Activation of the neuregulin/ErbB system during physiological ventricular remodeling in pregnancy

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Lemmens K, Doggen K, De Keulenaer GW. Activation of the neuregulin/ErbB system during physiological ventricular remodeling in pregnancy. Am J Physiol Heart Circ Physiol 300: H931–H942, 2011. First published December 24, 2010; doi:10.1152/ajpheart.00385.2010.—The neuregulin-1 (NRG1)/ErbB system has emerged as a paracrine endothelium-controlled system in the heart, which preserves left ventricular (LV) performance in pathophysiological conditions. Here, we analyze the activity and function of this system in pregnancy, which imparts a physiological condition of LV hemodynamic overload. NRG1 expression and ErbB receptor activation were studied by Western blot analyses in rats and mice at different stages of pregnancy. LV performance was evaluated by transthoracic echocardiography, and myocardial performance was assessed from twitches of isolated papillary muscles. NRG1/ErbB signaling was inhibited by oral treatment of animals with the dual ErbB1/ErbB2 tyrosine kinase inhibitor lapatinib. Analyses of LV tissue revealed that protein expression of different NRG1 isoforms and levels of phosphorylated ErbB2 and ErbB4 significantly increased after 1–2 wk of pregnancy. Lapatinib prevented phosphorylation of ErbB2 and ERK1/2, but not of ErbB4 and protein kinase B (Akt), revealing that lapatinib only partially inhibited NRG1/ErbB signaling in the LV. Lapatinib did not prevent pregnancy-induced changes in LV mass and did not cause apoptotic cell death or fibrosis in the LV. Nevertheless, lapatinib led to premature maternal death of ~25% during pregnancy and it accentuated pregnancy-induced LV dilatation, significantly reduced LV fractional shortening, and induced abnormalities of twitch relaxation (but not twitch amplitude) of isolated papillary muscles. This is the first study showing that the NRG1/ErbB system is activated, and plays a modulatory role, during physiological hemodynamic overload associated with pregnancy. Inhibiting this system during physiological overload may cause LV dysfunction in the absence of myocardial cell death.

neuregulin-1; ErbB receptor; lapatinib; ventricular remodeling; pregnancy

NEUREGULIN-1 (NRG1) is an epidermal growth factor (EGF)-related protein with cardioprotective and cardioregenerative properties (18). NRG1 is released by endothelial cells of the endocardium and of the myocardial microvasculature and binds to the membrane receptor tyrosine kinase ErbB3 and ErbB4 of adjacent cells in a paracrine fashion. Release of NRG1 from the endothelium is modulated by neurohormones and mechanical deformation (20). NRG1-induced activation of ErbB receptors triggers receptor dimerization, preferably with the coreceptor ErbB2. Dimerization leads to transphosphorylation of both receptor monomers and activation of downstream signaling cascades, including ERK1/2 mitogen-activated protein kinase, phosphoinositide (PI) 3-kinase and protein kinase B (Akt), and focal adhesion kinase (FAK) (10, 17).

These molecular events promote hypertrophic and even mitototic cardiomyocyte growth, cardiomyocyte resistance to apoptosis, reduced cardiomyocyte sensitivity to adrenergic stimulation, and regulation of myocardial cell-cell and cell-matrix interactions (19, 24).

The in vivo significance of NRG1/ErbB signaling in the adult heart has been demonstrated with conditional gene deletion studies in mice. These studies have revealed a dilated cardiomyopathy following conditional cardiac-specific deletion of ErbB2 and ErbB4 receptors (4, 13). Consistently, activation of ErbB signaling by in vivo administration of NRG1 in rodents improved left ventricular (LV) function and survival in ischemic, inflammatory, and toxic cardiomyopathy (21). Bersell and coworkers (1) showed that, in a mouse model of myocardial infarction, NRG1 induced proliferation of mononucleated cardiomyocytes and improved ventricular performance. Based on these preclinical results in rodents, NRG1 is currently tested in phase II clinical trials in human heart failure patients (12).

In conditions of LV damage and dysfunction, activation of NRG1/ErbB signaling is part of an adaptive compensatory program that preserves LV performance. At more advanced stages of LV dysfunction, however, activity of the NRG1/ErbB system is attenuated. This maladaptive change is the result of reduced ventricular expression of NRG1, reduced expression of ErbB receptors, and dysfunctional downstream ErbB signaling (6, 26). Attenuation of compensatory NRG1/ErbB signaling may be an important event in the progression of physiological to pathophysiological LV hypertrophy and irreversible heart failure.

