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Aminoglutethimide, a Steroidogenesis Inhibitor, Abolishes Hormonal Induction of Ornithine Decarboxylase in Steroidogenic Tissues: Evidence for Its Role as cAMP-Dependent Protein Kinase Inhibitor

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Aminoglutethimide (AMG), a potent inhibitor of steroidogenesis used in the treatment of breast cancer and some adrenal pathologies, abolished the induction of ornithine decarboxylase (ODC) elicited by peptide hormones and by dibutyryl-cAMP in steroidogenic tissues. This effect seems to be related to an inhibition of cAMP-dependent protein kinase (IC_{50} = 287 μM) rather than blockade of the steroidogenic pathway. This inhibition may explain some of the effects observed in AMG treatment which cannot be ascribed to its direct effect on the cytochrome P450scc complex or aromatase. Taking into account that ODC, the rate-limiting enzyme in polyamine synthesis, is elevated in many types of cancer and that overexpression of this enzyme is associated with cell transformation, one may speculate that the inhibitory action of AMG on protein kinase A represents a positive colateral effect of this drug in cancer therapy.

Key Words: **aminoglutethimide; steroid hormones; steroidogenesis; ornithine decarboxylase; hCG; ACTH; protein kinase A.**

Steroidogenic tissues such as adrenal glands and gonads synthesize and secrete steroid hormones in response to pituitary hormones such as corticotropin (ACTH) or luteotropin (LH) (1–3). The binding of these peptide hormones to their cognate receptors is coupled to the formation of cAMP and activation of the protein kinase A signaling pathway (4, 5). This promotes a rapid and acute steroidogenic response mediated by rapid activation of the complex that constitutes the rate limiting step in steroidogenesis which is followed by the ulterior stimulation of the transcription of some steroidogenic genes through both CRE-CREB-dependent and -independent mechanisms (5).

Many different *in vivo* and *in vitro* studies have shown that a common response of steroidogenic cells to ACTH and gonadotropins is the rapid induction on ornithine decarboxylase (ODC), the rate-limiting enzyme in the biosynthesis of polyamines (6, 7). These ubiquitous polycations are implicated in different aspects of cell physiology such as growth, differentiation, transformation, and death (8–12). Although there is clear evidence that the induction of ODC in steroidogenic cells is dependent on cAMP production (13) little is known about the components of the signaling cascade implicated in ODC activation. Moreover, the biological function of this induction is still an unanswered question. The fact that the activation of steroid synthesis precedes the rise in ODC activity suggests that steroids participate in the activation of ODC expression. Furthermore, in other tissues such as mouse kidney (15), rat prostate (16), and rodent uterus (17), sex steroids have a relevant role in the regulation of ODC activity.

In the present study we have compared the effect of human chorionic gonadotropin (hCG) and ACTH on ODC induction in gonads and adrenal glands, in the absence and presence of AMG or ketoconazole, two well known inhibitors of steroidogenesis (18–20). The results obtained indicate that AMG affects ODC induction not by decreasing steroid hormone concentration but by an inhibitory action on cAMP-dependent protein kinase.

MATERIALS AND METHODS

Animals and treatments. 26-day-old Swiss CD1 male mice were treated with 25 IU of hCG (Sigma Chemical Co., St. Louis, MO) or saline and killed 5 h after injection. The testes were dissected; one was used to measure ODC activity and the contralateral one for

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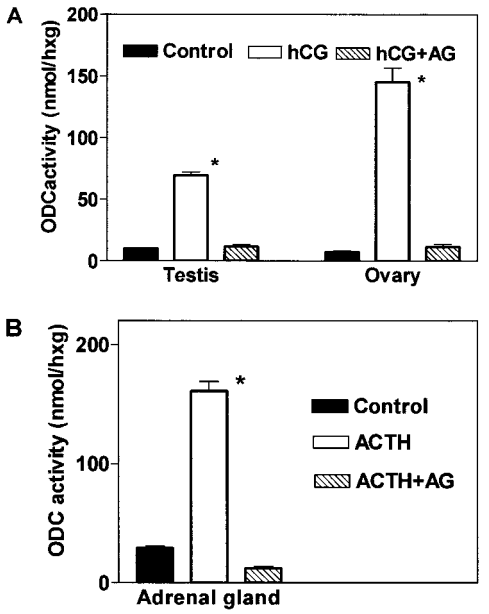

