Myocyte contractile activity modulates norepinephrine cytotoxicity and survival effects of neuregulin-1β

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Kuramochi, Yukio, Chee Chew Lim, Xinxin Guo, Wilson S. Colucci, Ronglih Liao, and Douglas B. Sawyer. Myocyte contractile activity modulates norepinephrine cytotoxicity and survival effects of neuregulin-1β. Am J Physiol Cell Physiol 286: C222–C229, 2004. First published October 1, 2003; 10.1152/ajpcell.00312.2003.—The purpose of this study is to test the hypothesis that mechanical and electrical activity in adult rat ventricular myocytes (ARVM) alters responses to proapoptotic and prosurvival ligands. We investigated the effect of electrical stimulation on myocyte survival, stress signaling, response to β-adrenergic receptor (β-AR)-stimulated apoptosis, and neuregulin-1β (NRG) in myocytes. NRG stimulation of Erk and Akt was similar between paced and quiescent cells. Pacing sensitized myocytes to β-AR-stimulated JNK phosphorylation and cell death with 0.1 μM norepinephrine (NE) in paced myocytes causing equivalent cytotoxicity to 10 μM NE in quiescent cells. NRG suppressed β-AR-induced apoptosis through a phosphatidylinositol-3-kinase-dependent pathway in both paced and quiescent cells, although it is overwhelmed by high-NE concentration in paced cells. Thus myocyte contractility modulates both NE cytotoxicity as well as the cytoprotective effect of NRG. These results demonstrate the feasibility and importance of using electrically paced cardiomyocytes in primary culture when examining the signaling pathways of cell survival.

METHODS

Cell preparation and electrical stimulation. ARVM were isolated as previously reported (8) and plated at densities of 80–150 myocytes/mm2 on four-well rectangular plates or 40 × 22-mm glass coverslips precoated with laminin (Becton-Dickinson). One hour after cell preparation, medium was changed to DMEM supplemented with albumin, creatine, carnitine, and taurine (ACCT media), with the addition of ascorbic acid (100 μM). ARVM were stimulated with carbon electrodes using a culture cell pacer system from IonOptix (Milton, MA). Stimulus parameters were 6.5 V/cm (48), and the duration was 2 ms, with alternating polarity. Electrical stimulation was performed at 0, 2, and 5 Hz. Under these conditions, we obtained ∼70% capture of myocytes.

Cell treatment. The recombinant NRG, glial growth factor 2 (courtesy of M. Marchionni), was used at 10 ng/ml. l-Norepinephrine (NE; Sigma) was used at 1 μM for α-AR stimulation (NEα) and at 10.0, 1.0, and 0.1 μM for β-AR stimulation (NEβ) after pretreatment with 0.1 μM NRG.

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with either propranolol (2 μM; Sigma) or prazosin (100 nM; Sigma), respectively. Preincubation with the PI3K inhibitor LY-294002 (Calbiochem) was for 60 min in culture room for study. For cell viability and apoptosis studies in response to β-AR stimulation, cell treatments were started 2 h after plating.

**Measurement of mitochondrial respiration.** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction was used as a measurement of mitochondrial respiration. Cells were incubated with 0.2 mg/ml of MTT in the culture media at 37°C for 2 h. Cells were lysed with DMSO, and absorbance was measured after addition of Sorensen’s glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5).

**Cell viability and apoptosis.** Cell viability was assessed by measurement of creatine kinase (CK) release into culture media (CK-10, Sigma) and by trypan blue uptake (22). Apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay using in situ cell death detection kit (Roche). The percentage of nonviable trypan blue-positive cells and TUNEL-positive cells was determined by randomly counting 300 cells in each well or coverslip.

**Western blot analysis.** Heat shock protein (HSP)70 and HSP90 antibodies were from Stressgen, whereas anti-actin was obtained from Sigma. Antibodies against phospho-Akt, Akt, phospho-Erk1/2, and phospho-p38 were from New England Biolab. Anti-Erk2, p38, phospho-JNK, and JNK were from Santa Cruz Biotechnology. Cytochrome c antibody was from Calbiochem.

