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INCREASE IN THE ATP SIGNAL AFTER TREATMENT WITH CISPLATIN IN TWO DIFFERENT CELL LINES STUDIED BY ^{31}P NMR SPECTROSCOPY*

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SUMMARY. We have compared the ^{31}P nuclear magnetic resonance (NMR) spectra of two different cisplatin resistant cell lines, one derived from human ovarian carcinoma and the other from rat lymphoma, and their respective cisplatin sensitive parental cells lines. Comparisons were made between the baseline spectra and after perfusion of the cells with 20 - 50 μM cisplatin for 16 - 20 hours. While no obvious differences were found between baseline spectra of sensitive and resistant cells, during cisplatin perfusion the sensitive cells had an increase in their ATP signals. The resistant cells also exhibited increases in their ATP signals during cisplatin perfusion but to a lesser extent than the sensitive cells. Although the significance of these ATP elevations towards the cellular pharmacology of cisplatin are not presently known, our studies demonstrate that ^{31}P NMR spectroscopy may be useful for elucidating differences in phosphate metabolism in cells expressing the cisplatin resistant phenotype.

Acquired resistance to chemotherapeutic agents is one of the most important problems in cancer treatment (1,2). Studies of metabolic changes which occur when tumor cells become drug resistant may contribute to the understanding of the resistant phenotype, and help in designing means to overcome it.

^{31}P NMR spectroscopy constitutes a non-invasive technique that is very useful for monitoring high energy and phospholipid metabolism. We have previously described a procedure for continuously perfusing cells under controlled conditions (3,4). This technique has been used to demonstrate alterations in basal levels of high energy phosphate and phospholipids in resistant cells and shifts in these levels in response to drug exposure (5,6).

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ABBREVIATIONS: NMR, nuclear magnetic resonance; cisplatin, *cis*-diamminedichloroplatinum(II); WR and WS, cisplatin resistant and sensitive Walker rat lymphoma cell lines; 2008, human ovarian carcinoma wild type cell line; C13*, cisplatin resistant 2008 cell line; UDPG, uridine diphosphoglycoside; PDE, phosphodiester; NAD, nicotinamide adenine dinucleotide.

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Cisplatin [*cis*-diamminedichloroplatinum(II)] has activity against many solid tumors (7). The mechanism of action of cisplatin is not completely understood, though its cytotoxicity is believed to result from the adducts it forms with nuclear DNA (8). It has been shown that mitochondrial morphology and function in some cisplatin resistant cell lines are different than that observed in the sensitive line (9). Recent studies also indicate that cisplatin and its analogues produce alterations in the structure and function of isolated mitochondria (10). In other cells, the post-treatment concentration of platinum in mitochondria was higher than in the other subcellular compartments (11). In the present study we have compared the ^{31}P NMR spectra of cisplatin-sensitive and -resistant 2008 human ovarian cancer cells and Walker 256 rat lymphoma cells before and after cisplatin perfusion. We have found that perfusion with 20-50 μM cisplatin caused an initial increase in the ATP signal of both the sensitive and resistant cells, but the effect on the resistant cells was muted.

MATERIALS AND METHODS

Cell Lines. The Walker 256 rat lymphoma cells have only recently been shown to be a lymphoma (12). The cell line was maintained in DMEM with glucose (H21), 10% fetal bovine serum and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively). The cells grew in suspension and were subcultured once a week at a ratio of 1:20. The resistant cells were initially obtained by chlorambucil treatment. These cell were 25-40 fold resistant to cisplatin as determined by the ratio of IC_{50} values.

The 2008 cell line had been established from a patient with serous cystadenocarcinoma of the ovary. The cisplatin-resistant 2008 cells were generated as previously described by monthly selection with cisplatin followed by chronic exposure to cisplatin and were designated C13* (13, 14). These C13* cells were 15-fold resistant as defined by the ratio of IC_{50} values determined by continuous exposure clonogenic assay. Cells were grown as monolayers in RPMI 1640 medium (Mediatech, Washington, DC) containing 5% heat-inactivated bovine calf serum supplemented with iron (Hyclone, Ogden, UT) and 50 μg/ml gentamicin sulfate (Gemini Bioproducts, Inc., Calabasas, CA). Cultures were equilibrated with humidified 5% CO_2 in air at 37 °C. Both 2008 cell types were routinely tested at 6-week intervals with a mycoplasma detection kit (Gen-Probe, San Diego, CA) and were mycoplasma negative throughout these studies.

