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

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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 cyclo-oxygenase-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 E_2 (PGE_2) 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.

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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, Gö6976, SP600125 and PD98059 were purchased from EMD Chemicals (San Diego, CA, USA). Stock solutions (10 mM) of tamoxifen, Gö6976, 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 (T^{202}/Y^{204}), phosphorylated p38MAPK (T^{183}/Y^{185}), phosphorylated Akt (S^{473}), phosphorylated c-Jun N-terminal kinase (JNK) (T^{183}/Y^{185}), 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 $4x10^5$ 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 DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Samples were electrophoresed on 12% polyacrylamide gels (Bio-Rad Laboratories), then transferred to nitrocellulose membranes (Bio-Rad Laboratories) for western blot analysis. Membranes were blocked in Tris-buffered saline (20 mM Tris pH 7.6, 150 mM NaCl) with 0.1% Tween-20 containing 5% non-fat dry milk (Bio-Rad Laboratories) at room temperature for 30 min. After washing, the membranes were incubated with primary antibodies (1:1,000 dilution) overnight at 4°C. The next day, membranes were washed and incubated with anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:1,000 dilution) for 2 h at room temperature. Proteins bands were detected via enhanced chemiluminescence (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Images were scanned by an AlphaImager densitometer (Alpha Innotech Corp., San Leandro, CA, USA). β-actin was used as a loading control. Membranes were incubated with anti-β-actin antibody (1:10,000 dilution) for 30 min at room temperature, washed, and anti-mouse secondary antibody (1:10,000 dilution) for another 30 min at room temperature.

To determine the effect of PKC inhibition on JNK phosphorylation, MCF-7/COX-2 cells were plated at $4x10^5$ cells/well in 6-well plates in DMEM/F-12 medium containing 5% FBS. Two days later, cells were treated with the PKC inhibitor Gö6976 (0, 25 and 50 nM) for 8 h. Untreated and treated cells were harvested and lysed. Western blot analysis was performed as described above.

Extraction of nuclear proteins. Nuclear proteins were extracted as previously described (23). MCF-7 and MCF-7/COX-2 cells were plated at $4x10^5$ 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). Nuclear protein concentrations were determined using the Bio-Rad DC protein assay. 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 preformed 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 ($4x10^5$ 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 SP600125 (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.
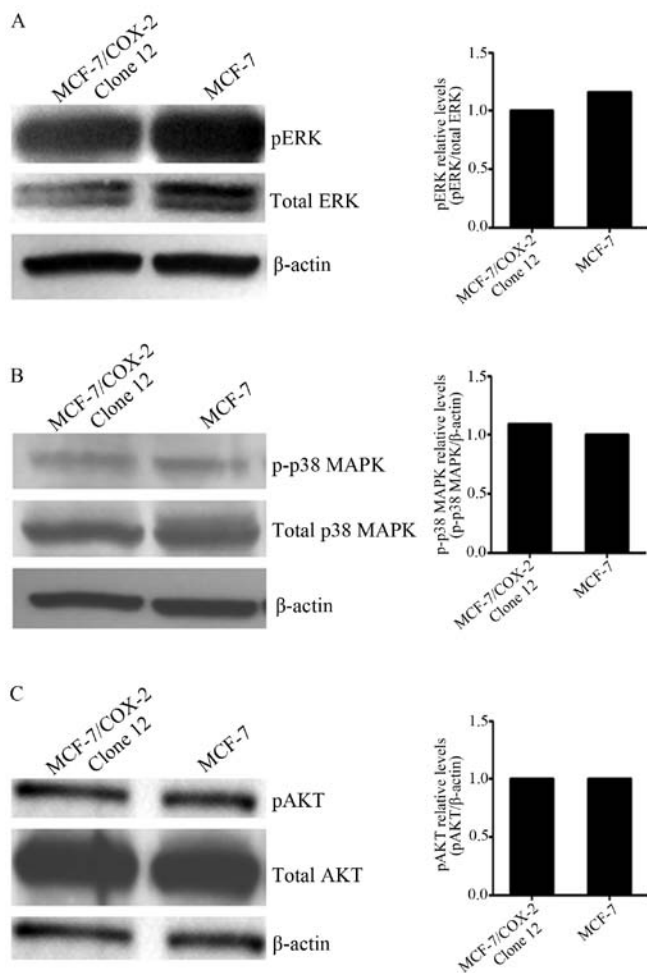
Figure 1. COX-2 does not enhance ERK, p38 MAPK or Akt phosphoryla-
tion in MCF-7 cells. COX-2 overexpression did not increase (A) ERK,
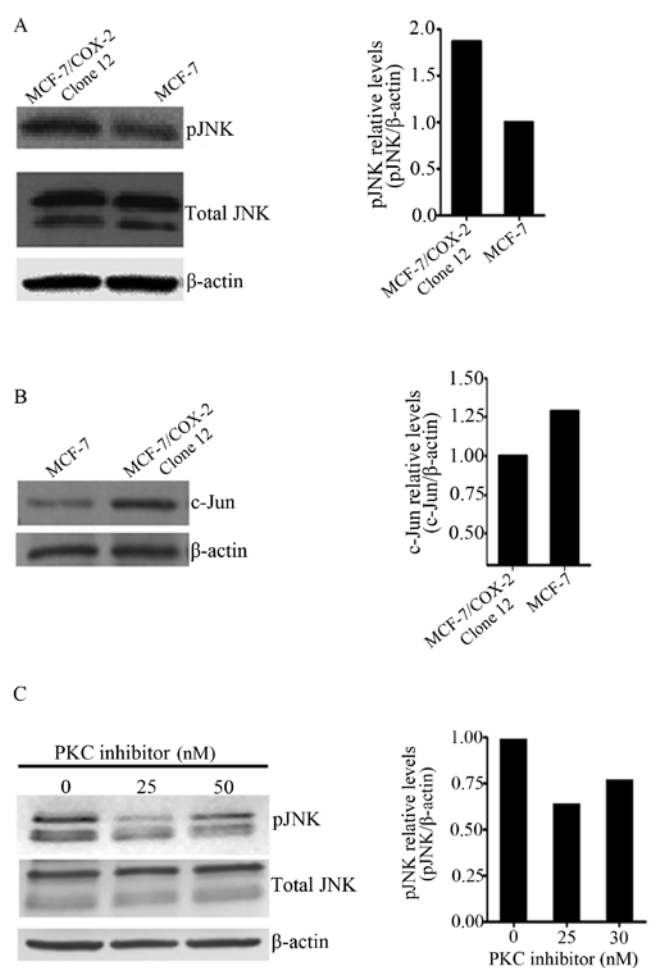(B) p38 MAPK or (C) Akt phosphorylation.

