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Cyclooxygenases in the Collecting Duct of Neonatal Rabbit Kidney

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Key Words
Kidney • Collecting duct • Epithelia • COX 1 and 2 • Differentiation • Perfusion culture • Electrolytes

Abstract
COX 1 and 2 expression during the terminal phase of kidney development is poorly understood. To obtain information about this process we followed the primary appearance of cyclooxygenases in collecting duct (CD) epithelia of the neonatal rabbit kidney with immunohistochemical and two dimensional electrophoretical methods. In the fully embryonic cortical zone immunohistochemical expression of COX 1 is seen in all cells of the CD ampulla, while COX 2 is lacking within the nephron inducer. Within the matured cortical collecting duct (CCD) COX 1 and 2 immunoreactivity could not be detected. In contrast, a heterogeneous expression profile for COX 1 and 2 is found in the outer medullary CD (OMCD), since not all cells showed immunohistochemical labeling. Within the inner medullary CD (IMCD) nearly all cells express both cyclooxygenases. As revealed by western blot experiments generated embryonic CD epithelia in perfusion culture demonstrate high COX 1 presence at the begin of culture, while COX 2 is found to a minor degree. From day 3 until day 14 continuous levels of COX 1 and 2 expression are detected. Administration of 1 x 10^{-7} mmol/l aldosterone does not influence COX expression, while application of 100 mmol/l NaCl increases COX 2 fourfold. The upregulation of COX 2 by a chronic NaCl load in embryonic epithelia suggests in part a constitutive and in part a facultative expression during CD cell differentiation.

Introduction

Cyclooxygenases (COX EC: 1.14.99.1) catalyse the conversion of arachidonic acid and oxygen into prostaglandin H_2 (PGH_2). PGH_2 is the precursor of various prostaglandins that are produced in subsequent reactions. Two isoforms of cyclooxygenase are known: COX 1 and COX 2 [1, 2]. COX 1 is considered to be expressed constitutively whereas COX 2 is known to be the inducible isoform of cyclooxgenases [3, 4]. The mRNA expression of both enzymes has been demonstrated in the renal cortex, medulla and papilla of rat, monkey and human [5]. In the kidney of rat and human COX 1 has been localized in all parts of the collecting duct (CD) beside medullary interstitial cells

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[5, 6]. In contrast, COX 2 has been shown to be expressed in the macula densa and in part of the thick ascending limb of Henle's loop [7]. The expression of COX 2 within the macula densa was reported to be increased by a low salt diet [7], while high oral salt intake led to an upregulation in the renal medulla [8]. The medullary localization of COX 2 to interstitial and/or collecting duct cells seems to be species-dependent and is discussed controversially up to date [8, 9, 10, 11, 12].

The collecting duct (CD) system of the adult kidney is heterogeneously composed and consists of principal (P) and different types of intercalated (IC) cells involved in the regulation of homeostasis [13, 14]. The different cell types develop during kidney development in parallel to the elongation phase of the collecting duct. In rabbit kidney they continue after birth to derive from the embryonic CD ampullae, which are localized underneath the capsule in the outer cortex, which contains fully embryonic tissue [15, 16]. To date little is known about the mechanism triggering development into a heterogeneously composed epithelium consisting of P and various IC cell types. In vitro experiments provided evidence that aldosterone [17] and the electrolyte environment [18, 19] are involved.

Recent reports about COX 2 knock out mice [20] and about experiments using COX 2 inhibitors during organogenesis [21] point to a role of COX 2 derived prostanoids in kidney development. For the neonatal kidney little is known about the synthesis of the different prostaglandins and the expression of their specific receptors. A recent paper describes a low COX activity and consequently a low release of prostaglandin E_2 (PGE_2) in the cortex of the newborn rat, whereas the medulla was a rich source of PGE_2, $PGF_{2á}$ and PGD_2 [22]. However, the expression of specific PGE_2 receptors was shown in the neonatal rat cortex [23]. It is not yet known, which of the four known PGE_2 receptors is present.

COX 1 and 2 expression during the terminal phase of kidney cell differentiation is poorly understood. Consequently, we focused in the present paper on the expression of cyclooxgenases during the terminal phase of the renal collecting duct development. First, we analyzed the primary expression of COX 1 and 2 along the maturing collecting duct in neonatal rabbit kidney with cell biological methods. Additionally, we investigated the influence of aldosterone and osmolytes on the expression of both COX 1 and COX 2 in embryonic collecting duct epithelia generated in serum-free perfusion culture.

Materials and Methods

Immunohistochemistry

Monitoring of cell development was performed in kidneys of neonatal rabbits (up to 1 day old) as described earlier [16]. 8 µm cryosections were prepared using a Cryostat HM 500 (Microm, Walldorf, Germany). Immunolabelling was started by fixing the cryosections for 10 minutes in ice-cold ethanol. Following several washes in phosphate buffered saline (PBS, pH 7.2) the sections were incubated with a blocking solution (PBS) containing 10% horse serum and 1% bovine serum albumin (BSA) for 30 min. The primary antibodies (anti-cyclooxygenase 1; sc-1752 and 2; sc-1745; Santa Cruz Biotechnology, California, USA diluted 1:100 in blocking buffer) were incubated for 1.5 h as described earlier [12]. Following several washes in PBS containing 1% BSA the sections were treated for 45 minutes with a donkey-anti-goat-IgG-fluorescein-isothiocyanate (FITC) - conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, USA) diluted 1:100 in blocking buffer. Following several washes in PBS the sections were embedded in Slow Fade Light Antifade Kit (Molecular Probes, Oregon, USA) and examined using an Axiovert 35 microscope (Zeiss, Oberkochen, Germany) and a Kodak Tx 400 film. Co-incubation experiments were performed using mab P_{CD} Amp1 diluted 1:10 in PBS as primary antibody. P_{CD} Amp1 is a fiber-associated extracellular matrix protein located in the embryonic epithelial - mesenchymal interface [24]. A donkey-anti-mouse-IgG-Texas-red-conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, USA) diluted 1:150 in blocking buffer was used as secondary antibody. Cryosections of cultured collecting duct epithelia were treated the same way.

Tissue isolation and generation of CD epithelia in culture

Generation of embryonic collecting duct epithelia was performed by isolating cortical explants from the kidneys of newborn New Zealand rabbits (up to 1 day old) according to methods described earlier [25]. The explants consisted of a piece of capsule fibrosa with adherent collecting duct ampullae, S-shaped bodies and nephrogenic blastema, which were mounted in tissue carriers (Fig. 1A). For generation of a CD epithelium the carriers were placed for 24 hours in a 24 - well plate in a CO_2 incubator (5% CO_2/95% air). During culture of the explants in Iscove´s modified Dulbecco´s medium (IMDM; Gibco BRL Life Technologies, Eggenstein, Germany) including 10% fetal bovine serum (Boehringer, Mannheim, Germany) an outgrowth of cells from the collecting duct ampullae was observed. Within 24 hours the entire surface of the explant (6 mm in diameter) was covered by a monolayer of polarized collecting duct cells. During perfusion culture the epithelium develops features of principal- (P-cells) and intercalated-cells (IC-cells) [17, 18, 26]. Serum supplemented medium was used for this limited period of time only.

