Loss of High-Energy Phosphate following Hyperthermia Demonstrated by 
in Vivo \(^{31}\)P-Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT

We have used in vivo \(^{31}\)P-nuclear magnetic resonance spectroscopy to study the changes in high-energy phosphates following hyperthermia. Immediately after heating, there is a fall in adenine triphosphate and apparent intracellular pH and an increase in inorganic phosphate. Following sublethal heating (40\(^\circ\) for 15 min), these changes were partial, and they resolved over the subsequent 45 hr. With tumors given severe hyperthermia (47\(^\circ\) for 15 min), there was complete disappearance of adenine triphosphate, with no recovery by 24 hr posttreatment. Qualitatively similar effects were seen after heating of normal leg muscle. The degree of fall of the adenine triphosphate/inorganic phosphate concentration ratio was directly proportional to the heat dose and to thermal cell kill. \(^{31}\)P-Nuclear magnetic resonance spectroscopy may be useful in thermal dosimetry and treatment evaluation following hyperthermia.

INTRODUCTION

Hyperthermia is a potential antitumor modality which is currently the subject of extensive investigation. Following exposure to heat, virtually every phase of metabolism becomes disrupted. No clear consensus exists, however, as to the primary metabolic events associated with thermal cell kill. Studies have demonstrated that respiration, anaerobic glycolysis, and DNA, RNA, and protein synthesis are inhibited at temperatures above 42\(^\circ\). These studies have been summarized in recent reviews (5, 11). The majority of the available data are derived from in vitro systems. Cellular metabolism depends greatly on environmental factors. Because of the variable metabolic states of cells within a solid tumor, as well as modifying host factors, it is sometimes difficult to extrapolate from in vitro data to the in vivo situation. Thus, the ability to study the metabolism of tumors in situ could help clarify which events are most important in thermal cell kill.

NMR\(^3\) is a noninvasive spectroscopic technique that can be used to study the metabolism of tissues both in vitro and in vivo. Isotopes that have been detected in living cells include \(^1\)H (6), \(^13\)C (27), \(^13\)N (21), \(^19\)F (13), \(^23\)Na (10), and \(^31\)P (8). \(^31\)P is the most extensively studied isotope in biological samples. It offers the advantages of moderately high sensitivity (detectable at concentrations \(\pm 1\) mm), 100\% natural abundance, relative spectral simplicity, and presence in a number of detectable key respiratory intermediates. The apparent intracellular pH can also be determined from the chemical shift of the P, peak in the \(^{31}\)P-NMR spectra (24).

The development of surface coils (1) and field focusing techniques (18) has led to the noninvasive measurement of NMR spectra of specific tissue and organs. These methods have been used to monitor the in vivo \(^{31}\)P-NMR spectra of muscle and brain (1), kidney (2), and liver (18) in experimental animals, as well as muscle in humans (26).

We have recently used a surface coil probe to obtain in vivo \(^{31}\)P-NMR spectra from s.c. murine tumors and human tumors in nude mice and to monitor the response of some of these tumors to radiation and chemotherapy (15, 23). In this paper, we report our use of \(^{31}\)P-NMR spectroscopy to record the changes in high-energy phosphates following hyperthermic treatment of a murine osteosarcoma and murine leg muscle and relate these spectral changes to heat dose and to an estimate of cell kill.

MATERIALS AND METHODS

Murine Tumor System. Female C3H mice (8 to 12 weeks old) were given s.c. inoculations of s.c. 1 to 3 \(\times 10^6\) trypan blue-negative Dunn osteosarcoma cells. The single-cell suspension was prepared from intact tumors by a Pronase digestion technique (17). Tumor damage from hyperthermia was quantitated by measuring the change in serum alkaline phosphatase, a tumor-associated marker, by a colorimetric procedure (17). This marker has previously been related quantitatively to the total number of viable tumor cells in the mouse (20). We have seen, after hyperthermia, a reproducible fall in serum alkaline phosphatase that is proportional to the heat dose and that parallels gross tumor regression (data not shown). Alkaline phosphatase measurements were made on Days -1 and 6 after treatment. Mice were anesthetized with Nembutal for all treatments and spectroscopic measurements. The skin overlying the tumor was shaved before spectroscopy.

