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Monitoring the transport and phosphorylation of 2-deoxy-D-glucose in tumor cells in vivo and in vitro by ^{13}C nuclear magnetic resonance spectroscopy

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We describe the use of 2-deoxy-D-[6-^{13}C]glucose to follow simultaneously, by ^{13}C NMR, both transport and phosporylation to its 6-phosphate form, in MCF-7 breast cancer cells in vitro and in vivo in subcutaneous tumors in nude mice.

NMR, ^{13}C-; Deoxy-D-glucose, 2-; Phosphorylation; Transport; Labeling, ^{13}C; Glycolysis; (Cancer cell, Tumor)

1. INTRODUCTION

The synthetic glucose analog, 2-deoxy-D-glucose (2DG), competes with glucose for carrier-mediated transport into cells where it is phosphorylated by hexokinase to a stable end product, 2-deoxy-D-glucose 6-phosphate (2DG6P). Radioisotopes of 2DG and its derivative 2-fluoro-2-deoxy-D-glucose (FDG) have been used extensively for monitoring regional glucose utilization in vivo. Simultaneous measurement of rates of glucose consumption in various components of rat brain using [^{14}C]2DG and quantitative autoradiography was developed by Sokoloff and co-workers [1]. With the advent of positron emission tomography (PET), this concept was extended to studies examining cerebral function in patients by monitoring [^{18}F]FDG and [^{11}C]2DG [2,3]. Monitoring 2DG utilization by PET is currently being developed for clinical diagnosis of tumors [4] and has provided a means of tumor grading [5] and differentiating between radiation necrosis and recurrent tumors [6]. These techniques measure a differential accumulation of 2DG6P, simulating glucose utilization, but do not differentiate between transport and phosphorylation.

Here, we describe a method for simultaneously monitoring both processes by ^{13}C magnetic resonance spectroscopy (MRS). The metabolism of 2DG has previously been monitored in vivo by ^{31}P, ^{19}F, and ^{13}C MRS. The phosphorylation of 2DG was observed in a suspension of ascites tumor cells [7], and in situ in rat brain by ^{31}P MRS [8], although the 2DG6P signal was poorly resolved from the phosphomonoester signals. We encountered the same problem in monitoring the metabolism of 2DG in tumor tissue in vivo by ^{31}P MRS. ^{19}F MRS has been used to follow the brain metabolism of FDG beyond the hexokinase reaction [9]. The ^{19}F chemical shifts of FDG and FDG 6-phosphate are so close in solution [10] that they could not be resolved in vivo. In a preliminary report, the metabolism of [^{13}C]2DG was followed in rat brain [11]. We have demonstrated that both 2DG and 2DG6P can be monitored in tumor tissue subcutaneously implanted in mice and in a suspension of the same tumor cells derived from culture. The signal from 2DG enriched with ^{13}C in the C6 position (the site of phosphorylation) is clearly

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resolved from the phosphorylated product, 2DG6P at 100 MHz.

2. MATERIALS AND METHODS

2.1. *Materials*

2-Deoxy-D-[6-^{13}C]glucose was kindly furnished as a gift from the NIH-sponsored Stable Isotope Resource at Los Alamos National Laboratory and by Dr Joseph J.H. Ackerman, Washington University, St. Louis, MO. 2DG was purchased from Sigma (St. Louis, MO). The cell growth medium was improved minimal Eagle's medium (IMEM, NIH Media Unit) with 5% fetal calf serum and penicillin-streptomycin (100 U/ml, 10 mg/l) and modified to contain 4 mM D-glucose.

