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Deoxycorticosterone inactivation by AKR1C3 in human mineralocorticoid target tissues

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Abstract

Aldosterone is the principal endogenous mineralocorticoid in humans and regulates salt and water homeostasis. Cortisol, the major glucocorticoid, has high affinity for the mineralocorticoid receptor; however, 11β-hydroxysteroid dehydrogenase type 2 converts cortisol to the inactive steroid cortisone in aldosterone target cells of the kidney, thus limiting the mineralocorticoid action of cortisol. Deoxycorticosterone (DOC) binds to the mineralocorticocoid receptor with high affinity and circulates at concentrations comparable to aldosterone. Severe DOC excess as is seen in 17α- and 11β-hydroxylase deficiencies causes hypertension, and moderate DOC overproduction in late pregnancy is associated with hypertension. Here, we demonstrate that DOC is inactivated by the 20-ketosteroid reductase activity of the human AKR1C3 isozyme. Immunohistochemical analyses demonstrate that AKR1C3 is expressed in the mineralocorticoid-responsive epithelial cells of the renal cortical and medullary collecting ducts, as well as the colon. Our findings suggest that AKR1C3 protects the mineralocorticoid receptor from activation by DOC in mineralocorticoid target cells of the kidney and colon, analogous to cortisol inactivation by 11β-hydroxysteroid dehydrogenase type 2.

Keywords: DOC; Mineralocorticoid; Inactivation; AKR1C3; Human; Kidney; Colon

1. Introduction

The mineralocorticoid receptor (MR) is expressed in the renal distal convoluted tubules and cortical collecting ducts where it acts to increase reabsorption of sodium from the urine (Agarwal and Mirshahi, 1999; Rogerson and Fuller, 2000). The MR possesses high affinity ($K_D \sim 1$ nM) for the physiological mineralocorticoid aldosterone as well as the glucocorticoid cortisol, but cortisol circulates at concentrations ∼3 orders of magnitude higher than aldosterone (1 μM versus 1 nM, respectively) (Arriza et al., 1987; Krozowski and Funder, 1983; Quinkler et al., 2002; Rupprecht et al., 1993a,b). To protect the MR from the mineralocorticoid activity of the more abundant cortisol, microsomal 11β-hydroxysteroid dehydrogenase (HSD) type 2, which catalyzes the conversion of cortisol to its inactive 11-keto derivative cortisone, is expressed in the same cell types that express the MR (Krozowski et al., 1995a,b, 1989; Shimojo et al., 1997; Smith et al., 1996). The physiological importance of cortisol metabolism in mineralocorticoid target cells is underscored by the fact that inactivating mutations in the 11β-HSD type 2 enzyme cause apparent mineralocorticoid excess syndrome, in which cortisol causes hypertension despite suppression of renin and aldosterone (White et al., 1997). The local inactivation of cortisol, which prevents activation of MR, is an example of pre-receptor regulation of steroid hormone action.

Several studies have shown that deoxycorticosterone (DOC) also is a potent mineralocorticoid (Ferrari and Bonny, 2003; Morrison et al., 2002; Mussig et al., 2005; Ortiz and Garvin, 2001; Verlander et al., 2003). DOC binds to and activates the MR with an affinity similar to that of aldosterone (Quinkler et al., 2002). In contrast to aldosterone, which is synthesized only in the zona glomerulosa of the adrenal cortex and whose synthesis is tightly regulated via the renin–angiotensin system, DOC is formed both as an intermediate of adrenal steroidogenesis and by extra-adrenal 21-hydroxylation of plasma-borne progesterone (Casey and MacDonald, 1982; Winkel et al., 1983). Excessive

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adrenal DOC production occurs in 17α- and 11β-hydroxylase deficiencies, causing circulating DOC concentrations to reach 1–20 nM, which will lead to hypertension in many, but not all afflicted subjects (Biglieri and Kater, 1991; Biglieri et al., 1989b; Kater and Biglieri, 1994). The rate of DOC formation by the peripheral pathway is proportional to the concentration of progesterone in plasma, and importantly, is not subject to negative feedback regulation (Casey and MacDonald, 1982). During pregnancy, circulating DOC concentrations rise from 0.1 nM and peak at the end of the third trimester in the range of 1.5–10 nM, concentrations sufficiently high to cause hypertension (Brown et al., 1972; Ehrlich et al., 1974; Nolten et al., 1978; Parker et al., 1980; Sippell et al., 1978). Despite these elevated levels, hypertension occurs in only 4% of pregnancies (Cunningham et al., 2001). This discrepancy raises the important question: are mineralocorticoid target tissues normally protected from DOC action by a mechanism similar to the cortisol to cortisone metabolism mediated by 11β-HSD type 2?

