Cyclooxygenase-2 utilizes Jun N-terminal kinases to induce invasion, but not tamoxifen resistance, in MCF-7 breast cancer cells

VIANEY GONZALEZ-VILLASANA1,2, YOLANDA GUTIÉRREZ-PUENTE1 and ANA M. TARI2,3

1Department of Biochemistry, Faculty of Biological Sciences, University of Nuevo Leon, San Nicolas de los Garza, Nuevo León, Mexico; 2Department of Experimental Therapeutics, University of Texas M.D. Anderson Cancer Center, Houston, TX; 3Department of Neuroscience, University of Florida, Gainesville, FL, USA

Received April 4, 2013; Accepted May 29, 2013

DOI: 10.3892/or.2013.2549

Abstract. Elevated cyclooxygenase-2 (COX-2) expression in breast tumors is associated with a lower survival rate in patients with estrogen receptor α (ERα)-positive tumors. We hypothesized that COX-2 reduces the survival rate of breast cancer patients with ERα-positive tumors since COX-2 increases the invasiveness of ERα-positive breast tumors and decreases tumor sensitivity to tamoxifen. Previously, we demonstrated that COX-2 stimulates the activity of protein kinase C (PKC) to increase the invasiveness of ERα-positive MCF-7 breast cancer cells and to decrease the sensitivity of MCF-7 cells to tamoxifen. High levels of COX-2 are associated with the activation of the mitogen-activated protein kinase (MAPK) family and the Akt kinase. However, it is not known whether these kinases mediate COX-2-induced invasive activity and tamoxifen resistance. In the present study, we report that COX-2 utilizes PKC to enhance the phosphorylation of Jun N-terminal kinases (JNKs), but not that of other MAPK family members or Akt. Inhibition aimed at JNKs reduced COX-2-induced invasion but not COX-2-induced tamoxifen resistance. We conclude that JNKs are essential for induced cell invasion by COX-2, but not tamoxifen resistance, in ERα-positive breast cancer cells.

Introduction

A number of studies have shown high levels of the cyclooxygenase-2 (COX-2) protein in solid tumors (1-6). In breast cancer, COX-2 expression is a predictor of poor disease-free and overall survival (4-9). In a retrospective study of 1,576 invasive breast tumors, Ristimaki et al (4) found that elevated COX-2 expression was associated with a lower survival rate in patients with estrogen receptor α (ERα)-positive breast tumors. Women whose invasive breast tumors were ERα-positive but had low levels of COX-2 had an 86% chance of 5-year distant disease-free survival, whereas women whose tumors were ERα-positive but had high levels of COX-2 had a 76% chance of 5-year distant disease-free survival (4).

Breast cancer patients who have ERα-positive breast tumors are typically treated with selective estrogen receptor modulators (SERMs). We previously demonstrated that transfection of the COX-2 gene into the tamoxifen-sensitive, ERα-positive MCF-7 breast cancer cell line (MCF-7/COX-2) reduced the sensitivity of MCF-7 cells to tamoxifen by ~5-fold (10). These data suggest that breast cancer patients who have ERα-positive and COX-2-overexpressing tumors may not benefit from tamoxifen as much as patients who have low levels of COX-2 in their ERα-positive breast tumors.

Elevated levels of COX-2 have also been associated with lymph node and distant metastasis (11,12). COX-2 has been shown to increase breast cancer cell invasion in vitro (13-15) and in vivo (16-18). We and others demonstrated that MCF-7/COX-2 cells are ~3-fold more invasive than parental MCF-7 cells (13,14). The decrease in tamoxifen sensitivity and increase in invasive activity by COX-2 may contribute to the reduced survival rate noted in patients with ERα-positive, COX-2-overexpressing breast tumors.