Hyperthermia Treatment. Three weeks after inoculation, the tumors measured 1.5 to 2.0 cm in diameter and contained 1 to 5 \(\times 10^8\) cells. Hyperthermia was then administered via a capacitive radiofrequency device of our own design. A signal generator (Model 3002; Wavetek, Inc., San Diego, Calif.) produced a 1.5-MHz signal, which was then fed to an amplifier (Model 310L; ENI, Inc., Rochester, N.Y.). The power was transmitted to the mouse via a coaxial cable, which terminated in 2 brass electrodes. These electrodes bracketed the tumor and were coupled to it by standard electrode jelly. Temperature measurements were made with an electronic thermometer (BAT-8; Bailey Instruments, Saddle Brook, N.J.) and Teflon-sheathed copper-constantan thermocouples (Bailey IT-21). The thermocouples were inserted along a 25-gauge needle track along the longest axis of the tumor. A small amount of radiofrequency power (0.5 watt) was applied, and the thermocouple was moved back and forth until it was at the point of maximum temperature. Power was then increased until the thermocouple registered the target temperature. Treatment duration was timed from the moment target temperature was reached. Power was adjusted manually to keep the tumor within \(\pm 0.1\)\(^\circ\) of target temperature. Similar procedures were used for heating of normal murine thigh muscle.

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2 To whom requests for reprints should be addressed, at Comprehensive Cancer Center, University of Alabama in Birmingham, Birmingham, Ala. 35294.
3 The abbreviations used are: NMR, nuclear magnetic resonance; PME, phosphomonoesters; PCr, phosphocreatinine; PCA, perchloric acid.

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NMR Spectroscopy. All in vivo 31P-NMR spectra were measured without 1H decoupling on a Bruker CXP-200/300 spectrometer (89-mm bore) operating at 80.96 MHz in the Fourier transform mode. We used the following spectral parameters: 25-μsec pulse (65° flip angle at the center of the coil); 4K data points with quadrature detection; and 3.2-sec recycle time per scan. Resolution was enhanced by the convolution difference method (line broadenings, 15 and 400 Hz). We confirmed absence of saturation by varying the recycle time. Tumor spectra were the sum of at least 512 scans, while those from muscle were the sum of at least 256 scans. Spectra of tumor extracts were measured by Fourier transform NMR on a Bruker WH-400 spectrometer.

The surface coil consisted of a 3-turn, flat, circular coil. It was wound from a 16-gauge, plastic-coated copper wire and had a diameter of 19 mm. The sensitivity (mapped with a sample of phosphoric acid in a 2-mm capillary) decreased monotonically from the center of the surface coil and was negligible beyond about 7 mm along an axis perpendicular to the coil and beyond about 8 mm along an axis in the plane of the coil. The anesthetized mouse was placed in a polyethylene cage which contained a hole through which the tumor or leg protruded. The cage was placed 1.2 cm from the surface coil, with the tissue positioned at the center of the coil. That only tumor resonances were detected was confirmed by the absence of signals when a normal mouse was placed in the probe.

The assignments of resonances to PME, P, PCr, ADP, ATP, and reduced and oxidized nicotinamide adenine phosphate (collectively referred to as NAD) were made from published values (7). The apparent vibrational difference of the chemical shift of the 31P, peak. This shift was calibrated with a solution of 12.5 mM phosphoric acid, 12.5 mM phosphocreatine, and 125 mM NaCl. The 31P chemical shift (7.5 ppm) serves as references. Relative concentrations were estimated from the integrated areas of the corresponding 31P-NMR resonances.

PCA Extraction of Solid Tumors. Immediately after recording of in vivo 31P-NMR spectra, an osteosarcoma (0.9 g) was freeze-clamped by compression between 2 blocks of lead which had been precooled to the temperature of liquid nitrogen. The mouse was then sacrificed by cervical dislocation, and the tumor was excised and weighed while still cold.