Despite the emerging cardioprotective actions of ErbB signaling, ErbB2 inhibitors are used in the treatment of cancer. Not surprisingly, cardiac side effects, including a fall of LV ejection fraction, have been observed. To date, these effects are most significant for the anti-ErbB2 monoclonal antibody trastuzumab, especially when administered concomitantly with or shortly after treatment with anthracyclines, which cause oxidative stress in the LV (16, 28). When applied several weeks after cardiotoxic chemotherapy, cardiac side effects are much less. These observations suggest that the injured heart may reside, at least transiently, in a state of “NRG1/ErbB dependency.” The conditions in which this occurs are only beginning to emerge.

In contrast to previous studies that focus on pathophysiological conditions and disease states, the present study investigates the activity and the functional importance of the NRG1/ErbB system in conditions of increased physiological LV hemodynamic loading, namely pregnancy. Functional importance of NRG1/ErbB signaling was evaluated by applying the ErbB1/ErbB2 tyrosine kinase inhibitor (and anti-cancer drug)
lapatinib. The inhibitory profile of lapatinib on NRG1/ErbB signaling in the LV was, therefore, characterized.

**METHODS**

**Antibodies.** Anti-ErbB2 antibody clone 21 was purchased from Lab Vision. Antibodies to ErbB1 clone 1005, phospho-ErbB2 (phosphotyrosine 1248), ErbB4 (clone C-18), ErbB3 (clone G-4) actin (clone C-110), and horseradish peroxidase-conjugated anti-phosphotyrosine antibody (PY20) were from Santa Cruz Biotechnologies. Antibodies to phospho-ErbB4 (phosphotyrosine 1284), phospho-Akt (serine 473), Akt, phospho-ERK1/2 (threonine 202/tyrosine 204, ERK1/2), cleaved caspase-3 (aspartate 175), phospho-FAK, and FAK were from Cell Signaling Technologies. Anti-Bcl-xl/S was from Novus Biologicals. Anti-phospho-phospholamban (serine 16) and anti-phospholamban (clone A1) antibodies were purchased from Upstate. Sarcoplasmic reticulum Ca2+/-ATPase (Serca) 2A antibody was from Affinity Bioreagents.

**Pregnancy model and study design.** Sprague Dawley rats (170–220 g, n = 36) and C56BL/6 mice (18–20 g, n = 15) were purchased from Charles River and randomly assigned to one of the following groups: nonpregnant, early pregnant (7–14 days), late pregnant (18–20 days), and postpartum. The presence of spermatozoa in the vaginal smear was considered as evidence of mating, and the day of their occurrence was designated as the first day of pregnancy. Mating of the different groups was designed such that age-matched animals were killed at the end of the protocol. Transthoracic echocardiograms were performed on lightly anesthetized rats and conscious mice using a Vingmed System 5 equipped with a 10-MHz transducer. LV anterior and posterior wall thickness and end-diastolic (EDD) and end-systolic (ESD) internal dimensions were measured on three consecutive cycles and averaged by a single observer in a blinded fashion. Fractional shortening (FS) was calculated as %FS: [(EDD – ESD)/EDD] × 100 (29). LV mass was obtained with the following formula: 1.04[(AW + PW + EDD)3 – EDD3] × 0.8 + 0.6 (5) where AW is anterior wall and PW is posterior wall.

At the time of death, LV tissue was snap-frozen in liquid nitrogen and stored at −80°C until analysis. In a separate protocol, pregnant and nonpregnant mice (n = 63) were treated with lapatinib (Tykerb, 80 mg/kg, daily), prepared from tablets to a suspension in 7.5% (wt/vol) gelatin, or with vehicle by oral gavage. Treatment was given daily, starting on the day of mating until the day of death, being 19 days after mating for late-pregnant mice or 7 days after delivery for the postpartum group. Echocardiography was performed at the start of the protocol, at late-pregnant stages, and on the day of death. Age-matched nonpregnant controls received vehicle, and echocardiograms were taken in parallel with pregnant groups.

