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Effect of Estrogenic Perturbations on δ-Aminolevulinic Acid-Induced Porphobilinogen Deaminase and Protoporphyrin IX Levels in Rat Harderian Glands, Liver, and R3230AC Tumors

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ABSTRACT. The Harderian gland in rodents highly expresses enzymes of the heme biosynthetic pathway that are responsible for porphyrin production. Interestingly, many of the steps in Harderian gland heme biosynthesis, including protoporphyrin production, are controlled hormonally. We hypothesized that estrogenic alterations, ovariectomy or tamoxifen administration, might also alter the response of porphobilinogen deaminase activity and/or protoporphyrin IX production to δ-aminolevulinic acid administration in the hormonally responsive R3230AC rat mammary adenocarcinoma. We also determined whether the response of the R3230AC tumor, borne on ovariectomized hosts, to δ-aminolevulinic acid-based photodynamic therapy was altered compared with tumors treated on intact hosts. Ovariectomy of female Fischer rats bearing the hormonally responsive R3230AC mammary adenocarcinoma caused a significant reduction in δ-aminolevulinic acid-induced protoporphyrin IX levels and porphobilinogen deaminase activity in tumors compared with levels in tumors from intact animals treated with δ-aminolevulinic acid. In contrast, although porphobilinogen deaminase activity in the Harderian gland from ovariectomized animals was reduced significantly compared with that in glands from intact animals, protoporphyrin IX levels were unaltered. Administration of the anti-estrogen tamoxifen to tumor-bearing rats resulted in a significant increase in porphobilinogen deaminase in both tumor and Harderian gland. Although administration of δ-aminolevulinic acid increased protoporphyrin IX levels in Harderian glands in tamoxifen-treated animals, tumor levels of protoporphyrin IX remained unaltered in the tamoxifen-treated rats. Treatment of R3230AC tumors with δ-aminolevulinic acid-based photodynamic therapy in ovariectomized rats resulted in a significantly reduced response compared with the same treatment regimen in intact animals, 4.9 ± 0.39 versus 10.6 ± 0.6 days to reach twice the initial tumor volume, respectively. These results indicate that the hormonal status of the host should be considered when treating hormonally sensitive tumors with δ-aminolevulinic acid-based photodynamic therapy. BIOCHEM PHARMACOL **58**;11:1821–1829, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. δ-aminolevulinic acid; photodynamic therapy; protoporphyrin IX

The heme biosynthetic pathway, long appreciated for its essential metabolic role, has gained interest recently due to its production of PPIX†, an endogenous photosensitizer that can be used in PDT of cancer [1–4]. This therapy consists of the systemic or topical administration of δ-ALA followed 1–5 hr later by exposure of malignant lesions to laser light in the 630–640 nm range of the visible spectrum. A photochemical reaction ensues, generating the toxic oxygen species singlet oxygen, which oxidizes intracellular biomolecules, leading to cellular demise and ultimately tumor necrosis [5, 6].

The process by which δ-ALA induces the accumulation of PPIX is complex, since control of the heme biosynthetic pathway is highly regulated [7, 8]. Introduction of δ-ALA circumvents the initial regulated enzyme, δ-ALA-S. This enzyme is feedback inhibited by heme produced in the last step of the pathway, where ferrochelatase inserts iron into PPIX. Circumvention of this control point results in the accumulation of PPIX, which is formed as the penultimate step of the heme pathway [7, 8]. It is this circumvention that has been exploited for use in PDT. There are reports that many tumors accumulate more PPIX than do normal tissues, the basis for which is yet to be defined [2, 9–12].

The Harderian gland is located behind the eye in most vertebrates that possess a nictitating membrane, e.g. reptiles, amphibians, birds, and mammals such as rodents, cats, and sheep [13, 14]. It is not found in humans, except in very early developmental stages [13]. Except for ferrochelatase, the Harderian gland maintains a high level of constitutively expressed heme biosynthetic enzymes and a correspond-

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† *Abbreviations*: PPIX, protoporphyrin IX; PDT, photodynamic therapy; PBGD, porphobilinogen deaminase; δ-ALA, δ-aminolevulinic acid; δ-ALA-S, δ-aminolevulinic acid synthase; and δ-ALA-D, δ-aminolevulinic acid dehydratase.

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ingly high endogenous concentration of PPIX [15]. These factors make the Harderian gland an excellent choice of tissue to study the regulatory elements of the heme biosynthetic pathway. In addition, the heme pathway in the Harderian gland is responsive to alterations in the hormonal milieu of the host [13, 16, 17]. Androgens, when present, appear to suppress the production of PPIX, whereas castration of the male hamster leads to about a 10-fold increase in the amount of PPIX produced by the Harderian gland [18]. In the female, removal of the major source of estrogens, the ovaries, results in a 25% decrease in PPIX production by the Harderian gland [13]. The hormonal responsivity of the Harderian gland is reminiscent of what is observed for glands that secrete steroid hormones, such as the testes and ovaries.

