ProNGF, a cytokine induced after myocardial infarction in humans, targets pericytes to promote microvascular damage and activation

Chia-Jen Siao,1 Christina U. Lorentz,1 Pouneh Kermani,1 Tina Marinic,1 John Carter,2 Kelly McGrath,1 Victoria A. Padow,1 Willie Mark,7 Domenick J. Falcone,5 Leona Cohen-Gould,3,4 Diana C. Parrish,6 Beth A. Habecker,6 Anders Nykjaer,8 Lora H. Ellenson,5 Lino Tessarollo,9 and Barbara L. Hempstead1

1Division of Hematology/Medical Oncology and 2Division of Cardiovascular Pathophysiology, Department of Medicine, 
3Department of Biochemistry, 4Department of Cell and Developmental Biology, and 5Department of Pathology, Weill Cornell Medical College, New York, NY 10065 
6Department of Physiology and Pharmacology, Oregon Health and Science University, Portland, OR 97239 
7Developmental Biology Program, Sloan-Kettering Institute, New York, NY 10065 
8Department of Medical Biochemistry, University of Aarhus, Aarhus DK-8000, Denmark 
9Mouse Cancer Genetics Program, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 21702

Treatment of acute cardiac ischemia focuses on reestablishment of blood flow in coronary arteries. However, impaired microvascular perfusion damages peri-infarct tissue, despite arterial patency. Identification of cytokines that induce microvascular dysfunction would provide new targets to limit microvascular damage. Pro–nerve growth factor (NGF), the precursor of NGF, is a well characterized cytokine in the brain induced by injury. ProNGF activates p75 neurotrophin receptor (p75NTR) and sortilin receptors to mediate proapoptotic responses. We describe induction of proNGF by cardiomyocytes, and p75NTR in human arterioles after fatal myocardial infarction, but not with unrelated pathologies. After mouse cardiac ischemia-reperfusion (I–R) injury, rapid up-regulation of proNGF by cardiomyocytes and p75NTR by microvascular pericytes is observed. To identify proNGF actions, we generated a mouse expressing a mutant Ngf allele with impaired processing of proNGF to mature NGF. The proNGF–expressing mouse exhibits cardiac microvascular endothelial activation, a decrease in pericyte process length, and increased vascular permeability, leading to lethal cardiomyopathy in adulthood. Deletion of p75NTR in proNGF–expressing mice rescues the phenotype, confirming the importance of p75NTR-expressing pericytes in the development of microvascular injury. Furthermore, deficiency in p75NTR limits infarct size after I–R. These studies identify novel, nonneuronal actions for proNGF and suggest that proNGF represents a new target to limit microvascular dysfunction.

The primary therapeutic goal after acute myocardial infarction (MI) is to limit the duration of ischemia and to establish reperfusion using angioplasty or thrombolysis. However, even with improved arterial flow, a significant proportion of patients experience microvascular damage that leads to decreased microvascular perfusion and chronically impaired heart function (Eltzschig and Collard, 2004; Bekkers et al., 2010). At this time, the proinflammatory cytokines induced by ischemia that mediate microvascular dysfunction or apoptosis after cardiac ischemia remain largely unknown.

We considered whether nerve growth factor (NGF), and specifically its uncleaved precursor...
ProNGF, could act as a potential proapoptotic and proinflammatory cytokine in the ischemic heart. NGF is initially synthesized as proNGF, which is normally cleaved intracellularly to release mature NGF (Heymach and Shooter, 1995). Mature NGF binds to the TrkA receptor tyrosine kinase to mediate survival and differentiative effects in neurons (Reichardt, 2006). Under pathological conditions, proNGF is secreted and acts as a distinct ligand to promote neuronal apoptosis by binding to the p75 neurotrophin receptor (p75NTR), a member of the tumor necrosis factor receptor family, and the transmembrane receptor sortilin (Lee et al., 2001; Nykjaer et al., 2004). This receptor complex activates stress and apoptotic signaling molecules, such as JNK (c-Jun N-terminal kinase) and caspase-3 (Nykjaer et al., 2005; Jansen et al., 2007; Volosin et al., 2008; Hempstead, 2009).

ProNGF and p75NTR are present at low to undetectable levels in normal, uninjured adult tissues (Fanburg-Smith and Miettinen, 2001; Harrington et al., 2004; Lommatzsch et al., 2005; Hempstead, 2009). However, they are rapidly induced after acute neuronal injury and mediate cell death or degeneration after seizures or axotomy (Harrington et al., 2004; Volosin et al., 2008). In addition, proNGF is up-regulated in neurodegenerative diseases and aging (Pedraza et al., 2005; Jansen et al., 2007). However, ngn1 mRNA is expressed in many organs, and secreted mature NGF promotes sympathetic innervation during development and regulates sympathetic...
tone in the adult (Donovan et al., 1995; Glebova and Ginty, 2005; Habecker et al., 2008). In the adult heart, mature NGF is secreted tonically by cardiac myocytes to modulate synaptic transmission by sympathetic neurons (Luther and Birren, 2006). Additionally, within hours of cardiac ischemia-reperfusion (I-R) injury in rodents, ngf mRNA is induced (Hiltunen et al., 2001), and immunoreactivity to the mature NGF domain increases in human hearts after acute MI (Meloni et al., 2010). These studies, however, do not distinguish whether mature NGF or proNGF is induced after cardiac ischemia. P75NTR expression is also induced in the vasculature after acute injury to the aorta (Donovan et al., 1995), and p75NTR activation promotes vascular smooth muscle and endothelial cell death in vitro (Wang et al., 2000; Kim et al., 2004). Genetic deletion of p75NTR in mice (p75−/−) results in reduced apoptosis of vascular smooth muscle cells after vascular injury, suggesting that locally produced neurotrophins regulate this response (Kraemer, 2002).

