Activation of Phosphatidylcholine Cycle Enzymes in Human Epithelial Ovarian Cancer Cells

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Abstract

Altered phosphatidylcholine (PC) metabolism in epithelial ovarian cancer (EOC) could provide choline-based imaging approaches as powerful tools to improve diagnosis and identify new therapeutic targets. The increase in the major choline-containing metabolite phosphocholine (PCho) in EOC compared with normal and nontumoral immortalized counterparts (EONT) may derive from (a) enhanced choline transport and choline kinase (ChoK)-mediated phosphorylation, (b) increased PC-specific phospholipase C (PC-plc) activity, and (c) increased intracellular choline production by PC deacylation plus glycerophosphocholine-phosphodiesterase (GPC-pd) or by phospholipase D (pdl)-mediated PC catabolism followed by choline phosphorylation. Biochemical, protein, and mRNA expression analyses showed that the most relevant changes in EOC cells were (a) 12-fold to 25-fold ChoK activation, consistent with higher protein content and increased ChoK\textsubscript{a} mRNA expression levels; and (b) 5-fold to 17-fold PC-plc activation, consistent with higher, previously reported, protein expression. PC-plc inhibition by tricyclohexadecan-9-yl-potassium xanthate (D609) in OVCAR3 and SKOV3 cancer cells induced a 30% to 40% reduction of PCho content and blocked cell proliferation. More limited and variable sources of PCho could derive, in some EOC cells, from 2-fold to 4-fold activation of pdl or GPC-pd. Phospholipase A\textsubscript{2} activity and isoform expression levels were lower or unchanged in EOC compared with EONT cells. Increased ChoK\textsubscript{a} mRNA, as well as ChoK and PC-plc protein expression, were also detected in surgical specimens isolated from patients with EOC. Overall, we showed that the elevated PCho pool detected in EOC cells primarily resulted from upregulation/activation of ChoK and PC-plc involved in PC biosynthesis and degradation, respectively. Cancer Res; 70(5): 2126–35. ©2010 AACR.

Introduction

Despite progress in clinical oncology, epithelial ovarian cancer (EOC) continues to be the gynecologic malignancy with the highest death rate in industrialized countries and a 5-year survival as low as 44% (1). Suitable molecular imaging approaches could facilitate the elucidation of mechanisms of EOC progression, improve clinical diagnosis and follow-up, and identify new therapeutic targets.

Detection of an aberrant phosphatidylcholine (PC) metabolism in tumors by magnetic resonance spectroscopy (MRS; refs. 2, 3) allowed for the identification of novel indicators of in vivo tumor progression by using choline-based MRS and positron emission tomography (4–8). An elevation of the \textsuperscript{1}H MRS resonance at 3.2 ppm, mainly due to headgroups of choline-containing metabolites (tCho), is a common feature in several cancers including EOC (9–17). Changes in the tCho spectral profile reflect altered contents and fluxes of phosphocholine (PCho), glycerophosphocholine (GPC), and free choline (Cho) in the PC cycle (Fig. 1A).

Major mechanisms of PCho accumulation in tumor cells include enhanced choline transport and choline kinase (ChoK)–mediated phosphorylation and activation of PC-specific phospholipases (13, 15, 17–19). These biochemical features could represent fingerprints of tumor progression and potential therapeutic targets (6, 20–22).

Previous studies have shown that ChoK is upregulated by oncogenes, growth factors, and carcinogens (22, 23) and that its ChoK\textsubscript{a} isoform could be constitutively activated in human tumor cells (24), in which it may act as a prognostic factor (25, 26). Furthermore, specific pharmacologic or small interfering RNA-ChoK inhibition have antiproliferative effects on cancer cells (18, 27).
A neutral-active PC-specific phospholipase C (PC-plc) is also activated in EOC compared with nontumoral EONT cells (15, 28), inhibition of this enzyme being associated with reduced response of EOC cells to mitogens (28).