FIG. 1. Effect of AMG on hormonal induction of ODC. (A) Gonadal ODC: 26-day-old male and adult females were treated with 25 IU hCG or hCG + AMG (150 mg/kg) and killed 5 h after injection. (B) Adrenal ODC: adult female mice were treated with 50 μg ACTH or ACTH + AMG and killed 5 h after injection. Results are the means ± SEM from 4–6 animals per group. *$P < 0.001$ vs control and hormone + AMG.

determination of testosterone concentration. Adult female mice were treated with 25 IU of hCG or 50 μg of human $ACTH_{1-24}$ (Calbiochem, Darmstadt, Germany) and the ovaries or adrenal glands were removed 5 h after injection. AMG, [3-(4-aminophenyl)-3-ethyl-2,6-piperidinedione)] (Sigma Chemical Co., St. Louis, MO), was given 30 min before hormone administration at dose of 150 mg/kg. Ketoconazole (300 mg/kg, Isdin, Barcelona) was given by gavage for 10 days before hCG administration. Progesterone (50 mg/kg) and estradiol (500 μg/kg) were administered sc 30 min before gonadotropin administration. In intra-bursa experiments N^6,2′-*O*-dibutyryl cAMP (20 μg) was administered directly into the ovary of mice under ether anesthesia. Blood samples were collected under light ether anesthesia by cardiac puncture. Plasma was obtained by centrifugation at 4°C and was kept frozen at −70°C until analysis.

Steroid measurements. Testosterone and progesterone were determined by ELISA with Enzymun Test kits supplied by Roche Diagnostic (Barcelona, Spain). Corticosterone was measured using a ^{125}I RIA kit (ICN Biomedicals Inc., Costa Mesa, CA). Plasma steroid concentrations were measured in duplicate. Tissue steroid concentrations were determined after homogenization of gonads or adrenals in ice-cold ethanol (1:20 wt/vol) using a polytron. The extracts were centrifuged at 10,000*g* for 20 min, the supernatant was diluted in 50% ethanol containing 0.9% NaCl and hormones were measured in duplicate.

Enzyme measurements. ODC activity was determined in the cytosolic fraction (12,000 g supernatant) by measuring the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine according to a previously described protocol (21) with certain modifications. In brief, tissues were homogenized in 20 vol ice-cold buffer containing 25 mM Tris (pH 7.2), 2 mM dithiothreitol, 0.1 mM pyridoxal phosphate, 0.1 mM EDTA, and 0.25 M sucrose. The extract was centrifuged at 12,000*g* for 20 min and enzyme activity was determined in the supernatant. The incubation mixture contained 0.25 μCi of L-[1-^{14}C]ornithine (56 Ci/mol, Moravek Biochemicals, CA) and 50 μl of tissue extract in a total volume of 62.5 μl.

Protein kinase activity was determined by means of a nonradioactive protein kinase assay kit (Calbiochem-Novabiochem Corp., La Jolla, CA). The assay kit employs an enzyme-linked immunosorbent assay which utilizes a synthetic PKC/PKA pseudosubstrate (–RFARKGSLRQKVV) and a monoclonal antibody that recognizes the phosphorylated form of the peptide. Protein kinase A, mouse recombinant catalytic subunit and rat brain protein kinase C catalytic subunit were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA).

Statistical analysis. Results are given as means ± SEM. Statistical comparisons were calculated by one-way ANOVA followed by the post-hoc Tukey multiple range test with a Prism program (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant.

RESULTS

The effects of AMG on ODC induction and steroid secretion in the ovary of adult female mice and the testis of immature male mice are presented in Fig. 1A. As reported by others, hCG (25 IU) produced a marked increase in ODC activity in the ovary and testis after 5 h of treatment, as well as a significant increase in plasma progesterone in female mice and plasma and testicular levels of testosterone in immature male mice (Table 1). The concurrent treatment with AMG (150 mg/kg) prevented the rise in ovarian or testicular ODC activity and almost depleted the gonads and plasma of progesterone or testosterone. While ACTH (50 μg/

TABLE 1

Effect of AMG on Steroid Secretion Elicited by hCG in Mouse Gonads

	26-day old male: Testosterone		Adult female: Progesterone	
Treatment	Plasma (ng/ml)	Testis (μg/g)	Plasma (ng/ml)	Ovary (μg/g)
Control	0.30 ± 0.05	0.060 ± 0.003	22.1 ± 1.4	11.88 ± 0.14
hCG	5.1 ± 0.23[a]	2.420 ± 0.091[a]	33.5 ± 1.5[a]	13.39 ± 0.13[a]
HCG + AMG	0.35 ± 0.03	0.141 ± 0.010	<0.1	<0.01

Note. 26-day-old male mice and adult female mice at the diestrus stage were treated with 25 IU of hCG or 25 IU hCG + 150 mg/kg AMG and killed 5 h after injection. Results are the means ± SEM from 4–6 animals per group.

