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Netrin-1 Abrogates Ischemia Reperfusion-induced Cardiac Mitochondrial Dysfunction via Nitric Oxide-dependent Attenuation of NOX4 Activation and Recoupling of NOS

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Abstract

Despite an established role of mitochondrial dysfunction in cardiac ischemia reperfusion (I/R) injury, the upstream activators have remained incompletely defined. We have recently identified an innovative role of exogenously applied netrin-1 in cardioprotection, which is mediated by increased nitric oxide (NO) bioavailability. Here, we tested the hypothesis that this "pharmacological" treatment of netrin-1 preserves mitochondrial function via novel mechanisms that are NO dependent. Freshly isolated C57BL6 mouse hearts were perfused using a Langendorff system, and subjected to a 20 min global ischemia/60 min reperfusion, in the presence or absence of netrin-1. I/R induced marked increases in infarct size, total superoxide and hydrogen peroxide production, activity and protein abundance of NADPH oxidase (NOX) isoform 4 (NOX4), as well as impaired mitochondrial integrity and function, all of which were attenuated by netrin-1. This protective effect of netrin-1 is attributed to cGMP, a downstream effector of NO. The protein levels of NOX1 and NOX2 were however unaffected, and infarct size from NOX1 and NOX2 knockouts were not different from wild type animals. Scavenging of NO with PTIO reversed inhibitory effects of netrin-1 on NOX4, while NO donor attenuated NOX4 protein abundance. *In vivo* NOX4 RNAi, or sepiapterin perfusion, resulted in recoupling of NOS, decreased infarct size, and blockade of dysfunctional mitochondrial swelling and mitochondrial superoxide production. These data demonstrate that netrin-1 induces cardioprotection through inhibition of NOX4 activity, which leads to recoupling of NOS, augmented NO bioavailability, reduction in oxidative stress, and ultimately preservation of mitochondrial function. The NO-dependent NOX4 inhibition connects with our previously established pathway of DCC/ERK1/2/eNOS/NO/DCC feed-forward mechanism, to maintain NOS in the coupling state to attenuate oxidative stress to preserve

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mitochondrial function. These findings may promote development of novel therapeutics for cardiac I/R injury.

Keywords

netrin-1; ischemia/reperfusion (I/R) injury; cardioprotection; nitric oxide (NO); NO synthase (NOS); NOS uncoupling; NADPH oxidase isoforms 4 (NOX4); mitochondrial dysfunction

1) Introduction

Myocardial ischemia reperfusion (I/R) injury describes the damage to the heart that occurs, paradoxically, during the reintroduction of blood flow into the heart after a brief period of ischemia [1]. Past studies have clearly shown that the mitochondria play a major role in the necrosis and apoptosis of cardiomyocytes during this event [2, 3]. The key mechanism seems to be the opening of the mitochondrial permeability transition pore (mPTP), which in turn leads to increased calcium-dependent swelling and rupture of mitochondria, and eventually cell death [4, 5]. One of the key events that control the fate of mitochondria is elevated reactive oxygen species (ROS) production [6-8]. While small amounts of ROS could result in cardioprotection via preconditioning[9], excessive production of ROS during reperfusion seems especially important in inducing injury. Besides its well-documented effects on DNA, lipid, and protein damage[10, 11], there are evidences that this increase in ROS levels contribute to mitochondrial dysfunction. For example, ROS has been shown to trigger the opening of mPTP [12]. Antioxidant enzymes such as manganese superoxide dismutase [13] and catalase [14], as well exogeneously applied agents such as vitamin E[15], have been shown to provide cardioprotection, implicating an intermediate role of oxidative stress in mediating cardiac I/R injury.

While the importance of mitochondrial dysfunction during I/R is acknowledged, mechanisms leading up to the dysfunction and the initial sources of ROS are less clear. In a previous study in our laboratory, we have shown that netrin-1, a secreted axon guiding molecule [16], has important impacts on cardiovascular system by serving as an angiogenic stimulator and a cardioprotective agent[17, 18],[20]. We have further shown that exogenously applied netrin-1 exerts robust cardioprotective effects against I/R injury, via an increase in nitric oxide (NO) production that is dependent on a novel DCC/ERK1/2/eNOS/DCC feed-forward pathway [19]. We therefore wondered if this endogenously increased NO production mediates cardioprotection by modulating oxidative stress and mitochondrial function. Using the Langendorff system to perfuse the heart *ex vivo* for an established I/R protocol, we observed increases in superoxide and hydrogen peroxide production, NOX4 activity, and NOX4 protein abundance, all of which were attenuated to baseline by netrin-1 perfusion. Through the use of NO inhibitors and NO donors, it was clear that NO modulates the reduction of NOX4 by netrin-1. *In vivo* RNAi to inhibit NOX4 pathway resulted in decreased infarct size, reduced superoxide production, recoupled NO synthase (NOS), and improved mitochondrial integrity and function. Further, recoupling of NOS alone, via the perfusion of sepiapterin, was also effective in decreasing infarct size and improving mitochondrial function. Taken together, our data demonstrate a novel pathway by which netrin-1 abrogates mitochondrial dysfunction during cardiac I/R injury, namely NO-

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dependent inhibition of NOX4 activation and consequent attenuation of NOS uncoupling. These findings reveal new mechanistic insights into I/R induced mitochondrial dysfunction, and may therefore promote development of novel therapeutics.

2) Methods

2.1) Materials

Unless otherwise noted, all chemicals and drugs in the highest purity were obtained from Sigma-Aldrich (St. Louis, MO). Custom made siRNA was obtained from Integrated DNA Technologies (Coralville, IA).

2.2) Animals

Six to eight weeks old male C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). The NOX1 null (NOX1-/y) founder mice were generously provided by Dr. Krause Karl-Heinz from the University of Geneva. NOX2 null (NOX2 -/y) mice breeders were originally obtained from Jackson Laboratories (Cat#002365). The use of animals and experimental procedures were approved by the Institutional Animal Care and Usage Committee at the University of California Los Angeles.

2.3) Langendorff perfusion for cardiac ischemia reperfusion injury

Langendorff perfusion of the heart was performed as previously describe[19]. A schematic of the perfusion protocols is shown in Figure 1A. Perfusion dosages of the interventions were: netrin-1, 100 ng/mL, PTIO, 60 μmol/L, and RP-8-Br-PET-cGMP, 10 μmol/L.

2.4) Preparation of heart tissue for biochemical assays

For superoxide measurement and Western blot analysis, hearts were homogenized in ice cold homogenization buffer (Tris 50 mmol/L, EDTA 0.1 mmol/L, EGTA 0.1 mmol/L, protease inhibitor cocktail, pH 7.4) on ice using a glass homogenizer (DUALL model, size 21, Kimble-Chase, Vineland, NJ). The homogenates were then centrifuged at 16,000 g for 15 min at 4°C, and the resulting supernatant used.

2.5) Superoxide and NOS uncoupling activity measurements with electron spin resonance

Superoxide production and NOS uncoupling activity were determined from heart homogenates using electron spin resonance (ESR) as previously described [20-27]. For the determination of NOS uncoupling activity, superoxide production was measured in the presence or absence of NOS inhibitor L-NAME (100 μmol/L). When NOS is healthy or "coupled", L-NAME reduces NO production to result in less buffering effect of superoxide, hence an increase in superoxide detected. When NOS is "uncoupled", L-NAME treatment leads to the reduction in superoxide production, which is derived from NOS.

2.6) Membrane fraction NOX activity assay

For isolation of membrane factions, hearts were homogenized on ice as described above. The homogenates were serially centrifuged at 1,200 g for 5 min at 4°C, 22,000 g for 20 min

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at 4°C, and 137,000 g for 90 min at 4°C. The pellet was collected and resuspended in homogenate buffer (100 μL per heart).

Superoxide production from membrane factions was determined in the presence or absence of NADPH substrate (100 μmol/L final concentration) using ESR, with 10 μg protein loaded per sample. Kinetic NOX activity was calculated as the difference between the two measurements, normalized by protein. NOX4 contribution to this signal was assessed using Fulvene-5, a specific NOX4 inhibitor (kindly provided by Dr. Jack L. Arbiser from Emory University) [28].

2.7) Western blot for protein level analysis of NOX isoforms

Western blotting was performed as per standard protocols. Primary antibody for NOX1 (SC-5821, Santa Cruz, Santa Cruz, CA) was used at a dilution of 1:1000; NOX2 (611415, BD Transduction Labs, Sparks, MD) at 1:1000; NOX4 (AB81967, Abcam, Cambridge, MA) at 1:1000; and actin (A2066, Sigma-Aldrich, St. Louis, MO) at 1:3000. Secondary antibody conjugated to HRP (Bio-Rad) was used at a dilution of 1:2000 for NOX1, NOX2, and NOX4, and 1:10000 for actin. In view of the controversies regarding a good NOX4 antibody, and the fact our NOX4 antibody worked well after titrating at different conditions, we have performed additional control experiments using overexpressed NOX4 as a positive control. As shown in Fig. 1D, the antibody was able to detect NOX4 specifically. The procedures for this control experiment were: bovine aortic endothelial cells cultured as previously described[29] at passage 4-5 were transfected with pCMV-XL5 human NOX4 plasmid (Origene , Cat#SC310253). NOX4 siRNA used is the same as the one described below. Transfection was done once per day for two days using Lipofectamine 2000 (Invitrogen) as per manufacturer's instructions.

