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Aromatase and cyclooxygenases: enzymes in breast cancer☆

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Abstract

Aromatase (estrogen synthase) is the cytochrome P450 enzyme complex that converts C_{19} androgens to C_{18} estrogens. Aromatase activity has been demonstrated in breast tissue in vitro, and expression of aromatase is highest in or near breast tumor sites. Thus, local regulation of aromatase by both endogenous factors as well as exogenous medicinal agents will influence the levels of estrogen available for breast cancer growth. The prostaglandin PGE_2 increases intracellular cAMP levels and stimulates estrogen biosynthesis, and previous studies in our laboratories have shown a strong linear association between aromatase (*CYP19*) expression and expression of the cyclooxygenases (*COX-1* and *COX-2*) in breast cancer specimens. To further investigate the pathways regulating *COX* and *CYP19* gene expression, studies were performed in normal breast stromal cells, in breast cancer cells from patients, and in breast cancer cell lines using selective pharmacological agents. Enhanced COX enzyme levels results in increased production of prostaglandins, such as PGE_2. This prostaglandin increased aromatase activity in breast stromal cells, and studies with selective agonists and antagonists showed that this regulation of signaling pathways occurs through the EP_1 and EP_2 receptor subtypes. *COX-2* gene expression was enhanced in breast cancer cell lines by ligands for the various peroxisome proliferator-activated receptors (PPARs), and differential regulation was observed between hormone-dependent and -independent breast cancer cells. Thus, the regulation of both enzymes in breast cancer involves complex paracrine interactions, resulting in significant consequences on the pathogenesis of breast cancer.

Keywords: Aromatase; Cyclooxygenase-1; Cyclooxygenase-2; Prostaglandin E_2; PPAR receptors

1. Introduction

Estrogens are involved in numerous physiological processes including the development and maintenance of the female sexual organs, the reproductive cycle, reproduction, and various neuroendocrine functions. These hormones also have crucial roles in certain disease states, particularly in mammary and endometrial carcinomas. Cancer is the leading cause of death among women between the ages of 30 and 54 years, with breast and uterine cancers comprising 28 and 10%, respectively, of all cancers in females per year. An estimated 203,000 new cases of breast cancer in women will be diagnosed in the US in 2001. Currently, one out of eight American women will develop breast cancer in her lifetime [1]. Approximately one-third of all breast cancer patients and two-thirds of postmenopausal breast cancer patients have hormone-dependent (estrogen-dependent) breast cancer, which contains estrogen receptors and requires estrogen for tumor growth. The possible biochemical role(s) of estrogens and related compounds in the development of estrogen-dependent breast cancer remains to be elucidated. Estrogens, as well as other steroid hormones, produce normal physiological effects by binding to specific nuclear receptor proteins. Following the binding of estrogen to its receptor, the steroid–receptor complex interacts with sequence specific hormone response elements (HREs) in target cell chromatin to induce gene expression, resulting in DNA transcription to produce enhanced mRNA and protein synthesis. Estrogens induce growth and proliferation of certain target cells, such as breast epithelial cells and estrogen-dependent mammary carcinoma cells.

Estradiol, the most potent endogenous estrogen, is biosynthesized from androgens by the cytochrome P450 enzyme complex called aromatase, with the highest levels of enzyme present in the ovaries of premenopausal women, in the placenta of pregnant women, and in the peripheral adipose tissues of postmenopausal women and men. Aromatase is the enzyme complex responsible for the conversion of androgens to estrogens during steroidogenesis [2]. Aromatase activity has also been demonstrated in breast tissue in vitro [3–6], and the importance of intratumoral aromatase and

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local estrogen production is being unraveled [4,7–9]. Aromatase has been measured in the stromal cell component of normal breast and breast tumors, but the enzyme has also been detected in the breast epithelial cells in vitro [3,7–11]. Furthermore, expression of aromatase is highest in or near breast tumor sites [9]. The increased expression of aromatase cytochrome $P450_{arom}$ observed in breast cancer tissues was associated with a switch in the major promoter region utilized in gene expression, and promoter II is the predominant promoter used in breast cancer tissues [12,13]. As a result of the use of the alternate promoter, the regulation of estrogen biosynthesis switches from one controlled primarily by glucocorticoids and cytokines to a promoter regulated through cAMP-mediated pathways [12]. The prostaglandin PGE_2 increases intracellular cAMP levels and stimulates estrogen biosynthesis [12], whereas other autocrine factors such as IL-1β do not appear to act via PGE_2 [14].

