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Reduction of Infarct Size by the Therapeutic Protein Tat-Ndi1 *in vivo*

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Abstract

Lethal myocardial ischemia-reperfusion injury has been attributed in part to mitochondrial respiratory dysfunction (including damage to Complex I) and the resultant excessive production of reactive oxygen species. Recent evidence has shown that Ndi1 (the single-subunit protein that in yeast serves the analogous function as Complex I), transduced by addition of the TAT-conjugated protein to culture media and perfusion buffer, can preserve mitochondrial function and attenuate ischemia-reperfusion injury in neonatal rat cardiomyocytes and Langendorffperfused rat hearts. However, this novel metabolic strategy to salvage ischemic-reperfused myocardium has not been tested *in vivo*. In this study, TAT-conjugated Ndi1 and placebo control protein were synthesized using a cell-free system. Mitochondrial uptake and functionality of TAT-Ndi1 was demonstrated in mitochondrial preparations from rat hearts after intraperitoneal administration of the protein. Rats were randomized to receive either TAT-Ndi1 or placebo protein, and two hours later all animals underwent 45 min coronary artery occlusion followed by 2 hours of reperfusion. Infarct size was delineated by tetrazolium staining and normalized to the volume of at-risk myocardium, with all analysis conducted in a blinded manner. Risk region was comparable in the two cohorts. Pre-ischemic administration of TATNdi1 was profoundly cardioprotective. These results demonstrate that it is possible to target therapeutic proteins to the mitochondrial matrix, and that yeast Ndi1 can substitute for Complex I to ameliorate ischemia/reperfusion injury in the heart. Moreover, these data suggest that cell-permeable delivery of mitochondrial proteins may provide a novel molecular strategy to treat mitochondrial dysfunction in patients.

INTRODUCTION

Myocardial ischemia/reperfusion (I/R) injury is attributed in part to mitochondrial damage and dysfunction, including I/R-induced defects in complex I activity (1). Complex I (NADH-quinone oxidoreductase) is the first component of the electron transport chain that catalyzes the transfer of electrons from NADH to ubiquinone. It is composed of 45 subunits and resides in the inner mitochondrial membrane. This enzyme complex plays a critical role in oxidative phosphorylation and contributes to the formation of the proton gradient which drives ATP synthesis.

There is considerable evidence that complex I is a major site of damage during I/R; its activity rapidly falls during both ischemia and reperfusion (2-7). The decline in complex I may be even greater during reperfusion (1). The defect in complex I activity in the I/R-injured rat heart has been linked to oxygen free radical-induced cardiolipin damage. Complex I (1) and complex III (8) are known sources of reactive oxygen species (ROS) and require cardiolipin in the inner mitochondrial membrane for functionality. Thus, in the setting of I/R injury, a vicious cycle is established in which ROS from complex I damages cardiolipin, which impairs normal electron flow through complex I and increases ROS production. Thus complex I represents a potential therapeutic target for amelioration of I/R injury.

One approach to restore normal NADH oxidation and electron flow at reperfusion is xenotransplantation of Ndi1 (NADH-quinone internal oxidoreductase) (9). Ndi1 is a single 56kDa polypeptide that serves as the entry site of electrons from NADH to the respiratory chain in yeast and bacteria. Structurally simpler than the multi-subunit complex I of mammalian mitochondria, Ndi1 is functionally analogous with respect to oxidation of NADH and reduction of quinone; however, it does not pump protons. Heterologous expression of this yeast polypeptide in complex I-deficient mammalian cells partially restores NADH oxidation and ATP production, which can proceed via protons pumped from complexes III and IV.

Ndi1 was first studied by Seo and colleagues (10) as a possible treatment for Parkinson disease, based on the rationale that excess ROS from complex I contributed to death of dopaminergic neurons in the substantia nigra (11-13). Neural cells transduced with virally-delivered Ndi1 exhibited reduced levels of ROS production after rotenone poisoning. Utilization of NADH by Ndi1 was suggested to limit availability of electrons to complex I for ROS production (13). Based on these findings, we hypothesized that Ndi1 could attenuate ROS production from complex I damaged by myocardial I/R. To overcome the limitations of gene therapy, we generated a cell-permeable fusion protein of Ndi1 and the 11aa transduction domain from HIV-TAT (TAT-Ndi1) and demonstrated that it entered cardiomyocytes and localized to the mitochondrial inner membrane (9). When the fusion protein was delivered to Langendorff-perfused rat hearts subjected to I/R, ROS production was suppressed, ATP levels were preserved, and infarct size was reduced by 62%. Similar production was observed when TAT-Ndi1 was infused at the onset of reperfusion (9). We concluded that Ndi1 could reduce I/R injury in buffer-perfused hearts. The purpose of the present study was to establish whether Ndi1 could be used to functionally replace complex I and reduce myocardial infarct size in vivo.

