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Gender differences in steroid modulation of angiotensin II-induced protein kinase C activity in anterior pituitary of the rat

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Abstract

To investigate whether the various steroid hormones can modulate the basal and angiotensin II-induced protein kinase C (PKC) activity in the anterior pituitary of the rat, female and male intact and ovariectomized female Wistar rats were treated in vivo with estradiol (E2), progesterone (P), dehydroepiandrostendione sulfate (DHEA-S), and pregnenolone sulfate (PREG-S). Estradiol caused the increase of basal PKC activity in intact and ovariectomized females, but did not change the enzyme activity in males. In ovariectomized animals the increase of PKC activity was lower than in intact females. Progesterone decreased PKC activity only in intact animals. DHEA-S strongly enhanced activity of PKC in ovariectomized females. Pregnenolone sulfate did not significantly change PKC function of all studied groups. Incubation with AngII enhanced the PKC activity in intact (without steroid treatment) animals of both genders. In females, AngII and estradiol together rise the PKC-stimulated phosphorylation in greater degree than used separately. Treatment with other investigated steroids reduced the effect of AngII. In intact males every examined hormone turned back the stimulatory effect of AngII on PKC activity. These data suggest that gender differences in PKC activity are likely related to hormonal milieu of experimental animals and may depend in part on the basic plasma level of estrogens.

Keywords: Angiotensin II; Inositol-1,4,5-trisphosphate; Pituitary; 17-β-Estradiol; Progesterone; Pregnenolone sulfate; DHEA-S

Angiotensin II (AngII), an octapeptide generated by the renin–angiotensin system, is mostly involved in regulating the fluid balance and the arterial pressure but can also mediate release of anterior pituitary hormones such as prolactin, luteinizing hormone, and adrenocorticotropic hormone [1]. The angiotensin II receptor in the anterior pituitary (AT1) belongs to the family of seven-transmembrane domain receptors that signals via heterometric G-proteins [2,3]. Ligand binding activates G_q, leading to the activation of phospholipase C, which hydrolyzes membrane phosphoinositides into, among others, inositol-1,4,5-trisphosphate (IP_3) and sn-1,2-diacyloglycerol (DAG) [4,5]. The consequence of generation of these second messengers is a rise of intracellular calcium. This, in turn, affects many physiological events such as cell contractility, secretion, gene transcription, and cell proliferation [6]. The changes in intracellular calcium concentration after AngII consist of two phases, fast and high initial rise of calcium and then plateau phase. AngII induces Ca^{2+} mobilization from the endoplasmic reticulum, evoking an early spike response in intracellular calcium. This initial rise of calcium is mediated by IP3. The second phase requires Ca^{2+} entry to the cells mainly through voltage-sensitive Ca^{2+} and is modulated by protein kinase C (PKC) [7]. These two phases can occur independently, and may be also independently regulated [8]. Angiotensin II is shown not only to generate IP3 but also to activate the membrane-bound protein kinase C activity in smooth muscle [9], intestinal epithelium [10] and adrenal cortex [11], and in the anterior pituitary [12] and thus, on this way, change the intracellular calcium concentration. Evidence for modulation of AngII effects by gonadal steroids has been obtained in vitro and in vivo. Angiotensin II release from the brain and AngII receptors number in

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the anterior pituitary [2,13] change during the oestrus cycle. Testosterone also affects the pituitary renin–angiotensin system [14,15]. Estrogens also modulate AngII action in the pituitary in ovariectomized rat [16]. Besides, the sex steroids can act directly at the pituitary and regulate cellular mechanisms controlling the intracellular calcium concentration. The action of sex hormones in the anterior pituitary implies the gender-specific differences in receptor density and postreceptoral changes. However, little is know about the effects of gender and status of the gonads on AngII-induced Ca^{2+} mobilization and protein kinase C activity in the anterior pituitary. In the previous papers we have described effects of various steroid hormones on AngII-induced IP3 concentration [12,17,18]. The purpose of this study was to investigate changes in angiotensin II-induced protein kinase C activity after the treatment with two major ovarian hormones; 17-β-estradiol and progesterone and two neurosteroids, pregnenolone sulfate and dehydroepiandrosterone sulfate in vivo in male and female rats. We have evaluated protein kinase C activity in anterior pituitary as a degree of phosphorylation of synapsin I, the phosphoprotein evoked the neurotransmitter release and found to be the brain substrate for various protein kinases, including PKC [19].

