Nicotinamide Phosphoribosyltransferase Regulates Cell Survival Through NAD⁺ Synthesis in Cardiac Myocytes

Chiao-Po Hsu, Shinichi Oka, Dan Shao, Nirmala Hariharan, Junichi Sadoshima

Rationale: NAD⁺ acts not only as a cofactor for cellular respiration but also as a substrate for NAD⁺-dependent enzymes, such as Sirt1. The cellular NAD⁺ synthesis is regulated by both the de novo and the salvage pathways. Nicotinamide phosphoribosyltransferase (Nampt) is a rate-limiting enzyme in the salvage pathway.

Objective: Here we investigated the role of Nampt in mediating NAD⁺ synthesis in cardiac myocytes and the function of Nampt in the heart in vivo.

Methods and Results: Expression of Nampt in the heart was significantly decreased by ischemia, ischemia/reperfusion and pressure overload. Upregulation of Nampt significantly increased NAD⁺ and ATP concentrations, whereas downregulation of Nampt significantly decreased them. Downregulation of Nampt increased caspase 3 cleavage, cytochrome c release, and TUNEL-positive cells, which were inhibited in the presence of Bcl-xL, but did not increase hairpin 2–positive cells, suggesting that endogenous Nampt negatively regulates apoptosis but not necrosis. Downregulation of Nampt also impaired autophagic flux, suggesting that endogenous Nampt positively regulates autophagy. Cardiac-specific overexpression of Nampt in transgenic mice increased NAD⁺ content in the heart, prevented downregulation of Nampt, and reduced the size of myocardial infarction and apoptosis in response to prolonged ischemia and ischemia/reperfusion.

Conclusions: Nampt critically regulates NAD⁺ and ATP contents, thereby playing an essential role in mediating cell survival by inhibiting apoptosis and stimulating autophagic flux in cardiac myocytes. Preventing downregulation of Nampt inhibits myocardial injury in response to myocardial ischemia and reperfusion. These results suggest that Nampt is an essential gatekeeper of energy status and survival in cardiac myocytes. (Circ Res. 2009;105:481-491.)

Key Words: NAD⁺ • apoptosis • myocardial ischemia

Nicotinamide adenine dinucleotide (NAD⁺) participates in redox reactions as a transfer molecule for electrons. Because of its involvement in the mitochondrial TCA cycle and the electron transport chain, NAD⁺ acts as a key cofactor for energy production. NAD⁺ also serves as the substrate for various enzymes, including the nuclear enzyme poly(ADP-ribose) polymerase (PARP)-1, and the class III histone deacetylases, i.e., the sirtuin family. Because the sirtuin family plays an essential role in mediating lifespan extension, stress resistance and regulation of metabolism, NAD⁺ may control the level of stress resistance in cells partly through regulation of sirtuins.

NAD⁺ can be freshly synthesized from amino acids, including tryptophan or aspartic acid, via the de novo pathway or taken up efficiently from the extracellular space. Importantly, NAD⁺ can also be resynthesized from NAD⁺ metabolites through the salvage pathway. In yeast, increased expression of pyrazinamidase/nicotinamidase 1, a nicotinamidase converting nicotinamide to nicotinic acid, is both necessary and sufficient for lifespan extension induced by calorie restriction and low-intensity stress, such as osmotic stress. Nicotinamide phosphoribosyltransferase (Nampt) is a rate-limiting enzyme in the mammalian NAD⁺ salvage pathway, and has been proposed to be a functional equivalent of pyrazinamidase/nicotinamidase 1 in mammals. Upregulation of Nampt increases the cellular NAD⁺ level and enhances the transcriptional regulatory activity of the catalytic domain of Sirt1 in mouse fibroblasts. In HEK293 cells, Nampt is an essential component of the mitochondrial NAD⁺ salvage pathway and promotes cell survival through stimulation of mitochondrial sirtuins, including Sirt3 and Sirt4. However, because the cellular content of NAD⁺ may be regulated by multiple mechanisms in a cell type–specific manner, it remains to be elucidated whether Nampt plays an essential role in regulating the cellular content of NAD⁺ in cardiac myocytes.

We have shown previously that Sirt1 protects cardiac myocytes from serum starvation-induced cell death in vitro, and that mild to modest overexpression of Sirt1 in the heart protects the heart from oxidative stress and retards the...
Nampt affects myocardial injury in response to ischemia and reperfusion in vivo. We examined how expression of Nampt is regulated in the heart under stress. Mice were subjected to clinically relevant forms of stress in the heart, including 24 hours of ischemia, 45 minutes of ischemia followed by 24 hours of reperfusion (I/R), and 2 or 4 weeks of pressure overload by transverse aortic constriction (TAC). Mice develop compensated hypertrophy 2 weeks after TAC and heart failure 4 weeks after TAC. Protein expression of Nampt was significantly downregulated after ischemia, I/R, and 2 or 4 weeks of TAC (Figure 1A). Nampt mRNA was also downregulated in response to these stimuli, as determined by quantitative RT-PCR (Figure 1B), suggesting that downregulation of Nampt protein expression is at least, in part, attributable to downregulation of Nampt mRNA. Downregulation of Nampt was observed within 6 hours of ischemia (Online Figure I, A). Reduced Nampt levels during pressure overload reverted to the control level when pressure overload was removed (Online Figure I, B). Downregulation of Nampt after I/R was confirmed by immunostaining at the level of individual myocytes in the heart (Figure 1C). On the other hand, expression of Nampt in the heart increased during aging (6-fold at 1 year), suggesting that regulation of Nampt expression is stimulus-dependent (Figure 1D).