Based on these findings, we queried whether components of the heme biosynthetic pathway and/or PPIX production in the hormonally responsive R3230AC rat mammary adenocarcinoma would also be affected by hormonal perturbations. Our studies of enzymes of the heme biosynthetic pathway and their relationship to δ-ALA-based PDT have focused mainly on PBGD [19, 20], because of its reported role as a key secondary regulatory step, after δ-ALA-S, in heme biosynthesis [8, 21]. Our findings demonstrated that the δ-ALA-induced increase in PBGD activity, apparently resulting from increased synthesis of PBGD *de novo*, correlated closely with the concomitant increased accumulation of PPIX [19, 20].

We undertook experiments to determine, first, whether alterations of the hormonal status of the host, e.g. ovariectomy or administration of the anti-estrogen tamoxifen, would modify the level of PBGD in R3230AC tumors, liver, or Harderian glands of the Fischer female rat. Second, we sought to determine whether alterations in the activity of PBGD were correlated with changes in the level of δ-ALA-induced PPIX. Finally, we ascertained whether alteration of the hormonal status of the host affected the response of R3230AC tumors to δ-ALA-based PDT.

The results of the present studies demonstrated that alterations in PBGD activity were different in R3230AC tumors, rat liver, and Harderian glands depending on the hormonal status of the host, i.e. ovariectomized or treated with tamoxifen. The tumor levels of PPIX were suppressed significantly in ovariectomized hosts, and these reductions were reflected in the lack of tumor response to δ-ALA-based PDT.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and reagents were purchased from the Sigma Chemical Co. unless otherwise noted. δ-ALA was obtained from Porphyrin Products. Tamoxifen time release pellets were purchased from Innovative Research Inc.

Animals and Tumors

The R3230AC mammary adenocarcinoma was maintained by transplantation in the abdominal region of 100- to 120-g female Fischer rats, using the sterile trochar technique described earlier [22]. All animals were cared for under the guidelines of the University Committee on Animal Resources at the University of Rochester.

Ovariectomy, Hormone, and δ-ALA Administration to Tumor-Bearing Animals

Ovariectomy was performed 1 week prior to tumor transplantation, using a standard surgical procedure approved by the University Committee on Animal Research and the Association for Assessment and Accreditation of Laboratory Animal Care. Briefly, animals were anesthetized with halothane, and the dorsal region was prepared by shaving the area and applying a coating of chlorhexidine and iodine. A small incision through the skin and body wall was made above the kidney region, and the ovary was externalized, clamped, and excised. The body wall was sutured with 5–0 chromic gut, and the skin was closed with staples that were removed 7–10 days later.

Tamoxifen was delivered from s.c. implanted time release pellets designed to deliver 0.83 μg/kg/day, when placed in rats weighing approximately 100 g. The pellets were inserted through a small incision made in the suprascapular area of the neck. This procedure was performed on the same day as tumor transplantation. The incision was closed with a single staple that was removed 7–10 days later. Pellets remained implanted throughout the experimental period, usually 7–14 days after their implantation.

Administration of δ-ALA was performed on animals whose tumors had reached approximately 1 g wet weight, usually 10–14 days after tumor transplantation and/or ovariectomy or tamoxifen administration. A dose of 300 mg/kg was delivered i.v. via the tail vein, and animals were killed 3 hr after δ-ALA injection; tissues were excised and stored frozen at −86° until used for PBGD or PPIX determinations.

Measurement of PBGD Activity in Tissue Preparations

The assay for PBGD activity measures the absorbance of uroporphyrin, formed after light-induced oxidation of uroporphyrinogen, which is a condensation product of hydroxymethylbilane, the immediate product of PBGD action. The procedure is essentially described by Grandchamp *et al.* [23]. Briefly, tissues were homogenized (1:5, w/v) in 0.05 M Tris–HCl (pH 7.4), and centrifuged at 1000 *g* for 15 min; portions of the supernatant containing 2 mg protein were incubated for 30 min at 45° in the dark with 1.0 mL of porphobilinogen at concentrations ranging from 0 to 500 μM. The reaction was stopped by the addition of 2 mL ethyl acetate/acetic acid (3:1, v/v). The mixture was centrifuged at 1000 *g* and then exposed to ambient light at

room temperature for 15 min. The porphyrin-containing upper layer (1.6 mL) was transferred to a tube containing 1 mL of 0.5 M HCl, thoroughly mixed, and centrifuged at 2500 *g* for 10 min. Then the lower layer was transferred to a cuvette, and the uroporphyrin absorption at 405 nm was measured. Activity is expressed as picomoles of uroporphyrin formed per milligram of protein for 30 minutes.

