Inhibition of Leydig Tumor Cell Steroidogenesis by 10-Propargylestr-4-ene-3,17-dione, an Irreversible Aromatase Inhibitor

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INTRODUCTION

Estrogens and androgens have been shown to be involved in the maintenance of several hormone-dependent tumors (1, 2). In fact, many Leydig cell tumors found in animals are actually induced by estrogens (3). These tumors possess high affinity estrogen receptors, some in direct proportion to their sensitivity to that steroid (4). Previous research has established that the murine Leydig cell tumor (M5480A) possesses estrogen receptors and responds to low doses of estradiol by altered steroidogenesis (5). However, the role of estrogens in neoplastic Leydig cells is still unresolved. By comparison, several endogenous steroids have also been implicated in the modulation of testosterone production in normal Leydig cells (6, 7). Estrogens, in particular, have long been known to reduce testosterone production in vivo (8), partly by direct testicular actions (9–11). Recently, Darney and Ewing (12) have demonstrated that testosterone autoregulates its secretion. The mechanisms by which these steroids alter steroidogenesis are uncertain.

The testicular tumor (M5480A) used in these studies produces primarily progesterone and reduced amounts of testosterone under basal and gonadotropin-stimulated conditions (13). Earlier studies have demonstrated that low levels of estrogens are also produced by these tumor cells (5). Estrogens are produced in normal Leydig cells as well (14). Several compounds have recently been synthesized that irreversibly inactivate the aromatase enzyme complex (15, 16) and thereby drastically reduce estrogen production. These agents are presently under investigation to determine their efficacy in the treatment of hormone-dependent tumors (1). In addition, aromatase inhibitors may be useful in delineating the role of estrogens in Leydig cell function.

In light of the demonstrated effects of estrogens in these tumors, we undertook the present studies to ascertain the presence of aromatase and to investigate its sensitivity to the potent aromatase inhibitor 10-propargylestr-4-ene-3,17-dione (17). The effects of PED on Leydig tumor cell steroidogenesis were also examined, and our results suggest that PED (or metabolites), in addition to its inhibition of aromatase, exhibits effects at other sites in the steroidogenic pathway.

MATERIALS AND METHODS

Materials. All unlabeled steroids used in these studies as standards for HPLC and RIA were obtained from Steraloids (Wilton, NH). PED was synthesized as described elsewhere (17). [1,2,6,7,16,17-3H]Progestosterone, [7-3H]pregnenolone, [1,2,6,7-3H]testosterone, [1,2,6,7,16,17-3H]testosterone, and [16,23,24-3H]testosterone were purchased from New England Nuclear (Boston, MA). The progesterone antiserum was kindly provided by Dr. T. O. Abney (Medical College of Georgia), while pregnenolone antiserum was purchased from Radioassay Systems Laboratories (Carson, CA). Purified hCG (CR-121) was supplied by Dr. R. E. Canfield under the auspices of the National Institute of Child Health and Human Development (Bethesda, MD). M-199 was purchased from Gibco (Grand Island, NY), and bovine serum albumin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), were obtained from Sigma Chemical Co. (St. Louis, MO). Linbro tissue culture plates (24 wells) were purchased from Flow Laboratories (McLean, VA). A Partisil PS-X 5/25 HPLC column was acquired from Whatman (Clifton, NJ), and the Cosorasil used in the HPLC guard column was obtained from Waters Associates (Framingham, MA). HPLC grade methylene chloride, acetonitrile, and 2-...
propanol were purchased from Fisher Scientific. The diethyl ether used in steroid extractions was purchased from Mallinckrodt (Paris, KY), and dioxane, xylene, naphthalene, and 2,5-diphenyloxazole were obtained from Research Products International (Mt. Prospect, IL).