**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AAR</td>
<td>area at risk</td>
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<tr>
<td>Ad</td>
<td>adenovirus</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>I/R</td>
<td>ischemia/reperfusion</td>
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<td>LV</td>
<td>left ventricular</td>
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<tr>
<td>MMS</td>
<td>methylmethane sulfonate</td>
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<tr>
<td>moi</td>
<td>multiplicity of infection</td>
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<tr>
<td>NAD⁺</td>
<td>nicotinamide adenine dinucleotide</td>
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<tr>
<td>Nampt</td>
<td>nicotinamide phosphoribosyltransferase</td>
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<tr>
<td>NTg</td>
<td>nontransgenic</td>
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<tr>
<td>PARP</td>
<td>poly(ADP-ribose) polymerase</td>
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<tr>
<td>sh</td>
<td>short hairpin</td>
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<tr>
<td>TAC</td>
<td>transverse aortic constriction</td>
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<td>Tg</td>
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**Results**

**Nampt Is Downregulated by Pathological Stimuli In Vivo**

We examined how expression of Nampt is regulated in the heart under stress. Mice were subjected to clinically relevant forms of stress in the heart, including 24 hours of ischemia, 45 minutes of ischemia followed by 24 hours of reperfusion (I/R), and 2 or 4 weeks of pressure overload by transverse aortic constriction (TAC). Mice develop compensated hypertrophy 2 weeks after TAC and heart failure 4 weeks after TAC. Protein expression of Nampt was significantly downregulated after ischemia, I/R, and 2 or 4 weeks of TAC (Figure 1A). Nampt mRNA was also downregulated in response to these stimuli, as determined by quantitative RT-PCR (Figure 1B), suggesting that downregulation of Nampt protein expression is at least, in part, attributable to downregulation of Nampt mRNA. Downregulation of Nampt was observed within 6 hours of ischemia (Online Figure I, A). Reduced Nampt levels during pressure overload reverted to the control level when pressure overload was removed (Online Figure I, B). Downregulation of Nampt after I/R was confirmed by immunostaining at the level of individual myocytes in the heart (Figure 1C). On the other hand, expression of Nampt in the heart increased during aging (6-fold at 1 year), suggesting that regulation of Nampt expression is stimulus-dependent (Figure 1D).

**Nampt Critically Regulates NAD⁺ and ATP Levels in Cardiac Myocytes**

To elucidate the function of Nampt in cardiac myocytes, either Ad-Nampt or Ad–short hairpin (sh)RNA-Nampt was transduced into cultured neonatal rat cardiac myocytes. Transduction of Ad-Nampt dose-dependently increased expression of Nampt in cardiac myocytes (Figure 2A). Upregulation of Nampt induced by Ad-Nampt (3 multiplicities of infection [mois]) modestly but significantly increased the cellular level of NAD⁺ (1.32±0.15-fold, *P*<0.01) compared to Ad-LacZ (3 mois) (Figure 2B), accompanied by a significant increase in the cellular ATP content (1.19±0.13-fold, *P*<0.01) (Figure 2C) in cardiac myocytes. The cellular level of NAD⁺ at baseline and in response to Nampt overexpression was greater when nicotinamide, a substrate of Nampt, was added to the culture medium (Figure 2B). Conversely, transduction of Ad-shRNA-Nampt significantly decreased expression of Nampt in cardiac myocytes (Figure 2D). Downregulation of Nampt induced by Ad-shRNA-Nampt significantly decreased the cellular level of NAD⁺ (0.67±0.36-fold, *P*<0.05), an effect which was reversed by supplementation of the culture media with NAD⁺ (500 μmol/L), which is delivered into cells through transporter proteins, such as connexin 43 (Figure 2E). Downregulation of Nampt also caused a significantly decrease in the cellular ATP content (0.84±0.15-fold, *P*<0.01) in cardiac myocytes, which was also reversed by addition of NAD⁺ (500 μmol/L) to the culture media (Figure 2F). As expected, glucose deprivation significantly reduced the ATP content, serving as a positive control of the assay.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**NAD⁺ and ATP Measurements**

NAD⁺ was measured using the EnzyChrom NAD⁺/NADH Assay Kit according to the protocol of the manufacturer (ECND-100, Bioassay Systems, Hayward, Calif).

**Transgenic Mice**

Nampt transgenic mice (Tg-Nampt) were generated using the α-myosin heavy chain promoter (courtesy of J. Robbins, University of Cincinnati, Ohio) to achieve cardiac-specific expression of the transgene on an FVB background. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey.

**Statistics**

Statistical analyses between groups were performed by 1-way ANOVA, and differences among group means were evaluated using Fisher’s project least significant difference post test procedure for group data with a probability value less than 0.05 considered significant.
Figure 1. Nampt is downregulated by pathological stimuli in vivo. A, Heart homogenates were prepared from mice subjected to ischemia for 24 hours, ischemia for 45 minutes and reperfusion for 24 hours, and TAC for 2 or 4 weeks. Expression of Nampt, GAPDH, and actin was evaluated by immunoblots. B, The effect of pathological stress on the expression level of Nampt mRNA in the heart. Nampt
Nampt Positively Regulates Survival in Cardiac Myocytes

We investigated whether Nampt affects cell survival in cardiac myocytes. Cardiac myocytes were transduced with either Ad-LacZ or Ad-Nampt at 3 mois. Forty-eight hours after transduction, myocytes were treated with methylmethanesulfonate (MMS), a DNA alkylating agent known to hyperactivate PARP-1 and induce necrotic cell death,15 or glucose deprivation for 4 hours. We then evaluated cell viability using the CellTiter-Blue assay. Myocytes transduced with Ad-Nampt were modestly but significantly more resistant to cell death than those with Ad-LacZ in the presence of MMS or glucose deprivation (Online Figure II, A). Downregulation of Nampt slightly increased the number of TUNEL-positive cardiac myocytes (Figure 3A), consistent with the notion that they may induce cardiac myocyte apoptosis via a common mechanism. Downregulation of Nampt did not significantly increase the number of necrotic myocytes, as evaluated by in situ ligation of hairpin probes with blunt ends (hairpin 2) (Figure 3D).17 Treatment of myocytes with 1.2 mmol/L MMS, induced a significant increase in hairpin 2 labeled cardiac myocytes, thereby serving as a positive control (Figure 3D). Propidium iodide staining of unpermeabilized myocytes also showed that Nampt downregulation alone did not induce necrotic cell death (Online Figure III, A). However, Nampt downregulation significantly increased propidium iodide–positive myocytes in the presence of glucose deprivation (Online Figure III, B and C). Taken together, these results suggest that downregulation of Nampt induces apoptotic, but not necrotic, cell death at baseline, and could facilitate necrotic cell death under stress. Downregulation of Nampt slightly increased the activity of PARP, as evaluated by immunoblotting of proteins containing poly(ADP-ribose) polymer (Online Figure IV), which would facilitate NAD+ depletion and cell death in cardiac myocytes.

