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Specific effect of arachidonic acid on 17β-hydroxysteroid dehydrogenase in rat Leydig cells

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Abstract It is well known that arachidonic acid (AA) acts as an intratesticular factor regulating luteinizing hormone-mediated testicular steroidogenesis. The present studies were conducted to determine the effect of AA on steroidogenic enzymes in rat Leydig cells. Exogenously added AA significantly inhibited 22(*R*)-hydroxy-cholesterol-stimulated testosterone production, which is a clear indication that AA is acting at some point after cholesterol transport to the inner mitochondrial membrane. AA failed to block the conversion of 22(*R*)-hydroxycholesterol to pregnenolone, indicating that the cytochrome P-450 side-chain cleavage enzyme complex is not the site of inhibition. The present results demonstrate that only 17β-hydroxysteroid dehydrogenase seems to be involved in the AA action, since nearly 60% inhibition of testosterone production was found when the cells were incubated with androstenedione. Furthermore, no effect of AA was found when androstenediol was used as substrate in the testosterone synthesis, which indicates that 3β-hydroxysteroid dehydrogenase is not affected by AA. The conversion of AA to its metabolites is not required for its action on 17β-hydroxysteroid dehydrogenase and the activation of protein kinase C is not involved in the inhibitory effect.

Key words: Arachidonic acid; Steroidogenic enzyme; Leydig cell

1. Introduction

Numerous studies suggest that arachidonic acid plays an important role in the regulation of steroidogenesis. Arachidonic acid, which is esterified in the *sn*-2 position of membrane phospholipids, is mainly directly released from phospholipids by activation of phospholipases A_2 (PLA_2). It can be also produced by activation of: phospholipases C (PLC) followed by diacylglycerol hydrolysis by diacylglycerol lipase; phospholipases D (PLD) followed by phosphatidic acid phosphohydrolase and diacyl glycerol lipase; and, finally, phospholipases A_1 (PLA_1) followed by lysophospholipase [1]. Arachidonic acid can be further metabolized via the cyclooxygenase-, lipoxygenase- or cytochrome P-450-dependent epoxygenase pathways to prostaglandins, leukotrienes and epoxyeicosatrienoic acids respectively [2]. Fatty acids can act on signal transduction pathways in a direct and/or indirect manner. However, several studies clearly show that fatty acids per se are messenger and modulator molecules mediating cell responses to extracellular signals [1]. Recent results have shown that luteinizing hormone (LH) causes a dose- and time-dependent release of arachidonic acid from Leydig cells [3] and that arachidonic acid metabolites are involved in LH-induced steroidogenesis [4–6]. Furthermore, arachidonic acid itself has been reported to act as an additional intracellular messenger associated with the hormonal action of LH [3,6,7] and to have an intermediary role in gonadotropin-releasing hormone (GnRH)-induced testosterone secretion [8,9].

Previous results from our group have shown that, in rat Leydig cells, arachidonic acid exerts a dose- and time-dependent biphasic effect on LH- and dibutyryl-cAMP-stimulated testosterone production [10]. At short periods of incubation this fatty acid inhibits testosterone synthesis by decreasing cAMP levels. Recently, we have shown that the effect of arachidonic acid on steroidogenesis is also beyond cAMP formation [11]. In the present study, we investigate whether arachidonic acid regulates testosterone production at some point after cholesterol transport to the inner mitochondrial membrane. Testosterone biosynthesis from cholesterol in Leydig cells involves the action of four steroidogenic enzymes (Fig. 1) [12]. Here we have examined the effect of arachidonic acid treatment on the activity of: cholesterol side-chain cleav-

