



## Highlighted Article

# Netrin-1 prevents ischemia/reperfusion-induced myocardial infarction via a DCC/ERK1/2/eNOS<sub>S1177</sub>/NO/DCC feed-forward mechanism

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## ARTICLE INFO

## Article history:

Received 14 October 2009

Received in revised form 28 November 2009

Accepted 30 November 2009

Available online 12 January 2010

## Keywords:

Netrin-1

Nitric oxide

eNOS

DCC

Myocardial infarction

Ischemia reperfusion

Cardioprotection

ERK1/2

Electron spin resonance

## ABSTRACT

We have recently shown that a novel endothelial mitogen netrin-1 potently stimulates nitric oxide (NO<sup>•</sup>) production via a DCC-ERK1/2 dependent mechanism. In view of the well-established cardioprotective role of NO<sup>•</sup>, the present study investigated whether netrin-1 is cardioprotective via NO<sup>•</sup> signaling in the heart. Netrin-1 receptor DCC was abundantly expressed in the C57BL/6J mouse hearts. Perfusion of heart with netrin-1 (100 ng/mL) using a Langendorff system significantly increased NO<sup>•</sup> production. Under ischemia/reperfusion (I/R), netrin-1 induced a substantial reduction in infarct size (21.8 ± 4.9% from 42.5 ± 3.6% in the controls), which was accompanied by an augmented production of NO<sup>•</sup>. Pre-perfusion with DCC-antibody, U0126 (MEK1/2 inhibitor), L-NAME or PTIO (NO<sup>•</sup> scavenger) attenuated protective effects of netrin-1 on infarct size and NO<sup>•</sup> production, indicating upstream roles of DCC and ERK1/2 in NO<sup>•</sup> production, as well as an essential role of NO<sup>•</sup> in cardioprotection. Netrin-1 induced reduction in infarct size was significantly attenuated in DCC+/- mice, confirming an intermediate role of DCC. In additional experiments we found netrin-1 increased ERK1/2 and eNOS<sub>S1177</sub> phosphorylation, and DCC protein expression, which was diminished by I/R. Furthermore, netrin-1-induced DCC upregulation was NO<sup>•</sup> and ERK1/2-dependent, implicating a feed-forward mechanism. DAF-AM staining revealed enhanced NO<sup>•</sup> production in both cardiac endothelial cells (ECs) and myocytes. In primarily isolated cardiomyocytes, netrin-1 also increased NO<sup>•</sup> production, DCC abundance and ERK1/2 phosphorylation. Of note, cardiac apoptosis was significantly attenuated by netrin-1, which was reversed by DCC-antibody, U0126, L-NAME or PTIO. In summary, our data clearly demonstrate that netrin-1 potently protects the heart from I/R injury by stimulating NO<sup>•</sup> production from cardiac ECs and myocytes. This potent effect is mediated by a DCC/ERK1/2/eNOS<sub>S1177</sub>/NO<sup>•</sup>/DCC feed-forward mechanism in both cell types.

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## 1. Introduction

Netrin-1 is a secreted molecule that is largely known to play a defined role in guiding vertebrate commissural axons in neuronal development [1–3]. Recent studies have further demonstrated a critical role of netrin-1 in endothelial cell proliferation, migration and angiogenic signaling [4–8], in addition to morphogenesis of epithelial cells [9,10]. At least eight netrin receptors have been characterized in neurons, vascular system and other cell types in mammals. These include deleted in colorectal cancer (DCC), UNC5A, B, C, D, neogenin, α6β4 and α3β1 integrins [10–14]. Netrin-1 binding to DCC mediates attractive outgrowth of axons, as well as positive angiogenic signalings in endothelial and vascular smooth muscle cells [4–6]. In contrast, the UNC5B receptor appears repulsive, mediating cellular effects such as filopodial retraction [15,16], particularly in developing capillaries. The overall expression profile of netrin-1 receptors in the heart however, has remained completely unknown.

In a recent study we found netrin-1 induces production of nitric oxide (NO<sup>•</sup>) to promote aortic endothelial cell migration and proliferation [6]. Uniquely, we found that netrin-1 induced NO<sup>•</sup> production is DCC-dependent, involving a feed-forward activation of ERK1/2-eNOS [6]. Binding of netrin-1 to DCC leads to an initial activation of ERK1/2, consequent phosphorylation and activation of eNOS as well as production of NO<sup>•</sup>, which in turn further activates ERK1/2 and more NO<sup>•</sup> production to prompt endothelial cell growth and migration [6]. In view of the potent cardioprotective effects of NO<sup>•</sup>, it is logical to speculate that netrin-1 might have beneficial effect in protecting cardiac muscles from ischemia/reperfusion (I/R) insult. It has been established that a deficiency in endothelial nitric oxide synthase (eNOS) exacerbates myocardial I/R injury, whereby eNOS overexpression, NO<sup>•</sup> donor, or dietary supplementation of nitrite significantly improved cardiac function in models of hypoxia and myocardial I/R injury [17–21]. In addition, netrin-1 has been shown to protect against renal I/R injury *in vivo*, with unknown molecular/signaling mechanisms [22].

Therefore in the present study we aimed to examine whether and how netrin-1, potentially via production of NO<sup>•</sup>, is cardioprotective.

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Three netrin-1 receptors, DCC, neogenin and UNC5B, are present in the endothelial cells (ECs) of various vascular beds, and have been examined to date for their roles in angiogenic signaling. We first found that despite an absence of UNC5B, DCC and neogenin abundantly expressed in C57BL/6J mouse heart, at both mRNA and protein levels. This was consistent with what was found earlier in adult ECs.<sup>6</sup> Hearts perfused using a Langendorff system had an excessive myocardial infarction (MI,  $42.5 \pm 3.6\%$ ) after global ischemia (20 min) and reperfusion (60 min), which was dramatically reduced by netrin-1 intervention (100 ng/mL, pre-perfusion 45 min, reperfusion 60 min) to  $21.8 \pm 4.9\%$ . This finding was accompanied by an augmented NO<sup>•</sup> production. We then further demonstrated that this cardioprotective effect of netrin-1 was dependent on DCC, and consequent activations of ERK1/2 and eNOS<sub>S1177</sub>. Both cardiomyocytes and cardiac ECs were responsible for an increase in NO<sup>•</sup> production, which feed-forwardly upregulated DCC expression. I/R-induced cardiac apoptosis was significantly attenuated by netrin-1. Taken together; these observations clearly demonstrate that netrin-1 potentially protects the heart from I/R injury via a DCC/ERK1/2/eNOS<sub>S1177</sub>/NO<sup>•</sup>/DCC feed-forward mechanism.

## 2. Methods

### 2.1. Materials

Purified mouse netrin-1 was purchased from R&D Systems. Polyclonal antibody for DCC was obtained from EMD Calbiochem. Polyclonal antibodies specific for phosphorylated ERK1/2, eNOS<sub>S1177</sub> were obtained from Cell Signaling Technology (CST). Polyclonal antibody specific for VEGFR2 was purchased from Abcam. Other chemicals were purchased from Sigma in the highest purity.

