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Apigenin Induces Apoptosis through Proteasomal Degradation of HER2/*neu* in HER2/*neu*-overexpressing Breast Cancer Cells via the Phosphatidylinositol 3-Kinase/Akt-dependent Pathway*

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Apigenin is a low toxicity and non-mutagenic phytopolyphenol and protein kinase inhibitor. It exhibits anti-proliferating effects on human breast cancer cells. Here we examined several human breast cancer cell lines having different levels of HER2/*neu* expression and found that apigenin exhibited potent growth-inhibitory activity in HER2/*neu*-overexpressing breast cancer cells but was much less effective for those cells expressing basal levels of HER2/*neu*. Induction of apoptosis was also observed in HER2/*neu*-overexpressing breast cancer cells in a dose- and time-dependent manner. However, the one or more molecular mechanisms of apigenin-induced apoptosis in HER2/*neu*-overexpressing breast cancer cells remained to be elucidated. A cell survival pathway involving phosphatidylinositol 3-kinase (PI3K), and Akt is known to play an important role in inhibiting apoptosis in response to HER2/*neu*-overexpressing breast cancer cells, which prompted us to investigate whether this pathway plays a role in apigenin-induced apoptosis in HER2/*neu*-overexpressing breast cancer cells. Our results showed that apigenin inhibits Akt function in tumor cells in a complex manner. First, apigenin directly inhibited the PI3K activity while indirectly inhibiting the Akt kinase activity. Second, inhibition of HER2/*neu* autophosphorylation and transphosphorylation resulting from depleting HER2/*neu* protein *in vivo* was also observed. In addition, apigenin inhibited Akt kinase activity by preventing the docking of PI3K to HER2/HER3 heterodimers. Therefore, we proposed that apigenin-induced cellular effects result from loss of HER2/*neu* and HER3 expression with subsequent inactivation of PI3K and AKT in cells that are dependent on this pathway for cell proliferation and inhibition of apoptosis. This implies that the inhibition of the HER2/HER3 heterodimer function provided an especially effective strategy for blocking the HER2/*neu*-mediated transformation of breast cancer cells. Our results also demonstrated that apigenin dissociated the complex of HER2/*neu* and GRP94 that preceded the depletion of HER2/*neu*. Apigenin-induced degradation of mature HER2/*neu* involves polyubiquitination of HER2/*neu* and subsequent hydrolysis by the proteasome.

Several cancers, including breast cancer, have a lower incidence in Asia than in Western countries (1). This may be attributed to the Asian dietary regimen rich in flavonoid-containing plants, which are thought to be anti-tumorigenic. Among the plant flavonoids, apigenin (4′,5,7,-trihydroxyflavone) is a chemopreventive compound (2, 3) and an inhibitor of certain signal transduction pathways (4). It has low toxicity, is non-mutagenic, and is widely distributed in many fruits and vegetables, including parsley, onions, oranges, tea, chamomile, wheat sprouts, and in some seasonings (5, 6). Apigenin has potential uses in cancer prevention and therapy (7), and it possesses growth inhibitory properties against many human cancer cell lines, including breast (8), colon (9), skin (10), thyroid (11), leukemia (12), and prostate carcinomas cells (13). Apigenin has been demonstrated to be a protein kinase inhibitor, and it achieves this inhibitory effect by competing with ATP (14). Our previous report (15) showed that apigenin inhibited TPA[1]-induced c-*jun* and c-*fos* expression and TPA-mediated tumor promotion of mouse skin.

Breast cancer is the most common cancer among women in the Western world and the second leading cause of cancer-related deaths in women (16). Gene amplification and/or overexpression of some oncogenes have been implicated in breast cancers. HER2/*neu* (also known as ErbB2) is among the most characterized oncogenes linked with poor prognosis in breast cancer (17). Overexpression of HER2/*neu* is found in ~30% of human breast cancers and correlates with more aggressive tumors and more resistance to cancer chemotherapy (18). An increase in HER2/*neu* expression also enhances malignant phenotypes of cancer cells, including those with metastatic potential (19–21). The association of HER2/*neu* overexpression in cancer cells with chemoresistance and metastasis provides a plausible interpretation for the poor clinical outcome of patients with HER2/*neu*-overexpressing cancers; it suggests that the enhanced tyrosine kinase activity of HER2/*neu* might play a critical role in the initiation, progression, and outcome of human tumors.

HER2/*neu* is a member of the class II receptor (ErbB) tyrosine kinase family, which in human includes the HER1 (epidermal growth factor receptor, ErbB1), HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). ErbB receptors are essential mediators of cell proliferation and differentiation. Their aberrant

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[1] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; GSK-3, glycogen synthase kinase-3; PDK, 3-phosphoinositide-dependent protein kinase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LLnL, *N*-acetyl-Leu-Leu-norleu-al; CQ, chloroquine; Hsp90, heat shock protein 90.

activation is associated with the development and severity of many cancers. Homo- and hetero-dimerization of ErbB receptors result in a wide variety of cellular signal transduction. Dimerization of HER2/*neu* and HER3 occurs frequently and is a preferred heterodimer (22). The HER2/HER3 dimer constitutes a high affinity co-receptor for heregullin, which is capable of potent mitogenic signaling (23). HER3 is a kinase-defective protein that is phosphorylated by HER2/*neu*. Tyrosine-phosphorylated HER3 is able to directly couple to PI3K (phosphatidylinositol 3-kinase), a lipid kinase involved in the proliferation, survival, adhesion, and motility of tumor cells (24–27).

Activation of PI3K and the generation of phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate *in vivo* are necessary for the activation of Akt/PKB, a downstream mediator of PI3K signaling, through phosphorylation of Thr-308 and Ser-473 by PDK1 and PDK2/integrin-linked kinase (28). In numerous cell types, it has been shown that Akt/PKB induces survival and suppresses apoptosis induced by a variety of stimuli, including growth factor withdrawal and loss of cell adhesion. The mechanisms by which Akt/PKB regulates cell survival involve the phosphorylation and inactivation of the apoptotic mediators BAD (29), caspase-9 (30), FKHRL1 (31), and IKK-α (32, 33). Akt/PKB is also involved in regulating cell proliferation (34, 35).

Studies performed in animal model have shown that down-regulating HER2/*neu* by repressing the HER2/*neu* promoter (36–38) or by using anti-HER2/*neu* antibodies (39–42) can suppress tumor growth and dissemination. One therapeutic approach that has already reached clinical application is the use of an unarmed monoclonal antibody called Trastuzumab (Herceptin^{TM}) (43). Studies have attributed the therapeutic potential of anti-HER2/*neu* antibodies to their ability to enhance intracellular degradation of the cell surface-localized oncoprotein (44). These findings suggest that manipulating HER2/*neu* may be of substantial value in the treatment of breast cancer.

Apigenin has been shown to efficiently inhibit the growth of MCF-7 and MDA-MB-468 breast carcinoma cell lines, and its growth inhibitory effects are mediated by targeting different signal transduction pathways (8). However, MCF-7 and MDA-MB-468 express only basal levels of HER2/*neu*. Here, we investigated the effectiveness of apigenin against a series of breast cancer cells having different levels of HER2/*neu* expression. We showed that apigenin efficiently inhibited the growth of MDA-MB-453 HER2/*neu*-overexpressing breast cancer cells. Induction of apoptosis was also observed in these HER2/*neu*-overexpressing breast cancer cells. In addition, to elucidate the molecular mechanism of apigenin-induced apoptosis in HER2/*neu*-overexpressing breast cancer cells, the apoptotic machinery and the expression of several cell survival genes were investigated. We demonstrated that HER2/*neu* was degraded in MDA-MB-453 HER2/*neu*-overexpressing breast cancer cells by proteasomal degradation, and that the inhibition of cell growth and induction of apoptosis by apigenin may be through suppression of HER2/HER3 signaling and disruption of the PI3K/Akt-dependent pathway.

EXPERIMENTAL PROCEDURES

Cell Culture—The human breast cancer cell lines used in this study were MDA-MB-453, BT-474, and SKBr-3, all of which overexpress HER2/*neu*, and MCF-7, which expresses the basal level of HER2/*neu*. We also used HBL-100 cell line, which is derived from a normal human breast tissue transformed by SV40 large T antigen and expresses a basal level of HER2/*neu*. All of the cells were grown in DMEM/F-12 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and gentamicin (50 mg/ml). Cells were grown in a humidified incubator at 37 °C under 5% CO_2 in air.