The induction of ngf mRNA by cardiomyocytes and of p75NTR by vascular cells after injury suggests a potential paracrine role for NGF or proNGF in modulating vascular integrity. Microvascular endothelial survival depends on reciprocal interactions with neighboring pericytes during development, and pericytes maintain microvascular structure and function in the adult animal (Gaengel et al., 2009). Disruption of endothelial cell–pericyte communication during development leads to vascular hemorrhage and embryonic death, as is readily observed in platelet-derived growth factor B (Pdgfb)− or platelet-derived growth factor receptor β (Pdgfrb)−/− deficient mice, where pericyte recruitment to specific vascular beds is impaired (Lindahl et al., 1997; Hellström et al., 1999; Bjarnegård et al., 2004). In adult mice, TGF-β and bone morphogenetic proteins play critical roles in maintaining pericyte survival and promoting microvascular integrity (El-Bizri et al., 2008; Walsh et al., 2009). These observations suggest that disruption of endothelial cell–pericyte interactions during cardiac microvascular maturation may lead to cardiac dysfunction later in life.

In this study, we examined the expression of proNGF and its receptors, p75NTR and sortilin-related VPS10 domain containing receptor 2 (SorCS2), a sortilin family member (Willnow et al., 2008), in the infarcted myocardium after cardiac ischemia in human autopsy material and in an established mouse model of I-R injury. We observed the induction of proNGF by cardiac myocytes and arterioles, and of p75NTR and SorCS2 by mural cells of arterioles in the peri-infarct region of individuals who died after a recent MI. Comparable induction patterns were observed in mouse tissue after I-R injury. To investigate the consequences of proNGF expression on the cardiac vasculature, we generated and analyzed a knock-in mouse model of I-R injury. We observed the induction of proNGF and its receptors in the human and mouse heart.

RESULTS
Myocardial ischemia induces expression of proNGF and its receptors in the human and mouse heart

To determine whether proNGF induction is observed in extraneural organs after ischemic injury, we examined proNGF immunoreactivity in human heart tissue from patients that succumbed to a fatal MI as compared with heart tissue from individuals that died of nonatherosclerotic causes. Using antisera specific to the prodomain of proNGF that detects proNGF but not mature NGF (Harrington et al., 2004), we rescued in mice that are deficient in p75NTR. Furthermore, deficiency in p75NTR limits the infarct size expansion after myocardial I-R injury, when compared with infarct size in wild-type mice. These observations identify proNGF and p75NTR as potential therapeutic targets to limit microvascular dysfunction in the ischemic heart.

![Figure 2](image-url)

Figure 2. P75NTR and SorCS2, to which proNGF also binds, are up-regulated in PDGFR-β+ pericytes after I-R in mice. (A) Immunoprecipitation–Western blot analysis from 293T cells transfected with the indicated plasmids. The experiment was performed three independent times. (B) p75NTR (red) expression on PDGFR-β+ (green) pericytes in the peri-infarct regions of injured hearts compared with pericytes of uninjured myocardium. (C) SorCS2 (red) expression in a population of pericytes coexpressing PDGFR-β (green) in the injured myocardium after I-R. (B and C) Bar, 25 µm. n = 3 mice/group.
observed increased proNGF immunoreactivity in cardiac myocytes in the peri-infarct region (Fig. 1, A [right] and C) but not in remote areas in hearts from patients with recent MI (not depicted). Little to no proNGF immunoreactivity was detected in the hearts of patients that died of noncardiac causes (Fig. 1, A [left] and C). Anti-proNGF immunoreactivity was also observed in the smooth muscle layer of most arterioles in the peri-infarct region from patients that had experienced a fatal MI but not in heart sections from noncardiac patients (Fig. 1, A [arrow] and D). ProNGF immunoreactivity was not observed in the mural wall of veins or venules (Fig. 1 E). We next examined the expression of p75NTR. In the heart tissue of noncardiac patients, p75NTR immunoreactivity was not detected (Fig. 1 B, left) except in nerve fibers in the adventitia of arteries (Fig. 1 B and not depicted). However, in the peri-infarct region of patients with recent MI, immunoreactivity was observed in mural cells of arterioles, but in few veins (Fig. 1, B [right, arrow], D, and E). Lastly, we examined the expression of SorCS2, a sortilin family member (Willnow et al., 2008). Increased SorCS2 immunoreactivity was observed in arterioles within the peri-infarct region of patients with recent MI but was not detected in the vasculature of noncardiac patients (Fig. 1 F).

