Vascular endothelial growth factor increases the ultrafiltration coefficient in isolated intact Wistar rat glomeruli

Andrew H. J. Salmon, Christopher R. Neal, David O. Bates and Steven J. Harper

Microvascular Research Laboratories, Department of Physiology, School of Veterinary Sciences, University of Bristol, Southwell Street, Bristol BS2 8EJ, UK

Vascular endothelial growth factor (VEGF) is expressed by the podocytes of renal glomeruli, and has profound influences on systemic microvascular permeability and haemodynamics. We describe an extensive refinement of a model that permits evaluation of the ultrafiltration coefficient (L_pA) of isolated mammalian glomeruli, in the absence of circulating and haemodynamic influences, and tested the hypothesis that VEGF influences glomerular L_pA via an effect on endothelial cells. Glomeruli were isolated by sieving Wistar rat renal cortical tissue, and individually loaded onto a suction micropipette. Flowing perifusate containing 1% bovine serum albumin (BSA) was rapidly switched to an oncopressive perifusate containing 8% BSA, eliciting transglomerular fluid efflux. The rate of the resultant reduction in glomerular volume was used to calculate glomerular L_pA (1.07 ± 0.53 nl min^{-1} mmHg^{-1} (mean $\pm$ s.d.), $n = 51$), which compares favourably with those reported in the same rat strain using different techniques. A significant relationship between L_pA and initial glomerular volume (V_i) ($r = 0.72$, $n = 41$, $P < 0.0001$) necessitated correction of L_pA for V_i. The initial rate of change of glomerular volume, normalized for V_i, showed a strong positive correlation with applied oncotic gradient (Pearson $r = 0.59$, $n = 28$, $P < 0.001$), as predicted by Starling's law of filtration. A 60 min exposure of glomeruli to 1 nm VEGF increased glomerular L_pA/V_i (1.19 ± 0.19 ($n = 10$) to 2.23 ± 0.33 ($n = 9$) min^{-1} mmHg^{-1} (mean $\pm$ s.e.m.); $P < 0.02$). Time- and concentration-dependent relations between VEGF and L_pA/V_i were observed. The VEGF-induced elevation of L_pA/V_i was blocked by the selective VEGF-R2 inhibitor ZM323881. We suggest that glomerular VEGF contributes to the high physiological permeability of mammalian glomeruli to water through an action on endothelial cells.

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Corresponding author D. O. Bates: Microvascular Research Laboratories, Department of Physiology, School of Veterinary Sciences, University of Bristol, Southwell Street, Bristol BS2 8EJ, UK. Email: dave.bates@bristol.ac.uk

Vascular endothelial growth factor (VEGF) was originally described by its ability to increase macromolecular extravasation in the systemic circulation (Senger *et al.* 1983), hence the initial term 'vascular permeability factor'. Subsequent investigations have shown that VEGF increases systemic microvessel permeability to both water (Bates & Curry, 1996) and albumin (Wu *et al.* 1996) and alters vessel diameter and compliance (Bates, 1998). VEGF also plays a fundamental role in systemic endothelial cell maintenance and angiogenesis (Ferrara, 2004). VEGF and its associated receptors are strongly expressed in renal glomeruli, and are traditionally considered to exhibit a trans-basement membrane distribution. *In situ* hybridization reveals strong VEGF expression by podocytes (Bailey *et al.* 1999), and the cognate VEGF receptors VEGFR-1 and

VEGFR-2 are found on glomerular endothelial cells (Simon *et al.* 1995). However, recent work suggests that podocytes also express VEGF receptors (namely VEGFR-1 and neuropilin-1, but not VEGFR-2), and that this coexpression of VEGF and VEGF receptors by podocytes permits podocyte-mediated alterations in podocyte behaviour, such as Ca^{2+} homeostasis (Foster *et al.* 2003) and nephrin phosphorylation (Foster *et al.* 2005), representing a functional autocrine VEGF effect. The components of the VEGF axis are disturbed in a wide variety of glomerular pathologies associated with altered glomerular permeability (such as proteinuria) (Schrijvers *et al.* 2004), and VEGF appears to have an important role in glomerulogenesis (Kitamoto *et al.* 1997) and glomerular repair from injury in some animal models of glomerular

disease (e.g. Masuda *et al.* 2001). VEGF induces endothelial cell fenestrations *in vitro* (Esser *et al.* 1998*b*), binds molecules which form important components of the glomerular basement membrane, such as heparan sulphate (Soker *et al.* 1993), and induces phosphorylation of the podocyte slit-diaphragm protein nephrin in conditionally immortalized human podocytes *in vitro* (Foster *et al.* 2005). The anatomical arrangement of the VEGF axis, and the ability of VEGF to interact with the major components of the glomerular filtration barrier *in vitro*, have led to the suggestion that VEGF may contribute to the normal control of glomerular permeability (Schrijvers *et al.* 2004).

In a recent phase II study of metastatic colon cancer (Kabbinavar *et al.* 2003), a significant excess of patients treated with monoclonal antibody therapy to VEGF experienced proteinuria compared with those in the control group. Although the occurrence of proteinuria was not replicated in the phase III study (Hurwitz *et al.* 2004), the threshold value for proteinuria of 500 mg day^{-1} in the latter study significantly exceeds that considered pathological in the normal population (Johnson & Feehally, 2003). Furthermore, VEGF monoclonal antibody therapy abrogated glomerular hyperfiltration and albuminuria in diabetic rats, but did not affect these parameters in control rats (de Vriese *et al.* 2001). Application of VEGF to the noradrenaline-preconstricted renal vascular bed of isolated perfused rat kidneys led to an increase in renal perfusion flow rate. However, no change in glomerular filtration rate or glomerular permeability to albumin was noted (Klanke *et al.* 1998). The role of VEGF in the control of glomerular permeability therefore remains unclear (Schrijvers *et al.* 2004).

Traditional, but infrequently attempted, techniques for examining glomerular permeability at the single-nephron level include direct glomerular capillary micropuncture (Brenner *et al.* 1971) and tubular stop-flow studies (Gertz *et al.* 1966). The profound alterations in haemodynamics induced by VEGF (Bates & Harper, 2003) are likely to complicate the assessment of glomerular ultrafiltration coefficient (L_pA) using these techniques. The model developed by Savin and colleagues (Savin & Terreros, 1981) permits the study of glomerular filtration characteristics in isolation from circulating and haemodynamic influences. The volumetric response of an isolated glomerulus to a transglomerular oncotic gradient allows determination of L_pA. Values obtained using this technique (Savin & Terreros, 1981) have been noted to correspond well with L_pA values obtained using *in vivo* techniques (Maddox *et al.* 1992). Using this model, Savin and coworkers have shown that various mediators possess the ability to alter L_pA, including angiotensin II (Wiegmann *et al.* 1990). We have therefore refined the methods of Savin to permit examination of the influence of VEGF on glomerular L_pA.

Methods

Glomerular isolation and solutions

All experiments were performed on adult male Wistar rats (weight 257–346 g), and conformed to UK animal legislation. Animals were anaesthetized with 5% halothane (Merial, Essex, UK), and killed by cervical dislocation. Laparotomy and bilateral nephrectomy were performed immediately post mortem, and kidneys were placed in mammalian Ringer solution (mM: 115 NaCl, 5 KCl, 10 sodium acetate, 1.2 Na_2HPO_4, 25 $NaHCO_3$, 1.2 $MgSO_4$, 1 $CaCl_2$, 5.5 D(+)glucose (all from VWR, Leics, UK), bubbled with 95%O_2–5%CO_2 (BOC, Manchester, UK) until pH 7.40–7.45), containing 1% bovine serum albumin (BSA) (Sigma, MO, USA). The outer 1–2 mm of superficial renal cortex was dissected from both kidneys, and glomeruli were isolated using a standard sieving technique (Savin & Terreros, 1981). The concentration of residual plasma proteins within glomerular capillaries equilibrates with the surrounding 1% BSA during this time. The glomerular harvest retained by the 100 μm mesh was transferred to either control or test solution at 37°C.

Perifusate containing mammalian Ringer solution and varying concentrations of BSA was adjusted to pH 7.45 $\pm$ 0.02 with NaOH or HCl accordingly. In each experiment, one perifusate contained 1% BSA (generically termed dilute perifusate), and the other contained a higher concentration of BSA (concentration 8%, other than in experiments examining the relationship between the initial rate of fluid flux and the applied oncotic gradient, in which the concentration was between 4 and 10%, generically termed concentrated perifusate). Mammalian Ringer solution was re-bubbled with 95%O_2–5%CO_2 until pH 7.40–7.45, and BSA solutions were re-made for each rat studied. The oncotic pressure of each BSA solution was determined with a modified Hanson's osmometer, using a semi-permeable membrane with 10 kDa cut-off (Amicon, Lexington, MA, USA). There was no change in the oncotic pressure of the perifusate solutions during the course of each experiment.
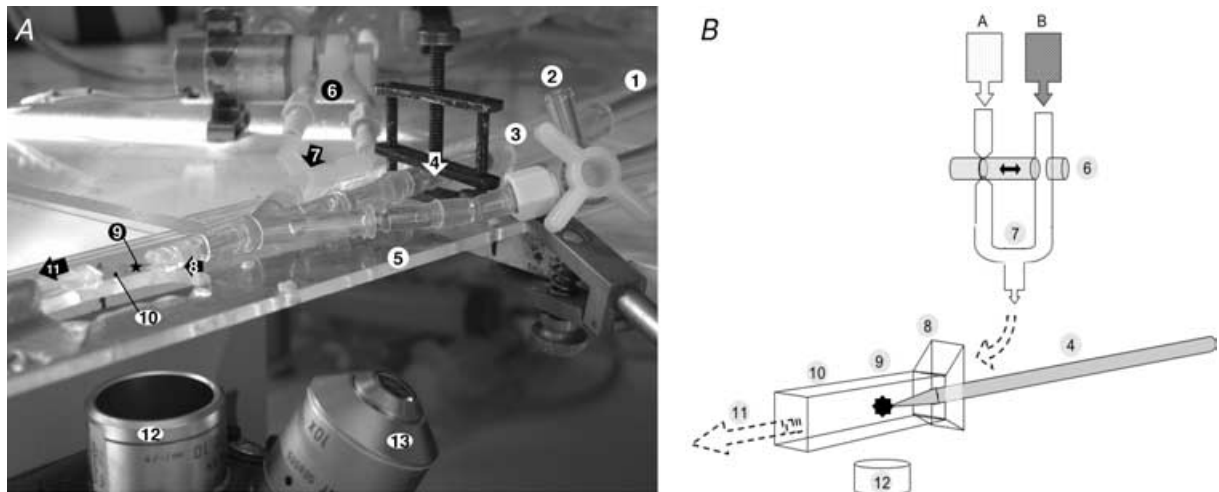
Apparatus

