Acute laminar shear stress reversibly increases human glomerular endothelial cell permeability via activation of endothelial nitric oxide synthase

Heather S. Bevan, Sadie C. Slater, Hayley Clarke, Paul A. Cahill, Peter W. Mathieson, Gavin I. Welsh, and Simon C. Satchell

Academic Renal Unit, University of Bristol, Southmead Hospital, Bristol, United Kingdom; and Vascular Health Research Centre, Faculty of Science and Health, Dublin City University, Dublin, Ireland

Submitted 9 August 2010; accepted in final form 15 July 2011

Laminar shear stress is a principal regulator of systemic endothelial cell gene expression, morphology, and the production of soluble mediators including nitric oxide (NO) (9, 21, 28) and hence is a key determinant of vascular health (9, 42). Its importance is highlighted by pathological processes associated with reduced or absent laminar shear stress, including atherosclerosis (5, 7, 31). The pattern of shear stress is important: prolonged (>24 h) exposure to laminar shear stress (but not turbulent or oscillatory shear) results in anti-inflammatory and vasoprotective effects (5, 7). Acute shear stress (<24 h) results in transient activation of signaling intermediaries including the endothelial nitric oxide synthase (eNOS)/NO pathway and is likely to be of physiological importance in vascular remodeling, reaction to injury, regulation of blood flow, and endothelial permeability (5).

Various studies have examined the effect of laminar shear stress on permeability of systemic endothelia (31, 41). In general, in vitro work shows an increase in hydraulic conductivity (23, 39) and a corresponding decrease in transendothelial electrical resistance (TEER) (10), but effects on macromolecular permeability, studied in different endothelia, have been conflicting (8, 23). Such results emphasize that there are important functional differences in endothelia from diverse vascular beds (1). In vivo, the situation is also complicated, with sites of elevated permeability present at both regions of low and high shear stress, suggesting that each condition may affect permeability via different mechanisms (31).

NO has wide-ranging effects on EnC in vitro and in vivo, depending on the species and the vascular bed (5). Altered bioavailability of NO modulates EnC barrier function both in normal physiological and in pathology (20, 37). In vitro, a threshold effect of NO can be observed; NO does not change endothelial barrier integrity at <10 μM but induces a rapid drop in TEER and an increase in fluorescein flux at >20 μM (22). Endothelial NOS activation and production of NO in systemic EnC in response to acute laminar shear stress occurs via activation of the phosphatidylinositol (PI3-kinase/Akt pathway and subsequent phosphorylation of eNOS at serine 1177 (13, 29, 38).

The expression of eNOS in the human kidney has been localized throughout the renal vasculature, including in the glomerulus, where its expression has been presumed to be endothelial (2, 17). Work on shear stress in bovine GEnC has shown expression of NO and endothelin (ET)-1 (4) and that chronic shear stress promotes GEnC differentiation (32) and regulates NF-κB and PDGF-B (11). Under normal conditions, the glomerulus is a high shear stress environment with shear of between 5 and 20 dyn/cm² likely in most capillary loops,
although some computer models have predicted a range of 1–95 dyn/cm² (3). However, real-time observations in rodents have shown intraglomerular blood flow to be almost laminar with few site differences in blood velocity within a single glomerulus (26). Increasing heterogeneity of glomerular blood flow, and therefore shear stress, is associated with progressive glomerulosclerosis (26).

In the present study, we have used human conditionally immortalized GEnC to test the hypothesis that acute laminar shear stress modulates GEnC barrier properties via the activation of eNOS. We used in vitro models including an automated electrical cell-substrate impedance sensor (ECIS) system as used previously (35, 40) but with the addition of a flow module. The ECIS system measures TEER which is inversely related to the fractional area of pathways across a cell monolayer open to water and small molecules and as such can be used as an index of hydraulic conductivity (permeability to water).

**MATERIALS AND METHODS**

Reagents were obtained from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

**Confocal microscopy on human renal cortex.** Human adult renal cortex was collected from the normal pole of unilateral, unipolar renal carcinoma nephrectomy specimens with Southmead Research Ethics Committee approval. Eight-micrometer frozen sections were incubated in blocking solution (5% FCS, 2.5% BSA, and 0.05% Tween 20 in PBS) and then with antibodies to vascular endothelial (VE)-cadherin (catalog no. SC9989, Santa Cruz Biotechnology, Santa Cruz, CA) and eNOS (SC136977, Santa Cruz Biotechnology). Primary antibody binding was detected using secondary antibodies labeled with different fluorophores (Molecular Probes, Paisley, UK). No primary and an appropriate IgG were used as controls. Sections were examined using a Leica confocal imaging spectrophotometer system (TCS-SP2, Leica Microsystems, Wetzlar, Germany).

**GEnC culture.** Human conditionally immortalized GEnC were used, as described and characterized in detail previously (36). These

---

**Fig. 1.** Endothelial nitric oxide synthase (eNOS) expression is localized to the glomerular endothelium in human kidney. Human kidney sections were stained for total eNOS (A) and counterstained for the endothelial cell marker VE-cadherin (B). Total eNOS was expressed within the endothelium of the glomeruli (C). Scale bar = 50 μm. Images are representative of sections from 3 kidneys.