To answer this question, we searched for an enzyme that converts DOC to a metabolite that lacks the ability to activate the MR. One set of candidate enzymes are the aldo–keto reductases (AKRs) of the subfamily, AKR1C1–AKR1C4 (Hyndman et al., 2003; Penning et al., 2000). Previously, these enzymes have been shown to function as 3α-, 3β-, 17β- and 20α-hydroxysteroid dehydrogenases in different ratios in a substrate dependent manner, and to display tissue specific expression patterns (Penning et al., 2000; Steckelbroeck et al., 2004). We show herein that the AKR1C3 enzyme converts DOC to the inactive mineralocorticoid 20α-hydroxy-DOC and that this enzyme is expressed in the mineralocorticoid target cells of the renal cortical and medullary collecting ducts, as well as in the epithelial cells of colon.

2. Materials and methods

2.1. Steroids

[4-^{14}C]Deoxycorticosterone (DOC) (58.3 mCi/mmol), [4-^{14}C]androstenedione (53.6 mCi/mmol), [1,2,6,7-^{3}H]progesterone (102.1 Ci/mmol) and [1,2,6,7-^{3}H]cortisol (78.0 Ci/mmol) were purchased from NEN Life Science Products (Shelton, CT). Aldosterone, cortisol, DOC (4-pregnen-21-ol-3,20-dione), 20α-hydroxy-DOC (4-pregnen-20α,21-diol-3-one), 20β-hydroxy-DOC (4-pregnen-20β,21-diol-3-one) and progesterone were from Steraloids (Newport, RI). Other reagents were purchased from Sigma–Aldrich (St. Louis, MO) and tissue culture medium from Invitrogen (Carlsbad, CA) unless otherwise specified.

2.2. Enzyme assay in vitro

Recombinant human AKR1C1, 1C2, 1C3 and 1C4 enzymes were expressed in *Escherichia coli* and purified as previously described (Burczynski et al., 1998). Assays contained 100 mM potassium phosphate, pH 7.0, 2.5 mM NADPH (N-7505; Sigma) and radioactive substrate with 2% (v/v) ethanol in a total volume of 0.1 ml. For substrate specificity analyses, 1 and 5 μg of the respective AKR isozyme were incubated with 1 μM radiolabeled DOC, cortisol and progesterone for 10 min at 37 °C. Kinetic analyses with 0.5 μg of AKR1C3 were performed in duplicate using steroid substrate concentrations of 0.625–20 μM for 10 min at 37 °C. The reactions were terminated by the addition of 1 ml chloroform/methanol (2:1, v/v) and extraction. The organic phase was evaporated, and substrate and products were separated on Whatman Partisil LK5D (Whatman, Clifton, NJ) chromatoplates in chloroform/ethyl acetate (3:1, v/v). Product quantitation was performed using a BioScan 200 Imaging System (Washington, DC), using a 6-mm collimator at high efficiency. Kinetic constants were derived by fitting the data to the Michaelis–Menten equation with non-linear regression analysis using GraFit Version 5.0.1 (Erithacus Software, Limited, Horley, UK). Values of k_{cat} were calculated using a molecular weight of 37 kDa for monomeric AKR1C3. To determine whether the enzymatic product of AKR1C3 with DOC (Rf = 0.74) as substrate was the 20α- or 20β-hydroxy epimer of DOC, the zone of the chromatoplate containing the enzymatic product (Rf = 0.31) was scraped off the chromatoplate and steroid was eluted off the silica using chloroform/methanol (9:1, v/v) for further analysis by HPLC. The residue was dissolved in 140 μl of methanol, and 40 μl was injected into a Breeze model 1525 binary pump HPLC system equipped with model 717 plus autoinjector and a 4.6 mm × 150 mm, 5 μm C_{18} Symmetry column (Waters Corp., Milford, MA). The column effluent was analyzed with a model 2487 dual-wavelength UV detector set to 254 nm, and a β-RAM model 3 in-line radioactivity detector (IN/US Systems, Inc., Tampa, FL). All samples were separated at 30 °C and a flow rate of 1 ml/min using 50% aqueous methanol for 10 min, followed by a linear gradient of 50–75% methanol in water over 20 min and re-equilibration at 50% methanol for 10 min. Retention times (radioactivity detector) were: 20α-hydroxy-DOC, 19.7 min; 20β-hydroxy-DOC, 23.4 min. The enzymatic product of AKR1C2 and 1C4 with cortisol as substrate was not further analyzed, but assumed to be 20α-hydroxycortisol.