Measurement of PPIX Content in Tissue Homogenates

The tissue concentrations of δ-ALA-induced porphyrins were determined by measuring the fluorescence of tissue homogenates 3 hr after δ-ALA injection. Animals were killed, and tissues were excised, rinsed in 0.9% NaCl, and frozen at −80° until used for fluorescence measurements. Tissue samples, approximately 0.1 to 0.15 g, were homogenized on ice in 2× vol. of 0.05 M phosphate buffer, pH 7.4, using a Polytron homogenizer (PCU 110, Brinkmann Inc.). Samples of 10- to 120-μL were transferred to a quartz cuvette containing 2 mL of 0.05 M phosphate buffer, the suspension was mixed thoroughly with a Pasteur pipet, and the cuvette was positioned in a spectrofluorimeter (Fluorolog 2, SPEX Industries). Samples were excited at 400 nm, and the fluorescence emission was scanned from 600 to 720 nm, resulting in the appearance of two distinct peaks positioned at 630 and 704 nm. Maximum fluorescence intensity was detected at 630 nm, and this peak was selected for measurement of the porphyrin content in tissues. Tissue concentrations were computed from the fluorescence values obtained by a titration of a PPIX standard (Porphyrin Products). PPIX was used as the standard since previous reports demonstrated that PPIX is the major contributor to the fluorescence detected after exposure of cells in culture to δ-ALA or after *in vivo* administration of δ-ALA [1, 24, 25]. Protein determinations were performed according to the method of Lowry *et al.* [26].

Irradiation of Tumors after δ-ALA Administration

Animals bearing tumors that had reached 0.5 cm in diameter, 0.2 cc or less in volume, were selected for δ-ALA-based PDT. Three hours after δ-ALA administration, animals were anesthesized using a combination of 75 mg/kg of ketamine dihydrochloride (Parke-Davis Inc.) and 6 mg/kg of xylazine (Butler Co.), the skin over the tumor was shaved, and tumors were irradiated at 100 mW/cm^2 for 30 min for a total fluence of 180 J/cm^2. Irradiation was delivered using an argon-ion pumped dye laser (Inova 90, Coherent Laser Inc.). The laser was tuned to 630 nm, and wavelength was verified using a grating monochrometer (model H10, Instruments SA). Laser light was delivered to tumors via a 400 μm i.d. fiber optic fitted with a GRIN lense (General Fiber Optics).

Measurement of Tumor Growth and Statistical Analysis

Calipers were used to obtain two perpendicular dimensions of tumors each day, and calculations of tumor volumes were made from these measurements. The endpoint used to determine treatment efficacy was the time required, in days, for tumors to reach double their initial pretreatment volume. Serving as controls were tumor-bearing intact rats, and rats that had been ovariectomized 1 week prior to tumor transplantation. Tumors borne on animals treated with δ-ALA without irradiation, or exposed to irradiation in the absence of δ-ALA treatment, displayed the same tumor doubling times as tumors borne on control, untreated animals [24]. Values obtained for each treatment group were compared to those obtained for controls and among treatment groups using the SAS statistical program (SAS Inc.). A P value of <0.05 was considered significant.

All other statistical comparisons were accomplished using Student's t-test, and a P value of <0.05 was considered significant.

RESULTS

Effect of Ovariectomy and/or δ-ALA Administration on PBGD Activity Levels

Basal levels of PBGD activity (pmol uroporphyrin formed/30 min/mg protein) varied in the tissues studied with the rank order from the highest to the lowest being: Harderian gland (1420 ± 137) > tumor (235 ± 24) > liver (101 ± 8.4) (see panels a–c of Fig. 1). Ovariectomy of the host did not produce a similar response in enzyme activity in these tissues. In both tumor and the liver, ovariectomy had little effect on PBGD activity compared with that measured in tissues from intact animals (Fig. 1a and b). In contrast, PBGD activity in the Harderian gland from ovariectomized animals was reduced to almost half that measured in glands from intact animals, 820 ± 60 versus 1420 ± 137 pmol uroporphyrin formed/30 min/mg protein, respectively (Fig. 1c).

Administration of δ-ALA had different effects on PBGD enzyme activity depending on the hormonal status of the host, i.e. intact or ovariectomized, and on the tissue assayed. In the liver and Harderian gland, PBGD activity was not altered significantly after the administration of δ-ALA to intact animals (Fig. 1b and c). Enzyme activities in the tumor, however, increased from 235 ± 24 pmol uroporphyrin formed/30 min/mg protein in untreated intact animals to 424 ± 22 pmol uroporphyrin formed/30 min/mg protein in intact animals injected with δ-ALA (Fig. 1a), results that are consistent with our previous report [24]. In contrast, treatment of ovariectomized animals with δ-ALA did not cause a significant effect on PBGD activity in any of the tissues studied (Fig. 1 a–c). These results imply that ovarian status may play a role in the tumor PBGD response to δ-ALA administration.

