Role of Neuregulin-1/ErbB2 Signaling in Endothelium-Cardiomyocyte Cross-talk*

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Neuregulin-1 (NRG-1), a cardioactive growth factor released from endothelial cells, has been shown to be indispensable for the normal function of the adult heart by binding to ErbB4 receptors on cardiomyocytes. In the present study, we have investigated to what extent ErbB2, the favored co-factor of ErbB4 for heterodimerization, participates in the cardiac effects of endothelium-derived NRG-1. In addition, in view of our previously described anti-adrenergic effects of NRG-1, we have studied which neurohormonal stimuli affect endothelial NRG-1 expression and release and how this may fit into a broader frame of cardiovascular physiology. Immunochemistry staining of rat heart and aorta showed that NRG-1 expression was restricted to the endocardial endothelium and the cardiac microvascular endothelium (CMVE); by contrast, NRG-1 expression was absent in larger coronary arteries and veins and in aortic endothelium. In rat CMVE in culture, NRG-1 mRNA and protein expression was down-regulated by angiotensin II and phenylephrine and up-regulated by endothelin-1 and mechanical strain. CMVE-derived NRG-1 was shown to phosphorylate cardiomyocyte ErbB2, an event prevented by a 24-h preincubation of myocytes with monoclonal ErbB2 antibodies. Pretreating cardiomyocytes with these inhibitory anti-ErbB2 antibodies signified the cardiotoxicity of ErbB2 antibodies. Accordingly, ErbB2 signaling participated in the paracrine survival and growth controlling effects of NRG-1 on cardiomyocytes in vitro, explaining the cardiotoxicity of ErbB2 antibodies in patients. Cardiac NRG-1 synthesis occurs in endothelial cells adjacent to cardiac myocytes and is sensitive to factors related to the regulation of blood pressure.

In the adult heart, the neuregulin (NRG) receptors ErbB2 and ErbB4, but not ErbB3, are found on cardiomyocytes, whereas NRG-1 has been detected in the endothelium (1). Binding of NRG-1 to its receptor induces the formation of homo- and heterodimers, which is crucial for signaling (2). Although NRG-1 does not bind directly to ErbB2, it is the favored co-receptor for heterodimerization (3). This means that, in the adult heart, NRG-1 signaling can occur through ErbB2/ErbB4 heterodimers and/or ErbB4/ErbB4 homodimers. The importance of NRG/ErbB signaling in the adult heart was revealed by an unforeseen side effect of trastuzumab (Herceptin), a monoclonal antibody against ErbB2 used in the treatment of breast cancer. Unexpectedly, trastuzumab induced dilated cardiomyopathy and heart failure in human patients when combined with a treatment of anthracycline (4, 5). In addition, postnatal conditional mutation of cardiac ErbB2 leads to dilated cardiomyopathy in the mouse (6).

Despite these observations, the specific role of ErbB2 in the cardioprotective actions of NRG-1 has remained controversial. Hence, the interpretation of the cardiotoxic effects of trastuzumab in patients has remained difficult. For example, Grazette et al. (7) indicate that inhibition of ErbB2 phosphorylation with a human inhibitory antibody that cross-reacts with the rat receptor homologue spontaneously activates the mitochondrial apoptosis pathway in neonatal rat cardiomyocytes. Similarly, Rohrbach et al. report that targeting ErbB2 with antisense technology activates the same mitochondrial apoptotic cascade as anthracycline (8). In contrast, Ozcelik et al. (6) have not observed increased myocardial apoptosis in cardi-specific ErbB2 knock-outs, and Fukazawa et al. report that the anti-apoptotic effect of NRG-1 in cardiomyocytes does not involve ErbB2 (9). In the latter study, the role of ErbB2 was studied with an ErbB2-stimulating antibody that failed to reveal a role of ErbB2 in the cell viability-modulating activities of NRG-1.

NRG biology is, besides its complexity at the receptor level, further complicated by the release of multiple spliced variants of the NRG gene product. Cote et al. (10) demonstrate that cardiac endothelial cells release various NRG-1 isotypes. Studying the NRG-ErbB signaling axis and the specific role of ErbB2 with exogenously administered recombinant NRG-1, as done in most studies, does therefore not mimic per se the molecular events induced by the “mixture” of ligands released by the endothelial organ.

In the present study, we have investigated the role of ErbB2 in the hypertrophic and anti-apoptotic effects mediated by the cardiac endothelium through experiments with endothelium-cardiomyocyte co-cultures or endothelium-conditioned medium and with recombinant NRG-1 as a positive control. Prior to these studies, we verified and confirmed that the endothelium is the main source of NRG-1 in the heart and studied to
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what extent NRG-1 synthesis and release from the endothelium responds to neurohormonal and biomechanical stimuli.