To investigate the expression of proNGF and p75NTR in a rodent I-R model that recapitulates many features of severe myocardial ischemia in humans, transient ischemia was induced in C57BL6/J mice by occlusion of the left anterior descending coronary artery, followed by reperfusion for 24 h or 3 d. Using this model, we found that proNGF immunoreactivity was up-regulated in cardiac myocytes in the infarct and peri-infarct regions of the injured myocardium after 24 h of reperfusion, whereas low levels of proNGF was detected in the hearts of sham-operated mice (Fig. 1 G). ProNGF immunoreactivity remained elevated in the peri-infarct myocardium after 3 d of reperfusion (unpublished data).

We next examined the expression of proNGF receptors, including p75NTR, sortilin (Nykjaer et al., 2004), and SorCS2 in the mouse heart after I-R. Although p75NTR is expressed by cardiac sympathetic fibers in both uninjured and injured myocardium (not depicted), cell-associated p75NTR expression was increased (Fig. 1 H) and colocalized with PDGFR-β+ pericytes (Fig. 2 B, right) but not isolecin B4 (IB4)+ endothelial cells (not depicted) in the peri-infarct region after 24 h of reperfusion. Induction of p75NTR was observed in the microvasculature but not in coronary arteries or veins. P75NTR immunoreactivity was rarely detected in the vasculature from the uninjured cardiac tissue from sham-operated animals (Fig. 2 B, left). We also evaluated the expression of sortilin and SorCS2, to which proNGF also binds (Fig. 2 A). SorCS2 (Fig. 2 C), but not sortilin (not depicted), was up-regulated in the infarcted heart, specifically in PDGFR-β-expressing pericytes. The coordinate induction of proNGF in cardiac myocytes, and of p75NTR and SorCS2 in pericytes after injury, led us to hypothesize that proNGF may act in a paracrine manner to modulate microvascular function.

In vivo effects of increased expression of proNGF
To directly evaluate the action of proNGF in the heart, we generated a mouse in which the ngf coding exon was replaced with a mutant allele with impaired furin cleavage (Lee et al., 2001).
using gene targeting. This mutant *ngf* allele contains a C-terminal HA tag to facilitate detection (*proNgf-HA/+*; Fig. 3 A). As a control, mice were also generated in which one endogenous *ngf* coding exon was replaced with a wild-type mouse *ngf* sequence with a C-terminal HA tag (*wtNgf-HA/+*; Fig. 3 B). The HA-epitope tag facilitates detection of proNGF or mature NGF proteins in these mice, as total NGF levels in normal tissues are at subnanomolar concentrations (Yang et al., 2009). *ProNgf-HA/+* mice express one allele of *proNgf-HA* and one endogenous *ngf* allele to maintain viability. *Ngf−/−* mice (Crowley et al., 1994) were analyzed in parallel for phenotypic comparison and to permit detection of potential gain-of-function phenotypes of proNGF.

*ProNgf-HA/+* mice expressed proNGF mRNA in the heart (Fig. 3 C), and the level of total NGF proteins was comparable to that of wild-type littermates, as measured by an ELISA that detects both proNGF and mature NGF (Fig. 3 E). Western blot analysis of tissue lysates confirmed that *proNgf-HA/+* mice express predominantly proNGF-HA (32 kD protein) from the proNGF-HA allele, whereas *wtNgf-HA/+* mice express predominantly mature NGF (13 kD protein) from the wtNgf-HA/+ allele (Fig. 3 D, right). ProNGF (32 kD) was detected in the lysates of hearts of *proNgf-HA/+* but not wild-type control mice (Fig. 3 D, left). These results indicate that the cleavage of proNGF to mature NGF was impaired in *proNgf-HA/+* mice, resulting in increased expression of the proNGF isof orm.

In the hearts of 8-mo-old *proNgf-HA/+* mice, we observed marked structural and histological changes when compared with age-matched wild-type (*Ngf+/+) littermates (Fig. 4, A, B, D, and E), as well as with *Ngf+/−* and *wtNgf-HA/+* mice (not depicted). *ProNgf-HA/+* mice exhibit biventricular enlargement with myocardial wall thinning (Fig. 4, A and B), extensive myocardial fibrosis (Fig. 4, D, E, G, H, and J), and increased numbers of infiltrating CD68-expressing histiocytes (not depicted) as compared with the myocardium of control mice. However, no evidence of myocyte cell death was observed in the *proNgf-HA/+* mice at early ages (4 wk of age) or at later ages (8 mo old) as measured by cleaved caspase-3 immunofluorescence (unpublished data). In addition, matrix metalloproteinase-2 and -9 activity was unchanged in the *proNgf-HA/+* mice (unpublished data). Quantitation of collagen deposition as detected by Masson’s trichrome within the hearts of *proNgf-HA/+* mice and wild-type littermates documented increased levels in *proNgf-HA/+* animals of 6–8 mo of age (5.77% *proNgf-HA/+* vs. 0.39% *Ngf+/+, n = 4 of each genotype; Fig. 4, G, H, and J). These phenotypic changes are reminiscent of the pathology observed in patients with dilated cardiomyopathy (Roura and Bayes-Genis, 2009).

