[CANCER RESEARCH 46, 4087–4090, August 1986]

Differences in Phosphate Metabolite Levels in Drug-sensitive and -resistant Human Breast Cancer Cell Lines Determined by ^{31}P Magnetic Resonance Spectroscopy[1]

J. S. Cohen, R. C. Lyon, C. Chen, P. J. Faustino, G. Batist,[2] M. Shoemaker, E. Rubalcaba, and K. H. Cowan

Clinical Pharmacology Branch, National Cancer Institute, NIH, Bethesda, Maryland 20892

ABSTRACT

^{31}P magnetic resonance spectra of perfused human breast cancer cells with the phenotype of pleiotropic drug resistance exhibit striking differences in the levels of phosphate metabolites from the wild-type, drug-sensitive parent cell line. Resistant cells demonstrated elevated levels of phosphocreatine and depressed levels of phosphomonoesters, phosphodiesters, and diphosphodiesters. These differences may reflect significant alterations in the control of bioenergetic metabolism between drug-resistant and -sensitive cells.

INTRODUCTION

One of the major problems in cancer chemotherapy is the phenomenon of PDR.[3] This is manifested as a resistance to a variety of drugs with different structures and apparently different mechanisms of action, following treatment with only one of them (1). Several cell lines have been developed that exhibit stable PDR following selection against a particular drug, usually an anthracycline or *Vinca* alkaloid, and these cell lines have been studied for their differences from the WT drug-sensitive parent cell line. While changes in the protein components in cell membranes (2, 3) and cytosol (4, 5) have been identified in cells with PDR, the precise role of these changes in the resistant phenotype is unknown.

^{31}P MRS has been used to study the metabolism of mammalian cells. We have described a method in which mammalian cells are embedded in an agarose gel thread to facilitate continuous perfusion under controlled conditions that can be made to mimic *in vivo* conditions (6, 7). In order to investigate the possible metabolic basis for PDR, we have applied this technique using ^{31}P MRS to an established human breast cancer cell line (MCF-7) and a line selected *in vitro* for resistance to Adriamycin which exhibits PDR. We have chosen MCF-7 cells for these studies because this is a well-characterized cell line that retains many of the characteristics of breast tissue, and Adriamycin is the preferred drug in the treatment of breast cancer.

There has been a great deal of inconsistency in reports of the presence of phosphocreatine in ^{31}P MRS studies of various cancer cells (8, 9) and tumors (for a review, see Ref. 10). Although *in vivo* experiments are complicated due to possible signal detection from peripheral muscle tissue, in some cases the presence of appreciable amounts of phosphocreatine in tumor cells could not be discounted (11, 12). Several explanations for the variability of phosphocreatine in these types of studies have been advanced, including differing degrees of hypoxia, differential vascularization, and infiltration by normal cells (10). A further possible explanation lies in the heterogeneity of genotype manifested in most tumors (13).

In the current work we have found that there are clear distinctions between the ^{31}P magnetic resonance spectra observed for WT MCF-7 breast cancer cells and a drug-resistant cell line. The resistant cells were found to contain elevated steady-state levels of phosphocreatine and depressed levels of phosphomonoesters, primarily G6P and F6P, phosphodiesters such as GPE and GPC, and diphosphodiester, *e.g.*, UDPG. Cancer cells are generally known to exhibit elevated levels of aerobic glycolysis (14, 15). However, we believe this is the first time specific differences in energy metabolism have been related to the phenomenon of PDR. These noninvasive magnetic resonance studies may have significance for future *in vivo* and clinical MRS studies of tumors.

MATERIALS AND METHODS

Tumor Cells. MCF-7 human breast cancer cells were grown in monolayers in RPMI-1640 on improved modified Eagle's medium (NIH Medium Unit) supplemented with penicillin-streptomycin (100 units/ml, 10 mg/liter), and 5% fetal calf serum (Grand Island Biological Co., Grand Island NY) under a 5% CO_2 environment as described elsewhere.[4] Adr^R cells were obtained by serial passage of the parental wild-type cells in stepwise increasing concentrations of Adriamycin until cells capable of growing in 10 μM were obtained. These cells are 192-fold more resistant to Adriamycin than the wild-type cells, and they exhibit high levels of cross-resistance to a number of other drugs, including vincristine, vinblastine, VP-16, and actinomycin D. Sensitivity was assayed by growth inhibition during continuous growth.[4] Prior to biochemical and MRS studies the cells were grown in drug-free medium for at least 6 wk. The resistant phenotype in Adr^R cells is stable when serially passed in drug-free medium for greater than 52 wk. For MRS studies, cells were harvested using 0.5% trypsin (Gibco)-0.02% EDTA in Dirk's saline, and they were washed in phosphate-buffered saline and resuspended as described below.