Perfusion culture

After initiation of culture the tissue carriers were placed in a perfusion culture container (Fig. 1B; Minucells and Minutissue, Bad Abbach, Germany). Fresh medium was continuously perfused for 14 days at a rate of 1 ml/h with an IPC N8 peristaltic pump (Fig. 1C; Ismatec, Wertheim, Germany). To maintain a constant

temperature of 37° C, the container was placed on a thermo plate (Medax, Kiel, Germany) and covered by a transparent lid (Fig. 1C). During perfusion culture Iscove's modified Dulbecco's medium (IMDM; order # 21980 - 032; Gibco BRL-Life Technologies, Eggenstein, Germany) without serum was used as the standard medium. Aldosterone was used at a concentration of 1 x 10^{-7} M (Sigma-Aldrich-Chemie, Deisenhofen, Germany). 1% antibiotic-antimycotic solution (Gibco BRL-Life Technologies) was added to all culture media. Furthermore, up to 50 mmol/l HEPES (Gibco BRL-Life Technologies) was used in the medium to maintain a constant pH of 7.4 in perfusion culture under atmospheric room air (0.3% CO_2).

RNA extraction

Total RNA was extracted from frozen tissue stored at -80°C according to the protocol of Chomczynski and Sacchi [27]. After homogenization in 2 ml solution D consisting of guanidine thiocyanate (4 M) containing 0.5 % N-lauryl-sarcosinate, 10 mmol/l EDTA, 25 mmol/l sodium citrate, 700 mmol/l bb-mercaptoethanol, 0.1 volume 2 M sodiumacetate (pH 4), 1 volume phenol (water saturated) and 0.2 volume chloroform were added sequentially to the homogenate. After cooling on ice for 15 min, samples were centrifuged. RNA in the supernatant was precipitated with an equal volume of isopropanol at -20°C for at least 1 h and centrifuged at 10.000 *g* for 15 min at 4°C. The resulting RNA pellets were resuspended in 0.3 ml of solution D, again precipitated with an equal volume of isopropanol at -20°C. Pellets were finally dissolved in diethylpyrocarbonate treated water and stored at -80°C until further processing.

Ribonuclease protection assay for COX 2 and cytoplasmic βb-actin

Phosphorus-32 labelled antisense probes were generated by in vitro transcription using PCR amplificated gene fragments of the investigated mRNAs. The used PCR primers were as follows: for COX 2 (accession no. U97696) we used fwd 5'attgatggagaggtatat3' and rev 5'caatcacaatcttaatag 3' yielding a 270 base long antisense transcript. For βb-actin (accession no.V01217) we used fwd 5'gcgcgccactgtcgagtc3' and rev 5'cgcaccaagcaggagcgtacc3' yielding a 306 base long antisense transcript. PCR inserts were cloned into the *Eco*RI cloning site of the transcription vector pCRII Topo using the Topo Cloning Kit (Invitrogen, Groningen, Netherlands) according to the manufacturers instructions. Correctness of the constructed plasmids was confirmed by sequence analysis done by Sequiserve, Vaterstetten, Germany. Linearization with *Eco*RV followed by vitro transcription with SP6 RNA polymerase yields the respective antisense transcripts plus 80 bases polylinker sequences of the pCRII Topo vector. As negative control 20µg yeast tRNA were analyzed in each assay. Moreover an aliquot of the undigested probe was analyzed on a separate lane to confirm correct length of the protected fragments. Transcripts were continuously labeled with [aa^{32}P]guanosine triphosphate (400Ci/mmol; Amersham, Freiburg, Germany) and purified on a Sephadex G50 spin column. For hybridization total RNA was dissolved in a buffer containing 80% formamide, 40mmol/I piperazine N,N¢¢-bis(2-ethane sulphonic acid), 400mmol/l NaCl, 1mmol/l EDTA (pH 8). 5 x 10^5 cpm of the cRNA probes hybridized with 20 µg of total RNA (COX 2), 2µg of total RNA (ββ-actin) or 20 µg tRNA (negative control) at 60°C for 16-18 h, then digested with RNase A/T1 (RT/30 min) and proteinase K (37°C/30 min). After phenol/chloroform extraction and ethanol precipitation, protected fragments were separated on a 8 % polyacrylamide gel. The gel was dried for 2 h, signals were quantitated in a Phosphoimager (Instant Imager 2024, Canberra-Packard, Dreieich, Germany) and autoradiography was performed at -80°C on Kodak X-Omat films for 2 days [28].

Sodium Dodecyl Sulfate-Polyacrylamid Gel Electrophoresis (PAGE) and Immunoblotting

Freshly isolated cortical renal explants or generated CD epithelia were homogenized in a sample buffer containing 2% sodium dodecyl sulfate (SDS), 10% glycerin, 125 mM Tris-HCl, 1mM EDTA (all obtained from Sigma-Aldrich-Chemie) and centrifuged at 10000 x g for 10 min. The supernatants were used in the following experiments. The amount of proteins was determined by a protein microassay (Bio-Rad Laboratories, Hercules, CA, USA). 15 µmg (Fig. 5) or 30 µmg (Fig. 3) protein samples were separated by SDS-PAGE in 10% Laemmli minigels according to methods described earlier [29], which were electrophoretically transferred to P-Immobilon membranes (Millipore, Eschborn, Germany) [30]. In order to detect immunoreactive proteins, the blots were first blocked (PBS, pH 7,2; 0,05% Tween, Sigma, Deisenhofen, Germany; 10% horse serum, Boehringer, Mannheim, Germany) followed by an incubation for 1 h at room temperature with a goat polyclonal antiserum raised against COX 1 or 2 diluted 1 : 200. A horseradish peroxidase-conjugated donkey anti-goat immunoglobulin antiserum (1: 1000, Dianova, Hamburg, Germany) served as detecting antibody applied for 45 min as described earlier. Blot development was started by addition of 0,5 mg/ml diaminobenzidine, 0,02% H_20_2 and 0,03% cobalt chloride dissolved in citrate buffer, pH 6,3. Washing the membrane in tap water stopped the reaction. Immunoblots were documented with a Scan Jet 6200 C (Hewlett Packard, Greely, USA). Determination of apparent molecular weight was performed in conjunction with broad range molecular weight standard proteins (Bio-Rad Laboratories).

Two-dimensional polyacrylamid gel electrophoresis (2-D-PAGE) and subsequent western blot

Tissue samples were homogenized in lysis buffer (pH 7,5) containing 8 M Urea, 4% CHAPS, 40mM Tris-HCL, 2 mM DTT and 0,5% carrier ampholytes. 50 µg solubilized protein samples of epithelia cultured in IMDM containing 1 x 10^{-7}M/l aldosterone were loaded for isoelectric focussing to each tube. The gels were run for 14 hours using increasing voltage (100-1000 V) in a Model 175 Tube Cell (Bio-Rad Laboratories). After 15 min equilibration in 2% sodium dodecyl sulfate (SDS), 10% glycerin, 125 mM Tris-HCL, 1mM EDTA , the isoelectric focused gel tubes were laid on the surface of 3 mm thick 10% Laemmli gels, the proteins were separated by SDS-PAGE [31]. After equilibration in a borate acid buffer, proteins were electrophoretically transferred to P-Immobilon membranes and incubated with the COX antibodies. Isoelectric point and molecular weight of detected proteins was determined by 2-D SDS-PAGE standards (Bio-Rad Laboratories).

