Different modulation of aromatase activity in frog testis in vitro by ACE and ANG II

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Miano, Antonino, Anna Gobbetti, Massimo Zerani, Luana Quassinti, Ennio Maccari, Oretta Murri, Domenico Amici, and Massimo Bramucci. Different modulation of aromatase activity in frog testis in vitro by ACE and ANG II. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R1261–R1267, 1999.—The aim of the present research was to study the role of angiotensin-converting enzyme (ACE) and ANG II in amphibian (Rana esculenta) testicular steroidogenesis and prostaglandin production. Hormonal effects of ACE, ACE inhibitors, synthetic bullfrog ANG I, and [Val5]ANG II were determined in frog testis of prereproductive period. Production of 17β-estradiol, progesterone, androgens, and PGE2 and PGF2α, was determined by incubating frog testes with ACE (2.5 mU/ml), captopril (0.1 mM), lisinopril (0.1 mM), [Val5]ANG II (1 μM), and synthetic bullfrog ANG I (1 μM). The analysis of the data showed an independent modulation of 17β-estradiol and androgen production by ACE and ANG II. The ACE pathway caused a decrease of 17β-estradiol production and an increase of androgen production in frog testes; on the other hand, the ANG II pathway increased 17β-estradiol production and decreased androgen production. The determination of testicular aromatase activity showed a positive regulation by ANG II and a negative regulation by ACE. As for prostaglandin production, only ANG II influenced PGF2α. These results suggest a new physiological role of ACE and ANG II in modulating steroidogenesis and prostaglandin production.

17β-estradiol; progesterone; androgens; prostaglandin E2; prostaglandin F2α; angiotensin-converting enzyme

ANGIOTENSIN-CONVERTING ENZYME (ACE; EC 3.4.15.1) is a glycosylated integral membrane protein located on the luminal surface of the cell membrane. Known primarily for its role in the regulation of blood pressure and hydromineral metabolism, it is found in a large variety of cells, tissues, and biological fluids including plasma, semen, proximal renal tubular cells, intestinal epithelial cells, stimulated macrophages, brain, lung, vascular endothelium, and the medial and adventitial layers of blood vessel walls (16). ACE is a peptidyl dipeptidase that removes the carboxy terminal His-Leu from ANG I to produce the octapeptide ANG II, and, in addition, inactivates bradykinin, a mediator of inflammation and vasodilator peptide, as well as substance P, enkephalins, and endorphins (17). There are two isoforms: a larger one of 100–110 kDa protein encoded by a 3-kb mRNA found in somatic cells and a smaller one of 100–110 kDa protein encoded by a 3-kb mRNA found in testicular germ cells. These two ACE isozymes are encoded by the same gene, which is transcribed in two different mRNAs (19). Cloning and sequencing of the human germinal ACE cDNA (8) have revealed that it corresponds to the COOH terminal of the somatic ACE cDNA. The transcription of germinal ACE occurs via a testis-specific promoter located within intron 12 of the ACE gene (17, 18, 22, 33). In addition, the germinal isoform possesses a specific NH2-terminal sequence transcribed from exon 13, which is absent in the somatic ACE cDNA due to an alternative splicing (17, 22, 33). In the mouse, the two ACE cDNAs (somatic and germinal) (1, 2, 23) revealed the same structure as the human cDNAs and a high degree of homology both in amino acid and nucleotide sequences. The germinal isoform of ACE was shown to be tissue and stage specific during spermatogenesis in mouse and rat testes: it is exclusively expressed in male germ cells after completion of meiosis and throughout spermiogenesis (31). In rat, a positive regulation of testicular ACE expression by androgens and luteinizing hormone was also observed (36), but in the prepubertal period the germinal isoform of ACE has not been detected. The function of testicular ACE is unknown, although studies with ACE-deficient mice demonstrated reduced male fertility in homozygous mutants (9, 13, 21).

