Neuregulin1 Improved Cardiac Function in Doxorubicin-Treated Mice with Cardiomyocyte-Specific over expression of a Dominant-Negative PI3Kp110α

Yun Bian1, Marcy Silver1, Jillian Onufruk1, Kalon K.L. Ho2, Mark A. Marchionni2, Peter M Kang3, David A Goukasian1, Joseph Carrozza1, James P Morgan1 and Xinhua Yan*1

1Cardiovascular Research, Department of Medicine, Steward St. Elizabeth’s Medical Center and Tufts University School of Medicine, Boston, USA
2Cardiovascular Division, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, USA
3Alzcor Pharmaceuticals Inc. Arlington, USA

Abstract
Neuregulin1s (NRG1s) are effective for protecting the heart from various stresses. Our previous studies show that heterozygous knockout of the NRG1 gene worsens while injections of a recombinant NRG1 improve cardiac function in Chemotherapy Drug Doxorubicin (DOX) - treated mice. In cultured cardiomyocytes, studies show that the Phosphoinositide 3-Kinase (PI3K) pathway is necessary for NRG1 to reduce DOX-induced apoptosis and loss of cardiac troponins. Here, we test whether PI3Kp110α, a Class IA PI3K isomor, is necessary for NRG1’s cardioprotective effects in DOX-treated mice. DOX was administered to mice with cardiomyocyte-specific overexpression of a constitutively active PI3Kp110α (CaPI3K), or a dominant negative PI3Kp110α (dnPI3K). Solvent or NRG1 was administered concurrently with DOX to dnPI3K mice. Our results showed that survival and cardiac function were improved in CaPI3K, but worsened in dnPI3K mice. Concurrent NRG1 injections significantly improved survival and cardiac function in dnPI3K mice, which were associated with increased activation of ERK1/2 and mTORC1 in the heart. These results have demonstrated that PI3Kp110α is important for protecting the heart from DOX. Further, the results suggest that PI3Kp110α is not necessary for NRG1 to protect the heart from DOX. NRG1 may protect the heart by activating alternative survival pathways, such as ERK1/2 and mTORC1, in DOX-treated dnPI3K mouse hearts.

Keywords: Neuregulin1; PI3K; Doxorubicin; Cardiotoxicity

Introduction
The Neuregulin1 (NRG1) gene is a member of the epidermal growth factor gene family [1]. It encodes at least fifteen isoforms of NRG1 proteins, which are ligands of the HER receptors [2]. The NRG1-HER signaling is pivotal for protecting the heart from various cardiac stresses [3-5]. Our results show that heterozygous knockout of the NRG1 gene worsens, whereas injections of a recombinant NRG1 alleviate, DOX-induced cardiac dysfunction in mice [3,6]. In the heart, NRG1 is synthesized and secreted by the endocardium and the endothelium of the cardiac microvasculature [7]. NRG1 activates HER2 and HER4 receptors on cardiomyocytes, thereby activating multiple signaling pathways and regulating key functions of cardiomyocytes [2,7]. A major signaling pathway activated by NRG1-HER is the phosphoinositide 3-kinase (PI3K) pathway [2]. Studies in isolated cardiomyocytes show that PI3Ks are necessary for multiple cardioprotective effects of NRG1, such as anti-apoptosis, antioxidative stress, maintaining cardiac troponins and cardiac repair [6,8]. In addition to PI3Ks, NRG1 also activates multiple signaling pathways, such as ERK, mTOR, PKC and STAT, to exert its functions [2,9].

