Molecular and Cellular Endocrinology 332 (2011) 253–260

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Localization and functional activity of cytochrome P450 side chain cleavage enzyme (CYP11A1) in the adult rat kidney

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ARTICLE INFO

Article history:
Received 8 September 2010
Received in revised form 20 October 2010
Accepted 27 October 2010

Keywords:
CYP11A1
Kidney
Steroidogenesis
Mitochondria
Pregnenolone

ABSTRACT

Cumulative evidence demonstrated effective downstream metabolism of pregnenolone in renal tissue. The aim of this study was to evaluate the expression and functional activity of cytochrome P450 side chain cleavage enzyme (CYP11A1), which converts cholesterol into pregnenolone, in adult rat kidney. Immunohistochemical labeling for CYP11A1 was observed in renal cortex and medulla, on structures identified as distal convoluted tubule and thick ascending limb of Henle's loop, respectively. Immunoblotting analysis corroborated the renal expression of the protein in inner mitochondrial membrane fractions. The incubation of isolated mitochondria with the membrane-permeant cholesterol analogue 22R-hydroxycholesterol resulted in efficient formation of pregnenolone, the immediate precursor for the synthesis of all the steroid hormones. The low progesterone production rate observed in these experiments suggested a poor activity of 3β-hydroxysteroid dehydrogenase enzyme in renal mitochondria. The steroidogenic acute regulatory protein (StAR), involved in the mitochondrial import of cholesterol, was detected in renal tissue at both mRNA and protein level. Immunostaining for StAR showed similar distribution to that observed for CYP11A1. The expression of StAR and CYP11A1 was found to be higher in medulla than in cortex. This enhanced expression of steroidogenesis-related proteins correlated with a greater pregnenolone synthesis rate and higher steroid hormones tissular content measured in medulla. In conclusion, we have established the expression and localization of StAR and CYP11A1 protein, the ability of synthesizing pregnenolone and a region-specific content of sex hormones in the adult rat kidney. These data clearly show that the kidney is a steroid hormones synthesizing organ. It is proposed that the existence in the kidney of complete steroidogenic machinery would respond to a physiological significance.

Abbreviations: CYP11A1, cytochrome P450 side chain cleavage enzyme; CYP17, cytochrome P450 17α-hydroxylase/17,20 lyase/17,20 desmolase; GAPDH, glyceraldehyde phosphate dehydrogenase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 11β-HSD, 11β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; 20α-HSD, 20α-hydroxysteroid dehydrogenase; 22R-OHC, 22R-hydroxycholesterol; P4, progesterone; P5, pregnenolone; StAR, steroidogenic acute regulatory protein; T, testosterone; TSPO, translocator protein of 18 kDa; RIA, radioimmunoassay.

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doi:10.1016/j.mce.2010.10.020

1. Introduction

Cumulative evidence attributes steroidogenic capabilities to the rat kidney. The expression and/or activity of different steroidogenic enzymes, such as 3β-hydroxysteroid dehydrogenase (3β-HSD) (Zhao et al., 1991), 11β-HSD (Zhao et al., 2010), 17β-HSD, 20α-HSD, cytochrome P450 17α-hydroxylase/17,20 lyase/17,20 desmolase (CYP17) (Dalla Valle et al., 2004a), 21-hydroxylase (Lajic et al., 1995) and 5α-reductase (Dalla Valle et al., 2004a) were detected in rat renal tissue. Different authors pointed out that locally formed steroids, previously termed nephrosteroids (Dalla Valle et al., 2004a), may act in a paracrine or autocrine fashion (Zhao et al., 1991; Dalla Valle et al., 2004a). Despite the steroid-metabolizing ability of rat kidney, the evidence for renal production of pregnenolone, the immediate precursor for the synthesis of all the steroid hormones, has not been clearly established yet.

The rate-limiting step in steroidogenesis is cholesterol delivery to the inner mitochondrial membrane where it is converted into pregnenolone by action of cytochrome P450 side chain cleavage enzyme (CYP11A1) (Stocco, 1999a, b). Cholesterol transport is mediated by a functional interaction between the steroidogenic acute regulatory protein (StAR) and the translocator protein of 18 kDa (TSPO) (Miller, 2007b,a). TSPO is the new term for peripheral benzodiazepine receptor (Papadopoulos, 2004). It is proposed that the "cholesterol recognition aminoacid concensus" domain of TSPO contains the reservoir of labile cholesterol available for steroidogenesis, and that StAR acts to mobilize this pool from the outer to the inner mitochondrial membrane (Miller, 2007a). Expression of StAR mRNA (Dalla Valle et al., 2004b) and TSPO protein (Beaumont et al., 1984) in rat renal tissue was described. Dalla Valle et al. (2004b) detected CYP11A1 protein in fetal and newborn rat kidney.

The significance of responses induced by steroid hormones in modulating normal renal physiology and modifying the progression to end stage renal disease has lately become evident (Thomas et al., 2007; Yanes et al., 2008; Maric, 2009). Pregnenolone has been shown to have cytoprotective effects against injury in isolated renal tubules (Waters et al., 1997). If pregnenolone is synthesized in renal tissue, it could act locally by itself or by means of its derived steroids, playing a potential physiological role.

The aim of the present study was to evaluate the ability of adult rat kidney to synthesize pregnenolone. The expression of proteins involved in cholesterol mitochondrial import was also assessed.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (3 months, 300–350 g body weight) were used in all experiments. The animals were housed in rooms with controlled temperature (21–23 °C) and regular light cycles (12 h). They were allowed free access to a standard diet and tap water. All experimental procedures were conducted in accordance with institutional guidelines and the recommendations for laboratory animals care and use as promulgated by the National Institute of Health (National Centre for Research Resources), Bethesda, MD, USA.

Animals were anesthetized with sodium thiopental (70 mg kg^{-1} b.w., I.P.). Blood was collected from inferior cava vein and serum was stored at −20 °C for steroid determinations. Serum progesterone (P4) and testosterone (T) were determined by radioimmunoassay (RIA) after two extractions with 5 ml ethyl ether (Merck) (Charreau et al., 1981). The kidneys were removed and washed in cold saline solution for further experiments. Cortical and medullary tissue was separated as required.

