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Modulation of cholinephosphotransferase activity in breast cancer cell lines by Ro5-4864, a peripheral benzodiazepine receptor agonist

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Abstract

Changes in phospholipid and fatty acid profile are hallmarks of cancer progression. Increase in peripheral benzodiazepine receptor expression has been implicated in breast cancer. The benzodiazepine, Ro5-4864, increases cell proliferation in some breast cancer cell lines. Biosynthesis of phosphatidylcholine (PC) has been identified as a marker for cells proliferating at high rates. Cholinephosphotransferase (CPT) is the terminal enzyme for the de novo biosynthesis of PC. We have addressed here whether Ro5-4864 facilitates some cancer causing mechanisms in breast cancer. We report that cell proliferation increases exponentially in aggressive breast cancer cell lines 11-9-1-4 and BT-549 when treated with nanomolar concentrations of Ro5-4864. This increase is seen within 24 h of treatment, consistent with the cell doubling time in these cells. Ro5-4864 also upregulates c-fos expression in breast cancer cell lines 11-9-1-4 and BT-549, while expression in non-tumorigenic cell line MCF-12A was either basal or slightly downregulated. We further examined the expression of the CPT gene in breast cancer (11-9-1-4, BT-549) and non-tumorigenic cell lines (MCF-12A, MCF-12F). We found that the CPT gene is overexpressed in breast cancer cell lines compared to the non-tumorigenic cell lines. Furthermore, the activity of CPT in forming PC is increased in the breast cancer cell lines cultured for 24 h. Additionally, we examined the CPT activity in the presence of nanomolar concentrations of Ro5-4864. Biosynthesis of PC was increased in breast cancer cell lines upon treatment. We therefore propose that Ro5-4864 facilitates PC formation, a process important in membrane biogenesis for proliferating cells.
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Regulation of PC metabolism is one of the vital aspects of the cell cycle with implications in the control of cell proliferation as well as in apoptosis [1]. Alterations in membrane phospholipids have been associated with malignant transformation [2], tumorigenicity [3], and metastasis [4,5]. Studies have shown that malignant transformations result in altered membrane choline phospholipid metabolism in human cancer including breast [6–8], prostate [9], and brain tumors [10,11]. In vivo and in vitro studies using NMR spectroscopy have also identified high levels of phosphocholine in breast cancer and lower levels in non-tumorigenic tissues [12]. These findings show that choline phospholipids and their precursors can be used as markers of tumor progression. Phosphocholine is a precursor and a product of the break down of phosphatidylcholine (PC). Cholinephosphotransferase (CPT) is the terminal enzyme in the biosynthesis of PC by the CDP-choline pathway [13]. We have recently reported that CPT activity is increased in breast cancer cell lines compared to non-tumorigenic ones [14]. It was observed that there was differential expression between the CPT gene in breast cancer cell line 11-9-1-4 and the non-tumorigenic cell line MCF-12A. Sequence analysis of the gene from these cell lines indicated the presence of a S323Y mutation in

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the cancerous cell line, indicating that a change in the profile of CPT function in breast cancer probably took place.

PC is the most abundant phospholipid in eukaryotic biological membrane. Along with other phospholipids such as phosphatidylethanolamine (PE), and neutral lipids, PC forms a bilayer that defines the plasma membrane. Membrane integrity is regulated and maintained not only by the presence of these essential lipids, but also in the presence of cholesterol that encompasses the lipid bilayer and maintains membrane fluidity [15]. PC metabolism is also essential in signal transduction processes leading to mitogenic activity found in many cancers. Products of the hydrolysis of PC such as phosphocholine and diacylglycerol can function as second messengers that are utilized in mitogenic signaling pathways such as in the activation of the ras-raf-MAPK [16–18] and protein kinase C cascades [19]. Furthermore, ras oncogene product directly or indirectly causes an increase in the turnover of phosphatidylcholine in C3H10T1/2 cells [20].

Many benzodiazepines including Ro5-4864, which interacts with the peripheral benzodiazepine receptor, have been implicated in the onset of breast cancer [21]. There are contradicting reports based on binding characteristics of agonist to PBR and cell proliferation mechanisms. Initial studies using high concentrations of drug ligands have indicated that their binding to PBR could inhibit DNA synthesis in different mammalian cell lines [22,23]. It has now been shown that concentrations of peripheral benzodiazepine PK11195 in the receptor binding range (nanomolar range) stimulate DNA synthesis concomitant with increased cholesterol transport in the nuclear membrane [24]. Interestingly, many studies have also determined that there are differences in the binding characteristics between breast cancer cell lines and non-tumorigenic cells [24–32,5].