To evaluate ultrastructural changes in the myocardium that result from increased proNGF expression, we examined left ventricular subendocardial heart sections from *proNgf-HA/+*, wild-type (*Ngf+/+*), and heterozygous (*Ngf+/−*) mice of 4 mo of age by transmission electron microscopy (Fig. 5, A–C). Although the hearts from *Ngf+/+* and *Ngf+/−* mice appeared normal, the myocardium from *proNgf-HA/+* mice was notable for focal myofibrillar damage and focal collagen deposition (unpublished data). In addition, many endothelial cells exhibited attenuation of the cytoplasm with occasional overt rupture and extravasation of erythrocytes from the vascular lumen.
(Fig. 5 C and not depicted). Perivascular edema was prominent in the capillary bed of proNgf-HA/+ mice, as was an increase in caveolae in endothelial cells (Fig. 5 C), observations which were not seen in Ngf+/+ or Ngf−/− mice. The abnormalities in endothelial cell morphology were restricted to capillaries because the endothelial cells lining arteries and veins appeared normal (unpublished data). These observations indicate that microvascular damage occurs by early adulthood and are consistent with ultrastructural changes observed in other mouse models of microvascular dysfunction (Hellström et al., 1999).

To determine the time course of functional impairment in proNgf-HA/+ mice, we serially examined a cohort of mice from 1.5 to 8 mo of age using transthoracic echocardiography (Fig. 4 K). Contractile dysfunction of proNgf-HA/+ mice was evident at 2 mo of age with a continued decline in proNgf-HA/+ mice as compared with control animals (Fig. 4 K, compare Ngf+/+ [filled blue diamonds; n = 18] to proNgf-HA/+ [filled red squares; n = 31]; P < 0.005). Left ventricular posterior wall thickness measured at diastole (LVPWd) and systole (LVPWs) was increased in Ngf+/+ mice (Fig. 4, A and B), consistent with development of dilated cardiomyopathy. Kaplan-Meier analysis of this cohort of serially imaged animals demonstrated a 50% mortality rate among the proNgf-HA/+ cohort at 5 mo of age when compared with wild-type Ngf+/+ mice (Fig. 4 L). Furthermore, most proNgf-HA/+ animals died before 8 mo of age as a result of progressive cardiomyopathy, although a minority of animals died before 6 mo of age, or lived to 12 mo, consistent with modest variations in disease onset. However, proNgf-HA/+ mice from three independently targeted embryonic stem cell clones exhibited this cardiac phenotype, which was not observed in wtNgf-HA/+ mice derived from two independent clones.

Genetic deletion of p75 rescues proNGF-mediated cardiomyopathy

To determine if proNGF acts through the p75NTR and/or sortilin receptors, we serially examined mice expressing proNGF that were deficient in either p75NTR (p75−/−;proNgf-HA/+; n = 14) or sortilin (sortilin−/−;proNgf-HA/+; n = 15). Using echocardiography, we found that p75NTR deficiency blocked the development of ventricular dilation and fibrosis (Fig. 4, C, F, and I) and cardiac hypocontractility (Fig. 4 K, empty red squares) and prevented premature death (Fig. 4 L). However, sortilin deficiency did not protect proNGF-expressing mice from developing cardiomyopathy (Fig. 4 K, green triangles; and histological data not depicted). Kaplan-Meier analysis demonstrated that 50% of the sortilin−/−;proNgf-HA/+
to the activated microvascular endothelial cells, and deposits of fibrin strands in the lumen of capillaries (not depicted). Some capillaries in the proNgf-HA/+ ventricular myocardium exhibited perivascular edema with a loss of close apposition of endothelial cells to the extracellular matrix (3/16 vessels examined; Fig. 5 I, asterisk), which was not observed in control mice. In addition, a loss of apposition in the processes of pericytes with endothelial cells was observed in the proNgf-HA/+ mice (Fig. 5 I, arrowhead). Abnormalities in microvascular endothelial cells in proNgf-HA/+ mice at 1 mo of age were extensive, as 40/75 endothelial cells examined at the ultrastructural level exhibited one or more signs of activation, in contrast to <5% of microvascular endothelial cells in hearts of control mice.

Biochemical and immunohistological changes were also evident in the microvasculature of proNgf-HA/+ mice. Increased platelet accumulation, detected by CD41+ immunoreactivity, was observed in the proNgf-HA/+ ventricular myocardium when compared with wild-type littermates (Fig. 6, A and B). This phenotype was rescued in p75NTR−/−;proNgf-HA/+ animals (Fig. 6 C, quantified in Fig. 6 J). Focal increase in intercellular adhesion molecule (ICAM) immunoreactivity was also observed in the proNgf-HA/+ mice as compared with Ngf+/− mice, again suggesting endothelial cell activation (Fig. 6, D and E).

Figure 6. Abnormalities in the microvasculature of hearts from proNgf-HA/+ mice. (A–C) Hearts from proNgf-HA/+ mice at postnatal day 9 analyzed for platelet deposition (as assessed by CD41 immunofluorescence, red) compared with Ngf+/− and with p75−/−;proNgf-HA/+ myocardium. (D–F) ICAM-1 expression (red) in the proNgf-HA/+ myocardium compared with the Ngf+/− and p75−/−;proNgf-HA/+ myocardium, at postnatal day 9. (G–I) FITC-dextran (green) accumulation and extravasation was examined in the myocardium from 3-mo-old proNgf-HA/+, Ngf+/+, and p75−/−;proNgf-HA/+ mice. (J and K) Quantification of CD41 and ICAM-1 immunofluorescence (n = 3–6 mice/condition, eight fields of view per mouse). *, P < 0.05; **, P < 0.01, mean ± SEM. Bar, 100 µm.