For the detection of cytochrome c release from mitochondria, cytosol fraction is extracted with digitonin lysis buffer (17). The residual organellae are lysed with modified RIPA buffer (1% NP-40, 50 mM Tris-HCl, 1 mM EDTA, 0.25% DOC, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 1 mM sodium orthovanadate). Aliquots representing 5 μg of protein from digitonin-permeabilized cytosolic and noncytosolic fractions were used. To detect other proteins, 50- to 100-μg aliquots of total cell lysates were used. Sample proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane (Bio-Rad). After membrane development with ECL reagent (Pierce), quantification was performed by densitometry (Molecular Analyst, Bio-Rad).

**RESULTS**

**Chronic electrical stimulation increases mitochondrial activity with minimal effect on cell survival.** The effects of electrical pacing on myocyte survival have not previously been reported. We therefore compared survival of ARVM in culture with or without electrical stimulation for up to 48 h. Electrical stimulation was performed at 0, 2, and 5 Hz using 2-ms pulse duration and 6.6-V/cm stimuli with alternating polarity. These conditions result in more than 70% capture of myocytes. MTT uptake was not different between unpaced and paced myocytes after 24 h of pacing. By 48 h, the MTT uptake was higher in the paced myocytes (P < 0.05 vs. 0 Hz. 0 vs. 2 and 5 Hz). Although MTT uptake is a measure of cell viability, we saw no systematic difference in trypan blue uptake and CK release in the culture media. The MTT results must therefore reflect an increase in mitochondrial respiration per cell in the paced myocytes. As CK release and trypan blue uptake do not detect early changes in apoptosis, we measured the number of TUNEL-positive cells. We found no difference in the number of TUNEL-positive myocytes among conditions at 24 h. By 48 h, myocytes paced at 2 Hz for 48 h demonstrated a small but significant reduction in apoptosis compared with both unpaced and paced myocytes. CK release and trypan blue uptake do not detect early changes in apoptosis.

**Chronic electrical pacing does not change MAPK activity and expression of HSP70/90 at baseline.** We examined the effect of electrical stimulation on cell “stress” using mitogen- and stress-activated protein kinases (Erk, JNK, p38) and HSP70/90 expression as stress indicators after 24 h of pacing. At this time point, there was no evidence of either kinase activation or changes in HSP expression (Figs. 2 and 3).

**Statistical analysis.** Results are expressed as means ± SD of at least three different experiments. One-way ANOVA was used for multiple comparison, with Bonferroni posttest analysis. A value of P < 0.05 was considered statistically significant.

**Fig. 1.** Chronic electrical stimulation of adult rat ventricular myocytes (ARVM) increases 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake with minimal change in myocyte viability. A: results of MTT assay are normalized to quiescent conditions (n = 8). *P < 0.05 vs. 0 Hz. B: percentage of apoptosis assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining (n = 5). *P < 0.05 vs. 0 and 5 Hz at 48 h. C: percentage of cell death of unfixed cells assessed by trypan blue uptake (n = 6). D. CPK assay of culture media (n = 6). P = not significant.
Electrical stimulation enhances NEβ-induced JNK phosphorylation without altering p38 activation. We examined short-term responses to NRG, NEα, or NEβ stimulation in quiescent and paced ARVM for 24 h. NRG-stimulated Erk1/2 and Akt activation as well as NEα-AR-stimulated Erk1/2 phosphorylation were similar in quiescent and paced cells (Fig. 2, B and C). However, pacing augmented NEβ-AR-stimulated activation of JNK (Fig. 3A). Phosphorylation of p38 in response to NEβ was not different in paced vs. quiescent cells (Fig. 3B).

Pacing augments NEβ cytotoxicity. The increased JNK activation by NEβ in paced myocytes suggests greater sensitivity and Akt activation as well as α-AR-stimulated Erk1/2 phosphorylation were similar in quiescent and paced cells (Fig. 2, B and C). In contrast, pacing augmented β-AR-stimulated activation of JNK (Fig. 3A). Phosphorylation of p38 in response to NEβ was not different in paced vs. quiescent cells (Fig. 3B).

