Fluorescence dilution technique for measurement of albumin reflection coefficient in isolated glomeruli

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RUNNING TITLE: Glomerular Permeability to Albumin
This study describes a high-throughput fluorescence dilution technique to measure the albumin reflection coefficient ($\sigma_{\text{Alb}}$) of isolated glomeruli. Rats were injected with fluorescein isothiocyanate–dextran 250 (75 mg/kg) and the glomeruli were isolated in a 6% bovine serum albumin solution. Changes in the fluorescence of the glomerulus due to water influx in response to an imposed oncotic gradient was used to determine $\sigma_{\text{Alb}}$. Adjustment of the albumin concentration of the bath from 6% to 5%, 4%, 3%, and 2% produced a 10%, 25%, 35%, and 50% decrease in the fluorescence of the glomeruli. Pretreatment of glomeruli with protamine sulfate (2 mg/ml) or TGF-β1 (10 ng/ml) decreased $\sigma_{\text{Alb}}$ from 1 to 0.54 and 0.48, respectively. Water and solute movement were modeled using the Kedem-Katchalsky equations and the measured responses closely fit the predicted behavior, indicating that loss of albumin by solvent drag or diffusion is negligible in comparison to the movement of water. We also found that $\sigma_{\text{Alb}}$ was reduced by 17% in Fawn Hooded Hypertensive rats, 33% in hypertensive Dahl Salt-sensitive (SS) rats, 26% in streptozotocin-treated diabetic Dahl SS rats and 21% in 6-month old Type II diabetic nephropathy rats relative to control SD rats. The changes in glomerular permeability to albumin were correlated with the degree of proteinuria in these strains. These findings indicate that the fluorescence dilution technique can be used to measure $\sigma_{\text{Alb}}$ in populations of isolated glomeruli and provides a means to assess the development of glomerular injury in hypertensive and diabetic models.

**KEY WORDS:** kidney, glomerulus, proteinuria, renal hemodynamics, renal disease
INTRODUCTION

Proteinuria and microalbuminuria have long been used as markers for early detection of chronic kidney disease (CKD). (2, 3, 5, 11, 14, 52) Elevated excretion of protein may be a consequence of injury to the glomerular protein permeability barrier, defects in tubular reabsorption of filtered protein, or combination of both. (11, 49, 52) However, the relative contributions of changes in renal hemodynamics versus alterations in the glomerular permeability barrier to protein in the development of proteinuria are difficult to determine in vivo. Thus, it is desirable to develop an in vitro model to study the barrier function of glomerulus in the absence of variation in perfusion pressure.

In 1992, Savin et al. described a method to measure the reflection coefficient to albumin ($\sigma_{Alb}$) in isolated glomeruli and the relative permeability of the glomerulus to albumin (Palb) defined as $1-\sigma_{Alb}$. (38) Changes in the diameter of isolated glomeruli due to water movement across the glomeruli capillaries were found to be proportional to the magnitude of an imposed oncotic gradient. (39) Using this approach, Savin and her group demonstrated that numerous mediators including cardiotrophin-like cytokine factor 1 (CLCF1), (43) endothelin, (36) transforming growth factor beta (TGF-β), (9) superoxide, (41, 44) focal glomerulosclerosis factor, (37, 42) tumor necrosis factor alpha (TNFα) (24) and platelet-activating factor (PAF) (46) increase Palb in isolated glomeruli, whereas epoxyeicosatrienoic acids (EETs), (40) 20-hydroxyeicosatetraenoic acid (20-HETE) (25) and nitric oxide (NO) (41, 44) protect the glomerular protein permeability barrier. One limitation of their technique is that the alterations in the diameter of the glomeruli are small (on the order of a few microns) as they are proportional to the cube root of the volume change. Thus, the glomeruli have to be imaged at high magnification which limits the number that can be studied in a given field. This also limits
the ability to study populations of glomeruli isolated from different animals which is necessary when studying hypertensive and diabetic strains with focal glomerular disease since the degree of injury is heterogeneous. Another complication is that the reduction in the compliance of the glomerulus in animals with focal glomerulosclerosis reduces the change in diameter of the glomerular capillaries leading to an overestimation of the fall in Palb.

The present study describes a modification of the technique of Savin to measure the \( \sigma_{\text{Alb}} \) in populations of isolated glomeruli. Rats or mice were treated with fluorescein isothiocyanate–dextran 250 (FITC-dextran 250 KDa) \textit{in vivo} and glomeruli were isolated in 6% bovine serum albumin (BSA) solution. The changes in the glomerular volume, rather than diameter, were directly measured by the dilution of the fluorescence signal in the glomerular capillaries due to water influx in response to an imposed oncotic gradient. The glomeruli were imaged at low power so that the changes in fluorescence of many glomeruli (20-50) could be simultaneously studied. Alterations in water movement in response to various imposed oncotic gradients were modeled using the Kedem-Katchalsky equations (1, 16, 17) and the measured responses fit the predicted behavior. The technique was validated by showing that protamine sulfate (2 mg/ml) and TGF-\( \beta \)1 (10 ng/ml) decreased \( \sigma_{\text{Alb}} \) as previously reported (9, 38, 39, 45, 51). Moreover, \( \sigma_{\text{Alb}} \) was reduced in hypertensive and diabetic strains of rats with various degrees of proteinuria.

**MATERIALS AND METHODS**

**GENERAL**

Experiments were performed on male C57BL/6 mice (The Jackson Laboratory), Sprague Dawley (SD) (Charles River Laboratories, Wilmington, MA), Dahl salt-sensitive (Dahl SS), Type 2 diabetic nephropathy (T2DN), (27) and Fawn Hooded Hypertensive (FHH) rats that were
obtained from inbred colonies maintained at the University of Mississippi Medical Center (UMMC). The animals were housed in the Animal Care Facility at UMMC that is approved by the American Association for the Accreditation of Laboratory Animal Care. The mice and rats had free access to food and water throughout the study. All protocols received approval by the Institutional Animal Care Committee at UMMC.