Cell preparation for ^{31}P NMR Studies. Sub-confluent 2008 cells were harvested from sixteen 150 mm tissue culture plates with 0.05% (w/v) trypsin-0.53 mM EDTA, centrifuged at 1000 x g for 5 min. and washed twice in growth medium. Walker rat lymphoma cells were harvested by centrifugation and washed as above. Approximately 1 ml packed cells (2-3 x 10^8 cells) were mixed with an equal amount of 1.8% (w/v) low gelling temperature agarose (Seaplaque agarose, FMC Bioproducts, Rockland, ME) in 10 mM HEPES buffer in 0.9% (w/v) saline at pH 7.4 and cast into agarose threads as described previously by extruding the mixture through chilled 0.5 mm internal diameter tubing into a screw cap Wilmad 10 mm NMR tube (4). The solid threads were concentrated without compression (no more than 2 ml of the mixture was used) at the bottom of the NMR tube by using an insert with inlet and outlet tubing. The cell thread mixture was continuously perfused with the appropriate medium. The perfusion medium employed with the Walker rat lymphoma cells was DMEM as described above. The 2008 and C13* cells were perfused with RPMI 1640 also described above. The medium was gently bubbled with 5% CO_2 / 95% compressed air (v/v) to ensure the availability of oxygen to cells as well as to maintain the pH of the media at 7.4. A peristaltic pump (Pharmacia P-3) maintained a constant perfusion rate of 1 ml/minute. The NMR tube containing the cells and perfusion apparatus was placed in a standard 10 mm NMR probe which was maintained at a constant temperature of 37 °C. Heating the NMR probe was enough to warm the media immediately surrounding the cells, so no external heat source was used (5).

After the acquisition of 2 baseline spectra (1 hour each) the cisplatin (National Cancer Institute, Bethesda, MD, or Bristol-Myers Co., Syracuse, NY) was added at the indicated concentration to the perfusion medium reservoir.

Magnetic Resonance Spectroscopy and Data Analysis. ^{31}P NMR spectra were recorded on a Varian XL-400 NMR spectrometer operating at 162 MHz. A 3 second repetition time and a 60 degree flip angle were used (5). In this study we compared the same phosphorus metabolites from different cell lines, therefore it was not necessary or practical to obtain quantitative results by allowing complete relaxation. Twelve hundred scans were accumulated for each spectrum totaling one hour. The cells were continously perfused with the growth medium during the experiment. ^{31}P chemical shifts were determined by standardizing ß-ATP to -18.7 ppm. ^{31}P

spectra were analyzed on a Varian 4000 ADS data station. Peaks heights were measured. The line width of the resonances did not change by more than 3% over the time course of our experiments. We have observed that peak measurements were reproducible with less than 5% variation (6). NMR data acquisition and processing were performed with identical parameters throughout all experiments. Since cell counts are not always precise and there can be substantial loss of cells during thread casting and placing of the perfusion insert, a protein assay was used to standardize the actual amount of cells used in each experiment and to make quantitative comparisons (15). The protein was determined by the bicinchoninic acid assay (Pierce, Rockfort, IL), based on the reaction of the peptide bonds of the proteins with Cu^{2+} and quantification of protein at 562 nm employing BSA as a standard. The agarose in the threads does not interfere with the assay (16).

Statistical Analysis. The mean and variance data from different cell lines were compared for significance with the Fisher-Behrens test and F-test respectively. All values reported represent the mean ± the S.D. of replicate experiments. The analysis was performed with the Statview program for Apple-Macintosh.

RESULTS

The baseline spectra of the Walker rat lymphoma sensitive (WS) and Walker rat lymphoma cisplatin resistant (WR) were similar, showing low levels of UDPG and PDE s (Figs. 1, 3). The baseline spectrum of the sensitive 2008 human ovarian carcinoma cells, was also similar to its cisplatin-resistant C13* sub-line in all resonances except UDPG which was increased in the C13* cells (Figs. 2, 4). The baseline spectra of the 2008 and C13* cells also resembled the WR and WS cells.

The 2008 and C13* cells were perfused with 50 μM cisplatin and the WR and WS cells were perfused with 20 μM cisplatin. All ATP signals for all of the cell lines started to increase within 4 hours after the addition of cisplatin and this increase in ATP continued for several hours (Fig. 5, only indicated for ß-ATP). However, in both the cisplatin sensitive cells the increase in the ATP signals was greater than in their resistant counterparts. The WS cells ultimately had a 50±16 % increase in ß -ATP (Fig. 3) over the baseline spectrum. The 2008 cells had a 30±10 % increase in ß -ATP (Fig. 4) as compared with the baseline spectrum. The ß -ATP

