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Induction of 5-aminolevulinate synthase by activators of steroid biosynthesis

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Abstract

Different cytochromes P450 are involved in steroid biosynthesis. These cytochromes have heme as the prosthetic group. We previously reported that ACTH, an activator of glucocorticoid biosynthesis in adrenal, requires heme biosynthesis for a maximal response. In the present study, we investigated the effect of ACTH, and the effect of two activators of the adrenal mineralocorticoid synthesis, endothelin-1 and low sodium diet on 5-aminolevulinate-synthase (ALA-s) mRNA. ALA-s is the rate-limiting enzyme in heme biosynthesis. It was found that infusion of rats with ACTH for 1 h caused an increase of adrenal ALA-s mRNA and activity accompanied by an increase in plasma corticosterone. CYP21, a cytochrome involved in the synthesis of both corticosterone and aldosterone, was not modified at the RNA level in adrenal glands by 1 h of ACTH infusion. Consistently, infusion of endothelin-1 for 1 h increased ALA-s mRNA and aldosterone content in adrenal gland without modifying CYP21 mRNA levels. To study if ALA-s is also regulated by the main physiological stimuli that increase adrenal mineralocorticoid secretion, we fed rats with low salt diet for 2 or 15 days. Low salt diet treatment increased adrenal gland ALA-s mRNA levels. On the other hand, the rapid stimulation of ALA-s mRNA by ACTH which acts through cyclic AMP was confirmed in H295R human adrenocortical cells, the only human adrenal cell line that has a steroid secretion pattern and regulation similar to primary cultures of adrenal cells. Our findings suggest that the acute activation of adrenal steroidogenic cytochromes by trophic hormones involves an increase in heme biosynthesis which will favor the production of active cytochromes.

Keywords: ACTH; Endothelin; Low sodium diet; Steroid biosynthesis; Ala-synthase (ALA-s); Cytochrome P450 21-hydroxylase (CYP21); Rat adrenal; H295R human adrenocortical cells

Introduction

Synthesis of glucocorticoids and mineralocorticoids takes place in the cortex of the adrenal gland. In mammals, the main mineralocorticoid is aldosterone and the main glucocorticoid is cortisol, except in rat and mouse, which lack 17-hydroxylase activity, and hence, their main glucocorticoid is corticosterone. Synthesis of these steroids from cholesterol involves several reactions; some of them are oxidation reactions catalyzed by cytochromes P450 (Hall, 1984).

Several cytochromes P450 are involved in the biosynthesis of these steroids: cytochrome P450 side-chain cleavage (SCC), which converts cholesterol to pregnenolone; cytochrome P450 21-hydroxylase (CYP21) that transforms progesterone into 11-desoxicorticosterone (DOC) and cytochrome P450 11β1 (CYP11B1) and β2 (CYP11B2) which catalyze the late pathway from DOC to corticosterone and aldosterone, respectively (Sander et al., 1994; Kawamoto et al., 1992).

Steroid biosynthesis in adrenal is regulated by: ACTH, the physiological activator of glucocorticoid synthesis, endothelin-1, a potent vasoconstrictor that stimulates aldosterone synthesis (Simpson and Waterman, 1983; Cozza et al., 1989) and sodium restriction, the physiological stimuli for aldosterone biosynthesis in adrenal gland (Adler et al., 1993).

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Steroidogenic cytochromes are holoenzymes with heme as the prosthetic group.

The first and rate-limiting enzyme of heme pathway is 5-aminolevulinate-synthase (ALA-s), which is regulated by the end-product, heme (De Matteis, 1988). Ferrochelatase is the last enzyme in heme biosynthesis which converts protoporphyrin IX and Fe to heme. Different inhibitors of ferrochelatase, like 3, 5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) and allylisopropilacetamide (AIA), cause a decrease in heme content and a subsequent induction of ALA-s (De Matteis, 1988; Cole and Marks, 1984; Marks et al., 1988).

