Role of Intramitochondrial Arachidonic Acid and Acyl-CoA Synthetase 4 in Angiotensin II-Regulated Aldosterone Synthesis in NCI-H295R Adrenocortical Cell Line

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Although the role of arachidonic acid (AA) in angiotensin II (ANG II)- and potassium-stimulated steroid production in zona glomerulosa cells is well documented, the mechanism responsible for AA release is not fully described. In this study we evaluated the mechanism involved in the release of intramitochondrial AA and its role in the regulation of aldosterone synthesis by ANG II in glomerulosa cells. We show that ANG II and potassium induce the expression of acyl-coenzyme A (CoA) thioesterase 2 and acyl-CoA synthetase 4, two enzymes involved in intramitochondrial AA generation/export system well characterized in other steroidogenic systems. We demonstrate that mitochondrial ATP is required for AA generation/export system, steroid production, and steroidogenic acute regulatory protein induction. We also demonstrate the role of protein tyrosine phosphatases regulating acyl-CoA synthetase 4 and steroidogenic acute regulatory protein induction, and hence ANG II-stimulated aldosterone synthesis. **(*Endocrinology* 153: 3284–3294, 2012)**

Steroid hormones, required for normal reproductive function and body homeostasis, are synthesized in steroidogenic cells of the adrenal, ovary, testis, placenta, and brain. Regulation of steroidogenesis is predominantly controlled by the trophic hormones: LH/chorionic gonadotropin (CG) in Leydig cells; ACTH in adrenal zona fasciculata (ZF) and angiotensin II (ANG II), potassium, and ACTH in adrenal zona glomerulosa (ZG).Transport of cholesterol from the outer to the inner mitochondrial membrane, the rate-limiting step of steroidogenesis (1, 2), is regulated by these hormones, acting through different signal transduction pathways. These hormones regulate phospho-dephosphorylation mechanisms through activation of different kinases and phosphatases. These processes finally allow the interaction of several proteins involved in cholesterol transport to the action site. Among these proteins the translocator protein (TSPO) (3) and the steroidogenic acute regulatory (StAR) protein, both mitochondrial proteins, are widely studied (4, 5). This protein complex contributes to the delivery of cholesterol from outer to inner mitochondrial membrane (6).

In steroidogenic cells, protein tyrosine phosphatases (PTP) are one of the targets of hormone-induced Ser/Thr phosphorylation (7). We previously described that *in vivo* ACTH treatment produces an increase in total PTP activity in adrenal ZF (8). This increase in enzymatic activity is accompanied by a decrease in phosphotyrosine proteins. We and other laboratories have also described that ACTH promotes rapid activation of more than one PTP in bovine and rat ZF cells (9, 10). In rat adrenal ZF and Leydig cells, both primary cell cultures and MA-10 cell line, we demonstrated that PTP inhibitors reduce induction of steroid synthesis in response to hormone or cAMP (8, 11–13).

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Abbreviations: AA, Arachidonic acid; ACOT2, acyl-CoA thioesterase; ACSL4, acyl-CoA synthetase 4; ANG II, angiotensin II; BPA, benzylphosphonic acid; 8Br-cAMP, 8-bromo-cAMP; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; CG, chorionic gonadotropin; CoA, coenzyme A; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; PAO, phenylarsine oxide; PKA, protein kinase A; PTP, protein tyrosine phosphatases; StAR, steroidogenic acute regulatory; ZF, zona fasciculate; ZG, zona glomerulosa.

It is accepted that hormone stimulation of steroid synthesis in adrenal ZF, ZG, and testicular Leydig cells involves the release of arachidonic acid (AA) (14). Subsequent AA metabolism by lipoxygenase or epoxygenase pathways has been implicated in the regulation of steroid synthesis in adrenal and Leydig cells through the induction of StAR (15, 16). We described a hormonally regulated pathway for the generation and exportation of AA in mitochondria in Leydig cells that is involved in the regulation of StAR protein induction and steroid synthesis (17–19). In this mechanism, free intramitochondrial AA is generated by the action of an acyl-coenzyme A (CoA) synthetase (ACSL4) and a mitochondrial acyl-CoA thioesterase (ACOT2). ACSL4 sterified free AA to AA-CoA, which could be delivered to ACOT2 and, in turn, releases AA in the mitochondria upon hormone treatment (17–19). Export of AA from mitochondria depends on energized and actively respiring organelle. In Leydig cells, mitochondrial ATP disruption affects the export of AA, StAR protein levels, and steroidogenesis (19, 20). After export from mitochondria, AA participates, through its lipoxygenated metabolites, in the induction of StAR protein in Leydig cells (15).

Hormone stimulation of AA release, StAR induction, and steroid production through cAMP-dependent phosphorylation involves, as an early step, new synthesis of ACSL4 (21). LH/CG and their second messenger (cAMP) increase ACSL4 protein levels in a time- and concentration-dependent manner. The induction of ACSL4 is dependent on PTP activity (22, 23). We described that the steroid production by hormone stimulation is linked to the sequential action of PTP, ACSL4, and StAR proteins (22, 23).

Although the role of AA in ANG II and potassium-stimulated steroid production in ZG cells is well documented (24–26), the mechanism responsible for AA release remains unknown. Although the hydrolysis of phospholipids by phospholipase A_2 results in the release of arachidonate, a precursor of eicosanoids, this enzyme is not activated in ANG II-stimulated rat ZG cells (24). We hypothesized that the mechanism of AA generation present in Leydig and ZF adrenal cells may also operate in ZG cells. Therefore the aim of this work was to demonstrate the participation of ACSL4, ACOT2, and intramitochondrial AA in ANG II and potassium-triggered steroid synthesis. Moreover, we also studied the role of PTP in the regulation of steroid synthesis in ZG cells.