2. Aromatase and cyclooxygenases in breast cancer

Local production of PGE_2 via the cyclooxygenase isozymes (constitutive COX-1 isozyme and inducible COX-2 isozyme) can influence estrogen biosynthesis and estrogen-dependent breast cancer. This biochemical mechanism may explain epidemiological observations of the beneficial effects of non-steroidal anti-inflammatory drugs (NSAIDs) on breast cancer [15,16]. Recent studies in our laboratory suggest a relationship between *CYP19* gene expression and the expression of *COX* genes [17]. Gene expressions of *CYP19*, *COX-1*, and *COX-2* were performed in 20 human breast cancer specimens and in 5 normal control breast tissue samples. Increased *CYP19* expression was observed in samples with a greater extent of breast cancer cellularity (Fig. 1), in agreement with literature reports showing

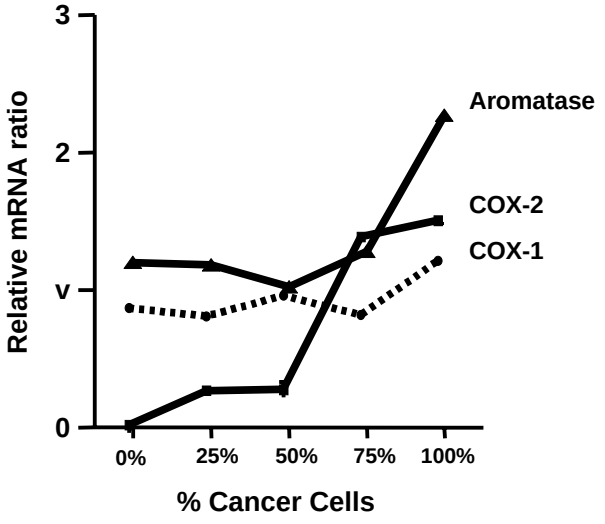

Fig. 1. Expression of aromatase (*CYP19*), *COX-1*, and *COX-2* gene expression in human breast tissue specimens.

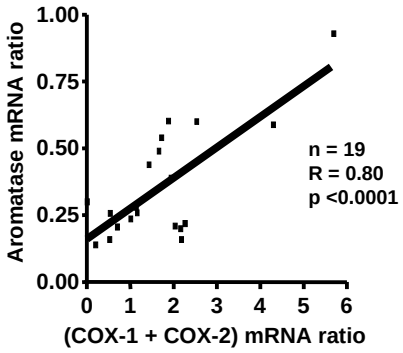

Fig. 2. Correlation of aromatase (*CYP19*) gene expression with *COX-1*, and *COX-2* gene expression in human breast tissue specimens.

that aromatase levels were higher in tumors than in normal tissue. In addition, *COX-2* mRNA expression rose significantly with increasing breast cancer cellularity in the samples (Fig. 1). Linear regression analysis using a bivariate model showed a strong linear association between *CYP19* expression and the sum of *COX-1* and *COX-2* expression ($R = 0.80$, $P < 0.0001$, Fig. 2) [17]. This significant relationship between the aromatase and cyclooxygenase enzyme systems suggests that autocrine and paracrine mechanisms may be involved in hormone-dependent breast cancer development via growth stimulation from local estrogen biosynthesis.

