Pulsatile Flow Increases the Expression of eNOS, ET-1, and Prostacyclin in a Novel In Vitro Coculture Model of the Retinal Vasculature

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PURPOSE. By the development of a novel retinal microvascular endothelial and pericyte cell coculture system, this study determined the effects of pulsatile flow on the activation of the endothelial cell markers nitric oxide (NO), prostacyclin (PGI₂), and endothelin (ET)-1.

METHODS. Monocultured bovine retinal endothelial cells (BRECs) and cocultured BRECs with bovine retinal pericytes (BRPs) were exposed to low flow (flow rate, 0.3 mL/min; pulse pressure, 6 mm Hg; shear stress, 0.5 dyne/cm²) or high flow (flow rate, 25 mL/min; pulse pressure, 56 mm Hg; shear stress, 23 dyne/cm²) for 24 hours, by using a novel perfused transcapillary culture system. The cells were characterized by immunohistochemistry and electron and confocal microscopy. Endothelial nitric oxide synthase (eNOS) and phosphorylated-eNOS (pp-eNOS) were determined by Western blot analysis. Nitrate, PGI₂, and ET-1 levels were quantified in the medium perfusate by using fluorometric and enzyme-linked immunosorbent assays, respectively. Activation of cyclooxygenase (COX)-2 in BRECs was determined by measuring COX-2 promoter activity with a luciferase reporter assay.

RESULTS. The presence of BRPs and BRECs was confirmed by Western blot, immunocytochemistry, and scanning electron microscopy. Phosphorylated eNOS (pp-eNOS) protein levels in BRECs were significantly increased from low to high flow in both mono- and cocultures, concomitant with a significant increase in nitrate levels in the conditioned medium after exposure to pulsatile flow. In parallel cultures, PGI₂ levels were also significantly enhanced concomitantly with an increase in the transactivation of a COX-2 promoter BREC after exposure to pulsatile flow. ET-1 levels were also increased in both mono- and cocultured cells.

CONCLUSIONS. In this study a novel, functioning, in vitro model of retinal microvascular endothelial and pericyte cells that respond to changes in pulsatile flow was established. (Invest Ophthalmol Vis Sci. 2005;46:375–382) DOI:10.1167/iovs.04-0806

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Perfusion of retinal vessels is dependent on the complex balance between opposing vasodilatory and vasoconstrictive forces in the retinal blood supply. Retinal pericyte and endothelial cell (EC) cross-talk in these vessels is largely influenced by mechanical stimuli due to the pulsatile nature of blood flow (i.e., pulse pressure [cyclic strain] and shear stress). These mechanical forces result in cellular signaling events mediated by growth factors and intracellular secondary messengers, G proteins, small guanosine triphosphatases (GTPases), and kinases. The response to fluid shear stress, the frictional tangential force imposed on the vessel wall due to blood flow, results in abbluminal release of factors from ECs, resulting in dilation or constriction of the underlying pericyte layer. Whereas ECs are the major recipient of shear stress, cyclic strain exerts its effect on both the endothelium and the pericyte cell layer, and both forces have been shown to modulate local autoregulation of vessel tone.

Several pathologic effects are associated with disturbed retinal blood flow. Vascular dysregulation or impaired autoregulation leads to vasospasm of retinal vessels and has been implicated in normal-tension glaucoma, in which decreased retinal blood flow correlates with increasing optic nerve head damage and reduced pulsatile ocular blood flow. Similarly, hyperglycemic retinas exhibit impaired flicker-induced vasodilation, also implicating vascular dysregulation in diabetic retinopathy.

ECs modulate vessel tone via the vasodilators nitric oxide (NO) and prostacyclin (PGI₂) and vasoconstrictors such as endothelin (ET)-1. One of the earliest events occurring in ECs placed under increased fluid shear stress is the activation of eNOS by phosphorylation at several sites and the subsequent release of the vasodilator NO. Phosphorylation of eNOS has been recognized as a critical regulatory mechanism that controls its activity with at least five specific phosphorylation sites having been recognized. Reductions in the bioavailability of NO, leading to impaired vasodilation, have been associated with risk factors for atherosclerosis, diabetes, and hypertension. Production of the vasodilator PGI₂ is controlled by the enzyme cyclooxygenase (COX), which catalyzes the formation of prostaglandins and thromboxanes from arachidonic acid. COX comprises two isoenzymes: COX-1, which is constitutively expressed, and COX-2, which is an inducible enzyme with expression that is regulated differently among cell types. COX-2 induction has been shown to be upregulated by laminar shear stress and in the production of PGI₂, an unstable prostaglandin released by mast cells and the endothelium. PGI₁ has been shown to be involved in several biological processes, including inhibition of platelet aggregation, vasodilation, and vascular permeability.

