Vascular Pharmacology 62 (2014) 82–87

Contents lists available at ScienceDirect

Vascular Pharmacology

journal homepage: www.elsevier.com/locate/vph

Flow-induced regulation of brain endothelial cells in vitro

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ARTICLE INFO

Article history:
Received 9 January 2014
Received in revised form 31 January 2014
Accepted 18 February 2014
Available online 28 February 2014

Chemical compounds studied in this article:
Mevastatin (PubChem CID: 64715)
Atorvastatin (PubChem CID: 60823)
Simvastatin (PubChem CID: 54454)
Losartan (PubChem CID: 3961)
Valsartan (PubChem CID: 60846)

Keywords:
Endothelial cell
Flow
Nitric oxide synthase
Statin
Angiotensin receptor blocker

ABSTRACT

Endothelial cell (EC) function and susceptibility to vascular disease are regulated by flow; this relationship has been modeled in systemic, but not cerebrovascular, EC culture. We studied the effects of unidirectional flow of medium, produced by orbital rotation of cultures, on morphology and protein expression in bEnd.3 mouse brain ECs. Flow altered the expression of key transcription factors and gasotransmitter-synthesizing enzymes, and increased NO production. Statins and angiotensin receptor blockers reproduced the effect of flow on endothelial nitric oxide synthase expression. Thus, flow modified brain EC properties and function in vitro, with similarities and possible differences compared to previous studies on systemic ECs. Thus, the effect of flow on brain ECs can be modeled in vitro and may assist the investigation of mechanisms of cerebrovascular disease.

1. Introduction

Endothelial cell (EC) dysfunction, characterized by impaired vasoregulatory, antithrombotic, anti-inflammatory, and antiproliferative activity, is an early feature of vasodegenerative disease due to atherosclerosis, hypertension and diabetes [1]. A clinical correlate is defective endothelium-dependent, nitric oxide (NO)-mediated vasodilation [2], which is also observed in animal models of vascular disease [3], and appears to result from reduced generation of NO by endothelial NO synthase (eNOS) [4]. eNOS-mediated NO production is likewise impaired at sites of predilection for atherosclerosis [5], which include arterial branch points and curvatures associated with diminished or disturbed blood flow [6], and can be restored by drugs used to treat vascular disease [7]. Thus, flow-sensitive endothelial eNOS activity provides a benchmark for investigating the pathophysiology and pharmacotherapy of vascular disease.

Effects of flow on EC function have been modeled in vitro using cultures maintained under various flow conditions. Flow of culture medium over EC monolayers can be induced using a cone-and-plate or parallel-plate apparatus or by growing cells on a mechanical platform shaker [8]. Cultures can thereby be exposed to unidirectional, laminar flow and associated shear stress [9], under which conditions they exhibit features of normal EC function, including alignment in the direction of flow [10], transcription factor induction [11], gene expression [12], and eNOS activation [13], that are absent in stationary EC cultures.

Most studies of the interaction between flow and EC function in vitro have used venous or aortic ECs, but other sites, such as the cerebral circulation, are also important targets of disease. In addition, the cerebral circulation may have unique features that influence EC function and disease pathophysiology or treatment [14–16]. To examine these issues and characterize brain EC responses to flow and drugs in vitro, we investigated the effects of stationary versus orbitally rotating culture conditions and selected cardiovascular drugs on the expression of eNOS and other key proteins in the bEnd.3 mouse brain EC line [17].

Abbreviations: EC, endothelial cell; eNOS, endothelial NO synthase; DiI-Ac-LDL, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate-labeled acetylated low-density lipoprotein; HO1, heme oxygenase 1; iNOS, inducible NO synthase; nNOS, neuronal NO synthase; NFκB, nuclear factor κB; CBS, cystathionine-β-synthase; CSE, cystathionine–γ-lyase; HO2, heme oxygenase 2; KLF2, Krüppel-like factor 2; DAPI, 4′,6-diamidino-2-phenylindole; vWF, von Willebrand factor; DAF-DA, 4,5-diaminofluorescein diacetate; HUVEC, human umbilical vein endothelial cell.

