[Methyl-^{3}H]-choline Incorporation into MCF-7 Cells: Correlation with Proliferation, Choline Kinase and Phospholipase D Assay

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Abstract. *Background: [Methyl-^{3}H]-choline is a promising new positron emission tomography (PET) agent used for cancer imaging whose mechanism has still not fully been elucidated. In this study, whether [methyl-^{3}H]-choline determined by measuring the activity of choline kinase (ChoK) and phospholipase D (PLD) in rapidly proliferating and confluent breast cancer MCF-7 cells is related to cell proliferation or not was investigated. Materials and Methods: The activity of ChoK and PLD were determined using ion exchange chromatography and transphosphatidylation assay respectively. Results: [Methyl-^{3}H]-PCho content expressed as pmol mg^{-1} protein^{-1} min^{-1} (n=6) was significantly higher in the exponentially growing (484.04±20.23) compared with confluent (70.35±9.83) cells using Student's t-test (p<0.001). Moreover, PLD activity expressed as the mean (n=6) (disintegration per minute (d.p.m.)/μg protein±SD (mean S phase ±SD)) showed significantly higher (p<0.001) activity in the exponentially growing cells (196.39±2.21 d.p.m./μg protein (39.69±4.00%)) compared with confluent cells (99.10±1.35 d.p.m./μg protein (9.33±0.82%)). Conclusion: This study indicates that the major water-soluble choline metabolite was phosphocholine (PCho) as a consequence of increased ChoK and PLD activity in the exponentially growing cells compared to confluent cells.*

The breakdown of choline-containing phospholipids such as phosphatidylcholine (PtdCho), the most abundant membrane phospholipid, is important in cell signal transduction (1, 2). The hydrolysis of PtdCho, a source of lipid second messengers, can be regulated by phospholipase A_2 (PLA_2), phospholipase C (PLC) and phospholipase D (PLD) (1). PtdCho hydrolysis by PLD leads to the formation of phosphatidic acid (PA) and choline, whereas PtdCho hydrolysis by PLC generates diacylglycerol (DAG) and phosphocholine (PCho) (3-4).

Most mammalian PLD activity is found associated with membranes and appears to be specific for PtdCho (5). PLD is considered a critical regulator of cell proliferation (6-7), survival signalling (8-9), and cell transformation and tumour progression (7, 10-12). Thus, there is some evidence that indicates that PLD activity is involved in enhancing cell proliferation and transformation. In addition, choline kinase (ChoK), a cytosolic enzyme, catalyzes the reaction of Mg^{2+}-ATP with choline yielding ADP and PCho. It was reported that increased levels of ChoK play a role in the generation of human tumours by *ras* oncogenes (13-16) and this might be related to cell proliferation. Yamashita and Hosaka (17) reported that ChoK mRNA and protein levels are highest in the exponential phase and decline in the stationary phase of tumour cell growth. Many studies demonstrated that choline incorporation is related to cellular proliferation and rapidly proliferating cells increase their demands for phospholipid synthesis. Therefore, the main objective of this study was to determine whether choline kinase (ChoK) and phospholipase D (PLD) activity is related to choline-containing phospholipids and tumour cell proliferation or not.

Materials and Methods

Cell culture and culture media. The human breast cancer (MCF-7) cells were obtained from the American Type Culture Collection (ATCC; Virginia, USA). MCF-7 cells were grown in RPMI-1640 medium containing glutamax-I, supplemented with 20 U/ml penicillin, 20 μg/ml streptomycin and 10% foetal calf serum (FCS; Gibco, Grand Island NY, USA). Cells were incubated at 37˚C (in 5% CO_2:95% air).

Radio-labelled compounds. [Methyl-^{3}H]-choline chloride (specific activity: 2.92 TBq/mmol) was obtained from Amersham Pharmacia

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Biotech UK limited (Buckinghamshire, UK). [9,10-^{3}H]-myristic acid (specific activity: 1.85 TBq/mmol) was obtained from Sigma-Aldrich (UK).

