ORIGINAL RESEARCH

Recombinant Extracellular Domain (p75ECD) of the Neurotrophin Receptor p75 Attenuates Myocardial Ischemia–Reperfusion Injury by Inhibiting the p-JNK/Caspase-3 Signaling Pathway in Rat Microvascular Pericytes

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BACKGROUND: Pro-NTs (precursor of neurotrophins) and their receptor p75 are potential targets for preventing microvascular dysfunction induced by myocardial ischemia–reperfusion injury (IRI). p75ECD (ectodomain of neurotrophin receptor p75) may physiologically produce neurocytoprotective effects by scavenging pro-NTs. We therefore hypothesized that p75ECD may have a cardioprotective effect on IRI through microvascular mechanisms.

METHODS AND RESULTS: Myocardial IRI was induced in Sprague-Dawley rats by occluding the left main coronary arteries for 45 minutes before a subsequent relaxation. Compared with the ischemia–reperfusion group, an intravenous injection of p75ECD (3 mg/kg) 5 minutes before reperfusion reduced the myocardial infarct area at 24 hours after reperfusion (by triphenyltetrazolium chloride, 44.9±3.9% versus 34.6±5.7%, P<0.05); improved the left ventricular ejection fraction (by echocardiography), with less myocardial fibrosis (by Masson's staining), and prevented microvascular dysfunction (by immunofluorescence) at 28 days after reperfusion; and reduced myocardial pro-NTs expression at 24 hours and 28 days after reperfusion (by Western blotting). A simulative IRI model using rat microvascular pericytes was established in vitro by hypoxia–reoxygenation (2/6 hours) combined with pro-NTs treatment (3 nmol/L) at R. p75ECD (3 μg/mL) given at R improved pericyte survival (by methyl thiazolyl tetrazolium assay) and attenuated apoptosis (by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling). In the reperfused hearts and hypoxia–reoxygenation +pro-NTs-injured pericytes, p75ECD inhibited the expression of p-JNK (phospho of c-Jun N-terminal kinase)/caspase-3 (by Western blotting). SP600125, an inhibitor of JNK, did not enhance the p75ECD-induced infarct-sparing effects and pericyte protection.

CONCLUSIONS: p75ECD may attenuate myocardial IRI via pro-NTs reduction-induced inhibition of p-JNK/caspase-3 pathway of microvascular pericytes in rats.

Key Words: c-Jun N-terminal kinase ■ extracellular domain ■ microvascular dysfunction ■ neurotrophin receptor ■ pericyte ■ reperfusion injury
Although modern reperfusion therapies for acute myocardial infarction can restore epicardial artery flow in most patients, up to one third of patients do not achieve normal myocardial reperfusion because of microvascular dysfunction (MVD) as a consequence of myocardial ischemia–reperfusion injury (IRI), which is associated with an adverse prognosis.\textsuperscript{1,2} Accordingly, an effective MVD prevention is of great clinical relevance,\textsuperscript{3} and strenuous efforts have been made to identify cytokines that may induce MVD or to seek new therapeutic targets that may improve MVD.\textsuperscript{4–7}

Current studies have demonstrated that neurotrophins, as a family of proteins that promote cardiomyocyte survival, can repair infarcted myocardium in mice.\textsuperscript{8,9} However, they are not considered ideal drug candidates because of a poor pharmacokinetic performance and potential side effects.\textsuperscript{10} Conversely, the precursors of neurotrophins, pro-NTs (precursor of neurotrophins), which mainly include pro-NGF (precursor of nerve growth factor) and pro-BDNF (precursor of brain-derived growth factor), may serve as harmful cytokines to induce apoptosis by activating their receptor p75 (p75NTR).\textsuperscript{10–13} During myocardial IRI, the upregulated pro-NTs in cardiomyocytes and vascular smooth muscle cells may activate their receptor p75NTR in microvascular pericytes, resulting in MVD.\textsuperscript{3} However, no effective strategies have been documented to inhibit this process.

Recent evidence suggests that the soluble ectodomain of p75NTR (p75ECD) may act as a neurotoxin scavenger to block pro-NTs-mediated neurite collapse in mice with Alzheimer disease and frontotemporal lobar degeneration.\textsuperscript{14–16} Nonetheless, whether p75ECD can serve as an endogenous bioactive factor to reduce myocardial IRI remains unknown and what the underlying cellular and molecular mechanisms are remain blurred. The current study adopted a 2-experiment strategy (an in vivo rodent myocardial IRI model and an in vitro simulative IRI model with pericyte hypoxia–reoxygenation and pro-NTs stimulation) to investigate the role of p75ECD in the cardioprotection and pericyte protection and that of p-JNK (phospho of c-Jun N-terminal kinase)/caspase-3 pathway as the downstream effector of p75NTR. It attempted to present a novel practical strategy to protect hearts against myocardial IRI.

**CLINICAL PERSPECTIVE**

What Is New?

- As a bioactive factor, p75ECD (ectodomain of p75 neurotrophin receptor), if applied after an ischemic event (posttreatment), may attenuate the myocardial ischemia–reperfusion injury in rats by inhibiting the p-JNK (phospho of c-Jun N-terminal kinase)/caspase-3 pathway of microvascular pericytes through scavenging pro-NTs (precursor of neurotrophins).

What Are the Clinical Implications?

- The injection of p75ECD can be performed after the occurrence of myocardial infarction, suggesting strong maneuverability and broad clinical application prospects.
- After myocardial ischemia, pro-neurotrophins and their receptor (p75NTR [p75 neurotrophin receptor]) may serve as the targets for repairing myocardial injury, providing new therapeutic insights into the treatment of myocardial injury.