Micropipettes were pulled from glass capillary tubes (o.d. 1.2 mm; Clark Electromedical Instruments, Reading, UK), and the 15 μm aperture tip was placed within a flange-ended, rectangular cross-section glass microslide (i.d. 400 μm × 4 mm; Camlab, Cambridge, UK) above the ×10 objective of an inverted microscope (Leica DM IL HC Fluo) (Fig. 1). Magnification was further adjusted using a ×1/×1.5/×2 zoom set in the body of the microscope. A monochrome video camera (Hitachi KP-M3AP) was attached to the top of the microscope to permit binocular visualization and simultaneous recording of

images of individual glomeruli loaded into the system, and it was held by gentle suction on the micropipette tip. The video camera was connected through a digital timer (FOR.A VT33) to a videocassette recorder (Panasonic AG7350) and monochrome monitor (Sony SSM-125CE). Perifusate was held in one of two heated glass reservoirs (Radnoti, CA, USA), placed at a height of between 40 and 140 cm above the microslide. The reservoirs were connected to the microslide via a series of heat-insulated Tygon tubes (Fisher, UK), which passed through either channel of an either/or rapid-response remote tap (075P3; Bio-Chem Valve, Inc.) placed as close as possible to the micropipette tip. Further tubing connected the exit of the microslide to a series of interchangeable collection chambers: one to waste, and one chamber per perifusate. Perifusate was thence recycled to the initial heated reservoirs by means of two peristaltic pumps. A water bath and pump (Techne, Cambridge, UK) were used to circulate warmed fluid through a series of tubes and jackets designed to heat regulate the entire pre-microslide apparatus. The bath temperature and flow rate were adjusted to achieve a flowing perifusate temperature (at the locus of the glomerulus) of $37 \pm 0.5°C$ in all experiments. Flow rates (typically $1\,\mathrm{ml\,s^{-1}}$) for each perifusate were calculated and equalized before introduction of glomeruli. There was no significant relationship between L_pA/V_i values (L_pA values normalized for initial glomerular volume (V_i)) elicited with perifusate flow rates between 0.75 and $1.75\,\mathrm{ml\,s^{-1}}$,

and the perifusate flow rate applied ($r = -0.14$, $n = 51$, $P > 0.3$).

Glomerular volumetric change

Glomeruli that were free of Bowman's capsule and arteriolar or tubular fragments, and which were macroscopically intact, were chosen for study. Isolated glomeruli exhibited normal morphology, both at the light and electron microscope level (Fig. 2), in keeping with previous reports (Savin & Terreros, 1981). The mean $\pm$ S.E.M. volume of glomeruli harvested from the superficial cortex of rat kidneys (1.05 ± 0.04 nl, $n = 51$) was not significantly different from that reported in the literature (1.08 ± 0.06 nl, $n = 20$, $P > 0.7$, unpaired t test) (Pinnick & Savin, 1986). All glomerular observations were performed within 3 h of nephrectomy, as there was no significant decline in L_pA/V_i values obtained from glomeruli incubated at $37°C$ for up to 3 h post mortem ($r = -0.13$, $n = 41$, $P > 0.4$). After a period of equilibration in flowing 'dilute' perifusate (approximately 2 min), the perifusate was rapidly changed to 'concentrated BSA' perifusate. Perifusate exiting the microslide and entering the collection chambers was diverted to waste for 2 s prior to, and 8 s after, the switch of perifusate, before perifusate recycling was re-started. Perifusate switch was confirmed by the appearance of lines of altered refraction around the glomerulus (the


Figure 1. Glomerular oncometry apparatus
A, photograph of the apparatus (note that the insulating apparatus required for paired studies has been removed for clarity): 1, aspiration line; 2, three-way tap for injection of glomeruli; 3, distal tip of suction micropipette; 4, micropipette within holder, clamped onto mount for stability; 5, Perspex mount; 6, head of remote tap bearing two perifusate-containing lines, and permitting rapid perifusate exchange; 7, confluence of perifusate lines; 8, flanged end of rectangular glass microslide; 9, locus of isolated glomerulus; 10, body of microslide; 11, route of perifusate efflux; 12, ×4 objective; 13, ×10 objective. *B*, illustration of the apparatus, with the same reference numbers as for *A*. In addition: A, heated reservoir containing 'dilute' perifusate; B, heated reservoir containing 'concentrated' perifusate.

Schlieren phenomenon). Following perifusate exchange, erythrocytes began to move longitudinally within glomerular capillaries, providing a useful index of changes in glomerular volume elicited by trans-filtration barrier fluid movement between the perifusate and glomerular capillaries.

Analysis of glomerular volumetric change

Perifusate switches were recorded on videotape at 50 fields s^{-1}. Video sequences were reviewed (off-line) using an Apple Video Player (Apple, USA), and a sequence of still images, straddling the time point at which perifusate switch occurred, were created. The glomerular outline in each image was replicated using the magnetic lasso tool in
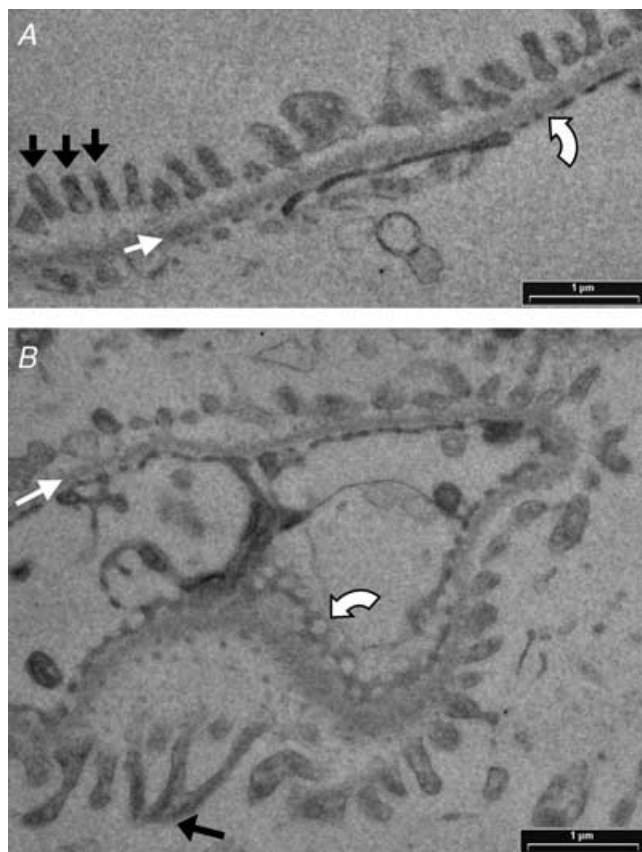
Figure 2. Transmission electron micrographs of isolated glomeruli
Glomeruli were isolated from the superficial renal cortex of Wistar rat kidneys, and processed for transmission electron microscopy (TEM). *A*, normal glomerular filtration barrier, displaying evenly distributed podocyte foot processes (black arrows), uniform glomerular basement membrane (white arrow) and endothelial fenestrae (curved white arrow). *B*, micrograph displaying an *en face* view of the glomerular filtration barrier. The uniform filtration barrier (white arrow) remains apparent. Fenestrae appear as circular discontinuities in the endothelium (curved white arrow). Branching of podocyte processes to form interdigitating foot processes (black arrow) is also apparent.

Adobe Photoshop 7.0 (Adobe Systems, Inc., CA, USA), and the area (A, μm^2) of the glomerular image calculated using Image J (US National Institutes of Health). Computer analysis of video images of volumetric changes of glomeruli enables measurement of the entire glomerular perimeter, rather than just four glomerular diameters at 45 deg intervals (Savin & Terreros, 1981). Glomerular volume (nl) was derived from these area measurements by substituting glomerular image area (A) into the formula

$$\text{Glomerular volume} = 4/3\pi r^3 \qquad (1)$$

(where r = glomerular radius) to reveal:

$$\text{Glomerular volume} = \left[4/3A(\sqrt{(A/\pi)})\right]/10^{-6} \quad (2)$$

Glomerular volume (nl) was plotted against time since first appearance of the Schlieren phenomenon (Fig. 3*A*). Two regression lines were then applied to these points (Fig. 3*B*). The slope of the first line was prescribed as zero, and this first line was applied to points covering the period before perifusate switch, when baseline glomerular volume was stable. The two lines were calculated to meet at their breakpoint. The breakpoint was determined iteratively, and not confined to the time of the video image at which the Schlieren phenomenon became evident. This defined the time point at which the glomerular volume starts to decline. The second line was applied to points covering a time period of at least 0.04 s from the breakpoint (i.e. at least three volume measurements), and points covering a time period of no more than 0.1 s (i.e. no more than six volume measurements). Within these confines, the points to which an applied regression line had the greatest slope were chosen. These time confines were applied to this line in order to minimize slope measurement error introduced by using too few time points, and in order that the slope should relate to the period of glomerular volume change before erythrocyte movement within glomerular capillaries began.