METHODS

Ndi1 Synthesis

TAT-conjugated Ndi1 protein was synthesized from a hemagglutinin (HA) - and histidine-(His)-tagged TAT-Ndi1 construct (provided by R. Gottlieb; prepared as described (9)) using a cell-free protein synthesis kit (EasyXpress; Qiagen) according to the manufacturer's instructions. This *E. coli* lysate system resulted in higher yields and more full-length protein than bacterial expression (**Fig. 1**). After low-speed centrifugation to remove debris, the

protein product was concentrated using a 50kDa cutoff, diluted with purification buffer (pH~7.8, 50mM Tris, 200mM NaCl), and the fraction containing full-length Ndi1 protein was separated by HPLC. Synthesis of tagged Ndi1 protein (molecular weight: ~75kDa) was confirmed by immunoblotting for HA and Ndi1 (gift from the Yagi lab) (**Fig. 1**). Control DNA was used with the same Qiagen cell-free system to generate the material for control injections. Protein was stored at -80°C until use (< 1mo).

TAT-Ndi1 Administration

The animal studies were approved by the Institutional Animal Care and Use Committee of Wayne State University and performed in accordance with the *Guide for the Care and Use of Laboratory Animals*. Female Sprague-Dawley rats received TAT-Ndi1 or placebo (2mg protein/kg in a volume of 0.5 ml via intraperitoneal (i.p.) injection). The dose was established on the basis of preliminary experiments in which TAT-Ndi1 was given at doses ranging from 1 to 4 mg protein/kg; the presence of tagged TAT-Ndi1 in the heart (detected by immunoblotting) and reduction of infarct size (compared to historical controls) served as the primary endpoints (data not shown). Two hours after i.p. injection rats were subjected to myocardial I/R as described below. After 2h reperfusion, hearts were harvested for determination of infarct size or for complex I assays.

Mitochondrial Isolation

Female Sprague-Dawley rats (weight: 368±28 grams) were randomized to receive TAT-Ndi1 (n=4) or placebo (n=3), followed 2h later by coronary artery occlusion. Two rats developed lethal ventricular fibrillation during the initial 20 minutes of ischemia. In the remaining 5 animals (2 TAT-Ndi1-treated rats, 3 placebo controls) the anterior LV wall was harvested for analysis at 1hr post-perfusion. Two additional rats were used as untreated and non-ischemic controls, with samples obtained under anesthesia in a time-matched manner from the anterior LV wall. Heart tissue was immersed in ice-cold mitochondrial isolation buffer (pH 7.4); (in mM: sucrose 250, Tris-HCl 50, Na-HEPES 10, EDTA 5, EGTA 10,; with a protease inhibitor cocktail and minced with scissors. The tissue suspension was transferred to an isolation buffer (pH 7.4) supplemented with BSA (0.5%), homogenized with three short pulses, and centrifuged at 1,000g for 10min at 4°C to remove debris. The supernatant was collected, centrifuged at 10,000xg for 15min at 4°C. The pellet was resuspended and re-centrifuged, and the final pellet was resuspended in the isolation buffer. Protein concentration in the crude mitochondrial fraction was determined by bicinchoninic acid assay.

Mitochondrial Complex I Activity Determination

Mitochondrial complex I activity (i.e., oxidation of NADH to NAD⁺) was quantified using an adaptation of spectrophotometric methods (14-16). We compared total NADH oxidoreductase activity to rotenone-insensitive activity (attributable to Ndi1) (17, 18). Aliquots of crude mitochondrial fraction (10µl, containing 20µg protein) were added to wells containing 120µl assay buffer (25mM K₂HPO₄, 5mM MgCl₂, 0.25% BSA, 3.7µM antimycin A, 2mM KCN, 62.8mM ubiquinone, pH 7.4). After 1min at 30°C reactions were initiated by the addition of NADH (5µl, 5.7mM) in the presence or absence of rotenone, a complex I

inhibitor (2 μ l, 0.36mM in DMSO). The decrease in absorbance at 340nm (oxidation of NADH) was monitored for 10min. For all samples (+/-rotenone) absorbance change reached a plateau within 3min. The rate of NADH oxidation over this 3min interval was reported as absorbance units/min/mg protein for total activity and rotenone-resistant activity (mean $\pm$ standard error).