**Nonstandard Abbreviations and Acronyms**

| Abbreviation | Definition                          |
|--------------|------------------------------------|
| BDNF         | brain-derived growth factor         |
| H/R          | hypoxia–reoxygenation               |
| IS           | infarct size                        |
| IRI          | ischemia–reperfusion injury        |
| JNK          | c-Jun N-terminal kinase             |
| MVD          | microvascular dysfunction           |
| p-JNK        | phospho of c-Jun N-terminal kinase  |
| pro-BDNF     | precursor of brain-derived growth factor |
| pro-NGF      | precursor of nerve growth factor    |
| pro-NTs      | precursor of neurotrophins          |
| p75ECD       | the ectodomain of p75NTR            |
| p75NTR       | p75 neurotrophin receptor           |

Methods

The data, analytic methods, and study materials will be made available to other researchers from the corresponding authors upon request, for purposes of reproducing the results or replicating the procedure.

**Animals, Cultured Cells, and Experimental Protocols**

A total of 88 male Sprague-Dawley rats weighing 200 to 220 g (8 weeks) (Beijing Huafukang Bioscience Co., Ltd., China) were enrolled in the study. The in vivo myocardial IRI model was established by occluding the left main coronary arteries for 45 minutes to induce ischemia (I) and subsequently relaxing them to achieve reperfusion (R).\textsuperscript{17,18} Seventy-two rats were randomly divided into 6 groups (Figure S1A): (1) Sham group, undergoing operation and snare placement without ligation; (2) I/R group, receiving I for 45 minutes and a subsequent R; (3–5) p75ECD groups, undergoing the same treatment as the I/R group and receiving a tail-vein injection of p75ECD (1, 3, or 9 mg/kg, respectively), a p75NTR extracellular domain-human IgG Fc fusion
protein, 5 minutes before R (posttreatment); and (6) p75ECD+SP600125 group, receiving I for 45 minutes and a subsequent R, and a tail-vein injection of 3 mg/kg p75ECD and 20 mg/kg SP600125 (JNK pathway inhibitor, MCE, USA) 5 minutes before R. Eight animals from each group received a 24-hour R. In the Sham, I/R, and 3 mg/kg p75ECD groups, another 8 animals received a 28-day R. To test the effects of SP600125 alone on myocardial IRI, an additional 16 wild-type rats were divided into 2 groups (n=8): I/R group, receiving I for 45 minutes and a subsequent 24-hour R; SP600125 group, receiving I for 45 minutes and a subsequent 24-hour R, and a tail-vein injection of 20 mg/kg SP600125. All animal experiments described in this article were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Committee of Experimental Animal Care of the Affiliated Union Hospital, Fujian Medical University.

Microvascular pericytes were used in vitro to simulate the myocardial IRI microenvironment, with a combination of hypoxia/reoxygenation (H/R) (2/6-hour) and pro-NTs stimulation at R.19 The in vitro study consisted of 5 groups (Figure S1B): Control group, normally cultured cells without hypoxia; H/R group, undergoing H for 2 hours and a sequent R for 6 hours; pro-NTs group (H/R combined with pro-NTs) to simulate IRI in vitro, receiving the same treatment as the H/R group and additional 3 nmol/L pro-NTs (including pro-NGF and pro-BDNF) (Alomone Labs, Israel) at R; pro-NTs+p75ECD group, receiving the same treatment as the pro-NTs group and additional 3 μg/mL p75ECD at R; pro-NTs+p75ECD+SP600125 group, receiving the same treatment as the pro-NTs+p75ECD group and additional 20 μmol/L SP600125 at R. The optimal concentrations of pro-NTs (3 nmol/L) and p75ECD (3 μg/mL) were determined by pilot dose-dependent survival studies as shown in Figures S2 and S3, respectively.

**Statistical Analysis**

All data were presented as mean±SD. Statistical analysis was performed using SPSS 17.0 for Windows. To compare multiple groups, data were analyzed by ANOVA. When a statistical difference appeared, the least significant difference procedure was applied. A value of *P*<0.05 was considered statistically significant.

For detailed methods, see Data S1.

**RESULTS**

Exclusion and Mortality

A total of 94 rats were initially enrolled into the study. Among them, 1 died because of an anesthetic accident, 2 during early reperfusion, and 3 during late reperfusion, resulting in a valid body of 88 rats.

**p75ECD Posttreatment In Vivo Reduces the Myocardial Infarct Size at 24 Hours After Reperfusion in Rats**

SD rats were used to measure p75ECD-induced cardioprotection in vivo, and the infarct size (IS) was evaluated at 24 hours after reperfusion (24 hour R) (Figure 1). No visible ischemic and necrotic areas were evident in the Sham group (Figure 2A.1) and no significant differences in area at risk/left ventricle were observed in the other groups (ranging from 49.1% to 51.2%). Compared with the I/R group, p75ECD at a dose of 3 mg/kg, applied 5 minutes before reperfusion (posttreatment), reduced AN/AAR. Data are expressed as mean±SD. *n*=8 rats/group. *P*<0.05, as compared with the I/R group. AAR indicates area at risk; AN, area of necrosis; I/R, ischemia–reperfusion; JNK, c-Jun N-terminal kinase; and p75NTR, p75 neurotrophin receptor.

*Figure 1. The reduced myocardial infarct size by in vivo p75ECD posttreatment at 24 hours after reperfusion in rats.*

