Nature of glomerular capillary permeability changes following acute renal ischemia/reperfusion (I/R) injury in rats

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Abstract

This study was performed to evaluate the alterations of the glomerular filtration barrier characteristics following acute renal ischemia/reperfusion (I/R). Ischemia was induced in anesthetized rats by unilateral renal artery occlusion for either 20 or 60 minutes, followed by reperfusion during 20 or 60 minutes, respectively, with the contralateral kidney serving as control. Sieving coefficients (θ) were obtained by analyzing Ficoll [mol.radius (a_e) 13-85 Å] in urine and plasma after 20 and 60 min I/R. Furthermore, θ for human serum albumin (HSA) was estimated using a tissue uptake technique after 20 and 60 min of I/R, while clearance of HSA compared to that for neutralized HSA (nHSA) was assessed after 20 min of I/R only. Glomerular filtration rate (GFR) was measured by ^{51}Cr-EDTA and inulin. I/R reduced GFR and increased θ for Ficoll molecules of a_e >55 Å and θ for albumin. θ for Ficoll *vs.* a_e, analysed using a two-pore model, demonstrated that, despite increases in θ, the large pore fractional ultrafiltration coefficient (α_L) was unchanged after 20 min of I/R, owing to the decline in GFR, but increased after 60 min of I/R. However, the apparent α_L for albumin, increased already after 20 min of I/R ($p<0.005$) and the nHSA/HSA clearance ratio was slightly reduced, possibly reflecting a diminished negative charge barrier. In conclusion, after 20 min of I/R indications of a reduced charge selectivity were noted, while after 60 min of I/R, there was mainly a reduction in size selectivity, compatible with an increased formation of large pores.

Key words: albumin, Ficoll, anoxia, fractional clearance, oxidative stress

Running head: Increased glomerular permeability after acute I/R.

Introduction

Ischemia represents the most common cause of acute renal failure (ARF). When renal blood flow is compromised, oxygen is depleted from the tissue resulting in a diminution of ATP. This reduces the capacity of the Na/K-ATPase, producing cell swelling. During reperfusion free oxygen radicals are formed, further contributing to cellular damage. The effects of ischemia/reperfusion (I/R) on tubular epithelial cell damage have been studied extensively, particularly the decreased capacity for tubular protein-reuptake, leading to proteinuria. Following I/R the function of the glomerular barrier is clearly also affected. However, this has only rarely been addressed previously.

The glomerular barrier is made up by three sequential layers: the fenestrated endothelium with its glycocalyx, the glomerular basement membrane (GBM), and finally, the podocytes with their interdigitating foot processes and the podocyte slit diaphragms (PSD) (6). All of these three layers are potential ischemic targets, and alterations in any of them would lead to an increased glomerular permeability. The first layer, the endothelial glycocalyx, is composed of negatively charged proteoglycans that, conceivably, contribute to the negative charge of the glomerular barrier. Morphological studies in heart capillaries have indicated that ischemia causes disruption and “clumping” of the endothelial glycocalyx (16, 29), hence suggesting that the disruption of glomerular glycocalyx may play a role during ischemia-induced proteinuria. However, the endothelial glycocalyx is not visualised by normal fixation procedures, and is therefore overlooked in many pathological situations. Since ischemia does not cause any obvious ultrastructural changes of the glomerular filter, it has been proposed that the proteinuria-induced by ischemia may, at least partly, be a result of injury to the glycocalyx.

The second layer, the glomerular basement membrane (GBM), is composed of collagen type IV, laminin-11, entactin and heparan sulphate proteoglycans (HSPGs), such as perlecan and agrin (9). Heparan sulphate proteoglycans are important components of the GBM, and their negative charge may be of both structural and functional importance. This is evident in animals overexpressing heparanase-1, an enzyme degrading HSPG chains, the ensuing alteration in the GBM resulting in proteinuria (33). Furthermore, increased concentrations of metalloproteinases have been detected in glomeruli after I/R (4), and may be a factor contributing to ischemic proteinuria, as these enzymes are important for normal degradation and remodelling of the GBM.

It has been shown that podocytes undergo flattening and spreading of their foot-processes in acute renal failure (20). These changes occur early, already during the ischemic period, and increase in severity with the ischemic duration. There is a close structural interaction between the GBM and the podocytes. In mice with altered GBM, the podocytes appear effaced with a reduced number of foot processes (33). Hence, following I/R, alterations clearly occur in all layers of the glomerular capillary wall.

