

Trastuzumab, but Not Pertuzumab, Dysregulates HER2 Signaling to Mediate Inhibition of Autophagy and Increase in Reactive Oxygen Species Production in Human Cardiomyocytes

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Abstract

Dysregulation of autophagy has been implicated in various cardiovascular diseases. Trastuzumab, a humanized monoclonal antibody, binds to HER2 domain IV and is approved for the treatment of HER2-positive breast cancer. Trastuzumab therapy is associated with considerable cardiotoxicity, the mechanism of which remains unclear. HER2 signaling plays a pivotal role in cardiomyocyte development and survival and is essential for the prevention of cardiomyopathy. However, a direct link has not been confirmed between trastuzumab-induced cardiomyopathy and impaired HER2 signaling. Our data reveal a novel mechanism by which trastuzumab dysregulates HER2 signaling and impairs basal autophagic process in human primary cardiomyocytes. Specifically, trastuzumab treatment leads to the phosphorylation of HER1-Y845 and HER2-Y1248 and the activation of Erk. This in turn results in upregulation of mTOR signaling pathway and

subsequently inhibition of autophagy in primary cardiomyocytes and C57BL/6 mice. Trastuzumab-induced downregulation of autophagy is further supported by the fact that trastuzumab treatment reduces protein levels of autophagosome-associated signaling molecules such as Atg 5-12, Atg 7, Atg 14, and Beclin 1. We further demonstrated that trastuzumab-mediated inhibition of autophagy resulted in the increased production of reactive oxygen species (ROS) in cardiomyocytes. Pertuzumab, another anti-HER2 therapeutic mAb binding to HER2 domain II, fails to modulate HER2 signaling and is unable to inhibit autophagy and to increase ROS production in cardiomyocytes. This study provides novel mechanistic insights into trastuzumab-induced cardiotoxicity, which may assist in formulating novel approaches for clinical management of trastuzumab-induced cardiomyopathy. *Mol Cancer Ther*; 15(6); 1321–31. ©2016 AACR.

Introduction

Trastuzumab is a humanized monoclonal antibody directed against the extracellular domain IV of HER2 and approved for the treatment of HER2-positive breast cancer and gastric cancer or gastroesophageal junction adenocarcinoma (1–3). Overexpression of HER2 is seen in 20% to 30% of breast cancer patients and is associated with poor survival, increased metastasis, and resistance to chemotherapy. Trastuzumab exhibits its anticancer attributes by binding to extracellular domain IV of HER2, and thereby directly modulating pro-survival and proliferative signaling cascades, including PI3K and MAPK pathways (4–6). Even though trastuzumab is clinically highly efficacious as monotherapy or in conjunction with anthracyclines, it has been shown to be associated with considerable cardiotoxicity and cardiomyopathy (2, 7, 8). A retrospective clinical study sought to estimate cardiac dysfunction, reported that incidences of cardiac dysfunctions

were about 3% to 7% when patients receive trastuzumab as a single agent; however, incidence was 27% when patients receive trastuzumab in combination with anthracyclines (2, 7). Pertuzumab, a humanized monoclonal antibody, is approved for use in combination with trastuzumab and docetaxel in patients with HER2-positive metastatic breast cancer (9). Unlike trastuzumab, this antibody binds to domain II of the HER2. A phase III clinical study reported that there was no evidence of additive cardiac toxicity when pertuzumab in combination with trastuzumab was used in breast cancer patients as compared with trastuzumab alone (10), although data from early clinical trials suggested that pertuzumab might increase the cardiotoxicity of trastuzumab (11).

Molecular mechanisms associated with trastuzumab-induced cardiotoxicity in breast cancer patients remain largely unknown. Using C57BL/6 mouse model, we have previously reported that trastuzumab treatment induced alteration in myocardial expression of genes that are critically involved in cardiac and mitochondrial functions, adaptation to stress, and DNA repair (12). We observed that these genetic changes are associated with increased myocardial oxidative and nitrative stress, and potentially activated apoptotic pathways, leading to elevated serum troponin-1 and cardiac myosin light chain-1 (cMLC1) levels (12). Another study revealed that administration of anti-HER2 antibody could cause cardiotoxicity in rat cardiomyocytes by activating mitochondrial apoptotic pathway and impairing mitochondrial membrane integrity via modulation of apoptotic proteins BCL-XL and BCL-XS (13).

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Autophagy is a highly conserved intracellular pathway, which is essential for lysosomal degradation of damaged cytoplasmic components and recycling of long-lived proteins (14, 15). Autophagy process involves sequestration of cytoplasmic constituents and dysfunctional organelles in an autophagosome, a double-membrane vesicle, which are then delivered to the lysosome by fusion followed by degradation (16, 17). Basal level of autophagy facilitates protein turnover and organelle maintenance in cells, and is imperative for maintaining cellular energy homeostasis under both normal and pathologic conditions (18, 19). Even though constitutive autophagy in the heart is a homeostatic mechanism for maintaining global cardiac function, it could also trigger cardiomyocyte death thus impairing cardiac function in other contexts, emphasizing its role as a double-edged sword that could be either protective or injurious depending on the cellular environment. Nakai and colleagues (20) reported that activation of autophagy played a beneficial role in the heart in response to pressure overload. They observed that deletion of temporally controlled cardiac-specific Atg 5 led to cardiac hypertrophy, left ventricular dilatation, and contractile dysfunction. In another study, LAMP2-deficient mice show excessive accumulation of autophagic vacuoles and impaired autophagic degradation of long-lived proteins, resulting in cardiomyopathy (21).

Numerous reports have endorsed the critical involvement of ErbB/HER family receptors in cardiomyocyte development and survival (22, 23). Using a mouse model deficient in cardiac HER2, Crone and colleagues (24) investigated the physiologic role of HER2 signaling in the adult heart. They observed that these mice have dilated cardiac chambers and myocardial wall thinning, and concluded that HER2 is essential for the prevention of dilated cardiomyopathy. Another study using conditional HER2 mutant mice specifically in ventricular cardiomyocytes reported that mice develop a severe dilated cardiomyopathy (25). Although the underlying mechanisms have not yet been sufficiently elucidated, disruption of the HER2 signaling cascade within the heart is thought to play a role in trastuzumab-induced cardiotoxicity.

In this study, we investigated the effect of trastuzumab and pertuzumab on autophagy modulation in human primary cardiomyocytes and mice model, and explored the implications of autophagy in inducing cardiotoxicity and cardiac dysregulations following trastuzumab therapy. We also evaluated the role of autophagy in enhancing the generation of free radicals in cardiomyocytes that are exposed to trastuzumab and pertuzumab treatment. Because we used human primary cells as model for this investigation, it provides a clinically relevant prototype to study trastuzumab-mediated cardiotoxicity.

