

## **Nicotinamide mononucleotide (NMN) affords cardioprotection by stimulating glycolysis**

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## ABSTRACT

**Rationale:** Nicotinamide adenine dinucleotide (NAD<sup>+</sup>) is a substrate for sirtuin (SIRT) lysine deacylases. Stimulation of SIRT1 is cardioprotective against ischemia-reperfusion (IR) injury, prompting interest in orally-available NAD<sup>+</sup> precursors such as nicotinamide mononucleotide (NMN) as potential cardioprotective agents. While the biological activity of NMN has been largely attributed to SIRT stimulation, NMN effects on metabolism, and any role these may play in cardioprotection, are less well understood. **Objective:** To investigate potential non-SIRT mechanisms for NMN cardioprotection, with a focus on metabolism. **Methods & Results:** NMN was protective in perfused mouse hearts (post-IR functional recovery: NMN 42±7% vs. vehicle 11±3%). However, protection was insensitive to the SIRT1 inhibitor splitomicin (recovery 47±8%), and NMN did not impact lysine acetylation in the cytosol where cardiac SIRT1 is located, thus suggesting NMN does not stimulate cardiac SIRT1 activity. Surprisingly, NMN was not protective in hearts perfused without glucose (palmitate as fuel source; recovery 11±4%). Since glycolysis requires NAD<sup>+</sup>, and is associated with some cardioprotective paradigms, we hypothesized NMN protection may be due to glycolytic stimulation. In primary cardiomyocytes, NMN induced cytosolic and extracellular acidification, and enhanced lactate generation, indicative of increased glycolysis. Finally, since extension of ischemic acidosis into early reperfusion (i.e., acid post-conditioning) is cardioprotective, we hypothesized that NMN delivery at reperfusion may protect, and indeed this was the case (recovery 39±8%). **Conclusions:** The acute cardioprotective benefit of NMN is mediated via glycolytic stimulation, and this effect of NMN may be worthy of investigation in other situations where NAD<sup>+</sup> precursor supplements are of therapeutic interest.

## KEY WORDS

Lactate, Acidosis, Ischemia, NAD<sup>+</sup>, Glycolysis

## INTRODUCTION

The SIRT family of NAD<sup>+</sup> dependent lysine deacylases are important regulators of metabolic health<sup>1,2</sup>. SIRT activity is known to decline with age<sup>3</sup>, and as such a number of NAD<sup>+</sup> biosynthetic precursors are under investigation as *nutriceuticals*, aimed at ameliorating diseases of aging<sup>4,5</sup>. Among these compounds, nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN) are orally bioavailable and are currently the subject of clinical trials (NCT02812238, NCT02835664, NCT02303483, NCT02678611, etc)<sup>4,6</sup>.

Cardiomyocyte SIRT1 is important in several cardioprotective paradigms, including ischemic preconditioning (IPC)<sup>7-9</sup>. Recently it was shown that NMN confers cardioprotection in a mouse model of ischemia-reperfusion (IR) injury, and this protection was lost in *Sirt1*<sup>-/-</sup> mice<sup>10</sup>. Although thus far the biological activity of NMN has been mostly attributed to the SIRT stimulation<sup>11</sup>, the NAD<sup>+</sup>/NADH redox couple is critically important for metabolic pathways including glycolysis and mitochondrial oxidative phosphorylation<sup>12</sup>. The acute effects of boosting cellular NAD<sup>+</sup> levels on these pathways are poorly understood, and the role that such metabolic perturbations may have in the protective effects of NMN are unknown. Herein, we investigated alternative cardioprotective mechanisms of NMN beyond SIRT1.

## METHODS

Chemicals and reagents were from Sigma (St. Louis MO). Wild-type male C57BL6/J mice were bred in-house, handled according to the "NIH Guide" with food and water *ad libitum*, and used at age 8-12 weeks. Following tribromoethanol anesthesia (200mg/kg ip), hearts were perfused in Langendorff constant flow mode (4 ml/min.) as previously described<sup>9</sup>. Krebs Henseleit (KH) buffer was supplemented with 5 mM glucose plus 100 μM palmitate-BSA, or palmitate-BSA alone (glucose-free)<sup>9</sup>. NMN was delivered via a port above the perfusion cannula from a 100x stock in KH. Hearts were freeze-clamped in liquid N<sub>2</sub> and cardiac metabolites extracted in 80% MeOH for LC-MS/MS based metabolomics as previously described<sup>13</sup>. Primary cardiomyocytes were isolated by collagenase digestion, and intracellular pH measured using fluorescence microscopy with the pH sensitive probe BCECF, as previously described<sup>14,15</sup>. Cardiomyocyte oxygen consumption and extracellular acidification were measured in a Seahorse™ XF24 analyzer as previously described<sup>14</sup>. Statistical significance between groups was determined by ANOVA assuming a normal distribution followed by post-hoc Student's t-test (significance threshold p<0.05).

