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^{31}P-NMR SPECTROSCOPY DEMONSTRATES DECREASED ATP LEVELS IN VIVO AS AN EARLY RESPONSE TO PHOTODYNAMIC THERAPY

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^{31}P-Nuclear magnetic resonance was used to monitor in situ phosphorus containing compounds in mammary tumors after photodynamic therapy, consisting of administration of hematoporphyrin derivative followed by photoradiation of the lesion. A rapid decrease in ATP along with an increase in Pi resonance intensities was observed. The beta-ATP/Pi ratio decreased by 1 hour, dropping in 2 to 8 hours to 0 to 20 percent of that found prior to photoradiation. Disrupted cells and pycnotic nuclei were observed 48 to 72 hours after photoradiation to a depth of approximately 5 mm. Together with previous studies in vitro, reduction in tumor ATP levels appears to be an early biochemical response to photodynamic therapy.

Systemic administration of hematoporphyrin derivative (Hpd) followed by exposure of the tumor to visible light, a regimen termed photodynamic therapy (PDT), is a promising new approach for cancer therapy (1). Hpd, a complex mixture of porphyrins, appears to be preferentially retained by tumors, with the ensuing cytotoxicity mediated by the production of singlet oxygen arising from a Type II photosensitization mechanism (2,3). Studies in vitro demonstrated subcellular damage occurring at the plasma membrane (4,5), microsomes (6,7), nucleus (8-10) and mitochondria (11-16) attributable to photosensitization with Hpd. Recently, we demonstrated that cellular levels of ATP were reduced following Hpd photosensitization of R3230AC tumor cells in vitro. These results, together with earlier studies, led us to propose that this response was due primarily to inhibition of mitochondrial production of ATP (16). Phosphorus-31 nuclear magnetic resonance spectroscopy (^{31}P-NMR) provides a useful non-invasive analytical method for monitoring levels of phosphorus-containing compounds in vivo and in situ in both normal and

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malignant tissues in hosts exposed to various therapeutic regimens (17-19). In the present study, we investigated the effect of Hpd-induced photosensitization on levels of phosphorus metabolites in the R3230AC mammary adenocarcinoma, by using ^{31}P-NMR spectra to detect changes in the metabolite levels in the tumor in situ.

MATERIALS AND METHODS

Fischer 344 female rats, each bearing two subcutaneously implanted R3230AC tumors (approximately 1 cm diameter), were injected i.p. with 25 mg/kg Photofrin II, a preparation of Hpd enriched in hydrophobic porphyrins (supplied by Photomedica Inc., Raritan, NJ). The animals were housed in the dark for specified periods (30 min, 24 hr, and 72 hr) after drug administration. ^{31}P-NMR spectra were taken with a General Electric 2T CSI spectrometer operating with a working magnet bore of 22 cm. The phosphorus spectra of the tumor were obtained using a specially constructed coil (20) fitted with a grounded copper foil shield around the base of the tumor to minimize contributions from surrounding non-tumor tissues (21). A 1000 W Xenon arc lamp was used for photoradiation, filtered to produce a broad spectrum (530-700 nm), focused to a 1 cm diameter beam, and a total fluence estimated to be 54 J/cm^2 at the porphyrin absorbing wavelengths. No elevation of the temperature at the tumor surface was observed during irradiation.

RESULTS

Phosphorus-31 NMR spectra of the treated tumors were obtained prior to irradiation, immediately after and at selected times up to 30 hrs following photoradiation. The spectrum obtained from the tumor after injection with Photofrin II, immediately prior to photoradiation, served as the control (Figure 1A). The major peaks in the spectrum are attributed to: I, beta ATP; II, alpha ATP; III, gamma ATP; IV, phosphocreatine; V, phosphodiesters; VI, Pi; and VII, mixed phospholipids. The ^{31}P-NMR spectrum of the same tumor 2 hrs after photoradiation is illustrated in Figure 1B. Compared to the control (1A), a significant reduction in the resonance intensities associated with ATP and a concomitant increase in the Pi resonance was observed. Controls, irradiation without drug and drug administration without irradiation, showed no change in ^{31}P-NMR spectra for up to 30 hrs after these manipulations.