Cell Transfection—The plasmid pSV2-erbB2, a constitutive expression vector, carries the 4.4-kb full-length human HER2/*neu* cDNA under the control of the SV40 promoter/enhancer sequence. Two million cells were transfected with 2 μg of DNA mediated by 10 μl of Lipofectin reagent (Invitrogen). Experiments were performed 24 h after transfection.

Western Blot Analysis—The cells (1.5×10^6) were seeded onto a 100-mm tissue culture dish in 10% FBS DMEM/F-12 and cultured for 24 h. The cells were then incubated in 1% FBS DMEM/F-12 treating with various dose of apigenin for various time periods. Cells were washed three times with PBS and then lysed in gold lysis buffer (10% glycerol, 1% Triton X-100, 137 mM NaCl, 10 mM NaF, 1 mM EGTA, 5 mM EDTA, 1 mM sodium pyrophosphate, 20 mM Tris-HCl, pH 7.9, 100 mM β-glycerophosphate, 1 mM sodium orthovanadate, 0.1% SDS, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin). Protein content was determined against a standardized control, using the Bio-Rad protein assay kit (Bio-Rad Laboratories). A total of 50 μg of protein was separated by SDS-PAGE and transferred to nitrocellulose filter paper (Schleicher & Schuell, Inc., Keene, NH). Nonspecific binding on the nitrocellulose filter paper was minimized with a blocking buffer containing nonfat dry milk (5%) and Tween 20 (0.1%, v/v) in PBS (PBS/Tween 20). Then the filter paper was incubated with primary antibodies followed by incubation with horseradish peroxidase-conjugated goat anti-mouse antibody (1:2500 dilution, Roche Applied Science, Indianapolis, IN). Reactive bands were visualized with an enhanced chemiluminescence system (Amersham Biosciences, Arlington Heights, IL). The intensity of the bands was scanned and quantified with National Institutes of Health Image software.

In Vitro HER2/neu Tyrosine Kinase Assay—Immunocomplex was precipitated from lysate of MDA-MB-453 cells with monoclonal anti-HER2/*neu* antibody c-*neu* (Ab-3) on protein-A-conjugated agarose beads (40 μl) (Roche Applied Science) and then washed three times with 50 mM Tris-HCl buffer containing 0.5 M LiCl (pH 7.5) and once in assay buffer (50 mM Tris-HCl (pH 7.5) and 10 mM $MnCl_2$). Radiolabeled ATP (10 μCi of [γ-^{32}P]ATP, Amersham Biosciences) and 10 μl of enolase (2.5 mg/ml, Sigma Chemical Co., St. Louis, MO) were added to the beads, followed by incubation for 20 min at room temperature. The reaction products were separated by 8% SDS-PAGE. The gel was dried and visualized by autoradiography.

In Vitro PI3K Assay—MDA-MB-453 cell extracts (500 μg) were immunoprecipitated with anti-PI3K(p85) antibody (Upstate Biotechnology, Inc.) and protein A-Sepharose beads (Repligen). Immunoprecipitation complexes were washed three times with 1% Triton X-100 in PBS, twice with a buffer containing 0.5 M LiCl, 0.1 M Tris, pH 7.5, and twice with 10 mM Tris, pH 7.5, 100 mM NaCl, and 1 mM EDTA. Complexes were incubated for 20 min at room temperature with 1 μM ATP, 5 μCi of [γ-^{32}P]ATP (Amersham Biosciences), and a 0.5 μM/ml lipid mix of phosphatidylinositol and phosphatidylserine (1:1) in 10 mM HEPES (pH 7.0), and 1 mM EGTA. The reaction was quenched by 1 M HCl, and lipids were extracted with $CHCl_3$:CH_3OH (1:1). The organic layer was analyzed by thin-layer chromatography developed with 1-propanol:methanol:glacial acetic acid (50:15:35) and detected by autoradiography.

In Vitro Akt Kinase Assay—Kinase activity was assayed using a New England Biolabs Akt Kinase Kit. Akt was immunoprecipitated, washed twice with lysis buffer, then twice with kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerolphosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$). 200 μM ATP and 1 μg of substrate (paramyosin fused to a GSK-3 crosstide) were added, and assays were performed at 30 °C for 30 min. Reaction mixtures were separated by 10% SDS-PAGE, and the p-GSK-3 reaction product was detected by immunoblotting.

In Vitro grp94 Autophosphorylation Assay—MDA-MB-453 cell extracts (500 μg) were immunoprecipitated with GRP94 (Upstate Biotechnology, Inc.). Mixtures were incubated for 3 h at 4 °C, and then 40 μl of protein-A-conjugated agarose beads was added. After rotation at 4 °C for overnight, immunocomplexes were washed 5 times with 800 μl each of a washing buffer (50 mM Tris, 100 mM NaF, 50 mM NaCl, 2 mM EDTA, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10% glycerol, 1% Nonidet P-40, pH 8.0) and finally resuspended in 50 mM Tris, pH 7.4. Ten μl of the immunocomplex beads was incubated in a buffer containing 30 mM Hepes, 400 kBq of 0.2 mM [γ-^{32}P]ATP, pH 7.5, in the presence of 5 mM $CaCl_2$ or $MgCl_2$ at 37 °C for 30 min with occasional mixings. The reaction was terminated by adding 5× SDS sample buffer and boiling for 5 min. The proteins eluted from the immunoaffinity resins were analyzed by SDS-PAGE and autoradiography.

Pulse-chase Labeling Assay—MDA-MB-453 cells were grown to 70% confluence in 100-mm dishes in DMEM/F-12 supplemented with 10%

fetal calf serum. Plates were washed and then incubated in DMEM lacking methionine and cysteine for 20 min and then pulsed for 30 min in 1 ml of deficient media containing 10% dialyzed fetal calf serum and 0.1 mCi of [^{35}S]methionine (Trans-Label, ICN). After pulsing, plates were washed once in complete media and then incubated in complete media containing either 40 μM apigenin or vehicle control (0.1% Me_2SO). After incubation, plates were washed three times in PBS, and the cells were lysed in gold lysis buffer. Cell lysates were cleared by a 10-min spin at 12,000 $\times$ *g*, and then an equal amount of protein (500 μg) was immunoprecipitated with monoclonal antibody Ab-3 as described above. Immunocomplexes were separated by 8% SDS-polyacrylamide gel. The gel was dried and visualized by fluorography.

Immunofluorescence Assay—MDA-MB-453 cells were plated on coverslips placed in six-well plates. Experiments were performed 24 h after cell attachment. Cells were fixed in PBS containing 4% paraformaldehyde for 10–15 min at room temperature. Cells were rinsed with PBS for 2–3 times followed by blocking with 1% normal goat serum for 30 min. Incubations were performed with primary antibodies diluted in blocking buffer at 4 °C for overnight, after which coverslips were washed and incubated for 30 min with the fluorescein isothiocyanate-conjugated secondary antibodies diluted in blocking buffer. Coverslips were washed and mounted in Vectashield (Vector Laboratories, Burlingame, CA) and viewed under a Leica TCS SP2 confocal laser-scanning microscope (Leica Microsystems, Heidelberg, Germany).

MTT Assay—Cells were seeded in a 96-well microtiter plate (1×10^4 cells/well) overnight, then treated with varying concentrations of apigenin, and incubated for an additional 72 h. The effect of apigenin on cell growth was examined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Briefly, 20 μl of MTT solution (5 mg/ml, Sigma Chemical Co.) was added to each well and incubated for 4 h at 37 °C. The supernatant was aspirated, and the MTT-formazan crystals formed by metabolically viable cells were dissolved in 200 μl of Me_2SO. Finally, the absorbance was monitored by a microplate reader at a wavelength of 595 nm.

DNA Extraction and Electrophoretic Analysis—Cells (4×10^5 cells/ml) were harvested, washed in PBS, and then lysed by digestion buffer containing 0.5% Sarkosyl, 0.5% mg/ml proteinase K, 50 mM Tris (pH 8.0), and 10 mM EDTA at 55 °C for 3 h. RNase A (0.5 mg/ml) was added, and the mixture was incubated at 55 °C for 24 h, after which the DNA was extracted by phenol/chloroform/isoamyl alcohol (25:24:1). Approximately 20 μg of DNA was loaded in each well, and electrophoresed into a 1.8% agarose gel (containing ethidium bromide) at 50 V for 120 min. The gel was then visualized under a UV light and photographed.