To further elucidate the mechanisms underlying the altered tCho profile in EOC cells, we report on the measurements of absolute activity rates of ChoK, PC-plc, phospholipase D (pld), and glycerophosphocholine-phosphodiesterase (GPC-pd), as well as on differential metabolic fluxes through the PC deacylation pathway in EOC and EONT cells. Comparative analyses of mRNA expression levels were also performed for ChoKα and ChoKβ isoforms, cytidylyltransferase (ct), and phosphocholine transferase (pct), pld1, pld2, and 19 phospholipase A2 isoforms. The role of PC-plc in the intracellular accumulation of PCho in EOC cells and the effect of its inhibition on cell growth have been investigated. Analyses are also reported on ChoKα mRNA expression and on ChoK and PC-plc protein expression in a set of surgical specimens from patients with EOC.

Materials and Methods

Epithelial Ovarian Nontumoral and EOC Cells

Ovary surface epithelial (OSE) cells, their stably immortalized nontumoral variants (IOSE and hTERT), and the serous EOC cell lines OVCAR3, SKOV3, CABAI, and IGROV1 were prepared and cultured as described (15). Four additional cell lines were used for microarray analysis: OVCA432 (15), INT-Ov1, INT-Ov2 (29), and OAW42 (kindly provided by Dr. A. Ullrich, Max-Planck Institute, Germany). Tumor lines were maintained as described (15), except for OAW42, which was maintained in MEM.

Chemicals

1,2-Dihexanoyl-sn-glycero-3-phosphocholine (C6PC) was purchased from Avanti Polar Lipids, Inc.; trimethylsilyl-propionic-2,2,3,3-d4 acid sodium salt was from Merck & Co.; tetramethylsilane, CDCl3, and CD3OD were from Cambridge Isotope Laboratories, Inc.; tricyclodecan-9-yl-potassium xanthate (D609), sn-glycero-3-phosphocholine, alkaline phosphatase (AP), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and other chemicals were purchased from Sigma-Aldrich.

Clinical Specimens

Clinical specimens, including OSE cells, were obtained with Istituto Nazionale dei Tumori-Milan Review Board approval. Informed consent to use leftover biological material for investigation purposes was obtained from all patients. EOC samples were taken at the time of initial surgery from 21 patients with histologically confirmed EOC (stages III–IV according to International Federation of Gynecology and Obstetrics criteria) who underwent exploratory laparatomy between 1998 and 2005. Samples were immediately frozen in liquid nitrogen and stored at −80°C until use.

Cell Extracts

Aqueous cell extracts were prepared as described (15). Lipid extracts were obtained and dissolved in CDCl3/CD3OD (2:1, v/v) as described (13).
Nuclear Magnetic Resonance Spectroscopy

High-resolution nuclear magnetic resonance (NMR) experiments (25°C) were performed at either 400 or 700 MHz (Bruker AVANCE Spectrometers). 1H and 31P NMR spectra of aqueous cell extracts were obtained using references, acquisition parameters, data processing, and data analysis as described (15, 30). Fully relaxed 1H NMR spectra of lipid extracts were acquired using a 30-degree flip angle, 12.7 s repetition time, 32K time domain data points, and 128 transients. Relative PC quantification was performed on the –N3(CH3)3 headgroup signal at 3.22 ppm.

Biochemical Assays of PC Cycle Enzymes in EOC and EONT Cells

Enzyme activities were determined at 25°C by NMR assays specifically developed and validated in our laboratory. Due to the difficulty of obtaining sufficient amounts of OSE cells, hTERT cells were used as EOC nontumoral counterparts. This choice was justified by the insignificant differences in the respective contents of PCho, GPC, free Cho, and tCho detected in OSE and hTERT cells (15).

ChoK activity. 1H and 31P NMR assays were performed upon the addition of exogenous choline chloride, ATP, and Mg2+ ions to cytosolic cell preparations in Tris-HCl (pH 8.0), as described (15, 30).

PC-specific phospholipases and GPC-phosphodiesterase.

Cell pellets (15–20 × 10⁶ cells) were resuspended, sonicated, and centrifuged as described (30, 31), and the assays performed on supernatants using a monomeric short chain PC, C6PC, as substrate, below the critical micellar concentration (ref. 32; 5.0 mmol/L in 10 mmol/L CaCl2; pH 7.2; ref. 33). ChoK activity.