2.8) Mitochondrial isolation

Heart tissue was rinsed twice in ice cold PBS immediately at the end of the Langendorff experiment, then homogenized in a pre-chilled glass homogenizer using isolation buffer I (sucrose 250 mmol/L, EGTA 1 mmol/L, HEPES 10 mmol/L, and Tris-HCl 10 mmol/L, pH 7.4, 1 ml buffer/0.1 g tissue) on ice. The homogenate was then centrifuged at 800 g for 7 min at 4°C, followed by 4,000 g for 15 min at 4°C. The pellet was rinsed using 1 ml of isolation buffer II (isolation buffer I without EGTA), then centrifuged again at 4,000 g for 15 min at 4°C. Further purification was performed by resuspending the pellet with 3 ml of a 19% percoll solution, which was then layered on top of a double layer of 30% and 60% percoll solution in a centrifugation tube, and finally centrifuged at 60 min at 4,500 g. The mitochondrial layer in the tube was carefully removed and washed by alternating centrifugation and resuspension with isolation buffer II. The final pellet was resuspended in isolation buffer II.

2.9) Mitochondrial swelling assay

Thirty μg of freshly isolated mitochondria were mixed in buffer containing 250 mmol/L sucrose and 10 mmol/L Tris (pH 7.4). The mixture was then incubated with 5 mmol/L succinate for 1 min at room temperature, then with 250 μmol/L of $CaCl_2$. Absorbance at 540

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nm was measured immediately for 20 min at 1 min intervals. Swelling was measured as a decrease in absorbance over time.

2.10) Mitochondrial respiration assay

Mitochondrial respiration was measured using XF24 analyzer (Seahorse Biosciences). Isolated mitochondria were mixed with mitochondrial assay solution (sucrose 70 mmol/L, mannitol 220 mmol/L, KH_2PO_4 5 mmol/L, $MgCl_2$ 5 mmol/L, HEPES 2 mmol/L, EGTA 1 mmol/L, FA-free BSA 0.2%) and placed onto the assay plate for quadruplicate measurements (2 μg protein per well). Respiratory control ratio, which measures the tightness of coupling between respiration and phosphorylation, was measured as the ratio between state 3 and state 4, where state 3 is the oxygen consumption rate (OCR) after stimulation with ADP (4 mmol/L final concentration), and state 4 is the OCR after blockage of complex V with oligomycin (2 umol/L final concentration).

2.11) Amplex red measurement of H_2O_2

Hearts were homogenized in a glass homogenizer with ice-cold Krebs-Ringer Buffer immediately after the perfusion protocol. Equal parts of the homogenates were mixed with Amplex Red reaction buffer, containing 50 μmol/L Amplex red and 0.16 U/mL of HRP in 50 mmol/L sodium phosphate buffer, pH 7.4. In separate tubes, PEG-catalase (100 U/mL final concentration) was added to assess non-H_2O_2 specific signal in the sample. The samples were incubated at 37°C for 1 hr in the dark. The samples were then centrifuged at 2,500 g for 5 min. The supernatants were loaded to a 96 well plate, and then measured using a fluorescent plate reader at 530 nm excitation and 590 emission. Freshly prepared H_2O_2 standards were used to calculate the amount of H_2O_2 produced by the samples.

2.12) TTC staining for inarct size analysis

At the end of Langendorff experiments, hearts were removed and sliced into 7 sections using a rodent heart slicing matrix (Holliston, MA). Heart tissues were then incubated in a TTC solution (1% TTC in PBS) for 20 min at 37°C, prior to being fixed overnight in 10% formalin for contrast.

2.13) Detection of S-nitrosylated proteins

S-nitrosylated proteins were detected using the S-nitrosylated protein detection kit from Cayman Chemical (Cat#10006518) as per manufacturer's instructions, based on the Jaffrey *et al.* ‘Biotin-switch’ method. Briefly, hearts after Langendorff perfusion were homogenized on ice with a glass homogenizer in the supplied buffer, followed by blocking free -SH groups, cleavage of any S-NO bonds, and biotinylation of new –SH groups. The resulting homogenate was then separated in a 10% SDS-PAGE and transferred to a nitrocellulose membrane, and subjected to streptavidin-based colorimetric detection.

2.14) Cardiomyocyte isolation and treatment with MAMANOATE

After anesthesia with isoflurane, the heart from mice was rapidly removed and placed in ice cold perfusion buffer (113 mmol/L NaCl, 4.7 mmol/L KCl, 0.6 mmol/L KH_2PO_4, 0.6 mmol/L Na_2HPO_4, 1.2 mmol/L $MgSO_4$-$7H_2O$, 0.032 mmol/L phenol red, 12 mmol/L

$NaHCO_3$, 10 mmol/L $KHCO_3$, 10 mmol/L HEPES, 30 mmol/L taurine, 10 mmol/L 2,3-Butanedione monoxime, 5.5 mmol/L glucose). The heart was then cleaned of connecting tissue, cannulated and perfused with 10 ml of perfusion buffer at 37°C. Afterwards, the heart was then perfused with digestion buffer (perfusion buffer plus 12.4 μM $CaCl_2$, 0.14 mg/mL trypsin, 7.7 mg/mL collagenase II) for 20 min at 37°C, and then cut into 4 pieces in digestion buffer and gently pulled apart using forceps. The larger pieces were further broken apart by pipetting using wide bore tips several times. Equal volume of myocyte stopping buffer 1 (perfusion buffer plus 10% fetal bovine serum, 12.5 μmol/L $CaCl_2$) was added to the solution, and the entire sample was allow to sediment for 10 min. The pellet was resuspended with myocyte stopping buffer 2 (perfusion buffer plus 5% fetal bovine serum and 12.5 μmol/L $CaCl_2$). $CaCl_2$ was slowly added to the suspension at RT in 4 min steps with final $CaCl_2$ concentration of 62 μmol/L, 112 μmol/L, 212 μmol/L, 500 μmol/L, and finally 1 mmol/L. The suspension was then allowed to sediment again for 10 min at RT, and the pellet was resuspended with myocyte culture medium (MEM with Hanks' salts and L-glutamine plus 0.1 mg/mL bovine serum albumin, 100 U/mL penicillin, and 2 mmol/L L-Glutamine). Cardiomyocytes were treated with either vehicle (sterile water), or MAMANOATE (1 mmol/L), for 2 hr in a cell incubator prior to being harvested for analysis of NOX4 protein abundance by Western blotting.

2.15) siRNA treatment

Five nmol of *in-vivo* grade siRNA (NOX4: sense: CAUGCUGCUGCUGUUGCAUGUUUCA, antisense: CCCUCUGAUGUAAUGGAACUCCGUA, Dharmacon; control: sense: UUCUCCGAACGUGUCACGUdTdt, antisense: ACGUGACACGUUCGGAGAAdTdT Dharmacon) was prepared with a nanoparticle based *in-vivo* transfection kit (Altogen Biosystems) as per manufacturer's instructions. Injections were made via tail vein, once every 24 hours for 2 days Experiment was performed 24 hrs after the last injection.

2.16) Nitric oxide measurement using electron spin resonance

Nitric oxide (NO) was measured using electron spin resonance (ESR) as previously described [19-27]. Briefly, whole heart homogenates were incubated in equal volume of freshly prepared NO-specific spin trap $Fe^{2+}(DETC)_2$ colloid (0.5 mmol/L) for 60 min at 37°C and treated with the calcium ionophore A23187 (10 μmol/L). The suspension was then snap-frozen with liquid nitrogen, then loaded into a finger Dewar for measurement in an eScan ESR spectrophotometer (Bruker), using the following settings: center field, 3410; field sweep, 100 G; microwave frequency, 9.73 GHz; microwave power, 13.26 mW; modulation amplitude, 9.82 G; 512 points resolution and receiver gain, 356.

2.17) Determination of H_4B content by HPLC

After the Langendorff experiment, the entire heart was homogenized on ice in H_4B lysis buffer (0.1 mol/L phosphoric acid, 1 mmol/L EDTA, 10 mmol/L DL-Dithiothreitol), and then centrifuged at 12,000g for 3 min. Lysates were then subjected to differential oxidation in acidic (0.2 mol/L trichloroacetic acid with 2.5% I2 and 10% KI) and alkalytic (0.1 mol/L NaOH with 0.9% I2 and 1.5% KI) solutions as described previously. After centrifugation, 20

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μL of the supernatant was injected into a HPLC system equipped with a fluorescent detector (Shimadzu). Excitation and emission wavelengths of 350 nm and 450 nm were used to detect H_4B and its oxidized species.

2.18) Statistical analysis

All statistical analyses were performed using Sigmastat. Data are presented as Mean±SEM. Student's *t*-test was used when testing for the difference between two groups of data, while ANOVA was used to compare multiple groups, both with a significant level of 0.05. Holm-Sidak test was used as the post-hoc test after the ANOVA

3) Results

3.1) Netrin-1 abolishes I/R-induced increase in superoxide production, NOX activity, NOX4 protein abundance, and mitochondrial dysfunction

We have previously shown that netrin-1 exerts potent cardioprotective effects via increased production of nitric oxide (NO) [19]. In view of the established role of mitochondrial dysfunction in cardiac I/R injury, the present study aimed to delineate whether netrin-1's cardioprotective effects are at least in part mediated by NO-dependent protection of mitochondrial function. One of the known upstream factors of mitochondrial dysfunction during I/R is oxidative stress. Therefore we first examined whether netrin-1 has any impact on superoxide production during cardiac I/R injury. A Langendorff perfusion system was used to perfuse heart *ex vivo* in the presence or absence of netrin-1 treatment. Figure 1A shows a schematic illustration of the experimental protocols.

