Poly(ADP-ribose) Polymerase-1-dependent Cardiac Myocyte Cell Death during Heart Failure Is Mediated by NAD\textsuperscript{+} Depletion and Reduced Sir2\textalpha{\textalpha} Deacetylase Activity\textsuperscript{*}

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Robust activation of poly(ADP-ribose) polymerase-1 (PARP) by oxidative stress has been implicated as a major cause of caspase-independent myocyte cell death contributing to heart failure. Here, we show that depletion of myocyte NAD levels and the subsequent reduction of Sir2\textalpha{\textalpha} deacetylase activity are the sequential steps contributing to PARP-mediated myocyte cell death. In both failing hearts and cultured cardiac myocytes, the increased activity of PARP was associated with depletion of cellular NAD levels and reduced Sir2\textalpha{\textalpha} deacetylase activity. Myocyte cell death induced by PARP activation was prevented by depletion of cellular NAD levels either by adding NAD directly to the culture medium or by overexpressing NAD biosynthetic enzymes. The beneficial effect of NAD repletion was seen, however, only when Sir2\textalpha{\textalpha} was intact. Knocking down Sir2\textalpha{\textalpha} levels by small interfering RNA eliminated this benefit, indicating that Sir2\textalpha{\textalpha} is a downstream target of NAD replenishment leading to cell protection. NAD repletion also prevented loss of the transcriptional regulatory activity of the Sir2\textalpha{\textalpha} catalytic core domain resulting from PARP activation. We also show that PARP activation and the concomitant reduction of Sir2\textalpha{\textalpha} activity in failing hearts regulate the post-translational acetylation of p53. These data demonstrate that, in stressed cardiac myocytes, depletion of cellular NAD levels forms a link between PARP activation and reduced Sir2\textalpha{\textalpha} deacetylase activity, contributing to myocyte cell death during heart failure.

Heart failure is a pathological state in which the heart is unable to pump blood at a rate commensurate with the requirements of the metabolizing tissue. It is usually associated with heart enlargement and chamber dilation, in which contractile functions deteriorate. Although several abnormalities involving calcium homeostasis, contractile protein function, energetics, cytoskeleton rearrangement, and loss of viable myocytes have been identified, the basic molecular and cellular mechanisms that mediate the pathogenesis of heart failure remains poorly understood (1).

Poly(ADP-ribose) polymerase-1 (PARP)\textsuperscript{2} is a multifunctional DNA-bound enzyme located in the nuclei of various cells, including cardiac myocytes (2). PARP is activated in response to DNA single-break stands, which could develop as a response to free radical and oxidant cell injury (3). In addition, nuclear accumulation of Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, and polyamines has been shown to trigger PARP activation (4). Once activated, it catalyzes transfer of ADP-ribose moieties from NAD\textsuperscript{+} to the target proteins, with the concomitant formation of nicotinamide (2, 3). Under physiological conditions, mild activation of PARP regulates many cellular processes, including DNA repair, gene expression, cell cycle progression, cell survival, chromatin remodeling, and genome stability (2, 3). PARP has also been shown to physically bind to cardiac and skeletal muscle-specific transcription factors, TEF-1 and MyoD, and to regulate their muscle gene activation potential (5). However, overactivation of PARP represses gene transcription and threatens cell survival (2). We (6) and others (7) have shown recently that robust activation of PARP occurs under conditions of pressure overload cardiac hypertrophy and heart failure. PARP overactivation in the heart induces muscle gene dysregulation and myocyte cell death, without activation of caspases (6). The molecular mechanism behind PARP-mediated myocyte cell death is not known. Although one possibility is that PARP overactivation depletes cellular NAD content during addition of extended chains of ADP-ribose moieties on cellular proteins, including PARP itself (poly-(ADP-ribosylation)), thereby suppressing the activity of other NAD-dependent cellular processes.

In addition to protein ribosylation, other NAD-dependent cellular processes include energy metabolism, ADP-ribose cyclase synthesis, and class III histone deacetylase activity (8, 9). As NAD is essential for the mitochondrial electron transport reaction, depletion of NAD by PARP overactivation has been shown to repress mitochondrial function, leading to energy deficit, release of apoptosis-inducing factors (AIF), and eventually cell death (10, 11). In non-proliferating cells (such as neurons and cardiac myocytes), however, NAD has been shown to be compartmentalized, and the mitochondrial NAD pool is not easily depleted by PARP activation (9, 12). In these cells, PARP overactivation is likely to threaten cell survival by inhibiting the activity of other NAD-dependent pathways.

One group of factors that are greatly affected by changes in cellular NAD levels is the class III histone deacetylases, also called sirtuins and SIRT proteins (13). They are homologous to the yeast SIR2 gene, which has been implicated in chromatin silencing, cell survival, and aging (14). In mammals, seven different SIRT isoforms have been identified. Human SIRT1, an ortholog of mouse Sir2\textalpha{\textalpha}, is primarily located in the cell nucleus, whereas other SIRT isoforms are located within the cytoplasm and mitochondria (14). The deacetylase activity of SIRT proteins is dependent upon the availability of cellular NAD content. They catalyze a unique reaction in which NAD is broken into nicotinamide and ADP-ribose. The acetyl group that is removed from the substrate is
transferred to an ADP-ribose moiety, giving rise to formation of O-acetyl-ADP-ribose, a novel metabolite (15). SIRT proteins are considered nuclear targets of redox signaling and function as energy sensors (16, 17). Reduced cellular levels of NAD attenuate the SIRT deacetylation activity, and this increases the activity of many apoptotic effectors, e.g., p53 and Ku70, causing cells to become senescent or apoptotic (18). Sir2α is also considered a longevity factor inasmuch as the life span of the organism is shortened by a null mutation of the sir2 gene, and it is extended by the presence of an extra copy (19, 20). In mice, deletion of the sir2 gene is lethal, and the rare surviving animals exhibit developmental defects of the retina and heart (21). A protective role of Sir2α has also been demonstrated in the brain, where overactivation of Sir2α was shown to protect the brain against axonopathy and neurodegeneration (22).

