Neuregulin-1 Induces a Negative Inotropic Effect in Cardiac Muscle

Role of Nitric Oxide Synthase

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Background—Deficient cardiac neuregulin/ErbB signaling increases susceptibility to heart failure. In this study, we examined the effects of neuregulin-1 (NRG-1) on myocardial contractility. Methods and Results—NRG-1 (α and β isoforms) induced a negative inotropic effect in isolated rabbit papillary muscles and a rightward shift of the dose-response curve to isoproterenol. Both effects were attenuated by L-NMMA, which suggests a role for NO synthase. In cultured rat cardiomyocytes, NRG-1β enhanced nitrite production and resulted in phosphorylation of endothelial NO synthase and the serine/threonine kinase Akt. Conclusions—NRG-1 has negative inotropic effects that are preserved during β-adrenergic stimulation and activates endothelial NO synthase in cardiomyocytes. (Circulation. 2004;109:324-326.)

Key Words: neuregulin ■ nitric oxide synthase ■ endothelium ■ contractility ■ heart failure

Monoclonal antibodies against the neuregulin (NRG) receptor ErbB2 (Trastuzumab), used in the treatment of breast cancer, may induce dilated cardiomyopathy and heart failure. From this observation, it was hypothesized that the NRG/ErbB pathway may participate in the pathogenesis of heart failure.

In the fetal heart, NRG-1 has been detected in endothelial cells, whereas ErbB2 and ErbB4 receptors are expressed throughout the myocardium. Targeted mutagenesis of NRG-1, ErbB2, or ErbB4 results in embryonic lethality due to failure of myocardial trabeculation. The ErbB3 receptor is present in endocardial cushion mesenchyme, and ErbB3-deficient mice exhibit cardiac cushion abnormalities. In the adult heart, ErbB2 and ErbB4 are found in the T-tubule system of cardiomyocytes, whereas NRG-1 is expressed in endocardial and microvascular endothelial cells. Conditional mutation of cardiac ErbB2 leads to dilated cardiomyopathy, which suggests a role for the NRG/ErbB pathway in heart failure.

Experimental studies have shown that NRG-1 promotes survival and hypertrophic growth of adult cardiac myocytes in vitro. In the present study, we characterized the direct effects of NRG-1 on contractility of adult cardiac muscle and the possible contribution of the Akt-NO synthase (NOS) pathway in these effects.

Methods

Mechanical Performance

Isometric contractions were recorded from papillary muscles isolated from the right ventricle of New Zealand White rabbits with an electromechanical length-force transducer as described previously. After determination of the length at which peak twitch active tension is maximal (Lmax), stabilized muscles were randomly assigned to one of the following treatment protocols: (1) muscles kept in baseline conditions (control group), (2) muscles in which NRG-1α2 epidermal growth factor (EGF) domain (500 ng/mL or 71 nmol/L, Sigma; hereafter referred to as NRG-1α) or NRG-1β EGF domain (100 ng/mL or 13 nmol/L, Sigma; hereafter referred to as NRG-1β) was added at time zero, or (3) muscles exposed to indomethacin (10 μmol/L, Merck Sharp & Dohme), L-NMMA (N\(^\text{G}\)-methyl-L-arginine, acetate salt, 50 μmol/L, Sigma), or the tyrosine kinase inhibitor genistein (25 μmol/L, Sigma) 30 minutes before NRG-1 administration. Drug treatments were added to the solution bathing the muscle and remained present to the end of the experiment. Separate experiments were performed for cumulative dose-response curves for NRG-1α (0.1 to 100 ng/mL). In separate experiments, cumulative concentration-response curves were constructed for isoproterenol (10 nmol/L to 10 μmol/L, Sigma) in control muscles and in muscles exposed to NRG-1α (100 ng/mL) or NRG-1β (20 ng/mL) for 5 hours in both the presence and absence of L-NMMA.

Cell Isolation and Culture

Neonatal rat cardiac myocytes (NRCMs) were isolated from 1- to 2-day-old Harlan Sprague-Dawley rats and cultured as described previously. After 24 hours, cells were washed with Hank’s balanced salt solution and serum-starved for 24 hours before the experiments were begun.

Measurement of NO Production

NRCMs plated in 12-well culture plates at a density of 5×10⁵ cells/well were exposed to NRG-1 for 24 hours, and medium was assayed for nitrite with the Griess reaction. L-NMMA was added to determine the role of NOS. Acetylcholine (Sigma) was used as a positive control. Protein content was determined with the Bradford assay.
Immunoprecipitation
Immunoprecipitation experiments were performed as described previously with slight modifications. Briefly, NRCMs were plated at a density of 4×10^5 cells per 100-mm dish and treated with NRG-1β. Cells were harvested in lysis buffer with 0.2% Triton X-100, and lysates were incubated with anti-eNOS (endothelial NOS) antibody (Transduction Technologies) and secondary horseradish peroxidase–conjugated antibodies (Amersham). The reactive proteins were detected by chemiluminescent reaction on Hyperfilms (Amersham).

Statistics
All data are expressed as mean±SEM. Repeated-measures ANOVA (SPPS 10.0) were performed on raw data (after logarithmic transformation for homoscedasticity) to analyze differences between different treatments.

The EC_50 (the concentration at which the effect is half-maximal) of NRG-1 and of the isoproterenol dose-response curves was calculated on raw data of active tension development with Graph Pad. EC_50 of isoproterenol dose-response curves of the different treatments were analyzed with ANOVA followed by Bonferroni correction for multiple comparisons (SPPS 10.0). Differences were considered significant at P<0.05.

