Advances in renal (patho)physiology using multiphoton microscopy

Doctoral theses

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INTRODUCTION

Multiphoton fluorescence microscopy is a state-of-the-art confocal imaging technique ideal for deep optical sectioning of living tissues. It is capable of performing real time, noninvasive, quantitative imaging of organ functions in health and disease with high spatial and temporal resolution which other imaging modalities cannot achieve. Using this novel technique we aimed to revisit one, classical field of renal physiology: the tubuloglomerular feedback (TGF), seeking evidences supporting the role of the tubular fluid flow in the TGF mechanism. Also, we aimed to study the effector response of the TGF: the vasoconstriction of the afferent arteriole. Gathering evidences seemed to support the hypothesis that there are two distinct parts of the AA that produced reduction in the diameter of the vessel during TGF: the proximal AA and the terminal intraglomerular part of the AA. In addition to identifying the connection between the tubular fluid flow and the control of the glomerular filtration as well as the function of the AA, we examined the effect of tubular flow on the late part of nephron, in the control of the sodium reabsorption in the principal cells of the cortical collecting duct.
For these reasons my thesis consist the following main chapters:

- Evidence for tubular fluid flow sensing by macula densa cells
- Macula densa control of the intraglomerular precapillary sphincter
- Luminal ATP release and pressure natriuresis in Connexin 30 knockout mice

1 EVIDENCE FOR TUBULAR FLUID FLOW SENSING BY MACULA DENSA CELLS

Tubuloglomerular feedback is a major regulatory process which function is to control glomerular filtration rate and glomerular blood flow. Deviations of luminal NaCl concentration at the macula densa (MD) serves as an input signal initiating the fine control of glomerular arteriolar vascular tone that result in opposite changes of glomerular filtration rate. The morphological basis of TGF is a close anatomical link between the macula densa segment of the early distal tubule and the glomerular vessels. This creates the possibility of a feedback mechanism: to balance the tubular salt and water delivery by adjusting the pre- and postglomerular vascular tone. The most important vascular adjustment is a vasoconstriction of the afferent arteriole as NaCl concentration at the macula densa increases.

TGF is the hallmark of the kidney function which was always in the focus of the renal physiologists. Despite of the remarkable attention, several details of the exact mechanism of the TGF is still debated. One of this so far less understood component of the existing model is the role of the tubular flow in the TGF. The flow-effect was proposed long ago. Though it is

controversial, current paradigm describes that MD cells respond to elevation in luminal [NaCl] but it lacks to reflect the flow-effect. It is recognized that changes in the NaCl delivery also accompanied by the alterations in fluid flow. If these two components of the tubular fluid are dependent on one another it would assume that the sensor (MD cells) could respond to both. Therefore we aimed to study the role of the tubular fluid flow in the control of the TGF.

1.1 Aims

Our specific aims were:

1. To examine if the TGF can be initiated by changing the tubular flow rate per se without causing any alteration in the tubular [NaCl]
2. To find the tubular flow sensor in the distal tubule
3. To determine the effect of the changing tubular [NaCl] on the fluid flow induced TGF
4. To identify the signaling pathway that is involved in the flow induced TGF

1.2 Methods

Immunohistochemistry:

C57/BL6 mice were anesthetized with Inactin. Animals were infused with PBS to wash the blood cells out of the circulation. Kidneys were fixed in situ by perfusion of paraformaldehyde in PBS. The tissues were harvested and coronal kidney sections were then post-fixed overnight in paraformaldehyde and embedded in paraffin. Subsequently, 5-µm sections of the paraffin block were deparaffinized in toluene and rehydrated through graded ethanol. Monoclonal antibody against acetylated alpha tubulin, Alexa 594-conjugated secondary antibody and Vectashield mounting medium containing DAPI for nuclear labeling was used on paraffin-embedded mouse sections.

