In Vivo Visualization of the Anti-albuminuric Effects of the Angiotensin Converting Enzyme Inhibitor Enalapril

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glomerular filtration rate, GFR
Abstract

Angiotensin converting enzyme (ACE) inhibitors are commonly used antiproteinuric drugs. Here we assessed the effect of the ACE inhibitor enalapril on the glomerular sieving coefficient of albumin (GSC_A) using intravital multiphoton microscopy. Munich Wistar Frömter (MWF) rats were used as a model of hypertension-related glomerular lesions. Young (9-week-old) MWF rats were non-proteinuric, similar to what was observed in control Wistar rats. However, the urinary albumin excretion in the MWF rats gradually increased during aging, averaging 0.00062±.0001 at the age of 9 weeks and 0.0054±.0003 (mg/mosmol/l) at the age of 52 weeks (p<.0001). The albuminuria in aged MWF rats was accompanied by structural changes, which were indicative of glomerular lesions. The GSC_A was low in young MWF rats but increased markedly during aging, averaging 0.00057±4.7*10^-5 (n=25) in young and 0.0027±0.00036 in 52-week-old MWF rats (n=36; p<.0001). The treatment of proteinuric 12-month-old MWF rats with enalapril over a 4-week-period reduced the GSC_A from 0.0027±0.00036 to 0.00139±0.00013 (p=.0005). Similarly, the urinary albumin excretion was reduced, averaging 0.0051±0.0003 and 0.0036±.0005 (mg/mosmol/l) before and after enalapril administration, respectively (p=.0089). In parallel, enalapril treatment reduced the mean arterial blood pressure (144.6±6.5 mmHg in untreated vs. 110.9±.6 mm Hg in enalapril-treated MWF rats) and increased the GFR from 1.64±0.3 ml/min to 3.58±0.3 ml/min (p=.0025 vs. baseline). In summary, enalapril reduced the GSC_A in proteinuric MWF rats, which was paralleled by a similar reduction in the urinary albumin excretion. These data suggest that glomerular rather than tubular mechanisms account for the beneficial antiproteinuric effects of the ACE inhibitor.
Introduction

Albuminuria, defined as the urinary excretion of more than 30 mg of albumin per day in humans, is considered a hallmark of kidney diseases of various etiologies. Furthermore, the magnitude of albumin excretion is a prognostic marker for the progression of kidney diseases. An inadequate function of the glomerular filtration barrier is the most likely cause for the development of albuminuria (Haraldsson et al., 2008), but compromised albumin retrieval by the tubular system may also be involved (Russo et al., 2002). In addition to being a symptom of various glomerular diseases, there is accumulating evidence that an enhanced filtration and excretion of albumin *per se* acts a risk factor for the progression of renal diseases (Remuzzi and Bertani, 1998; Remuzzi et al., 2005) and the occurrence of cardiovascular events (Wagner et al., 1994). Thus, when the filtration of albumin exceeds the normal level, it is partly compensated for by tubular uptake processes (Christensen and Birn, 2001), and the intracellular accumulation of albumin and its degradation products cause a detrimental local inflammatory response (Bertani et al., 1989).

Independent of the confounding causes for albuminuria, inhibitors of the renin-angiotensin system (RAS) in many cases reduce the degree of albuminuria (Barnett, 2005). This beneficial effect is presumably related to hemodynamic, *i.e.*, blood pressure lowering effects and to direct effects on the filtration barrier (Taal and Brenner, 2000). Thus, angiotensin II alters the function of the glomerular filtration barrier, resulting in increased leakiness for proteins, even when the renal perfusion pressure was kept constant (Axelsson et al., 2012; Schießl and Castrop, 2013).

Despite the compelling clinical evidence for an anti-albuminuric effect of RAS inhibitors, a direct evaluation of their effect on the filtration barrier is difficult. Thus, the evaluation of the morphologic effects of RAS inhibitors on the filtration barrier in kidney specimens, such as those prepared for the use in electron microscopy, is an indirect approach and is aggravated by possible fixation artifacts. Whole organ approaches, such as the
measurement of albumin in the urine, do not allow direct conclusions in terms of the function of the filtration barrier because the effects may be, at least partially, masked by the tubular uptake and degradation. These technical obstacles contribute to the recent controversy regarding the amount of filtered albumin in the healthy glomerulus and the contribution of proximal tubular dysfunction in the pathogenesis of albuminuria (Russo et al., 2002; Russo et al., 2007; Peti-Peterdi, 2009; Tanner, 2009; Schießl and Castrop, 2013; Castrop, 2014; Sandoval and Molitoris, 2014).