Here, we show that PARP activation in cardiac myocytes during heart failure results in myocyte cell death by decreasing the cellular NAD levels, consequently reducing the activity of Sir2α deacetylation. PARP-mediated myocyte cell death is protected by replenishing cellular NAD levels as well as by activation of Sir2α. These results are the first to demonstrate a link between poly(ADP-ribosylation) and Sir2α-mediated deacetylation pathways and document a novel mechanism of nuclear signaling contributing to myocyte cell death during heart failure.

**MATERIALS AND METHODS**

**Cell Culture and Transfection**—Primary cultures of cardiac myocytes were prepared from 2-day-old neonatal rat hearts as described previously (6). Myocytes were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and 5 mg/ml each penicillin and streptomycin (Invitrogen). Typically 1 × 10⁵ myocytes were transfected 48 h after plating using the TfxTM-20 reagent (22). 24 h after plating. The luciferase and medium and transfected with the Lipofectamine reagent (Invitrogen) peroxidase-conjugated anti-rabbit IgG secondary antibody (1:100 dilution; Upstate Biotechnology catalog no. 07-131) primary antibodies and phycoerythrin-conjugated anti-rabbit IgG secondary antibody (1:500 dilution; Santa Cruz Biotechnology catalog no. sc-7150) and anti-Sir2α (1:100 dilution; Upstate Biotechnology catalog no. 07-131) primary antibodies and phycoerythrin-conjugated anti-rabbit IgG secondary antibody (1:500 dilution; Santa Cruz Biotechnology catalog no. sc-3739). Myocytes were maintained under serum-free conditions, and cell death was induced by treatments with angiotensin (20 μM) and H2O2/FeSO4 (0.1 mM each). Treatment with resveratrol (0.5 μM) was performed under serum-free conditions for 18 h before transfection. H9C2 and COS-7 cells were grown in Dulbecco’s modified Eagle’s medium and transfected with the Lipofectamine reagent (Invitrogen) 24 h after plating. The luciferase and β-galactosidase assays were performed as described (6).

**Aortic Banding**—Aortic banding was carried out in adult mice to produce pressure overload hypertrophy/heart failure as described (6). Adult mice weighing ~30 g were anesthetized and ventilated. The chest was opened at the second intercostal space. The aorta was isolated from the adjacent tissue and banded between carotid arteries over a 27-gauge needle, which was immediately removed. Animals with sham surgery underwent the identical procedure with the exception of band placement. Animals were killed 6 weeks post-surgery, and their hearts were removed and analyzed for the development of cardiac hypertrophy and heart failure. In 6-week banded mice, the heart weight/tibia length ratio increased by 70% compared with control mice, and they had clear signs of ventricular dilation, a clinical symptom of heart failure. The expression of the atrial natriuretic peptide, brain natriuretic peptide, and β-myosin heavy chain hypertrophy marker genes was elevated in the hearts of banded mice. These hearts were also analyzed by immunohistochemical analysis to determine the extent of fibrosis, and massive fibrosis was detected in the hearts of banded mice compared with the sham-operated control mice. The detailed characterization of the hypertrophy of these mice has been published previously (6).

**Western Blot Analysis**—Whole cell extracts from mouse and human left ventricles were made in 2 volumes of extraction buffer containing 62.5 mM Tris (pH 6.8), 6 mM urea, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.003% bromphenol blue, and protein inhibitors. The samples were then sonicated for 20 s (Microtipset limit 40% duty cycle sonicator, Sonics & Materials, Inc., Newtown, CT). Protein concentration was determined using the BCA protein assay kit (Pierce), and samples were either stored frozen in a ~80 °C freezer or analyzed the same day. Typically, 75 μg of protein sample (unless otherwise specified) was heated at 65 °C for 15 min and resolved on an 8% SDS-polyacrylamide gel. Western blot analysis was performed according to standard procedures described previously (6). The primary antibodies used for the analysis were as follows: mouse anti-poly(ADP-ribose) monoclonal antibody (Alexis Biochemicals catalog no. ALX-804-220), rabbit anti-Sir2α antibody, anti-Lys373/Lys382 p53 antibody (Upstate Biotechnology catalog no. 06-758), rabbit anti-p53 polyclonal antibody (Santa Cruz Biotechnology catalog no. sc-6243), rabbit anti-PARP antibody, and goat anti-glyceraldehyde-3-phosphate dehydrogenase antibody (Santa Cruz Biotechnology catalog no. sc-20357). Primary antibodies were used at 1:1000 dilution, and horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse antibodies were used at 1:3000 dilution.

**Immunoprecipitation Analysis**—Cells (or tissue) were washed with ice-cold phosphate-buffered saline (PBS) and lysed (homogenized) in radioimmunoprecipitation assay buffer (1× PBS, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 10 mg/ml phenylmethylsulfonyl fluoride, 100 mM sodium orthovanadate, and protease inhibitors). Protein (500 μg) from each group was incubated overnight with rabbit anti-p53 antibody (1:150 dilution) at 4 °C with gentle rocking. Protein A/G PLUS-agarose beads (50 μl; Santa Cruz Biotechnology catalog no. sc-2003) were added and incubated for 4 h at 4 °C. The beads were then pelleted by centrifugation, washed repeatedly with PBS, and analyzed by Western blotting.