Cell Perfusion. Cells were prepared for perfusion by casting in an agarose gel thread matrix as previously described (7). Between 10^7 and 5×10^8 cells were used in 1–3 ml of final gel solution. The gel-cell threads were perfused in a screw cap Wilmad MRS tube (10 mm). The perfusion apparatus used was an improved version of that described previously (7), with a fixed internal volume (10–20 ml) that could be closed from the external reservoir by a series of valves. The perfusate was usually RPMI-1640 medium without serum but with 4-(2-hydroxyethyl l)-1-piperazineethanesulfonic acid buffer (20 mM, pH 7.5). The pH was maintained at 7.5 with a Radiometer pH-stat, which could be adjusted to any pH required. Oxygen (100%) was bubbled through the perfusate in the pH-stat cup, and the oxygen content was monitored continuously. Further details of this system will be described elsewhere (16).

^{31}P Magnetic Resonance Spectra. ^{31}P magnetic resonance spectra of perfused cells were recorded on a Varian XL-400 at 162 MHz at 22 or

Received 12/10/85; revised 4/14/86; accepted 4/16/86.

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[1] Part of this work was presented at the third meeting of the Society of Magnetic Resonance in Medicine, London, August 1985.

[2] Present address: Montreal General Hospital Research Institute, Room 960, 1650 Cedar Avenue, Montreal, Canada H3G-1A4.

[3] The abbreviations used are: PDR, pleiotropic drug resistance; MRS, magnetic resonance spectroscopy; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; UDPG, uridine 5′-diphosphoglucose; GPE, glycerophosphoethanolamine; GPC, glycerophosphocholine; WT, wild type; Adr^R, Adriamycin resistant; PL, phospholipid; SP, sugar phosphate.

[4] G. Batist, A. Tulpule, B. K. Sinha, A. Katki, E. Rubalcaba, C. E. Myers, and K. H. Cowan, manuscript submitted for publication.

37°C. Usually 4000 transients were accumulated with a 0.6-s repetition time and a 65°C (33 μs) pulse. ^{31}P chemical shifts are reported relative to α-ATP as −11.3 ppm. Quantitative spectra were collected every 40 s using a 90°C pulse (51 μs). Spectra of cell extracts were recorded at 5°C using the deuterium lock. Relative peak areas were obtained by integration. Peak assignments in the cell extracts were confirmed by the addition of known phosphates, and the observation that the signals of these compounds remained superimposed with the specific resonance peak with pH titration.

Extraction Procedure. Cell extracts were prepared immediately following cell harvest by treatment of cell pellets with 1.0 ml of cold D_2O and 0.5 ml of cold perchloric acid (30%). The mixture was sonicated on ice for 5 min at the beginning and end of a 30-min period. The pH was neutralized with KOH (10 N) and centrifuged to remove $KClO_4$ precipitate. EDTA and diphenyl phosphate were added to a final concentration of 10 mM and 1 mM, respectively. To reduce hydrolysis the extracts were kept cold, and spectra were collected immediately following preparation.

Creatine Kinase Assay. Creatine kinase was assayed according to the method of Bergmeyer (17) using cell homogenates. Cell were harvested as described above and were then sonicated for three 10-s periods.

RESULTS

Cells in Gel Threads. Wild-type cells in gel threads were suspended in medium, and loose cells were decanted off. This procedure was repeated daily, and after 6 days photomicrographs were taken of individual threads. As shown in Fig. 1 cells were growing out of the gel matrix, indicating that cell division was occurring on the surface of the thread, if not within it. As shown previously (7) the gel threads can be seen to contain a high density of apparently unaffected cells. The high density of cells in the packed gel threads can be considered to approximate the environment of a tumor, although in this case the cells have been grown in culture and selected for a particular phenotype.