Components of the prorenin-renin-ANG system (PRAS) are present locally within the male reproductive tissue (27). PRAS may be considered an important member of the local regulatory system, producing ANG II needed for paracrine functions. ANG II receptors have been demonstrated to be present in the testis of rat and several primate species including humans (25). In all species examined, Leydig cells possessed specific ANG binding sites. ANG II inhibited adenylate cyclase activity in Leydig cell membranes and reduced basal and human chorionic gonadotropin-stimulated cAMP pools and testosterone production in intact cells (20). In Sertoli cells, ANG II increased cytosolic calcium through AT2-receptor subtypes in a cAMP-independent pathway (12).

In a previous report, we studied the role of ACE and ANG II in ovarian steroidogenesis and prostaglandin production in the water frog, Rana esculenta (4). 17β-Estradiol, progesterone, and PGE2 production was modulated by ovary ACE; on the other hand, [Val5]ANG II modulated the production of progesterone and PGF2α, whereas androgen production was not influenced. These studies suggested the existence of two pathways, independently regulated by ACE and ANG II, modulating ovarian steroidogenesis and prostaglandin production.

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Preparation of Crude Homogenates, Tissue Membranes, and Testicular Trypsin Extraction

The preparation of crude homogenates, tissue membranes, and testicular trypsin extraction followed a method previously described (4). Protein content was evaluated by the method of Bradford (3), with bovine serum albumin as standard. Six adult male frogs were used for each extraction.

Experimental Protocol

In vitro studies. To study the testicular steroidogenesis and prostaglandin production, male frogs from the preproductive period were captured and killed in the field by decapitation. The testes were removed, placed in cold DMEM containing 10 mM Hepes, 0.1 mg/ml penicillin G, and 0.1 mg/ml streptomycin, and transferred to the laboratory where they were distributed over incubation wells (2 tests/well) each containing 2 ml of incubation medium (10). Each incubation set of wells was divided into six experimental groups (each consisting of 4 wells): 1) medium alone; 2) medium plus 2.5 µM rabbit lung ACE; 3) medium plus 0.1 µM captopril; 4) medium plus 0.1 mM lisinopril; 5) medium plus 0.1 mM captopril plus 2.5 µM rabbit lung ACE; and 6) medium plus 0.1 mM lisinopril plus 2.5 µM rabbit lung ACE. [Val5]ANG II and synthetic bullfrog ANG I, at the final concentration of 1 µM, were added to a second and third incubation set, respectively. In a fourth incubation set, 2.5 µM rabbit lung ACE were replaced with 2.5 µM trypsinized frog testis ACE. Acetylsalicylic acid, at the final concentration of 2.5 µM, was added to a fifth and sixth incubation set containing 1 µM [Val5]ANG II and 1 µM synthetic bullfrog ANG I, respectively. In a seventh incubation set, PGF2α was added at the final concentration of 100 nM. Culture plates were wrapped in aluminum foil and incubated at room temperature. The incubation medium was removed after 6 h and stored at −20°C until hormone assays. The control experiment was repeated with incubation media without testicular activity.

Aromatase activity determination. Testes were weighed and homogenized in cold buffer (50 µl/ml fresh weight tissue: 20 mM KH2PO4, 1 mM EDTA, 3 mM NaCl, 10% glycerol, and 10 mM β-mercaptoethanol, pH 7.4). The determination of aromatase activity was performed as reported by Zerani et al. (38).

Determination of FAPGG Hydrolyzing Activity and Kinetic Parameters

The determination of FAPGG hydrolytic activity and kinetic parameters of testis ACE followed a method previously described for ovary ACE (4).