Flow Cytometry—Cells (2×10^5) were cultured in 60-mm Petri dishes and incubated for various times. Then cells were harvested, washed with PBS, resuspended in 200 μl of PBS, and fixed in 800 μl of iced 100% ethanol at −20 °C. After being left to stand overnight, cell pellets were collected by centrifugation, resuspended in 1 ml of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/ml RNase), and incubated at 37 °C for 30 min. Then 1 ml of propidium iodide solution (50 μg/ml) was added, and the mixture was allowed to stand on ice for 30 min. Fluorescence emitted from the propidium iodide-DNA complex was quantitated after excitation of the fluorescent dye by FAC-Scan cytometry (BD Biosciences, San Jose, CA).

RESULTS

Apigenin Preferentially Inhibited the Proliferation of HER2/neu-overexpressing Breast Cancer Cells—The growth of the tested cell lines was inhibited by apigenin in a dose-dependent manner but to varying extents (Fig. 1). At a 40 μM concentration, apigenin blocked 48% of growth in HER2/*neu*-overexpressing MDA-MB-453 cells. However, under the same conditions, it inhibited only 31%of growth in MCF7 (basal HER2/*neu* levels). Apigenin had little effect on the immortalized noncancerous HBL-100 breast cell line even at 70 μM. These results suggest that apigenin preferentially suppresses growth of the HER2/*neu*-overexpressing breast cancer cell lines.

Apigenin Induced Apoptosis in the HER2/neu-overexpressing Breast Cancer Cells—Apigenin-treated MDA-MB-453 cell lines underwent apoptosis in a dose- and time-dependent manner as measured by flow cytometry using propidium-iodide staining

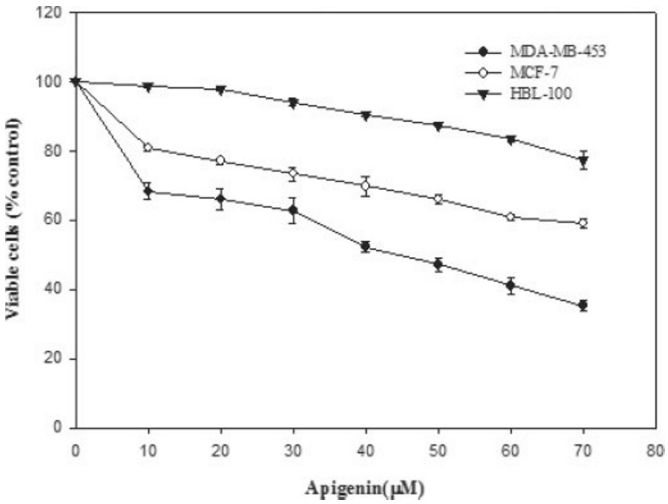

FIG. 1. **Effect of apigenin on the proliferation of human breast cancer cells expressing different levels of HER2/neu.** MDA-MB-453 cells overexpress HER2/*neu*, whereas MCF-7 and HBL-100 cells express normal levels of HER2/*neu*. After incubation with different concentrations of apigenin at 37 °C for 72 h, the effect on cell growth was examined by MTT assay. The number of viable cells after treatment is expressed as a percentage of the vehicle-only control. This experiment was repeated three times. *Bars* represent the S.E.

(Fig. 2*A*). A significant number of the cells (55.37%) started to undergo apoptosis as early as 36 h after treatment with 40 μM apigenin. A lower concentration of apigenin (20 μM) resulted in apoptosis in fewer cells (~20% at 36 h). As shown in Fig. 2*B*, by comparing with vehicle control, apigenin treatment (20 and 40 μM for 48 h) resulted in DNA fragmentations in MDA-MB-453 cells. Similarly, treatment with apigenin at 40 μM for 24 and 48 h resulted in the formation of a DNA ladder.

Apigenin Inhibited PI3K Activity and Akt Kinase in HER2/neu-overexpressing Breast Cancer Cells—A key mechanism by which HER2/*neu* overexpression stimulates tumor cell growth and renders cells chemoresistant is through the HER2/*neu* receptor. This mechanism involves the PI3K/Akt signaling pathway, and human breast cancer cells with overexpression and amplification of HER2/*neu* have been shown to make increased use of the signaling pathway mediated by PI3K/Akt (17, 45). Activated Akt is considered the focal point of a survival pathway known to protect cells from apoptosis by several stimuli, whereas in a recent report, apigenin displayed potent inhibitory effects on PI3K activity (46). As shown in Fig. 3*A*, our results also indicated that apigenin possessed inhibitory effects on PI3K activity in the HER2/*neu*-overexpressing breast cancer cells. Furthermore, we found that in the HER2/*neu*-overexpressing breast cancer cell lines MDA-MB-453, BT-474, and SKBr-3, treatment with apigenin had no effect on steady-state levels of total PI3K protein, whereas its downstream effector of phosphorylated Akt was inhibited in a dose- and time-dependent manner (Fig. 3*B*). Wortmannin and LY294002 are known to be irreversible PI3K inhibitors and were used here as positive controls (Fig. 3*B*). Treatment of the HER2/*neu*-overexpressing breast cancer cell lines with wortmannin almost completely inhibited Akt phosphorylation at 2 h, whereas the reduced inhibition at 16 h post-treatment (Fig. 3*B*) is presumably attributable to wortmannin having a relatively short half-life. Akt kinase has been shown to phosphorylate several key substrates that regulate protein translation, apoptosis, and cellular proliferation (47, 48), and phosphorylation of its substrate, glycogen synthase kinase-3 (GSK-3), was demonstrated here in MDA-MB-453 cells (Fig. 3*C*). Apigenin caused dephosphorylation of GSK-3 at concentrations associated with inhibition of