PI3 kinases are conserved lipid kinases that phosphorylate the 3’-hydroxyl group of phosphoinositides [10]. Class I PI3Ks are most studied in both cancer and the heart. They are further divided into class IA and class IB. Class IA are heterodimers comprised of a regulatory subunit (p85α, p55α, p50α, p85β, p55γ) and a catalytic subunit (p110α, p110β, p110δ). In response to the Receptor Tyrosine Kinase (RTKs) activation, class IA PI3Ks are recruited to the tyrosine phosphate motifs on the activated RTKs via the regulatory subunits. Class IB PI3Ks are composed of a regulatory subunit p101 and a catalytic subunit p110y, and activated by G Protein Coupled Receptors (GPCRs) [11,12]. PI3Kp110α is important for protecting the heart from cardiac stress [13,14]. Studies have shown that cardiomyocyte-specific overexpression of a dominant negative PI3Kp110α (dnPI3K) exacerbates pressure-overload induced hypertrophic cardiomyopathy and myocardial infarction induced heart failure [13,15]. With ongoing clinical trials using PI3K inhibitors for cancer therapy it is important to know whether inhibition of PI3Kp110α will aggravate chemotherapy, such as Doxorubicin (DOX), induced cardiotoxicity; and if so, whether there are means to alleviate this type of cardiotoxicity [16].

We hypothesize that inhibition of PI3Kp110α may exacerbate DOX cardiac dysfunction. NRG1, by activating alternative signaling pathways, may be capable of circumventing the loss of PI3Kp110α to provide cardioprotective effects against DOX. To study the specific effects of PI3Kp110α inhibition in DOX-injured hearts, we tested our hypotheses by using transgenic mouse models with cardiomyocyte-specific overexpression of a constitutively active PI3Kp110α (CaPI3K) or a dominant negative PI3Kp110α (dnPI3K). First, we tested whether cardiomyocyte-specific overexpression CaPI3K would improve cardiac function in DOX-treated mice; second, whether cardiomyocyte-specific overexpression...
of dnPI3K would exacerbate cardiac dysfunction in DOX-treated mice; and third, whether NRG1 injections would alleviate DOX cardiac dysfunction in dnPI3K mice.

Methods

Animal model

The generation of CaPI3K and dnPI3K mice were described previously [17]. Three month old WT and CaPI3K male mice (in total, n=106) were treated with a single dose of DOX (15 mg/kg, i.p. Bedford Laboratories, Bedford, OH). Three-month-old WT and dnPI3K female mice (in total, n=201) were treated with a single dose of DOX (20 mg/kg, i.p.), or a concurrent treatment of NRG1 and DOX. Solvent (20 mM sodium acetate, 100 mM sodium sulfate, 1% mannitol and 100 mM L-arginine, pH 6.5) or recombinant NRG1β (recombinant human glial growth factor 2 (rhGGF2), 0.75 mg/kg s.c.; a gift from Acorda Therapeutics Inc.) was administered one day before and daily after the DOX administration. 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 Steward St. Elizabeth’s Medical Center IACUC.

Left ventricular function

Function was measured by LV catheterization using Millar MicroTip Blood Pressure System (AD Instruments, Inc., Colorado Springs, CO). In brief, a Millar Micro-Tip catheter transducer was inserted into the LV via the right carotid artery after mice were anesthetized with urethane (1000mg/kg) and α-chloralose (50mg/kg). LV systolic and diastolic pressures were recorded. The time point chosen was based on pilot experiments in each mouse line.

Western blot analysis

Mice LV tissue (n=4-6 per group) was lysed in a buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10 mM sodium pyrophosphate, 20mM β-glycerophosphate, 10 mM Na3VO4, 1 mM NaF, 1 mM PMSF, and protease inhibitor cocktail tablet (Roche Diagnostics Corporation, Indianapolis, IN). LV tissue was homogenized by PowerGene homogenizer (Fisher Scientific, Pittsburgh, PA). Membranes were probed with antibodies against phosphorylated and total Akt (Thr 308 or Ser 473), ERK1/2 (Thr202/Tyr204) and S6 (Ser240/244) (Cell Signaling Technology, Danvers, MA). Protein bands were visualized using enhanced chemiluminescence (Amersham, Arlington Heights, IL). Membranes were re-probed with antibodies against β-actin (Fisher Scientific, Pittsburgh, PA) as loading control.