Materials and Methods

Materials

Acrylamide, bis-acrylamide, AA, agarose, formaldehyde, BSA, fatty acid-free BSA, 8-bromo-cAMP (8Br-cAMP), and phenylarsine oxide (PAO) were purchased from Sigma Chemical Co. (St. Louis, MO). Benzylphosphonic acid bis-acetoxymethyl ester (BPA), a cell-permeable derivative of benzylphosphonic acid, came from ICN Biomedicals, Inc. (Aurora, OH). DMEM/Ham's F-12 1:1 cell culture media, antibiotics, trypsin-EDTA, and TriZol reagent were from Life Technologies, Inc. (Gaithersburg, MD); plastic flasks and dishes were provided by Orange Scientific (Braine-l'Alleud, Belgium). Cosmic calf bovine serum was from HyClone (Tauranga, New Zealand). Polyclonal antibodies against StAR protein, ACOT2, and ACSL4 were previously produced in our laboratory (21, 27). Anti-β-tubulin monoclonal antibody was from Upstate Group Co. (Temecula, CA), and anti-OxPhos Complex III core 2 subunit monoclonal antibody was from Invitrogen (Carlsbad, CA). Aldosterone antibody was generously provided by Alain Bélanger (Laval University, Quebec, Canada). Electrophoresis supplies, polyvinylidendifluoride membrane, and secondary antibodies (horseradish peroxidase-conjugated goat antibody) were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). An enhanced chemiluminescence kit was from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other chemicals were commercial products of the highest grade available.

Cell line

The NCI-H295R cell line is a clonal strain of human adrenal carcinoma (28). The cell line was from American Type Culture Collection (ATCC, Manassas, VA) and was handled as originally described (29). The growth medium consisted of DMEM/Ham's F-12 1:1 containing 1.1 g/liter $NaHCO_3$, 20 mM HEPES, 200 IU/ml penicillin, 200 μg/ml streptomycin sulfate, and 5% COSMIC serum. Flasks and multiwell plates were maintained at 37 C in a humidified atmosphere containing 5% CO_2. ANG II was used to treat the cells (100 nM) for the times indicated. Potassium was used to treat the cells (14 mM) for the times indicated. Aldosterone production was measured in the culture medium by RIA.

Bovine adrenal ZG cell culture and treatments

Bovine adrenal glands were obtained from a local slaughterhouse. ZG cells were prepared by enzymatic dispersion with dispase and purified on Percoll density gradients (30). Primary cultures of purified ZG cells were established as described in detail elsewhere (30) and kept in serum-free DMEM/Ham's F-12 1:1 containing 1.1 g/liter $NaHCO_3$, 20 mM HEPES, 200 IU/ml penicillin, and 200 μg/ml streptomycin sulfate medium for 1 d before experiments, which were performed on the third day of culture. Cells cultured in 10-cm plastic Petri dishes (10^7

cells per dish) or in 24-well plates (5×10^5 cells per well) were washed twice with modified Krebs-Ringer (136 mM NaCl; 5 mM $NaHCO_3$; 1.2 mM KH_2PO_4; 1.2 mM $MgSO_4$; 1.8 mM KCl; 0.2 mM EGTA; 5.5 mM D-glucose; 20 mM HEPES; pH 7.4). At the end of the incubation period, cells were scraped and sedimented at $200 \times g$ for 15 min before subcellular fractionation as described below. ANG II (100 nM), Potassium (KCl) (14 mM), and 8Br-cAMP (1 mM) were used to treat cells for the times indicated.

Isolation of mitochondria

Isolation of mitochondria was done as described elsewhere (31). Briefly, cell cultures were washed with PBS, scraped in 10 mM Tris-HCl (pH 7.4), 250 mM sucrose, 0.1 mM EDTA, 10 μM leupeptin, 1 μM pepstatin A, and 1 mM EGTA (buffer A), homogenized with a Pellet pestle motor homogenizer (Kimble Kontes, Vineland, NJ), and centrifuged at $600 \times g$ for 15 min. The supernatant was centrifuged at $10{,}000 \times g$ for 15 min and rendered a mitochondrial pellet that was washed once with buffer A and resuspended in 10 mM Tris-HCl (pH 7.4), 10 μM leupeptin, 1 μM pepstatin A, and 1 mM EGTA. Fractions were subjected to enzymatic analysis to assess their purity according to Ref. 32. The purity of each fraction was at least 90%, a value similar to that of previous publications (33).

Western blot

Total or mitochondrial proteins (20 μg) were separated on 12% SDS/PAGE and electrotransferred to polyvinylidendifluoride membranes as described previously (17). Membranes were then incubated with 5% fat-free powdered milk in 500 mM NaCl, 20 mM Tris-HCl (pH 7.5), and 0.5% Tween 20 for 60 min at room temperature with gentle shaking. Membranes were then rinsed twice in 500 mM NaCl, 20 mM Tris-HCl (pH 7.5), and 0.5% Tween 20 and incubated overnight with appropriate dilutions of primary antibody at 4 C: 1:4,000 rabbit polyclonal anti-ACOT2, 1:1,500 rabbit polyclonal anti-ACSL4, 1:1,000 rabbit polyclonal anti-StAR protein, 1:5,000 mouse monoclonal anti-β-tubulin, and 1:10,000 mouse monoclonal anti-OxPhos Complex III core 2 subunit. Bound antibodies were developed by incubation with secondary antibody (1:5,000 goat antirabbit and 1:5,000 goat antimouse horseradish peroxidase conjugated) and detected by chemiluminescence.

[1-^{14}C]AA incorporation in NCI-H295R cells

NCI-H295R cells were labeled by a previously described method (31) with minor modifications. [1-^{14}C]AA (NEN Life Science Products, Boston, MA; specific activity, 53 mCi/mmol) was added to the cultures (1 μCi/ml per well; 1 well = 2×10^6 cells) in serum-free DMEM/Ham's F-12 1:1 medium containing 0.5% fatty acid-free BSA. After 3 h incubation at 37 C in a humidified atmosphere containing 5% CO_2, cells were incubated for 3 h in the presence or absence of ANG II 10^{-7} M. When indicated, cells were incubated with antimycin A, oligomycin, or carbonyl cyanide m-chlorophenyl hydrazone (CCCP) together with ANG II. After these treatments, the cells were washed with serum-free DMEM/Ham's F-12 1:1 medium containing 0.5% fatty acid-free BSA. Mitochondrial pellets obtained were resuspended as described above. Protein concentration was measured and lipids were extracted from equal amounts of mitochondrial proteins (500 μg) from each treatment after the previous addition of 500 ng of unlabeled AA. Lipid extraction was performed twice with ethyl acetate (six volumes per one volume of mitochondrial fraction). The organic phase was then collected and dried in nitrogen at 25 C and analyzed by two successive thin-layer chromatographies on silica gel. Radioactive spots were developed with a Storm phosphorimager (Amersham Biosciences, Stockholm, Sweden) after 1 wk of exposition, and spot intensities were analyzed with ImageQuant 5.2 software (Amersham Biosciences).