2.2. Tissue steroid extraction

The content of progesterone (P4) and testosterone (T) in renal cortex (CX) and medulla (M) was assessed by RIA. Fragments of CX and M (approximately 500 mg) were homogenized with 2 ml of acetone and then centrifuged. The supernatant was added with 2000 cpm (counts per minute) 1,2,6,7-^{3}HProgesterone (^{3}HP4) 80.2 Ci $mmol^{-1}$ (Amersham International, Buckinghamshire, GB) to evaluate the recovery of steroids after the extraction, and then was dried with N_2. The steroids were extracted using ethyl ether and splitting the phases by freezing at −70 °C, and then dried with N_2. The pellet was resuspended in 1.4 ml of methanol and then 0.6 ml of distilled water and 2 ml hexane were added. After a centrifugation, the upper hexane phase, containing mainly lipids, was removed and the aqueous phase was mixed with 6 ml of dichloromethane. The organic phase containing the steroids was dried with N_2. The remaining pellet was resuspended in 350 μl of RIA buffer (Na_2HPO_4 40 mM, NaH_2PO_4 30 mM, NaCl 150 mM, sodium azide 0.01%, gelatine 0.1%, pH 7.0). A volume of 50 μl of each sample was transferred to a tube containing scintillation liquid Hisafe 3 (Wallac, England) for counting in a Perkin Elmer 2800 TR Liquid Scintillation Counter. The extraction procedure efficiency was calculated as the rate between the cpm measured in the samples at the end of the procedure and the cpm of the ^{3}HP4 added at the beginning of the extraction. The RIA measurements of P4 and T in renal tissue were corrected for the extraction efficiency (40–50%) and tissue weight.

2.3. Reverse transcription polymerase chain reaction

TSPO and StAR mRNA levels were measured by reverse transcription polymerase chain reaction (RT-PCR). Total RNA from CX and M tissue was extracted using the Trizol Reagent method (Invitrogen, Life Technologies) according to manufacturer's instructions. The synthesis of cDNA by oligo dT-primed reverse transcription was performed using the Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT) (Invitrogen, Life Technologies).

PCR was performed in a master mix: 3 μl of cDNA solution, 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.25 mM dNTPs and 1 U Platinum DNA polymerase (Invitrogen, Life Technologies). Each product was co-amplified in the same PCR tube with glyceraldehyde phosphate dehydrogenase (GAPDH). To validate the use of this RT-PCR assay as a tool for the semiquantitative measurement of mRNAs, cycle number curves were established, and the samples were quantitated in the curvilinear phase of the PCR amplification. All the amplification conditions were assayed in order to use, in each sample, the minimal number of amplification cycles that allowed the analyses of products in agarose gels.

The amplification profile consisted of initial denaturization at 94 °C for 5 min, followed by cycles of 94 °C for 1 min, Tm for 1 min, 72 °C for 1 min and a final extension at 72 °C for 5 min. The primers and Tm used were: TSPO: Tm 58 °C, product length: 234 bp, sense 5′-CCATGCTCAACTACTATGTATGGC-3′ and antisense 5′-GTACAACTGTCCCCGCATG-3′; StAR: Tm 57 °C, product length: 746 bp, sense 5′-CCAGGAGCTGTCCTACATCCAG-3′ and antisense 5′-TACTACCCCTCTCGTTGTCCT-3′; GAPDH: product length: 452 bp, sense 5′-ACCACAGTCCATGCCATCAC-3′ and antisense 5′-TCCACCACCCTGTTGCTGTA-3′. Primer concentration for amplification and number of cycles of the different mRNAs were: TSPO 0.2 μM and GAPDH 0.01 μM, 30 cycles; StAR 0.4 μM and GAPDH 0.03 μM, 33 cycles. Amplified PCR products were electrophoresed in 1.2% agarose gels, stained with ethidium bromide, and analysed using a Kodak Electrophoresis Documentation Analysis System. The intensity of bands was measured by densitometry for semiquantitation. The intensity of TSPO and StAR bands was normalized to the corresponding band intensity of GAPDH. Comparisons were performed between samples run on the same gel.

2.4. Isolation of mitochondria

Fragments of CX and M tissue were homogenized (1 g of tissue per 5 ml of buffer) in isolation buffer (IB) containing sucrose 0.27 M, EDTA 1 mM, Tris–HCl 5 mM and a mixture of protease inhibitors (phenylmethysulphonyl fluoride 1 mM, leupeptin 1 mM), pH 7.40. The homogenates were centrifuged at 500 × *g* for 10 min at 4 °C and the pellet, consisting of nuclei and unbroken cells, was discarded. The resulting supernatant (total homogenate, H) was centrifuged at 5500 × *g* for 15 min at 4 °C. The pellet, consisting of mitochondrial fraction (MIT), was resuspended in 800 μl of IB for isolation of mitochondrial membranes or in reaction buffer for pregnenolone synthesis as described in Section 2.8. Protein content of MIT fraction was assessed by Bradford's method (Bradford, 1976).

2.5. Isolation of inner mitochondrial membranes

Inner mitochondrial membranes (IMM) were isolated with digitonin as described previously by Calamita et al. (2005). Protein content of IMM fraction was assessed by Bradford's method (Bradford, 1976). The enrichment in IMM was confirmed by immunoblotting using an antibody against prohibitin, an IMM marker (Calamita et al., 2005).