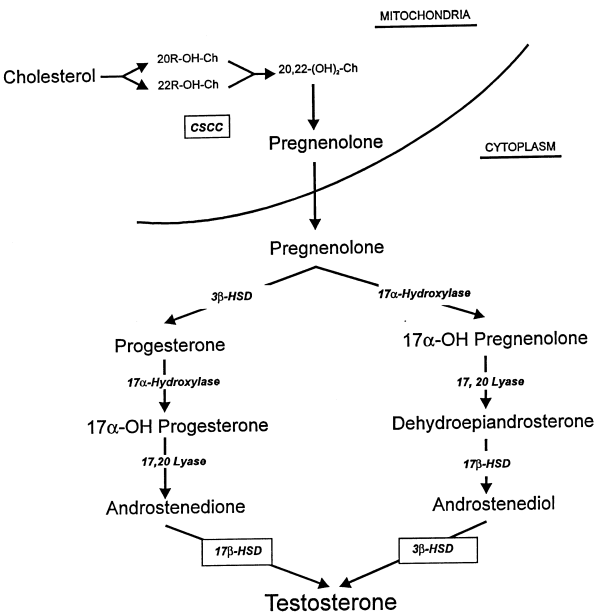

Fig. 1. Schematic pathways for the conversion of cholesterol to testosterone. The enzymes considered in this study are in a box. CSCC: Cholesterol side-chain cleavage enzyme; 3β-HSD: 3β-hydroxysteroid dehydrogenase; 17β-HSD: 17β-hydroxysteroid dehydrogenase.

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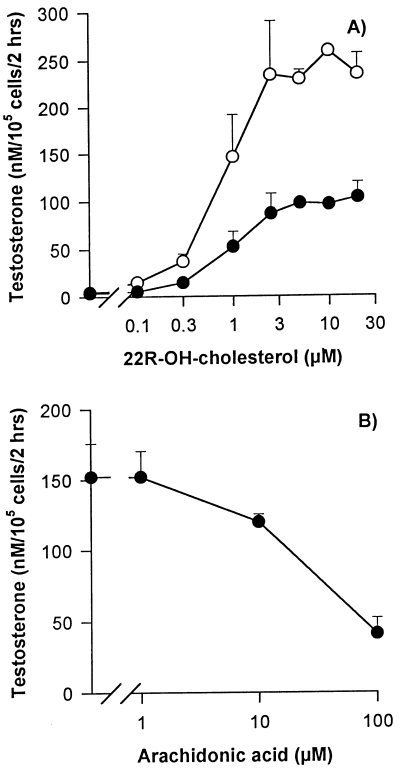

Fig. 2. Effect of arachidonic acid (AA) on the conversion of 22(*R*)-hydroxycholesterol to testosterone. The cells (10^5 cells/well/ml) were incubated with: (A) increasing concentrations of 22(*R*)-hydroxycholesterol in the absence (○) and presence (●) of AA (100 μM), (B) increasing concentrations of AA and 22(*R*)-hydroxycholesterol (1 μM). Testosterone accumulation was measured in the incubation medium, as described in Section 2. All data points are the mean ± S.E.M. of six experiments, each performed in triplicate. When S.E.M. values are not shown, these are smaller than the respective symbols.

age (CSCC), 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) enzymes, in rat testis Leydig cells.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's minimum medium (DMEM) was obtained from Gibco Ltd. (Middlesex, UK). Collagenase (type I) was purchased from Worthington Biochemical (Freehold, NJ, USA). Cyanoketone and SU-10603 (inhibitors of pregnenolone metabolism) were obtained from Sterling Research Group Europe (Guildford, Surrey, UK). Arachidonic acid (free acid, sealed ampoule), bovine serum albumin (essentially fatty acid-free) (BSA-FAF), Percoll, trypsin inhibitor (1% sterile-filtered solution), pregnenolone, progesterone, androstenediol, androstenedione and 22(*R*)-hydroxycholesterol were purchased from Sigma Quimica (Alcobendas, Spain). All other chemicals used were of analytical reagent grade.

2.2. Cell isolation and purification

Leydig cells were obtained from 200–300 g Sprague-Dawley rats. All animals received care according to the guidelines of the University Committee of Animal Resources at the University of Alcalá. The testes were decapsulated and subjected to longitudinal shaking (65 strokes/min) with collagenase (0.5 mg/ml) and trypsin inhibitor (20 μl/ml) for 40 min at 37°C. The cells were filtered through 60 μm nylon gauze to remove fragments of seminiferous tubules, and subjected to centrifugal elutriation followed by Percoll density gradient (0–90% v/v) centrifugation [13]. Leydig cell purity was routinely >95%, as determined by 3β-HSD cytochemistry [13]. Viability of the cells was determined by diaphorase histochemistry [13] and was not affected by any of the treatments (the viability was >90%).