[a] $P < 0.001$ vs control and hCG + AMG.

TABLE 2

Effect of AMG on Corticosterone Secretion Elicited by ACTH on Mouse Adrenal Glands

Treatment	Plasma corticosterone (ng/ml)
Control	161 ± 31
ACTH	464 ± 40[a]
ACTH + AMG	26 ± 3

Note. Adult female mice were treated with 50 μg of human $ACTH_{1-24}$ or 50 μg of human $ACTH_{1-24}$ + 150 mg/kg AMG and killed after 30 min of injection. Results are the means ± SEM from 4–6 animals per group.

[a] $P < 0.01$ vs control or ACTH + AMG.

TABLE 4

Effect of AMG on Intrabursa Dibutyryl-cAMP Mediated ODC Induction in Mouse Ovary

Treatment	Ovarian ODC activity (nmol/h × g)
Saline	10.1 ± 2.5
dbc-AMP	31.0 ± 2.9[a]
dbc-AMP + AMG	5.8 ± 1.1

Note. Adult female mice were injected with AMG (150 mg/kg) or vehicle 30 min before intrabursa administration of dbc-AMP (20 μg) or saline, and were killed after 5 h. Results are the means ± SEM from 4–6 animals per group. Statistical significance: [a] $P < 0.001$ vs saline or dbc-AMP + AMG.

animal) significantly increased adrenal ODC activity and corticosterone secretion in adult female mice; AMG blocked the rise in adrenal decarboxylase (Fig. 1B) and produced a marked decrease in plasma corticosterone levels (Table 2). These results indicated that AMG abolished the hormonal induction of ODC in steroidogenic tissues where the synthesis and secretion of steroids is severely inhibited. This suggested either that steroid hormones acting through autocrine or paracrine mechanisms or the presence of an active steroidogenic system is required for the induction of ODC in these tissues. Another possibility is that AMG may inhibit some step in the signaling pathway that leads to ODC induction, independently of its action on steroidogenesis. To test these possibilities we administered exogenous progesterone and estradiol to determine whether these steroids prevent the effect of AMG. In addition, another inhibitor of steroidogenesis, ketoconazole (20), was used to compare its effects with those AMG.

Table 3 shows that concurrent administration of progesterone and estradiol did not prevent the effects produced by AMG on hCG action on the ovary of adult mice. Moreover the treatment with ketoconazole produced a marked decrease in plasma progesterone concentration without affecting the increase of ODC in response to hCG treatment. These results reveal that neither steroid hormones nor steroidogenesis are required for ODC induction in response to gonadotropins.

To assess whether the observed effect of AMG on ODC induction is related to the inhibition of a particular step in the signaling pathway stimulated by gonadotropins we tested the effect of this drug on the increase of ovarian ODC activity elicited by direct administration of dibutyryl-cAMP to the ovary by means of intrabursa experiments. Table 4 shows that AMG severely reduced the increase in ovarian ODC elicited by cAMP, suggesting that some step downstream cAMP production in the signaling pathway is affected by AMG. This result is also in harmony with previous reports claiming that AMG does not have a significant effect on cAMP formation (22). To assess the possible action of AMG on protein kinase A, we used an *in vitro* system designed to test putative effectors of protein kinase A and protein kinase C activity. Figure 2 shows that while AMG did not significantly inhibit mouse protein kinase C, it produced a dose-dependent inhibi-

TABLE 3

Effects of AMG, Steroid Hormones, and Ketoconazole on ODC Induction in Adult Mouse Ovary and Progesterone Secretion