^{31}P Magnetic Resonance Spectra of Intact Cells. ^{31}P magnetic resonance spectra of perfused cells were recorded numerous times for sensitive and resistant cell lines over a period of 1 yr with qualitatively the same pattern of cellular components. In order to quantitate the relative differences in the levels of phosphate metabolites in the WT and resistant cells, up to 5×10^8 cells were used, and spectra were accumulated with a long repetition rate (40 s), in order to ensure complete spin relaxation (T_1 for P_i is 8–10 s; those for the intracellular ATP signals are approximately 300–500 ms). Spectra were collected from four different harvests of WT and Adr^R cells each (Fig. 2). Nine distinct peaks were integrated from each spectrum, and the mean concentration of each component relative to that of ATP is given in Table 1. The Adr^R cells exhibited elevated levels of phosphocreatine (3-fold) and depressed levels of GPE (8-fold), GPC (13-fold), and UDPG (2-fold). A standard t test based on the means ± standard deviations in Table 1 indicated that the statistical significance of the results for GPE and UDPG was high ($P < 0.01$), while for GPC and phosphocreatine the significance was somewhat less ($P < 0.05$). However, the appropriateness of applying statistical analysis to such measurements is unclear. Because of the inherent low sensitivity of the ^{31}P MRS method, the standard deviation calculated for such measurements will always be large. Further, the extensive time required to accumulate these spectra is such that few statistical analyses of ^{31}P MRS measurements can be found in the litera-

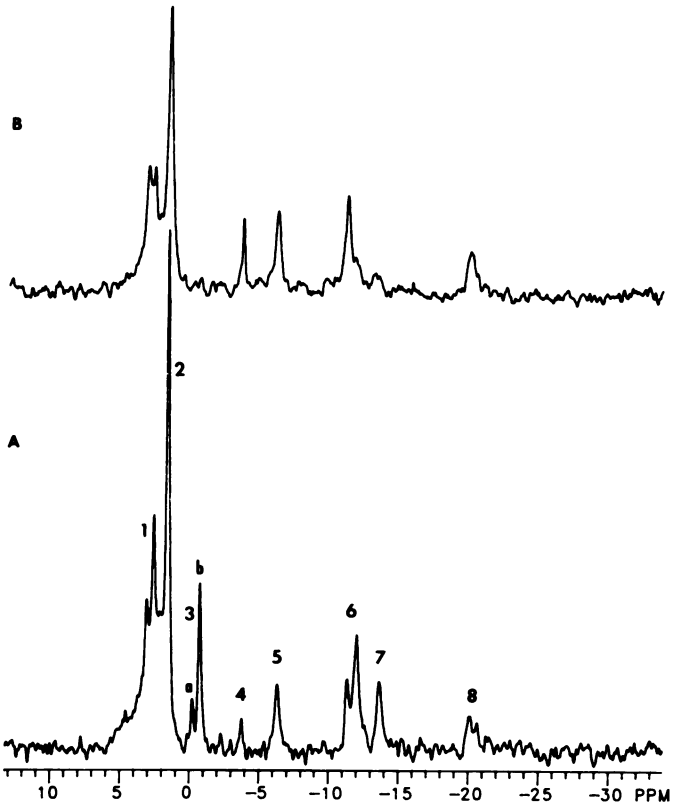

Fig. 2. Quantitative ^{31}P magnetic resonance spectra at 162 MHz of perfused MCF-7 cells: WT cells (*A*); Adr^R cells (*B*). Two hundred scans were accumulated with a 40-s repetition time at 22°C. Perfusion was with RPMI-1640 medium with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (20 mM) at pH 7.5. Peak assignments are: *1*, SP; *2*, P_i; *3a*, GPE; *3b*, GPC; *4*, phosphocreatine; *5*, γ-ATP; *6*, α-ATP, NAD, and UDPG; *7*, UDPG; and *8*, β-ATP.

