Effect of 4-Hydroxyandrostenedione on Murine Leydig Tumor Cell Steroidogenesis

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ABSTRACT

The murine Leydig cell tumor (M548OA) possesses high levels of estrogen receptors and is known to produce estrogens. In these studies we examined the effects of the potent aromatase inhibitor 4-hydroxyandrostenedione (4-OHA) on Leydig tumor cell steroidogenesis both in vitro and in vivo. The addition of 4-OHA to Leydig tumor cells in primary culture resulted in a dose- and a time-dependent decrease in media progesterone levels. The observed decrease was most likely due to impaired synthesis of progesterone, inasmuch as no alteration in progesterone metabolism was seen when progesterone levels were diminishing. However, 4-OHA inhibited progesterone conversion to testosterone following 1 h of incubation, but this effect disappeared coincident with 4-OHA metabolism. Analysis of pregnenolone production revealed a biphasic dose-dependent effect of 4-OHA. At low doses (0.01-0.1 μM), 4-OHA was found to decrease pregnenolone concentrations, while at higher doses (1-10 μM) pregnenolone levels were elevated. Therefore, the actions of 4-OHA on Leydig cell steroidogenesis in vitro appear to be multifocal. Other experiments were performed to evaluate the effects of 4-OHA on tumor-bearing male mice in vivo. In these studies, the predominant effects of 4-OHA were to act as an aromatase inhibitor and to inhibit progesterone production. Thus, while 4-OHA is a potent aromatase inhibitor, we have found that this compound may alter steroidogenesis in Leydig tumor cells at several sites prior to aromatization.

INTRODUCTION

Estrogens have been implicated in the induction and maintenance of many types of tumors (1–3). Many Leydig cell tumors possess estrogen receptors and have the capacity to aromatize androgens to estrogens (4, 5). The gonadotropins, luteinizing hormone and hCG, can increase estrogen production in Leydig cells both by stimulating the activity of endogenous aromatase and by the production of new enzyme (6–8). Although it has been suggested that intracellular estrogen production is involved with the maintenance of Leydig cell responsiveness (9), considerable uncertainty remains as to the specific role of estrogens within Leydig cells (10).

As an aid in delineating the functions of estrogens in cells in which they are also produced, specific inhibitors have been used to inactivate the aromatase cytochrome P-450 (11). Several of these agents, specifically 4-OHA, androstatrienedione, and PED, have been identified as putative suicide substrates for aromatase and have been reported to irreversibly inactivate this enzyme (12). By drastically reducing endogenous aromatization, the impact of intracellular estrogens on cellular functions may become more obvious. Determining the role of estrogens in cells is complicated by the purported dose-dependent biphasic action of estrogens both in normal cells and in estrogen-dependent tumors (13–15).

Several aromatase inhibitors, most notably 4-OHA, are currently being evaluated as potential therapeutic agents in the treatment of estrogen-dependent tumors (11, 16). Recent clinical studies suggest that 4-OHA may be effective in the management of human breast cancer (17). Furthermore, other studies have suggested that aromatase inhibitors may also be useful in the treatment of benign prostatic hypertrophy and possibly some ovarian cancers (16, 18).

Although these agents are considered highly specific, several studies have suggested that some aromatase inhibitors (or their metabolites) may have secondary effects, particularly at relatively high concentrations (19, 20). Some of the aromatase-independent effects of 4-OHA may in part be explained by influences on androgen synthesis and/or action (19, 21). Therefore, the present study was undertaken to examine the effects of 4-OHA on Leydig tumor cell (M548OA) steroidogenesis. Previous studies from our laboratory suggested that exogenous estradiol inhibits steroidogenesis in these cells (4). Furthermore, another aromatase inhibitor, PED, was found to have multiple effects on Leydig cell steroidogenesis at μM concentrations (20). In the current studies we examined the effects of 4-OHA in Leydig tumor cells under in vitro and in vivo conditions. Our results suggest that, at μM concentrations, 4-OHA also differentially acts at multiple sites in the steroidogenic pathway.