Counterbalancing these vasodilatory effects is the endothelin family, which includes peptides that are the most potent vasoconstrictors known to date. Three vasoactive endothelin isoforms have been identified—ET-1, -2, and -3—and have been shown to exert their effects through two G protein-coupled receptors, ET₄ and ET₅, to produce various physiological responses. ET₄ receptors are found on cells including vessel...
and airway smooth muscle cells, cardiomyocytes, and fibroblasts and show higher affinity for ET-1 and -2, whereas ET<sub>a</sub> receptors are found predominately localized to ECs and smooth muscle cells and have affinity for all three ET peptides. Binding of ET-1 to ET<sub>a</sub> receptors on smooth muscle cells results in vasoconstriction, cell growth, and cell adhesion. However, binding of the same peptide to ET<sub>b</sub> receptors on ECs causes vasodilation by stimulating the release of NO and PGI<sub>2</sub>.

A large proportion of our knowledge and understanding of the retinal microvascular system has been obtained by drawing comparisons with the more extensively researched macrovascular system, and both shear stress and cyclic strain have been shown independently to modulate EC production of these vasoactive agents in macrovascular cells.\textsuperscript{28–32} In the present study, we developed a novel in vitro model of microvascular retinal endothelial and pericyte cells in culture and studied the interaction between these cells when exposed to pulsatile fluid flow in a perfused transcapillary coculture system. Our study presents the effect of varying pulsatile flow rates on the vasoactive cell markers NO, PGI<sub>2</sub>, and ET-1.

### MATERIALS AND METHODS

**Endothelial and Pericyte Cell Culture**

Bovine retinal endothelial cells (BRECs) and bovine retinal pericytes (BRPs) were kindly donated by Alan Stitt (Queens University, Belfast, Northern Ireland, UK). BRECs were grown in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1× ITS. Both cell lines were maintained in a humidified atmosphere of 5% CO<sub>2</sub>/95% air and routinely used between passages 5 and 9.

**Perfused Transcapillary Cocultures**

The perfused transcapillary culture apparatus (CELLMAX QUAD artificial capillary culture system; Spectrum Laboratories Inc., Santa Clara, CA) consisted of an enclosed bundle of 50 semipermeable, coated (Pronectin-F; Sanyo Chemical Industries, Ltd., Kyoto, Japan) polypropylene capillaries (capillary length, 13 cm; outer diameter, 630 μm; wall thickness, 150 μm; luminal area, 70 cm<sup>2</sup>; outer surface area, 100 cm<sup>2</sup>; extracapillary volume 1.4 mL; 95% molecular weight cutoff [MWCO] 0.5 μm) through which medium from a reservoir is pumped, at a chosen flow rate, via silicone rubber tubing. As the gear pump rotates, the motor shaft forces the pump pins to depress the pump tubing on the capillary module, thereby forcing culture medium to flow in a pulsatile fashion through the gas-permeable silicone flow path into the capillary (Fig. 1a). By altering the flow rate using an electronic control unit that is housed outside the humidified incubator, varying pulsatile flow rates and hence pulse heights (pressure) can be achieved in this system (Fig. 1a; Table 1). To maintain the pH, pCO<sub>2</sub>, and pO<sub>2</sub> of the culture medium at constant levels, we housed the perfused transcapillary culture system in a humidified atmosphere in a standard CO<sub>2</sub> incubator, thereby allowing gaseous exchange to occur through the silicone rubber tubing. Before the addition of cells, the module was equilibrated for 3 days by circulation of culture medium through the capillaries and tubing. The outer surface area of the capillaries was 100 cm<sup>2</sup>. BRPs from culture flasks of equivalent or greater surface area were harvested by adding 0.125% trypsin-EDTA and injected into the extracapillary space (ECS) at a density of 2 × 10<sup>5</sup> cells/cm<sup>2</sup> by a double-syringe method.\textsuperscript{3} Briefly, BRPs were introduced with a syringe into one ECS port, and the displaced medium was withdrawn from the opposite ECS port with another syringe (Fig. 1a). Cells were allowed to adhere for 3 hours, after which the pump was set to low flow (0.5 mL/min; pulse pressure, 6 mm Hg; shear stress, 0.5 dyne/cm<sup>2</sup>) and returned to the incubator for 3 days.