In vitro isolated and microperfused glomerulus – JGA:

Individual glomeruli with afferent arteriole (AA) and attached MD segment were freehand dissected from mouse (C57/BL6) kidneys and perfused in vitro. AA was canulated, stained with Fluo-4 (1 µM, 25˚C, 15 min.) for intracellular Ca^{2+} imaging and perfused with Ringer's solution in each experiment. In the first approach, the cTAL was removed so that the MD plaque remained intact and accessible from the bath, as opposed to the double perfusion model, in which the cTAL remained intact and attached to the glomerulus. Whenever the MD plaque was unable to be visualized or was damaged, those tissues were excluded from our study. The tissue was bathed in Ringer's solution and the temperature was maintained at 37°C. TGF was induced by either of the following stimuli: (A) Laminar flow applied directly onto the apical surface of the MD cells using different [NaCl] concentration solutions (0, 10,

80, 135mM [NaCl]). When removing NaCl from the solution, NaCl was isosmotically substituted with NMDG cyclamate, KCl with potassium gluconate, and $CaCl_2$ with calcium gluconate to achieve a [NaCl] of 0, 10 or 80mM. In separate groups, furosemide was also added to 0mM NaCl–containing Ringer's solution to inhibit NKCC2; separately tempol to eliminate the flow-induced generation of reactive oxygen species; and finally suramin to block the purinergic signaling. Fluid flow was 20nL/min. (B) Bending MD cilia directly with a glass micropipette under no-flow condition. Primary cilium of the MD cells were bended by moving a micropipette parallel with and close to the luminal membrane of the MD cells without touching the apical surface of the cells. (C) Perfusing the intact cTAL by different [NaCl] solutions (10, 80mM [NaCl]). In a separate group furosemide was also added to 10mM NaCl–containing Ringer's solution. Fluid flow was 20nL/min.

In all approaches, TGF was observed as an elevation in the intracellular Ca^{2+} concentration ($[Ca^{2+}]ic$) of vascular smooth muscle cells (VSMC) of the AA, and as a consequence a reduction in the diameter of the AA. Data were collected in every 2 sec by Stallion imaging software (Intelligent Imaging Innovations, Inc.).

Statistics

Data were analyzed using Microsoft Excel (Microsoft). P values were calculated using two-tailed Student's t-test. (* $p<0.05$)

1.3 Results

The flow induced feedback mechanism.

In order to examine if MD cells are able to respond to changes in tubular fluid flow a single glomerulus with attached AA was dissected. The intact MD region was explored by removal of the adjacent cTAL parts. This way the MD cells become freely accessible from the bath. Applying fluid flow directly onto the surface of the MD cells induced significant $[Ca^{2+}]ic$ elevation in the VSMCs of the AA that was followed by a subsequent vasoconstriction of the AA. This response could be evoked regardless of the [NaCl] of the applied solution: as the [NaCl] of the perfusion solution was reduced the $[Ca^{2+}]ic$ responses measured in the VSMC of the AA become slightly smaller, however statistically significant difference between the stimuli was not found.

Interestingly, in some experiments it was possible to identify two separate responsive regions of the AA: a juxtaglomerular or proximal and a distal region. These two regions were connected by a vascular segment identified as the renin positive part of the AA based on the highly granulated morphology of the smooth muscle cells. After repeated flow stimuli the

elevation in the [Ca^{2+}]ic of the VSMCs and the vasoconstriction did not show any sign of desensitization.

TGF related [Ca^{2+}]ic elevations were also detected in the podocytes. The [Ca^{2+}]ic elevations in the podocytes were identical to those which have been observed in the AA VSMCs, in terms of the absence of desensitation. In addition to the involvement of the preglomerular artery and the podocytes in the flow-induced TGF, the whole mesangium responded to flow stimulus. Simultaneously with the Ca^{2+} elevations the diameter of the whole glomerular tuft also reduced.

The inhibition of the NKCC2 cotransporter (0mM [NaCl] + furosemide) resulted in the same [Ca^{2+}]ic elevation of the VSMCs as was achieved by the 0mM [NaCl] solution alone. Moreover, scavenging the superoxide anions failed to alter the [Ca^{2+}]ic elevation of the VSMCs achieved after the applications of 10mM or 80mM [NaCl]. As opposed to the preincubation with the non-selective P2 purinergic blocker, suramin the flow-induced responses were significantly blunted.

Comparing the salt and the flow effect.