MATERIALS AND METHODS

Materials. A Partisil PX S 5/25 HPLC column was purchased from Whatman (Clifton, NJ). Corasil was obtained from Waters Assoc. (Framingham, MA), and HPLC grade methylene chloride, acetonitrile, and 2-propanol were from Fisher Scientific (Pittsburgh, PA). The aromatase inhibitor, 4-OHA, was synthesized as described earlier (22).

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The abbreviations used are: hCG, human chorionic gonadotropin; 4-OHA, 4-hydroxyandrostenedione; PED, 10-propargylestr-4-ene-3,17-dione; HPLC, high performance liquid chromatography; BSA, bovine serum albumin; 3Δ-HSD, 3Δ-hydroxysteroid dehydrogenase.

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Effects of 4-OHA on Leydig Tumor Cell Progesterone Levels. Since progesterone is the major steroid product of this tumor (4), we examined the effects of 4-OHA (10 μM) on progesterone production. At various times after the addition of 4-OHA (1–48 h), the culture media were recovered and subsequently analyzed for progesterone production by specific radioimmunoassay. Because of variations in basal steroidogenesis between separate experiments, the data shown are the results of a typical study. 4-OHA had no apparent effect on media progesterone levels for up to 6 h in culture (Fig. 1). However, after that time a decrease of about 25% in progesterone concentration was observed. By 48 h, progesterone concentrations had returned to control levels (data not shown).

Incubation of cells with graded doses of 4-OHA for 12 h resulted in significant reductions in media progesterone, e.g., 12 and 23% for the 1 and 10 μM doses, respectively (Fig. 2). Although dose-dependent lowering of progesterone levels was also observed in the presence of hCG, on the whole, progesterone concentrations were reduced to a lesser degree. For example, the 10 μM dose of 4-OHA significantly lowered progesterone values by approximately 10% in the presence of hCG, while no significant effect was observed at 0.1 or 1 μM 4-OHA (data not shown).

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Statistics. All experiments were performed at least twice in triplicate, and the data are reported as mean ± SEM. Differences between experimental groups were evaluated using Student’s t test. P < 0.05 was selected as the limit of statistical significance.

RESULTS

Effects of 4-OHA on Leydig Tumor Cell Progesterone Levels. Since progesterone is the major steroid product of this tumor (4), we examined the effects of 4-OHA (10 μM) on progesterone production. At various times after the addition of 4-OHA (1–48 h), the culture media were recovered and subsequently analyzed for progesterone production by specific radioimmunoassay. Because of variations in basal steroidogenesis between separate experiments, the data shown are the results of a typical study. 4-OHA had no apparent effect on media progesterone levels for up to 6 h in culture (Fig. 1). However, after that time a decrease of about 25% in progesterone concentration was observed. By 48 h, progesterone concentrations had returned to control levels (data not shown).

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Effect of 4-OHA on the Conversion of Progesterone to Testosterone. Since progesterone is an intermediate in the Leydig cell steroidogenic pathway, the decreased progesterone levels observed in the above experiments could be due to either decreased synthesis or increased metabolism. To determine which mechanism is operative, Leydig tumor cells were incubated in medium containing [3H]progesterone and the effects of 4-OHA on the conversion of labeled progesterone to testosterone examined by HPLC. The radioactive steroid profile from ether extracts of culture media incubated for 1 h under basal conditions is shown in Fig. 3. Following scintillation counting of column fractions, two major peaks of radioactivity could be correlated with definite steroid absorbance maxima. The larger of the two radioactive peaks comigrated with the progesterone standard, while the other major peak comigrated with testosterone. Another minor (less than 3% of recoverable counts), yet reproducible, radioactive peak was observed which comigrated with the estradiol standard. The magnitude of the estradiol peak was insufficient to evaluate estrogen production by HPLC.