BRECs were introduced into the luminal compartment, again using the double-syringe method at a density of 2 × 10<sup>5</sup> cells/cm<sup>2</sup> and allowed to attach for 3 hours before the medium was circulated at low flow for a further 5 days (see Fig. 1b for a diagrammatic representation). Low serum (1%) was used to enhance BRECs attachment to the cell culture pronectin-coated capillaries. In addition, to prevent BRECs from being flushed out of the capillaries and to promote their adherence immediately after cell loading, we rerouted the perfusion medium (now also containing 1% FBS) for 6 hours via the ECS, using the side ports. After this period, the perfusion circuit was returned to its original path, whereby medium, containing 20% FBS, was again perfused through the lumen of each capillary that was now lined with BRECs. The cells were seeded at an appropriate density to guarantee full coverage of all capillaries. The number of cells that did not adhere were routinely counted to measure seeding density and adherence.

### TABLE 1. Pulse Pressures within the Luminal and Extracapillary Space under Low- and High-Pulsatile Flow Conditions

<table>
<thead>
<tr>
<th>Pulse Pressures</th>
<th>Amplitude</th>
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<tbody>
<tr>
<td>ECS Low Flow</td>
<td>Max: 24</td>
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<tr>
<td></td>
<td>Min: 18</td>
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<tr>
<td>ECS High Flow</td>
<td>Max: 70</td>
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<td></td>
<td>Min: 14</td>
</tr>
<tr>
<td>Inlet Low Flow</td>
<td>Max: 11</td>
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<tr>
<td></td>
<td>Min: 0</td>
</tr>
<tr>
<td>Inlet High Flow</td>
<td>Max: 80</td>
</tr>
<tr>
<td></td>
<td>Min: 10</td>
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</table>

Data are in mm Hg.
after the pump had been turned back on to ensure maximum coverage of each capillary. The harvested cells were then routinely counted at the end of each experiment to confirm uniform seeding density and adherence.

To obtain “high flow” the flow rate was increased steadily over approximately 5 hours until the desired high flow rate was reached \((t = 0)\). After 24 hours’ exposure to high flow, cells were harvested from their separate compartments by first washing the cells with Hanks’ balanced salt solution (HBSS), by using the double-syringe method, and removing the remaining cells by treatment with 0.125% trypsin-EDTA. The circulating medium was also harvested at the end of each experiment. For BREC monolayers, no BRPs were seeded into the ECS, and the BREC were seeded into the luminal compartment as described earlier. Pulse pressures were monitored simultaneously intralaminally at the inlet port and extra-luminally (ECS) at the side port, by using pressure transducers connected to a recorder (models 7 and 7E; Grass-Telefactor Instrument Co., W. Warwick, RI; Fig. 1c). In the present study, the low pulsatile flow rate used was 0.5 mL/min, corresponding to a shear stress of 0.5 dyne/cm\(^2\) and a pulse pressure of 24/18 mm Hg with a frequency of 0.2 Hz and an amplitude of 6 mm Hg in the ECS. The high pulsatile flow rate was 23 mL/min, corresponding to a shear stress of 23 dyne/cm\(^2\), a pulse pressure of 70/14 mm Hg with a frequency of 2 Hz, and an amplitude of 56 mm Hg in the ECS.

**Detection of Secreted Proteins by Enzyme-Linked Immunosorbent Assay**

ET-1 and PGI\(_2\) levels in the medium were measured by enzyme immunoassay (EIA) kit, according to the manufacturer’s instructions. Briefly, ET-1 was measured with an ET-1/acyetylcholinase-conjugated antibody, with detection limit of 1.5 pg/mL (Cayman Chemical Co., Ann Arbor, MI). PGI\(_2\) levels were similarly measured by a 6-keto-prostaglandin F\(_{1\alpha}\)-alkaline phosphatase-conjugated antibody with a detection limit of 1.4 pg/mL (Assay Designs, Ann Arbor, MI).

