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Urea Restrains Aldosterone-Induced Development of Peanut Agglutinin–Binding on Embryonic Renal Collecting Duct Epithelia

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Abstract. Peanut agglutinin (PNA) represents a commonly used marker for β-type intercalated (IC) cells and their distribution in the corticomedullary course of the collecting duct (CD) in the mature rabbit kidney. It has been shown that aldosterone is able to generate >90% of PNA-binding cells in an embryonic CD epithelium *in vitro*. In adult kidney, a maximum of only 25% PNA-positive cells is found in the cortical segment of the CD, and PNA-binding completely disappears in the inner-medullary CD. Molecules that regulate the gradual development of CD-specific cells during organ growth are unknown. In the present experiments, it was found that addition of physiologic concentrations of urea to the culture medium is able to restrain the action of aldosterone in embryonic CD epithelia. Urea antagonizes in a concentration-dependent manner the action of aldosterone finally leading to only 10% of PNA-binding cells. The data point to a urea-specific effect, because osmolytes such as NaCl and mannitol did not affect PNA binding. In addition, urea did not influence expression of principal-cell typical markers such as AQP2 and 3. The findings may explain that a higher number of PNA-positive cells is found in the cortical region of the kidney correlated with a low concentration of urea as compared with only few PNA-binding cells in the medullary CD, where a high concentration of urea occurs. Thus, an increasing concentration of urea may trigger the number of PNA-positive cells in the cortical-medullary course of the CD during organ development.

Embryonic development of the metanephros starts by the morphogenic interaction between the ureteric bud and the surrounding mesenchyme (1,2). Dichotomous branchings of the ureteric bud epithelium determine first the kidney poles, then the anterior and posterior half of the organ, the number of pyramids, and finally the amount of collecting ducts (CD) that reach the future area cribrosa (1,2). In a subsequent process, the induction of the nephrons takes place. This happens in a highly coordinated process between the CD ampullae as derivatives of the ureteric bud and the cap condensates (3,4). By the exchange of morphogenic signals between both tissues, part of the cap condensate separates and forms at the lateral side of each CD ampulla the pretubular aggregates as a first visible sign of nephron development (4–8). Organ growth proceeds when the CD ampulla tip divides dichotomously and elongates toward the capsule. By this mechanism, each tip of the CD ampulla determines the complete future microarchitecture of the kidney.

The functional development of the CD epithelium occurs in an antiparallel manner as opposed to the growth direction of the CD ampulla (Figure 1). Each CD ampulla in the embryonic part of the developing rabbit kidney consists of three different zones (9). Zone 1 is located within the tip of the ampulla. It is the niche for epithelial stem cells (9), which are capable of initiating nephrogenesis in the surrounding mesenchymal stem cells of the cap condensate (8,10). Zone 2 comprises the neck of the ampulla. This area appears gray in the light microscope and contains multiple dividing cells, which cause the elongation of the CD tubule during the dichotomous branching process. Zone 3 comprises the shaft of the ampulla and is recognized by the primary appearance of principal (P) and various types of intercalated (IC) cells (9,11,12).

While the morphogenic signals between the CD ampulla and the cap condensate are being investigated intensively (4–8), little knowledge is available about the mechanism that leads to the functional development of the CD epithelium. During maturation, the embryonic epithelium within the ampulla shaft develops into a heterogeneously composed epithelium consisting of functional P and various types of IC cells (11). This process was investigated earlier by morphologic (13), immunohistochemical (9), and histochemical (12,14) methods. Especially peanut agglutinin (PNA) served as a histochemical marker to follow the development of β-type IC cells (12,14). Although it has been shown in culture experiments that β-type IC cells may undergo conversion to P cells (15), data obtained from tissue sections of neonatal rabbit kidney indicated that P and IC cells derive from a common precursor cell located within the CD ampulla by asymmetrical cell division (9,16).

The cell biologic mechanism that leads from a precursor cell within the ampulla to functional P and various types of IC cells is unknown (17). It has been shown that alkali feeding of

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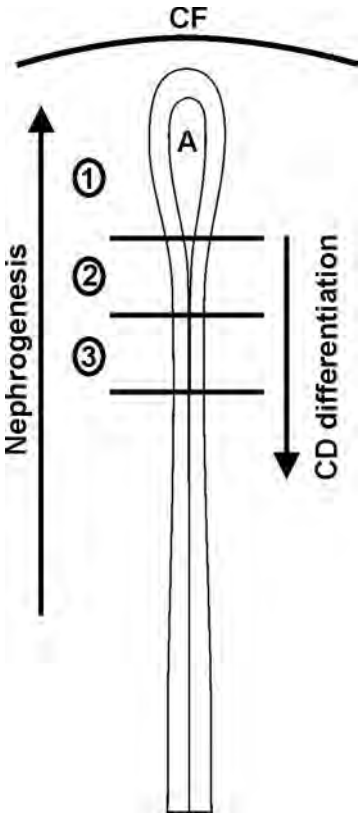

Figure 1. The antiparallel direction of collecting duct (CD) ampulla growth and CD cell maturation. Zone 1, ampulla tip; zone 2, ampulla neck; zone 3, ampulla shaft.

pregnant mothers triggers an earlier appearance of β-type IC cells compared with controls (18). Furthermore, it was demonstrated that the electrolyte environment of cultured embryonic CD epithelia is able to influence the development of P and IC cell features (19). Experiments with a tissue-specific model additionally showed that application of aldosterone in the culture medium increased the amount of PNA-positive cells in maturing CD epithelia (20). More than 90% PNA-positive cells were consistently found, indicating that a feature of β-type IC cells is developed (20). However, this high number of PNA-positive cells is an artificial overexpression under *in vitro* conditions and is never found within the adult rabbit kidney (12). Here, a maximum of 10 to 25% PNA-positive cells was detected in the cortical collecting duct (CCD). Thus, on the one side, aldosterone promotes the development of PNA-binding cells under *in vitro* conditions to a high degree, but, on the other side, this amount of cells is not found in the adult kidney despite the presence of the hormone. Consequently, the action of aldosterone must be restrained by a growth factor or by the environment during kidney development.