Results
Effects of NRG-1 on Myocardial Contractility
Ninety minutes after its addition, NRG-1β (100 ng/mL) significantly decreased peak twitch active tension by 18.6±2.6% versus baseline (from 42.6±8.0 mN/mm², n=7, P<0.001) and peak rate of tension development by 11.0±5.0% versus baseline (from 286±49 to 255±45 mN·mm⁻²·s⁻¹, n=7, P<0.001), whereas in control muscles, these parameters remained unchanged. The onset of this negative inotropic response appeared within 30 minutes and persisted for at least 90 minutes (Figure 1A). Indomethacin, an inhibitor of the prostaglandin pathway, did not affect this negative inotropic effect (eg, at 90 minutes: active tension −26.1±3.4%, n=6, P=1 versus NRG-1β alone, P=0.001 versus indomethacin alone). L-NMMA, an inhibitor of NOS significantly attenuated the response to NRG-1 (eg, at 90 minutes: active tension −2.0±3.7%, n=9, P=0.04 versus NRG-1β alone, P=1.00 versus L-NMMA alone; Figure 1A), whereas genistein completely blocked the effect of NRG-1 (data not shown, n=5). After NRG-1β was washed out, muscle performance partially recovered (NRG-1β washed out at t=60 minutes; active tension at 60 minutes decreased 19.2±2.2% from baseline; 30 minutes after washout, active tension decreased only 9.2±1.2% from baseline; n=8). The response to NRG-1β was dose dependent, with EC_50 at 22.9±2.3 nmol/L (Figure 1B). Similar results were obtained for NRG-1α (Figure 1C).

NRG-1 also influenced myocardial performance under β-adrenergic activation. Figure 1D shows that NRG-1β significantly shifted the dose-response curve for isoproterenol to the right (logEC_50 −6.91±0.08, n=10, in control versus −6.23±0.16, n=9, in the presence of NRG-1β; P=0.01). This rightward shift was attenuated by L-NMMA (logEC_50 −6.66±0.17, n=9, P=1.00 versus control, P=0.26 versus NRG-1β), whereas L-NMMA in the absence of NRG-1β had no effect (logEC_50 −6.87±0.14, n=9). Similar results were observed for NRG-1α (data not shown).

NRG-1 Induces NO Production Through eNOS Activation
In NRCMs, NRG-1β enhanced nitrite production from 73±3.3 to 90±5.4 nmol/mg protein (n=12, P=0.008; Figure 2A). Furthermore, immunoblot analyses showed that NRG-1β induced a time-dependent phosphorylation of eNOS (Figure 2B) and of the serine/threonine kinase Akt (Figure 2C).

Discussion
In the present study, we demonstrated that NRG-1 affects myocardial contractility and activates NOS in cardiomyocytes. These observations add important information to the physiology of the NRG1/ErbB pathway.

First, we observed that both the α- and β-isoforms of NRG-1 induced a negative inotropic response in adult cardiac muscle. At first glance, this negative inotropic effect may appear in conflict with the putative protective actions of NRG-1 in the heart. A negative inotropic effect, however, should not be regarded as detrimental per se. It rather suggests that the NRG1/ErbB signaling pathway may have a modulatory role and could be activated in conditions of enhanced cardiac inotropism, such as in myocardial hypertrophy or during β-adrenergic stimulation.

Consistent with this conjecture, we observed that the inotropic effect of NRG-1 was preserved during adrenergic stimulation and desensitized the myocardium to isoproterenol. This negative
response to stretch while attenuating the β-adrenergic response in caveolae that harbor β-adrenoceptors. Consistent with the present report, activation of eNOS can thus depress contractility and reduce β-adrenergic responsiveness. Not surprisingly, ErB receptors colocalize with eNOS and β-adrenergic receptors in caveolae. With respect to overall cardiac pump performance, activation of eNOS can have distinct beneficial effects through hastening of the onset of ventricular relaxation, enhancement of early rapid filling, and improvement in diastolic compliance. However, L-NMMA blocks other NOS isoforms; thus, we cannot attribute the inotropic effects of NRG-1 to a specific NOS isoform.

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References

14. Sawyer DB, Zuppinger C, Miller TA, et al. The activation of eNOS is observed that NRG-1 activated Akt in cultured cardiomyocytes. Akt is a serine/threonine kinase involved in the phosphorylation of eNOS. The activation of eNOS is consistent with the observed negative inotropic effect, although the role of NO in the regulation of cardiac contractility appears somewhat conflicting. The inconsistent results in different studies can be explained in part by the recent discovery that the subcellular compartmentalization of NO isoforms with effector molecules is important for NO signaling. eNOS can increase excitation-contraction coupling in T-tubular caveolae close to the sarcoplasmic reticulum in

Figure 2. A, Nitrite in culture medium of neonatal rat cardiac myocytes in control conditions and after 24 hours stimulation with acetylcholine (positive control, 50 μmol/L), NRG-1α (500 ng/mL), or NRG-1β (20 ng/mL), L-NMMA (100 μmol/L), added simultaneously with NRG1, was used to determine role of NOS. *P<0.05. Data are mean of at least 4 independent triplicate experiments. B, Representative experiment (n=3) on effect of NRG-1α (20 ng/mL) on eNOS phosphorylation. After eNOS immunoprecipitation (IP), immunoblots were probed with anti-phospho-eNOS antibody. C, Representative experiment (n=3) on effect of NRG-1β (20 ng/mL) on Akt phosphorylation. Immunoblots were probed with anti-phospho-Akt antibody. WB indicates Western blot.
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