Single glomerulus with attached AA and intact cTAL was dissected and both were perfused. When the cTAL was perfused with 80mM [NaCl] solution at flow rate of 20nL/min significant [Ca^{2+}]ic elevation in VSMCs and vasoconstriction of the AA was observed. If [NaCl] was reduced to 10mM and the same maneuver was repeated similar elevation in [Ca^{2+}]ic and reduction in the vessel diameter were observed. However, this response was significantly reduced compared to that recorded using 80mM [NaCl] solution. Besides, when 10mM [NaCl] solution was supplemented with furosemide (100μm) the [Ca^{2+}]ic elevation in VSMCs was the same (Fluo-4 F/Fo: 1.23±0.08) compared to that achieved only with the application of the 10mM [NaCl] solution.

The identification of the flow sensor.

As described earlier in mouse, rat and human tissue we wanted to confirm that the MD cells have cilia. Labeling mouse sections with α-tubulin we identified 5-8μm long cilia on the apical surface of the MD cells. Each MD cell possesses one, primary cilium.

Bending the cilium of the MD cell.

Using the open MD approach the primary cilium of the MD cells was bended by a thin micropipette. This stimulus resulted in significant [Ca^{2+}]ic elevation in the AA VSMCs and subsequent vasoconstriction of the AA. This [Ca^{2+}]ic elevation in the VSMC was specific to this maneuver since direct mechanical stimulus (poking of the MD cells) did not produce any response. Repeating the stimulus did not result in desensitization.

1.4 Conclusions

We confirmed that the MD cells possess one, apical primary cilium. Applying fluid flow to the MD apical surface or bending the primary cilia directly induces TGF regardless of the [NaCl] of the applied solution. The feedback mechanism evoked by these maneuvers involves the vascular smooth muscle cells of the afferent artery, the podocytes and the whole glomerular tuft as well. Even repeated stimuli did not result in desensitization. To identify the signaling mechanism that is responsible for conducting the flow-effect reactive oxygen species was scavenged though it has no effect on flow-induced TGF. However, inhibition of the P2 purinergic receptors significantly reduced the TGF response. Our data suggest that the primary cilium of the MD cell act as a flow sensor. Additionally to the tubular salt sensing mechanisms, MD cells are equipped with a tubular flow sensing mechanism which may contribute to MD cell function and TGF.

2 MACULA DENSA CONTROL OF THE INTRAGLOMERULAR PRECAPILLARY SPHINCTER

Tubuloglomerular feedback (TGF) is a homeostatic control mechanism which function is to stabilize NaCl delivery into the late part of the nephron and thereby to stabilize NaCl excretion. Deviations of luminal NaCl concentration at the macula densa (MD) serves as an input signal initiating fine control of glomerular arteriolar vasomotor tone that result in inverse changes of glomerular filtration rate. Preliminary findings indicated swelling/contraction of certain cells in the terminal, intraglomerular part of the AA during TGF. This contraction appeared to cause a very significant reduction of vessel diameter, nearly to the point of complete closure of the AA. Therefore, we have named it the “glomerular sphincter” based on similarities with the precapillary sphincter in other vascular beds.

2.1 Aims

Our specific aims were:

1. To visualize the preglomerular capillary sphincter both in vitro and in vivo using two photon microscopy
2. To identify the cellular components involved in the formation of the preglomerular capillary sphincter
3. To study the regulation of the preglomerular capillary sphincter
4. To examine if a similar postglomerular capillary sphincter exists at the juxtaglomerular part of the efferent artery

2.2 Methods

In vitro isolated and microperfused afferent arteriole-JGA-glomerulus

A superficial afferent arteriole with its glomerulus and attached distal tubule containing the MD was microdissected from kidneys of New Zealand white female rabbits. The arteriole was perfused with a modified Krebs-Ringer-HCO_3 buffer containing (in mM) 115 NaCl, 5 KCl, 25 $NaHCO_3$, 0.96 NaH_2PO_4, 0.24 Na_2HPO_4, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 5.5 D-glucose, and perfusion pressure was maintained at around 50 mmHg throughout the experiment. The tubular perfusate was an isosmotic, low NaCl containing Ringer's solution consisting of (in mM): 10 NaCl, 135 N-methyl-D-glucamine (NMDG) cyclamate, 5 KCl, 1 $MgSO_4$, 1.6 Na_2HPO_4, 0.4 NaH_2PO_4, 1.5 $CaCl_2$, 5 D-glucose, and 10 N -2-hydroxyethyl-piperazine-N' -2-ethanesulfonic acid (HEPES). TGF was initiated by switching to a similar Ringer's solution in which [NaCl] was increased from 10 to 80 mM. Isosmolality of this solution was maintained by reducing NMDG cyclamate to 65 mM. The tubule segment was cannulated and perfused at a baseline rate of approx. 10 nl/min. The bath was identical to the arteriolar perfusate.