2.3. Cell incubation, testosterone and pregnenolone determinations

Purified Leydig cells were resuspended in DMEM containing 10 mM HEPES pH 7.5 and 0.1% BSA-FAF and plated into 24-well Costar culture plates at a density of 100 000 cells/well in a final volume of 1 ml. The cells were incubated in monolayer for 2 h at 34°C in an air incubator in the presence and absence of different compounds. 22(*R*)-Hydroxycholesterol was used as substrate instead of cholesterol because it is more water-soluble. For pregnenolone measurement, the cells were pre-incubated for 30 min in the presence of 20 μM SU-10603 and 5 μM cyanoketone as potent inhibitors of pregnenolone metabolism (more than 95% in adult rats) [14]. The steroids used as substrates were dissolved in ethanol. The stock solutions were diluted with incubation medium before addition to the wells. Arachidonic acid was first dissolved in ethanol at a concentration of 80 mM and further diluted in incubation medium before its addition to the cells. All the manipulations were done under a nitrogen atmosphere. A new ampoule of arachidonic acid was used in each experiment. The final concentration of ethanol was always less than 0.1% (v/v) and did not alter basal or stimulated steroid release. The reactions were stopped with perchloric acid (final concentration 0.12 M) and the samples were stored at −20°C. The samples were thawed and neutralized with tripotassium phosphate (final concentration 0.11 M) before radioimmunoassay of testosterone [15] and pregnenolone [16].

All data are expressed as mean ± S.E.M. from at least three experiments, all of them performed in triplicate. Student's *t*-test was used for statistical analysis and differences were considered significant when $P < 0.05$.

3. Results

Arachidonic acid decreased 22(*R*)-hydroxycholesterol-stimulated testosterone production (Fig. 2A) and this effect was dose-dependent (Fig. 2B). The testosterone production was inhibited by about 60% with 100 μM of arachidonic acid at all the cholesterol concentrations studied.

The next experiment was designed to investigate the involvement of CSCC enzyme in the arachidonic acid action. Leydig cells were incubated with increasing concentrations of cholesterol (up to 20 μM) and SU-10603 (20 μM) and cyanoketone (5 μM) as inhibitors of pregnenolone metabolism in the presence and absence of 100 μM arachidonic acid. The levels of pregnenolone produced by Leydig cells were similar both in the presence and in the absence of arachidonic acid (Fig. 3).

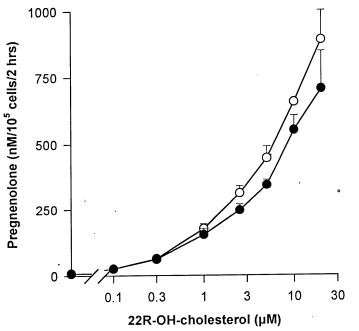

Fig. 3. Lack of effect of arachidonic acid on 22(*R*)-hydroxycholesterol-stimulated pregnenolone production. Leydig cells (10^5 cells/well/ml) were incubated with increasing concentrations of 22(*R*)-hydroxycholesterol, in the absence (○) and presence (●) of arachidonic acid (100 μM). Pregnenolone accumulation was measured in the incubation medium, as described in Section 2. All data are the mean ± S.E.M. of six experiments, each performed in triplicate.

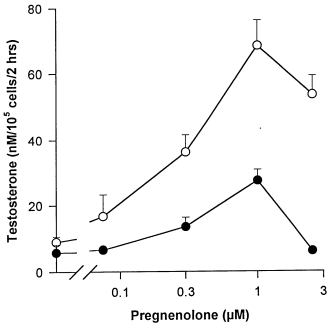

Fig. 4. Effect of arachidonic acid (AA) on the conversion of pregnenolone to testosterone. The cells (10^5 cells/well/ml) were incubated with increasing concentrations of prenenolone in the absence (○) and presence (●) of AA (100 μM). Testosterone accumulation was measured in the incubation medium, as described in Section 2. All data points are the mean ± S.E.M. of five experiments, each performed in triplicate. When S.E.M. values are not shown, these are smaller than the respective symbols.