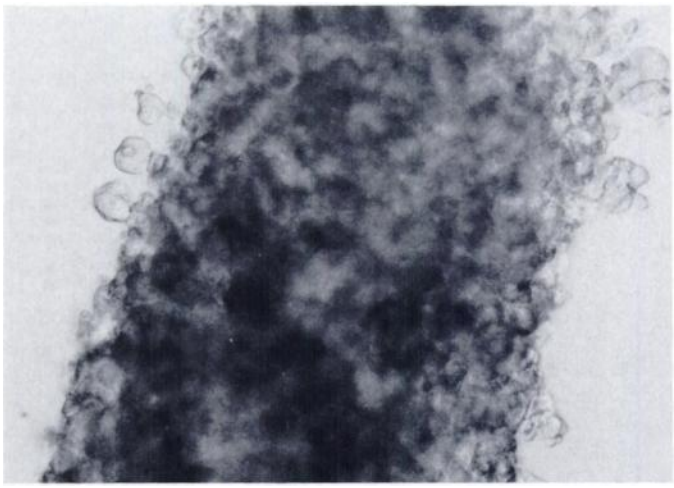

Fig. 1. Photomicrograph of surface of agarose gel thread (0.5-mm diameter) with embedded MCF-7 breast cancer cells after 6 days, following daily serial transfer to fresh RPMI-1640 medium.

Table 1 *Concentration of phosphate metabolites in intact cells (relative to β-ATP)*

Compound	Peak no.	Wild type	Adr^R	WT/Adr^R
SP region	1	5.4 ± 2.0[a]	2.8 ± 0.9	1.9
GPE	3a	0.6 ± 0.1	0.08 ± 0.08	8.0[c]
GPC	3b	1.3 ± 0.5	0.1 ± 0.6	13[d]
Phosphocreatine	4	0.2 ± 0.1	0.6 ± 0.2	0.3[d]
γ-ATP[b]	5	1.2 ± 0.2	1.3 ± 0.09	0.9
α-ATP, UDPG, NAD[b]	6	3.0 ± 0.5	2.3 ± 0.4	1.3
UDPG	7	1.2 ± 0.2	0.5 ± 0.06	2.4[c]
β-ATP	8	1.0	1.0	1.0

[a] Mean ± SD of four experiments. P_i is not reported since it is not intracellular.
[b] These peaks could also contain small contributions from ADP and other nucleoside triphosphates.
[c] $P < 0.01$, statistical significance t-test.
[d] $P < 0.05$.

ture. The significance of the differences observed is far better indicated by comparison with other results, for example, from extracts of cells (see below). Although our measurements are quantitative, the conclusions based upon them must be regarded as qualitative.

Except for the pH-induced chemical shift change of the external P_i, changes in external pH (7.5–5.5) and temperature (22°–37°C) had little effect on the ^{31}P spectra of the WT cells (Fig. 3). From the position of the peak presumed to be intracellular P_i the intracellular pH was approximately 7.4. Samples of cells cast in gel threads and stored overnight at 4°C retained their phosphocreatine peak on reperfusion.

^{31}P MRS of Cell Extracts. To identify and measure the absolute levels of phosphate metabolites, cell extracts were prepared in the cold from three different harvests of wild-type and Adr^R cells (Fig. 4). The acid-soluble phosphate concentrations in solution were quantitated from ^{31}P magnetic resonance spectra using 1 mM diphenylphosphate as a standard. This resonates in a region of the spectrum (−8.9 ppm) between the γ-ATP and α-ATP resonances at pH 7. As a result of the extraction procedure enhanced spectral resolution could be obtained, permitting improved quantitation of the components, particularly G6P and F6P. Extraction also released components previously immobilized in the intact cells, permitting observation of a PL peak. The concentrations (in μM) were normalized to 10^8 cells, averaged, and are given in Table 2. As with the perfused cells, phosphocreatine was elevated in the extracts of Adr^R cells compared to extracts of WT cells. The levels of creatine kinase activity measured (3 samples) were WT, 78.2 ± 13.4, and Adr^R, 92.6 ± 3.7 IU/mg of protein, which are not significantly different. The levels of UDPG (10-fold), GPE and GPC (5-fold), and the sugar phosphates (2-fold) were generally consistent in the cell extracts with the intact perfused cells.