In vivo imaging of the kidney

Male Munich-Wistar rats were used for in vivo experiments. Under thiobutabarbital sodium anesthesia the right femoral vein was cannulated for fluorescent dye administration (quinacrine and rhodamine-conjugated 70,000 MW dextran, to label the renin content of the kidney and the intravascular space, serum respectively). From a dorsal incision, the kidney was exteriorized with intact blood flow and innervation. The animal was placed on the stage of an inverted microscope with the exposed kidney placed in a coverslip-bottomed heated chamber bathed in normal saline.

Immunhistochemistry

Sprague-Dawley rats were anesthetized with Inactin. Kidneys were fixed in situ. Coronal kidney sections were then post-fixed overnight and embedded in paraffin. Subsequently, sliced sections were then incubated with a mouse monoclonal α-smooth muscle actin antibody. Sections were labeled with an Alexa Fluor 594 conjugated goat anti-mouse antibody. Sections were counterstained with quinacrine. Next, sections were mounted with Vectashield mounting media containing the nuclear stain DAPI.

Statistics

Statistical analysis was performed with paired t-test and ANOVA with Statistica 6.0 Statsoft. The significance level was set at a value of $P<.05$. All data are presented as mean±SE.

2.3 Results

The vascular morphology of the microperfused AA-attached glomerulus preparation was examined before and after the activation of the TGF mechanism by increasing tubular salt content at the MD from 10 to 80 mM. The internal diameter of the non-stimulated AA was uniform along the entire extraglomerular segment (10.4±0.5 μm), however, the ID of the terminal intraglomerular part was much smaller (5.1 ± 0.6 μm). TGF activation resulted in a significant vasoconstriction in the proximal AA. Vessel ID decreased by 34.2 ± 8.0 %. However, TGF stimulation caused a proportionally larger increase in vascular resistance in the intraglomerular AA segment, ID decreased by 69.3 ± 5.9 %. Renin granular cells in the wall of the intraglomerular sphincter did not produce any significant contraction during TGF.

Two-photon imaging of the intact whole kidney was performed. There were segmental differences in the vascular diameter of proximal versus intraglomerular AA. The presence of a preglomerular sphincter was evident in all AAs observed. Also, in some experiment the temporary but complete shut down of the glomerulus was observed due to the vasoconstriction of the preglomerular capillary sphyincter.

Immunohistochemical labeling of α-smooth muscle actin on rat kidney sections identified the proximal, extraglomerular portion of the AA. The labeling was strong, throughout the cytoplasm in all vascular smooth muscle cells. In contrast, cells of the efferent arteriole were only weakly stained. No smooth muscle cells were found at the site of the glomerular sphincter, based on actin labeling.

Pharmacological experiments were performed using the in vitro microperfused experimental model to study the mechanism of the glomerular sphincter. The addition furosemide to the tubular perfusate completely abolished the TGF-mediated closure of the sphincter. Interestingly, the non-selective P2 purinergic receptor blocker suramin, added to the AA perfusate and bath not only prevented the TGF-induced vasoconstriction, but it caused a slight relaxation of the sphincter. Addition of the adenosine A1 receptor inhibitor DCPCX to the arteriolar perfusate and bath had no effect on sphincter activity.

2.4 Conclusion

These studies describe the morphology, function, and mechanism of the terminal AA, an intraglomerular precapillary sphincter. The results confirm previous preliminary data that the principal effector site of TGF-mediated increase in vascular resistance occurs in the area of this glomerular sphincter. For the first time, imaging techniques with high temporal and spatial resolution provided real-time observation of the sphincter activity during TGF using the in vitro microperfused JGA preparation. Surrounding extraglomerular mesangial cells, and

not renin granular cells, contracting in concert with the entire intraglomerular mesangium appear to be responsible for the generation of sphincter activity. Also a novelty in this study is the first-time demonstration of a functional glomerular sphincter in the intact rat kidney, using two-photon microscopy. Moreover, the exciting finding that MD control of sphincter activity was purely ATP-mediated provides further support that ATP is directly involved in TGF, and not only through its breakdown to adenosine.