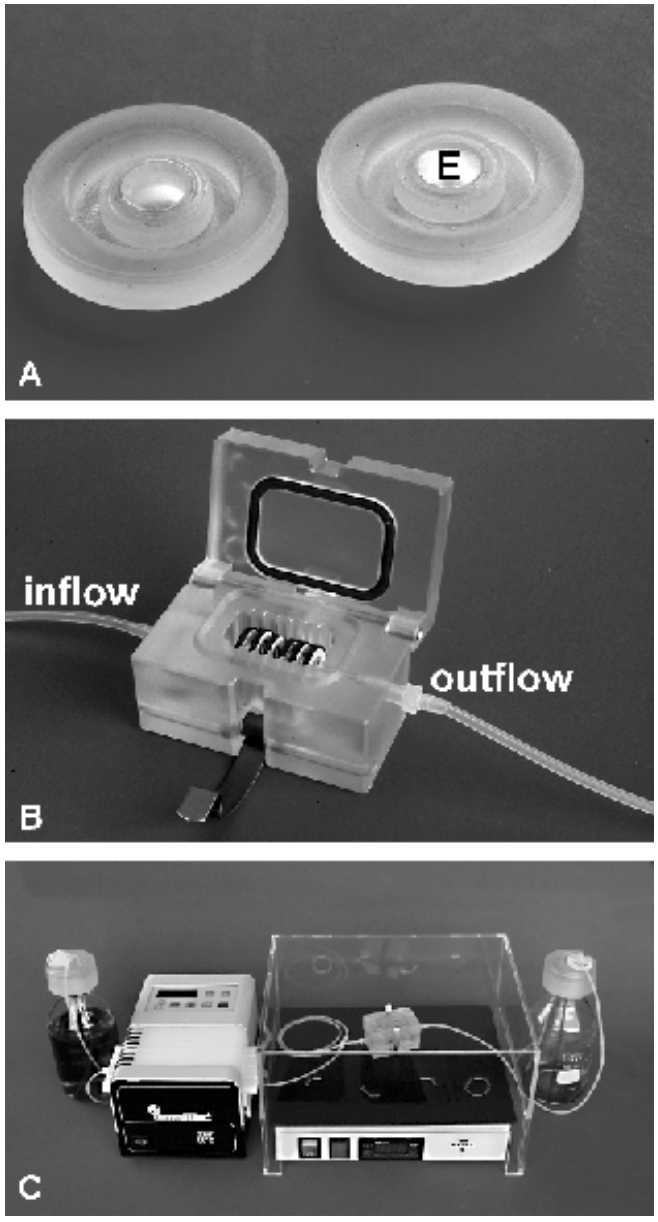


Fig. 1. Embryonic CD epithelia in perfusion culture. (A) Renal explants (6 mm in diameter, E) with embryonic CD ampullae are mounted in tissue carriers. After 24h a polarized collecting duct epithelium has developed in medium containing serum. (B) 6 carriers with the generated epithelia are placed in a perfusion culture container. (C) A peristaltic pump transports the medium (1 ml/h) and a thermo plate maintained a constant temperature of 37^0 C during the 14-d culture period under serum free conditions.

D*ensitometric analysis of western blotting*

Labeled bands of western blotting were semiquantitatively analyzed using a photounit (Kaiser, Hamburg, Germany) and the Bioprofil Bio 1d software (Ltf Labortechnik, Wasserburg, Germany). Optical densities of the bands were given as relative densitometric units.

Results

Immunodetection of COX in the collecting duct within the developing rabbit kidney. The outer cortex of the neonatal rabbit kidney consists of a fully embryonic zone, while half matured and matured tissue is found in the mid and inner cortical part, respectively within the medulla. Along this cortico - medullary axis of development we performed immunohistochemical experiments to elucidate the primary appearance of COX 1 and 2 (Fig. 2 and Table 1).

Embryonic zone: In the fully embryonic zone in the outer cortex we found COX 1 immuno-labeling within the ampulla (Fig. 2A). All the cells within the ampullar tip, neck and shaft region revealed an intensive cytoplasmatic reaction for COX 1. Incubation of the same tissue slice with mab P_{CD} Amp1 (Fig. 2B) allowed us to clearly localize the basal aspect of the CD ampulla and to relate the site of COX 1 expression. In contrast to the ampullar epithelium neighbouring S- shaped bodies and developing microvasculature revealed no immunolabelling for COX 1. Using COX 2 antibody a cellular reaction within the collecting duct ampulla could not be observed (Fig. 2C). However, a faint reaction was seen in the cell layer surrounding the ampulla. Control incubation with mab P_{CD} Amp1 demonstrated the basal aspect of the same branching CD ampulla (Fig. 2D).

CCD: Further downwards along the developmental axis in the mid cortical region the CCD showed no detectable cytoplasmatic reaction after COX 1 immunolabeling, while the parietal cells of Bowman‘s capsule demonstrated a strong labeling (Fig. 2E). In contrast to observations on the maturing rat kidney [32], COX 2 was prominently found in cells of Bowman‘s capsule and in mesangial cells of the glomerulus (Fig. 2F). Only few cells of the macula densa were labeled by the COX 2 antibody (Fig. 2F, arrow), while matured cortical CD cells were completely negative.

OMCD: In the outer medullary CD (OMCD) only part of cells showed an intensive label after COX 1 (Fig. 2G) and COX 2 (Fig. 2H) antibody incubation. Coincubation experiments with PNA (Peanut agglutinin), which binds to B-type intercalated cells in the CD [33], displayed no colocalization with COX 1 and 2 at this site (no figure).

IMCD: In the inner medullary collecting duct (IMCD) nearly all of the cells revealed a positive reaction for COX 1 and 2 with a pronounced signal in the perinuclear region. In addition, medullary interstitial cells were immunoreactive for COX 1 (Fig. 2I) and COX 2