Calculation of L_pA

The slope of the second regression line describes the greatest initial rate of change of glomerular volume. Starling's law of filtration dictates that:

$$J_v/A = L_p[(P_c - P_i) - \sigma(\Pi_c - \Pi_i)] \qquad (3)$$

where J_v is the rate of fluid flux, L_p is the hydraulic conductivity, P_c is the capillary hydrostatic pressure, P_i is the interstitial hydrostatic pressure, σ is the reflection coefficient, Π_c is the capillary oncotic pressure, and Π_i is the interstitial oncotic pressure. Glomerular volume change indicates fluid efflux from the glomerulus driven by the oncotic pressure gradient applied by introduction of the 'concentrated' perifusate in the vicinity of a glomerulus, the capillary contents of which have

equilibrated with the initial 'dilute' perifusate. The second regression line, determined as outlined above, describes the greatest rate of fluid efflux from the glomerulus; this equates with the term J_v in Starling's equation. A net hydrostatic force ($P_c - P_i$) acting across the isolated glomerulus can be assumed to be negligible: the arteriolar remnants are open, there is an absence of flow within the glomerular capillaries (as indicated by the absence of movement of erythrocytes in the first 0.1 s after perifusate switch), and equilibration of the hydrostatic forces necessary to induce flow of each perifusate is rigorously ensured in each experiment. Previous work has demonstrated that σ of isolated glomeruli is not significantly different from 1 (Savin *et al.* 1992). One can therefore rearrange the Starling equation to show that:

$$L_p A = J_v / -\Delta\Pi \qquad (4)$$

(in nl min^{-1} mmHg^{-1}) (where $\Delta\Pi$ is the difference between capillary and interstitial oncotic pressure).

Determination of mean fractional filtration rate

As described above, a plot of glomerular volume against time was generated for each glomerulus studied. In addition to the calculation of $L_p A$, these plots were also used to determine the mean fractional filtration rate ($J_v(f)$) from populations of glomeruli exposed to the same experimental conditions (Fig. 4). For each glomerulus, volumes calculated from each video frame were normalized to the 'breakpoint' volume (i.e. the volume at the time of initiation of glomerular shrinkage; see above), and time zero was prescribed as the 'breakpoint' time. Hence the fold change in glomerular volume could be calculated for all glomeruli exposed to the same experimental conditions, and at all time points between 0.1 s prior to, and at least 0.1 s after, the onset of glomerular shrinkage (Fig. 4A). This information was used to generate a plot of fold change in glomerular volume *versus* time. To calculate the mean $J_v(f)$, a linear regression line was applied to the initial period of mean glomerular shrinkage (Fig. 4B). This is in contrast to the application of the initial slope of a single exponential curve fitted to the mean volumetric change (Savin, 1986). We have noted better indices of accordance (standard deviation of residuals) of glomerular volumetric change during this initial phase to linear (0.0017) rather than non-linear (0.0041) models. We also suggest that the use of linear modelling is more in keeping with the hypothesis that the rate of change of glomerular volume during this initial time period represents fluid efflux whilst capillary contents are conserved and luminal hydrostatic pressures remain negligible. Examination of control glomeruli revealed that application of the line to the first 0.08 s provided the most appropriately fitting line (as determined by a 'runs test')

Figure 3. Representative plots of glomerular volume change *versus* time since perifusate switch
A, volume change displayed by one glomerulus, in response to 'dilute to concentrated', then 'concentrated to dilute' switch of solutions. i–ii, glomerulus volume is stable when surrounded by dilute perifusate (1% BSA); ii, perifusate switch to concentrated BSA (8%) reduces glomerular volume by 10% (iii); glomerular volume then stabilizes (iv) (equilibration); v, glomerular swelling on re-introduction of 'dilute' perifusate (vi). B, fluid egress from isolated glomerulus (filtration): initial rate of reduction of glomerular volume when exposed to 8% BSA (oncotic gradient 29.2 mmHg). The slope of regression line is set at zero (a). The direction of fluid movement out of the glomerulus is in the same direction as glomerular filtration (slope of line b = −1.125 nl s^{-1}). C, fluid ingress into isolated glomerulus (reabsorption): application of a 9.8 mmHg oncotic gradient induces glomerular swelling (slope of line b = 0.240 nl s^{-1}).
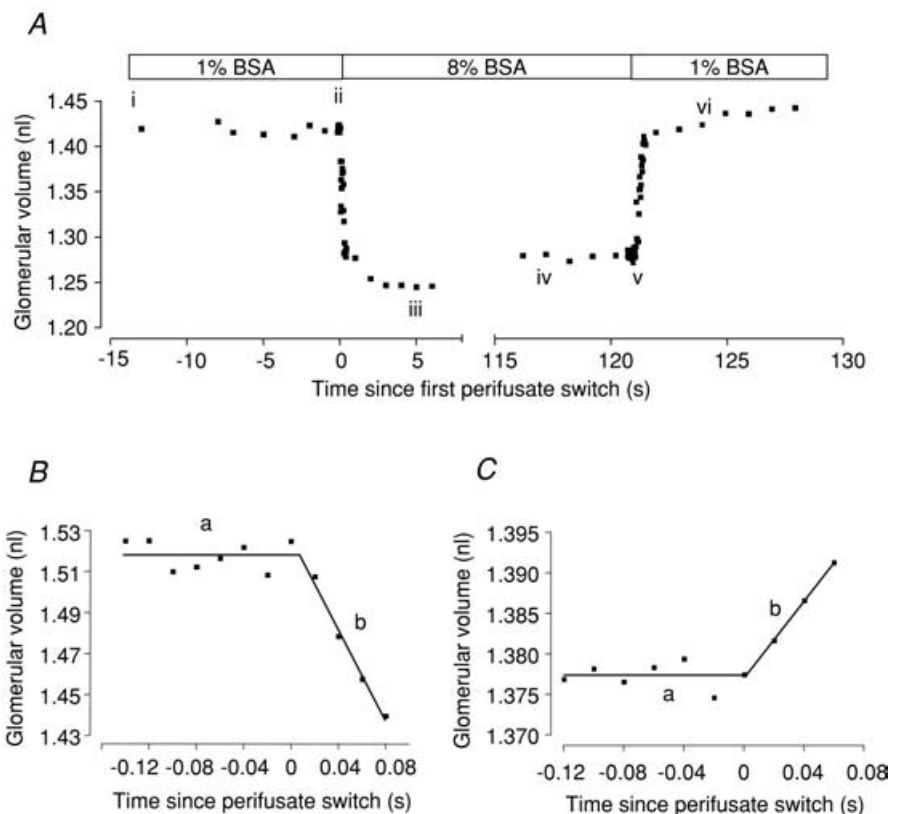
applied to the maximum number of volumetric points possible (thereby providing the best estimate of mean $J_v(f)$). The slopes of these lines can be used to compare mean fractional change in volume per unit time, or fractional rate of fluid efflux. This mean $J_v(f)$ is therefore used to compare populations of glomeruli exposed to different experimental conditions.

### Repetitive filtration (paired) experiments

The ability to compare sequential measurements of L_pA in the same glomerulus, rather than comparing different populations of glomeruli, has been postulated to permit evaluation of small mediator-induced changes in L_pA (Savin, 1986). Such repetitive evaluations also enable examination of L_pA values immediately following short periods of mediator incubation (up to approximately 15 min, given the time constraints of the experimental procedure). To this end, the apparatus and methodology were adjusted to permit repetitive evaluation of L_pA values obtained from one glomerulus.

The portion of the microslide containing the glomerulus was heat regulated with flow of fluid from the water bath through large-calibre (700 μm $\times$ 7 mm) rectangular cross-section glass microslides. The relative flow of fluid through these heat-regulating microslides, as compared with that through the remainder of the heat-regulating system, was adjusted to achieve a temperature of $36.5 \pm 0.5°C$ for static perifusate solutions in the immediate vicinity of the glomerulus, whilst maintaining the temperature of flowing perifusate, at the locus of the glomerulus, at $37 \pm 0.5°C$.

Paired glomerular oncometry studies have been reported (Savin, 1986) in glomeruli in which afferent and efferent arteriolar remnants were crimped in the holding micropipette. That procedure was employed in order that intracapillary plasma proteins, which are critical in the establishment of a transglomerular oncotic gradient, are not lost during the expansion in glomerular volume, which occurs when 'dilute' perifusate is applied to a glomerulus that has been equilibrated in 'concentrated' perifusate. We elected to adopt two experimental protocols in the evaluation of repetitive filtration studies in the same glomerulus to test the hypothesis that conservation of capillary contents is an essential element of repetitive filtration studies. These two protocols differed in the duration of time between the first 'dilute' to 'concentrated' switch and restoration of the initial 'dilute' perifusate. The mean $\pm$ s.e.m. interval between application of the 'concentrated' perifusate and restoration of the initial 'dilute' perifusate was 40.6 ± 4.4 s in the 'delayed replacement' protocol; this interval was 1.2 ± 0.1 s in the 'rapid replacement' protocol. Evaluation of glomerular volume immediately prior to each switch, erythrocyte movement, and ultrafiltration coefficient corrected for glomerular volume (L_pA/V_i) and mean $J_v(f)$, was made for each repetition of filtration using both of the protocols.

