Cyclooxygenase-2 Is Essential for HER2/neu to Suppress N-(4-Hydroxyphenyl)retinamide Apoptotic Effects in Breast Cancer Cells

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Abstract

We reported that HER2/neu reduces the sensitivity of breast cancer cells to N-(4-hydroxyphenyl)retinamide (4-HPR) by suppressing nitric oxide production. We showed that HER2/neu uses Akt to induce cyclooxygenase-2 (COX-2) expression and that inhibition of Akt or COX-2 increases 4-HPR-induced apoptosis and nitric oxide production. Apoptosis induced by the 4-HPR and COX-2 inhibitor combination, although unaffected by an anti-HER2/neu antibody, was reversed by the COX-2 product prostaglandin E2, indicating that COX-2 is a major mechanism by which HER2/neu suppresses 4-HPR apoptosis in breast cancer cells. Combining 4-HPR with COX-2 inhibitors may be a novel chemopreventive strategy against HER2/neu-overexpressing breast tumors.

Introduction

The oncogene HER2/neu is overexpressed in 25–30% of breast cancers and is associated with aggressive disease and increased resistance to a variety of cancer therapies (1). We reported recently that HER2/neu increases the resistance of breast cancer cells to N-(4-hydroxyphenyl)retinamide (4-HPR) by suppressing the production of the apoptotic molecule nitric oxide (NO; Ref. 2). However, the identities of the downstream signaling proteins used by HER2/neu to induce these effects are not known. The association between Akt activity and HER2/neu overexpression in breast cancer cells has been well established (3). Akt promotes breast cancer cell survival and therapeutic resistance to chemotherapy, trastuzumab, and tamoxifen (3). There is also increasing evidence that cyclooxygenase-2 (COX-2) may mediate the effects of HER2/neu. COX-2 catalyzes the conversion of arachidonic acid to prostaglandins (PGs). High levels of COX-2 and its main product, PGE2, have been found in human breast cancer cells and tumors that overexpress HER2/neu but not in normal breast tissue (4, 5). COX-2 overexpression increases resistance to apoptosis, particularly NO-mediated apoptosis (4, 6, 7). There also appears to be a link between Akt activity and COX-2 expression (8–11). Therefore, our aim was to determine whether HER2/neu uses Akt and COX-2 to suppress 4-HPR apoptotic effects in breast cancer cells. We report that HER2/neu uses Akt to increase COX-2 expression to reduce the sensitivity of breast cancer cells to 4-HPR. Furthermore, one mechanism by which COX-2 reduces the sensitivity of HER2/neu-overexpressing breast cancer cells to 4-HPR is via PGE2.

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Materials and Methods

Reagents. Trastuzumab (Herceptin) was provided by Genentech (San Francisco, CA). 4-HPR, PGE2, and β-actin monoclonal antibody were purchased from Sigma Chemical Co. (St. Louis, MO). HER2/neu monoclonal antibody was purchased from Oncogene (Boston, MA). Phospho-Akt (Ser473) and phospho-GSK-3β (Ser21) antibodies were purchased from Cell Signaling (Beverly, MA). The Akt inhibitor IL-6-hydroxymethyl-chiro-inositol2-(k)-2-O-methyl-3-O-octadecycarbonate was purchased from Calbiochem (San Diego, CA). The selective COX-2 inhibitor NS398 and COX-2 monoclonal antibody were purchased from Cayman Chemical (Ann Arbor, MI). Stock solutions of 4-HPR (10 μM), the Akt inhibitor (10 μM), NS398 (1 μM), and PGE2 (10 μM) were prepared in DMSO and stored at −20°C.