**PROTOCOL 1. GLOMERULAR ISOLATION AND IMAGING**

The rats or mice were anesthetized with 2% isoflurane. FITC-dextran 250 KDa (Sigma-Aldrich, St. Louis, MO) at a dose of 75 mg/kg in a 0.9% NaCl solution was injected into the femoral vein. Other fluorescently labeled Cy3, Cy5 and rhodamine high molecular weight dextrans work equally as well. After 3-5 minutes, the kidneys were harvested and placed in ice-cold isotonic Hank’s Balanced Salt Solution (HBSS, Life Technologies, Grand Island, NY) containing 6% BSA (Sigma-Aldrich, St. Louis, MO) and 10 mM HEPES (Sigma-Aldrich, St. Louis, MO), pH 7.4. Glomeruli were isolated as previously described. (38, 39) Briefly, the kidney was hemisected on a sagittal plane and the renal cortex was separated from the medulla, chopped into fine pieces, and passed through stainless steel filter with decreasing pore sizes from 150 μm to 106 μm (USA standard sieve No. 100 and No. 140, respectively, Thermo Fisher Scientific, Waltham, MA) into a petri dish. The glomeruli were captured on a 70-μm cell strainer (BD Bioscience, San Jose, CA) and washed off of the sieve with ice-cold Hank’s solution containing 6% BSA. The mixture was transferred to a 15 ml tube and stored on ice prior to an experiment.

A small aliquot (50 μl-100 μl) of the isolated glomeruli was loaded onto a coverslip, pre-coated with Poly-L-lysine hydrobromide (10 mg/ml, Sigma-Aldrich, St. Louis, MO), that formed the bottom of a fluid exchange chamber (RC-24, Warner Instruments, Hamden, CT). The inflow
line to the chamber was attached to two peristaltic pumps so that the bath could be rapidly exchanged in less than 10 seconds. The effluent was collected through a vacuum line. The isolated glomeruli were imaged using a fluorescent microscope (Nikon TS-100, Nikon Instruments Inc., Melville, NY) equipped with a high sensitivity camera and a 175-watt Xenon Arc Lamp (Intracellular Imaging, Cincinnati, OH) and filter wheel (Excitation/emission: 480/510-550 μm). The glomeruli were observed using a low numerical aperture 5X lens (Nikon Instruments Inc., Melville, NY) with a large depth of field (>50 μm) so that the fluorescence signal from the entire glomerulus could be collected. Approximately 30 glomeruli per field were selected for study based on their morphological appearance. A recording area slightly larger than the circumference of the glomeruli was defined using Image 1 fluorescent imaging software (Incyte, Cincinnati, OH) so that all of the label remains in the recorded volume when the glomeruli expand following a reduction in the oncotic pressure of the bath. We excluded glomeruli that had intact Bowman’s capsules, visible arteriolar fragments, attached proximal tubules or were deformed or torn, (39) as well as those were poorly labeled or too bright with intensities near the saturation level of the camera.

Fluorescent intensities were individually recorded and the values were expressed as percent of the control intensity measured at time zero for each glomerulus. There was more variation in the time course of the changes in fluorescent intensity between glomeruli than between rats of a given strain, so we typically averaged all the individual data and expressed the results as number of glomeruli studied per strain or group rather than calculating the group mean from the average value recorded from each rat.

Additional studies were performed to determine the distribution of the labeled dextran in the glomerular capillaries following i.v. administration. The glomeruli were labeled in vivo with
FITC-Dextran 250 as described above. The kidneys were harvested and immediately placed in 10% neutral buffered formalin. Paraffin sections (3 μm) were prepared and counterstained with Evans blue (0.001% for 5 minutes) that exhibits red fluorescence. Images were obtained using a fluorescent microscope (Olympus BH-2) equipped with a highly sensitivity Q-Imaging digital camera (400 X magnification, W. Nuhsbaum Inc., McHenry, IL). Overlaid images were created to visualize the localization of the dyes by using FITC (Excitation/emission: 480/510-550 μm) and rhodamine (Excitation/emission: 550/610 μm) filters, respectively.

**PROTOCOL 2. RESPONSE TO DIFFERENT ONCOTIC GRADIENTS**

Glomeruli were isolated from SD rats or C57BL/6 mice as described above. After selecting the glomeruli to be studied, the chamber was perfused at 0.5 ml/min with a solution containing 6% BSA and baseline fluorescent signals were recorded at a rate of 1 image per second for 60 seconds. The solution was switched to one containing 5%, 4%, 3% or 2% BSA and the fluorescent signals from the glomeruli were recorded at 1 second intervals for an additional 180 seconds. The initial fluorescence value (time 0) was converted to 100% and the subsequent values were expressed as a percentage of the initial value.