EXPERIMENTAL PROCEDURES

Reagents—Anti-ErbB2 antibody (Ab-9, clone B10) was purchased from Neomarkers. All other chemicals were purchased from Sigma.

Cell Culture—Cardiac microvascular endothelial cells (CMVE) and rat aortic endothelial cells were isolated and cultured as previously described (11, 12). Only second passage endothelial cells were used for experiments. Confluent cell cultures were serum-starved for 24 h prior to the start of the experiments. Purity of the cell cultures has been demonstrated previously (12) and confirmed at several points throughout the study. Neonatal rat cardiac myocytes were isolated from 1- to 2-day-old Sprague-Dawley rats and cultured as previously described (13). AD293 cells were purchased from Stratagene (La Jolla, CA) and cultured following instructions of the vendor.

Conditioned Medium and Co-Culture—To obtain conditioned medium, CMVE was grown to confluence on 75-cm² culture flasks using Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. At confluence, the medium was changed to Dulbecco’s modified Eagle’s medium containing 0.1% fetal calf serum. The medium was collected 48 h later and stored at −20 °C until further use. For co-culture experiments, CMVE was grown to confluence on 6-well plate inserts (Biocoat®, serum-starved (0.1% fetal calf serum) for 24 h, and inserted into wells in which neonatal rat cardiac myocytes were grown and serum-starved for 24 h.

Strain Experiments—For stretch experiments, cells were cultured on collagen-coated silicone membranes that allow controlled cyclic biaxial uniform strain (14).

Rat Aortic Banding—Left ventricular hypertrophy was induced in adult Sprague-Dawley rats (224 g mean body weight) by a transverse aortic constriction (TAC) (15). Briefly, rats were anesthetized with fentanyl (intramuscular, 0.05 mg/kg, Janssen-Cilag), diazepam (5 mg/kg, Roche Applied Science), and haloperidol (3 mg/kg, Janssen-Cilag) and subsequently intubated endotracheally and ventilated. A left lateral thoracotomy was performed exposing the heart and ascending aorta. A 5–0 black braided silk suture (Deknatel) ligature was tied around the ascending aorta between the innominate artery and the left carotid artery against an 18-gauge needle, and the needle was then removed. At least four rats in each group were killed at 2, 4, and 8 weeks after surgery. Age-matched control animals underwent a sham operation, including thoracotomy and intubation endotracheally and ventilated. A left lateral thoracotomy was performed exposing the heart and ascending aorta. A 5–0 black braided silk suture (Deknatel) ligature was tied around the ascending aorta between the innominate artery and the left carotid artery against an 18-gauge needle, and the needle was then removed. At least four rats in each group were killed at 2, 4, and 8 weeks after surgery. Age-matched control animals underwent a sham operation, including thoracotomy but without banding of the aorta. Hypertrophy was assessed from the heart weight/body weight ratio and echocardiographic parameters. Echocardiograms were performed on lightly anesthetized animals using a Vingmed System 5 equipped with a 10-MHz transducer before surgery and at the time of killing. Left ventricular anterior and posterior wall thickness and end-diastolic and end-systolic internal dimensions (end diastolic dimension (EDD), end systolic dimension (ESD)) were measured on three consecutive cycles and averaged by a single observer in a blinded fashion. Left ventricular end diastolic volume was obtained using the Teichholz method (16), \((7/(2.4 + EDD))×100\). Fractional shortening was calculated as the % of fractional shortening, \(((EDD – ESD)/ EDD)\)×100. (17).

Real-time Quantitative Reverse Transcription-PCR—Cells were harvested, and rat heart tissue (100 mg) was mixed in TRIzol® reagent (Invitrogen). RNA was isolated following instructions of the manufacturer. Real-time PCR was performed with the TaqMan® one-step reverse transcription-PCR system (Applied Biosystems) in a 25-μl reaction volume containing 5 μl of total RNA (10–100 ng), 12.5 μl of one-step reverse transcription-PCR Master Mix, 0.5 μl of RNase inhibitor, 100–800 nm both primers and 400 nm probe. After initial incubation for 30 min at 48 °C and 10 min at 95 °C, 45 PCR cycles were performed that consisted of 15 s of denaturation at 95 °C and 60 s of annealing and extension at 60 °C on an ABI Prism 7700 sequence detection system. TaqMan® probes were labeled with 6-carboxy-fluorescent reported dye and 6-carboxy-tetramethylrhodamine quencher dye. Expression was normalized to GAPDH expression. Sequences for specific primers and probes were as follows: rat brain natriuretic peptide (BNP, GenBank™ accession number NM_031545) sense, 5’-TGGGCAGAAGATAGACCGGA-3’; antisense, 5’-ACAAC-
CTAGCCCGTAC-3 and probe, 5'-CCAAGCGACTGGCCG-3'; rat GAPDH (GenBank® accession number NM_017008) sense, 5'-GCCTCGTCTAGACAGATGGT-3' and probe, 5'-GAAGGCAGCCCTGGTAACC-3'. To assess NRG-1 expression, the rat NRG Assay-on-demand (Applied Biosystems) was used consisting of premixed primers and TaqMan™ probe. This assay recognizes the NRG-1 gene but does not discriminate between its isoforms.

