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Noninvasive imaging identifies new roles for cyclooxygenase-2 in choline and lipid metabolism of human breast cancer cells

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Abstract

The expression of cyclooxygenase-2 (COX-2) is observed in approximately 40% of breast cancers. A major product of the COX-2-catalyzed reaction, prostaglandin E₂, is an inflammatory mediator that participates in several biological processes, and influences invasion, vascularization and metastasis. Using noninvasive MRI and MRS, we determined the effect of COX-2 downregulation on the metabolism and invasion of intact poorly differentiated MDA-MB-231 human breast cancer cells stably expressing COX-2 short hairpin RNA. Dynamic tracking of invasion, extracellular matrix degradation and metabolism was performed with an MRI- and MRS-compatible cell perfusion assay under controlled conditions of pH, temperature and oxygenation over the course of 48 h. COX-2-silenced cells exhibited a significant decrease in invasion relative to parental cells that was consistent with the reduced expression of invasion-associated matrix metalloproteinase genes and an increased level of the tissue inhibitor of metalloproteinase-1. We identified, for the first time, a role for COX-2 in mediating changes in choline phospholipid metabolism, and established that choline kinase expression is partly dependent on COX-2 function. COX-2 silencing resulted in a significant decrease in phosphocholine and total choline that was detected by MRS. In addition, a significant increase in lipids, as well as lipid droplet formation, was observed. COX-2 silencing transformed parental cell metabolite patterns to those characteristic of less aggressive cancer cells. These new functional roles of COX-2 may identify new biomarkers and new targets for use in combination with COX-2 targeting to prevent invasion and metastasis.

Keywords

COX-2; choline kinase; lipid droplets; invasion; MRS; breast cancer

INTRODUCTION

Cyclooxygenase-2 (COX-2), the inducible isoform of prostaglandin (PG) H synthase or cyclooxygenase, converts arachidonic acid into prostaglandin H₂ (PGH₂), the common

precursor for various PGs (1). Its expression is induced by proinflammatory cytokines, such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α , and its promoter contains a cyclic AMP response element, a nuclear factor- κ B binding site, two nuclear factors for IL-6 target sequences and hypoxia response elements (HREs) (2). Persistent expression of COX-2 has been linked to tumorigenesis and metastasis in solid tumors, including breast cancers, where it is overexpressed in approximately 40% of cases (3). The induction of COX-2 expression increases the biosynthesis of PGE₂, a secondary lipid mediator central to the inflammatory cascade that participates in several biological processes, including development, pain, immunity and angiogenesis (1,4), and cancer (5,6). Following its secretion, PGE₂ binds to extracellular G-protein-coupled receptors initiating a signaling cascade that results in the expression of genes with strong carcinogenic properties (7). Further, COX-2 has been implicated in the inhibition of apoptosis (8,9), alteration of cell adhesion (10), promotion of metastasis (11) and stimulation of neovascularization (12,13). COX-2 overexpression has been found to be sufficient to transform normal tissues in an animal model of carcinogenesis (14).

Because of its importance in cancer progression and metastasis, the discovery of multiple roles of COX-2 in cancer can provide new insights into the mechanisms by which COX-2 mediates a more aggressive and metastatic phenotype. These insights can be used to identify new biomarkers and pathways to exploit in combination with COX-2 targeting. We have observed previously that the silencing of COX-2 by a COX-2-specific short hairpin RNA interference molecule reduces tumor onset, inhibits extrapulmonary colonization, reduces angiogenesis and decreases the secretion of [H⁺] and lactate by highly metastatic, poorly differentiated MDA-MB-231 breast cancer cells (15,16).

In this study, we used a noninvasive MRI- and MRS-compatible cell perfusion assay to dynamically track the invasiveness and metabolism of MDA-MB-231 cells with and without COX-2 silencing. COX-2 silencing resulted in a profound decrease in invasion and extracellular matrix (ECM) degradation, as well as significant changes in the levels of choline and lipid metabolites, and choline kinase α (Chk α) expression. These results have identified new functional roles for COX-2 in mediating changes in choline and lipid metabolism.

MATERIALS AND METHODS

Cell culture and immunoblotting

Parental MDA-MB-231 breast cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). For cell perfusion and lipid droplet experiments, MDA-MB-231 human breast cancer cells were maintained in minimal essential medium (RPMI-1640, Sigma, St. Louis, MO, USA) supplemented with 8.25% fetal bovine serum plus 100 units/mL penicillin and 100 μ g/mL streptomycin. Cells were cultured in standard cell culture incubator conditions at 37 °C in a humidified atmosphere containing 5% CO₂. Two groups of COX-2-silenced MDA-MB-231 cells were used in our experiments, termed 'Clone 2' and 'Pooled'. Clone 2 cells were obtained from a single clone of COX-2 short hairpin RNA-transfected cells. Pooled cells were obtained from the pooling of four separate clones that could not be induced to express COX-2 (16). Whole-cell extracts were prepared

by lysing cells with M-PER™ reagent (ThermoFisher, Rochester, NY, USA) buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA).

For immunoblotting, cells were cultured as above, but without the use of antibiotics. MDA-MB-231 cells (1.4×10^6) were treated with IL-1 β (10 ng/ μ L; R&D Systems, Minneapolis, MN, USA) for the times indicated. Clone 2 cells (1.4×10^6) were treated with PGE₂ (50 nM; CaymanChem, Ann Arbor, MI, USA) for the times indicated. Cells were washed twice in phosphate-buffered saline and lysed for 30 min using M-PER™ (ThermoFisher). Cytoplasmic proteins were separated by centrifugation and their amount was assayed using the Bradford protein assay (Biorad, Hercules, CA, USA). Fifty micrograms of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using standard protocols and probed with a custom-made Chk-specific antibody (17) and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody. The signal was amplified using anti-rabbit and anti-mouse secondary antibodies (GE Healthcare, Piscataway, NJ, USA) and visualized by chemiluminescence using Supersignal WestPico™ (ThermoFisher).