Coronary Artery Occlusion and Infarct Size Determination

Adult female Sprague-Dawley rats (n=21) weighing 327 $\pm$ 62 grams (mean $\pm$ SD) were randomized to receive either TAT-Ndi1 or placebo control protein (2 mg protein/kg in a volume of 0.5 ml i.p.). Ninety minutes after i.p. administration of protein, rats were anesthetized with sodium pentobarbital (50mg/kg i.p.), intubated via tracheostomy, and ventilated with air. The heart was exposed through a left lateral thoracotomy at the fourth intercostal space, and the proximal left coronary artery was snare-occluded for 45min (initiated exactly 2h after i.p. administration of protein) followed by 2h reperfusion as previously described (19, 20). Hearts were harvested for mitochondrial isolation (using the anterior LV wall, see above) or for infarct size determination. The area at risk of infarction (AR: the primary determinant of infarct size in this model) and area of necrosis (AN) were assessed using standard methods (20-24). Briefly, AR was delineated by ligating the left coronary artery and injecting Monastral blue pigment into the vasculature via a cannula inserted in the femoral vein. Rats were then euthanized under deep pentobarbital anesthesia by injection of KCl. The hearts were rapidly excised, cut into 5-6 transverse slices and photographed. To distinguish necrotic from viable myocardium, the heart slices were incubated in triphenyltetrazolium chloride for 10min at 37°C and re-photographed. AR and AN were quantified using image analysis software (SigmaScan Pro, SPSS Inc.), corrected for tissue weight and summed for each heart. Risk regions and infarct sizes were quantified by a single investigator who was blinded with regard to the group assignments. AR was expressed as a % of the total LV weight, and rats in either group with a negligible risk region (prospectively defined as <10% of the LV (22)) were excluded from further analysis. For remaining animals, AN was calculated and expressed as a % of the AR. For the 21 rats studied, 1 died at the time of anesthesia, 3 were excluded because of technical failures, and an additional 2 rats were excluded because of small risk regions occupying <10% of the LV. Data are reported for the 15 animals that successfully completed the protocol: 8 treated with TATNdi1 and 7 placebo-controls. Results are reported as mean $\pm$ standard error, and AR/LV and AN/AR were compared between TAT-Ndi1- and placebo-treated groups by t-test.

RESULTS

TAT-Ndi1 Transduction and Complex I Activity

Western blot analysis of heart mitochondria confirmed the presence of TAT-Ndi1 in heart mitochondria from rats that received TAT-Ndi1 by i.p. injection (**Fig. 2**). Complex I activity (NADH oxidation) in non-ischemic shams, assessed in the absence of rotenone, averaged 1.28 $\pm$ 0.04 U/min/mg. As expected, addition of rotenone inhibited complex I activity by 90 $\pm$ 5%, to a mean of 0.13U/min/mg (**Fig. 2**). In all rats subjected to ischemia/reperfusion, complex I activity in the absence of rotenone was attenuated when compared with shams, averaging 0.72 and 0.86 U/min/mg in the placebo-controls and TAT-Ndi1-treated groups. In

the placebo control cohort, addition of rotenone inhibited complex I activity to the same degree as seen in the shams: i.e., by $85\pm5\%$, to a mean of 0.13 U/min/mg. In contrast, in TAT-Ndi1-treated rats, complex I activity was only *partially* inhibited by rotenone, to a mean of 0.44 U/min/mg (**Fig. 2**). Rotenone-insensitive NADH oxidase activity in cardiac mitochondria from TAT-Ndi1-treated rats reflects the contribution of Ndi1. Because of the limited number of samples obtained, statistical analysis of the three groups was not done.