Chemicals and instrumentations. All chemical reagents used were supplied by Sigma-Aldrich (UK) unless otherwise specified. AG-50W-X8 Dowex cation [H^+] exchange resin (200-400 mesh; Bio-Rad Econo-column; 0.75 bed volume) columns, dye reagent and bovine serum albumin (BSA) were obtained from Bio-Rad (UK). Ultima Gold scintillant fluid was obtained from Meridian (Scotland, UK). A liquid scintillation counting analyser (LSC-1900CA; Tri-CARB) was obtained from Packard (Frankfurt, Germany). Flexible plates with a plastic (polyfluorocarbon) sheet were purchased from Avicel Analtech (Scotland, UK). Glass-backed silica gel 60 Å (20 cm x 20 cm; 250-μm thick) TLC plates were purchased from Whatman (Clifton, NJ, USA). The surface autoradiographic enhancer, EN^{3}HANCE, for spraying TLC plates was purchased from Perkin Elmer (USA), and X-ray film (X-OMAT AR) was purchased from Kodak (Rochester, NY, USA). The phosphatidylbutanol (PtdBut) standard was purchased from Affiniti Research Products (Scotland, UK).

Cell extract and choline kinase activity. MCF-7 cell extract from exponentially growing and confluent cells was prepared separately as follows: the activity of ChoK was determined after homogenizing an MCF-7 cell pellet in ice-cold 25 mM Tris/HCl buffer (1.5 ml), pH 7.4, using a Dounce homogenizer (Scotland, UK). The homogenate was centrifuged at 500 xg for 5 min to remove cell nuclei and debris, and the protein content was determined for each sample. ChoK assay mixture containing 40 mM Tris/HCl with unlabelled carrier of 0.25 mM choline, pH 7.4, [methyl-^{3}H]-choline (37 kBq/sample), 2 mM ATP, and 10 mM $MgCl_2$ were added to 96 μg of homogenate protein in a total volume of 0.1 ml. After incubation for 20 min at 37˚C, the reaction was stopped by the addition of an ice-cold mixture of methanol and chloroform in a total volume of 1.5 ml. Lipid-metabolite extraction was then carried out (18). The total radioactivity in each aqueous phase was determined. Separation of [methyl-^{3}H]-PCho from [methyl-^{3}H]-choline was carried out using ion exchange chromatography on Dowex H^+ columns.

Measurement of PLD activity. PLD activity was assayed using a modification from Shen *et al.* (19) as follows: exponentially growing and confluent MCF-7 cells were grown in 25 cm^2 flasks in triplicate. Two sets of these flasks were cultured, one set in the absence (control) and one in the presence of 1-BtOH (experimental) respectively. Cells were prelabelled with 37 kBq of [9,10-^{3}H]-myristic acid per 1 ml of medium at 37˚C (in 5% CO_2:95% air) for 5 h. In the last hour, 0.3% 1-BtOH was added to the medium. MCF-7 cells were then washed 6 times with ice-cold phosphate-buffered saline (PBS), trypsinized, with trypsin EDTA, then neutralized with medium and centrifuged. The media were then decanted and lipid-metabolite extraction was carried out on each cell pellet (20). Lipid phase samples were lyophilised to dryness using Speed Vac Plus (SC110 A Savant, Frankfurt, Germany). The lipidic pellets were resuspended in 10 μl chloroform/methanol (4:1, v/v) and spotted onto thin layer chromatography (TLC) soft-coat silica gel plates.

