Choline containing metabolites during cell transfection: an insight into magnetic resonance spectroscopy detectable changes

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Abstract Increases in choline containing metabolites have been associated with a number of disorders, including malignant cell growth. In this study, high resolution magic angle spinning ^{1}H nuclear magnetic resonance spectroscopy was employed to monitor metabolite changes during cell transfection, and an increase in phosphocholine was detected. This increase appears to be correlated with cell membrane disruption associated with the insertion of plasmid DNA into cells, since the level of phosphocholine in mock transfected cells was comparable to that of control cells. These data suggest choline containing metabolite changes detected in vivo using magnetic resonance spectroscopy relate to cell membrane disruption.

Key words: High resolution magic angle spinning; Metabolic profiling; Metabolomics; Phosphocholine

1. Introduction

Increased resonance intensities of choline containing metabolites have been associated with a number of pathologies using ^{1}H magnetic resonance spectroscopy (MRS) in vivo, including tumour growth, multiple sclerosis, Duchenne muscular dystrophy and AIDS dementia [1–4]. However, ^{1}H MRS in vivo has poor spectral resolution and it is not possible to distinguish between different choline containing metabolites.

High resolution ^{1}H nuclear magnetic resonance (NMR) spectra of cell extracts does distinguish between the singlets associated with choline (δ 3.20), phosphocholine (δ 3.23), glycerophosphocholine (δ 3.26) and phosphatidylcholine (δ 3.30), and Bhakoo and co-workers have used this methodology to detect increases in the ratio of phosphocholine to glycerophosphocholine in aqueous extracts of cancerous cell lines [5]. These data suggest that the increased levels of choline containing metabolites commonly detected in MRS studies may be associated with aqueously soluble pools. However, one caveat is that spectra of extracts reflect the relative solubilities of the metabolites within an extraction media as well as the tissue content of the metabolite. Further support that MRS detects predominantly cytosolic choline and its derivatives arises from the chemical environment that cell membrane metabolites occur within. These molecules experience large dipole–dipole couplings caused by their restricted environment, broadening resonances, with dipolar couplings being of the order of 50 kHz in lipid bilayers [6]. Thus, cell membrane bound choline would produce a broad component, poorly detectable above the baseline in most spectra obtained in vivo. Furthermore, spectra obtained in vivo often rely on T_2 editing to remove resonances from molecules within constrained environments, such as cell membranes.

High resolution magic angle spinning (HRMAS) ^{1}H NMR spectroscopy produces high resolution spectra of intact tissue, circumventing the need to extract metabolites [7–9]. The technique is particularly effective at distinguishing malignant tissue from benign growths, as demonstrated in renal tissue [10], the brain [7], adipocytes [8,11] and prostate tissue [9]. In these studies, cancerous tissue was in part characterised by increased choline containing metabolites, suggesting that these compounds are biomarkers for cell membrane manufacture and uncontrolled cell growth. However, unlike solid state MAS NMR spectroscopy, the modest rotation speeds used in HRMAS ^{1}H NMR spectroscopy (< 6000 Hz) are less than the dipole–dipole interactions of the cell membrane [6], and hence, membrane bound choline containing metabolites are expected to be poorly observed.

Here we demonstrate the use of HRMAS ^{1}H NMR spectroscopy as an ideal technique to readily follow metabolic changes in intact cells expressing a recombinant protein following transfection. We also show that the relative intensity of phosphocholine increases in the HRMAS ^{1}H NMR spectra of such cells, and suggest an explanation for the increased levels of choline containing metabolites in the ^{1}H MRS spectra of many diverse pathological conditions.

2. Materials and methods

2.1. Mammalian expression system and transfection

The T-Rex™ mammalian expression system [12] was used. The tetracycline repressor expression vector (pcDNA6/TR) stably transformed in the rat hepatoma McRH7777 cell line was a kind gift from Drs Boren and Gustafsson (Wallenberg Laboratory, Goteborg University, S-41345 Goteborg, Sweden). The *lac*Z gene, pcDNA4/TO/lacZ, and the Calcium Phosphate Transfection kit were obtained from