Pacing augments NEβ cytotoxicity. The increased JNK activation by NEβ in paced myocytes suggests greater sensitivity
to NEβ in the setting of electrical pacing. We therefore examined the morphological alterations and apoptosis of ARVM with or without NEβ (10 μM NE) for 18 h. Cell shape did not change by electrical stimulation alone, but the combination of NEβ and pacing increased the number of rounded cells with membrane-blebs (Fig. 4A) as well as TUNEL-positive cells in a frequency-dependent manner (Fig. 4B). We examined the effect of pacing on the cytotoxic threshold of [NEβ]μM, comparing NEβ-induced cell death at 18 h as well as activation of JNK and p38 at 15 min. In quiescent myocytes, cell death was only increased at the high [NEβ]μM tested (10 μM), whereas in paced myocytes cell death occurred at [NEβ]μM as low as 0.1 μM (Fig. 4C). Similarly, while 0.1 μM NEβ did not activate JNK in quiescent ARVM, there was robust activation of JNK in ARVM paced at 5 Hz (Fig. 5A). Furthermore, we treated cells with the Ca2+ ionophore ionomycin at 10 μM to determine if JNK activation is in response to increased [Ca2+]i and observed immediate JNK phosphorylation (Fig. 5B). This result suggests the possibility that the increased JNK activation seen in paced myocytes in response to NEβ is due to a rise in [Ca2+]i.

NRG protects ARVM from NEβ-induced apoptosis through PI3K pathway activation. We examined the effect of NRG on β-AR-stimulated apoptosis. At [NEβ]0.1μM in paced myocytes, NRG protected ARVM from apoptosis (Fig. 6, A–C). At [NEβ]10μM, however, quiescent but not paced cells were rescued by NRG pretreatment (Fig. 6D). Thus NRG suppression of NEβ-induced cell apoptosis is dependent on the pacing frequency and [NEβ]μM.

NRG protection from NEβ apoptosis was prevented by the PI3K inhibitor LY-294002 (Fig. 6, A–C). NEβ-induced cytochrome c release from mitochondria and caspase-3 activation (Fig. 7), steps required for the apoptotic cascade (38), were also suppressed by NRG in a PI3K-dependent manner.

Fig. 4. Pacing sensitizes ARVM to NEβ-induced apoptosis. Myocytes are plated and paced at 0, 2, and 5 Hz. One hour after starting electrical stimulation, β-AR stimulation (with 10 μM NE) of myocytes is performed for 18 h. A: microscopic images with or without β-AR stimulation show increased numbers of rounded cells under paced conditions (original magnification: ×200). B: percentage of TUNEL-positive myocytes with or without β-AR stimulation (n = 4; *P < 0.05 vs. 0 and 5 Hz with NEβ; †P < 0.05 vs. 0 and 2 Hz with NEβ). C: myocytes were treated with NEβ at 0.1, 1.0, and 10.0 μM for 18 h. Then, cell viability was accessed by trypan blue uptake (n = 4; *P < 0.05 between 0 and 5 Hz, †P < 0.05 among 3 frequencies).

Fig. 5. Pacing sensitizes ARVM to NEβ-induced JNK activation. Myocytes are left quiescent or electrically paced for 24 h. Phosphorylated-JNK, phosphorylated-p38, and total JNK are examined by Western blot analysis with [NEβ]0.1μM for 15 min (A) and after incubation with ionomycin (10 μM) for indicated periods of times (B). Blots are representative of different 3 experiments.
DISCUSSION

The ARVM contract about five times per second in vivo, but upon isolation and placement in cell culture they beat rarely. These nonphysiological conditions acutely lower oxygen consumption (40) and chronically lead to changes in metabolic enzyme expression (51), decline in contractile properties (13, 20, 23), and change in fatty acid uptake (27). Electrical field stimulation has been used to maintain contractile function and calcium transients in ARVM and adult feline cardiomyocytes (4, 19, 23). However, the effects of electrical field stimulation have not been considered systematically in studies of myocyte survival. Some of these pacing systems require higher voltage or longer duration, and in earlier work there appeared to be deleterious effects on cell stress (24) and survival (Sawyer DB, unpublished observation). In the present study, using a commercially available system, we were able to find stimulus parameters that allowed field stimulation in the absence of molecular evidence of cell stress, without worsening myocyte survival.