Microvascular endothelial activation and increased vascular permeability precede the development of cardiac fibrosis

To better understand the pathophysiological mechanisms that underlie proNGF:p75NTR-mediated cardiomyopathy, we performed histological and ultrastructural analysis of heart tissue obtained from 1-mo-old mice, when cardiac compromise is not yet apparent. Although the hearts of proNgf-HA/+ mice have a normal histological appearance (Fig. 5, D–F), ultrastructural analysis (n = 2 of each genotype) revealed that microvascular endothelial cells were abnormal in proNgf-HA/+ but not in Ngf+/− or Ngf−/− myocardium (Fig. 5, G–I). Morphological changes included many prominent endothelial projections into the capillary lumen (Fig. 5 I, arrow), increased platelet adherence to the activated microvascular endothelial cells, and deposits of fibrin strands in the lumen of capillaries (not depicted). Some capillaries in the proNgf-HA/+ ventricular myocardium exhibited perivascular edema with a loss of close apposition of endothelial cells to the extracellular matrix (3/16 vessels examined; Fig. 5 I, asterisk), which was not observed in control mice. In addition, a loss of apposition in the processes of pericytes with endothelial cells was observed in the proNgf-HA/+ mice (Fig. 5 I, arrowhead). Abnormalities in microvascular endothelial cells in proNgf-HA/+ mice at 1 mo of age were extensive, as 40/75 endothelial cells examined at the ultrastructural level exhibited one or more signs of activation, in contrast to <5% of microvascular endothelial cells in hearts of control mice.

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Surprisingly, we were unable to detect p75NTR in the cardiac microvasculature or in cardiac myocytes by immunofluorescence microscopy in mice from 1 to 4 wk of age (unpublished data), despite immunological and ultrastructural evidence of microvascular damage in the proNgf-HA/+ mice. However, immunofluorescence microscopy confirmed the colocalization of p75NTR and PDGFR-β in perinatal hearts (Fig. 7, A and B), supporting our hypothesis formulated from the cardiac I-R studies (Fig. 2 B) that PDGFR-β–expressing pericytes are the target of cardiac myocyte-derived proNGF. Furthermore, we detected SorCS2 expression in a subpopulation of PDGFR-β+/p75NTR+ pericytes (Fig. 7, D and E; and not depicted) in the perinatal heart.

To elucidate the effects of proNGF on pericytes in the perinatal heart, we used NG2DsRedBAC–transgenic mice, which express the red fluorescent protein DsRed under the control of...
the Cspg4 promoter, allowing selective detection of pericytes and smooth muscle cells in the periphery (Zhu et al., 2008; Lebrin et al., 2010). These mice were crossed with the proNgf-HA/+ mice to generate NG2dsRedBAC;proNgf-HA/+ mice. Using a morphometric approach, we quantitated pericyte process length in 1-mo-old NG2dsRedBAC;proNgf-HA/+ mice and NG2dsRedBAC;Ngf+/+ littersates. Pericytes from NG2dsRedBAC;proNgf-HA/+ mice had significantly shorter pericyte processes compared with NG2dsRedBAC;Ngf+/+ control mice (Fig. 8). These data suggest that proNGF targets p75NTR present on perinatal pericytes to decrease pericyte process length and therefore reduce pericyte coverage of the cardiac microvasculature.

These observations support the hypothesis that up-regulation of p75NTR and SorCS2 in pericytes in the peri-infarct myocardium (Fig. 1, B–E) may be an important step in the induction of microvascular endothelial pathology by reducing pericyte process length and coverage of the microvasculature. Collectively, these studies suggest that proNGF activation of p75NTR and SorCS2 receptors on pericytes impairs mechanical and trophic support of microvascular endothelial cells, leading to endothelial activation, platelet adhesion, enhanced vascular permeability, and ultimately cardiac fibrosis.

**p75NTR deficiency provides protection from infarct expansion**

To determine if proNGF/p75NTR signaling modulates microvascular dysfunction and the expansion of infarct size after myocardial I-R injury, we measured infarct size in wild-type and p75−/− mice 10 d after reperfusion. Infarct size was quantified after Masson’s trichrome staining and normalized to the area at risk. Infarct size was significantly smaller in p75−/− mice compared with wild-type mice (Fig. 9). These results suggest that elevated proNGF, acting through the receptor p75NTR induced in pericytes after I-R, may contribute to post-MI microvascular deficiency and increase of infarct size. We have previously demonstrated that infarct size is not different 24 h after I-R in p75−/− mice compared with wild-type mice (Lorentz et al., 2011). However, analysis of infarct size 24 h after I-R injury primarily measures the area of necrosis induced by cardiac ischemia, whereas the expression of p75NTR and proNGF are maintained for several days (unpublished data). Collectively, these results suggest that p75NTR is not directly involved in the necrotic lesion induced by I-R but rather plays a role in infarct expansion via microvascular injury.