The percentage change in the intensity of glomerular fluorescence signal at equilibrium was plotted against the % change in the protein concentration of the bath that is proportional to the magnitude of the oncotic pressure gradient. In addition, the results were fit to a model of water movement across the glomerular capillaries using the following equations (1, 16, 17):

\[
\frac{dV}{dt} = -J_v = L_p A \cdot \sigma_{Alb} RT \left(\frac{q}{v} - C_0(t)\right);
\]

\[
\frac{dq}{dt} = \left[\frac{-q}{v} - C_0(t)\right]/\ln (q/v \cdot C_0) \cdot (1 - \sigma_{Alb}) J_v - k \left(\frac{q}{v} - C_0(t)\right).
\]
where: \( \frac{dV}{dt} \) = the change in glomerular volume per unit time; \( J_v \) = net fluid movement; 

\( L_p A \) = filtration coefficient; \( A \) = glomerular area; \( \sigma_{Alb} \) = reflection coefficient for albumin; \( R \) = a modified gas constant expressed in mmHg; \( T \) = the bath temperature in degrees Kelvin; \( q/v \) = concentration of BSA in the glomeruli; \( C_0(t) \) = concentration of BSA in the bath; \( 1-\sigma_{Alb} \) = inverse of the reflection coefficient; \( k \) = diffusional permeability of capillary wall to solute. These equations assume that the initial hydrostatic pressure gradient across the isolated glomerulus is negligible and the reflection coefficient for small solutes is zero. The nonlinear differential equations were solved using the finite difference least squares method using a MATLAB computing environment and the three parameters (\( L_p A \), \( \sigma_{Alb} \), and \( k \)) were estimated by an iterative best fit of the experimental data.

**PROTOCOL 3. REVERSIBILITY OF THE WATER MOVEMENT**

To test whether the changes in the water movement and the fluorescence of the glomeruli is bidirectional, glomeruli were isolated from two groups of 12 week old normal SD rats. In one group, the glomeruli were isolated in 6% BSA and the fluorescence was recorded for 20-40 seconds to obtain a baseline value. An oncotic gradient was imposed by changing BSA concentration in bath from 6% to 4% for 5 minutes, and then back to 6% for another 5 minutes. In the second group, the glomeruli were isolated in 4% BSA and the oncotic pressure was increased by changing BSA in the bath from 4% to 6% for 5 minutes, and then back to 4% for another 5 minutes.

**PROTOCOL 4. EFFECT OF TEMPERATURE AND STORAGE TIME ON THE MEASUREMENT OF \( \sigma_{Alb} \)**
Glomeruli were isolated in 6% BSA and stored at room temperature or on ice for various times and $\sigma_{Alb}$ was measured at room temperature. The data were recorded for 20-40 seconds to obtain the baseline value. An oncotic gradient was imposed by rapidly changing BSA in medium from 6% to 4% for 3 minutes. An aliquot of the isolated glomeruli was studied every 15 minutes for up to 90 minutes after isolation.

**PROTOCOL 5. RESPONSE TO PROTAMINE SULFATE AND TGF-β1**

Male SD rats at age of 12-week old were anesthetized with isoflurane and received a 50 mg/kg i.v. injection of protamine sulfate or vehicle which has been shown to increase glomerular permeability to albumin both *in vivo* and *in vitro* (38, 39, 51). After 15 minutes, the glomeruli were labeled by an *i.v.* injection of FITC-Dextran 250 kDa (75 mg/kg) and were isolated in 6% BSA containing 2 mg/ml protamine sulfate as described above. The change in fluorescence in response to a decrease in the BSA concentration of the bath from 6 to 4% was recorded. Additional experiments were performed with glomeruli isolated from 12-week old SD rats. These glomeruli were incubated *in vitro* with TGF-β1 (10 ng/ml, Sigma-Aldrich, St. Louis, MO) or vehicle for 15 min at 37 °C that also has been reported to increase Palb. (34, 38, 45)

**PROTOCOL 6. RELATIONSHIPS BETWEEN $\sigma_{Alb}$ AND PROTEIN EXCRETION IN HYPERTENSIVE AND DIABETIC RATS**

*Hypertensive models*

These experiments were performed on 12-15 week old male FHH and Dahl SS rats. The FHH rats begin to develop progressive proteinuria as early as 9 weeks of age and chronic kidney disease by 21 weeks of age. (10, 19-23, 28, 29, 35) They were maintained on a normal rodent diet containing 0.4% NaCl. The Dahl SS rat is a genetic model of salt sensitive hypertension that rapidly develops proteinuria and glomerulosclerosis when fed with high salt (HS) diet. (6-8, 30,
Two groups of Dahl SS rats were studied: one group was fed a low salt (LS) diet containing 0.4% NaCl from birth, while the other was switched to a HS diet containing 8% NaCl starting at 9-week old of age for 3 weeks. Prior to measurement of $\sigma_{\text{Alb}}$, the FHH and Dahl SS rats were placed in metabolic cages and urine was collected to measure protein excretion. Then the rats were anesthetized, the glomeruli were labeled and isolated and $\sigma_{\text{Alb}}$ was measured as described above.

**Diabetic models**

Nine-week old male Dahl SS rats were treated with streptozotocin (STZ) (50 mg/kg, *i.p.*) to induce type I diabetes and were studied 3 weeks later. (13, 15, 31, 50) Six-month old T2DN rats were used as a model of type II diabetes that develops proteinuria. (26, 54) The rats were placed in metabolic cages and urine was collected to measure protein excretion. Glomeruli were harvested and $\sigma_{\text{Alb}}$ was measured as described above.

**STATISTICS**

Data were presented as mean ± SEM. The significance of the differences in mean values between two groups of rats or treatments was evaluated using a paired or unpaired T-test. The significance of the differences in mean values between and within multiple groups over time was tested using an analysis of variance (ANOVA) for repeated measures and a Holm Sidak test for preplanned comparisons. A $P$ value < 0.05 was considered to be statistically significant.

