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Choline metabolism in breast cancer; ^{2}H-, ^{13}C- and ^{31}P-NMR studies of cells and tumors

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Abstract

Choline metabolism in breast cancer cells and tumors has been investigated by multinuclear NMR in order to provide the biochemical basis for the presence of high phosphocholine in breast carcinoma relative to benign breast tumors and normal breast tissue. Choline was found to be transported into MCF7 human breast cancer cells and rapidly phosphorylated to phosphocholine which was then accumulated in the cells to high concentrations. The increased level of phosphocholine did not affect the rate of synthesis of phosphatidylcholine, indicating tight regulation of this pathway. The incorporation of [1,2-^{13}C]choline (100 μM) into phosphocholine and phosphatidylcholine after 24 h was 69.5 and 36% of the total respective pools. Incorporation of $^{2}H_9$-choline to tumors implanted in nude mice was achieved by infusing the deuterated choline to the blood circulation. The metabolism of deuterated choline was then monitored by ^{2}H localized MRS. The blood level of choline before the infusion was 58.6 ± 10.3 μM (measured by ^{1}H-NMR of plasma samples) and increased $\sim$ 5-fold during the infusion (measured by ^{2}H-NMR). This increase in the blood level resulted in a gradual increase of a signal at 3.2 ppm due to deuterated choline metabolites. It appears that the increased availability of choline in the blood circulation leads to accumulation of phosphocholine in the tumors by the same mechanism as in the cells.

Keywords: Choline; Phosphocholine; MCF7 human breast cancer cells; Deuterium-NMR; Transport; Metabolism

1. Introduction

Since the early days of in vivo ^{31}P spectroscopy it was shown that malignant tumors contain high levels of phosphomonoesters (PME) as opposed to normal counterpart tissues [1]. The predominant metabolites contributing to the PME signal were found to be phosphocholine (PCho) and phosphoethanolamine (PEtn) [1–4]. Specifically in breast carcinoma, high PME content was detected in vivo [5–7] and in perfused biopsies of breast cancer [8], while in normal tissue and perfused biopsies of benign breast tissue this level was low or undetected.

Recently, increased levels of PCho were found in perchloric acid extracts of human breast tumors by ^{1}H spectroscopy [9,10], indicating that the predominant contribution to the PME signal in breast carcinoma was from PCho. Moreover, in vivo ^{1}H spectroscopy of the breast demonstrated clearly in tumors the presence of a signal at 3.2 ppm corresponding to the chemical shift of choline metabolites, while only water and lipid signals where detected in control subjects [11]. Localized in vivo proton spectroscopy of human breast masses revealed significant amounts of choline metabolites in carcinomas as opposed to benign processes with the exception of one tubular adenoma (a benign neoplasm arising from epithelial cells) [12]. Infiltrating ductal carcinomas were shown to have high PCho content, whereas in normal breast tissue of the same patients PCho was not detectable [13]. ^{1}H spectroscopy of fine-needle biopsy specimens also indicated the presence of

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high levels of choline metabolites in invasive cancer relative to that found in benign breast lesions [14].

NMR studies of human breast cancer cell lines and of primary cell cultures of mammary epithelial cells confirmed the above findings, showing that breast cancer cells contain at least ten times more PCho than normal human mammary epithelial cells [15]. This marked difference in metabolism appeared to be predominantly due to high choline transport rates and high choline kinase activity in the cancer cells [16]. In normal mammary epithelial cells of pregnant rats, most of the choline that was taken up by the cells was found to remain in the form of free choline [17].

Choline is an essential nutrient [18], abundant in most common foods [19]. It is metabolized in vivo through three distinct pathways [19], which are illustrated in Fig. 1. The phosphorylation of choline to yield PCho, is catalyzed by choline kinase using Mg^{2+} and ATP. This enzyme is widely distributed in mammalian tissues. Phosphorylation of choline is the first step in the Kennedy pathway which is the major pathway for the biosynthesis of phosphatidylcholine (PtdCho). CTP: phosphocholine cytidylyltransferase catalyses the synthesis of CDP-choline from CTP and PCho, and is the rate limiting step in the synthesis of PtdCho. CDP-choline is rapidly combined with diacylglycerol, to form PtdCho, in a reaction catalyzed by CDP-choline:1,2,diacylglycerol choline phosphotransferase (Fig. 1(box A)). Another major use of choline is via irreversible oxidation to form betaine, an important methyl donor. This reaction occurs mainly in the liver and kidney. Once betaine is formed it cannot be reduced to form choline. However, it can donate a methyl group to homocysteine, thereby producing dimethylglycine and methionine. The oxidative pathway (Fig. 1(box B)) acts to diminish the choline in tissues while, at the same time, scavenging some methyl groups. Only a small fraction of dietary choline is metabolized by a third pathway of acetylation, catalyzed by the activity of acetylcholine transferase. This enzyme is highly concentrated in the terminals of cholinergic neurons, but it is also found in the placenta. The acetylation product is acetylcholine (Fig. 1(box C)) which is a well known neurotransmitter.

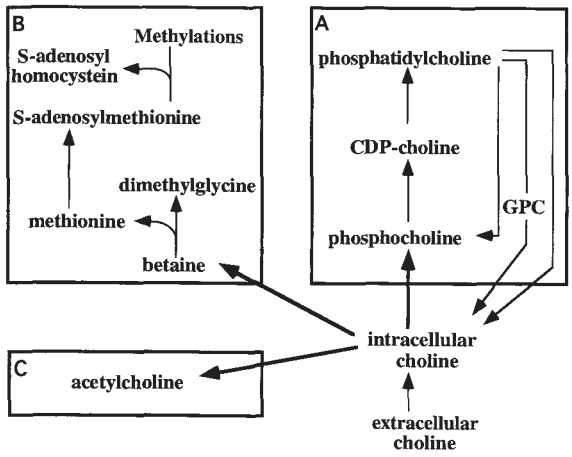

Fig. 1. Choline metabolism in mammalian cells through the Kennedy pathway (A), the oxidative pathway (B) and the acetylation pathway (C).