### 2.2. Animal

Male C57BL/6J mice (6–8 weeks old) were obtained from the Jackson Laboratories (Bar Harbor, ME). DCC<sup>+/-</sup> mice were kindly provided by Dr. Marc Tessier-Lavigne from Genentech. Mice were housed under a pathogen-free condition. 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. RT-PCR and Western blot

Total RNA was extracted from mouse hearts using TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed in standard fashion with iScript cDNA synthesis Kit (Bio-Rad). PCR was performed using the following primer pairs: DCC, fw: CAGCAAAAAGTGTGCAAGGA and rev: CGCAAAGTTCAGAATCGTCA; UNC5B, fw: AGTGTAATGGCGAGTGGGTC and rev: CGAAGAGTTCCTC-CACTTGC; Neogenin, fw: TGAACCAGTTGTGGGAAACA and rev: GCCACT-CATTGGAGGTTTGT; UNC5A, fw: CGTGTCTGCACTTCAAAGA and rev: CCTGGTAGCTGACAAGGAGC; UNC5C, fw: CACATCTGGAGTGGCTCTCA and rev: GCATAGCTTCTGCCGATAG; UNC5D, fw: GTAAAGCAGCT-CAAGGTGGC and rev: ATGCAGCAGCTTTGGTTCTT; α6, fw: GTGTGTGAACATCAGGTGCC and rev: ATATTCTGAGCAGCAGCGGT; α3, fw: GCTGACCTGATCATCTGCAA and rev: GCAGTAGACAGGAAGGCAG; β4, fw: GAGAGCGAGAGGGTGTCTATC and rev: ATATCTC-CATTGGGCCTCTCT; PCRs were carried out on an icycler (Bio-Rad) including primers generated for GAPDH, [(95 °C/2 min), (95 °C/25 s, 57 °C/5 s, 68 °C 5 min) × 35, (72 °C/10 min)]. Western blot analysis was performed as previously published [23].

### 2.4. Langendorff perfusion

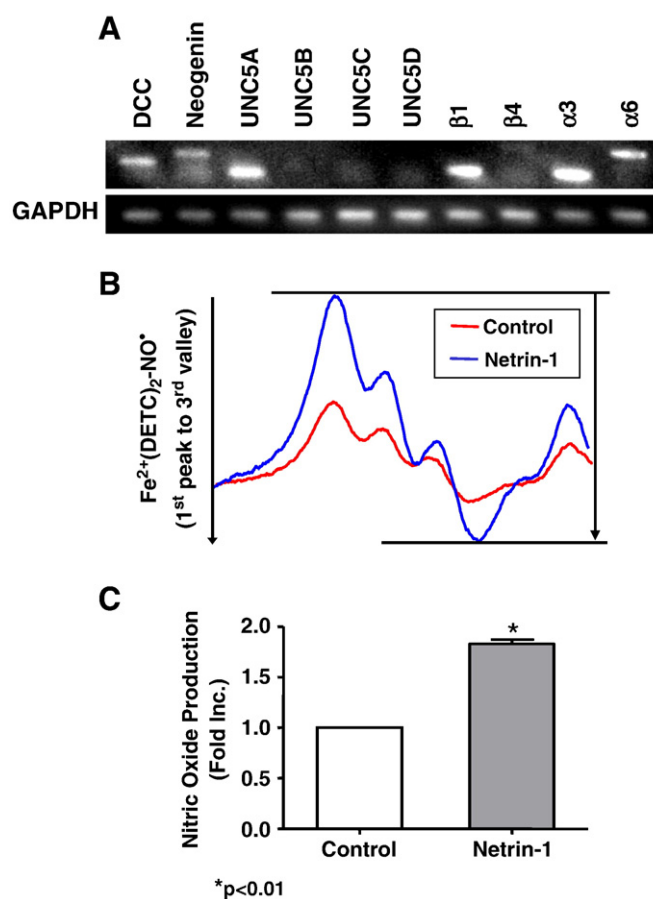
The mice were anesthetized with intraperitoneal pentobarbitone (60 mg/kg). Hearts were harvested and rapidly transferred into ice-

cold Krebs–Henseleit buffer (KHB). The aorta was cannulated with a 20-gauge stainless steel blunt needle and transferred to the Langendorff rig and perfused retrograde instantly with modified Krebs–Henseleit buffer, which contained (in mmol/L): NaCl 118.0, KCl 4.7, CaCl<sub>2</sub> 2.5, MgSO<sub>4</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25.0, D-Glucose 10 at constant pressure ( $80 \pm 1$  mm Hg) using a peristaltic pump and feedback system.

For no I/R setting, hearts were perfused with KHB or netrin-1 for 120 min. For I/R setting, hearts were subjected to 30 min of perfusion with DCC-antibody (1 μg/mL), U0126 (50 μmol/L), L-NAME (100 μmol/L), PTIO (60 μmol/L) or KHB only, followed by 45 min netrin-1 (100 ng/mL) perfusion. Then I/R injury was consistently produced by subjecting the hearts to 20 min of normo-thermic ischemia, followed by reperfusion for 60 min with netrin-1. These experimental protocols are illustrated in supplemental Figure 1.

### 2.5. Electron spin resonance detection of nitric oxide radical

Bioavailable nitric oxide radical (NO<sup>•</sup>) from cells or tissues was detected using electron spin resonance (ESR) as described [6,23]. In brief, whole heart homogenates or cardiomyocytes were incubated with equal volume of freshly prepared NO<sup>•</sup>-specific spin trap Fe<sup>2+</sup>(DETC)<sub>2</sub> colloid (0.5 mmol/L) for 60 min. Gently collected homogenates or cell suspensions were snap-frozen in liquid nitrogen and loaded into a finger Dewar for analysis with an eScan



**Fig. 1.** Expression of Netrin-1 receptors in the heart and netrin-1 stimulation of cardiac nitric oxide (NO<sup>•</sup>) production: Whole mouse hearts (C57BL/6J) were homogenated for RNA extraction. (A) Expression of DCC, neogenin, UNC5A–D, integrin β1, β4, α3, and α6 mRNA in the mouse heart was measured by RT-PCR and normalized to levels of GAPDH. In additional experiments, mouse hearts were freshly isolated and perfused with netrin-1 (100 ng/mL) on the Langendorff rig for 120 min. Hearts were then homogenated and incubated with the NO<sup>•</sup>-specific spin trap Fe<sup>2+</sup>(DETC)<sub>2</sub> for 60 min prior to ESR analysis of NO<sup>•</sup> production. (B) Representative ESR spectra. (C) Grouped data from six independent experiments (Means ± SEM, n = 6), \*p < 0.01.

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.

## 2.6. Infarct size analysis

Infarct size was determined by triphenyl tetrazolium chloride (TTC) staining. In brief, at the end of the experimental protocol hearts were infused with 1% TTC in phosphate-buffered solution (pH 7.4) for 10 min prior to snap freezing at  $-20^{\circ}\text{C}$ . While frozen, the hearts were sliced perpendicular to the long-axis of the heart at 1-mm intervals and de-stained in 10% formaldehyde solution to increase contrast between necrotic and viable myocardium. The heart slices were then digitally photographed for planimetry using NIH Image 1.62. Infarct size is expressed as an infarct-to-risk zone ratio (the risk zone is the whole ventricular volume in this global ischemic model).

## 2.7. Creatine kinase (CK) release

The effluent was collected during the entire 60 min of reperfusion. The amount of CK was determined using a CK Reagent Set (Diagnostic Chemicals). CK reagent was reconstituted in 10 mL of buffer provided by the manufacturer. 40  $\mu\text{L}$  of each sample was mixed with 1 mL of reconstituted reagent, incubated for 2 min at  $37^{\circ}\text{C}$  and read at 340 nm by a Microplate Reader (BioTek). The results were normalized by protein concentration, which was determined by DC protein assay (Bio-Rad).

## 2.8. Spatial localization of $\text{NO}^{\cdot}$

In order to visualize  $\text{NO}^{\cdot}$  and to analyze its spatial localization in heart sections, the level of  $\text{NO}^{\cdot}$  concentration was monitored using a fluorescent  $\text{NO}^{\cdot}$  probe, DAF-FM Diacetate. Sections were loaded with DAF-FM Diacetate (10  $\mu\text{mol/L}$  for 1 h in DMSO), washed to remove excessive probe, and soaked in fresh buffer for an additional 30 min incubation to complete de-esterification of the intracellular diace-

tates. The sections were then mounted and visualized with a confocal microscope.

