Heat Shock Protein 90 and ErbB2 in the Cardiac Response to Doxorubicin Injury

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Abstract

A major drawback to doxorubicin as a cancer-treating drug is cardiac toxicity. To understand the mechanism of doxorubicin cardiac toxicity and the potent synergic effect seen when doxorubicin is combined with anti-ErbB2 (trastuzumab), we developed an in vivo rat model that exhibits progressive dose-dependent cardiac damage and loss of cardiac function after doxorubicin treatment. The hearts of these animals respond to doxorubicin damage by increasing levels of ErbB2 and the ErbB family ligand, neuregulin 1/3, and by activating the downstream Akt signaling pathway. These increases in ErbB2 protein levels are not due to increased ErbB2 mRNA, however, suggesting post-transcriptional mechanisms for regulating this protein in the heart. Accordingly, levels of heat shock protein 90 (HSP90), a known ErbB2 protein stabilizer and chaperone, are increased by doxorubicin treatment, and coinmunoprecipitation reveals binding of HSP90 to ErbB2. Isolated cardiomyocytes are more susceptible to doxorubicin after treatment with HSP90 inhibitor, 17-(allylamino)-17-demethoxygeldanamycin, suggesting that the HSP90 is protective during doxorubicin treatment. Thus, our results provide one plausible mechanism for the susceptibility of the heart to anti-ErbB2 therapy post-doxorubicin therapy in subclinical and clinical conditions. Additionally, these results suggest that further testing is needed for HSP90 inhibitors under various conditions in the heart. [Cancer Res 2007;67(4):1436–41]

Introduction

Doxorubicin is an effective agent for treatment of cancer, but dosing is limited by irreversible, long-term cardiac damage. For treatment of breast cancers with HER2 amplification, doxorubicin is commonly administered in combination with anti-ErbB2 (Herceptin-trastuzumab) because these two drugs act synergistically to kill cancer cells. Yet, this combination results in even more frequent (up to 30%), and sometimes fatal, cardiomyopathy (1, 2). The mechanism of this doxorubicin cardiac toxicity and the synergy with trastuzumab is not sufficiently understood to help identify patients at high risk for cardiac damage before treatment or during the early stages of treatment.

The effects of trastuzumab on the heart would suggest an impairment role of ErbB2 signaling in the heart, and this is supported by experiments using genetically engineered mice. Knockout mice of ErbB2, ErbB4, or its ligand neuregulin 1β (NRG1β) die in utero due to cardiac development impairment (3–5). Additionally, cardiomyocytes isolated from mice with reduced cardiac ErbB2 have an increased sensitivity to doxorubicin (6). Consistent with this, heterozygote NRG1β-deficient mice are more sensitive to doxorubicin showing ErbB2 pathway importance in the heart (7). One unanswered question in this field, however, is how doxorubicin exposure affects ErbB2 expression and Akt activation in the heart. If this occurred, as a protective response, it could explain why ErbB2 inhibition would put the heart at risk for doxorubicin toxicity.

Because ErbB2 and downstream signaling seem protective during doxorubicin treatment, another protein that should be considered in the context of doxorubicin injury is heat shock protein (HSP) 90 (HSP90), a modulator and chaperone of ErbB2. Inhibitors of HSP90, which are being developed for cancer therapy, induce the release of ErbB2 from HSP90, resulting in subsequent ErbB2 protein degradation (8, 9). This protein:protein association is also important in cultured cardiomyocytes because HSP90 inhibitor geldanamycin induces ErbB2 protein degradation (10), but it is not known if ErbB2 and HSP90 associate in vivo in the heart or if doxorubicin induces HSP90 in this setting.

Investigation of an animal model may help address some of these issues. Therefore, we developed a rat model of doxorubicin injury to the heart to investigate cellular and molecular responses to this drug.