**DISCUSSION**

Events that lead to persistent cardiac microvascular compromise after transient ischemic injury remain incompletely understood (Eltzschig and Collard, 2004; Bekkers et al., 2010). Early studies demonstrated impaired microvascular perfusion which led to the proposal that luminal obstruction by thromboembolic debris and endothelial cell edema is the main cause of the pathology observed (Kloner et al., 1974). However, it is now established that inflammatory cytokines play a crucial role in initiating microvascular damage (Nian et al., 2004). Cytokines such as TNF, IL-1, and IL-6 are induced soon after myocardial ischemia, triggering leukocyte transmigration, platelet adhesion, and local generation of reactive
studies will be required to determine whether all of these activations, and extracellular matrix deposition, although further studies will be required to determine whether all of these effects are direct or result from cytokine amplification after transmigration of leukocytes.

One important aspect of our study is the identification of p75NTR-expressing pericytes as the cell type activated by proNGF. This observation underscores the critical role of pericytes to establish and maintain stabilization of the cardiac microvasculature (Gaengel et al., 2009). Pericytes release cytokines locally to induce endothelial cells to exit the cell cycle and differentiate via angiopoietin-1:Tie2 signaling (Dumont et al., 1994; Sato et al., 1995; Suri et al., 1996). Conversely, endothelial cells produce TGF-β that locally activates Alk5 on pericytes to stimulate proliferation and differentiation (Oh et al., 2000; Larsson et al., 2001). These reciprocal interactions promote pericyte ensheathment to stabilize endothelial junctions and reduce vascular permeability. Most studies of pericyte–endothelial cell interactions focus on developmental time points (Armulik et al., 2005). This study identifies a subpopulation of cardiac pericytes that transiently express p75NTR in late gestation, although expression is down-regulated postnatally.

The current studies support the hypothesis that after ischemia, cardiomyocyte–derived proNGF, acting through p75NTR, which is induced by pericytes, causes pericyte injury and pericyte process retraction, resulting in a lack of trophic support of the microvascular endothelium. Indeed, ultrastructural abnormalities observed in the proNgf-HA+/mice, which include increased caveolae, numerous luminal projections, loss of apposition of pericytes and endothelial cells, and perivascular edema, are highly reminiscent of the ultrastructural changes in mice deficient in pericytes (Lindahl et al., 1997; Hellström et al., 1999). Furthermore, vessel damage in the proNGF-expressing heart is restricted to capillaries, precapillary arterioles, and postcapillary venules, with normal coronary arteries and veins. This pattern corresponds to vessels with pericyte ensheathment (Attl and Lawrenson, 2001). Although proNGF induces apoptosis of p75NTR-expressing neurons in other injury models, we did not detect overt pericyte apoptosis. This may reflect difficulties in quantitating pericyte loss, as pericyte coverage of the abluminal endothelium is estimated to be only 10–20% in the cardiac microvasculature (Shepro and Morel, 1993). However, proNGF-induced activation of p75NTR-expressing pericytes may induce more subtle effects, such as process retraction, to reduce pericyte ensheathment. Indeed, examination of pericyte process length revealed shorter pericyte processes in the proNgf-HA+/mice compared with littermate control mice, suggesting that inhibition of pericyte process outgrowth or process retraction is occurring when cardiac proNGF levels are elevated. These data are reminiscent of neuronal studies in which proNGF induces growth cone collapse (Deinhardt et al., 2011) and proBDNF, a related proneurotrophin ligand, induces retraction of p75NTR-expressing neuronal processes (Koshimizu et al., 2009; Yang et al., 2009). Collectively these data suggest that proNGF activation of pericytes expressing p75NTR results in endothelial cell activation, leukocyte transmigration, fibrin and platelet deposition, and microvascular thrombosis, culminating in myocardial ischemia and fibrosis.
These findings are consistent with prior reports of local microvascular loss that result in dilated cardiomyopathy (Roura and Bayes-Genis, 2009). The current study strongly suggests that pericytes play a crucial role in maintaining microvascular function after ischemic injury.

ProNGF has been best characterized as a cytokine induced after neuronal injury in the central and peripheral nervous systems (Hempstead, 2009). Under these conditions, proNGF promotes neuronal apoptosis, and infusion of function-blocking antibodies specific for proNGF rescues most p75NTR-expressing neurons after central neuron axotomy or seizures (Harrington et al., 2004; Volosin et al., 2006). Results presented here extend the pathological actions of proNGF to include induction of vascular injury after MI in humans and cardiac I-R injury in mice. The coordinate up-regulation of proNGF and p75NTR within 24 h after I-R injury (Hiltunen et al., 2001; Hasan et al., 2006; this paper) is consistent with the rapid induction observed in the central nervous system. The progressive decrease in cardiac contractility in proNGF-HA/+ mice, but not Ngf+/- mice, indicates that sustained levels of proNGF, rather than haploinsufficiency of mature NGF, induces cardiac microvascular dysfunction.

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verified using a second pair of PCR primers specific for the neomycin-resistant gene of bacteria: 5′-GAGATCCACTGTTCTAGCCTGAG-3′ and 5′-CCACACACTGTTGCACTG-3′.