The uptake of choline as well as its metabolism are tissue dependent. All tissues accumulate choline, but uptake by liver, kidney, mammary gland, and brain are of special importance, and were extensively studied [20]. In a recent study, phosphonium choline (a choline analog) incorporation and metabolism to the corresponding phospholipid in mammary adenocarcinoma implanted in mice was followed by ^{31}P-NMR [21].

In an effort to understand the biochemical basis underlying the observation of high PCho in breast carcinoma, we have initiated a study of choline metabolism in human breast cancer cells and tumors implanted in nude mice [22]. We performed studies on perfused MCF7 cells and MCF7 cell extracts of the water-soluble and the lipid metabolites, utilizing deuterated and carbon-13 labeled choline. In addition, in vivo studies of deuterated choline metabolism were performed on MCF7 human breast tumors.

2. Materials and methods

2.1. Animals

CD-1 nude female and male and CD-1 female and male mice, 6–8 weeks old (Weizmann Institute Animal Services) were housed in a controlled environment (24°C and daily cycles of 12 h of light, and fed a semi-synthetic diet of 50% corn supplemented with minerals, vitamins, 0.2% choline chloride, and 0.2% methionine. Food and water were supplied ad libitum.

2.2. Cell culture

MCF7 human breast cancer cells were cultured routinely as monolayers in Dulbeco's modified Eagle's medium (DMEM) supplemented with 6% FCS and antibiotics as previously described [23]. The standard choline concentration of this medium is 28 μM, with additional 2 μM of choline from serum.

2.3. Studies of perfused cells

For the NMR measurements the cells were grown on agarose polyacrolein microspheres, 300–500 μm in diameter as previously described [16,24]. 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, as previously described [24]. Then the medium was replaced with a DMEM without choline chloride (containing 2 μM of choline from serum). After 6 h in the choline deficient medium, 50 μM of $^2H_9$-choline chloride (Cambridge Isotope Laboratories, CIL) were added. After 17 h, additional 50 μM of $^2H_9$-choline chloride were added, to a total of 100 μM of $^2H_9$-choline in the medium.

2.4. Cell extracts

MCF7 cells were cultivated before the extraction in two ways.

1. Control MCF7 cells were cultivated in the standard growth medium that contains 28 μM choline of DMEM with additional 2 μM of choline from serum.
2. MCF7 cells grown in the presence of 100 μM labeled choline were cultured for one day either with medium containing 100 μM $^2H_9$-choline chloride (CIL) or with medium containing 100 μM 1,2-^{13}C-choline chloride (CIL).

Extraction of the water-soluble metabolites and of the lipids from the same cell culture preparation was performed by the dual phase extraction (DPE) method as previously described [25]. The number of cells for each extract was 1.5×10^8. The dried residue of the water-soluble metabolites was dissolved in 0.5 ml of 25 mM TRIS (Sigma) buffer solution, containing 10 mM of the disodium salt of ethylenediaminetetraacetic acid (Na_2H_2 EDTA) (Fluka). The pH was adjusted to 8–8.3. The dried lipid samples were dissolved in a mixture of 0.4 ml chloroform and 0.2 ml of methanolic EDTA [25]. Extract solutions were transferred to 5 mm NMR tubes for the NMR measurements.

2.5. Tumors

MCF7 cells, cultured routinely as describe above, were inoculated s.c. ($\sim 10^7$ cells per mouse) in the right flank of CD-1 female athymic mice. Before the cell inoculation a pellet of 17β-estradiol (0.72 mg/pellet, 60 days release time; Innovative Research of America) was implanted underneath the skin [26]. The studies were performed when the volume of the tumors reached 1.5 cm^3.

2.6. Plasma samples

Blood was withdrawn by a retro orbital sinus puncture into an heparinized Eppendorf tube (1 ml). The samples were placed on ice and then centrifuged for 10 min. After centrifugation, the supernatant (plasma) was separated, its volume measured (50–440 μl) and then lyophilized at 10 μm Hg to dryness. Dried plasma samples were dissolved in 500 μl of 99.99% enriched D_2O containing 1 mM methanol for ^{1}H spectroscopy, and in 500 μl of H_2O for ^{2}H spectroscopy, and were transferred to a 5 mm NMR tube. The samples were kept at −20°C, until the NMR measurements.

2.7. Infusion

$^2H_9$-choline chloride (CIL) in saline was infused to mice at a dose of 16 μmol kg^{-1} min^{-1} [27] for 2 h, using a home build slow infusion pump. To determine the concentration of $^2H_9$-choline in the plasma, blood samples were taken from mice ($n = 5$) infused with $^2H_9$-choline as described above, starting at 11 min of infusion, during 1.5 h of infusion, and up to 12 min after cessation of the infusion.

2.8. MRS and MRI

High resolution NMR spectra of plasma samples and extracts were recorded on Bruker AMX-500 or DMX-400 or AMX-400 spectrometers. ^{1}H spectra of plasma were recorded at 500 MHz by applying 90° pulses and a repetition time of 10 s. ^{2}H-NMR spectra of plasma were recorded at 500 and 400 MHz using a broadband probe by applying 90° pulses and a repetition time of 4.3 s. ^{2}H-NMR spectra of extracts were recorded at 400 MHz by applying 90° pulses and a repetition time of 3 s. These acquisition parameters provided fully relaxed conditions. Proton decoupled ^{31}P spectra of cell extracts were recorded at 400 MHz by applying 45° pulses, and a repetition time of 2.5 s and continuous composite pulse proton decoupling. ^{13}C spectra of cell extracts were recorded at 400 MHz by applying 60° pulses, repetition time of 2.4 s, and continuous composite pulse proton decoupling.

