NRG-1-induced cardiomyocyte hypertrophy. Role of PI-3-kinase, p70^{S6K}, and MEK-MAPK-RSK

RAGAVENDRA R. BALIGA, DAVID R. PIMENTAL, YOU-YANG ZHAO, WILLIAM W. SIMMONS, MARK A. MARCHIONNI, DOUGLAS B. SAWYER, AND RALPH A. KELLY

Cardiovascular Division, Brigham and Women’s Hospital and Harvard Medical School, Boston 02115; Cambridge Neurosciences, Cambridge, Massachusetts 02139; and Department of Medicine, University of California at San Diego School of Medicine, La Jolla, California 92093

Baliga, Ragavendra R., David R. Pimental, You-Yang Zhao, William W. Simmons, Mark A. Marchionni, Douglas B. Sawyer, and Ralph A. Kelly. NRG-1-induced cardiomyocyte hypertrophy. Role of PI-3-kinase, p70^{S6K}, and MEK-MAPK-RSK. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H2026–H2037, 1999.—Neuregulins are a family of growth-promoting peptides known to be important in neural and mesenchymal tissue development. Targeted disruption of neuregulin (NRG)-1 or one of two of its cognate receptors, ErbB2 or ErbB4, results in embryonic lethality because of failure of the heart to develop. Although expression of NRGs and their receptors declines after midembryogenesis, both ErbB2 and ErbB4 are present in cardiac myocytes, and NRG-1 expression remains inducible in primary cultures of coronary microvascular endothelial cells from adult rat ventricular muscle. In neonatal rat ventricular myocytes, a soluble NRG-1, recombinant human glial growth factor-2, increased [H]phenylalanine uptake and induced expression of atrial natriuretic factor (ANF) and sarcomeric F-actin polymerization. The effect of NRG-1 on [H]phenylalanine uptake and sarcomeric F-actin polymerization was maximal at 20 ng/ml but declined at higher concentrations. NRG-1 activated p42/p44 mitogen-activated protein kinase (MAPK) [extracellular signal-regulated kinase (ERK)-2/ERK1] and ribosomal S6 kinase (RSK)-2 (90-kDa ribosomal S6 kinase), both of which could be inhibited by the MAPK/ERK kinase-1 antagonist PD-098059. NRG-1 also activated 70-kDa ribosomal S6 kinase, which was inhibited by either rapamycin or wortmannin. Activation of these pathways exhibited the same “biphasic” response to increasing NRG-1 concentrations. Wortmannin and LY-294002 blocked sarcomeric F-actin polymerization but not [H]phenylalanine uptake or ANF expression, whereas PD-098059 consistently blocked both [H]phenylalanine uptake and ANF expression, thereby suggesting that NRG-1-induced cardiomyocyte hypertrophy is mediated by both PI-3-kinase and MAPK-RSK pathways.

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hypertrophic response in neonatal rat ventricular myocytes is characterized by a series of phenotypic changes, including an increase in protein synthesis, increased organization of contractile proteins into sarcomeric units, and reexpression of a number of “fetal genes” (34). A number of growth-promoting peptide and nonpeptide growth-promoting mediators are now known to activate one or more nonredundant intracellular signaling pathways. Use of PD-098059, a specific inhibitor of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK)-1, has confirmed that the activation of a hypertrophic program by ANG II and α-adrenergic agonists involves, at least in part, increased ERK activity (2, 6, 32). Moreover, dominant negative forms of ras have been shown to inhibit α-adrenergic agonist (phenylephrine)-induced hypertrophic responses (such as increase in cell size, transcriptional activation of atrial natriuretic peptide, and organization of actin filaments), thereby indicating that ras is essential for phenylephrine-induced hypertrophy (22, 43). However, multiple parallel signaling pathways must be involved, since transfection or microinjection of a dominant negative form of MAPK or Raf-1 did not suppress organization of actin filaments induced by phenylephrine (44, 45).

Cardiac muscle hypertrophy also requires an accelerated rate of protein synthesis, which is regulated in part by signaling proteins that interact with the translational machinery of the ribosome (21). Among them, S6, a component of 40S ribosomal proteins, is located at the interface between 40S and 60S ribosomal proteins and may interact directly with the mRNA (39). Accumulating evidence suggests that multiple serine phosphorylations of S6 at the carboxy terminus regulate the rate of protein synthesis by stimulating initiation and elongation of protein translation (39). The S6 phosphorylation at the carboxy terminus is mediated by a family of serine/threonine kinases, known as S6 kinases, which consist of two distinct families, a 90-kDa ribosomal S6 protein kinase (RSK; p90S6K) and a 70-kDa protein kinase (p70S6K) (4, 15, 39).