MRI/MRS-compatible cell perfusion assay

Four days prior to the MR experiments, cells were seeded on Biosilon (Nunc, Roskilde, Denmark) beads at a cell density of 1.5×10^6 cells per 0.5 mL of beads in Petri dishes (Nunc) and grown to approximately 60% confluence. A schematic diagram of the cell perfusion assay is shown in Fig. 1, and a detailed description of the MR cell perfusion system can be found in Pilatus *et al.* (18) and Ackerstaff *et al.* (19). A chamber containing Matrigel® (Sigma-Aldrich) at a concentration of 8.8mg/mL, which was part of the MR-compatible cell perfusion assay, was used to determine the degradation and invasion of ECM by cancer cells. Two layers of perfluorocarbon-doped alginate beads were interspersed within the layers of cancer cells on Biosilon beads to monitor the oxygen tension in the sample using ¹⁹F MR relaxometry.

The following series of MR experiments was performed on a 9.4-T MR spectrometer (Bruker BioSpin Corp., Billerica, MA, USA) every 12 h and up to 48 h. ¹H MRI was performed to evaluate the overall sample preparation, to visualize the geometry of the ECM gel and to detect changes in the integrity of the ECM gel as a result of invasion and degradation by cancer cells. Two-dimensional images were acquired using a spin-echo imaging sequence with a field of view of 40mm, TR= 1 s and TE= 30ms from a 2-mm-thick central slice of the sample. One-dimensional (1D) ¹H MR profiles of intracellular water, with a spatial resolution of 62.5 μ m, were acquired along the length (z-axis) of the sample by diffusion-weighted (DW) 1D ¹H MRI using gradient pulses with a duration of 3ms and gradient strength of 18G/cm, and employing a diffusion weighting time of 100ms. These profiles were used to derive an invasion index by quantifying the number of cells invading into the ECM, as the signal from slow-diffusing water, which represents intracellular water, is directly proportional to the number of cells (20). The invasion index $I(t)$ at time t was calculated as follows:

$$I(t) = I_{p,7mm}(t) / I_p(t) - I_{p,7mm}(t_0) / I_p(t_0)$$

where $I_{p,7mm}(t)$ and $I_{p,7mm}(t_0)$ are the integral values of the signal at time t and t_0 , respectively, obtained by integrating the intracellular water signal over a 7-mm region starting at the base of the ECM chamber, and $I_p(t)$ and $I_p(t_0)$ are the integrals of the profile of the entire sample at time t and t_0 , respectively. t_0 refers to the first imaging time point, which is typically 2 h after the sample is loaded.

Intracellular levels of total choline (tCho), i.e. signals from phosphocholine (PC) + glycerophosphocholine (GPC) + free choline (fCho), total creatine (tCr), i.e. signals from creatine + phosphocreatine, and lactate and lipids (Lac/lipid) were derived from global, DW 1D ^1H MR spectra. ^1H spectra were acquired with a DW stimulated echo pulse sequence using chemical shift-selective (CHESS) water suppression with TR = 2 s, a sweep width of 4 kHz, 2 K data points and 128 scans that were processed with a line broadening of 5 Hz. DW water-suppressed 1D ^1H MR spectra with TR = 2 s, a sweep width of 4 kHz, 2 K data points, 256 averages and line broadening of 8 Hz were acquired using lactate editing (21) to quantify changes in lactate. DW 1D ^1H MR spectra were acquired with four averages without water suppression to determine cell proliferation and normalize the metabolite signals to the cell number. Localized DW 1D ^1H chemical shift imaging (CSI) MR spectra with and without water suppression were acquired to obtain metabolic information from 310- μm -thick slices along the z -axis of the sample. Localized 1D ^1H CSI and ^{19}F MR spectra were acquired every 24 h. The oxygen tension was obtained from slice-selective 1D ^{19}F inversion recovery MR experiments. Energy metabolites, pH and the choline phospholipid metabolites PC and GPC were obtained from unlocalized ^{31}P MR spectra. ^{31}P spectra were acquired by accumulating 4000 scans using a 45° radiofrequency pulse, TR = 1 s, a sweep width of 10 kHz, 2 K data points and line broadening of 15 Hz.

Cell extract studies

Water-soluble and lipid extracts were obtained from approximately 1.5×10^7 cells using the dual-phase extraction method described previously (17). Samples were dissolved in deuterated solvents containing 3-(trimethylsilyl)propionic-2,2,3,3- d_4 acid (Sigma-Aldrich) in the case of water-soluble fractions, and tetramethylsilane (Cambridge Isotope Laboratories, Inc., Andover, MA, USA) in the case of lipid fractions, to serve as concentration standards and chemical shift references. Fully relaxed ^1H MR spectra of the extracts were acquired on a Bruker Avance 500 spectrometer (Bruker BioSpin Corp.). Integrals of lactate methyl protons at 1.3 ppm, methyl $\text{N}(\text{CH}_3)_3$ signals of fCho at 3.209 ppm, PC at 3.227 ppm and GPC at 3.236 ppm in the ^1H MR spectra of water-soluble metabolites, and of phosphatidylcholine at 3.20 ppm and the methylene and methyl groups of fatty acids (F_{mix}) at 1.20–1.30 ppm and 0.9 ppm in the ^1H MR spectra of lipids, were determined. Signal integrals of the metabolites were normalized to cell numbers and quantified relative to the standard.