## 2.9. Isolation of adult mouse cardiomyocytes

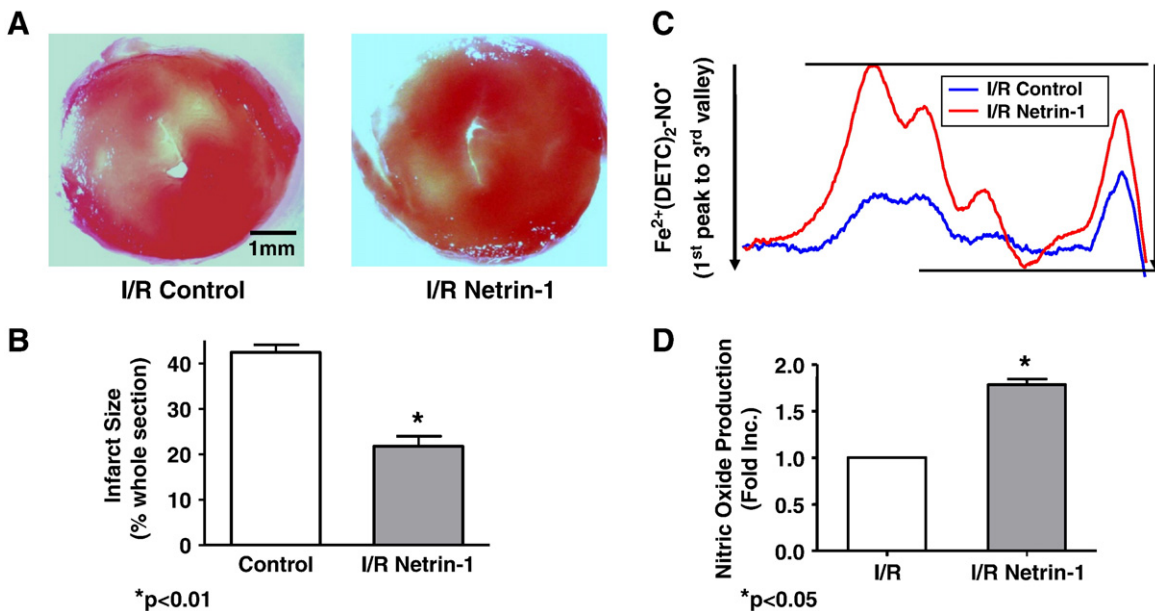
The heart was retrogradely perfused ( $37^{\circ}\text{C}$ ) at a constant pressure of 80 mm Hg for 5 min with a  $\text{Ca}^{2+}$ -free Tyrode buffer containing (in mmol/L): NaCl 130, KCl 5.4,  $\text{MgSO}_4$  1,  $\text{NaH}_2\text{PO}_4$  0.6, D-Glucose 10, HEPES 10, which was continuously bubbled with 95%  $\text{O}_2$  + 5%  $\text{CO}_2$ . The enzymatic digestion was commenced by adding collagenase type II (Worthington, 0.56 mg/mL each) to the perfusion buffer and continued for 20 min. The heart was cut down and placed in the Petri dish with perfusion solution. The digested ventricular tissues were cut into 2–3 mm pieces and gently aspirated with a transfer pipette to facilitate cell dissociation. The myocytes were allowed to sediment by gravity for 8–10 min in the 15-mL Falcon tubes.

## 2.10. Detection of apoptosis

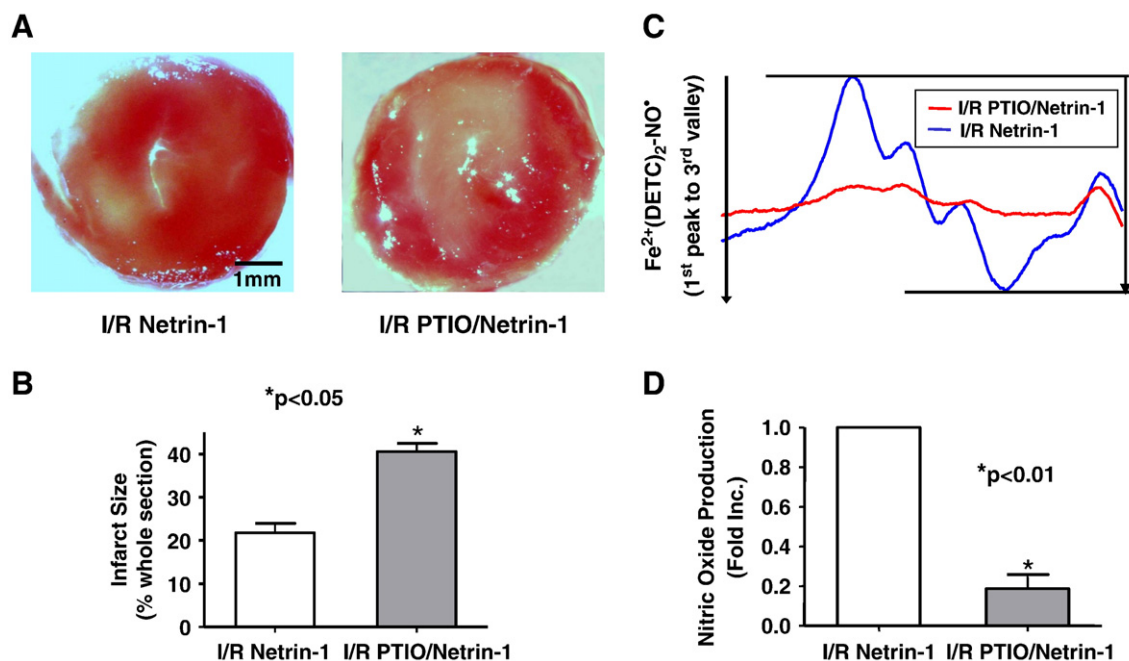
Apoptosis was detected by the apoptosis detection kit (Chemicon International) according to the manufacturer's protocol. At the end of the experimental protocols frozen heart sections (12  $\mu\text{m}$ ) were fixed with 1% formalin for 10 min at room temperature (RT) and washed twice in PBS, permeabilized with ethanol/acetic acid (2:1) for 5 min at  $-20^{\circ}\text{C}$ , washed twice in PBS, and then incubated for  $>10$  s with the equilibration buffer. Subsequently, the sections were incubated for 1 h with DIG-conjugated dUTP and TdT enzymes at  $37^{\circ}\text{C}$ , and then for 10 min in the stop buffer at RT, and finally washed three times in PBS. The sections were then incubated with FITC-conjugated anti-DIG antibody for 30 min at RT, and washed three times in PBS. Coverslipped sections were mounted with medium containing 0.5  $\mu\text{g/mL}$  of Propidium Iodide.

## 2.11. Statistical analysis

All data are presented as mean  $\pm$  SEM from four to six independent experiments (different individual mouse or different passage of Bovine aortic endothelial cells (BAECs) used on different days).  $\text{NO}^{\cdot}$



**Fig. 2.** Netrin-1 protects heart from ischemia/reperfusion injury. Hearts were pre-perfused for 45 min with netrin-1 (100 ng/mL) before ischemia/reperfusion (20 min ischemia, 60 min reperfusion with netrin-1). Sections of hearts were stained with 2,3,5-TTC and infarct area calculated as % of risk area. (A) Infarct size was significantly decreased in netrin-1 treated hearts compared to that of controls ( $21.8 \pm 4.9\%$  vs.  $42.5 \pm 3.6\%$ , respectively,  $p < 0.01$ ); (B) Infarct size shown in quantitative grouped data (Means  $\pm$  SEM,  $n = 5$ ),  $*p < 0.01$ . (C) Representative ESR spectra of  $\text{NO}^{\cdot}$  production from hearts perfused with netrin-1; (D) Grouped data from four independent experiments.  $*p < 0.05$ .