Hydrolysis of ANG I by Homogenate and Membrane Suspension of Testis

Homogenate (5 µg) or membrane suspension (1 µg) of testis was added to 100 µM synthetic bullfrog ANG I solution in 80 mM borate buffer (pH 8.2) containing 300 mM NaCl in a total volume of 20 µl. The solution was incubated at 37°C for 5 min. TFA was used to acidify to pH 2.0, stopping the incubation, and the solution was injected for reverse-phase HPLC analysis using a 5-µm Supelcosil LC-318 column protected with a 5-µm Supelcosil LC-318 guard column (2 cm × 4.6 mm ID). The elution was performed with a linear gradient from 15 to 35% of 0.1% TFA in water and 0.1% TFA in acetonitrile at a flow rate of 1 ml/min. Eluate absorbance was monitored by ultraviolet absorbance at 214 nm. Identification of the ANG I peak was facilitated by adding 1 nmol of [Val5]ANG II to the...
mixture after incubation and before injection of the sample for reverse-phase HPLC analysis or by incubating the mixture with 0.1 mM captopril.

**Determination of Progesterone, Androgens, 17β-Estradiol, PGF$_{2\alpha}$, and PGE$_2$**

Concentrations of progesterone, androgens, 17β-estradiol, PGF$_{2\alpha}$, and PGE$_2$ were measured in incubation media by RIA as described previously (10). Intra- and interassay coefficients of variation and minimum detectable doses were: progesterone, 9%, 16%, 12 pg; androgens, 12%, 18%, 19 pg; 17β-estradiol, 8%, 19%, 11 pg; PGF$_{2\alpha}$, 9%, 18%, 15 pg; and PGE$_2$, 7%, 17%, 17 pg. Testosterone was not separated from 5α-dihydrotestosterone and, therefore, as the antiserum used is not specific, the data are expressed as androgens.

**Statistical Analysis**

ANOVA followed by Duncan’s multiple range test (7, 32) was used to analyze the data.

**RESULTS**

The ACE activity contents of lung, kidney, and testis of frog (R. esculenta) were measured by following the hydrolyzing activity of crude tissue homogenates on FAPGG, a synthetic substrate of ACE. As reported in Table 1, the FAPGG hydrolyzing activity is present in lung and kidney of male frog with values very close to those reported for the female frog (4). The hydrolyzing activity in testis was ~70–90 times less compared with the FAPGG hydrolyzing activity of ovary tissue (in reference to the weight).

To compare the kinetic parameters of ovary ACE with testis ACE, a partial purification of the enzyme from testicular tissue was carried out after the partial purification procedure of ovary ACE. Linear regression analysis of FAPGG hydrolysis by tissue membrane preparation gave a Michaelis-Menten constant ($K_m$) of 0.297 ± 0.044 mM and a maximum velocity of 5.164 ± 0.352 nmol·min$^{-1}$·mg protein$^{-1}$. Captopril and lisinopril, two specific ACE inhibitors, inhibited the enzyme activity at low concentration. The IC$_{50}$ values, obtained from inhibition curves, were 26.600 ± 0.322 nM for captopril, and 1.326 ± 0.575 nM for lisinopril using FAPGG as substrate.

Frog testicular membrane suspension was incubated at 37°C in the presence of synthetic bullfrog ANG I. Aliquots of incubation mixture were drawn at different times and analyzed by reverse-phase HPLC. The chromatographic elution profiles showed the presence of a peak that corresponds to [Val$^5$]ANG II by comparison with a standard sample of synthetic [Val$^5$]ANG II. Captopril and lisinopril at the concentration of 10$^{-4}$ M inhibited the production of [Val$^5$]ANG II almost completely. Comparison of these data from testis ACE with those obtained from ovary ACE shows a high degree of homology in kinetic parameters.

To study the physiological function of ACE on steroidogenesis and prostaglandin synthesis in frog testis, the production of 17β-estradiol, progesterone, androgens, PGE$_2$, and PGF$_{2\alpha}$ was determined by incubating in vitro testicular tissue in the presence of captopril, lisinopril, rabbit lung ACE, [Val$^5$]ANG II, synthetic bullfrog ANG I, and frog testicular ACE.