Tumor Preparation. Murine Leydig cell tumors (M5480A) were maintained by serial transplantation of C57BL/6J mice as previously described (13). Tumors were excised at Day 14 posttransplantation from mice sacrificed by cervical dislocation. Tumors to be used for cell culture were dissociated nonenzymatically by passage through a 30-mesh tissue sieve (Belco Glass, Vineland, NJ) under sterile conditions. Dispersed cells were washed in sterile M-199 containing 0.1% bovine serum albumin and incubated for 5 min at room temperature to allow cell aggregates to settle. Suspended cells were removed by aspiration and diluted in M-199-0.1% bovine serum albumin, and cell concentrations were determined by a Coulter counter (Hialeah, FL).

For the detection of aromatase, tumors were homogenized in 20 mM Tris-HCl buffer containing 250 mM sucrose and 1 mM EDTA, pH 7.4. The homogenate was centrifuged at 9,000 x g for 15 min in a Sorvall RC-2B centrifuge, and the resultant supernatant was centrifuged at 105,000 x g for 1 h in a Beckman L2-65B ultracentrifuge. The pellet from this centrifugation was resuspended in the same buffer without sucrose and centrifuged again. This crude microsomal pellet was then suspended in 10% glycerol:20 mM Tris-HCl buffer:250 mM sucrose:1 mM EDTA (pH 7.4) and either stored at -70°C or used immediately to assess aromatase activity. Microsome preparations from human placenta were also prepared by the same method and used in the aromatase assay as a positive control.

Aromatase Assay. Aromatase activity in tumor microsome preparations was assayed according to a method modified from Rabe et al. (18). Briefly, aliquots of the microsome preparation (0.7 ml of a solution containing about 7 mg of microsomal protein per ml) were added to 2.5 ml of assay buffer (30 mM Tris-HCl:250 mM sucrose:6.25 mM MgCl2:1 mM CaCl2, pH 7.4), containing 1 mM NAPDH, an NAPDH regenerating system (6.5 mM glucose-6-phosphate and 0.5 units of glucose-6-phosphate dehydrogenase per ml), and varying amounts of tritiated testosterone. In the inhibition experiments, the inhibitor or its carrier solvent was added to the assay buffer prior to addition of the microsome preparation. These samples were incubated in a shaking water bath at 37°C. At various times, 0.1-ml aliquots were taken from the vials, added to 10 ml of scintillation cocktail (666 ml of dioxane, 330 ml of xylene, 80 g of naphthalene, and 5 g of 2,5-diphenyloxazole), and counted, yielding "total counts." Water (2 ml) was then added to each vial, causing a phase separation in which the aqueous phase contained all the tritiated water produced as a result of aromatization. The vials were then counted again yielding "steroid counts." The amount of tritiated water was determined as (total counts) minus (steroid counts), and a plot of this parameter (which is proportional to the amount of estrogen produced) versus time yields enzyme velocity. The velocity thus derived was corrected for the 25% of the tritium in the a-position of the 1,2,3-tritiated testosterone used as substrate.

To validate the aromatase assay in this Leydig cell tumor, an experiment was performed using the method of Thompson and Sitteri (19). Aliquots (0.2 ml) of the incubation mixture from Leydig cell tumor microsomes were added to 1 ml of chloroform, vortexed, and centrifuged, and then 0.1 ml of the aqueous layer was counted. Alternatively, the above method was modified using Whatman PS phase separator filter papers to separate the organic and aqueous phases. Within experimental error, the results from these methods were comparable to those obtained using the technique of Rabe et al. (18).

Primary Cell Culture. Aliquots of dispersed Leydig tumor cells were added to culture wells to attain a final concentration of 1-1.5 x 10⁶ cells/ml. Cells were subsequently incubated for 1, 3, 6, 12, or 24 h in a humidified CO2 incubator maintained at 37°C under 95% air:5% CO2. PED (0.1-10 μM) was first dissolved in ethanol, then diluted in culture medium, and finally added to selected culture wells either alone or in the presence of hCG (50 ng/ml). Preliminary experiments were performed to ensure that the small percentage of ethanol present in cultures was innocuous. The 50-ng/ml dose of hCG had previously been determined to stimulate steroidogenesis maximally. After incubating the cultures for the designated times, the culture medium was aspirated from the wells and stored at -20°C for subsequent analysis. In the studies in which steroidogenesis was monitored by HPLC, 1 ng of [3H]progesterone and 100 ng of unlabeled progesterone were added to the culture wells 1 h before the termination of the experiment. Preliminary experiments were performed with various concentrations of unlabeled progesterone to establish a proper ratio of labeled to unlabeled progesterone to ensure that the labeled substrate was not limiting. In other experiments, [3H]testosterone was added to primary cultures in the presence or absence of various doses of PED. Metabolism of this steroid was evaluated by HPLC. All studies were performed at least twice with triplicate measurements at each data point.