SD rats were used to measure p75ECD-induced cardioprotection in vivo, and the infarct size (IS) was evaluated at 24 hours after reperfusion (24 hour R) (Figure 1). No visible ischemic and necrotic areas were evident in the Sham group (Figure 2A.1) and no significant differences in area at risk/left ventricle were observed in the other groups (ranging from 49.1% to 51.2%). Compared with the I/R group, p75ECD at a dose of 3 mg/kg, applied 5 minutes before reperfusion (posttreatment), significantly reduced the IS (area of necrosis/area at risk) (44.9±3.9% versus 34.6±5.7%, *P*<0.05) (Figures 1 and 2A, A.3). The p75ECD-induced reduction of the IS was dose dependent, but a higher dose of 9 mg/kg did not achieve further reduction (34.4±3.3%) when compared with that of 3 mg/kg. Therefore, 3 mg/kg was used in the histological, molecular biological, and echocardiographic studies. The results suggest that the application of p75ECD before reperfusion (posttreatment) can reduce reperfusion injury (the myocardial IS at 24 hour R as an indicator).
Echocardiography was further used to evaluate cardiac function, and collagen-specific Masson’s trichrome was used to detect myocardial fibrosis at 28 days after reperfusion (Figure 2B through 2F). Compared with the Sham group, cardiac function in the I/R group was significantly decreased (left ventricular ejection fraction, 61.9±7.6% versus 31.0±1.6%, \( P < 0.01 \); left ventricular fractional shortening, 35.1±5.6% versus 15.6±0.9%, \( P < 0.01 \)); the left ventricle was significantly enlarged (left ventricular end-diastolic diameter, 8.8±0.6 mm versus 11.4±0.5 mm, \( P < 0.01 \); left ventricular end-systolic diameter, 5.8±0.8 mm versus 9.6±0.4 mm, \( P < 0.01 \)). Compared with the I/R group, p75ECD posttreatment at a dose of 3 mg/kg significantly improved the cardiac function (left ventricular ejection fraction, 43.1±5.2%, \( P < 0.01 \); left ventricular fractional shortening, 22.5±3.2%, \( P < 0.05 \)) and reduced the left ventricular enlargement (left ventricular end-diastolic diameter, 10.0±0.3 mm, \( P < 0.01 \); left ventricular end-systolic diameter, 7.7±0.5 mm, \( P < 0.01 \)) (Figure 2B, 2D, and 2E). No visible myocardial fibrosis was evident in the Sham group (Figure 2C). Compared with the I/R group,
the fibrosis area was significantly reduced in the p75ECD group (34.3±3.2% versus 21.4±2.3%, \( P < 0.01 \)) (Figure 2F). The results suggest that the reduction of the myocardial IS during an early reperfusion (24 hours R) may further lead to a reduction in late (28 days R) myocardial fibrosis and the left ventricular remodeling, improving cardiac function.

**p75ECD Posttreatment In Vivo Alleviates Myocardial IRI-Induced Microvascular Dysfunction at 28 Days After Reperfusion in Rats**

Pericytes play an important role in regulating microvascular function. To evaluate the effects of p75ECD on microvascular injury in vivo, the coverage of pericytes/endothelial cells and microvascular leakage was detected by immunofluorescence staining at 28 days R (Figure 3). Positive cells of platelet-derived growth factor receptor-\( \beta \)/CD31 co-expression were located in microvasculature of hearts (Figure 3A). Compared with the Sham group, ischemia–reperfusion (the I/R group) resulted in a significant reduction in the ratio of platelet-derived growth factor receptor-\( \beta \)+ (a marker of pericytes)/CD31+ (a marker of endothelial cells) cells (Figure 3A and 3D), and a significant increase in the area with CD68+ (a marker of macrophages) and ICAM-2+ (Figure 3B, 3E and 3C, 3F), suggesting an increase in the microvascular leakage. The microvascular dysfunction was reduced

![Figure 3](image-url)
by the post-treatment of p75ECD at a dose of 3 mg/kg (the p75ECD group) (Figure 3A through 3F).

p75ECD Posttreatment In Vivo Reduces the Myocardial Expression of Pro-NTs (Pro-NGF and Pro-BDNF) at 24 Hours and 28 Days After Reperfusion in Rats

To evaluate the effect of p75ECD on harmful pro-NTs, their protein expression (pro-NGF and pro-BDNF) in reperfused hearts was detected by Western blotting (Figure 4). At 24 hours and 28 days after reperfusion, compared with the Sham group, ischemia–reperfusion (the I/R group) resulted in a significant increase in the protein expression of pro-NGF and pro-BDNF. The effect was blocked by p75ECD posttreatment at a dose of 3 mg/kg (the p75ECD group) (Figure 4). These results indicate that pro-NTs are produced in vivo by reperfused hearts and dislodged by p75ECD, which was confirmed by a subsequent in vitro evaluation.

p75ECD Posttreatment In Vitro Inhibits H/R- and Pro-NTs-Induced Cell Death and Apoptosis in Microvascular Pericytes

To further evaluate the effects of p75ECD on pro-NTs and microvascular injury in vitro, microvascular pericytes were cultured with a combined stimulation of H/R and pro-NTs to simulate myocardial IRI. Pericyte viability was analyzed by methyl thiazolyl tetrazolium assay and apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling immunofluorescence staining (Figure 5). As shown in Figure 5A, consistent with the in vivo myocardial IRI study, the in vitro H/R study demonstrated that compared with the Control group, the survival of microvascular pericytes was reduced by hypoxia-reoxygenation (the H/R group), significantly enhanced by pro-NTs stimulation (the pro-NTs group), but markedly abolished by p75ECD posttreatment (the proNTs+p75ECD group). As shown in Figure 5B, the apoptosis of pericytes was also increased by H/R, significantly enhanced by pro-NTs, but abolished by p75ECD. These results demonstrate the inhibitive effects of p75ECD on pro-NTs and its protective effects on microvascular pericytes.

p-JNK/Caspase-3 Pathway Is Involved in p75ECD Posttreatment-Induced In Vivo and In Vitro Cardioprotection

Compared with the I/R group, SP600125 (the inhibitor of JNK pathway) alone reduced the IS (44.8±4.1% versus 35.7±2.6%, P<0.05). Moreover, as shown in Figures 1 and 5, SP600125 did not alter p75ECD-induced cardioprotection in vivo and in vitro, suggesting that p75ECD may block the JNK pathway to reduce the IS and protect pericytes. Furthermore, the protein expression of p-JNK/Caspase-3 was detected

Figure 4. Reduced protein expression of pro-NTs (pro-NGF and pro-BDNF) at 24 hours and 28 days after reperfusion by in vivo p75ECD posttreatment in rats. Western blotting was used to detect the protein expression of pro-NTs (pro-NGF and pro-BDNF) in hearts with I/R. Representative graphs for pro-NGF and pro-BDNF protein expression are shown in (A.1 and B.1). Quantitative Western blotting demonstrated that in vivo p75ECD posttreatment inhibited I/R-induced upregulation of pro-NGF and pro-BDNF (A.2 and A.3 for 24 hours R, B.2 and B.3 for 28 days R). Data are expressed as means±SD, n=8 rat/group. *P<0.01, as compared with the I/R group. I/R indicates ischemia–reperfusion; pro-BDNF, the precursors of brain-derived growth factor; p75ECD, ectodomain of pro-NTs receptor p75NTR; pro-NGF, the precursors of nerve growth factor; pro-NTs, the precursors of neurotrophins; R, reperfusion.
The inhibited in vitro simulative myocardial IRI-induced cell death and apoptosis of microvascular pericytes by p75ECD posttreatment.

