Endothelium-Derived Neuregulin Protects the Heart Against Ischemic Injury

Nadia Hedhli, PhD*; Qunhua Huang, PhD*; April Kalinowski, MS; Monica Palmeri, MS; Xiaoyue Hu, MD; Raymond R. Russell, MD, PhD; Kerry S. Russell, MD, PhD

Background—Removal of cardiac endothelial cells (EC) has been shown to produce significant detrimental effects on the function of adjacent cardiac myocytes, suggesting that EC play a critical role in autocrine/paracrine regulation of the heart. Despite this important observation, the mediators of the protective function of EC remain obscure. Neuregulin (NRG, a member of the epidermal growth factor family) is produced by EC and cardiac myocytes contain receptors (erbB) for this ligand. We hypothesized that NRG is an essential factor produced by EC, which promotes cardioprotection against ischemic injury.

Methods and Results—We demonstrate that human cardiac EC express and release NRG in response to hypoxia–reoxygenation. Under conditions where hypoxia–reoxygenation causes significant cardiac myocyte cell death, NRG can significantly decrease apoptosis of isolated adult ventricular myocytes. Coculturing adult murine myocytes with human umbilical vein, murine lung microvascular, or human coronary artery EC can also protect myocytes against hypoxia–reoxygenation–induced apoptosis. These protective effects are abolished by NRG gene deletion or silencing of NRG expression in EC. Finally, endothelium-selective deletion of NRG in vivo leads to significantly decreased tolerance to ischemic insult, as demonstrated by impaired postischemic contractile recovery in a perfused whole-organ preparation and larger infarct sizes after coronary artery ligation.

Conclusion—Together, these data demonstrate that EC-derived NRG plays an important role in cardiac myocyte protection against ischemic injury in the heart and supports the idea that manipulation of this signaling pathway may be an important clinical target in this setting. (Circulation. 2011;123:2254-2262.)

Key Words: apoptosis ■ cardioprotection ■ endothelium-derived factors ■ ischemia

Endothelial cells (EC) in the heart play a variety of important roles both during cardiac development and in the adult human and animal heart. Cardiac EC (vascular and endocardial) are uniquely poised to act as sensors of systemic stress (by virtue of exposure to circulating factors) as well as sensors and regulators of cardiac stress and function (by virtue of their close proximity to cardiac myocytes).1 The idea that cardiac EC play a critical role in autocrine/paracrine regulation of the heart is supported by the finding that removal of either of these types of EC has been shown to have significant detrimental effects on the contractile function of the adjacent cardiac myocytes.2,3 Despite these important observations, the mechanisms and mediators of these functions of the EC are largely undefined.

Clinical Perspective on p 2262

Neuregulins (NRGs) and their receptors (erbB tyrosine kinases) have been shown to play crucial roles in both the developing and adult cardiovascular system. One of the earliest indications that erbB receptors are important in the adult human heart was the finding that the incidence of left ventricular dysfunction is increased in cancer patients being treated with a humanized monoclonal antibody directed against erbB2 (trastuzumab).4 It has also been shown that expression of erbB2 and erbB4 is decreased in left ventricular tissue from patients with end-stage dilated cardiomyopathy (both ischemic and nonischemic). However, this effect was found to be reversible in a subset of patients who underwent temporary unloading with a left ventricular assist device.5 Elevated serum levels of NRG are also correlated with poor outcomes in patients with heart failure.6 These investigators propose that increased NRG in this setting may represent an inadequate compensatory response.

In animal models, homozygous germline deletion of NRG, erbB2, or erbB4 results in embryonic lethality because of the failure of proper cardiac ventricular and valvular development.7–9 Mice in which cardiomyocyte-selective erbB2 or erbB4 expression is significantly reduced survive to birth but rapidly develop heart failure within the first few months of life.10–12
Animals with heterozygous germline deletion of NRG can survive to birth, and reportedly have no significant baseline defects in cardiac function; however, after doxorubicin injury, these animals have significantly worse cardiac dysfunction and poorer survival than wild-type controls. Of note, cardiac tissue–selective deletion of NRG has not previously been reported.

