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Arachidonic Acid Regulation of Steroid Synthesis: New Partners in the Signaling Pathway of Steroidogenic Hormones

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ABSTRACT

Although the role of arachidonic acid (AA) in trophic hormone-stimulated steroid production in various steroidogenic cells is well documented, the mechanism responsible for AA release remains unknown. We have previously shown evidence of an alternative pathway of AA generation in steroidogenic tissues. Our results are consistent with the hypothesis that, in steroidogenic cells, AA is released by the action of a mitochondrial acyl-CoA thioesterase (MTE-I). We have shown that recombinant MTE-I hydrolyses arachidonoyl-CoA to release free AA. An acyl-CoA synthetase specific for AA, acyl-CoA synthetase 4, has also been described in steroidogenic tissues. In the present study we investigate the new concept in the regulation of intracellular levels of AA, in which trophic hormones can release AA by mechanisms different from the classical PLA_2-mediated pathway. Inhibition of ACS4 and MTE-I activity by triacsin C and NDGA, respectively results in a reduction of StAR mRNA and protein abundance. When both inhibitors are added together there is a synergistic effect in the inhibition of StAR mRNA, StAR protein levels and ACTH-stimulated steroid synthesis. The inhibition of steroidogenesis produced by the NDGA and

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triacsin C can be overcome by the addition of exogenous AA. In summary, results shown here demonstrate a critical role of the acyl-CoA synthetase and the acyl-CoA thioesterase in the regulation of AA release, StAR induction, and steroidogenesis. This further suggests a new concept in the regulation of intracellular distribution of AA through a mechanism different from the classical PLA_2-mediated pathway that involves a hormone-induced acyl-CoA synthetase and a hormone-regulated acyl-CoA thioesterase.

Key Words: Acyl-CoA thioesterase; Acyl-CoA synthetase; StAR; ACTH.

INTRODUCTION

Although the role of arachidonic acid (AA) in trophic hormone-stimulated steroid production in various steroidogenic cells is well documented (1–10), the mechanism responsible for AA release remains unknown. Previous studies have reported that phospholipase A_2 (PLA_2) inhibitors abrogate the effect of LH- and ACTH-stimulated steroid production thereby suggesting the involvement of PLA_2 in the mechanism of action of trophic hormones (2,3,7). However, no evidence has been reported demonstrating the activation of PLA_2 by steroidogenic hormones.

We have presented data showing evidence of an alternative pathway of AA generation in steroidogenic tissues (11). Our results are consistent with the hypothesis that, in steroidogenic cells, AA is released by the action of an acyl-CoA thioesterase. We have shown that mitochondrial acyl-CoA thioesterase (MTE-I) hydrolyses arachidonoyl-CoA (AA-CoA) to release free AA and that ACTH increases the activity of MTE-I in Y1 cells. We also demonstrated that commonly used PLA_2 inhibitors, as well as inhibitors of AA release and metabolism, such as nordihydroguayaretic acid (NDGA, a lipoxigenase inhibitor), effectively impair the activity of recombinant MTE-I.

A possible explanation for the effect of classical PLA_2 inhibitors on thioesterase activity could be the existence of a serine-histidine-aspartic acid catalytic triad normally present in $\alpha\beta$ hydrolases as determined by site-directed mutagenesis (12). The amino acids of the catalytic triad are all conserved and located within the third exon, even though this exon shows the lowest degree of sequence conservation. This is in agreement with our previous results showing that antibodies raised against a synthetic peptide matching a sequence that contains the serine included in the catalytic triad inhibit steroid synthesis in a recombinant cell-free assay (13).

The protein was first identified as a hormone-dependent phosphoprotein involved in steroid synthesis through the release of AA (14). This protein was identified by its capacity to increase mitochondrial steroidogenesis in a cell-free assay (15,16). The activity of the protein was dependent of cAMP and PKA and blocked by the use of 4-bromophenacyl-bromide (BPB) and NDGA, both inhibitors of AA release. Importantly, this inhibition could be overcome by the addition of AA (16,17), an indication that this protein regulates steroid synthesis through the (direct or indirect) activation of AA release. The protein was later purified to homogeneity and identified as a 43-kDa phosphoprotein (p43) (14). Further cloning and sequencing of a cDNA encoding p43 revealed its primary structure (18). The protein was 100% homologous to a mitochondrial–peroxisome-proliferator-induced acyl-CoA thioesterase (MTE-1) and 92.5%

homologous to a cytosolic thioesterase (CTE-1) (19,20). CTE-1 and MTE-1 are members of an acyl-CoA thioesterase family with very long chain acyl-CoA thioesterase activity (19,20) that includes four isoforms with different subcellular locations and high degree of homology (21). The family includes a cytosolic (CTE-1), a mitochondrial (MTE-1), and peroxisomal (PTE-Ia and Ib) forms of the enzyme (21). Recently, this gene family was cloned and characterized in mice showing that all isoforms are encoded by three exons spaced by two introns (21,22).