Nile red staining for lipid droplets

Cells were grown on glass chamber slides (ThermoFisher) to 60–70% confluence, washed with phosphate-buffered saline and fixed with 3% (w/v) paraformaldehyde. Cells were washed with phosphate-buffered saline and incubated with Nile red (1 $\mu\text{g/mL}$; Sigma-Aldrich) for 10 min at room temperature. Cell nuclei were counterstained with Hoechst

H-33342 (Invitrogen, Carlsbad, CA, USA). Cells were washed and mounted using Faramount aqueous mounting medium. Fluorescence microscopy was performed with a Zeiss LSM 710NLO-Meta confocal laser scanning microscope (Carl Zeiss, Inc., Thornwood, NY, USA) using a C-Apo 40X/1.1W LD water immersion lens. Nile red-stained lipid droplets and Hoechst-stained nuclei were excited at 488 and 880 nm, respectively, and fluorescence emission was detected using 560-nm long-pass and 450–500-nm bandpass filters, respectively. Confocal *z*-sections, 1 μ m thick, were imaged. The number and size of lipid droplets per cell were quantified using customized in-house software, as described previously (22). Approximately 20–40 cells per field of view from five randomly selected fields of view obtained from two slides were analyzed for each cell line.

Statistical analysis

The Kolmogorov–Smirnov test was used to verify that the data were normally distributed, after which an unpaired *t*-test was applied to determine the significance of the differences between the groups. We also ran the Mann–Whitney *U*-test which does not assume a normal distribution of the data. Data comparisons with $p < 0.05$, obtained using an unpaired *t*-test, and $p < 0.057$, obtained using the Mann–Whitney *U*-test, were considered to be significantly different. Tests were run using SigmaPlot software (Systat Software, San Jose, CA, USA).

Microarray analysis

The experimental steps describing the isolation of total RNA and the statistical analysis of microarray data from MDA-MB-231 cells have been described previously (15). Briefly, RNA from COX-2-containing (MDA-MB-231) and COX-2-silenced (Clone 2 and Pooled) cells was isolated as described previously using a Qiagen RNA extraction kit (Qiagen, Valencia, CA, USA). All samples were run in commercial arrays from Affymetrix (Affymetrix, Santa Clara, CA, USA), using Human Genome U133Plus 2.0 GeneChip arrays, as described in the Affymetrix website (<http://www.affymetrix.com>). These descriptions include all information currently considered under the ‘Minimum Information About a Microarray Experiment’ supportive guidelines, with which the Johns Hopkins Medical Institutions Microarray Core Facility abides in all of its procedures. Expression signals were obtained by robust multiarray analysis. A criterion of posterior probability > 0.9 was used to produce a list of genes altered significantly in COX-2-containing and COX-2-silenced cells unless otherwise indicated.

RESULTS

COX-2 silencing reduces invasion and expression of the invasion-related transcriptome

Representative ^1H MR images of the ECM acquired over 48 h demonstrated a reduction in the degradation of reconstituted ECM by COX-2-silenced cells compared with parental MDA-MB-231 cells, as shown in Fig. 2a. As both Clone 2 and Pooled cells are COX-2-silenced derivatives of the same parental MDA-MB-231 cells, and demonstrate similar invasion indices, data obtained from individual experiments using both were combined for statistical analysis. Quantitative time-dependent invasion indices $I(t)$ obtained from intracellular DW water profiles demonstrated that the invasion of COX-2-silenced cells was

reduced significantly relative to COX-2-containing MDA-MB-231 parental cell lines, as shown in Fig. 2b.

Reduced mRNA expression of the invasion-related matrix metalloproteinase genes 3 (MMP-3) and 10 (MMP-10) was observed in COX-2-silenced cells, as shown in Fig. 2c. Expression of MMP-3 and MMP-10 was reduced by 4.6- and 2.1-fold, respectively, in COX-2-silenced Clone 2 cells, and by 4.9- and 2.0-fold, respectively, in Pooled cells. The urokinase plasminogen activator (u-PA) was reduced by 1.7- and 2.3-fold in Clone 2 and Pooled cells, respectively, and the tissue plasminogen activator (t-PA) by 3.81- and 5-fold in Clone 2 and Pooled cells, respectively, relative to parental MDA-MB-231 cells. The mesotrypsin (PRSS3) message was also reduced by 2.6- and 3.4-fold in Clone 2 and Pooled cells, respectively, relative to parental cells. Expression levels of tissue inhibitor of metalloproteinase-1 (TIMP1) mRNA were increased by 2.9- and 3.4-fold in Clone 2 and Pooled cells, respectively, compared with parental MDA-MB-231 breast cancer cells. In addition, 2.1- and 2.5-fold decreases in vascular endothelial growth factor A (VEGFA) mRNA were observed in Clone 2 and Pooled COX-2-silenced cells, respectively (Fig. 2c).

COX-2 silencing alters choline and lipid metabolites to those typical of differentiated poorly aggressive cancer cells

Representative ^1H MR spectra from MDA-MB-231 parental and COX-2-silenced cells, obtained from intact perfused cells, revealed reduced tCho levels in Pooled COX-2-silenced cells relative to MDA-MB-231 cells (Fig. 3a). Representative high-resolution ^1H MR spectra from the water-soluble fraction of cell extracts further confirmed the decrease in PC and lactate with COX-2 silencing observed with our real-time perfusion experiments (Fig. 3a, insets). The Lac/lipid signal, which consists of signal from both lactate and lipids, did not change significantly, as the lactate signal decreased significantly and the lipid signal increased significantly, as discussed subsequently. Consistent with the changes observed in the ^1H spectra, ^{31}P spectra identified a reduction in PC levels in perfused COX-2-silenced cells relative to MDA-MB-231 cells (Fig. 3b). Quantitative data acquired over the course of 2 days from multiple experiments of intact cells are summarized in Fig. 4. Levels of tCho and PC were significantly lower in COX-2-silenced cells relative to parental cells at all time points (Fig. 4a, b). Lactate levels determined with lactate editing were reduced significantly in COX-2-silenced cells relative to parental cells by 24 h (Fig. 4c) in perfused cells.

