Neuregulins promote survival and growth of cardiac myocytes

PERSISTENCE OF ErbB2 AND ErbB4 EXPRESSION IN NEONATAL AND ADULT VENTRICULAR MYOCYTES*

You-yang Zhao‡, Douglas R. Sawyer‡§, Ragavendra R. Baliga§, Douglas J. Opel, Xinqiang Han, Mark A. Marchionni§, and Ralph A. Kelly†

From the Cardiovascular Division, Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts 02115 and ¶Cambridge Neuroscience Inc., Cambridge, Massachusetts 02139

Neuregulins (i.e. neuregulin-1 (NRG1), also called new differentiation factor, heregulin, glial growth factor, and acetylcholine receptor-inducing activity) are known to induce growth and differentiation of epithelial, glial, neuronal, and skeletal muscle cells. Unexpectedly, mice with loss of function mutations of NRG1 or of either of two of their cognate receptors, ErbB2 and ErbB4, die during midembryogenesis due to the aborted development of myocardial trabeculae in ventricular muscle. To examine the role of NRG and their receptors in developing and postnatal myocardium, we studied the ability of a soluble NRG1 (recombinant human glial growth factor 2) to promote proliferation, survival, and growth of isolated neonatal and adult rat cardiac myocytes. Both ErbB2 and ErbB4 receptors were found to be expressed by neonatal and adult ventricular myocytes and activated by rhGGF2. rhGGF2 (30 ng/ml) provoked an approximate 2-fold increase in embryonic cardiac myocyte proliferation. rhGGF2 also promoted survival and inhibited apoptosis of subconfluent, serum-deprived myocyte primary cultures and also induced hypertrophic growth in both neonatal and adult ventricular myocytes, which was accompanied by enhanced expression of prepro-atrial natriuretic factor and skeletal α-actin. Moreover, NRG1 mRNA could be detected in coronary microvascular endothelial cell primary cultures prepared from adult rat ventricular muscle. NRG1 expression in these cells was increased by endothelin-1, another locally acting cardiotropic peptide within the heart. The persistent expression of both a neuregulin and its cognate receptors in the postnatal and adult heart suggests a continuing role for neuregulins in the myocardial adaption to physiologic stress or injury.

An increasing number of locally acting biologic mediators have now been implicated in regulating myocardial development as well as the subsequent adaptive growth response of postnatal cardiac muscle to physiologic stress. Aside from acetylcholine and the biogenic amines, most of these mediators are locally acting peptide growth factors that are synthesized by cardiac muscle cells themselves or by other parenchymal cells within the heart, such as endothelial cells of the microvasculature and endocardium (1–6). Endothelins and angiotensins, for example, both of which have been shown to induce a hypertrophic growth in cardiac myocytes, are synthesized and released by endothelial cells of the coronary microvasculature (7–10).

Another growth factor signaling system that has recently been implicated in cardiac development is the “neuregulins,” a family of locally acting peptide autacoids known to be important in the development of the central and peripheral nervous system. Targeted disruption of the neuregulin-1 (NRG1) gene or of either of two neuregulin receptors (ErbB2 or ErbB4) led unexpectedly to death during midembryogenesis from aborted development of trabeculation in ventricular muscle of the fetal myocardium (11–15). These reports indicated that neuregulins, released from the endocardial endothelium, are essential for the growth and phenotypic adaptation of subjacent cardiac myocytes during development. Neuregulins include the growth-regulatory proteins: glial growth factor (GGF), Neu differentiation factor, heregulin, and acetylcholine receptor-inducing activity. All of these proteins are encoded by a single gene but exist in at least 15 isoforms, including both integral membrane and soluble signaling proteins (16). Recently, two additional genes coding for neuregulin-like signaling proteins have been identified that have been termed “neuregulin-2” (NRG2), and “neuregulin-3” (NRG3) (17–19). Products of the originally identified neuregulin family, now termed neuregulin-1 (NRG1), share about 40 and 20% amino acid sequence identity with NRG2 and NRG3, respectively. Unlike NRG1-derived mRNAs, which within the developing heart are limited largely to the endocardial endothelium of ventricular muscle, NRG2 mRNAs are found primarily in the endothelium of developing atrial muscle, while no NRG3 mRNA has been detected by in situ hybridization in the heart at midembryogenesis (18, 19). Neuregulins, which are produced by either neuronal or mesenchymal cells, mediate their effects by binding to and signaling via the ErbB family of receptors, including ErbB1/epidermal growth factor receptor, Neu/ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4 (16, 20). All neuregulins (i.e. NRG1, NRG2, and NRG3) identified to date bind to either ErbB3 or ErbB4, subsequently recruiting another ErbB recep-

* This work was supported by National Institutes of Health Grant HL61641 (to R. A. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ These two authors contributed equally to this work.
§ Present address: Cardiology Division, Boston University Medical Center, 88 E. Newton St., Boston, MA 02118.
¶ To whom correspondence should be addressed: Cardiology Division, Brigham and Women’s Hospital, 75 Francis St., Boston, MA 02115. Tel.: 617-732-7503; Fax: 617-732-5132; E-mail: rakelly@bics.bwh.harvard.edu.