The purpose of the present study was to investigate the functional nature of the lesions occurring in the glomerular capillary wall following acute I/R injury. To determine if acute I/R leads to an alteration in the size selective barrier we investigated the urine excretion of FITC-Ficoll, a neutral polydisperse polysaccharide, allowing the assessment of sieving coefficients (fractional clearances, θ) for a broad spectrum of molecular sizes in one single experiment. To get further insights into the potential charge dependent nature of permeability changes, we also assessed the fractional clearance of native (neg.) albumin. Moreover, when permeability changes were very

discrete, i.e. after 20 min I/R, we compared the clearance of native (neg. charged) albumin to that of neutralized albumin.

Glossary

α_L	Fractional ultrafiltration coefficient accounted for by large pores
$A_0/\Delta x$	Effective area for diffusion over unit path length
$\Delta\pi$	The colloid osmotic pressure difference across the glomerular capillary wall
Jv_L	Large pore volume flow
LpS	The total glomerular UF coefficient (hydraulic conductivity)
θ	Sieving coefficient, fractional clearance (primary urine-to-plasma concentration ratio)
σ_L	Large pore reflection coefficient
r_s	Small pore radius
r_L	Large pore radius

Experimental Procedures

Experiments were performed in male Wistar rats (Møllegaard, Lille Stensved, Denmark). The experimental groups are presented in Table 1. In addition, one group of animals (n=7) were used for assessing the clearance ratio of neutralized albumin to native (neg.) albumin after 20 min of I/R. The rats were kept on standard chow and were allowed free access to food and water until the day of the experiments. The experiments were approved by the Animal Ethics Committee at Lund University and conducted according to the *APS's Guiding Principles in the Care and Use of Animals.*

Surgery

Anaesthesia was induced with pentobarbital sodium (60 mg/kg i.p.) and body temperature was kept at 37°C by a thermostatically controlled heating pad. The tail artery was used for continuous monitoring of arterial pressure on a polygraph (Grass instruments Co. Model 7B, Quincy Mass., USA). A tracheotomy was performed to facilitate breathing. The left carotid artery and the left jugular vein were cannulated (PE-50) for blood sampling and infusion purposes respectively. Access to the abdominal cavity was obtained through a midline abdominal incision. The urethers were dissected free and cannulated (PE-10) for continuous urine sampling. Furosemide (Impugan®, Dumex-Alpharma, 0.5 mg) was administered in the tail artery to increase urine production and facilitate cannulation of the ureters.

Experimental protocols

GFR was measured in both kidneys separately during the pre-ischemic resting period (20-30 min), using ^{51}Cr-EDTA. A priming dose of ^{51}Cr-EDTA (100 μl, 0.37 MBq, Amersham, Biosciences, Buckinghamshire, UK) was administered and followed by a continuous infusion (3 ml/hour) of ^{51}CrEDTA (0.37MBq/ml in 0.9% NaCl) throughout the experiment. Ischemia of the left kidney was induced with a temporary 20 min or 60 min clamp of the left renal artery. Urine was collected from the urethers (no urine was produced from the clamped kidney during the artery obstruction). After the ischemic period (20 or 60 min) the artery clamp was removed, followed by a 20 or 60 min reperfusion period (respectively). During the reperfusion period, urine production resumed in the previously obstructed kidney. Ten minutes before the end of the reperfusion period a bolus dose containing fluorescein isothiocyanate (FITC) labelled Ficoll-70

(42 μg), FITC-Ficoll-400 (1 mg) and FITC-Inulin (0.5 μg) (TdB Consultancy, Uppsala, Sweden) was given followed by an infusion of FITC-Ficoll-70 (94.5 μg/min), FITC-Ficoll-400 (3 mg/min), FITC-Inulin (1.5 μg/min) and ^{51}Cr-EDTA (n=7 at 20 min and n=9 at 60 min). At the end of the reperfusion period, urine was collected for 5 minutes and a midpoint plasma sample was aspirated for analysis of Ficoll sieving coefficients.