DOI of original article: http://dx.doi.org/10.1016/j.vph.2014.05.007.

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http://dx.doi.org/10.1016/j.vph.2014.02.003
1537-1891/

2. Material and methods

2.1. Cell culture

bEnd.3 mouse brain ECs (derived by transformation with polyoma middle T antigen; #CRL-2299) were purchased from ATCC (Manassas, VA). Cells were cultured in 100-mm uncoated plastic dishes in humidified 95% air/5% CO_2 at 37 °C and DMEM containing 10% FBS and 1× penicillin/streptomycin, with medium changed 2–3 times per week. Near-confluent cultures were split 1:4–6 and given 4 h for attachment before further manipulation.

2.2. DiI-labeled acetylated low-density lipoprotein uptake

DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate)-labeled acetylated low-density lipoprotein (DiI-Ac-LDL; Kalen Biomedical, Montgomery Village, MD) was added at 20–100 μg/ml and cultures were incubated for 2 h at 37 °C [18]. Medium was removed, cultures washed with phosphate-buffered saline, and DiI detected by fluorescence microscopy with excitation at 514 nm and emission at 565 nm using a Gemini XPS Fluorescence Microplate Reader (Molecular Devices, Sunnyvale, CA).

2.3. Flow experiments

Cultures on 100-mm plates were placed on an orbital shaker (Barnstead Lab-Line 1314R; VWR International, Radnor, PA) in an incubator containing humidified 95% air/5% CO_2 at 37 °C. For stationary cultures, the orbital shaker was not turned on. For orbitally shaken cultures used to model unidirectional flow, the shaker was set at 80 rpm. In some experiments a reciprocal shaker (ADV 3750 Recipro Shaker; VWR, Radnor, PA; setting 100) was used to model bidirectional flow. Cultures were maintained under these conditions for 7 days, with 1–2 medium changes, before being assayed.

2.4. Drug treatment

Mevastatin, atorvastatin, simvastatin, losartan, and valsartan were from Sigma (St. Louis, MO). Stationary cultures were treated as described above, except drugs were added at the onset of the 7-day culture period and with each medium change.

2.5. Western blotting

Protein (30 μg) was run on 4–12% SDS-polyacrylamide gels at 200 V for 35–40 min and transferred to polyvinylidene fluoride membranes at 75 V for 90–120 min at room temperature. Membranes were incubated with 5% non-fat milk in 1 × PBS-Tween 20 (PBST) for 45 min at room temperature, then with primary antibody in 5% non-fat milk at 4 °C overnight. Membranes were washed 3 times for 10 min each with 1 × PBST, and secondary antibody in 5% non-fat milk was added for 1 h at room temperature. Membranes were washed again, treated with Western Lightning Ultra (PerkinElmer, Waltham, MA) for 2 min, and blotted dry. Protein was measured by densitometry with Quantity One (Biorad, Hercules, CA), with mouse monoclonal anti-β-actin (1:10,000; Sigma) used to detect differences in loading.

Primary antibodies were rabbit polyclonal anti-heme oxygenase 1 (HO1; 1:2000) and anti-inducible NOS (iNOS; 1:200) (Abcam, Cambridge, MA); rabbit polyclonal anti-neuronal NOS (nNOS; 1:1000) and anti-nuclear factor κB (NFκB; 1:1000) (Cell Signaling, Danvers, MA); rabbit polyclonal anti-eNOS (1:2000) and anti-Ser1177-phospho-eNOS (Ser1177P-eNOS; 1:1000) (Millipore, Billerica, MA); mouse monoclonal anti-cystathionine-β-synthase (CBS; 1:1000), anti-cystathionine-γ-lyase (CSE; 1:500), and anti-heme oxygenase 2 (HO2; 1:200) (Santa Cruz Biotechnology, Santa Cruz, CA); and rabbit polyclonal anti-Krüppel-like factor 2 (KLF2; 1:400) (Sigma). Secondary antibodies were HRP-conjugated donkey anti-rabbit and sheep anti-mouse (GE Healthcare, Pittsburgh, PA) and donkey anti-goat (Santa Cruz) IgG (1:10,000–15,000).