3.1.1) Netrin-1 abolishes I/R-induced increase in superoxide production— Superoxide production was measured using ESR in heart homogenates from hearts that underwent I/R injury using the Langendorff system, with and without perfusion of netrin-1 (100 ng/ml). As shown in Figure 1B, I/R injury induced a ~2.5 fold increase in superoxide production compared to no I/R sham controls (p=0.001). Markedly, perfusion with netrin-1 attenuated this increase back to baseline.

3.1.2) Netrin-1 reverses I/R-induced increase in NOX activity—It has become clear that NOXs are the major enzymatic sources of ROS in the vasculature, although their roles in the heart have been less clear. Here, we examined whether NOX activity was regulated by netrin-1 during I/R. Figure 1C shows NOX activity under control, I/R, and netrin-1 perfused I/R conditions. The data here show that NADPH-driven NOX activity in the membrane fraction was significantly increased with I/R, while netrin-1 perfusion reduced this activation to near control levels.

3.1.3) Netrin-1 attenuates I/R-induced increase in NOX4 protein abundance— Western blotting was used to examine whether certain NOX isoform(s) was(were) regulated at the protein level to contribute to this increase in NOX activity. Figure 1D shows the validation of the NOX4 antibody, using NOX4 plasmid overexpression and NOX4 siRNA knockdown. The results show that the NOX4 antibody used was specifically detecting the correct protein in the Western blot. Figure 1E shows that while the protein levels of NOX1

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and NOX2 remained unchanged under the different conditions, NOX4 was significantly and reproducibly upregulated by I/R, and attenuated by netrin-1. Further, specific NOX4 activity was assessed by measuring NOX activity with and without the presence of fulvene-5, a specific NOX4 inhibitor[28]. The results, shown in Figure 1F, indicate that I/R significantly increased NOX4 activity, while netrin-1 abolished this increase. Taken together, these data suggest that NOX4 is specifically responsible for the increase in NOX activity observed during I/R, and that abrogation of NOX4 protein abundance and activity by netrin-1 at least in part explains its inhibitory effect on oxidative stress.

3.1.4) Netrin-1 abolishes I/R-induced mitochondrial dysfunction—To determine effects of netrin-1 on cardiac mitochondrial integrity and function, mitochondrial swelling assay was performed using freshly isolated, purified mitochondria from I/R-injured hearts with or without netrin-1 perfusion. While I/R induced significant dysfunctional swelling of the mitochondria, this was completely attenuated to baseline by netrin-1 perfusion (Figure 1G), likely consequent to the beneficial effects of netrin-1 in attenuating NOX4-dependent oxidative stress as described above. We later found that indeed, inhibition of NOX4 signaling was able to preserve mitochondrial integrity as reflected by reduced swelling (below in section 3.3.3)

To further assess mitochondrial function, oxygen consumption rate of the isolated mitochondria was determined, and the respiratory control ratio (RCR), a measure of mitochondrial function, was calculated. The results show that I/R significantly reduced RCR, while the perfusion of netrin-1 brought the RCR back to control levels (Figure 1H, n=4, $p<0.05$). Taken together, these data show that netrin-1 perfusion prevents the decline of mitochondrial function from I/R.

3.1.5) Netrin-1 reduces I/R induced increase in H_2O_2 levels—To further examine netrin-1's effect on the redox state of the I/R-injured heart, H_2O_2 levels were also measured using the Amplex Red assay. NOX4 has been previously shown to produce H_2O_2 directly in some cell types, although it may also be due to the fact only permeable H_2O_2 can be detected from these cells. Figure 1I shows that in our system, production of H_2O_2 had a similar trend as superoxide production, where I/R significantly increased H_2O_2 production, and netrin-1 completely abolished this increase.

3.1.6) Netrin-1 induced cardioprotection is mediated by cGMP—We next examined ether whether netrin-1's cardioprotective effect is also mediated via cGMP, a downstream effector of NO. Isolated mice hearts were perfused with the cGMP inhibitor Rp-8-Br-PET-cGMP, then netrin-1 before I/R, followed by infarct size analysis via TTC. The results in Figure 1J show that inhibition of cGMP completely abolished netrin-1's cardioprotective effect, suggesting that the increased NO from netrin-1 perfusion exerts its cardioprotective effect via cGMP ($p<0.01$, n=4 each). In addition, we further examined S-nitrosylation of proteins, which has been shown to play an important role in protection against I/R injury [30-32], as a possible downstream regulator of netrin-1's cardioprotective effect. However, netrin-1 did not seem to regulate this process significantly (n=4 each, Figure 1K). Since netrin-1 attenuates ROS production through NO-dependent inhibition of

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NOX4/uncoupled NOS/mitochondria pathway (see below), these data may indeed imply lack of nitrosative stress that often increases protein nitrosylation.

3.2) NO mediates NOX4 reduction during netrin-1 provoked cardioprotection against I/R injury

We have previously shown that netrin-1 protects the heart against I/R damage via a DCC/ERK1/2/eNOS/NO pathway[19]. Here, we examined whether NO produced in this pathway was involved in the attenuation of NOX4 afforded by netrin-1 administration. We examined NOX4 protein abundance after perfusing the heart with a potent NO scavenger PTIO, along with netrin-1. The results, shown in Figure 2A, indicate that PTIO completely abolished netrin-1's downregulating effects on NOX4 protein level.

In addition, we subjected freshly isolated cardiomyocytes to the NO donor MAMANOATE prior to analysis of NOX4 protein abundance by Western blotting. As is obvious, NO is indeed highly effective in attenuating NOX4 protein levels in primary cardiomyocytes (Figure 2B). Taken together, these data suggest that netrin-1 downregulation of NOX4 protein abundance during I/R injury is mediated by endogenously increased NO production.

3.3) Role of attenuated NOX4 in netrin-1 induced cardioprotection

Data described above implicate that NOX4 may be involved in netrin-1's cardioprotective effects. Here, we further tested this hypothesis by examining effects on cardiac protection of silencing NOX4 pathway using *in vivo* RNAi.

3.3.1) Validating the use of NOX4 siRNA—Figure 3A shows Western blot data from mice that were transfected with control or NOX4 siRNA. The top two panels indicate that the protein levels of NOX1 and NOX2 in the heart remained unchanged, while the protein level of NOX4 was significantly depressed with the NOX4 siRNA treatment.

3.3.2) NOX4 siRNA treatment protects against I/R damage—TTC staining was used to assess infarct size in mice transfected of control or NOX4 siRNA prior to I/R injury. The summarized data shown in Figure 3B indicate that infarct size decreased significantly from 47.8±6.0% to 15.0±5.3% in response to *in vivo* NOX4 RNAi, implicating an important role of NOX4 in I/R induced cardiac damage. This seems consistent with the previously established role of NOX4 in mediating hypertropic injury of the heart[33].

To further validate that NOX4 is the NOX isoform responsible for this protective mechanism, NOX1-KO and NOX2-KO mice were subjected to I/R damage and determination of infarct size. The results, shown in Figure 3C, indicate that these animals were not protected against I/R damage (infarct size at 43.4±5.0% and 42.3±5.9% for NOX1-KO and NOX2-KO, respectively), suggesting that NOX4 is the NOX isoform specifically involved in the process.

3.3.3) NOX4 siRNA treatment improves mitochondrial function in I/R-injured heart—As was shown earlier, netrin-1 perfusion improved mitochondrial function (Figure 1G). We further tested whether suppressing the protein abundance of NOX4 alone was able to also improve mitochondrial function. Figure 3D demonstrates that NOX4 siRNA

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treatment resulted in largely restored mitochondrial integrity and function, as assessed by calcium induced swelling assay. These data suggest that mitochondrial dysfunction lies downstream of NOX4 activity in I/R injured heart.

3.3.4) NOX4 siRNA treatment reduces mitochondrial ROS production in I/R-injured heart

3.3.4) NOX4 siRNA treatment reduces mitochondrial ROS production in I/R-injured heart—To further validate the improvement of mitochondrial function after NOX4 siRNA treatment, we measured superoxide production directly from the isolated mitochondria via ESR. Figure 3E indicates superoxide production from isolated mitochondria. In the control siRNA treated hearts, superoxide production increased significantly under I/R when compared to the no I/R group (from 0.1689±0.019 to 0.4683±0.047 μM/min/mg protein), p<=0.001). However, in NOX4 siRNA treated hearts, this increase was largely attenuated (from 0.142±0.032 to 0.209±0.027 μM/min/mg protein), non significant).

3.3.5) NOX4 siRNA treatment recouples NOS in I/R-injured heart—In previous studies, we and others have elucidated that NOX activity lies upstream of eNOS uncoupling[20, 24, 34, 35]. We also know that the activation of coupled eNOS by the DCC/ERK dependent pathway is important for the production of NO in response to netrin-1. Therefore we wondered if the inhibition of the NOX pathway would be sufficient in protecting NOS to stay in its coupled state to produce NO rather than more superoxide if uncoupled. Here, we inhibited NOX4 pathway using *in vivo* RNAi, and assessed the NOS coupling state via determination of L-NAME sensitive superoxide production. Summarized data in Figure 3F demonstrate that with control siRNA treatment, I/R uncouples NOS, as shown by the reduction of superoxide production with the addition of L-NAME. With NOX4 siRNA treatment, the addition of L-NAME in the measurement did not decrease superoxide production, suggesting that NOS is recoupled. This shows that inhibiting NOX4 through the use of NOX4 siRNA was able to prevent NOS uncoupling, implicating an upstream role of NOX4 in NOS uncoupling which has been previously implicated in models of heart failure.