In the present experiments, we show data of embryonic CD epithelia cultured in the presence of aldosterone and urea. We demonstrate that embryonic CD epithelia tolerate a long-term application of urea over 14 d. Physiologic concentrations of urea are able to modulate the development of PNA binding on cells of the developing CD epithelium and restrain the effect of aldosterone.

Materials and Methods

Tissue Preparation for Histochemistry

One-day-old New Zealand rabbits were anesthetized with ether and killed by cervical dislocation. Both kidneys were removed immediately. The kidneys were then cut precisely along the corticomedullary axis, frozen in liquid nitrogen, and stored at −80°C up to the subsequent treatments.

Tissue Preparation for Generation of Embryonic CD Epithelia

Generation of embryonic CD epithelia was performed by isolating cortical embryonic explants from the kidneys of newborn New Zealand rabbits (up to 1 d old) according to methods described earlier (21). The explants consisted of a piece of capsule fibrosa with adherent CD ampullae, S-shaped bodies, and nephrogenic blastema, which were mounted in tissue carriers. For generation of an embryonic CD epithelium, the carriers were placed for 24 h 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; Life Technologies BRL Life Technologies, Eggenstein, Germany) including 10% FBS (Boehringer, Mannheim, Germany), an outgrowth of cells from the CD ampullae was observed. Within 24 h, the entire surface of the explant (6 mm in diameter) was covered by a monolayer of polarized CD cells that were positive for cytokeratin-19.

Perfusion Culture with Generated CD Epithelia

After initiation of culture, the tissue carriers were placed in a perfusion culture container (Minucells and Minutissue, Bad Abbach, Germany, www.minucells.de). Fresh medium was continuously perfused for 14 d at a rate of 1 ml/h with an IPC N8 peristaltic pump (Ismatec, Wertheim, Germany). For maintaining a constant temperature of 37°C, the container was placed on a thermo plate (Medax, Kiel, Germany) and covered by a transparent lid. During perfusion culture, IMDM (order no. 21980-032) without serum was used as the standard medium. Aldosterone was used at a concentration of 1×10^{-7} M (Sigma-Aldrich-Chemie, Deisenhofen, Germany). Antibiotic-antimycotic solution (1%; Life Technologies BRL-Life Technologies) was added to all culture media. Furthermore, up to 50 mmol/L HEPES (Life Technologies 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). Urea was added at concentrations of 4.5 (serum value), 9, 13.5, and 18 mmol/L. In additional experiments, the same concentrations of urea were used in combination with 20 mmol/L NaCl. In detail: 20 mmol/L NaCl + 4.5 mmol/L urea, 20 mmol/L NaCl + 9 mmol/L urea, 20 mmol/L NaCl + 13.5 mmol/L urea, and 20 mmol/L NaCl + 18 mmol/L urea. Resulting osmolalities are shown in Table 1.

Table 1. Measured osmolality in the culture medium after addition of 18 mmol/L urea, 20 mmol/L NaCl, and after simultaneous addition of urea and NaCl to the culture medium

	Osmolality
IMDM (control)	334 mosmol/kg
IMDM + urea	352 mosmol/kg
IMDM + NaCl	371 mosmol/kg
IMDM + NaCl + urea	385 mosmol/kg

Lectin Incubation

Corticomedullary-oriented cryosections (8 μm) of neonatal rabbit kidney and of the generated epithelium were prepared using a cryomicrotome (Microm, Heidelberg, Germany). The cryosections were fixed in ice-cold ethanol and then washed with PBS. After incubation in blocking solution (PBS + 1% BSA + 10% horse serum) for 30 min, the sections were exposed to rhodamine-conjugated PNA or soybean agglutinin (SBA; Vector Laboratories, Burlingame, CA) diluted 1:1000 in PBS for 45 min. After several washes in PBS, the specimens were embedded with Slow Fade Light Antifade Kit (Molecular Probes, Eugene, OR) and analyzed using an Axioskop 2 plus microscope (Zeiss, Oberkochen, Germany). Images were made by a digital camera and thereafter processed with Photoshop 5.5 (Adobe Systems, San Jose, CA).

Immunohistochemistry

Cryosections (8 μm thick) of 1-d-old rabbit kidneys were fixed in ice-cold ethanol. After washing with PBS, the sections were blocked with PBS containing 1% BSA and 10% horse serum for 30 min. Cox-1, Cox-2, Aquaporin 2 (AQP2), AQP3 (all obtained from Santa Cruz Biotechnologies, Santa Cruz, CA), mAb anti–Na/K-ATPase, and mAb anti-Troma1 (Development Studies Hydroma Bank; University of Iowa, Department of Biological Sciences, Iowa City, IA, under contract NO1-HD-7-3263 from the NICHD), mAb anti-occludin and anti-Ki67 (Zymed, CA) mAb anti-P_{CD} Amp1 (22), and mAb anti–cytokeratin-19 (gift from Prof. Dr. R. Moll, Marburg, Germany) were applied as primary antibodies for 1 h in blocking solution. The specimens were incubated for 45 min with donkey anti-mouse or donkey anti-goat IgG FITC-conjugated secondary antibodies diluted 1:200 in PBS containing 1% BSA (Jackson Immunoresearch Laboratories, West Grove, PA). The sections were then analyzed using an Axioskop 2 plus microscope. Images were made by a digital camera and thereafter processed with Photoshop 5.5.