**Immunohistochemistry**—Immunohistochemistry was performed with anti-NRG-1α or -NRG-1β antibody (Santa Cruz Biotechnology) and anti-PECAM (platelet endothelial cell adhesion molecule) antibodies (SeroFec). Cryostat sections of rat hearts and aortas were obtained as previously described (18). Briefly, following fixation with acetone for 10 min, cryo-sections were incubated with blocking solution (goat IgG, 0.2% bovine serum albumin in phosphate-buffered saline) at room temperature. After 30 min, the sections were incubated with primary antibody overnight at 4 °C. Subsequently, the secondary antibody was incubated for 2 h at 37 °C and after three washes, the sections were mounted in Slowfade plus glycerol (Molecular Probes). Images were made on an Olympus fluorescence microscope with a Sensicam charge-coupled device camera and stored on a computer for later analysis with Photoshop software.

**Immunoprecipitation and Western Analysis**—Immunoprecipitation followed by Western blotting was performed (19, 20) with the following modifications. Cells were harvested in lysis buffer containing 0.2% Triton X-100 and protease inhibitor mixture (Sigma). Heart tissue was mixed in the same lysis buffer (100 mg/ml) with a Polytron homogenizer (Pt 2100, Kinematica, Littau, Switzerland). Equal amounts of cell lysates were incubated with primary antibody at 4 °C overnight, in which, thereafter, protein A/G plus agarose beads (Santa Cruz Biotechnology) were added. Proteins were separated on NuPAGE® BisTris gels (Invitrogen) and electrotransferred to a polyvinylidene difluoride membrane ( Pierce). The membranes were blocked in 5% nonfat dry milk with 0.1% Tween 20 and incubated with primary antibody overnight at 4 °C and with secondary horseradish peroxidase-conjugated antibody for 2 h at room temperature. The signal was revealed with Supersignal West Pico chemiluminescent substrate (Pierce). NRG-1 in CMVE cell lysates (300 μg) was immunoprecipitated with an antibody
targeting a common extracellular domain (Ab-1, clone 7D4; Neomarkers), on which, thereafter, Western analysis was performed with an antibody against the carboxyl terminus of NRG-1 (C-20, Santa Cruz Biotechnology). Equal volumes of CMVE culture medium were condensed using Centriplus YM-10 centrifugal filter units (Millipore) to 1.5 ml; proteins were further precipitated with trichloroacetic acid and immunoblotted with Ab-1. Activation of ErbB2 was determined in the cell lysates of neonatal rat cardiac myocytes (500 μg) by immunoprecipitation with anti-ErbB2 antibody (Cell Signaling Technologies) and immunoblotting with anti-phosphotyrosine antibody (P-Tyr-100, Cell Signaling Technologies). A phosphatase inhibitor specific for tyrosine protein phosphatases was added to the lysis buffer (phosphatase inhibitor mixture 2, Sigma).

Cardiomyocyte Surface Area—Neonatal rat cardiac myocytes were cultured on culture slides in 6-well plates at a density of 5 × 10^5 cells/well and stimulated for 72 h by co-culture with CMVE or by medium conditioned by CMVE. After stimulation, the cells were incubated with BCECF-AM (2′,7′-bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein acetoxymethyl ester) (Molecular Probes) and fixed in 4% paraformaldehyde. The cells were visualized using an Olympus fluorescence microscope with a Sensicam charge-coupled device camera, and the surface area was calculated with image analysis software (SigmaScan Pro 5). For each group, at least 80–100 cardiomyocytes were examined in eight randomly selected fields at 20× magnification in three independent experiments. During the analyses, the investigator was unaware of the treatment the cells had received.