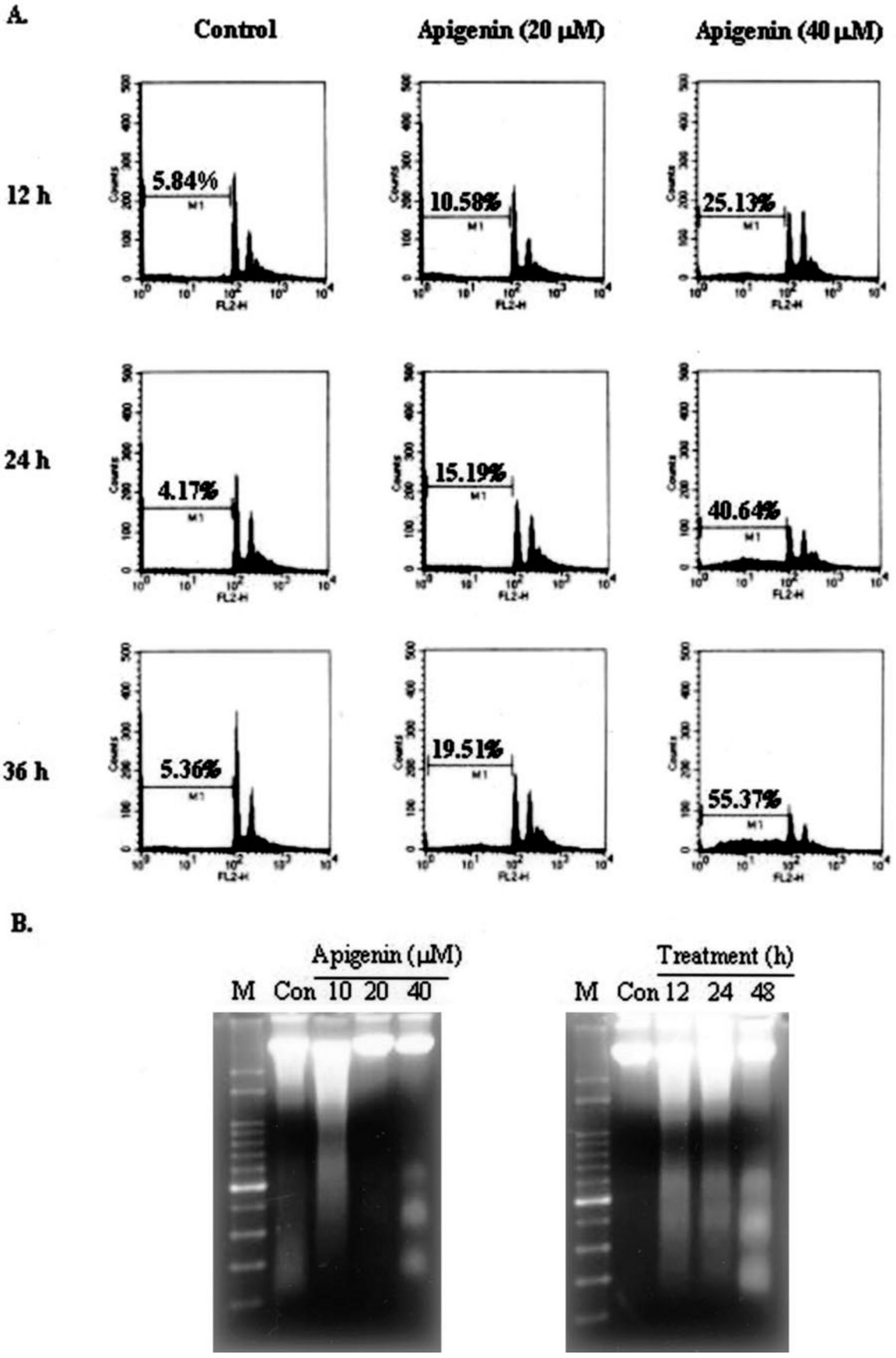

FIG. 2. **Apigenin-induced apoptosis in HER2/*neu*-overexpressing breast cancer cell lines.** *A*, MDA-MB-453 cells were treated with Me_2SO (*Con*) (*left column*) or 20 (*middle column*) and 40 (*right column*) μM apigenin for 12, 24, and 36 h and analyzed by flow cytometry as described under "Experimental Procedures." *B*, DNA ladder formation in MDA-MB-453 cells incubated with Me_2SO (*Con*) or apigenin (40 μM) at 37 °C for various times (*right column*) and various doses for 48 h (*left column*).

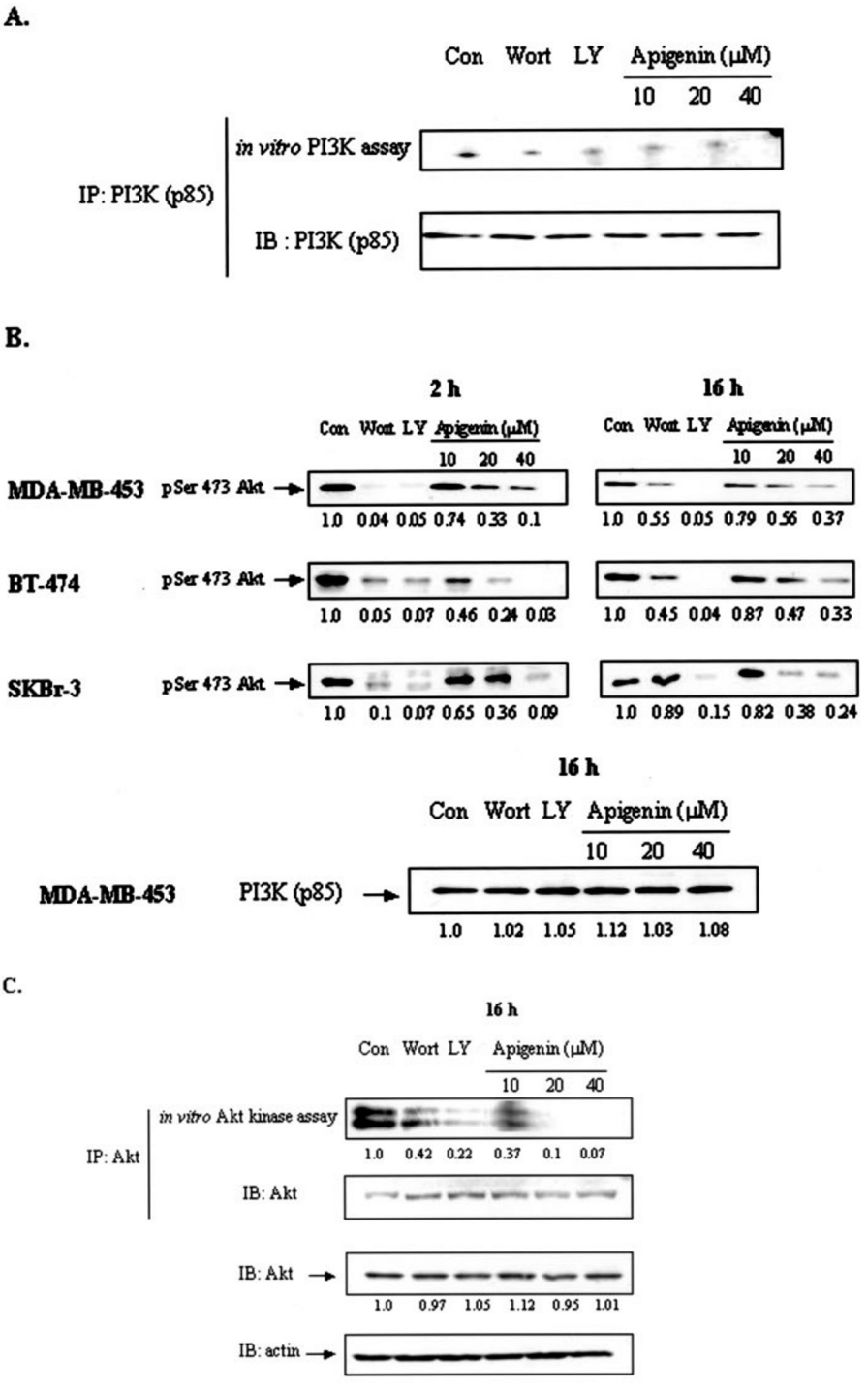

FIG. 3. **Effect of apigenin on the activity of PI3K and Akt kinase in HER2/*neu*-overexpressing breast cancer cell lines.** *A*, MDA-MB-453 cell lysates were immunoprecipitated with p85 subunit of PI3K and following treatment with PI3K inhibitors wortmannin (*Wort*; 500 nM) or LY294002 (*LY*; 20 μM) or apigenin (10, 20, and 40 μM). Kinase reaction products were separated by thin layer chromatography. Immunoblotting with PI3K(p85) antibody demonstrate equivalent protein in treated and untreated lanes. *B*, HER2/*neu*-overexpressing breast cancer cell lines MDA-MB-453, BT-474, and SKBr-3 were treated with the PI3K inhibitors wortmannin (*Wort*; 500 nM) or LY294002 (*LY*; 20 μM) or apigenin (10, 20, and 40 μM) at 37 °C for 2 h (*left column*) or 16 h (*right column*). Levels of phosphorylated Akt (pSer 473 Akt) and PI3K (p85) were analyzed by immunoblotting. *C*, MDA-MB-453 cells were treated as above, and Akt kinase activity was measured by *in vitro* phosphorylation of GSK-3-α/β. Cell lysates were immunoprecipitated with Akt antibody, and kinase activity was detected by blotting with an anti-pGSK-3-α/β antibody. Immunoblotting with Akt antibody demonstrate equivalent protein in treated and untreated lanes. Levels of Akt and actin were analyzed by immunoblotting. The values below the figures represent change in protein expression of the bands normalized to actin.

Akt activation, but at the same time, treatment with apigenin had no effect on steady-state levels of total Akt kinase protein (Fig. 3*C*). To test whether apigenin directly inhibited the Akt kinase, Akt was immunoprecipitated from untreated MDA-MB-453 cells. After treatment of the precipitates with various concentrations of apigenin, measurement of the Akt kinase activity showed that apigenin have no inhibitory effect on Akt activity (data not shown).