Tissue uptake technique:

After 20 or 60 min of I/R, albumin θ was measured using a tissue uptake technique (17). ^{125}I-human serum albumin (HSA) was given as a bolus (0.2 MBq, Institute for Energy Technique, Kjeller, Horten, Norway) in the tail artery (n=11 at 20 minutes and n=6 at 60 minutes). Six blood samples (25 μl) and one urine sample were collected during 8 min. Thereafter, a whole body washout was performed via the carotid artery (20 ml/min) for 8 minutes. The inferior vena cava was freed and cut open for collection of the rinse fluid. The washout fluid mixture contained equal amounts of 0.9% saline and heparinized horse serum (SVA, Uppsala, Sweden). Thereafter, the kidneys were removed and the cortex was dissected and assessed with respect to radioactivity. Sieving coefficients for neutralized human serum albumin (nHSA) were measured after 20 min of I/R (n=6) as described above for ^{125}I-HSA. In seven additional experiments, we simultaneously compared the clearance of ^{125}I-HSA to that of ^{131}I-nHSA after 20 min ischemia. The simultaneous measurement of ^{125}I-HSA and ^{131}I-nHSA did not allow the assessment of GFR by ^{51}Cr-EDTA. Hence, only a clearance ratio, but not absolute values of θ, could be obtained in these experiments. nHSA was prepared by Dr. Olav Tenstad by a graded modification of the COOH-groups using a procedure modified from Hoare and Koshland (11) and earlier described at some length (17). nHSA was labelled with ^{131}I, using 1,3,4,6-tetrachloro-3α,6α-

diphenylglycouril (Iodo-Gen) as described in (17). All radioactivity measurements were performed in a gamma scintillation counter (Wizard 1480, LKP Wallac, Turku, Finland). Radioactive decay and spill over from the ^{51}Cr to the ^{125}I channel, or from the ^{131}I channel to the ^{125}I channel, were appropriately accounted for.

High Performance Size Exclusion Chromatography (HPSEC)

Size exclusion was achieved by using an Ultrahydrogel-500 column (Waters) and a phosphate buffer with 0.15 M NaCl (pH 7.4). Fluorescence was detected with a fluorescence detector with a $\lambda_{exitation}$ at 492 nm and a $\lambda_{emission}$ at 518 nm (Waters 2475). The system was controlled by Breeze software 3.2 (Waters). The column was calibrated using five narrow FITC-Ficoll standards, seven narrow FITC-dextran standards and a few protein standards. A calibration curve was achieved using the relationship: $y = -475.45x^3+1059.4x^2–878.78x +294.41$ and is described in detail in a forthcoming publication (Asgeirsson, D *et al*, unpublished data).

Calculations:

For the tissue uptake technique, renal tracer protein clearance was calculated from the amount of tracer radioactivity accumulated in both kidneys (cortex) plus the TCA-precipitable urine tracer activity (collected during the tracer infusion period) divided by the area under the curve of the plasma tracer concentration *vs.* time function. Protein θ values were calculated by dividing the measured protein clearance by the simultaneously assessed GFR (17, 27). Ficoll θ were obtained by analyzing the HPSEC-curves obtained from the urine and the plasma sample (Cp_F) for each experiment. The urine concentration *vs.* a_e curve was divided by the inulin concentration, to obtain the primary urine concentrations (Cu_F). θ for each a_e was calculated by dividing Cu_F by

Cp_F (Cu_F/ Cp_F). The two-pore model (17, 22, 27) was used to analyze the θ data for Ficoll (15-80Å). A non-linear least squares regression analysis was used to obtain the best curve fit, using scaling multipliers as described previously (17). An apparent α_L was calculated for the HSA data, as obtained from the following equation (cf eq 4 in (27) and eq 25 in (22)):

$$Jv_L / GFR = \frac{\alpha_L(1-\alpha_L)\Delta\pi\ L_pS(1-\sigma_L)}{GFR} + \alpha_L \qquad \text{Eq 1}$$

Because $(1-\alpha_L)$ is very close to unity we have:

$$\alpha_L = \frac{Jv_L}{\Delta\pi\ L_pS(1-\sigma_L) + GFR} \qquad \text{Eq 2}$$

where Δπ was set at 28 mm Hg, L_pS was calculated assuming a filtration pressure of 9 mm Hg.

Statistical analysis

Values are presented as means ±SEM. Differences between groups were calculated using one-way ANOVA with Bonferroni`s multiple-comparison correction. A paired t-test was used for comparisons between HSA and nHSA in the same kidney. Significance levels were set at $p<0.05$*, $p<0.001$** and $p<0.005$***.