In this study, we used intravital multiphoton microscopy (MPM) to assess the effects of the ACE inhibitor enalapril on albumin filtration in an animal model of chronic albuminuria. Enalapril was used because of its well established renoprotective effects and to modulate the effects of angiotensin II on both AT1 and AT2 receptors (Schießl and Castrop, 2013). Munich Wistar Frömter (MWF) rats were used as a hypertensive model with age-dependent albuminuria and deterioration of kidney function, and the influence of enalapril on the glomerular sieving coefficient for albumin (GSCA) was determined in the live animal. We hypothesized that enalapril would reduce the leakiness of the filtration barrier and, consequently, the amount of albumin excreted in the urine. We further aimed to determine, if the antiproteinuric effect of enalapril was related to changes in the GFR.

We found that enalapril markedly reduced the GSCA, which was paralleled by a similar decline in urinary albumin excretion. The reduction in the GSCA during enalapril treatment was accompanied by considerable improvement of the GFR. Our data suggest that the antiproteinuric effect of ACE inhibitors is primarily mediated by modulation of the permeability of the glomerular filtration barrier.
Methods

Animals. Animal experiments were performed using male Munich Wistar Frömter (MWF) rats from a breeding colony at the University of Regensburg. For the experiments, we examined rats from the age of 6-8 until 52 weeks. The animals were fed standard rodent chow (# E15000, ssniff Spezialdiäten, Soest, Germany) and kept on a 12:12-h light-dark cycle. The animals had free access to tap water. All of the animal care procedures and experiments were approved by the IACUC of the University of Regensburg and were conducted according to the National Institutes of Health’s Guidelines for the use of laboratory animals.

MWF rats were used in the experiments because this strain has numerous surface glomeruli in the region of <100 µm below the kidney capsule and these glomeruli are readily accessible for MPM. The tubular system of the surface glomeruli of the MWF are characterized by short loops of Henle.

Animal preparation. For the multiphoton microscopy experiments, rats were anesthetized with 113 mg/kg thiobutabarbital (Inactin, 5-sec-butyl-5-ethyl-2-thioxodihydropyrimidine-4,6(1H,5H)-dione, Sigma Aldrich) by intraperitoneal injection. The body temperature was maintained at 37°C by placing the animals on an operating table with a servo-controlled heating plate, as described recently (Schießl and Castrop, 2013). The right carotid artery was cannulated using hand-drawn polyethylene tubing so that the arterial blood pressure and heart rate could be measured continuously. A cannula was inserted into the right jugular vein for the intravenous injection of fluorescent dye. For the imaging session, the left kidney was exposed by making a small flank incision. At the end of the experiments, animals were euthanized by the injection of a lethal dose of thiobutabarbital (300 mg/kg, i.v.).

Multiphoton microscopy. The experiments were performed using a Zeiss LSM 710 NLO confocal fluorescence microscope (Carl Zeiss Jena GmbH, Jena, Germany) equipped with a servo-controlled warming plate to maintain the body temperature of the animal at 37°C. Excitation was achieved using a Chameleon Ultra-II MP laser (Coherent Deutschland
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GmbH, Dieburg, Germany) at 860 nm with a laser power of 22% of 3200 mW. Sixteen-bit 512x512 pixel images (providing a theoretical dynamic range for intensities measurements of 0-65,536 pixel intensity) were obtained using a pixel dwell time of 1.27 µs and a line average of one by applying a 40x long distance (LD) C-Apochromat 40/1.1 water objective. The emissions were collected using external detectors (nondescanned detectors with filter set 1 (green channel): beamsplitter 500-550, longpass (LP) 555 and filter set 2 (red channel): beamsplitter P 565-610 including mirror) and internal detectors (blue channel: 420 – 440/main beamsplitter: 458). The detector settings were kept constant for all measurements: for the green, red and blue channels, respectively, the master gain was 500/500/500, the digital gain was 10/10/10 and the offset was -4000/-3500/-3000. The photodetector offset was chosen to reduce background fluorescence and, at the same time, keep an adequate detector sensitivity. For an analysis of the offset/albumin GSC ratios see the supplemental figure 1.

To label the vasculature, a 5 mg/ml solution of Alexa Fluor 594 BSA conjugate (Invitrogen GmbH, Darmstadt, Germany) dissolved in PBS was first purified by dialysis for three days (Spectra/Por Float-A-Lyzer, 50 kDa, Spectrum Europe B.V. Breda, Netherlands), then concentrated using Nanosep® Centrifugal (VWR International GmbH, Darmstadt, Germany) and injected i.v. (1.3 µl/g). The fluorescence was detected using the red channel. Consequently, only the labeled exogenously administered albumin was measured in the experiments. The proximal tubules were visualized by collecting autofluorescence (green channel), and the collagen was visualized using second harmonic generation (SHG, blue channel).