3 IMPARED LUMINAL ATP RELEASE AND PRESSURE NATRIURESIS IN CONNEXIN 30 KNOCKOUT MICE

It is well established that renal tubular epithelial cells release ATP, which then binds to purinergic receptors along the nephron and collecting ducts to regulate salt and water reabsorption. However, the molecular mechanism of ATP release and the identity of ATP channels are less clear. Previous work also suggested that the release of ATP in epithelia may be triggered by mechanical stimulation, including elevations in tubular fluid flow rates.

Connexin 30 (Cx30) is a member of the connexin (Cx) family of transmembrane proteins. Various Cx proteins can form large pores in the nonjunctional plasmamembrane of cells (gap junction hemichannels) before the assembly of two Cx hemichannels into complete gap junction channels between adjacent cells. Cx hemichannels are large, mechanosensitive ion channels that allow the passage of a variety of small molecules and metabolites, including ATP. Our laboratory localized Cx30 in the nonjunctional apical plasma membrane of cells in the distal nephron, suggesting that Cx30 may function as an ATP-releasing, luminal membrane hemichannel in this nephron segment. The physiologic significance of this mechanically induced Cx30-mediated luminal ATP release was studied by assessing its involvement in pressure natriuresis, a multifactorial, multimechanistic intrarenal phenomenon that causes diuresis and natriuresis in response to elevations in systemic BP. Because pressure natriuresis is related to mechanical factors and Cx hemichannels are mechanosensitive, we hypothesized that Cx30-mediated ATP release is involved, at least in part, in pressure natriuresis, a critically important phenomenon in the maintenance of body fluid balance and BP.

3.1 Aims

Our specific aims were:

1. To study if Cx30 hemichannel is capable releasing ATP using imaging approach (biosensor technique and confocal microscope)
2. To examine the role of Cx30 in pressure natriuresis and diuresis phenomenon in Cx30 animal model

3. To explore the contribution of the cortical collecting duct to the pressure natriuresis
4. To test if the Cx30 is involved in the regulation of the pH homeostasis

3.2 Methods

Immunohistochemistry

The Cx30 mouse model was established. After fixation and blocking, wild-type and Cx30 mouse kidney sections were incubated with Cx30 antibodies. Sections were then incubated with horseradish peroxidase–conjugated goat anti-rabbit IgG and enhanced with Alexa Fluor 594–labeled tyramide signal amplification. Finally, sections were mounted with Vectashield mounting medium containing the nuclear stain DAPI.

ATP Biosensor Technique

Animals were anesthetized by Inactin. Kidneys were harvested and cortical collecting ducts (CCDs) were dissected freehand. CCDs were partially split open to provide access to the luminal cell surface and placed into a temperature-controlled chamber containing oxygenated Krebs-Ringer solution. Then, tubules were perfused in the 0- to 20-nl/min range with a solution containing (inmM) 25NaCl, 5 KCl, 1MgSO4, 1.6 NaHPO4, 0.4 NaH2PO4,5 D-glucose, 1.5 CaCl2, 110 NMDG-cyclamate, and 10 HEPES. In some experiments, bath osmolality was reduced from 300 to 270 mOsm/kg. Intercalated cells (ICs) were labeled by Alexa Fluor 594–peanut lectin and principal cells (PCs) by quinacrine. After cannulation and perfusion of a microdissected CCD, a small group of Fluo-4– and Fura Red loaded PC12 cells were gently positioned in direct contact with the apical surface of PCs or ICs. These PC12 cells were used as biosensors of freshly released extracellular ATP on the basis of changes in their $[Ca^{2+}]$ic. In some experiments, PC12 cells were preincubated with the purinergic receptor blocker suramin to identify the capability of Cx30 hemichannels to release ATP. Each perfused CCD was dissected from a different mouse.