**Measurement of Myocyte Cell Death**—Myocytes were washed three times with PBS and then stained with two DNA-binding dyes, Hoechst 33342 and propidium iodide (Molecular Probes), according to the manufacturer’s protocol. Hoechst 33342 readily penetrates the cell membrane and stains the nuclei of all (both live and dead) cells. Propidium iodide does not penetrate the cell membrane and stains the nuclei of dead cells only. Cells were incubated for 5 min with the dye reagent, washed with PBS, and visualized under a fluorescence microscope. To determine the extent of death in both the adherent and detached populations of cells, fluorescence-activated cell sorting (FACS) analysis was performed. For this analysis, cells were trypsinized, and the enzyme was inactivated by addition of an equal volume of fetal bovine serum. Cells were collected by centrifugation, and a single cell suspension of myocytes was prepared in PBS and stained with both Hoechst 33345 and propidium iodide for 5 min. Cells that detached spontaneously were also collected and included for the analysis. Cells were washed twice, resuspended in PBS, and analyzed by FACS analysis (LSR II, BD Biosciences). Cell debris was gated out using forward scatter versus side scatter, and propidium iodide versus Hoechst 33345 fluorescence was...
analyzed by gating propidium iodide-negative and propidium iodide-positive cells.

**Human Failing Heart Samples**—Left ventricular samples were obtained from patients with end stage heart failure at the time of heart transplantation. Control samples were obtained from patients with non-failing hearts who underwent valve surgery. The patients studied suffered from ischemic cardiomyopathy (**n** = 7), idiopathic dilated cardiomyopathy (**n** = 5), or restrictive cardiomyopathy (**n** = 2). Control myocardial specimens were obtained from five patients with non-failing hearts who underwent valve surgery. All samples were immediately frozen in liquid nitrogen and stored at −80 °C until analyzed. Patients received different combinations of the following pharmacological agents: digoxin, dobutamine, angiotensin-converting enzyme inhibitors, calcium channel blockers, β-adrenergic blockers, and diuretics. These heart samples were also analyzed previously to detect the activity of PARP (6).

**Recombinant DNA**—The luciferase reporter plasmid with multiple Gal4 DNA-binding sites in the promoter region and the expression plasmids encoding the Gal4 DNA-binding domain (Gal4DBD), Gal4DBD fused to the Sir2α catalytic core domain (Gal4DBD-mCORE), Gal4DBD fused to the catalytic core domain with a mutation that eliminates deacetylase activity (Gal4DBD-mCORE(H355A)), Namp1, and Nmnat have been described (23). The PARP expression plasmid was a gift from Dr. W. Dawson (Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD). The wild-type Sir2α and mutant Sir2α(H355A) expression plasmids were provided by Dr. W. Gu (Department of Pathology, College of Physician and Surgeons, Columbia University, New York, NY). His-tagged pcDNA3.1-Nmnat1 and His-tagged pcDNA3.1-Nmnat1(W170A) were provided by Dr. Jeffrey Milbrandt (Washington University School of Medicine, St. Louis, MO). Small interfering RNA (siRNA)-mediated silencing of Sir2 was carried out by transfection using the pSUPER RNAi System™ (Oligo-Engine catalog no. VEC-PBS-0003/0004) with incorporation of 20 nucleotides of Sir2α (gaagttgacctcctcattgt) (22).

**NAD and ATP Estimation**—The NAD levels were measured according to the method described previously by Jacobson and Jacobson (24) with slight modification. An average of 1 × 10^5 cells or 50 mg of frozen

![FIGURE 1. PARP overactivation in cardiac myocytes depletes cellular NAD levels. **A**, heart failure was produced in mice by aortic banding (band) for 6 weeks. Control (Cont) and failing heart muscle samples were analyzed by Western blotting using anti-PARP and anti-poly(ADP-ribose) (PAR) antibodies. The blots shown are representative tracings of an experiment done seven times. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **B**, NAD content in the same heart samples used in A was determined as described under “Materials and Methods.” **C and D**, cardiac myocytes (1 × 10^5) were transfected with varying amounts of PARP expression plasmid. Forty-eight hours following transfection, cells were harvested, and NAD and ATP contents were measured. Values are means of 3 separate experiments. *p*, significantly different from control (**p** < 0.01, test).

**RESULTS**

**PARP Activation Depletes Cellular NAD Levels**—Robust PARP activation in both animal and human failing hearts has been implicated as a major cause of caspase-independent myocyte cell death during heart failure (6). To examine the mechanism of PARP-mediated myocyte cell death, we first measured the NAD content in mouse failing hearts, in which massive poly(ADP-ribosylation) of nuclear proteins was observed. We found that the increased PARP activity was associated with a significant reduction (30%) of the NAD content in each of the seven failing heart samples analyzed (Fig. 1, A and B). To demonstrate a direct link between PARP activation and the change in cellular NAD content, we transfected primary cultures of neonatal rat cardiac myocytes or H9C2 myocytes with varying amounts of PARP expression plasmid. Cells transfected with the β-galactosidase expression plasmid...
were used as a reference control. Forty-eight hours following transfection, cells were harvested and ATP, and the NAD content was measured. As shown in Fig. 1C, the cellular NAD content declined gradually with increasing amounts of PARP, but not with β-galactosidase expression in the cells. However, the cellular ATP content declined at a much slower rate (Fig. 1D). When massive PARP expression was introduced, both NAD and ATP levels declined significantly compared with their respective controls. Data obtained from the time course study with the PARP expression plasmid indicated that the maximum decline in NAD levels (~50%) occurred within the first 24 h, with the remaining NAD levels maintained even up to 4 days after cell transfection (data not shown). Hoechst 33345 and propidium iodide staining of cells indicated that PARP overexpression resulted in concentration-dependent cell death. More than 50% of the cells died at 48 h following administration of 2.0 μg of PARP expression plasmid, when a nearly 50% reduction of NAD content was observed (Fig. 1E).