Quantitation of the results obtained was performed by expressing the data as ratios of the area under the Pi peak (VI) and the total area under the observed phosphorus resonances (Pi/total), and of the area under the beta ATP peak (I) versus the Pi peak (VI) (beta ATP/Pi). These data are depicted in

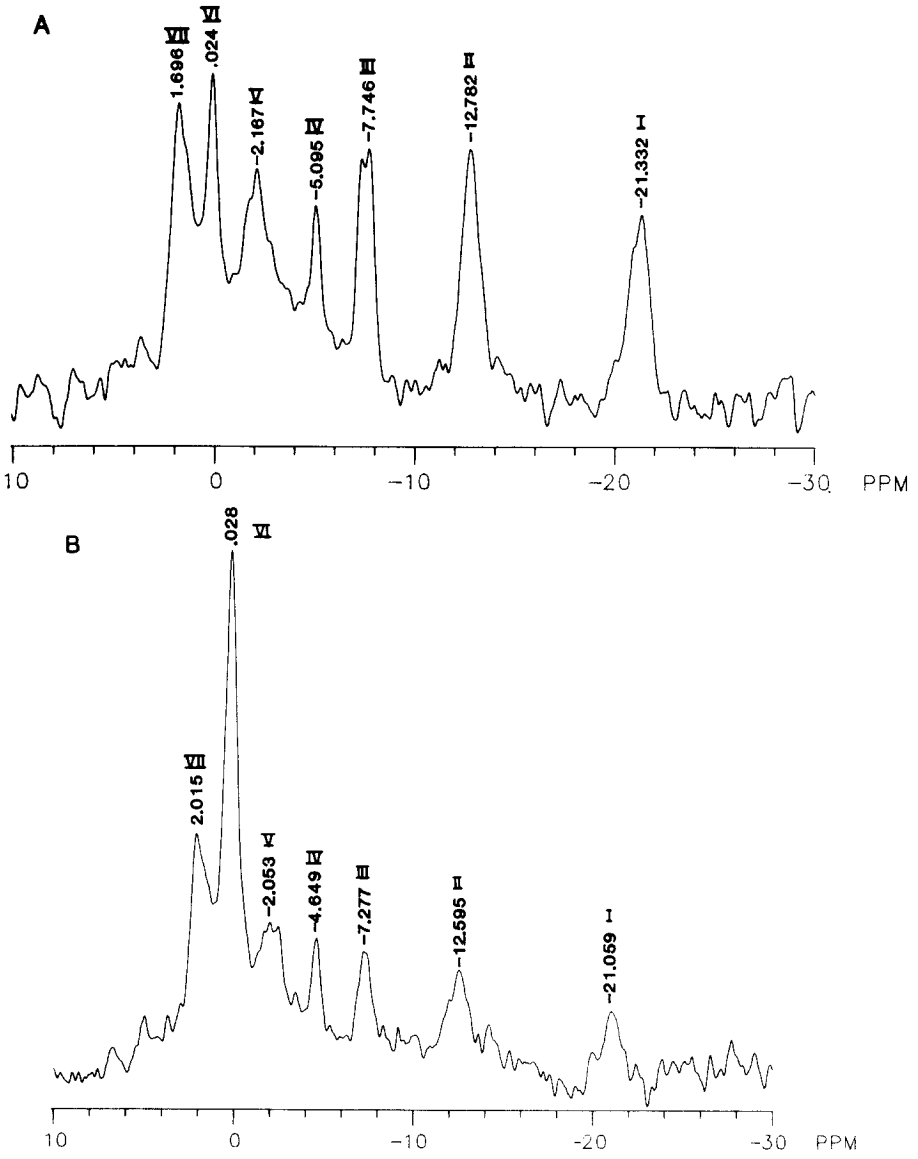

Figure 1. ^{31}P-NMR spectra of R3230AC tumor 24 hrs after injection of 25 mg/kg Photofrin II immediately prior to (A) and 2 hr after (B) photoradiation of the tumor. Spectra were obtained using approximate Ernst angle conditions with a 45° pulse width set at 4.5 μsec and a repetition rate of 2 sec. The sweep width was ± 1,000 Hz, using quadrature detection. Each spectrum is the sum of 512 scans. Total accumulation time was approximately 17 min. An exponential linebroadening of 10 Hz was applied to the spectra.