MTE-I was identified in all steroidogenic tissues including brain and placenta (18). On the other hand, a report by Kang (23) implicates the participation in steroid synthesis of an AA-preferring acyl-CoA synthetase named ACS4, in steroidogenic tissues. The expression of ACS4 was observed in adrenal cortex cells, luteal and stromal cells of the ovary and Leydig cells of the testis (21). Moreover, it was demonstrated that ACTH induced the expression of ACS4 in the murine adrenocortical tumor cell line Y1, an effect that is suppressed by glucocorticoids (24).

In agreement with those results we have proposed the concerted regulation of steroidogenesis exerted by MTE-I and ACS4 based on experiments performed in the presence of triacsin C, an inhibitor of acyl-CoA synthetases and NDGA, which inhibits the activity of the acyl-CoA thioesterase (11). Our results clearly showed that addition of triacsin C in combination with ineffective doses of NDGA produced a marked reduction of the IC_{50} for NDGA (11).

In the present study we further investigate the new concept in the regulation of intracellular levels of AA, in which trophic hormones can release AA by mechanisms different from the classical PLA_2-mediated pathway.

RESULTS AND DISCUSSION

Taking into account that NDGA and triacsin C produced a significant inhibition on steroidogenesis as previously described (11), here we report that inhibition of MTE-I

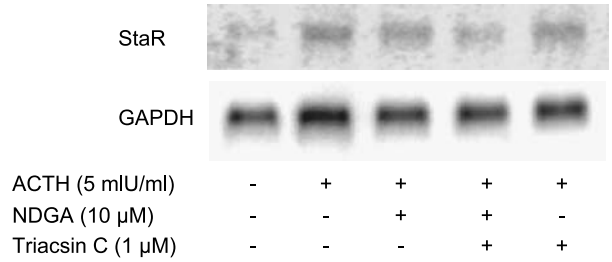

Figure 1. Combined effect of triacsin C and NDGA on StAR mRNA expression. Y1 cells were preincubated in the presence or the absence of triacsin C (4 h, 37°C) and NDGA (15 min, 37°C). Cells were then incubated for 60 min with 2 mIU/ml of ACTH or its vehicle. StAR mRNA expression was assayed by Northern blot. For this purpose, total RNA was isolated by the guanidinium isothiocyanate method using TriZol reagent (GIBCO-Life Technologies), according to the manufacturer's protocol. Twenty-four micrograms total RNA were separated by electrophoresis on 1.5% agarose gels and blotted onto Hybond N+ (Amersham). StAR mRNA was detected using a specific ^{32}P-labeled cDNA probe.

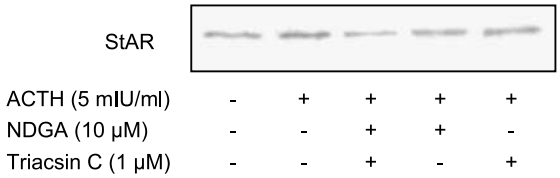

Figure 2. Combined effect of triacsin C and NDGA on StAR protein expression. Y1 cells were preincubated in the presence, or the absence, of triacsin C (4 h, 37°C) and NDGA (15 min, 37°C). Cells were then incubated for 60 min with 2 mIU/ml of ACTH or its vehicle. StAR protein content was assessed by Western blot analysis. Proteins (50 µg per sample) were separated by SDS-PAGE 12% and electrophoretically transferred to PVDF membranes (Bio-Rad Laboratories) according to the manufacturer recommendations. StAR protein was detected using anti-StAR antibodies and immunoreactive bands were visualized by enhanced chemiluminescence.

and ACS4 activity by these compounds results in a reduction of Steroidogenic Acute Regulatory protein (StAR) (a protein that is induced by AA and that controls the rate-limiting step in steroidogenesis) and its mRNA abundance (Figs. 1 and 2).