Perfused MCF7 cells were monitored in the AMX-500 spectrometer (Bruker). Alternating ^{2}H and ^{31}P spectra were recorded using a broadband probe. ^{2}H spectra were recorded by accumulating 300 transients with 90° pulses and 4 s repetition time. Proton decoupled ^{31}P spectra were recorded by accumulating 900 transients with 45° pulses, 2 s repetition time and continuous composite pulse proton decoupling. The chemical shifts in the ^{31}P spectra were assigned in reference to α-NTP at −10.03 ppm, and in ^{2}H spectra to HDO at 4.8 ppm.

In vivo studies were performed on a 4.7 T Biospec spectrometer (Bruker). ^{1}H-MR images for localization were recorded utilizing a ^{1}H-RF volume coil, 7.5 cm in diameter. ^{2}H localized spectra were recorded from a 1 cm^3 volume utilizing a ^{2}H-RF surface coil, 1 cm in diameter, with the ISIS pulse sequence [28]. The ISIS sequence consists of a combination of 8 RF pulses and magnetic field gradients in which three orthogonal slices are selectively inverted. We used adiabatic 180° pulses for the selective inversion and repeated each eight

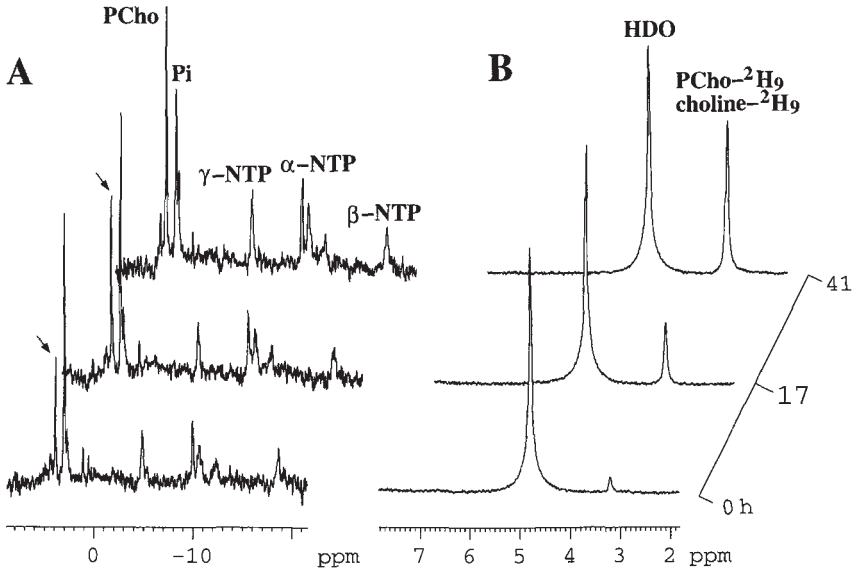

Fig. 2. (A) ^{31}P and (B) ^2H spectra of MCF7 cells perfused with medium containing deuterated chorine (36°C). The cells were perfused in the NMR spectrometer with a choline deficient medium for the first 6 h followed by perfusion (time = 0 h) with medium containing 50 μM of $^2H_9$-choline chloride for 17 h and then with medium containing 100 μM $^2H_9$-choline chloride. Proton decoupled ^{31}P spectra were obtained by accumulating 900 transients with 45° pulses and 2 s repetition time and applying a line broadening of 10 Hz. ^2H spectra were obtained by accumulating 300 transients with 90° pulses and a 4 s repetition time applying a line broadening of 0.5 Hz.

pulses 40 times (320 transients). The delay between RF pulses and between the series was 3 s.

NMR spectra were analyzed with a Bruker software XWIN-NMR. Lorentzian/Gaussian line deconvolution was applied in the analysis of the in vivo spectra. The concentration of deuterated choline metabolites was determined using the natural abundance of deuterium in water (16.4 mM) as a concentration standard.

3. Results

Transport of choline and its metabolism in perfused MCF7 cells were monitored by alternating ^{31}P and ^2H-NMR spectra, in the presence of $^2H_9$-choline chloride in the medium. The initial ^{31}P spectrum recorded prior to the addition of deuterated choline (0 h) exhibited intracellular phosphate signals including α, β, and γ-NTP, and PCho as well as extracellular inorganic phosphate (Pi) (Fig. 2(A), bottom spectrum). The other signals in this spectrum were previously assigned too [15]. Following the addition of deuterated choline to the medium a gradual increase in the intensity of the PCho signal was observed (Fig. 2(A)). After 17 h of perfusion with a medium containing 50 μM $^2H_9$-choline, the PCho increased by 50% and additional 24 h of perfusion with a medium containing 100 μM $^2H_9$-choline led to a two fold increase in this signal with respect to time $t = 0$. Throughout this experiment the NTP signals increased by $\sim 35\%$, possibly due to growth. The parallel ^2H spectra recorded in this experiment (Fig. 2(B)) revealed an increase in the signal at 3.2 ppm, assigned to choline and phosphocholine. Initially, in the spectrum recorded immediately after adding deuterated choline to the medium (0 h), this signal was due to the presence of external deuterated choline. The HDO natural abundance signal at 4.8 ppm was used as a concentration standard (16.4 mM) for determining the concentration of the deuterated choline metabolites. Throughout this experiment the intensity increase in the 3.2 ppm signal intensity was predominantly due to the synthesis of deuterated PCho, in accord with the ^{31}P results and with ^{13}C results described previously [16].