1 The abbreviations used are: NRG, neuregulin; ANF, atrial natriuretic factor; ARVM, adult rat ventricular myocyte(s); GGF, glial growth factor; rhGGF2, recombinant human glial growth factor 2; MHC, myosin heavy chain; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide; NRVM, neonatal rat ventricular myocyte(s); TRITC, tetramethylrhodamine isothiocyanate; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; DMEM, Dulbecco’s modified Eagle’s medium; PBS, fetal bovine serum; CMEC, coronary microvascular endothelial cell(s); PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR.
routinely obtained primary cultures with stated, all experiments were performed 36–48 h after changing to a cultures used for thymidine uptake measurements. Unless otherwise 24–48 h to prevent proliferation of non-myocytes, with the exception of (32). Rod-shaped cardiac myocytes were plated in culture medium on

primary cultures was carried out using techniques previously described

Neuregulins have been shown to regulate the proliferation, differentiation, and survival of Schwann cells and oligodendrocytes (23–26), neurite extension of retinal neurons (27), the maturation of skeletal muscle myoblasts (28), and expression of acetylcholine receptors in skeletal muscle (29, 30). However, direct evidence for effects of neuregulins on cardiac myocytes has not been reported. Here, we examine the ErbB receptor expression in neonatal and adult rat ventricular myocytes. We demonstrate that a soluble recombinant form of a human NRG1, glial growth factor 2 (rhGGF2), promotes embryonic cardiac myocyte proliferation and the growth and survival of both neonatal and adult ventricular myocytes in vitro. We also examine the expression of NRG1 in primary cultures of coronary microvascular endothelial cells. These data support the concept that the neuregulin-ErbB signaling system plays an important role in the onset of myocardial trabeculation and cardiac morphogenesis by promoting the proliferation, survival, and maturation of cardiomyocytes and suggest that this system may remain an important pathway for phenotypic adaptation in both neonatal and adult myocardium.

EXPERIMENTAL PROCEDURES

Preparation of Cardiac Myocyte and Non-myocyte Primary Cultures—Embryonic and neonatal rat ventricular myocyte (NRVM) primary cultures were prepared as described previously (31). To selectively enrich for myocytes, dissociated cells were centrifuged twice at 500 rpm for 5 min, pelleted twice for 75 min, and finally plated at low density (0.7–1 × 10^6 cells/cm^2) in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies Inc.) supplemented with 7% fetal bovine serum (FBS; Sigma). Cytosine arabinoside (10 μM; Sigma) was added during the first 24–48 h to prevent proliferation of non-myocytes, with the exception of cultures used for thymidine uptake measurements. Unless otherwise stated, all experiments were performed 36–48 h after changing to a serum-free medium, DMEM plus ITS (Sigma). Using this method, we routinely obtained primary cultures with >95% myocytes, as assessed by microscopic observation of spontaneous contraction and by immunofluorescence staining with a monoclonal antocardiac myosin heavy chain antibody (anti-MHC; Biogenesis, Sandown, NH).

Primary cultures of cellular fractions isolated from neonatal hearts enriched in non-myocytes were prepared by passing twice cells that adhered to the tissue culture dish during the preplating procedure. These non-myocyte cultures, which contained few anti-MHC-positive cells, were allowed to grow to subconfluence in DMEM supplemented with 20% FBS before switching to DMEM plus ITS for a subsequent 36–48 h.

Isolation and preparation of adult rat ventricular myocyte (ARVM) primary cultures was carried out using techniques previously described (32). Rod-shaped cardiac myocytes were plated in culture medium on
Neuregulin/ErbB Signaling in Postnatal Myocardium

10263

ng/ml) or 7% FBS. DNA synthesis was assessed by [3H]thymidine incorporation, and the data are expressed as relative cpm/dish normalized to the mean cpm/dish of control cells in each experiment (mean ± S.D. of triplicate analyses from independent experiments; *, p < 0.05 versus control).

Coronary microvascular endothelial cells (CMEC) from adult rat hearts were isolated as described by Nishida et al. (33). The isolated cells were plated at a density of 2500 cells/cm². After 1 h of plating, the cells were washed twice with DMEM to remove loosely adherent cells and then maintained in DMEM supplemented with 20% FBS. These primary isolates have been documented to contain >90% endothelial cells, with a phenotype at low passage number consistent with their microvascular origin, as described previously (33).