Steroid Radioimmunoassays. Progesterone and pregnenolone RIAs were performed on unextracted dilutions of the culture medium according to the method of Nieschlag and Loriaux (20). Preliminary assays were run to ensure that neither PED nor the culture medium significantly altered the results of the specific RIAs. However, in preliminary testosterone and estradiol RIAs, it was noted that native PED exhibited significant cross-reactivity with these two particular antisera.

High-Performance Liquid Chromatography. Prior to HPLC analysis, steroids in culture medium were extracted with 5 volumes of diethyl ether (v/v). The organic phase was separated from the aqueous medium following freezing in a dry ice:acetone bath. The ether extracts were subsequently dried under N2 and reconstituted in 50 μl of HPLC solvent [methylen chloride:acetonitrile:propanol (88:10:1)]. Steroid separation was achieved at ambient temperature on a Whatman Partisil column (0.46 x 25 cm) using an isocratic solvent system. Solvent flow rate was 1 ml/min with a back pressure of 900 psi. UV absorbance was monitored at 235 nm by an LDC SpectroMonitor III (Riviera Beach, FL). Standard solutions of progesterone, androstenedione, estradiol, 17α-hydroxyprogesterone, and testosterone were periodically applied to the column both singly and in combination to monitor column stability, and relative retention times of 6.7, 7.5, 8.8, 10.6, and 13.0 min, respectively, were observed. Chart 1 depicts a representative chromatogram of equimolar steroid standards of progesterone (P), androstenedione (A), 17β-estradiol (E), 17α-hydroxyprogesterone (17α), and testosterone (T). Oxidoreduction of steroids was monitored at 236 nm. All steroids were dissolved in HPLC solvent consisting of methylene chloride, acetonitrile, and 2-propanol (88:10:1).

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amounts of these standards. In addition, a standard solution of PED was chromatographed to determine its respective retention and absorbance in comparison to the steroid standards. The PED chromatographed as a single peak with a retention time of 7.8 min. HPLC column fractions were collected and monitored for radioactivity in a Beckman liquid scintillation spectrometer.

RESULTS

Leydig Tumor Aromatase Activity. Murine Leydig cell tumor microsomal preparations were examined to determine the presence of aromatase activity, and Chart 2 shows the results of a typical experiment. A significant increase in tritiated water production was observed over time in tumor microsomal preparations; this amounts to approximately 100 pmol/g of protein/h. Control incubations containing only assay buffer or heat-inactivated microsomes did not exhibit any time-dependent change in tritiated water production.

To assure that the tritiated water production was indeed due to aromatase, and secondarily to examine the efficacy of the aromatase inhibitor PED, various doses of PED were incubated with tumor microsomes. It was found that PED did inhibit tritiated water production by the tumor microsomes, but it appeared much less potent in the tumor than in human placenta (Table 1). Variability was noted in the apparent aromatase activity of the primary tumor microsomes, and this led to differing efficacies of PED in independent assays.

Effects of PED on Progesterone Synthesis. In order to investigate the effects of PED on Leydig tumor cell steroidogenesis, dispersed cells were maintained in primary culture and incubated with graded doses of PED for varying periods of time. Culture medium steroid levels were evaluated by specific RIA. A dose- and time-dependent decrease in basal progesterone concentrations was observed following the addition of PED (Chart 3A). The lowest dose of PED examined (0.1 μM) was effective only at 1 h, reducing progesterone levels by 11%. In contrast, 1 μM PED inhibited progesterone levels throughout the incubation period and, paradoxically, appeared more efficacious than the higher dose of PED at early time points.

