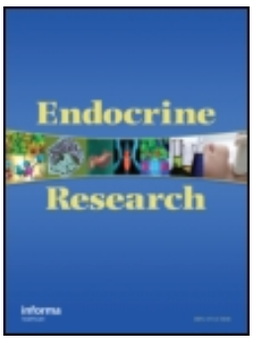

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ACTH REGULATION OF MITOCHONDRIAL ACYL-CoA THIOESTERASE ACTIVITY IN Y1 ADRENOCORTICAL TUMOUR CELLS

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ABSTRACT

We have previously purified and cloned a phosphoprotein, *A*rachidonic acid-*R*elated *T*hioesterase *I*nvolved in *St*eroidogenesis (ARTISt), involved in steroid synthesis through Arachidonic Acid (AA) release. Arachidonic acid-related thioesterase involved in steroidogenesis resulted to be a member of a new family of acyl-CoA thioesterases. The protein was identified by its biocapacity to increase mitochondrial steroidogenesis in a cell free bioassay. In the present study we measure the activity of ARTISt using arachidonoyl-CoA (AA-CoA) as substrate. We demonstrate that ACTH significantly stimulates endogenous mitochondrial thioesterase activity as early as 5 min after ACTH stimulation of Y1 cells. Nordihydroguaiaretic acid (NDGA), an inhibitor of AA release known to affect steroidogenesis, affects the in vitro activity of recombinant ARTISt and also the endogenous mitochondrial acyl-CoA thioesterases. ACTH activation of the enzyme protected ARTISt to the inhibitory effect of NDGA. These results show that an enzyme that release AA from AA-CoA can be regulated in intact cells by steroidogenic hormones.

Key Words: Acyl-CoSA thioesterase; ACTH; Mitochondria

INTRODUCTION

We have previously identified a hormone-dependent phosphoprotein involved in steroid synthesis through the release of arachidonic acid (AA).

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The protein was identified by its capacity to increase mitochondrial steroidogenesis in a cell-free bioassay.[1,2] The activity of the protein was dependent on cAMP and PKA and blocked by the use of 4-bromophenacyl-bromide and nordihydroguaiaretic acid (NDGA), both inhibitors of AA release. Importantly, this inhibition could be reverted by the addition of AA,[2,3] an indication that this protein regulates steroid synthesis through the (direct or indirect) activation of AA release.

Like StAR, p43 is targeted to the inner mitochondrial membrane.[4,5] In accordance with the postulated obligatory role of the protein in steroidogenesis, we detected the protein and its mRNA in all steroidogenic tissues including placenta and brain.[4] Given the obligatory role of the protein in the activation of steroidogenesis through the release of AA, we proposed the name *A*rachidonic acid-*R*elated *T*hioesterase *I*nvolved in *St*eroidogenesis (ARTISt) for p43.[4] Here we address the question of whether ACTH can regulate the activity of the mitochondrial enzyme using Arachidonoyl-CoA (AA-CoA) as a substrate. We demonstrated that ACTH significantly stimulates ARTISt activity in intact Y1 cells.

MATERIALS AND METHODS

Tissue Culture

Murine Y1 adrenocortical tumor cells, were maintained in Ham-F10 medium, supplemented with 12.5% heat-inactivated horse serum and 2.5% heat-inactivated fetal bovine serum, 1.2 g/L $NaHCO_3$, 200 IU/mL penicillin, and 200 μg/mL streptomycin sulfate. When appropriate, ACTH was added to the culture medium in the presence of 0.1% bovine serum albumin. Steroids produced were measured by radioimmunoassay (RIA). Determination of progesterone production by RIA and of steroids by fluorometry showed comparable results. Therefore, data are shown as progesterone production (ng/mL) in the incubation medium.

Thioesterase Activity Determination

Acyl-CoA thioesterase activity was determined using 15 μM [1-^{14}C]AA-CoA (Specific activity: 51.6 mCi/mmol, NEN Life Science Products, Boston, MA) as substrate in a buffer containing 10 mM Hepes, 50 mM KCl, pH 7.4. AA released during the reaction was extracted from the aqueous phase with *n*-hexane and quantified by scintillation counting.