Cell Death Assays—Apoptosis was assessed using TUNEL staining with the in situ Cell Death Detection Kit (Roche Applied Science) according to the manufacturer’s protocol. In

![Figure 3](image3.png)  **FIGURE 3.** Effect of endothelin-1 and cyclic stretch on NRG-1 protein levels in CMVE. (A) CMVE was treated with endothelin-1 (100 ng/ml) or 5% cyclic stretch for the indicated time periods. Equal amounts of cell lysates (300 μg) were immunoprecipitated (IP) with Ab-1 and immunoblotted (IB) with C-20. The same cell lysates were also used for the immunoblotting with actin antibody. (B), the supernatant of treated CMVE was condensed using Centriplus filter units (Millipore), and proteins were further precipitated with trichloroacetic acid and immunoblotted with Ab-1. Blots are representative of at least three different experiments. Results were quantified by densitometry. *

![Figure 4](image4.png)  **FIGURE 4.** NRG-1 mRNA expression during pressure overload in rat. (A) NRG-1 mRNA expression was assessed with real-time PCR in hearts of rat with a transverse aortic constriction (TAC) during 2, 4, 8, and 16 weeks. Aged-matched sham-operated animals served as the control (sham). *, p < 0.05 versus control. B, equal amounts of cell lysates from rat heart at 8 weeks (left) and 16 weeks (right) after sham operation or TAC were immunoblotted (IB) with NRG Ab-1. The same cell lysates were immunoblotted with actin antibody to control for equal loading. *, p < 0.05 versus control.
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FIGURE 5. CMVE activates ErbB2 and ERK in cardiomyocytes. Cardiomyocytes were incubated with NRG-1β (20 ng/ml)- or CMVE-conditioned medium (CoMe) for 10 min. Anti-ErbB2 (1 µg/ml) antibody was administered to cardiomyocytes 24 h in advance. A, effect of NRG-1β- and CMVE-conditioned medium on ErbB2 phosphorylation in cardiomyocytes. Cells lysates (500 µg) were first immunoprecipitated (IP) with anti-ErbB2 and then Western blotted (IB) with anti-phosphotyrosine antibody. The same cell lysates were also used for immunoblotting with actin antibody. B, effect of NRG-1β- and CMVE-conditioned medium on ERK phosphorylation (pERK) in cardiomyocytes. Immunoblotting (IB) was performed for pERK and actin. Blots are representative of three different experiments.

RESULTS

Endothelial NRG-1 Expression—
Immunohistochemical staining of rat hearts and aortas was performed to study the endothelial expression of NRG-1 in vivo. Fig. 1A shows that a distinct staining of NRG-1β was detected in the endocardial endothelium and in the endothelium of the myocardial microvasculature (CMVE), but it was negative in aortic endothelium. Staining was also negative in the endothelium of larger coronary arteries and veins. Similar results were obtained for NRG-1α (not shown). Also, expression of NRG-1 mRNA was abundant in cultured CMVE and endocardial endothelium and remained nearly undetectable in cultured aortic endothelium (Fig. 1B).

NRG-1 mRNA expression in CMVE significantly changed upon exposure of CMVE to a number of physiological stimuli. Endothelin-1 (100 nmol/liter) and cyclic mechanical strain (5%, 1 Hz) time-dependently up-regulated endothelial NRG-1 mRNA expression with a maximal increase of 11.8 ± 6.7-fold (n = 4, p = 0.01) after a 6-h incubation of endothelin-1 and of 3.5 ± 0.4-fold (n = 4, p = 0.04) after 4 h of cyclic mechanical strain (Fig. 2A). Fig. 2B shows that the up-regulation of NRG-1 mRNA by cyclic mechanical strain was amplitude-dependent following a bell-shaped curve with a maximum at 1% strain. NRG-1 expression in CMVE was time-dependently down-regulated by angiotensin II (maximum 4.0-fold, n = 4, p = 0.03) and phenylephrine (maximum 1.9-fold, n = 4, p = 0.02) (Fig. 2C). The down-regulation by angiotensin II was dose-dependent with a maximal effect at 100 nmol/liter (Fig. 2D). NRG-1 expression was unaffected by tumor necrosis factor-α and interleukin-1β (data not shown).

Up-regulation of NRG-1 expression by endothelin-1 and cyclic mechanical strain was confirmed at the protein level by immunoblot analysis. Fig. 3A shows that endothelin-1 and strain significantly increased the expression of a 115-kDa immunoreactive protein detected by immunoprecipitation with NRG Ab-1 and immunoblotting with C-20. This band corresponds to the transmembrane NRG-1 pro-protein (10). In addition, endothelin-1 and cyclic mechanical strain increased the presence in the culture medium of an ~30-kDa protein detected by the NRG Ab-1 (Fig. 3B), suggesting that both stimuli also induce cleavage and release as between group factors, with post hoc Bonferroni testing for comparison of expression across the group.
of NRG-1. Endothelin-1 and mechanical strain also increased the abundance of a 60-kDa immunoreactive protein (not shown), but it is not completely certain whether this band corresponds to a NRG-1 product (10).