Exogenous administration of NRG has been shown to protect cardiac myocytes against apoptosis in response to serum deprivation, chemotherapy agents, and H2O2. Long-term treatment of mice with exogenous NRG after myocardial infarction has been shown to improve cardiac remodeling and function. Because the endogenous source of NRG in the heart is believed to largely be microvascular and endocardial endothelium, we and others have hypothesized that endotheli derived NRG plays an important role in cardiac protection against ischemic injury, though this has yet to be directly tested in vivo. To examine whether EC can provide cardiac protection against ischemic insult and whether NRG is essential for this effect, we used a combination of in vitro coculture experiments and an in vivo model of endothelium-selective NRG deletion. Our findings provide direct evidence that EC are a crucial source of NRG for cardioprotection against ischemic insult and suggest that endothelium may be an important target for therapeutics in this setting.

**Methods**

**Cells and Reagents**

Human coronary artery EC (HCAEC) were purchased from Lonza (Walkersville, MD). Human umbilical vein EC were isolated from pooled donors in our core facility and cultured as previously described. Lung microvascular EC (LMVEC) were harvested from adult mice by collagenase digestion and immunopurification. Myocytes were harvested from adult (6-week-old to 12 week-old) mice by collagenase perfusion and plated on laminin-coated (10 mg/mL, BD Biosciences) glass coverslips (as previously described). Additional methodological details can be found in the online-only Data Supplement.

**Immunofluorescence and Western Blotting**

Human cardiac autopsy specimens were obtained from deidentified donors through our tissue bank. This investigation conforms to the principles outlined in the Declaration of Helsinki, 1997. Immunoprecipitation and immunoblotting of NRG from cell culture media was performed as previously described.

The following anti-NRG antibodies were used for these experiments: Antibodies 7D5 (Labvision/NeoMarkers) and Ab-1 (R&D Systems), which detect the extracellular epidermal growth factor domain of NRG, were used for immunofluorescence staining, immunoprecipitation and immunoblotting of cleaved/released NRG from cell media; and SC-348 (Santa Cruz Biotechnology), which detects the intracellular domain of transmembrane/cell-associated NRG, was used for immunoblotting of full-length NRG. Additional antibodies included antierbB4 (SC-283, Santa Cruz Biotechnology), anti-CD45 (BD Biosciences), anti-CD31 (BD Biosciences), and antiphosphotyrosine (PY20, Santa Cruz Biotechnology). Lectin staining was performed using biotinylated lectin (Sigma Aldrich) and streptavidin-labeled Alexa 488 (Invitrogen).

**Coculture and Hypoxia–Reoxygenation Experiments**

Adherent human cardiac myocytes plated on coverslips were placed in the bottom of a 6-well plate. For coculture experiments, EC were placed in gelatin-coated transwells (0.4 µm pore size, Becton Dickinson) and placed above the myocytes at the beginning of the hypoxic exposure period. Purified recombinant NRGβ3 (Sigma-Aldrich) was added to myocytes at a final concentration of 3 µmol/L in PBS at the time they were placed in the hypoxia chamber, remaining present throughout the experiment. Cells were then placed in a hypoxia chamber purged with 95% N2/5% CO2 for 15 to 20 minutes. Reoxygenation was performed by removing the plates from the chamber and allowing equilibration with room air in a 37°C incubator with 5% CO2. At the end the hypoxia–reoxygenation (HR) treatment, the transwells were removed and cardiomyocytes on glass coverslips were stained for TUNEL, annexin V, propidium iodide, or DAPI.

**Animal Derivations and Treatments**

All animal protocols were approved by the Yale Institutional Animal Care and Use Committee. Inducible and endothelium-selective NRG knockdown mice were generated using the VE-cadherin promoter-driven creERT2 mouse (VEcad-creERT2, generously provided by Dr Luisa Iruela-Arispe). These mice were crossed with mice carrying homozgyously floxed alleles (exons 7 to 9 deleted in presence of cre recombinase) of the neuregulin-1 gene (NRG F/F, generously provided by Dr Carmen Birchmeier). We used 3 separate controls: C→+ (VEcad-creERT2, NRG wild-type) mice were treated with vehicle or tamoxifen and used to control for both the effects of cre expression and tamoxifen treatment. CFF (VEcad-creERT2, NRG F/F) mice were treated with vehicle as controls for the presence of floxed NRG alleles. Mice were given daily intraperitoneal injections with vehicle (10% EtOH in sunflower oil) or 1 mg tamoxifen for 9 days, a time point when we determined that endothelial protein expression of NRG has decreased to <10% of controls. After a short gene induction period (3 days tamoxifen injection), NRG depletion was achieved by injecting animals daily with 2.5 µg of recombinant human NRG (rNRG, R&D Systems) throughout the remainder of the tamoxifen induction period (total 9 days, as above).