Choline formation was measured by 1H NMR as a product of the following catabolic pathways:

(1) pld-mediated C6PC hydrolysis

\[
\text{C6PC} \xrightarrow{\text{pld}} \text{Cho} \tag{a}
\]

and

(2) phosphodiesterase (pd)-mediated hydrolysis of GPC formed by the combined action on C6PC of pla (plA1 and plA2) and lysophospholipases (lpl).

\[
\text{C6PC} \xrightarrow{\text{pla}} \text{lysoC6PC} \xrightarrow{\text{lpl}} \text{GPC} \tag{b}
\]

plus

\[
\text{GPC} \xrightarrow{\text{GPC–pd}} \text{Cho} \tag{c}
\]

The overall activity rate of Cho released by the pathways in (a) and (b) plus (c) is referred to here as C6PC-plied.

The PC-plc activity was determined from the additional increase in Cho production in the same cell lysates as above, in the presence of exogenous alkaline phosphatase (AP), according to the reactions:

\[
\text{C6PC} \xrightarrow{\text{plc}} \text{PCho} \tag{d}
\]

plus

\[
\text{PCho} \xrightarrow{\text{AP}} \text{Cho} \tag{e}
\]

For these assays, each cell lysate was divided into four aliquots (15 × 10⁶ cells each): two prepared in the presence or absence of C6PC (to determine, by subtraction, the contribution of endogenous Cho) and two in the presence or absence of AP (to determine the contributions of either pld or PC-plc activity to the final Cho content).

31P NMR analyses allowed the evaluation of differential pla2-mediated and plA1-mediated production of 1-hexanoyl-α-glycerophosphocholine (2-lyso-C6PC, 0.31 ppm) and 2-hexanoyl-α-glycerophosphocholine (1-lyso-C6PC, 0.47 ppm) in the C6PC (0.01 ppm) deacylation pathway.

The activity of GPCpd was determined by adding exogenous GPC (5 mmol/L) to the cell lysate, in the presence of 10 mmol/L of MgCl₂, as previously described (34).

No differences in phospholipase assays were observed in cell lysates incubated in the presence or absence of protease inhibitors, indicating that Cho and PCho were not produced by protein degradation.

Microarray Analysis

Gene expression of the enzymes and transporters demonstrated or proposed to be involved in choline metabolism were searched using a data set generated in an independent study at the Istituto Nazionale dei Tumori.2 Human genome U133 Plus 2.0 GeneChip (Affymetrix), covering 47,000 transcripts and variants, was used. Briefly, total RNA from eight cell lines and OSE cells (four different preparations, one to three passages at maximum) were extracted using TRIzol Reagent (Life Technologies) and cleaned-up on mini-columns (RNAeasy Mini Kit, Qiagen). A similar protocol was used for 20 frozen EOC surgical specimens in some experiments, as specified. Total RNA (3 μg) was reverse-transcribed, labeled, and hybridized to the chip at 45°C for 16 h. After washing, chips were scanned (Affymetrix GeneChip Scanner3000 7G) and images were analyzed using GeneChip Operating Software v1.4 (GCOs1.4). Probe set intensities were calculated using the Microarray Analysis Suite (MAS v5.0, Affymetrix). Data analysis and exploration of probe set intensity levels were performed using Bioconductor open source⁵ under the R Software environment (35). The reading of probe level

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2 I. De Cecco, M. Bagnoli, P. Alberti, E. Marchesi, F. Raspagliesi, D. Mezzanzanica, and S. Canevari, unpublished data.

⁵ http://www.bioconductor.org/
data, background correction, normalization by MASS “global scaling” procedure, and summarizing the probe set values into one expression measure were performed using the affy package. All microarray data are available in Gene Expression Omnibus, accession number GSE19352.

**Real-time PCR**

Total RNA was extracted from OSE and IGROV1, SKOV3, and OVCAR3 cell lines using the RNAspinn Mini Isolation Kit (GE Healthcare) and reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems). Quantitative real-time PCR was performed by an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) using TaqMan Gold RT-PCR Reagents. Three independent RNA preparations were reverse transcribed and at least two quantitative PCR (qPCR) reactions were done using each reverse transcription (RT) product. The primer pairs and the TaqMan probes for the target mRNAs were from Applied Biosystems (Assay ID: CHKA, Hs00608045_m1; CHKB, Hs00993897_g1; PCYT1A, Hs00192339_m1; PCYT1B, Hs00191464_m1; CHPT1, Hs00220348_m1; PLD1, Hs00160118_m1; and PLD2, Hs00160163_m1).