Effect of Apigenin on Tyrosine Phosphorylation and Protein Level of HER2/neu—We next examined the effect of apigenin on the tyrosine kinase activity of HER2/*neu*. MDA-MB-453 human breast cancer cells were treated with various concentrations of apigenin or control vehicle at 37 °C for 24 h, and then both HER2/*neu* protein and tyrosine phosphorylation levels were measured by Western blotting. Apigenin inhibited tyrosine phosphorylation and depleted HER2/*neu* in a dose-dependent manner (Fig. 4, *A* and *B*). An immunocomplex assay was then carried out to examine whether the reduced tyrosine phosphorylation affected the tyrosine kinase activity of HER2/*neu*. As shown in Fig. 4*C*, the autophosphorylation ability of HER2/*neu* from MDA-MB-453 cells treated with apigenin for 24 h was inhibited, and the transphosphorylation ability of HER2/*neu* for enolase, an exogenous substrate for tyrosine kinase, was also significantly decreased compared with untreated cells. To address further whether apigenin directly inhibited the intrinsic tyrosine kinase activity of HER2/*neu*,

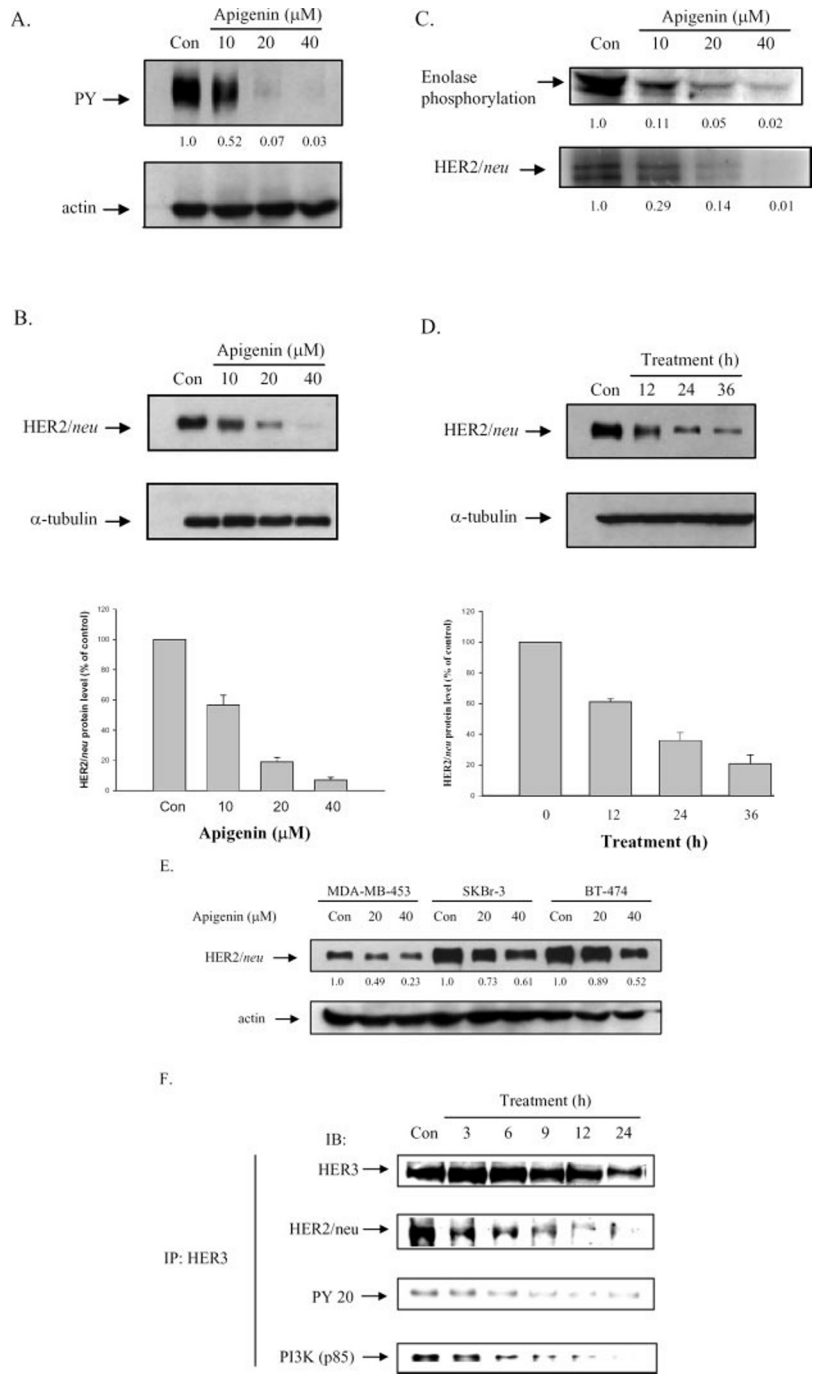

FIG. 4. **Effect of apigenin on tyrosine phosphorylation and protein level of HER2/*neu* in HER2/*neu*-overexpressing human breast cancer cell lines.** MDA-MB-453 cells were incubated with Me_2SO (*Con*) or apigenin (10, 20, and 40 μM) at 37 °C for 24 h. Immunoblotting was used to measure levels of (*A*) phosphotyrosine (*PY*) and actin and (*B*) HER2/*neu* and α-tubulin (*top panel*). The histogram shows the averaged results of three independent experiments (*lower panel*). *Bars* represent the S.E. *C*, MDA-MB-453 cells were incubated as above and cell lysates were immunoprecipitated with anti-HER2/*neu* antibody. Kinase activity was measured by incubation with [γ-^{32}P]ATP and enolase at room temperature. Reactants were resolved on 8% SDS-PAGE. The gel was dried, and the phosphorylation products were visualized by autoradiography. *D*, MDA-MB-453 cell lines were incubated with Me_2SO (*Con*) or apigenin (20 μM) at 37 °C for various times and levels of HER2/*neu* and α-tubulin were analyzed by immunoblotting (*upper panel*). The histogram shows the averaged results of three independent experiments (*lower panel*). *Bars* represent the S.E. *E*, hER2/*neu*-overexpressing breast cancer cell lines MDA-MB-453, BT-474, and SKBr-3 were incubated with Me_2SO (*Con*) or apigenin (20 and 40 μM) at 37 °C for 36 h and levels of HER2/*neu* and α-tubulin were analyzed by immunoblotting. The values *below the figures* represent change in protein expression of the bands normalized to control. *F*, MDA-MB-453 cells were treated with 40 μM apigenin for various times, cell lysates were immunoprecipitated with HER3 and immunoblotted for HER3, HER2/*neu*, PY20, and p85 subunit of PI3K.

HER2/*neu* was immunoprecipitated from untreated MDA-MB-453 cells. After treatment of the precipitates with various concentrations of apigenin, measurement of the tyrosine kinase activity showed that neither autophosphorylation nor transphosphorylation of HER2/*neu* was inhibited by apigenin (data not shown). To investigate the kinetics of depletion of HER2/*neu*, we treated MDA-MB-453 cells with 20 μM apigenin for different time periods and then harvested them for Western blot analysis of HER2/*neu*. The HER2/*neu* protein levels decreased in a time-dependent manner after apigenin treatment (Fig. 4*D*). To further confirm that the depletion of the HER2/*neu* protein levels by apigenin is a general phenomenon for HER2/*neu*, we also demonstrated similar results in two other breast cancer cell lines that overexpress HER2/*neu* (*SKBr-3* and *BT-474*; Fig. 4*E*).

Apigenin Inhibited Akt Kinase Activity by Preventing the Docking of PI3K to HER2/HER3 Heterodimers—Akt kinase associated with the plasma membrane by binding to phosphatidylinositol 3-phosphates via its pleckstrin homology domain (49). At the membrane, Akt kinase is activated as a result of the phosphorylation of Thr-308 and Ser-473 by 3-phosphoinositide-dependent protein kinase 1 (PDK1) and perhaps by other kinases (50). Thus, Akt kinase activation is dependent on PI3K activity. Activated HER2/*neu* forms heterodimers with other members of the HER kinase family and transphosphorylates its dimerization partner (51). One of these, HER3, has no endogenous tyrosine kinase activity but contains multiple tyrosines that when phosphorylated are docking sites for PI3K (52). We have already shown that apigenin inhibits PI3K activity (Fig. 3*A*) and the tyrosine kinase activity of HER2/