Results

20 minutes of I/R

Whilst GFR remained unchanged in the control kidney during both the ischemic and reperfusion period, GFR was markedly reduced (~45%) after the reperfusion period in the ischemic kidney (Table 1). From the relationship of log θ for Ficoll *vs.* SE- radius (15-80 Å) it is evident that θ for FITC-Ficoll increased for molecules >55Å in the post-ischemic kidney (2.7 fold) as compared to the control kidney, as shown in Fig 1. The best curve fit of θ vs. a_e according to the two-pore theory, was obtained for the parameters listed in Table 3. Even though θ was significantly higher for large size Ficoll (a_e>55Å) after 20 minutes of I/R, the fractional ultrafiltration coefficient accounted for by the large pores (α_L) was not significantly increased, indicating that the reduced GFR post-ischemia may have been the prime factor responsible for the increase in Ficoll θ (Fig 2)(cf (23)).

The θ for neutralized HSA was 9 fold higher than that for native HSA in the control kidney ($p<0.005$) (Table 2). Both HSA and nHSA increased after the I/R insult, the increase however not reaching statistical difference for nHSA (Table 2). In the control kidney 99% ±0.083% of the nHSA and 88%±2.8% of the native HSA was reabsorbed in the proximal tubules, while in the ischemic kidney the fractional reabsorption was 97%±0.49% and 81%±3.4%, respectively.

In a separate set of experiments, in which native (neg.) and neutral albumin were analyzed together simultaneously (control kidney *vs*. ischemic kidney), the ratio of clearance for nHSA to HSA was slightly reduced ($p=0.049$) in the kidneys exposed to 20 min of I/R compared to control (Fig 3), indicative of a reduction in the negative charge barrier. This was further evidenced by the

calculation of an "apparent" α_L for HSA according to the two pore theory (assuming a large pore radius of 100Å). After 20 min I/R there was an increase in this parameter (Table 2, Fig 2). An increase in apparent α_L for native HSA, but not for the uncharged Ficoll, may thus reflect a change in the glomerular charge barrier characteristics (Fig 3). The small pore radius (r_s) and large pore radius (r_L) were unchanged after the I/R insult (Table 3).

60 minutes of I/R

Ischemia and reperfusion of the contralateral kidney did not affect hemodynamics (GFR) in the ipsilateral control kidney. 60 min of I/R, however, markedly reduced the GFR in the post-ischemic kidney (~75%)(Table 1). The θ for Ficoll molecules of a_e>55Å increased ~5 fold in the I/R kidney, the increase being 2.4-fold larger than for 20 min I/R (Fig 1). The r_s and r_L were not affected by 60 min I/R. However, there was significant increases in α_L, indicating that 60 min of I/R increased the number of large pores in the glomerular filter (Fig 2 and Table 3), without affecting the large pore radius. $A_0/\Delta X$ was reduced 3 fold ($p<0.005$) after 60 min of I/R, i.e. largely in parallel with the fall in GFR (Table 3), indicating a reduced effective pore area for solute diffusion after 60 min of I/R.

Discussion

Despite extensive experimental and clinical research on acute renal failure (ARF), only modest progression have been made to improve the mortality during the last 20 years. ARF is common in critically ill patients, the most frequent cause being an ischemic injury to the kidney, initiated by, for example, congestive heart failure, hypotension, septic shock, volume depletion, the use of aminoglycosides or radiocontrast procedures (25). There exist a spectrum of treatments for ARF, but they are all currently only supportive. Commonly practiced treatments that are used to inhibit development of ARF include: volume expansion, loop-diuretics and mannitol, dopamine, N-Acetylcysteine and atrial natriuretic peptide (ANP). However, recent clinical studies show no clear therapeutic benefit of any of the pharmacologic therapies for ARF (19), and further research is needed to increase the knowledge in this field.

The effects of I/R on glomerular barrier selectivity have only been rarely investigated. Hence, this is the first study, to our knowledge, in which a detailed analysis has been performed to evaluate the nature of the glomerular permeability changes occurring after acute I/R *in vivo*. The major result of the present study is that acute I/R markedly reduces the size selectivity of the glomerular barrier by the functional appearance of an increased number of large pores (cf. "shunt pathways"). This pattern of alterations of the glomerular barrier function mimics what has been previously seen in a number of different pathophysiologic disorders. The increased number of large pores in the present study after 60 min of I/R, as determined by Ficoll, was mirrored by a large increase in θ for albumin (a_e=36Å) (Fig 1). According to the two-pore theory, native albumin, due to its net negative charge, is for its transglomerular passage more or less totally confined to the large pore pathway. This is because of the high size and charge selectivity of the

glomerular barrier to proteins (21, 23, 27, 30), normally excluding (neg.) native albumin from the small pore pathway. An increase in the large pore number (mirrored by α_L) should thus be well reflected by increases in θ for albumin, as noted in the present study.