2.3. Enzyme assay in transfected HEK-293 cells

The full-length cDNA encoding human AKR1C3 (SwissProt accession number P42330) was isolated by hybridization cloning of a human testis cDNA library in a pCMV mammalian expression vector (Geissler et al., 1994). Human embryonic kidney 293 cells, HEK-293 (American Type Culture Collection CRL no. 1573) were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin sulfate and 10 mM HEPES, pH 7.0, (complete DMEM). On day 0, 100,000 HEK-293 cells were plated into 24-well Costar dishes (Corning Inc., Corning, NY) containing 0.5 ml complete DMEM per well. On day 1, cells were transfected with 1 μg/well of the pCMV-AKR1C3 or pCMV plasmids (0.9 μg) and pVA_1 (0.1 μg) (Svensson and Akusjarvi, 1985) using a calcium phosphate method (Sambrook and Russell, 2001). Forty-eight hours after addition of plasmid DNA, radioactive substrate (1 μM final concentration) was added to the individual wells, and samples were collected at the indicated time-points. Extraction of steroids and analysis of product formation was performed as described above.

2.4. MR transactivation assay

The MR/MMTV-luciferase receptor reporter system was composed of pXM4-MR which specifies the full-length human MR with a C-terminal nonapeptide hemagglutinin epitope tag (gift from Alex Odermatt) (Odermatt et al., 2001), a mouse mammary tumor virus luciferase reporter vector (pMMTV-luc), and a control β-galactosidase plasmid (pCMX-β-gal) (gifts from David Mangelsdorf) (Willy et al., 1995). On day 0, 100,000 HEK-293 cells/well were plated in 24-well Costar dishes in a volume of 0.5 ml of complete DMEM supplemented with 10% (v/v) charcoal/dextran treated fetal bovine serum (Hyclone, Logan, UT). On day 1, cells were transfected with a mixture of plasmid DNA (1 μg/well) consisting of pXM4-MR (50 ng), pMMTV-luc (200 ng), pCMV-β-gal (100 ng), pVA_1 (100 ng) and pCMV-AKR1C3 or pCMV (550 ng) using a calcium phosphate method (Sambrook and Russell, 2001). Forty-eight hours after transfection, DOC or aldosterone (1000× stock solutions in ethanol) were added to final concentrations of 1 pM to 300 nM. After an additional 20 h incubation, the cells were lysed and assayed for luciferase activity (Promega, Madison, WI) and β-galactosidase activity (Tropix, Bedford, MA) according to the manufacturers' instructions using a Cambridge Technology 7715 Microplate Luminometer (Cambridge, MA). Relative level of transactivation was calculated by dividing luciferase units by β-galactosidase units. The concentration of ligand that resulted in 50% of maximal activation of MR (EC_{50}) was calculated using GraFit Version 5.0.1.

2.5. Production of monoclonal antibody

A synthetic peptide [C]LHYFNSDSFASHPNYPYSDEY corresponding to amino acid residues 303-323 in human AKR1C3 (SwissProt accession number P42330), was coupled to keyhole limpet hemocyanin and used for immunization of mice. Hybridomas were established and screened for antibody production by an enzyme-linked immunosorbent assay (ELISA) using the AKR1C3 peptide as previously described (Lindqvist and Andersson, 2002; Moghrabi et al., 1997). Positive hybridomas were subsequently counter-screened by ELISA using a peptide corresponding to amino acid residues 303-323 in human AKR1C4 (SwissProt accession number P17516). Hybridoma #5-28 was chosen since it secreted an antibody, designated MAb-5-28 (subclass IgG2a/κ), that recognized the 1C3 peptide and not the 1C4 peptide. For ELISA using purified AKR1C1-4 proteins, wells were coated with 5 μg of protein/ml in PBS (50 μl/well). Mice were maintained and treated in accordance with the guidelines set forth by the Animal Welfare Information Center. Protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center at Dallas.

2.6. Immunoblotting

Immunoblotting of purified human AKR1C1, 1C2, 1C3, and 1C4 enzymes using the anti AKR1C3 specific monoclonal antibody MAb-5-28 was performed as previously described (Lindqvist and Andersson, 2002).

