearts. Mitochondrial protein acetylation was increased in both SIRT3+/− adult and WT aged hearts (relative to WT adult), suggesting similar activities of SIRT3. Also, enzymatic activities of two SIRT3 targets, Cx I and MnSOD, were similarly and significantly inhibited in SIRT3+/− adult and WT aged cardiac mitochondria. In conclusion, decreased SIRT3 may increase the susceptibility of cardiac-derived cells and adult hearts to IR injury and may contribute to a greater level of IR injury in the aged heart.

SIRT3; heart; aging; ischemia; reperfusion; mitochondria; acetylation

MYOCARDIAL INFARCTION (MI) remains a major clinical problem in the Western world. Much of the morbidity and mortality associated with MI is due to the lack of adequate protection of the myocardium when ischemic areas are reperfused after surgical or catheter-based interventions (23). The heart is an energetically demanding organ, and its function relies on mitochondrial ATP generation. Irreversible damage to mitochondria during MI results in a shortage of ATP supply, oxidative stress, and eventually, cardiomyocyte death. This pathologic process is termed ischemia-reperfusion (IR) injury.

Aging is a major risk factor for MI, and recovery from IR injury is greatly complicated in aged hearts (6, 25). A number of observations draw a link among the loss of mitochondrial function, aging, and increased susceptibility of aged hearts to IR injury (6, 11, 25, 28, 58). However, the underlying mechanisms of this link remain ambiguous.

Within the aging field, a great deal of attention has been directed toward the silent information regulator of transcription (SIRT) family of proteins (4, 44). SIRTs are class III NAD+-dependent histone deacetylases involved in gene silencing (12a). Importantly, SIRT-mediated protein deacetylation has emerged as an important post-translational modification involved in signaling in several cell compartments (30). Although a direct role for nuclear/cytosolic SIRT1 in cardioprotection against IR injury has been reported (22, 39, 40), the role of other SIRTs in modulating IR injury is less clear.

Since mitochondria and metabolism are sensitive targets for damage during IR injury (36), the role of mitochondrial SIRTs in cardioprotection is of particular interest. SIRT3 is the major mitochondrial deacetylase (31), and several proteins implicated in regulating the response to IR have been identified as SIRT3 targets (47). They include cyclophilin D (18, 49), isocitrate dehydrogenase 2 (ICDH2) (59), Ku70 (52), MnSOD (46), and complex I (Cx I) (3). Whereas the number of SIRT3 targets that may be involved in cardiac IR injury continues to grow, the interplay between those targets and SIRT3 in the context of IR is not known.

SIRT3 protein expression is lower in old, sedentary adults (27). In addition, SIRT3 activity may be compromised by low levels of NAD+, as seen in aged hearts (7, 50). Notably, SIRT3 can be inhibited by oxidants (15), which may be generated at higher rates in aged hearts (5). Therefore, if the protective effects of SIRT3 rely on its enzymatic activity, this might be impaired in aged individuals. Indeed, adult SIRT3−/− mice share a number of cardiac phenotypes with aged wild-type (WT) mice, such as cardiac hypertrophy and fibrosis (18, 51). Nevertheless, direct inhibition of SIRT3 activity in aged hearts has not been reported, and the relationship between downregulation of SIRT3 and poor recovery of aged hearts from IR injury is unclear. In this study, we examined the connection between SIRT3 and IR injury in the context of aging. Our data support a link between SIRT3 downregulation and vulnerability of both adult and aged hearts to IR injury.
METHODS

Animals and materials. Male WT [7 mo ("adult") and 18 mo ("aged")]) and SIRT3+/− [7 mo (adult)] mice, on a C57BL/6 background, were used in this study. All mice were maintained in a pathogen-free vivarium under recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with a 12:12-h, light-dark cycle and food and water available ad libitum. All experimental protocols were approved by the Association for Assessment and Accreditation of Laboratory Animal Care-accredited University of Rochester Committee on Animal Resources. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise mentioned.