3.4) Role of NOS recoupling in netrin-1 induced cardioprotection

Notably, the enzyme eNOS or NOS can serve as a significant source of superoxide in the hypertropic and failing hearts[36, 37]. Under certain pathological conditions, the normally NO producing NOS can become uncoupled and instead produces superoxide. Previous studies have shown that eNOS is uncoupled under I/R[38, 39]. Data described in the above paragraph revealed that reduction of NOX4, via *in vivo* RNAi, was effective in recoupling eNOS activity that was uncoupled during I/R. We next examined whether recoupling of NOS, via the perfusion of sepiapterin, is effective in abrogating I/R induced cardiac injury.

3.4.1) Both netrin-1 and Sepiapterin recouples NOS in I/R-injured hearts— Sepiapterin, one of the stable precursors of NOS cofactor tetrahydrobiopterin (H_4B), has been shown in the past to recouple NOS[40]. Here, we first examined whether netrin-1 and sepiapterin recouples NOS in Langendorff perfused hearts. Figure 4A shows results of L-NAME sensitive superoxide production determined by ESR, which indicate that both netrin-1 and sepiapterin perfusion potently recoupled NOS.

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We further measured NO production from these hearts using ESR. Figure 4B show that I/R significantly reduces the amount of NO when compared to controls, suggesting dysfunctional NOS. Perfusion with netrin-1 or sepiapterin completely restored NO bioavailability under I/R, indicating restoration of NOS function.

3.4.2) Netrin-1 improves H_4B levels in I/R injured hearts—To further elucidate effects of netrin-1 on NOS coupling, we measured bioavailability of NOS cofactor H_4B. The results, shown in Figure 4C, indicate that I/R reduces tissue levels of H_4B, while the perfusion of netrin-1 restores this back to control levels. These data strongly suggest that netrin-1 is effective in preserving H_4B bioavailability to improve NOS function under I/R.

3.4.3) Sepiapterin decreases infarct size in I/R-injured heart—Next, we tested whether NOS recoupling alone via the use of sepiapterin offers cardioprotection. Summarized data in Figure 4D demonstrate that sepiapterin perfusion significantly decreased infarct size from 46.6±4.3% to 18±6.4% when compared to non-treated controls.

3.4.4) Sepiapterin improves mitochondrial function in I/R-injured heart—We further tested whether NOS recoupling via the use of sepiapterin can improve mitochondrial function. The summarized results in Figure 4E show that mitochondrial integrity assessed by calcium induced swelling assay was markedly improved by sepiapterin perfusion. This would suggest that NOS uncoupling lies upstream of mitochondrial dysfunction in I/R-injured heart, and that prevention of NOS uncoupling via mechanisms such as attenuation of NOX4 activation, is beneficial in preserving mitochondrial function. Taken together, these data seem to implicate a NOX4/NOS uncoupling/mitochondria pathway that is attenuated by netrin-1-derived NO production, resulting in cardioprotection.

3.4.5) Sepiapterin reduces mitochondrial superoxide production in I/R-injured heart—Superoxide production from isolated mitochondria from I/R-injured hearts was also measured to assess mitochondrial function. Figure 4F shows that I/R significantly increases superoxide production from isolated mitochondria from 0.143±0.05 to 0.430±0.017 μM/min/mg protein ($p<0.01$, n=3), while the recoupling of NOS by sepiapterin attenuated this response to 0.235±0.87 μM/min/mg protein.

Discussion

The most significant finding of the present study is the identification of a novel pathway of NOX4/NOS uncoupling/mitochondrial dysfunction in I/R injury, which can be attenuated by netrin-1 in a NO-dependent fashion to result in cardioprotection. NOX4 protein abundance and activity were found to be increased in I/R-injured hearts, which would increase superoxide and hydrogen peroxide production. This increase then leads to uncoupling of NOS, further accumulation of reactive oxygen species and consequently mitochondrial dysfunction. Perfusion of netrin-1 abolished NOX4 activation, leading to the recoupling of NOS and preservation of mitochondria function, ultimately reduced infarct size. Netrin-1 reduction of NOX4 was abolished by NO scavenger PTIO, while NO donor MAMANOTE also downregulated NOX4 protein abundance in primarily cultured cardiomyocytes, altogether suggesting an intermediate role of NO in mediating netrin-1 attenuation of NOX4.

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We have previously established that netrin-1 exerts cardioprotection by a DCC/ERK1/2/eNOS/NO/DCC feed-forward pathway that results in markedly elevated NO production. Our current study extends on these findings to have further pinpointed that this endogenously elevated NO exerts its cardioprotective effects by targeting NOX4 and uncoupled NOS to preserve mitochondrial function, which is a central mediator of survival signaling under I/R.

As shown in Figure 1B, I/R dramatically increased superoxide production in perfused hearts, which was completely abrogated by the addition of netrin-1. As it is well established that abnormal increases in oxidative stress can lead to tissue damage, this clearly shows that netrin-1's cardioprotective effects are at least in part mediated via the reduction in oxidative stress. Further, we show here that this increase is related to an increase in NOX4 protein abundance and NOX4 activity in these hearts, but not changes in other NOX isoforms. In a study using cardiac specific NOX4 transgenic mice, the investigators have demonstrated that upregulation of NOX4 stimulates apoptosis in cardiac myocytes, and induces mitochondrial superoxide production and mitochondrial damage [33]. Our findings seem to be consistent with these observations to show that a NO dependent attenuation of NOX4 by netrin-1 was able to provide protection against mitochondrial dysfunction and ultimately cardiac damage. Indeed, as shown in Figure 1G, netrin-1 markedly attenuated I/R induced mitochondrial swelling.

To further examine the importance of regulations of NOX4 in I/R damage and netrin-1 induced cardioprotection, we used siRNA to decrease protein abundance of NOX4 *in vivo*. We have shown that silencing NOX4 was able to reduce infarct size (Figure 3B), and improve mitochondrial function (Figure 3D & E). Further, NO-producing activity of NOS was restored in NOX4 inhibited hearts, suggesting that NOX4 activation can lead to detrimental NOS uncoupling (Figure 3F, 4B&C.) A previous study using mice deficient in NOX activity has shown that NOX activity did not contribute to I/R injury[41]. However, it should be noted that in that study, mice used were deficient in p47phox[41], an essential component of most NOX isoforms including NOX2 that is abundant in the heart. However, studies into the function of NOX4 have shown that p47phox is not necessary for NOX4 activation[42]. Our data also indicated that NOX2 and NOX1 were not regulated by I/R or netrin-1. NOX1 seems much less abundant than NOX2 and NOX4 in the heart, although our Western blot data cannot compare their relative abundance quantitatively. Taken together, this may suggest that NOX4 is the only, specific isoform of NOXs that is involved in early response to I/R injury. Of note, it was reported that NOX2 was upregulated under I/R in rabbits[43]. Other than being a different animal and experimental model, the major difference between this report and our study is the length of reperfusion. The previous study in rabbits has a reperfusion time of 12 hours, while in the current study it is 1 hour. These observations may suggest a temporal involvement of different NOX isoforms under I/R, which however require further investigation.

To further elucidate the sequence of events that leads to netrin-1 induced cardioprotection, we examined the importance of NOS coupling activity in improving mitochondria function. Sepiapterin is a stable precursor of NOS cofactor tetrahydrobiopterin (H_4B), which has been shown to restore NO generating activity from uncoupled NOS, or “recouple NOS”. Using sepiapterin perfusion, we were able to show decreased infarct size (Figure 4D), restoration

of NOS function (Figure 4A, B, C), as well as improved mitochondrial function (Figure 4E & F). Taken together, these data clearly illustrate two key points. First, uncoupled NOS is one of the major sources of ROS in I/R injured heart. The second point is that uncoupled NOS lies upstream of mitochondrial dysfunction that ultimately leads to cardiac cell death during I/R. As described above, we found that recoupling of NOS via the reduction of NOX4 also occurs in response to netrin-1 perfusion (Figure 3F). These findings are consistent with previous observations implicating an important protective role of NO during I/R injury [8, 44]. Our data uniquely further demonstrate that endogenous production of NO in response to netrin-1, from NOS that is maintained in the coupling state, exerts potent cardioprotection via preservation of mitochondrial function.

It should be noted that previous literatures have reported that NOX2 and NOX4 are the two major NOX isoforms in the heart [45, 46]. In the present study we clearly observed NOX1 protein expression in our samples of heart homogenates. The exposure time for the NOX1 blots was however much longer than those for the NOX2 and NOX4 blots, although this is perhaps more likely affected by efficacies of different antibodies. Previous studies have shown that while NOX1 is present in the heart, it is in much lower abundance than NOX2 and NOX4 [33, 47]. In the present study, the TTC data indicate that NOX1 KO mice did not protect against I/R damage, implicating that NOX1 is likely not involved in mediating I/R injury. It is important to note however, other evidences exist that NOX1 is important in the ischemic response in other organs, such as during stroke[48]

Of note, NOX4 has been shown to be upregulated by hypoxia in many different tissues, such as kidneys [49], brain [50], pulmonary vasculature [51], and the failing heart [45]. In all these conditions NOX4 was found to play a damaging role. However, in a previous study NOX4 was found to play a protective role in chronically stressed hearts through an enhancement of angiogenesis[52]. It is important to note that the difference between this study and the present study is the time frame, where in the current model the hearts were harvested 1 hour post ischemia, while in the aforementioned study the tissue was harvested after 6 weeks.

