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Kinetics of Choline Transport and Phosphorylation in Human Breast Cancer Cells; NMR Application of the Zero Trans Method

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Abstract. *The mechanism and kinetics of choline transport and phosphorylation in MCF7 human breast cancer cells was studied by ^{31}P, ^{13}C and ^{2}H NMR, applying the zero trans method. Choline was transported by a Michaelis-Menten like mechanism with a maximum transport rate $T_{max}=13.5 \pm 2.6$ nmol/hour/mg protein (3.06 ± 0.6 fmol/cell/hour) and choline concentration at half maximal transport rate of $K_t=46.5 \pm 2.8$ μM. The rate of choline phosphorylation was more than two orders of magnitude faster than the rate of its transport (T_{max}) maintaining the ratio [phosphocholine]/[choline] higher than 100. The results demonstrated enhanced choline transport and choline kinase activity in breast cancer cells.*

Numerous *in vitro* and *in vivo* ^{31}P NMR studies have revealed the high content of phosphocholine (PC) or phosphoethanolamine (PE) or both in most cancers, while in the corresponding normal tissues these metabolites were present at low levels, occasionally below detection (1,2 and references cited therein). Specifically, ^{31}P NMR spectra of human breast cancer in patients and in animal models exhibited high levels of PC and PE (3-8). Furthermore, human breast cancer cells grown in culture (*e.g.* MCF7, T47D) maintained elevated levels of PC, while the content of this metabolite was more than ten times lower in normal human mammary epithelial cells cultivated under similar conditions (8 and Ting *et al* in this issue).

Phosphocholine is the first intermediate in the stepwise incorporation of choline into phospholipids by the Kennedy pathway, and serves as a precursor of several phospholipids, including PtdCho, choline plasmalogen and sphingomyelin (10 and references cited therein). From ^{13}C studies of human breast cancer spheroids, using ^{13}C enriched choline it was concluded that PC level in breast cancer cells is determined by the concentration and availability of choline in the medium (11,12). This implied that most of the PC is derived from external choline. Substantial transport of choline was also demonstrated in mammary epithelial cells of the lactating rat (13). However, in these cells, despite the concentrative uptake of choline to high intracellular levels, the concentration of PC was extremely low, 10-500 lower than choline (13). These results suggest that malignant transformation in mammary epithelial cells might be associated with the induction of choline kinase activity resulting in augmented levels of PC. To further test this hypothesis we have measured the rate and mechanism of choline transport and the rate of choline phosphorylation relative to choline transport in MCF7 human breast cancer cells. The measurements included monitoring of three nuclei: ^{13}C, ^{2}H and ^{31}P, and employment of ^{13}C and ^{2}H enriched choline. We also demonstrated NMR methodology to measure transport based on the zero trans method (14). The results indicated that the rate of phosphorylation of choline was more than two orders of magnitude faster than the rate of transport. Transport was shown to proceed by a Michaelis-Menten kinetics. Analysis of the transport data yielded the maximum rate of choline transport (T_{max}) and the concentration of choline required to reach half T_{max} (K_t). It was concluded that faster active transport and induction of choline kinase activity are responsible for the presence of high PC in breast cancer cells.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium, FCS, fetal calf serum, NMR, nuclear magnetic resonance, PC, phosphocholine, PE, phosphoethanolamine, PtdCho, phosphatidylcholine.

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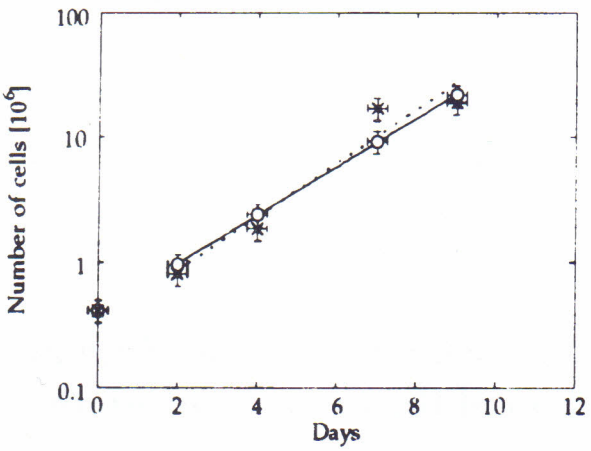