Materials and Methods

Antibodies and reagents

Antibodies directed against LC3 I/II, p62/SQSTM1, Atg 5 (this antibody is capable of detecting Atg 5 conjugated to Atg12), Beclin 1, Atg 7, Atg 14, HER2 (29D8), phospho-HER2 (pY1248), phospho-HER1 (pY845), Erk1/2, phospho-ERK1/2 (202T/204Y), phospho-mTOR, total mTOR, phospho-Ulk1, total Ulk1, phospho-S6K1, total S6K1, phospho-S6, total S6, phospho-Akt and total Akt were obtained from Cell Signaling Technology. Antibody against actin was obtained from Sigma-Aldrich. All secondary antibodies for Western blotting and fluorochrome-conjugated secondary antibodies for immunofluorescence studies were purchased from Invitrogen/Molecular Probes. Trastuzumab

(Genentech), Cetuximab (ImClone LLC), and Pertuzumab (Genentech) were purchased from the pharmacy at the National Institutes of Health (NIH). Human IgG was purchased from Life Technologies and was dialyzed in PBS before the usage to remove a preservative. Rapamycin, Lapatinib, and U0126 were obtained from Sigma-Aldrich. ROS detection reagent 2'-7'-Dichlorodihydrofluorescein diacetate (DCFDA) was purchased from Life Technologies. Human EGFR phosphorylation antibody array kit was obtained from Ray Biotech Inc.

Human primary cardiomyocytes, cell culture conditions, and treatment

Human primary cardiomyocytes (cat#6200) were obtained from ScienCell research laboratories. Cells were cultured in human cardiac myocytes media (ScienCell) containing 5% FBS, and 1% penicillin/streptomycin solution and 1% cardiac myocytes growth supplement (ScienCell) and used within 6 months. These cells were not authenticated by the authors. For trastuzumab and pertuzumab treatment, cardiomyocytes were seeded in 6-well plates at 1×10^5 density in 2-mL pre-warmed growth media. Next day, cells were treated with either trastuzumab or pertuzumab at 50 μ g/mL concentration or left untreated. Cells were harvested at 6, 24, and 48 hours for further experiments. For trastuzumab + rapamycin experiments, cells were pre-treated with rapamycin (10 μ mol/L) for 1 hour, and then incubated with trastuzumab for indicated time points. For lapatinib + trastuzumab treated EGFR antibody array experiments, cells were treated with lapatinib (200 nmol/L) for 2 hours or trastuzumab (4 μ g/mL) for 1 hour or combination of two agents for 2 hours.

LC3 staining

Human primary cardiomyocytes were cultured in 4-well chamber slides and treated with trastuzumab for indicated time duration in humidified incubator. Cells were then stained with anti-LC3 II antibody according to the manufacturer's protocol (Cell Signaling Technology). Slides were mounted with coverslips using Prolong Gold AntiFade Reagent with DAPI. Images were captured on a Zeiss LSM-510 Meta microscope using a 40 \times /1.4NA plan Apo objective. For each treatment group, 50 cells were randomly selected and LC3 puncta in a single cell were manually counted under the microscope.

Human EGFR phosphorylation antibody array

Human EGFR phosphorylation antibody array allows for simultaneously detecting the relative level of phosphorylation of 17 specific sites for human EGFR family in cell lysate. The array was obtained from Ray Biotech, Inc. and the assays were performed according to the manufacturer's instructions.

ROS production measurement

DCFDA is one of the most widely used techniques for directly measuring the redox state of a cell. DCFDA, a cell permeable, non-fluorescent precursor of DCF can be used as an intracellular probe for oxidative stress. DCFDA was reconstituted in DMSO by adding 8.6 μ L DMSO in 50 μ g dye to prepare 10 mmol/L stock solution. Following treatments, cardiomyocytes were harvested and washed with PBS and suspended again in 0.5 mL PBS. DCFDA was then added to cells at concentration of 10 μ mol/L, and cells were incubated at 37°C in the dark for 30 minutes. Cells were washed, suspended in PBS, and analyzed by flow cytometry with excitation at 488 nm and emission at 530 nm wavelength. Human

IgG-treated cardiomyocytes were used as negative control and tert-butyl-hydroperoxide (tBHP), a potent ROS inducer, -treated cells were used as positive control.

Animal experiments

All animal experiments were approved by and conducted in accordance with the regulations of the FDA IACUC guidelines. We have previously established murine model of trastuzumab-induced cardiotoxicity using C57BL/6 mice (12). Mice at age 8 to 9 weeks (National Cancer Institute, Frederick MD) were randomly assigned into three groups; trastuzumab, pertuzumab, and vehicle (solvent provided by the manufacturer to dissolve trastuzumab). Both trastuzumab and pertuzumab were purchased from the pharmacy at the National Institutes of Health (NIH; Bethesda, MD). Ten mice in each treatment group received intraperitoneal injection of 10 mg/kg/d of trastuzumab, pertuzumab or comparable volume of vehicle for 6 consecutive days. All groups of mice were euthanized on day 7 and hearts were harvested for autophagy assays using Western blotting.

Statistical analysis

GraphPad Prism and Microsoft Excel software were used for statistical studies. Statistical significance was determined by the Student *t* test (*, $P < 0.05$; **, $P < 0.01$). Data are expressed as mean \pm SEM.

Results

Trastuzumab, but not pertuzumab, inhibits autophagy in human primary cardiomyocytes

