Phospholipid Metabolism in Cancer Cells Monitored by $^{31}$P NMR Spectroscopy*

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Peter F. Daly, Robb C. Lyon, Patrick J. Faustino, and Jack S. Cohen

From the Clinical Pharmacology Branch and Pediatric Oncology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Addition of choline, ethanolamine, or hemicholinium-3 (a choline kinase inhibitor) to the perfusate of human breast cancer cells monitored by $^{31}$P NMR spectroscopy resulted in significant changes to phosphomonoester (PME) and phosphodiester (PDE) signals. These results enable us to assign the PMEs to phosphocholine (PC) and phosphoethanolamine (PE), the PDEs to glycerophosphorylcholine and glycerophosphoryl-ethanolamine, and to define the pathways producing them. The PMEs are products of choline and ethanolamine kinases, the first steps in phospholipid synthesis; and the PDEs are substrates of glycerophosphorylcholine phosphodiesterase, the last step in phospholipid catabolism. Furthermore, PC and PE peaks are twice as intense in cells at log phase versus confluence. We also observed these signals in vivo in human colon and breast tumors grown in mice. Since PMEs are low in most nonproliferating tissues, they could form a basis for noninvasive diagnosis. Also, PE and PC are situated between the control enzymes of two major synthetic pathways and will allow noninvasive $^{31}$P NMR studies of these pathways in intact cells and in vivo.

The phosphomonoester (PMEs) observed in $^{31}$P NMR spectra of malignant cells were originally assigned to phosphocholine (PC) and phosphoethanolamine (PE) (1), but subsequently were referred to as sugar phosphates both in normal (2) and malignant tissues (3–8). Some identified AMP as one of the PME peaks (9, 10). Because of the proximity of most PME peaks, it is difficult to distinguish among them. A few recent publications (11, 12) have again assigned the PMEs to PC and PE in acid extracts of cells by NMR chemical shift behavior with changing pH, two-dimensional NMR, and chromatography. However, the effect of extraction on levels of metabolites is not known. The PME peak became potentially important when it was described as a possible diagnostic marker for tumors (13–15). Prominent PMEs have been observed in human in vivo studies of thyroidomyosarcoma (6), neuroblastoma (13), bone (16), and brain tumors (17). In addition, peaks due to phosphodiesters (PDEs) in tumors (14, 15) have been assigned to glycerophosphorylcholine (GPC) and glycerophosphorylethanolamine (GPE). Applications of localized $^{31}$P NMR are being actively investigated including diagnosis (16), correlation of PDE pool sizes with estrogen sensitivity in breast cancer (18), and monitoring of therapy (19). A previous publication from this laboratory (19) demonstrated significant differences in the PME and PDE resonances between the drug-sensitive MCF-7 breast tumor cell line and the drug-resistant AdrR line derived from MCF-7.

We have studied the energy metabolites (ATP, P, and phosphocreatine) observed in NMR spectra of metabolizing cancer cells in vitro (20, 21) using a perfusion system. In cells (19) and human tumors grown in mice (22) we have also seen prominent PME and PDE peaks. Apart from the uncertain designation of the PME peak(s) as sugar phosphates, AMP, or PE plus PC, there is disagreement over which pathways lead to PE and PC in malignant cells. Some speculate they arise from choline and ethanolamine kinases (9, 11) but others propose phospholipid breakdown by a phospholipase C (12, 23). Because the pathways to the PMEs have not been experimentally identified, their significance in tumors remains unknown (12, 13). To distinguish the origins of these peaks, avoid extraction artifacts, and elucidate the pathways leading to PMEs we activated or inhibited the enzymes which metabolize PMEs in functioning cells. We have done this using our system, where the perfusate is controlled and the cell population is homogenous. This avoids the ambiguity when observing tumors in vivo which are composed of heterogeneous populations of normal and malignant cells. We compared our spectra from cells with in vivo spectra from human breast and colon tumors in mice and find them to be highly similar. In our laboratory high ATP and low P, peaks correlate with cell survival proven by clonogenic assay (24). Using those criteria we maintained cells in the spectrometer for up to 24 h without signs of cell death. This allowed prolonged observation of the effects of substrates and inhibitors on peak intensities enabling clear identification of the pathways producing them.