In conclusion, this work identifies a novel pathway of NOX4 activation and NOS uncoupling in mediating mitochondrial dysfunction during cardiac I/R injury. Netrin-1 exploits this pathway by reducing the protein abundance and activity of NOX4, which leads to recoupled NOS and improved mitochondrial function, ultimately reducing infarct size to afford cardioprotection. The inhibition of NOX4 was further found to be mediated by NO, which connects the present results with our previously established notion of a DCC/ERK1/2/eNOS/NO/DCC feed-forward mechanism of netrin-1 mediated cardioprotection[19]. These overall molecular mechanisms of netrin-1 induced cardioprotection are summarized schematically in Figure 5.

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Highlights

- Netrin-1 attenuates ischemia reperfusion (I/R) injury-induced oxidative stress
- Netrin-1 recouples nitric oxide synthase (NOS) during I/R
- Netrin-1 preserves mitochondrial function through NO-dependent inhibition of NADPH oxidase isoforms 4 (NOX4) and NOS uncoupling
- In vivo RNAi inhibition of NOX4 or perfusion of sepiapterin to recouple NOS result in cardioprotection against I/R
- NO inhibition of NOX4 connects to DCC/ERK1/2/eNOS/NO pathway for cardioprotection, by maintaining NOS in the coupling state

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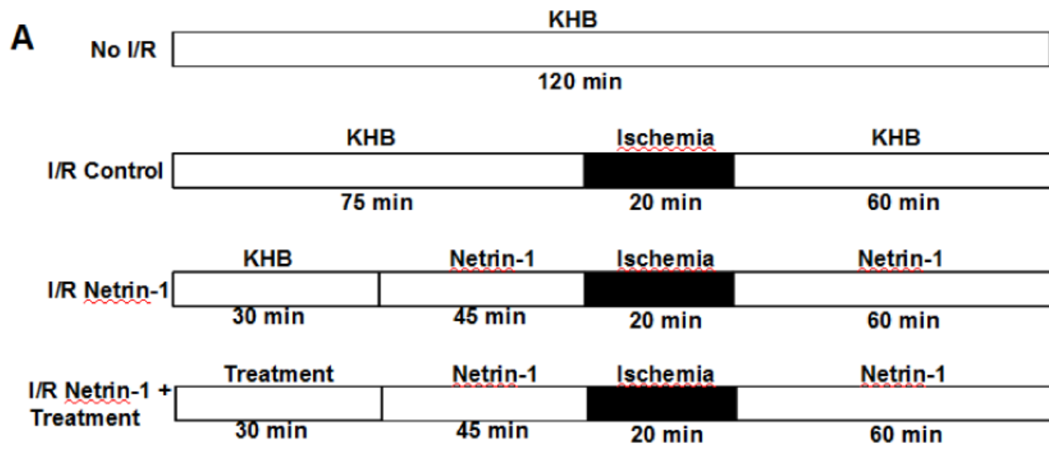

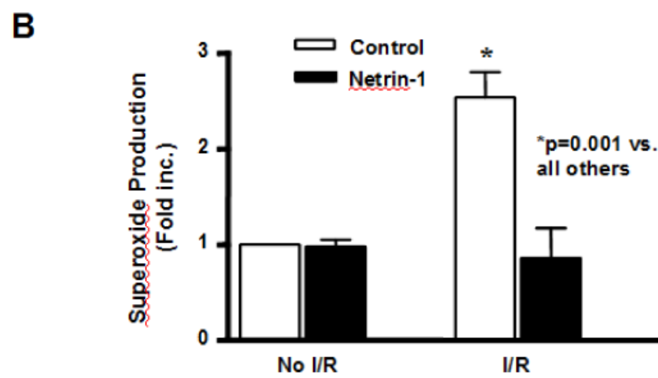

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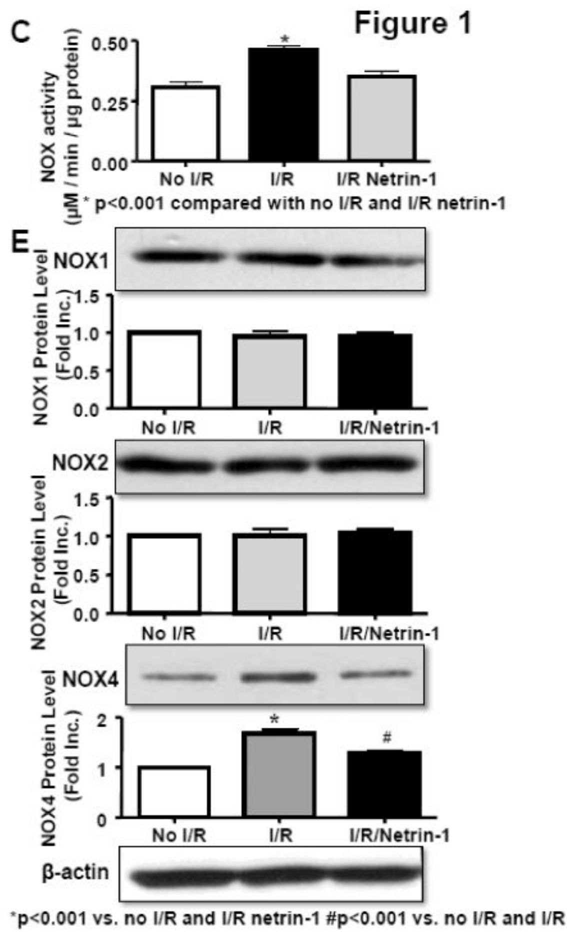

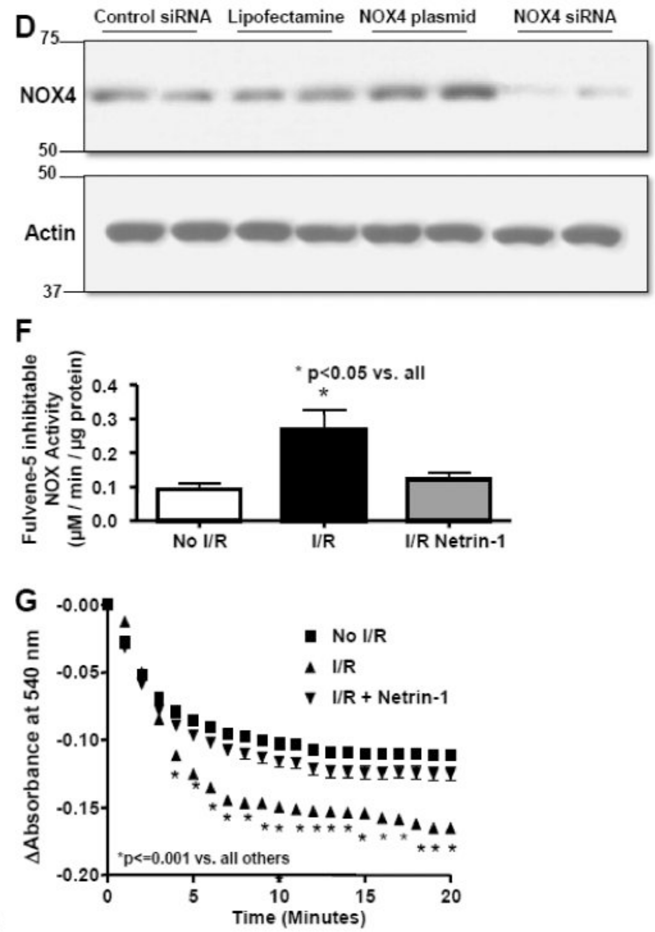

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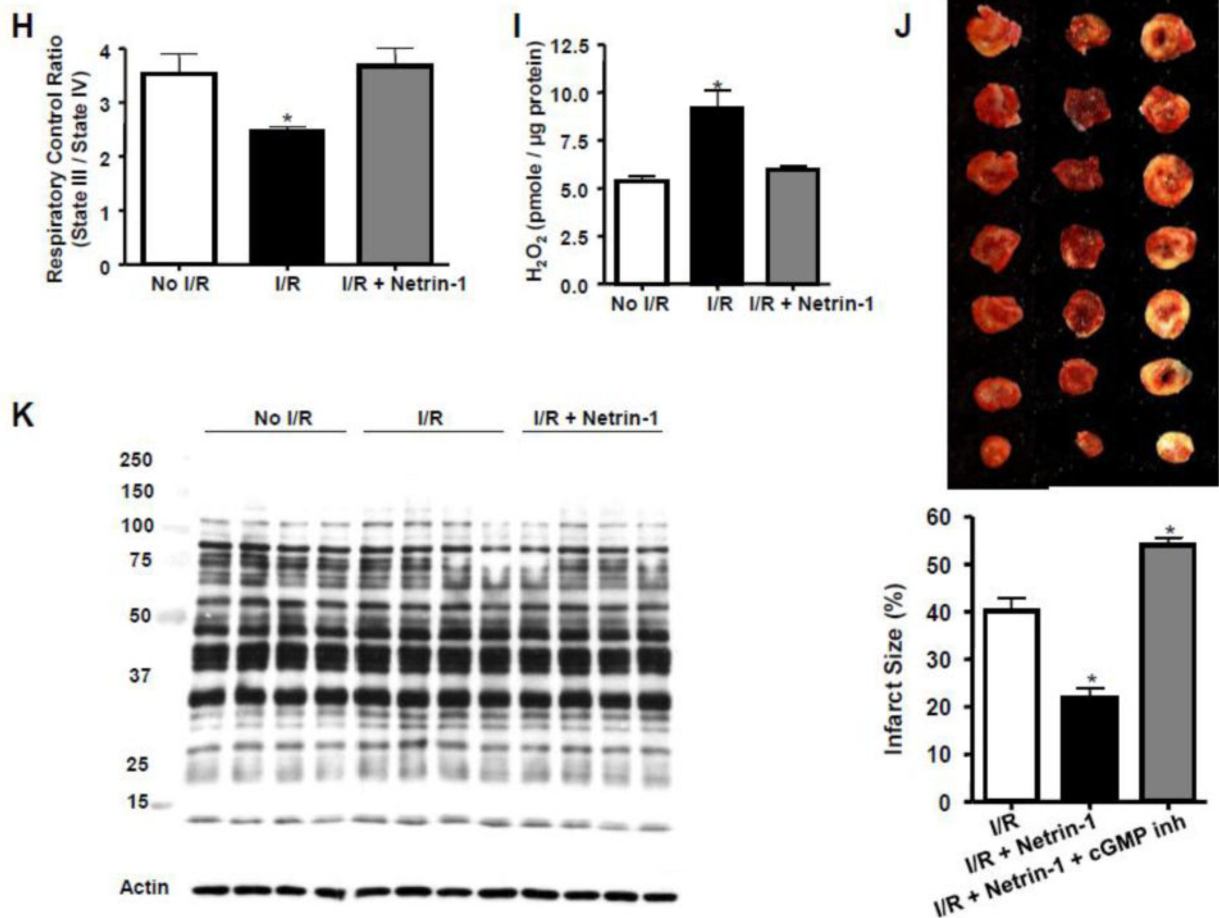