RSK consists of three isoforms: RSK-1, RSK-2, and RSK-3, of which, RSK-2 (also known as cAMP response element-binding protein kinase) is a well-recognized signaling protein that is activated by a variety of growth factors in several tissues (10, 32, 40, 51). Several lines of evidence suggest that RSK and p70S6K are regulated by distinct signaling pathways. For example, RSK but not p70S6K is phosphorylated and activated by the ERKs (3–5, 11). p70S6K has been shown to be activated in response to ANG II and phenylephrine in cardiac myocytes (7, 13, 31). Rapamycin, a p70S6K inhibitor, selectively inhibits phenylephrine- and ANG II-induced protein synthesis and total RNA levels but does not affect the induction of the fetal gene program characteristic of hypertrophy induced by either phenylephrine or ANG II (7, 31). Moreover, increased protein content stimulated by phenylephrine and ANG II is rapamycin sensitive, whereas that stimulated by fetal calf serum is not (7, 31).

Recently, class IA phosphoinositide 3-kinases (PI-3-kinases), heterodimeric proteins composed of an ~85-kDa adapter and ~110-kDa catalytic subunits that catalyze the synthesis of 3-phosphorylated phosphoinositide, have been shown to be associated with receptors that stimulate cellular growth and reorganization of actin, indicating a role for this lipid kinase in mitogenic signaling (47). However, the role of PI-3-kinases in mediating cardiac myocyte hypertrophy has not been extensively evaluated. Wortmannin, a fungal derivative, and the drug LY-294002 have been identified as relatively specific inhibitors of PI-3-kinase in cardiac myocytes. At concentrations of 10–50 M and 50 µM/l, LY-294002 inhibits p70S6K and has been shown to affect phenylephrine-induced protein synthesis in cardiac myocytes.

Therefore, the present study was designed to examine 1) whether NRG-1-induced myocyte protein synthesis, atrial natriuretic factor (ANF) gene expression, and sarcomeric actin reorganization, associated with hypertrophic growth, were sensitive to inhibitors of MEK, p70S6K, and PI-3-kinase; 2) whether NRG-1 activates p42/p44 (ERK) MAPKs; and 3) the effects of NRG-1 on RSK-2 (p90S6K) and p70S6K in cardiac myocytes.

METHODS

Materials. All culture reagents were purchased from Gibco BRL (Gaithersburg, MD). All radiochemicals were obtained from NEN (Boston, MA) and Amersham. PD-098059 was obtained from New England Biolabs. LY-294002 was obtained from Biomol. NRG-1 was a gift from Cambridge Neuroscience, Cambridge, MA. Rapamycin was a gift from Dr. Suren Sehgal of Wyeth-Ayerst Pharmaceuticals. All other chemicals were from Sigma Chemical (St. Louis, MO).

Preparation of neonatal rat ventricular myocyte cultures. Primary cultures of the neonatal rat ventricular myocytes (NRVMs) were prepared as described previously (37). To selectively enrich for myocytes, the dissociated cells were preplated twice for 2 h each, during which time nonmyocytes attached readily to the bottom of the culture dish. This was followed by centrifugation at 500 g for 5 min. The centrifuged cells were then resuspended in 10% FCS containing DMEM. The resultant suspension of myocytes was plated on 100-mm culture plates at a density of 1.5 × 10⁶ cells/cm² in 7% bovine calf serum with cytosine arabinoside (10 µM) for the first 24 h. The medium was then changed to a serum-free condition for 24–48 h with 100 µM of bromodeoxyuridine. Using this method, we routinely obtained myocyte-rich cultures with 90–95% myocytes, as assessed by microscopic observation and by immunofluorescence staining with a monoclonal antibody (MF20) against α-myosin heavy chain.