Pericyte viability was analyzed by MTT and apoptosis by TUNEL immunofluorescence staining. A, Bar graphs represent pericyte viability (survival) relative to the Control group by MTT. B, Bar graphs represent the apoptosis ratio, a percentage of the occurrence of TUNEL-positive nuclei over the total nuclei by fluorescence microscopy. C, Representative confocal fluorescence micrographs. The nuclei were stained blue by DAPI, and TUNEL-positive pericytes were stained green, and then they were shown as merge (white arrows). Data are expressed as means±SD, n=5/group. *P<0.01, as compared with the Control group; †P<0.01, as compared with the H/R group; ‡P<0.01, as compared with the pro-NTs group. DAPI indicates 4,6-diamidino-2-phenylindole; H/R, hypoxia–reoxygenation; IRI, ischemia–reperfusion injury; MTT, methyl thiazolyl tetrazolium assay; pro-NTs, pro-neurotrophins; p75ECD, ectodomain of pro-NTs receptor p75NTR; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling.

Similarly, SP600125 (the pro-NTs+p75ECD+SP600125 group) failed to enhance the effect of p75ECD on p-JNK/caspase-3 pathway (Figure 6C.1 through C.3). These in vivo and in vitro results demonstrate that p75ECD may confer cardioprotection by blocking pro-NTs and the p-JNK/caspase-3 pathway of microvascular pericytes.

**p75ECD Posttreatment In Vivo Does Not Reduce Protein Expression of p75NTR in Reperfused Rat Hearts**

Western blotting was conducted to detect the protein expression of p75NTR in rat hearts and microvascular pericytes. As shown in Figure S4A and S4B, at in vivo 24 hours and 28 days after reperfusion,
compared with the Sham group, the protein expression of p75NTR from reperfused hearts was significantly upregulated by ischemia–reperfusion (the I/R group). The in vitro experiment further confirmed that in cultured microvascular pericytes, the protein expression of p75NTR was also upregulated by hypoxia–reoxygenation stimulation (Figure S4C). However, the upregulated p75NTR was not downregulated by in vivo p75ECD posttreatment (the p75ECD group) at 24 hours after reperfusion (Figure S4A), suggesting that p75ECD cannot normalize p75NTR upregulation after the myocardial infarction and that p75NTR may have a longer impact on the cardiac remodeling.

DISCUSSION

The current study, using 2 experiments (in vivo rat myocardial IRI model and in vitro simulative IRI model in cultured rat microvascular pericytes by H/R combined with pro-NTs stimulation), first demonstrated that the recombinant extracellular domain of p75NTR
(p75ECD) fused with Fc fragment of immunoglobulin G (p75ECD-Fc), applied after an ischemic event (before reperfusion or at reoxygenation) (posttreatment), may be cardioprotective by inhibiting the p-JNK/caspase-3 pathway of microvascular pericytes through scavenging pro-NTs.

Myocardial IRI may lead to MVD with impaired heart function. The NTs are a family of growth factors that exert beneficial effects on the cardiovascular system, and may serve as a therapeutic target for heart repair. Previous strategies including NGF gene delivery and recombinant NGF-based experimental therapy were hindered by undefined effects, safety concerns, and side effects. Recently, pro-NGF has been identified as a new target to reduce MVD. However, effective strategies have not yet been developed.

p75NTR activation is subject to regulated intramembrane proteolysis, and the cleavage of regulated intramembrane proteolysis is taken over by the metalloproteinase ADAM17/TACE that leaves a membrane-bound COOH-terminal fragment and liberates the extracellular domain (p75ECD), a bioactive factor after the cleavage of the full-length p75NTR by α-secretase TACE. More recently, p75ECD as the extracellular domain of p75NTR has been demonstrated to be physiologically neuroprotective by scavenging pro-NTs in mice. However, the effect of p75ECD on cardiac repair remains unknown. Given the above evidence, it is reasonable to assume that p75ECD may exert cardioprotection against myocardial IRI, as pro-NGF, an apoptotic ligand of p75NTR, is upregulated after cardiac infarction in animals. As a bioactive factor, p75ECD may serve as a novel, safe, and effective candidate to protect hearts from IRI.

In this study, we designed a 2-experiment strategy to confirm the involvement of pro-NTs and MVD in p75ECD-induced cardioprotection. An in vivo rat myocardial IRI model was routinely established. Then, to independently evaluate the effect of p75ECD on pericytes, pro-NTs, and p75NTR, we developed an in vitro simulative myocardial IRI model with rat microvascular pericytes through a combined scheme of H/R and pro-NTs stimulation. This novel model is based on the following considerations: pro-NTs are mainly upregulated by cardiomyocytes and vascular smooth muscle cells, and p75NTR as the pro-NTs receptor is not expressed in healthy or diseased cardiomyocytes, but mainly expressed in microvascular pericytes in response to ischemic stimulus. The combination of H/R and pro-NTs stimulation may better evaluate the effects of p75ECD on MVD and the signaling pathways involved, and the potential effects of pro-NTs. Moreover, in comparison with the use before ischemia, it is clinically more feasible to administer p75ECD in vivo after the onset of ischemia 5 minutes before reperfusion (posttreatment) because of the predictability of reperfusion events.