To test the effect of endogenous PARP activation, we analyzed the effect of an alkylating DNA-damaging agent, N-methyl-N’-nitro-N-nitrosoguanidine (MNNG), which is known to stimulate PARP activity (12). As shown in Fig. 2, cells treated with 10 μM MNNG showed a nearly 15% decline in cellular NAD levels; however, no significant cell death could be detected at this dose of MNNG even after 3 days of treatment (Fig. 2B). When cells were treated with a higher dose of MNNG (100 μM), cellular NAD levels reduced gradually in a time-dependent manner (Fig. 2A). Almost 50% of the NAD level dropped by 4–8 h of MNNG treatment, and thereafter, most of the remaining NAD level was maintained. In these plates, cell death reached a plateau following 16 h of MNNG treatment, when nearly 60% of the cells were found dead (Fig. 2B). To examine whether PARP was indeed involved in MNNG-mediated cell death, one batch of cells was pretreated with a PARP inhibitor (3-aminobenzamide). The results indicated that 3-aminobenzamide treatment markedly reduced the MNNG-induced cell death, thus confirming a role of PARP in this type of cell death. These results together demonstrate that PARP activation in cardiac myocytes leads to fast depletion of cellular NAD levels, and this might contribute to PARP-mediated myocyte cell dysfunction and death.

**NAD Repletion Protects Myocytes against PARP-mediated Cell Death**—Knowing that PARP-mediated myocyte cell death is associated with reduced NAD content, we next investigated whether repletion of NAD could offer cell protection. Previous reports have indicated that, similar to neuronal cells, cardiac myocytes express membrane channels that can bind and transport extracellular NAD (25). This encouraged us to examine whether exogenously added NAD could prevent cardiac myocyte cell death. To test this possibility, we added different concentrations of NAD (0.1–0.5 mM) to the culture medium immediately following treatment of cells with MNNG or transfection of cells with the PARP expression plasmid. As shown in Fig. 3A, inclusion of 100 μM NAD in the culture medium significantly reduced (50%) MNNG-mediated myocyte cell death. Likewise, cell death induced by PARP overexpression was found to be significantly less (~50%) in culture plates with varying amounts of NAD (0.1–0.5 mM) compared with control cells receiving vehicle (Fig. 3B). Maximum protection against PARP-mediated myocyte cell death.
A Linkage between PARP and Sir2α Deacetylase

Reduced Sir2α activity contributes to PARP-mediated myocyte cell death—Because NAD is essential for Sir2 deacetylase activity, we next examined whether PARP activation attenuates Sir2α deacetylase activity. Mouse failing heart samples with robust PARP activation were also analyzed to determine Sir2α deacetylase activity. We found that the failing heart samples with increased poly(ADP-ribose)ylation of proteins were consistently associated with significantly reduced Sir2α deacetylase activity (Fig. 4A). To confirm these results, we measured Sir2α activity in myocytes treated with the PARP inhibitor, 3-aminobenzamide. As shown in Fig. 4B, treating cells with 3-aminobenzamide elevated the levels of Sir2α, suggesting that PARP levels modulate the activity of Sir2α. We next investigated whether PARP-mediated cell death could be prevented by activation of Sir2α. We cotransfected myocytes with a Sir2α expression plasmid or treated them with a Sir2 activator (resveratrol). Sir2α activation in these cells was verified by immunostaining or Western analysis, and the extent of cell death was measured by Hoechst 33345 and propidium iodide staining, followed by FACS analysis. Interestingly, we found that PARP-mediated cell death was markedly reduced by overexpression of wild-type Sir2α, but not by the mutant Sir2α(H355A), which lacks deacetylase activity (Fig. 4C). Likewise, resveratrol treatment prevented cell death induced by PARP overexpression as well as by MNNG treatment of cells (Fig. 4, C and E).

To examine whether the reduced level of cell death correlated with NAD levels, we also measured cellular NAD levels in these cells. As shown in Fig. 4C (lanes 2 and 3), PARP overexpression alone significantly reduced the cellular NAD content, as expected. However, much to our surprise, we found that the NAD content of cells treated with resveratrol or cells overexpressing Sir2α was not significantly reduced even when the same amount of PARP was overexpressed in these cells, which alone resulted in a nearly 50% reduction of the NAD content (Fig. 4C, lanes 4–6 and 8–10). These experiments were repeated at least three times with different cell cultures and plasmid preparations, and same results were found. These results suggest that the activation of Sir2α deacetylase can prevent PARP-mediated NAD depletion.