## RESULTS

We recently showed that the SIRT1 inhibitor splitomicin (Sp) causes mild cellular alkalinization in primary mouse cardiomyocytes<sup>16</sup>. In companion experiments, we also tested the effects of the SIRT1 activator NMN, and surprisingly found that 1 mM NMN induced cellular acidification (Figure 1A/B). NAD<sup>+</sup> is a substrate for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in glycolysis, and under normal or hypoxic conditions either the malate/aspartate shuttle or lactate dehydrogenase respectively serve to regenerate NAD<sup>+</sup> from NADH, to permit continued glycolysis and prevent reductive stress<sup>17</sup>. Given this dependence of glycolysis on NAD<sup>+</sup> availability, we hypothesized that NMN-induced acidification may be due to glycolytic stimulation. Figure 1C shows that delivery of 1 mM NMN to perfused mouse hearts resulted in a significant elevation of NAD<sup>+</sup> and a similar increase in NADH, the latter suggesting NAD<sup>+</sup> reduction by metabolism. NMN also drastically elevated cardiac lactate, with a moderate but significant rise in pyruvate. Furthermore, Figure 1D shows that 1 mM NMN elicited a ~3-fold increase in cardiomyocyte extracellular acidification rate (ECAR), without significantly impacting oxygen consumption rate (OCR). Notably, although SIRT1 is known to regulate glycolysis<sup>18</sup>, splitomicin was without effect on NMN-induced ECAR enhancement (Figure 1D). Together these data indicate that NMN stimulates cardiomyocyte glycolysis in a SIRT1 independent manner.

Although enhanced glucose utilization is associated with cardiac pathology and heart failure<sup>19-23</sup>, more recently parallels have been drawn between cardiac glycolysis and hypoxic

survival<sup>13,24</sup>. Thus we hypothesized NMN-induced glycolytic stimulation may play a role in the reported cardioprotective benefits of this molecule<sup>10</sup>. Figure 2A/B shows that pre-ischemic delivery of 1 mM NMN afforded significant cardioprotection against IR injury (post-IR functional recovery: NMN 42±7% vs. vehicle 11±3%; infarct size: NMN 33±5% vs. vehicle 67±5%; means ± SEM, n=5-6, p<0.05 for both parameters). However, in contrast with previous reports of a requirement for SIRT1<sup>10</sup>, we observed no effect of splitomicin on NMN-induced cardioprotection (p=0.64 vs. NMN alone). This result is consistent with the lack of splitomicin effect on NMN-induced glycolytic stimulation (Figure 1D). Notably, splitomicin inhibits cardioprotection by IPC<sup>9</sup>, suggesting the inhibitor is functional in this system. Furthermore, cardiac SIRT1 is primarily cytosolic<sup>25</sup>, and acetyl-lysine western blots revealed no effect of NMN on cytosolic lysine acetylation (Figure 2C). Together these results suggest no role for SIRT1 in NMN-induced cardioprotection. Although this contrasts with reports of a requirement for SIRT1 in NMN protection<sup>10</sup>, *Sirt1*<sup>-/-</sup> mice show enhanced baseline IR injury (~50% greater infarct vs. wild-type) which may over-ride the ability of NMN to protect via other mechanisms.

To test the requirement for glycolysis in NMN-induced cardioprotection, hearts were perfused without glucose (i.e., with palmitate as the sole metabolic substrate). Figure 3A/B shows that such restriction of glucose availability ablates the protective benefit of NMN. Importantly, glucose withdrawal had no significant effect on baseline injury alone (infarct 67±5 % with glucose plus fat, vs. 73±4% with fat only), indicating this loss of NMN cardioprotection was not due to hearts being damaged beyond repair.