2.6. Immunoblotting analysis

Samples from CX and M tissue of MIT (60 μg of protein) for StAR detection or IMM (10 μg of protein) for CYP11A1 detection were run on sodium dodecyl sulphate 12% polyacrylamide gels (SDS-PAGE) (Bio-Rad Mini Protean 3, Hercules, CA, USA) (Laemmli, 1970). The amount of protein was chosen after the linearity of detection had been verified. The separated proteins were transferred onto nitrocellulose membranes (Bio-Rad) in Tris–glycine transfer buffer with methanol 20% in a mini-blotter (Sigma–Aldrich). Uniform protein loading and transference to the membrane was verified by staining with Ponceau 2R before blocking procedures with 5% non-fat dry milk in Tris-buffered saline with 0.05% Tween 20. Membranes were then incubated overnight with a rabbit polyclonal antibody against StAR (1:5000 dilution) (Bose et al., 1999), or with a rabbit polyclonal antibody against CYP11A1 (1:2000 dilution) (Hales et al., 2000; Allen et al., 2006) and developed using anti-rabbit horse radish

peroxidase labeled secondary antibody (1:4000 dilution, Amersham Bioscience) and a luminal-chemiluminescent substrate (ECLPlus, Amersham Bioscience), according to the manufacturer's protocols. Samples of adrenal gland mitochondria (MIT AG, 10 μg of protein) obtained as described in Section 2.4 were used as positive control in StAR and CYP11A1 Western blots. HepG2 cell lysate (HepG2, 20 μg of protein) was used as negative control in StAR Western blots (Christenson et al., 1999). To verify the protein loading, membranes were incubated with a rabbit anti-prohibitin antibody (1:5000 dilution, Abcam, Cambridge, UK) and revealed as described above. Densitometry analysis of the Western blot bands intensity was performed. StAR and CYP11A1 antibodies were a gift from Dr. Walter L. Miller (Department of Pediatrics, University of California, San Francisco) and Dr. Dale B. Hales (Department of Physiology, Southern Illinois University Carbondale, School of Medicine, Carbondale, IL), respectively.

2.7. StAR and CYP11A1 immunohistochemistry

Kidney slices were fixed in 10% (v/v) phosphate buffer (PB)-formalin solution pH 7.40 and embedded in low melting point paraffin. Briefly, 5 μm paraffin sections were placed in 3% hydrogen peroxide in PB for 15 min to inhibit the endogenous peroxidase activity and blocked for 1 h at room temperature in 1% bovine serum albumin in PB containing 0.1% Triton X-100. The sections were incubated with polyclonal antibody against StAR (1:70 dilution) or CYP11A1 (1:150 dilution) at 4 °C for 24 h and subsequently incubated with biotinylated goat anti-rabbit immunoglobulin and streptavidin-HRP (UtraMarque™ HRP Detection System). Immunoreactivity was detected with 2.7 mM 3,3′-diaminobenzidine tetrahydrochloride in PB with 0.03% hydrogen peroxide (w/v). The reaction was stopped by adding distilled water. Sections were lightly counterstained with hematoxylin. As a negative control, the primary antibody was replaced with normal rabbit serum in PB. Slices of rat adrenal gland were processed in the same way and used as positive controls.

2.8. Pregnenolone and progesterone synthesis

Pregnenolone (P5) and P4 synthesis protocol was modified from Espinosa-Garcia et al. (2000). Transformation of cholesterol into P5 and P4 was measured in reaction buffer containing 250 mM sucrose, 5 mM $MgSO_4$, 20 mM KH_2PO_4, 25 mM Tris–HCl, 0.2 mM EDTA, 1 mg ml^{-1} bovine albumin, 20 mM glucose-6-phosphate, 2 U glucose-6-phosphate dehydrogenase and in the absence or in the presence of 50 μM 22R-hydroxycholesterol (22R-OHC), a membrane-permeant cholesterol analogue. Mitochondria suspensions from renal cortex and medulla were incubated at 37 °C for 5 min in a shaking bath. The reaction was started by the addition of 5 mM sodium isocitrate, 0.5 mM $NADP^+$ and 0.5 mM NADPH (final volume 50 μl for medullary mitochondria and 100 μl for cortex mitochondria). After 45 min, the reaction was stopped with methanol. After methanol was evaporated, the samples were used to determine the P5 and P4 concentration by RIA.

2.9. RIA measurements

The evaporated residues obtained as described in the previous sections were resuspended in RIA buffer (see above) and appropriate aliquots were assayed for P5, P4 and T by RIA, as previously described (Del Punta et al., 1996; Mondillo et al., 2009).

RIAs were performed using ^{3}HPregnenolone (50.5 Ci mmol^{-1}), ^{3}HProgesterone (80.2 Ci mmol^{-1}) and ^{3}HTestosterone (80 Ci mmol^{-1}) (Amersham International, Buckinghamshire, GB). A volume of 100 μl of each sample was incubated for 16 h at 4 °C with 100 μl of ^{3}HP4, ^{3}HT or ^{3}HP5 and 100 μl of the dilute corresponding antibody. After the incubation, the free hormone was separated from the complex hormone–antibody using a carbon suspension Norit A 0.5% (w/v)-dextran 70 0.05% (w/v) in RIA buffer and then centrifuged at 1400 × *g* for 10 min. The supernatants were transferred to counting vials containing 2.5 ml of scintillation liquid Hisafe 3. The radioactivity was measured with Perkin Elmer 2800 TR Liquid Scintillation Counter. The utility range of the assay was 25–1600 pg P4 or P5/tube and 12.5–800 pg T/tube (final volume 500 μl). The intraassay and interassay variations were 7.5 and 15.1% for P5, 8.0% and 14.2% for P4 and 7.3% and 13.2% for T, respectively.

2.10. Statistics

All results were expressed as mean ± SEM of 5–6 observations. All the experiments were performed by duplicate with equal results. Statistics was performed using the one way analysis of variance followed by *t*-Student or Newman Keuls test as adequate. The 0.05 level of probability was used as the criterion of significance in all cases.

3. Results

3.1. Serum concentration and renal content of progesterone and testosterone

Serum concentration of P4 was 4.02 ± 1.32 ng ml^{-1} and of T was 1.15 ± 0.35 ng ml^{-1}. Tissue content of P4 and T were measured after extraction as indicated in Section 2. P4 content was significantly higher in M as compared to CX (CX: 13.7 ± 2.6, M: 28.7 ± 4.8*, pg mg^{-1}, *$p < 0.05$). Renal T content showed a tendency to be augmented in M but no statistical significance was reached (CX: 2.9 ± 1.9, M: 4.3 ± 1.7, ng mg^{-1}).