2.7. Immunohistochemistry

Normal human tissues were obtained during routine surgical biopsies or autopsies (ProPath Laboratory, Inc., Dallas, TX), fixed in neutral buffered formalin, and processed routinely into paraffin blocks. Representative histologic sections of each specimen stained with hematoxylin and eosin were examined by experienced diagnostic pathologists to confirm the preservation of the histological structure and to validate the normalcy. For immunostaining, the blocks were cut at 4 μm, and sections were mounted on charged adhesive slides and dried in a 1000 W microwave oven (set on "high") for 3 min, followed by 10 min in a 56 °C oven. Slides were then deparaffinized in xylene and rehydrated in graded alcohols to distilled water. Endogenous peroxidase activity was quenched by placing the slides in 0.3% hydrogen peroxide with 0.1% sodium azide at room temperature for 10 min. Slides were then subjected to epitope retrieval by placing the slides in 250 mM Tris–HCl, pH 9.0 (MAb-5-28) or 1 mM Na-EDTA, pH 8.0 (anti-cytokeratin), epitope retrieval solution in a pressure cooker (BioCare Medical, Walnut Creek, CA) for 5 min at full pressure, followed by cool down (total time in pressure cooker from start to finish is 48 min). After rinsing three times with PBS containing 0.1% Tween-20, slides were covered with MAb5-28 in tissue culture medium or mouse anti-human cytokeratin cocktail AE1/AE3 (Cat. No. AM071-5M, BioGenex, San Ramon, CA) and incubated for 30 min in a 25 °C incubation oven using gentle orbital rotation at 40 rpm. For negative controls, irrelevant mouse monoclonal antibody in tissue culture medium was used in place of primary antibody. After rinsing 3 times in PBS, slides were incubated with PowerVision Polymerized Horseradish Peroxidase-conjugated anti-Mouse IgG (ImmunoVision Technologies Co., Daly City, CA) for 30 min at 25 °C. Finally, the slides were immersed for 5 min in 25 °C diaminobenzidine (DAB) (Research Genetics, Huntsville, AL), enhanced with 0.5% copper sulfate in PBS for 5 min at 25 °C, counterstained in hematoxylin, dehydrated in graded alcohols, cleared in xylene, and coverslipped for visualization.

3. Results

3.1. Steroid metabolism by purified AKR1C isozymes

The 20-ketosteroid reduction of progesterone is the major metabolizing activity in human kidney homogenates (Quinkler et al., 2001, 1999), and all four members of the human AKR1C subfamily have been shown to possess 20α-HSD activity with progesterone as substrate to varying degrees (Penning et al., 2000). Since the mRNAs encoding the AKR1C1, C2 and C3 enzymes are expressed in human kidney (Nishizawa et al., 2000; Quinkler et al., 2003), we determined whether any of the AKR1C isozyme(s) have the capacity to act as 20-ketosteroid reductases on DOC and cortisol. Using in vitro assays with purified recombinant AKR1C1-1C4 enzymes, we confirmed that progesterone is a substrate for all four isozymes (Table 1), in accordance with previous studies (Penning et al., 2000). Further experiments revealed that only AKR1C3 utilized DOC as substrate (Table 1), and HPLC analysis (not shown) confirmed that the metabolite formed was 20α-hydroxy-DOC. Cortisol was a poor substrate for AKR1C4 and AKR1C2, but was not a substrate for AKR1C1 and AKR1C3.

Table 1
Substrate specificity of purified human AKRs

Substrate	AKR1C (nmol/min per mg)			
	1	2	3	4
DOC	<0.02	0.18	3.33	<0.02
Cortisol	<0.02	0.07	<0.02	0.29
Progesterone	5.5	1.3	4.1	2.0

We next determined the kinetic constants for AKR1C3 in vitro with DOC as substrate. The K_m and V_{max} of the enzyme with this substrate were 4.3 ± 1.5 μM and 36.5 ± 10.4 nmol/min per mg, respectively (Table 2). These values correspond to a turnover number (k_{cat}) of 1.35 ± 0.38 min^{-1} and a catalytic efficiency (k_{cat}/K_m) of 314 min^{-1} mM^{-1}. For comparison, the K_m and V_{max} values for the 20-ketosteroid reduction of progesterone were 2.8 ± 0.8 μM and 28.0 ± 8.5 nmol/min per mg, respectively, corresponding to a turnover number of 1.04 ± 0.31 min^{-1} and a catalytic efficiency of 370 min^{-1} mM^{-1}. The AKR1C3 enzyme also possesses 3-ketosteroid and 17-ketosteroid reductase activity and is also referred to as 3α-HSD type 2 and 17β-HSD type 5 in the literature (Penning et al., 2000). Of these activities, the 17β-HSD activity is more pronounced and is assumed to be important for the extra-glandular conversion of androstenedione into testosterone, and this activity was examined in this comparative study. The K_m and V_{max} of the enzyme with androstenedione substrate were 13.4 ± 2.7 μM and 23.5 ± 6.6 nmol/min per mg, respectively. These values correspond to a turnover number of 0.87 ± 0.24 min^{-1} and a catalytic efficiency of 65 min^{-1} mM^{-1}. Taken together, in vitro the AKR1C3 isozyme is equally efficient in utilizing DOC and progesterone as substrates, and its 20α-HSD activity with DOC as

Table 2
Catalytic constants of purified AKR1C3

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
DOC	4.3 ± 1.5	1.35 ± 0.38	314
Progesterone	2.8 ± 0.8	1.04 ± 0.31	370
Androstenedione	13.4 ± 2.7	0.87 ± 0.24	65

Each K_m and k_{cat} value represents the average ± S.D. of three independent experiments performed in duplicate. k_{cat}/K_m, catalytic efficiency.

substrate is superior to its 17β-HSD activity with androstenedione as substrate.