Materials and Methods

Animals. Female Sprague-Dawley 10-week-old rats (200 g) were acquired from Zivic Miller (Pittsburgh, PA) and acclimated for 1 week. All procedures associated with this study were reviewed and approved by the Institutional Animal Care and Use Committee and done in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Study design. Animals were randomly assigned to one of three treatment groups: doxorubicin treatment of 2.5 mg/kg for 6 weekly doses and a cumulative dose of 15 mg/kg ($n = 10$); Novaplus, Bedford Laboratories, Bedford, OH), doxorubicin treatment of 2.5 mg/kg for 3 weekly doses and cumulative dose of 7.5 mg/kg ($n = 10$), or saline treatment for 6 weekly doses ($n = 10$). Rats were monitored for changes in cardiovascular variables via echocardiography from weeks 8 to 10 postinduction of treatment. Cardiac toxicity was defined by a decline of cardiac fractional shortening percentage to ≤45%. Treatments were given via jugular injections under ketamine (75 mg/kg) and xylazine (10 mg/kg) anesthesia.

Transsthoracic echocardiography. Transsthoracic echocardiography was done using a Sequoia Acuson (Malvern, PA) C256 or Visualsonics (Toronto, Ontario, Canada) Vevo 660 Echocardiography System. Ultrasound machine operators were blinded to the experimental groups. Rats were habituated before study to immobilization inside a modified disposable rodent restrainer (Decapicone, BrainTree Scientific, Inc., Braintree, MA). This allowed imaging of an anesthetized rat in –3 min. The heart was first imaged in two-dimensional mode in parasternal short axis for left
ventricle at sweep speed of 200 mm/s. The cursor was positioned perpendicular to interventricular septum and left ventricular posterior wall at the level of the papillary muscles of left ventricle, whereas the two-dimensional image was converted to M-mode echocardiogram. From the M-mode echocardiogram image, left ventricular chamber diameters were measured at the end of diastole (left ventricular end-diastolic internal diameter) and systole (left ventricular end-systolic internal diameter). Fractional shortening represents percentage change in left ventricular chamber dimension with systolic contraction. For each rat, three values for each measurement were obtained and averaged.

Necropsy. Rats were euthanized and received postmortem examination and midwall left ventricle was sectioned and frozen for molecular studies. The rest of the heart was fixed for histology and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) evaluation. In addition, other rats were perfused via aortic cannulation with 0.2% glutaraldehyde and 4% paraformaldehyde fixation for immunoelectron microscopy.

Western analysis. Frozen left ventricle (40 mg) was rapidly homogenized in ~500 μL buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 0.5% NP40, 10 mmol/L sodium molybdate, 8.8 g/mL sodium pyrophosphate, 4 g/L Na/F). One milligram of protein was precleared with protein G-agarose beads (Invitrogen, Carlsbad, CA) and then incubated with 4 μg antibody (HSP90 or ErbB2) for 2 h on a rotary shaker at 4°C. After incubation, protein G-agarose beads were added for an additional hour, then washed with fresh buffer, boiled for 5 min in Laemmli SDS sample buffer, and frozen until used for Western blotting.

RNA analysis. Total RNA was isolated from 40 mg heart left ventricle tissue using a Trizol RNAeasy protocol (Qiagen, Valencia, CA), with an in-column DNase treatment (RNase-Free DNase Set, Qiagen). Quality and concentration of the samples were evaluated using a NanoDrop ND-1000 and 2100 Bioanalyzer, respectively. To generate RNA for real-time quantitative PCR (qPCR) standards, a 661-bp region of the rat ErbB2 gene and a 363-bp region of the rat ribosomal 28s gene were generated by PCR and cloned into the pcRII Topo plasmid (Invitrogen) using the following primers: 5'-GACTCGTCCTCCTGTGTGGC-3' (forward) and 3'-GACCGCAGGATGACGGAGCC-5' (reverse). The following antibodies were used: ErbB2 (sc-285; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), ErbB4 (sc-283; 1:500; Santa Cruz Biotechnology), NRG-1 (sc-1792; 1:500; Santa Cruz Biotechnology), phospho–mammalian target of rapamycin (mTOR; 1:1,000; Cell Signaling, Boston, MA), phospho-p70s6k (1:1,000; Cell Signaling), phospho-Akt (1:1,000; Cell Signaling), total Akt (1:1,000; Cell Signaling), HSP90 (1:1,000; BD Transduction Laboratories, San Diego, CA), and HSP70 (1:5,000; Stressgen, San Diego, CA). After incubation in antirabbit or antimouse horseradish peroxidase secondary antibody (1:2500; Amersham, Piscataway, NJ), blots were exposed to chemiluminescent substrate (Pierce, Rockford, IL) and exposed to CL-Xposure film (Pierce, Rockford, IL). Protein levels were normalized to total Akt.