**Determination of the albumin glomerular sieving coefficient (GSC_{A}).** The GSC was defined as the ratio of the albumin concentration in the filtrate (in Bowman’s space) to the albumin concentration in the plasma (in the glomerular capillaries). In this study, the albumin GSC was determined using multiphoton microscopy to determine the fluorescence intensity ratio of the Alexa Fluor 594 BSA conjugate.
For each animal, 2-4 superficial glomeruli were selected using visible (green) light from a mercury arc lamp. All of the measurements were taken at a depth of 30 µm underneath the kidney capsule (identified by the SHG of the collagen) to keep the intensity values between measurements comparable. For each measurement, six images were collected with a time lapse of 10 seconds to minimize laser-induced tissue damage. A z-stack was constructed for each of the selected glomeruli to assess the 3-D structure of glomerular capillaries and to ensure that the fluorescent values measured in the Bowman’s space were not artificially increased by out-of-focus fluorescence from the capillaries in the immediate vicinity below or above the focal plane. To prevent the tissue from photo-bleaching, the laser power used to generate the z-stacks was reduced to 18%.

To determine the albumin GSC, the fluorescent intensity in three regions of interest (ROIs, 150 pixels each) within the glomerular capillary loops and two ROIs in the Bowman’s space were measured for each of the six images. For the fluorescence intensity in the plasma, the ROIs were placed within the outer margins of the capillaries showing the brightest fluorescence in the field. The ROIs in the Bowman’s space were selected in regions away from capillaries.

To correct for background noise, six background images for each glomerulus were collected, before the dyes were injected, using identical recording settings. To measure the background fluorescence intensities, four ROIs were selected in Bowman’s space and two ROIs were selected within the capillary loops of each analyzed image.

GSC calculations were based on the average intensity values after subtracting the background fluorescence for the plasma and Bowman’s space using the following formula:

\[
\text{albumin GSC} = \frac{\text{intensity Bowman’s space} - \text{intensity background Bowman’s space}}{\text{intensity capillaries} - \text{intensity background capillaries}}.
\]

Lower limit of detection (LLD) for the fluorescent intensity in Bowman’s space. The LLD for the intensity values in the Bowman’s space was calculated for each experiment as
previously described (Anderson, 1989; Tanner, 2009). The calculated values for the albumin GSC were only used in further analyses if the mean fluorescence intensity value of the Alexa 594-BSA in the Bowman’s space exceeded the value of the LLD.

*Imaging of glomerular morphology using MPM:* The vasculature was stained by injecting rhodamine B, which binds to plasma proteins. To visualize podocytes, negative imaging was performed during a continuous infusion of 3 µl/min of a 5 mg/ml Lucifer Yellow (LY) solution (Peti-Peterdi and Sipos, 2010). This small molecular weight dye is freely filtered into Bowman space and stains the primary filtrate. Podocytes are excluded from this staining and appear as dark objects at the outer margins of the glomerular capillaries. By contrast, mesangial cells take up Lucifer Yellow.

*Proximal tubular albumin uptake.* The proximal tubular albumin uptake was quantified as the intensity of Alexa Fluor 594 BSA per area. In detail, 3-4 images of S1 proximal tubule segments were acquired 20 minutes after the injection of Alexa Fluor 594 BSA and proximal tubular fluorescence intensities were measured in 3-4 regions of interest (ROI) for each image. The values were corrected for background autofluorescence. The values are given as intensities/area of the ROI. The average ROI was 360±14 µm².

*Determination of the glomerular filtration rate (GFR).* To monitor GFR during enalapril treatment of 12-month-old MWF rats, GFR was determined as the creatinine clearance. To gain the required plasma samples, blood was obtained by tail-bleed at baseline and after 2 and 4 weeks of enalapril administration. Urine samples were collected in metabolic cages (6 hours) after animals were accustomed to the metabolic cages for one week. Urine collection was done between 9 am and 3 pm (6 hours) to eliminate possible circadian variations. After measurement of creatinine concentrations in urine and plasma (conducted by the IDEXX veterinarian laboratory by the use of the CREAplus enzymatic assay), the GFR was calculated using the following formula: GFR= (creatinine concentration in urine * urinary volume per minute)/(creatinine concentration in plasma).
**Quantification of the urinary albumin excretion.** To quantify the urinary albumin content, the urine samples were collected in metabolic cages and diluted 1:10. The diluted samples were separated on a 10% SDS-PAGE gel. Albumin BSA standard solutions (0.25/0.5/1.0/2.0 µg/µl) were prepared and separated on the gel. A Coomassie staining was performed to visualize the albumin content, and the amount of albumin in the samples was analyzed by densitometry. Because the urinary albumin concentration for a given albumin filtration and tubular re-uptake depends on the degree of volume reabsorption along the tubular system, the urine albumin content was normalized against the urine osmolarities, which were determined using the freezing point depression method, as described (Oppermann et al., 2010). This normalized concentration of albumin in the urine was used as a surrogate for renal albumin excretion per time.