PCR Analysis of ErbB Receptors in Rat Heart—cDNA sequences encoding portions of the C termini of ErbB receptors were amplified by using the following synthetic oligonucleotide primers: ErbB2A (5′-TTG-GCTAGTCAGAGTCCACCAC-3′; sense) and ErbB2B (5′-CCCT-TCTCTCGGTACTAAGTATTCCG-3′; antisense) for amplification of ErbB2 codon positions 857–1207 (34); ErbB3A (5′-GCTTAAAGTGCT-TGGCTGGGTCGTC-3′; sense) and ErbB3B (5′-TCTTACACACTGA-CACCTTTCTCTT-3′; antisense) for amplification of ErbB3 codon positions 712–1085 (35); ErbB4A (5′-AAATTCACCATCAGAGTGGTGAAGC-3′; sense) and ErbB4B (5′-TCTTACGTTGAAGTCCCAACCAC-3′; antisense) for amplification of ErbB4 codon positions 896–1262 (36). RNA samples (1 μg) from rat hearts or freshly isolated neonatal and adult rat ventricular myocytes were reverse transcribed to generate first-strand cDNA. The PCR reactions were performed for 30 cycles. Each cycle included 30 s at 94 °C, 75 s at 63 °C, and 120 s at 72 °C. The PCR products were directly cloned into the TA cloning vector (Invitrogen Co., San Diego, CA) and verified by automatic DNA sequencing.

Analysis of ErbB Receptor Phosphorylation—To analyze which receptor subtypes were tyrosine-phosphorylated, neonatal and adult ventricular myocyte cells were maintained in serum-free medium for 24–48 h and then treated with rhGGF2 at 20 ng/ml for 5 min at 37 °C. Cells were quickly rinsed twice with ice-cold PBS and lysed in cold lysis buffer containing 1% Nonidet P-40, 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 10 mM sodium molybdate, 8.8 μM sodium pyrophosphate, 4 μM NaF, 1 μM phenylmethanesulfonyl fluoride, 10 μg/ml aprotinin, and 20 μM leupeptin. Lysates were centrifuged at 12,000 × g at 4 °C for 20 min, and aliquots of 1500 μg (ErbB4 detection) or 3000 μg (ErbB2 detection) of supernatant were incubated with antibody specific to ErbB3 or phosphotyrosine, respectively, overnight at 4 °C and precipitated with protein G plus agarose (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA). Immunoprecipitates were collected and released by boiling in SDS sample buffer. Samples were fractionated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Bio-Rad), and probed with a PY99 anti-phosphotyrosine antibody or with an antibody specific to

Fig. 2. GGF2 provokes proliferation of cardiac myocytes but not nonmyocyte cells in vitro. Panel A, effects of GGF2 on rat embryonic cardiac myocyte DNA synthesis. Embryonic cardiac myocytes (embryonic day 17) maintained in serum-free medium for 3 days were treated either without (control; C) or with the indicated concentrations of rh-GGF2 (ng/ml). Recombinant human fibroblast growth factor-2 (25 ng/ml) was used as a positive control. Panel B, effects of GGF2 on rat embryonic cardiac myocyte cultures proliferation in vitro. Embryonic cardiac myocytes (embryonic day 17) were plated at a density of 0.6 × 10⁶ cells/6-well plate. After 2 days in serum-free medium (day 0), the cells were treated either without (control; C) or with 40 ng/ml of GGF2 (GGF2) for the indicated time periods. The medium was changed once on day 3. At 2, 4, and 6 days following treatment, the cells were trypsinized, stained, and counted under microscopy. The data are expressed as mean ± S.D. of triplicate analyses from three independent experiments (*, p < 0.05 versus control).

Panel C, effects of GGF2 on DNA synthesis in myocyte-enriched and non-myocyte fractions from neonatal rat ventricular myocyte primary isolate. NRVM-enriched primary isolates or a non-myocyte-enriched fraction (see "Experimental Procedures") were exposed to serum-free medium alone (control; C) or to medium containing the indicated concentrations of GGF2 (in ng/ml) or 7% FBS. DNA synthesis was assayed by [3H]thymidine incorporation, and the data are expressed as relative cpm/dish normalized to the mean cpm/dish of control cells in each experiment (mean ± S.D. of triplicate analyses from independent experiments; *, p < 0.02 versus control).
ErbB2. All of these antibodies were purchased from Santa Cruz Biotechnology.

**Incorporation of 
\(^3\)HThymidine and \(^3\)HLeucine—As an index of DNA synthesis, \(^3\)Hthymidine incorporation was measured as described previously (37). After incubation for 48–72 h in serum-free medium (PBS), the cells were stimulated with different concentrations of rhGGF2 (Cambridge NeuroScience Co., Cambridge, MA) for 20 h. \(^3\)Hthymidine (0.7 Ci/mmol; DuPont) was then added to the medium at a concentration of 5 (NRVM) or 15 \(\mu\)Ci/ml (embryonic myocytes), and the cells were cultured for another 8 h. Cells were washed with PBS twice and 10% trichloroacetic acid (Sigma) once, and 10% trichloroacetic acid was added to precipitate protein at 4 °C for 45 min. Parallel cultures of myocytes not exposed to rhGGF2 were harvested under the same conditions as controls. The precipitate was washed twice with 95% ethanol, resuspended in 0.15 M NaOH, and saturated with 1 M HCl, and then aliquots were counted in a scintillation counter. The results are expressed as relative cpm/dish normalized to the mean cpm of control cells in each experiment. For antibody-blocking experiments, the same procedure was applied except that the cells were preincubated with an antibody (0.5 \(\mu\)g/ml) specific for each neuregulin receptor (c-Neu Ab-2, Oncogene Research Products; and ErbB3 or ErbB4, Santa Cruz Biotechnology), for 2 h before the addition of either rhGGF2 or recombinant human fibroblast growth factor-2.