RNA extraction and semiquantitative RT-PCR

Total RNA from the different treatment groups was extracted using TriZol reagent following the manufacturer's instructions (Life Technologies, Inc.-BRL, Grand Island, NY). The RT and PCR analyses were made with 2 or 4 μg of total RNA of NCI-H295R cells. The cDNA generated were further amplified by PCR under optimized conditions using the primer pairs listed below. Primers used for *ACOT2* amplification (amplicon size 870 bp) were: sense primer, 5′-AGATCATTAGGGTTCCTGC TCG-3′, and antisense primer, 5′-TTGATGCGATT TCTGTTGACG-3′. For *ACSL4* amplification (amplicon size 115 bp), sense primer, 5′-GAAGGTAAAAAGTTAA-CAGGCAAACAT-3′, and antisense primer, 5′-TCA-GAGTTTAAATCTCTTTCCCAGGTT-3′, were used. The amplified *L19* ribosomal protein product of each sample (amplicon size 500 bp) was used as housekeeping gene (34). Specific primers for *L19* were: sense: 5′-AG-TATGCTCAGGCTTCAGAA-3′, and antisense: 5′-TTC-CTTGGTCTTAGACCTGC-3′. Reaction conditions were one cycle of 94 C for 5 min followed by 27 cycles for *ACSL4* and *L19* of 94 C for 30 sec, 60 C for 30 sec, and 72 C for 45 sec, 33 cycles for *ACOT2* of 94 C for 30 sec, 60 C for 30 sec, and 72 C for 1 min. The number of cycles used was optimized for each gene to fall within the linear range of PCR amplification. PCR for *ACOT2* amplification included 5% dimethylsulfoxide. PCR products were resolved on 1.5% agarose gel containing ethidium bromide. The gel images were digitally recorded and amplicon

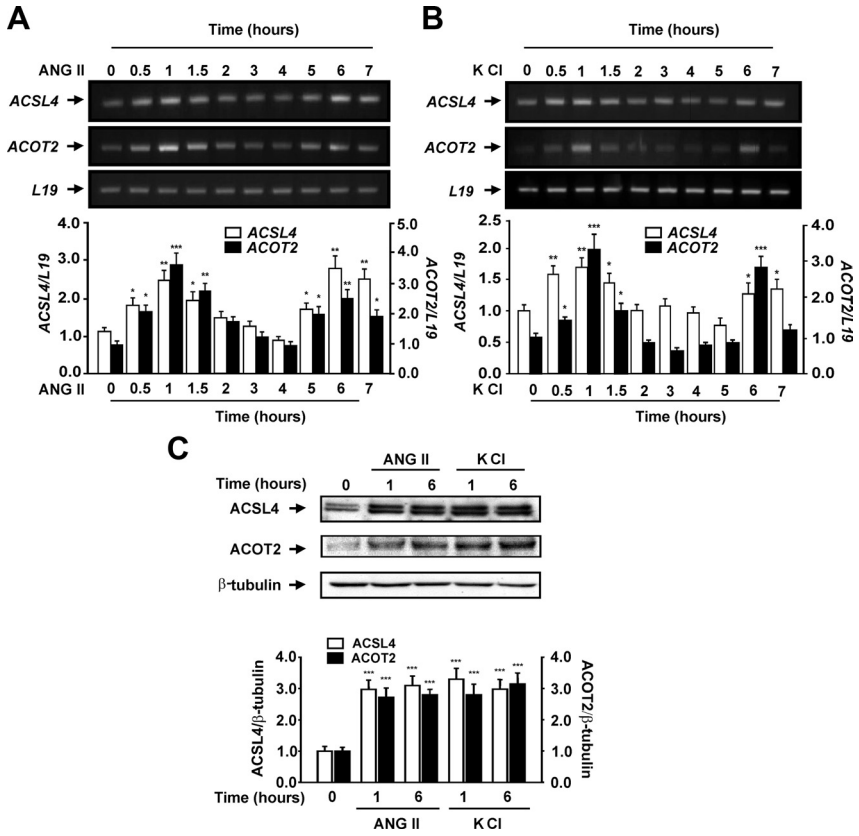

FIG. 1. Regulation of ACSL4 and ACOT2 expression levels by ANG II and potassium in NCI-H295R cells. A and B, Cells were stimulated with (A) ANG II (100 nM) or (B) KCl (14 mM) for the times indicated (0–7 h). Total RNA was extracted from different treatment groups and subjected to semiquantitative RT-PCR analysis of *ACSL4* and *ACOT2* mRNA expression. The RT-PCR products were resolved in ethidium bromide-stained agarose gels (1.5%). Images of gels were acquired, and a representative image is shown. The integrated OD of *ACSL4* and *ACOT2* mRNA expression was quantified for each band and normalized with corresponding *L19* mRNA bands. Data represent the means ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* untreated cells. C, Cells were stimulated with ANG II (100 nM) for 1 and 6 h. Cell proteins were subjected to SDS-PAGE, and Western blot analysis was performed using sequentially anti-ACSL4, anti-ACOT2, and anti-β-tubulin antibodies. Specific bands were detected by chemiluminescence. A representative Western blot and the quantitative representation of three independent experiments are shown. The integrated OD of ACSL4 and ACOT2 protein levels was quantified for each band and normalized with the corresponding β-tubulin signal. Results are expressed as means ± SD of three independent experiments. ***, $P < 0.001$ *vs.* untreated cells.

levels were quantified with the Gel-Pro computer-assisted image analyzer (IPS, North Reading, MA).

Protein quantification and statistical analysis

Protein was determined by the Bradford method (35) using BSA as a standard. Statistical analysis was performed by Student's *t* test or ANOVA followed by the Student-Newman-Keuls test.