2.2. *Cultured cells*

Wild-type MCF-7 human breast cancer cells were grown in monolayers in the described growth media under a 5% CO_2 environment. Cells were harvested using 0.5% trypsin, 0.2% EDTA in Dirk's saline (Gibco), centrifuged at 4°C at 730 × *g* for 10 min and washed three times in the growth media. Cell suspensions for in vitro MRS were prepared as follows. Following centrifugation, the cell pellet (0.4 ml, approx. 8×10^7 cells) was transferred to a 10 ml NMR tube and diluted to 1.8 ml with cold growth media and maintained at 4°C. Cold D_2O (0.1 ml) and 1.65 mg [6-^{13}C]2DG in 0.1 ml growth media were added to make a final concentration of 5 mM 2DG in 2.0 ml suspension of cells. Oxygen was continuously bubbled in the bottom of the tube to oxygenate the cells and to prevent settling. During the 5 min tuning and shimming period, the suspension was allowed to warm to 22°C, which initiated the uptake of 2DG.

2.3. *Tumor-bearing mice*

Athymic nude mice which had been implanted subcutaneously with MCF-7 cells were obtained from Frederick Cancer Research Facility (Frederick, MD). Mice were sedated for MRS studies by i.p. injection of chloral hydrate in 0.9% saline at a dose of 430 mg/kg.

2.4. *Spectroscopy*

^{31}P spectra at 162 MHz and ^{13}C spectra at 100 MHz with proton decoupling were recorded on a Varian XL-400 NMR spectrometer. All spectra of solid tumors in vivo were collected using a custom-designed probe as in [12]. In vitro spectra of suspended cells were collected using a Varian 10-mm high-resolution probe at 22°C. The spectral parameters are listed in the figure legends.

3. RESULTS AND DISCUSSION

In vivo ^{31}P spectra of an MCF-7 tumor subcutaneously implanted in an athymic mouse are shown in fig.1. The mouse was fasted for 18 h prior to administration of 2 g/kg 2DG i.p. Except for the reduction in P_i, the uptake and phosphorylation of 2DG had little effect on the observed phosphates. 2DG6P resonates in the phosphomonoester (PME) region of the spectra at

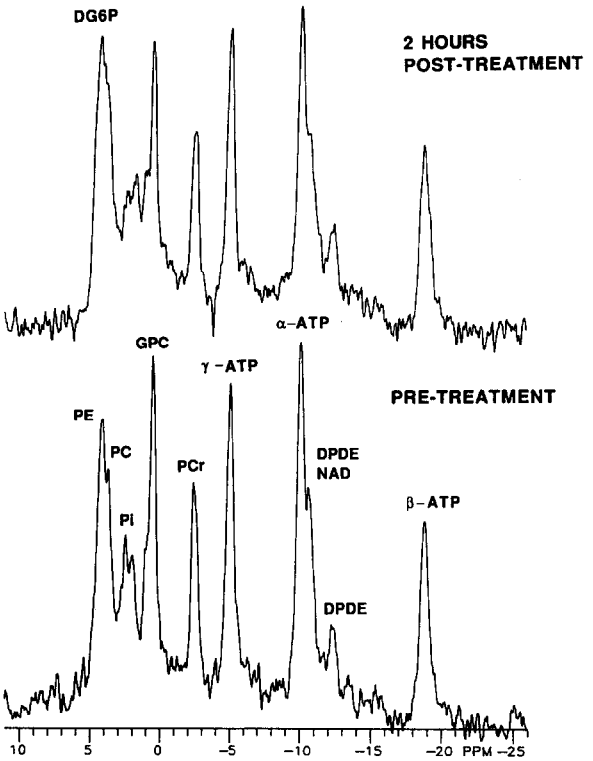


Fig.1. In vivo ^{31}P spectra of human breast tumor (MCF-7) subcutaneously implanted in a nude mouse (fasted for 18 h); pretreatment and 2 h following i.p. administration of 2 g/kg dose of 2DG. Peaks identified as: PE, phosphoethanolamine; PC, phosphocholine; Pi, inorganic phosphate; PCr, phosphocreatine; DPDE, diphosphodiester; DG6P, 2-deoxyglucose 6-phosphate. Each spectrum was collected for 60 scans at a 5 s repetition time, using 20 μs pulse width (70°) and 20 Hz line broadening.