Characterization of phospholipids. The lipid phases from exponentially growing and confluent MCF-7 cells were separated on TLC soft-coat silica gel plates using the following solvent: chloroform/methanol/glacial acetic acid/water (25/15/4/2, v/v/v/v) (20). TLC plates were dried in a fume hood using a warm air dryer, then placed in a chromatography tank saturated with iodine vapour, until lipids were visualized as yellow spots. The areas corresponding to authentic standards (PtdCho, PtdEth, PtdSe and PtdBut) were marked and traced lightly with a pencil, and following sublimation of iodine from the plates at room temperature, radioactive areas were scraped into scintillation vials, and counted after the addition of 10 ml of scintillation fluid. In some experiments, prior to scraping, the plates were sprayed with EN^{3}HANCE spray and submitted to autoradiography, being exposed for 30 min at –80˚C using X-OMAT AR Kodak film. Relative optical densities in selected regions of interest were determined using an image display system and computer (Densitometer Fluor-S Max multi imager, Bio-Rad) with Quantity One software (Bio-Rad).

Results

Choline kinase (ChoK) assay. Ion exchange chromatography was carried out to separate [methyl-^{3}H]-PCho formed during the assay from [methyl-^{3}H]-choline substrate. The measured eluate of [methyl-^{3}H]-PCho was standardised to its protein values and expressed as pmol mg^{-1} $protein^{-1}$ min^{-1}: [Methyl-^{3}H]-PCho (mean±SD) was 484.04±20.23 and 70.35±9.83 for exponentially growing and confluent cells (n=6), respectively. ChoK activity and [methyl-^{3}H]-PCho content were significantly higher in the exponentially growing cells compared with the confluent cells using Student's *t*-test (t=45, p<0.001).

Measurement of PLD activity and characterization of phospholipids. The TLC technique was used to characterize and identify the presence of [^{3}H]-phosphatidylbutanol ([^{3}H]-PtdBut) in exponentially growing and confluent populations of MCF-7 cells after incubation with [9,10-^{3}H]-myristic acid. After running the TLC plates of the lipid phase samples, several lipid metabolites and phospholipids were identified by co-chromatography with standards. Figure 1 shows the locations of authentic standards. The transphosphatidylation [^{3}H]-PtdBut reaction results were normalized to their corresponding protein contents and expressed as the mean (n=6) (disintegrations per minute (d.p.m.)/μg protein±SD (mean S phase±SD)). [^{3}H]-PtdBut accumulation was significantly higher (p<0.001) in the exponentially growing cells (196.39±2.21 d.p.m./μg protein (39.69±4.00%)) compared with the confluent cells (99.10±1.35 d.p.m./μg protein (9.33±0.82%)), which is a measure of PLD activity. The results from both control samples of the exponentially growing cells and the confluent cells showed that 98.59±0.28% and 97.39±0.22% respectively of [9,10-^{3}H]-myristic acid was incorporated into PtdCho.

Figure 2 shows the autoradiogram of labelled lipid metabolites and phospholipid in the presence of 1-BtOH of exponentially growing (lane 1) and confluent (lane 2) MCF-7 cells.