Fig. 3 also displays the radioactivity profiles of Leydig cell cultures incubated with 10 µM 4-OHA for 1 h. These profiles were qualitatively similar to those obtained under control conditions, but the magnitude of the testosterone peak was dramatically reduced. After a 1-h incubation of 4-OHA with the cells, significant inhibition (approximately 65%) of conversion occurred. Under basal conditions 19% of [3H]progesterone was converted to testosterone, while in the presence of 4-OHA conversion dropped to less than 7% (6.6 ± 0.1%; P < 0.001).

These results were the same regardless of hCG stimulation. Examination of progesterone conversion following 12 h of culture revealed 4-OHA to be ineffective in either control or hCG-stimulated Leydig tumor cells (data not shown). The loss of this inhibitory effect coincided with the appearance of a new nonradioactive absorbance peak unique to cultures containing 4-OHA, suggesting that 4-OHA was metabolized by the Leydig tumor cells.

Effect of 4-OHA on Pregnenolone Levels. To evaluate the effects of 4-OHA on progesterone synthesis, pregnenolone levels were monitored in the presence or absence of 10 µM 4-OHA. In contrast to basal progesterone levels, significant increases in pregnenolone concentrations were observed from 1 to 12 h of culture in the presence of 4-OHA (Fig. 4). Similarly, hCG-stimulated pregnenolone levels were also elevated over the same period of culture when 4-OHA was present in the medium (data not shown).

Moreover, the effect of 4-OHA on media pregnenolone levels was evident at 1 h. Interestingly, the incubation of various doses of 4-OHA (0.01–10 µM) with Leydig tumor cells for 1 h revealed a biphasic response (Fig. 5). Low doses (0.01–0.1 µM) slightly inhibited media pregnenolone values, while high doses (10 µM) increased the media levels of this steroid. These effects on pregnenolone levels by 4-OHA were observed even though there were no significant effects on progesterone levels at this time regardless of dose (Fig. 1).6

Effect of 4-OHA on Leydig Tumor Cell Steroidogenesis in Vivo. Studies were undertaken to determine the predominant effects of 4-OHA on solid Leydig cell tumors in male mice. Serum progesterone levels were examined from tumor-bearing mice given injections of 4-OHA. Table 1 shows that even after 12 h, 4-OHA inhibits basal progesterone levels by more than 20% but was not effective in the presence of maximally stimulating doses of hCG. These results are comparable to those observed following 12 h of primary culture (cf. Fig. 1).

Since we have previously demonstrated that these tumors possess low levels of aromatase activity and produce estradiol (20), the effect of in vivo administration of 4-OHA on tumor...
aromatase activity was also evaluated. 4-OHA was found to significantly inhibit tumor aromatase activity 12 h after the second injection (Fig. 6). The observed activity in 4-OHA-treated animals was 39 ± 6% (averaged from two separate experiments) lower than controls. Similar inhibition was observed under hCG-stimulated conditions (data not shown).

DISCUSSION

These studies demonstrate that 4-OHA leads to dose and time-dependent alterations in Leydig tumor cell steroidogenesis both in vivo and in vitro. The multiplicity of effects, resulting from the addition of 4-OHA to Leydig tumor cells, suggests that this agent has actions at several sites in the steroidogenic pathway. Moreover, the finding that some of these enzymatic sites were either unaffected by exogenous estrogen or affected similarly (4) suggests that some of the actions of 4-OHA in the Leydig cell tumor may be independent of aromatase inhibition.

Previously, 4-OHA has been shown to be a potent aromatase inhibitor and to be effective in altering estrogen-responsive processes, including the regression of some breast tumors (26). Recent evidence suggests that this agent may also be effective in the treatment of androgen-dependent disorders in that it is a weak androgenic activity, its potency as an aromatase inhibitor more than compensates for this action.

We undertook the present studies to examine the effects of 4-OHA in a steroidogenic tumor model. As expected from earlier studies (11, 12), 4-OHA was observed to inhibit aromatase activity. Furthermore, in primary cultures of Leydig tumor cells and in tumor-bearing mice, 4-OHA was observed to significantly reduce progesterone levels after 12 h. In culture this effect persisted for more than 24 h under both basal and hCG-stimulated conditions. 4-OHA also inhibited progesterone conversion to testosterone, presumably at 17-hydroxylase/17,20-lyase. These results are consistent with our earlier results with another aromatase inhibitor, PED (20).