To determine a link between PARP and Sir2α during stimulation of cells with a natural hypertrophic stimulus, we examined the effect of angiotensin II (Ang-II) on these nuclear targets of redox signaling. Ang-II is known to produce cardiac hypertrophy and cell death by stimulating the synthesis of reactive oxygen/nitrogen species (26). To test whether PARP is a downstream target of Ang-II-mediated hypertrophy, we treated primary cultures of cardiac myocytes with 20 μM Ang-II, a dose known to induce cell death. For a positive control, cells were stimulated with a free radical-generating mixture of H2O2 and FeSO4 (0.1...
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mM each). On the third day after treatment, cells were harvested, and the cell lysate was prepared and analyzed by Western blotting to detect PARP activation. As shown in Fig. 6A, stimulation of cells by Ang-II or H2O2/FeSO4 increased the activity of PARP, as evident from the elevated levels of poly(ADP-ribosylated) proteins. We then investigated whether oxidative stress-induced cell death resulting from PARP activation could be protected by Sir2α activation. Cells were treated with the Sir2α activator resveratrol together with Ang-II or H2O2/FeSO4. On the third day following treatments, cells were stained with Hoechst 33342 and propidium iodide, indicating massive myocyte cell death. When cells were treated with resveratrol, a large number of cells became resistant to H2O2/FeSO4- and Ang-II-mediated cell death (Fig. 6, A and B). Resveratrol-treated cells were widely spread out and had no signs of cell shrinkage. They also made better connections among themselves and appeared in synchronously contracting islands of cells. In contrast, cells treated with the Sir2α inhibitor sirtinol shrank; the majority of them detached from the bottom of the plate and almost all of the remaining cells stained positive for propidium iodide, indicating massive cell death. Thus, these results collectively demonstrate that (i) Sir2α activation protects cardiac myocytes from oxidative stress- and Ang-II-mediated cell death and that (ii) PARP-mediated cell death results in part from reduced Sir2α deacetylase activity.

PARP Activation Eliminates the Transcriptional Regulatory Activity of the Sir2α Catalytic Core Domain—To further establish a link between PARP activation and reduced Sir2α deacetylase activity, we utilized a reporter gene transcription assay in which the Gal4DBD fusion system is used to analyze the activity of histone deacetylases in vivo. This system, the expression plasmid encodes a fusion protein with the Gal4DBD fused to the Sir2α catalytic core domain (Gal4DBD-mCORE), and the reporter plasmid has a luciferase reporter gene that is driven by the thymidine kinase minimum promoter and four tandem repeats of Gal4 DNA-binding sites (23). In this assay, repression of the reporter gene (luciferase) activity reflects the NAD-dependent deacetylase activity of the expression plasmid. Cardiac myocytes and/or COS-7 cells were cotransfected with different combinations of plasmids. Forty-eight hours following transfection, cells were harvested, and luciferase activity was determined. Expression of cytomegalovirus-β-galactosidase was used as a reference control. As shown in Fig. 7A, the reporter gene activity was significantly repressed by overexpression of Gal4DBD-mCORE compared with the activity of the Gal4DBD control. However, when the H355A mutation, which destroys NAD-dependent Sir2α deacetylase activity, was introduced into the core domain, this repressive activity was abolished. The repressive activity of Gal4DBD-mCORE was also abolished by addition of nicotinamide, a chemical inhibitor of Sir2α (data not shown). These results demonstrate that NAD-depend-
ent deacetylase activity is required for the repressive activity of the Gal4DBD-mCORE fusion protein. To investigate the effect of PARP on Sir2α deacetylase activity, cells were cotransfected with a constant amount of Gal4DBD-mCORE expression plasmid and increasing amounts of PARP expression plasmid. We found that PARP overexpression completely blocked the transcriptional repressive activity of Gal4DBD-mCORE in a concentration-dependent manner (Fig. 7A).

This blockage of the transcriptional activity of Sir2α was reversed, however, by overexpressing cells with the NAD biosynthetic enzyme Nmnat or Nampt, which restored the cellular NAD levels (Fig. 7B). Thus, these data strongly indicate that the poly(ADP-ribosylation) and Sir2α deacetylase reactions are linked by alterations in cellular NAD levels.

PARP Activation Promotes Acetylation of p53—Finally, to identify a target protein that could be subject to post-translational modification by both the PARP and Sir2α enzymes, we examined the acetylation of p53. p53 is known to be deacetylated by Sir2α, which in turn silences the pro-apoptotic activity of p53 (18, 21). To demonstrate the impact of PARP activation on the acetylation of p53, COS-7 cells were transfected with varying amounts of PARP expression plasmid. Cells treated with the Sir2 inhibitor nicotinamide or with the class I and II histone deacetylase inhibitor trichostatin A were utilized as positive and negative controls, respectively. On the second day following transfection, cells were harvested, and p53 was immunoprecipitated and analyzed by Western blotting using anti-acetylated Lys373/Lys382 p53 antibody. We found that PARP overexpression elevated the acetylation level of p53 in a concentration-dependent manner. As expected, the activity of acetylated p53 was also increased by nicotinamide, but not by the trichostatin A treatment of cells (Fig. 8A). However, no change was found in the expression levels of p53 in any groups of these cells, suggesting that the increased content of acetylated p53 was due to protein modification and not to altered protein levels.

Increased PARP Activity and p53 Acetylation in Human Failing Hearts—A previous study from this laboratory has shown activation of PARP in human failing hearts (6). To examine whether increased PARP activity changes the acetylation status of p53, we measured the levels of Sir2α and acetylated p53 in the same human failing heart samples, in which PARP was heavily activated. As shown in Fig. 8B, we found that the increased activity of PARP was accompanied by reduced levels of Sir2α and increased activity of acetylated p53 in human failing heart samples. However, no change was observed in p53 protein content.

