Netrin-1 improves post-injury cardiac function in vivo via DCC/NO-dependent preservation of mitochondrial integrity, while attenuating autophagy

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A B S T R A C T

Reperfusion injury of the heart is a severe complication of angioplasty treatment of acute myocardial ischemia, for which no therapeutics are currently available. The present study aimed to identify whether and how a novel protein, netrin-1, induces cardioprotection in vivo during ischemia/reperfusion (I/R) injury. Wild type (WT) C57BL6/J mice were subjected to a 30 min coronary occlusion followed by a 24 h reperfusion with vehicle (normal saline), netrin-1, UO126 (MEK1/2 inhibitor), PTIO (nitric oxide/NO scavenger), netrin-1/UO126 or netrin-1/PTIO intraventricularly. Some were injected of netrin-1 via tail vein. Netrin-1 at 5 μg/kg induced a substantial reduction in infarct size (19.7 ± 5.0% from 41.3 ± 1.8% in the controls), and markedly improved cardiac function as measured by ejection fraction and fractional shortening from echocardiography. Experiments with mice deficient in netrin-1 receptor DCC (deleted in colorectal cancer, DCC +/-), or reperfusion with netrin-1/UO126 or netrin-1/PTIO, attenuated the protective effects of netrin-1, implicating intermediate roles of DCC, ERK1/2 and NO. Netrin-1 induced phosphorylation of ERK1/2 and eNOS was abolished in DCC +/- mice. Electron spin resonance (ESR) determination of NO production from isolated left ventricles demonstrated that netrin-1 improves NO bioavailability, which was attenuated by UO126 or in DCC +/- mice, suggesting upstream roles of DCC and ERK1/2 in NO production. Netrin-1 further reduced mitochondrial swelling and mitochondrial superoxide production, which was absent when co-treated with PTIO or UO126, or in DCC +/- mice, indicating critical roles of DCC, ERK1/2 and NO in preserving mitochondrial integrity. In a permanent coronary ligation model of myocardial infarction (MI) to assess post-MI remodeling, netrin-1 abolished the marked increase in autophagy. In summary, our data demonstrate robust cardioprotective effect of netrin-1 in vivo, as shown by reduced infarct size and improved cardiac function. Mechanistically, this protection is mediated by netrin-1 receptor DCC, and NO dependent preservation of mitochondria. This work clearly establishes a therapeutic potential of netrin-1 for acute treatment of MI, perhaps also for chronic post-MI remodeling. This article is part of a Special Issue entitled: Autophagy and protein quality control in cardiometabolic diseases.

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1. Introduction

Acute myocardial infarction (MI) is one of the leading causes of morbidity and mortality in the adult population that strikes 7.6 million American adults, or about one person every 44 seconds in the United States [1]. The mainstay of current therapy for acute MI is the restoration of blood flow (reperfusion) to the affected area through angioplasty, sometimes in conjunction with thrombolytic therapy to remove/prevent thrombi formation. However, the reperfusion of ischemic tissue can itself lead to severe myocardial damage [2]. Intensive research efforts have been focused on the amelioration of various pathophysiological components of ischemia/reperfusion (I/R) injury to limit the extent of cell death [3]. However, data accumulated in the past 20 years have shown that there is no clinically effective therapy for the prevention of I/R injury. Currently, while there are some potential agents under trials for this condition, such as cyclosporine A, there are no clinically approved treatments for the prevention of cardiac I/R injury.

Netrin-1 has been originally identified as a midline-derived chemoattractant that guides axons to the midline during development [4-6]. Its functions are mainly mediated by the receptors deleted in colorectal cancer (DCC) and the uncoordinated-5 (UNC5) families [7-10].
More recently, netrin-1 has been shown to mediate angiogenesis [11–13]. In our laboratory, we have shown that netrin-1 can induce angiogenesis through an increase in endothelial nitric oxide (NO) synthase (eNOS) activation and NO production [13]. Further, we have shown that netrin-1 induces cardioprotection against I/R via a DCC/ERK1/2/eNOS/NO/DCC feedback pathway, using an ex-vivo model of Langendorff perfused heart [14].

