Heterozygous knockout of neuregulin-1 gene in mice exacerbates doxorubicin-induced heart failure


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Liu, Fen-Fen, James R. Stone, Adam J. T. Schuldt, Katashi Okoshi, Marina P. Okoshi, Masaharu Nakayama, Kalon K. L. Ho, Warren J. Manning, Mark A. Marchionni, Beverly H. Lorell, James P. Morgan, and Xinhua Yan. Heterozygous knockout of neuregulin-1 gene in mice exacerbates doxorubicin-induced heart failure. Am J Physiol Heart Circ Physiol 289: H660–H666, 2005. First published April 15, 2005; doi:10.1152/ajpheart.00268.2005.—Neuregulins and their erbB receptors are essential for cardiac development and postulated to be cardioprotective in the presence of injury in the postnatal heart. We tested the hypothesis that the development of doxorubicin-induced cardiotoxicity in vivo is more severe in mice with heterozygous knockout of the neuregulin-1 gene (NRG-1+/−) compared with wild-type mice (WT). Three-month old NRG-1+/− and WT mice were injected with a single dose of doxorubicin (20 mg/kg ip). Survival was analyzed by the Kaplan-Meier approach. Left ventricular (LV) function and signaling pathways were analyzed 4 days after treatment. Fifteen days after treatment, survival was significantly lower in doxorubicin-treated NRG-1+/− mice (NRG-1+/−-Dox) compared with doxorubicin-treated WT mice (WT-Dox) (15% vs. 33%, P < 0.01). LV mass was significantly lower in NRG-1+/−-Dox but not in WT-Dox compared with nontreated animals. LV systolic pressure and LV midwall fractional shortening were significantly lower in NRG-1+/− mice (NRG-1+/−-Dox) compared with doxorubicin-treated WT mice (WT-Dox) (15% vs. 33%, P < 0.01). LV mass was significantly lower in NRG-1+/−-Dox but not in WT-Dox compared with nontreated animals. LV systolic pressure and LV midwall fractional shortening were significantly lower in NRG-1+/− mice (NRG-1+/−-Dox) compared with WT-Dox mice. LV protein levels of NRG-1, erbB2, and erbB4 receptors were similar in WT-Dox and NRG-1+/−-Dox mice. However, levels of phosphorylated erbB2, Akt, and ERK-1/2 were significantly decreased in NRG-1+/−-Dox compared with WT-Dox mice. A significant decrease in phosphorylated P70S6K levels was also observed in NRG-1+/−-Dox compared with nontreated NRG-1+/− mice. These results demonstrate that heterozygous knockout of the neuregulin-1 gene worsens survival and LV function in the presence of doxorubicin-induced cardiac injury in vivo. This is associated with the depression of the erbB2 receptor as well as Akt, p70S6K, and ERK-1/2 pathways.

derB2; receptors; transgenic mice

NEUREGULINS ARE A FAMILY of growth factors which promote cell survival and growth via activation of their erbB receptors (3, 11). Neuregulins and their erbB receptors are essential for cardiac development. Complete deletion of the neuregulin-1 gene, or either the erbB2 or the erbB4 receptor, causes death during embryogenesis from aborted development of ventricular trabeculae (14, 21, 23). In the adult heart, neuregulin-erbB signaling may also be required for maintaining cardiac function, especially in the presence of another insult. Cardiomyopathic conditional knockout of the erbB2 gene produces a dramatic decrease in the erbB2 protein level in myocytes and dilated cardiomyopathy in adult mice (7, 27). Studies from our laboratory have demonstrated that left ventricular (LV) erbB2 and erbB4 levels are downregulated at the early stage of heart failure induced by chronic pressure overload in rodents (29). Partial interference of neuregulin-erbB signaling in mice with heterozygous knockout of the neuregulin-1 gene (23, 25) or in humans treated with anti-erbB2 antibody alone (34) does not cause cardiac dysfunction in vivo. However, trastuzumab, an anti-erbB2 antibody, exacerbates doxorubicin cardiac toxicity and increases the risk of severe heart failure in humans (12, 33). Studies using neonatal and adult rat cardiac myocytes indicate that neuregulin-1 promotes myocyte growth and survival (40) and protects against doxorubicin-induced myofilament disarray (30). In vitro studies (13) in neonatal rat cardiac myocytes have also shown that neuregulin-1β prevents doxorubicin-induced apoptosis via activation of the erbB4-phosphatidylinositol 3-kinase (PI3K)-Akt pathway.