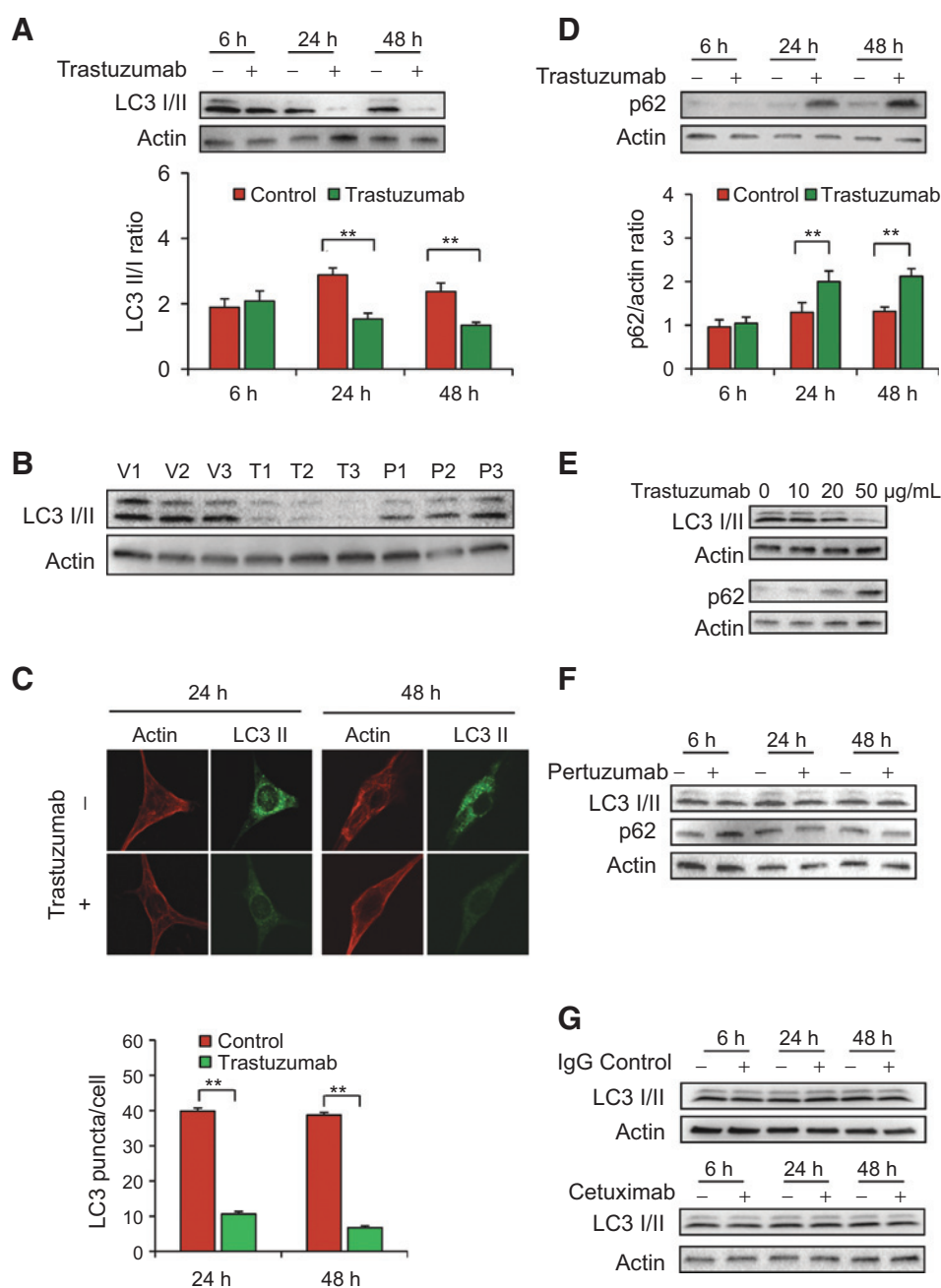
During autophagy, LC3 is cleaved at the carboxy terminus yielding the cytosolic LC3 I form, which is subsequently trans-formed to LC3 II through lipidation by an ubiquitin-like system (15). This enables LC3 II to become associated with autophagic vesicles, which serves as indicator for autophagy. To determine the induction or inhibition of autophagy after trastuzumab treatment, we assessed the level of autophagic marker protein LC3 I/II in primary human cardiomyocytes after 6, 24, and 48 hours of trastuzumab treatment by Western blot analysis. Time-dependent Western blot data showed that LC3 I/II expression, especially LC3 II expression, was remarkably downregulated in human primary cardiomyocytes after 24 and 48 hours of trastuzumab treatment as compared with untreated cells (Fig. 1A). LC3 I/II expression levels remained unchanged at 6 hours of trastuzumab treatment. Quantitative analysis of LC3 I/II blots revealed that ratio of LC3 II/LC3 I was significantly decreased in cardiomyocytes treated with trastuzumab for 24 and 48 hours. To further substantiate our results from cell culture model, we extended our studies to trastuzumab-induced cardiotoxicity mice model. We performed Western blotting to assess the changes in LC3 I/II expression levels in heart tissues collected from mice that were treated with trastuzumab or pertuzumab at 10 mg/kg/d, or vehicle for 6 days. Analysis from western blotting data showed that LC3 I/II expression markedly decreased in mice heart tissues in trastuzumab treatment group but not in pertuzumab and control groups (Fig. 1B). To further confirm biochemical findings, cells treated with trastuzumab were stained for immunofluorescent studies using anti-LC3 II antibody to examine the formation of autophagosomes (punctate LC3 II staining; Fig. 1C). Confocal microscopy imaging showed that LC3 II punctate structures were abundantly present in untreated control cardiomyocytes, but significantly decreased after treatment with trastuzumab for 24 and 48 hours (Fig. 1C).

Alterations in levels of p62 (also known as SQSTM1 or sequestosome 1) are also used to measure autophagy activity. The p62 is an ubiquitin-binding scaffold protein that interacts with poly-ubiquitinated protein aggregates through an ubiquitin-binding domain and with LC3 through its LC3-interacting region (LIR; ref. 26). p62 accumulation is a good measure of defects in selective autophagy of ubiquitinated aggregates. Although at 6 hours, the levels of p62 remained the same in the treated and non-treated cells, a drastic accumulation of p62 levels was found in cardiomyocytes treated with trastuzumab for 24 and 48 hours (Fig. 1D). Densitometry analysis of p62 ratio versus loading control (actin) revealed that p62/actin was significantly enhanced at 24 and 48 hours of trastuzumab treatment but not at 6 hours. To test whether trastuzumab inhibited autophagy in dose-dependent manner, we exposed human cardiomyocytes to 10, 20, and 50 μ g/mL doses for 24 hours. Figure 1E showed dose-dependent inhibition of autophagic activity in human cardiomyocytes by trastuzumab with most efficient inhibition of autophagy at 50 μ g/mL of trastuzumab (Fig. 1E). We did not see any alterations in expression levels of LC3 I/II and p62 in cardiomyocytes treated with pertuzumab for 6, 24 and 48 hours (Fig. 1F), implying that autophagic machinery essentially remains unaffected by pertuzumab treatment. For controlling our trastuzumab-mediated inhibition of autophagy, we incubated human cardiomyocytes with purified human IgG and cetuximab, a therapeutic monoclonal antibody directed against EGFR, at 50 μ g/mL concentrations for 6, 24, or 48 hours and observed no alternations in autophagic activity with those treatments (Fig. 1G).

Trastuzumab downregulates autophagosome-associated proteins

Extensive studies from yeast and mammalian models indicated that two ubiquitin-like conjugation systems, Atg 8 (LC3 II) and Atg 12, are predominantly needed for autophagosome synthesis and maturation (27). The covalent reaction of Atg 12-Atg 5 complex is mediated by ubiquitin E1-like enzyme Atg 7 and the E2-like enzyme Atg 10 (27). The Atg 12-Atg 5 conjugate acts as an E3-like enzyme, which is required for lipidation of Atg 8 proteins and their association to the vesicle membranes. In our study, we monitored the expression levels of Atg 5-Atg 12 complex in human cardiomyocytes after treatment with trastuzumab for 6, 24, and 48 hours using a rabbit monoclonal antibody, which is capable of detecting the Atg 5 when it is in conjugation with Atg 12. As depicted in Fig. 2, Atg 5-Atg 12 conjugate expression was completely obliterated at 24 and 48 hours of trastuzumab treatment in cardiomyocytes; however, at 6 hours, there was no noticeable decrease in protein levels of Atg 5-Atg 12 (Fig. 2). Likewise, E1-like enzyme Atg 7, which activates Atg 5-Atg 12 complex, was slightly downregulated after 24 hours trastuzumab treatment, and further reduced at 48 hours in human cardiomyocytes (Fig. 2).