Examination of medium progesterone levels from gonadotropin-stimulated cells revealed a similar dose- and time-dependent phenomenon (Chart 3B). Again, a significant reduction of progesterone levels was observed only at 1 h when 0.1 μM PED was added to cultures. An apparent lack of dose dependence also occurred with the higher doses of PED for short periods of culture. However, by 3 h, 10 μM PED reduced progesterone concentrations to a greater degree. Even after 24 h, medium progesterone levels were less than 30% of control levels for the highest dose of PED. By 48 h, the 1 μM dose was no longer effective, while 10 μM still significantly reduced medium progesterone (data not shown). The addition of estradiol to cultures did not reverse the effects of PED.

Effects of PED on Pregnenolone Synthesis. Since the observed reductions in medium progesterone could be due to either increased metabolism or decreased synthesis, experiments were undertaken to examine other steroids. Preliminary RIAs revealed a significant cross-reactivity of PED with the testosterone and estradiol antisera, thereby precluding any examination of those steroids by this technique. However, no interference from PED was observed in a pregnenolone RIA. Therefore, medium pregnenolone levels were examined as an indicator of steroid synthesis. In these experiments, 10 μM PED was incubated with Leydig tumor cells for various periods of time under basal or hCG-stimulated conditions. As can be seen in Chart 4A, basal medium pregnenolone values were not affected at 1 and 3 h, but thereafter a significant reduction in pregnenolone was observed. A similar result was found when hCG was added to the cultures (Chart 4B).

Effects of PED on Progesterone Metabolism. Despite the concordance in media levels of both progesterone and pregnenolone, the observed decreases may have been due to increased metabolism of these steroids. In order to examine this possibility, studies were undertaken to determine the fate of labeled progesterone added to Leydig cell cultures, with metabolism being monitored by HPLC. The absorbance profiles (235 nm) obtained from HPLC of media extracts from basal and hCG-stimulated cultures were similar and have been omitted. One major difference in PED-treated cultures was a prominent absorbance peak comigrating with native PED. Another significant absorbance peak unique to the PED-treated cultures comigrated with testosterone.
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Chart 3. A, basal progesterone levels following incubation of dispersed Leydig tumor cells with 0 (○), 0.1 μM (●), 1 μM (△), and 10 μM (▲) PED. Cells were incubated for various times at 37°C, and the culture medium was aspirated and frozen for future analysis by specific progesterone radioimmunoassay. Statistically significant decreases in medium progesterone levels were observed under the following conditions: 0.1 μM PED, 1 and 6 h; 1 μM, all times; 10 μM, all times except 1 h. B, progesterone levels from gonadotropin-stimulated Leydig tumor cells incubated with graded doses of PED. Statistically significant decreases in progesterone levels were observed at 1 h for the 0.1 μM dose of PED and at all time points for the 1 and 10 μM doses. Bars, SE.

Chart 4. A, medium pregnenolone levels obtained from cultures of Leydig tumor cells incubated with 10 μM PED. ●, medium pregnenolone levels as determined by RIA under control conditions; ▲, results observed upon addition of 10 μM PED. Significant reductions in medium pregnenolone levels were observed at 6, 12, and 24 h. B, medium pregnenolone levels from cultured Leydig tumor cells coincubated with a maximally stimulating dose of hCG (50 ng/ml) and 10 μM PED. Significant decreases in pregnenolone levels were seen at 6, 12, and 24 h. Bars, SE.

Chart 5 shows the radioactive steroid profiles of extracted media from cultures incubated for 1 h with and without a maximally stimulating dose of hCG. A distinct radioactive progesterone peak was observed in Fraction 13 (retention time = 6.7 min). Although other absorbance peaks were noted, the only other radioactive peak observed was one comigrating with testosterone. No significant difference in the rate of testosterone production between basal and hCG-stimulated cultures was observed at either 1 or 12 h. Even though 17α-hydroxyprogesterone and androstenedione are intermediates in the Δ4-pathway, no other major radioactive peaks were detected. One minor, yet reproducible, peak comigrated with estradiol and represented less than 3% of the recovered counts. However, part of this component may represent labeled dihydrotestosterone which was found to elute very near estradiol. No evidence for altered or enhanced progesterone metabolism was detected when hCG was added to the cell cultures.