Discussion

PCho serves as a precursor for the synthesis of phospholipids such as PtdCho and phosphatidylserine (PtdSe) (21). PtdSe is a substrate for the synthesis of phosphatidylethanolamine (PtdEth). Therefore, regulating ChoK is crucial for the synthesis of most cellular phospholipids. These phospholipids are the major components of cellular membranes and are also precursors for signal transduction (1). The activation of ChoK is essential for building membranes, cell growth, cell proliferation, and for rebuilding phospholipids that are degraded in the process of signal transduction. Thus, ChoK and PCho have an essential function in cell proliferation. In this study, ChoK activity and choline water-soluble metabolites such as PCho content were investigated in exponentially growing and confluent MCF-7 cells incubated with [methyl-^{3}H]-choline for 10 min, which were further identified using TLC. Here ChoK showed higher activity in exponentially growing cells compared to confluent cells. This is consistent with other studies (20, 22-23) and presumably is in response to demands from rapidly proliferating tumour cells for increased phospholipid synthesis. This study supports the nuclear magnetic resonance spectroscopy (NMRs) results of high PCho contents in tumour cells and growth-promoted cells (24-26). In addition, high levels of PCho were previously observed in NMRs from breast cancer tissue (27-29) due to increased ChoK activity (24, 30-32). Up-regulation of ChoK activity with increased PCho content was observed in 3T3 fibroblasts treated with mitogenic growth factors (20, 23, 33-35), and in human colon cancer, adenoma tissue (36), human tumour-derived cell lines, and in human breast, as well as in prostate cancer (37-39). Here we have demonstrated that higher [methyl-^{3}H]-PCho content and ChoK activity in cells incubated with [methyl-^{3}H]-choline was related to cell proliferation. Smith *et al.* (40-43) showed that the concentration of PCho in tumour cells was strongly associated with the S phase fraction. It was also shown that ChoK is an essential regulator for DNA synthesis, cell proliferation and PCho production (14-15, 20, 23, 44-45). Chung *et al.* (46) demonstrated a significant correlation between PCho production and DNA synthesis in NIH3T3 fibroblasts sublines expressing high human ChoK levels without increasing PtdCho synthesis. They demonstrated that ChoK up-regulates insulin and insulin-like growth factor I (IGF-I), increasing cellular PCho production by MAPK-regulated mechanisms that ultimately result in DNA synthesis and enhanced cell proliferation. But this study indicated that PtdCho did not play a role in the mediation of the induced ChoK/PCho effects since its synthesis was not significantly altered. On the other hand, Nishijima *et al.* (47) reported that ChoK is a regulatory enzyme for PtdCho synthesis. By means of an *in situ* autoradiographic assay for the base-exchange reaction of phospholipids with L-serine in Chinese hamster ovary cell colonies, they demonstrated that the activity of ChoK and the enzyme that catalyses the base-exchange of phospholipids with choline (choline-exchange enzyme) *in vitro* in mutant extracts were strikingly reduced, while the specific activities of other enzymes of PtdCho synthesis were normal compared to their parent controls. In parallel, they also demonstrated that PtdCho synthesis and PtdCho turnover rates as well as PCho synthesis were also reduced approximately two-fold in these mutant extracts *in vivo* and this was suggested to be due to the primary defect in ChoK levels. In addition, from a theoretical point of view, Infante (48) suggested that ChoK is a rate-limiting enzyme for PtdCho synthesis in rat liver.

The phospholipid composition of cell membranes represents a balance between their synthesis and degradation. Here the increased generation of [methyl-^{3}H]-PCho in the exponentially growing cells may be as a consequence of the up-regulation of ChoK in response to demands from these cells for increased phospholipid synthesis and their role as phospholipid precursors. Preliminary results suggested that a simple mechanism, involving either activation of a PtdCho-specific PLC (40, 44, 49), or direct generation of PCho by phosphorylation of choline either from intracellular reservoirs or originating from specific hydrolysis of PtdCho by PLD could explain the high levels of PCho associated with proliferation.

However, many studies have demonstrated that the generation of PCho may be unrelated to the activation of a PtdCho-specific PLC and that the enzymatic activity of PLC was not involved in the induction of the DNA synthesis of NIH3T3 cells (50-51). PtdCho-specific PLD hydrolysis will result in phosphatidic acid (PA), which was found to be a relevant signalling molecule in some signalling pathways and choline which can then be phosphorylated to PCho (52). PLD stimulates RAF-1 signalling and induces cell proliferation (53). Since there is no specific inhibitor available for either mechanism (activation of a PtdCho-specific PLC or PLD) to study their role in signal transduction and cell proliferation, HC-3 was used and proven to block ChoK activity as well as PCho production by interfering with the activation of RAF-1/MAPK pathway without affecting the PtdCho-specific phospholipases. This block of PCho production after stimulation of normal cells with serum or different growth factors suggests in this case that PCho is not generated by activation of a PtdCho-specific PLC (34). On the other hand, many studies demonstrated that the elevated constitutive levels of PCho result from a PLD/ChoK pathway (34, 16, 54-55) that is essential for DNA synthesis and cell proliferation. Moreover, PCho itself is mitogenic and it has a role as a crucial messenger for DNA synthesis. Cell proliferation in NIH3T3 cells can be triggered off by the generation of PCho (54). Several studies pointed out that an increase in ChoK activity results in elevated levels of PCho, a putative novel