In another study, it was observed that peripherally active benzodiazepines in the presence of nerve growth factor caused an induction of c-fos [33]. Inducible transcription factor c-fos has been studied as a cytoplasmic regulator of the biosynthesis of phospholipids [34]. C-fos was shown to activate the metabolism of phospholipids in the cytoplasm by means of an AP-1-independent activity where by a rapid induction upon cell stimulation, it associates to the endoplasmic reticulum. In these reports, it was also suggested that this is where c-fos first regulates the synthesis and replenishment of phospholipids required for signal transduction pathways. Subsequently, c-fos regulates enzymes involved in the genesis of the new membrane necessary for cell growth. In addition, blocking c-fos expression by addition of antisense oligonucleotides showed a marked inhibition of phospholipid activation in NIH 3T3 cells [34].

In this study, we measured PC formation in breast epithelial cells. Our studies correlate reports of high expression of PBR and ligand-induced cell proliferation to c-fos induction and PC synthesis in different breast epithelial cell lines. We used PBR ligand Ro5-4864 to determine the authenticity of our current findings that PBR ligands can increase proliferation in breast cancer.

Materials and methods

Cell proliferation assay. Cells were cultured on 96-well plates at a concentration of 5000 cells/well in DMEM-F12 supplemented with 10% FBS with various concentrations of PBR ligands in a final volume of 100 μl and incubated for 24 h. Cell proliferation was analyzed by the WST-1 assay (Roche Biochemicals, Indianapolis, IN) by measuring the absorbance at the wavelength 420–480 nm.

Western blot analysis of c-fos. Cells were plated and cultured to confluence in DMEM/F-12 containing 10% FBS. Different concentrations of the PBR agonist ligand Ro5-4864 (Sigma, St. Louis, MO) were used to stimulate the cells for 24 h. After treatment, cells were trypsinized in 0.05% trypsin and then lysed in buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 10 μg/ml aprotinin). The protein samples were sonicated and the protein concentration was determined by the Lowry method [35]. Protein samples were then solubilized in SDS–PAGE buffer (0.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 1% of 2-mercaptoethanol, and 0.1% bromophenol blue) and electrophoresed on a 15% SDS–PAGE. The protein sample was electrically transferred to PVDF membrane at 100 V for 2 h in transfer buffer (25 mM Tris–HCl, 192 mM glycine, and 20% methanol). The PVDF membrane was blocked with 5% milk in TBS–Tween (10 mM Tris–HCl, pH 8.0; 150 mM NaCl, 0.05% Tween 20). After blocking, the membrane was washed three times with TBS–Tween. The membrane was incubated (1:1000) in mouse polyclonal anti-c-fos antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) in TBS–Tween containing 5% milk for 1 h at room temperature. The membrane was washed again three times and a secondary HRP conjugated goat anti-mouse antibody (1:5000), (Santa Cruz Biotechnologies, Santa Cruz, CA) was used to detect the binding of the primary antibody by incubation for 1 h. The PVDF membrane was again washed three times (10 min/wash) in TBS–Tween. Immunoreactive proteins were visualized by using chemiluminescence Western blot detecting reagents. All procedures for chemiluminescence were performed according to manufacturer's protocol (Perkin-Elmer, Boston, MA). Resulting protein on the membrane was exposed to X-ray film. To normalize the experiment and to ensure that the autoradiographic density at 67 kDa was due to stimulation by benzodiazepine Ro5-4864 of c-fos, blots were reprobed using anti β-actin antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). Blots were first washed in stripping buffer containing 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl, pH 7.4, at 50 °C for 30 min and subsequently washed, and the membranes were blocked for 2 h at room temperature in blocking buffer (5% non-fat milk, 10% TBS, and 10% Tween 20). Blots were then washed with TBS–Tween and incubated with mouse monoclonal β-actin antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) (1:1000 dilution in TBS–Tween containing 5% milk) for 1 h at room temperature. Blots were then washed and subsequently incubated with HRP conjugated goat anti mouse antibody (1:5000) for 1 h at room temperature in TBS–Tween containing 5% milk. Blots were washed and immunoreactive proteins were visualized by chemiluminescence as described. Membranes were exposed to Kodak scientific imaging X-ray film and processed for analysis.