The data show that the basal production of progesterone (127 ± 45 pg/testis) in frog testis remained unchanged after treatment of frog testicular tissue with captopril, lisinopril, rabbit lung ACE, [Val$^5$]ANG II, synthetic bullfrog ANG I, and frog testis ACE. Figure 1 shows the 17β-estradiol production by frog testis incubated in vitro. The 17β-estradiol basal value of 151.3 ± 26.11 pg/testis was inhibited by adding rabbit lung ACE at the final concentration of 2.5 μM/ml (Fig. 1A). Treatment with specific ACE inhibitors captopril (0.1 mM) and lisinopril (0.1 mM) increased the production of 17β-estradiol approximately fourfold over the basal level (Fig. 1A). Addition of 1 μM [Val$^5$]ANG II to the incubation medium caused an increase of 17β-estradiol (450%) that was nullified by addition of rabbit lung ACE (Fig. 1B). The presence of ACE inhibitors with ANG II amplified 17β-estradiol production by about nine times for captopril and about 10 times for lisinopril, even with the addition of rabbit lung ACE. In Fig. 1C, addition of 1 μM ANG I to the incubation medium stimulated 17β-estradiol as reported for addition of ANG II. The presence of rabbit lung ACE nullified the
stimulus of ANG I. ACE inhibitors prevented conversion of ANG I to ANG II, obtaining an increase of 17β-estradiol production due only to the presence of inhibitors. To avoid contamination in the commercial rabbit lung ACE, frog testis ACE was partially purified as described in MATERIALS AND METHODS. The amount of frog testis ACE used in the test was 2.5 mU/ml. The data confirm the results obtained with rabbit lung ACE (Fig. 1D) and suggest the presence in the frog testis of two pathways controlling 17β-estradiol production. The first involves an ACE pathway and the second an ANG II pathway independent of ACE conversion.

Figure 2 shows androgen production (basal value 2,601 ± 254.1 pg/testis) by frog testis incubated in vitro. Unlike in the case of 17β-estradiol production, the addition of rabbit lung ACE (2.5 mU/ml) to the incubation medium increased the androgen production approximately twofold compared with the basal value. Treatment with ACE inhibitors caused a decrease of androgen production (~65%) also in presence of rabbit lung ACE (Fig. 2A). Addition of ANG II to the incubation medium also led to a decrease in androgen production (~58%), which was nullified by rabbit lung ACE but enhanced by ACE inhibitors. These data confirm the results obtained with 17β-estradiol, in which two pathways modulating hormone production in frog testis are present. Addition of 1 μM synthetic bullfrog ANG I to the incubation medium confirmed the data obtained with ANG II (Fig. 2C). ANG I inhibited androgen production via ANG II conversion by endogenous ACE, whereas the presence of rabbit lung ACE nullified the stimulus of ANG I. ACE inhibitors prevented conversion of ANG I to ANG II and obtained a decrease of androgen production due only to the presence of ACE inhibitors. The addition of partially purified frog testis ACE confirmed the data obtained with rabbit lung ACE.

The analysis of results regarding the influence of ACE and ANG II on 17β-estradiol and androgen production revealed an opposite effect. Addition of ACE to testicular tissue caused a decrease of 17β-estradiol and an increase of androgen production, whereas ANG II showed an increase of 17β-estradiol and a decrease of androgen production. These data suggest an influence of the ACE and ANG II pathways in modulating aromatase activity, the enzyme complex that catalyzed the conversion of androgens to estrogens. With this in view, aromatase activity was determined in homogenates of frog testis after treatment with rabbit lung ACE, captopril, lisinopril, [Val5]ANG II, synthetic bullfrog ANG I, and frog testis ACE. Figure 3 shows the aromatase activity of frog testis homogenates after incubation in vitro. The data show the same pattern seen in 17β-estradiol production, confirming the influence of ACE and ANG II on aromatase activity in frog testicular tissues.