In the present study we aimed to examine whether and how netrin-1, potentially via production of NO, is cardioprotective in vivo. We examined whether netrin-1 improves cardiac function by measuring ejection fraction and fraction shortening using echocardiography at day 1 and day 3 after reperfusion, using animals treated with netrin-1 both intraventricularly and intravenously via the tail vein. Treatment with netrin-1 dose-dependently reduced infarct size and improved cardiac function measured by echocardiography. Experiments using pharmacological inhibitors (UO126 as ERK1/2 inhibitor, PTIO as NO scavenger), animals deficient in netrin-1 receptor DCC, and experiments measuring p-eNOS, p-ERK1/2, NADPH oxidase isoform 4 (NOX4) expression and activity, levels of NO and superoxide by electron spin resonance (ESR), as well as mitochondrial damage via mitochondrial swelling, showed that mechanistically netrin-1’s cardioprotective effect is mediated by DCC and NO dependent suppression of NOX4-derived oxidative stress, as well as protection of mitochondria. Using an additional permanent myocardial infarction (MI) model we have also observed robust effects of netrin-1 in attenuating autophagy. This work clearly shows the efficacy of netrin-1 as a potent cardioprotective agent against I/R damage in vivo, hence the potential as a novel therapeutic for acute treatment of MI, or perhaps also for chronic post-MI remodeling.

2. Materials and methods

2.1. Materials

Purified recombinant mouse netrin-1 (R&D Systems, Minneapolis, MN, USA) was administered at doses ranging from 1 to 50 μg/kg, dissolved in normal saline, via injection into the LV lumen at the onset of reperfusion. Some mice were subjected to tail vein injection with netrin-1 (5 μg/kg) also immediately at the onset of reperfusion. UO126, a MEK1/2 inhibitor (200 μg/kg, dissolved in DMSO < 0.01%), and PTIO (1 mg/kg, dissolved in normal saline), a specific NO chelator, were purchased from Sigma-Aldrich in highest purity (St. Louis, MO, USA), and administered via injection into the LV lumen at the onset of reperfusion.

Polyclonal antibodies specific for phosphorylated and total forms of ERK1/2 and eNOS were obtained from Cell Signaling Technology (CST, Danvers, MA, USA). NOX4 antibody was purchased from Abcam (Abcam, Cambridge, MA, USA). Anti-actin antibody, and other chemicals in highest purity were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA).

2.2. Animals

Male C57BL6/J mice (6–8 weeks old) were obtained from the Jackson Laboratories (Bar Harbor, ME). The DCC +/- breeding colony was kindly provided by Dr. Marc Tessier-Lavigne from The Rockefeller University. Mice were housed under pathogen-free conditions. The use of animals and experimental procedures were approved by the Institutional Animal Care and Usage Committee at the University of California Los Angeles (UCLA).

2.3. In vivo murine model of myocardial ischemia–reperfusion injury

Briefly, mice were pre-medicated with heparin (1000 IU/kg, i.p.) and anesthetized 5 min later with sodium pentobarbital (60 mg/kg, i.p.). An additional dose of pentobarbital (50 μL; 20 mg/kg, i.p.) was given as needed to maintain anesthesia. After an adequate depth of anesthesia was attained the mouse is fixed in a supine position with tape. Mice were then orally intubated and ventilated mechanically with a Harvard Apparatus Rodent Ventilator (model 845). A mix of oxygen and carbon dioxide (95:5%) was supplied, and body temperature was monitored using a rectal probe thermometer and controlled with a heating pad. Left thoracotomy was performed in order to reveal the left coronary artery (LCA). Myocardial ischemia was achieved by tying a 7-0 Prolene thread around the LCA, which was then subsequently confirmed by the occurrence of regional cyanosis. The LCA was completely occluded for 30 min, and reperfusion was initiated by removal of the 7-0 suture. Reperfusion was confirmed by visualization of a hyperemic response. The chest wound was then reapproximated, and mice were extubated and allowed to recover with supplemental oxygen until mobile. All mice received buprenorphine (0.1 mg/kg) subcutaneously to minimize pain. The I/R protocol and the experimental procedures involving treatments with netrin-1 and pharmacological inhibitors are summarized in Suppl. Fig. 1.

2.4. Infarct size analysis

After 24 h reperfusion, mice were given heparin (1000 IU/kg, i.p.), after which they were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). The coronary artery was re-occluded at the original site of occlusion, while a solution of Evans blue dye (5% solution) was injected through the apex. The auricle and the right ventricle were removed and the left ventricle (LV) was excised into 6 slices using a mouse heart matrix (Harvard Apparatus, Holliston, MA, USA). Before 2,3,5-triphenyltetrazolium chloride (TTC) incubation, each slice is photographed in order to determine the area at risk (AAR) identified by the absence of Evans blue staining. The infarct area (IA) was identified by incubating each slice with TTC (1% solution, 5 min at 37 °C). The AAR identified by the absence of Evans blue staining was expressed as a percentage of the left ventricle weight and the IA was expressed as a percentage of the AAR. Each transverse slice was fixed in 10% neutral buffered formaldehyde and 1 h later, weighed and photographed. The different areas (AAR, IA, LV circumference, LV cavity circumference) were measured by computerized videoplanimetry (NIH ImageJ-1.37), and from these measurements infarct size was calculated as a percentage of the AAR.