A key event in IR injury is the opening of the mitochondrial permeability transition (PT) pore. During ischemia, acid pH maintains the pore in a closed state, but pH recovery upon reperfusion triggers pore opening<sup>26-28</sup>. As such, reperfusion with acidic media (i.e., acid post-conditioning) is cardioprotective via maintaining PT pore closure into early reperfusion<sup>29</sup>. Since acidosis is a key effect of NMN (Figure 1), we hypothesized that NMN delivery at reperfusion may be cardioprotective. Figure 3C/D shows that, indeed, 1 mM NMN at reperfusion was significantly protective (recovery 39±8%, infarct 28±5%, means ± SEM, n=6). Overall, our data suggest that NMN acutely stimulates cardiac glycolysis, which may contribute to its cardioprotective effects.

## DISCUSSION & CONCLUSIONS

The heart is conventionally viewed as a “metabolic omnivore”, but under normal conditions the bulk of cardiac ATP requirements are furnished by mitochondrial β-oxidation of fatty acids<sup>19</sup>. As such, a switch to favor glucose utilization is typically associated with cardiac pathology and heart failure<sup>19-23</sup>. However, several studies have also linked glycolysis to cardioprotection: A screen for compounds that enhance glycolysis yielded hits that were protective in models of cardiac IR injury<sup>24</sup>. Glycolysis is also up-regulated in IPC<sup>13,30</sup> and the presence of glucose is necessary for IPC<sup>13</sup>. Furthermore, the *pH hypothesis* of ischemic post-conditioning (IPoC) posits that protection by IPoC is afforded by extending ischemic acidosis into early reperfusion, maintaining PT pore closure<sup>29</sup>. Together with our data showing NMN stimulates glycolysis, these findings suggest that NMN-induced cardioprotection may proceed via metabolic acidosis.

While acidosis is a response of all eukaryotes to hypoxia/ischemia, the potential of acidic pH to drive protective molecular signaling events has only recently become appreciated. For example, acidic pH imparts *de-novo* activities to several metabolic enzymes, resulting in generation of unique metabolites<sup>15,31</sup>. In particular, the *oncometabolite* 2-hydroxyglutarate (2-HG) is generated by lactate and malate dehydrogenases under acidic conditions<sup>15,31</sup>. 2-HG is a competitive inhibitor of the α-ketoglutarate dependent dioxygenase family of epigenetic regulators, which includes the JmjC domain-containing histone demethylases, the TET 5-methylcytosine hydroxylases, and the EGLN prolyl-hydroxylases that regulate hypoxia inducible factor (HIF)<sup>32,33</sup>. As such, acid pH can induce HIF-1α via a pathway that requires 2-HG generation<sup>15</sup>.

The observation that NMN rapidly induces metabolic acidosis raises the possibility that at least some of the benefits reported for dietary NMN supplementation<sup>4-6,12</sup> may be attributed to such effects. A wide variety of health benefits are reported for NMN and NR supplementation, including neurological, cardiac, obesity/diabetes-related, and anti-aging<sup>12</sup>. In many cases, such

benefits may be linked to enhancing glycolysis. For example, NMN treatment increases performance in whole body glucose tolerance testing<sup>34</sup>. Similarly, NR enhances stem cell function, with *stem-ness* being generally associated with a glycolytic metabolic state<sup>5</sup>.

Despite the reported benefits of dietary NMN/NR supplementation, our results urge caution regarding the widespread human use of these nutraceuticals. For example, the loss of SIRT1 activity in aging is associated with HIF stabilization and a *pseudo-hypoxic* metabolic state<sup>35</sup>. As such, acute acidosis resulting from NMN supplementation may activate HIF, worsening pseudo-hypoxia. Similarly, HIF activation and metabolic acidosis<sup>36</sup> are well-known hallmarks of cancer, and indeed the same screen that identified glycolytic stimulators as protective against hypoxia found numerous anti-cancer drugs as glycolytic suppressors<sup>24</sup>. Acid pH is also known to promote the reverse reaction of isocitrate dehydrogenase (i.e., reductive carboxylation of  $\alpha$ -ketoglutarate to citrate), which is an important driver of lipid biosynthesis in cancer. Together with the apparent ability of NMN to promote stem-ness<sup>5</sup>, these findings suggest that NAD<sup>+</sup> boosting supplements may promote tumor growth.