**Echocardiographic Measurements**

Left ventricular size and function were assessed by echocardiography using the Vevo770 system (VisualSonics, Toronto, Canada) with a 40 MHz probe. Examinations were performed on mice under light anesthesia with inhaled isoflurane (<5.0% to 2% vaporized in O2). A total of 4 measurements was made in both short- and long-axis views and averaged together to obtain functional and anatomic data.

**Langendorff Perusions**

Responses to ischemia/reperfusion injury were assessed in the retrogradely perfused isolated mouse heart as previously described.

**Infarct Model**

Mice were anesthetized and ventilated (Rodent Ventilator, Harvard Instruments) with room air supplemented with oxygen. After left thoracotomy, the left coronary artery was ligated 2 mm below the atrial appendage. After 30 minutes of the VCD coronary occlusion, the ligature was removed and the heart was repurposed for 4 hours. Core temperature was monitored and maintained at 37°C, and the ECG was monitored to document ST-segment elevation during coronary occlusion.

**Statistical Analysis**

Data were analyzed using nonparametric ANOVAs (Kruskal-Wallis) in cases where conformity to a normal distribution could not be confirmed using the Kolmogorov-Smirnov test. In normally distributed samples, ANOVAs were performed to test for differences between multiple groups using a Bonferroni post test to determine intergroup differences. All statistical analyses were performed using Prism 4 software (GraphPad Software Inc, La Jolla, CA).

**Results**

Human Cardiac Endothelial Cells Express Neuregulins and Release It in Response to Hypoxia

To demonstrate that human cardiac EC are the primary cell source of NRG, cardiac autopsy specimens were stained by...
immunofluorescence using antibodies against NRG and lectin (Figure 1A through 1F). These samples were taken from the subendocardial region of the left ventricle and demonstrate that endocardial endothelium (Figure 1A through 1C), as well as small subendocardial vessels (Figure 1D through 1F) express NRG. These microvascular and endocardial EC are likely to provide an important local source of NRG, being adjacent to a large area of myocardium. Isolated HCAEC also express abundant full-length NRG (Figure 1G, top). In response to H/R, there is loss of full-length NRG from cell-membrane lysates and accumulation of the extracellular N-terminal ligand domain in the tissue culture media from these cells (Figure 1G, bottom). On the basis of these findings, we hypothesized that during periods of cardiac ischemia, NRG may be an important factor released by EC to provide protective effects in the heart.

**Neuregulins Protects Cardiac Myocytes Against Apoptosis Induced by Hypoxia–Reoxygenation**

As described above, NRG has been shown to protect cardiac myocytes against apoptosis in response to several types of injury. We tested whether hypoxia-induced apoptosis could also be mitigated by NRG. Adult murine cardiac myocytes were placed on glass coverslips in a hypoxia chamber for 4 hours, followed by 18 hours reoxygenation. The coverslips were then stained by TUNEL with an antibody directed against annexin V, with propidium iodide and with DAPI. To assess myocyte apoptosis, TUNEL or annexin-positive rod-
shaped cells were counted and normalized to the total number of cells (on the basis of DAPI-positive nuclear staining). For quantification of total cell necrosis, propidium iodide–positive cells were also counted at normalized-to-total DAPI-positive cells. Adult myocytes subjected to isolation and incubation for equivalent times under normoxic conditions exhibit low-level ongoing apoptosis and necrosis (Figure 2C, control). However, the number of apoptotic cells is significantly increased after H/R (4 hours/18 hours). In the presence of NRG, significantly fewer myocytes undergo apoptosis and necrosis in response to H/R injury, demonstrating the protective effect of NRG against hypoxia-induced cell death in this model.