**COX-2 Promoter Activity**

pG-UCOX2 was the kind gift of Hiroyasu Inoue (Department of Pharmacology, National Cardiovascular Centre Research Institute, Japan). In this construct, luciferase (Luc) mRNA is expressed under the control of the human COX-2 enhancer/promoter. BREC were transiently transfected with this plasmid (LipoFectamine; Invitrogen) according to the manufacturer’s specifications. DEMM (1500 \(\mu\)L) containing 33.75 \(\mu\)g COX-2 Luc reporter vector and 11.25 \(\mu\)g LacZ (a plasmid encoding \(\beta\)galactosidase activity) was mixed with 750 \(\mu\)L DEMM containing 50 \(\mu\)L of the transfection reagent. The DNA/reagent mix was added to cells harvested from 5 \(\times\) 75-cm \(^2\) flask and resuspended in 12.5 mL DEMM. Cells were left to incubate for 15 to 20 minutes at room temperature after which, the volume was split evenly and seeded onto two separate capillary modules. After exposure to increased fluid shear stress using the apparatus described earlier, the cells were trypsinized from the capillaries. Transactivation of reporter genes was evaluated by the luciferase assay (Promega, Madison, WI) and normalized to the \(\beta\)-galactosidase activity. The latter was performed according to the manufacturer’s instructions (High-Sensitivity \(\beta\)galactosidase Assay; Stratagene, La Jolla, CA).

**NO/Nitrate Assay**

Measurement of nitrate in culture medium was performed by fluorometric assay, as described previously. In brief, the reaction of 2,3-diaminonaphthalene (DAN) with nitrate results in the formation of 1-(H)-naphthotriazole, a fluorescent product. The reaction is initiated by the addition of 10 \(\mu\)L of DAN solution (0.05 mg/mL in 0.62 M HCl) to 100 \(\mu\)L of standard or medium sample and allowing the reaction to continue for 10 minutes. The reaction is terminated by the addition of 5 \(\mu\)L of 2.8 M NaOH. Samples were read with a spectrometer (Luminance LS 50B; Perkin Elmer, Boston, MA) at excitation 365 nm and emission 450 nm, with nitrate activity recorded as picomoles nitrate per milligram protein.

**SDS-PAGE and Immunoblot Analysis**

Cell pellets harvested from the transcapillary culture apparatus (Cellmax; Spectrum Laboratories Inc.) were treated with lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM Na\(_2\)EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM \(\beta\)-glycerophosphate, 1 mM sodium orthovanadate, and 1 \(\mu\)g/mL leupeptin). Following centrifugation to pellet insoluble material, soluble extracts equalized by bichoninic acid (BCA) protein assay were added to equal volume of electrophoresis sample buffer and fractionated by SDS-PAGE on 10% (wt/vol) polyacrylamide resolving gels. After transfer to nitrocellulose membrane (Amersham, Buckinghamshire, UK), nonspecific protein binding sites were blocked by a 60-minute incubation in PBS-T (phosphate-buffered saline, 0.1% Tween-20) containing 5% (wt/vol) nonfat skim milk. Membranes were then incubated overnight at 4°C with either eNOS (1:2000; Cayman Chemical Co.), or pp-eNOS\(^{177}\) (1:500; specific to phosphorSer\(^{177}\) human sequence; Cell Signaling, Beverly, MA) antibodies diluted in PBS-T with 2.5% BSA. After two 10-minute washes with PBS-T, membranes were incubated with horseradish peroxidase-conjugated rabbit polyclonal IgG antibody for 90 minutes at room temperature (1:10,000 eNOS; 1:1000 pp-eNOS; Amersham). After two further washes with PBS-T, immunoreactive proteins were identified by enhanced chemiluminescence (West Pico SuperSignal; Pierce, Cheshire, UK). Scanning densitometry was performed with image-analysis software (1D; Eastman-Kodak, Rochester, NY).