Statistical analysis

Results are presented as Mean ± SEM. Statistical analysis was conducted using SigmaStat (Systat Software Inc. San Jose, CA). Comparison between groups was performed by ANOVA followed by Tukey’s test. Kaplan-Meier estimates of survival were computed using SigmaStat (Systat Software Inc. San Jose, CA). Differences were considered significant with P<0.05.

Results

Cardiomyocyte-specific overexpression of CaPI3K improved survival and cardiac function in DOX-treated mice

DOX significantly reduced two-week survival to 14% in WT-DOX (5 out of 37 mice survived, P<0.05) compared to non-treated WT and CaPI3K mice (20 out of 20 mice survived per group). CaPI3K overexpression significantly improved survival to 38% in CaPI3K-DOX (6 out of 16 mice survived, P<0.05) compared to WT-DOX mice (Figure 1A).

In non-DOX treated mice, heart weight normalized by tibial length (HW/TL) was significantly higher in CaPI3K vs. WT mice; cardiac function was similar between CaPI3K and WT mice (Figures 1B-1F). DOX significantly reduced LV systolic pressure (LVSP), dP/dtmax, and dP/dtmin in WT-DOX mice, these indices were maintained in CaPI3K-DOX mice and were significantly higher in CaPI3K-DOX compared to WT-DOX mice (Figures 1D-1F and Table 1).

We measured the activation of PI3Ks (readout: pAkt- Thr308, ERK1/2, mTORC1 (readout: pS6) and mTORC2 (readout: pAkt-Ser 473) in mouse hearts [18-21]. Without DOX, the PI3K activity was increased 6.5-fold in CaPI3K hearts [17]. pAkt (Thr 308 and Ser473) and pS6 were similar in CaPI3K vs. WT; pERK1/2 were similar in CaPI3K and WT.

DOX reduced BW and HW/TL in mice. These indices were significantly lower in CaPI3K vs. WT (Figures 1G and 1H).

Taken together, these results demonstrated that cardiomyocyte-specific overexpression of CaPI3K improved survival and cardiac function in DOX-treated mice. These were associated with increased activation of PI3K, ERK1/2 and mTORC2 in CaPI3K-DOX vs. WT-DOX mouse hearts.

Cardiomyocyte-specific overexpression of dnPI3K exacerbated cardiac dysfunction in DOX-treated mice

DOX significantly reduced two-week survival to 21 % in WT-DOX (6 out of 28 mice survived, P<0.05) and 16% in dnPI3K-DOX mice (3 out 19 mice survived, P<0.05), compared to non-treated WT and dnPI3K mice respectively (20 out of 20 mice survived per group; Figure 2A).

In non-DOX treated mice, HW/TL was lower in dnPI3K vs. WT mice; cardiac function was similar between dnPI3K and WT mice (Figures 2B-2F). DOX reduced BW and HW/TL in mice. These indices were significantly lower in dnPI3K-DOX vs. WT-DOX mice (Figures 2B and 2C). Six days after the DOX treatment, DOX significantly reduced heart rate (HR), LVSP, dP/dtmax and dP/dtmin in dnPI3K-DOX but not in WT-DOX mice (Figures 2D-2F).

Without DOX treatment, PI3K activity was decreased 77% in dnPI3K hearts [17]. pAkt (Thr308 and Ser473) were lower, while pERK1/2 and pS6 were similar, in dnPI3K vs. WT hearts. DOX significantly reduced pERK1/2 and pS6 in dnPI3K-DOX hearts compared to dnPI3K and WT-DOX hearts (Figures 2G and 2H).

These results demonstrated that cardiomyocyte-specific overexpression of dnPI3K exacerbated DOX cardiac dysfunction in mice. This was associated with decreased activation of ERK1/2 and mTORC1 in dnPI3K-DOX compared to WT-DOX hearts.