In this study, we tested the hypothesis that interference with neuregulin-erbB signaling in vivo exacerbates doxorubicin-induced heart failure using a well-characterized transgenic mouse model with heterozygous knockout of the neuregulin-1 gene (NRG-1+/−) (23, 25). We demonstrated that the survival and LV function were severely impaired in neuregulin-1 knockout mice in the presence of doxorubicin-induced cardiac injury in vivo. This was associated with the inhibition of activation of the erbB2 receptor as well as the depression of activation of the Akt, p70S6K, and ERK-1/2 pathways.

MATERIALS AND METHODS

Animal model. The transgenic mouse model with neuregulin-1 gene knockout was generated by Meyer and Birchmeier (23) through deletion of exons 7, 8, and 9, which encode the carboxy-terminal of the EGF domain of all known neuregulin-1 isoforms. Mice with homozygous neuregulin-1 knockout die during embryogenesis from failure of development of ventricular trabeculae. However, NRG-1+/− mice, which are used in this study, exhibit normal postnatal viability and cardiac development (25). The well-established protocol of Myers et al. (24) was used to produce subacute doxorubicin injury in mice. Three-month-old wild-type mice (WT, n = 39) and NRG-1+/− mice (n = 62) were treated with a single dose of doxorubicin (20 mg/kg ip) for 14 days (24). LV systolic pressure and LV midwall fractional shortening were measured 4 days after treatment. LV unhindered fractional shortening was measured 15 days after treatment. LV systolic pressure and LV midwall fractional shortening were significantly lower in doxorubicin-treated NRG-1+/− mice (NRG-1+/−-Dox) compared with doxorubicin-treated WT mice (WT-Dox) (15% vs. 33%, P < 0.01). LV mass was significantly lower in NRG-1+/−-Dox but not in WT-Dox compared with nontreated animals. LV systolic pressure and LV midwall fractional shortening were significantly lower in NRG-1+/− mice (NRG-1+/−-Dox) compared with WT-Dox mice. LV protein levels of NRG-1, erbB2, and erbB4 receptors were similar in WT-Dox and NRG-1+/−-Dox mice. However, levels of phosphorylated erbB2, Akt, and ERK-1/2 were significantly decreased in NRG-1+/−-Dox compared with WT-Dox mice. A significant decrease in phosphorylated P70S6K levels was also observed in NRG-1+/−-Dox compared with nontreated NRG-1+/− mice. These results demonstrate that heterozygous knockout of the neuregulin-1 gene worsens survival and LV function in the presence of doxorubicin-induced cardiac injury in vivo. This is associated with the depression of the erbB2 receptor as well as the Akt, p70S6K, and ERK-1/2 pathways.

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mg/kg ip, WT-Dox and NRG-1-/-Dox, respectively). Nontreated WT (n = 20) and NRG-1-/- (n = 20) mice were used as controls. Survival of mice 15 days after doxorubicin treatment was analyzed using Kaplan-Meier methods and the log-rank test. All animal studies were in compliance with the Guide for the Care and Use of Laboratory Animals (National Institute of Health Publication, 1996) and approved by Beth Israel Deaconess Medical Center IACUC.

Creatine kinase level. In a separate cohort of mice, serum creatine kinase (CK) levels were measured 4 days after doxorubicin treatment in NRG-1-/-Dox (n = 5) and WT-Dox (n = 4) mice, as well as nontreated WT and NRG-1-/- mice (n = 5/group) using a commercially available kit (Diagnostic Chemicals). In this and in subsequent experiments described below, the time point was selected based on the published studies (24) in this doxorubicin-injury model, as well as the abrupt decrease in survival at 4–5 days postdoxorubicin treatment in this study.

