Dynamic, size-selective effects of protamine sulfate and hyaluronidase on the rat glomerular filtration barrier in vivo

Kristinn Sverrisson, Josefin Axelsson, Anna Rippe, Daniel Asgeirsson, and Bengt Rippe

Department of Nephrology, Lund University, Lund, Sweden

Submitted 2 April 2014; accepted in final form 8 September 2014

Sverrisson K, Axelsson J, Rippe A, Asgeirsson D, Rippe B. Dynamic, size-selective effects of protamine sulfate and hyaluronidase on the rat glomerular filtration barrier in vivo. Am J Physiol Renal Physiol 307: F1136–F1143, 2014.—The proteinuric actions of protamine sulfate (PS) have classically been, at least partly, attributed to alterations of the negatively charged glomerular endothelial glycocalyx. To investigate whether the charge-selective properties of the glomerular filtration barrier (GFB) would be altered by PS, we assessed the glomerular sieving of conventional, uncharged, polydispersed Ficoll (n-Ficoll) compared with charge modified, conformationally intact, anionic (carboxymethylated) Ficoll (a-Ficoll) before and after systemic infusions of PS in rats. For comparison, we also investigated the impact of hyaluronidase (hyase), which partially degrades the glycocalyx, on GFB permeability. In anesthetized Wistar rats, blood access was achieved, and the left ureter was cannulated for urine collection. Rats were infused with either n-Ficoll or a-Ficoll before and during systemic infusions with either PS or hyase. Plasma and urine samples were taken repeatedly and analyzed by high-performance size exclusion chromatography to assess glomerular sieving coefficients (θ) for Ficoll (radius 10–80 Å). The GFB showed a significant glomerular charge selectivity for Ficoll molecules of radius 20–35 Å. PS and hyase infusions reversibly increased θ for large Ficoll molecules (Ficoll molecules of radius 50–80 Å). Thus, for PS, θ for a-Ficoll molecules of radius 70 Å increased from 2.47 ± 10^{-5} ± 1.1^{-5} to 7.25 ± 10^{-5} ± 1.1^{-5} (P < 0.05) at 15 min. For hyase, changes in a-Ficoll molecules of radius 50–80 Å were, however, not statistically significant. Neither PS nor hyase had any effect on θ for n-Ficoll molecules of radius 20–45 Å or a-Ficoll molecules of radius 20–45 Å. It is concluded that systemically administered PS and hyase in moderate doses dynamically decreased the size selectivity of the rat GFB without affecting its charge selective properties.

endothelial glycocalyx; Ficoll; sieving coefficient; carboxymethylated Ficoll

EXPOSURE of the glomerular filtration barrier (GFB) to polymers such as protamine sulfate (PS) can cause proteinuria and foot process effacement (30, 49). These actions have classically, at least partly, been attributed to alterations of the negatively charged components of the GFB (3, 8, 24, 33, 48, 49, 52, 57). PS exposure has frequently been used as a proteinuria model, and at least the albuminuric effects of PS have been assumed to be based on neutralization of the GFB (12, 35, 47, 56). The possibility that other actions of PS are involved as well, such as dynamic changes in the barrier dependent on the dynamics of the contractile cytoskeleton of podocytes and/or endothelial cells, has also been frequently advanced during the last few decades (12, 30, 55). Thus, to what extent PS causes increases in GFB permeability due to charge modification, or to what extent size-selective actions are also of importance warrant further elucidation.

The endothelial surface layer (ESL) consists of a membrane-bound layer of proteoglycans, glycosaminoglycans, and sialoglycoconjugates, the glycocalyx (GC), and a loosely attached endothelial cell coat (ECC) (15, 22). Sialoglycoconjugates and sulfated glycosaminoglycans, such as heparan sulfate, chondroitin sulfate, and, to some extent, hyaluronan, are molecules in the ESL that are negatively charged (4, 25, 31, 53). Investigations that have tried to modify the charge of the GC (15, 54) or to reduce the impact of it via enzymatic ESL degradation have demonstrated increases in the glomerular permeability to albumin as a consequence of such perturbations (27, 28, 51). For example, hyaluronidase (hyase), which cleaves hyaluronan, chondroitin, and chondroitin sulfate, has been demonstrated to decrease the ESL and increase the permeability of peripheral and glomerular capillaries (16, 21, 25, 27, 28, 36). In some of these studies, negatively charged albumin (radius 36 Å and sieving coefficient θ ~ 0.0001 (22)), which according to pore theory (the two-pore model) would permeate the GFB exclusively through large pores (radius ~115–120 Å), has been compared with hyperpermeable neutral Ficoll (radius 36 Å) passing mainly through small pores (radius ~37.5 Å) and having a high θ (~0.1) (2). Since the glomerular permeability to albumin has been shown to be increased by hyase, with permeation of Ficoll molecules of radius 36 Å remaining unchanged, this has been taken as evidence that charge selectivity is specifically affected by perturbing the GC. Another, more likely, interpretation is that hyase and PS exclusively affect the large pore permeation of macromolecules and, hence, glomerular albumin transport, whereas small pore transport of Ficoll molecules of radius 36 Å, according to the two-pore theory, would remain unchanged.