The rate of \(^3\)Hleucine uptake was used as an index of protein synthesis. For these experiments, 10 \(\mu\)m cytosine arabinoside was added to the culture medium. Cells were grown in serum-free medium for 36–48 h and then stimulated with different doses of rhGGF2. After 40 h, \(^3\)Hleucine (5 \(\mu\)Ci/ml) was added for 8 h, and cells were washed with PBS and harvested with 10% trichloroacetic acid. Trichloroacetic acid-precipitable radioactivity was determined by scintillation counting as above.

**Indirect Immunofluorescence Staining of Cardiac Myocytes—For examination of changes in myocyte phenotype with rhGGF2, cells were fixed in 4% (w/v) paraformaldehyde for 30 min at room temperature, rinsed with PBS, permeabilized with 0.1% Triton X-100 for 15 min, and then incubated with 1% PBS for another 15 min, followed by incubation with anti-MHC antibody (1:300) and visualized with TRITC-conjugated (NRVM) or fluorescein isothiocyanate-conjugated (ARVM) second antibody. NRVM were examined using indirect immunofluorescence microscopy, while ARVM were examined using a MRC 600 confocal microscope (Bio-Rad) with a krypton/argon laser.

**Cell Survival Assay and Detection of Apoptosis—Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) cell respiration assay, which is dependent on mitochondrial activity in living cells (38). Primary culture myocytes were reverse transcribed and analyzed by RT-PCR. For RT-PCR, an equal amount of cDNA per sample was amplified with specific primers. The PCR products were electrophoresed in a 1% agarose gel and visualized by ethidium bromide staining. The intensity was determined by densitometry (Ultrascan XL, Amersham Pharmacia Biotech).

**Results

**Expression of Neuregulin Receptors in the Heart—To examine the expression profile of the NRG receptors (i.e. ErbB2, ErbB3, and ErbB4) in rat myocardium, RNA from rat heart tissues at midembryogenesis (embryonic day 14) or postnatal day 1 and from freshly isolated neonatal and adult ventricular myocytes were reverse transcribed and analyzed by RT-PCR. Primers that flank the variable C termini of ErbB receptors were used to amplify the products shown in Fig. 1A. In freshly isolated neonatal and adult rat ventricular myocyte primary cultures, both ErbB2 and ErbB4 mRNA were readily detectable by RT-PCR, although ErbB4 expression levels were consistently higher than those of ErbB2. Expression of ErbB3 mRNA was not detected in either whole heart at postnatal day 1 or freshly isolated cardiomyocytes, but ErbB3 mRNA was detected in the developing rat heart at embryonic day 14. Furthermore, when using receptor-specific cDNA probes for ErbB2, ErbB3, and ErbB4, only transcripts for ErbB4 were readily detectable in freshly isolated neonatal and adult rat ventricular myocytes but not in myocyte-depleted, non-myocyte-enriched primary cultures by Northern blot (data not shown).

To determine whether ErbB2 and ErbB4 were tyrosine-phosphorylated following neuregulin treatment, rhGGF2 (20 ng/ml) was briefly (5 min) added to cultures of either neonatal or adult rat ventricular myocytes. Lysates were immunoprecipitated and probed with an anti-phosphotyrosine antibody (for detection of ErbB4) or an anti-ErbB2 antibody. As shown in Fig. 1, B and C, phosphorylated forms of ErbB2 and ErbB4 were detected in both neonatal and adult cardiomyocytes, but phosphorylated ErbB4 was more easily detected than phosphorylated ErbB2. All of these antibodies were purchased from Santa Cruz Biotechnology.

**Isolation and Hybridization of RNA—Total cellular RNA was isolated by a modification of the acid guanidinium/thiocyanate phenol/chloroform extraction method (39) using the TRIZOL reagent (Life Technologies). The following cDNA probes labeled with [a-\(^32\)P]dCTP by random priming were used: rat prepro-atrial natriuretic factor (prepro-ANP) (0.6 kilobase pair of coding region) (40), rat skeletal a-actin (240 base pairs of a 3'-untranslated region) (41), and rat heart neuregulin-1 (0.67 kilobase pair of coding region spanning the Ig-like and epidermal growth factor-like domains) (42). Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (240 base pairs of the coding region) (43) was used as control for loading and transfer efficiency. Signal intensity was determined by densitometry (Ultrascan XL, Amersham Pharmacia Biotech).

