

Constitutive and Inducible Akt Activity Promotes Resistance to Chemotherapy, Trastuzumab, or Tamoxifen in Breast Cancer Cells

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Abstract

To evaluate the role of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in breast cancer cell survival and therapeutic resistance, we analyzed a panel of six breast cancer cell lines that varied in erbB2 and estrogen receptor status. Akt activity was constitutive in four cell lines and was associated with either PTEN mutations or erbB2 overexpression. Akt promoted breast cancer cell survival because a PI3K inhibitor, LY294002, or transient transfection of a dominant-negative Akt mutant inhibited Akt activity and increased apoptosis. When combined with therapies commonly used in breast cancer treatment, LY294002 potentiated apoptosis caused by doxorubicin, trastuzumab, paclitaxel, or etoposide. Potentiation of apoptosis by LY294002 correlated with induction of Akt by doxorubicin or trastuzumab alone that occurred before the onset of apoptosis. Similar results were observed with tamoxifen. Combining LY294002 with tamoxifen in estrogen receptor-positive cells greatly potentiated apoptosis, which was correlated with tamoxifen-induced Akt phosphorylation that preceded apoptosis. To confirm that the effects of LY294002 on chemotherapy-induced apoptosis were attributable to inhibition of Akt, we transiently transfected breast cancer cells with dominant-negative Akt and observed increased doxorubicin-induced apoptosis. Conversely, stably transfecting cells with constitutively active Akt increased Akt activity and attenuated doxorubicin-induced apoptosis. These studies show that endogenous Akt activity promotes breast cancer cell survival and therapeutic resistance, and that induction of Akt by chemotherapy, trastuzumab, or tamoxifen might be an early compensatory mechanism that could be exploited to increase the efficacy of these therapies.

Introduction

Akt (or protein kinase B) is a well-characterized serine/threonine kinase that promotes cellular survival. Akt is activated in response to many different growth factors, including IGF²-I, epidermal growth factor, basic fibroblast growth factor, insulin, interleukin 3, interleukin 6, heregulin, and vascular endothelial growth factor (reviewed in Ref. 1). Akt is the cellular homologue of the product of the *v-akt* oncogene (2–4) and has three isoforms, Akt1, Akt2, and Akt3 (or protein kinases B α , B β , and B γ). Activation of all three isoforms is similar in that phosphorylation of two sites, one in the activation domain and one in the COOH-terminal hydrophobic motif, are necessary for full activity. For Akt1, phosphorylation of T308 in the activation domain by phosphoinositide-dependent kinase 1 is dependent on the products of PI3-K, PIP₂, and PIP₃. Cellular levels of PIP₂ and PIP₃ are controlled by the tumor suppressor, dual-phosphatase PTEN, which dephosphorylates PIP₂ and PIP₃ at the 3' position. The mechanism of S473 phosphorylation is less clear. Kinases potentially responsible for S473 phosphorylation include phosphoinositide-dependent kinase 1 (5), integrin-linked kinase or an integrin-linked kinase-associated kinase (6, 7), Akt itself (8), or an as yet uncharacterized PDK2. Once activated, Akt exerts antiapoptotic effects through phosphorylation of substrates that directly regulate the apoptotic machinery such as Bad (9, 10) or caspase 9 (11), or phosphorylation of substrates that indirectly inhibit apoptosis such as the human telomerase reverse transcriptase subunit (12), forkhead transcription family members (13, 14), or I κ B kinases (15, 16).

Previous studies have demonstrated that Akt plays an important role in survival when cells are exposed to different apoptotic stimuli such as growth factor withdrawal, UV irradiation, matrix detachment, cell cycle discordance, DNA damage, and administration of anti-Fas antibody, transforming growth factor- β , glutamate, or bile acids (17–31). Recently, we have shown that Akt is constitutively active in >90% of non-small cell lung cancer cell lines and contributes to both chemotherapeutic resistance and radiation resistance (32). The importance of Akt in promoting therapeutic resistance in other solid tumor cell systems such as breast cancer has not been established, however.

Genetic and biochemical evidence suggests that activation of the PI3K/Akt pathway contributes to breast cancer tumorigenesis. Patients with familial syndromes character-

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² The abbreviations used are: IGF, insulin-like growth factor; PI3-K, phosphatidylinositol 3-kinase; PIP₂, phosphatidylinositol 3,4-bisphosphate; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; PTEN, phosphatase and tensin homologue deleted on chromosome 10; HA, hemagglutinin; ER, estrogen receptor; GFP, green fluorescent protein; FBS, fetal bovine serum; LS, low serum (0.1% FBS); GSK, glycogen synthase kinase; ROS, reactive oxygen species; PARP, poly(ADP ribose) polymerase.

ized by germ-line PTEN mutations (Cowden's, Bannayan-Zonana) are predisposed to breast cancer development (33, 34). In spontaneous breast cancers, PTEN mutations are found in ~5% of samples, and loss of heterozygosity of the PTEN locus is present in 40% (35–37). *In vitro*, transfecting wild-type PTEN into PTEN-negative breast cancer cells can decrease Akt activity and cause cell cycle arrest, apoptosis, or anoikis (38, 39). Amplification of the *Akt2* gene is found in 3% of spontaneous breast cancers (40), and an inverse relationship between ER status and Akt3 activity has been described in both breast cancer cell lines and tumor specimens (41). Akt is a downstream target of many receptor-stimulated pathways involved in breast cancer, including ER α , IGF-IR, epidermal growth factor receptor, and erbB2. In addition, constitutively active Akt contributes to breast tumor formation *in vivo* when it is expressed in mice under the control of an Moloney murine tumor virus promoter and in conjunction with either polyomavirus middle T antigen or IGF-II (42, 43).

In these studies, we used a small molecule inhibitor of the PI3K/Akt pathway and transfection of Akt mutants to show that Akt promotes breast cancer cell survival and resistance to chemotherapy, trastuzumab, or tamoxifen. Most interestingly, activation of Akt by doxorubicin, trastuzumab, or tamoxifen alone was observed before the onset of apoptosis. This is the first report to identify early induction of Akt activity by therapeutic agents as a possible new mechanism of therapeutic resistance and to show that exploitation of inducible Akt activity might increase therapeutic efficacy in breast cancer cells.

Materials and Methods

Materials. Cell culture reagents were purchased from Life Technologies, Inc. (Rockville, MD). Protease inhibitor mixture was obtained from Sigma Chemical Co. (St. Louis, MO). Human recombinant IGF-I was purchased from R&D Systems (Minneapolis, MN). LY294002 was from Alexis Biochemicals (San Diego, CA). Protran pure nitrocellulose membranes were purchased from Schleicher & Schuell (Keene, NH). Phospho-specific and total Akt antibodies, PARP antibodies, GSK3 α/β antibodies, and Akt kinase assay kits were purchased from Cell Signaling (Beverly, MA). HA probe clone Y-11 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). GFP antibody was obtained from Zymed Laboratories (South San Francisco, CA). Paclitaxel, etoposide, and doxorubicin were purchased from Calbiochem (La Jolla, CA). Trastuzumab (Herceptin) was from Genentech (San Francisco, CA). Tamoxifen was from Sigma. Plasmids encoding GFP, pEGFP-F, were obtained from Clontech (Palo Alto, CA). The dominant-negative Akt (Akt-CAAX; Ref. 44) and pSG5 constructs were generous gifts from Dr. B. M. T. Burgering (Leuven, Belgium). pSR α and myristolated (Myr) Akt plasmids were kind gifts from Dr. P. Tsichlis (Thomas Jefferson University Medical Center, Philadelphia, PA). The Cell Death Detection ELISA kits were purchased from Roche Diagnostics (Mannheim, Germany).