The ΔΔCT method was used to determine the quantity of the target sequences in EOC cell lines relative both to OSE cells (calibrator) and to an endogenous control (GAPDH). Analyses were performed using SDS software 2.2.2 (Applied Biosystems). Expression levels were presented as the relative fold change and calculated as:

\[
2^{-\Delta\Delta CT} = 2^{-[\Delta CT(\text{target})-\Delta CT(\text{calibrator})]} \quad \text{and each}
\]

\[
\Delta CT = \Delta CT(\text{target sequence}) - \Delta CT(\text{GAPDH})
\]

**Western Blotting in Cells and Tissues**

Total cell and tissue lysates were obtained as described (36). Lysates were separated by 10% SDS-PAGE and transferred to nitrocellulose (Hybond C-Super; Amersham) or polyvinylidene fluoride membranes (Immobilon PVDF, Millipore). Membranes were incubated in 1% nonfat dry milk overnight at 4°C with rabbit polyclonal anti-PC-PLC antibody (28), custom-made rabbit polyclonal anti-ChoK antibody (27), or the goat polyclonal antibody anti-ChoK (Santa Cruz Biotechnology, Inc.). Mouse monoclonal antibodies against β-actin or GAPDH were used as loading controls. Horseradish peroxidase–labeled secondary antibodies (Amersham) were added for 1 h at room temperature. Immunoblots were developed using the SuperSignal West Pico chemiluminescence substrate kit (Pierce Biotechnology, Inc.). Densitometry analyses were performed with a Bio-Rad apparatus (Bio-Rad Laboratories Srl) using Quantity One software.
One software or using ImageJ (Wayne Rasband, NIH, Washington, DC).

Statistical Analysis
Data were analyzed using GraphPad Software version 3.03 or using JMP Software Package (Brooks/Cole-Thomson Learning). Statistical significance of differences was determined by one-way ANOVA or by Student’s t test, as specified. Differences were considered significant at \( P < 0.05 \).

Results

Levels of Choline-Containing Metabolites in EONT and EOC Cells
In agreement with our previous study (15), the tCho content in EONT cells was 5.4 ± 0.6 nmol/10^6 cells, comprising a PCho content of 2.6 ± 0.3 nmol/10^6 cells (Fig. 1B). Significantly higher tCho (10.3–20.3 nmol/10^6 cells) and PCho levels (8.0–14.0 nmol/10^6 cells) were detected in the set of four analyzed EOC cell lines (Fig. 1B; one-way ANOVA, \( P < 0.001 \)). The contribution of PCho to the tCho resonance increased from 53.2 ± 4% in EONT to 75 ± 5% in EOC cells (\( P < 0.001 \)), with a resulting decrease in the GPC/PCho ratio. The overall PC content increased 1.3-fold to 1.6-fold (\( P < 0.04 \)) in EOC compared with EONT cells (Fig. 1C).

Choline Transport
Gene expression analyses of members of the three Cho transporter families (37) showed no differential mRNA levels for the high-affinity Cho transporter CHT1 in EOC compared with OSE cells. Among organic cation transporters, OCT1 and OCT2 remained below the expression threshold, whereas OCT3, the most highly expressed one, was substantially downregulated in cancer cells (Fig. 2A). Among Cho transporter–like proteins (CTL1–5), only CTL3 showed some increase in mRNA expression in EOC cells. Choline might also be transported by a Cho/H^+ antiport system involving Na^+}/H^+ exchangers (NHE; ref. 37); however, in our analysis, the overall expression of NHE1–5 did not reveal any difference between EOC and OSE cells (Fig. 2A).

Overexpression and Activation of ChoK in EOC Cells
The mRNA expression analyses of eight EOC cell lines compared with OSE cells showed ChoKα (CHKA gene) but not ChoKβ (CHKB gene) upregulation (data not shown). Data were independently validated by RT-qPCR on three EOC cell lines (OV CAR3, IGROV1, and SKOV3) compared with three different OSE preparations (Fig. 2B). These results, together with the downregulation of ct (PCYT1A and PCYT1B genes) and pct (CHPT1 gene) in cancer cells (Fig. 2B), point to a major role of ChoKα in the build-up of the PCho pool. Indeed, a significant 2.7 ± 0.3-fold increase in ChoK protein expression was observed by Western blotting of EOC cell lysates (Fig. 3A and B). Also in agreement with our previous study (15), assays on cytosolic preparations (Fig. 3C) showed a 12-fold to 25-fold ChoK activation in EOC (7.0–16.0 nmol/10^6 cells/h, i.e., 28.5–62.3 nmol/mg protein/h) compared with hTERT cells (0.6 ± 0.2 nmol/10^6 cells/h, i.e., ~3.0 nmol/mg protein/h).