**Materials and Methods**

**Tumor Cells**—MDA-MB-231 human breast cancer cells were obtained from Dr. Marc Lipman (Medicine Branch, National Cancer Institute) and grown in monolayers in National Institutes of Health improved minimal essential media (IMEM) supplemented with penicillin/streptomycin (100 units/ml, 10 mg/liter) and 10% fetal calf serum under a 5% CO₂ environment. IMEM contains 40 μM choline and no ethanolamine. For most experiments IMEM was modified to contain 15 μM choline and is designated as “IMEM (low choline).” IMEM (low choline) plus 10 μM ethanolamine simulated physiologic levels for human females of these two amines (25, 26). Cells were grown to log phase (30 to 50% confluency) or to 95% confluency, harvested with 0.5% trypsin, 0.2% EDTA (GIBCO), washed twice in HEPES-buffered IMEM with 10% fetal calf serum, and then suspended in agarose.

**Cell Perfusion**—Cells were suspended in agarose gel threads by mixing 1.3 ml of cells (3–5 × 10⁷) with 1.3 ml of 1.8% agarose at 37°C and extruding the mixture through a 0.5-mm internal diameter tubing into a screw-cap Wilmad NMR tube (10 mm). The threads were then concentrated but not compressed by placing into the NMR tube an insert with inlet and outlet tubing for perfusate. The perfusion system was an improved version of that described previously (19). Perfusion from a 500-ml reservoir entered through 0.5-mm tubing which opened at the bottom of the NMR tube, and after flowing upwards through the threads exited via openings in the insert to a waste bottle. The rate of perfusion was 0.5 ml/min. The perfusate consisted of Buffer
A (50 mM HEPES-Na salt at pH 7.5, 105 mM NaCl, 5 mM KCl, 2 mM MgCl2) and 11 mM glucose. Ethanolamine HCl, chloride, and HC-3 were obtained from Sigma.

\[ ^{31}P \text{ NMR Spectra of Perfused Control Cells} \]

Spectra of MDA-MB-231 human breast cancer cells at pH 7.5 and 37 °C were recorded over a period of 5 months and gave reproducible spectra when grown to the same extent of confluency. As controls, cells were perfused for 18 to 24 h with Buffer A with glucose and the peaks remained at a constant steady state level. The assignments for the PME resonances to PE and PC are based on the results of the present work. The other resonances are assigned by reference to the literature where there has been general agreement. The 32.5-s repetition time ensured complete T1 spin relaxation, and therefore all spectra were recorded over a period of several months and gave reproducible results.

**RESULTS**

**Effect of Choline**—Cells were grown in IMEM (low choline) plus 10 μM ethanolamine to confluency (Fig. 1A) and to log phase (Fig. 1B). At confluency the 3.0 (PE) and 2.5 ppm (PC) resonances are only 50 and 40% their intensities at log phase, respectively, whereas the other peaks remain unchanged. Reproducible spectra have been obtained under identical conditions three times at both confluency and log phase. The media was changed frequently so that the amount of choline and ethanolamine supplied per confluent cell was as much or more than cells harvested at log phase to avoid effects produced by depletion of these nutrients.

**Effect of Ethanolamine**—Fig. 1, B and C, shows the results of cells grown to log phase with or without ethanolamine in the culture media. On four separate occasions when grown with ethanolamine there is a prominent PE peak, whereas none was present when grown without ethanolamine. When cells grown without ethanolamine are perfused with 2 mM ethanolamine for 16 h, there is the appearance and rapid growth of a PE peak (Fig. 2). Simultaneously there is a marked reduction of the PC resonance until at hour 16 it was only a shoulder with its intensity artificially elevated above the base line by the large PE peak. The integrated area of this shoulder at hour 16 represents less than 20% of the original peak at hour 1 and is less than 10% of the original area at hour 19 (not shown). The peak intensities versus time fit an exponential decay (e^−t); PC gave a half-life of 7.1 h. A saturation curve (1 − e^−t) for the PE increase gave a time constant of 3.0 h. In addition to the changes in the PME resonances there was a marked linear increase in the GPE (−0.3 ppm) and GPC (−0.8 ppm) peaks. The reduction of the PC peak intensity by 2 mM ethanolamine in the perfusate is slower when cells are grown to log phase in IMEM (which has 400 μM choline) and reduces by only 30% after 16 h.