3. PGE_2 regulation of aromatase via prostanoid receptors

Prostaglandins such as PGE_2 are capable of activating both protein kinase A (PKA) and protein kinase C (PKC) pathways, and PGE_2 binds to four main prostanoid receptors, designated EP_1 [18], EP_2 [19], EP_3 [20,21] and EP_4 [22,23] based on their different pharmacological properties and secondary messenger pathways [24,25]. These G-protein-coupled receptor pathways are classified as either stimulatory or inhibitory. EP_1 is linked to a stimulatory pathway that activates protein kinase C and initiates a cascade of serine/threonine phosphorylation events. Both the EP_2 and EP_4 receptors are coupled to the protein kinase A signal transduction system. However, in contrast to the EP_2 receptor, the signal produced by the EP_4 receptor is lost within a few minutes due to agonist-induced desensitization [26]. EP_3 is the only inhibitory receptor identified thus far. It is also coupled to the adenylate cyclase–PKA signal transduction system, but its effects are mediated by an inhibitory G-protein subunit. This pathway represents an inherent mechanism of terminating PGE_2 signaling.

Selective agonists and antagonists were used to probe pathways to determine their involvement in aromatase regulation [27]. PGE_2 (1 μM) significantly increased aromatase activity levels after 24 h of treatment (Fig. 3). Two

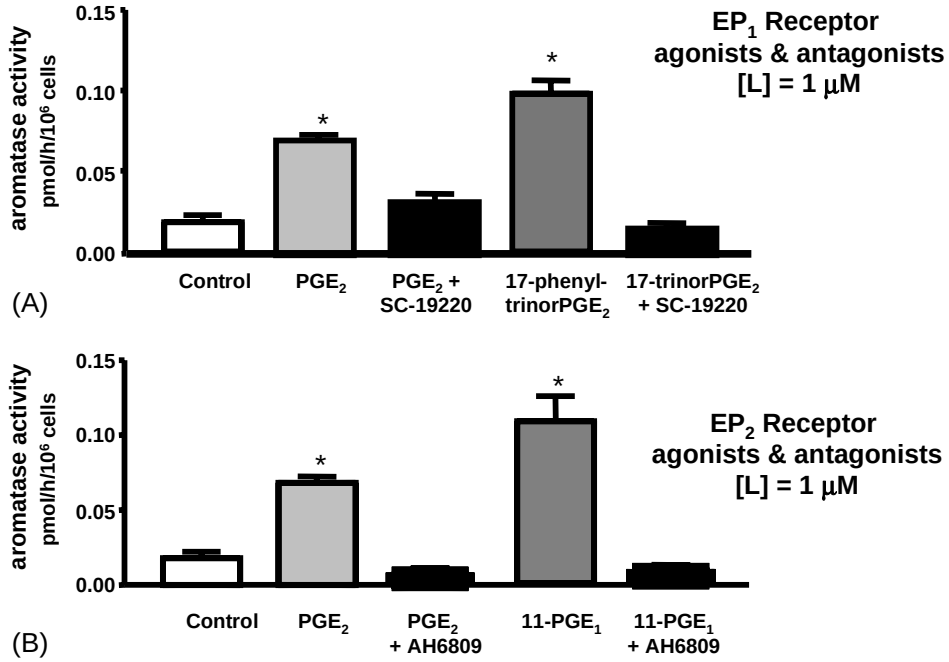

Fig. 3. (A) Regulation of aromatase activity by the EP_1 pathway. PGE_2 and 17-phenyl trinor PGE_2 (17-PGE_2) were tested at 1 μM. SC-19220 was added simultaneously at 10 μM with 17-PGE_2 or PGE_2. (B) Regulation of aromatase activity by the EP_2 pathway. PGE_2 and 11-deoxy PGE_1 (11-PGE_1) were tested at 1 μM. AH 6809 was added simultaneously at 10 μM with 11-PGE_1 or PGE_2. Values are expressed as pmol of ^3H_2O formed per hour incubation time per million cells and reported as mean ± S.D. $^*P < 0.05$ by unpaired Student's *t*-test ($n = 3$).

synthetic analogs of PGE_2, 17-phenyl trinor prostaglandin E_2 and 11-deoxyprostaglandin E_1, were used in these experiments. 17-Phenyl trinor prostaglandin E_2 is an EP_1 agonist [28] and 11-deoxyprostaglandin E_1 is a selective EP_2 agonist [29]. Both ligands were tested at 1 μM for 24 h to evaluate their effects on aromatase activity. Both significantly increased aromatase activity over control levels ($P < 0.05$) (Fig. 3A). The activity levels induced by these agonists were comparable to those induced by PGE_2.