Overall our results suggest that the nutraceutical use of NMN and NR should not overlook the classical role that NAD<sup>+</sup> plays in glycolysis. GAPDH is one of the most highly and stably expressed proteins in the cell, such that it is often used as a “housekeeping protein” for experimental loading controls. The levels of GAPDH are likely to be several orders of magnitude higher than those of NAD<sup>+</sup> consuming signaling enzymes such as SIRTs, PARPs, and CD38. Thus, the biological effects of large-scale and acute elevations in [NAD<sup>+</sup>] are likely to involve glycolytic stimulation. It remains to be determined whether long term beneficial effects of NAD<sup>+</sup> precursors are mediated by repetitive transient metabolic acidosis.

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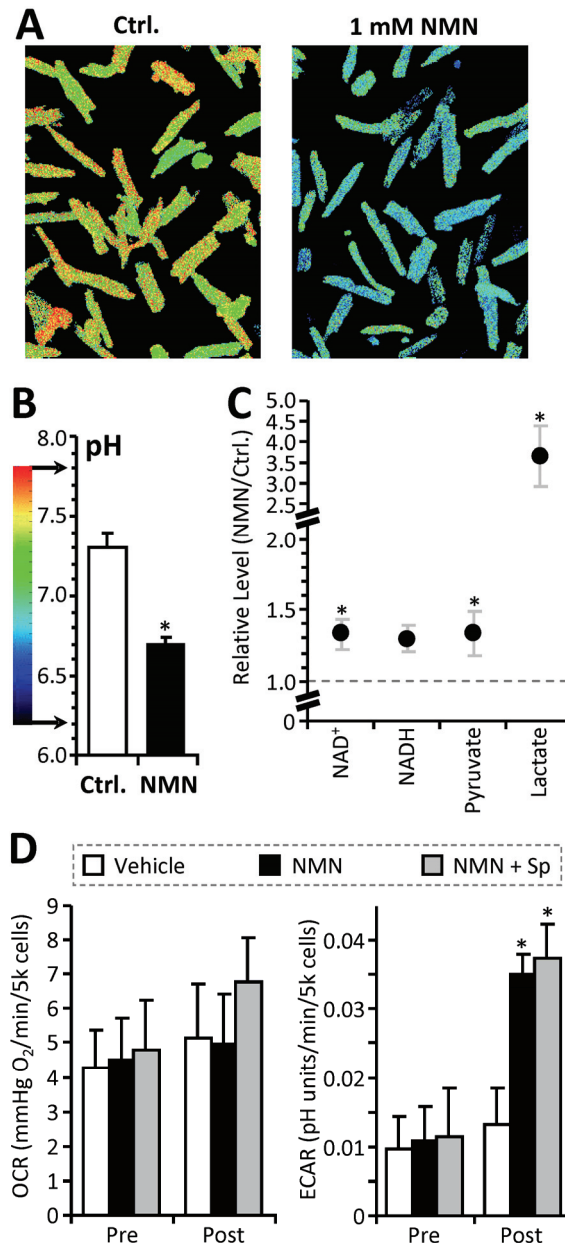
## DISCLOSURES

The authors declare no conflicts of interest.