Figure 2. COX-2 utilizes PKC to enhance JNK phosphorylation and nuclear
c-Jun levels in MCF-7 cells. (A) JNK phosphorylation and (B) nuclear c-Jun
levels were higher in the MCF-7/COX-2 cells when compared with levels in
the MCF-7 breast cancer cells. (C) Inhibition of PKC decreased JNK phos-
phorylation in the MCF-7/COX-2 cells.

Results

*COX-2 overexpression increases phosphorylated JNK levels
and nuclear c-Jun levels.* High levels of COX-2 or PGE_2,
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 inva-
sion 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 phos-

phorylation 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 phos-
phorylation, 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
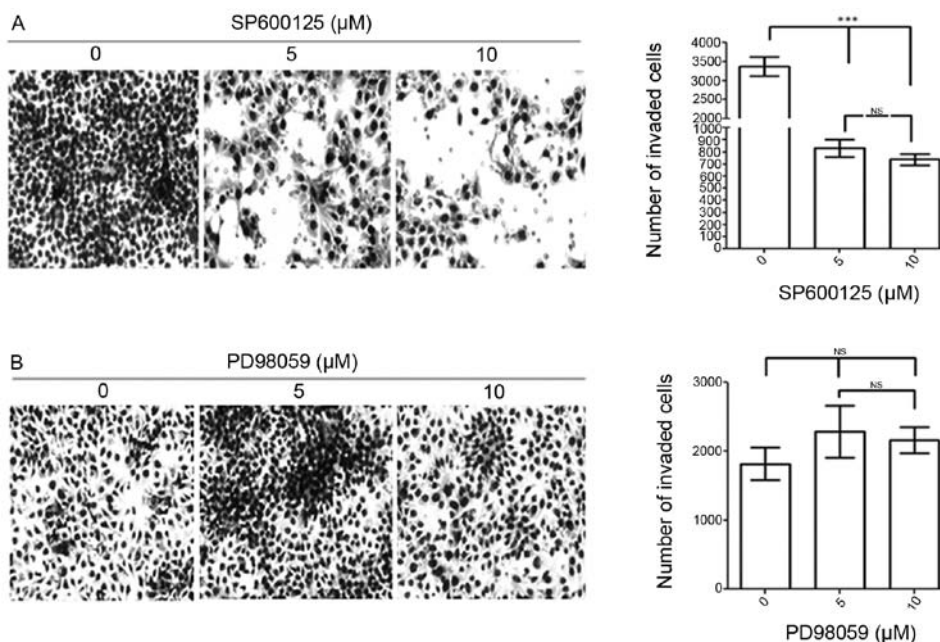essential for COX-2 to induce tamoxifen resistance. As shown

Figure 3. Inhibition aimed at JNK, but not ERK, decreases the invasion of MCF-7/COX-2 cells. Matrigel invasion assay was used to assess the invasiveness of MCF-7/COX-2 cells treated with (A) a JNK inhibitor or (B) an ERK inhibitor.
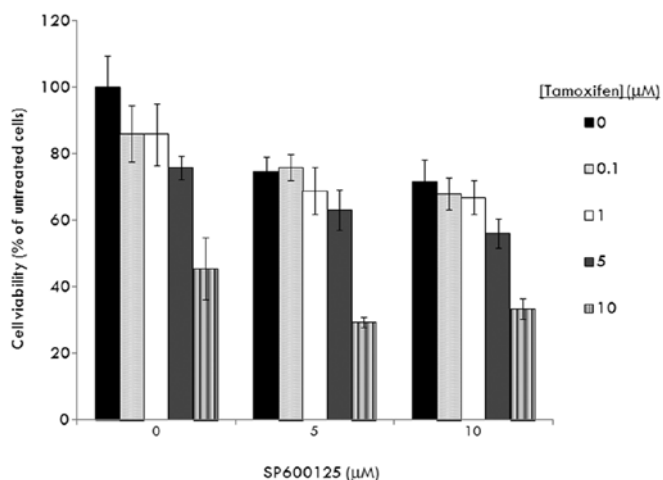

Figure 4. JNK inhibition does not alter the tamoxifen sensitivity of MCF-7/COX-2 cells. MTS assay was used to evaluate the effect of SP600125 on the sensitivity of MCF-7/COX-2 cells to tamoxifen.

by various proteins, including interleukin-8 (13), leptin (23) and transglutaminase (25). Activated JNKs can translocate to the nucleus where they regulates 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 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

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 ERα-positive COX-2-overexpressing breast tumors by reducing tumor invasiveness and metastatic potential.

Acknowledgements

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