Reverse transcriptase-polymerase chain reaction of CPT. Total RNA was isolated from 2.0×10^7 cells using the TRIZOL method (Invitrogen, Fredrick, MD). Experiments were carried out according to manufacturer's protocol. Cells were first washed with sterile PBS

three times and resuspended in 1 ml TRIZOL. The mixture was kept at RT for 5 min and 200 μl of chloroform was added and the mixture was vigorously stirred. The phases were separated by centrifuging at 10,000*g* for 15 min, at 4 °C. The RNA was then precipitated from the aqueous phase with isopropanol (25 °C). The pellet was washed with ample amounts of sterile 70% (25 °C) ethanol. The concentration and the purity of the RNA were analyzed in a UV spectrophotometer. RT-PCR was performed using 2 μg RNA from each sample, using the one-step RT-PCR kit (Invitrogen, Fredrick, MD). The primer sequences were acquired from the NCBI GenBank sequence of human CPT. The primer sequences used for CPT RT-PCR were as follows; forward primer 5′TTGCGCTCATTGGCAGACTTATGT3′ and reverse primer 5′TCTCTTCAATCCATGTTATTCTGA3′ (GenBank Accession No. NM_020244). GAPDH was also used as an internal control to ensure equal loading of RNA. The RT-PCR products were subjected to electrophoresis on a 1% agarose and visualized under UV light.

Assay of CPT activity. Cholinephosphotransferase activity was measured by monitoring the incorporation of [methyl-^{14}C]CDP-choline into phosphatidylcholine [14]. The dioleoylglycerol suspension was prepared by dissolving 100 mg of the lipid in 0.15 ml ethyl alcohol and 2.25 ml of 0.01% Triton X-100. The mixture was sonicated at 0 °C in a Model 185 Branson sonifier cell disruptor. The final reaction mixture contained the following: 10 mM $MgCl_2$, 5 mM reduced glutathione, 50 mM Tris–HCl (pH 8.5), 80 μM [methyl-^{14}C]CDP-choline (specific activity 40 Ci/mol), 6 mM 1,2-dioleoyl-glycerol, and the protein in the total volume of 100 μl. The reaction was started by adding 20 μl (20 μg protein) of samples and incubated at 37 °C for 2 min. The reaction was stopped by adding 550 μl of *n*-butanol. Lipids were extracted by adding 500 μl of butanol-saturated water. The mixture was then allowed to equilibrate for 10 min and was centrifuged in a Beckman model TJ-6 tabletop centrifuge at 3000 rpm for 10 min. Approximately 350 μl of the butanol layer was removed carefully and placed in a counting vial. The radioactivity was determined after adding 5 ml of hydrofluor scintillation fluid and counted in a Beckman LS-355 scintillation counter. In separate experiments, the same protocol was followed for the CPT assay. In separate experiments, 10 μM of PBR ligand Ro5-4864 was used to treat 3.0×10^6 cells for 24 h. Cells were trypsinized, washed, and finally resuspended in 1× PBS. CPT activity was determined as mentioned previously [14].

Results

Our data show that PBR agonist Ro5-4864 induces cell proliferation in breast epithelial cells. Using WST-1 cell proliferation reagent, we measured the abundance of proliferating cells in breast epithelial cells (Fig. 1). We show that this ligand-induced proliferation is enhanced in breast cancer cells compared to the non-tumorigenic cells. The stimulation of proliferation was minimal with optimal effects seen after 24 h. Proliferation was seen within the nanomolar range. This supports the reported data that PBR specific ligand-induced proliferation at low concentrations [24].

We have also determined that Ro5-4864 induced the expression of c-fos. Lower nanomolar concentrations induced c-fos expression in breast cancer cells, while induction in non-tumorigenic cells was either reduced or remained basal (Fig. 2). We also observed that c-fos expression was generally increased in breast cancer cells compared to non-tumorigenic cells. These changes in expression observed at the nanomolar range, were consistent with other observations where Ro5-4864 has been shown to promote biological activity [24]. Optimal c-fos expression in breast cancer cells 11-9-1-4 and BT-549 was observed at 0.1 μM. At the same concentration, the non-tumorigenic cells MCF-12A showed a decrease in expression.