Prior validation of in vivo inhibitory effects of lapatinib was performed in female mice (n = 48) by administration of a single dose of lapatinib or vehicle and subsequent randomization to one of the following intraperitoneal treatments: PBS, NRG1 (10 μg/kg; Preprotech), or EGF (100 μg/kg; Preprotech). Animals were killed 15 min after the intraperitoneal injection to analyze ErbB signaling in LV tissue.

All study protocols were approved by the local animal care committee and conform to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996).
cleaved caspase-3. After primary antibody incubation, sections were incubated with species-appropriate horseradish peroxidase-conjugated secondary antibodies (Vector Laboratories). Staining with 0.1% Sirius red F3BA saturated in picric acid (picro Sirius red staining) was used to assess interstitial fibrosis (percent rate of Sirius red-stained collagen area to total myocardial area in digitalized microscopic images).

Real-time PCR. Heart tissue was homogenized with a Polytron homogenizer in TRIzol Reagent (Invitrogen, Life Technologies). mRNA was isolated following instructions of the manufacturer. Reverse transcription was performed with TaqMan reverse transcription reagents (Applied Biosystems), and samples were used for real-time PCR in a 25-μl reaction containing 12.5 μl SYBR Green PCR Reaction Mix (Applied Biosystems) and 100–900 nmol/l of both primers and antisense 5′-GCT AAG CAG TTG GTG GTG CA-3′ and antisense 5′-GAGCAGAGAGACAGGAA-3′. mRNAs of interest were amplified for 45 cycles at 95°C and a primer annealing and elongation step of 1 min at 60°C, with and without blocking peptide, a NRG1-α antibody (sc-348), and a NRG1-β antibody [anti-β, epidermal growth factor (EGF) domain]. Isomers detected by a specific antibody are stated above the correspondingly blocked band. Immunoblots reveal multiple bands. The 44-kDa protein likely corresponds to NRG1-α, whereas the 55- and 80-kDa bands correspond to NRG1-β isoforms. The 115-kDa protein can be both NRG1-α2α and NRG1-β2α.

Fig. 1. Expression of neuregulin-1 (NRG1) isoforms in left ventricular (LV) tissue. NRG1 protein expression in LV tissue of rats (R) and mice (M) was examined with Western blot analyses using NRG1 antibody (Ab)-1 (clone 7D4), a cytoplasmic antibody (sc-348) with and without blocking peptide, a NRG1-α antibody (sc-1794), and a NRG1-β antibody [anti-β, epidermal growth factor (EGF) domain]. Isomers detected by a specific antibody are stated above the corresponding blot. Immunoblots reveal multiple bands. The 44-kDa protein likely corresponds to NRG1-α, whereas the 55- and 80-kDa bands correspond to NRG1-β isoforms. The 115-kDa protein can be both NRG1-α2α and NRG1-β2α.

Table 1. Cardiac parameters at different stages of pregnancy in rat

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Early Pregnancy</th>
<th>Late Pregnancy</th>
<th>Postpartum</th>
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<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>13</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>262 ± 8</td>
<td>285 ± 12*</td>
<td>375 ± 6*</td>
<td>290 ± 7*</td>
</tr>
<tr>
<td>Heart wt, mg</td>
<td>782 ± 20</td>
<td>882 ± 20</td>
<td>927 ± 31*</td>
<td>898 ± 20</td>
</tr>
<tr>
<td>Heart wt-to-body wt ratio</td>
<td>3.1 ± 0.02</td>
<td>3.2 ± 0.2</td>
<td>2.5 ± 0.1*</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>Echocardiogram</td>
<td></td>
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<tr>
<td>Heart rate, beats/min</td>
<td>463 ± 17</td>
<td>477 ± 14</td>
<td>460 ± 14</td>
<td>490 ± 13</td>
</tr>
<tr>
<td>IVS, mm</td>
<td>1.53 ± 0.07</td>
<td>1.57 ± 0.04</td>
<td>1.63 ± 0.09</td>
<td>1.55 ± 0.05</td>
</tr>
<tr>
<td>LV PW, mm</td>
<td>1.57 ± 0.06</td>
<td>1.56 ± 0.05</td>
<td>1.89 ± 0.15</td>
<td>1.72 ± 0.07</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>5.70 ± 0.06</td>
<td>6.17 ± 0.07*</td>
<td>6.25 ± 0.13*</td>
<td>6.55 ± 0.14*</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>1.58 ± 0.27</td>
<td>1.43 ± 0.10</td>
<td>1.30 ± 0.16</td>
<td>1.87 ± 0.20</td>
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<tr>
<td>FS, %</td>
<td>72 ± 4</td>
<td>77 ± 2</td>
<td>79 ± 2</td>
<td>72 ± 3</td>
</tr>
<tr>
<td>LV mass, mg</td>
<td>416 ± 23</td>
<td>477 ± 21</td>
<td>569 ± 49*</td>
<td>559 ± 22*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals in each group. IVS, intraventricular septum; IV, left ventricular posterior wall; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; FS, fractional shortening; LV mass, left ventricular mass. *P < 0.05 vs. control by one-way ANOVA with post hoc Bonferroni.