We previously reported that heme stimulates steroid biosynthesis. We also reported that DDC, which inhibits ferrochelatase activity in rat adrenal, blocks ACTH-mediated aldosterone and corticosterone biosynthesis and heme partially restores the inhibition produced by DDC (Martini et al., 1997). These findings prove the requirement of heme synthesis for ACTH action.

Heme has also been proved to stimulate another steroidogenic cytochrome P450, the estrogen synthetase (Bellino and Hussa, 1985).

Phenobarbital, an activator of cytochrome content, is known to induce both apocytochrome P450 and ALA-s in liver, the latter induction will ensure the availability of the prosthetic group, heme (Srivastava et al., 1989; Dwarki et al., 1987; Rangarajan and Padmanaban, 1989).

In the present study we want to investigate if different activators of steroid biosynthesis in adrenal, ACTH, endothelin-1 and low sodium diet, stimulate heme synthesis. It is known that activators of ALA-s, the rate-limiting enzyme in heme pathway, cause an increase in ALA-s mRNA (Srivastava et al., 1988). Thus, we analyzed ALA-s mRNA levels in the adrenal gland of rats treated with ACTH, endothelin-1 or low sodium diet.

Materials and methods

Materials

Adrenocorticotropin (ACTH) 1–24 was a gift from Ciba Geigy (Switzerland). DDC (3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine) was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). AIA was a gift from Hoffmann, La Roche and Co. (Switzerland). Endothelin-1 and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals

Male Sprague Dawley rats (3 months old) were maintained on standard rat chow (Teklad, Harlan, Indianapolis, IN) and tap water in an environment with 12:12-h light–dark cycles. Animal protocols were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Infusion of rats with ACTH and endothelin-1

Rats were anesthetized with pentobarbital (60 mg/kg). A cannula was placed in the trachea and, the jugular vein was isolated and cannulated for hormone infusion. All animals remained anesthetized throughout the infusion. After a stabilizing period of 15 min, saline was infused for 15 min, and then vehicle (saline), ACTH (100 ng/kg × min) or endothelin-1 (80 ng/kg × min) was infused for an additional 60 min. At the end of the infusion period, rats were sacrificed, adrenal glands removed, flash frozen in liquid nitrogen and stored at −80 °C.

Treatment of rats with normal, low or high salt diet

For salt intake manipulation studies, rats ($n=4$ per group) were fed ad libitum a standard normal salt diet (NS; 0.3% NaCl; Harlan Teklad, Madison, WI), low salt diet (LS, 0.03% NaCl), or high salt diet (HS, standard chow plus 0.9% saline to drink).

At the end of the experimental protocols, rats were anesthetized with isoflurane, adrenal glands removed, freed of fat, flash frozen in liquid nitrogen and stored at −80 °C.

Cell culture

H295R human adrenocortical cells (Bird et al., 1993) (a generous gift from Dr. W.E. Rainey, University of Texas Southwestern, Dallas, TX) were cultured in H295R complete media containing DMEM:F12 (1:1) supplemented with 2% Ultroser G (Biosepra, Villeneuve-la-Garenne, France), ITS-Plus (Discovery Labware, Bedford, MA) and antibiotic/antimycotic mixture (Invitrogen, Carlsbad, CA) as we previously described (Romero et al., 2004). Cells were cultured in 6-well plates until subconfluent, media removed and replaced with fresh media containing 10 μM forskolin. Cells were incubated for 3 h, media removed and total RNA extracted as described below.

Determination of aldosterone and corticosterone

Radioimmunoassay was used for aldosterone (Gomez-Sanchez et al., 1987) and corticosterone (Gomez-Sanchez et al., 1975) determination. Aldosterone was measured in supernatants obtained by centrifugation at 6000 ×g of adrenal homogenates prepared from part of the adrenal gland obtained before RNA preparation. Corticosterone was measured in blood as previously described (Gomez-Sanchez et al., 1975).