**RESULTS**

**PROTOCOL 1: GLOMERULAR ISOLATION AND IMAGING**

A schematic summary of the fluorescence dilution technique for measurement of $\sigma_{\text{Alb}}$ is presented in *Figure 1A*. Rats are injected with a high molecular weight FITC-Dextran 250 that
remains in the vasculature. The kidneys are collected and glomeruli are isolated by passing the tissue through a series of sieves. The glomeruli are placed in a rapid exchange recording chamber connected to two inflow pumps to allow for rapid exchange of the bathing media. The effluent is collected by vacuum and the glomeruli are imaged on a fluorescent inverted microscope equipped with a 5X (0.3 NA) fluorescent lens with a large depth of field (>50 μm). The glomeruli are visualized and the fluorescence in an area encompassing each glomerulus defined using the Image 1 fluorescent imaging software.

The left panel of **Figure 1B** presents a representative field of view of the fluorescently labeled isolated glomeruli. The field contains >30 glomeruli and most are well filled with the FITC-Dextran. Twelve glomeruli were selected for study (circled) and the fluorescent intensity was individually recorded. The right panel presents a renal section that was counterstained with Evans blue and indicates that the FITC-Dextran 250 was confined to the glomerular capillaries. **Figure 1C** presents typical traces from the 12 circled glomeruli as indicated in **Figure 1B** left panel. The average value for this group of glomeruli is plotted as a black bold line. **Figure 1D** presents the time course of changes in the fluorescence in the bath following switching the bath solution between a control solution and one that contains FITC-Dextran. These data demonstrate that the bath can be completely exchanged within seconds.

**PROTOCOL 2. RESPONSE TO DIFFERENT ONCOTIC GRADIENTS**

A simplified schematic of the theoretical basis of the fluorescence dilution technique is presented in **Figure 2A**. Glomeruli were isolated in an isotonic HBSS solution containing 6% BSA. Following a rapid step change in the concentration of the bath from 6% to 4%, an oncotic gradient of about 9 mm Hg is generated which drives water into the capillaries. As results, the concentration and fluorescence intensity of the FITC-dextran in the glomerular capillaries is
reduced. Assuming the glomerulus was impermeable to albumin and the reflection coefficient is 1, a reduction in the protein concentration of the media from 6 to 4% would reduce the oncotic pressure in the bath by 33% and should increase the volume of the glomerulus and reduce the fluorescent signal by a similar amount.

A mathematic model derived from Kedem–Katchalsky equations (1, 16, 17) that describes the expected movement of water and solute (BSA) in response to oncotic pressure gradient is presented in Figure 2B. In this model, water flux (formula 1) is equal to the product of \( L_pA \) (hydraulic permeability), \( \sigma_{Alb} \) (reflection coefficient), and sum of the oncotic and hydrostatic pressure gradients across the glomerular capillaries. The oncotic pressure gradient is defined by the difference in the concentration of albumin in the glomerular capillaries and the bath. Equation 2 describes the diffusion and filtration of albumin across the glomerular capillaries. If we assume that the initial hydrostatic pressure gradient, at least at time 0, is negligible, the albumin reflection coefficient (\( \sigma_{Alb} \)) can be determined by the measuring change in volume of the glomerulus, whereas \( \sigma_{Alb} = \frac{\% \text{ change in } \Delta v}{\% \text{ predicted change in } \Delta v} \) which should be equal to the percentage change in oncotic pressure determined by the imposed protein concentration gradient. The range of values for \( \sigma_{Alb} \) is 0 and 1. A relative \( Palb \) can be calculated as \( 1-\sigma_{Alb} \).

In the current study, a high molecular weight of fluorescent FITC-Dextran which is impermeable to the glomeruli capillaries was used as a volume marker. Under these conditions the fluorescence is the glomerular capillaries is directly proportional to the concentration of FITC-Dextran and inversely proportional to the glomerular volume. Therefore, we substituted the percentage change in fluorescence for \( \Delta v \) and defined the \( \sigma_{Alb} = \frac{\% \text{ change in fluorescence}}{\% \text{ change in } \Delta v} \) and defined the \( \sigma_{Alb} = \frac{\% \text{ change in fluorescence}}{\% \text{ change in } \Delta v} \).
fluorescence / expected % change in volume that is equal to the % change in the albumin concentration of the bath.

The time course of the change in fluorescent intensity in FITC-Dextran 250 labelled glomeruli isolated from 9 week old young SD rats in response to different oncotic gradients imposed by changes of BSA concentration from 6% to 5%, 4%, 3% and 2% is presented in Figure 2C. This data was iteratively fit to a mathematical models of equations 1 and 2 as described in detail previously by Beard et al. (1) by first assuming that the glomerular capillaries were impermeable to albumin ($\sigma_{Alb}=1$) to obtain the best fit estimates for $L_pA=3.8$ dl/mg x sec and $K = 0.05$ sec$^{-1}$. We then varied $\sigma_{Alb}$ to 0.83 to obtain the best fit for magnitude the responses. Lowering $\sigma_{Alb}$ decreases the magnitude of the response, whereas altering $L_pA$ affects the shape of the curve and how rapidly equilibrium is attained. The model was relatively insensitive to changes in $K$, as would be expected since diffusive loss of protein across the capillaries is negligible relative to the rapid movement of water.

Additional glomeruli were isolated from 9 week old young SD rats to better understand the changes in fluorescent intensity in response to different oncotic gradients imposed by changes of BSA concentration as presented in Figure 3A. The expected changes in the fluorescence intensity of the glomeruli in response to changing the bath from 6% BSA to 4%, 3% and 2% are 33.3%, 50% and 67.7%, respectively. The experimental results indicated the measured changes in fluorescent signal were 27.9%, 33.5% and 50.2%. The relationship between % change in fluorescence and the expected % change in fluorescence was linear, with a slope of 0.992 and an adjusted $R^2$ of 0.983 (Figure 3B).