Beclin 1 is part of a Class III PI3K complex that participates in autophagosome formation, mediating the localization of other autophagy proteins to the pre-autophagosomal membrane (28). Atg 14, an autophagy regulatory protein, contains two coiled-coil domains at its N-terminus that are required for binding of CCD regions of Beclin 1 and class III PI3k. Atg 14 plays a key role in autophagosome biogenesis, knockdown of which has been shown to suppress autophagy. Our Western blotting results showed that trastuzumab inhibited Beclin 1 and Atg 14 expression levels in human cardiomyocytes at 24 and 48 hours exposure,

**Figure 1.**

Trastuzumab, but not pertuzumab, inhibits autophagy in human primary cardiomyocytes. A, human primary cardiomyocytes were seeded in 6-well plates and treated with trastuzumab for 6, 24, or 48 hours or left untreated. Whole-cell lysate (WCL) was subjected to Western blot analysis. Top, the expression levels of LC3 I/II determined by Western blot analysis using anti-LC3 I/II antibody. Actin Western blot analysis of WCL was done to control for equal loading (note: from here on, actin Western blots were used to control for equal loading in all figures). Graph shows the quantitative analysis of ratio of LC3 II versus LC3 I obtained in top. B, Western blotting analysis shows the expression of LC3 I/II in heart tissues of mice that were treated with vehicle (V1, V2, and V3), trastuzumab (T1, T2, and T3), and pertuzumab (P1, P2, and P3). C, top, representative confocal microscopy images of LC3 II staining and LC3 puncta in human cardiomyocytes treated with trastuzumab or left untreated. The graph shows quantitative analysis of LC3 II puncta/cell from 50 randomly selected cells in each group. D, experiments were performed similar to those described in A. Top, the expression levels of p62 as determined by Western blotting after treatment with trastuzumab at the indicated time points. The graph shows the quantitative evaluation of the ratio of p62 and actin. E, Western blotting analysis shows the expression levels of LC3 I/II and p62 in WCL of human cardiomyocytes that were exposed to 10, 20, and 50 µg/mL of trastuzumab for 24 hours. F, Western blotting analysis shows LC3 I/II and p62 expression levels after treatment with pertuzumab at indicated time points. G, Western blotting analysis shows the expression levels of LC3 I/II and p62 in WCL of human cardiomyocytes that were exposed to 50 µg/mL of human IgG or cetuximab. Data in this figure are representative of at least two or more independent experiments and expressed as mean \pm SEM. Statistical significance was determined by the Student *t* test; **, $P < 0.01$.

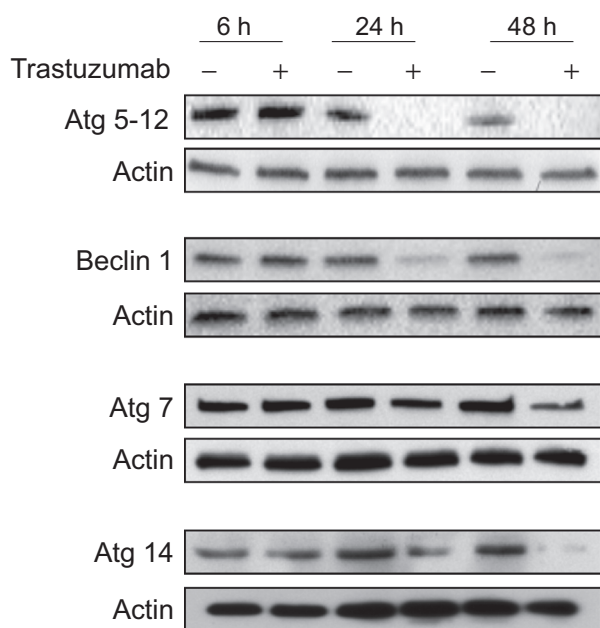


Figure 2.

Trastuzumab downregulates autophagosome-associated proteins and disrupts autophagosome assembly machinery. Human cardiomyocytes were seeded in 6-well plates and treated with trastuzumab at indicated time or left untreated. WCL was collected and Western blotting was performed to determine the expression levels of autophagosome-associated proteins. Total levels of Atg 5-12, Beclin 1, Atg 7, and Atg 14 were determined by using anti-Atg 5, anti-Beclin 1, anti-Atg 7, and anti-Atg 14 antibodies, respectively. Western blotting data are representative of two or more independent experiments.

thus effectively disrupted the assembly of complexes that are required for autophagosome formation (Fig. 2). Taken together, these data collectively suggest that trastuzumab effectively inhibited autophagosome assembly complex proteins and subsequently impaired autophagic machinery.

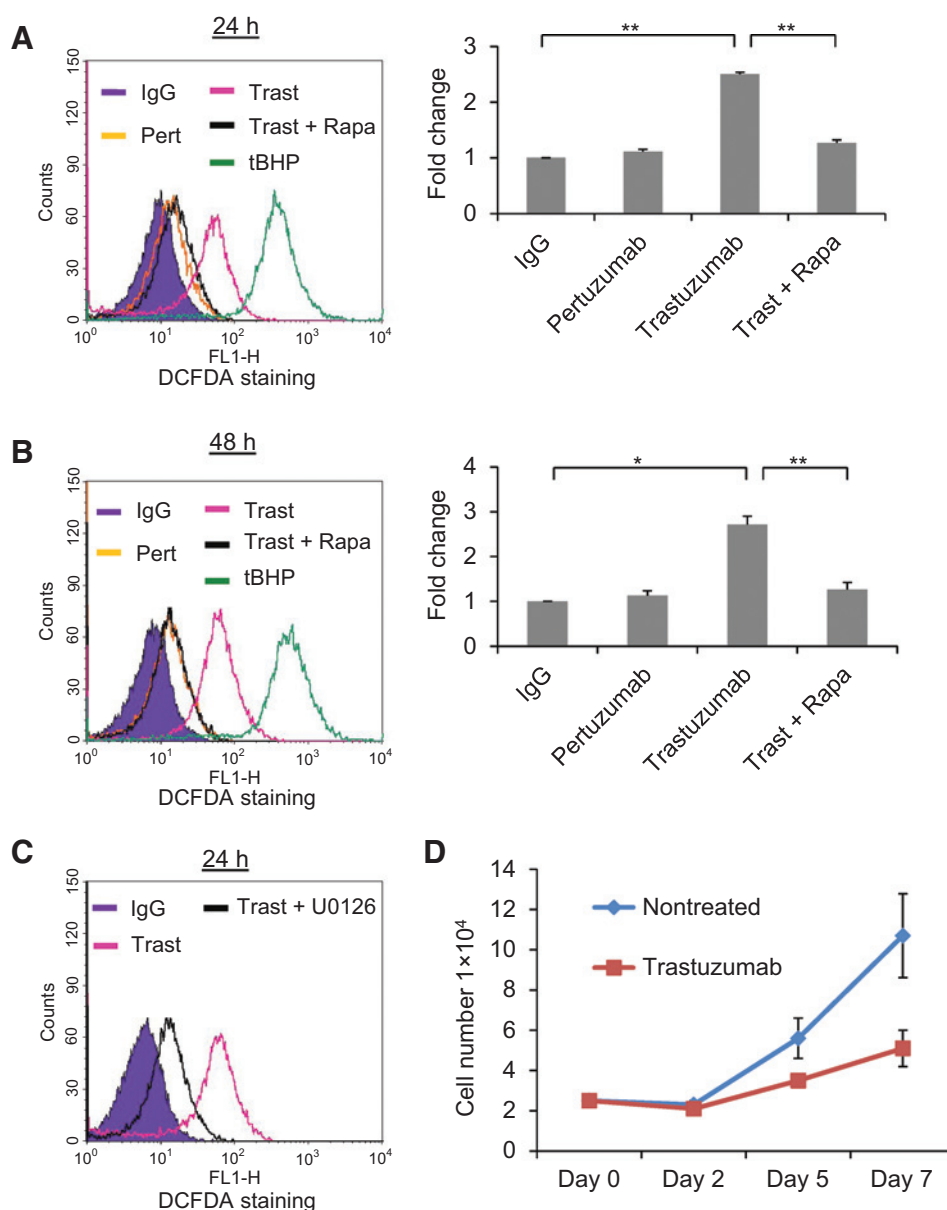
Trastuzumab-mediated autophagy inhibition leads to enhanced ROS production in human cardiomyocytes