Pressure-Natriuresis Measurements

Ten- to 13-wk-old Cx30 mice (n 8) and Cx30 littermates (n 6) were anesthetized using the combination of ketamine and inactin. Trachea was cannulated to facilitate breathing, and cannulas were inserted into the carotid artery to perform continuous blood pressure (BP) measurements and into the femoral vein for intravenous infusion. The abdominal aorta (distal to the renal artery) and later the mesenteric artery were ligated to elevate BP in a stepwise manner. A catheter was placed into the bladder to collect urine. The operation was followed by a 15-min equilibration period. Renal functions were then determined during three consecutive 20-min periods: (1) Control, (2) first elevation period in which the abdominal aorta was compressed, and (3) the second elevation period in which the mesenteric artery was

additionally ligated. In each period, urine and blood samples were collected to perform volume, Na^+ and K^+ measurements. Immediately after blood collection, mice received an equivalent amount of donor mouse whole blood, collected a few hours before the experiment. See figure 1 for details.

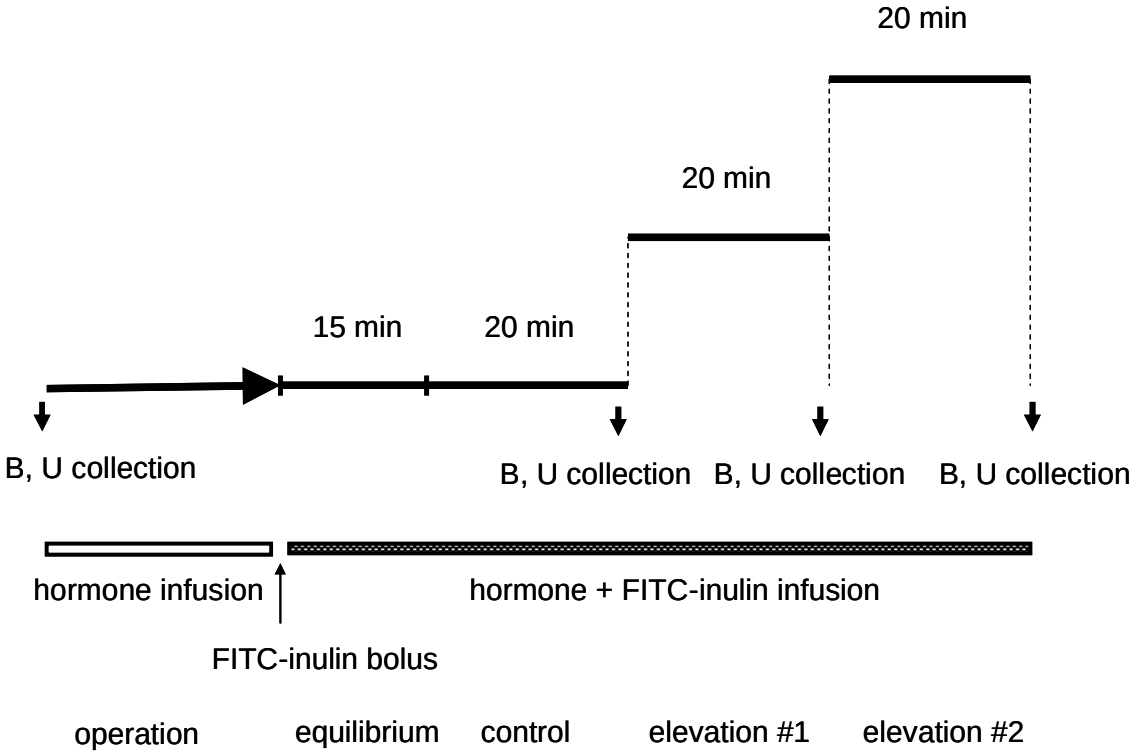

Figure 1. Experimental setup of the in vivo study. After the operation and the 15-min. equilibrium there were 3 consecutive 20-min period. In the elevation #1 the abdominal aorta, in the elevation #2 the mesenteric arteries were ligated. B: blood, U: urine

After femoral vein cannulation, the mice were given continuous infusion containing BSA and a "hormone cocktail" (norepinephrine, arginine vasopressin, hydrocortisone, aldosterone). Hematocrit was measured. GFR was calculated by using the FITC-inulin technique. Serum and urine Na^+ and K^+ concentrations; plasma Cl^-, HCO_3, aldosterone and arginine-vasopressin levels were also determined.

High-Salt Diet

Wild-type and Cx30 mice were kept on normal or high-salt diet for 2 wk. Some mice received benzamil, a selective inhibitor of the ENaC, during the second week. Mice were anesthetized, and BP was measured through the cannulated carotid artery, as described above.