Materials and methods

All chemicals were obtained from Sigma, Germany. [γ-^{32}P]ATP was purchased from DuPont NEN, USA.

Animals and experimental protocol. Male and randomly chosen cycling female, Wistar rats weighing 180–220 g were used. The rats had been kept in light- and temperature-controlled rooms with tap water and food available ad lib. Animals were divided into three main groups; intact females, intact males, and ovariectomized females.

A. Intact animals. Intact animals were divided into examined groups, dependently on received substance. Every examined group of intact animals (females or males, 8 animals per group) received for five days intraperitoneally injections of one of the following steroid hormone: 1. 17-β-estradiol benzoate (E_2), 2. progesterone (P), 3. pregnenolone sulfate (PREG-S), 4. dehydroepiandrosterone sulfate (DHEA-S) in a dose of 50 g per animal per day, the dose allowing to abolish the cycling activity of the ovaries. The control group was injected with the same volume of oil. Twenty-four hours after the last injection the rats were sacrificed.

B. Ovariectomized females. Ovariectomy was done 14 days before experiment. The examined groups of ovariectomized animals received all examined steroids in one dose—50 μg, 24 h before decapitation. The control group was injected with the same volume of oil.

After the decapitation, the pituitary gland was collected, the posterior lobe was removed, anterior lobe was weighed and homogenized at 0–4 °C in medium containing aprotinine as proteases inhibitor. The control group was injected with similar volume of oil. Protein content in the samples was estimated according to the method of Ohnishi and Barr [20], with bovine serum albumin as a standard. The angiotensin II effects on PKC activity was evaluated in vitro. The homogenates of pituitaries were incubated with angiotensin in dose 10^{-9} M for 10 min. The time of incubation—10 min—was chosen for dose-dependent experiments, based on the results of time-dependent studies. The homogenates incubated without angiotensin II served as control.

Determination of protein kinase C activity. The standard phosphorylation assay medium contained 50 mM Tris–HCl, pH = 7.4; 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM EGTA, 1 mM $CaCl_2$, and 1 mM vanadyl sulfate as phosphatase inhibitor. Medium contained also 1 μM phorbol 12-myristate-13-acetate (PMA) which is a specific activator of the protein kinase C. The reaction was started by adding [γ-^{32}P]ATP to the reaction mixture. After incubation for 2 min at 37 °C the reaction was stopped by the addition of dissociation buffer containg 50 mM Tris–HCl, pH = 6.8, 20% (v/v) glycerol, 0.1% β-mercaptoethanol, 6% SDS, and 0.002% bromophenol blue and heating at 100 °C for 2 min. The solubilized proteins were resolved on 10% SDA–polyacrylamide gel as described by Laemmli [21]. Simultaneously, the pattern proteins of molecular weights 24–100 kDa were separated. After electrophoresis, the gels were silver-stained [22] and dried. The radioactivity of the band was detected by autoradiography. Protein phosphorylation was quantified from densitometric scans of autoradiograms by measuring peak areas and comparing to the control values. Synapsin I was identified by limited proteolysis (proteasis from Staphylococcus aureus V8) [23], and using monoclonal antibody (Calbiochem).

The analysis of data. The results are expressed as percentage of basal—100% (animals injected with oil) PKC activity. Data were analyzed and expressed as means ± SE. The comparison between means was performed using ANOVA followed by Newman–Keul's test with classification criteria (gender and treatment). Differences were considered statistically significant at $p < 0.002$.