RNA extraction and RNase protection assay (RPA)

Total RNA was extracted from rat adrenal with acid phenol/guanidine thiocyanate reagent (TRIzol reagent, Life Technologies, Inc.). The amount of RNA extracted was estimated by measuring the absorbance at 260 nm. Ribonuclease protection assays were performed using RPA III Kit (Ambion Inc., Austin, TX). ALA-s antisense riboprobe was constructed from a plasmid that contains the rat liver c-DNA generously provided by M. Yamamoto (Yamamoto et al., 1988). This gene encodes a housekeeping isozyme that is also expressed in adrenal (Srivastava et al., 1988; Srivastava et al., 1992). PCR primers were designed to obtain a product of 413 bp from ALA-s c-DNA and a T7 phage promoter was added to the 5′end of the

Table 1
Effect of ACTH on serum corticosterone

Corticosterone (ng/ml serum)	
Control	23±1
ACTH	207±21*

Corticosterone was measured in serum of rats infused with saline (control) or ACTH, as indicated in Materials and methods. Results are means±S E of six determinations. *Significantly different from control, $p<0.05$ (Student's t test).

antisense primer. This PCR product was used as DNA-template to prepare a biotinylated antisense RNA probe with the BIOTINscript non-isotopic in vitro transcription kit (Ambion). The transcript was purified in a denaturing 5% polyacrylamide gel containing 8 M urea.

We found that quantification of ALA-s mRNA by RPA using this probe was able to show the increase in hepatic ALA-s mRNA in rats treated with AIA and DDC, two known inducers of this enzyme (Yamamoto et al., 1988) (data not shown).

CYP21 biotinylated probe was synthesized as previously reported (Zhou et al., 1997).

Ten micrograms of total RNA, quantified by spectrophotometry, were hybridized overnight with the biotinylated probe and afterwards, the unprotected RNA was digested with RNases, and the protected RNA was precipitated and separated on a 5% polyacrylamide gel containing 8 M urea (RPA III kit). The separated RNA was electroblotted onto Zeta-probe GT nylon membranes (BioRad) and detected by BrightStar Biodetected kit (Ambion). Films were scanned and bands quantified using Scion image analysis software version 4.03 (Scion Corp., Frederick, MD).

Determination of ALA-synthase activity

ALA-s was assayed in adrenal homogenates, according to the method of (Marver et al., 1966) modified by the addition of 0.5 mM pyridoxal phosphate to the incubation media. Homogenates obtained from 40 mg of rat adrenal were added per assay.

RNA extraction and RT-PCR

ALA-s mRNA was quantified as previously described (Romero et al., 2004; Yanes et al., 2005). Briefly, total RNA was extracted with Tri-Reagent (MRC, Cincinnati, OH), resuspended in diethyl pyrocarbonate-H_2O, DNase treated with Turbo DNAfree kit (Ambion, Austin, TX), and quantified by spectrophotometry. Five micrograms of RNA were reverse transcribed (RT) with 0.5 μg of $T_{12}VN$ primer and Superscript III (Invitrogen, Carlsbad, CA) in a final volume of 20 μl. The reaction was carried out for 60 min at 50 °C and terminated by incubation at 75 °C for 15 min. Primers were designed with Primer3 software (Rozen and Skaletsky, 2000). ALA-s primers were: forward 5′-CTTCAGGATAACTTGCCCAAAG-3′ and reverse 5′-AAACTCGGTAGGTGTGGTCATTT-3′. GAPDH primers were previously described (Gomez-Sanchez et al., 2004). Real-time polymerase chain reaction (PCR) contained 1 μl of RT product, 0.1 μM of each primer, 0.2 mM dNTPs, SYBR green I (1:20,000 final concentration; Molecular Probes, Eugene, OR), and 1 μl of titanium Taq DNA polymerase (Clontech, Palo Alto, CA). Amplifications were performed in a real-time thermal cycler (iCycler, Bio-Rad Laboratories). Cycling conditions were 1 min at 95 °C, followed by 50 cycles of 15 s at 95 °C, 15 s at 60 °C, and 60 s at 72 °C. Fluorescence data were collected during the elongation step. After PCR amplification, the specificity of the PCR was confirmed by melting temperature determination of the PCR product and electrophoretic analysis in 4% NuSieve 3:1 agarose gels (Cambrex, Rockland, ME). PCR product quantification was performed by the relative quantification method (Pfaffl, 2001), standardized against GAPDH. Efficiency for each primer pair was assessed by using serial dilutions of pooled RT product.