Microscopic assessment of cardiac injury. Four days after doxorubicin treatment, ventricular tissues of NRG-1-/-Dox (n = 5), WT-Dox (n = 5), nontreated NRG-1-/- (n = 6), and nontreated WT (n = 4) mice were saved immediately in 2% glutaraldehyde, 2% paraformaldehyde, and 4% glucose in phosphate buffer. To prevent any deterioration of the cardiac tissue, the entire procedure was performed rapidly within 1 to 2 min. For light microscopy examination, 1-μm sections were taken from the epon-embedded blocks and stained with toluidine blue O. Specimens were examined by a pathologist who was blinded to the identity of the samples. The severity of cardiac damage in the myocardial cells was graded according to a scoring system described by Billingham et al. (2) as follows: normal myocardial

![Graph](image_url)

Fig. 1. Fifteen-day survival was analyzed in nontreated wild-type mice (WT) and mice with heterozygous knockout of the neuregulin-1 gene (NRG-1-/-) (n = 20/group), and those treated with doxorubicin (Dox) (WT-Dox, n = 39; and NRG-1-/-Dox, n = 62, respectively). Survival was analyzed by the Kaplan-Meier approach and the log-rank test.
morphology (grade 0), myocytes affected by distended sarcotubular system (vacuolization) or myofibrillar loss affecting morphology (grade 1.0), damage to 6–15% of myocytes (grade 1.5), damage to 16–25% of myocytes (grade 2.0), damage to 26–35% of myocytes (grade 2.5), or damage to >35% of myocytes (grade 3.0).

**LV function.** LV function was measured by two-dimensional targeted M-mode echocardiography 4 days after doxorubicin treatment (WT, n = 5; NRG-1+/−, n = 7; WT-Dox, n = 5; NRG-1+/−-Dox, n = 5) (10). LV pressure was measured by direct LV catheterization using a customized micromanometer at the same time point as previously described (WT, n = 6; NRG-1+/−, n = 5; WT-Dox, n = 5; NRG-1+/−-Dox, n = 8) (10).

Markers of apoptosis. First, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining was performed at 1, 2, 3, and 4 days after doxorubicin treatment in WT mice to assess the time course of the appearance of positive staining on 5-μm heart sections using the ApopTag kit (Intergen) according to the manufacturer’s instructions. TUNEL staining was then performed 2 days after treatment in NRG-1+/−-Dox and WT-Dox (n = 3 mice/group). For each sample, 32 fields (about 800 nuclei/field) were counted. Caspase-3 activity was measured at 4 h and 12 h and 1, 2, and 3 days after doxorubicin treatment using synthetic peptide acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin (Ac-DEVD-AMC) (Calbiochem). Ac-DEVE-AMC and 50 μg of the sample protein were incubated in reaction buffer [20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1% Nonidet P (NP-40), and 10% glycerol] at 37°C for 1 h. Fluorescence was measured on a spectrophotometer with an excitation wavelength of 380 nm and emission wavelength of 460 nm (n = 3 mice/group). Dexamethasone-treated mouse thymus was used as a positive control for apoptosis and demonstrated the expected TUNEL-positive cells as well as caspase-3 activation.

Western blot analysis. In additional cohorts, LV tissues (n = 4/group) were removed 4 days after doxorubicin treatment, immediately frozen in liquid nitrogen, and then homogenized in lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM NaVO₄, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 4 μg/ml pepstatin, and 1 mM PMSF]. Fifty micrograms of LV protein were loaded on SDS-PAGE gels, transferred to nitrocellulose membrane, and probed with antibodies against phospho-erbB2 (Tyr877, Cell Signaling), erbB2, erbB4 (Santa Cruz), heregulin (HRG) (Santa Cruz), phospho-Akt (Ser473), Akt, phospho-p70S6K (Thr389), p70S6K, phospho-ERK-1/2 (Thr202-Tyr204), ERK-1/2 (Cell Signaling), and GAPDH (Chemicon) (n = 4/group). The levels of signaling proteins were normalized to the levels of GAPDH.

**RESULTS**

Baseline characteristics of NRG-1+/− mice. Consistent with the prior reports (23, 25) in this model, NRG-1+/− mice were viable and fertile. There was no difference in survival between NRG-1+/− and WT animals in the absence of doxorubicin treatment during the study. No differences in LV function were observed through either echocardiography or LV hemodynamic measurements in the absence of doxorubicin treatment (Tables 1 and 2).