Activity of PC-Specific Phospholipases in Choline-Producing PC Degradation Pathways
PCho production in cancer cells might also be derived from the phosphorylation of intracellular Cho released by PC catabolism.

The overall rate of Cho production by PC hydrolytic pathways (defined as pld^α activity in Materials and Methods)
measured in cell lysates in 10 mmol/L of CaCl2 was 6.7 ± 0.7 nmol/10^6 cells/h in EONT cells, increased 2-fold to 3-fold in IGROV1 and OVCAR3, but remained unaltered in CABA1 and SKOV3, respectively (Fig. 4A, white columns).

To dissect the individual contributions to the rate of Cho formation (pld*) respectively given by pld and by the deacylation pathway, we performed two types of experiments. GPC-pd assays (34) carried out on cell lysates under the same conditions of ionic strength (CaCl2, 10 mmol/L) showed that this enzyme gave only a minor, if any, contribution (maximum, 5–10%) to the rate of Cho production (Fig. 4A, black columns). Therefore, under these conditions, the pld* and pld activity rates were practically identical. We could then conclude that pld was significantly activated (∼2-fold to 4-fold) only in some (IGROV1 and OVCAR3) but not in all EOC cells. On the other hand, gene expression analyses (data not shown) and RT-qPCR experiments (Fig. 4B) performed on pooled EOC compared with EONT cells, did not show differential expression for PLD1 and showed only a moderate, if any, overexpression for the PLD2 gene.

When the GPC-pd assay was performed under optimal conditions of ionic strength (MgCl2, 10 mmol/L; pH 7.2; ref. 34) the rate of Cho production was ∼3.8 nmol/10^6 cells/h in EONT cells, and increased 2-fold to 4-fold in some, but not in all EOC cells (Fig. 4A, gray columns). Moreover, because no PCho was formed in the reaction mixture, we could exclude any additional contribution to GPC degradation from the alternative GPC phosphodiesterase.

Regarding the deacylation pathway, 31P NMR analysis of cell lysates in the presence of C6PC (in CaCl2, 10 mmol/L), showed that the 1-lyso-C6PC level was <5% that of 2-lyso-C6PC in both EOC and EONT cells (data not shown) and the content of 2-lyso-C6PC was, at 1 hour, significantly lower in cancer cells (Fig. 4C). The concentration of this derivative results from the balance between upstream plA2-mediated C6PC deacylation and downstream lpl-mediated 2-lyso-C6PC degradation into GPC. Because the latter remained below detection at any time point of incubating the cell lysates with C6PC (data not shown), and the GPC-pd activity was very low in 10 mmol/L of CaCl2 (see above), we concluded that the deacylation pathway was dominated by plA2 and the activity of this enzyme was lower in EOC than in EONT cells. Indeed, mRNA expression analyses showed that PLA1A was practically below the expression threshold in both EONT

Figure 4. Enzymes of pld-mediated and deacylation pathways in EOC and EONT cells. A, pld* and GPC-pd activity (mean value ± SD, n ≥ 6). B, RT-qPCR analysis of PLD1 and PLD2 in EOC cells (OSE used as an internal calibrator; horizontal line at quantification level = 1). A representative experiment of three performed is shown. For each gene, mean value ± SD is reported. C, relative 2-Lyso-C6PC formation (±SD, n ≥ 3) in EOC and hTERT cell lysates at 1 h of exposure to C6PC. D, gene expression of plA1 and of plA2 isoforms. The dotted line represents sensitivity threshold.
and EOC cells. Only 4 out of 19 PLA2 isoforms seemed to be differentially expressed in EOC compared with EONT cells, but the global difference in overall PLA2 expression was not significant (Fig. 4D).

**Activation of PC-Specific Phospholipase C in EOC Cells**

The activity of PC-plc was ∼0.45 ± 0.30 nmol/10⁶ cells/h in EONT cells and increased 5-fold to 17-fold (one-way ANOVA, P < 0.03) in the investigated EOC cells (Fig. 5A).