Cell Culture. Cell lines were obtained from Dr. S. Lipkowitz at the National Cancer Institute/Navy Medical Oncology and were maintained in RPMI 1640 supplemented with 10%

(v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin in an incubator calibrated to 37°C, 6% CO₂ in 75-cm² flasks. Stock flasks were split on a weekly basis at a 1:4, 1:10, or 1:20 ratio.

Pharmacological Treatments. To examine effects of serum starvation and IGF-I stimulation on Akt activation, cells were plated at 5×10^5 cells/well in 6-well dishes in 10% RPMI 1640 and incubated for 24 h. Medium was changed to RPMI 1640 supplemented with 10% FBS or 0.1% FBS overnight. Some samples were treated with IGF-I (10 nM) for 15 min before lysing. To examine the effect of LY294002 on Akt activity, cells were plated at 5×10^5 and incubated 24 h. Medium was changed to RPMI 1640 with 0.1% FBS overnight. LY294002 (25 μ M) was added 2 h before cell lysis for *in vitro* kinase assays. To study the effect of LY294002 on apoptosis, cells were plated at 1.25×10^5 in a 24-well dish and incubated 24 h. Medium was changed to RPMI 1640 supplemented with 0.1% FBS and incubated with or without LY294002 (25 μ M) for 48 h. For combination experiments with chemotherapy or tamoxifen, LY294002 was added simultaneously, and samples were incubated 48 h before quantification of apoptosis. To study the effect of doxorubicin or trastuzumab treatment on Akt phosphorylation, cells were plated at 5×10^5 per well in 6-well dishes and incubated overnight in LS. Cells were treated for 1, 6, or 24 h before lysing for immunoblotting. Immunoblotting experiments were repeated at least three times, and apoptosis experiments were done in triplicate and repeated at least three times.

Immunoblotting. Preparation of cell extracts, protein assays, and immunoblotting were performed as described previously (32). Equivalent loading was confirmed by staining membranes with fast green as described previously (45).

Akt Kinase Assays. Akt kinase assays were performed as recommended by the manufacturer, with some exceptions as described below. Cells were plated at 5×10^5 cells/well in 6-well plates and treated with LY294002 as described above. Cells were washed once with PBS and then incubated on ice for 10 min in 200 μ l cell lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride. Lysates were sonicated and cleared, and equal amounts of lysate protein were immunoprecipitated for 2 h at 4°C with anti-phospho-Akt antibody. Immunoprecipitates were washed twice with cell lysis buffer and twice with kinase buffer. Kinase reaction was performed for 30 min at 30°C in 40 μ l kinase buffer supplemented with 1 μ g of glutathione S-transferase-GSK fusion protein and 200 μ M ATP. Reactions were terminated upon addition of 3 \times SDS Sample Buffer. Samples were boiled 5 min and loaded into 12% SDS-polyacrylamide gels. Experiments were repeated at least three times.

Transient Transfections. Cells were plated at 3×10^5 per well in 6-well dishes (~70% confluency) and transfected in triplicate using the FuGENE 6 Transfection Reagent according to the manufacturer's protocol (Roche Diagnostics, Inc., Indianapolis, IN). For gating purposes in flow cytometry, one well/plate was transfected with GFP alone. For all other wells, plasmids encoding pSG5 or Akt-CAAX were cotransfected with a plasmid encoding GFP. Only cells expressing GFP were analyzed for cell cycle distribution via flow cytometry.

etry. Each experimental condition was analyzed in parallel for immunoblotting and flow cytometry. Experiments were repeated at least three times.

Stable Transfections. Cells were plated at 2×10^5 cells/well in a 6-well dish and transfected with 4 μg of DNA encoding pSR α or Myr-Akt with LIPOFECTamine Transfection Reagent according to the manufacturer's protocol (Life Technologies, Inc.). Cells were diluted and transferred to multiple 150-mm dishes and were selected in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 800 $\mu\text{g}/\text{ml}$ G418. Single colonies were selected using cloning rings and/or filter paper, expanded in medium containing 200 $\mu\text{g}/\text{ml}$ G418, and analyzed by immunoblotting as described above.

CellDeath ELISA Assay. Assays were performed as recommended by the manufacturer with exceptions as described below. Cells were plated at 1×10^4 per well in triplicate in 96-well plates and treated as described. Cells were lysed in 200- μl cell lysis buffer for 30 min at room temperature. Lysates were transferred to reaction wells and incubated with immunoreagent for 2 h. Wells were washed three times with incubation buffer, ABTS reagent was added, and the plates were read on a Titertek multiscan ELISA reader at multiple time intervals. Experiments were performed at least three times.

Flow Cytometry/Apoptosis Assays. Floating cells were collected, and adherent cells were harvested by trypsinization and then centrifuged at $1000 \times g$ for 5 min. Cells were fixed in ice-cold 70% methanol added dropwise and then incubated at -20°C for 30 min. Cells were centrifuged and incubated with propidium iodide (25 $\mu\text{g}/\text{ml}$) supplemented with RNase A (30 $\mu\text{g}/\text{ml}$) for 30 min at room temperature. Quantification of sub-2N DNA was determined by flow cytometry analysis using a Becton-Dickinson FACSsort and by manual gating using CellQuest software. Gating was performed on blinded triplicate samples.

Statistical Analysis. Statistical comparison of mean values was performed using the Student *t* test. All *P*s are two tailed. Interactions between LY294002 and chemotherapeutic agents were classified by the fractional inhibition method as follows: when expressed as the fractional inhibition of cell viability, additive inhibition produced by LY294002 and chemotherapy (*i*) occurs when $i_{1,2} = i_1 + i_2$; synergism when $i_{1,2} > i_1 + i_2$; and antagonism when $i_{1,2} < i_1 + i_2$ (46).