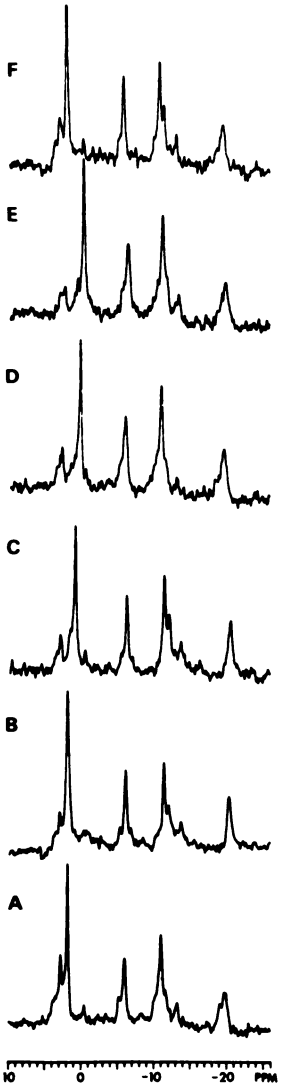

Fig. 3. Effect of pH and temperature on the ^{31}P magnetic resonance spectra at 162 MHz of perfused wild-type MCF-7 cells. Each spectrum is 1000 scans at 0.6-s repetition time. *A*, pH 7.5, 22°C; *B*, pH 7.5, 37°C; *C*, pH 6.5, 22°C; *D*, pH 6.0, 22°C; *E*, pH 5.5, 22°C; *F*, returned to pH 7.5, 22°C.

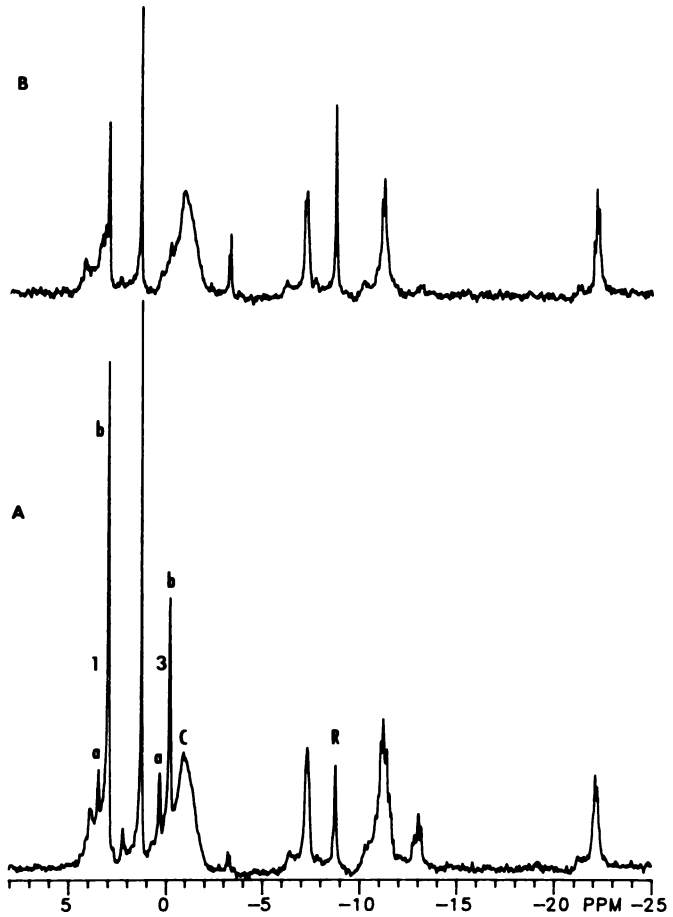

Fig. 4. Quantitative ^{31}P magnetic resonance spectra at 162 MHz of perchloric acid extracts of MCF-7 cells: WT cells (1022 scans) (*A*); Adr^R cells (1500 scans) (*B*), in D_2O at pH 7.0 with 40-s repetition time at 5°C. Peak assignments are the same as in Fig. 2, with the SP peak identified as G6P (*1a*) and F6P (*1b*), and the phosphodiester peaks assigned to GPE (*3a*) and GPC (*3b*). Additional signals arise from PL (Peak *C*), and the reference standard 1 mM diphenyl phosphate (Peak *R*).