In contrast to our studies with PED (20), however, media pregnenolone levels did not correspond to those of progesterone following 4-OHA administration; in fact, a biphasic dose-dependent response was observed. At low doses, 4-OHA slightly lowered media pregnenolone levels, while at 10 μM, 4-OHA increased pregnenolone levels, even as progesterone levels remained unchanged. The dramatic increase in media pregnenolone levels may in part arise from an inhibition of androgen production in these cells and an accumulation of precursors. However, since alterations in pregnenolone levels were observed prior to any significant rise in progesterone levels this conclusion is unlikely. Rather these results suggest that 4-OHA has effects both prior to and at the level of 3β-HSD/isomerase.

Numerous studies have suggested that other aromatase inhibitors and steroid hormones may alter steroidogenesis at an early site (20, 28, 29). Our previous studies suggested that estradiol may affect Leydig tumor cells at 3β-HSD (4), and a similar site of action has been observed in other steroidogenic cells (30, 31). However, the magnitude of the effect of 4-OHA was somewhat greater than that previously observed in these cells with other aromatase inhibitors and with estradiol (4, 20).

As mentioned in the results, the restoration of progesterone conversion to testosterone was coincident with the appearance of a new absorbance peak unique to cultures incubated with 4-OHA. These results suggest that not only is 4-OHA metabolized by these cells but also that the effect of 4-OHA on progesterone conversion is probably attributable to the native compound and not a metabolite. In comparison, the aromatase

![Graph](image-url)
inhibitor PED was observed to impair progesterone conversion even at 12 h, a time at which it had been only partially metabolized (20). Since these aromatase inhibitors are androstenedione derivatives, they may, at high doses, act as competitive substrates for 17-ketosteroid reductase. Alternatively, these agents may be acting indirectly at the late steroidogenic regulatory site, e.g., the 17α-hydroxylase-17,20-lyase described by Catt et al. (32). Since this latter enzyme has been shown to be estrogen-responsive (33), it is uncertain whether the observed effects of 4-OHA are direct or indirect.

In summary, 4-OHA has multiple sites of action on Leydig tumor cell steroidogenesis. At 1 h, 4-OHA inhibited the conversion of progesterone to testosterone, possibly at 17-hydroxylation, while progesterone levels remained unaltered. Irrespective of the effects of 4-OHA on isolated cells, its predominant actions in vivo may prove most interesting. Brodie et al. (34) have clearly demonstrated that 4-OHA has prolonged effects in vivo. In our study, using a different animal model, but a comparable dose, Leydig cell tumor aromatase activity was significantly inhibited by 4-OHA even after 12 h. In addition, consistent with the cell culture results, serum progesterone levels were also decreased. Recently, Pickles et al. (35) have reported that continued administration of 4-OHA to patients resulted in a transient reduction in some adrenal steroids in addition to its effects on estrogen production, while serum progesterone levels were minimally reduced after 2 weeks. Several studies have observed 4-OHA to be metabolized by cells both in culture and in vivo (36, 37). However, some of the effects of 4-OHA persist despite this metabolism, suggesting that bioactive metabolites may be produced (36). The prolonged effects of 4-OHA in mice, rats, and humans are consistent with the proposal that bioactive metabolites of 4-OHA exist.

In conclusion, our studies have shown that the potent aromatase inhibitor 4-OHA is also an antisteroidogenic compound acting at several sites in the steroidogenic pathway. The sites of action displayed differential sensitivity to 4-OHA in a dose- and time-dependent manner; furthermore, 4-OHA was found to be efficacious both in vitro and in vivo. Our results suggest that 4-OHA may possess antisteroidogenic actions which may complement its actions as an aromatase inhibitor.

REFERENCES


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