TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Cigarette Smoke Regulates Calcium-Independent Phospholipase A₂ Metabolic Pathways in Breast Cancer

Shannon Kispert, Theresa Schwartz, and Jane McHowat

From the Department of Pathology, Saint Louis University School of Medicine, St. Louis, Missouri

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Address correspondence to
Jane McHowat, Ph.D., Department
of Pathology, Saint Louis
University School of Medicine,
1402 S Grand Blvd, St. Louis,
MO 63104. E-mail:
mchowaj@slu.edu.

Phospholipase A₂ (PLA₂)—dependent pathways are important in the regulation of cell proliferation, differentiation, motility, and immune responses, and can be dysregulated during tumor development and progression. We show herein, for the first time, that cigarette smoking leads to an increase in platelet-activating factor (PAF) content and PAF receptor expression in human breast cancer cells and tissue. PAF production could be abrogated in triple-negative breast cancer cells by inhibition of calcium-independent PLA₂ (iPLA₂). We also demonstrate that cigarette smoke induces the expression of cyclooxygenase-2 and microsomal prostaglandin E synthase-1 and reduces 15-hydroxyprostaglandin dehydrogenase, resulting in prostaglandin E₂ release in human breast cancer. Increased cyclooxygenase-2 expression and prostaglandin E₂ release could be abrogated in metastatic breast cancer cells by inhibition of iPLA₂. These studies indicate that iPLA₂-dependent metabolic pathways play an important role in tumor initiation or progression in smokers, representing novel therapeutic targets for breast cancer patients who smoke. (*Am J Pathol* 2017, 187: 1855–1866; <http://dx.doi.org/10.1016/j.ajpath.2017.04.003>)

There are many risk factors for cancer development and subsequent metastasis, which include adverse lifestyle choices such as diet, alcohol consumption, and cigarette smoking.^{1,2} Despite a decline during the past three decades, cigarette smoking among adults in the United States remains widespread and year-to-year decreases in prevalence have been seen only intermittently in recent years.¹ The Centers for Disease Control and Prevention estimate that 40 million Americans continue to smoke, which is approximately one in every five adults.³ Cigarette smoking is responsible for 480,000 deaths each year and continues to be the leading cause of preventable disease and death.¹

Several cohort studies have described the link between patients with breast cancer and those with long-term cigarette smoking history.¹ In addition, several studies have demonstrated that components of cigarettes are found in human breast milk, as well as nipple aspirate, of smokers, advocating for the direct involvement of cigarette smoke in breast tissue injury.^{4,5} Despite these claims of correlation from cohort studies and verification of the presence of cigarette components in the breast, the issue remains

controversial because of lack of consistent data and underlying mechanisms.

Phospholipase A₂ (PLA₂) enzymes collectively contribute to a wide variety of pathways involved in cell differentiation, proliferation, and apoptosis, all of importance in tumor development and progression.⁶ Calcium-independent PLA₂ (iPLA₂) enzymes are responsible for the hydrolysis of membrane phospholipids at the *sn*-2 fatty acid to produce a free fatty acid and a lysophospholipid (Figure 1). Free arachidonic acid can be modified by cyclooxygenase enzymes (COX) to produce prostaglandin H₂ (PGH₂) (Figure 1). Modification of PGH₂ by microsomal prostaglandin E synthase-1 (mPGES-1) leads to the production of prostaglandin E₂ (PGE₂), which plays a relevant role in inflammation as well as cancer progression^{7–15} (Figure 1). The remaining lysophospholipid from alkyl ether phosphatidylcholine can be acetylated to form

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platelet-activating factor (PAF), which has been implicated in oncogenic pathways^{16–25} (Figure 1).

Smoking has been associated with breast cancer development and progression, yet the mechanism underlying this correlation remains unclear. In this study, we used normal mammary epithelial cells, MCF-10A, hormone-positive breast tumor cells, MCF-7, and triple-negative breast tumor cell lines, MDA-MB-468 and MDA-MB-231, as well as human breast tumor tissue to identify the result of cigarette smoking in iPLA₂-initiated metabolic pathways in breast cancer. We show herein, for the first time, that cigarette smoking leads to increased PAF accumulation and PAF receptor (PAF-R) expression in human breast cancer cells and human tumor tissue. We observed increased PGE₂ release in breast cancer cells as well as increases in the expression of enzymes responsible for PGE₂ production, COX-2 (which catalyzes the reaction of arachidonic acid to the precursor PGH₂), and mPGES-1 (responsible for the conversion of PGH₂ to the active form PGE₂), in breast cancer cells and human tumor tissue after exposure to cigarette smoke. In addition to increased expression of these enzymes, we saw decreased expression of 15-hydroxyprostaglandin dehydrogenase (15-PGDH), which is responsible for PGE₂ catabolism. The increase in COX-2 could be abrogated by treatment with an iPLA₂ inhibitor, bromoenol lactone (BEL), suggesting therapeutic potential for manipulation of this pathway in current smokers.