The combined effects of EP agonists and antagonists on aromatase activity were also examined. SC-19220, a selective antagonist at the EP_1 receptor [30], and AH 6809, an antagonist of the EP_1 and EP_2 receptors [31], were used simultaneously with their respective agonists at 10 μM. SC-19220, when used in combination with 17-phenyl trinor PGE_2, decreased activity levels to that of control (Fig. 3A). The same effect was observed when added along with PGE_2. Likewise, when AH 6809 was added in conjunction with the EP_2 agonist 11-deoxy PGE_1 or PGE_2, aromatase activity levels were decreased to basal levels (Fig. 3B). These levels were not significantly different from that of control. These results are particularly important since PGE_2, 17-phenyl trinor PGE_2, and 11-deoxy PGE_1 all significantly induced aromatase activity levels over control ($P < 0.05$). On the other hand, 1 μM sulprostone, an EP_3 agonist [32], had no apparent effect on aromatase activity. These results suggest that PGE_2 induces aromatase activity and expression through at least two prostanoid receptor subtypes, EP_1 and EP_2.

4. Regulation of aromatase and cyclooxygenases by growth factors

Knowledge of the signaling pathways that regulate the expression and enzyme activity of aromatase and cyclooxygenases in stromal and epithelial breast cells will aid in understanding the interrelationships of these two enzyme systems and potentially identify novel targets for regulation. The effects of epidermal growth factor (EGF), transforming growth factor-β (TGFβ), and tetradecanoyl phorbol acetate (TPA) on aromatase and cyclooxygenases were studied in primary cultures of normal human adipose stromal cells and in cell cultures of normal immortalized human breast epithelial cells MCF-10F, estrogen-responsive human breast cancer cells MCF-7, and estrogen-unresponsive human breast cancer cells MDA-MB-231 [33].

Selected agonists of signaling pathways associated with tumorigenesis were used to assess COX isozyme inducibility in the estrogen-responsive MCF-7 and estrogen-independent MDA-MB-231 cell lines. A 24 h dose with 50 pM TGFβ or 4 nM EGF did not alter COX-1 or COX-2 levels relative to untreated controls. However, the phorbol ester, TPA, which stimulates PKC pathways, induced COX-2 levels by 75% in MDA-MB-231 cells (Table 1). The estrogen-responsive MCF-7 cells showed no significant change in COX-1 or COX-2 following treatment with any experimental agent used. Aromatase activity was significantly increased by EGF and TPA in MCF-7 cells, but not TGFβ (Fig. 4A). Although aromatase activity was much lower in MDA-MB-231 cells

Table 1
COX-1 and COX-2 regulation in breast cancer cell lines through multiple signaling pathways

Treatment	COX-2		COX-1	
	MCF-7	MDA-MB-231	MCF-7	MDA-MB-231
Control	100.0 ± 5.0	100.0 ± 5.8	100.0 ± 19.8	100.0 ± 9.9
4 nM EGF	112.1 ± 18.7	63.6 ± 38.8	133.4 ± 40.7	77.0 ± 5.3
50 pM TGFβ	83.1 ± 3.5	106.0 ± 13.4	106.5 ± 42.7	64.6 ± 26.7
50 nM TPA	86.0 ± 19.5	174.3 ± 37.9*	136.4 ± 19.9	111.0 ± 22.6

Hormone-responsive (MCF-7) and hormone-independent (MDA-MB-231) breast cancer cell lines were grown to subconfluence in growth medium and refreshed with reduced serum media for 24 h. At this time, cells were treated in triplicate with 50 pM TGFβ, 4 nM EGF, 50 nM TPA, or ethanol for an additional 24 h. Total extracted proteins were subjected to Western analysis for COX-2 and COX-1. Band luminescence was quantified and normalized to β-actin. Values are expressed as percent change relative to control lanes from the respective cell line, and reported as mean ± S.D. ($n = 3$).