Western Blot Analysis of Renal Epithelial Salt Transporters

Manually dissected slices of kidney cortex were homogenized in protease inhibitor cocktail. A total of 40g of protein was separated on a 4t o2 0 % SDS-PAGE and transferred

onto polyvinylidene difluoride membrane. This was followed by an incubation of the primary antibody of ENaC, NaKCC2, NHE3 and NCC, along with-actin. After washing, blots were incubated with either a goat anti-mouse or goat anti-rabbit secondary antibody.

RT-PCR

Total RNA was purified from whole kidneys of $Cx30^{+/+}$ and $Cx30^{-/-}$ mice. RNA was quantified using spectrophotometry and reverse-transcribed to single-stranded cDNA using avian reverse transcriptase and random hexamers. cDNA was amplified by Taqpolymerase and specific primers. The primer sequences and expected band sizes for various Cx and ATP receptors were described previously. The PCR product was analyzed on a 2% agarose gel.

Statistical Analysis

Statistical analysis was performed by ANOVA and post hoc comparison by Bonferroni test. P 0.05 was considered significant.

3.3 Results

Localization of Cx30 in the Mouse Kidney

Immunolocalization studies found intense Cx30 labeling exclusively in the luminal, nonjunctional cell membrane of a select population of cells in the connecting tubule (CNT) and CCD.

Luminal ATP Release Measurements

We measured Cx30-mediated ATP release into the tubular fluid using freshly dissected, in vitro microperfused CCDs from wild-type and Cx30 mice and an established ATP biosensor technique. In response to increasing tubular flow rate from 2 to 20 nl/min, the biosensor PC12 cells produced elevations in $[Ca^{2+}]$ic when positioned next to ICs, indicating ATP release from these cells. Substantial but significantly reduced flow-induced $[Ca^{2+}]$ic responses were observed when the biosensor cell was attached to PCs' apical membrane. Importantly, the ATP biosensor signal was almost completely abolished when ICs in preparations dissected from Cx30 mice were used. Preincubation of the biosensor cells with the purinergic receptor inhibitor suramin served to ensure ATP specificity, and it completely blocked the biosensor $[Ca^{2+}]$ic signal. Similarly, no response was detected when the biosensor PC12 cell was positioned at the exit of the open CCD without any direct cell membrane contact with either ICs or PCs. Similar to the effect of increased tubular flow, reducing bath osmolality from 300 to 270 mOsm/kg (creating an interstitium-to-lumen pressure gradient) caused significant ATP release from ICs in a Cx30-dependent manner.

Pressure Natriuresis and Diuresis in Cx30 Mice

Wild-type and $Cx30^{-/-}$ mice were instrumented for pressure natriuresis measurements. The two-step elevations in mean arterial pressure resulted in the resting value of 115.0±4.5 to 131.0±4.1 and then to 151±4.8 mmHg. There were no significant differences in BP levels between Cx30 and $Cx30^{-/-}$ mice at any given time during these experiments. The increased renal perfusion pressure-induced natriuresis was significantly blunted in $Cx30^{-/-}$ mice. There was no difference in pressure-induced kaliuresis in wild-type and $Cx30^{-/-}$ mice. The urine output increased in response to elevations in renal perfusion pressure in both groups, however, the magnitude of pressure diuresis was markedly blunted in $Cx30^{-/-}$ mice. GFR was maintained at a constant level during these studies and was not different between wild-type and $Cx30^{-/-}$ mice. Also, hematocrit did not change during these experiments.

Collecting Duct Specificity

Mice were treated with benzamil, a selective inhibitor of the collecting duct–specific epithelial sodium channel (ENaC). Because there was no difference in BP between wild-type and $Cx30^{-/-}$ mice under normal conditions, we placed mice on high-salt diet for 2 wk. BP in the wild-type mice (75.0±1.7 mmHg) did not change; however, high-salt diet caused a significant BP elevation in $Cx30^{-/-}$ mice (103.0±2.4 mmHg). Administration of the ENaC blocker benzamil had no effect in $Cx30^{+/+}$ mice, but it returned BP to normal levels in $Cx30^{-/-}$ mice on high-salt diet.