PROTOCOL 3. REVERSIBILITY OF THE WATER MOVEMENT
The results of experiments to determine whether water movement in the glomerulus was bidirectional are presented in Figure 4A. The fluorescence intensity increased by 8% (P<0.05) in FITC-Dextran 250 labelled glomeruli isolated from SD rats in 4% BSA after the bath was replaced with 6% BSA. It decreased by 20% (P<0.05) after medium was switched back to 4% BSA. Similarly, Figure 4B demonstrates that changing medium containing 6% BSA to 4% BSA resulted in a decrease in fluorescence intensity of 20% (P<0.05) and increased by 4% (P<0.05) after medium was changed back to 6% BSA.

PROTOCOL 4. EFFECT OF TEMPERATURE AND STORAGE TIME ON THE MEASUREMENT OF $\sigma_{\text{Alb}}$

These studies were performed to determine the effect of temperature and storage time on the measurement of $\sigma_{\text{Alb}}$. FITC-Dextran 250 labelled glomeruli were isolated from young 9 week old SD rats and stored at room temperature or on ice. The intensity of the fluorescence signal of the glomeruli was recorded every 15 minutes for 90 minutes after glomeruli isolation. As displayed in Figure 5, there was a time-dependent decrease in the magnitude of the $\sigma_{\text{Alb}}$ response in glomeruli isolated and stored at room temperature (Figure 5A) relative to glomeruli isolated with cold media and studied immediately at room temperature (Figure 5B). Glomeruli isolated and stored on ice exhibited a better sigmoid curve and the fall in fluorescence intensity ranged from 25 ± 1% at time 0 in control glomeruli versus only 20 ± 1 and 15 ± 1 in glomeruli isolated and stored at room temperature for 0 and 90 minutes. The relative intensity of the fluorescence of glomeruli isolated and stored on ice at time 0 most closely matched the expected change (75% vs. 67%). There is no significant difference in the response of glomeruli isolated and stored on ice for 15 or 30 minutes, but the response was diminished when glomeruli were stored on ice for longer periods (60 to 90 minutes).
PROTOCOL 5. RESPONSE TO PROTAMINE SULFATE AND TGF-β1

Previous studies reported that protamine sulfate and TGF-β1 increase the albumin permeability of the glomerulus, (38, 45) therefore experiments were performed to determine if the fluorescence dilution assay can detect the expected changes in $\sigma_{Alb}$. The results are presented in Figure 6. The fluorescence of the glomeruli decreased by about 25% in response to a decrease protein concentration of the bath from 6 to 4% in glomeruli isolated from control rats. The magnitude of the response was significantly reduced in glomeruli pretreated with protamine sulfate (Figure 6A right panel) or TGF-β1 (Figure 6A left panel) in vitro. Correspondingly, Palb increased from 0.20 ± 0.03 to 0.54 ± 0.02 in glomeruli treated with protamine sulfate (Figure 6B right panel) and to 0.48 ± 0.04 in TGF-β1 (Figure 6B left panel) treated group.

PROTOCOL 6. RELATIONSHIPS BETWEEN $\sigma_{Alb}$ AND PROTEIN EXCRETION IN HYPERTENSIVE AND DIABETIC STRAINS OF RATS

The results of these experiments are presented in Figure 7. The fluorescence intensity in response to changes in the albumin concentration of the bath from 6% to 4% fell by 29% in glomeruli isolated from young SD rats, and was significantly greater in FHH rats (23%) and Dahl SS rats fed a HS diet (19%), but not in Dahl SS rats fed a LS diet (25%). Similarly, the fall in fluorescence intensity in response to changes in oncotic gradient in diabetic animal models was significantly reduced in STZ treated Dahl SS rats (21%) and 6-month old T2DN rats (23%) than that seen in SD rats (Figure 7A).

A comparison of Palb measured in these various groups is presented in Figure 7B. Palb was significantly higher in glomeruli isolated from FHH (0.28 ± 0.03), Dahl SS rats fed a HS diet (0.42 ± 0.03), STZ-treated Dahl SS rats with type I diabetes (0.36 ± 0.02) and T2DN rats (0.31 ± 0.02) as compared with SD rats (0.13 ± 0.02). The difference in Palb was not significant
in SD rats versus in Dahl SS rats fed a LS diet (0.27 ± 0.02). A comparison of the protein excretion in the various groups presented in Figure 7C. Protein excretion was significantly elevated in 12-week old Dahl SS rats fed a LS diet (74 ± 8 mg/day) and 6 months old T2DN rats (51 ± 10 mg/day) as compared with 12 week old normal SD rats (19 ± 1 mg/day). Protein excretion was much higher in FHH rats (93 ± 9 mg/day), Dahl SS rats fed a HS diet (287 ± 40 mg/day) and STZ-treated type I diabetic Dahl SS rats (183 ± 23 mg/day). Figure 7D presents the relationship between proteinuria and Palb measured in these different strains. Urine protein excretion well correlated with the measurement of Palb of glomeruli isolated from these various strains. (R^2 = 0.75).

**DISCUSSION**

Proteinuria has long been used as markers for early detection of chronic kidney disease. However, increases in the excretion of protein may be due to injury to the glomerular permeability barrier or defects in tubular reabsorption of filtered protein. (11, 49, 52). Moreover, it is difficult to dissect the relative contributions of changes in blood pressure and renal hemodynamics versus alterations in the barrier function of the glomerulus *in vivo*. Thus, it is desirable to study the barrier function of glomerulus *in vitro* in the absence of potential differences in renal hemodynamics. In this regard, Savin et al. first described a technique to measure the relative permeability of isolated glomeruli to albumin based on the change in volume of the glomerulus in response to an imposed oncotic gradient. Alterations in the volume of fluid in the glomerular capillaries were assessed by measuring the increase in diameter of the glomerulus before and after imposing an oncotic gradient by lowering the protein concentration of the incubation media from 6 to 2%. This method has proven to be extremely useful to identify factors that increase the glomerular permeability to albumin. However, it is more difficult to
study the changes in permeability associated with hypertension and diabetes in which the
glomerular injury is very heterogeneous because the glomeruli have to be imaged at high
magnification using this method to detect the small differences in diameter. This also limits the
number of glomeruli that can be studied in a given experiment. Another problem is that renal
fibrosis which reduces the compliance of the glomerulus can lead to an overestimation of the
increase in Palb.