2.5. Echocardiography

Cardiac morphology and function were assessed using anesthetized (0.6–0.8% isoflurane in 95% oxygen, heart rate: 420–450 beats/min) mice by transthoracic echocardiography (Veo2100 echocardiograph with MS-400 probe, VisualSonics, Toronto, Ontario, Canada). Two-dimensional images and M-mode tracing were recorded from the parasternal short axis view at the midpapillary level to determine the left ventricular internal diastole diameter (LVDD) and left ventricular internal systolic diameter (LVDS). Fractional shortening and ejection fraction were calculated directly from the short axis view of heart contraction.

2.6. Western blot analysis

At the end of the 10 min or 30 min of reperfusion, the heart was removed and the LV was immediately frozen in liquid nitrogen. Subsequently, the tissue was powdered and homogenized in lysis buffer (Tris 50 mmol/L, EDTA 0.1 mmol/L, EGTA 0.1 mmol/L, protease inhibitor cocktail, phosphatase inhibitors cocktail 2 and 3, pH 7.4). The homogenates were vortexed and centrifuged at 17,600 g for 30 min at 4 °C. The supernatants were used for Western blot analysis. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard (Bio-Rad protein assay kit, Bio-Rad, Hercules, CA, USA).
2.7. Electron spin resonance detection of nitric oxide radical

Bioavailable nitric oxide radical (NO) from LV tissues was detected using electron spin resonance (ESR) as described [13–16]. In brief, the LV heart homogenates were incubated with equal volume of freshly prepared NO-specific spin trap Fe$^{57}$T (DETC)$_2$ colloid (0.5 mMm/L) for 60 min, in the presence of calcium ionophore A23187 (10 μM/L). Gently collected homogenates suspensions were snap-frozen in liquid nitrogen and loaded into a finger Dewar for analysis with an eScan electron spin resonance (ESR) spectrophotometer (Bruker) at 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.

2.8. Electron spin resonance measurement of mitochondrial superoxide production

For isolation of heart mitochondria, fresh left ventricular tissues were placed in buffer containing 220 mM/L mannitol, 70 mM/L sucrose, 10 mM/L HEPES, 1 mM/L EGTA (Sigma–Aldrich, St. Louis, MO, USA), pH 7.4 at 4 °C. The tissues were minced with scissors and homogenized on ice using a Teflon Potter homogenizer and were centrifuged at 1000 g for 10 min. Supernatants were centrifuged at 10,000 g for 10 min. The final pellets were resuspended in homogenization buffer including 0.01 mM/L EGTA. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard (Bio–Rad protein assay kit, Bio–Rad, Hercules, CA, USA). The specific superoxide spin trap methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH, 500 μM/L, Alexis) solution was prepared freshly in nitrogen gas bubbled Krebs/HEPES buffer containing diethyldithiocarbamic acid (DETC, 5 μM/L Sigma) and deferoxamine (25 μM/L Sigma). Three microliters of the isolated mitochondria was then mixed with spin trap solution and loaded into glass capillary (total volume of 100 μL, Fisher Scientific) for analysis of superoxide signal (CM$^+$ formed after trapping O$_2^{-}$) using eScan ESR spectrometer (Bruker) at 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. The final superoxide value was normalized by the amount of mitochondrial protein loaded.

2.9. Mitochondrial swelling assay

The activation of the mitochondrial permeability transition pore was determined by Ca$^{2+}$-induced swelling of isolated cardiac mitochondria. Opening of the pore causes mitochondrial swelling, which was measured spectrophotometrically as a decrease in absorbance at 540 nm. Isolated cardiac mitochondria (30 μg) were incubated for 1 min in the swelling buffer, which contained 250 mM/L sucrose, 10 mM/L Tris·HCl (pH 7.4) and energized with 2 mM/L of succinate, to a final protein concentration of 0.3 mg/L. Pore opening was induced by 250 μM/L of CaCl$_2$ and the decrease in absorbance was measured at 540 nm every minute for 20 min using a 96-well plate.

2.10. NOX4 activity assay

NOX4 activity was determined using isolated membrane fraction. For the isolation of the membrane fraction, hearts were isolated from animals after 10 min of reperfusion, then homogenized on ice in a glass homogenizer in lysis buffer as described above. The homogenate was serially centrifuged at 1200 g for 5 min, 22,000 g for 20 min, and 13,700 g for 90 min at 4 °C. The final pellet was resuspended in lysis buffer (100 μL per heart). Superoxide production was measured as described above, with 10 μg protein loaded as measured using the Bradford method. Kinetic NADPH-driven (100 μM/L final concentration) NOX4 activity was measured in the presence or absence of Fulvene-5, a specific NOX4 inhibitor (kindly provided by Dr. Jack L. Arbiser from Emory University) [17].