3.2. TSPO and StAR mRNA expression

Two fragments of the expected size for TSPO and StAR were consistently detected in agarose gels after RT-PCR using GAPDH as a housekeeping gene. TSPO mRNA expression was detected in cortex and medullary tissue as seen in Fig. 1A. No differences in TSPO mRNA abundance were observed between CX and M tissue. The expression of StAR mRNA was also detected in CX and M tissue. Levels of StAR mRNA were 3-fold greater in M tissue than in CX tissue (Fig. 1B).

3.3. StAR and CYP11A1 protein expression

StAR protein was detected in isolated mitochondria from CX and M tissue by Western blot. The abundance of the protein was significantly higher in M compared with CX samples (Fig. 2). A sample of adrenal gland mitochondria (MIT AG) was used as positive control while a HepG2 cell lysate was used as negative control.

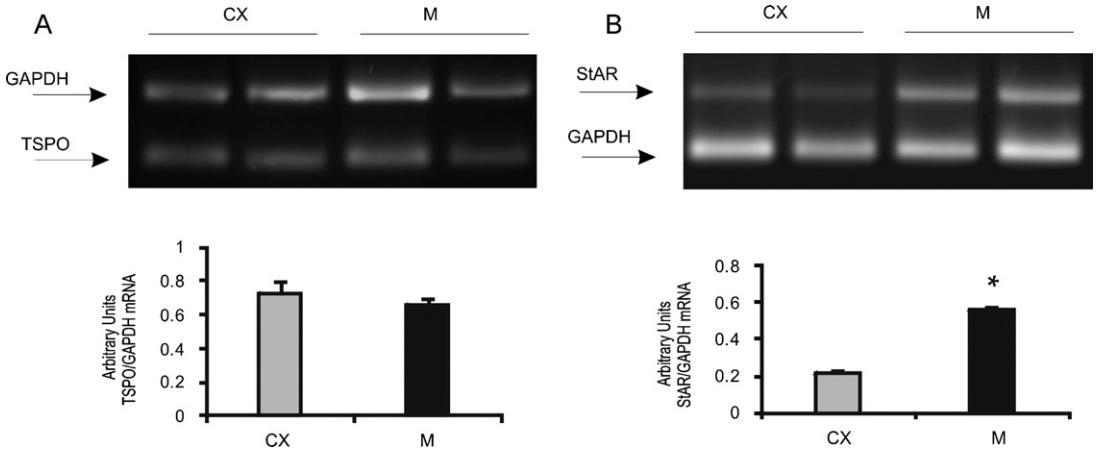

Fig. 1. mRNA expression of (A) TSPO and (B) StAR in renal cortex (CX) and medulla (M) from adult rats. mRNA levels were determined by semi-quantitative RT-PCR. Results are shown as the ratio of TSPO or StAR mRNA to GAPDH mRNA and expressed in arbitrary units. Experiments were done in duplicate, using independently extracted RNA samples from different animals with equivalent results. Bars represent means ± SEM of 6 observations, *$p < 0.05$ vs. CX.

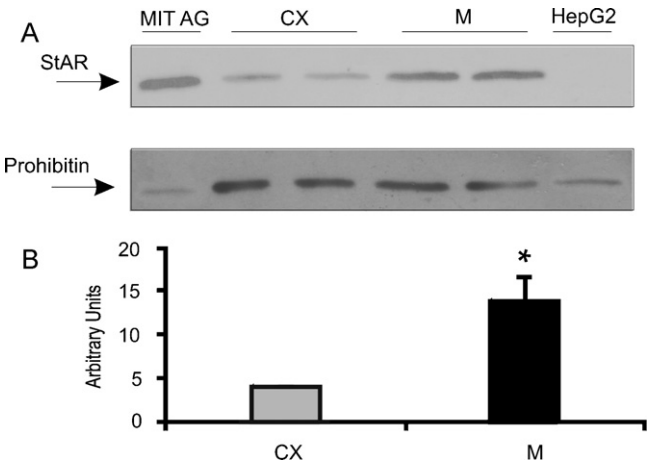

Fig. 2. Abundance of StAR in mitochondrial fraction from renal cortex (CX) and medulla (M) from adult rats. Representative Western blot showing immunodetectable StAR protein. Each lane was loaded with 60 μg of mitochondrial protein. Adrenal gland mitochondria (MIT AG, 10 μg of protein) were used as positive control while HepG2 cell lysate (HepG2, 20 μg of protein) was used as negative control. StAR band density was normalized for the corresponding prohibitin band density and expressed in arbitrary units. Bars represent means ± SEM of 5 observations, $^{*}p < 0.05$ vs. CX.

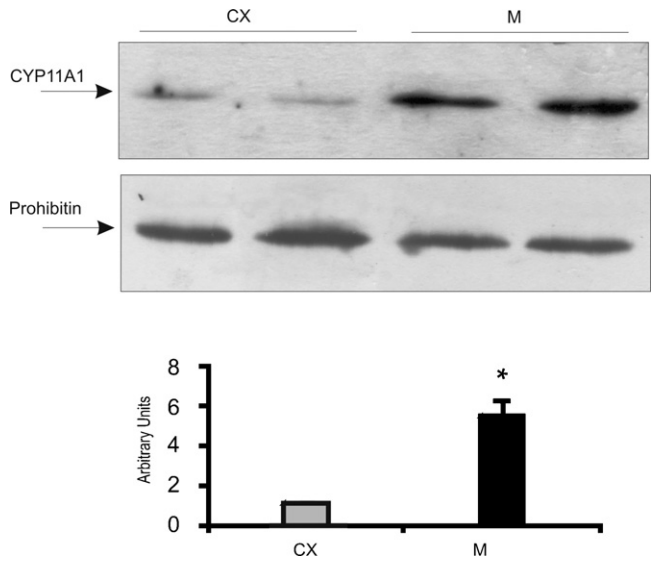

Fig. 4. Abundance of CYP11A1 protein in inner mitochondrial membrane (IMM) fraction from renal cortex (CX) and medulla (M) of adult rats. Representative Western blot showing immunodetectable CYP11A1 protein. Each lane was loaded with 10 μg of IMM protein. CYP11A1 band density was normalized for the corresponding prohibitin band density and expressed in arbitrary units. Bars represent means ± SEM of 5 observations, $^{*}p < 0.05$ vs. CX.