Quantitative Analysis of Labeled Cells in the Cultured CD Epithelium

To determine the number of immunopositive or lectin labeled cells in the cultured epithelia, we applied a double-labeling procedure. The epithelia were first labeled with the nuclear marker propidium iodide (4 μg/ml in PBS; Sigma-Aldrich-Chemie) and then exposed to the respective cellular markers. By this method, the number of labeled and unlabeled cells within the epithelium could be determined easily. The mean of immunopositive cells within the epithelium is given in the text.

SDS-PAGE and Western Blotting Experiments

Generated CD epithelia were homogenized in a sample buffer containing 2% SDS, 10% glycerin, 125 mM Tris-HCl, and 1 mM EDTA (all obtained from Sigma-Aldrich-Chemie) and centrifuged at 10,000 $\times$ *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). Thirty-microgram protein samples were separated by SDS-PAGE in 10% Laemmli minigels according to methods described earlier, which were electrophoretically transferred to P-Immobilon membranes (Millipore, Eschborn, Germany). For detecting immunoreactive proteins, the blots were first blocked (PBS, pH 7.2; 0.05% Tween; Sigma; 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, Cox-2, AQP2, and AQP3 diluted 1:200. A horseradish peroxidase–conjugated donkey anti-goat or anti-mouse Ig 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). Determination of apparent molecular weight was performed in conjunction with broad-range molecular weight standard proteins (Bio-Rad Laboratories).

Statistical Analyses

All values are presented as mean $\pm$ SEM. Levels of significance were calculated by ANOVA followed by Bonferroni's test for multiple comparisons. Differences were considered significant at $P < 0.05$.

Results

The gradual process of kidney development can be visualized clearly by histochemical markers. For example, lectin histochemistry demonstrates the developmental gradient between the outer cortex and the medulla. In the outer cortex, PNA is bound at the basal aspect of each CD ampulla (Figure 2A), whereas in the inner cortex, label is detected at the luminal cell side of numerous cells demonstrating the presence of matured β-type IC cells (Figure 2B). The inner-medullary CD fails to bind PNA (Figure 2C), because only P cells populate this tubular segment. In contrast, APQ2 cannot be detected in the CD ampulla (Figure 2D) but is found at P cells in the maturing cortical (Figure 2E) and matured medullary CD (Figure 2F).

In perfusion culture experiments of embryonic CD epithelia, it has been shown that aldosterone is able to induce >90% PNA-positive cells within the cultured epithelia (20). However, because in the matured CD of the kidney only 10 to 25% of the cells become PNA positive (12,13), the existence of an antagonizing mechanism must be assumed. In the present experimental series, urea was used to examine its influence on differentiation.

Differentiation Status of the Cultured CD Epithelia

To asses the development of urea-treated embryonic epithelia, we performed immunohistochemistry (Figure 3). Tissue-specific proteins such as Troma1 (Figure 3A) and cytokeratin-19 (Figure 3B) were found in CD epithelia cultured for 14 d in IMDM containing 18 mmol/L urea. Detection of occludin (Figure 3C) indicated the establishment of a polarized CD epithelium with a luminal and basolateral compartment. The lack of P CD Amp1 (Figure 3D) indicated a downregulation of embryonic features, whereas the absence of Ki67 label (Figure 3E) demonstrated a postmitotic status of the cells. All of these features reveal a cell biologic status of the CD epithelium that is identical to characteristics found in adult CD.

Culture of Embryonic CD Epithelia in IMDM Containing Aldosterone

Embryonic CD epithelia were cultured for 14 d in serum-free IMDM containing constantly aldosterone ($n = 7$). As revealed by histochemistry in this experimental series, >90% PNA-positive cells were detectable in the generated CD epithelia (Figure 4A), whereas <10% AQP2-positive (Figure 5A)