Materials and Methods

Reagents

Human breast cancer lines and MCF-10A cells were obtained from ATCC (Manassas, VA). Cigarette smoke extract (CSE) was obtained from Murty Pharmaceuticals (Lexington, KY). COX-2, iPLA₂β, PAF-R, mPGES-1, and 15-PGDH antibodies were obtained from Cayman (Ann Arbor, MI). PAF antibody was obtained from Abbiotec (San Diego, CA). PGE₂ and iPLA₂γ antibodies were obtained from Abcam (Cambridge, UK). All other chemicals were obtained from Sigma Chemical Co (St. Louis, MO).

Cell Culture

Cells were grown to confluence in Dulbecco's modified Eagle's medium or mammary epithelium basal medium (Lonza, Mapleton, IL) with 10% fetal bovine serum and penicillin/streptomycin/amphotericin. Cells were incubated at 37°C, with an atmosphere of 95% O₂ and 5% CO₂. Cells were subcultured using 0.05% trypsin/EDTA and passaged in a 1:3 ratio. Cells were incubated in certain experiments with 20 μg/mL CSE or 5 μmol/L iPLA₂ inhibitor BEL, as noted.

Figure 1 Proposed effects of cigarette smoke exposure on calcium-independent phospholipase A₂ (iPLA₂)-dependent metabolic pathways in breast cancer. We hypothesize that cigarette smoke exposure leads to the increase of cyclooxygenase-2 (COX-2), prostaglandin H₂ (PGH₂), prostaglandin E synthase (PGES), and prostaglandin E₂ (PGE₂) release. The increase of PGE₂ release is further exacerbated by 15-hydroxyprostaglandin dehydrogenase (15-PGDH) reduction by cigarette smoke exposure. In addition to prostaglandin modification, we hypothesize cigarette smoke also causes the increase of platelet-activating factor (PAF) and reduction of PAF-acetylhydrolase (PAF-AH).

Breast Tissue

Biopsy specimens were obtained from patients with low-grade (Nottingham score, ≤4) and high-grade (Nottingham score, ≥8) breast tumors, as identified by a Saint Louis University Hospital (St. Louis, MO) pathologist. Control tissue was obtained from patients with no evident breast tumors ($n \geq 3$ for each group in this study). All of the participants provided written informed consent under a protocol approved by the Saint Louis University Institutional Review Board.

Immunoblot Analysis

Cells were suspended in lysis buffer containing 20 mmol/L HEPES (pH 7.6), 250 mmol/L sucrose, 2 mmol/L dithiothreitol, 2 mmol/L EDTA, 2 mmol/L EGTA, 10 mmol/L β-glycerophosphate, 1 mmol/L sodium orthovanadate, 2 mmol/L phenylmethylsulfonyl fluoride, 20 μg/mL leupeptin, 10 μg/mL aprotinin, and 5 μg/mL pepstatin A. Cells were sonicated on ice and centrifuged at 20,000 × *g* at 4°C for 20 minutes to remove cellular debris and nuclei. Cytosolic protein was separated by SDS/PAGE and electrophoretically transferred to nitrocellulose membranes (Bio-Rad, Richmond, CA). The blocked nitrocellulose membrane was incubated with primary antibodies (COX-2, mPGES-1, and 15-PGDH) and horseradish peroxidase-conjugated secondary antibodies. Regions of antibody binding were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL) after exposure to film (Hyperfilm; Amersham). Equal loading was verified by immunoblot analysis for actin. Protein content was determined by densitometric quantification using TotalLab Quant (Total Lab, Newcastle, UK).

Immunofluorescence

Paraffin-embedded human breast tissue sections were cut and rehydrated in xylene and alcohol. After antigen retrieval, slides were incubated in blocking buffer (1× phosphate-buffered saline/5% normal serum/0.3%

Triton X-100) and subsequently incubated with a primary antibody (PAF-R) overnight at 4°C. Slides were washed with phosphate-buffered saline and incubated with an Alexa Fluor–conjugated secondary antibody. Slides were coverslipped with Prolong Gold Antifade-Reagent (Life Technologies, Carlsbad, CA) with DAPI and allowed to cure overnight, protected from light, and viewed under an epifluorescence microscope.