**Fig. 3.** Nitric oxide is required for netrin-1 induced cardiac protection from ischemia/reperfusion injury. Hearts were pre-perfused with PTIO for 30 min prior to netrin-1 perfusion (100 ng/mL, 45 min), followed by ischemia/reperfusion (20 min ischemia, 60 min reperfusion with netrin-1). Sections of hearts were stained with 2,3,5-TTC and infarct area calculated as % of risk area. (A and B) Representative TTC stainings of I/R Netrin-1, I/R PTIO/Netrin-1 and quantitative grouped data (means ± SEM,  $n = 5$ ),  $*p < 0.05$ . (C) Representative ESR spectra for NO $\cdot$ ; (D) Grouped data from three independent experiments,  $*p < 0.001$ .

data have been normalized by protein prior to statistical analysis. ANOVA was used to compare means of different experimental groups. Statistical significance is set as  $p < 0.05$ .

### 3. Results

#### 3.1. Netrin-1 stimulates NO $\cdot$ production from mouse heart

RNA and protein samples were prepared from freshly isolated C57BL/6J mouse hearts. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) and western blot were used to determine mRNA and protein levels of netrin-1 receptors respectively. Relative abundance of each mRNA expression, indexed by endogenous GAPDH, was used for comparison. Whereas DCC, UNC5A, integrin  $\beta 1$  and  $\alpha 3$  were abundantly expressed at mRNA levels, UNC5B, C, D and  $\beta 4$  was undetectable (Fig. 1A). Neogenin and  $\alpha 6$  were weakly expressed. We have also confirmed DCC and neogenin expression at protein level (data not shown). Given the fact that DCC is present in the mouse heart, we wondered whether netrin-1 stimulates DCC-dependent changes in NO $\cdot$  signaling. Importantly, perfusion of heart with netrin-1 (100 ng/mL, 120 min) resulted in a significant increase in NO $\cdot$  production, as demonstrated by representative ESR spectra and grouped data from six independent experiments (Figs. 1B and C). Subsequent experiments indicate that netrin-1 also stimulated DCC-dependent NO $\cdot$  production from hearts exposed to I/R injury (see below).

#### 3.2. Nitric oxide mediates netrin-1 induced cardiac protection from ischemia/reperfusion injury

In view of the potent effect of netrin-1 on cardiac NO $\cdot$  production (Figs. 1B and C) and the potentially significant role of NO $\cdot$  in mediating cardioprotection, we next examined whether netrin-1 induces cardioprotection during I/R injury. Hearts pre-perfused for 45 min with netrin-1 (100 ng/mL) were subjected to 20 min global ischemia, followed by a 60-min reperfusion with netrin-1. The experimental protocol is illustrated in supplemental Figure 1. Infarct

size was quantitated as the area not stained red with tetrazolium red. Netrin-1 treated hearts had a substantial reduction in infarct size compared to untreated controls ( $21.8 \pm 4.9\%$  vs.  $42.5 \pm 3.6\%$  for netrin-1 treated I/R vs. I/R alone,  $p < 0.01$ ) (Figs. 2A and B). This was accompanied by an augmented production of NO $\cdot$  in netrin-1 treated, I/R-ed hearts ( $1.78 \pm 0.12$  fold higher than the untreated I/R controls, Figs. 2C and D).

To determine whether the cardioprotective effect of netrin-1 was dependent on NO $\cdot$ , we subjected mouse hearts to NO $\cdot$  scavenger PTIO (60  $\mu\text{mol/L}$ , 30 min) prior to netrin-1 perfusion and I/R injury. While NO $\cdot$  production was attenuated (Figs. 3C and D), the infarct size in PTIO-pre-treated, netrin-1-perfused hearts was also reversed to near control levels of  $40.6 \pm 4.2\%$  (Figs. 3A and B), implicating that the cardioprotective effects of netrin-1 is indeed, NO $\cdot$ -dependent.

#### 3.3. DCC/ERK1/2 is required for netrin-1 induced cardioprotection

DCC-dependent activation of the mitogen activated protein kinase ERK1/2 has been found a signal transduction pathway for netrin-1 stimulated NO $\cdot$  production in vascular ECs [6]. We next examined whether a similar signaling cascade is the mechanism whereby netrin-1 increases NO $\cdot$  production in the heart. Hearts were pre-perfused with antibody neutralizing DCC, MEK1/2 inhibitor U0126 or NOS inhibitor L-NAME for 30 min prior to netrin-1 perfusion. The apparent cardioprotective effects of netrin-1 were reversed ( $n = 4$  for all): the infarct size was increased upon addition of DCC antibody ( $32.7 \pm 4.5\%$ ), U0126 ( $41.3 \pm 4.1\%$ ) or L-NAME ( $40.9 \pm 7.2\%$ ), compared to netrin-1 treatment alone ( $21.8 \pm 4.9\%$ ) (Figs. 4A and B). In addition, creatine kinase (CK) release, as an index of cardiac muscle damage, showed identical responses (Fig. 4C). Concomitantly, increased NO $\cdot$  production by netrin-1 ( $1.78 \pm 0.12$  fold vs. untreated I/R controls) was also attenuated by DCC antibody ( $1.31 \pm 0.21$  fold vs. untreated I/R controls), U0126 ( $1.12 \pm 0.15$  fold vs. untreated I/R controls) or L-NAME ( $0.90 \pm 0.13$  fold vs. untreated I/R controls) (Figs. 4D and E). Taken together; these results indicate that NO $\cdot$ -



dependent cardioprotective effect of netrin-1 is mediated by DCC-dependent activation of ERK1/2 and eNOS.

In additional experiments hearts from wild type (WT) and DCC+/- mice were subjected to I/R and netrin-1 perfusion. Netrin-1 provoked NO<sup>-</sup> production in I/R-ed DCC+/- hearts was significantly reduced (0.66 fold  $\pm$  0.15,  $p < 0.05$ , Figs. 4F and G) comparing to age-matched WT hearts. Netrin-1 induced reduction in infarct size in WT hearts was also markedly attenuated (infarct size: 21.8  $\pm$  4.9% vs. 35.9  $\pm$  3.2% for WT vs. DCC+/- respectively,  $p < 0.05$ , Figs. 4H and I). These data further implicate an intermediate role of DCC in mediating netrin-1 induced cardioprotection.

Furthermore, DCC protein abundance, ERK1/2 phosphorylation and eNOS<sub>S1177</sub> phosphorylation were all increased, as evidenced by representative Western blots and quantitative data (Figs. 5A–D). Interestingly, I/R alone induced a rapid decline in DCC protein and mRNA expression (Figs. 5B and H). Netrin-1 perfusion of I/R hearts led to a significant restoration of DCC protein expression, which was abolished by DCC-antibody, U0126, or L-NAME (Fig. 5B), indicating a potential feed-forward loop of NO<sup>-</sup>-ERK1/2-DCC. BAECs and isolated cardiomyocytes were treated with NO<sup>-</sup> donor MAMANOATE (1 mmol/L, 2 h) prior to analysis of DCC expression. Of note, NO<sup>-</sup> donor increased DCC protein expression in both cultured ECs and cardiomyocytes (Fig. 5I). In additional experiments hearts were perfused with netrin-1 and harvested at 0, 5, 10 and 30 min. Netrin-1 stimulation resulted in a rapid, time-dependent increase in ERK1/2 and eNOS<sub>S1177</sub> phosphorylations (for 0, 5 and 10 min time points), which lasted till up to 30 min (longest time point examined, Fig. 5J). Of note, RNA samples extracted from netrin-1-perfused I/R hearts demonstrated an elevation in DCC mRNA levels (Fig. 5H) as well, indicating that transcriptional regulation may have occurred to contribute to protein changes.

Moreover, a clear increase in phosphorylated ERK1/2 (Fig. 5C) and eNOS<sub>S1177</sub> (Fig. 5D) was observed with netrin-1 treatment ( $n = 6$ ), which was inhibited by DCC-antibody or U0126, but not by addition of L-NAME. These results seem to indicate that netrin-1 initiates its signaling by binding to DCC, resulting in ERK1/2/eNOS activation to produce NO<sup>-</sup>, which in turn, upregulates DCC to form a positive feedback loop. Furthermore, protein expression of eNOS was upregulated by netrin-1 perfusion under I/R condition, whereas iNOS and nNOS expression were unaffected (Fig. 5A, E–G).