In the present study, we attempted to interact with the ESL of the GFB in vivo by infusing PS to produce charge neutralization or by inducing (partial) enzymatic degradation of the ESL via infusion of hyaluronidase. This was done under conditions of largely maintained systemic blood pressure. The main issue addressed is whether charge selectivity or size selectivity of the GFB, or both, would be affected by these manipulations of the GC. For that purpose, we assessed θ values of conformationally unchanged, anionic FITC-Ficoll (a-Ficoll) versus neutral, conventional FITC-Ficoll (n-Ficoll) compared over a wide range of molecular radii (10–80 Å) [relative molecular weight (Mr): 70,000 and 400,000]. Ficoll is a polysaccharide that is not significantly reabsorbed by the proximal tubules and therefore can be used as a direct probe of glomerular permeability to macromolecules via analysis of its steady-state urine-to-plasma concentration ratio. We used a fine-tuned high-performance size exclusion chromatography (HPSEC) system, whereby we are able to detect the very...
low 0 values (~10⁻⁵) for high-molecular-weight Ficoll, characterizing the normal GFB macromolecule permeability.

MATERIALS AND METHODS

Animals and Surgery

Experiments were performed in 29 male Wistar rats (Möllegård, Lille Stensved, Denmark) with an average body weight of 260.0 ± 2.3 g. Rats had access to water and standard chow ad libitum. Experiments were approved by the Malmö/Lund Committee for Animal Experiment Ethics. Anesthesia was induced with pentobarbital sodium (60 mg/kg ip). Body temperature was kept stable at 37°C with a heating pad. Breathing was facilitated by performing a tracheotomy. The tail artery was cannulated [polyethylene (PE)-50 cannula] for the maintenance administration of anesthesia and for monitoring mean arterial blood pressure (MAP) and heart rate (MP150 system, BIOPAC, with AcqKnowledge for Mac). The left carotid artery was cannulated for blood sampling, and the right and left jugular veins were cannulated for infusion purposes (PE-50 cannulas). Via a small abdominal incision, the left ureter was cannulated (PE-10 cannula connected to a PE-50 cannula) for urine sampling. To facilitate cannulation of the ureter, urine production was temporarily increased by administration of furosemide (0.375 mg/kg, Furix, Takeda Pharma, Solna, Sweden) via the tail artery.

FITC-Ficoll and Elugrams

n-Ficoll 70 (M, 70,000), n-Ficoll 400 (M, 400,000), and Inulin, all labeled with FITC, were obtained from TdB Consultancy (Upsala, Sweden). FITC-labeled a-Ficoll 70 and 400 were gifts from Dr. William Fissell (Vanderbilt University, Nashville, TN). Carboxyethylphosphonate labeled with FITC, were obtained from TdB Consultancy (Uppsala, Sweden). FITC-labeled a-Ficoll 70 and 400 were gifts from Dr. William Fissell (Vanderbilt University, Nashville, TN). Carboxyethyl- phosphonate labeled with FITC, were obtained from TdB Consultancy (Uppsala, Sweden). FITC-Ficoll 70, (20 mg FITC-labeled inulin, and 80 μl 51Cr-labeled EDTA (51Cr-EDTA; 3.7 MBq/ml), given as a priming bolus dose, followed by a constant infusion (12 ml kg⁻¹·h⁻¹) of FITC-Ficoll 400 (0.48 mg/ml), FITC-Ficoll 70 (20 μg/ml), FITC-inulin (0.5 mg/ml), and 51Cr-labeled EDTA (0.296 MBq/ml) continued throughout the experiments.

