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The Human 11β-Hydroxysteroid Dehydrogenase Type II Enzyme: Comparisons with Other Species and Localization to the Distal Nephron

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Effective glucocorticoid inactivation is currently thought to be an indispensable feature of mineralocorticoid target cells. The enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) inactivates glucocorticoids and prevents them from binding to the non-selective mineralocorticoid receptor. In the kidney it is the NAD dependent high affinity isoform (11β-HSD2) which is thought to endow specificity on the receptor. The recent cloning of the human, sheep and rabbit 11β-HSD2 enzymes permits a comparison of the enzyme from the three species. Human and rabbit enzymes are 87% identical and of similar length, while the human and sheep enzymes have only 75% identity. The last 12 residues in all three species were found to be highly divergent, but most of the ovine dishomology can be accounted for by the deletion of a single nucleotide toward the C-terminus of the protein resulting in a shift in reading frame generating a protein 27 residues longer than the human isoform. Numerous other deletions were also observed in this region of the sheep cDNA sequence. Furthermore, the rabbit cDNA also displayed a large degree of dishomology with the human sequence a short distance downstream from the termination codon. Conserved overlapping cytoplasmic translocation signals were observed in all three species, suggesting a topology whereby the enzyme is anchored into the endoplasmic reticulum by multiple hydrophobic regions in the N-terminus and the bulk of the 11β-HSD2 peptide is sited in the cytoplasm. A polyclonal antibody generated against the C-terminus of human 11β-HSD2 was used to localize the enzyme within the kidney. A high level of immunoreactivity was observed in distal tubules and collecting ducts, localizing the enzyme to the same part of the nephron as the mineralocorticoid receptor. Moderate levels of staining were also seen in vascular smooth muscle cells. These results support the notion that 11β-HSD2 is an autocrine protector of the mineralocorticoid receptor and that it plays an important role in cardiovascular homeostatic mechanisms.

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INTRODUCTION

The study of the regulation of active hormone concentrations at the level of the target cell has recently become an important subject. In the kidney glucocorticoids are inactivated [1, 2] by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) allowing aldosterone to occupy the non-selective mineralocorticoid receptor [3]. A low affinity isoform of the enzyme (11β-HSD1) exists in the liver where it performs the reverse reaction, producing active glucocorticoid by converting cortisone to cortisol. In the distal tubule of the kidney, however, a high affinity unidirectional 11β-HSD isoform (11β-HSD2) converts glucocorticoids to their inactive 11-keto metabolites [4]. Inhibition of 11β-HSD activity by licorice leads to sodium retention, hypokalemia and elevated blood pressure [5], symptoms similar to those observed in the congenital syndrome of apparent mineralocorticoid excess (AME) where patients also display low cortisone to cortisol

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ratios and reduced 5β-reductase metabolites in the urine [6]. The parallel modulation of these two enzymes has led some workers to suggest that a common circulating factor may be responsible [7]. However, recent clinical data has failed to show any correlation between inhibitors extracted from urine, and mineralocorticoid receptor activation and 11β-HSD activity [8].

Numerous studies have provided evidence for a high affinity NAD-dependent 11β-HSD enzyme distinct from the NADP dependent 11β-HSD1 [9, 10–13]. An early study showed that the rat kidney contained an NAD-dependent activity localized to the distal nephron [9], clearly distinct from the medullay localization of 11β-HSD1. In retrospect, the rat kidney proved to be a complex tissue to study with both isoforms of the enzyme present in high amounts, while human kidney appears not to contain appreciable amounts of the 11β-HSD1 isoform [14]. While the role of 11β-HSD1 in the rat medulla remains unknown it is possible that, consistent with transfection studies with the cloned cDNA [15], this enzyme serves as an oxidoreductase in the proximal tubule.