The metabolism of choline in MCF7 cells was further investigated in cell extracts. The cells were cultured in the presence of deuterated choline. The water-soluble metabolites and the lipid metabolites were extracted from the same samples (see Section 2). The ^{31}P spectrum of the water-soluble metabolites (Fig. 3(A)) showed the phosphate metabolites including α, β, and γ-NTP, and PCho. The ^2H spectrum of the same sample (Fig. 3(B)) showed a dominant signal of $^2H_9$-choline metabolites at 3.2 ppm as well as the natural abundant HDO signal. Resolution enhancement indicated the presence of a small signal at 0.04 ppm down field from PCho, of about 10% of the major 3.2 ppm signal (Fig. 3(B)). The chemical shift of this signal corresponds to that of $^2H_9$-betaine, but further studies are required in order to verify this tentative assignment. As expected, in the ^2H spectrum of the water phase extract of control cells only an HDO signal was detected (not shown). The ^{31}P spectrum of the lipid phase (Fig. 3(C)) showed the various signals due to MCF7 phospholipids including PtdCho [29]. A similar profile and relative amounts

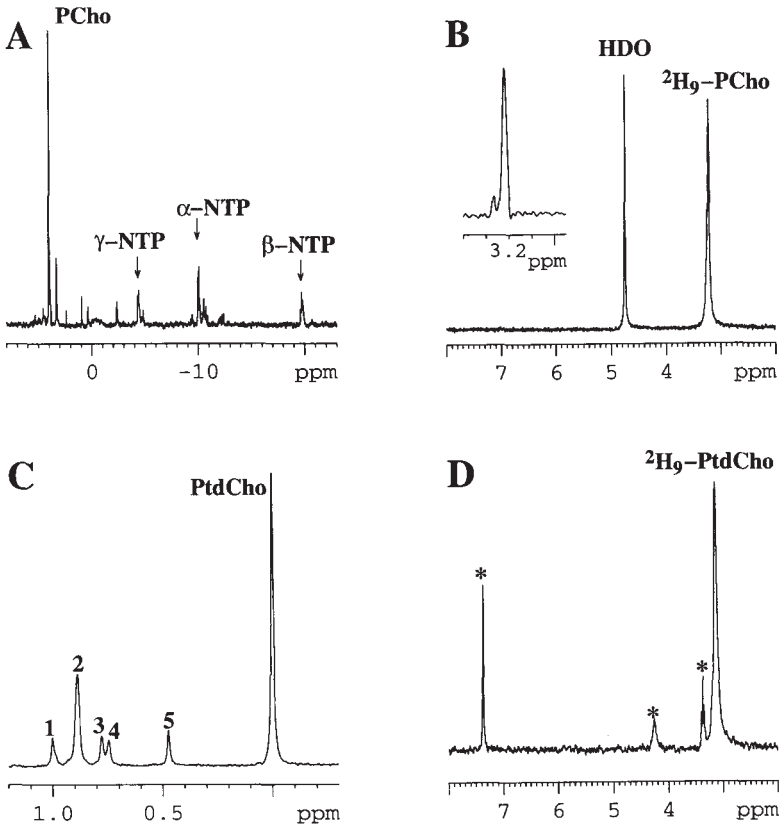

Fig. 3. ^{31}P and ^2H spectra of extracts of MCF7 cells cultured in the presence of 100 μM ^2H labeled choline. The extracts included the water and lipid phases of the same cell preparation. The cells were cultivated prior to the extraction for 1 day in growth medium containing 100 μM $^2H_9$-choline chloride. Proton decoupled ^{31}P spectra were recorded by applying 45° pulses and a 2.5 s repetition time. ^2H spectra were recorded by applying 80° pulses and a 3 s repetition time. (A) ^{31}P spectrum of the aqueous phase, number of scans = 880, processed with a line broadening of 3 Hz. (B) ^2H spectrum of the same sample as in A, number of scans = 600 processed with a line broadening of 1 Hz processed with resolution enhancement. (C) ^{31}P spectrum of the lipid phase, number of scans = 2400, processed with a line broadening of 0.5 Hz. Assignments: (1) cardiolipin; (2) phosphatidylethanolamine; (3) phosphatidylserine; (4) sphingomyelin; (5) phosphatidylinositol. (D) ^2H spectrum of the same sample as in (C), number of scans = 600; processed with a line broadening of 0.5 Hz. Signals arising from the solvents.

of phospholipids were found in the lipid extracts of control cells. Thus, the phospholipid composition and the relative content of the phospholipids did not alter due to the increased level of choline in the medium, in accord with previous results [15]. The ^2H spectrum of the lipid phase (Fig. 3(D)) showed a distinct signal at 3.2 ppm attributed to $^2H_9$-PtdCho and several signals due to the solvent, while the lipid phase extract of control cells exhibited only the solvent signals (not shown). Thus, extracts of cells grown in the presence of $^2H_9$-choline have confirmed the observation made in perfused cells and demonstrated the presence of deuterated PtdCho labeling in the lipid phase which could not be detected in intact cells.

Further quantitative analysis of the incorporation of labeled choline to PCho and PtdCho was performed in cell extracts using 1,2-^{13}C-choline. The water-soluble metabolites and the lipid metabolites were extracted from the same samples (see Section 2). The ^{31}P spectrum of the water-soluble metabolites (Fig. 4(A)) showed the same profile as in Fig. 3(A). However, the signal of 1,2-^{13}C-PCho (double doublet) was superimposed on the unlabeled PCho signal (singlet), as can be seen in the insert of Fig. 4(A). The splitting of the PCho is due to J coupling of the phosphorous nucleus with the two ^{13}C carbons of the choline moiety. The ^{13}C spectrum of the same sample (Fig. 4(B)) showed two signals with the chemical shifts of carbon 1 and 2 of 1,2-^{13}C-PCho [30]. As expected, in parallel ^{31}P and ^{13}C spectra of extracts of control cells neither ^{13}C splitting nor ^{13}C signals, respectively, were observed. Therefore, by adding to the culture medium 1,2-^{13}C-choline, it was ascertained that the predominant labeled signal in the water phase was PCho (69) and not choline. The percentage of labeled PCho was determined from the ^{31}P spectrum (Fig. 4(A)) by dividing the integrated intensity of the double doublet, due to the interaction with the ^{13}C carbons of the ^{13}C labeled PCho, to that of the singlet of non labeled PCho.