4.6 ppm. As shown in an earlier study [8], 2DG6P was not resolved from the PME region containing phosphoethanolamine (PE) and phosphocholine (PC). During post-treatment the signal intensity of the PME region increased with a concurrent decline in P_i. This increasing peak was observed and assigned in extracts to 2DG6P.

In order to follow the uptake of 2DG and its phosphorylation ^{13}C MRS was used to monitor [6-^{13}C]2DG. Since phosphorylation occurs at position 6, the chemical shifts of the carbon in this position are affected the most. The ^{13}C chemical shifts of carbon 6 in 2DG are 63.82 ppm (α-anomer) and 63.88 ppm (β-anomer) vs 65.88 and 66.01 ppm for the α- and β-anomers at pH 7.1

following phosphorylation (note chemical shifts are pH-dependent).

The phosphorylation of ^{13}C-enriched 2DG was first demonstrated in vitro using a suspension of intact MCF-7 cells. ^{13}C spectra were collected every 4.3 min following the addition of 1.65 mg [6-^{13}C]2DG to 2.0 ml of cell suspension containing 8×10^7 cells. Since the course of the process was sufficiently shorter in duration, and ^{13}C material very scarce, it was not considered necessary to perfuse these cells [13]. As shown in fig.2, 2DG6P was present following the addition of 2DG and resonances of its α- and β-anomers were clearly resolved from those of 2DG. The time course for production of 2DG6P is shown in fig.3. The rate of decrease in 2DG is somewhat greater than the rate of increase in 2DG6P. A linear net loss in total ^{13}C label was observed. This could be attributed to loss of [^{13}C]2DG, since this signal would not fit well to an exponential decay, but ran parallel to the sum of the total signal at long times, and was fitted well with an exponential plus a linear decay with the same slope derived from the sum. By contrast, the signal for 2DG6P was fitted well by an exponential increase. The rates derived from the fits were corrected for the amount of protein in the cell preparation, and gave values of 8.3×10^{-4} and 6.5×10^{-4} mM/min per mg protein for 2DG and 2DG6P, respectively. The net loss of 2DG at long times could be attributed to 2DG6P being further metabolized [9,14], and/or gradual damage to cell integrity, which is shown by the loss of most of the ATP in the ^{31}P NMR spectra after 4 h.

A proton-decoupled natural-abundance ^{13}C spectrum of an MCF-7 tumor subcutaneously implanted in an athymic mouse is shown in fig.4a. The spectrum is characterized by a large envelope of methyl and methylene carbons (14–46 ppm) with a peak at 32 ppm, trimethylamine groups of