Increased oxidative stress is implicated in the pathophysiology of wide range of cardiac disorders, including congestive heart failure and cardiomyopathy, which are typical symptoms of trastuzumab-induced cardiotoxicity. It has been reported that inhibition of autophagy results in the accumulation of damaged mitochondria and increases concentrations of ROS, and as a consequence activates the inflammasome (29). Previously, we showed that trastuzumab increased the levels of nitrotyrosine and 4-hydroxynonenal in heart tissues of trastuzumab-treated mice, thereby triggering oxidative stress (12). Here, we carried out experiments to assess the production of ROS in human primary cardiomyocytes treated with trastuzumab, pertuzumab, and combination of trastuzumab + rapamycin. We found that 24 and 48 hours trastuzumab treatment considerably enhanced the fluorescent intensity of ROS detection reagent DCFDA-loaded cardiomyocytes, hence, indicating increased ROS production in those cells in comparison with IgG-treated cells (Fig. 3A and B). We noticed that pertuzumab treatment did not significantly increase the production of ROS at 24 or 48 hours time points in human

cardiomyocytes as compared with IgG-treated cells (Fig. 3A and B). Furthermore, we tested whether rapamycin, which is an mTOR inhibitor and prominent autophagy enhancer, can reverse the ability of trastuzumab to induce oxidative stress in cardiomyocytes. As shown in Fig. 3A and B, trastuzumab + rapamycin treatment significantly reduced ROS generation in human cardiomyocytes compared with trastuzumab alone. Likewise, U0126, which is a potent Erk1/2 inhibitor, also significantly inhibited the trastuzumab-induced ROS production ($P < 0.05$; Fig. 3C). Taken together, these data suggest that mTOR is downstream of HER2 in modulating trastuzumab-induced ROS production in human primary cardiomyocytes. We next addressed whether trastuzumab was able to inhibit the growth of human primary cardiomyocytes. As shown in Fig. 3D, which is the representative of three independent experiments, although we observed that cardiomyocytes treated with trastuzumab grew slower than non-treated cardiomyocytes in all three independent experiments, no significant differences ($P > 0.05$) were detected between cells treated with trastuzumab and cells left untreated.

Trastuzumab, but not pertuzumab, is capable of inducing HER1 and HER2 tyrosine phosphorylation

To investigate whether the binding of trastuzumab or pertuzumab to HER2 on the cell surface of cardiomyocytes has the effects on the receptor phosphorylation, we analyzed 17 potential phosphorylation sites among the HER family receptors. We have previously reported that binding of trastuzumab to cell surface HER2 activates HER2 kinase activity and enhanced phosphorylation of HER1 at tyrosine 845 (Y845) and HER2 at tyrosine 1248 (Y1248) in trastuzumab-sensitive breast cancer cells (30). As shown in Fig. 4A, trastuzumab also induced phosphorylation of HER1-Y845 (a pair of dots in the blue rectangle) and HER2-Y1248 (a pair of dots in the red rectangle) as compared with control. In contrast, pertuzumab did not induce phosphorylation at either HER1-Y845 or HER2-Y1248 site (Fig. 4A). Figure 4B showed that trastuzumab-induced receptor tyrosine phosphorylation (HER1-Y845 and HER2-Y1248) was blocked by preincubation with lapatinib, a dual HER1 and HER2 kinase inhibitor, in human cardiomyocytes. These data suggest that binding of trastuzumab to HER2 activates kinase activity of HER2 or both HER1 and HER2, which mediates receptor tyrosine phosphorylation. Figure 4C showed that pertuzumab did not mediate receptor phosphorylation and the combination of trastuzumab with pertuzumab gave rise to a similar phosphorylation profiles as compared with that induced by trastuzumab alone. We recently also reported that trastuzumab induced phosphorylation of Erk, a downstream signaling molecule of HER2, in breast cancer cells (30). To address whether trastuzumab also impacts on downstream of HER2 signaling, human primary cardiomyocytes were serum-free overnight and then preincubated with either lapatinib or Erk inhibitor (U0126) for 1 hour or left untreated, and then treated with trastuzumab together with either lapatinib or U0126 for additional 1 hour. As shown in Fig. 4D, trastuzumab induced Erk phosphorylation in 1 hour under serum-free condition in human primary cardiomyocytes. Moreover, the enhanced Erk phosphorylation induced by trastuzumab was diminished by lapatinib, suggesting that the upregulated Erk activity is mediated by trastuzumab-induced HER2 or EGFR/HER2 activation (Fig. 4D). Erk is downstream of HER family molecules and has been implicated in upregulation of mTOR (31). Figure 4E and F showed that trastuzumab induced activation of mTOR as measured by the

**Figure 3.**

ROS production in human primary cardiomyocytes. Cardiomyocytes were seeded in 6-well plates and treated with trastuzumab, pertuzumab, trastuzumab + rapamycin for 24 and 48 hours or left untreated. Cells then were harvested by trypsinization and washed with PBS and suspended in 0.5 mL PBS. DCFDA was then added to cells at a concentration of 10 $\mu\text{mol/L}$, and cells were incubated at 37°C in the dark for 30 minutes. Cells were washed, suspended in PBS, and analyzed by flow cytometry with excitation at 488 nm and emission at 530 nm wavelength. Human IgG-treated cardiomyocytes were used as negative control and tBHP-treated cells were used as positive control. Left, the flow cytometric histogram of cells at 24 hours (A), and 48 hours (B) treated with IgG, trastuzumab, pertuzumab, trastuzumab + rapamycin, and tBHP. Right, quantitative analysis of the fold change in trastuzumab, pertuzumab, and trastuzumab + rapamycin-treated cells relative to IgG-treated cells. C, flow cytometric histogram showing the cardiomyocytes treated with IgG, trastuzumab and trastuzumab + U0126 for 24 hours. Data are representative of three or more independent experiments and expressed as mean \pm SEM. Statistical significance was determined by the Student *t* test; *, $P < 0.05$; **, $P < 0.01$. D, cardiomyocytes were seeded in 12-well plates at 2.5×10^4 density in media supplemented with 1% FBS. Cells were treated with trastuzumab at 50 $\mu\text{g/mL}$ for 2, 5, and 7 days or left untreated. Cell numbers were quantified and statistical significance was determined by the Student *t* test.

phosphorylation levels of S6 at Ser 235/236, which is commonly used as downstream effector of mTOR. However, trastuzumab-induced activation of mTOR was blocked by both U0126 and lapatinib (Fig. 4E and F). Taken together, these data indicate that trastuzumab-mediated activation of mTOR is via HER2 to Erk signaling pathway.