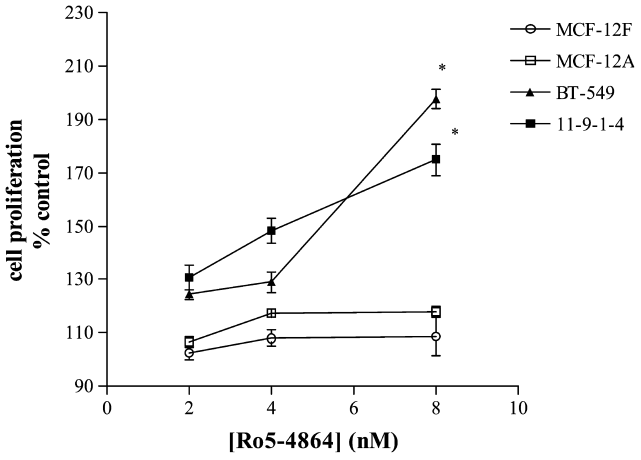

Fig. 1. PBR agonist Ro5-4864-induced cell proliferation in breast cancer cells. Cells (5000) were plated in DMEM-F12 for 24 h with Ro5-4864 (1–8 nM) or without ligand, and cell proliferation was measured by adding 10 μl WST-1 reagent to 100 μl of proliferated cells in culture. The absorbance of samples was measured with an ELISA plate reader at 450 nm. $N = 3$, $^*P \leqslant 0.01$.

CPT gene expression was also increased according to our findings (Fig. 3). The CPT gene expression was increased in breast cancer cell lines 11-9-1-4 and BT-549 compared to non-tumorigenic cells MCF-12A and MCF-12F. CPT activity was also enhanced by determining the incorporation of [methyl-^{14}C]CDP-choline to DAG to form PC; this is the terminal step in PC biosynthesis catalyzed by CPT (Fig. 4). We report in these studies that PBR agonist Ro5-4864 enhanced c-fos expression (Fig. 2) and CPT activity in breast cancer cells compared to non-tumorigenic cells (Fig. 5).

Discussion

Aberrant proliferation is one of the defining factors during malignant transformation. A characteristic feature of malignant neoplasm is its ability to invade and metastasize to other locales in the body. Dysregulation of normal cell growth and differentiation are the main defining events during carcinogenesis. These events are marked by malfunctioning signal transduction pathways that would normally confine the cell to "normal" growth. Numerous studies have shown that PBR agonist Ro5-4864 regulates cell proliferation in a number of cell models [26–30]. Malignant breast tumors are primarily characterized by cell proliferation, tumor invasion, and metastasis [36].