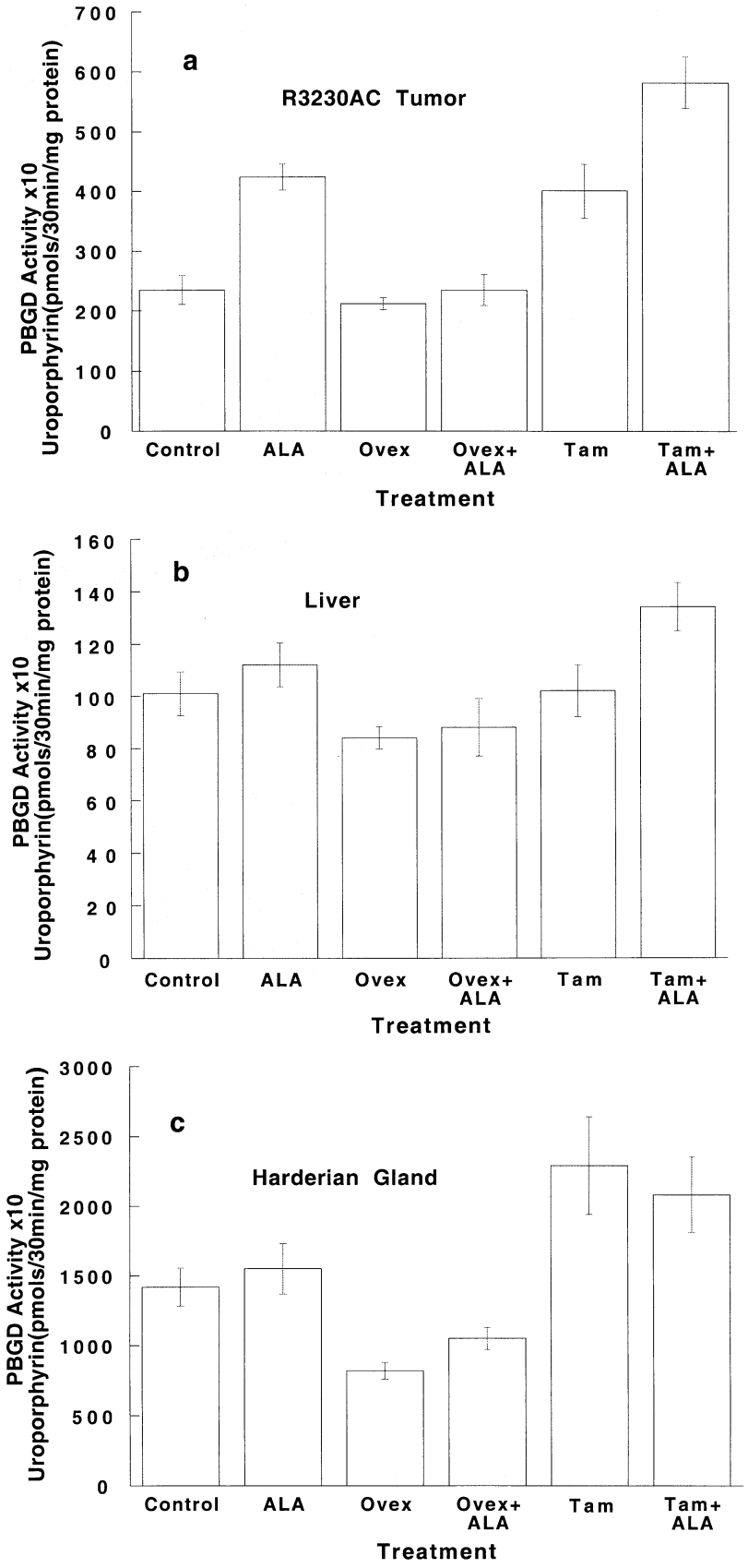

FIG. 1. Effect of estrogenic alterations on δ-ALA-induced PBGD activity. R3230AC tumors (a), liver (b), or Harderian glands (c) were excised from either intact, ovariectomized, or tamoxifen-treated rats, and PBGD determinations were performed (experimental conditions are detailed in Materials and Methods). Data are expressed as picomoles of uroporphyrin formed per 30 minutes per milligram of protein. Each column represents the mean PBGD activity obtained for 7 to 23 separate determinations; bars are the SEM.

Effect of Tamoxifen Administration and/or δ-ALA Administration on PBGD Activity Levels

Delivery of tamoxifen at 0.83 μg/kg for 7–10 days to intact animals had no significant effect on PBGD activity in the liver compared with enzyme activity in livers harvested from intact untreated rats (Fig. 1b). However, PBGD activity in R3230AC tumors and Harderian glands excised from tamoxifen-treated hosts was significantly higher than PBGD activity measured in these tissues from untreated animals, $P < 0.005$ for tumors and $P < 0.05$ for Harderian glands (Fig. 1a and c).