**RESULTS

Expression of Neuregulin Receptors in the Heart—To examine the expression profile of the NRG receptors (i.e. ErbB2, ErbB3, and ErbB4) in rat myocardium, RNA from rat heart tissues at midembryogenesis (embryonic day 14) or postnatal day 1 and from freshly isolated neonatal and adult ventricular myocytes were reverse transcribed and analyzed by RT-PCR. Primers that flank the variable C termini of ErbB receptors were used to amplify the products shown in Fig. 1A. In freshly isolated neonatal and adult rat ventricular myocyte primary cultures, both ErbB2 and ErbB4 mRNA were readily detectable by RT-PCR, although ErbB4 expression levels were consistently higher than those of ErbB2. Expression of ErbB3 mRNA was not detected in either whole heart at postnatal day 1 or freshly isolated cardiomyocytes, but ErbB3 mRNA was detected in the developing rat heart at embryonic day 14. Furthermore, when using receptor-specific cDNA probes for ErbB2, ErbB3, and ErbB4, only transcripts for ErbB4 were readily detectable in freshly isolated neonatal and adult rat ventricular myocytes but not in myocyte-depleted, non-myocyte-enriched primary cultures by Northern blot (data not shown).

To determine whether ErbB2 and ErbB4 were tyrosine-phosphorylated following neuregulin treatment, rhGGF2 (20 ng/ml) was briefly (5 min) added to cultures of either neonatal or adult rat ventricular myocytes. Lysates were immunoprecipitated and probed with an anti-phosphotyrosine antibody (for detection of ErbB4) or an anti-ErbB2 antibody. As shown in Fig. 1, B and C, phosphorylated forms of ErbB2 and ErbB4 were detected in both neonatal and adult cardiomyocytes, but phosphorylated ErbB4 was more easily detected than phosphorylated ErbB2.
ated ErbB2 and quite prominent in neonatal myocytes, which is consistent with the expression profile we observed above.

**GGF2 Provokes Cardiac Myocyte Proliferation in Vitro**—To investigate the ability of GGF2 to stimulate the proliferative response of embryonic cardiac myocytes, myocytes maintained in serum-free medium for 3 days were subsequently treated with rhGGF2 for 30 h. As displayed in Fig. 2A, GGF2 provoked an approximately 2-fold increase in the DNA synthesis of rat embryonic cardiac myocytes (day 17), as assessed by \[^{3}H\]thy-midine incorporation. The maximal effective concentration was ~30 ng/ml (i.e., 0.54 nM). Exposure to recombinant human fibroblast growth factor-2 (25 ng/ml) had a similar effect. Since embryonic cardiac myocytes still possess active proliferative capacity, we next counted the cell number after a treatment with GGF2 (40 ng/ml) for the indicated time periods (Fig. 2B). The GGF-treated cells proliferated day by day, and the cell number peaked by day 4 and was increased by about 3-fold. However, as shown in Fig. 2C, GGF2 (50 ng/ml) had little

---

**FIG. 4.** GGF2 diminishes apoptotic cell death in neonatal rat ventricular myocyte primary cultures. Primary cultures of NRVM 2 days in serum-free medium were maintained in either the absence of rhGGF2 (A, B, and C) or in the presence of 20 ng/ml of rhGGF2 (E, F, and G) for 4 days. Cells were then fixed and stained with anti-MHC antibody and a TRITC-conjugated secondary antibody to visualize myocytes (A and E) or with fluorescein-conjugated dUTP (i.e., TUNEL) to reveal apoptotic cells (B and F). The TUNEL-positive myocytes displayed cell shrinkage and chromatin condensation, which were also identified by Hoescht 33258-staining (C and G). Apoptosis was quantified either by counting the number of TUNEL-positive myocytes (D) or by flow cytometry analysis of the sub-G1 fraction following propidium iodide staining of primary NRVM cultures that had been treated for 4 days with the indicated concentrations of rhGGF2 (H). The data shown for (D) and (H) are given as mean ± S.D. for three independent experiments. Bar, 10 μm.
mitogenetic effect on cardiac non-mycyte fractions obtained following the preplating steps of the neonatal rat ventricular myocyte isolation procedure, while 7% FBS could induce a nearly 10-fold increase in [3H]thymidine incorporation into these cell populations. In neonatal ventricular myocytes, GGF2 could induce DNA synthesis to a lesser extent than in embryonic cardiac myocyte (Fig. 2, A and C). Therefore, GGF2 shows a relatively specific mitogenic effect on cardiac myocytes compared with a myocyte-depleted cell population, which, using the method of myocyte isolation we employed here, is composed largely of fibroblasts and endothelial cells (33).

**GGF2 Promotes Cardiac Myocyte Survival in Vitro**—During development, the net increase in the number of functional embryonic myocytes is dependent on both myocyte proliferative capacity and survival. Therefore, it was of interest to determine whether GGF2 could promote survival of cardiac myocytes in addition to proliferation. Primary cultures of neonatal rat ventricular myocytes maintained in serum-free media exhibited a gradual decrease in cell number. Using the MTT cell respiration assay to measure cell viability, we have observed that approximately 25% of cells die by day 4. In contrast, the addition of GGF2 resulted in a 30% increase in MTT activity compared with controls. The effect was concentration-dependent with an EC50 of 0.2 ng/ml (Fig. 3). This survival effect was observed in NRVM primary cultures for up to 7 days (data not shown); it was also observed in the presence of cytosine arabinoside, an antiproliferative agent. As shown in Fig. 3, the survival effect of GGF2 was greater in 4 days in the continuous presence of cytosine arabinoside, with about 90% myocyte viability in the presence of 50 ng/ml rhGGF2 compared with approximately 70% viability in control cultures. In contrast, GGF2 had no significant effect on the survival of myocyte-depleted, non-myocyte-enriched primary isolates at 4 days (data not shown).