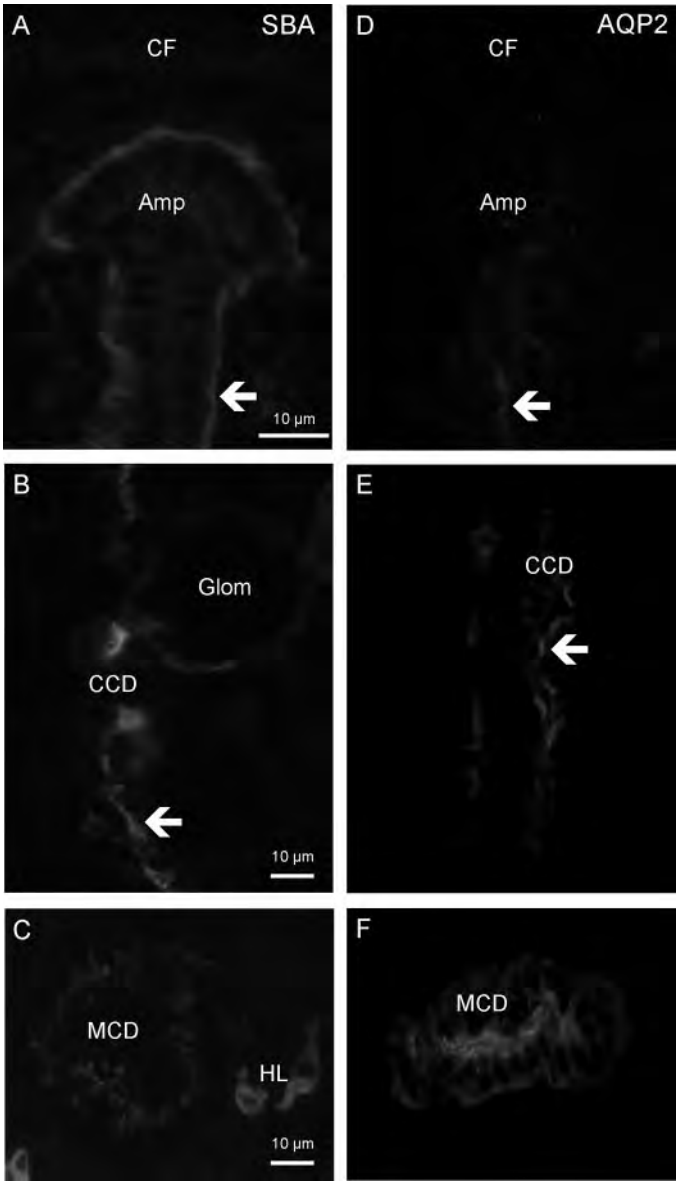

Figure 2. Development gradient within the neonatal rabbit kidney. (A) PNA binding is detectable at the basal aspect of the CD ampulla tip, whereas Aquaporin 2 (APQ2) expression is lacking at this site (D). A faint luminal reaction occurs at the distal end of the CD ampulla shaft region (D, arrow). (B) β-type intercalated (IC) cells in the cortical collecting duct (CCD) revealing luminal binding to peanut agglutinin (PNA; arrow). (C) PNA binding is lacking in the medullary collecting duct (MCD), because principal (P) cells expressing AQP2 channels are populating the MCD (F). (E) AQP2 expression in P cells of CCD (arrow). CF, capsula fibrosa; Amp, CD ampulla; Glom, glomerulus; HL, thin limb of loop of Henle.

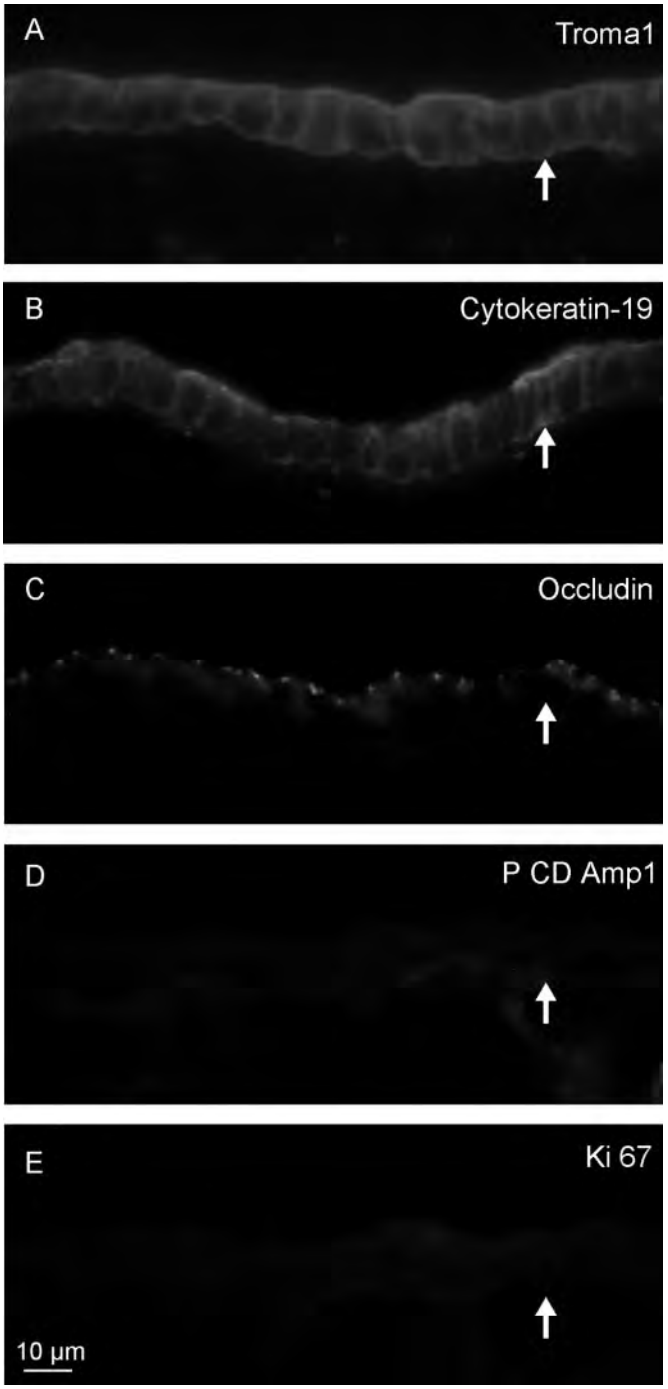

Figure 3. Developmental status of CD epithelium after culture for 14 d in the presence of 18 mmol/L urea. CD-specific marker such as Troma1 (A) and cytokeratin-19 (B) are found in all cells. Occludin (C) is present, indicating the establishment of a polarized epithelium. The lack of P CD Amp1 (D) shows the downregulation of embryonic features. The absence of Ki67 protein (E) displays the postmitotic status of the epithelial cells. Arrows mark the basal aspect of the CD epithelium.

and >90% Cox-1–positive (Figure 5B) cells were observed. Labeling with SBA for control showed that all cells within the epithelium revealed lectin binding (Figure 4B).