The present study describes a high throughput fluorescence dilution technique that is a
modification of the original method (38) to measure albumin reflection coefficient ($\sigma_{Alb}$) in a
population of glomeruli. The major difference is that in the present study the rats were injected
with a high molecular weight FITC-Dextran 250 in vivo to label the plasma in the glomerular
capillaries and serve as a volume marker. The change in the fluorescent signal in isolated
glomeruli due to water influx in response to an imposed oncotic gradient was used to determine
the $\sigma_{Alb}$. The results indicate that changing the albumin concentration of the bath from 6% to
5%, 4%, 3%, and 2% produced a linear decrease in the fluorescent signal of the glomeruli. The
alterations in water and solute movement were modeled using an approach previously described
by Beard et al. (1) using the Kedem-Katchalsky equations and the measured responses closely fit
the predicted behavior, indicating that loss of solute by solvent drag or diffusion of albumin is
negligible in comparison to the rapid movement of water. Changing the $\sigma_{Alb}$ reduces the driving
force for water movement and the magnitude of the response, whereas changes in $L_{pA}$ affect the
shape of the response and the time to reach a new steady state. The oncotic pressure of the
dextran is minimal based on the molar concentration in the plasma. This can be demonstrated in
*Figure 3A* from the fact that the fluorescent signal did not decrease in glomeruli bathed with 6%
albumin. Water would move into the glomerulus and lower the fluorescent signal if the oncotic
pressure of dextran in the plasma was significant. The generation of a hydrostatic pressure in the glomerulus is the most likely responsible for the deviation from the expected Palb closer to 0.1 which we could achieve when studying glomeruli isolated from SD rats (Figures 2-7) and in mouse glomeruli (Figure 1C) using ideal isolation and storage conditions.

We also examined the ability of the fluorescent dilution assay to detect the effects of protamine sulfate and TGF-β1 on $\sigma_{Alb}$ of isolated glomeruli. Protamine sulfate is a polyanion that has been report to increase albumin permeability of the glomerulus (38) and cause proteinuria by reducing the effective negative charge of the slit pores. We found that $\sigma_{Alb}$ decreased in glomeruli isolated from SD rats treated with protamine sulfate compared with vehicle-treated rats, consistent with previously reported results (38). Similarly, incubation of isolated glomeruli with TGF-β1 also reduced $\sigma_{Alb}$. Thus the results obtained using the fluorescent dilution assay are very consistent with previous results by Sharma et al. (45) and others (34, 38, 39, 45, 51) indicating that TGF-β1 increases Palb in isolated glomeruli.

The fluorescent dilution assay offers many advantages over the original method. First, since the glomeruli are fluorescently labeled, damage to individual glomeruli can be visualized by the loss of the label from the capillaries or a decline in the fluorescent signal with time. Second, the magnitude of the change in fluorescence or volume is much larger and easier to detect than the change in diameter which is proportional to the cube root of the change in volume. Third, the time course of the changes in the fluorescent signal can be continuously monitored, whereas typically only steady state changes in diameter at two time points are studied using the original technique. The most important advantage is that the glomeruli can be imaged using a low power lens so that measurements can be made from many glomeruli simultaneously (up to 50 per field) and the entire measurement can be completed in 120 seconds. This allows one to
measure $\sigma_{Alb}$ in a population of glomeruli isolated from a single kidney over a 30-minute time frame which is helpful when studying renal disease models in which the degree of glomerular hypertrophy, sclerosis and podocyte loss is very heterogeneous. However, there are limitations to this technique. Some glomeruli in animals with severe sclerosis will not fill and therefore cannot be studies. Exclusion of these severely damaged glomeruli would lead to an underestimate the overall increase in permeability within a strain. On the other hand, glomeruli isolated from severely diseased animals could be more susceptible to tearing and damage during the isolation procedure leading to an overestimation of the degree of injury. These problems can be minimized by close visual inspection of each glomerulus prior to selecting them for study.