MAPK assays. MAPK activity was assessed both by in-gel kinase and by immune kinase assays. To assess the activation of MAPKs, in-gel myelin basic protein (MBP) kinase assays were carried out. Myocyte extracts were prepared with the use of buffer A (1% Triton X-100, 150 mM NaCl, 10 mM potassium phosphate, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40). Total protein content was measured with the use of the Bradford assay (Bio-Rad). A quantity of 60–100 µg of protein was resolved with the use of 10% SDS-PAGE polymerized with 0.4 mg/ml of MBP. Each gel was then washed with 20% isopropanol in buffer B (100 mM Tris at pH 8.0 and 5 mM 2-mercaptoethanol) and then denatured by buffer B containing 6 M guanidine-HCl for 1 h, followed by renaturation in...
buffer B containing 0.04% Tween 40 at 4°C for 16 h, with five to six changes of this buffer over the time period. The gel was then incubated in kinase buffer A (20 mM HEPES, pH 7.2, 10 mM MgCl₂, and 2 mM 2-mercaptoethanol) for 30 min, followed by another incubation in kinase buffer A containing 250 μCi of [γ-³²P]ATP (NEN) and 50 μM ATP at room temperature for 1 h. The gel was then washed several times with 1% sodium deoxycholate in 5% TCA, and radiolabeled MBP was detected by autoradiography.

All experiments assessing MAPK activity were confirmed by immune complex kinase assay with the use of cellular lysates prepared from ventricular myocytes. Lysates were immunoprecipitated with anti-ERK2 antibodies (Santa Cruz Biotechnology), and immune complexes were washed three times with an immunoprecipitation buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM dithiothreitol) and once with kinase buffer B (30 mM Tris, pH 8, 20 mM MgCl₂, and 2 mM MnCl₂). MAPK activity was assayed by the addition of kinase assay kit (Upstate Biotechnology, Lake Placid, NY). The assay kit is based on the phosphorylation of MBP by MAPK activity assay kit (Upstate Biotechnology). p70S6K activity was measured with p70S6K antibody (Santa Cruz Biotechnology) for 16 h at 4°C. Protein A-Sepharose was then added, and the immunoprecipitates were washed with buffer A three times. The immunoprecipitates were then mixed with 2x sample buffer (125 mM MgCl₂, Tris pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.003% bromphenol blue) and electrophoresed on a 7.5% SDS-polyacrylamide gel (Bio-Rad) for 30 min. The phosphorylated substrate was separated from the residual [γ-³²P]ATP with the use of P81 phosphocellulose paper, and radioactivity was quantified by scintillation counting (protocol designed by Upstate Biotechnology).

Incorporation of [³H]phenylalanine and [³H]thymidine. As an index of protein synthesis, [³H]phenylalanine incorporation was measured as described previously (38). After incubation in serum-free medium for 48 h, cells were stimulated with rhGGF2 (20 ng/ml) for 24 h in the presence of [³H]phenylalanine (10 μCi/ml) and unlabeled phenylalanine (0.36 mM) in the medium. Cells were washed with PBS, and 10% TCA was added at 4°C for 60 min to precipitate protein. Under control conditions, parallel cultured cells were harvested without NRG-1 stimulation. The precipitate was washed three times with 95% ethanol and then resuspended at 0.15 N NaOH. Aliquots were counted in a scintillation counter, and the results are expressed as counts per minute per dish. The effects of rapamycin, PD-098059, wortmannin, and LY-294002 on NRG-1-induced protein synthesis were also determined.

[³H]Thymidine uptake measurement and cell counts were performed in the culture medium in the absence of bromodeoxyuridine. Cells were grown in serum-free media for 24 h and then stimulated with 20 ng/ml of NRG-1. After 18 h, [³H]thymidine (5 μCi/ml) was added for 6 h. Cells were then washed with PBS and harvested with 10% TCA. TCA-precipitable counts were measured as above.

Regulation of RSK-2 activity. RSK-2 activity was measured by an immune complex kinase assay with the use of S6 peptide (RRRLSSLRA) as a substrate (5). Cell-free lysates were prepared similar to those for MAPK assay, except that buffer C was used. Buffer C contained 10 mMol/l KPO₄ (pH 7.4), 1 mMol/l EDTA, 5 mMol/l EGTA, 10 mMol/l MgCl₂, 50 mMol/l β-glycerophosphate, 1 mMol/l sodium orthovanadate, 1 µg/ml p70S6K antibody, and 2 µM cold ATP and 0.5% Triton X-100 at 4°C. The immunoprecipitates were then electrophoretically transferred to polyvinylidene difluoride membranes at 5 V/cm for 16–20 h at 4°C. The membranes were blocked with 5% nonfat milk in PBS for 4 h and washed twice in PBS before incubation with the same anti-p70S6K antibody for 16–20 h. This was followed by three more washes and incubation for 1 h with a 1:10,000 dilution of secondary antibody (goat anti-rabbit IgG conjugated with horseradish peroxidase). Antibody binding was detected with the use of the enhanced chemiluminescence method, according to the manufacturer's instruction (Amersham).