Results

The effects of examined steroids on basal (without AngII and steroids) PKC activity differ dependently on the gender and gonadal status of animals (Fig. 1). The effects of various steroid hormones on PKC activity are illustrated in Fig. 2. E2 treatment caused the increase of basal PKC activity in intact and ovariectomized females, but did not change the enzyme activity in males. In ovariectomized animals the increase of PKC activity was statistically lower than in intact females. Progesterone decreased PKC activity only in intact animals, in the same degree in both sexes. In OVX females this hormone did not alter the enzyme activity. DHEA-S strongly enhanced the activity of PKC in ovariectomized

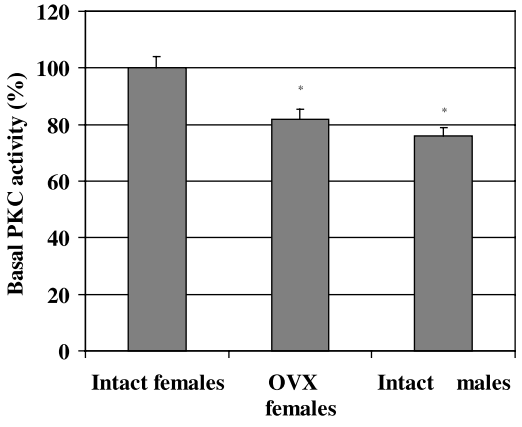

Fig. 1. Basal (without AngII and steroid treatment) PKC activity in intact females, ovariectomized females and intact males. The asterisk means the statistical significance to the values of intact females.

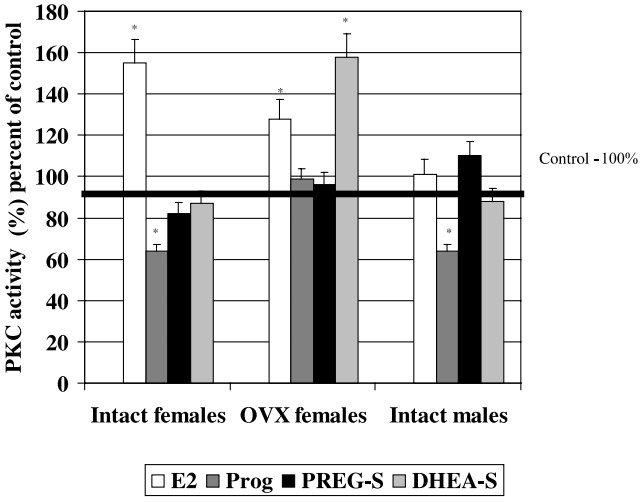

Fig. 2. The changes in PKC activity in anterior pituitary of the examined groups of rats previously treated with all examined steroids, 17-β-estradiol, progesterone, pregnenolone sulfate, and DHEA-S. The results are expressed as a percentage of control activity = 100%. Control values regard to group received oil. The asterisk means the statistical significance to the control values (without steroids) $p < 0.002$.

females, whereas in the intact animals the activity of the enzyme was decreased. Pregnenolone sulfate did not change PKC function in all studied groups.

PKC activity was enhanced after incubation with AngII in group without steroid treatment of intact females (Fig. 3) and males (Fig. 4). The stimulation was bigger in females. In this group, AngII and estradiol together rise the PKC-stimulated phosphorylation in greater degree than used separately. Treatment with other investigated steroids; progesterone, pregnenolone sulfate, and DHEA-S reduced the effect of AngII (Fig. 2).

In intact males all the examined hormones including E2, turned back the stimulatory effect of AngII on PKC activity (Fig. 3).