Metabolic changes observed in intact cells were confirmed by the quantification of metabolites obtained from high-resolution ^1H MR spectra of water-soluble cell extracts. A significant reduction in tCho, PC and lactate was observed in COX-2-silenced cells relative to parental MDA-MB-231 cells (Fig. 4d).

Changes in signals from neutral fatty acids were identified from ^1H MR spectra of the lipid fraction of cell extracts. Representative ^1H MR spectra of the lipid fraction from MDA-MB-231 parental and COX-2-silenced cell extracts are shown in Fig. 5a and demonstrate the profound effect of COX-2 silencing on the fatty acid signals. A significant increase in the mixed fatty acid signal (F_{mix}) at 1.3ppm (FCH_2) and 0.9ppm (FCH_3) was observed with COX-2 silencing, as shown in the quantitative data summarized in Fig. 5b. No significant differences in phosphatidylcholine were observed between the two groups. To identify the

mechanisms underlying this increase in lipid signals, we carried out Nile red staining for lipid droplets in these cells. Representative images of Nile red-stained lipid droplets in parental MDA-MB-231 and COX-2-silenced cells are shown in Fig. 5c, and demonstrate a significant increase in the number of lipid droplets with COX-2 silencing. Median values of the number of lipid droplets increased significantly in COX-2-silenced cells (21.06 ± 2.5) relative to parental control MDA-MB-231 cells (13.24 ± 3.1), as shown in Fig. 5d. There were no significant differences in the size of lipid droplets.

Immunoblotting for Chk expression revealed a reduction in Chk in untreated COX-2-silenced cells (Fig. 6, bottom panel) relative to untreated COX-2-containing parental MDA-MB-231 cells (Fig. 6, top panel). Treatment of COX-2-containing MDA-MB-231 cells with IL-1 β (10 ng/mL), a potent inducer of COX-2 in these cells (16), increased Chk protein levels by an average of 2.3-fold, 6 h following treatment, an effect that was mitigated within 24 h of induction (Fig. 6, top panel). To ascertain that the increase in the Chk level was specifically related to COX-2 signaling, we supplemented COX-2-silenced cells with exogenous PGE₂ (50 nM) and observed an increase in Chk protein levels by 1.7-fold, 24 h post-treatment (Fig. 6, bottom panel).

DISCUSSION

Noninvasive functional imaging characterization of intact perfused MDA-MB-231 cells revealed a significant decrease in invasion, tCho, PC and lactate, and a significant increase in mixed fatty acid signals, following COX-2 silencing in MDA-MB-231 cells. The changes in choline metabolites and mixed fatty acid signals were consistent with the decrease in Chk and increase in lipid droplets observed in COX-2-silenced cells. Molecular characterization detected reduced expression of the invasion-related genes MMP-3, MMP-10, t-PA, u-PA, PRSS3 and VEGFA. In addition, a significant increase in TIMP-1 was observed with COX-2 silencing.

COX-2 silencing resulted in a significant reduction in the ability of cells to degrade and invade the ECM, consistent with our earlier observation of a failure of COX-2-silenced cells to colonize the lung in an experimental model of metastasis (16), and further highlight the role of COX-2 in invasion and the metastatic cascade. This decrease in ECM invasion and degradation is also consistent with the reduced expression of the invasion-associated genes MMP-3, MMP-10 and VEGFA. MMPs have been identified as the major enzymes responsible for the modeling and degradation of the ECM (23). Previously, we observed a significant decrease in MMP-1, or collagenase-1, with COX-2 silencing (16). In addition to a decrease in MMP-1 here, we observed a significant decrease in MMP-3 and MMP-10 mRNA levels. MMP-3, or stromelysin-1, has broad substrate specificity and degrades many ECM proteins, such as fibronectin, gelatin, laminin and proteoglycans, and is also involved in the activation of the gelatinases and MMP-13 (23). It has been linked to the development of arthritis, asthma, impaired wound healing, invasion and metastasis (23). The involvement of MMP-3 is consistent with the widely accepted involvement of COX-2 in these diseases (4,24–26). MMP-10, or stromelysin-2, shares an 82% sequence homology with MMP-3, as well as similar substrate specificity. Huang *et al.* (27) have shown that MMP-3 and MMP-10 are upregulated in the premetastatic lung. Lentiviral inhibition of MMP-3 and MMP-10

attenuates pulmonary vascular permeability and inhibits lung metastasis, which is also in agreement with our earlier observation that COX-2-silenced cells fail to colonize the lung in an experimental model of metastasis.

u-PA and t-PA are plasminogen activators associated with the breakdown of ECM and cellular adhesion molecules. Plasminogen is converted to the active enzyme plasmin by t-PA or u-PA, which further converts latent pro-MMPs into active MMPs (28). TIMP1 is an inhibitor of MMPs and inhibits collagenases, stromelysins and gelatinases (29). The reduction in MMP-3 and MMP-10 and plasminogen activators (u-PA and t-PA) and the elevated level of TIMP1, in COX-2-silenced cells relative to parental MDA-MB-231 cells, confirm the important role of COX-2 in ECM degradation.

