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Localization of the prostacyclin receptor in human kidney

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Background. Prostacyclin is an important mediator of renal hemodynamics. Furthermore, recent studies argue for a role of this arachidonic acid metabolite in the regulation of salt and water handling in the distal nephron. To gain insight into the network of prostacyclin signal transduction, we analyzed the intrarenal distribution of the prostacyclin receptor (IP receptor) in adult human kidney.

Methods. Specific polyclonal antibodies against a synthetic peptide of the human IP receptor were generated. By means of immunohistology the localization of IP receptor protein was studied. The mRNA expression for IP receptor was analyzed by *in situ* hybridization using specific cRNA probes.

Results. In human kidney sections both IP receptor-immunoreactive protein and mRNA were expressed in smooth muscle cells and endothelial cells. Expression of the IP receptor was observed in glomerular cells, namely mesangial cells, endothelial cells, and podocytes. Both mRNA and protein expression for IP receptor was observable in Tamm-Horsfall-negative distal tubules and collecting ducts.

Conclusions. The vascular expression of the IP receptor is consistent with the known vasodilatory effect of prostacyclin in vascular beds. Glomerular expression argues for a role of this autacoid in the regulation of glomerular hemodynamics. The tubular distribution might point towards the involvement of prostacyclin in renal salt and water handling.

Prostacyclin (PGI_2) is a labile metabolite of arachidonic acid produced by the cyclooxygenase pathway. The actions of this and other prostanoids are mediated via specific cell surface receptors that are members of the G protein-coupled receptor gene superfamily. The human prostacyclin receptor (IP receptor) has recently been cloned and sequenced by us and others [1, 2]. Among human tissues Northern analysis showed highest expression of IP receptor mRNA in kidney, arguing for an important role of prostacyclin in renal physiology. PGI_2 accounts for elevation of cAMP concentration in target cells. Consistent with well characterized pharmacological studies [3] the transient expression of the IP receptor in COS-M6 cells resulted in an increase in intracellular cAMP levels upon a challenge with the stable prostacyclin analog, iloprost. Interestingly, recent works provided some pharmacological evidence for the existence of additional IP receptor subtypes in rabbit kidney and in mast cells, whose activation are presumably not linked to an increase in cAMP generation [4, 5]. However, the existence of these subtypes awaits confirmation at the molecular level.

PGI_2 plays a major physiological role in systemic circulation as a potent mediator of vasodilation and inhibitor of platelet aggregation. In a recent study we have demonstrated the intrarenal expression of prostacyclin synthase in vessels and intraglomerularly in mesangial and endothelial cells [6]. The effects of prostacyclin on renal functions, however, are less well understood. In humans the importance of renal prostaglandins becomes evident upon treatment with nonsteroidal anti-inflammatory drugs (NSAID). NSAID, which inhibit cyclooxygenase and therefore block prostaglandin synthesis, can precipitate renal failure in susceptible individuals [7]. This is probably due to inhibition of vasodilatory prostaglandins, such as PGI_2 and PGE_2. Furthermore, various studies analyzed the interaction between PGI_2 and the renin-angiotensin system. PGI_2 synthesis is thought to be stimulated by a decrease in glomerular perfusion pressure in parallel with an increase in renin secretion [8], and this interaction has been termed intrarenal baroreceptor pathway [9]. It is thought to counteract the vasoconstrictive effects of angiotensin II on glomerular perfusion. However, a recent study of Oida and coworkers failed to demonstrate glomerular expression of the IP receptor in mouse kidney [10]. Other potential roles for PGI_2 include regulation of arginine vasopressin (AVP)-induced Na^+ absorption in cortical collecting ducts [5], regulation of water flow [11] and stimulation of matrix synthesis [12].

Our study was designed to identify the intrarenal target of PGI_2. By means of immunohistology and non-radioactive *in situ* hybridization, we examined the intrarenal distribution of the IP receptor in human kidney.

Key words: kidney, IP receptor, distal tubule, podocyte, prostaglandin, renal function, vasodilation, hemodynamics.

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METHODS

Immunohistochemistry

Monoclonal antibodies directed against von Willebrand-antigen, and polyclonal antibodies against Tamm-Horsfall and cytokeratin were purchased from Boehringer/Mannheim and Genzyme (Germany), antibodies to smooth muscle cell actin were obtained from Sigma (Germany). Secondary antibodies were obtained from Dako A/S (Denmark) and Dianova (Germany). Polyclonal antibodies against the IP receptor were gained by the immunization with the peptide RNLTYVRGSVGPAT, located at the N-terminus of the receptor protein, which was synthesized onto a lysine matrix. The immune serum was affinity purified using the lysine coupled peptide. The specificity of the polyclonal anti-IP receptor antibody was demonstrated by the following control experiments: (*1*) no staining was observed using the preimmune serum; (*2*) the specificity of the labeling obtained in kidney sections was controlled by coincubation of the IP receptor antibodies with an excess of the synthetic peptide used to produce the antibodies; (*3*) Western blot analysis using human platelets revealed a single band with a molecular weight of approximately 52 kD as determined by SDS-PAGE analysis.

Human kidneys were freshly obtained from tumor nephrectomy surgery. The duration between vascular clamping of the renal artery and fixation was kept to less than 30 minutes. Immunohistological processing of cryosections was performed as described by us recently [13]. Briefly, frozen tissue was cut nominally at 4 μm, thaw-mounted on poly-L-lysine-coated glass slides, air-dried and fixed in acetone for 10 minutes at 4°C. The slides were subsequently incubated 45 minutes at 37°C with the primary antibodies. Primary antibodies were detected with the alkaline phosphatase anti-alkaline phosphatase method or with fluorescent secondary antibodies. Immunofluorescence sections were viewed in a Leitz UV microscope with epi-illumination using band pass filter combinations for selective red or green fluorescence.