Cell Lines and Culture Conditions. MCF-7 breast cancer cells and the HER2/neu-overexpressing breast cancer cell lines SKBr-3 and MDA-MB-453 were obtained from American Type Cell Culture (Manassas, VA). Dr. Mien-Chie Hung (Houston, TX) provided MCF-7 cells transfected with the HER2/neu gene (MCF-7/HER2). Cells were cultured in DMEM/F12 medium supplemented with 5% heat-inactivated fetal bovine serum (FBS) at 37°C under 5% CO2 in a humidified incubator. The MCF-7/HER2 cells were cultured in DMEM/F12 medium supplemented with 5% FBS and G418 (500 μg/ml).

Western Blot Analysis. Protein lysates (50 μg) from exponentially growing MCF-7, MCF-7/HER2, SKBr-3, and MDA-MB-453 cells were loaded onto a 10% polyacrylamide gel to determine the HER2/neu and COX-2 levels of these breast cancer cell lines. Protein lysates from MCF-7/HER2 and SKBr-3 cells treated with trastuzumab for 4 days were used to determine the effect on COX-2 expression. Protein lysates from SKBr-3 cells treated with NS398 (1, 5 μM) or the Akt inhibitor (10, 20 μM) for 4 days were used to determine the effect on phospho-Akt, phospho-GSK-3β, and COX-2 levels. Western blot analyses were performed as described by Simeone et al. (2, 12).

Cell Growth and NO Assay. MCF-7/HER2, SKBr-3, and MDA-MB-453 cells were plated at 4000, 3000, and 2000 cells/well, respectively, in 96-well plates in 0.1 ml of DMEM/F12 medium supplemented with 5% FBS. The next day, the cells were treated with 4-HPR (0.1, 1.0 μM) in the presence and absence of the Akt inhibitor (10, 20 μM). After 5 days of incubation, cell growth was measured by the Celltiter 96 Aqueous nonradioactive proliferation assay (Promega, Madison, WI). Values are reported as means (average of six wells) ± SD. Each experiment was performed in triplicate.

MCF-7/HER2, SKBr-3, and MDA-MB-453 cells were plated at 75,000, 100,000, 75,000 cells/well, respectively, in six-well plates in 2 ml of DMEM/F12 medium supplemented with 5% FBS. Twenty-four h later, cells were treated with 4-HPR (0.1, 1.0 μM) in the presence and absence of the selective COX-2 inhibitor NS398 (1, 5 μM). Exogenous PGE2 also was added to some wells. MCF-7/HER2 and SKBr-3 cells were treated with 10 μM PGE2, and MDA-MB-453 cells were treated with 0.1 μM PGE2. After 5 days of incubation, cell growth was determined by total live cell counts using trypan blue exclusion. Values are reported as means (± SD) of experiments performed in triplicate.

For NO determination, supernatants were collected from the treated cells, and aliquots were stored at −80°C. Total NO was determined by quantifying nitrite using a colorimetric nonenzymatic NO assay kit (Oxford Biomedical Research, Oxford, MI) as described previously (2, 12). Nitrite values were normalized for total cell counts and expressed as μmol/million cells. Values are reported as means (± SD) of experiments performed in triplicate.
were treated with 4-HPR (0.1, 1.0 μM) in the presence and absence of the Akt inhibitor (10, 20, or 30 μM). After a 5-day incubation, cell growth was measured by the CellTiter 96 Aqueous nonradioactive proliferation assay (Promega, Madison, WI). Values are reported as means (average of six wells) ± SD. Each experiment was performed in triplicate.

Apoptosis Analysis. The effect of 4-HPR alone or in combination with NS398 on apoptosis was analyzed by flow cytometry. MCF-7/HER2, SKBr-3, and MDA-MB-453 cells were plated at 75,000, 100,000, and 75,000 cells/well, respectively, in 2 ml of DMEM/F12 medium supplemented with 5% FBS. After 24 h, cells were treated with 4-HPR (1 μM) alone or in combination with NS398 (5 μM). Cells also were treated with 4-HPR and NS398 in the presence and absence of trastuzumab. MCF-7/HER2, SKBr-3, and MDA-MB-453 cells were treated with 1.0, 0.1, and 1.0 μM trastuzumab, respectively. After 5 days of incubation, cells were harvested and prepared for flow cytometric analysis as described previously (2, 12).