Administration of δ-ALA to tumor-bearing animals 7–10 days after implantation of tamoxifen pellets caused no significant change in PBGD activity in livers compared with enzyme activity measured in livers from intact animals or in livers from intact animals given δ-ALA alone (Fig. 1b). However, enzyme activity in tumors removed from hosts treated with tamoxifen plus δ-ALA was significantly higher ($P < 0.001$) than PBGD activity in tumors from intact untreated animals as well as tumors from intact animals treated with δ-ALA alone ($P < 0.005$) (Fig. 1a). These findings imply that tamoxifen action on PBGD activity in different tissues may not be the same.

Effect of Ovariectomy on δ-ALA-Induced PPIX Accumulation

PPIX was not detectable in tumors or livers obtained from intact animals not treated with δ-ALA. The Harderian glands had an average basal level of 186 ± 15 pmol PPIX/mg protein without δ-ALA administration (Fig. 2c). Tumors borne on animals ovariectomized 7–10 days prior to δ-ALA administration had significantly lower amounts of PPIX than that measured in tumors from intact animals administered δ-ALA alone ($P < 0.001$), 2.5 ± 0.21 versus 5.5 ± 0.6 pmol PPIX/mg protein, respectively (Fig. 2a). Livers from ovariectomized animals displayed significantly higher PPIX levels than livers from animals injected with δ-ALA alone ($P < 0.01$), 24.2 ± 2.7 versus 14.9 ± 1.4 pmol PPIX/mg protein, respectively. In the Harderian gland, a modest reduction in PPIX was observed when glands from ovariectomized hosts were compared with those from intact untreated animals or those injected with δ-ALA alone (Fig. 2c). Injection of δ-ALA 7–10 days after ovariectomy had little effect on the PPIX levels in Harderian glands (Fig. 2c). These data demonstrate that ovariectomy of hosts had varying effects on the levels of δ-ALA-induced PPIX accumulation in the tissues studied.

Effect of Tamoxifen Administration on δ-ALA-Induced PPIX Accumulation

Tumors borne on animals with tamoxifen pellets implanted 7–10 days prior to δ-ALA accumulated equivalent amounts of PPIX as those administered δ-ALA alone. Treatment of animals with δ-ALA after tamoxifen implantation increased PPIX levels significantly ($P < 0.001$) in liver tissue compared with livers from animals injected with δ-ALA alone, 29 ± 3.1 versus 14.9 ± 1.4 pmol PPIX/mg protein, respectively (Fig. 2b). In Harderian glands excised from hosts implanted with tamoxifen pellets, PPIX levels were significantly higher ($P < 0.001$) than those in glands from intact, ovariectomized, δ-ALA-treated, or ovariectomy/δ-ALA-treated hosts. However, administration of δ-ALA 7–10 days after tamoxifen pellet implantation did not increase PPIX levels in Harderian glands significantly compared with levels in those glands excised from hosts treated with tamoxifen alone. These data demonstrated that tamoxifen had different effects on δ-ALA-induced PPIX accumulation in the various tissues studied, but, in general, PPIX levels increased over those levels induced by δ-ALA alone.

Effect of δ-ALA-Induced PDT on R3230AC Tumor Growth

Ovariectomy resulted in a significant reduction in both δ-ALA-induced PBGD activity and PPIX accumulation in tumors compared with tumors on animals administered δ-ALA alone. Therefore, experiments were performed to determine if these changes would alter the response of tumors to δ-ALA-induced PDT. The data in Fig. 3 demonstrate that ovariectomy significantly impaired the tumor response to δ-ALA-based PDT. The tumor volume doubling time of lesions on intact animals treated with δ-ALA and light was 10.6 ± 0.6 days, a growth that was delayed three times longer than that observed for untreated tumors (Table 1). The volume doubling time of tumors treated with δ-ALA-based PDT on intact animals was significantly longer than the volume doubling time of tumors treated on ovariectomized animals (Table 1). Ovariectomy alone did not alter tumor growth significantly compared with tumors monitored on intact untreated animals. These data demonstrate that ovariectomy, i.e. removal of the major source of endogenous estrogens, significantly inhibited the response of R3230AC tumors to δ-ALA-induced PDT.