Generation of cRNA probes

cRNA probes for human IP receptor were prepared as follows: a 782 bp *Apa*I - *Dra*II fragment of phIPR1 was subcloned into pBluescript vector (Stratagene). After linearization of the template DNA with *Apa*I and *Eco*RI antisense and sense cRNA were synthesized and labeled with digoxigenin using a nonradioactive nucleic acid synthesis kit (Boehringer Mannheim, Germany). The integrity of the cRNA-probes was determined by electrophoresis through a 3% polyacrylamide/6 M urea denaturing gel and ethidium bromide staining. Labeling efficiency of the riboprobes was checked by dot blots. To determine the specificity of the hybridization protocol the following control experiments were conducted. First, incubation of tissue sections with RNase prior to hybridization with cRNA-probe completely prevented staining. Second, we used an EP3 receptor cRNA probe (kindly provided by A. Schmid) [14], which is similar in both, length (800 bp) and GC-content to the IP receptor probe used in this study. The EP3-probe was run in parallel and resulted in a different staining pattern that matched with data on EP3 receptor distribution in kidney presented by Breyer et al [15]. Third, hybridization with the digoxigenin labeled IP receptor sense probe was conducted in parallel. No specific staining reaction was detectable.

In situ hybridization with digoxigenin-labeled probes

In situ hybridization was essentially carried out as described previously [13]. Cryostat sections of 4 μm thickness were cut and fixed for one hour with 4% paraformaldehyde in PBS, pH 7.2. Sections were treated with 0.2 N HCl, acetylated in 0.1 M triethanolamine and 0.25% acetic anhydride, and finally dehydrated in 100% alcohol. Digoxigenin-labeled riboprobes (1 ng/μl) were added to the hybridization solution containing 50% deionized formamide, 0.3 M NaCl, 10 mM Tris-HCl, pH 8, 1 mM EDTA, 0.2 mg/ml salmon sperm DNA, 1 mg/ml yeast transfer RNA, 1 $\times$ Denhardt's solution, and 10% dextran sulfate and incubated for 18 hours at 57°C. The slides were washed sequentially with 1 $\times$ SSC at room temperature for one hour, 15 minutes in 0.5 $\times$ SSC at 55°C and 10 minutes in 1 $\times$ SSC. The sections were then treated with 20 μg/ml RNase A (Boehringer, Mannheim, Germany) in 0.5 M NaCl/10 M Tris-HCl, pH 7.4/1 mM EDTA for 30 minutes at 37°C. Sections were washed 5 $\times$ in 1 $\times$ SSC and were then equlilibrated in buffer 1 (0.1 M Tris-HCl and 0.15 M NaCl, pH 7.5) and incubated with buffer 1 containing 2% blocking reagent (Boehringer) and 1% BSA (Sigma, Germany) for 30 minutes at room temperature. The sections were incubated with alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (Boehringer Mannheim) diluted 1:500 in buffer 1 containing 1% normal sheep serum and 2% blocking reagent for 60 minutes at room temperature. The slides were washed twice with buffer 1 and rinsed in buffer 3 (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.75). Then, 9 μl 4-nitro blue tetrazolium chloride, 7 μl 5-bromo-4-chloro-3-indoylphosphate (Boehringer Mannheim) and 0.5 mg levamisole (Sigma) in 2 ml of buffer 3 were added. The color reaction was stopped with 0.01 M Tris-HCl, 1 mM EDTA, pH 8.0. Slides were rinsed with distilled water and covered with aqueous mounting medium.

RESULTS

Characterization of anti-IP receptor polyclonal antibodies

We raised a polyclonal antiserum against the hydrophilic amino terminus of human IP receptor. To assess specificity of the antibody, we performed Western immunoblot of

membrane fractions from human platelets. Platelets are known to express IP receptor for counteracting the proaggregatory action of thromboxane [16]. As shown in Figure 1A the anti-IP receptor antibodies identified a diffuse band with an apparent molecular mass of approximately 52 kD.

Furthermore, the antibody specificity was confirmed by preabsorption of the purified anti-IP receptor antibodies prior to immunohistochemistry with an excess of IP peptide against which they were raised. The labeling was specific as it could be blocked by preincubation of the antibodies with the synthetic peptide (Fig. 1 B, C). Coincubation with an unrelated peptide has had no effect on labeling (data not shown).

Expression of IP-immunoreactive protein in human kidney

The regional distribution and cellular localization of IP receptor protein was investigated in human kidney sections. In renal cortex IP receptor immunoreactive protein was most prominent in endothelial cells and smooth muscle cells of small arteries (Fig. 2A) and veins (data not shown). A consistent labeling was observed in peritubular capillaries (exemplarily indicated by arrowhead in Fig. 2A). Expression of IP receptor immunoreactive protein also localized in smooth muscle cells of interlobular arteries (Fig. 2B). In addition to these vascular structures we observed immunoreactive protein in glomeruli (Fig. 2C). The intraglomerular staining pattern observed was diffuse, suggesting that IP receptor protein is expressed in all glomerular cells, that is, mesangial cells, endothelial cells and visceral epithelial cells. Staining of podocytes could be identified by light microscopy.