For immunoblotting of RSK-2 antibody (Santa Cruz Biotechnology) for at least 2 h at 4°C. The immunoprecipitates were then washed in buffer C at least three times. Ten microliters of immunoprecipitate were incubated with substrate and inhibitors with the use of a kit from Upstate Biotechnology and [γ³²P]ATP (10 Ci/mmol). To terminate the reaction, samples were spotted onto Whatman P81 phosphocellulose paper (2.5 cm) and washed five times (5 min each) with 0.5% phosphoric acid and once with acetone. The papers were then placed into scintillation vials, and the radioactivity was counted.

For immunoblotting of RSK-2, cell lysates from myocytes treated with NRG-1 were prepared by addition of 1 ml of ice-cold lysis buffer A containing (in mmol/l) 10 potassium phosphate (pH 7.4), 1 EDTA, 5 EGTA, 10 MgCl₂, and 50 β-glycerophosphate, along with 10 mMol/l okadaic acid, 100 μmol/l leupeptin, 10 μmol glycerol, 100 μmol/l okadaic acid, and 0.003% bromphenol blue) and boiled. The immunoprecipitates were mixed with 2x sample buffer (125 mMol/l Tris, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.003% bromphenol blue) and boiled. The immunoprecipitates were
then loaded into each lane of a 7.5% SDS-polyacrylamide gel and electrophoresed at a constant current of 20 mA. Proteins were then electrophoretically transferred to polyvinylidene difluoride membranes at 5 V/cm for 16–20 h at 4°C. The membranes were blocked with 5% nonfat milk in PBS for 4 h and washed in PBS three times before incubation with the same anti-RSK-2 antibody for 16–20 h, followed by three more washes and incubation for 1 h with a 1:10,000 dilution of secondary antibody (goat anti-rabbit IgG conjugated with horseradish peroxidase). Antibody binding was detected with the use of the enhanced chemiluminescence method, according to the manufacturer’s instruction (Amersham).

Effect of NRG-1 on sarcomeric F-actin organization. To determine the effect of the pharmacological inhibitors of MEK/ERK, p70S6K, and PI-3-kinase activities, followed by NRG-1 on reorganization of F-actin, the cells were pretreated with PD-098059, rapamycin, wortmannin, and LY-294002 in separate plates and then treated with NRG-1 (20 ng/ml). Wortmannin (Sigma) was dissolved in DMSO to a final concentration of 10 mM, dispensed into 5-µl aliquots, and stored at 4°C. Wortmannin aliquots were diluted in 1:1,000 ice-cold PBS to a concentration of 10 µM. Aliquots from this diluted stock were added directly to the cells to achieve the final concentration of 100 nM. Cells were incubated for 24 h before fixation. Cells were washed with PBS, fixed with 3.6% formaldehyde in PBS, lysed with 0.3% Triton X-100 in PBS, and blocked with 10% goat serum in PBS plus 0.1% Tween 20. Next, cells were stained with FITC-conjugated phalloidin (Sigma; 40 µg/ml in PBS, 0.5% Nonidet P-40, and 2 mg/ml BSA) and washed in PBS plus 0.1% Tween 20. The cells were then incubated with anti-myosin heavy chain (1:300) as described previously (53) and mounted for indirect fluorescence microscopy (44).

Effect of NRG-1 on ANF gene expression. Total cellular RNA was isolated from cultured ventricular myocytes treated with NRG-1 for 24 h by a modification of the acid guanidinium thiocyanate-phenol-chloroform extraction method with the use of the Trizol reagent (Life Technologies), as described in detail previously (53). After denaturation in formaldehyde and formaldehyde, equal amounts of total RNA (10 µg/lane) were size-fractionated by electrophoresis through 1% agarose gels containing 3% formaldehyde. The fractionated RNA was electrophoretically transferred to nylon membranes (GeneScreen Plus; NEN) at 5 V/cm, cross-linked by ultraviolet radiation at 9,120 mJ (UV Stratalinker 1800, Stratagene, La Jolla, CA), and then hybridized at 63.5°C with [32P]-labeled oligonucleotide and rat preproANF probe (0.6-kb pair of coding region). Complementary DNA probes were radiolabeled with the use of the random-priming method (Boehringer Mannheim, Indianapolis, IN). Oligonucleotides were radiolabeled by terminal deoxynucleotide transferase with [α-32P]dATP. Signal efficiency was determined by densitom-
etry (GS-700; Bio-Rad, Hercules, CA). All blots were reprobed with the 32P-oligonucleotide complementary to 18S ribosomal RNA, washed, and autoradiographed with a laser densitometer. Levels of mRNA reported here are normalized to the level of 18S rRNA to correct for potential differences in the amount of RNA loaded and transferred.