Results

To evaluate the effect of activators of steroid biosynthesis on ALA-s mRNA, we first investigated the effect of ACTH, the main stimulator of glucocorticoid production.

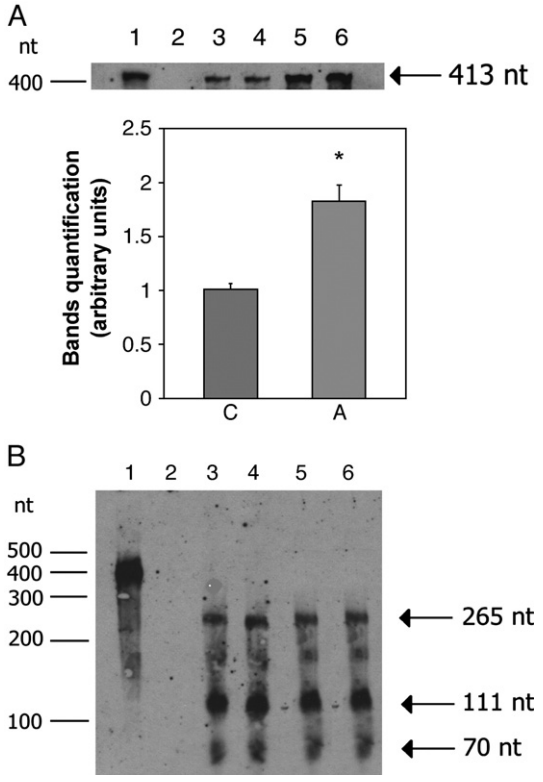

Fig. 1. Effect of ACTH treatment on ALA-s and CYP21 mRNA in rat adrenal. Rats ($n=6$ per group) were infused with vehicle (control) or ACTH for 1 h. Animals were killed, total RNA was extracted from adrenal and ALA-s (A) and CYP21 (B) mRNAs were analyzed by RPA, as indicated in Materials and methods. Lane 1: 10 μg of yeast RNA without Rnases digestion, lane 2: 10 μg of yeast RNA with Rnases digestion, lanes 3–6: each lane resulted from one rat, lanes 3 and 4: 10 μg of total control adrenal RNA, lanes 5 and 6: 10 μg of total treated adrenal RNA. The protected fragments are indicated by arrows. The positions of RNA size markers are indicated at the left. ALA-s mRNA bands from control (C) and ACTH-treated (A) rats were quantified using Scion image analysis software version 4.03 and are presented as bars graph. Results are expressed relative to control which is set to 1 and represent means±SE of six rats per group. *Significantly different from control, $p<0.05$ (Student's t test).

Table 2
Effect of ACTH on ALA-synthase activity in rat adrenal

ALA-synthase activity (nmol ALA/mg of adrenal per h)	
Control	13.3±1.0
ACTH	27.9±3.7*

ALA-synthase activity was measured in adrenal homogenates of rats infused with saline (control) or ACTH as indicated in Materials and methods. Results are means±S E of three determinations. *Significantly different from control, $p<0.05$ (Student's t test).

Infusion of rats with ACTH for 1 h provoked a significant increase in serum corticosterone as shown in Table 1.

We then quantified ALA-s mRNA levels in the adrenal gland by RPA. ACTH infusion increased adrenal ALA-s mRNA as shown in Fig. 1A.

ACTH treatment also increased ALA-s activity in adrenal (Table 2), which indicates that ALA-s mRNA was translated into active protein.