In this study, for the first time, in an in vivo rat model, myocardial infarct–sparing effects of p75ECD at different doses were evaluated 5 minutes before reperfusion. We found that, to protect hearts against in vivo IRI, 3 mg/kg of p75ECD may be a minimal effective dose. Here, at 24 hours R, a myocardial infarct–sparing effect was achieved, when the area of necrosis was primarily measured. Meanwhile, the protein expressions of pro-NGF, pro-BDNF, and p75NTR were simultaneously upregulated by I/R stimulation, and pro-NGF and pro-BDNF, but not p75NTR, were downregulated by p75ECD. Together with the fact that in vitro H/R-induced pericyte injury was enhanced by pro-NTs, in this study, p75ECD may suppress the activation of p75NTR pathway, without affecting the protein expression of p75NTR, to reduce myocardial IRI by scavenging pro-NTs and/or by competitively binding p75NTR to replace pro-NTs. Thus, the upregulation of pro-NTs may not play a major role in p75NTR expression, since an ischemic event is a primary factor triggering the up-regulation of p75NTR.

Consistent with the in vivo study, the in vitro study showed that H/R also induced p75NTR expression in microvascular pericytes, which is consistent with the previous study. p75NTR-expressing pericytes have been identified as the cell type activated by pro-NGF, and may serve as a major cellular effector for pro-NTs-mediated pathogenesis. A previous study has demonstrated that the IS is not different at 24 hours R in p75NTR−/− mice when compared with that of the wild-type mice, suggesting that p75NTR is not directly involved in the necrotic lesion but plays a role in infarct expansion via microvascular injury. Two mechanisms can explain p75ECD-induced chronic cardioprotection (the improved cardiac function and attenuated left ventricular remodeling) at 28 days R: for one, p75ECD may block microvascular injury–induced infarct expansion by scavenging pro-NTs, as p75ECD has a long half-time of about 7 days in vivo; for another, reduced myocardial IS at early reperfusion (24 hours) may attenuate the left ventricular remodeling, therefore improving the cardiac function at a later reperfusion (28 days).

Pericytes, which are mural cells of the microcirculation that wrap around the endothelial cells, play an important role in regulating microvascular permeability. The survival of microvascular endothelial cells depends on a reciprocal interaction with adjacent pericytes, and contraction of the pericytes following ischemic injury will lead to a loss of nutritional support of microvascular endothelium, thus increasing vascular permeability and leading to MVD and in return exacerbating myocardial IRI. Siao et al have demonstrated...
that the pro-NGF-expressing mouse may activate cardiac microvascular endothelium, decrease pericyte process length, and increase vascular permeability, with platelet-derived growth factor receptor-β-positive pericytes as the target of cardiomyocyte-derived pro-NGF. In this study, we focused on the role of pericytes. The reduced ratio of platelet-derived growth factor receptor-β-positive pericytes/CD31-positive endothelial cells by in vivo I/R stimulation may indicate an injury and loss of pericytes, which may lead to MVD. This is also confirmed by the in vitro experiment, in which H/R led to an increase in pericyte death and apoptosis, which was aggravated by pro-NTs. In the in vivo microenvironment, the pro-NTs released from cardiomyocytes may act in a paracrine manner to modulate microvascular function. Thus, the in vitro model used in this study closely mimics the in vivo microenvironment by simulating pericyte injury via a combined scheme of H/R and pro-NTs stimulation.

In the myocardial IRI model, some studies have observed that pro-NGF promotes myocardial inflammation by recruiting leukocytes, endothelial activation, and extracellular matrix deposition. In this study, p75ECD in vivo preserved the microvascular dysfunction by increasing the coverage of pericytes/endothelia and reducing microvascular leakage with fewer inflammatory CD68-positive macrophages and less ICAM-2 infiltrating into the ischemic region. A similar pattern was found in the in vitro experiment, where p75ECD inhibited cell death and apoptosis induced by H/R and pro-NTs in cultured pericytes. The current study demonstrates that during H/R, pro-NTs may induce apoptosis and reduce the survival of pericytes. Thus, p75ECD may protect pericytes from pro-NTs-induced inflammation and injury in vitro. In this study, given the upregulation of p75NTR in cultured H/R pericytes and in reperfused hearts, scavenging pro-NTs by p75ECD and suppression of the p75NTR signaling pathway may be involved in the cardioprotection.

It has been well established that mature neurotrophins bind to Trk alone or complexed with p75NTR for prosurvival signals, while pro-NGF and pro-BDNF bind exclusively to a complex of p75NTR with sortilin and trigger apoptotic events. p75NTR can induce apoptosis in the absence of a ligand. The p75NTR signaling upon binding of pro-NTs, which has been extensively studied in neurons, ultimately results in the activation of JNK and caspase-3 and can lead to neuronal death. It has also been reported that by binding to and activating p75NTR, pro-NTs recruit signaling proteins via ligands, including TRAF6, RIP2, and TRADD, and trigger downstream signals, including nuclear factor kappa-B, finally leading to the activation of JNK and caspase-3 and subsequent neuronal death.

The in vivo and in vitro experiments of the current study support the hypothesis that after ischemia, cardiomyocyte-derived pro-NTs, acting on p75NTR, which is upregulated in pericytes, cause pericyte injury and MVD. Furthermore, the downstream signaling proteins JNK/caspase-3 of p75NTR were detected, and SP600125, an inhibitor of JNK, was used in this study. In fact, it has been demonstrated that SP600125 alone can reduce myocardial IS. In this study, SP600125 alone reduced the IS and did not alter p75ECD-induced cardioprotection (by reducing myocardial IS in vivo and improving the survival of pericytes with reduced apoptosis in vitro), suggesting that p75ECD may block the activation of JNK/caspase pathway to reduce the IS and protect pericytes. This was further confirmed by the fact that SP600125 failed in vivo and in vitro to enhance the effect of p75ECD on the activation of the p-JNK/caspase-3 pathway. These in vivo and in vitro results demonstrate that p75ECD may confer cardioprotection by blocking pro-NTs and p-JNK/caspase-3 pathway of pericytes. More recently, the activation of p-JNK/caspase-3 is also involved in pro-BDNF-induced H/R injury in myocardial microvascular endothelial cells. Thus, increased pro-NTs in response to ischemia/hypoxia can activate JNK/caspase-3 to cause cell death and tissue damage. p75ECD only blocks pro-NTs/p75NTR signaling to protect cells from injury, likely via blocking JNK/caspase-3 signals, and does not affect the expression of p75NTR.