Statistical analysis. Data are given as means ± SE. Statistical analyses were performed with the use of ANOVA or unpaired Student’s t-test, as appropriate. P values were adjusted by the Bonferroni method for multiple comparisons. A value of P < 0.05 was considered significant.

RESULTS

NRG-1-induced growth of NRVMs is inhibited by rapamycin and PD-098059 but not by wortmannin or LY-294002. We examined the effects of a NRG-1 (rhGGF2) on protein synthesis and the rate of DNA synthesis in primary NRVM cultures. In myocytes, rhGGF2 (20 ng/ml) caused a significant increase in protein synthesis, as reflected by [3H]phenylalanine incorporation over 24 h (Fig. 1A). The magnitude of increase in [3H]phenylalanine induced by rhGGF2 (20 ng/ml) was comparable with that induced by phenylephrine (10 µM), a well-characterized hypertrophic stimulus for the neonatal rat myocyte phenotype (data not shown). In contrast, rhGGF2 (20 ng/ml) did not significantly increase DNA synthesis, as measured by [3H]thymidine uptake over 24 h (data not shown). Interestingly, a higher concentration of rhGGF2 (100 ng/ml) did not increase protein synthesis compared with control (Fig. 1B). Rapamycin and PD-098059 inhibited NRG-1-induced protein synthesis, whereas wortmannin had no significant effect (Fig. 1A). Interestingly, when the cells were pretreated with LY-294002, NRG-1 caused a 3.5-fold increase in [3H]phenylalanine uptake.

NRG-1-mediated preproANF expression is inhibited by PD-098059 but not by rapamycin, wortmannin, or LY-294002. We examined the effects of a NRG-1 (rhGGF2) on preproANF gene expression in primary NRVM cultures. In myocytes, rhGGF2 (20 ng/ml) caused a 1.8-fold increase in preproANF gene expression over 24 h (Fig. 2). The magnitude of increase in preproANF gene expression induced by rhGGF2 (20 ng/ml) was comparable with that induced by phenylephrine (10 µM). PD-098059 inhibited NRG-1-induced ANF gene expression, whereas wortmannin had no significant effect (Fig. 2). Pretreatment with LY-294002 resulted in NRG-1 causing a 3.9-fold increase in ANF compared with control (Fig. 2).

Rapamycin, wortmannin, and LY-294002 but not PD-098059 inhibit NRG-1-induced reorganization of sarcomeric F-actin. We also examined the effect of NRG-1 on reorganization of contractile proteins into sarcomeric units by examining the filamentous actin within myocytes with fluorescently labeled phalloidin. Figure 3A shows control myocytes (i.e., in the absence of serum), and Fig. 3B shows myocytes treated with 20 ng/mg rhGGF2 for 24 h. The actin of control myocytes is generally not organized into parallel myofibrillar bundles, whereas after NRG-1 treatment, the number of myocytes that show organized sarcomeric actin (visible as discrete fluorescent bands) significantly increased. NRG-1-induced organization of actin was also observed in myocytes treated with NRG-1 in the presence of 50 µM PD-098059 (Fig. 3C) but not in myocytes treated with NRG-1 in the presence of either rapamycin (10 ng/ml; Fig. 3D), wortmannin (100 nM; Fig. 3E), or LY-294002 (10 µM; Fig. 3G). None of these agents had any effect on F-actin polymerization in the absence of NRG-1 (data not shown). Interestingly, as in the case of the protein synthesis data noted above, a fivefold higher concentration of NRG-1 (i.e., 100 ng/ml rhGGF2) had only minimal effects on actin reorganization (Fig. 3, F compared with B).

NRG-1 activates ERK1/ERK2 MAPKs. To examine whether ERK activities are activated by rhGGF2 (20 ng/ml), we examined the effect of rhGGF2 on ERK1/ERK2 phosphorylation in NRVMs. Representative Western blot (A) and aggregate data from 2 independent experiments (B) are shown, describing effects of PD-098059, LY-294002, rapamycin, and wortmannin on ERK1/ERK2 phosphorylation. Figure 4A shows the effects of NRG-1 on ERK1/ERK2 phosphorylation in NRVMs. The magnitude of increase in phosphorylation induced by NRG-1 was comparable with that induced by phenylephrine (10 µM). PD-098059 inhibited NRG-1-induced ERK1/ERK2 phosphorylation, whereas wortmannin had no significant effect (Fig. 4A). Pretreatment with LY-294002 resulted in NRG-1 causing a 4.2-fold increase in ERK1/ERK2 phosphorylation compared with control (Fig. 4B).