Experimental Procedures

Four experimental groups were investigated. Rats were infused with either n-Ficoll or anionic a-Ficoll before and during systemic infusion with either PS or hyase. n-Ficoll or a-Ficoll was infused in a mixture containing 960 μg FITC-Ficoll 400, 40 μg FITC-Ficoll 70, 500 μg FITC-labeled inulin, and 80 μl 51Cr-labeled EDTA (51Cr-EDTA; 3.7 MBq/ml), given as a priming bolus dose, followed by a constant infusion (12 ml kg⁻¹·h⁻¹) of FITC-Ficoll 400 (0.48 mg/ml), FITC-Ficoll 70 (20 μg/ml), FITC-inulin (0.5 mg/ml), and 51Cr-labeled EDTA (0.296 MBq/ml) continued throughout the experiments.

After an initial resting period of 20–30 min, to achieve steady-state concentrations of Ficoll and allow the animals to recover from surgery, blood and urine were sampled for a control “baseline” period. Rats were then infused with either PS or hyase after a priming bolus dose of either compound. To minimize the hemodynamic effects of PS or hyase, some experiments were carried out to find the highest PS or hyase dose that could be given without lowering MAP by 20–25% and at what rate the bolus dose and infusion could be given. Blood and urine were sampled at 5, 15, 30, and 60 min. At each sampling time, urine from the left kidney was collected for 5 min, and a plasma sample was collected in the middle of the urine collection period (at 2.5 min).

PS-treated groups. Rats were infused with PS (10 mg/ml, LEO Pharma, Ballerup, Denmark) together with either n-Ficoll (n = 8) or a-Ficoll (n = 7). A bolus dose of PS (8 mg/kg) was given over a prolonged period of time (15 min) to minimize hemodynamic effects. An infusion of 0.08 mg·kg⁻¹·min⁻¹ was then continued throughout the experiment. The infusion was stopped if the MAP fell by 20% (to avoid pressure drops below 75% of baseline value). In a total of five experiments, the infusion was stopped temporarily, during 2 min and up to 15 min in total, with little effect on the total dose of PS given in each experiment.

Hyaluronidase-treated groups. Hyase (hyaluronidase from bovine testes, 850 U/mg, Sigma-Aldrich) was given together with either n-Ficoll (n = 8) or a-Ficoll (n = 6). A bolus of 5 mg/kg hyase (4,250 U/kg) was injected in the beginning of each experiment. Due to the fact that hyase is an enzyme, a priming period of 10 min was instituted after the bolus dose, allowing the enzyme to circulate to exert its effect.

Control experiments. In six rats, an L-type Ca²⁺ channel blocker, nimodipine (0.4–1.5 μg·kg⁻¹·min⁻¹, Nimotop, Bayer, Solna, Sweden), was infused during 20 min to lower MAP by ~20%, to mimic the blood pressure fall obtained in PS-treated animals (see below). 0 values to Ficoll were assessed before start of the infusion and at 5 and 15 min.

Glomerular Sieving of FITC-Ficoll

Plasma and urine samples were assessed using HPSEC system (Waters, Milford, MA) using an Ultrahydrogel 500 column with a guard column (Waters) and calibrated as previously described at length (2). The mobile phase was driven by a pump (Waters 1525), and fluorescence was detected with a fluorescence detector (Waters 2475) at an excitation wavelength of 492 nm and an emission wavelength of 518 nm. Samples were loaded to the system with an autosampler (Waters 717 plus). The system was controlled by Breeze Software 3.3 (Waters).

0 values were obtained by analyzing HPSEC curves from plasma and urine samples during each experiment. The 0 value of Ficoll was determined as the “fractional clearance” according to the following formula: \( \theta = (C_{PU} \times C_{IP})/(C_{PU} \times C_{IP} + C_{PIU}) \), where \( C_{PU} \) is the urine Ficoll concentration, \( C_{IP} \) is the inulin concentration in plasma, \( C_{IPU} \) is the Ficoll concentration in plasma, and \( C_{PIU} \) is the inulin concentration in urine.

Transmission Electron Microscopy of Rat Glomeruli Exposed to PS

In each of two rats in which an increase in 0 values for n-Ficoll molecules of radius 55–80 Å had occurred after 15 min of PS infusion, 7–10 glomeruli were examined by electron microscopy. Kidney biopsies were obtained with a biopsy needle (True Core II biopsy instrument, Angiotech Medical Device Technologies, Gainesville, GA). Biopsies were fixed with 1% osmium tetroxide and dehydrated with ethyl alcohol and acetone, after which they were embedded in Agar resin and left for 2 days. Sections (60 nm) were cut using an ultramicrotome (EM UC7, Leica Microsystems), placed on copper grids, and stained with 4% uranyl acetate and lead citrate. Specimens were examined using a transmission electron microscope (Technai Spirit Biotwin 120 kV, FEI).