Figure 1. *Growth curves of MCF7 cells cultivated in standard medium (o) and in medium containing low (2 μM) choline (x).*

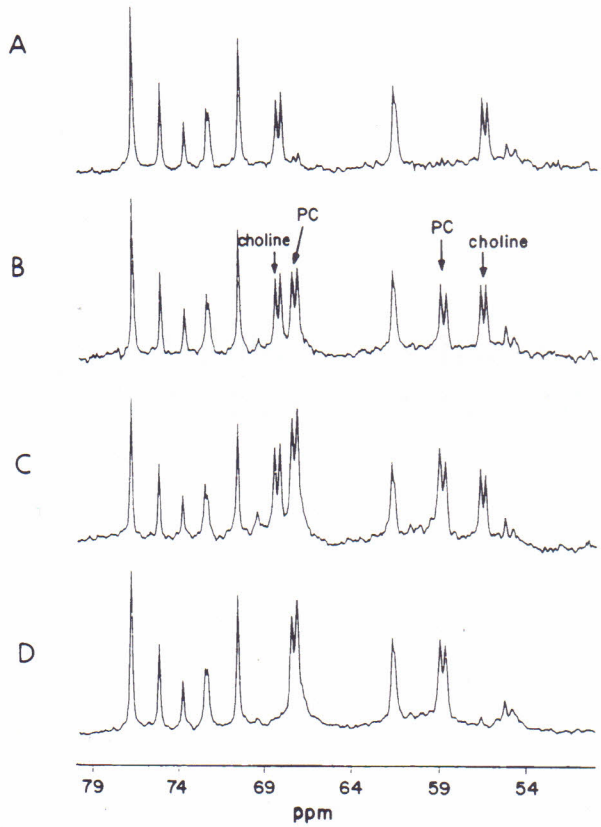

Figure 2. *^{13}C spectra of MCF7 cells perfused at 36°C with medium containing 200 μM [1,2-^{13}C]choline. A. Recorded within the first 90 min after the addition of ^{13}C labeled choline. B. Recorded 7.5 h later. C. Recorded 30.5 h later. D. Recorded immediately after replacing the medium with ^{13}C labeled choline with medium containing 200 μM of choline at natural abundance. Spectra were recorded as described in Materials and Methods and processed with a line broadening of 10 Hz.*

Materials and Methods

MCF7 cells were cultured routinely in Dulbecco's Minimal Eagle medium (DMEM) supplemented with 6% fetal calf serum (FCS) and antibiotics as previously described (15). The amount of protein *per* cell was determined by the Bradford method (16) using BSA as standard, and was found to be 221 ± 27 pg/cell.

For NMR measurements the cells were grown on agarose polyacrolein microspheres, 300-500 μm in diameter as previously described (9). Cell adhesion was improved by precoating the microspheres with collagen (from calf skin, type 1, Sigma). After ~4 days of culture on beads, before reaching a stationary phase, the microspheres (2-2.5 ml) with cells were placed in a 10 mm NMR tube and were perfused with oxygenated DMEM + 6% FCS at 36 ± 1°C or 32 ± 1°C as previously described (9). The cells used in kinetic studies were cultured with choline free medium which contained only ~2 μM choline from serum. We have found that this change in medium composition did not affect the rate of cell growth. Cells cultured for 10 days in medium containing low choline concentration showed the same growth rate as cells cultured in medium with standard choline concentration (28 μM in DMEM + ~2 μM choline from serum) (Figure 1).

The NMR recordings were performed with a Bruker AM-500 spectrometer. Alternating ^{31}P and ^{13}C or ^{31}P and ^{2}H spectra were recorded using a broadband probe. Proton decoupled ^{31}P NMR spectra were recorded at 202.5 MHz by applying 45° pulses, 2 second repetition time and continuous composite pulse proton decoupling of ~1 Watt. Each spectrum was acquired for 0.5 hour (900 transients). The chemical shifts of the ^{31}P signals were assigned in reference to α-NTP at -10.03 ppm. Concentrations were calculated using the integrated intensity of Pi in the medium (1.0 mM) as a reference, taking into account saturation effects due to the short repetition time relative to T_1. The Pi concentration of the medium was measured independently for each experiment by referencing it to a known concentration of added PCr.