Small interfering RNA transfection in H9c2 cells and in vitro-simulated IR injury. H9c2 cardiac-derived cells (American Type Culture Collection, Manassas, VA) were transfected with SIRT3 small interfering RNA (siRNA) and subjected to in vitro IR injury; the major steps of this procedure have been described previously (41). Briefly, H9c2 cells were plated on 22-mm, 12-well plates (Coster Bio-One, Monroe, NC) and transfected with 50 nM SIRT3 siRNA [SIRT3 knockdown (SIRT3kd); Qiagen, Valencia, CA] or scrambled control siRNA (Ctrl). Successful knockdown was verified after 72 h using immunoblotting.

For in vitro-simulated IR injury, cells were incubated in normoxic buffer (DMEM, 10 mM glucose, 10 mM HEPES, pH 7.4, at 37°C). Simulated ischemia (4 h) started when cells were transferred into a hypoxic chamber with 0% O2 (Coy, Grass Lake, MI), and media were replaced with hypoxic buffer (glucose-free DMEM, 10 mM HEPES, pH 6.5, at 37°C). Upon reperfusion, the media were replaced with normoxic buffer, and cells were incubated at 37°C (room air + 5% CO2) for 2 h. Cell death was then assayed by measuring lactate dehydrogenase (LDH) release using a Cytotoxicity Detection Kit (Roche Diagnostics, Indianapolis, IN), according to the manufacturer’s protocol.

Cx I activity and O2 consumption rate measurements. Cx I activity was measured by monitoring rotenone-sensitive NADH oxidation at 340 nm (8) in freeze/thawed Ctrl and SIRT3kd H9c2 cells and in mitochondria isolated from WT adult, SIRT3+/− adult, and WT aged hearts, before and after IR injury. For O2 consumption rate (OCR) measurements, H9c2 cells were plated on V7-PS 24-well plates (Seahorse Bioscience, Billerica, MA). One-half of the wells was transfected with 50 nM SIRT3 siRNA (Qiagen) and the other one-half with Ctrl siRNA. Seventy-two hours later, OCR was measured using a Seahorse Bioscience XF24 Flux Analyzer, as described previously (17), with a standard baseline, oligomycin (1 μg/ml), FCCP (500 nM), antimycin A (5 μM; "BOFA" protocol). Reserve capacity was calculated as a difference in OCR between baseline and FCCP-uncoupled respiration rate.

Mouse Langendorff-perfused heart. Following avertin anesthesia, hearts were excised and perfused in constant flow mode (4 ml/min−1, 100 mg−1) with Krebs-Henseleit (KH) buffer, comprised of 118 mM NaCl, 4.7 mM KCl, 25 mM NaHCO3, 10 mM glucose, 1.2 mM MgSO4, 1.2 mM KH2PO4, and 2.5 mM CaCl2 (buffer was equilibrated with 95% O2 + 5% CO2, pH 7.4, at 37°C). Global ischemia (25 min) was induced by stopping flow and immersing the heart in deoxygenated KH buffer. Reperfusion (1 h) was begun by raising flow back to the pre-ischemic rate over a period of 1 min. Functional parameters measured included heart rate ([HR] = beats/min), left ventricular-developed pressure ([LVDP] = left ventricular pressure (LVP)−ystolic − LVPdiastolic), rate pressure product ([RPP] = LVP×HR), and ischemic hypercontracture (differences between pre-ischemic LVPdiastolic and maximum LVP during ischemia). For infarct size measurements (using the same hearts as for the functional measurements), the heart was cut transversely into five sections, which were incubated in 1.0% 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C for 20 min, followed by fixation in 10% neutral-buffered formalin for 24 h. The area at risk was the whole heart, and infarct was indicated by the areas unstained with TTC. Slices were photographed, and infarct/whole heart ratios were determined with Photoshop software (39). For IR experiments, the following groups were used: adult WT (n = 9), adult SIRT3+/− (n = 7), and aged WT (n = 12).

Isolated mitochondrial preparation. Following euthanasia, hearts were removed and immersed in 2 ml ice-cold isolation medium (IM), comprised of 300 mM sucrose, 20 mM Tris-HCl, 2 mM EGTA, pH 7.35, at 4°C. Tissue was chopped, then washed, and homogenized in 2 ml IMM. The homogenate was centrifuged at 1,000 g for 5 min. The pellet was discarded and the supernatant centrifuged at 7,000 g for 10 min. The pellet was resuspended in 1.5 ml IMM and then centrifuged at 10,000 g for 5 min. The final pellet was resuspended in 50 μl IM. Protein was determined by the Lowry method against a standard curve constructed using BSA.