The frozen tumor (submerged in liquid nitrogen) was pulverized with a precooled mortar and pestle, and cold (4°C) 0.5 N PCA was added (1 mliter PCA per g of tumor weight). Following evaporation of the cryogen, the PCA-tumor suspension was allowed to stand for 5 to 10 min at 4°C and then centrifuged to remove cellular debris. The supernatant was removed and neutralized with 2.5 N potassium bicarbonate. Following another centrifugation to remove precipitated potassium perchlorate, the supernatant was lyophilized to dryness. The residue was dissolved in a minimal amount of water and centrifuged again, and the supernatant was passed through a column (1 x 8 cm) of Chelex 100 (sodium form; Bio-Rad Laboratories, Richmond, Calif.). The eluate was again lyophilized and dissolved in D2O, and the pH of the solution was adjusted to that of the in vivo tumor.

RESULTS

Chart 1 presents 31P-NMR spectra from a murine osteosarcoma. The upper spectrum was obtained from the tumor in vivo. The major peaks are derived from PME (primarily phosphocholine and phosphoethanolamine), P, and ATP. Several other nucleoside triphosphates may contribute to the resonances labeled ATP, but their concentrations are much lower. For this reason, the nucleoside triphosphate peaks are labeled only as ATP. Several minor peaks are seen between -2 and 4 ppm. The lower spectrum is from a PCA extract of the same tumor. The freeze-clamp process appears to have prevented loss of ATP after death, as evidenced by the lack of significant amounts of ADP. While the 2 spectra are basically similar, certain differences are apparent. There is an obvious difference in resolution between the extract and in vivo spectra, with quite a bit of broadening seen in the latter. This difference is because the phosphorus metabolites in vivo are largely bound to paramagnetic ions or large molecules. Preparation of the PCA extract removes most of these paramagnetic ions and produces sharper peaks. These changes impair the ability of in vivo NMR spectroscopy to detect and quantify changes in minor phosphorus-containing species. For instance, the small peak labeled II (possibly uridine diphosphoglucose) on the extract spectrum is not distinguished from the much larger peak III (primarily the alpha-phosphorus of ATP) on the in vivo tracing. Peak V (PCr) on the in vivo spectrum appears to have 2 components, whereas only one is present in the extract. This difference is likely due to the poorer signal/noise ratio seen with in vivo tracing, though we cannot exclude the occurrence of a labile phosphorus-containing species which was destroyed during preparation of the extract. We have rarely seen 2 peaks in this area on spectra of PCA extracts from other tumor lines. The relative heights of the peaks differ between the 2 spectra. For instance, the in vivo spectrum shows much less PCr (peak V) than does ATP (peak I). In the extract spectrum, however, peak V is taller than peak I. These results are not discordant, however, when one examines the area under the peaks. The integral curve clearly shows that the relative amounts

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of PCr and ATP are similar to those measured in vivo. A final difference is in the chemical shift of the ATP peaks. The relative up-field (more negative) shift of the ATP peaks in the extract is due to the removal of Mg\(^{2+}\) during preparation.

Charts 2 and 3 show serial \(^{31}\)P-NMR spectra of tumors in 2 mice. The bottom spectrum in each chart presents the pretreatment condition of the tumor. Peaks I, II, and III are derived primarily from adenine nucleotides, while Peak V represents P\(_i\). The tumor studied in Chart 1 was heated to 40\(^\circ\) for 15 min. This heat dose produced no apparent cell kill, as demonstrated by steady tumor growth and rise in serum alkaline phosphatase. There was an immediate decrease in peaks representing ATP and an increase in the P\(_i\) peak, reflecting a 50% fall in the ATP/P\(_i\) ratio. Five hr later, there was slight recovery of the ATP/P\(_i\) ratio. Forty-five hr after hyperthermia, the ATP/P\(_i\) ratio had returned to pretreatment values, with little change thereafter. The apparent intracellular pH, as reflected in the shift of the P\(_i\) peak, changed synchronously with the ATP/P\(_i\) ratio. Immediately after treatment, there was a drop in intracellular pH from 7.2 to 6.7. Subsequently, the pH also increased to a maximum at 45 hr after treatment.