Culture of Embryonic CD Epithelia in IMDM Containing Aldosterone and Urea

In a next experimental series, generated CD epithelia were cultured in IMDM containing urea concentrations between 4.5 mmol/L (normal serum level) to 18 mmol/L urea ($n = 7$ in each group). Exposure to increasing urea concentrations resulted in a decreasing number of PNA-positive CD cells (Fig-

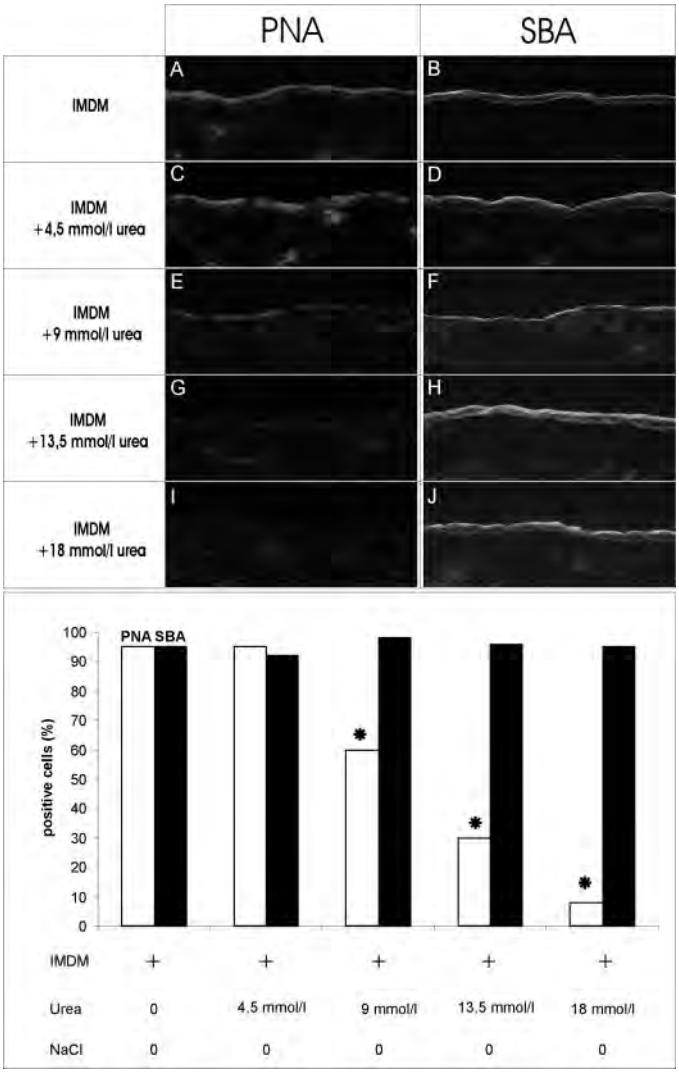

Figure 4. Influence of urea on PNA and soybean agglutinin (SBA) binding in CD cells after 14 d of culture. (A) In the control (Iscove's modified Dulbecco's medium [IMDM] containing aldosterone), PNA binding is visible in >90% of all CD cells (C). Increasing urea concentration decreases PNA binding drastically (E,G). (I) Fewer than 10% of the CD cells are positive after 18 mmol/L urea application, whereas SBA binding is not affected by urea (B, D, F, H, and J). *$P < 0.05$ compared with control (A).

ure 4, C, E, G, and I; *$P < 0.05$ compared with control), whereas SBA binding remained unaffected (Figure 4, D, F, H, and J). In detail, as compared with controls (Figure 4A), culture of the epithelia in IMDM with 4.5 mmol/L urea did not show alterations (Figure 4C), whereas 9 mmol/L urea leads to 60% (Figure 4E), 13.5 mmol/L urea leads to 30% (Figure 4G), and 18 mmol/L urea leas to only 10% PNA-binding cells (Figure 4I).

For excluding the possibility that the reduced PNA binding was caused by the interference of urea with the PNA-binding sites, detection of lectin binding was performed in PBS containing 18 mmol/L urea. Compared with control experiments in pure PBS, the presence of urea in the incubation solution did not lead to a reduced PNA binding. This finding clearly demonstrates that reduced PNA binding obtained in our experiments is caused by a reduced development of glycoproteins or glycolipids.

As shown in Figures 2 and 5, maturing CD epithelia contain AQP2 channels and Cox-1 enzyme. To test whether urea is involved in the development of both proteins, we performed immunohistochemistry. However, the expression of both AQP2 (Figure 5, C, E, G, and I) as well as Cox-1 protein (Figure 5, D, F, H, and J) was unchanged by different urea concentrations.

Culture of Embryonic CD Epithelia in IMDM Containing Aldosterone and NaCl

It was shown in earlier investigations that addition of NaCl to IMDM modulates differentiation of embryonic CD epithelia (19). Consequently, 20 mmol/L NaCl was added to IMDM to

