Cardiac ErbB-1/ErbB-2 mutant expression in young adult mice leads to cardiac dysfunction

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Cardiac ErbB-1/ErbB-2 mutant expression in young adult mice leads to cardiac dysfunction. Am J Physiol Heart Circ Physiol 295: H543–H554, 2008; doi:10.1152/ajpheart.91436.2007.—Multiple factors lead to the development and maintenance of chronic heart failure. Blockade of ErbB-2 or ErbB-4 tyrosine kinase receptor signaling leads to dilated cardiomyopathy. ErbB-1 may protect the heart against stress-induced injury and its ligand; epidermal growth factor (EGF) increases myocardial contractility, whereas heparin-binding EGF is essential for normal cardiac function. However, the role of ErbB-1 in control of cardiac function is not clear. We hypothesized that ErbB-1 is essential for maintaining adult cardiac function. Using the edcsyne-inducible gene expression system, we expressed humanized cardiomyocyte-specific dominant-negative ErbB-1 mutant receptors (hErbB-1-mut) in young adult mice that block endogenous cardiac ErbB-1 signaling. Molecular, morphological, and physiological tests (under anesthesia) were performed. As a result, hErbB-1-mut was expressed selectively in cardiomyocytes leading to the blockade of endogenous ErbB-1 phosphorylation and ErbB-2 transphosphorylation. An increase in left ventricular mass, atrial natriuretic factor expression, and histological changes were indicative of cardiac hypertrophy. Cardiac dilation, numerous cardiac lesions, and the loss of the clear boundary between cardiac fibrils were noted histologically. Early and long-term hErbB-1-mut induction led to a significant decrease in fractional shortening and to significant increases in left ventricular end-systolic diameter and volume. The treatment of adenyl cyclase activator (forskolin analog) normalized the depressed cardiac function. Resting cardiac function returned to normal after reversing mutant expression. A 4-day survival rate of transverse-aortic constricted hErbB-1-mut mice was only 20% compared with 100% in controls. In conclusion, these observations indicate that the blockade of cardiac ErbB-1 signaling leads to the blockade of ErbB-2 signaling and that together they result in cardiac dysfunction.

The recent evidence that ErbB receptor tyrosine kinases alter myocardial function offers exciting new therapeutic possibilities (9, 17, 31, 34, 44, 50, 56). Treatment with neuregulin-1 (ligand for ErbB-2 and ErbB-4) improved cardiac function in ischemic, dilated, and viral-induced cardiomyopathy in canine and rodent models (34). The expression and activation of the neuregulin receptors ErbB-2 and ErbB-4 are depressed in failing human myocardium (50). Interestingly, breast cancer chemotherapy, in combination with the humanized monoclonal ErbB-2 antibody trastuzumab, results in significant cardiac dysfunction (14, 19, 56). The ventricle-restricted gene deletion of ErbB-2 and ErbB-4 and the conditional mutation (MLC2vcre/+ ErbB2Δlox/−) of ErbB-2 receptors result in dilated cardiomyopathy (9, 17, 44).

The evolutionarily conserved epidermal growth factor (EGF) receptor (EGFR) family of ErbB tyrosine kinase receptors is a ubiquitously expressed family of proteins involved in complex signaling cascades in higher organisms (3, 7). The growth factor binding to the receptor ectodomain activates the cytoplasmic tyrosine kinase initiated by receptor homo- and/or heterodimerization, thus stimulating signaling pathways that direct cellular responses (33). The transactivation among ErbB receptors has been demonstrated in a variety of cell types and tissues (20). The four subtypes of ErbB receptors and their cognate ligands are indispensable for embryonic and cardiovascular development since receptor knockout in mice is embryonically lethal (2, 6, 7, 12, 18, 26, 30, 37). The prototype ErbB-1 receptor (or EGFR) knockout mice display developmental abnormalities in a strain-dependent manner (7). ErbB-1 mutant mice (Egfrwa2/wa2:Ptpn11+/−/− mice and hEGFRKIKI mice) develop cardiac hypertrophy and semilunar valve developmental abnormalities (6, 54).

Using a conditional gene knockout strategy, Iwamoto et al. (27) have shown that heparin-binding EGF (ligand for ErbB-1 and ErbB-4) is essential for normal cardiac function. EGFinduced contraction is evident in coronary, aortic, and other smooth muscle systems (23). In the heart, EGF increases contractility by elevations in cAMP levels in cardiac myocytes (39, 46). Phosphorylation of Gs on tyrosine residues by the tyrosine kinase activity of ErbB-1 activates Go in vitro. This increases its ability to stimulate adenyl cyclase, which in turn elevates cAMP levels (46). Lorita et al. (35) have also shown that EGF administration leads to positive inotropic effects in perfused rat hearts and cardioprotective effects against the harmful effects of epinephrine. Endogenous or exogenous ligand-activated ErbB-1 may protect the heart against stress-induced injury (45). A number of reports have concluded that ERK1/2 and Akt are important for ErbB-1-mediated cardiomyocyte survival (15, 16, 36). Recently, Howes et al. (25) showed that increased Goq activity can provide antiapoptotic signals by eliciting ErbB-1 phosphorylation and subsequent Akt activation, independent of the well-known ability of Goq signaling to elicit hypertrophy. Unexpectedly, a different model of cardiac-specific dominant-negative EGFR, which is discussed later, did not show cardiac phenotype; however, the model needs detailed characterization (65).