2.11. Animal model of MI and assessment of autophagy

Male C57BL/6 mice (8–10 weeks old) were used to induce myocardial infarction (MI) by permanent left anterior descending (LAD) coronary artery ligation. Two days before the surgery, the mice were infused with netrin-1 (15 ng/kg per day) or vehicle using subcutaneously implanted osmotic pumps (14 days, Diret Corp). Then the mice were anesthetized with pentobarbital (30 mg/kg, i.p.) and fixed in the supine position with positive pressure respiration. The left thorax was opened and the LAD coronary artery was located and ligated with a 8–0 silk suture 2–3 mm from origin. The ligation was deemed successful when the anterior wall of the LV turned pale. After the ligation, the chest was closed in layers, and the mice were removed from the ventilator when awake. Sham-operated animals were subjected to similar surgery, except that no ligature was placed.

Two weeks later, isolated hearts were subjected to protein isolation and western blotting analysis as described earlier, using specific antibodies to LC3 (1:3000, Sigma) and β-actin. LC3 expression levels were analyzed using ImageJ program and normalized by β-actin.

2.12. Statistical analysis

All data are presented as mean ± SEM. Comparisons between two groups were made using student’s t-test, while multiple comparisons with more than two groups were made using ANOVA followed by Newman–Keuls’s post-hoc test. Statistical significance was defined as p < 0.05.

3. Results

3.1. Netrin-1 attenuates I/R induced myocardial infarction in vivo

To examine whether netrin-1 administration is effective in inducing cardioprotection against I/R damage in vivo, wild type C57BL/6j mice were subjected to a 30 min ischemia by coronary occlusion, followed by a 24 h reperfusion. Netrin-1 or vehicle (normal saline) was injected into the LV lumen at the onset of reperfusion at doses ranging from 1 to 50 μg/kg. Mice receiving vehicle injection displayed a 41.3 ± 1.8% infarct size per area at risk (Inf/AAR), and a 17.1 ± 1.5% infarct size per LV size (Inf/LV). Treatment with netrin-1 at 5 μg/kg and 10 μg/kg significantly decreased infarct size (19.7 ± 5.0% and 29.9 ± 1.5% respectively for Inf/AAR; and 8.1 ± 2.0% and 11.9 ± 0.8% respectively for Inf/LV, Fig. 1). At the high dose of 50 μg/kg, the cardioprotective effect of netrin-1 disappeared (47.0 ± 1.0% for Inf/AAR, and 20.1 ± 0.6% for Inf/LV). The percent AAR per LV (AAR/LV) was similar among all study groups, indicating similar severities of myocardial ischemia.
3.2. Netrin-1 improves cardiac function after ischemia–reperfusion in vivo

To further validate netrin-1’s cardioprotective properties in vivo, we measured LV dilatation (LVID:d and LVID:s) and cardiac function via echocardiography on animals that underwent I/R injury. The results, shown in Fig. 2A and C, illustrated that both fractional shortening and ejection fraction were significantly decreased in vehicle treated hearts at both 1 and 3 days after I/R. Treatment with netrin-1 through the LV resulted in significantly increased ejection fraction and fractional shortening, suggesting an improvement in cardiac function. Fig. 2A and B indicated that LV structures were significantly improved in netrin-1 treated hearts at both 1 and 3 days after I/R. These data show that netrin-1 can functionally rescue I/R injured heart in vivo.

Additionally, separate experiments were performed where the treatment of vehicle or netrin-1 was injected through the tail vein. The results, shown in Fig. 2D and F, indicate a similar trend in the recovery of cardiac function as compared to LV injected hearts. Likewise, LV structures were also improved as compared to LV injected hearts. This shows that netrin-1 is effectively protective against I/R injury when given through a more easily accessible route.

Fig. 1. Netrin-1 attenuates I/R induced myocardial infarct in vivo. Myocardial infarction was induced by a 30 min left coronary artery (LCA) ligation followed by a 24 h reperfusion in wild type C57BL6 mice in vivo. The mice were treated at the onset of reperfusion with either netrin-1 (1–50 μg/kg) or vehicle (normal saline). Evans blue was used to visualize the non-ischemic area. A) Representative TTC-stained heart slices from I/R (vehicle) group, and I/R+netrin-1 (1–50 μg/kg) groups. The white area indicates infarct zone, while the blue area indicates non-infarcted area. The red and white areas represent area at risk. B) Infarct size analyzed by: percentage of area at risk divided by left ventricular (AAR/LV), infarct size divided by area at risk (Inf/AAR), and infarct size divided by left ventricular (Inf/LV). The results are presented as mean ± SEM. The number of animal per group was seven. ***p < 0.001 vs. control I/R (vehicle).