2.6. Immunocytochemistry

Cells were cultured in 4- or 24-well plates containing 500 μl/well of medium and fixed by adding 500 μl/well of 4% paraformaldehyde (PFA) for 15 min at room temperature. Supernatant was aspirated and 500 μl/well of PFA was added for 15 min at room temperature. Cultures were washed 3 times with 1 × PBS, and blocking buffer (2% horse serum, 1% BSA and 0.1% Triton 100 in 1 × PBS) was added for 1 h at room temperature. Cultures were incubated with primary antibody at 4 °C overnight, washed 3 times with 1 × PBS at room temperature, incubated with secondary antibody for 1 h at room temperature, and washed 3 times with 1 × PBS. 4′,6-Diamidino-2-phenylindole (DAPI) was added to stain nuclei. Primary antibodies were rabbit polyclonal anti-von Willebrand factor (vWF; 1:500) (Abcam) and rabbit polyclonal anti-F-actin (1:500) (Millipore). Secondary antibody was donkey anti-rabbit IgG (1:400) (Millipore).

2.7. NOS activity assay

NOS activity was measured using a commercial kit (Sigma #FCANOS1). Cells grown on 100-mm dishes were detached with 0.25% trypsin, centrifuged at 1000 rpm for 5 min and resuspended in 5 ml of medium. Resuspended cells (10^5/200 μl/well) were added to black, clear-bottomed, 96-well culture plates and maintained in humidified 95% air/5% CO_2 at 37 °C for 4 h. Medium was removed and replaced with 190 μl of reaction buffer (Sigma #R2525), 10 μl of arginine substrate solution, and 0.1 μl of 4,5-diaminofluorescein diacetate (DAF-2 DA) solution. Cells were incubated for 2 h at room temperature in the dark, and fluorescence was measured with excitation at 470 nm and emission at 510 nm, using a Gemini XPS Fluorescence Microplate Reader (Molecular Devices). Controls included omitting cells, substrate, or DAF-2 DA.

2.8. Statistics

Each experiment was repeated at least 3 times with different platings of cells. Statistical significance was determined by t-tests with a threshold of $p < 0.05$.

3. Results

Stationary bEnd.3 cultures showed phenotypic attributes of ECs, including expression of von Willebrand factor (vWF) and uptake of DiI-Ac-LDL (Fig. 1A). Under these conditions, cells grew without directional alignment (Fig. 1B). In contrast, cells grown for 7 days on an orbital shaker platform, which produced unidirectional (circular) flow of culture medium, were aligned in the direction of rotation at the periphery (but not the center) of culture dishes (Fig. 1B), consistent with similarly rotated bovine aortic ECs [8] and the relationship between shear stress and orbital radius [8,9]. Cells grown on a reciprocal shaker, which produced direction-changing flow, remained unaligned (Fig. 2A), as observed for stationary cultures. Cell alignment in orbitally rotated cultures was also evident in cultures immunostained for F-actin (Fig. 1C), which reorganizes in response to shear stress to promote EC elongation and alignment [19].

The beneficial effect of flow-induced shear stress on EC function is mediated partly by altered activity of transcription factors [20], including induction of KLF2 [11] and suppression of NFκB [21]. Accordingly, we compared expression of these proteins in stationary and orbitally rotated bEnd.3 cultures. Rotation increased KLF2 expression to 341 ± 113% ($p < 0.05$, $n = 6$) and reduced the ratio of nuclear to cytoplasmic NFκB to 17 ± 7% ($p < 0.05$, $n = 4$) of values in stationary cultures (Fig. 3).