Furthermore, we observed labeling of tubular epithelial cells with anti-IP receptor polyclonal antibodies. As judged by light microscopy we tentatively identified these tubular structures as distal tubules (Fig. 2D) and cortical collecting ducts (data not shown). A faint staining was also observed in medullary collecting duct cells (Fig. 2E). Compared to the staining of cortical tubules the signals obtained in medullary tubules were less strong.

Double-immunofluorescence allowed us to colocalize definitively the IP receptor immunopositivity with von Willebrand factor in endothelial cells (Fig. 3 A, B). To identify the specific segment of the distal tubule we performed double-immunofluorescence with anti-Tamm-Horsfall antibodies. Tamm-Horsfall-protein is known to be expressed in medullary and cortical thick ascending loops of Henle [17]. The expression of IP receptor protein was only present in Tamm-Horsfall-negative distal tubules (Fig. 3 C, D). In renal medulla immunoreactivity towards anti-IP receptor antibodies was detectable in vasa recta. Colocalization with smooth muscle cell actin allowed the positive identification of these structures (Fig. 3 E, F).

Expression of IP receptor mRNA in human kidney

To confirm the expression pattern observed with the anti-IP receptor antibodies, we analyzed expression of IP receptor mRNA in human kidney cryosections.

In renal cortex processed with IP receptor antisense riboprobe staining is visible in glomeruli and vascular structures. In arteries (Fig. 4A) and veins (data not shown), IP receptor mRNA is expressed in both endothelial and smooth muscle cells.

In the glomeruli intense staining was observed at the margins of the glomerular lobules, which suggests an abundant expression of the IP-receptor mRNA in podocytes (Fig. 4B). In addition a faint staining throughout the glomerulus was observed. Note that staining was also detectable in endothelial and smooth muscle cells of the glomerular arteriole. No staining was found in cells of macula densa.

Apart from glomeruli and vessels we detected expression of IP receptor mRNA in cells of the distal tubule (Fig. 4C). Tubular staining was observed consistently; although staining was less intense compared to vessels and glomeruli. These structures could be identified as Tamm-Horsfall negative distale tubules since serial sections processed for IP receptor mRNA and Tamm Horsfall protein revealed no colocalization (data not shown). In renal medulla expression of IP receptor mRNA was confined to collecting tubules (Fig. 4D) and vasa recta (Fig. 4E).

DISCUSSION

Western blot analysis

To test the specificity of the affinity purified, polyclonal anti-human IP receptor antibodies we used two different approaches: competition immunohistochemistry and Western blot analysis. In immunohistochemistry the staining could be blocked if the antibodies were preincubated with the IP receptor peptide. Immunoblotting was performed using a human platelet membrane fraction. A slightly diffuse band with an apparent mobility of about 52 kD is visible, and the molecular weight predicted from the open reading frame of the human IP receptor cDNA is 41 kD [2]. The deduced amino acid sequence of the IP receptor displays two potential N-linked glycosylation consensus sequences (Asn-X-Ser/Thr) at Asn^{7} and Asn^{203}. A similar difference between the calculated molecular weight (37 kD) and the observed weight as judged by SDS-PAGE (55 kD) has been reported for the human thromboxane receptor, which contains two N-linked glycosylation sites as well (Asn^{4} and Asn^{16}) [18, 19]. Therefore, we speculate that the discrepancy between the predicted and apparent molecular weight may be due to glycosylation of the IP receptor