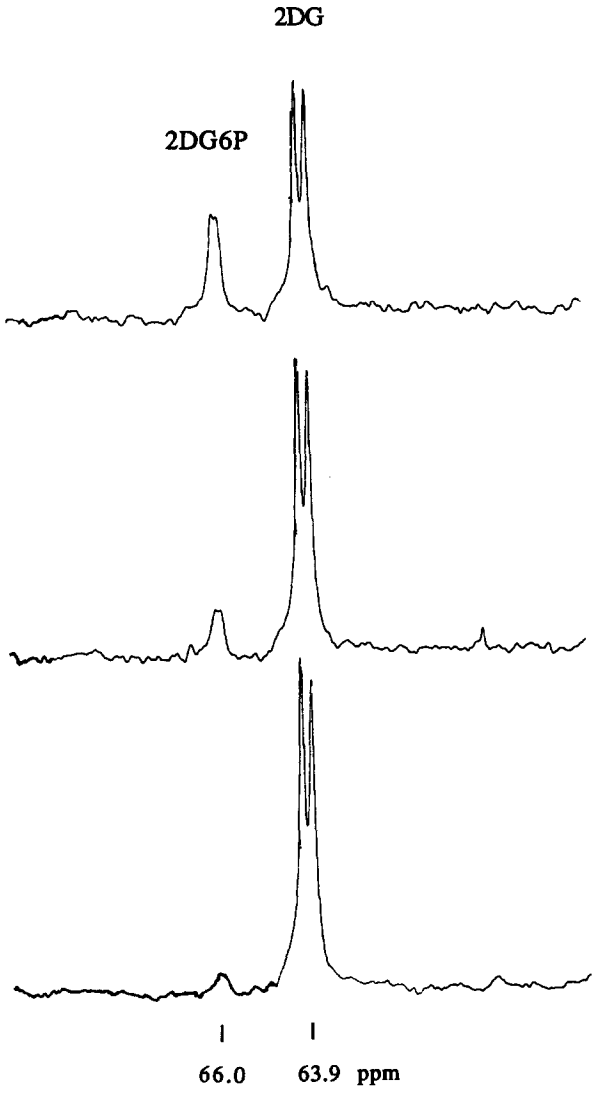


Fig.2. In vitro ^{1}H-decoupled ^{13}C spectra of a suspension of MCF-7 cells in a Varian 10 mm high-resolution probe at 22°C. Spectra are shown at various times following the addition of 1.65 mg [6-^{13}C]2DG to 8×10^7 cells in 2.0 ml media. Peaks are identified as α- and β-anomers of [6-^{13}C]2DG and 2DG6P. Each spectrum was collected for 200 scans at 1.4 s repetition time using 15 μs pulse width (66°) and 7 Hz line broadening.

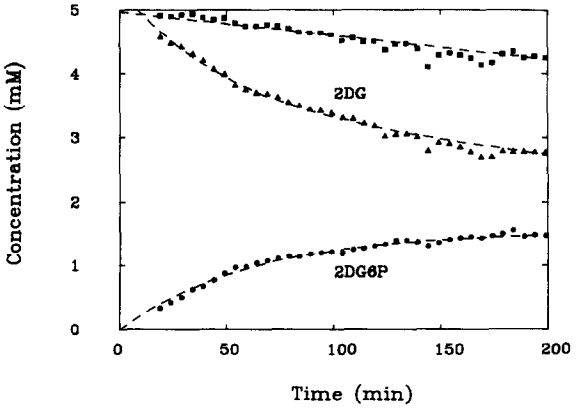
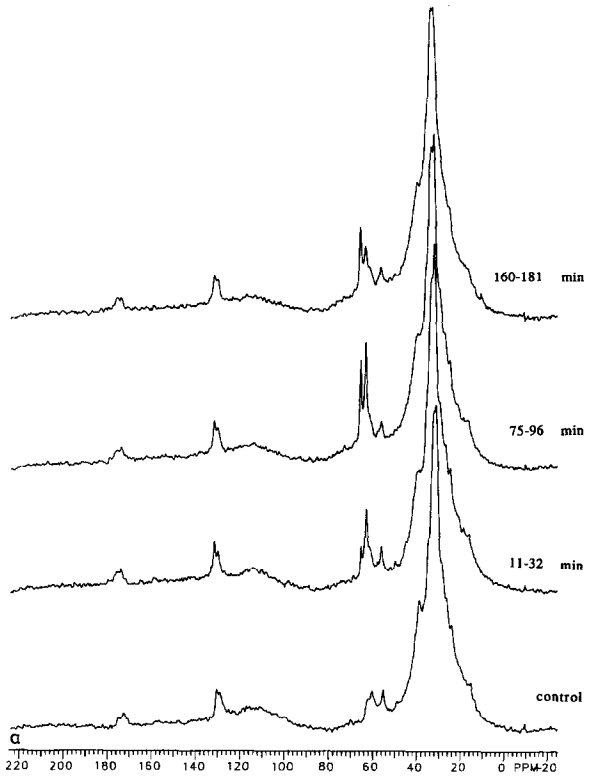


Fig.3. Time course of utilization of 2DG and production of 2DG6P by MCF-7 cells in vitro. Data from signal intensities of ^{13}C spectra described in fig.2.