DISCUSSION

The gland of Harder, commonly referred to as the Harderian gland, is located in the orbit of the eye in terrestrial vertebrates possessing nictitating membranes. The Harderian gland is not present in adult humans or in some other adult mammals such as bats, cows, horses, and higher primates. Its numerous functions, some of which are species-specific, include lubrication of the nictitating membrane, which is universal among all species, storage of immunocompetent cells, pheromone production, thermoregulation, and photoprotection of the retina [13]. The latter function, photoprotection of the retina, is of interest because, in the female rodent Harderian gland, all the enzymes of the heme biosynthetic pathway, except ferrochelatase, are highly constitutively expressed [13, 16]. This

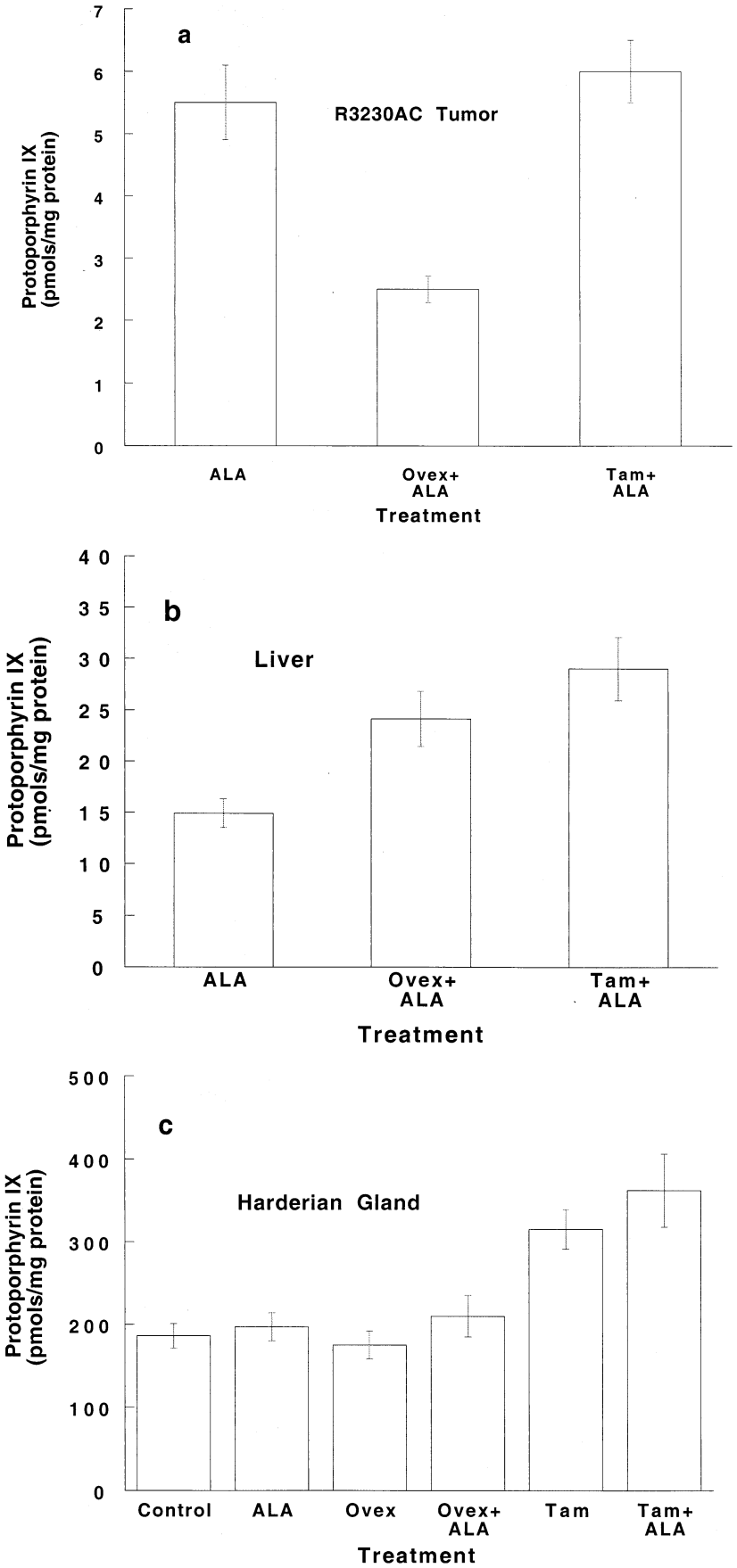

FIG. 2. Effect of estrogenic alterations on δ-ALA-induced PPIX accumulation. R3230AC tumors (a), liver (b), or Harderian glands (c) were excised from either intact, ovariectomized, or tamoxifen-treated rats, and PPIX determinations were performed (experimental conditions are detailed in Materials and Methods). Data are expressed as picomoles of PPIX accumulated per milligram of protein. Each column represents the mean obtained from 7 to 13 separate determinations; bars are the SEM.