We found a difference in modulation of prostaglandin production by ACE and ANG II in frog ovary (4). The ACE pathway acted on PGF2α, whereas that of ANG II regulated PGF2α. To confirm these data, PGF2α and PGF2α production were determined in frog testis after treatment with rabbit lung ACE, captopril, lisinopril, [Val5]ANG II, synthetic bullfrog ANG I, and frog testis ACE. Figure 4 shows PGF2α production (expressed as pg/testis) by frog testicular tissue incubated in vitro. The data show the same pattern as seen in PGF2α production in frog ovary. The basal value of PGF2α (275 ± 41.5 pg/testis) remained unchanged in the presence of rabbit lung ACE, captopril, and lisinopril (Fig. 4A). ANG II (1 μM) increased the production of PGF2α (331%) without any influence of rabbit lung ACE and/or ACE inhibitors (Fig. 4B). The production of PGE2 (basal value 485 ± 37 pg/testis) was not affected...
by rabbit lung ACE, captopril, lisinopril, [Val⁵]ANG II, synthetic bullfrog ANG I, or frog testis ACE.

In the present paper, we show that ANG II is able to increase PGF₂α production and to enhance aromatase activity in frog testis. Gobbetti and Zerani (11) reported that aromatase activity is influenced by PGF₂α in the brain of the newt Triturus carnifex during male courtship. Therefore, frog testis was incubated in vitro with ANG II and with ANG I in presence of an inhibitor of prostaglandin synthesis (2.5 µM acetylsalicylic acid) and with PGF₂α (100 nM). The results exclude the involvement of PGF₂α in modulating aromatase activity.

**DISCUSSION**

The aim of the present study was to examine the role of ACE and ANG II in testicular steroidogenesis and prostaglandin production in Rana esculenta. Male frogs appear to be potentially continuous breeders, producing spermatozoa throughout the entire year, with cysts of all spermatogenetic stages being always present (24). Temperature is the primary factor in the regulation of the testicular cycle quiescent phase during winter. Rana esculenta (southern European populations) shows a new wave of spermatogenesis in spring immediately before the spring-summer breeding commences (28). Therefore, we studied the hormonal effects of ACE, ACE inhibitors, ANG I, and ANG II in the prereproductive period.

The ACE activity present in homogenate of frog testis is very low compared with other tissues of frog, but the data are in agreement with what was reported for Rana catesbeiana (37). The low value of ACE activity found for frog testis is also in agreement with what was reported for humans, in which the testis ACE activity is approximately threefold less than lung ACE activity (34). However, the data are in contradiction with the findings for rat and mouse testis (6, 14), in which ACE activity is higher in the testis than in the lung. These differences may be explained by the different procedures used for sample preparation; in fact, rat and mouse testis homogenates were repeatedly dialyzed.

With regard to the kinetic parameters, the frog testis ACE shows a Kₘ value very close to that of frog ovary for the synthetic substrate FAPGG, whereas IC₅₀ values of captopril and lisinopril are slightly lower than those of frog ovary ACE (4).

In a previous report, we gave evidence for two pathways independently regulated by ACE and ANG II and modulating the steroidogenesis and prostaglandin production in amphibian ovary. More precisely, ovary ACE was involved in modulating 17β-estradiol, progesterone, and PGE₂, whereas ANG II modulated the production of progesterone and PGF₂α (4). Data obtained in in vitro incubation of testis tissue with ACE and ACE inhibitors suggest the involvement of ACE activity reducing 17β-estradiol production; instead, ANG II, exogenous or derived from ANG I hydrolysis, showed positive results on 17β-estradiol production. The experiments performed to study the effect of ACE, ANG I, and ANG II on androgen secretion showed opposite results compared with data obtained for 17β-estradiol. Indeed, the increase of androgen production was due to the ACE activity, whereas ANG II caused negative modulation. The observation that ANG II produced an inhibitory effect on androgen production was in agreement with data reported in mammals by Khanum and Dufau (20), where ANG II inhibited adenylate cyclase activity in rat Leydig cell membranes and reduced basal and human chorionic gonadotropin-stimulated cAMP pools and testosterone production in intact cells. The experiments performed to study the effects of ACE, ANG I, and ANG II on 17β-estradiol and androgen production suggest the presence, also in the frog testis, of two pathways independently regulated by ACE and ANG II, as seen in frog ovary.