**Determination of plasma renin activity (PRA).** PRA was measured in seven young (8 weeks of age) and six old MWF rats (52 weeks of age). Blood samples were collected from rats by tail bleeding. Approximately 60 µL of blood were collected into an EDTA-containing microhematocrit tube. Red blood cells and plasma were separated by centrifugation; the plasma was frozen until used for renin measurements. Using a 2-fold dilution of 12 µl of plasma, renin activity was measured by radioimmunoassay (DiaSorin, Stillwater, MN), as described in detail elsewhere (Mederle et al., 2013).

**Experimental protocols** To characterize the urinary albumin excretion in aging rats, urine samples from four MWF rats and two control Wistar rats were collected in metabolic cages at the ages of 9, 12, 14, 16, 18, 22, 25 und 52 weeks and the albumin/osmolality-ratio was analyzed.

In order to analyze the glomerular albumin filtration in young and aged MWF rats, the GSC₂ was determined in five young male MWF rats (<9 weeks of age) and in five 52-week-old MWF rats.
Enalapril ((2S) – 1 - [(2S) – 2 - {{(2S) – 1 – ethoxy – 1 – oxo – 4 – phenylbutan – 2 - yl] amino} propanoyl] pyrrolidine – 2 - carboxylic acid, Sigma Aldrich) was dissolved in water and added to the drinking water of six 48-week-old male MWF rats to a final concentration of 100 mg/L. The drinking volume was monitored daily and, if necessary, the concentration was adjusted to obtain a daily dosage of approximately 10 mg/kg. The duration of the enalapril treatment was 1, 2, 3, and 4 weeks. Urine samples were collected in metabolic cages at baseline and every week during the enalapril treatment for the determination of the urinary albumin excretion and the GFR. Plasma samples for the GFR measurement were obtained under baseline conditions and during the enalapril administration at the time points of 2 and 4 weeks.

Quantitative image analyses and statistics. Multiphoton microscopy images were analyzed using the ZEN 2010 64-bit software (Carl Zeiss Jena GmbH, Jena, Germany). The Coomassie-stained SPS-PAGE gels were analyzed by densitometry using Image J 1.47 (W. Rasband, NIH).

Data were further analyzed using paired or unpaired t-tests in Graph Pad Prism 5 (GraphPad Software, Inc., La Jolla, USA) or by ANOVA with the Bonferroni post hoc test when necessary. All data are given as the mean±SEM (standard error of the mean). A p-value <0.05 was considered significant.
Results

Changes in the urinary albumin excretion in MWF rats and Wistar control rats during aging. As a model of low nephron number, MWF rats gradually develop glomerulosclerosis, hypertension and proteinuria (Remuzzi et al., 1988; Hackbarth et al., 1991). An in vivo inspection of the morphology of the glomeruli of 9-week-old MWF rats revealed no apparent abnormalities (fig. 1). In contrast, the glomeruli of 52-week-old MWF rats showed marked morphological alterations, as shown in figure 2. Thus, in some glomeruli, podocyte detachment from the basement membrane was observed, which was accompanied by an intense fluorescence of rhodamine B-labeled plasma proteins in the urinary space, indicating massive local protein filtration. In addition, the subpodocyte space occasionally was expanded, forming pseudocysts (Peti-Peterdi and Sipos, 2010) (fig. 2). Furthermore, as shown in figure 2, we found evidence for mesangial matrix expansion.