In the present study, some findings deviated from the expected behavior. The results presented in Figure 4 indicate that the movement of water is bidirectional, but the increase in fluorescence when the oncotic pressure was increased was much less than expected. This may be due to two factors. First, when the oncotic pressure of the bath is increased and the glomerular capillaries collapse, the transmission of both excitation and emitted light in the glomerulus would be reduced to limit the increase in fluorescence. Secondly, we defined the recording volume with the Image one fluorescent imaging software to be slightly larger than the diameter of the glomerulus so that all of the fluorescent signal would remain within the recording volume when the glomeruli swell in response to a reduction in the oncotic pressure of the bath. However, when the oncotic pressure of the bath is increased, the size of the glomerulus contracts and the contribution of the background to the average signal within the recorded area increases and attenuates the increase in fluorescence. This problem can be partially mitigated by redefining a smaller recording volume after the oncotic pressure of the bath is increased but it cannot address the issue with the decrease in transmission of light in the glomerulus.
Another difference in the measured versus expected results was that the change in fluorescence decreased by 25%, 35%, 50% as compared to the expected change of 33.3%, 50%, 66.7% when the protein concentration of the bath was lowered from 6% to 4%, 3% and 2%. We believe there are two factors contributing to this difference. The first is that the hydrostatic pressure in the glomerulus increases from 0 to a few mm Hg as water moves into the capillaries in response to a decrease in oncotic pressure of the bath. This opposes the dilution of the fluorescent label and contributes to the deviation from the expected behavior especially when using large gradients which cause greater distention of the glomerular capillaries. One can readily appreciate that hydrostatic pressure in the glomerulus does increase in these experiments as the volume of the capillaries expand because we can sometimes see expulsion of the fluorescent dextran from the capillaries of some glomeruli if the collapsed arterioles open up which typically occurs if the pressure in the capillaries increases by a few mm Hg. For this reason, we typically use a step change of 6 to 4% in the albumin concentration of the bath instead of establishing a larger gradient to minimize the potential for a rise in hydrostatic pressure. The other complicating factor is the effect of temperature and storage time on the measurements. The results presented in Figure 5 indicate that the change in fluorescence in response to a step change in oncotic pressure is significantly larger in glomeruli isolated and stored in ice cold media than in those isolated and stored at room temperature. Moreover, the change in fluorescence is reduced even in glomeruli stored on ice for longer than 30 minutes. In this regard, we did store the glomeruli on ice for longer periods in some of our initial studies and this may be the reason why the change in fluorescence was less than expected.

Additional studies were performed to determine if the fluorescence dilution assay could detect differences in $\sigma_{Alb}$ in hypertensive and diabetic animal models with varying degrees of
proteinuria. We have previously reported that Dahl SS rats develop severe hypertension, proteinuria and glomerular injury when fed with a HS diet and diabetic nephropathy when treated with STZ to induce type I diabetes. (47, 53) They have less renal injury when fed with a LS diet. We also reported that male FHH rats start to develop proteinuria and glomerular injury at about 9-week of age and exhibit severe renal injury by 21-week of age. (4) T2DN rats begin to exhibit a mild degree of proteinuria at 6 months of age and develop severe diabetic nephropathy between 12-18 months of age. (18, 54) In the present studies, we found that the fall in fluorescence intensity in isolated glomeruli was significantly reduced following a reduction in BSA from 6% to 4% in glomeruli isolated from Dahl S rats treated with STZ or fed a HS diet and in FHH and T2DN rats in comparison to the response seen in 12-week old SD rats. The magnitude of the rise in Palb ($1-\sigma_{Alb}$) in these strains was correlated with the degree of proteinuria. The only strain that deviated from the regression line was the T2DN strain. Proteinuria was lower than expected given the increase in Palb. This may reflect a higher capacity of this strain to reabsorb filtered protein, at least early in the early stages of the disease process.

In summary, we have developed a high throughput method to measure $\sigma_{Alb}$ in a large number (>30 glomeruli/experiment, 3-4 experiments/30 min) of glomeruli isolated from a single animal. The measured change in $\sigma_{Alb}$ fits with the predicted behavior defined in a model of water movement in isolated glomeruli using the Kedem-Katchalsky equations (16, 17) previously described by Beard et al. (1) and allows one to study differences in $\sigma_{Alb}$ in populations of glomeruli derived from normal and diseased animals. The responses to different molecules that influence the glomerular permeability barrier can also be studied. Based on these results, we recommend that glomeruli should be isolated and stored at 4 °C for no more than 30
minutes to measure Palb. They can be incubated at room temperature or at 37 °C to study the
effects of drugs and hormones on Palb as long as they are compared with appropriate control
glomeruli studied under the same storage and incubation conditions. In conclusion, the present
fluorescence dilution method provides a means to assess the development of glomerular disease
in hypertensive and diabetic models and to study the effectiveness of various treatments to slow
the progression of CKD.

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**FIGURE LEGENDS**

**FIGURE 1. GLOMERULAR ISOLATION AND IMAGING**

**(A).** Schematic of the fluorescence dilution $\sigma_{Alb}$ measurement technique. Rats were injected with FITC-Dextran 250. The kidneys were harvested and the cortex was separated and passed through filters into a petri dish. The glomeruli were collected using a 70-$\mu$m nylon filter in ice-cold Hank’s Balanced Salt Solution (HBSS) containing 6% BSA and were loaded onto a fast fluid exchange electrophysiological chamber and imaged using an inverted fluorescent microscope. **(B).** The left panel presents a typical low power image of glomeruli filled with FITC-Dextran 250. Twelve glomeruli in the field were selected for study (circled) and the fluorescent intensity was individually recorded. The right panel displays the intrarenal localization of FITC-Dextran 250 in a paraffin renal section that was counterstained with Evans blue. FITC-Dextran 250 was confined to the glomerular capillaries. **(C).** Presents typical traces from the 12 circled glomeruli as indicated in *Figure 1B* left panel. The average value for this
group of glomeruli is plotted in a black bold line. \textbf{(D)}. A typical trace showing the changes in fluorescence intensity in the bath when the perfusion solution was changed from media containing FITC-dextran to one without indicates that the bath is fully exchanged in a few seconds.

\textbf{FIGURE 2. THE THEORETICAL BASIS of the fluorescence dilution $\sigma_{Ah}$ measurement.} \textbf{(A)}. Mechanism of water flux after changing oncotic gradient from 6\% to 4\% BSA. Rapid exchange of medium to a lower BSA concentration imposes an oncotic gradient across the capillary wall that drives water into the capillary and dilutes the fluorescent intensity. \textbf{(B)}. Kedem-Katchalsky equations for water and solute flux across the glomerular capillaries. Water and solute (BSA) flux are described in formulas (1) and (2), respectively. \textbf{(C)}. Validation of the fluorescence dilution technique. The dotted lines are experimental results with FITC-Dextran 250 labelled glomeruli isolated from SD rats using our new fluorescence dilution technique. The solid lines are of a best fit of the experimental data based on a computer model of water movement in isolated glomeruli using the Kedem-Katchalsky equations previously described by Beard \textit{et al.} (1) Data are presented as mean ± SEM. Numbers in parentheses indicate the number of rats and glomeruli studied in each group.