The role of myocardial ErbB-1 receptors in the regulation of adult cardiac function in vivo is not clear. We hypothesized that ErbB-1 is essential for normal cardiac function and, thus,
that compromised ErbB-1 signaling will lead to a diminished functional capacity of the adult heart. Since ErbB-1 is expressed in a variety of cell types and tissues, pharmacological interventions may complicate the results. Therefore, we employed the established edcsyone-inducible gene expression system that expresses dominant-negative ErbB-1 mutant receptors specifically in cardiomyocytes selectively in young adult mice. These humanized ErbB-1 mutant receptors (hErbB-1-mut) lack the intracellular domain (this domain is essential for phosphorylation and intrinsic tyrosine kinase activity) but retain the ability to dimerize with normal endogenous ErbB-1 receptors (1, 29, 47). Thus endogenous ErbB-1 signal transduction is blocked selectively in cardiomyocytes of young adult mice in a dominant-negative fashion. The present study demonstrates that selective disruption of adult cardiac ErbB-1 receptor-mediated signaling, along with compromised ErbB-2 function, leads to compromised cardiac function at rest and during stress. This negative impact on the heart can be abrogated by activating the adenylyl cyclase system in vivo.

MATERIALS AND METHODS

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, and all experiments involving animals were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. Experiments were performed in both sexes. Once we established that ponasterone A (PonA, inducer) wild-type controls (WT + PonA group) did not show any significant difference compared with the mutant controls (Mut + placebo), we continued to use only the mutant controls. It is important to emphasize that PonA is known to be neither toxic nor teratogenic and does not interfere with any mammalian physiological function (1).

Ecdysone-Inducible Expression System

The cardiac-specific mutant receptor expression was accomplished via the ecdysone-inducible expression system (1, 32, 41, 48, 51), which was modified by 1) replacing the original cyto-megalovirus promoter in the pVgRXR plasmid with the mouse α-miosyn heavy chain (α-MHC) gene promoter (Pα-MHC-pVgRXR) and 2) cloning a mutant gene that encodes a human ErbB-1 receptor lacking the intracellular domain, into the pIND plasmid (pIND-hErbB-1-mut). The molecular principle of this expression system is that upon administering edcsyone (an insect molting hormone) or a synthetic steroid analog PonA, this steroid binds to ecdysone receptors (EcRs) that are the expressed products driven by the α-MHC promoter, resulting in the formation of a heterodimer composed of the ecdysone-retinoid receptor complex, EcR-RXR. The DNA binding domain of this heterodimer will bind to the EGREX hybrid response element in the second transgene, leading to the expression of a gene of interest. Because mammalian cells are not responsive to PonA due to the absence of the EcR (64), transgene expression is highly specific and inducible. The α-MHC promoter is able to specifically direct transgene expression in cardiomyocytes (21, 58). Hence, the EcR expression would be restricted to cardiomyocytes, and thus the humanized mutant ErbB-1 (hErbB-1-mut) receptors should be expressed in the same cell population at a selected time. Two transgenic lines [Pα-MHC-pVgRXR (M+/+ /E−/−) and pIND-hErbB-1-mut (M−/− /E+/+)] are required for producing double-transgenic animals (M+/− /E+/−) in which the endogenous cardiomyocyte ErbB-1-mediated signal transduction can be disrupted. Selective expression of hErbB-1-mut in cardiomyocytes can be initiated in M+/− /E+/− animals by administering PonA that binds to the EcR as described earlier.

Generation and Identification of Transgenic Animals

Transgenic line 1 (Pα-MHC-pVgRXR, the α-MHC promoter was a gift from Dr. Jeffrey Robbins, University of Cincinnati, Cincinnati, OH) and line 2 (pIND-hErbB-1-mut, the mutant construct was provided by Dr. Axel Ullrich, Max-Planck Institute, Martinsried, Germany) were produced by the Transgenic Animal Facility at the University of Nebraska Medical Center (Omaha, NE). Briefly, the individual expression cassette was isolated and injected into the pronuclei of fertilized mouse eggs [obtained from C57BL/6 X DBA2 (F1) mice]. Therefore, the eggs were transferred to the uterus of surrogate pseudopregnant mice, allowing them to develop to term. Genotyping was performed using PCR with primer FMHC3 (5′-CAGAGAAGCAGGCACTTTAC-3′), RVgEcR-A2-1 (5′-CGTCTAAGTGAGTTCGGTC-3′), EcR-F-S (5′-CTGAATTCAATTTCAACAAGGTTA-3′), and hErbB-1 (5′-CAAATTTCTTTCTTCCAGA-3′). FMHC3 (forward) and VgEcR-A2-1 (reverse) amplify a 230-bp transgene fragment encompassing the 3′-end of the mouse α-MHC promoter and 5′-end of the edcsyone receptor. EcR-F-S (forward) and hErbB-1 (reverse) amplify a 360-bp transgene fragment encircling part of pIND vector and extracellular domain of the human ErbB-1 receptor. ErbB-1 PCR was carried out on genomic DNA (100 ng) derived from tail biopsies for 45 cycles of 94°C for 15 s, 55°C for 1 min, and 72°C for 2 min in a 25-μl reaction volume containing 50 pmol of primer and 20 pmol of reverse primer, 2.5 μl of 10× Taq PCR buffer (Promega, Madison, WI), 2 μl of 25 mmol/l MgCl2 (Promega), 1 μl of 10 mmol/l deoxynucleotide triphosphates (dNTPs—dATP, dCTP, dGTP, and dTTP; Invitrogen, Carlsbad, CA), and 0.125 μl of Taq polymerase (Promega). Four independent founders of each transgenic line were identified. The ErbB-1 PCR was run for 25 cycles and showed a 300-bp band for the positive control. Western blots of 2-mo-old WT and M+/- mice were collected and stored at −80°C until the analysis was performed using procedures previously described (24).