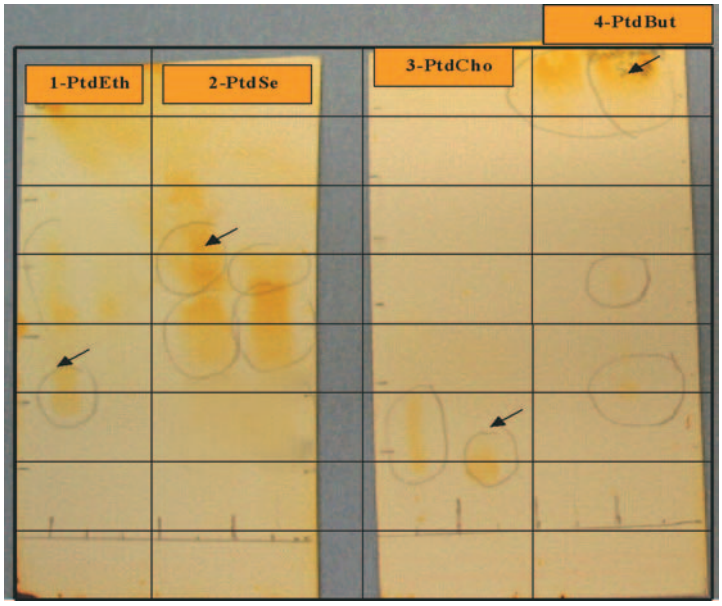

Figure 1. *TLC plates of the lipid phase samples with authentic standards following sublimation of iodine: phosphatidylethanolamine (PtdEth): lane 1, phosphatidylserine (PtdSe): lane 2, phosphatidylcholine (PtdCho): lane 3, and phosphatidylbutanol (PtdBut): lane 4. The arrows indicate the locations of the standards.*

second messenger involved in cell proliferation (34, 49, 54, 56). The TLC results of the aqueous metabolites showed that exponentially growing cells have higher [methyl-^{3}H]-choline and [methyl-^{3}H]-PCho content compared with confluent cells. Moreover, in this study we provide evidence that [methyl-^{3}H]-PCho is the major metabolite in the exponentially growing cells compared to the confluent cells and here over 98% of the activity in the lipid phase in MCF-7 cells was identified as PtdCho. Taken together, our results provide evidence that the increased [methyl-^{3}H]-PCho content, which was identified with TLC as the major component of choline water-soluble metabolites, and ChoK activity in the exponentially growing cells compared to the confluent cells is related to cell proliferation that may be involved in membrane synthesis and signalling. Moreover, the elevated [methyl-^{3}H]-PCho content in the exponentially growing cells compared with the confluent cells may be in part due to the elevated ChoK and PLD activity.

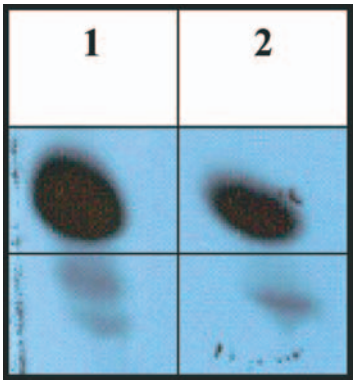

Figure 2. *Autoradiogram of labelled lipid metabolites and phospholipids in the presence of 1-BtOH. Lane 1: exponentially growing cells and lane 2: confluent cells.*

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