Fig. 6. NRG protects ARVM from NEβ-induced apoptosis. A: cells paced at 5 Hz are incubated with indicated treatment for 18 h, and apoptosis is examined by TUNEL staining. NRG treatments are started 30 min before NEβ stimulation. Phase-contrast views of TUNEL staining (top, arrowhead means TUNEL-positive cell), light microscopic view (middle), and DAPI (bottom) staining are shown (original magnification: ×200, scale bar = 100 μm). B: TUNEL staining at high magnification (×400, scale bar = 100 μm). Arrow means the TUNEL-positive cell in which chromatin condensation is obvious on DAPI staining. Top: TUNEL staining; bottom: DAPI staining. C: quantification of TUNEL-positive myocytes. In 5-Hz paced cells, [NEβ]0.1μM causes almost the same percentage of apoptosis as quiescent cells treated with [NEβ]0.1μM. NRG protects cells from apoptosis under both conditions. The protective effect of NRG is completely blocked by phosphatidylinositol-3-kinase inhibitor LY-294002 (LY; n = 5). *P < 0.05 vs. control (no treatment), NEβ, LY + NEβ, and LY + NRG + NEβ at the same frequency; †P < 0.05 vs. NEβ, LY + NEβ, and LY + NRG + NEβ at the same frequency; ‡P < 0.05 vs. control, NEβ, LY + NEβ, and LY + NRG + NEβ at the same frequency. D: pretreatment with NRG for 30 min suppresses NEβ (10 μM)-induced apoptosis in quiescent cells but not in paced cells (n = 4). *P < 0.05 vs. control, NRG, and NRG + NEβ at the same frequency; †P < 0.05 vs. control and NRG.
or without LY-294002 and/or NRG after pacing overnight, followed by caspase-3 processing induced by overstimulation in the stimulation protocols and/or culture conditions. We anticipate that it makes sense that myocyte survival would improve in vitro under more physiological conditions. We found that mRNA expression of muscle-specific carnitine palmitoyltransferase-I, an enzyme for long-chain fatty acid oxidation in mitochondria, is increased in ARVM after 48 h of pacing by real-time PCR analysis (Kuramochi Y, unpublished observation). However, higher mitochondrial uptake of MTT did not completely correlate with cell viability and apoptosis in this study. Further work is needed, perhaps with metabolic substrate alterations, to better optimize cell survival in paced myocytes.

Chronic electrical stimulation with this system at frequencies up to 5 Hz did not induce changes in HSP70/90 expression nor activation of Erk1/2, p38, and JNK, which we interpret as evidence for minimal cell stress induced by pacing. This is in contrast to reports that short-term pacing induces JNK activation in NRVM (30, 46). This discrepancy may be explained by differences in the cell pacer device, timing of experiments, and conditions employed (11), or cell phenotype studied. Certainly, electrical stimulation at sufficient currents will induce oxidation of lipid and media, with deleterious effects on cell stress. The lack of baseline activation of stress signaling with preserved responses to ligand-activated signaling, in this case NRG and NE, shows the feasibility of using this pacing system to study myocyte hypertrophy under these more physiological conditions.