Results

We previously demonstrated that generation and exportation of AA in the mitochondria in steroidogenic cells is effected by the concerted action of two enzymes: ACSL4 and ACOT2 with hormone stimulation (18, 19, 27). First we analyzed the effect of ANG II and potassium on ACSL4 and ACOT2 expression in an adrenocortical cell line, NCI-H295R. We determined the kinetics of *ACSL4* and *ACOT2* mRNA expression after ANG II (100 nM) (Fig. 1A) or potassium (14 mM) (Fig. 1B) treatment. NCI-H295R cells stimulated either by ANG II or potassium showed a biphasic *ACSL4* mRNA expression pattern. The magnitude of the response was significant ($P < 0.01$) at 30 min and peaked at 1 h. At 2–3 h it reached values similar to the control and increased again later, approximately 1.5–2.5 fold at 6 h. Cells stimulated with either ANG II or potassium also showed a biphasic pattern of *ACOT2* mRNA expression at 1 h and 6 h (a 2.5- and 3.5-fold increase in the level of *ACOT2* mRNA over control cells, respectively). The magnitude of the response was significant ($P < 0.05$) at 30 min. In contrast, cAMP-stimulated NCI-H295R cells showed an increase in *ACSL4* mRNA only at 1 h of stimulation whereas *ACOT2* mRNA remained unchanged (Fig 2 A). These results are similar to those observed for cAMP-stimulated Leydig cells (21).

Next we studied whether the increment in *ACSL4* and *ACOT2* mRNA levels by ANG II and potassium shown above was also accompanied by an increment of ACSL4 and ACOT2 protein levels (Fig. 1C). In fact, cells stimulated with ANG II and potassium also showed an increase in *ACSL4* and *ACOT2* protein expression pattern with increments in protein levels (3-fold over control cells) at 1 and 6 h (Fig. 1C). As observed for *ACSL4* and *ACOT2* mRNA, cAMP-stimulated NCI-H295R cells showed an increase in ACSL4 protein level only at 1 h of stimulation whereas ACOT2 remained unchanged (Fig 2B).

In systems dependent on cAMP/protein kinase A (PKA), induction of StAR protein expression is regulated by AA generated in the mitochondria (19). In LH-stimulated Leydig cells, disruption of mitochondrial ATP synthesis was also described to inhibit export of AA and steroidogenesis (19). This was demonstrated in experiments

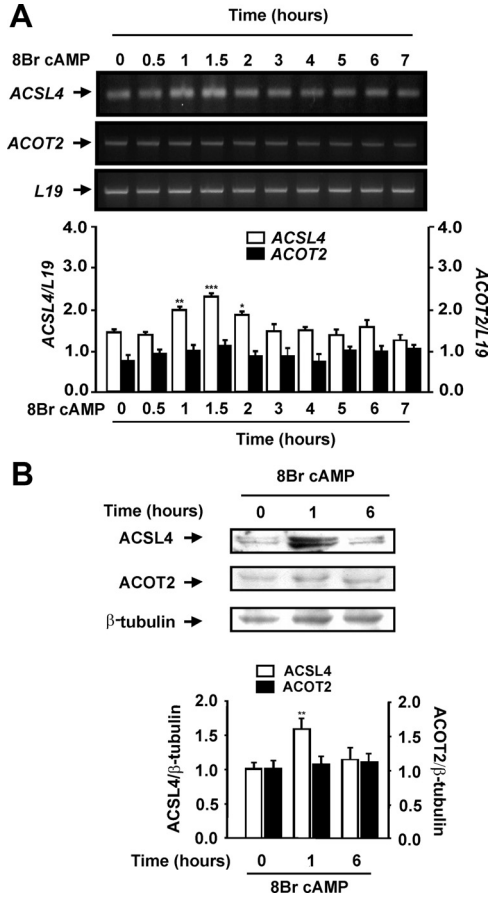

FIG. 2. Regulation of ACSL4 and ACOT2 expression levels by 8Br-cAMP in NCI-H295R cells. A, Cells were stimulated with 8Br-cAMP (1 mM) for the times indicated (0–7 h). Total RNA was extracted from different treatment groups and subjected to semiquantitive RT-PCR analysis of *ACSL4* and *ACOT2* mRNA expression. The RT-PCR products were resolved in ethidium bromide-stained agarose gels (1.5%). Images of gels were acquired, and a representative image is shown. The integrated OD of *ACSL4* and *ACOT2* mRNA expression was quantified for each band and normalized with corresponding *L19* mRNA bands. Data represent the means ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* untreated cells. B, Cells were stimulated with 8Br-cAMP (1 mM) for 1 and 6 h. Cell proteins were subjected to SDS-PAGE, and Western blot analysis was performed using sequentially anti-ACSL4, anti-ACOT2, and anti-β-tubulin antibodies. Specific bands were detected by chemiluminescence. A representative Western blot and the quantitative representation of three independent experiments are shown. The integrated OD of ACSL4 and ACOT2 protein levels was quantified for each band and normalized with the corresponding β-tubulin signal. Results are expressed as means ± SD of three independent experiments. ***, $P < 0.001$ *vs.* untreated cells.

that used antimycin A, an agent that inhibits mitochondrial complex III, thereby slowing down the transport of electrons through the chain of the inner mitochondrial membrane, and oligomycin, an agent that inhibits F_0/F_1 ATPase and prevents the export of ATP.

To determine whether an AA generation/export system in mitochondria could also be involved in the action of ANG II in NCI-H295R cells, we analyzed intramitochondrial AA levels in cells treated with antimycin A or oligomycin with ANG II stimulus. As shown in Fig. 3A, ANG II did not affect AA levels in mitochondria. However, antimycin A and oligomycin produced a significant increase in ANG II-induced mitochondrial AA accumulation (Fig. 3A, lanes 4 and 5 *vs.* lane 2). As a control, we used CCCP, a $\Delta\psi_m$ uncoupler that does not affect AA accumulation in Leydig cells (19). Treatment of NCI-H295R cells with CCCP (Fig. 3A, lane 3 *vs.* lane 2) did not affect AA accumulation in these cells.