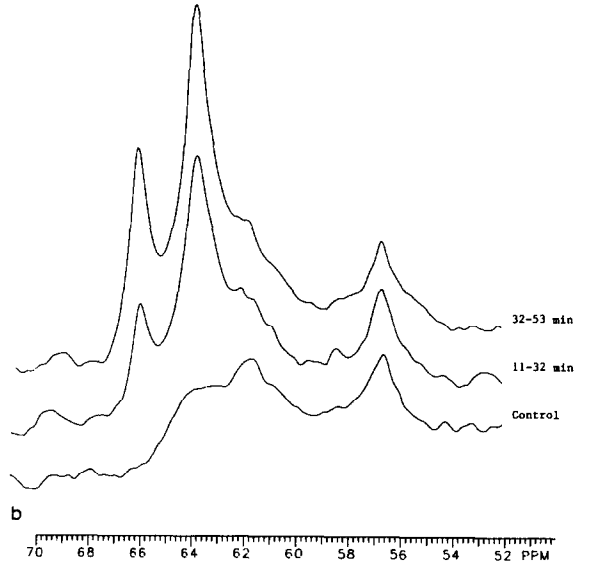


Fig.4. In vivo ^{1}H-decoupled ^{13}C spectra of MCF-7 cells subcutaneously implanted in a nude mouse (fasted for 18 h); pretreatment (control) and following i.p. administration of 1 g/kg dose of [6-^{13}C]2DG. Peaks identified in b as 2DG6P (66 ppm) and 2DG (64 ppm).

phosphatidylcholine at 54.8 ppm, olefinic carbons at 130 and 132 ppm, and carbonyl carbons at 173 ppm [12]. The fasted mouse (18 h) was injected i.p. with a 1 g/kg dose of [6-^{13}C]2DG. Both 2DG and 2DG6P appeared in the first spectrum (11–32 min). These peaks are clearly resolved from each other and the background (natural-abundance spectrum) as shown in fig.4b. The time course for the appearance of 2DG and 2DG6P in the tumor in vivo is shown in fig.5. This represents a composite of several processes including uptake and elimination of 2DG, phosphorylation of 2DG, and elimination of 2DG6P. The maximum signal intensity for 2DG occurs at 60 min and for 2DG6P at 110 min.

It has been shown that the multidrug-resistant (mdr) line of MCF-7 tumor cells, known as AdrR, has a 3-fold higher rate of glycolysis vs wild-type cells [15]. It has been suggested that 2DG may be used as a metabolic inhibitor of tumor growth [16–18]. In order to evaluate the effectiveness of a combination of 2DG and adriamycin as therapeutic agent against the mdr cell line, it is necessary to be able to compare the rates of uptake and phosphorylation, and we are currently performing these studies. The present results demonstrate the efficacy of using ^{13}C-labelled

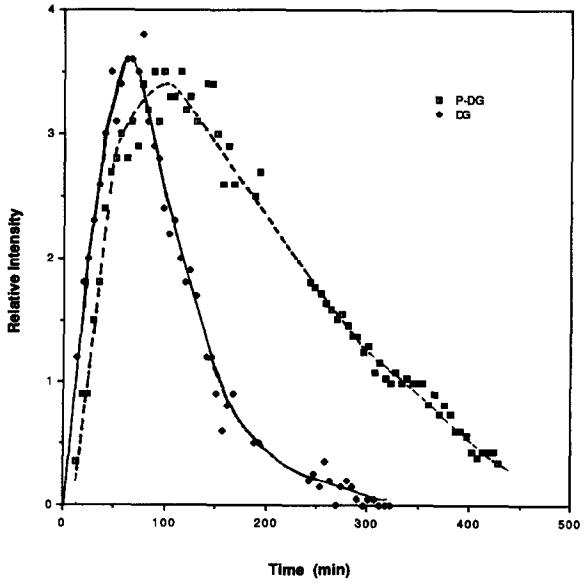


Fig.5. Time course of utilization of 2DG and production of 2DG6P by an MCF-7 tumor in vivo. Data from signal intensities of ^{13}C spectra described in fig.4.