In frog testis, the pathways regulated by ACE activity and ANG II seem to involve the modulation of...
ametase activity. In Fig. 5, we propose a schema, derived from analysis of our experimental data, in which ACE and ANG II have an opposite effect in modulating aromatase activity in frog testis. This model explains some paracrine functions activated by ACE via 17β-estradiol modulation of reproductive processes. Rana esculenta displayed high estrone peaks in plasma and testes concomitantly with a sharp androgen decrease in postreproductive period (35). According to our model, decrease of testicular ACE in frog testis, due to discharge of spermatazoa during the breeding period, may increase aromatase activity with a consequent rise in 17β-estradiol level and decrease in androgen level. The 17β-estradiol peak in postreproductive period may induce cellular proliferative activity in primary spermatogonial cells, as reported by Minucci et al. (26).

In mammals, testicular ACE was expressed exclusively in the haploid germ cell of mouse and rat testis. In both species, the highest level of expression was associated with the elongation of spermatids at steps 10–11, although in prepubertal animals, hardly any signals were seen. The first signal appeared from day 23 of age; its intensity gradually increased until, at days 28–35, an adult level of ACE mRNA expression was reached. A dose correlation between the germ cell-specific formation of testicular ACE and maturation of the germ cell exists (31). Recently, in adult male bonnet monkeys (Monkey radiata) treated with a long-acting nonsteroidal aromatase inhibitor (CGP 47645), a marked reduction in sperm counts with inhibition in spermiogenic processes was observed (30). In humans, mutation dysfunction in estradiol receptor α and aromatase decreased sperm counts and resulted in poor sperm viability (5). These data, explained with our model, suggest the involvement of testicular ACE activity in a feedback control of spermatogenetic progression regulating the production of estrogen via aromatase activity modulation in the testis.

Recently, Hess et al. (15) have reported that estrogen also regulates the reabsorption of luminal fluid in the head of the epididymis. Schill et al. (29) observed that infertile men suffering from oligozoospermaia and/or asthenozoospermia showed higher sperm concentrations after 3 mo of captopril therapy. Also in this case, these results can be explained with our model, in which ACE inhibitors increase 17β-estradiol production, permitting a higher reabsorption of luminal fluid in the head epididymis.

Our data are not in agreement, however, with those reported by Hagaman et al. (13), in which mice lacking somatic and testicular ACE produced a normal number of sperm that were indistinguishable from wild-type sperm in assays of viability, motility, capacitation, and induction of acrosome reaction. These discrepancies require more investigation in view of the different experimental models.

In regards to prostaglandin production in frog testis, ANG II influences PGF2α synthesis, whereas PGE2 is not affected. In newt brain, PGF2α influences aromatase activity (11), whereas in frog testis there is no correlation between PGF2α, stimulated by ANG II, and aromatase activity. The influence on PGF2α production by ANG II confirms what has been reported for frog ovary (4).

We conclude that ACE activity and ANG II independently influence aromatase activity, modulating estrogen production in a negative and positive way, respectively. The variations of estradiol concentration could influence the spermatogenesis in a feedback mechanism as reported in literature. Our results suggest a new physiological role for ACE and ANG II, still today considered mainly involved in regulation of blood pressure and hydromineral metabolism.

Perspectives

The study of the ACE-ANG II system in frog testis confirmed what was observed in frog ovary, in which ACE and ANG II independently regulate steroidogenesis and prostaglandin production. Moreover, in the case of frog testis, the two pathways seem to modulate aromatase activity and then estradiol production. In literature, estrogens have been reported to be involved in the inhibition of spermatogenesis and, furthermore, in humans an increase of estradiol level has been demonstrated in patients suffering from infertility. Our results, obtained in amphibian testis, if confirmed in mammals, may help to elucidate mechanisms for paracrine regulation of mammal testis steroidogenesis.

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