---

**Fig. 2.** Effects of laminar shear stress on glomerular endothelial cells (GEnC) morphology. Shown are representative immunofluorescent images of GEnC under no-flow (A and C) and flow (10 dyn/cm² for 24 h; B and D) conditions using the orbital rotator system. Phalloidin staining for F-actin shows alignment of stress fibers parallel to the direction of shear. Arrow indicates direction of flow. Images are representative of 3 independent experiments. Scale bars = 250 μm (A and B) and 50 μm (C and D).
cells are the best-characterized human GEnC line available and express similar levels of endothelial markers and exhibit similar typical behaviors compared with primary culture GEnC (36). At the permissive temperature of 33°C, the temperature-sensitive SV40 transgene is activated, causing cell proliferation. At 37°C, the transgene is inactivated, rendering the cells quiescent. GEnC were cultured in endothelial growth medium 2 microvascular (EGM2-MV; Lonza, Berkshire, UK) containing 5% FCS and growth factors as supplied excepting vascular endothelial growth factor (VEGF). GEnC were seeded and maintained at 33°C for 4–6 days, and then at 37°C for 2–7 days before the experiments.

Induction of laminar shear stress in an orbital shaker model. GEnC, seeded in 10-cm petri dishes at 7,000 cells/cm², were exposed to laminar shear stress using an orbital shaker (SSM1, Stuart, Staffordshire, UK). Shear stress was calculated using the equation \( \tau_w = \alpha \sqrt{\rho \eta / (2\pi f)^3} \), where \( \tau_w \) is shear stress at the dish wall in dyn/cm², \( \alpha \) is the radius of rotation (cm), \( \rho \) is density of liquid (1.005 g/ml), \( \eta \) is fluid viscosity (0.0075 dyn/cm² at 37°C), and \( f \) is rotations per second (8). Cells were exposed to 0, 10, 15, or 20 dyn/cm² laminar shear stress for 24 h and then lysed, centrifuged, and soluble extracts were stored at -80°C. The medium was stored at -80°C for 3 months or NO analysis. As a positive control, cells were treated with 1 nM VEGF (R&D Systems, Oxfordshire, UK) for 10 min.

In experiments to block laminar shear stress-induced intracellular pathways, cells were pretreated with potent inhibitors of the PI3-kinase/Akt pathway: 100 nM wortmannin (Calbiochem, Nottinghamshire, UK) or 0.5 \( \mu \)M PI-103 (Alexis Biochemicals, Devon, UK), both of which are PI3-kinase inhibitors, or 1 \( \mu \)M Akt1/2 kinase inhibitor (Sigma), before exposure to 10 dyn/cm² laminar shear stress for 10 min. In experiments to block laminar shear stress-induced NO production, cells were pretreated with PI3-kinase/Akt pathway inhibitors as above or with the NOS inhibitors 100 \( \mu \)M l-NG-nitroarginine methyl ester (l-NAME) or 100 \( \mu \)M l-NG-monomethyl arginine (l-NMMA) before exposure to 10 dyn/cm² of laminar shear stress for 24 h.

Immunofluorescence on cultured GEnC. GEnC were seeded onto plastic coverslips (22-mm diameter, Fisher, Leicestershire, UK) in six-well plates at 7,000 cells/cm² and maintained as above. Coverslips with confluent monolayers of GEnC were then attached to the bottom of 10-cm petri dishes using a small amount of vacuum grease. For the effects of laminar shear stress on cell morphology (actin staining), cells were exposed to 10 dyn/cm² laminar shear stress for 24 h. For the effects of laminar shear stress on eNOS, cells were exposed to 10 dyn/cm² for 10 min before fixation. Cells were incubated in blocking solution and then with antibodies to eNOS, ENOS phosphorylated at serine 1177 (9571S, Cell Signaling, Hertfordshire, UK), or a phalloidin-Alexa Fluor 488 conjugate (Molecular Probes). Secondary antibodies and controls were as above. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole and then examined using a cell-imaging system (Leica AF6000LX, Leica Microsystems).

NO assay. Left unbound in solution, NO decays rapidly after its synthesis, with a half-life of <5 s; therefore, measurement of the NO metabolites nitrate and nitrite by biochemical assay is a more accurate strategy for quantification of NO production. Nitrate in culture medium was measured by fluorometric enzymatic assay, according to the manufacturer’s instructions (R&D Systems). The assay is based on the conversion of nitrate to nitrite by nitrate reductase, and subsequent analysis of total nitrite using Griess’s reagent. The optical density was determined using a microplate reader (Multiskan Plus, Labsystems, Finland).

---

**Fig. 3.** Immunofluorescence showing total (TeNOS; A and C) and phosphorylated (P-eNOS; B and D) eNOS expressed in GEnC under control conditions (A and B) or laminar shear stress (10 dyn/cm² for 10 min; C and D) using the orbital rotator system. Both total and phosphorylated eNOS are labeled in red. Nuclei are counterstained with 4,6-diamidino-2-phenylindole (blue). Images are representative of 3 independent experiments. Scale bar = 50 \( \mu \)m.
Vantaa, Finland) set at 540 nm/690 nm and concentration of nitrite from a standard curve.