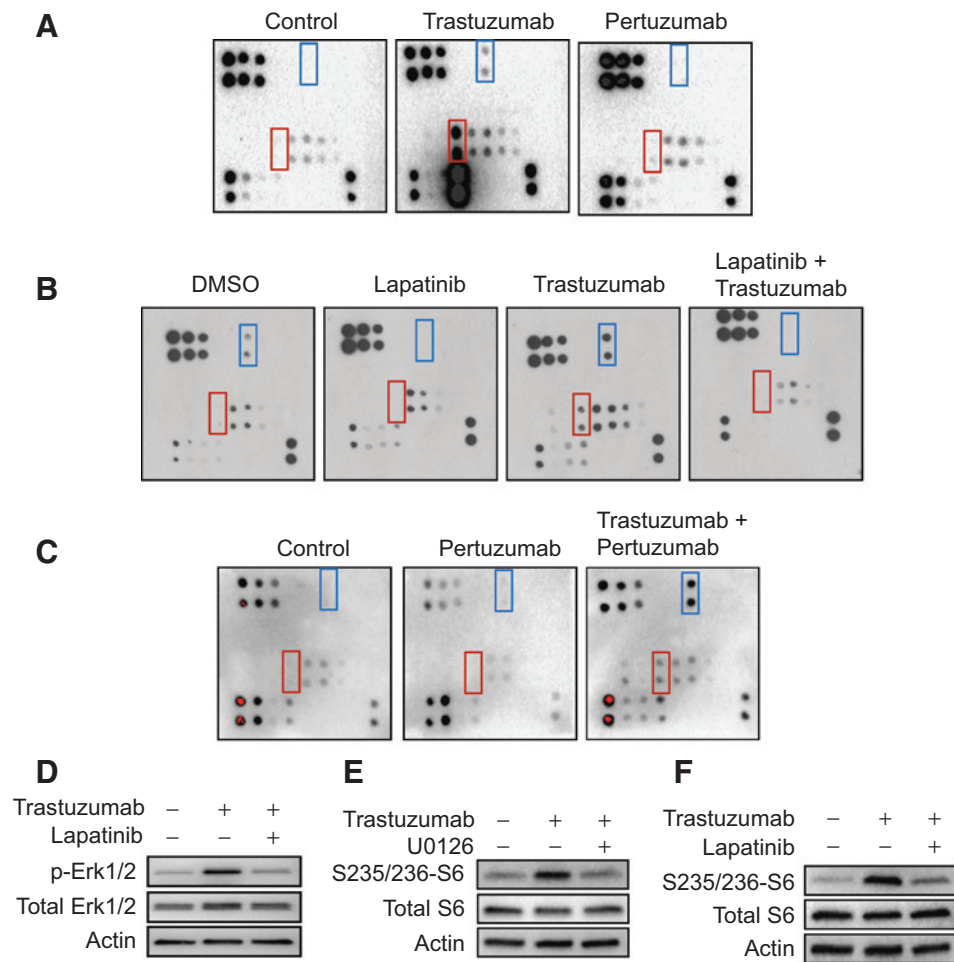
Dysregulation of HER2 signaling by trastuzumab leads to the activation of Erk/mTOR/Ulk1 signaling pathway to mediate autophagy inhibition in human cardiomyocytes

We next studied the underlying signaling mechanisms that led to the inhibition of autophagy in human cardiomyocytes treated with trastuzumab. The mTOR pathway has been well documented to play a central role in the regulation of autophagy. Enhanced mTOR activity disrupts Ulk1 and AMPK interaction by phos-

phorylating Ulk1 at Ser 757 residue, resulting in suppression of autophagy (32). We explored Erk/mTOR/Ulk1 signaling in autophagy regulation by trastuzumab in human primary cardiomyocytes. Figure 5A showed that Erk1/2 was phosphorylated after 24 and 48 hours treatment of trastuzumab in cardiomyocytes. We observed that trastuzumab treatment increased phosphorylation of mTOR in human cardiomyocytes at 24 and 48 hours under 1% serum (FBS) condition (Fig. 5B). Next, we assessed the phosphorylation of Ulk1, which is downstream of mTOR and is critical for the regulation of autophagy activity in cells, after trastuzumab treatment. Using the antibody specific to phosphorylated Ser 757 residue in Ulk1, we found that Ulk1 was phosphorylated after cardiomyocytes were treated with trastuzumab for 24 and 48 hours, suggesting disruption of AMPK–Ulk1 interaction leading to autophagy suppression (Fig. 5C).

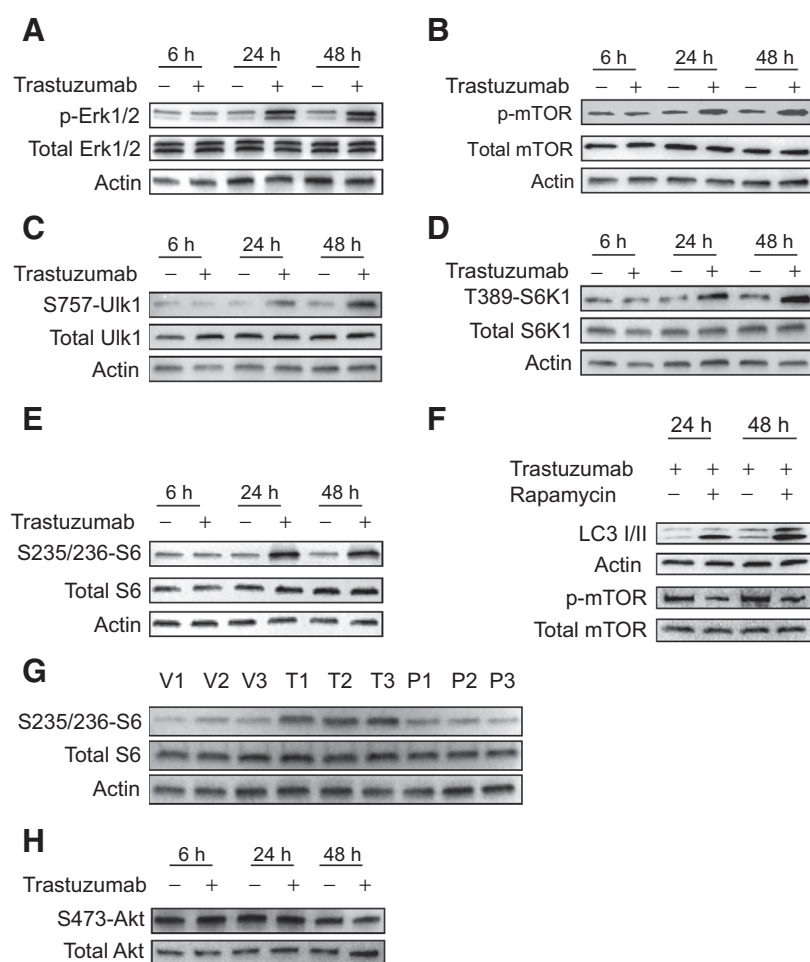
Figure 4.