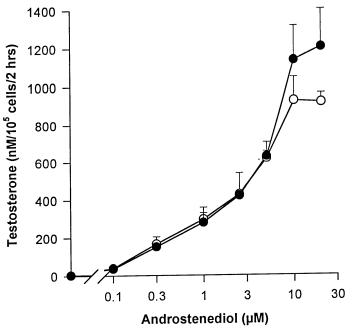

Fig. 5. Lack of effect of arachidonic acid (AA) on androstenediol-stimulated testosterone production. Leydig cells (10^5 cells/well/ml) were incubated with increasing concentrations of androstenediol, in the absence (○) and presence (●) of AA (100 μM). Testosterone accumulation was measured in the incubation medium, as described in Section 2. All data are the mean ± S.E.M. of six experiments, each performed in triplicate. When S.E.M. values are not shown, these are smaller than the respective symbols.

We then investigated the action of arachidonic acid in pregnenolone metabolism. First, Leydig cells were incubated with increasing concentrations of pregnenolone (up to 2.5 μM) with or without arachidonic acid, and the levels of testosterone were measured. In this case, the fatty acid inhibited the pregnenolone-stimulated testosterone production by about 60% at all pregnenolone concentrations studied (Fig. 4). Several experiments were conducted to determine whether arachidonic acid affects testosterone production by modification of 17β-HSD and/or 3β-HSD activity. Incubation of Leydig cells with androstenediol, as substrate of 3β-HSD, produced a dose-dependent stimulation of testosterone production and non-significant changes were found when the incubations were performed in the presence of arachidonic acid (100 μM) (Fig. 5). In order to investigate whether arachidonic acid modulates testosterone production by modification of 17β-HSD activity, Leydig cells were incubated with increasing concentrations of androstenedione (up to 10 μM) in the presence and absence of arachidonic acid (100 μM). As is shown in Fig. 6A, arachidonic acid inhibited androstenedione-stimulated testosterone production and this effect followed a dose-dependent pattern (Fig. 6B). The highest percentage of inhibition was found at 100 μM arachidonic acid. To examine if the inhibition of 17β-HSD activity by arachidonic acid is due to the fatty acid itself or to its transformation to active metabolites, we added inhibitors of cyclooxygenase (indomethacin), lipoxygenase (NDGA) and epoxygenase P-450 (clotrimazole) enzymes together with arachidonic acid (Fig. 7). The levels of testosterone produced by Leydig cells incubated with androstenedione (1 μM) were inhibited by arachidonic acid by about 60%. However, when inhibitors of arachidonic acid metabolism were present, the percentage of inhibition was significantly higher.

Finally, the role of protein kinase C (PKC) in arachidonic acid action was investigated since it has been shown that in Leydig cells, PKC can be stimulated by arachidonic acid [10]. Activation of PKC using the tumor-promoting phorbol ester phorbol-12-myristate-13-acetate (PMA) did not modify the levels of testosterone produced by androstenedione as suggested by the corresponding results: 4.6 ± 1.1, 241.5 ± 35.7, 79.7 ± 24.7, and 237.1 ± 39.8 nM testosterone/10^5 cells/2 h, for cells incubated in basal conditions or in the presence of androstenedione (1 μM) alone and in combination with arachidonic acid (100 μM) or PMA (0.3 μM), respectively.