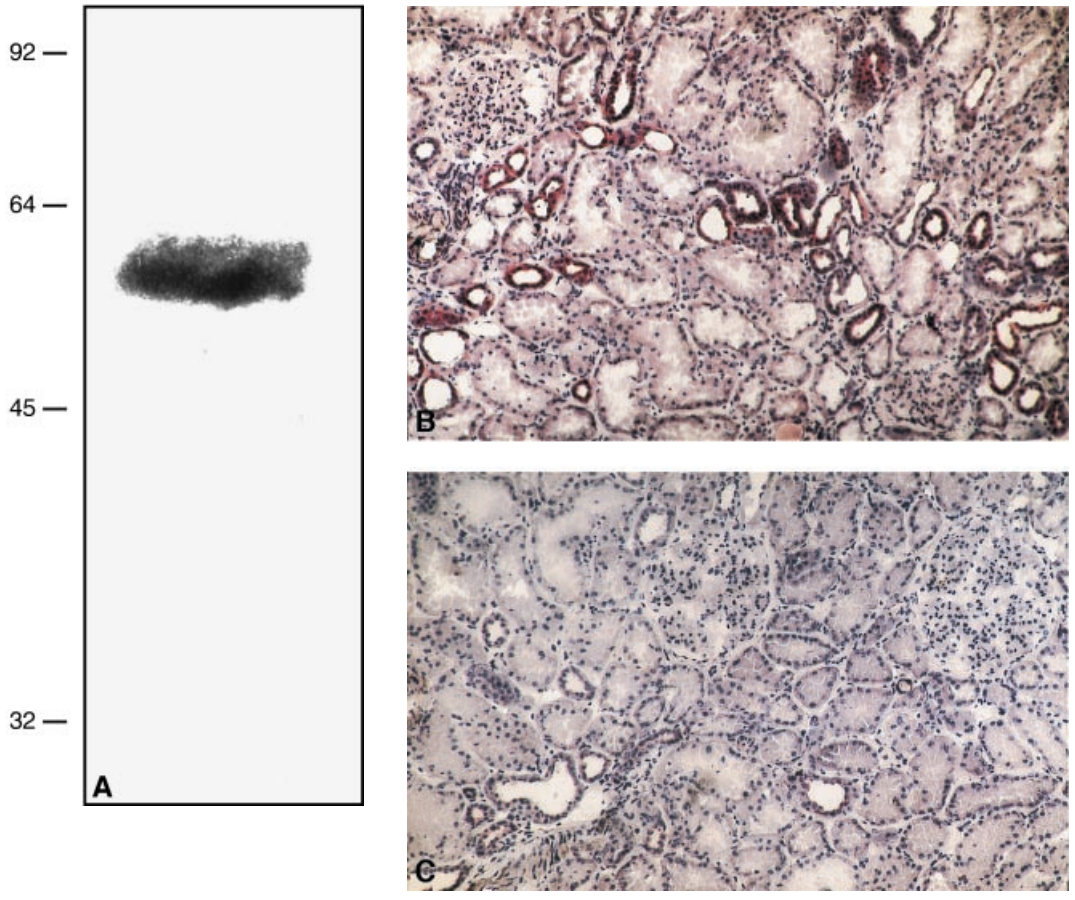


Fig. 1. Specificity of the anti-IP receptor antibody. (*A*) Western blot analysis of platelets using anti-IP receptor antibodies. Ten percent SDS-PAGE of platelet proteins showing a diffuse band of about 52 kD are identified by affinity purified anti-IP receptor antibodies. (*B*) Staining of kidney cortical section with anti-IP receptor antibodies (magnification ×100). (*C*) Preabsorption of the anti-IP receptor antibody with an excess of IP receptor peptide prior to immunostaining (magnification ×100). No or negligible signals are observed. Publication of this figure in color was made possible by a grant from KfH Kuratorium für Dialyse und Nierentransplantation e.V., Marburg, Germany.

protein with subsequent alteration of migration on SDS-PAGE. In summary, these data present strong evidence for the specificity of the generated IP receptor antibodies.

Vascular expression of the IP receptor

The involvement of PGI_2 in renal regulatory mechanisms are becoming increasingly apparent, but many questions remain unanswered. An important aspect is the distribution of the target of PGI_2, and thus cells expressing IP receptors. In human kidney, IP receptor expression was present in endothelial cells of arteries and veins. We speculate that PGI_2 might act in a feedback manner on endothelial cells to counteract the action of thromboxane. Human endothelial cells are known to express the thromboxane receptor [20]. PGI_2 and TXA_2 have a dominant role in the hemostatic balance, which involves interaction between platelets and endothelial cells [21].

Expression in smooth muscle cells, however, was confined to all vascular beds. Identical to mouse kidney, we observed the expression of both IP receptor mRNA and IP receptor immunoreactive protein in interlobular arteries and afferent arterioles. These findings are in agreement with various studies on renal hemodynamics [22, 23]. In humans, infusion of prostacyclin resulted in a decrease of renal vascular resistance and an increase of renal plasma flow [11]. Moreover, prostaglandins, especially PGI_2, have been shown to antagonize the constrictor action of a variety of pressure hormones, including angiotensin II and norepinephrine. Furthermore, the expression of the IP receptor in the vasa recta suggests it has an important role in regulating renal microcirculation. The relevant target site is thought to be the renal medulla, thereby protecting the highly vulnerable medulla against ischemic damage under pathophysiological conditions.

Glomerular expression of IP receptor

In renal cortex, we observed significant glomerular expression of both IP receptor immunoreactive protein and IP receptor mRNA. Double-immunofluorescence revealed that IP receptor is expressed in all glomerular cell types. In accordance with its expression *in vivo* we observed expression of mRNA for IP receptor in cultured human podocytes