To determine the progression of proteinuria in MWF rats of our colony, we characterized the urinary albumin excretion during aging. As a control, the urinary albumin of Wistar rats was measured. Urine from MWF rats and control Wistar rats was collected in metabolic cages at 9, 12, 14, 16, 18, 22, 25 and 52 weeks of age and the albumin/osmolality-ratio was determined. The urinary albumin excretion of Wistar rats did not change over time and was low at all investigated time points, averaging 0.00079±6.5*10^{-5} [mg/mL]/[mOsmol/kg] (n=16). There was no difference in the urinary albumin excretion of the 9-week-old MWF rats when compared with the non-proteinuric Wistar rats. However, as shown in figure 3, the albumin/osmolality-ratio in the MWF rats increased from 0.00062±0.0001 at 9 weeks of age to 0.0054±0.0003 [mg/mL]/[mOsmol/kg] at 52 weeks of age (linear regression: p<0.0001; r^2=0.59; n=5). When compared with the Wistar rats, the urinary albumin content was significantly higher in the MWF rats at the age of 18, 22, 25 and 52 weeks (p=.0189, .0001, .0015 and <.0001, respectively).
Effect of enalapril on the urinary albumin excretion in aged MWF rats. Because angiotensin II is known to be involved in the progression of renal failure and proteinuria (Remuzzi et al., 2005), the plasma renin activity (PRA) was determined in 8 and 52 week old MWF rats. The PRA in the old rats was lower compared with the young animals, averaging 5.4±1.2 and 1.3±0.1 ng Ang I/mL/hr, respectively (p=.008; n=7 and 6, respectively; fig. 4).

To address the anti-proteinuric effects of ACE inhibition on the urinary albumin excretion, 12-month-old proteinuric MWF rats received enalapril (100 mg/L) in the drinking water over a period of 4 weeks. Enalapril treatment caused a significant reduction in the mean arterial blood pressure (MAP) in 12-month-old MWF rats. The MAP in untreated MWF rats was 144.6±6.5 mmHg (n=5). Following 4 weeks of enalapril treatment, MAP was reduced to 110.9±6 mm Hg (n=6; p=.0011). To determine the effects of enalapril on the urinary albumin excretion, urine was collected every week in metabolic cages, and the albumin excretion was determined. As shown in fig. 5, the urinary albumin excretion decreased significantly in response to enalapril. The albumin/osmolality-ratio was 0.0051±0.0003 [mg/mL]/[mOsmol/kg] (n=6) during baseline conditions, and declined to 0.0023±.0004, 0.0033±.0003, 0.0041±.0003, and 0.0036±.0005 [mg/mL]/[mOsmol/kg] after 1, 2, 3 and 4 weeks of enalapril administration, respectively (p=0.0002, 0.0056, 0.0304, and 0.0089, respectively). Thus, albumin excretion was reduced by approximately 50% after one week of enalapril treatment and remained almost constant during the following 3 weeks.

GSC\textsubscript{A} in young and old MWF rats. Considering the ongoing discussion regarding the physiological extent of glomerular albumin filtration (Russo et al., 2007), we aimed to determine whether the observed proteinuria in 12-month-old MWF rats was due to an increased glomerular albumin filtration or rather due to an insufficient proximal tubular reabsorption of the filtered albumin. To investigate the glomerular albumin filtration in young (<9 weeks) and old (12 months) MWF rats, the glomerular sieving coefficient of albumin (GSC\textsubscript{A}) was determined using MPM. We found that the GSC\textsubscript{A} in young MWF rats (<9 weeks
of age) was low, averaging $0.00057 \pm 4.7 \times 10^{-5} \text{ (n=25)}$. At the age of 12 months, the GSCA rose to $0.0027 \pm 0.00036 \text{ (n=36, p<0.0001)}$ in the MWF rats, an increase of almost 400% (fig. 6).

**Effect of enalapril on the GSCA of proteinuric old MWF rats.** Because the urinary albumin excretion in old MWF rats was significantly reduced during administration of enalapril, we next investigated, if the antiproteinuric effects of enalapril are related to changes in the permeability of the glomerular filtration barrier. As shown in fig. 7, after 4 weeks of enalapril treatment, the GSCA of 12-month-old MWF rats decreased by approximately 50%, averaging $0.0027 \pm 0.00036$ before and $0.00139 \pm 0.00013$ after enalapril administration (n=36 and 39 glomeruli, respectively; p=.0005). Thus, the relative reduction of the GSCA caused by the enalapril treatment was quantitatively similar to what was observed for the urinary albumin excretion.

**Effect of enalapril on proximal tubular albumin uptake in old MWF rats.** Urinary albumin excretion depends on albumin filtration and tubular re-uptake. Therefore, we next determined the effect of enalapril on the uptake of filtered albumin in the S1 segment of the proximal tubule. Tubular Alexa Fluor 594 BSA was markedly reduced after enalapril treatment averaging $44.6 \pm 4.9$ and $13.7 \pm 1.9/\mu m^2$ in untreated and treated old MWF rats, respectively (p<.0001; fig. 8).