Figure 1. Netrin-1 attenuates I/R induced increases in superoxide production, NOX activity, NOX4 protein abundance and mitochondrial dysfunction

A) Schematic illustration of experimental protocols. **B)** Superoxide production from I/R injured hearts with and without netrin-1 perfusion. Superoxide production from heart homogenates was measured using electron spin resonance (ESR), shown as the amount inhibited by Mn-SOD, and normalized to no I/R condition. Superoxide production from the I/R control was significantly higher than all other conditions (p=0.001 vs. all others, n=3). Of note, the 2.5-fold increase in superoxide production provoked by I/R was completely attenuated by netrin-1 perfusion. **C)** NADPH-driven NOX activity assessed using purified membrane faction of perfused hearts. Measurements shown are superoxide production under control (without addition of NADPH substrate, top), with NADPH (middle), and the difference between the two (bottom), which is an indication of NOX activity. Data indicate that under I/R, NOX activity was significantly increased compared to no I/R, which was completely attenuated by netrin-1 perfusion ($p<0.001$ vs. all others, n=4). **D)** Western blot showing the specificity of the NOX4 antibody used. NOX4 protein expression was increased in the plasmid overexpressed cells, while decreased in the NOX4 siRNA treated cells. **E)** Protein levels of NOX1, NOX2 and NOX4 in no I/R, I/R, and netrin-1 perfused I/R-injured hearts. NOX1 and NOX2 protein levels were unchanged. NOX4 was significantly and reproducibly upregulated in I/R-injured hearts, which was abolished by netrin-1 perfusion

(p<0.001 vs. all others, n=4). **F)** NOX4 activity assessed using the NOX activity assay with fulvene-5, a specific NOX4 inhibitor. Data show that NOX4 activity was significantly increased by I/R, and reduces to baseline with netrin-1 perfusion (p<0.05, n=3) **G)** Mitochondrial swelling assay from I/R-injured hearts with or without netrin-1 perfusion. Mitochondrial swelling was measured as an assessment of mitochondria integrity. Summarized data show that during the monitoring time of 20 min, calcium induced swelling of mitochondria was markedly increased in I/R-injured heart. Perfusion with netrin-1 attenuated this response to baseline (p<=0.001 vs. all others, n=4). **H)** Respiratory control ratio, a measure of mitochondrial function, was measured as the ratio of state III (ADP stimulated) verses state IV (oligomycin inhibited) oxygen consumption rate (n=4, p<0.05). **I)** H_2O_2 as measured with Amplex red (n=4 each, p<0.01 vs all) shows I/R significantly increasing H_2O_2 levels compared with controls, while netrin-1 treatment abolishes this response. **J)** TTC from I/R-injured hearts after being perfused with netrin-1 or co-perfused with the cGMP inhibitor Rp-8-Br-PET-cGMP (n=4). The results show that inhibition of cGMP completely eliminated netrin-1's cardioprotective effect. **K)** Detection of S-nitrosylated proteins from I/R-injured hearts, with or without netrin-1 perfusion. The results show that there were no significant changes in S-nitrosylation of proteins with I/R or perfusion of netrin-1.

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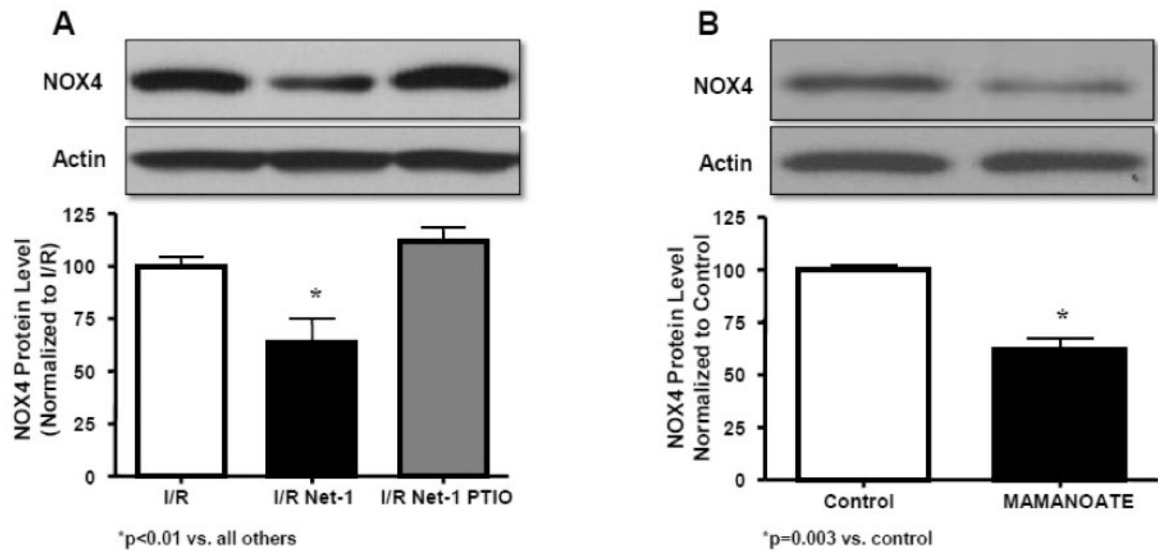

Figure 2. Nitric oxide (NO) mediates netrin-1 downregulation of NOX4 protein abundance

A) NOX4 protein level in I/R injured hearts that were subjected to netrin-1 with or without NO scavenger PTIO. The reduction in NOX4 protein level in netrin-1 treated hearts was abolished by co-treatment with PTIO ($p<0.01$ vs. all others, n=3) . **B)** NOX4 protein level in freshly isolated adult cardiomyocytes was significantly downregulated by the NO donor MAMANOATE (1 mmol/L) ($p=0.003$ vs. control, n=3) .

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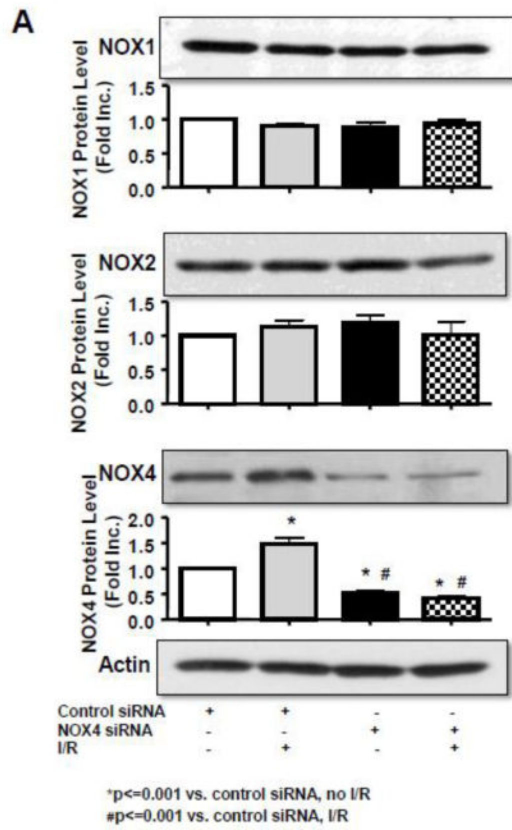