Finally, regulation of NRG-1 mRNA expression in vivo was assessed during pressure overload in rat. At 8 weeks, TAC-induced pressure overload in rat resulted in concentric left ventricular hypertrophy (heart weight/body weight 5.1 ± 0.6 versus 3.4 ± 0.1 in sham, p < 0.05; left ventricular end diastolic volume 250 ± 26 versus 220 ± 20 in sham, p = 1.00, n = 5). At this stage, NRG-1 mRNA was 13.7 ± 5.5-fold increased in TAC versus sham (p = 0.02, n = 5) (Fig. 4A), and NRG-1 protein was 3.8 ± 0.1-fold increased in TAC versus sham (p < 0.01, n = 3) (Fig. 4B). At 16 weeks, TAC-induced pressure overload in rat resulted in eccentric left ventricular hypertrophy (heart weight/body weight 4.3 ± 0.3 versus 3.3 ± 0.17 in sham, p < 0.05; left ventricular end diastolic volume 350 ± 26 μl versus 226 ± 13 μl in sham, p < 0.05, n = 11) with reduced fractional shortening (fractional shortening 47 ± 4% versus 58 ± 2 in sham, p < 0.05, n = 11), hallmarks of left ventricle dysfunction and failure. At this stage, NRG-1 mRNA and protein expression levels fell to base-line values upon the transition to eccentric left ventricular hypertrophy and left ventricular dysfunction (Fig. 4).

**Activation of ErbB2**—Fig. 5A shows that CMVE-conditioned medium induced phosphorylation of ErbB2 and subsequent activation of ERK1/2 in cardiomyocytes to the same extent as recombinant NRG-1β. A 24-h pretreatment of cardiomyocytes with anti-ErbB2 antibody was able to block both effects. In contrast with the known acute effects of this antibody, a 24-h treatment with anti-ErbB2 antibody did not induce receptor phosphorylation or activation of ERK.

**NRG-1 and the Hypertrophic Effects of CMVE on Cardiomyocytes**—It has been shown previously that exogenous recombinant NRG-1β promotes hypertrophic growth of adult cardiac myocytes in vitro through ErbB4 (1). We investigated the role of endothelium-derived NRG-1β in the paracrine hypertrophic effects of CMVE on cardiomyocytes by analyzing (i) cardiomyocyte hypertrophy induced by medium conditioned by CMVE and (ii) cardiomyocyte hypertrophy induced by co-culture with CMVE (communication through Transwell filters), both in the presence and absence of the inhibitory anti-ErbB2 antibody.

Cardiomyocyte hypertrophic growth responses were assessed from BNP mRNA expression and cardiomyocyte surface area. As shown in Fig. 6A, a significant increase in BNP mRNA expression was identified in cardiomyocytes exposed for 24 h to NRG-1 (5.1 ± 0.9-fold increase, n = 5, p = 0.003 versus control), in cardiomyocytes exposed to CMVE-conditioned medium (3.7 ± 0.8-fold increase, n = 6, p = 0.01 versus control), and in cardiomyocytes co-cultured with CMVE (2.5 ± 1.1-fold increase, n = 8, p = 0.04 versus control). Consistently, NRG-1, CMVE-conditioned medium, and co-culture increased cell surface area (control, 1032 ± 41 μm², n = 6; 72 h of NRG-1, 1418 ± 62 μm², n = 6, p = 0.04; conditioned medium, 1856 ± 116 μm², n = 6, p < 0.001; co-culture, 1525 ± 172 μm², n = 4, p = 0.01). Co-culture of cardiomyocytes with aortic endothelial cells or AD293 cells had no effect (data not shown). Importantly, pretreating cardiomyocytes during 24 h with an anti-ErbB2 antibody (1 μg/ml) completely abolished the increase of BNP mRNA expression and of cell surface area induced by CMVE-conditioned medium and co-culture (Fig. 6).

**NRG-1 and the Anti-apoptotic Effects of CMVE on Cardiomyocytes**—It has been shown previously that exogenous recombinant NRG-1β protects adult cardiac myocytes against anthracycline and β-adrenergic receptor-induced apoptosis in vitro. We investigated the role of endothelium-derived NRG-1β in the paracrine anti-apoptotic effects of CMVE on cardiomyocytes.