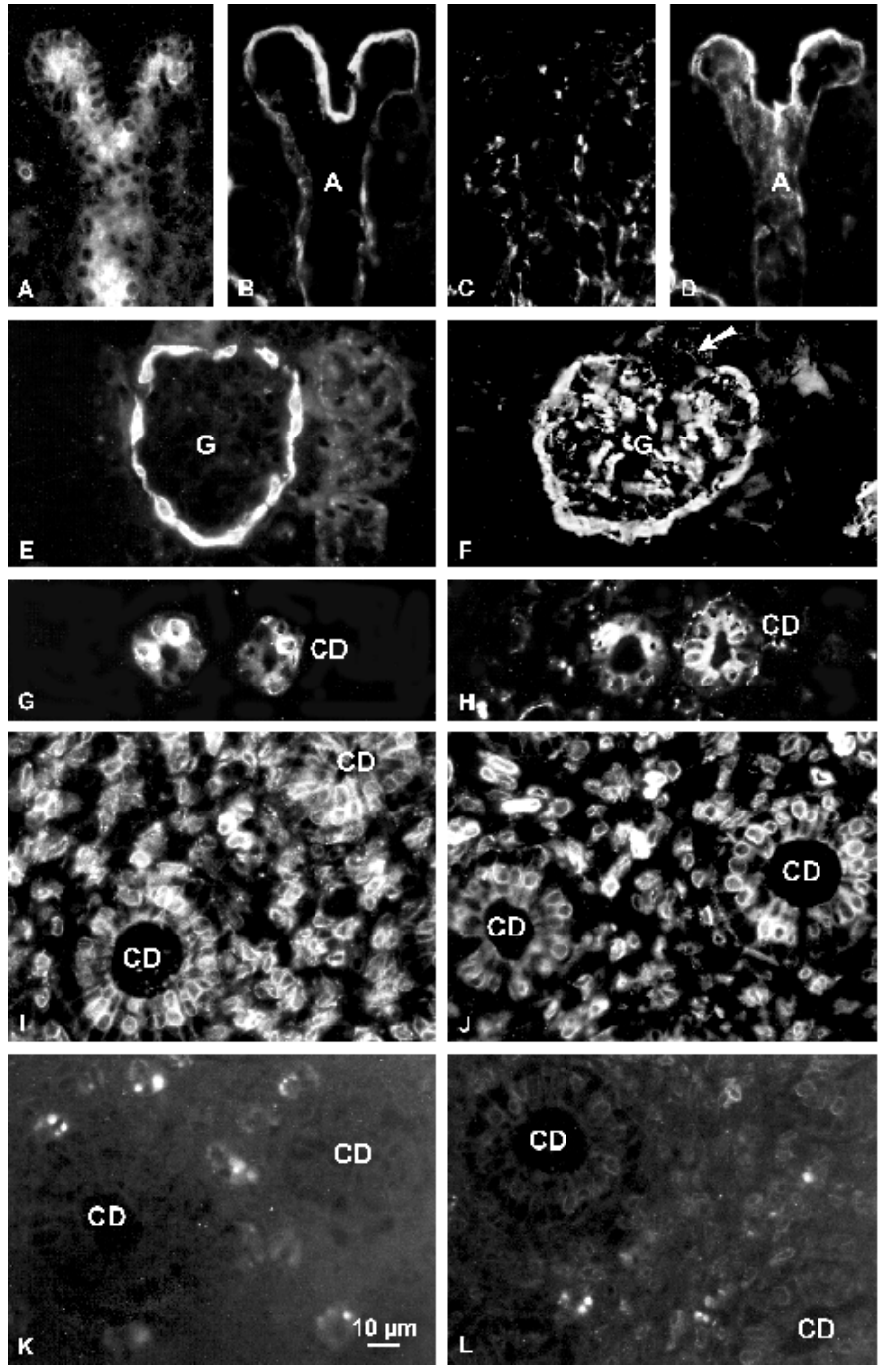


Fig. 2. Immunohistochemical localization of COX 1 and 2 in the neonatal rabbit kidney. Embryonic outer cortex with CD ampulla: (A) COX 1 in the CD ampulla tip, neck and shaft region. (B) Labeling the same tissue with mab P_{CD}Amp1 illuminates the basal aspect of the ampulla. (C) COX 2 labeling is absent in the same ampulla on a consecutive cryosection, but appears in interstitial cells surrounding the ampulla. (D) Labeling with mab P_{CD}Amp1. Midcortex: (E) COX 1 labeling is present in the parietal cells of Bowman's capsule of the glomerulus, but not on the CCD. (F) COX 2 labeling is found in the mesangial cells and in the parietal cells of Bowman's capsule of the glomerulus, but not on the CCD. The cytoplasm of few macula densa cells is labelled by COX 2 antibody (arrow in F). Outer medulla: (G) Part of the CD cells are labeled for COX 1. (H) Same findings for COX 2. Inner medulla: COX 1 (I) and COX 2 (J) are found in nearly all cells of the inner medullary collecting duct with a perinuclear enhancement of signal. The label is also found in interstitial cells. Peptide competition experiments revealed for COX 1 (K) and COX 2 (L) a specific binding in CD cells and in interstitial cells. (A = ampulla; G = glomerulus; A, B, C, D: longitudinal sections through the ampulla; E – L: cross sections through the collecting duct).

(Fig. 2J). Labeling of the antibody on segments of Henle's loop can be excluded since no co-reaction with PNA at this site was found (no figure). Incubation of COX 1 (Fig. 2K) and COX 2 (Fig. 2L) antibodies in the presence of competitive blocking peptides revealed an inhibition of reaction on the antigenic site of CD cells and interstitial cells. These results demonstrate the specificity of labeling on the tissue section.

Immunodetection of COX in the collecting duct within the adult rabbit kidney. COX 1 (Fig. 3A) and 2 (Fig. 3B) immunoreactivity was observed in the cytoplasm of cells of Bowman's capsule. COX 2 labeling was present in the glomeruli (Fig. 3B). As demonstrated for COX 1 in figure 3C and for COX 2 in figure 3E, both antibodies displayed binding to collecting duct cells without binding to all cells. Cells revealing a lack of

Table 1. Localization and expression of COX 1 and 2 protein in the collecting duct of neonatal rabbit kidney and embryonic CD epithelium cultured in a perfusion container (- no reaction, +++++ strong reaction); Number of investigated tissues stated in parentheses.

	COX 1	COX 2
Neonatal rabbit kidney (n = 28):		
CD ampulla cells	++	-
cortical CD cells (CCD)	-	-
outer medullary CD cells (OMCD)	+++	+++
Inner medullary CD cells (IMCD)	+++	++
Cultured CD epithelium (n = 58):		
Tissue before culture (n = 24)	+++	(+)
IMDM without supplements (n = 18)	+++	+++
Aldosterone treatment (n = 20)	+++	+++
100 mmol/l NaCl (n = 12)	+++	+++++
100 mmol/l Mannitol (n = 8)	+++	+++

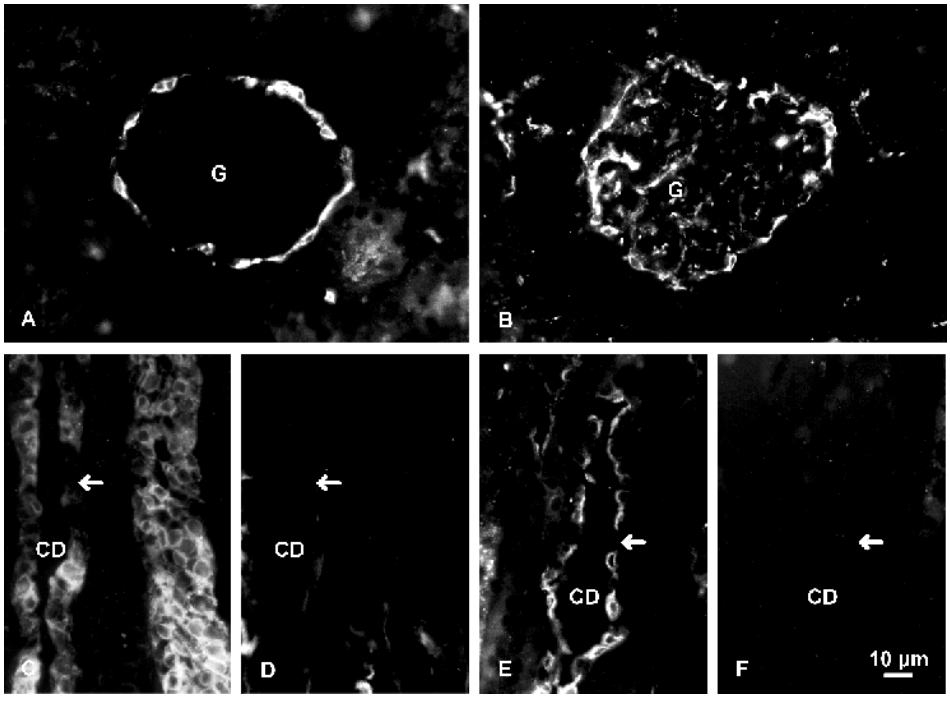


Fig. 3. Immunohistochemical localization of COX in the collecting duct of the adult rabbit kidney. COX 1 (A) and 2 (B) immunoreactivity was observed in the cytoplasm of cells of Bowman's capsule. Additional COX 2 labeling was present in the glomeruli (B). As demonstrated for COX 1 (C) and for COX 2 (E), both antibodies displayed binding to the collecting duct without binding to all cells. Cells revealing a lack of COX 1 (C) and COX 2 (E) immunoreaction showed in the same tissue slices no PNA (peanut agglutinin) binding (D,F). (G = glomerulus, CD = collecting duct, asterisks = cells negative for COX and PNA).