3.2. Steroid metabolism by AKR1C3 in HEK-293 cells and the effect of AKR1C3 on MR transactivation by DOC

To confirm that AKR1C3 can metabolize DOC in living cells and to determine the physiological consequences of this activity, we expressed AKR1C3 in HEK-293 cells and assayed enzyme activity and MR activation. Fig. 1 shows that endogenous enzymes in mock-transfected HEK-293 cells demonstrate a negligible capacity to metabolize DOC. In contrast, cells expressing AKR1C3 converts both DOC and progesterone to their 20α-hydroxy counterparts, as well as androstenedione to testosterone, thus confirming the data obtained with purified recombinant AKR1C3.

We next asked the question whether co-expression of AKR1C3 impairs the ability of DOC to activate MR. A transactivation assay was utilized in which human MR was expressed in HEK-293 cells harboring a MR-responsive mouse mammary tumor virus (MMTV) promoter/reporter plasmid. Fig. 2 shows dose-response curves for MR activation by DOC in the absence or presence of recombinant AKR1C3. The EC_{50} for DOC is 0.6 nM, and when AKR1C3 is co-expressed with MR, the EC_{50} for DOC increases approximately 1000-fold. Hence, AKR1C3 is in living cells capable of inactivating DOC at physiological concentrations. We also performed control experiments demonstrating that 20α-hydroxy-DOC does not activate MR at concentrations up to 1 μM (not shown). To compare the relative activity of DOC and aldosterone as MR agonists, and to determine whether AKR1C3 inactivates aldosterone, we expanded the transactivation experiments to include aldosterone. The EC_{50} for MR activation by aldosterone is 0.5 nM, and co-expression of AKR1C3 enzyme increases the EC_{50} for aldosterone less than three-fold, suggesting that the attenuation of MR activation by AKR1C3 is selective for the DOC ligand.

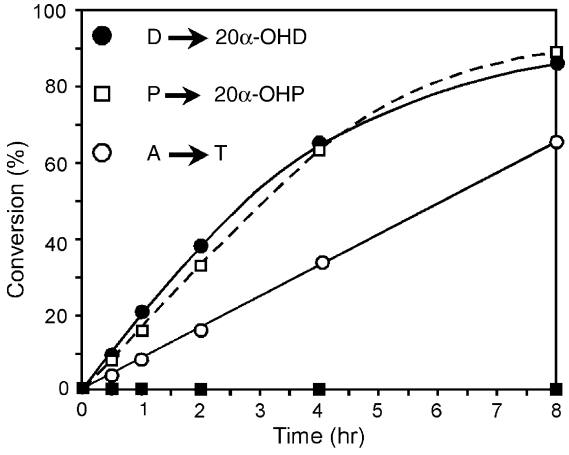

Fig. 1. Expression analysis of human AKR1C3 in HEK-293 cells. A cDNA encoding AKR1C3 or empty vector control were transfected into HEK-293 cells on day 1. The ability of the AKR1C3 transfected cells to convert DOC to 20α-hydroxy-DOC (D → 20α-OHD), progesterone to 20α-hydroxyprogesterone (P → 20α-OHP), and androstenedione to testosterone (A → T) was determined on day 3. Conversion of DOC, progesterone and androstenedione to products was also determined in wells transfected with empty vector control (■). Product quantitation was performed by thin layer chromatography and radioactivity scanning as described under Section 2. Data are representative of two independent experiments.

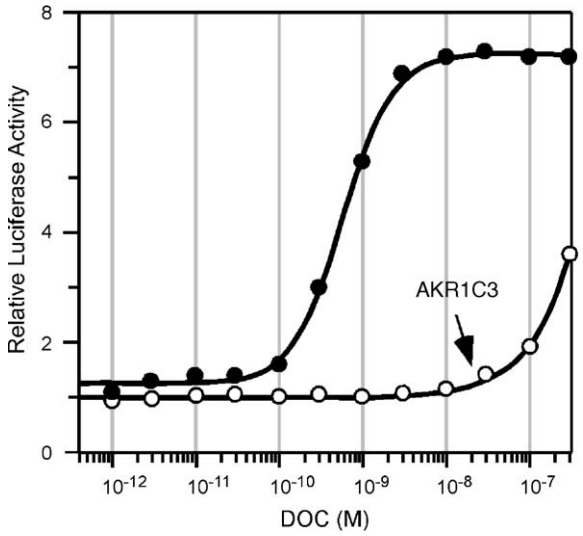

Fig. 2. Co-expression of AKR1C3 decreases MR transactivation by DOC in HEK-293 cells. Expression plasmids for human MR, β-galactosidase, and human AKR1C3 (◯) or empty vector control (●) were transfected into HEK-293 cells together with a MMTV-luciferase reporter plasmid. Luciferase and β-galactosidase enzyme activities were measured in cell lysates after incubation with DOC for 20 h. Mean values based on data from triplicate wells for each concentration of DOC were plotted.