Endothelial Cell Coculture Protects Cardiac Myocytes Against Apoptosis Induced by Hypoxia–Reoxygenation

Because endothelium is a primary source of NRG in the heart, we next wanted to test whether EC could provide NRG in the setting of hypoxic injury to protect cardiac myocytes. We selected 3 different sources of EC: human umbilical vein EC, LMVEC, and HCAEC. These EC were plated in transwells at near confluence, and the transwells were placed in coculture with primary adult murine cardiac myocytes. These cocultures were then subjected to the same H/R (4 hours/18 hours) protocol, and the number of annexin V, TUNEL, or propidium iodide–positive myocytes was quantitated (as for Figure 2). All 3 EC types were able to provide protection to myocytes against H/R-induced apoptosis (Figure 3). Interestingly, HCAEC (which we have demonstrated to release NRG in this experimental protocol, Figure 1B) provided excellent protection in this assay, which supports the idea that this mechanism may play a role in the human heart during ischemic injury. However, NRG is only 1 of several potential factors that may be released from EC and provide myocyte protection in this setting. Therefore, we wished to directly test whether EC expression of NRG is required for protection of cardiac myocytes against hypoxia-induced apoptosis in this model.

Endothelial Expression of Neuregulins Is Required for Protection of Cardiac Myocytes Against Hypoxia-Induced Apoptosis

We used 2 independent methods to decrease NRG expression in EC to test whether loss of NRG expression would abolish the protective effects of EC on cocultured myocytes. First, we isolated LMVEC from mice carrying homozygously floxed alleles of NRG. These cells were then infected with adenoviral expression constructs for red fluorescent protein (AdenoRed, to serve as a marker for transduction efficiency and as a negative control) or cre recombinase (AdenoCre, to allow recombination of the floxed NRG alleles resulting in loss of NRG expression). As shown in the inset panel for Figure 4A, there was a dose-dependent decrease in NRG
expression in these EC that correlated with increasing expression of cre recombinase, as expected. Lung microvascular EC were then placed in coculture transwells with adult murine cardiac myocytes and subjected to the H/R protocol. Untreated LMVEC or LMVEC infected with AdenoRed express abundant NRG (inset panel 4A) and are very effective at protecting cardiac myocytes against H/R-induced apoptosis. However, after transduction with AdenoCre, LMVEC express significantly less NRG and lose their ability to confer protection during H/R.

To further confirm this finding in human endothelium, we used a small interfering RNA (siRNA) strategy to decrease expression of NRG in HCAEC. Transduction of HCAEC with control scrambled siRNA did not affect expression of NRG under normoxic culture conditions (Figure 4B inset, top) or release of NRG into tissue culture media after H/R (Figure 4B inset, bottom). Human coronary artery EC transduced with siRNA directed against NRG (but not scrambled control siRNA) fail to protect cardiac myocytes. (Inset: immunoblotting of HCAEC lysates or media (40 kD, cleaved product detected by immunoprecipitation) from H/R-exposed HCAEC after siRNA transfection.) *P<0.01 versus normoxic control, control siRNA-treated cocultured or nontreated HCAEC cocultured myocytes; †P<0.05 versus control siRNA-treated or nontreated HCAEC cocultured myocytes; ‡P<0.001 versus normoxic control, control siRNA-treated or nontreated HCAEC cocultured myocytes. MOI indicates multiplicity of infection; NRG, neuregulin; Cre, creatinine; H/R, hypoxia and reoxygenation; LMVEC, lung microvascular endothelial cells; EC, endothelial cells; and siRNA, small interfering RNA.

Loss of Endothelial Neuregulins Expression Decreases Ischemic Tolerance in the Intact Heart