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Fig. 2. Increased NRG1-ErbB signaling in the LV during pregnancy in the rat. NRG1 protein expression (A and B), ErbB2 (C) and ErbB4 (D) receptor expression and phosphorylation, and phosphorylation of downstream ErbB targets ERK1/2 (E) and protein kinase B (Akt, F) were examined in LV tissue of rats at different stages of pregnancy. Expression levels were determined from the density of blotted bands of at least 6 independent experiments. NRG1 expression was normalized to actin expression. Phosphorylated ErbB receptor levels were normalized to total receptor levels. Phosphorylated protein levels of Erk1/2 and Akt were normalized to total protein levels. Graphs show relative expression vs. nonpregnant controls. *P < 0.05 vs. control; n = 6 mice.
Fig. 3. In vivo effects of NRG1 and EGF on tyrosine phosphorylation of LV ErbB1, ErbB2, and ErbB4 receptors and inhibitory effect of lapatinib. Mice were treated with a single dose of lapatinib and 6 h later injected with either NRG1 (10 μg/kg) or EGF (100 μg/kg). Animals were killed after 15 min, and LV lysates were investigated for ErbB phosphorylation using immunoprecipitation (IP) for ErbB receptors and Western blotting (WB) for phospotyrosine (pY). Representative blots of NRG1-induced (left) and EGF-induced (right) phosphorylation of ErbB1 (A), ErbB2 (B), and ErbB4 (C) are shown. Bar graphs show relative expression vs. vehicle-treated controls after densitometric analyses. AU, arbitrary units. *P < 0.05 after Bonferroni post hoc test; n = 6 mice.
load. The above measurements were also performed in mice, and data were qualitatively similar (data not shown).

Expression of NRG1 proteins during pregnancy. Expression levels of different NRG1 isoforms in rat LV tissue were analyzed at different stages of pregnancy and postpartum. As shown in Fig. 2, LV expression of the 44-kDa NRG1-α increases 1.7 ± 0.2-fold at early stages of rat pregnancy compared with nonpregnant controls (P < 0.05, n = 6; Fig. 2A). At late pregnancy, this increase was less pronounced and statistically nonsignificant. The 115-kDa NRG1-α/β isoform increased 1.5 ± 0.2-fold at the early stage and 1.7 ± 0.3-fold at late stages of rat pregnancy (P < 0.05, n = 6; Fig. 2B). Changes in the 80- and 55-kDa isoforms were not significant.

Activation of ErbB2 and ErbB4 receptors and downstream signaling during pregnancy. At late pregnancy, levels of phosphorylated ErbB2 and ErbB4 increased 1.9 ± 0.3- and 1.9 ± 0.2-fold, respectively, whereas total receptor levels remained unchanged (Fig. 2, C and D). Downstream ErbB targets Akt and Erk1/2 were also mildly activated (Fig. 2, E and F). ErbB3 receptor expression was detected in rat LV, but phosphorylation levels remained unchanged during pregnancy (data not shown). Similar results were observed in mice (also see Fig. 5).