Immunoprecipitation. Frozen left ventricle was rapidly homogenized in ~500 μL buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 0.5% NP40, 10 mmol/L sodium molybdate, 8.8 g/mL sodium pyrophosphate, 4 g/L Na/F). One milligram of protein was precleared with protein G-agarose beads (Invitrogen, Carlsbad, CA) and then incubated with 4 μg antibody (HSP90 or ErbB2) for 2 h on a rotary shaker at 4°C. After incubation, protein G-agarose beads were added for an additional hour, then washed with fresh buffer, boiled for 5 min in Laemmli SDS sample buffer, and frozen until used for Western blotting.

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Figure 1. Effect of doxorubicin on heart function and cardiac cell death. A, representative M-mode echocardiograms of left ventricle from each treatment group. Cumulative dose of 15 mg/kg doxorubicin induces contractility deficits at 10 wks from the initial injection. B, histogram of fractional shortening percentage (index of contractility). Columns, mean (n = 10 per group); bars, SD. *** P < 0.001. C, histogram of TUNEL-positive nuclei. Columns, mean; bars, SD. Rats were euthanized at 10 wks and hearts were analyzed by TUNEL assay. Apoptotic cells were determined using TUNEL according to the manufacturer’s instructions (In situ Cell Death Detection kit, Fluorescein, Roche Applied Science, Indianapolis, IN) using antigen retrieval methods. TUNEL-positive cells were determined by direct fluorescence of nuclei incorporated dUTP in cardiomyocytes. Total number of cells per ×20 field was quantified by H&E staining and TUNEL-positive nuclei were expressed as a percentage of total cells (nuclei) in each heart with five fields per heart analyzed. ** P < 0.01; *** P < 0.001. D, representative images of TUNEL fluorescence (converted to black/white) for saline, 7.5 mg/kg, and 15 mg/kg doxorubicin-treated hearts.
5'-CTCGGACATGGTCCAGAAGGC-3' (reverse) for ErbB2 and 5'-CTAACCA-GGATTCCTCAGTAACG-3' (forward) and 5'-CTCTTAACGGTTTCGCCTC-3' (reverse) for ribosomal 28s. In vitro transcribed RNA (T7 Megascript, Ambion, Austin, TX) was quantitated by spectrometry and used as standard for the real-time PCR assay. Two micrograms of total RNA from each sample were reverse transcribed using a SuperScript II kit (Invitrogen) in a 20-μL reaction. cDNA was quantitated by real-time PCR using specific sets of primers and probe either for ErbB2 (forward 5'-ATCCTAATTCAACAAACGAGACAG-3', reverse 5'-GATGTGTGTTCTCTCAACACCTTG-3', and probe 5'-/56-FAM/GAGTTAGTGGAGCCGGCTGAC/3BHQ_1/-3') or for