A chicken β-actin cre-deleter mouse strain (Sakai and Miyazaki, 1997) was used to remove the neo-resistance cassette, thus permitting expression of the knocked-in allele under its endogenous ngf gene promoter elements. The following primer pairs were used to identify the presence of the cre-recombinase allele in mice: 5′-TTATACACCGCTGAGTATAGC-3′ and 5′-TATCCTGACGAGTCTCCTTAG-3′. p75+/- mice were purchased from The Jackson Laboratory and backcrossed to C57BL/6J mice until the N8 generation before breeding with other mouse strains. NG2DrRedBAC mice (Zhu et al., 2008) were also purchased from The Jackson Laboratory and were crossed to the proNgf-HA+/− mice to generate NG2DrRedBAC; proNgf-HA/− mice and NG2DrRedBAC; Ngf+/− littermate control mice for pericyte process analysis. Other control strains include mice until the N8 generation before breeding with other mouse strains.

For immunohistochemical analysis, embryonic or perinatal tissues/organs were fixed overnight in 4% paraformaldehyde at 4°C and then cryoprotected with 30% sucrose. Adult hearts were similarly processed and cryoprotected with sucrose before embedding in OCT/30% sucrose (1:1; Sakura). For protein and RNA analyses, heart tissues were stored at −80°C until processing. To analyze vascular permeability, FITC-dextran (70 kDa; Sigma-Aldrich) was injected as a bolus through the tail vein of 3- to 5-month-old mice (Cannell et al., 1983). After perfusion for 10 min, the animals were sacrificed and their hearts were harvested and processed for immunofluorescence microscopy.

Masson’s trichrome demonstration of collagen deposition was performed according to the manufacturer’s instructions (Polysciences Inc. PA Corp.) on cryosections. Quantification of cardiac fibrosis was performed using ImageJ (National Institutes of Health). Three to four fields of view from the subendocardium of the heart were analyzed from two to three 10 µm sections from mice 6–8 mos of age. In brief, a binary image was generated using Photoshop (Adobe), and blue pixels were converted to white pixels whereas pixels of all other colors were converted to black. The area of fibros (white pixels) was measured using a set threshold (ImageJ) and normalized to the total area of tissue in the field of view. RT-PCR was performed according to standard protocols and reagents (Invitrogen) using the HA primers described. The NGF Emax kit (Promega) was used for all ELISA measurements, according to the manufacturer’s instructions.

**Immunoprecipitation/Western blotting**. Frozen hearts and brains were homogenized in lysis buffer (0.1 M Tris, pH 7.4/1% Triton X-100/0.1% NP-40/0.05% SDS/30% glycerol/protease inhibitor cocktail [Sigma-Aldrich]). Proteins tagged by an HA epitope were enriched by binding to an anti-HA antibody (Sigma-Aldrich) at 4°C. Protein A–Sepharose beads (Sigma-Aldrich) were added to capture the immune complex, washed extensively, and boiled in SDS-PAGE buffer. Blotted proteins were detected using HA.11 monoclonal antibody (Covance) and developed with enhanced chemiluminescence (GE Healthcare). 293T cells were transfected using pcDNA3.1 constructs expressing N-terminal HA-tagged and C-terminal FLAG-tagged mouse proNGF, and/or N-terminal myc-tagged human sortilin and SorCS2 (provided by Z.Y. Chen and F.S. Lee, Weil Cornell Medical College). The myc epitope tags were inserted after the propeptide cleavage site to ensure the expression of the tag. Cell lysates were immunoprecipitated and Western blotted using an antibody to FLAG conjugated to protein A–Sepharose beads (Sigma-Aldrich). The myc-tagged sortilin or SorCS2 proteins were detected using a rabbit anti-myc antibody (Covance) and the HA-tagged proNGF was detected with mouse anti-HA (Covance). The extracts were centrifuged (14,000 rpm for 15 min) at 4°C to remove insoluble debris, and the supernatants were collected for matrix metalloproteinase analysis. Equal amounts of supernatant (80 µl; 20 µg) were mixed with 20 µl 5% SDS sample buffer (without mercaptoethanol) and incubated for 30 min at 37°C. Samples and molecular weight markers were electrophoresed in a 10% polyacrylamide gel containing 0.25% gelatin. The gel was then washed (2×) in 2.5% Triton X-100 to remove SDS, incubated at 37°C for 18 h in 200 mM NaCl containing 40 mM Tris-HCL and 10 mM CaCl2, pH 7.5, and stained with Coomassie Blue. The presence of gelatinolytic activity was identified as clear bands on a uniform blue background after destaining.