Chart 3 shows spectra from an osteosarcoma exposed to a larger thermal dose, 47\(^\circ\) for 15 min. This heat dose produced at least 2 logs of cell kill. This was apparent from the shrinkage of the tumor (~3 x 10\(^6\) cells) to below the limits of palpation (~10\(^6\) cells) and from the fall in the serum alkaline phosphatase to below background levels. Immediately after treatment, there was complete conversion of ATP to P\(_i\). No recovery of ATP occurred by 24 hr, at which time the tumor grossly necrosed and sloughed off, preventing further spectroscopic measurements. Small amounts of PME and NAD (peak at ~8 ppm on the 0-hr spectrum) may have persisted for a few hr after treatment but eventually disappeared.

Charts 4 and 5 show pre- and posttreatment \(^{31}\)P-NMR spectra from the thigh muscle of 2 mice. The pretreatment spectrum in each case shows the expected large PCr peak, with smaller peaks from ATP and little P\(_i\). Following mild hyperthermia of 40\(^\circ\) for 15 min (Chart 4), there was some deterioration in the signal/noise ratio. There was no fall in the ATP/P\(_i\) ratio. The P\(_i\) peak did show an upfield shift, suggesting a fall in the muscle pH from 7.3 to 7.0. No functional impairment ensued. After heating to 47\(^\circ\) for 15 min (Chart 5), the leg muscle shows nearly complete conversion of high-energy phosphates (both PCr and ATP) to P\(_i\), with a corresponding fall in intracellular pH. No further spectra were recorded and, several hr later, the leg had become an eschar, without functional ability. These changes in phosphorus metabolites following heating of muscle are qualitatively similar to those seen after tumor hyperthermia. The tumor is clearly more sensitive at lower heat doses but, at higher temperatures, the magnitude of P\(_i\) loss is similar.

The changes seen by NMR spectroscopy in ATP and P\(_i\) may be useful in the study of thermal dosimetry. Chart 6 presents the mean change in the ATP/P\(_i\) ratio over the period of a single hyperthermia treatment, at various heat doses. There is an obvious dose effect, with the higher heat doses producing greater reduction in ATP and increase in P\(_i\). It can be seen that even sham-heated animals had a slight decrease in P\(_i\). This likely results from a decrease in tumor tissue perfusion during spectroscopy as a result of the combined effects of pentobarbital anesthesia and bleeding into the tumor from thermocouple placement.
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6 Days Post-Treatment pH = 7.2

A/P = 0.6

45 hr Post-Treatment pH = 7.4

A/P = 0.7

5 hr Post-Treatment pH = 7.1

A/P = 0.4

0 hr Post-Treatment pH = 6.7

A/P = 0.3

Chart 3. Serial 31P-NMR spectra from a Dunn osteosarcoma treated at 47°C for 15 min. Identity of peaks: (I) ATP; (II) ATP + ADP + NAD; (III) ATP + ADP; (IV) PCr; (V) P; ATP/P is similar in both spectra, but pH shows a drop from 7.3 to 7.0.

Chart 4. 31P-NMR spectra from murine thigh muscle before and after treatment at 40°C for 15 min. Pretreatment spectrum is at top. Identity of peaks: (I) ATP; (II) ATP + ADP + NAD; (III) ATP + ADP; (IV) PCr; (V) P; ATP/P is similar in both spectra, but pH shows a drop from 7.3 to 7.0.

Chart 5. 31P-NMR spectra from murine thigh muscle before and after treatment at 47°C for 15 min. Pretreatment spectrum is at bottom. Identity of peaks is the same as in Chart 4.

Chart 6. Change in ATP/P, following increasing heat dose. Ordinate shows the percentage of change in the ratio of ATP/P, from 1 day before to immediately after hyperthermia treatment. Columns, mean of at least 5 animals; bars, S.E.

Table 1 relates the change in [ATP]/[Pi] to tumor cell kill as reflected in the serum alkaline phosphatase. Here, again, there is a dose effect, with those animals experiencing the greatest cell kill (and, hence, the greatest fall in alkaline phosphatase) also showing greater reduction in [ATP]/[Pi].
DISCUSSION

Several workers have pointed out differences in the response of tumors heated in vivo compared to that of those treated in vitro (14). These differences include a more rapid cell death rate in excess of that expected from direct thermal injury (22). Some have speculated that vascular damage may be one of the primary mechanisms of cell death following in vivo hyperthermia (28). We have demonstrated a prompt, dose-related loss of high-energy phosphate, increase in P_i, and fall in intracellular pH in murine osteosarcomas treated with hyperthermia. Such changes are known to occur in devascularized tissues (4, 23) and are consistent with previous reports of impaired blood flow and oxygen and glucose utilization (3, 29) following hyperthermia.