Trastuzumab induces phosphorylation of HER1-Y845 and HER2-Y1248 in human primary cardiomyocytes. Cardiomyocytes were plated in 10-cm dishes, serum-starved overnight and either treated with trastuzumab, pertuzumab, trastuzumab + pertuzumab, or left untreated. After harvesting, the WCL were subjected to RayBio human EGFR phosphorylation antibody array 1 according to the instructions provided by the manufacturer. The pair of dots in blue rectangles indicates phosphorylated HER1-Y845; the pair of dots in red rectangles indicates HER2-pY1248. A, changes in phosphorylation of HER family receptors following treatment of human primary cardiomyocytes with trastuzumab or pertuzumab. B, detection of phosphorylation level in HER family receptors following treatment of cardiomyocytes with DMSO, lapatinib (5 $\mu\text{mol/L}$), trastuzumab, or lapatinib (5 $\mu\text{mol/L}$) + trastuzumab. C, analysis of phosphorylation levels of HER family receptors after cells were treated with pertuzumab alone or in combination with trastuzumab. D, p-Erk1/2 and total Erk1/2 were evaluated in human cardiomyocytes that were either treated with trastuzumab or trastuzumab + lapatinib (5 $\mu\text{mol/L}$) or left untreated. E, phosphorylation of S6 at Ser 235/236 and total S6 were determined in WCL of human primary cardiomyocytes treated with either trastuzumab or trastuzumab + U0126 (1 $\mu\text{mol/L}$; E) or lapatinib (5 $\mu\text{mol/L}$; F).



Although the well-defined role of S6K1, a direct substrate of mTOR, in autophagy regulation is not yet known, previous reports have suggested that S6K1 negatively regulates autophagy (33). It has also been reported that the phosphorylation of S6K1 correlates with the suppression of autophagy (33). Our results showed that there was an increase in the phosphorylation of S6K1 kinase at Thr 389 site after treatment with trastuzumab for 24 and 48 hours in human cardiomyocytes (Fig. 5D). Ribosomal protein S6 is direct and most studied downstream substrate of S6K1, which predominantly phosphorylates S6. A study on rat hepatocytes concluded that S6 phosphorylation was directly involved in the autophagy and that autophagic and protein synthetic pathways were regulated in an opposite manner by the degree to which S6 was phosphorylated (34). Figure 5E showed that trastuzumab induced dramatic phosphorylation of S6 at Ser 235/236 after 24 and 48 hours exposure, consistent with the data shown in Fig. 4E and F. This is most likely the consequence of trastuzumab-induced activation of mTOR and S6K1 (Fig. 5B and D). To establish that activation of the mTOR pathway by trastuzumab is responsible for autophagy inhibition, we pre-treated human cardiomyocytes with rapamycin for 1 hour followed by trastuzumab treatment for 24 and 48 hours. We found that rapamycin

pre-treatment reversed trastuzumab's ability to suppress autophagy as measured by LC3 I/II expression (Fig. 5F). Phosphorylation of mTOR was also inhibited in cells treated with the combination of rapamycin and trastuzumab compared with trastuzumab alone, consistent with data shown in Fig. 3. To confirm our *in vitro* data, we performed Western blotting to assess the changes in phosphorylation levels of S6 in heart tissues collected from mice treated with trastuzumab, pertuzumab, or vehicle for 6 days at 10 mg/kg/d. As shown in Fig. 5G, S6 phosphorylation was notably increased in the trastuzumab treatment group, but not in the pertuzumab treatment group, as compared with the vehicle group, indicating that mTOR/S6K1 signaling was activated by trastuzumab treatment in mouse heart tissue, resulting in autophagy inhibition. Taken together, our data suggest that trastuzumab dysregulates HER2 activity, leading to the activation of Erk to mTOR signaling to mediate inhibition of autophagy in human primary cardiomyocytes. Figure 5H showed little or no changes in the levels of phospho-Akt at Ser 473 when cardiomyocytes were treated with trastuzumab, suggesting that trastuzumab-induced activation of mTOR is independent of Akt in human primary cardiomyocytes. These data also indicate that impact of trastuzumab on Akt signaling in human cardiomyocytes is different

**Figure 5.**

Trastuzumab-mediated autophagy inhibition is mediated via the HER2–Erk–mTOR–Ulk1 pathway in human primary cardiomyocytes. A, cardiomyocytes were seeded in 6-well plates and incubated in 1% FBS overnight. Cells then were treated with trastuzumab (50 $\mu\text{g}/\text{mL}$) at 6, 24, and 48 hours or left untreated. Western blotting was performed to detect the phosphorylated and total proteins in WCL. Levels of phosphorylated Erk1/2 (p-Erk1/2) and total Erk1/2. B, the levels of phosphorylated mTOR (p-mTOR) and total mTOR. C, phosphorylated Ulk1 at Ser 757 and total Ulk1. D, phosphorylated S6K1 at Thr 389 and total S6K1. E, phosphorylated S6 at Ser 235/236 and total S6. F, cardiomyocytes were treated with trastuzumab alone or pretreated with rapamycin for 1 hour then with trastuzumab for 24 and 48 hours. Expression levels of LC3 I/II, and phosphorylated and total mTOR levels were detected in WCL. G, Western blot analysis shows the phosphorylation of S6 at Ser 235/236 and total S6 in heart tissues of mice that were treated with vehicle (V1, V2, and V3), trastuzumab (T1, T2, and T3) or pertuzumab (P1, P2, and P3). H, phospho-Akt at Ser 473 and total of Akt in WCL of cardiomyocytes treated with trastuzumab for the indicated time points. Western blotting data are representative of two or more independent experiments.

from that in HER2-positive breast cancer cells (SKBR3 and BT474) in that the Akt activity is upregulated and trastuzumab strongly suppresses Akt activity (30).

Pertuzumab does not interfere with the mTOR-dependent pathway in human cardiomyocytes

Because autophagy activity remained largely unaltered in cardiomyocytes treated with pertuzumab (Fig. 1F), we hypothesized mTOR-dependent autophagy pathways might not be targeted by pertuzumab treatment. To validate this hypothesis, we monitored phosphorylation profiles of Erk1/2, mTOR and S6 in human cardiomyocytes treated with pertuzumab for 6, 24, and 48 hours. Figure 6A showed that phosphorylation of Erk1/2 was not changed after pertuzumab treatment. Similarly, phosphorylated mTOR remained unaltered in cardiomyocytes treated with pertuzumab as compared with the control (Fig. 6B). The levels of S6 phosphorylation also remained unchanged in cells treated with pertuzumab or left untreated (Fig. 6C), consistent with data shown in Fig. 5G. Taken together, these results indicate that the impact of pertuzumab on HER2 and its downstream signaling pathway is different from that of trastuzumab, which may explain why pertuzumab is unable to downregulate autophagy in human cardiomyocytes.