Just a minor (non-significant) increase in the fractional ultrafiltration coefficient accounted for by the large pores (α_L) was observed after 20 min of I/R for the Ficoll sieving data. By contrast, α_L for Ficoll increased significantly (4-fold) after 60 min of I/R ($p<0.05$) (Fig 2). Assessed from θ for native albumin, however, an increase in apparent α_L was observed already at 20 min of I/R, but no further increase was observed after 60 min I/R. Since Ficoll data predicted only a minor, non-significant increase in large pore number after 20 min of I/R, the increase in apparent α_L for HSA may be due to a small charge defect in the glomerular barrier appearing after 20 min of I/R, making albumin partly permeable also through the small pore pathway. This was further corroborated by the fact that the clearance ratio of nHSA to HSA was slightly reduced at 20 min I/R (Fig 3). However, after 60 min of I/R, the increase in large pore number, as interpreted from the increase in α_L for Ficoll, apparently superseded the small charge defect observed at 20 min I/R, because α_L for albumin (in absolute and relative terms) did not differ from that calculated from Ficoll data in this situation.

There were no changes in Ficoll θ among small Ficoll molecules ($a_e<50$ Å) after the I/R injury. The reduction in GFR following upon I/R would *per se* lead to an increase in θ for these Ficoll molecules provided that the capillary pore area ($A_0/\Delta x$) and small pore radius (r_s) had remained unchanged, leading to a "steeper" sieving curve (23). However, this effect was canceled by the simultaneous parallel reduction in $A_0/\Delta x$, thus leaving the Peclet numbers (ratio of GFR to

$A_0/\Delta x$), and hence, the sieving coefficients in the 20-50Å a_e-interval, unchanged. The parallel decrease in $A_0/\Delta x$ and GFR could be attributed to alterations in renal hemodynamics. After ischemia, swelling of endothelial cells and a reduction of the capillary lumen have been reported. The ensuing decline in glomerular blood flow has been denoted the "no-reflow" phenomenon (26). There are also indications of a reduced NO production in the ischemic endothelium, related to the formation of ROS (1), also contributing to the "no-reflow" phenomenon. Indeed, intravital videomicroscopy of glomerular vessels during reperfusion of a previously obstructed kidney, has demonstrated an instantaneous recovery of glomerular blood flow, followed by an oscillating flow pattern of cessation and partial recovery of glomerular perfusion (31).

Whilst the increment in θ for native (neg. charged) albumin (a_e=36 Å) in the present study largely paralleled the increase in θ for large MW (a_e>55 Å) Ficoll molecules, θ for Ficoll of 36Å radius ($Ficoll_{36Å}$), remained largely unchanged. This may be explained by the fact that $Ficoll_{36Å}$ was two orders of magnitude more permeable than native albumin and one order of magnitude more permeable than neutralized albumin of equivalent molecular radius across the glomerulus, as discussed at some length previously (23, 30). The relative "hyperpermeability" of $Ficoll_{36Å}$ will make this molecule relatively insensitive to changes in large pore number and large pore volume flow (23, 30). In the present study 60 min of I/R caused the large pore fractional fluid flow (Jv_L/GFR) to increase from $3.6 \cdot 10^{-4}$ to $16 \cdot 10^{-4}$ i.e. by 0.0012. Such changes would theoretically alter θ for $Ficoll_{36Å}$ insignificantly, i.e. by only ~0.5 % (from ~0.10 to 0.1005), assuming an unchanged small and large pore radius. At the same time one would observe a two-fold increase in θ for HSA (from 0.0005 to 0.001), provided that no charge alterations had occurred, and that

albumin transport is confined to large pores. Again, the fact that θ_{alb} increased more than two-fold, may be ascribed to slight reductions in microvascular charge barrier.