VEGF experiments

Recombinant human VEGF (rhVEGF165; R & D Systems, MN, USA) was used. PTK787/ZK222584 was obtained from Novartis Pharma AG, Basel, Switzerland, and
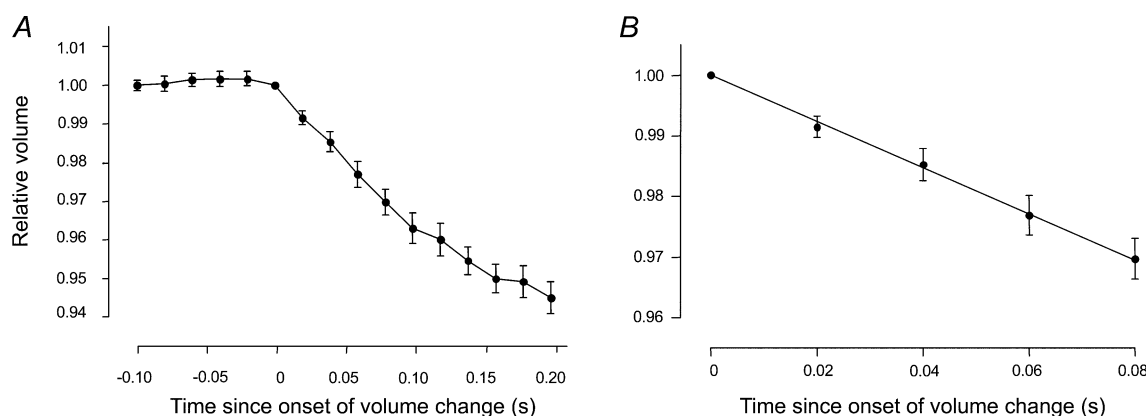
Figure 4. Calculation of mean fractional filtration rate ($J_v(f)$)
A, for each individual glomerulus, glomerular volume is normalized to that at the onset of the shrink phase of filtration, and time is normalized to the onset of the shrink phase of filtration. Relative glomerular volume can then be plotted against time for a population of glomeruli exposed to the same experimental conditions. *B*, a linear regression line is applied to the change in relative volume that occurs in the first 0.08 s after the onset of the shrink phase of glomerular filtration. The magnitude of the slope of this line (-0.38 ± 0.01 s^{-1}) describes the mean filtration rate for this representative control population ($n = 10$).

dissolved in *N*,*N*-dimethylformamide (Sigma, MO, USA) at a final concentration of 100 nM, which is a concentration previously shown to cause inhibition of VEGF-R1 and -R2 signalling (Whittles *et al.* 2002). ZM323881 was obtained from Calbiochem, CA, USA, and dissolved in *N*,*N*-dimethylformamide at a final concentration of 10 nM, which is a concentration previously shown to cause selective inhibition of VEGF-R2 signalling in systemic microvessels, thereby blocking VEGF-mediated increases in hydraulic conductivity in that model (Whittles *et al.* 2002).

Following isolation, glomeruli were incubated at 37°C in either 1% BSA solution, or 1% BSA solution containing test solutions, in unpaired experiments. Glomeruli from each solution were then individually loaded into the micro-slide observation chamber after the prescribed exposure period. In paired experiments, the 'delayed replacement' protocol was used. Flow was halted, and sufficient volume of VEGF-containing 'dilute' solution was injected into the microslide to ensure that the glomerulus was entirely bathed in VEGF-containing solution. Control paired studies performed at the same time employed BSA-containing 'dilute' solution for microslide injection. Subsequent 'dilute' to 'concentrated' perifusate switches were performed as soon as possible after re-initiation of flow (typically under 5 s). Blinding to the experimental condition was maintained from the time of solution creation until completion of regression line application.

Statistical analysis

The frequency distribution of L_pA/V_i values was not significantly different from the normal distribution (mean ± S.E.M. 1.02 ± 0.06 min^{-1} mmHg^{-1}; Kolmogorov-Smirnov distance 0.10, $n = 51$, $P > 0.1$). Two-sided parametric tests were therefore employed: Pearson's rank for examination of correlation (r), unpaired *t* test for unpaired experiments, paired *t* test for paired experiments, and repeated-measures ANOVA test for repeated paired observations. Unpaired *t* tests were used to compare mean $J_v(f)$. All experiments were designed with sufficient power to detect a twofold change in both L_pA and mean $J_v(f)$ (assuming $\alpha = 0.05$ and $1 - \beta = 0.8$), using the statistical package G-Power (http://www.psychologie.uni-trier.de//pub/gpowermac/gpower212.sit.hqx) (unpaired experiments) or the formulae described by Machin & Campbell (1987) (paired experiments). A *P* value of less than 0.05 was considered statistically significant. Results are reported as means ± S.E.M. unless otherwise stated.

In order to facilitate comparison of the results in this paper with those reported in the literature using Wistar rats, direct glomerular capillary micropuncture (Aukland *et al.* 1977) and stop-flow studies (Arendshorst

& Gottschalk, 1974) were reviewed. In order to calculate L_pA values from direct glomerular capillary micro-puncture reports, glomerular capillary pressure (P_{gc}) measurements (Aukland *et al.* 1977) have been combined with the most concordant results reported in the literature, predominantly comparing rat strain and volume status of the animal. Single-nephron glomerular filtration rate (SNGFR) values are from Table 3 of Davis *et al.* (1988), in which the loop of Henle inflow rate was within the 'normal range', i.e. 10–25 nl min^{-1} (Davis *et al.* 1988), and Π_a (afferent arteriolar oncotic pressure) is from Table 2 of the same paper. Π_e (efferent arteriolar oncotic pressure) has been calculated from Table 2 of the review by Maddox *et al.* (1992). $<\Pi_{gc}>$ (mean glomerular oncotic pressure) is calculated using the formula $<\Pi_{gc}> = (\Pi_a + \Pi_e)/2$, according to the convention of Ott *et al.* (1976). L_pA has been calculated from the quotient of SNGFR and $(P_{gc} - <\Pi_{gc}>)$.

Results

Validation of method

Control glomeruli. Mean ± S.E.M. L_pA values from control glomeruli were 1.07 ± 0.07 nl min^{-1} mmHg^{-1} ($n = 51$). Mean $J_v(f)$ was -0.41 ± 0.02 s^{-1} ($n = 51$). L_pA values demonstrated a significant correlation with initial glomerular volume ($r = 0.72$, $n = 41$, $P < 0.0001$, $L_pA = 1.23 - 0.33$ nl min^{-1} mmHg^{-1}, Fig. 5). Henceforth, L_pA values are corrected for initial glomerular volume (V_i): L_pA/V_i (min^{-1} mmHg^{-1}). The initial rate of change of glomerular volume ($\Delta V/\Delta t$), corrected for V_i (($\Delta V/\Delta t)/V_i$; s^{-1}), significantly and linearly increased
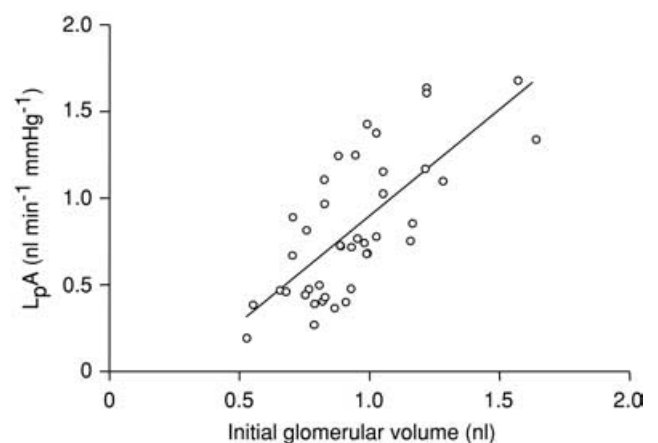
Figure 5. Relationship between L_pA and initial glomerular volume
The ultrafiltration coefficient (L_pA) was calculated for glomeruli of different sizes under control conditions. A strong positive linear correlation was observed between L_pA and initial glomerular volume (V_i) ($r = 0.72$, $n = 41$, $P < 0.0001$, regression line $L_pA = 1.23 - 0.33$ nl min^{-1} mmHg^{-1}).

Table 1. Effect of oncotic pressure on glomerular ultrafiltration rate

BSA concentration (mg ml^{-1})	Oncotic pressure difference (mmHg)	Rate of change of volume (($\Delta V/\Delta t)/V_i$) (s^{-1})	Number of glomeruli examined
40	9.8	0.26 ± 0.05	6
60	18.7	0.43 ± 0.09	4
80	29.2	0.53 ± 0.08	14
100	41.8	0.81 ± 0.09	4

with the oncotic pressure difference ($r = 0.59$, $n = 28$, $P < 0.001$, $(\Delta V/\Delta t)/V_i = 0.02\Delta\Pi + 0.1$ s^{-1}; Table 1 and Fig. 6).

**Repetitive filtration (paired) control studies.** Glomerular volume before the first repetition of filtration (0.95 ± 0.06 nl; $n = 16$) was slightly, but significantly greater than glomerular volume before the second repetition (0.90 ± 0.06; $n = 16$) ($P < 0.005$, paired t test) using the 'delayed replacement' protocol (Fig. 7A). In addition, the number of erythrocytes ejected on re-application of the initial 'dilute' perifusate using the 'delayed replacement' protocol was 42.4 ± 7.8 ($n = 5$), a figure significantly different from zero ($P < 0.01$, one-sample t test *versus* zero) (Fig. 7B). In contrast, using the 'rapid replacement' protocol, glomerular volume was unchanged prior to the first (0.97 ± 0.16; $n = 4$) and second (0.97 ± 0.16; $n = 4$) repetition of filtration ($P > 0.8$; paired t test) (Fig. 7A), and the number of erythrocytes ejected (0.2 ± 0.2; $n = 5$) was not significantly different from zero ($P > 0.35$, one-sample t test *versus* zero) (Fig. 7B).