Fig. 2. Intraventricular and intravenous delivery of netrin-1 improves cardiac function after I/R injury in vivo. Echocardiography was performed on wild type C57BL6/J mice reperfused for 24 h or 72 h after a 30 min left coronary artery (LCA) ligation. Left ventricular internal diastole diameter (LVID:D), left ventricular internal systolic diameter (LVID:S), ejection fraction and fractional shortening were measured. A) Representative echocardiography data from mice with netrin-1 (5 μg/kg) injected through the LV at the onset of reperfusion. B) Grouped data for left ventricular internal dimension (LVID) during systole (p < 0.05, **p < 0.01 vs. I/R(vehicle)-1 day, ***p < 0.001 vs. I/R(vehicle)-3 days) and diastole (p < 0.05 vs. I/R(vehicle)-1, **p < 0.05 vs. I/R(vehicle)-3 days) for mice with netrin-1 injected through LV. ***p < 0.001 vs. I/R(vehicle)-1 day, **p < 0.01 vs. I/R(vehicle)-3 days, n = 3–7/group. D) Representative echocardiography data from mice with netrin-1 (5 μg/kg) injected through tail vein. E) Grouped LVID during systole (**p < 0.01 vs. I/R(vehicle)-1 day, ***p < 0.001 vs. I/R(vehicle)-3 days) and diastole (p < 0.05, **p < 0.01 vs. I/R(vehicle)-1 day, ***p < 0.001 vs. I/R(vehicle)-3 days) from mice with netrin-1 (5 μg/kg) injected through tail vein, n = 3–9/group. F) Grouped ejection fraction (p < 0.05, **p < 0.01 vs. I/R(vehicle)-1 day, ***p < 0.001 vs. I/R(vehicle)-3 days) and fractional shortening (p < 0.05, ***p < 0.001 vs. I/R(vehicle)-1 day, **p < 0.01 vs. I/R(vehicle)-3 days) from mice with netrin-1 (5 μg/kg) injected through tail vein, n = 3–9/group. Data are presented as mean ± SEM.
A

Wild type with I/R 1 day

Sham
Saline, injected through LV
Netrin-1, injected through LV

Wild type with I/R 3 day

Saline, injected through LV
Netrin-1, injected through LV

B

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*p<0.05 vs. I/R (vehicle)-1, # p<0.05 vs. I/R (vehicle)-3 day
N=3-7/group.

C

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*** p<0.001 vs. I/R (vehicle)-1 day
n=3-7/group
D

Wild type with I/R 1 day

Sham
Saline, injected through tail vein
Netrin-1, injected through tail vein

Wild type with I/R 3 day

Saline, injected through tail vein
Netrin-1, injected through tail vein

E

1 day 3 day 1 day 3 day

* p<0.05, ** p<0.01 vs. I/R (vehicle) -1 day
# p<0.05, ## p<0.01 vs. I/R (vehicle) -3 day
N=3-9/group.

0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0

LVId:d (mm)

F

1 day 3 day 1 day 3 day

* p<0.05 vs. I/R (vehicle) -1 day
# p<0.05, ### p<0.001 vs. I/R (vehicle) -3 day
n=3-9/group.

0 10 20 30 40 50

Fractional Shortening (%)

0 10 20 30 40 50 60 70 80 90

Ejection Fraction (%)

Fig. 2 (continued).
followed by a 24 h of reperfusion. Mice received either 5 μg/kg netrin-1 at the onset of reperfusion. Netrin-1 yielded significant protection in DCC+/+ and not in DCC+/− animals. A) Representative TTC-stained heart slices from DCC+/+, I/R + netrin-1 group, and DCC+/−, I/R + netrin-1 group. B) Infarct size analyzed by percentage of area at risk divided by left ventricle (AAR/LV), infarct size divided by area at risk (Inf/AAR), and infarct size divided by left ventricle (Inf/LV). The results are represented as mean ± SEM. ***p < 0.001 vs. DCC+/+, I/R + netrin-1, n = 7/group.

3.3. The cardioprotective effect of netrin-1 in vivo is dependent on DCC

To examine whether netrin-1’s cardioprotective effect is dependent upon its receptor deleted in colorectal cancer (DCC), DCC+/+ and DCC+/−/ mice were subjected to a 30 min of myocardial ischemia followed by a 24 h of reperfusion. Mice received either 5 μg/kg netrin-1 or vehicle at the onset of reperfusion. In DCC+/−/ mice, netrin-1 failed to reduce infarct size (44.4 ± 1.4% vs. 24.7 ± 3.2% in DCC+/+ mice for Inf/AAR, and 18.3 ± 0.6% vs. 11.0 ± 1.2% in DCC+/+ mice for Inf/LV) (Fig. 3). AAR/LV was similar for both groups. These data clearly implicate an intermediate role of DCC in mediating netrin-1 induced cardioprotection in vivo.