Statistical Analysis. Synergistic and additive effects of 4-HPR in combination with NS398 were determined as described previously (2). Statistical analyses of the PGE2 experiments were determined as described previously (2). Results and Discussion

Inhibition of Akt Enhanced 4-HPR Inhibitory Effects in HER2/neu-Overexpressing Breast Cancer Cells. To determine whether Akt plays a role in increasing the resistance to 4-HPR in HER2/neu-overexpressing cells, an Akt inhibitor was used to block Akt activity. MCF-7/HER2, SKBr-3, and MDA-MB-453 cells were treated with 4-HPR (0.1, 1.0 μM) in the presence and absence of the Akt inhibitor (10, 20 μM). Clinically relevant doses of 4-HPR were used in these experiments (13). At the concentrations used, the Akt inhibitor alone did not inhibit the growth of the HER2/neu-overexpressing cell lines (Fig. 1). The growth of all three of the cell lines was not affected by the 0.1-μM dose of 4-HPR alone. The MCF-7/HER2, SKBr-3, and MDA-MB-453 cells also were relatively resistant to the 1.0-μM dose of 4-HPR (<20% growth inhibition). Addition of the Akt inhibitor increased the sensitivity of all three of the cell lines to 4-HPR-induced growth inhibition (Fig. 1). The Akt inhibitor combined with either 0.1 or 1 μM 4-HPR resulted in a dose-dependent increase in growth inhibition in the MCF-7/HER2 cells. The combination of the Akt inhibitor with 0.1 μM 4-HPR did not decrease cell growth in the MDA-MB-453 or SKBr-3 cells; however, the combination of the Akt inhibitor with 1 μM of 4-HPR increased growth inhibition in a dose-dependent manner in both cell lines. These results indicate that HER2/neu uses Akt to increase the resistance of breast cancer cells to 4-HPR.

HER2/neu Uses Akt to Induce COX-2 Expression in Breast Cancer Cells. Increased levels of COX-2 have been found in HER2/neu-overexpressing breast cancer cells (7). We confirmed that parental MCF-7 cells, which express low levels of HER2/neu (Fig. 2A), have lower COX-2 levels than do MCF-7/HER2 cells (Fig. 2B). Furthermore, blocking HER2/neu with the anti-HER2/neu antibody trastuzumab decreased COX-2 expression in the MCF-7/HER2 and SKBr-3 cells (Fig. 2B).

Studies indicate that there is a relationship between Akt activity and COX-2 expression. Activation of Akt and subsequent inactivation of GSK-3β was found to be essential for the transcriptional activation of the COX-2 gene in human keratinocytes in response to UVB (10). To determine whether HER2/neu uses Akt to increase COX-2 levels, SKBr-3 cells were treated with the Akt inhibitor (10, 20 μM) or the selective COX-2 inhibitor NS398 (1, 5 μM). The Akt inhibitor effectively suppressed Akt activity, as shown by the decreased phosphorylation of its downstream target GSK-3β (Fig. 2C). Inhibition of Akt reduced COX-2 expression in SKBr-3 cells (Fig. 2C). In a human lung adenocarcinoma cell line, COX-2 promoted cell survival by activating an Akt-dependent pathway (9). In the current study, however, inhibition of COX-2 by NS398 did not affect the activity of Akt or its downstream target GSK-3β in SKBr-3 cells (Fig. 2C). These results indicate that COX-2 is a downstream target of Akt in breast cancer cells that overexpress HER2/neu.