L_pA/V_i, examined repetitively in the same glomerulus using the 'delayed replacement' protocol, was unaltered between the first (1.05 ± 0.08 min^{-1} mmHg^{-1}) and second (1.15 ± 0.07 min^{-1} mmHg^{-1}) repetition of

filtration ($P > 0.25$, paired t test; $n = 16$) (Fig. 7C). This was also true when the 'rapid replacement' protocol was employed (first L_pA/V_i 0.6 ± 0.09 min^{-1} mmHg^{-1}; second L_pA/V_i 0.71 ± 0.14 min^{-1} mmHg^{-1}; $P > 0.35$, paired t test; $n = 4$). However, there was a statistically significant 11% increase in the magnitude of mean $J_v(f)$ for the second repetition of filtration as compared with the first repetition (first $-0.42 ± 0.01$; second $-0.46 ± 0.02$; $n = 5$ pairs, $P < 0.05$, unpaired t test) using the 'delayed replacement' protocol (Fig. 7D), and a 37% elevation using the 'rapid replacement' protocol (first $-0.23 ± 0.01$; second $-0.31 ± 0.01$; $n = 5$ pairs, $P < 0.005$, unpaired t test). Therefore paired analysis of mean $J_v(f)$ can only determine agonist-induced increases in filtration rate greater than that induced by the experimental protocol alone, or reductions in filtration rate.

Effect of VEGF on ultrafiltration coefficient

Time course. VEGF has been shown to alter systemic microvascular hydraulic conductivity in a biphasic manner – acutely over a few seconds, and chronically over many minutes up to 24 h (Bates & Curry, 1996). To determine whether VEGF increased hydraulic permeability in glomeruli, glomerular filtration was examined after four different durations of VEGF exposure: 30 s, 15 min, 23 ± 1.8 min and 56 ± 5.4 min (Fig. 8). A 30 s exposure to VEGF did not increase L_pA/V_i (0.92 ± 0.09 to 1.04 ± 0.10 min^{-1} mmHg^{-1}; $n = 10$ pairs; $P > 0.35$, paired t test) (Fig. 8A). The 17% increase in mean $J_v(f)$ after exposure to VEGF for 30 s (from $-0.38 ± 0.005$ to 0.44 ± 0.01 s^{-1}; $n = 5$) (Fig. 8B) was no different from the 15% increase seen after exposure to control solution for 30 s (from $-0.38 ± 0.01$ to $-0.44 ± 0.02$ s^{-1}; $n = 5$). Similarly, 15 min exposure to VEGF did not increase L_pA/V_i (1.20 ± 0.22 to 1.10 ± 0.25 min^{-1} mmHg^{-1}; $n = 5$
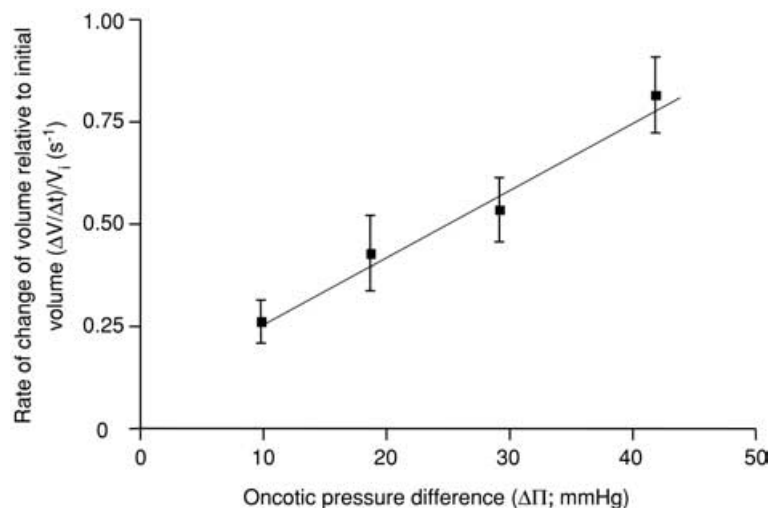
Figure 6. Relationship between initial rate of change of glomerular volume per unit initial glomerular volume and oncotic gradient applied
Populations of glomeruli were exposed to different oncotic gradients, by altering the BSA concentration of the 'concentrated' perifusate. As predicted by Starling's equation, the magnitude of the rate of change of initial glomerular volume per unit initial glomerular volume was linearly related to the magnitude of the oncotic gradient applied. (Linear regression $(\Delta V/\Delta t)/V_i = 0.02$ mmHg + 0.1 s^{-1}; $r = 0.59$, $n = 28$, $P < 0.001$).

pairs; $P > 0.75$, paired t test). There was also no difference in mean $J_v(f)$ after exposure to VEGF for 15 min (-0.48 ± 0.03 compared with $0.41 \pm 0.02 \, s^{-1}$; $n = 5$; $P > 0.1$, unpaired t test) or after exposure to control solution for 15 min (-0.50 ± 0.03 to $-0.52 \pm 0.02 \, s^{-1}$; $n = 5$; $P > 0.5$, unpaired t test).

$L_p A / V_i$ values after exposure to VEGF for 23 min ($1.14 \pm 0.29 \, min^{-1} \, mmHg^{-1}$; $n = 6$) were not significantly greater than control ($0.92 \pm 0.06 \, min^{-1} \, mmHg^{-1}$; $n = 21$; $P > 0.25$, unpaired t test); however, the mean $J_v(f)$ after 23 min VEGF exposure ($-0.44 \pm 0.02 \, s^{-1}$) was significantly greater than that observed after exposure to control solution ($-0.36 \pm 0.003 \, s^{-1}$, $n = 5$, $P < 0.005$, unpaired t test). When compared with controls, 56 min exposure to VEGF elicited a significant, approximately twofold, elevation in both $L_p A / V_i$ (1.19 ± 0.19 ($n = 10$) to 2.24 ± 0.33 ($n = 9$) $min^{-1} \, mmHg^{-1}$, $P < 0.02$, unpaired t test) and mean $J_v(f)$ (-0.47 ± 0 to $-0.88 \pm 0.03 \, s^{-1}$; $n = 5$, $P < 0.0001$, unpaired t test).

Concentration–response effect of VEGF. To determine whether a concentration–response effect of VEGF

on glomerular hydraulic permeability could be discerned, glomerular filtration was examined after 60 min exposure to four different concentrations of VEGF. Exposure to 10 PM VEGF and 100 PM VEGF elicited non-significant elevations in $L_p A / V_i$ and mean $J_v(f)$ when compared with control glomeruli (control glomeruli, $L_p A / V_i$ $0.85 \pm 0.05 \, min^{-1} \, mmHg^{-1}$, mean $J_v(f)$ $-0.32 \pm 0.01 \, s^{-1}$; 10 PM VEGF, $L_p A / V_i$ $0.92 \pm 0.08 \, min^{-1} \, mmHg^{-1}$, mean $J_v(f)$ $-0.42 \pm 0.03 \, s^{-1}$; 100 PM VEGF, $L_p A / V_i$ $1.10 \pm 0.09 \, min^{-1} \, mmHg^{-1}$, mean $J_v(f)$ $-0.46 \pm 0.02 \, s^{-1}$; all $P > 0.05$ versus control, one-way ANOVA, Bonferroni correction). Exposure to 1 NM VEGF and 3 NM VEGF elicited significant elevations in $L_p A / V_i$ and mean $J_v(f)$ when compared with control glomeruli (1 NM VEGF, $L_p A / V_i$ $1.67 \pm 0.23 \, min^{-1} \, mmHg^{-1}$, mean $J_v(f)$ $-0.66 \pm 0.04 \, s^{-1}$; 3 NM VEGF, $L_p A / V_i$ $1.74 \pm 0.17 \, min^{-1} \, mmHg^{-1}$, mean $J_v(f)$ $-0.66 \pm 0.07 \, s^{-1}$; all $P < 0.001$ versus control, one-way ANOVA, Bonferroni correction). A plot of $L_p A / V_i$ against the logarithm of the concentration of VEGF is shown in Fig. 9; this plot reveals an EC_{50} of VEGF on $L_p A / V_i$ of 296 PM. An EC_{50} of 299 PM is obtained when mean $J_v(f)$ is plotted against the logarithm of the concentration of VEGF.
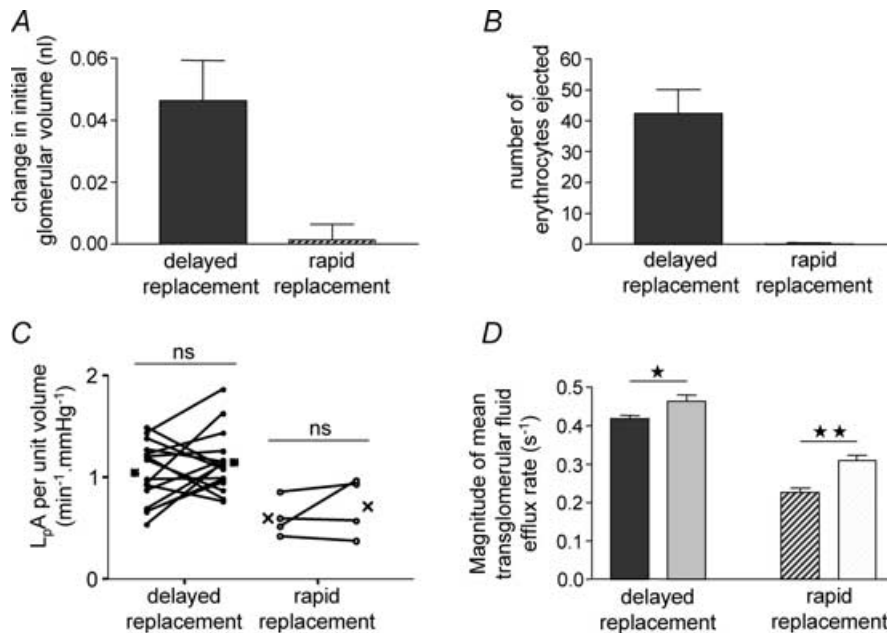
Figure 7. Comparison of 'delayed replacement' (40 s delay) and 'rapid replacement' (1.2 s delay) paired filtration study protocols