With this evidence, we then examined the effects of 10 μM PED on the metabolism of labeled progesterone by the cells. Except for the previously mentioned absorbance peaks arising from PED and a metabolite, no alteration in the steroid absorbance profile was observed upon addition of PED.

Despite the observation that comparable amounts of labeled progesterone were metabolized regardless of treatment, the amount of radioactivity migrating as labeled testosterone was dramatically reduced when PED was added to cell cultures (Chart 5). While approximately 22% of total counts were associated with testosterone under basal or hCG-stimulated conditions, only 5–6% of the radioactivity appeared as labeled testosterone following the addition of 10 μM PED. This represents a reduction of greater than 70% in the conversion of progesterone to testo-
treated cultures, but none represented more than 4% of the total column radioactivity. Because of their small size, it was difficult to correlate these peaks with any of the standards. The effects of PED could not be reversed by the coinubcation of 17β-estradiol and PED with Leydig tumor cells.

To examine further the effects of PED on Leydig tumor cells, we added labeled testosterone to cells in primary culture in order to determine whether PED altered the conversion of that steroid. Results of control experiments revealed very little metabolism of testosterone. Less than 1% of the radioactivity comigrated with estradiol, and therefore we cannot evaluate the effects of PED on the aromatase activity in cultured cells.

**DISCUSSION**

These studies have demonstrated that PED or a metabolite inhibits both basal and hCG-stimulated steroidogenesis at multiple sites in the M5480 Leydig tumor cells in vitro. In addition to the inhibition of microsomal aromatase activity, another action was manifested by a decreased conversion of labeled progesterone to testosterone. However, even with this diminished progesterone metabolism in the presence of PED, medium progesterone levels continued to fall. This observation indicates that the addition of PED serves to inhibit progesterone synthesis. A multiplicity of effects has been described for antisteroidogenic compounds such as aminogluthethimide, and these studies demonstrate multiple effects of a potent aromatase inhibitor such as PED within a hormone-responsive tumor.

PED has previously been shown to be the most potent aromatase inhibitor in several tissues (17, 21), and we have confirmed this in human placenta. By comparison, placental microsomes exhibited approximately 5000 times as much aromatase activity as this Leydig cell tumor (e.g., 80 pg of estradiol per mg of tumor microsomal protein per 3 h). The inability of PED to inhibit all of the apparent aromatase activity in this tumor may in part be due to the presence of water-soluble metabolites of the labeled testosterone.

The most pronounced effect of PED in the Leydig tumor cells is the inhibition of progesterone synthesis. The concomitant decrease in medium pregnenolone concentrations further implies that the early site of action for PED occurs prior to 3β-hydroxysteroid dehydrogenase, suggesting an effect either within the mitochondrion or upon cholesterol transport or synthesis. There is also considerable evidence suggesting that gonadotropins and several steroids act at a site prior to progesterone synthesis (22-24). In addition, aminogluthethimide inhibits steroidogenesis at a level preceding progesterone formation (25), and Donaldson et al. (26) reported that ATD displayed antisteroidogenic effects in male rats. We have also found that ATD reduces progesterone levels in cultured Leydig tumor cells. Recently, we have shown that estrogens similarly inhibit progesterone synthesis in Leydig tumor cells (5). However, the current studies demonstrate that PED decreases medium progesterone levels to a much greater degree than estrogen. It is therefore unlikely that the effects of PED are due solely to its action as an aromatase inhibitor.