Immunohistochemistry and Analysis

Breast tissue for immunohistochemistry was fixed in 10% buffered formalin, embedded in paraffin, and cut into sections (5 µm thick). Tissue was deparaffinized and rehydrated in xylene and decreasing concentrations of reagent alcohol. Antigen retrieval was performed, and tissue was incubated with blocking buffer and primary antibody (PAF) overnight. Immunohistochemistry was completed with use of the Vectastain Elite Universal ABC system and diaminobenzidine (Biogenex, Fremont, CA). Slides were counterstained with filtered Gills III hematoxylin, blued in saturated lithium carbonate solution, and viewed under a light microscope. Immunohistochemistry was quantified using image analysis by ImageJ FIJI (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>) wherein percentage area of diaminobenzidine-positive signal was calculated. $n \geq 3$ patients per group and $n \geq 3$ microscopic fields per patient for quantification.

Measurement of PAF Production

PAF production was measured using an enzyme-linked immunosorbent assay kit (Biotang Inc., Waltham, MA). Breast cell monolayers were washed with ice-cold Dulbecco's phosphate-buffered saline and frozen at –20°C. After two freeze-thaw cycles, aliquots of the suspension were added to microtiter plates with a biotin-conjugated polyclonal antibody specific for PAF. PAF content in samples was determined spectrophotometrically at 450 nm using a Synergy 2 microplate reader (Biotek, Winooski, VT).

PGE₂ Release

Cells were grown to confluence in 16-mm tissue culture dishes. Cells were washed twice with Hanks' balanced salt solution containing 135 mmol/L NaCl, 0.8 mmol/L MgSO₄, 10 mmol/L HEPES (pH 7.6), 1.2 mmol/L CaCl₂, 5.4 mmol/L KCl, 0.4 mmol/L KH₂PO₄, 0.3 mmol/L Na₂HPO₄, and 6.6 mmol/L glucose. After washing, 0.5 mL of Hanks' balanced salt solution with 0.36% bovine serum albumin was added to each culture well. Cells received pretreatment with BEL or no pretreatment and then were incubated with CSE or media alone. The surrounding buffer was removed from the cells after selected time intervals, and PGE₂ release was measured immediately using an immunoassay kit (R&D Systems, Minneapolis, MN). The protein content for the confluent monolayers was determined in three

representative cell culture wells and was assumed to be constant between wells for each experiment.

Statistical Analysis

All cell studies were repeated with three separate cell cultures. All human studies were repeated with at least three separate patients using at least three objective fields for analysis. Data were analyzed using *t*-test and are given as means + SEM. Differences were regarded as significant at $P < 0.05$.

Results

iPLA₂ in Human Breast Cancer

iPLA₂ is responsible for cleavage of membrane phospholipids to form free arachidonic acid and a lysophospholipid. We investigated the effects of CSE on the two predominant mammalian isoforms of iPLA₂, iPLA₂β and iPLA₂γ. Incubation with CSE had little effect on protein expression of either isoform in any of the cell lines tested (Figure 2, A and B, and Supplemental Figure S1). Immunohistochemistry for iPLA₂ isoforms was performed on human breast tissue biopsy specimens (Figure 2, C–F). Patients were from four groups: low-grade carcinoma with no smoking history, low-grade carcinoma with long-term smoking history, high-grade carcinoma with no smoking history, or high-grade carcinoma with long-term smoking history. The patient groups labeled as long-term smoking were defined as patients who reported a 12 pack-year or longer history of smoking. Quantification of immunohistochemical staining showed significantly increased iPLA₂β expression in all groups when compared to normal mammary tissue. High-grade tumor tissue from long-term smokers had the highest expression of iPLA₂β, which was significant compared to all other groups, including other high-grade tumors from nonsmokers (Figure 2E). No changes in iPLA₂γ were detected by immunohistochemistry (Figure 2F).