Survival analysis. Survival of NRG-1+/−-Dox and WT-Dox mice was analyzed by the Kaplan-Meier approach and the log-rank test (Fig. 1). Fifteen days after doxorubicin treatment, survival was significantly lower in NRG-1+/−-Dox compared with WT-Dox mice (15% vs. 33%, P < 0.01). The survival was also significantly lower in doxorubicin-treated mice compared with nontreated controls (WT-Dox vs. WT, 33% vs. 100%, P < 0.01; NRG-1+/−-Dox vs. NRG-1+/−, 15% vs. 100%, P < 0.001). An abrupt decrease in survival was found in both WT-Dox and NRG-1+/−-Dox mice at day 4 after treatment. It has been reported (35) that doxorubicin-treated mice consume less food. To exclude the possibility that impaired substrate availability was contributory, we measured serum glucose levels in doxorubicin-treated mice 4 days after
Cardiac myocardial injury. Cardiac myocardial injury was assessed by both measurement of CK release in the serum and microscopic analysis of ventricular tissue 4 days after doxorubicin treatment. Both WT-Dox and NRG-1/PD-Dox mice showed elevation of serum CK levels compared with WT and NRG-1/PD mice, respectively (WT-Dox vs. WT, 3,000 ± 1,200 vs. 153 ± 48 U/l, P < 0.05; NRG-1/PD-Dox vs. NRG-1/PD, 3,475 ± 493 vs. 98 ± 23 U/l, P < 0.05). However, there were no differences in CK levels following doxorubicin treatment between WT-Dox and NRG-1/PD-Dox mice. This indicates that the magnitude of the initial doxorubicin injury was similar in WT-Dox and NRG-1/PD-Dox mice. Figure 2 displays the mean scores of myocardial damage using the Billingham scoring system (2). Myocardial damage was evident in all doxorubicin-treated mice compared with untreated WT and NRG-1/PD mice (score = 0). There was a statistically insignificant trend of increased myocardial damage scores in NRG-1/PD-Dox hearts compared with WT-Dox hearts (1.7 ± 0.6 vs. 1.4 ± 0.7, P = 0.75). No morphological damage was found in the liver, lung, kidney, spleen, and skeletal muscles in doxorubicin-treated animals.

Echocardiographic and hemodynamic measurements. The effects of doxorubicin on LV remodeling and function in WT-Dox and NRG-1/PD-Dox mice were assessed by in vivo echocardiography and direct LV catheterization (Tables 1 and 2). Four days after doxorubicin treatment, a significant loss of body weight (BW) was observed in NRG-1/PD mice compared with NRG mice (loss of 27% of BW, P < 0.001) compared with untreated NRG mice. There was a trend of decrease in BW in WT-Dox mice (loss of 11% of BW, P = 0.34) compared with untreated WT mice. Both heart weight (HW) and LV weight (LVW) were significantly decreased in NRG-1/PD mice (loss of 29% of BW and 27% of LVW, P < 0.01 and P < 0.001, respectively) compared with NRG-1/PD mice. The HW and LVW were not significantly decreased in WT-Dox mice (loss of 15% of HW and 13% of LVW, P = 0.30 and P = 0.35, respectively) compared with WT mice. LV contractile function was assessed by echocardiography showed that LV midwall fractional shortening, a physiologically appropriate measure of LV systolic performance that is minimally influenced by LV size (3, 32), was significantly depressed in NRG mice compared with NRG and WT-Dox mice. Consistent with this, LV systolic pressure as measured by direct LV catheterization was significantly depressed in NRG mice compared with NRG and WT-Dox mice. No depression in LV contractility was found in WT-Dox compared with WT mice. Taken together, the absence of any increase in LVW or wall thickness in the presence of depression of LV function suggests a deficiency of the expected early initiation of compensatory hypertrophy.

TUNEL staining. In WT-Dox mice, a significant increase in TUNEL-positive LV nuclei was detected 2 and 3 days after treatment compared with untreated WT mice, although the prevalence of TUNEL-positive nuclei was very low (≈0.025%, Fig. 3A). Two days after doxorubicin treatment, the time point at which we observed TUNEL-positive nuclei in WT-Dox mice, we did not find a significant difference in TUNEL-positive nuclei in NRG mice compared with WT-Dox mice (Fig. 3B). Consistent with this, it was very low level of TUNEL-positive nuclei, we did not detect an increase in caspase-3 activity in doxorubicin-treated mice compared with untreated controls (data not shown).