**Immunocytochemistry**

BRPs seeded onto capillaries were washed twice with PBS and fixed by the addition of 3% paraformaldehyde for 15 minutes at room temperature. The plastic casing housing the capillaries was then removed and the capillaries cut into 1-cm sections. Cells were permeabilized by the addition of 0.2% (vol/vol) TX-100 in PBS followed by two washes with PBS and incubation for 30 minutes at room temperature in blocking solution (1% BSA in PBS). Capillaries were then washed twice with PBS, followed by incubation for 2 hours at room temperature with a 1:200 dilution of anti-c-skeletal muscle cell-specific actin (Sigma-Aldrich) primary antibody in blocking solution. After they were washed for 5 minutes with three changes of PBS, sections were incubated with a 1:400 dilution of Alexa 488-conjugated rabbit anti-mouse IgG in blocking solution for 60 minutes at room temperature (Molecular Probes, Leiden, The Netherlands). Capillary sections were then washed with PBS, before being mounted onto microscope slides for fluorescence microscopy analysis.

**Confocal Microscopy**

Visualization of BRP anti-smooth muscle cell-specific actin was performed on cells grown on capillaries and prepared as described for immunocytochemistry. BRP nuclei were stained for 5 minutes with 0.2 \(\mu\)g/mL propidium iodide (Molecular Probes). Control procedures included unstained cells, to allow for autofluorescence, and secondary antibody only to control for nonspecific binding of the fluorescent secondary antibody. Alexa-modified antibodies were excited at 488 nm using argon laser and detected with 505–550 nm band pass filter. Propidium iodide was excited at 543 nm and detected with a long pass band filter 590 nm. All images were acquired by confocal microscope (model LSM 510; Carl Zeiss Meditec, Jena, Germany).

**Scanning Electron Microscopy**

Media was removed from the capillary system and replaced with 2.5% glutaraldehyde in \(1 \times\) PBS and incubated at room temperature for 1 hour. Cells were then washed in 0.1 M cacodylate buffer (pH 7.4; Sigma-Aldrich) and incubated for a further 90 minutes in 1% osmium tetroxide in cacodylate buffer at 4°C. After a brief wash with cacodylate buffer, a graded series of ethanol solutions were perfused to
dehydrate the capillaries (50%, 60%, 70%, 80%, and 90% ethanol for 10-15 minutes each). The capillaries are then incubated in 100% ethanol twice for 10 minutes and dried in a standard bell chamber in a vacuum overnight.

**Statistical Analysis**

Results are expressed as mean ± SEM. Statistical significance was assessed by Wilcoxon Signed Rank Test, with significance values of *P* ≤ 0.05.

**RESULTS**

**Characterization of Pericytes and ECs Exposed to Pulsatile Flow**

The presence of BRP cells on the surface of the capillaries was determined by immunocytochemistry and scanning electron and confocal microscopy of individual capillaries removed from the bundle. Staining for the presence of α-smooth muscle cell–specific actin, a known cytoskeletal marker of pericyte cells, showed BRPs to be positive for this marker (Fig. 2a, 2b) and negative for eNOS and the astrocyte-specific marker glial fibrillary acidic protein (GFAP, results not shown). Visualization of BRPs with anti-α-smooth muscle cell–specific actin was obtained on subconfluent cells by confocal microscopy, and the presence of BRPs on the capillaries was confirmed. Moreover, the actin staining appeared more punctate under high flow as the cells begin to orient in the direction of flow (Fig. 2c), confirming that these changes induced a clear reorganization of the cytoskeleton. Scanning electron microscopy revealed that the pericyte cell layer at confluence maintained a stellar morphology under low-flow conditions similar to those observed in cells grown in static cultures. Increases in medium flow rate led to orientation of the BRPs along the direction of flow, forming elongated striated cells (Fig. 3). The presence of functional microvascular ECs was further confirmed by Western blot of phospho-eNOS in these cells (Fig. 4a).

**Pulsatile Flow–Induced Activation of eNOS**

We investigated the effect of low (0.3 mL/min; 6 mm Hg; 0.5 dyne/cm²) and high (25 mL/min; 56 mm Hg; 23 dyne/cm²) pulsatile flow on the total eNOS protein levels and the phosphorylation state of eNOS in BRECs cultured alone or in coculture with BRPs over a 24-hour period. Under these conditions, no changes in total eNOS expression were observed in either mono- or cocultures of BRECs (Fig. 4a) as determined by Western blot analysis. However, a significant increase in the phosphorylation state of the eNOS protein was detected in both monocultured (1.903 ± 0.332-fold, *n* = 6) and cocultured (1.932 ± 0.199-fold, *n* = 6) BRECs (Fig. 4a). Metabolism of NO and release of nitrate into the circulating medium was further confirmed by a fluorometric DAN assay. There was a significant increase in nitrate levels (2.306 ± 0.276 and 2.404 ± 0.38-fold, *n* = 5) in mono- and cocultures of BRECs, respectively (Fig. 4b).