On the basis of our in vitro data described above, we next wished to test whether EC-derived NRG is required for cardioprotection in vivo. As previously described, germline deletion of NRG results in embryonic lethality. Thus, we wished to create a model of NRG deletion in which NRG could be selectively deleted from the endothelium of adult animals. To accomplish this, we bred mice carrying a tamoxifen-inducible endothelium-selective cre recombinase transgene (VEcad-cereERT2)24 with those carrying CFF.25 Isolated purified cardiac EC from CFF mice treated with tamoxifen expressed significantly reduced NRG protein compared to vehicle-treated C++ or CFF or tamoxifen-treated C++ EC (Figure 5A). There was no significant difference in wall thickness, left ventricular chamber size, or systolic function (assessed by echocardiography; Table) in the endothelium-specific NRG knockout mice (CFF) compared with controls either before (pre) or after (post) vehicle or tamoxifen treatment. There was also no significant difference in the baseline histological appearance of these hearts after vehicle or tamoxifen treatment, including capillary density (assessed...
ischemic injury, hearts from these animals were subjected to
30 minutes of low-flow ischemia followed by reperfusion. There was no effect of tamoxifen treatment or cre expression (as shown by the C++ tamoxifen mice) on postischemic recovery compared to vehicle-treated controls (Figure 5B and 5C). However, hearts with EC deletion of NRG demonstrated a significant impairment in systolic function during reperfusion compared with all control groups. This finding suggests that NRG may provide important cardioprotection in the intact heart.

Loss of Endothelial Neuregulins Expression Decreases Ischemic Tolerance in the Intact Heart

We next tested whether loss of EC NRG would also result in impaired ischemic tolerance in vivo. Using the same murine model of EC NRG deletion as described above, we performed ligation of the left coronary artery (20 minutes) followed by reperfusion (4 hours). There was no significant difference in the area at risk between the groups. However, the infarct area (either expressed as percentage total left ventricle or percentage area at risk) was significantly larger in the EC NRG-deleted animals compared with the control groups (Figure 6). In addition, the number of TUNEL-positive cells was significantly larger in the infarct regions of the EC NRG-deleted animals (3.8 ± 0.9/mm² versus 3.0 ± 0.5/mm² in C++ tamoxifen controls; P < 0.05), as was the number of infiltrating leukocytes (CD45 positive, 268 ± 32/mm² versus 170 ± 28/mm² in C++ tamoxifen controls; P < 0.05), both confirming more severe injury in animals with decreased EC NRG expression.

To test whether downstream erbB receptor activation is impaired in EC NRG-deleted hearts during ischemic injury, we isolated left ventricles from control (noninfarcted) and infarcted animals. The expression of erbB4 was examined by immunoblotting and activation of erbB4 was determined by immunoprecipitation and immunoblotting with antiphosphotyrosine and antierbB4 antibodies. There was no significant effect of EC NRG deletion on basal erbB4 expression or activation (Figure 6B). However, there was significantly less erbB4 activation in the infarcted left ventricles from EC NRG-deleted mice than control animals. These data support the idea that EC-derived NRG is an important activator of cardioprotective erbB4 signaling in the setting of ischemic injury.

To confirm that loss of NRG was indeed the cause of the increased infarct-related injury in the EC NRG-deleted animals, we administered rNRG to CFF tamoxifen-treated animals and performed left anterior descending artery ligation as before. Administration of exogenous rNRG fully reversed the effects of EC-selective NRG deletion, leading to significantly smaller infarct sizes that did not differ significantly from those seen in C++ tamoxifen or CFF vehicle controls. Taken together, the whole-organ perfusion data and in vivo infarct findings support the idea that loss of endothelial NRG expression significantly increases the susceptibility of the heart to injury in response to ischemic insults and further extends our observations made in the 2-cell coculture system.

Discussion

Much data have been published demonstrating that exogenous administration of NRG results in activation of erbB

Figure 5. Endothelial deletion of NRG impairs postischemic recovery in Langendorff perfused hearts. A, Western blot of pooled (3 to 4 hearts/sample), isolated, purified cardiac endothelial cells from either vehicle or tamoxifen-treated mice demonstrating a significant reduction of NRG expression after tamoxifen induction. A reprobe of the same blot using Actin as a loading control is shown in lower panel. B, Left ventricular developed pressures (LVDP) in perfused hearts from vehicle or tamoxifen-treated mice. Baseline and ischemic LVDP were not significantly different between groups. Reperfusion (postischemic, taken at end of 30 minutes reperfusion) LVDP was significantly impaired in CFF NRG knockout animals (CFF tamoxifen). *P < 0.05 versus all other groups. C, Rate–pressure products (RPP) in the same perfused hearts. Reperfusion RPP was also significantly impaired in the NRG knockout animals. *P < 0.05 versus all other groups. CFF indicates homozygously floxed NRG alleles.