3.4. Role of NO and ERK1/2 in netrin-1 induced cardioprotection in vivo

We have previously identified a DCC-ERK1/2/eNOS-NO pathway in mediating netrin-1’s potent cardioprotective effect against I/R injury ex-vivo[14]. Here, we examined whether similar mechanisms underlie netrin-1 induced cardioprotection in vivo. Mice were subjected to a 30 min myocardial ischemia, followed by a 24 h reperfusion, and injected with vehicle (normal saline); netrin-1; U0126 (MEK1/2 inhibitor); PTIO (NO scavenger); netrin-1/U0126, or netrin-1/PTIO (at same doses described earlier) at the onset of reperfusion. As shown in Fig. 4, U0126 or PTIO alone had no significant effect on infarct size. Co-treatment of U0126 or PTIO with netrin-1 completely prevented netrin-1’s cardioprotective effect, as shown by the infarct size measurements (Fig. 4). These data indicate that ERK1/2 and NO are required for netrin-1 induced cardioprotection in vivo. AAR/LV was similar for both groups (Fig. 4), showing that I/R injury was comparable.

3.5. Netrin-1 activation of ERK1/2 and NO in vivo is DCC dependent

As shown above, ERK1/2 and NO are involved in netrin-1 induced cardioprotection against I/R damage in vivo. Here, we examined whether mechanistically they lie downstream of DCC. Western blotting of p-ERK1/2 and p-eNOS was performed from DCC+/+ and DCC+/−/ mice subjected to a 30 min ischemia followed by a 24 h reperfusion, and treated at the onset of reperfusion with vehicle (normal saline) or netrin-1. In DCC+/+ mice, treatment with netrin-1 significantly activated ERK and eNOS in cardiac tissue under I/R. This activation was abolished in DCC−/−/ mice, where netrin-1 resulted in no significant change in ERK and eNOS activation compared with untreated controls (Fig. 5A, B). These results demonstrate that netrin-1 activation of ERK1/2 and eNOS activation in vivo are dependent on DCC. We also examined iNOS expression after reperfusion of netrin-1 (Fig. 5C), which showed no significant changes between I/R or I/R/netrin-1 group with sham condition. This further support the observation that eNOS is the major NOS isoform that is responsible for cardioprotective effect of netrin-1.

3.6. Netrin-1 induces NO production in vivo in a DCC and ERK1/2 dependent manner

Data described above suggest that both ERK1/2 and eNOS activation lie downstream of DCC. We next measured NO directly using ESR to elucidate the exact relationship between the three components. Wild type C57BL6 mice subjected to I/R injury was treated with vehicle, netrin-1, or netrin-1/U0126 at the onset of reperfusion. The heart was harvested for NO measurements from the LV. The data, shown on Fig. 6A, demonstrate that vehicle treated hearts had significantly reduced NO bioavailability, while treatment with netrin-1 markedly corrected this deficiency. Co-treatment of netrin-1 and U0126 abolished the beneficial effects of netrin-1 on NO production, suggesting that activation of ERK1/2 proceeds eNOS/NO activation.

Furthermore, we measured LV NO production using I/R injured DCC+/+ and DCC+/−/ mice. The results, shown on Fig. 6B, indicate that netrin-1 mediated NO increase shown in DCC+/+ group completely abolished in DCC+/−/ animals. Taken together, these data confirm that netrin-1 stimulates NO production in I/R injured hearts through a DCC/ERK1/2/eNOS pathway in vivo.

3.7. Netrin-1 attenuates mitochondrial superoxide production in vivo via DCC and ERK1/2

Previous studies have shown that the large burst of oxidants in the mitochondria observed during early reperfusion contributes strongly to I/R damage[18]. Mice were subjected to a 30 min myocardial ischemia, injected with vehicle (normal saline); netrin-1 or U0126 (at same doses described earlier) into the LV at the onset of reperfusion. The heart was then harvested for mitochondrial isolation and subsequent ESR detection of superoxide production from mitochondria. The results, shown in Fig. 7A, illustrate that mitochondria from vehicle treated hearts produced ~4 times more superoxide after I/R injury when compared to mitochondria isolated from sham surgery controls.
Treatment with netrin-1 significantly reduced this increase to ~2.5 times, while co-treatment of netrin-1 with UO126 abolished this reduction, suggesting the role of ERK1/2 in netrin-1 mediated reduction in I/R induced mitochondrial superoxide production.