Preparation of Mitochondrial Fractions

Isolation of mitochondria was done as described.[5]

RESULTS

We have previously demonstrated the presence of ARTISt in mitochondria from adrenal and from Y1 cells. In order to study the effect of ACTH on mitochondrial thioesterase activity, Y1 adrenal cells were incubated in the presence or the absence of 5 mIU ACTH for 5 and 30 min. Following incubation, the mitochondria were isolated from control and ACTH-treated cells. Enzyme activity was then determined as AA released from AA-CoA as described in Materials and Methods. As shown in Fig. 1, ACTH produced a 1.8-fold stimulation of mitochondrial thioesterase activity already after 5 min of hormone stimulation.

Since NDGA strongly inhibits both steroid production, StAR protein expression[6–8] and recombinant ARTISt activity[9] we tested here whether NDGA inhibits ARTISt activity after ACTH stimulation. For that purpose, NDGA was added to the enzyme assay where it effectively reduced the activity of the enzyme isolated from mitochondria of both ACTH-stimulated and control cells. The extent of the inhibition was 15 and 20% vs. 22 and 39% for 50 and 100 μM NDGA in ACTH-stimulated and control mitochondria respectively (Fig. 2). These results are in agreement with those reporting that the inhibitory effect of NDGA is manifested only when the inhibitor is added prior to ACTH in the assay.[10]

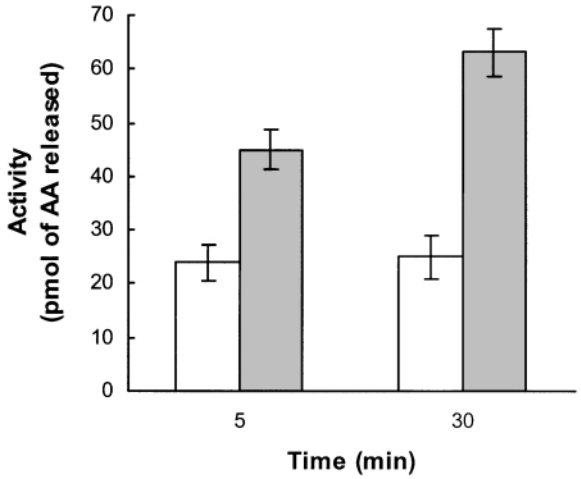

Figure 1. *Mitochondrial acyl-CoA thioesterase activity on Y1 cells.* Acyl-CoA thioesterase activity was determined in the mitochondrial fraction of Y1 cells using [1-^{14}C]AA-CoA as substrate. The mitochondrial fraction was obtained as described in Materials and Methods from cells that were incubated in the presence (filled bars) or the absence (open bars) of 5 mIU/mL ACTH. Results are expressed as the mean ± SD of one representative experiment performed in triplicate.

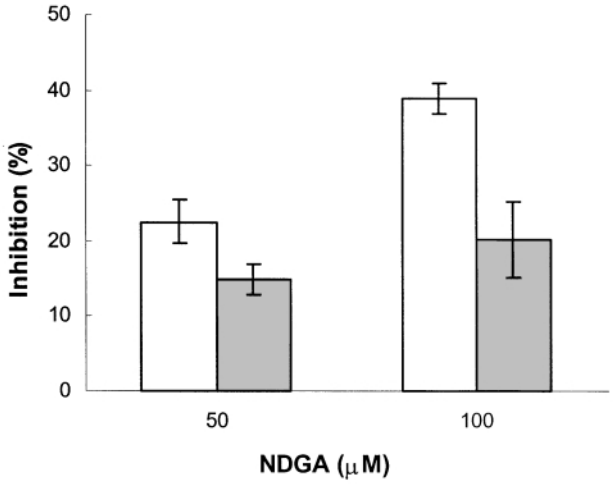

Figure 2. *Effect of NDGA on Mitochondrial acyl-CoA thioesterase activity on Y1 cells.* Acyl-CoA thioesterase activity was determined in the mitochondrial fraction of Y1 cells using [1-^{14}C]AA-CoA as substrate and in the presence of NDGA. The mitochondrial fraction was obtained as described in Materials and Methods from cells that were incubated in the presence (filled bars) or the absence (open bars) of 5 mIU/mL ACTH for 30 min. Results are expressed as the mean ± SD of one representative experiment performed in triplicate.