The ^{31}P spectrum of the lipid phase showed the same phospholipid profile as in Fig. 3(C) except for splitting in the PtdCho signal due to 1,2-^{13}C-PtdCho (double doublet) superimposed on the unlabeled PtdCho signal (see insert in C). The ^{13}C spectrum of the lipid phase

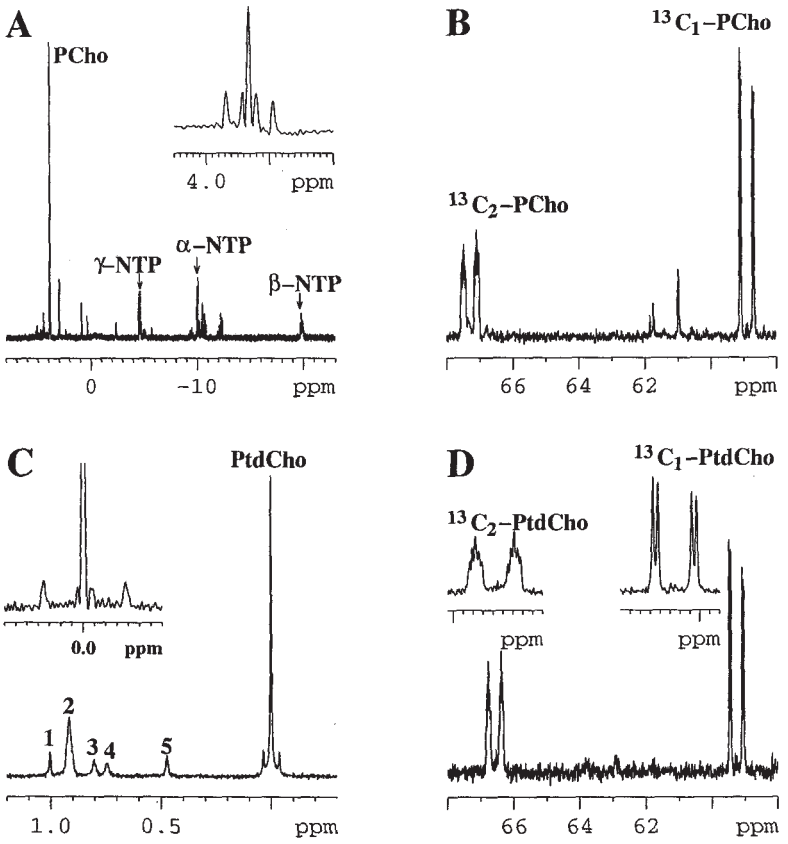

Fig. 4. ^{31}P and ^{13}C spectra of extracts of MCF7 cells cultured in the presence of 100 μM ^{13}C labeled choline. The extracts included the water and lipid phases of the same cell preparation. The cells were cultivated prior to the extraction for 1 day in growth medium containing 100 μM [1,2-^{13}C]-choline chloride. Proton decoupled ^{31}P spectra were recorded by applying 45° pulses and a 2.5 s repetition time. ^{13}C spectra were recorded by applying 60° pulses and a 2.4 s repetition time. (A) ^{31}P spectrum of the aqueous phase, number of scans = 2008, processed with a line broadening of 1.5 Hz, insert: expansion of the region of the PCho signal, J ($^{31}P - ^{13}C_2$) = 7.9 Hz, J ($^{31}P - ^{13}C_1$) = 4.3 Hz. (B) ^{13}C spectrum of the aqueous phase. number of scans = 800 processed with a line broadening of 0.1 Hz. (C) ^{31}P spectrum of the lipid phase, number of scans = 800, processed with no line broadening, insert: expansion of the PtdCho region, processed with resolution enhancement by a Gaussian multiplication, J ($^{31}P - ^{13}C_2$) = 6.8 Hz, J ($^{31}P - ^{13}C_1$) = 4.9 Hz. Assignment of signals 1–5 is the same as in Fig. 3(C). (D) ^{13}C spectrum of the lipid phase, number of scans = 800, processed with a line broadening of 0.1 Hz, inserts: on the right-expansion of the region of $^{13}C_1$-PtdCho and on the left-expansion of the region of $^{13}C_2$-PtdCho.

(Fig. 4(D)) showed the signals of carbon 1 and 2, at chemical shifts indicative of the presence of 1,2-^{13}C-PtdCho [31]. The percentage of labeled PtdCho (36%) was determined from the ^{31}P spectrum of the lipid phase (Fig. 4(C)) by dividing the integrated intensity of the double doublet ^{31}P signal, due to interaction with the ^{13}C labeled carbons of the choline moiety, to that of the ^{31}P singlet signal of the non labeled PtdCho.