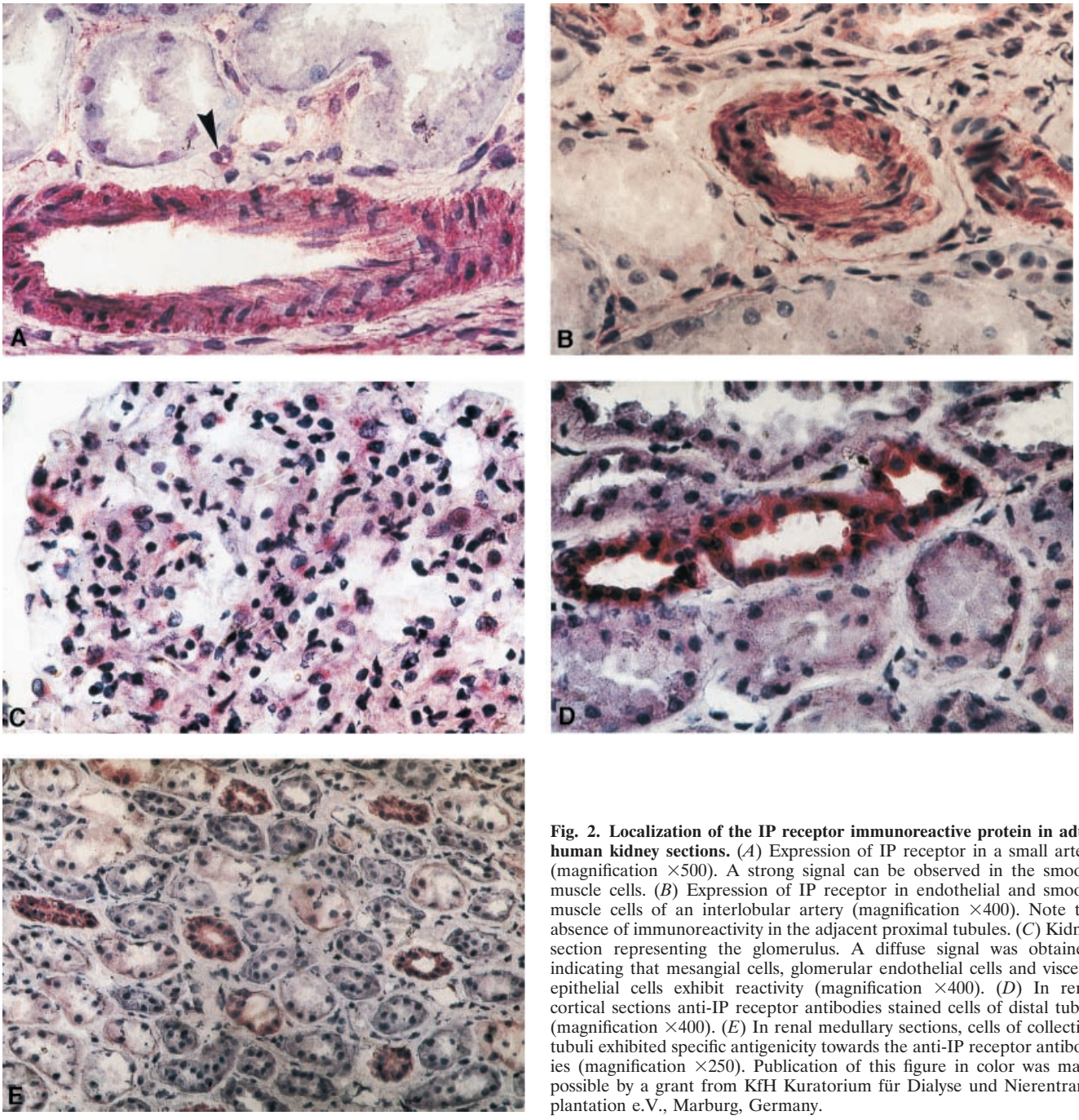


Fig. 2. Localization of the IP receptor immunoreactive protein in adult human kidney sections. (*A*) Expression of IP receptor in a small artery (magnification ×500). A strong signal can be observed in the smooth muscle cells. (*B*) Expression of IP receptor in endothelial and smooth muscle cells of an interlobular artery (magnification ×400). Note the absence of immunoreactivity in the adjacent proximal tubules. (*C*) Kidney section representing the glomerulus. A diffuse signal was obtained, indicating that mesangial cells, glomerular endothelial cells and visceral epithelial cells exhibit reactivity (magnification ×400). (*D*) In renal cortical sections anti-IP receptor antibodies stained cells of distal tubuli (magnification ×400). (*E*) In renal medullary sections, cells of collecting tubuli exhibited specific antigenicity towards the anti-IP receptor antibodies (magnification ×250). Publication of this figure in color was made possible by a grant from KfH Kuratorium für Dialyse und Nierentransplantation e.V., Marburg, Germany.

as well as in mesangial cells (manuscript in preparation). These findings are in contrast with data from Oida et al [10], who observed IP receptor mRNA expression limited to interlobular arteries and afferent arterioles in mouse kidney. However, no mRNA expression was detectable in glomeruli. Apart from technical differences (radioactive vs. non-radioactive *in situ* hybridization) the different pattern of IP receptor expression we observed in human kidney may reflect the relative abundance of prostacyclin synthase expression in human glomeruli [24] or glomerular cells [25]. Although the expression of prostacyclin synthase has not been analyzed in murine glomeruli, it is worth mentioning that PGI_2 is the main prostanoid in human glomeruli, in contrast to rodents where PGE_2 appears to be the main metabolite of arachidonic acid [26]. We detected a low but significant expression of prostacyclin synthase in glomerular endothelial cells and in mesangial cells and, consistently, we observed that PGI_2 is the main