COX 1 and COX 2 immunoreaction were negative for PNA (peanut agglutinin) indicating that the COX 1 and 2 negative cells do not express B-type intercalated cell features (Fig. 3 D and F).

Time dependent appearance of COX 1 and 2. Immunohistochemistry demonstrated that COX 1 and 2 do not appear at the same time in the embryonic collecting duct (Fig. 2A and C). To gain information about the time course of COX 1 and 2 upregulation, embryonic CD epithelium was isolated and cultured up to 14 days. The appearance of COX 1 and 2 was registered by western blotting analysis (Fig. 4A and B). Immunoreaction for COX 1 was detected already after isolation of the embryonic tissue and was continuously present until day 14 in culture (Fig. 4A). This early expression of COX 1 was already described in the CD ampulla by immunhistochemistry (Fig. 2A). In contrast, only a very faint signal for COX 2 immunoreactivity was present after isolation of the embryonic tissue (Fig. 4B). This low degree of expression of COX 2 in the CD ampulla was confirmed by immunohistochemistry in the cortex of neonatal kidney (Fig. 2C). Then at day 3 the signal for COX 2 increased and was expressed to the same intensive level from day 4 until day 14. On the transcriptional level the time dependent expression could be confirmed by a ribonuclease protection assay (Fig. 4C). A low signal for COX 2 mRNA was detected after tissue isolation, while intensive signals were found after 14 days of culture.

COX 1 and 2 expression in generated CD epithelia after 14 days of culture. The next series of experiments should reveal in how far the expression of COX 1 and 2 occurs in cultured CD epithelium derived form the embryonic zone of neonatal kidney. After isolation the embryonic CD epithelia were cultured under permanent perfusion of medium and under serum free conditions. Immunohistochemistry showed the presence of COX 1 (Fig. 5A) and 2 (Fig. 5B) in all cells of epithelia cultured in IMDM containing 1 x 10^{-7} M aldosterone after 14 days. Immunohistochemistry indicated no clearly visible difference in the level of protein expression. Peptide competition experiments revealed a complete reduction of immunoreaction for both antibodies (COX 1: Fig. 5C; COX 2: Fig. 5D).

Modulation of COX 1 and 2 expressions in embryonic CD epithelia. In a further set of experiments we tested in how far the expression of COX 1 and 2 can be modulated under culture conditions (Table 1). As revealed in western blot experiments COX 1 reaction was found as a 70 kDa and 72 kDa band (Fig. 6, lane 1), while COX 2 was detected as a single 73 kDa band (Fig. 6, lane 6) testing cultured epithelia. Control western blotting experiments with COX 1 antibody confirmed labeling of the 70 kDa and 72 kDa band in the adult rabbit kidney (Fig. 6, lane 4).

Regarding immunohistochemistry (Fig. 5), we were not able to determine whether a change in COX reactivity induced by NaCl could be seen. For that reason western blotting experiments were performed. The same degree of expression for COX 1 (Fig. 7B lane 1and 2) and COX 2 (Fig. 7D lane 1and 2) was observed after 14 days in the presence of 1 x 10^{-7} M aldosterone or without hormonal application. In contrast, as revealed by densitometric analysis a fourfold and maximal increase of COX 2 immunoreactivity was obtained after chronic application of 100 mmol/l NaCl to the basal culture medium (Fig. 7D lane 3). A combination of aldosterone and increasing NaCl concentration did not show a further increasing effect (Fig. 7D lane 4).

The addition of NaCl to the culture medium is combined with an increase of osmolarity of the culture medium. Consequently, mannitol was used as substance to control the possible influence of osmolarity on COX 1 and 2 expression. Addition of 100 mmol/l mannitol to the culture medium did not influence COX 2 expression in the cultured epithelia (Fig. 7D lane 5 and 6).

Immunohistochemistry of COX 1 and COX 2 protein revealed that the NaCl load induced COX 2 expression was exclusively restricted to the cultured CD epithelia (Fig. 5F). No immunoreaction could be detected in the mesenchymal tissue underneath the epithelium.

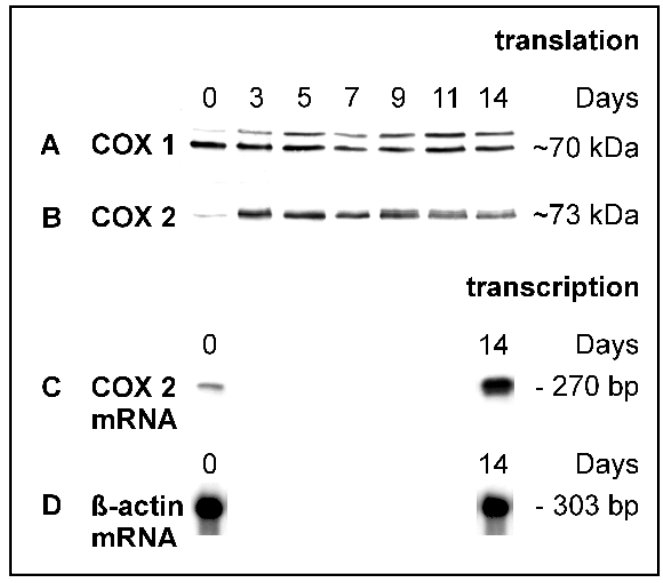


Fig. 4. (A,B) Western blot analysis and (C) COX 2 protection assay of embryonic CD epithelia cultured up to 14 days. 30 µg protein was loaded in each lane. (A) COX 1 is already present in the isolated tissue and remains unchanged until 14 days. (B) COX 2 is upregulated at day 3. (C) The amount of mRNA for COX 2 was low in the freshly isolated tissue and is increased in the tissue cultured 14 days. (D) ß-actin mRNA as control.

Two dimensional electrophoresis and subsequent western blot experiments. Blocking experiments of COX 1 antibody with epitope peptide for COX 2 (Fig. 6, lane 3) and vice versa incubations of COX 2 antibody with COX 1 epitope peptide (Fig. 6, lane 7) revealed binding of both antibodies to the same bands as detected by COX 1 and 2 antibody without blocking peptides. This result suggests specificity of epitope binding without cross reactivity. The specificity further could be confirmed by two dimensional electrophoresis and subsequent western blot experiments. To the best of our knowledge two dimensional electrophoresis with subsequent western blotting for COX 1 and 2 antibodies were not previously published. Both antibodies revealed binding on definetively different protein spots (Fig. 8). The COX 1 antibody (Fig. 8A) recognized 5 spots (spot number 1: 70 kDa, pI 6,8; spot number 2: 70 kDa, pI 6,6; spot number 3: 47 kDa, pI 8,4; spot number 4: 47 kDa, pI 6,6; spot number 5: 47 kDa; pI 5,7). The COX 2 antibody (Fig. 8B) showed staining of 6 spots (spot number 1: 73 kDa, pI 7,2; spot number 2: 72 kDa, pI 6,9; spot number 3: 45 kDa, pI 7,1; spot number 4: 45 kDa, pI 6,4; spot