The simultaneous addition of triacsin C and NDGA produced a synergistic inhibitory effect on StAR mRNA and protein levels. The same inhibitory effect was also observed for steroid synthesis (11). The inhibition of steroidogenesis produced by the NDGA and triacsin C was overcome by the addition of exogenous AA (Fig. 3).

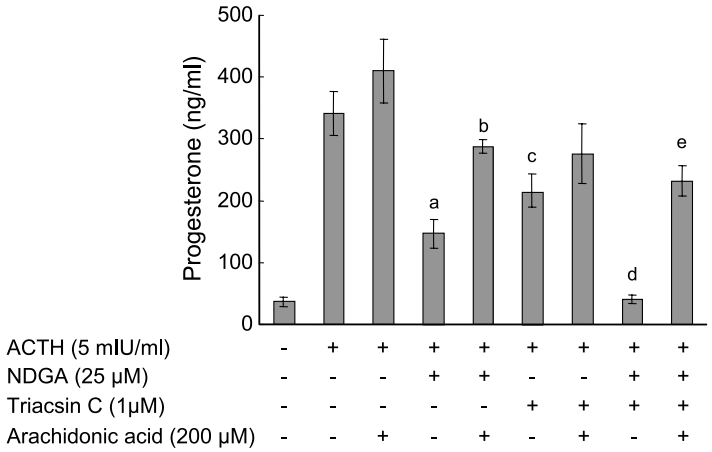

Figure 3. Effect of AA on steroidogenesis inhibition produced by ACS4 and MTE-I inhibitors. Y1 cells were preincubated in the presence or the absence of triacsin C (4 h, 37°C), NDGA (15 min, 37°C) and AA (200 µM). Cells were then incubated for 60 min with 2 mIU/ml of ACTH or its vehicle. Progesterone produced was determined in the incubation medium by RIA. Data are expressed as the mean ± S.D. (n = 3). a: $P < 0.001$ *vs.* ACTH-stimulated cells; b: $P < 0.001$ *vs.* NDGA treated cells and stimulated by ACTH; c: $P < 0.05$ *vs.* ACTH-stimulated cells; d: $P < 0.001$ *vs.* ACTH stimulated cells, and e: $P < 0.001$ *vs.* NDGA, Triacsin C treated cells and stimulated by ACTH.

The fact that AA can overcome the inhibitory effect produced by NDGA at the concentration used in this experiment suggests that this compound is working at the level of the acyl-CoA thioesterase and not at the level of the lipoxygenase.

In view of the fact that AA release is linked to hormone-dependent StAR induction, our results demonstrate that the acyl-CoA synthetase and the acyl-CoA thioesterase are both involved in StAR induction and steroidogenesis through the release of AA.

In addition, we have recently described that silencing the expression of mitochondrial acyl-CoA thioesterase I and of acyl-CoA synthetase 4 inhibits hormone-induced steroidogenesis. The reduction of MTE-I and ACS4 expression levels produced a marked reduction of steroid output in cAMP-stimulated Leydig and adrenal cells. The inhibition of steroidogenesis was overcome by addition of exogenous AA. Knocking down the expression of MTE-I led to a significant reduction in the expression levels of StAR (paper submitted for publication in Molecular and Cellular Endocrinology).

The role of cAMP-dependent protein kinase phosphorylation events is widely accepted in ACTH- and LH-mediated stimulation of cholesterol transport from the outer to the inner mitochondrial membrane and steroid output in adrenal and Leydig cells. These phosphorylation events trigger a multistep signal transduction pathway that in adrenal and Leydig cells generates AA to regulate the expression of StAR.

Recently, we have presented data that led us to propose that hormone stimulation of AA release, StAR induction and steroid production through cAMP-dependent phosphorylation involves *de novo* synthesis of ACS4 as an early step (manuscript submitted for publication at Journal of Molecular Endocrinology). That hypothesis is supported by: 1) ACS4 is rapidly induced by hormones acting via cAMP-dependent processes, 2) this process is inhibited by cycloheximide treatment, an established inhibitor of protein synthesis that inhibits steroid synthesis. The high turnover rate of ACS4 is in line with the tight control of the intracellular concentrations of fatty acyl-CoA esters due to their strong detergent-like nature and their important role in lipid metabolism.

On the other hand, the synthesis of the acyl-CoA thioesterase appears not to be controlled by hormone treatment (manuscript submitted for publication at Journal of Molecular Endocrinology). However, as we described, the hormones have a direct effect on enzyme activity and since this activity requires an acyl-CoA pool as a source of AA, we cannot rule out an enzyme activation by a hormone increased availability of its substrate.