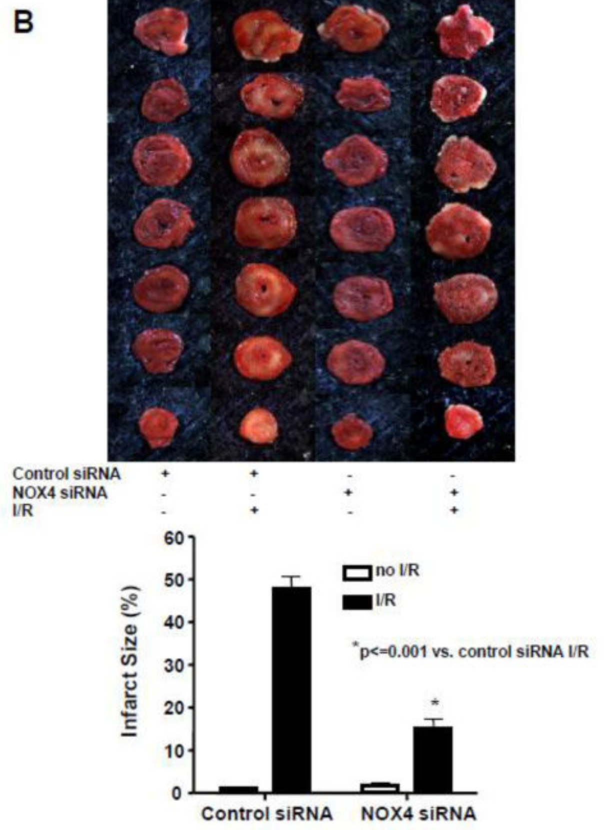

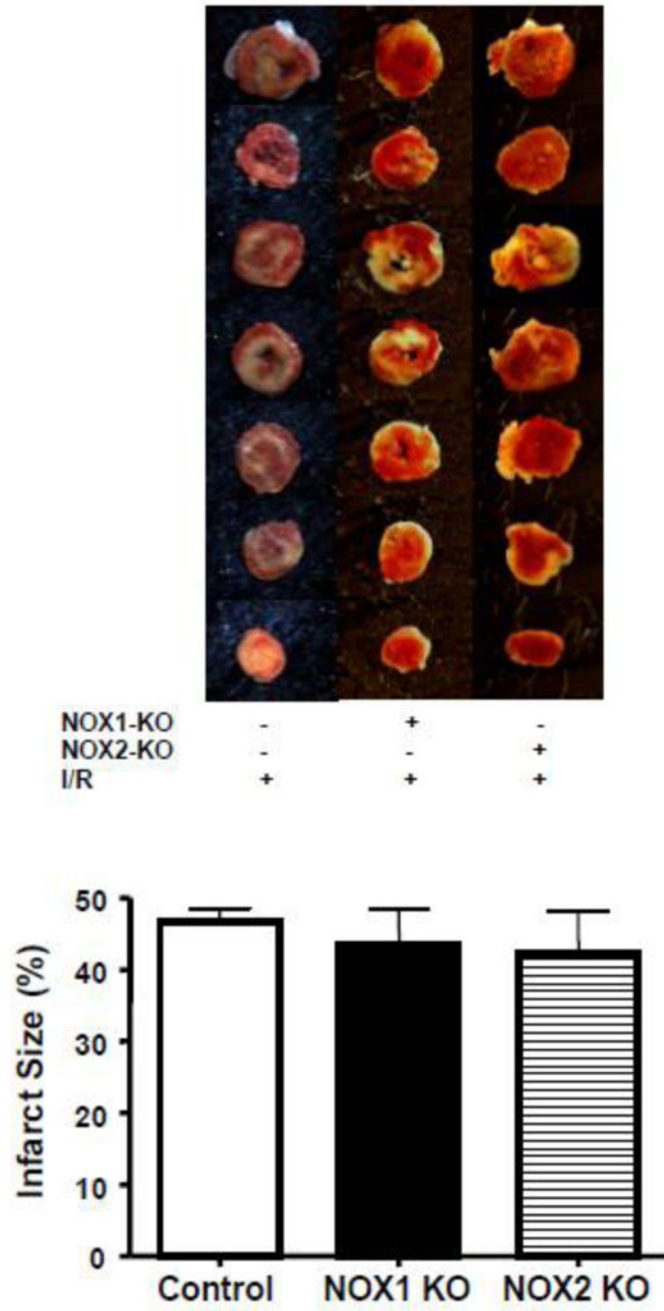

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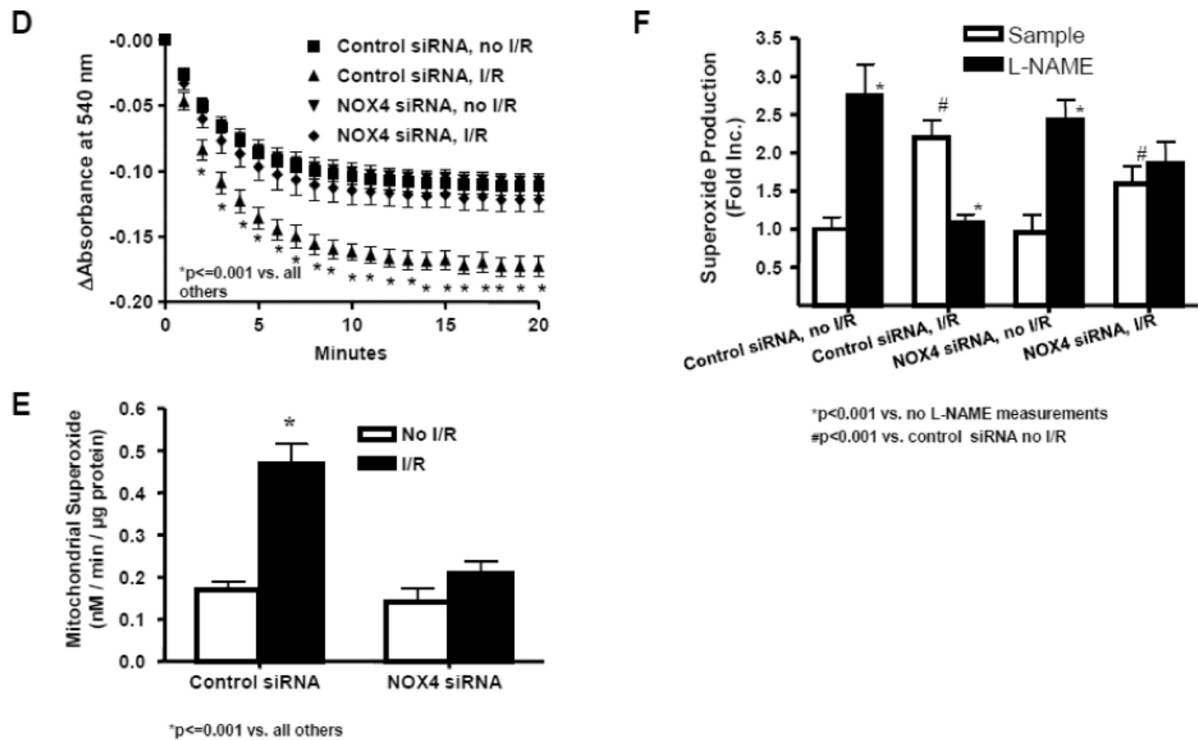

Figure 3. Role of attenuated NOX4 in cardioprotection: Protection of NOS and mitochondrial function

A) Protein levels of NOX1 (top), NOX2 (middle), and NOX4 (bottom) in I/R injured hearts isolated from mice transfected with NOX4 siRNA. The summarized results demonstrate the efficacy of NOX4 siRNA in reducing NOX4 protein levels while leaving the other isoforms intact (*p<=0.001 vs. control siRNA no I/R; #p<=0.001 vs. control siRNA I/R, n=3). **B)** Infarct size in I/R-injured hearts isolated from mice transfected with NOX4 siRNA. Representative and summarized TTC data indicate decreased infarct size in response to I/R in NOX4 siRNA treated group (p<0.001 vs. control siRNA I/R, n=5). **C)** Infarct size in I/R-injured hearts isolated from NOX1 and NOX2 knockout mice compared with controls. Representative and summarized TTC data show that NOX1 and NOX2 knockout had no significant effect on infarct size (n=5 each). **D)** Mitochondrial swelling in I/R-injured hearts isolated from mice transfected with NOX4 siRNA. Mitochondrial swelling was reduced in I/R-injured hearts treated with NOX4 siRNA (p<=0.001 vs. all others, n=5). **E)** Mitochondrial superoxide production in I/R-injured hearts isolated from mice transfected with NOX4 siRNA. Results show that mitochondria isolated from control siRNA treated hearts had significantly increased superoxide production under I/R, which was abolished in NOX4 siRNA treated mice (*p<=0.001 vs. all others). **F)** NOS uncoupling activity in I/R-injured hearts isolated from mice transfected with NOX4 siRNA. In control siRNA treated hearts, I/R induced a significant uncoupling of NOS as reflected by an increase in L-NAME-inhibitable superoxide production. This response was however completely prevented by NOX4 siRNA treatment (*p<0.001 vs. no L-NAME measurements, #p<0.001 vs. control siRNA no I/R control, n=3-4).