Following exposure of OVCAR3 cells to the PC-plc inhibitor D609, PC-plc activity decreased from 4.3 ± 1.2 nmol/10⁶ cells/h to 0.3 ± 0.3 nmol/10⁶ cells/h (n = 5, P < 0.001) and the average PCho level decreased by 37 ± 8% (P < 0.04; Fig. 5B and C), whereas GPC and Cho were not significantly altered. Similar effects were found in SKOV3 cells, in which exposure to D609 induced a PCho decrease of 44% (data not shown). These data provided the first direct demonstration that PC-plc could substantially contribute to PCho accumulation in EOC cells.

Our previous studies showed that cell exposure to D609 induced long-lasting G0/G1 cell cycle arrest in platelet-derived growth factor–stimulated fibroblasts (38) and blocked S phase fraction recovery in OVCAR3 cells re-exposed to complete medium after serum depletion (28). Experiments in the present study showed that incubation with D609 induced a long-lasting (at least up to 72 hours) cell proliferation arrest in OVCAR3 cells, an effect comparable with that of FCS deprivation (Fig. 5D).

**ChoK and PC-plc Detection in Clinical Specimens**

Because upregulation of both ChoK and PC-plc was found in all investigated EOC cell lines, preliminary experiments were conducted to evaluate the expression of these enzymes in a set of EOC surgical specimens. The mRNA relative expression levels of ChoKα measured in clinical tissue samples were significantly higher than those in OSE cells. Overall, these levels were, however, lower than those detected in EOC cell lines (ANOVA analysis for all the three groups, P < 0.001; Fig. 6A). The increased, albeit heterogeneous, expression of ChoKα was also confirmed at the protein level by Western blot analysis of lysates of surgical specimens randomly selected from those analyzed for gene expression (Fig. 6B).

At present, only protein expression data could be obtained for PC-plc, whose gene has not yet been identified.
in mammalian cells. Also, the expression of this enzyme was higher (although variable) in lysates of surgical specimens compared with those of EONT cells (see Fig. 6C; ref. 28).

**Discussion**

By combining $^1$H MRS studies with biochemical assays and mRNA and protein expression analyses, this study showed that the elevated PCho pool in EOC cells primarily resulted from the upregulation/activation of two enzymes, ChoK and PC-plc, which were involved in de novo biosynthesis and PC degradation, respectively.

Changes in choline transport and ChoK activity may both be responsible for enhanced radioactive choline uptake and PCho accumulation in cancer cells (15, 17, 39, 40). These mechanisms have a direct implication on choline-based positron emission tomography examinations (7, 8). Due to reported effects on cell proliferation, choline transport may represent a potential target for therapy (8, 17, 37). No differential changes were, however, observed in mRNA expression of CHT1, OCT, or CTL proteins, except for downregulation of OCT2 and moderate upregulation of CTL3 in EOC compared with EONT cells. Further investigations are needed to clarify whether posttranscriptional modifications of choline transporters may contribute to enhance choline uptake by EOC cells (15).

In the Kennedy pathway, ChoK$\alpha$ was overexpressed at the mRNA level in EOC cells. Similar or even higher ChoK$\alpha$ upregulation was reported in breast and bladder cancer cell lines (17, 26). The elevated ChoK$\alpha$ expression in these cancer cells suggests an increase in ChoK$\alpha$/ChoK$\beta$ at the expense of ChoK$\alpha$/ChoK$\beta$ and ChoK$\beta$/ChoK$\beta$ aggregates, a condition reported in liver carcinogenesis (24). It is worth noting that rather uniform increases in ChoK$\alpha$ mRNA (3.8 ± 0.2-fold) and ChoK protein expression (2.7 ± 0.3-fold) were associated with EOC with a strong and variable amplification (12-fold to 25-fold) of ChoK activity, which reached the highest values thus far reported for epithelial cancer cell lines (17, 26). These results indicate that other factors, likely depending on oncogene-driven signaling alterations, might influence ChoK activity, in agreement with evidence obtained from other tumor systems (23, 41).