Characterization of Transgenic Animals

Detection of EcR expression in transgenic animals. The EcR expression was detected by Western blot analysis as described (32). Tissues from 2-mo-old WT and M+/- /E−/− mice were collected and homogenized. The total protein concentration of each lysate was determined using a Bradford assay kit (Bio-Rad, Hercules, CA). Lysates with an equal amount of protein (3 mg) were then immunoprecipitated overnight at 4°C with an EcR antibody (15C3, 1:50 dilution, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). The immunoprecipitated proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel, transferred to nitrocellulose membranes, and probed with an EcR antibody (10F1, 1:500, Hybridoma Bank). The reactions were developed using the enhanced chemiluminescence system (No. 34075, Pierce Chemical).

Detection of hErbB-1-mut expression in transgenic animals. Two-mo-old WT and M+/- /E+/+ mice were subcutaneously implanted with three PonA pellets (150 μg/pellet per 3-wk release duration) for 2 wk. Tissue samples were collected and stored at −80°C until the preparation of the whole cell lysate was prepared and Western blot analysis was performed using procedures previously described (24).
Immunoprecipitation was performed using human-specific ErbB-1 antibody (EGFR-Ab-13, 1:50, Labvision, Fremont, CA), whereas membranes were probed with a human-specific ErbB-1 antibody (EGFR-Ab-14, 1:500, Labvision).

In two PonA-treated animals, the hearts were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and prepared for immunohistochemistry by sectioning at 10 μm. The procedure was performed as described by Li et al. (32) but using a human-specific hErbB-1 antibody (1:500, c-erb-1, Ab-1, Neomarkers) and repeated for α-MHC immunodetection using α-MHC antibody (1:30, Y-20; sc-12117; Santa Cruz Biotechnology).

Detection of endogenous phosphorylated mouse ErbB receptor in cardiomyocytes of transgenic animals. Young adult double-transgenic hErbB-1-mut mice carrying the Pα-MHC-pVgRXR and pLND-hErbB-1-mut transgenes (60-day old, PonA-treated, male, two per PonA dosage treatment) were used for either subcutaneous injection (1 mg PonA/20 μl DMSO/100 μl corn oil) of PonA (dosage: 0, 1, 2, and 4 mg/mouse) or subcutaneous implantation of PonA containing pellets (dosage: 0, 1, 3, and 6 pellets/mouse; 150 μg/pellet per 3-wk release duration). After 15 h (injection) or 2 wk (implantation) of PonA treatment, heart tissues were collected for protein lysate preparation. Each lysate sample containing 2 mg of protein was first treated with EGF (100 ng/ml) for 5 min to induce phosphorylation of ErbB receptors. After electrophoresis followed by electrotransfer of total proteins from SDS-PAGE gel to membrane, a sequential immunodetection was performed as described by Li et al. (32) but using a human-specific anti-ErbB-1 antibody (sc-12117; Santa Cruz Biotechnology).

Histology

Hearts were fixed in 10% formalin and embedded in paraffin for serial sectioning at 10 μm thickness. The sections were stained with hematoxylin-eosin.

Determination of Atrial Natriuretic Factor mRNA Level by RT-PCR

Animals. The hearts from each group of animals were collected and used for RNA preparation according to the procedure reported previously (24). The atrial natriuretic factor (ANF) mRNA expression was determined using sense 5'-GAGTGGACGACTGGAGAAG-3' and antisense 5'-CCCTTTACCCGAAGCTGTG-3' primers that amplifies a 270-bp transgene fragment as described by Li et al. (32).

In Vivo Transthoracic Echocardiographic Imaging

In vivo cardiac imaging in mice was performed using a high-frequency ultrasound imaging system. The VEVO 770 high-resolution in vivo imaging system (VisualSonics, Toronto, ON, Canada) has a RMV-707B scan head designed for high frame rate, real-time small animal imaging applications with a frequency of 30 MHz used in this study (v2.2.3 and v2.3.0). Experimental mice were anesthetized with isoflurane at a concentration of 3% and then maintained at 1% isoflurane using nasal prongs during the whole procedure. Mice were placed supine onto a 37°C imaging platform, and body temperature was monitored with a rectal thermometer. The hair on the precordial region was cleanly removed with a Nair lotion hair remover (Church and Dwight, Princeton, NJ), and the region was covered with a prewarmed ultrasound transmission gel, Aquasonic clear (Parker Laboratory, Fairfield, NJ). Following every mouse scan, the table was cleaned with T-Spray II disinfectant cleaner (Pharmaceutics Innovative, Newark, NJ). The measurements were taken from the parasternal short-axis view in M-mode to view the LV movement during diastole and systole corresponding to the electrocardiogram. All data and imaging were analyzed by the VisualSonics Cardiac Measurements Package (version 10). Based on the dosage studies in Fig. 3, 600 μg of PonA or placebo were used to induce sufficient hErbB-1-mut expression in physiological studies involving echocardiographic assessment. For acute intraperitoneal injections of dobutamine or NKH-477, the probe was fixed and kept stationary during the experiment.