A, rapid replacement resulted in no significant change of initial glomerular volume ($P > 0.8$, one-sample t test versus 0), in contrast to delayed replacement (filled column). *B*, rapid replacement, in contrast to delayed replacement, resulted in no ejection of erythrocytes ($P > 0.35$, one-sample t test versus 0). *C*, there was no significant change in $L_p A$ per unit volume ($L_p A / V_i$, $min^{-1} \, mmHg^{-1}$) between the first and second filtrations using both the 'delayed replacement' and 'rapid replacement' protocols. *D*, there was a significant change in the mean rate of transglomerular fluid efflux between the first (dark filled column) and second (light filled column) repetition of filtration in populations of glomeruli subjected to both the 'delayed replacement' (*$P < 0.05$, unpaired t test, $n = 5$) and 'rapid replacement' (**$P < 0.005$, unpaired t test, $n = 5$) protocols.

A

B
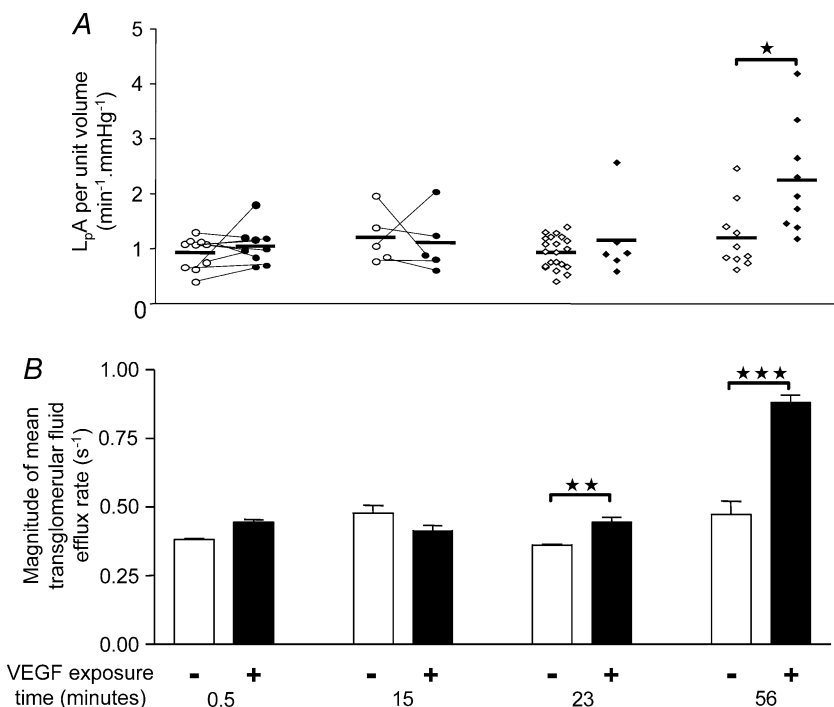
Figure 8. Effect of VEGF on glomerular hydraulic permeability

Glomeruli were exposed to VEGF (1 nM) (filled symbols/bars) or control solution (open symbols/bars) for between 0.5 and 56 min. *A*, effect of VEGF on L_pA/V_i. Circles connected by lines represent L_pA/V_i values from the same glomerulus before and after exposure to VEGF. Diamond-shaped symbols represent single L_pA/V_i measurements in unpaired studies. Horizontal bars represent the mean of each group. VEGF exposure for 56 min elicited an approximate doubling of L_pA/V_i, *$P < 0.02$, unpaired *t* test. *B*, bars represent mean ± S.E.M. transglomerular fluid efflux rates ($J_v(f)$) from populations of glomeruli exposed to control or VEGF (1 nM) solution. VEGF exposure for 30 s or 15 min increased mean $J_v(f)$ by no more than that seen in control-exposed glomeruli. VEGF exposure for 23 min (**$P < 0.005$, unpaired *t* test) and 56 min (***$P < 0.0001$, unpaired *t* test) elicited a significant increase in mean $J_v(f)$.

Blockade of VEGF effect with selective receptor inhibitors. In an attempt to discern the VEGF receptor through which the VEGF-induced elevation of L_pA/V_i is mediated, we co-incubated glomeruli for 60 min with 1 nM VEGF with either of the VEGF receptor inhibitors PTK787/ZK222584 (at 100 nM, a non-selective inhibitor of VEGF receptors R1 and R2) or ZM323881 (at 10 nM, a selective inhibitor of VEGF-R2) (Fig. 10). Application of 1 nM VEGF for 60 min again approximately doubled L_pA/V_i as compared with control (control, 0.85 ± 0.05 min^{-1} mmHg^{-1}, $n = 41$; VEGF, 1.67 ± 0.23 min^{-1} mmHg^{-1}, $n = 16$; one-way ANOVA with Bonferroni correction $P < 0.001$).

This effect was eliminated by co-application of the pan-VEGF receptor inhibitor 100 nM PTK787/ZK222584 (0.92 ± 0.08 min^{-1} mmHg^{-1}, $n = 20$; one-way ANOVA with Bonferroni correction $P < 0.001$ *versus* VEGF alone, $P > 0.05$ *versus* control), and by co-application of the VEGF-R2 inhibitor, 10 nM ZM323881 (0.76 ± 0.07 min^{-1} mmHg^{-1}, $n = 24$; one-way ANOVA with Bonferroni correction $P < 0.001$ *versus* VEGF alone, $P > 0.05$ *versus* control).
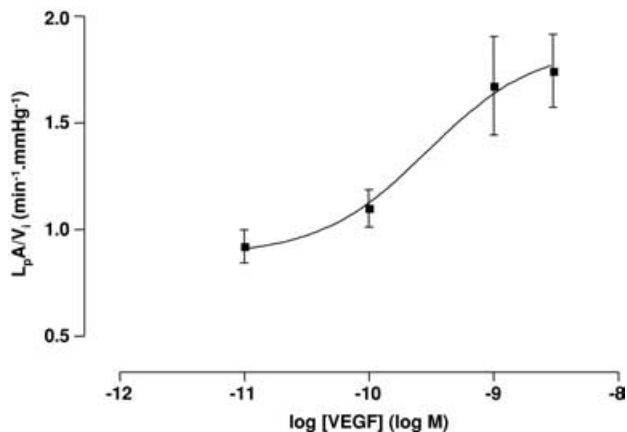
Figure 9. Dose-related elevation of L_pA/V_i by VEGF

Populations of glomeruli were exposed to different conentrations of VEGF for 60 min. Concentrations of VEGF between 10 pM and 3 nM elicited dose-dependent elevations of L_pA/V_i, revealing an EC$_{50}$ of 295 pM.
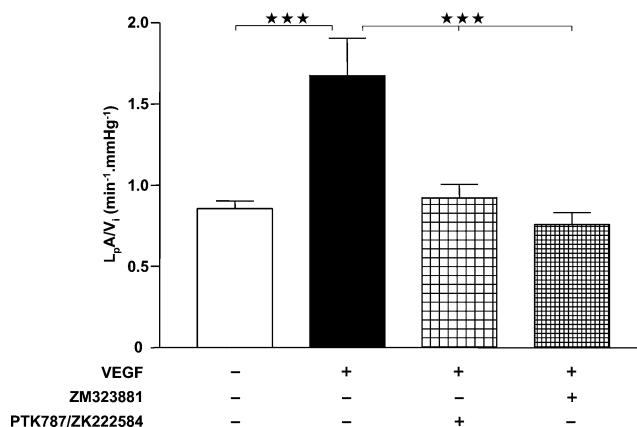
Figure 10. Blockade of VEGF-induced elevation of L_pA/V_i by VEGF-R2 inhibition

Populations of glomeruli were exposed for 60 min to control solution (open bar), 1 nM VEGF alone (filled bar), 1 nM VEGF plus the pan-VEGF receptor inhibitor (100 nM PTK787/ZK222584) (loosely cross-hatched bar), or 1 nM VEGF plus the selective VEGF-R2 inhibitor (10 nM ZM323881) (tightly cross-hatched bar). ★★★$P < 0.001$, one-way ANOVA with Bonferroni correction.

Table 2. Summary of the major differences between the work of Savin *et al.* and the current study

	Savin *et al.*[*]	Current study
Apparatus	Room temperature, open bath	37°C, closed system
Application of oncotic gradient	Gravity-fed replacement of static solution at uncontrolled rate	Controlled, continuous flow; rapid/reproducible switch
Direction of transglomerular fluid flux	Into glomerulus – non-physiological	Leaving glomerulus – physiological
Repetitive filtration studies	Crimped arterioles (rarely published)	Double perifusate switch
Estimation of glomerular volume	Four equally spaced glomerular diameters	Glomerular perimeter
Estimation of initial rate of change of glomerular volume	Greatest difference in glomerular volume occurring in 0.017 s	Actual rate of change in glomerular volume occurring in 0.04–0.1 s
Units of measurement	L_pA	L_pA/V_i, mean $J_v(f)$

[*]Savin & Terreros (1981); Pinnick & Savin (1986).