^{13}C NMR spectra were recorded at 125.7 MHz by applying 45° pulses, 2 second repetition time and continuous composite pulse proton decoupling of ~1 Watt. Each spectrum was acquired for 1.5 hours (2700 transients). The ^{13}C natural abundance signal of C1-ß-glucose in the medium served as a reference for chemical shift determination (96.8 ppm). The integrated intensity of ^{13}C-choline signal in the medium (0.2 mM) was used as a concentration reference. In order to confirm that ^{13}C-choline concentration was not significantly altered during the experiment we have determined the total choline consumption and conversion to PC during the experiment. We found that after 32 hours the initial ^{13}C-choline concentration in the total volume of the perfusion medium (100 ml) was reduced by 7%. Since this reduction from initial concentration was smaller than the experimental error (including integration error), it was justified to neglect it in the data analysis.

^{2}H NMR spectra were recorded at 76.8 MHz by applying 90° pulses and 4 second repetition time and acquiring 300 transients for 20 min. Concentration was determined from the integrated intensities of choline-D_9 referenced to natural abundant deuterium in water (16.4 mM).

Data analysis. Spectra were analyzed using UXNMR (standard Bruker software package). PC concentration in the sample was computed from the integrated intensity of its signal (^{2}H or ^{31}P or ^{13}C) and concentration calibration, as described above. The initial rates were calculated by a fit using the linear range of the concentration dependent curves in the course of the experiment.

The determination of transport kinetics was based upon plots of initial rate (v), rate constant [K (= v/S)], and the inverse of the rate constant (1/K) *vs.* choline concentration (S). It was previously demonstrated that a distinct pattern in each of the three plots defines the transport mechanism (14). The selection of the mechanism of

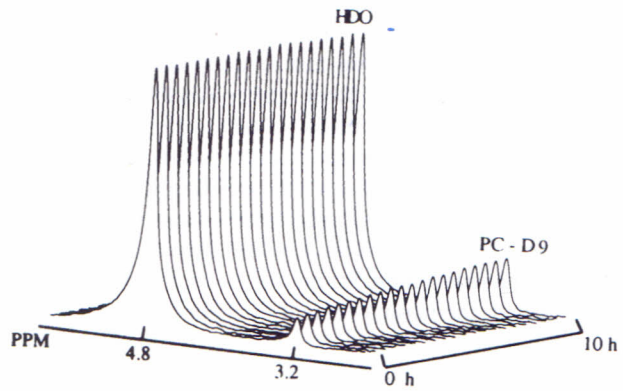

Figure 3. *Deuterium spectra of MCF7 cells perfused at 32°C with medium containing 30 μM choline-D9. Spectra were accumulated as described in Materials and Methods every 30 min, 20 min acquisition and additional 10 min, time interval. Spectra were processed with 3 Hz line broadening.*

choline transport was based on the best fit of the experimental data, to all three plots. The rate constants are presented with the standard error of estimation from the three plots.

Results

Estimation of the rate of choline phosphorylation. In order to estimate the rate of choline phosphorylation relative to the transport rate we initially perfused the cells with a high level of [1,2^{13}C]-choline (200 μM). The choline in the medium could be easily detected and a gradual increase in the intensity of [1,2^{13}C]-PC signals, due to phosphorylation of [1,2^{13}C]-choline, was observed (Figure 2, A-C). Then the perfusion medium was replaced with medium containing 200 μM choline at the natural abundance of ^{13}C. The signals due to the intracellular ^{13}C-PC remained high and the signals due to ^{13}C labeled choline in the medium disappeared completely as expected, however, no traces of any internal ^{13}C choline could be observed (Figure 2D). Although intracellular choline was not detected, it was possible to estimate from the signal to noise ratio of the ^{13}C-PC signal an upper limit for the ratio of [PC]/[choline] of >100. We have thus concluded that the phosphorylation rate was by at least two orders of magnitude faster than the transport rate. Throughout this experiment, choline metabolism was also followed every ~6 hours by recording ^{31}P spectra. The values of PC content obtained from the ^{31}P spectra were equal to those obtained from the preceding ^{13}C spectra.