Results

Akt Activity Is Constitutive, PI3-K Dependent, and Promotes Survival of Breast Cancer Cells. To determine whether Akt is active in breast cancer cells, we analyzed a panel of six breast cancer cell lines that varied in erbB2 and ER status. Cells were grown under normal growth conditions (10% FBS) or deprived of serum overnight (0.1% FBS), and Akt activity was assessed by immunoblotting with phospho-specific antibodies against phosphorylated S473 and T308. Fig. 1A shows that all but MCF7 and MB231 cells exhibited phosphorylation of S473 under normal growth conditions. Patterns for T308 phosphorylation were similar to those observed with S473 phosphorylation, except for ZR75-1 cells, which displayed S473 phosphorylation but not T308 phos-

phorylation. Serum starvation decreased S473 phosphorylation only in SKBR3 cells, suggesting that the other cell lines did not depend on serum-derived growth factors for S473 phosphorylation. Of note, ZR75-1, SKBR3, and MB453 cells increased T308 phosphorylation in response to serum starvation. Whether this induction of Akt phosphorylation is part of a general cellular stress response is unclear, but selective increases in T308 phosphorylation have been observed in animal models of hypoglycemic coma (47). To determine whether IGF-I administration would increase basal levels of Akt phosphorylation, IGF-I was added under conditions of serum starvation. IGF-I had little effect in cells with high levels of endogenous Akt phosphorylation. In MCF7 and MB231 cells, phosphorylation of S473 and T308 was induced by IGF-I (T308 phosphorylation in MB231 cells was evident on longer exposures; data not shown), indicating that the IGF-IR signaling pathway that leads to Akt activation can be stimulated in cells with little basal Akt phosphorylation. Levels of total Akt did not change under any experimental condition. Our immunoblotting data therefore predicted that the four breast cancer cell lines with S473 and T308 phosphorylation would have constitutively active Akt.

Because Akt phosphorylation did not decrease with serum starvation and PTEN normally regulates Akt activity, we used immunoblotting to test whether Akt phosphorylation correlated with PTEN protein expression (Fig. 1B). Of the four cell lines with the highest levels of phosphorylated Akt, MB468 and ZR75-1 cells did not express PTEN protein, consistent with previous studies (38). The other two cell lines with high levels of constitutive Akt phosphorylation (SKBR3 and MB453) have wild-type PTEN but overexpress erbB2 (38), indicating that constitutive Akt phosphorylation is associated with PTEN mutations or erbB2 overexpression in these cell lines.

To demonstrate that phosphorylated Akt is enzymatically active and that phosphorylation of Akt depends on PI3K, we performed *in vitro* kinase assays in the presence and absence of a PI3K inhibitor, LY294002, using GSK3 α/β as a substrate. Fig. 1C shows that the four cell lines with the highest levels of Akt phosphorylation displayed the highest levels of Akt kinase activity. The greatest level of kinase activity was observed with the MB468 cells, which have the highest ratio of phosphorylated S473 and T308 to total Akt. Of note, breast cancer cells that showed little phosphorylated Akt (MCF7 and MB231) contained small amounts of active Akt that could be detected once Akt was immunoprecipitated. LY294002 dramatically decreased Akt kinase activity, regardless of endogenous Akt levels. In MB468 cells and MB453 cells where the inhibition appeared incomplete, relative inhibition of Akt activity by LY294002 was 89 and 73%, respectively (densitometry not shown). These results agree with most of the manuscripts in the Akt literature in that Akt kinase activity correlated with Akt phosphorylation, and that Akt activation was predominantly PI3K dependent.

To test whether activation of the PI3K/Akt pathway promotes breast cancer cell survival, we treated cells with LY294002 and assessed apoptosis. Morphological changes characteristic of apoptosis including membrane blebbing, increased refractoriness, chromatin condensation, and cell

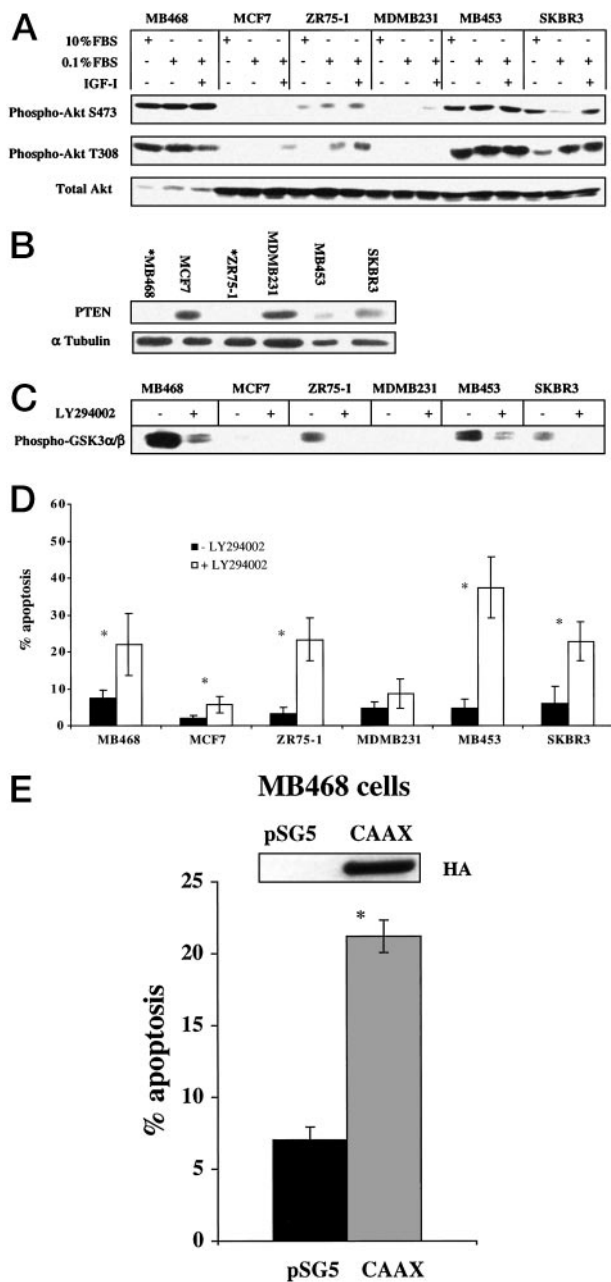


Fig. 1. Status of the Akt pathway and effect of PI3K inhibition in breast cancer cells. **A**, cells were plated at 5×10^5 per well and exposed to HS (10% FBS) or LS (0.1% FBS) overnight or LS + IGF-I (50 ng/ml) for 15 min. Immunoblotting was performed as described. The upper panel shows levels of S473 phosphorylation, middle panel levels of T308 phosphorylation, and lower panel levels of total Akt. **B**, levels of PTEN protein under normal growth conditions (HS). *, cell lines with mutant PTEN. α -Tubulin levels are shown to indicate comparable loading. **C**, cells were plated as above and exposed to LS or LS + LY294002 (25 μ M) for 2 h. Active Akt was immunoprecipitated with anti-phospho S473 antibodies, and *in vitro* kinase assays were performed as described using GSK3 α/β as a substrate. Levels of phosphorylated GSK3 α/β are shown. **D**, cells were plated at 1.25×10^5 per well and exposed to LS (■) or LS + 25 μ M LY294002 (□) for 48 h. Cells were harvested, and apoptosis was quantified via flow cytometry as described. Columns are the means from four independently performed experiments; bars, SE. *, two-tailed *P*s comparing LY294002 treatment to control were <0.05 . **E**, cells were plated at 3×10^5 per well in triplicate, and parallel samples were transiently cotransfected with plasmids encoding GFP and pSG5 (■) or GFP and Akt-CAAX (□) as described. Subsequently, cells were exposed to LS for 48 h. Whole cell

shrinkage (48) were induced by LY294002 and evident at 48 h (data not shown). Other evidence for LY294002-induced apoptosis included increased annexin V binding to cells as assessed by flow cytometry (data not shown). To measure apoptosis quantitatively, we assessed sub-2N DNA content by staining cellular DNA with propidium iodide, followed by analysis using flow cytometry. Fig. 1D shows that basal levels of apoptosis remained $<10\%$ for all cells that were serum starved for 48 h. LY294002 increased apoptosis the greatest in cell lines with the highest levels of Akt phosphorylation and Akt activity. The fact that apoptosis was greater in MB453 cells than in MB468 cells after LY294002 treatment (Fig. 1C). The small increases in apoptosis observed with MCF7 cells may reflect other effects of LY294002 or inhibition of the small amount of Akt activity detected in the kinase assays. The differences in apoptosis with MB231 cells were not statistically significant ($P > 0.05$). Together, these studies show that LY294002 increased breast cancer cell apoptosis in cells with active Akt and suggest that activation of the PI3K/Akt pathway promotes survival under conditions of serum starvation.