PRSS3 is a serine protease that has been shown to be involved in transendothelial migration in non-small-cell lung carcinoma and its poor prognosis (30). PRSS3 has also been shown to be involved in the promotion of breast cancer progression (31).

VEGFA has been observed to act in an autocrine fashion to promote breast cancer invasion, and antisense VEGFA oligos significantly reduce invasion into Matrigel by MDA-MB-231 cells (32). Consistent with our finding that less aggressive COX-2-silenced cells contain less VEGFA, the overexpression of COX-2 in MCF-7 cells results in increased VEGFA mRNA levels and increased invasion of ECM (33). Our finding that COX-2 silencing results in a significant reduction in VEGFA mRNA levels may further explain the reduced invasiveness of these cells. A significant association between high tumor COX-2 mRNA expression and high VEGFA mRNA expression has been observed previously in patients with non-small-cell lung cancer, together with a significant difference in survival and relapse time between patients with high and low tumoral COX-2 and VEGFA mRNA expression (34).

Increased PC and tCho are consistently observed in cancers, especially breast cancer (35–37), and only now are we beginning to uncover the complexity of factors that regulate choline metabolism in cancer. PC is primarily a membrane phospholipid precursor which is formed by the phosphorylation of free choline by Chk (35). Chk expression has been associated with increased invasion, drug resistance and a more aggressive phenotype (35,38,39). As tCho and PC are being evaluated as biomarkers in cancer diagnosis, and following treatment, it is important to understand the role of inflammation in the elevated tCho/PC phenotype. We have observed previously that choline metabolism is altered in MDA-MB-435 cells following pharmacological inhibition with indomethacin, a nonspecific COX inhibitor (19). Here, we have shown, for the first time, the role of COX-2-specific silencing in decreasing PC and, consequently, tCho. We have further characterized the molecular mechanism underlying this alteration and confirmed a close association between the COX-2 product PGE₂ and Chk, explaining the decrease in PC and tCho with COX-2 silencing. Exogenous supplementation of PGE₂ restored Chk expression in COX-2-silenced cells, demonstrating a hitherto unknown role of COX-2 in mediating changes in Chk and choline metabolism. Previously, we have observed significant hypoxia-inducible factor-1 (HIF-1) α stabilization in response to inflammatory stimuli, such as treatment with IL-1 β , which was abolished in COX-2-silenced cells (40). Our previously published studies have identified HIF-1 binding sites in the Chk promoter region (41). PGE₂-mediated stabilization

of HIF-1 and its binding to HREs in the Chk promoter, which increases Chk transcription, may be one possible mechanism by which Chk expression increases with the induction of COX-2. These data also suggest that the response to COX-2 inhibitors may be detected noninvasively by a decrease in tCho using a clinically translatable technique, such as ^1H spectroscopic imaging.

The decrease in lactate observed is consistent with our earlier findings that COX-2 silencing results in reduced lactate production or secretion (15). The decrease in lactate production or secretion is probably a result of the inhibition of PGE₂ synthesis, because lactate transport can be mediated, in part, through a PGE₂–lactate exchange mechanism (42). The increased fatty acid signal observed in COX-2-silenced cells is consistent with the increase in cytoplasmic lipid droplets observed in these cells following Nile red staining. Nile red or similar lipophilic stains are used to visualize neutral lipids, which have been shown to correlate with NMR-visible neutral lipids (43,44). Increased intracellular lipids have been detected in response to differentiation therapy (45). The changes in lipid signals are identical to those observed following Chk small interfering RNA treatment of MDA-MB-231 cells (17), suggesting that the changes in lipid metabolism observed herein are probably mediated through the effect of COX-2 on Chk, and reflect the acquisition of a more differentiated phenotype.

COX-2 function has been the target of pharmaceutical intervention in various cancers, such as breast, colon, lung and gastric cancer (46–49). We have discovered new functional roles of COX-2 that may identify new biomarkers and new targets for use in combination with COX-2 targeting to prevent invasion and metastasis.

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Abbreviations used

1D	one-dimensional
CHESS	chemical shift-selective
Chkac	choline kinase ac
CSI	chemical shift imaging
COX-2	cyclooxygenase-2
DW	diffusion-weighted
ECM	extracellular matrix
fCho	free choline
F_{mix}	mixed fatty acids

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPC	glycerophosphocholine
HIF-1	hypoxia-inducible factor-1
HRE	hypoxia response element
IL-1β	interleukin-1 β
Lac/lipid	lactate + lipids
MMP	matrix metalloproteinase
PC	phosphocholine
PG	prostaglandin
PGH₂	prostaglandin H ₂
PRSS3	mesotrypsin
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
tCho	total choline
tCr	total creatine
TIMP1	tissue inhibitor of metalloproteinase-1
t-PA	tissue plasminogen activator
u-PA	urokinase plasminogen activator
VEGFA	vascular endothelial growth factor A

References

1. Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem.* 2000; 69:145–182. [PubMed: 10966456]
2. Chun KS, Surh YJ. Signal transduction pathways regulating cyclooxygenase-2 expression: potential molecular targets for chemoprevention. *Biochem Pharmacol.* 2004; 68:1089–1100. [PubMed: 15313405]
3. Ristimäki A, Sivula A, Lundin J, Lundin M, Salminen T, Haglund C, Joensuu H, Isola J. Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. *Cancer Res.* 2002; 62:632–635. [PubMed: 11830510]
4. Narumiya S, FitzGerald GA. Genetic and pharmacological analysis of prostanoid receptor function. *J Clin Invest.* 2001; 108:25–30. [PubMed: 11435452]
5. Howe LR. Inflammation and breast cancer. Cyclooxygenase/prostaglandin signaling and breast cancer. *Breast Cancer Res.* 2007; 9:210. [PubMed: 17640394]
6. Singh-Ranger G, Salhab M, Mokbel K. The role of cyclooxygenase-2 in breast cancer: review. *Breast Cancer Res Treat.* 2008; 109:189–198. [PubMed: 17624587]
7. Wang D, Dubois RN. Eicosanoids and cancer. *Nat Rev Cancer.* 2010; 10:181–193. [PubMed: 20168319]
8. Sheng H, Shao J, Morrow JD, Beauchamp RD, DuBois RN. Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. *Cancer Res.* 1998; 58:362–366. [PubMed: 9443418]