Table 2 *Absolute concentration of phosphate metabolites in cellular extracts (μmol/10^8 cells)*

Compound	Peak no.	Wild type	Adr^R	WT/Adr^R
Unknown SP		1.5 ± 1.5[a]	0.7 ± 0.7	2.1
G6P	1a	3.0 ± 2.9	1.3 ± 1.2	2.3
F6P	1b	5.0 ± 2.4	2.9 ± 2.4	1.9
GPE	3a	1.0 ± 0.05	0.2 ± 0.1	5
GPC	3b	2.8 ± 1.1	0.6 ± 0.5	4.7
PL	c	5.9 ± 2.1	6.6 ± 1.9	0.9
Phosphocreatine	4	0.1 ± 0.09	0.4 ± 0.3	0.2
γ-ATP[b]	5	2.4 ± 0.2	2.5 ± 0.9	1.0
α-ATP, UDPG, NAD[b]	6	4.5 ± 1.1	3.3 ± 1.1	1.4
UDPG	7	1.0 ± 0.8	0.1 ± 0.1	10
β-ATP	8	1.7 ± 0.3	2.2 ± 0.9	0.8

[a] Mean ± SD of three experiments.
[b] See Table 1.

DISCUSSION

It has been known for many years that malignant cells have altered energy metabolism compared to normal cells. In particular, cancer cells exhibit a greater capacity for aerobic glycolysis than usually found in normal somatic cells (14). One explanation advanced to explain this property is the higher level of hexokinase found in certain kinds of malignant cells (15). Given this alteration in bioenergetic properties it should not perhaps surprise us to find differences in bioenergetic properties between different sublines of malignant cells. Nevertheless, it was something of a surprise to find consistent and reproducible differences in the levels of phosphocreatine, SP, GPE, GPC, and UDPG between drug-sensitive and -resistant breast cancer cells.

The observed differences in phosphocreatine, GPE, and GPC are obvious due to the clear resolution of their ^{31}P signals in unambiguous chemical shift positions (18). However, other potential differences between the ^{31}P spectra of the breast cancer cells *in vitro* could be masked by the lack of resolution between different species, such as phosphate monoesters, and the broadening of signals due to reduced mobility within the cell. In order to test for further differences it was necessary to obtain extracts of cells and to prepare these under the most mild conditions to prevent inadvertent hydrolysis of phosphate esters. It is easier to obtain well-resolved spectra of these extracts because they exhibit sharper signals and are more stable than the cells from which they derive, and hence they can be kept in the spectrometer for longer periods. While the total amounts of phosphomonoesters appeared to be approximately the same in both cell types during perfusion, the cell extracts revealed differences in specific sugar phosphates within that region of the spectrum. The major component, identified as fructose 6-phosphate, and the minor component, glucose 6-phosphate, were 2-fold higher in the WT compared to the Adr^R cells.

The finding of differences in levels of major phosphate-containing metabolites between drug-sensitive and -resistant breast cancer cells suggests differences in the control of important metabolic pathways. The differences in SP and UDPG levels indicate differences in glucose utilization, possibly related to the control of glycolysis. It has also been found that the regulation of the hexose monophosphate shunt by oxidation-reduction mediators is different between WT and Adr^R cells (19). The differences observed in GPC and GPE levels, which are degradation products of PL, may arise from differences in phospholipase activity.

Phosphocreatine is usually a subsidiary means of energy storage of cells, such as muscle cells, able to undergo anaerobic glycolysis. We have shown that the differences in phosphocreatine levels observed here do not arise from differences in creatine kinase levels between WT and Adr^R cells. The significance of the differences in metabolite concentrations we have described is unclear, and we do not know whether these differences are basic or secondary to the phenomenon of PDR. Nevertheless this study indicates what kind of information is potentially available from *in vitro* ^{31}P MRS studies of available cell lines. Further, there is the important possibility that the differences observed noninvasively in intracellular components in this study could be used in *in vivo* ^{31}P MRS studies of tumors as markers of such properties as drug resistance.

ACKNOWLEDGMENTS

We thank Charles E. Myers for helpful discussions.

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