There were some limitations in our study. First, the study did not adopt p75NTR knockout animals and the evidence for the involvement of JNK/caspase 3 is indirect, despite the use of SP600125, a JNK signaling pathway inhibitor. Second, no signaling pathways other than JNK/caspase were detected in this study. Third, p75NTR was not detected more than 28 days after myocardial IRI in this study. Finally, we did not measure the area of the rat heart without backflow in vivo, nor did we examine in vivo the roles of other cells such as microvascular endothelial cells and smooth muscle cells.

In conclusion, we established an in vitro simulative IRI model in cultured rat microvascular pericytes by a combined scheme of H/R and pro-NTs stimulation, and first demonstrate that p75ECD as a bioactive factor, applied after an ischemic event (posttreatment), may attenuate the myocardial IRI in rats by inhibiting the p-JNK/caspase-3 pathway of microvascular pericytes through scavenging pro-NTs. It provides a novel, safe, and effective strategy for cardioprotection from IRI.
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Disclosures

None.

Supplementary Materials

Data S1

Figures S1–S4

References 3, 17, 18, and 36–38

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SUPPLEMENTAL MATERIAL
Data S1. Supplementary Methods

Animals, cultured cells and experimental protocols

This study enrolled a total of 88 male Sprague-Dawley rats weighed 200-220 g (8 weeks) (Beijing Huafukang Bioscience Co., Ltd., China). The in vivo myocardial IRI model was established by occluding the left main coronary arteries for 45 min to induce ischemia (I) and subsequently relaxing them to achieve reperfusion (R) [17, 18]. Seventy-two rats were randomly divided into 6 groups (Figure S1A): (1) Sham group, undergoing operation and snare placement without ligation; (2) I/R group, receiving I for 45 min and a subsequent R; (3)-(5) p75ECD groups, undergoing the same treatment as the I/R and receiving a tail-vein injection of p75ECD, a p75NTR extracellular domain-human IgG Fc fusion protein (Tiantai Medicine Technology Co., Ltd. China) with 1, 3, or 9 mg/kg 5 min before R (post-treatment); (6) p75ECD+SP600125 group, receiving I for 45 min and a subsequent R, and a tail-vein injection of 3 mg/kg p75ECD and 20 mg/kg SP600125 (JNK pathway inhibitor, MCE, USA) 5 min before R. Eight animals from each group received a 24-h R. In the Sham, I/R, and 3 mg/kg p75ECD groups, another eight animals received a 28-d R. To test the effects of SP600125 alone on myocardial IRI, additional 16 WT rats were divided into two groups (n = 8): I/R group, receiving I for 45 min and a subsequent 24-h R; SP600125 group, receiving I for 45 min and a subsequent 24-h R, and a tail-vein injection of 20 mg/kg SP600125. All animal experiments described in this article were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Committee of Experimental Animal Care of the Affiliated Union Hospital, Fujian Medical University.

Since pro-NTs were mainly up-regulated by cardiomyocytes and vascular smooth muscle
cells, and p75NTR by microvascular pericytes during in vivo myocardial IRI [3], we used microvascular pericytes in vitro to simulate myocardial IRI microenvironment, with a combination of hypoxia/reoxygenation (H/R) (2 h/6 h) and pro-NTs stimulation at R. As shown in Fig S1B, the in vitro study consisted of 5 groups: Control group, normal cultured cells without hypoxia; H/R group, subject to H for 2 h and followed by R for 6 h; pro-NTs group (H/R combined with pro-NTs) to in vitro simulate IRI, receiving the same treatment as the H/R group and addition of 3 nmol/L pro-NTs (including pro-NGF and pro-BDNF) (Alomone Labs, Israel) at R; pro-NTs+p75ECD group, receiving the same treatment as the pro-NTs group and addition of 3 μg/ml p75ECD at R; pro-NTs+ p75ECD+ SP600125 group, receiving the same treatment as pro-NTs+ p75ECD group and addition of 20 μmol/L SP600125 at R. The optimal concentrations of pro-NTs (3 nmol/L) and p75ECD (3 μg/ml) were determined by pilot dose-dependent survival studies as shown in Fig S2 and Fig S3, respectively.

**Myocardial ischemia-reperfusion and pericycle hypoxia-reoxygenation models**

To establish the in vivo model of myocardial ischemia-reperfusion (I/R), rats were intraperitoneally anaesthetized with 75 mg/kg ketamine and 7.5 mg/kg diazepam before surgery and ventilated by a rodent ventilator (tidal volume, 1.0 ml/100 g body weight; respiratory rate, 50-60 cycles per minute). The chest was opened via a left thoracotomy through the fourth intercostal space to expose the heart. After pericardiotomy, a silk 7-0 suture was placed under the left main coronary artery and the ends of the tie were threaded through small polyethylene tubes to form snares for reversible artery occlusion [36]. At 24 h or 28 d R, rats were sacrificed by an intravenous bolus injection of 10 % KCl under deep anesthesia. In each group, at 24 h R, hearts from 8 rats were used to assess the area at risk by injecting 1 ml of
0.1 % Evan’s blue (Sigma, USA) after the left main coronary artery was ligated again. Then the heart tissues from the apex were stored at -80 °C for assessment of histological studies and western blotting. The rest of the heart was cut transversely into three slices parallel to the atrioventricular groove at a thickness of 1-2 mm for 2, 3, 5-triphenyltetrazolium chloride staining. In Sham, Control, and 3 mg/kg p75ECD groups, at 28 d R, another 8 rats were used for echocardiographic assessment. The hearts were harvested and stored at -80 °C for histological studies and western blotting.