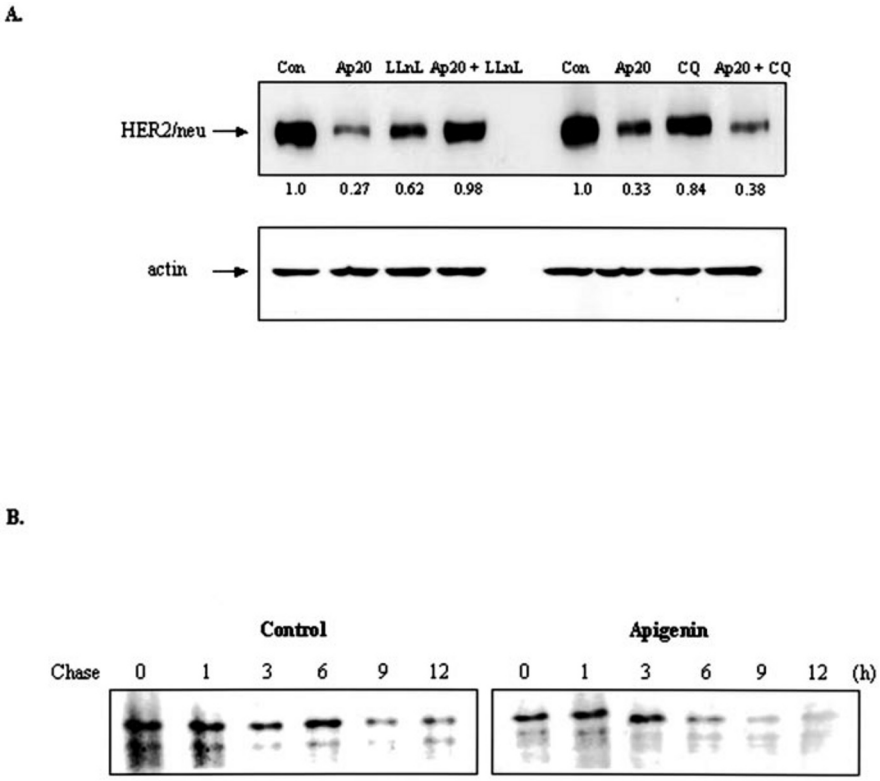

FIG. 5. **Apigenin-induced HER2/*neu* degradation and loss of HER3-associated p85 and PI3K activity.** *A*, MDA-MB-453 cells were treated with (*1*) 1% FBS DMEM/F-12 media for 9 h (*Con*); (*2*) 20 μM apigenin for 9 h (*Ap20*); (*3*) 100 μM *N*-acetyl-Leu-Leu-norleu-al (*LLnL*) or chloroquine (*CQ*) for 9 h; (*4*) 100 μM LLnL or CQ pretreatment for 2 h followed by 20 μM apigenin for 9 h. Anti-HER2/*neu* antibodies were used to determine HER2/*neu* protein levels in the cell lysates. This experiment was repeated three times with similar results. The values *below the figures* represent change in protein expression of the bands normalized to actin. *B*, MDA-MB-453 cells were pulsed with 0.1 mCi of [^{35}S]methionine for 30 min and chased in the absence or presence of 40 μM apigenin. Equal protein amounts were immunoprecipitated with HER2/*neu* antibody and separated by 8% SDS-polyacrylamide gel. The gel was dried and analyzed by fluorography.

neu (Fig. 4*A*); we now investigate whether apigenin inhibits Akt activation by preventing the docking of PI3K to HER2/HER3 heterodimers. After 24 h of treatment, HER3 levels declined with longer exposure to apigenin (Fig. 4*F*). However, association of HER3 with HER2 declined after 3 h and HER2/*neu* protein was barely detectable after 24 h of treatment (Fig. 4*F*). Phosphorylation of HER3 declined in parallel, whereas decreased phosphorylation of HER3 led to a coordinate decrease in its binding to the p85 regulatory subunit of PI3K (Fig. 4*F*).

Apigenin Depleted HER2/neu by Proteasomal Degradation—HER2/*neu* protein has been shown to be degraded in the proteasome by the antibiotic benzoquinone asamycin (53); here we examine whether the apigenin-induced depletion of HER2/*neu* also occurs in the proteasome. We found that pretreatment of MDA-MB-453 cells with the proteasome inhibitor *N*-acetyl-Leu-Leu-norleu-al (LLnL) blocked the depletion of HER2/*neu* protein levels by apigenin (Fig. 5*A*). Pretreatment with the lysosome inhibitor chloroquine (CQ), on the other hand, had no effect (Fig. 5*A*). These results suggest that apigenin decreases HER2/*neu* protein levels by promoting the degradation of HER2/*neu* protein in the proteasome. In addition, the pulse-chase labeling assay also showed that 40 μM apigenin did enhance the depletion of mature HER2/*neu* protein (Fig. 5*B*).

Apigenin Changed the Subcellular Distribution of HER2/neu—An immunofluorescence study with anti-HER2/*neu* antibody (Ab-3) showed that the control cells had strong immunofluorescence at the plasma membrane (Fig. 6, *A* and *E*). After apigenin treatment, the immunofluorescence at the plasma membrane disappeared and was replaced by diffuse cytoplasmic punctate staining (Fig. 6, *B* and *F*), which might be compatible with localization in the endoplasmic reticulum or the Golgi apparatus. Cells transiently transfected with a human cDNA encoding HER2/*neu* (pSV2-erbB2) recovered the immunofluorescence at the membrane (Fig. 6*D*). This phenomenon was not observed in cells transfected with control vector (Fig. 6*C*). Addition of Actinomycin D (Fig. 6*G*) or cycloheximide (Fig. 6*H*) did not significantly alter the effect of apigenin on the immunofluorescence pattern, indicating that apigenin treatment did not alter HER2/*neu* mRNA levels or change the rate of *de novo* synthesis of HER2/*neu*.

Dissociation of HER2/neu from GRP94 Preceded the Depletion of HER2/neu—Recent study has demonstrated that curcumin, may act as an ATP competitor, inhibited HER2/*neu* tyrosine kinase activity *in vitro* and depleted HER2/*neu* protein *in vivo* by disrupting its binding with a chaperone, GRP94 (66). To further study the mechanism of HER2/*neu* depletion, we treated the MDA-MB-453 cells with either the control vehicle (Me_2SO) or 40 μM apigenin at varying periods and studied the binding of HER2/*neu* with GRP94. Equal amounts of fractionated proteins were immunoprecipitated with 2 μg of anti-HER2/*neu* monoclonal antibody, and the immunoprecipitates were then blotted with HER2/*neu*, GRP94, and ubiquitin, respectively. After 1-h treatment, because the HER2/*neu* protein level was not significantly changed, the binding of HER2/*neu* with GRP94 had already markedly decreased (Fig. 7*A*). In the companion Western blot from anti-HER2/*neu* immunoprecipitations of the same samples, the ubiquitin signal was increased by apigenin treatment, suggesting that ubiquitination of the protein occurred prior to HER2/*neu* degradation (Fig. 7*A*). GRP94 is an ATP-binding protein and has Mg^{2+}-dependent ATPase activity (54). To assess further if apigenin disrupted

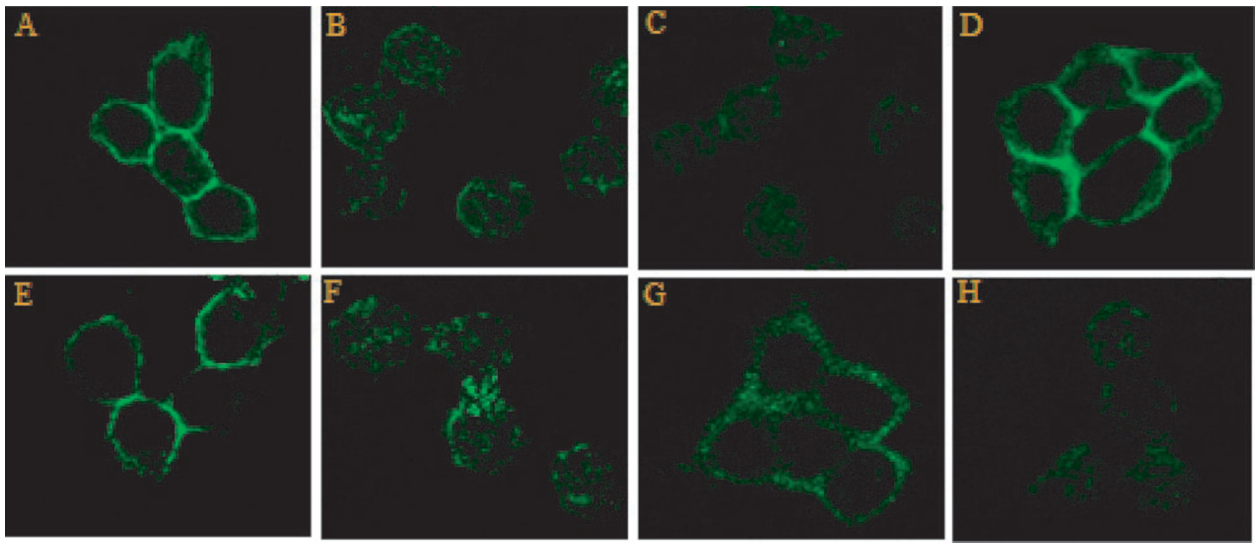

FIG. 6. **Changes in subcellular distribution of HER2/*neu* after the 9-h exposure to apigenin.** MDA-MB-453 cells grown on coverslips were treated with control vehicle (*A* and *E*) or 40 μM apigenin (*B* and *F*) for 9 h. Cells transfected with empty vector (*C*) or pSV2-erbB2 (*D*) were incubated with 40 μM apigenin for 9 h. Prior to adding 40 μM apigenin, cells were pretreated with actinomycin D (*G*) or cycloheximide (*H*). Cells were fixed with 4% paraformaldehyde and stained with HER2/*neu* antibody followed by fluorescein isothiocyanate-conjugated secondary antibody (*green*). Analysis of subcellular distribution was performed by confocal microscopy.

the association of GRP94 with HER2/*neu* through competition with ATP, an *in vitro* GRP94 ATPase activity assay was then performed. As shown in Fig. 7*B*, the autophosphorylation ability of GRP94 from MDA-MB-453 cells treated with apigenin was inhibited.