To examine the role of the DCC receptor in this phenomenon, identical I/R protocol was performed using DCC+/+ and DCC+/- animals. The results, shown in Fig. 7B, illustrate that netrin-1's effect on mitochondrial superoxide production was completely abolished in DCC+/-, implicating a dependency on the DCC receptor.

3.8. Netrin-1 preservation of mitochondrial Integrity in vivo: dependence on DCC and ERK 1/2

One of the major events in I/R induced cardiac injury is mitochondrial damage and increased superoxide production from mitochondria. We further examined impact on mitochondrial integrity of netrin-1 by employing a calcium dependent swelling assay. As shown in Fig. 8A, netrin-1 perfusion resulted in a marked attenuation of the swelling activity, which was reversed by co-treatment with the ERK1/2 inhibitor UO126. Similar experiments were performed using DCC+/- and DCC+/-+ animals. The results, shown in Fig. 8B, indicate that while netrin-1 is protective of mitochondrial damage in DCC+/- animals, DCC+/- animals had a complete loss of the protection. Taken together, these data indicate that DCC and ERK1/2 are required for mitochondrial preservation induced by netrin-1 during I/R injury in vivo.

3.9. Netrin-1 down-regulates NOX4 expression and activity in a NO and ERK1/2 dependent manner

In a previous study using an ex vivo model of Langendorff perfused heart, we observed an increase in NOX4 expression and activity from I/R injured hearts, which was abolished by treatment with netrin-1 [19]. Here, we examined if this also holds true in the in vivo condition and the signaling pathway involved. Western blots for NOX4 and actin were performed on hearts treated with vehicle, netrin-1, netrin-1/UO126 and harvested 30 min after reperfusion. Fig. 9A and B showed that treatment with netrin-1 significantly reduced NOX4 expression in LV, which was reversed by co-treatment with PTIO and UO126. These results show that netrin-1 reduction of NOX4 is dependent on ERK1/2 and NO. Given that ERK1/2 activation occurs prior
to NO production in response to netrin-1, these data suggest a DCC: ERK1/2:eNOS:NO:NOX4 pathway. To further examine changes in NOX4 activity, we measured NOX4 specificity using ESR in the presence of Fulvene-5, an inhibitor for NOX4 [17]. The results, shown on Fig. 9C, indicate that I/R significantly increased NOX4 activity, while netrin-1 perfusion largely abrogated this effect. Further, treatment with PTIO, a specific NO scavenger, completely abolished netrin-1’s inhibitory effect on NOX4 activation, again implicating an upstream role of NO in silencing NOX4 pathway of oxidative activation.

3.10. Netrin-1 attenuates autophagy in post-MI remodeled heart

In additional experiments we performed permanent LAD ligation to induce myocardial infarction (MI). Chronically, autophagy was markedly increased in post-MI remodeled heart, which was completely attenuated by netrin-1 perfusion (Fig. 10). Microtubule-associated proteins 1A/1B light chain 3, LC3, a mammalian homolog of yeast Atg8, is a major mediator of autophagy. During autophagy, LC3-I, a cytosolic form of LC3, is converted into LC3-II which is a specific marker of autophagosomes, and the ratio of LC3-II/LC3-I is an established indicator of autophagy. In this study, we found that LC3-II was upregulated in the post-MI heart and the myocardial LC3-II/LC3-I ratio was significantly increased in MI animals. However, in mice infused with netrin-1 for 14 days using subcutaneously embedded osmotic minipumps, the myocardial LC3-II/LC3-I ratio decreased to the level similar to the sham group.

4. Discussion

The most significant findings of the present study are: 1) establishment of a potent cardioprotective effect of netrin-1 in vivo; 2) establishment that netrin-1 not only effectively reduces infarct size after I/R injury, but also markedly improves cardiac function; 3) establishment that netrin-1 exerts cardioprotection in vivo via preservation of mitochondrial integrity that is dependent on a DCC/ERK1/2/eNOS/NO pathway; 4) establishments that netrin-1 attenuates oxidative stress in I/R injured heart via ERK1/2/NO-mediated downregulation of NOX4 expression and activity; 5) establishment that in addition to its potent, acute cardioprotective effects during I/R injury, netrin-1 may also be
beneficial in treating post-MI remodeling of the heart by attenuating autophagy. These findings mechanistically highlight a therapeutic potential of netrin-1 in treating reperfusion dependent or independent myocardial infarction when delivered to animals in vivo.