The human 11β-HSD2 enzyme was recently cloned from a kidney expression library [16]. Characterization of the cloned species showed the enzyme to be NAD-dependent, have a high affinity for glucocorticoids, to be exclusively dehydrogenase in directionality and inhibitable by glycyrrhetinic acid, the active component of licorice. Northern blot analysis showed the 11β-HSD2 gene to be highly expressed in kidney and placenta, with undetectable levels in the liver. These results are in excellent agreement with previous *in vitro* studies, confirming the authenticity of the cloned enzyme [9, 11–13]. The 11β-HSD1 and 11β-HSD2 enzymes belong to the short-chain alcohol dehydrogenase superfamily [17, 18] but there is only 14% identity between the two isoforms. Instead, 11β-HSD2 is almost closely related to 17β-HSD2 [19] with which it is 34% identical. Ovine and rabbit 11β-HSD2 enzymes [20, 21] have also been cloned recently and this study will compare all three species.

Further work from our laboratory has focused on the localization of the enzyme within various tissues. We have recently raised a polyclonal antibody to human 11β-HSD2 and performed immunohistochemicial studies in the kidney and placenta [22]. The present report also includes additional localization studies in the kidney.

MATERIALS AND METHODS

Cloning strategy

Full details of the cloning strategy have been presented elsewhere [16]. Briefly, a human kidney cDNA library in pcDNA1 was transfected into CHOP [23] cells. Three days later radioactive corticosterone was added to the culture medium, incubated overnight and the medium assayed for the presence of steroid metabolites by thin layer chromatography. Positive pools were identified and a single clone isolated by sibling selection.

Immunohistochemistry

A peptide corresponding to the last 15 residues of human 11β-HSD2 was synthesized. Generation of the antiserum, immunopurification of the antibody and immunohistochemistry were performed as previously described [22].

RESULTS

The human 11β-HSD2 cDNA clone isolated by expression screening is 1872 bases in length and contains an open reading frame of 405 amino acids (Fig. 1). Two features of the cDNA sequence are immediately apparent. First, the 5′ untranslated region is highly GC rich, suggesting the possibility of significant mRNA secondary structure. However, computer analysis for secondary structure revealed only one pair of seven nucleotide elements which may form a significant stem in the classical stem and loop structure. Secondly, nucleotides surrounding the initiator codon (GGCCGCCATGG) conform well with the Kozak [24] consensus sequence (GCCAGCCAUGG).

A comparison of the human, rabbit and sheep 11β-HSD2 enzymes is shown in Fig. 2. The respective lengths of these proteins are 405, 406 and 427 residues. The extra length of the ovine protein is due to frame shifts resulting from the deletions of nucleotides starting at a position corresponding to 1181 nucleotides in the human cDNA. There is an 87% identity between human and rabbit sequences and a 75% identity between human and sheep. The highest degree of sequence dissimilarity is seen in the C-terminal region, in particular the last 12 residues in each sequence are highly divergent. However, despite frame shifts in the ovine sequence comparison of the three proteins by the Clustal4 algorithm [25] shows an interesting alignment of Proline residues in this region. Five positions contain conserved prolines in all three species over a stretch of 25 amino acids. Potential N-linked glycosylation sites are not conserved and occur at residues 394–396 in the human, at 272–274 in the rabbit, and at 96–98 and 245–247 in the sheep. In the human enzyme there are cytoplasmic translocation signals, Arg–Arg, at positions 335, 336 and 359, 360. The motif at position 335 is conserved in the rabbit while the one at position 359 is conserved in the sheep sequence.

We further explored the divergence of the ovine and human C-terminal regions by aligning the corresponding cDNA sequences (Fig. 3). The ovine sequence was found to contain numerous deletions and one insertion of three nucleotides when compared to the human cDNA. Overall the region 1164–1330 nucleotides in the sheep contains the highest degree of divergence with the human cDNA while sequences immediately 5′ and

3′ remain highly conserved. The overall degree of conservation suggests that the differences are not due to splicing events. Comparison of the rabbit and human sequences over the same region showed a high degree of dishomology beginning at nucleotide 1380 in the human sequence, well beyond the termination codons in both species (results not shown). The high degree of dissimilarity here may be due to either a gene insertion event or to RNA splicing.

As part of on-going studies to characterize the 11β-HSD2 enzyme we have raised a polyclonal antibody (HUH23) to a synthetic peptide deduced from the human cDNA. Figure 4 shows a low power view of a section of human kidney cortex stained with the immunopurified antiserum. Intense staining was observed in distal convoluted tubules and collecting ducts. Occasionally populations of distal tubules showed only moderate staining.

In Fig. 5 is shown a high power magnification of a section of human kidney cortex. Again intense staining was observed in distal convoluted tubules while smooth muscle cells of an artery showed moderate reactivity.

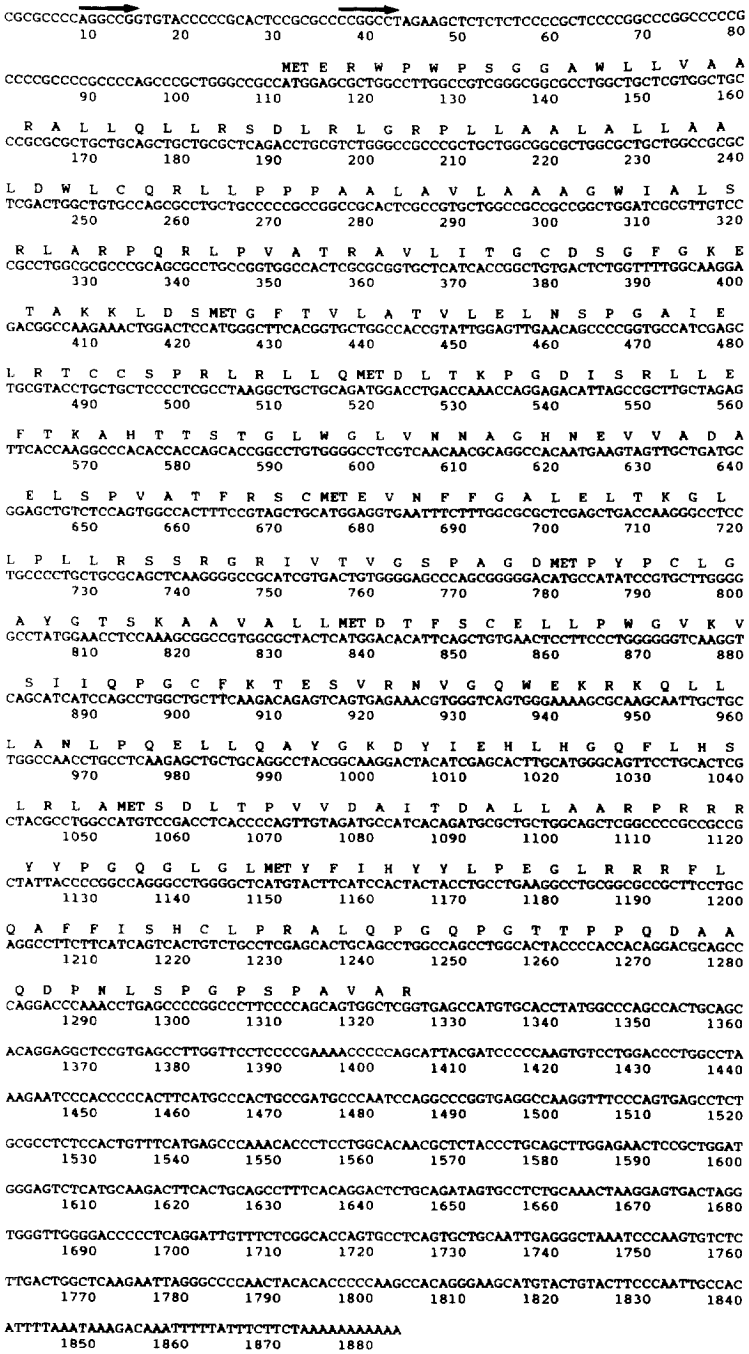

Fig. 1. Complete cDNA and deduced protein sequences of the human 11β-HSD2 enzyme. Arrows indicate the position and extent of secondary structure formation in the 5′-untranslated region.

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HUM-HSD2        MERWPWPSGGAWLLVAARALLQLLRSDLRLGRPLLAALALLAALDWLCQRLLPPPAALAV
RAB-HSD2        MERWPWPSGGAWLLVAARALIQLLRADLRLGRPLLAALALLAALDWLCQSLLPPSAALAV
SHE-HSD2        MESWPWPSGGAWLLVAARALLQLLRADLRLGRPLLAALALLAALDWLCQRLLPPLAALAV
                **.*****************.****.************************.**** *****

HUM-HSD2        LAAAGWIALSRLARPQRLPVATRAVLITGCDSGFGKETAKKLDSMGFTVLATVLELNSPG
RAB-HSD2        LAAAGWIALSRLARPQRLPVATRAVLITGCDSGFGKETAKKLDAMGFTVLATVLEMNGPG
SHE-HSD2        LAATGWIVLSRLARPQPLPVATRAVLITGCDSGFGNATAKKLDAMGFTVLATVLDLNSPG
                ***.***.********.******************..******.**********..*.**

HUM-HSD2        AIELRTCCSPRLRLLQMDLTKPGDISRLLEFTKAHTTSTGLWGLVNNAGHNEVVADAELS
RAB-HSD2        ALELRACCSPRLKLLQMDLTKPADISRALEFTKAHTTSTGLWGLVNNAGHNDVVADVELS
SHE-HSD2        ALELRACCSSRLQLLQMDLTKPADISRVLEFTKVHTASTGLWGLVNNAGQNIFVADAELC
                *.***.***.**.**********.**** *****.**.************.*  ***.**.

HUM-HSD2        PVATFRSCMEVNFFGALELTKGLLPLLRSSRGRIVTVGSPAGDMPYPCLGAYGTSKAAVA
RAB-HSD2        PVATFRNCMEVNFFGALELTKGLLPLLHHSRGRIVTLGSPAGEMPYPCLAAYGTSKAAMA
SHE-HSD2        PVATFRTCMEVNFFGALEMTKGLLPLLRRSSGRIVTVSSPAGDMPFPCLAAYGTSKAALA
                ******.***********.********. *.*****..****.**.***.********.*

HUM-HSD2        LLMDTFSCELLPWGVKVSIIQPGCFKTESVRNVGQWEKRKQLLLANLPQELLQAYGKDYI
RAB-HSD2        LLMDAFSCELLPWGVKVSVIQPGCFKTESVSNVSHWEQRKQLLLANLPGELRQAYGEDYI
SHE-HSD2        LLMGNFSCELLPWGVKVSIILPACFKTESVKDVHQWEERKQQLLATLPQELLQAYGEDYI
                ***..*************.* *.*******..* .**.*** ***.** ** ****.***

HUM-HSD2        EHLHGQFLHSLRLAMSDLTPVVDAITDALLAARPRRRYYPGQGLGLMYFIHYYLPEG-LR
RAB-HSD2        EHLHREFLHSLRLALPDLSPVVDAITDALLAARPRPRYYPGRGLGLMYFIHYYLPEG-LR
SHE-HSD2        EHLNGQFLHSLSQALPDLSPVVDAITDALLAAQPRRRYYPGHGLGLIYFIHYYLPEGCGR
                ***. .*****. *..**.*************.**.*****.****.**********  *

HUM-HSD2        RRFLQAFFISHC-----LP--RALQPGQ-PGTTPPQDAAQD-PNLSPGPSPAVAR-----
RAB-HSD2        RRFLQSFFIIPC-----LP--RALRPGQ-PGATPAPDTAQDNPNPNPDPSLVGAR-----
SHE-HSD2        VSCSPSSSVPMCQEHYRLPAWPYLCPGHSPGPRPQTGPLSHCPVSRAHVEQLQQRRFLVP
                 .  ..  .  *     **  . * **. **. *. ..  . *  ..  .   .*

HUM-HSD2        -------
RAB-HSD2        -------
SHE-HSD2        LLFFQVF
```

Fig. 2. Multiple alignment of the human (HUM-HSD2), rabbit (RAB-HSD2) and sheep (SHE-HSD2) 11β-HSD2 protein sequences [16, 21, 20]. Asterisks denote positions of identity in all three enzymes and dots indicate conservative changes. Alignment was performed by the CLUSTAL4 algorithm [25].

Furthermore, HUH23 also showed some staining of visceral epithelial cells of the outer capillary loop of a glomerulus. Figure 6 shows a high powered view of a renal artery stained with HUH23 more clearly illustrating staining of all smooth muscle cells surrounding the blood vessel. We have previously validated the specificity of HUH23 immunoreactivity in the kidney by preincubation of the antibody with the immunizing peptide [22].

DISCUSSION

The recent expression cloning of the 11β-HSD2 enzyme from three species has permitted a comparison

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HUM-HSD2    1209 TTCATCAGTCACTGTCTGCCTCGAGCACTGCAGCCTGGCCAGCCTGGCACTACCCCACCA
SHE-HSD2    1097 TTCATCAGTCCCTATGTGCCAAGAGCACTACAGGCTG-CCAGCCTGGCCTTACCTCTGCC
                 ********** ** * ****  ******* *** *** **********  **** *  *

HUM-HSD2    1269 CAGGACGCAGCCCAGGACCCAAACCTGAGCCCCGGCCCTTCCCCAGCAGTGGCTCGGTGA
SHE-HSD2    1156 CGGGACATA-CCCAGGACCAAGGCCCCAGACTGGACCCCTCTCC--CACTG-CCCAGTGA
                 * ****  * ********* *  **  ** *  * *** ** **  ** ** * * ****

HUM-HSD2    1329 GCCATGTGCACCTATGGCCCAGCCACTGCAGCACAGGAGGCTCCGTGAGCCTTGGTTCCT
SHE-HSD2    1212 GC-AGG-GCACATGTAG---AGCAGCTCCAGCAGAGGAGGTTCCTTGTGCCCTTGCTCTT
                 ** * * **** * * *   ***  ** ***** ****** *** ** *** * * ** *

HUM-HSD2    1389 CCCCGAAAACCCCCAGCATTACGATCCCCCAAGT--GTCCTGGACCCTGGCCTAAAGAAT
SHE-HSD2    1267 CTTCCA--------GGTATTCTGACCCCCAAGGTCTGCCCTAGAGCCTGGCCCAAAGGAC
                 *  * *          * ***  ** **** * **  * *** ** ******* **** *

HUM-HSD2    1447 CCCACCCCCACTTCATGCCCACTGCCGATGCC-CAATCCAGGCCCGGTGAGGCCAAGGTT
SHE-HSD2    1319 CCCAACCC------ATGC--ACTGCCGATGCCACAGGCCAGGCCTGGTGAGGTGAAGGCT
                 **** ***       ****  ************ **  ******* *******  **** *
```

Fig. 3. Alignment of human (HUM-HSD2) and sheep (SHE-HSD2) cDNA sequences surrounding termination codons. Stop codons are boxed. Alignment was performed by the CLUSTAL4 algorithm [25].

of protein sequences. Human and rabbit sequences are of similar length and most highly conserved save for 12 residues at the C-terminus. The sheep sequence differs from both these proteins in that it is significantly longer due to single nucleotide deletions which result in frame shifts in the coding sequence. These deletions may have arisen as part of the same evolutionary process which gave rise to the more extensive divergence further downstream. The frame shifts in the sheep cDNA sequence produce a non-conserved peptide of 69 amino acids starting from residue 358. However, despite the changes in reading frames comparison of 11β-HSD2 from all three species showed an interesting alignment of prolines in the C-terminal region. Proline residues are often found at β-turns, suggesting that 11β-HSD2 is extensively folded in this region.

Little is currently known about the topology of the microsomal 11β-HSD2 enzyme. Recent studies on the 11β-HSD1 isoform have revealed a single transmembrane segment at the N-terminus, with the bulk of the polypeptide chain projecting into the lumen of endoplasmic reticulum [26]. Hydropathy analysis of the 11β-HSD2 protein sequence suggests that the enzyme is anchored by three transmembrane segments at the N-terminus [27]. The identification of conserved cytoplasmic translocation signals suggests that 11β-HSD2 projects into the cytoplasmic side of the endoplasmic reticulum. The two isoforms of 11β-HSD thus appear to be localized within different intracellular compartments.

Previous studies on the 11β-HSD1 enzyme in the rat showed that it was localized in proximal tubules [28] while the mineralocorticoid receptor was sited in distal tubular elements [29], leading workers to suggest a paracrine mode of protection. However, other studies have shown that 11β-HSD1 is unlikely to protect the receptor on the grounds of its proximal tubular localization, low affinity for glucocorticoids and the observation that it acts as an oxidoreductase in mineralocorticoid responsive toad bladder cells [15]. In the present study we used a highly specific antibody [22] to demonstrate that 11β-HSD2 colocalizes with the mineralocorticoid receptor in distal tubules and collecting ducts; these results suggest a mechanism consisting of autocrine protection of the receptor.

Occasionally distal tubules showed only moderate staining. This suggests that the concentration of the enzyme varies between populations of tubules. This variation may be a reflection of a normally low expression in some distal tubules or it may be due to the

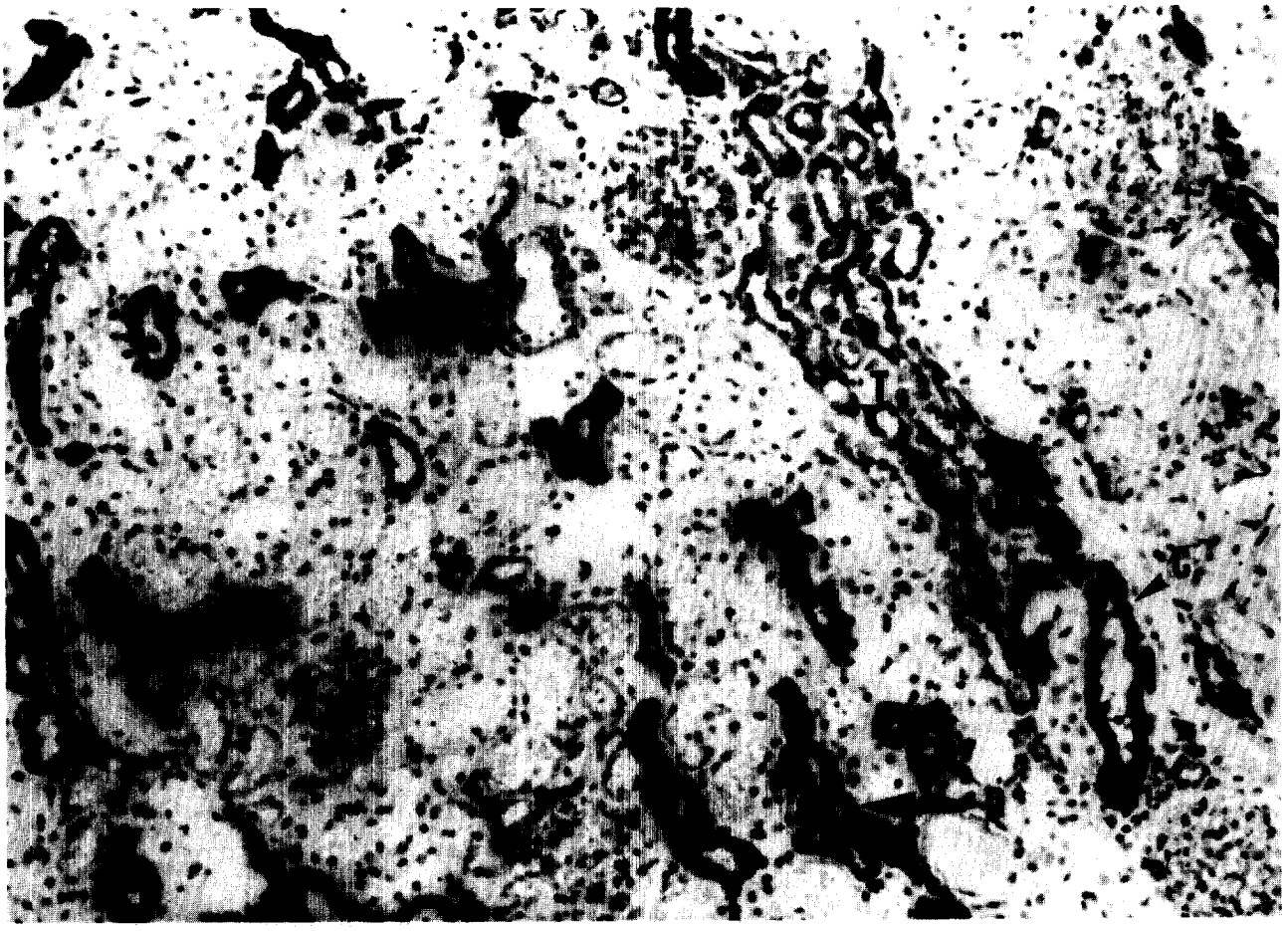

Fig. 4. Immunohistochemical localization of 11β-HSD2 in a section of human kidney cortex using the HUH23 antibody. D, distal convoluted tubule; C, collecting duct; G, glomerulus; T, distal tubules (magnification ×124).

modulation of enzyme expression by the surrounding milieu. The role of the low amounts of 11β-HSD2 staining in the visceral epithelial cells of the outer capillary loop of the glomerulus must remain speculative, but it is interesting to note that the mesangial cells do not appear to be immunoreactive with the HUH23 antiserum. Studies on the 11β-HSD activity in isolated tubules and glomeruli from the human kidney have also shown considerable variability [30].

Smooth muscle cells of the vasculature also showed moderate staining with the HUH23 antibody. The rat mesenteric vascular arcade has previously been shown to be highly aldosterone-selective *in vivo*, and *in vitro* shows considerable levels of 11β-HSD activity [31]. These observations are consistent with a more recent study suggesting that glucocorticoids and mineralocorticoids modulate vascular smooth muscle cell contractility by affecting the Na^+ and/or Ca^{2+} transport systems in these cells [32]. *In vivo* studies in human subjects also support an important role for 11β-HSD2 activity in the vasculature [33]. By including AME patients in their study, and the use of enzyme inhibitors, these authors concluded that 11β-HSD modulates the access of cortisol to vascular receptors and thereby influences vascular sensitivity to noradrenaline, confirming previous results obtained in a study on cortisol induced hypertension in man [34]. In another study, 11β-HSD activity was found to be defective in a proportion of patients with essential hypertension. The defect was not associated with mineralocorticoid excess and it was suggested that it may cause hypertension by increasing exposure of vascular steroid receptors to cortisol [35]. Given the present identification of 11β-HSD2 in both the distal tubule and vascular smooth muscle cells the above observation in hypertensive patients would suggest that the enzyme may be differentially regulated at the two sites.

The recent expression cloning of 11β-HSD2 from the kidneys of three species and the localization of the enzyme to the distal nephron and vasculature has implications for the type I form of AME. The fact that the same protein was cloned in all three instances suggests that it is the most abundant renal 11β-HSD in these species. The identification of 11β-HSD2 in mineralocorticoid target cells and vascular smooth muscle cells is consistent with a defect in this enzyme leading to sodium retention and the development of hypertension in these patients. What remains to be explained is why 5β-reductase activities are also compromised in this syndrome and in some hypertensives

Fig. 5. HUH23 localization of 11β-HSD2 in human kidney cortex. A, artery; D, distal convoluted tubule; E, visceral epithelial cells; G, glomerulus; V, vascular smooth muscle cells (magnification ×310).

Fig. 6. Staining of an interlobular renal artery with the HUH23 antibody. A, artery; V, vascular smooth muscle cells (magnification ×310).

[36]. One plausible explanation may be that elevated levels of cortisol down-regulate expression of the 5β-reductase gene. In the type II form of AME, 11β-HSD activity appears normal, but decreased 5β-reductase activity results in a lower clearance of cortisol producing symptoms identical to those seen in type I patients [6]. This implies that mutations in the 5β-reductase gene may result in the AME type II syndrome and suggests that 5β-reduction is also part of the mechanism protecting the mineralocorticoid receptor from occupation by glucocorticoids. Studies are currently underway in our laboratory to identify mutations in the 11β-HSD2 gene in AME patients and to determine whether glucocorticoids modulate 5β-reductase gene expression.

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