Figures 2A and B for the control (zero time) and at those times when ^{31}P-NMR spectra were obtained subsequent to photoradiation. A rapid and marked increase in the Pi/total ratio, representing a 3.5 to 5.0-fold increase by 2 to 8 hr, was observed (Figure 2A). The elevated Pi/total ratio remained high

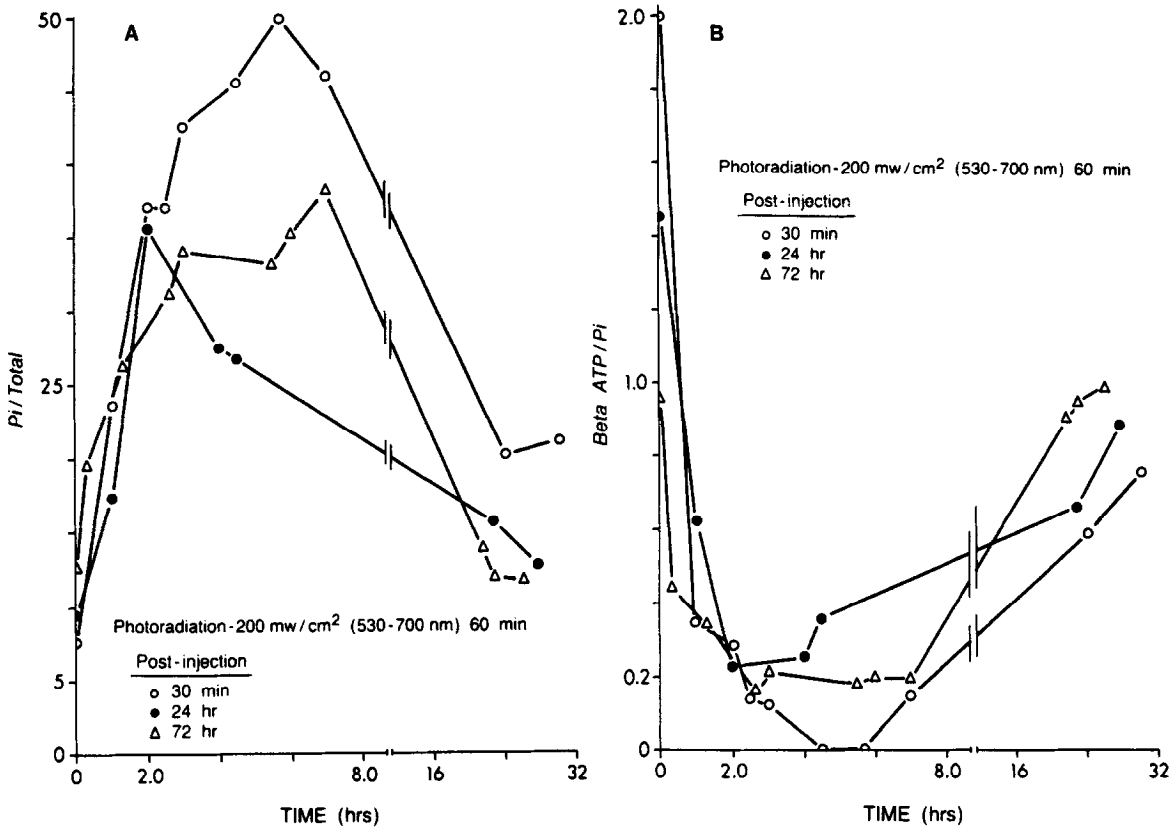

Figure 2. Ratios of Pi/total (A) and beta ATP/Pi (B) calculated from ^{31}P-NMR spectra of tumors from hosts injected with 25 mg/kg Hpd (Photofrin II) 30 min (o), 24 hr (•), or 72 hr (Δ) prior to photoradiation. Each point is the ratio calculated from spectra obtained immediately prior to and at selected times (up to 30 hrs) after photoradiation.

for approximately 2 hr followed by a return to basal levels for the tumor photoradiated 24 hr after porphyrin injection. This elevated Pi level lasted up to 6 hr in tumors photoradiated either 30 min or 72 hr after drug administration. The Pi/total ratio returned to or neared the control levels by 24 hr after photoradiation. Data obtained from ^{31}P-NMR spectra were also expressed as ratios of beta ATP/Pi (Figure 2B). It is clear that all three therapy protocols (photoradiation at 30 min, 24 hr, or 72 hr post-injection) produced a similar pattern, consisting of a marked decrease in the beta ATP/Pi ratio, compared to control levels (zero time), lasting up to about 6 hr after photoradiation. Subsequently, the beta ATP/Pi ratio returned to the values obtained prior to photoradiation (in 7 different control animals, beta ATP/Pi ratio prior to photoradiation was 1.25 ± 0.14, mean ± SEM). It is interesting