3.3. Immunohistochemical analysis of AKR1C3 in mineralocorticoid target tissues

If DOC metabolism by AKR1C3 is a physiologic mechanism for impeding MR activation by DOC, then AKR1C3 should be expressed in the mineralocorticoid-responsive cells of the kidney and colon. To determine the cell type-specific expression of AKR1C3 in human kidney, we developed a monoclonal antibody against a synthetic peptide that is unique to the AKR1C3 protein. Fig. 3A shows a Coomassie-blue stained polyacrylamide gel of purified AKR1C1, 1C2, 1C3 and 1C4 enzymes, and the immunoblot in Fig. 3B shows that the monoclonal antibody MAb-5-28 specifically recognizes the AKR1C3 protein (lane 3). The antibody also specifically recognized the AKR1C3 isozyme as judged in an enzyme-linked immunosorbent assay using the purified AKR1C1-4 proteins (not shown).

Immunohistochemical analyses of human kidney sections using MAb-5-28 indicate that AKR1C3 protein is expressed in both cortex and medulla of the human kidney (Fig. 4). In cortex, strong and diffuse cytoplasmic staining is observed in the proximal convoluted tubules and S_3 segments. A majority of the distal convoluted tubules are negative for AKR1C3 protein, although occasional tubules display focal and weak positivity. Scattered thick ascending limbs demonstrate focal and weak signals. Collecting ducts in the cortex and medulla show moderate staining in both principal and intercalated cells; the latter cell type appears to stain stronger with an apical and cytoplasmic pattern. The thin limbs of Henle also were moderately

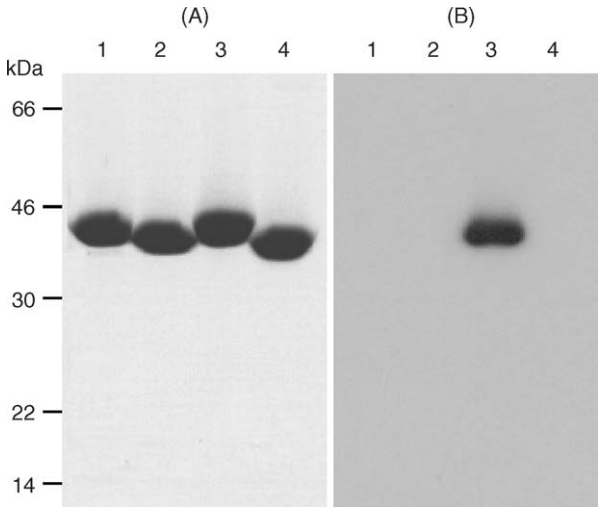

Fig. 3. Coomassie stain and immunoblotting of purified human AKR1C1-4. AKR isozymes were purified as described (Burczynski et al., 1998). Aliquots of protein (lane 1, AKR1C1; lane 2, AKR1C2; lane 3, AKR1C3; lane 4, AKR1C4; (A) 7.5 μg each; (B) 25 ng each) were subjected to SDS-polyacrylamide gel electrophoresis. (A) Protein detection was performed by Coomassie Brilliant Blue R staining. (B) Proteins were transferred to a nitrocellulose membrane and incubated with 5 μg/ml of MAb-5-28, and the antibody–antigen complexes were visualized by a chemiluminescence method as described under Section 2. The film was exposed for 10 s. The positions of prestained molecular size markers are shown on the left; kDa, kilodalton.

positive. No staining is seen in glomeruli and vascular structures. As expected, distal convoluted tubules and both cortical and medullary collecting ducts were strongly positive whereas thin limbs of Henle were weakly positive for low-molecular-weight cytokeratin (AE1/AE3), which is considered as a marker of the above tubular segments (Silva et al., 1993). Comparison of serial sections stained with the antibodies against AE1/AE3 and AKR1C3 helped us to accurately identify the tubular segments which were positive for AKR1C3. No difference in cell type-specific staining for AKR1C3 was observed when kidney sections from men and women were compared. In the colon, AKR1C3 is found in both surface and crypt epithelium, and both absorptive and goblet cells are positive (Fig. 4). The staining is more intense in the surface epithelium than in the crypt epithelium. These results confirm that AKR1C3 is expressed in mineralocorticoid target cells of the kidney and colon.

4. Discussion

DOC is a high-affinity ligand and agonist for the MR, based on in vitro ligand-binding and cell-based transactivation assays. In contrast, DOC is not as potent a mineralocorticoid in vivo as predicted from these assays. The current studies provide an explanation for this conundrum by demonstrating that human AKR1C3 converts DOC to the inactive metabolite 20α-hydroxy-DOC and that AKR1C3 is expressed in mineralocorticoid responsive cells of target tissues. The other three human AKR1C isozymes, which exhibit 20α-HSD activity with progesterone, metabolize DOC to 20α-hydroxy-DOC at <5% the rate of AKR1C3 (Table 1). Furthermore, AKR1C3 metabolizes DOC five times more efficiently than androstenedione (Table 2). These observations suggest that DOC metabolism to 20α-hydroxy-DOC is an important activity of AKR1C3, and that AKR1C3 expression in target cells is a pre-receptor mechanism for attenuating DOC action. This 20α-HSD inactivation pathway provides an explanation for why DOC is a less potent mineralocorticoid than aldosterone.