Inhibitory effects of lapatinib on ventricular NRG1 signaling in vivo. To evaluate the functional importance of ErbB receptor signaling in pregnancy, mice were treated with the ErbB1/ErbB2 inhibitor lapatinib. Before these experiments, we vali-

Fig. 4. In vivo effects of NRG1 and EGF on phosphorylation of LV Akt and ERK1/2 and inhibitory effect of lapatinib. Mice were treated with a single dose of lapatinib and 6 h later injected with either NRG1 (10 μg/kg) or EGF (100 μg/kg). Animals were killed after 15 min, and LV lysates were investigated for phosphorylation of downstream ErbB targets Akt (A) and ERK1/2 (B). NS, not significant. *P < 0.05 after Bonferroni post hoc test; n = 6 mice.
dated to what extent orally administered lapatinib inhibited NRG1-induced receptor signaling in the heart. Effects of lapatinib on EGF-induced signaling were performed for comparison. We first observed that oral doses ≥ 80 mg/kg produced maximal inhibition of NRG1-induced ErbB2 receptor phosphorylation and that these effects were most pronounced at 6–8 h after treatment (data not shown). Inhibition gradually declined but was still present after 24 h. Based on these results, further validation experiments were performed in which mice were first treated for 6–8 h with a single dose of lapatinib and then stimulated with NRG1 of EGF, both administered intraperitoneally.

As shown in Fig. 3, 15 min after intraperitoneal administration, NRG1 induced ErbB2 and ErbB4 tyrosine phosphorylation. Unexpectedly, NRG1 also variably induced ErbB1 tyrosine phosphorylation. EGF led to phosphorylation of ErbB1 and ErbB2, but not of ErbB4. Pretreatment with lapatinib (100 mg/kg) completely inhibited EGF- and NRG1-induced phosphorylation of ErbB1 and ErbB2. In contrast, NRG1-induced tyrosine phosphorylation of ErbB4 remained unchanged. At the postreceptor level, NRG1 induced phosphorylation of Akt and of ERK1/2, whereas EGF only led to phosphorylation of ERK1/2 and not of Akt (Fig. 4). Strikingly, lapatinib abrogated NRG1- and EGF-induced phosphorylation of ERK1/2, but phosphorylation of Akt by NRG1 remained unaffected.

Taken together, these experiments indicate that lapatinib, through inhibition of ErbB1 and ErbB2, leads to abrogation of EGF/ErbB1–2/ERK and of the NRG1/ErbB2/ERK signaling in the LV in vivo. By contrast, lapatinib does not inhibit NRG1-induced activation of ErbB4 and Akt. Hence,

Fig. 5. Inhibitory effect of chronic lapatinib treatment in pregnant mice. Phosphorylation of ErbB2 (A) and ErbB4 (B) was investigated with immunoprecipitation for ErbB receptors and Western blotting for phosphotyrosine in nonpregnant and pregnant vehicle-treated and lapatinib-treated mice. Phosphorylation of ERK1/2 (C) and Akt (D) was analyzed with Western blot analyses using phosphospecific antibodies. *P < 0.05 vs. nonpregnant control. #P < 0.05 vs. vehicle-treated pregnant control; n = 6–8 mice.
applied lapatinib only partially inhibits NRG1-induced signaling in the LV.

In pregnant mice, prolonged treatments with lapatinib induced a comparable partial inhibitory profile. Lapatinib prevented increased phosphorylation of ErbB2 and ERK1/2 at late pregnancy and did not affect the increased phosphorylation of ErbB4 and Akt (Fig. 5).

Lapatinib increases maternal and offspring mortality. First and importantly, lapatinib treatment led to premature death of pregnant mice (26%, \( P = 0.025 \) vs. pregnant vehicle-treated mice; Fig. 6A), whereas none of the nonpregnant mice died during the study. As a side observation, we also detected a significantly higher mortality among pups from lapatinib-treated mothers compared with pups from vehicle-treated mice (15 of 17 mice or 88%; \( P = 0.01 \); Fig. 6B). As shown in Fig. 6C, phosphorylation of ErbB2 was decreased in LV tissue of pups from lapatinib-treated mothers, suggesting that lapatinib crossed the placental barrier. In addition, pregnancy rate after mating appeared to be lower in lapatinib-treated females (16 of 24 mice or 66%) compared with vehicle-treated mice (15 of 17 mice or 88%). Whether this was due to more deaths in utero or to reduced fertility could not be elucidated.

Effects of lapatinib on cardiac function during pregnancy. The effect of lapatinib (80 mg·kg\(^{-1}\)·day\(^{-1}\), oral gavage) on LV function during pregnancy was evaluated by treating mice throughout pregnancy and during the first week postpartum. LV function was evaluated by transthoracic echocardiography; cardiac muscle performance was evaluated from isolated papillary muscle in vitro.