Fig. 2. NRG-1 increases prepro-atrial natriuretic factor (preproANF) expression in NRVMs. Representative Northern blot (A) and aggregate data from 2 independent experiments (B) are shown, describing effects of PD-098059, LY-294002, rapamycin, and wortmannin on preproANF expression from total RNA (20 µg/ml) from NRVM incubated with NRG-1 for 24 h. Only PD-098059 inhibited NRG-1-induced ANF expression at 24 h. Pretreatment with LY-294002 resulted in a 3.9-fold increase in ANF expression that was nearly double that induced by NRG-1 alone. Levels of mRNA reported here were normalized to level of 18S rRNA to correct for potential differences in amount of RNA loaded and transferred (means ± SE; * P < 0.05).
ng/ml), we measured their activities with the use of SDS-PAGE gels containing MBP. As shown in Fig. 4, A–C, NRG-1 (rhGGF2) activates MBP kinase activities of proteins at 44 and 42 kDa in NRVM lysates, consistent with the activation of ERK1 and ERK2, respectively, with a peak in ERK2 activity at 10 min. To confirm the identity of the 44- and 42-kDa kinase activities on ERK1/ERK2, respectively, kinase assays were performed after immunoprecipitation with specific MAPK antibodies. The results in Fig. 4D are expressed as the relative increase compared with baseline MAPK activity. In immunoprecipitates with an anti-ERK1 antibody, three- to fourfold increases in myocyte MAPK activity were observed after a 10-min exposure to NRG-1. The maximal effects of rhGGF2 were observed in the 10-ng/ml range; higher concentrations (i.e., 100 ng/ml) did not induce MAPK activation. The selective MEK1 inhibitor PD-098059 (50 µM) inhibited the NRG-1-induced increase in p42/p44 MAPK, confirming that NRG-1 activates the MEK-ERK pathway (Fig. 4E).

NRG-1 activates RSK-2. The phosphorylation of the 40S ribosomal protein (S6) occurs during increased protein synthesis and growth. At least two families of S6 kinases have been identified, a 70- to 85-kDa S6 kinase (p70S6K) and p90S6K (RSK). Because p42/p44 MAPKs activate RSK, we examined whether NRG-1 activates RSK-2, using an immune complex RSK-2 assay with an S6 peptide (RRLSSLRA) as substrate. As shown in Fig. 5, rhGGF2 (20 ng/ml) activated RSK-2 in cardiac myocytes. The time course of activation of RSK-2 (Fig. 5A) was similar to that of p42/p44 MAPKs. Phosphorylation of the S6 peptide in this assay condition was specific to RSK-2, because no significant increase in S6 peptide phosphorylation was observed when the anti-RSK antibody was preabsorbed with an excess amount of antigen peptide. The activity of RSK-2 kinase could be inhibited by PD-098059, confirm-
Fig. 4. NRG-1 activates myocyte ERK1/ERK2. A: NRVMs were exposed to rhGGF2 (20 ng/ml) for 10 min, after which cell lysates were immunoprecipitated with an anti-ERK1 antibody, followed by analysis of MAPK activity. FCS, fetal calf serum. B and C: neonatal myocytes were exposed to 20 ng/ml rhGGF2 for indicated times, and cell lysates were prepared and applied to SDS-PAGE gels containing myelin basic protein. D: concentration-effect curve is shown for rhGGF2 (0.1–100 ng/ml) on activation of p44 MAPK activity (the blot shows representative immunoprecipitation for ERK1 at each concentration). E and F: MEK inhibitor PD-098059 (50 µM, E) inhibited activation of p44 MAPK (F) at 10 min induced by 20 ng/ml rhGGF2. All data were normalized to MAPK activities in control, untreated cells (means ± SE; *P < 0.05).
ing that NRG-1 activation of RSK-2 is mediated by the MEK-p44/p42 ERK pathway (Fig. 5B). As with the activation of ERK1/ERK2, the NRG-1-induced increase in RSK-2 activity was maximal at 20 ng/ml; higher concentrations of NRG-1 resulted in less activation of these MAPKs. Indeed, at 100 ng/ml rhGGF2, there was no increase in RSK-2 activity compared with initial levels (Fig. 5C).