9. Tang X, Sun YJ, Half E, Kuo MT, Sinicrope F. Cyclooxygenase-2 overexpression inhibits death receptor 5 expression and confers resistance to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human colon cancer cells. *Cancer Res.* 2002; 62:4903–4908. [PubMed: 12208739]
10. Dormond O, Bezzi M, Mariotti A, Ruegg C. Prostaglandin E2 promotes integrin alpha Vbeta 3-dependent endothelial cell adhesion, rac-activation, and spreading through cAMP/PKA-dependent signaling. *J Biol Chem.* 2002; 277:45 838–45 846.
11. Li G, Yang T, Yan J. Cyclooxygenase-2 increased the angiogenic and metastatic potential of tumor cells. *Biochem Biophys Res Commun.* 2002; 299:886–890. [PubMed: 12470662]
12. Leahy KM, Koki AT, Masferrer JL. Role of cyclooxygenases in angiogenesis. *Curr Med Chem.* 2000; 7:1163–1170. [PubMed: 11032965]
13. Liu XH, Kirschenbaum A, Yao S, Stearns ME, Holland JF, Claffey K, Levine AC. Upregulation of vascular endothelial growth factor by cobalt chloride-simulated hypoxia is mediated by persistent induction of cyclooxygenase-2 in a metastatic human prostate cancer cell line. *Clin Exp Metastasis.* 1999; 17:687–694. [PubMed: 10919714]
14. Liu CH, Chang SH, Narko K, Trifan OC, Wu MT, Smith E, Haudenschild C, Lane TF, Hla T. Overexpression of cyclooxygenase-2 is sufficient to induce tumorigenesis in transgenic mice. *J Biol Chem.* 2001; 276:18 563–18 569.
15. Stasinopoulos I, Mori N, Bhujwalla ZM. The malignant phenotype of breast cancer cells is reduced by COX-2 silencing. *Neoplasia.* 2008; 10:1163–1169. [PubMed: 18953425]
16. Stasinopoulos I, O'Brien DR, Wildes F, Glunde K, Bhujwalla ZM. Silencing of cyclooxygenase-2 inhibits metastasis and delays tumor onset of poorly differentiated metastatic breast cancer cells. *Mol Cancer Res.* 2007; 5:435–442. [PubMed: 17510310]
17. Glunde K, Raman V, Mori N, Bhujwalla ZM. RNA interference-mediated choline kinase suppression in breast cancer cells induces differentiation and reduces proliferation. *Cancer Res.* 2005; 65:11 034–11 043. [PubMed: 15665274]
18. Pilatus U, Ackerstaff E, Artemov D, Mori N, Gillies RJ, Bhujwalla ZM. Imaging prostate cancer invasion with multi-nuclear magnetic resonance methods: the Metabolic Boyden Chamber. *Neoplasia.* 2000; 2:273–279. [PubMed: 10935513]
19. Ackerstaff E, Gimi B, Artemov D, Bhujwalla ZM. Anti-inflammatory agent indomethacin reduces invasion and alters metabolism in a human breast cancer cell line. *Neoplasia.* 2007; 9:222–235. [PubMed: 17401462]
20. Pilatus U, Shim H, Artemov D, Davis D, van Zijl PC, Glickson JD. Intracellular volume and apparent diffusion constants of perfused cancer cell cultures, as measured by NMR. *Magn Reson Med.* 1997; 37:825–832. [PubMed: 9178232]
21. Hetherington HP, Avison MJ, Shulman RG. ^1H homonuclear editing of rat brain using semiselective pulses. *Proc Natl Acad Sci USA.* 1985; 82:3115–3118. [PubMed: 2987910]
22. Glunde K, Guggino SE, Solaiyappan M, Pathak AP, Ichikawa Y, Bhujwalla ZM. Extracellular acidification alters lysosomal trafficking in human breast cancer cells. *Neoplasia.* 2003; 5:533–545. [PubMed: 14965446]
23. Klein T, Bischoff R. Physiology and pathophysiology of matrix metalloproteases. *Amino Acids.* 2011; 4:271–290. [PubMed: 20640864]
24. Carey MA, Germolec DR, Langenbach R, Zeldin DC. Cyclooxygenase enzymes in allergic inflammation and asthma. *Prostaglandins Leukot Essent Fatty Acids.* 2003; 69:157–162. [PubMed: 12895598]
25. Lee KH, Kim HS, El-Sohehy A, Cornelis MC, Uhm WS, Bae SC. Cyclooxygenase-2 genotype and rheumatoid arthritis. *J Rheumatol.* 2006; 33:1231–1234. [PubMed: 16821264]
26. Liclican EL, Nguyen V, Sullivan AB, Gronert K. Selective activation of the prostaglandin E2 circuit is a key component of chronic injury-induced pathological angiogenesis. *Invest Ophthalmol Vis Sci.* 2010; 51:6311–6320. [PubMed: 20610836]
27. Huang Y, Song N, Ding Y, Yuan S, Li X, Cai H, Shi H, Luo Y. Pulmonary vascular destabilization in the premetastatic phase facilitates lung metastasis. *Cancer Res.* 2009; 69:7529–7537. [PubMed: 19773447]