by anti-CD31 staining, C++ vehicle: 141 ± 37 vessels/high power field; C++ tamoxifen: 188 ± 32/high power field; CFF vehicle: 151 ± 88/high power field; CFF tamoxifen: 151 ± 27/high power field, n = 3 to 4/group) To test whether animals with reduced EC expression of NRG are more susceptible to ischemic injury, hearts from these animals were subjected to
receptors that is cardioprotective. Because endothelium appears to be the major source of NRG in the heart, many investigators have speculated that these endothelium-derived NRG play a key role in vivo in the setting of cardiac injury. However, direct evidence that endothelium-derived NRG is the key factor mediating EC protection of myocytes against ischemic injury has been lacking. Our data now provide the crucial link to complete this important hypothesis.

The specific vascular beds that express NRG in the heart is of interest. Our data support the idea that endocardial, microvascular, and subendocardial coronaries express NRG. EC isolated from human epicardial (pooled left and right coronaries) coronaries also express abundant NRG in culture. This is in distinction to findings from other authors who have shown that rat epicardial coronaries do not appear to express NRG. The table below provides the echocardiographic evaluation of transgenics before and after treatment with tamoxifen or vehicle.

### Table. Echocardiographic Evaluation of Transgenics Before and After Treatment

<table>
<thead>
<tr>
<th>Echo Parameter*</th>
<th>Vehicle (n=5)</th>
<th>Tamoxifen (n=5)</th>
<th>Vehicle (n=13)</th>
<th>Tamoxifen (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVIDd, mm</td>
<td>3.09±0.20</td>
<td>3.03±0.10</td>
<td>3.04±0.16</td>
<td>3.27±0.28</td>
</tr>
<tr>
<td>LVIDs, mm</td>
<td>1.73±0.16</td>
<td>1.71±0.11</td>
<td>1.68±0.08</td>
<td>1.79±0.04</td>
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<tr>
<td>LVPWd, mm</td>
<td>0.74±0.13</td>
<td>0.75±0.03</td>
<td>0.71±0.09</td>
<td>0.76±0.04</td>
</tr>
<tr>
<td>LVAWd, mm</td>
<td>0.76±0.05</td>
<td>0.75±0.12</td>
<td>0.80±0.06</td>
<td>0.75±0.04</td>
</tr>
<tr>
<td>FS, %</td>
<td>44.15±1.77</td>
<td>43.58±1.73</td>
<td>44.76±1.74</td>
<td>45.06±4.27</td>
</tr>
<tr>
<td>EF, %</td>
<td>76.82±2.08</td>
<td>76.01±2.06</td>
<td>76.93±1.36</td>
<td>77.17±4.18</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>483±30</td>
<td>487±30</td>
<td>445±39</td>
<td>446±37</td>
</tr>
</tbody>
</table>

*Values are Mean±SD. LVIDd, left ventricular end-diastolic internal dimension; C++, (VEcad-creERT2), express cre recombinase only in the presence of tamoxifen and are NRG wild type; CFF, (VEcad-creERT2, NRG F/F), express cre recombinase only in the presence of tamoxifen and are NRG knockouts only in the presence of tamoxifen; LVIDs, ventricular end-systolic internal dimension; LVPWd, left ventricular diastolic posterior wall thickness; LVAWd, left ventricular diastolic anterior wall thickness; FS, fractional shortening; EF, ejection fraction; and HR, heart rate.

The specific vascular beds that express NRG in the heart is of interest. Our data support the idea that endocardial, microvascular, and subendocardial coronaries express NRG. EC isolated from human epicardial (pooled left and right coronaries) coronaries also express abundant NRG in culture. This is in distinction to findings from other authors who have shown that rat epicardial coronaries do not appear to express NRG. Because microvascular and endocardial EC provide...
the most extensive contact area with cardiac myocytes, it would seem logical that these EC play a critical role in EC-myocyte signaling during cardiac injury (eg, ischemia).