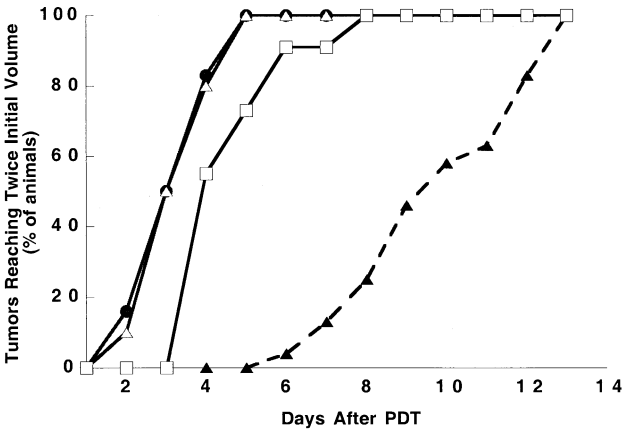

FIG. 3. Effect of δ-ALA-induced PDT on the growth of R3230AC tumors. Treatment conditions and the methods used to calculate tumor volumes and statistically analyze the results are detailed in Materials and Methods. Data are presented as percent of animals on which tumors reached twice their initial pretreatment volume. Symbols represent data collected for untreated tumors (●), tumors borne on intact rats treated with δ-ALA and light (▲), tumors borne on ovariectomized rats (△), and tumors borne on ovariectomized rats treated with δ-ALA and light (□).

combination of high levels of enzymes involved in porphyrin synthesis and low ferrochelatase activity, the enzyme responsible for forming heme from PPIX, results in high concentrations of PPIX. Interestingly, major differences in the porphyrin biosynthetic pathway between male and female Harderian glands of rodents exist, suggesting that porphyrin production is controlled by the hormonal milieu of the host [16, 17]. Studies have shown that basal δ-ALA-S levels are five times greater in the female hamster Harderian gland than in the male gland and uroporphyrinogen decarboxylase (URO-D) is 50 times greater in the female than in the male [17]. On the other hand, ferrochelatase and δ-ALA-D activities are equivalent in the male and female [17]. Concomitant with these findings, PPIX levels in the female hamster Harderian glands were 70 times higher than PPIX concentrations in the male [13]. Furthermore, altering the hormonal status of the male hamster by castration increased PPIX levels 10-fold over basal levels in intact animals [13]. This elevation was reversed by androgen administration to castrated males [18]. Conversely, ovariectomy of female hamsters decreased Harderian gland PPIX levels by approximately 25% compared with intact controls [13]. These studies indicate that androgens may play a major role in down-regulating porphyrin biosynthesis in rodent Harderian glands. The presence of androgen, as well as estrogen, receptors in Harderian glands is in keeping with a likely regulatory role for these steroids [13].

TABLE 1. Response to PDT

Treatment	N	Days to double initial volume	
		Mean ± SEM	Median
Control	12	3.8 ± 0.2*	4.0
PDT	12	10.6 ± 0.6	10.0
Ovex	10	3.6 ± 0.3	3.5
Ovex + PDT	11	4.9 ± 0.39	4.0
Statistical comparisons:			
Control vs PDT			$P < 0.0001$†
Control vs ovex			$P = 0.77$
Control vs ovex + PDT			$P = 0.03$†
PDT vs ovex			$P < 0.0001$†
PDT vs ovex + PDT			$P < 0.0001$†
Ovex vs ovex + PDT			$P = 0.027$†

* Values are number of days required for tumors to double in volume.
† $P < 0.05$ indicates a significant difference.

The evolutionary basis for hormonal control of heme biosynthesis in the Harderian gland and the reason for the dramatic gender differences are not well understood, but such observations are documented for a variety of conditions. In one report, hexachlorobenzene administration induced hepatic porphyria, and excess uroporphyrin in the urine of female rats, but not in males [27]. Another investigation showed that a single dose of estradiol benzoate to male rats reduces microsomal heme and cytochrome P450 production by approximately 75% [28]. In contrast, it was demonstrated that porphyrin administration alters hormone levels. Injection of cobalt–protoporphyrin into male rats increased androgen metabolism, resulting in dramatically reduced testosterone levels and a prolonged induction of heme oxygenase [29].

These studies, describing hormonal control of heme biosynthesis in tissues other than the Harderian gland, prompted us to ask whether similar characteristics might persist in hormonally responsive malignant lesions. We addressed this question by investigating the effects of estrogen perturbations of the host on δ-ALA-induced PBGD and PPIX levels in the hormonally responsive R3230AC mammary adenocarcinoma of the rat. PBGD was selected because of its reported role as a secondary control step in heme biosynthesis [8, 21] and because our previous results suggested that a relationship between PBGD activity and PPIX levels *in vivo* and *in vitro* might exist [19, 20]. In the present study, we compared PBGD and PPIX levels in tumors with those in Harderian glands and liver from the same animals to determine whether any similarities or differences existed in the response of these tissues to hormonal alterations and δ-ALA administration. We also performed studies to determine whether hormonal alterations would impact the effectiveness of δ-ALA-based PDT of the R3230AC tumor.

Results from these studies demonstrated that PBGD activity in tumor and liver tissue from ovariectomized animals was reduced slightly compared with the enzyme activity in tissues from intact control animals. Enzyme activity in the Harderian gland from ovariectomized animals, however, was reduced significantly compared with that in glands from intact animals. Subsequent administration of δ-ALA to ovariectomized animals did not increase PBGD activity significantly in any of the tissues studied.