In order for the renin–angiotensin–aldosterone axis to regulate salt and water balance, the MR must be protected from activation by other, more abundant steroids with comparable affinities for MR. The best-studied example is cortisol, a high-affinity ligand for MR that circulates at concentrations 1000-fold higher than aldosterone. Mineralocorticoid target tissues express the 11β-HSD type 2 enzyme, which protects the MR by converting cortisol to the inactive steroid cortisone. Loss of 11β-HSD type 2 activity cause apparent mineralocorticoid excess syndrome, characterized by hypertension that is responsive to suppression of cortisol synthesis. Severe endogenous cortisol excess (Cushing's disease) also causes hypertension by overwhelming the capacity of 11β-HSD type 2 to catabolize this potent MR agonist (White et al., 1997). Thus, pre-receptor regulation of cortisol action on MR is an important clinical phenomenon.

Here, we found that AKR1C3 efficiently utilizes DOC as substrate and that the product of the reaction is 20α-hydroxy-DOC. In cell-based MR transactivation assays, co-expression of AKR1C3 increases the EC_{50} for DOC 1000-fold, and 20α-hydroxy-DOC does not activate MR. Furthermore, AKR1C3 does not utilize cortisol as substrate, underscoring that inactivation of DOC by AKR1C3 and of cortisol by 11β-HSD type 2 in mineralocorticoid target tissues are two distinct mechanisms that limit MR activation by steroids other than aldosterone. It should be mentioned that other metabolic pathways that render DOC less active at the MR may exist.

In subjects with genetic deficiencies of either 11β-hydroxylase (White et al., 1994) or 17α-hydroxylase (Auchus, 2001), elevated ACTH production drives adrenal hyperplasia and excess steroid synthesis. Consequently, large amounts of steroid precursors, particularly DOC, are secreted into the circulation, and DOC concentrations rise to 1–20 nM, which is 10–100-times higher than normal. While most of these subjects develop hypertension, others have normal blood pressure, and the magnitude of plasma DOC elevations do not appear to correlate with the development of hypertension. One explanation for the different responses might be inter-individual differences in local DOC metabolism mediated by AKR1C3 within mineralocorticoid target tissues.

Analogously, approximately 4% of women develop hypertension during pregnancy, particularly in the third trimester, when increased placental progesterone production drives plasma DOC concentrations up to 1.5–10 nM, comparable to concentrations found in 17α- and 11β-hydroxylase deficiencies. During pregnancy, plasma-borne progesterone is constitutively 21-hydroxylated to DOC in extra-adrenal tissues by enzymes other than the steroid 21-hydroxylase CYP21, and hence DOC production is independent of ACTH and angiotensin II regulation. It is known that DOC administration to normal volunteers can cause hypertension when concentrations exceed 3 nM (Biglieri et al., 1989a), but it is not known why only