Glomerular Filtration Rate

The glomerular filtration rate (GFR) was assessed using the steady-state clearance of 51Cr-EDTA (Amersham Biosciences, Buckinghamshire, UK) from plasma to urine. Urine was collected from the left ureter repeatedly during the experiment, and blood samples using microcapillaries (25 μl) were taken for the calculation of GFR. The radioactivity in blood and urine was measured using a γ-counter (Wizard 1480, Wallac, Turku, Finland). Hematocrit was assessed throughout the experiments to be able to convert blood radioactivity into plasma radioactivity. GFR was calculated by dividing the urinary excretion (\( U_i \times V_i \)) of 51Cr-EDTA per minute by the plasma tracer concentration, where \( U_i \) is the tracer concentration in urine and \( V_i \) is the flow of urine per minute.
Two-Pore Analysis

A two-pore model (34, 44) was used to analyze the data for Ficoll molecular radius 10 – 80 Å. A nonlinear least-squares regression analysis was used to obtain the best curve fit, using scaling multipliers, as previously described at some length (34).

Statistics

Values are presented as means ± SE. Differences among groups were tested using nonparametric ANOVA with the Kruskal-Wallis test and post hoc tested using the Mann-Whitney U-test. Bonferroni corrections for multiple comparisons were made when applicable. Significant levels were set at P < 0.05. All statistical calculations were made using IBM SPSS statistics version 20.0.0 for Windows (SPSS, Chicago, IL).

RESULTS

Elugrams

Figure 1, A and B, shows elugrams for n-Ficoll 70 versus a-Ficoll 70 (n = 2) and n-Ficoll 400 versus a-Ficoll 400, respectively. In the elugrams, there was almost no difference in elution time, demonstrating no difference in Stoke-Einstein radius for a-Ficoll versus n-Ficoll.

Glomerular Sieving of n-Ficoll and a-Ficoll During Baseline Conditions

During baseline, the glomerular sieving curve for a-Ficoll (n = 13) was displaced to the left compared with that of n-Ficoll (n = 16), indicating the presence of glomerular charge selectivity for Ficoll molecules of radius (a_e) ~20–35 Å (Fig. 2), supporting our previous findings (7). The largest difference occurred at SE radius 27 Å. Thus, the θ value for a-Ficoll molecules of radius 27 Å was 0.37 ± 0.01 and that for n-Ficoll molecules of radius 27 Å was 0.45 ± 0.01 (P = 0.01). For molecules larger than 45 Å, there were no differences in θ values between a-Ficoll and n-Ficoll during baseline conditions.

Infusion of PS

After systemic infusion of PS, the glomerular permeability for both a-Ficoll and n-Ficoll increased for large molecules (50 – 80 Å) after 5 and 15 min (Fig. 3) but not for smaller Ficoll molecules. For n-Ficoll molecules of radius 70 Å, θ values increased from 2.85 ± 0.88 × 10^-5 to 1.18 ± 0.28 × 10^-4 at 5 min (P < 0.05) and for a-Ficoll molecules of radius 70 Å, θ values increased from 2.47 ± 1.1 × 10^-5 to 7.26 ± 1.1 × 10^-5 (P < 0.05) at 15 min (Fig. 4). There were, however, no statistically significant increases in θ values for n-Ficoll molecules of radius 70 Å at 5 or 15 min during infusions of nimodipine, which reduced systemic MAP to the same extent as did PS (data not shown).

Electron Microscopy of the Glomerular Architecture of PS-Treated Rats

No morphological changes could be detected in the GFB in the 7–10 glomeruli examined in each of 2 rats exposed to PS for 15 min. Figure 5, A and B, shows samples from one such glomerulus at two different magnifications.

Fig. 2. Glomerular sieving coefficients (θ) versus Stokes-Einstein radii for n-Ficoll and a-Ficoll during baseline. There was a slight, but significant, reduction in θ values for a-Ficoll of radius ~20–35 Å.