We examined next whether the survival effect of GGF2 was mediated by inhibition of programmed cell death (apoptosis). After 6 days in serum-free medium, about 17% of NRVM maintained under control conditions at low density (i.e., subconfluent) exhibited evidence of apoptosis as detected by TUNEL staining, with small condensed nuclei and cell shrinkage consistent with apoptotic cell death (Fig. 4). In the presence of 20 ng/ml rhGGF2, the number of TUNEL-positive myocytes declined to about 8% (Fig. 4D). The effect of GGF2 on inhibiting apoptosis was also quantified using flow cytometric analysis of propidium iodide-labeled NRVM primary cultures. After 4 days in serum- and insulin-free medium, 22% of NRVM were hypodiploid, consistent with initiation of programmed cell death. In the presence of rhGGF2 at concentrations above 10 ng/ml, less than 10% of NRVM exhibited evidence of apoptosis (Fig. 4H).

The survival and antiapoptotic effects of GGF2 on the ARVM were also examined by MTT cell respiration assay and TUNEL staining (Fig. 5). When compared with untreated ARVM primary cultures, in which more than 10% of cells were positive for TUNEL labeling, rhGGF2 (20 ng/ml)-treated adult myocyte cultures exhibited only about 3% TUNEL-positive staining (Fig. 5B). These results indicate neuregulins act as survival factors at least in part by preventing programmed cell death in both neonatal and adult ventricular myocytes.

**GGF2 Induces Hypertrophic Growth of Cardiac Myocytes**—To investigate whether neuregulin signaling can induce a hypertrophic (growth) response in cardiac myocytes, we examined the effects of GGF2 on induction of myocyte hypertrophy in both neonatal and adult rat ventricular myocyte primary cultures. As shown in Fig. 6, A and B, after a 72-h incubation in serum-free medium with 20 ng/ml (i.e., 0.36 nM) of rhGGF2, NRVM exhibited a significant increase in cell size and in myofibrillar development. A hypertrophic response in cardiac myocytes is characterized by a number of phenotypic changes in addition to an increase in cell size, such as an increase in contractile protein content without cellular proliferation and the reactivation of an “embryonic” gene program (44). Therefore, we examined the effects of neuregulin on levels of prepro-ANF and skeletal α-actin mRNA (transcripts normally found in relatively low abundance in neonatal and adult ventricular myocytes) and on [3H]leucine incorporation as an index of protein synthesis in NRVM primary cultures. As shown in Fig. 6C, rhGGF2 (20 ng/ml) increased mRNA levels for prepro-ANF and skeletal α-actin within 60 min, approximately doubling by 16 h. GGF2 also stimulated [3H]leucine incorporation, with about a 120% increase at 48 h, at a concentration of 5 ng/ml. To minimize possible confounding effects of GGF2 on the rate of [3H]leucine uptake into non-mycyte contaminant cells, these experiments were repeated in the continuous presence of cytosine arabinoside with similar results (data not shown).

GGF2 also caused hypertrophic responses in cultured ARVM. By 72 h in the continuous presence of 20 ng/ml rhGGF2, some adult myocytes had begun to develop “pseudopod-like” extensions, primarily from the region of the intercalated discs, and by 5 days, more than 60% of the GGF-treated adult cardiomyocytes displayed phenotypic changes consistent with those illustrated in Fig. 7, B and C, while more than 80% of untreated ARVM maintained the phenotype exhibited in Fig. 7A. As shown in Fig. 7D, rhGGF2 (20 ng/ml) doubled prepro-ANF mRNA abundance in ARVM primary cultures after 8 h, and this had increased 3–4-fold within 20 h. An increase in skeletal α-actin mRNA abundance was also observed that was greater than that seen with phenylephrine (10 μM), an α-adrenergic agonist known to induce hypertrophic growth and reexpression of a number of fetal genes in adult rat ventricular myocytes. Within 7 h, skeletal α-actin mRNA levels were easily detectable, and they increased by an additional 250% by 30 h of treatment with GGF2. Neither GGF2 nor phenylephrine had any effect on GAPDH mRNA abundance under the conditions employed here. As illustrated in Fig. 7E, GGF2 induced a dose-dependent increase in [3H]leucine incorporation, with a 70% increase at a concentration of 5 ng/ml. Thus, this neuregulin induces phenotypic changes consistent with hypertrophic
adapation in both neonatal and adult rat ventricular myocyte phenotypes at subnanomolar concentrations.