This latter result is especially notable because PBGD activity in the tumor increases when δ-ALA is administered to intact tumor-bearing animals. Taken together, these results suggest that at least one step in heme biosynthesis in the R3230AC tumor is responsive to hormonal alteration of the host.

PPIX levels in Harderian glands from ovariectomized hosts were reduced slightly (by 6%) compared with levels in glands from intact animals. Administration of δ-ALA did not increase these levels appreciably. This result is curious because our previous studies have suggested a strong correlation between the level of cellular PBGD activity and PPIX accumulation in response to exogenously administered δ-ALA [19, 20]. The present data showed that δ-ALA-induced PBGD activity and PPIX levels in the Harderian gland do not display a strong correlation when studied under different hormonal conditions, a finding that demonstrates the complexity of the regulatory mechanisms involved in heme biosynthesis. The effect of ovariectomy on δ-ALA-induced PPIX levels in tumors was much more dramatic. Levels were reduced by more than half that obtained in tumors from intact animals treated with δ-ALA alone. However, the effect of ovariectomy on tumor PPIX synthesis may not be attributable solely to the removal of estrogens and progesterone. Reduction in circulating levels of these steroid hormones may also affect the production and/or release of pituitary hormones, specifically prolactin. Marr *et al.* [30] found that when bromocriptine, an inhibitor of prolactin secretion, was administered to castrated male hamsters, the expected increase in PPIX in the Harderian gland did not occur. These results suggest that hormones, other than androgens or estrogens, may play a regulatory role in PPIX biosynthesis in hormonally responsive tissues.

The results obtained with tamoxifen, a well-documented anti-estrogenic agent, were expected to emulate either the effects of ovariectomy or androgen administration, i.e. suppression of porphyrin production in Harderian glands. These expectations were not realized. In fact, in all three of the tissues studied, PBGD was increased after tamoxifen administration. Subsequently, δ-ALA administration further enhanced enzyme activity in the tumors and liver. Levels of δ-ALA-induced PPIX, on the other hand, were not changed significantly in tumors from animals treated with tamoxifen compared with those from animals treated with δ-ALA alone. In contrast, significant increases in δ-ALA-induced PPIX were observed for liver and Harderian glands from animals treated with tamoxifen. The differences in δ-ALA-induced PPIX among the tissues obtained from tamoxifen-treated animals may be attributed to a number of factors, of which the presence or absence and/or functionality of estrogen receptors could play a major role. Another possibility is that R3230AC tumor cells may have a threshold capacity for the production of PPIX, above which any further manipulation would have no effect. In other work, Momma *et al.* [31] observed androgen sensitivity in a prostate cancer cell line. The addition of 5α-dihydrotestosterone to cell culture media resulted in a 70% increase in PPIX accumulation compared with an androgen-insensitive tumor cell line treated under the same conditions. The effects of androgens or androgenic analogues on δ-ALA-induced PPIX accumulation will need further investigation to define their action on heme biosynthesis. Studies of the hormonally sensitive endometrium and myometrium of the rat have not produced a clear consensus of results. Yang *et al.* [32] showed that the endometrium displays δ-ALA-induced PPIX, whereas the myometrium does not. In a more recent study, Roy *et al.* [33] demonstrated that both uterine tissues in the rat produce δ-ALA-induced PPIX, with the endometrium accumulating approximately two times more PPIX than the myometrium at most of the doses of δ-ALA used. Interestingly, in contrast to our studies, they showed that ovariectomy of rats prior to δ-ALA treatment results in a significant increase in the amount of δ-ALA-induced PPIX. The disparity between their results and ours could be due to the type of tissue studied, normal uterus versus malignant mammary tissue, the route of administration of ALA, or the time course.

The most dramatic result in the present study is that tumor response to δ-ALA-based PDT was inhibited significantly when tumors were treated on ovariectomized animals. The most obvious reason appears to be the significant reduction of PPIX in these tumors. The significance and relevance of this finding are that one might be able to alter porphyrin biosynthesis by manipulating the hormonal milieu of a malignant lesion. With the proper combination of hormonal treatment, δ-ALA-based PDT might be enhanced greatly. On the other hand, caution must be taken because these data suggest that the hormonal status of the host could limit δ-ALA induction of heme biosynthesis and porphyrin accumulation, hence impacting the efficacy of δ-ALA-based PDT.

These studies demonstrating that the hormonal status of the host may modulate the effects on δ-ALA-induced biosynthesis of PPIX strongly support the need for additional research in this area. Does hormonal regulation of heme biosynthesis occur in all hormonally-responsive or -dependent malignancies? Are hormone receptors necessary mediators for hormonal regulation of heme biosynthesis, or can steroid regulation of heme biosynthesis occur without traditional receptor mediation? Further investigations in our laboratory are planned to address and answer these questions.

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