* $P < 0.05$ by unpaired Student's t-test ($n = 3$).

than in MCF-7 cells, stimulation by growth factors was still observed. In contrast to MCF-7 cells, TGFβ and TPA significantly increased activity in MDA-MB-231 cells (Fig. 4B), while only a modest increase with EGF was observed.

Untreated normal adipose stromal cells exhibited high basal levels of COX-1 but low to undetectable levels of COX-2. A dramatic induction of COX-2 was observed in the adipose stromal cells by EGF, TGFβ, and TPA. Collectively, this data suggests higher constitutive expression of COX-2 in epithelial cells, which is inducible through PKC and smad signaling pathways. Modulation of aromatase enzyme activity by signaling factors in adipose stromal cells was determined using the tritiated water-release assay (Fig. 5). EGF,

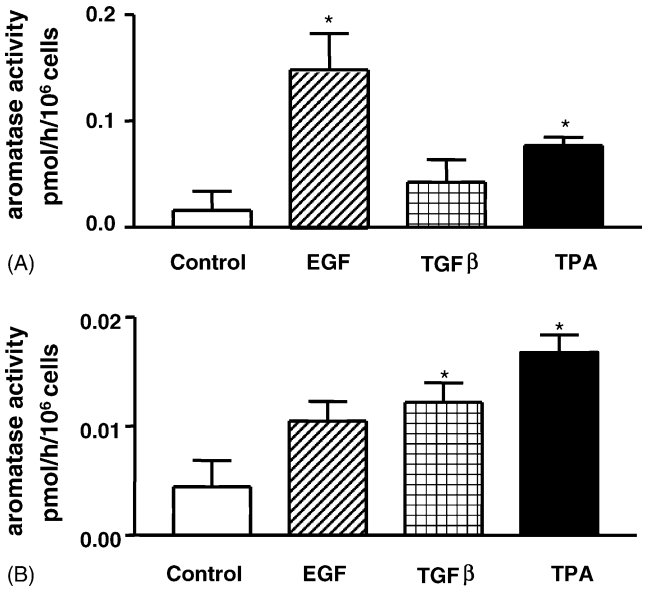

Fig. 4. Modulation of aromatase enzyme activity in human breast cancer cell lines by signaling factors. MCF-7 cells (A) and MDA-MB-231 cells (B) in T-25 flasks were pre-treated for 18 h with 4 nM EGF, 50 pM TGFβ, 50 nM TPA, 100 nM APTA or 0.1% ethanol (control) in media. Subsequently, the cells were incubated with 2 μCi per flask of [1β-^{3}H]-androst-4-ene-3,17-dione (50 nM) for an additional 4 h. Activity levels were normalized to the number of cells in each flask. Values are expressed as pmol of ^3H_2O formed per hour incubation time per million cells and reported as mean ± S.D. * $P < 0.05$ by unpaired Student's t-test ($n = 3$).