**Figure 2.** Effects of doxorubicin on ErbB2 pathway expression in the heart. Midwall left ventricle samples from control or doxorubicin-treated rats were collected 10 wks after the initial injection, and proteins were isolated for Western blotting (n = 10 per group). A, representative Western blots indicate increased expression of the ErbB2 and NRG1β by doxorubicin treatment. Phosphorylated forms of downstream ErbB2 pathway proteins are increased in both doxorubicin treatment groups compared with saline. B, histogram of relative protein expression (relative to saline) or activation (phosphorylation) normalized to total Akt. Columns, mean; bars, SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001. C, electron micrograph of hearts from a saline-treated rat (a and c) compared with a 15 mg/kg doxorubicin-treated rat (b and d). Top, a saline-treated rat heart (a) compared with a doxorubicin-treated rat with dilated sarcoplasmic reticulum and dilated t-tubules (b) characteristic changes in doxorubicin cardiomyopathy. Magnification, ×10,000. Bottom, immunoelectron micrographs of hearts from a saline-treated rat (c) and doxorubicin-treated rat (d). Magnification, ×200,000. Increased immunogold labeling of ErbB2 is observed with doxorubicin treatment versus saline with subcellular localization of ErbB2 to cardiomyocyte t-tubules adjacent to Z band.
ErbB2 and HSP90 Induction in Heart by Doxorubicin

**Results and Discussion**

Two cumulative doses of doxorubicin (7.5 or 15 mg/kg) or saline were given to 10-week-old female Sprague-Dawley rats over a 6-week period to develop a rat model to study the effects of doxorubicin on heart function, morphology, and molecular pathway activation. Fractional shortening percentage was used as global systolic function indicator, and representative M-modes of left ventricle and summary histograms (Fig. 1A and B) show reduced contractility only in the 15 mg/kg group at 10 weeks from the initial injection. Euthanasia and tissue harvesting were done at 10 weeks or when fractional shortening percentage was ≤45% in these animals. Time-matched rats (10 weeks) were also euthanized from the 7.5 mg/kg and control groups.

The number of TUNEL-positive nuclei in left ventricles was compared between three treatment groups: saline control, 7.5 mg/kg, and 15 mg/kg (Fig. 1C and D). The 15 mg/kg treatment group showed a higher level of cell death (30 ± 6.7%; P < 0.05 TUNEL-positive nuclei) accompanying the decline in contractility. Surprisingly, the 7.5 mg/kg group also showed a marked increase in cell death (15 ± 1.2%) compared with the saline control rats (5.7 ± 0.3%), although the 7.5 mg/kg rats did not show reduced contractility by echocardiography. Interestingly, the 7.5 mg/kg group developed contractility deficits and heart failure at 15 weeks in two subsequent longitudinal studies (data not shown), indicating that the dose of doxorubicin and timing of heart failure presentation are related.
We then evaluated the effects of doxorubicin chemotherapy on heart ErbB2 expression and downstream signaling pathways in experimental groups. As seen in Fig. 2, doxorubicin treatment results in dose-dependent increases in ErbB2 protein levels and parallel increases in phosphorylation of the downstream Akt, mTOR, and 70S6k proteins. Interestingly, NRG1β, the most likely ligand for ErbB2 and ErbB4 heterodimerization in the heart, was also induced in both doxorubicin treatments. ErbB4 protein expression did not vary between treatments. ErbB2 or ErbB4 was not found differentially phosphorylated between the treatment groups.

We also evaluated ErbB2 expression and subcellular location by immunoelectron microscopy in hearts from doxorubicin- (15 mg/kg) and saline-treated rats. ErbB2 is known to localize to cardiomyocyte

![Figure 4](https://cancerres.aacrjournals.org/content/67/4/1440/F4.large.jpg)

**Figure 4.** Effect of doxorubicin and HSP90 inhibitor (17AAG) on rat cultured cardiomyocytes. A, cell viability of cardiomyocytes was assessed by LDH, MTT, and live/dead cell assays on 96-well plates by manufacturer’s directions. Columns, mean of three separate experiments with 24 wells per treatment; bars, SD. 17AAG alone is not toxic at low doses (1 μmol/L). However, cotreatment with 17AAG markedly increases the toxicity of doxorubicin. **,** *P < 0.001. B, characteristic morphology of cardiomyocytes after 48 h of treatment before viability assays were done.
t-tubules, a tubular network of cell membrane invaginations (14). Consistent with immunoblot results, ErbB2 immunogold–labeled antibody complexes were elevated by doxorubicin treatment and localized to cardiomycocyte t-tubules (Fig. 2C). Electron micrographs also show doxorubicin-induced dilation of t-tubules and sarcoplasmic reticulum, a characteristic finding in doxorubicin heart toxicity.