NRG1 improved survival and cardiac function in DOX-treated dnPI3K mice

NRG1 injections significantly improved two-week survival to 48%
in NRG1-dnPI3K-DOX (15 out of 31 mice survived, $P<0.05$) compared to 16% in dnPI3K-DOX mice. NRG1 also improved survival to 44% in NRG1-WT-DOX (11 out 25 mice survived, $P=0.25$) compared to 21% in WT-DOX mice. NRG1 alone did not alter survival rate in non-DOX treated mice [6].

NRG1 injections did not alter the adverse effects of DOX on BW and HW/TL in dnPI3K-DOX mice (Figures 2B and 2C, Table 2). NRG1, however, significantly improved HR, LVSP, dp/dtmax and dp/dtмин in NRG1-dnPI3K-DOX vs. dnPI3K-DOX mice (Figures 2D-2F and Table 2).

NRG1 injections significantly increased pERK1/2 and pS6 in NRG1-dnPI3K-DOX vs. dnPI3K-DOX hearts (Figure 2G and 2H).

These results demonstrated that NRG1 was capable of improving cardiac function in DOX-treated mice with cardiac-specific inhibition of PI3Kp110α. This effect of NRG1 was associated with increased activation of ERK1/2 and mTORC1.

Discussion

This study demonstrated that inhibition of PI3Kp110α exacerbated DOX-induced cardiac dysfunction in mice; NRG1 injections alleviated DOX cardiac dysfunction in mice with decreased PI3Kp110α activity in the heart. These results suggest that caution must be taken when PI3K inhibitors are used in combination with DOX in cancer patients. The results also suggest that NRG1 could be used as an adjuvant agent to reduce the incidence and severity of heart failure caused by anti-cancer therapies that use PI3K inhibitors and DOX.

Since its discovery more than 40 years ago, DOX continues to be used as a first-line anti-neoplastic drug for a wide variety of cancers, despite that it causes a dose-related cardiotoxicity [22]. Chemotherapy, such as DOX, is often inevitable in certain cancer patients previously treated with kinase inhibitors. Combination cancer chemotherapy using kinase inhibitors and DOX, however, can cause a high incidence of a severe of heart failure [23,24].

Clinical studies have shown that the incidence of New York Heart Association class III/IV heart failure is 16% among patients treated with Trastuzumab (a monoclonal antibody that blocks HER2) and

**Table 1:** Cardiac hemodynamic measurements of DOX-treated CaPI3K mice.
Improved Survival and Cardiac Function in DOX-Treated dnPI3K Mice

Figure 2: Cardiomyocyte-Specific Overexpression of dnPI3K Exacerbated Cardiac Dysfunction in DOX-Treated Mice; Concurrent NRG1 Injections Improved Survival and Cardiac Function in DOX-Treated dnPI3K Mice (A) Survival analysis. DOX reduced two-week survival to 21% in WT-DOX (n=28, 6 out of 28 mice survived) and 16% in dnPI3K-DOX (n=19, 3 out of 19 mice survived). NRG1 injections improved survival to 44% in NRG1-WT-DOX mice (n=25, 11 out of 25 mice survived) and 48% in NRG1-dnPI3K-DOX mice (n=31, 15 out of 31 mice survived). (B) - (C) Body weight (BW) and heart weight to tibia length ratio (HW/TL) in solvent-treated, DOX-treated and NRG1-treated mice six days after the DOX injection. (D) - (F) Hemodynamic measurements. Function was measured by LV catheterization six days after the DOX treatment. HR=heart rate; LVSP=left ventricular systolic pressure. BW, HW/TL and function were measured in WT (n=8), dnPI3K (n=8), WT-DOX (n=13), dnPI3K-DOX (n=12), NRG1-WT-DOX (n=8) and NRG1-dnPI3K-DOX (n=10) mice. (G) - (H) Western blot analysis. Phosphorylated and total Akt, ERK1/2 and S6 were measured in WT (n=6), dnPI3K (n=5), WT-DOX (n=6), dnPI3K-DOX (n=6), NRG1-WT-DOX (n=6) and NRG1-dnPI3K-DOX (n=6) mouse hearts six days after the DOX injection. The representative blots (G) and quantitative analysis (H) were shown. *P<0.05 vs. WT; †P>0.05 vs. WT-DOX; §P<0.05 vs. dnPI3K-DOX; ¶P<0.05 vs. NRG1-WT-DOX.