NRG-1 activates p70S6K. To examine whether p70S6K is activated on stimulation with NRG-1, in vitro kinase assays were performed with S6 peptide used as a substrate, with the use of myocyte lysates that had been immunoprecipitated with an antibody against p70S6K. As shown in Fig. 6A, the NRG-1-induced increase in p70S6K activity was observed at 1 ng/ml, reached a peak at ~20 ng/ml, and then declined at higher concentrations, similar to what was observed with RSK and ERK1/ERK2 activation. Pretreatment of myocytes with rapamycin prevented any increase in NRG-1-mediated activation of p70S6K, as shown in Fig. 6B. The time course of p70S6K activation was slower than that of ERK1/ERK2, as shown in Fig. 6C. There was an increase in p70S6K within 10 min of stimulation with rhGGF2 (20 ng/ml), which peaked at ~25 min and disappeared within 60 min.

Activation of PI-3-kinase has also been demonstrated to regulate p70S6K activity. To determine whether this signaling pathway mediates the NRG-1-induced increase in p70S6K activity, we incubated cardiac myocytes with wortmannin (100 nM) for 30 min before treatment with rhGGF2 (20 ng/ml). As shown in Fig. 6D, wortmannin inhibited the NRG-1-mediated increase in p70S6K activity.

DISCUSSION

In this report, we demonstrate that a soluble NRG-1, rhGGF2, induces increased protein synthesis, as reflected by a net increase in [3H]phenylalanine incorporation; increases ANF gene expression; and initiates organization of actin myofilaments into myofibrils in NRVMs in primary culture. Unlike embryonic cardiac muscle cells isolated from rat hearts in midembryogenesis, which exhibit a robust proliferative response to NRG-1 (53), neonatal myocytes exhibited no significant increase in [3H]thymidine uptake with rhGGF2.

The MEK1 (MAPK kinase) inhibitor PD-098059 effectively suppressed NRG-1-mediated [3H]phenylalanine uptake and ANF gene expression but had no effect on the organization of sarcomeric actin. This is consistent with the results reported for ANG II in NRVMs, in which PD-098059 blocked ANG II-mediated activation of ERK1/ERK2 and ANF gene expression but did not affect the ability of the peptide to trigger sarcomeric actin reorganization (2). Note that the absence of new protein synthesis or ANF gene expression is not required for organization of sarcomeric actin, which, in response to ANG II, is essentially complete within 30 min of addition to neonatal myocyte in culture (1).

Interestingly, the low-molecular-weight GTP-binding protein RhoA has been shown to play an intermediary role in myofibrillogenesis in NRVMs in response to...
activation of either the ANG II receptor (i.e., AT$_1$) or the $\alpha_1$-adrenergic receptor (1, 33). Nevertheless, RhoA appears not to be essential for mature myofibrillogenesis, even in response to ANG II, implicating several possibly redundant signaling pathways (1, 46).

The inability to detect an effect of wortmannin or LY-294002 on NRG-1-induced activation of $[^{3}H]$phenylalanine uptake does not completely exclude a role for PI-3-kinase in NRG-1-mediated protein synthesis. Duckworth and Cantley (14) have commented recently on the “conditional” inhibition of MEK1/ERK signaling by wortmannin, demonstrating that a wortmannin-inhibitable response in selected cell types can be overridden at higher agonist concentrations, probably by a
protein kinase C (PKC)-mediated pathway. Our data cannot exclude this potential mechanism.

The MEK1 (MAPK kinase) inhibitor PD-098059 effectively suppressed NRG-1-induced ANF gene expression, an effect similar to phenylephrine-induced cardiomyocyte hypertrophy but in contrast to ANG-II-induced hypertrophy. Like ANG-II-induced ANF gene expression, rapamycin did not suppress NRG-1-induced ANF gene expression. Wortmannin also did not affect ANF gene expression, despite inhibition of p70S6K, suggesting that NRG-1-induced ANF expression is not entirely dependent on p70S6K activation or that wortmannin did not completely inhibit p70S6K. Finally, pretreatment with LY-294002, another specific PI-3-kinase inhibitor, appeared to accentuate the increase in ANF gene expression in response to NRG-1, the mechanism for which remains unclear.