Selectivity: θ values for Ficoll molecules of radius (a_e) ~20–35 Å (Fig. 2), supporting our previous findings (7). The largest difference occurred at SE radius 27 Å. Thus, the θ value for a-Ficoll molecules of radius 27 Å was 0.37 ± 0.01 and that for n-Ficoll molecules of radius 27 Å was 0.45 ± 0.01 (P = 0.01). For molecules larger than 45 Å, there were no differences in θ values between a-Ficoll and n-Ficoll during baseline conditions.

Infusion of PS

After systemic infusion of PS, the glomerular permeability for both a-Ficoll and n-Ficoll increased for large molecules (50 – 80 Å) after 5 and 15 min (Fig. 3) but not for smaller Ficoll molecules. For n-Ficoll molecules of radius 70 Å, θ values increased from 2.85 ± 0.88 × 10^-5 to 1.18 ± 0.28 × 10^-4 at 5 min (P < 0.05) and for a-Ficoll molecules of radius 70 Å, θ values increased from 2.47 ± 1.1 × 10^-5 to 7.26 ± 1.1 × 10^-5 (P < 0.05) at 15 min (Fig. 4). There were, however, no statistically significant increases in θ values for n-Ficoll molecules of radius 70 Å at 5 or 15 min during infusions of nimodipine, which reduced systemic MAP to the same extent as did PS (data not shown).

Electron Microscopy of the Glomerular Architecture of PS-Treated Rats

No morphological changes could be detected in the GFB in the 7–10 glomeruli examined in each of 2 rats exposed to PS for 15 min. Figure 5, A and B, shows samples from one such glomerulus at two different magnifications.
Infusion of Hyase

After systemic infusion of hyase, the glomerular permeability to large neutral Ficoll molecules (50–80 Å) increased after 15 min (Fig. 6). Values for n-Ficoll molecules of radius 70 Å thus increased from $2.46 \pm 1.2 \times 10^{-5}$ to $1.24 \pm 0.31 \times 10^{-4}$ ($P < 0.05$). For a-Ficoll, however, there was no statistically significant increase in glomerular permeability (Fig. 7).

Sieving of Ficoll Molecules of Radius 25–45 Å After PS or Hyase

Values for a-Ficoll molecules of radius 25–45 Å and n-Ficoll molecules of radius 25–45 Å over time remained unaltered compared with baseline during PS or hyase infusion. This indicates that there was no change in charge selectivity of the GFB across the small pore pathway after either PS or hyase, as shown in Fig. 8.

Hemodynamic Parameters

The blood pressure in animals treated with PS fell by 13–15% from baseline ($P < 0.05$; Fig. 9A). In animals treated with hyase, the blood pressure reduction was not significant (Fig. 9B). There were no significant changes in heart rate during any of the experimental periods.

GFR

Throughout the experiments, GFR had tendency to decline, but this only reached statistical significance in n-Ficoll animals treated with hyase (Fig. 10, A and B). At 15 min, GFR thus fell from $0.76 \pm 0.10$ to $0.48 \pm 0.04$ ml·min$^{-1}$.g$^{-1}$ ($P = 0.047$).

Two-Pore Modeling

The best curve fits of $\theta$ versus $a_e$ for Ficoll according to the two-pore model were obtained using the parameters shown in Table 1.

DISCUSSION

The permselectivity of the GFB has been a matter of debate for several decades. To what extent size, shape, charge, and deformability (flexibility) of molecules influence their glomerular permeability through the GFB has thus been partly controversial (58). In the 1970s, Brenner and colleagues (11) published their seminal work on the differential glomerular transport of anionic, neutral, and cationic dextrans, strongly indicating that the GFB is negatively charged. Charge selectivity is thought to be a consequence of the presence of “fixed” negative charges in the GFB, preventing negatively charged...
proteins, such as albumin, from entering the primary urine. Hence, modification of the fixed GFB charges would result in albuminuria (12). The extent and location of the negative charge barrier have, however, remained controversial. Negatively charged proteoglycans in the GBM, such as agrin and perlecan (both heparan sulfate-containing proteoglycans), have been thought to be pivotal (29, 37, 43), but animals deficient in agrin and perlecan have not been found to present with albuminuria (19, 23). Furthermore, in vitro studies (10, 14) of the isolated GBM have indicated that the GBM does not contribute to the charge selectivity of the entire GFB. It has therefore been suggested that the charge selectivity of the GFB must reside in the ESL (and/or epithelial cell surface layer) (10, 22, 38, 39). This is apparently partly conflicting with the classic studies by Farquhar et al. and Venkatachalam et al. (29, 37, 43, 48, 49) based on electron microscopic assessments of cationic ferritin or native ferritin to trace the glomerular charge barrier. In these studies, the fixed negative charges of the GFB seemed to be localized in the laminae rarae of the GBM.