**Effect of enalapril on the GFR of old MWF rats.** Because glomerular albumin filtration is dependent on the GFR (Haraldsson et al., 2008), we next evaluated changes in the GFR during enalapril treatment. The GFR of the animals was estimated by the creatinine clearance method. The GFR of 12-month-old MWF rats was determined before and after 2 and 4 weeks of enalapril therapy. GFR was $1.64 \pm 0.3 \text{ ml/min}$ before the enalapril administration and increased to $3.44 \pm 0.1 \text{ ml/min} \text{ (n=6, p=.0022 vs. baseline)}$ after two weeks of enalapril administration. After 4 weeks of enalapril treatment, the GFR averaged $3.58 \pm 0.3 \text{ ml/min} \text{ (n=6, p=.0025 vs. baseline)}$. Thus, the RAS inhibition significantly improved the GFR in 12-month-old MWF rats (fig. 9).
Discussion

In this study we investigated the glomerular albumin filtration and the antiproteinuric effects of ACEi treatment in aged MWF rats using intravital multiphoton microscopy. MWF rats were used in the study as a disease model of hypertension with chronic albuminuria. We found that enalapril markedly reduced the albuminuria in aged MWF rats and that this antiproteinuric effect was directly related to changes in the glomerular albumin filtration.

To address the antiproteinuric effect of enalapril, the glomerular sieving coefficient of albumin was determined in vivo using multiphoton microscopy (MPM). This technique allows for the simultaneous determination of the glomerular function and morphology in the live animal (Peti-Peterdi et al., 2012). A limitation is that in mammalian kidneys the majority of glomeruli are too deep to be visualized by MPM (Schießl et al., 2013). Due to their high number of superficial glomeruli, MWF rats are particularly suited for the investigation of glomerular function by MPM, and, similarly, by micropuncture (Hackbarth et al., 1983). For the investigation of the normal function of the glomerulus, however, only young MWF rats appear to be suitable because aged MWF rats develop hypertension, proteinuria, and glomerulosclerosis (Hackbarth et al., 1991). This pathology is most likely related to the low nephron number of this rat strain (Rothermund et al., 2011). In accordance with recent ex vivo evaluations of the glomerular morphology of aged MWF rats (Macconi et al., 2006), we found marked structural alterations in vivo, including the detachment of podocytes from the basement membrane, formation of pseudocysts, and evidence for mesangial matrix expansion. As a limitation of the MWF strain, the surface glomeruli of the MWF have very short loops of Henle and it remains to be determined what the contribution of these nephrons is to total kidney albuminuria.

In young MWF rats, the GSC for albumin was very low (<0.0006), suggesting that the glomerular filtration of albumin in the normal kidney is minimal. The limited glomerular filtration of large proteins, such as albumin, is generally considered to be related to the
charge- and size-selective properties of the glomerular filtration barrier (Haraldsson et al., 2008; Jeansson et al., 2009). The low GSC values of albumin are in agreement with recent data from our and other groups using MPM to determine the GSC of albumin (Peti-Peterdi, 2009; Tanner, 2009; Schießl and Castrop, 2013). Similar low values for the GSC of albumin were determined using micropuncture techniques. Thus, the GSC for albumin in the rat was found to be in the range of 0.0003 to 0.0006 (Oken and Flamenbaum, 1971; Stolte et al., 1979; Tojo and Endou, 1992). These data are in contrast to recent reports, which challenged the classic view that albumin is largely restricted from the glomerular filtration. Thus, Russo et al. reported, using MPM, that the normal kidney filters albumin at near nephrotic levels, with GSC values for albumin of approximately 2 orders of magnitude higher than what was found in our study (Russo et al., 2007). It was suggested, that this massive filtration of albumin is subsequently compensated for by proximal tubular uptake and transcytosis of intact albumin (Dickson et al., 2014). These findings sparked a heated debate regarding the magnitude of glomerular albumin filtration in the normal kidney and its implications for albuminuria (Christensen et al., 2007; Russo et al., 2007; Peti-Peterdi, 2009; Tanner, 2009; Schießl and Castrop, 2013; Castrop, 2014; Sandoval and Molitoris, 2014). The common understanding of the integrity of the filtration barrier implies that albumin is largely restricted from glomerular filtration (Haraldsson and Sorensson, 2004). Albuminuria therefore is usually considered to be caused by alterations in the properties of the glomerular filtration barrier (Salvetti et al., 1999; Haraldsson et al., 2008). Conversely, other researchers have proposed extensive glomerular albumin filtration suggesting that albuminuria is primarily an issue of inadequate tubular albumin retrieval (Dickson et al., 2014). Our data from the aging MWF rats, however, do not support this assumption because the increase in urinary albumin excretion in the aged rats was paralleled by a similar increment in the GSC for albumin, suggesting that the primary cause for albuminuria is a glomerular one.
The antiproteinuric effects of ACEi are well known, but the underlying mechanisms responsible for these effects have not yet been fully elucidated. As expected, the administration of enalapril to aged MWF reduced the mean arterial blood pressure and caused a considerable reduction in the urinary albumin excretion. It should be noted that, in this context, the plasma renin activity of the aged MWF rats was low when compared with young animals, confirming the clinical observation that ACEi has antiproteinuric effects also in patients with low-renin hypertension (Jafar et al., 2003; Arnold et al., 2013). Apparently, when the function of the glomerular filtration barrier is compromised due to various insults, even low angiotensin II levels are sufficient to promote albuminuria. In this context, an acute increase in the filtration of macromolecules in response to angiotensin II infusion without pre-existing glomerular structural impairments has been shown (Axelsson et al., 2012; Schießl and Castrop, 2013). These acute effects of angiotensin II on the GSC of albumin were largely blunted in the presence of the AT1 receptor antagonist losartan, suggesting that the effect of enalapril observed in our study was primarily related to an alteration of AT1 rather than AT2 receptor signaling (Schießl and Castrop, 2013).