To demonstrate that the effects of LY294002 on breast cancer cell apoptosis were specific for inhibiting Akt activity, we transiently cotransfected MB468 cells with HA-tagged dominant-negative Akt (Akt-CAAX) and GFP or vector alone (pSG5) and GFP and assessed apoptosis in the GFP-positive cells. Fig. 1E shows that in MB468 cells with high Akt activity, transfection of Akt-CAAX increased apoptosis from 7 to 22%. Similar results were obtained with transient transfection of an ATP binding mutant of Akt, K179M (data not shown). Insets show the expression of HA-tagged Akt-CAAX (top inset). The induction of apoptosis by Akt-CAAX was similar to that observed with LY294002 in MB468 cells, suggesting that the proapoptotic effects of LY294002 were attributable to Akt inhibition. The fact that a small molecule inhibitor of the PI3K/Akt pathway or dominant-negative Akt induced breast cancer cell apoptosis demonstrates that breast cancer cells depend on Akt for survival and that if active, Akt is an exploitable therapeutic target.

Effects of LY294002 on Chemotherapy- or Trastuzumab-induced Apoptosis in Breast Cancer Cells. To determine whether activation of the PI3K/Akt pathway affects the sensitivity of breast cancer cells to chemotherapy, we added LY294002 and individual therapeutic agents to three cell lines that had low erbB2 levels but varied in basal Akt activity and measured apoptosis. Fig. 2A shows that in MB468 cells, LY294002 increased basal apoptosis from 6 to 19%. When combined with paclitaxel, the effects of LY294002 were additive. Greater than additive effects with LY294002 were apparent with etoposide, trastuzumab, or doxorubicin in MB468 cells. In ZR75-1 cells, LY294002 in-

lyates from ungated samples were harvested for immunoblotting to assess HA levels (inset). Samples were also fixed, stained with propidium iodide, and prepared for flow cytometry. Apoptosis was quantified by gating on GFP-positive cells and performing cell cycle analysis. Columns are the means from three independently performed experiments; bars, SE. *, two-tailed *P* comparing apoptosis with CAAX to vector alone was <0.05 .

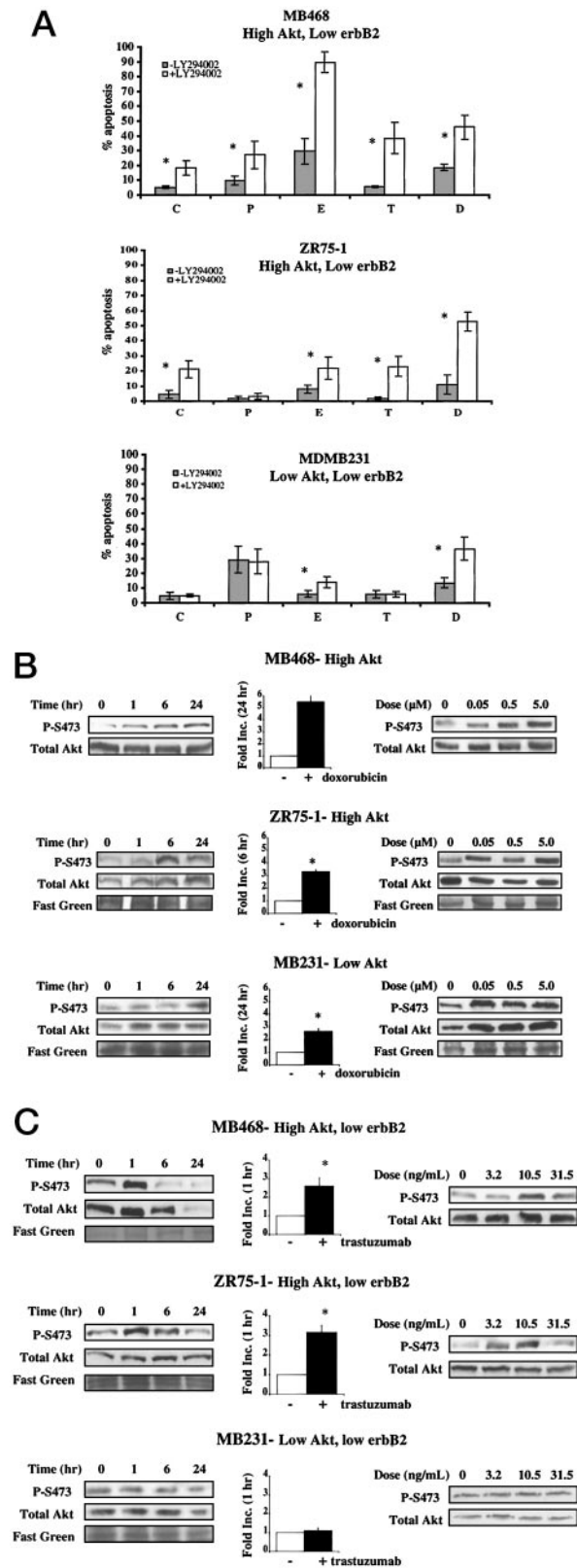


Fig. 2. LY294002 potentiates chemotherapy-induced apoptosis and correlates with early induction of Akt activity by chemotherapeutic agents. **A**, cells were plated at 1.25×10^5 /well and placed in 0.1% FBS. LY294002 and chemotherapeutic agents were added simultaneously, and cells were

increased basal apoptosis from 5 to 20%. Paclitaxel or etoposide antagonized the effects of LY294002. Trastuzumab added singly decreased basal apoptosis ($P < 0.05$) and caused a G_0 - G_1 arrest (data not shown), but in combination with LY294002, greater than additive effects on apoptosis were observed. Greater than additive effects were also evident when LY294002 and doxorubicin were combined. In MB231 cell lines (with low levels of Akt activity), there was no effect of LY294002 on basal apoptosis or paclitaxel- or trastuzumab-induced apoptosis, but greater than additive effects were observed when LY294002 was combined with either etoposide or doxorubicin. Across the three cell lines tested, the most effective combinations with LY294002 were with doxorubicin, trastuzumab, or etoposide.