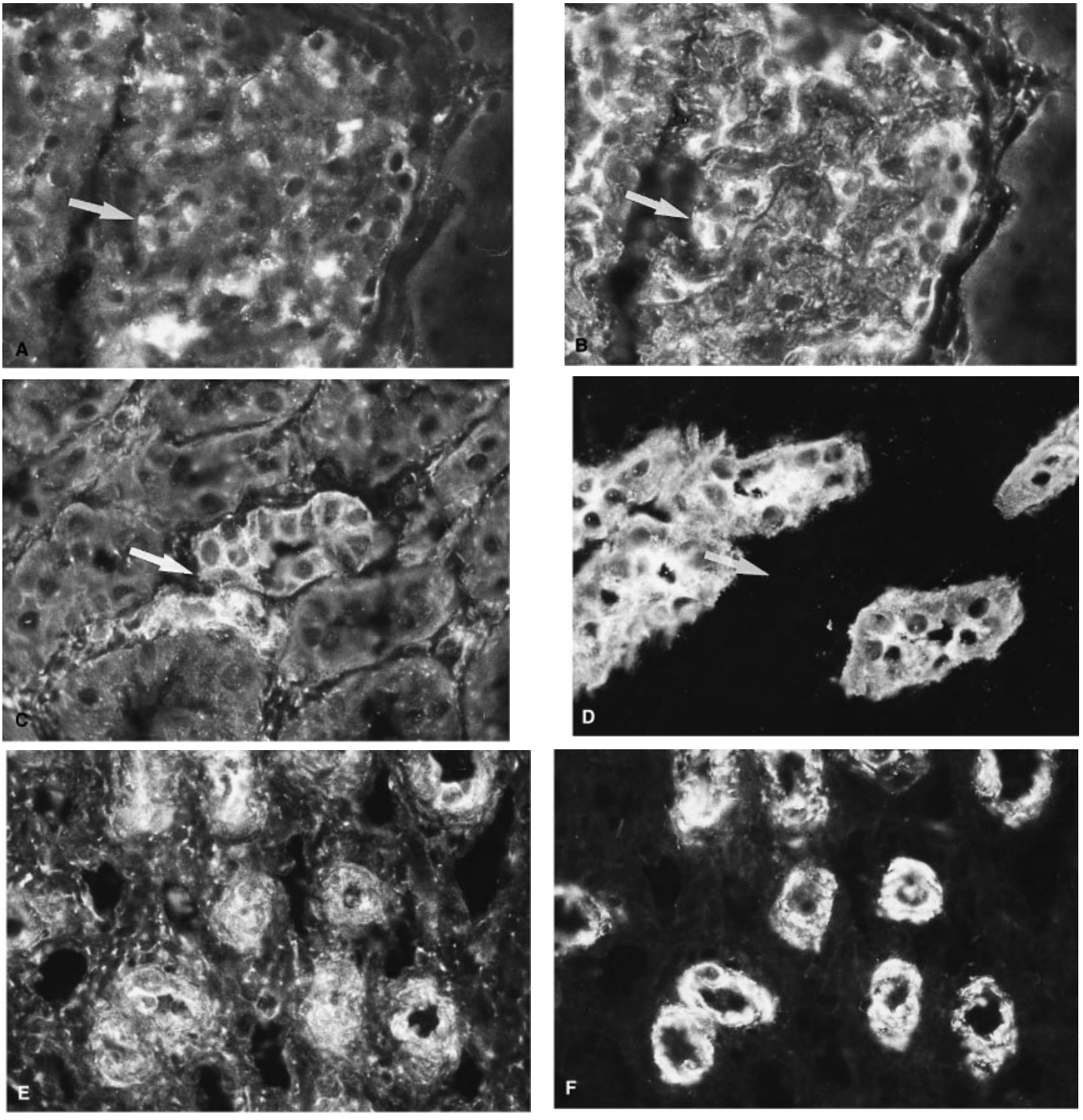


Fig. 3. Colocalization of the IP immunoreactive protein with various antigens. Colocalization of IP receptor (*A*) with von Willebrand factor (*B*). A monoclonal antibody directed to von Willebrand factor was used to identify endothelial cells (exemplary indicated by white arrow). No colocalization of IP receptor (*C*) with Tamm-Horsfall protein (*D*). Location of the distal tubule stained by anti-IP receptor antibody is indicated by white arrow. In this area no expression of Tamm-Horsfall protein is visible. Colocalization of IP receptor (*E*) with smooth muscle actin (*F*) in vasa recta (magnification A-F, ×500).

metabolite in human cultured mesangial cells (unpublished observations). In line with our finding of glomerular IP receptor expression, the prostacyclin analog iloprost has been shown to antagonize the cyclosporine A-induced constriction in human glomeruli [27]. The constitutive expression of prostacyclin synthase and the IP receptor probably serves to antagonize signals conveyed tonically by angiotensin II and other vasoconstritive agents.