In order to extend the cell culture studies of choline metabolism to tumors in vivo we measured initial choline plasma levels in mice used for tumor implantation. Furthermore, we monitored changes in blood choline levels during infusion of choline. The level of choline in the plasma of the mice studied here (CD-1 athymic immunodeficient female mice) was determined by proton spectroscopy and was found to be 58.6 ± 10.3 μM ($n = 5$). Further determinations in CD-1 athymic immunodeficient male mice and in CD-1 female and male mice yielded similar results with an average of 62.3 ± 5.3 μM ($n = 22$). Changes in the plasma level of choline were induced by i.v. infusion of $^2H_9$-choline chloride (see Section 2). Samples of blood withdrawn in intervals of 11 min in the course of this infusion showed an initial rise in deuterated choline levels reaching a constant level at 22 min, until infusion was stopped (at 88 min). The constant level in the plasma amounted to 221.7 ± 21.9 μM ($n = 8$). This level was 4 fold higher from that prior to the infusion. Halting the infusion led to a rapid decrease of about 70% in 7 min. Modulation of choline plasma levels of rats during infusion of choline chloride yielded a similar trend [26,31].

Choline metabolism of implanted MCF7 tumors was monitored in vivo in the course of infusion of deuterated choline using ^2H localized spectroscopy. ^2H spectra were recorded before, during and after the infusion ($n = 4$). An example is shown in Fig. 5: at the top, an image is presented with the localized volume within the

tumor (white square) from which ^2H spectra (Fig. 5(A and B)) were accumulated. The first spectrum, accumulated during the first 16 min of infusion, exhibit only a signal of HDO at natural abundance (Fig. 5(A)). From 40 to 60 min on, a gradual increase at the region of 3.2 ppm was observed (Fig. 5(B)). This signal was tentatively assigned to free $^2H_9$-choline (3.2 ppm), $^2H_9$-PCho (3.22 ppm), and $^2H_9$-betaine (3.261 ppm). The concentration of these deuterated metabolites amounted to 1.4 mM (Fig. 5(C)). An additional small signal was tentatively resolved with the aid of a Lorentzian line shape deconvolution and appeared to be rising at 3.6 ppm. This signal, consistently present in most of the spectra that included the 3.2 ppm signal of deuterated choline

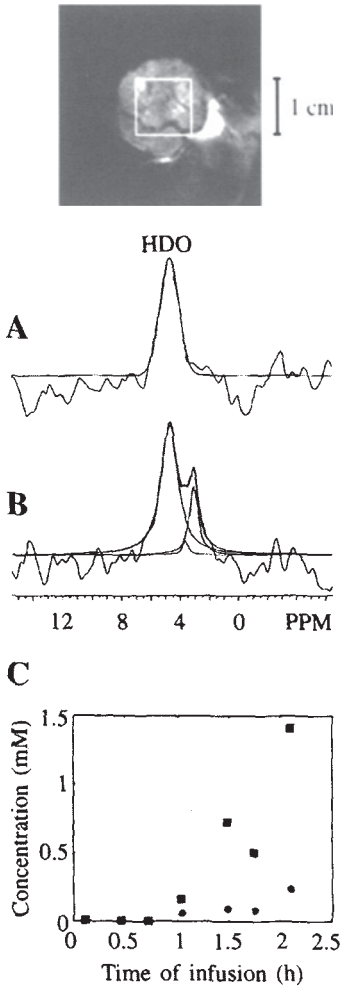

Fig. 5. In vivo deuterium MRS of MCF7 tumor implanted in athymic nude mice in the course of infusion of deuterated choline. Top; ^1H image of the tumor demonstrating the localized volume from which 2H spectra were recorded [white square]. A and B: In vivo ^2H spectra from the localized volume recorded at 8 and 105 min, respectively, after starting the infusion of $^2H_9$-choline. Spectra were recorded and simulated as described in Materials and Methods. Each spectrum consisted of 320 scans (16 min), and was processed with a line broadening of 4 Hz. (C) Changes in the concentration of deuterated-choline metabolites in the tumor, derived from deconvolution and integration of the signals at 3.2 ppm (■) and 3.6 ppm (●).

metabolites, could not be attributed to most of the water soluble choline metabolites (dimethylglycine, sarcosine, methionine, trimethylamine, and acetylcholine) [32]. Spectra recorded after terminating the infusion revealed a decrease in the intensity of the signal at 3.2 ppm, while the intensity of the signal at 3.6 ppm continued to increase. In Fig. 5(C) the changes with time in the content of the deuterated choline metabolites are summarized.

4. Discussion

Previous studies have indicated that the levels of choline and ethanolamine metabolites, predominantly the phosphorylated metabolites, change markedly as a result of malignant transformation. In addition choline phospholipids and their metabolites have been suggested to be involved in signal transduction and carcinogenesis [33]. The pathways responsible for these changes and inductions have not been yet fully characterized. However, studies of breast cancer cells have indicated that both the rates of the transport and the phosphorylation of choline are elevated in the cancer cells [15,16,30]. In this study we have further verified this mechanism and proceeded to find whether it holds in vivo too. We have chosen to investigate choline metabolism in MCF7 human breast cancer cells grown in culture and as tumors implanted in mice. This line has been extensively studied in the past [34] and serves as a model for human breast cancer. Multinuclear NMR spectroscopy has been used as the main tool in this study, with the aid of choline labeled with deuterium or with carbon-13.

Continuos monitoring of deuterated choline uptake and phosphorylation in perfused MCF7 cells by alternating ^2H and ^{31}P recordings showed that choline is transported and rapidly phosphorylated accumulating in the cells as PCho. Thus, high levels of PCho in breast cancer cells result from efficient transport and high activity of choline kinase. ^2H spectroscopy of deuterated choline provided quantitative kinetic data in perfused cells due to the use of HDO as an internal concentration standard. The trend shown in perfused, living cells was confirmed by extract studies of cells cultured in the presence of deuterated choline. The kinetic of the transport of choline into MCF7 cells was found to follow a Michaelis–Menten mechanism, with a Michaelis–Menten rate constant (K_t) for the transport of 46.5 ± 2.8 μM and a maximum transport velocity (T_{max}) of 13.5 ± 2.6 nmol h^{-1} mg^{-1} protein [16].