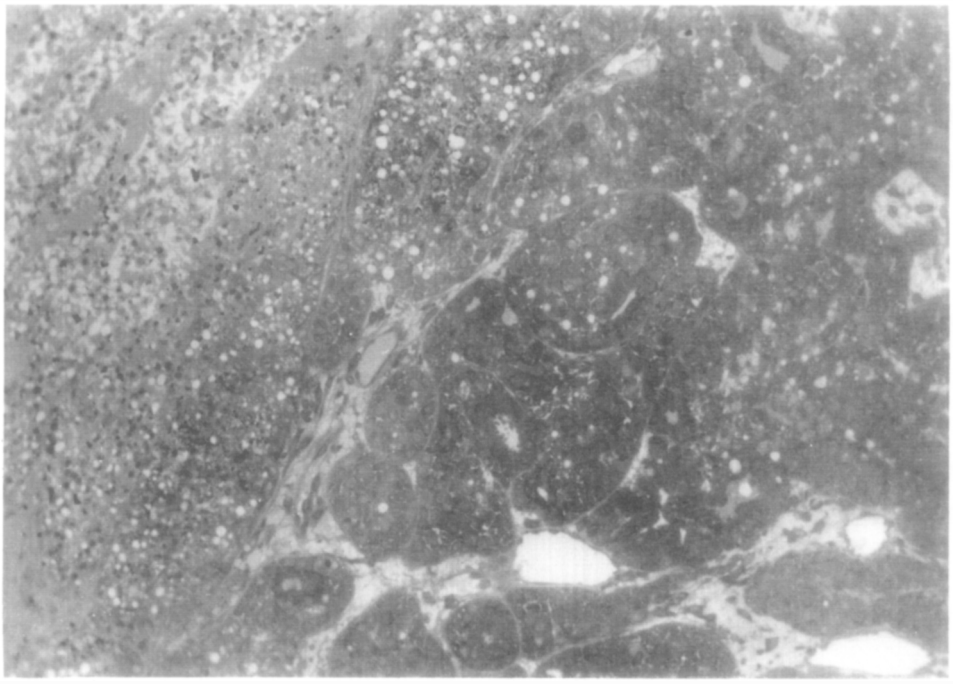

Figure 3. Photomicrograph of a section of tumor obtained at 72 hrs after photoradiation (96 hrs after administration of Photofrin II). Viable tumor is at the right, with a necrotic area at the left. The line of demarcation between the two areas is sharp. Magnification, 150X.

that the beta ATP concentration dropped to undetectable levels in the tumor photoradiated 30 min after drug administration, whereas the beta ATP/Pi ratio remained at approximately 0.2 for the other protocols. The ^{31}P-NMR data show that the effects of porphyrin photosensitization of the R3230AC mammary tumor display a reciprocal relationship of the levels of ATP and Pi, and that this response is rapid and large in magnitude.

Morphological analysis was performed on both the photoradiated and light shielded tumors. The tumors were excised, fixed in 2.5 percent gluteraldehyde, embedded in Immunobed, sectioned for light microscopy and stained with toluidine blue and azure II. Light microscopy of the photoradiated tumor (Figure 3) presented a zone of necrosis with a rather sharp demarcation where viable tumor cells were evident. This line of demarcation between necrotic and viable cells suggests a threshold for effective light penetrance. The necrotic region is characterized by disrupted cells, pycnotic nuclei, and cell debris. In the light-shielded tumor, the

morphology is representative of viable tumor cells, organized in an acinar pattern with secretory vesicles and no marked parenchymal cellular degradation. These morphological data indicate that the photoradiation employed provided efficaceous penetration to a depth of 5-7 mm, resulting in cell death and necrosis, whereas at greater depths, regions not exposed to sufficient photoradiation, ATP levels recovered and tumor cells remained viable.

DISCUSSION

The phosphorus-31 NMR spectra obtained from R3230AC tumors subjected to PDT demonstrate that this treatment regimen produced a rapid and dramatic decrease in the levels of ATP and a concomitant marked increase in Pi by 2-6 hrs throughout the entire tumor mass. These levels returned to near control levels at about 24 hrs post-photoradiation suggesting a damaging but reversible effect on the tumor. Since no alterations were observed in the absence of photoradiation, we suggest that cellular sites responsible for the production of ATP were affected by PDT. When considered with our earlier results *in vitro* (13-16), these ^{31}P-NMR data are interpreted as additional support that mitochondrial oxidative phosphorylation is an important target for porphyrin photosensitization *in vivo*. Although the data presented do not exclude effects on other metabolic sources of ATP, i.e., glycolysis, it is apparent that decreases in tumor ATP levels are an early event *in situ*, effects that could result in subsequent cytotoxicity. One explanation for the return of ATP and Pi to levels approaching those seen prior to photoradiation by 24 hr following photoradiation is that lethal damage did not occur throughout the tumor but was limited to regions of sufficient fluence. Effects of PDT on tumor vasculature have been reported (22,23), and deprival of metabolic substrates may have also contributed to a temporary reduction in ATP synthesis. Tumor cells not sufficiently affected by PDT would be expected to return to normal metabolism, with subsequent increases of ATP levels in the ^{31}P-NMR spectrum.