Discussion

Here we describe an extensive refinement of a very powerful, yet not widely used, method for measurement of glomerular ultrafiltration coefficient. There are very few techniques with which it is possible to study the filtration characteristics of renal glomeruli at the single-nephron level. The use of an oncometric technique in isolated glomeruli offers the possibility of examining the filtration characteristics of glomeruli, independent of circulating and haemodynamic influences, from a wide variety of species, including man (Savin & Terreros, 1981). We have refined the model of Savin and coworkers in an attempt to improve approximation with the *in vivo* situation, and to address some of the potential sources of error in the model. The major differences between the two models are summarized in Table 2.

Limitations and advantages of this refinement of the Savin oncometric method for measurement of ultrafiltration coefficient

Experimental methods. Fluid egress from glomeruli has been studied here, rather than fluid ingress as in Savin's method (Savin & Terreros, 1981), as the former is the direction of fluid movement in the production of the primary glomerular ultrafiltrate *in vivo*. The similarity of L_pA values for fluid movement in either direction under control conditions (Savin & Terreros, 1981) does not necessarily imply that both will be altered in the same way by mediators that affect L_pA. Thermoregulation of the apparatus permits evaluation of L_pA in glomeruli incubated and examined at 37°C. Tissue compliance, fluid viscosity and the cellular effects of mediators are all more likely to be in keeping with the *in vivo* situation at this temperature. Pre-determination of the flow rates of both perifusate solutions optimizes the repeatability of the characteristics of new perifusate introduction. In addition, the use of two flowing perifusate solutions with equal flow rates eliminates the potential influence of shear rate introduced by injecting new perifusate in the

vicinity of the isolated glomerulus at an indeterminable and non-reproducible rate (Savin & Terreros, 1981). The only force driving transglomerular fluid flux in this model will be the oncotic pressure difference between the two perifusate solutions.

Analysis methods. The two methods for determination of L_pA also differ considerably in the manner of estimation of the greatest initial rate of change in glomerular volume. We have elected to apply a regression line to glomerular volume measurements from at least three, and no more than six, time points. This substantially reduces the calculated slope when compared to the use of the greatest difference between two points within the same time frame, as employed by Savin *et al.* (see Fig. 11) (Savin & Terreros, 1981). Nevertheless, the use of multiple time points reduces error attributable to single-point variation. We have also chosen to limit the period of examination to the first 0.1 s after introduction of an oncotic gradient, as we have replicated the finding of Savin *et al.* that this is the earliest time at which erythrocytes are observed to move within capillaries. It is important to limit the period of examination to that prior to longitudinal movement of intracapillary contents, since this is the only period of the examination during which the capillary contents can be assumed not to have been affected by changes induced by fluid movement across the open ends of the afferent and efferent arterioles.

Determination of the mean $J_v(f)$ for populations of glomeruli provides an alternative index of mean glomerular permeability to water. We have elected to examine fold changes in glomerular volume. This is in contrast to the use of absolute changes in glomerular volume employed in the only previous report of population mean glomerular volumetric change (Savin, 1986). We believe the former to be more appropriate given the strong positive correlation between L_pA and V_i.

The coefficient of variation for L_pA values derived from 10 repetitive evaluations of the shrink phase of two glomeruli were 21.2 and 16.1% (mean 18.7%). This is similar but lower than the coefficient of variation for
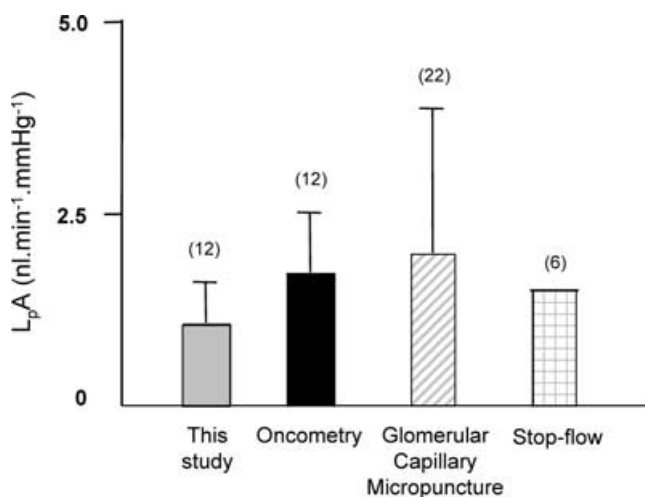
L_pA obtained by Savin & Terreros (1981) (24.86%, $n = 17$ rats). The finding that the coefficient of variation for L_pA measurements is in the order of 20% identifies this as the lower limit of resolution of this technique. The coefficient of variation for mean $J_v(f)$ for five independent control populations was 12.7%. This lower coefficient of variation for mean $J_v(f)$ (as compared with those for L_pA or L_pA/V_i) suggests that more subtle changes in glomerular hydraulic permeability characteristics can be discerned with this analysis method.

Other possible confounding errors in the system. Assumptions inherent to any oncometric method have been extensively discussed elsewhere in the literature (Savin & Terreros, 1981). However, one principal assumption is that the reflection coefficient (σ) 'is not significantly different from one' (Savin *et al.* 1992). Any change in σ, particularly that induced by mediators such as VEGF, would change the applied oncotic pressure gradient. Future studies regarding the potential influence of VEGF on σ will address this assumption.

The characteristics of the perifusate switch will have a significant influence on the extent to which the glomerulus is exposed to the intended oncotic gradient. It is likely that less than 100% of the glomerular filtration barrier is exposed to the oncotic gradient immediately. The L_pA/V_i value obtained using this technique may therefore not include a filtration contribution from the deepest portions of the glomerulus, leading to further underestimation of the true L_pA. However, recent studies have shown that it is unlikely that all parts of the glomerulus contribute equally to the filtration of plasma across the basement membrane, due to the three-dimensional relationship between the glomerular filtration barrier and the position of surrounding podocyte cell bodies (Neal *et al.* 2005).

Single glomerulus repetitive filtration studies. In one previous report (Savin, 1986), paired glomerular experiments have been possible, but only in glomeruli in which afferent and efferent arteriolar fragments have been crimped in the holding micropipette. We have employed a different strategy to ensure that capillary contents are conserved between repetitions of filtration. The replacement of the initial 'dilute' perifusate very shortly after application of the initial 'dilute' to 'concentrated' switch ('rapid replacement' protocol) provides sufficient video frames for the analysis of the initial phase of reduction in glomerular volume, whilst still permitting conservation of capillary contents during the subsequent increase in glomerular volume (as evinced by the absence of change in glomerular volume, and absence of capillary content expulsion). Our apparatus permits this protocol because the use of two flowing perifusate solutions ensures complete solution exchange around the glomerulus, thereby allowing repetitive application of a new perifusate of known oncotic composition under identical repetitive conditions. We concur with Savin in finding that ultrafiltration coefficient values do not alter between repetitions of filtration in the same glomerulus (Savin, 1986). However, this is true whether a protocol that does ('rapid replacement') or does not ('delayed replacement') conserve capillary contents is employed. More striking is the finding that a non-significant, mean 19%, elevation in L_pA/V_i is accompanied by a significant, mean 16%, elevation in mean $J_v(f)$ in consecutive repetitions of filtration, suggesting that this more discriminatory test is able to discern subtle changes in glomerular hydraulic permeability which occur as a consequence of repetitive filtration studies.

Comparison of results obtained using this modification with those in the literature

This modification of the Savin method results in measurements of ultrafiltration coefficient that conform to two principal requirements that indicate a relatively high degree of confidence in the results. The first is that Fig. 6 demonstrates that the initial rate of change of glomerular volume per unit volume conforms to Starling's prediction of filtration, being linearly dependent on the applied pressure difference. This is only likely to be the case if the glomerular swelling rate is proportional to

Figure 11. Comparison of ultrafiltration coefficient (L_pA) values obtained in the current study with those reported in the literature for Wistar rats

L_pA values from this report (grey bar) have been compared with the results of this paper, but analysed according to Savin's 'greatest difference' method (black bar; see Discussion; Savin & Terreros, 1981), with results from direct glomerular micropuncture studies (hatched bar; see Methods; Aukland *et al.* 1977), and with results from stop-flow studies (Arendshorst & Gottschalk, 1974). Numbers in parentheses represent number of animals studied. Error bars represent standard deviation (where reported). L_pA values were similar to those published in the literature.

the ultrafiltration coefficient. Secondly, we report L_pA values that are broadly comparable with those derived using the same technique and with those derived using other techniques in this strain of rats (Fig. 11).