DISCUSSION

It is already known that AA plays an important role in the regulation of steroidogenesis.[6,11–13] Previous studies have also reported that phospholipase A_2 (PLA_2) inhibitors abrogate the effect of ACTH- and LH-stimulated steroid production, thereby suggesting the involvement of PLA_2 in the mechanism of action of trophic hormones. However, no evidence has been reported demonstrating the activation of PLA_2 by steroidogenic hormones. In the present study we describe for the first time that ACTH can regulate the activity of a mitochondrial thioesterase enzyme that releases AA from AA-CoA. It has been reported that inhibitors commonly used to inhibit PLA_2 also inhibit Acyl-CoA thioesterase activity.[9] Thus, our results support the notion that AA release in steroidogenic cells is regulated through the activation of ARTISt, a mitochondrial Acyl-CoA thioesterase that belongs to a new family of acyl-CoA thioesterases specific for very long chain Acyl-CoA.

In addition, Kang et al. demonstrated the expression of an AA-preferring acyl-CoA synthetase named ACS4, in steroidogenic tissues.[14] The expression of ACS4 was observed in adrenal cortex cells, luteal and stromal cells of the ovary and Leydig cells of the testis.[14] Moreover, the expression of ACS4 was demonstrated in the murine adrenocortical cell line (Y1) where it is induced by ACTH and suppressed by glucocorticoids.[15] Taking together, those results

suggest that AA-CoA is an important intermediate in steroidogenesis. In this regard we have recently demonstrated that triacsin C, an inhibitor of AA-CoA synthetase activity, affects hormone-induced steroid synthesis in Y1 cells.[9] The participation of the AA-CoA-mediated pathway for AA release in steroidogenesis was further demonstrated by the combined inhibitory effect of triacsin C and NDGA.[9] Our results clearly show that addition of triacsin C in combination with ineffective concentrations of NDGA produced a marked reduction of the IC_{50} for NDGA. Therefore, the regulatory role of AA in steroidogenesis requires the concerted action of the acyl-CoA synthetase and the thioesterase.[9]

The question then arises as to why free cytosolic AA has to be re-esterified in order to stimulate steroidogenesis. One possible explanation is the need for AA in a special compartment of the cell (e.g. mitochondria). The compartmentalization of long-chain acyl-CoA esters is an important unsolved problem, and the actual cytosolic concentration of free long-chain acyl-CoA esters is not known for any tissue.[16] The high degree of sequestration of CoA into long chain acyl-CoA suggests that AA is likely to become limiting for diverse roles in specific compartments of the cell.[16]

It is known that an acyl-CoA binding protein (ACBP) known also as DBI (diazepam binding inhibitor) is reported to be expressed in high concentrations in specialized cells such as steroid producing cells of the adrenal cortex and testis.[17,18] Thus it can be proposed that AA-CoA binds to AA-CoA-binding protein (DBI), which in turns binds to an 18 kDa protein located in the outer mitochondrial membrane,[18,19] the peripheral benzodiazepine receptor (PBR). This will possibly lead to facilitated transfer of AA-CoA into the mitochondria. Peripheral benzodiazepine receptor is expressed at particularly high levels in adrenal cells and is also essential for steroidogenesis.[18,19] This supply of AA-CoA to the acyl-CoA thioesterase may be an additional mechanism for the activation of the enzyme.

Another important issue is the origin of cytosolic free AA to be esterified into AA-CoA. As already mentioned AA could derive from plasma membrane phospholipids or from cholesterol esters. The major source of cholesterol in the rat adrenal is the cholesterol esterified in high density lipoprotein (HDL).[20,21] In adrenocortical cells, HDL enhances steroid production and increases cellular cholesterol content. Rat HDL contains a high amount of arachidonate in its cholesterol esters fatty acids. This is an agreement with the suggestion that free AA, which will be esterified to acyl-CoA may come from the hydrolysis of cholesterol ester.[14] Nevertheless, since it has been shown that dexamethasone inhibits cholesterol metabolism and that this effect is reverted by free AA, we can not rule out the possibility that the free AA that is esterified into acyl-CoA may come from membrane phospholipids by the action of PLA_2.

In summary, a possible model for the regulation of intracellular AA levels includes acyl CoA synthetases and thioesterases. ACS4 would act sequestering free AA by esterification into AA-CoA which will then bind ACBP/DBI thus forming an intracellular pool that could then be delivered to an acyl-CoA

thioesterase that will, in turn, release AA in a specific compartment of the cell upon hormone treatment.

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