Transverse-Aortic Constriction

The procedure was followed as described (49) in three different groups of mice, viz., WT treated with PonA and double-transgenic-positive mutants with or without treatment of PonA. To minimize surgery-induced variation, only those animals that survived after 48 h post-transverse-aortic constriction were included in this analysis.

Detection of β1-Adrenergic Receptor Expression in ErbB-1 Mutants

With a similar Western blot protocol as mentioned earlier, and including 3% BSA with blocking solution and antibody treatment, β1-adrenergic receptor (β1-AR) expression was studied using β1-AR antibody (V-19: 1:1,000; sc-568, Santa Cruz). The reactions were developed using the enhanced chemiluminescence system (No. 34075, Pierce Chemical).

Detection of Endogenous ErbB-1 and ErbB-4 Receptor Transphosphorylation In Vivo

BWEM cells (11) were seeded as 1 × 10^6 cells/10-cm dish culture dish overnight followed by serum-starving for 2 h before EGF (100 μg/ml) or neuregulin (100 μg/ml) treatment for 5 min at room temperature. Cells were then harvested using 0.5 ml radioimmunoprecipitation assay solution and incubated on ice for 10 min followed by centrifugation at 10,000 rpm for 5 min at 4°C. Supernatant was collected and the protein concentration was determined using Bio-Rad protein assay kit. Lysates with equal amount of protein (1 mg) were immunoprecipitated overnight at 4°C with the ErbB-1 (sc-03, Santa Cruz)- or ErbB-4 (sc-283, Santa Cruz)-specific antibody (1:500). The immunoprecipitated proteins were separated with the 8% SDS-PAGE gel, transferred onto nitrocellulose membrane (Schleicher and Schuell), and blotted with PY20 (1:500, Santa Cruz). The total content of ErbB-1 was detected with the ErbB-1-specific antibody sc-03 (1:500, Santa Cruz), and the total content of ErbB-4 receptors was detected with the ErbB-4-specific antibody (sc-283, Santa Cruz). The reactions were developed using the enhanced chemiluminescence system (No. 34075, Pierce Chemical).

Statistical Analyses

Statistical analyses were performed with GraphPad Prism (version 3.02 for Windows, GraphPad Software, San Diego, CA) using t-tests (for 2 groups), one-way ANOVA (for 3 or more groups) followed by Newman-Keuls post hoc tests, and results were tabulated as means ± SE. P < 0.05 was considered statistically significant.

RESULTS

Characterization of Transgenic Animals

Cardiac-restricted EcR expression. To determine whether the α-MHC promoter is able to direct the expression of EcR, tissues from WT and M^{+/-}\text{E/-}\text{E/-} mice were subjected to Western blot analysis. The nonmammalian EcR expression was evident only in the hearts of M^{+/-}\text{E/-}\text{E/-} transgenic animals (Fig. 1A) but not in other organ tissues, including skeletal muscle and brain, or in WT mice. This indicates that the α-MHC promoter drives EcR expression selectively in the heart as shown elsewhere (21, 58).

Heart-restricted hErbB-1-mut expression. To examine whether the hErbB-1-mut receptors are expressed in the heart, adult double-transgenic mice were implanted subcutaneously...
with three PonA pellets for 2 wk (150 μg/pellet per 3-wk release duration). The expression of the hErbB-1-mut was tested in selected tissues from both M\(^+/+\)/E\(^+/+\) and WT animals that were either treated (PonA or vehicle) or not treated. Western blot analysis (Fig. 1B) demonstrates that hErbB-1-mut is expressed solely in the transgenic hearts induced with PonA and not in other tissues, such as skeletal muscle or brain, or in WT mice. This clearly concurs with the

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**Fig. 1.** Detection of ecdysome receptor (EcR) and humanized ErbB-1 receptor mutant (hErbB-1-mut) expression. A: detection of EcR protein expression. EcR is expressed only in the hearts of transgenic animals (TAs; M\(^+/+\)/E\(^+/+\)) but not in wild-type (WT) hearts or in other tissues of WT and TAs (representative data from skeletal muscle and brain are shown). B: detection of hErbB-1-mut receptor protein expression in hErbB-1-mut mice. hErbB-1-mut is detected only in the hearts of TAs (M\(^+/+\)/E\(^+/+\)) but not in WT hearts or in other tissues of WT and transgenic mice (representative data from skeletal muscle and brain are shown). NT, no treatment; v, vehicle; p, ponasterone A (PonA).

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**Fig. 2.** Immunohistochemical detection of colocalization of expressed hErbB-1-mut with α-myosin heavy chain (α-MHC)-positive cardiac myocytes around cardiac fibrils. In hErbB-1-mut transgenic mice (TA + PonA), a red dashed line-framed area is enlarged (400X; left, bottom, red arrows) and a black dashed line-framed region is similarly amplified (right, bottom, black arrowheads). Control samples (WT + PonA) do not show colocalization.
data seen in Fig. 1A inferring that both EcR and hErbB-1-mut are expressed exclusively in the transgenic hearts. These results demonstrate the specificity of the α-MHC promoter-driven hErbB-1-mut expression system in the transgenic animals. To further examine whether the expression of the hErbB-1-mut receptor was indeed restricted to cardiomyocytes, an antibody recognizing only the extracellular domain of human ErbB-1 was used to colocalize hErbB-1-mut with α-MHC-positive cardiomyocytes. As expected, all hErbB-1-mut receptor immunofluorescence-positive signals were detected only in the cardiomyocytes from M+/E+/E+ animals treated with PonA and not in the controls (Fig. 2). These results clearly demonstrate that the α-MHC promoter-controlled EcR and hErbB-1-mut expression system is spatially and temporally specific with respect to the cardiomyocytes.