Knockdown of Nampt Inhibits Autophagic Flux

FK866, a potent inhibitor of Nampt, induces autophagy, but not apoptosis, in SH-SY5 neuroblastoma cells.6 Because downregulation of Nampt decreases the ATP content in cardiac myocytes, we examined whether downregulation of Nampt induces autophagy, a compensatory mechanism which preserves cellular ATP levels.18 Knockdown of Nampt by Ad-shRNA-Nampt increased LC3-II, a lipided form of LC3 that is an indicator of autophagosome accumulation, in a dose-dependent manner (Figure 4A). Importantly, however, knockdown of Nampt also induced a significant increase in p62 (SQSTM1/sequestosome 1), a protein known to be degraded by autophagy (Figure 4B),19–21 suggesting that Nampt downregulation inhibits autophagic flux. Nampt knockdown did not affect mRNA expression of p62 (Online Figure V, A), suggesting that increased p62 expression is unlikely to be attributable to increased production. To further examine whether downregulation of Nampt affects autophagic flux, we evaluated the effect of chloroquine, which inhibits autophagosome–lysosome fusion, on accumulation of autophagosomes.22 Chloroquine treatment alone increased LC3-II and Ad-shRNA-Nampt failed to further increase LC3-II, suggesting that downregulation of Nampt does not increase autophagosome formation (Online Figure V, B). Accumulation of autophagosomes was also evaluated by observation of green fluorescent protein (GFP)-LC3 dots. Downregulation of Nampt induced accumulation of GFP-LC3 dots in cardiac myocytes, consistent with the results of the LC3-II immunoblot analysis. Accumulation of GFP-LC3 dots was not attributable to adenovirus transduction because it was not observed when control adenovirus was used. As expected, accumulation of GFP-LC3 dots was induced by chloroquine because of suppression of autophagosome–lysosome fusion. As with the LC3-II immunoblot analysis, downregulation of Nampt in the presence of chloroquine did not further increase autophagosome accumulation, again
suggesting that downregulation of Nampt does not stimulate autophagic flux, but rather inhibits either autophagosome-lysosomal fusion or lysosomal degradation of autophagosome cargos (Figure 4C).

Figure 2. Nampt regulates NAD\(^+\) and ATP levels in cardiac myocytes. Cardiac myocytes were transduced with Ad-LacZ, Ad-shRNA-scramble, Ad-Nampt, or Ad-shRNA-Nampt at indicated multiplicities of infection (MOI). B. − indicates Ad-LacZ and + Ad-Nampt. In A and D, cell lysates were subjected to immunoblot analyses with anti-Nampt, anti-GAPDH, and anti-tubulin antibodies. In B, C, E, and F, cellular [NAD\(^+\)] and [ATP] were measured using the EnzyChrom NAD\(^+\)/NADH Assay Kit and ATP Bioluminescent Assay Kit, respectively. In B, cells were cultured with either normal culture media or those supplemented with 20 mmol/L nicotinamide for 16 hours. In C through F, the level of Nampt, [NAD\(^+\)], and [ATP] in control virus transduced myocytes is expressed as 1. In E and F, cardiac myocytes were cultured with or without 500 \(\mu\)mol/L NAD\(^+\) or glucose-free medium for 24 hours. Data represent the mean of 4 experiments ± SEM.

Expression and/or activation of several proteins (mTOR, AMPK, p70S6K, 4E-BP1, and Beclin-1) associated with autophagy were not significantly affected by knockdown of Nampt (Online Figure VI). Exogenously applied NAD\(^+\)
significantly reversed the accumulation of autophagosomes induced by knockdown of Nampt (Figure 4C), suggesting that the effect of Nampt downregulation was mediated through decreases in NAD⁺.

The level of proteins containing acetyl-lysine was significantly increased after knockdown of Nampt in cardiac myocytes. The level of acetyl-lysine in SirT1 substrates, including Histone H3 and H4, was also increased by Nampt knockdown (Online Figure VII). These results are consistent with the notion that the downregulation of Nampt inhibits the activity of SirT1. Because SirT1 stimulates autophagy in HeLa and MEF cells,²³ we examined the effect of SirT1 knockdown on autophagy in cardiac myocytes. Downregulation of SirT1 by adenovirus harboring Ad-shRNA-SirT1 caused accumulation of LC3-II and p62 (Figure 4D), suggesting that, like downregulation of Nampt, downregulation of SirT1 inhibits autophagic flux. Furthermore, Ad-shRNA-Nampt and Ad-shRNA-SirT1 did not show additive effects on accumulation of LC3-II and p62 (Figure 4D). These results are consistent with the notion that downregulation of Nampt inhibits autophagic flux through suppression of SirT1.

**Generation of Transgenic Mice With Cardiac-Specific Overexpression of Nampt**

To examine the in vivo function of Nampt, transgenic mice with cardiac specific overexpression of Nampt (Tg-Nampt) were generated. Line nos. 8 and 9 expressed Nampt 2-fold and 3.5-fold in the heart, respectively, compared to respective control nontransgenic (NTg) mice (Online Tables I and II). The level of NAD⁺, NADH, and NAD/NADH in the heart was significantly greater in Tg-Nampt than in NTg (Figure 5C). Overexpression of Nampt in the heart in vivo induced greater increases in NAD⁺ content than that in cardiac myocytes in vitro (Figure 2B). Although endogenous Nampt in the heart is downregulated in response to ischemia, the level of Nampt in Tg-Nampt mice after ischemia was equivalent to the basal level of Nampt in NTg (Figure 5D).

To examine the effect of Nampt on myocardial injury attributable to I/R, Tg-Nampt and NTg mice were subjected
to 45 minute of ischemia and twenty-four hours of reperfusion, and the size of myocardial infarction was evaluated by triphenyltetrazolium chloride staining (Figure 6A). The size of the area at risk (AAR) was not significantly different between Tg-Nampt and NTg mice (Figure 6A). However, the size of myocardial infarction/AAR after I/R was significantly smaller in Tg-Nampt mice than in NTg mice (Figure 6A), and the number of TUNEL-positive cells in the ischemic border zone was also smaller in Tg-Nampt mice than in WT mice (Figure 6B). Protection against I/R injury in Tg-Nampt was observed in both line nos. 8 and 9. These results suggest that Nampt plays a protective role against I/R and that downregulation of Nampt may play an important role in mediating myocardial injury in response to ischemia and I/R.