In addition, the effect of ACTH infusion on the amount of apocytochrome CYP21 mRNA was also studied. CYP21 is a cytochrome involved in the synthesis of both glucocorticoids and mineralocorticoids. It has been previously reported that CYP21 levels are not modified 1 h after ACTH injection in rat adrenal glands (Lehoux et al., 1998). To test if 1 h of ACTH infusion provoked a similar response, we quantified CYP21 mRNA levels by RPA in the same samples. The CYP21 probe used for RPA, detected two transcripts in rat adrenal gland, protecting a 265 nt fragment in one of them that contains intron 9 and two fragments of 111 and 70 nt from the other transcript with no introns, as previously reported (Zhou et al., 1997). ACTH infusion did not modify CYP21 mRNA levels (Fig. 1B) despite the increase observed in ALA-s mRNA. Thus, CYP21 could be used as an internal control.

On the other hand, rats were infused with an activator of mineralocorticoid biosynthesis, endothelin-1. Infusion of rats with endothelin-1 for 1 h provoked a significant increase in aldosterone content in adrenal (Table 3) as it was previously reported (Pecci et al., 1994). Endothelin-1 infusion also provoked a significant increase in ALA-s mRNA in adrenal while no significant change in CYP 21 mRNA was found (Fig. 2).

Another known regulator of mineralocorticoid biosynthesis in adrenal is salt intake. Low salt intake upregulates aldosterone biosynthetic protein expression and concomitantly aldosterone secretion by the adrenal gland while high salt diet intake has the opposite effect (Quinn and Williams, 1988; Stewart, 2003). To further analyze the effect of steroid biosynthesis activators on the rate-limiting enzyme of heme biosynthesis, we investigated if salt intake regulates ALA-s expression in adrenal gland. Rats were placed in low or high salt diet for 2 or 15 days and the amount of ALA-s mRNA was quantified by real-time RT-PCR. Low salt diet caused a 4-fold increase in ALA-s mRNA expression after 2 days of treatment (Fig. 3) and ALA-s mRNA levels remained elevated for up to 15 days. In contrast, high salt diet did not modify ALA-s mRNA expression during the course of the treatment.

To test if these results could be extended to a model different from rat adrenal and to further evaluate the effect of ACTH and cyclic AMP signaling on ALA-s mRNA, we used H295R human adrenocortical cells. The H295R human adrenocortical cell line (Bird et al., 1993) is a widely used in vitro model for the study of adrenal cell physiology and metabolism because it is the only human adrenal cell line that has a steroid secretion pattern and regulation similar to primary cultures of adrenal cells (Rainey et al., 2004). H295R cells do not respond to ACTH treatment due to a deficiency in its receptor transduction pathway. ACTH mainly acts through the cyclic AMP-PKA

Table 3
Effect of endothelin-1 on aldosterone accumulation in rat adrenal

Aldosterone (ng/mg of adrenal)	
Control	0.13±0.01
Endothelin-1	3.52±0.48*

Aldosterone was measured in adrenal homogenates of rats infused with saline (control) or endothelin-1, as indicated in Materials and methods. Results are means±S E of six determinations. *Significantly different from control, $p<0.05$ (Student's t test).