The effect produced by antimycin A and oligomycin on mitochondrial AA accumulation in NCI-H295R paralleled the inhibitory effect of these two compounds on aldosterone secretion (Fig. 3B). The effect of this inhibitors in the process of ANG II-stimulated steroid synthesis was reversed by the addition of AA in combination with ANG II (Fig. 3B). This effect was not observed when cells were treated with a combination of CCCP, ANG II, and AA (Fig. 3B). Steroidogenesis supported by the permeable analog of cholesterol, 22(R)HO-cholesterol, was not affected by antimycin A, oligomycin, or CCCP (data not shown). These results suggest that ATP synthesis might be necessary for free fatty acid export and steroid synthesis as was shown in Leydig cells (19, 20, 36).

In the next step we studied the effect of mitochondrial disruptors on StAR protein induction. As shown in Fig. 3C, the three compounds inhibited StAR protein induction promoted by ANG II. The addition of AA restored StAR induction in the presence of antimycin A and oligomycin but not in the presence of CCCP (Fig. 3C).

To confirm results for NCI-H295R cells, we studied the effect of mitochondrial disruptors on freshly isolated ZG cells. Results shown in Fig. 4 indicate that disruption of the mitochondrial function affected aldosterone production in a similar fashion to the inhibition observed in the NCI-H295R cell line. Similarly, antimycin A, oligomycin, and CCCP inhibited StAR protein induction in these cells (Fig. 4, *inset*).

We previously demonstrated that both ACSL4 and StAR induction by cAMP depend on PTP activity in Leydig cells (12, 37). To determine whether steroidogenesis stimulated by ANG II or K^+ involves PTP action, we analyzed the effect of two different inhibitors of PTP activity, PAO and benzylphosphonic acid (BPA), on aldosterone production in NCI-H295R cells. As shown in Fig. 5A, 0.125 μM was sufficient to detect inhibitory action of PAO on aldosterone levels (Fig. 5A), whereas aldosterone synthesis levels were reduced to control levels by using 2 μM of PAO (Fig. 5A). Figure 5B shows that 0.025 μM BPA is sufficient to detect its inhibitory action on stimulation of steroidogenesis. These results support that PTP are involved in steroidogenesis stimulated by ANG II or K^+.

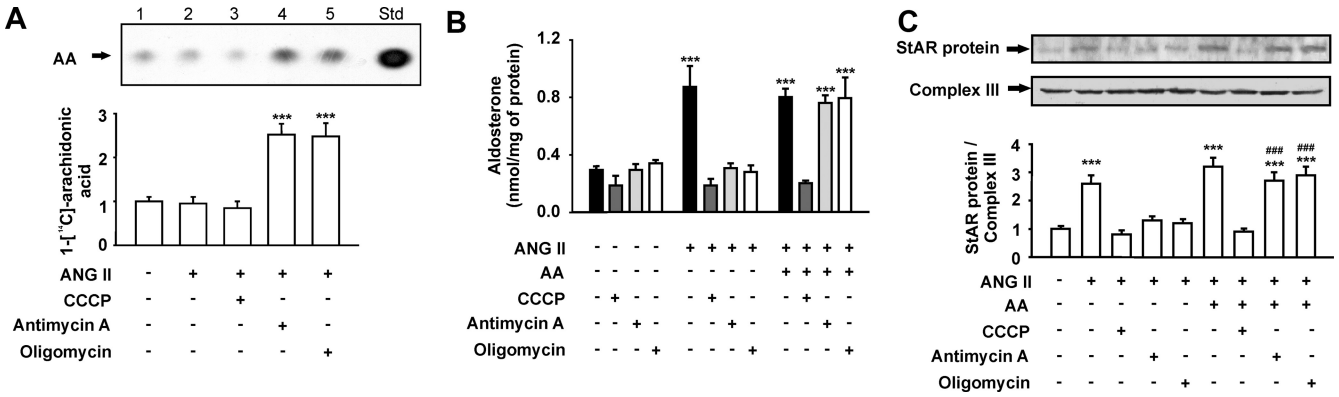

FIG. 3. Effect of antimycin A, oligomycin, and CCCP and exogenous AA on mitochondrial AA accumulation, aldosterone synthesis, and StAR protein induction in NCI-H295R cells. A, Cells were labeled with 1-[^{14}C]AA as described in *Materials and Methods*. Then, cells were stimulated with ANG II (100 nM) in the presence and in the absence of CCCP (5 μM), antimycin A (10 μM), or oligomycin (1 μM) for 3 h. Then the mitochondrial fraction was obtained as described in *Materials and Methods*, and lipids were extracted with ethyl acetate. The organic phase was dried and dissolved in chloroform-methanol (9:1, vol/vol) and analyzed by thin-layer chromatography on silica gel plates. Representative autoradiography showing AA spots in mitochondrial fraction is shown. *Bars* denote mitochondrial AA levels (in arbitrary units). Data represent the means ± SD of three independent experiments. ***, $P < 0.001$ *vs.* untreated cells. B, Cells were stimulated with ANG II (100 nM) for 6 h in the presence and in the absence of the inhibitors (CCCP, 5 μM; antimycin A, 10 μM; oligomycin, 1 μM) and AA (100 μM) in the culture medium containing 0.1% fatty acid-free BSA. Aldosterone production in the incubation media was measured by RIA. Results are expressed as the means ± SD from the three independent experiments. ***, $P < 0.001$ *vs.* untreated cells. Then the mitochondrial fraction was obtained as described in *Materials and Methods*, proteins were subjected to SDS-PAGE, and Western blot analysis was performed using sequentially anti-StAR and anti-complex III antibodies. Specific bands were detected by chemiluminescence. A representative Western blot and the quantitative representation of three independent experiments are shown (panel C). The integrated OD of StAR protein levels was quantified for each band and normalized with the corresponding complex III signal. Results are expressed as means ± SD of three independent experiments. ***, $P < 0.001$ *vs.* untreated cells; ###, $P < 0.001$ *vs.* ANG II without AA. Std, Standard.

Steroidogenesis supported by the permeable analog of cholesterol (22(R) HO-cholesterol) was not affected by PAO or BPA treatment (data not shown).