sugar analogs, particularly 2DG that is labelled at the position of phosphorylation, in order to follow a metabolic process in vitro and in vivo.

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REFERENCES

[1] Sokoloff, L., Reivich, M., Kennedy, C., Des Rosiers, M.H., Patlak, C.S., Pettigrew, K.D., Sakurada, O. and Shinohara, M. (1977) J. Neurochem. 28, 897–916.
[2] Reivich, M., Kuhl, D., Wolf, A., Greenberg, J., Phelps, M., Ido, T., Casella, V., Fowler, J., Hoffman, E., Alavi, A., Som, P. and Sokoloff, L. (1979) Circ. Res. 44, 127–137.
[3] Phelps, M.E., Mazziotta, J.C. and Huang, S.C. (1982) J. Cereb. Blood Flow Metab. 2, 113–162.
[4] Beaney, R.P. (1984) Seminars Nucl. Med. 14, 324–341.
[5] Di Chiro, G., DeLaPaz, R.L., Brooks, R.A., Sokoloff, L., Kornblith, P.L., Smith, B.H., Patronas, N.J., Kufta, C.V., Kessler, R.M., Johnston, G.S., Manning, R.G. and Wolf, A.P. (1982) Neurology 32, 1323–1329.
[6] Patronas, N.J., Di Chiro, G., Brooks, R.A., DeLaPaz, R.L., Kornblith, P.L., Smith, B.H., Rizzoli, H.V., Kessler, R.M., Manning, R.G., Channing, M., Wolf, A.P. and O'Connor, C.M. (1982) Radiology 144, 885–889.
[7] Navon, G., Ogawa, S., Shulman, R.G. and Yamane, T. (1977) Proc. Natl. Acad. Sci. USA 74, 87–91.
[8] Deuel, R.K., Yue, G.M., Sherman, W.R., Schicker, D.J. and Ackerman, J.J.H. (1985) Science 228, 1329–1331.
[9] Nakada, T., Kwee, I.L. and Conboy, C.C. (1986) J. Neurochem. 46, 198–201.
[10] Nakada, T., Kwee, I.L., Rao, G.A. and Conboy, C.C. (1985) Biochem. Arch. 1, 163–166.
[11] Kotyk, J.J., Rust, R.S., Deuel, R.K. and Ackerman, J.J.H. (1987) Proc. Soc. Magn. Reson. Med. Sixth Annu. Meet., p.445.
[12] Lyon, R.C., Tschudin, R.G., Daly, P.F. and Cohen, J.S. (1988) Magn. Reson. Med. 6, 1–14.
[13] Lyon, R.C., Faustino, P.J. and Cohen, J.S. (1986) Magn. Reson. Med. 3, 636–672.
[14] Renner, E.D., Plageman, P.G.W. and Bernlohr, R.W. (1972) J. Biol. Chem. 247, 5765–5776.
[15] Lyon, R.C., Cohen, J.S., Faustino, P.J., Megnin, F. and Myers, C.E. (1988) Cancer Res. 48, 870–877.
[16] Woodward, G.E. and Hudson, M.T. (1954) Cancer Res. 14, 599–605.
[17] Jain, V.K., Kalia, U.K., Sharma, R., Maharajan, V. and Menon, M. (1985) Int. J. Radiat. Oncol. Biol. Phys. 11, 943–950.
[18] Halanka, M. (1974) Biochim. Biophys. Acta 355, 77–104.