In contrast to a report that rapamycin had little effect on ANG II-induced myofibrillogenesis (31), rapamycin inhibited the increase in protein synthesis in response to NRG-1, and rapamycin, wortmannin, and LY-294002 inhibited the organization of sarcomeric F-actin by NRG-1. These results indicate that activation of a PI-3-kinase and subsequent activation of p70S6K are necessary for rapid myofibrillogenesis in response to NRG-1, although the role of this signal transduction pathway in the induction of protein synthesis in response to either ANG II or NRG-1 appears to differ. These discrepant results are explained in part by the different classes of PI-3-kinase isoenzymes activated by each extracellular signaling peptide. ANG II activates class Iα PI-3-kinases, which are coupled to G protein β-subunit signaling, whereas receptor tyrosine kinases are linked to class Iα PI-3-kinases (47). Activation of p70S6K by class Iα PI-3-kinases is known to be independent of Raf-MEK1/ERK signaling and might involve mammalian target of rapamycin mTOR and possibly a PKC (8, 47). In contrast, as shown in Fig. 5, activation of p90S6K by NRG-1 was clearly mediated by the MEK1/ERK pathway. Also, although not explicitly examined in the experiments here, other parallel MAPK-mediated pathways might also play a role in the hypertrophic response of cardiac myocytes. Both the stress-activated protein kinase/c-J un amino-terminal kinase pathway and the p38-MAPKs clearly play a role in mediating phenotypic adaptation of cardiac muscle to specific forms of stress [e.g., reactive oxygen species induced by ischemic perfusion (52) as well as by well-characterized hypertrophic stimuli, e.g., the activation of p38MAPK by α1-adrenergic agonists (12, 17, 50, 52)].

The mechanism(s) responsible for the decline in NRG-1-mediated [3H]phenylalanine incorporation, MEK1/ERK, and p70S6K activation by higher (i.e., >10 ng/ml) concentrations of rhGGF2 is unclear. This was not observed in the proliferative response of e17 embryonic rat ventricular myocytes exposed to this concentration of rhGGF2, in which the rate of increase in [3H]thymidine incorporation had reached plateau at 30 ng/ml rhGGF2 but did not decline at higher concentrations (53). Moreover, the anti-apoptotic effect of NRG-1 on both neonatal and adult rat ventricular myocytes cultured in serum-free medium was sustained at 100 ng/ml (2 h), although, again, no additional improvement in survival was seen at concentrations >10 ng/ml rhGGF2 in either the neonatal or adult phenotype (53). One possible explanation for the suppression of MEK1/ERK activation by 100 ng/ml rhGGF2 is suggested by recent reports by Vecchi et al. (48) and Vecchi and Carpenter (49). They demonstrated that activation of a phorbol ester-activated PKC isoenzyme in a number of cell types that constitutively express ErbB4, including the AT-1 cardiac muscle cell line, results in rapid and extensive proteolytic cleavage of the 120-kDa ectodomain of the receptor by a metalloprotease. Cleavage of the ectodomain results in ubiquination of the membrane-anchored cytoplasmic domain and targeting to the proteosome, a pathway that is clearly distinct from that of the EGF (ErbB1) receptor, which contains a lysosomal targeting motif and is internalized by a clathrin-mediated pathway. Thus the concentration of rhGGF2 above those found to induce a maximal response in MEK1/ERK signaling in neonatal myocytes might, by virtue of triggering activation of a PKC, result in a rapid decline in NRG-1-coupled receptors (predominantly ErbB4 in postnatal cardiac myocytes).

The demonstration that at least two NRG receptors (ErbB2 and ErbB4) are expressed in neonatal and adult ventricular myocytes (53) and are functionally coupled to downstream signaling pathways in cardiac myocytes, as demonstrated here, suggests that NRG-1/ErbB signaling, essential for normal myocardial development, remains intact in postnatal myocardium. This, coupled with the observation that NRG-1 expression can be rapidly induced by endothelins in the coronary microvascular endothelial cells (53), suggests that NRGs might, in part, mediate myocardial hypertrophy and remodeling in the intact heart.

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Present addresses: R. R. Baliga, Division of Cardiology, UT Southwestern Medical Center, Dallas, TX 75235; D. R. Pimental and D. B. Sawyer, Division of Cardiology, Boston University Medical Center, Boston, MA 02118.

Address for reprint requests and other correspondence: R. A. Kelly, Division of Cardiology, Brigham and Women’s Hospital, 75 Francis St., Boston, MA 02115 (E-mail: rakelly@rics.bwh.harvard.edu).

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