**SDS-PAGE and Western blotting.** Protein concentration was measured by BCA assay (Thermo Scientific, Northumberland, UK). Samples were resolved by SDS-PAGE under reducing conditions and blotted onto polyvinylidene difluoride membranes (Millipore, Hertfordshire, UK). For eNOS Western blots, an eNOS recombinant protein electrophoresis standard was included (Cambridge Bioscience, Cambridge, UK). The membranes were air-dried and blocked in 5% BSA before incubation with primary antibodies to eNOS (CAY160880, Cambridge Bioscience), phospho-serine 1177-eNOS as above, Akt, phospho-serine 473-Akt, AMPKα, anti-phospho-threonine 172-AMPKα (9272, 4058, 2603, 2535, respectively, all Cell Signaling) and actin. Bands were detected using horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Buckinghamshire, UK) and the ECL chemiluminescence system (Amersham Biotech, Buckinghamshire, UK), and images were acquired using a ChemiDoc-It imager (UVP, Cambridge, UK). Densitometry was performed using Image v1.62 software (National Institutes of Health, Bethesda, MD).

**Effects of laminar shear stress on electrical resistance of GEnC monolayers.** Effects of laminar shear stress on permeability of GEnC monolayers were assessed by real-time measurement of TEER using an automated ECIS system with flow module (ECIS 1600R, Applied Biophysics). This apparatus consists of a flow array with a flow chamber (0.5 × 0.04 × 5 cm), two media reservoirs (one at either end), and eight gold microelectrodes in the chamber base through which media is pumped via gas-permeable silicone tubing. Media pumped from the reservoir bottle enters the flow chamber at the inlet reservoir, flows over the GEnC seeded onto the microelectrodes, and exits via the outlet reservoir. The flow rate is determined by the tubing diameter and the pump head rotation speed (controlled by a PC running ECIS software). Flow throughout all but the very ends of the channel is laminar, and shear stress is calculated using the equation \( \tau = (6U\mu)/H \), where \( \tau \) is shear stress in dyn/cm\(^2\), \( U \) is the flow velocity in cm/s, \( \mu \) is the viscosity of the culture medium, and \( H \) is the height of the chamber.

GEnC were seeded into the flow chamber at 50,000 cells/cm\(^2\) in 100 μl of medium and allowed to attach for 4 h, after which 80 μl of cell-free medium was added to each reservoir. The medium was...

---

**Fig. 4. ENOS phosphorylation, at serine 1177 in response to laminar shear stress results in an increase in NO production.**

- **A:** exposure to 10, 15, or 20 dyn/cm\(^2\) laminar shear stress for 24 h significantly increased the level of eNOS phosphorylation (n = 8, \( P < 0.05 \) 1-way ANOVA, \(* P < 0.05\) compared with the no-flow controls). An eNOS electrophoresis standard and GEnC treated with or without 1 nM VEGF for 10 min served as controls. Bands were detected using horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Buckinghamshire, UK) and the ECL chemiluminescence system (Amersham Biotech, Buckinghamshire, UK), and images were acquired using a ChemiDoc-It imager (UVP, Cambridgeshire, UK). Densitometry was performed using Image v1.62 software (National Institutes of Health, Bethesda, MD).

- **B:** NO production. Exposure to 10, 15, or 20 dyn/cm\(^2\) laminar shear stress for 24 h significantly increased the level of total nitrate in the media (indicative of NO production) compared with the no-flow controls (n = 6, \( P < 0.01 \) 1-way ANOVA, \(** P < 0.05\) compared with the no-flow controls). Exposure to 10 dyn/cm\(^2\) of shear for 24 h in the presence of the NOS inhibitors l-NMMA (100 μM) and l-NAME (100 μM) abolished the laminar shear stress-induced increase in NO (n = 4, \( P < 0.01 \) 1-way ANOVA, \(** P < 0.001\) compared with the no-flow controls). Numbers to the left of blots indicate molecular mass of bands (kDa).

---

The effects of laminar shear stress on electrical resistance of GEnC monolayers were assessed by real-time measurement of TEER using an automated ECIS system with flow module (ECIS 1600R, Applied Biophysics). This apparatus consists of a flow array with a flow chamber (0.5 × 0.04 × 5 cm), two media reservoirs (one at either end), and eight gold microelectrodes in the chamber base through which media is pumped via gas-permeable silicone tubing. Media pumped from the reservoir bottle enters the flow chamber at the inlet reservoir, flows over the GEnC seeded onto the microelectrodes, and exits via the outlet reservoir. The flow rate is determined by the tubing diameter and the pump head rotation speed (controlled by a PC running ECIS software). Flow throughout all but the very ends of the channel is laminar, and shear stress is calculated using the equation \( \tau = (6U\mu)/H \), where \( \tau \) is shear stress in dyn/cm\(^2\), \( U \) is the flow velocity in cm/s, \( \mu \) is the viscosity of the culture medium, and \( H \) is the height of the chamber.