### 3.4. Cardiomyocytes and ECs-derived NO<sup>-</sup> production in response to netrin-1

The NO<sup>-</sup>-specific fluorescent probe DAF-AM was used to estimate changes in NO<sup>-</sup> production in left ventricle. In netrin-1 perfused, post-I/R heart NO<sup>-</sup> staining was clearly increased (supplemental Figure 2A, D, G, J). As shown in supplemental Figure 2C, F, I, L, some of the increase in NO<sup>-</sup> staining was detected specifically in the ECs that line cardiac microvessels, which also labeled positive for VEGFR2 fluorescent antibody (supplemental Figure 2B, E, H, K). The rest of the NO<sup>-</sup> staining seemed to come primarily from cardiomyocytes and showed an increase in netrin-1 treated myocardium.

To further investigate a specific role of cardiomyocytes in netrin-1 signaling in the heart, cardiomyocytes were freshly isolated from

hearts and treated with netrin-1 (30 min or 60 min) in Tyrode's solution. There was a significant increase in NO<sup>-</sup> production from cardiomyocytes treated with netrin-1, as demonstrated by representative ESR spectra and grouped data from three independent experiments (supplemental Figure 3A–D). Protein abundance of DCC and ERK1/2 phosphorylation were also significantly elevated in netrin-1-treated cardiomyocytes (supplemental Figure 3E), echoing results found in lysed cardiac tissues (Fig. 5B). These results seem to suggest that netrin-1-stimulated NO<sup>-</sup> production from cardiomyocytes is also DCC-dependent and requires activation of ERK1/2 and eNOS.

### 3.5. Netrin-1 protects cardiac cells from apoptosis

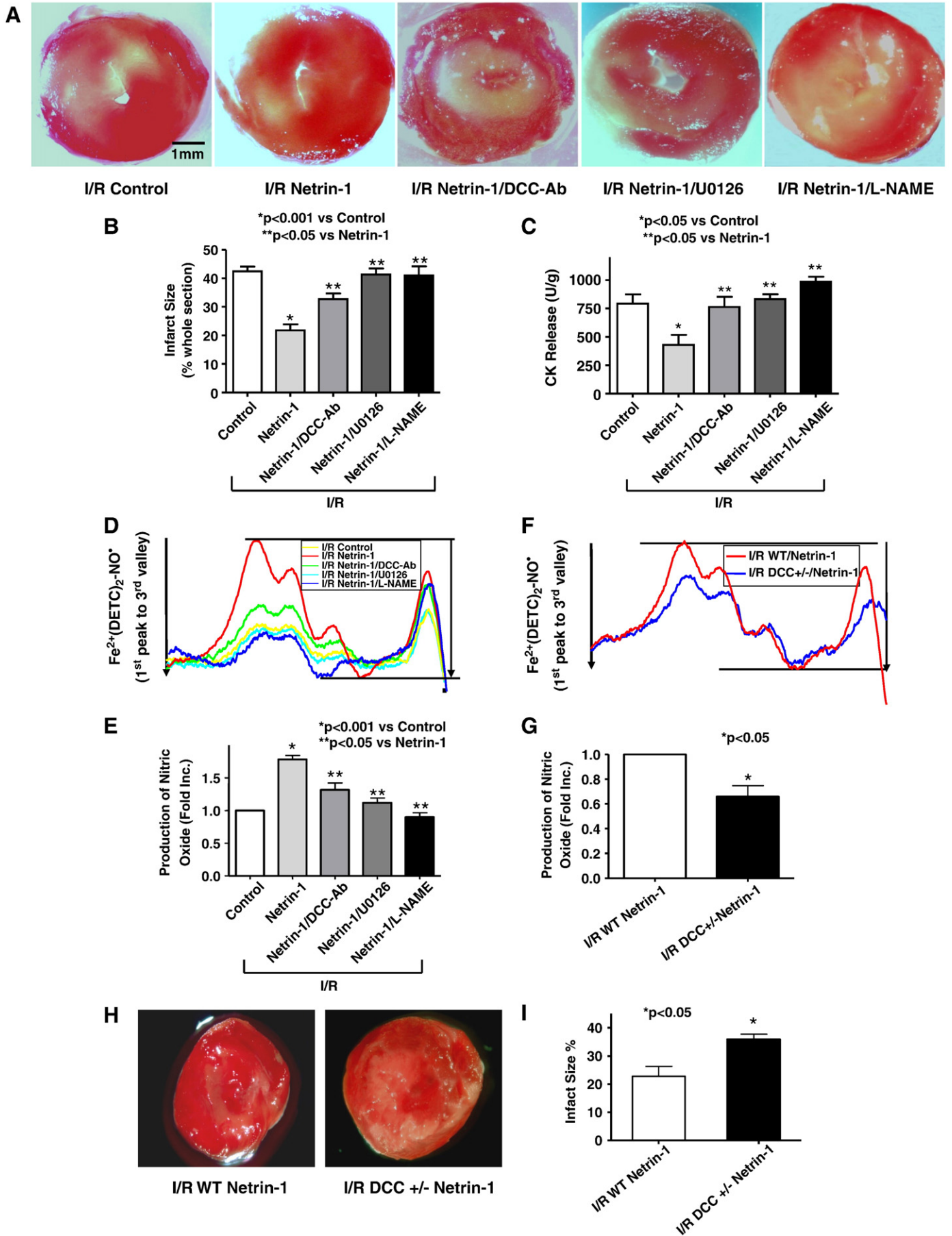
NO<sup>-</sup> protects ECs and cardiomyocytes from apoptosis induced by oxidant stress, inflammatory cytokines and chemotherapeutic agents [24–27]. TUNEL assay was performed to determine whether netrin-1 inhibits apoptotic cell death induced by I/R. Left ventricular sections were incubated with FITC-conjugated anti-DIG antibody and coverslipped with the mounting medium containing 0.5  $\mu$ g/mL of Propidium Iodide (PI). Representative TUNEL (green) and PI (red) stained sections and quantitative analysis are shown in Figs. 6A and B. I/R injury led to a significant increase in the percentage of TUNEL-positive cells compared to the non-I/R sections (18.2  $\pm$  1.45% vs. 0.33  $\pm$  0.5%). The heart pre-treated with netrin-1 exhibited a significant decrease in TUNEL-positive cells (6.78  $\pm$  0.93%). The decrease in apoptosis was completely attenuated by DCC-antibody (17.83  $\pm$  2.87%), U0126 (19.24  $\pm$  2.4%) or L-NAME (17.82  $\pm$  2.19%), again consistently indicating intermediate roles of DCC, ERK1/2 and NO<sup>-</sup>.