Figure 7 shows the effect of lapatinib on cardiac dimensions and function, both in nonpregnant or pregnant conditions. Importantly, the increases in heart weight and LV mass observed during pregnancy were similar in vehicle-treated and lapatinib-treated mice (Fig. 7, A and B). However, lapatinib-treated mice showed a slight but highly significant increase of pregnancy-induced LV dilatation compared with vehicle-treated pregnant controls (LVEDD at late pregnancy: 3.2 ± 0.1 mm in lapatinib-treated vs. 2.8 ± 0.1 mm in vehicle-treated pregnant mice, \( P < 0.001 \); Fig. 7C). This effect was accompanied by a small but significant decrease in LV fractional shortening (73.5 ± 0.9% in lapatinib-treated vs. 77.3 ± 1.1% in vehicle-treated pregnant mice, \( P < 0.05 \); Fig. 7D). Lapatinib did not change myocardial BNP transcript levels (data not shown).

Interestingly, as shown in Table 2, lapatinib induced an increase in twitch duration (time to half relaxation, \( t_{1/2} \)) of isolated mouse papillary muscles, irrespective of pregnant state (\( P < 0.05 \) by 2-way ANOVA, no interaction with pregnant state). Peak isometric twitch active tension and onset of isometric twitch relaxation (time to peak active tension, \( t_{AT} \)) were, however, not influenced by lapatinib treatment. In addition, pregnancy itself induced a significant delay in onset of twitch relaxation (\( t_{AT} \)) and a significant increase in twitch duration (\( t_{1/2} \)) (\( P < 0.01 \) by 2-way ANOVA, no interaction with lapatinib treatment).

Effects of lapatinib on ventricular cell death and fibrosis during pregnancy. Figure 8 shows the effect of lapatinib on parameters of LV apoptosis and fibrosis. Protein expression levels of the proapoptotic Bcl-xS and the antiapoptotic Bcl-xL were determined. No change in the Bcl-xS-to-Bcl-xL ratio was observed after lapatinib treatment in vivo, independent of the state of pregnancy (Fig. 8A). In addition, cleavage of caspase-3 was examined by Western blot analysis and immunohistochemical staining of LV tissue, both showing no increase in cleaved caspase-3, independent of the state of pregnancy (Fig. 8B). Finally, Sirius staining of LV cross sections failed to show any notable fibrosis upon lapatinib treatment in all conditions (Fig. 8C).
Effects of lapatinib on cardiomyocyte calcium handling and cardiomyocyte coupling. Because NRG1 has been shown to activate phospholamban (PLB) phosphorylation (2), we analyzed the effects of lapatinib on calcium-regulating proteins SERCA2a and PLB in pregnancy. NRG1 has also been shown to activate FAK, essential for maintenance of sarcomeric organization (17), in an ErbB2-dependent manner. Therefore, FAK phosphorylation was compared in vehicle-treated and lapatinib-treated mice. As shown in Fig. 9, lapatinib significantly decreased PLB phosphorylation in pregnant mice ($47 /H11005 3\%$ reduction vs. vehicle-treated pregnant controls, $P /H11021 0.05$, $n /H11005 4$). There was also a borderline significant trend toward decreased phosphorylation of PLB in nonpregnant lapatinib-treated animals ($P /H11005 0.06$ vs. vehicle-treated nonpregnant mice). SERCA2a expression remained unaltered in all groups. Phosphorylation of FAK was not influenced by pregnancy state or lapatinib treatment.

DISCUSSION

The ventricular NRG1/ErbB system has cardioprotective and cardioregenerative properties that are activated in conditions of pathophysiological stress, such as myocardial ischemia, systemic anthracycline treatment, and chronic heart failure. In the present study, we showed that the NRG1/ErbB system in the LV becomes activated during pregnancy, which imparts a condition of physiological ventricular overload and remodeling. Partial inhibition of this pathway by lapatinib led to premature death of pregnant (but not nonpregnant) mice and accentuated pregnancy-induced ventricular dilatation with a small drop in LV fractional shortening, suggesting a major modulatory role for the NRG1/ErbB system during LV adaptation to physiological stress.