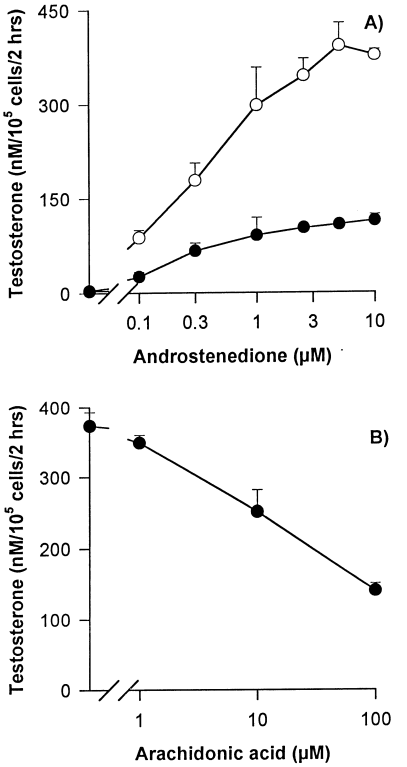

Fig. 6. Effect of arachidonic acid (AA) on the conversion of androstenedione to testosterone. The cells (10^5 cells/well/ml) were incubated with: (A) increasing concentrations of androstenedione in the absence (○) and presence (●) of AA (100 μM), (B) increasing concentrations of AA and androstenedione (1 μM). Testosterone accumulation was measured in the incubation medium, as described in Section 2. All data points are the mean ± S.E.M. of six (A) and four (B) experiments, each performed in triplicate. When S.E.M. values are not shown, these are smaller than the respective symbols.

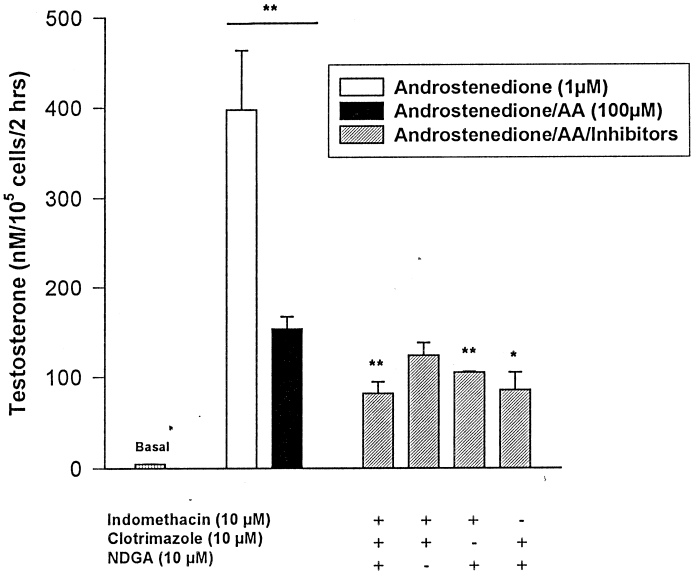

Fig. 7. Effect of arachidonic acid (AA) metabolism inhibitors on androstenedione-stimulated testosterone production. The cells (10^5 cells/well/ml) were incubated with medium alone (basal) or androstenedione in the absence and presence of AA and its metabolism inhibitors. The data represent the mean ± S.E.M. of three different experiments each performed in triplicate. $*P < 0.05$, $**P < 0.01$, vs. testosterone production with androstenedione/AA.

4. Discussion

Previous results from our group have indicated that arachidonic acid regulates LH-stimulated testosterone production by decreasing cAMP production. However, this fatty acid seems to act also at some point below cAMP formation [11]. The results presented here show that administration of exogenous arachidonic acid to rat Leydig cells resulted in a decrease of 22(*R*)-hydroxycholesterol-stimulated testosterone production, which is a clear indication that the post-cAMP effects of this fatty acid are directly concerned, at least in part, with steroidogenic enzymes. Similar results have been observed in other steroidogenic cells, like granulosa [17] and theca [18] cells, in which the inhibitory effect of arachidonic acid on steroidogenesis occurs by decreasing the conversion of cholesterol to progesterone or androstenedione, respectively.

The first step in the biosynthesis of testosterone from cholesterol is its transport to the inner mitochondrial membrane, where it is acted upon by CSCC enzyme to produce pregnenolone. In our system, arachidonic acid failed to block the conversion of 22(*R*)-hydroxycholesterol to pregnenolone, indicating that the cytochrome P-450 side-chain cleavage enzyme complex is not the site of inhibition. However, the effect of arachidonic acid on CSCC enzyme seems to be dependent on the cell type studied. While in Leydig (present results) and theca [18] cells this fatty acid has no effect on this rate-limiting step of steroidogenesis, in granulosa cells arachidonic acid decreases the ability of cells to convert cholesterol to pregnenolone [17].