Expressed hErbB-1-mut Inhibits Endogenous Mouse ErbB-1 Phosphorylation and ErbB-2 Transphosphorylation in the Heart

The ability of hErbB-1-mut to block endogenous ErbB-1 receptor signaling has been documented both in vitro and in vivo (29, 32, 47). Nevertheless, it is imperative to test whether cardiomyocyte-specific hErbB-1-mut mice are able to block endogenous ErbB-1 receptor phosphorylation, an event required for initiating signal transduction (4) in our model.

Fig. 3. In vivo induction of hErbB-1-mut expression and blockade of endogenous ErbB-1/2 phosphorylation. Western blot analysis (A) shows that injection of PonA (>2 mg) for 15 h is able to initiate a high expression level of hErbB-1-mut receptor that abolishes EGF-induced endogenous ErbB-1/2 phosphorylation. B: implantation of PonA containing pellets (≥3 pellets, 150 μg/pellet per 3 wk-release duration) leads to a high expression level of the hErbB-1-mut receptor sufficient to completely block the phosphorylation of ErbB-2 receptors. pErbB-1, endogenous phosphorylated ErbB-1; tErbB-1, total endogenous ErbB-1; +, presence; −, absence.

Fig. 4. Dilation of ventricles in hErbB-1-mut hearts. A: histological detection of ventricular dilation in TA + PonA are shown (TA-hErbB-1-mut TAs) compared with the WT controls treated with PonA. B: representative transverse sections show ventricular dilation and hypertrophy of hErbB-1-mut hearts (TA + PonA) compared with the controls (TA-PonA). Bar = 1 mm. Part of the right ventricular wall of the hErbB-1-mut hearts (TA + PonA) is missing due to sectioning problem. C: atrial natriuretic factor (ANF) mRNA expression increased in hearts of TA + PonA compared with WT + PonA. Both ANF and cyclophilin (p1B15) mRNAs were codetected by performing RT-PCR (n = 4 mice). PonA treatment consisted of 150 μg/pellet per 3-wk-release duration for 6 wk.
Hence, transgenic hearts were tested for hErbB-1-mut expression by two methods: subcutaneous injection or pellet implantation of PonA. Western blot analysis shows that the injection (Fig. 3A) of PonA (≥2 mg) for 15 h was able to initiate a high level of expression of hErbB-1-mut, which is sufficient to completely abolish EGF-induced transphosphorylation of endogenous ErbB-2 receptors. EGF-induced phosphorylation of endogenous ErbB-1 receptors was blocked with 4 mg PonA. The implantation (Fig. 3B) of PonA-containing pellets also leads to a high expression level of the hErbB-1-mut sufficient to completely block the phosphorylation of ErbB-2 receptors. This indicates that the dominant-negative effect also affects transphosphorylation of ErbB-2 by ErbB-1. Thus the hErbB-1-mut is able to disrupt endogenous ErbB-1/2 receptor-mediated signal transduction in cardiomyocytes.

**Cardiac Phenotypes**

Gross histological analysis (Fig. 4A) indicates that the hearts of double-transgenic mice bearing P_{aMHIC}pVgRXR and pIND-hErbB-1-mut transgenes treated with PonA are dilated compared with the WT controls. Transverse sectioning (Fig. 4B) also shows ventricular dilation and hypertrophy in hErbB-1-mut hearts compared with placebo controls. ANF mRNA expression (Fig. 4C) was significantly increased in the hErbB-1-mut hearts, indicating that these mice demonstrate cardiac dilation and hypertrophy.

Higher magnifications of histological samples demonstrate the presence of numerous cardiac lesions (Fig. 5A) in double-transgenic mice expressing hErbB-1-mut in the heart following PonA implantation for 5 mo. Longitudinal sections show the loss of a clear boundary between cardiac fibrils in hErbB-1-mut transgenic mice compared with the mutant controls (Fig. 5B).

**hErbB-1-mut Mice Display Abnormal Cardiac Function**

Consistent with the histological assessment, high-frequency echocardiography performed in young adult mice (n = 8 to 9/group) revealed (Table 1) significantly decreased fractional shortening and significantly increased left ventricular (LV)
Table 1. Diminished cardiac function in resting hErbB-1-mut mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT + PonA</th>
<th>Mut + Placebo</th>
<th>Mut + PonA</th>
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<tr>
<td>FS, %</td>
<td>33.2±1.3</td>
<td>39.8±4.0</td>
<td>21.6±0.9**</td>
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<td>LVIDs, mm</td>
<td>2.37±0.07</td>
<td>2.17±0.19</td>
<td>2.94±0.10**</td>
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<td>LVIDd, mm</td>
<td>3.56±0.12</td>
<td>3.57±0.14</td>
<td>3.75±0.11</td>
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<tr>
<td>IVSs, mm</td>
<td>1.4±0.07</td>
<td>1.34±0.11</td>
<td>1.69±0.18</td>
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<tr>
<td>IVSd, mm</td>
<td>1.05±0.07</td>
<td>0.94±0.089</td>
<td>1.47±0.15**</td>
</tr>
<tr>
<td>LVPWs, mm</td>
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<td>1.65±0.13</td>
<td>1.02±0.03**</td>
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<tr>
<td>LVPWd, mm</td>
<td>1.2±0.08</td>
<td>0.95±0.07</td>
<td>0.8±0.03*</td>
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Values are means ± SE; n = 8 to 9 mice/group. Cardiac function appears to be diminished in humanized ErbB-1-mutant (hErbB-1-mut) mice [Mut + poasterone A (PonA)] compared with the controls [wild-type (WT) + PonA and Mut + placebo] in both early and long-term implanted mice. FS, fractional shortening; LVIDs and LVIDd, LV internal diameters in systole and diastole, respectively; IVSs and IVSd, interventricular septal dimension in systole and diastole, respectively; LVPWs and LVPWd, LV posterior wall thickness in systole and diastole, respectively. *P < 0.05 vs. WT + PonA; †P < 0.05 vs. Mut + placebo; ‡P = 0.0515 vs. all controls.