Our data on ATP fluxes after hyperthermia are at variance with those of Herman et al. (19). Using Chinese hamster ovary cells in culture, they found no significant change in ATP concentration, even after substantial heat exposure. An obvious explanation of this discrepancy is that the loss of high-energy phosphate results primarily from vascular impairment, a parameter not present in tissue culture systems. Our findings of a direct correlation between loss of ATP, cell kill, and heat dose, when compared with the previous in vitro data (19), are consistent with the view that vascular factors may be of extreme importance in in vivo thermal cell kill.

These observations also reinforce the importance of selecting clinically appropriate models for combined modality trials involving hyperthermia. In vivo and in vitro studies may differ markedly in the effects of treatment on levels of ATP, a substance of importance to most cellular processes. Hypoxia and impaired blood flow may greatly modify tumor responses to radiation and chemotherapy. The application of in vivo NMR spectroscopy to humans is at hand (26). Such technology may therefore help elucidate the metabolic events occurring during clinical hyperthermia treatment and may help clarify which tumor models most closely resemble their human counterparts metabolically.

The clinical development of hyperthermia has been hindered by imprecise dosimetry. Inhomogenous heat distribution, variable metabolic states of the cells, and electrical interference with thermocouples have impeded attempts to define a heat "dose" in physical terms. Much of the recent progress in chemotherapy has resulted from the use of biological end points (of either antitumor effect or toxicity) to define drug dose. The practice of treating embryonal carcinomas until disappearance of human chorionic gonadotropin from the blood is a well-known example. More recently, it has been shown that patients undergoing adjuvant chemotherapy for breast cancer have fewer relapses if enough drug is given to produce a constant level of myelosuppression (9). Changes in the levels of several glycolytic and respiratory intermediates have been used to predict tissue viability in organ transplant (4) and tumor therapy (12) studies. The ability to express thermal effects in terms of cell damage, rather than time at temperature, may improve dosimetry. 31P-NMR spectroscopy offers that potential.

In spite of its considerable promise for monitoring phosphorus-containing species noninvasively, in vivo 31P-NMR spectroscopy does suffer from some technical limitations. This analytical tool is relatively insensitive. Quantitative information is limited to only a few of the most abundant phosphorus species. Peaks derived from ATP and P_i are large and present in most spectra. Previous data suggest that the S.D. from measurement of these peaks is around ±10%. Minor peaks, such as PCr, may be present only in very small tumors that are rapidly growing. The variance in measurement of those peak areas may be as high as ±50%. We have also noted deterioration of the signal/noise ratio following interventions that disturb tissue homogeneity. This is a particular problem after hyperthermia treatment, which is associated with hemorrhage from thermocouple insertion, edema, and necrosis of tissues. This variation in signal/noise ratio among spectra explains our use of a ratio, ATP/P_i, to express quantitative information. Such a ratio is less likely to be affected by variations in noise than is the absolute area under any particular peak. At times, it is also necessary to record many more scans per spectrum after treatment than before. We are currently exploring several modifications of the surface coil probe to improve the signal/noise ratio. Our preliminary data suggest that a shielded solenoidal probe may improve the signal/noise ratio by a factor of 3.

The precise biological events leading to thermally induced loss of ATP remain to be defined. While some of the ATP changes are transient, we have still seen a clear-cut dose effect when NMR spectra are compared with either heat dose or cell kill. Thus, we feel that the changes in 31P-NMR spectra after hyperthermia reflect metabolically important events. NMR spectroscopy has the advantages of being rapid, noninvasive, and nonperturbing. We feel that in vivo 31P-NMR spectroscopy may be able to provide prompt feedback on the effectiveness of hyperthermia treatment. Studies to investigate this possibility are now in progress.

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ADDENDUM

A recent publication by Findly et al. (16) has shown that in vivo 31P-NMR spectroscopy can show loss of ATP following heat shock of Tetrahymena.

REFERENCES


* Unpublished observations.
7 Unpublished observation.


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