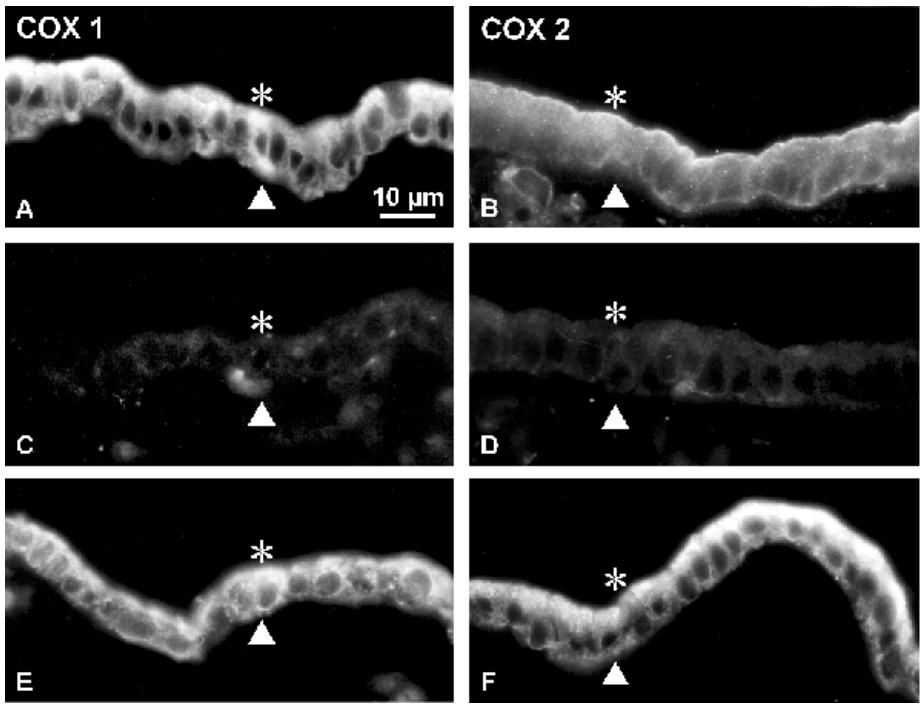


Fig. 5. Immunohistochemistry of COX 1 and COX 2 in the generated collecting duct epithelia after 14 days of culture. COX 1 (A) and COX 2 (B) immunoreaction was observed in all cells of the CD epithelia. (C) Simultaneous exposure of COX 1 antibody and blocking peptide during incubation leads to a complete loss of immunoreaction in the epithelia and demonstrates the specificity of labeling. (D) Same effect was found for COX 2. Restricted immunoreaction to the epithelial cells could be detected for COX 1 (E) and COX 2 (F) after 100 mmol/l NaCl addition to the culture medium. (asterisk = luminal side, arrow = basal aspect of the epithelium).

number 5: 46 kDa; pI 5,8; spot number 6: 45 kDa; pI 4,7). It is evident that both antibodies reveal binding not to a single but to several spots. Spot 1 and 2 of COX 2 antibody label is in accordance to earlier published molecular weight data [11]. Spot 3 - 6 belong to up to date unknown proteins and were not detected in such intensity in one dimensional electrophoresis (Fig. 7). COX 2 label shows on SDS-PAGE a single band with ~73 kDa, but in two dimensional electrophoresis it consists of spot 1 and 2. The lower bands, around 45 kDa, spread into spot 3, 4, 5 and 6 (Fig. 8A). According to the demonstrated isoelectric points COX 1 (6,6;6,8) and 2 (6,9; 7,2) can be discriminated as different antigens in 2-D electrophoresis.

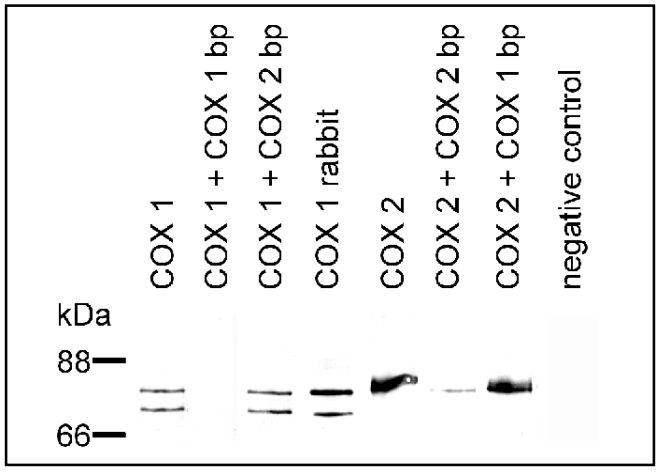
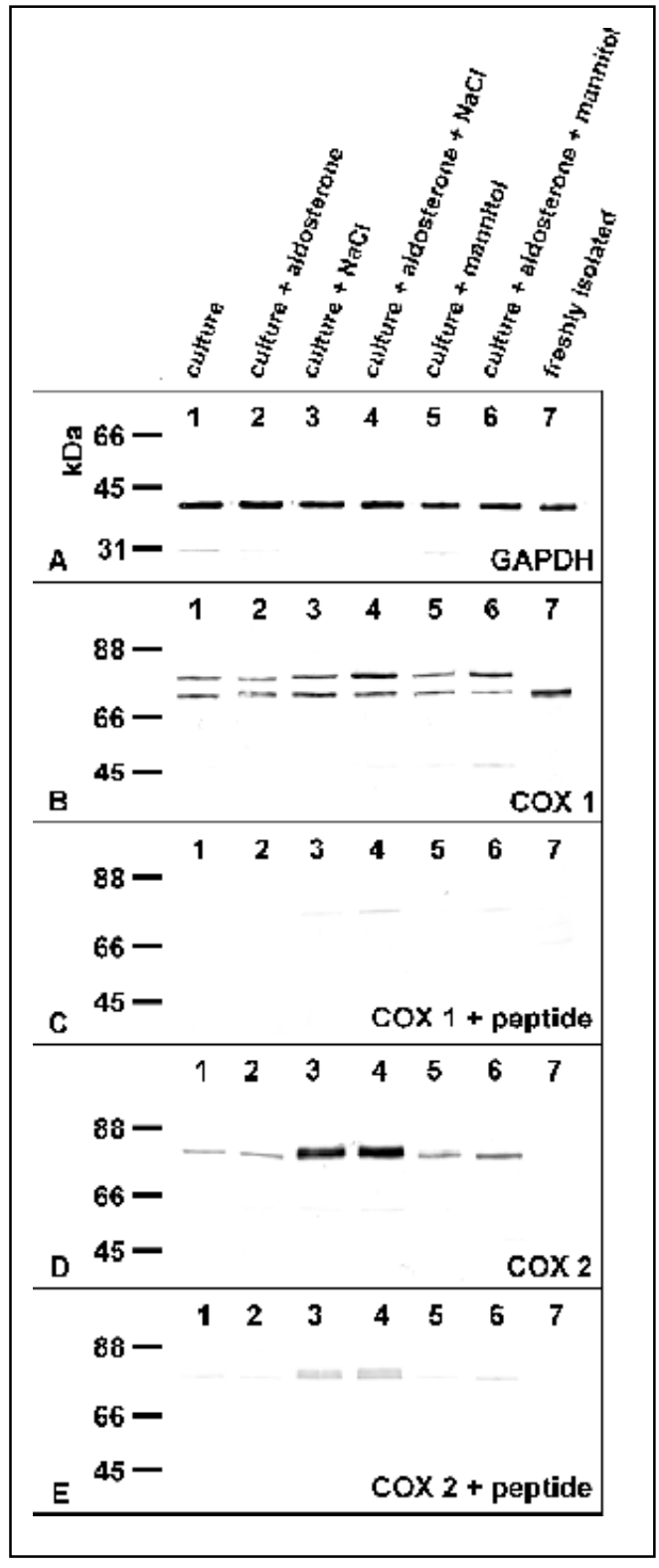