To establish in vitro hypoxia/reoxygenation model, rat primary microvascular pericytes (Procell Life Science and Technology Co., Ltd. China) were cultured in a growth medium of 1:1 mixture of DMEM/Ham’s F12 added with 10% fetal bovine serum, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 1% antibiotic-antimycotic mixture in a 5% CO₂ humified incubator at 37 °C. In a hypoxia chamber (MIC-101, Billups-rothenberg, USA), 95% N₂ and 5% CO₂ were added to induce hypoxia for 2 h, then reoxygenated with normal gas for 6 h [37]. At an end concentration of 3 nmol/L, pro-NTs (including pro-NGF and pro-BDNF) were added at R. At the end of culture, pericytes were collected for cell viability assessment, histological studies and western blotting.

**Determination of area at risk and the infarct size**

The heart slices from 24 h R were incubated at 37 °C in 1 % pH 7.4 triphenyltetrazolium chloride (Sigma, USA) for 15 min, fixed in 10 % formaldehyde solution for 24 h and photographed, and quantified by planimetry using ImageJ 1.43 software (NIH) in a blind fashion. Area at risk (AAR) was composed of the viable ischemic and necrotic area, and expressed as a percentage of the left ventricle (AAR/LV). Area of necrosis (AN) (infarct size,
IS) was expressed as a percentage of AAR (AN/AAR).

**Determination of the cardiac function by echocardiography**

Cardiac function was blindly assessed by transthoracic echocardiography at 28 d R. Short-axis 2-dimensional view of the left ventricle was taken at the level of the papillary muscles to obtain the M-mode recordings with a 17.5 MHz electronic-phased-array transducer (Vevo-770, Visualsonics, CA). The left ventricular end-systolic diameter (LVESd) and end-diastolic diameter (LVEDd) were measured by averaging the measurements from 3 continuous cardiac cycles. The left ventricular fractional shortening (LVFS) (%) was calculated as (LVEDd-LVESd)/ LVEDd×100; end-diastolic volume (LVEDv) as 7.0×LVEDd³/ (2.4+ LVEDd); end-systolic volume (LVESv) as 7.0×LVESd³/ (2.4+LVESd); and ejection fraction (LVEF) (%) as (LVEDv-LVESv)/ LVEDv×100 [38].

**Analysis of in vitro pericyte viability by MTT and apoptosis by TUNEL**

Cell viability was measured by methyl thiazolyl tetrazolium (MTT) assay (Xin fan Biotechnology Co., Ltd. China). Briefly, at the end of hypoxia-reoxygenation, MTT diluted in PBS (0.5mg/ml) was added to the culture medium and the cells were sequentially cultured at 37 °C for 4 hours, and then insoluble purple formazan crystals produced by mitochondrial dehydrogenases in viable cells were dissolved for 15 min at 37°C in 100 µl of a 10% (w/v) Dimethyl sulfoxide solution in 0.01 mol/L HCl. Optical density was measured at 490 nm with a microplate reader (Bio-Rad, Thermo, USA). The experiment was repeated three times.

TUNEL Assay Kit (Calbiochem, Germany) was used to analyze apoptosis. Briefly, Pericytes on slides were washed 3 times with PBS for 5 min and permeabilized with 0.1% (v/v) Triton X-100 for 2 min on ice. Samples were incubated in 50 µl of TUNEL reaction mixture at 37 °C
for 1 h in a dark humidified atmosphere. After 3 washes with PBS, apoptotic pericytes were observed under a fluorescence microscope using 488 nm excitation filters by counting the positive nuclei and negative stained cell in each of the random five fields of vision. The apoptosis ratio, a percentage of the occurrence of TUNEL-positive nuclei over the total nuclei, was used to evaluate the extent of apoptosis.

**Histological studies**

The coverage of pericytes/endothelial cells and microvascular leakage were detected at 28 d R by immunofluorescence staining [3]. Platelet-derived growth factor receptor (PDGFR)-β as a marker of pericytes, and cluster of differentiation 31 (CD31), were used to evaluate the coverage of pericytes/endothelial cells. Intercellular adhesion molecule-2 (ICAM-2) combined with CD68, a marker of macrophages, was used to evaluate microvascular leakage. In brief, frozen sections were incubated with primary antibodies (anti-CD31, anti-PDGFR-β, anti-CD68 and anti-ICAM-2 from Abcam, UK) and secondary antibody (IgG-Texas-Red for CD31 and ICAM-2; FIFC for PDGFR-β and CD68 ), and the nuclei were stained with 4, 6-diamino-2-phenylindole (DAPI). For each heart, 3 non-successive sections were analyzed under a laser scanning confocal microscope (LSM510, Zeiss, Japan). For each section, 5 high-power fields were randomly selected for analysis. The coverage of pericyte/endothelial cells was expressed as the ratio of PDGFR-β-positive cells/CD31-positive cells. The microvascular leakage was expressed as the CD68-positive and ICAM-2-positive area (pixel). The quantification analysis was performed by two observers blinded to the treatment of the animals.

Myocardial fibrosis at 28 d R was observed by collagen-specific Masson's trichrome staining according to the manufacturer’s instructions (Xin Fan Biotechnology Co., Ltd. China). Normal
myocardium is red, nuclei black, and collagen matrix (fibrosis area) blue. Quantification of myocardial fibrosis was performed using ImageJ 1.43 software (NIH). The area of fibrosis (blue pixel) was measured and normalized to the total area of the left ventricle.