PAF in Human Breast Cancer

Herein, we show the effect of CSE incubation on the accumulation of PAF using MDA-MB-231 and MCF-10A cell lines (Figure 3). PAF is significantly increased in triple-negative MDA-MB-231 cells after 24 and 48 hours of CSE treatment; however, we observed no significant changes with MCF-10A cells incubated with CSE (Figure 3A). PAF accumulation could be abrogated via pretreatment with the racemic iPLA₂ inhibitor BEL in MDA-MB-231 cells. MCF-10A cells exhibit no significant changes with BEL pretreatment when compared with controls (Figure 3A).

To verify PAF accumulation in human breast cancer, we performed immunohistochemistry on samples from patients with infiltrating ductal carcinoma (Figure 3D). We detected PAF in all patient samples (Figure 3D). When quantified, we observed an increased PAF signal that correlated with

tumor grade and long-term smoking history (Figure 3B). The presence of PAF was highest in the group with high-grade tumor and long-term smoking history.

PAF-R Expression in Human Breast Cancer

For PAF to exert its biological activity, it binds to the PAF-R. We previously reported increased PAF-R expression after CSE exposure in MDA-MB-231 breast cancer cells.²⁰ In addition to *in vitro* experiments, we performed immunohistochemistry and immunofluorescence on human breast tumor tissue from patients with and without long-term smoking status. Our data show increased expression of the PAF-R in long-term smokers versus non-smoking patients (Figure 3, C and E). We also observed increased PAF-R expression in high-grade tumor tissue when compared to low-grade tissue (Figure 3, C and E). An interesting feature of the PAF-R staining was localization to the nucleus, which can be seen via immunohistochemistry and immunofluorescence (Figure 3E).

COX-2 and mPGES-1 Expression

In addition to PAF production, the free arachidonic acid released from iPLA₂ hydrolysis of membrane phospholipids can be metabolized by COX-2 to form prostaglandins. PGE₂ is the major prostaglandin hallmark of tumor progression. The production of PGE₂ is dependent on mPGES-1, which acts on the COX-2 product, PGH₂, for its production. We examined COX-2 expression via immunoblot analysis in MCF-10A, MCF-7, MDA-MB-468, and MDA-MB-231 cells (Figure 4A and Supplemental Figure S1). After 24 and 48 hours of CSE exposure, we observed no change in COX-2 expression in MCF-10A, MCF-7, or MDA-MB-468 cells when compared to controls (Figure 4A). However, we observed significant increases in COX-2 expression in MDA-MB-231 cells after 24 and 48 hours of CSE treatment (Figure 4A). This increased COX-2 expression could be reduced by pretreatment with BEL, the racemic iPLA₂ inhibitor, suggesting membrane phospholipid hydrolysis is required (Figure 4B). In human tissue, we observed strong COX-2 expression in all breast tumor tissue, with the highest expression in patients with long-term smoking history (Figure 4C).

Quantification of immunohistochemistry revealed significant increases in COX-2 expression in low- and high-grade smokers compared with normal tissue controls (Figure 4D). The expression of mPGES-1 in MCF-10A and MDA-MB-231 cells was measured via immunoblotting after 24 and 48 hours of CSE exposure (Figure 5A). Our data show significant increases of mPGES-1 after 24 and 48 hours of CSE exposure in both MCF-10A and MDA-MB-231 cell lines (Figure 5A and Supplemental Figure S1). In human breast tissue, immunohistochemistry showed expression of mPGES-1 in low- and high-grade tumors, with the highest expression seen in patients with high-grade tumors and long-term smoking history (Figure 5, B and C). Quantification of immunohistochemistry revealed significant increases in mPGES-1 expression in high-grade tumors from smokers and nonsmokers. More important, we saw a significant increase in mPGES-1 expression in high-grade tumors from smokers when compared to high-grade tumors from nonsmokers.

PGE₂ Release from Human Breast Cell Lines and Content in Human Breast Biopsy Specimens

After measurement of mPGES-1, PGE₂ release was measured under the same conditions. PGE₂ release was significantly increased after 24 hours of CSE exposure in MCF-10A, MCF-7, MDA-MB-468, and MDA-MB-231 cell lines (Figure 6A). After 48 hours of CSE exposure, PGE₂ release was increased even further in MDA-MB-231 cells. Pretreatment with BEL abrogated the effects of 24 and 48 hours of treatment in all cell lines. We observed changes in PGE₂ similar to those of COX-2 and mPGES-1 via immunohistochemistry (Figure 6, B and C). PGE₂ was detected in both low- and high-grade tumors, with the highest expression seen in patients with long-term smoking history (Figure 6, B and C). In addition, we saw a significant increase in mPGES-1 expression in high-grade tumors from smokers when compared to low-grade tumors from smokers.