The reduction of albuminuria in aged MWF rats during ACEi was accompanied by a similar decrease in the GSC of albumin. Thus, during enalapril treatment, the GSC of albumin and the urinary albumin excretion were similarly reduced by approximately 50%, suggesting again that the proteinuric effect of angiotensin II is related to glomerular rather than to tubular effects. The proximal tubular uptake of Alexa-albumin was markedly reduced after administration of enalapril suggesting that the ACE inhibition reduced the tubular load of albumin. As an alternative explanation, enalapril administration may have inhibited the proximal tubular albumin uptake. The latter explanation, however, appears less likely, because it was recently shown that blockade of the renin-angiotensin system promotes the tubular uptake of plasma proteins (Tojo et al., 2003) and, furthermore, reduced tubular albumin uptake would lead to increased, not decreased, albuminuria.
AT1 receptors are expressed in several structures of the glomerulus, including podocytes and mesangial cells (Sharma et al., 1998; Miyata et al., 1999). AT1 receptors have been shown to regulate the intracellular calcium level, cAMP concentration and membrane potential of cultured podocytes, and angiotensin II may modulate the podocytes’ cytoskeleton and, consequently, the structure and function of the foot processes (Gloy et al., 1997; Gloy et al., 1998; Gekle, 2007). Contractions of podocytes may increase the leakiness of the filtration barrier for macromolecules such as albumin (Lapinski et al., 1996; Kriz, 2004). In addition, when podocytes contract the dimensions of the subpodocyte space will be reduced. The subpodocyte space is the area confined by the basement membrane and the podocytes, and this area restricts the movement of large molecules, such as albumin, across the glomerular capillary wall (Neal et al., 2005; Salmon et al., 2007). Similar to what has been observed for podocytes, there is considerable evidence that cultured mesangial cells constrict in response to AT1 receptor activation (Stockand and Sansom, 1998). Angiotensin II therefore may reduce the size of the effective filtration area and this would cause a reduction in the single nephron GFR for a given net filtration pressure. Changes in the snGFR, in turn, may influence the albumin GSC, as outlined below.

The inhibition of the RAS by ACEi and ARBs occasionally causes a transient reduction of the GFR, and this appears to be particularly relevant in patients with an activated RAS, such as during severe hypovolemia or renal artery stenosis (Campanacci et al., 1989; Bakris and Weir, 2000). Nevertheless, in the long run, ACEi and ARBs preserve the magnitude of renal filtration and delay the progression of the deterioration of renal function in several diseases, such as diabetes and hypertension (Barnett, 2005; Ruggenenti et al., 2012). In our model of low-renin hypertension, treatment of the aged MWF rats with enalapril over 4 weeks even increased the GFR. Considering the impact of changes in the GFR on the filtration of albumin, Lund et al. reported that the glomerular GSC of macromolecules rose when the single nephron GFR was markedly reduced (Lund et al., 2003). The authors
suggested that the increased GSC was related to the dominant contribution of diffusion to the transport of macromolecules across the filtration barrier, when the single nephron GFR approaches values of zero (Lund et al., 2003). According to these data, the rise in GFR observed during enalapril treatment may have contributed to the reduction of the albumin GSC. The exact quantitative contribution of the increased GFR to changes in the albumin GSC remains to be determined. It appears likely that the increase in total kidney GFR during enalapril treatment was not mediated homogeneously by all glomeruli. Thus, a correlation between albumin GSC and the GFR would need to be established at the single nephron level.