In contrast to pathophysiological remodeling, physiological remodeling is characterized by preserved LV hemodynamic pump performance and by the absence of myocardial cell death and tissue fibrosis (22). Whereas pathophysiological LV remodeling is associated with activation of neurohormonal pathways that signal through G protein-coupled receptors, physiological LV remodeling seems more associated with activation of growth factors that signal through tyrosine kinase receptors and PI 3-kinase/Akt (7, 15). Consistent with this premise, we here identify the activation of the receptor tyrosine kinases

### Table 2. Contractile parameters of mice papillary muscle

<table>
<thead>
<tr>
<th>Nonpregnant</th>
<th>Pregnant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>Lapatinib</td>
</tr>
<tr>
<td>$n$</td>
<td></td>
</tr>
<tr>
<td>AT, mN/mg</td>
<td>5.37 ± 0.86</td>
</tr>
<tr>
<td>tHR, ms</td>
<td>124 ± 2</td>
</tr>
<tr>
<td>tAT, ms</td>
<td>81.4 ± 2.3</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n$, no. of animals in each group. Active tension (AT) was normalized to muscle weight. tHR, time from stimulus to half isometric relaxation; tAT, time from stimulus to peak active tension development. Lapatinib significantly affects tHR; pregnancy significantly increases tAT and tHR. *$P < 0.05$ by two-way ANOVA. There is no interaction between lapatinib treatment and the pregnant state.
ErbB4 and ErbB2, together with increased LV expression of the ErbB3/4 ligand NRG1, in physiological remodeling in pregnancy. Although expressed, the activity of LV ErbB3 remained unchanged. Whether these findings can be generalized to other conditions of physiological hemodynamic stress such as exercise training needs to be confirmed.

During pregnancy, maternal hemodynamic conditions are associated with an expansion of circulatory volume leading to LV dilatation (9). Applying Laplace’s law, a ventricular dilatation in the presence of normal ventricular wall thickness will result in increased wall strain. During pregnancy, LV wall strain will increase proportional to LV end-diastolic volume, as wall thickness and contractility remain unchanged (also see Tables 1 and 2). Based on our previous in vitro finding that cardiac endothelial NRG1 expression increases in response to cyclic strain, we speculate that the increased expression of LV NRG1 protein is related to this increase in LV wall strain (20). Other load-independent stimuli such as increasing circulating estrogen levels can, however, not be excluded.

Given the paracrine activity of NRG1, increased expression and shedding of NRG1 in the LV lead to intensified cell communication in the myocardium. Most likely, this communication imparts activation of ErbB receptors on cardiomyocytes, as previously described (20). An autocrine activation of ErbB receptors on cardiac endothelial cells, or a paracrine activation of ErbB receptors on other nonmyocyte cells, like cardiac fibroblasts, Purkinje fibers, or cardiac stem cells, is likely, but poorly, explored.

Although not specifically addressed in this study, activation of the LV NRG1/ErbB system in pregnancy seems to be milder than in disease models like chronic heart failure (20). Also, in some models of pathophysiological stress, the initial robust activation of NRG1/ErbB signaling is followed by a subsequent attenuation (6). Such a biphasic response was not observed during the duration of pregnancy.

Lapatinib, an ErbB1/ErbB2 tyrosine kinase inhibitor, induced premature death and affected LV performance in pregnant mice, but not in nonpregnant mice. This observation indicates that the LV NRG1/ErbB system has a physiological role in pregnancy. A number of aspects require specific discussion.

First, oral treatment of mice with lapatinib led to a mere partial inhibition of the NRG1/ErbB system in the LV. Consistent with its pharmacological actions as an ErbB1/ErbB2 antagonist, lapatinib abrogated activation of ErbB2 and downstream ERK1/2. By contrast, lapatinib did not affect the activation of ErbB4 and Akt, both when triggered by either exogenous NRG1 or by pregnancy. Although straightforward from a pharmacological point of view, this observation is intriguing. It is generally believed that, upon stimulation by ligand binding, ErbB4 first heterodimerizes with ErbB2 and then becomes transphosphorylated by ErbB2. To what extent the observation of a preserved ErbB4 phosphorylation in the presence of lapatinib (and hence, inhibited ErbB2 activity) can be explained by ErbB4 homodimerization and subsequent ErbB4-ErbB4 transphosphorylation or, alternatively, by residual ErbB2 activity despite the presence of lapatinib needs further research. In any case, lapatinib only partially blocks the NRG1/ErbB system, leaving the activation of ErbB4 and Akt unaffected. Therefore, the effects of a complete ErbB inhibition that includes ErbB4 inhibition in physiological LV overload remain to be elucidated. Unfortunately, specific pharma-