15-PGDH Expression

15-PGDH is responsible for prostaglandin inactivation and is down-regulated in several cancers.^{26–28} We investigated the

Figure 2 **A:** Calcium-independent phospholipase A₂β (iPLA₂β) expression determined by immunoblot in MCF-10A, MCF-7, MDA-MB-468, and MDA-MB-231 cells incubated with 20 μg/mL cigarette smoke extract (CSE) for 24 hours, 48 hours, or medium only. Values shown are for three different cell cultures. **B:** iPLA₂γ expression determined by Western blot in MCF-10A, MCF-7, MDA-MB-468, and MDA-MB-231 cells incubated with 20 μg/mL CSE for 24 hours, 48 hours, or room air control only. Values shown are for three different cell cultures. **C:** Representative iPLA₂β immunohistochemical expression in high- and low-grade breast tumors from smokers and nonsmokers. **D:** Representative iPLA₂β immunohistochemical expression in high- and low-grade breast tumors from long-term smokers and nonsmokers. **E:** Image analysis for iPLA₂β immunohistochemistry. Data are from non-smoking patients with low-grade tumors or high-grade tumors and from long-term smokers with low-grade tumors and high-grade tumors compared with control normal mammary tissue. **F:** Image analysis for iPLA₂γ immunohistochemistry. Data are from non-smoking patients with low-grade tumors or high-grade tumors and from long-term smokers with low-grade tumors and high-grade tumors compared with control normal mammary tissue. Data are expressed as means + SEM (**A**, **B**, **E**, and **F**). ***P* < 0.01 versus control; †*P* < 0.05 versus high-grade tumors from nonsmokers. Scale bars: 200 μm (**C** and **D**, columns 1 and 3); 20 μm (**C** and **D**, columns 2 and 4). Original magnification: ×10 (**C** and **D**, columns 1 and 3); ×60 (**C** and **D**, columns 2 and 4).

Figure 3 **A:** Platelet-activating factor (PAF) accumulation in MDA-MB-231 and MCF-10A cells incubated with 20 μ g/mL cigarette smoke extract (CSE) for up to 48 hours. MDA-MB-231 cells pretreated with bromoenol lactone (BEL) show a significant decrease in PAF production in the presence of CSE. Values shown are for three different cell cultures. **B:** Image analysis for PAF immunohistochemistry. Data are from non-smoking patients with low-grade tumors or high-grade tumors and from long-term smokers with low-grade tumors and high-grade tumors compared with control normal mammary tissue. **C:** Image analysis for PAF receptor (PAF-R) immunohistochemistry. Data are from non-smoking patients with low-grade tumors or high-grade tumors and from long-term smokers with low-grade tumors and high-grade tumors compared with control normal mammary tissue. **D:** Representative PAF immunohistochemical expression in high- and low-grade breast tumors from smokers and nonsmokers. **E:** Representative PAF-R immunohistochemical expression in high- and low-grade breast tumors from smokers and nonsmokers. Data are expressed as means $\pm$ SEM (**A–C**). * P < 0.05, ** P < 0.01 versus controls; † P < 0.05 versus low-grade tumors from nonsmokers; †† P < 0.01 versus no BEL pretreatment. Scale bars: 200 μ m (**D** and **E**, columns 1 and 3); 20 μ m (**D** and **E**, columns 2 and 4). Original magnifications: $\times 10$ (**D** and **E**, columns 1 and 3); $\times 60$ (**D** and **E**, columns 2 and 4).

expression of 15-PGDH in response to CSE exposure in human breast cell lines. In MCF-10A cells, exposure to 24 hours of CSE caused a decrease in 15-PGDH, which was attenuated after 48 hours of CSE (Figure 7A and Supplemental Figure S1). In MDA-MB-468 and MDA-MB-231 breast cancer cells, 24 hours of CSE exposure did not significantly alter 15-PGDH expression; however, after 48 hours of CSE exposure, 15-PGDH was significantly decreased. MCF-7 cells were unaffected by CSE exposure. Immunohistochemistry revealed expression of 15-PGDH in all tumor tissue, with the highest expression seen in control tissue as well as low-grade tumor tissue (Figure 7, B and C). High-grade tumor tissue from both smokers and nonsmokers had significantly decreased expression of 15-PGDH (Figure 7, B and C).