In summary, albuminuria in aged MWF rats was related to an increased glomerular albumin filtration. The ACE inhibitor enalapril reduced the degree of albuminuria, and this was accompanied by a quantitatively similar decrease in the GSC of albumin. The increase in GFR during enalapril treatment may have contributed to the decrease in the albumin GSC. Furthermore, enalapril treatment reduced the proximal tubular uptake of albumin suggesting a reduced tubular albumin load. These data suggest that the antiproteinuric effects of the ACE inhibitor are related to changes in glomerular albumin filtration rather than to a modulation of the tubular albumin retrieval.
Authorship contributions

Participated in research design: Schießl, Castrop

Conducted experiments: Schießl, Kattler, Castrop

Contributed new reagents or analytic tools: N/A

Performed data analysis: Schießl, Kattler, Castrop

Wrote or contributed to the writing of the manuscript: Schießl, Castrop
References


Footnote

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Figures

Figure 1: Healthy glomerulus in a 9-week-old male MWF rat. Plasma was stained by rhodamine b, which binds to plasma proteins and therefore remains within the renal vasculature. Podocytes are visualized by negative imaging during a continuous infusion of LY and appear as dark objects at the outer margin of the glomerular capillaries (thin arrows). Mesangial cells absorb the LY over time and obtain a bright green staining (thick arrows).

Figure 2: Glomeruli of old MWF rats showed marked morphological alterations. (A) In a few glomeruli, extended areas of the glomerular basement membrane lacked podocytes (tag) and this was accompanied by a massively increased permeability of the filtration barrier for rhodamine B-labeled plasma proteins (marked red fluorescence in the urinary space). (B) In some areas the typical green staining of mesangial cells by LY was expanded along the capillary wall indicating expansion of the mesangium (arrows). (C) Glomerular pseudocyst containing rhodamine B-labeled plasma proteins (asterisk).

Figure 3: Characterization of the urinary albumin excretion in aging MWF and Wistar rats. Young MWF rats (< 9 weeks) are non-proteinuric when compared with Wistar rats. Urinary albumin content in MWF rats increases linearly with age.

Figure 4: Plasma renin activity in male, young (8 weeks of age) and old MWF rats (52 weeks of age). Old MWF rats show significantly lower levels of renin when compared with young animals.

Figure 5: Effects of enalapril on the urinary albumin excretion in old MWF rats. Albumin/osmolality-ratio in old MWF rats, before and during the treatment with enalapril (n=6). * indicates p<.05 vs. baseline.
Figure 6: Albumin GSC was determined in young (< 9 weeks of age) and old MWF rats (52 weeks of age). The glomerular albumin filtration was significantly elevated in old MWF rats.

Figure 7: Effects of enalapril on the GSCA in old MWF rats. The albumin GSC was reduced by the application of enalapril.

Figure 8: Effect of enalapril on the proximal tubular albumin uptake in old MFW rats. (A) Proximal tubular uptake of albumin (red, arrows) in an untreated old MWF rat (52 weeks of age). The proximal tubular albumin uptake (S1 segment) was reduced by the application of enalapril (B). (C) Proximal tubular Alexa-albumin fluorescence intensities in untreated and enalapril-treated old MWF rats.

Figure 9: Effects of enalapril on the GFR in old MWF rats.
Figure 1
Figure 4

The diagram shows a comparison of AngI levels between the MWF young and MWF old groups. The x-axis represents the groups, with MWF young on the left and MWF old on the right. The y-axis represents the concentration of AngI in ng AGL/mL/h, ranging from 0 to 15.

Significant difference between the groups is indicated by the p-value of 0.008.
Figure 6
Figure 7

![Graph showing GSCA with two groups: MWF old and MWF old + enalapril. The p-value is 0.0005.](image-url)
Figure 8

A

B

C Proximal tubular albumin uptake

![Graph showing proximal tubular albumin uptake](image)

- **Proximal tubular albumin uptake**
- Graph indicates a significant difference between untreated and treated conditions with a p-value of <0.0001.
Photodetector offset calibration for the determination of albumin GSC in old MWF rats. Large negative offsets were applied to reduce background fluorescence. Extremely large negative offsets (-6500) result in a false GSC values of zero (i.e., the measured intensity in the Bowman’s space equals zero). Large positive offsets lead to a saturation of the fluorescence signal in the glomerular capillaries, which causes falsely high albumin GSC values. The offset used in this study (-3500) is in the lower range of constant GSC/offset ratios and appears to be a suitable compromise between sensitivity and background fluorescence reduction. N=6 glomeruli for each data point.