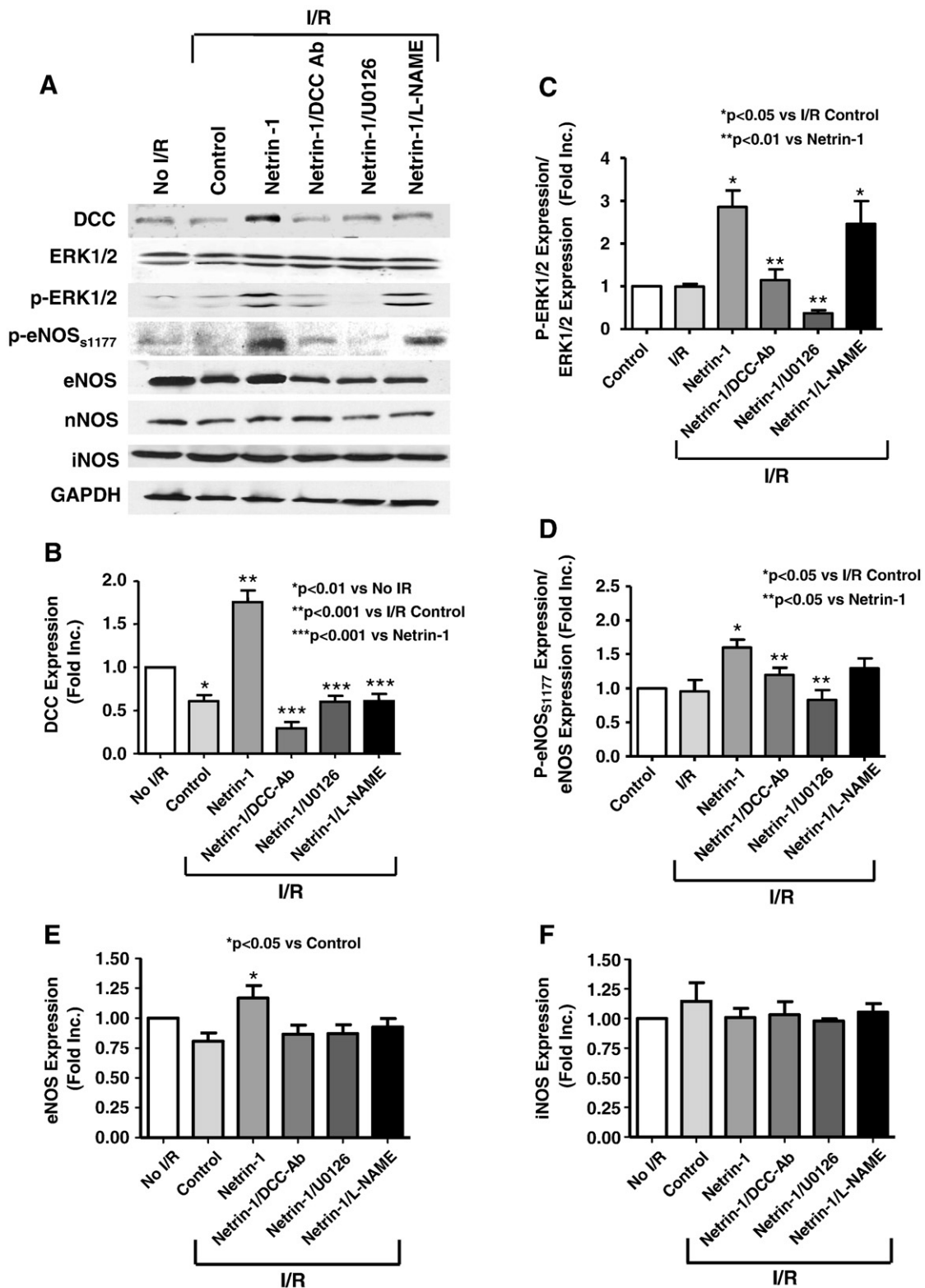
## 4. Discussion

The most significant finding of the study is the innovative identification of a DCC/ERK1/2/eNOS<sub>S1177</sub>/NO<sup>-</sup>/DCC/NO<sup>-</sup> pathway that protects the heart from I/R induced apoptotic cell death and infarction. NO<sup>-</sup> is produced following a DCC-dependent ERK1/2-eNOS<sub>S1177</sub> activation in both cardiomyocytes and cardiac ECs, and that elevation in DCC expression was abolished by inhibition of ERK1/2 or NO<sup>-</sup>, revealing a feed-forward loop of DCC-ERK1/2-NO<sup>-</sup>-DCC. I/R induced a rapid loss in DCC protein and mRNA expression, which was recovered by netrin-1 to a level that was two fold of the control. Exogenous NO<sup>-</sup> donor also upregulated DCC expression in both cultured myocytes and BAECs.

Despite having been well characterized for its role in regulating axonal guidance [1–3], any potential effects of circulating netrin-1 on the pathophysiology of the cardiovascular system remain largely elusive. While recent studies identified an angiogenic role of netrin-1 [4–8], whether it affects cardiac function is completely unknown. In the present study for the first time we delineated effects of netrin-1 perfusion on cardiac protection. Also for the first time we examined expressional profiles in the heart of the eight known netrin-1 receptors. As demonstrated in Fig. 1, DCC, neogenin, UNC5A, integrin  $\beta$ 1 and  $\alpha$ 3 were found highly expressed in C57BL/6J mouse hearts (mRNA detected by RT-PCR). Among these DCC and neogenin are attractive receptors that mediate axonal outgrowth, and both expressed in cultured endothelial cells; however only DCC mediates

**Fig. 4.** DCC/ERK1/2 activation is required for netrin-1-induced cardioprotection. Hearts were perfused with DCC antibody (1  $\mu$ g/mL), MEK1/2 inhibitor U0126 (50  $\mu$ mol/L) or L-NAME (100  $\mu$ mol/L) for 30 min prior to 45 min netrin-1 perfusion. Ischemia/reperfusion injury was consistently produced by subjecting the hearts to 20 min of ischemia, followed by reperfusion for 60 min (with or without netrin-1). Sections of hearts were stained with 2,3,5-TTC and infarct area calculated as % of risk area. (A) Representative TTC stainings of control hearts, and hearts receiving netrin-1, DCC-antibody/netrin-1, U0126/netrin-1 and L-NAME/netrin-1; (B) Infarct size shown in quantitative grouped data of A (means  $\pm$  SEM,  $n = 5$ ), \* $p < 0.001$  vs. control; \*\* $p < 0.05$  vs. netrin-1; (C) Creatine kinase (CK) release was measured by collecting effluent during the entire 60 min of reperfusion from control hearts, and hearts receiving netrin-1, DCC-antibody/netrin-1, U0126/netrin-1 and L-NAME/netrin-1 (Means  $\pm$  SEM,  $n = 3$ ). \* $p < 0.05$  vs. control; \*\* $p < 0.05$  vs. netrin-1; (D) Representative ESR spectra of NO<sup>-</sup> production of control hearts, and hearts receiving netrin-1, DCC-antibody/netrin-1, U0126/netrin-1 and L-NAME/netrin-1; (E) Grouped data of NO<sup>-</sup> production of D (means  $\pm$  SEM,  $n = 5$ ). \* $p < 0.001$  vs. control, \*\* $p < 0.05$  vs. netrin-1; (F) Representative ESR spectra of NO<sup>-</sup> production from IR-ed hearts of WT or DCC+/- mice perfused with netrin-1; (G) Grouped data of NO<sup>-</sup> production of F (means  $\pm$  SEM, \* $p < 0.05$ ); (H) Representative TTC stainings of netrin-1 perfused, IR-ed hearts from WT or DCC+/- mice; (I) Infarct size shown in quantitative grouped data of H (means  $\pm$  SEM,  $n = 3$ ), \* $p < 0.05$ .





**Fig. 5.** DCC-ERK1/2-eNOS<sub>1177</sub>-NO-DCC feed-forward loop upon netrin-1 activation. Hearts were perfused as described in Fig. 4. All quantitative data are expressed in Means  $\pm$  SEM for statistical analysis. (A) Representative Western blots; (B) Grouped densitometric data of DCC protein expression ( $n=6$ ), \* $p < 0.01$  vs. no I/R; \*\* $p < 0.001$  vs. I/R control. \*\*\* $p < 0.001$  vs. netrin-1; (C) Grouped densitometric data of ERK1/2 phosphorylation that is normalized by ERK1/2 ( $n=3$ ), \* $p < 0.05$  vs. I/R control, \*\* $p < 0.01$  vs. netrin-1; (D) Grouped densitometric data of eNOS<sub>s1177</sub> phosphorylation that is normalized by eNOS ( $n=4$ ), \* $p < 0.01$  vs. I/R control, \*\* $p < 0.01$  vs. netrin-1; (E) Grouped densitometric data of eNOS protein expression ( $n=5$ ), \* $p < 0.05$  vs. I/R control; (F) Grouped densitometric data of iNOS protein expression ( $n=4$ ); (G) Grouped densitometric data of nNOS protein expression ( $n=3$ ); (H) Representative and grouped mRNA levels of RT-PCR analysis of DCC in post netrin-1-perfused hearts ( $n=3$ ), \* $p < 0.05$ ; (I) Representative and grouped Western blot data of DCC expression in NO<sup>-</sup> donor-treated BAECs and cardiomyocytes. (J) Representative and grouped Western blots data of ERK1/2 and eNOS<sub>s1177</sub> phosphorylation at different time points. Hearts were perfused with netrin-1 (100 ng/mL) and harvested at 0, 5, 10 and 30 min for Western blot analysis.