COX-2 utilizes its product prostaglandin E2 (PGE2) to stimulate protein kinase C (PKC) activity. We demonstrated that activation of PKC reduced the anti-proliferative effects of tamoxifen (10), and increased the invasiveness of MCF-7 cells across a Matrigel basement membrane (13). Although high levels of COX-2 have been associated with activation of the mitogen activated protein kinase (MAPK) family (19-21) and the Akt kinase (22), it is not known whether these kinases mediate COX-2-induced tamoxifen resistance and invasive activity. In the present study, we report that COX-2 utilizes PKC to increase the activity of Jun N-terminal kinases (JNKs) to mediate invasion, but not tamoxifen resistance, in MCF-7 breast cancer cells.
Materials and methods

**Cell lines and culture conditions.** The MCF-7 human breast cancer cell line was obtained from the American Type Cell Culture (ATCC, Manassas, VA, USA). MCF-7/COX-2 cells were generated by stably transfecting plasmids encoding the COX-2 gene into ERα-positive MCF-7 cells (10,13). MCF-7/COX-2 cells were obtained from individual colonies, and continuously cultured in DMEM/F-12 medium containing 5% FBS and 500 µg/ml G418. We selected clone 12, which expressed higher levels of COX-2 than the parental MCF-7 cells (10,13) for our studies.

**Chemical reagents.** Tamoxifen citrate, G66976, SP600125 and PD98059 were purchased from EMD Chemicals (San Diego, CA, USA). Stock solutions (10 mM) of tamoxifen, G66976, SP600125 and PD98059 were prepared in DMSO and stored at -20°C. All reagents were diluted in culture medium to the indicated final concentration. Matrigel was purchased from BD Biosciences (Bedford, MA, USA). Antibodies specific for phosphorylated ERK (T185/Y185), phosphorylated p38MAPK (T182/Y185), phosphorylated Akt (S473), phosphorylated c-Jun N-terminal kinase (JNK) (T183/Y185), ERK, p38MAPK, Akt, JNK, and c-Jun were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies specific for β-actin and Histone H3 were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Anti-mouse and anti-rabbit secondary antibodies conjugated with horseradish peroxidase were purchased from Amersham Life Sciences (Cell Signaling Technology).

**Western blotting.** Western blotting was performed as previously described (10,23). MCF-7 parental and MCF-7/COX-2 cells were plated at 4x10⁵ cells/well in 6-well plates in DMEM/F-12 medium containing 5% FBS. Two days later, cells were harvested and cell pellets were lysed. The protein concentration was determined using the Bio-Rad DC protein assay. Nuclear proteins were extracted as previously described (23). MCF-7 and MCF-7/COX-2 cells were plated at 4x10³ cells/well in 6-well plates in DMEM/F-12 medium containing 5% FBS. Cells were harvested, and cell pellets were lysed with 250 µl buffer (10 mM HEPES, 10 mM KCl, 0.5% Nonidet P-40, pH 7.9) on ice for 15 min. Nuclei were pelleted by centrifugation at 13,000 rpm for 1 min, and nuclear proteins were extracted with 50 µl nuclear extraction buffer (20 mM HEPES, 400 mM NaCl, pH 7.9).

**Western blot analysis.** Membranes were incubated with the anti-Histone H3 antibody (1:5,000 dilution) overnight at 4°C. Histone H3 was used as a loading control. Membranes were incubated with the anti-mouse secondary antibody (1:5,000 dilution) for another 30 min at room temperature.

**Nuclear proteins.** Nuclear proteins were extracted from individual colonies of MCF-7/COX-2 cells as described above. Nuclear proteins (50 µg) were electrophoresed on 12% polyacrylamide gels (Bio-Rad Laboratories), transferred to nitrocellulose membranes (Bio-Rad Laboratories), and western blot analysis for c-Jun was performed (primary antibody was added at a 1:500 dilution). Histone H3 was used as a loading control. Membranes were incubated with the anti-Histone H3 antibody (1:5,000 dilution) for 30 min at room temperature, washed, and with the anti-mouse secondary antibody (1:5,000 dilution) for another 30 min at room temperature.