**Pulsatile Flow–Induced PGI₂ Release**

To examine the effect of pulsatile flow on the production of PGI₂, we exposed mono- and cocultures of BRECs and BRPs to low- and high-flow rates, as has been described. The levels of PGI₂ present in the circulating medium were determined by enzyme immunoassay. There was a significant increase in PGI₂ levels in cells exposed to high flow compared with low flow (2.260 ± 0.257-fold, *n* = 6, and 2.015 ± 0.372-fold, *n* = 7) in both mono- and cocultures, respectively (Fig. 5a).

To determine whether the increase in PGI₂ levels was a direct consequence of the induction of COX-2 expression, we examined the effect of pulsatile flow on COX-2 promoter activity in BRECs. In cells transiently transfected with a plasmid encoding the COX-2 promoter, there was a significant increase in COX-2 transactivation (5.066 ± 1.07-fold, *n* = 5) in mo-
nocultured BRECs after 24 hours’ exposure to pulsatile flow (Fig. 5b).

**Pulsatile Flow–Induced Changes in ET-1 Release**

To examine the relationship between pulsatile flow and ET-1 release, we exposed BRECs and BRPs to low and high pulsatile flow rates as has been described. Exposure of cells to high pulsatile flow resulted in a 1.850 ± 0.338- and 1.961 ± 0.205-fold (n = 5) increase in medium ET-1 levels in mono- and cocultured BRECs, respectively, compared with low flow (Fig. 6).

**DISCUSSION**

The present study describes the use of a novel transcapillary coculture system as a basis for mimicking the effects of hemodynamic forces on microvascular endothelial and pericyte cells cultured in vitro. Blood vessels are continually exposed to hemodynamic forces generated in the vasculature due to fluid-induced shear and circumferential stresses. Changes in these conditions result in alterations in the signal-transduction pathways and release of vasoactive substances from vascular ECs that are ultimately responsible for regulating vascular tone.3–7 Retinal blow flow, as measured by various noninvasive techniques, ranges from 60 to 100 μL/min in larger retinal vessels, corresponding to a shear stress level of ∼50 dynes/cm².34,35 As smaller ocular vessels receive ∼10% of retinal blood flow, the levels of shear stress for microvascular ECs are within the range of 0.5 to 23 dynes/cm² used in the present study. Changes in hemodynamics can result in endothelial dysfunction, a process that has been described in macrovascular disease states including the pathogenesis of atherosclerosis and thrombosis36 and is also of particular interest in the microvasculature in conditions such as proliferative diabetic retinopathy (PDR)37–39 and normal-tension glaucoma40–42 in which there is growing evidence of endothelial dysfunction as a major risk factor.1,18 The present study combined for the first time the relevant physiologic forces of pulse pressure and shear stress with coculture to evaluate their effects on the expression and activity of the vasoactive substances NO, PGI₂, and ET-1 in a novel retinal microvascular coculture model.

Immunocytochemistry, scanning electron microscopy, and confocal microscopy, in combination with Western blot analysis, confirmed the presence and growth of microvascular retinal ECs and pericytes in the perfused transcapillary culture system. Moreover, there was a single layer of pericytes oriented along the direction of flow on the abluminal side of the capillaries. The distinct compartmentalization of actin observed in pericytes after exposure to high pulsatile flow, with actin less evenly distributed throughout the cytoplasm to accommodate altered cell function, suggests that phenotypic modulation may involve not only quantitative changes in contractile proteins, but also reorganization of these proteins. Because the cytoskeleton acts as a spatial regulator of intracellular signaling, reorganization of the cytoskeleton may lead to realignment of signaling molecules, which, in turn, may mediate the changes in function associated with phenotypic modulation after exposure to flow. Culturing of the BRECs alone or as cocultures with pericytes did not significantly enhance or diminish the effects of pulsatile flow on eNOS and COX activity or ET-1 levels, suggesting that the major source of pulsatile-flow–induced changes in these vasoactive substances was the retinal microvascular EC.