\textbf{FIGURE 3. RESPONSE TO DIFFERENT ONCOTIC GRADIENTS} \textbf{(A)}. Fluorescence dilution curves. Changes in the fluorescent intensity in FITC-Dextran 250 labelled glomeruli isolated from SD rats exposed to different oncotic gradients are presented. The expected fall in fluorescence based on the change in oncotic pressure is presented in red. \textbf{(B)}. Relationship be the measured change in fluorescence versus the percentage fall in oncotic pressure. The % change of fluorescence intensity in the isolated glomeruli is linearly related to...
the % change of oncotic pressure. Data were presented as mean ± SEM. Numbers in parentheses indicate the number of rats and glomeruli studied per group.

FIGURE 4. REVERSIBILITY OF THE WATER MOVEMENT

(A). Time course of changes in fluorescence in labeled glomeruli after changing the albumin concentration of the bath from 4 % to 6 % and then back to 4 % BSA (B). Time course of the changes in fluorescence of labeled glomeruli after changing the albumin concentration of the bath from 6 % to 4 % and then back to 6 % BSA. Data were presented as mean ± SEM. Numbers in parentheses indicate the number of rats and glomeruli studied per group. * indicates a significant difference (P<0.05) compared to the time 0 control point. # indicates a significant difference (P<0.05) compared to the switch in the oncotic pressure gradient.

FIGURE 5. EFFECT OF TEMPERATURE AND STORAGE TIME

(A). The change of fluorescence intensity in glomeruli isolated and stored at room temperature (RT) compared to glomeruli isolated on ice and studied at time 0 (control). (B). The change of fluorescence intensity in glomeruli isolated and stored on ice for various lengths of time. All glomeruli were isolated in isotonic HBSS solution with 6% BSA. The change in fluorescence intensity in an aliquot of the stored glomeruli in response to a fall in the albumin concentration of the bath from 6-4% was recorded every 15 minutes up to 90 minutes after glomeruli isolation. The expected change in fluorescence based on the fall in oncotic pressure of the bath is plotted in red. Data were presented as mean ± SEM. Numbers in parentheses indicate the number of SD rats and glomeruli studied per group. * indicates a significant difference (P<0.05) compared to the change in fluorescence of glomeruli isolated on ice and studied immediately after isolation at time 0.
FIGURE 6. RESPONSE TO PROTAMINE SULFATE AND TGF-β1

(A). Response to protamine sulfate and TGF-β1. Time course of changes in fluorescence in glomeruli isolated from control SD rats and treated with protamine sulfate (right panel) or TGF-β1 (left panel). (B). Effect of protamine sulfate (right panel) and TGF-β1 (left panel) on Palb of isolated glomeruli. All glomeruli were isolated and stored on ice in isotonic HBSS solution with 6% BSA. The glomeruli in the protamine sulfate experiments were studied at room temperature while the glomeruli in the TGF-β1 experiments were incubated with TGF-β1 or vehicle for 15 minutes at 37°C prior to study. Data were presented as mean ± SEM. Numbers in parentheses indicate the number of SD rats and glomeruli studied per group. * indicates a significant difference (P<0.05) compared to the corresponding value in the control glomeruli treated with vehicle.

FIGURE 7. RELATIONSHIP BETWEEN CHANGES IN σAlb AND PROTEINURIA IN HYPERTENSIVE AND DIABETIC STRAINS of RATS

(A). Changes in the fluorescence intensity of glomeruli isolated from SD, FHH, Dahl SS rats fed a LS and HS diet and a streptozotocin (STZ) treated type I diabetic Dahl SS rats and type II diabetic (T2DN) rats in response to a fall in the protein concentration of the bath albumin concentration from 6 to 4%. (B). Presents Palb calculated from the σAlb measure in the various strains of rats. (C). Comparison of proteinuria in the various strains of rats. Urinary protein excretion was compared in age matched SD, FHH, Dahl SS rats fed a low salt (LS) and high salt (HS) diet or STZ induced type I diabetic Dahl SS rats and T2DN rats. (D). Relationship between protein excretion and Palb in the various hypertensive and diabetic strains of rats. Urine protein excretion is positively correlated with Palb in different strains of rats. All glomeruli were isolated and stored on ice in isotonic HBSS solution with 6% BSA and were measured within 30
mins from isolation. Data were presented as mean ± SEM. Numbers in parentheses indicate the number of rats and glomeruli studied per group at each time period. * indicates a significant difference compared to SD control rats.
\[ \frac{dV}{dt} = -J_v = L_p A \sigma_{Alb} RT \left( \frac{q}{v} - c_0(t) \right) \quad (1) \]

water flux  \quad \text{hydraulic perm.}  \quad \text{reflection coef.}  \quad \text{oncotic gradient}

\[ \frac{dq}{dt} = \left[ \ln \frac{q}{v c_0} \right] (1-\sigma_{Alb}) J_v - k \left( \frac{q}{v} - c_0(t) \right) \quad (2) \]

solute flux  \quad \text{solute gradient}  \quad \text{solvent drag}  \quad \text{diff. permeability to alb.}
Figure 2C

![Graph showing relative intensity over time for different BSA concentrations](image2c.png)

Figure 3A

![Graph showing percent fluorescence over time for different BSA concentrations](image3a.png)
Figure 7D