Many questions regarding potentiation of apoptosis by LY294002 arose from these experiments. How could combinations of LY294002 and chemotherapy have greater than additive effects on apoptosis? How could LY294002 potentiate doxorubicin-induced apoptosis in cells with low endogenous levels of Akt activity? Likewise, how could LY294002 potentiate trastuzumab-induced apoptosis in cells with low levels of erbB2 that do not respond to trastuzumab when used singly? Although potentiation by LY294002 might not be related to inhibition of the PI3K/Akt pathway, data from Fig. 1 suggested that the biological effects of LY294002 in breast cancer cells were related to inhibition of the PI3K/Akt pathway. A mechanism was proposed. Because LY294002 increased apoptosis greatest in cells with high Akt activity, we hypothesized that the greater than additive effects of combining LY294002 with otherwise less effective agents might be related to increased Akt activity caused by the agents themselves. Precedent for this hypothesis relies partially on earlier data that showed that different forms of cellular stress could increase Akt activity (49–52). If breast cancer cells were to increase Akt activity in response to therapeutic agents, they might be more susceptible to inhibition of the PI3K/Akt pathway by LY294002, thereby result-

incubated for 48 h. Apoptosis was measured using flow cytometry as described. *Lanes C*, control; *Lanes P*, paclitaxel ($5 \mu\text{M}$), *Lanes E*, etoposide ($100 \mu\text{M}$ for all but MB468 cells; $1 \mu\text{M}$ for MB468 cells); *Lanes T*, trastuzumab (10.5 ng/ml); *Lanes D*, doxorubicin ($5 \mu\text{M}$). \square , control; \square , LY294002 ($25 \mu\text{M}$). *Columns* are the means from four independently performed experiments for each cell line; *bars*, SE. *, two-tailed *P*s comparing LY294002 + chemotherapy to chemotherapy alone were <0.05 . **B**, *left panels*, time-dependent induction of Akt phosphorylation. Cells were plated at 5×10^5 per well and were treated with doxorubicin ($5 \mu\text{M}$) in LS for 0, 1, 6, or 24 h. Samples were harvested for immunoblotting as described. Representative experiments showing levels of phosphorylated S473 and total Akt are shown. Where levels of total Akt differed significantly, regions of fast green-stained membranes that encompassed M_r 60,000 (molecular weight of Akt) are shown to demonstrate comparable loading. *Middle panels*, quantification of band intensity at the time point of maximal induction of Akt phosphorylation with $5 \mu\text{M}$ doxorubicin for each cell line was performed using NIH Image software. *Columns* are the means from three independently performed experiments; *bars*, SE. *, $P < 0.05$. *Right panels*, dose-dependent induction of Akt phosphorylation. Cells were incubated in LS with the doses of doxorubicin shown for the period of time that yielded maximal induction of Akt phosphorylation for each cell line (24 h for MB468 and MB231 cells; 6 h for ZR75-1). Representative immunoblots showing levels of phospho-S473 and total Akt are shown. *C*, cells were treated as in **B** but with trastuzumab (10.5 ng/ml) for the time course (*left panels*). Dose responses were assessed at 1 h for all cell lines (*right panels*).

ing in increased levels of apoptosis that could not have been predicted by measuring the effects of each agent alone.

Early Induction of Akt Phosphorylation by Doxorubicin or Trastuzumab. To test the hypothesis that cancer therapies themselves could modulate Akt, doxorubicin was added to MB468, ZR75-1, or MB231 cells, and immunoblotting for S473 phosphorylation and total Akt levels was performed at time points when apoptosis could not be detected. Fig. 2B shows that in the three cell lines that had greater than additive apoptotic responses to combinations of LY294002 and doxorubicin, doxorubicin (5 μM) increased S473 phosphorylation over the course of 24 h. In MB468 cells, phosphorylated Akt was increased 5.4-fold at 24 h, with no change in total Akt levels (*left and middle panels*). Increased Akt phosphorylation was observed with doses as low as 50 nM (*right panels*). In ZR75-1 cells, phosphorylated Akt was increased 3.3-fold at 6 h., and total Akt levels increased over 24 h (*left and middle panels*). Increased Akt phosphorylation was observed at a dose of 50 nM (*right panels*). In MB231 cells, total Akt levels rose before increased S473 phosphorylation, which was increased 2.4-fold at 24 h (*left and middle panels*). Similar to MB468 and ZR75-1 cells, increased Akt phosphorylation was observed in MB231 cells with doses as low as 50 nM (*right panels*). Whether the delay in S473 phosphorylation in MB231 cells is related to the fact they are the only cell line of this group with wild-type PTEN is unclear, but the slow time course of increased Akt phosphorylation in MB231 cells suggests that the mechanism is not likely to involve direct stimulation of growth factor receptor signaling pathways. Moreover, the effects of doxorubicin on total Akt levels suggest that doxorubicin might increase Akt synthesis and/or stability. Together, these studies support our hypothesis in that cells that had a greater than additive apoptotic response to the combination of doxorubicin and LY294002, doxorubicin increases Akt activity before the detection of apoptosis.

Fig. 2C shows the results of similar experiments performed with trastuzumab. When trastuzumab (10.5 ng/ml) was added to MB468 or ZR75-1 cells (that did not die in response to trastuzumab alone but died in response to trastuzumab combined with LY294002), increased S473 phosphorylation was observed, but the responses were biphasic. In MB468 cells, maximal induction of Akt phosphorylation (2.6-fold) was observed at 1 h, but at 6 h, levels of S473 phosphorylation dropped below baseline (*left and middle panels*). Total Akt levels also dropped below baseline by 24 h, but no apoptosis and no lower molecular weight bands suggestive of Akt degradation (53) were detected at this time (data not shown). A biphasic phosphorylation response in terms of dose of trastuzumab was also observed (*right panels*), in that less Akt phosphorylation was observed with administration of 3.2 or 31.5 ng/ml than with 10.5 ng/ml. In ZR75-1 cells, maximal induction of Akt phosphorylation was 3.2-fold at 1 h (*left and middle panels*), but levels fell below baseline by 24 h. Levels of total Akt increased by 6 h and remained elevated at 24 h. With ZR75-1 cells, increased Akt phosphorylation was observed with doses as low as 3.2 ng/ml and reached a maximum at 10.5 ng/ml but was decreased at 31.5 ng/ml (*right panels*). The faster induction and biphasic nature of Akt phosphorylation by trastuzumab compared with doxorubicin

in MB468 and ZR75-1 cells suggest that these agents may use different mechanisms to activate Akt. In MB231 cells that did not undergo apoptosis in response to combining LY294002 and trastuzumab, no increase in Akt phosphorylation was observed in response to trastuzumab, and levels of both phosphorylated and total Akt dropped below baseline by 24 h (*left and middle panels*). Doses as high as 31.5 ng/ml were ineffective in increasing Akt phosphorylation (*right panels*). These results are consistent with those obtained with doxorubicin, in that a correlation was established between induction of Akt by trastuzumab and greater than additive effects on apoptosis with combinations of LY294002 and trastuzumab. In contrast to doxorubicin-induced Akt phosphorylation, however, which seemed to occur independently of endogenous Akt activity, trastuzumab only increased Akt phosphorylation in cells with high endogenous levels of Akt activity.