TAT-Ndi1 Effect on Infarct Size

To determine if pretreatment with TAT-Ndi1 could ameliorate I/R injury, we administered TAT-Ndi1 or placebo 2h before I/R. The two groups were well-matched with regard to risk region: AR/LV averaged $32\pm5\%$ and $29\pm3\%$ in control and TAT-Ndi1-treated rats, respectively ($p=0.800$ [ns]). However, infarct size was significantly smaller in rats that received TAT-Ndi1 *versus* placebo controls ($33\pm6\%$ *versus* $60\pm5\%$; $p=0.005$) (**Fig. 3**). Thus i.p. administration of TAT-Ndi1 was able to protect cardiac mitochondria from I/R injury and reduce infarct size in an *in vivo* model.

DISCUSSION

We provide novel evidence that TAT-Ndi1 reduced infarct size in an *in vivo* model of ischemia/reperfusion injury. Administration of Ndi1, the yeast polypeptide equivalent of mammalian complex I, contributed to the NADH oxidase activity of isolated cardiac mitochondria. Immunoblotting for TAT-Ndi1 and the presence of rotenone-insensitive NADH oxidase activity indicate that enzymatically active protein was present in the mitochondria.

Our studies confirm reports by others that complex I activity is reduced after I/R (25-32). Our measurement of complex I activity did not show an increase in activity in the cardiac mitochondria from animals treated with TAT-Ndi1, in contrast to our previous findings in the *ex vivo* model. This may be related to the assay method (respirometry in the Perry study (9) and isolated NADH oxidase activity in the present work). In both studies, however, the contribution of Ndi1 to overall complex I activity was modest. The previous findings suggested that TAT-Ndi1 protected the heart by accelerating NADH oxidation, thus decreasing the availability of these electrons for ROS production by damaged complex I and other NAD(P)H oxidases. Thus, TATNdi1 may reduce I/R injury by supporting electron flow through the respiratory chain, and perhaps more importantly by preventing ROS production from excess NADH. The mechanism of TAT-Ndi1 cardioprotection was previously addressed (9), and therefore was not re-examined in the study. The primary purpose of this study was to demonstrate cardioprotective efficacy *in vivo*.

Another approach to cardioprotection involving NADH:ubiquinone oxidoreductase is the reduction of ROS production by endogenously inhibiting Complex I. Using mitochondria-targeted – nitrosothiols (MitoSNOs), Prime *et al* (33) reported that infusion of the compound MitoSNO1 mimicked ischemic preconditioning and was protective against ischemia/reperfusion (I/R) injury in Langendorff perfused mouse hearts when administered at reperfusion. Hearts had better recovery of function as assessed by rate pressure product and a decrease in infarct size. They surmised that the protection conferred by MitoSNO1 was

likely a consequence of the persistent S-nitrosylation of complex I. Subsequently, Chouchani et al (34) directly showed that MitoSNO S-nitrosylation of Cys 39 of the ND3 subunit of complex I was associated with reversible inhibition of the activity of complex I during ischemia, with benefits during the early minutes of reperfusion. Their studies indicated that the S-nitrosylation interfered with electron transfer to ubiquinone but not the interaction with NADH at the flavin center. Interestingly, S-nitrosylation of ND3 does not result in ROS production, in contrast to rotenone, a complex I inhibitor which binds to the ND1 subunit (not to be confused with yeast Ndi1). This may be due to differential effects on retrograde electron flow (complex II → ubiquinone → complex I) which conceivably may be prevented by S-nitrosylation of ND3 but not by rotenone. Ndi1, by providing an alternative path for electron flow to ubiquinone that does not involve a reactive semiquinone intermediate, protects the mitochondria during reperfusion while supporting ATP production and NADH oxidation. While Chouchani et al did not measure ATP or NADH/NAD⁺ ratio, their findings suggest that S-nitrosylating agents could be beneficial in the setting of ischemia/reperfusion injury. It is possible that concurrent administration of TAT-Ndi1 and MitoSNO could have additive benefit, assuming MitoSNO does not inhibit Ndi1. Ndi1 supports electron transfer from NADH to ubiquinone to support OXPHOS, whereas S-nitrosylation of ND3 would mitigate ROS production from endogenous complex I.