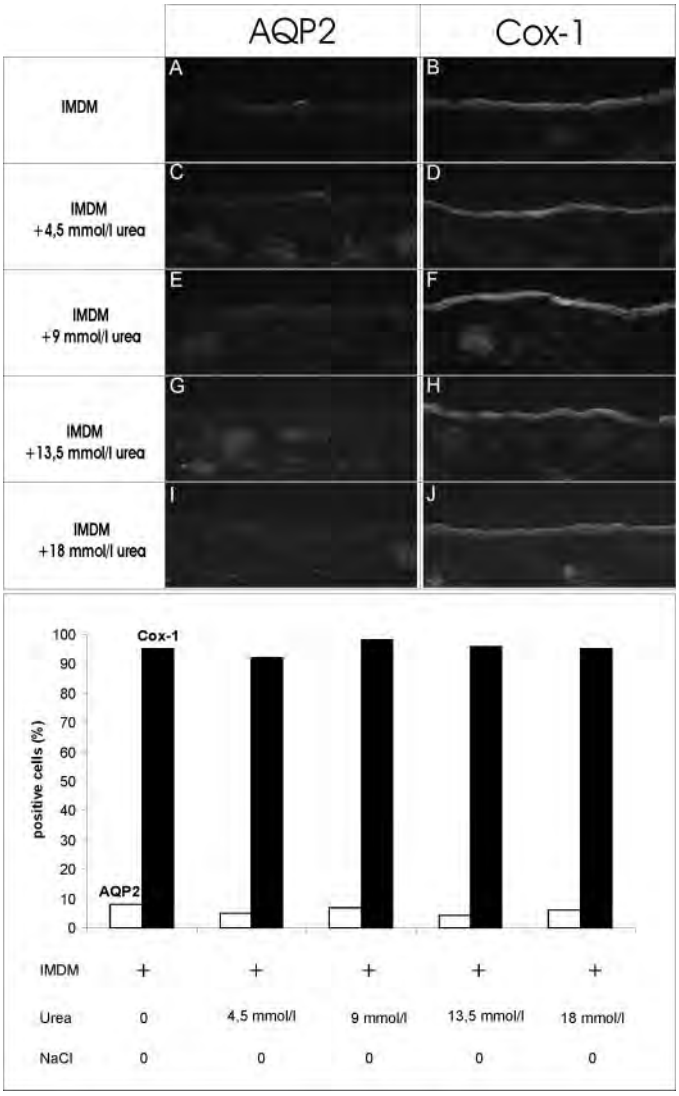

Figure 5. Influence of urea on AQP2 and Cox-1 expression in CD cells after 14 d of culture. (A) In the control (IMDM containing aldosterone), <10% of CD cells are positive for AQP2, whereas nearly all CD are positive for Cox-1 (B). Increasing urea concentrations do not affect expression of both molecules (C–G).

adapt the serum level for Na^+ in the culture medium (Figure 6; $n = 7$ in each group). The histochemical profile yielded that application of additional NaCl in the culture medium did not influence the binding pattern of PNA (Figure 6A), SBA (Figure 6B), and Cox-1 (Figure 7B) in CD epithelia as compared with controls. However, the amount of AQP2-positive cells was moderately increased. In the control group with pure IMDM, <10% AQP2-positive cells were found (Figure 5A), whereas addition of 20 mmol/L NaCl showed up to 20% of fluorescence cells (Figure 7A).

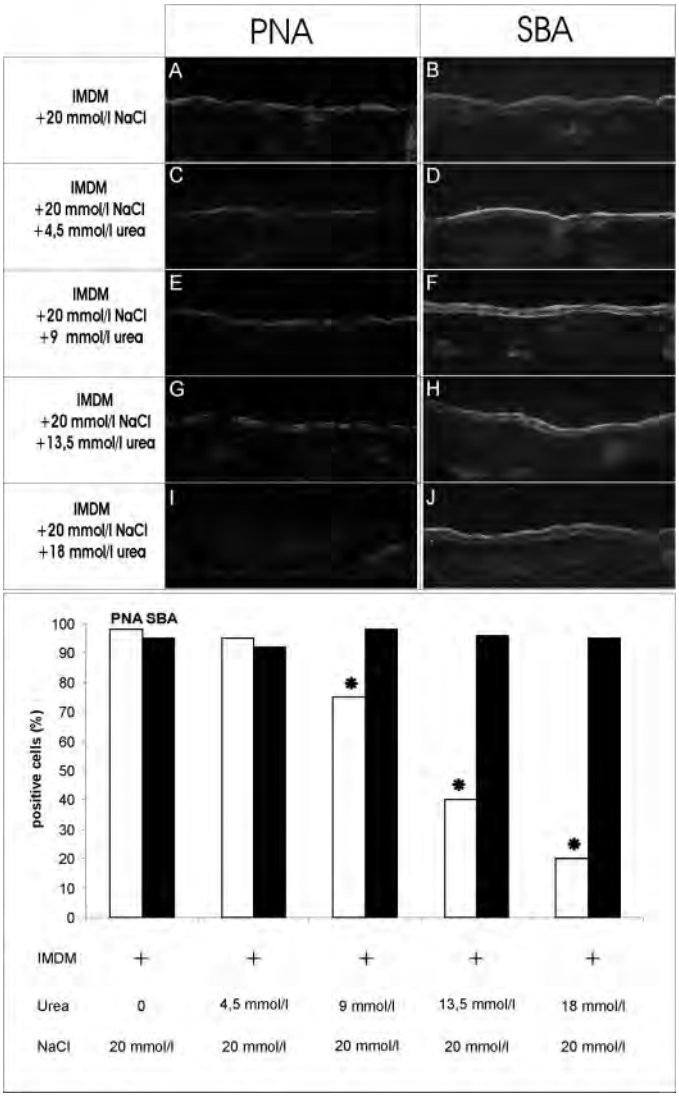

Figure 6. Influence of NaCl and urea on PNA and SBA binding in CD cells after 14 d of culture. (A) In the control (IMDM containing aldosterone and 20 mmol/L NaCl), PNA binding is visible in >90% of all CD cells (C). Increasing urea concentrations decrease PNA binding drastically despite NaCl addition to the culture medium (E, G). (I) Fewer than 10% of the CD cells are positive after 18 mmol/L urea application, whereas SBA binding is affected neither by urea nor by NaCl (B, D, F, H, and J). *$P < 0.05$ compared with control (A).