FIGURE 7. PARP regulates the transcriptional regulatory activity of the Sir2α catalytic core domain by modulating the cellular NAD levels. A, increasing doses of PARP inhibit the transcriptional activity of the Sir2α core domain recruited into a reporter gene. COS-7 cells were cotransfected with the luciferase reporter plasmid (1.0 μg) together with different combinations of expression plasmids (0.5 μg) as indicated. The PARP expression plasmid was used in increasing amounts (0.2–5 μg). Forty-eight hours following transfection, cells were harvested, and luciferase activity was measured. Expression of β-galactosidase was used as a reference control. Values are means ± S.E. of four to eight separate experiments. *, significantly different (p < 0.001; t test) compared with the control (cells receiving the Gal4DBD vector), mt, mutant. B, overexpression of NAD biosynthetic enzymes (Nampt and Nmnat) restores the transcriptional repressive activity of the Sir2α core domain. Cells were cotransfected with different combinations of plasmids (5.0 μg) as indicated. Luciferase activity was determined 48 h after transfection and normalized to β-galactosidase expression in the same samples. Parallel plates of cells were also used to determine cellular NAD levels. Values are means ± S.E. of three separate experiments. * and [carat], luciferase and NAD values, respectively, were significantly different (t test) compared with the control (cells receiving the Gal4DBD vector).

FIGURE 8. PARP overactivation induces p53 acetylation. A, COS-7 cells were transfected with increasing amounts of PARP expression plasmid or treated with nicotinamide (NAM) or trichostatin A (TSA). After 24 h of transfection or treatment, cells were harvested; the cell lysate was prepared; and 500 μg of protein was subjected to p53 immunoprecipitation. Acetylated p53 and total p53 levels were determined by Western analyses using specific antibodies. C, control. B, human non-failing (Control) and failing heart (Failing H.) samples were analyzed by Western blotting to measure the levels of poly(ADP-ribosylation) (PAR), Sir2α, and p53. Acetylated p53 levels were determined by immunoprecipitation of p53, followed by Western analysis using anti-acetylated p53 antibody. The blots shown are from two separate non-failing (control) and failing hearts of patients suffering from dilated ischemic cardiomyopathy.
between control and failing hearts. These results suggest that the change in PARP activity regulates the post-translational acetylation of p53 and provide further support for a linkage between PARP activation and reduced Sir2α deacetylase activity.

DISCUSSION

The main objective of this study was to determine the mechanism of cardiac myocyte cell death associated with PARP overactivation. Several lines of evidence presented here show that PARP activation depletes cellular NAD levels, consequently repressing Sir2α deacetylase activity and leading to myocyte cell death. For instance, (i) increased poly(ADP-riboseylation) of nuclear proteins in failing hearts was accompanied by reduced levels of NAD. Also, overexpression of PARP in primary cultures of cardiac myocytes depleted the cellular NAD levels. (ii) PARP-mediated cell death was prevented by NAD replenishment. (iii) PARP activation resulted in reduced Sir2α deacetylase activity. (iv) PARP-mediated cell death was exacerbated and prevented by reducing and increasing the activity of Sir2α, respectively. Thus, these data strongly demonstrate a linkage between PARP and Sir2α activities, which appear to be inversely related at a higher magnitude of PARP activation, and suggest that myocyte cell death occurring during the progression of cardiac hypertrophy to heart failure might be mediated in part by reduced Sir2α deacetylase activity.

Role of PARP in Cardiac Hypertrophy and Cell Death—In a heart with a sustained work load, myocytes undergo a hypertrophic growth response, the initiating events of which are similar to those that drive cell cycle progression in proliferating cells. A continuous growth signal in myocytes causes cells at some point to malfunction and leads to cell death. As cells die, the work load of the remaining cells increases, which further aggravates this process and eventually leads to organ failure (27). In both humans and animals with different cardiac disorders, myocyte cell death has been implicated as a common cause of cardiac dysfunctions (28). However, the mechanism of cell death in a failing heart remains highly disputed (28, 29). With regard to the role of caspases in myocyte cell death, conflicting data have been reported. Although activation of caspases in ischemic heart disease seems fairly accepted, its participation in non-ischemic diseases remains controversial (Refs. 27 and 28 and references therein). During the early stages of cardiac hypertrophy (or physiological hypertrophy), no activation of caspases has been reported, whereas PARP activation and apoptosis have been observed accompanying the adaptive phase of hypertrophy (6, 30). Some investigators have shown that, during sustained pressure overload, caspase activation peaks only during hypertrophy of the myocardium; thereafter, it declines in failing hearts (31). Others have presented even stronger evidence against caspase-mediated cell death during heart failure. Knaapen et al. (32) have shown that cardiomyocytes undergo caspase-dependent cell death in embryonic hearts, whereas they go through caspase-independent autophagic cell death in adult failing hearts. Recently, we (6) and others (33) reported that PARP is not cleaved (marker of caspase activation), but rather its expression is progressively increased in relation to the degree of cardiac hypertrophy, suggesting that hemodynamic stress endangers cardiac myocytes through a mechanism that is different from the conventional caspase-mediated apoptosis. The data presented here show that activation of PARP during hypertrophy consumes cellular NAD levels and that loss of the NAD pool beyond a critical level threatens the activity of Sir2α deacetylase, which consequently leads to myocyte cell death.

In a recent study, Alcendor et al. (34) showed that Sir2α activation protects cardiac myocytes from apoptosis. Paradoxically, they found increased levels of Sir2α in pacing-induced heart failure. However, even after studying a large number of banded mouse hearts and human failing hearts, we failed to detect induction of Sir2α in failing heart samples. The reason for this discrepancy is likely to be the different model systems used. The mechanism of evolution of cardiac hypertrophy by continuous pacing and pressure overload differs significantly in many aspects, including stability of the disease and the final outcome of the myocyte phenotype. It will be interesting to test whether PARP levels are also changed in pacing-induced failing heart samples, in which Sir2α levels were found to be elevated.