LY294002 Increases Tamoxifen-induced Apoptosis in Cells That increase Akt Phosphorylation in Response to Tamoxifen. To extend our findings with LY294002 to other therapeutic agents used in breast cancer treatment, we tested whether LY294002 would increase tamoxifen-induced apoptosis. In these experiments, we quantitatively measured apoptosis with two separate assays, the flow cytometry-based assay used above and an ELISA-based assay that measures histone release that we have found to be more sensitive than flow cytometry-based assays (data not shown). Fig. 3A depicts fold increases in apoptosis of ZR75-1 cells, as measured by histone release from apoptotic cells. When tamoxifen was added to ER+ ZR75-1 cells, there was no increase in apoptosis at 40 nM, but there was a 2-fold induction of apoptosis at 80 nM. When LY294002 was added, basal apoptosis increased 4-fold, which is consistent with the high endogenous levels of Akt activity. When combined with tamoxifen at 40 nM, LY294002 increased apoptosis 10-fold. When combined with tamoxifen at 80 nM, apoptosis increased 15-fold. Similar quantification of apoptosis was observed when sub-2N DNA was measured using flow cytometry (data not shown). To determine whether induction of S473 phosphorylation could be correlated with the greater than additive effects of combining tamoxifen and LY294002, tamoxifen (80 nM) alone was added, and levels of total and phospho-S473 were determined at various time points by immunoblotting (Fig. 3A, *left panels, inset*). Similar to the increases in Akt phosphorylation observed with doxorubicin or trastuzumab in ZR75-1 cells, tamoxifen (80 nM) increased Akt phosphorylation 4.8-fold at 6 h, and phosphorylation remained elevated at 24 h (*left and middle panels, inset*). Dose responses of Akt phosphorylation to tamoxifen were biphasic (*right panels, inset*). Although increased Akt phosphorylation was observed at 40 nM, maximal induction occurred at 80 nM (*right panels*). At higher doses of tamoxifen (160 nM), phosphorylation of Akt decreased below baseline, consistent with other reports showing that blocking ER can reduce activity of the PI3K/Akt pathway.

In the ER+ MCF7 cells that have low levels of Akt activity (Fig. 3B), tamoxifen alone at 40 nM had little effect on apoptosis but increased apoptosis 2-fold at 80 nM. LY294002 alone slightly increased basal apoptosis ($P < 0.05$), consist-

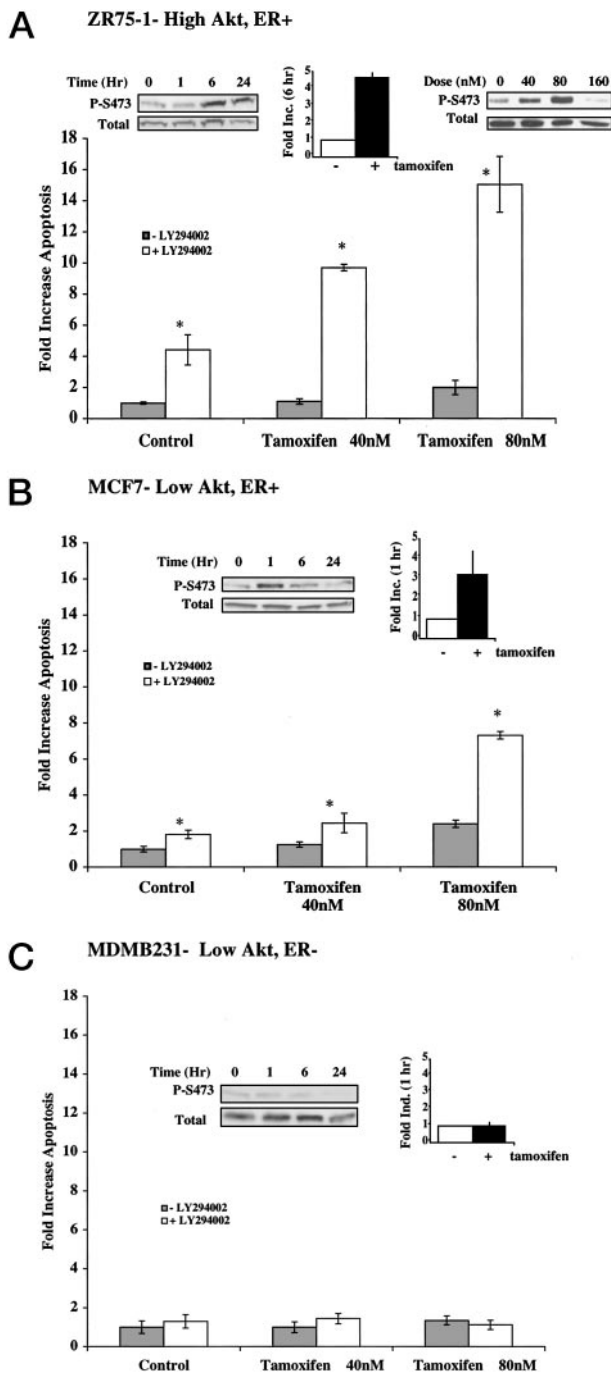


Fig. 3. LY294002 potentiates tamoxifen-induced apoptosis in ER+ cells and correlates with early induction of Akt activity by tamoxifen. Cells (A, ZR75-1; B, MCF7; C, MB231) were plated at 1×10^4 /well and exposed to LS alone (Control) or 40 or 80 nM tamoxifen with (□) or without (■) LY294002 (25 μ M). Cell Death ELISAs were performed at 48 h as described. Ratios of apoptosis were established using untreated cell extracts as having a value of 1. Columns are the means from three experiments; bars, SE. Representative immunoblots from three experiments for phospho-S473 and total Akt in cells treated with 80 nM tamoxifen in LS for 0, 1, 6, or 24 h (or ZR75-1 cells treated with various doses of tamoxifen for 6 h (A, right panels, inset) are shown in left insets. Middle insets, quantification of band intensity at the time point of maximal induction of Akt phosphorylation with 80 nM tamoxifen for each cell line was performed using NIH Image software. Bars are the means from three independently performed experiments; bars, SE. *, $P < 0.05$.

ent with low endogenous Akt levels in these cells. When combined, tamoxifen (40 nM) and LY294002 increased apoptosis 2.4-fold. Importantly, when combined with tamoxifen at 80 nM, LY294002 increased apoptosis >7-fold. When Akt phosphorylation was measured before the onset of apoptosis (left insets), tamoxifen alone increased S473 phosphorylation 3.2-fold at 1 h (middle inset) without changing total Akt levels. At 24 h, levels of S473 phosphorylation had dropped to baseline.

In the ER- MB231 cells with low levels of Akt activity, neither tamoxifen nor LY294002 alone, nor the combination, had any effect on apoptosis (Fig. 3C). Correspondingly, tamoxifen did not increase phosphorylation of S473 (inset). In fact, tamoxifen decreased S473 phosphorylation at 24 h, which is similar to the decreased S473 phosphorylation observed with trastuzumab in these cells (trastuzumab also did not induce apoptosis in MB231 cells). The mechanism responsible for decreased S473 phosphorylation by these agents in MB231 cells is unknown. Taken together, these studies show that inhibition of the PI3K/Akt pathway sensitizes ER+ breast cancer cells to tamoxifen, and that potentiation of tamoxifen-induced apoptosis by LY294002 is associated with an induction of Akt activity by tamoxifen that precedes apoptosis.