In order to study CYP11A1 protein expression, IMM fractions were obtained. The enrichment of these membrane fractions was verified by the higher expression found for the specific marker prohibitin (Fig. 3A). As expected, CYP11A1 abundance was also higher in IMM fraction than in MIT fraction (Fig. 3B). A sample of adrenal gland mitochondria (MIT AG) was used as positive control (Fig. 3B). CYP11A1 protein abundance was significantly greater in M than in CX IMM samples (Fig. 4).

3.4. Immunohistochemical localization of StAR and CYP11A1 protein

In order to evaluate the localization of StAR and CYP11A1 protein in different kidney structures, immunohistochemical studies were performed. Inmunohistochemical labeling detected StAR protein in epithelial cells of distal tubules, showing positive staining in cortical distal convoluted tubules (Fig. 5D andF) and in outer medullary thick ascending limb of Henle's loop (Fig. 5C and E).

Immunohistochemical studies for CYP11A1 showed a pronounced abundance of this protein in the outer medulla relative to other regions (Fig. 6B). High magnification images clearly showed positive staining for CYP11A1 in cortical distal convoluted tubules (Fig. 6D and F) and in outer medullary thick ascending limb of Henle's loop (Fig. 6C and E). This distribution pattern was similar to that found for StAR protein. Slight staining for CYP11A1 in proximal tubules could not be disregarded. No specific reaction was seen in collecting ducts or glomeruli.

Cortical adrenal gland tissue, used as positive control, exhibited a positive staining reaction both with StAR antibody (Fig. 5A) and with the CYP11A1 antibody (Fig. 6A) used, corroborating their specificity. The incubation of kidney (Figs. 5G and H and 6G and H) and adrenal gland slices (not shown) with normal rabbit serum in replacement of StAR and CYP11A1 antibodies yielded no specific staining.

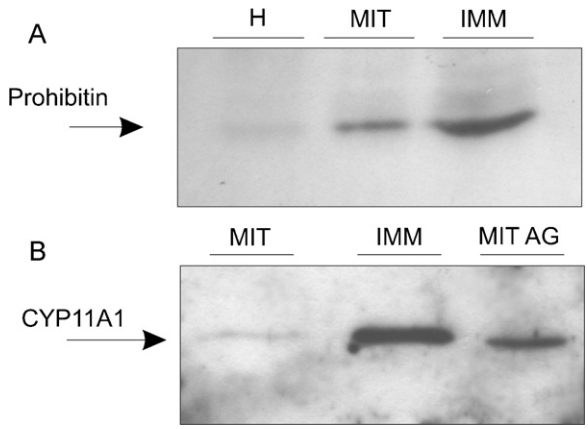

Fig. 3. Relative abundance of (A) prohibitin and (B) CYP11A1 in inner mitochondrial membrane fraction from adult rat kidneys. (A) Western blot analysis of prohibitin expression in renal cortex (CX) samples. Each lane was loaded with 10 μg of protein as follows, lane 1: total homogenate (H), lane 2: mitochondrial fraction (MIT), lane 3: inner mitochondrial membrane fraction (IMM). (B) Western blot analysis of CYP11A1. Lanes were loaded as follows, lane 1: 30 μg of protein of MIT from CX, lane 2: 10 μg of protein of IMM from CX, lane 3: 10 μg of protein of MIT from adrenal gland (MIT AG), obtained in the same way as MIT from renal CX, was used as positive control.

3.5. Pregnenolone and progesterone synthesis

The synthesis of P5 and P4 was achieved by incubating isolated mitochondria in a reaction buffer containing NADH/NADPH-regenerating system in the presence of 22R-OHC. Pregnenolone synthesis rate in the absence of 22R-OHC was $0.682 \pm 0.226\ \text{pg}\,\mu\text{g}^{-1}\,\text{h}^{-1}$ in CX and $0.960 \pm 0.363\ \text{pg}\,\mu\text{g}^{-1}\,\text{h}^{-1}$ in M mitochondria suspensions. No statistical differences were found. The addition of 22-OHC to the reaction buffer significantly increased the amount of P5 synthesized in mitochondria isolated from cortical and medullary tissue (Fig. 7). In cortical mitochondria the synthesis of P5 increased 5-fold compared to control without 22R-OHC, while in medullary mitochondria the synthesis of P5 increased 50-fold over control, indicating a greater enzymatic activity of CYP11A1 in renal medulla.

Progesterone synthesis rate measured in the absence of 22R-OHC was $1.772 \pm 0.151\ \text{pg}\,\mu\text{g}^{-1}\,\text{h}^{-1}$ in CX and $2.913 \pm 0.235\ \text{pg}\,\mu\text{g}^{-1}\,\text{h}^{-1}$ in M mitochondria suspensions. The