Daunorubicin (1 μmol/liter) induced a significant increase in TUNEL-positive nuclei from 10.6 ± 0.3% in control to 30.5 ± 2.1% after 24 h of daunorubicin treatment (n = 3, p = 0.001). When cardiomyocytes were pretreated with CMVE-conditioned medium or exogenous NRG-1β for 1 h, the apoptotic effect of daunorubicin was completely abrogated (TUNEL assay; daunorubicin plus conditioned medium, 10.3 ± 2.9%, n = 3, p = 0.001 versus daunorubicin alone; daunorubicin plus NRG-1β, 17.3 ± 0.4%, n = 3, p = 0.04 ver-
Importantly, this anti-apoptotic effect of CMVE-conditioned medium was completely abolished by pretreating cardiomyocytes with anti-ErbB2 antibody (TUNEL assay, 31.3 ± 1.1%, n = 3, p = 0.001 versus control) (Fig. 7, A and B). Anti-ErbB2 antibody alone did not induce apoptosis and did not enhance anthracycline-induced apoptosis (not shown). Similar observations were made when apoptotic responses were analyzed with annexin labeling (Fig. 7C).

**DISCUSSION**

During fetal maturation, NRG-1 plays a crucial role in cardiac development through activation of ErbB2 and ErbB4 oncogene receptors on cardiomyocytes (22–26). Previous studies established a role for NRG-1 beyond cardiac development, but inconsistent conclusions have been made on the specific role of ErbB2 in the anti-apoptotic effects of NRG-1 in postnatal cardiomyocytes. Given the fact that monoclonal inhibitory antibodies against ErbB2 are efficient in the treatment of breast cancer but also induce cardiomyopathy, we further investigated the role of ErbB2 in the regulation of cardiomyocyte viability. After confirming that the cardiac endothelium is the main source of NRG-1 in the heart, we studied NRG-ErbB signaling by using experimental models of endothelium-cardiomyocyte cross-talk. Next, we showed that inhibition of ErbB2 phosphorylation and subsequent signaling with inhibitory monoclonal antibodies abrogated endothelium-induced growth and survival of cardiomyocytes, providing direct proof for a role of ErbB2 in endothelium-cardiomyocyte cross-talk and in the preservation of myocardial integrity.

Our results on the obligatory role of ErbB2 in the anti-apoptotic effects of NRG-1 may appear somewhat surprising at first glance, especially because previous experiments with the same monoclonal antibody led to opposite conclusions. Closely comparing our experiments with those from Fukazawa et al. (9) provide, however, a plausible explanation. First, Fukazawa et al. use this monoclonal antibody as a stimulatory antibody to selectively activate ErbB2. Indeed, consistent with our experiments, the authors show that the monoclonal antibody induces ErbB2 phosphorylation on cardiomyocytes, at least within the first minutes after administration. When subsequently assessing the anti-apoptotic effects of this antibody on cardiomyocytes, however, Fukazawa et al. preincubate the antibody for 24 h prior to

**FIGURE 7. Anti-apoptotic effects of CMVE-derived NRG-1 on cardiomyocytes.** Cardiomyocytes were incubated for 24 h with daunorubicin (1 μmol/liter). 1 h prior to daunorubicin administration, cells were treated with NRG-1β (20 ng/ml)- or CMVE-conditioned medium. To some cultures, anti-ErbB2 antibody was administered 24 h in advance. Control cardiomyocytes were not exposed to any treatment. A, representative neonatal rat cardiomyocytes stained with the TUNEL technique (top panel). The total number of cells was counted by staining with DAPI (lower panel). B, bar graph showing the quantitative data from the TUNEL assays. C, bar graph showing the quantitative data from the annexin labeling. All values are means ± S.E. of three independent experiments. *, p < 0.05 versus control; §, p < 0.05 versus daunorubicin.
exposing the cardiomyocytes to daunorubicin in the assumption that the stimulatory effects of the antibody on ErbB2 and subsequent signaling would last for so long. In contrast to this assumption, in our experiments, performed 24 h after administration, ErbB2 was indeed no longer phosphorylated and neither was downstream ERK-1. Instead, the antibody had become inhibitory, preventing the phosphorylation of ErbB2 and ERK-1 phosphorylation by cardiac endothelial cells and recombinant NRG-1. It has been previously demonstrated (27–29) that inhibitory ErbB2 antibodies can transiently stimulate receptor phosphorylation but function only as partial agonists. The prolonged inhibitory effect is due to the acceleration of endocytosis and degradation of the ErbB2 receptor.

Second, Fukazawa et al. (9) investigate the effects of the antibody on daunorubicin-induced apoptosis per se, i.e. in the absence of other stimuli, and observe no effect of the antibody. This result is identical to ours; yet in parallel experiments, we showed that the antibody inhibited the protective effects of cardiac endothelial cells and of recombinant NRG-1. Based on these results, we conclude that ErbB2 does participate in cardiomyocyte anti-apoptotic NRG-1 signaling and believe we have resolved at least some of the controversy.