Although Ficoll seems to overestimate the clearance of proteins in the a_e interval of 25-55Å, there is a remarkable similarity in sieving coefficients for proteins and Ficoll in the a_e interval 55-75Å. As discussed in a previous paper, this probably is due to the fact that the a_e/r_L ratio for this molecular size range is approximately <0.5-0.7, in which the anomalous glomerular permeation behavior of Ficoll is not manifested (2, 23). Because both proteins and $Ficoll_{>55Å}$ can be used to assess the large pore radius and α_L, we have tried to reconcile albumin θ with those for $Ficoll_{55-75Å}$. This analysis yielded a slightly higher value for α_L and a slightly lower value for the large pore radius than that obtained from Ficoll data alone (Fig 4). It thus seems that θ for albumin may have been (slightly) higher than expected from just extrapolating $Ficoll_{55-75Å}$ θ data back to 36 Å. The reason for this discrepancy is not obvious. It may be due to a slight overestimation of θ for albumin, especially in the ischemic situation, partly due to post-ischemic interstitial trapping of tracer that may not be easily washed out (7). θ could also be slightly overestimated due to trapping of proteins in the endothelium and in the mesangium. However, we believe that the fraction of trapped proteins/polysaccharides is, after all, very small since our θ data comply with a recent, very carefully performed micropuncture study (28). Alternatively, the large pore radius was slightly overestimated from Ficoll data alone. One reason for such an overestimation could be the predicted Ficoll hyperpermeability, for very large Ficoll molecules, i.e. when $a_e/r_L>0.7$ (for a_e>70-75Å).

The major rationale for using polysaccharides as molecular markers for glomerular transport is that their tubular reabsorption is negligible (24) so that the final urine can be used to investigate the sieving properties of the glomerular filter. During ischemic conditions, the tubular epithelial cells become damaged and the tubular reabsorption decreases, which will influence the reabsorption, primarily of proteins. In the present study, 88% of the HSA and 99% of the nHSA was reabsorbed in the control kidney, while only 81% of the HSA and 97% of the nHSA was reabsorbed in the ischemic kidney (20 min of I/R). The larger amount of tubular reabsorption of the neutralized HSA is in agreement with earlier studies, showing that the higher the isoelectric point of a protein, the larger is its proximal tubular reuptake (5). However, since both the urine excretion and the renal epithelial uptake of protein tracer was used to calculate the clearance and θ in our study, the measured θ was insensitive to the differences in proximal tubular reabsorption among the different tracers.

The endothelial glycocalyx is currently thought to represent the major charge barrier in renal and systemic capillaries (10). Modifying the glycocalyx by enzymatic digestion using hyaluronidase, increased the glomerular permeability to both albumin and large size Ficoll molecules *in vivo* (12). Further, treatment with another glycocalyx-degrading enzyme, chondroitinase, in the cooled isolated perfused kidney (cIPK), apparently reduced the thickness of the glycocalyx while increasing the permeability (θ) to albumin. However, in those experiments the increases in θ for large size Ficolls were not significant (13). The results mimics the changes observed in the present study after 20 min of I/R (mild ischemia), suggesting that this relatively short I/R period may have led to alterations that could, at least partly, be attributed to alterations in the endothelial glycocalyx. The reactive oxygen species (ROS) formed during the reperfusion period (8), can

alter molecular structures and induce an inflammatory response. According to a previous study infusion of H_2O_2 for a period of 1 hour, indeed induced an increase in urine albumin excretion and in θ for large (>42 Å) dextrans, without causing significant changes in GFR, again indicating the induction of an increased number of large pores in the glomerular filter (32). Although the authors did not find any apparent alterations in the anionic barrier of the GBM, assessed by staining with polyethyleneimine, there could still have been marked alterations in the endothelial glycocalyx in addition to the reduced size selectivity.

The pathophysiology of the permeability changes occurring after severe ischemia (60 min of I/R) is not exactly known, but could be ascribed to a number of different mechanisms. One scenario could be a remodelling of the GBM. In isolated renal endothelial cells from rats which had undergone 30 min of ischemia, there was an increase in the proteolytic activity of matrix metalloproteinases (MMPs)(3), enzymes responsible for matrix (collagen) remodelling. Furthermore, after renal I/R both mRNA and protein levels of MMP-9 increased (4), indicating that renal ischemia could lead to degradation of the GBM, which in turn could be responsible for the increase in glomerular permeability. Heparan sulphate proteoglycans (HSPG´s) are important structural contributors in the GBM, and gene modifications leading to either loss of HSPG´s, or their degradation, indeed leads to proteinuria (18, 33). Thus, both HSPG´s and collagen may be targets of ischemic damage.