**CMEC Express NRG1: Regulation by Endothelin—NRG1 expression has been identified in the developing murine (embryonic day 10 and 17) endocardial endothelium by *in situ* hybridization (11, 45). To determine if NRG1 expression could be detected in an endothelial cell phenotype obtained from the heart of adult animals, CMEC was isolated from adult rat ventricle and cultured for analysis of NRG1 expression. As shown in Fig. 8, a basal level of NRG1 mRNA was detected in confluent primary cultures of CMEC using a NRG1-specific cDNA probe that included both the Ig-like and epidermal growth factor-like domains. Consistent with a previous report (46), this probe recognizes three transcripts of 10, 7, and 3 kilobase pairs. In addition, as shown in Fig. 8 and in contrast to FBS, endothelin-1 increased NRG1 expression in primary CMEC cultures. We observed an induction of NRG1 mRNA (approximately 2-fold) that peaked at ~16 h and then decreased to basal levels by 24 h.

**DISCUSSION**

The characterization of mice with targeted disruption of either the Ig-like or epidermal growth factor-like domains of the NRG1 gene and of mice expressing loss of function mutations of either the ErbB2 or ErbB4 gene, highlight the importance of neuregulins in cardiac development (11–14). In this report, we have documented that two neuregulin receptors, ErbB2 and ErbB4, continue to be expressed in the postnatal and adult myocardium. Further, we demonstrated the soluble neuregulin GGF2-induced phenotypic changes in neonatal as well as the "terminally differentiated" adult myocytes in culture.

The regulated expression of ErbB receptors presumably determines in part the developmental role of neuregulins during embryogenesis and in the postnatal heart. Using *in situ* hybridization, it had been reported that myocardial cells in the fetal heart expressed both ErbB2 and ErbB4 (11, 13) and that expression of ErbB3 was localized in the mesenchymal cells of the endocardial cushion and bulbus cordis (11) in midembryogenesis (embryonic day 10.5). The present study extends this observation to show that while all three receptors are expressed during midembryogenesis in the rat heart, only expression of ErbB3 is markedly down-regulated later during development. We also found that both ErbB2 and ErbB4 were rapidly and specifically phosphorylated in response to the addition of GGF2 to primary cultures of both neonatal and adult rat ventricular myocytes, suggesting ErbB2 and ErbB4 may form heterodimer to signal in postnatal myocardium.

In addition to their essential role during cardiac ontogeny, neuregulins join a growing list of stimuli that induce hypertrophic growth of cardiac myocytes, including α1-adrenergic agents, mechanical stretch, thyroid hormone, interleukin-1α, fibroblast growth factors, heparin-binding epidermal growth factor-like growth factor, endothelins, and angiotensins among others (2, 3, 6, 47, 48). What role GGF2 or other neuregulins play in late myocardial embryogenesis or in the postnatal animal remains to be elucidated. Since we observed not only myocyte growth but also reemergence of a pattern of embryonic gene transcription in neonatal and adult ventricular myocytes exposed to GGF2 that is characteristic of myocyte hypertrophic growth *in situ* in the myocardium, it is possible that neuregulins participate with other growth stimuli in some forms of cardiac adaptation to physiologic stress in the postnatal animal.

Unlike most other hypertrophic stimuli, however, GGF2 promoted the survival of adult and neonatal cardiac myocytes, at least in part by inhibiting apoptosis in low density, subconfluent myocyte cultures maintained in serum-free medium. This result is similar to that reported for CT-1, which, at least in neonatal rat ventricular myocytes, also has been shown to reduce apoptosis induced by the removal of serum (49). In contrast to the results reported for CT-1, GGF2, at least under the conditions and at the concentrations we employed, did not
completely prevent apoptosis in neonatal myocytes, although this may be due to methodological differences. The antiapoptotic action of CT-1 has been shown to require the activation of the Ras/Raf/MEK/mitogen-activated protein kinase pathway, since inhibition of extracellular signal regulated kinase 1 and 2 activation with the MEK inhibitor PD98059 prevented CT-1's antiapoptotic activity (49). Interestingly, GGF2 also activates these mitogen-activated protein kinase pathways in myocytes. However, activation of this mitogen-activated protein kinase pathway alone cannot be sufficient for the antiapoptotic effects of either of these ligands, since this signal transduction cascade is also activated in cultured cardiac myocytes by angiotensin II (5), as well as interleukin-1β and IFNγ (50), all of which have been shown to promote apoptosis in both adult (51) and neonatal3 rat ventricular myocytes.

This apparent paradox also is reflected in the effects of these myocardial growth factors on the expression of ANF. Increased expression of ANF is one of the most reliable markers of hypertrophy of ventricular myocytes, both in vivo and in vitro (52). Interestingly, ANF has recently been reported to induce apoptosis of neonatal rat ventricular myocytes when added to primary cultures at sufficient concentrations (53). In contrast, while CT-1 and GGF2 increase the expression of ANF in neonatal myocyte cultures to levels that are similar to those achieved with the hypertrophic but pro-apoptotic stimuli, angiotensin II or interleukin-1β/IFNγ, both CT-1 and GGF2 clearly protect against apoptotic myocyte death, at least in vitro.