**Effects of Choline and Hemicholinium 3**—As shown in Fig. 3, when cells grown to confluency were perfused with 2 mM choline there is a 60% increase of the PC peak, a linear increase of the GPE and GPC resonances, while the ATP peaks remained unchanged. Cells grown to confluency in IMEM had a prominent PC peak and only a shoulder at 3.0 ppm as compared to cells grown to confluency in IMEM (low choline) plus 10 μM ethanolamine where there was over a 70% smaller PC resonance plus the appearance of a prominent PE peak (not shown). Fig. 4 shows that when cells grown to log phase in IMEM (low choline) plus 10 μM ethanolamine are perfused with 100 μM HC-3 there is no significant change in the PE resonance whereas the PC peak is reduced exponen-

![Fig. 1. Effects of growth conditions on spectra. Shown are quantitative \(^{31}P \) NMR spectra of cells obtained immediately after being harvested and while being perfused with Buffer A plus 11 mM glucose at 37 °C. Each spectrum represents a 1-h accumulation. A, cells grown in IMEM modified to 15 μM choline and 10 μM ethanolamine, harvested at 35% confluency. B, cells grown in IMEM modified to 15 μM choline and 10 μM ethanolamine, harvested at log phase. C, cells grown in IMEM modified to 15 μM choline and no ethanolamine, harvested at log phase. UDPG, uridine diphosphoglucose.](image1)

![Fig. 2. Effect of ethanolamine in perfusate. Shown are quantitative \(^{31}P \) NMR spectra of cells grown in IMEM with 15 μM choline and no ethanolamine, harvested at log phase, and then perfused with Buffer A, 11 mM glucose, plus 2 mM ethanolamine at 37 °C. Each spectrum represents a 1-h accumulation. Hours 2 to 16 are shown. The initial spectrum (hour 1) obtained without ethanolamine in the perfusate is shown in Fig. 1C.](image2)
spectra of cells grown in IMEM with 15 μM choline and 10 μM ethanolamine, harvested at 95% confluency, and then perfused with Buffer A, 11 mM glucose, plus 100 μM HC-3. Each spectrum represents a 1-h accumulation.

Fig. 3. Effect of choline in perfusate. Quantitative 31P NMR spectra of cells grown in IMEM with 15 μM choline and 10 μM ethanolamine, harvested at 95% confluency, and then perfused with Buffer A, 11 mM glucose, plus 2 mM cholines are shown. Each spectrum represents a 1-h accumulation.

Fig. 4. Effect of HC-3 in perfusate. Quantitative 31P NMR spectra are depicted of cells grown in IMEM with 15 μM choline and 10 μM ethanolamine, harvested at log phase, and then perfused with Buffer A, 11 mM glucose, plus 100 μM HC-3. Each spectrum represents a 1-h accumulation.

**DISCUSSION**

Essentially 31P NMR creates a window where key intermediates of phospholipid turnover can be observed in metabolizing cells. We have identified the two prominent PMEs as PE and PC since their levels depend on regulation of choline and ethanolamine kinase. In phospholipid turnover (27) the membrane is maintained by a balance of anabolic and catabolic pathways or increased by a greater net flux through the synthetic pathways. In synthesis the sequential steps choline to phosphocholine to CDP-choline to phosphatidylcholine are catalyzed by choline kinase (EC 2.7.1.32), choline-phosphate cytidylyltransferase (EC 2.7.7.19), and cholinephosphotransferase (EC 2.7.8.2), respectively. Analogously, ethanolamine kinase (EC 2.7.1.82), ethanolamine-phosphate cytidylyltransferase (EC 2.7.7.14), and ethanolaminephosphotransferase (EC 2.7.8.1) produce phosphatidylethanolamine. These two pathways synthesize the majority of these phospholipids in breast (28) and most other mammalian tissues (29, 30). The visibility of PC and PE peaks, located between the kinases and cytidylyltransferases (the controlling enzymes of synthesis (29, 31)), and the visibility of ATP, the source of phosphorus for the kinases, are particularly fortunate. The degradative pathways are via phospholipases A1 (EC 3.1.1.32) and A2 (EC 3.1.1.4) to GPE and GPC and then to choline and ethanolamine by glycerophosphorylcholine phosphodiesterase (EC 3.1.4.2) (30, 32). Thus, choline and ethanolamine are the start of synthesis and the end of degradation for these two major membrane components. Due to structural similarity, choline (trimethyllethanolamine) is a competitive inhibitor of ethanolamine kinase and ethanolamine is a competitive inhibitor of choline kinase (28, 33, 34). Both choline and ethanolamine are inhibitors of GPC phosphodiesterase (32).