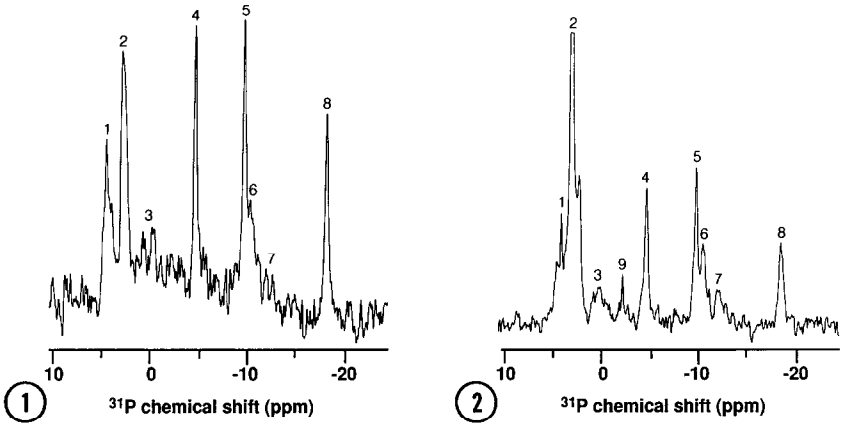

Figure 1. The baseline ^{31}P NMR spectrum for the cisplatin-resistant Walker rat lymphoma cells. The peak assignments are: [1] phosphomonoesters; [2] inorganic phosphate; [3] phosphodiesters; [4] γ-ATP; [5] α -ATP; [6] NAD(P) and UDPG; [7] UDPG; and [8] ß -ATP.

Figure 2. The baseline ^{31}P NMR spectrum of the cisplatin-resistant C13* human ovarian carcinoma cells. The peak assignments are as in Fig. 1, and [9] phosphocreatine.

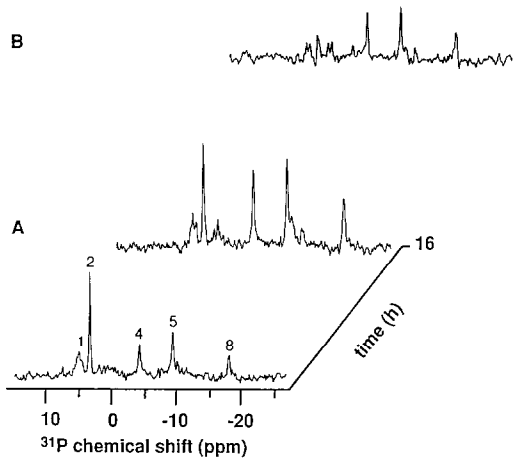

Figure 3. (A) Baseline ^{31}P spectrum of the cisplatin-sensitive Walker rat lymphoma (WS) cells and spectrum after 16 hours of perfusion with 20 µM cisplatin. (B) Difference spectrum demonstrating the changes in the ATP levels of the cells during this time. The peak assignments are as in Figs. 1 and 2.

increase over baseline levels for the WR cells was 21±6 % and for the C13* cells was 16±7 % (Table 1). The differences in the increase of the ATP signal (calculated only for ß-ATP) between the sensitive and resistant cells of each type were significant for both kinds: $p< 0.01$ for WR and WS, and $p<0.05$ for the 2008 and C13* (Table 1).

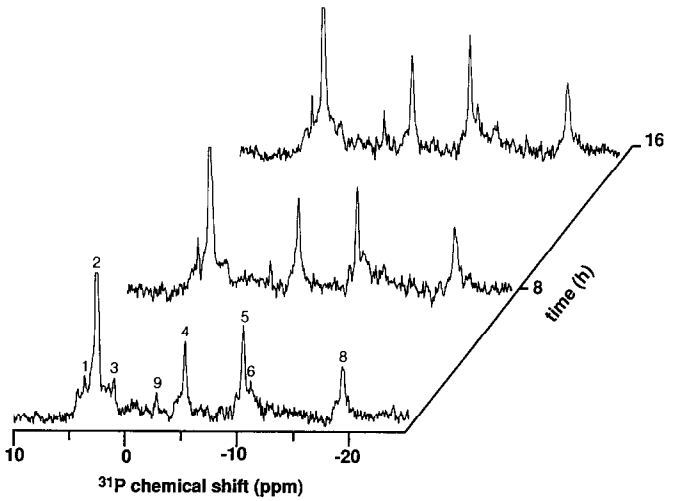

Figure 4. ^{31}P spectra of the cisplatin-sensitive 2008 cells starting with a baseline spectrum and after 8 and 16 hours of perfusion with 50 µM cisplatin. The peak assignments are as in Figs. 1 and 2.