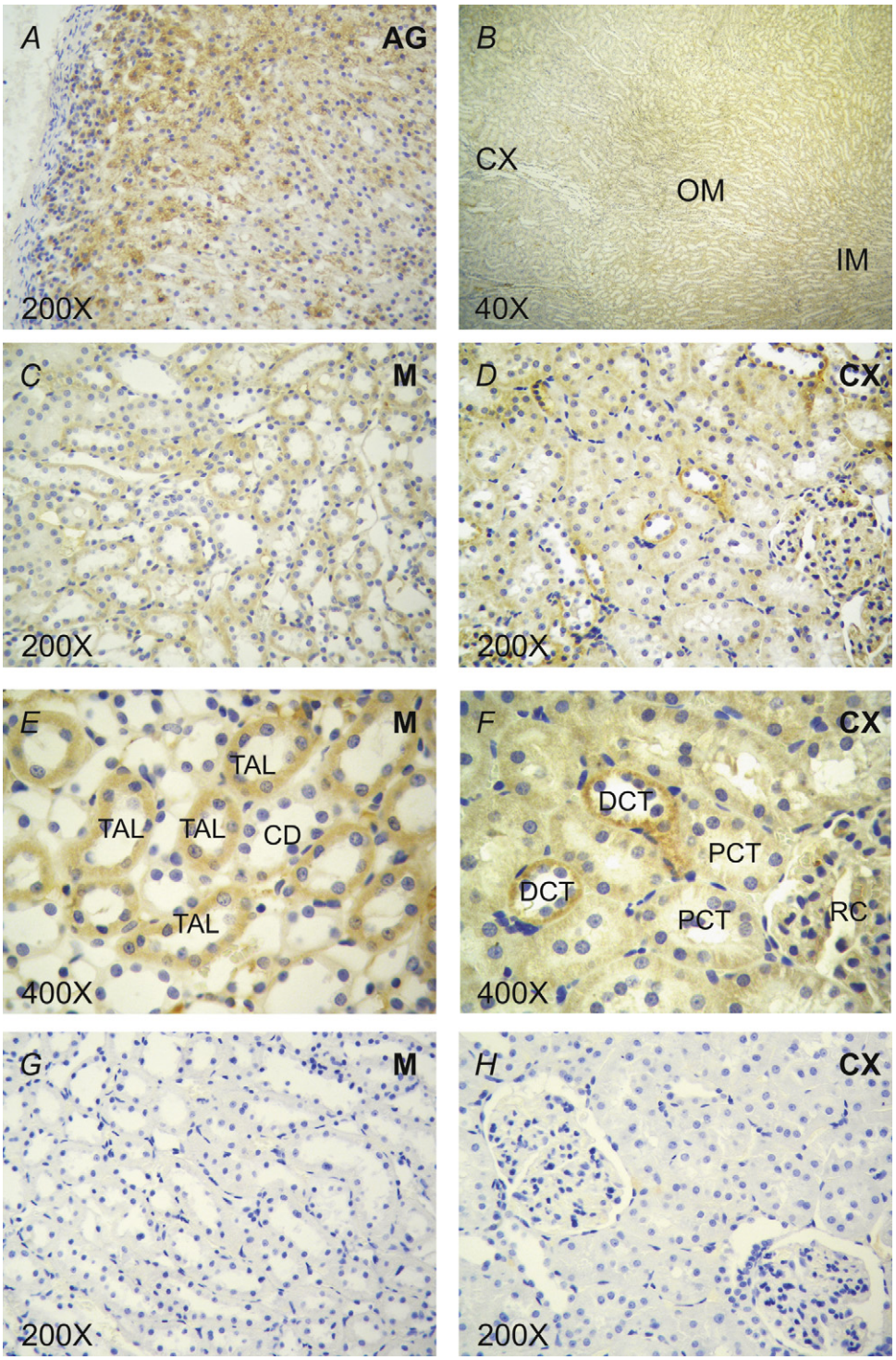

Fig. 5. Immunohistochemical staining for StAR in kidney tissue from adult rats. Sections were exposed to an antiserum against rat StAR. A positive immune response was localized in thick ascending limb of Henle's loop (TAL) cells in the outer medulla (OM) (C and E) and in distal convoluted tubule (DCT) cells in cortex (D and F). The incubation of kidney slices with normal rabbit serum in replacement of StAR antibody yielded no specific staining in medulla (G) or cortex (H). Cortical adrenal gland cells exhibited a positive staining reaction (A) with the StAR antibody used, corroborating its specificity. CD: collector duct, RC: renal corpuscle, PCT: proximal convoluted tubule, IM: inner medulla.

incubation with 22R-OHC significantly increased P4 synthesis in mitochondria isolated from renal medulla, while there were no changes in P4 synthesis in cortical mitochondria (Fig. 7). Despite being statistically significant, the increase of P4 synthesis in medullary mitochondria incubated with 22R-OHC was only 30% higher than those incubated without the cholesterol analogue.

4. Discussion

In the present study, a steroid biosynthetic pathway in rat renal mitochondria mediated by CYP11A1 was demonstrated by the conversion of cholesterol into pregnenolone. This reaction represents the first step in the overall steroid hormone biosynthesis. To our