Because downregulation of Nampt inhibits autophagy, we hypothesized that increased expression of Nampt during

Figure 4. Knockdown of Nampt affects autophagic flux, in a manner similar to chloroquine treatment. A and B, Myocytes were transduced with Ad-shRNA-scramble (Ad-sh-Scr.) or Ad-shRNA-Nampt (Ad-sh-Nampt) at indicated multiplicities of infection (MOI) and cultured for 96 hours. Cell lysates were subjected to immunoblot analyses with anti-LC3 (A), anti-p62 (B), and anti-tubulin (B) antibodies. The values of LC3-II/LC3-I and p62 expression in myocytes transduced with Ad-shRNA-scramble are designated as 1. Experiments were conducted 3 times. C, Myocytes were transduced with Ad-GFP-LC3 (10 mois) and either Ad-shRNA-scramble or Ad-shRNA-Nampt at 3 mois and cultured for 96 hours. Cell lysates were subjected to immunoblot analyses with anti-LC3, anti-p62, anti-Sirt1, anti-Nampt, and anti-tubulin antibodies. The results shown are representative of 3 experiments.
ischemia may protect the heart by stimulating autophagy. The size of myocardial infarction/AAR after 2 hours of ischemia was significantly smaller in Tg-Nampt than in NTg (Figure 6C). Furthermore, the level of autophagic flux after 2 hours of ischemia was greater in Tg-Nampt than in NTg, as indicated by less accumulation of p62 in Tg-Nampt (Figure 6D). Taken together, these results are consistent with the notion that downregulation of Nampt during ischemia may be detrimental, in part, because of inhibition of autophagic flux.

Discussion

Expression of Nampt and its contribution to NAD\(^+\) synthesis are cell type–dependent. Our results clearly suggest that Nampt is both necessary and sufficient for the regulation of the intracellular level of NAD\(^+\) in cardiac myocytes. It should be noted, however, that a 75% reduction of Nampt expression by shRNA-induced knockdown causes only a 30% reduction in intracellular NAD\(^+\) content, suggesting that the NAD\(^+\) may also be synthesized from alternative sources, including nicotinic acid and tryptophan, in cardiac myocytes.\(^5\) Alternatively, only a small amount of Nampt may be sufficient to maintain the cellular level of NAD\(^+\) in cardiac myocytes. It should be noted that Nampt overexpression induced greater increases in NAD\(^+\) contents in the heart in vivo than in cardiac myocytes in vitro. We speculate that the availability of NAD\(^+\) precursors, which could be greater in the heart in vivo than in cardiac myocytes kept in culture medium in vitro, may affect the relative importance of Nampt mediated NAD\(^+\) production among the various mechanisms of NAD\(^+\) synthesis.

Expression of Nampt is significantly downregulated under stress in the heart in vivo, at both protein and mRNA levels. Downregulation of Nampt may contribute to decreases in NAD\(^+\) during pathological conditions in the heart. During heart failure, enzymes consuming NAD\(^+\), such as PARP\(^{24}\) and Sirt1, are upregulated,\(^10\) whereas Nampt is downregulated. Nampt downregulation also stimulates PARP (Online Figure IV). This would induce conditions in which the cellular level of NAD\(^+\) is decreased. It has been shown that expression of Nampt is upregulated by hypoxia in cultured cardiac myocytes in vitro.\(^4\) However, upregulation was not observed in vivo at either the mRNA or protein level. Interestingly, expression of Nampt was significantly upregulated during aging in the mouse heart in vivo, although the level of NAD\(^+\) was similar between 1 to 2 months and 12 to 14 months of age (Online Figure VIII). Upregulation of Nampt during aging may be a compensatory mechanism to maintain the cardiac level of NAD\(^+\). Thus, expression of Nampt is regulated in a stimulus-dependent manner. The molecular mechanism regulating expression of Nampt remains to be elucidated.

One of the most prominent actions of Nampt in cardiac myocytes is to regulate the cellular ATP level. A positive correlation was observed between the cellular Nampt level and both NAD\(^+\) and ATP. Decreases in the cellular ATP content attributable to knockdown of Nampt were normalized by exogenously supplied NAD\(^+\). Transfer of electrons is
among the most important functions of NAD$^+$ as a coenzyme, and the energy produced by glycolysis and the citric acid cycle is transferred to NAD$^+$ by its reduction to NADH. Our results suggest that Nampt could be an important target for modulation of the energy status in cardiac myocytes.

Our results suggest that the downregulation of Nampt causes increases in cell death in cardiac myocytes (Figure 7). The cell death induced by Nampt knockdown has characteristic features of apoptosis mediated through a mitochondrion-dependent mechanism but not necrosis or autophagic cell death because (1) the cell death was accompanied by cytochrome $c$ release and inhibited by Bcl-xL; (2) there was no increase in propidium iodide–positive nuclei or hairpin 2 ligation–positive cells$^{17}$; and (3) the cell death was not accompanied by increases in autophagy. The fact that downregulation of Nampt induces apoptosis, which is an energy consuming process, despite the fact that Nampt knockdown decreases the cellular ATP content, is intriguing. It is possible that decreases in cellular ATP content may not be severe enough to induce either autophagy or necrosis. In fact, decreases in the cellular ATP content attributable to Nampt knockdown were not as severe as those attributable to glucose deprivation. Downregulation of Nampt does increase necrotic cell death during glucose starvation, suggesting that more severe ATP depletion causes necrosis. One mechanism me-

Figure 6. I/R injury is attenuated in Tg-Nampt mice. A, Tg-Nampt and NTg mice were subjected to 45 minutes of ischemia and 24 hours of reperfusion. Upper, Gross appearance of LV myocardial sections after Alcian blue and triphenyltetrazolium chloride staining. Lower left, The AAR (percentage of LV) was comparable between NTg and Tg-Nampt. Lower right, The infarction area/AAR was significantly smaller in Tg-Nampt than in NTg mice. B (left), LV myocardial sections were subjected to TUNEL and DAPI staining. Representative images of the staining in the border zone are shown. Right, The number of TUNEL-positive myocytes is expressed as a percentage of total nuclei detected by DAPI staining. C and D, Tg-Nampt and NTg mice were subjected to 2 hours of ischemia. C (upper), Gross appearance of LV myocardial sections after Alcian blue and triphenyltetrazolium chloride staining. Lower left, The AAR (percentage of LV) was comparable between NTg and Tg-Nampt. Lower right, The infarction area/AAR was significantly smaller in Tg-Nampt than in NTg. D, Expression of p62 and tubulin at the baseline and after 2 hours of ischemia was determined by immunoblot analyses. The results shown are representative of 5 experiments.