MnSD activity. Isolated heart mitochondria were suspended at 50 μg/ml in phosphate buffer (pH 7.35, 25°C) containing xanthine oxidase (2 U/ml), xanthine (10 mM), cytochrome c (2 mM), and KCN (1 mM). Xanthine/xanthine oxidase produces superoxide, which reduces cytochrome c at a linear rate (measured spectrophotometrically at 550 nm). SOD interferes this reduction, and the SOD content of mitochondrial samples was determined from a standard curve constructed using known amounts of SOD. The presence of KCN both inhibits Cu/ZnSOD, rendering the assay specific for MnSOD, and inhibits mitochondrial cytochrome c oxidase, preventing reoxidation of reduced cytochrome c.

Western blotting. For immunoblotting, mitochondria and H9c2 cell pellets were lysed in SDS-containing sample loading buffer. Samples were resolved on 12% or 16% SDS-PAGE gel, transferred to nitrocellulose, stained with Ponceau S, and probed with antibodies [anti-SIRT3 and anti-acyetylation lysine (Cell Signaling Technology, Danvers, MA); anti-NADH dehydrogenase (ubiquinone) 1 α subcomplex subunit 9 (NDUFA9) and anti-adenine nucleotide translocase 1 (Abcam-MitoSciences, Cambridge, MA); and anti-β-actin and anti-voltage-dependent anion channels (Calbiochem-Millipore, Darmstadt, Germany)] at 1:1,000 dilutions. In some cases, membranes were stripped and reprobed. A horseradish peroxidase-linked secondary antibody (GE Biosciences, Pittsburgh, PA) was used at 1:2,500 dilution, with enhanced chemiluminescence detection (Pierce, Rockford, IL, or GE Biosciences).

Statistics. Significance between the two groups was determined using Student’s t-test. Multiple comparisons were performed assuming a normal distribution using ANOVA with Tukey’s multiple comparison test (Prism 6.0 for Windows; GraphPad, La Jolla, CA).

RESULTS

SIRT3-deficient H9c2 cells (SIRT3kd) were used to test a role for SIRT3 in simulated IR injury. Figure 1A demonstrates that 50 nM of siRNA decreased the SIRT3 protein level down to 10% vs. nonsilencing siRNA (Ctrl), with no changes in the β-actin level. To check whether downregulation of SIRT3 modulates simulated IR injury, both Ctrl and SIRT3kd cells were subjected to 4 h of simulated ischemia, followed by 2 h of reperfusion. Figure 1B demonstrates that LDH release, measured at the end of the simulated IR protocol, was significantly higher in SIRT3kd cells (vs. Ctrl IR), indicating higher cell death in the SIRT3kd group.

In addition to being vulnerable to simulated IR injury, SIRT3kd cells exhibited remarkable metabolic disturbances. Indeed, despite no changes in the levels of NDUF9 (Fig. 1C), SIRT3kd cells exhibited significantly less Cx I activity (compared with Ctrl; Fig. 1D). A link between Cx I and cardioprotection is suggested by the observation that fully functional Cx I is necessary for endogenous cardioprotection (38). Further
supporting a role for SIRT3 in mitochondrial metabolism, SIRT3kd cells exhibited a lower OCR compared with Ctrl (Fig. 1E). A similar bioenergetic profile has been shown with SIRT3kd in C2C12 skeletal myoblasts (24, 54). A significant reduction in the reserve capacity in SIRT3kd cells (171 ± 20 pMoles/min vs. 287 ± 18 pMoles/min in Ctrl, P < 0.05; Fig. 1E) may predict poor tolerance to stress, and this may further exacerbate recovery from IR injury.

Next, with the use of a more physiologic approach of the Langendorff-perfused heart, we investigated if SIRT3+/−/adult hearts were more susceptible to IR injury compared with the WT adult. We also performed experiments using aged (18 mo) WT hearts with the objective of determining whether the biochemical and physiological changes seen in SIRT3+/− hearts (see below) were phenocopied by aging.

Pre-ischemic values of LVDP and HR are shown in Table 1. No significant differences were seen, although both LVDP and HR were slightly increased in SIRT3+/− hearts. During ischemia, the magnitude of hypercontracture was similar across all three groups (Table 1).