Ischemia has been shown to cause flattening and spreading of the podocyte foot processes, a condition that occurs early in the ischemic response (20). There are tight connections between the GBM and the podocytes. The architecture of the foot processes is dependent upon integrin-

($\alpha3\beta1$) and α-dystroglycan-mediated interactions with the GBM, α-dystroglycan being linked to laminin and agrin. Recently, it has been shown that these linkages could be disrupted by ROS in *in vitro* experiments (14). Hence, the increased amount of ROS associated with ischemia could in part explain the detachment of the podocyte processes from the GBM. Whether the detachment is a primary phenomenon or a secondary consequence occurring due to remodelling of the GBM is not clear. In mice overexpressing heparanase-1, the podocytes appear flattened and have a reduced number of foot processes (33), probably secondary to the degradation of the GBM. Likewise, mice lacking the $\alpha3$-integrin are born with disorganized GBM and non-differentiated podocytes lacking foot-processes (15). Thus it seems that any condition in which GBM and the podocytes loose their interconnections may lead to alterations in the glomerular size selective barrier.

In conclusion, 60 min of I/R induced an increased formation of large pores in the glomerular filter, reflected by increases in α_L for both Ficoll and native albumin. However, after 20 min of I/R, the insult to the glomerular filter was less pronounced and compatible with reductions in charge selectivity, and to a lesser extent, in size selectivity, reflected by a larger increment in the glomerular permeability to albumin than to Ficoll.

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Figure legends

Fig. 1

Sieving coefficients (θ) v.s SE-radius (a_e) for Ficoll and HSA in the control kidney and in the ischemic kidney at 20 and 60 min of I/R. Control (20 min) is shown in red, control (60 min) in blue, ischemia (20 min) in purple and ischemia (60 min) in green. Ficoll θ are given for 400 data points between 15 and 80 Å and θ for HSA is given at 36 Å.

Fig.2

Relative fractional ultrafiltration coefficient accounted for by large pores (α_L) in % for Ficoll and native albumin (HSA). α_L for Ficoll was obtained from the two pore model. α_L for HSA was calculated assuming a filtration pressure of 9 mm Hg (in order to assess the LpS) and $\Delta\pi$ was set at 28 mmHg while the large pore radius was assumed to be 100 Å as obtained from the Ficoll data. Absolute values for α_L are shown in Table 2 and 3.

Fig. 3

The clearance of nHSA and HSA were determined simultaneously using the tissue uptake technique in a separate series of experiments (n=7) in order to assess the nHSA/HSA ratio in the ischemic and non-ischemic kidneys. The reduction in nHSA/HSA ratio in the ischemic kidney after 20 min of I/R was of borderline statistical significance (p=0.049).

Fig. 4

Both native albumin and Ficoll$_{>55Å}$ are, according to the two pore theory, confined for their glomerular passage to the large pore pathway because neg. albumin cannot pass 38Å (charged) small pores. Reconciling θ for albumin with θ for Ficoll$_{55\text{-}75Å}$ and fitting the data to a single large pore yielded a slightly higher value for α_L (control$_{20}$: 3.3×10^{-4} , ischemia$_{20}$: 6.3×10^{-4}, control$_{60}$: 2.9×10^{-4} ischemia$_{60}$: 18×10^{-4}) and at the same time a slightly decreased large pore radius (~92 Å compared to ~100Å), than what was obtained from Ficoll data alone (Table 3). Solid lines represent the best fit of control groups, dotted line represents 20 min of I/R and dashed line represents 60 min of I/R. Data points for control$_{20}$ are shown as circles, ischemia$_{20}$ as solid triangles, control$_{60}$ as open stars and ischemia$_{60}$ as open pentagons.

Table 1

GFR and body weight for the different experimental groups

		20 min I/R				**60 min I/R**		
		Control		***I/R***		***Control***		***I/R***
GFR (ml/min/g kidney)	**n**	During Ischemia	After Reper-fusion	After Reperfusion	**n**	During ischemia	After Reper-fusion	After Reperfusion
Ficoll	7	0.45±0.07	0.58±0.04	0.38± 0.08	9	0.72±0.10	0.75±0.07	0.11±0.03***
HSA	11	0.67±0.05	0.67±0.05	0.30±0.06***	6	0.69±0.09	0.60±0.12	0.24±0.07*
nHSA	6	0.58±0.07	0.89±0.10	0.32±0.08***				
Body weight (g)								
Ficoll			283 ± 8.4				281 ± 10	
HSA			308 ± 9.4				270 ± 9.8	
nHSA			295 ± 12				-	

Values are given as means±SE. * represents statistical difference between control and I/R kidney after reperfusion. $p<0.05$*, $p<0.005$***

Table 2

Sieving coefficients (θ) for HSA and nHSA, and apparent α_L for HSA at 20 and 60 min of I/R, respectively