GEnC were seeded into the flow chamber at 50,000 cells/cm\(^2\) in 100 μl of medium and allowed to attach for 4 h, after which 80 μl of cell-free medium was added to each reservoir. The medium was...
exchanged daily by slowly aspirating the reservoirs and chamber and then adding 160 μl of fresh media. In all experiments, an ECIS flow array under no-flow conditions was used as a control.

Arrays were attached to the ECIS array holders and with the use of the ECIS attachment mode, resistance (R) was measured from each electrode at regular time intervals, while the ECIS flow module was used to control the medium flow rate. Cultures were allowed to stabilize for at least 2 h before initiation of intermittent laminar shear stress, as follows: 10 dyn/cm² (5.9 ml/min) for 30 min followed by 4 h of no shear. This was repeated over a 20- to 24-h period, and in some cases either a PI3-kinase/Akt pathway inhibitor or NOS inhibitor as above was then added to the culture medium 30 min before the next laminar shear stress period, and the experiment continued. The ratio of the resistance at each time point to the resistance at the onset of flow (in that cycle) was calculated for each electrode.

Fig. 5. A: Akt phosphorylation, at serine 473, in response to laminar shear stress. Exposure to 10, 15, or 20 dyn/cm² laminar shear stress for 24 h significantly increased the level of Akt phosphorylation compared with the no-flow controls. (n = 5, P < 0.05 1-way ANOVA, *P < 0.05 compared with the no-flow controls). B: AMPK phosphorylation in response to laminar shear stress. Exposure to 10, 15, or 20 dyn/cm² laminar shear stress for 24 h significantly decreased the level of AMPK phosphorylation compared with the no-flow controls. (n = 3, P < 0.0005 1-way ANOVA, *P < 0.001). As a positive control, GEnC were also treated with 1 nM VEGF for 10 min.

Fig. 6. Akt/phosphatidylinositol 3-kinase (PI3K) inhibitors inhibit laminar shear stress-induced Akt (serine 473) and eNOS (serine 1177) phosphorylation and NO production. GEnC were pretreated with either 1 μM Akt1/2, 0.5 μM PI-103, or 100 nM wortmannin for 30 min before exposure to laminar shear stress at 10 dyn/cm² for 10 min. A: phosphorylation of Akt at serine 473 and [n = 3, P = not significant (NS)]. B: eNOS at serine 1177 were determined by Western blot analysis using phospho-specific antibodies (n = 3, P = NS). C: shear-induced NO production is abolished when cells are pretreated with either 1 μM Akt1/2, 0.5 μM PI-103, or 100 nM wortmannin before exposure to laminar shear stress at 10 dyn/cm² for 24 h (n = 4, P < 0.001 1-way ANOVA, **P < 0.01).
RESULTS

Endothelial NOS is localized to GEnC in vivo. Confocal microscopy for eNOS on sections of normal human renal cortex demonstrated the expression of eNOS in glomeruli (Fig. 1). Endothelial NOS colocalized with the endothelial-specific marker VE-cadherin, confirming expression in GEnC.

Effects of laminar shear stress on GEnC morphology. Following exposure of GEnC to either 0 or 10 dyn/cm² laminar shear stress, cellular realignment was examined by F-actin staining. In the unsheared cells, cellular alignment was random and multidirectional (Fig. 2, A and C) whereas sheared cells had realigned in the direction of flow, with redistribution of actin bundles parallel to the direction of flow (Fig. 2, B and D).

Flow-induced activation of eNOS and NO release. Following exposure of GEnC to either 0 or 10 dyn/cm² laminar shear stress, the subcellular localization of total and phosphorylated eNOS was monitored by immunofluorescence. Both total and phosphorylated eNOS were detected in the cytoplasm of control cells and those exposed to laminar shear stress (Fig. 3). Phosphorylated eNOS expression increased and became more concentrated in perinuclear regions in response to laminar shear stress (Fig. 3D).

In response to 10, 15, or 20 dyn/cm² laminar shear stress over 24 h, there was no change in the level of total eNOS (data not shown) but a significant increase in the phosphorylation of eNOS, at serine 1177, regardless of the degree of shear (Fig. 4A). There was a corresponding increase in NO production measured by nitrate assay, again independent of the laminar shear stress level (Fig. 4B). Furthermore, pretreatment with either 100 μM l-NMMA or 100 μM l-NAME blocked the laminar shear stress-induced increase in NO production (Fig. 4C).

Flow-induced effects on Akt and AMPK phosphorylation. Having shown that phosphorylation of the serine 1177 residue corresponds to increased activity of eNOS, we next investigated the underlying mechanism. Ten, 15, or 20 dyn/cm² laminar shear stress over 24 h significantly increased in Akt phosphorylation at serine 473 regardless of the degree of shear (Fig. 5A) whereas laminar shear stress reduced phosphorylation of AMPKα (Fig. 5B).