Addition of ethanolamine is then readily seen as causing an increase in PE by acting as a substrate for ethanolamine kinase, a decrease in PC by inhibition of choline kinase, and an increase in GPE and GPC by inhibition of GPC phosphodiesterase (Fig. 2). HC-3 blocks membrane transport of ethanolamine and choline and inhibits choline kinase (but not ethanolamine kinase) (35-37). HC-3 had the predicted result of decreasing PC while not changing PE (Fig. 4). The perfusate contained no choline or ethanolamine so the effect cannot be attributed to blocking membrane transport and indicates that HC-3 can cross the cell membrane. The increase in GPE and GPC is interpreted as inhibition of the diesterase by this choline analog or an indirect effect due to an increase in intracellular choline. It is unlikely that a significant proportion of the PC pools in these cells could be explained by a phospholipase C (EC 3.1.4.3) as has been suggested (12, 23). Ethanolamine is not a known inhibitor of phospholipase C and since the reduction of the PC peak by ethanolamine was over 90% after 19 h this precludes any major contribution to the concentration of PC to result from this enzyme. Similarly, the almost complete disappearance of this peak by inhibiting choline kinase indicates little if any contribution to this resonance from sugar phosphates.

It is difficult to raise the intracellular concentration of choline by passive diffusion across cell membranes due to its positive charge as compared to ethanolamine, which is neutral. The transport of choline across mammalian cell membranes plateaus after 0.2 mM (34, 37) and only doubles intracellular choline (34). The transport of ethanolamine however does not saturate (37). To have corresponding effects on the PC versus PE pools requires incubating liver cells with 20 times the amount of choline as compared to ethanolamine (38). Our results show that choline significantly changed pool sizes of PMEs (Fig. 3), but to a lesser extent than an equal amount of ethanolamine (Fig. 2). In our experiments PE continued to increase after 16 h, but the effect of choline on PE pool size leveled off after 6 h. Also choline did not reach sufficient intracellular concentration to competitively inhibit ethanolamine kinase, although it did inhibit GPC phosphodiesterase.

Cells grown in media with varying concentrations of ethanolamine and choline had consistent effects on the PME peaks. Media without ethanolamine produced cells with no visible PE resonance, whereas this is one of the largest peaks when 10 μM ethanolamine is in the media (Fig. 1). This indicates that the 3.0 ppm resonance does not derive from sugar phosphates. IMEM produced a large PC peak but when IMEM was readjusted to 15 μM choline this peak decreased by more than a factor of 3. Since cells grown without ethanolamine still produce phosphatidylethanolamine (39, 40) by decarboxylation of phosphatidylserine (39), the lack of a PE peak in cells grown without ethanolamine cannot be attributed to a decrease in amount of substrate for phospholipase C but indicates a lack of substrate for ethanolamine kinase.

Increased concentrations of PE and PC correlate with situations requiring increased synthesis of the membrane. The
MDA-MB-231 cell line is known to slow its growth as it nears confluency and to produce transforming growth factor-β (41) which is autoinhibitory for further growth (42). At log phase, when net phospholipid synthesis is necessary, there was a 2-fold increase in PE and PC compared to confluency. This increase with increased synthesis has been reported and estradiol, fetal calf serum, and growth factors cause an increase in the amount and activity of choline kinase with a corresponding doubling in both PC pool size and rate of synthesis of phosphatidylcholine (31, 43, 44). Published spectra of tumors in vivo in humans and animals consistently demonstrate higher PME resonances when compared to normal tissue or benign growths (6, 13, 16, 17, 45, 46). These observations lead us to hypothesize that the PMEs observed in tumors represent increased concentrations of PC and PE and reflects the abnormal overexpression of growth factors resulting in increased choline and ethanolamine kinase. Work is in progress to test this hypothesis.

Human colon and MDA-MB-231 breast tumors implanted in nude mice have been observed in vivo with the use of a probe designed in our laboratory (22) and show two large clearly resolved resonances at 3.0 and 2.5 ppm which we attribute to the rapid clearance of these compounds from circulation by the liver, kidney, and possibly other tissues. The liver is particularly efficient at converting choline to betaine (29). This demonstrates distinct advantages of the cell perfusion system where concentrations in the perfusate are well controlled and the cell population is homogenous. Future in vivo 31P NMR experiments with 13C-enriched substrates are being planned.

In summary we have shown that in intact functioning human breast cancer cells the 31P NMR PME resonances (which are absent or much less prominent in many nonmalignant tissues) can be assigned to known intermediates of major pathways in phospholipid metabolism, and these peaks alter with different states of growth. Consequently 31P NMR signals may be useful, interpretable markers for tumor growth and diagnosis in vivo. Furthermore, the ability to directly observe and manipulate the peaks in metabolizing cells in a controlled perfusion system should facilitate a basic understanding of the biochemical factors controlling the concentration of metabolites visible by NMR methods. This will allow a better understanding of spectra currently being published on tumors in vivo in humans and animals. The knowledge gained from malignant tumors and cells will also shed light on the regulation of these phospholipid pathways by hormones, growth factors, substrates, and cofactors in normal tissue as well.

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REFERENCES