PARP activation during increased cardiac demand is not always bad. PARP has been shown to play a role in cell survival/growth and gene regulation. PARP has also been shown to participate in cardiac muscle-specific gene transcription by binding to transcription enhancer factor-1 (5). In the brain, stress-mediated atrophy of neurons was shown to be accompanied by reduced levels of PARP, implying that PARP has a role in the normal cell growth and development of neurons (35). We have recently shown that a mild activation of PARP also occurs during exercise-induced physiological hypertrophy, in which no decline in heart function and myocyte cell death is detectable (6). Thus, it appears that PARP plays a role in the development of both physiological and pathological cardiac hypertrophy depending upon the magnitude of PARP activation.

PARP Activation Depletes Myocyte NAD Levels—In this study, we found that, in response to PARP activation, myocyte NAD levels dropped sharply, but never went below 50% of the control values, indicating that a large pool of NAD remains inaccessible to PARP activation in cardiac myocytes. It is also important to note that a nearly 15% decline in NAD content upon MNNG treatment was not sufficient to cause myocyte cell death, suggesting that a small portion of the NAD pool is liberally available for the poly(ADP-ribosylation) reaction and that utilization of this pool does not fatally hamper the activity of other NAD-dependent reactions. This is consistent with our observation that PARP activation during physiological hypertrophy was not accompanied by down-regulation of Sir2α activity (data not shown). One explanation for this incomplete depletion of NAD could be derived from studies with astrocytes and nerve cells, in which only a partial depletion of the NAD pool was observed upon activation of PARP by DNA-damaging agents (12, 36). A previous report has indicated that intracellular NAD is segregated into cytosolic and mitochondrial pools that do not readily inter-change (37). It has also been shown that, in neurons, PARP activation leads to preferential depletion of the cytosolic NAD pool without altering the mitochondria; NAD pool, unless mitochondrial permeability is compromised (12). A similar situation is likely to exist for cardiac myocytes, as a considerable amount (>50%) of NAD in myocytes has been shown to be present within the mitochondria, and it is impermeable to cytosol (9). Thus, in this study, the observed decline in NAD content following PARP activation is likely to be of cytosolic and nuclear origin. Loss of this NAD pool is expected to impede the activity of NAD-dependent reactions in these compartments. Our data presented here demonstrate that depletion of NAD and the consequent suppression of the activity of the longevity factor Sir2α are the sequence of events of PARP activation that lead in part to myocyte cell death during heart failure.

PARP activation is also known to kill cells by translocation of AIF from mitochondria to the nucleus, where it induces DNA degradation without activation of caspases (10). The mechanism of cell death by AIF translocation seems to come in play when mitochondrial integrity is compromised. Yu et al. (10) have reported that, when mitochondria are isolated in NAD-free buffer, no AIF release can be detected, suggesting that loss of cytosolic and nuclear NAD pools is not sufficient to trigger
the release of AIF from mitochondria. Instead, severe NAD consumption, which could limit substrate delivery to the mitochondria, might act as a signal to initiate the death program through AIF translocation (36). At least two lines of evidence obtained from our myocyte cultures argue against the role of AIF as a primary component of PARP-mediated cell death. (i) PARP activation does not allow NAD levels to go below 50% of the control levels. (ii) Complete rescue of myocytes from PARP-mediated cell death could be achieved by NAD replenishment and by Sir2α deacetylase activation. Still, unlike in cultured myocytes, in a mechanically overloaded heart, a role of AIF in myocyte cell death cannot be excluded. In fact, in a mouse model of heart failure accumulation of AIF in the nuclear fraction of the heart has been demonstrated (7). Recently, in astrocytes, PARP activation-dependent release of AIF from mitochondria was shown to be prevented by NAD replenishment, suggesting that severe decrement of NAD levels is an upstream mechanism of AIF translocation (12). Thus, it appears that PARP activation could kill cells by either mechanism, by reducing Sir2α deacetylase activity and/or by AIF translocation, depending upon the degree of loss of the NAD pool. The preference of the mechanism will certainly lie with the magnitude of PARP activation. During moderate PARP activation, when mitochondrial functions are preserved, cells will die preferably due to reduced Sir2α deacetylase activity, and under conditions of severe PARP activation, when mitochondrial integrity is compromised, they will die by AIF translocation. In a decompensated failing heart, it is very likely that both mechanisms contribute to myocyte cell death when massive PARP activation occurs. However, as the reduced activity of Sir2α seems to be an earlier event than the AIF release (guided by the extent of NAD loss), contributing to myocyte cell death, it seems reasonable to believe that the progression of heart failure associated with cell death could be delayed or prevented by activation of Sir2α deacetylase.

Enhanced Activity of Sir2α Protects Myocytes against PARP-mediated Cell Death—in mammals, at least seven different SIRT isoforms have been identified. SIRT1 (human ortholog of mouse Sir2α) is localized primarily in the nucleus, whereas other SIRT isoforms are located within the cytoplasm and/or mitochondria (14). Sir2α regulates chromatin remodeling, gene transcription, and the activity of many apoptotic effectors, including p53 (18). To determine which Sir2 isoform is involved in protecting cells with NAD replenishment, experiments were performed with siRNA constructs to knockdown the Sir2α transcripts. The suppression of Sir2α expression eliminated the protection offered by NAD replenishment as effectively as sirtinol treatment, indicating that Sir2α is the main contender of increased NAD supply that prevents myocyte cell death by PARP activation. The myocyte protection observed with resveratrol treatment also supports the involvement of Sir2α, as knocking down Sir2α eliminated this protection. These results are in agreement with a recent report showing that increased NAD biosynthesis activates SIRT1 to effectively block axonal self-destruction (22).