**Matrigel invasion assay.** Matrigel invasion assay was performed as previously described (13,23,24) by counting the number of cells that invaded through Transwell inserts coated with the Matrigel artificial basement membrane. Six-well plate Transwell inserts with 8-µm pore-size polycarbonate filters (Thermo Fisher Scientific, Middleton, VA, USA) were coated with Matrigel (0.7 mg/ml) and placed at room temperature for 40 min. MCF-7/COX-2 cells (4x10³ in 500 µl) were pretreated with the JNK inhibitor SP600125 or the ERK inhibitor PD98059 (0, 5 or 10 µM) in DMEM/F-12 medium containing 5% FBS for 30 min before being added to the Matrigel-coated Transwell inserts. Seventy-two hours later, cells that invaded through the Matrigel onto the lower side of the filter were fixed, stained with Hema-3 and photographed. The invaded cells from each filter were counted in five fields under a light microscope at magnification x40. The invasiveness of MCF-7/COX-2 cells was expressed as the mean number of cells that had invaded to the lower side of the filter. The experiments were performed in triplicate wells.

**CellTiter 96 Aqueous non-radioactive proliferation assay.** The inhibitory effects of tamoxifen on MCF-7/COX-2 cells were studied as previously described (10). MCF-7/COX-2 cells were plated at 1,000 cells/well in 96-well plates in 0.1 ml of DMEM/F-12 medium supplemented with 5% FBS. The next day, the medium was replaced with DMEM/F-12 medium supplemented with 5% charcoal-stripped serum (CSS). Twenty-four hours later, cells were pretreated with 500125 (0, 5 and 10 µM) before being treated with various concentrations of tamoxifen for 5 days. At the end of the incubation, cell proliferation was determined by the Promega (Madison, WI, USA) CellTiter 96 Aqueous non-radioactive proliferation (MTS) assay and was expressed as the percentage of proliferating cells relative to the untreated cells.
Results

COX-2 overexpression increases phosphorylated JNK levels and nuclear c-Jun levels. High levels of COX-2 or PGE,
have been associated with activation of MAPKs (19-21) and Akt (22). We sought to determine whether MAPK and/or Akt are utilized by COX-2 to induce breast cancer cell invasion and tamoxifen resistance. First, we determined whether COX-2 overexpression alters the phosphorylation levels of the MAPK family or that of Akt. Phosphorylation levels of ERK (Fig. 1A), p38 MAPK (Fig. 1B) and Akt (Fig. 1C) were very similar between the MCF-7/COX-2 and MCF-7 parental cells. However, higher JNK phosphorylation levels were observed in MCF-7/COX-2 cells when compared with levels in the MCF-7 cells (Fig. 2A). This corresponded to higher nuclear c-Jun levels in MCF-7/COX-2 cells than levels in MCF-7 cells (Fig. 2B). Previously we showed that PKC plays an essential role in mediating COX-2-induced invasion and tamoxifen resistance in MCF-7 breast cancer cells (10). To determine whether JNK is downstream of PKC in the COX-2 pathway, we treated MCF-7/COX-2 cells with the PKC inhibitor Gö6976 and utilized western blotting to analyze JNK phosphorylation levels. Inhibition of PKC resulted in decreased JNK phosphorylation in the MCF-7/COX-2 cells (Fig. 2C). These data indicate that PKC regulates COX-2-induced JNK activation.