Figure 7. Present hypothesis. The proposed Nampt pathway in cardiac myocytes under stresses.
diating apoptosis induced by downregulation of Namp is suppression of Sirt1, an NAD⁺-dependent enzyme. Although Namp downregulation causes only a partial reduction in NAD⁺, Namp may directly channel NAD⁺ into the active site of Sirt1.23 We have shown previously that downregulation of Sirt1 induces apoptosis in cardiac myocytes.9 Activation of PARP-1, an NAD⁺-consuming enzyme, induces death of cardiac myocytes through suppression of Sirt1.24 The fact that downregulation of both Namp and Sirt1 failed to show an additive effect on myocyte death is consistent with the notion that these 2 molecules are in the same signaling pathway inducing death of cardiac myocytes.

Our results suggest that downregulation of Namp inhibits autophagic flux in cardiac myocytes. Because downregulation of Namp potently induces accumulation of p62 and mimics the action of chloroquine, a chemical inhibitor of autophagosome lysosome fusion,22 we speculate that downregulation of Namp inhibits either autophagosome–lysosomal fusion or degradation of the cargos in the lysosome, which would explain the accumulation of LC3-II in the presence of Namp downregulation. Because inhibition of autophagic flux is normalized by addition of NAD⁺, basal autophagy in cardiac myocytes must be NAD⁺-dependent, which is intriguing because autophagy is inhibited even in the presence of mild energy starvation, a condition which favors autophagy. Downregulation of Namp during myocardial ischemia may possibly exacerbate energy deprivation through suppression of autophagy. Consistent with this notion, inhibition of Namp downregulation reduced the size of myocardial infarction after prolonged ischemia.

FK866, an inhibitor of Namp, induces autophagy in neuroblastoma cells.6 Although FK866 induced increases in LC3-positive vesicles in neuroblastoma cells, whether or not FK866 also stimulates lysosomal degradation of autophagosome cargo has not been tested. FK866 treatment did not induce apoptosis in neuroblastoma cells, whereas downregulation of Namp induces apoptosis in cardiac myocytes. We speculate that the role of NAD⁺ in mediating different forms of cell death may be cell type–dependent. Alternatively, inhibition of Namp may affect the cellular NAD⁺ level differently in different cell types.

Because the histone deacetylase activity of Sirt1 is NAD⁺-dependent, downregulation of Namp causes suppression of Sirt1 activity. Recent evidence suggests that Sirt1 stimulates autophagy through deacetylation of Atgs in cancer cells.23 Downregulation of Sirt1 increased accumulation of p62, suggesting that it suppresses autophagic flux, thereby mimicking the effect of Namp downregulation in cardiac myocytes. Furthermore, downregulation of both Namp and Sirt1 did not show additive effects on inhibition of autophagic flux, consistent with the notion that Namp may inhibit autophagic flux through suppression of Sirt1. Further investigation is required to elucidate the role of Sirt1 in mediating the effect of Namp downregulation on autophagy in cardiac myocytes. An electron transport chain present in lysosomes maintains a proton gradient at the expense of NADH.26 Decreases in NAD⁺ may directly inhibit the function of lysosomes, which may also contribute to the suppression of autophagy induced by downregulation of Namp.

Namp can act as a secreted hormone, termed visfatin, and may exert insulin-mimetic effects.27 However, overexpression of Namp in cardiac myocytes stimulates neither Akt/p70S6K signaling in vitro nor cardiac hypertrophy in vivo, suggesting that the action of Namp as an extracellular ligand may be negligible at a level of up to 4-fold overexpression in the heart.

Namp expression in the heart significantly reduced the size of myocardial infarction after ischemia and I/R, suggesting that Namp has a protective function in the heart in vivo. Because downregulation of endogenous Namp after ischemia and I/R is normalized in Tg-Namp, the result suggests that downregulation of Namp has a causative role in mediating myocardial injury during ischemia or I/R. Because downregulation of Namp decreases cellular ATP content, increases apoptosis, and inhibits autophagy, we speculate that all of these contribute to myocardial injury after I/R. Reperfusion after prolonged ischemia causes release of NAD⁺ from mitochondria through mPTP opening and subsequent downregulation of NAD⁺,28 which would exacerbate myocardial damage by I/R. Importantly, because the detrimental effects of Namp downregulation in cardiac myocytes appear to be rescued by exogenously applied NAD⁺, increasing the cellular level of NAD⁺ by targeting Namp may be a promising strategy to reduce injury from I/R. Increased Namp provides protection against cell death and requires an intact mitochondrial NAD⁺ salvage pathway, as well as the mitochondrial NAD⁺-dependent deacetylases SIRT3 and SIRT4, in vitro.4 Whether or not this holds true for the in vivo heart has not been tested. Further studies should elucidate the downstream molecular mechanism by which NAD⁺ protects the heart from ischemic injury.

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Disclosures

None.

References

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Expanded Materials and Methods

Antibodies

Anti-Nampt antibody was purchased from Bethyl and Abcam. Other antibodies include anti-Sirt1 (Upstate Biotechnology), anti-Bcl-xL (Pharmingen), anti-tubulin (Sigma), anti-cleaved-caspase 3 (Cell Signaling), anti-LC3 (MBL), and anti-p62 (American Research Products, Inc.) anti-cytochrome C (Sigma), anti-cytochrome c oxidase I (COX I) (Sigma), anti-hyper-acetylated Histone H4 (Millipore), anti-phospho-AMPKα (Thr172), anti-AMPK, anti-phospho-4E-BP1 (Thr37/46), anti-4E-BP1, anti-phospho-p70 S6K (Thr389), anti-p70 S6K, anti-phospho-mTOR (Ser2448), anti-mTOR, anti-phospho-Akt (Ser473), anti-Akt, anti-acetyl-lysine, anti-acetyl-Histone H3 (Lys 23) (Cell Signaling), anti-Beclin 1 (BD Biosciences), and anti-GAPDH (Chemicon) antibodies.

Adenovirus vectors

We made replication-defective human adenovirus type 5 (devoid of E1) harboring Nampt and shRNA-Nampt. Adenovirus harboring β-galactosidase or scramble shRNA was used as a control. Adenoviruses harboring Sirt1, shRNA-Sirt1, Bcl-xL, and GFP-LC3 have been described 1,2.

Primary cultures of neonatal rat ventricular myocytes

Primary cultures of ventricular cardiac myocytes were prepared from 1-day-old Crl: (WI) BR-Wistar rats (Harlan). A cardiac myocyte-rich fraction was obtained by centrifugation through a discontinuous Percoll gradient. Cells were cultured in complete medium (CM) containing Dulbecco's modified Eagle's medium (DMEM)/F-12 supplemented with 5% horse serum, 4 μg/mL transferrin, 0.7 ng/mL sodium selenite (Life Technologies, Inc.), 2 g/L bovine serum albumin.
(fraction V), 3 mmol/L pyruvic acid, 15 mmol/L HEPES, 100 µmol/L ascorbic acid, 100 µg/mL ampicillin, 5 µg/mL linoleic acid, and 100 µmol/L 5-bromo-2'-deoxyuridine (Sigma) 3.