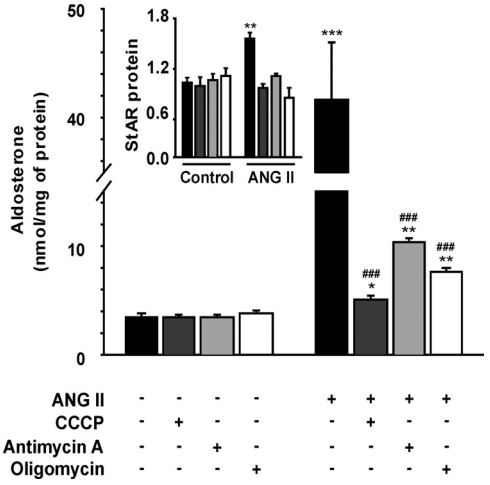

FIG. 4. Effect of antimycin A, oligomycin, and CCCP on aldosterone synthesis in rat freshly isolated ZG cell culture. Bovine ZG cells were stimulated with ANG II (100 nM) in the presence and absence of CCCP (5 μM), antimycin A (10 μM), or oligomycin (1 μM) for 2 h. Aldosterone production in the incubation media was measured by RIA. Results are expressed as the means ± SD of four independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* untreated cells. ###, $P < 0.001$ *vs.* ANG II alone. Mitochondrial proteins were obtained and subjected to SDS-PAGE and Western blot analysis using anti-StAR antibody. Specific bands were detected by chemiluminescence. The integrated OD of StAR protein levels was quantified for each band (*inset*). Results are expressed as means ± SD of four independent experiments. **, $P < 0.01$ *vs.* untreated cells.

We studied the effect of PTP inhibition on ACSL4 and ACOT2 induction by 2 μM PAO and 0.4 μM BPA with ANG II stimulus. Both PAO and BPA inhibited ANG II-stimulated induction of ACSL4 protein but had no effect on ANG II-stimulated induction of ACOT2 protein (Fig. 5C). Similar results were found when mRNA levels were analyzed (Fig. 5D).

Next, we tested the ability of AA to reverse reduction in steroid production produced with inhibition by PTP. Cells were stimulated with ANG II for 6 h in the presence and absence of PAO or BPA together with AA. In the presence of PTP inhibitors and AA, cells restored their capacity of steroid secretion (Fig. 6A).

As expected, PTP inhibition also impaired stimulation of StAR protein synthesis (Fig. 6B), and AA also overcame inhibition produced by PAO and BPA on ANG II-stimulated StAR protein levels (Fig. 6B).

SHP2 has been shown to participate in ANG II signal transduction pathway in vascular smooth muscle cells (38, 39). We thus studied the effect of a specific inhibitor of SHP2 activity on aldosterone synthesis in glomerulosa cells. As shown in Fig. 7, NSC-87877 significantly inhibited aldosterone synthesis in a concentration-dependent fashion with maximal inhibition at 100 μM of the inhibitor.

Discussion

In previous works we demonstrated the fundamental role of intramitochondrial AA in the regulation of the synthesis

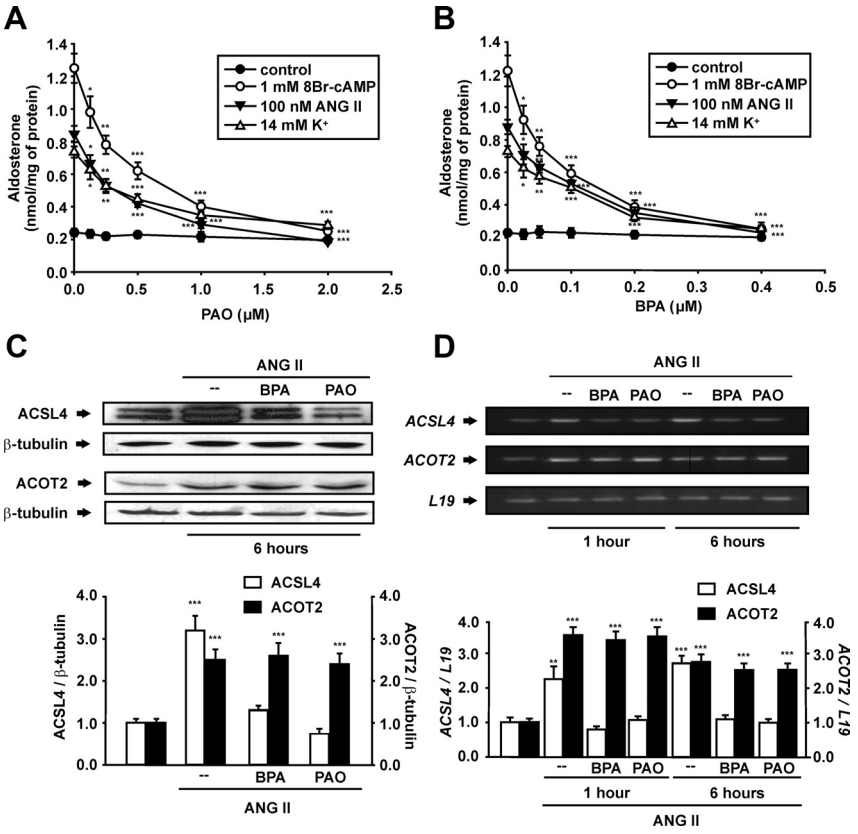

FIG. 5. Effect of PTP inhibitors on ANG II and 8Br-cAMP-stimulated aldosterone production and ACSL4 and ACOT2 induction in NCI-H295R cells. A and B, Cells were incubated with or without 8Br-cAMP (1 mM), ANG II (100 nM), or KCl (14 mM) for 6 h in the presence or absence of the indicated concentrations of PAO (A) or BPA (B). Aldosterone production was determined by RIA. Values represent the means ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* 8Br-cAMP, ANG II, or KCl alone. C, Cells were stimulated with ANG II (100 nM) for 6 h. Cell proteins were subjected to SDS-PAGE, and Western blot analysis was performed using sequentially anti-ACSL4, anti-ACOT2, and anti-β-tubulin antibodies. Specific bands were detected by chemiluminescence. A representative Western blot and the quantitative representation of three independent experiments are shown. The integrated OD of ACSL4 and ACOT2 protein levels was quantified for each band and normalized with the corresponding β-tubulin signal. Results are expressed as means ± SD of three independent experiments. ***, $P < 0.001$ *vs.* untreated cells. D, Cells were stimulated with ANG II (100 nM) for 1 h or 6 h. Total RNA was extracted from different treatment groups and subjected to RT-PCR analysis of *ACSL4* and *ACOT2* mRNA expression. The RT-PCR products were resolved in ethidium bromide-stained agarose gels (1.5%). Images of gels were acquired, and a representative image is shown. The integrated OD of *ACSL4* and *ACOT2* mRNA expression was quantified for each band and normalized with corresponding *L19* mRNA bands. Data represent the means ± SD of three independent experiments. **, $P < 0.01$; ***, $P < 0.001$ *vs.* untreated cells.