Neuregulin-ErbB signaling may represent a generalized system for maintaining close apposition between distinct cell lineages that are necessary for normal tissue architecture and function in certain organs. Neuronally expressed neuregulins appear to drive the proliferation and growth and survival of adjacent Schwann cells during axonal generation (23, 26), and apoptosis of Schwann cells distal to mechanically injured motor axons can be prevented by application of a soluble neuregulins (25). Similarly, neuregulins expressed by motor neurons at the neuromuscular junction appear to drive the formation of the acetylcholine-responsive postsynaptic endplates in skeletal muscle (54). The expression of neuregulin by coronary microvascular endothelial cells suggest that these cells may play a role in the regulation of NRG synthesis and secretion in the postnatal myocardium that is analogous to that played by the endocardial endothelium in the heart during development. The effects of the soluble neuregulin, GGF2, on cardiac myocyte proliferation, growth, and survival in vitro offer additional insight into the role the neuregulin-ErbB signaling system plays in myocardial development. Persistent expression of neu-

---

1 R. Baliga, D. Sawyer, Y.-Y. Zhao, R. Kelly, manuscript in preparation.
2 M. A. Arstall, D. B. Sawyer, and R. A. Kelly, submitted for publication.
Regulation of NRG1 mRNA levels by endothelin in primary cultures of CMEC. A, confluent serum-starved CMEC were treated either without (C) or with endothelin-1 (ET-1, 2 × 10⁻⁷ M) for the time periods indicated, or with 10% FBS for 16 h. Total RNA was extracted, and 25 μg of the RNA was analyzed by Northern blot using 3²P-labeled NRG1 and GAPDH probes. B, the results in A were quantified by normalizing each of the three transcripts to the GAPDH standard and expressing the sum of the three transcripts as a ratio to untreated controls. This experiment was repeated twice with similar results.

REFERENCES

1. Simpson, P. (1985) Circ. Res. 56, 884–894
2. Housh, D. G., Stewart, A. F., Chang, K. C., Bailey, B. A., Karliner, J. S., Camacho, S. A., Long, C. S., and Simpson, P. C. (1996) J. Biol. Chem. 271, 5839–5843
3. Ramirez, M. T., Sah, V. P., Zhao, X.-L., Hunter, J. J., Chien, K. R., and Brown, J. (1997) J. Biol. Chem. 272, 14057–14061
4. Ramirez, M. T., Post, G. R., Sulakhe, P. V., and Brown, J. (1995) J. Biol. Chem. 270, 4446–4451
5. Sadashima, J., Qiu, Z., Morgan, J. P., and Irazu, S. (1995) Circ. Res. 75, 1–15
6. Kaye, D., Pimental, D., Prasad, S., Maki, T., Berger, H.-J., McNeil, P. L., Smith, T. W., and Kelly, R. A. (1996) J. Clin. Invest. 97, 1–11
7. Nishida, M., Carley, W. W., Gerrisen, M. E., Ellingsen, O., Kelly, R. A., and Smith, T. W. (1993) Am. J. Physiol. 264, H639–H652
8. Nishida, M., Springhorn, J. P., Kelly, R. A., and Smith, T. W. (1993) J. Clin. Invest. 91, 1984–1994
9. Shubeita, H. E., McDonough, P. M., Harris, A. N., Knowlton, K. U., Glembocki, C. C., Brown, J. H., and Chien, K. R. (1990) J. Biol. Chem. 265, 20555–20562
10. Fischer, T. A., Ungureanu-Longrois, D., Singh, K., de Zengotita, J., Alali, A., Gardbut, A. P., Lee, M.-A., Balligand, J.-L., Kifor, I., Smith, T. W., and Kelly, R. A. (1997) Am. J. Physiol. 273, H1058–H1068
11. Meyer, D., and Birchmeier, C. (1995) Nature 378, 386–390
12. Gassmann, M., Casagranda, F., Orioli, D., Simon, H., Lai, C., Klein, R., and Lemke, G. (1995) Nature 378, 390–394
13. Lee, K. F., Simon, H., Chen, B., Bates, H., Hwang, M.-C., and Hauser, C. (1995) Nature 378, 394–398
14. Kramer, R., Buyac, N., Kane, D. J., Martin, L. E., Tarpley, J. E., and Theil, L. E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4833–4838
15. Marchionni, M. A. (1995) Nature 378, 334–335
16. Carraway, K. L., and Burden, S. J. (1995) EMBO J. 14, 585–596
17. Nishida, M., Careley, W. W., Meggs, L. G., and Smith, T. W. (1993) Am. J. Physiol. 264, H639–H652
18. Carraway, K. L., III, Weber, J. L., Unger, M. J., Ledesma, J., Yu, N., Carraway, K. L., and Burden, S. J. (1995) EMBO J. 14, 239–249
19. Levi, A. D. O., Bunge, R. P., Lofgren, J. A., Hefti, F., Nikolics, K., and Yaffe, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9562–9567
20. Sheng, Z., Knowlton, K. U., Zhu, H., and Chien, S. (1996) J. Cell Biol. 132, 1033–1044
21. Chien, K. R., Knowlton, K. U., Zhu, H., and Chien, S. (1997) J. Cell Biol. 136, 1201–1213