Regulation of eNOS phosphorylation by Akt. To determine further the role of Akt in laminar shear stress-induced phosphorylation of eNOS, GEnC were pretreated with potent inhibitors of the PI3-kinase/Akt pathway. The trend toward phosphorylation of Akt (Fig. 6A) and eNOS (Fig. B) in response to laminar shear stress in control cells in these experiments was not seen in the presence of the Akt1/2 kinase inhibitors PI-103 or wortmannin, suggesting that these inhibitors block phosphorylation in response to shear. Furthermore, these inhibitors also blocked the production of NO in response to shear (Fig. C), suggesting that Akt is upstream of eNOS phosphorylation and NO production in the response of GEnC to acute laminar shear stress.

Flow-induced changes in GEnC barrier properties. In view of the increase in eNOS activity, we hypothesized that laminar shear stress may modulate GEnC permeability through increased NO production. Figure 7 shows an example of the variation in TEER monitored during repeated exposure to 10-dyn/cm² laminar shear stress in a single experiment. A sharp decrease in the mean TEER was observed at the onset of flow, a maximal reduction was reached at 10–15 min, and then TEER slowly increased for 15 min before flow ceased. Thereafter, TEER continued to increase before stabilizing at approximate...
approximately baseline values until flow was initiated again, repeating the cycle.

To investigate a role for NO in this response, experiments were repeated in the presence of 100 μM L-NMMA, or 100 μM L-NAME, both NOS inhibitors, or a vehicle-only control. Exposure to 10 dyn/cm² with or without vehicle resulted in a transient decrease in TEER, similar to that observed previously (Fig. 8A). Pretreatment with either L-NMMA or L-NAME, respectively, completely or substantially abolished the laminar shear stress-induced transient decrease in TEER (Fig. 8, B–D). The maximal reduction observed in TEER following the onset of flow was minimized following treatment with L-NMMA and L-NAME compared with pretreatment and the vehicle control (Fig. 8D).

To determine further the role of the PI3K/Akt pathway in laminar shear stress-induced TEER changes, experiments were repeated in the presence of Akt1/2 kinase or PI-103 in place of NOS inhibitors (Fig. 9). Pretreatment with Akt1/2 completely blocked the laminar shear stress-induced decrease in TEER (Fig. 9, B–D). The maximal reduction observed in TEER following the onset of flow was significantly lower with Akt1/2 compared with pretreatment with vehicle (Fig. 9D).

DISCUSSION

We have described the use of sophisticated model systems to define the morphological, signaling, and functional effects of...
acute laminar shear stress on GEnC in vitro. We have confirmed our hypothesis by demonstrating activation of the Akt-eNOS-NO pathway, reduction of TEER, and dependence of the TEER reduction on NO.

Confocal microscopy on human renal cortex was consistent with previous reports of glomerular eNOS expression (2, 17). By colocalization with an endothelial-specific marker, we confirmed expression of eNOS by GEnC in vivo, suggesting that it has a physiological role in the glomerulus and reinforcing the rationale for study of its biology in cultured GEnC.

We successfully employed an orbital shaker model for exerting laminar shear stress and demonstrated that GEnC respond to acute laminar shear stress by orientation parallel to the direction of shear stress and with corresponding redistribution of actin filaments. By immunofluorescence, we confirmed that total and phosphorylated eNOS is expressed in cultured human GEnC. Phospho-eNOS appeared in the perinuclear region in response to laminar shear stress, consistent with the dissociation of eNOS from caveolin and delocalization from caveolae to the Golgi complex, an important step in eNOS activation and implying that this perinuclear pool is the source of the increased NO production observed (5).

Western blot analysis confirmed that laminar shear stress phosphorylates eNOS at serine 1177 but not in a laminar shear stress level-dependent manner (across the range tested). Moreover, exposure of GEnC to varying levels of laminar shear stress stimulated NO release into the media. These results are in good agreement with previous studies that have used either macro- or microvascular endothelial cells (21, 33, 42). The range of shear stress tested was designed to encompass estimated mean levels of glomerular shear stress (3). A laminar shear stress level-dependent effect on eNOS phosphorylation and NO release may have been demonstrated with the inclusion of a lower level of laminar shear stress (<2 dyn/cm²) as shown in other EnC (21, 33).

Fig. 9. Reduction in TEER induced by laminar shear stress is blocked by inhibitors of PI3K/Akt. A: arrays were treated with vehicle (DMSO) only. Laminar shear stress causes a rapid reduction in TEER over 15 min followed by a gradual recovery. Vehicle does not affect TEER of control or laminar shear stress-treated array. B: arrays were treated with 1 μM Akt1/2. The reduction in TEER induced by laminar shear stress is almost completely abrogated by Akt1/2. C: arrays were treated with 0.5 μM PI-103. The reduction in TEER induced by laminar shear stress is completely abrogated by PI-103. D: maximal decline in TEER in response to laminar shear stress, pre- and posttreatment, was calculated, and the graph displays mean data from multiple arrays (for vehicle treatment: 3 independent arrays, 20 electrodes total; for Akt1/2: 3 independent arrays, 20 electrodes total; for PI-103: 3 independent arrays, 17 electrodes total), confirming blocking of TEER reduction in response to laminar shear stress by eNOS inhibition (vehicle NS, Akt1/2 P < 0.001, PI-103 P < 0.001 by paired t-tests).
The regulation of eNOS activity by its phosphorylation is complex, involving a number of protein kinases including Akt and AMPKα. The present study demonstrated that laminar shear stress phosphorylates Akt in GEnC and is in agreement with previous studies using microvascular endothelial cells (13, 29). Experiments to inhibit the PI3-kinase/Akt pathway, by Akt1/2, PI-103 and wortmannin suggested that laminar shear stress-induced eNOS phosphorylation is dependent on Akt in GEnC, further supported by the blockade of increased NO production in response to laminar shear stress by Akt inhibition.