Netrin-1 is cardioprotective in vivo when given at modest doses (Fig. 1). Further, this protective effect is accompanied by improvement in the physiological function of the heart as measured by ejection fraction and fractional shortening via echocardiography (Fig. 2). While netrin-1 has been shown by our laboratory in the past to confer cardioprotection in an ex-vivo model [14], the use of netrin-1 in an in vivo, physiological setting in the present study further confirms its potency and consistency in cardioprotection. The improvement in cardiac function after netrin-1 treatment again supports the use of netrin-1 in a physiological setting. Taken together, these data firmly establish netrin-1 as a possible therapeutic agent against I/R injury.

The molecular pathway through which netrin-1 exerts cardioprotection in vivo is thoroughly characterized in this study. The use of DCC+/- animals (Fig. 3) and inhibitors for ERK1/2 and NO (Fig. 4) all abolished the cardioprotective effect of netrin-1, which clearly shows involvement of these signaling mediators. To elucidate the precise pathway for this protection, we first examined phosphorylation of ERK1/2 and eNOS in DCC+/+ and DCC+/- mice (Fig. 5), which shows that the activation of both ERK1/2 and eNOS by netrin-1 in DCC+/+ animals is abolished in DCC+/- mice, suggesting that...
Fig. 8. Netrin-1 preservation of mitochondrial integrity during I/R in vivo is DCC and ERK 1/2 dependent. Calcium induced mitochondrial swelling was measured from freshly isolated mitochondria after I/R injury. A) Hearts were injected with vehicle, netrin-1 (5 μg/kg) or netrin-1+UO126 (200 μg/kg), at the onset of reperfusion. The data show that netrin-1 markedly reduced mitochondrial swelling, while co-treatment with UO126 abolished this protective effect. ***p < 0.001 vs. sham, ###p < 0.001 vs. I/R+netrin-1, n=5/group. B) Same experiments performed on DCC+/+ and DCC+/- animals. Data show that netrin-1's protective effect against mitochondrial swelling was absent in DCC+/- animals. ***p < 0.001 vs. DCC+/+, n=5/group.

Fig. 9. Netrin-1 downregulates NOX4 expression and activity in vivo during I/R. Shown are representative western blots and grouped densitometric data from mice subjected to sham surgery, permanent LAD ligation for myocardial infarction (MI), or MI surgery with netrin-1 perfusion in osmotic minipumps. A) Western blots showing NOX4 expression. B) Grouped densitometric data of NOX4 protein expression that is normalized by Actin. Results are mean ± S.E.M. #p < 0.05 vs. I/R+netrin-1, n=5/group. C) Grouped data shows that Fulvene-5 (a specific inhibitor of NOX4) sensitive NOX activity was increased with I/R, and netrin-1 perfusion eliminated this increase. Co-treatment with PTIO, a NO scavenger, abolished this effect of netrin-1. *p < 0.05 vs. sham, #p < 0.05 vs. I/R+netrin-1, n = 4/group.
results of our current study, where netrin-1, which was shown here to preserve mitochondrial integrity via NO dependent ATP generation, but also involved in cell death and loss of functions such as maintaining cardiomyocyte structure and function. However, cardiac autophagy is increased in response to oxidative stress, ATP depletion and mPTP opening [43, 44]. Of note, such metabolic changes are involved in both I/R and post-MI remodeling, and previous studies have shown that significantly increased cardiac autophagy is observed during I/R [43, 45], and post-MI remodeling [46, 47]. In this study, by employing a permanent LAD ligation model of MI, we consistently found that cardiac autophagy, assessed by the ratio of LC3-II to LC3-I, was dramatically upregulated in the post-MI remodeled heart, and this response was completely attenuated by netrin-1 infusion. These data indicate that netrin-1 might be highly protective chronically as well, via modulation of autophagy-dependent post-MI remodeling. Of note, the limitation of the study here is the employment of a permanent MI model rather than a more acute I/R model. However our hypothesis was that autophagy might be much more importantly modulated by netrin-1 during the chronic process to limit excessive remodeling of heart and enable better repair.

In summary, this study clearly illustrates that netrin-1 effectively protects the heart from I/R injury in vivo, when delivered intravenously or intravenously via tail vein. This is mediated by DCC dependent ERK1/2/eNOS activation and NO production, and subsequent preservation of mitochondrial integrity that is essential for the survival of cardiomyocytes and the improved cardiac function. These results here establish netrin-1 as a potent therapeutic agent against I/R injury and elucidate several novel mechanisms to this function. In addition to this therapeutic application to acute MI, netrin-1 may also be beneficial in treating post-MI remodeling of the heart chronically, together making netrin-1 a particularly valuable therapeutic option for MI management.

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Disclosures

The authors have no conflicts of interest to disclose.

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