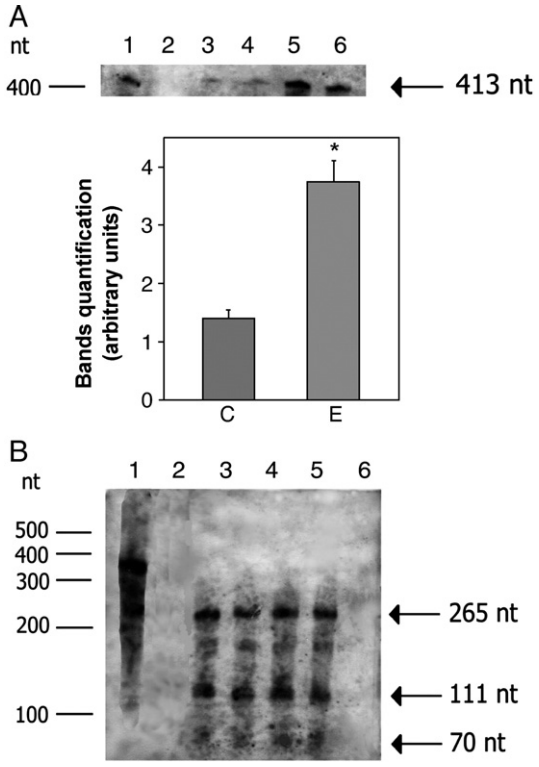

Fig. 2. Effect of endothelin-1 treatment on ALA-s and CYP21 mRNA in rat adrenal. Rats ($n=6$ per group) were treated with vehicle (control) or with endothelin-1 for 1 h. Animals were killed, total RNA was extracted from adrenal and ALA-s (A) and CYP21 (B) mRNAs were analyzed by RPA, as indicated in Materials and methods. Lane 1: 10 μg of yeast RNA without Rnases digestion, lane 2: 10 μg of yeast RNA with Rnases digestion, lanes 3–6: each lane resulted from one rat, lanes 3 and 4: 10 μg of total control adrenal RNA, lanes 5 and 6: 10 μg of total endothelin-1-treated adrenal RNA. The protected fragments are indicated by arrows. The positions of RNA size markers are indicated at the left. ALA-s mRNA bands from control (C) and endothelin-1-treated (E) rats were quantified using Scion image analysis software version 4.03 and are presented as bars graph. Results are expressed relative to control which is set to 1 and represent means±SE of six rats per group. *Significantly different from control, $p<0.05$ (Student's t test).

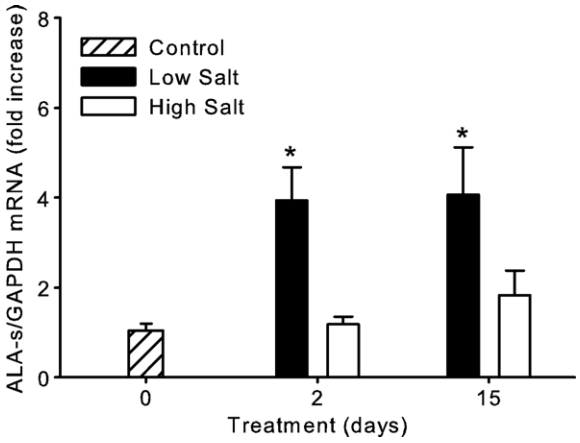

Fig. 3. Effect of low and high sodium diet treatment on ALA-s mRNA in rat adrenal. Rats ($n=4$ per group) were treated with normal (crossed bars), low (black bars) or high salt diet (white bars). The days of salt treatments are indicated in the figure. After treatments, adrenals glands were removed, RNA was obtained and ALA-s mRNA was quantified by real-time polymerase chain reaction as indicated in Materials and methods. Results are expressed relative to control which is set to 1 and represent means±SE of four determinations. ∗Significantly different from control, $p<0.01$, (ANOVA).

intracellular signaling pathway (Sewer and Waterman, 2003; Gallo-Payet and Payet, 2003) and is mimicked in vivo by the use of the adenylyl cyclase activator, forskolin. Forskolin caused 2.6-fold upregulation of ALA-s mRNA expression quantified by real-time RT-PCR in H295R cells after 3 h of treatment (1.00±0.04 vs. 2.67±0.50, $n=3$, $p<0.05$).

Discussion

In the present manuscript we showed that one hour infusion of rats with ACTH or endothelin-1, two activators of adrenal steroid biosynthesis, provoked a rapid increase of ALA-s mRNA. ALA-s is the first and rate-limiting enzyme in heme biosynthesis and all the activators described so far, induce the enzyme (De Matteis, 1988; Marks et al., 1988; Srivastava et al., 1988). In keeping with our findings, it was reported that a chronic treatment of rats with ACTH once daily for three days increases ALA-s activity in adrenal (Condie et al., 1976) and it was also reported that dexamethasone, an inhibitor of ACTH release, decreases ALA-s mRNA in rat adrenal (Srivastava et al., 1992).