Discussion

Although several studies have shown a possibility of modulation of angiotensin action by sex hormones [17,24–26] little is know about the effect of gender and status of gonads on various cellular responses evoked by AngII. Our previous studies concerning the influence of steroid hormones on IP_3 formation after AngII showed that not only main ovarian hormones as estradiol and progesterone, but also pregnenolone sulfate and DHEA-S can change modulation in the generation of this second messenger by AngII [12,17,18]. All of them were shown to act within the brain as neurosteroids and cause non-genomic effects on pituitary cells membrane. Neurosteroids action evokes the different effects depending not only on specificity of steroid but also on the gender of the

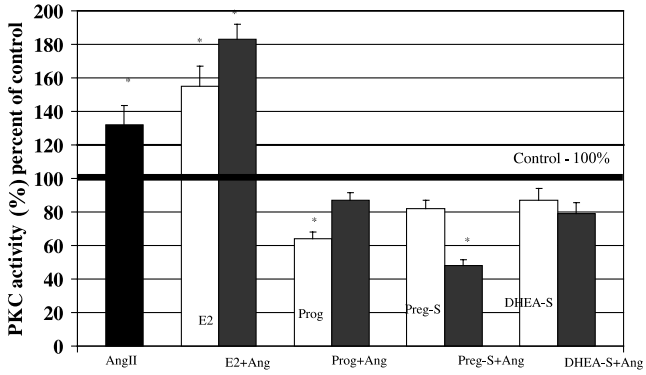

Fig. 3. The effects of angiotensin II alone and with steroids on phosphorylation of synapsin I catalyzed by PKC in intact females. Control values regard to group received neither examined steroids nor AngII. The results are expressed as a percentage of control activity = 100%. The asterisk means the statistical significance to the control values (without angiotensin) $p < 0.002$.

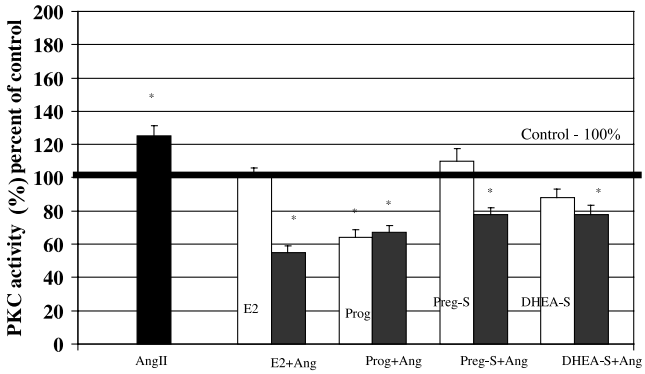

Fig. 4. The effects of angiotensin II alone and with steroids on phosphorylation of synapsin I catalyzed by PKC in intact males. Control values regard to group received neither examined steroids nor AngII. The results are expressed as a percentage of control activity = 100%. The asterisk means the statistical significance to the control values (without angiotensin) $p < 0.002$.

animal [27]. It has been shown that administration of 17-β-estradiol to intact rats results in increased PKC activity in normal and hyperplastic pituitaries [28]. In rat with pituitary hyperplasia caused by estrogen implantation the level of diacyloglycerol and expression of various forms of PKC are also increased [28–30]. Moreover, estrogens can mediate, probably via PKC, similarly to AngII, the LH release through the facilitation of GnRH secretion and responses to GnRH [31]. It is also been shown that estradiol increases the number of functioning channels in the membrane of pituitary cells [32].

Our current results show that basal PKC activity was greater in intact females than in OVX females and in intact males, suggesting that the gender differences are less likely related to the level of androgens but more likely related to the concentration of estrogens. This is supported by observation that E2 treatment of intact females and males was associated with the enhancement