28. Nyberg P, Ylipalosaari M, Sorsa T, Salo T. Trypsins and their role in carcinoma growth. *Exp Cell Res.* 2006; 312:1219–1228. [PubMed: 16457812]
29. Lijnen HR. Matrix metalloproteinases and cellular fibrinolytic activity. *Biochemistry (Moscow).* 2002; 67:92–98. [PubMed: 11841344]
30. Diederichs S, Bulk E, Steffen B, Ji P, Tickenbrock L, Lang K, Zanker KS, Metzger R, Schneider PM, Gerke V, Thomas M, Berdel WE, Serve H, Muller-Tidow C. S100 family members and trypsinogens are predictors of distant metastasis and survival in early-stage non-small cell lung cancer. *Cancer Res.* 2004; 64:5564–5569. [PubMed: 15313892]
31. Hockla A, Radisky DC, Radisky ES. Mesotrypsin promotes malignant growth of breast cancer cells through shedding of CD109. *Breast Cancer Res Treat.* 2010; 124:27–38. [PubMed: 20035377]
32. Bachelder RE, Wendt MA, Mercurio AM. Vascular endothelial growth factor promotes breast carcinoma invasion in an autocrine manner by regulating the chemokine receptor CXCR4. *Cancer Res.* 2002; 62:7203–7206. [PubMed: 12499259]
33. Prosperi JR, Mallery SR, Kigerl KA, Erfurt AA, Robertson FM. Invasive and angiogenic phenotype of MCF-7 human breast tumor cells expressing human cyclooxygenase-2. *Prostaglandins Other Lipid Mediat.* 2004; 73:249–264. [PubMed: 15290792]
34. Yuan A, Yu CJ, Shun CT, Luh KT, Kuo SH, Lee YC, Yang PC. Total cyclooxygenase-2 mRNA levels correlate with vascular endothelial growth factor mRNA levels, tumor angiogenesis and prognosis in non-small cell lung cancer patients. *Int J Cancer.* 2005; 115:545–555. [PubMed: 15704107]
35. Eliyahu G, Kreizman T, Degani H. Phosphocholine as a biomarker of breast cancer: molecular and biochemical studies. *Int J Cancer.* 2007; 120:1721–1730. [PubMed: 17236204]
36. Katz-Brull R, Lavin PT, Lenkinski RE. Clinical utility of proton magnetic resonance spectroscopy in characterizing breast lesions. *J Natl Cancer Inst.* 2002; 94:1197–1203. [PubMed: 12189222]
37. Negendank W. Studies of human tumors by MRS: a review. *NMR Biomed.* 1992; 5:303–324. [PubMed: 1333263]
38. Iorio E, Mezzanzanica D, Alberti P, Spadaro F, Ramoni C, D'Ascenzo S, Millimaggi D, Pavan A, Dolo V, Canevari S, Podo F. Alterations of choline phospholipid metabolism in ovarian tumor progression. *Cancer Res.* 2005; 65:9369–9376. [PubMed: 16230400]
39. Shah T, Wildes F, Penet MF, Winnard PT Jr, Glunde K, Artemov D, Ackerstaff E, Gimi B, Kakkad S, Raman V, Bhujwalla ZM. Choline kinase overexpression increases invasiveness and drug resistance of human breast cancer cells. *NMR Biomed.* 2010; 23:633–642. [PubMed: 20623626]
40. Stasinopoulos I, O'Brien DR, Bhujwalla ZM. Inflammation, but not hypoxia, mediated HIF-1 α activation depends on COX-2. *Cancer Biol Ther.* 2009; 8:31–35. [PubMed: 19390242]
41. Glunde K, Shah T, Winnard PT Jr, Raman V, Takagi T, Vesuna F, Artemov D, Bhujwalla ZM. Hypoxia regulates choline kinase expression through hypoxia-inducible factor-1 α signaling in a human prostate cancer model. *Cancer Res.* 2008; 68:172–180. [PubMed: 18172309]
42. Chan BS, Endo S, Kanai N, Schuster VL. Identification of lactate as a driving force for prostanoid transport by prostaglandin transporter PGT. *Am J Physiol Renal Physiol.* 2002; 282:F1097–F1102. [PubMed: 11997326]
43. Delikatny EJ, Cooper WA, Brammah S, Sathasivam N, Rideout DC. Nuclear magnetic resonance-visible lipids induced by cationic lipophilic chemotherapeutic agents are accompanied by increased lipid droplet formation and damaged mitochondria. *Cancer Res.* 2002; 62:1394–1400. [PubMed: 11888911]
44. Delikatny EJ, van Holst Pellekaan CJ, King NJ. Autologous lymphocyte-monocyte co-culture increases NMR-visible and cytoplasmic lipids in the absence of increased markers of lymphocyte activation. *Biochim Biophys Acta.* 2001; 1533:243–254. [PubMed: 11731334]
45. Milkevitch M, Shim H, Pilatus U, Pickup S, Wehrle JP, Samid D, Poptani H, Glickson JD, Delikatny EJ. Increases in NMR-visible lipid and glycerophosphocholine during phenylbutyrate-induced apoptosis in human prostate cancer cells. *Biochim Biophys Acta.* 2005; 1734:1–12. [PubMed: 15866478]

46. Diaz-Cruz ES, Brueggemeier RW. Interrelationships between cyclooxygenases and aromatase: unraveling the relevance of cyclooxygenase inhibitors in breast cancer. *Anticancer Agents Med Chem.* 2006; 6:221–232. [PubMed: 16712450]
47. Koehne CH, Dubois RN. COX-2 inhibition and colorectal cancer. *Semin Oncol.* 2004; 31:12–21. [PubMed: 15252926]
48. Krysan K, Reckamp KL, Sharma S, Dubinett SM. The potential and rationale for COX-2 inhibitors in lung cancer. *Anticancer Agents Med Chem.* 2006; 6:209–220. [PubMed: 16712449]
49. Wang D, Mann JR, DuBois RN. The role of prostaglandins and other eicosanoids in the gastrointestinal tract. *Gastroenterology.* 2005; 128:1445–1461. [PubMed: 15887126]

Figure 1. Schematic illustration of the MR-compatible cell perfusion assay used to derive an invasion index and to quantify choline and lipid metabolites. ECM, extracellular matrix.