We also found that AMPKα was dephosphorylated 24 h after the application of laminar shear stress. Although previous studies have demonstrated AMPKα phosphorylation following exposure to laminar shear stress, the reported duration of this response is inconsistent (13, 46). One group has reported that AMPKα phosphorylation is transient, being downregulated within 20-min exposure to flow (46), yet others have reported that AMPKα phosphorylation is sustained for 24 h (44). The reason for the apparent discrepancies is not clear; however, several factors including the endothelial cell type and experimental design differ. Of note, however, is the evidence that suggests AMPKα can transiently respond to changes in the magnitude and plasticity of shear stress (46). But, further studies are required to decipher the role of AMPKα in the shear-induced eNOS activation in GEnC.

Permeability of GEnC monolayers in response to laminar shear stress was assessed by TEER, an indicator of the pathways across a cell monolayer open to water and small solutes. Our results show a reversible reduction in TEER, providing evidence of a dynamic functional response of GEnC to laminar shear stress. These results are consistent with experiments performed by DePaul et al. (10) using bovine aortic EnC although in these experiments an initial increase in impedance was observed. Other groups have measured hydraulic conductivity (Lp) in bovine aortic EnC and retinal microvascular EnC (6, 27) and shown an increase in Lp, corresponding to the decrease in TEER seen here. The abrogation of the laminar shear stress-induced TEER reduction by eNOS inhibitors and by inhibition of the PI3-kinase/Akt pathway directly demonstrates a central autocrine role for NO in this response, consistent with in vivo findings in rat mesenteric micro vessels (24). While the extent to which these responses observed in vitro would be manifest in the fenestrated glomerular endothelium in vivo is not certain, these results suggest an important role for laminar shear stress, eNOS, and NO in regulating the contribution of GEnC to the glomerular filtration barrier. Furthermore, this axis may also regulate other glomerular cells, most obviously podocytes, in a manner analogous to EnC-mural cell interactions in other vessels. Recently, one group has demonstrated that acute shear stress (1 h) increases permeability to labeled albumin in porcine aortic EnC, while chronic shear stress (1 wk) has the opposite effect (43). Such observations suggest that the physiological effects of laminar shear stress on permeability are time-dependent, acute changes being important in rapid adjustment of microcirculatory permeability while chronic exposure to shear contributes to the characteristics of particular circulations (41). It would therefore be instructive to study effects of chronic exposure to laminar shear stress on GEnC.

Caution must be exercised in translating findings from in vitro studies to the intact glomerulus in situ, but there is accumulating corresponding in vivo evidence for the importance of glomerular eNOS although the role of laminar shear stress in its regulation hasn't been directly addressed. Knock-out studies have demonstrated that an absence of eNOS leads to distinct glomerular lesions (14) and markedly increased susceptibility to diabetic glomerular disease (25, 30, 47). In humans, loss of glomerular eNOS expression has been identified in glomerulonephritis, further suggesting that eNOS plays a protective role (17, 19, 20), and eNOS gene polymorphisms have been associated with more advanced diabetic nephropathy (45). Disturbance of NO regulation has been put forward as a major contributory mechanism in the development of diabetic glomerular disease (30, 37). Given the importance of eNOS and NO expression level in normal glomerular physiology and in disease states, we propose that shear stress plays a significant role in fine-tuning the bioavailability of NO to maintain glomerular health in general as well as the endothelial contribution to the glomerular filtration barrier.

ACKNOWLEDGMENTS
Some of the data presented have previously appeared in abstract form (J Am Soc Nephrol 19: 216A, 2008).