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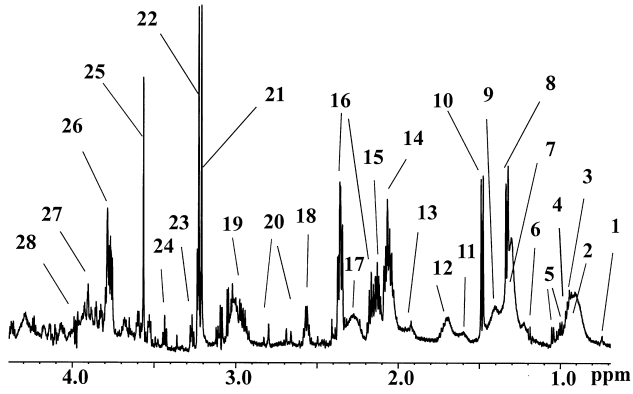

Fig. 1. HRMAS ^{1}H NMR spectrum of intact mock transfected cells showing aliphatic region using a conventional solvent suppressed pulse sequence. Key: (1) Cholesterol, (2) C**H**$_3$CH$_2$- lipid, (3) isoleucine, (4) leucine, (5) valine, (6) ethanol, (7) CH$_2$C**H**$_2$CH$_2$ lipid, (8) lactate, (9) CH$_2$C**H**$_2$CH$_2$ lipid (extracellular?), (10) alanine, (11) C**H**$_2$CH$_2$CO lipid, (12) C**H**$_2$CH$_2$C=C lipid, (13) acetate, (14) glutamate/glutamine, (15) glutamate, (16) glutamine, (17) C**H**$_2$CO, (18) glutathione, (19) lysine, (20) citrate, (21) choline, (22) phosphocholine, (23) glycerophosphocholine/phosphatidylcholine, (24) taurine, (25) glycine, (26) choline/phosphocholine/phosphatidylcholine, (27) creatine, (28) glucose/sugars containing region.

Invitrogen. Cells were grown in DMEM containing 20% fetal calf serum, 2 mM L-glutamine, 2 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 3 μg/ml blasticidin. In all, there were five groups of cells; control cells (untreated, $n=3$), mock transfected (Ca^{2+} treatment only, $n=4$), mock transfected+tetracycline ($n=4$), transfected with pcDNA4/TO ($n=2$) or pcDNA4/TO/lacZ ($n=4$). Transfections were performed as recommended by Invitrogen, in the absence and presence of pcDNA4/TO or pcDNA4/TO/lacZ (30 μg/1×10^6 cells). Tetracycline was added to the media (1 mg/ml) of transfected cells after an overnight incubation. Sixty five hours post-transfection, cells were harvested in phosphate-buffered saline, extensively washed and snap-frozen in liquid nitrogen. The efficiency of transfection was verified by β-galactosidase staining.

2.2. HRMAS ^{1}H NMR spectroscopy

Cells (0.5×10^6) were extensively washed in 0.9% NaCl in D_2O, and resuspended in 50 μl of this solution. Cells were pipetted into a 4 mm outer diameter zirconium oxide rotor, and the rotor filled with ~10 μl D_2O spiked with 10 mM TSP. Rotors were inserted into a high resolution ^{1}H–^{13}C-MAS probe interfaced with an AVANCE spectrometer and a 9.6 T superconducting magnet (Bruker GmbH, Karlsruhe, Germany). HRMAS ^{1}H NMR spectra were acquired at the magic angle (54.7°) and 300 K with a rotor spin speed of 4 kHz.

Solvent suppressed and T_2 edited Carr–Purcell–Meiboom–Gill (CPMG) spectra were acquired with 256 scans, into 64 k data points across a spectral width of 6410.3 Hz, using a relaxation delay of 2 s [13]. For the acquisition of CPMG spectra, a total spin–echo delay of 42 ms was used. Line-broadening apodisation function of 1.0 Hz was applied to all HRMAS ^{1}H NMR FIDs prior to Fourier transformation. Spectra were analysed using standard Bruker software (XWIN-NMR version 2.5). Assignments were made using literature values [8,11] and using ^{1}H–^{1}H correlation spectroscopy.

Spectra were initially compared visually. Following this, principle component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) was applied to the spectra to identify patterns of variation resulting from the treatments [14]. ^{1}H NMR spectra of the cell culture media acquired using a standard flow probe and the solvent pre-saturation pulse sequence described above were similarly compared using pattern recognition.

Results are presented as means ± standard error of the mean and were compared using Student's t-test and the ANOVA test for variance with a Tukey post-test within Instat (Graphpad Inc., CA, USA).