The enhanced choline phosphorylation could lead to modulation of the rate of PtdCho synthesis, the final product of choline metabolism, which is the predominant component of cellular membranes. To monitor changes in the level of PtdCho we had to use cell

extracts since the line width of the nuclei of PtdCho in intact membranes is usually broadened and therefore difficult to observe. By applying an extraction procedure that simultaneously yielded an extract of the water soluble metabolites and an extract of the lipids we related the level of PCho to that of PtdCho in the same cells. The analysis of the spectra of the extracts clearly indicated high regulation of PtdCho synthesis. Although the PCho was elevated the amount of PtdCho relative to the amount of other phospholipids remained the same in MCF7 cells cultivated in the presence of either 30 or 130 μM choline in the growth medium. These results were in accord with the tight regulation of PtdCho synthesis by the CTP:phosphocholine cytidylyltransferase, the rate limiting enzyme in the Kennedy Pathway [35].

The ^{13}C chemical shifts of the methylenes of the three water soluble cholines: choline, PCho and GPC are resolvable [16,36]. We have therefore chosen to label the methylenes of choline with carbon-13 and use high resolution ^{13}C-NMR spectroscopy as well. This labeling also enabled us to determine, from high resolution ^{31}P spectra of these extracts, the ratio of labeled to unlabeled phosphorylated choline metabolites through the splitting of the ^{31}P nuclei. The results have indicated a higher labeling fraction of PCho than of PtdCho, again demonstrating the regulation of PtdCho synthesis and the capacity of the cancer cells to accumulate high amounts of PCho.

In summary, these results indicate that in cancer cells part of the reactions within the same pathway are markedly changed while part retain their regulation. The exact role of elevated PCho in cancer cells is still not known and further studies at the molecular level may elucidate this puzzling phenomenon.

It is important to characterize choline metabolism in breast cancer in vivo, in order to further understand the clinical observations of high PCho in breast carcinoma. We used MCF7 tumors implanted in mice as a model and altered choline concentration by infusing it into the blood circulation. The tumors included breast cancer epithelial cells of human origin, and stroma and blood vessels of mice origin. Thus, unlike cell cultures, where choline metabolism is uniform, in the tumors, this metabolism can vary depending on the tissue components.

The level of choline in the plasma of the mice was initially determined by ^{1}H-NMR. The changes due to choline infusion were determined by using deuterated choline and ^{2}H-NMR. The level of choline in the plasma (58 μM) was slightly above the K_t for choline transport to the cancer cells (46.5 μM). Following infusion of deuterated choline a five fold increase in the total blood concentration of choline had occurred, predominantly of the deuterated species. The fate of choline in the tumor due to the high rise in deuterated choline level in the blood was monitored in vivo with localized ^{2}H-MRS. Again, the natural abundance HDO signal served as a reference of concentration. The results clearly indicated a rise in deuterated choline metabolites in the tumor. The signal of deuterated choline metabolites appeared to have a major component at 3.2 ppm and a small component at 3.6 ppm. The low resolution of the deuterium spectrum did not permit specific assignment of the choline metabolites. Based on the cell culture work we propose that deuterated PCho is the predominant species. However, we can not exclude the presence of metabolites with a very similar chemical shift such as free choline and betaine as was previously found in tumors of mice fed with phosphonium choline [21] and in normal rat mammary epithelial cells [17]. It is clear that by infusing choline labeled with ^{13}C carbons in the methylenes it would be possible to obtain a full assignment of these metabolites as was shown in the cell cultures.

To conclude, we have followed choline metabolism in human breast cancer cells and tumors. The studies of cells clearly indicated rapid uptake and phosphorylation of choline as the main mechanism responsible for high PCho in breast cancer. Deuterium MRS using $^{2}H_9$-choline was found suitable for quantitative in vivo measurements of the uptake and accumulation of metabolites using HDO as an internal standard for concentration. The apparent disadvantage of the deuterium sensitivity was compensated by its fast T_1 relaxation which allowed fast acquisition. Further characterization of the nature of the choline metabolites in vivo are currently under investigation.

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References

[1] Negendank W. Studies of human tumors by MRS: a review. NMR Biomed 1992;5:303–24.
[2] Oberhaensli RD, Hilton-Jones D, Bore PJ, Hands LJ, Rampling RP, et al. Biochemical investigation of human tumors in vivo with phosphorus-31 magnetic resonance spectroscopy. Lancet 1986;2:8–11.
[3] Steen RG. Response of solid tumors to chemotherapy monitored by in vivo ^{31}P nuclear magnetic resonance spectroscopy: A review. Cancer Res 1989;49:4075–85.
[4] Negendank W, Li CW, Padavic-Shaller K, Murphy-Boesch J, Brown TR. Phospholipid metabolites in ^{1}H-decoupled ^{31}P MRS in vivo in human cancer: implications for experimental models and clinical studies. Anticancer Res 1996;16:1539–44.