Discussion

The results of the present study demonstrate that cigarette smoke components alter downstream metabolites of

iPLA₂-catalyzed hydrolysis of membrane phospholipids, which are involved in tumor formation and metastasis.^{6,12,15,16,18} iPLA₂ enzymes hydrolyze membrane phospholipids at the *sn*-2 fatty acid position to produce a free fatty acid and a lysophospholipid. iPLA₂ can cleave arachidonated membrane phospholipids to yield arachidonic acid, which can be further modified by COX and mPGES-1 enzymes to produce PGE₂.²⁹ The remaining alkyl ether phosphatidylcholine from the iPLA₂ cleavage can be modified to form PAF, which has been implicated in oncogenic pathways.^{16–25} In addition to PAF, COX-2, PGE₂, and 15-PGDH have all been implicated in tumorigenesis or progression. In addition, iPLA₂γ is primarily associated with mitochondria and may play a direct role in cancer because of aberrant oxidative stress regulation, signaling, and metabolism.^{30–32} However, in this study, we detected no change in iPLA₂γ expression, suggesting that it is not involved in mitochondrial changes implicated in tumorigenesis and progression.

Figure 4 **A:** Cyclooxygenase-2 (COX-2) expression determined by immunoblot in MCF-10A, MCF-7, MDA-MB-468, and MDA-MB-231 cells incubated with 20 µg/mL cigarette smoke extract (CSE) for 24 hours, 48 hours, or medium control only. Values shown are for three different cell cultures. **B:** MDA-MB-231 cells pretreated with bromoenol lactone (BEL) show a significant decrease in COX-2 expression in the presence of CSE. Values shown are for three different cell cultures. **C:** Representative COX-2 immunohistochemical expression in high- and low-grade breast tumors from smokers and nonsmokers. **D:** Image analysis for COX-2 immunohistochemistry. Data are from non-smoking patients with low-grade tumors or high-grade tumors and from long-term smokers with low-grade tumors and high-grade tumors compared with control normal mammary tissue. Data are expressed as means + SEM (**A**, **B**, and **D**). * $P < 0.05$, ** $P < 0.01$ versus controls; † $P < 0.05$, †† $P < 0.01$ versus no BEL pretreatment. Scale bars: 200 µm (**C**, columns 1 and 3); 20 µm (**C**, columns 2 and 4). Original magnifications: $\times 10$ (**C**, columns 1 and 3); $\times 60$ (**C**, columns 2 and 4).

In the current study, cigarette smoke had little effect on iPLA_{2γ} expression, yet significant alterations are seen with iPLA_{2β} expression via immunohistochemistry (Figure 2). iPLA_{2β} increases with tumor grade, with the highest expression seen in high-grade smokers (Figure 2E). A study by Dasgupta et al³³ found that nicotine-induced breast cancer cell motility, and a study by Calderon et al³⁴ found that nicotine-induced motility, was mediated in part by iPLA_{2β}-dependent matrix metalloproteinase 9 production. Both of these studies support our findings that iPLA_{2β} is increased in high-grade tumors from long-term smokers and may be a major contributing factor to tumor development and progression. The observed increase in iPLA_{2β} could

account for the increases in PAF accumulation because of the fact that iPLA_{2β} is the isoform responsible for PAF production in endothelial cells.³⁵ Discrepancies between immunoblot and immunohistochemistry data may be because of the fact that the cell lines undergo only 48 hours of incubation with CSE, yet the patients are long-term smokers. In addition, the patient tumors are classified as low and high grade, which do not account for differences in receptor status, HER2 (*ERBB2*) gene amplification, or BRCA mutation.

Previously, we reported that cigarette smoke extract mediated PAF and PAF-R expression *in vitro* in breast tumor cells.^{20,36} We have shown these cigarette

Figure 5 **A:** Microsomal prostaglandin E synthase-1 (mPGES-1) expression determined by immunoblot in MCF-10A, MCF-7, MDA-MB-468, and MDA-MB-231 cells incubated with 20 μ g/mL cigarette smoke extract (CSE) for 24 hours, 48 hours, or medium control only. Values shown are for three different cell cultures. **B:** Image analysis for PGES immunohistochemistry. Data are from non-smoking patients with low-grade tumors or high-grade tumors and from long-term smokers with low-grade tumors and high-grade tumors compared with control normal mammary tissue. **C:** Representative mPGES-1 immunohistochemical expression in high- and low-grade breast tumors from smokers and nonsmokers. Data are expressed as means $\pm$ SEM (**A** and **B**). * P < 0.05, ** P < 0.01 versus controls; † P < 0.05 versus high-grade nonsmoker. Scale bars: 200 μ m (**C**, columns 1 and 3); 20 μ m (**C**, columns 2 and 4). Original magnifications: $\times 10$ (**C**, columns 1 and 3); $\times 60$ (**C**, columns 2 and 4).

