Assessing choline kinase activity in cancer cells using a 31P NMR enzymatic assay

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Introduction

Choline metabolites are widely studied in oncology as a diagnostic marker of malignancy [1]. Choline is incorporated into membrane phospholipids through the three-step Kennedy pathway. The first step of this process, the ATP-dependent phosphorylation of choline to phosphocholine, is catalyzed by choline kinase in the presence of Mg^{2+}. Malignant transformations are often associated with altered membrane phospholipid metabolism and increased choline kinase activity, resulting in increased levels of phosphocholine in almost all forms of cancer. Inhibitors of choline kinase are being investigated as therapeutic agents in cancer research, [2, 3] and implementation of non-invasive endpoints to monitor their activity is critical for the clinical development and evaluation of this novel therapy. Here we present an effective NMR method to quantitatively assess choline kinase activity in cancer cells using ^{31}P NMR.

Methods and Results

NMR experiments were conducted on a Bruker Avance 500MHz spectrometer (Bruker Instruments, Germany). ^{31}P NMR spectra were acquired at 202.45MHz using a 30 degree pulse-and-acquire sequence, ns=128, at 25° C temperature. The acquisition time was 5min 39s per spectrum, which gave the time resolution for the experiments.

Human MDA-MB-231 breast and PC-3 prostate cancer cells were cultivated in DMEM containing 10% fetal calf serum and maintained at 37° C in a 5% CO_2 atmosphere. MDA-MB-231 ($8x10^6$) and PC-3 ($11x10^6$) cells were harvested by trypsinization. The cellular cytoplasm of the cells was extracted using a lysis buffer for separate extraction of cytoplasm and nucleus (NE-PER cytoplasmic and nuclear extraction reagents, Perbio), as choline kinase is present in the cell cytoplasm. Using this method nuclear proteins were not released into solution, allowing higher concentration of cytoplasmic proteins without the risk of precipitation and degradation of the enzyme. Immediately before the start of the experiment, exogenous choline chloride, ATP, and $MgCl_2$ were added to the cell lysate, with final concentrations Cho 5mM, ATP 10mM, $MgCl_2$ 10mM, pH = 8.0 [4].

The absolute concentration of the metabolites was quantified using the initial concentration of exogenous ATP (10mM) as a reference and normalized to the number of extracted cells. The identity of the peak assigned to PCho was verified at the end of the experiment by spiking the solution with additional compound.

In Fig. 1 a ^{31}P NMR spectrum of PC-3 cell cytoplasm 3 hours after the beginning of the experiment is shown. PCho peak is increased 7-fold compared with the normal level. The growing peak at 4.8ppm has been tentatively assigned to Adenosine Monophosphate (AMP), as a consequence of ATP breakdown.

In Fig. 2 we report the dynamics of the phosphorylation of choline in the cytoplasm of PC-3 cells. PCho, AMP, and inorganic phosphates (PI) increase over time in both breast and prostate cancer cell lines, while ATP decreases.

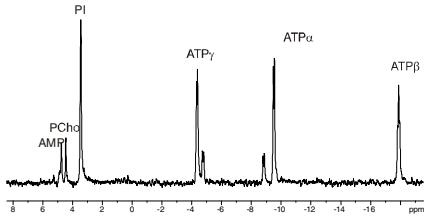

Fig. 1 ^{31}P NMR spectrum of cytoplasm of prostate PC-3 cancer cells at the end of the experiment. PCho and AMP increased.

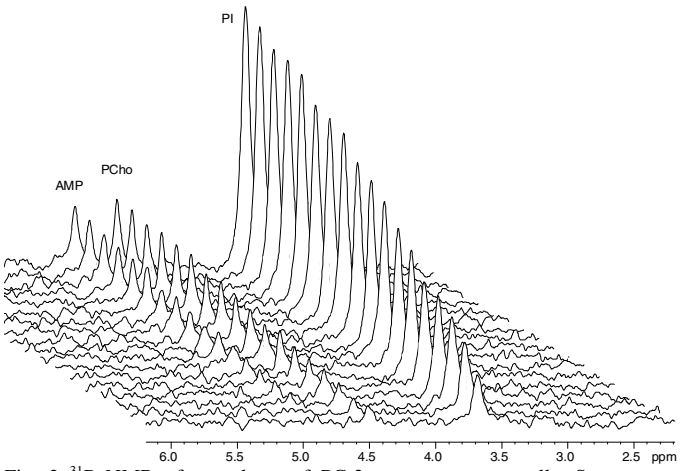

Fig. 2 ^{31}P NMR of cytoplasm of PC-3 prostate cancer cells. Spectra are reported with 11.3min time interval. PCho and PI increase over the time. The peak tentatively assigned to AMP appears as a consequence of ATP breakdown.

The increase of PCho (per million cells) over the time is shown in Fig. 3 for PC-3 and MDA-MB-231 cells, and is used to estimate the activity of choline kinase. For MDA-MB-231 and PC-3 cells choline kinase activity is respectively 0.10±0.01 nmol/min and 0.26±0.01 nmol/min per 10^6 cells. The lower choline kinase activity in breast cells suggests that the higher initial concentration of endogenous PCho in MDA-MB-231 cells with respect to PC-3 cells (~6-fold higher from Fig. 3) is not due to greater enzyme activity, but more likely reflects differences in transport rates of choline into the cell.

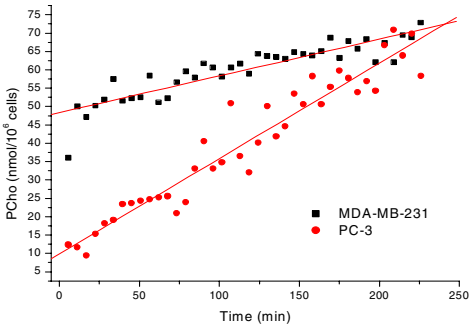

Fig. 3 The increase of PCho peak integral over the time in cytoplasm of breast MDA-MB-231 and prostate PC-3 cancer cells after addition of exogenous choline, ATP, MgCl2.

Discussion and Conclusion

Dynamic measurements of choline kinase activity by ^{31}P NMR are of increasing interest in view of the current efforts to focus anticancer drug research on specific pharmacodynamic markers. Choline kinase activity is usually assessed by ^{14}C choline radioactive assays, which require a separate sample for every time point and is not always hazard-free. We have presented a ^{31}P NMR assay, which offers an easy and robust way to dynamically measure choline kinase activity in the same sample, thereby increasing reproducibility and reliability.

Acknowledgements

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References

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