HER2/neu-mediated Resistance to Apigenin-induced Apoptosis—Apigenin decreased HER2/*neu* protein levels and induced apoptosis in the HER2/*neu*-overexpressing breast cancer cells. However, the expression of HER2/*neu* is an important mechanism for cell survival. To examine whether the HER2/*neu* could mediate the resistance to the apigenin-induced apoptosis, MDA-MB-453 cell lines were transiently transfected with a human cDNA encoding HER2/*neu* (pSV2-erbB2) and treated with apigenin. Expression of pSV2-erbB2 in MDA-MB-453 cells did not induce the degradation of HER2/*neu* in a time-dependent manner after apigenin treatment (Fig. 8*A*). PI3K activity was also elevated in pSV2-erbB2-transfected cells (Fig. 8*B*). In addition, as shown in Fig. 8*C*, pSV2-erbB2-transfected MDA-MB-453 cells demonstrated high resistance to apigenin-induced apoptosis (more than 50% of cell survival), whereas the untransfected cells progressively underwent cell death in a dose- and time-dependent manner (59.83% of cells started to undergo apoptosis as early as 36 h after treatment with 40 μM apigenin). Lower concentration of apigenin (20 μM) treatment also started to undergo apoptosis (20%) at 36 h (Fig. 8*C*).

DISCUSSION

We have demonstrated that apigenin preferentially inhibited the growth of HER2/*neu*-overexpressing breast cancer cell lines but not the lines expressing basal levels of HER2/*neu* (Fig. 1). Previous cell cycle studies using fluorescence-activated cell sorting showed that both MCF-7 and MDA-MB-468 cells were arrested in the G_2/M phase (8, 55). Both of these cell lines express basal levels of HER2/*neu*, here we further showed that apigenin also induced apoptosis in HER2/*neu*-overexpressing MDA-MB-453 cells (Fig. 2). We demonstrated here for the first time that apigenin induces cell growth inhibition of HER2/*neu*-overexpressing breast cancer cell lines accompanied by the induction of apoptosis processes. Investigation of the signal molecules that may be involved during the induction of apoptotic processes showed that components of the cell survival pathways are affected in apigenin-treated HER2/*neu*-overexpressing breast cancer cell lines (Figs. 3 and 4). Because Akt/PKB, a serine/threonine kinase, is known to be an important survival factor in signal transduction pathways involved in cell growth, this kinase is a possible target for anticancer drug-induced apoptosis. Overexpression of Akt has been reported to be involved in drug resistance (56), and treatment with anticancer drugs such as CPT-11 and growth factor deprivation have been shown to suppress the activity of Akt, leading to loss of cell viability and apoptosis (57). The mechanism by which Akt protects cells from death is likely to be multifactorial, because Akt directly phosphorylates several components of the cell-death machinery (58). We found that apigenin inhibits Akt phosphorylation at serine 473 without significantly affecting PI3K protein levels (Fig. 3*B*); in addition, Akt kinase activity was inhibited in apigenin-treated HER2/*neu*-overexpressing breast cancer cell lines (Fig. 3*C*). We further examined the effect of apigenin on Akt kinase activity, using cell lysates from MDA-MB-453 cells in a protein kinase assay. The results showed that, under the conditions used, apigenin was unable to directly inhibit Akt kinase activity (data not shown).

Studies with breast cancer cell lines and human tumors have demonstrated constitutive phosphorylation of HER2/*neu* is associated with resistance to systemic therapies and local radiation therapies. Activation of HER2-containing heterodimers results in receptor autophosphorylation on COOH-terminal tyrosine residues, which become the docking sites for a number of signal transducers and adaptor molecules that initiate a plethora of signaling programs leading to cell proliferation, differentiation, migration, adhesion, protection from apoptosis, and transformation, among other effects. The PI3K-Akt pathway is one of the signaling pathways activated by HER2/*neu*. For this reason, we tested whether apigenin inhibited the tyrosine phosphorylation of HER2/*neu*. We found that apigenin repressed the PY levels of HER2/*neu* and also depleted the HER2/*neu* protein levels (Fig. 4, *A* and *B*). However, apigenin did not directly inhibit intrinsic tyrosine kinase activity of HER2/*neu* (data not shown); the apigenin-induced inhibition of the tyrosine phosphorylation of HER2/*neu* must be caused by depleting the HER2/*neu* protein levels (Fig. 4,

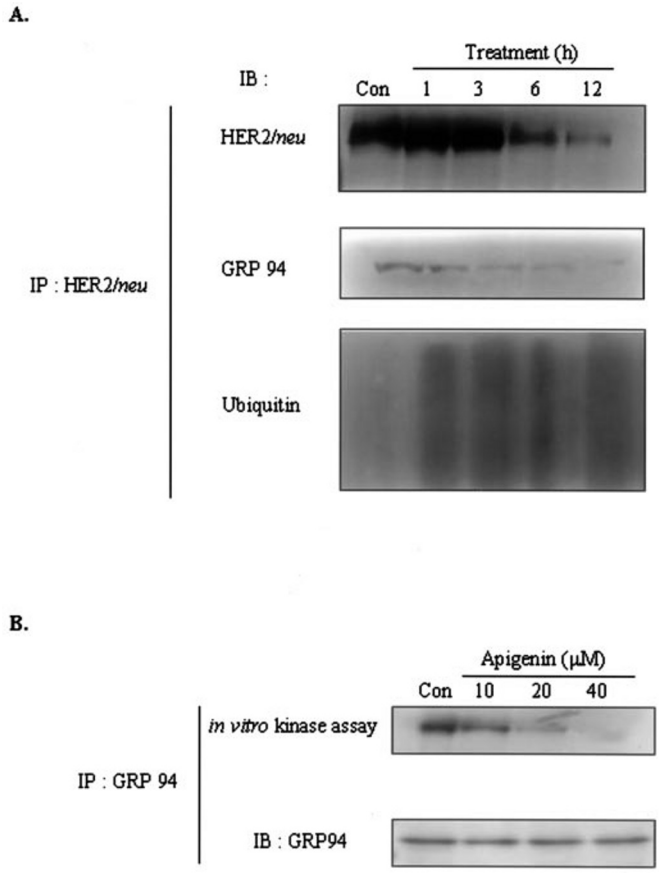

FIG. 7. **Dissociation of the HER2/*neu*/GRP94 heterocomplex by apigenin and induction of polyubiquitination of HER2/*neu*.** *A*, MDA-MB-453 cells were treated with 40 μM apigenin for various times, cell lysates were immunoprecipitated with HER2/*neu* and immunoblotted for HER2/*neu*, GRP94, and ubiquitin. *B*, MDA-MB-453 cell lysates were immunoprecipitated by GRP94 antibodies and incubated with Me_2SO (*Con*) or apigenin (10, 20, and 40 μM) in the presence of 5 mM $MgCl_2$ and 0.2 mM [γ-^{32}P]ATP in 50 mM Hepes buffer, pH 7.4, at 37 °C for 30 min. Samples were subjected to SDS-PAGE. The gel was dried, and the phosphorylation products were visualized by autoradiography.