ErbB2, erbB4 receptors, and downstream signaling. There were no differences in baseline LV protein levels of erbB2 and erbB4 receptors between NRG mice and WT mice or between doxorubicin-treated and nontreated animals (erbB2 levels are shown in Fig. 4; erbB4, WT: 100 ± 15, NRG: 100 ± 12; WT-Dox: 100 ± 15, NRG-Dox: 123 ± 18%, P = no significance between groups). However, LV protein levels of phosphorylated erbB2 (Tyr 877) were significantly decreased in NRG mice compared with WT-Dox and nontreated NRG mice (Fig. 4). No differences in protein...
levels of LV neuregulin-1 were detected between NRG-1-/- and WT mice or in doxorubicin-treated and nontreated animals (data not shown). In untreated mice, protein levels of phosphorylated Akt (Ser473) and phosphorylated ERK-1/2 (Thr202-Tyr204) were similar between NRG-1-/- and WT mice (Figs. 5 and 6). Four days after doxorubicin treatment, no decreases in the levels of phosphorylated Akt and ERK-1/2 were observed in WT-Dox mice; however, LV levels of phosphorylated Akt and ERK-1/2 were significantly decreased in NRG-1-/-Dox mice compared with NRG-1-/- and WT-Dox mice. There were no differences in the LV levels of total Akt and ERK-1/2 between the groups. Phosphorylated levels of p70S6K (Thr389), a downstream molecule of PI3K and/or the Akt pathway (28), were similar between WT and NRG-1-/- mice without treatment. However, 4 days after doxorubicin treatment, levels of phosphorylated p70S6K (Thr389) were significantly decreased in NRG-1-/-Dox mice compared with NRG-1-/- mice (Fig. 7). There was a trend toward a decrease in phosphorylated p70S6K levels in WT-Dox compared with WT mice. No differences in the levels of total p70S6K were found between the groups (data not shown).

DISCUSSION

This study shows that subacute doxorubicin cardiac injury is associated with increased mortality in NRG-1-/- compared with WT mice. In addition, neuregulin-1 knockout causes more severe loss of HW and LVW, as well as LV contractile dysfunction after doxorubicin injury. These changes in NRG-1-/- mice occur rapidly after doxorubicin injury and are associated with downregulation of phosphorylated erbB2, cardioprotective Akt, p70S6K, and ERK-1/2 pathways in vivo.

Neuregulin-erbB signaling and cardiac function. In the heart, neuregulins are produced by the endocardium and the endothelium of the cardiac vasculature and bind to erbB2 and erbB4 receptors on cardiac myocytes via a paracrine mode of signaling (11). Neuregulins and their erbB receptors are required for normal development of the heart (14, 21, 23). In the adult heart, neuregulin-erbB signaling is also required in maintaining cardiac function, especially in the presence of another insult. In mouse models with cardiac-specific conditional knockout of the erbB2 gene, mice survived to adulthood but developed dilated cardiomyopathy at the age of 3 mo (7, 27).

Neuregulin-erbB signaling and doxorubicin cardiotoxicity. Doxorubicin induces cardiac myocardial injury via several mechanisms, including free radical generation (20, 38), increases in sarcomeric protein synthesis (17, 18), cytoskeletal abnormality (5, 31, 36), depletion of intracellular ATP (18), and apoptosis (6, 19, 22). Interference of cardioprotective Akt and ERK-1/2 pathways has also been implicated in mediating doxorubicin cardiotoxicity (37, 41). In vitro studies in cardiac myocyte culture have shown that neuregulin-1 promotes myocyte viability via activation of Akt, MAPK, and p70S6K (1) and reduces doxorubicin-induced myofibrillar disarray by activation of Akt and ERK-1/2 pathways (30). It has also been shown (13) that neuregulin-1B prevents doxorubicin-induced apoptosis in neonatal rat cardiac myocytes via activation of the erbB4-PI3K-Akt pathway. In this study, we tested the hypo-

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neuregulin-erbB signaling and treatment with doxorubicin would interfere with similar survival pathways in vivo.