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**Chart 5.** Chromatographic profile of radioactive steroids extracted from Leydig tumor cell cultures under basal and hCG-stimulated conditions in the presence or absence of 10 μM PED. The column was standardized with progesterone (P), androstenedione (A), 17β-estradiol (E), 17α-hydroxyprogesterone (17α), and testosterone (T) dissolved in the column solvent. Steroid standards are marked by arrows. In A, dispersed cells were incubated with [3H]progesterone for 1 h, and excess unlabeled progesterone was added to the cultures to limit metabolism of the labeled progesterone. Three radioactive peaks were evident. The major peak comigrated with progesterone, the next largest peak comigrated with testosterone standard, and a third peak eluting prior to progesterone is as yet unidentified. In B, dispersed cells were incubated with [3H]progesterone and hCG (50 ng/ml) for 1 h. The conversion of progesterone to testosterone was similar to that found under basal conditions. In addition to the peaks observed in basal cultures, a more distinct radioactive peak comigrating with estradiol was observed; however, tritiated dihydrotestosterone was found to migrate very near this peak and may therefore contribute to this radioactive peak. In C, dispersed cells were incubated under basal conditions for 1 h with labeled progesterone and 10 μM PED. The addition of PED to cell cultures reduced the conversion of progesterone to testosterone by 75%. In D, gonadotropin-stimulated cell cultures were incubated for 1 h with labeled progesterone in the presence of 10 μM PED. Results of these incubations were quantitatively and qualitatively similar to those under basal conditions.

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ever, it is still unknown whether this enzyme from gonadal tissue may be affected by PED in vivo or in vitro.

The delayed reductions in medium progesterone levels observed in these studies may be in part explained by the apparent inhibition of testosterone production by PED. This possibility is supported by the observed increases in radioactive peaks co-migrating with androstenedione and progesterone upon the addition of PED to cultures (Chart 5). The eventual decreases in medium progesterone levels may result from a prolonged inhibition of progesterone synthesis coupled with an incomplete inhibition of testosterone production. One possible site of action for PED may be at the level of the late regulatory site described by Catt and coworkers (28, 29). They localized this site to the 17α-hydroxylase:17,20-lyase complex and found it to be sensitive to gonadotropins and steroids. Since most aromatase inhibitors, including PED, are androstenedione derivatives, the observed decrease in testosterone levels may in part be due to a competitive inhibition at the level of 17-ketosteroid reductase. The partial compensation in radioactive peaks co-migrating with androstenedione following PED treatment would support this hypothesis. We are uncertain whether the effects of PED are due to action on the regulatory site, on the enzyme, or both.

It is difficult to reconcile the effects of PED in Leydig tumor cells solely with its putative role as an antiaromatase. As mentioned previously, another aromatase inhibitor, ATD, has been reported to display effects independent of its actions on the aromatase complex (26, 30). Brodie et al. (15) have also presented evidence to suggest that some of the effects of 4-hydroxandrostenedione cannot be reversed by estradiol. Consequently, the actions and effects of these aromatase inhibitors may be more complex than first thought. Even though they act quite effectively as aromatase inhibitors, several of these compounds or their metabolites exert other effects that appear to be independent of estrogens. These multiple effects may, in part, explain the lack of dose dependency of PED observed after short incubation times in our studies. The additional actions of these inhibitors or their metabolites may serve to heighten their efficacy as antitumor agents. It is well established that many endocrine-dependent tumors may require several hormones for growth (31), and several novel therapeutic regimens have exploited this situation (32, 33). Therefore, a multiplicity of actions by the aromatase inhibitors, resulting in a reduction not only of estrogens but other steroids as well, may be quite beneficial.

In summary, these studies have demonstrated that the addition of PED to primary cultures of M5480A Leydig tumor cells inhibits steroidogenesis at multiple sites. In addition to inhibiting aromatase activity, PED impairs the conversion of progesterone to testosterone, possibly by competitive inhibition of steroidogenic enzymes. Furthermore, PED reduces both progesterone and pregnenolone production. It is unknown whether PED is acting at an early steroid-sensitive regulatory site or whether it is having other actions, such as altering cholesterol synthesis or transport. Regardless of the mechanism of action of this compound, its effects on tumor cells may prove quite valuable in inhibiting growth of hormone-dependent tumors.

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