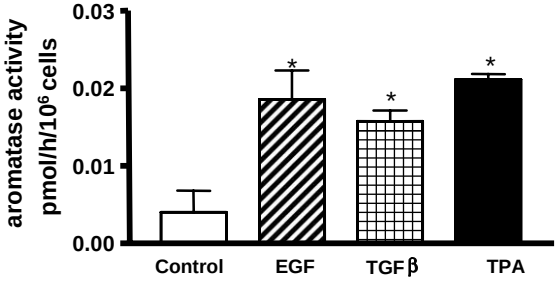

Fig. 5. Modulation of aromatase enzyme activity in adipose stromal cells by signaling factors. Adipose stromal cells in T-25 flasks were pre-treated for 24 h with 4 nM EGF, 50 pM TGFβ, 50 nM TPA or 0.1% ethanol (control) in defined media. Subsequently, the cells were incubated with 2 μCi per flask of [1β-^{3}H]-androst-4-ene-3,17-dione (50 nM) for an additional 18 h. Activity levels were normalized to the number of cells in each flask, determined by the diphenylamine DNA assay. Values are expressed as pmol of ^3H_2O formed per hour incubation time per million cells and reported as mean ± S.D. * $P < 0.05$ by the unpaired Student's t-test ($n = 3$).

TGFβ and TPA all significantly increased enzyme activity after 24 h of treatment ($P < 0.05$). EGF and TPA produced higher activity levels than TGFβ. The concentration of EGF used in this study (4 nM) corresponds to 25 ng/ml.

5. Regulation of cyclooxygenases by PPARs in breast cancer

Peroxisome proliferators are a broad class of structurally heterogeneous compounds that include herbicides, plasticizers, eicosanoids, polyunsaturated fatty acids and non-steroidal anti-inflammatory drugs. They were originally described as inducers of both the size and number of peroxisomes [34], membrane-bound organelles that perform β-oxidation of fatty acids, among other diverse metabolic functions [35]. The first described compounds were hypolipidemic drugs of the fibrate class, which caused hepatocarcinogenesis in rodents after chronic exposure [34,36–38]. Since then, they have been linked to different physiological events such as adipocyte differentiation, angiogenesis [39] lipid metabolism and metabolic disorders such as obesity

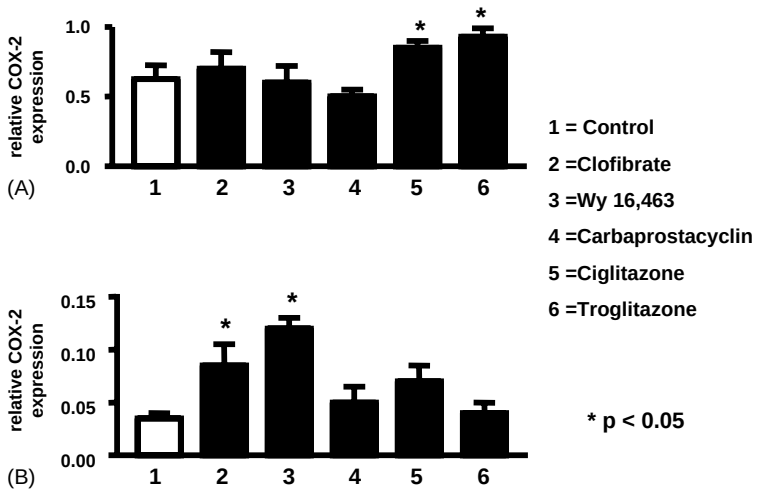

Fig. 6. Regulation of COX-2 protein expression in MCF-7 cells (A) and in MDA-MB-231 (B) by synthetic PPAR ligands. Whole cell lysates were prepared from cells treated for 18 h with 0.1% ethanol (control), 300 μM clofibrate, 50 μM Wy 14,643, 100 nM carbaprostacyclin, 6 μM ciglitazone or 2 μM troglitazone. Thirty micrograms of whole cell lysates were subjected to Western blotting analysis with antibodies to COX-2 and β-actin. Quantified levels are expressed as relative COX-2/β-actin protein expression and reported as mean ± S.D. $^{*}P < 0.05$ by the unpaired Student's *t*-test ($n = 3$).

and type II diabetes [40,41]. Although structurally diverse, peroxisome proliferators selectively bind a set of nuclear hormone receptors called peroxisome proliferator-activated receptors (PPARs), consisting of three known isoforms of human PPARs: α, β, and γ. The molecular mechanism(s) by which PPARs mediate their diverse effects involves binding DNA as heterodimers with the retinoid X receptors (RXRs) α, β or γ, nuclear receptors for 9-*cis*-retinoic acid [41,42]. Ligand binding induces a conformational change in the receptor that is necessary for heterodimerization and interaction with coactivators. The DNA binding domain enables the recognition of peroxisome proliferator response elements (PPREs), specific DNA sequences in the promoters of target genes [43–46].