ErbB-1-mutants demonstrate low dD/dt, which is consistent with the impaired LV fractional shortening and, hence, impaired cardiac function. In addition, a relatively load-independent parameter, midwall shortening fraction was studied using the method of Dong (10), and we found that the hErbB-1-mut mice receiving PonA showed significantly decreased (P < 0.05; n = 3 to 4) shortening (8.75 ± 0.28%) compared with mutant mice receiving placebo (14.89 ± 2.75%). Although the LV posterior wall thickness was decreased in the Mut + PonA group, the interventricular septal wall thickness increased significantly in the Mut + PonA group compared with the control mice (Fig. 7). Consistent with the increased ANF expression and histological changes, LV mass was also significantly (P < 0.05; n = 4) increased in these hErbB-1-mut receiving PonA (4.95 ± 0.39 mg/g body wt) compared with the placebos (3.76 ± 0.03 mg/g body wt). The electrocardiographic period between the beginning and end of T-wave is significantly prolonged in the PonA-induced mutants compared with the WT and mutant controls, indicative of impaired ventricular relaxation. However, the cause of impaired relaxation is yet to be determined. Together, these results indicate that ErbB-1/ErbB-2 receptor-mediated signaling in cardiomyocytes is important for maintaining normal resting adult cardiac function.

Increased Mortality in hErbB-1-mut Mice Following Pressure Overload

To determine the effect of cardiac stress in hErbB-1-mut mice, aortic banding was carried out in both control (WT + PonA; transgenic animal-PonA) and hErbB-1-mut (transgenic animal + PonA) animals (60–70 days old). Animals that died within the first 48 h were excluded to minimize surgery-induced variation. Survival rate (Fig. 8) was significantly decreased (80% mortality) in the first 4 days following transverse-aortic constriction in transgenic mice expressing hErbB-1-mut receptors compared with no deaths in the controls observed over a period of 11 days (log rank test, P < 0.05). This result suggests that the disruption of ErbB-1 receptor-mediated signaling in cardiomyocytes leads to an inability to respond to the biomechanical stress of pressure overload.

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Fig. 6. Echocardiographic changes in rate of change of LV dimension and LV relaxation time. Transgenic mice induced with or without PonA and WT controls induced with PonA were studied (n = 8 to 9 mice). The rate of change of LV dimension (dD/dt) was measured as the difference in LV dimensions during the electrocardiographic period between the peak of the R-wave and the end of the S-wave. The PonA-induced mutants were significantly diminished compared with both the controls. This is a time interval where the heart is least influenced by the prevailing load. Ventricular relaxation time, considered as the electrocardiographic period between the beginning and end of T-waves, is significantly prolonged in the PonA-induced mutants compared with the controls. ***P < 0.001.
Cardiac Dysfunction Is Reversible in hErbB-1-mut Mice

In separate groups of placebo mutant control (Mut/H11001 placebo) and PonA mutant (Mut/H11001 PonA) mice (n/4/group) implanted for 2 mo, we discontinued subsequent implantations and assessed LV function. Interestingly, the impairment in resting fractional shortening and LV internal diameter reversed back to control levels in the Mut/H11001 PonA group 3.5 wk after stopping the PonA treatment. The LV mass is relatively decreased in the PonA-induced ErbB-1-mutant mice after discontinuation of PonA. There is neither any significant difference between controls before and after discontinuation of placebo nor between controls (with or without placebo) and mutants after discontinuation of PonA. This suggests a reversal of the hypertrophic phenotype following the cessation of ErbB-1-mutant induction (Fig. 9). Together, these data strongly indicate a cause-effect relationship of hErbB-1-mut and the role of ErbB-1 in the maintenance of adult cardiac function.

Altered Inotropic Responses in hErbB-1-mut Mice Following Dobutamine Challenge

To determine pharmacological stress response in hErbB-1-mut mice, we examined the cardiac effects to β1-AR stimulation using dobutamine (Dobu; Bedford Laboratories, Bedford, OH) in control (WT + PonA and Mut + placebo) and hErbB-1-mut mice (Mut + PonA) induced with 600 mg PonA per mouse for at least 9 wk (n = 3 to 4 per group). Following baseline echocardiographic measurements, control and hErbB-1-mut mice were injected intraperitoneally with 1 mg/kg (63) of Dobu. Saline injection in WT mice served as controls for Dobu effects with no significant increase in heart rate, whereas in all other groups, Dobu increased the heart rates (data not shown). The 1-min peak responses were significantly increased in the controls, whereas in the hErbB-1-mut mice, the responses were not statistically significant from the baseline (Fig. 10). Western blot analysis showed that β1-AR expression is unchanged, indicating that the β1-AR may not be involved in this deranged cardiac ErbB-1 signaling system.