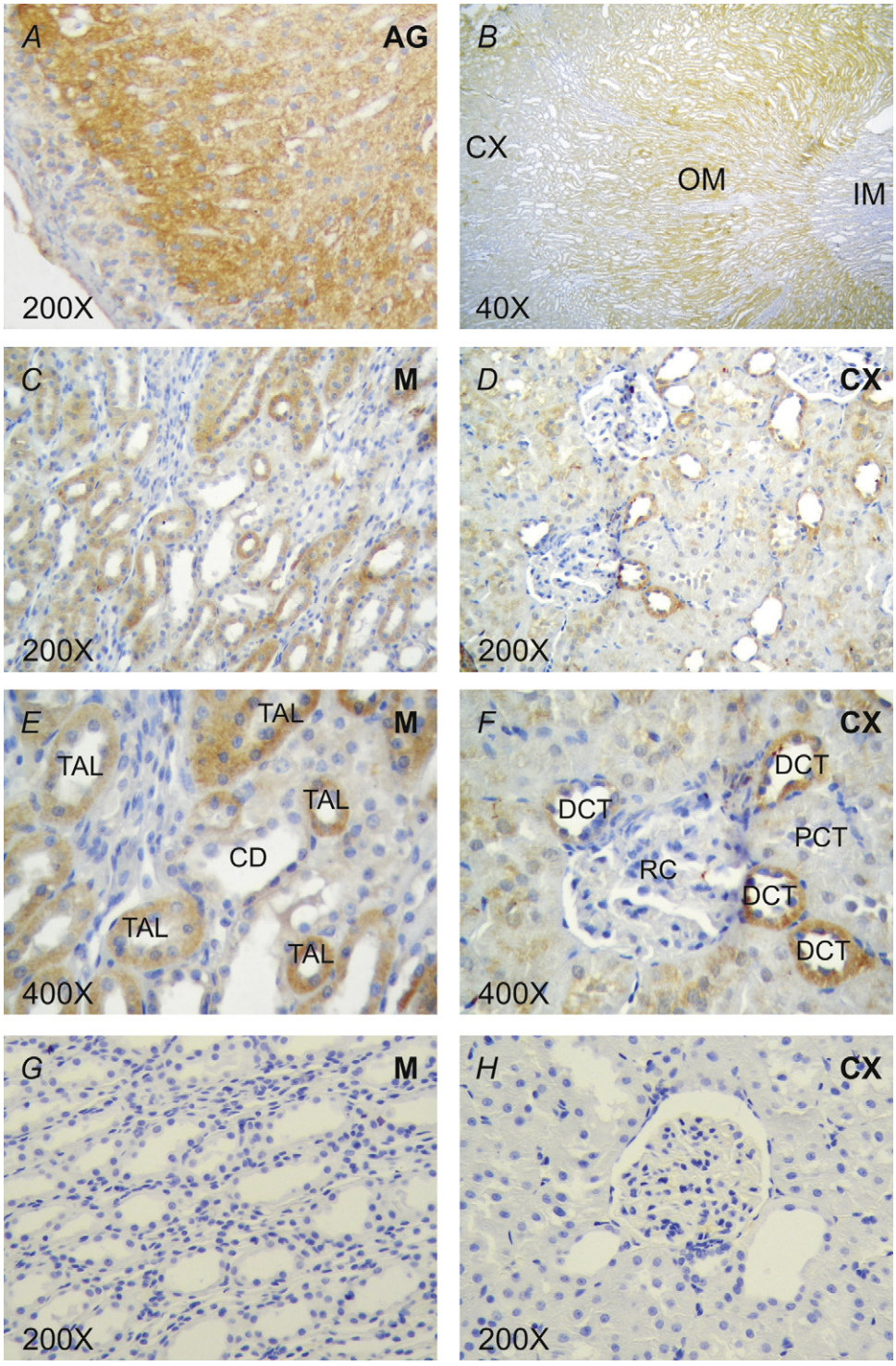

Fig. 6. Immunohistochemical staining for CYP11A1 in kidney tissue from adult rats. Sections were exposed to an antiserum against rat CYP11A1. A positive immune response was localized in thick ascending limb of Henle's loop (TAL) cells in the outer medulla (OM) (C and E) and in distal convoluted tubule (DCT) cells in cortex (D and F). The incubation of kidney slices with normal rabbit serum in replacement of CYP11A1 antibody yielded no specific staining in medulla (G) or cortex (H). Cortical adrenal gland cells exhibited a positive staining reaction (A) with the CYP11A1 antibody used, corroborating its specificity. CD: collector duct, RC: renal corpuscle, PCT: proximal convoluted tubule, IM: inner medulla.

knowledge, these results constitute first evidence for the presence and enzymatic activity of CYP11A1 in the adult rat kidney. In accordance to this finding, expression of other protein component of the system that transfers electrons from NADPH to cholesterol, ferredoxin (Driscoll and Omdahl, 1986), was previously found in rat kidney (Mellon et al., 1991). It was also reported the presence of the third component of this system, ferredoxin reductase, in kidney mitochondria of other species such as chick (Pedersen et al., 1976) and pig (Driscoll and Omdahl, 1986). CYP11A1 mRNA expression in the kidney of young adult rats had been previously reported by using RT-PCR (Dalla Valle et al., 2004b) or RNase protection assays (Mellon et al., 1991). CYP11A1 protein has been detected in cortical distal tubules by immunostaining and in mitochondrial fraction by Western blot in kidneys from newborn, but it could not be

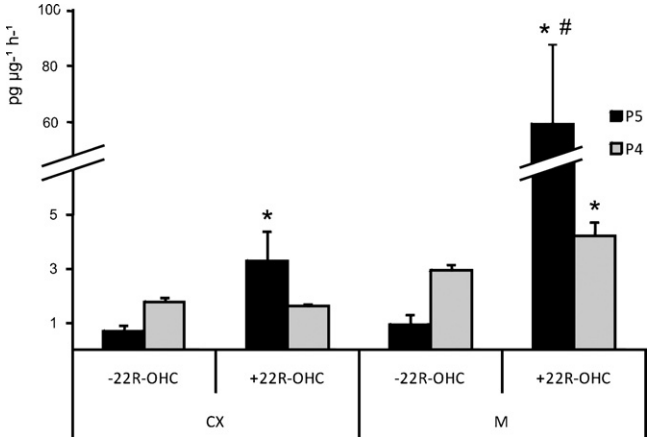

Fig. 7. Synthesis of pregnenolone (P5) and progesterone (P4) in isolated mitochondria from renal cortex (CX) and medulla (M) of adult rats. Mitochondrial samples were incubated in reaction buffer in the presence (+22R-OHC) or absence (−22R-OHC) of 22R-hydroxycholesterol for 45 min. P5 and P4 were assessed by RIA. Experiments were done in duplicate with equal results. Bars represent means ± SEM of 6 observations. $^{*}p < 0.05$ vs. corresponding group in the absence of 22R-OHC, $\#p < 0.05$ vs. CX in the presence of 22R-OHC.

detected in samples from 60-day-old rats (Dalla Valle et al., 2004b). These authors suggested that nephrosteroids could be involved in the regulation of kidney development and differentiation. In our study, IMM samples obtained from 90-day-old rats were assessed and CYP11A1 was detected by Western blot. The enrichment of CYP11A1 in IMM membrane fraction (Fig. 3A) and the different sources of the antibodies utilized could contribute to the differences between ours and the mentioned results. In the present study, immunohistochemical analyses showed that the expression of this protein is primarily concentrated in cortical and medullary distal tubules. The pronounced abundance of CYP11A1 in outer medulla, in structures identified as ascending limb of Henle's loop, indicates that the pattern of protein distribution in adult rat kidney is different from that found in the post natal developmental period (Dalla Valle et al., 2004b). This fact, associated to the functional expression of CYP11A1 in the adulthood suggests that renal steroidogenesis would not play a role only in developmental processes, but also in the maintenance of physiological functions.