of glucocorticoids and androgens (18, 19). Here we describe for the first time that in adrenocortical cells ANG II regulates the generation and export of AA in mitochondria and that these events are implicated in StAR induction and aldosterone synthesis. This study was performed with the adrenocortical human cell line NCI-H295R as experimental model. These cells typically transport cholesterol across the mitochondrial membrane, which is regulated by three different stimuli: ACTH, mainly through the cAMP/protein kinase A (PKA) system, and potassium and ANG II, which exert their actions through cAMP/PKA-independent pathways. Therefore, these results demonstrate that the generation and exportation of intramitochondrial AA is a common step in steroid synthesis stimulated by cAMP-dependent and -independent pathways. Here we also demonstrate, for the first time, that ANG II and potassium regulate the expression of both ACSL4 and ACOT2 proteins. These enzymes are involved in the mitochondrial AA generation/export system and are regulated by ACTH through cAMP/PKA pathway in other steroidogenic cells. Therefore, PKA-independent signals are also able to regulate the expression of ACSL4 and ACOT2 in glomerulosa cells.

Regulation of ACSL4 and ACOT2 by ANG II and potassium is evidenced by an increase in their mRNA and protein levels. Detailed kinetic analysis reveals that ANG II- and potassium-induced *ACSL4* and *ACOT2* mRNA expression is biphasic. This pattern is similar to that produced by epidermal growth factor in Leydig cells (14) and differs from the effect produced by ACTH and LH on adrenocortical and Leydig cells, respectively. In fact, the latter two hormones increase the expression of *ACSL4* but not of *ACOT2* (21).

Our experiments show two bursts of mRNA at 1 and 6 h after ANG II and potassium treatment (Fig. 1). We also show here that ACSL4 and ACOT2 protein levels are increased both at 1 and 6 h after ANG II and potassium stimulation. This result allows us to suggest that the increase in mRNA and protein at 1 h contributes to the regulation of aldosterone synthesis occurring 6 h after ANG II treatment. The second burst of ACSL4 mRNA observed 6 h after ANG II and potassium treatment coincides with what we reported for epidermal growth factor -stimulated MA-10 Leydig cells (14), although it differs with that observed for hCG-, or cAMP-stimulated MA-10 Leydig cells and cAMP-stimulated NCI-H295R cells (Fig. 2), that stimulates the initial ACSL4 increase but not the second; and do not affect ACOT2 (21). Thus, it is possible that ANG II and potassium differentially regulate two signaling pathways to render the same final effect in a different time frame. Given that we did not analyze the signal transduction pathway

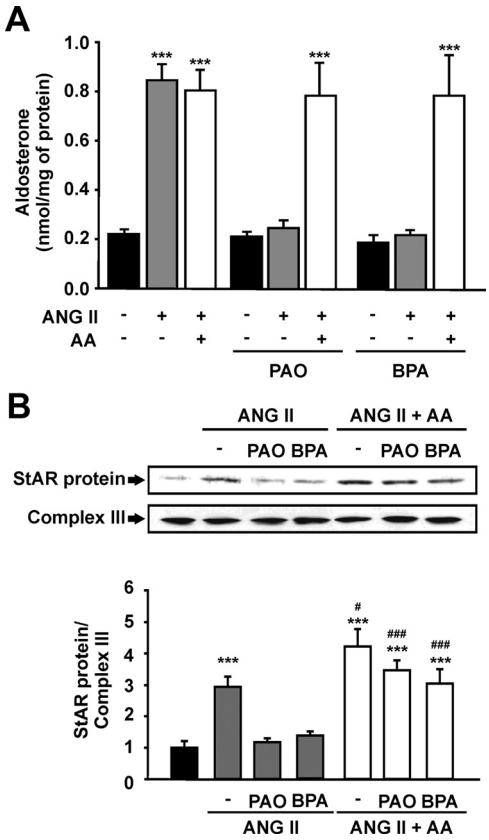

FIG. 6. Effect of AA on ANG II-stimulated steroid production and StAR protein levels inhibited by PTP inhibitors. Cells were stimulated with ANG II (100 nM) for 6 h in the presence or absence of the PTP inhibitors (PAO, 2 μM; BPA, 0.4 μM) and AA (100 μM) in the culture medium containing 0.1% fatty acid-free BSA. Aldosterone production was determined by RIA (panel A). Values represent the means ± SD of three independent experiments. ***, $P < 0.001$ *vs.* untreated cells. Mitochondrial proteins were obtained and subjected to SDS-PAGE and Western blot analysis using sequentially anti-StAR and anti-complex III antibodies. Specific bands were detected by chemiluminescence. A representative Western blot and the quantitative representation of three independent experiments are shown (panel B). The integrated OD of StAR protein levels was quantified for each band and normalized with the corresponding complex III signal. Results are expressed as means ± SD of three independent experiments. ***, $P < 0.001$ *vs.* untreated cells; #, $P < 0.05$, ###, $P < 0.001$ *vs.* ANG II without AA.

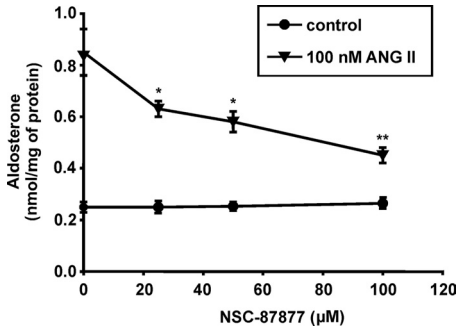

FIG. 7. Effect of NCS-87877 on ANG II-stimulated steroid production. Cells were incubated in the presence or absence of ANG II (100 nM) for 6 h with the indicated concentrations of NCS-87877. Aldosterone production was determined by RIA. Values represent the means ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$ *vs.* respective control.

involved in the effect observed in NCI-H295R cells, further studies are required to define the actual signaling pathway involved.