**TUNEL staining in cultured cardiac myocytes**

Myocytes were fixed in PBS containing 3.7% paraformaldehyde. Staining was performed using the In Situ Cell Death Detection kit (Roche) 4.

**Hairpin2**

A 196 bp double stranded DNA fragment was prepared using primers 5′-CCCTGTAGCCGCTTAAG-3′ and 5′-GGTCGAGGTGCCGTAAAGCA-3′ complementary to pBluescript SK(−) (Stratagene). Polymerase chain reaction (PCR) with *PfuUltra* polymerase was performed with 16.6 µmol/L Texas Red-12-dUTP (Molecular Probes), 16.6 µmol/L dTTP, 50 µmol/L dATP, 50 µmol/L dCTP and 50 µmol/L dGTP. Agarose gel electrophoresis of an aliquot of the reaction showed a single product. Cells were fixed and permeabilized in methanol and acetone. After washing with PBS, a mix of 50 mmol/L Tris-HCl, pH 7.8, 10 mmol/L MgCl₂, 10 mmol/L DTT, 1 mmol/L ATP, 25 µg/mL BSA, 15% polyethylene glycol (8,000 mol wt, Sigma), Texas red-labeled DNA fragment at 1 µg/mL and DNA T4 ligase (Boehringer Mannheim) at 25 U/mL was added. Sections were covered with glass coverslips and placed in a humidified box for 1 h. The sections were thoroughly washed in 70°C water. The samples were observed under a fluorescent microscope immediately after counterstaining with 10 µg/mL 4,6-diamidino-2-phenylindole (DAPI) 5.

**NAD⁺ measurement**

*Data supplement page 2*
We measured the level of NAD$^+$ in cardiac myocytes and in the heart, using the EnzyChrom NAD$^+$/NADH Assay Kit, according to the manufacturer's protocol (#ECND-100, Bioassay Systems, Hayward, CA). The method is based on an alcohol dehydrogenase cycling reaction, in which a tetrazolium dye is reduced by NADH in the presence of phenazine methosulfate. The intensity of the reduced product color, measured at 565 nm, is proportionate to the NAD$^+$/NADH concentration in the sample. This assay method allows one to measure NAD$^+$ and NADH from duplicate samples. Although we measured NAD$^+$ in all samples, we only measured NADH in heart homogenates because the level of NADH was within the detection range of the assay in the in vivo heart samples but it was sometimes below the reliable detection range in cardiac myocyte samples. For in vitro samples, myocytes cultured on a 10 cm dish were treated with trypsin, washed twice with cold PBS, and pelleted through centrifugation. Myocytes were separated into two parts, one for NAD$^+$ measurement and the other for protein concentration. Cell pellets were resuspended in 1.5 mL eppendorf tubes with 100 μL NAD$^+$ extraction buffer (containing 0.40% hydrochloric acid). For the heart samples, the heart (20 mg) was homogenated with 100 μL NAD$^+$ extraction buffer for NAD$^+$ determination or with 100 μL NADH extraction buffer for NADH determination. Extracts were heated at 60°C for five minutes. Twenty μL of assay buffer (containing Tris (hydroxymethyl) aminomethane 3.0% and BSA 0.10%) was added to the extracts followed by 100 μL NADH extraction buffer for NAD$^+$ measurement or 100 μL NAD$^+$ extraction buffer for NADH measurement (to neutralize the extracts). The samples were vortexed and centrifuged at 13,200 rpm for five minutes. Supernatants (120 μL) were then mixed with Working Reagent, containing 50 μL assay buffer, 1 μL alcohol dehydrogenase, 10 μL 1% (vol) ethanol, 14 μL phenazine methosulfate, and 14 μL tetrazolium dye. Optical density at 565 nm was recorded at time zero (OD$_0$) and at 15 minutes (OD$_{15}$) with a 96-well plate reader spectrophotometer. The difference in the absorbances at
time zero and 15 minutes (OD₀-OD₁₅) of the test sample was compared with that of the standard solution to determine the NAD⁺ or NADH concentration. Myocytes (the other half) or the heart samples were lysed with RIPA buffer to measure protein concentration. The amount of NAD⁺ and NADH was normalized to the protein concentration.

**Measurement of intracellular ATP contents**

Intracellular ATP contents were measured using an ATP Bioluminescent Assay Kit (Sigma)². Cells were lysed directly in the somatic-cell ATP-releasing agent, and the lysates were assayed according to the manufacturer's instructions, using a 1:625 dilution of the ATP assay mix. Light emitted was measured using a luminometer. ATP contents were calculated by comparison with a standard curve derived from known concentrations of ATP, ranging from 0.01 to 10 pmol/L.

**Construction of short hairpin RNA (shRNA) adenoviral expression vectors**

pSilencer 1.0-U6 expression vector was purchased from Ambion. The U6 RNA polymerase III promoter and the polylinker region were subcloned into the adenoviral shuttle vector pDC311 (Microbix). The hairpin-forming oligo, corresponding to bases 54-72 (5'-CAAGGTTACTCACTATAAAATTTCAAGAGATTATATAGTGGATAACCTTTTTTTTTTT-3’) of the rat nampt cDNA and its antisense with ApaI and Hind III overhangs were synthesized, annealed, and subcloned distal to the U6 promoter. The loop sequence is underlined. A recombinant adenovirus was generated using homologous recombination in 293 cells.

**Immunoblot analysis**

For immunoblot analysis, cells were lysed in lysis buffer (50 mmol/L Tris-HCl pH 7.4, 0.1% SDS,
1% IGEPAL CA-630, 0.15 mol/L NaCl, 0.25% Na-deoxycholate, and 1 mmol/L EDTA supplemented with protease inhibitors). Densitometric analysis was performed using Scion Image software (Scion).

**Evaluation of GFP-LC3 dots**
Cardiac myocytes were grown on gelatinized coverslips. Myocytes were transduced with Ad-GFP-LC3 for 48 hours. Samples were mounted using SlowFade Light Antifade Kit (Molecular Probes) and the fluorescence of GFP-LC3 was observed under a fluorescence microscope. The number of cells with GFP-LC3 dots was counted in five independent visual fields.