Expression of Renal Salt Transporters and ENaC in Cx30 Mice

We studied the expression of the main renal salt transporters: NaHE3, NKCC2, NCC, and ENaC, in wild-type and $Cx30^{-/-}$ mice using immunoblotting of whole-kidney homogenates. There was no statistically significant difference in the expression of these salt transporters or ENaC between Cx30 and Cx30 mice, with the exception of NCC, which was significantly less abundant in kidneys of $Cx30^{-/-}$ mice.

Cx and Purinergic Receptor Expression Profile in $Cx30^{-/-}$ Mice

The expression profile of well-established renal Cx isoforms in kidneys of $Cx30^{+/+}$ and $Cx30^{-/-}$ mice were compared using reverse transcriptase PCR of whole kidney samples. Expression of Cx30.3, Cx37, Cx40, Cx43, and Cx45 was found in both wild-type and $Cx30^{-/-}$ mice, with the exception that $Cx30^{-/-}$ mice were Cx30 deficient, as expected. Similarly, a variety of P2X and P2Y purinergic receptors, including the main luminal membrane isoform P2Y2, seemed to be expressed in kidneys of both $Cx30^{+/+}$ and $Cx30^{-/-}$ mice.

Other Systemic and Renal Parameters in Cx30 Mice

All mice were age matched, and body weight was not different between $Cx30^{+/+}$ and $Cx30^{-/-}$ groups. Also, plasma electrolyte levels were the same. Because Cx30 hemichannels were localized to the apical membrane of ICs, which regulate pH homeostasis, plasma [Cl^-]

and [HCO_3^-] were measured but did not show any difference between the two groups. Plasma aldosterone and vasopressin levels were not different between $Cx30^{+/+}$ and $Cx30^{-/-}$ mice.

3.4 Conclusion

This study identified functional Cx30 hemichannels in the intact kidney tissue as an important source of luminally released ATP in the collecting duct. Cx30 hemichannel opening at the luminal cell membrane of the CCD was triggered mechanically by elevations in tubular fluid flow rate or an interstitium-to-lumen osmotic pressure gradient, resulting in significant amounts of released ATP (up to 50μM) in the luminal microenvironment. Physiologic significance of the Cx30-mediated luminal ATP release was confirmed in vivo in a mouse model of pressure natriuresis, an important renal physiologic mechanism that maintains body fluid and electrolyte balance and BP. $Cx30^{-/-}$ mice expressed a salt retention phenotype indicated by their reduced ability to excrete urinary salt and water in response to acute elevations in BP. This was due to the dysfunction of the Cx30-expressing collecting duct, because high-salt diet–induced elevations in BP (in $Cx30^{-/-}$ mice) were prevented by pharmacologic inhibition of salt reabsorption in the CCD. This report describes a mechanically induced ATP-releasing mechanism in the intact CCD and its partial involvement in the pressure-natriuresis phenomenon. Also, this article further emphasizes the in vivo physiologic significance of purinergic regulation of renal tubular salt and water reabsorption.

LIST OF PUBLICATIONS

Publications related to present thesis:

1. **Sipos A**, Vargas SL, Toma I, Hanner F, Willecke K, Peti-Peterdi J. Connexin 30 deficiency impairs renal tubular ATP release and pressure natriuresis. J Am Soc Nephrol 20: 1724-32, 2009. (PMID: 19478095) IF: 7.111.

2. **Sipos A,** Toma I, Kang JJ, Rosivall L, Peti-Peterdi J. Advances in renal (patho)physiology using multiphoton microscopy. Kidney Int 72:1188-91, 2007. (PMID: 17667980), IF: 4.922.

3. Kang JJ, Toma I, **Sipos A**, McCulloch F, Peti-Peterdi J. Imaging the renin-angiotensin system: An important target of anti-hypertensive therapy. Adv Drug Deliv Rev 58:824-33, 2006. (PMID: 16979787) IF: 7.977.

4. Rosivall L, Mirzahosseini S, Toma I, **Sipos A**, Peti-Peterdi J. Fluid flow in the juxtaglomerular interstitium visualized in vivo. Am J Physiol Renal Physiol 291:F1241-7, 2006. (PMID: 16868308) IF: 4.199.

5. Kang JJ, Toma I, **Sipos A**, McCulloch F, Peti-Peterdi J. Quantitative imaging of basic functions in renal (patho)physiology. Am J Physiol Renal Physiol 291:F495-502, 2006. (PMID: 16609147) IF: 4.199.

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