Inhibition of COX-2 Enhances 4-HPR-Induced NO Production and Apoptosis in HER2/neu-Overexpressing Breast Cancer Cells. MCF-7/HER2, SKBr-3, and MDA-MB-453 cells were treated with 4-HPR (0.1, 1.0 μM) in the presence and absence of the COX-2 inhibitor NS398 (1, 5 μM). Clinically achievable doses of the COX-2 inhibitor were used in these experiments (14). In all three of the cell lines, NS398 alone and the 0.1-μM dose of 4-HPR did not affect (P > 0.05) NO production (Fig. 3). NO production was not induced (P > 0.05) by the 1.0-μM dose of 4-HPR in the MDA-MB-453 cells, whereas small but significant increases (P < 0.01) in NO production were observed in the MCF-7/HER2 and SKBr-3 cells. In the MCF-7/HER2 and SKBr-3 cells, the addition of NS398 enhanced 4-HPR-mediated NO production in a dose-dependent manner at both doses of NO
4-HPR (Fig. 3). In the MCF-7/HER2 and SKBr-3 cells, an additive and synergistic effect ($P < 0.01$) on NO production was observed when 0.1 μM 4-HPR was combined with 1 and 5 μM NS398, respectively (Fig. 3). Synergistic effects were obtained when 1 μM 4-HPR was combined with either 1 or 5 μM NS398 in MCF-7/HER2 and SKBr-3 cells. The 4-HPR and COX-2 inhibitor combination also was effective in increasing NO production in MDA-MB-453 cells, which express lower levels of COX-2 than do MCF-7/HER2 and SKBr-3 cells. However, in the MDA-MB-453 cells, 1 μM NS398 was as effective as the 5-μM dose in increasing 4-HPR-mediated NO production at both doses of 4-HPR (Fig. 3). In the MDA-MB-453 cells, additive effects were observed when the 0.1 and 1 μM doses of 4-HPR were combined with either 1 or 5 μM NS398.

Flow cytometry was used to compare apoptosis induced by 4-HPR (1 μM) in the presence and absence of NS398 (5 μM). In the presence of NS398, the proportion of apoptotic cells induced by 4-HPR was increased from 2.6%, 4.4%, and 3.4% to 23.7%, 25.5%, and 6.9% in MCF-7/HER2, SKBr-3, and MDA-MB-453 cells, respectively (Tables 1 and 2). Thus, NO production was correlated directly with increased apoptosis induced by the 4-HPR and NS398 combination in all three of the cell lines.

We have shown previously that trastuzumab enhances 4-HPR-induced apoptosis (2). To prove further that HER2/neu uses COX-2 to decrease the sensitivity to 4-HPR, the three cell lines were treated with 4-HPR and NS398 in the presence and absence of trastuzumab. Clinical doses of trastuzumab were used in this experiment (15). The addition of trastuzumab did not affect the number of apoptotic cells induced by the 4-HPR and NS398 combination in any of the
cell lines (Table 1A), suggesting that COX-2 is a major mechanism by which HER2/neu suppresses the effects of 4-HPR. Mann et al. (16) reported that the combination of trastuzumab with the selective COX-2 inhibitor celecoxib was more effective in vitro and in vivo against colorectal carcinoma growth than either agent alone. Differences in cell type, status of HER2/neu overexpression and/or amplification, and the doses of trastuzumab and COX-2 inhibitor used may explain the discrepancy between our results and those of Mann et al. (16).

**PGE2 Suppresses Apoptosis Induced by the 4-HPR and COX-2 Inhibitor Combination.** To determine whether PGE2, a major product of COX-2, mediates the COX-2-induced 4-HPR resistance, exogenous PGE2 was added to HER2/neu-overexpressing cells treated with 4-HPR and the COX-2 inhibitor NS398. The three cell lines were relatively resistant to 1 μM 4-HPR alone (≤30% growth inhibition) and to 5 μM NS398 alone (≤20% growth inhibition; Table 2). However, these small decreases in cell counts induced by 4-HPR alone and NS398 alone were statistically significant (P < 0.05) in MCF-7/HER2 and SKBr-3 cells. At the concentrations used, PGE2 induced <25% cell growth inhibition; however, these decreases in cell counts were statistically significant (P < 0.05) in SKBr-3 and MDA-MB-453 cells. Addition of NS398 increased (P < 0.05) the sensitivity of MCF-7/HER2, SKBr-3, and MDA-MB-453 cells to 4-HPR-induced apoptosis (Table 2). The apoptotic effects of the 4-HPR and NS398 combination were significantly (P < 0.05) reduced in all of the cell lines in the presence of exogenously added PGE2 (Table 2). NO produced by the 4-HPR and NS398 combination also was significantly (P < 0.05) decreased in all three of the cell lines by the addition of exogenous PGE2 (data not shown). These data indicate PGE2 as one mechanism by which COX-2 induces 4-HPR resistance in HER2/neu-overexpressing breast cancer cells.