After doxorubicin injury, the NRG-1+/−-Dox mice exhibited a reduction in BW as well as LV mass. In contrast with the very rapid increase in LV mass, which is observed following injury with depressed contractile function in other rodent injury models such as infarction, the NRG-1+/−-Dox mice with depressed systolic function failed to recruit compensatory hyper trophy. Neuregulin-1 promotes protein and sarcomere synthesis and growth of myocytes. Consistent with this, our results suggest that impaired neuregulin-1 signaling contributes to the more severe loss of LV mass in the presence of doxorubicin injury in vivo. More severe loss of LV mass may worsen LV contractile function in NRG-1+/−-Dox mice. Because no damage or atrophy was observed in other organs and because serum glucose levels were normal, we concluded that the decrease in survival was not related to the damage of other organs or starvation but more likely was related to the early development of heart failure in these mice. Doxorubicin in this and other models (8) usually causes a delayed and long-term decrease in cardiac function in vivo. This may explain why no cardiac dysfunction was observed 4 days after doxorubicin treatment in WT-Dox mice.

Two days after doxorubicin treatment, the time point at which a very low level of TUNEL-positive nuclei was detectable in WT-Dox cardiac myocardium, there was no clear increase in TUNEL staining in NRG-1+/−-Dox compared with WT-Dox mice. Furthermore, the magnitude of TUNEL-positive staining was scant, and no increase in caspase-3 activity was detected. This result suggests that extensive apoptosis might not be the major contributor to the decreased survival and LV function observed in NRG-1+/−-Dox mice. This was consistent with the prior findings (7, 27) in mice with conditional knockout of erbB2 receptors, in which there was not a clear relationship, if any, between cardiac dysfunction and apoptosis. However, it cannot be excluded that doxorubicin may cause more severe loss of mitochondrial potential and depletion of intracellular ATP (16) in NRG-1+/−-Dox mice, in which the erbB2 signaling is impaired. Further studies are needed to address this.

At baseline in nontreated NRG-1+/− and WT mice, there were similar levels of neuregulin-1 and erbB2, erbB4 receptors, as well as phosphorylated erbB2, Akt, p70S6K, and ERK-1/2. After doxorubicin treatment, the NRG-1+/−-Dox mice exhibited pronounced depression of the levels of phosphorylated erbB2, Akt, p70S6K and ERK-1/2. Neuregulin-1, via binding to its erbB receptors, causes activation of various signaling pathways, including PI3K-Akt and MAPK (26, 39). Activation of these pathways then stimulates distinct transcriptional programs in the nucleus, affecting cell growth, differentiation, adhesion, migration, and apoptosis (3, 26, 39). Activation of Akt, p70S6K, as well as ERK-1/2 by neuregulin-1 is implicated in sarcomeric actin polymerization and myofibrillar protein synthesis in cardiac myocytes (1). This may provide a cardioprotective role in the presence of doxorubicin cardiac injury, in which doxorubicin may promote a decrease of sarcomeric protein by either inhibition of muscle gene transcription (17) or myofibril degeneration (36), as well as cytoskeletal abnormalities (31). In addition, neuregulin-1 via activation of the PI3K-Akt pathway may protect the heart from oxidative stress induced by doxorubicin as suggested by in vitro studies (15).

Limitations of the study. The present study demonstrates that neuregulin-1 knockout is associated with impaired activation of Akt, p70S6K, and ERK-1/2 pathways following doxorubicin injury in the heart. Akt regulates cell growth and functions via multiple mechanisms (4). Further studies are needed to identify specific pathways downstream of Akt that are important in the protection of the heart from doxorubicin injury (37). Studies are also needed to determine whether the Akt or the MAPK pathway is essential and sufficient for the actions of neuregulin-1 in the protection of the heart from doxorubicin injury in vivo.

In summary, heterozygous knockout of the neuregulin-1 gene is associated with the very early development of depressed survival, reduced LV mass, and severe LV dysfunction following doxorubicin injury in vivo. Our observations support the concept that the neuregulin-1 signaling is cardioprotective in the presence of cardiac injury and may act via the PI3K-Akt and the activation of the p70S6K and ERK-1/2 pathways.

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