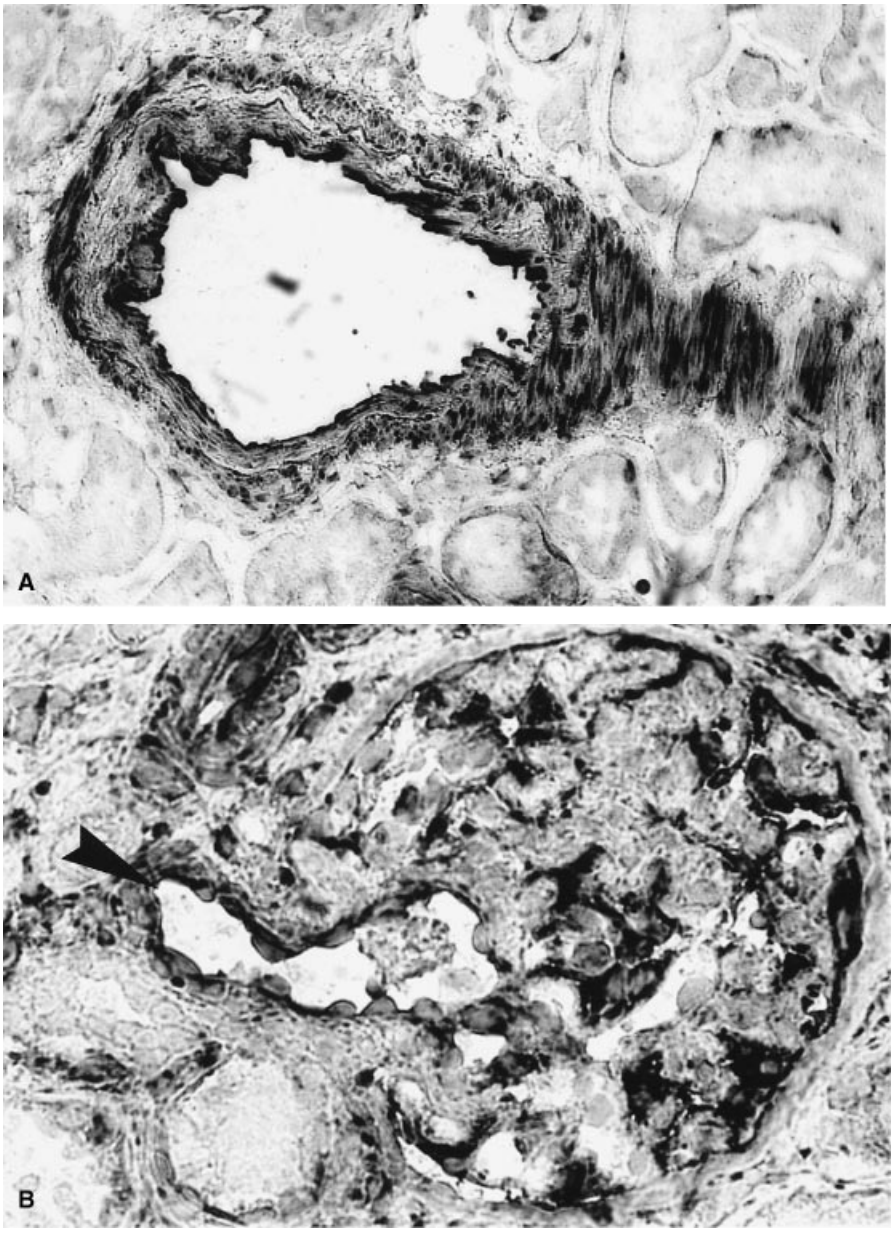
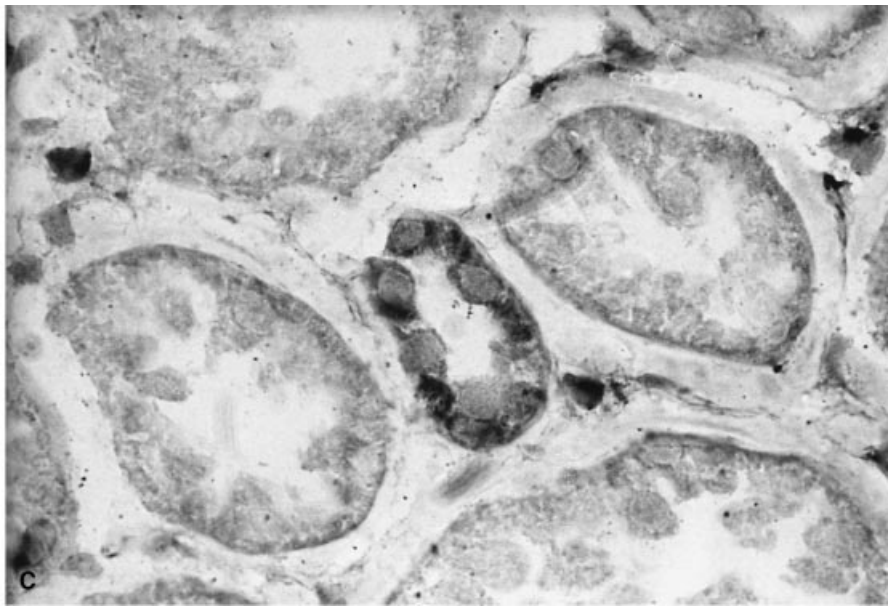
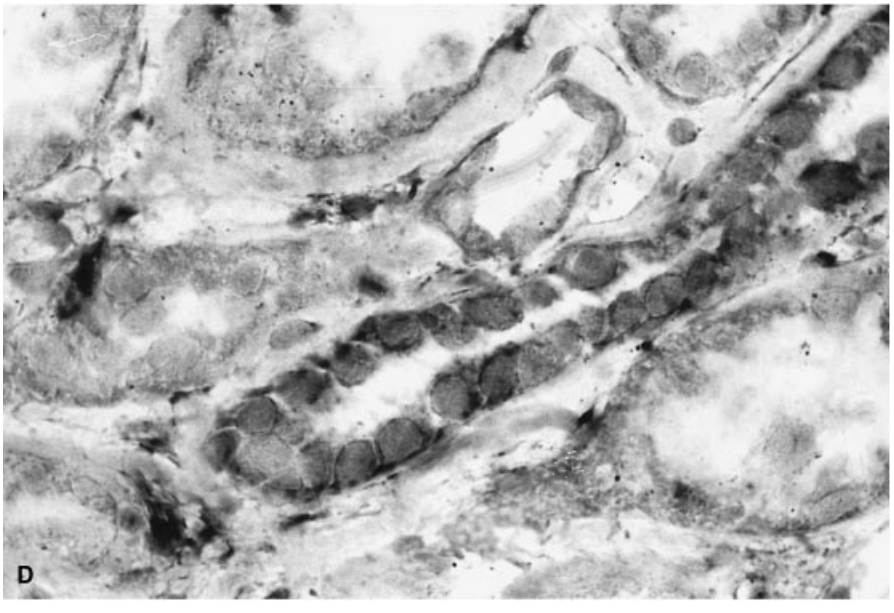


Fig. 4. Localization of IP receptor mRNA in human kidney sections. (*A*) Expression of IP receptor mRNA in a renal artery. Strong staining of endothelial and smooth muscle cells is observed. Note absence of staining in surrounding proximal tubules. (*B*) Presence of IP receptor mRNA in a glomerulus. In the glomerulus strong labeling is observed in podocytes. In addition, expression of IP receptor mRNA in endothelial and smooth muscle cells of a glomerular arteriole (indicated by arrowhead) is visible. (*C*) Staining of IP receptor mRNA is visible in cells of distal tubule. (*D*) Staining of IP receptor mRNA is visible in cells of collecting duct. (*E*) In renal medulla a strong signal for IP receptor mRNA was detected in vasa recta. Note the absence of staining in the surrounding tubular epithelial cells (magnification A-E, ×400).