The regulation of NRG expression and release from EC is complex. Cleavage and release of NRG can be mediated by several metalloproteinases in response to different stimuli. We have confirmed that human cardiac EC express NRG and release this ligand in response to hypoxic stress. This important observation extends that of Sawyer et al, who were the first to show that NRG is released from murine hearts in a whole-organ model of ischemia/reperfusion. The idea that ischemia itself can promote NRG release from EC is an important distinction because in vivo or whole-organ models of ischemic injury, multiple other factors that can modulate NRG release may also come into play. For example, we have recently shown that some inflammatory cytokines are important activators of NRG release from EC. In the setting of chronic injury, such as that seen after myocardial infarction, inflammation is an important modulator of cardiac remodeling. Endothelial cell expression of NRG is also increased by endothelin-1 and mechanical strain and decreased by angiotensin II and phenylephrine. Thus, there may be multiple mechanisms in vivo that affect release of NRG from EC in response to ischemic injury in both the acute and chronic setting; however, our data support the idea that hypoxia alone is sufficient to activate this response in isolated EC.

The mechanisms by which NRG promotes cardiomyocyte protection have been studied primarily using isolated neonatal rat ventricular myocytes (NRVM), which may have important differences from adult cardiac myocytes. For this reason, we chose to use isolated adult myocytes in these studies. In other studies, activation of erbB4 with subsequent signaling through the phosphoinosotide 3-kinase/AKT pathway has been shown to be crucial for cardiac myocyte protection against anthracycline-induced apoptosis. Our data also support that erbB4 activation in response to ischemia may be an important consequence of EC NRG signaling. In addition to NRG, EC can produce several other potential cardioactive factors that could modulate in this process (eg, endothelin-1, nitric oxide or prostacyclins). Previous data using coculture or conditioned media models do not directly demonstrate that NRG is the factor secreted by EC that mediates cardioprotection during chemotherapeutic injury or oxidant stress (H2O2). By specifically and significantly reducing NRG expression using transgenic and siRNA techniques, we clearly demonstrate that NRG is a crucial cardioprotective factor provided by EC in the setting of ischemic injury, closing the loop between EC production of NRG and the requirement for erbB activation by EC for EC-mediated cardioprotection.

Previous studies demonstrating the importance of erbB2 and 4 receptors on maintenance of cardiac function fail to address the role of cardioprotection during ischemic insult because of the fact that sustained deletion of either of these receptors leads to spontaneous dilated cardiomyopathy, complicating use of these models to further study additional cardiac insults. The finding that germline deleted NRG heterozygotes have impaired tolerance to anthracycline toxicity also fails to address the important question about the role of endothelial NRG in the setting of ischemic insult, due to potential developmental and postnatal changes in the myocytes seen in these animals as well as inability to determine the specific cell type responsible for NRG production in this model. Our model has allowed us to delete NRG in an endothelium-selective manner in adult animals. By keeping our gene induction protocol short, we were able to study animals without baseline cardiac dysfunction after NRG deletion and demonstrate that EC-derived NRG provides an important cardioprotective effect in the setting of ischemic insult both in whole-organ and intact animal models.

In summary, we have shown that endothelium is a critical source of NRG for protection of myocytes against hypoxic/ischemic injury. The finding that human arterial endothelium can provide this factor (as shown in our coculture experiments) adds support to the notion that strategies aimed at modifying EC expression and release of NRG during ischemic insult may provide important clinical benefits.

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Disclosures

None.

References

It has long been known that cardiac endothelium provides more than just a conduit for delivery of oxygen and nutrients to cardiac myocytes. The removal of cardiac endothelium has important negative consequences for myocyte function. A number of potential factors responsible for this role of endothelium have been proposed, one of which is the epidermal growth factor family member neuregulin. Neuregulin is made by endothelial cells as a transmembrane precursor and can be released from endothelium after several types of stimuli, including hypoxic injury. In this article, we provide direct evidence that endothelium-derived neuregulin is an important protective factor for cardiac myocytes in the setting of ischemic injury using both in vitro and in vivo models. Importantly, in the setting of loss of neuregulin expression by endothelium, administration of exogenous recombinant neuregulin can rescue the phenotype of increased sensitivity to ischemic injury. This places neuregulin in a small group of endothelium-derived cardioprotective factors that may be clinically useful targets for therapy in ischemic cardiovascular diseases.
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SUPPLEMENTAL MATERIAL