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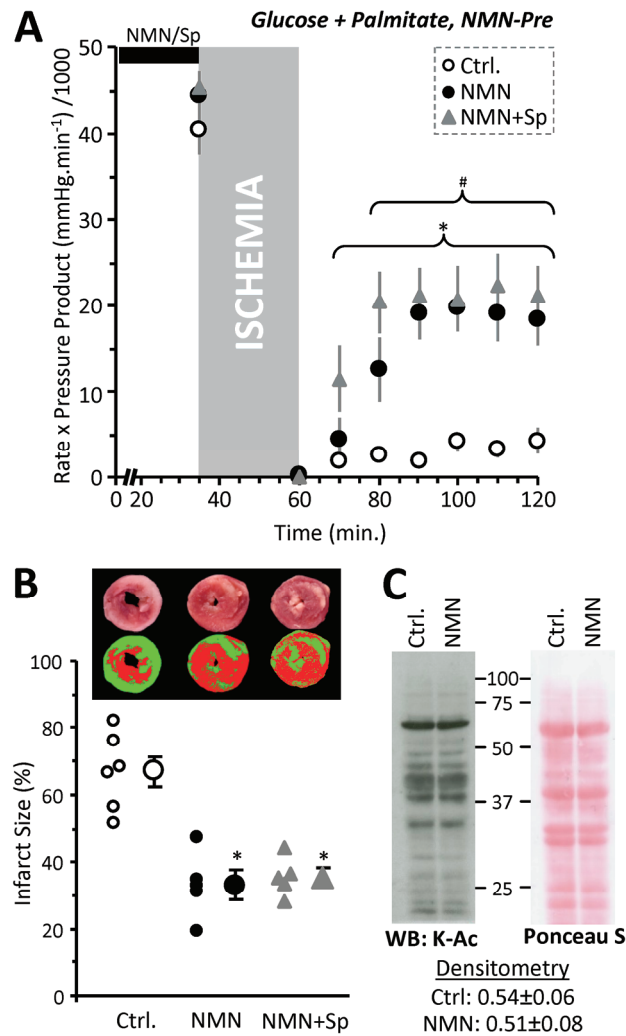
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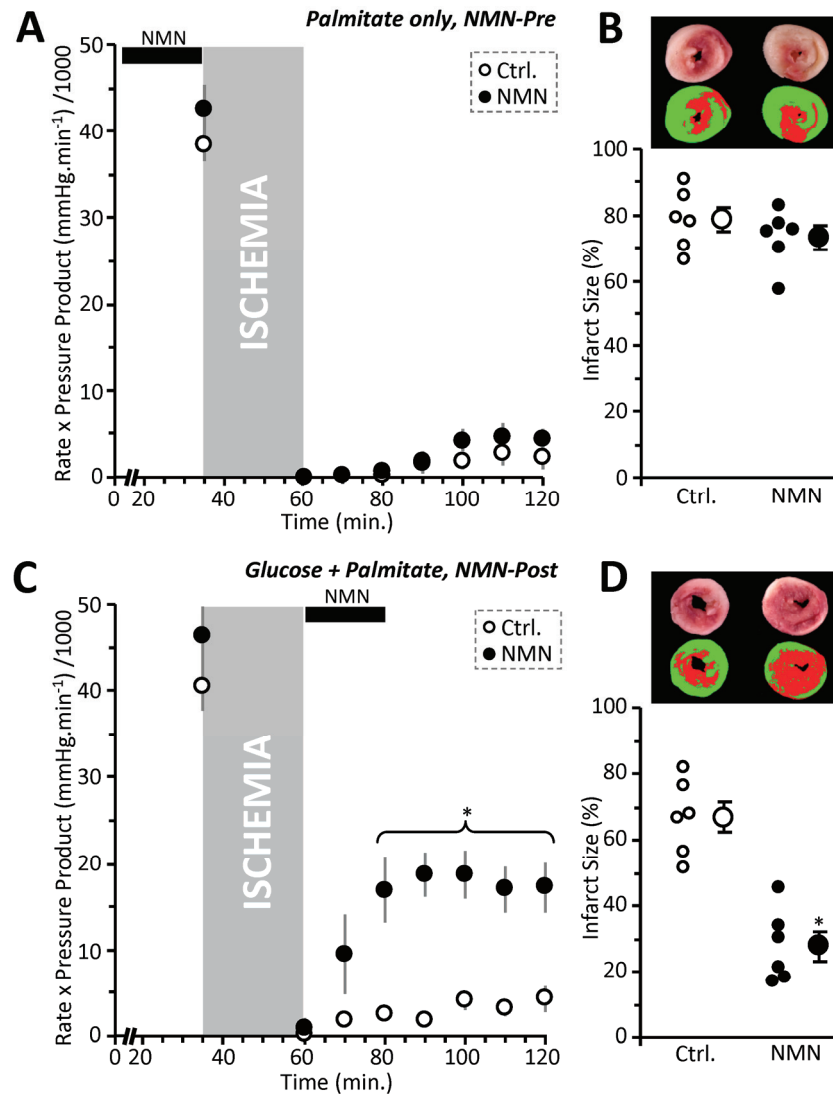