Fig. 6. Western blotting experiments with blocking peptides to control specificity of particular antibodies. 30 µg protein was loaded in each lane. Lane 1: COX 1 antibody probed with cultured tissue. Lane 2: COX 1 antibody in addition with COX 1 blocking peptide probed with cultured tissue. Lane 3: COX 1 antibody in addition with COX 2 blocking peptide probed with cultured tissue. Lane 4: COX 1 antibody probed with rabbit kidney. Lane 5: COX 2 antibody probed with cultured tissue. Lane 6: COX 2 antibody in addition with COX 2 blocking peptide probed with cultured tissue. Lane 7: COX 2 antibody in addition with COX 1 blocking peptide probed with cultured tissue. Lane 8: negative control (Only secondary antibody was applied).

Discussion

The aim of the present immunohistochemical and biochemical experiments was to analyze the transition from an embryonic to a matured collecting duct epithelium. The neonatal rabbit kidney is an ideal model organ to follow this development, since on a clearly orientated cortico - medullary tissue section increasingly matured zones can be compared. Fully embryonic structures are found in the outer cortex, while maturing and matured tissues are present in the inner cortex and medulla. On such tissue sections we tested the immunohistochemical appearance of COX 1 and 2 by commercially available antibodies (Fig. 2). The same antibodies were tested earlier by other authors in we-

Fig. 7. Western blot experiments to investigate modulation of COX 1 and 2 expression in embryonic CD epithelia after 14 days of culture. 15 µg protein was loaded in each lane. Lane 1: IMDM; Lane 2: IMDM + aldosterone; Lane 3: IMDM + 100 mmol/l NaCl; Lane 4: IMDM + aldosterone + 100 mmol/l NaCl; Lane 5: IMDM + 100 mmol/l mannitol; Lane 6: IMDM + aldosterone + 100 mmol/l mannitol; Lane 7: Freshly isolated tissue before culture. (A) GAPDH expression in the different treated CD epithelia as control. (B) No differences in COX 1 expression were found after 14 days of culture. (C) Nearly complete inhibition of immunoreaction was revealed after addition of the blocking peptide. (D) A clear upregulation of COX 2 is detectable after 100 mmol/l NaCl addition to the culture medium independent of aldosterone treatment. (E) Densitometric analysis showed that the immunoreaction was reduced to 30% after addition of COX 2 blocking peptide. The probe with freshly isolated tissue showed COX 1 immunoreactivity with only one band (B), while COX 2 is lacking (D).



stern blot experiments in rabbit kidney without showing morphological data on the protein level [12]. Mostly, detection of COX 1 and 2 was performed on formaline fixed and paraffine embedded tissue in rat kidney with antibodies generated in rabbit [7, 32]. The up to date published literature shows that the expression of cyclooxygenases is species-dependent within the kidney [5]. The occurrence of these enzymes within the collecting duct system is described for COX 1 in all CD parts in dog, rat, monkey and human [5]. In contrast, COX 2 expression is found within the CD in adult rats only after chronic dehydration, while it is not found in controls [8]. In situ hybridization experiments of adult rabbit kidney showed COX 2 upregulation in medullary interstitial cells and not within the collecting duct in water deprivated animals [12].

Altering antigenic sites during formalin fixation and paraffin embedding may cause the inherent localization of COX within kidney. Consequently, we investigated in the present paper the appearance of COX 1 and 2 on cryosections of neonatal kidneys with minimal fixation in ethanol. Using the same COX 2 antibody as described by Hao et al. [12], we found that both enzymes are constitutively expressed in the matured medullary collecting duct without labeling the cortical CD (Fig. 2). Further we found COX 1 immunolabel in the embryonic zone at most cells of the collecting duct ampulla (Fig. 2A). This particular site of occurrence seems to be interesting, since COX 1 colocalizes with proteins such as Wnt-11 [34], Pax-2 [35], BMP-7 [36], which are involved in the induction process of nephrogenesis at the tip of the ampulla [37]. In contrast, no immunoreaction was found for COX 2 in the collecting duct ampulla (Fig. 2C). A faint label is found at the epithelial interface outside of the ampulla on mesen-

chymal cells. This result contrasts to experiments on the developing rat kidney, where a diffuse COX 2 immunoreaction was found on the branching collecting duct ampulla [32], however it confirms data on fetal human kidney [6]. The exclusive presence of COX 1 within the CD ampulla (Fig. 2A) points out a prostaglandin synthesis pathway involved in nephron induction or in CD maturation. Because of the lack within the ampulla such a role cannot be described to COX 2. A role of the immunopositive interstitial cells surrounding the ampulla cannot be explained yet (Fig. 2C).

Previous studies showed that COX 2, but not COX 1 gene disruption in mice led to disorders in renal development [20]. Investigations with COX 2 inhibitors confirmed this role of COX 2 in renal development of rodents [21]. In rabbit the renal development is not restricted to the gestation period. At the day of birth an immature zone in the subcapsular region still can be observed. However, at the day of birth we found only very weak signals for COX 2 (Fig. 4B,C) and clear signals for COX 1 (Fig. 4A). Others studies revealed a decline of COX 2 protein expression during four days after birth in mice and rat, but a constant expression of COX 1 from birth until six weeks after birth [21]. For that reason it remains unlikely that only COX 2 isoform has a functional role during tubulogenesis.

Several publications have shown that in rat COX 2 is located within the macula densa region and the adjacent thick ascending limb [7]. Salt restriction in animal models yielded an increased expression of COX 2 in the cortex region [7]. Other experiments showed in the medullary collecting duct and in IMCD-K2 cells that COX 2 is upregulated by hyperosmolarity [8]. It is assumed that produced prostaglandins act as functional antagonists of vasopressin induced water absorption in the collecting duct [38]. As revealed by mRNA protection assay and western blot, it is well established that the expression of COX 2 in the kidney medulla is increased by a NaCl load [8, 9]. However the cellular localization is contradictory discussed. Most of the experiments were performed on cultured cells and not in kidney. Hao et al. found that the expression of COX 2 in medullary interstitial cells of the rabbit kept in medium containing serum was stimulated by NaCl as well as by mannitol whereas urea showed no effect [8, 12]. Yang et al. described an increased expression of COX 2 in MDCK cells after addition of NaCl, mannitol, raffinose and sucrose and demonstrated an increased immunoreactivity