Treatment	Ovarian ODC activity (nmol/h × g)	Plasma progesterone (ng/ml)
Control	8.3 ± 1.1	21.2 ± 1.4
hCG	126 ± 6.3[a]	29.4 ± 1.6[c]
hCG + AMG	11.3 ± 3.3	<1
hCG + AMG + P + E2	10.5 ± 0.7[b]	92.1 ± 1.8
hCG + ketoconazole	137 ± 4.6[a]	1.5 ± 0.6

Note. Female mice were treated as indicated in Table 1. Progesterone (P), 50 mg/kg, and estradiol (E2), 500 μg/kg, were given 30 min before hCG administration. Ketoconazole (300 mg/kg) was administered for 10 days before hCG administration. Results are the means ± SEM from 4–10 animals per group. Statistical significance: [a] $P < 0.001$ vs control and hCG + AMG; [b] $P < 0.001$ vs hCG; [c] $P < 0.001$ vs the other groups.

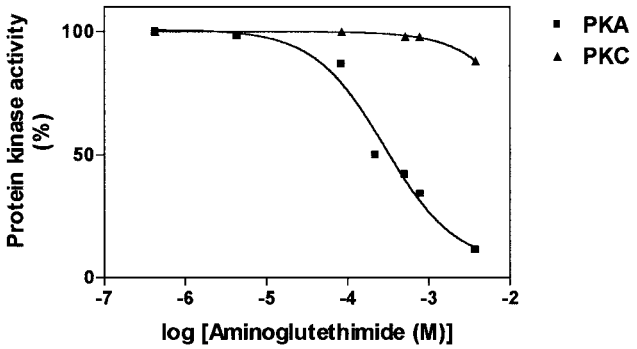

FIG. 2. Effect of AMG on protein kinase (PK) activity. PKA: cAMP-dependent protein kinase (mouse catalytic subunit). PKC: protein kinase C (rat brain). Results are the mean of duplicate determinations.

tion of mouse recombinant protein kinase A, with IC_{50} = 287 μM.

DISCUSSION

The results indicate that AMG, a potent inhibitor of steroidogenesis, abolishes the induction of ODC activity promoted by peptide hormones such as hCG or ACTH in steroidogenic tissues. They further show that steroid hormones do not seem to be required for the induction of ODC that follows the onset of steroidogenic stimulation by hCG or ACTH in these tissues. This is in contradiction to results obtained in other rodent tissues such as kidney, prostate or uterus, where the stimulation of this enzyme is largely dependent of the presence of steroid hormones and steroid receptors (23). Our results also indicate that the presence of an active steroidogenic pathway is not essential for ODC induction, since this enzyme is fully induced in tissues in which steroidogenesis is blocked by ketoconazole. This does not support a hypothesis based in the contention that the stimulation of polyamine biosynthesis is useful to increase the antioxidant capacity of tissues such as gonads and adrenals which possess a high rate of generation of reactive oxygen species (24–27).

To our knowledge this is the first report showing that AMG inhibits murine protein kinase A. Although the calculated inhibitory constant is higher than the values reported for other well known protein kinase inhibitors (28, 29), the inhibitory effect observed on ODC induction was essentially complete at the pharmacological dose used. One may speculate that either the protein kinase A holoenzyme is more sensitive to the inhibitory action of AMG than the recombinant and non-myristylated catalytic subunit (30) used in the inhibition test or that *in vivo* the drug is metabolized to produce derivatives more potent than AMG. Our results suggest that this drug may affect the steroidogenic pathway not only by a direct inhibitory action on some cytochrome P450 enzymes such as aromatase or cytochrome P450 scc complex (31–33) but also through an indirect route mediated by its action on the expression of steroidogenic genes that are regulated by cAMP (5). The inhibition of protein kinase A by AMG may also explain some biological effects of this drug which cannot be ascribed to its action on the steroidogenic pathway (34, 35) as well as some of the multiple adverse effects described for this inhibitor used in the treatment of certain adrenal pathologies and breast cancer (36, 37). Moreover, the inhibitory action on ODC induction shown by AMG in our experiments may be considered in principle as a beneficial collateral effect when using this compound in oncotherapy, since there is strong evidence that cell transformation, invasiveness and angiogenesis are dependent on ODC activity and polyamines (8–12). In this regard it has been shown recently that breast cancer ODC is an independent prognostic factor for recurrence and death in breast cancer patients (38). Finally, the use of AMG may prove to be a valuable tool for better understanding of the signaling pathways implicated in ODC induction in cells different to those steroidogenic tissues.

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