There is substantial experimental evidence that cardiac endothelial cells in the endocardium and in the coronary microvasculature directly affect the cardiac mechanical performance, growth, and rhythmicity of the adjacent myocardium. These effects appear to be mediated by diffusible substances (such as nitric oxide, prostacyclin, and endothelin-1) and as more recently demonstrated by NRG-1 (30). Non-paracrine interactions between endothelial cells and cardiomyocytes, involving endothelium-dependent electrochemical gradients, have also been described, at least for the endocardial endothelium (31). Endothelial activation and subsequent dysfunction occur in response to physiological and pathophysiological stimuli and are early events in cardiovascular homeostasis and disease (30). In this study, we have shown that endothelial NRG-1 synthesis is sensitive to modulation by angiotensin-II and phenylephrine. These hormones are adaptively released in the circulation in conditions of low blood pressure to enhance cardiac output and to increase peripheral resistance. The suppressive actions of both hormones on endothelial NRG-1 signaling fit with these blood pressure-enhancing actions, as they will reduce the anti-adrenergic activity of NRG-1 on the heart (32, 33). Consistently, increased mechanical deformation and endothelin-1, characteristic for hypertensive states, have opposite effects on endothelial NRG-1. Finally, to what extent beneficial effects of pharmacological angiotensin II and phenylephrine blockade in chronic heart failure (34, 35) are related to the effects on endothelial NRG-1 is unknown but deserves further investigation.

In pressure overload-induced hypertrophy, NRG-1 was up-regulated, but this up-regulation faded away upon the transition to left ventricular dysfunction and failure. We speculate that the increase in NRG-1 expression is an adaptive response to mechanical overload (consistent with the up-regulation of NRG-1 by cyclic mechanical strain in vitro) but that subsequent activation of neurohormonal factors, such as angiotensin II and phenylephrine, exerts opposing effects on NRG-1 expression, thereby decreasing the expression levels. Therefore, it would be interesting to investigate whether pharmacological inhibition of angiotensin II and phenylephrine preserves high protective levels of NRG-1 in the cardiac endothelium during progression of heart failure and to what extent this effect contributes to the beneficial effects of these drugs.

Cardiac endothelial cells release several isoforms of NRG-1. Consistently, in our experiments, several bands were detected by immunoblotting NRG-1 from endothelial cell lysates. Interestingly, apart from a 115-kDa band and several somewhat smaller bands known to correspond to the transmembrane NRG pro-protein (10, 36–38), an additional 60-kDa band was observed, whose expression also increased in response to endothelin-1 and mechanical deformation (not shown). Whether this band represents immature preglycosylated NRG proteins or carboxyl-terminal fragments resulting from cleavage of pro-proteins (10, 39, 40) or instead has to be considered as a protein unrelated to NRG-1 (10) was not further addressed in this study. Immunoblotting NRG-1 in the medium of cardiac endothelial cells, however, only generated one band of ~30 kDa corresponding to cleaved NRG-1, confirming previous observations that cardiac microvascular endothelial cells contain the proteases necessary for cleavage of NRG-1 (38).