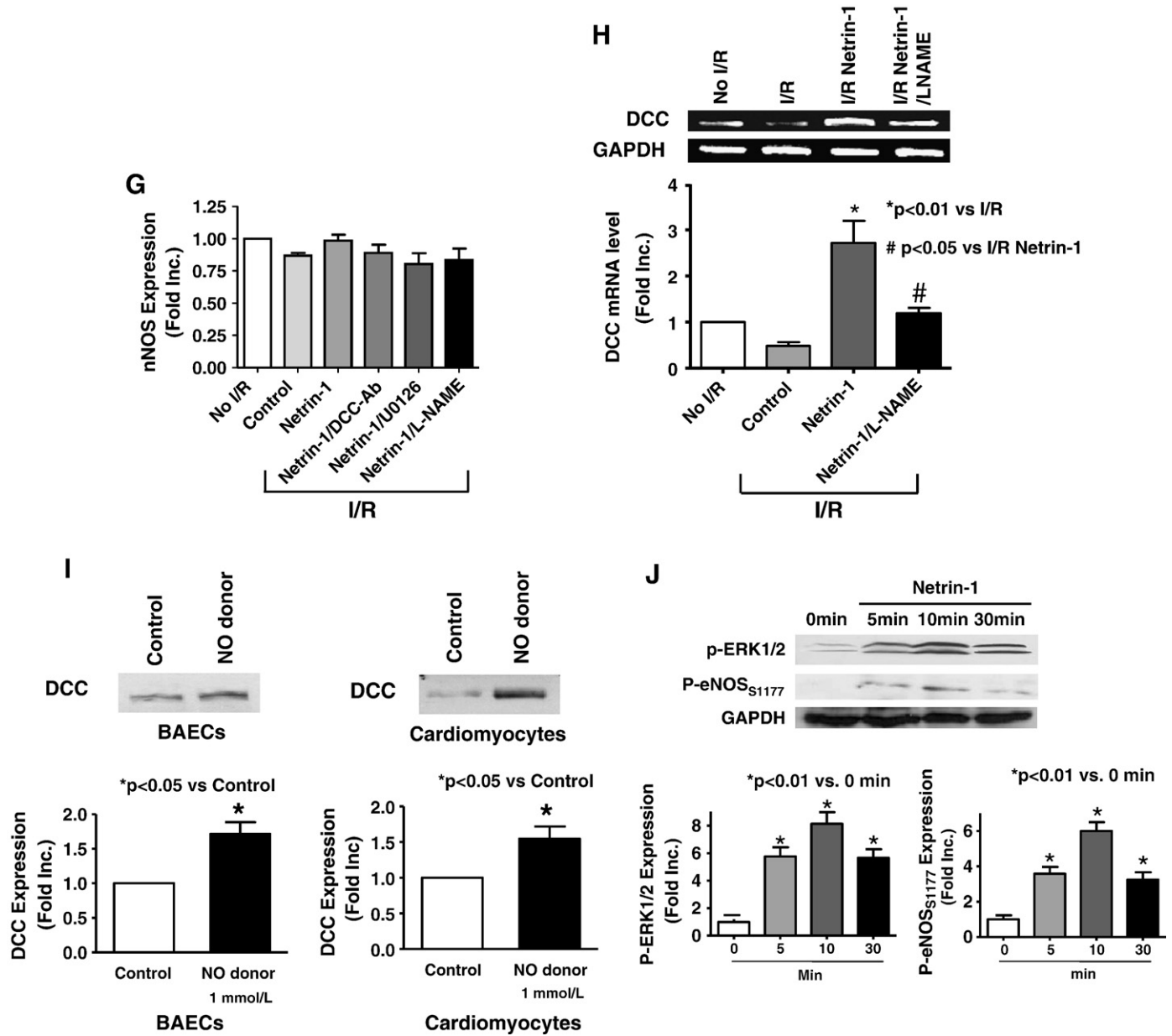


Fig. 5 (continued).

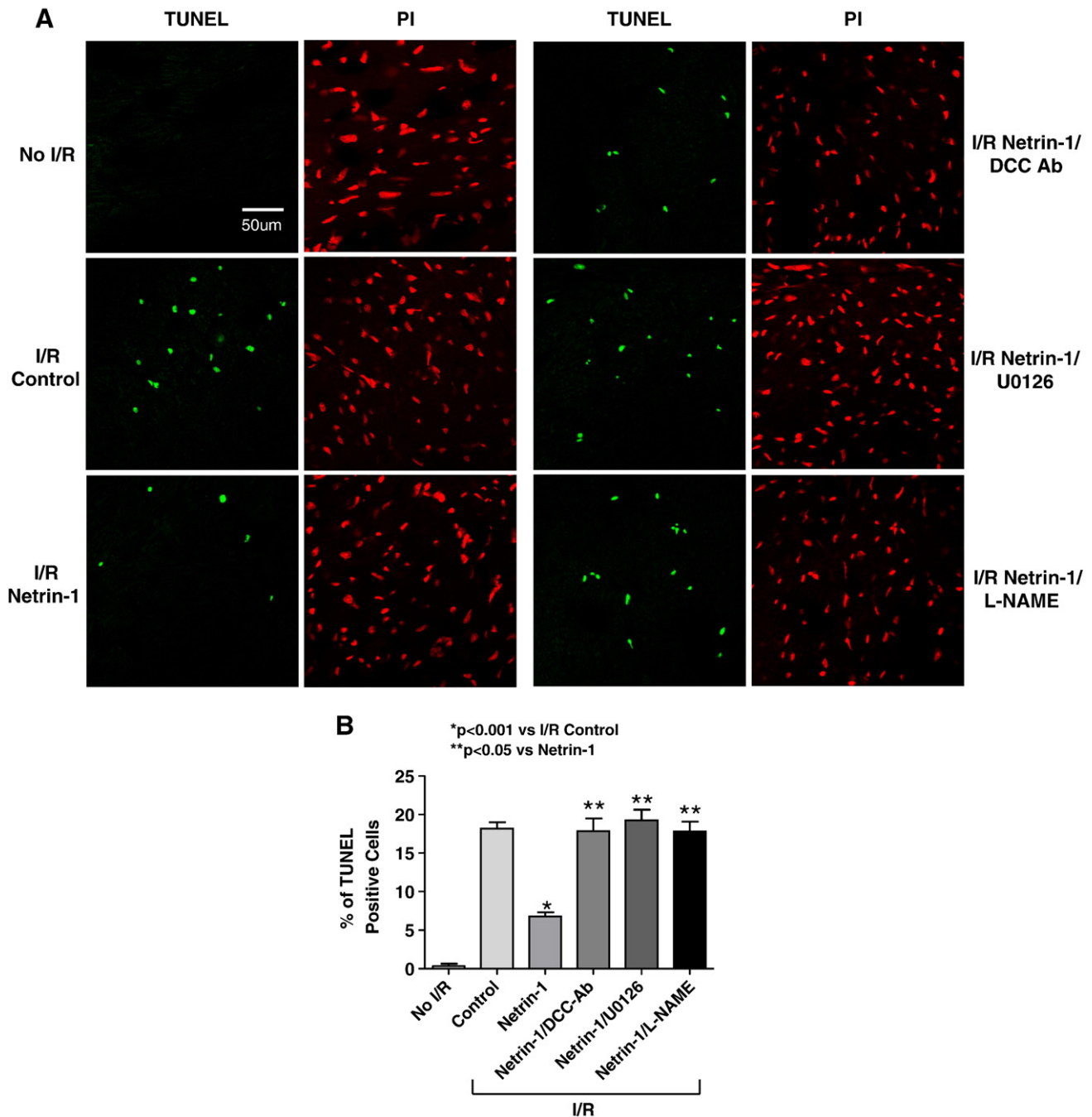
netrin-1 stimulated NO<sup>-</sup> production and endothelial cell growth and migration [6]. The repulsive receptor UNC5B however was not expressed in mouse heart (Fig. 1), although found in ECs previously [15,27]. The mouse hearts we used for these analyses were rinsed out of blood completely using Krebs–Henseleit buffer, so that the mRNA extracted was not contaminated by those of blood cells. If using uncleaned hearts or monocytes, abundant UNC5B mRNA was detected (data not shown), which is consistent to previous findings that monocytes express large amount of UNC5B [28].