Probing glomerular charge selectivity requires that the probe molecules differ only with respect to charge and not with respect to size, shape, or flexibility. Dextran and, to some extent, Ficoll, are, compared with globular proteins, flexible molecules with an extended diameter and showing a relative glomerular “hyperpermeability” (1). Therefore, they should not be used as uncharged reference molecules versus, e.g., albumin. Furthermore, modification of the molecular charge may result in changes in molecular conformation. Indeed, in a previous study (2), we found that charge modification of Ficoll by carboxymethylation resulted in an extended (increased) molecular radius and, conceivably, an increased molecular “flexibility” (58), and, hence, permeability, of the charge modified molecule across the GFB. In that study and in a previous study by Guimarães et al. (20), comparison of native neutral Ficoll with charge modified Ficoll (a-Ficoll) thus showed increased, not decreased, glomerular permeability. In the present study, however, we used a preparation of a-Ficoll that turned out to be conformationally unaltered compared with n-Ficoll. The results clearly indicate that the GFB is negatively charged, although not to the extent previously thought. The present study thus confirms and extends our previous study (7) of the charge selectivity of the GFB using differently charged Ficoll molecules as probes, which yielded a calculated surface charge density (in an assumed negatively charged fiber matrix barrier) of \(-0.02 \text{ C/m}^2\) (38).

The present study further demonstrates that systemically administered PS did not affect rat GFB charge selectivity but rather had dynamic and partly reversible size-selective effects. Thus, \(\theta\) values for both a-Ficoll molecules of radius 50–80 Å and n-Ficoll molecules of radius 50–80 Å rapidly increased after PS. This suggests that PS may have directly affected the glomerular endothelial cell or podocyte structure and/or contractility. Already in 1978, Kerjaschki et al. (30) demonstrated that PS induced permeability alterations involved podocyte actin filament reorganization, which could be ameliorated by cooling, cytochalasin B treatment, affecting the cellular F-actin cytoskeleton, or Ca\(^{2+}\) depletion (by EDTA). Furthermore, Barnes et al. (8) demonstrated that polycations appeared not only to neutralize the anionic charge of the GFB and to

![Fig. 7. \(\theta\) values for Ficoll70Å as a function of time during infusion of hyase. \(\theta\) values increased for n-Ficoll70Å at 15 min, but there was no significant increase in \(\theta\) values for a-Ficoll70Å (*\(P < 0.05\)).](image)

![Fig. 8. \(\theta\) values for Ficoll molecules of radius 27 Å (Ficoll27Å) as a function of time for all experimental groups. \(\theta\) values for both a-Ficoll27Å and n-Ficoll27Å remained unaltered over time compared with baseline (0 min) during PS and hyase infusions.](image)

![Fig. 9. A: mean arterial pressure (MAP) as a function of time for animals treated with PS. MAP fell by 13–15% from baseline (*\(P < 0.05\)). B: MAP as a function of time for animals treated with hyase.](image)
increase its permeability to an anionic tracer but also increased the permeability to cationic tracers. Increases in \( \theta \) values to both albumin and neutral IgG after systemic polycation infusion in rats were also noticed by Hunsicker et al. (26) and by Bertolatus et al. (9). This can be explained by size-selective changes of the glomerular filter. In a recent study (55), the cellular \( \text{Ca}^{2+} \) signaling events leading to reorganization of the cytoskeleton in podocytes after high doses of PS (600 \( \mu \)g/ml during 40 min) were investigated. Calcineurin inhibitors (FK-506 and cyclosporin A) and the cathepsin L inhibitor E64 all inhibited the PS-induced glomerular barrier changes. In addition, rapamycin, a mammalian target of rapamycin inhibitor, was barrier protective, indicating that both calcineurin-independent and calcineurin-dependent components of cellular \( \text{Ca}^{2+} \) signaling may be involved in the size-selective effects of PS on the GFB.