The novel finding in our study is the demonstration for the first time that it is possible to deliver a cell-permeable functional enzyme to the mitochondrial matrix *in vivo*. TAT-Ndi1 persists in heart tissue for at least 48h, when administered *i.p.* to mice (**see Data Supplement**), so its beneficial effects might persist long enough to permit replacement of endogenous complex I damaged by ischemia/reperfusion injury. It may also be possible to treat inheritable mitochondrial diseases such as complex I deficiencies with therapeutic protein delivery. TATNdi1 could offer benefit in the setting of acute poisoning with rotenone or the parkinsonian mimetic 1-methyl-4-phenylpyridinium (MPP⁺). The administration of a yeast protein may theoretically elicit an immunologic response that could limit its use for chronic therapy, but long-term expression of Ndi1 (mediated by viral gene transfer) did not elicit an immune response whereas cytosolic expression of GFP did. They attributed the lack of immune response to Ndi1 to its intramitochondrial localization (35). It remains to be determined whether TAT-Ndi1 will elicit an immune response if given repeatedly, which could restrict its use to one-time administration.

TAT-Ndi1—even as a one-time intervention—could be useful in the setting of acute MI. Perry et al. showed that TAT-Ndi1 could reduce infarct size when administered at reperfusion (9), although the present study did not examine infusion of the protein at reperfusion. TAT-Ndi1 could also improve organ preservation for transplantation, and might enhance bridge to recovery after placement of a left ventricular assist device.

In summary, this study demonstrates feasibility and efficacy of therapeutic protein administration for mitochondrial dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Paradies G, Petrosillo G, Pistolese M, Ruggiero FM. Reactive oxygen species affect mitochondrial electron transport complex I activity through oxidative cardiolipin damage. *Gene*. 2002; 286:135–141. [PubMed: 11943469]
2. Aldakkak M, Stowe DF, Chen Q, Lesnefsky EJ, Camara AK. Inhibited mitochondrial respiration by amobarbital during cardiac ischaemia improves redox state and reduces matrix Ca²⁺ overload and ROS release. *Cardiovasc Res*. 2008; 77:406–415. [PubMed: 17900548]
3. Cairns CB, Ferroggiaro AA, Walther JM, Harken AH, Banerjee A. Postischemic administration of succinate reverses the impairment of oxidative phosphorylation after cardiac ischemia and reperfusion injury. *Circulation*. 1997; 96(II):260–265. [PubMed: 9236443]
4. Hardy DL, Clark JB, Darley-USmar VM, Smith DR. Reoxygenation of the hypoxic myocardium causes a mitochondrial complex I defect. *Biochem Soc Trans*. 1990; 18:549. [PubMed: 1703502]
5. Maklashina E, Sher Y, Zhou HZ, Gray MO, Karliner JS, Cecchini G. Effect of anoxia/reperfusion on the reversible active/de-active transition of NADH-ubiquinone oxidoreductase (complex I) in rat heart. *Biochim Biophys Acta*. 2002; 1556:6–12. [PubMed: 12351213]
6. Solaini G, Harris DA. Biochemical dysfunction in heart mitochondria exposed to ischaemia and reperfusion. *Biochem J*. 2005; 390:377–394. [PubMed: 16108756]
7. Veitch K, Maisin L, Hue L. Trimetazidine effects on the damage to mitochondrial functions caused by ischemia and reperfusion. *Am J Cardiol*. 1995; 76:25B–30B.
8. Chen Q, Moghaddas S, Hoppel CL, Lesnefsky EJ. Ischemic defects in the electron transport chain increase the production of reactive oxygen species from isolated rat heart mitochondria. *Am J Physiol Cell Physiol*. 2008; 294:C460–466. [PubMed: 18077608]
9. Perry CN, Huang C, Liu W, Magee N, Carreira RS, Gottlieb RA. Xenotransplantation of mitochondrial electron transfer enzyme, Ndi1, in myocardial reperfusion injury. *PLoS One*. 2011; 6:e16288. [PubMed: 21339825]
10. Seo BB, Wang J, Flotte TR, Yagi T, Matsuno-Yagi A. Use of the NADH-quinone oxidoreductase (NDI1) gene of *Saccharomyces cerevisiae* as a possible cure for complex I defects in human cells. *J Biol Chem*. 2000; 275:37774–37778. [PubMed: 10982813]
11. Marella M, Seo BB, Matsuno-Yagi A, Yagi T. Mechanism of cell death caused by complex I defects in a rat dopaminergic cell line. *J Biol Chem*. 2007; 282:24146–24156. [PubMed: 17581813]
12. Marella M, Seo BB, Nakamaru-Ogiso E, Greenamyre JT, Matsuno-Yagi A, Yagi T. Protection by the NDI1 gene against neurodegeneration in a rotenone rat model of Parkinson's disease. *PLoS One*. 2008; 3:e1433. [PubMed: 18197244]
13. Seo BB, Marella M, Yagi T, Matsuno-Yagi A. The single subunit NADH dehydrogenase reduces generation of reactive oxygen species from complex I. *FEBS Lett*. 2006; 580:6105–6108. [PubMed: 17055488]
14. Tatarkova Z, Kuka S, Racay P, Lehotsky J, Dobrota D, Mistuna D, Kaplan P. Effects of aging on activities of mitochondrial electron transport chain complexes and oxidative damage in rat heart. *Physiol Res*. 2011; 60:281–289. [PubMed: 21114360]
15. Janssen AJ, Trijbels FJ, Sengers RC, Smeitink JA, van den Heuvel LP, Wintjes LT, Stoltenberg-Hogenkamp BJ, Rodenburg RJ. Spectrophotometric assay for complex I of the respiratory chain in tissue samples and cultured fibroblasts. *Clin Chem*. 2007; 53:729–734. [PubMed: 17332151]
16. de Wit LE, Sluiter W. Chapter 9 Reliable assay for measuring complex I activity in human blood lymphocytes and skin fibroblasts. *Methods Enzymol*. 2009; 456:169–181. [PubMed: 19348888]
17. Estornell E. Mitochondrial complex I: new insights from inhibitor assays. *Protoplasma*. 2000; 213:11–17.