smoke—mediated effects for the first time *in vivo* in human breast tumor tissue via immunofluorescence and immunohistochemistry. PAF and PAF-R expression was positively correlated with tumor grade and smoking history (Figure 3, C and E). PAF-R expression was significantly increased in low-grade smokers when compared to low-grade nonsmokers (Figure 3E). This same trend is seen between high-grade smokers when compared to nonsmokers as well for PAF and the receptor (Figure 3, C and E). Cigarette smoke—induced PAF accumulation and PAF-R expression could promote tumor progression and metastasis by several mechanisms, which could include increased cell motility and invasiveness, as we have demonstrated previously.²⁰ Our findings in human breast tumor tissue support our previous *in vitro* studies and suggest that cigarette smoke

exposure may lead to tumor progression, in part by increased cell motility.²⁰ Considering higher-grade/stage tumors are generally associated with worsened prognosis and PAF is increased in higher-grade tumors, it is probable that PAF antagonists could offer a therapeutic target for patients with metastatic disease who smoke.³⁷

In addition to cigarette smoke—induced alterations in PAF/PAF-R expression, CSE alters other enzymes in the iPLA₂ pathway, such as COX-2, which may be involved in breast tumorigenesis and progression.^{38,39} COX-2 expression is not only an important enzyme for PGE₂ production, but overexpression is an important marker for aggressive tumors and an unfavorable prognostic factor in high-grade breast cancers.^{39,40} We observed no alteration in COX-2 expression after 24 and 48 hours of CSE exposure in

Figure 6 **A:** Prostaglandin E₂ (PGE₂) release measured in MCF-10A, MCF-7, MDA-MB-468, and MDA-MB-231 cells incubated with 20 µg/mL cigarette smoke extract (CSE) for up to 48 hours. Pretreatment with bromoenol lactone (BEL) shows a significant decrease in PGE₂ release despite CSE incubation. Values shown are for three different cell cultures. **B:** Image analysis for PGE₂ immunohistochemistry. Data are from non-smoking patients with low-grade tumors or high-grade tumors and from long-term smokers with low-grade tumors and high-grade tumors compared with control normal mammary tissue. **C:** Representative PGE₂ immunohistochemical expression in high- and low-grade breast tumors from smokers and nonsmokers. Data are expressed as means + SEM (**A** and **B**). **P* < 0.05, ***P* < 0.01 versus controls; †*P* < 0.05 versus high-grade nonsmoker and low-grade smoker; ‡*P* < 0.01 versus no BEL pretreatment. Scale bars: 200 µm (**C**, columns 1 and 3); 20 µm (**C**, columns 2 and 4). Original magnifications: ×10 (**C**, columns 1 and 3); ×60 (**C**, columns 2 and 4).

Figure 7 **A:** 15-Hydroxyprostaglandin dehydrogenase (15-PGDH) expression determined by immunoblot in MCF-10A, MCF-7, MDA-MB-468, and MDA-MB-231 cells incubated with 20 μ g/mL cigarette smoke extract (CSE) for 24 hours, 48 hours, or medium control only. Values shown are for three different cell cultures. **B:** Image analysis for 15-PGDH immunohistochemistry. Data are from non-smoking patients with low-grade tumors (blue bars) or high-grade tumors and from long-term smokers with low-grade tumors and high-grade tumors compared with control normal mammary tissue. **C:** Representative 15-PGDH immunohistochemical expression in high- and low-grade breast tumors from smokers and nonsmokers. Data are expressed as means + SEM (**A** and **B**). * $P < 0.05$, ** $P < 0.01$ versus control. Scale bars: 200 μ m (**C**, columns 1 and 3); 20 μ m (**C**, columns 2 and 4). Original magnifications: $\times 10$ (**C**, columns 1 and 3); $\times 60$ (**C**, columns 2 and 4).

normal mammary epithelial cells MCF-10A as well as hormone-positive cells MCF-7 (Figure 4A). We observed slight increases in triple-negative MDA-MB-468 cells after 24 hours, which returns to baseline at 48 hours (Figure 4A). However, significant increases in COX-2 expression were seen in triple-negative metastatic MDA-MB-231 cells after only 24 hours of CSE exposure, which is further increased after 48 hours. This increase could be abrogated by BEL, an iPLA₂ inhibitor (Figure 4B). In addition, COX-2 expression in patients was increased with tumor presence, as expected (Figure 4D). There was no significant difference in expression between low- and high-grade tumors from non-smokers, yet tumors from smokers revealed significant changes in COX-2 expression when compared to their nonsmoker counterparts (Figure 4D). In addition to the

PAF/PAF-R antagonists, COX-2 could be a potentially beneficial therapeutic target for breast cancer patients with a long-term smoking history.