In addition, in this paper, the increase of ALA-s mRNA by ACTH, which is known to act through cyclic AMP and PKA (Sewer and Waterman, 2003; Gallo-Payet and Payet, 2003), was evaluated in H295R human adrenocortical cells. Due to a deficiency in ACTH receptor transduction pathway in these cells, the hormone action is mimicked in vivo by the use of the adenylyl cyclase activator, forskolin. Forskolin caused a rapid upregulation of ALA-s mRNA after 3 h of treatment. These results further prove the involvement of ACTH and cyclic AMP in ALA-s induction and are in agreement with a previous report that shows that cyclic AMP stimulates ALA-s transcription of the housekeeping gene (Varone et al., 1999; Giono et al., 2001).

Herein, we found that induction of ALA-s in rat adrenal occurred rapidly, after treatment of rats with either ACTH or endothelin-1 for only 1 h, when the synthesis of steroids was stimulated but no increase in CYP21 mRNA levels was detected.

Accordingly, ACTH is known to induce CYP21 after chronic treatments (John et al., 1986) but it was also reported that when rats were given a single ACTH injection, their plasma corticosterone and aldosterone were maximally elevated 0.5 and 1 h after the injection, with no increase in cytochromes P450 SCC, CYP21 or CYP11B1 protein content in rat adrenal (Lehoux et al., 1998).

In our experiments, rats were infused with ACTH for only 1 h. This treatment with ACTH did not increase CYP21 mRNA and was probably not long enough for any apocytochrome induction. If this is the case, the rapid increase in heme synthesis would produce holoenzyme from pre-existing apocytochromes as it was previously reported (Cozza et al., 1993).

Consistently, we previously found that the synthesis from DOC of aldosterone, in calf and rat adrenal homogenates and the synthesis from DOC of corticosterone in rat adrenal homogenate, is increased about two fold by the in vitro addition of hemin (Martini et al., 1997). This would indicate that half of the cytochromes involved in these reactions, are in the apocytochrome form in basal condition. Similar concentration of free apocytochrome was reported for estrogen synthetase in human choriocarcinoma cells (Bellino and Hussa, 1985).

In the present manuscript, we also evaluated the effect of a chronic treatment on ALA-s mRNA, we observed that treatment of rats with a low sodium diet for 2 days, which is known to stimulate aldosterone biosynthesis, increased ALA-s mRNA in adrenal gland and this increase remained after 15 days of sodium restriction.

In keeping with the fact that activators of steroidogenic cytochromes in adrenals stimulate heme biosynthesis, it was previously reported that an activator of steroid biosynthesis in testis, HCG, increased ALA-s mRNA in that tissue (Srivastava et al., 1988).

Other cytochromes stimulators, such as: FB, a known activator of different cytochromes P450 in liver (Srivastava et al., 1989) or uncoupling protein (UCP-1) that stimulates mitochondrial proliferation and hence, respiratory cytochromes (Li et al., 1999), were also reported to induce ALA-s, therefore increasing heme availability.

Accordingly, activators of adrenal steroidogenic cytochromes induce ALA-s and this will contribute to generate active cytochromes.

In addition, it has been reported that 5 beta-reduced steroids increase Ala-s activity and cytochromes P450 in chick embryo liver and adrenal (Incefy and Kappas, 1974; Aragones et al., 1991). This will further prove the interaction between heme and steroid metabolism. Thus, 5 beta steroids may contribute to the activation of ALA-s, although the importance of this mechanism in mammals and the relationship with the effect of the activators evaluated in this paper, remain to be investigated.

On the other hand, the acute stimulation of steroidogenesis by trophic hormones requires the synthesis of steroidogenic acute regulatory protein (StAR), which delivers cholesterol to the inner mitochondrial membrane, where SCC converts cholesterol to pregnenolone (Strauss et al., 1999). Induction

of StAR protein to ensure cholesterol availability at mitochondria and induction of ALA-s to ensure heme availability, might be important events mediating the acute effects of stimulators of steroid biosynthesis.

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