ECs are known to modulate vessel tone via the production and release of the vasodilators NO42 and PGI₂43 and the vasoconstrictor ET-1.44 One of the earliest events occurring in ECs placed under increased hemodynamic constraints is the activation of eNOS through phosphorylation at several sites and the subsequent release of the vasodilator NO.16 A large number of in vitro and in vivo studies have demonstrated that NO plays an important role in regulation of regional ocular blood flow.45–49 Indeed, NO has been implicated in the etiology of several ocular diseases that result in altered ocular blood flow.47–49 Because phosphorylation of eNOS has been recognized as a critical regulatory mechanism controlling its activity,

**FIGURE 3.** Representative scanning electron micrographs of BRPs cultured on capillaries and subjected to (a) low- and (b) high-flow conditions. In low-flow conditions, pericytes were more stellar/rounded in their morphology. Arrows: bovine retinal pericytes. In high-flow conditions, the pericytes changed their morphology and oriented themselves along the direction of flow, giving a more striated appearance. Cell contacts became tighter, and the capillary was no longer visible. Magnification, ×450.

**FIGURE 4.** The effect of pulsatile flow on NO production. (a) The effect of pulsatile flow on phospho-eNOS expression. The levels of phospho-eNOS and total eNOS in mono- and cocultured BRECs after exposure for 24 hours to low and high pulsatile flow rates were quantified by Western blot analysis. Representative examples of Western blots are shown. (b) The effect of pulsatile flow on BREC nitrate release. Nitrate released into the circulating medium after eNOS activation was determined by DAN assay. Data are the mean ± SEM of results in at least five independent experiments (*P ≤ 0.05 versus low-flow control).
we examined the expression of pp-eNOS in mono- and cocultured BRECs after exposure to pulsatile flow. In agreement with previous studies using macrovascular ECs, the present study demonstrated that pulsatile flow can activate eNOS through the increased expression of phosphorylated eNOS in microvascular BRECs over similar periods. Moreover, as shear stress alone has been shown to stimulate NO release in retinal microvascular ECs, these experiments further suggest that NO may be a key signaling molecule in elevating vascular transport in ocular diseases such as diabetic retinopathy.

Previous studies have suggested that prostaglandins, synthesized by COX-1 or -2, may contribute to normal physiological and homeostatic functions in the retina. We therefore investigated the role of pulsatile flow in modulating microvascular retinal endothelial PGI2 release and the contributory role of the COX-2 isozyme in mediating this response. As previously observed in macrovascular ECs, pulsatile flow increased the levels of PGI2, concomitant with a significant increase in the transactivation of the COX-2 promoter in these cells. Although previous studies have reported that expression of COX-2 is increased in human umbilical vein ECs (HUVECs) exposed to laminar shear stress, and that this form of the enzyme has a higher affinity for the PGI2 precursor, arachidonate, and PGI2 synthase than does COX-1, this is the first study to demonstrate COX-2–mediated prostaglandin production in response to pulsatile flow in retinal microvascular ECs.

Exposure of human glomerular microvascular ECs (HGMECs) to low levels of laminar shear stress has shown an initial increase in ET-1 followed by a decrease at 24 hours. In contrast, in the present study we demonstrated a sustained increase in ET-1 levels secreted by BRECs in response to high pulsatile flow conditions in both monoculture and in coculture with pericytes after a similar period. Several studies have suggested that patients with glaucoma have increased circulating plasma ET-1 levels, which may be the source of retinal vessel vasoconstriction and ischemia of the optic nerve head vessels. Indeed, a strong immunoreactivity for ET-1 converting enzyme (ECE)-1 can be found in the blood vessels of the retina, optic nerve, and choroids, suggesting an important role for ET-1 during autoregulation within the eye. Because ocular blood flow alterations in patients with glaucoma seem, at least partly, to be related to a systemic vascular dysregulation and are exhibited as altered responsiveness to ET-1, it is possible that flow-induced changes in ET-1 production within the eye contributes to the pathogenesis of glaucomatous damage.

It is clear that the model does not allow for pericyte-to-endothelial physical interactions, such as gap junctions, which are likely to be important in the regulation of the microvascular response to changes in blood flow. Intercellular communication through gap junctions (GJIC) is most likely relevant to maintaining the integrity of the blood-retinal barrier and high-glucose-induced downregulation of Cx43 expression and inhibition of GJIC in retinal pericytes may play a role in the disruption of vascular homeostasis in diabetic retinopathy. However, despite this apparent limitation, the coculture system allows for the specific examination of individual cell-signaling pathways in either ECs or pericytes without having any cross-contamination of either cell type, which would prevail if the cells physically interacted with each other.