**Western blotting**

The protein expressions of pro-NGF, pro-BDNF, p75NTR, JNK and caspase-3 in reperfused myocardium and cultured pericytes were assessed by western blotting. A volume of 10 μl of heart or pericyte extracts mixed with an appropriate volume of loading sample buffer was loaded and electrophoresed on 10% SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes, followed by incubation with anti-pro-NGF (Merck Millipore, Germany), anti-pro-BDNF (Merck Millipore, Germany), anti-p75NTR (Abcam, UK), anti-T-JNK (Abcam, UK), anti-P-JNK (Abcam, UK), anti-caspase-3 (Abcam, UK) and subsequently with secondary antibodies, and scanned by Odyssey fluorescent scanner. β-actin was used as an internal control. Immunoreactive bands were detected by enhanced chemiluminescence plus reagent (Amersham). Target signals were normalized relative to the β-actin expression and assessed by ImageJ 1.43 software (NIH).
**Figure S1**

| A) In vivo rat experiment | B) In vitro cell experiment |
|--------------------------|-----------------------------|
| **Strategies**           | **Control**                 |
|                         | **Cultured 8 h under normoxia** |
| I/R                     | H/R                         |
| p75ECD 1mg/kg           | proNTs                      |
| p75ECD 3mg/kg           | proNTs + p75ECD             |
| p75ECD 9mg/kg           | proNTs + p75ECD + 5P600125  |
| Perfusing 24 h (n=8), 28 d (n=8) | H 2 h, R 6 h, R 0 min       |

**Figure S2**

![Cell Viability Graph](image)
Figure S3

![Figure S3](image)

Figure S4

![Figure S4](image)
Supplemental Legends

Figure S1. Experimental strategies and groups for p75ECD-induced cardioprotection. A: **Strategy 1: in vivo rat experiment:** To establish an *in vivo* model of myocardial ischemia-reperfusion injury (IRI), Sprague-Dawley rats were exposed to ischemia (I, dark bar) by ligating the left main coronary arteries and subsequent reperfusion (R, white bar) by relaxing them. Then animals were divided into six groups. The Sham group was operated on without ligating coronary arteries. Different doses of p75ECD (1, 3, 9 mg/kg) and SP600125 (20 mg/kg an inhibitor of c-Jun N-terminal kinase, JNK) were given by tail vein injection at I 40 min, 5 min before R (arrows). **B:** **Strategy 2: in vitro cell experiment.** To establish an *in vitro* model of hypoxia/reoxygenation, rat microvascular pericytes were exposed to hypoxia (H, dark bar) by adding 95% N₂ and 5% CO₂ and reoxygenation (R, white bar) by normoxia. Then pericytes were divided into five groups. Control was treated under normoxia. Pro-NTs (3 nmol/L of pro-NGF and pro-BDNF, respectively), p75ECD (3 μg/ml) and SP600125 (20 μmol/L) were given at the onset of R (arrows). The pro-NTs group served as an in vitro simulative myocardial IRI model with a combination of H/R and pro-NTs stimulation. pro-NTs, the precursors of neurotrophins; p75ECD, ectodomain of pro-NTs receptor p75NTR. pro-NGF, the precursors of nerve growth factor; pro-BDNF, the precursors of brain derived growth factor.

Figure S2. Determination of the best concentration of pro-NTs. Using rat microvascular pericytes cultured with hypoxia-reoxygenation (H/R) (2 h/6 h), different final concentrations of pro-NTs (0.3, 1, 3, 9 nmol/L pro-NGF and pro-BDNF) were used at reoxygenation to determine the best concentration to simulate *in vitro* IRI model. The Control group was cultured under normoxia, and other groups were subjected to 2-h hypoxia followed by 6-h reoxygenation. Cell viability was measured by methyl thiazolyl tetrazolium assay. Data were normalized against the Control group. The dose of 3nM (nmol/L) was determined as the best
concentration to induce the injury of microvascular pericytes under hypoxia-reoxygenation. pro-NTs, the precursors of neurotrophins; pro-NGF, the precursors of nerve growth factor; pro-BDNF, the precursors of brain derived growth factor. * \( p<0.05 \), as compared with the Control group; \( ^{*} p<0.05 \), as compared with the H/R group; \( ^{\dagger} p<0.05 \), as compared with the 1 nM pro-NTs group.

**Figure S3. Determination of the best concentration of p75ECD.** Using rat microvascular pericytes, in an *in vitro* model with a combination of hypoxia/reoxygenation (H/R) (2 h/6 h) and pro-NTs (3 nmol/L) stimulation at reoxygenation, different final concentrations of p75ECD (0.3, 1, 3, 9 ug/ml) were used at reoxygenation to determine the best concentration to protect microvascular pericytes. The Control group was cultured under normoxia, and other groups underwent 2-h hypoxia followed by 6-h reoxygenation. Cell viability was measured by methyl thiazolyl tetrazolium assay. Data were normalized against the Control group. The dose of 3 ug/ml was determined as the best concentration to confer cytoprotection under hypoxia-reoxygenation. p75ECD, ectodomain of pro-NTs receptor p75NTR; pro-NTs, the precursors of neurotrophins. * \( p<0.05 \), as compared with the Control group; \( ^{*} p<0.05 \), as compared with the H/R group; \( ^{\dagger} p<0.05 \), as compared with the 1 ug/ml p75ECD group.

**Figure S4. Protein expression of pro-NTR in reperfused rat hearts and reoxygenated pericytes.** Western blotting was performed to detect the protein expression of pro-NTR in rat hearts and microvascular pericytes. **A:** Western blotting showing p75NTR protein expression in rat hearts at 24 h after reperfusion. **B:** Western blotting showing p75NTR protein expression in rat hearts at 28 d after reperfusion. **C:** Western blotting showing p75NTR protein expression in cultured microvascular pericytes at 6 h after reoxygenation. The stimulation of ischemia-
reperfusion or hypoxia-reoxygenation significantly upregulated p75NTR protein expression in vivo and in vitro. However, in vivo p75ECD post-treatment did not downregulate it at 24 h after reperfusion. Data are expressed as mean ± SD, n = 8 or 5. * p < 0.01, as compared with the I/R or Control group.