Normal Inotropic Responses in hErbB-1-mut Mice Following Activation of Adenylyl Cyclase

To determine whether the attenuated response to β1-AR stimulation was due to alterations in downstream signaling, we activated adenylyl cyclase acutely using the forskolin analog NKH-477 (Tocris Biosciences, Ellisville, MO; forskolin, 0.5 mg/kg ip; n = 4/group) in separate groups of hErbB-1-mut mice and controls. Following intraperitoneal administration of NKH-477, the inotropic responses were similar in all groups of mice. The fractional shortening increased and LV internal dimensions decreased in Mut + PonA mice to the same degree as in WT and Mut + placebo (Fig. 11). Thus dysfunctional hErbB-1-mut hearts exhibited normal responses to the activation of adenylyl cyclase.

DISCUSSION

Salient Findings

Using a cardiac-specific conditional gene expression system, we performed initial characterization of a hErbB-1-mut mouse model and demonstrated an important effect of ErbB proteins on the cardiac function and induction of dilated cardiomyopathic phenotype. This effect is the result of mutant ErbB-1 expression leading to compromised ErbB-2 function, and,
subsequently, the two together affect the cardiac function. Taken together with the echocardiographic, histological, and ANF data, under conditions not dependent on prevailing load or heart rate, these hErbB-1-mut mice displayed cardiac dilation, hypertrophy, and depressed cardiac function that was reversed upon the discontinuation of PonA. The depressed function is normalized by adenylyl cyclase stimulation. In addition, they exhibited increased mortality in response to pressure overload. Reversibility of the depressed cardiac function upon cessation of PonA indicates a direct correlation between mutant transgene induction and phenotypic changes. These results may also shed light on the role of ErbB-1/ErbB-2 receptors in adaptive and potentially reparative mechanisms used during regression of dilated cardiomyopathy/heart failure (13).

The ErbB Signaling Network and Cardiac Function

The cardiac defects in our model are similar to the dilated cardiomyopathic phenotypes reported in gene knockout/mutant mice of other ErbB receptor subtypes including ErbB-2 (9, 40, 44). However, none of our hErbB-1-mut mice died prematurely as opposed to death observed before reaching the age of 6 mo in conditional ErbB-2 mutant (MLC2vcre/+ ErbB2flox/flox) mice (15).

Fig. 9. Reversible responses in hErbB-1-mut mice. Placebo (Mut + placebo) and PonA (Mut + PonA) implantations were stopped in separate sets of double-transgenic mice (n = 4 mice/group). Fractional shortening, LV systolic internal diameter, and LV Mass showed normalized responses in PonA-stimulated mice similar to the controls. Stop indicates discontinuation of pellets. *P < 0.05, NS, not significant; sys, system.

Fig. 10. Altered inotropic responses in hErbB-1-mut mice following dobutamine (Dobu) challenge. Left: mice were injected with equal volume of saline (Saln; control) or 1 mg/kg ip Dobu (Mut + placebo control; WT + PonA control; and Mut + PonA experimental). Saline injections did not show any significant change as expected. Following acute Dobu injection, the inotropic responses of controls were significantly increased. However, in the PonA-induced mutants, the responses were not significantly different from the baseline (BL). Right: Western blot analysis results showed no change in β-adrenergic type 1 receptor protein expression in the mutant mice induced with PonA compared with placebo group (n = 3 to 4 mice). ***P < 0.001; **P < 0.01.
ErbB-4 knockout mice (17, 44). The cardiac dysfunction in our hErbB-1-mut mice was mild (with no overt signs of decompensation) and remained the same over the time studied since there was no difference between early and long-term induced groups. This suggests that although important, ErbB-1 may not be the dominant pathway for maintaining cardiac function as opposed to ErbB-2/ErbB-4 receptor-mediated pathways. Also, cardiotoxicity following anti-ErbB-2 therapy is more prominent than anti-ErbB-1 therapy in breast cancer patients (14), consistent with the idea that ErbB-1 may play a minor role compared with ErbB-2 in maintaining cardiac function. Our results indicate an obligatory functional interaction of ErbB-1 with ErbB-2 in the adult heart. The decrease/lack of ErbB-2 phosphorylation following mutant ErbB-1 expression seen in our study is supported by a previous in vitro study (57) where the binding of EGF to transfected cells coexpressing ErbB-2 together with a similar ErbB-1 mutant (a deletion mutant lacking most of the cytoplasmic domain of ErbB-1) caused heterodimerization and the absence of tyrosine phosphorylation and thus an inhibitory influence on tyrosine kinase activity. It is also interesting to observe that ErbB-2 phosphorylation is reduced to a much greater extent than ErbB-1 in the hErbB-1-mut hearts. This may mean that ErbB-1 predominantly acts via the ErbB-2 pathway, or it may be due to the fact that ErbB-2 is generally the preferred heterodimerization partner for the ErbB receptor family (7). The other possibility is that the hErbB-1-mut receptor may heterooligomerize (5, 8) with endogenous ErbB-1 and ErbB-2 following ligand stimulation. On the other hand, preliminary evidence from our laboratory suggests that ErbB-1 and ErbB-4 receptors transphosphorylate each other in the presence of EGF and neuregulin-1 in cardiomyocytes in vitro (Fig. 12). Taken together, it is tempting to postulate that all three ErbB receptors (-1, -2, and -4) work in concert to maintain normal mammalian cardiac function. More detailed analyses would be interesting but beyond the immediate scope of this study. The pathophysiology of an integrative signaling network can be clearly explained only with models of simultaneous loss-of-function mutations of more than one ErbB receptor.