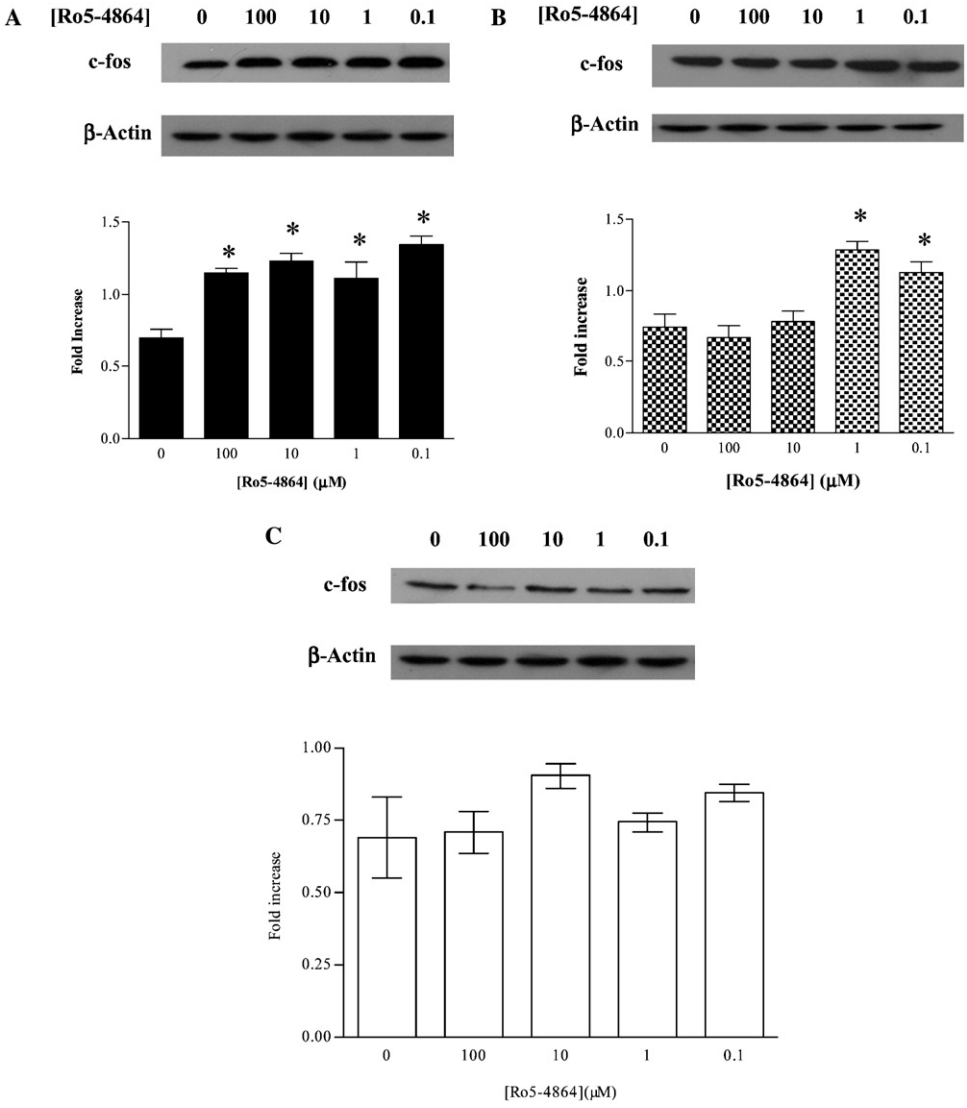

Fig. 2. Effect of PBR ligand on c-fos expression in 11-9-1-4, BT-549, and MCF-12A cells. Breast epithelial cells were cultured to confluence. Cells were then incubated with or without indicated concentrations of Ro5-4864 for 1 h. C-fos expression was determined as indicated in the Western blot protocol under Materials and methods. Data are shown as c-fos expression where basal expression is defined by unstimulated cells. $N = 3$, $^{*}P \leqslant 0.001$.

Cells that are undergoing transformation require a new environment to encourage further growth. In our study, we focused on membrane biogenesis as a way to determine whether PBR ligand Ro5-4864 induces cell proliferation. Our findings that PBR ligand agonist enhances breast cancer cell proliferation are further supported by our observations that CPT activity is increased as shown by an increase in the biosynthesis of PC. In addition, our studies show that increase in membrane phospholipid (PC) biosynthesis is enhanced by Ro5-4864.

We have reported that the CPT gene is overexpressed in breast cancer cells compared to non-tumorigenic ones (Fig. 3). CPT catalyzes the final reaction in the biosynthesis of PC, an essential membrane component. PC, together with other phospholipids like PS, PE, fatty acids, and cholesterol, forms the lipid bilayer. We also showed that CPT activity is increased in breast cancer cells. The increase in proliferation is further mediated by PBR agonist Ro5-4864 as seen in our studies and those of others [24]. This implicates a role for PBR in carcinogenic mechanisms that are still yet to be elucidated. In a previous study, we analyzed the sequences of CPT gene in 11-9-1-4 and MCF-12A cells. We identified a critical mutation S323Y, in the partial sequence [14]. This tyrosine substitution could cause a phosphorylation leading to signaling cascades that may lead to cancer.