Fig. 8. Effects of lapatinib on ventricular cell death and fibrosis during pregnancy in mice. A: protein levels of the proapoptotic Bcl-xS and the antiapoptotic Bcl-xL were determined with Western blot analyses in vehicle- and lapatinib-treated nonpregnant and pregnant mice. Bar graph shows the ratio of Bcl-xS to Bcl-xL after densitometric analysis. B: cleavage of caspase-3 was examined by Western blot analysis and immunohistochemical staining of LV tissue. Images are representative sections of vehicle- and lapatinib-treated pregnant mice. C: representative images of Sirius red-stained LV sections in pregnant mice. Bar graph shows %rate of Sirius red-stained collagen area to total myocardial as quantified in minimum 5 digitalized microscopic images/animal.
ological ErbB4 inhibitors are not available, and gene deletions of ErbB4 are lethal.

Second, lapatinib was associated with maternal death during pregnancy and with postnatal death of their offspring. To what extent mortality of both can be causally linked to effects on the heart is not clear. Of note, also vehicle treatment was associated with offspring, but not maternal, mortality, although offspring mortality during treatment with vehicle was still lower than during treatment with lapatinib. This effect is, most likely, because of the manipulations of the pregnant rats during daily oral gavages, which leads to increased postnatal rejection of the pups.

Third, the effects of lapatinib on LV performance occurred without signs of increased LV cell death. Consistently, ErbB2 inhibition in vitro, with an anti-ErbB2 antibody or with the tyrosine kinase inhibitor PKI166, causes disturbances of cardiomyocyte myofibrillar structure and function, without affecting cardiomyocyte cell viability (23, 11, 27). Hence, one of the major observations of this study is that inhibition of the NRG1/ErbB system may induce LV dysfunction, even when ErbB-controlled and Akt-mediated survival pathways are preserved. Of note, recent clinical trials have reported that cardiotoxic side effects of lapatinib are minor (25). The data of this study urge, however, for a continued monitoring of cardiac side effects during lapatinib treatment, even when this treatment is given in the absence of pathophysiological cardiovascular stress.

Finally, lapatinib diminished levels of phosphorylated PLB, the principal regulator of SERCA in cardiomyocytes. This effect may explain the abnormalities of twitch relaxation of the papillary muscles, isolated from lapatinib-treated mice. It remains uncertain, however, whether this effect is sufficient to explain the effects of lapatinib on LV performance in vivo, for a number of reasons. First, lapatinib influenced PLB both in pregnant and nonpregnant conditions, but affected LV performance in vivo only in pregnant conditions. Second, a significant depression of SERCA function would be expected to also affect cardiac muscle twitch amplitude in vitro, which remained unaffected by lapatinib. The contrast between the depressed fractional shortening of the dilated LV in pregnant, lapatinib-treated mice in vivo and the preserved contractile performance of the isolated papillary muscles from the heart in vitro may suggest that external loading conditions (in combination with a depressed NRG1/ErbB system), rather than intrinsic contractile defects, may underlie the observed cardiac side effects caused by lapatinib in vivo.

In summary, our data show that the NRG1/ErbB system is activated during physiological LV overload associated with pregnancy. These observations suggest that activation of the NRG1/ErbB system is more than a stress response to myocardial damage and also functions as a modulatory system of ventricular function in physiological conditions.

Fig. 9. Effects of lapatinib on calcium-handling proteins during pregnancy in mice. A: expression levels of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) 2a, phosphorylated phospholamban (PLB), and total PLB were determined using Western blot analyses in nonpregnant and pregnant vehicle-treated and lapatinib-treated mice. Expression levels of SERCA2a were normalized to actin to correct for loading differences. Phosphorylated PLB was normalized to total PLB. Densitometric analyses was performed using Image J. B: phosphorylated focal adhesion kinase (FAK) and total FAK protein levels were assessed by Western blot in nonpregnant and pregnant vehicle-treated and lapatinib-treated mice. \#P < 0.05 vs. vehicle-treated pregnant control.
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DISCLOSURES

No conflicts of interest are declared by the authors.

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