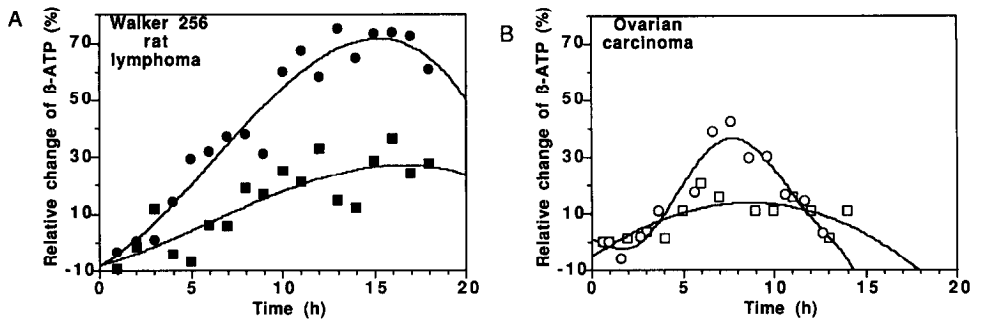

Figure 5. Relative changes of ß-ATP signal after cisplatin perfusion for (A) WS [●] and WR [■], (B) 2008 [○] and C13* [□] cell lines. The perfusion with cisplatin was started after two hours of baseline acquisition. Results are plotted from a single experiment.

There were no significant changes after cisplatin perfusion in any of the other metabolites observed in the ^{31}P spectrum. In the 2008 cells, after 18 hours of cisplatin perfusion all signals were depleted, although the C13* cells were still viable. The ATP signals during cisplatin perfusion in the WS cells continued to increase for a longer time period (maximum 12-16 hours) than the 2008 cells and were not completely depleted by the end of the experiment (18 hrs). Other than the rise in ATP levels, the WR cells did not change significantly from their baseline value during the cisplatin perfusion.

There were no significant changes in the ATP signals in the control experiments with no drug added to the perfusate of either the WR or WS cells or the 2008 or C13* cells.

Table 1. Final percent change in ß -ATP signal after 16 hours of perfusion with cisplatin-containing medium relative to cells perfused with medium alone

Cell line	Percent increase	Standard Deviation	cisplatin concentration	P values[a]
Walker rat lymphoma sensitive (WS)	50	16	20 µM	< 0.01
Walker rat lymphoma-resistant (WR)	21	6	20 µM	
Ovarian carcinoma-sensitive (2008)	30	10	50 µM	< 0.05
Ovarian carcinoma-resistant (C13*)	16	7	50 µM	

[a] The P values show a significant difference between the sensitive and resistant cells from each cell line within the confidence levels indicated. The values were calculated from 2-3 experiments with 6-9 measurements each, starting after the ATP reaches a plateau at 6-8 hours of perfusion with cisplatin and continuing for 12-16 hours. Control values were not significantly different from baseline ($p < 0.1$).

DISCUSSION

Prior data has demonstrated that multi-drug resistant cancer cells invariably give ^{31}P NMR spectra significantly different from those of drug-sensitive lines, pointing to major differences in metabolic control (17). However, the data presented here showed no substantial baseline spectral differences between both cisplatin resistant and sensitive cell lines. Multi-drug resistance is a different phenomenon than cisplatin resistance. Our results agree with those recently found in vivo with three hypopharynx carcinoma cell lines with increasing cisplatin resistance, where there were no substantial spectral differences between the resistant and sensitive cells (18).

Several in vivo ^{31}P NMR studies have described an initial increase in the ratios of high energy phosphates to other metabolites when the tumors were treated with chemotherapeutic agents (19-21). Both activation and decline can be produced in tumors treated with chemotherapeutics. We have studied only tumor cells in an isolated perfusion system in an attempt to determine the basis of this response.

The increase in bioenergetic status can be associated with the effect of trypsinization (22). We have addressed this concern by using two different cell types in our study. We have observed spectra from both attached cells that require trypsinization and from suspension cells which are not trypsinized. We obtained similar results from both cell lines, with the effect being greater in the latter. We did not see any initial increase in the baseline signals of either type of cell when perfused without drugs. Nor did we see any outstanding difference in the baseline spectra of either cell type, demonstrating that the extrinsic glycoproteins which are removed during trypsinization do not contribute significantly to the NMR spectra.

Some cisplatin resistant cells have altered mitochondrial morphology and function (9). Cisplatin and its derivatives have also been shown to cause altered mitochondrial function and structure in isolated mitochondria (10). Preliminary studies show that the cisplatin resistant cells (C13*) do not recover from treatment with 2-deoxy-glucose after reperfusion with glucose containing medium as the sensitive 2008 cells do (data not shown). This, along with the previous information may indicate that mitochondria of the cisplatin resistant cells do not have the same high energy metabolic function as the sensitive cells. The increase in the NMR ATP signal may be due to the mitochondria expelling previously 'NMR invisible' pools of ATP in response to cisplatin and/or may also be due to many other factors including inhibition of energy-utilizing functions or an increase in glycolysis or oxidative phosphorylation. Our results suggest that the study of high energy phosphate metabolism may provide important clues to the mechanisms of cisplatin resistance.

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