**Immunohistochemistry and immunofluorescence**. Cryostat sections of heart tissues were fixed in acetone (−20°C) or in 4% paraformaldehyde (at room temperature) for 10 min. For immunohistochemistry, sections were treated for 30 min in 0.1% H2O2/methanol at −20°C, and then blocked (5% serum of host of secondary antibody/5% nonfat dry milk/0.1% Triton X-100/PBS) and incubated with primary antibody overnight at 4°C. The primary antibodies used were: p75 (extracellular domain-directed; R&D Systems, 1:1,000; intracellular domain-directed; Promega, 1:500), proNGF (prodomain-specific, 1:100, Harrington et al., 2004), sortilin (R&D, 1:400), SorCS2 (intracellular domain-directed; generated by immunizing rabbits against huSorCS2 aa 1138–1159, coupled to KLH, 1:300; or extracellular domain directed: R&D Systems, 1:1,000), CD140B (PDGFR-β; bE Biosciences, 1:200), IB4 (preconjugated to biotin, Vector Laboratories, 1:300), activated caspase-3 (Cell Signaling Technology, 1:200), tyrosine hydroxylase (Millipore, 1:150), CD31 (BD, 1:100), CD41 (BD, 1:100), fibrinogen (FITC-conjugated, Dako, 1:250), CD68 (TRITC-conjugated, Serotec, 1:100), CD45 (BD, 1:200), and Gr1 (BD, 1:200). Biotinylated secondary antibodies were conjugated to ABC reagent and detected using the VIP Peroxidase Substrate kit (Vector Laboratories). Immunofluorescence was performed as described, except within peroxide treatment. Alexa Fluor–conjugated secondary antibodies (Invitrogen) were used to detect primary antibodies. Light microscopy was performed on a microscope (Olympus) and a QImaging Retiga EXi camera and a BX50 microscope (Olympus) and MicroFire digital camera (Optronics). Immunofluorescent microscopy was performed using either a Observer.Z1 (Carl Zeiss) or an Eclipse 80i (Nikon). Digital images were acquired with an AxioCam MRm or AxioCam MRc camera and AxioVision 4.8 software or with a QImaging Retiga1300i camera and Q Capture or Elements software (Nikon).
reversibly ligated with an 8–0 suture for 45 min and then reperfused by re-
lease of the ligature. Occlusion was confirmed with ST segment elevation on
the electrocardiogram, regional cyanosis, and wall motion abnormalities. Reper-
fusion was confirmed by return of color to the myocardium distal to the liga-
tion and disappearance of ST elevation. The suture remained within the
wound for identification of the ligature site, and the chest and skin were
layered in layers. After surgery, animals were returned to individual cages and
given regular food and water for 24 h or 3 d before euthanasia and tissue
harvest. 0.1 mg/kg Buprenex was administered as needed to ensure that
the animals were comfortable after surgery. All surgical procedures were per-
formed under aseptic conditions.

Infarct/area at risk analysis. 10 d after the onset of reperfusion, the mice
were anesthetized with 4% isoflurane. Once unconscious, the mice were intu-
lated and mechanically ventilated and anesthesia was maintained with 2% in-
haled isoflurane. The chest cavity was then reopened and the left anterior
descending coronary artery was reocluscled using the same suture from the
I-R procedure. Evans blue dye (1 ml of 1.5% [wt/vol] solution made with
0.9% NaCl) was infused through a polyethylene tube (PE10) in the right jugu-
lar vein to delineate the area at risk. The heart was then excised for infarct size
analysis and cut into transverse sections 1 mm thick using a cutting block. Both
sides of all slices were photographed for measurement of area at risk (ImageJ).
The slices were then placed in 10% neutral buffered formalin and fixed over-
night at 4°C. The slices were then thoroughly washed with PBS and cryopro-
tected with 30% sucrose before embedding in OCT:30% sucrose (1:1, Sakura).
Masson’s trichrome demonstration of collagen deposition was performed ac-
cording to the manufacturer’s instructions (PolyScientific R&D Corp.) on
cryosections from each of the 1 mm sections to identify the infarcted area.
Sections stained by Masson’s trichrome were photographed using a micro-
scope (Olympus) with a 4× objective and a QImaging Retiga EXi camera.
Composite images of the entire sections were made using Photoshop. Total area,
area at risk, and infarct areas for each slice were traced (ImageJ), and the area of
myocardium at risk and infarcted myocardium were calculated from the mea-
sured areas. Infarct size was normalized as a fraction of the area at risk.

Electron microscopy. All reagents were purchased from Electron Microscopy
Sciences. Hearts were dissected from mice, rinsed well in cold PBS, and im-
nersed in modified Karnovsky’s fixative (2.5% glutaraldehyde, 4% paraformalde-
yde, and 0.02% picric acid in 0.1 M sodium cacodylate buffer, pH 7.3) over-
night. 1-mm thick blocks were post-fixed in 1% osmium tetroxide/1.5%
kataonium-ferricyanide, stained with 1.5% uranyl acetate, and dehydrated
through a graded ethanol series. After embedding in Spurr’s resin, sections
were cut at 55–60 nm thickness using a diamond knife (Diatome) on an ul-
tramicrotome (Ultracut S; Leica). Sections were contrasted with lead citrate
and viewed on a JEM 100 CX-II electron microscope (JEOL) operated at
80 kV. Images were recorded on 4489 Electron Image film (Kodak) and then
digitized at 900 dpi for publication.

Estimation of pericyte process length. Hearts from 1-mo-old
NG2DrdsRedBAC; proNgf+/+ mice and NG2DrdsRedBAC; Ngf+/−
littermates were sectioned at 30 µm thickness and cover slipped. The sections
were numbered blindly before quantitative analysis. To be selected for analy-
sis, DiRed labeled pericytes had to meet the following characteristics: have
a single cell body having primary processes growing out from the cell body
and relatively isolated from neighboring pericytes; be positioned in the sub-
epicardial layer of the heart, running in a longitudinal direction; and have
intact processes with consistent NG2DrdsRed labeling. 20 pericytes from two
sections from each animal were traced at 40× using NeuroExplorer software.
Total process length was determined using NeuroExplorer software.

Online supplemental material. Table S1 describes patient information.
Online supplemental material is available at http://www.jem.org/cgi/
content/full/jem.201111749/DC1.

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