	HSA		nHSA	
	Control	I/R	Control	I/R
	$\times 10^{-4}$	$\times 10^{-4}$	$\times 10^{-4}$	$\times 10^{-4}$
θ	4.9 ± 0.34	20.6 ± 3.5 ***	45 ± 4.5††	185 ± 88
20 min I/R				
θ	4.9 ± 0.63	32.7 ± 14 *	-	-
60 min I/R				
α_L	2.91±0.21	11.3±2.16	-	-
20 min I/R				
α_L	3.23±0.43	10.4±4.41	-	-
60 min I/R				

Values are given as means±SE. α_L denotes the *apparent* (calculated) fractional LpS accounted for by the large pores (of radius 100 Å). * indicates statistical difference between contol and I/R kidney and † indicates statistical difference between HSA and nHSA control kidneys.

Table 3

Two-pore analysis

Kidney	Small pore radius (r_s) (Å)	Large pore radius (r_L) (Å)	$A_0/\Delta X$ (cm)/ g ($\times 10^5$)	Jv_L/GFR ($\times 10^{-4}$)	α_L ($\times 10^{-4}$)
20 min control	46.5± 0.21	100 ± 4.8	4.47 ± 0.37	3.19±0.56	1.14 ± 0.29
20 min Ischemia	46.2 ± 0.27	106 ± 3.9	3.07 ± 0.33	6.07 ±1.24	2.08 ± 0.43
60 min control	46.1 ± 0.17	99 ± 3.6	6.38 ± 0.29	3.61 ± 0.35	1.29 ± 0.13
60 min Ischemia	46.4 ± 0.39	104 ± 3.4	1.95± 0.19 ***	16.4 ± 5.0 *	5.74 ± 1.81 *

Values are given as means±SE. * indicates statistical difference between control and I/R kidney. *p<0.05, ***p<0.005. There were no statistical differences between the 20 min control and I/R kidneys for any of the parameters. α_L=fractional ultrafiltration coefficient accounted for by large pores.

Figure1

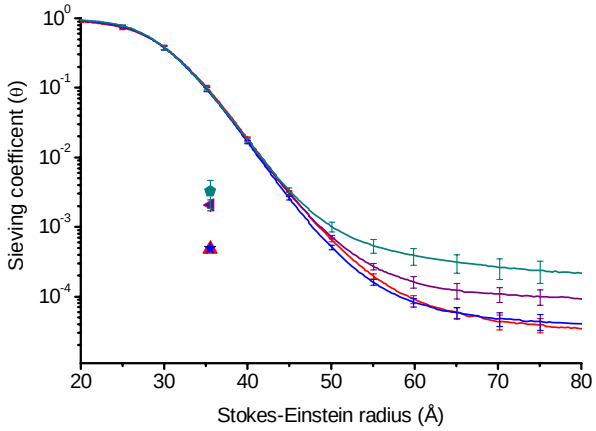

Figure 2

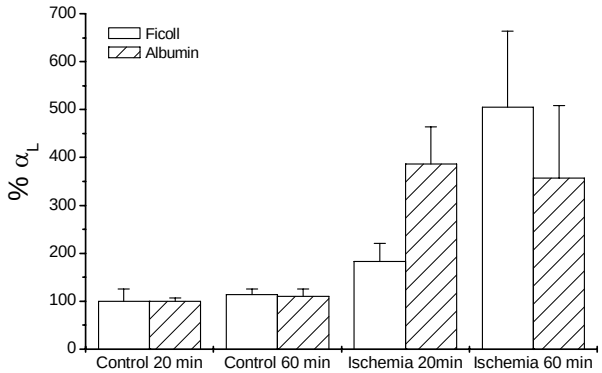

Figure 3

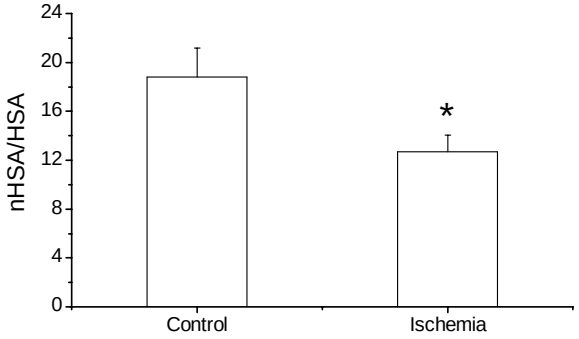

Figure 4