3. Results and discussion

HRMAS ^{1}H NMR spectroscopy of the McRH7777 cell line stably transformed with the tetracycline repressor expression vector (pcDNA6/TR) produced spectra of a resolution (Fig. 1) comparable with those previously obtained from aqueous extracts of cancerous cell lines [5], despite the relatively small number of cells (0.5×10^6) used. To examine the impact of transfection on cell metabolites on this cell line during transient transfection experiments, we performed a mock transfection. No metabolic changes were observed, including the resonance intensities of phosphocholine or choline (Table 1). Further, when the mock transfected cells were treated with tetracycline, as would be required to induce gene expression in the expression system used, the spectra were comparable to control cells. This was confirmed using both PCA and PLS-DA methods of pattern recognition.

Next, we examined the impact of transfecting the T-Rex inducible vector pcDNA4/TO/lacZ into the stably transformed McRH7777 cells. A significant increase in phosphocholine, relative to the resonance intensity of glycine, was detected (Fig. 2). This was independent of the transcription of the LacZ gene as these changes were also observed when the LacZ gene was absent from the plasmid vector. The phosphocholine and choline resonances were in a relatively unrestricted environment, and readily observable in T_2 weighted CPMG spectra (Table 1). PLS-DA also demonstrated that transient transfection of pcDNA4/TO/lacZ DNA into our cell system produced an increased resonance from phosphocholine (Fig. 3). This resonance was associated with an increase in PLS factor t3 and a decrease in factor t1. These factors had a combined $Q^2=0.43$ for the goodness of fit to the predicted model, and the changes were correlated with a decrease in CH_2CH_2, CH_3CH_2 and $CH=CHCH_2CH_2$ lipid moieties detected within the cells. However, these changes were the only differences in the spectra that separated the transfected cells (i.e. those receiving plasmid DNA) from the mock transfected cells, and control cells. PCA of spectra ob-

Table 1
Metabolite ratios for integrals relative to the glycine resonance at δ 3.56 for the cell treatment groups

	Control ($n=3$)	Mock transfected ($n=4$)	Mock transfected and tetracycline ($n=3$)	Transfected with pcDNA4/TO ($n=2$)	Transfected with pcDNA4/TO/lacZ ($n=4$)
Phosphocholine (1)	1.43 ± 0.41	1.41 ± 0.43	1.26 ± 0.56	5.12 ± 1.30*,†,‡	5.84 ± 0.64***,††,‡‡
Choline (1)	0.63 ± 0.45	1.27 ± 0.31	0.73 ± 0.23	0.85 ± 0.33	1.10 ± 0.25
Phosphocholine (2)	1.52 ± 0.56	1.47 ± 0.56	2.46 ± 0.57	5.33 ± 0.95*,†,‡	5.62 ± 0.61**,†,‡
Choline (2)	0.63 ± 0.37	1.84 ± 0.81	1.87 ± 0.25	1.10 ± 0.45	1.04 ± 0.26

The resonances at δ 3.21 and δ 3.23 for choline and phosphocholine, respectively, were used for the ratios. Ratios are included for both pulse sequences used: (1) conventional solvent pre-saturation, (2) CPMG pulse sequence. $^*P<0.01$, $^{**}P<0.01$ and $^{***}P<0.005$ for difference between control cells and treated cells, $^\dagger P<0.05$ and $^{\dagger\dagger}P<0.01$ for difference between mock transfected and other groups, $^\ddagger P<0.05$ and $^{\ddagger\ddagger}P<0.01$ for difference between mock transfected and tetracycline using Tukey post-test following ANOVA test of variance

tained from the cell culture media failed to separate the cells into treatment groups, indicating there were no metabolic changes within the media. This indicates that flux into and out of the cells was the same for all five groups.

Transfection of cells relies on the membrane being made permeable to the transfection vector by electroporation or chemicals such as calcium phosphate, DEAE-dextran or DMSO [15]. All of the cells examined in this study were treated with Ca^{2+} apart from the control group. This suggests that the increase in phosphocholine observed in transfected cells is specific to transfection with a plasmid vector. As we know of, no mechanism where DNA replication can cause a change in phosphocholine metabolism and similar cell counts were used in these experiments, thus, it seems reasonable to imply that the changes in phosphocholine are caused by extra cell membrane disruption by the plasmid vector. Furthermore, the increased phosphocholine detected in these transfected cells was most likely released from the '^{1}H MRS poorly visible' cell membrane into the '^{1}H MRS visible' cytosol, as the spinning speeds involved were not great enough to resolve cell membrane lipids, and no difference in influx/efflux of cell media metabolites were detected. We have also noted increases in phosphocholine following the addition of DMSO to stable cell lines (data not shown), further supporting our hypothesis that the increased resonance intensity of phosphocholine in transfected cells detected in this study is caused by cell membrane disruption. Intriguingly, the increase resonance intensity of phosphocholine was not accompanied by an increase in the resonance intensity of choline. This suggests that the cell membrane disruption effects phosphocholine more greatly, possibly caused by the molecules more polar nature.