18. Yamashita T, Nakamaru-Ogiso E, Miyoshi H, Matsuno-Yagi A, Yagi T. Roles of bound quinone in the single subunit NADH-quinone oxidoreductase (Ndi1) from *Saccharomyces cerevisiae*. *J Biol Chem*. 2007; 282:6012–6020. [PubMed: 17200125]
19. Himori N, Matsuura A. A simple technique for occlusion and reperfusion of coronary artery in conscious rats. *Am J Physiol*. 1989; 256:H1719–1725. [PubMed: 2735441]
20. Li YW, Whittaker P, Kloner RA. The transient nature of the effect of ischemic preconditioning on myocardial infarct size and ventricular arrhythmia. *Am Heart J*. 1992; 123:346–353. [PubMed: 1736569]
21. Ytrehus K, Liu Y, Tsuchida A, Miura T, Liu GS, Yang XM, Herbert D, Cohen MV, Downey JM. Rat and rabbit heart infarction: effects of anesthesia, perfusate, risk zone, and method of infarct sizing. *Am J Physiol*. 1994; 267:H2383–2390. [PubMed: 7528994]
22. Whittaker P, Kloner RA, Przyklenk K. Intramyocardial injections and protection against myocardial ischemia. An attempt to examine the cardioprotective actions of adenosine. *Circulation*. 1996; 93:2043–2057. [PubMed: 8640981]
23. Whittaker P, Rakusan K, Kloner RA. Transmural channels can protect ischemic tissue. Assessment of long-term myocardial response to laser- and needle-made channels. *Circulation*. 1996; 93:143–152. [PubMed: 8616921]
24. Sala-Mercado JA, Wider J, Undyala VV, Jahania S, Yoo W, Mentzer RM Jr, Gottlieb RA, Przyklenk K. Profound cardioprotection with chloramphenicol succinate in the swine model of myocardial ischemia-reperfusion injury. *Circulation*. 2010; 122:S179–184. [PubMed: 20837911]
25. Ambrosio G, Zweier JL, Flaherty JT. The relationship between oxygen radical generation and impairment of myocardial energy metabolism following post-ischemic reperfusion. *J Mol Cell Cardiol*. 1991; 23:1359–1374. [PubMed: 1811055]
26. Gorenkova N, Robinson E, Grieve DJ, Galkin A. Conformational Change of Mitochondrial Complex I Increases ROS Sensitivity During Ischemia. *Antioxid Redox Signal*. 2013; 19:1459–1468. [PubMed: 23419200]
27. Lesnefsky EJ, Chen Q, Moghaddas S, Hassan MO, Tandler B, Hoppel CL. Blockade of electron transport during ischemia protects cardiac mitochondria. *J Biol Chem*. 2004; 279:47961–47967. [PubMed: 15347666]
28. Lesnefsky EJ, Slabe TJ, Stoll MS, Minkler PE, Hoppel CL. Myocardial ischemia selectively depletes cardiolipin in rabbit heart subsarcolemmal mitochondria. *Am J Physiol Heart Circ Physiol*. 2001; 280:H2770–2778. [PubMed: 11356635]
29. Paradies G, Petrosillo G, Pistolese M, Di Venosa N, Federici A, Ruggiero FM. Decrease in mitochondrial complex I activity in ischemic/reperfused rat heart: involvement of reactive oxygen species and cardiolipin. *Circ Res*. 2004; 94:53–59. [PubMed: 14656928]
30. Paradies G, Petrosillo G, Pistolese M, Di Venosa N, Serena D, Ruggiero FM. Lipid peroxidation and alterations to oxidative metabolism in mitochondria isolated from rat heart subjected to ischemia and reperfusion. *Free Radic Biol Med*. 1999; 27:42–50. [PubMed: 10443918]
31. Petrosillo G, Ruggiero FM, Di Venosa N, Paradies G. Decreased complex III activity in mitochondria isolated from rat heart subjected to ischemia and reperfusion: role of reactive oxygen species and cardiolipin. *FASEB J*. 2003; 17:714–716. [PubMed: 12586737]
32. Tompkins AJ, Burwell LS, Digerness SB, Zaragoza C, Holman WL, Brookes PS. Mitochondrial dysfunction in cardiac ischemia-reperfusion injury: ROS from complex I, without inhibition. *Biochim Biophys Acta*. 2006; 1762:223–231. [PubMed: 16278076]
33. Prime TA, Blaikie FH, Evans C, Nadochiy SM, James AM, Dahm CC, Vitturi DA, Patel RP, Hiley CR, Abakumova I, et al. A mitochondria-targeted S-nitrosothiol modulates respiration, nitrosates thiols, and protects against ischemia-reperfusion injury. *Proc Natl Acad Sci U S A*. 2009; 106:10764–10769. [PubMed: 19528654]
34. Chouchani ET, Methner C, Nadochiy SM, Logan A, Pell VR, Ding S, James AM, Cocheme HM, Reinhold J, Lilley KS, et al. Cardioprotection by S-nitrosation of a cysteine switch on mitochondrial complex I. *Nat Med*. 2013; 19:753–759. [PubMed: 23708290]
35. Marella M, Seo BB, Flotte TR, Matsuno-Yagi A, Yagi T. No immune responses by the expression of the yeast Ndi1 protein in rats. *PLoS One*. 2011; 6:e25910. [PubMed: 21991386]