PPAR signaling, in recent years, has been implicated in the transcriptional regulation of COX-2 [47–50], the inducible enzyme that catalyzes the synthesis of prostaglandins from arachidonic acid. This development is strengthened by the discovery of a response element (PPRE) in the COX-2 promoter of human mammary epithelial cells [47]. Compelling evidence for the pathological significance of COX-2 in several cancers including breast cancer, indicates that upregulation of COX-2 is critical [51]. Expression of COX-1, the constitutive form of the enzyme, does not change appreciably during tumorigenesis. High levels of prostaglandins, particularly PGE_2, promote tumor growth and development. Furthermore, the prostanoid 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, is reportedly a PPARγ ligand [52,53], that induces COX-2 expression [47]. This suggests that COX-2 may be directly involved in its own upregulation.

The effects of various PPAR ligands on cyclooxygenase protein expression were evaluated in breast cancer cell lines. Regulation of COX-2 by synthetic ligands in MCF-7 cells was investigated. Whole cell lysates were prepared from cells treated with 300 μM clofibrate, 50 μM Wy 14,643, 100 nM carbaprostacyclin, 6 μM ciglitazone, and 2 μM troglitazone. The PPARα ligands, clofibrate and Wy 14,643 did not induce COX-2 protein (Fig. 6A). In contrast, statistically significant induction was obtained with both ciglitazone and troglitazone ($P < 0.05$). Interestingly, the carbaprostacyclin treatment reduced the amount of COX-2 protein below control levels ($P < 0.05$). These results suggest that COX-2 protein expression in MCF-7 cells is regulated by PPARs β and γ but not by PPARα. Whole cell lysates prepared from MDA-MB-231 cells treated with synthetic ligands were analyzed by Western blotting (Fig. 6B). The PPARα ligands clofibrate (300 μM) and Wy 14,643 (50 μM) both significantly induced COX-2 protein expression over control levels ($P < 0.05$, clofibrate; $P < 0.001$, Wy 14,643). Neither carbaprostacyclin (100 nM) nor the PPARγ ligands ciglitazone (6 μM) and troglitazone (2 μM) had any significant effect on COX-2 protein levels. In contrast to COX-2, expression levels of the COX-1 protein did not change in either MDA-MB-231 or MCF-7 cells with any treatment. Thus, cyclooxygenase-1 expression is not controlled by PPAR signaling in breast cancer epithelial cells.

6. Summary

Local regulation of aromatase by both endogenous factors as well as exogenous medicinal agents will influence the levels of estrogen available for breast cancer growth. The prostaglandin PGE_2 increases intracellular cAMP levels and stimulates estrogen biosynthesis, and previous studies in our laboratories have shown a strong linear association between aromatase (*CYP19*) expression and expression of the cyclooxygenases (*COX-1* and *COX-2*) in breast cancer

specimens. To further investigate the pathways regulating *COX* and *CYP19* gene expression, studies were performed in normal breast stromal cells, in breast cancer cells from patients, and in breast cancer cell lines using selective pharmacological agents. Enhanced COX enzyme levels results in increased production of prostaglandins, such as PGE_2. This prostaglandin increased aromatase activity in breast stromal cells, and studies with selective agonists and antagonists showed that this regulation of signaling pathways occurs through the EP_1 and EP_2 receptor subtypes. *COX-2* gene expression was enhanced in breast cancer cell lines by ligands for the various peroxisome proliferator-activated receptors, and differential regulation was observed between hormone-dependent and -independent breast cancer cells. Thus, the regulation of both enzymes in breast cancer involves complex paracrine interactions, resulting in significant consequences on the pathogenesis of breast cancer.

Acknowledgements

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