However, in contrast to the COX-2 data, CSE elicits mPGES-1 overexpression in both the normal and triple-negative cell lines after only 24 hours of exposure (Figure 5A). The discrepancy seen between COX-2 and mPGES-1 increased expression in MCF-10A cells could be because of differences in nuclear PAF-R expression. Stimulation of PAF nuclear receptors has been shown to induce the expression of COX-2.⁴¹ We have shown previously that MCF-10A cells do not express detectable levels of PAF-R, and incubation with CSE did not induce its expression.³⁶ We have also shown that PAF accumulation is at basal levels in MCF-10A and cannot be induced after CSE

exposure, in contrast to breast cancer cells MCF-7, MDA-MB-231, and MDA-MB-468.²⁰ The fact that MCF-10A cell lines lack PAF/PAF-R expression could explain why after CSE exposure, they do not show increased expression of COX-2, yet show increases in mPGES-1 and PGE₂. Patient data show that PGES expression is in all tumor cell lines, with the highest level of expression in high-grade tumors from long-term smokers, which is significant when compared to high-grade tumors from nonsmokers, supporting the hypothesis that increased PGES expression can contribute to tumor progression, particularly through PGE₂ release (Figure 5B). Differences in expression of COX-2, PGES, and PGE₂ release between the triple-negative cell lines may be because of the differences in claudin expression. MDA-MB-231 cells are a claudin low subtype of breast cancer cell characterized by low expression of epithelial-mesenchymal transition markers and are often less responsive to chemotherapy when compared to MDA-MB-468 cells.⁴² Studies have found that the transforming growth factor- β pathway induced the regenerative capacity of tumor-initiating cells *in vivo* in only claudin low subtypes of breast cancer.⁴³ This transforming growth factor- β induction of progression can be regulated by COX-2 and PGE₂.⁴⁴ Therefore, the differences we see herein from claudin low MDA-MB-231 may be regulated by transforming growth factor- β .

Despite increased PGE₂ release in all cell lines after 24 hours of CSE exposure, MCF-10A and MCF-7 PGE₂ levels fall back down after 48 hours of CSE exposure (Figure 6A). This temporary increase in MCF-10A PGE₂ release corresponds to decreased levels of 15-PGDH, the enzyme responsible for PGE₂ degradation, after 24 hours of exposure. After 24 hours of CSE exposure, when PGE₂ release nears control levels in MCF-10A and MCF-7 cell lines, 15-PGDH is balanced near control levels as well (Figure 6A). 15-PGDH is tightly regulated in normal mammary cell lines, yet significant decreases are seen after 48 hours of CSE exposure in both invasive triple-negative breast tumor cells, which corresponds with increased PGE₂ release (Figures 6A and 7A). This is reflected in human tissue, wherein 15-PGDH expression disappears with tumor presence and long-term smoking history (Figure 7C). These data are supported by other studies, wherein 15-PDGH expression was down-regulated in lung, bladder, and colon cancers.^{27,28,45,46} Of particular interest, studies have shown that 15-PGDH may serve as a tumor suppressor gene in breast cancer and modulate the estrogen receptor pathway.²⁶

These studies show, for the first time, dysregulation of iPLA₂-dependent pathways in the breast after cigarette smoke exposure. We observed increased expression of PAF and the PAF receptor after cigarette smoke exposure may contribute to tumor progression directly as well as increased COX-2 expression in tumors, leading to PGE₂ release. Regardless of mechanism, CSE causes increased PGE₂ release in both normal mammary cell lines and metastatic triple-negative cell lines. Treatment of MDA-MB-231 cells

with BEL abrogated the effects of CSE on COX-2 expression and PGE₂ release, both involved in tumor progression, suggesting that iPLA₂ may be a potential therapeutic target option in the management of metastatic breast cancer.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2017.04.003>.

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