Figure 1. TAT-Ndi1 preparation

Immunoblot of TAT-Ndi1 purified from cell-free expression system. Lane 1: Preparation using control DNA; lane 2-5: preparation using cDNA for TATNdi1; lane 6: Lysate from cell-free expression system; lane 7: E. coli bacterial expression of TAT-Ndi1. Upper panel is probed with anti-HA antibody; lower panel is probed with anti-Ndi1 antibody. Arrowheads indicate 75kDa, the expected MW of TAT-Ndi1.

Figure 2. Complex I and Ndi1 activity in rat heart mitochondria

A. Total and rotenone-resistant Complex I activity was measured in rat heart mitochondria from control rats (n=2); placebo protein-treated rats subjected to I/R (Ctrl+I/R), (n=3); and TAT-Ndi1-treated rats subjected to I/R (Ndi1+I/R), (n=2). B. Blot of cytosol and mitochondria from rat heart 2hr after i.p. administration of TAT-Ndi1, probed with anti-HA antibody. Statistical analysis was not done due to limited number of samples obtained.

Figure 3. Effect of TAT-Ndi1 on infarct size

Infarct size as a percentage of the risk region (28-32% of total LV weight: no difference between groups). Solid circles represent animals treated with control protein, and shaded squares represent animals treated with TAT-Ndi1. Open symbols indicate mean $\pm$ SE; * $p=0.005$. Representative TTC-stained heart sections are shown from rats treated with control protein or Tat-Ndi1.