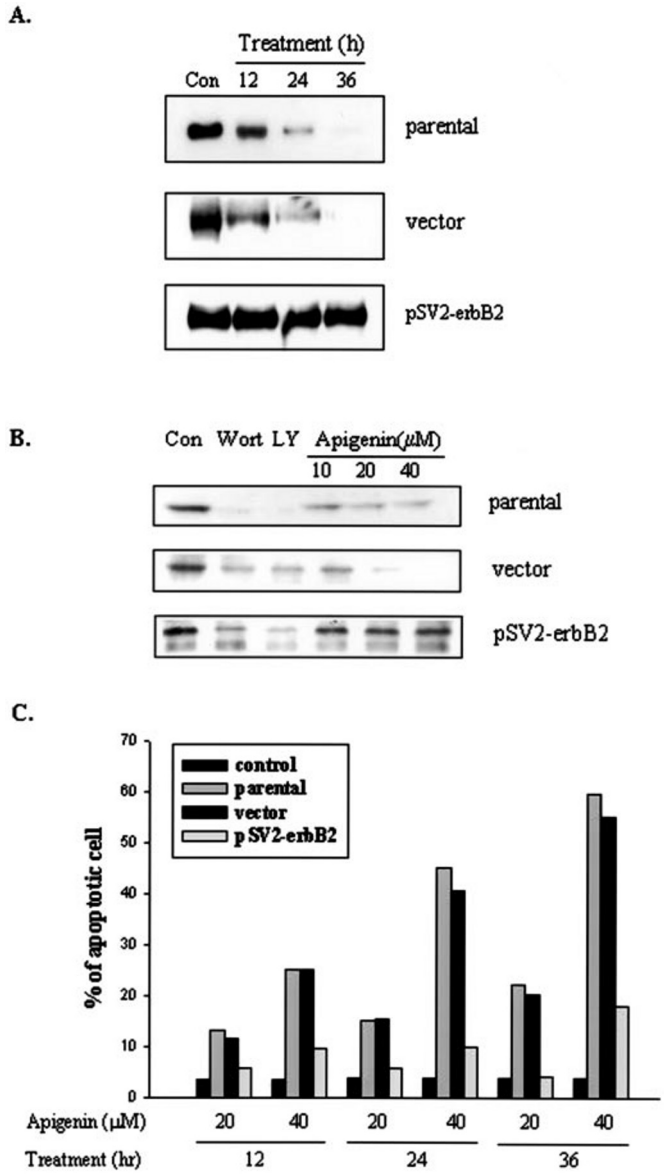

FIG. 8. **HER2/*neu*-mediated resistance to apigenin-induced apoptosis.** MDA-MB-453 cells were transfected with empty vector or pSV2-erbB2. *A*, transfected cells were incubated with Me_2SO (*Con*) or apigenin (20 μM) at 37 °C for various times and levels of HER2/*neu* were analyzed by immunoblotting. *B*, transfected cells were treated with PI3K inhibitor (500 nM wortmannin (*Wort*); 20 μM LY294002 (*LY*)) or apigenin (10, 20, and 40 μM) at 37 °C for 2 h. Levels of phosphorylated Akt (pSer 473 Akt) were analyzed by immunoblotting. *C*, transfected cells were treated with Me_2SO (*Con*) or apigenin (20 and 40 μM) for 12, 24, and 36 h and analyzed by flow cytometry as described under "Experimental Procedures."

C–E). This is the first report showing that apigenin can inhibit protein kinase activity by depleting the protein kinase itself.

Another important observation pertaining to HER heterodimer collaboration during tumor development is that expression of HER3 is seen in many of the same tumor types that overexpress HER2/*neu*, including breast and bladder cancers and melanomas (59–62). Furthermore, many HER2/*neu*-overexpressing breast tumors display elevated levels of phosphotyrosine on HER3 (63), probably as a result of spontaneous dimerization with HER2/*neu*. All of these suggest that HER2/*neu* and HER3 function together to stimulate mitogenic signaling networks. HER3 has multiple binding sites for p85, which makes it the most efficient activator of PI3K, and HER2/*neu* signaling through HER2/HER3 with activation of PI3K and Akt has been suggested by other investigators (27, 64). Here we have shown that degradation of HER2/*neu* in cells exposed to apigenin led to HER3 dephosphorylation (Fig. 4*F*), loss of its association with PI3K (Fig. 4*F*), and a rapid decline in Akt activity (Fig. 3*C*). Functional inhibitors of Akt might be expected to inhibit tumor cell growth and increase their sensitivity to stimuli that induce apoptosis. Here, we showed that the apigenin inhibits Akt function in tumor cells in a complex manner. First, apigenin directly inhibited the PI3K activity (Fig. 3*A*), upstream mediator of Akt, and indirectly caused an inhibitory effect on Akt kinase activity. In addition, we proposed that the apigenin-induced cellular effects result from loss of HER2/*neu* and HER3 expression with subsequent inactivation of PI3K and Akt in cells that are dependent on this pathway for cell proliferation and inhibition of apoptosis.

Apigenin exhibits a variety of effects, including inhibition of malignant cell growth. It can inhibit multiple protein kinases (14, 65), and, in common with virtually all of the natural phosphotyrosine-receptor kinase blockers (66–69), it acts by competing with ATP (14). Therefore, naturally occurring apigenin has been proposed to exert biological effects on cells through inhibition of these different key enzymes. For these reasons, to identify the molecular mechanism of apigenin-induced apoptosis in HER2/*neu*-overexpressing breast cancer

cells, several kinases involved in signal transduction were investigated. Apigenin was found to directly inhibit the PI3K activity but did not directly inhibit those of Akt kinase and HER2/*neu* tyrosine kinase, suggesting that apigenin is rather a specific inhibitor of protein kinases. Subsequently, the effects of structurally related flavonoids on those kinases involved in HER2/*neu*-overexpressing signal transduction mediators could be assessed.

A recent report (66) demonstrated that curcumin dissociates the complex between HER2/*neu* and GRP94, a molecular chaperone, in the endoplasmic reticulum. This dissociation precedes the depletion of mature HER2/*neu* at the plasma membrane. The depletion of mature membrane HER2/*neu* and the concomitant accumulation of HER2/*neu* in the cytoplasmic organelles are compatible with the notion that the complex of HER2/*neu* with GRP94 is necessary for its maturation and subsequent transport to the plasma membrane (66, 70). In this study, we demonstrated that apigenin depletes mature HER2/*neu in vivo*. After 12 h of apigenin treatment, the HER2/*neu* protein was almost undetectable. Apigenin also dissociated the complex of HER2/*neu* and GRP94 and preceded the depletion of HER2/*neu* (Fig. 7*A*). We thus hypothesized that apigenin may also disrupt the association of HER2/*neu* and the chaperone complex through competition with ATP, and this may explain why apigenin can deplete HER2/*neu* protein (Fig. 7*B*). Our results indicated that apigenin can also deplete other members of the class II receptor (ErbB) tyrosine kinase family, such as epidermal growth factor receptor (HER1) (data not shown) and HER3 (Fig. 4*F*), although much less than HER2/*neu*. Moreover, Gupta *et al.* (13) reported that apigenin significantly decreased serum-induced AR protein expression in human prostate carcinomas cells, perhaps through a similar mechanism. However, the nature of GRP94 function is still not well understood, although along with other chaperones, it is thought to participate in the maturation of transmembrane and secreted proteins.

Recent studies identified that the benzoquinone ansamycins such as geldanamycin enhanced intracellular degradation of HER2/*neu* and involved targeting of the heat shock protein 90 (Hsp90) (71). Hsp90 forms complexes with HER2/*neu* and other client proteins. Once geldanamycin blocks ATP binding to Hsp90, the chaperone complex associated with the client protein is biased toward a degradative fate, resulting in polyubiquitylation and subsequent destruction of the client. The mature HER2/*neu* requires Hsp90 association with its kinase domain to maintain the conformation necessary to heterodimerize with other ligand-activated ErbB proteins. Investigations on the possible involvement of Hsp90 in apigenin-induced degradation of HER2/*neu* are currently in progress.

Our present study shows that apigenin-induced degradation of mature HER2/*neu* involves polyubiquitination of HER2/*neu* (Fig. 7*A*) and subsequent hydrolysis by the proteasome (Fig. 5*A*). Apigenin-stimulated ubiquitination of HER2/*neu* occurred rapidly and was easily detectable on anti-ubiquitin immunoblots within 1 h of adding apigenin to cells at 40 μM. The ubiquitination of HER2/*neu* occurred prior to any measurable decrease in HER2/*neu* protein levels, suggesting that conjugation of HER2/*neu* to ubiquitin was a prerequisite to its degradation (Fig. 7*A*).

In conclusion, the results of this study provide mechanistic evidence that apigenin induces apoptosis by depleting HER2/*neu* protein and, in turn, suppressing the signaling of the HER2/HER3-PI3K/Akt pathway. The apoptosis-inducing ability of apigenin, in conjunction with its low toxicity and non-mutagenic nature, makes it a potentially effective chemopreventive and therapeutic agent against HER2/*neu*-overexpressing breast cancers.

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