Figure 2.

(a) Representative ¹H MR images showing reduced degradation of extracellular matrix (ECM) by Pooled and Clone 2 cyclooxygenase-2 (COX-2)-silenced MDA-MB-231 cells relative to parental MDA-MB-231 cells. (b) Quantitative time-dependent invasion indices $I(t)$ were obtained from diffusion-weighted intracellular water profiles of parental MDA-MB-231 cells (filled squares) and COX-2-silenced cells (open squares). Values are mean $\pm$ standard deviation. * $p < 0.05$, unpaired t -test; $p < 0.057$, Mann-Whitney U -test; $n = 4$. Data for COX-2-silenced cells were pooled from Clone 2 ($n = 2$) and Pooled cells ($n = 2$). (c) Fold change in expression of invasion-related transcripts in COX-2-silenced Clone 2 cells (gray) and Pooled cells (black) relative to MDA-MB-231 parental cells. #Reduction in u-PA in MDA-MB-231 *versus* Clone 2 cells did not fulfill the posterior probability criterion of >0.9 . However, this transcript is shown because this criterion was met in MDA-MB-231 *versus* Pooled cells and because u-PA levels were consistently reduced in Clone 2 cells in three separate microarray experiments with a consistency of 1.71-fold (shown), 1.79-fold and 1.53-fold (not shown). MMP, matrix metalloproteinase; PRSS3, mesotrypsin; TIMP1, tissue inhibitor of metalloproteinase-1; t-PA, tissue plasminogen activator; u-PA, urokinase plasminogen activator; VEGFA, vascular endothelial growth factor A.

Figure 3.

(a) Representative ^1H MR spectra displayed at comparable signal-to-noise levels from intact perfused parental MDA-MB-231 cells (top) and Pooled cyclooxygenase-2 (COX-2)-silenced cells (bottom) at 24 h. The insets in the corresponding top and bottom panels display representative high-resolution ^1H MR spectra from the water-soluble fraction of cell extracts, and confirm a decrease in phosphocholine (PC) and lactate (Lac) with COX-2 silencing. (b) ^{31}P MR spectra acquired from intact parental MDA-MB-231 cells (top) and Pooled COX-2-silenced cells (bottom) at 24 h, showing the decrease in PC with COX-2 silencing. DPDE, diphosphodiester; Glx, glutamine + glutamate; GPC, glycerophosphocholine; NTPs, nucleoside triphosphates; Pi, inorganic phosphate; tCho, total choline; tCr, total creatine.

Figure 4.

Quantification of data from ^1H and ^{31}P MR spectra of intact perfused cells, demonstrating significant decreases in total choline (tCho) (a), phosphocholine (PC) (b) and lactate (Lac) (c) in cyclooxygenase-2 (COX-2)-silenced (open squares) relative to parental (filled squares) MDA-MB-231 cells during perfusion. (d) Metabolite concentrations obtained from high-resolution ^1H MR spectra of water-soluble cell extracts, demonstrating significant decreases in tCho, PC and Lac in COX-2-silenced (open bars) relative to parental (filled bars) MDA-MB-231 cells. Values are means $\pm$ standard deviation. * $p < 0.05$, unpaired t -test; $p < 0.057$, Mann-Whitney U -test; $n = 4$.

Figure 5.

(a) Representative high-resolution ^1H MR spectra from the lipid fraction of cell extracts, demonstrating the increase in the mixed fatty acid signal F_{mix} at 1.3 ppm [$\text{F}(\text{CH}_2)$] and 0.9 ppm [$\text{F}(\text{CH}_3)$] in Pooled cyclooxygenase-2 (COX-2)-silenced cells relative to parental MDA-MB-231 cells. (b) Summary of the quantified F_{mix} in arbitrary units, demonstrating a significant increase in F_{mix} with COX-2 silencing. Values are means $\pm$ standard deviation. $*p < 0.05$, unpaired t -test; $p < 0.057$, Mann-Whitney U -test; $n = 4$. (c) Representative confocal images of lipid droplets visualized by Nile red staining (red), with Hoechst-stained nuclei (blue), in COX-2-silenced and COX-2-containing parental MDA-MB-231 breast cancer cells. (d) Summary of the number of lipid droplets, demonstrating a significant increase in lipid droplets in Clone 2 COX-2-silenced cells relative to parental MDA-MB-231 cells. Values are means $\pm$ standard deviation. $*p < 0.05$, unpaired t -test; $p < 0.057$, Mann-Whitney U -test; $n = 5$.

Figure 6.

Representative immunoblots of parental MDA-MB-231 cyclooxygenase-2 (COX-2)-containing and Clone 2 COX-2-silenced cells treated at the times indicated with interleukin-1 β (IL-1 β) (10 ng/mL) and prostaglandin E₂ (PGE₂) (50 nM), and probed for choline kinase (Chk) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).