GRANTS
This work was funded by a Wellcome Trust fellowship (S. C. Satchell, 075731), Kidney Research UK (RP13/106), and by the Biotechnology and Biological Sciences Research Council (BB/G012776/1).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES
1. Aird WC, Mechanisms of endothelial cell heterogeneity in health and disease. Circ Res 98: 159–162, 2006.
2. Bachmann S, Bosse HM, Mundel P, Topography of nitric oxide synthesis by localizing constitutive NO synthases in mammalian kidney. Am J Physiol Renal Fluid Electrolyte Physiol 268: F885–F898, 1995.
3. Ballermann BJ, Dardik A, Eng E, Liu A, Shear stress and the endothelium. Kidney Int 54: 100–108, 1998.
4. Ballermann BJ, Marsden PA, Endothelium-derived vasoactive mediators and renal glomerular function. Clin Invest Med 14: 508–517, 1991.
5. Balligand JL, Feron O, Desy C, eNOS activation by physical forces: from short-term regulation of contraction to chronic remodeling of cardiovascular tissues. Physiol Rev 89: 481–534, 2009.
6. Chang YS, Yaccino JA, Lakshminarayanan S, Frangos JA, Tarbell JM, Shear-induced increase in hydraulic conductivity in endothelial cells is mediated by a nitric oxide-dependent mechanism. Arterioscler Thromb Vasc Biol 20: 35–42, 2000.
7. Chiu JJ, Usami S, Chien S, Vascular endothelial responses to altered shear stress: pathologic implications for atherosclerosis. Ann Med 41: 19–28, 2009.
8. Colgan OC, Ferguson G, Collins NT, Murphy RP, Meade G, Cahill PA, Cummins PM, Regulation of bovine brain microvascular endothelial tight junction assembly and barrier function by laminar shear stress. Am J Physiol Heart Circ Physiol 292: H3190–H3197, 2007.
9. Davies PF, Spanu JA, Krauss RM, Shear stress biology of the endothelium. Ann Biomed Eng 33: 1714–1718, 2005.
10. DePaola N, Phelps JE, Florez L, Keese CR, Minnear FL, Giaever I, Vincent P, Electrical impedance of cultured endothelium under fluid flow. Ann Biomed Eng 29: 648–656, 2001.
11. Eng E, Ballermann BJ, Diminished NF-kappaB activation and PDGF-B expression in glomerular endothelial cells subjected to chronic shear stress. Microvasc Res 65: 137–144, 2003.
12. Eremina V, Jefferson JA, Kowalewska J, Hochster H, Haas M, Weisstuch J, Richardson C, Kopp JB, Kabir MG, Backx PH, Gerber
ACUTE SHEAR STRESS AND THE GLOMERULAR ENDOTHELIUM

HP, Ferrara N, Barisoni L, Alpers CE, Quaggin SE. VEGF inhibition and renal thrombotic microangiopathy. N Engl J Med 358: 1129–1136, 2008.

Forbes MS, Thornhill BA, Park MH, Chevalier RL. Lack of endothelial nitric oxide synthase leads to progressive focal renal injury. Am J Pathol 167: 1087–1099, 2005.

Foster RR, Hole R, Anderson K, Satchell SC, Coward RJ, Mathieson PW, Gillatt DA, Saleem MA, Bates DO, Harper SJ. Functional evidence that vascular endothelial growth factor may act as an autocrine factor on human podocytes. Am J Physiol Renal Physiol 284: F1263–F1273, 2003.

Foster RR, Slater SC, Seckley J, Kerjaschki D, Bates DO, Mathieson PW, Satchell SC. Vascular endothelial growth factor-C: a potential paracrine regulator of glomerular permeability, increases glomerular endothelial cell monolayer integrity and intracapillary calcium. Am J Pathol 173: 938–948, 2008.

Furusu A, Miyazaki K, Ozono Y, Koji T, Harada T, Sakai H, Kohno S. Expression of endothelial and inducible nitric oxide synthase in human glomerulonephritis. Kidney Int 53: 1760–1768, 1998.

Haraldson B, Axtrom J, Deen WM. Properties of the glomerular barrier and mechanisms of proteinuria. Physiol Rev 88: 451–487, 2008.

Heeringa P, Bijl M, de Jager-Krikken A, Zandvoort A, Dijkstra G, Heeringa P, Steenbergen E, van Goor H. Renal expression of endothelial and inducible nitric oxide synthase, and formation of peroxynitrite-modified proteins and reactive oxygen species in Wegener’s granulomatosis. J Pathol 193: 224–232, 2001.

Hurst RR, Breen Bergen E, van Goor H. A protective role for endothelial nitric oxide synthase in glomerulonephritis. Kidney Int 61: 822–825, 2002.

Hendrickson RJ, Cappadona C, Yankah EN, Sitzmann JV, Cahill PA, Redmond EM. Sustained pulsatile flow regulates endothelial nitric oxide synthase and cyclooxygenase expression in co-cultured vascular endothelial and smooth muscle cells. J Mol Cell Cardiol 31: 619–629, 1999.

Hurst RD, Clark JB. Nitric oxide-induced blood-brain barrier dysfunction is not mediated by inhibition of mitochondrial respiratory chain activity and/or energy depletion. Nitric Oxide 1: 121–129, 1997.

Jo H, Dull RO, Hollis TM, Tarbell JM. Endothelial albumin permeability is shear dependent, time dependent, and reversible. Am J Physiol Heart Circ Physiol 260: H1992–H1996, 1991.

Kajimura M, Michel CC. Flow modulates the transport of K+ through the walls of single perfused mesenteric venules in anaesthetised rats. J Physiol 521: 665–677, 1999.

Kanetsuna Y, Takahashi K, Nagata M, Gannon MA, Breyer MD, Ott MJ, Olson JL, Ballermann BJ. Chronic in vitro flow promotes ultrastructural differentiation of endothelial cells. Endothelium 3: 21–30, 1995.