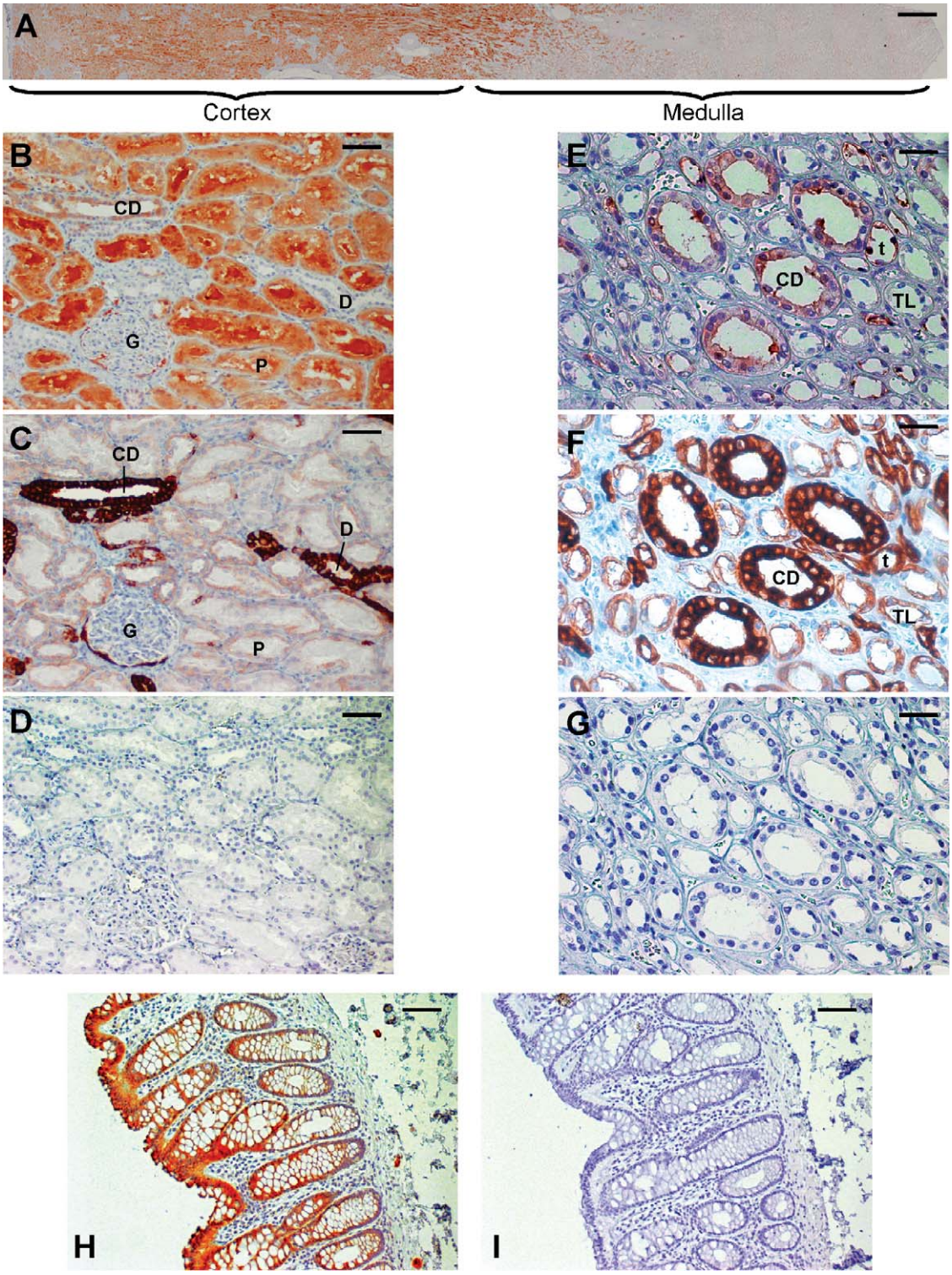

Fig. 4. Immunohistochemical localization of AKR1C3 in sections of human kidney and colon. (A, B and E) Positive (red) staining for AKR1C3 is seen in the epithelial cells of proximal convoluted tubule (P), collecting ducts (CD), and thin limbs of Henle (t) of the kidney. No immunostaining is observed in distal convoluted tubules (D), glomeruli (G) and thick limbs of Henle (TL). (C and F) Positive staining for low-molecular-weight cytokeratin (AE1/AE3) is seen in distal convoluted tubules, cortical and medullary collecting ducts, and thin limbs of Henle. (D and G) No staining is observed when kidney sections are incubated with an irrelevant antibody. (H) Positive staining is seen in the columnar epithelium lining the colon. (I) No staining is observed when colon sections are incubated with an irrelevant antibody. The blue color is hematoxylin counterstaining. Bars = 750 μm (A); 60 μm (B–D, H, I); 30 μm (E–G).

a minority of pregnant women develop hypertension. The variable response to DOC is consistent with our hypothesis that DOC is inactivated in mineralocorticoid target cells and that the ability to locally metabolize DOC varies amongst individuals.

The cell type-specific expression of AKR1C3 in the human kidney corresponds to the distribution expected for an enzyme that protects the kidney from a circulating mineralocorticoid. Mineralocorticoid action (reabsorption of sodium and water) occurs only in the epithelia of the distal convoluted tubules and collecting ducts, and AKR1C3 immunoreactivity is found abundantly in the water-absorptive principal cells of the collecting ducts. Hence, AKR1C3 is expressed in the same cell types that are known to express the MR and 11β-HSD type 2. In addition, AKR1C3 immunoreactivity is also found in epithelia of proximal tubules, which may relate to the other substrates and activities of this enzyme. AKR1C3 also metabolizes prostaglandin D_2 to 9α,11β-prostaglandin F_2 (Suzuki-Yamamoto et al., 1999), which defends the proximal nephron against the vasoconstrictive actions of mast cell prostanoids. AKR1C3 is expressed in various tissues and probably serves several physiological roles, including the metabolism of androstenedione to testosterone outside the adrenals and gonads. The immunolocalization of AKR1C3, its ability to utilize DOC as substrate five times more efficiently that androstenedione, and the poor metabolism of DOC by other human AKR1C enzymes, suggest that the 20α-HSD activity with DOC described here is a physiologically significant, rather than gratuitous activity of AKR1C3.

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