PS has many well-known side effects, such as hypotension, pulmonary vasoconstriction, and anaphylaxis (32, 40, 50). While PS can induce dose-dependent proteinuria (35, 52, 55), high doses of PS can also cause oliguria (35). PS-induced hypotension can be blocked by inhibitors of nitric oxide, indicating that the effects of protamine on blood pressure are mediated, in part, by the vascular endothelium (41, 42). Robson et al. (57) used higher PS doses infused in awake restrained animals and concluded that albuminuria was not related to hemodynamic factors. In the present experiments, the dose of PS administered (calculated to be \( \sim 50 \mu \)g/ml) was adjusted to avoid any substantial hemodynamic effects resulting in rather moderate effects of PS on glomerular permeability, not leading to foot process effacement.

Hyaluronan is an important structural component of the GC, and an intact endothelial GC is required for normal GFB function (13, 17). Hyaluronidase not only degrades the hyaluronan of the surface layer of the endothelium but also, to some extent, degrades chondroitin and chondroitin sulfate of the glomerular GC (18, 25, 36). In the present study, systemic infusion of hyaluronidase increased the glomerular permeability to large neutral Ficoll molecules (n-Ficoll molecules of radius 50–80 Å) after 15 min, and, as after PS, this permeability increase was transient. Although we used a 10-min priming period after the hyase bolus, the optimal time for the enzyme effects on the GC may be longer, conceivably explaining the lag in response time. Contrary to our expectations, however, hyase did not increase the permeability for large a-Ficoll molecules (a-Ficoll molecules of radius 50–80 Å). The reason for this finding is not clear. If the (potential) charge selectivity of the large pores had been affected, then \( \theta \) values for a-Ficoll molecules of radius 50–80 Å would have increased slightly, whereas \( \theta \) values for n-Ficoll molecules of radius 50–80 Å would have stayed more or less unchanged. However, the opposite occurred. Anyway, it seems safe to avoid any substantial hemodynamic effects resulting in rather moderate effects of PS on glomerular permeability, not leading to foot process effacement.

### Table 1. Two-pore parameters at baseline and at 15 min of PS and hyase infusion

<table>
<thead>
<tr>
<th>Two-Pore Parameter</th>
<th>n-Ficoll PS</th>
<th>a-Ficoll PS</th>
<th>n-Ficoll Hyase</th>
<th>a-Ficoll Hyase</th>
</tr>
</thead>
<tbody>
<tr>
<td>( r_s ), Å</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>43.3 ± 0.3</td>
<td>42.6 ± 0.5</td>
<td>42.6 ± 0.5</td>
<td>43.9 ± 0.3</td>
</tr>
<tr>
<td>15 min</td>
<td>43.2 ± 0.6</td>
<td>41.5 ± 0.4</td>
<td>42.6 ± 0.3</td>
<td>42.8 ± 0.3</td>
</tr>
<tr>
<td>( r_L ), Å</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>124.7 ± 5.0</td>
<td>118.5 ± 7.9</td>
<td>115.8 ± 8.2</td>
<td>106.7 ± 7.2</td>
</tr>
<tr>
<td>15 min</td>
<td>161.8 ± 12.3</td>
<td>150.5 ± 6.5*</td>
<td>162.1 ± 7.7*</td>
<td>114.4 ± 11.3</td>
</tr>
<tr>
<td>( \Delta X ), cm × 10⁻⁸</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>4.80 ± 0.19</td>
<td>3.41 ± 0.44†</td>
<td>5.52 ± 0.73</td>
<td>2.61 ± 0.11</td>
</tr>
<tr>
<td>15 min</td>
<td>3.30 ± 0.54*</td>
<td>3.43 ± 0.42</td>
<td>2.53 ± 0.48*</td>
<td>3.12 ± 0.30</td>
</tr>
<tr>
<td>( J_{\Delta}/\text{GFR} ) × 10⁵</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>9.66 ± 2.00</td>
<td>9.97 ± 3.02</td>
<td>13.60 ± 4.56</td>
<td>9.55 ± 1.13</td>
</tr>
<tr>
<td>15 min</td>
<td>13.66 ± 2.92</td>
<td>15.2 ± 1.93</td>
<td>22.39 ± 7.54</td>
<td>9.90 ± 3.16</td>
</tr>
<tr>
<td>( \alpha_L ) × 10²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>3.01 ± 0.61</td>
<td>3.26 ± 0.97</td>
<td>4.71 ± 1.84</td>
<td>3.25 ± 0.43</td>
</tr>
<tr>
<td>15 min</td>
<td>3.85 ± 0.80</td>
<td>4.42 ± 0.54</td>
<td>6.38 ± 1.52</td>
<td>3.24 ± 0.40</td>
</tr>
</tbody>
</table>