Upon reperfusion, we observed significantly worse recovery of the RPP in all three groups (vs. pre-ischemic level; Fig. 2A). Importantly, both adult SIRT3+/− and aged WT hearts were more susceptible to IR injury (worse RPP recovery) compared with adult WT hearts (Fig. 2A). This corresponded to an increase in the size of infarcted area in SIRT3+/− adult and WT aged hearts compared with adult WT (Fig. 2B). These results indicate that IR injury caused marked functional disturbances in SIRT3+/− adult and WT aged hearts.

Having established that adult SIRT3+/− and WT aged hearts possessed increased sensitivity to IR injury relative to young WT controls, we proceeded to investigate whether SIRT3 was downregulated in WT aged hearts. First, we examined the level of SIRT3 protein using immunoblot analysis of mitochondrial extract from WT adult, SIRT3+/− adult, and WT aged hearts. As expected, SIRT3 protein expression was decreased in SIRT3+/− adult hearts; however, no changes between WT adult and aged hearts were found (Fig. 3A). These results, however, do not eliminate the possibility that SIRT3 enzymatic activity might be impaired in WT aged hearts. Although the reliability of enzymatic SIRT activity assays has been questioned recently (42), mitochondrial protein acetylation has been used previously as a surrogate marker for SIRT3 activity in the heart (26, 55); thus we analyzed protein acetylation in mitochondrial samples isolated from WT adult, SIRT3+/− adult, and WT aged hearts. Figure 3B demonstrates that mitochondrial proteins were more acetylated in both SIRT3+/− adult and WT aged hearts compared with WT adult, with the most marked changes in protein acetylation observed in the 37- to 50-kDa range. The densitometry profiles revealed that acetylation in SIRT3+/− hearts was more pronounced than in WT hearts, indicating that IR injury caused marked functional disturbances in SIRT3+/− adult and WT aged hearts.
related patterns in the SIRT3+/− adult group matched those found in the WT aged group (Fig. 3B), indicating a link between downregulation of SIRT3 activity and protein acetylation in WT aged hearts. Figure 3C shows equal protein loading across the groups, indicating that increased signals observed in SIRT3+/− adult and WT aged heart mitochondria (Fig. 3B) were exclusively due to protein post-translational modifications in the form of acetylation.

Next, we tested the effect of IR injury on mitochondrial protein expression and lysine acetylation. After IR injury, SIRT3 protein level was similar in adult and aged WT hearts (Fig. 3D). Mitochondrial acetylation, normalized to corresponding loading controls, was significantly higher in adult SIRT3+/− and aged WT hearts relative to adult WT (Fig. 3E and F). These data indicate that IR injury did not change the pre-ischemic profiles of SIRT3 protein expression and mitochondrial acetylation.

To provide additional support for the possible downregulation of SIRT3 activity in aged WT hearts, we demonstrated (Fig. 4, A and B) that pre-ischemic activities of SIRT3 targets, Cx I and MnSOD, were significantly depressed in both SIRT3+/− adult and WT aged cardiac mitochondria compared with the WT adult. Figure 4, C and D, demonstrates that mitochondria isolated from both SIRT3+/− adult and WT aged hearts after IR injury also showed reduction in the activities of Cx I and MnSOD relative to adult WT.

**DISCUSSION**

The main findings of this study were as follows: 1) SIRT3-deficient (SIRT3−/−), cardiac-derived cells were more susceptible to simulated IR injury and exhibited lower Cx I activity and functional reserve capacity than control cells; 2) under IR injury conditions, SIRT3+/− adult and WT aged hearts showed a similar phenotype of injury, resulting in significant depression of RPP and a larger MI compared with WT adult hearts; 3) mitochondria isolated from WT aged hearts possessed a higher level of protein acetylation (vs. WT adult), and those acetylated bands matched with those detected in mitochondria isolated from SIRT3+/− hearts; and 4) activities of SIRT3 targets Cx I and MnSOD were diminished significantly in mitochondria isolated from both adult SIRT3+/− and aged WT hearts.