It was not possible to reliably estimate the resonance intensities of glycerophosphocholine or phosphatidylcholine within these cells as these resonances were very broad, presumably caused by residual dipolar couplings which were not removed by the process of spinning. Weybright and co-workers [16] observed two lipid environments within adipocyte cells using gradient HRMAS spectroscopy, suggesting one set to be freely moving within the intracellular space, and the other in a more restricted environment. This study also used modest spin speeds, insinuating that while certain lipids may diffuse slowly, they still possess a high degree of rotational motion in order to be visible at slow spin speeds.

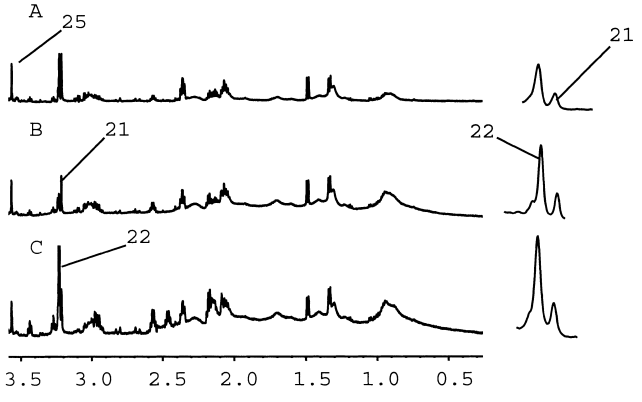

Fig. 2. HRMAS ^{1}H NMR spectra of intact mock transfected cells (A), following treatment with tetracycline (B) and following transfection with a vector containing LacZ (C). The resonance intensity of glycine (δ 3.58) has been adjusted so to be constant for the three cell types. While in these spectra some variation in other metabolites are apparent, PC analysis only highlighted the change in phosphocholine (δ 3.23) as being significant for all the samples. The phosphocholine and choline region is expanded to the right of the full spectra. Key as in Fig. 1.

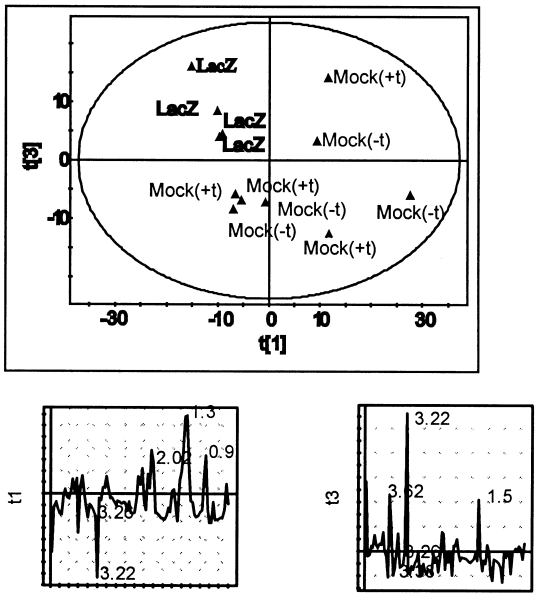

Fig. 3. PLS-DA of HRMAS ^{1}H NMR spectra (top), demonstrating the separation of spectra from cells transfected with LacZ compared with mock transfected cells with (+) and without (−) tetracycline. Transfected cells had a higher loading in terms of component t3, but lower in t1 compared with the other two groups. Phosphocholine (δ 3.22) contributed to the loading plots of both components t1 and t3.

The results from this study suggest that increased detection of choline containing metabolites during MRS in vivo, especially in malignant tissue [17], may correlate not with choline synthesis [5] or cellular proliferation [1,18] but with a change in the distribution of the metabolites caused by cell membrane disruption. Correlating increases in the NMR detectability of choline containing metabolites with cell membrane disruption also may explain why a number of studies concerning noncancerous pathologies have detected rises in choline containing metabolites. In multiple sclerosis, increased choline detection [2] may result from demyelination altering neuronal cell membrane integrity. Similarly, increases in the detectability of choline metabolites in the brains of sufferers of Duchenne muscular dystrophy [3] and related animals models [19,20] may correlate with reduced cell membrane integrity caused by a failure to express dystrophin, a protein known to stabilise the cell membrane.

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