Influence of VEGF on glomerular ultrafiltration coefficient

Comparison with previous reports. This is the first report to suggest that VEGF may influence glomerular ultrafiltration characteristics from normal animals. Previous reports using isolated kidney preparations have demonstrated no effect of VEGF on surrogate markers of glomerular permeability to water (Klanke *et al.* 1998). It is notable that the concentration of VEGF used in that study (6.6 ng ml^{-1}; 165 pM) is a concentration similar to that which revealed no significant change in L_pA/V_i in the current study (100 pM). Unfortunately concentration–response information in that system was not possible because of limited availability of VEGF. In addition, the use of whole-kidney glomerular filtration rate as the primary outcome measure of glomerular permeability to water would have added further difficulties when attempting to discern any VEGF-induced elevation in L_pA in that study. Not only does this term encompass a number of variables, such as the number of glomeruli recruited and the net ultrafiltration pressure in addition to the glomerular ultrafiltration coefficient, but most significantly it has been noted that, in many circumstances, 'increases in K_f [L_pA] above normal will have no effect on single-nephron glomerular filtration rate' (Maddox *et al.* 1992). Similar comments also apply to other investigations of the role of VEGF on glomerular permeability in whole animals (de Vriese *et al.* 2001). Whilst the absence of transcapillary hydrostatic pressure in our system undoubtedly misrepresents the *in vivo* situation, and therefore does not incorporate any alterations in ultrafiltration coefficient that may be brought about by VEGF-induced haemodynamic alterations *in vivo*, this model does permit selective examination of the role of mediators on glomerular ultrafiltration coefficient without masking or alteration by haemodynamic or circulating influences. As discussed above, this is both a strength and limitation of this model.

Time course of VEGF effect. Exposure of systemic microvessels to VEGF for 30 s elicits a median elevation of L_p by eightfold, followed by a secondary increase in L_p that is present 24 h after 10 min exposure to VEGF (Bates & Curry, 1996). This secondary increase in L_p begins to appear about 1 h after initial VEGF exposure (D. O. Bates, unpublished observations), as is seen in culture (Cohen *et al.* 1999). The absence of a VEGF-induced elevation of glomerular L_pA/V_i when exposure times are at or below 15 min may reflect differences between the two micro-

vascular beds. For instance, the constitutive fenestration of glomerular endothelial cells, and the 50-fold higher resting permeability of glomerular, as compared with systemic, capillaries (Deen *et al.* 1973) may mean that very brief exposure to VEGF elicits no further elevation in the hydraulic permeability of glomerular capillaries. The time course of our results are more consistent with those seen in other glomerular permeability examinations, such as the reduction in transendothelial electrical resistance (a marker of glomerular permeability to water and small solutes) of monolayers of human glomerular endothelial cells in response to VEGF, which occurs over 30–60 min (Chen *et al.* 2002; Satchell *et al.* 2004), and the similar time course of increases in the number of vesicular organelles in VEGF-treated immortalized rat glomerular endothelial cells (Chen *et al.* 2002). The fact that L_pA/V_i values of control glomeruli do not alter for three hours after glomerular isolation suggests that the elevation of L_pA/V_i values induced by longer (>20 min) exposure to VEGF is not mediated by a survival-related permeability effect on glomerular cells. In addition, given the observed time course, it is unlikely that the alterations in endothelial cell behaviour which are important in angiogenesis (e.g. proliferation; Ferrara, 2004) will be involved in this system. The signalling pathways involved in the acute VEGF-induced alterations in permeability are still undergoing elucidation, but appear to involve phospholipase C, diacylglycerol, store-independent elevations in intracellular Ca^{2+}, nitric oxide and cyclic GMP (Bates & Harper, 2003). Increased endothelial nitric oxide synthase (eNOS) phosphorylation and consequently elevated nitric oxide levels have recently been demonstrated to be induced by 30 min exposure of isolated glomeruli to 100 ng ml^{-1} VEGF (Wang *et al.* 2004), and this may represent an important pathway for VEGF-mediated alterations in glomerular permeability to water.

Concentration-dependent effect of VEGF. In this study, we report that the VEGF-induced alterations in glomerular permeability are concentration dependent. This is in keeping with the findings of Eremina and colleagues, who have elegantly demonstrated a VEGF dose-sensitivity of the glomerulus with regard to glomerular development and pathology using podocyte-specific VEGF genetic manipulation studies (Eremina *et al.* 2003), and also in keeping with the concentration-dependent increase in eNOS expression following 24 h exposure of isolated glomeruli to VEGF (Wang *et al.* 2004).

VEGF, VEGF-R2 and glomerular endothelium. We have demonstrated that the VEGF-mediated elevations in glomerular permeability to water are mediated via VEGF-R2, which is in keeping with the majority of actions of VEGF (Bernatchez *et al.* 1999), including VEGF-induced elevations in systemic microvessel

permeability to water (Whittles *et al.* 2002). A role for VEGF-R2-mediated signalling in glomerular pathology is suggested by the repeated demonstration of alterations in VEGF-R2 levels in animal models (Horita *et al.* 1998; Cooper *et al.* 1999; Kanellis *et al.* 2004) and human disease (Thomas *et al.* 2000; Bortoloso *et al.* 2004), but direct examination of a functional effect of VEGF-R2 on the glomerular filtration barrier has not previously been demonstrated.

In addition, a number of reports from different laboratories have repeatedly demonstrated the selective location of VEGF-R2 on glomerular endothelial cells (Simon *et al.* 1995; Cooper *et al.* 1999; Kitamoto *et al.* 2001; Foster *et al.* 2003). This identifies glomerular endothelial cells as the location (within the tri-layered glomerular filtration barrier) for the VEGF-induced alteration in permeability seen in this system. VEGF alters the trans-endothelial electrical resistance, a marker of permeability to water and small solutes, of monolayers of glomerular endothelial cells *in vitro* (Satchell *et al.* 2004). Genetic alteration of VEGF expression in podocytes leads to ultrastructural alterations in glomerular endothelial cells (Eremina *et al.* 2003). Renal lesions in pre-eclampsia are almost entirely confined to the glomerular endo-thelium, and are associated with elevated levels of the VEGF-antagonist soluble fms-like tyrosine kinase 1 (sFlt1) (Karumanchi *et al.* 2005), and administration of this protein to pregnant rats recapitulates the glomerular lesions of pre-eclampsia (Maynard *et al.* 2003). It is noteworthy that pre-eclampsia is associated with a reduction in glomerular filtration rate (Karumanchi *et al.* 2005). These studies provide supporting evidence for the hypothesis that VEGF may alter glomerular permeability to water via an action on glomerular endothelial cells.

VEGF causes the appearance of vesiculo-vacuolar organelles in glomerular endothelial cells in culture (Chen *et al.* 2002), with a concurrent increase in surrogate markers of monolayer permeability to water. VEGF can elicit the appearance of fenestrations in endothelial cells derived from non-fenestrated microvascular beds *in vitro* (Esser *et al.* 1998*b*) and *in vivo* (Roberts & Palade, 1995), but whether antagonism of VEGF alters glomerular endo-thelial fenestration remains debated (Gerber *et al.* 1999; Ostendorf *et al.* 1999). VEGF has also been shown to precipitate the formation of transcellular gaps (Michel & Neal, 1999) and induce alterations in adherens junction proteins, an effect likely to be mediated via VEGF-R2 (Esser *et al.* 1998*a*). Mathematical models predict a 'negligible' contribution of endothelial cells to overall glomerular hydraulic resistance (Drumond & Deen, 1994), unless endothelial cell fenestrae are modelled as containing gel-like glycocalyx, for which improvements in fixation technique are providing increasing evidence (Rostgaard & Qvortrup, 1997; Hjalmarsson *et al.* 2004). Under these circumstances, models suggest that the endothelial

cell layer may contribute up to 24% of the overall glomerular hydraulic resistance (Drumond & Deen, 1994). The significant endothelial-cell-mediated, VEGF-induced elevation in L_pA/V_i observed in this model therefore supports the glycocalyx-filled fenestral model postulated by Drumond & Deen (1994). Rapid VEGF-induced changes in glycocalyx have previously been postulated (Fu & Shen, 2003), and alterations in endothelial-cell glycocalyx have been shown to effect a 2.5-fold change in hydraulic conductivity in other microvascular beds (Adamson, 1990) and postulated to alter glomerular permeability (Ciarimboli *et al.* 2003). Alternatively, VEGF may modulate an alternative characteristic of endothelial fenestrae (e.g. fenestral density) (Gerber *et al.* 1999), thus eliciting an alteration in normalized ultrafiltration coefficient. The overwhelming contribution of endothelial fenestrae to the hydraulic permeability of the glomerular endothelial cell layer makes it unlikely that changes in intercellular junctions would be of sufficient magnitude to double the overall ultrafiltration coefficient of the filtration barrier.

A doubling of the hydraulic conductivity of the glomerular filtration barrier, due solely to an increase in endothelial-mediated VEGF-R2 signalling, would result in a doubling of protein clearance purely by convective means, independently of GFR changes, reflection coefficient reductions (which cannot be ruled out in these experiments), or alterations in slit diaphragm permeability. Therefore these results suggest that changes in glomerular endothelial permeability to water could contribute significantly to proteinuria when VEGF concentrations are raised. Future investigations into the effects of VEGF on glomerular ultrastructure and signalling pathways should yield further insights into the contributions of VEGF and endothelial cells to the regulation of glomerular permeability.

In summary, we report refinement of a model that permits examination of the permeability characteristics of glomeruli from a wide variety of animals, including humans. L_pA values obtained in the present study compare favourably with those observed using a variety of other *in vivo* techniques. Whilst a plethora of information supports the hypothesis that VEGF in the glomerulus promotes end-othelial cell proliferation and maintenance (Schrijvers *et al.* 2004), and the increase in prevalence of proteinuria seen in patients treated with anti-VEGF monoclonal antibody (bevacizumab) for disseminated colorectal carcinoma (Kabbinavar *et al.* 2003), has already highlighted the possibility that interference with the VEGF axis may perturb glomerular selective-permeability characteristics, this is the first report to confirm the speculation that VEGF may regulate glomerular permeability to water (Schrijvers *et al.* 2004). Our observations that this effect is mediated via VEGF-R2, selectively expressed by glomerular endothelial cells, highlights important issues regarding contributions

of the individual components of the glomerular filtration barrier to water permeability.

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