How does Sir2α activation protect cells against PARP-mediated cell death? It is important to note that Sir2α activation protects cells against PARP-mediated cell death when Sir2α is expressed (activated) either before or together with PARP activation, but not after PARP activation. Also, the Sir2α mutant (which lacks deacetylase activity) provides no protection, indicating that Sir2α deacetylase activity is essential for its protective effects. Another important finding worth noting is that PARP-mediated NAD depletion was prevented in cells overexpressing Sir2α. These findings suggest that Sir2α activation (either directly or through metabolites of its deacetylation reaction) might inhibit the activity of PARP, leading to preservation of cellular NAD levels. In this context, it will be worthwhile to test whether the novel metabolite of the Sir2 deacetylation reaction, O-acetyl-ADP-ribose, which has physiological activity (15), has the potential to regulate the activity of PARP. This needs to be formally demonstrated, but if it turns out to be true, it will mean that the by-products of both poly(ADP-ribosylation) (nicotinamide) and NAD-dependent deacetylation (O-acetyl-ADP-ribose) reactions regulate each other’s activity. This will also explain how the PARP-Sir2 axis of signaling is intertwined to control the balance between cell survival and death in a given physiological situation. Other possibilities for the opposite effects of PARP and Sir2α on cell survivability could be envisioned based upon their many common targets of post-translational modification, e.g. Ku70, p53, and NF-κB. Both p53 and Ku70 have been shown to bind to PARP and to be poly(ADP-ribosylated), a change that enhances their pro-apoptotic activity, whereas Sir2α-dependent deacetylation suppresses their apoptotic activity (17, 18, 21, 38, 39). Likewise, PARP activation poly(ADP-ribosylates) both p65 and p50 subunits of NF-κB and, together with p300, synergistically activates NF-κB-dependent gene expression (40). On the other hand, Sir2α deacetylase inhibits the transactivation potential of NF-κB by directly deacetylating the RelA/p65 subunit (41). Thus, Sir2α and PARP could exert an opposite effect on cell survivability by modifying one or more of these common nuclear targets. Future studies directed toward identifying the cardiac targets of poly(ADP-ribosylation) should be able to discriminate between these possibilities and explain more precisely how PARP and Sir2α regulate myocyte cell survivability.

In summary, in this study, we have identified a novel mechanism of myocyte cell death attributed to depletion of NAD and subsequent down-regulation of Sir2α deacetylase activity. A model of PARP activation and its downstream signaling mechanism contributing to myocyte

FIGURE 9. Schematic model of PARP activation and downstream sequence of events leading to cardiac myocyte cell death. Cell stress activates Ca2+/R (ON) S, which adds massive negative charge to the transcription factors, it could interfere with their transcriptional activity, which could limit substrate delivery to the mitochondria, might act as a signal to initiate the death program through AIF translocation (36). At least two lines of evidence obtained from our myocyte cultures argue against the role of AIF as a primary component of PARP-mediated cell death. (i) PARP activation does not allow NAD levels to go below 50% of the control levels. (ii) Complete rescue of myocytes from PARP-mediated cell death could be achieved by NAD replenishment and by Sir2α deacetylase activation. Still, unlike in cultured myocytes, in a mechanically overloaded heart, a role of AIF in myocyte cell death cannot be excluded. In fact, in a mouse model of heart failure accumulation of AIF in the nuclear fraction of the heart has been demonstrated (7). Recently, in astrocytes, PARP activation-dependent release of AIF from mitochondria was shown to be prevented by NAD replenishment, suggesting that severe decrement of NAD levels is an upstream mechanism of AIF translocation (12). Thus, it appears that PARP activation could kill cells by either mechanism, by reducing Sir2α deacetylase activity and/or by AIF translocation, depending upon the degree of loss of the NAD pool. The preference of the mechanism will certainly lie with the magnitude of PARP activation. During moderate PARP activation, when mitochondrial functions are preserved, cells will die preferably due to reduced Sir2α deacetylase activity, and under conditions of severe PARP activation, when mitochondrial integrity is compromised, they will die by AIF translocation. In a decompensated failing heart, it is very likely that both mechanisms contribute to myocyte cell death when massive PARP activation occurs. However, as the reduced activity of Sir2α seems to be an earlier event than the AIF release (guided by the extent of NAD loss), contributing to myocyte cell death, it seems reasonable to believe that the progression of heart failure associated with cell death could be delayed or prevented by activation of Sir2α deacetylase.

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In summary, in this study, we have identified a novel mechanism of myocyte cell death attributed to depletion of NAD and subsequent down-regulation of Sir2α deacetylase activity. A model of PARP activation and its downstream signaling mechanism contributing to myocyte deacetylase activity by resveratrol treatment to prevent cell dysfunction and death during heart failure.
cell dysfunction and death during heart failure is given in Fig. 9. Our data support many previous studies showing that myocardial oxidative stress contributes to the development of cardiac hypertrophy and myocyte cell death independent of caspase activation. These data also suggest that alterations of the NAD biosynthetic pathway and/or changes in Sir2 deacetylase activity will provide new therapeutic opportunities for the management of cardiac diseases involving myocyte cell death.

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