Kanetsuna Y, Takahashi K, Nagata M, Gannon MA, Breyer MD, Ballermann BJ. Chronic in vitro flow promotes ultrastructural differentiation of endothelial cells. Endothelium 3: 21–30, 1995.

Kotani K, Sasaki O, Takahashi T, Kikuchi T, Oka H, Nagase H, Nishiyama Y, Yonezawa K, Yamada T, Hino K, Momma K, Ohno H, Kume N, Hasegawa T, Honjo K, Inagami T. The Akt kinase signals directly to endothelial nitric oxide synthase. Curr Biol 9: 845–848, 1999.

Kotani K, Sasaki O, Takahashi T, Kikuchi T, Oka H, Nagase H, Nishiyama Y, Yonezawa K, Yamada T, Hino K, Momma K, Ohno H, Kume N, Hasegawa T, Honjo K, Inagami T. The Akt kinase signals directly to endothelial nitric oxide synthase. Curr Biol 9: 845–848, 1999.

Lack of endothelial nitric oxide synthase knockout mice develop advanced diabetic nephropathy. J Am Soc Nephrol 18: 539–550, 2007.

Ogumrinade O, Kameya GT, Truskey GA. Effect of fluid shear stress on the permeability of the arterial endothelium. Ann Biomed Eng 30: 430–446, 2002.

Ott MJ, Olson JL, Ballermann BJ. Chronic in vitro flow promotes ultrastructural differentiation of endothelial cells. Endothelium 3: 21–30, 1995.

Rossi J, Rouleau L, Tardif JC, Leask RL. Effect of simvastatin on Kruppel-like factor2, endothelial nitric oxide synthase and thrombomodulin expression in endothelial cells under shear stress. Life Sci 87: 92–99, 2010.

Satchell SC, Anderson KL, Mathieson PW. Angiopoietin I and vascular endothelial growth factor modulate human glomerular endothelial cell barrier properties. J Am Soc Nephrol 15: 566–574, 2004.

Satchell SC, Buchatska O, Khan SB, Bhangal G, Tasman CH, Saleem MA, Baker DP, Lobb RR, Smith J, Cook HT, Mathieson PW, Pusey CD. Interferon-beta reduces proteinuria in experimental glomerulonephritis. J Am Soc Nephrol 18: 2875–2884, 2007.

Satchell SC, Tasman CH, Singh A, Ni L, Geelen J, von Ruhland CJ, O’Hare MJ, Saleem MA, van den Heuvel LP, Mathieson PW. Conditionally immortalized human glomerular endothelial cells expressing nestinestrations in response to VEGF. Kidney Int 69: 1633–1640, 2006.

Satchell SC, Tooke JE. What is the mechanism of microalbuminuria in diabetes: a role for the glomerular endothelium? Diabetologia 51: 714–725, 2008.

Sessa WC. eNOS at a glance. J Cell Sci 117: 2427–2429, 2004.

Sill HW, Chang YS, Artman FR, Frangos JA, Hollis TM, Tarbell JM. Shear stress increases hydraulic conductivity of cultured endothelial monolayers. Am J Physiol Heart Circ Physiol 268: H535–H543, 1995.

Singh A, Satchell SC, Neal CR, McKenzie EA, Tooke JE, Mathieson PW. Glomerular endothelial glycoalyx constitutes a barrier to protein permeability. J Am Soc Nephrol 18: 2885–2893, 2007.

Tarbell JM. Shear stress and the endothelial transport barrier. Cardiovasc Res 87: 320–330, 2010.

Walshe TE, Ferguson G, Connell P, O’Brien C, Cahill PA. Pulsatile flow increases the expression of eNOS, ET-1, and prostacyclin in a novel in vitro coculture model of the retinal vasculature. Invest Ophthalmol Vis Sci 46: 375–382, 2005.

Warboys CM, Eric Berson R, Mann GE, Pearson JD, Weinberg PD. Acute and chronic exposure to shear stress have opposite effects on endothelial permeability to macromolecules. Am J Physiol Heart Circ Physiol 298: H1850–H1856, 2010.

Zanchi A, Mocuzzi DK, Hanna LS, Wantman M, Warram JH, Krolewski AS. Risk of advanced diabetic nephropathy in type 1 diabetes is associated with endothelial nitric oxide synthase gene polymorphism. Kidney Int 57: 405–413, 2000.

Zhang Y, Lee TS, Kolb EM, Sun K, Lu X, Sladek FM, Kassab GS, Garland T Jr, Shyy JY. AMP-activated protein kinase is involved in diabetes: a role for the glomerular endothelium? J Cell Sci 117: 2427–2429, 2004.

Zhang Y, Lee TS, Kolb EM, Sun K, Lu X, Sladek FM, Kassab GS, Garland T Jr, Shyy JY. AMP-activated protein kinase is involved in diabetes: a role for the glomerular endothelium? J Cell Sci 117: 2427–2429, 2004.

Zhao HI, Wang S, Cheng H, Zhang MZ, Takahashi T, Fogo AB, Breyer MD, Harris RC. Endothelial nitric oxide synthase deficiency produces accelerated nephropathy in diabetic mice. J Am Soc Nephrol 17: 2664–2669, 2006.