SIRT3 is important in stress responses, but its role may vary depending on the stressor. For example, SIRT3 plays a vital role in the accommodation of cellular metabolism to nonfatal stress conditions, such as dietary restriction (20). In contrast, SIRT3−/− mice were able to tolerate dobutamine-induced stress related to robust inotropic stimulation in a manner similar to their WT littermates (54). Since SIRT3 activity has been linked to regulation of mitochondrial function (21, 33), such discrepancies in stress tolerance might be related to the degree of mitochondrial involvement in each particular type of stress. Importantly, SIRT3 is required to maintain an endogenous cellular resistance to more severe cardiac stress, resulting in hypertrophy (18, 51). In this study, SIRT3 was shown to play an important role in a clinically relevant model of severe cardiac stress associated with IR injury (Figs. 1, A and B, and 2).

The downstream mechanisms by which SIRT3 appears to afford protection against IR injury remain unclear and might be related to one or more of the SIRT3-regulated, stress-response pathways. One SIRT3 target, which may impact IR injury, is the permeability transition pore, a nonselective mitochondrial channel that opens early during cardiac reperfusion and initiates mitochondrial swelling via membrane disruption (19). SIRT3 deacetylates cyclophilin D, a component of the permeability transition pore and prevents its opening (18, 49). SIRT3 may also enhance antioxidant defense via deacetylation and activation of MnSOD (46). Furthermore, deacetylation and activation of mitochondrial ICDH2 lead to an increase of NADPH pools that enhance antioxidant capacity (59). In addition, SIRT3 deacetylates Ku70, which promotes its interaction with BAX protein, preventing translocation to mitochondria (52). Previously, an important role of the electron transport chain, in particular for Cx I and II, in IR injury was demonstrated (9, 37, 57). SIRT3 interacts with and deacetylates several subunits of Cx I and II to regulate their activity (3, 10).
Even these limited observations suggest that SIRT3, via regulation of mitochondrial enzymes, can play an important role in recovery from IR injury.

Since the importance of these pathways might be tissue specific (13), we examined two of them and found both to be relevant for SIRT3+/− and aged cardiomyocytes. First, MnSOD activity was depressed significantly in mitochondria isolated from both adult SIRT3+/− and aged WT compared with adult WT (Fig. 4, B and D), and this is likely to increase the superoxide level. Second, Cx I activity was decreased at...
both the cellular (SIRT3kd vs. Ctrl; Fig. 1D) and organ (adult SIRT3+/− and aged WT hearts vs. adult WT; Fig. 4, A and C) levels. In this regard, a number of studies demonstrated that the SIRT3 downregulation and high mitochondrial protein acetylation coincided with cardiac mitochondrial disturbances associated with electron transport chain defects (26, 55). Specifically, increased mitochondrial protein acetylation has been shown in Cx I-deficient mice with cardiac-specific deletion of the NADH dehydrogenase (ubiquinone) Fe-S protein 4 (NDUSF4) (26) and in a mouse model of Freidreich’s ataxia, which has multiple defects in the electron transport chain (55). Furthermore, mice harboring defects in the electron transport chain and SIRT3−/− mice both developed heart failure faster than control mice (18, 26, 55) and recapitulated the cardiac phenotype of WT aged hearts (12). With the further establishment of a connection between increased susceptibility of aged hearts to cardiac stress and the role of downregulated SIRT3 in this process, we demonstrated that IR injury resulted in less functional recovery upon reperfusion and larger infarctions in both SIRT3+/− adult and WT aged hearts compared with WT adult hearts (Fig. 2).

Although SIRT3 protein levels are the same in WT adult vs. aged hearts (Fig. 3A), it is possible that SIRT3 enzymatic activity can be attenuated by the age-related increase in 4-hydroxynonenal adduction to the cysteine residue (15, 35) or other unidentified modifications. Such an impairment of SIRT3 activity is suggested by the high level of mitochondrial protein acetylation detected in WT aged hearts (Fig. 3B). Also, since IR injury did not alter the patterns or levels of increased protein acetylation in adult SIRT3+/− and aged WT mitochondria relative to adult WT (Fig. 3E), acute changes in protein acetylation may not be a primary modulator of IR injury.

Hyperacetylation of mitochondrial proteins has been widely used as a marker of SIRT3 activity in noncardiac tissues (13, 14, 21, 24, 31, 56). Hence, we demonstrated increased mitochon-
SIRT3, AGING, AND CARDIOPROTECTION

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DISCLOSURES

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS


REFERENCES