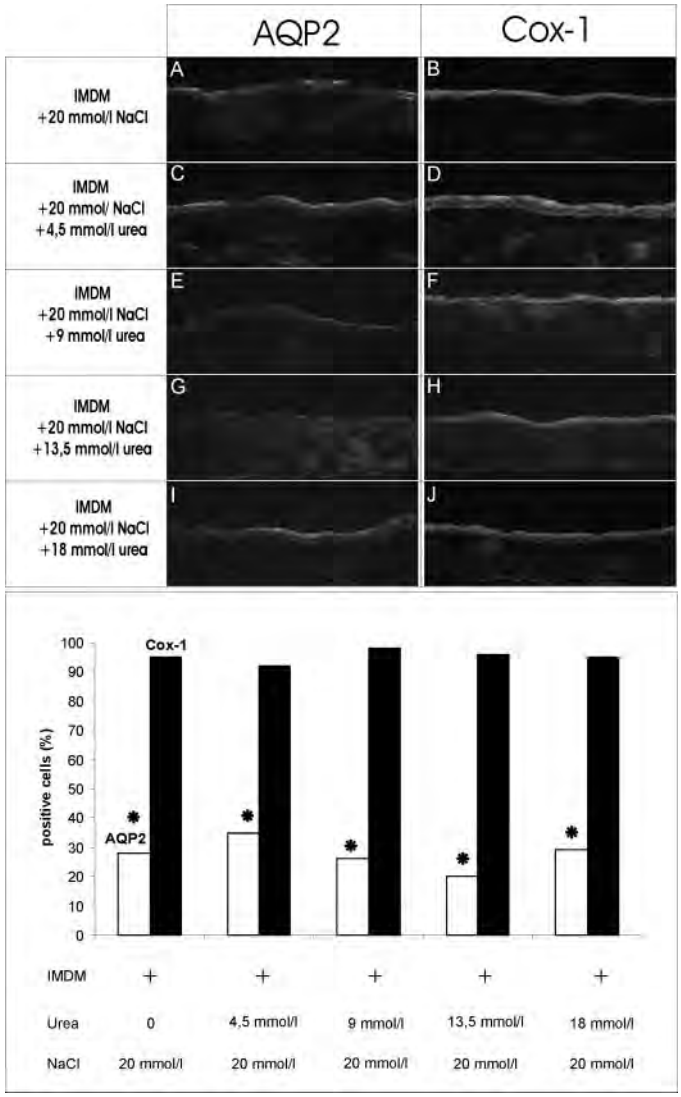

Figure 7. Influence of urea and NaCl on AQP2 and Cox-1 expression in CD cells after 14 d of culture. (A) After addition of 20 mmol/L NaCl to the culture medium, a moderate increase of up to 25% AQP2-positive CD cells are visible (*$P < 0.05$ compared with IMDM without NaCl; Figure 5A), whereas Cox-1 expression is not altered by NaCl alone or in combination with urea (B, D, F, H, and J). The increase of AQP2 expression is not further affected by increasing urea concentrations (C, E, G, I).

Culture of Embryonic CD Epithelia in IMDM Containing Aldosterone, NaCl, and Urea

Addition of both 4.5 mmol/L urea and 20 mmol NaCl to IMDM reflects the concentration status, which is found for these molecules in serum ($n = 7$ in each group). Under these culture conditions, we found the same data as obtained without urea (Figures 6, C and D, and 7, C and D). However, addition of higher concentrations of urea (9, 13.5, or 18 mmol/L) in combination with 20 mmol/L NaCl leads to the same effect (Figure 6, E, G, and I) as seen without 20 mmol/L NaCl (Figure 4, E, G, and I), where the amount of PNA-binding CD cells was drastically reduced. In comparison with PNA-binding occurrence of SBA binding (Figure 6), AQP2 (Figure 7) and

Cox-1 enzyme (Figure 7) was not affected under this experimental set-up.

As shown in Table 1, addition of urea or NaCl alone or in combination with the culture medium resulted in changes of osmolality. Because addition of NaCl did not reduce the amount of PNA-binding cells (Figure 6A), the decrease of PNA binding in urea-containing medium must be a specific effect that is not be caused by osmolality. Control experiments with addition of 20 mmol/L mannitol to IMDM also showed no effect on PNA binding.

It can be concluded that aldosterone induces and urea restrains the development of PNA binding on cells of the embryonic CD epithelium. As shown in Western blotting experiments, the expression of Na/K-ATPase (Figure 8A), Cox-1 (Figure 8B), Cox-2 (Figure 8C), and AQP3 (Figure 8E) was not altered by addition of increasing urea as well as by 20 mmol/L NaCl or by simultaneous application. The moderate 20% increase of AQP2-positive cells after NaCl addition to IMDM shown by immunohistochemistry (Figure 6A) could not be demonstrated in related immunoblotting experiments (Figure 8D).