The fast phosphorylation of choline also enabled us to apply the zero trans method for measuring choline transport. In order to use this method it was necessary to prove that the measurement is carried out under conditions in which the trans face of the membrane (the intracellular compartment) contains very low concentrations of the substrate (close to zero) while in the external face (the medium) the concentration can be varied in a controlled manner. The analysis of the above ^{13}C data demonstrated clearly that choline was rapidly phosphorylated to PC and therefore its intracellular concentration remained always very low, despite its high external concentration. Thus the main condition for measuring choline transport by the zero trans approach was found to hold in MCF7 cells, namely, in the trans face (inside the cells) the choline concentration was invariably much lower than in the cis face (the medium outside the cells) where it could be varied during the experiment.

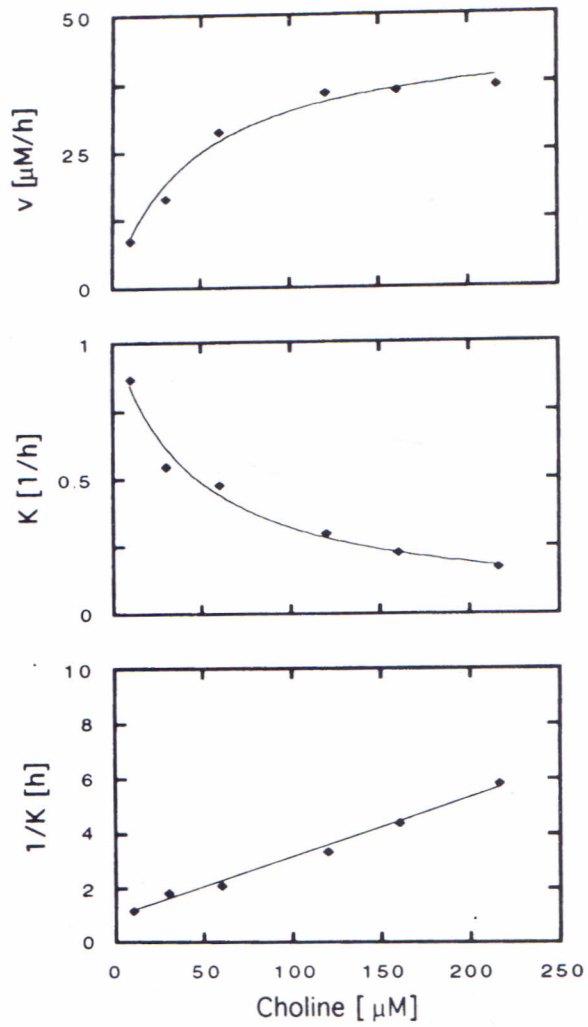

Figure 4. *Variations in initial rate of choline transport, K (= v/S) and 1/K with choline concentration (S). Results obtained from deuterium spectra which monitored the changes in time in the signal intensity of deuterated PC after the addition of increasing amounts of choline-D9 (10 to 210 μM). These three plots show distinct patterns of a Michaelis-Menten transport mechanism.*

Choline transport mechanism and rate constants. Transport rates were measured by deuterium NMR using deuterated choline (choline-D9) and monitoring the synthesis of

deuterated PC. The cells were initially cultivated on beads with medium containing low choline (~2 μM). Then, while perfused in the spectrometer, increasing amounts of deuterated choline (choline-D_9) were added to the medium. Two distinct signals were observed in the ^2H spectra of the cells perfused with choline-D_9: a constant deuterium signal of water at natural abundance (16.4 mM) and an increasing signal of intracellular PC-D_9 (3.2 ppm) superimposed on the medium choline-D_9 which remained constant (see Materials and Methods for explanation of this statement) for each addition of choline (Figure 3). The contribution from intracellular choline was too low to be detected as was shown above in the ^{13}C studies. The initial change with time in the PC signal was linear and yielded directly the initial rate of choline transport and phosphorylation with the former being the rate limiting step. PC levels determined from ^{31}P spectra, recorded at various time points throughout this experiment, were similar to those obtained from subsequent deuterium spectra.

Plots of v, of K = v/S and of 1/K *vs.* choline concentration, S (Figure 4) indicated that the transport of choline proceeded *via* Michaelis-Menten mechanism. The fitting of the experimental data to the Michaelis-Menten equation yielded the values of the rate constants: a maximum transport rate T_{max}=13.5 ± 2.6 nmol/h/mg protein (3.06 ± 0.6 fmol/cell/h) and choline concentration at half maximal transport rate K_t=46.5 ± 2.8 μM.