of PKC activity. These data suggest that gender differences in PKC activity may be dependent in part on the basal plasma level of estrogen. Probably for this reason the stimulatory effect of exogenous estradiol is smaller in OVX females than in intact animals. This is not surprising that the effects of pre-treatment with E2 on AngII-induced PKC activation are different depending on gender of animals. It is known that in male rats AngII increased cytosolic calcium concentration more than in females. The same effect concerns the AngII induced growth of vascular smooth muscle cells [33,34]. Janik et al. [35] reported that gender plays an important role in the prolactin secretory response to physiological doses of AngII and that is reflected in inositol phosphate accumulation. As is already reported, a sexual dimorphism was apparent in estrogen-induced pituitary adenomas, suggesting the animal's pre-existing hormonal milieu may play an important role in setting the sensitivity to exogenous estrogen [36,37]. As is reported by Piroli et al. [36], continuous stimulation by estrogen influenced the proportion of different types of pituitary cells depending on the sex of the animal and the hormonal background. Depending on type of cell the intracellular mechanism of steroid hormone action can vary. Sex steroid concentration as well as their receptor levels are obviously different in all three groups; intact females, intact males, and OVX females, causing a different sensitivity to administrated hormones. This especially concerns the levels of circulating estrogen as well as its receptor. Estradiol is rather transported to the brain than synthesized within. In our study, estradiol in males turns back the stimulatory effects of AngII, whereas in females AngII and estradiol given together rise the PKC activity in a greater degree than given separately. The gender differences observed by us in PKC modulation could be due to a multitude of effects of sex hormones in vivo. There is a hypothesis that sex specific differential regulation of different PKC isozymes is function of sexual maturation [38]. It is suggested that the expression of various isozymes and activity of PKC are gender-related, and differentially respond to sex hormones [39,40]. Other signaling pathways, i.e., calcium entry, may also differ in different sexes, and the administration of exogenous steroids causes different, sometimes opposite effects depending on gender [41]. All these observations taken together with our results allow to draw the conclusion that the effects of E2 on PKC activity are depending not only on the short treatment with exogenous hormones, but also on long-term exposure to estrogen, related to the gonadal status of the animal.

Interestingly, in ovariectomized females DHEA-S strongly stimulates PKC activity, and this effect is not observed in intact females and males. Ovariectomy in females reproduces the hormonal situation observed in old rats, because the castration is associated with the loss of estradiol and progesterone [41]. Age and stress are associated with vulnerability to degeneration of various structures and intracellular messenger systems. It has been hypothesized that several neurosteroids, among others DHEA-S, might have a protective function against ischemia or neurodegeneration. Our results are in concordance with several studies reporting the positive effects of the acute administration of DHEA or its sulfates in rodents and humans, restoring, among others, memory performances in old animals to the levels observed in young ones [42]. Racchi et al. [43] showed that DHEA-S can restore the defective PKC signal transduction machinery in castrated rats.

The exposure to progesterone caused a decrease of PKC-stimulated phosphorylation. This fact may be caused by opposite to estradiol genomic or non-genomic effects of progesterone on calcium current [44]. The new observation, for the first time described by us, is the influence of PREG-S and progesterone on AngII-induced PKC activity. Interestingly, exposure to progesterone and PREG-S turned back the stimulatory effect of AngII. Although PREG-S separately did not change PKC activity, it strongly diminished the AngII action. This phenomenon was observed in both sexes. The molecular basis of this observation is not clear. It is possible that partially PREG-S acts as precursor of progesterone, and in the presence of AngII it elicits non-genomic effects characteristic for progesterone. It is also possible that the exposure to different hormones changes the expression of various cytosolic and membrane proteins, on the way of genomic action of steroid hormones.

The modulation of genomic or non-genomic steroid action in the brain might be a consequence of particular hormonal milieu—for example, pregnancy, ovarian cycle, or castration [27,40]. The differences in response to AngII observed by us in both sexes could also result from gender differences in AT1 receptor number and sensitivity, PKC modulation or expression, or all of them. However, we are not able to draw conclusions on whether the examined steroids act in genomic (i.e., increasing the expression of AT1 receptors or calcium channels) or non-genomic (modulation of function of ion channels or enzyme activity) manner, but any of these ways cannot be excluded. In conclusion, gender specific changes in AngII-stimulated PKC activity in female rats with intact gonadal function compared with ovariectomized females and intact males are possibly related with long-term influence of the endogenous estrogen. Further study is needed to assess the molecular basis of gender differences in response to AngII.

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