**Reverse Transcription–Polymerase Chain Reaction**
Total RNA was subjected to reverse transcription–quantitative polymerase chain reaction (RT-qPCR) as described previously.

**Immunohistochemistry**
The heart specimens were fixed with formalin, embedded in paraffin, and sectioned at 6-μm thickness. The method of immunostaining has been described.

**Echocardiography**
Mice were anesthetized using 12 μL/g BW of 2.5% avertin (Sigma-Aldrich), and echocardiography was performed using ultrasonography (Acuson Sequoia C256; Siemens Medical Solutions) as previously described.
Ischemia/reperfusion and prolonged ischemia

Mice were housed in a temperature-controlled environment with 12-hr light/dark cycles where they received food and water ad libitum. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). A rodent ventilator (model 683; Harvard Apparatus Inc) was used with 65% oxygen during the surgical procedure. The animals were kept warm using heat lamps. Rectal temperature was monitored and maintained between 36 and 37°C. The chest was opened by a horizontal incision through the muscle between the ribs (third intercostal space). Ischemia/reperfusion was achieved by ligating the anterior descending branch of the left coronary artery (LAD) using an 8-0 prolene suture, with a silicon tubing (1 mm OD) placed on top of the LAD, 2 mm below the border between left atrium and LV. Regional ischemia was confirmed by ECG change (ST elevation). After occlusion for 45 min, the silicon tubing was removed to achieve reperfusion and the rib space and overlying muscles were closed. When recovered from anesthesia, the mice were extubated and returned to their cages. They were given water and standard mouse food and housed in a climate-controlled environment. Prolonged ischemia was achieved by ligating the anterior descending branch of the left coronary artery (LAD) using an 8-0 prolene suture, and closing the wound. Evaluation of infarction size was done by TTC staining 24 hour after ischemia 2. In order to evaluate the effect of Nampt upon prolonged ischemia, some mice were subjected to prolonged ischemia (2 hours) and euthanized without reperfusion.

Assessment of area at risk and infarct size

After intervention, the animals were reanesthetized and intubated, and the chest was opened. After arresting the heart at the diastolic phase by KCl injection, the ascending aorta was canulated and perfused with saline to wash out blood. The LAD was occluded with the same suture, which had
been left at the site of the ligation. To demarcate the ischemic area at risk (AAR), Alcian blue dye (1%) was perfused into the aorta and coronary arteries. Hearts were excised, and LVs were sliced into 1-mm thick cross sections. The heart sections were then incubated with a 1% triphenyltetrazolium chloride solution at 37°C for 15 min. The infarct area (pale), the AAR (not blue), and the total LV area from both sides of each section were measured using Adobe Photoshop (Adobe Systems Inc.), and the values obtained were averaged. The percentage of area of infarction and AAR of each section were multiplied by the weight of the section and then totaled from all sections. AAR/LV and infarct area/AAR were expressed as percentages.

**Evaluation of apoptosis in tissue sections**

DNA fragmentation was detected *in situ* using TUNEL, as described. Briefly, deparaffinized sections were incubated with proteinase K, and DNA fragments were labeled with fluorescein-conjugated dUTP using TdT (Roche Molecular Biochemicals). Nuclear density was determined by manual counting of DAPI-stained nuclei in six fields for each animal using the 40x objective, and the number of TUNEL-positive nuclei was counted by examining the entire section using the same power objective.
Supplemental Figures

Figure S1

(A) Time course of Nampt expression in response to myocardial ischemia. C57/B6 mice were subjected to myocardial ischemia by coronary ligation. Heart samples were collected at indicated time points. Expression of Nampt and tubulin was evaluated by immunoblot analyses. (B) Reversibility of Nampt downregulation after pressure overload. Mice were subjected to sham operation, aortic banding for one week, or aortic banding for one week followed by debanding for one week. Expression of Nampt and tubulin was evaluated by immunoblot analyses.

Figure S2

Cardiac myocytes were transduced with either Ad-LacZ or Ad-Nampt (A) or Ad-shRNA-scramble (control) or Ad-shRNA-Nampt (B) and cultured for 48 hours. Cell viability was measured after 4-hours of treatment with 0.6 or 1.2 mM methyl methanesulfonate (MMS) or glucose deprivation. Cell viability was evaluated by CellTiter-Blue Cell Viability Assays. Cell viability of myocytes transduced with Ad-LacZ or Ad-scramble without stress was expressed as 100%.

Figure S3

(A) The effect of Nampt knockdown on necrotic cell death. Neonatal rat cardiac myocytes were transduced with Ad-shRNA-Nampt (shNampt) or Ad-shRNA-scramble (ShControl) (3MOI) and cultured for 5 days. Myocytes treated with chelerythrine (CE, 100 μM for 30 min) were used as a positive control for necrotic cell death. Cells were stained with propidium iodide for 5 min without cell permeabilization, then counterstained with DAPI after permeabilization. (B) The effect of Nampt knockdown on necrotic cell death in response to glucose deprivation. Cardiac myocytes were transduced with Ad-shRNA-Nampt (shNampt) or Ad-shRNA-scramble (shControl or sh-con) (3MOI) and cultured for 5 days. Myocytes were then incubated with glucose free medium for 4
hours. Cells were stained with propidium iodide for 5 min without cell permeabilization. (C) Cultured neonatal rat cardiac myocytes were transduced with Ad-shRNA-scramble (Ad-sh-Scr.) or Ad-shRNA-Nampt (Ad-sh-Nampt) (3MOI) and cultured for 5 days. Chelerythrine (CE) (100 μM for 30 minutes) was used as positive control. Samples were subjected to immunoblot analyses with PAR antibody.

Figure S4
The effect of Nampt knockdown on PARP activity. Cultured neonatal rat cardiac myocytes were transduced with Ad-shRNA-scramble (sh-scr) or Ad-shRNA-Nampt (sh-Nampt) (3MOI) and cultured for 5 days. Chelerythrine (100 μM) was used as positive control. Samples were subjected to immunoblot analyses with anti-poly ADP-ribose polymer (PAR) antibody.