Accruing evidence points to the role of ChoK in cell proliferation, transformation, and carcinogenesis and supports the use of this enzyme as a novel target for the treatment of different types of tumors (22, 23, 27, 41–43). In fact, a ChoK inhibitor, Mn58b, was found to reduce tumor growth and tCho in human carcinoma models (18, 41). Transient ChoK$\alpha$ downregulation by small interfering RNA-ChoK$\alpha$ induced cell differentiation, decreased cell proliferation, and reduced PCho and tCho levels in breast cancer cells (27). The combination of small interfering RNA-ChoK$\alpha$ with 5-fluorouracil resulted in a larger reduction of cell viability/proliferation in breast cancer than in nontumoral breast epithelial cells (42). Finally, lentivirus-mediated systemic delivery of the analogous short hairpin RNA resulted in a significant reduction in tumor growth along with reduced PCho and tCho levels in breast tumor xenografts in vivo (43). These findings suggest that EOC may also be a candidate for similar treatment strategies.

Among other PC cycle pathways possibly contributing to PCho accrual, only PC-plc-mediated PC degradation was substantially and consistently activated in all EOC cell lines investigated. In fact, in the deacylation pathway, the mRNA expression of 19 plA2 isoforms was either unaltered or reduced and the overall PlA2 activity decreased in EOC compared with EONT cells, in agreement with the plA2 group IVA underexpression reported for a breast cancer cell line.
(13). At the end of the deacylation pathway, a 2-fold to 4-fold activation of GPC-pd was limited to only some EOC cell lines. Moreover, preliminary results in our laboratory showed unaltered mRNA expression in EOC versus EONT for GDPD5, a glycerol-phosphodiesterase recently indicated as a plausible candidate for mediating GPC-pd activity (44). Even the activity of pld, an enzyme playing a critical role in cell proliferation and neoplastic processes (45, 46), was enhanced in only some of the investigated EOC cells and the average mRNA expression levels of its major isoforms, pld1 and pld2, were substantially unaltered in EOC compared with EONT cells. Similar variable patterns of pld activity and isoform expression were reported in breast cancer cells (17).

Growing evidence indicates the relevant role of PC-plc in mitogenesis, differentiation, and apoptosis (38, 47–49). Although isolated from some mammalian cells, this enzyme has not yet been cloned. Evidence of possible PC-plc activation was initially reported by Glunde and colleagues in breast cancer cells (13). Following detection and characterization of differential subcellular localization of a 66 kDa PC-plc (28), the present study shows 5-fold to 17-fold increases in PC-plc activity in EOC compared with EONT cells. Furthermore, our preliminary experiments showed that ChoK silencing in breast cancer cells resulted in compensatory PC-plc upregulation, suggesting links between pathways responsible for the activation of these two enzymes (50).

Inhibition of PC-plc by D609 induced a 30% to 40% decrease in the PCho content of OVCAR3 and SKOV3 cells, providing direct evidence of the contribution of PC-plc activity to the intracellular PCho pool in these cells. This result, together with the antiproliferative effects induced on these cells by D609, point to the possible use of the MRS PCho resonance to monitor the effects of treatments directed against these enzymes in ovarian and other cancers. Although a recent study suggests that PC-plc activity may also be conferred by sphingomyelinases (51), this mechanism unlikely contributes to PC-plc activation in EOC cells because PC-plc activity was abolished in OVCAR3 cells by D609, a very poor sphingomyelinase inhibitor.

The data reported here on upregulation of ChoK and PC-plc in EOC cells, led us to investigate the expression levels of these enzymes in EOC surgical specimens. ChoK overexpression has been reported and proposed as an indicator of reduced patient survival in human lung and bladder carcinomas (25, 26). Our study shows for the first time that ChoK mRNA expression, as well as ChoK and PC-plc protein expression, are elevated in EOC surgical specimens. The difference between in vitro and in vivo gene expression levels of ChoK might be derived from stromal contamination and/or from in vitro culture conditions. Independently of absolute levels of gene or protein expression, we can envisage that upon validation, the parallel detection of ChoK and PC-plc in the same specimens may facilitate future investigations on their role as predictive or prognostic factors.

In conclusion, the ChoK spectral metabolite provides an endogenous reporter on the activity of these enzymes, and can be used to detect and monitor the effects of downregulation or inhibition of these enzymes as novel therapeutic strategies in ovarian cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Phosphatidylcholine Metabolism in Ovarian Cancer


Activation of Phosphatidylcholine Cycle Enzymes in Human Epithelial Ovarian Cancer Cells

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