Advancing our understanding of retinal blood flow autoregulation through changes in microvascular EC function and the subsequent interaction with the retinal pericyte is of crucial importance to the understanding and origin of events within the eye that lead to ocular diseases such as glaucoma and retinopathies. Using our novel perfused transcapillary microvascular retinal EC-pericyte culture system, under physiological pulsatile flow conditions in coculture, we can now begin to establish the contribution of retinal microvascular ECs and retinal pericytes to changes in the vascular cell fate typical of ocular disease in humans.

**References**


3. Haefliger IO, Flammer J, Beny JL, Luscher TF. Endothelium-depen-
dent vasoactive modulation in the ophthalmic circulation. Prog

4. Traub O, Berk BC. Laminar shear stress: mechanisms by which
endothelial cells transduce an atheroprotective force. Arterioscler

suppression by shear stress: pharmacological evaluation of the role
of tyrosine kinase, intracellular calcium, cytoskeleton, and mech-

6. Redmond EM, Cahill PA, Sittmann JZ. Flow-mediated regulation of
G-protein expression in co-cultured vascular smooth muscle and

7. Ikeda M, Kito H, Sumpio BE. Phosphatidylinositol-3 kinase depen-
dent MAP kinase activation via p21ras in endothelial cells exposed
to cyclic strain. Biochem Biophys Res Commun. 1999;257:668–
671.

8. Lehoux S, Tdgui A. Signal transduction of mechanical stresses in

9. Nagaoka T, Sakamoto T, Mori F, Sato E, Yoshida A. The effect of
nitric oxide on retinal blood flow during hypoxia in cat. Invest

10. Patrick CW Jr, McIntire LV. Shear stress and cyclic strain modula-

Regulation of human retinal blood flow by endothelin-1. Am J

dysfunction in normal pressure glaucoma. Invest Ophthalmol Vis

71–88.


GT. Reduced response of retinal vessel diameters to flicker stimu-
891.

dependent endothelial nitric-oxide synthesis phosphorylation sites
by mass spectrometry and regulation of phosphorylation and nitric
oxide production by the phosphatidylinositol 3-kinase inhibitor

caracterization of the constitutive bovine aortic endothelial cell

endothelium-dependent relaxation by activating protein kinase C. J

19. Ohara Y, Peterson TE, Harrison DG. Hypercholesterolemia in-
1993;91:2546–2551.

20. Smith WL, Langenbach R. Why there are two cyclooxygenase

21. Herschman HR. Function and regulation of prostaglandin synthase

22. Topper JN, Cai J, Fad D, Gimbrone MA Jr. Identification of vascu-
lar endothelial genes differentially responsive to fluid mechanical
stimuli: cyclooxygenase-2, manganese superoxide dismutase, and
endothelial nitric oxide synthase are selectively up-regulated by
steady laminar shear stress. Proc Natl Acad Sci USA. 1996;93:
10417–10422.

23. Inoue H, Umesono K, Nishimori T, Hirata Y, Tanabe T. Glucoco-
toid-mediated suppression of the promoter activity of the cyclo-
oxigenase-2 gene is modulated by expression of its receptor in
vascular endothelial cells. Biochem Biophys Res Commun. 1999;

MM, Sanchez de la Cuesta F. Effects of the selective inhibition of
platelet thromboxane synthesis on the platelet-subendothelium

25. Hata Y, Clermont A, Yamauchi T, et al. Retinal expression, regu-
lation, and functional bioactivity of prostacyclin-stimulating factor.

family: three structurally and pharmacologically distinct isopep-
tides predicted by three separate genes. Proc Natl Acad Sci USA.

27. Haefliger IO, Meyer P, Flammer J, Luscher TF. The vascular endo-
thelium as a regulator of the ocular circulation: a new concept in
341.

expression within the superior mensorcular artery of portal hyper-

constrictor peptide produced by vascular endothelial cells. Na-

30. Davis ME, Cai H, Drummond GR, Harrison DG. Shear stress regu-
lates endothelial nitric oxide synthase expression through c-Src by