The conversion of cholesterol into pregnenolone was previously thought to be the rate-limiting step in steroidogenesis. Nevertheless, it is now known that the critical step is cholesterol transport to the inner mitochondrial membrane where CYP11A1 resides (Stocco, 1999a,b). Mitochondrial import of cholesterol requires a functional interaction between StAR and TSPO (Miller, 2007b,a). The present study shows StAR and TSPO mRNA expression in rat renal tissue. Other authors had detected StAR mRNA in males from birth to 60 days of age (Dalla Valle et al., 2004b). TSPO mRNA and its protein expression were reported by other investigators in adult rat medullary and cortical renal tissue (Bribes et al., 2002; Zhang et al., 2006). The results presented in these experiments introduce evidence for the expression of StAR protein in adult rat kidney. Immunohistochemistry for StAR showed positive staining in thick ascending limb of Henle's loop and in the distal convoluted tubule. Similar distribution pattern found in the kidney for StAR and CYP11A1, involved in transport and conversion of cholesterol, respectively, suggest a functional interaction of these proteins directed to pregnenolone synthesis. This finding strengthens the hypothesis of the functionality of the steroidogenic elements found in the kidney. The existence of complete steroidogenic machinery in the kidney, including the expression and activity of other steroidogenic enzymes previously described, would respond to a physiological significance.

The expression of StAR and CYP11A1 was found to be higher in the medulla than in cortical tissue. This enhanced expression of steroidogenesis-related protein in medulla correlated with CYP11A1 activity, judging from the augmented formation of pregnenolone. The greater pregnenolone synthesis in medulla than in cortex is consistent with the higher level of testosterone and progesterone found in the former renal region. These findings suggest that the content of steroid hormones in the kidney could be influenced by local steroidogenesis.

Progesterone formation was detected in medullary mitochondrial suspensions incubated with 22-OHC. A scarce progesterone synthesis was observed even when pregnenolone synthesis increased nearly 50-fold over samples incubated in absence of 22-OHC. These results suggest a poor activity of 3β-hydroxysteroid dehydrogenase in renal mitochondria. Despite microsomal contamination could not be disregarded, it is important to consider that a dual location for 3β-HSD, at microsomal and mitochondrial level, has been described in other tissues (Cherradi et al., 1993, 1994). 3β-HSD expression in adult rat kidney was previously reported and formation of [^{14}C]progesterone from [4-^{14}C]pregnenolone in whole renal tissue was measured (Zhao et al., 1991). Present results of sex steroid measurements showed that renal content of progesterone is nearly three orders of magnitude lower than testosterone. Renal synthesis of pregnenolone could favour androgens formation from dehydroepiandrosterone pathway. The conversion of [4-^{14}C]dehydroepiandrosterone into [4-^{14}C]Δ^4-dione in adult rat kidney (Zhao et al., 1991) seems to sustain this suggestion. Further investigation will be required to elucidate this matter.

Pregnenolone formation from cholesterol has already been described in extraglandular sites, like rat glial cells (Schumacher et al., 2004). Steroids produced by glia regulate different functions and exert neuroprotective and reparative roles (Schumacher et al., 2004; Garcia-Ovejero et al., 2005). These actions of neurosteroids are principally mediated by interaction with $GABA_A$ receptor. The presence of $GABA_A$ receptor was also demonstrated in rat kidney (Amenta et al., 1988). Previous studies from our laboratory suggested that endogenous GABA exerts a vasodilatory modulation, via $GABA_A$ receptors, in the isolated perfused rat kidney (Monasterolo et al., 1996). Other investigators demonstrated that steroids, including pregnenolone, and the $GABA_A$ agonist muscimol exhibit cytoprotective effects against mitochondrial inhibition and anoxia in rabbit renal proximal tubules (Waters et al., 1997). These data strengthen the hypotheses that local formation of pregnenolone, and downstream synthesized nephrosteroids, could play a role in the maintenance of renal physiological functions and/or in the progression of pathological situations.

Steroidogenic pathways were described in human kidney (Winkel et al., 1980; Quinkler et al., 1999, 2003). Physiological significance of renal steroidogenesis was inferred from different studies. In cultured human mesangial cells, angiotensin II induced enhancement of pregnenolone and aldosterone production. It was proposed that the latter steroid could regulate the permeability/filtration function (Nishikawa et al., 2005). Other authors suggested that progesterone metabolism and androgen synthesis in human kidney could be involved in water balance during the menstrual cycle and pregnancy and in the sex-dependent differences in renal blood pressure regulation (Quinkler et al., 2003). The role that sex steroids play in chronic kidney disease and in the progression to end stage renal disease in humans is not clear. Elucidation of the mechanisms underlying the impact of steroids on renal disease will allow improvement of treatment paradigms. Since surgical and pharmacological tools are suitable in animal models, these are still the best studies to evaluate mechanisms responsible for sex steroids impact in renal disease (Yanes et al., 2008).

5. Conclusions

In conclusion, we have established the expression and localization of StAR and CYP11A1 protein, the capability of pregnenolone and progesterone synthesis and a region-specific content of sex steroids in the adult rat kidney. These data clearly show that the rat kidney is a steroids synthesizing organ and encourage further investigation of the role of local steroidogenesis, previously limited to development and differentiation, in the modulation of physiological functions.

Acknowledgements

This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 05299 to LAM), by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT 2005 5-38281 to OPP) and by the University of Buenos Aires (X814 to OPP). The authors wish to acknowledge Casandra Monzón for technical assistance.

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