We have postulated that the second burst of *ACSL4* mRNA and the increase in protein levels at 6 h may correspond to cell growth regulation (14). We have shown that ACSL4 and the intramitocondrial AA contribute, not only to the regulation of steroid synthesis but also to the regulation of cell proliferation. Thus, we postulate that the second burst of *ACSL4* mRNA may correspond to the regulation of cell growth by ANG II. Several lines of evidence indicate that ANG II can regulate cell growth in glomerulosa cells (40, 41).

ANG II stimulation of aldosterone synthesis in adrenal *ZG* cells involves the release of AA (42, 43). In this study, when we studied levels of free AA in mitochondria, no significant increases were observed in response to hormone treatment. However, when AA export was inhibited, free fatty acid in mitochondria increased. This result may suggest that AA is generated in mitochondria by ANG II stimulation but is quickly exported. In heart and Leydig cells, antimycin A and oligomycin inhibit the export of fatty acid from mitochondria (19, 20, 44). In this paper we show that these agents also inhibited ANG II-induced export of mitochondrial AA.

Another agent used was the ionophore, CCCP. In the concentrations used in this work, this agent produces dissipation of the electrochemical gradient without altering ATP synthesis (19). Although steroidogenesis is inhibited in the presence of CCCP, AA does not accumulate in mitochondria after stimulation with ANG II, which suggests that this agent does not affect the export system of mitochondrial AA as occurred in Leydig cells and heart (19, 20, 44). Other authors have shown that mitochondrial $\Delta\psi_m$ must not be altered to allow StAR to enter mitochondria for its subsequent processing (20). This would explain why a low content of this mitochondrial protein and therefore a reduction in steroid synthesis were observed in cells exposed to CCCP.

In cAMP/PKA-dependent systems, StAR protein expression is induced by export of AA from mitochondria. Here we show that in NCI-H295R cells as well the intramitochondrial AA produced has to be exported to induce expression of the StAR protein. Consequently, we show that the generation and export of AA in mitochondria is a universal mechanism necessary for StAR protein induction, an intermediary in the transport of cholesterol from the outer to the inner mitochondrial membrane.

Concerning the role of AA, rat *ZG* cells metabolized arachidonate to hydroxyeicosatetraenoic acids (HETE) by lipoxygenases and epoxyeicosatrienoic acids (EET) by cytochrome P-450 epoxygenase, although no leukotrienes were detected (45). 12-HETE itself induces Ca^{2+} release from intracellular stores as well as Ca^{2+} influx from extracellular fluid, whereas lipoxygenase inhibitors reduce the effect of ANG II on $[Ca^{2+}]_c$ and aldosterone production (42). 12-HETE leads to aldosterone production in NCI-H295R cells, partly through activation of cAMP response element binding protein/activating transcription factor 1, which may induce StAR transcription (46). Moreover, EET produced by cytochrome P450 enzymes are also able to induce StAR protein expression in Leydig cells (15). Recently Kopf *et al.* (47) demonstrated that ANG II stimulates ZG cells to release EET and dihydroxyeicosatrienoic acids, resulting in potassium channel activation and relaxation of adrenal arteries. We suggest that the mitochondrial AA import/export system could be involved in the production of these eicosanoid compounds implicated in the ANG II action mechanism. In human arterial smooth muscle cells, ACSL4 is known to modulate prostaglandin E2 release (48). Recently, we demonstrated that ACSL4 and intramitochondrial AA are involved in the aggressive phenotype of human breast cancer cells through the production of lipoxygenase compounds and prostaglandin production (49).

PTP inhibition is involved in regulation of ACSL4 expression in the different steroidogenic systems. This was demonstrated by the use of inhibitors of PTP activity with different action mechanisms: PAO and BPA. PAO is an oxidizing agent that modifies the thiol group of a cysteine present in all active PTP sites and blocks its activity. BPA is a structural nonhydrolysable analog of the substrate of PTP. The use of two different inhibitors is a useful tool to specifically attribute involvement of PTP on steroidogenesis, ACSL4 and StAR protein expression. Therefore, this PTP activity could be the point of convergence of different signal transduction pathways.

Our present results showed that the site of action of PTP in the stimulation of the aldosterone synthesis by ACTH, potassium, or ANG II stimuli precedes activation of cholesterol transport into the mitochondria. We also determined that PTP action is located between the activation of signal transduction systems and the release of AA. This conclusion is supported by the fact that inhibitors of PTP activity do not disrupt the steroid synthesis sustained by a permeable analog of cholesterol (22(R)HO-cholesterol) and that exogenous AA reversed the inhibition produced by PTP inhibitors on aldosterone synthesis. This is consistent with the action site of PTP in the stimulation of glucocorticoids and androgens by ACTH and LH, respectively (8, 11–13). These results demonstrate, for the first time, the involvement of PTP activity in the regulation of steroidogenesis stimulated by ANG II and potassium.

It has been demonstrated that ACSL4 is the limiting enzyme in the mechanism of mitochondrial AA generation/export system (17), and this fatty acid is necessary for StAR protein induction (16, 50) and, consequently, also for stimulation of steroidogenesis. We demonstrated that PTP activity is involved in the expression of ACSL4 but not of ACOT2.

Overall, these results suggest that, when stimulation is performed in the presence of PTP inhibitors, reduced levels of ACSL4 could be the cause of a decrease of both the induction of StAR protein and steroid synthesis stimulated by ANG II. This conclusion is supported by the fact that, with the addition of exogenous AA in the presence of these inhibitors, StAR protein levels and synthesis of aldosterone are recovered. We recently identified SHP2 as one of the PTP involved in ACSL4 and StAR protein induction in Leydig cells (37). We have previously demonstrated the participation of SHP2 in the regulation of steroid synthesis in Leydig cells (37). Here, we demonstrate the participation of SHP2 in ANG II-mediated regulation of steroidogenesis in glomerulosa cells.

In conclusion, our study has shown the existence of common intermediaries shared by different signal transduction pathways in the regulatory mechanism of steroidogenesis. Together, these results confirm that the generation and export of AA in mitochondria is a universal event in steroidogenic systems to regulate the induction of StAR protein, essential for intramitochondrial transport of cholesterol and the rate-limiting step in steroidogenesis.

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