Concurrent DOX therapy, compared to 3% in patients treated with DOX alone (fivefold increase) [5]. Compared to DOX mono-therapy induced heart failure, which may appear years after the cessation of the therapy [25], cardiac dysfunction caused by Tnsztuzumab and DOX combination therapy is frequently detected during the course of the treatment and may be irreversible [5,26].

Like the HER2 receptor, the PI3K pathway is one of the most mutated pathways in cancer [27]. PIK3CA, the gene encoding the PI3Kp110α, is found mutated in 27% of breast, 24% of endometrial and 15% of colorectal cancers, therefore, is a major drug target for cancer therapy [27]. PI3Ks, on the other hand, are pivotal for maintaining cardiac physiological function, especially in the presence of cardiac stress. It is important to determine whether inhibition of PI3Ks could cause cardiac side-effects, especially in the presence of DOX. We used a transgenic mouse model with cardiomyocyte-specific overexpression of a dominant negative PI3Kp110α, and treated them with DOX. This could mimic the clinical setting where DOX is used followed by PI3Kp110α inhibitors. We have demonstrated that although inhibition of PI3Kp110α itself does not cause cardiac dysfunction; these hearts are more susceptible to DOX cardiac toxicity. In addition, we have demonstrated that PI3Kp110α is not necessary for NRG1 to improve cardiac function in DOX-injured mice. Our results suggest that NRG1 could improve cardiac function in patients treated with DOX and PI3Kp110α-specific inhibitors.

This study has focused on the complex signaling pathways downstream of NRG1-HER in the heart. We have shown that alternative pathways exist downstream of NRG1 to compensate for the loss of PI3Kp110α. The activation of ERK1/2 and mTORC1 are decreased in dnPI3K-DOX mouse hearts, but are maintained in NRG1-dnPI3K-DOX hearts, suggesting that NRG1 may circumvent the loss of PI3Kp110α by activating these ERK1/2 and mTORC1.

mTORC1 is pivotal for protein synthesis and cell growth [28]. Activation of mTORC1, however, inhibits Akt [29]; on the other hand, inhibition of mTORC1 activates Akt and ERK1/2 [18,19]. We have observed that increased activation of mTORC1 and mTORC2 is associated with either an exacerbation or an improvement of cardiac function in DOX-treated mice. Further studies are needed to clarify the role of mTOR in DOX-treated hearts. Although in non-DOX treated mice, the PI3K activity is lower in dnPI3K vs. WT mouse hearts [17], it is increased in dnPI3K-DOX vs. dnPI3K hearts (Figure 2G and 2H). This could be caused by the feedback activation of Akt and mTORC2 caused by decreased activation of mTORC1 in dnPI3K-DOX hearts [20,30].

The role of NRG1 on tumor growth and progression is controversial. Studies have shown that NRG1 is either an oncogene or a tumor-suppressor gene [31,32]. Accumulating evidence has pointed out that the outcome of NRG1 stimulation in tumor cells depends on the integration of the biological effects of NRG1 on tumor cells, as well as the cell type, the expression and density of the HER receptors and their ligands, and the dosage and the frequency of NRG1 stimulation [33-35]. Studies have also shown that NRG1 increases breast cancer sensitivity to DOX. The mechanism may be related to NRG1’s effect on inducing the activity of topoisomerase II, which is a major target of DOX [36,37].

Taken together, we have demonstrated that hearts with decreased activation of PI3Kp110α are more susceptible to DOX-induced cardiac dysfunction. NRG1 is capable of circumventing the partial loss of PI3Kp110α to improve cardiac function in DOX-injured hearts by...
activating alternative survival pathways, such as ERK1/2 and mTORC1.

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References