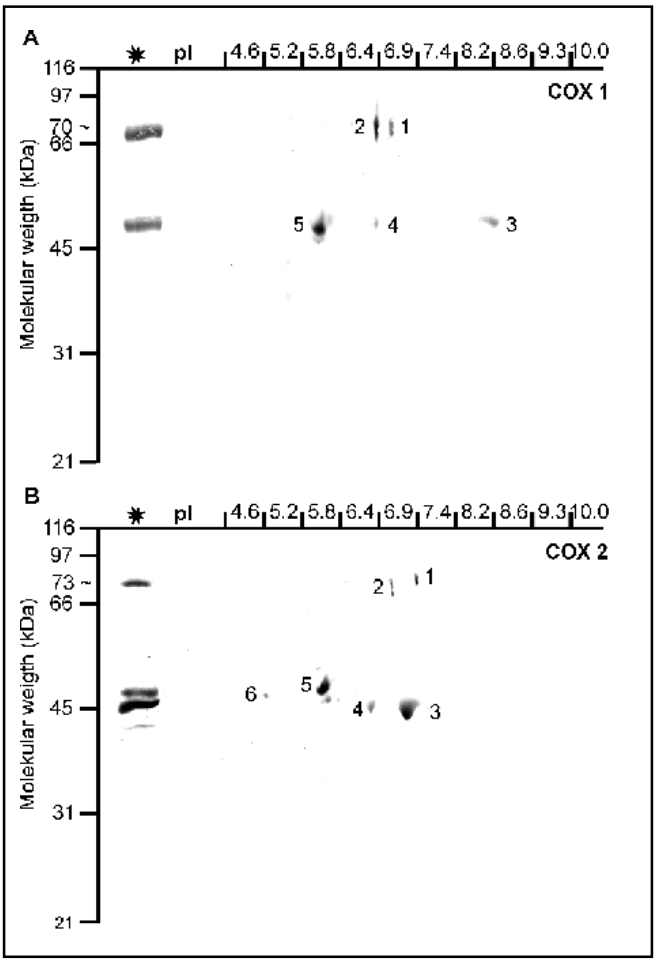


Fig. 8. 2-D electrophoresis with subsequent western blot to localize COX 1 (A) and COX 2 (B) immunoreaction. The COX 1 antibody recognized 5 spots (spot number 1: 70 kDa, pI 6,8; spot number 2: 70 kDa, pI 6,6; spot number 3: 47 kDa, pI 8,4; spot number 4: 47 kDa, pI 6,6; spot number 5: 47 kDa; pI 5,7). The COX 2 antibody showed staining of 6 spots (spot number 1: 73 kDa, pI 7,2; spot number 2: 72 kDa, pI 6,9; spot number 3: 45 kDa, pI 7,1; spot number 4: 45 kDa, pI 6,4; spot number 5: 46 kDa; pI 5,8; spot number 6: 45 kDa; pI 4,7). It is evident that both antibodies reveal binding not to a single but to several spots. Spot 1 and 2 label is in accordance to literature data of COX 1 and 2 protein, while spot 3 - 6 belong to unknown proteins. According to the isoelectric points COX 1 and 2 can be discriminated as different antigens. The lane beyond the asterisks shows an one dimensional separation of COX enzymes, which was run for control in the same plate during two dimensional separation. (A) The 70 kDa COX 1 band consists in two dimensional electrophoresis not of one compound but of two spots (spot 1, 2). The lower 47 kDa band consists of spot 3, 4 and 5. (B) COX 2 shows on SDS-PAGE a single band with 73 kDa, but in two dimensional electrophoresis it splits into spot 1 and 2. The lower bands, around 45 kDa, split into spot 3, 4, 5 and 6.

for COX 2 in the medullary collecting duct of water deprived rats [8]. Similarly, urea has been shown to stimulate the expression of COX 2 in a cell line of the inner-medullary collecting duct of the mouse (mIMCD-K2) [8]. Taken together these data suggest that the expression of COX 2 both in medullary interstitial cells and in collecting duct cells is stimulated by an increase of the osmolarity.

In contrast, in our present experiments we found that the expression of COX 2 in the generated collecting duct epithelia was increased by an elevated concentration of NaCl (Fig. 7D lane 3 and 4). However, the expression of COX 2 was not influenced by mannitol (Fig. 7D lane 5 and 6) pointing to a NaCl load specific effect. Further, we performed culture experiments with embryonic CD epithelia under chronic hormonal treatment and increased NaCl exposure to elucidate possible mechanisms triggering the development from an embryonic towards a functional CD epithelium. The origin and functionality of the cultured epithelium was shown in previous investigations [25]. In perfusion culture an embryonic epithelium can be promoted into a matured form, which has lost proliferating activity and stays in the interphase as it is observed in the adult kidney. This is the only method to investigate terminal differentiation on such a model as compared to proliferating cells in a culture dish. Earlier experiments further showed that differentiation can be initiated by a permanent luminal NaCl load over 14 days in gradient perfusion culture, which resulted in an upregulation of facultatively expressed proteins, while constitutive protein synthesis was not affected [26]. The antiparallel downregulation of the embryonic P_{CD} Amp 1 antigen shows the maturation process of the epithelia after a chronic NaCl load [24]. The present data show an upregulation of COX 2 protein in the first 3 days of culture and a constant expression until day 14. COX 1 expression in the cultured CD epithelia remains stable during the whole culture period (Fig. 4). COX 2 expression on the protein level was clearly enhanced after addition of 100 mmol/l NaCl to the culture medium (Fig. 7D lane 3 and 4), while no increased COX 1 was found (Fig. 7B lane 3 and 4).

Earlier experiments have shown that aldosterone is an important differentiation factor for embryonic collecting duct epithelia kept in perfusion culture [17]. Although aldosterone is able to upregulate different proteins, the present experiments show that the hormone does not affect the COX 1 (Fig. 7B lane 1 and 2) and COX 2 expression (Fig. 7D lane 1 and 2). In contrast to experiments on adrenalectomiced adult rats [39], we could not notice such a modulation effect in embryonic epithelia provoked by a mineralocorticoid. The upregulation of COX 2 and maintenance of COX 1 in the cultured epithelia reflects the status, which is found in the matured IMCD of neonatal rabbit kidney and demonstrates a step in CD cell differentiation. The facultative character of the COX 2 expression in embryonic CD epithelia further suggests the influence of electrolytes in the late phase of collecting duct cell development.

In the one-dimensional western blot experiments COX 1 immunolabel was found on 70 and 72 kDa band (Fig. 6, lane 1), while COX 2 was detected on a 73 kDa band (Fig. 6, lane 6). In contrast, performing two-dimensional electrophoresis, the COX 1 antibody labeled spot 1 - 5 (Fig. 8A) and the COX 2 antibody labeled spot 1 – 6 (Fig. 8B). This experiment clearly shows that the two-dimensional electrophoresis revealed for COX 1 and 2 antibodies much more labeled spots as compared to one-dimensional electrophoresis. At the moment we can only show but not explain this results. However, it must be taken into consideration that the same epitope within different molecules is recognized by both antibodies. Crossreactivity of both antibodies could be excluded by blocking peptide assays in immunohistochemistry (Fig. 5C,D) and in western blot experiments (Fig. 6, Fig. 7C,E). Independent of this fact, the results further show that the frequently used antibodies recognize not a single band respectively spot, but different molecules in neonatal rabbit kidney. In the future, it would be interesting to investigate if beside COX 1 and 2 further isoforms of cycloxygenases exist, which may be related to the detected molecules.

In summary, our data show the primary expression of COX 1 and 2 during the terminal phase of collecting duct epithelium development using neonatal rabbit kidney as model system. The localization of only COX 1 in the CD ampulla indicates a possible functional role of the enzyme during tubulogenesis. The COX 2 expression is retarded and is inducible by increasing NaCl application in embryonic CD epithelia. Finally, we found COX 1 and 2 to be colocalized in cells of the medullary collecting duct.

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