We observed numerous necrotic lesions (Fig. 5) in the hErbB-1-mut hearts, which is consistent with lesions observed by the restraint-and-cold exposure stress model reported by Pareja et al. (45). These investigators showed that EGF-induced protection was abolished by the ErbB-1 inhibitor AG-1478, indicating that stress-induced injury was protected by activated ErbB-1 in mice. Several studies have also clearly shown that ERK1/2 and Akt are important for ErbB-1/ErbB-2-mediated cardiomyocyte survival (15, 16, 25, 36, 40).

**ErbB in Contraction Function and Cardiac Hypertrophy**

In cardiomyocytes (35, 39, 46), it has been shown that EGF elevates cAMP accumulation by augmenting adenylyl cyclase activity resulting in increased contractility in intact hearts. In vitro it appears that the activation of adenylyl cyclase by EGF requires the intrinsic protein tyrosine kinase activity of the EGF receptor ErbB-1 (38, 46). Our model shows that ErbB-1/2 may be important for normal cardiac contractility mediated by adenylyl cyclase in vivo. We used NKH-477 (a forskolin analog), which is a potent activator of adenylyl cyclase with selectivity to the cardiac (type V) enzyme. Since the drug does not effectively pass the blood-brain barrier (28), it should not stimulate the nervous system as much as forskolin and hence avoid neurally mediated effects (53). Studies suggest that the absence of adenylyl cyclase V is associated with increased basal function (59) and protection against apoptosis and deteriorating function (43) under chronic catecholamine stress. However, our model differs by using NKh-477 only as an acute treatment in resting cardiac ErbB-1 mutants while the chronic effects are unknown. We also found that the β1-AR expression is unchanged in these mutant mice (Fig. 10). It may be possible that an intermediary G protein is involved, which is not clear. Our results also support an earlier study (52) on the beneficial effects of NKh-477 on cardiac function in chronic...
heart failure model, which also suggested that NKH-477 may help to reverse the cardiac dysfunction associated with chronic heart failure. Alternatively, as seen in a different study (42), it is yet to be determined about the possibility that the ErbB-1-ERK-mediated cardioprotection (conferred by β-arrestin-mediated β1-AR transactivation of ErbB-1) may be impaired in our ErbB-1 mutant model following stimulation with Dobut. On the other hand, the role of ErbB-1 has also been implicated to be important in cardiac glycoside therapy for heart failure (22, 62).

The hErbB-1-mut hearts indicate hypertrophic changes as seen by the increase in the LV mass, ANF expression, and histological changes. Studies have shown that ErbB-1 is important for angiotensin-II (60, 65) and other ErbB-1 knockin (55) models of hypertrophy. However, others have observed hypertrophic phenotypes even in the absence of ErbB receptors (9, 17). In fact, the idea of angiotensin II–ErbB-1-mediated hypertrophy has been challenged by a recent study in which angiotensin II-stimulated dominant-negative ErbB-1 did not inhibit hypertrophy in cardiomyocytes in vitro and showed only an increasing trend towards hypertrophy (5).

Zhai et al. (65) concluded that no obvious baseline cardiac phenotype has been observed in a cardiac-specific dominant-negative ErbB-1 mouse model. A variety of factors may explain the discrepancy in results with respect to our study. First, ErbB-1 gene-knockout mice phenotypes are clearly known to differ in a strain-dependent manner (55, 61). Ours is from the C57BL/6 X DBA2, whereas the study of Zhai et al. (65) used mice from FVB background. Therefore, it is possible the phenotypes will differ. The other important factor is that ours is an inducible expression system that has the advantage of expressing mutant ErbB-1 specifically in the adult heart and not since birth (unlike their animal model). We believe that this model has more relevance to the adult with acquired cardiovascular disease. Contrasting to our result, their result did not observe any change in ErbB-2 phosphorylation, which may also be a factor involved in their lack of phenotype. As discussed earlier, in addition to the idea that ErbB-2 may play a dominant role, these can be conclusively understood only with future models of simultaneous loss-of-function mutation of ErbB-1 along with gain-of-function mutation of ErbB-2 receptors. Finally, the study by Zhai et al. (65) only examined resting phenotypes. We believe it is important to examine stress-induced phenotypes as was done in our aortic constriction model.

In summary, cardiac ErbB-1 receptors affect cardiac ErbB-2 receptors, and the two together affect normal adult cardiac function in vivo and thus compromised ErbB signaling leads to a dilated cardiomyopathic phenotype. Future studies are needed to focus on further understanding the relative contribution of each of the cardiac ErbB receptor subtypes in control of adult cardiac function and dissecting the different roles of ErbB-1 in cardiomyocyte survival, hypertrophy in vivo at rest and following chronic stress. As discussed earlier, more pronounced cardiac phenotypes might be observed by expressing ErbB-1 mutants along with ErbB-2 and/or ErbB-4 mutants.

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