The concept that NO<sup>-</sup> is a powerful cardioprotectant against I/R injury has become well accepted [17,20,29]. Whereas in eNOS-deficient mice the infarct size is significantly augmented following I/R injury [30], overexpression of eNOS is associated with reduced infarct size [17,18,31]. Nitrite supplementation results in a 48% reduction in infarct size post I/R insult [32–35]. Indeed, NO<sup>-</sup> has been shown to mediate cardioprotective effects of estrogen [36], statins [37], moderate alcohol [38], peroxisome proliferator-activated receptor- $\alpha$  [39] and sildenafil [40]. In our study, netrin-1 induced an approximate 2-fold increase in cardiac NO<sup>-</sup> production, which was associated with a 49% decrease in

infarct size following I/R injury. The specific, NO<sup>-</sup>-mediated cardioprotective effect of netrin-1 was abolished by NO<sup>-</sup> scavenger PTIO (Fig. 3) and NOS inhibitor L-NAME (Fig. 4). Taken together, our data further establishes a cardioprotective role of NO<sup>-</sup> and reveals the mechanisms whereby netrin-1 exhibits cardioprotection.

Our findings support an essential role of DCC in mediating netrin-1 induction of NO<sup>-</sup> in cardiac ECs and myocytes. DCC-antibody abolishes netrin-1 induction of NO<sup>-</sup> production in I/R-ed hearts. This finding shares similarities with our previous observation that netrin-1 stimulates NO<sup>-</sup> production in mature ECs in a DCC-dependent manner [6]. DCC protein abundance and mRNA expression were increased in netrin-1-perfused, I/R-ed hearts, although I/R alone induced a decline in DCC protein and mRNA expression (Figs. 5A and B and I). NO<sup>-</sup> donor also upregulated DCC protein expression in both cultured ECs and cardiomyocytes (Fig. 5H). Taken together; these data reveal a feed-forward loop of DCC-NO<sup>-</sup>-DCC, and a predominant role of DCC in mediating netrin-1-dependent cardioprotection. Though the expression of neogenin was abundant, neogenin-antibody had no effect on netrin-1 stimulated NO<sup>-</sup> production or cardioprotection (data not shown).





**Fig. 6.** Netrin-1 reduces cardiomyocyte apoptosis in I/R-ed hearts: hearts perfused as described in Fig. 4. (A) Representative detection of I/R-induced apoptotic cells by TUNEL (green), and of all the cells by Propidium iodide (PI, red), in heart sections of the following conditions: control, ischemia/reperfusion (I/R), netrin-1 treated I/R, netrin-1/DCC I/R, netrin-1/U0126 I/R or Netrin-1/L-NAME I/R; (B) Grouped data from three independent experiments. \* $p < 0.001$  vs I/R control, \*\* $p < 0.05$  vs netrin-1.

Netrin-1 perfusion resulted in marked increases in ERK1/2 (Fig. 5C) and eNOS<sub>s1177</sub> (Fig. 5D) phosphorylations, which were inhibited by DCC-antibody or U0126, but not by addition of L-NAME. These seem to suggest that netrin-1/DCC/ERK1/2/eNOS pathway is turned on first, as inhibition of NO<sup>•</sup> production was ineffective in preventing DCC/ERK activation, whereas, the secondary feed-forward mechanism of NO<sup>•</sup>/DCC/ERK1/2 occurs later, as DCC-antibody and U0126 blocked eNOS phosphorylation. The role of ERK1/2 in mediating netrin-1 activation of eNOS is similar to previous observations that ERK1/2 is involved in transient activation of eNOS by Reactive oxygen species (ROS) [41,42]. It is interesting to speculate that this may share similarities with ROS-dependent preconditioning. Recent studies by Das et al. [43] demonstrated that ERK1/2 phosphorylation mediates

sildenafil-induced myocardial protection against I/R injury in mice. Previous studies by Li et al. [44] also demonstrated that ERK1/2 mediates NO<sup>•</sup> donor stimulated apoptotic reduction during I/R. Transgenic mice with activated MEK1/ERK2 have protected myocardium when exposed to I/R insult *in vivo* [45]. Confirming a cardioprotective role of ERK1/2, our data also neatly identify a netrin-1/DCC/ERK1/2/eNOS/NO<sup>•</sup>/DCC feed-forward loop that mediates the cardioprotective effects of netrin-1.

We have also further evaluated a potential role of PI3K/AKT pathway in mediating netrin-1 induced cardioprotection. Following the same I/R protocol outlined in suppl. Fig. 1, we found that after the entire I/R procedure, AKT phosphorylation was minimal and unaffected by DCC-Ab, U0126 or L-NAME (data not shown), although

our data do not rule out the possibility that AKT was more active at earlier time points. LY294002 pretreatment also failed to reduce netrin-1 stimulated NO<sup>-</sup> production (data not shown), implicating that PI3K/AKT pathway is not required for netrin-1 stimulation of NO<sup>-</sup> production in this particular Langendorff perfusion model.

Netrin-1 induced reduction in myocardial injury, is at least in part, mediated by NO<sup>-</sup>-dependent decrease in myocardial apoptosis. We observed that TUNEL-positive cardiomyocytes were decreased by 62.7% in netrin-1-treated hearts than that of controls. Apoptosis has been observed previously in hearts subjected to either continuous ischemia or ischemia followed by reperfusion [46,47]. One of the major protective mechanisms of NO<sup>-</sup> has been shown to be prevention of myocardial apoptosis/death [31,48]. Indeed, myocardial apoptosis was significantly increased in eNOS-deficient mice during fetal and neonatal heart development, implicating that basal NO<sup>-</sup> release from eNOS protects cardiomyocytes from apoptosis [49]. Weiland et al. [50] have also shown that inhibition of endogenous eNOS potentiates I/R induced myocardial apoptosis via caspase 3 pathway. Consistent to these observations, our data demonstrated that inhibition of netrin-1 signaling to attenuate NO<sup>-</sup> production resulted in loss of the cardioprotective effects of netrin-1.

In summary, our innovative findings characterized a signaling mechanism whereby netrin-1 exerts its powerful cardioprotective effect during I/R injury. Upon netrin-1 perfusion, its attractive receptor DCC is activated, resulting in ERK1/2/eNOS<sub>1177</sub> activation, which in turn, produces NO<sup>-</sup> to upregulate DCC expression, forming a feed-forward loop to maintain DCC activity and additional NO<sup>-</sup> production. A persistent supply of NO<sup>-</sup> may thus underlie the marked reductions in infarct size and cardiac apoptosis. Only previously known as a neuronal developmental protein and a regulator of angiogenesis, netrin-1, here gains a new role as a potent cardioprotective agent. Additional investigations focusing on the therapeutic potential of netrin-1 in managing ischemic heart disease may prove highly rewarding.

## 5. Disclosure

None.

## Acknowledgments

The authors' work has been supported by National Heart, Lung and Blood Institute (NHLBI) Grant HL077440 (HC), HL057244 (PLL and HC), HL080111 (PPP and HC), an American Diabetes Association Award 7-08-RA-18 (HC), and a Start-up Fund from the University of California Los Angeles (HC).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmcc.2009.11.020.

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