Our data demonstrate that Akt also plays a vital role in increasing the 4-HPR resistance in HER2/neu-overexpressing breast cancer cells. The Akt-dependent pathway may be independent of COX-2. This is because despite a dose-dependent increase in 4-HPR-mediated growth inhibition with the addition of the Akt inhibitor, decreases in COX-2 expression were similar at both doses of the Akt inhibitor. These data suggest that Akt, whether operating in a COX-2-dependent or -independent manner, is essential for HER2/neu to decrease the sensitivity of breast cancer cells to 4-HPR.

Breast cancers that overexpress HER2/neu are resistant to a variety of cancer therapies (1). Although trastuzumab represents a significant development for the management of HER2/neu-positive breast cancer, its use is limited by low response rates and the development of drug resistance (17). COX-2 inhibitors have shown promise as chemopreventive agents against HER2/neu-positive tumors. Selective COX-2 inhibitors have been shown to inhibit the development of mammary tumors in transgenic mice that express high levels of HER2/neu in the mammary gland (18) and to reduce the growth rate of a HER2/neu-positive colon cancer cell line (16). In the present study, the 4-HPR plus COX-2 inhibitor combination resulted in additive and synergistic effects on apoptosis and NO production in HER2/neu-overexpressing breast cancer cells, whereas the trastuzumab plus COX-2 inhibitor combination did not result in increased apoptosis. The well-documented long-term tolerability and chemopreventive properties of 4-HPR make it an extremely advantageous agent to be used in combination therapies (19). Pharmacologically achievable doses of 4-HPR and the COX-2 inhibitor also were used in these studies (13, 14). Thus, our findings indicate that the combination of 4-HPR with selective COX-2 inhibitor may be a novel and effective adjuvant

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### Table 1 Inhibition of COX-2 enhanced 4-HPR-induced apoptosis in HER2/neu-overexpressing breast cancer cells

<table>
<thead>
<tr>
<th>4-HPR (1 μM)</th>
<th>MCF-7/HER2 cells</th>
<th>% Apoptotic cells</th>
<th>SKBr-3 cells</th>
<th>% Apoptotic cells</th>
<th>MDA-MB-453 cells</th>
<th>% Apoptotic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>-</td>
<td>NS398 (5 μM)</td>
<td>Trastuzumab (1.0 μM)</td>
<td>-</td>
<td>NS398 (5 μM)</td>
<td>Trastuzumab (1.0 μM)</td>
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### Table 2 PGE2 suppressed the growth inhibition induced by the 4-HPR and COX-2 inhibitor combination in HER2/neu-overexpressing breast cancer cells

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<th>4-HPR (1 μM)</th>
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<td>42.5 ± 1.7</td>
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<td>45.5 ± 2.9</td>
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<td>40.8 ± 1.4</td>
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<table>
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<th>Cell count (×10^5)</th>
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<tr>
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4-HPR, N-(4-hydroxyphenyl)retinamide.

5 Selective cyclooxygenase-2 (COX-2) inhibitor.

6 Percentage of apoptotic cells (i.e., cells in the sub-G1 peak) was evaluated by flow cytometric analysis of propidium iodide staining.

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chemopreventive strategy for breast cancer patients with HER2/neu-overexpressing tumors.

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