Detailed Methods

Cells and reagents

Endothelial cell isolation from lungs (lung microvascular EC, LMVEC) was performed by collagenase digestion (2mg/ml, ~172U/mg, Type I Collagenase, Sigma-Aldrich). EC were purified (to >90%, as assessed by FACS analysis) by serial passage over anti-mouse CD 102 (ICAM-2, Pharmigen)-coated magnetic beads (M450 sheep anti-rat IgG Dynal beads (Invitrogen Dynal AS, Osb, Norway) and grown on 0.1% gelatin-coated flasks in low-glucose DMEM/F12 (Invitrogen) with 20% fetal bovine serum. LMVEC were infected using AdenoCre or control virus for 24 hours at an MOI of 100:1 and returned to normal culture without virus for an additional 24 hours prior to use in co-culture experiments. For in vitro experiments, LMVEC were isolated from NRG F/F mice and treated with AdenoCre as described above to achieve NRG gene deletion.

Monolayers of HCAEC (60% confluent) in 6 well plates were transfected with scrambled control siRNA (Ambion, Inc.) or NRG siRNA (100nM, Ambion, Inc.) using Lipofectamine 2000™ (Invitrogen). Adenoviruses containing CMV promoter driven cre recombinase expression (AdenoCre) or control adenovirus expressing DS-Red (AdenoRed) were a kind gift from Dr. Frank Giordano (Yale School of Medicine).\(^1\) Cells lysates from siRNA or adenovirus treated EC were analyzed by Western blot for cre expression with anti-cre antibody (Novagen), anti-βtubulin (Santa Cruz Biotechnology) or NRG expression with SC-348 (Santa Cruz Biotechnology).

TUNEL, annexin V and propidium iodide (PI) staining

Mouse myocytes were subjected to terminal deoxynucleotidytransferase dUTP nick end labeling (TUNEL) using an in situ cell death detection kit (Roche). Annexin V was detected using a FITC-labeled antibody-based staining kit (BD Pharmingen). PI staining was performed by incubating
sections in 1 μg/ml PI (Sigma-Aldrich) for 30 minutes at room temperature. Slides were rinsed with PBS and fixed in 4% paraformaldehyde. All slides were mounted in medium with DAPI (Vector Laboratories, Inc) and analyzed by fluorescence microscopy.

**Animal derivations and treatments**

All animals were in a C57Bl6 background. Based on previously published information, we performed dose/timecourse testing to ensure cre expression after tamoxifen treatment in reporter mice (generated from VEcad-creERT2 mice crossed into a ZEG reporter line). We confirmed that endothelial-selective cre expression and gene recombination can be efficiently achieved after i.p. tamoxifen injection in 3-5 days in animals less than 6 weeks of age (data not shown), as previously reported. Tamoxifen (Sigma-Aldrich) stock was prepared by sonication at a concentration of 100mg/ml in ethanol. A working solution was prepared by diluting the stock 1:10 in autoclaved sunflower oil (Sigma-Aldrich). rNRG (R&D Systems) was prepared in PBS with 0.1% BSA and injected i.p. in the opposite flank from that injected with the tamoxifen preparation.

**Langendorff perfusions**

Hearts were perfused with Krebs-Henseleit buffer containing 7 mM glucose, 0.4 mM oleate, and 1% BSA for 30 minutes under normal flow conditions (4 ml/min) followed by 30 minutes of low-flow (0.75 ml/min) ischemia and then 30 minutes of reperfusion at 4 ml/min. A fluid-filled balloon, attached to a solid-state pressure transducer (Millar Instruments, Houston, TX), was inserted into the left ventricle via a left atriotomy to measure left ventricular developed pressure and heart rate using a digital acquisition system (ADInstruments, Colorado Springs, CO) at a balloon volume that resulted in a baseline left ventricular end-diastolic pressure of 5 mm Hg.

**Infarct models and staining**
To measure infarct size, unfixed hearts were stained with TTC leaving necrotic tissue white and viable tissue red. The in vivo non-ischemic region was stained by infusing Evan’s blue dye after transient re-occlusion of the left coronary artery (to determine area at risk). Heart were fixed overnight in formalin and sectioned the following day into 1mm thick slices and photographed. Images were analyzed using NIH Image J software (http://rsbweb.nih.gov/ij/).

References: