

Localization of the polyol pathway in the human kidney

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Summary. Sorbitol plays an important role in the osmotic regulation of the mammalian kidney. Sorbitol synthesis is regulated by the enzyme aldose reductase (AR) and its degradation to fructose is catalyzed by the enzyme sorbitol dehydrogenase (SDH). Various data exist on the polyol pathway on the rat kidney, but little is known about the distribution of the polyol pathway enzymes in the human kidney. Determination of enzyme activities and a semiquantitative determination of mRNA expression, immunohistochemistry and in-situ hybridisation in healthy human kidney tissue was carried out. The enzyme activity of AR showed a fourfold increase from cortex to papilla, while SDH-activity dropped from cortex to papilla by a factor of four. Corresponding data was obtained at the mRNA level from the semiquantitative polymerase chain reaction (PCR). Additional differentiation at the cellular level reveals both enzymes in cells of the proximal and distal tubules, thick ascending loop, thin loop and collecting duct. Studies of enzyme activity and expression by immunohistochemistry, PCR and in-situ hybridization presented corresponding results with respect to the localization of the enzymes, which match the experimental data obtained from rats very well. Thus, the established rat model might well represent the situation in the human kidney, too.

Key words: Polyol pathway, Aldose reductase, Sorbitol dehydrogenase, Human kidney

Introduction

The polyol (or sorbitol) pathway consists of two enzymes, aldose reductase (AR) and sorbitol dehydrogenase (SDH). AR (EC 1.1.1.21) catalyzes the

reduction of glucose to sorbitol by NADPH and SDH (EC 1.1.1.21) converts sorbitol to fructose dependent on NAD⁺ (Jeffery and Joernvall, 1983; Kador et al., 1985; Yabe-Nishimura, 1998).

The physiological role of aldose reductase in the kidney, the rate limiting enzyme of the polyol pathway, is the intracellular accumulation of the osmolyte sorbitol as an important mechanism in the long-term adaptation of the cell to a rise in the extracellular osmolarity (Grunewald and Kinne, 1989). High extracellular osmolarities induce renal sorbitol synthesis by increasing AR activity and vice versa (Garcia-Perez et al., 1989; Grunewald and Kinne, 1989).

However, the polyol pathway plays an important role in the pathogenesis of diabetic nephropathy (Dunlop, 2000; Forbes et al., 2007). Under normoglycaemic conditions, glucose utilization by the polyol pathway is limited in tissues taking up glucose independent of insulin, such as in the kidney (Tomlinson, 1993). This is due to the relatively low intracellular glucose concentration and the low affinity of AR for glucose (Swidan and Montgomery, 1998). Only 3% of the glucose is utilized by AR (Morrison et al., 1970). Here, most of the cellular glucose is phosphorylated into glucose 6-phosphate by hexokinase and glucokinase (Philips and Steadman, 2003).

In the case of hyperglycaemia, however, when hexokinase and glucokinase appear to be saturated, the proportion of glucose utilized by the polyol pathway is about 30% of the total, but a corresponding rise of SDH does not occur, causing an intracellular rise of sorbitol concentration (Gonzales et al., 1984; Kador et al., 1985).

Diabetic nephropathy is the main reason for terminal renal insufficiency and for 30-40% of all kidney transplantations (Philips and Steadman, 2003). An important co-factor is the glucose-stimulated activation of the polyol pathway, which influences the sorbitol and myo-inositol pathway (Greene et al., 1987; Meyer et al., 2005). This activation of the polyol pathway produces several effects. The osmotic balance of the tissue is

disturbed by an accumulation of sorbitol, while consumption of NAD(P)H in these processes may alter the redox potential of the cells, making them less resistant to oxidative stress. Finally, sorbitol may be converted to fructose, which promotes nonenzymatic glycation of proteins (Lane, 2002).

To understand the effect of the polyol pathway, it is important to know something about the localization of the enzymes. Several investigations on the enzyme localization of AR and SDH and their expression in the rat have been carried out, but few data exist on the human kidney. Corder et al. (1977) were the first to localize AR and SDH in rat kidneys by immunohistochemistry and showed that the main localization of AR was in the inner medulla, whereas SDH was detected with an opposite distribution pattern along the cortico-papillary gradient. The aim of our investigations was to localize protein and mRNA of both enzymes in individual structures of the normal human kidney along the cortico-papillary gradient in order to compare this data with the results of the animal model. For the first time, we describe the localization of polyol pathway enzymes in the human kidney as a whole at the mRNA and protein levels.

Materials and methods

Tissue

The human kidney tissue was taken from tumor nephrectomies (Dept. of Urology, University Hospital Goettingen). From the tumor-free region, a tissue piece, macroscopically containing all parts from cortex to papilla, was removed within 2 hours after kidney resection. Immediately after preparation, the tissue was either frozen or formalin-fixed or used for determination of enzyme activity. Microscopically, the tissue did not show any tumorous areas. The various nephron segments were identified by their difference in length, cellular morphology and distribution in the renal sections (Pfaller, 1982; Kriz and Bankir, 1988).

All patients had normal creatinine and blood glucose levels before kidney resection. 4 of 20 patients showed high blood-pressure.

On the basis of the limited amount of tissue, it was not possible to perform all investigations on every kidney.

Determination of aldose reductase activity (AR)

The determination of AR activity is based on the reduction of DL-glyceraldehyde to glycerol by NADPH in the presence of AR. The assay contained 50 mmol/l phosphate buffer (pH 6.2), 400 mmol Li₂SO₄, 10 mmol/l DL-glyceraldehyde and 0.1 mmol/l NADPH (Grunewald et al., 2001a; Sato and Kador, 1993). The decrease in absorbance was monitored at 340 nm at 37°C for 5 minutes in the presence or absence of DL-glyceraldehyde to correct for unspecific NADPH

dehydrogenase activity. Li₂SO₄ stimulates AR and not aldehyde reductase (Das and Srivastava, 1985).

Determination of sorbitol dehydrogenase activity (SDH)

This assay is based on the reduction of fructose to sorbitol by NADH in the presence of SDH. It was performed in 106 mmol/l triethanolamine buffer pH 7.4, containing 1.2 mmol/l NADH and 1.19 mmol/l D-fructose. The decrease in absorbance at 340 nm was measured at 37°C for 5 minutes. Unspecific oxidation of NADH was corrected by parallel monitoring of the sample in the absence of fructose (Gerlach, 1983).

PAP immunodetection

Here, we used 5 µm thick, paraffin-embedded tissue sections for the peroxidase-antiperoxidase (PAP) technique (Sternberger et al., 1970). First, the tissue sections were deparaffinized in xylol and rehydrated with ethanol/H₂O. All tissue sections were consecutively incubated with polyclonal antibodies against AR (provided by Prof. Dr. WG Guder, Munich) (Moeckel et al., 1995; Grunewald et al., 2001a) or SDH (Dako, Hamburg, Germany, antibodies were verified by western blots on rat and human kidney cell proteins) in a dilution of 1:400 (one hour, 22°C), swine anti-rabbit bridge antibody (Dako, Hamburg, Germany) (1:50, 30 minutes, 22°C) and a peroxidase anti-peroxidase complex (Dako, Hamburg, Germany) (1:150). The presence of AR or SDH was indicated by the precipitation of DAB (3,3'-diaminobenzidine tetrahydrochloride). To minimize the unspecific background, the endogenous peroxidase was blocked with a methanol/H₂O₂-mixture before antigen-antibody binding. In tests, the primary antiserum was omitted.

RNA-purification

Total RNA from the tissue was isolated by using a commercial silica-membrane based system following the manufacturer's protocol (RNeasy, Quiagen, Hilden, Germany). Tissue was homogenized (Ultra-Turrax, Janke and Kunkel, Staufen, Germany) after separating the different kidney sections macroscopically. RNA yield and purity were estimated spectrophotometrically by determining absorbance at 260 and 280 nm. The integrity of the RNA was checked on a non-denaturing agarose gel.

Reverse transcription polymerase chain reaction (RT-PCR)

1 µg of total-RNA from the tissue was reversely transcribed by the Superscript™ II reverse transcriptase at 42°C for 50 minutes. A 5 µl aliquot of the RT was used for the PCR reaction. The following specific primers were added were added to the assay: AR: forward, 5'-AAG CGT GAG GAG CTC TTC ATC G-

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3'; reverse, 5'-TTA TTG TGC TTG GCT GCG ATC G-3', product length 523 bp; SDH: forward, 5'-CAC TAC TGG GAG TAT GGT CG-3'; reverse 5'-GAC CTT CTA CTT TCC TGG CG-3'; product length 567 bp; the housekeeping gene beta actin: forward, 5'-AGC CAT GTA CGT TGC TAT-3'; reverse, 5'TGC CAA TGG TGA TGA CCT-3'; product length 364 bp. Using Taq polymerase purchased from Qiagen (Hilden, Germany), the PCR reaction was performed (1 minute, 94°C denaturing; 2 minutes, 55°C for AR or 51°C for SDH annealing; 3 minutes, 72°C elongation). The adequate number of PCR cycles to reach the log-phase amplification was determined in experiments with 25, 30 and 35 PCR cycles. The signal generated by the 30-cycle PCR was significantly lower than that generated by the 35-cycle PCR. This result demonstrated that at 30 cycle-PCR, the amplification of AR, SDH and beta actin was at log-phase and was adopted in this investigation. Following PCR, an aliquot was separated on a 1.8% agarose gel.

For further specification, PCR products were cut by specific restriction enzymes (PST1 (AR, SDH), EcoRI (SDH)). Furthermore, a sequencing of the PCR-product with the automatic sequencer ABI PRISM 310 (Perkin Elmer, Ueberlingen, Germany) was performed.

Additionally, a semiquantitative analysis of mRNA expression by using beta actin as external standard was carried out by densitometric analysis of the bands with the computer-based Molecular Analyst (Bio-Rad, Munich, Germany).

Synthesis of ribonucleotide probes

The synthesis of the ribonucleotide probes (AR, SDH) was carried out by in-vitro transcription of PCR products. The PCR was performed as described with specific primers, containing an additional binding sequence for a T7- (forward) and T3-polymerase (reverse).

The probes for the ribonuclease protection assay and the in-situ hybridisation were psoralen-biotin-labeled following the manufacturer's protocol (Nonisotopic Labeling Kit, Ambion, Austin, USA).

In-situ hybridization

The paraffin sections (5 µm) were deparaffinized in xylol and rehydrated in decreasing concentrations of ethanol followed by a pretreatment with proteinase K in 100 mM Tris-HCl, pH 8.0, and 50 mM EDTA at 37°C for 30 minutes. The sections were postfixed for 5 minutes at 4°C in 4% paraformaldehyde, 0.1 M phosphate buffer, pH 7.4, followed by an acetylation procedure for 2x5 minutes in 100 mM triethanolamine and 0.25% acetic anhydride. The tissue sections (2 min., 85°C) and probes (5 min, 55°C) were denatured and hybridized overnight at 48°C in a hybridization buffer (Amersham, Buckinghamshire, England) containing the biotin-labeled probes at a final concentration of 4 ng/µl.

After hybridization, the sections were immersed in 5% 20x standard saline citrate (SSC) and 1% SDS (10%) for 2x10 minutes. Finally, 20 µg/ml RNase A in NTE buffer were added and incubated for 30 minutes, followed by another immersion, as previously described for 2x10 minutes at 50°C. After being washed in Tris-HCl buffer, the sections were incubated in a blocking buffer (Roche, Mannheim, Germany) for 30 minutes. The detection was identically performed for all tissue sections with 4-nitroblue tetrazolium chloride (NBT) plus 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). Nuclei were stained briefly with Meyer's haemalaun (Merck, Darmstadt, Germany). Negative controls were performed with sense probes.

Statistical analysis

All results are expressed as mean +/- standard deviation (SD). Statistical significance was evaluated using analysis of variance (ANOVA). The difference between the kidney sections was tested with an analysis according to Tukey. Difference was considered statistically significant at $p < 0.05$.

Results

The enzyme activities of AR and SDH in the human kidney show diametric maximums (Fig. 1). One enzyme unit defined the amount of enzyme consuming or producing one micromole of NADH per minute. Thus, AR has the lowest activity in the cortex increasing towards the papilla (cortex 15.2 ± 6.0 U/g; outer medulla 29.6 ± 22.6 U/g; inner medulla 52.9 ± 25.4 U/g; papilla 61.6 ± 37.6 U/g; $n=10$) with a ratio of 1:4. SDH activity is highest in the cortex decreasing towards the papilla (cortex 106.0 ± 58.8 U/g; outer medulla 38.7 ± 33.1 U/g; inner medulla 29.7 ± 28.3 U/g; papilla 24.2 ± 18.7 U/g; $n=14$) with a ratio of 4:1. These results correspond to the

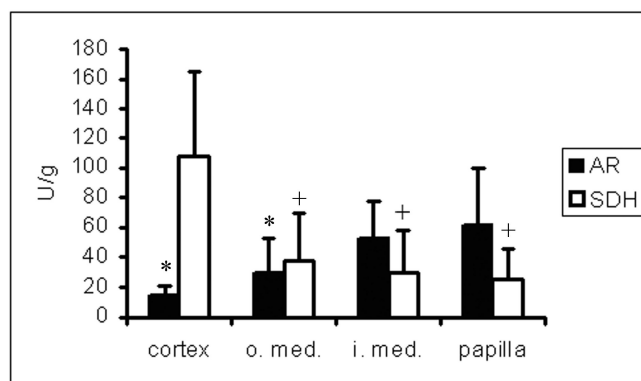


Fig. 1. Enzyme activities [U/g] of AR and SDH from cortex to papilla. AR activity is highest in papilla, dropping to cortex, whereas SDH activity is highest in cortex. Mean ± SD (AR: 10 experiments; SDH: 14 experiments) (* $p < 0.05$ versus papilla; + $p < 0.05$ versus cortex).

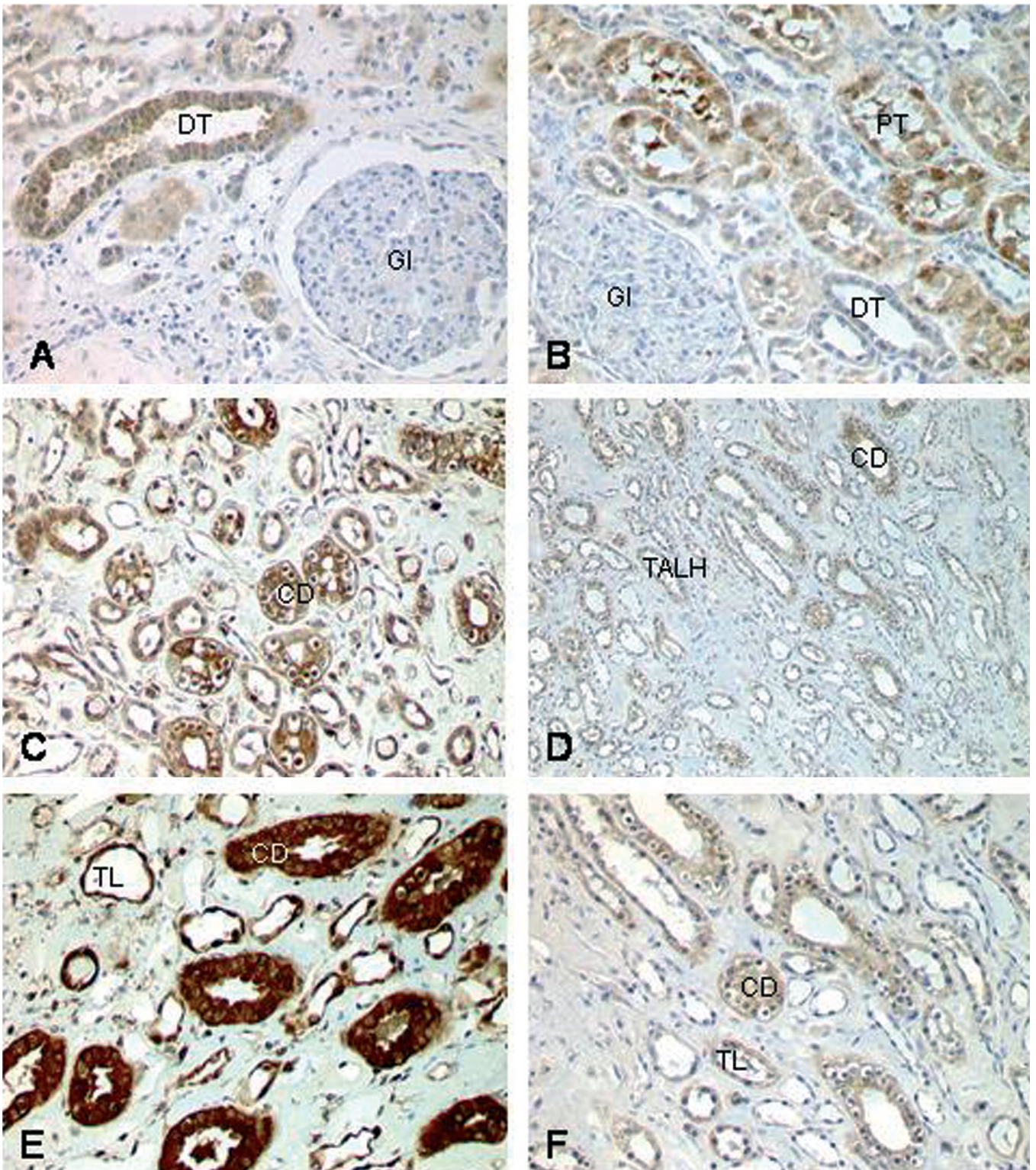


Fig. 2. Visualization of aldose reductase (AR) and sorbitol dehydrogenase (SDH) in renal cortex (**A**: AR, 200x; **B**: SDH, 200x), renal outer medulla (**C**: AR, 400x; **D**: SDH, 200x) and papilla (**E**: AR, 400x; **F**: SDH, 200x) by immunohistochemistry. Detection of both enzymes by specific antibodies was seen in cells of proximal tubule (PT), distal tubule (DT), thick ascending loop (TALH), thin loop (TL) and collecting ducts (CD), while no signals were detected in cells of glomerulus, interstitium and endothelium.

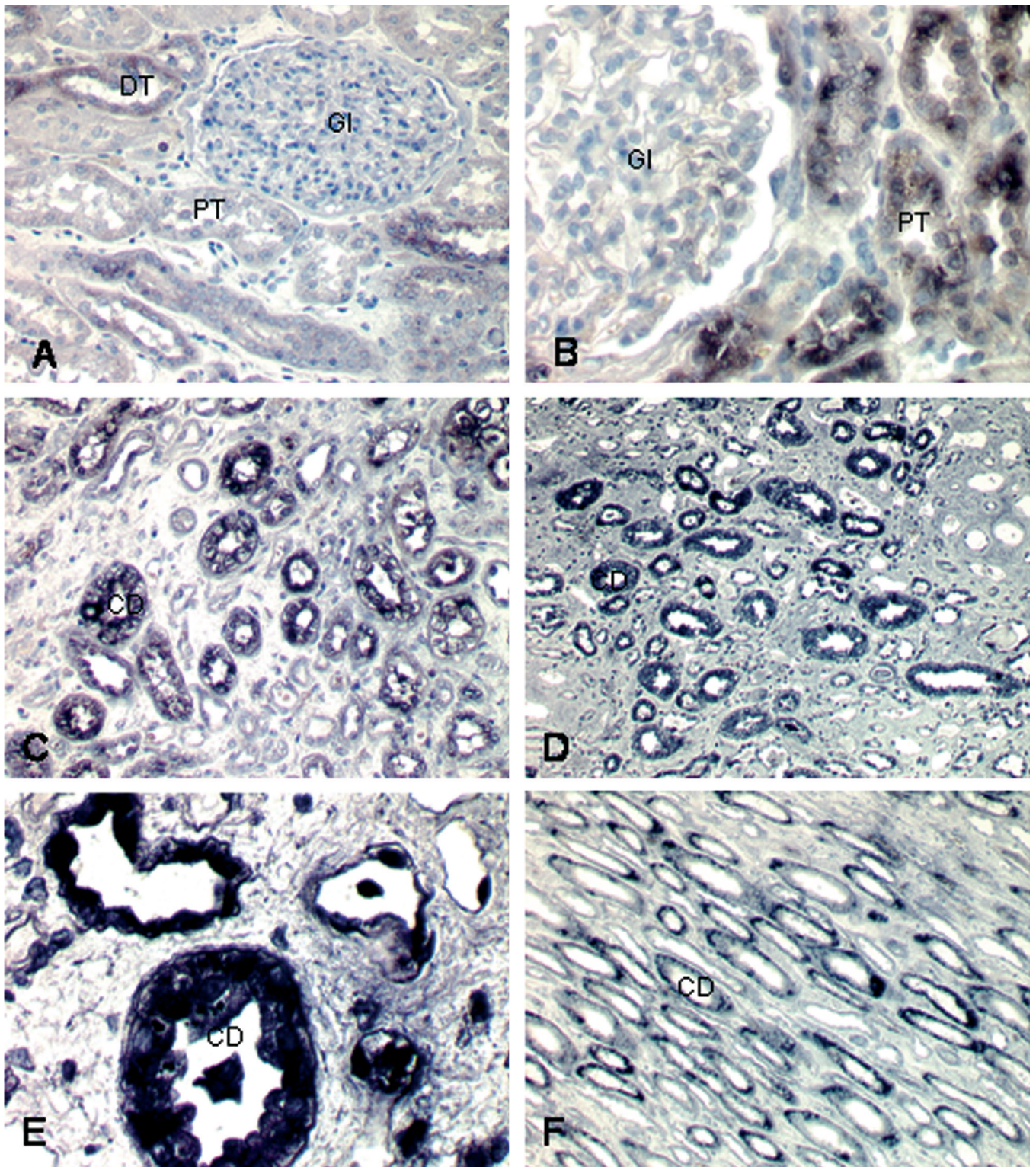


Fig. 3. Detection of aldose reductase (AR) and sorbitol dehydrogenase (SDH) in renal cortex (**A**: AR, 200x; **B**: SDH, 400x), renal outer medulla (**C**: AR, 200x; **D**: SDH, 100x) and papilla (**E**: AR, 400x; **F**: SDH, 200x) by *in-situ* hybridization. The *in-situ* hybridization revealed both mRNAs in cells of proximal tubule (PT), distal tubule (DT), thick ascending loop (TALH), thin loop (TL) and collecting ducts (CD), while no signals were detected in cells of glomerulus, interstitium and endothelium.

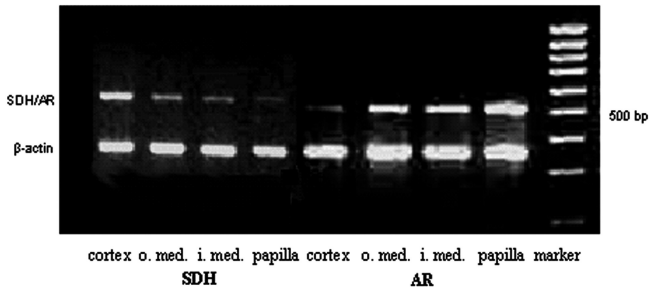


Fig. 4. Semiquantitative RT-PCR of aldose reductase (AR) and sorbitol dehydrogenase (SDH) mRNA with beta actin as standard (30 cycles). Highest SDH mRNA was seen in cortex dropping towards papilla, where AR mRNA expression is the weakest, rising towards papilla.

enzyme localization visualized by immunohistochemistry. Detection of the enzymes by specific antibodies was seen for AR (n=10) and SDH (n=11) in cells of the proximal tubule (PT), distal tubule (DT), thick ascending loop (TALH), thin loop (TL) and collecting ducts (CD), while no signals were detected in cells of the glomerulus, interstitium and endothelium. Here, AR showed an increasing amount of staining from cortex to papilla, with the highest intensity in collecting ducts and thick ascending loop. This rise in signal intensity was not seen for SDH, where the collecting ducts and proximal tubules showed the highest intensity (Fig. 2).

Furthermore, the in-situ hybridization experiments revealed the mRNA of AR (n=7) and SDH (n=7) in the same structures as the proteins shown by immunohistochemistry, again with an opposite gradient, as previously described (Fig. 3). The AR signal intensity was strongest in collecting ducts and thin loop, while the highest signal of SDH was detected in collecting ducts, proximal tubule and thick ascending loop. Analysis by RT-PCR presented a single band for AR and SDH each at the expected size (AR, 523 bp; SDH 567) (Fig. 4). For specification of the PCR products, the restriction digestion generated two fragments of the expected size (AR, PST1; SDH, PST1, EcoRI), while the sequencing of the PCR-products showed an identity corresponding to the sequences published.

AR- and SDH-mRNA-expression, each compared to beta actin, presented opposite gradients in the distribution, as previously described by enzyme activities. AR displayed the lowest mRNA expression in the cortex increasing towards the papilla, while SDH mRNA expression declined from cortex to papilla.

A correspondence between enzyme activity, protein localization and mRNA expression of both enzymes along the cortico-papillary gradient was observed.

Discussion

For the first time, we presented the localization of the polyol pathway enzymes (AR, SDH) throughout the

entire nephron of the normal human kidney using a combination of methods. Various investigators described the localization of these two key enzymes, AR and SDH, in human or rat kidneys as the most frequently used animal model. Several investigations were performed doing in vitro cell experimental techniques in single kidney cell types under special osmotic conditions. This fact is important to consider when comparing different results concerning single nephron segments or interstitial cells.

Regarding enzyme activities, we demonstrated the highest AR activity in the papilla, which is the innermost kidney zone, with a decrease towards the cortex. These results were comparable with the detection of AR activity and protein localization in the rat kidney (Corder et al., 1977; Nishimura et al., 1993; Robinson et al., 1993; Chauncey et al., 1998). In contrast, SDH activity decreased from the cortex towards the papilla in our investigations, as was also shown for the rat model (Heinz et al., 1975; Corder et al., 1977; Chauncey et al., 1988). These specific enzyme activities imply that sorbitol in the polyol pathway is more strongly synthesized in the papilla and less widely in the cortex. In conclusion, the distribution of the enzyme activities measured is similar in human and rat kidneys. These results correlate with the physiologic role of sorbitol in osmoregulation, the intermediate product of the polyol pathway, and reflect the osmotic gradient of the kidney (Beck et al., 1988).

The localization of both proteins along the nephron in specific cell types was performed using specific polyclonal antibodies (Moeckel et al., 1995; Grunewald et al., 2001a) and PAP-DAB detection. We were able to detect both enzymes in the whole nephron, with the exception of the glomerula. In the rat model, conflicting results of immunohistochemical detection of AR, depending on the antibodies used, have been reported.

For example, the use of antibodies against rat lens AR and rat placenta AR localized the enzyme in rat kidneys in cells of the nephron of inner and outer medulla, whereas immunohistochemical detection failed in the cortex (Terubayashi et al., 1989). However, detection of AR with an antibody against seminal vesicle AR succeeded only in glomerulus, distal tubule, thin loop and epithelium of the kidney pelvis. (Ludvigson and Sorenson, 1980; Terubayashi et al., 1989). On the other hand, AR was detected immunohistochemically in all parts of the rat kidney nephron (Corder et al., 1977; Wirth and Wermuth, 1985). These different results point out the high dependence of the PAP-technique on the antibody used. Aida et al. (2000) localized AR in the mouse model from an inner stripe of outer medulla to inner medulla (including papilla), where they detected a strong staining with the avidin-biotin-peroxidase complex. They used the same antibody in an AR knockout mouse as well, where no staining was visible.

With regard to SDH, hardly any immunohistochemical investigations have been undertaken for the rat kidney, although Corder et al. (1977) detected

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SDH in the whole nephron by this method. Wohlrab and Krautschick (1983) verified the presence of SDH by the PAP-DAB technique in all nephron segments except for the glomerula and thin loop, while with other detection systems a positive staining for SDH in the glomerulus was possible (Beck et al., 1988; Krautschick and Wohlrab, 1984).

On the basis of the various antibodies used and different osmotic conditions to be expected during detection of both enzymes, the different results detecting AR and SDH in specific cell types can be explained. Therefore, in human renal papillary and rat medullary interstitial cells, in which we were not able to detect AR and SDH by PAP-technique, the existence of both enzymes was shown by determining the enzyme activity. Enzyme activities were influenced by osmolarity (Grunewald et al., 2001b; Steffgen et al., 2003). AR was especially affected by higher osmolarity due to a higher sorbitol synthesis (Steffgen et al., 2003). These changes in enzyme activity are accompanied by a corresponding different expression of the enzymes. In immunohistochemical investigations of healthy human kidneys in comparison with diabetic kidneys with higher osmotic levels, no staining was found in the glomerula of healthy kidneys, whereas a staining of both enzymes was detected in mesangial cells of the diabetic kidney. Furthermore, in normal human kidneys, including normal osmolarity, AR and SDH staining was strong for AR in the medulla, but weaker in the cortex and, in contrast to this, SDH showed the strongest protein expression in the cortical tubule system and a weaker expression in the medulla, as shown in our investigations on normal kidneys (Kasajima et al., 2001).

The immunohistochemically observed, divergent localization of both enzymes along the nephron was reflected in the mRNA expression levels in our investigations, where the expression of AR is highest in the papilla and drops towards the cortex, whereas SDH is expressed most strongly in the cortex. On rat and human kidneys, similar expression patterns were demonstrated. Grunewald et al. (1998) described the highest AR mRNA by PCR investigations on rat kidneys in the inner medulla, followed by the outer medulla and cortex, and showed the strongest SDH mRNA expression in the cortex and the weakest in the outer medulla. Northern-blot analysis performed on rat kidneys demonstrated a similar distribution of mRNA with an AR maximum in the inner medulla, while the SDH presented its maximal expression in the cortex (Nishimura et al., 1988; Burger-Kentischer et al., 1999a).

The accurate localization of mRNA was demonstrated in our non-radioactive in-situ hybridization experiments. As in our immunohistochemistry experiments, these experiments also detected both enzymes in all segments except for the glomerula. In rat kidney, AR showed the strongest signals in the papilla, especially in the collecting ducts (CD), while positive signals were found in thin loop

(TL), endothelial and even interstitial cells. AR was localized in all CD-cells from papilla to cortex, even in the epithelium of Bowman's capsule and macula densa cells, but not in the proximal tubule (PT) and thick ascending loop (TALH) (Burger-Kentischer et al., 1998, 1999a,b).

In rat kidneys, SDH was detected in the whole nephron. In the cortex, SDH signals were localized in Bowman's capsule, glomerular cells, collecting duct, endothelial and interstitial cells, where the signal in the proximal tubule was the lowest. Compared to endothelial and collecting duct cells, the cells of proximal tubule and thick ascending loop showed only weak signals in the outer medulla. Only a moderate SDH expression in epithelial, interstitial and collecting duct cells was seen in the papilla, whereas the endothelial cells presented a stronger SDH expression (Burger-Kentischer et al., 1999a).

Under changing osmotic conditions, mRNA expression changes, as seen in protein detection. Hypoosmolarity during acute diuresis reduced AR mRNA expression in rat kidneys, while SDH mRNA was not affected significantly due to a lower sorbitol synthesis (Burger-Kentischer et al., 1999a; Grunewald et al., 1998). Nevertheless, investigations on normal and diabetic rat kidneys presented a decrease in SDH mRNA under hyperglycaemic conditions in contrast to an increase in AR mRNA (Hoshi et al., 1996; Hodgkinson et al., 2001). These findings reflect the relevance of experimental conditions by interpreting mRNA expression patterns of the enzymes described. Against this backdrop, our human and the existing rat data of mRNA expression are in conformity with the detection in the different kidney zones, but there are differences with regard to the mRNA detection in the different cell types based on the previously mentioned causes.

In conclusion, we were able to identify AR and SDH in all parts of the nephron at the protein and the mRNA expression levels except for the glomerula. AR showed its maximum in the inner kidney zones, whereas SDH showed its maximum in the outer kidney zones. In the rat model, a detection of both enzymes in glomerula and even interstitial cells was possible depending on the techniques and terms applied.

The protein localization corresponds to the mRNA expression of the enzymes along the cortico-papillary gradient. This reflects the physiological role of the osmolyte sorbitol as an intermediate product of the polyol pathway in osmoregulation with the highest concentrations of osmotic active substances in the innermost kidney zones (Beck et al., 1998).

In diabetic nephropathy, the expression of the polyol pathway enzymes in diabetic kidneys may be induced by excessive osmotic stress or by an accumulation of oxidative stress-related aldehydes (Baynes and Thorpe, 1999). Further investigations are necessary to compare our results in healthy human kidneys with the polyol pathway enzyme localization in humans with diabetic nephropathy.

Recapitulating, our results in human healthy kidneys are consistent with the rat kidney, with species differences, so that many research results from the rat model might be valid for human tissues as well.

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