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REFERENCES

1. Zhao, Y. Y., Sawyer, D. R., Baliga, R. R., Opel, D. J., Han, X., Marchionni, M. A., and Kelly, R. A. (1998) J. Biol. Chem. 273, 10261–10269
2. Holbro, T., and Hynes, N. E. (2004) Annu. Rev. Pharmacol. Toxicol. 44, 195–217
3. Burden, S., and Yardon, Y. (1997) Neuron 18, 847–855
4. Feldman, A. M., Lorell, B. H., and Reis, S. E. (2000) Circulation 102, 272–274
5. Slamon, D. J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., Baselga, J., and Norton, L. (2001) N. Engl. J. Med. 344, 783–792
6. Ozcelik, C., Erdmann, B., Pitz, B., Wettscureck, N., Britsch, S., Hubner, N., Chien, K. R., Birchmeier, C., and Garrant, A. N. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 8880–8885
7. Grazette, L. P., Boecker, W., Matsu, T., Semigran, M. J., Force, T. L., Hajjar, R. J., and Rosenzweig, A. (2004) J. Am. Coll. Cardiol. 44, 2231–2238
8. Rohrbach, S., Muller-Werdan, U., Werdan, K., Koch, S., Gellerich, N. F., and Holtz, J. (2005) J. Mol. Cell. Cardiol. 38, 485–493
9. Fukazawa, R., Miller, T. A., Kuramochi, Y., Frantz, S., Kim, Y. D., Marchionni, M. A., Kelly, R. A., and Sawyer, D. B. (2003) J. Mol. Cell. Cardiol. 35, 1473–1479
10. Cote, G. M., Miller, T. A., Lebrassere, N. K., Kuramochi, Y., and Sawyer, D. B. (2005) Exp. Cell Res. 311, 135–146
11. Nishida, M., Carley, W. W., Gerritsen, M. E., Ellingsen, O., Kelly, R. A., and Smith, T. W. (1993) Am. J. Physiol. 265, H639–H652
12. Hendrickx, J., Doggen, K., Weinberg, E. O., Van Tongelen, P., Fransen, P., and De Keulenaer, G. W. (2004) Physiol. Genomics 19, 198–206
13. Yamamoto, K., Dang, Q. N., Kennedy, S. P., Osathanondh, R., Kelly, R. A., and Lee, R. T. (1999) J. Biol. Chem. 274, 21840–21846
14. Cheng, G. C., Briggs, W. H., Gerson, D. S., Libby, P., Grodzinsky, A. J., Gray, M. L., and Lee, R. T. (1997) Circ. Res. 80, 28–36
15. Condorelli, G., Morisco, C., Stassi, G., Notte, A., Farina, F., Sparagemonella, G., de Rienzo, A., Roncarati, R., Trimarco, B., and Lenzo, G. (1999) Circulation 99, 3071–3078
16. Teichholz, L. E., Kreulen, T., Herman, M. V., and Gorlin, R. (1976) Am. J. Physiol. 231, 223–228
17. Coucelo, J., Joaquim, N., and Coucelo, J. (2000) J. Exp. Zool. 286, 585–595
18. Fransen, P., Hendrickx, J., Brutsaert, D. L., and Sys, S. U. (2001) Cardiovasc. Res. 52, 487–499
19. Ungureanu-Longrois, D., Balligand, J. L., Simmons, W. W., Okada, I., Kobzik, L., Lowenstein, C. J., Kunkel, S. L., Michel, T., Kelly, R. A., and Smith, T. W. (1995) Circ. Res. 77, 494–502
20. Martinet, W., Abbeloos, V., Van Acker, N., De Meyer, G. R., Herman, A. G., and Kockx, M. M. (2004) J. Pathol. 202, 382–388
21. Kuramochi, Y., Lim, C. C., Guo, X., Colucci, W. S., Liao, R., and Sawyer, D. B. (2004) Am. J. Physiol. 286, C222–C229
22. Meyer, D., and Birchmeier, C. (1995) Nature 378, 386–390
23. Kramer, R., Bucay, N., Kane, D. J., Martin, L. E., Tarpley, J. E., and Theill, L. E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4833–4838
24. Lee, K. F., Simon, H., Chen, H., Bates, B., Hung, M. C., and Hauser, C. (1995) Nature 378, 394–398
25. Gassmann, M., Casagranda, F., Orioli, D., Simon, H., Lai, C., Klein, R., and Lemke, G. (1995) Nature 378, 390–394
26. Erickson, S. L., O’Shea, K. S., Ghaboosi, N., Loverro, L., Frantz, G., Bauer, M., Lu, L. H., and Moore, M. W. (1997) Development (Camb.) 124, 4999–5011
27. Yarden, Y. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2569–2573
28. Klapper, L. N., Vaisman, N., Hurwitz, E., Pinkas-Kramarski, R., Yarden, Y., and Sela, M. (1997) Oncogene 14, 2099–2109
29. Yip, Y. L., and Ward, R. L. (2002) Cancer Immunol. Immunother. 50, 569–587
30. Brutsaert, D. L. (2003) Physiol. Rev. 83, 59–115
31. Fransen, P., Lamberts, R. R., Hendrickx, J., and De Keulenaer, G. W. (2004) Cardiovasc. Res. 63, 700–708
32. Lemmens, K., Fransen, P., Sys, S. U., Brutsaert, D. L., and De Keulenaer, G. W. (2004) Circulation 109, 324–326
33. Lemmens, K., Segers, V. F., and De Keulenaer, G. W. (2005) Circulation 111, e175
34. McMurray, J. J. (2004) J. Renin. Angiotensin. Aldosterone. Syst. 5, Suppl. 1, S17–S22
35. Clark, A. L., and Cleland, J. G. (2000) Heart Fail. Rev. 5, 101–114
36. Herrlich, A., Leitch, V., and King, L. S. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 15799–15804
37. Shirakabe, K., Wakatsuki, S., Kurisaki, T., and Fujisawa-Sehara, A. (2001) J. Biol. Chem. 276, 9352–9358
38. Kuramochi, Y., Cote, G. M., Guo, X., LeBrasseur, N. K., Cui, L., Liao, R., and Sawyer, D. B. (2004) J. Biol. Chem. 279, 51141–51147
39. Frenzel, K. E., and Falls, D. L. (2001) J. Neurochem. 77, 1–12
40. Montero, J. C., Yuste, L., Diaz-Rodriguez, E., Esparis-Ogando, A., and Pandiella, A. (2000) Mol. Cell. Neurosci. 16, 631–648