We then evaluated ErbB2 mRNA levels in the saline and 15 mg/kg treatment groups (Fig. 3A). Unexpectedly, ErbB2 mRNA was not affected by doxorubicin treatment, leading us to consider a post-transcriptional mechanism as an alternative means to increase ErbB2 cardiac protein levels in injured cells. Because HSP90 has been reported to stabilize ErbB2 in cancer cells and cultured cardiomycocytes (8–10), we considered the possibility that HSP90 is also responsible for stabilizing ErbB2 in the heart after doxorubicin injury. As shown in Fig. 3B and C, HSP90 protein levels were significantly increased in both of our doxorubicin groups, and immunoprecipitation studies revealed that HSP90 associates with ErbB2 in the setting of doxorubicin injury but not in saline-treated rats (Fig. 3D). We also observed significant increases in the inducible form of HSP70 after doxorubicin injury, leading us to conclude that these cells experience a generalized HSP response. Because in vivo increases in HSP90 and ErbB2 cardiac proteins occur even before cardiac dysfunction is detected by echocardiography, we considered the consequence of HSP90 function inhibition in isolated cardiomycocytes using doxorubicin and the HSP90 inhibitor, 17AAG, separately and in combination.

As shown by three different methods used to evaluate toxicity (Fig. 4A–D), 17AAG alone is not toxic at low doses (1 μmol/L). However, cotreatment with 17AAG markedly increases the toxicity of doxorubicin. A higher dose of 17AAG is cardiotoxic, although it is unlikely that this concentration is physiologically relevant.

To our knowledge, this is the first report showing ErbB2 elevation in the heart in response to injury and this observation suggests that the increase in ErbB2 may initially be a survival response to injury. However, as observed in both doxorubicin cumulative doses studied here, injury above a yet undefined threshold level cannot be rescued by ErbB2 or Akt activation. Importantly, this elevation of ErbB2 (and activation of downstream signaling) occurs before evidence of functional systolic deficits. If a similar relationship occurs in humans, this molecular change could potentially be used to predict which patients receiving anti-ErbB2 treatment are at risk for developing cardiac symptoms. Anti-ErbB2 is now used to treat patients in the adjudgent setting in early (15) and in advanced cases of breast cancer.

Another important finding is the elevation of HSP90 and HSP70 in the heart responding to doxorubicin injury. HSPs are also induced by exercise, a known protective factor for doxorubicin cardiotoxicity (16, 17), and HSP70 protects the heart from hypoxia/reoxygenation and postinfarction stresses in the heart (18, 19). Presently, the protective role of HSP90 in oxidative stress conditions has not been fully explored due to the lack of HSP90-overexpressing mice. One issue that needs to be addressed is whether other oxidative stress-induced cardiac injuries (e.g., ischemia/reperfusion or chemical oxidants) can effect in vivo HSP90 cardiac expression and secondary modulation of stress-related signal transduction. In addition to our findings with doxorubicin, chronic cyclosorpin A treatment also induces in vivo HSP90 expression in the heart and is associated with modulation of protective endothelial nitric oxide synthase signaling (20), reflecting the importance of cardiac HSP90. HSP90 inhibitors are in clinical development for cancer therapy, and our finding suggests that further testing should be done to evaluate the potential cardiac side effects of HSP90 inhibitors in vivo, especially in situations in which the heart relies on HSP90 for protection. Alternatively, devising strategies to selectively increase cardiac HSP90 could offer a means of protecting the heart from doxorubicin and other cardiotoxic drugs.

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