[5] Ng TC, Grundfest S, Srinivasan V, Baldwin NJ, Majors AW, et al. Therapeutic response of breast carcinoma monitored by ^{31}P-MRS in situ. Magn Reson Med 1989;10:125–34.
[6] Sijens PE, Wijredeman HK, Moerland MA, Bakker GJG, Vermoulen JWA, et al. Human breast cancer in vivo: ^{1}H- and ^{31}P-MR spectroscopy at 1.5 T. Radiology 1988;161:53–5.
[7] Glanholm J, Leach MO, Collins DJ, Mansi J, Sharp JC, et al. In vivo ^{31}P magnetic spectroscopy for monitoring treatment response in breast cancer. Lancet 1989;1:1326–7.
[8] Degani H, Horowitz A, Itzchak Y. Breast tumors: Evaluation with ^{31}P-MR spectroscopy. Radiology 1986;161:53–5.
[9] Gribbestad IS, Fjosne HE, Kvinnsland S. In vitro proton NMR spectroscopy of extracts from human breast tumours and non-involved breast tissue. Anticancer Res 1993;13:1973–80.
[10] Gribbestad IS, Petersen SB, Fjosne HE, Kvinnsland S, Krane J. ^{1}H-NMR spectroscopic characterization of perchloric acid extracts from breast carcinomas and non-involved breast tissue. NMR Biomed 1994;7:181–94.
[11] Gribbestad IS, Nilsen G, Fjosne HE, Haugen OA. Combined ^{1}H-MR spectroscopy and imaging of normal human breast and breast tumors. J Magn Reson Imag 1994;4:107.
[12] Roebuck JR, Lenkinski RE, Schnall MD. Spatially localized proton magnetic resonance spectroscopy of human breast disease at 1.5 T. Proc Int Soc Magn Resonance Med 1996;2:1247.
[13] Barry PA, Mackinnon WB, Malycha P, Gillett D, Mountford CE. Benign breast lesions distinguished from invasive cancer by ^{1}H magnetic resonance spectroscopy on fine needle biopsy. Proc Int Soc Magn Resonance Med 1996;2:266.
[14] Mackinnon WB, Barry PA, Malycha PL, Gillett DJ, Russell P, et al. Fineneedle biopsy specimens of benign breast lesions distinguished from invasive cancer ex vivo with proton MR spectroscopy. Radiology 1997;204:661–6.
[15] Ting YT, Sherr D, Degani H. Variations in the energy and phospholipid metabolism in normal and cancer human mammary epithelial cells. Anticancer Res 1996;16:1381–8.
[16] Katz-Brull R, Degani H. Kinetics of choline transport and phosphorylation in human breast cancer cells; NMR application of the zero trans method. Anticancer Res 1996;16:1375–80.
[17] Chao CK, Pomfret EA, Zeisel SH. Uptake of choline by rat mammary-gland epithelial cells. Biochem J 1988;254:33–8.
[18] Zeisel SH, Costa KAD, Franklin PD, Alexander EA, lamont JT, et al. Choline, an essential nutrient for humans. FASEB 1991;5:2093–8.
[19] Zeisel SH. Choline deficiency. J Nutr Biochem 1990;1:332–49.
[20] Zeisel SH. Choline. Modern Nutrition in Health and Disease, 8th Edition. Philadelphia: Lea and Febiger, 1994.
[21] Street JC, Szwergold BS, Matei C, Kappler F, Mahmood U, et al. Study of the metabolism of choline and phosphatidylcholine in tumors in vivo using phosphonium choline. Magn Reson Med 1997;38:769–75.
[22] Katz-Brull R, Bendel P, Margalit R, Degani H. Quantitative in vivo kinetics of choline metabolism in breast cancer-a localized deuterium MRS study. MAGMA 1997;5:459.
[23] Furman E, Rushkin E, Margalit R, Bendel P, Degani H. Tamoxifen induced changes in MCF7 human breast cancer: in vitro and in vivo studies using nuclear magnetic resonance spectroscopy and imaging. J Steroid Biochem Mol Biol 1992;43:189–95.
[24] Degani H, Ronen SM, Furman E. Breast cancer: Spectroscopy and imaging of cells and tumors. In: Gillies R, editor. Magnetic resonance in physiology and medicine. San Diego: Academic Press, 1994.
[25] Tyagi RK, Azrad A, Degani H, Salomon Y. Simultaneous extraction of cellular lipids and water-soluble metabolites: evaluation by NMR spectroscopy. Magn Reson Med 1996;35:194–200.
[26] Haran EF, Maretzek AF, Goldberg I, Horowitz A, Degani H. Tamoxifen enhances cell death in implanted MCF7 breast cancer by inhibiting endothelium growth. Cancer Res 1994;54:5511–4.
[27] Freeman JJ, Choi RL, Jenden DJ. Plasma choline: its turn over and exchange with brain choline. J Neurochem 1975;24:729–34.
[28] Ordidge RJ, Connelly A, Lohman JAB. Image-selected in vivo spectroscopy (ISIS). A new technique for spatially selective NMR spectroscopy. J Magn Reson 1986;66:283–94.
[29] Sherr D, Rivenson D, Rushkin E, Degani H. Differences in the composition and metabolism of the phospholipids in normal and cancerous mammary epithelial cells. Proc Soc Magn Resonance 1994;1:452.
[30] Ronen SM, Rushkin E, Degani H. Lipid metabolism in large T47D human breast cancer spheroids: ^{31}P- and ^{13}C-NMR studies of choline and ethanolamine uptake. Biochim Biophys Acta 1992;1138:203–12.
[31] Ronen SM, Degani H. The application of ^{13}C-NMR to the characterization of phospholipid metabolism in cells. Magn Reson Med 1992;25:384–9.
[32] Lynch MJ, Masters J, Pryor JP, Lindon JC, Spraul M, et al. Ultra high field NMR spectroscopic studies on human seminal fluid, seminal vesicle and prostatic secretions. J Pharm Biomed Anal 1994;12:5–19.
[33] Zeisel SH. Choline phospholipids: signal transduction and carcinogenesis. FASEB 1993;7:551–7.
[34] Levenson AS, Jordan VC. MCF–7: The first hormone-responsive breast cancer cell line. Cancer Res 1997;57:3071–8.
[35] Pelech SL, Vance DE. Regulation of phosphatidylcholine biosynthesis. Biochim Biophys Acta 1984;779:217–51.
[36] Ronen SM, Rushkin E, Degani H. Lipid metabolism in T47D human breast cancer cells: ^{31}P and ^{13}C-NMR studies of choline and ethanolamine uptake. Biochim Biophys Acta 1991;1095:5–16.