Values are means ± SE. PS, protamine sulfate; hyase, hyaluronidase; n-Ficoll, neutral Ficoll; a-Ficoll, anionic Ficoll; \( r_s \), small-pore radius; \( r_L \), large-pore radius; \( \Delta X \), effective pore area over unit diffusion path-length; \( J_{\Delta}/\text{GFR} \), fractional fluid flow through large pores; GFR, glomerular filtration rate; \( \alpha_L \), fractional ultrafiltration coefficient accounted by large pores. *\( P < 0.05 \), statistical differences between values at 15 min compared with baseline values in each group; †\( P < 0.05 \), statistical difference between baseline conditions for a-Ficoll compared with baseline for n-Ficoll.
conclude that glomerular charge selectivity was not affected by hyase. If hyase had created a more “open” (negatively charged) GC matrix, enabling molecules to penetrate deeper into the GC (25), then n-Ficoll molecules of radius 50–80 Å would indeed show increased GC permeation, while, conceivably, anionic molecules may have been partly prevented to do so. Contradicting this hypothesis is, however, the dynamics and reversibility in the changes in θ values for n-Ficoll molecules of radius 50–80 Å. These dynamic changes would favor the concept of an active, dynamic alteration of the GFB after hyase, conceivably by reorganization of the cytoskeleton in podocytes and/or endothelial cells, rather than changes affecting GC function.

The rather moderate effects of PS and hyase on the permeability of the GFB found in the present study may be explained by the rather moderate systemic doses of PS and hyase administered so as to avoid large hemodynamic effects. Still, the doses administered were large enough to produce size-selective changes in the GFB. However, there were no overt morphological alterations of the GFB found by transmission electron microscopy in the present study after PS infusion. In the pioneering morphological and tracer studies by Venkatachalam and colleagues (48, 49), PS in buffer solution was directly infused intrarenally (20–50 μg/ml), which produced foot process effacement after only 10 min. In the study of Hunsicker et al. (26) on the effects of systemic polycation infusions on rat glomerular permeability, reversible foot processes effacement occurred only after 45 min of polycation infusion. In the present study, however, the permeability response occurred with a maximum at ~5 and 15 min, at which time the morphology of the GFB appeared to be largely unaltered. Our data thus strongly suggest that PS and hyase given in moderate doses affect glomerular permeability in a manner neither involving the charge selective properties of the GFB nor its (gross) morphology, but, conceivably, induce subtle alterations in the contractility and/or adhesion of the cells of the GFB.

In the present investigation, we chose not to study the leakage of albumin across the GFB due to unexpectedly high levels of free iodine and denaturated protein in the radiolabeled albumin preparations at our disposal. However, in a number of previous studies, we have been able to simultaneously assess high-molecular-weight Ficoll and θ values for radiolabeled (125I-labeled) albumin, and we have demonstrated a more or less perfect coupling between these two parameters (5, 6, 45, 46). In fact, θ values for Ficoll molecules of radius 55 Å and that for albumin show identical sieving coefficients, as also demonstrated by another group (39). Therefore, given the close coupling of θ values for albumin to those of Ficoll molecules of radius 50–80 Å, we have considered it safe to rely on the latter as an indicator of glomerular permeability.

In summary, PS and hyase, given in doses that did not markedly reduce systemic blood pressure or GFR, were both able to reduce the size selectivity of the GFB in vivo without changing its charge selectivity. Thus, interacting with the glomerular GC and the ESL, PS and hyase conceivably produce changes in GFB dynamics. Our results are thus in agreement with recent findings underpinning the importance of active cellular (Ca2+ signaling) mechanisms in causing proteinuria after perturbations of the glomerular GC while leaving the charge-selective function of the GFB largely intact.

ACKNOWLEDGMENTS

The authors acknowledge Kerstin Wihlborg for skillful typing of the manuscript and thank Dr. William Fissel (Vanderbilt University, Nashville, TN) for granting the FITC-labeled anionic Ficoll. The authors also thank Carolina Cranford for the help with the preparation of kidney biopsies for electron microscopy.

GRANTS

This work was supported by Swedish Medical Research Council Grant 08285, the Swedish Heart and Lung Foundation, and the Medical Faculty at Lund University (ALF Grant).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