Discussion

Autophagy is a catabolic process aimed at recycling cellular components and damaged organelles in response to diverse conditions of stress and has been implicated in the regulation of cardiac development (35). Dysregulation of autophagy has been implicated in various cardiovascular diseases including cardiomyopathy and heart failure. However, its precise role in cardiac pathology remains unclear. Inhibition of autophagy has been reported to contribute to the progression of cardiac hypertrophy (36, 37). Clinical study has demonstrated significant cardiotoxicity in patients treated with trastuzumab (2), mechanisms of which remain elusive. The results described in this study reveal a novel mechanism by which trastuzumab dysregulates HER2 to Erk and mTOR signaling, leading to the impairment of basal autophagic process in human cardiomyocytes. Specifically, trastuzumab treatment interferes with cardiac HER2 signaling that leads to the phosphorylation of HER1-Y845/HER2-Y1248 and the activation of Erk. This in turn results in upregulation of the mTOR–Ulk1 pathway to mediate inhibition of autophagy in cardiomyocytes, such that cells displayed a time-dependent decrease in expression levels of LC3 I/II and increase in p62 after trastuzumab treatment. The trastuzumab-induced downregulation of autophagy is further supported by the fact that

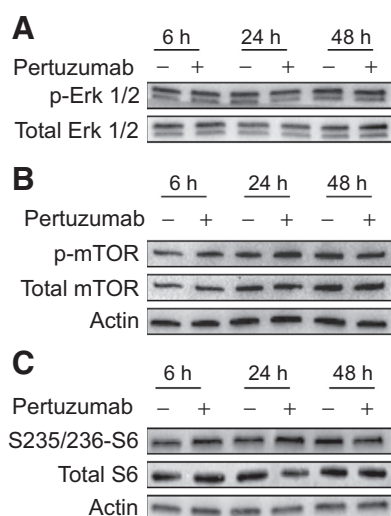


Figure 6.

Pertuzumab does not interfere with the mTOR-dependent pathway in human primary cardiomyocytes. Cardiomyocytes were seeded in 6-well plates and treated with pertuzumab for 6, 24, and 48 hours or left untreated. After treatments, WCL was collected, and Western blotting was done to determine phosphorylated and total proteins. A, levels of p-Erk1/2 and total Erk1/2. B, levels of p-mTOR and total mTOR. C, levels of phosphorylated S6 and total S6. Western blotting data in this figure are representative of two or more independent experiments.

trastuzumab treatment reduces protein levels of autophagosome-associated signaling molecules such as Atg 5-12, Atg 7, Atg 14, and Beclin 1. To the best of our knowledge, this study reports for the first time that trastuzumab is capable of inhibiting autophagy in human cardiomyocytes via dysregulation of HER2 signaling pathway.

Doxorubicin (Dox; adriamycin) is effective in the treatment of a broad range of solid malignancies. However, the clinical use of Dox is limited by its dose-dependent cumulative cardiotoxicity and fatal drug-induced congestive heart failure (38, 39). The role of autophagy in Dox-induced cardiotoxicity has been investigated extensively; however, inconsistent reports on the effects of Dox on autophagy and its role in cardiotoxicity exist (40). Most of the studies suggest that Dox may enhance cardiac autophagy, which contributes to the pathogenesis of Dox-induced cardiotoxicity (40). In the myocardium, autophagy functions by removing protein aggregates and damaged organelles as a prosurvival pathway and is enhanced in pathologic conditions, including cardiac hypertrophy, cardiomyopathy, and heart failure (41). However, a growing number of studies have underscored the downregulation of autophagy in pathogenesis of cardiovascular and neurodegenerative disorders. Mice deficient in Atg 5 in neural cells developed progressive deficits in motor function, accompanied with increased levels of ubiquitinated protein due to lack of clearance of diffuse cytosolic proteins through basal autophagy (42). Similarly, another report indicated that Atg 7 deficiency in mice triggered significant neuronal loss in the cerebral and cerebellar cortices of the central nervous system because of massive accretion of inclusion bodies (43). Recently, Thomas and colleagues (44) demonstrated that cardiac-specific ablation of MCL-1, an antiapoptotic BCL-2 protein, resulted in impairment of autophagy, rapid cardiomyopathy, and onset of heart failure. Our study supports these findings and provides evidence that trastuzumab treatment inhibits autophagy

in cardiomyocytes, which may hinder the efficient degradation of damaged cytoplasmic materials.

The relationship between trastuzumab and anthracyclines is now established. Clinical studies indicate that incidences of cardiac dysfunctions are significantly increased when patients receive anthracyclines following the trastuzumab treatment as compared with patient treated with anthracyclines alone (7). It is proposed that a patient who has previously experienced cardiac damage induced by anthracyclines is more vulnerable to trastuzumab-induced cardiotoxicity (45). The role of trastuzumab in this proposed model is that it may interfere with repair of cardiac cells that have been damaged by anthracycline exposure (45, 46). It was also reported that in the absence of anthracyclines trastuzumab may be given for at least with low levels of cardiotoxicity (47). HER2 signaling plays pivotal role in cardiomyocytes for the prevention of dilated cardiomyopathy (24). Mice with conditional HER2 mutations develop a severe dilated cardiomyopathy (25). It was reported that myocardial HER2 is upregulated after anthracycline exposure (48), which may create a pool of vulnerable cardiomyocytes that are dependent on intact HER2 signaling for recovery (48). However, a direct link has not been confirmed between trastuzumab-induced cardiomyopathy and impaired HER2 signaling (45). Our study provides evidence at the molecular level that binding of trastuzumab to HER2 leads to dysregulation of HER2 signaling in cardiomyocytes, leading to the inhibition of autophagy, which may interfere with repair of anthracycline-damaged cardiomyocytes.

The myocardium is extremely prone to oxidative damage due to its lower levels of catalase activity and superoxide dismutase compared with other tissues (49, 50). Dox-induced free radical production is the most widely studied mechanism to mediate mitochondrial damage, which contributes to Dox-induced cardiotoxicity (50, 51). Many studies have shown that ROS are the upstream modulators of autophagy and may act as the intracellular signaling molecules that transmit the extracellular stimuli by signaling their presence to the autophagic machinery (35). However, it has also been reported that inhibition of autophagy by autophagy inhibitor (3-methyladenine) results in the accumulation of damaged mitochondria and the increased concentration of mitochondrial ROS (29), suggesting that the enhanced production of ROS is the consequence of the inhibition of autophagy by 3-methyladenine. Our data suggest that trastuzumab-induced increase in ROS production is likely due to inhibition of autophagy. Collectively, this study provides novel mechanistic insights into trastuzumab-induced cardiotoxicity, which may assist in formulating novel approaches for clinical management of trastuzumab-induced cardiomyopathy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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