Because CSCC enzyme was not affected by arachidonic acid, a decrease of testosterone levels is likely when Leydig cells are incubated with pregnenolone in the presence of this fatty acid. In fact, our results indicate that pregnenolone-stimulated testosterone production is inhibited (about 60%) by arachidonic acid. Three enzymes are involved in the biosynthesis of testosterone from pregnenolone: 3β-HSD, 17α-hydroxylase/lyase and 17β-HSD [12]. Results from the present study demonstrate that only 17β-HSD seems to be involved in the arachidonic acid action, since nearly 60% inhibition of testosterone production was found when the cells were incubated with androstenedione. Furthermore, no arachidonic acid effect was found when androstenediol was used as substrate in testosterone synthesis, which is a clear indication that 3β-HSD is not affected by the fatty acid. Similar results have been obtained in granulosa cells, where the conversion of pregnenolone to progesterone was not inhibited in the presence of arachidonic acid [17].

The action of arachidonic acid on signal transduction pathways can be achieved directly and/or indirectly by catabolic conversion to eicosanoids. The inhibitory effect of arachidonic acid on 17β-HSD was shown to be not dependent on metabolism to eicosanoids since cyclooxygenase, epoxygenase and lipoxygenase inhibitors potentiated this inhibitory effect. In agreement with this, Romanelli et al. [7] and Lin [9] found that conversion of arachidonic acid to its metabolites is not required for its steroidogenic effects in rat Leydig cells. However, Mele et al. [6] recently stated that the lipoxygenase pathway is involved in the mechanism of action of LH on testis Leydig cells and these products seem to be important in the later conversion of pregnenolone to testosterone [19]. Our results strongly support the previous concept that the arachidonic acid effect on Leydig cells is not due to its transformation to active metabolites. Furthermore, the presence of inhibitors of arachidonic acid metabolism in the incubation medium together with the fatty acid results in higher inhibition of androstenedione-stimulated testosterone production than that obtained in the absence of the inhibitors. This fact can be explained by the blocking effect of indomethacin, clotrimazole and NDGA, on the further arachidonic acid metabolism, thus increasing its concentration into the cells and therefore its inhibitory effect.

In a number of studies, phosphorylation has been shown to

be involved in regulating enzyme activity, including the NAD- and NADP-dependent dehydrogenases. Various potential phosphorylation sites for PKC, casein kinase (CK) and protein kinase A (PKA) have been detected in recently cloned 17β-HSD isoenzymes [20] and it has been postulated that multiple kinases may play a role in the regulation of 17β-HSD [21]. Furthermore, we have previously shown that arachidonic acid stimulated PKC activity in rat Leydig cells [10], so it is likely that PKC could mediate the action of arachidonic acid. However, our results clearly indicate that PKC activation has no effect on 17β-HSD activity.

The mechanisms of the inhibitory effect of the fatty acid on 17β-HSD remain to be determined, but they seems to be specific to this enzyme. It has been postulated that in the rat testis, the enzymes of androgen biosynthesis, associated with the smooth endoplasmic reticulum, have specific requirements for optimal activity such as the membrane lipid composition [22]. Alterations of the structure of the membrane environment produced by arachidonic acid could be responsible for the inhibition of 17β-HSD. In agreement with this, Cooke and Robaire [23] demonstrated that steroid biosynthetic enzymes differ in their requirements with respect to the polar and non-polar regions of the membrane and that changes in the membrane environment can regulate the activity of steroidogenic enzymes. In our system, 17β-HSD appears to be sensitive to changes in the structure of the membrane environment.

In conclusion, the present results demonstrate that 17β-HSD activity is under arachidonic acid control and support the idea that this fatty acid acts as an additional intracellular messenger associated with the hormonal action of LH.

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