Modulation of Akt Activity with Transfection of Akt Mutants Alters Sensitivity of Breast Cancer Cells to Chemotherapy. To demonstrate that the effects of LY294002 on chemotherapy-induced apoptosis were specific for inhibiting Akt activity, we transiently cotransfected cells with HA-tagged dominant-negative Akt (Akt-CAAX) and GFP or vector alone (pSG5) and GFP, treated with doxorubicin, and assessed apoptosis in the GFP-positive cells using flow cytometry. (The CellDeath ELISA was not used in these experiments because of the need to gate on transfected cells.) Fig. 4A shows that in MB468 cells with high Akt activity, transfection of Akt-CAAX increased basal apoptosis from 6 to 17%, as assessed by formation of sub-2N DNA. The observed increase in basal apoptosis correlated well with increased PARP cleavage (lower left insets). (Upper insets show the expression of HA-tagged Akt-CAAX.) When doxorubicin was added to cells transfected with vector alone, apoptosis increased from 6 to 11%. When added to cells transfected with the Akt-CAAX mutant, doxorubicin increased apoptosis from 17 to 24%. Differences in PARP cleavage in doxorubicin-treated MB468 cells transfected with pSG5 or Akt-CAAX were less apparent (lower right insets), because doxorubicin alone increased PARP cleavage. Doxorubicin also causes rapid loss of GFP in transfected cells,³ which may explain why a greater than additive response was seen with cells treated with LY294002 and doxorubicin (Fig. 2) but was not as apparent with cells transiently transfected with mutant Akt and treated with doxorubicin. Cells that die from CAAX but lose GFP would not be counted in the apoptotic fraction. Nonetheless, these studies indicate that dominant-negative Akt potentiates doxorubicin-induced apoptosis.

³ A. Clark, unpublished observation.

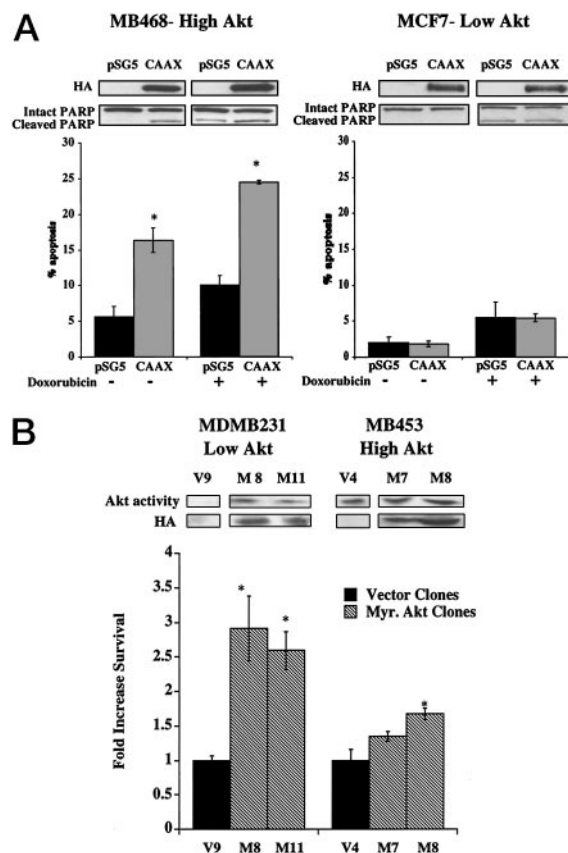


Fig. 4. Modulating Akt activity with dominant-negative or constitutively active Akt mutants alters the sensitivity of breast cancer cells to chemotherapy. **A**, cells were plated at 3×10^5 per well, and parallel samples in triplicate were transiently cotransfected with plasmids encoding GFP and pSG5 (■) or GFP and Akt-CAAX (□) or GFP alone (not shown) as described. Cells were allowed to recover from transfection for 24 h and then exposed to LS \pm doxorubicin ($5 \mu\text{M}$) for 48 h. Whole cell lysates from ungated samples were harvested for immunoblotting to assess HA levels (*top insets*) and cleavage of PARP (*lower insets*). Samples were also fixed, stained with propidium iodide, and prepared for flow cytometry. Apoptosis was quantified by gating on GFP-positive cells and performing cell cycle analysis. Levels of apoptosis were equivalent in cells transfected with GFP alone or with GFP and pSG5 (data not shown). **Columns** are the means from triplicate samples; **bars**, SD. A representative experiment from three independent experiments is shown for each cell line. **B**, cells were stably transfected, and individual clones were expanded under antibiotic selection as described. Kinase assays were performed and are shown in *top panels*. Immunoblotting was performed to assess HA expression (*middle panels*). To measure apoptosis, clones were plated in triplicate at 1.25×10^5 /well, exposed to 0.1% FBS, and treated with or without doxorubicin (500 nM) for 48 h, and CellDeath ELISAs were performed as described. Increased viability in the Myr Akt clones is expressed in relation to the vectors clones, where viability after doxorubicin treatment was set to 1. **Columns** are means from triplicate samples; **bars**, SD. A representative experiment from three independent experiments is shown for each cell line. *, $P < 0.05$.

In contrast to the potentiation of apoptosis by Akt-CAAX in cells with high Akt activity, transfecting MCF7 cells with low levels of Akt activity with Akt-CAAX had little effect on basal levels of apoptosis or doxorubicin-induced apoptosis, as assessed by flow cytometry or PARP cleavage (*lower insets*), even although expression of HA-tagged Akt-CAAX was easily detected (*upper insets*). Together, these studies confirm and extend the data generated with LY294002 by demon-

strating that inhibition of Akt with either LY294002 or Akt-CAAX selectively increases basal- and chemotherapy-induced apoptosis in cells with high levels of endogenous Akt.

We then performed the converse experiments; we stably transfected MB231 cells (low Akt levels) or MB453 cells (high Akt levels) with constitutively active Akt or empty vector to determine whether increasing Akt activity would attenuate chemotherapy-induced apoptosis. Fig. 4B shows that transfection of MB231 cells with HA-tagged Myr Akt resulted in expression of the HA epitope tag (*bottom insets*) and increased Akt activity (*top insets*) in the two Myr-Akt clones shown. When doxorubicin was added to the representative vector clone and two Myr-Akt clones, viability in the two Myr-Akt clones was greater by 2.9- or 2.6-fold, respectively, compared with the vector clone. These results show that increasing Akt activity in breast cancer cells with low levels of endogenous Akt attenuates chemotherapy-induced apoptosis. When the same set of stable transfections was performed with MB453 cells that contain high levels of Akt activity, there was no additional increase in Akt activity (*top inset*), despite expression of the HA epitope. Viability after doxorubicin exposure was slightly greater for the two clones expressing Myr-Akt compared with the vector clone (1.3-fold for M7 and 1.7-fold for M8), but the potentiation of survival was less than that observed in MB231 cells that have low endogenous levels of Akt. These studies with active Myr-Akt complement the studies of dominant-negative Akt-CAAX in cells with high Akt activity by showing that increasing Akt activity in breast cancer cells that exhibit little endogenous activity resulted in greater cell survival when cells were exposed to chemotherapy. The fact that two opposing genetic approaches to modulating Akt activity yielded opposite, predictable responses in terms of sensitivity to doxorubicin supports earlier results with LY294002 and emphasizes the importance of the Akt pathway in breast cancer cell survival and chemotherapeutic resistance.