Figure S5
(A) Downregulation of Nampt does not affect p62 mRNA expression. Neonatal rat cardiac myocytes (NRCMs) were transduced with either Ad-shRNA-scramble or Ad-shRNA-Nampt at 3 MOI and cultured in serum-free conditions for 96 hours. mRNA expression of p62 was measured by RT-qPCR and normalized by S15RNA. The value in myocytes transduced with Ad-shRNA-scramble was expressed as 1.(B) Downregulation of Nampt does not further increase accumulation of LC3-II in the presence of chloroquine. Cardiac myocytes were transduced with Ad-shRNA-scramble or Ad-shRNA-Nampt at 3 MOI and cultured in serum-free conditions for 96 hours. Some myocytes were incubated with chloroquine (6 μM) for 2 hours. Cell lysates were subjected to immunoblot analyses with anti-LC3 and anti-tubulin antibodies. Results are representative of three experiments.

Figure S6
Downregulation of Nampt does not significantly affect AMPK, p70S6K, 4E-BP1, Akt or mTOR
phosphorylation in cardiac myocytes. Cardiac myocytes were transduced with Ad-shRNA-scramble or Ad-shRNA-Nampt at 3 MOI and cultured in serum or serum-free conditions for 96 hours. Cell lysates were subjected to immunoblot analyses with antibodies against phosphor-AMPK, AMPK, phospho-P70, P70, phospho-4E-BP1, 4E-BP1, phospho-mTOR, mTOR, phospho-Akt, Akt, Beclin-1 and actin. Results are representative of four experiments.

**Figure S7**

Knockdown of Nampt decreases the activity of Sirt1. Myocytes were transduced with Ad-shRNA-scramble or Ad-shRNA-Nampt. Cell lysates were subjected to immunoblot analyses of acetylated-lysine proteins, acetyl-Histone H3 and hyperacetylated Histone H4 (Ponceau S staining shown as a loading control).

**Figure S8**

The level of [NAD$^+$], [NADH], [NAD$^+$]/[NADH] in the heart was measured using either 1-2 or 12-14 months old mice. Data are mean ± SEM obtained from 5-6 experiments.
References


### Table S1

Gravimetric Parameters of Tg-Nampt (Line 9) at 3 Months of Age

<table>
<thead>
<tr>
<th>Group</th>
<th>WT (N=5)</th>
<th>Tg (N=5)</th>
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<tr>
<td>HW/BW (mg/g)</td>
<td>3.98±0.02</td>
<td>3.84±0.01</td>
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<td>LV/BW (mg/g)</td>
<td>2.75±0.03</td>
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<tr>
<td>RV/BW (mg/g)</td>
<td>0.69±0.01</td>
<td>0.66±0.01</td>
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<tr>
<td>Atrium/BW (mg/g)</td>
<td>0.37±0.02</td>
<td>0.31±0.02</td>
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<tr>
<td>Lung/BW (mg/g)</td>
<td>4.99±0.06</td>
<td>4.80±0.07</td>
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HW, heart weight; BW, body weight; LV, left ventricle; RV, right ventricle.

### Table S2

Echocardiographic Analysis of Tg-Nampt (Line 9) at 3 Months of Age

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<td>DSEP WT (mm)</td>
<td>0.85±0.03</td>
<td>0.83±0.03</td>
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<td>LVEDD (mm)</td>
<td>3.70±0.08</td>
<td>3.68±0.09</td>
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<td>DPW WT (mm)</td>
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<td>SSEP WT (mm)</td>
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<td>LVESD (mm)</td>
<td>2.43±0.06</td>
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<tr>
<td>SPW WT (mm)</td>
<td>1.11±0.02</td>
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<tr>
<td>EF (%)</td>
<td>71.6±2.1</td>
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<tr>
<td>FS (%)</td>
<td>34.5±1.6</td>
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<tr>
<td>HR (bpm)</td>
<td>474±31</td>
<td>470±19</td>
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</table>

DSEP, diastolic septal; WT, wall thickness; LVEDD, left ventricular end-diastolic dimension; DPW, diastolic posterior wall; SSEP, systolic septal; LVESD, left ventricular end-systolic dimension; SPW, systolic septal wall; EF ejection fraction; FS, fractional shortening; HR, heart rate.
Supplemental Figure S1

### A

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<tr>
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<td>Nampt</td>
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<tr>
<td>tubulin</td>
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### B

<table>
<thead>
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<th>S</th>
<th>B</th>
<th>D</th>
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<tr>
<td>tubulin</td>
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**Legend:**
- **S** = sham
- **B** = banding
- **D** = debanding

**Graphs:**
- **Nampt** and **tubulin** expression levels over time.
- **Bar graphs** showing relative expression levels.
Supplemental Figure S2

A

Relative cell viability (%)

Ad-LacZ  Ad-Nampt  Ad-LacZ  Ad-Nampt  Ad-LacZ  Ad-Nampt

1.2mM MMS  Glucose deprivation

P<0.005  P<0.01

B

Relative cell viability (%)

Ad-sh-Scr  Ad-sh-Nampt  Ad-sh-Scr  Ad-sh-Nampt  Ad-sh-Scr  Ad-sh-Nampt

0.6mM MMS  Glucose deprivation

P<0.001  P<0.001  P<0.001
Supplemental Figure S3

A

B

C

Supplemental Figure S3

Glucose deprivation

shControl  shNampt  CE

shControl

shNampt

CE

PI

DAPI

20 μm

*P<0.01 vs sh-con

PI positive cell %

sh-con  sh-Nampt

glucose deprivation

*
Supplemental Figure S4

- sh-scr
- sh-Nampt
- chele-rythrine

(kD)

- 100
- 25

anti-PAR

anti-tubulin
Supplemental Figure S5

Ad-sh-Scr. (N=5)       Ad-sh-Nampt (N=5)   3MOI

LC3-I

LC3-II

Tubulin

Relative mRNA expression
(p62/S15RNA)

0.00          0.25          0.50          0.75          1.00          1.25          1.50

Ad-sh-Scr. (N=5)       Ad-sh-Nampt (N=5)   3MOI

-+ -+

6 μM chloroquine

Ad-sh-Scr.  Ad-sh-Nampt  3MOI

-  +  -  +  6 μM chloroquine

LC3-I

LC3-II

Tubulin
## Supplement

### Figure S6

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Supplemental Figure S7

Acetylated protein

Ponceau S stain

Ad-sh-Scr.   Ad-sh-Nampt

Ad-sh-Scr.   Ad-sh-Nampt

Acetyl-H3

Hyperacetyl-H4

Ad-sh-Scr.   Ad-sh-Nampt
Supplemental Figure S8

![Bar graphs showing NAD(pmol/mg tissue weight) and NADH(pmol/mg tissue weight) for 1-2 and 12-14 groups.](image)