The sensitivity of myocytes to the cytotoxic effects of NEβ was markedly altered by electrical pacing, with ~100-fold decrease in the [NEβ]M necessary to induce cell death at physiological frequencies. Our findings support the notion that NEβ may contribute to adverse myocardial remodeling through the induction of apoptosis in vivo. Interestingly, the [NEβ]M that induced apoptosis in paced cells was much closer to that reported in animal models of cardiac failure than that in quiescent cells (34, 36, 42), suggesting the pacing system in more closely approximating in vivo conditions. Although the exact mechanism for increased NEβ sensitivity in paced ARVM remains to be elucidated, we suspect it is related, at least in part, to an increase in [Ca2+]i, that occurs in electrically stimulated myocytes (4, 19). In quiescent ARVM, NEβ-induced apoptosis can be inhibited with an L-type Ca2+ channel blocker (8). It is well known that prolonged [Ca2+]i elevations can induce cell death (29, 41, 45, 54). Electrical stimulation alone increases free Ca2+ content only during systole (4, 19), whereas the combination of β-AR stimulation and pacing acts synergistically to increase both systolic and diastolic [Ca2+]i through several mechanisms (54). Prolonged increases in [Ca2+]i act through one or more pathways including potentiation of calmodulin-dependent protein kinase II (54), increases in sarcoplasmic reticulum Ca2+ (44), and frequency-dependent Thr17 phosphorylation of phospholamban (18). Moreover, mitochondrial Ca2+ also increases with pacing frequency in the presence of NEβ (33). These mechanisms may raise endoplasmic reticulum and mitochondrial Ca2+ load and perhaps lead to apoptosis by influencing mitochondrial permeability transition pore (9, 10, 49).

We found that JNK phosphorylation in response to NEβ was markedly augmented in paced ARVM. The increased JNK activation paralleled the rise in cell death and is consistent with our recent finding that JNK activation is required for β-AR-induced apoptosis in ARVM (38). The calcium ionophore ionomycin also induced JNK activation, suggesting a link between increases in [Ca2+]i, and JNK activation. In contrast to JNK, p38 phosphorylation after β-AR stimulation was like an “on/off” switch, independent of pacing, and by extension [Ca2+]i and metabolism. These data imply that p38 activation is more tightly coupled to the β-AR activation than JNK and hence is not influenced by environmental conditions. Further...
studies are necessary to fully elucidate the interaction between Ca\(^{2+}\) homeostasis, reactive oxygen species (ROS), and activated JNK/p38, which appear to collectively regulate the life or death of a myocyte in the presence of β-AR stimulation (25, 47, 52).

In the intact heart, of course, there are many other factors that mediate myocyte fate, including NRG. Acting through the erbB2 and erbB4 receptor tyrosine kinases, NRG activates both PI3K/Akt and Erk1/2 pathways, both of which have been implicated in modulation of cell survival (16, 53). NRG is expressed in cardiac microvascular endothelial cells (53). The finding that NRG prevents β-AR-stimulated apoptosis in ARVM is consistent with the idea that NRG is among the endogenous cardiac growth factors that act to preserve cardiac structure and function in the presence of stress. The protective effect of NRG in ARVM occurs through a PI3K-dependent pathway, although it is overwhelmed by high [NE\(^{+}\)]/min in paced myocytes. Furthermore, pretreatment of NRG suppressed the cytochrome c release from mitochondria and caspase-3 processing. β-AR-stimulated apoptosis in ARVM can be inhibited by antioxidants (35, 36), suggesting one potential mechanism for NRG protection is the modulation of oxidative stress (16), either through suppressing generation of ROS or increasing the activity of endogenous antioxidant enzymes. Thus NRG cytoprotection may be similar to the actions of insulin and insulin-like growth factor-I (IGF-I), which activate the PI3K/Akt pathway (1, 31) and inhibit ROS-stimulated apoptosis in other experimental systems.

Although the NRG/erbB signaling system shares many features with insulin, IGF-I, and other growth factors, there is an absolute requirement for NRG and its receptors for the maintenance of normal cardiac structure and function. In mice lacking erbB2 or erbB4 in the myocyte, cardiac failure develops in the absence of overt stress (15, 26, 32). These mice appear to have increased levels of myocyte loss from apoptosis. Interestingly, in a pressure overload model of myocardial failure, downregulation of erbB2 and erbB4 receptors was observed (39). Our current observations support the thesis that downregulation of NRG receptors, and therefore NRG signaling, might be mechanistic in the progression of cardiac failure in these models. Moreover, strategies to augment NRG/erbB signaling in the heart may be beneficial in preventing the progression of heart failure.

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