Discussion

These studies are the first to show that Akt is a survival factor for breast cancer cells under conditions of serum starvation or with administration of chemotherapy, trastuzumab, or tamoxifen. Using phospho-specific antibodies and *in vitro* kinase assays, we showed that Akt is constitutively active in four of six breast cancer cell lines, and that Akt activity was associated with mutant PTEN status or erbB2 overexpression. Although other mechanisms such as autocrine growth factor loops may contribute to activation of Akt in breast cancer cells, >30% of breast cancers exhibit PTEN mutations or erbB2 overexpression, and Akt may therefore be constitutively active in tumors bearing these molecular alterations. The fact that inhibition of the PI3K/Akt pathway with LY294002 or dominant-negative mutant Akt increased apoptosis suggests that measuring Akt activity *in vivo* would identify patients most likely to benefit from approaches that target this pathway.

In addition to showing that Akt promotes breast cancer cell survival under conditions of serum deprivation, we used the small molecule inhibitor LY294002 as well as transfection of

Akt mutants to demonstrate that Akt promotes resistance to therapies commonly used in the treatment of breast cancer patients. To our knowledge, these studies are the first to demonstrate that a single small molecule kinase inhibitor can modulate sensitivity to such diverse therapies as traditional cytotoxic chemotherapy, immune therapy, and endocrine-based therapy. With cell line specificity, LY294002 potentiated apoptosis caused by doxorubicin, trastuzumab, etoposide, or tamoxifen. These results are similar to our earlier studies that showed that combining LY294002 with trastuzumab or etoposide had greater than additive effects on apoptosis in lung cancer cells (32). Thus, approaches that inhibit the PI3K/Akt pathway may have utility when combined with traditional forms of chemotherapy. Previously, LY294002 had been shown to potentiate gemcitabine-induced (54) and farnesyltransferase inhibitor-induced apoptosis (55) in a small number of cell lines, but neither study addressed Akt phosphorylation. In the only report to assess direct effects of chemotherapy on Akt phosphorylation, Nakashio *et al.* (56) showed that the topoisomerase I inhibitor, topotecan, decreased Akt phosphorylation after 48 h of exposure, but measurement of Akt phosphorylation at earlier time points was not performed. Therefore, ours is the first report to show that the potentiation of apoptosis caused by combining LY294002 with different therapies is associated with an early induction of Akt phosphorylation by these therapies that precedes apoptosis.

Induction of Akt activity was observed with doxorubicin, trastuzumab, or tamoxifen. Doxorubicin increased Akt phosphorylation slowly (over 24 h) and did so independently of endogenous Akt activity. What biological properties of doxorubicin might contribute to Akt induction? Anthracyclines have pleiotropic actions within cells, including inhibition of topoisomerase II, generation of ROS, and induction of DNA damage. Interestingly, greater than additive effects of combining LY294002 with the topoisomerase II inhibitor, etoposide, were also observed in our studies, but a mechanistic connection between Akt activity and topoisomerase II function has not been established. Doxorubicin-induced ROS may also contribute to Akt activation, because oxidative stress caused by administration of hydrogen peroxide can activate Akt in fibroblasts and smooth muscle cells (49–52), but activation of Akt in these studies occurred within minutes, was transient, and was observed only in untransformed cells. Likewise, doxorubicin-induced DNA damage may also contribute to Akt activation, because UV B irradiation has been shown to transiently increase Akt activity before the onset of DNA damage (57, 58). Current studies are identifying mechanisms of Akt activation by doxorubicin.

Patterns of Akt activation by trastuzumab differed from those with doxorubicin. In cells with high levels of Akt activity and mutant PTEN (MB468, ZR75-1), trastuzumab induced biphasic responses, with early potentiation of Akt phosphorylation followed by attenuation hours later. In cells with low levels of Akt activity and wild-type PTEN (MB231), no increases in phosphorylated Akt were detected. These studies suggest that early activation of Akt by trastuzumab depends on high endogenous levels of Akt activity that might be

related to mutant PTEN status. Moreover, they are consistent with observed biphasic agonistic and antagonistic properties of antibodies such as trastuzumab targeted against erbB2 (59). Early agonistic effects of trastuzumab on erbB2 in cells with mutant PTEN could explain increased Akt phosphorylation. Subsequent antagonist effects on erbB2 could result in decreased Akt phosphorylation, which we have observed previously 48–72 h after trastuzumab administration (60). An interesting potential clinical application of these observations is that trastuzumab, when combined with approaches that target the PI3K/Akt pathway, might have clinical utility in patients whose tumors have low erbB2 levels yet have high levels of Akt activity or bear PTEN mutations (*e.g.*, Cowden's syndrome patients).

Tamoxifen also increased Akt activity, but only in cells that were ER+. Greater than additive effects on apoptosis were observed with tamoxifen and LY294002 in ZR75-1 cells, and this correlated with a 4.8-fold increase in Akt phosphorylation by tamoxifen at 6 h. In ER+ cells with lower levels of endogenous Akt activity because of wild-type PTEN (MCF-7), activation of Akt was nonetheless still observed, suggesting that tamoxifen might act as an agonist on ER to stimulate Akt and that tamoxifen-induced Akt activity is not dependent on mutant PTEN status. This hypothesis is supported by the fact that the ER– MB231 cells did not induce Akt phosphorylation in response to tamoxifen and is consistent with other studies that have shown that stimulation of ER α can stimulate the PI3K/Akt pathway (61, 62).

Our results are the first to demonstrate early activation of Akt by different therapies used in breast cancer treatment. In that vein, Akt may be considered as a molecular “crutch” that breast cancer cells rely on early to escape cell death once they are exposed to toxic stimuli. In addition to being activated by growth stimuli or ROS, Akt can be activated by other cellular stresses such as hypoglycemia (47), heat shock (63, 64), and hypoxia (65, 66). Cells may activate Akt by different mechanisms with different kinetics, depending on whether the stimulus is initially perceived as a growth stimulus or cellular insult. In that regard, the patterns of Akt induction in our studies suggest that doxorubicin might have been sensed as a toxic insult, whereas trastuzumab and tamoxifen may have been initially perceived as growth stimuli. Other mechanisms may also have contributed to altering Akt levels in breast cancer cells. For example, the observed changes in total Akt protein levels induced by these therapeutic agents suggest that Akt synthesis and/or Akt turnover may also be affected. Taken together, our data emphasize the potential importance of Akt as a therapeutic target in breast cancer. The constitutive activation of Akt in breast cancer cells suggests that exploiting tumor cell activation of Akt may directly decrease tumor cell survival. Moreover, inhibiting the induction of Akt caused by traditional forms of therapy may eliminate a “crutch” that tumor cells “lean on” to escape cell death, thereby directly influencing the decision to undergo apoptosis and increasing therapeutic effectiveness of standard agents.

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