Curran et al. [33] reported that c-fos expression was induced by peripherally active benzodiazepines. C-fos is a transcription factor that constitutes DNA-binding AP-1 complexes that regulate gene expression promoting many cellular changes, including cell growth and differentiation in many cells [37]. It was discovered that

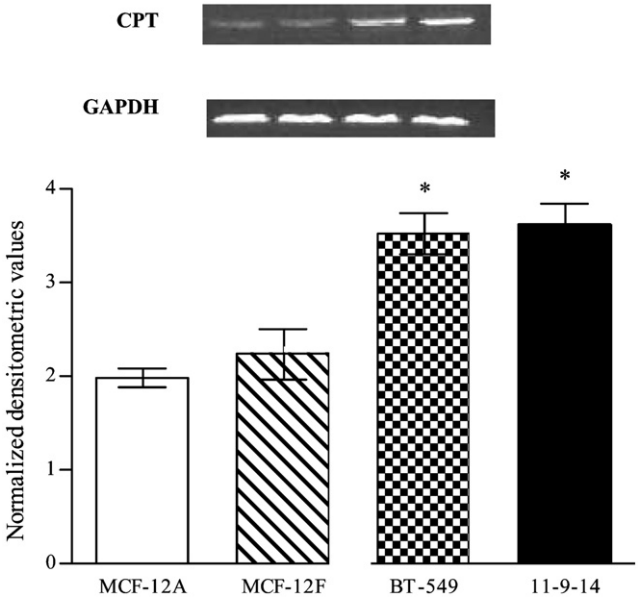

Fig. 3. CPT gene expression. RT-PCR was used to determine PBR expression levels. Lanes 1 and 2, MCF-12A and MCF-12F cell lines, respectively. Lanes 3 and 4, BT-549 and 11-9-1-4, respectively. Two micrograms of RNA was used for RT-PCR. GAPDH was used as an internal control. Experiments were carried out as indicated under Materials and methods. Data shown are expressed as PBR expression, normalized with GAPDH levels. $N = 3$, $^{*}P \leqslant 0.001$.

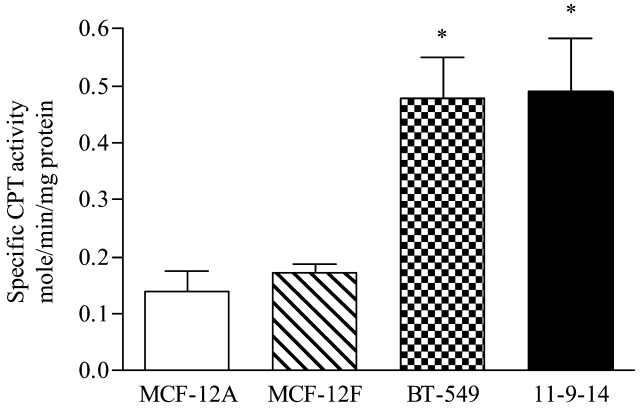

Fig. 4. CPT activity in breast epithelial cells. Cells were grown to confluence following growth for 24 h and CPT activity was determined. The incorporation of [methyl-^{14}C]CDP-choline to form PC was determined. The procedure for CPT activity determination is given under Materials and methods. $N = 3$, $^{*}P \leqslant 0.001$.

c-fos expression was induced in the presence of nerve growth factor [33]. Interestingly, in another study [34], it was revealed that c-fos regulates the metabolism of phospholipids cytoplasmically through its interactions with the endoplasmic reticulum. Experiments demonstrated that in the fibroblast cell line NIH 3T3, c-fos expression was a determinant in the rate of synthesis of phospholipids. This action of c-fos was abrogated using antisense oligonucleotide resulting in a dramatically reduced phospholipid synthesis. We have support-

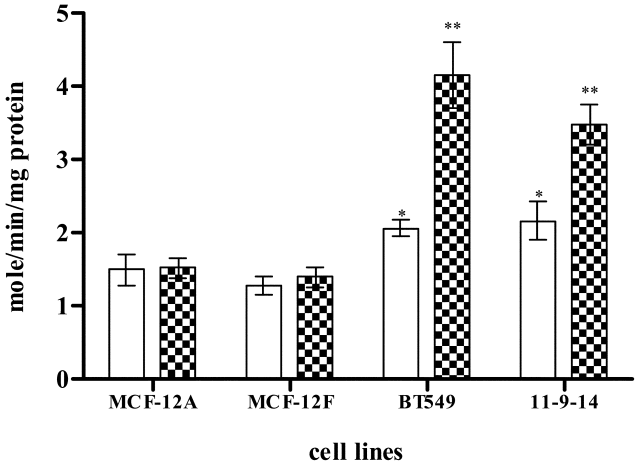

Fig. 5. Effect of PBR ligand Ro5-4864 on CPT activity in breast epithelial cells. Ligand-induced CPT activity was determined by incubating cells with 10 nM Ro5-4864 for 24 h. The incorporation of [methyl-^{14}C]CDP-choline to form PC was determined. $N = 3$, $^{*}P \leqslant 0.001$.