Microscopic examination of the R3230AC tumor following PDT demonstrates that this mammary adenocarcinoma is responsive to this therapy *in vivo*. The

ease of obtaining ^{31}P-NMR spectra should provide a method for pursuing a number of fundamental studies on drug and light dose relationships, as well as on pharmacokinetics, together providing data for optimization of these parameters to achieve maximum therapeutic efficacy.

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REFERENCES

1. Dougherty, T.J. (1984) Porphyrin Localization and Treatment of Tumors, pp. 75-90, Alan R. Liss, New York.
2. Weishaupt, K.R., Gomer, C.J., and Dougherty, T.J. (1976) Cancer Res. 36, 2326-2329.
3. Gibson, S.L., and Hilf, R. (1985) Photochem. Photobiol. 42, 367-373.
4. Kessel, D. (1977) Biochemistry 16, 3443-3449.
5 Kohn, K., and Kessel, D. (1979) Biochem. Pharmacol. 28, 2465-2470.
6. Dixit, R., Mukhtar, H., and Bickers, D.R. (1983) Photochem. Photobiol. 37, 173-176
7. Das, M., Dixit, R., Mukhtar, M. and Bickers, D.R. (1985) Cancer Res. 45, 608-615.
8. Gomer, C.J. (1980) Cancer Lett. 11, 161-167.
9. Moan, J., Watsvik, H., and Christensen, T. (1980) Cancer Res. 40, 2915-2918
10. Fiel, R.J., Datta-Gupta, N., Mark, E.H., and Howard, J.C. (1981) Cancer Res. 41, 3543-3545.
11. Berns, M.W., Dahlman, A., Johnson, F.M., Burns, R.G., Sperling, D., Guiltin, M., Siemens, A., Walter, R., Wright, W., Hammer-Wilson, M., and Wile, A. (1982) Cancer Res. 42, 2325-2329.
12. Sandberg, S., and Romslo, I. (1980) Biochim. Biophys. Acta 593, 187-195.
13. Gibson, S.L., and Hilf, R. (1983) Cancer Res. 43, 4191-4197.
14. Hilf, R., Smail, D.B., Murant, R.S., Leakey, P.B., and Gibson, S.L. (1984) Cancer Res. 44, 1483-1488.
15. Perlin, D.S., Murant, R.S., Gibson, S.L., and Hilf, R. (1985) Cancer Res. 45, 653-658.
16. Hilf, R., Murant, R.S., Narayanan, U., and Gibson, S.L. (1986) Cancer Res. 46, 211-217.
17. Sijens, P.E., Bovee, W.M.M.J., Seijkens, D., Los, G., and Rugers, D.H. (1986) Cancer Res. 46, 1427-1432.
18. Chopp, M., Helpern, J.A., Frinak, S., Hetzel, F.W., Ewing, J.R., and Welch, K.M.A. (1985) Med. Phys. 12, 256-258.
19. Lilly, M.B., Ng, T.C., Evanochko, W.T., Katholi, C.R., Kumar, N.G., Elgavish, G.A., Durant, T.-R., Hiramoto, R., Ghanta, V., and Glickson, J.D. (1984) Cancer Res. 44, 633-638.
20. Schnall, M.D., Subramanian, V.H., Leigh, J.S., and Chance, B. (1985) J. Magn. Reson. 65, 122-127.
21. Ng, T.C., and Glickson, J.D. (1985) Magn. Reson. Med. 2, 169-175.
22. Selman, S.H., Kreimer-Birnbaum, M., Klaunig, J.E., Goldblatt, P.J., Keck, R.W., and Britton, S.L. (1984) Cancer Res. 44, 1924-1927.
23. Henderson, B.W., Waldow, S.M., Mang, T.S., Potter, W.R., Malone, P.B., and Dougherty, T.J. (1985) Cancer Res. 45, 572-576.