Discussion

A zero trans method to measure transport kinetics, based on NMR spectroscopy ,was demonstrated by measuring choline transport into living cells. The method was verified by using three different nuclei of choline and phosphocholine: ^{31}P, ^{13}C and ^2H. Each nucleus also provided unique advantages: a) The use of ^{13}C labeled choline made it possible to monitor simultaneously choline and phosphocholine signals; b) A substantial improvement in the signal to noise ratio and temporal resolution was provided by ^2H NMR of choline labeled with a large number of deuterons (replacing the nine protons of the trimethylamine group and permitting relatively fast acquisition since T1 of the deuterons was about one order of magnitude shorter than that of ^{31}P or ^{13}C); c) The natural abundance of deuterium in water served as an internal constant reference of concentration, thus, providing accurate determination of deuterons concentration; d) ^{31}P spectra served to measure the total PC pools and to monitor continuously the viability of the cells.

Generally it is essential to demonstrate for each transporter and cellular system that the zero trans method is suitable for measuring transport. This was achieved for choline by using both ^{13}C enriched choline and choline at ^{13}C natural abundance and showing that the intracellular choline level is kept low due to the rapid phosphorylation of choline. The following concentration dependent measurements using deuterated choline as a substrate and monitoring both the deuterons and ^{31}P of PC yielded the kinetic constants of choline transport. We have also employed the useful data analysis developed by Stein (14) which demonstrated transport mechanism (Figure 4).

The K_t of choline in human breast cancer cells was found to be of a median value with respect to the physiological concentration of human blood choline, ranging between 10 to 100 μM (17-19). Thus, if the K_t of MCF7 is typical of breast cancer in patients, then PC in such lesions may fluctuate markedly with the plasma content of choline. Factors such as diet (20) as well as age and hormonal status can also influence the choline blood levels. These fluctuations can further cause variations in PC of the tumor.

It was previously demonstrated that choline transport into normal mammary-gland epithelial cells, isolated from lactating female rats, follows saturable Michaelis-Menten kinetics coupled to non-saturable linear behaviour. The saturable component of the transport mechanism was found to be predominant at the physiological range of choline concentration with a Kt of 35 μM (13). This value is close to the value we found for breast cancer cells. However, in the rat mammary cells most of the accumulated choline remained in the form of free choline keeping the ratio [PC]/[choline] very low (0.1-0.002). Low levels of PC were also demonstrated in primary cultures of human mammary epithelial cells (8), while the results here have shown that in human mammary cancer cells most of the choline is immediately phosphorylated to form PC and therefore [PC]/[choline]>100. Furthermore, the maximum rate of choline transport into MCF7 cells (T_{max}), which was found to be 13.6 nmol/h/mg protein, is about one order of magnitude faster than that into the normal rat mammary cells (T_{max}=1.24 nmol/h/mg protein) (13). Thus both the capacity to transport choline as well as the activity of choline kinase appear to be augmented as a result of malignant transformation of breast epithelial cells.

It is interesting to note that C6 and 9L glioma cells showed about the same K_t as MCF7 cells (47.6 and 42.3 μM respectively) (21). Although the value of T_{max} for these cells was not reported, their high PC level suggests a fast transport rate as well as high activity of choline kinase. Regulation of choline kinase with increased levels of PC were also observed in 3T3 fibroblasts treated with mitogenic growth factors (22,23), implying that choline kinase is a crossover point and serves to regulate choline phospholipids. These phospholipids, in addition to their essential function as structural components of membranes and lipoproteins, also serve as sources of mediators and modulators of transmembrane signal trunsductory pathways, thereby influencing cell proliferation and differentiation (24). Although studies of human breast cancer *in vivo* (5) have indicated that the source of phosphocholine is unlikely to be directly related to cell signaling, the activation of choline kinase together with activation of a specific PtdCho

phospholipase C (25) might be related to enhanced transmembrane signaling.

In summary, we have shown that it is possible to apply a zero trans NMR method to determine the mechanism and kinetic parameters of choline transport in perfused cells. The method can be extended to study transport of a broad range of metabolites, provided zero trans conditions are shown to hold. The results demonstrated that breast cancer cells exhibit fast choline transport and phosphorylation which results in the accumulation of high levels of phosphocholine.

Acknowledgements

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