ing data showing that Ro5-4864 induces the expression of c-fos in breast epithelial cells. It can be elucidated that c-fos induction can function in the events that lead to turnover of phospholipids that participate in signal transduction pathways. The expression level of c-fos in untreated breast cancer cells was lower and increased with an increase in the concentration of the ligand that plateaus at higher concentrations. This implies that the induction of c-fos by Ro5-4864 is effective for c-fos induction at low nanomolar concentrations as seen in proliferation experiments [24].

C-fos-dependent phospholipid activation can affect the turnover of phosphoinositides, which, when hydrolyzed, can cause the generation of second messengers [38]. We propose that the presence of cholesterol transported by nuclear PBR can cause induction of c-fos by affecting the nuclear environment that may favor transcription. Furthermore, there is increasing evidence of nuclear lipid signaling. Tamiya-Koizumi [39] reported that choline phospholipid metabolism also occurs in the cell nucleus. Phosphatidylcholine and sphingomyelin were reported to be present in the nuclear membrane, chromatin, and nuclear matrix. PC metabolic enzymes such as CTP: phosphocholine cytidyltransferase (CT), PC specific phospholipase D (PC-PLD) were all found to be present in the nucleus. Of all these enzymes reported, only CT contained a nuclear localization signal (NLS). CT is localized to the nucleus after dephosphorylation upon treatment with oleate in HeLa cells [39]. The role cholesterol may play in the nucleus is not clear. However, CPT serine–tyrosine mutation shown in our earlier reports may explain its probable involvement in phosphorylation–dephosphorylation events critical in cell signaling.

It is possible that c-fos may also be involved in the regulation of key enzymes involved in the generation

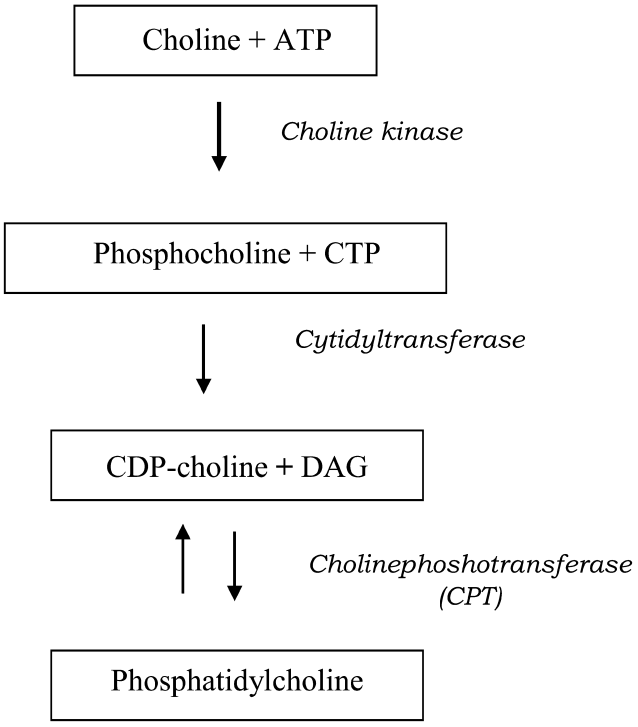

Fig. 6. Cholinephosphotransferase catalyzed biosynthesis of PC.

of second messengers. CPT catalyzes the reaction that produces PC by transferring choline onto diacylglycerol (DAG) in the favored forward reaction (Fig. 6) [13]. This reaction is reversible. During tumorigenesis, many proteins are altered by up-or downregulation of the gene. This alteration could have functional consequences for CPT. CPT malfunction may result in the reaction being reversed to favor the production of DAG. DAG accumulation and diversion can activate many signaling cascades including that of PKC resulting in cancer causing mechanisms in the cell.

Many molecular and cellular changes are currently used as a factor in diagnosing breast cancer as prognostic indicators. Effective anticancer therapies are key to treating breast cancer. In this study, we believe that our data present PC as a phospholipid whose metabolism is an important factor to consider in understanding the causes of breast cancer.

Acknowledgment

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