Inhibition of JNKs decreases the invasiveness of MCF-7/COX-2 cells. Since we observed that COX-2 stimulates JNK phosphorylation, we hypothesize that COX-2 utilizes JNKs to induce invasion. MCF-7/COX-2 breast cancer cells were pretreated with SP600125, a chemical inhibitor against JNKs, to determine whether JNK inhibition would decrease COX-2-mediated invasion. At 5 and 10 µM concentration, SP600125 decreased MCF-7/COX-2 invasive activity by ~75 and 80%, respectively (Fig. 3A). On the other hand, since COX-2 did not increase ERK phosphorylation, we did not expect ERK to mediate COX-2-induced invasion. Indeed, when we treated MCF-7/COX-2 cells with PD98059, a chemical inhibitor against ERK, PD98059 did not affect MCF-7/COX-2 invasive activity (Fig. 3B). These data indicate that JNKs, not ERK, mediate COX-2-induced breast cancer cell invasion.

Inhibition of JNKs does not affect tamoxifen sensitivity in MCF-7/COX-2 cells. We determined whether JNKs are also essential for COX-2 to induce tamoxifen resistance. As shown
in our previous report (10), MCF-7/COX-2 cells were very insensitive to tamoxifen. At 5 µM concentration, tamoxifen inhibited MCF-7/COX-2 cell growth by only 20% (Fig. 4). In the presence of 5 and 10 µM SP600125, tamoxifen (at 5 µM) inhibited MCF-7/COX-2 cell growth by 30 and 40%, respectively (P>0.05) (Fig. 4). These data indicate that JNKs do not mediate COX-2-induced tamoxifen resistance.

**Discussion**

In the present study, we demonstrated that COX-2 utilizes PKC to stimulate JNK activity, which is essential for COX-2 to induce breast cancer cell invasion. JNKs have been shown to mediate the invasive activity of breast cancer cells induced by various proteins, including interleukin-8 (13), leptin (23) and transglutaminase (25). Activated JNKs can translocate to the nucleus where they regulate transcription factors such as c-Jun, ATF-2, Elk-1, p53 and c-Myc, resulting in enhanced expression and/or activity of proteases, such as urokinase plasminogen activator (13), metalloproteinase-2 (23), or reduced expression of programmed cell death 4 (26), a candidate tumor suppressor gene, thereby enhancing the ability of breast cancer cells to invade through the basement membrane. Wang et al (27) also demonstrated that JNKs mediate epithelial-mesenchymal transition, survival and proliferation of breast cancer cells. These pro-tumorigenic properties of JNKs may explain why elevated levels of phosphorylated JNKs have been correlated with a poorer prognosis in breast cancer patients (28,29).

Tamoxifen is the most widely used drug for breast cancer treatment. Unfortunately, many patients with advanced ERα-positive disease fail to respond to tamoxifen, and many responsive patients acquire resistance to tamoxifen, leading to disease progression. Although upregulation of JNK activity, c-Jun phosphorylation and AP-1 DNA binding activity have been found in ERα-positive breast tumors with the acquired tamoxifen resistance phenotype (30,31), JNKs have not been shown to mediate tamoxifen resistance. Here, we demonstrated that inhibition of JNKs did not resensitize COX-2-overexpressing breast cancer cells to tamoxifen. Our data suggest that either JNKs are not involved in regulating tamoxifen sensitivity, or that inhibition of JNKs alone is not sufficient to reverse tamoxifen resistance. Indeed several mechanisms, including loss or modification of ERα expression, regulation of signal transduction pathways, altered expression of specific microRNAs, balance of co-regulatory proteins and genetic polymorphisms involved in tamoxifen metabolism, could contribute to the development of tamoxifen resistance (32-34).
In conclusion, we found that COX-2 increased the activity of JNKs to mediate invasion, but not tamoxifen resistance, in MCF-7 breast cancer cells. In breast cancer, COX-2 expression is a predictor of poor disease-free and overall survival (4-9) and has been implicated as a marker of high metastatic potential (11,12). Pharmacological inhibition aiming at JNKs may have potential therapeutic benefit in patients with ER-positive COX-2-overexpressing breast tumors by reducing tumor invasiveness and metastatic potential.

Acknowledgements

The present study was supported by the Susan G. Komen Breast Cancer Foundation.

References