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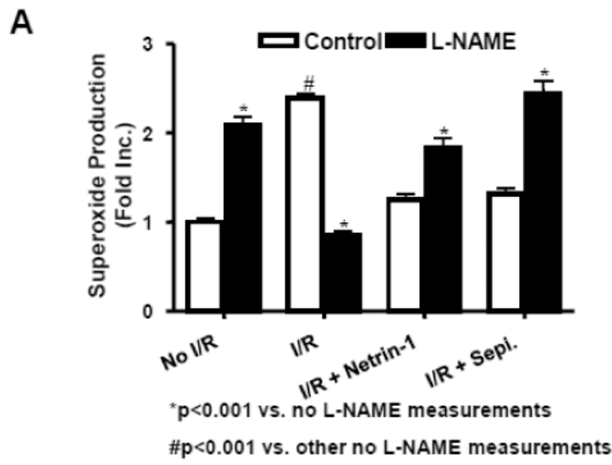

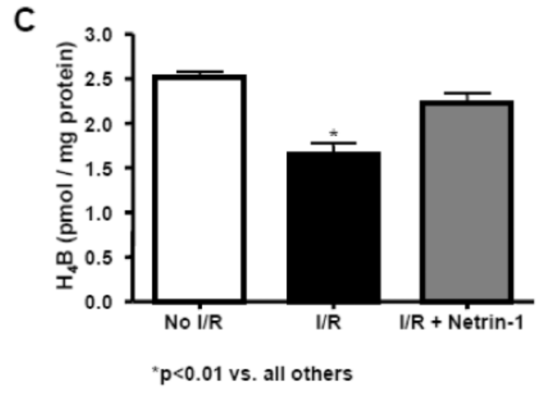

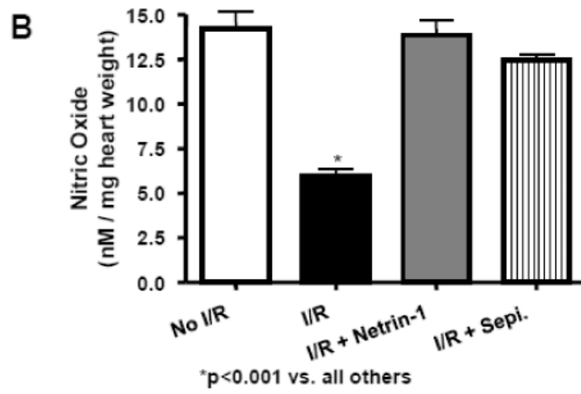

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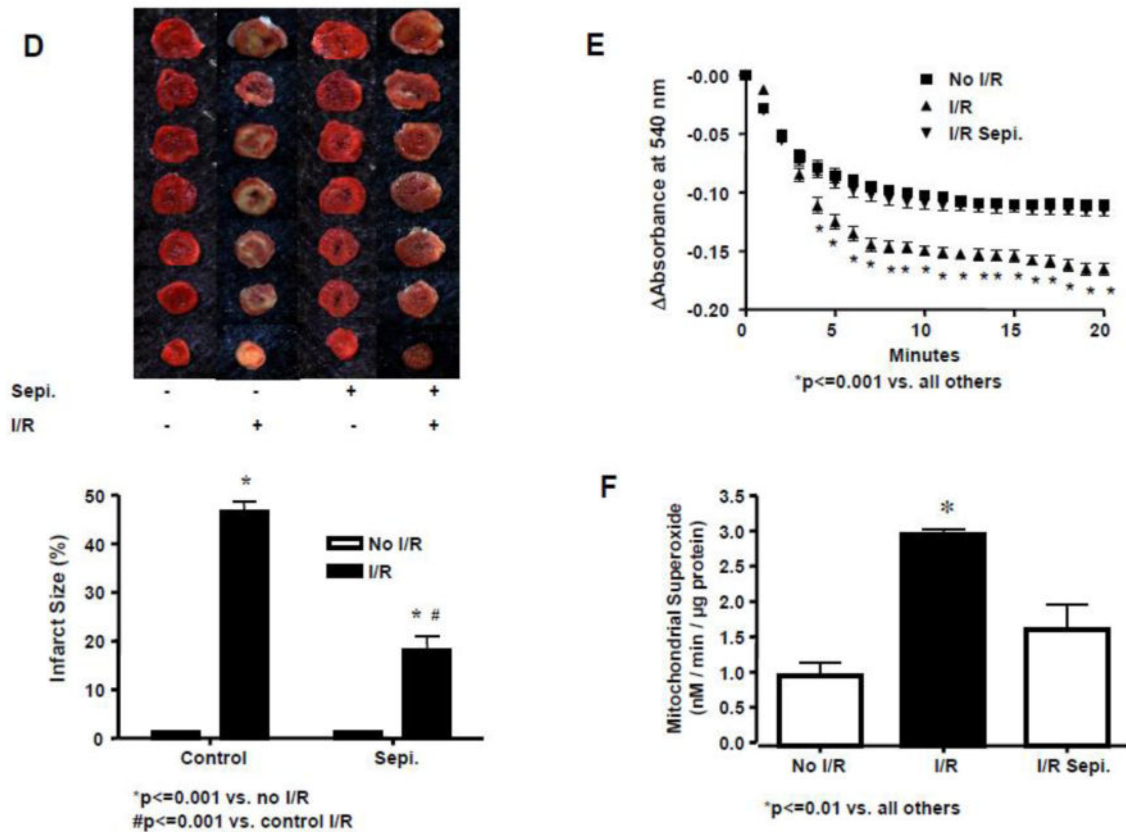

Figure 4. Sepiapterin recoupling of NOS reduces infarct size and improves mitochondrial function

A) NOS uncoupling activity in I/R-injured hearts with or without netrin-1 or sepiapterin (sepi.) perfusion. Superoxide production was measured by ESR, with and without addition of L-NAME to assess the (un)coupling state of NOS. Data indicate that increased NOS uncoupling activity provoked by I/R was attenuated by either netrin-1 or sepiapterin perfusion (p<0.001 vs. no L-NAME measurements, #p<0.001 vs. no I/R control, I/R netrin-1, and I/R sepiapterin, n=4). **B)** NO levels measured from controls, I/R-injured hearts with or without netrin-1 or sepi perfusion. Data indicate that NO was reduced with I/R, but restored back to control levels with either netrin-1 or sepi. perfusion (n=4 each, p<0.001). **C)** H_4B levels from I/R-injured hearts, with or without netrin-1 perfusion, as measured by HPLC. Data indicate reduced tissue levels of H_4B with I/R, which was significantly improved by netrin-1 perfusion. **D)** Infarct size in I/R-injured hearts with or without sepi. perfusion. As is obvious, sepi. perfusion substantially attenuated infarct size assessed by TTC staining (p<=0.001 vs. no I/R, #p<0.001 vs. control I/R). **E)** Mitochondrial swelling assay in I/R-injured hearts with or without sepi. perfusion. Sepi. significantly attenuated calcium induced mitochondrial swelling in I/R-injured hearts (p<=0.001 vs. all others, n=4). **F)** Mitochondrial superoxide production in I/R-injured hearts with or without sepi. perfusion. Mitochondrial superoxide production was measured directly from purified cardiac mitochondria using ESR. As is obvious, sepi. markedly abrogated I/R induced increase in mitochondrial superoxide production (p<=0.01 vs. all others, n=3).

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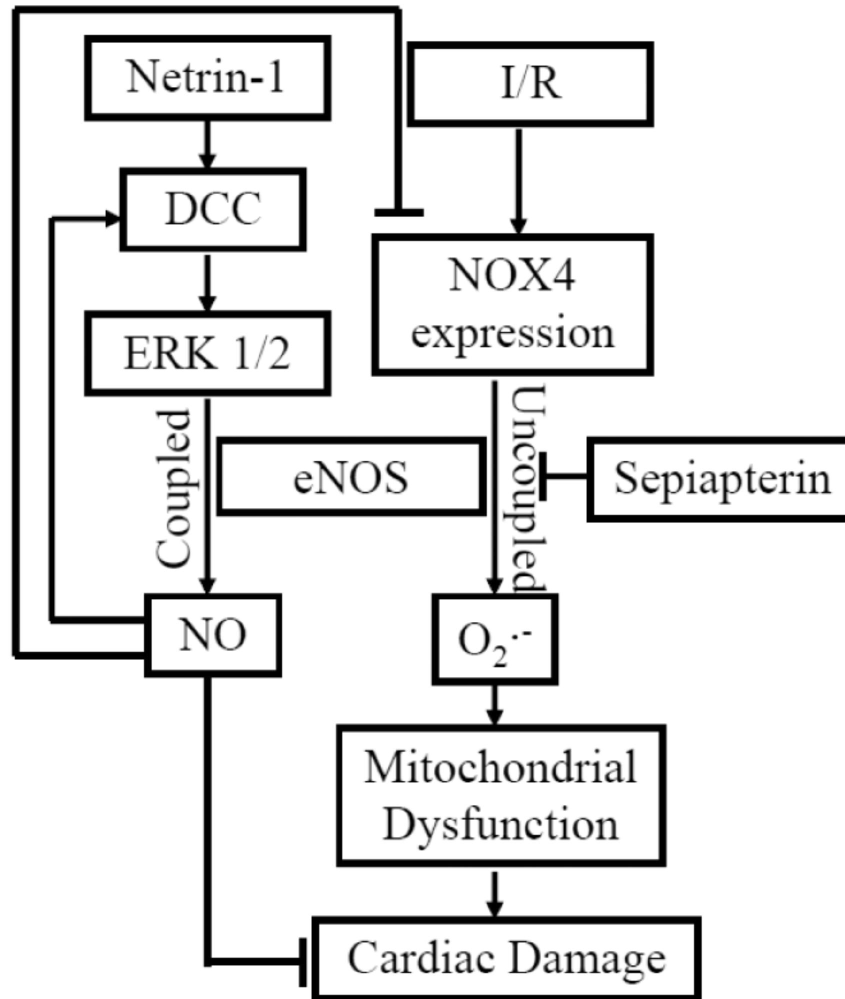

Figure 5. Molecular mechanisms underlying netrin-1 induced cardioprotection

Netrin-1 perfusion activates its transmembrane receptor DCC, leading to ERK-dependent activation of eNOS phosphorylation and NO production, which in turn exerts direct cardioprotective effects that are NO-mediated. On the other hand, netrin-1 derived NO attenuates I/R activation of NOX4, resulting in recoupled NOS, and preserved mitochondrial function. All together, these pathways maintain a positive feed-forward loop of sustained endogenous production of NO, which protects cardiomyocytes againist I/R injury to markedly reduce necrosis and apoptosis.