**Figure 1: NMN Stimulates Cardiomyocyte Glycolysis Leading to Cell Acidification. (A):** Representative fluorescent microscopic images of primary adult mouse cardiomyocytes stained with the pH sensitive vital dye BCECF and treated for 40 min. with 1 mM NMN or vehicle control. Images are pseudo-colored according to the pH scale in panel B. **(B):** Graph shows average pH values from 9 independent plates of cells (means  $\pm$ SEM, \* $p$ <0.05 between treatment groups). **(C):** Relative levels of selected metabolites in freeze-clamped perfused mouse hearts treated for 20 min. with 1 mM NMN or vehicle control. Note y-axis breaks. Data are presented as the metabolite level in NMN hearts normalized to that in control hearts (means  $\pm$  SEM, N=7, \* $p$ <0.05 between treatment groups). **(D):** Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) values measured via Seahorse™ XF analysis on primary adult mouse cardiomyocytes before and 5 min. after addition of 1 mM NMN (black bars) or vehicle (white bars) to the media. Where indicated, splitomicin (Sp, 10  $\mu$ M) was present throughout (gray bars). Data are means  $\pm$  SD for 7-8 wells per treatment group, on a single XF-24 plate. \* $p$ <0.05 between Pre and Post, within a given treatment group.





**Figure 2: NMN Induced Acute Cardioprotection is Independent of SIRT1. (A):** Cardiac functional data (rate x pressure product) for mouse hearts subjected to 25 min. global ischemia and 60 min. reperfusion. Prior to the onset of ischemia (black bar at top), hearts were perfused for 20 min. with vehicle (Ctrl, white circles), 1 mM NMN (black circles), or 1 mM NMN plus 10  $\mu$ M splitomicin (gray triangles). Perfusion media contained both glucose (5 mM) and palmitate-BSA (100  $\mu$ M) as metabolic substrates. Data shown are means  $\pm$  SEM, N=5-6, \* $p$ <0.05 for all points indicated, between Ctrl. and NMN+Sp, # $p$ <0.05 for all points indicated between Ctrl. and NMN. **(B):** Infarction data for the hearts from panel A, determined by tetrazolium chloride staining. Images above the graph show representative images (upper), and pseudo-colored images (lower) used for quantitation by planimetry. In graph, individual data points are shown on the left, and means  $\pm$  SEM on the right, for each treatment group. \* $p$ <0.05 vs. Ctrl. **(C):** Acetyl-lysine western blot on cytosolic fraction from NMN perfused hearts. Hearts were perfused for 20 min. with 1 mM NMN under normoxic conditions then freeze-clamped and the cytosolic fraction isolated. Left: pan acetyl-lysine blot, right: matching Ponceau S stained membrane for loading control. Numbers below indicate densitometric quantitation of acetyl-lysine intensity down whole lanes, normalized to protein loading, for several independent experiments (means  $\pm$  SEM, N=4).



**Figure 3: NMN Induced Acute Cardioprotection Requires Glucose and is Effective at Reperfusion.** **(A):** Cardiac functional data (rate x pressure product) for mouse hearts subjected to 25 min. global ischemia and 60 min. reperfusion. Prior to the onset of ischemia (black bar at top), hearts were perfused for 20 min. with vehicle (Ctrl, white circles), or 1 mM NMN (black circles). Experiments were performed essentially as in Figure 2A, except that perfusion media contained zero glucose, such that palmitate was the sole metabolic substrate. Data shown are means  $\pm$  SEM, N=6. No significant differences noted between Ctrl. and NMN groups. **(B):** Infarction data for the hearts from panel A, determined by tetrazolium chloride staining. Images above the graph show representative images (upper), and pseudo-colored images (lower) used for quantitation by planimetry. In graph, individual data points are shown on the left, and means  $\pm$  SEM on the right, for each treatment group. No significant differences noted between Ctrl. and NMN groups. **(C):** Cardiac functional data (rate x pressure product) for mouse hearts subjected to 25 min. global ischemia and 60 min. reperfusion. At the onset of reperfusion (black bar at top), hearts were perfused for 20 min. with vehicle (Ctrl, white circles), or 1 mM NMN (black circles). Perfusion media contained both glucose (5 mM) and palmitate-BSA (100  $\mu$ M) as metabolic substrates. Data shown are means  $\pm$  SEM, N=6. \* $p$ <0.05 for all points indicated, between Ctrl. and NMN groups. **(D):** Infarction data for the hearts from panel C, determined by tetrazolium chloride staining. Images above the graph show representative images (upper), and pseudo-colored images (lower) used for quantitation by planimetry. In graph, individual data points are shown on the left, and means  $\pm$  SEM on the right, for each treatment group. \* $p$ <0.05 between Ctrl. and NMN groups. N.B. Control data in panels C/D are the same as those in Figure 2A/B.