Expression of the IP receptor in distal tubule and collecting duct cells

In contrast to mouse, we observed in human kidney expression of IP receptor in the distal tubules and in collecting ducts. To our knowledge this is the first study providing direct evidence for the expression of IP receptor protein in human tubular epithelial cells. Although modulation of renal salt and water handling has been mainly attributed to the tubular actions of prostaglandin E_2 (PGE_2) via various PGE_2 (EP) receptors, some studies have shown that prostacyclin also exerts direct effects on the modulation of Na^+ absorption and hydraulic conductivity along the nephron. PGI_2 has been shown to be natriuretic in dogs when infused into the renal artery at low concentrations [28], and PGI_2 inhibits both transepithelial voltage and Na^+ reabsorption in rabbit isolated perfused cortical collecting ducts [29]. These data were recently corroborated and extended by Hebert, Regnier and Peterson [5]. Apart from the natriuretic effects and inhibition of the AVP-induced increase of the hydraulic conductivity by PGI_2 in rabbit collecting ducts, the authors demonstrated that PGI_2 signal was not transduced by an increase in cAMP levels. Instead, activation of phospholipase C and inhibition of adenylate cyclase activity were observed, and therefore, the existence of new receptor subtypes was concluded. Using the PGI_2 analogs iloprost and carbaprostacyclin, the presence of a G_i-coupled PGI_2-receptor was also observed in isolated cells of rat medullary thick





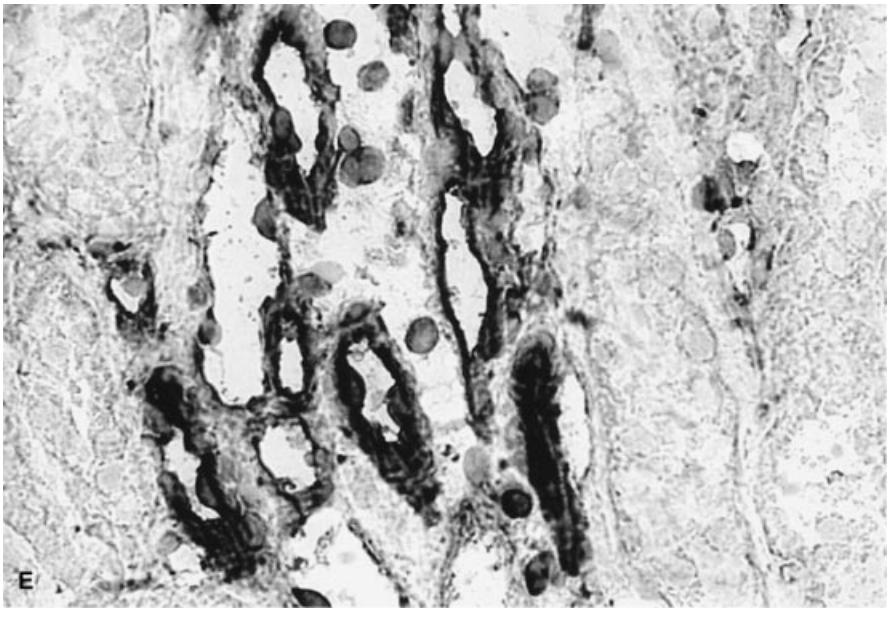


Fig. 4. *Continued.*

ascending limbs of Henle [30, 31]. Additionally, recent data support a role for PGI_2 as a mediator of increased distal tubule HCO_3^- secretion [32]. This effect might be mediated by an increase of intracellular cAMP levels. Our study did not address the intracellular signal transduction system, but to our knowledge there is currently no proof for the existence of IP receptor subtypes at the molecular level.

Concerning the physiological significance of IP receptor expression in human collecting ducts, the study of Natov and coworkers is particularly relevant to our findings [11]. Infusion of PGI_2 in healthy volunteers with normal kidney induced a slight, but significant natriuretic effect. Although it has been largely accepted in the literature that PGI_2 induces natriuresis primarily through a decrease in filtration fraction or through "a washout" of the corticopapillary gradient by enhanced medullary blood flow, a decrease in filtration fraction has not been consistently followed by a rise in natriuresis [33]. In this regard, a direct effect of PGI_2 via a tubular IP receptor may account for the observed natriuresis.

In summary, we found prominent expression of IP receptor immunoreactive protein and mRNA in endothelial cells, glomeruli and in smooth muscle cells of interlobular arteries, afferent arterioles and small veins. These findings are in agreement with numerous physiological studies analyzing the effects of prostacyclin infusion on renal hemodynamics and glomerular perfusion. Although direct effects of prostacyclin on renal salt and water handling have already been described in the rabbit cortical collecting duct, and *in vivo* effects of prostacyclin in humans are compatible with our findings, the expression of IP receptor protein and mRNA in distal tubules and collecting ducts points towards new functions of PGI_2 in human kidney that are currently poorly defined. Further work is needed to unravel the physiological significance of prostacyclin on renal salt and water handling in humans, and to clarify the signal transduction systems involved.

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APPENDIX

Abbreviations used in this article are: AVP, arginine vasopressin; COX, cyclooxygenase; EP receptor, prostaglandin E_2 receptor; GFR, glomerular filtration rate; IP receptor, prostacyclin receptor; NSAID, nonsteroidal antiinflammatory drug; PGE_2, prostaglandin E_2; PGI_2, prostacyclin; RT-PCR, reverse transcriptase-polymerase chain reaction; TXA_2, thromboxane A_2.

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