An important issue is the origin of cytosolic free AA to be esterified into arachidonoyl-CoA. The cytosolic free AA could derive from plasma membrane phospholipids or cholesterol esters. The major source of cholesterol in the rat adrenal is the cholesterol esterified into high-density lipoproteins (HDL) (25,26). It has been shown that dexamethasone inhibits cholesterol metabolism and that this effect is reversed by free AA. These results may indicate that the AA esterified into acyl-CoA may come from membrane phospholipids by the action of PLA_2. However, no demonstration of an inhibitory effect of dexamethasone on PLA_2 activity was reported in those studies. On the contrary, glucocorticoids are known to inhibit ACS4 induction. Moreover, as shown in Table 1, arachidonyl trifluoromethylketone (ATK), a specific inhibitor of cytosolic PLA_2(27), had no effect on hormone-induced steroid synthesis.

Table 1. Effect of ATK on steroids synthesis.

	ACTH (5 mIU/ml)	
ATK (μM)	−	+
0	36.3 ± 4.6	173.5 ± 8.2
1	28.2 ± 6.7	157.3 ± 1.8
10	31.2 ± 2.3	163.9 ± 6.0
100	39.1 ± 4.3	143.1 ± 3.9

Y1 cells were preincubated in the presence or the absence of different concentrations of ATK (10 min, 37°C) and then incubated for 60 min with 5 mIU/ml of ACTH or its vehicle. Progesterone produced was determined in the incubation medium by RIA. Data are expressed as the the mean ± S.D. (n = 3).

Together, those results strongly support the hypothesis that the free cytosolic AA to be esterified into AA-CoA by the acyl-CoA synthetase is derived from cholesterol esters. In adrenocortical cells, HDL enhances steroid production and increases cellular cholesterol content. Rat HDL contains a high amount of arachidonate in its cholesterol esters. This is in agreement with the suggestion that free AA, which will be esterified to acyl-CoA, is derived from the hydrolysis of cholesterol esters (20).

In summary, results shown here demonstrate a critical role of the acyl-CoA synthetase and the acyl-CoA thioesterase in the regulation of AA release, StAR induction, and steroidogenesis. This further suggests a new concept in the regulation of intracellular distribution of AA through a mechanism different from the classical PLA_2-mediated pathway that involves a hormone-induced acyl-CoA synthetase and a hormone-regulated acyl-CoA thioesterase. A scheme depicting the possible regulation of AA release that will result in StAR induction and steroid synthesis is shown in Fig. 4.

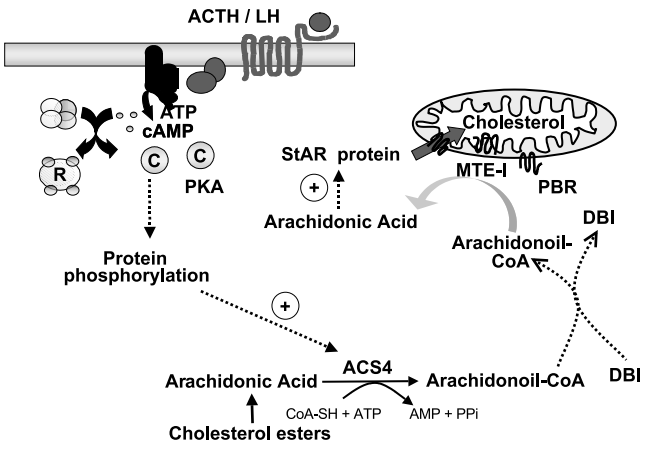

Figure 4. Scheme depicting the possible regulation of AA release in hormone stimulated steroid synthesis.

Hormones will rapidly induce the acyl-CoA synthetase, an effect exerted by protein phosphorylation events, which will esterify AA derived from cholesterol esters into arachidonoyl-CoA. In its turn, AA-CoA may bind to the diazepam binding inhibitor (DBI, an acyl-CoA binding protein), which will bind to the peripheral benzodiazepam receptor (PBR) located in the outer mitochondrial membrane. This possibly will lead to facilitated transfer of arachidonoyl-CoA to the mitochondria that will serve as substrate for the MTE-I located in the mitochondria. The AA generated by this pathway will induce StAR protein, which will increase the transport of cholesterol from the outer to the inner mitochondrial membrane, the rate limiting steep step in steroid synthesis.

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