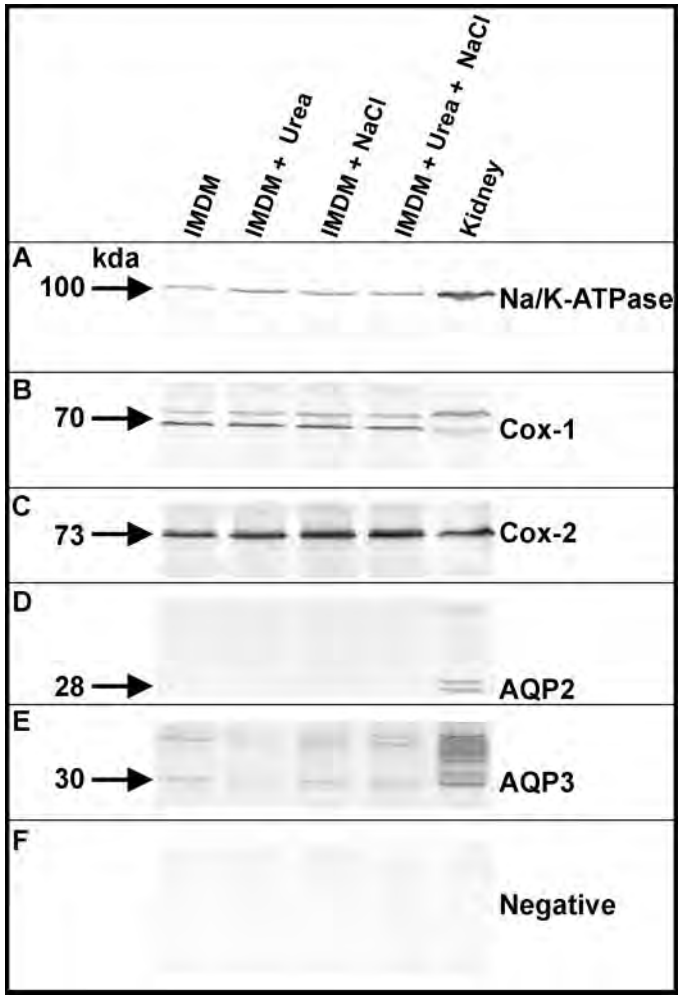

Figure 8. Western blotting results obtained from CD epithelia cultured in the presence of urea or NaCl or in simultaneous application after 14 d of perfusion culture. As positive control served a total kidney extract. Na/K-ATPase (A), Cox-1 (B), Cox-2 (C), and AQP3 (E) are detectable in the cultured epithelia under different conditions, and their expression is not affected by urea or NaCl. Reaction for AQP2 in the CD epithelia is not observed in the Western blotting experiments; however, it is found in the kidney extract (D).

Discussion

Urea is the end product of nitrogen metabolism and has to be eliminated through the urine to avoid damage to the tissues within the organism. The interstitial accumulation of urea in the kidney is an important prerequisite for the formation and elimination of a concentrated urine (23). This capacity develops until the end of organ growth (24). In the adult cortex, peritubular urea concentration should be fairly equal to plasma urea, except in the medullary rays, where it might be a little higher. In the outer stripe of the medulla, however, the concentration is much higher than in the cortex because of the urea returning from the inner medulla in the ascending vasa recta, which are very abundant in this region. In the inner stripe, the interstitium surrounding the tubules has a higher urea concentration than in the cortex. Finally, in the inner medulla, the urea concentration is much higher than in the outer part. Typically, urea concentration in the papilla may vary from 500 to 1500 mmol/L according to the intensity of the urine concentrating activity (25,26).

During the development of the organism, tissues are exposed to different concentrations of urea. After birth, renal CD epithelia have to withstand the highest urea concentrations within the organism. Pilot experiments were conducted recently to evaluate the osmotic tolerance of adult renal inner medullary epithelial cells. In these experiments, cells were isolated from adult animals and kept in culture under numerous passages. Experimental exposure was performed by adding NaCl and urea to the culture medium until 1640 mosmol/kg H_2O was reached (27). During this treatment, only 30% of cells survived for 24 h. However, when the exposure to urea was made in a linear increase over 20 h, nearly 90% of the cells stayed viable 24 h later. It was concluded that gradual changes in osmolality allow cells to tolerate high amounts of urea up to 1640 mosmol/kg H_20.

In the present experiments, we used a culture protocol mimicking tissue-specific characteristics. Renal CD epithelia were generated from embryonic CD ampulla cells on a kidney-specific collagenous support. During perfusion culture, a polarized CD epithelium was established, which in analogy to the adult kidney did not show any more mitotic cells (Figure 3E) (19). Applying physiologic concentrations of urea over 14 d, we analyzed in the present experiments the histochemical profile of generated CD epithelia. The cultured embryonic CD epithelia were able to tolerate an environment that contained 4.5 to 18 mmol/L urea for at least 14 d in culture in excellent condition. According to morphologic criteria, qualitative differences were not observed between urea-treated specimens and controls.

We initially exposed the epithelia to 4.5 mmol/L urea, which corresponds to physiologic serum values. As shown in Figure 4C, this urea concentration does not influence the aldosterone-induced development of PNA binding in the CD epithelium. However, increasing concentrations of 9, 13.5, or 18 mmol/L urea lead to a downregulation of PNA binding (Figures 4 and

6). Whereas culture media without urea showed 90% PNA-positive cells, application of urea (Figure 4, E, G, and I) or urea in combination with additional NaCl (Figure 6, E, G, and I) to the medium resulted in a clear decrease of 10% PNA-positive cells. Thus, long-term application of urea over 14 d to the culture medium is a suitable method to affect the aldosterone-induced development of PNA-binding cells in generating renal CD epithelia. Because we do not have functional data yet, we cannot ascribe the generated PNA binding exclusively to adult β-type IC cells (12). The cell biological mechanism underlying this development remains unclear. It has been shown that the typical IC-cell distribution in the CD of adult kidneys is the result of apoptosis (28). However, it is unlikely that urea affects the apoptotic pathway in the presented experimental set-up. PNA binding develops continuously, and the maximum is reached at day 10 of the culture period (16). At this time, proliferation has stopped in the epithelium and the cell number remains constant during the proceeding culture period.

The number of PNA-positive cells varies in the different regions of the rabbit kidney. In the outer cortex, a maximum of PNA-positive cells is found, whereas their number decreases toward the inner cortex (12,13). In contrast, in the medulla, PNA-positive cells cannot be detected. It is unknown by which mechanisms the cellular gradient is developed, showing numerous PNA-binding cells in the outer CCD, few in the inner CCD, and none in the medulla of the kidney (Figure 2). The present culture experiments point out that an area-dependent urea accumulation may be one of the factors influencing the amount of PNA-binding cells in the respective segment. Urea concentration is low in the cortex but increases toward the medulla. Thus, low urea concentration in the outer cortex parallels with numerous PNA-positive cells, whereas increasing urea concentration in the inner cortex shows a decline. It remains unclear whether urea is the only substance restraining the amount of PNA-positive cells during development. Although we did not yet perform physiologic experiments to test typical functional parameters of the generated epithelia, it is obvious that PNA binding on CD cells is modulated by increasing concentrations of urea under *in vitro* conditions. However, whether the same mechanism is acting in the developing organ remains to be elucidated.

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